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The Danger Signal, Extracellular ATP, Is a Sensor for an Airborne Allergen and Triggers IL-33 Release and Innate Th2-Type Responses

Hideaki Kouzaki,*† Koji Iijima,* Takao Kobayashi,* Scott M. O’Grady,‡§ and Hirohito Kita*‡

The molecular mechanisms underlying the initiation of innate and adaptive proallergic Th2-type responses in the airways are not well understood. IL-33 is a new member of the IL-1 family of molecules that is implicated in Th2-type responses. Airway exposure of naive mice to a common environmental aeroallergen, the fungus *Alternaria alternata*, induces rapid release of IL-33 into the airway lumen, followed by innate Th2-type responses. Biologically active IL-33 is constitutively stored in the nuclei of human airway epithelial cells. Exposing these epithelial cells to *A. alternata* releases IL-33 extracellularly in vitro. Allergen exposure also induces acute extracellular accumulation of a danger signal, ATP; autocrine ATP sustains increases in intracellular Ca^{2+} concentration and releases IL-33 through activation of P2 purinergic receptors. Pharmacological inhibitors of purinergic receptors or deficiency in the *P2Y2* gene abrogate IL-33 release and Th2-type responses in the *Alternaria*-induced airway inflammation model in naive mice, emphasizing the essential roles for ATP and the P2Y_{2} receptor. Thus, ATP and purinergic signaling in the respiratory epithelium are critical sensors for airway exposure to airborne allergens, and they may provide novel opportunities to dampen the hypersensitivity response in Th2-type airway diseases such as asthma. *The Journal of Immunology*, 2011, 186: 4375–4387.

Infection and tissue injury induce inflammation and immune responses (6). During an infection, microorganisms initiate a series of inflammatory responses through their pathogen-associated molecular pattern molecules. Th2-type immune responses can be induced by an experimental allergen, OVA, contaminated with a low-dose LPS (7) or by an allergen’s structural capacity to stimulate the TLR4 (8). Alternatively, immune cells react to tissue injury by recognizing the molecules that are normally located inside the cell, but are released by injured cells (9). These damage-associated molecular patterns (DAMPs) interact predominantly with host pattern recognition receptors and induce inflammation and immune responses. A classic Th2-type adjuvant, alum, likely uses this injury-mediated mechanism by inducing the release of uric acid (10). However, airborne allergens or environmental pollutants generally do not infect hosts and are unlikely to injure tissues. Thus, when mammals are exposed to allergens or pollutants, do these infection or tissue injury models apply to the development of Th2-type immune responses?

Among the most potent molecules of the innate immune system are the IL-1 family members (11); these cytokines, such as IL-1, IL-18, and IL-33, are evolutionarily ancient and are involved in regulating innate and adaptive immune responses. IL-33 (or IL-1F11) is a ligand of the orphan T1/ST2 receptor (12); it was first described as a NF abundantly expressed in the nucleus of endothelial and epithelial cells (13–16). In vivo systemic administration of IL-33 to mice profoundly alters immunity and inflammation, including lung and gastrointestinal eosinophilia and increased levels of serum IgE and IgA (12). IL-33 is implicated in diseases, for example, asthma (17), anaphylactic responses (18), and cardiovascular and rheumatoid diseases (19). Cells in barrier tissues constitutively express and store IL-33, suggesting its central role in mucosal immunity (19). IL-33 drives production of cytokines and chemokines by Th2 cells, mast cells, basophils, eosinophils, NKT cells, and NK cells (12, 20–29). More recent studies reported that IL-33 can induce proliferation of and Th2 cytokine production by a novel non-T/non-B cell population (30–32). Thus, IL-33 may...
be important in innate and adaptive Th2-type immune responses. However, the mechanisms for the synthesis, processing, and release of IL-33 remain poorly understood (19). Unlike IL-1β, pro-IL-33 (i.e., full-length 31-kDa protein) has biological activity (33–35), and IL-33 does not require proteolytic maturation by caspase-1 (33, 34). Thus, IL-33 may be an “alarmin” that is released during necrotic cell death and associated with infection or tissue injury (33, 36).

Lung, skin, and intestinal epithelial cells that interact with the external environment produce antimicrobial molecules, cytokines, and chemokines that are essential for innate and adaptive immunity (37). In this study, we sought to identify the mechanisms for the innate immune regulation of airway Th2-type responses that are induced by exposure to natural environmental allergens. In humans, an association between fungal exposure, in particular to *Alternaria alternata*, and asthma is recognized clinically and epidemiologically (38, 39). Severe asthma and life-threatening acute exacerbations of asthma in humans have been associated with increased airborne exposure to *Alternaria* (40–42). Thus, we used *Alternaria* to provoke Th2-type immune responses relevant to human diseases. Airway exposure of naive mice to *Alternaria* induces rapid secretion of IL-33 into the airways and subsequent Th2-type cytokine production. In response to *Alternaria* allergens, airway epithelial cells translocate nuclear IL-33 and actively release it into the extracellular milieu. ATP-mediated activation of a P2 purinergic receptors and sustained increases in intracellular calcium concentration ([Ca²⁺]i) are required for this IL-33 secretion in vitro and in vivo. Thus, the airway epithelium responds to noncytolytic “stress” induced by components within our atmospheric environment by releasing ATP, leading to Th2-type inflammatory or possibly homeostatic immune responses.

**Materials and Methods**

**Mice and cells**

Animals were bred and maintained under specific pathogen-free conditions; all animal experiments were done with the approval of and in accordance with regulatory guidelines and standards set by the Institutional Animal Care and Use Committee of Mayo Clinic–Rochester. BALB/c, C57BL/6, Rag2^−/−^-Rag1^−/−^ (B6.129S7-RagFltm1Sv/J), P2X7^−/−^ (B6.129P2-P2x7tm1Yeu/J), and P2Y2^−/−^ (B6.129P2-P2y2tm1Hkl/J) were from The Jackson Laboratory. Female mice (7–13 wk old) were used. Normal human bronchial airway epithelial (NHBE) cells were from MBL International and Abcam, respectively. Rabbit-anti-human IL-33 and rabbit anti-human high mobility group box-1 son ImmunoResearch Laboratories. (BEGM) (both from Lonza); cells were used within three passages.

**Detection of IL-33 by immunohistochemistry and by confocal microscopy**

To detect IL-33 in tissue specimens, formalin-fixed paraffin-embedded sections were deparaffinized and rehydrated. Ag retrieval was performed by heating the sections for 30 min in Tris-EDTA buffer (pH 9.0; Dako). The sections were stained using HRP/3-amin-9-ethyl-carbazole detection kits from Lab Vision or R&D Systems, using manufacturers’ instructions; rabbit anti-human IL-33 or normal rabbit IgG were used as primary Abs. Sections were counterstained with Vectastain hematoxylin QS and mounted in Faramount (Dako).

NHBE cells were cultured on Lab-Tek 2 chamber slides (Fisher Scientific). After stimulation with Alternaria extract (50 μg/ml), LPS (1 μg/ml), poly(I:C) (10 μg/ml), ionomycin (1 μM), or thapsigargin (3 μM) for 18 h, the cells were incubated with BD Golgiplug (BD Pharmingen) for 30 min and then washed with BD Perm/Wash buffer for 30 min at room temperature. Fixed cells were blocked with 5% normal rabbit serum (Sigma-Aldrich) for 1 h and then washed with BD Perm/Wash buffer. 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 for 30 min at room temperature. Fixed cells were blocked with 5% normal rabbit serum (Sigma-Aldrich) for 1 h and stained overnight with rabbit anti-human IL-33, rabbit anti-HMGBl, or normal rabbit IgG at 4˚C.

To detect P2 purinergic receptors, cells were stained overnight with anti-P2X-R, anti-P2Y-R, or normal rabbit IgG at 4˚C. For immunofluorescence, the cells were incubated with FITC-conjugated goat anti-rabbit IgG for 2 h at room temperature, washed in BD Perm/Wash buffer for 30 min, and mounted in Vectashield mounting medium with DNA-binding dye, DAPI (Vector Laboratories). Fluorescent images were visualized using a confocal microscope (LSM510 confocal microscope), and digital images (512 × 512 pixels, ×800 magnification) were captured by using the KS400 image analysis system (both Carl Zeiss). The threshold for each negative control image was calibrated to a baseline value without positive pixels. All images were processed using the Zeiss LSM image browser.

**Cytokine production and release by NHBE cells**

NHBE cells were seeded (3 × 10⁴ cells/well) in a 24-well tissue culture plate and grown until 80% confluence, usually 4 d. The cells were then stimulated with Alternaria extract (50 μg/ml), Oriental cockroach extract (50 μg/ml), zymosan (200 μg/ml), poly(I:C) (10 μg/ml), LPS (1 μg/ml), ionomycin (1 μM), or thapsigargin (3 μM) for 2 h (for IL-33 release) or 8 h (for IL-6 production). Cell-free supernatants were collected and analyzed for IL-6 or IL-33 by ELISA (R&D Systems). Lactate dehydrogenase (LDH) activity in the supernatants was measured using a cytotoxicity detection kit (Roche Applied Science). In some experiments, NHBE cells were preincubated with apyrase (30 U/ml), oATP (100 μM), or suramin (300 μM) for 30 min at 37˚C before adding *Alternaria* extract. Total cellular IL-33 was recovered with five freeze/thaw cycles or treating cells with 0.5% Nonidet P-40.

**Gene knockdown in NHBE cells**

When 30–50% confluent, NHBE cells were transfected with small interfering RNAs (siRNAs) targeting P2X2-R or P2Y2-R or control RNA at 5 nM using HiPerFect transfection reagent (Qiagen). The transfected cells were grown for 48 h and then stimulated with Alternaria extract (50 μg/ml) for 2 h. Target gene knockdown was verified by examining expression of P2X2-R and P2Y2-R mRNA by real-time RT-PCR. Briefly, total RNA was purified with TRIzol (Invitrogen); DNase digestion used DNase I amplification grade (Invitrogen). cDNAs were synthesized from 1 μg purified RNA using an iScript CDNA synthesis kit (Bio-Rad). The reaction used incubation at 45˚C for 5 min and was stopped by heating to 85˚C for 5 min. The real-time RT-PCR reactions contained 1 μl CDNA, 12.5 μl TaqMan Universal PCR Master Mix, and 1.25 μl TaqMan gene expression assay of the target genes: P2X2-R (Hs01175721_m1), P2Y2-R (Hs01856611_s1), and 18S (Hs099999901_s1) (18S; as an endogenous control) (Applied
BioBiosystems); the final reaction volume was 20.0 μl with sterile water. Amplification and detection of specific products were performed using the IQ5 multicolor real-time PCR detection system (Bio-Rad). The protocol was as follows: denaturation by a hot start at 95°C for 10 min, followed by 40 cycles of a two-step program (denaturation at 95°C for 15 s and annealing/extension at 60°C for 1 min). Transcription was normalized to the 18S rRNA transcription.

**Immunoblot for IL-33**

NHBE cells (1 × 10^5 cells/ml) were lysed with RIPA lysis buffer (Santa Cruz Biotechnology). Culture supernatants from NHBE cells, which were treated with five freeze/thaw cycles (45) or stimulated with Alternaria extract for 2 h, were also obtained. Cell lysates and supernatants were concentrated by lyophilization. The samples were boiled for 10 min and aliquots equivalent to 1.3 × 10^5 cells were electrophoresed on a 10% NuPAGE Novex Bis-Tris gel with MOPS buffer (Invitrogen). After protein transfer, the nitrocellulose membrane was cut, blocked in milk buffer, and incubated with normal rabbit IgG or rabbit anti-human IL-33 (1 μg/ml). HRP-conjugated goat anti-rabbit IgG was used for detection, and the blot was developed using an ECL kit (Amersham Pharmacia Biotech), followed by exposure to BioMax film (Kodak).

**Cell viability assays**

Viability of NHBE cells was examined with the LIVE/DEAD cellular viability/cytotoxicity kit (Invitrogen) that uses calcine acetoxymethyl (calcine AM) and ethidium homodimer-1 (EthD-1) dyes to detect active esterase and compromised membrane integrity, respectively. After incubation with medium or Alternaria extract (50 μg/ml) for 2 or 8 h, NHBE cells were incubated with 2 μM calcine AM and 4 μM EthD-1 for 30 min at room temperature. Using fluorescence microscopy, intact (calcine AM positive and EthD-1 negative) and damaged (EthD-1 positive) cells in five randomly chosen fields were counted and expressed as percentage of cells over the total cells (≥500 cells were counted).

**Measurement of [Ca2+]i**

NHBE cells were seeded at low density on coverslip chamber slides for 48–72 h in BEGM. In some experiments, cells were treated with P2X-R siRNA, P2Y2R siRNA, or control RNA, as described above. To measure calcium, the BEGM was replaced with HBSS containing 10 mM HEPES buffer (pH 7.4), and the cells were loaded with 10 μM fura 2-AM (Invitrogen) for 90 min, washed in HBSS, and mounted onto the stage of a Nikon Diaphot inverted microscope with an epifluorescence attachment. Fluorescence in single cells was visualized using a Nikon ultraviolet Fluor 3 × 20 or ×40 oil-immersion objective. The fluorescence excitation, image acquisition, and real-time data analyses were controlled by Image-1 Metamorph software (Universal Imaging). [Ca2+]i was measured as the ratio of fluorescence emitted at 510 nm when the cells were alternately excited at 340 and 380 nm. Alternaria extract and P2R antagonists were introduced by single-pass, continuous-flow perfusion. [Ca2+]i was calculated using the fura 2-AM calcium imaging calibration kit (Invitrogen).

**ATP measurements in cell culture media**

To measure ATP levels in cell culture supernatants, NHBE cells were treated with medium alone, Alternaria extract (50 μg/ml), or Oriental cockroach extract (50 μg/ml) for periods ≤32 min in a 24-well tissue culture plate. Cell culture media was collected and immediately placed on ice. ATP concentrations were measured with an ATP determination kit (Bioassy Systems) and a luminometer, following the manufacturer’s instructions.

**CD4+ T cell cytokine production in response to NHBE cell lysates**

Isolated CD4+ T cells were cultured with BM-derived DCs and cell lysates as previously described (29). Briefly, CD4+ T cells isolated from spleens of BALB/c mice were seeded at 1 × 10^5 cells/ml in round-bottom 96-well tissue culture plates and incubated with bone marrow-derived DCs from BALB/c mice at a 1:10 DC/T cell ratio with medium or with a 10% freeze/thaw lysate of NHBE cells for 10 d. To block the IL-33–ST2 pathway, goat anti-human IL-33 (10 μg/ml), goat anti-mouse ST2 Ab (10 μg/ml), or normal goat IgG (control Ab, 10 μg/ml) were included. Supernatants were collected and concentrations of IL-5, IL-13, and IFN-γ were measured by ELISA (R&D Systems).

**Statistical analysis**

All data are reported as the mean ± SEM from the indicated number of replicates. Two-sided differences between two samples were analyzed with the Mann–Whitney U test or Student t test. Multiple comparisons between treatment and control conditions were performed by ANOVA. The p values < 0.05 were considered significant.

**Results**

*Exposure to Alternaria induces IL-33 release and Th2 cytokine production in mouse airways*

To investigate the immunological mechanisms of asthma and Th2-type airway immunity, we used a naive mouse model and administered *Alternaria* extract (50 μg/dose) into the airways of mice. A bacterial product and TLR4 agonist, namely LPS (1 μg/dose), acted as control. The *Alternaria* extract contained minimal endotoxin (3 ng/mg, 0.15 μg/dose). Six hours later, Th2 cytokine levels, including IL-5 and IL-13, were increased in *Alternaria*-exposed lungs of BALB/c mice (Fig. 1A); by 24 h, these cytokine levels decreased. IL-5 and IL-13 levels did not increase in LPS-exposed mice. IL-4 was undetectable in LPS- and *Alternaria*-exposed BALB/c mice. Interestingly, within 1 h after receiving *Alternaria* extract, IL-33 levels increased markedly in BAL fluids, preceding the increases in IL-5 and IL-13; LPS did not induce IL-33 in BAL fluids. After receiving LPS, IL-1β levels in BAL fluids increased from 6 to 24 h; *Alternaria* induced small increases in IL-1β levels. Similarly, in C57BL/6 mice (Fig. 1B), IL-33 levels increased markedly in BAL fluids within 1 h after receiving *Alternaria* extract; the levels of IL-5 and IL-13 were increased by 6 and 12 h, respectively. Thus, respiratory exposure to the fungus increases airway IL-33 levels that are followed by increases in Th2 cytokines in both BALB/c and C57BL/6 mice; LPS increases IL-1β levels, but not IL-33 or Th2 cytokines.
To examine the role of IL-33 in Th2 cytokine responses, we used mice deficient in the IL-33 receptor T1/ST2 (ST2\(^{-/-}\)) (46). Alternaria-induced increases in IL-5 and IL-13 were reduced to baseline levels (i.e., PBS) in ST2\(^{-/-}\) mice (Fig. 2A), but increases in IL-6 or IL-33 were not affected. The production of IL-5 and IL-13 in Alternaria-exposed mice was similar in Rag1\(^{-/-}\) and wild-type mice (Fig. 2B), suggesting that these Th2-type cytokine responses are independent of adaptive immunity. The production of IL-33 was not significantly affected in Rag1\(^{-/-}\) mice. An adaptor protein, MyD88, mediates signal transduction by most TLRs (47); MyD88 is also required for signaling of IL-1 family cytokines, such as IL-1\(\beta\) and IL-33 (48). Exposing MyD88\(^{-/-}\) mice to Alternaria extract abolished IL-5 and IL-13 production (Fig. 2C), consistent with the potential involvement of IL-33, but the increase in BAL IL-33 was not affected. Thus, when mice are exposed to Alternaria, the MyD88-independent release of IL-33 likely mediates production of Th2-type cytokines in the airway mucosa through an innate mechanism or mechanisms.

Biologically active full-length IL-33 is localized in nuclei of airway epithelial cells

In resting conditions, epithelial cells in mucosal organs and vascular endothelial cells are probably the dominant cells expressing and storing IL-33 in their nuclei (15, 16). We used immunohistochemistry to verify localization of IL-33 in the nuclei of airway epithelial cells in human nasal tissues, especially in basal layer cells (Fig. 3A). To dissect the roles of epithelial cell-derived IL-33 in Th2-type immune responses, we cultured NHBE cells. By double-staining NHBE cells for IL-33 and DAPI, IL-33 was mainly localized in each nucleus with faint staining in the cytoplasm (Fig. 3B). As a control, we used HMGB1 protein (45), which is a prototypic nuclear alarmin (49). HMGB1 localized in the nuclei of NHBE cells (Fig. 3B). Immunoblot analysis revealed that IL-33 in NHBE cell lysates migrated as a 30- to 31-kDa band (Fig. 3C), suggesting that it is the full-length proform and unprocessed by caspase-1 (33–35). To examine whether this full-length IL-33 is biologically active, we incubated cocultures of
CD4+ T cells and DCs with NHBE cell lysates. These lysates induced production of IL-5, IL-13, and IFN-γ by the CD4+ T cells (Fig. 3D). Treatment with the lysate and anti-ST2 (i.e., IL-33 receptor) Ab or anti–IL-33 Ab abolished IL-5 production (p < 0.01, n = 4); these Abs partially inhibited IL-13 production (p < 0.05, n = 4). In contrast, IFN-γ production was unaffected, implicating roles for non–IL-33 molecules (e.g., HMGB1, uric acid) in the lysates that promote IFN-γ production.

**IL-33 is released extracellularly when airway epithelial cells are exposed to allergen extracts, but not to TLR agonists**

To examine whether NHBE cells release nuclear IL-33 extracellularly after innate immunological stimulation, we incubated cells with extracts of common airborne allergens, *Alternaria* and Oriental cockroach (38, 50), or with TLR ligands, including zymosan (TLR2 ligand), poly(I:C) (TLR3 ligand), and LPS (TLR4 ligand). Immunoreactive IL-33 was released rapidly and reached a plateau by 2 h (Fig. 4B); in contrast, IL-6 production did not reach a plateau by 8 h. NHBE cells incubated with *Alternaria* extract, IL-33 was released rapidly and reached a plateau by 2 h (Fig. 4B); in contrast, IL-6 production did not reach a plateau by 8 h. NHBE cells incubated with *Alternaria* or Oriental cockroach extracts, IL-33 levels increased in the supernatants (p < 0.01); in contrast, exposure to various TLR ligands did not induce IL-33 release. When NHBE cells were incubated with zymosan or poly(I:C) for 8 h, they produced high IL-6 levels (p <

**FIGURE 3.** Biologically active full-length 30-kDa IL-33 protein is stored in nuclei of airway epithelial cells. A, Nasal specimens from 12 normal individuals were stained with rabbit anti-human IL-33 or normal rabbit IgG. Results shown are representative. Scale bars, 50 μm. Insets show higher magnifications of the epithelium. B, NHBE cells were cultured, fixed, permeabilized, and stained with rabbit anti-human IL-33 (upper panels) or rabbit anti-HMGB1 (lower panels), followed by FITC-conjugated goat anti-rabbit IgG. After mounting and staining with a nuclear staining dye, DAPI, cells were visualized by confocal microscopy. Left panels show FITC-images with anti–IL-33 or anti-HMGB1; middle panels show nuclear staining with DAPI (pseudocolored); right panels show overlays of left and middle panels. Results are representative of four independent experiments. Scale bar, 20 μm. C, Immunoblot analysis of NHBE cell lysate (left lane) and cell culture supernatant (right lane) used anti-human IL-33. Results are representative of three independent experiments. D, Isolated CD4+ T cells were cultured with DCs at a 1:10 DC/T cell ratio with medium alone or with a 10% freeze/thaw lysate of NHBE cells for 10 d. To block the IL-33–ST2 pathway, goat anti-human IL-33, goat anti-mouse ST2, or normal goat IgG was included. Concentrations of IL-5, IL-13, and IFN-γ were measured in supernatants by ELISA. Bars represent the mean ± SEM (six independent experiments). *p < 0.05, **p < 0.01 compared with the samples with lysate but without Abs by Mann–Whitney U test.
FIGURE 4. IL-33 is released extracellularly in a noncytotoxic manner by NHBE cells exposed to allergen extracts. A, NHBE cells were stimulated with medium (upper panels), Alternaria extract (left and middle lower panels), or poly(I:C) (right lower panel) for 4 h. Cells were stained and analyzed as in Fig. 3. In upper row, left panel shows FITC images with anti–IL-33, middle panel shows nuclear staining with DAPI (pseudocolored), and right panel shows overlay of left and middle panels. In lower row, left panel shows overlay of anti–IL-33 and DAPI images, middle panel shows overlay of anti-HMGB1 and DAPI images, and right panel shows overlay of anti–IL-33 and DAPI images. Results are representative of four independent experiments. Original magnification ×1000. B, NHBE cells were incubated with medium alone or Alternaria extract (50 μg/ml) for up to 8 h. IL-33 and IL-6 concentrations in the cell-free supernatants were analyzed by ELISA. Error bars represent the mean ± SEM (six independent experiments). *p < 0.05, **p < 0.01 compared with cells stimulated with medium alone by Mann–Whitney U test. C, NHBE cells were stimulated with medium, Alternaria extract, Oriental cockroach extract, zymosan, poly(I:C), or LPS for 2 (for IL-33 release, left panel) or 8 h (for IL-6 production, right panel). Cell-free supernatants were analyzed for IL-6 or IL-33 by ELISA. Bars represent the mean ± SEM (six independent experiments). *p < 0.05, **p < 0.01 compared with medium alone by Mann–Whitney U test. D, Culture supernatants from NHBE cells, which were treated with five freeze/thaw cycles (left lane) or stimulated with Alternaria extract for 2 h (right lane), were analyzed by immunoblot with anti-human IL-33. Results are representative of three independent experiments. E, NHBE cells were stimulated with medium or Alternaria extract for 2 h or treated with five freeze/thaw cycles or 1% Nonidet P-40. Cell-free supernatants were analyzed for LDH activity and for IL-33. Bars represent the mean ± SEM (six independent experiments). **p < 0.01 compared with medium alone by Mann–Whitney U test. F, NHBE cells were incubated with medium or Alternaria extract for 2 or 8 h and then incubated with calcein AM and EthD-1 for 30 min. Upper
when cells were incubated with *Alternaria*, Oriental cockroach, or LPS, they produced low levels of IL-6 (p < 0.05). The IL-33 in the NHBE cell supernatants migrated as a 30- to 31-kDa band (Fig. 4D), suggesting that IL-33 is released as a noncleaved, full-length protein. Thus, IL-33 and IL-6 are released from NHBE cells by allergens and TLR ligands, respectively.

IL-33 has been considered an alarmin that is released during cellular injury or necrotic cell death (33, 36). To address whether injury was responsible for extracellularly released IL-33 induced by *Alternaria*, we used indicators of cell membrane integrity, including LDH activity in cell-free supernatants and staining with the membrane-impermeable nucleic acid dye, EthD-1. When NHBE cells were exposed to medium or to *Alternaria* extract for 2 h, LDH activity was undetectable in the supernatants (Fig. 4E); when the cells were treated with freeze/thaw cycles or were exposed to a detergent, Nonidet P-40, LDH release was robust. No differences were observed in the permeability to EthD-1 between the cells incubated with medium alone or those incubated with *Alternaria* extract for ≤8 h (Fig. 4F). About 90% of NHBE cells were also alive with medium alone or *Alternaria* extract, as judged by conversion of calcein AM to fluorescent calcein. Overall, when NHBE cells are exposed to *Alternaria*, stored IL-33 was rapidly and actively released without apparent cellular injury.

Allergen exposure increases [Ca2+]i, through extracellular release of ATP

We next addressed how airway epithelial cells might “sense” the allergens and release stored IL-33. Under stress conditions, many plant and animal cells release ATP via lytic or nonlytic mechanisms, and extracellular ATP can be a danger signal and a mediator of inflammation (51). When we exposed NHBE cells to fresh medium, within 60 s after exchange of medium we detected ATP in the cell supernatants (Fig. 5A). ATP levels returned to baseline levels within 4 min, likely because of ecto-ATPase activity (52). Importantly, when we added allergen extracts (e.g., *Alternaria* and Oriental cockroach) to NHBE cells, the peak extracellular ATP levels were ~2.5-fold higher than with medium alone or *Alternaria* extract, as judged by conversion of calcein AM to fluorescent calcein. Overall, when NHBE cells are exposed to *Alternaria*, stored IL-33 was rapidly and actively released without apparent cellular injury.

ATP-mediated increases in [Ca2+]i, induce IL-33 release

To examine the roles of ATP-induced [Ca2+]i increases in IL-33 release, NHBE cells were incubated with *Alternaria* extract in the presence of the Ca2+ chelators EGTA or BAPTA-AM. By immunohistochemistry, EGTA treatment blocked the *Alternaria*-induced transport of IL-33 from nucleus to cytosol (Fig. 7A). EGTA and BAPTA-AM inhibited the *Alternaria*-induced IL-33 release into the extracellular media (p < 0.01 and p < 0.05, respectively, n = 5), whereas basal IL-33 release with medium was unaffected (Fig. 7B). Conversely, to examine whether increases in [Ca2+]i are sufficient to induce IL-33 release, NHBE cells were incubated with a calcium ionophore, ionomycin, or with a Ca2+-ATPase inhibitor, thapsigargin, which depletes Ca2+ from reticular stores, leading to the activation of Ca2+ channels and Ca2+ entry (59). Ionomycin treatment localized IL-33 to cytoplasmic organelles with an apparent vesicular structure (Fig. 7C); in contrast, ionomycin did not affect the localization of nuclear HMGB1. Thapsigargin treatment showed IL-33 localization in the cytoplasm similar to ionomycin and did not affect localization of HMGB1. Furthermore, IL-33 was detected in the supernatants of NHBE cells treated with ionomycin or thapsigargin (p < 0.05) (Fig. 7D). Thus, increased [Ca2+]i is likely necessary and sufficient to release IL-33 from NHBE cells. Importantly, treatment of NHBE cells with wide-spectrum inhibitors for P2 purinergic receptors (i.e., oATP and suramin) (60) or enzymatic removal of ATP from the incubation medium by exogenous ATPase (i.e., apyrase) abrogated *Alternaria*-induced extracellular release of IL-33, suggesting critical roles for ATP and P2 purinergic receptors in IL-33 release (Fig. 7E). Indeed, depletion of specific transcripts for P2X7R or P2YR by siRNA inhibited IL-33 release from NHBE cells in response to *Alternaria* (p < 0.05, n = 5) (Fig. 7F).

**ATP mediates IL-33 release and Th2-type innate immune responses in the airways of mice exposed to Alternaria**

To test the importance of ATP as an innate sensor for allergen exposure in vivo, we used an airway model that exposed nonsensitized naive mice to *Alternaria*. As shown in Fig. 1 above, intranasally administered *Alternaria* extract rapidly increased BAL IL-33 within 1 h, followed by increased levels of lung IL-5 and IL-13 from innate sources by 6 h. We now used this model to
FIGURE 5. Exposure of NHBE cells to allergen extracts induces extracellular ATP release and increases [Ca^{2+}]. A, NHBE cells were treated with medium, *Alternaria*, or Oriental cockroach extracts. The kinetics of ATP concentrations in cell-free supernatants were measured. Bars represent the mean ± SEM (four independent experiments). *p < 0.05, **p < 0.01 compared with medium alone by Mann–Whitney U test. B, NHBE cells were loaded with fura 2-AM and exposed to *Alternaria* extract. Fluorescence in single cells was monitored and analyzed by inverted microscopy, as described in Materials and Methods. Upper panels show [Ca^{2+}], in NHBE cell monolayer with pseudocolours (green < yellow < orange < red < white in the order of [Ca^{2+}]). Left panel shows before exposure, and right panel shows 300 s after exposure to *Alternaria*. Original magnification ×400. Lower panels use pseudocolours to show the kinetic changes in [Ca^{2+}], in a randomly selected NHBE cell. Images 1 through 6 were taken at 285, 293, 301, 317, 333, and 347 s after exposure to *Alternaria*. Results are representative of five independent experiments. Original magnification ×1000. C, NHBE cells were loaded with fura 2-AM, preincubated with or without EGTA, exposed to *Alternaria* extract (arrow), and 600 s later 10 μM UTP (arrow) was added to the cells with EGTA. Fluorescence and [Ca^{2+}], in single cells were analyzed as described above. Left panel shows kinetic changes in [Ca^{2+}], in one experiment from 50 randomly selected cells, representative of three experiments. Right panel shows [Ca^{2+}], before (basal) and 400 s after exposure to *Alternaria*. Bars represent the mean ± SEM (three independent experiments each with 50 cells). **p < 0.01 compared with basal [Ca^{2+}], by Student t test.

Examine the effects of blocking of P2 purinergic receptors in vivo with the same inhibitors as in the in vitro studies. Intranasally administered 0ATP plus *Alternaria* inhibited the *Alternaria-*induced increases in BAL IL-33 levels (p < 0.05) (Fig. 8A) and reduced both IL-5 and IL-13 production in the lungs (p < 0.05) (Fig. 8B). Similarly, mice exposed intranasally to *Alternaria* plus suramin showed minimal IL-33 release and IL-5 and IL-13 production.

To determine whether deficiency in one of the P2 purinergic receptors is sufficient to modulate the innate Th2-type response to *Alternaria*, we administered *Alternaria* extract into the airways of *P2X7*−/− and *P2Y2*−/− mice (Fig. 8C). Production of both IL-5 and IL-13 was not affected in *P2X7*−/− mice. In contrast, production of IL-5 in the lungs was reduced in *P2Y2*−/− mice by 90% as compared with wild-type mice (p < 0.01). Similarly, IL-13 levels decreased by 70% in *P2Y2*−/− mice (p < 0.05). Thus, disrupting the ATP signaling pathway at the receptor level impairs allergen-induced IL-33 release and subsequent Th2-type immune responses in the airway mucosa.

**Discussion**

IL-1 family cytokines (e.g., IL-1β, IL-33) may be sorted into two groups, depending on their production, regulation, extracellular release, and their cellular sources (11). Pro–IL-1β is synthesized when monocytes or macrophages are exposed to microbial or tissue injury products, and it is released extracellularly after proteolytic processing to mature IL-1β by caspase-1. In contrast, IL-33 is constitutively expressed and stored in the nucleus of epithelial and endothelial cells. IL33 and IL1RL1 (i.e., ST2) genes have been implicated in asthma in a recent large-scale genome-wide association scan study (61). Increased expression of IL-33 by epithelial cells is also observed in patients with asthma (62). However, the molecular mechanisms involved in IL-33 production and release remain enigmatic. The major finding in this report is that when airway epithelial cells are exposed to environmental allergens, they actively and rapidly release IL-33. Sustained increases in [Ca^{2+}], are sufficient and necessary to translocate and release IL-33. Furthermore, extracellular accumulation of ATP appears pivotal in regulating the Ca^{2+} response and subsequent IL-33 release. In mice exposed to an environmental allergen, in vivo blockade of the P2 purinergic receptor pathways inhibited IL-33 release and Th2 cytokine production, suggesting biological significance of the ATP-mediated IL-33 release. Our findings are consistent with reports that showed biological activity for unprocessed full-length pro–IL-33 and showed that the processing and release of IL-33 probably does not involve caspase-1 (35).
Thus, airway epithelial cells may use ATP to sense noncytolytic stress in the air we breathe and to initiate Th2-type immune responses by releasing preformed, full-length IL-33.

Previous models proposed IL-33 as an alarmin, which is released during necrotic cell death associated with tissue damage or infection, similarly to a prototypic alarmin, HMGB1 (36). In theory, potential cytotoxic effects of allergen extracts might release IL-33 from airway epithelial cells. However, direct toxic effects appear unlikely because cell membrane integrity was not compromised after cells were exposed to *Alternaria* extract and after they released IL-33. Pharmacological inhibitors of P2 purinergic receptors and Ca\(^{2+}\)-chelating agents also abolished IL-33 release in vitro and in vivo.

Extracellular ATP serves as a danger signal to alert the immune system of tissue stress or damage (63). Increased levels of ATP are observed in BAL fluids of patients with asthma or sensitized mice challenged with an Ag (64). Neutralization of ATP inhibits Th2 sensitization to inhaled Ags and ongoing Th2-type airway inflammation (64). We also found that exposure to Aeroallergens induces rapid extracellular release of ATP (Fig. 5), and neutralization of ATP or blocking of the P2 purinergic pathway inhibits IL-33 release and early innate Th2 type immune responses to an

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**FIGURE 6.** Blockade of P2 purinergic receptors by pharmacologic agents or siRNA attenuates Ca\(^{2+}\) response in NHBE cells exposed to *Alternaria* extract. A, NHBE cells were loaded with fura 2-AM, preincubated with or without 300 µM oATP or 50 µM PPADS, and exposed to *Alternaria* extract (see arrows). NHBE cells were fixed, permeabilized, and stained with rabbit anti-P2X7R or anti-P2Y2R, followed by FITC-conjugated goat anti-rabbit IgG. After mounting and staining with DAPI, cells were visualized by confocal microscopy. Results are representative of three independent experiments. Scale bar, 20 µm. C, NHBE cells were transfected with siRNA against P2X7R or P2Y2R or control RNA and grown for 48 h. Expression levels of P2X7R and P2Y2R mRNA by NHBE cells were examined by real-time RT-PCR. In replicate wells, transfected NHBE cells were loaded with fura 2-AM and exposed to *Alternaria* (see arrows). Fluorescence and [Ca\(^{2+}\)]\(_i\) in single cells were analyzed as in Fig. 4. Left upper panels show expression of P2X7R and P2Y2R mRNA. Data were normalized to the amounts of P2X7R and P2Y2R mRNA in NHBE cells without siRNA transfection as 100%. Results are representative of three experiments. Left lower panels show kinetic changes in [Ca\(^{2+}\)]\(_i\) in one experiment from 50 randomly selected cells, representative of three experiments. Right panel shows [Ca\(^{2+}\)]\(_i\) before (basal) and 400 s after exposure to *Alternaria*. Bars represent the mean ± SEM (three independent experiments each with 50 cells). **p < 0.01 compared with [Ca\(^{2+}\)]\(_i\) after exposure to *Alternaria* in the cells treated with control siRNA by ANOVA.
inhaled allergen (Figs. 7, 8). Taken together, these findings suggest that extracellular ATP plays pivotal roles in various stages of Th2-type immune responses and inflammation in the airways.

By immunohistochemistry, both HMGB1 and IL-33 were localized in the nuclei of NHBE cells (Fig. 3). However, HMGB1 did not translocate to the cytoplasm when epithelial cells were exposed to Alternaria extract or to ionomycin, suggesting that the Ca\textsuperscript{2+} signal is unlikely to regulate HMGB1 release. Activated macrophages secrete HMGB1 after hyperacetylation of lysine residues (65), and necrotic cells release HMGB1 during cellular damage (45). Thus, distinct intracellular mechanisms may regulate the extracellular release of alarmin-like proteins, and different classes of molecules may be released depending on the cells’ conditions or cell types. Generally, in vivo cell necrosis triggers an acute inflammatory response by releasing DAMPs (66). Our freeze/thaw airway epithelial cell lysates were highly immunogenic and induced production of Th1 (IFN-γ) and Th2 cytokines (IL-5, IL-13) in the DC and CD4\textsuperscript{+} T cell coculture system. Thus,

**FIGURE 7.** ATP-mediated increase in [Ca\textsuperscript{2+}], is necessary and sufficient to translocate and release nuclear IL-33. A, NHBE cells were preincubated without (left panel) or with (right panel) 1 mM EGTA and stimulated with Alternaria extract for 4 h. The cells were stained with anti-human IL-33 and DAPI and analyzed as in Fig. 2. Panels show overlay of anti–IL-33 and DAPI images and are representative of four independent experiments. Original magnification ×1000. B, NHBE cells were preincubated with medium, EGTA, or BAPTA-AM and stimulated with medium or Alternaria extract for 2 h. IL-33 in cell-free supernatants was analyzed by ELISA. Bars represent the mean ± SEM (six independent experiments). *p < 0.05, **p < 0.01 compared with cells stimulated with Alternaria without inhibitors by Mann–Whitney U test. C, NHBE cells were treated with medium (left), ionomycin (middle), or thapsigargin (right) for 4 h. The cells were stained with anti-human IL-33 (upper panels) or anti-HMGB1 (lower panels) and DAPI and analyzed as in Fig. 2. Upper panels show overlay of anti–IL-33 and DAPI, and lower panels show overlay of anti-HMGB1 and DAPI. Results are representative of four independent experiments. Original magnification ×1000. D, NHBE cells were incubated with medium, ionomycin, or thapsigargin for 2 h. IL-33 in cell-free supernatants was analyzed by ELISA. Bars represent the mean ± SEM (four independent experiments). *p < 0.05 compared with cells incubated with medium alone by Mann–Whitney U test. E, NHBE cells were preincubated with medium, oATP, suramin, or apyrase and stimulated with medium or Alternaria extract for 2 h. IL-33 in cell-free supernatants was analyzed by ELISA. Bars represent the mean ± SEM (six independent experiments). *p < 0.05 compared with cells stimulated with Alternaria without inhibitors by Mann–Whitney U test. F, NHBE cells were untreated (control) or transfected with siRNA against P2X7R or P2Y2R or control siRNA and cultured for 48 h. Cells were stimulated with medium or Alternaria extract for 2 h. IL-33 in cell-free supernatants was analyzed by ELISA. Bars represent the mean ± SEM (six independent experiments). *p < 0.05 compared with control siRNA-treated cells stimulated with Alternaria extract by Mann–Whitney U test.
because aeroallergens induced ATP release, but no apparent cellular injury, they may trigger IL-33 release without DAMP release, resulting in a Th2-type immune response. Conversely, massive cellular damage to airway epithelial cells by microbial infection or toxic chemicals may release DAMPs as well as IL-33, resulting in Th1 and Th2 responses or a Th1-dominant response. The selective release of alarmin-like molecules from the airway epithelium appears to modulate the magnitude and nature of inflammation.

IL-33 release by airway epithelial cells may involve different innate immunological stimuli other than conventional TLRs. In this study, while TLR agonists induced IL-6 production by airway epithelial cells, TLR agonists in vitro or LPS in vivo did not induce IL-33 release. Furthermore, in mice exposed to Alternaria, IL-33 release was induced even in MyD88-deficient mice. Proinflammatory stimuli, such as IL-1β and TNF-α, inhibit transcription of IL-33 in endothelial cells (16). Thus, IL-33 production and release are independent of, or rather negatively regulated by, the TLRs and the NF-κB signaling pathway. Instead, in airway epithelial cells, the ionomycin- or thapsigargin-induced increases in [Ca^{2+}], were sufficient to translocate IL-33 from nucleus to cytoplasm and to the extracellular milieu. In human embryonic kidney (HEK-293) cells transfected with IL-1β genes, sustained increases in [Ca^{2+}], produced rapid secretion of mature IL-1β and unprocessed pro–IL-1β (67). In monocytes and macrophages, Ca^{2+} was involved in P2X7R-mediated secretion of mature IL-1β, while K+ efflux was involved in enzymatic processing of pro–IL-1β to mature IL-1β (67). Thus, the Ca^{2+}-dependent secretory pathway/apparatus for IL-1 family cytokine release may be ubiquitously expressed regardless of the cell type, and increases in [Ca^{2+}], may be critical in secretion, but not processing, of IL-1 family molecules. Because IL-33 requires no proteolytic processing by caspase-1, the increases in [Ca^{2+}], may be sufficient to mobilize and release IL-33 from airway epithelial cells.

As potential therapeutic targets, the molecules playing major roles in Ca^{2+} signal regulation and IL-33 release are of interest. We found that ATP is released within 60 s when airway epithelial cells are exposed to aeroallergens. Generally, ATP is stored at millimolar concentrations within cells, and disrupting plasma mem-

FIGURE 8. Administration of P2 purinergic receptor antagonists or deficiency in P2Y2 gene attenuates IL-33 release and innate Th2-type responses in mice. A, BALB/c mice received intranasal PBS or Alternaria extract mixed with PBS, αATP, or suramin. After 1 h, mice were killed, and IL-33 in BAL fluid supernatants was measured by ELISA. Bars represent the mean ± SEM (five mice per group). *p < 0.05 compared with mice receiving Alternaria without inhibitors by Mann–Whitney U test. B, BALB/c mice received intranasal PBS or Alternaria extract mixed with PBS, αATP, or suramin. After 12 h, mice were killed, and IL-5 and IL-13 in lung homogenates were measured by ELISA. Bars represent the mean ± SEM (five mice per group). *p < 0.05 compared with mice receiving Alternaria without inhibitors by Mann–Whitney U test. C, Wild-type C57BL/6 mice, P2X7−/−, or P2Y2−/− mice received intranasal PBS or Alternaria. After 12 h, mice were killed and IL-5 and IL-13 in lung homogenates were measured by ELISA. Error bars represent the mean ± SEM (six mice per group). *p < 0.05, **p < 0.01 compared with wild-type mice receiving Alternaria by Mann–Whitney U test.
bran integrity leads to ATP extracellular release (68). In both animals and plants, ATP is also released in noncytolytic conditions, such as membrane deformation, mechanical stress, osmotic stress, and exposure to proteases (69–71). Thus, ATP can be used to communicate tissue distress or injury to the immune system, promoting appropriate responses, for example, inflammation, restoration of homeostasis, and adaptation to the altered cellular conditions or environment. Activation of P2X7-R by ATP facilitates the rapid influx of extracellular Na+ and Ca2+ and efflux of intracellular K+ (72). In optic nerve glia, low ATP concentrations mediate the increase in [Ca2+]i through P2Y2-R and, at high concentrations, P2X7-R is also activated, resulting in robust and sustained increases in [Ca2+]i (73). In our study of human airway epithelial cells, both P2X7-R and P2Y2-R were involved in the Ca2+ response and IL-33 release in vitro. Broad-spectrum inhibitors of P2 purinergic receptors and mice deficient in the P2Y2-R gene showed a marked inhibition of innate Th2 cytokine production in response to Alternaria in vivo. Thus, the P2Y2-R and perhaps the P2X7-R play major roles in detecting the danger signal, extracellular ATP, and in triggering IL-33 release and the subsequent Th2-type responses. Some caution may be necessary to interpret the roles of specific receptors, however. Whereas the involvement of P2Y2-R was observed in both human epithelial and mouse in vivo models, there appears to be discrepancy regarding the involvement of P2X7-R. Gene-deficient mice might have unknown immunological abnormalities that we may not be aware of, or they might compensate for the deficiency in one gene (e.g., P2X7-R) by upregulating other genes. The coupling of receptors to their effector molecules may be different between mice and humans. Furthermore, the mouse in vivo system may involve other cell types more than airway epithelial cells, which may overcome the deficiency in one cell type. Any of these mechanisms may produce the different observations between the mouse and human models.

We were surprised by the rapid (by 12 h after allergen exposure) production of IL-5 and IL-13 in the lungs of naive mice; this cytokine response was independent of adaptive immunity. Endogenous IL-33 and its receptor, ST2, mediated this “innate” Th2 cytokine response. Robust IL-6 production was also observed within 12 h of Alternaria exposure (Fig. 2), suggesting that this cytokine may be involved in Th2-type immune responses of the airways to inhaled allergens (74). A major question remains regarding the cellular source of innate IL-5 and IL-13. Recently, exogenous IL-25 and IL-33 or infection with helminths induced proliferation of novel non-T/non-B cells that produced abundant exogenous IL-25 and IL-33 or infection with helminths induced proliferation of novel non-T/non-B cells that produced abundant cytokine that signals via the IL-1 receptor-related protein ST2 and induces T helper cell type 2 responses to inhaled antigen. J. Exp. Med. 196: 1645–1651.


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