

Increased extracellular maspin levels after mechanical compression *in vitro* or allergen challenge *in vivo*



To the Editor:

During asthma exacerbations, bronchoconstriction causes buckling of the airway, which imposes compressive mechanical stress on airway epithelial cells.¹ In our previous studies using *in vitro* mechanical compression that mimics the mechanical environment of buckled epithelium, we have shown that mechanical compression of well-differentiated human bronchial epithelial (HBE) cells leads to recapitulation of key features of airway remodeling, including collagen deposition, goblet cell hyperplasia, and contraction and proliferation of airway smooth muscle cells, even in the absence of inflammatory cells.^{2,3} Some of these *in vitro* findings were validated in studies performed in animal models and in human subjects.^{4,5} These studies suggest that compressed bronchial epithelial cells initiate and propagate airway remodeling during asthma exacerbations. However, the effect of asthma exacerbations on pathophysiologic mediator production from bronchial epithelial cells and how these mediators could contribute to airway remodeling remain to be elucidated.

Maspin, a noninhibitory member of the mammary serine protease inhibitor (serpin) superfamily, was originally identified as a tumor suppressor protein in human mammary epithelial cells.⁶ Maspin is suggested to contribute to tissue-remodeling processes through modulation of cell-cell interactions and the extracellular matrix (ECM) by means of inhibition of collagen degradation and promotion of epithelial adhesion to the ECM.^{7,8} Here, using both mechanically compressed well-differentiated primary HBE cells and bronchoalveolar lavage (BAL) fluid collected from a mouse model of allergic asthma and patients with mild asthma challenged with an allergen, we identified secreted maspin as a potential mediator of airway remodeling in asthmatic patients (see the [Methods](#) section in this article's Repository at www.jacionline.org for detailed methodology).

Culture of primary HBE cells seeded in transwells was maintained under submerged conditions until confluence and switched to the air-liquid interface (ALI). In submerged conditions, which are comprised solely of basal stem cells, intracellular maspin was detectable, suggesting that maspin is expressed in basal stem cells ([Fig 1, A](#)). During differentiation in ALI conditions, the level of intracellular maspin progressively increased and plateaued on ALI day 7 ([Fig 1, A](#)), suggesting that maspin might also be abundantly expressed in differentiated cells. In well-differentiated HBE cells secreted maspin was barely detectable in the basolateral media from the control condition. After compression, the level of secreted maspin was noticeably increased as early as 3 hours and continuously increased up to 24 hours ([Fig 1, B](#)). Mechanical compression with 5 or 10 cm H₂O pressure did not induce secretion of maspin to greater than baseline values, whereas compression with 20 or 30 cm H₂O markedly induced maspin secretion ([Fig 1, C](#)). Maspin mRNA expression was modestly and transiently increased up to 1.5-fold at 8 hours after initiation of compression (see [Fig E1](#) in this article's Repository at www.jacionline.org). Blocking of epidermal growth factor receptor (EGFR) activity using AG1478 did not inhibit compression-induced maspin secretion (data not shown). However, blocking of protein kinase C (PKC)

activity using bisindolylmaleimide 1 significantly attenuated compression-induced maspin secretion by 53% ([Fig 1, D and E](#)). Although mechanical compression activates both the EGFR and PKC pathways,³ maspin secretion was independent of EGFR but dependent on a partially PKC pathway.

To determine whether maspin might be implicated in asthma pathogenesis, we examined maspin levels in BAL fluid collected from a mouse model of asthma using house dust mite (HDM). Compared with that from control mice, the amount of maspin was significantly greater in BAL fluid collected from sensitized and challenged mice ([Fig 2, A and B](#)).

Lastly, to determine whether extracellular maspin secretion was increased during asthma exacerbation induced by allergen challenge, we measured maspin levels in BAL fluid collected from patients with mild asthma before and at 48 hours after exposure to an allergen challenge (see [Fig E1, F](#), and [Table E1](#) in this article's Repository at www.jacionline.org). Compared with BAL fluid collected before allergen challenge, maspin protein levels were significantly increased in BAL fluid collected after allergen challenge ([Fig 2, C and D](#)).

Recent evidence suggests that maspin possesses multiple functions through its interaction with adhesion molecules and ECM proteins.⁸ Maspin is detected in multiple organs, but its expression is primarily detected in epithelial cells in the breast, skin, prostate, and airway tissues.⁹ The most-well recognized function of maspin is as a tumor suppressor that acts by increasing tumor cell apoptosis or by inhibiting angiogenesis and tumor cell invasion and metastasis.⁸ In tumor metastasis the inhibitory role of maspin has been strongly supported by studies showing that extracellular maspin inhibits cancer cell metastasis through regulation of cell migration, cell-cell adhesion, and ECM degradation.⁸ Although functions of extracellular maspin have been primarily linked to cancer metastasis, data from previous studies suggest a potential role for maspin in tissue remodeling.^{7,8} For example, extracellular maspin binds to and thereby inhibits degradation of type I and III collagens.⁷

Interestingly, type III collagen is a major ECM component, expression of which is increased by mechanical compression and is also increased in the subepithelium of the remodeled airway in asthmatic patients.⁵ Increased deposition of type III collagen in patients with asthma could be the result of increased maspin secretion, which acted to inhibit collagen degradation. Together, these data indicate that extracellular maspin can affect functions of multiple cell types in multiple contexts and support our hypothesis that airway epithelially derived maspin can play a role during airway remodeling and asthma pathogenesis.

This is the first study to recognize the potential implication of maspin in the pathophysiology of asthma. Here we identify that HBE cells abundantly express maspin and that secretion of maspin is substantially increased by mechanical compression, mimicking bronchoconstriction during asthma exacerbations. Levels of maspin secretion depended on the elapsed time after mechanical compression and on the magnitude of pressure applied. Mechanical compression-induced maspin secretion depended in part on PKC signaling.

Furthermore, in both a mouse model of allergic asthma and asthmatic patients, the level of maspin detected in BAL fluid was significantly increased after allergen challenge. We do not yet

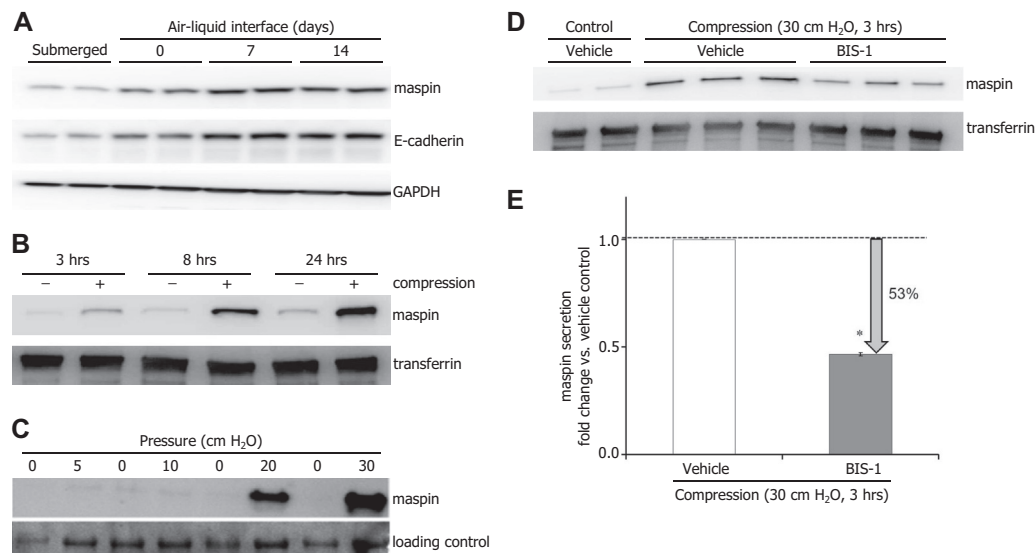


FIG 1. Mechanical compression induces maspin secretion from HBE cells. **A**, Representative Western blot of maspin in protein lysates collected from cells in submerged culture and over ALI days from 3 independent experiments. E-cadherin and GAPDH were loading controls. **B**, Time course of maspin secretion after mechanical compression (30 cm H₂O for 3 hours) representative of 3 independent experiments. **C**, Secreted maspin detected in conditioned media after mechanical compression at various magnitudes of pressure. **D**, Western blot of secreted maspin detected in conditioned media after compression of HBE cells pretreated with vehicle or the selective PKC inhibitor bisindolylmaleimid-1 (BIS-1). **E**, Quantitative analysis of compression-induced maspin secretion shows inhibition by pretreatment with BIS-1 (mean $\pm$ SEM from 3 independent experiments). * $P < .01$, Student t test.

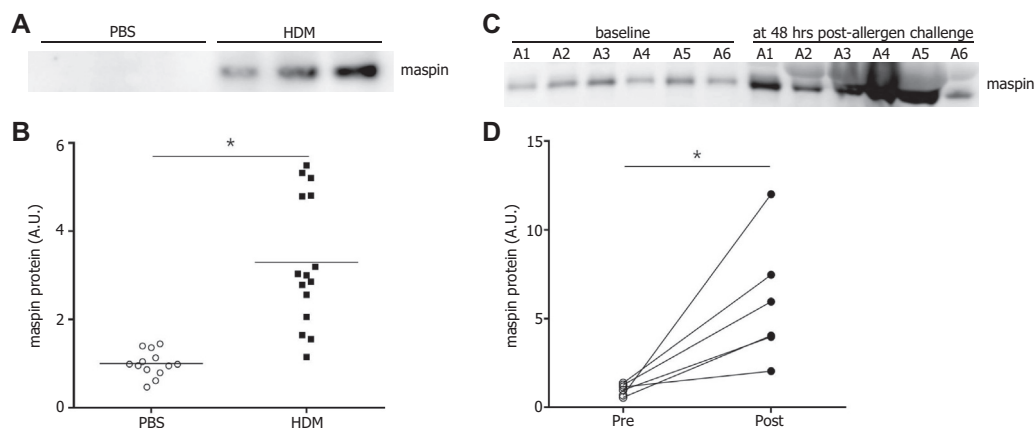


FIG 2. Maspin secretion is increased in BAL fluid from a mouse model of HDM-sensitized allergic asthma and in BAL fluid after an allergen challenge in patients with asthma. **A**, Western blot of maspin in BAL fluid collected from control and HDM-exposed mice. **B**, Quantification of maspin protein in BAL fluid from mice. * $P < .01$, Student t test. **C**, Western blot analysis of maspin in BAL fluid collected from asthmatic patients before and after allergen challenge ($n = 6$ patients). **D**, Quantification of maspin protein in BAL fluid. * $P < .05$, paired t test. A.U., Arbitrary units.

know the function of extracellular maspin in airway remodeling in asthmatic patients. However, given the role of maspin in previous studies, we speculate that maspin secreted from airway epithelial cells can promote airway remodeling through inhibition of collagen degradation or enhancement of epithelial adhesion to ECM. In this study we used an *in vitro* compression model to identify the modulation of airway epithelial cells by mechanical force, as exerted during asthma exacerbations, without the confounding effects of the many immunologic mediators associated with asthma. Thus remaining questions should include how or which immunologic or inflammatory factors modulate the effect of

bronchoconstriction-induced mechanical compression on the production of maspin in the airway.

A limitation of our study was the use of materials and data collected from a small number of patients challenged with an allergen. Therefore the effect of our findings would be amplified by immediate follow-up studies, including detection of extracellular maspin in BAL fluid or serum collected from a large cohort of patients with various endotypes to determine whether maspin could be a potential biomarker for asthma.

In conclusion, we found that bronchial epithelial cells are a source of maspin, which is increased in the airway after allergen

exposures or during bronchoconstriction, suggesting that extracellular maspin might contribute to the pathophysiology of asthma.

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Dendritic cell recognition by group 3 innate lymphoid cells through DNAX accessory molecule 1 triggers proinflammatory reciprocal cell activation



To the Editor:

Innate lymphoid cells (ILCs) represent a heterogeneous family of lymphocytes that includes, in addition to classic cytotoxic

natural killer (NK) cells, the more recently described non-cytotoxic ILC populations.

We have previously shown that human NK cells are able to recognize dendritic cells (DCs) through the NKp30 receptor, a member of the natural cytotoxicity receptor family, and that this recognition results in the activation of NK cells¹ and in a process of DC “editing” able to select DCs with the most efficient antigen-presenting capability.^{2,3} Our current results unveil a similar but even more effective bridge between innate and adaptive immunity in which group 3 innate lymphoid cells (ILC3s), potent effectors of the very early immune response, can receive an activating signal by DCs through cellular ligands able to engage the activating receptor DNAX accessory molecule 1 (DNAM-1) and in turn act as early and strong activators of DCs.

ILC3s are characterized by expression of the transcription factor retinoic acid–related orphan receptor (ROR) γ t. In addition to ROR γ t, they homogeneously express the IL-7 receptor (CD127) and the stem cell factor receptor c-Kit (or CD117) and can be further dissected according to NKp44 expression (see Fig E1, A, in this article's Online Repository at www.jacionline.org).

DNAM-1 is known to be expressed on NK cells, monocytes, and subsets of T cells and has recently been described also on the surface of mouse ILC3s.⁴ We have here shown that human ILC3s express DNAM-1 at levels comparable with those on NK cells (Fig 1, A) and provided first evidence that engagement of DNAM-1 receptor on ILC3s is required for inducing GM-CSF production by ILC3s during DC/ILC3 interaction, whereas neither NKp44 nor NKp30 are involved (Fig 1, B). In turn, GM-CSF released by ILC3s stimulates IL-1 β release by the interacting DCs (Fig 1, C and D), and remarkably, this latter cytokine can be responsible for DC maturation in an autocrine and, most likely, paracrine fashion on surrounding DCs, as assessed by upregulation of costimulatory/activation molecules on DC surfaces (Fig 1, E). Of note, DC activation induced by ILC3s was, at least in this experimental setting, significantly more efficient than that induced by autologous NK cells (Fig 1, E). On the other hand, IL-1 β produced by DCs during their cross-talk with ILC3s plays a relevant role also for ILC3 proliferation and production of IL-22 (Fig 1, F), thus resulting crucial for inducing reciprocal cell activation during the cross-talk between DCs and ILC3s.

It is noteworthy that during coculture with autologous DCs, ILC3s proliferate more vigorously than NK cells, which are known to proliferate upon interaction with DCs (Fig 1, G).¹ The robust proliferation of ILC3s occurring upon interaction with DCs provides a possible explanation for accumulation of these innate lymphocytes in secondary lymphoid organs, in mucosal sites, and at epithelial barriers, where DCs are present as sentinel cells and abundantly recruited during inflammation. Accordingly, the frequency of NKp44⁺ ILC3s in human inflamed tonsils apparently correlated with the degree of inflammation and *in vitro* appeared to be the prominent ILC subset activated by DCs (see Fig E1, B-E). Remarkably, we showed that IL-1 β –producing DCs and ILC3s harbor in the same area of human inflamed tonsils (Fig 1, H), thus suggesting that early release of IL-1 β by DCs encountering ILC3s at these sites would play a central role in the initiation and also maintenance of inflammatory processes.

Hence, in the context of the early phase of the immune response, the role played by ILC3s in activating the subsequent adaptive response might be more relevant than previously appreciated, particularly in settings in which activation of

METHODS

Culture of primary normal HBE cells

Primary normal human bronchial epithelial (NHBE) cells at passage 2 were plated on 12-well transwell plates (Corning, Corning, NY) coated with 50 ng/mL type 1 rat tail collagen (BD Biosciences, San Jose, Calif). Cells were grown for 4 to 6 days in submerged conditions and, after reaching confluence, were exposed to air by removing media from the apical surfaces of cells. NHBE cells were fed a 1:1 mixture of bronchial epithelial basal medium and high-glucose (4.5 g/L) Dulbecco modified Eagle medium supplementation. After 14 to 16 days in ALI culture, cells were well differentiated, as confirmed by staining for MUC5AC and β -tubulin IV, and then used for application of mechanical compression.

Exposure of NHBE cells to mechanical compression

Compressive mechanical stress was applied with a custom pressure setup, which allowed for application of compressed air balanced with 5% CO₂ to the apical side of cells in ALI culture. Unless noted otherwise, in all experiments well-differentiated NHBE cells were exposed to a pressure of 30 cm H₂O. This is comparable with the pressure generated during maximum bronchoconstriction and is an order of magnitude greater than that experienced by the airway epithelium during normal breathing or nonasthmatic coughing. NHBE cells were exposed to pressure for 3 hours, and then cells and conditioned media were collected immediately or at indicated time points at 3, 8, or 24 hours after initiation of pressure. Control cells were connected with a sham setup identical to that of compressed cells, except that they were not connected to pressurized air. In some experiments, to determine the role of TGF- β 2, NHBE cells were treated with vehicle control or TGF- β 2 at 10 ng/mL for 3 days and then subsequently exposed to compression.

Inhibitor experiments

A selective inhibitor for EGFR (0.5 μ M AG1478) or PKC (1 μ M bisindolylmaleimide-1) was used to identify intracellular signaling pathways involved in compression-induced maspin secretion. Inhibitors or vehicle were spiked into basal media 30 minutes before application of compression. All inhibitors were initially dissolved in dimethyl sulfoxide as stock solutions and then dissolved to a final concentration in minimal media depleting bovine pituitary extract and epidermal growth factor from HBE cell-culture media. Dimethyl sulfoxide was used as a vehicle control.

Western blot analysis

Protein lysates were prepared from cell lysates to detect intracellular protein and from basolateral conditioned media to detect secreted protein by using Western blot analysis. Primary antibodies against maspin (1:1,000; Cell Signaling Technology, Danvers, Mass), E-cadherin (1:1,000; BD Biosciences, Billerica, Mass), glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 1:5,000; Cell Signaling Technology), and transferrin (1:10,000; Thermo Fisher Scientific, Waltham, Mass) were used for Western blot analyses. We used GAPDH for intracellular maspin and transferrin for secreted maspin as

loading controls. From Western blot images, maspin bands were quantified by using ImageJ software (National Institutes of Health, Bethesda, Md). Using recombinant human maspin protein (Sigma, St Louis, Mo), we semiquantitatively determined the amount of secreted maspin by means of linear interpolation.

Quantitative RT-PCR

An RNeasy Mini Kit (Qiagen, Germantown, Md) was used to isolate total RNA. MultiScribe reverse transcriptase (Applied Biosystems, Foster City, Calif) was used to synthesize cDNA by using 1 μ g of RNA. Twenty nanograms of cDNA was used for quantitative real-time PCR by using 2 $\times$ SYBR Green Master Mix (Life Technologies, Grand Island, NY). Primers for maspin and GAPDH were generated by using Primer Blast or Primer Express 3.0 software (Table E2). The fold change of maspin mRNA expression was calculated by using the $\Delta\Delta$ cycle threshold method.

Allergen-sensitized and allergen-challenged mice

All experiments were performed under protocols approved by the Harvard Medical Area Standing Committee on Animals (Harvard T.H. Chan School of Public Health, Boston, Mass). For all experiments, we used both male and female C57BL/6J mice at 4 to 5 weeks of old age purchased from the Jackson Laboratory (Bar Harbor, Me). HDM extracts (product no. XPB70D3A2.5, lot nos. 343206 and 2875, endotoxin units/2.5 mL sized vial) were purchased from Greer Laboratories (Lenoir, NC) and dissolved in PBS. As previously described, mice were exposed to 50 μ g of HDM extracts dissolved in 30 μ L of PBS intranasally. Levels of endotoxin contained in the installed HDM was less than 10.8 endotoxin units/mg of protein. Control mice received 30 μ L of PBS administered intranasally. At 48 hours after the last HDM challenge, BAL fluid was collected. In BAL fluid proteins were precipitated with 20% trichloroacetic acid and were used for Western blot analysis.

Collection of BAL fluid from asthmatic patients

BAL fluid was collected from patients who had received a previous diagnosis of mild asthma. The Partners Human Research Committee at Brigham and Women's Hospital approved the protocol, which was in accordance with ethical standards (Institutional Review Board #2012P001029). Written informed consent was obtained from each patient by a physician. No patients were undergoing corticosteroid treatment. For each patient, BAL fluid was collected immediately before an allergen challenge and also at 48 hours after the challenge. As clinical parameters, eosinophil percentages and FEV₁ were measured.

Statistical analysis

Data are presented as means $\pm$ SEMs or means $\pm$ SDs. Statistical analysis was performed with the Student *t* test. A Spearman correlation test was performed between the extent of increased maspin secretion and the clinical parameters FEV₁ (percentage of baseline) and eosinophil percentage. A *P* value of less than .05 was considered significant.

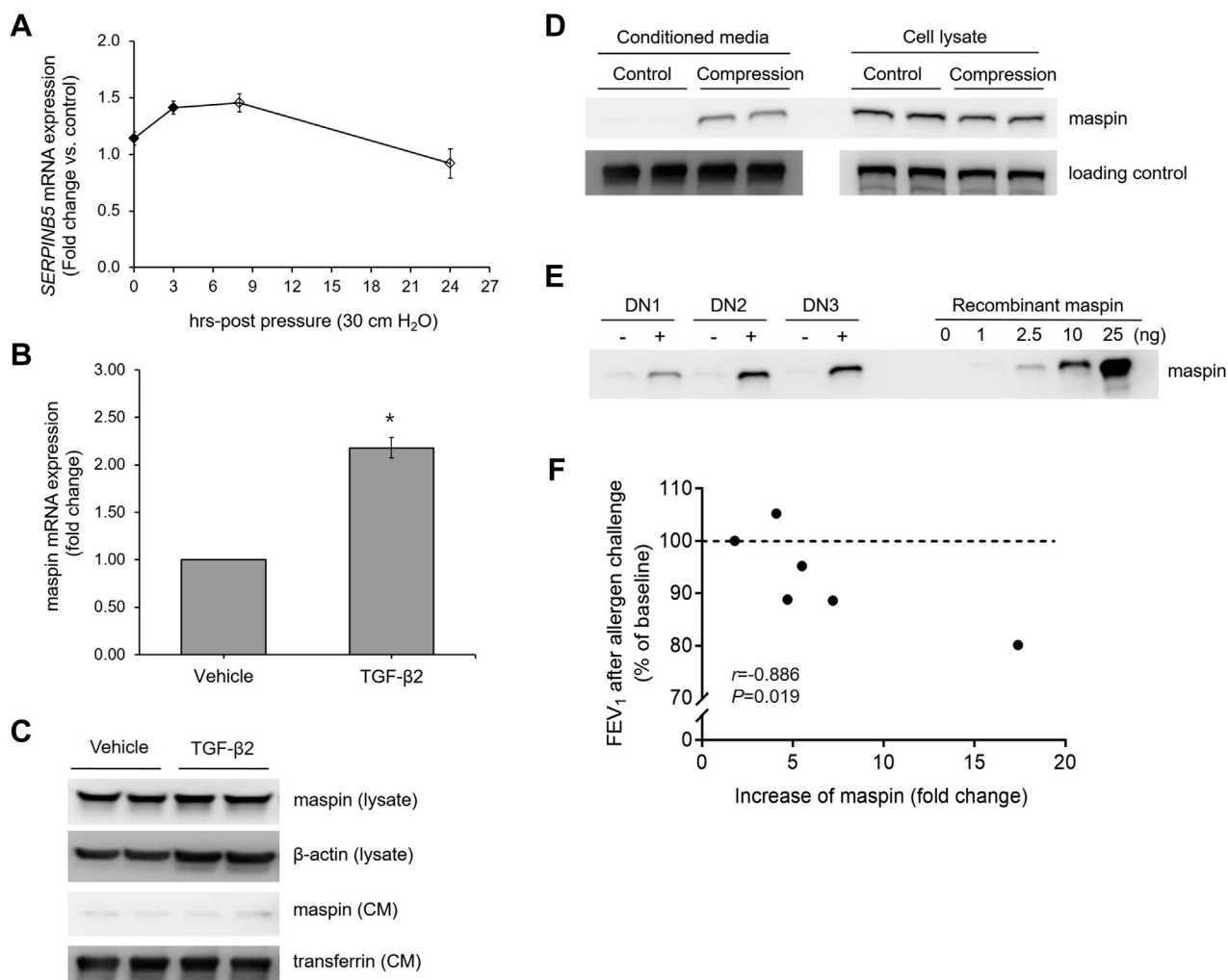


FIG E1. **A**, Time course of maspin mRNA expression after initiation of mechanical compression (30 cm H₂O for 3 hours; means $\pm$ SDs; $n = 3$ independent wells). *Solid squares* indicate time points for cells under compression, and *open squares* indicate time points for cells released from compression. **B**, Effect of TGF- β 2 on maspin mRNA expression in NHBE cells. After incubation with TGF- β 2 for 3 days, maspin mRNA expression was increased (means $\pm$ SEMs from 3 independent experiments). * $P < .01$. **C**, Effect of TGF- β 2 on intracellular and secreted maspin in NHBE cells. After incubation with TGF- β 2 for 3 days, intracellular maspin in the cell lysates and secreted maspin in conditioned media (CM) were detected. β -Actin was used for cell lysates, and transferrin was used for CM as loading controls. **D**, Maspin detected in CM and cell lysates after mechanical compression. **E**, Comparison of secreted maspin from NHBE cells of 3 different donors (DN1-DN3) after mechanical compression. Recombinant human maspin was used as a positive control and for quantification based on linear interpolation. **F**, Correlation between the degree of FEV₁ reduction and the degree of increased maspin secretion in BAL fluid after allergen challenge in asthmatic patients.

TABLE E1. Patient information for materials collected at 48 hours after allergen challenge

Donor no.	Age (y)	Sex	Ethnicity	Eosinophils (%) [*]	FEV ₁ (% of baseline)	Fold change of maspin detected in BAL fluid
A1	29	F	White	5	88.6	7.2
A2	21	F	Hispanic	76.1	105.2	4.1
A3	28	M	White	84.9	88.8	4.7
A4	19	F	White	88.5	80.1	17.4
A5	24	F	White	86.2	95.2	5.5
A6	28	M	White	82.1	100	1.8

F, Female; M, male.

^{*}For all patients, less than 0.5% of total cells were eosinophils before allergen challenge. Eosinophil numbers and FEV₁ values were determined at 48 hours after allergen challenge and compared with values determined before allergen challenge.

TABLE E2. Information on primers

Gene	Forward (5' → 3')	Reverse (5' → 3')	Reference
<i>SERPINB5</i>	AGACAGACACCAAACCAAGTGCAGA	TCCCATACAGAACGTGGCCTCCA	Primer Blast
<i>GAPDH</i>	TGGGCTACACTGAGCACCAG	GGGTGTCGCTGTTGAAGTCA	Primer Express 3