

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

Caroline J. Dietz, PhD; Hua Sun, PhD; William C. Yao, MD ; Martin J. Citardi, MD; David B. Corry, MD; Amber U. Luong, MD, PhD 

**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 GB83, 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

Laryngoscope, 00:1–6, 2019

## 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.<sup>1</sup> Described as a predominantly type 2 inflammatory disease,<sup>2</sup> CRSwNP is characterized by increased

levels of type 2 cytokines such as IL-4, IL-5, and IL-13,<sup>3</sup> 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.<sup>4–6</sup> 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.<sup>7,8</sup> 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.<sup>6,9</sup> IL-33 also acts as a chemoattractant of Th2 cells<sup>10</sup> and promotes Th2 polarization of naïve CD4<sup>+</sup> cells.<sup>11</sup> 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)<sup>12</sup> as well as upregulation of IL-33 expression.<sup>6</sup> 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.<sup>13</sup> Fungi contain and secrete several immunogenic components, including chitin,  $\beta$ -glucans, and proteases. Fungal proteases are of particular interest because they possess the potential to act as adjuvants in driving prolonged type

From the Department of Otolaryngology–Head and Neck Surgery (C.J.D., H.S., W.C.Y., M.J.C., A.U.L.); the Center for Immunology and Autoimmune Diseases, Institute of Molecular Medicine (C.J.D., H.S., A.U.L.), McGovern Medical School at the University of Texas Health Science Center; and the Department of Medicine and the Biology of Inflammation Center, Baylor College of Medicine (D.B.C.), Houston, Texas, U.S.A.

Additional supporting information may be found in the online version of this article.

Editor's Note: This Manuscript was accepted for publication on March 26, 2019.

This study was partially funded by a Triological Society Research Career Development grant and an American Academy of Otolaryngic Allergy Foundation grant to A.U.L. M.J.C. received consulting fees from Acclarent (Irvine, CA), Arrinex (Redwood City, CA), Biosense Webster (Haifa, Israel), Factory CRO (Bilthoven, The Netherlands), Intersect ENT (Menlo Park, CA), Medical Metrics (Houston, TX), Medtronic (Jacksonville, FL), and Stryker (Kalamazoo, MI). A.U.L. received consulting fees from Aerin Medical (Sunnyvale, CA), Arrinex (Redwood City, CA), Intersect ENT (Menlo Park, CA), Medtronic (Jacksonville, FL), and Stryker (Kalamazoo, MI). She is on the advisory board for ENTvantage (Austin, TX) and Lyra Therapeutics (Watertown, MA). W.C.Y. serves as a speaker for Optinose (Yardley, PA). The Department of Otolaryngology received industry research funding from Astrogeneca and Genetech. The authors have no other funding, financial relationships, or conflicts of interest to disclose.

Send correspondence to Amber Luong, MD, PhD, FACS, Associate Professor of Otorhinolaryngology–Head and Neck Surgery, McGovern Medical School at the University of Texas Health Science Center, 6431 Fannin Street, MSB 5.036, Houston, TX 77030. E-mail: amber.u.luong@uth.tmc.edu

DOI: 10.1002/lary.28000

2 inflammation.<sup>14</sup> Protease exposure has been linked to airway inflammation<sup>15,16</sup>; recent studies indicate that fungal serine protease activity drives early release of IL-33, promoting pulmonary inflammation and a Th2 response.<sup>17</sup>

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,<sup>18</sup> and patients with asthma have a 40% to 75% chance of having concurrent CRS.<sup>19</sup> 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.<sup>20</sup> 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 (50 U/mL; Sigma-Aldrich) as described previously.<sup>21</sup> After filtration through a 40 µm strainer, 2 mM EDTA was added to inhibit enzymes, and the remaining cells were washed and resuspended in Bronchial Epithelial Cell Growth Basal Medium (BEGM) (Lonza, Basel, Switzerland) with penicillin/streptomycin (1X; Corning, Corning, NY). The cell suspension was plated in a Petri dish and adherent cells allowed to attach for 2 hours at 37°C. The remaining adherent cell-free supernatant was then transferred to a collagen-coated flask. Epithelial cells were plated into 96-well plates and, upon confluence, cultured with antibiotic- and steroid-free BEGM (Lonza) 24 hours prior to stimulation. Epithelial cells were stimulated for 24 hours with media alone, chitin (1000 µg/mL; Sigma), curdlan AL (100 µg/mL; Invivogen, San Diego, CA), scleroglucan (200 µg/mL; Invivogen), papain (papaya latex, 500 ng/mL; Sigma), extracts from house dust mite (HDM) (*D. pteronyssinus*, 200 µg/mL; Greer Laboratories, Lenoir, NC), or *A. fumigatus* (25 µg/mL; Greer Laboratories), after which cells were collected in RLT buffer (Qiagen, Hilden, Germany) containing β-mercaptoethanol. The serine protease inhibitor, 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF; Sigma), was preincubated for 2 hours at 37°C at increasing concentrations

TABLE I.  
Clinical Characteristics of Enrolled Participants.

| Phenotype           | HC (n = 13) | CRSwNP (n = 49) |
|---------------------|-------------|-----------------|
| Age, years (median) | 60          | 46              |
| Female              | 77          | 45              |
| Male                | 23          | 55              |
| Asthma              | 0           | 39              |
| Atopy               | 0           | 57              |
| ASA sensitivity     | 0           | 0               |
| Previous surgery    | 23          | 43              |
| Systemic steroids   | 0           | 0               |
| Intranasal steroids | 0           | 49              |

\*Values are presented as percentages unless noted otherwise.

ASA = aspirin; CRSwNP = chronic rhinosinusitis with nasal polyps; HC = healthy control.

(0.0025 mM to 1 mM) followed by *A. fumigatus* extract added at a final concentration of 25 µg/mL. 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*.

### RNA Isolation for Polymerase Chain Reaction Analysis

Inflamed mucosa collected at the time of surgery was placed into RNeasy lysis buffer (Qiagen). Total RNA was isolated from SNECs collected in RLT Buffer (Qiagen), and inflamed mucosa using RNeasy Mini Kit (Qiagen) using the manufacturer's protocol and complementary DNA (cDNA) was synthesized with iScript cDNA Synthesis Kit (Biorad, Hercules, CA) using manufacturer's instructions.

### 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 primers at a concentration of 250 nM. Primer sequences for IL-33 were forward 5'-CAAAGAAGTTTGGCCCATGT-3' and reverse 5'-AAGGCAAAGCACTCCACAGT-3'; PAR2 were forward 5'-GAGTTGGATTGGACAGTAG-3' and reverse 5'-GCTAGGATTACAGGCATGAG-3' and for actin were forward 5'-GGATGCAGAAGGAGATCACTG-3' and reverse 5'-CGATCCACACGGAGTACTTG-3'. Reactions were incubated for 2 minutes at 50°C, denatured for 3 minutes at 95°C, and subjected to 40 two-step amplification cycles with denaturation at 95°C for 15 seconds, followed by annealing-extension at 60°C for 45 seconds. A dissociation curve was run to ensure that the correct amplicon was detected. The threshold cycle (Ct) was determined for the gene of interest and the housekeeping gene β-actin, and these values were used to calculate relative Ct using the delta-delta-Ct algorithm with β-actin as a reference gene.

### 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

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.<sup>6</sup> 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  $\beta$ -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.<sup>17</sup> 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

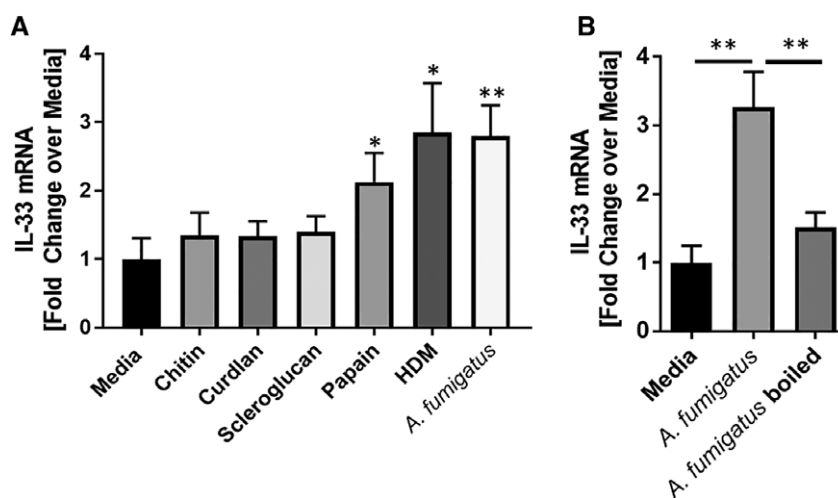


Fig. 1. IL-33 expression is enhanced by papain, HDM, and *A. fumigatus*. (A) Fold increase of IL-33 mRNA expression in primary sinonasal epithelial cells from patients with CRSwNP (*n* = 8–10) cultured for 24 hours in medium alone, chitin (1000  $\mu$ g/mL), curdlan (100  $\mu$ g/mL), scleroglucan (200  $\mu$ g/mL), papain (500 ng/mL), HDM extract (200  $\mu$ g/mL), *A. fumigatus* extract (25  $\mu$ g/mL), or (B) boiled *A. fumigatus* extract (25  $\mu$ g/mL). Data was normalized to actin and presented as fold change  $\pm$  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 polyps; HDM = house dust mite; IL-33 = interleukin-33; SEM = standard error of the mean.

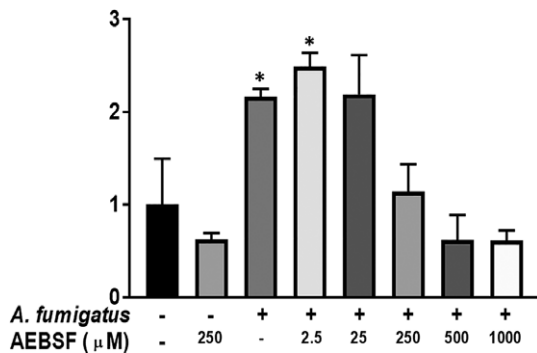


Fig. 2. *A. fumigatus*-mediated induction of IL-33 expression is prevented by serine protease inhibition. Fold increase of IL-33 mRNA expression in HSNECs from patients with CRSwNP (n = 3) cultured for 24 hours in medium alone or AEBSF (2.5–1000 μM) in the absence or presence of *A. fumigatus* extract (25 μg/mL). Data are normalized to actin and presented as fold change ± SEM. \*\*\**P* < .001, 1-way ANOVA. \**P* < .05, Student *t* test vs. media. *A. fumigatus* = *Aspergillus fumigatus*; AEBSF = 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride; ANOVA = analysis of variance; CRSwNP = chronic rhinosinusitis with nasal polyps; HSNEC = human sinonasal epithelial cells; IL-33 = interleukin-33; SEM = standard error of the mean.

CRSwNP compared to HC (Fig. 3A). We further examined CRSwNP SNECs 24 hours following *A. fumigatus* extract stimulation to determine if PAR2 was expressed by SNECs and if exposure to fungal extract enhanced expression of PAR2. We verified PAR2 expression by human SNECs using qRT-PCR but found that *A. fumigatus* exposure had no significant effect on PAR2 expression by CRSwNP SNECs (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.<sup>17</sup>

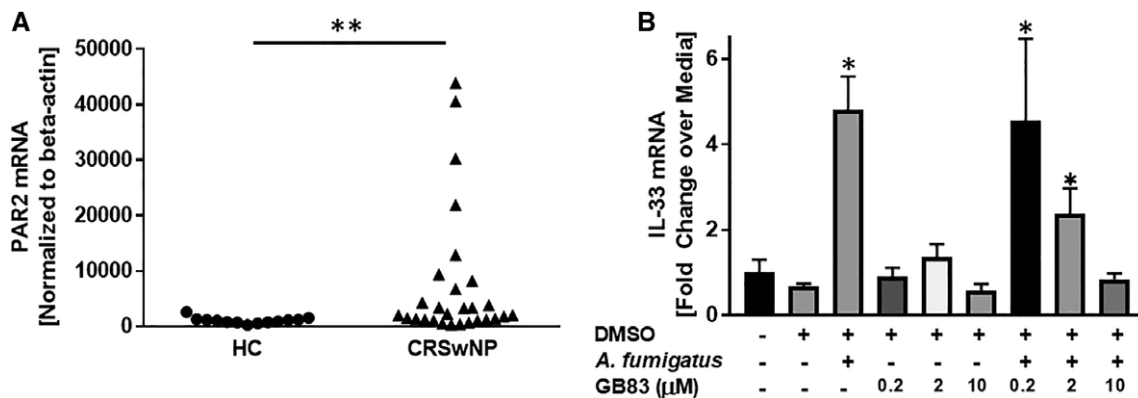


Fig. 3. Inhibition of PAR2 prevents the *A. fumigatus*-mediated increase in IL-33 expression. (A) PAR2 mRNA expression is elevated in inflamed mucosa from CRSwNP (n = 28) patients in comparison to healthy controls (n = 13). (B) Fold increase of IL-33 mRNA expression in HSNECs from patients with CRSwNP (n = 4) cultured for 24 hours in medium alone; DMSO; and *A. fumigatus* extract (25 μg/mL) in the absence or presence of GB83, a PAR2 reversible antagonist (0.2–10 μM). Data are normalized to actin and presented as (A) individual values or (B) fold change ± SEM. (A) \*\**P* < 0.01, Mann-Whitney vs. HC. (B) \*\**P* < 0.01, Kruskal-Wallis. \**P* < 0.05, Mann-Whitney vs. DMSO. *A. fumigatus* = *Aspergillus fumigatus*; CRSwNP = chronic rhinosinusitis with nasal polyps; DMSO = dimethyl sulfoxide; HSNEC = human sinonasal epithelial cells; IL-33 = interleukin-33; PAR2 = protease activated receptor; SEM = standard error of the mean.

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<sup>22</sup> that previously has been found to inhibit peptide agonist- and protease-induced activation of PAR2.<sup>22,23</sup> 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.<sup>6</sup> 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.<sup>13</sup> 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*,<sup>6</sup> 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,<sup>24</sup> epithelial cell lines,<sup>25</sup> and osteoclasts.<sup>26</sup> In particular, the effects of the beta-glucans are attributed to the pattern recognition receptor, Dectin-1,<sup>26,27</sup> which is highly expressed on macrophages and lymphocytes. Limited studies have demonstrated the presence of Dectin-1 on human epithelial cell lines<sup>28,29</sup> and in tissue from lung cancer patients.<sup>30</sup> Induction of Dectin-1 in mouse airway epithelial cells via adenovirus has antifungal effects,<sup>31</sup> 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.

Snelgrove 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.<sup>17</sup> 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.<sup>32</sup> Endogenous protease inhibitors, Cystatin A, a cysteine protease inhibitor, and SPINK 5, a serine protease inhibitor, have been found to be deficient in CRSwNP patients.<sup>33</sup> 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.<sup>33</sup> 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 chemoattractant protein 1, highlighting the importance of serine protease activity in the downstream induction of cytokines by *A. fumigatus*.<sup>34</sup> 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 PAR2<sup>17,35,36</sup> 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 NF $\kappa$ B signaling in HEK293T cells.<sup>37</sup> 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.

PARs 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.<sup>38,39</sup> 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.<sup>40</sup> 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.<sup>41</sup> 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 CRSwNP<sup>42</sup> 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.

## Acknowledgment

The authors would like to thank Dr. Ryan Drake, Ameerah Wishahy, Robyn Reed, Yi-Dong Li, and Dr. Faramarz Ashoori

for excellent technical assistance. Additional thanks to Drs. Kenneth Altman and Melissa Pynnonen for their suggestions on the manuscript.

Accepted as a Triological Society Thesis (no. 2019-14).

## BIBLIOGRAPHY

- Barry JY, McCrary HC, Kent S, Saleh AA, Chang EH, Chiu AG. The Triple Aim and its implications on the management of chronic rhinosinusitis. *Am J Rhinol Allergy* 2016;30:344–350.
- Van Zele T, Claeys S, Gevaert P, et al. Differentiation of chronic sinus diseases by measurement of inflammatory mediators. *Allergy* 2006;61:1280–1289.
- Tyler MA, Russell CB, Smith DE, et al. Large-scale gene expression profiling reveals distinct type 2 inflammatory patterns in chronic rhinosinusitis subtypes. *J Allergy Clin Immunol* 2017;139:1061.e4–1064.e4.
- Mjosberg JM, Trifari S, Crellin NK, et al. Human IL-25- and IL-33-responsive type 2 innate lymphoid cells are defined by expression of CCR2 and CD161. *Nat Immunol* 2011;12:1055–1062.
- Reh DD, Wang Y, Ramanathan M Jr, Lane AP. Treatment-recalcitrant chronic rhinosinusitis with polyps is associated with altered epithelial cell expression of interleukin-33. *Am J Rhinol Allergy* 2010;24:105–109.
- Shaw JL, Fakhri S, Citardi MJ, et al. IL-33-responsive innate lymphoid cells are an important source of IL-13 in chronic rhinosinusitis with nasal polyps. *Am J Respir Crit Care Med* 2013;188:432–439.
- Liew FY, Pitman NI, McInnes IB. Disease-associated functions of IL-33: the new kid in the IL-1 family. *Nat Rev Immunol* 2010;10:103–110.
- Chackerian AA, Oldham ER, Murphy EE, Schmitz J, Pflanz S, Kastelein RA. IL-1 receptor accessory protein and ST2 comprise the IL-33 receptor complex. *J Immunol* 2007;179:2551–2555.
- Barrett NA, Austen KF. Innate cells and T helper 2 cell immunity in airway inflammation. *Immunity* 2009;31:425–437.
- Komai-Koma M, Xu D, Li Y, McKenzie AN, McInnes IB, Liew FY. IL-33 is a chemoattractant for human Th2 cells. *Eur J Immunol* 2007;37:2779–2786.
- Kurowska-Stolarska M, Kewin P, Murphy G, et al. IL-33 induces antigen-specific IL-5+ T cells and promotes allergic-induced airway inflammation independent of IL-4. *J Immunol* 2008;181:4780–4790.
- Mulligan JK, Mulligan RM, Atkinson C, Schlosser RJ. Human sinonasal epithelial cells direct dendritic function and T-cell T helper 1/T helper 2 skewing following *Aspergillus* exposure. *Int Forum Allergy Rhinol* 2011;1:268–274.
- Porter PC, Lim DJ, Maskatia ZK, et al. Airway surface mycosis in chronic TH2-associated airway disease. *J Allergy Clin Immunol* 2014;134:325–331.
- Porter PC, Yang T, Luong A, et al. Proteinases as molecular adjuvants in allergic airway disease. *Biochim Biophys Acta* 2011;1810:1059–1065.
- Reed CE, Kita H. The role of protease activation of inflammation in allergic respiratory diseases. *J Allergy Clin Immunol* 2004;114:997–1008; quiz 1009.
- Shen HD, Tam MF, Chou H, Han SH. The importance of serine proteinases as aeroallergens associated with asthma. *Int Arch Allergy Immunol* 1999;119:259–264.
- Snelgrove RJ, Gregory LG, Peiro T, et al. *Alternaria*-derived serine protease activity drives IL-33-mediated asthma exacerbations. *J Allergy Clin Immunol* 2014;134:583.e6–592.e6.
- Promsopa C, Kansara S, Citardi MJ, Fakhri S, Porter P, Luong A. Prevalence of confirmed asthma varies in chronic rhinosinusitis subtypes. *Int Forum Allergy Rhinol* 2016;6:373–377.
- Spector SL, Bernstein IL, Li JT, et al. Parameters for the diagnosis and management of sinusitis. *J Allergy Clin Immunol* 1998;102:S107–S144.
- Orlandi RR, Kingdom TT, Hwang PH, et al. International consensus statement on allergy and rhinology: rhinosinusitis. *Int Forum Allergy Rhinol* 2016;6(suppl 1):S22–S209.
- Shaw JL, Ashoori F, Fakhri S, et al. Increased percentage of mast cells within sinonasal mucosa of chronic rhinosinusitis with nasal polyp patients independent of atopy. *Int Forum Allergy Rhinol* 2012;2:233–240.
- Barry GD, Suen JY, Le GT, Cotterell A, Reid RC, Fairlie DP. Novel agonists and antagonists for human protease activated receptor 2. *J Med Chem* 2010;53:7428–7440.
- Hennessey JC, McGuire JJ. Attenuated vasodilator effectiveness of protease-activated receptor 2 agonist in heterozygous par2 knockout mice. *PLoS One* 2013;8:e55965.
- Van Dyken SJ, Mohapatra A, Nussbaum JC, et al. Chitin activates parallel immune modules that direct distinct inflammatory responses via innate lymphoid type 2 and gammadelta T cells. *Immunity* 2014;40:414–424.
- Khosravi AR, Erle DJ. Chitin-induced airway epithelial cell innate immune responses are inhibited by carvacrol/thymol. *PLoS One* 2016;11:e0159459.
- Zhu X, Zhao Y, Jiang Y, et al. Dectin-1 signaling inhibits osteoclastogenesis via IL-33-induced inhibition of NFATc1. *Oncotarget* 2017;8:53366–53374.
- Adams EL, Rice PJ, Graves B, et al. Differential high-affinity interaction of dectin-1 with natural or synthetic glucans is dependent upon primary structure and is influenced by polymer chain length and side-chain branching. *J Pharmacol Exp Ther* 2008;325:115–123.
- Bertuzzi M, Hayes GE, Icheoku UJ, et al. Anti-*Aspergillus* activities of the respiratory epithelium in health and disease. *J Fungi (Basel)* 2018;4. doi: <https://doi.org/10.3390/jof4010008>.
- Sun WK, Lu X, Li X, et al. Dectin-1 is inducible and plays a crucial role in *Aspergillus*-induced innate immune responses in human bronchial epithelial cells. *Eur J Clin Microbiol Infect Dis* 2012;31:2755–2764.
- Heyl KA, Klassert TE, Heinrich A, et al. Dectin-1 is expressed in human lung and mediates the proinflammatory immune response to nontypeable *Haemophilus influenzae*. *MBio* 2014;5:e01492–e01514.
- Liu ZC, Wang M, Sun WK, et al. Up-regulation of Dectin-1 in airway epithelial cells promotes mice defense against invasive pulmonary aspergillosis. *Int J Clin Exp Med* 2015;8:17489–17497.
- Boitano S, Flynn AN, Sherwood CL, et al. *Alternaria alternata* serine proteases induce lung inflammation and airway epithelial cell activation via PAR2. *Am J Physiol Lung Cell Mol Physiol* 2011;300:L605–614.
- Kouzaki H, Matsumoto K, Kikuoka H, et al. Endogenous protease inhibitors in airway epithelial cells contribute to eosinophilic chronic rhinosinusitis. *Am J Respir Crit Care Med* 2017;195:737–747.
- Tomei JF, Wierenga AT, Hiemstra PS, Kauffman HK. Proteases from *Aspergillus fumigatus* induce release of proinflammatory cytokines and cell detachment in airway epithelial cell lines. *J Infect Dis* 1997;176:300–303.
- Millien VO, Lu W, Shaw J, et al. Cleavage of fibrinogen by proteinases elicits allergic responses through Toll-like receptor 4. *Science* 2013;341:792–796.
- Ebert CS Jr, McKinney KA, Urrutia G, et al. Expression of protease-activated receptors in allergic fungal rhinosinusitis. *Int Forum Allergy Rhinol* 2014;4:266–271.
- Rallabhandi P, Nhu QM, Toshchakov VY, et al. Analysis of proteinase-activated receptor 2 and TLR4 signal transduction: a novel paradigm for receptor cooperativity. *J Biol Chem* 2008;283:24314–24325.
- Jairaman A, Yamashita M, Schleimer RP, Prakriya M. Store-operated Ca<sup>2+</sup>-release-activated Ca<sup>2+</sup> channels regulate PAR2-activated Ca<sup>2+</sup> signaling and cytokine production in airway epithelial cells. *J Immunol* 2015;195:2122–2133.
- Jairaman A, Maguire CH, Schleimer RP, Prakriya M. Allergens stimulate store-operated calcium entry and cytokine production in airway epithelial cells. *Sci Rep* 2016;6:32311.
- O'Grady SM, Patil N, Melkamu T, Maniak PJ, Lancto C, Kita H. ATP release and Ca<sup>2+</sup> signaling by human bronchial epithelial cells following *Alternaria* aeroallergen exposure. *J Physiol* 2013;591:4595–4609.
- Balenga NA, Klichinsky M, Xie Z, et al. A fungal protease allergen provokes airway hyper-responsiveness in asthma. *Nat Commun* 2015;6:6763.
- Tyler MA, Padro Dietz CJ, Russell CB, et al. Distinguishing molecular features of allergic fungal rhinosinusitis. *Otolaryngol Head Neck Surg* 2018;159:185–193.

## Appendix

**Article Title:** *Aspergillus fumigatus* induction of IL-33 expression in Chronic Rhinosinusitis is PAR2-dependent

### Methods

#### *Epithelial Cell Culture and Stimulation*

As described in the main text, human sinonasal epithelial cells (SNECs) were isolated from ethmoid sinus tissue collected at the time of surgery. Epithelial cells were plated into 96-well plates and, upon confluence, cultured with antibiotic- and steroid-free BEGM 24 hours prior to stimulation. Epithelial cells were then stimulated for 24 hours with media alone, fibrinogen alone, *A. fumigatus* extract, *A. fumigatus*-derived fibrinogen cleavage product (FCPs) in the absence or presence of AEBSF, e64 or LPS-RS as detailed in the figure legends.

The serine protease inhibitor, 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF; Sigma) and cysteine protease inhibitor, trans-epoxysuccinyl-L-leucylamido(4-guanidino)butane (e-64; Sigma) were preincubated for 2 hours at 37°C with and without *A. fumigatus* extract followed by addition of fibrinogen for FCP generation for 2 hours at 37°C.

For the experiments with LPS-RS, SNECs were preincubated with LPS-RS for 2 hours at 37°C prior to addition of *A. fumigatus*-derived FCPs.

#### *RNA Isolation for PCR analysis*

Inflamed mucosa collected at the time of surgery was placed into RNeasy Lysis Buffer (Qiagen). Total RNA was isolated from SNECs collected in RLT Buffer (Qiagen) and inflamed mucosa using RNeasy Mini Kit (Qiagen) using the manufacturer's protocol and complementary DNA (cDNA) was synthesized using iScript cDNA Synthesis Kit (BioRad, Hercules, CA) using manufacturer's instructions.

### *Quantitative Real-Time PCR*

Real-time 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 primers at a concentration of 250nM. Primer sequences for IL-33 were forward 5'- CAAAGAAGTTTGCCCCATGT-3' and reverse 5'- AAGGCAAAGCACTCCACAGT-3', PAR2 were forward 5'- GAGTTGGGATTGGACAGTAG-3' and reverse 5'-GCTAGGATTACAGGCATGAG-3', TLR4 were forward 5'- AGGATGAGGACTGGGTAAG-3' and reverse 5'- CGGGAATAAAGTCTCTGTAGTG-3' and for actin were forward 5'- GGATGCAGAAGGAGATCACTG-3' and reverse 5'- CGATCCACACGGAGTACTTG-3'. Reactions were incubated for 2 minutes at 50°C, denatured for 3 minutes at 95°C, and subjected to 40 two-step amplification cycles with denaturation at 95°C for 15 seconds followed by annealing-extension at 60°C for 45 seconds. A dissociation curve was run to ensure that the correct amplicon was detected. The threshold cycle (Ct) was determined for the gene of interest and the housekeeping gene  $\beta$ -actin and these values were used to calculate relative Ct using the DDCT method with  $\beta$ -actin as a reference gene.

### *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  $\pm$  SEM over media control. Normally distributed data were analyzed by 1-way Analysis Of Variance (ANOVA) and between conditions comparisons were made by student's t-test. Non-normally distributed data were analyzed by Kruskal-Wallis and by Mann-Whitney and  $p < 0.05$  was considered significant.

## Figure legends

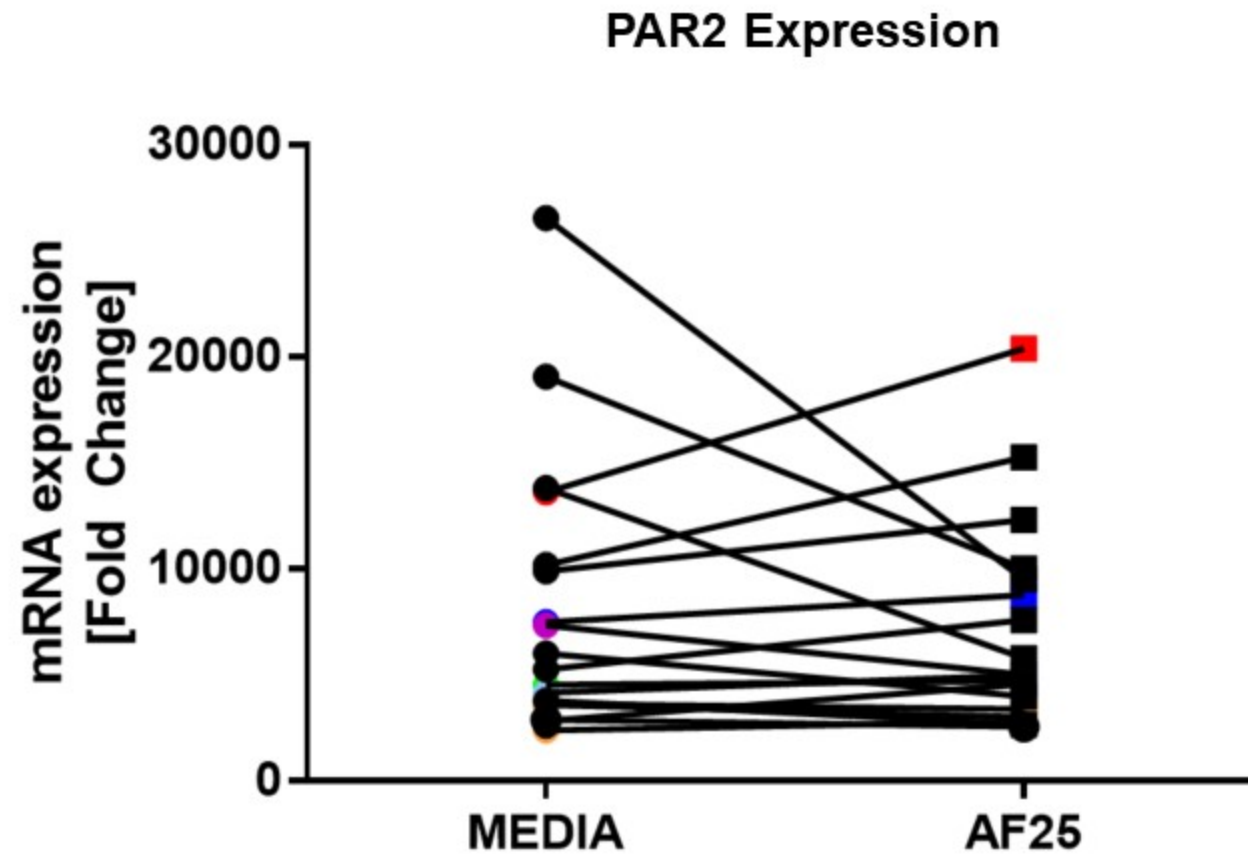
**Appendix Figure 1.** IL-33 enhancement in human SNECs by *A. fumigatus*-derived FCPs is serine protease- but not cysteine protease-dependent. Fold increase of IL-33 mRNA expression in SNECs from patients with CRSwNP (n=6) cultured for 24 hours in medium alone, fibrinogen alone, AEBSF (a serine protease inhibitor) at low (2.5  $\mu$ M) and high (250  $\mu$ M) concentrations preincubated with or without *A. fumigatus* extract (25  $\mu$ g/ml) prior to FCP generation, e64 (an irreversible cysteine protease inhibitor) at low (1 nM) and high (10 nM) concentrations preincubated with or without *A. fumigatus* extract prior to FCP generation, *A. fumigatus*-derived FCPs (50  $\mu$ g/ml), or *A. fumigatus* extract (25  $\mu$ g/ml). Data are normalized to actin and presented as fold change + SEM. \*,  $p < .05$ , \*\*,  $p < .01$ , unpaired t test versus fibrinogen alone.

**Appendix Figure 2.** Induction of PAR2 expression in sinonasal epithelial cells from patients with CRSwNP in response to stimulation with *Aspergillus fumigatus* extract. Fold increase of PAR2 mRNA expression in SNECs from patients with CRSwNP (n=18) cultured for 24 hours in medium alone or *A. fumigatus* extract (25  $\mu$ g/ml). Data are normalized to actin and presented as fold change + SEM.

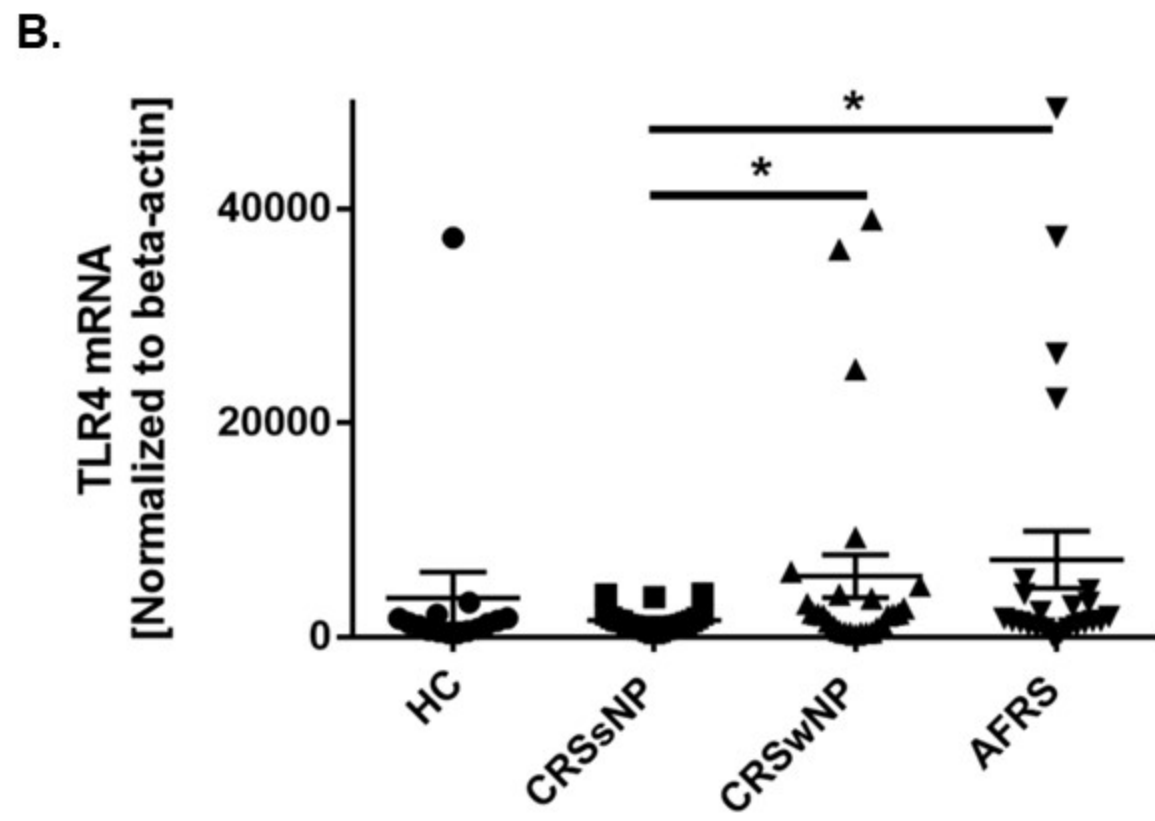
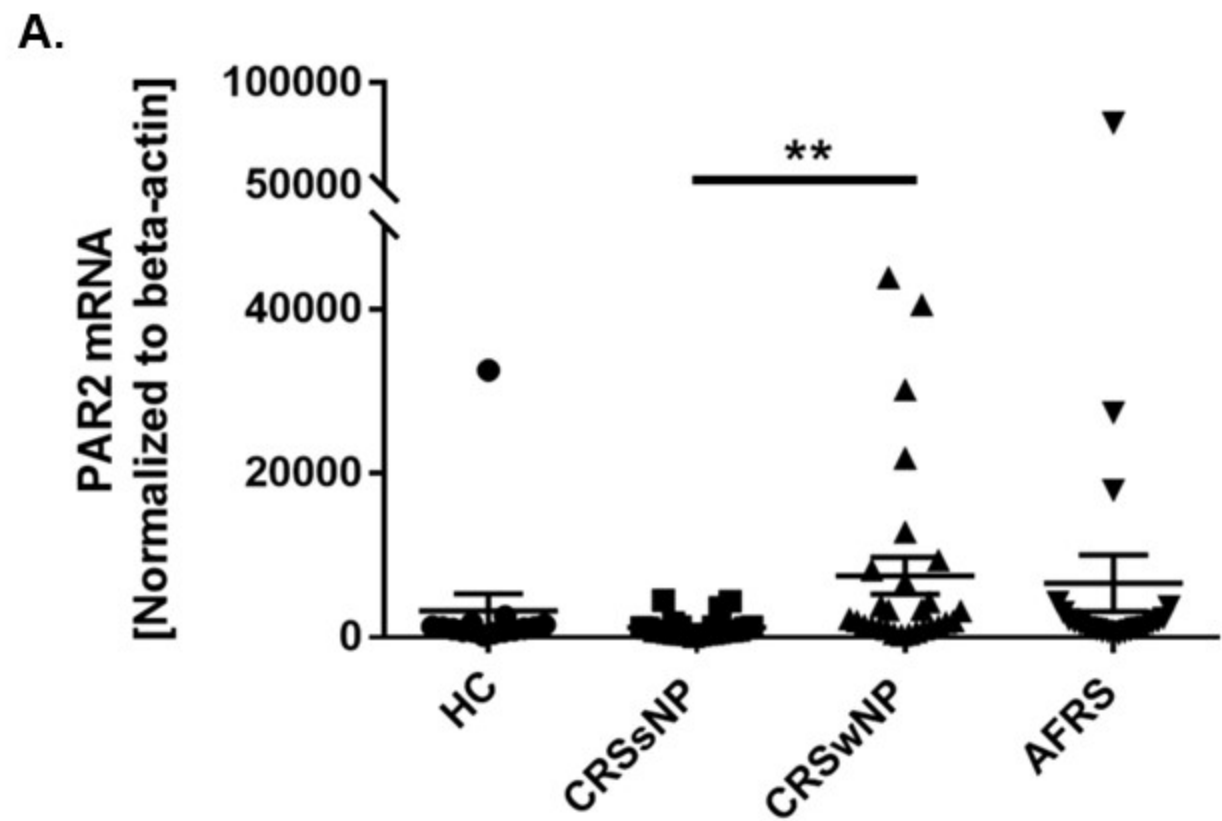
**Appendix Figure 3.** PAR2 and TLR4 expression increased in CRSwNP and AFRS. (A) PAR2 and (B) TLR4 mRNA expression standardized to beta-actin in inflamed sinus mucosa from healthy control (HC) (n=14), chronic rhinosinusitis without nasal polyps (CRSsNP) (n=24), chronic rhinosinusitis with nasal polyps (CRSwNP) (n=28), and allergic fungal rhinosinusitis (AFRS) (n=24). \*,  $p < .05$ , \*\*,  $p < .01$ , independent t-test.

**Appendix Figure 4.** Inhibition of TLR4 does not prevent the *A. fumigatus*-derived FCPs increase in IL-33 expression. Fold increase of IL-33 mRNA expression in human SNECs from patients with CRSwNP (n=5) cultured for 24 hours in medium alone, fibrinogen alone (50 µg/ml), and *A. fumigatus*-derived FCPs (50 µg/ml) in the absence or presence of LPS-RS, a TLR4 antagonist, (1-10 µg/ml). Data are normalized to actin and presented as fold change + SEM. \*\*, p<.01, Kruskal-Wallis. \*, p < .05, Mann-Whitney vs LPS-RS alone.

## Appendix Figure 2



### Appendix Figure 3



Appendix Figure 4

