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Airway uric acid is a sensor of inhaled protease allergens and initiates type 2 immune responses in respiratory mucosa[1]

Kenichiro Hara[*,†], **Koji Iijima**[*], **Martha K. Elias**[*], **Satoshi Seno**[‡], **Ichiro Tojima**[‡], **Takao Kobayashi**[*], **Gail M. Kephart**[*], **Masahiko Kurabayashi**[†], and **Hirohito Kita**[*,§]

[*]Division of Allergic Diseases, Department of Internal Medicine, Mayo Clinic, Rochester, MN 55905

[†]Department of Medicine and Biological Science, Gunma University Graduate School of Medicine, Maebashi, Gunma, Japan

[‡]Department of Otorhinolaryngology, Shiga University of Medical Science, Otsu, Shiga, Japan

[§]Department of Immunology, Mayo Clinic, Rochester, MN 55905

Abstract

While type 2 immune responses to environmental antigens 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. Here we report that IL-33 and thymic stromal lymphopoietin (TSLP) were produced quickly in the lungs of naïve mice exposed to cysteine proteases, such as bromelain and papain, as a model for allergens. IL-33 and TSLP sensitized naïve animals to an innocuous airway antigen OVA, which resulted in production of type 2 cytokines and IgE antibody and eosinophilic airway inflammation when mice were challenged with the same antigen. 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 naïve 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.

Introduction

Asthma and allergic airway diseases have increased over the past 50 years. Globally, 300 million and 400 million people suffer from asthma and allergic rhinitis, respectively (1).

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Contact: Dr. Hirohito Kita, Division of Allergic Diseases, Department of Immunology, Mayo Clinic Rochester, 200 First Street SW, Rochester, MN 55905. kita.hirohito@mayo.edu; Phone, 507-284-6109; FAX, 507-284-5045.

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Allergic asthma is generally caused by type 2 immune responses to innocuous airborne antigens, leading to eosinophilic airway inflammation, mucus 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 antigens 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 antigens 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 antigen. 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 omega-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.

Here, 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 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, UK). *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, Switzerland). All knockout or transgenic mice were bred in the Mayo Clinic animal care facility, and female mice aged 6–10 weeks 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), peridinin-chlorophyll protein (PerCP)-conjugated anti-CD44 (IM7), and allophycocyanin (APC)-conjugated anti-CD25 (PC61) antibodies 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 (EU)/mg protein] was purified from specific pathogen-free chicken eggs under sterile conditions. Recombinant mouse IL-33 (Ser109-Ile266, <0.01 EU/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 less than 0.005 EU/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 less than 2 EU/mg protein.

Acute airway inflammation model

To examine acute airway immune responses, 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 naïve wild-type (WT) mice or *ST2*$^{-/-}$ mice that were lightly anesthetized using isoflurane inhalation. 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 Hanks balanced salt solution. 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; more than 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

To examine the effects of proteases or MSU crystals on adaptive type 2 immune response development, naïve 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. In some experiments, bromelain or papain were pretreated with the cysteine protease inhibitor E64 (10 μM) for 30 minutes 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 antibodies. 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 above. 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, naïve mice were gavaged once daily for 16 days with febuxostat (5 mg/kg/dose) or distilled water two days 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 days/week for 2 weeks, 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/day, daily for 3 days, to *Il5*$^{+/venus}$ and *Il13*$^{+/eGFP}$ mice or wild-type 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, Canada) for 1 hr at 37°C. After digestion, single lung cells were hemolyzed with ammonium-chloride-potassium (ACK) buffer, and washed with phosphate buffered saline (PBS) containing 0.1% sodium azide and 1% bovine serum albumin. To examine the expression of cytokines by group 2 innate lymphoid cells (ILC2s), lung single cell suspensions were stained with a PE-conjugated lineage cocktail [CD3

(145-2C11), CD14 (rmC5-3), CD16/32 (2.4G2), B220 (RA3-6B2)], APC-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 antigen-specific IgE, IgG1, and IgG2a

To quantitate the levels of OVA-specific IgE antibody in plasma specimens, ELISA plates (Immulon 4; Thermo Labsystems) were coated with 5 μg/ml rat anti-mouse IgE mAb (Serotec) in 0.1 M carbonate buffer (pH 9.5) for 2 hr 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 hr at room temperature. Thereafter, the plates were incubated for 1 hr 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. TMB (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, ELISA plates were coated with 10 μg/ml OVA, blocked with BSA, and incubated 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 TMB 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 antibody concentrations and OD values up to 1.5.

Measurement of cytokines, uric acid (UA), and high mobility group box protein B1 (HMGB-1) 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, Amplex® Red fluorogenic substrate uric acid/uricase assay kits (Invitrogen, Grand Island, NY) were used. HMGB-1 levels in BAL fluid supernatants were analyzed using HMGB-1 ELISA kits (IBL International, Toronto, Ontario). UA levels in lung homogenates were measured using colorimetric uric acid 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 days). Cells were stimulated with serial dilutions of MSU crystal suspensions or 100 μg/ml *Alternaria* extract for 3 hr. 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 hr 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-positive and EthD-1-negative) and damaged (EthD-1-positive) 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 hr, 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 hr 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 hr 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, Inc.). 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's t tests. Multiple comparisons between treatment and control conditions were performed using one-way analysis of variance. Values of p less than 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 naïve wild-type (WT) BALB/c mice. Substantial amounts of IL-33 and IL-25, but not TSLP, were detectable in lung homogenates of naïve non-treated animals (Figure 1A; please note y-axis scales). Upon exposure to the proteases, the lung levels of

IL-33 and TSLP quickly increased within 3 hr, peaking at 3 to 6 hr (Figure 1A). The 10 μg/dose of bromelain appeared to be more potent than the 50 μg/dose of 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 antigens, we administered endotoxin-free OVA protein with or without bromelain into the airways of naive BALB/c mice on days 0 and 7 (Figure 1B). On day 14, plasma levels of OVA-specific IgE and IgG1 antibodies increased significantly in mice exposed to OVA plus bromelain (Figure 1C). OVA alone or bromelain alone did not induce these antibody responses. No increase in IgG2a antibody was observed in mice exposed to OVA plus bromelain.

When these mice were challenged i.n. with OVA antigen (without bromelain) on days 21 through 23, mice previously exposed to OVA plus bromelain demonstrated marked airway eosinophilia, mucus hyperplasia, and peribronchial infiltration with inflammatory cells (Figures 1D and 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 OVA alone or bromelain alone (Figure 1F). These immunological responses to OVA were abolished in *Rag1*$^{-/-}$ mice (Figure S1A), 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 (Figure S1B and S1C). 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 antigens.

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 above, mice deficient in cytokine receptors were exposed i.n. to endotoxin-free OVA plus bromelain and challenged with OVA alone. Mice deficient for IL-33 receptor (*ST2*$^{-/-}$ mice) showed more than 80% reduction in BAL eosinophils and BAL IL-5 and IL-13 levels as compared to WT mice (Figure 2A). Anti-OVA IgE antibody was significantly inhibited, and an apparent decrease in airway mucus hyperplasia was observed in *ST2*$^{-/-}$ mice (Figure 2B). Mice deficient for TSLP receptor (*Tslpr*$^{-/-}$ mice) and mice deficient for IL-25 receptor (*Il17rb*$^{-/-}$ mice) also showed significant decreases in BAL eosinophils, lung IL-5 and IL-13 levels, and serum IgE antibody (Figure 2C and 2D). In contrast, no differences in these immunological parameters were observed in mice deficient in IL-1 receptor (*Il1r1*$^{-/-}$), the receptor for IL-1α and IL-1β (Figure 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 uric acid (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 to WT mice when they were exposed to OVA plus bromelain and challenged with OVA (Figure S2A). 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 (Figure S2B). 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 (i.e., BAL fluids) were collected and analyzed quantitatively, UA levels increased rapidly within 3 hr after a single airway exposure of mice to bromelain (Figure 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 Figure 1B, naïve mice were exposed i.n. to endotoxin-free OVA plus bromelain with or without uricase and 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 antibodies (Figure 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 non-specific inhibitory effects of uricase on the development of type 2 immune responses in the airways, IL-33 was used 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 (Figure S3). 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-1 receptor 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 naïve BALB/c mice. Lung levels of IL-33 and TSLP, but not IL-25, increased significantly 3hr after a single airway administration of MSU crystals (Figure 4A) to naïve mice. Increased BAL levels of IL-33 (Figure 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 (Figure S4A). 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 (Figure 4C). IL-5 and IL-13 production was inhibited and IL-17 production was enhanced in *ST2*$^{-/-}$ mice, suggesting involvement of the IL-33 pathway. The possible cellular source(s) of IL-5 and IL-13 were further examined by using IL-5venus (51) and IL-13eGFP (52) cytokine reporter mice. Lineage-negative(Lin^{-})CD25^{+}CD44^{hi} lung ILC2s (25) increased their expression of IL-5 and IL-13 when exposed to MSU crystals *in vivo* (Figure 4D), suggesting that IL-33-responsive ILC2s are likely involved. Production of these type 2 cytokines was also observed when naïve C57BL/6 mice were exposed to MSU crystals (Figure S4B).

To examine whether the adaptive arm of type 2 immunity can be initiated by UA, we administered OVA with or without MSU crystals into the airways of naïve Balb/c mice and then challenged them 2 weeks later with OVA antigen (Figure 5A). Mice previously exposed to OVA with MSU crystals developed marked airway eosinophilia as well as increased BAL levels of IL-5 and IL-13 (Figures 5B and 5C); no or minimal increases in IL-17 or IFN-γ were observed. These Th2-type immune responses to OVA antigen were significantly inhibited in *ST2*$^{-/-}$ mice (Figures 5B and 5C) as well as in *Il17rb*$^{-/-}$ and *Tslpr*$^{-/-}$ mice (Figures 5D and 5E). Taken together, these data suggest that airway exposure to exogenous UA provokes both innate and adaptive type 2 immune responses in respiratory mucosa and that IL-33, as well as IL-25 and TSLP, play key roles in these responses.

Airway epithelial cells secrete IL-33 in response to UA

Our knowledge of the immunological mechanisms involved in production and/or secretion of IL-33 is limited. Airway epithelial cells are considered one of the major sources of IL-33 in respiratory mucosa (35–37, 53). IL-33 is constitutively produced and stored within epithelial cells, particularly in nuclear compartments, and it has been considered a DAMP molecule (54, 55).

To investigate whether IL-33 is secreted by airway epithelial cells in response to UA, we turned to an *in vitro* model. By confocal microscopy, as reported previously (56, 57), IL-33 protein was localized mainly within the nuclei of non-stimulated NHBE cells (Figure 6A); minimal IL-33 was also detectable in the perinuclear region. When the cells were exposed to

MSU crystals, IL-33 was mobilized throughout the entire cytoplasmic compartment in a granular pattern. Furthermore, IL-33 protein was detected in cell-free supernatants of NHBE cells after they were incubated for 3 hr with MSU crystals (Figure 6B). The stimulatory effects were observed with MSU crystals as low as 1 μg/ml. At 100 μg/ml, MSU crystals induced extracellular release of IL-33 at levels comparable to those of *Alternaria* extract, a potent agonist of IL-33 secretion (56). In addition, when NHBE cells were stained with the membrane-impermeable nucleic acid dye, EthD-1, no apparent difference was observed in cell membrane integrity between the cells incubated with media alone and those incubated with 100 μg/ml MSU crystals (Figure 6C), suggesting that IL-33 can be secreted from NHBE cells without apparent cell death.

Blockade of UA synthesis inhibits allergic airway inflammation induced by exposure to natural allergens

The experiments described above used model allergens, namely cysteine proteases, to demonstrate the critical role for UA to induce IL-33 and initiate type 2 immune responses. However, whether UA is involved in allergic airway inflammation induced by natural 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 weeks to several allergens that are relevant to human asthma (Figure 7A).

Uricase is a potent agent that depletes UA. However, it has a short half-life *in vivo* and can elicit neutralizing antibodies 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 days before allergen exposure and continuing throughout the experiment (Figure 7A). When naïve BALB/c mice were repeatedly exposed i.n. to a cocktail of *Alternaria*, *Aspergillus*, and HDM extracts, they developed marked airway eosinophilia as well as increased lung levels of IL-5 and IL-13 (Figures 7B and 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 (Figure 7D). Mice exposed to a cocktail of allergens also showed mucous hyperplasia (Figures 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 demonstrated that DCs activated by endogenous UA are involved in Th2-type immune responses (4). 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 as to 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 the current study, we used proteases as a model “allergen”. Our results show that TLR4 or IL-1α were 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 subcutaneous 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 the current 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 (Figure 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 (Figure 3), possibly due to the presence of residual UA following 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 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 (XOR), 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 XOR (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 (Figure 4) and recapitulate potent type 2 adjuvant activities of cysteine proteases (Figure 5). The innate type 2 response was observed in both Balb/c mice and C57BL/6 mice (Figure S4). As a model of human gout, intraperitoneal injection of MSU crystals induced IL-1β production by tissue resident cells, resulting in robust neutrophilic inflammation through activation of IL-1 receptor (42, 48, 75). In contrast, our results showed that the IL-33, but not the IL-1, receptor was involved in the eosinophilic responses to the proteases (Figure 2). Thus, the different organs may preferentially use distinct IL-1-family molecules, and the route of administration (i.e., airway lumen vs. 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 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 (Figure S4A). 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 naïve non-sensitized 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 antigen-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 (Figure 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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New or special abbreviations

ACK	ammonium chloride/potassium
APC	allophycocyanin
BAL	bronchoalveolar lavage
DAMP	damage-associated molecular pattern
DC	dendritic cells
EU	endotoxin unit

HDM	house dust mite
HMGB-1	high mobility group box protein B1
ILC	innate lymphoid cells
ILC2	group 2 ILC
i.n	intranasal
Lin	lineage
LN	lymph node
MSU	monosodium urate
NHBE	normal human bronchial epithelial
PAS	periodic acid-Schiff
PAR2	protease-activated receptor 2
SNP	single nucleotide polymorphism
TSLP	thymic stromal lymphopoietin
UA	uric acid
WT	wild-type
XOR	xanthine oxidoreductase

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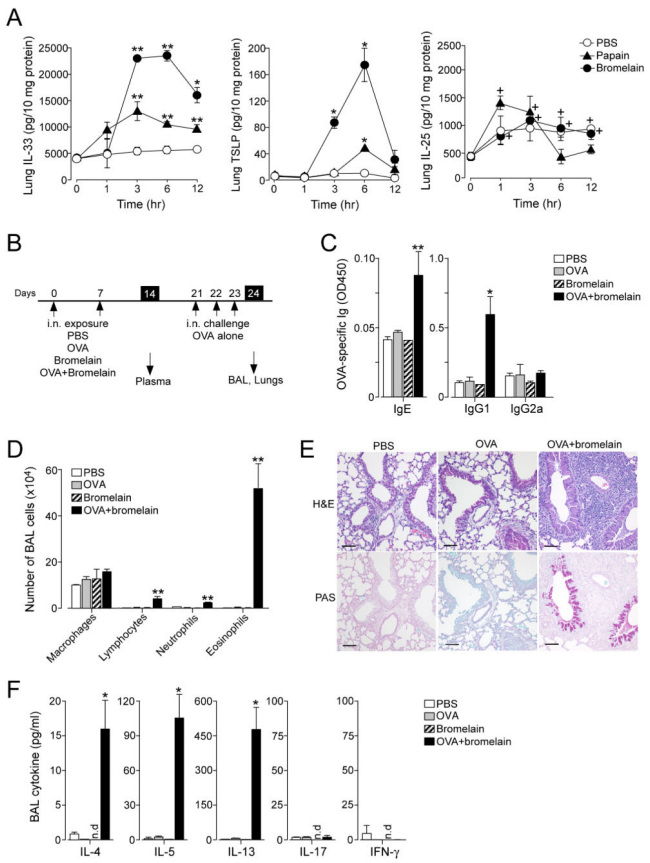

Figure 1.

Airway exposure of naïve mice to cysteine proteases promotes IL-33 and TSLP production and induces Th2-type immune responses to innocuous antigens. (A) Naïve BALB/c mice were intranasally (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 to PBS, $^{+}$ $p<0.05$, compared to non-treated mice (i.e., 0 hr), n=5–8 mice/group. Experiments were repeated three times; data shown are one representative experiment. (B) Experimental protocol to study antigen-specific immune responses. On days 0 and 7, naïve 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 antibodies 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 stain. Scale bars represent 100 μm. (F) Concentrations of cytokines in BAL fluids were analyzed using ELISA. Data shown are the mean±SEM, * $p<0.05$, compared to PBS group, n=6 mice/group. Experiments were repeated twice; data shown are one representative experiment. n.d., not determined.

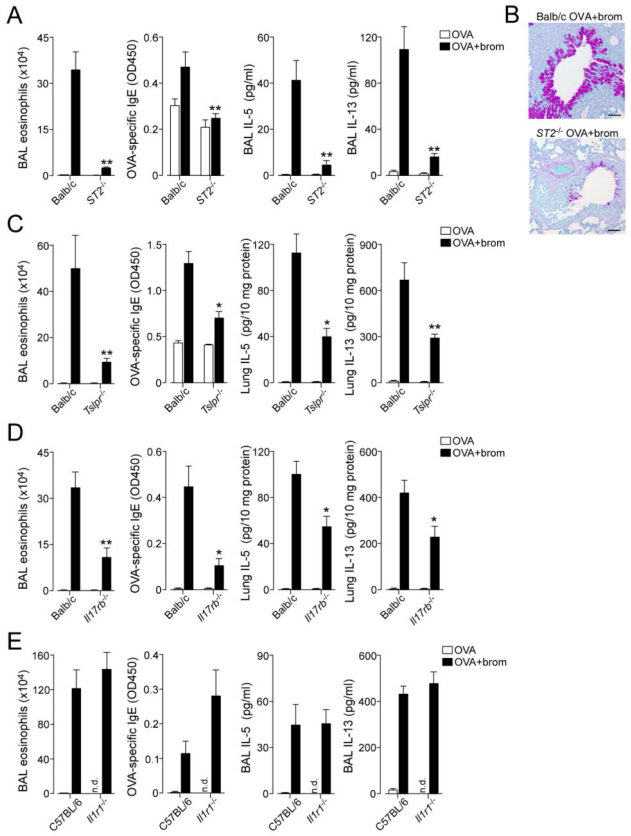

Figure 2.

Bromelain-induced antigen-specific Th2-type immune responses to OVA are dependent on IL-33, TSLP and IL-25. Naïve WT BALB/c, C57BL/6 mice, or *ST2*$^{-/-}$ mice (A and 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 Figure 1B. Total numbers of eosinophils in BAL fluids, plasma levels of anti-OVA IgE antibody, and BAL or lung levels of cytokines were determined. Lung sections were stained with PAS. Data shown are the mean±SEM, * $p<0.05$, ** $p<0.01$, compared to WT mice, n=5–6 mice/group. Experiments were repeated twice (A, B, D) or once (C, E); data shown are one representative experiment. n.d., not determined.

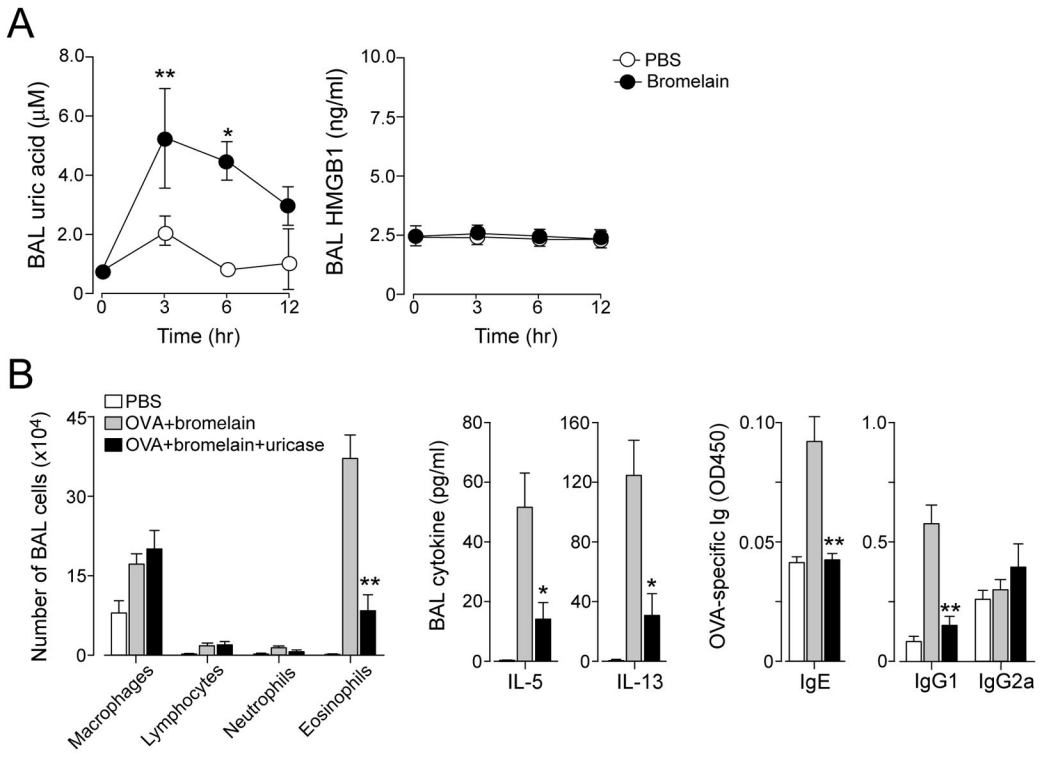

Figure 3.

Endogenous UA in the airways plays a pivotal role in type 2 immune responses induced by bromelain. (A) Naïve 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 to PBS, n=6 mice/group. Experiments were repeated twice; data shown are pools of two experiments. (B) Using the same protocol as shown in Figure 1B, naïve 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 antibodies. 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 to OVA plus bromelain, n=5–6 mice/group. Experiments were repeated twice; data shown are one representative experiment.

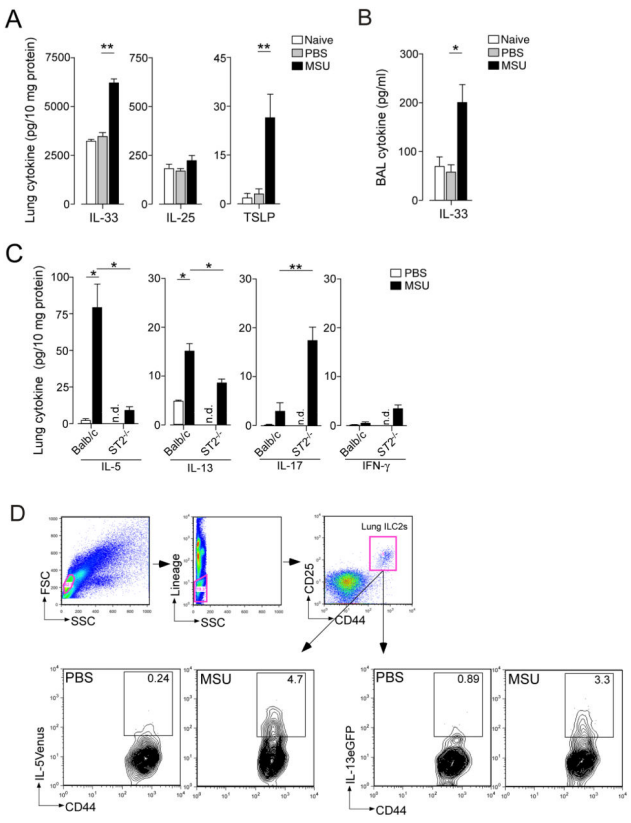

Figure 4.

Airway administration of MSU crystals induces IL-33 and TSLP production in the lungs and trigger innate type 2 responses. (A and B) Naïve WT BALB/c mice were untreated or administered once i.n. with MSU crystals (1 mg/dose) or PBS. After 3 hr, 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) Naïve WT BALB/c mice or *ST2*$^{-/-}$ mice were administered once i.n. with MSU crystals. After 3 hr, 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 days. 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.

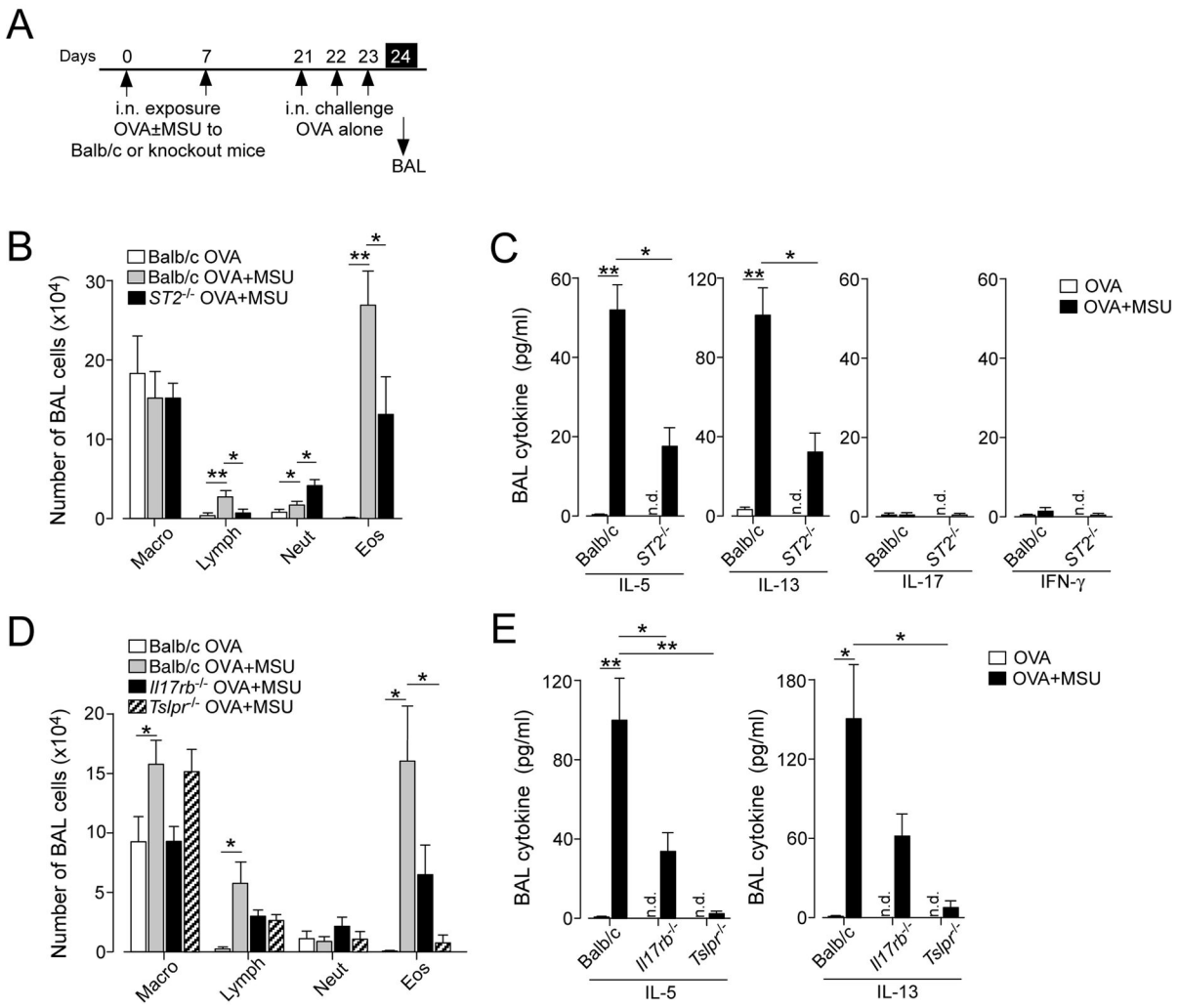

Figure 5.

MSU crystals induce adaptive type 2 responses to innocuous antigens in the airways.

(A) Experimental protocol. Naïve WT BALB/c mice, *ST2*$^{-/-}$ mice, *Il17rb*$^{-/-}$ mice, or *Tslp*$^{-/-}$ mice were exposed to OVA alone (100 μg/dose) or OVA plus MSU crystals (1 mg/dose) on days 0 and 7. All mice were challenged with OVA alone on days 21, 22, and 23. BAL fluids were collected on day 24. (B, C, D and E), The number of cells in BAL fluids (B and D) and the levels of cytokines in the supernatants (C and E) were analyzed. Data shown are the mean±SEM, *p<0.05, **p<0.01, n=6 mice/group. (B and C) Experiments were repeated twice; data shown are one representative experiment. (D and E) Experiments were performed once. n.d., not determined.

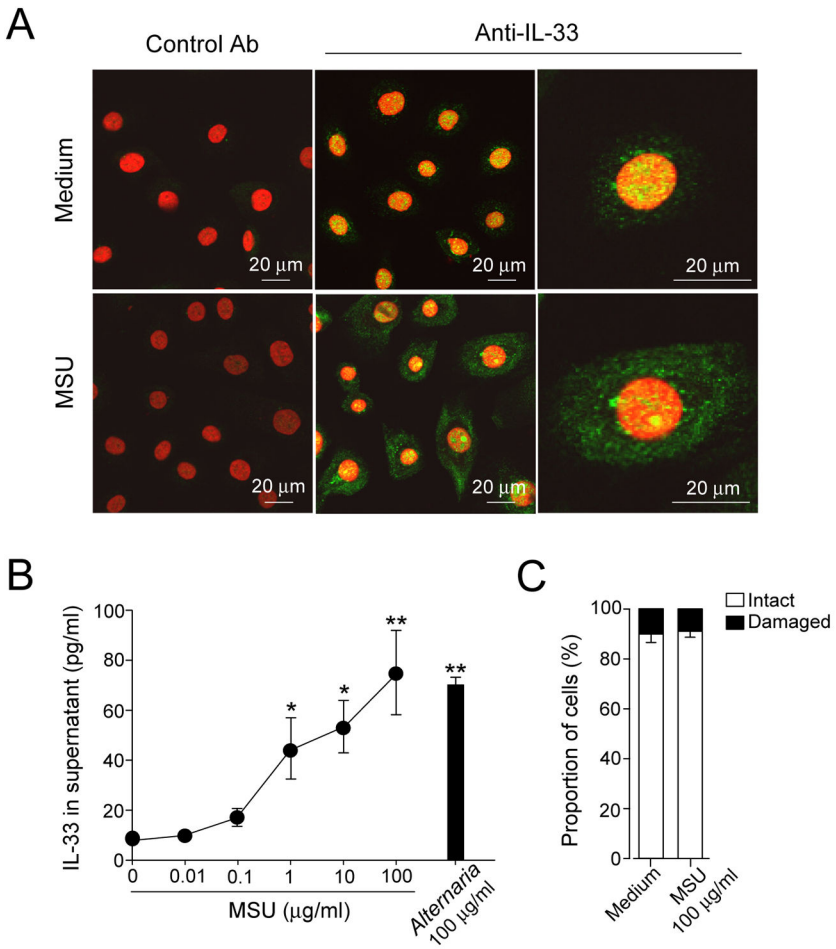

Figure 6.

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 hr, 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 represent 20 μm. (B) NHBE cells were exposed for 3 hr 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 to media alone. Data shown are a pool of six experiments. (C) NHBE cells were exposed for 3 hr 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.

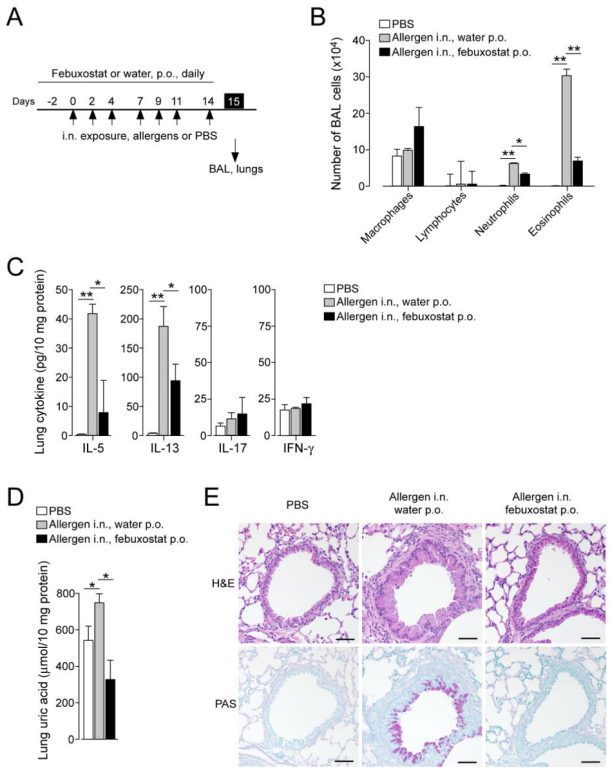

Figure 7.

The UA synthesis inhibitor febuxostat attenuates eosinophilic airway inflammation and asthma-like pathology in mice exposed repeatedly to allergen extracts. (A) Experimental protocol. Naïve BALB/c mice were orally administered febuxostat (5 mg/kg/dose) or distilled water daily starting on day −2 for 16 days. Mice were exposed i.n. to PBS or a cocktail of allergen extracts (*Alternaria*, *Aspergillus*, and HDM, 10 μg each/dose), 3 times per week for 2 weeks. 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.