

PATHOPHYSIOLOGY

Trefoil Factor 2 Regulates Airway Remodeling in Animal Models of Asthma

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Background. Epithelial denudation and metaplasia are important in the pathogenesis of airway remodeling and asthma. Trefoil factor 2 (TFF2) is a member of a family of peptides involved in protection and healing of the gastrointestinal epithelium but which are also secreted in the airway mucosa. **Methods.** We investigated the role of TFF2 in airway remodeling by histological and morphometric analysis of lung tissue from TFF2-deficient mice subjected to two relevant animal models of asthma: an ovalbumin model of allergic airways disease and an *Aspergillus fumigatus* antigen sensitization model. **Results.** In the ovalbumin model TFF2-deficient mice had increased goblet cell hyperplasia, but not epithelial thickening compared to wild-type (WT) counterparts. In the *Aspergillus* model TFF2-deficient mice also had increased goblet cell hyperplasia, and epithelial thickness was also increased in the *Aspergillus*-sensitized mice compared to WT controls. TFF2 deficiency was also associated with increased subepithelial collagen layer thickness. **Discussion.** The current study demonstrates a role of TFF2 in airway remodeling in mouse models of airway disease. Further studies into the mechanisms of action of TFF2 and its role in asthma are warranted.

Keywords trefoil factor 2, asthma, airway remodeling, allergic airways disease, epithelium

INTRODUCTION

Asthma affects more than 300 million people in the world. The economic costs of asthma are one of the highest of all chronic diseases. These costs are predominantly directed to the care of children and adults with severe asthma who have ongoing symptoms despite maximal therapy with available anti-inflammatory agents (1). Ongoing symptoms are thought to reflect persistent airway hyperresponsiveness (AHR) and progressive airway remodeling which is thought to play a major role in corticosteroid refractory asthma (1).

Array and genome-wide studies have identified numerous candidate genes for asthma (2). Of particular interest are those genes which may have a role in asthma independent of airway inflammation. A genome-wide linkage study in mice (3,4) demonstrated an association between a small number of genes and the common clinical endpoint in asthma, AHR. These genes included trefoil factor 2 (TFF2) and relaxin. We have previously identified an important role for relaxin in mouse lung structure and airway remodeling in allergic airways disease (AAD) (5). TFF2 has also been postulated as important in the lung and had already become well established as a protective molecule in the gut (6). Since their identification, relaxin and TFF2 have been the subject of further

genetic and functional analyses. Polymorphisms have been identified which lead to altered protein structure (4). Both proteins are involved in processes fundamental to airway remodeling and maintain normal airway structure.

The critical factor underlying the initiation of airway remodeling in asthma is a fundamental defect of epithelial repair. Interactions between injured epithelium and underlying mesenchymal elements, comprising the epithelial mesenchymal trophic unit (EMTU), form the nexus for downstream events in the airway remodeling process (7).

TFF2 is well established as a protective molecule in the gut (6). It is produced by intestinal epithelium and promotes epithelial repair by mediating epithelial restitution (cell migration) and inhibiting apoptosis (8). Recent studies have suggested a similar protective role for TFF2 in lung epithelium (4). It is therefore plausible that TFF2 plays a key role in the initiation and/or progression of airway remodeling in asthma.

In addition to the studies linking TFF2 with lung function in the mouse, other groups have suggested a role for TFF2 in human asthma. Kuperman et al. (2) in a comprehensive functional genomic study made particular reference to TFF2 as the third highest out of 11 genes increased in epithelial cells from asthma sufferers versus controls. In addition they listed TFF2 as highly increased by both array and PCR techniques in three murine AAD models. Another study in a short-term ovalbumin-induced AAD (OVA) mouse model showed TFF2 to be increased (9) and Greene et al. (10) and Zimmermann et al. (11) showed TFF2 to be one of only five genes to have

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a twofold or greater increase in expression with OVA, trimellitic anhydride and *Aspergillus* models (10,11).

Nikolaidis and colleagues (9) have investigated TFF2 expression in OVA and *Aspergillus* models of AAD and found TFF2 to be allergen induced and differentially regulated by Th2 cytokines and signal transducer and activator of transcription 6 (STAT6). In a later study the OVA and *Aspergillus* models were applied to TFF2-deficient mice. TFF2 did not affect induction and resolution of mucous cell metaplasia, mucus levels, and tissue inflammation in this study (9).

In the current study we investigated the role of TFF2 in airway remodeling by histological and morphometric analysis of lung tissue from TFF2-deficient mice subjected to an OVA model of AAD and an *Aspergillus fumigatus* antigen sensitization model.

METHODS

TFF2-KO Mice Lung Tissue

Tissue blocks of wild-type (WT) saline mice, WT *Aspergillus*-sensitized mice, TFF2-deficient saline mice, and TFF2-deficient *Aspergillus*-sensitized mice were obtained from Professor Marc E. Rothenberg and Dr Simon Hogan (Department of Paediatrics, University of Cincinnati College of Medicine, OH, USA). TFF2-deficient mice were generated and backcrossed onto a *Balb/c* background for four generations (TFF2 knockout (KO)) (12). An OVA AAD model of asthma was previously induced in these mice (12). Briefly, mice were sensitized with two intraperitoneal injections of 50 mg OVA and 1.0 mg alum in 200 mL saline on days 0 and 14, followed by two intranasal challenges with 50 μ L of 50 μ g OVA on days 28 and 31.

The *Aspergillus fumigatus* antigen model of allergic lung disease was induced as previously described (12). Briefly, mice were lightly anesthetized by methoxyflurane inhalation, held in the supine position during administration of 100 μ g *A. fumigatus* in 50 μ L of saline into the nares, and were held upright until consciousness was regained. Saline control mice were administered with 50 μ L saline. After three treatments per week for 3 weeks, mice were killed between 18 and 20 hours after the last intranasal challenge.

Lung Histopathology

The right lung lobes were fixed in formalin, embedded in paraffin, and routinely processed. Sections were stained with Masson trichrome for assessment of epithelial and subepithelial collagen thickness, and Alcian blue-periodic acid Schiff (AB-PAS) for assessment of goblet cells (5,13,14).

Morphometric Analysis of Structural Changes

Morphometric evaluation of lung tissue sections was determined as described previously (13–15). Images of lung tissue sections were captured using a digital camera (Q Imaging, Burnaby, BC, Canada). A minimum of five

bronchi measuring 150–350 μ m luminal diameter were analyzed per mouse for the parameters described below using Image Pro-Discovery software (Media Cybernetics, Silver Spring, MD, USA), which was calibrated with a reference micrometer slide. The thickness of the bronchial epithelial layer was measured by tracing around the basement membrane and the luminal surface of epithelial cells and calculating the area between these lines, using a digitizer (Aiptek, Irvine, CA, USA). Total collagen thickness was similarly measured by tracing around the outer extent of the total collagen layer in the submucosal region and around the basement membrane and the area between these lines calculated. Total areas were calculated by subtracting the inner area from the outer area. These areas were expressed per length (μ m) of basement membrane to account for variation in bronchial diameters. Goblet cells were counted in AB-PAS-stained sections and expressed as number of cells per 100 μ m of basement membrane.

Statistical Analysis

Morphometry was expressed as median with 95% confidence interval and analyzed using the Mann–Whitney test.

RESULTS

Role of Endogenous TFF2 on airway remodeling (AWR) in the OVA AAD Model and the Aspergillus Model

To determine the role of endogenous TFF2 on airway remodeling changes in asthma, mouse lung sections of TFF2-deficient mice were stained with AB-PAS and Masson trichrome stains and analyzed in comparison to their WT and saline controls.

Effect on Epithelial Thickening in the OVA AAD Model

The OVA AAD model did not cause significant thickening of airway epithelium; there was no apparent epithelial thickening between OVA and saline mice. There was also no significant difference in epithelial thickness between WT and TFF2-deficient mice (Figure 1).

Endogenous TFF2 Attenuates Goblet Cell Hyperplasia in the OVA AAD Model

AB-PAS staining of sections from WT saline and WT mice given the ovalbumin-induced AAD (OVA) and TFF2-deficient saline and TFF2-deficient OVA mice was performed and mucin-positive cells enumerated. The number of goblet cells in the airways of WT OVA mice was significantly increased ($p < .001$, Figure 2) as compared to WT saline mice. TFF2-deficient OVA mice also had significantly more goblet cells in their airways as compared to TFF2-deficient saline mice ($p < .001$, Figure 2). Interestingly, goblet cell number in the airways of TFF2-deficient OVA mice was significantly increased ($p = .0036$, Figure 2) in comparison to WT OVA mice.

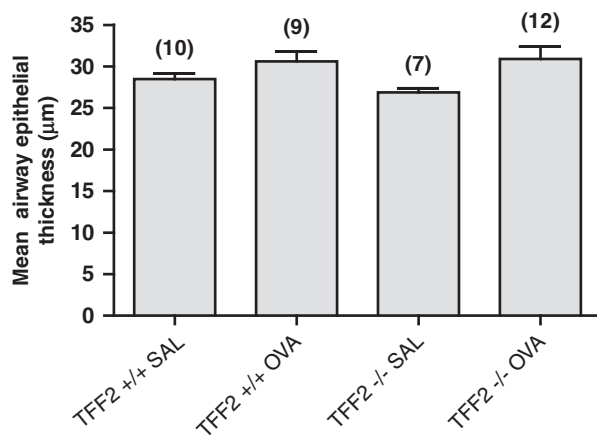


FIGURE 1.—Effect of trefoil factor 2 (TFF2) deficiency on epithelial thickening in ovalbumin-induced allergic airways disease (OVA AAD). Masson trichrome-stained airways from saline-treated and OVA-treated wild-type and TFF2-deficient mice were analyzed by morphometric image analysis. Epithelial thickness of four to five airways per mouse were measured and expressed as mean epithelial thickness. Numbers in parentheses represent number of mice. Errors bars are $\pm$ SEM.

Endogenous TFF2 Plays a Role in Reducing Subepithelial Fibrosis in the OVA AAD Model

Image analysis of Masson trichrome-stained sections showed that WT OVA mice had significantly more subepithelial thickening as compared to WT saline mice ($p < .001$, Figure 3), which was expected of an AAD model. However, TFF2-deficient OVA mice had no apparent increase in subepithelial thickening as compared to TFF2-deficient saline mice (Figures 4 and 5). Interestingly, TFF2-deficient saline mice had significantly increased subepithelial thickening as compared to WT saline mice ($p = .0001$, Figure 3) and had similar airway subepithelial thickness to WT OVA mice ($p = .5360$, Figure 3). TFF2-deficient OVA mice were also found to have no significant difference in subepithelial thickening as compared to OVA mice ($p = .9151$).

Effect on Epithelial Thickening in the Aspergillus AAD Model

Saline-sensitized TFF2-deficient mice show no increase in epithelial thickness when compared to saline-sensitized WT mice (Figure 4). *Aspergillus*-sensitized TFF2-deficient mice showed a marked increase in epithelial thickness ($p < .05$) when compared to saline-sensitized TFF2-deficient mice. An increase in epithelial thickness was seen in *Aspergillus*-sensitized TFF2-deficient mice when compared to *Aspergillus*-sensitized WT mice ($p < .05$).

Effect of Endogenous TFF2 on Goblet Cell Hyperplasia in the Aspergillus AAD Model

Saline-sensitized TFF2-deficient mice showed no increase in goblet cell numbers when compared to saline-sensitized WT mice (Figure 5). *Aspergillus*-sensitized TFF2-deficient mice showed a marked increase in goblet

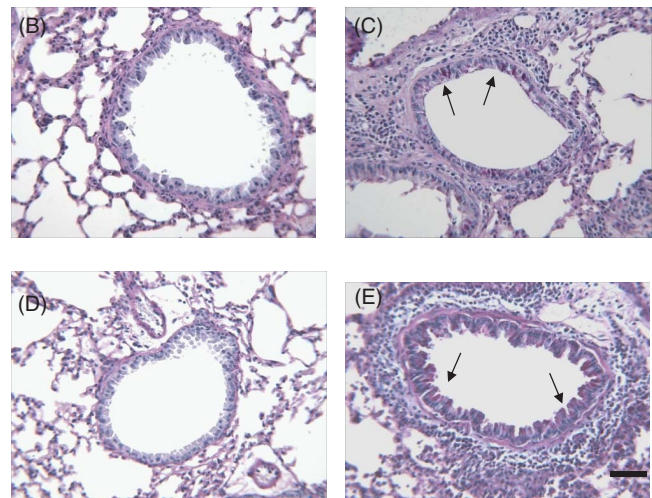
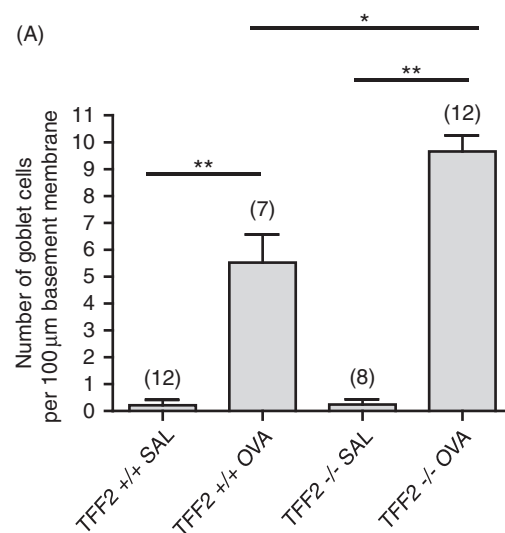


FIGURE 2.—Effect of trefoil factor 2 (TFF2) deficiency on goblet cell metaplasia in ovalbumin-induced allergic airways disease (OVA AAD). (A) Saline-treated and OVA-treated wild-type and TFF2-deficient mouse sections were stained with Alcian blue-periodic acid Schiff (AB-PAS) and assessed for number of goblet cells per 100 μ m basement membrane. Numbers in parentheses represent number of mice in each group. Error bars are $\pm$ SEM. ** $p < .001$ when compared to saline-treated wild-type mice, ** $p < .001$ when compared to saline-treated TFF2-deficient mice; and * $p = .0036$ when compared to OVA-treated wild-type mice. Representative photomicrographs of AB-PAS stained airways in OVA AAD from (B) saline-treated wild-type, (C) OVA-treated wild-type, (D) saline-treated TFF2-deficient, and (E) OVA-treated TFF2-deficient mice showing effect of TFF2 deficiency on goblet cell numbers (indicated by arrows) in OVA AAD. Scale bar = 100 μ m.

cell numbers ($p < .05$) when compared to saline-sensitized TFF2-deficient mice. An increase in goblet cell number was seen in *Aspergillus*-sensitized TFF2-deficient mice when compared to *Aspergillus*-sensitized WT mice ($p < .05$).

Endogenous TFF2 Plays a Role in Reducing Subepithelial Fibrosis in the Aspergillus AAD Model

Saline-sensitized TFF2-deficient mice showed a significant increase in subepithelial thickness when compared to saline-sensitized WT mice ($p < .05$) (Figure 6).

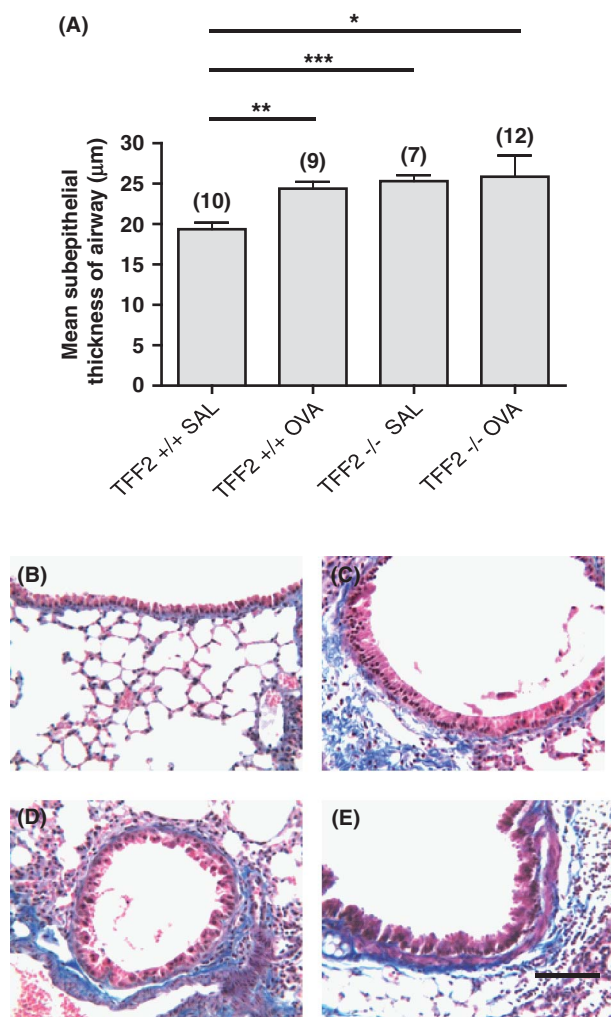


FIGURE 3.—Effect of trefoil factor 2 (TFF2) deficiency on subepithelial fibrosis in Alcian blue-periodic acid Schiff (OVA AAD). (A) Sections from wild-type and TFF2-deficient mice were stained with Masson trichrome and analyzed for collagen deposition. Subepithelial thickness of four to five airways were measured by morphometric image analysis and expressed as mean thickness per airway. Numbers in parentheses represent numbers of mice. Error bars are $\pm$ SEM. * p < .05 as compared to saline-treated wild-type mice. ** p < .001 as compared to saline-treated wild-type mice. *** p = .0001 as compared to saline-treated wild-type mice. Representative photomicrographs of Masson trichrome-stained airways in OVA AAD (B) saline-treated wild-type mice, (C) OVA-treated wild-type mice, (D) saline-treated TFF2-deficient mice, and (E) OVA-treated TFF2-deficient mice airways showing effects of TFF2 deficiency on airway subepithelial thickness. The subepithelial collagen-stained region of saline-treated TFF2-deficient mice was thickened as compared to its wild-type control. That thickness was no different from its OVA-sensitized counterparts. Scale bar = 300 μ m.

Aspergillus-sensitized TFF2-deficient mice showed no significant increase in subepithelial thickness (p = .07) when compared to saline-sensitized TFF2-deficient mice. An increase in subepithelial thickness was seen in *Aspergillus*-sensitized TFF2-deficient mice when compared to *Aspergillus*-sensitized WT mice (p < .05).

DISCUSSION

In the current study, we investigated the role of TFF2 in the development of airway remodeling in asthma by

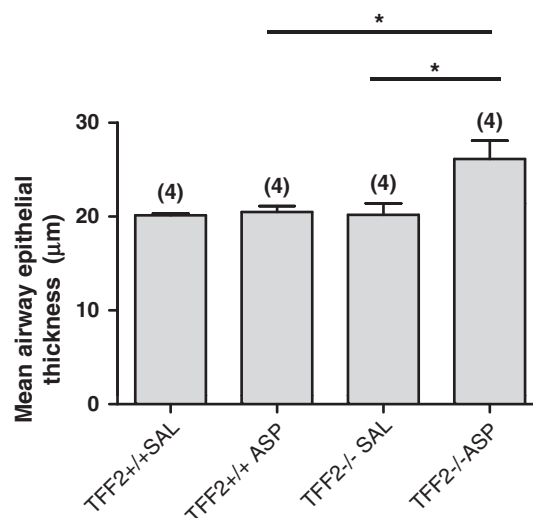


FIGURE 4.—Airway epithelium thickness of *Aspergillus*-treated mice. Lung sections were stained with Masson trichrome for assessment of epithelial thickness. The epithelial thickness of *Aspergillus*-treated trefoil factor 2 (TFF2)-deficient mice were significantly increased when compared to saline-treated controls (p < .05). Data are shown as mean $\pm$ SE. * p < .05 compared to saline-treated TFF2-deficient mice; * p < .05 compared to *Aspergillus*-treated wild-type mice.

histochemistry and morphometric analysis of lung tissue from TFF2-deficient and WT mice subjected to OVA- and *Aspergillus*-induced models of AAD. We identified an important role for TFF2 in the progression of airway remodeling changes – specifically, endogenous TFF2 was shown to attenuate changes in epithelial structure, goblet cell metaplasia, and subepithelial collagen deposition associated with allergic airway inflammation. Furthermore, we confirmed a critical homeostatic role for TFF2 in maintaining normal airway structure in the healthy state. Absence of endogenous TFF2 resulted in increased deposition of subepithelial collagen, presumed to have occurred as a result of chronic epithelial injury/impaired epithelial integrity related to defective epithelial repair.

In WT mice, the OVA and *Aspergillus* AAD models induced differential effects on airway remodeling. The OVA model induced goblet cell metaplasia and subepithelial collagen deposition but did not alter epithelial thickness, while the *Aspergillus* model resulted in epithelial thickening without goblet cell metaplasia or subepithelial collagen deposition, indicating that the OVA AAD model induces a more aggressive allergic inflammatory response in the airways. These differences are not surprising since these models of experimental asthma have been shown to induce distinct gene signatures by transcript expression profiling, with 44% and 37% of genes induced by the OVA and *A. fumigatus* models, respectively, being unique to each model (16). Despite these differences, the absence of endogenous TFF2 in the setting of these AAD models revealed a consistent role for TFF2 in limiting the extent of airway remodeling changes associated with allergic inflammation. Both models identified a role for TFF2 in protecting against goblet cell metaplasia while at least one of the two models revealed a role for TFF2 in

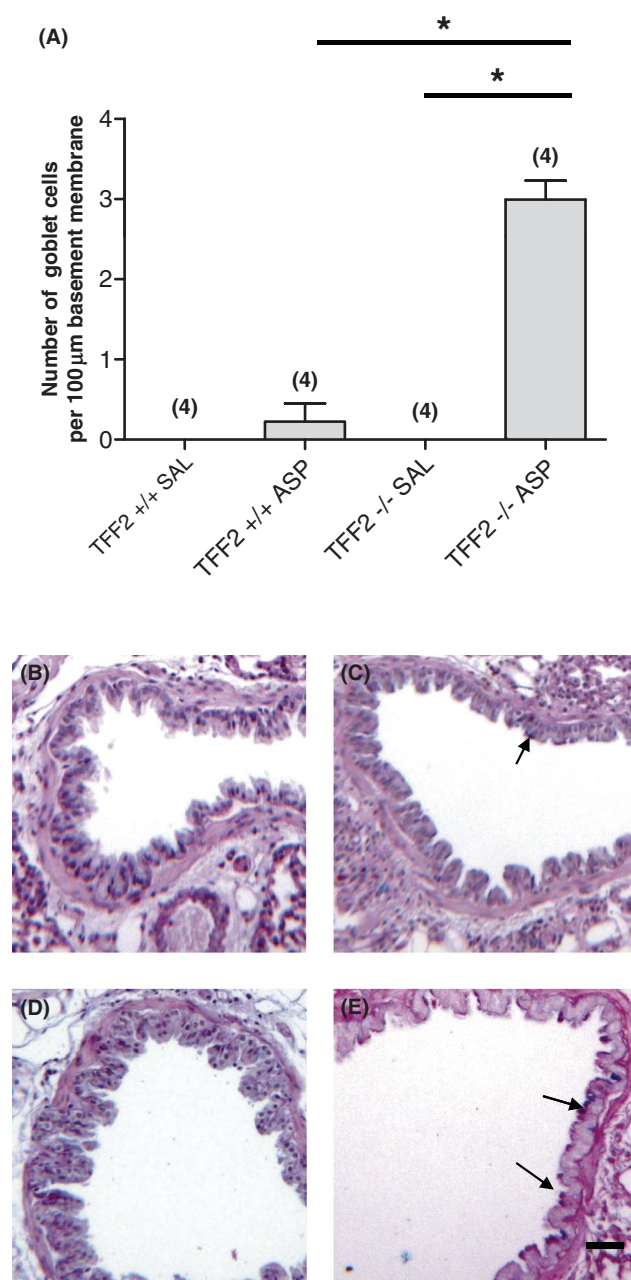


FIGURE 5.—Goblet cell metaplasia in *Aspergillus*-treated mice. (A) Lung sections were stained with Alcian blue-periodic acid Schiff (AB-PAS) for assessment of goblet cell metaplasia. A significant increase in goblet cell number in *Aspergillus*-treated trefoil factor 2 (TFF2)-deficient mice was seen when compared to saline-treated TFF2-deficient controls ($p < .05$). * $p < .05$ compared to saline-treated TFF2-deficient mice; * $p < .05$ compared to *Aspergillus*-treated wild-type. Representative photomicrographs of AB-PAS-stained sections from the *Aspergillus* model (B) saline-treated wild-type mice, (C) *Aspergillus*-treated wild-type mice, (D) saline-treated TFF2-deficient mice, and (E) *Aspergillus*-treated TFF2-deficient mice. Goblet cell numbers were increased in *Aspergillus*-treated TFF2-deficient mice (arrows). No goblet cells were seen in other groups. Scale bar = 300 μm.

protecting against epithelial thickening and fibrosis. An interesting observation was that absence of endogenous TFF2 resulted in exaggerated inflammation-induced subepithelial fibrosis in the *Aspergillus* but not in the OVA AAD model. This may reflect the fact that OVA

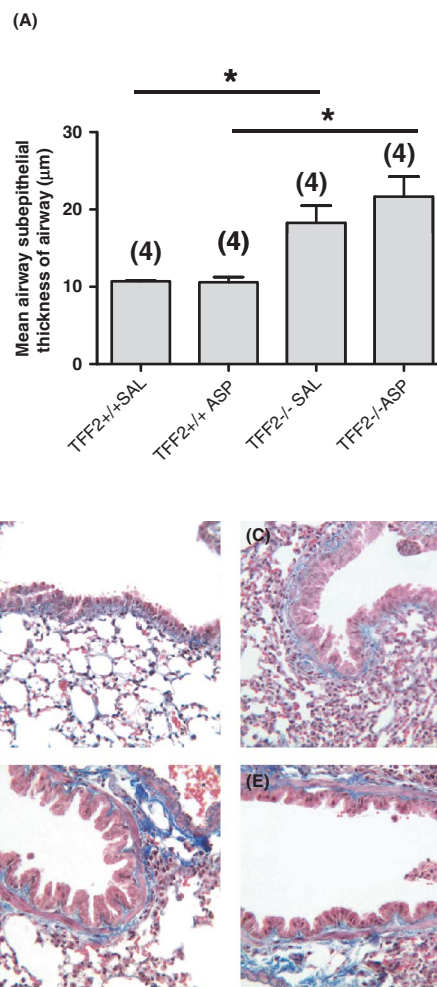


FIGURE 6.—Airway subepithelium thickness of *Aspergillus*-treated mice. (A) Lung sections were stained with Masson trichrome for assessment of subepithelial collagen thickness. The subepithelial thickness of *Aspergillus*- and saline-treated trefoil factor 2 (TFF2)-deficient mice was significantly increased when compared to wild-type controls ($p < .05$). There was a trend toward increased subepithelial thickness in *Aspergillus*-treated TFF2-deficient mice when compared to saline-treated TFF2-deficient controls ($p = .07$). * $p < .05$ to saline-treated wild-type; * $p < .05$ to *Aspergillus*-treated wild-type. Representative photomicrographs of Masson trichrome-stained *Aspergillus*-sensitized mice (B) saline-treated wild-type mice, (C) *Aspergillus*-treated wild-type mice, (D) saline-treated TFF2-deficient mice, and (E) *Aspergillus*-treated TFF2-deficient mice. Epithelial thickness was increased in *Aspergillus*-sensitized TFF2-deficient mice. Saline- and *Aspergillus*-sensitized TFF2-deficient mice had an increase in subepithelial thickness. Scale bar = 300 μm.

treatment induced significant fibrosis in the WT mice whereas *Aspergillus* treatment did not; therefore, any further increases in fibrosis associated with absence of TFF2 in the setting of allergic inflammation may have been masked in the OVA model, but readily observed in the *Aspergillus* mice. The mechanisms by which TFF2 might protect against epithelial thickening and goblet cell metaplasia require further elucidation. We performed Ki-67 staining of lung tissue sections and did not identify increased epithelial cell proliferation with AAD (data not shown) which suggests that epithelial remodeling changes do not relate to increased proliferation. This is consistent with a recent study (17).

Importantly, our findings delineate a direct relationship between epithelial injury and subepithelial collagen deposition. This would support the current paradigm of airway remodeling in asthma, which suggests that increased deposition of subepithelial collagen occurs as a common downstream consequence of chronic epithelial injury and release of secondary repair signals to underlying mesenchymal fibroblasts. Dysregulated secretion of growth factors and cytokines (particularly TGF- β) from injured epithelium is suggested to lead to reactivation of the EMTU (18,19), and to drive remodeling changes of fibrosis, angiogenesis, and smooth muscle hypertrophy. TGF- β is a potent inducer of fibrosis in this process, acting to inhibit synthesis and activity of matrix metalloproteinases (20).

In a previous study, TFF2 deficiency did not influence goblet cell numbers in these experimental AAD models, where goblet cell numbers were expressed as a percentage of total epithelial cells (12). However, that approach would not have identified an increased number of goblet cells if the number of epithelial cells and goblet cells increased in parallel. We assessed the absolute number of goblet cells per length of basement membrane and found that absence of TFF2 was associated with increased goblet cell metaplasia in the setting of airway inflammation in both models of AAD. This confirms a role for TFF2 in preventing airway remodeling associated with airway inflammation. Taken together, our findings indicate that TFF2 is not involved in the generation of allergic airway inflammation, but has an important role in promoting epithelial repair and restitution during the inflammatory process, which translates to a central role in protecting against downstream remodeling events driven by epithelial injury.

Importantly, in this study, we showed for the first time that endogenous TFF2 can attenuate subepithelial collagen deposition in the healthy state. Remarkably, the absence of endogenous TFF2 was associated with significantly increased subepithelial fibrosis in saline-treated TFF2-deficient mice in both AAD models. These findings suggest that under normal conditions, TFF2 can modulate subepithelial collagen deposition, either directly or indirectly through its effects on epithelial repair. As the first line of defense at the host–environment interface, the airway epithelium is continuously challenged by low-grade inflammatory signals from diverse stimuli including non-pathogenic microbial flora. Therefore, efficient repair/restitution processes are likely to play a crucial role in regulating and maintaining normal epithelial structure and function. Continuous low-grade inflammatory signals in the absence of TFF2 would result in chronic epithelial insult, which in turn leads to secondary repair signals that drive subepithelial collagen deposition.

In summary, this study has demonstrated that TFF2 (1) plays a vital homeostatic role in the regulation of first line defense at the host–environment interface within the lung and in maintenance of normal airway epithelial structure and function in this setting; (2) plays

an important role in protection against airway epithelial injury following an acute allergic inflammatory insult and thereby limits unwanted downstream remodeling changes within the airway wall; and (3) can attenuate airway collagen deposition and remodeling associated with allergic inflammation, supporting the link between epithelial damage and remodeling changes in AAD. The TFF2-deficient mouse offers a novel model in which to delineate EMTU interactions and the relationship between the epithelium and structural changes within the airway wall. Further studies investigating the application of TFF2 as a novel therapy that targets airway remodeling in asthma would be warranted.

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DECLARATION OF INTEREST

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