

Trefoil Factor-2 Reverses Airway Remodeling Changes in Allergic Airways Disease

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Trefoil factor 2 (TFF2) is a small peptide with an important role in mucosal repair. TFF2 is up-regulated in asthma, suggesting a role in asthma pathogenesis. Given its known biological role in promoting epithelial repair, TFF2 might be expected to exert a protective function in limiting the progression of airway remodeling in asthma. The contribution of TFF2 to airway remodeling in asthma was investigated by examining the expression of TFF2 in the airway and lung, and evaluating the effects of recombinant TFF2 treatment on established airway remodeling in a murine model of chronic allergic airways disease (AAD). BALB/c mice were sensitized and challenged with ovalbumin (OVA) or saline for 9 weeks, whereas mice with established OVA-induced AAD were treated with TFF2 or vehicle control (intranasally for 14 d). Effects on airway remodeling, airway inflammation, and airway hyperresponsiveness were then assessed, whereas TFF2 expression was determined by immunohistochemistry. TFF2 expression was significantly increased in the airways of mice with AAD, compared with expression levels in control mice. TFF2 treatment resulted in reduced epithelial thickening, subepithelial collagen deposition, goblet-cell metaplasia, bronchial epithelium apoptosis, and airway hyperresponsiveness (all $P < 0.05$, versus vehicle control), but TFF2 treatment did not influence airway inflammation. The increased expression of endogenous TFF2 in response to chronic allergic inflammation is insufficient to prevent the progression of airway inflammation and remodeling in a murine model of chronic AAD. However, exogenous TFF2 treatment is effective in reversing aspects of established airway remodeling. TFF2 has potential as a novel treatment for airway remodeling in asthma.

Keywords: trefoil factor 2; asthma; airway remodeling; allergic airways disease; epithelium

Asthma is characterized by allergic airway inflammation, airway remodeling, and airway hyperresponsiveness (AHR). In the majority of patients with asthma, bronchodilators and corticosteroids effectively control airway inflammation and AHR, allowing good symptom control. However, some patients experience persistent symptoms because of sustained AHR, despite optimal steroid treatment. In these individuals, airway remodeling is the major factor contributing to continuing AHR and irreversible airway obstruction (1, 2).

The major changes of airway remodeling include goblet-cell metaplasia, increased epithelial thickness, subepithelial fibrosis, smooth muscle hyperplasia, and neovascularization (3, 4). All of these are directly linked with AHR and reduced airway caliber. Remodeling changes in asthma were suggested to be initiated

and propagated by epithelial damage and inherent defects in epithelial repair (5). Furthermore, airway epithelial remodeling may drive other airway remodeling changes (6). These findings suggest that treatments to prevent or reverse airway epithelial damage and remodeling may be effective in minimizing airway remodeling, the resultant AHR, and loss of lung function.

Trefoil factor-2 (TFF2) is one of three related peptides, each characterized by three disulfide-linked cysteine pairs in a common structural element called the trefoil motif. Trefoil factors play major roles in epithelial repair and homeostasis, particularly in the gut and lung (7–9). In the gastrointestinal tract they have been shown to be induced after epithelial damage, and facilitate short-term restitution and long-term glandular re-epithelialization during the repair process (10). Studies have documented TFF2 expression by epithelial cells in the human and murine lung (11, 12). Furthermore, TFF2 expression was shown to be increased in human asthma and murine models of allergic airway disease (AAD), whereas genome-wide linkage studies identified an association between *Tff2* gene polymorphisms and AHR in mice (13, 14), suggesting a role for TFF2 in the pathogenesis of asthma. TFF2 was shown to be rapidly induced but not contributory in the regulation of airway inflammation in a murine model of acute airway inflammation (12, 15). In addition, TFF2 deficiency resulted in increased goblet-cell metaplasia and epithelial thickening in models of short-term AAD (16). The role of exogenous TFF2 treatment in the regulation of airway remodeling and AHR, and the expression of TFF2 in the setting of chronic airway inflammation and remodeling (which more closely mimics human asthma), have not been investigated previously, to the best of our knowledge.

We aimed to delineate further the contributions of TFF2 to the pathogenesis of asthma. We determined the protein expression and tissue distribution of TFF2 in the airway and lung in a chronic model of AAD associated with airway remodeling and AHR, and evaluated the effects of intranasal TFF2 treatment on established changes of airway remodeling in a murine model of AAD.

MATERIALS AND METHODS

Animals

Six-week-old female BALB/c mice were used in these studies (17–19). Experimental procedures were approved by the Animal Ethics Committee of Murdoch Children's Research Institute, and followed the Australian Guidelines for the Care and Use of Laboratory Animals for Scientific Purposes.

Murine Model of Chronic AAD

An established model of ovalbumin (OVA)-induced chronic AAD was used as previously described (1, 4, 20, 21). Mice were sensitized with OVA (Sigma Chemical, St. Louis, MO) on Day 0 and Day 14, and were then challenged with OVA 3 days per week for 6 weeks. OVA-exposed mice were then treated with either glycosylated human recombinant TFF2 (OVA TFF2) vehicle control (OVA vehicle), or were left untreated

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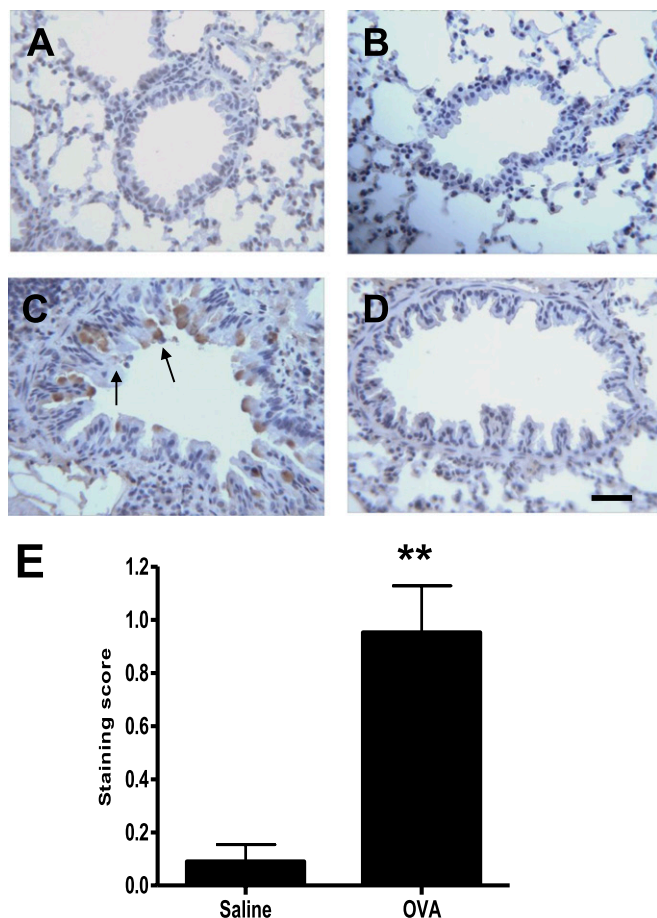


Figure 1. Trefoil factor-2 (TFF2) protein expression and localization in chronic allergic airways disease (AAD). Saline (A) and ovalbumin (OVA)-sensitized and challenged (C) murine airways were stained for TFF2 by immunohistochemistry, using a rabbit polyclonal anti-human TFF2 antibody, and were scored for staining intensity. TFF2 expression was found mainly in bronchial epithelial cells (arrows). Staining intensity was scored from 0 (no staining) to 3 (intense staining). (E) Significantly more staining was observed in OVA-sensitized and challenged mice, compared with saline mice. Negative control sections from saline (B) and OVA (D) mice are also shown. Scale bar = 50 μ m. ** $P < 0.01$, versus saline group.

(OVA). Mice exposed to saline instead of OVA served as additional controls (SAL). For treatment experiments, 12 mice were included in each of the four experimental groups: OVA mice treated with TFF2 (TFF2), OVA mice treated with vehicle (vehicle), OVA mice not treated (OVA), and saline mice not treated (saline). For TFF2 expression experiments, 15 mice in each of the OVA and saline groups were examined.

Intranasal TFF2 Treatment

Fifty microliters of human glycosylated recombinant TFF2 peptide (0.5 mg/ml) (22) or vehicle (PBS) were administered to OVA-sensitized mice once daily for 2 weeks from Days 64–78 ($n = 12$). Briefly, mice were lightly anesthetized with isoflurane and held in a supine position, and 50 μ l of TFF2 or vehicle were administered intranasally, using an autopipette.

Methacholine-Induced AHR

Twenty-four hours after TFF2 or vehicle treatment, AHR was measured by invasive plethysmography, using a murine plethysmograph (Buxco Electronics, Troy, NY) as described previously (20).

Bronchoalveolar Lavage

After measurements of airway reactivity, bronchoalveolar lavage (BAL) was performed, and differential cell counts of BAL cells were determined (20, 23). Total viable cell counts were determined using a hemocytometer with trypan blue exclusion. Differential cell counts were determined on cytospin smears of BAL samples (4×10^5 cells) from individual mice stained with Diff-Quik (Life Technologies, Auckland, New Zealand) and identified by standard morphological criteria after counting 300 cells.

Quantitation of Serum OVA-Specific IgE Concentrations

Serum was obtained by the lethal cardiac puncture of anesthetized mice after AHR measurement, and stored at -70°C for the measurement of OVA-specific IgE by ELISA (1, 20, 24). OVA-specific IgE concentrations were expressed as arbitrary units (AUs), where 1 AU = optical density of 1:50 dilution of positive control serum.

Tissue Collection

Lung tissues were weighed (i.e., total lung weight) and then separated into individual lobes for hydroxyproline analysis and histological analysis (1, 20, 25).

Lung Histopathology

The right lung lobe and trachea were fixed in formalin, embedded in paraffin, and routinely processed (1, 20, 25). Sections were stained with Masson trichrome for the assessment of epithelial and subepithelial collagen thickness, or with Alcian blue–periodic acid Schiff (AB-PAS) for the assessment of goblet cells.

Morphometric Analysis of Structural Changes

Morphometric evaluation of lung tissue sections was determined as described previously (1, 20, 26). At least five bronchi measuring 150 to 350 μ m in luminal diameter were analyzed per mouse.

Hydroxyproline Analysis of Lung Collagen

A portion of each lung sample was treated as described previously to determine hydroxyproline content (27). Total hydroxyproline contents (in micrograms) were converted by comparison with a linear standard curve of 4-hydroxyproline (Sigma-Aldrich, Sydney, New South Wales, Australia).

TABLE 1. EVALUATION OF ALLERGIC RESPONSE BY BAL DIFFERENTIAL COUNT ($\times 10^4$ CELLS/ML) AND OVA-SPECIFIC IGE ELISA

Mouse Group:	Saline	OVA*	Vehicle	TFF2
Total cells*	12.33 $\pm$ 1.89	25 $\pm$ 2.74 [†]	24.5 $\pm$ 4.10 [†]	31 $\pm$ 7.65 [†]
Eosinophils*	0.08 $\pm$ 0.31	0.69 $\pm$ 0.48 [†]	0.71 $\pm$ 0.33 [‡]	0.78 $\pm$ 0.48 [‡]
Neutrophils*	0.28 $\pm$ 0.56	0.63 $\pm$ 0.29	0.71 $\pm$ 0.75	0.65 $\pm$ 0.23
Lymphocytes*	2.38 $\pm$ 2.04	5.31 $\pm$ 1.31	5.53 $\pm$ 2.35	7.01 $\pm$ 1.19
Monocytes*	9.56 $\pm$ 2.61	18.38 $\pm$ 1.44	17.5 $\pm$ 2.06	22.69 $\pm$ 1.38
OVA-specific IgE (AU)a	0 $\pm$ 0.01	0.28 $\pm$ 0.08 [‡]	0.32 $\pm$ 0.05 [‡]	0.43 $\pm$ 0.06 [‡]

*Values are means $\pm$ SE.

[†] $P < 0.03$ when compared with its saline control.

[‡] $P < 0.005$ when compared with its saline control.

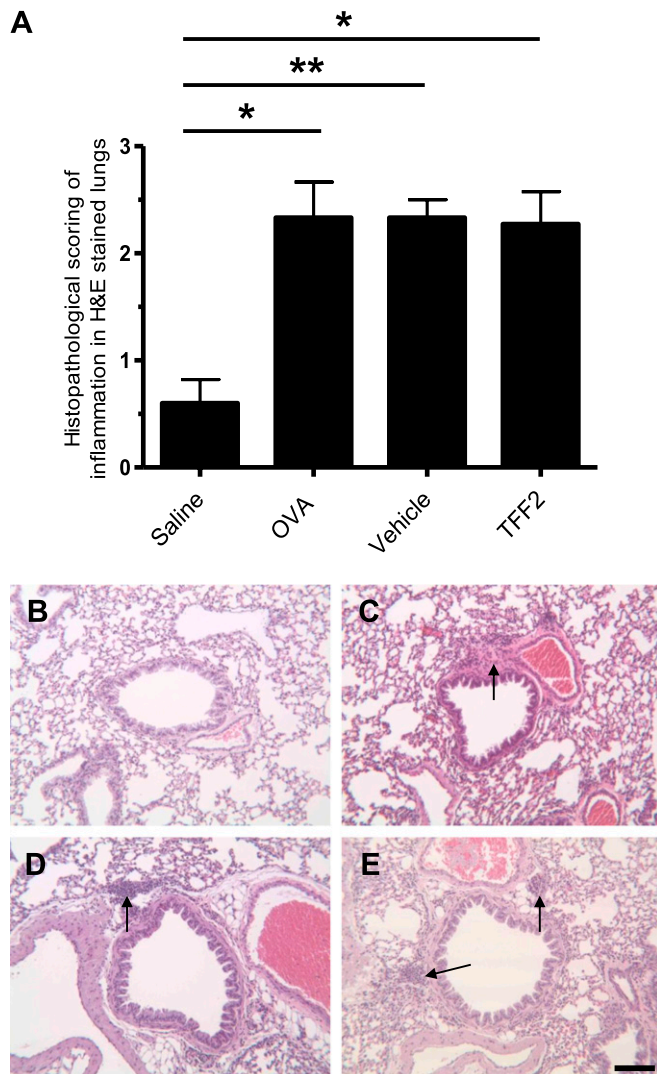


Figure 2. Effect of TFF2 intranasal treatment on overall lung inflammation in chronic AAD. (A) Sections from saline and OVA-sensitized and challenged mice (treated with TFF2 or vehicle) were stained with hematoxylin and eosin (H&E), and scored for numbers and distribution of inflammatory aggregates on a scale of 0 (no apparent inflammation) to 4 (severe inflammation). * $P < 0.05$ and ** $P < 0.01$, versus saline group. Hematoxylin and eosin-stained sections show effects of TFF2 intranasal treatment on overall lung inflammation in chronic AAD. Sections from saline, OVA vehicle, and TFF2-treated mice were stained with hematoxylin and eosin. No inflammatory infiltrate is seen in saline mice (B), but aggregates of inflammatory cells are present peribronchially in OVA mice (C), vehicle-treated mice (D), and TFF2-treated mice (E) (arrows). Scale bar = 100 μ m.

Zymography Assay of Gelatinases

Gelatin zymography was performed on a portion of lung as previously described (28). Bands on the zymograph, indicative of matrix metalloprotease (MMP)-9 and MMP-2 concentrations, were then analyzed by densitometry, using a GS 710 densitometer (Bio-Rad Laboratories, Richmond, CA) and Quantity-One software (Bio-Rad Laboratories). The average density of each sample was expressed as a relative ratio to saline control concentrations.

Immunohistochemistry

For TFF2 localization, antigen retrieval with citrate buffer (pH 6.0) was performed before incubation with rabbit anti-human recombinant TFF2 polyclonal antibody, which cross-reacts with murine TFF2 (29).

Bound TFF2 antibody was detected using a horseradish peroxidase conjugated anti-rabbit secondary antibody. Epidermal growth factor receptor (EGF-R) was detected with a polyclonal antibody (SC-03; Santa Cruz Biotechnology, Santa Cruz, CA) with EDTA antigen retrieval. Transforming growth factor (TGF)- β was detected with a polyclonal antibody (SC-146; Santa Cruz). α -Smooth muscle actin (SMA) was detected with a monoclonal antibody and the Animal Research Kit (1A4; DAKO, Carpinteria, CA). Staining intensity was scored from 0 (no staining) to 3 (intense staining). α -SMA muscle thickness was measured by morphometry.

Immunofluorescence of Apoptotic Staining

Immunofluorescence staining for annexin V was performed as described previously (30). Fluorescence associated with the membrane of epithelial cells was quantitated using Fiji Image J, version 1.46a.

Statistical Analysis

Lung function studies were analyzed using two-way ANOVA, with the Bonferroni *post hoc* test. Morphometry was expressed as mean values with 95% confidence intervals, and analyzed using the Mann-Whitney test.

RESULTS

Expression of TFF2 Protein in a Murine Model of Chronic AAD

TFF2 protein expression was localized to occasional bronchial epithelial cells in control mice (saline group) (Figures 1A and 1E). TFF2 protein expression was markedly increased in mice with AAD (OVA) compared with saline mice ($P = 0.0003$), and staining was localized to the cytoplasm and mucous vacuoles of bronchial epithelial cells (Figure 1C). No epithelial staining was observed in negative control sections stained with an irrelevant rabbit primary antibody or with no primary antibody (Figures 1B and 1D, respectively).

Validation of the Chronic Model of AAD

The chronic AAD model used in the present study showed airway inflammation changes consistent with those reported previously (1, 4). IgE concentrations in OVA-sensitized mice were significantly increased, compared with saline control mice ($P < 0.01$; Table 1). Numbers of eosinophils in lung washouts were significantly increased in OVA-sensitized mice, compared with saline control mice ($P < 0.05$; Table 1), indicative of allergic sensitization. This model of chronic AAD manifests many of the pathological features of human asthma, including increased OVA-specific IgE, airway inflammation, AHR, and airway remodeling changes such as epithelial thickening, goblet-cell hyperplasia, subepithelial fibrosis, and neovascularization. However, it does not display smooth muscle thickening (4, 20).

Effects of TFF2 Treatment on Airway Inflammation, Airway Remodeling, and AHR in a Murine Model of Chronic AAD

Airway inflammation. OVA-sensitized mice that were untreated, vehicle-treated, or TFF2-treated all showed significantly higher total cell counts in BAL, compared with saline control mice (all $P < 0.01$, versus saline group). The numbers of eosinophils, neutrophils, lymphocytes, and monocytes were all significantly higher in the untreated, vehicle-treated, and TFF2-treated OVA-sensitized groups, compared with the saline control group. However, no differences were evident in inflammatory cell counts between the untreated, vehicle-treated, and TFF2-treated OVA-sensitized groups (Table 1).

Significantly higher numbers of inflammatory cells were present in hematoxylin and eosin-stained lung sections from untreated,

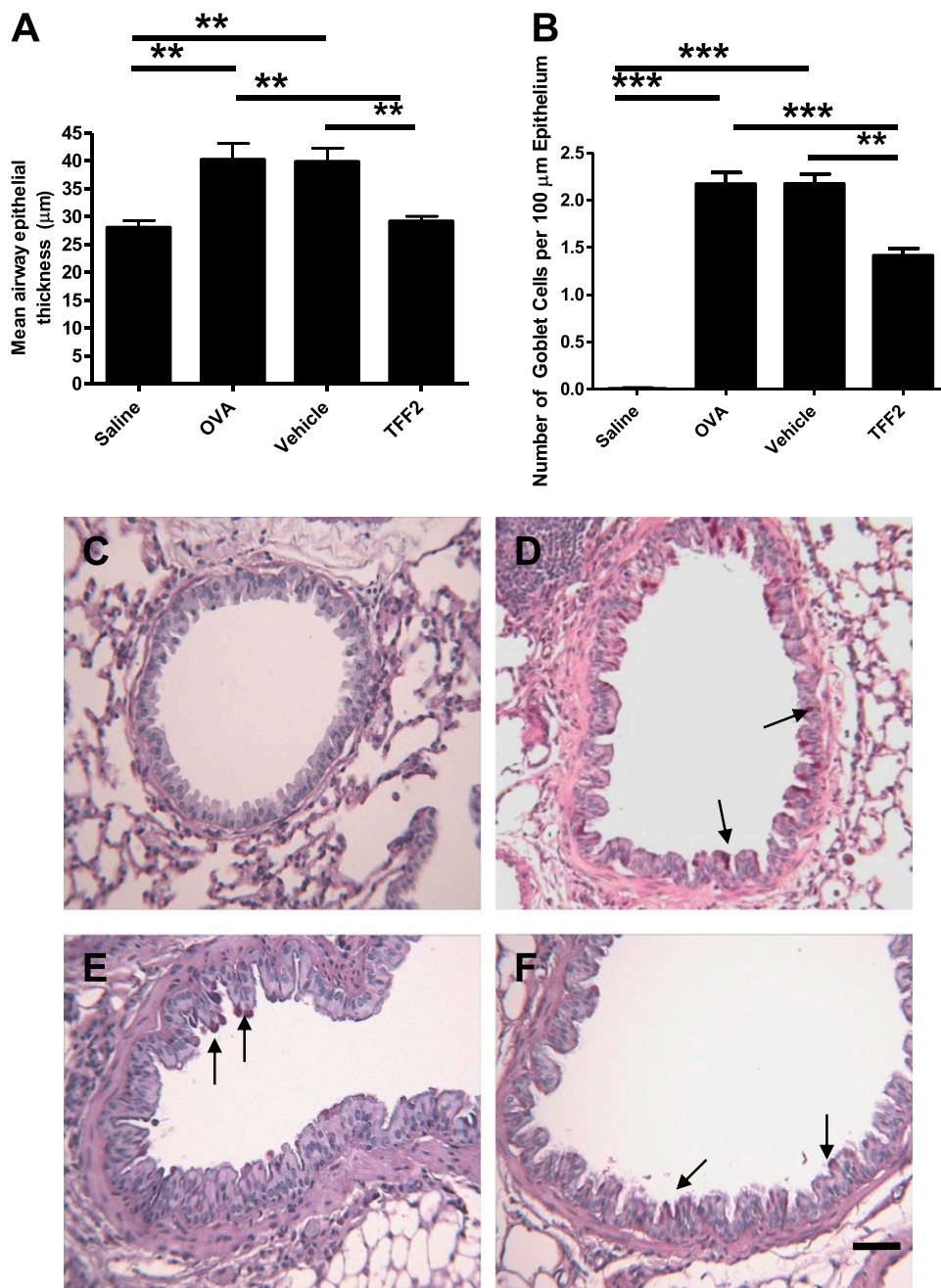


Figure 3. Effect of TFF2 intranasal treatment on airway epithelia in chronic AAD. (A) Airway epithelial thicknesses of Masson trichrome-stained sections from saline, OVA, vehicle-treated, and TFF2-treated mice were measured by morphometric image analysis and assessed for thickness of epithelia. $**P < 0.01$, between groups. (B–F) Sections from saline, OVA, vehicle, or TFF2 mice were stained with Alcian blue–periodic acid Schiff (AB-PAS) and assessed for goblet-cell numbers. Numbers in parentheses represent number of mice in each experimental group. $**P < 0.01$, $***P < 0.001$ versus the saline group. (C–F) Representative photomicrographs of AB-PAS-stained airways from saline (C), OVA (D), vehicle-treated (E), and TFF2-treated mice (F). OVA-sensitized challenged mice had increased goblet cell numbers (arrows), compared with saline mice. Scale bar = 50 μm.

vehicle-treated, and TFF2-treated OVA groups, compared with the saline control group ($P < 0.05$, $P < 0.01$, and $P < 0.05$ versus the saline group, respectively; Figure 2). Again, no significant difference was evident in the inflammation score between the untreated, vehicle-treated, and TFF2-treated OVA groups.

Epithelial thickening. Airway epithelial thickness was significantly elevated in the untreated and vehicle-treated OVA mice compared with the saline control mice, consistent with previous findings (both $P < 0.01$, versus saline group; Figure 3) (1, 4). However, TFF2-treated OVA mice had substantially reduced airway epithelial thickness, compared with vehicle-treated and untreated OVA mice ($P < 0.01$, versus both OVA and vehicle-treated groups; Figure 3), with TFF2 treatment reducing airway epithelial thickness to that observed in the saline control mice.

Goblet-cell metaplasia. Goblet-cell numbers were significantly elevated in the untreated, vehicle-treated, and TFF2-treated OVA

mice, compared with the saline control mice, consistent with previous findings (all $P < 0.05$, versus saline group) (1, 4). TFF2-treated OVA mice had substantially reduced goblet-cell numbers compared with vehicle-treated and untreated OVA mice ($P < 0.01$ and $P < 0.001$, versus OVA and vehicle-treated groups, respectively; Figure 3).

Subepithelial thickening. Airway subepithelial collagen thickness was significantly elevated in the untreated and vehicle-treated OVA mice compared with the saline mice, consistent with previous findings (both $P < 0.01$, versus the saline group; Figure 4) (1, 4). TFF2-treated OVA mice demonstrated significantly reduced subepithelial fibrosis compared with the vehicle-treated and untreated OVA mice ($P < 0.05$, versus both OVA and vehicle-treated groups; Figure 4). However, subepithelial collagen thickness in TFF2-treated mice remained significantly higher than in the saline control mice ($P < 0.05$, versus saline group).

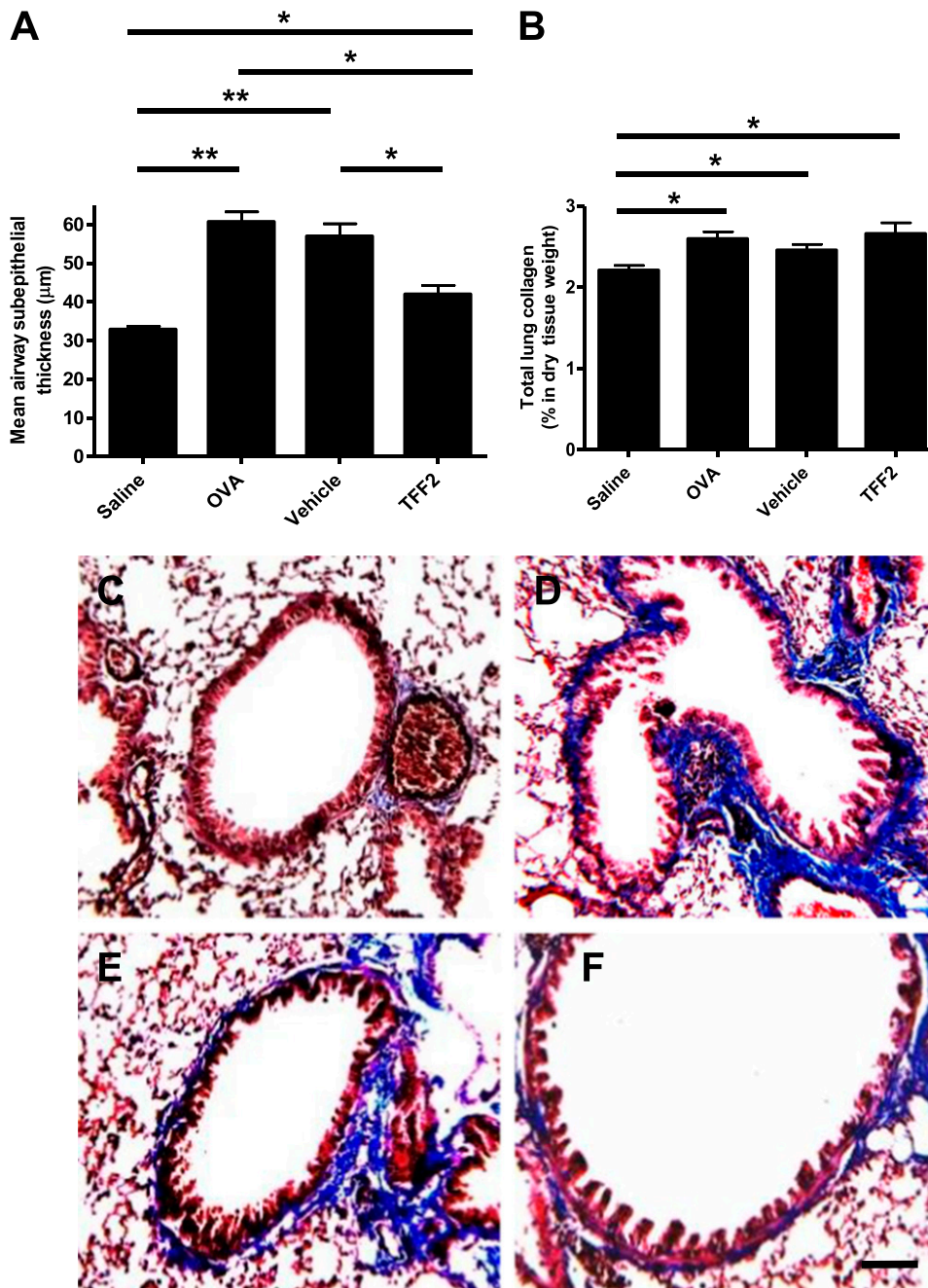


Figure 4. Effect of TFF2 intranasal treatment on subepithelial fibrosis. (A) Masson trichrome-stained airways from saline, OVA, vehicle-treated, or TFF2-treated mice were analyzed for subepithelial thickening attributable to collagen. * $P < 0.05$ and ** $P < 0.01$, between groups. (B) Effect of TFF2 intranasal treatment on total lung collagen content. Collagen from lung lobes of saline, OVA, vehicle-treated, and TFF2-treated mice was extracted and analyzed by hydroxyproline assay. Total collagen content was calculated from hydroxyproline, and expressed as a percentage of lung dry weight. * $P < 0.05$. Representative photomicrographs of Masson trichrome-stained airways from saline (C), OVA (D), vehicle-treated (E), and TFF2-treated (F) mice show effects of TFF2 treatment on epithelial and subepithelial thickness. TFF2-treated mice demonstrated a significant reduction in airway epithelial and subepithelial thickness, compared with OVA and vehicle-treated mice. Scale bar = 50 μm .

Total lung collagen estimation. Hydroxyproline estimation of total lung collagen content showed elevated collagen concentrations in untreated, vehicle-treated, and TFF2-treated OVA mice compared with saline control mice ($P < 0.05$), with no significant differences between the OVA mice that were untreated or treated with TFF2 or vehicle (Figure 4).

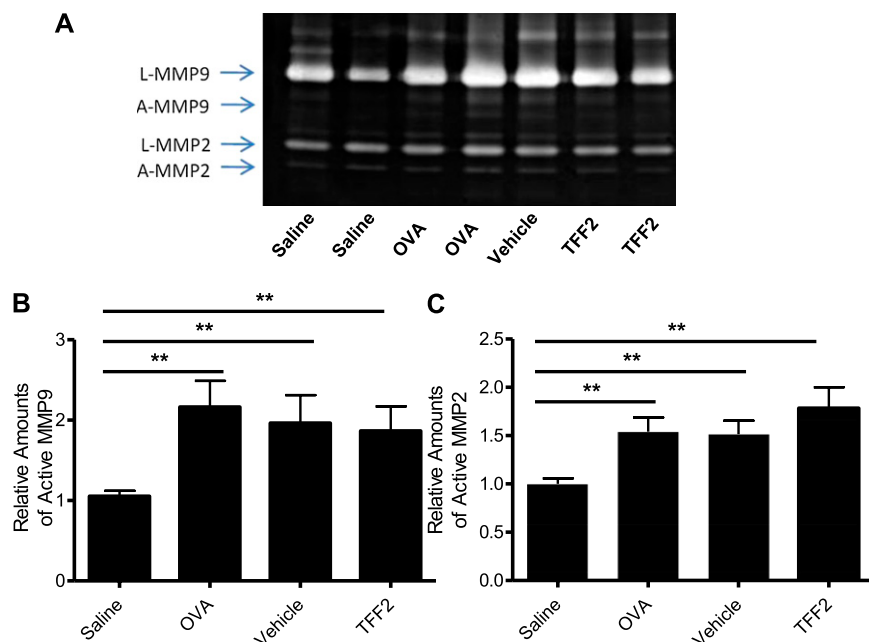
Active MMP concentrations in the lung. Gelatin zymography was performed on a portion of lung tissue. The resultant bands on the zymograph, indicative of active MMP-9 and MMP-2 concentrations, were analyzed by densitometry (Figure 5). Concentrations of active MMP-9 and MMP-2 were significantly increased in all treatment groups of OVA-sensitized mice, compared with saline control mice ($P < 0.01$). No significant differences in active MMP-9 and MMP-2 concentrations were seen between any of the treatment groups.

TGF- β 1 immunohistochemistry. Little constitutive staining for TGF- β 1 was observed in the saline mice (Figures 6A and 6B). In

untreated (Figure 6C), vehicle-treated (Figure 6D), and TFF2-treated (Figure 6E) OVA mice, staining for TGF- β 1 was markedly increased compared with saline control mice (all $P < 0.001$, versus saline group). Strong cytoplasmic staining was localized to bronchial epithelial cells, connective tissue cells of the lamina propria and adventitia, and smooth muscle cells (Figures 6C–6E).

α -SMA immunohistochemistry. No significant difference was evident in α -SMA staining between that found in saline mice (Figures 6F and 6G) and that found in untreated (Figure 6H), vehicle-treated (Figure 6I), and TFF2-treated (Figure 6J) OVA mice in the smooth muscle layer, as assessed by immunohistochemistry and morphometry. Thus, TFF2 treatment did not appear to affect smooth muscle thickness.

EGF-R immunohistochemistry. In untreated (Figures 6K and 6M) and vehicle-treated (Figure 6N) OVA mice, staining for EGF-R was markedly increased compared with saline control mice (Figure 6L) (both $P < 0.01$, versus saline group). However,



between treatment groups. The average density of each sample was expressed as the relative ratio to saline control samples. Data are presented as mean $\pm$ standard error. $**P < 0.01$, versus group. Scale bar = 50 μ m. $**P < 0.01$ and $***P < 0.001$, compared with saline mice.

no statistically significant difference was evident in EGF-R staining between TFF2-treated (Figures 6K and 6O) OVA mice and saline mice, or between TFF2-treated OVA mice and untreated or vehicle-treated OVA mice. Staining was localized mainly to the airway epithelial cells (Figures 6M–6O).

Annexin V immunofluorescence. Annexin V immunofluorescence staining was used to detect bronchial epithelium apoptosis in saline and OVA-induced AAD mice (Figure 7). Strong staining of annexin V was observed in the bronchial epithelia of mice treated with OVA (Figure 7B). On the other hand, annexin V staining was not observed in the bronchial epithelia of saline control mice (Figure 7A), and was minimal in mice treated with OVA-TFF2 (Figure 7C). Weak staining of annexin V was observed in the peribronchial inflammatory cells within all treatment groups, and weak background staining was evident in the subepithelial layer of OVA-TFF2 treated mice (Figure 7).

Airway hyperresponsiveness. Untreated, vehicle-treated, and TFF2-treated OVA mice showed significantly increased methacholine-induced AHR compared with saline control mice at the four highest doses of methacholine (Figure 8) (all $P < 0.05$, versus the saline group). However, airway resistance in TFF2-treated mice was significantly reduced compared with that in vehicle-treated and untreated OVA mice at the highest three doses of methacholine (at least $P < 0.05$, versus OVA and vehicle-treated groups).

DISCUSSION

In this study, we demonstrated that TFF2 expression was significantly increased in mice with chronic AAD, which is associated with airway inflammation, remodeling, and AHR. Moreover, exogenous TFF2 was effective at reversing the established remodeling changes of epithelial thickening, goblet-cell hyperplasia, subepithelial fibrosis, bronchial epithelial apoptosis, and AHR. These findings have important implications. First, the relative increase in TFF2 expression in the AAD-affected airway and lung suggests that TFF2 expression is increased in response to epithelial injury, and that this increased TFF2 expression may contribute to airway epithelial protection or repair in asthma. Nevertheless, the

Figure 5. Effects of TFF2 on active matrix metalloproteinase (MMP) concentrations in lungs of OVA-sensitized mice. (A) Representative image of MMP gelatin zymography. (B and C) Concentrations of MMP in a portion of lung were determined by gelatin zymography. MMP-9 and MMP-2 have a latent (L) (92 kD and 72 kD, respectively) and an active (A) form that can be visualized as separate bands. (B) Concentrations of active MMP-9 were significantly increased in all OVA-sensitized mice, compared with saline control mice ($P < 0.01$). No significant difference in active MMP-9 concentrations was evident between treatment groups. The average density of each sample was expressed as the relative ratio to saline control samples. Data are presented as mean $\pm$ standard error. $**P < 0.01$, versus saline group. (C) Effects of TFF2 treatment on active MMP-2 concentrations in lungs of OVA-sensitized mice. Concentrations of active MMP-2 were significantly increased in all OVA-sensitized mice, compared with saline control mice ($P < 0.01$). No significant difference in inactive MMP-2 concentrations was evident between treatment groups.

increased endogenous expression of TFF2 failed to prevent the progression of airway inflammation and epithelial remodeling changes in a murine model of AAD, suggesting that the host up-regulation of TFF2 is insufficient to prevent epithelial injuries caused by continued exposure to OVA, or else that other overriding proinflammatory and pro-remodeling mechanisms are also up-regulated in AAD. Third, exogenous TFF2 treatment was shown to reverse established airway epithelial thickening and goblet-cell hyperplasia, and remarkably to reverse airway fibrosis and bronchial epithelial apoptosis in murine AAD, which contributed to the ability of TFF2 to suppress AHR. This highlights the important role of TFF2 in promoting reparative epithelial processes in the airway and lung, along with the central role of epithelial damage/repair in the progression of airway remodeling in AAD. Importantly, these dramatic, beneficial effects of TFF2 treatment in our murine model of AAD indicate the exciting potential for exogenous TFF2 to be applied as an anti-remodeling therapy in human asthma.

TFF2 is a major peptide product of the gut epithelium, where it plays a reparative and cytoprotective role in maintaining local mucosal homeostasis. It promotes epithelial restitution (cell migration), and is antiapoptotic in the context of epithelial repair (31). A recent analysis of TFF2 function in the gut suggests that it exerts focal anti-inflammatory actions (31), and inhibits metastatic and neoplastic cell proliferation (32). Much of the published research on TFF2 concerns the gut, where the importance of epithelial repair in ulceration is well characterized, whereas the role of TFF2 in the lung was not, to the best of our knowledge, studied in detail previously. With attention now focused on the epithelial mesenchymal trophic unit (EMTU) and epithelial damage as a major etiology in asthma and airway remodeling (33), the potential roles of mediators in airway epithelial defense and remodeling have emerged as important areas for research.

Recent studies have suggested a role for TFF2 in human asthma. TFF2 has been shown to exert mitogenic actions on human bronchial epithelial cells, mediated via the extracellular signal-regulated kinases/p42/p44 (ERK1/2), p38 MAPK, and c-Jun N-terminal kinase (JNK) pathway (34). This may be consistent

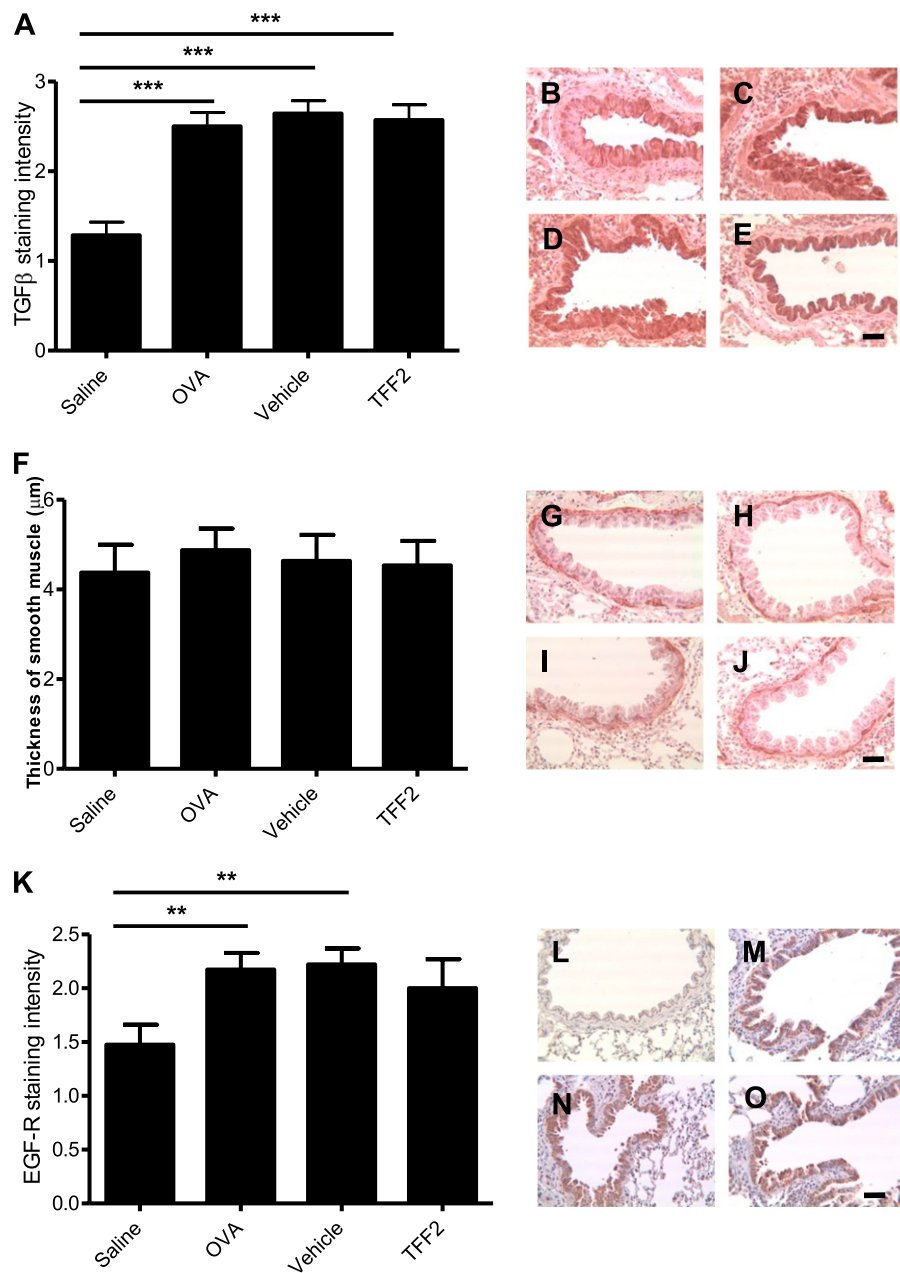


Figure 6. Immunohistochemical staining of transforming growth factor (TGF)-β1 (A–E), α-smooth muscle actin (SMA) (F–J), and epidermal growth factor receptor (EGF-R) (K–O). (B) Little constitutive staining for TGF-β1 was observed in saline mice. In OVA (C), vehicle-treated (D), and TFF2-treated OVA (E) mice, staining for TGFβ1 was markedly increased compared with saline control mice ($P < 0.001$). Strong cytoplasmic staining was localized to bronchial epithelial cells, connective tissue cells of the lamina propria and adventitia, and smooth muscle cells. (F–J) No significant difference was evident between saline mice, and untreated, vehicle-treated, and TFF2-treated OVA mice in smooth muscle thickness as assessed by α-SMA immunohistochemistry and morphometry. TFF2 treatment exerted no effect on smooth muscle thickness. (K–O) EGF-R immunohistochemistry. In untreated and vehicle-treated OVA mice, staining for EGF-R was markedly increased compared with saline control mice ($P < 0.01$). No statistically significant difference was evident between TFF2-treated OVA mice and saline mice, or between TFF2-treated OVA mice and untreated or vehicle-treated OVA mice. Staining was localized mainly to airway epithelial cells. Scale bar = 50 μm. $**P < 0.01$ and $***P < 0.001$, compared with saline mice.

with the repair function of TFF2 in the gastrointestinal tract, and may be of particular relevance in asthma, where epithelial injury and the EMTU play major roles in the progression of airway inflammation and remodeling (35). Furthermore, epithelial barrier function protects against allergen entry and therefore host immune responses to allergens (35). Kuperman and colleagues (11), in a functional genomic study, showed that TFF2 is strongly up-regulated in epithelial cells from patients with asthma compared with control subjects. This observation has been mirrored in three different murine AAD models (12, 36, 37). TFF2 expression was increased in a short-term murine OVA model (12), and the *Tff2* gene was one of only five genes to increase by twofold or greater in OVA, trimellitic anhydride, and aspergillus models of AAD (36, 37). Using antibodies that recognize TFF2 but not other trefoil factors, we have demonstrated the strong up-regulation of protein staining in lung epithelial cells from mice with chronic AAD, compared with control mice. Strong cytoplasmic staining was localized to occasional cells or groups of cells in the airway surface epithelium. This suggests an up-regulation of

endogenous TFF2 production in response to epithelial injuries caused by airway inflammation. Endogenous TFF2 likely acts to promote epithelial repair and inhibit airway inflammation locally, but the expression levels are insufficient to prevent the progression of airway inflammation as well as epithelial and other remodeling changes. Consistent with our present findings, Nikolaidis and colleagues reported that TFF2 was rapidly induced by allergen exposure (12). However, TFF2 deficiency did not alter tissue inflammation in an acute (short-term allergen exposure) murine model of AAD (15). Further research is required to elucidate the mechanisms limiting the ability for endogenous TFF2 to protect against epithelial injury, inflammation, and remodeling in asthma, and to establish how this peptide acts in the lung.

Our study contained the exciting finding that exogenous TFF2 dramatically reversed established epithelial thickening, goblet-cell hyperplasia, and subepithelial fibrosis in a murine model of AAD. In asthma, epithelial hyperplasia is believed to develop as a result of chronic inflammatory insult, mediated by the increased

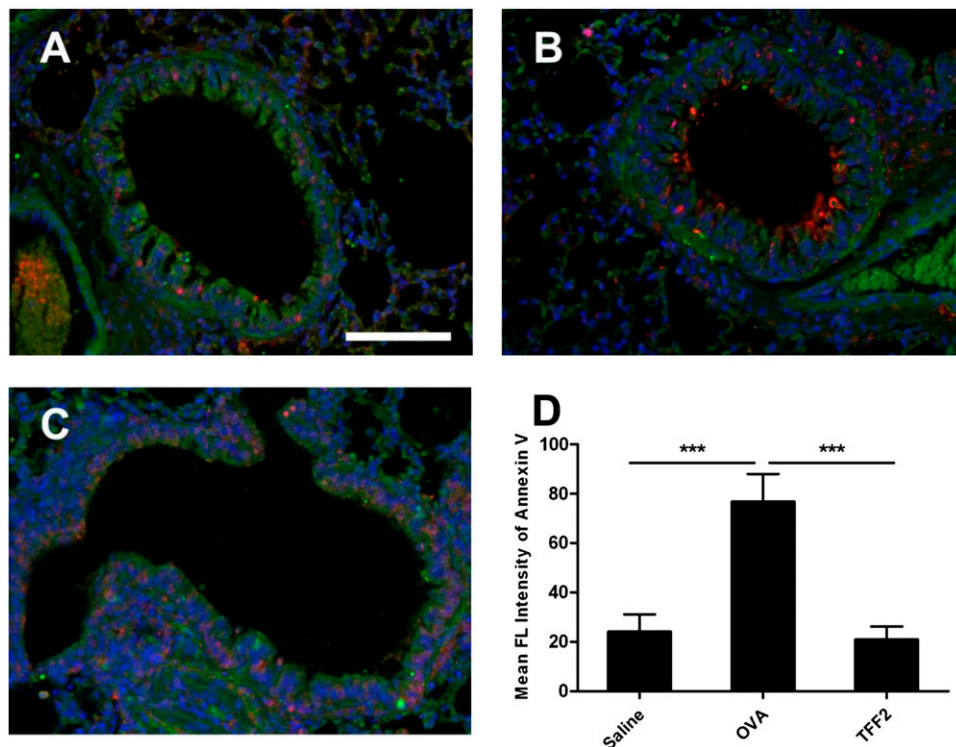


Figure 7. Immunofluorescence staining for annexin V, a membrane marker of apoptosis. Annexin V (red) is shown in representative murine tissue sections from saline control (A), OVA-PBS (B), and OVA-TFF2 (C) mice. Mouse monoclonal anti-histone deacetylase 8 (HDAC8; green) was used to highlight tissue structure. Strong staining of annexin V was found largely in bronchial epithelia among mice treated with OVA-vehicle, representing apoptotic effects in the chronic model of AAD. Annexin V staining was absent in the bronchial epithelia of mice treated with OVA-TFF2 and in saline control mice. Weak staining is observed in the subepithelial layer of OVA-TFF2 mice and in the peribronchial inflammatory cells of all treatment groups. *Merged image:* Nucleus stained with DAPI (54), annexin V (red), and HDAC8 (green). Images were acquired with an Olympus BX61 fluorescence microscope with an automated FV10 camera (Olympus, Center Valley, PA). Bar = 100 μ m. (D) Immunofluorescence analysis of annexin V was used to highlight statistically significant

strong staining in OVA-PBS mice, compared with OVA-TFF2 and saline control mice. Analysis was performed using Image J (Fiji). Values represent the mean $\pm$ SD ($n = 3$). Statistical analysis was performed using one-way ANOVA ($P < 0.001$). FL, fluorescence.

proliferative capacity or inhibition of apoptosis. The airway epithelium of asthma sufferers may present increased susceptibility to damage, and reduced epithelial integrity leads to shedding (33). Based on these observations and the known actions of TFF2 in promoting epithelial repair/restitution and inhibiting focal inflammation in the intestinal epithelium, our finding that exogenous TFF2 reduced airway epithelial thickening was not unexpected. TFF2 has also been shown to exert antiproliferative effects in the context of gastric cancer in mice (32), which may involve relevance to epithelial cell proliferation and remodeling in the airway (38). Interestingly, however, we did not identify increased epithelial cell proliferation in Ki-67-stained lung tissue sections from OVA-sensitized mice, because Ki-67 staining was similar in TFF2-treated, vehicle-treated, and untreated OVA mice (data not shown). This suggests that epithelial thickening in AAD does not relate to proliferation, and that the reduced epithelial thickness associated with TFF2 treatment was not the result of attenuated proliferation. Increased goblet-cell numbers in asthma and AAD are now recognized as attributable to metaplasia, rather than cell proliferation or hyperplasia (39). Goblet-cell metaplasia will result in epithelial thickening. Cells producing large quantities of mucin will exhibit an enlarged mucigen vacuole. The correlation between epithelial thickness and goblet-cell number has been established in human biopsies (40). Our morphometric evaluation of goblet cells in AB-PAS-stained sections was consistent with this. TFF2 has been shown to regulate epithelial repair via binding to EGF-R, and has been shown to increase EGF-R expression in cancer cells (41). EGF-R plays an important role in the regulation of epithelial proliferation, and EGF-R-deficient mice have reduced AHR and airway smooth muscle thickening in a model of AAD (42). EGF-R is also involved in the regulation of mucin production and goblet-cell metaplasia (43). In the current study, EGF-R was increased significantly in mice with AAD, but was unaltered by TFF2 treatment, suggesting that EGF-R was not

involved in the TFF2-mediated effects demonstrated in airway/epithelial remodeling.

Another important observation indicated that TFF2 treatment reversed subepithelial collagen deposition in chronic AAD (without necessarily affecting total collagen concentrations). No evidence demonstrates that TFF2 can directly regulate fibrosis or directly act on lung fibroblasts or profibrotic factors to inhibit fibrosis progression. We therefore believe that exogenous TFF2 may have indirectly influenced airway fibrosis via its protective effects in promoting epithelial repair. Damage to the airway epithelium is associated with the release of growth factors and cytokines from epithelial cells that contribute to the proliferation and differentiation of fibroblasts and the deposition of extracellular matrix in the subepithelial basement membrane region, whereas epithelial injury and dysfunction constitute an important driver of airway remodeling (33). Hence, TFF2 may indirectly prevent the progression of subepithelial fibrosis by limiting the release of secondary repair signals from the damaged epithelium. In the present study, we showed reduced bronchial epithelial apoptosis in mice treated with TFF2. Antiapoptosis is a known property of TFF2 (44), and is very important in AAD and asthma, where apoptosis is a feature of biopsies from asthma sufferers (45). Our findings strongly support the current paradigm of asthma pathogenesis, which suggests that initial epithelial injury or a primary defect of epithelial repair is the fundamental driver of chronic inflammation, in turn leading to the activation of secondary tissue repair processes and ensuing structural changes. In this paradigm, strategies that promote epithelial repair and restitution may be expected to limit epithelial injury, and limit the progression of airway remodeling. In the present study, we examined three important factors released from the epithelium that are known to modulate fibrosis. The expressions of TGF- β (a profibrotic factor) and of MMP-9 and MMP-2 (collagen-degrading enzymes) were unaltered by TFF2 treatment, suggesting that TFF2 acts directly on the epithelium, and that the decreased subepithelial fibrosis measured

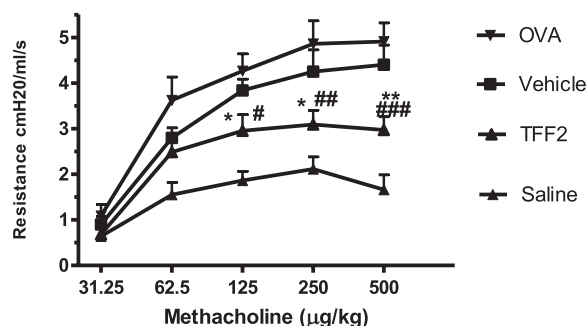


Figure 8. Effect of TFF2 intranasal treatment on airway hyperresponsiveness (AHR). Increasing concentration of B-methacholine was administered to TFF2-treated, TFF2 vehicle-treated, and saline mice by invasive plethysmography, and airway resistance (as a measure of AHR) was evaluated. * $P < 0.05$ and ** $P < 0.01$, compared with the vehicle-treated OVA group. ## $P < 0.01$ and ### $P < 0.001$, compared with the OVA-alone group.

in TFF2-treated mice resulted indirectly from the ability of TFF to reduce apoptosis, enhance healing, and repair the epithelium.

In the present study, the reduction in airway subepithelial thickness was not associated with a reduction in total lung collagen. According to one explanation for this, TFF2 is expected to exert actions locally within the airway mucosa, because TFF2 was shown to be expressed selectively by airway epithelial cells. Furthermore, TFF2 was administered to mice intranasally, and would therefore be expected to exert actions primarily in the large and medium airways. Because the majority of collagen and extracellular matrix is present within the distal lung parenchyma, we are not surprised that the local delivery of TFF2 into the airway lumen would not affect total lung collagen content.

Importantly, in the present study, TFF2 treatment suppressed AHR. We previously demonstrated a strong association between AHR and both epithelial thickness and subepithelial collagen thickness (1, 46, 47). Furthermore, we demonstrated that treatment with the anti-remodeling and antifibrotic hormone relaxin resulted in reduced epithelial and subepithelial thickness and the attenuation of AHR (21). Mathematical models have shown that increased thicknesses in each component of the airway wall, both internal and external to the smooth muscle layer, contribute to AHR (48–50), resulting in a cumulative impact of airway wall thickening on AHR. In the present study, the reduction in epithelial and subepithelial thickness achieved with TFF2 treatment translated to a suppression of AHR.

The goal of asthma therapy is to obtain long-term control of asthma symptoms, to prevent exacerbations, and to obtain the best possible lung function for patients (50). Corticosteroids exert limited efficacy in the prevention and reversal of airway remodeling (51). The present study provides evidence of a novel anti-remodeling therapy that can reverse established airway remodeling in AAD.

We have demonstrated that TFF2 protein expression was increased and more widely distributed in lung tissue from mice with chronic AAD, compared with control mice. However, this increased expression of TFF2 was insufficient to prevent the progression of airway remodeling. Importantly, we have shown for the first time, to the best of our knowledge, that intranasal TFF2 was able to reverse established airway remodeling changes of epithelial remodeling and subepithelial fibrosis in an experimental model of AAD (when repeatedly applied over a 2-week period). These results provide strong evidence for an important protective role of exogenous TFF2 in airway epithelial homeostasis and in the prevention or reversal of airway fibrosis in a murine model of chronic AAD. Other investigators have

suggested that trefoil peptides could be used in short-term therapeutic interventions for acute lung injury and acute respiratory distress syndrome (52). A TFF2 therapy for asthma could potentially slow disease progression, particularly if used as an adjunct therapy in conjunction with inhaled corticosteroids. Further pre-clinical studies evaluating the dosage, delivery, and potential benefits of TFF2 therapy are warranted.

Author disclosures are available with the text of this article at www.atsjournals.org.

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