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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 naïve mice exposed to cysteine proteases, such as bromelain and papain, as a model for allergens. IL-33 and thymic stromal lymphopoietin sensitized naïve 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 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 phenotype 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.

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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; IL-2, group 2 ILC; i.n., intranasally; MSU, monosodium urate; NHEBE, 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 stressors in respiratory mucosa.

**Materials and Methods**

**Mice and cells**

BALB/c, C57BL/6, BALB/cByL, Th2REo-2g-I (Th2-4), BALB/cByl background, Rag1−/− mice (BALB/c background), Il1r−/− mice (C57BL/6 background), and PAR2−/− mice (C57BL/6 background) were purchased from The Jackson Laboratory (Bar Harbor, ME). ST2−/− (Il1r−/−) mice (BALB/c background), Il17rb−/− mice (BALB/c background), and Il13Rα2−/− mice (BALB/c background) were provided by Dr. Andrew McKenzie (Medical Research Council Laboratory of Molecular Biology, Cambridge, U.K.). Tg flp (mice (BALB/c background) were provided by Dr. Stono Ziegler (Benaroya Institute, Seattle, WA). IL5vevoGFP mice (BALB/c background) were provided by Dr. Kiyoshi Takatsu (University of Tohoku, Tohoku, 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 (NBHE) cells were purchased from Lonza (Allendale, NJ) and maintained in serum-free bronchial epithelial cell growth medium (Lonza). NBHE cells were used within three passages.

**Reagents**

PE-conjugated anti-CD3 (17A2), anti-CD14 (rmC5-3), anti-CD61/62 (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 cytotoxic 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 (Ser106Lys101) <0.01 endotoxin units/mg 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 μg 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 with the cytotoxic 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 IL5vevoGFP and Il13Rα2−/− 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 a mixture of 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−CD44hi cells as previously described (25). The expression levels of IL-5, IL-13, and IL-33eGFP 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 specimens, 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 washed with PBS containing 1 μg/mL biotin-conjugated OVA, followed by 1:5,000 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 reader (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 fluorescent 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, Tokyo, Japan).
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 x 10⁴ 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 Cell 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.
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. onto 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 non-treated 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
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 (Il1r1−/− 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 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 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.
(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...
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-33 reporter mice. Lin−CD25−CD44hi lung ILC2s increased their expression of IL-5 and IL-13 when exposed to MSU crystals in vivo (Fig. 4D), suggesting that IL-33–responsive ILC2s are likely involved. Production of these type 2 cytokines was also observed when naive C57Bl/6 mice were exposed to MSU crystals (Supplemental Fig. 4B).

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 naive BALB/c mice and then challenged them 2 wk later with OVA Ag (Fig. 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 (Fig. 5B, 5C); no or minimal increases in IL-17 or IFN-γ were observed. These Th2-type immune responses to OVA Ag were significantly inhibited in ST2−/− mice (Fig. 5B, 5C), as well as in Il17rb−/− and Tslpr−/− mice (Fig. 5D, 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 used an in vitro model. By confocal microscopy, as reported previously (56, 57), IL-33 protein was localized mainly within the nuclei of nonstimulated NHBE cells, as well as increased BAL levels of IL-5 and IL-13 (Fig. 5B, 5C); no or minimal increases in IL-17 or IFN-γ were observed. These Th2-type immune responses to OVA Ag were significantly inhibited in ST2−/− mice (Fig. 5B, 5C), as well as in Il17rb−/− and Tslpr−/− mice (Fig. 5D, 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.

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

The experiments described earlier 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
Alternate text here
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 adhesive 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
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 patients with chronic rhinosinusitis during disease exacerbation (80) increased in patients with asthma after allergen exposure (4) and in several natural allergens (Fig. 7). The airway levels of UA are in-}

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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 patients with chronic rhinosinusitis during disease exacerbation (80) increased in patients with asthma after allergen exposure (4) and in several natural allergens (Fig. 7). The airway levels of UA are in-


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.
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. Uracase 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<sup>−/−</sup> 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.