A fungal protease allergen provokes airway hyper-responsiveness in asthma

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Asthma, a common disorder that affects >250 million people worldwide, is defined by exaggerated bronchoconstriction to inflammatory mediators including acetylcholine (ACh), bradykinin and histamine—also termed airway hyper-responsiveness. Nearly 10% of people with asthma have severe, treatment-resistant disease, which is frequently associated with immunoglobulin-E sensitization to ubiquitous fungi, typically Aspergillus fumigatus (Af). Here we show that a major Af allergen, Asp f13, which is a serine protease, alkaline protease 1 (Alp 1), promotes airway hyper-responsiveness by infiltrating the bronchial submucosa and disrupting airway smooth muscle (ASM) cell-extracellular matrix (ECM) interactions. Alp 1-mediated ECM degradation evokes pathophysiological RhoA-dependent Ca2+ sensitivity and bronchoconstriction. These findings support a pathogenic mechanism in asthma and other lung diseases associated with epithelial barrier impairment, whereby ASM cells respond directly to inhaled environmental allergens to generate airway hyper-responsiveness.

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In asthma, chronic lung inflammation due to the immune response to inhaled allergens leads to desquamation of the respiratory epithelium, increased bronchial smooth muscle mass and mucous gland hypertrophy\(^1\,^2\). However, the mechanisms by which such airway ‘remodelling’ brings about enhanced contraction of airway smooth muscle (ASM) cells to pro-contractile ligands are poorly understood\(^3\). Intrapерitoneal immunization of mice with a diverse array of allergens including ovalbumin (OVA), house dust mite (HDM) or Aspergillus fumigatus (Af) followed by respiratory mucosal challenge induces what is termed as ‘allergic sensitization’: expansion of allergen-specific T helper type 2 (T\(_{\text{H}2}\)) cells, synthesis of allergen-specific immunoglobulin-E (IgE) and production of cytokines in lung including interleukin-4 (IL-4), IL-5 and IL-13. These events result in allergic inflammation including goblet cell metaplasia, increased mucous production, leukocyte infiltration (predominantly eosinophils) and collagen deposition in the bronchial submucosa\(^4\,^7\).

Protease activity is a common and important feature of many allergens capable of inducing asthma. A secreted protease of Aspergillus oryzae (Ao) was shown to elicit T\(_{\text{H}2}\) immunity and allergic inflammation in mice through proteolytic cleavage of fibrinogen. Fibrinogen cleavage products can act as Toll-like receptor 4 ligands on respiratory epithelial cells, innate lymphoid cells and macrophages to elicit cytokine production and upregulation of mucin genes that promote the asthma phenotype\(^8\). Serine protease allergens can both activate and impair respiratory epithelial functions. An alkaline serine protease from the allergenic fungus Penicillium citrinum (Pen c13) induced proinflammatory cytokine release in airway epithelial cells through activation of coagulation factor II (thrombin) receptor and coagulation factor II-like receptor 2 (F2R and F2RL1, previously known as protease-activated receptors (PAR) 1 and 2)\(^9\). Both Pen c13 and a serine protease component of the ubiquitous mold Alternaria alternata induce respiratory epithelial barrier dysfunction through altered cell–cell junctions and actin cytoskeletal rearrangements\(^10\,^11\).

Induction of allergic sensitization and airway hyper-responsiveness (AHR) in mice by allergens generally requires priming with both the allergen and an adjuvant at sites distant from the lung. However, short-term respiratory mucosal exposure of mice to protease-containing allergens such as Af or Ao may evoke AHR without prior remote priming with allergen and adjuvant\(^12\). Inhalation of proteolytically active Ao, but not its protease-inactive counterpart, elicited nearly equivalent AHR in the presence or absence of OVA, which did not correlate strictly with the magnitude of allergic inflammatory response (IgE response and airway eosinophilia). Likewise, the purified, secreted Af protease, which itself is poorly immunogenic\(^13\), induced AHR in the presence of OVA despite recruiting markedly fewer airway eosinophils to the lung than OVA plus crude Af allergen. These results suggest that proteolytic activity of certain allergens, while not sufficient to elicit AHR in the absence of lung inflammation, nonetheless contribute to AHR through mechanisms independent of allergic sensitization.

Whether allergens have a direct and pathogenic impact on ASM contraction in asthma has not been explored. Here we investigate the hypothesis that lung epithelial destruction associated with asthma permits penetration of allergen components into the bronchial submucosa to promote ASM contraction. We detect an Af-derived protease (Alp 1) in the bronchial smooth muscle layer of human subjects with asthma and allergen-challenged mice but not in control subjects or naïve mice, and we provide a potential mechanism by which Alp 1 induces a pro-contractile phenotype of ASM cells.

### Results

**Af protease activity promotes AHR.** Proteolytic enzymes secreted by Af cause epithelial desquamation and have an integral function in tissue invasiveness\(^14\,^15\). We found that a commercially available and clinically used Af extract had readily detectable protease activity, which was abolished by heat inactivation or preincubation with inhibitors of serine proteases (phenylmethylsulfonyl fluoride or antipain), but not cysteine proteases (E-64) (Fig. 1a). To determine the relative importance of Af protease activity for the induction of AHR, we sensitized and challenged mice with either native or heat-inactivated (HI-)

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**Figure 1 | Af protease activity promotes AHR.** (a) Protease activity in crude Af extracts incubated with vehicle alone, PMSF (serine protease inhibitor, 250 μM), E-64 (cysteine protease inhibitor, 10 μM) or heat-inactivated (HI)-Af was determined as described in Methods. Bar graph shows mean ± s.e.m. of three independent experiments (**,**,**,** P<0.0001, one-way analysis of variance (ANOVA)). (b) Lung resistance (R\(_{\text{L}}\)) in response to aerosolized methacholine (MCh) was measured in naïve, Af or HI-Af-sensitized and challenged BALB/c WT mice 24 h after the final challenge. Data in b–e are represented as mean ± s.e.m. of four to six mice per group measured in a single experiment (* P=0.04, ** P=0.001,**,**,** P=0.0006, two-way ANOVA, comparing Af versus HI-Af). (c) Peribronchial infiltration and goblet cell metaplasia of paraffin-embedded mouse lungs were evaluated by using haematoxylin/eosin and Periodic acid-Schiff staining, respectively (Scale bar, 100 μm, original magnification, 10 ×). (d) Total cell counts and (e) differentials were assessed in BALF cytospin preparations (** P<0.01 two-way ANOVA).
Af allergen extracts and measured total lung resistance ($R_L$) in anesthetized mice following methacholine inhalation. As expected, mice challenged with untreated Af had significantly increased $R_L$ compared with naive mice (Fig. 1b). Mice challenged with HI-Af had significantly reduced $R_L$ values compared with mice that received untreated Af. However, native or HI-Af induced comparable sensitization, as evidenced by equivalent peribronchial inflammation, goblet cell metaplasia (Fig. 1c) and total cell counts in bronchoalveolar lavage fluid (Fig. 1d), although the composition of BAL fluid differed modestly between the two groups. Challenge with HI-Af elicited slightly fewer airway eosinophils and a greater influx of neutrophils than did challenge with untreated Af (Fig. 1e). These results suggest that Af protease activity also contributes to AHR through mechanisms distinct from the inflammatory response.

Af enhances Ca$^{2+}$ mobilization in ASM cells. Our results suggested that Af promotes AHR by augmenting ASM contraction partially through inflammation-independent mechanisms. Agonist stimulation of GPCRs induces bronchoconstriction initially by increasing cytosolic Ca$^{2+}$ levels$^{18,19}$. To determine whether Af affected GPCR-evoked Ca$^{2+}$ signalling, we incubated cultured human ASM cells (HASM) with Af extract for 24 h prior to quantification of cytosolic Ca$^{2+}$ by fluorescence microscopy. Af pre-treatment led to an increase in basal cytosolic Ca$^{2+}$ in a concentration-dependent manner (Fig. 3a) and significantly enhanced intracellular Ca$^{2+}$ flux in response to GPCR ligands bradykinin or histamine compared with vehicle-treated cells (Fig. 3b–e). In contrast, vehicle and Af-pre-treated HASM displayed similar responses to ionomycin (Fig. 3f), which increase cytosolic Ca$^{2+}$ through receptor-independent mechanisms. GPCR-mediated Ca$^{2+}$ mobilization is dependent on PLCβ-mediated generation of inositol 1, 4, 5-trisphosphate (InsP3), which in turn stimulates InsP3 receptors on sarcoplasmic reticulum to release Ca$^{2+}$ into the cytosol. Accordingly, we observed increased amounts of bradykinin-evoked InsP3 (a stable metabolite of InsP3) in Af-pre-treated HASM cells compared with controls (Fig. 3g).

Addition of Af extract to HASM immediately prior to measurement of Ca$^{2+}$ concentrations (that is, without prolonged preincubation) failed to induce any Ca$^{2+}$ mobilization, and simultaneous stimulation of cells with Af extract and bradykinin did not potentiate Ca$^{2+}$ signalling induced by bradykinin alone (Fig. 3h). These results suggested that Af does not activate Ca$^{2+}$ signalling through a protease-activated receptor, such as F2R or F2LR1. In support of this hypothesis, we found that 24 h pre-treatment of ASM with Af actually abolished responses to thrombin and resulted in profoundly diminished levels of F2R at the plasma membrane (Fig. 3i,j). Thus, chronic exposure of HASM cells to Af may potentiate GPCR-evoked Ca$^{2+}$ spikes in a receptor-independent manner.

### Figure 2 | Af induces bronchoconstriction in PCLS

Mouse lung slices were pre-treated with vehicle or Af (5 or 10 μg ml$^{-1}$) for 24 h. (a) Bright field images of airways in PCLS treated with vehicle or Af (5 μg ml$^{-1}$) were obtained and used to calculate baseline airway luminal areas as per the Methods. (mean ± s.e.m. of 26–45 airways evaluated in 2–3 experiments; *P = 0.03, t-test). (b) Left panel: bright field images were obtained before (NT) and 4 min after carbachol (CCCh, 100 μM) stimulation. Scale bar, 300 μm; original magnification, 5 x . Arrows denote contracting airways. Right panel: slices were stimulated with increasing concentrations of CCCh, and percent contraction was calculated based on the airway luminal area. Data are expressed as mean ± s.e.m. of three independent experiments (23–25 airways per group) fitted to a curve using the least-squares fit method. $E_{\text{max}}$ was compared between groups by extra sum-of-square F test (**P < 0.01, ***P < 0.001, ****P < 0.0001, two-way analysis of variance). (c) PCLS were prepared as in a and stimulated with the indicated concentrations of KCl. Data are represented as mean ± s.e.m. of two independent experiments (9–15 airways per group).
**Af** degrades extracellular matrix. Exposure of ASM cells to **Af** could also affect expression of receptors or elements of the excitation–contraction signalling pathway that elicit bronchoconstriction. We failed to detect increased expression of components of the excitation–contraction signalling pathway that elicit bronchoconstriction. We failed to detect increased expression of con- traction-inducing receptors or intracellular contractile proteins in HASM following **Af** treatment (Supplementary Fig. 1a). However, incubation with **Af** induced profound changes in HASM cell morphology, including elongation and detachment, disassembly of vinculin-rich focal adhesions (adhesomes), reduced actin stress fibres (Fig. 4a,b) and haptotaxis (Supplementary Movies 1,2), without altering vinculin expression (Supplementary Fig. 1b) or eliciting changes in cell viability, apoptosis or proliferation (Supplementary Fig. 2). These results suggested that **Af** perturbs integrin-mediated extracellular matrix (ECM) attachments of HASM.

ECM activation of membrane-bound integrins elicits autophosphorylation of focal adhesion kinase (FAK), actin polymerization and assembly of a signalosome of cytoskeletal proteins including vinculin, paxillin and α-actinin, which are essential for actomyosin crossbridging and force generation. Surprisingly, however, deficiency of α9β1 integrin in ASM by conditional genetic deletion of α9 (Iga9) in mice resulted in spontaneous AH. We observed markedly reduced surface expression and increased cytosolic localization of α9 integrin in **Af**-treated cells compared with controls by immunofluorescence staining (Fig. 4c). Heat inactivation or preincubation of **Af** extract with a serine protease inhibitor prior to addition to HASM cells prevented **Af**-induced morphological change and detachment (Fig. 4a) as did pre-coating plates with exogenous ECM proteins including collagen I and fibronectin (Fig. 4d,e). Together, these

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**Figure 3 | **Af** modulates Ca^{2+} mobilization in ASM cells.** Serum-starved HASM cells were left untreated or pre-incubated with **Af** extract for 24 h prior to measurement of cytosolic Ca^{2+} by fluorescence microscopy as outlined in the Methods. (a) Baseline Ca^{2+} levels after treatment are shown as mean ± s.e.m. of a minimum of 60 cells per condition measured in three independent experiments (****P<0.0001, one-way analysis of variance (ANOVA)). (b–f) Agonist-induced Ca^{2+} mobilization. Line graphs in (b) (bradykinin, 100 nM), (e) (histamine, 10 μM) and (f) (ionomycin, 2 μM) show normalized Ca^{2+} measurements over time (mean ± s.e.m. of 16–80 cells measured in 2–3 independent experiments) at baseline and following addition of agonist (arrows). (c) Peak and area under curve (d) of Ca^{2+} spikes in (b) were calculated (***P<0.0005, ****P<0.0001, one-way ANOVA). (g) Serum-starved HASM cells were pre-treated with vehicle or **Af** (2 μg ml⁻¹) for 24 h. Accumulation of InsP_{1} was measured after addition of the indicated concentrations of bradykinin. Data are represented as mean ± s.e.m. of three independent experiments (**P<0.01, ***P<0.001, two-way ANOVA). (h) HASM cells were stimulated acutely with **Af** bradykinin (100 nM) or **Af** + bradykinin (100 nM) followed by measurement of Ca^{2+} by microscopy as in (b–f). Graph represents mean ± s.e.m. of 7–60 cells per condition. (i) HASM cells were pre-treated with vehicle or **Af** (5 μg ml⁻¹) for 24 h followed by stimulation with thrombin and measurement of Ca^{2+} mobilization by fluorimetry. Normalized Ca^{2+} values are represented as mean ± s.e.m. of a single experiment representative of two independent experiments. (j) Surface and total F2R receptor expression in vehicle or **Af**-treated HASM cells was measured by flow cytometry. Representative histograms from two to three independent experiments are shown.
results indicated that one or more serine proteases present in Af allergen extracts might digest ECM components to disrupt integrin-mediated attachments in ASM cells.

A prior study demonstrated that a serine protease purified from Af culture supernatants degraded multiple ECM proteins, including elastin, collagen types I–III and fibronectin. Hence, we found that the Af allergen extract used here reduced the collagen content of HASM cells in culture (Fig. 5a) and of PCLS (Fig. 5b) and digested several purified ECM components (Fig. 5c,d). We also determined whether exposure of HASM cells to Af induced production of matrix metalloproteinases (MMPs) or tissue inhibitor of metalloproteinases (TIMPs) that are expressed by HASM and could affect ECM composition. Af treatment of HASM increased secretion of MMP1 and MMP3 compared with vehicle treatment, but not any of the others tested including MMP2, 8, 9, 10 or TIMPs 1, 2 and 4 (Fig. 5e).

Notably, we detected substantially less protease activity in extracts of another common allergen, HDM, than in Af solutions with equivalent protein concentrations. HDM extract induced morphological changes and detachment of HASM cells only at much higher concentrations than did active Af, and these effects were abolished by addition of cysteine protease inhibitors (Supplementary Fig. 3).

Af modifies Ca²⁺ signalling in ASM cells. Integrin-mediated ECM interactions and the formation of focal adhesion plaques may affect the metabolism of membrane phospholipids that

**Figure 4 | Af serine protease activity induces morphological changes in ASM cells.** (a) Serum-starved HASM cells were treated with vehicle, unmodified Af (5 µg ml⁻¹), Hi-Af or Af incubated with PMSF (250 µM) or E-64 (10 µM) protease inhibitors. Scale bar, 200 µm; original magnification, 10 ×. (b,c) Serum-starved HASM cells (b) or mouse ASM cells (c) were treated with vehicle or Af (5 µg ml⁻¹) for 24 h. Fixed cells were stained with phalloidin (red) and anti-vinculin antibody (green) (b) or α9-integrin antibody (green) (c). Nuclei were counterstained with DAPI. Images in (b,c) are from a single experiment representative of 2-3 similar experiments. Scale bar, 20 µm; original magnification, 63 ×. (d) HASM cells were cultured on plates coated with collagen I (0.2-1 mg ml⁻¹) followed by treatment with Af (5 µg ml⁻¹) for 48 h. DIC images are from a single experiment representative of three similar experiments. Scale bar, 200 µm; original magnification, 10 ×. (e) HASM cells were seeded on plates coated with collagen I (1 mg ml⁻¹), collagen III (0.1 mg ml⁻¹), collagen IV (0.5 mg ml⁻¹) or fibronectin (0.1 mg ml⁻¹). After serum starvation, cells were treated with Af (5 µg ml⁻¹) and visualized by microscopy 48 h post treatment. Scale bar, 200 µm; original magnification, 10 ×. Images are from a single experiment representative of two to four independent experiments.
**(a)** Total collagen content in cultured HASM cells left untreated or incubated with Af for the indicated times. Graph shows mean ± s.e.m. from a single experiment representative of three independent experiments (**P = 0.006, ****P < 0.0001, two-way analysis of variance (ANOVA)). **(b)** Collagen content in PCLS left untreated or incubated with Af for the indicated times; data are mean ± s.e.m. of three independent experiments (**P = 0.007; ****P < 0.0001, two-way ANOVA)). **(c)** Vehicle, Af (5, 10 or 20 μg) or collagenase D (50 μg) was incubated with collagen I (20 μg) for the indicated times at 37 °C. Samples were resolved by SDS-PAGE and visualized by Coomassie staining. α1, α2 and β subunits of collagen I are denoted by red arrows. **(d)** Vehicle, Af (20 μg ml−1) or heat-inactivated Af (HI-Af, 20 μg ml−1) were incubated with collagen I, III, IV or fibronectin (20 μg) for 30 min at 37 °C. **(e)** Secretion of MMPs and TIMPs in cell supernatants was measured by antibody array. Bar graph depicts relative expression (mean ± s.e.m. of three independent experiments) in Af-treated HASM cells compared with control (**P = 0.003, *P = 0.01, t-test).**

**Figure 5** | Af degrades the extracellular matrix. (a) Total collagen content in cultured HASM cells left untreated or incubated with Af for the indicated times. Graph shows mean ± s.e.m. from a single experiment representative of three independent experiments (**P = 0.006, ****P < 0.0001, two-way analysis of variance (ANOVA)). (b) Collagen content in PCLS left untreated or incubated with Af for the indicated times; data are mean ± s.e.m. of three independent experiments (**P = 0.007; ****P < 0.0001, two-way ANOVA)). (c) Vehicle, Af (5, 10 or 20 μg) or collagenase D (50 μg) was incubated with collagen I (20 μg) for the indicated times at 37 °C. Samples were resolved by SDS-PAGE and visualized by Coomassie staining. α1, α2 and β subunits of collagen I are denoted by red arrows. (d) Vehicle, Af (20 μg ml−1) or heat-inactivated Af (HI-Af, 20 μg ml−1) were incubated with collagen I, III, IV or fibronectin (20 μg) for 30 min at 37 °C. (e) Secretion of MMPs and TIMPs in cell supernatants was measured by antibody array. Bar graph depicts relative expression (mean ± s.e.m. of three independent experiments) in Af-treated HASM cells compared with control (**P = 0.003, *P = 0.01, t-test).**
Alp 1 protease mediates the bronchoconstriction induced by Af crude allergic extracts.

Alp 1 is present in human and murine bronchi. To determine the extent to which Alp 1 reaches the airway submucosa to induce AHR in vivo, we performed immunostaining on mouse and human lung sections with anti-Alp 1 antiserum. We sensitized mice intraperitoneally with Af followed by repeated intranasal challenge with Af extracts. We detected Alp 1 in bronchial smooth muscle of mice sensitized and challenged with Af, but not in naive mice, or in animals simply inoculated intranasally with Af for 4 h.
or overnight prior to lung harvest in the absence of prior sensitization (Fig. 8a and Supplementary Fig. 5d). We also detected Alp 1 in the lung tissue (Fig. 8b) and BAL fluid (Fig. 8c) of Af-sensitized and challenged mice by immunoblotting but not in naïve mice. Based on quantitative immunoblotting, Alp 1 concentrations in BAL fluid were \( \approx 0.005 \mu g \text{ml}^{-1} \) (Supplementary Fig. 5b), suggesting that we used physiologically relevant concentrations for in vitro assays. Collectively, these results demonstrated that both the allergic inflammatory response to Af and allergen protease activity seemed to be required for Alp 1 dissemination in the bronchial submucosa in mice.

To extend these findings to humans, we examined lung sections from control subjects or those patients with mild to moderate asthma for the presence of Alp 1. Clinical characteristics of the patient groups are summarized in Table 1. While we detected minimal Alp 1 staining in bronchial smooth muscle bundles of lungs from healthy subjects, Alp 1 immunostaining was present in the ASM layer of lung sections from subjects with non-atopic asthma (Fig. 8d), and further elevated levels of Alp 1 immunoreactivity were observed in ASM of patients with asthma and Af sensitization (epicutaneous reactivity) (Fig. 8d,e).

Figure 7 | Alp 1 serine protease regulates Ca\(^{2+}\) sensitivity in ASM. (a) HASM cells were treated with purified Alp 1 (3 \( \mu g \text{ml}^{-1} \)) in the presence or absence of serine (PMSF, 250 \( \mu M \)) or cysteine (E-64, 10 \( \mu M \)) protease inhibitors for 48 h. DIC images are from a single experiment representative of three similar experiments. Scale bar, 200 microns; original magnification, 10 × . (b) Serum-starved HASM cells were pre-incubated with vehicle, untreated Af or purified Alp 1 at the concentrations indicated for 24 h prior to measurement of cytosolic Ca\(^{2+}\) in individual cells following bradykinin (100 nM) stimulation as in Fig. 3. Data are represented as mean ± s.e.m. of 12–25 cells from 2 individual donors measured in 2 independent experiments (\( P < 0.0001 \), two-way analysis of variance (ANOVA), vehicle versus Af or Alp 1). (c) HASM cells were treated with vehicle, Af or Hi-Af (3 \( \mu g \text{ml}^{-1} \)) for the indicated times, and phosphorylation of MYPT1 and MLC2 was assessed by immunoblotting. Images are from a single experiment representative of three independent experiments. (d) PCLS were prepared from naïve mice and pre-treated with vehicle, Af or Hi-Af (5 \( \mu g \text{ml}^{-1} \)) for 24 h. Slices were stimulated with increasing concentrations of CCh and percent contraction calculated as in Fig. 1. Data (mean ± s.e.m.) are from a single experiment using 4 mice representative of 2 similar experiments (7–11 airways per group) fitted to a curve using the least-squares fit method. \( E_{\text{max}} \) was compared between groups by extra sum-of-square F-test; NS, not significant (\( ** P = 0.001; **** P < 0.0001 \), two-way ANOVA). (e) HASM cells were treated with vehicle or Alp 1 (1 \( \mu g \text{ml}^{-1} \)) for the indicated times, and phosphorylation of MYPT1 and MLC2 was assessed by immunoblotting. Images are from a single experiment representative of three independent experiments. (f) PCLS were prepared from naïve mice and pre-treated with vehicle or purified Alp 1 (0.1 or 0.5 \( \mu g \text{ml}^{-1} \)) for 24 h. Slices were stimulated with increasing concentrations of CCh and analyzed as in d. Data (mean ± s.e.m.) are from three experiments using 2–4 mice in each (30–80 airways per condition; \( * P = 0.03; **** P < 0.0001 \), two-way ANOVA).
concentration that induced a 20% decrease in the forced expiratory volume in 1 s (PC20FEV1) for each subject. There was a significant negative correlation between the amount of Alp 1 detected in the bronchial submucosa and the PC20FEV1 (Fig. 8f).

Discussion

ECM composition and deposition are radically altered in both allergic and non-allergic asthma26–28. Our results suggest a novel pathophysiological mechanism whereby in inflammation associated with respiratory mucosal exposure to Af allergen leads to ECM degradation through direct contact between a protease component and the bronchial submucosa. The serine protease Alp 1 secreted by Af digested ECM components directly and stimulated MMP1 release from ASM cells. We established that Alp 1 serine protease activity present in Af allergen extracts enhanced Ca2+ sensitivity (reduced MYPT1 activity and increased MLC phosphorylation) in cultured ASM cells by disrupting integrin-dependent ASM-ECM attachments and increasing RhoA and ROCK activity. Consequently, Af failed to augment airway contraction in PCLS pre-treated with a ROCK inhibitor, suggesting that it induced bronchoconstriction primarily through pathogenic Ca2+ sensitization.

Af also evoked substantial increases in the magnitude of GPCR agonist-induced Ca2+ transients in cultured ASM cells. We now know that intracellular Ca2+ concentrations fluctuate repeatedly following agonist stimulation, in the form of a series of Ca2+ oscillations29. The force of sustained airway contraction appears to correlate primarily with the persistence and frequency of these Ca2+ oscillations, rather than the magnitude of the Ca2+ spikes. However, published work has shown that while serotonin (5-HT) induced greater airway contraction of airways in PCLS than ACh, 5-HT evoked a Ca2+ oscillation frequency that was slower than ACh in HASM cells30. These results suggest that the correlation between contraction and oscillation frequency depends on the agonist studied. Thus, future measurements of Ca2+ oscillations and elucidation of the impact of Ca2+ sensitivity (inhibition of MLCP) on the frequency-contraction relationship in PCLS will allow us to better determine the significance of our in vitro findings for bronchial contraction in vivo.
How ECM–integrin interactions regulate ASM contraction in vivo also requires further study. Serine protease (elastase)-mediated airway destruction contributes to cigarette smoke-induced airway obstruction. Treatment of lung slices from naïve mice with a serine protease (human neutrophil elastase) led to significantly increased airway contraction. In a separate study, intratracheal administration of elastase to guinea pigs augmented bronchoconstriction in response to inhaled ACh through an unknown mechanism. Although it has been argued that integrin activation by the ECM and resultant actin filament interactions at the plasma membrane promote force generation in smooth muscle cells by increasing membrane tension and cytoskeletal rigidity, recent work has demonstrated that integrin signalling may regulate bronchial constriction in complex ways.

For example, based on their finding that loss of α9β1 integrin in mice increased asthma by stimulating PIP2 generation, Chen et al. also demonstrated that addition of cell-permeable PIP2 (equivalent to the effect of Af shown here) increased carbachol-induced constriction of human airways, which was prevented by integrin-blocking antibodies. A separate study found that mice lacking the integrin-binding glycoprotein milk fat globule-EGF factor 8 (Mgf8 or lactadherin in humans), which is secreted by ASM, had exaggerated AHR following allergen challenge despite mounting an inflammatory response that was similar to control mice. Their work suggested that in a physiological setting Mgf8–integrin binding in ASM served to suppress Ca2+ sensitivity through decreased expression and activation of RhoA and ROCK. In another work, integrin–collagen 1 attachments were shown to promote switching of bovine tracheal smooth muscle cells towards a hyperproliferative and hypocontractile phenotype in a FAK-dependent manner. Collectively, these observations are consistent with our results demonstrating that perturbation of ECM–integrin–FAK interactions by Af leads to increased RhoA and MLC activation and bronchoconstriction.

Although Af protease activity was required for elicitation of maximal AHR following sensitization and challenge, our results do not exclude the possibility that allergen protease activity contributes to AHR through multiple mechanisms. Previous studies demonstrated that sensitization and intranasal challenge with purified Af 1 alone is sufficient to induce T4,2-mediated lung inflammation and AHR. Simultaneous challenge with Af 1 purified from Af culture supernatants and a separate recombinant Af allergen (Asp f2) induced a synergistic inflammatory response but did not further enhance AHR compared with challenge with Af 1 alone. These results are consistent with our finding that Af 1 has the capacity to affect AHR through inflammation-dependent and independent mechanisms. None of these studies addressed whether or not Af 1 protease activity was required for the asthmatic phenotype and should be interpreted cautiously since both purified and recombinant Af 1 have weak protease activity (the study by Moser et al. and this study). Loss of activity may occur as a result of alterations in buffer pH and/or composition during purification, protein instability or the presence of endogenous activators or co-factors in the crude allergen extract. Finally, Asp f13/Af 1 may promote inflammation through direct effects on epithelium. Af 1 has been shown to cause epithelial barrier dysfunction, increase Muc5ac gene expression and induce cytokine production (IL-6 and IL-8).

Since respiratory exposure to Af is quite common, our results indicate that an additional lung insult—allergic inflammation, a respiratory irritant or environmental exposure in the case of non-astotic disease—may require that promote Af 1 penetration into the bronchial smooth muscle submucosa. Several factors may account for the presence of Af 1 (and probably other protease allergens) in the bronchial submucosa in people with asthma but not in healthy people. Recent clinical studies have demonstrated higher rates of surface airway mycotic in subjects with asthma (74%) than in controls (16%), and fungal-induced Tq2 immunity is restricted to asthma. Lungs of asthmatic subjects with Af sensitivity (positive skin prick test) may be more susceptible to germination of Af spores and have a higher burden of Af recoverable from sputum. Airway epithelium in subjects with asthma may be more susceptible to the permeability-inducing effects of protease allergens than that of healthy control. Taken together, these results and our work suggest that chronic pulmonary inflammation and epithelial desquamation associated with asthma promote dissemination of allergens such as Af 1 into the bronchial smooth muscle layer. We found that extracts of another perennial allergen, HDM, contained 75% less protease activity than did Af extract at equivalent protein concentrations and did not induce ASM detachment or morphological changes similar to those elicited by Af/Alp1. As prior studies have demonstrated that HDM induces proliferation and cytokine production in ASM cells, partially through activation of F2LR1 (ref. 42), it is unclear whether other environmental allergens will infiltrate the bronchial submucosa and engender AHR through mechanisms equivalent to those described here.

The inverse correlation between Af 1 staining and a parameter of AHR (PC20FEV1) suggests that analysis of Af 1 in the bronchial submucosa is clinically relevant. Future studies of patients with severe asthma and/or certain disease endotypes (for example, eosinophilic versus neutrophilic inflammation) may be necessary to determine whether Af 1/Asp f13 burden in ASM, or possibly in sputum, can be used as a biomarker to classify patients with fungal-associated asthma. Although our anti-Af 1 antibody did not inhibit Af 1 protease activity or protease-induced morphological changes of HASM cells (Supplementary Fig. 6), Af 1 remains a potential therapeutic target in this subset of patients pending development of an appropriate biologic. Our findings further suggest a rationale for serum protease, ROCK or PI3K inhibitors for the treatment of specific asthma phenotypes.

**Methods**

**Reagents and antibodies.** Carbachol, ACh, bradykinin, histamine, ionomycin, thapsigargin, bovine serum albumin (BSA), E-64, PDGF-BB, PMSF, collagen IV, elastase, recombinant Alp 1 have weak protease activity (the study by Moser et al. and this study). Loss of activity may occur as a result of alterations in buffer pH and/or composition during purification, protein instability or the presence of endogenous activators or co-factors in the crude allergen extract. Finally, Asp f13/Af 1 may promote inflammation through direct effects on epithelium. Af 1 has been shown to cause epithelial barrier dysfunction, increase Muc5ac gene expression and induce cytokine production (IL-6 and IL-8). Since respiratory exposure to Af is quite common, our results indicate that an additional lung insult—allergic inflammation, a respiratory irritant or environmental exposure in the case of non-astotic disease—may be required to promote Af 1 penetration into the bronchial smooth muscle submucosa. Several factors may account for the presence of Af 1 (and probably other protease allergens) in the bronchial submucosa in people with asthma but not in healthy people. Recent clinical studies have demonstrated higher rates of surface airway mycosis in subjects with asthma (74%) than in controls (16%), and fungal-induced Tq2 immunity is restricted to asthma. Lungs of asthmatic subjects with Af sensitivity (positive skin prick test) may be more susceptible to germination of Af spores and have a higher burden of Af recoverable from sputum. Airway epithelium in subjects with asthma may be more susceptible to the permeability-inducing effects of protease allergens than that of healthy control. Taken together, these results and our work suggest that chronic pulmonary inflammation and epithelial desquamation associated with asthma promote dissemination of allergens such as Af 1 into the bronchial smooth muscle layer. We found that extracts of another perennial allergen, HDM, contained 75% less protease activity than did Af extract at equivalent protein concentrations and did not induce ASM detachment or morphological changes similar to those elicited by Af/Alp1. As prior studies have demonstrated that HDM induces proliferation and cytokine production in ASM cells, partially through activation of F2LR1 (ref. 42), it is unclear whether other environmental allergens will infiltrate the bronchial submucosa and engender AHR through mechanisms equivalent to those described here.

The inverse correlation between Af 1 staining and a parameter of AHR (PC20FEV1) suggests that analysis of Af 1 in the bronchial submucosa is clinically relevant. Future studies of patients with severe asthma and/or certain disease endotypes (for example, eosinophilic versus neutrophilic inflammation) may be necessary to determine whether Af 1/Asp f13 burden in ASM, or possibly in sputum, can be used as a biomarker to classify patients with fungal-associated asthma. Although our anti-Af 1 antibody did not inhibit Af 1 protease activity or protease-induced morphological changes of HASM cells (Supplementary Fig. 6), Af 1 remains a potential therapeutic target in this subset of patients pending development of an appropriate biologic. Our findings further suggest a rationale for serum protease, ROCK or PI3K inhibitors for the treatment of specific asthma phenotypes.

**Table 1 | Clinical characteristics of patients studied**

|                      | Controls | Asthma (no Af sensitivity) | Asthma (Af sensitivity) |
|----------------------|----------|---------------------------|-------------------------|
| Number               | 14       | 10                        | 12                      |
| Age                  | 28(2.8)  | 29(2.7)                   | 41(4.4)                 |
| Male/female          | 5/9      | 6/4                       | 6/6                     |
| PC20FEV1 (1.015)     | ≥ 30 (33–219) | 3.6 (1.8–8.6)   | 0.67 (0.25–1.8)         |
| Pre-BD FEV1 (%Predicted) | 101.5 (97–106) | 90.4 (71–115) | 85.1 (64–126) |
| Inhaled corticosteroids | None | 5(400)                | 5(600)                  |
| LABA*               | None | 3(83)                     | 3(83)                   |
| Positive Af skin test | 0      | 0                         | 12                      |

Af: Asperillus fumigatus; CI, confidence interval.

*Mean (± s.e.m.).

1 Geometric mean (95% CI).

2 Mann–Whitney t-test versus Af sensitive group.

3 Number (range).

4 Number of subjects (equivalent of flucetacoin, mg).

5 Number of subjects (equivalent of salmeterol, mg).

6 Control group FEV1 and PC20 values do not differ from sensitive group.

7; CI, confidence interval.
fibroactin, stauroporine and Triton X-100 were purchased from Sigma. Collagen I and III were purchased from BD Biosciences. ROCK inhibitor Y27632 was from EMD Millipore, and Collagenase D was from Roche. Methanolic Texas Red-conjugated phalloidin was obtained from Molecular Probes. Antibodies used in this study were purchased from the following sources: mouse anti-

**Immunoblotting and immunofluorescence**. HASM cells were serum starved for 48 h followed by Af pre-treatment for the indicated times. Lysates were prepared from cells, lungs or BAL fluid using radioimmunoprecipitation lysis buffer supplemented with protease inhibitor and phosphatase inhibitor cocktails (Roche) and sonication. Primary antibody staining was detected using near-infrared conjugated secondary antibodies and visualized with the Li-Cor Odyssey Imaging System and Image Studio software (Li-Co Biosciences). For immunofluorescent staining, cells were fixed in 3.7% paraformaldehyde and permeabilized with PBS containing 0.2% vol/vol Triton X-100. Cells were then blocked in 2% (wt/vol) BSA in PBS. Cells were incubated with mouse anti-actin antibody (1:100) or rabbit anti-9B integrin (Santa Cruz, 1:75) overnight at 4 °C in blocking buffer. Cells were then incubated with AlexaFluor488-conjugated goat anti-mouse or goat anti-rabbit (1:200) secondary antibody (Molecular Probes). Cells were mounted on coverslips with Pro-Long Gold antifade reagent with DAPI. Images were obtained using the Leica DMI4000 light microscope equipped with Retiga2000R camera (QImaging, Canada) and analyzed by QCapture software. For analysis of BALF, mouse lungs were perfused with 1.5 ml ice-cold Hank’s balanced salt solution, and lavage fluid was collected and centrifuged at 1,500 r.p.m. at 4 °C. Cells were then incubated with 0.2% BSA in PBS for 30 min, washed with PBS and air-dried prior to cell seeding.

**Video microscopy**. Cells were seeded on chambered coverglass slides (Lab-Tek, Nunc) and serum starved for 48 h. Differential interference contrast images were recorded every 5 min using a Leica AF6000 XL confocal microscope with the 20× dry objective. Images were visualized per each condition 6 min before and 28 h after addition of vehicle or Af (10 µg ml⁻¹). Images and movies were recorded using Imairis software.

**ECM proteolytic activity assay**. Collagen I (20 µg) was prepared in buffer (50 mM Tris-HCl, 100 mM NaCl and 1.5 mM CaCl₂) and incubated with Af (20 µg) for 10 or 30 min or with collagenase D for 30 min at 37 °C. Purified collagens I, III, IV or fibroactin (20 µg) were incubated with vehicle or Af extract for 30 min at 37 °C. Samples were boiled with sample buffer at 95 °C, electrophoresed on 10% Tris-glycine gels, and visualized by Coomassie Blue staining. HASM cells or mouse PCLS were incubated with Af extract for the indicated times. Acid-pepsin soluble collagen content was measured by Sircol assay (Biocolor).

**MMP array**. Serum-starved HASM cells were treated with vehicle or Af (2 µg ml⁻¹) for 24 h. Relative amounts of MMPs and TIMPs in cell supernatants were determined using a Human MMP Antibody Array (Abcam), according to the manufacturer’s instructions. Bio-Plex Reader (Biorad) was used for reading the plates.

**Intracellular calcium measurements**. HASM cells were seeded at low density (30–50% confluence) onto polystyrene vessel tissue culture-treated glass slides (BD Falcon) in Ham’s F-12 medium supplemented with 10% FBS and 10% PBS for 24 h at 37 °C in 5% CO₂. Cells were starved in serum-free medium followed by treatment with vehicle or Af extract diluted in serum-free medium for 24 h at 37 °C. F-12 media was replaced with 1.5 Fluo-8 (100 µl per chamber; Abcam Fluo-8 No Wash Calcium Assay Kit) in Ham’s F-12 media. Cells were incubated in Fluo-8 solution for 30 min then at room temperature in the dark for 30 min. Optical imaging (Nikon Eclipse TE2000-U Inverted Research Microscope) was performed immediately after the final incubation. Bradykinin (100 nM), histamine (100 µM), ionomycin (2 µM) or thapsigargin (100 nM) was added manually to the imaged chamber after 10 frames had been recorded, and images were recorded every second for 120 s. Mean, maximum and minimum grayscale measurements were obtained for a cytoplasmic region of interest (ROI) for each responding cell in each frame using FIJI. Mean grayscale measurements were adjusted for background and divided by the first frame. Results were plotted as mean normalized relative fluorescence units (fold basal) ± s.e.m. versus time in seconds. In Fig. 3i, intracellular Ca²⁺ was measured in response to thrombin in cells plated in 96-well plates by fluorimetry as previously described.

**Ins₆₉ measurements**. HASM cells were seeded in 96-well plates and starved in serum-free media for 48 h followed by 24 h pre-treatment with vehicle or Af (2 µg ml⁻¹). Cells were then stimulated with increasing concentrations of bradykinin (100 nM) for 2 min. Ins₆₉ concentrations were measured by enzyme-linked immunosorbent assay (ELISA) as described by the manufacturer (IP-One ELISA, Cusbio Biosciences).

**P(4,5)PEL ELISA assay**. HASM cells were seeded in 6-cm dishes and starved in serum-free media for 48 h followed by pre-treatment with vehicle or Af (2 µg ml⁻¹) for indicated times. Extraction of P(4,5)PEL-rich fractions and competitive ELISA assay were performed according to the manufacturer’s instructions (Echelon Biosciences, kit# K-4500).

**RhoA-GTP activation assay**. HASM cells grown in 10-cm dishes were pre-treated with Af (2 µg ml⁻¹) for 24 h and then stimulated with bradykinin (1 µM) for the indicated times. Cells were scraped into ice-cold MLR lysis buffer (Millipore) supplemented with NaF, Na₂VO₃, protease and phosphatase inhibitor cocktails. Lysates were clarified by centrifugation at 14,000 g for 5 min. Supernatants were incubated with GST– rhoetkin Rho-binding domain according to the manufacturer’s instructions (Millipore). Pull downs and total cell lysates were electrophoresed on 10% Tris-glycine gels and immunoblotted with mouse anti-RhoA antibody. In a separate series of experiments, quantities of active RhoA and total RhoA were determined in cells treated as above using the appropriate G-LISA assay (Cytoskeleton). RhoA-GTP values were normalized to total RhoA for each sample.

**ROCK and MLCK activation assay**. Cells were seeded onto Cellstar tissue culture plates and grown to confluence in Ham’s F-12 media supplemented with 1-glutamine and 10% FBS at 37 °C in 5% CO₂. Cells were starved in serum-free media for 24 h at 37 °C in 5% CO₂, then treated with vehicle, Af extract or purified
Alp 1 diluted in serum-free medium for 24 h. Lysates were prepared in radio-immunoprecipitation buffer and immunoblotted using the following primary antibodies directed against Alp 1 (Abcam, 1:1,500), anti-γ-tubulin (γ-Tub) (Millipore, anti-γTuC2 (S187T19) (Cell Signaling Technologies) and anti-tubulin (Cell Signaling Technologies). The following secondary antibodies were used: donkey anti-mouse (Jackson ImmunoResearch Laboratories), peroxidase-conjugated or near-infrared dye-conjugated anti-mouse IgG or donkey anti-rabbit (Cell Signaling Technologies). Immunoblotting were visualized using standard chemiluminescence protocols or an Odyssey near-infrared imaging system (LI-COR Biosciences).

Flow cytometry. Serum-starved HASM cells were treated with Af (5 μg ml−1) for 24 h and then detached from the plate using a non-enzymatic solution (Cellstripper, Corning). For surface staining of FZR, cells were incubated with PE-conjugated mouse anti-FZR (1:15; BD Systems) or the PE-conjugated isotype control (IgG2b 1:15) for 20 min in FACs buffer at 4°C. Cells were then fixed in PBS containing 1% paraformaldehyde, pelleted and resuspended in PBS containing 0.1% saponin and 0.05% BSA prior to staining. Fluorescence data were collected using an LSRII flow cytometer (BD Biosciences) and analyzed with Flowjo software (Tree Star, Inc., Ashland, OR, USA).

Precision-cut lung slice airflow cytometry. Isolation and contraction of mouse PCLS were performed exactly as previously described with minor modifications. After collection of lung slices in serum-free medium, slices were treated with Af of vehicle for 24 h. The next day, slices were washed with serum-free medium and used for the contraction assay. Images were obtained using a Leica DM1400 light microscope equipped with Retiga2000R camera and analyzed using Image-Pro Plus software (Media Cybernetics).

Immunohistochemistry. De-identified human lung specimens and clinical parameters were obtained from the Respiratory Health Network Tissue Bank of the Fonds de recherche québécois en santé, IUCPQ site (www.tissuebank.ca). The IUCPQ Ethics Committee approved the collection of tissue samples by the tissue bank and their use for these studies. All subjects gave informed consent for the tissue sampling and future use of samples for research purposes. Subjects with asthma were classified as having mild to moderate asthma based on criteria established by the Global Initiative for Asthma (GINA). Human and mouse lung sections were deparaffinized and incubated with anti-Alp 1 rabbit antiserum (1:1,500) followed by DAB staining and haematoxylin counterstaining. In Image-Pro Plus software, the Alp 1 positive pixels within ASM bundles were chosen using the ‘Segmentation’ tool, and their area was measured by ‘Count and Measure Objects’ tool and then normalized to the total ASM bundle area.

Protease activity assay. Protease activity was assessed using the Fluoro Protease Assay kit (G-Biosciences) according to the manufacturer’s instructions with minor modifications. Briefly, tissues were homogenized in serum-free medium, slices were treated with Af or vehicle for 24 h. The next day, slices were washed with serum-free medium and used for the contraction assay. Images were obtained using a Leica DM1400 light microscope equipped with Retiga2000R camera and analyzed using Image-Pro Plus software (Media Cybernetics).

Purification of Alp 1 from Af extracts. Af crude extract (50 ml) was diluted in 25 mM acetic acid (200 ml) and applied to a HiPrep CM FF 16/10 column (GE Healthcare Bio-science) pre-equilibrated with 25 mM NaOAc-HAc, pH 4.8, with a peristaltic pump at 4°C. The column was then attached to an AKTA chromatography system. After initial washing with equilibration buffer, bound proteins were eluted with a NaCl gradient (0–0.5 M NaCl) and fractions were collected and kept on ice. Aliquots of each fraction (1 μl) were analysed for protease activity as above. Proteolytically active fractions were combined and diluted with equilibration buffer then applied to a second HiTrap CM FF column (GE Healthcare Bio-sciences) at 4°C. The column was then attached to a Tecan Infinite M200 fluorescent reader at excitation/emission of 488/530 nm. Buffer without protease was used as a blank for background subtraction.

Mass spectrometry and protein identification. Identification of all SDS–PAGE separated proteins was performed on reduced and alkylated, tryptic-digested samples and identified using mass spectrometry protocols. The gels were stained with colloidal Coomassie blue and destained with methanol and acetic acid. Each lane was cut into 200 μl polypropylene auto-sampler vials (Sun Sri, Rockwood, TN). Recovered peptides were re-suspended in 5 μl of Solvent A (0.1% formic acid, 2% acetonitrile and 97.9% water). Prior to mass spectrometry analysis, the re-suspended peptides were chromatographed directly on column, without trap cleanup. The bound peptides were separated using AQ C18 reverse phase media (3 μm particle size and 200 μm pore; 500 nl per min generating 80–120 bar pressure) packed in a pulled tip, nano-chromatography column (0.1 mm ID × 500 mm) from Precision Capillary Columns. Chromatography was performed in-line with an LTQ-Velos Orbitrap mass spectrometer (Thermo Fisher Scientific), and the mobile phase consisted of a linear gradient prepared from solvent A and solvent B (0.1% formic acid, 2% water and 97.9% acetonitrile) at room temperature. Nano LC–MS (liquid chromatography–tandem mass spectroscopy (LC–MS/MS)) was performed with a ProXeon Easy-nLC II multi-dimensional liquid chromatograph and temperature controlled Ion Max Nanospray source (Thermo Fisher Scientific) in-line with the LTQ-Velos Orbitrap mass spectrometer. Computer-controlled data-dependent automated switching to MS/MS by Xcalibur 2.1 software was used for data acquisition and provided peptide sequence information. Data processing and Catabat searching were performed with PD 1.2 and Mascot software (Matrix Science). Data were searched against protein sequences deposited in the National Center for Biotechnology Information Non-redundant protein database (NCBI nr, 04/2011) and a reverse sequence decoy database. Proteins were identified using a 1% false discovery rate cutoff and two peptides per protein minimum.
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Author contributions

N.A.B. and K.M.D. designed the project and wrote the manuscript. N.A.B. designed and performed most of the experiments. M.K. performed experiments shown in Figs 3a–h and 6a,b. J.J. contributed to the experiments in Figs 1b–e, 6h and 7b. M.Z. performed most of the experiments. N.A.B and K.M.D. designed the project and wrote the manuscript. N.A.B. designed and performed experiments shown in Fig. 1a and Supplementary Figs 3a,b and 4. Z.X. performed experiments shown in Fig. 4c and 5e. I.C.C. contributed to experiments shown in Figs 2a, 6h and 7d.f. R.A.P. designed the experiments, analyzed the data, provided critical cell lines and edited the manuscript. M.L. provided clinical samples and data, assisted in interpretation of data in Table 1 and Fig. 8, and edited the manuscript.

Additional information

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