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Segmental Allergen Challenge Enhances Chitinase Activity and Levels of CCL18 in *Mild* Atopic Asthma

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Abstract

Background—Allergic airway inflammation contributes to the airway remodeling that has been linked to increased obstruction and morbidity in asthma. However, the mechanisms by which allergens contribute to airway remodeling in humans are not fully established. CCL18, chitotriosidase (CHIT1) and YKL-40 are readily detectable in the lungs and contribute to remodeling in other fibrotic diseases, but their involvement in allergic asthma is unclear.

Objective—We hypothesized that CCL18, YKL-40, and CHIT1 bioactivity are enhanced in allergic asthma subjects after segmental allergen challenge and are related to increased profibrotic and Th2-associated mediators in the lungs.

Methods—Levels of CCL18 and YKL-40 protein and CHIT1 bioactivity in bronchoalveolar lavage (BAL) fluid, as well as CCL18, YKL-40, and CHIT1 mRNA levels in BAL cells, were evaluated in patients with asthma at baseline and 48 h after segmental allergen challenge. We also examined the correlation between CCL18 and YKL-40 levels and CHIT1 activity with the levels of other profibrotic factors and chemokines previously shown to be up-regulated after allergen challenge.

Results—CHIT1 activity and YKL-40 and CCL18 levels were elevated after segmental allergen challenge and these levels correlated with those of other profibrotic factors, T cell chemokines, and inflammatory cells after allergen challenge. CCL18 and YKL-40 mRNA levels also increased in BAL cells after allergen challenge.

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Conclusions & Clinical Relevance—Our results suggest that CCL18 and YKL-40 levels and CHIT1 activity are enhanced in allergic airway inflammation and thus may contribute to airway remodeling in asthma.

Keywords

asthma; segmental bronchoprovocation; CHIT1; YKL-40; CCL18

INTRODUCTION

Asthma is marked by airway obstruction, hyperresponsiveness, inflammation, and remodeling [1, 2]. Allergen exposure up-regulates the adaptive and innate immune responses, leading to the production of proinflammatory and profibrotic factors that may ultimately contribute to airway remodeling [2–4]. However, the relationship between inflammatory mediators and the observed structural changes seen in asthma is still unclear.

Chitinases have been linked to fibrotic diseases and Th2-mediated inflammation and are associated with asthma severity. Human chitinases, such as acidic mammalian chitinase (AMCase) and chitotriosidase (CHIT1), are evolutionarily conserved glycoproteins that cleave polysaccharide chitin found in several lower life forms including fungi, parasites, and crustaceans [5, 6]. Chitinase-like proteins, such as YKL-40, are structurally similar proteins that do not possess chitinase activity. Both chitinases and chitinase-like proteins have diverse immunological functions especially within the realm of Th2-mediated inflammation [7]. Increased YKL-40 levels in plasma and its presence in the lungs correlate with disease severity and degree of airway remodeling in asthma [8, 9]. Furthermore, polymorphisms in *CHI3L1*, the gene encoding YKL-40, have been associated with asthma [10]. Of the two known active human chitinases, CHIT1 is the most predominant in the lungs and its activity has been linked to other pulmonary disorders such as chronic obstructive pulmonary disease (COPD) and sarcoidosis [11, 12]. An association between atopy and CHIT1 activity is beginning to emerge [13, 14], but it is unclear how allergens affect the activity of CHIT1 in human lungs.

There have been links between CHIT-1 and the expression of a chemokine strongly expressed in the lungs, Pulmonary and Activated Regulated Chemokine (PARC or CCL18), in the context of sarcoidosis and Gaucher's disease [12, 15]. Increased levels of CCL18 have been reported in fibrotic lung diseases, including hypersensitivity pneumonitis, sarcoidosis, and idiopathic pulmonary fibrosis, with many of these studies implicating alveolar macrophages as the primary source of this chemokine [16–19]. CCL18 mRNA has been reported to be increased in the sputum of subjects with asthma compared to atopic and non-atopic controls [20]. Furthermore, basal CCL18 concentrations were higher in the serum and BAL fluid of allergic asthma patients who were untreated compared to those who either received inhaled corticosteroids or were non-allergic control subjects [21]. In a primate model, CCL18 mRNA expression in the lungs was increased after allergen inhalation [22], supporting a role for antigen-mediated up-regulation of CCL18. Furthermore, CCL18 expression can be induced in peripheral blood mononuclear cells (PBMCs) by allergen exposure *ex vivo* [21]. However, it has not been determined how *in vivo* exposure to allergen affects airway expression of CCL18 in asthma.

There is emerging evidence for a role of CCL18 and chitinases in asthma pathophysiology. Given evidence of their involvement in allergic asthma individually, the objective of this study was to elucidate the relationship between CHIT1 activity, YKL-40 and CCL18 levels, in the context of Th2-mediated inflammation and factors associated with remodeling, which

was investigated using an established model of segmental airway allergen challenge in allergic asthmatic patients. (PROOFED)

METHODS

Subjects

BAL fluid analysis was performed on 18 atopic subjects (skin prick test positive) with a history of mild asthma (median FEV₁ was 97% of predicted and median methacholine PC₂₀ was 5.7 mg/ml), including 9 males and 9 females (ages 19 to 32) (*See Table 1*). Analysis of BAL cell mRNA was performed on an additional group of four females and three males (ages 19–27) with a history of mild allergic asthma (mean $\pm$ SEM for FEV₁ was $92 \pm 4\%$ of predicted and methacholine PC₂₀ was 7.6 ± 2.9 mg/ml). All subjects were non-smokers, did not have a respiratory infection or asthma exacerbations within 30 days of the study, and had not received long-acting β -agonists within 2 days, antihistamines or leukotriene antagonists within 7 days, or corticosteroids within 30 days of study enrollment. The University of Wisconsin-Madison Health Sciences Human Subjects Committee approved the study, and informed consent was obtained from all subjects.

Segmental Bronchoprovocation with Antigen (SBP-Ag) and BAL

SBP-Ag and BAL (D0) were performed in two different bronchopulmonary segments as previously described [23]. Allergens used in SBP-Ag were cat antigen (Fel d1, Bayer Allergy Products, Spokane, WA, USA), ragweed (GS Ragweed mix, Greer Labs, Lenoir, NC, USA) and house dust mite (HDM, Dermatophagoides farinae, Miles Allergy Products, Spokane, WA, USA) based on each subject's skin test reactivity and correlative history. The allergen dose for SBP-Ag was based on the subject's physiological response to a previous inhaled allergen challenge (i.e., the provocative dose of allergen required to achieve a 20% fall in FEV₁ (AgPD₂₀)). A total dose of 30% of the subject's AgPD₂₀ was administered incrementally to insure subject's safety: 10% of the AgPD₂₀ in the first segment and, when this dose was well tolerated, 20% in the second segment. A second bronchoscopy with BAL (D2) was performed 48 h later. A total of 160 ml of sterile 0.9% NaCl warmed to 37°C was instilled in each segment in 4 aliquots. BAL fluid recovered from the two segments was pooled for analysis.

Analysis of BAL fluid

Cells were recovered by centrifugation and total and differential cell counts were performed [24]. BAL fluid was kept on ice throughout processing and stored at -80°C before analysis.

CHIT1 activity was measured utilizing the fluorogenic substrate 4-methylumbelliferyl- β -D-N,N',N''-triacetylchitotrioside (Sigma-Aldrich, St. Louis, MO, USA) in McIlvain buffer (100 mmol/L citric acid and 200 mmol/L sodium phosphate, pH 7.0). This assay was performed under optimal conditions for CHIT1, versus AMCase (whose optimal enzymatic activity is at pH of 4.0 [11]). Substrate was incubated with BAL fluid for 15 minutes at 37°C. The reaction was stopped with 2 ml of 0.3 mol/L glycine-NaOH buffer (pH 10.6). The substrate hydrolysis product 4-methylumbelliferone was quantified with a fluorometer using an excitation wavelength of 366 nm and an emission wavelength of 446 nm, and the results were compared to a standard 4-methylumbelliferone calibration curve. CHIT1 activity is expressed as nanomoles of substrate hydrolyzed per hour per milliliter of BAL fluid (nmol/ml/h). Thrombin activity was measured utilizing the chromogenic substrate H-D-hexahydrotyrosol-alanyl-arginine-para-nitroanilide diacetate (American Diagnostica, Greenwich, CT, USA) as previously described [25]. Thrombin activity is expressed as National Institutes of Health (NIH) U/ml of 1x BAL fluid.

“In-house” sandwich ELISAs were utilized as previously described [26] with the following modifications. After thawing, 1 ml aliquots of BAL fluid in Eppendorf tubes were centrifuged at $5,000 \times g$ for 5 min to remove any precipitates or debris. BAL fluid was diluted in LowCross-Buffer (Boca Scientific, Boca Raton, FL, USA) to reduce nonspecific low-affinity binding. Four serial dilutions of each BAL fluid were analyzed on the same plate and the dilutions that resulted in identical calculated values were averaged for the absolute concentration. The antibodies (ab) used for YKL-40 include a mouse monoclonal capture ab and a biotinylated goat polyclonal detection ab from an R&D Systems DuoSet kit (R&D Systems, Minneapolis, MN, USA). The capture ab for CCL18 (clone 64507), interferon-induced protein of 10kDa (IP-10)/CXCL10 (clone 33036.211), matrix metalloproteinase (MMP) 9 (clone 36020), and thymus and activation-regulated chemokine (TARC)/CCL17 (clone 54026) were all mouse monoclonal ab from R&D Systems. The detection ab were biotinylated goat polyclonal detection ab from R&D Systems. Ab for fibronectin included mouse monoclonal capture ab, clone 1601 (Bioscience Resource Project, Saco, ME, USA), and a horseradish peroxidase (HRP)-conjugated rabbit polyclonal detection ab (DakoCytomation, Carpinteria, CA, USA). Insulin-like growth factor binding protein-3 (IGFBP-3) was measured using a commercial kit from R&D Systems. The minimum detection limits were as follows: FN 30 ng/ml, CCL18 and YKL-40 30 pg/ml, IP-10 and TARC 12 pg/ml, and MMP9 3 pg/ml.

Real-time PCR of BAL cells

Total RNA was extracted from BAL cells isolated from BAL fluid before (D0) and 48 h after SBP-Ag (D2) using the RNeasy Mini kit (Qiagen, Valencia, CA, USA). The reverse transcription reaction was performed using the Superscript III system (Invitrogen/Life Technologies, Carlsbad, CA, USA). YKL-40 (forward: attgacagcagctatgacattgc, reverse: aaatcgtaggtcatgatgctaatgaa), CCL18 (forward: cccaagccaggtgtcatc, reverse: tgtatttctggaccactcttattg), CHIT1 (forward: ggctggcaggttcat, reverse: tgaagtagcagaccagttttgca), IP-10 (forward: gatttgctgccttatcttctgact, reverse: caggtacagcgtacagttctagagaga), and TARC (forward: agcacatccacgcagctcga, reverse: tgaagtactccaggcagcactccc) specific primers were designed with Primer Express 3.0 (Applied Biosystems/Life Technologies, Carlsbad, CA, USA) and blasted against the human genome to determine specificity using: <http://www.ncbi.nlm.nih.gov/tools/primer-blast>. Expressions were determined by quantitative real-time PCR (qPCR) using SYBR Green Master Mix (SABiosciences/Qiagen, Frederick, MD, USA). The housekeeping gene β -glucuronidase (GUSB) (forward: caggacctgcgacaagag, reverse: tcgcacagctgggtaag) was used to normalize the samples. Standard curves were performed and efficiency was determined for each set of primers. Efficiency was typically above 94%. The data are expressed as fold change using the comparative cycle threshold ($\Delta\Delta Ct$) method ($\Delta Ct = Ct_{\text{gene}} - Ct_{\text{GUSB}}$ and $\Delta\Delta Ct = \Delta Ct_{\text{of BAL cells at D0}} - \Delta Ct_{\text{of BAL cells at D2}}$). Values shown are fold change 48 h after SBP-Ag ($2^{-\Delta\Delta Ct}$) with the level in BAL cells before challenge fixed at 1.

Statistical Analysis

Baseline and post-SBP-Ag challenge measurements were compared using the paired t-test. Measurements were log-transformed where appropriate. Associations among chemokines and between chemokines and cell counts/percentages were examined using the Spearman's rank correlation coefficient. Measurements are summarized using the median and 1st and 3rd quartiles. A two-sided p-value < 0.05 was regarded as statistically significant.

RESULTS

SBP-Ag increases YKL-40 and CCL18 levels and CHIT1 activity in BAL fluid

General characteristics of the subjects are shown in Table 1. YKL-40 and CCL18 in BAL fluid quantified by ELISA are shown in Figure 1A. YKL-40 levels significantly increased from a median of 23 (1st and 3rd quartiles 16, 30) ng/ml at baseline to 150 (105, 354) ng/ml 48 h after antigen challenge ($p < 0.0001$). CCL18 levels also significantly increased after challenge (1.2 (0.7, 1.9) ng/ml at baseline to 16 (12, 21) ng/ml, $p < 0.0001$). The increased release of YKL-40 and CCL18 were allergen-specific, as challenge with antigen significantly increased the release of these cytokines compared to donor-matched segments that were saline-challenged (see Supplemental Figure 1).

Chitinase activity in BAL fluid, which is due to CHIT1 [11], increased significantly from 0.1 (0.1, 0.2) nmol/ml/h at baseline to 1.3 (0.5, 2.7) nmol/ml/h after SBP-Ag ($p < 0.0001$). Higher levels of YKL-40 at 48 h correlated with both higher CCL18 levels ($r=0.51$, $p=0.03$) and greater CHIT1 activity ($r=0.71$, $p=0.001$) (Figure 1B). Higher CCL18 levels tended to be associated with greater CHIT1 activity 48 h post-SBP-Ag ($r=0.46$, $p=0.06$).

CHIT1 activity and YKL-40 and CCL18 levels correlate with levels of remodeling factors in BAL fluid 48 h after SBP-Ag

As shown in Table 2, thrombin activity and the levels of fibronectin (FN), insulin-like growth factor binding protein-3 (IGFBP-3), and matrix metalloproteinase 9 (MMP9) are significantly higher in BAL fluid 48 h post-SBP-Ag, which is consistent with previous studies [24, 25, 27, 28]. Because CCL18 and chitinases have been implicated in other fibrotic diseases, we also explored the correlation of various fibrotic factors with CCL18/YKL-40 levels and CHIT1 activity in BAL fluid (Figure 2).

FN levels are correlated with those of CCL18 ($r=0.47$, $p=0.05$) and YKL-40 ($r=0.75$, $p<0.001$) as well as with CHIT1 activity ($r=0.54$, $p=0.02$). These relationships were dependent on allergen challenge, as none of these factors correlate to basal (D0) fibronectin levels in the BAL fluid (data not shown). Likewise, IGFBP-3 levels in BAL fluid only correlated with CCL18 levels ($r=0.64$, $p=0.004$), YKL-40 levels ($r=0.75$, $p<0.001$) and CHIT1 activity ($r=0.50$, $p=0.04$) 48 h post-SBP-Ag. MMP9 levels correlated with YKL-40 levels ($r=0.79$, $p<0.001$) and CHIT1 activity ($r=0.58$, $p=0.01$) but not significantly with CCL18 levels ($r=0.43$, $p=0.08$) 48 h post-SBP-Ag. Thrombin activity correlated with YKL-40 levels ($r=0.55$, $p=0.03$) and CHIT1 activity ($r=0.53$, $p=0.03$) but not significantly with CCL18 levels ($r=0.44$, $p=0.08$) 48 h post-SBP-Ag. Interestingly, a relationship between thrombin and CHIT1 activity in BAL fluid was also observed at baseline prior to SBP-Ag ($r=0.68$, $p=0.004$).

CHIT1 activity and CCL18/YKL-40 levels correlate with levels of inflammatory cytokines in BAL fluid after SBP-Ag

We and others have previously reported increased chemokines after SBP-Ag [29–31]. To determine if CCL18/YKL-40 levels after SBP-Ag correlated with the levels of selected inflammatory mediators, the levels of thymus and activation-regulated chemokine (TARC)/CCL-17 and interferon- γ -inducible protein-10 (IP-10) were determined and compared to the levels of CCL18/YKL-40 and CHIT1 activity. As reported previously [30, 31], IP-10 and TARC levels in BAL fluid significantly increased after SBP-Ag (Table 2). In addition, 48 h after SBP-Ag, TARC and IP-10 levels in BAL fluid correlated with YKL-40 levels ($r=0.68$, $p = 0.002$ vs. TARC; $r=0.71$, $p<0.001$ vs. IP-10) and CHIT1 activity ($r=0.62$, $p=0.006$ vs. TARC; $r=0.47$, $p = 0.05$ vs. IP-10), but only TARC, not IP-10, correlated with CCL18 levels ($r=0.82$ $p<0.001$ vs. TARC; $r=0.33$, $p=0.18$ vs. IP-10) (Figure 3).

Relationship between BAL cell composition and allergen-elicited mediators in BAL fluid

Alveolar macrophages are a major source of CCL18 and CHIT1 in the lungs [12, 32–34], whereas YKL-40 is expressed by alveolar macrophages, neutrophils, and epithelial cells [8, 35, 36]. To assess the relationship between allergen challenge-induced CCL18/YKL-40 levels or CHIT1 activity with different cell populations, we determined the cellular profile of the BAL fluid before and after SBP-Ag (Table 3). Before SBP-Ag, CCL18 and YKL-40 levels were detectable in the BAL fluid, but did not correlate with BAL cells. Conversely, CHIT1 activity did correlate with both the total number of BAL cells recovered ($r = 0.39$, $p = 0.04$) and the number of macrophages present ($r = 0.40$, $p = 0.04$) in the BAL fluid. It was also observed that IP-10 levels in BAL fluid were inversely correlated with total BAL cells ($r = -0.51$, $p = 0.03$) and total macrophage counts ($r = -0.59$, $p = 0.01$). Basal levels of IP-10 were also inversely correlated with the percentage of macrophages ($r = -0.55$, $p = 0.02$) but coordinately correlated with the percentage of lymphocytes ($r = 0.66$, $p = 0.003$) present in BAL fluid prior to SBP-Ag. Basal IGFBP-3 levels also correlated with the percentage of eosinophils in the BAL fluid ($r = 0.47$, $p = 0.05$), whereas basal MMP9 levels correlated with both total neutrophil numbers ($r = 0.52$, $p = 0.03$) and the percentage of neutrophils ($r = 0.51$, $p = 0.03$) in the BAL fluid.

In contrast, the levels of many of the measured mediators in BAL fluid correlated significantly with BAL cells 48 h post-SBP-Ag (Table 4). As shown in Table 4, CCL18 levels after allergen challenge significantly correlated with the number of macrophages, lymphocytes and eosinophils present. Furthermore, YKL40 levels correlated with lymphocyte and eosinophil numbers, and CHIT1 activity correlated with lymphocyte and neutrophil counts. Overall, most of the factors measured (except CHIT1 activity and MMP9 and IP-10 levels) correlated with cellular influx into the antigen challenged segment (*i.e.*, fold change in total BAL cell numbers), supporting that these recruited cells are related to the increased presence of factors in the lungs. When considering the concentration of cells per milliliter of BAL fluid, CHIT1 activity also correlated with the increased number of BAL cells. Many of the other examined factors also correlated with the concentrations of eosinophils, macrophages, and lymphocytes present in the BAL. Of note, besides CHIT1 activity, only MMP9 levels in BAL fluid correlated with the number of neutrophils present in the BAL fluid 48 h after SBP-Ag, and IP-10 was the only factor that did not significantly correlate with any of the BAL leukocytes.

To further support that BAL cells are a potential source of these up-regulated mediators, mRNA was extracted from isolated BAL cells before and after SBP-Ag and the mRNA expression of CCL18, YKL-40, CHIT1, TARC, and IP-10 was determined. As shown in Figure 4, mRNA levels for CCL18, YKL-40, and TARC are significantly increased in BAL cells after SBP-Ag. Consistent with the lack of BAL cell correlations, IP-10 expression did not significantly change after SBP-Ag. Interestingly, mRNA expression for CHIT1, the active chitinase in human lungs [11], was decreased in BAL cells after SBP-Ag.

Overall, these data support a relationship between leukocytes in the airway and the release of profibrotic factors and proinflammatory cytokines after allergen challenge in the lungs.

DISCUSSION

Using an established *in vivo* model for allergic airway inflammation in asthma, we demonstrated that BAL fluid levels of CCL18 and YKL-40 and CHIT1 activity increase significantly 48 h following allergen segmental airway challenge. Furthermore, the individual increases in these factors not only correlate with each other, but also with other profibrotic and inflammatory mediators after allergen challenge, suggesting a related role in

allergic asthma pathophysiology. Moreover, we established that BAL cells express CCL18, YKL-40, and CHIT-1 mRNA, suggesting that they are a potential source of these factors.

CCL18 is a chemokine unique to primates and does not yet have a known receptor [17]. It is constitutively expressed in the lungs and is primarily produced by alveolar macrophages and dendritic cells [21]. It is chemotactic for lymphocytes, including Th2 polarized cells and naïve T and B cells, and immature dendritic cells [21, 37]. Earlier reports have noted increased CCL18 expression in diseases associated with Th2 inflammation, such as atopic dermatitis [38, 39]. Our data also reveal a strong correlation between CCL18 levels and the Th2-associated chemokine TARC in the BAL fluid after allergen challenge.

Nadaï et al. [21] previously suggested the involvement of CCL18 in allergic asthma. They found that PBMCs treated *in vitro* with patient-specific allergens release CCL18, and non-medicated allergic asthmatics have elevated CCL18 levels in their BAL fluid/macrophage content versus non-allergic or steroid-treated allergic asthmatics. Our study demonstrates for the first time that CCL18 can be induced in human lungs following *in vivo* allergen challenge, further providing evidence of its role in allergic asthma and Th2-mediated inflammation.

Levels of CCL18 have been shown to be increased in scleroderma lung disease, hypersensitivity pneumonitis, idiopathic pulmonary fibrosis, and sarcoidosis fibrosis [16–19]. CCL18 has also been shown to promote collagen production from pulmonary fibroblasts using *in vitro* models [40, 41]. CCL18 has been shown to increase collagen production in human lung fibroblasts [42–44], and to be a sensitive indicator of active pulmonary fibrosis [45]. Furthermore, CCL18 production from BAL cells has been enhanced in patients with pulmonary fibrosis [33]. Our data further support a relationship between CCL18 and fibrotic factors, as levels of fibronectin and IGFBP-3 in the BAL fluid after allergen challenge correlated with the levels of CCL18.

Both chitinases and chitinase-like proteins have diverse immunological functions [7]. These evolutionarily conserved enzymes undoubtedly have a role in the anti-parasitic responses of the human immune system, but growing evidence also suggests that they might have other important immunomodulatory roles, especially in the context of unregulated inflammation. Although the exact function of chitinases as immunomodulators is still being elucidated, a growing body of literature shows involvement of chitinases in a variety of disease processes, including COPD, Gaucher disease, chronic inflammation (e.g., sarcoidosis, arthritis), and infections [12, 15, 34, 46].

Although the major chitinases in murine lungs are AMcase and YM1/2, CHIT1 is the predominant active chitinase in humans [11, 47]. Subjects homozygous for CHIT1 deficiency are less likely to suffer from atopic conditions [13]. Furthermore, increased levels of CHIT1 mRNA and protein are detectable in nasal mucosa from allergic donors compared with normal donors [14]. The role of CHIT1 in human asthma is unclear, but it has been argued that only smokers with COPD, but not asthmatics, have higher chitinolytic activity, increased levels of CHIT1 in BAL fluid, and more CHIT1-positive cells in bronchial biopsies [11, 35]. However, it is important to note that these studies did not screen for the 24 base pair duplication of CHIT1, which renders CHIT1 inactive. Therefore, the expression/activity of CHIT1 in asthmatics may have been masked in prior studies. It is also possible that there is sufficient active AMcase expression in the lungs to lead to the increased chitinase activity after allergen challenge, although it has been reported that all of the detectable AMcase in human lungs is in an inactive form [11].

Our data are the first to show that CHIT1 activity is increased in human lungs after *in vivo* allergen exposure and support the notion that allergen challenge leads to increased CHIT1

activity and correlates with the increased presence of profibrotic factors in the lungs. Interestingly, although CHIT1 activity increases, the levels of CHIT1 mRNA detected from BAL cells decreases 48 h after allergen challenge. There are many potential explanations for this decrease in CHIT1 mRNA: 1) CHIT1 mRNA may be more transient than the other factors measured; therefore, its mRNA accumulation may diminish while protein and activity is still present; 2) the cellular source of CHIT1 may be exclusively alveolar macrophages [11, 48], which are a lower percentage of the cells after allergen challenge, thus comparing the levels of CHIT1 before and after challenge is skewed against observing macrophage-derived endpoints; 3) the increased CHIT1 activity observed may not be linked to changes in CHIT1 expression. During post-translational processing, a fraction of CHIT1 is intracellularly converted by carboxyl-terminal proteolytic processing into a 39-kDa isoform that accumulates in lysosomes and has no chitinase activity [48]. Allergen challenge may block the accumulation of CHIT1 in the lysosome, resulting in increased levels of secreted active CHIT1 and 4) other factors besides CHIT1 are involved in the increased CHIT1 activity seen after allergen challenge. Regardless, CHIT1 activity is potentiated after allergen challenge and likely plays a role in the up-regulation of Th2-mediated inflammation.

YKL-40, a chitinase-like protein, has been shown to be increased in the serum and lungs of allergic asthmatics, and these YKL-40 levels correlate with severity of disease, asthma exacerbations, eosinophil levels, and degree of airway remodeling measured as the thickness of the subepithelial basement membrane [8, 49]. In addition, genetic polymorphisms in the chitinase 3-like 1 gene encoding for YKL-40 are associated with increased YKL-40 levels, bronchial hyperresponsiveness, risk for asthma, and reduced lung function [6, 9].

YKL-40, which is also increased in the BAL fluid and serum of COPD patients, can promote the release of proinflammatory mediators and remodeling factors, such as MMP9 from alveolar macrophages, suggesting a role for YKL-40 in airway inflammation and remodeling [35]. Our data show a strong correlation between YKL-40 and MMP9 levels (and all of the other fibrotic factors tested) after an *in vivo* allergen challenge of atopic asthmatics, further supporting that these two factors are linked in pulmonary inflammatory diseases.

In a letter to the editor, Kuepper et al. reported increased serum and BAL YKL-40 levels 24 h following a segmental allergen challenge in allergic asthma patients [50]. Leukocyte recruitment/composition in the lungs varies over time, with the peak of total leukocytes and alveolar macrophages occurring after two days [51], so our detection of YKL-40 levels after 48 h captured the peak of leukocyte recruitment to the lungs. Our data extend Kuepper's observations by demonstrating an increase in YKL-40 48 h after allergen challenge, and they also reveal new correlations between YKL-40 levels in the lungs with other allergen-elicited mediators and leukocytes recruited to the site of allergen challenge.

There are some limitations to our study. First, these patients may not represent the full spectrum of asthma patients; to ensure patient safety, only patients with mild asthma were selected for SBP-Ag. Therefore, it is not known if our findings can be directly extrapolated to patients with more severe disease. Second, our model is based on allergic inflammatory response, thus the findings are not applicable to patients with non-allergic asthma or to non-allergic stimuli such as viral respiratory tract infections. Third, with segmental airway allergen challenge, we only have lung samples at two time points. While in theory, more frequent sampling might be feasible, subject safety and comfort precluded obtaining more frequent samples. Since the inflammatory process occurs along a time continuum, it is possible that the levels of some of the cytokines would be different either before or after the 48 h time point when the second BAL sampling is performed. Therefore, our sampling may

have missed peaks of some of the measured factors. Finally, we did not examine tissue biopsies in the current study, thus we can't ascertain if remodeling process was initiated following antigen challenge. However, the increase in soluble matrix protein (*i.e.*, FN) that we were able to demonstrate suggests that there is a potential for such outcome.

In conclusion, we provide new evidence for the involvement of CCL18, YKL-40, and CHIT1 in allergic airway inflammation and suggest that these factors might be linked to airway remodeling, which is an important component of asthma pathophysiology. Better understanding of how these factors relate to each other and to the recruited inflammatory cells in the airway could provide necessary rationale for new therapeutic approaches to prevent structural airway changes that are potentially linked to allergen exposure. Such advances could also have implications to other Th2 inflammatory-fibrotic conditions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. BAL fluid concentrations of YKL-40 and CCL18 and CHIT1 activity are increased 48 h after SBP-Ag

(A) Data represent the concentrations of specific analytes in BAL fluid obtained immediately before (D0) and 48 h after (D2) SBP-Ag. Each symbol represents the same individual subject across assays. (B) Correlations between YKL-40, CCL18 levels and CHIT1 activity in BAL fluid 48 h post SBP-Ag were plotted together and correlations were determined. r_s = Spearman's correlation coefficients. Each symbol represents the same individual subject across assays (n = 18).

Figure 2. Associations between concentrations of remodeling factors with CHIT1 activity and YKL-40 and CCL18 concentrations in BAL fluid
 r_s = Spearman's correlation coefficients. Specific symbols represent individual subjects across correlations (n = 18).

Figure 3. Associations between concentrations of Th-associated chemokines with CHIT1 activity and YKL-40 and CCL18 concentrations in BAL fluid

r_s = Spearman's correlation coefficients. Specific symbols represent individual subjects across correlations ($n = 18$).

Figure 4. Mediator mRNA expression by BAL-derived cells before and after SBP-Ag
 (A) YKL-40, (B) CCL18, (C) CHIT1, (D) TARC, and (E) IP-10 mRNA expression in BAL cells immediately before (D0) and 48 h after (D2) SBP-Ag (n = 8, except CCL18 (n = 7)).

Table 1

Characteristics for subjects in the BAL fluid study *

Number	18
Sex	9 F, 9 M
Age (yrs)	22 (20, 23)
Baseline FEV ₁ (% predicted)	97 (90, 102)
Methacholine PC ₂₀ (mg/ml)	1.7 (0.8, 5.2)

* median (1st and 3rd quartiles)

Table 2

Select profibrotic and BAL fluid (BALF, 1x) protein profile before and 48 h after SBP-Ag

	D0	D2
CHIT1 activity (nmol/ml/h)	0.1 (0.1, 0.2)	1.3 (0.5, 2.7) *
YKL-40, ng/ml	22.7 (15.6, 29.8)	150.1 (104.8, 354.4) *
CCL18, ng/ml	1.2 (0.7, 1.9)	16.0 (11.9, 21.4) *
FN, µg/ml	1.0 (0.5, 2.4)	36.4 (15.4, 108.0) *
IGFBP3, ng/ml	0.7 (0.5, 2.0)	27.1 (13.2, 64.6) *
MMP9, ng/ml	0.2 (0.1, 0.3)	2.1 (1.3, 6.4) *
thrombin activity, NIH U/ml	0.4 (0.3, 0.5)	1.1 (0.7, 1.5) *
TARC, pg/ml	0 (0, 0)	1867 (987, 4310) *
IP10, pg/ml	35 (15, 42)	176 (80, 245) *

Data are expressed as median (1st and 3rd quartiles).*
p < 0.001 (D0 vs. D2)

Table 3

Cell Differentials in BAL fluid before and after SBP-Ag

	D0	D2
Total cells per ml, ($\times 10^4$)	13.6 (10.9, 15.9)	121.1 (56.6, 265.9) *
Eosinophils, %	0.7 (0.3, 1.2)	57.3 (39.2, 65.6) *
Macrophages, %	91.2 (82.4, 94.2)	29.6 (23.0, 45.4) *
Lymphocytes, %	6.0 (4.5, 10.5)	7.3 (5.2, 10.8)
Neutrophils, %	1.0 (0.6, 2.0)	3.5 (2.0, 4.6) *

Data are expressed as median (1st and 3rd quartiles).*
p < 0.001 (D0 vs. D2)

Table 4
Correlations between BAL Cellular and Protein Fluid Levels 48 h after SBP-Ag

The number of leukocytes (#) collected in the BAL fluid was compared to the levels of selected proteins measured 48 h after segmental allergen challenge. The Spearman's rank correlations and corresponding p-values are displayed. The boxes in white indicate significant correlations and the boxes in grey indicate no significant correlation. Cell.fold: fold change in cell numbers from D0 to D2; mac: macrophage; lym: lymphocyte; pmn: polymorphonuclear (neutrophil); eos: eosinophils.

	#cell	cell.fold	#mac	#lym	#pmn	#eos	
Chitinase Act	0.48	0.45	0.45	0.57	0.64	0.42	r_s
	0.04	0.06	0.06	0.01	0.005	0.08	p value
YKL40	0.52	0.51	0.31	0.59	0.44	0.51	r_s
	0.03	0.03	0.20	0.009	0.06	0.03	p value
CCL18	0.66	0.64	0.55	0.72	0.35	0.68	r_s
	0.003	0.004	0.02	0.0008	0.16	0.002	p value
FN	0.76	0.70	0.65	0.82	0.43	0.73	r_s
	0.0002	0.001	0.003	<0.0001	0.07	0.0006	p value
MMP9	0.42	0.45	0.30	0.57	0.56	0.40	r_s
	0.08	0.06	0.22	0.01	0.02	0.10	p value
TARC	0.75	0.74	0.65	0.86	0.47	0.73	r_s
	0.0003	0.0005	0.004	<0.0001	0.05	0.0006	p value
IP-10	0.18	0.25	0.08	0.34	0.41	0.16	r_s
	0.47	0.32	0.75	0.17	0.09	0.52	p value
IGFBP3	0.76	0.73	0.63	0.86	0.44	0.74	r_s
	0.0003	0.0006	0.005	<0.0001	0.06	0.0004	p value
Thrombin Act	0.76	0.61	0.81	0.76	0.40	0.66	r_s
	0.0007	0.01	0.0001	0.0006	0.13	0.005	p value