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Impaired induction of allergic lung inflammation by *Alternaria alternata* mutant MAPK homologue Fus3

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Abstract

The fungal allergen *Alternaria alternata* is associated with development of asthma, though the mechanisms underlying the allergenicity of *Alternaria* are largely unknown. The aim of this study was to identify whether the MAP kinase homologue Fus3 of *Alternaria* contributed to allergic airway responses. Wild-type (WT) and Fus3 deficient *Alternaria* extracts were given intranasal to mice. Extracts from Fus3 deficient *Alternaria* that had a functional copy of Fus3 introduced were also administered (CpFus3). Mice were challenged once and levels of BAL eosinophils and innate cytokines IL-33, thymic stromal lymphopoeitin (TSLP), and IL-25 (IL-17E) were assessed. *Alternaria* extracts or protease-inhibited extract were administered with (OVA) during sensitization prior to ovalbumin only challenges to determine extract adjuvant activity. Levels of BAL inflammatory cells, Th2 cytokines, and OX40-expressing Th2 cells as well as airway infiltration and mucus production were measured. WT *Alternaria* induced innate airway eosinophilia within 3 days. Mice given Fus3 deficient *Alternaria* were significantly impaired in developing airway eosinophilia that was largely restored by CpFus3. Further, BAL IL-33, TSLP, and Eotaxin-1 levels were reduced after challenge with Fus3 mutant extract compared with WT and CpFus3 extracts. WT and CpFus3 extracts demonstrated strong adjuvant activity in vivo as levels of BAL eosinophils, Th2 cytokines, and OX40-expressing Th2 cells as well as peribronchial inflammation and mucus production were induced. In contrast, the adjuvant activity of Fus3 extract or protease-inhibited WT extract was largely impaired. Finally, protease activity and Alt a1 levels were reduced in Fus3 mutant extract. Thus, Fus3 contributes to the Th2-sensitizing properties of *Alternaria*.

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Keywords

allergy; *Alternaria*; asthma; Fus3

INTRODUCTION

Sensitization to the fungal allergen *Alternaria alternata* has been implicated in the development and severity of asthma including an association with fatal/near-fatal attacks after *Alternaria* exposure [1–7]. Dispersion of *Alternaria* spores during warm dry weather periods has been known to be a source of outdoor allergens for sensitized individuals, but *Alternaria* has also recently been detected at high-level indoors and correlates with active asthma symptoms suggesting that this fungal allergen may be more ubiquitous and pathogenic than previously thought [8]. The unique associations with *Alternaria* and asthma are intriguing, but the mechanisms behind the unique pathogenesis of *Alternaria* are not well understood.

The allergenicity of *Alternaria* has largely been attributed to the strong protease activity similar to other fungal allergens and cockroach [9–11]. In vitro studies with human bronchial epithelial cells stimulated with *Alternaria* in the presence of protease inhibitors have demonstrated that thymic stromal lymphopoeitin (TSLP) production and calcium influx were dependent on the protease activity of *Alternaria* as well as epithelial protease-activated receptor 2 (PAR-2) [9, 10]. We have previously demonstrated that the innate eosinophilic lung response to *Alternaria* in vivo was not dependent on PAR-2 suggesting alternative protease or non-protease pathways contribute to innate inflammatory events [12]. Consistent with this, a very recent report showed that the non-protease activities of *Alternaria* are largely required for allergic lung inflammation in vivo [13]. Though these investigations suggest that different components (protease and non-protease) of *Alternaria* contribute to the initiation of type-2 lung inflammation, no reports have used a gene-deficient approach to identify fungal molecular pathways crucial to *Alternaria*'s ability to promote allergic sensitization.

Here, we have identified a role for the MAP kinase homologue Fus3 of *Alternaria* in promoting allergic lung inflammation through studies with gene-deficient *Alternaria* extracts. MAP kinases transduce extracellular signals and are critical for a variety of responses in eukaryotic cells including regulation of cell growth and differentiation. MAP kinase homologues have been discovered in fungal pathogens and include Fus3 and Slr2 [14–16]. The Fus3 pathway of *Alternaria* has been shown to be necessary for conidial development, resistance to copper fungicides, and melanin biosynthesis [16]. In this study, we utilized *Alternaria alternata* extracts from isolates with Fus3 gene disruption and investigated the innate airway response as well as allergic sensitization in mice administered the mutant *Alternaria* extract.

METHODS

Mice

Female C57BL/6J mice (The Jackson Laboratory, Bar Harbor, ME) were used when they reached 8–10 weeks of age. All animal experimental protocols were approved by the University of California, San Diego Animal Subjects Committees.

Alternaria Extracts and OVA

The wild-type strain of *Alternaria alternata* was cultured from citrus leaves and has been characterized elsewhere [15, 16]. The Fus3-deficient mutant and the CpFus3 strain expressing a functional Fus3 were created as previously reported [16]. Fus3 was inactivated by targeted gene disruption, using two fusion DNA fragments overlapping within the bacterial hygromycin phosphotransferase gene (HYG) that confers resistance to hygromycin. Successful disruption of Fus3 was validated by Southern blotting. Fungi (wild type, Fus3 mutant, and complementation strain CpFus3) were grown on potato dextrose broth (Difco, Sparks, MD) for 3 days under light. The mycelia were harvested and ground prior to extraction with cold 10 mM ammonium bicarbonate buffer, pH 8.2. The supernatants were collected after centrifugation at 8000×g for 30 minutes, mixed thoroughly with six volumes of pre-chilled (−20°C) acetone in glass bottle, and incubated overnight at −20°C. After centrifugation at 10,000×g for 60 minutes, extracts were resuspended in water and dialyzed against distilled water in 4°C overnight. Protein concentrations of each extract were determined by BCA assay (Pierce, IL) with BSA standard. OVA (Lot No. 51M12980), purified ovalbumin free of LPS, was obtained from Worthington (NJ).

Mouse Airway Inflammation Models

To elucidate the innate airway immune response induced by *Alternaria* extracts, mice were given a single intranasal administration of *Alternaria* extract (normalized to 40 µg of protein) or PBS under isoflurane (Vedco, Inc. St. Joseph, MO) anesthesia and euthanized 6 hours or 3 days later. To test the adjuvant activity of *Alternaria* extracts, mice were intranasally administered 100 µg OVA in the presence or the absence of *Alternaria* extracts (protein level 20 µg) in 60 µL of PBS on days 0 and 7. On days 21–23, the mice were challenged intranasally with 100 µg of OVA in 40 µL PBS. Mice were euthanized 24 hours after the final challenges.

Protease Inhibitor Studies

WT *Alternaria* extract was incubated with a protease inhibitor cocktail in DMSO (Calbiochem #539136) or DMSO alone and diluted 1:10 with extract for 30 minutes at 37°C. Inhibition of protease activity was confirmed prior to in vivo use. The extract was then combined with OVA (100 µg/mouse) and administered intranasally to mice.

Lung Processing and BAL Cellular Analysis

Lung and BAL processing was performed as previously reported [17]. The BAL fluid was obtained by intratracheal insertion of a catheter and five lavages with 0.5 mL of PBS containing 2% filtered bovine serum albumin (Sigma). The BAL fluid was centrifuged at

1500 rpm for 5 minutes and supernatants collected and frozen until ELISA was performed. Total BAL cells were counted with the Accuri C6 Flow Cytometer (BD). Cells were then incubated with a monoclonal antibody to CD16/CD32 (24G.2) for 10 minutes to block Fc receptors prior to staining with PE-conjugated Siglec-F, APC-conjugated Gr-1, and FITC-conjugated CD11c (eBioscience). BAL eosinophils were identified as Siglec F-positive CD11c-negative cells [18]. Neutrophils were identified as GR-1 positive Siglec-F negative cells. For evaluation of BAL lymphocytes from OVA challenged mice, BAL cells were stained with FITC-conjugated CD4 (eBioscience), biotinylated T1/ST2 (MD Bioscience, clone DJ8), and PE CD134 (OX40) (eBioscience) followed by Streptavidin APC. Flow cytometry was performed using an Accuri C6 Flow Cytometer (BD Biosciences) and sample data were further analyzed with Flow Jo software (Tree Star). For lung processing, the lungs were tied off at the trachea with surgical suture and were preserved in 10% buffered formalin (EMS, PA) before being embedded in paraffin for subsequent sectioning and staining.

Cytokine Assays

BAL supernatants were assessed for levels of IL-5, IL-13, IL-33, IL-17E, Eotaxin-1, and TSLP by ELISA according to the manufacturer's instructions and read with a BioRad Model 680 microplate reader. All ELISA kits used for BAL studies were from R&D Systems (Minneapolis, MN).

Periodic Acid Schiff (PAS) Expression

The paraffin-embedded lungs were sectioned at 5 μ m onto microscope slides. Periodic acid schiff (PAS) stained airway goblet cells were enumerated under light microscopy examination (X200 magnification). The number of PAS-positive as well as total airway epithelial cells was assessed in 8–10 randomly selected medium-sized closed circular bronchi (defined by having approximately 100–150 luminal airway epithelial cells). At least eight bronchioles were counted in each slide. Results are expressed as the ratio of PAS positive cells/bronchiole which is calculated from the number of PAS positive epithelial cells per bronchus divided by the total number of epithelial cells of each bronchiole. All slides were blinded during analysis.

Inflammation Index

After paraffin embedding, sections were stained with Hematoxylin and Eosin (H&E). An index of pathologic changes was utilized as previously reported [17] by scoring the inflammatory cell infiltrates (including eosinophils and mononuclear cells) around airways for greatest severity (0, normal; 1, <3 cell diameter thick; 2, 3–10 cells thick; 3, >10 cells thick) and overall extent (0, normal; 1, <10% of sample; 2, 10%–25%; 3, >25%) in the visual field. The index was calculated by multiplying severity by extent, with a maximum possible score of 9.

Protease Assay

Protease assays of each extract were carried out with the QuantiCleave™ Protease Assay Kit (Pierce). Briefly, 50 μ L of each extract or standard sample (serial diluted trypsin solution)

were incubated with 100 μ L of succinylated casein solution as substrate for 20 minutes at room temperature (RT) in a 96-well microplate followed by incubation with 50 μ L of 5% 2,4,6-trinitrobenzene sulfonic acid in methanol for 20 minutes at RT. The absorbances in wells were subsequently measured in a plate reader set to 450 nm.

Alt a1 Level Assay

The Alt a1 assay was performed with an Alt a1 ELISA kit (Indoor Biotech, VA) according to manufacturer's instruction.

Chitin Assay

For chitin purification, fungal mycelium was extracted with a buffer containing 50 mM Tris-HCl (pH 7.8), 2% SDS, 0.3 M β -mercaptoethanol, and 1 mM EDTA. After centrifugation at 8000 $\times g$ for 10 minutes, chitin was acidified in 6 M HCl, boiled at 100°C for at least 4 hours. Chitin was quantified by measuring the acid-released glucosamine from chitin using *p*-dimethylaminobenzaldehyde as a chromogen and measuring spectrophotometrically at A₅₂₀ [15].

Statistical Analyses

All results are presented as mean $\pm$ SEM. A statistical software package (Graph Pad Prism, San Diego, CA) was used for the analysis. *P*-values of <.05 were considered statistically significant.

RESULTS

Fus3 is Required by *Alternaria* for Induction of Innate Type 2 Responses

We have previously reported that administration of a single intranasal challenge of *Alternaria* extract to unsensitized mice results in recruitment of eosinophils as early as 24 hours [12, 19]. To test whether extracts from WT, Fus3 mutant, and complementation Fus3 (CPFus3) strains of *Alternaria alternata* could induce an innate eosinophilia and cytokine production, we generated extracts from these strains that have been previously reported [16]. Mice were challenged with a single intranasal administration of each extract as well as PBS (Figure 1). Mice receiving one challenge with WT *Alternaria* extract, but not PBS control, developed airway eosinophilia within 3 days (Figure 1A). In contrast, the BAL eosinophil levels were significantly reduced in mice receiving the same dose of Fus3 mutant extract. The CPFus3 extract generated from mutant Fus3 *Alternaria* with introduction of a wild-type Fus3 gene was able to rescue much of the eosinophil reduction found in mice receiving Fus3 extract. Thus, Fus3 is required by *Alternaria* for induction of innate eosinophilia.

Cytokines that initiate allergic responses include IL-33, TSLP, and IL-25. Within 3–6 hours after one *Alternaria* challenge in mice, we have previously reported that IL-33 levels are dramatically increased in the BAL and the innate eosinophilia requires IL-33 signaling [12, 19]. Therefore, we measured IL-33, TSLP, and IL-25 levels by ELISA 6 hours after one challenge with WT, Fus3 mutant, and CpFus3 extracts (Figure 1B). Mice that received WT and CPFus3 extracts mounted increases in IL-33 that were significantly higher compared with mice receiving Fus3 mutant extract or PBS. Further, levels of TSLP were increased

after WT extract and CPFus3 extract challenge, though to a much lower overall extent compared with IL-33. Measurement of IL-25 levels revealed no differences among the groups. We have previously shown that BAL Eotaxin-1 (CCL11) is increased after one challenge with *Alternaria* [12]. We therefore measured Eotaxin-1 levels that may have accounted for differences in eosinophil recruitment into the lung after challenge with the different extracts (Figure 1B). We found that mice challenged with WT extract had increased Eotaxin-1 in the BAL, whereas mice receiving Fus3 mutant extract had undetectable levels of Eotaxin-1 similar to PBS challenged mice. Therefore, the innate eosinophilia as well as IL-33, TSLP, and Eotaxin-1 production after *Alternaria* challenge require the presence of intact Fus3.

Fus3 is Required by *Alternaria* for Th2 Lung Inflammatory Responses

Alternaria alternata has been shown to induce strong Th2 sensitization to bystander proteins such as OVA and ragweed, and can thus act as a Th2-sensitizing adjuvant [20]. We hypothesized that the Fus3 mutant *Alternaria* may be impaired in generating Th2 responses to OVA protein compared to WT extract. Mice receiving intranasal sensitization with WT *Alternaria* and pure OVA protein on days 0 and 7 that were subsequently challenged with OVA alone 2 weeks later (Figure 2A) developed a robust Th2 response including increased BAL cellularity with predominately eosinophils (Figures 2B and C). Mice administered OVA alone intranasally without systemic sensitization developed tolerance [21, 22] and did not develop eosinophilic airway inflammation (Figures 2B and C). Th2 cytokines IL-5 and IL-13 were increased in the BAL of mice receiving WT *Alternaria* during sensitization, but not from mice receiving OVA alone (Figure 2D). Impressively, mice receiving Fus3 mutant *Alternaria* extract failed to develop airway eosinophilia and Th2 cytokine production (Figures 2C and D). In contrast, administration of CPFus3 *Alternaria* restored levels of airway eosinophils and Th2 cytokines similar to mice receiving WT *Alternaria*.

Peribronchial infiltration and epithelial mucus production are features of chronic asthma. We next determined whether mice undergoing the protocol in Figure 2A developed these features. As expected, mice receiving OVA alone without systemic sensitization developed very little, if any, peribronchial infiltration of cells (Figure 3A) or epithelial mucus production as measured by PAS+ staining (Figure 3B). In contrast, lung sections from mice that were administered WT *Alternaria* revealed robust peribronchial inflammation and epithelial mucus production (Figures 3A and B). Similar to the reductions in airway eosinophilia and Th2 cytokine levels, the Fus3 mutant *Alternaria* given with OVA induced very reduced peribronchial infiltration and mucus production that was largely restored with CPFus3 administration (Figures 3A and B). Thus, *Alternaria* requires Fus3 to generate a Th2 inflammatory response to a bystander protein compared with WT extract.

Fus3 is Required by *Alternaria* for Accumulation of Activated Lung Th2 Cells

One of the hallmarks of allergic asthma is the accumulation of activated Th2 cells in the lung that are thought to orchestrate the allergic inflammatory response. Th2 cells can be identified in mice by expression of T1/ST2, the receptor for IL-33 [23, 24]. Further, CD4 T cells that express the TNF receptor family member OX40 identify activated T cells that have been shown to contribute critically to allergic airway inflammation through OX40 signaling

[25, 26]. Mice undergoing the protocol in Figure 2A were assessed for levels of Th2 cells that express OX40. BAL cells were stained for CD4, T1/ST2, and OX40 and analyzed by FACS. CD4 cells were gated from BAL lymphocytes and analyzed for expression of T1/ST2 and OX40. Mice receiving OVA alone intranasally without systemic sensitization had very few T1/ST2 positive CD4 cells in the BAL with absent OX40 expression (Figures 4A and B). In contrast, mice receiving WT *Alternaria* plus OVA during sensitization had a significant increase in BAL CD4+ T1/ST2+ cells with over 15% positive for OX40 suggesting an activated phenotype (Figures 4A and B). The percent of OX40+ cells within the CD4 population was approximately three times higher in mice receiving WT *Alternaria* compared with Fus3 *Alternaria* (15.2% versus 6.33%). The total numbers of OX40-expressing Th2 cells were more dramatically decreased (approximately 10-fold) in mice receiving Fus3 mutant *Alternaria*, though CPFus3 restored the numbers of OX40+ Th2 cells to levels similar to mice receiving WT *Alternaria*. Thus, Fus3 is required by *Alternaria* for accumulation of activated Th2 cells in the lung.

Fus3 Contributes to *Alternaria* Protease Levels and Major Allergen Alt a1

The allergenicity of *Alternaria alternata* has been attributed to multiple factors including protease content and levels of the major allergen *Alt a1* [9–11, 27]. We sought to identify whether Fus3 mutation had an effect on protease, chitin, or *Alt a1* levels. We detected an approximately 35% reduction in the levels of protease in Fus3 mutants compared with WT *Alternaria* (Figure 5A). Further, CPFus3 restored levels of protease above WT levels. Levels of the major allergen *Alt a1* were also reduced in Fus3 mutants and were restored in the CPFus3 strain (Figure 5B). Administration of chitin to mice has been shown to result in eosinophilic inflammation [28, 29]. However, there was no significant difference at levels of fungal chitin (data not shown). Thus, the ability for Fus3 to promote the allergenicity of *Alternaria* may be in part due to protease content and levels of major allergen.

Alternaria Protease Inhibition Impairs Allergic Sensitization

To determine whether the protease activity of *Alternaria* contributes to allergic sensitization in our model, we incubated WT *Alternaria* with or without a protease inhibition cocktail prior to performing the OVA adjuvant protocol (Figure 6A). As in Figure 2, OVA given with WT *Alternaria* during sensitization induced high levels of BAL eosinophils compared with OVA alone (Figure 6B). Impressively, mice receiving *Alternaria* extract that had been incubated with protease inhibitor displayed very reduced BAL eosinophilia. Further, accumulation of BAL Th2 cells and activated OX40-expressing Th2 cells were also significantly reduced in mice receiving *Alternaria* extract pretreated with protease inhibitor compared to *Alternaria* without inhibitor (Figure 6C). This suggests that the protease activity of *Alternaria* contributes to allergic sensitization to a bystander antigen.

DISCUSSION

We have identified a role for the MAP kinase homologue Fus3 of *Alternaria* in promoting allergic lung inflammation. We utilized *Alternaria alternata* extracts from isolates with Fus3 gene disruption and investigated the innate airway response as well as allergic sensitization in mice administered mutant *Alternaria* extract with harmless protein. We found that Fus3

mutant *Alternaria* extracts were impaired in induction of innate lung eosinophilia, IL-33, and TLSP production. Further, Fus3 was required by *Alternaria* as an adjuvant to promote activated Th2 cell accumulation and lung inflammatory responses. Finally, we detected reduced levels of protease and major allergen content in the mutant extract.

We determined that mice receiving one challenge with intranasal Fus3 mutant *Alternaria* developed reduced levels airway eosinophils, IL-33, TSLP, and Eotaxin-1 compared with mice challenged with WT *Alternaria*. We have previously reported that *Alternaria* extract administered to the airways of mice induced an innate eosinophilic response dependent on IL-33R signaling and activation of type-2 innate lymphoid cells (ILC2) [12, 19]. Recent studies have also demonstrated that the protease allergen papain induces IL-33 production and innate eosinophilic lung responses dependent on ILC2s, suggesting that protease activity alone is sufficient to drive innate lung eosinophilia [30, 31]. Thus, in our current studies, the reduced levels of IL-33 in mice challenged with Fus3 mutant *Alternaria* are likely responsible for the impaired eosinophilic lung response that may be, in part, dependent on intact *Alternaria* protease levels.

Importantly, we demonstrated that Fus3 mutation abrogated the adjuvant activity of *Alternaria* in promoting Th2 responses to a bystander protein. A previous report showed that *Alternaria* administered with OVA protein during sensitization could induce an OVA-specific Th2 lung response and implicated activation of dendritic cells by *Alternaria* as a likely mechanism [20]. Fus3 mutation likely regulates many aspects of *Alternaria*'s ability to induce allergic responses including effects on levels of protease activity. Several studies have determined that protease allergens activate dendritic cells through a PAR-2 pathway resulting in DC migration to lymph nodes and initiate Th2 cell priming [32–34]. Whether products of Fus3 pathways directly induce dendritic cell responses that favor Th2 cell priming remains to be investigated in future studies.

The capacity for the *Alternaria* Fus3 pathway to regulate the allergenicity of *Alternaria* has not been reported. MAP kinase homologues in *Alternaria* include Fus3 and Slf2 and the Fus3 pathway has been shown to be important for conidial development, resistance to copper fungicides, and melanin biosynthesis [14–16]. Studies of *Alternaria alternata* have demonstrated that IgE-binding allergens are present in both conidia and fragmented hyphae [35, 36]. The extracts we have used in our studies are from fungal cultures that are predominately filamentous hyphae and contain very few spores. Though human inhalation of spores is largely thought to trigger asthma symptoms in sensitized individuals, there is evidence that airborne fungal hyphae contain allergen at high levels suggesting that environmental hyphal elements may also a source of allergen [37]. Despite this, one limitation of our work is that Fus3 is required for conidial development and thus has pleiotropic effects on the development and normal life-cycle of *Alternaria alternata* that may be responsible for allergic sensitization.

In our studies, we detected reduced levels of protease in the mutant Fus3 extract. Extracts from *Alternaria alternata* are complex and likely have multiple pathogen-associated molecular patterns that may activate innate immune pathways leading to Th2 responses. Protease activity of allergens including *Alternaria* has been implicated in allergic

sensitization and TSLP production [9–11]. Consistent with this, we detected reduced protease levels and airway TSLP levels in our in vivo studies utilizing the Fus3 mutant extract though we cannot conclude that the reduction in TSLP is a direct effect of reduced protease activity. We also determined that protease activity of *Alternaria* contributes to allergic sensitization to OVA protein suggesting that the reduced protease activity of the Fus3 mutant extract may be partially responsible for the development of an impaired Th2 response to OVA. However, the reduction in protease activity of the Fus3 extract was approximately 35% compared with WT extract and likely does not account for the demonstrated impairment in allergic sensitization. This strongly suggests that non-protease pathways regulated by Fus3 also contribute to the adjuvant properties of *Alternaria*.

Finally, we detected reduced levels of the major allergen Alt a1 in the mutant Fus3 extract. Interestingly, the dominant IgE binding major allergen Alt a1 is associated with increased asthma severity when present at high levels within *Alternaria* [38]. We also assessed for levels of chitin differences among the extracts as administration of chitin to mice has been shown to result in eosinophilic inflammation [28, 29]. We did not find differences in chitin levels (not shown) and chitin alone is not likely to be primarily responsible for the unique pathogenicity of *Alternaria alternata* as other fungi contain ample chitin as well [39]. Though we detected reduced levels of protease as well as Alt a1 in the Fus3 mutant extracts, there are likely other pathways regulated by Fus3 that are critical to the allergenicity of *Alternaria* to be investigated in future studies.

CONCLUSIONS

We have utilized an allergen gene deletion strategy to demonstrate the novel finding that the MAP kinase homologue Fus3 of *Alternaria* promotes Th2 lung inflammation. Airway instillation of *Alternaria alternata* extracts from isolates with the Fus3 gene disruption led to reduced innate lung eosinophilia as well as impairment in Th2 adjuvant activity. Thus, these investigations provide insight into how *Alternaria*, an allergen associated with severe human asthma, may promote allergic lung inflammation.

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FIGURE 1.

Fus3 is required by *Alternaria* for induction of innate type 2 responses. BAL cellular analysis at 3 days after single airway exposure of *Alternaria* extracts (WT, Fus, CpFus) or PBS challenge. (A) Eosinophils were identified as Siglec-F⁺ CD11c⁻ cells by flow cytometry (right) and enumerated (left). The results are expressed as the mean $\pm$ SEM ($n = 4-6$ in each group), $*P < .05$, $**P < .01$, Mann-Whitney. (B) Levels of BAL IL-33, TSLP, IL-25, and Eotaxin-1 6 hours after a single airway challenge with *Alternaria* extracts. The results are from two independent experiments and expressed as the mean $\pm$ SEM ($n = 6$ in each group), $**P < .01$, Mann-Whitney.

FIGURE 2.

Fus3 is critical for the adjuvant activity of *Alternaria* to induce Th2 lung responses. Mice received intranasal OVA with or without various *Alternaria* extracts on days 0 and 7 followed by OVA only challenges days 21–23. (A) Protocol showing a mouse airway sensitization and challenge model. (B) BAL total cells and (C) eosinophils enumerated 1 day after last OVA challenge. Eosinophil percent shown on right. (D) Levels of BAL IL-5 and IL-13 from mice undergoing the protocol in panel (A). The results are expressed as the mean $\pm$ SEM ($n = 4$ mice in each group), $*P < .05$, Mann-Whitney. One of the two independent experiments are shown.

FIGURE 3.

Alternaria requires Fus3 to initiate lung inflammation and mucus production. Lung sections from mice undergoing the protocol in Figure 2A were stained for H&E (A) and PAS (B) and scored (right). Scale bars 100 μ m. Results from at least 8 airways per mouse, 4 mice per group, mean $\pm$ SEM * P < .05, ** P < .01, *** P < .001, Mann–Whitney.

FIGURE 4.

Fus3 is required by *Alternaria* for accumulation of activated lung Th2 cells. BAL cells from mice undergoing the protocol in Figure 2A were stained for CD4, T1/ST2, and OX40 prior to FACS analysis. (A) BAL CD4+ lymphocytes from mice receiving OVA with or without various *Alternaria* extracts were analyzed for T1/ST2 and OX40 expression. (B) BAL CD4+T1/ST2+ T cells, CD4+OX40+ T cells, and CD4+T1/ST2+OX40+ T cells enumerated 1 day after last OVA challenge. The results are expressed as the mean $\pm$ SEM ($n = 4$ mice in each group), $*P < .05$, Mann-Whitney. One of the two independent experiments are shown.

FIGURE 5.

Protease and Alt a1 levels are reduced in *Alternaria* Fus3 mutants. Levels of protease (A) and Alt a1 (B) in *Alternaria* extracts. Six wells/group protease assay and 3–4 wells per group Alt a1 repeated twice. $P < .05$, Mann–Whitney U.

FIGURE 6.

Alternaria protease activity is critical for allergic sensitization and induction of Th2 lung responses. Mice received intranasal OVA with or without WT *Alternaria* extract pre-treated with or without protease inhibitor on days 0 and 7 followed by OVA only challenges days 21–23. (A) Protocol showing a mouse airway sensitization and challenge model. (B) BAL eosinophils percent and total numbers enumerated one day after last OVA challenge. (C) BAL CD4⁺ lymphocytes from mice undergoing protocol in panel (A) were analyzed for T1/ST2 and OX40 expression and enumerated. The results are expressed as the mean $\pm$ SEM ($n = 3-4$ mice in each group), * $P < .05$, t -test. PI = protease inhibitor.