

IL-13 and T_H2 cytokine exposure triggers matrix metalloproteinase 7-mediated Fas ligand cleavage from bronchial epithelial cells

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Background: Bronchial epithelial damage and activation likely contribute to the inflammatory and airway-remodeling events characteristic of severe asthma. Interaction of Fas receptor (CD95) with its ligand (FasL; CD95L) is an important mechanism of cell-mediated apoptosis. Bronchial epithelial FasL expression provides immune barrier protection from immune cell-mediated damage.

Objectives: Membrane FasL (mFasL) is a cleavage target of matrix metalloproteinases (MMPs). We investigated whether the asthmatic T_H2 environment might influence disease processes by increasing airway epithelial MMP-mediated cleavage of mFasL into proinflammatory soluble FasL.

Methods: We used human airway epithelial cell lines and primary cells to model the human airway epithelium *in vitro*. Airway tissue from healthy subjects and patients with severe asthma was used to investigate MMP expression patterns in diseased airways.

Results: We demonstrate that active MMP-7 is present in the ciliated epithelial cells of normal human airways. In patients with severe asthma, MMP-7 levels are increased in basal epithelial cells. Airway epithelial cell lines (1HAEo[−] and 16HBE14o[−]) *in vitro* express constitutively high levels of MMP-2 and MMP-9 but relatively low levels of MMP-7. T_H2 cytokine (IL-4, IL-9, and IL-13) treatment of 1HAEo[−] cells increased MMP-7 mRNA and activity, triggered colocalization of intracellular MMP-7 with FasL, and caused mFasL cleavage with soluble FasL release. Small interfering RNA knockdown shows that cytokine-induced mFasL cleavage is dependent on MMP-7 activity.

Conclusions: MMPs serve multiple beneficial roles in the lung. However, chronic disordered epithelial expression of MMP-7 in

patients with asthma might increase mFasL cleavage and contribute to airway epithelial damage and inflammation. (J Allergy Clin Immunol 2010;126:366-74.)

Key words: Asthma, airway epithelial cells, immune barrier, T_H2 cytokines, Fas ligand, matrix metalloproteinase 3, matrix metalloproteinase 7, inflammation

Asthma is a disease of increasing prevalence, but the cause remains elusive. The immune response in patients with asthma is skewed toward a T_H2-type response,¹ and T_H2 cytokines, including IL-4, IL-9, and IL-13, are thought to play crucial roles in the development of chronic airway inflammation and structural remodeling events.^{2,3} The airway epithelium is the interface between inspired air and the internal milieu, providing a physical barrier against inhaled allergens, pathogens, and other noxious airborne substances. In pathological studies epithelial damage and shedding are cardinal features of chronic airway remodeling in patients with asthma and correlate with disease severity and airway hyperreactivity.⁴⁻⁸ It is currently unknown how epithelial damage contributes to airway remodeling, although several studies suggest a link between activation of an epithelial “repair” phenotype in asthma and the development of other structural changes in the airway.⁹⁻¹¹

Studies have suggested that the interaction between Fas (CD95) and Fas ligand (FasL; CD95L) regulates remodeling in non-lymphoid tissues and airway epithelial cells (AECs) in patients with cystic fibrosis.¹² Fas is an ubiquitously expressed apoptosis-inducing receptor, and FasL is expressed on immune cells, including eosinophils, polymorphic neutrophils, and dendritic cells. Fas ligation by FasL leads to apoptosis of the Fas-presenting cell through trimerization of Fas and subsequent caspase activation. FasL is also expressed in several nonimmune tissues, such as the anterior chamber of the eye, testis, and brain, and confers “immune privilege” by protecting structural cells against immune cell-mediated cell death.¹³ The human airway epithelium has also been shown to express FasL and thus might function as an “immune barrier” in the airway.¹⁴ Airway epithelial FasL levels are increased in patients with severe asthma after steroid treatment, although it is possible that this increase in FasL levels reflects a more severe stage of disease.¹⁵ Cell-surface membrane FasL (mFasL) can be cleaved into soluble FasL (sFasL), which cannot trigger Fas trimerization and apoptosis and instead functions as a proinflammatory chemokine for polymorphonuclear neutrophils.¹⁶⁻¹⁸

The balance between the 2 biological forms of FasL is thought to be regulated by the matrix metalloproteinase (MMP) family of Zn²⁺- and Ca²⁺-dependent endopeptidases. Each MMP family member is capable of cleaving a variety of extracellular matrix (ECM) and non-ECM substrates (reviewed in Chakraborti et al¹⁹), and the cleavage of mFasL into sFasL is MMP

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Abbreviations used

ADAM:	A disintegrin and metalloproteinase
AEC:	Airway epithelial cell
DAPI:	4',6-Diamidino-2-phenylindole
ECM:	Extracellular matrix
FasL:	Fas ligand
FITC:	Fluorescein isothiocyanate
FKHR:	Forkhead
HB-EGF:	Heparin-binding EGF
HBEC:	Human bronchial epithelial cell
HPRT:	Hypoxanthine phosphoribosyltransferase
ISZ:	<i>In situ</i> zymography
mFasL:	Membrane Fas ligand
MMP:	Matrix metalloproteinase
rhMMP-7:	Recombinant human active MMP-7
r _p :	Pearson correlation of colocalization
sFasL:	Soluble Fas ligand
siRNA:	Small interfering RNA
TBS:	Tris-buffered saline
TIMP:	Tissue inhibitor of metalloproteinases
TRITC:	Tetramethylrhodamine isothiocyanate

dependent.²⁰ Shedding of sFasL from human FasL-expressing cells can be regulated by MMP-3 (stromelysin 1) expression, and MMP-3 activity correlates with sFasL levels in the synovial fluid of patients with rheumatoid arthritis.²¹ MMP-7 (matrilysin) can also cleave recombinant mFasL from the cell surface of transfected cell lines^{22,23} and is associated with inflammation in interstitial lung diseases, including idiopathic pulmonary fibrosis.²⁴ MMPs probably play essential roles in human airway defense and repair; for example, in wounded airway epithelium MMP-7 is found on the basal aspect of flattened migrating cells,²⁵ and murine knockout experiments show that MMP-7 is vital for tracheal epithelial repair.²⁶

Acute inflammation is a necessary process during wound repair, yet prolonged inflammation can lead to further tissue damage. Release of sFasL from the epithelium by means of MMP cleavage might trigger inflammation and contribute to immune cell-mediated epithelial injury through loss of mFasL-mediated immune privilege. As potential regulators of sFasL release, MMP-3 and MMP-7 might therefore contribute to the pathogenic changes observed in patients with asthma.

This study investigates the hypothesis that the T_H2 cytokine environment in the airways of asthmatic patients causes aberrant expression of epithelial MMPs. Furthermore, dysregulated MMP activity is responsible for cleaving mFasL from the epithelial surface, downregulating the epithelial immune barrier, and releasing proinflammatory sFasL.

METHODS

Primary human tissue and cell culture

Lungs from asthmatic patients and lungs from age-matched control subjects were obtained from collaboration between the International Institute for the Advancement of Medicine (Jessup, Pa) network and the iCAPTURE Centre (ethics approval H00-50100). Lung tissue was dissected for fixation or cell isolation (by T.-L. H.). Primary human bronchial epithelial cells (HBECs) isolated from tissue explants or purchased from a commercial source (Lonza, Walkersville, Md) were grown in bronchial epithelial growth media (Lonza). The 1HAEO[−] and 16HBE14o[−] SV40-transformed human AEC lines^{27,28} were cultured as described previously.²⁹ For experiments, cells were incubated with IL-4 (2 ng/mL), IL-9 (2 ng/mL), and IL-13 (10 ng/mL), alone and in

combination, or with recombinant human active MMP-7 (rhMMP-7; Calbiochem, San Diego, Calif).

MMP-7 immunohistochemistry and fluorescent *in situ* zymography

MMP-7 immunohistochemistry was performed according to a standard protocol by using a murine monoclonal anti-MMP-7 antibody (ID-2, 1:50, Calbiochem). Numbers of MMP-7⁺ apical and basal cells were calculated by observer-blinded manual point counting and expressed per millimeter of basement membrane length (Image-Pro Plus; Media Cybernetics, Bethesda, Md). Fluorescent *in situ* zymography (ISZ) was performed on OCT frozen airway sections by using 1 μmol/L PB-M7VIS (courtesy of J. O. M.), a substrate with a relatively high rate of cleavage by MMP-7.³⁰ Fluorescein isothiocyanate (FITC; sensor), tetramethylrhodamine isothiocyanate (TRITC; reference), and 4',6-diamidino-2-phenylindole (DAPI) images were captured by using wide-field fluorescence microscopy (Eclipse TE300; Nikon, Tokyo, Japan), and quantified with ImageJ software (National Institutes of Health), and the epithelial FITC/TRITC ratio was calculated. Reaction kinetics were simultaneously measured in 96-well format; PB-M7VIS substrate was incubated with rhMMP-7 (1,000 ng/mL, Calbiochem), and FITC and TRITC signals were measured with a plate reader (Genios; Tecan, Mannedorf, Switzerland).

Quantitative real-time PCR

Quantitative real-time PCR for MMPs/tissue inhibitors of metalloproteinases (TIMPs) and FasL was performed by using standard techniques with custom-made primers (Oligo [Molecular Biology Insights, Cascade, Colo] and Primer Express [Applied Biosystems, Foster City, Calif]). Primer sequences (see Table E1 in this article's Online Repository at www.jacionline.org), and specific reaction conditions are listed in the Methods section of this article's Online Repository at www.jacionline.org. Results are expressed as fold expression relative to untreated cells, normalized to β-actin (for MMPs/TIMPs) or hypoxanthine phosphoribosyltransferase (HPRT; for FasL) housekeeping controls.

Western blot analysis

Cell protein extracts and media supernatants were used for Western blot analysis, according to standard techniques,²⁹ with anti-MMP-7 (ID2, 1:200, Calbiochem) or anti-MMP-3 (Ab-2, 1:500; Mediacorp, Montreal, Quebec, Canada) antibodies.

Gel zymogram MMP assay

Media supernatant from cultured cells was centrifuged to remove dead cells, and MMP activity was measured by using casein-gel zymography. If required, samples were concentrated by means of centrifugation at 10,000 rpm at 4°C with 4K Nanosep concentration tubes (Pall, Port Washington, NY). Supernatant MMP-3 and MMP-7 activity was analyzed by using ready-made casein gels (Invitrogen, Carlsbad, Calif), according to standard protocols (see the Methods section in this article's Online Repository), and quantified by means of densitometric imaging of proteolytic bands (ImageJ software).

sFasL ELISA

sFasL protein concentrations were measured in supernatants by using Maxisorp ELISA plates (Fisher, Ontario, Canada) and a sFasL sandwich ELISA kit (Bender Medsystems, Burlingame, Conn), according to the manufacturer's instructions.

mFasL FACS assay

After experimental treatments, 1HAEO[−] cells were washed (PBS) and enzymatically dissociated (0.25% trypsin EDTA, Invitrogen). Cells were labeled with 1 μg of FITC-labeled rat anti-mFasL antibody (H11, Bender Medsystems) or unconjugated murine anti-mFasL antibody (BMS199/2, 1:100, Bender Medsystems) with an Alexa-488 anti-mouse secondary

antibody (Molecular Probes, Eugene, Ore). Isotype controls were FITC-conjugated rat IgG2a κ (Biosource International, Camarillo, Calif) and unconjugated murine IgG (MCA928, 1:50; Serotec, Raleigh, NC). Cells were analyzed with an EPICS XL FACS machine (Beckman Coulter, Fullerton, Calif) with appropriate software (Summit V4.3, Dako, Glostrup, Denmark).

Fluorescent confocal microscopy

1HAEo[−] cells grown in 8-chamber glass slides were treated with IL-4, IL-9, and IL-13 in combination. Cells were dual labeled with polyclonal rabbit anti-FasL (SC-834, 1:50; Santa Cruz Biotechnology, Santa Cruz, Calif) and monoclonal murine anti-MMP-7 (ID-2, 1:200, Calbiochem) primary antibodies (or isotype controls) and imaged with laser-scanning confocal microscopy.²⁹ Detailed methods are presented in the Methods section of this article's Online Repository. Three-dimensional stacks of 3 to 7 random fields were captured for each experimental condition, with 4 to 10 cells per field. Three-dimensional MMP-7/FasL colocalization was quantified in individual cells by using Volocity software (Improvision, Coventry, United Kingdom) to calculate the Pearson correlation of colocalization (r_p ; r_p of 0–0.5, random colocalization; r_p of 0.5–1.0, specific interaction [within approximately 100 nm³ voxels]).³¹

Small interfering RNA-targeted knockdown of MMP-7

1HAEo[−] cells were transfected with a pool of 4 individual *MMP7*-specific small interfering RNA (siRNA) sequences (ON-TARGETplus SMARTpool; Dharmacon, Lafayette, Colo) by using standard lipid transfection protocols (see the Methods section in this article's Online Repository).

Statistical analysis

Statistical analyses were performed with PRISM software (GraphPad Software, Inc, San Diego, Calif). Graphic data are expressed as means $\pm$ SDs. Means were compared with Student *t* tests or, for multiple comparisons, 1-way ANOVA with appropriate *post hoc* tests. Significance is noted at a *P* value of less than .05.

RESULTS

MMP-7 protein expression is abnormal in asthmatic airway epithelium

Representative images demonstrate that healthy subjects have a well-differentiated airway epithelium (Fig 1, A and C), with basal cells, parabasal cells, and apical cells, including ciliated cells and mucus-producing goblet cells. In contrast, the epithelium from asthmatic airways was often poorly differentiated and devoid of ciliated apical cells (Fig 1, B and D). MMP-7 is strongly expressed in ciliated cells of normal epithelium, particularly adjacent to the luminal surface (Fig 1, A). In asthmatic patients MMP-7 expression was found throughout the epithelium, including basal cells (Fig 1, B). The proportion of MMP-7⁺ apical epithelial cells was significantly lower in the airways of patients with severe asthma (30.3 ± 11.5 cells/mm) compared with those seen in healthy subjects (41.5 ± 11.5 cells/mm; Fig 1, E). In contrast, the number of MMP-7⁺ basal epithelial cells was significantly higher in asthmatic patients (22.4 ± 13.2 cells/mm) compared with that seen in healthy subjects (11.7 ± 9.3 cells/mm). Total apical and basal cell counts were similar in both groups.

ISZ indicates MMP-7 activity in normal human airway epithelium

Airway tissue incubated with MMP-7-selective PB-M7VIS substrate exhibits a strong FITC cleavage signal in the apical region of ciliated epithelial cells (Fig 1, F), which is absent from

mucus-producing goblet cells, reflecting the immunohistochemical MMP-7 staining pattern in normal airways. Data in the Online Repository show DAPI- and hematoxylin and eosin-stained sections from frozen airway tissue from a nonasthmatic subject (see Fig E1, A and B, in this article's Online Repository at www.jacionline.org). FITC and TRITC images obtained by means of fluorescent ISZ with PB-M7VIS are shown for serial sections (see Fig E1, C and D). Quantification of epithelial fluorescence shows a rapid increase in FITC/TRITC ratio, peaking at 2 hours of incubation, demonstrating MMP activity in airway epithelium (see Fig E1, E). Control sections incubated without PB-M7VIS substrate or without divalent enzymatic cofactors show no significant change in FITC/TRITC ratio over time (data not shown). Incubation of PB-M7VIS substrate with recombinant active MMP-7 (1,000 ng/mL) alone shows similar reaction kinetics to ISZ tissue slices (see Fig E1, F).

MMP expression in AECs

The constitutive expression of MMP-2, MMP-3, MMP-7, MMP-9, TIMP-1, TIMP-2, a disintegrin and metalloproteinase (ADAM) 10, and ADAM-17 mRNA in the 1HAEo[−] bronchial epithelial cell line was measured (see Table E2 in this article's Online Repository at www.jacionline.org). MMP-3 and MMP-7 are constitutively expressed at lower levels than gelatinases MMP-2 and MMP-9 and the biological MMP inhibitors TIMP-1 and TIMP-2.

MMP-3 and MMP-7 mRNA expression and activity are increased by T_H2 cytokine treatment

1HAEo[−] cells treated with IL-4, IL-9, and IL-13 individually and in combination show increased expression of MMP-3 and MMP-7 mRNA (Fig 2, A and B). MMP-3 mRNA is significantly upregulated at 4 hours by IL-13 treatment (Fig 2, A). MMP-7 mRNA was upregulated at 4 hours by IL-13 alone or in combination with IL-4 or IL-9 (Fig 2, B). Neither IL-4 nor IL-9 alone induced a significant change in MMP-7 expression.

Proteinase activities of MMP-3 and MMP-7 were quantified by means of casein zymography (Fig 2, C and D). A significant increase in MMP-3 activity was observed at 2 and 4 hours after IL-9 plus IL-13 treatment and 4 hours after IL-4 plus IL-13 treatment (Fig 2, C). T_H2 cytokines also increased MMP-7 activity (Fig 2, D); IL-13 in combination with IL-9 caused a significant increase in activity at 4 hours.

T_H2 cytokines trigger a transient increase in AEC mFasL presentation with release of sFasL

MMP-3, MMP-7, or both potentially cleave mFasL, causing release of sFasL from AECs. Supernatants of 1HAEo[−] cells contain active MMP-3 and both pro and active forms of MMP-7, whereas 16HBE14o[−] cells release only active MMP-3 (Fig 3, A). Previous experiments demonstrate that combinations of cytokines stimulate MMP synthesis and activity to a greater extent than individual cytokines. Subsequent experiments therefore used the combination of T_H2 cytokines to mimic the T_H2 environment in patients with asthma and to stimulate MMP activity. 1HAEo[−] cells treated with IL-4, IL-9, and IL-13 in combination release increased sFasL by means of ELISA at 8 hours compared with untreated control cells (Fig 3, B). In contrast, cytokine-treated 16HBE14o[−] cells show no detectable release of sFasL compared with that seen in control cells (data not shown).

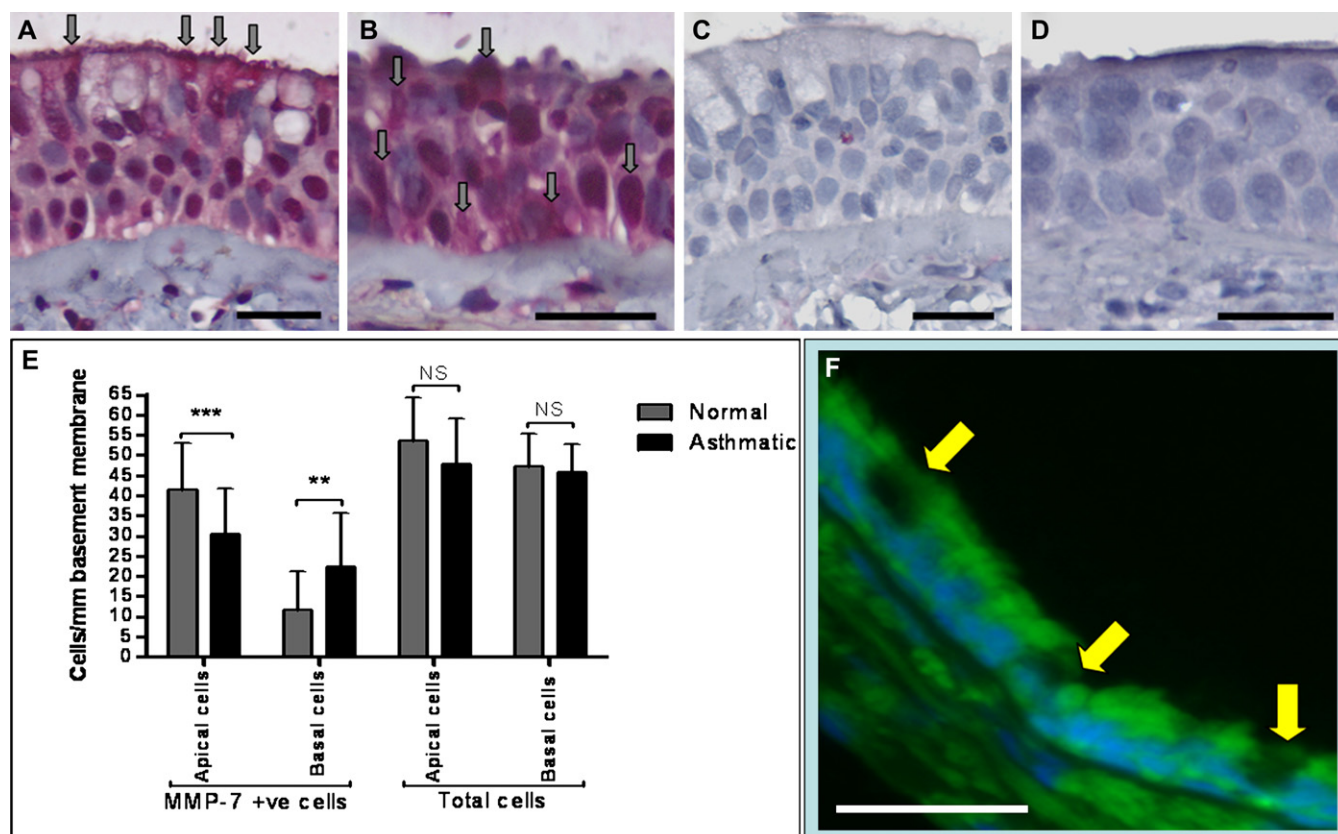


FIG 1. MMP-7 expression in normal (A and C) and asthmatic (B and D) airway epithelial tissue. Fig 1, A, In healthy subjects MMP-7 (pink) is present in ciliated cells (arrows). Fig 1, B, Patients with severe asthma express MMP-7 throughout the epithelium, including basal cells (arrows). Fig 1, C and D, Isotype controls for normal and asthmatic tissue, respectively. E, Cell-specific MMP-7 positivity per millimeter of basement membrane. ** $P < .01$ and *** $P < .001$, ANOVA ($n = 5$). NS, Not significant. F, ISZ with PB-M7VIS showing a cleaved FITC signal (arrows, goblet cells). Scale bars = 25 μm .

We next used FACS to measure cell-surface mFasL expression (Fig 3, C, and see Fig E2, A, in this article's Online Repository at www.jacionline.org). Untreated 1HAEo⁻ cells show mFasL expression levels of $13.2\% \pm 6.4\%$, which increase significantly 1.6-fold to $19.7\% \pm 6.9\%$ (4 hours after cytokine treatment; Fig 3, C). Cytokine-induced increase in mFasL presentation at the cell surface is transient, returning to control levels at 16 hours after treatment ($12.2\% \pm 5.0\%$).

We investigated whether the transient increase in mFasL levels and release of sFasL from T_H2 cytokine-treated 1HAEo⁻ cells was preceded by increased FasL gene transcription (see Fig E2, B). We demonstrate a significant reduction in FasL transcripts at 4 hours (0.69-fold $\pm$ 0.14-fold vs untreated control cells), 8 hours (0.61-fold $\pm$ 0.22-fold vs untreated control cells), and 16 hours (0.50-fold $\pm$ 0.11-fold vs untreated control cells) after treatment, returning to control levels at 24 hours. The absence of increased FasL gene expression suggests a cytoplasmic pool of FasL is translocated to the cell membrane before shedding as sFasL.

Cellular MMP-7 and FasL distribution after cytokine addition

We used confocal microscopy to test whether the transient increase in mFasL levels and subsequent release from the cell surface between 4 and 16 hours after cytokine treatment was due

to increased colocalization of FasL with MMP-7 at the cell membrane (Fig 4). In unstimulated cells (0 hours) MMP-7 is distributed throughout the cytoplasm of the cell, and at the cell membrane, FasL is present in the cell nuclei and cytoplasm; however, colocalization is low ($r_p = 0.460 \pm 0.045$). Four hours after cytokine addition, cell-surface MMP-7 staining is increased, but colocalization with FasL remains low ($r_p = 0.396 \pm 0.082$). At 8 hours, strong MMP-7 and FasL staining is present throughout the cell. Yellow signal indicates that FasL and MMP-7 are colocalized within the cell cytoplasm, and Z-plane cross-sections of the merged image show colocalization also at the cell membrane, confirmed by a significant increase in r_p (0.685 ± 0.097). This effect persists through 24 hours ($r_p = 0.696 \pm 0.109$). These results show that T_H2 cytokine exposure induces colocalization of MMP-7 with FasL in the cytoplasm and at the cell surface from 8 to 24 hours after treatment.

Recombinant active MMP-7 causes release of sFasL

To show that MMP-7 is capable of directly cleaving mFasL, we treated 1HAEo⁻ cells (Fig 5) and primary HBECs (see Fig E3 in this article's Online Repository at www.jacionline.org) with active rhMMP-7. Six hours of treatment with rhMMP-7 caused a concentration-dependent release of sFasL, reaching significance at 1 $\mu\text{g/mL}$ in 1HAEo⁻ cells (Fig 5, A) and primary HBECs. A time course shows maximal sFasL release from 1HAEo⁻ cells

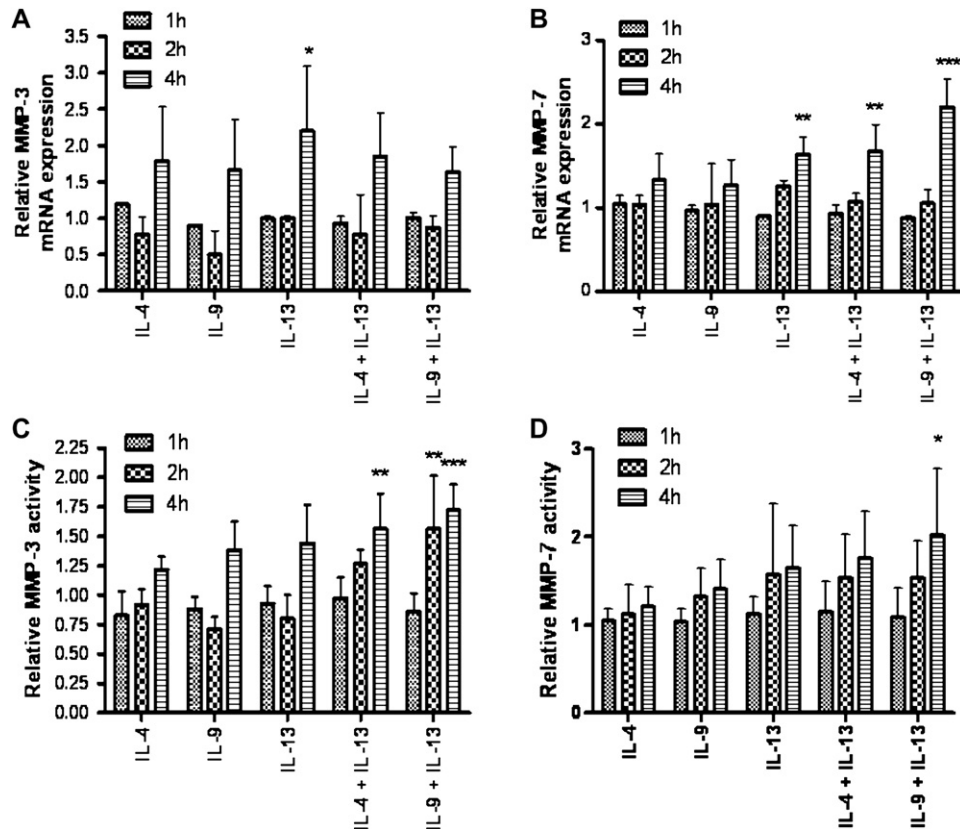


FIG 2. T_H2 cytokines increase MMP-3/MMP-7 expression and activity in 1HAEo⁻ cells. **A** and **B**, MMP-3 (Fig 2, A) and MMP-7 (Fig 2, B) mRNA expression levels measured by means of real-time PCR (n = 7). **C** and **D**, MMP-3 (Fig 2, C) and MMP-7 (Fig 2, D) activity in cell supernatants measured by means of casein zymography (n = 4-5). ***P* < .01 and **P* < .05 versus untreated control, ANOVA.

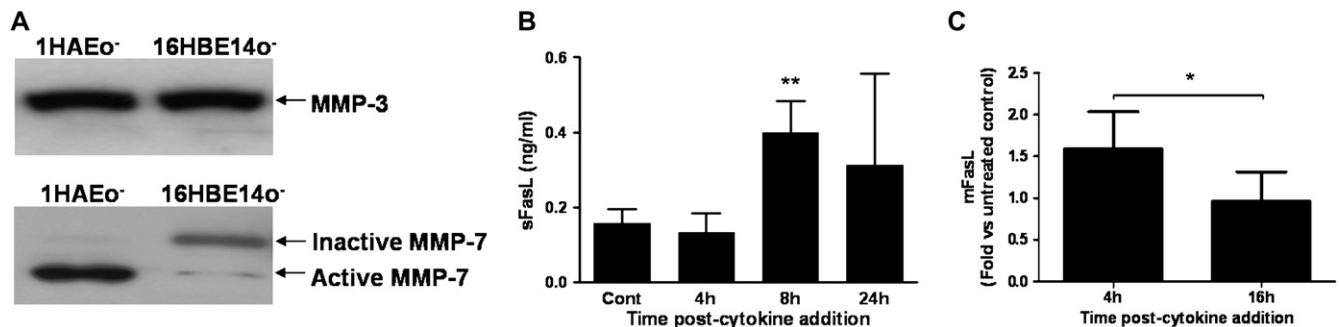


FIG 3. T_H2 cytokines cause sFasL release and a transient increase in mFasL presentation. **A**, Western blots showing MMP-3 and MMP-7 expression in 1HAEo⁻ and 16HBE14o⁻ cells. **B**, sFasL ELISA of supernatants from cytokine-treated 1HAEo⁻ cells. ***P* < .01 versus untreated control, ANOVA (n = 3). **C**, mFasL expression on cytokine-treated 1HAEo⁻ cells, as determined by means of FACS analysis. **P* < .05, Student *t* test (n = 5-8).

at 4 hours (Fig 5, B). rhMMP-7–induced release of sFasL corresponds to reduced mFasL presentation by means of FACS analysis (Fig 5, C).

MMP-7–specific knockdown by siRNA prevents loss of mFasL

We used siRNA technology to test the hypothesis that endogenous MMP-7 is necessary for cytokine-stimulated cleavage of mFasL from AECs (Fig 6). Sixteen hours after cytokine treatment,

1HAEo⁻ cells transfected with 100 nmol/L MMP7 siRNA show less caseolytic activity in supernatants at a molecular weight that corresponds to MMP-7 by means of Western blotting compared with scrambled siRNA-transfected control cells (Fig 6, A and B). After gene silencing of MMP-7, mFasL presentation measured by means of FACS remains increased compared with that seen in control cells at 16 hours after cytokine treatment (Fig 6, C, and see Fig E4 in this article's Online Repository at www.jacionline.org), suggesting endogenous MMP-7 activity is required for mFasL cleavage from the cell surface after cytokine treatment.

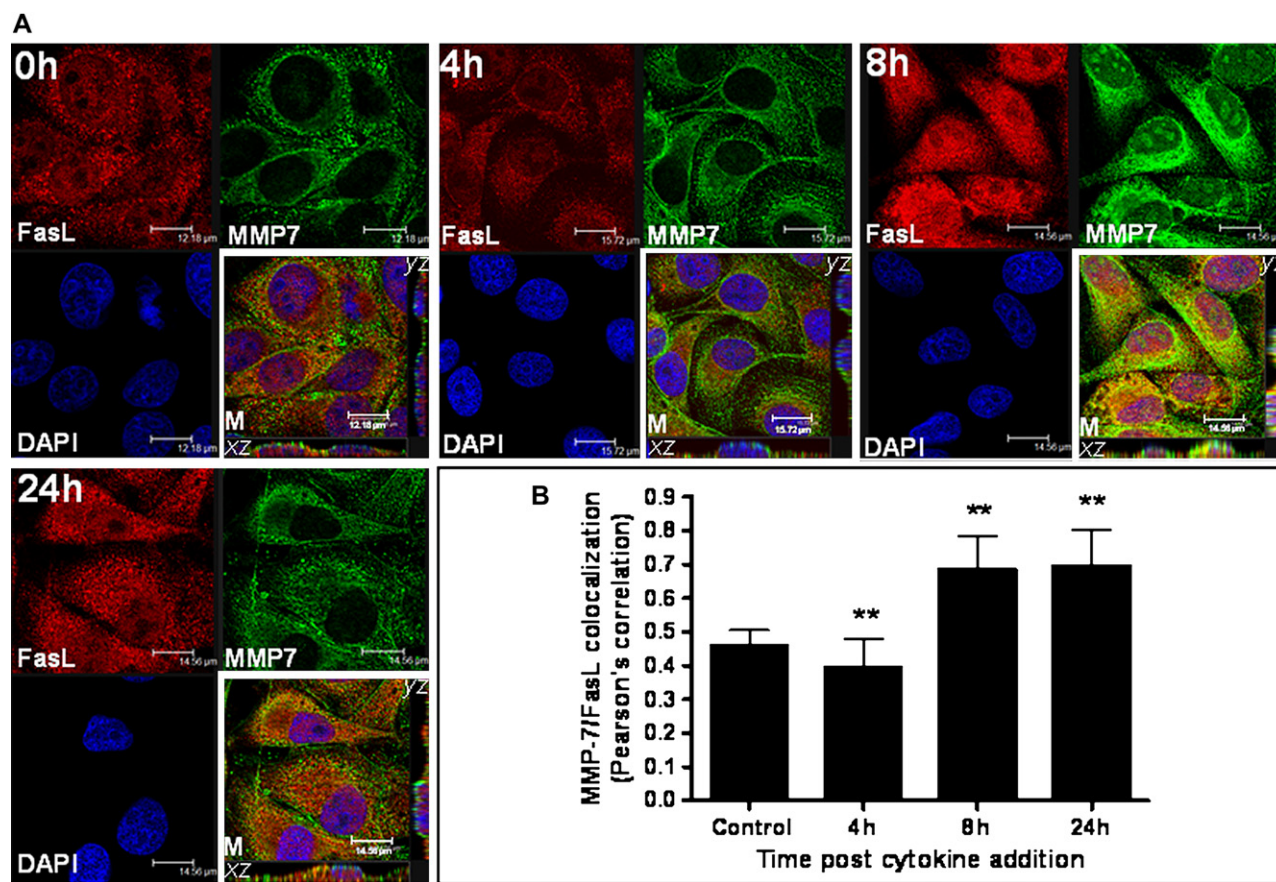


FIG 4. T_H2 cytokines trigger MMP-7/FasL colocalization. **A**, Confocal images of 1HAEo⁻ cells from 0 (untreated) to 24 hours after cytokine treatment stained for MMP-7 (green), FasL (red), nuclei (blue), and a 3-dimensional merged image (*M*). **B**, Yellow MMP-7/FasL colocalization signal was quantified by using Pearson correlation values. ** $P < .01$ versus untreated control, ANOVA ($n = 3-7$ fields).

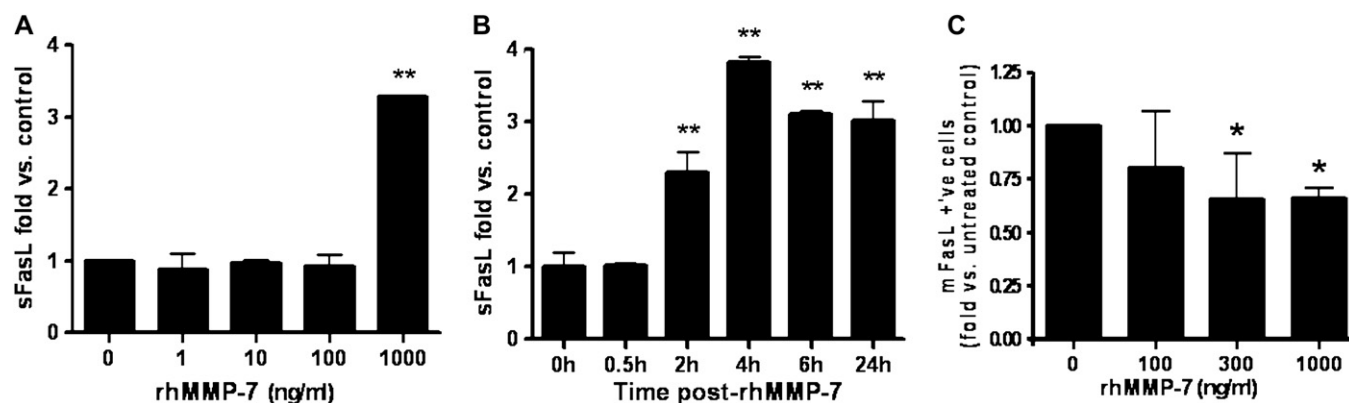


FIG 5. Recombinant MMP-7 causes AEC sFasL release and reduces cell-surface mFasL levels. **A**, sFasL ELISA of 1HAEo⁻ supernatants after 6 hours of incubation with rhMMP-7. **B**, Time course of sFasL release with 1 μ g/mL rhMMP-7. ** $P < .01$ versus untreated controls, ANOVA ($n = 3-4$). **C**, FACS shows that 4 hours of rhMMP-7 treatment reduces 1HAEo⁻ cell mFasL levels. * $P < .05$ versus untreated control, ANOVA ($n = 4$).

DISCUSSION

How chronic damage and activation of an epithelial repair phenotype might contribute to airway remodeling and inflammation in asthma is largely unknown. The effects of the asthmatic T_H2 cytokine environment on airway epithelial function are likely numerous and also undetermined. MMP-type proteases

are increasingly recognized as specific modulators of cell-signaling pathways important in inflammatory disease through the targeted cleavage of non-ECM substrates, including cytokines, chemokines, growth factors, and their receptors. MMP gene transcription and activity is regulated by complex and probably cell-specific pathways¹⁹; however, to date, there are few

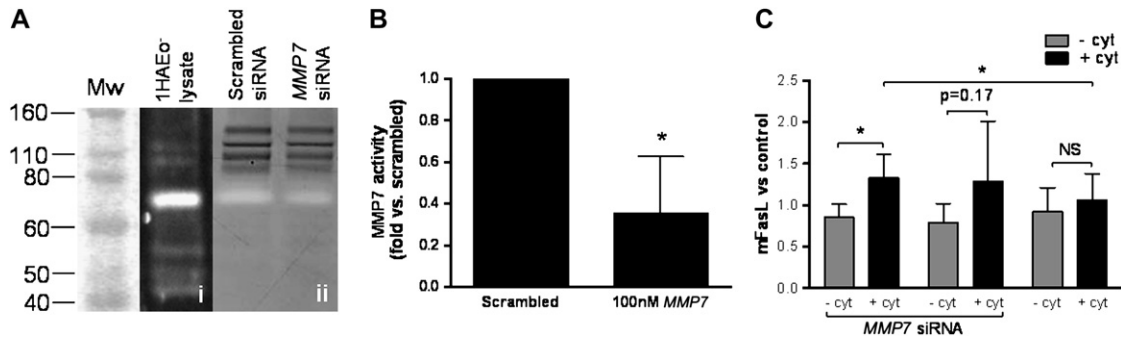


FIG 6. siRNA knockdown of MMP-7 inhibits mFasL cleavage. **A**, Casein zymograms demonstrate proteolytic activity in 1HAEo⁻ supernatants corresponding to the major band in MMP-7-stained cell lysate (*i*). Fig 6, **A** (*ii*), and **B**, Transfection with *MMP7*-specific siRNA reduced MMP-7 activity compared with that seen in scrambled siRNA-transfected controls. **C**, FACS to show mFasL expression in *MMP7* siRNA and scrambled siRNA-transfected 1HAEo⁻ cells 16 hours after cytokine treatment. **P* < .05, ANOVA (*n* = 7).

published data concerning the regulation of airway epithelial MMPs by an asthma-like T_H2 environment. Here we demonstrate dysregulated MMP-7 expression in the airway epithelium from asthmatic patients. In our epithelial model system, T_H2 cytokines specifically increase the transcription and activity of MMP-3 and MMP-7. We show MMP-7 cleaves mFasL, causing release of the potent neutrophil chemoattractant FasL,¹⁶ and thus cytokine-induced MMP-7 might prove to be an important contributor to airway inflammation in patients with asthma.

In our study the epithelium from the large airways of non-asthmatic subjects was well differentiated, with MMP-7 expression and activity strongest in ciliated apical cells. Constitutive secretion of MMP-7 into the airway lumen confirms previous studies suggesting that MMP-7 has a role in the innate mucosal immunity of the airway,³² perhaps by activating antibacterial defenses.³³ Other studies also show that MMP-7 is rarely expressed in basal epithelial cells of the normal airway, except during wound repair, when it is found on the basal aspect of migrating cells.^{25,26,34} We found MMP-7 throughout a poorly differentiated epithelium in asthmatic patients, including basal cells. This might be a normal response to injury in asthma and might reflect a lack of terminal differentiation and activation of an epithelial repair phenotype thought to be important in the remodeling response of the asthmatic airway.¹⁰

For *in vitro* experiments, we created a model of the airway epithelium in asthma by treating the 1HAEo⁻ and 16HBE14o⁻ AEC lines with a mix of T_H2 cytokines (IL-4, IL-9, and IL-13). 1HAEo⁻ and 16HBE14o⁻ cells remain as relatively undifferentiated cells with basal cell–like phenotypes in submerged culture^{27,28,35,36} and are therefore suitable models for the basal epithelial cells that often remain in the damaged epithelium of asthmatic patients. The natural dedifferentiation of ciliated epithelial cells during wound repair³⁷ would also suggest that a basal-like cell is the appropriate cellular phenotype for our *in vitro* studies. It should be noted that T_H2 cytokines, including IL-13 and IL-4, have been shown to influence the differentiation of AECs in air-liquid interface cultures,³⁸ which is consistent with changes identified in the airways of asthmatic patients *in vivo*.

Here we show that exposure of 1HAEo⁻ AECs to an asthmatic cytokine milieu increases endogenous MMP-7 activity, which results in the release of sFasL. Several of our observations indicate the endogenous regulation of MMP activity and FasL cleavage is more complex than upregulation of gene transcription and protease

release. Neutrophils contain cytoplasmic stores of gelatinases and collagenases,¹⁹ and our data suggest that release of preformed stored MMPs similarly occurs in 1HAEo⁻ cells; MMP-3 and MMP-7 activity is increased in cell supernatants before measurable increases in MMP mRNA, and immunolabeling demonstrates that cytoplasmic MMP-7 is already present (albeit at lower levels) in unstimulated 1HAEo⁻ cells. Increased gene expression after initial MMP release might function to replace the intracellular pool of MMPs.

The colocalization of MMP-7/FasL we observed in cells after cytokine treatment will serve to increase their local concentrations, suggesting cleavage of mFasL from the cell surface by endogenous MMP-7 requires a microenvironment in which enzyme and substrate are at high concentrations. Although MMP-7 does not possess a transmembrane domain, studies with mammary and uterine epithelium demonstrate that MMP-7 physically associates with the hyaluronan receptor (CD44) at the cell surface, where it cleaves and activates pro-HB-EGF.³⁹ Thus MMP-7 might be presented to substrates at the cell surface, including mFasL, in local domains of high concentration. Compartmentalization of MMP-7 through CD44 might explain why relatively high concentrations of exogenous MMP-7 were required for mFasL cleavage in our assays.

In other cell systems MMP-3 has been shown to cleave mFasL directly²¹ and, as a potential activator of MMP-7, might be upstream of the MMP-7-mediated FasL cleavage that we have demonstrated.¹⁹ We observed no sFasL release in a cell system that lacks active MMP-7 (16HBE14o⁻ cells). Our data clearly show that MMP-7 expression is dysregulated in the epithelium of asthmatic patients; however, we cannot discount MMP-3 as a potential mediator of FasL release *in vivo*. The *in vitro* studies demonstrate that MMP-7 is sufficient and necessary for FasL release; recombinant MMP-7 is capable of cleaving mFasL from epithelial cells, whereas siRNA targeted against *MMP7* reduces the loss of mFasL from the epithelial cell after cytokine treatment. *In vivo* studies confirm that MMP-7 can regulate inflammation neutrophilic inflammation, is common in patients with acute severe asthma,⁴⁰ and MMP-7 plays a vital role in controlling the transepithelial influx of neutrophils in a murine model of colon injury through the luminal release of the neutrophil chemokines.⁴¹ MMP-7 activity might also trigger eosinophilic airways inflammation; *MMP7*-null mice have reduced bronchial hyperresponsiveness to allergen, fewer eosinophils, and lower levels of IL-25 and eotaxin in the bronchoalveolar lavage fluid

compared with wild-type mice.⁴² Our study elucidates a novel mechanism whereby MMP-7 might play a similarly important role in regulating airway inflammation *in vivo* in patients with asthma through release of the pro-inflammatory chemokine, sFasL.

In our *in vitro* model cytokine-induced FasL release and increased mFasL presentation was accompanied by a decrease in *FasL* gene expression, which might reflect an attempt to control the resulting inflammatory signal. *FasL* gene expression is known to be regulated by a variety of transcription factors; however, the most likely candidate for mediating cytokine-induced inhibition of *FasL* transcription might be the Forkhead (FKHR) family. When dephosphorylated, FKHR translocates to the nucleus and binds a FKRH-responsive element within the *FasL* promoter, activating gene transcription.⁴³ When Akt signaling is activated, such as after IL-4 or IL-13 treatment,^{44,45} FKHR is phosphorylated and therefore does not bind to the *FasL* promoter.⁴³ T_H2 cytokines might thus inhibit FasL promoter activity in AECs through an Akt/FKHR-dependent mechanism.

The discovery of novel substrates for MMP-7 suggests the molecule has evolved to serve a variety of roles. Many are undoubtedly beneficial (eg, activation of antibacterial defensins or promotion of epithelial repair), whereas other proinflammatory functions are useful in the acute-phase response to injury. The studies presented here demonstrate that MMP-7 might be important in airway epithelial health and control of inflammation through direct cleavage of mFasL from the epithelial surface. It is probable that the increased basolateral expression of MMP-7 in the airway epithelium of asthmatic patients leads to cleavage and release of sFasL, contributing to inflammation through neutrophil influx and epithelial damage through removal of the mFasL-mediated immune barrier.¹⁴ If the cytokine environment in the airways of asthmatic patients generates a persistent increase in epithelial MMP-7 expression and activity, then the short-term benefits of acute inflammation and epithelial repair might develop into the pathological tissue remodeling and chronic inflammation associated with severe disease.

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Key messages

- Epithelial expression of mFasL acts as a protective immune barrier. mFasL can be cleaved from AECs by MMP-7, which is dysregulated in the asthmatic epithelium.
- FasL cleavage by MMP-7 might contribute to the asthma phenotype. sFasL acts as a neutrophil chemokine, and loss of the cell-surface FasL immune barrier exposes epithelial cells to immune cell-mediated death.
- T_H2 cytokines upregulate MMP-7 expression in AECs and stimulate colocalization of MMP-7 with FasL, triggering FasL cleavage and release. The inflammatory environment of the asthmatic airway might therefore contribute to disease through stimulation of specific epithelial proteases.

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METHODS

MMP-7 immunohistochemistry

Tissue from the tracheas and large bronchi of donor lungs was dissected, formalin fixed, and paraffin wax embedded, and 5- μ m sections were cut onto glass slides. Immunohistochemistry was performed according to standard protocol. Briefly, slides were dewaxed, rehydrated, and blocked in universal protein block (Dako) for 30 minutes. Slides were stained overnight at 4°C with a murine monoclonal anti-MMP-7 antibody (ID-2, Calbiochem) at 4 μ g/mL in Tris-buffered saline (TBS) plus 1% BSA, followed by a biotin-conjugated goat anti-mouse IgG secondary antibody (BA-9200; Vector Laboratories, Burlingame, Calif) at a 1:250 dilution. Bound antibody was labeled with an Avidin-Biotin Complex (DakoCytomation ABC Complex/AP) amplification step. Cell nuclei were counterstained with hematoxylin. MMP-7 positivity was assessed manually; 5 to 6 $\times$ 20 bright-field images (Nikon Eclipse E600 microscope) of sections from the large airways of 5 pediatric asthmatic patients and 5 healthy subjects were digitally captured (SpotFlex camera; Diagnostic Instruments, Sterling Heights, Mich), with the observer blinded to patient status. A digital grid was overlaid onto each image (ImageProPlus, Media Cybernetics), MMP-7⁺ apical cells (cells touching the airway lumen) and basal cells (cells touching the basement membrane) were manually counted, and the basement membrane length was calculated for each image. Cell numbers are expressed as the number of cells per millimeter of basement membrane length.

MMP-7-selective fluorescent ISZ

Frozen lung tissue from adult patients without asthma was obtained from the iCAPTURE tissue registry. Five- to 10- μ m-thin sections were cut from OCT (Tissue-Tek, Torrance, Calif) inflated and embedded airways. Fluorescent ISZ was performed with PB-M7VIS, an MMP-7-selective substrate with a relatively high rate of cleavage by MMP-7 (K_{cat}/K_m [$M^{-1}s^{-1}$] = $1.9 \pm 0.2 \times 10^5$).^{E1} Tissue sections on slides were quickly warmed to room temperature and overlaid with 40 μ L of liquefied 1% low-gelling-temperature agarose (Invitrogen) containing PB-M7VIS (1 μ mol/L) in ISZ buffer (100 mmol/L NaCl, 10 mmol/L CaCl₂, 20 mmol/L ZnCl₂, 100 mmol/L Tris [pH 7.5], and 0.05% Brij35) with DAPI (5 μ g/mL). Negative controls were prepared either in ISZ buffer without PB-M7VIS or with PB-M7VIS in saline buffer without divalent cofactors. After placing a coverslip, the agarose solution was allowed to set, and slides were incubated at 37°C in a dark humidified chamber for 6 hours. FITC (sensor), TRITC (reference), and DAPI images were captured at several time points by means of wide-field fluorescence microscopy (Eclipse TE300) with a color digital camera (SpotFlex camera, Diagnostic Instruments) and associated imaging software (Spot, Image Diagnostics). Exposure times and gain settings remained constant throughout each experiment. FITC and TRITC signal intensities in the epithelium were quantified with ImageJ image analysis software (<http://rsbweb.nih.gov/ij/>), and the FITC/TRITC ratio was calculated for each time point. Reaction kinetics were monitored with a Genios fluorescent plate reader (Tecan, Mannedorf, Switzerland); in a black, flat-bottomed 96-well plate (Corning, Corning, NY), 100 μ L of PB-M7VIS substrate in ISZ buffer (as above) was incubated with recombinant human active MMP-7 (1,000 ng/mL, Calbiochem) in a dark humidified chamber at 37°C. FITC (Ex λ , 485 nm; Em λ , 535nm) and TRITC (Ex λ , 530 nm; Em λ , 595 nm) signals were measured. MMP-7 was also incubated with a pan-specific MMP substrate, ES010 (10 μ mol/L, R&D Systems; (Ex λ , 360 nm; Em λ , 465 nm) to confirm activity.

Fluorescent confocal microscopy

IHAEO⁻ cells grown in 8-chamber glass slides were treated with IL-4, IL-9, and IL-13 in combination. Cells were fixed in 4% paraformaldehyde at several time points, permeabilized, and blocked in TBS plus 1% BSA. Cells were dual labeled sequentially with polyclonal rabbit anti-FasL (SC-834, 1:50, Santa Cruz Biotechnology) and monoclonal murine anti-MMP-7 (ID-2, 1:200, Calbiochem) primary antibodies (or appropriate isotype controls), with corresponding secondary antibodies (anti-rabbit Alexa-Fluor 594, anti-mouse Alexa-Fluor 488, both 1:200; Molecular Probes, Eugene, Ore). Nuclei were counterstained with DAPI (2-4 μ g/mL), placed under a coverslip, and imaged

with an AOBs Leica Confocal TCS SP2 microscope (63 $\times$ /NA1.2; Leica, Heidelberg, Germany). Lambda scans were performed to confirm specificity of Alexa fluor emission profiles. Image stacks (512 $\times$ 512 pixels, 3-frame average) were collated with Improvision Volocity software, with photomultiplier (PMT) levels constant throughout each experiment. Three to 7 random fields were captured for each experimental condition, with 4 to 10 cells within each field. Three-dimensional MMP-7/FasL colocalization was quantified in individual cells with Volocity software; thresholds were automatically set by using mean FITC and TRITC channel intensities, and values are expressed by using the Pearson correlation as an unbiased means of quantifying colocalization (r_p of 0-0.5, random colocalization; $r_p > 0.5$, specific colocalization).^{E2} Voxel sizes are approximately 100 nm $\times$ 100 nm $\times$ 100 nm for each 3-dimensional Z-stack.

Reverse transcription and real-time PCR

For MMP experiments, reverse transcriptase was used for first-strand cDNA synthesis after priming with oligo-dT (Qiagen, Ontario, Canada). The reaction was performed in 21- μ L reactions containing 5 μ g of RNA, 1 $\times$ standard buffer, 12.5 ng of oligo-dT, 5 mmol/L dithiothreitol, 0.5 mmol/L deoxyribonucleoside triphosphates, 0.6 U of Superscript II (Invitrogen, Ontario, Canada), and 1 U of RNase (Promega, Madison, Wis). The reaction was incubated at 38°C for 60 minutes, heated at 90°C for 10 minutes, and cooled to 4°C. The cDNA sample was purified by means of Montage PCR.

Quantitative real-time PCR for MMPs was performed in a LightCycler (Roche, Mannheim, Germany) with a 20- μ L volume containing 1 μ L of cDNA, 10 μ L of Mastermix (Qiagen), and 0.5 μ g of both primers (designed by Oligo software, see Table E1 for primer sequences). Preincubation was 15 minutes at 95°C to activate Taq polymerase, with amplification by 50 cycles of 20 seconds at 95°C, 20 seconds at 50°C, and 20 seconds at 72°C. After amplification, the temperature was increased by 0.2°C/s from 55°C to 95°C for melting curve analysis. Products were confirmed by means of electrophoresis on a 3% agarose gel stained with ethidium bromide. Gene expression was quantified with LightCycler software and expressed as fold relative to a β -actin housekeeping control. For FasL mRNA quantification, 1 μ g of RNA was Trizol extracted from IHAEO⁻ cells at different time points after cytokine treatment and DNaseI treated (10 minutes at 60°C, Invitrogen). Sixty-six nanograms of RNA was loaded to a 15- μ L reaction mix in a 384-well plate in triplicate, containing products from the SuperScript III Platinum SYBR Green one-step qRT-PCR kit with ROX reference dye (Invitrogen) and either FasL or HPRT housekeeping forward and reverse primers at 10 μ mol/L (see Appendix 1). The cDNA synthesis step was 3 minutes at 50°C and Taq polymerase activation for 5 minutes at 95°C, with PCR amplification by means of 40 cycles of 15 seconds at 95°C, 3 seconds at 60°C, and 60 seconds at 40°C. After amplification, the temperature was increased by 0.2°C/s from 55°C to 95°C for melting curve analysis. Data were analyzed with ABI software, and results were expressed as fold expression relative to untreated (control) cells and normalized to HPRT: $\Delta\Delta CT = [(CT_{FasL} - CT_{HPRT})^{TREATED} - (CT_{FasL} - CT_{HPRT})^{CONTROL}]$. Fold expression change is defined as $2^{-\Delta\Delta CT}$.

Western blot analysis

Cell protein extracts and media supernatants were used for Western blot analysis. Forty micrograms of nonreduced samples were electrophoresed in 12% SDS-PAGE gel and then transferred to nitrocellulose membrane. The membrane was blocked for 1 hour at room temperature in TBST (10 mmol/L Tris-HCl, 150 mmol/L NaCl, and 0.05% Tween-20) containing 5% skimmed milk. The membrane was then incubated overnight at 4°C in TBST containing 2.5% skimmed milk with a 1:200 dilution of monoclonal murine anti-MMP-7 antibody (ID2, Calbiochem). The membrane was washed 3 times with TBST, followed by an incubation step with a 1:2,000 dilution of horseradish peroxidase-labeled goat anti-mouse IgG (BD Biosciences Canada, Mississauga, Ontario, Canada). Samples were detected with enhanced chemiluminescence (Supersignal West Femto; Pierce, Cheshire, United Kingdom).

Casein gel zymogram MMP assay

Unboiled samples were diluted in sample-reducing buffer (2 $\times$) consisting of 0.5 mmol/L Tris (pH 6.8), 2% SDS, 20% glycerol, and 0.02% bromophenol

blue and loaded onto a 12% polyacrylamide gel containing 0.05% casein (Invitrogen). Electrophoresis was carried out at 125 V for 100 to 120 minutes in SDS-containing zymogram running buffer (Invitrogen), gels were washed in 100 mL of renaturing buffer (Invitrogen) for 30 minutes at room temperature and then incubated with 100 mL of developing buffer (Invitrogen) for 2 days at 37°C, fresh developing buffer was added, and gels were incubated for a further 2 days. After incubation, gels were stained in 40 mL of Coomassie Blue Stain (5% Coomassie; Diversified Biotech, Boston, Mass), 5% acetic acid, and 15% methanol for at least 1 hour, and destained in 30% acetic acid and 10% methanol until a transparent band was detected against a blue background.

siRNA-targeted knockdown of MMP-7

Briefly, cells were seeded into 24-well plates and cultured overnight to 30% to 40% confluency. Medium was replaced with 500 μ L per well Dulbecco minimum essential medium plus 10% FBS, and 100 μ L per well of

MMP-7-specific siRNA (ON-TARGET $plus$ SMARTpool, Dharmacon) was added at final concentrations of 10 to 100 nmol/L with 3 μ L per well of Hi-PerFect transfection reagent (Qiagen; later experiments used Accel serum-free siRNA delivery medium [Dharmacon]). Control cells were transfected with a pool of scrambled control siRNAs (ON-TARGET $plus$ negative control, Dharmacon) or treated with transfection media alone.

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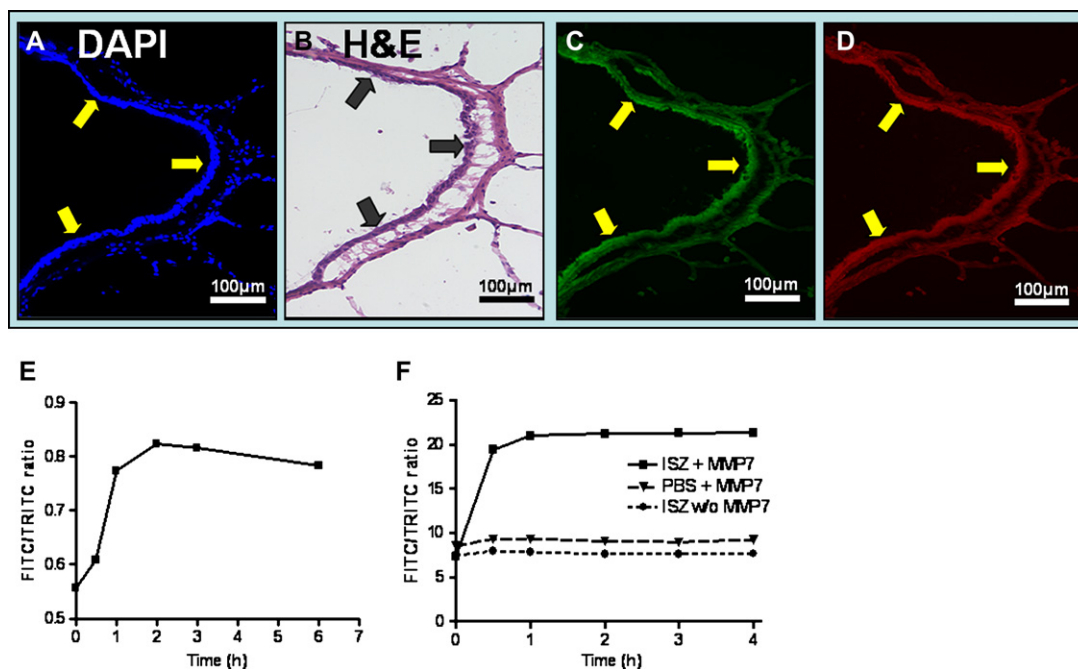


FIG E1. Fluorescence ISZ demonstrates airway epithelial MMP-7 activity. **A** and **B**, Airway epithelium stained with DAPI and hematoxylin and eosin (*H&E*), respectively. **C**, Airway sections incubated with PB-M7VIS substrate demonstrate substrate cleavage in the epithelium (*arrows*) by means of FITC signal at 2 hours. **D**, TRITC reference. **E**, Epithelial FITC/TRITC ratio was quantified at several time points. **F**, A fluorescent plate assay using rhMMP-7 and PB-M7VIS shows similar FITC/TRITC kinetics to tissue, with no change in negative controls.

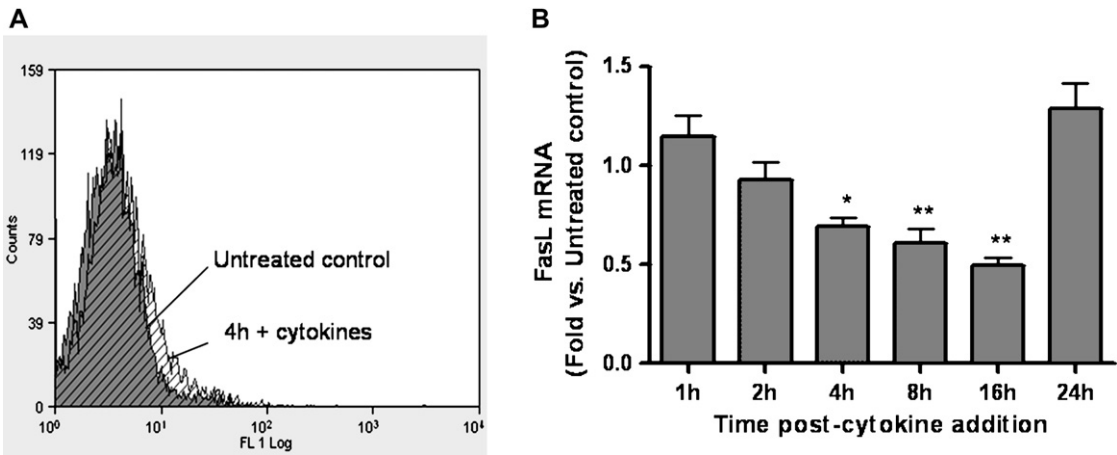


FIG E2. 1HAEo⁻ cell mFasL expression by means of FACS analysis and FasL mRNA quantification after cytokine addition. **A**, mFasL FACS traces of 1HAEo⁻ cells 4 hours after cytokine treatment. **B**, Postcytokine FasL mRNA expression determined by means of real-time PCR. **P* < .05 and ***P* < .01 versus untreated control, ANOVA (*n* = 3).

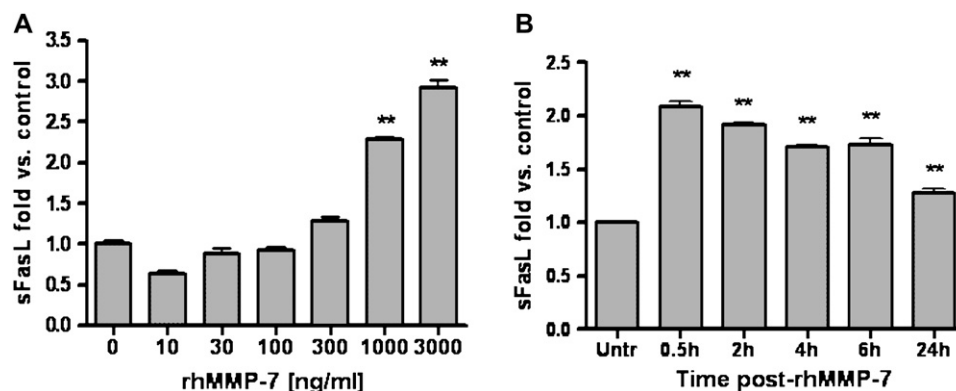


FIG E3. Recombinant MMP-7 causes sFasL release from primary HBECs. **A**, sFasL ELISA of supernatants from primary HBECs after 6 hours of incubation with rhMMP-7. **B**, MMP-7 (1 μ g/mL) causes rapid sFasL release. ** $P < .01$ versus untreated controls, ANOVA ($n = 3-4$).

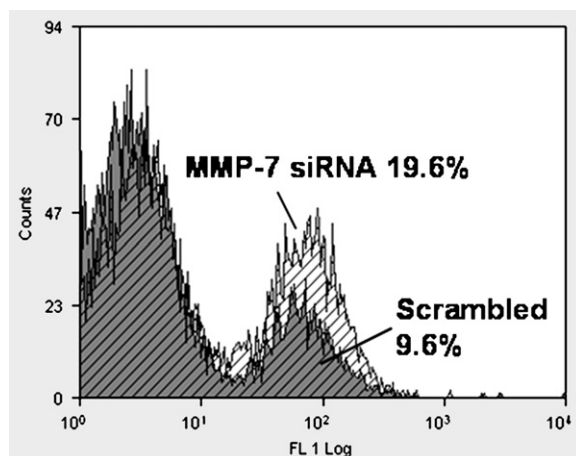


FIG E4. MMP -7 siRNA inhibits mFasL cleavage after cytokine treatment. FACS traces show mFasL expression by 1HAEo⁻ cells transfected with 100 nmol/L MMP-7 or scrambled siRNA 16 hour after cytokine treatment.

TABLE E1. MMP and TIMP primer sequences

Primer	Sequence
MMP-2	5'-TTTCCATTCCGCTTCCAGGGCACAT 5'-TCACTGCCTTTCTACACCACACGCT
MMP-3	5'-AATCCTACTGTTGCTGTG 5'-ACAAGGTTCATGCTGGTG
MMP-7	5'-TCTCCATTTCATAGGTTG 5'-TTGATGGGCCAGGAAACA
MMP-9	5'-GGCAGGACCGTCTCTACTGGCGCGT 5'-CAGAACAGAATACCAGTTTGTATC
TIMP-1	5'-TGGACTCTTGACATCACTACC 5'-CCAACAGTGTAGGTCTTGGTGA
TIMP-2	5'-CGTTTTGCAATGCAGATGTAGT 5'-CTGGATCCTCTTGATAGGGTTG
ADAM-10	5'-TACGAATGAAGAGGGACACTTCCC 5'-GCTCTTCGACACTAACGGGTCTAT
ADAM-17	5'-ATGAGGCAGTCTCTCCTATTCC 5'-GGTGGACTTCTCGAACAAGTAG
FasL	5'-TGCCACCCCTGAAGAAGAGA 5'-CTACCAAGGCAACCAGAACCA
HPRT-1	5'-TGACACTGGCAAAACAATGCA 5'-GGTCCTTTTCACCAGCAAGCT

TABLE E2. Baseline MMP mRNA expression in 1HAEo[−] cells

	mRNA expression relative to β-actin ($\times 10^{-3}$) in 1HAEo [−] cells
MMP-2	4.367 $\pm$ 0.0030 [SD]
MMP-3	0.720 $\pm$ 0.0023
MMP-7	0.081 $\pm$ 0.0001
MMP-9	1.664 $\pm$ 0.0012
TIMP-1	12.433 $\pm$ 0.1709
TIMP-2	12.155 $\pm$ 0.0103
ADAM-10	7.181 $\pm$ 0.0055
ADAM-17	16.090 $\pm$ 0.0098

mRNA expression for various MMPs and their inhibitors in resting 1HAEo[−] cells was measured by means of semiquantitative PCR. Relative expression levels are expressed as fold versus the β-actin housekeeping gene (n = 8-25).