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Segmental allergen challenge increases levels of airway follistatin-like 1 in patients with asthma



To the Editor:

We have used a proteomic approach to identify novel mediators of inflammation in the sputum of a subject with asthma compared with a healthy control subject (see this article's [Methods](#)^{E1,E2} section in the Online Repository at www.jacionline.org). Using this approach, we determined that follistatin-like 1 (FSTL1) is more than 200-fold highly expressed in the subject with asthma compared with the control subject and was the most highly expressed of the 508 proteins we examined in sputum. FSTL1, a 308 amino acid extracellular glycoprotein that shares 94% identity in man and mouse,^{1,2} is generated by nonhematopoietic cells such as cells of the mesenchymal lineage (fibroblasts, chondrocytes, osteocytes, adipocytes, cardiomyocytes) by stimuli including TGF- β , IL-1 β , TNF- α , and IL-6.^{1,2} FSTL1 released from these mesenchymal cells targets immune cells (monocytes, macrophages, and T cells) to express proinflammatory cytokines

(IL-1 β , TNF- α , IL-6, IFN- γ) and chemokines (IL-8, MCP1, IP10).¹ FSTL1 has been studied in embryogenesis,^{E1} tumor development,^{E2} cardiac disease,^{E3} arthritis,^{E4,E5} bleomycin-induced lung fibrosis,^{E6} and wound healing.^{E7} The predominant effect of FSTL1 appears to be proinflammatory,^{E4,E5,E8} although anti-inflammatory effects of FSTL1 have also been described.^{E9} Using a mouse model, we recently demonstrated that allergen challenge induced mouse lung macrophages to highly express FSTL1, which was associated with airway remodeling.³ Therefore, this study examined whether in humans, like in mice, allergen challenge could also induce expression of FSTL1 in the airway, and whether human bronchoalveolar lavage (BAL) macrophages like mouse macrophages expressed FSTL1 and were also activated by FSTL1 to express proremodeling mediators such as matrix metalloproteinase 9 (MMP9).⁴

To determine whether allergen challenge induced lung expression of FSTL1, stored BAL fluid samples from 12 subjects with mild allergic asthma (see [Table E1](#) in this article's Online Repository at www.jacionline.org) were assayed for FSTL1 by ELISA (R&D, Minneapolis, Minn) in a protocol approved by the University of Wisconsin-Madison Health Sciences Institutional Review Board. In brief, segmental bronchoprovocation with allergen (SBP-Ag) and BAL (preallergen and postallergen) was performed (see this article's [Methods](#) section^{E3,E4}) as previously described.⁵ SBP-Ag in subjects with asthma induced a significant increase in levels of BAL FSTL1 protein ($P < .03$) (preallergen vs postallergen) ($n = 12$ subjects) ([Fig 1, A](#)). Nine additional subjects with asthma previously described^{E10} who had sufficient BAL cells were included to analyze FSTL1 mRNA expression before and after SBP-Ag. There was an increase in FSTL1 mRNA in BAL cells after SBP-Ag (assessed by real time quantitative PCR [RT-qPCR]) (see this article's [Methods](#) section^{E4-E6}), which approximated statistical significance ($P = .057$) (preallergen vs postallergen) ($n = 9$ subjects) ([Fig 1, B](#)), and was positively associated with the percentage of BAL macrophages (see [Table E2](#) in this article's Online Repository at www.jacionline.org). Levels of BAL cell FSTL1 mRNA correlated significantly with levels of BAL cell MMP9 mRNA ($r = 0.67$; $P = .04$) ([Fig 1, F](#)), but not with BAL MMP9 protein (not shown). The negative correlation between FSTL1 and eosinophils ([Table E2](#)) could indicate that FSTL1 may also have anti-inflammatory effects on eosinophils. In this regard, at least 2 studies of FSTL1 administration in mouse models of arthritis have demonstrated that FSTL1 has anti-inflammatory effects as demonstrated by reduced joint inflammation and reduced expression of IL-6 and prostaglandin E₂.^{E11,E12}

To determine whether human lung BAL macrophages expressed FSTL1, we used 2 approaches, immunostaining of postmortem human lungs of those with asthma to detect whether FSTL1 was expressed by alveolar macrophages, and investigation of whether postmortem human BAL macrophages expressed FSTL1. We immunostained postmortem lung sections (asthma and normal control) ($n = 3$ /group) obtained from the National Disease Research Interchange (Philadelphia, Pa) with an anti-FSTL1 antibody (R&D) (see this article's [Methods](#) section^{E7}) ([Fig 1, C-E](#)). These studies demonstrated that FSTL1 was highly expressed in lung alveolar macrophages in asthma ([Fig 1, E](#)), but was not significantly expressed in control lung alveolar macrophages ([Fig 1, C](#)). The number of FSTL1+ cells was significantly higher in the airway of lungs of subjects with asthma compared with controls ($P < .001$) ($n = 3$) ([Fig 1, E](#)).

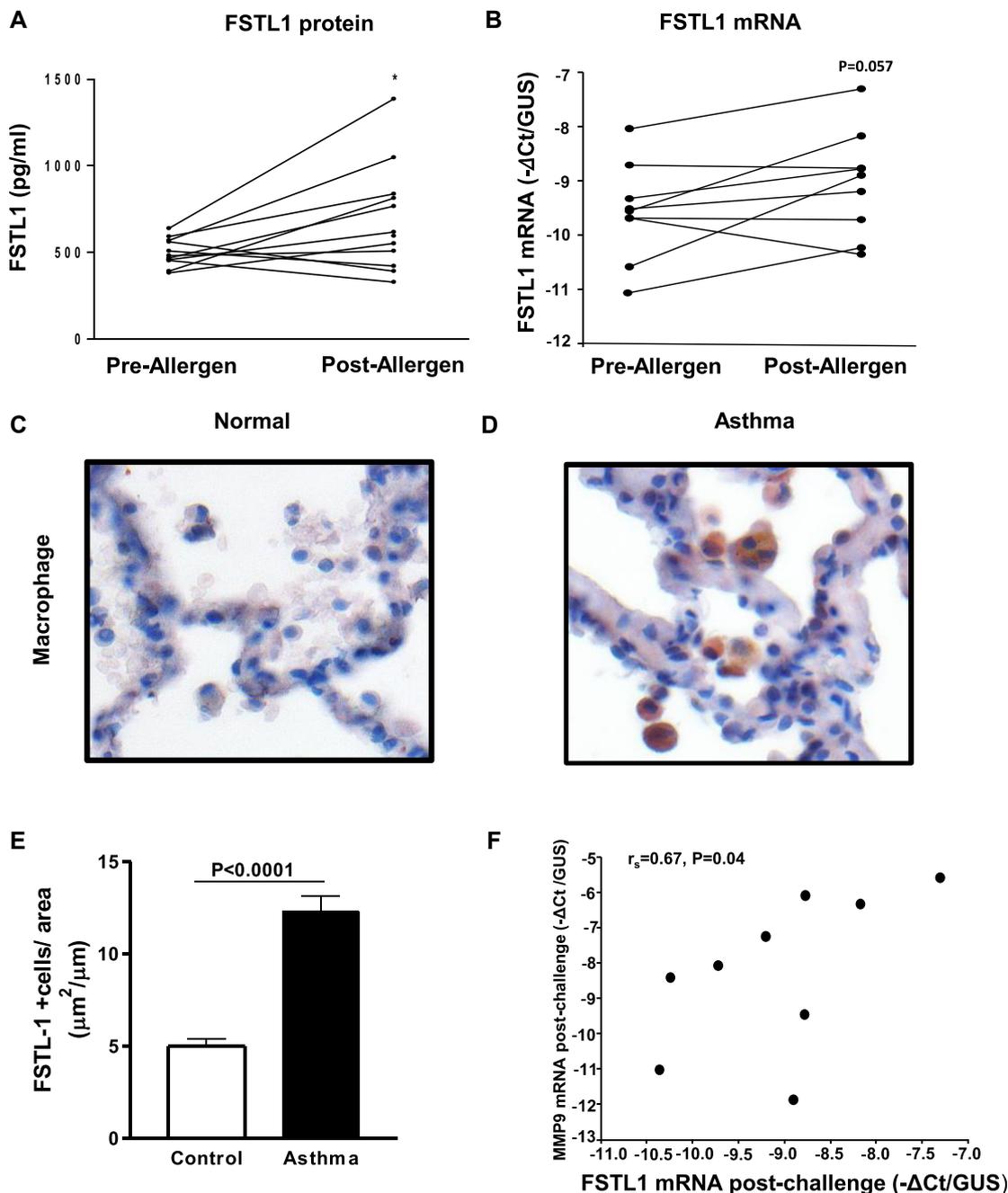


FIG 1. Segmental allergen challenge increases levels of FSTL1. **A**, Levels of FSTL1 were quantitated by ELISA in BAL fluid obtained before and after segmental allergen challenge in subjects with allergic asthma ($P < .03$) ($n = 12$ subjects). **B**, Levels of FSTL1 mRNA were quantitated by RT-PCR in BAL cells obtained before and after segmental allergen challenge in subjects with allergic asthma ($P = .057$) ($n = 9$ subjects). **C-E**, Lung sections from a normal subject and a subject with asthma were immunostained with an anti-FSTL1 antibody and demonstrate FSTL1+ alveolar macrophages in the subject with asthma (Fig 1, *D*), but not in the normal subject (Fig 1, *C*). The number of FSTL1+ alveolar macrophages quantitated by image analysis (Fig 1, *E*) was significantly higher in the lungs of subjects with asthma compared with controls ($P < .001$) ($n = 3$ subjects/group). **F**, BAL cell FSTL1 and MMP9 mRNA levels after segmental allergen challenge were determined by RT-PCR, and correlation calculated using Spearman correlation ($r_s = 0.67$; $P = .04$) ($n = 9$ subjects).

To determine whether human BAL macrophages responded to FSTL1, BAL macrophages were obtained from human lungs by lavage postmortem (see this article's [Methods](#) section^{E8}) as previously described.⁶ Incubation of BAL macrophages with FSTL1 (100 ng/mL) induced a significant increase in levels of

macrophage FSTL1 mRNA as assessed by RT-qPCR as compared with macrophages cultured in media alone ($P < .05$) (Fig 2, *A*). TGF- β 1, a known inducer of FSTL1, also significantly induced FSTL1 mRNA expression by BAL macrophages ($P < .05$) (Fig 2, *A*). These studies suggest that macrophage-derived

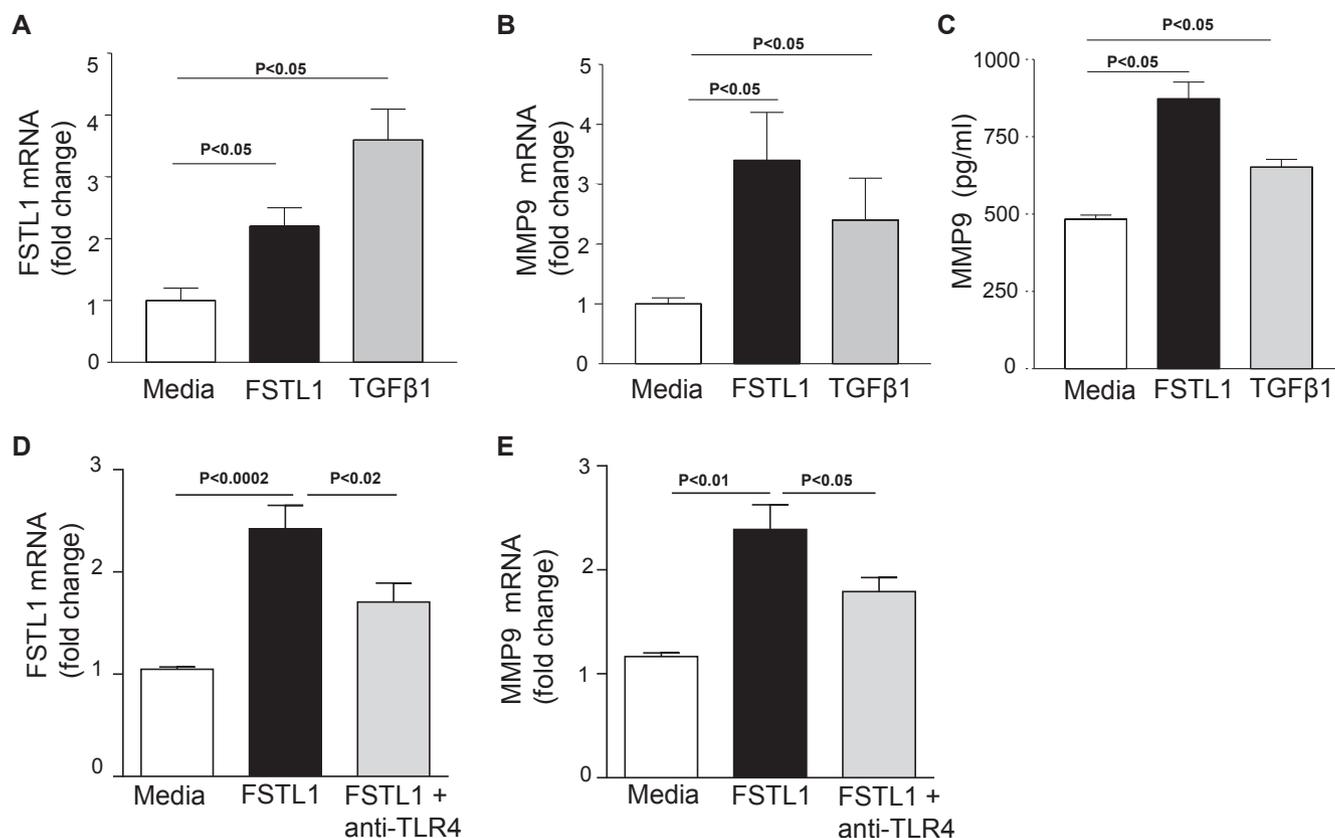


FIG 2. FSTL1 induces expression of FSTL1 and MMP9 by BAL macrophages *in vitro*. **A-C**, Human BAL macrophages ($n = 3$ subjects) were incubated *in vitro* with either FSTL1 (100 ng/mL) or TGF- β 1 (100 ng/mL). Levels of FSTL1 mRNA (Fig 2, A) and MMP9 mRNA (Fig 2, B) were quantitated by RT-qPCR, and levels of MMP9 protein (Fig 2, C) by ELISA. **D** and **E**, Human BAL macrophages ($n = 6$ subjects) were incubated *in vitro* with either an anti-hTLR4-IgG-neutralizing antibody, or a species and isotype control mouse IgG antibody, for 1 hour before stimulation with FSTL1 (100 ng/mL) for 24 hours. Levels of FSTL1 mRNA (Fig 2, D) and MMP9 mRNA (Fig 2, E) were quantitated by RT-qPCR.

FSTL1 can either through paracrine or autocrine mechanisms induce further FSTL1 expression by macrophages, similar to what we have noted in studies of mouse macrophages.³ In addition, FSTL1 induced BAL macrophages to express MMP9 mRNA as assessed by RT-qPCR ($P < .05$) (Fig 2, B), as well as MMP9 protein as quantitated by ELISA (R&D) ($P < .05$) (Fig 2, C). Previous studies have demonstrated that FSTL1 can activate Toll-like receptor 4 (TLR4)-dependent cytokine responses in cell types other than human BAL macrophages.^{E13} Accordingly, we incubated BAL macrophages ($n = 6$ donors) with FSTL1 and an anti-TLR4 antibody and noted significantly reduced levels of FSTL1 mRNA ($P < .02$) (Fig 2, D) and MMP9 mRNA ($P < .05$) (Fig 2, E). Thus, blocking only TLR4 signaling inhibits FSTL1 activation of BAL macrophages *in vitro*. Further studies are needed to determine whether other TLR4-expressing cells in the lung that respond to FSTL1 do so through TLR4 and/or other known FSTL1 signaling pathways (ie, bone morphogenetic protein, protein kinase B, adenosine monophosphate-activated protein kinase, Na/K-ATPase membrane potential).^{E13-E16}

In summary, we have made the novel observation that segmental allergen challenge increases levels of FSTL1 protein in the airway of subjects with asthma and that levels of human BAL cell FSTL1 mRNA correlate with levels of BAL cell MMP9 mRNA, a remodeling mediator we demonstrate is induced in

macrophages by FSTL1 *in vitro*. In addition, we show that human lung airway macrophages express and respond to FSTL1, potentially through paracrine or autocrine pathways. This study also demonstrates that human lung alveolar macrophages can be induced to express FSTL1, which suggests a different cellular source of FSTL1 in asthma compared with other diseases (arthritis, autoimmune disease, coronary disease) where macrophages and myeloid cells are not a significant source of FSTL1.^{1,2} FSTL1 in these diseases is generated in particular by nonhematopoietic cells such as cells of the mesenchymal lineage (fibroblasts, chondrocytes, osteocytes, adipocytes, cardiomyocytes).¹ The potential functional significance of allergen challenge inducing FSTL1 is suggested from our studies, demonstrating that in humans FSTL1 can induce BAL macrophages to express MMP9, a metalloproteinase associated with remodeling in asthma.⁷⁻⁹ The present human study demonstrates that allergen challenge of subjects with asthma induces FSTL1 expression, and extends our observations in mice in which we have demonstrated that mouse Fstl1 promotes airway remodeling.³ Further study is needed to determine whether targeting FSTL1 would inhibit airway remodeling in humans with asthma.

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A novel Rab27a mutation binds melanophilin, but not Munc13-4, causing immunodeficiency without albinism

To the Editor:

The small GTPase Rab27a regulates a number of exocytic processes. In melanocytes Rab27a interacts with melanophilin to

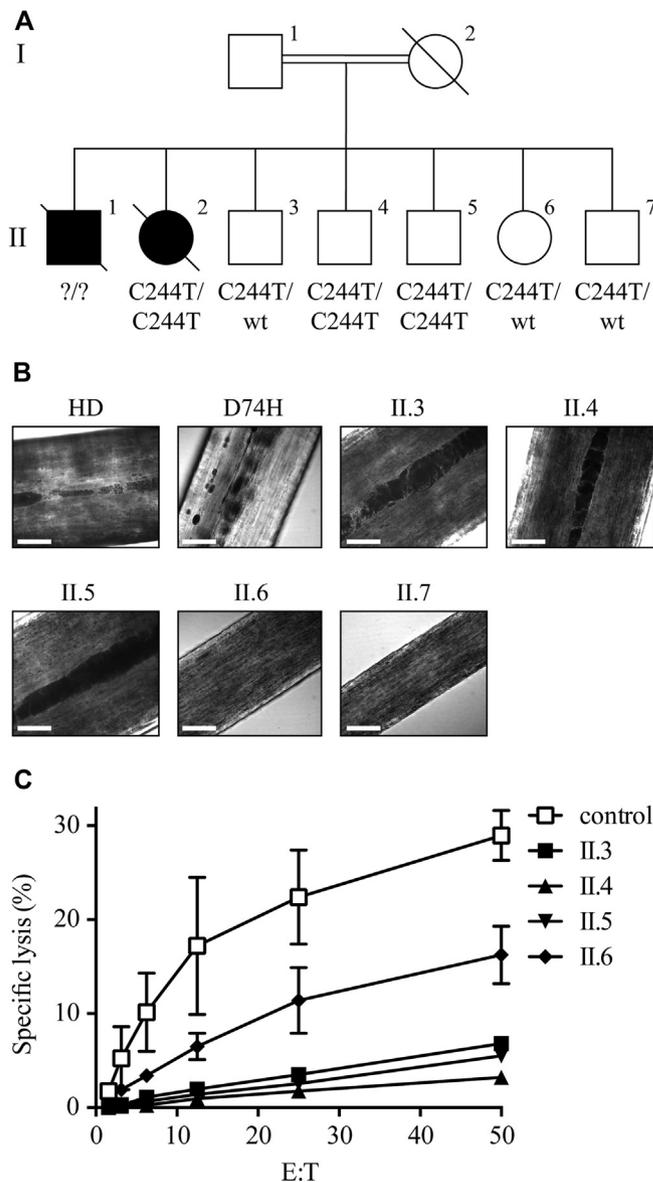


FIG 1. Rab27a R82C does not alter hair pigmentation but decreases NK cell-mediated cytotoxicity. **A**, Family pedigree. Parents are first-degree cousins. Patients II.1 and II.2 died. *wt*, Wild-type. **B**, Differential interference contrast images of hair from patients and a healthy donor (*HD*; $\times 63$ magnification). Scale bar = 25 μ m. **C**, NK cell cytotoxicity in PBMCs from patients and a healthy control subject measured in a standard 4-hour ⁵¹chromium release assay against K562 target cells (n = 2). *E:T*, Effector cell/target cell ratio.

mediate the melanosome transport required for pigmentation.¹ In natural killer (NK) cells and cytotoxic T lymphocytes, degranulation and thereby cell-mediated cytotoxicity require interaction of the active form of Rab27a with Munc13-4.^{2,3} Mutations in the human *RAB27A* gene cause the autosomal recessive Griscelli syndrome type 2 (GS2) with diluted pigmentation and immunodeficiency, which can progress to hemophagocytic lymphohistiocytosis (HLH).⁴ Recently, 6 patients with GS2 and normal pigmentation have been reported, identifying Rab27a mutations that selectively disrupt Munc13-4 binding when re-expressed in HEK293 cells.⁵ Here we present a new case of GS2 with no

METHODS

Sputum induction

Sputum induction and processing was performed according to a standardized protocol.^{E17} In brief, a subject with asthma and a control subject were exposed for 20 minutes to an aerosol of 3% hypertonic saline solution using a NOUVAG Ultrasonic nebulizer (Nouvag USA Inc, Lake Hughes, Calif) and sputum was collected into 50-mL sterile ampoules. The volume of the induced sputum was determined, and an equal volume of dithiothreitol (0.1% in saline; Sigma Chemical Co, St Louis, Mo) was added. After homogenization, sputum samples were centrifuged at 2000g for 5 minutes to separate the supernatants from the cell pellet. The supernatants were then aspirated and frozen at -80°C in separate aliquots for subsequent analysis.

Proteomics

A glass-slide-based proteomic array capable of detecting 508 proteins (Raybiotech, Norcross, Ga) was used to detect proteins in human sputum from a subject with asthma and a control subject according to the manufacturer instructions.

Segmental allergen challenge: Determination of the provocative dose of allergen leading to a 20% fall in FEV₁

To establish the dose of allergen to be administered by segmental challenge, a graded inhalation challenge was initially performed to determine the participant's provocative dose of allergen leading to a 20% fall in FEV₁ (AgPD₂₀) as described earlier.^{E10} Allergens used were *Dermatophagoides farinae* (Der p 1, dust mite), *Felis catus domesticus* (Fel d 1, cat), or *Ambrosia artemisiifolia* (Amb a 1, ragweed), obtained from Greer Laboratories (Lenoir, NC). The segmental allergen challenge was performed at least 1 month after the inhalation allergen challenge.

Bronchoscopy, segmental allergen challenge, and processing of BAL fluid

A baseline bronchoscopy with BAL was performed (4×40 mL aliquots of sterile 0.9% NaCl) in 2 bronchopulmonary segments (BAL preallergen). The preallergen BAL fluid was pooled for analysis. Segmental challenge at a total dose of 10% of the AgPD₂₀ was performed in one bronchopulmonary segment and if well tolerated, a dose of 20% of the AgPD₂₀ was administered in a second segment. Bronchoscopy with BAL was repeated 48 hours later and fluid from the 2 segments was pooled (BAL postallergen).

Segmental allergen challenge: BAL cells analysis

BAL cells were recovered from the lavage fluid by centrifugation at 400g for 10 minutes at 4°C . BAL cells were washed twice with Hanks' balanced salt solution containing 2% newborn calf serum. For differential cell counts, blood smears and cytospin preparations of BAL cells were stained with the Giemsa-based Diff-Quik stain (Baxter Scientific Products, McGaw Park, Ill). Cells were lysed in RLT Buffer (Qiagen, Valencia, Calif) and stored at -80°C . BAL fluids were stored in 1 mL aliquots at -80°C until analyzed.

Segmental allergen challenge: BAL cell RT-qPCR

Total RNA was extracted from BAL cells using the RNeasy Mini Kit (Qiagen). The reverse transcription reaction was performed using the Superscript III system (Invitrogen/Life Technologies, Grand Island, NY). mRNA expression was determined by quantitative PCR using SYBR Green Master Mix (SABiosciences, Frederick, Md) for human FSTL1 (forward, ggttctctaaaggcagcaactacag; reverse, caggcgagaatcaccattatca) and TaqMan for human MMP9 (reference sequence NM_004994.2, ABI ID Hs00234579_m1). FSTL1-specific primers were designed using Primer Express 3.0 (Applied Biosystems, Carlsbad, Calif) and blasted against the human genome to determine specificity using <http://www.ncbi.nlm.nih.gov/tools/primer-blast>. The reference

gene, β -glucuronidase (forward, caggacctgcgcacaagag; reverse, tcgacagctggggtaag), was used to normalize the samples. Standard curves were drawn, and efficiencies were determined for each set of primers. Data are expressed as $-\Delta\text{CT}$. Fold change after compared with before SBP-Ag was calculated using the comparative cycle threshold ($\Delta\Delta\text{CT}$) method, with fold change = $(2^{-\Delta\Delta\text{CT}})$.

National Disease Research Interchange postmortem lungs: FSTL1 immunohistochemistry

Postmortem human lungs from subjects with asthma or controls without asthma were obtained from the National Disease Research Interchange (Philadelphia, Pa) in a protocol approved by the UCSD Human Research Protections program. Lung sections were immunostained with an anti-FSTL1 antibody (R&D) or species and isotype control antibody. The number of lung Fstl1+ airway cells was quantitated with image analysis (Image-Pro, Rockville, Md) in each lung and results expressed as FSTL1+ cells/mm².

Postmortem lung BAL cells

Lung BAL was prepared from postmortem lungs at the Arkansas Children's Hospital Research Institute in an institutional review board-exempted protocol by cannulating the main intrapulmonary bronchus and inflation with 500 mL PBS and 1 mM glutathione using a peristaltic pump. Following collection, the BAL was centrifuged for 10 minutes at 1000g and the BAL cell pellet resuspended in 1/4 volume freezing buffer (10% dimethyl sulfoxide, 20% FBS, 70% Dulbecco modified Eagle medium:F12 medium), with aliquots frozen at $1^{\circ}\text{C}/\text{min}$ and stored in liquid nitrogen.

FSTL1 incubation with BAL macrophages

In these *in vitro* experiments, primary BAL macrophages from postmortem donor lungs were incubated with FSTL1 (100 ng/mL) or TGF- β 1 (100 ng/mL) for 24 hours. Levels of BAL macrophage FSTL1 and MMP9 mRNA were quantitated by quantitative PCR, and of MMP9 protein in supernatants by ELISA (R&D).

Incubation of BAL macrophages with an anti-TLR4 antibody and FSTL1

Human BAL macrophages ($n = 6$ donors) were incubated *in vitro* with either an anti-hTLR4-IgG-neutralizing antibody (10 $\mu\text{g}/\text{mL}$, InvivoGen, San Diego, Calif), or a species and isotype control mouse IgG antibody, for 1 hour before stimulation with FSTL1 (100 ng/mL) for 24 hours. Levels of FSTL1 mRNA and MMP9 mRNA were quantitated by RT-qPCR.

Statistical analysis

Statistical analyses were performed using *t* tests and results reported as mean \pm SEM unless otherwise indicated. *P* values of less than .05 were considered statistically significant.

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TABLE E1. Characteristics of asthma study subjects in segmental allergen challenge study

Characteristic	Value
n	12
Sex: female	9
Age (y)	21.8 ± 2.8
Baseline FEV ₁ (% of predicted)	93.2 ± 11.4
Methacholine PC ₂₀ (mg/mL)	2.4 (1.3-2.7)

Data are means ± SD or median (25th and 75th percentiles). All subjects were atopic (positive allergy skin prick test result), nonsmokers, and not on inhaled or oral corticosteroids. Asthma was defined using clinical criteria and confirmation with either a methacholine PC₂₀ < 8 mg or reversibility to beta agonist (>10%). PC₂₀, Provocative concentration of methacholine producing a 20% fall in FEV₁.

TABLE E2. Correlation of BAL cell FSTL1 mRNA fold change (postallergen vs preallergen challenge) with BAL cells

BAL cells	FSTL1 mRNA (fold change, <i>P</i> value)
Macrophages	0.668, .049
Eosinophils	-0.660, .053
Neutrophils	0.473, .199
Lymphocytes	-0.152, .697

Correlation of BAL cell FSTL1 mRNA fold change assessed by RT-PCR (postallergen vs preallergen challenge) with % BAL cells present after segmental allergen challenge. Pearson correlations and *P* values are indicated (n = 9).