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Aspergillus Fumigatus Induction of IL-33 Expression in Chronic Rhinosinusitis Is PAR2-Dependent

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Objective: In the pathophysiology of chronic rhinosinusitis with nasal polyps (CRSwNP), Aspergillus fumigatus (A. fumigatus) can upregulate IL-33 from human sinonasal epithelial cells (SNECs), which then activates innate lymphoid cells causing release of IL-13, an important driver of allergic inflammation. However, the mechanism by which A. fumigatus mediates the induction of IL-33 expression remains to be elucidated. The objectives of this study were to determine the specific fungal component(s) and the receptor responsible for mediating the A. fumigatus induced increase in IL-33 expression in SNECs from patients with CRSwNP.

Methods: SNECs from CRSwNP patients were cultured and stimulated with various fungal components in the absence or presence of 4-(2-Aminoethyl)benzenesulfonyl fluoride hydrochloride, an irreversible serine protease inhibitor, or GB603, a reversible protease-activated receptor 2 (PAR2) inhibitor. IL-33 expression was evaluated using quantitative real-time polymerase chain reaction (qRT-PCR). PAR2 expression was examined in inflamed mucosa from nonatopic control and CRSwNP patients.

Results: Elevation of IL-33 expression in primary SNECs was found in response to fungal protease but not fungal cell wall components. PAR2 expression was elevated in inflamed mucosa from CRSwNP patients in comparison to controls. The A. fumigatus fungal protease-mediated elevation in IL-33 expression by human SNECs was serine protease- and PAR2-dependent.

Conclusion: These data suggest that serine protease activity of A. fumigatus is capable of inducing IL-33 expression in CRSwNP SNECs via PAR2, a potential therapeutic target in the treatment of CRSwNP.

Key Words: Nasal polyps, fungal protease, IL-33, chronic rhinosinusitis, human sinonasal epithelial cells.

Level of Evidence: NA

INTRODUCTION

Chronic rhinosinusitis with nasal polyps (CRSwNP) is a chronic inflammatory disease of the sinonasal mucosa that results in significant healthcare costs associated with persistent medical therapy, recurrent sinus surgery, and loss of productivity. Described as a predominantly type 2 inflammatory disease, CRSwNP is characterized by increased levels of type 2 cytokines such as IL-4, IL-5, and IL-13, as well as immune cells including eosinophils, mast cells, and type 2 innate lymphoid cells (ILC2s).

Interleukin-33 (IL-33) is a cytokine that has recently been implicated in the pathophysiology of CRSwNP. IL-33 is produced primarily by epithelial and endothelial cells. It coordinates type 2 immune responses by signaling through the IL-33 receptor on immune cells, including T helper 2 (Th2) cells, macrophages, mast cells, basophils, eosinophils, and ILC2s. In CRS, IL-33 has been shown to stimulate the release of IL-5 and IL-13 by ILC2s and to promote type 2 innate and adaptive immune responses. IL-33 also acts as a chemottractant of Th2 cells and promotes Th2 polarization of naive CD4+ cells. However, the molecular specifics of IL-33 regulation and release are unclear.

Fungi, one of several microbes suspected as a possible etiology behind CRSwNP, are ubiquitous in the environment. Mold spores are frequently inhaled and cleared from the respiratory tract. Fungal exposure is linked to a type 2 immune response in sinonasal epithelial cells (SNECs) as well as upregulation of IL-33 expression. Furthermore, patients with CRSwNP, as compared to CRS without nasal polyps (CRSSNP), are more likely to have positive fungal cultures from their sinus lavage fluid and fungal-specific IL-4 secreting immune cells in their peripheral blood. Fungi contain and secrete several immunogenic components, including chitin, β-glucans, and proteases. Fungal proteases are of particular interest because they possess the potential to act as adjuvants in driving prolonged type

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2 inflammation.  Protease exposure has been linked to airway inflammation, recent studies indicate that fungal serine protease activity drives early release of IL-33, promoting pulmonary inflammation and a Th2 response.

The upper and lower airways are believed to be linked by comorbid pathologies because patients with CRSwNP have higher incidence of asthma in comparison to patients with CRSsNP, and patients with asthma have a 40% to 75% chance of having concurrent CRS. Despite evidence of the role of fungal protease in asthma, the specific component and mechanism of fungal interaction with SNECs responsible for upper respiratory inflammation remain unknown. The objective of this study is to determine the specific fungal component(s) responsible for inducing IL-33 gene expression and, consequently, the type 2 immune response in CRSwNP.

MATERIALS AND METHODS

Patients

The research protocol for this study was approved by the University of Texas Health Science Center’s institutional review board. Informed consent was obtained from patients aged between 16 and 75 years undergoing routine endoscopic sinus surgery for CRSwNP and not currently using systemic steroids, pregnant, or previously diagnosed with an immune system disorder. Patients with aspirin-exacerbated respiratory disease and allergic fungal rhinosinusitis (AFRS) were excluded from the CRSwNP group. The definition of CRSwNP was based on the criteria outlined by the International Consensus Statement on Allergy and Rhinology. Asthma status was based on a prior diagnosis of asthma by a pulmonologist, allergist, and/or positive pulmonary function testing. Atopy was determined by history of allergic rhinitis symptoms that was consistent with positive skin or blood test. Aspirin sensitivity was based on history of intake of aspirin or nonsteroid inciting worsening respiratory symptoms. Healthy controls (HC) were nonatopic and nonasthmatic patients undergoing endoscopic sinus surgery for the treatment of cerebrospinal fluid leaks or benign pituitary tumors. Patient characteristics are described in Table I.

Epithelial Cell Culture and Stimulation

Cells were dissociated from inflamed sinonasal mucosa using Collagenase D (2 mg/mL; Sigma-Aldrich, St. Louis, MO) and DNAse I/C14 incubated for 2 hours at 37°C. All sera, antibiotics and steroid-free BEGM (Lonza, Basel, Switzerland) were preincubated with human SNECs for 2 hours at 37°C prior to the addition of A. fumigatus.

RNA Isolation for Polymerase Chain Reaction Analysis

Inflamed mucosa collected at the time of surgery was placed into RNUlter (Qiagen). Total RNA was isolated from SNECs collected in RLT Buffer (Qiagen), and in RNAlater (Qiagen), after which cells were collected in RLT buffer (Qiagen). The protease activated receptor 2 (PAR2) inhibitor, GB83 (Axon Medchem, Groningen, Netherlands) was resuspended in dimethyl sulfoxide (DMSO), and increasing concentrations (0.2–10 μM) were preincubated with human SNECs for 2 hours at 37°C prior to the addition of A. fumigatus.

Quantitative Real-Time Polymerase Chain Reaction

Real-time polymerase chain reaction (PCR) was performed with an ABI Prism 7500 Sequence Detection system (Applied Biosystems, Foster City, CA) using iTaq SYBR Green Supermix (BioRad, Hercules, CA) and primer sets to a concentration of 250 nM. Primer sequences for IL-33 were forward 5'- AAAGAAATTTGCCCCACTG-3' and reverse 5'- AAGCAGAAAACCTGCTCAG-3'; PAR2 were forward 5'- GAGTTCGACATCGAGAC-3' and reverse 5'- GCTAGCGTCTTAAACGCAG-3' and for actin were forward 5'- CGATGCGAGAGCAGGATCTC-3' and reverse 5'- CGATCAGAGGAGATCG-3'. 

Statistical Analysis

Data were analyzed with Prism software version 6.03 (Graphpad Prism, La Jolla, CA). Data from multiple experiments are combined and are represented as fold change ± standard error of the mean over media control. Normally distributed data were analyzed by one-way analysis of variance, and between conditions were analyzed by one-way analysis of variance.
comparisons were made by Student t test. Non-normally distributed data were analyzed by Kruskal-Wallis and Mann-Whitney, and P < 0.05 was considered significant.

RESULTS

A. Fumigatus-Mediated Elevations of IL-33 Expression in Primary Human SNECs Are Mediated by Protease But Not Fungal Cell Wall Components

A. fumigatus extract is capable of enhancing IL-33 expression in comparison to media alone in SNECs from patients with CRSwNP. To determine the fungal extract component responsible for increasing IL-33 expression, SNECs were challenged individually with possible fungal components, chitin (a component of the fungal cell wall), curdlan and scleroglucan (both β-glucans and known polysaccharide components of the fungal cell wall), the cysteine protease papain, HDM (with protease activity similar to A. fumigatus), and A fumigatus extracts were cultured with primary SNECs from CRSwNP patients (n = 8–10) for 24 hours. IL-33 expression was assessed by quantitative real time (qRT)-PCR. In comparison to media alone and all cell wall components, papain, HDM, and A. fumigatus extracts were capable of significantly enhancing IL-33 mRNA expression in human SNECs (Fig. 1A). In contrast, the cell wall components chitin, curdlan, and scleroglucan did not affect IL-33 expression.

Furthermore, boiling of A. fumigatus extract prior to in vitro cell culture abrogated the fungal extract-induced increase in IL-33 expression (Fig. 1B) in comparison to media alone. The ability of multiple extracts with protease activity to upregulate IL-33 expression in combination with the heat-labile properties of the component responsible for upregulating IL-33 suggests that protease activity is important in driving IL-33 expression in SNECs.

Serine Protease Activity Drives A. Fumigatus-Mediated IL-33 Induction in Human SNECs

Although we determined that the component of the A. fumigatus extract responsible for inducing IL-33 expression was heat-sensitive and likely a protease, the nature of the protease activity that drove IL-33 expression remained to be elucidated. Recent work with Alternaria, another fungus commonly associated with the etiology of CRS, determined that there was a significant serine protease component of the fungal extract capable of driving allergic asthma in vivo, and that this phenotype was preceded by an early release of IL-33 in a PAR2-dependent manner. We sought to determine if serine protease inhibition of A. fumigatus would prevent the induction of IL-33 in human SNECs. A fumigatus extract was preincubated in the absence or presence of the irreversible serine protease inhibitor, AEBSF, at increasing concentrations for 2 hours at 37°C and then added to CRSwNP SNECs. After 24 hours, RNA was isolated, and IL-33 expression was assessed. We found that serine protease inhibition of A. fumigatus extract reduced the ability of the extract to increase IL-33 expression by SNECs in a concentration-dependent manner (Fig. 2). We were unable to prevent the A. fumigatus-mediated increase in IL-33 expression with the cysteine protease inhibitor, e64 (Appendix Fig. 1; available online). Thus, the upregulation in IL-33 expression by A. fumigatus was determined to be serine protease-dependent.

PAR2 Expression Is Elevated in CRSwNP Inflamed Mucosa

PARs are a class of G-protein coupled receptors that are activated by protease cleavage of an extracellular domain. Because PARs 1 to 4 have been found previously to be responsive to serine proteases, we sought to determine the expression of PARs 1 to 4 within inflamed mucosa of CRSwNP versus HC using qRT-PCR. Whereas there was a subset of patients in both groups with high PAR1, 3, and 4 expression (data not shown), PAR2 was the most consistently upregulated PAR in

![Image](https://example.com/image.png)

Fig. 1. IL-33 expression is enhanced by papain, HDM, and A. fumigatus. (A) Fold increase of IL-33 mRNA expression in primary sinonasal epithelial cells from patients with CRSwNP (n = 8–10) cultured for 24 hours in medium alone, chitin (1000 μg/mL), curdlan (100 μg/mL), scleroglucan (200 μg/mL), papain (500 ng/mL), HDM extract (200 μg/mL), A. fumigatus extract (25 μg/mL), or (B) boiled A. fumigatus extract (25 μg/mL). Data was normalized to actin and presented as fold change ± SEM. (A) **P < 0.01 Kruskal-Wallis. *P < 0.05. **P < 0.01 Mann-Whitney vs. media. (B) **P < 0.01, 1-way ANOVA. P < 0.01, Student t test vs. A. fumigatus. A. fumigatus = Aspergillus fumigatus; ANOVA = analysis of variance; CRSwNP = chronic rhinosinusitis with nasal polyposis; HDM = house dust mite; IL-33 = interleukin-33; SEM = standard error of the mean.)
expression at 24 hours in CRSwNP SNECs. A. fumigatus as a potential target of the serine protease activity of 4-(2-Aminoethyl) benzenesulfonyl error of the mean.

= human sinonasal epithelial cells; IL-33 = interleukin-33; SEM = standard error of the mean; CRSwNP = chronic rhinosinusitis with nasal polyps; HSNEC (Appendix Fig. 2, available online). Thus, we identified PAR2 as a potential target of the serine protease activity of A. fumigatus that was responsible for the induction of IL-33 expression at 24 hours in CRSwNP SNECs.

**IL-33 Induction by A. Fumigatus Is PAR2 Dependent**

PAR2 is a known target of serine proteases and has been shown previously to be targeted by fungal serine proteases. Because PAR2 was specifically upregulated in CRSwNP inflamed mucosa, we examined the role of PAR2 in the A. fumigatus-mediated induction of IL-33 in CRSwNP SNECs. GB83 is a reversible PAR2-selective antagonist that previously has been found to inhibit peptide agonist- and protease-induced activation of PAR2. GB83 was pre-incubated with confluent CRSwNP SNECs at increasing concentrations for a minimum of 2 hours at 37°C prior to the addition of A. fumigatus extract, and cells were collected 24 hours later for RNA analysis. In comparison to media alone and DMSO carrier control, PAR2 inhibition with GB83 prevented the A. fumigatus-induced increase of IL-33 in a concentration-dependent manner, indicating that PAR2 is an essential mediator of fungal protease-induced expression of IL-33 (Fig. 3B). Thus, the induction of IL-33 gene expression at 24 hours by A. fumigatus is regulated in a serine- and PAR2-dependent manner in CRSwNP SNECs.

**DISCUSSION**

In this study, we sought to identify the critical component(s) of A. fumigatus fungal extract driving published observation of increased IL-33 expression upon challenging SNECs. We demonstrated that active protease activity within the fungal extract was responsible. Collectively, our data suggest that fungal protease is sufficient to promote an IL-33-induced type 2 immune response observed in SNECs from CRSwNP patients through serine protease activity in a PAR2-dependent manner.

These findings complement previous studies linking fungal exposure to a type 2 immune response in CRS and IL-33 gene expression in human SNECs. In Porter et al., they demonstrated a higher prevalence of fungi from sinus lavage fluid of CRSwNP versus of nonatopic healthy controls, with Alternaria, A. fumigatus, and A. niger being the most common species identified. In addition, the presence of Alternaria and A. fumigatus were associated with having the most reactive IL-4 producing peripheral blood cells upon fungal challenge. In another study, it was demonstrated that...
SNECs from CRSwNP patients produced high levels of IL-33 mRNA in response to challenges with A. fumigatus, Alternaria, and Cladosporium herbarum, with A. fumigatus stimulating higher relative expression of IL-33 mRNA in SNECs from patients with CRSwNP in comparison to CRSsNP, a type 1-skewed disease. Thus, based on the in vivo relevance of A. fumigatus in CRSwNP pathophysiology and SNECs, we used A. fumigatus to examine the role of fungi in IL-33 expression by SNECs.

In order to narrow down the component of A. fumigatus extract that mediated the induction of IL-33 expression, we divided the potential inciting components of the extract into cell wall and protease components. Chitin, as well as the beta-glucans curdlan and scleroglucan, have previously been shown to induce IL-33 production in mouse models. 

Epithelial cell lines and osteoclasts. In particular, the effects of the beta-glucans are attributed to the pattern recognition receptor, Dectin-1,26,27 which is highly expressed on macrophages and lymphocytes. Limited studies have demonstrated the presence of Dectin-1 on human epithelial cell lines28,29 and in tissue from lung cancer patients.30 Induction of Dectin-1 in mouse airway epithelial cells via adenosine has antifungal effects,31 and the induction of IL-33 in osteoclasts was Dectin-1-dependent; this suggests that IL-33 production could potentially be induced by beta-glucans. However, in primary SNECs from CRSwNP patients, beta-glucans had no effect on IL-33 production, possibly indicating that Dectin-1 is not present in primary human SNECs or that Dectin-1 expression is suppressed in CRSwNP sinonasal epithelial cells in comparison to other CRS subtypes.

Snegrove et al. found that Alternaria-specific serine protease in a murine allergic asthma model induced rapid release of IL-33 in the airways of mice and promoted a robust type 2 response, as indicated by cellular infiltration of eosinophils and type 2 cytokine production.32 The type 2 immune response was dependent on the presence of ST2; the IL-33 receptor. Alternaria mediates these effects in a serine protease- and PAR2-dependent manner.32 Endogenous protease inhibitors, Cystatin A, a cysteine protease inhibitor, and SPINK5, a serine protease inhibitor, have been found to be deficient in CRSwNP patients.33 The lack of control of fungal protease activity is thought to contribute to the type 2 phenotype of CRSwNP.

Indeed, pretreatment of fungal extracts with Cystatin A and SPINK5 was associated with reduced IL-33 production in a mouse model of rhinosinusitis.34 Whereas most studies examining the interaction of fungi with respiratory epithelium involve Alternaria, A. fumigatus pretreatment with serine protease inhibitors had reduced cytokine production of IL-6, IL-8, and monocyte chemotactic protein 1, highlighting the importance of serine protease activity in the downstream induction of cytokines by A. fumigatus.34 In this study, we demonstrate for the first time that A. fumigatus is capable of inducing IL-33 expression in a serine protease-dependent manner in primary SNECs, indicating a shared pathway between Alternaria and A. fumigatus that can potentially be targeted to treat CRSwNP. Our findings also extend previous studies based on the lower airway by observing the effect of fungal protease on SNECs from the upper airway and provide a link to human type 2 disease.

Despite our understanding that the fungal serine protease activity is important in the observed upregulation of IL-33 expression in SNECs, it remained unclear which receptor or receptors are activated by the fungal protease activity. Recent evidence identified TLR4 and PAR217,35,36 as potential mediators of this response. Indeed, we found elevations in PAR2 and TLR4 (Appendix Fig. 3, available online) in the inflamed mucosa of CRSwNP and AFRS patients in comparison to HC patients and a novel cooperative role between TLR4 and PAR2 has been reported for downstream NFκb signaling in HEK293T cells.37 However, inhibition of TLR4 with LPS-RS, a potent competitive antagonist of TLR4, failed to prevent the A. fumigatus-mediated induction of IL-33 (Appendix Fig. 4, available online), whereas inhibition of PAR2 with GB83, a reversible PAR2 antagonist, did prevent the IL-33 induction, leading us to conclude that PAR2 is essential for the A. fumigatus-mediated induction of IL-33 expression in CRSwNP SNECs.

PAR2s can signal through a number of different pathways. In airway epithelial cells, Alternaria, can drive type 2 immune responses in a PAR2-dependent manner using calcium release-activated channels (CRAC), which result in oscillations of intracellular calcium.38,39 However, Jairaman et al. did not find the CRAC signaling pathway to be important in the Alternaria or A. fumigatus-mediated upregulation of intracellular calcium via PAR2 associated with the type 2 pathways in BEAS-2B cells.40 Balenga et al. recently demonstrated in an A. fumigatus-driven mouse model of allergic asthma that the type 2 phenotype was serine protease-driven and linked to calcium oscillations, indicating that A. fumigatus, similar to Alternaria, is capable of stimulating the calcium-mediated pathways.41 Because the induction of intracellular calcium signaling in primary SNECs by A. fumigatus has not been explored, it remains to be determined if A. fumigatus also upregulates IL-33 expression by using PAR2 to activate CRACs. The upregulation of several calcium-related signaling pathways and receptors in CRSwNP42 was recently reported; this signaling modality will be the subject of future investigations. Calcium-related signaling pathways in response to fungal protease interactions represent potential novel therapeutic targets for the treatment of CRSwNP.

CONCLUSION

This study significantly augments our understanding of the role of fungus in the pathogenesis of CRSwNP. Specifically, we demonstrated that 1) protease is the fungal component responsible for the A. fumigatus-enhanced IL-33 expression in SNECs; 2) A. fumigatus-enhanced IL-33 expression by SNECs is serine protease-dependent; 3) PAR2 is upregulated in CRSwNP inflamed mucosa; and 4) A. fumigatus-enhanced IL-33 expression is PAR2-dependent. Given this information, it may be prudent in future studies to target the protease-PAR2-IL-33 axis therapeutically for treatment of CRSwNP.

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