An extracellular matrix fragment drives epithelial remodeling and airway hyperresponsiveness

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It is anticipated that bioactive fragments of the extracellular matrix (matrikines) can influence the development and progression of chronic diseases. The enzyme leukotriene A₄ hydrolase (LTA₄H) mediates opposing proinflammatory and anti-inflammatory activities, through the generation of leukotriene B₄ (LTB₄) and degradation of pro-neutrophilic matrikine Pro-Gly-Pro (PGP), respectively. We show that abrogation of LTB₄ signaling ameliorated inflammation and airway hyperresponsiveness (AHR) in a murine asthma model, yet global loss of LTA₄H exacerbated AHR, despite the absence of LTB₄. This exacerbated AHR was attributable to a neutrophil-independent capacity of PGP to promote pathological airway epithelial remodeling. Thus, we demonstrate a disconnect between airway inflammation and AHR and the ability of a matrikine to promote an epithelial remodeling phenotype that negatively affects lung function. Subsequently, we show that substantial quantities of PGP are detectable in the sputum of moderate-severe asthmatics in two distinct cohorts of patients. These studies have implications for our understanding of remodeling phenotypes in asthma and may rationalize the failure of LTA₄H inhibitors in the clinic.

INTRODUCTION

Tissue inflammation and remodeling are cardinal features of many chronic diseases, as exemplified within the lungs, whereby they are hallmarks of genetic disorders such as cystic fibrosis (CF), as well as common lung diseases such as asthma and chronic obstructive pulmonary disease (COPD), which have emerged as a leading cause of morbidity and mortality worldwide. It had traditionally been hypothesized that chronic inflammation was driving the airway remodeling, but it is increasingly accepted that these features of disease can develop in parallel and that remodeling can even develop independently of the inflammation (1). Consequently, the mediators and mechanisms that instigate airway remodeling are still incompletely understood but are critical in dictating future efforts to develop effective treatment strategies.

Leukotriene B₄ (LTB₄) is generated intracellularly by the enzyme leukotriene A₄ hydrolase (LTA₄H) (2) and upon release binds to leukotriene B₄ receptor 1 (BLT1) (3). LTB₄ functions as a potent chemo tactic factor and activator for various inflammatory cells and instigates pathological inflammation observed in a multitude of chronic diseases (4–6). Consequently, there has been a concerted pharmaceutical effort to develop LTA₄H inhibitors to ameliorate LTB₄-mediated pathology, but these inhibitors have failed to demonstrate efficacy in the clinic (7). Most recently, the LTA₄H inhibitor JNJ-40929837 was assessed in a human bronchial allergen challenge model of asthma, but despite demonstrating clear target engagement and reducing LTB₄, this drug failed to show any clinical benefit over placebo (8).

The extracellular matrix (ECM) is the noncellular component of tissues that provides a scaffold for constituent cells and is critical in the provision of biological cues that dictate development, homeostasis, inflammation, and repair. Degradation of the ECM can liberate biologically active fragments, termed matrikines, which can dictate the progression of inflammation and injury seen in chronic lung diseases (9). One such matrikine is the tripeptide Pro-Gly-Pro (PGP) that is liberated from ECM collagen via the sequential enzymatic activity of matrix metalloproteinases (MMPs) and prolylendopeptidase (10). Once liberated, PGP can subsequently be chemically acetylated through the action of reactive aldehydes to a species that displays enhanced in vivo stability, AcPGP (N-acetylated PGP) (11). PGP and AcPGP function as neutrophil chemoattractants by mimicking key sequences found in glutamic acid, leucine, arginine–positive (ELR₃) chemokines and binding to CXCR1/2 (10, 12). Previously, we demonstrated that LTA₄H has a second anti-inflammatory activity, whereby it degrades PGP to facilitate the resolution of neutrophilic inflammation (11, 13, 14). Conversely, AcPGP is resistant to this LTA₄H-mediated degradation (11). Accordingly, it seems that the LTA₄H-PGP degradation pathway is perturbed in chronic lung diseases leading to the accumulation of PGP, which may subsequently be converted to AcPGP (11, 15), with both species subsequently driving inflammation and pathology (10, 15, 16). LTA₄H therefore represents a highly unusual enzyme with opposing proinflammatory and anti-inflammatory activities that dictate the amplitude and persistence of inflammation, potentially accounting for the failure of LTA₄H inhibitors in a clinical setting.

Here, we assessed this dual functionality of LTA₄H in defining the pathogenesis of asthma. We demonstrate that global deletion of LTA₄H abolished LTB₄-driven inflammation but paradoxically exacerbated airway hyperresponsiveness (AHR) owing to PGP accumulation and a
novel, neutrophil-independent activity for this peptide in promoting a profound pathological epithelial remodeling. Subsequently, we demonstrated that substantial quantities of AcPGP were present in the sputum of severe asthmatics from two separate patient cohorts, thus emphasizing the potential importance of this ECM-derived fragment in driving pathological airway remodeling in a clinical setting.

RESULTS
An absence of LTA₄H leads to exacerbated AHR yet reduced airway inflammation in a house dust mite model of allergic airways disease
To dissect the significance of the dual roles of LTA₄H in the context of allergic airways disease, we used a well-established murine house dust mite (HDM) model (Fig. 1A) (17). To define the specific role of LTβR in the pathogenesis of disease, we used mice deficient in its receptor, BLT1 (fig. S1A). HDM-treated bli²⁻/⁻ mice displayed a significant reduction in airway resistance relative to littermate controls (P < 0.05; fig. S1, B and C). Furthermore, analysis of hematoxylin and eosin (H&E)-stained lung sections demonstrated a reduction in pulmonary inflammation in HDM-exposed bli²⁻/⁻ mice relative to controls (fig. S1D). Thus, in our murine model, LTβR contributes to the inflammatory and AHR changes during allergic airways disease.

We subsequently investigated the phenotype of HDM-exposed lta4h⁻/⁻ mice, which cannot generate LTβR but simultaneously cannot degrade PGP (Fig. 1B). Despite being unable to produce LTβR, HDM-administered lta4h⁻/⁻ mice displayed a marked increase in airway resistance, both at baseline and to increasing doses of methacholine, relative to littermate controls (Fig. 1C and D, and fig. S2, A and B) and a modest yet significant reduction in airway compliance (P < 0.01; fig. S2, C and D). Despite the exacerbated AHR, HDM-exposed lta4h⁻/⁻ mice exhibited reduced total cellular infiltrate into their airways (Fig. 1E) and comparable cell numbers in their lung tissue relative to littermate controls (fig. S2E). A cardinal feature of allergic airways disease in this model is a robust eosinophilic infiltrate, but numbers of eosinophils in the airways of lta4h⁻/⁻ mice were reduced relative to controls (Fig. 1F), and lung eosinophil numbers were comparable (fig. S2F). T helper 2 (T2) cells expressing interleukin 1 receptor-like 1 (T1/ST2) (Fig. 1G) and IL-13 + (interleukin-13–positive) CD4⁺ T cells (Fig. 1H) were also reduced in the airways of HDM-treated lta4h⁻/⁻ mice relative to controls, although lung numbers were similar (fig. S2, G and H). Type 2 innate lymphoid cell (ILC2) numbers were equivalent between HDM-treated lta4h⁻/⁻ mice and littermate controls (fig. S2, I and J). Moreover, numbers of CD11c⁺ airway macrophages were comparable between HDM-administered lta4h⁻/⁻ mice and littermate controls (fig. S2K), and Ly6C hi monocytes were actually reduced in lta4h⁻/⁻ mice (fig. S2L).

HDM-exposed lta4h⁻/⁻ mice exhibit a reduction in classical drivers of AHR
T₂ cytokines IL-4, IL-5, and IL-13 are implicated in driving the inflammation, remodeling, and ultimately AHR in our murine model of allergic airways disease (18). These T₂ cytokines were increased in bronchoalveolar lavage fluid (BALF; Fig. 2, A to C) and lung homogenate (fig. S3, A to C) of wild-type mice upon HDM exposure, but their concentrations were markedly lower in HDM-exposed lta4h⁻/⁻ mice. Similarly, concentrations of the eosinophil chemoattractant eotaxin-2 were reduced in the BALF (Fig. 2D) and lung homogenate (fig. S3D) of HDM-treated lta4h⁻/⁻ mice relative to littermate controls.

Furthermore, lung homogenate concentrations of proinflammatory cytokines tumor necrosis factor–α (fig. S3E) and IL-6 (fig. S3F) were reduced in HDM-exposed lta4h⁻/⁻ mice, whereas the concentration of IL-17 was comparable between wild-type and knockout mice (fig. S3G). Mast cells are a hallmark feature of allergic airway disease, but the concentration of mast cell protease-1 (MCPT-1) was lower in the
HDM-treated lta4h−/− mice exhibit PGP accumulation but comparable neutrophil numbers

To further investigate what may underlie the changes in lung function in these mice, we considered the secondary PGP-degrading action of LTA4H. Administration of HDM resulted in an elevation in BALF concentrations of PGP-generating enzymes MMP-9 and prolylendopeptidase (Fig. 3, A and B), but their concentrations were comparable between lta4h−/− mice and littermate controls. Consequently, both lta4h−/− mice and littermate controls have a comparable capacity to generate PGP from collagen. However, whereas no PGP or AcPGP was detectable in the BALF of wild-type mice, substantial concentrations of PGP (but not of AcPGP) were detectable in HDM-treated lta4h−/− mice (Fig. 3C), due to an inability of lta4h−/− animals to degrade PGP (Fig. 3, D and E).

This model of allergic airways disease is predominantly eosinophilic with a minimal neutrophil infiltrate (fig. S5, A and B). Given the capacity of PGP to function as a neutrophil chemoattractant, we questioned whether PGP accumulation in the HDM-treated lta4h−/− mice promoted a more neutrophilic asthma—a phenotype associated with more severe forms of the disease in humans (22–24). Surprisingly, PGP accumulation in this setting did not lead to enhanced neutrophil numbers in the lung tissue (Fig. 3F) or airways (Fig. 3G) of HDM-treated lta4h−/− mice at 3 weeks or earlier time points (fig. S5, A and B). Although the neutrophil chemoattractant PGP is elevated in HDM-treated lta4h−/− mice, these animals cannot generate neutrophil chemoattractant LTB4 (fig. S5C); thus, the two mediators may differentially compensate. The concentration of classical mouse neutrophil chemokines, keratinocyte chemoattractant (KC) and macrophage inflammatory protein-2 (MIP-2), was modestly elevated in HDM-exposed animals, but no differences were apparent between wild-type and lta4h−/− mice (fig. S5, D and E). Finally, we considered whether neutrophil activation may be altered because of the presence of PGP, leading to augmented release of potentially pathological products. However, the concentration of both primary granule-derived myeloperoxidase (MPO; Fig. 3H and fig. S5F) and tertiary granule-derived MMP-9 (Fig. 3I) was increased in all groups, and the concentration of neutrophils as determined by the number of myeloperoxidase (MPO; Fig. 3I) and tertiary granule-derived MMP-9 (Fig. 3I) was increased in all groups.

PGP drives airway epithelial remodeling and AHR in the context of allergic airways disease

It was postulated that airway structural changes may account for the exacerbated airway resistance observed in the lta4h−/− mice administered HDM. Accordingly, observation of H&E-stained lung sections...
revealed that airway epithelial cell height was significantly augmented in the HDM-treated lta4h−/− mice relative to littermate controls, with epithelial cells projecting out to occlude the lumen of the airways \( (P < 0.001; \text{Fig. 4, A and B, and fig. S6}) \). Furthermore, some of the airways of lta4h−/− mice administered HDM were filled with mucus (Fig. 4A and fig. S6), and periodic acid–Schiff (PAS) staining demonstrated enhanced mucus production by the airway epithelia of HDM-treated lta4h−/− mice relative to littermate controls (Fig. 4, C and D). Subsequently, we questioned whether other aspects of airway remodeling were also altered in the lta4h−/− mice, but no differences were apparent in airway smooth muscle or collagen deposition (fig. S7).

The tripeptide arginine-threonine-arginine (RTR) functions to bind and neutralize PGP both in vitro and in vivo (25). HDM-exposed wild-type and lta4h−/− mice were therefore administered RTR or control peptide, alanine-serine-alanine (ASA), to directly probe the role of PGP in driving the remodeling and lung function changes (Fig. 5A). Administration of RTR to HDM-exposed lta4h−/− mice markedly reduced airway resistance (Fig. 5, B and C), epithelial height (Fig. 5, D and E), and epithelial mucus production (Fig. 5, F and G), demonstrating that all these changes were mediated through the action of PGP. Thus, PGP accumulation in HDM-treated lta4h−/− animals can drive pathological epithelial remodeling and ensuing AHR even in the absence of the important pathological mediator LTB4.

As would be anticipated, no PGP was detectable in the BALF of HDM-exposed bllt1−/− mice that have functional LTA4H (fig. S8A). Furthermore, epithelial height was actually reduced in HDM-exposed bllt1−/− mice relative to wild-type controls (fig. S8B) in keeping with the general reduction in inflammation, remodeling, and ameliorated AHR in these knockout mice that presents as a consequence of the loss of LTB4. This further validates the concept that the exacerbated AHR observed in HDM-exposed lta4h−/− mice is attributable to the accumulated PGP-driven epithelial pathology.

**Direct application of AcPGP induces epithelial remodeling and mucus production**

We subsequently assessed whether this epithelial remodeling effect of PGP was a consequence of a direct, sustained interaction of the peptide with epithelial cells. Normal human bronchial epithelial cells at air-liquid interface (ALI) were therefore cultured in media or media supplemented with AcPGP for 7 days. AcPGP as opposed to PGP was used in these studies because it is functionally comparable to PGP but is resistant to any endogenous LTA4 H that may be released from the bronchial epithelial cells. Analysis of H&E-stained sections of these cells after 7 days demonstrated that AcPGP treatment resulted in an increase in epithelial height (Fig. 6, A and B). In addition, AcPGP supplementation enhanced mucus production in apical supernatants collected at day 7 of culture (Fig. 6, A and B). These changes elicited by AcPGP were of a comparable magnitude to those observed after sustained stimulation of normal human bronchial epithelial cells at ALI with IL-13, a potent driver of goblet cell hyperplasia and mucus production (Fig. 6, A to C).

To further define the effect of PGP on epithelia, normal undifferentiated primary bronchial epithelial cells were cultured with different concentrations of AcPGP and visualized over a period of 72 hours. AcPGP caused an increase in epithelial cell confluence at all concentrations of the peptide tested (fig. S9, A to D), with this phenotype prominent at AcPGP concentrations as low as 10 ng/ml (Fig. 6, D and E).
The increase in confluence was, in part, attributable to an increase in cellular proliferation in response to AcPGP, with a significant increase in the proportion Ki-67–positive cells after 72 hours of peptide stimulation ($P < 0.001$; Fig. 6F and fig. S9, E and F). Biological effects of PGP on other cells have been demonstrated to be CXCR1/2-dependent (12, 26, 27). Anti-CXCR1/2 blocking antibodies significantly reduced the AcPGP-driven changes in epithelial confluence ($P < 0.01$; Fig. 6D and E) and proliferation ($P < 0.001$; Fig. 6F), demonstrating that these effects were also imparted via CXCR1/2 signaling. In addition, AcPGP-treated epithelial cells displayed enhanced radial spreading that would also contribute to the augmented confluency observed (movie S1). At later time points, in particular, AcPGP-treated cells also displayed marked morphological changes with prominent lamellipods, indicative of enhanced cellular motility, and some evidence of a loss of contact inhibition of locomotion (Fig. 6G and movie S1). Thus, AcPGP directly elicits functional and morphological changes in bronchial epithelial cells that could account for the epithelial phenotype observed in HDM-treated lta4h−/− animals. These studies highlight the capacity of a matrikine, generated during inflammation, to subsequently drive pathological airway remodeling, which, in turn, accounts for the marked disconnect between airway inflammation and lung function.

AcPGP is elevated in the sputum of severe asthmatic patients

Whereas no PGP or AcPGP was detectable in HDM-exposed wild-type mice, human severe asthma is a heterogeneous disease with variable inflammatory, remodeling, and clinical phenotypes, and PGP/AcPGP may naturally be present and contributing to pathology in a subset of patients. The concentrations of PGP and AcPGP were subsequently assessed in the sputum of a cohort of 42 patients with moderate-severe asthma and 15 healthy controls (table S1). Although AcPGP was undetectable in the sputum of any of the healthy controls, all of the asthma patients presented with substantial and remarkably similar quantities of the peptide (Fig. 7A). To corroborate these findings, we assessed the concentration of AcPGP in the sputum of a secondary, independently processed and analyzed, cohort of eight severe asthmatics and nine nonsevere asthmatics (table S2). Once again, all severe asthmatics had substantial concentrations of AcPGP in their sputum, which were significantly higher than those observed in nonsevere asthmatics ($P < 0.01$; Fig. 7B). Specifically, in this secondary cohort, no

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**Fig. 4. HDM-treated lta4h−/− mice exhibit augmented airway epithelial remodeling relative to littermate controls.** Wild-type and lta4h−/− mice were administered HDM or PBS intranasally three times per week for 3 weeks, and 24 hours after the final HDM/PBS exposure, lungs were removed for histological examination. (A) Representative H&E-stained lung sections from wild-type and lta4h−/− mice administered PBS or HDM. (B) Epithelial cell height around medium-sized conducting airways was assessed from H&E-stained lung sections. Data presented are an average per mouse. (C) Representative PAS-stained lung sections from wild-type and lta4h−/− mice administered PBS or HDM. (D) Goblet cells were scored from PAS-stained sections. The sum of airway scores from each lung was divided by the number of airways examined and expressed as mucus cell score in arbitrary units. Figures present combined data from two independent experiments with four to six mice per group in each experiment. Results depicted as means ± SEM. *$P < 0.05$, **$P < 0.001$, using Mann-Whitney statistical test. Scale bars, 20 μm.
DISCUSSION

Here, we dissected the dual roles of LTA₄H in driving the inflammatory and remodeling aspects of allergic airways disease. Selective abrogation of LTB₄ signaling ameliorated inflammation and AHR in our murine HDM model, supportive of previous assertions that LTB₄ is an important pathological mediator of asthma (20, 28–31). Conversely, global loss of LTA₄H resulted in an exacerbated AHR—seemingly paradoxical given the absence of LTB₄ and the ensuing reduction in inflammatory cells and mediators. We subsequently revealed that this exacerbated AHR was attributable to accumulation of PGP and a neutrophil-independent capacity of this peptide to elicit a thickening of the airway epithelial layer and elevated mucus production, leading to occlusion of the airways and limitation of airflow. Thus, we demonstrate a clear potential to disconnect airway inflammation from the remodeling and clinical aspects of allergic airways disease and highlight the critical importance of specific remodeling changes in driving lung function. We went on to demonstrate that AcPGP is elevated in two separate cohorts of asthmatic patients, highlighting the potential physiological relevance of this matrikine in defining clinical disease.

Whereas various cytokines and growth factors are recognized as instigators of inflammation and remodeling, the ECM is well-placed to sense tissue injury and initiate an appropriate localized response. Thus, the release of biologically active matrikines from the ECM is not only effectively able to function as damage-associated molecular patterns to promote inflammation but also appropriately placed to dictate and influence the course of wound repair. Consequently, a measured and controlled matrikine system could function to ensure an appropriate response at the specific site of injury. However, excessive matrikine production or persistence, as seen in many chronic lung diseases, would result in pathological sequelae. PGP/AcPGP is classically viewed as a neutrophil chemoattractant, but it is increasingly apparent that this peptide can also modulate the behavior of other cells. For example, recent studies have demonstrated that PGP/AcPGP acts via CXCR2 on endothelial cells to promote vascular permeability (26) and on endothelial progenitor cells to promote
**Fig. 6. AcPGP induces remodeling of human bronchial epithelial cells.** Normal human bronchial epithelial cells were cultured at ALI. Respective wells were treated with media or media supplemented with AcPGP (10 ng/ml) and/or IL-13 (10 ng/ml) for 7 days. Apical supernatants were collected, and cells were fixed for histological analysis. (A) Representative H&E- and PAS-stained sections of ALI culture epithelium after 7 days of treatment. (B) Epithelial cell height was assessed from H&E-stained sections, with data presented as an average per well. (C) Apical supernatant Muc5AC was assessed by ELISA at day 7 after treatment. Undifferentiated normal human bronchial epithelial cells were cultured in media or media supplemented with AcPGP (10 ng/ml) and visualized over a period of 72 hours using a JuLI Stage automated cell imaging system. In some groups, AcPGP-treated cells were preincubated with either anti-CXCR1/2 antibodies or IgG2a isotype control antibody. (D) Intragroup fold change in percent cell confluence of cells at 72 hours after each treatment relative to 0 hours. (E) Fold change in cell confluence over 72-hour period depicted for individual wells in each treatment group. (F) After 72 hours, the cells were fixed and stained for Ki-67, with Ki-67–positive cells expressed as a percentage of 4′,6-diamidino-2-phenylindole (DAPI)–positive cells. (G) Bright-field image of epithelial cells treated with media or AcPGP (10 ng/ml) after 72 hours. Figures (A) to (C) present combined data from two independent experiments with two wells per group in each experiment. Figures (D) to (F) represent data combined from two independent experiments with five wells per group in each experiment. Results depicted as means ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001, using Mann-Whitney statistical test (B and C) or analysis of variance (ANOVA) with Bonferroni correction (E and F). Scale bars, 20 μm.
migration, proliferation, and tube-forming activity (27). Airway epithelial cells, such as their endothelial counterparts, have been shown to express CXCR2 (32), and PGP liberated from the proximally located basement membrane is positioned to modulate epithelial behavior. We demonstrate that direct application of AcPGP to primary human bronchial epithelial cells promoted CXCR1/2-dependent proliferation and radial spreading and recapitulated the in vivo phenotype when the cells were cultured at ALI. Furthermore, AcPGP induced morphological changes in epithelial cells with prominent lamellipods indicative of enhanced cellular motility and some evidence of a loss of contact inhibition of locomotion. It is feasible that PGP elicits an epithelial response that is conducive to localized wound repair; however, if PGP persists as a result of aberrant LTA₄H activity or is converted to PGP-generating MMP-9 and PGP-degrading LTA₄H in our cohorts remains a limitation of this study. No correlation was detected within the moderate-severe asthma cohort between any lung function parameters and levels of AcPGP. However, a limitation of this patient group is that spirometry was performed at the time of patient enrollment, which was often many months before sputum acquisition. AcPGP is a dynamic, changeable readout that has previously been demonstrated in mice. Previous studies have highlighted the significance of specific pathways in driving AHR in mice but have not been recapitated in human disease. Thus, the significance of PGP in defining AHR in patients in severe asthma is yet to be validated and remains a limitation of this study. No correlation was detected within the moderate-severe asthma cohort between any lung function parameters and levels of AcPGP. Whereas no PGP or AcPGP was detectable in HDM-exposed wild-type mice, we demonstrated that AcPGP was present in the sputum of severe asthmatics, rationalizing the physiological relevance of our findings to a clinical setting. Although AcPGP was absent in all healthy controls, it was detectable in all severe asthmatics across two distinct cohorts of patients. In addition, the concentration of AcPGP was greater in severe asthmatics compared to those patients with non-severe disease in the second cohort, highlighting the potential usefulness of AcPGP as an indicator and instigator of severe pathological inflammation and remodeling. Given the function of PGP/AcPGP, it is pertinent that severe asthma phenotypes that exhibit pronounced neutrophilic inflammation (22–24) and greater airway epithelial thickness and proliferation that contribute to progressive decline in lung function have been described (33). Although concentrations of AcPGP detectable in the sputum of severe asthmatics were relatively similar across the two cohorts, it should be noted that differences in sputum processing limit the ability to make direct intercohort comparisons.

Furthermore, although PGP accumulates in \( \text{lta}4\text{H}^{--} \) mice, LTB₄ is absent and may thus counteract the increase in PGP in the context of allergic airways disease. Given that the BALF concentrations of PGP described in this study are lower than those reported previously in neutrophilic inflammatory models or diseases, it is also feasible that the concentration of the peptide generated is insufficient to promote a robust neutrophil infiltrate. Conversely, we demonstrate that PGP can drive the observed epithelial changes even at very low concentrations.

**Fig. 7. The matrikine AcPGP is elevated in the sputum of severe asthmatics.** (A) The concentration of AcPGP peptide in the sputum of patient cohort 1 (UK cohort), consisting of healthy controls (n = 15) and moderate-severe asthmatics (mod-sev asthma; n = 42), as determined by mass spectrometry. (B) The concentration of AcPGP peptide in the sputum of patient cohort 2 (US cohort), consisting of severe (n = 8) and nonsevere asthmatics (n = 9), as determined by mass spectrometry. The horizontal bar depicts the median of each group. Results depicted as means ± SEM. **P < 0.01, ***P < 0.001, using Mann-Whitney statistical test.
demonstrate efficacy (7) and which we have recently shown to abrogate PGP-degrading activity of LTA₄H (37). The recent failure of JNJ-40929837 to demonstrate efficacy in a human bronchial allergen challenge model of asthma, despite clearly reducing the concentration of LTB₄ (8), could be attributable to a confounding increase in PGP. Finally, we have previously demonstrated that PGP is elevated in chronic neutrophilic lung diseases such as COPD and CF (10, 12, 38). Aberrant epithelial remodeling with excessive mucus production is hallmark of these diseases that could in part be attributable to the previously unappreciated role for PGP now identified in this study.

In conclusion, we have identified a role for the matrikine PGP in driving pathological airway epithelial remodeling. Accordingly, we demonstrate that a failure of lta4h−/− mice to degrade PGP in a physiologically relevant murine model of allergic airways disease leads to severe adverse effects and exacerbated AHR, despite the absence of LTB₄. These studies have implications for future assessment of LTA₄H inhibitors in the clinic, as well as, more broadly, our understanding of inflammatory and remodeling changes in severe asthma, COPD, and CF.

MATERIALS AND METHODS

Study design

The primary objective of this study was to define the importance of the proinflammatory (LTB₄ generation) and anti-inflammatory (PGP degradation) activities of LTA₄H in defining the inflammatory and remodeling features of allergic airways disease. All mouse experiments were performed in accordance with the recommendations in the Guide for the Use of Laboratory Animals of Imperial College London, with the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines. All animal procedures and care conformed strictly to the UK Home Office Guidelines under the Animals (Scientific Procedures) Act 1986, and the protocols were approved by the Home Office of Great Britain. In all experiments, littermate controls were used to all knockout strains, and mice were reared under the same environmental conditions and were age-matched. Adult female mice were randomly placed in either the control group or the experimental group. Authors were blinded for cell counts and histology analysis. The number of mice in each group was determined by power calculations based on extensive previous experience with the model system and is defined in the respective figure legends. The number of independent replicates for each experiment is defined within the respective figure legends. No samples or animals were excluded from data analyses.

The human component of the study was designed to define the presence and levels of PGP and/or AcpGP in sputum derived from asthmatic patients and healthy controls. PGP/AcPGP levels were assessed by mass spectrometry and relevant proteases by ELISA, as described elsewhere in Materials and Methods. There were no previous data to inform power calculations in asthmatic patients, and thus, sample size was opportunistic and used historical sample sets. PGP/AcPGP levels were assessed in two independently acquired, processed, and analyzed patient cohorts. Authors were blinded for analysis of PGP/AcPGP levels. All research ethics and patient consent were in place and are detailed in the "Patient populations and sample collection" section, along with patient inclusion criteria and the number of patients within each cohort. Primary data are in table S4.

Patient populations and sample collection

For the primary patient cohort, sputum samples (spontaneous or induced by using hypertonic saline) were collected from 15 healthy individuals and 42 patients with moderate-severe asthma fulfilling the British Thoracic Society criteria (disease steps 3 to 5). Sputum samples were processed as previously described (39), and supernatants were stored at ~80°C. Clinical characteristics of patients are presented in table S1. All samples were collected at the Manchester University NHS Foundation Trust/North West Lung Centre under the framework of the Manchester Allergy, Respiratory and Thoracic Surgery Biobank (study nos. M2014–17 and M2015–32) with ethical approval of the National Research Ethics Service (Research Ethics Committee reference 10/H1010/7). All patients provided written informed consent before participation in the study. Spirometry was performed at the time of patient recruitment and thus may not be reflective of lung function at the time of sputum acquisition.

The secondary cohort was derived through the asthma clinic at the University of Alabama at Birmingham (UAB). The asthma clinic maintains a database, which includes demographics, spirometric values, and laboratory data. For those who consent to participate, blood and induced sputum samples are stored in a clinical specimen biorepository (UAB institutional review board number F150812003). Sputum was induced in patients using the Hargreaves protocol (40). Sputum samples were processed as previously described (41), and supernatants were stored at ~80°C. Clinical characteristics of patients are presented in table S2. The asthmatic populations are defined as severe versus nonsevere based on the need for high-dose inhaled corticosteroids (ICS) plus a second controller agent to maintain control of their asthma or remained uncontrolled despite use of these medications (42) at the time of sputum collection. High-dose corticosteroids are defined according to the Global Initiative for Asthma steroid equivalency table (www.ginasthma.org) or as the highest-dose ICS combined with long-acting beta agonist in the U.S. Food and Drug Administration–approved medication available in the United States.

Allergic airway disease model

Respective wild-type and lta4h−/− mice, on a 129/S6 background, were administered 25 μg of HDM extract (Greer Laboratories) in 50 μl of sterile PBS intranasally three times a week for 3 weeks. Respective wild-type and blt1−/− mice, on a C57BL/6 background, were administered 25 μg of HDM extract in 50 μl of sterile PBS intranasally five times a week for 3 weeks. These differing dosing protocols used in 129/S6 and C57BL/6 mice have been optimized to elicit comparable inflammatory, remodeling, and AHR in these distinct inbred strains. Control mice were administered 50 μl of sterile PBS intranasally at analogous time points. All mice were culled 24 hours after the final dose of HDM or PBS.

For the PGP neutralization studies, respective wild-type and lta4h−/− mice were administered HDM and either 50 μg of RTR or control peptide, ASA (AnaSpec peptides) intranasally three times a week for 3 weeks. All mice were culled 24 hours after the final dose of HDM.

Lung function

Measurements of dynamic resistance, elastance, and compliance were performed in anesthetized and tracheotomized mice using a flexiVent system (SCIREQ) in response to increasing concentrations (0, 3, 10, 30, 100, and 300 mg/ml) of methacholine (Sigma-Aldrich), as described previously (43).
Tandem mass spectrometry for PGP/AcPGP detection
For peptide quantification in BALF, PGP/AcPGP was measured using a MDS Sciei (Applied Biosystems) API-4000 spectrometer equipped with a Shimadzu high-performance liquid chromatography (HPLC). For peptide quantification from degradation experiments, PGP was measured using a Thermo Accela Pump and Autosampler coupled to a Thermo TSQ Quantum Access. HPLC was performed using a 2.0 mm x 150 mm Jupiter 4u Proteo column (Phenomenex) with A (0.1% HCOOH) and B (MeCN + 0.1% HCOOH): 0 to 0.5 min 5% buffer B/95% buffer A, then increased over 0.5 to 2.5 min to 100% buffer B/0% buffer A. Background was removed by flushing with 100% isopropanol/0.1% formic acid. Positive electrospray mass transitions were at 270/70, 270/116, and 270/173 for PGP and 312/112 and 312/140 for AcPGP. Peak area was measured, and peptide concentrations were calculated relative to heavy-labeled PGP and AcPGP internal standards using a relative standard curve method, as previously described (12).

Histological assessment of lung sections
The inferior lobe of the right lung was inflated with and stored in 10% neutral buffered formalin for 24 hours before paraffin wax embedding. The paraffin-embedded sections (4 μm thick) were subsequently stained for H&E to evaluate inflammation and general morphology. Epithelial cell height around medium-sized conducting airways (150 and 250 μm in diameter) was assessed, with 10 measurements made per airway and a minimum of 10 airways measured per section. Data presented are an average per mouse.

Goblet cells were visualized on PAS-stained sections and scored double-blind using a numerical scoring system (0, <5% goblet cells; 1, 5 to 25%; 2, 25 to 50%; 3, 50 to 75%; 4, >75%). The sum of airway scores from each lung was divided by the number of airways examined (20 to 50 airways per mouse) and expressed as mucus cell score in arbitrary units, as previously described (17).

Collagen deposition was assessed on Sirius red-stained sections. Image analysis was performed using Scion Image (Scion Corporation).

Smooth muscle actin staining was performed using a rabbit anti-smooth muscle actin (Thermo Fisher Scientific) antibody diluted 1:1500 using an avidin/biotin staining strategy. Tissue sections were visualized using a Leica DM2500 microscope and QWin software (Leica Microsystems Ltd.).

Assessment of human bronchial epithelial cell proliferation
Primary human normal bronchial epithelial cells (Lonza) were cultured for expansion in a 75-cm² flask (Corning), coated with type 1 collagen (30 μg/ml; Sigma-Aldrich), in Lonza bronchial epithelial growth medium (BEGM). Once more than 90% confluence was achieved, the cells were removed with trypsin (TrypLE Express Enzyme, Gibco) and plated onto a 96-well plate at a density of 5000 cells per well. The cells were supplemented with BEGM or with BEGM containing AcPGP (10 μg/ml, 1 μg/ml, 100 ng/ml, or 10 ng/ml). For assessment of a CXCR1/2-dependent mechanism, epithelial cells were incubated for 1 hour at 37°C with anti-CXCR1 and anti-CXCR2 antibodies (1 μg/ml; R&D Systems) or IgG2a isotype control antibodies (2 μg/ml; R&D Systems) before addition of AcPGP (10 ng/ml). The cells were placed in an incubator for 4 hours for acclimatization to avoid potential condensation on the lid. Snapshots of each well were recorded at 30-min intervals over 72 hours using the JuLI Stage Real-Time Cell History Recorder (NanoEnTek Inc.). Percentage cell confluence was automatically recorded for every snapshot of each respective well. At the end of the experiment, the cells were fixed in 10% neutral buffered formalin (Sigma-Aldrich) for subsequent analysis. Ki-67 staining was performed using a rat anti-mouse Ki-67 primary antibody diluted 1:500 (eBioscience). A Cy3-conjugated goat anti-rat antibody (Jackson ImmunoResearch) diluted 1:500 was subsequently added to enumerate Ki-67 positively stained cells. The cells were then counterstained with a DAPI dye (Sigma-Aldrich) diluted 1:1000. The number of DAPI-positive cells and the number of DAPI and Ki-67 double-positive cells were counted to determine the percentage Ki-67-positive cells per well.

ALI culture studies
Polarized human bronchial epithelial cell cultures (MucilAir) were provided by Epithelix (Sarl Epithelix). The primary cells were generated from a single healthy donor (71-year-old female with no smoking history). The MucilAir cell culture medium was changed every 2 days until experimentation. Respective wells were treated with MucilAir cell culture medium containing AcPGP (10 μg/ml; AnaSpec peptides), human recombinant IL-13 (10 ng/ml; Peprotech), or a combination of AcPGP (10 μg/ml) and human recombinant IL-13 (10 ng/ml). MucilAir cell culture medium was used for media control wells. The respective wells were treated apically overnight and then continuously fed basolaterally every 2 days with medium containing respective treatments. Apical supernatant was collected by washing the apical side of the Transwell with 200 μl of PBS on day 7 and stored at −80°C for subsequent analysis. At the end of the experiment, inserts were fixed in 4% paraformaldehyde and processed for histological analysis.

Statistical analysis
Statistical significance was calculated with a nonparametric Mann-Whitney statistical test (two-sided) and GraphPad Prism software (GraphPad Software Inc.). ANOVA with Bonferroni correction was used for multiple comparisons. Results are depicted as means ± SEM unless stated otherwise. All P values of <0.05, <0.01, and <0.001 were considered significant and are referred to as such in the text.


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An extracellular matrix fragment drives epithelial remodeling and airway hyperresponsiveness

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Extracellular matrix extends its influence in asthma

The interplay between immune cells and smooth muscle cells in asthma pathogenesis and response to treatment is under increasing scrutiny. Patel et al. focused on how leukotrienes can modulate immune inflammation and airway hypersensitivity. Mice lacking leukotriene A4 hydrolase had exaggerated epithelial remodeling but dampened immune responses in a house dust mite model of asthma. This was due to release of Pro-Gly-Pro (PGP), a component of the extracellular matrix, which was also elevated in the sputum from severe asthmatics in two clinical cohorts. Their results showcase how the extracellular matrix is a dynamic player in disease and reveal a mediator to be targeted in asthma therapy.

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