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Fstl1 Promotes Asthmatic Airway Remodeling by Inducing Oncostatin M

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Chronic asthma is associated with airway remodeling and decline in lung function. In this article, we show that follistatin-like 1 (Fstl1), a mediator not previously associated with asthma, is highly expressed by macrophages in the lungs of humans with severe asthma. Chronic allergen-challenged Lys-CreR Fstl1−/− mice in whom Fstl1 is inactivated in macrophages/myeloid cells had significantly reduced airway remodeling and reduced levels of oncostatin M (OSM), a cytokine previously not known to be regulated by Fstl1. The importance of the Fstl1 induction of OSM to airway remodeling was demonstrated in murine studies in which administration of Fstl1 induced airway remodeling and increased OSM, whereas administration of an anti-OSM Ab blocked the effect of Fstl1 on inducing airway remodeling, eosinophilic airway inflammation, and airway hyperresponsiveness, all cardinal features of asthma. Overall, these studies demonstrate that the Fstl1/OSM pathway may be a novel pathway to inhibit airway remodeling in severe human asthma. The Journal of Immunology, 2015, 195: 3546–3556.

Asthma is a disease characterized by airway inflammation, airway remodeling, and airway hyperresponsiveness (1). Features of airway remodeling in humans with asthma include increases in epithelial mucous cells, peribronchial fibrosis, and smooth muscle (SM) (1), which can be modeled in mice subjected to chronic allergen challenge (2). To identify potential novel mediators of airway remodeling in asthma, we measured levels of mediators in lungs from wild-type (WT) mice acutely challenged with allergen (which is not associated with induction of airway remodeling) and compared this with levels of mediators in lungs from WT mice chronically challenged with allergen (which is associated with induction of airway remodeling). This strategy identified that follistatin-like 1 (Fstl1), a mediator not previously associated with either asthma or airway remodeling in asthma, is highly expressed in WT mice chronically but not acutely challenged with allergen.

Fstl1 is a 308-aa extracellular glycoprotein that shares 94% identity in human and mouse (3, 4). Although Fstl1 is part of the follistatin family, it has very low protein sequence homology to follistatin (7%), as well as several key structural and functional differences. Fstl1 was initially identified as a TGF-β inducible gene and has been cloned in mouse (3) and human (4). Fstl1 is generated in particular by nonhematopoietic cells such as cells of the mesenchymal lineage (fibroblasts, chondrocytes, osteocytes, adipocytes, cardiomyocytes) by stimuli including TGF-β, IL-1β, TNF-α, IL-6, and LPS (5). Fstl1 released from these mesenchymal cells targets immune cells (monocytes, macrophages, and T cells) to express proinflammatory cytokines (IL-1β, TNF-α, IL-6, IFN-γ) and chemokines (IL-8, MCP-1, IP10) (5). Fstl1 binds to target cells through at least one defined cell-surface receptor (i.e., discoreinteracting protein 2 homolog A) (6). The mechanism by which Fstl1 influences signaling events in target cells has been the focus of several studies that have identified at least five potential Fstl1 signaling pathways including Fstl1 influencing the bone morphogenic protein (BMP) pathway to inhibit Smad signaling (7), as well as Fstl1 influencing the AKT (or protein kinase B) pathway, the AMP activated protein kinase pathway, the TLR4/ CD14 pathway, and Na/K-ATPase membrane potential (8, 9). Fstl1 has been implicated in cellular functions including survival, proliferation, differentiation, migration, and organ development (5). Fstl1 has not previously been reported in asthma or allergy but has been studied in embryogenesis (10, 11), tumor development (12), cardiac disease (13, 14), arthritis (5, 15, 16), and wound healing (17). The predominant effect of Fstl1 appears to be proinflammatory (15, 16, 18), although anti-inflammatory effects of Fstl1 have also been described (inhibits matrix metalloprotease (MMP)1, MMP2, MMP3, MMP9, and PGE2 expression) (8, 19). Fstl1 transfection into cells increases levels of IL-1β, TNF, and IL-6 (15).

In this study, we show that Fstl1, a mediator not previously associated with asthma, is highly expressed by macrophages in the lungs of humans with severe asthma as compared with normal control subjects. Although humans with severe asthma comprise only ~5% of all asthmatics, they use ~50% of the $20 billion/year in direct and indirect healthcare costs spent on all asthmatics in the United States (20), underscoring the need for novel therapies in...
these severe asthmatics. Using a mouse model of chronic asthma, we demonstrate that lung macrophages in mice, like lung macrophages in humans with severe asthma, highly express Fstl1, and that chronic allergen-challenged Lys-Cre'ESerFstl1' mice in which Fstl1 is inactivated in macrophages/myeloid cells had significantly reduced airway remodeling and significantly reduced expression of oncostatin M (OSM), a cytokine previously unrecognized to be downstream of the Fringe gene. The importance of the Fringe gene's role in airway remodeling has previously been described (22). We also found that mice challenged daily with Fstl1 protein upregulated oncostatin M (OSM), a cytokine previously unrecognized to be downstream of the Fringe gene. The importance of the Fringe gene's role in airway remodeling has previously been described (22).

Materials and Methods
Expression of Fstl1 in human asthma and control lungs
Human asthma lungs. Postmortem human lungs from asthmatics were obtained from National Disease Research Interchange in a protocol approved by the University of California, San Diego Human Research Protection program. Lung sections were immunostained with an anti-Fstl1 Ab (R&D) or species and isotype control Ab. To determine the contribution of lung macrophages to levels of Fstl1 detected, we immunostained lung sections with Abs to Fstl1 (R&D) and to CD68 (R&D), a macrophage marker. In these experiments, the different primary Abs were detected using two different HRP enzyme-labeled secondary Abs with tyramide signal amplification (Molecular Probes) according to the manufacturer's instructions as previously described (21).

Bronchial biopsy. The protocols for using bronchoscopy to obtain bronchial biopsies from patients with asthma (severe, mild) and control nonasthmatics at McGill University and Université de Montréal with the approval of the respective Institutional Review Boards have previously been described (22). The methods for processing the bronchial biopsies and immunostaining lung sections using the ABC immunoperoxidase method have also been described (22). In this study, biobanked bronchial biopsy sections from severe asthmatics (n = 10), mild asthmatics (n = 10), and nonasthmatic controls (n = 10) were immunostained with an anti-Fstl1 Ab (R&D) or species and isotype control Ab. The number of bronchial biopsy Fstl1' cells was quantitated with image analysis (Image-Pro) in each subject, and results are expressed as Fstl1' cells per square millimeter.

Animal care and use
All the mouse experimental protocols were approved by the University of California, San Diego Institutional Animal Care and Use Committee.

WT mouse acute and chronic OVA challenge and lung Fstl1 expression
We performed initial experiments to detect whether Fstl1 was expressed in WT mouse lung after acute or chronic OVA allergen challenge using two groups of mice (WT OVA; WT no OVA; eight female C57BL/6 mice/group aged 12 wk). In the acute OVA protocol, WT mice were sensitized and challenged with OVA (Worthington, Lakewood, NJ) as previously described (23). In brief, mice were sensitized i.p. with 100 µg OVA and 2 mg aluminum hydroxide (Inject Alum; Thermo Fisher Scientific, Waltham, MA) in a total volume of 200 µl PBS on days 0 and 10 followed by intranasal administration of 200 µg OVA in 20 µl PBS on days 21, 23, and 25. In the acute OVA protocol, mice were sacrificed on day 26 as previously described (2, 23). In the chronic OVA protocol, mice were initially sensitized and challenged with OVA as described for the acute OVA protocol, and from day 28 mice continued challenges with intranasal OVA twice a week for an additional 1 mo (2, 23). Non-OVA-challenged mice were sensitized and challenged with PBS only. Twenty-four hours after the last challenge, bronchoalveolar lavage (BAL) fluid and lungs were collected as previously described (2, 23) to assess levels of Fstl1 by immunohistochemistry, RTPCR, and ELISA.

WT mouse BAL fluid collection
BAL fluid was collected by lavaging the lung with 1 ml PBS via a tracheal catheter as previously described (2). BAL fluid was centrifuged, and the supernatant was frozen at −80°C for subsequent Fstl1 analysis. BAL total and differential cell counts were quantitated in Wright-Giemsa-stained slides.

WT mouse lung processing to detect Fstl1
Lungs were processed for immunohistochemistry (paraffin-embedded lung sections), as well as protein and RNA extraction, as previously described in this laboratory (24, 25). For protein and RNA extractions, lungs were initially snap-frozen in liquid nitrogen and stored at −80°C.

WT mouse lung immunohistochemistry to detect Fstl1 and M2 macrophages
For paraffin-embedded sections, lungs were equivalently inflated with an intratracheal injection of the same volume of 4% paraformaldehyde solution (Sigma Chemicals, St. Louis, MO) to preserve the pulmonary architecture. Lung sections were processed for immunohistochemistry to detect Fstl1 (anti-mouse Fstl1 Ab; Abcam). To determine the contribution of lung macrophages to levels of Fstl1 detected, we immunostained lung sections with Abs to Fstl1, as well as to F4/80 (anti-mouse F4/80 Ab; AbD Serotec). In these experiments, the two different primary Abs were detected using two different HRP enzyme-labeled secondary Abs with tyramide signal amplification (Molecular Probes) according to the manufacturer’s instructions as previously described (21). The anti-Fstl1 Ab was detected with an HRP-labeled secondary Ab (Alexa 546, red), whereas the anti-F4/80 Ab was detected with a different HRP-labeled secondary Ab (Alexa 488, green). Cells coexpressing F4/80 and Fstl1 stained a blended yellow color. To determine whether the macrophages in the lung had an M2 phenotype, we similarly used double-immunostaining combining an Ab to a mouse macrophage M2 marker [i.e., arginase 1 (26) (Abcam) with an anti-F4/80 Ab to detect macrophages. The number of cells in this study, biobanked bronchial biopsy sections from severe asthmatics (n = 10), mild asthmatics (n = 10), and nonasthmatic controls (n = 10) were immunostained with an anti-Fstl1 Ab (R&D) or species and isotype control Ab. The number of bronchial biopsy Fstl1' cells was quantitated with image analysis (Image-Pro) in each subject, and results are expressed as Fstl1' cells per square millimeter.

Detection of lung cells that express Fstl1
Because previous studies have not reported that macrophages or airway epithelium are significant sources of Fstl1 (5), we examined levels of Fstl1 by qRT-PCR in unstimulated and stimulated macrophages and epithelial cells compared with fibroblasts, a known source of Fstl1 (5). Pure populations of bone marrow–derived macrophages were cultured from mouse bone marrow as previously described (27). Pure populations of mouse primary lung fibroblasts and mouse lung epithelial cells were obtained from ScienCell. Stimuli used for all cell types included TGF-β1 (50 ng/ml; R&D Systems), a known inducer of Fstl1 in fibroblasts (5), and Fstl1 (100 ng/ml; Sino Biological), which has not previously been investigated as an autocrine stimulus for its production by macrophages. Macrophages were also stimulated with either IL-4 (100 ng/ml; R&D Systems) or IL-13 (100 ng/ml; R&D Systems). Cells (10^5/ml) were cultured for 24 h at 37°C in complete media (ScienCell) with or without the earlier stimuli, at which time RNA from the cells was extracted and processed for qRT-PCR to quantify Fstl1 mRNA expression.

WT mice challenged with Fstl1
To determine whether Fstl1 administration to the mouse airway can influence either airway inflammation, airway remodeling (mucus, fibrosis, SM changes), or AHR, we used two groups of WT mice (WT mice administered Fstl1, WT mice no Fstl1; eight female C57BL/6 mice aged 12 wk/group). The Fstl1-challenged WT mice were administered 10 µg Fstl1 (Sino Biological) in 50 µl PBS intranasally daily for 15 d and sacrificed on day 16.

WT mice challenged with Fstl1 and administered an anti-OSM Ab
In these experiments, WT mice challenged intranasally with 10 µg Fstl1 (n = 4 mice/group; female C57BL/6 mice aged 12 wk/group) received either 10 µg anti-OSM Ab or 10 µg anti-OSM Ab + 10 µg Fstl1. WT mice administered 10 µg anti-OSM neutralizing Ab and no Fstl1. Mice were sacrificed on day 16.
Detection of airway remodeling in WT mice challenged with Fstl1

Airway mucus expression: periodic acid–Schiff. To quantitate the level of mucus expression in the airway, we counted the number of periodic acid–Schiff (PAS)- and PAS+ epithelial cells in individual bronchioles as previously described in this laboratory (24, 25). At least 10 bronchioles were counted in each slide. Results are expressed as the percentage of PAS+ cells per bronchiole, which is calculated from the number of PAS+ epithelial cells per bronchiole divided by the total number of epithelial cells of each bronchiole.

Airway mucus expression: lung qRT-PCR Muc5AC. qRT-PCR was performed as described earlier for Fstl1 to detect the mouse lung mucous gene Muc5AC using Muc5AC primers (Applied Biosystems).

Peribronchial fibrosis: lung trichome staining. To detect peribronchial fibrosis, we outlined and quantified the area of peribronchial trichrome staining in paraffin-embedded lungs under a light microscope (Leica DMLS; Leica Microsystems) attached to an image analysis system (Image-Pro plus; Media Cybernetics) as previously described (24, 25). Results are expressed as the area of trichrome staining per micrometer length of basement membrane of bronchioles 150–200 μm of internal diameter.

AHR in WT mice challenged with Fstl1

AHR to methacholine was assessed in intubated and ventilated mice (WT Fstl1; WT no OVA) (n = 8 mice/group) (flexiVent ventilator; Scireq) anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg) i.p. as previously described (24, 25). The dynamic airway resistance was determined using Scireq software in mice exposed to nebulized PBS and methacholine (0 and 24 mg/ml). The following ventilator settings were used: tidal volume (10 ml/kg), frequency (150/min), and positive end-expiratory pressure (3 cm H2O).

IgE levels in WT mice challenged with Fstl1

Serum total IgE was quantitated with an IgE ELISA kit (BD Biosciences). ELISA plates were read with a BioRad Model 680 microplate reader.

In vitro effects of Fstl1 on target structural cells in lung (i.e., epithelium, fibroblasts, and SM)

Because Fstl1 administration to WT mouse lung in vivo induced mucus, peribronchial fibrosis, and AHR, we examined whether any of the effects of Fstl1 could be occurring by direct effects of Fstl1 on either fibroblasts, epithelial cells, or SM. In these in vitro experiments, either primary mouse lung fibroblasts, primary human bronchial epithelial cells (we used primary human bronchial epithelial cells because we were not able to obtain sufficient numbers of primary mouse bronchial epithelial cells for in vitro studies), or primary mouse lung SM cells (all obtained from Sciencell) were incubated with Fstl1 (100 ng/ml) for 24 h. End points measured for lung fibroblasts were collagen synthesis (collagen genes I, III, V by qRT-PCR), lung epithelial cells (mucous gene Muc5AC by qRT-PCR), and SM (contraction). For each cell type, a positive control was used (TGF-β1).

Lung SM contraction assay

Primary mouse lung SM cells (Sciencell) were cultured according to the manufacturer’s instructions for use in an in vitro SM gel contraction assay, which we have adapted from studies of human airway SM (28), as well as from our studies with esophageal SM contraction (29). SM cells were cultured in basal medium without growth factors for 24 h before seeding in collagen gels free of LPS (Advanced BioMatrix, San Diego, CA). After overnight incubation in collagen gels, SM cells were cultured in the presence or absence of Fstl1 (100 ng/ml). Control mediators used in the gel contraction assay included TGF-β1 (50 ng/ml; R&D Systems) a cytokine we have previously demonstrated to induce slow-onset SM contraction in this assay (29). With agonist-induced SM contraction, the area of the gel decreases significantly, as described in studies of airway SM (28). The area of the gels was quantitated by using a Bio-Rad ImageDR transilluminator and Versadoc scanner (Bio-Rad Laboratories, Hercules, CA) with an accompanying image-capture and analysis program to generate area in square millimeters.

Fstl1lox/lox and Lys-Cre6 mice

Because our initial studies in WT mice challenged with OVA allergen demonstrated that lung macrophages were a significant source of Fstl1, we used cre-lox techniques as previously described in this laboratory (30) to inactivate Fstl1 in macrophages and myeloid cells. Fstl1Cre/lox mice were kindly provided by X. Zhang (Shanghai Institute for Biological Sciences, Chinese Academy of Sciences), and X. Gao (Nanjing University, Nanjing, China) as described previously (7, 9). Lys-Cre6 mice (Cre expression in macrophages and myeloid cells) were acquired from Jackson Laboratories. To delete the Fstl1Cre allele in myeloid cells, we crossed Fstl1Cre mice (background strain C57/B6) with transgenic Lys-Cre6 mice to generate Lys-Cre6Fstl1Cre/lox, progeny in which the Fstl1Cre allele is selectively deleted in macrophages and myeloid cells. Mice were genotyped with cre- and Fstl1-specific primers, and the PCR product was run on a 1.5% agarose gel.

Lys-Cre6Fstl1Cre/lox OVA allergen challenge

Lys-Cre6Fstl1Cre/lox and littermate control mice (hereafter referred to as WT mice; n = 8 mice/group) were sensitized and challenged with OVA as described earlier for the chronic OVA challenge protocol. Outcomes measured included lung eosinophils, features of remodeling (mucus, fibrosis, SM), and remodeling mediators (MMP9, OSM). Eosinophils were quantitated by image analysis in lung sections immunostained with an anti–major basic protein (MBP) Ab (Dr. James Lee, Mayo Clinic, Scottsdale, AZ). MMP9 and OSM were quantitated by qRT-PCR.

Statistical analysis

All results are presented as mean ± SEM. A statistical software package (GraphPad Prism, San Diego, CA) was used for the analysis. The p values <0.05 were considered statistically significant.

Results

Fstl1 is highly expressed in humans with severe asthma

Immunofluorescence microscopy of lungs from human asthmatics demonstrated that Fstl1 was highly expressed (Fig. 1A) and that many of the Fstl1+ cells coexpressed CD68, a macrophage marker (Fig. 1B, 1C). We also used immunohistochemistry to quantitate levels of expression of Fstl1 in bronchial biopsies obtained from the lungs of severe asthmatics compared with control subjects. These studies demonstrated that the number of Fstl1+ cells in the lungs of severe asthmatics was significantly greater than the number of Fstl1+ lung cells in normal control subjects (p < 0.005; Fig. 1D). M2 macrophages highly express Fstl1

Having detected high levels of expression of Fstl1 in humans with severe chronic asthma, we used a mouse model of chronic asthma to determine the role of Fstl1 in contributing to the pathogenesis of asthma. WT mice challenged chronically with OVA allergen have a significant increase in lung Fstl1 mRNA as assessed by qPCR (p < 0.05; chronic OVA versus no OVA; Fig. 2A) and BAL Fstl1 protein as assessed by ELISA (p < 0.05; chronic OVA versus no OVA; Fig. 2B). In contrast, WT mice challenged acutely with OVA allergen do not have increases in lung Fstl1 mRNA (Fig. 2A) or BAL Fstl1 protein (Fig. 2B). We have previously demonstrated that chronic OVA, but not acute OVA challenge, induces airway remodeling (2).

Lung sections from WT mice not challenged with OVA had low numbers of F4/80+ cells (Fig. 2C) and which did not express Fstl1 (24, 25). Lung sections from WT mice challenged chronically with OVA allergen demonstrated a significant increase in the number of Fstl1+ cells that coexpressed the macrophage marker F4/80 (24, 25). The Fstl1+ cells also coexpressed the M2 macrophage marker arginase (24–27). The percentage of lung Fstl1+ cells that coexpressed arginase was significantly higher in WT mice chronically challenged with OVA compared with non-OVA–challenged mice (p < 0.005; Fig. 2L).

Prior studies have not reported macrophages as a significant source of Fstl1 (5). We therefore performed in vitro studies with macrophages to compare their ability to express Fstl1 with that of fibroblasts, a known source of Fstl1 (5). Macrophages stimulated in vitro with TGF-β1 (a known stimulus for Fstl1 induction in
Lys-

to determine the role of macrophage-derived Fstl1 in mediating

Because we had made the novel observation that lung macrophages remodel (Fig. 2M–O). Fstl1 expression by macrophages, fibroblasts, or epithelium consequently through an autocrine or paracrine pathway induce further Fstl1 stimulation (Fig. 2M–O). Thus, macrophages, fibroblasts, fibroblasts and epithelium) express Fstl1 mRNA in response to Cretg 0.05) or TGF-

expressed Fstl1 mRNA when incubated with either Fstl1 (5), we also examined whether lung epithelial cells expressed Fstl1. Like macrophages and fibroblasts, epithelial cells also expressed Fstl1 mRNA when incubated with either Fstl1 (p < 0.05) or TGF-β1 (p < 0.05) (Fig. 2O).

We also made the novel observation that macrophages (as well as fibroblasts and epithelium) express Fstl1 mRNA in response to Fstl1 stimulation (Fig. 2M–O). Thus, macrophages, fibroblasts, and epithelium are both cellular sources of Fstl1 that can subsequently through an autocrine or paracrine pathway induce further Fstl1 expression by macrophages, fibroblasts, or epithelium (Fig. 2M–O).

Inhibition of macrophage Fstl1 expression inhibits airway remodeling

Because we had made the novel observation that lung macrophages express high levels of Fstl1 in vitro (Fig. 2M) and in vivo (Fig. 2L), to determine the role of macrophage-derived Fstl1 in mediating the features of airway remodeling noted in chronic allergen-challenged WT mice, we chronically allergen-challenged Lys-Cretg/+/Fstl12/2a mice in which Fstl1 is inactivated in macrophages and myeloid cells. These studies demonstrated that chronically allergen-challenged Lys-Cretg/+/Fstl12/2a mice had reduced features of airway remodeling including reduced mucus (p < 0.05; Fig. 3A), reduced peribronchial fibrosis (p < 0.005; Fig. 3B), as well as reduced eosinophilic lung inflammation (p < 0.005; Fig. 3C), reduced expression of remodeling pathways including reduced lung OSM (p < 0.05; Fig. 3D), and reduced BAL MPP9 (p < 0.02; Fig. 3E). There was no change in AHR (data not shown). In separate in vitro experiments, we demonstrated that Fstl1 directly induces WT macrophages from bone marrow to express OSM (Fig. 3F).

Chronic Fstl1 induces airway remodeling

Chronic administration of Fstl1 to WT mice induced a significant increase in mucus as assessed by PAS staining (p < 0.005; chronic Fstl1 versus no Fstl1; Fig. 4A–C), Muc5AC immunostaining (p < 0.005; Fig. 4D, 4E), and by qPCR for the mucous gene Muc5AC (p < 0.05; Fig. 4F), as well as increased peribronchial fibrosis as assessed by image analysis of the peribronchial area of trichrome staining (p < 0.005; Fig. 4G) and AHR (p < 0.05; chronic Fstl1 versus no Fstl1; Fig. 4H).

Chronic Fstl1-challenged WT mice had increased numbers of lung MBP+ eosinophils (p < 0.0001; Fig. 4I) and BAL eosinophils (p < 0.005; Fig. 4J), without changes in the numbers of BAL macrophages, lymphocytes, or neutrophils (p = ns; Fig. 4I). The increase in lung and BAL eosinophils was associated with increased levels of the eosinophil active cytokines IL-5 mRNA (p < 0.05; Fig. 4K) and the eosinophil chemoattractant eotaxin-1 mRNA (also known as CCL11; p < 0.05; Fig. 4L) as assessed by qPCR. In addition, chronic Fstl1-challenged WT mice had increased levels of TNF-α mRNA (p < 0.05; Fig. 4M), lung OSM mRNA (p < 0.05; Fig. 4N), and IgE (p < 0.05; Fig. 4O) with no change in levels of TGF-β1 (data not shown).

Blocking OSM inhibits Fstl1-induced airway remodeling, eosinophilic inflammation, and AHR

Chronic administration of Fstl1 to WT mice pretreated with an anti-OSM Ab resulted in a significant reduction in airway remodeling (mucus and fibrosis) as assessed by PAS staining (p < 0.005; chronic Fstl1 + anti-OSM Ab versus chronic Fstl1; Fig. 5A–D), as well as significantly reduced peribronchial fibrosis as assessed by image analysis of the peribronchial area of trichrome staining (p < 0.005; Fig. 5E–H), lung eosinophilic inflammation (p < 0.05; Fig. 5I–L), BAL eosinophils (p < 0.05; Fig. 5M), and AHR (p < 0.05; Fig. 5N). Immunohistochemistry demonstrated that OSM was highly expressed by lung macrophages in chronic OVA-challenged, but not in non-OVA-challenged WT mice (Fig 5O, 5P).

Fstl1 can also directly induce remodeling in vitro

In vitro Fstl1 directly induced lung fibroblasts to express collagen genes known to be expressed in asthma (1), including collagen I (p < 0.05), collagen III (p < 0.05), and collagen V (p < 0.05) (Fig. 6A), suggesting potential direct effects of Fstl1 in vivo on inducing peribronchial fibrosis. Fstl1 also induced lung epithelial cells to express the mucous gene Muc5AC mRNA as assessed by qPCR (p < 0.05; Fig. 6B), as well as RANTES (p < 0.05; Fig. 6C), but not eotaxin-1 (data not shown). Fstl1 increased lung SM contractility (Fig. 6D, 6E), with slow-onset kinetics, similar to what we have previously demonstrated for TGF-β1–induced esophageal SM contraction (29) and TGF-β1–induced lung smooth contraction in this study (Fig. 6D, 6E).

Discussion

In this study, we have identified that Fstl1 is highly expressed by macrophages in the lungs of humans with severe asthma and, based on our studies using a mouse model of chronic asthma, that Fstl1 is a novel mediator of airway remodeling in asthma via induction of OSM, a previously unknown downstream pathway of Fstl1.

FIGURE 1. Fstl1 is highly expressed in lungs of humans with severe asthma. Lungs from human asthmatics were processed for immunofluorescence staining and microscopy (original magnification ×200) to detect either Fstl1 (A), CD68 (B), or Fstl1 and CD68 (C). Bronchial biopsies from human subjects with severe asthma, mild asthma, or no asthma were processed for immunohistochemistry with an anti-Fstl1 Ab (n = 10 subjects/group) (D). The number of peribronchial Fstl1+ cells were quantitated by light microscopy and image analysis in each group.

For 0.0001; Fig. 4I) and BAL eosinophils (p < 0.05; Fig. 4O) with no change in levels of TGF-β1 (data not shown).
There are several additional novel observations in this study, in particular the demonstration using Lys-Cre\textsuperscript{\#}Fstl\textsuperscript{\#\#} mice that macrophages/myeloid cells are a significant source of Fstl1 in asthma (previous studies have not noted that macrophages/myeloid cells are a significant source of Fstl1 in other diseases) (5), that administration of Fstl1 to WT mice induces airway remodeling in a mouse model of asthma, and that humans with severe asthma have increased expression of Fstl1 in bronchial biopsies compared with mild asthmatics and nonasthmatic controls, underscoring the relevance of the findings in a mouse model of asthma to human disease. To our knowledge, our study is the first to report that OSM, a member of the IL-6 family of cytokines (31), is induced by Fstl1. We demonstrated this in vitro (i.e., that Fstl1 can directly induce macrophages to express OSM), as well as in vivo in studies using an anti-OSM Ab that inhibited the ability of Fstl1 to induce airway remodeling (peribronchial fibrosis, mucus) in WT mice. Our studies of Lys-Cre\textsuperscript{\#}Fstl\textsuperscript{\#\#} mice demonstrated that macrophage
and myeloid cells expressing Fstl1 are key in vivo regulators of OSM expression, as chronic allergen-challenged Lys-Cre\(^{tg}\)/Fstl1\(^{D/D}\) mice did not generate OSM. The profibrotic effect of OSM has been appreciated in studies showing that OSM stimulates human lung fibroblast proliferation and collagen production (31, 32). In addition, adenoviral-mediated overexpression of OSM in the lungs of WT mice (33) results in the features we have noted to be induced by Fstl1 in this study including increased fibrosis, goblet cell hyperplasia, eosinophilic inflammation, and AHR, supporting our observations that OSM mediates the effects of Fstl1 on airway remodeling, eosinophilic inflammation, and AHR noted in this study. OSM upregulates VCAM and induces eotaxin expression, which can contribute to eosinophilic inflammation (34). Thus, overall, our studies of Fstl1 and OSM suggest a model in which chronic allergen challenge induces lung macrophages to express Fstl1, which then through a subsequent autocrine or paracrine pathway induces lung macrophages to express OSM, which stimulates fibroblast proliferation and collagen production (32), as well as goblet cell hyperplasia, eosinophilic inflammation, and AHR as previously described (33, 34). Support for a role of OSM in human asthma and airway remodeling is derived from studies demonstrating increased levels of OSM in the sputum of asthmatics with incompletely reversible airway obstruction (35). The OSM receptor has also been detected in the airways of fatal asthmatics (31). Although our in vivo studies with an anti-OSM Ab demonstrated that it blocked the vast majority of the effect of Fstl1 on airway remodeling (peribronchial fibrosis, mucus), eosinophilic inflammation, and AHR, we also made the novel observation that Fstl1 in vitro can directly influence the lung fibroblast expression of collagen genes associated with remodeling in asthma (collagen I, III, V) (1), mucous gene expression by airway epithelium, and lung SM contraction, suggesting a potential direct effect of Fstl1 on airway remodeling.

**FIGURE 3.** Inhibition of macrophage Fstl1 expression inhibits airway remodeling. Lys-Cre\(^{tg}\)/Fstl1\(^{D/D}\) or WT mice (eight mice/group) were sensitized with OVA allergen followed by chronic exposure to OVA allergen. Levels of lung mucus were quantitated by PAS staining (A). Levels of peribronchial trichrome staining were quantitated by image analysis (B). The number of peribronchial eosinophils was quantitated by MBP immunostaining and image analysis (C). Levels of OSM were quantitated by qPCR (D). Levels of BAL MMP9 were quantitated by ELISA (E). In separate experiments, WT mouse bone marrow–derived macrophages were incubated for 24 h with either Fstl1 (100 ng/ml) or media, and levels of OSM mRNA were quantitated by qPCR (F).
FIGURE 4. Chronic Fstl1 induces airway remodeling. WT mice (eight mice/group) were administered Fstl1 intranasally daily for 15 d before sacrifice (WT + Fstl1 group). A control WT group did not receive Fstl1 (WT). Lungs from the different groups of WT mice were processed for PAS staining (A–C), Muc5ac immunostaining (D and E), assessment of expression of the mucous gene Muc5AC by qPCR (F), quantitation of peribronchial fibrosis by trichrome staining and image analysis (G), measurement of AHR (H), quantitation of lung MBP+ eosinophils (I), BAL inflammatory cells (J), and assessment of cytokine gene expression by qPCR including IL-5 (K), eotaxin-1 (L), TNF-α (M), and OSM (N). Levels of serum IgE were quantitated by ELISA (O). Original magnification ×400.
Our study also identified that the M2 macrophage was a significant source of Fstl1 in mouse models of chronic asthma. In contrast, most prior studies of Fstl1 have not considered the macrophage or hematopoietic lineage cells to be a source of Fstl1 (5). Prior studies have demonstrated that mesenchymal cells (fibroblasts, synoviocytes, chondrocytes, osteocytes, adipocytes, cardiomyocytes, endotheliocytes) are a significant source of Fstl1 (5). The reasons for our study, but not prior studies, demonstrating a significant contribution of macrophages to Fstl1 generation may relate to the diseases studied (asthma compared with past studies of arthritis, autoimmune disease, coronary disease), the stimulus studied (chronic allergen), the organ studied (lung versus joint or heart), or other factors. We demonstrated that in vitro macrophages expressed Fstl1 mRNA when stimulated with TGF-β1 (a known inducer of Fstl1) and that the level of Fstl1 induced by TGF-β1 in macrophages was not significantly different from levels of Fstl1 induced in fibroblasts (a major known source of Fstl1) by TGF-β1 (5). We also demonstrated that in macrophages Fstl1 mRNA could be induced via an autocrine or paracrine pathway (Fstl1 stimulates macrophages to express Fstl1 mRNA).

FIGURE 5. Blocking OSM inhibits Fstl1-induced airway remodeling. WT mice (four mice/group) were administered Fstl1 intranasally daily for 15 d, with or without pretreatment with an anti-OSM Ab (anti-OSM). A control WT group received the anti-OSM Ab and no Fstl1. Levels of lung mucus were quantitated by PAS staining (A–D). Levels of peribronchial trichrome staining were quantitated by image analysis (E–H). The number of MBP⁺ peribronchial eosinophils were quantitated by image analysis (I–L). The number of Wright-Giemsa–stained BAL eosinophils was quantitated by light microscopy (M). Levels of AHR to methacholine were assessed by flexiVent (N). In a separate experiment, lungs from either WT mice subjected to chronic OVA challenge (WT + OVA) or WT mice not challenged with OVA (WT + No OVA) were immunostained with an anti-OSM Ab to detect OSM⁺ cells in the lung (O and P). Original magnification ×200 (A–H); ×400 (I–L, O, and P).
The use of homozygous Fstl1-deficient mice to study the role of Fstl1 in models of asthma or other diseases has not been possible because homozygous Fstl1-deficient mice die at birth because of respiratory failure (7). To our knowledge, our study is the first to use conditional inactivation of Fstl1 in macrophage/myeloid cells to study its influence on a disease phenotype in vivo. Recent studies have used heterozygous Fstl1<sup>+/−</sup>-deficient mice to demonstrate that inhibiting Fstl1 does not inhibit lung inflammation, but does attenuate bleomycin-induced pulmonary fibrosis in mice through a TGF-β-dependent pathway (36). In the study of bleomycin-induced pulmonary fibrosis, the cellular source of Fstl1 was fibroblasts (36), a well-known mesenchymal source of Fstl1 (5). Our study differs from the study of bleomycin-induced pulmonary fibrosis in that we demonstrate that in chronic allergen-induced asthma, nonmesenchymal cells such as macrophages (not considered a significant source of Fstl1) are a significant source of Fstl1, that inhibiting Fstl1 inhibits allergen-induced airway eosinophilic inflammation (no effect on inhibiting bleomycin-induced lung inflammation), and that the downstream pathway of Fstl1 in macrophages is OSM (in the bleomycin model it is TGF-β). Furthermore, we use conditional Lys-Cre<sup>+/−</sup>/Fstl1<sup>Δ/Δ</sup> mice, an approach not used in prior Fstl1 research, to demonstrate the importance of macrophage and myeloid cell–derived Fstl1 to asthma outcomes. Thus, pathways used by Fstl1 may differ in different diseases depending on which cell expresses and responds to Fstl1. In asthma, the autocrine/paracrine macrophage Fstl1 oncostatin pathway is important, whereas in bleomycin-induced pulmonary fibrosis, fibroblast-derived Fstl1 targets a different TGF-β-dependent pathway in epithelial cells. In addition, our studies of chronic allergen-challenged Lys-Cre<sup>+/−</sup>/Fstl1<sup>Δ/Δ</sup> mice demonstrate that these mice have reduced eosinophilic inflammation, whereas studies using bleomycin show no effect of Fstl1 on lung inflammation. Thus, this study has evidence for differentially activated Fstl1 downstream pathways (OSM versus TGF-β), with resultant differential Fstl1 effects on lung inflammation (Fstl1 mediates eosinophilic inflammation versus no effect of Fstl1 on lung inflammation in bleomycin-induced fibrosis), depending on the disease stimulus (chronic allergen-induced asthma versus bleomycin-induced lung fibrosis) and predominant cell expressing (macrophage versus fibroblast) or responding (macrophage versus epithelium) to a ligand such as Fstl1.

Although lung macrophages highly express Fstl1 in the mouse model of asthma, as well as in the lungs of human asthmatics, other cell types such as airway epithelium, but not fibroblasts, were also noted to have lower levels of immunostaining for Fstl1 in both OVA-challenged WT mice and Lys-Cre<sup>+/−</sup>/Fstl1<sup>Δ/Δ</sup> mice, as well as human lungs (data not shown). Although allergen-challenged Lys-Cre<sup>+/−</sup>/Fstl1<sup>Δ/Δ</sup> mice had significantly reduced eosinophilic airway inflammation, mucus, and fibrosis, they did not have reduced AHR. As WT mice challenged with Fstl1 develop increased AHR, this suggests that alternative cellular sources of Fstl1 in Lys-Cre<sup>+/−</sup>/Fstl1<sup>Δ/Δ</sup> mice may be contributing alone or in combination with macrophages to Fstl1-induced AHR. Future studies could examine whether inactivating Fstl1 in epithelium or other cells known to express Fstl1 such as fibroblasts alone or in combination with inactivation of Fstl1 in macrophages had effects on AHR. The innate immune response is also implicated in the production of eosinophil active cytokines IL-5 and eotaxin-1 in WT mice administered Fstl1. Currently, we do not know the cellular source(s) of IL-5 and eotaxin-1, which will require further study.

Several cytokines/mediators have been implicated in airway remodeling in asthma including TGF-β, LIGHT, IL-5, IL-13, MMP9, and LTC4 (1, 37). More recently, we have demonstrated that ORMDL3, an endoplasmic reticulum protein when expressed...
as a human transgene in mice, can induce airway remodeling in the absence of inflammation (24, 25). Thus, although it is increasingly appreciated that there are several asthma clinical endotypes (e.g., Th2, Th17) (38, 39), it is also likely that there are several different asthma endotypes that contribute to airway remodeling through either direct effects on target structural cells (epithelium, SM, fibroblast) or through indirect effects on inflammatory cells that subsequently influence target cells. In this regard, Fstl1 is an example of a cytokine that we have demonstrated induces airway remodeling, eosinophilic inflammation, and AHR through OSM, and may also have direct effects on target structural cells (epithelium, fibroblasts, SM).

In summary, we demonstrated that Fstl1, a mediator not previously associated with asthma, is highly expressed by macrophages in the lungs of humans with severe asthma and by M2 macrophages in the lungs of mice with chronic allergen-induced remodeling. Chronic allergen-challenged Lys-CreR+/Fstl1−/− mice in whom Fstl1 is inactivated in macrophages/myeloid cells had significantly reduced airway remodeling and expression of OSM. The importance of the Fstl1 induction of OSM to airway remodeling was demonstrated in studies in which administration of Fstl1 to the mouse airway induced airway remodeling and increased levels of lung OSM, whereas administration of an anti-OSM Ab blocked the effect of Fstl1 on inducing airway remodeling, eosinophilic inflammation, and AHR. The importance to human asthma is evident from the demonstration that Fstl1 is highly expressed in the lungs of severe human asthmatics compared with controls. Recent studies have also demonstrated that OSM levels are increased in BAL fluid of allergic asthmatic patients after segmental allergen challenge (40).

Overall, these studies demonstrate that the Fstl1/OSM pathway may be a novel pathway to inhibit airway remodeling in severe asthma, the subset of asthmatics most in need of novel therapies (41).

Disclosures

The authors have no financial conflicts of interest.

References


