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***Aspergillus fumigatus* alkaline protease 1 (Alp1/*Asp f13*) in the airways correlates with asthma severity**

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Summary

A fungal protease (*Alp1/Asp f13*) from *Aspergillus fumigatus* was detected in the airways of subjects with asthma but not controls, which correlated strongly with disease severity, respiratory dysfunction, and steroid use.

Keywords

Asthma; molds; *Aspergillus fumigatus*; protease; allergen; bronchial smooth muscle

To the Editor

Although fungi are ubiquitous in the environment and respiratory exposure to airborne spores occurs on a daily basis, sensitization and disease due to such exposure is relatively infrequent. *Aspergillus fumigatus* (*Af*) has been linked to severe, uncontrolled asthma. A potentially unique phenotype, termed “severe asthma with fungal sensitization” (SAFS) is

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Disclosures

The authors declare no conflicts of interest.

thought to describe approximately 20–25% of individuals with uncontrolled, persistent disease.¹ Previously, we detected a known allergen serine protease from *Af*, Alkaline protease 1 (Alp1), in the bronchial submucosa of subjects with mild/moderate asthma but not healthy controls and in allergen-challenged but not naïve mice.² We determined that Alp1 disrupted airway smooth muscle (ASM)-extracellular matrix (ECM) interactions through its protease activity, which augmented Ca²⁺ signaling and RhoA activation. Based on these initial findings, we hypothesized that: 1) Alp1 protease contributes the pathogenesis of asthma in some patients; 2) Alp1 abundance in the airways correlates with disease severity.

To further delineate the role of Alp1 in the lower respiratory tract in asthma, we evaluated Alp1 immunoreactivity in airway sections from 36 asthmatics and 10 healthy controls and in induced sputum supernatants from another 29 asthmatics and 10 healthy controls. The demographics of these subjects are described in Tables E1 and E2 in this Letter's Online Repository. There were an approximately equal number of male/female subjects, and the median age was 34 years (range 18–68 years). The group with severe asthma was significantly older than the healthy control group (median age 49, range 20–68 years; $p=0.01$). Subjects with asthma were classified as mild/moderate or severe based on GINA criteria. Immunostaining of airway sections with anti-Alp 1 antisera detected Alp1 immunoreactivity in bronchial smooth muscle bundles of lungs of subjects with asthma but not in healthy subjects, and Alp1 quantities in the bronchial smooth muscle layer increased progressively with asthma severity (Figure 1A–B). Little to no staining of was observed when airway sections from subjects with severe asthma were incubated with non-specific rabbit antisera (Figure E1). Surprisingly, Alp1 abundance was roughly equivalent in patients with and without sensitivity to *Af*(Figure 1C). We also observed similar patterns of Alp1 staining within the bronchial epithelium (Figure E2).

Alp1 immunoreactivity in ASM negatively correlated with pre-bronchodilator FEV₁ (analysis of the cohort as a whole or restricted to the asthma group only) and PC₂₀ (the provocative concentration of inhaled methacholine inducing a 20% decrease in FEV₁) (Figure 2A–B). Alp1 immunostaining in airways from patients receiving short or long acting β -agonists only (SABA/LABA, respectively) was significantly less than in the airways of patients requiring LABA plus inhaled corticosteroids (ICS), and Alp1 quantities correlated with ICS requirement (fluticasone equivalent dose) (Figure 2C–D).

Because we previously detected Alp1 in bronchoalveolar lavage fluid (BALF) of *Af* sensitized and challenged mice, but not naïve mice, by immunoblotting, we determined whether it could also be measured non-invasively in sputum from human subjects by immunoblotting. We detected a ~33 kDa band corresponding to the protease purified from commercial *Af* allergen extracts or from supernatants of *Af* cultures (Figure E3A **left panel**). In sputum samples from subjects with asthma, but not healthy controls, we observed an additional band at ~42–45 kDa, potentially representing an unprocessed precursor³ as detection of either band was strongly reduced or eliminated when blots were incubated with Alp1 antibody preadsorbed with antigen (Figure E3A **right panel**). This band was present only in sputum from subjects with asthma but not controls (Figure E3B). To determine whether Alp1 quantities in induced sputum correlated with disease severity, we generated a

standard curve based on quantitative immunoblotting of defined amounts of purified Alp1 (Figure E4A). However, Alp1 quantities were similar in sputum from patients with mild/moderate and severe asthma (Figure E3B). Alp1 quantities were significantly higher in sputum from patients with *Af* sensitivity than those without, regardless of clinical severity (Figure E4C). Alp1 levels were also significantly correlated with sputum neutrophil, but not eosinophil counts (Figure E4D and data not shown). We did not detect Alp1 in sera from healthy subjects or patients with asthma. Collectively, these results suggest that Alp1 protease may contribute to severe asthma through two distinct mechanisms: 1) allergenicity; and 2) proteolytic destruction of lung tissue, which could promote influx of neutrophils into the airway lumen.

Several observations support a pathogenic contribution of fungal colonization of the airways to severe asthma. Chronic fungal exposure is linked to asthma exacerbations in both children and adults.⁴ In experimental models of asthma in mice, chronic respiratory inoculation with fungi induces a more severe asthma phenotype than inhalation of other allergens such as house dust mite (HDM) through IL-33-dependent yet steroid-independent mechanisms.⁵ Alp1 is the major serine protease secreted by *Af*, and serine proteinase activity is crucial for *Af* to induce allergic airway inflammation and AHR.^{6, 7} Here we found equivalent Alp1 quantities in the airways regardless of *Af* sensitivity and no correlation between sputum Alp1 and eosinophils, suggesting that an IgE/type 2 response to *Af* is not strictly required for the pathogenicity of Alp1 in asthma. Fungal protease allergens may exert direct pathogenic effects on epithelial barrier function by disrupting cell-cell junctions, inducing cytoskeletal rearrangements, and promoting cytokine (e.g. IL-8) secretion.^{8, 9} Alp1 in sputum correlated well with sputum neutrophils, suggesting that it might increase epithelial secretion of neutrophil chemoattractants and thereby actively promote neutrophil migration to the airway lumen.

Alp1 may persist in the airways of patients with severe asthma due to impaired mucociliary clearance mechanisms, i.e. asthma-induced alteration of epithelium morphology and/or corticosteroid use. Alp1 also activates mucin gene expression in airway epithelial cells, which could further impede fungal clearance. Because our study was retrospective and cross-sectional, we could not address the utility of Alp1 measurements to evaluate longitudinal parameters including disease control and progression, or responses to specific treatments. However, Alp1 could be explored as a biomarker for endotype classification and/or surrogate for assessment of therapeutic interventions as it is easily identified in the airways from subjects with asthma but is virtually undetectable in samples from healthy controls, and it strongly correlates with hard functional endpoints (FEV1, PC₂₀).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used

Af	<i>Aspergillus fumigatus</i>
Alp1	Alkaline protease 1
SAFS	severe asthma with fungal sensitization
AHR	airway hyperresponsiveness
ABPA	allergic bronchopulmonary aspergillosis
ECM	extracellular matrix
IHC	immunohistochemistry
BALF	bronchoalveolar lavage fluid
ICS	inhaled corticosteroid
GINA	Global Initiative for Asthma
SABA	short acting β -agonist
LABA	long acting β -agonist

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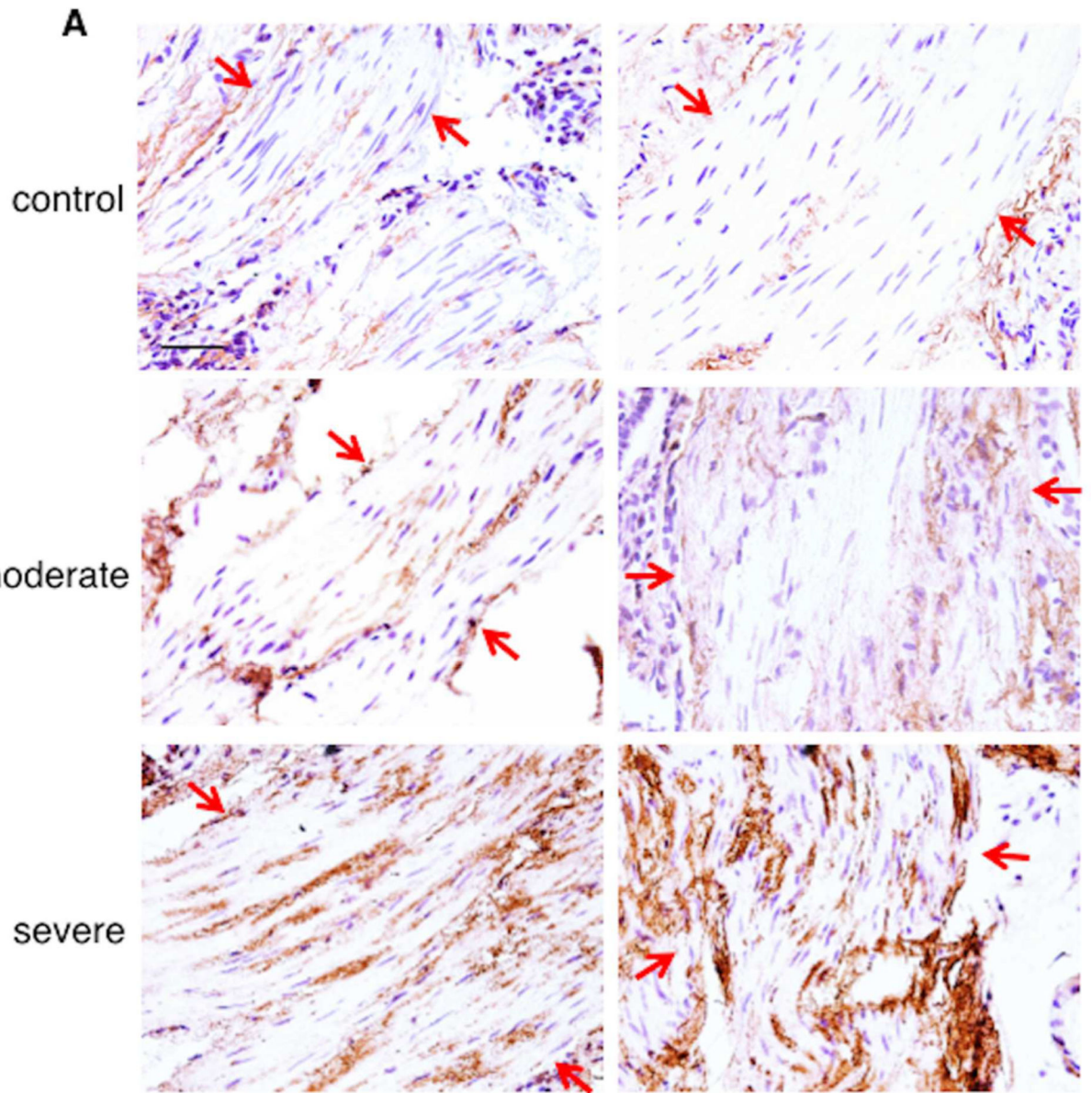
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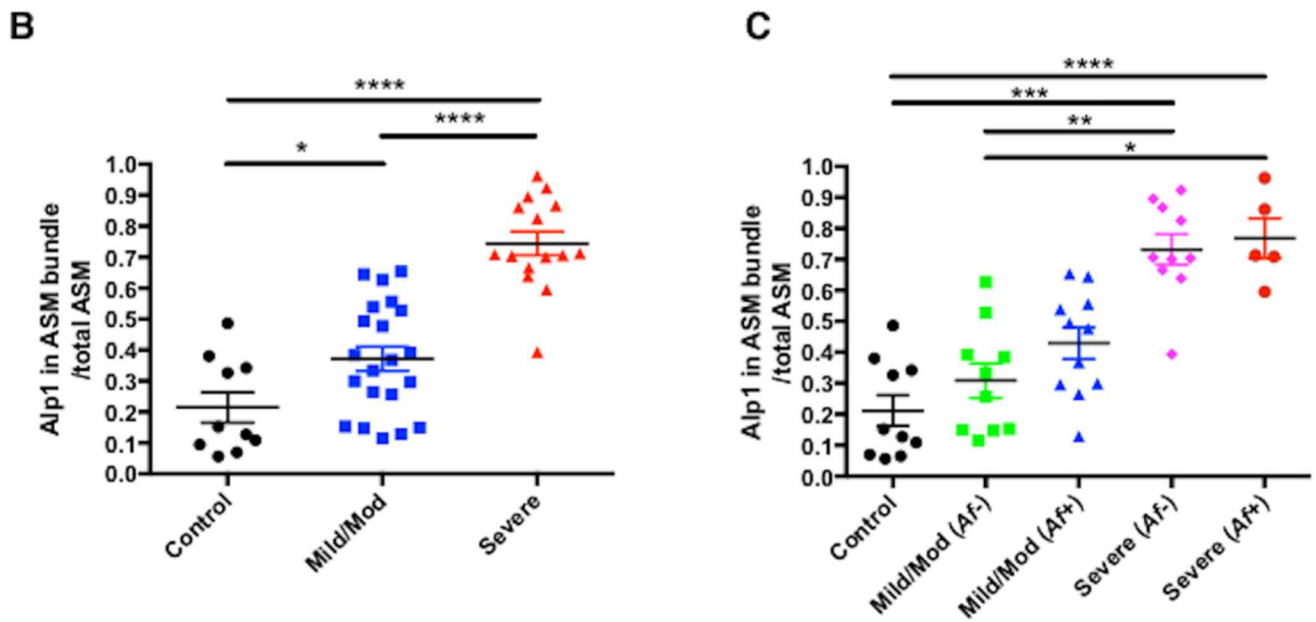


Figure 1. Alp1 is present in human airways and correlates with asthma severity

(A) Airway sections were immunostained with anti-Alp1Ab (representative images from 10–21 subjects/group (original magnification, 40×, bar=50 μm). Arrows delineate smooth muscle areas. (B–C) Alp1 staining in ASM increases with asthma severity (B) but not sensitivity to *Af*(C). Quantified using ImageJ and represented as Alp1⁺ ASM per total ASM area. (A) **p*=0.04, *****p*<0.0001, one way ANOVA. (B) *, **, ***, **** *p*=0.01; 0.004; 0.0008; <0.0001, Kruskal Wallis.

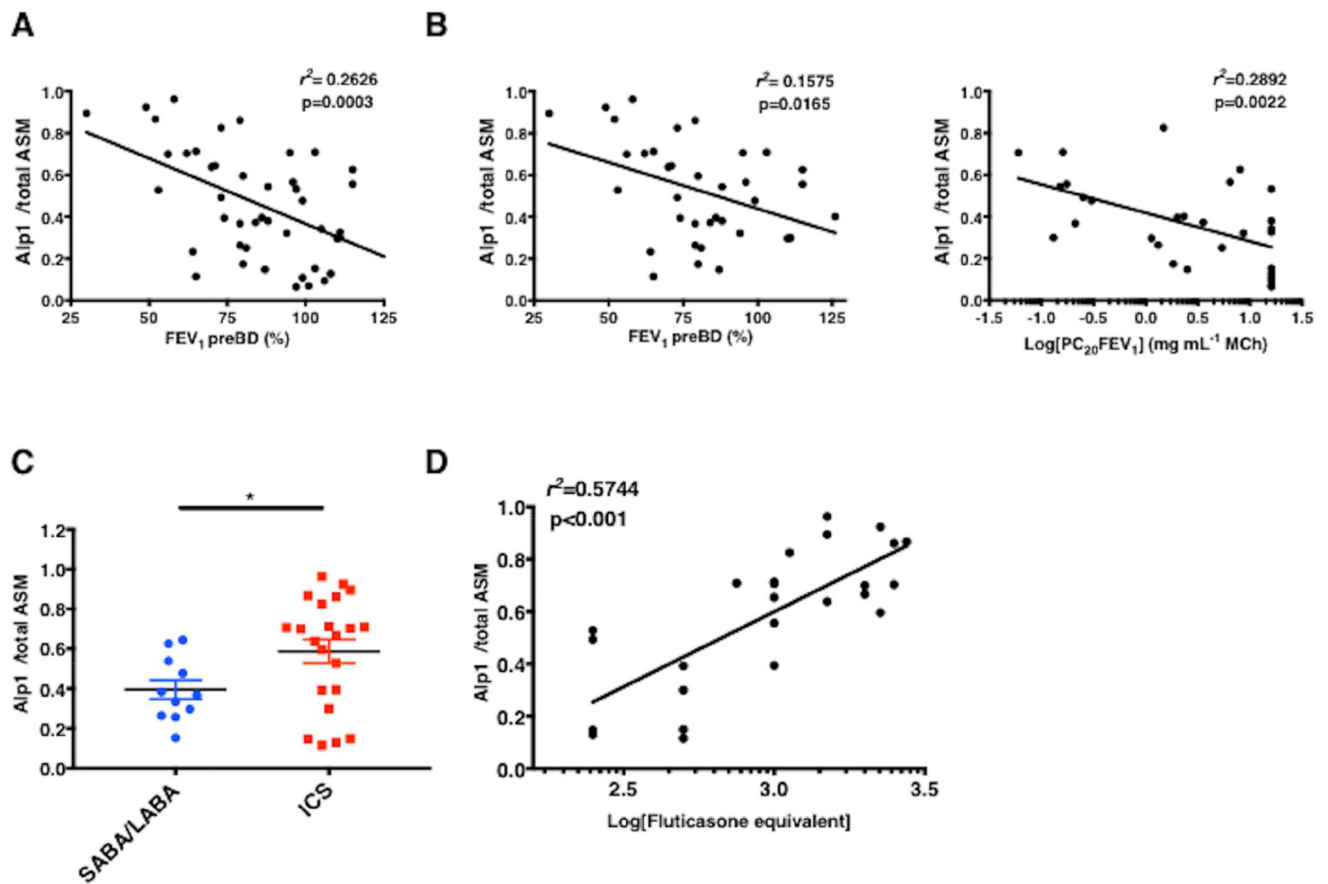


Figure 2. Alp1 immunostaining in ASM correlates with lung functional impairment (A–C) Correlation between Alp1 staining and FEV₁ pre-bronchodilator (BD) in the entire cohort (A) or among subjects with asthma (B); Alp1 correlation between Log(PC₂₀FEV₁) (C). Alp1 immunostaining in airways of subjects with asthma treated with ICS or SABA/LABA (* $p=0.03$, Mann-Whitney). (D) Correlation between Alp1 staining in airways and ICS requirement [Log(fluticasone equivalent)].