# *Alternaria*-derived serine protease activity drives IL-33 mediated asthma exacerbations☆

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**Background: The fungal allergen *Alternaria alternata* is implicated in severe asthma and rapid onset life-threatening exacerbations of disease. However, the mechanisms that underlie this severe pathogenicity remain unclear.**
**Objective: We sought to investigate the mechanism whereby *Alternaria* was capable of initiating severe, rapid onset allergic inflammation.**
**Methods: IL-33 levels were quantified in wild-type and ST2$^{-/-}$ mice that lacked the IL-33 receptor given inhaled house dust mite, cat dander, or *Alternaria*, and the effect of inhibiting allergen-specific protease activities on IL-33 levels was assessed. An exacerbation model of allergic airway disease was established whereby mice were sensitized with house dust mite before subsequently being challenged with *Alternaria* (with or without serine protease activity), and inflammation, remodeling, and lung function assessed 24 hours later.**
**Results: *Alternaria*, but not other common aeroallergens, possessed intrinsic serine protease activity that elicited the rapid release of IL-33 into the airways of mice through a mechanism that was dependent upon the activation of protease activated receptor-2 and adenosine triphosphate signaling. The unique capacity of *Alternaria* to drive this early IL-33 release resulted in a greater pulmonary inflammation by 24 hours after challenge relative to the common aeroallergen house dust mite. Furthermore, this *Alternaria* serine protease–IL-33 axis triggered a rapid, augmented inflammation, mucus release, and loss of lung function in our exacerbation model.**
**Conclusion: *Alternaria*-specific serine protease activity causes rapid IL-33 release, which underlies the development of a robust $T_H2$ inflammation and exacerbation of allergic airway disease. (J Allergy Clin Immunol 2014;■■■:■■■-■■■.)**

***Key words:*** Alternaria alternata*, allergic airway disease, asthma exacerbation, protease, IL-33*

*Abbreviations used*
AEBSF: 4-(2-Aminoethyl)benzenesulfonyl fluoride hydrochloride
ALT: *Alternaria*
BALF: BAL fluid
CAT: Cat dander
HDM: House dust mite
MCPT-1: Mast cell protease-1
MMP-9: Matrix metalloproteinase-9
PAP: Papain
PAR-2: Protease activated receptor 2
RAG: Ragweed
TRYP: Trypsin

Asthma is characterized by reversible airway obstruction as a result of a predominantly $T_H2$-driven airway inflammation and pulmonary remodeling. For individuals who exhibit a genetic predisposition, asthma can be induced or exacerbated by an abnormal response to environmental stimuli, such as allergens, infectious agents, or airborne pollutants. In addition to activation of pattern recognition receptors, many allergens possess intrinsic proteolytic activities that play an important role in development of allergic airway disease, in part, by activation of protease-activated receptors (PAR). IL-33 has been associated with the development and maintenance of allergic asthma via ligation of its receptor ST2.[1] IL-33 is located within the nucleus of Type II epithelial cells in the lung, but mechanisms that dictate its release remain ambiguous. It has been suggested that IL-33 can be released during necrosis in response to infection or trauma, and subsequently functions as an "alarmin."[2,3] Pulmonary IL-33 expression is elevated with individuals with asthma, which correlates with asthma severity.[4,5] Modulation of the IL-33–ST2 axis in murine models of allergic airway disease has supported a prominent role for this cytokine in asthma.[6-12] Genetic analysis also has linked polymorphisms in human IL-33–ST2 to the incidence of asthma.[13]

Severe asthma with fungal sensitization is characterized by the presence of severe asthma, fungal sensitization, and the exclusion of bronchopulmonary aspergillosis.[14] Epidemiologic studies have identified sensitivity to fungal allergens as a prominent cause of allergic asthma.[15] An association exists between sensitivity to the widely distributed fungus *Alternaria alternata* and asthma severity, hospital admission, and fatal asthma exacerbations.[14,16-23] High *Alternaria* spore counts are detected in late summer and/or early autumn, where dispersion of spores is associated with thunderstorms and leads to increased morbidity and mortality.[18,19,24-26] The prevalence of severe asthma with fungal sensitization has been estimated to be as frequent as 30%, although why molds are implicated in severe asthma compared with other aeroallergens has not been elucidated.

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Fungal allergens, such as *Alternaria*, possess intrinsic proteolytic activities that have the potential to act as adjuvants in driving a prolonged $T_H2$ inflammation.[27,28] Although the exact mechanism that defines this potential remains poorly defined, TLR4 activation has been implicated.[29] *Alternaria*-specific serine protease activity has previously been demonstrated to elicit epithelial cell increases in intracellular calcium through protease activated receptor 2 (PAR-2) activation and to drive pulmonary inflammation.[30] In the present study, we demonstrate that *Alternaria*-specific serine protease activity promotes the release of IL-33 in a murine model, which subsequently drives a robust release of early innate mediators and $T_H2$ pulmonary inflammation. Importantly, this serine protease–mediated IL-33 release was shown to underlie *Alternaria*-driven severe exacerbations of allergic airway disease.

## METHODS

### Mice

Female BALB/c mice (Charles River, Margate, United Kingdom) and $ST2^{-/-}$ mice on a BALB/c background (a kind gift from Andrew McKenzie, MRC Laboratory of Molecular Biology, Cambridge, United Kingdom), 6 to 8 weeks old received 10 μg house dust mite (HDM) (*Dermatophagoides pteronyssinus*), *Alternaria alternata*, cat dander, or ragweed (*Ambrosia artemisiifolia*) extract (Greer, Lenoir, NC); papain or trypsin (Sigma-Aldrich, Dorset, United Kingdom); or 50 μL of vehicle, PBS intranasally. Mice were culled either 1 or 24 hours after challenge. In some experiments, the *Alternaria* extract was preincubated with either 4-(2-Aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF) (25 mg/mL) or suramin (2 mM) (Sigma-Aldrich). FSSLYR-amide (Bachem AG, Bubendorf, Switzerland) was administered 30 minutes before allergen challenge (100 μg intraperitoneal and 12.5 μg intranasal). In the exacerbation protocol, mice were treated with either 15 μg HDM or PBS 3 times a week for 3 weeks before receiving single challenge with 10 μg *Alternaria*. All the experiments were performed in accordance with UK Home Office guidelines. Airway responsiveness was determined by direct measurements of lung function in anesthetized and tracheostomized mice 24 hours after final challenge.

### Tissue processing

Serum, BAL fluid (BALF) and lung tissue were collected.[31] Paraffin-embedded sections (4 μm) were stained with hematoxylin and eosin and periodic acid–Schiff. Paraffin sections were stained with goat anti-mouse IL-33 (R&D Systems, Abingdon, United Kingdom) by using an avidin-biotin staining method.

### Mediator analysis

BALF was analyzed by ELISA: IL-4, IL-5 (PharMingen, Oxford, United Kingdom), IL-13, IL-33, IL-25, matrix metalloproteinase-9 (MMP-9) (R&D Systems), IL-1β (eBioscience, Hatfield, United Kingdom), and albumin (Bethyl Laboratories, Montgomery, Tex). Uric acid was measured using an Amplex red uric acid/uricase assay kit (Invitrogen, Paisley, United Kingdom). Lactate dehydrogenase was measured by using an In Vitro Toxicology Assay kit (Sigma-Aldrich). Serum mast cell protease (MCP-1) was measured by ELISA (eBioscience, Hatfield, United Kingdom). IL-33 size was determined by Western blot. MMP-9 activity was determined by using Novex 10% zymogram gelatin gels (Invitrogen). MUC5AC transcript levels were determined by quantitative PCR.

### Flow cytometric analysis

Disaggregated lung cells were restimulated with 500 ng/mL of ionomycin and 50 ng/mL of phorbol 12-myristate 13-acetate in the presence of brefeldin A (BD Pharmingen, Oxford, United Kingdom). Cells were stained for CD3, CD4, CD8, IL-13, IL-17, or IFN-γ (eBioscience). In addition, cells were stained with Ly-6G, SiglecF, CD11b, CD11c, F4/80, CD45, lineage negative cocktail (eBioscience), T1/ST2 (Morwell Diagnostics, Zurich, Switzerland), or ICOS (Biolegend, London, United Kingdom). Labeled cells were acquired on a BD Fortessa (BD Bioscience, Oxford, United Kingdom), and analyzed by using FlowJo (Treestar, Ashland, Ore).

### Statistical analysis

Data were analyzed by using Prism 4 (GraphPad Software Inc, La Jolla, Calif). Multiple comparisons were performed by using the Kruskal-Wallis test. A 2-tailed $P$ value was determined by the Mann-Whitney test when comparing between 2 groups. Additional detail on the methods used in the present study are provided in this article's Online Repository at www.jacionline.org.

## RESULTS

### *Alternaria*-specific serine protease activity drives an early IL-33–mediated inflammation

Mice were treated intranasally with a panel of allergen extracts and the response to challenge determined after 1 hour. Papain and trypsin also were tested as examples of cysteine and serine proteases, respectively. Strikingly, *Alternaria* treatment resulted in a robust early IL-33 release, which was not observed in heat-treated *Alternaria* or with any other allergens and/or proteases (Fig 1, *A*). The failure to detect IL-33 in response to these other allergens was not a consequence of them eliciting IL-33 release and subsequently cleaving the cytokine into immunologically undetectable fragments (data not shown). From Western blots of the BALF, we determined that released IL-33 was a full-length protein (Fig 1, *B*). There was no significant increase in inflammatory cells to the lung or BALF (see Fig E1, *A* in this article's Online Repository at www.jacionline.org), which suggests that IL-33 release was from lung resident cells. The source of IL-33 in the BALF of *Alternaria*-exposed mice appeared to be Type II epithelial cells because they stained strongly for IL-33 in control mice, whereas the number of these $IL\text{-}33^+$ cells was significantly reduced 1 hour after *Alternaria* exposure (Fig 1, *C*). The release of preformed IL-33 into the airways in response to *Alternaria* was confirmed by the concomitant reduction of IL-33 levels in lung tissue (Fig E1, *B*).

Cellular damage results in lactate dehydrogenase release, and levels were modestly elevated in BALF in response to *Alternaria*, but, greater still after papain exposure, an enzyme that failed to elicit IL-33 release (Fig E1, *C*). Similarly, although the alarmin uric acid also was released in response to *Alternaria,* papain again induced greater release (Fig E1, *D*). Thus, taken together, the *Alternaria*-induced increase in IL-33 release appears to be independent of cellular damage. BALF albumin levels were significantly elevated by *Alternaria* but also by papain (Fig E1, *E*), which suggests that loss of epithelial barrier function alone and influx of extrapulmonary mediators also is not sufficient to account for the *Alternaria*-induced IL-33. As with IL-33, IL-1β was only detectable in *Alternaria*-exposed animals (Fig 1, *D*). Similarly, MMP-9 secretion was observed in *Alternaria* and to a lesser extent papain and cat dander–treated mice (Fig 1, *E*). Furthermore, serum mast cell protease (MCPT-1) levels also were significantly increased in response to *Alternaria* (Fig 1, *F*).

*Alternaria*-induced IL-33 release *in vitro* has previously been reported to be ATP signalling dependent.[32] Accordingly, blockade of ATP signaling *in vivo* through administration of P2 receptor antagonist suramin resulted in a 50% reduction in IL-33 release in response to *Alternaria* at 1 hour (Fig 2, *A*). Similarly, blocking PAR-2 receptor activation by endogenous proteases in the allergen inhibited IL-33 release into the BALF in response to *Alternaria* by 68% (Fig 2, *A*). The failure to completely abrogate IL-33 release by these inhibitors and/or antagonists may reflect redundancy within pathways. Because