

Mechanistic Insights into the Contribution of Epithelial Damage to Airway Remodeling

Novel Therapeutic Targets for Asthma

Simon G. Royce^{1,2}, Xuelei Li¹, Stephanie Tortorella¹, Liana Goodings², Bryna S. M. Chow^{3,4}, Andrew S. Giraud¹, Mimi L. K. Tang^{*1,5}, and Chrisan S. Samuel^{*2,3,4}

¹Department of Allergy and Immune Disorders, Murdoch Children's Research Institute, Melbourne, Victoria, Australia; ²Department of Pharmacology, Monash University, Melbourne, Victoria, Australia; ³Florey Neuroscience Institutes and ⁴Department of Biochemistry and Molecular Biology, University of Melbourne, Melbourne, Victoria, Australia; and ⁵Department of Paediatrics, Royal Children's Hospital, The University of Melbourne, Melbourne, Victoria, Australia

Abstract

It has been suggested that an inherent airway epithelial repair defect is the root cause of airway remodeling in asthma. However, the relationship between airway epithelial injury and repair, airway remodeling, and airway hyperresponsiveness (AHR) has not been directly examined. We investigated the contribution of epithelial damage and repair to the development of airway remodeling and AHR using a validated naphthalene (NA)-induced murine model of airway injury. In addition, we examined the endogenous versus exogenous role of the epithelial repair peptide trefoil factor 2 (TFF2) in disease pathogenesis. A single dose of NA (200 mg/kg in 10 ml/kg body weight corn oil [CO] vehicle, intraperitoneally) was administered to mice. Control mice were treated with CO (10 ml/kg body weight, intraperitoneally). At 12, 24, 48, and 72 hours after NA or CO injection, AHR and various measures of airway remodeling were examined by

invasive plethysmography and morphometric analyses, respectively. TFF2-deficient mice and intranasal treatment were used to examine the role of the epithelial repair peptide. NA treatment induced denudation and apoptosis of airway epithelial cells, goblet cell metaplasia, elevated AHR, and increased levels of endogenous TFF2. Airway epithelial changes peaked at 12 hours after NA treatment, whereas airway remodeling changes were observed from 48 hours. TFF2 was protective against epithelial damage and induced remodeling and was found to mediate organ protection via a platelet-derived growth factor-associated mechanism. Our findings directly demonstrate the contribution of epithelial damage to airway remodeling and AHR and suggest that preventing airway epithelial damage and promoting epithelial repair may have therapeutic implications for asthma treatment.

Keywords: trefoil factor 2; asthma; airway remodeling; naphthalene; epithelium

The primary role of the superficial airway epithelium is to provide a defensive barrier between the environment and the body. It plays an important part in a number of respiratory diseases, including asthma, where epithelial susceptibility to damage has been proposed as an important etiological factor (1). Large genome-wide association studies of asthma have identified many candidate genes expressed in epithelium and lacking overlap with genes regulating IgE levels (2). It has been suggested that

epithelial damage and dysregulated repair can account for asthma pathology independent of inflammation, driving airway remodeling and subsequent airway hyperresponsiveness (AHR) (1). The effects of corticosteroid therapy on epithelial development and homeostasis remain controversial, and there is evidence of deleterious effects (3). Therefore, there is a need for adjunct therapies using agents that protect and repair the airway epithelium (3).

A recent genome-wide association study in mice identified four novel genes associated with AHR (4, 5), including the genes for the antifibrotic hormone relaxin (6) and for trefoil factor 2 (TFF2). TFF2 is a protective molecule released in response to injury at the edge of gastric ulcers as part of the so-called gastrointestinal repair kit. Although its antiinflammatory effects have been well documented (7–10), one study has shown that it plays a role in promoting a Th2 response in a hookworm model (11).

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*Co-corresponding authors.

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Correspondence and requests for reprints should be addressed to Simon G. Royce, Ph.D., Royal Children's Hospital, Immunology, Flemington Rd, Parkville, Australia 3052. E-mail: simon.royce@monash.edu; Mimi L.K. Tang, M.D., Ph.D., F.R.A.C.P., F.R.C.P.A., F.A.A.A.I. E-mail: mimi.tang@rch.org.au; or Chrisan S. Samuel, Ph.D. E-mail: chrisan.samuel@monash.edu

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TFF2 mRNA expression is also increased in asthma and in a chronic mouse model of allergic airways disease (AAD) (12). Mice treated with intranasal recombinant TFF2 for 2 weeks in the chronic AAD model had decreased subepithelial collagen thickening, other features of airway remodeling, and AHR. TFF2-deficient mice treated with an acute AAD model had increased remodeling parameters (13). However, the TFF2 did not affect inflammation in the same AAD model (14). These results suggest that TFF2 may influence airway remodeling and AHR via mechanisms independent of airway inflammation.

There is no cell culture or animal model that truly replicates the pathology of human asthma. Perhaps the best models available are the chronic allergen challenge models. However, these models do not replicate airway epithelial damage and repair. In human asthma, the epithelium is inherently susceptible to disease; that is, in genetically susceptible individuals, impaired epithelial barrier function leaves the airways vulnerable to viral infection and environmental insults (15). As such, epithelial damage may precede other asthma symptoms, appear independent of atopy, and be associated with airway remodeling and asthma severity. The naphthalene (NA) model of acute epithelial damage and repair has been well characterized (16) and is based on selective cytotoxicity of NA to Clara cells in mice. Because these cells make up a much higher proportion of the superficial epithelial cell population compared with that in humans, their ablation leads to the formation of lesions in the airways resembling epithelial damage and denudation observed in endobronchial biopsies from patients with severe asthma (16).

In the current study, we investigated the mechanisms by which epithelial damage and/or aberrant repair can drive structural and functional changes in the murine NA model. In addition, we investigated the role of the epithelial protective molecule TFF2 in protecting the murine airway from NA-induced epithelial and subepithelial remodeling changes.

Materials and Methods

Study Design

The study was divided into three parts. Part A involved characterization of the murine

NA model of airway epithelial damage and repair; in part B, we studied the effect of endogenous TFF2 deficiency in the murine NA model; and in part C we studied the effect of exogenous recombinant TFF2 treatment in the murine NA model. For part A, mice were culled at 12, 24, 48, and 72 hours after NA administration. For parts B and C, mice were culled at time points representing severe epithelial damage (24 h) and repair and airway reepithelialization (72 h), as determined from part A.

Animals

Six-week-old female C57B6J mice were used in these studies. This mouse strain has been shown to be susceptible to NA-induced airway epithelial damage (17). Experimental procedures were approved by the Murdoch Children's Research Institute and Monash University Animal Ethics Committees and followed the Australian Guidelines for the Care and Use of Laboratory Animals for Scientific Purposes. For part B, TFF2-deficient animals and wild-type littermates on C57B6J background were used as previously described (18).

Mouse Model of NA Airway Injury

Mice were injected with NA (200 mg/kg in 10 ml/kg body weight corn oil [CO] vehicle) (Sigma Chemical Co., St. Louis, MO) intraperitoneally on Day 1. Control mice were injected with CO (10 ml/kg body weight CO, intraperitoneally) (19). All injections were performed between 8:00 and 10:00 A.M. to control for circadian rhythms (17). NA- and CO-treated mice were culled at four time points: 12, 24, 48, or 72 hours ($n = 10$ mice per group and time point).

Intranasal Treatment

Human glycosylated recombinant TFF2 peptide (0.5 mg/ml) (20) or vehicle (PBS) was administered to mice once daily from Day 0. For comparison, the corticosteroid dexamethasone (DEX) was administered according to a previously optimized dosage (21). For intranasal treatments, mice were lightly anesthetized with isoflurane and held in a supine position, and 50 μ l of TFF2, DEX, or vehicle was administered intranasally using an autopipette.

Methacholine-Induced AHR

At the conclusion of the experiments outlined in parts A, B, and C, AHR was measured in anesthetized, tracheotomized

mice by invasive plethysmography using a mouse plethysmograph (Buxco Electronics, Troy, NY) in response to increasing doses of nebulized methacholine, as described previously (22).

Tissue Collection

Lung tissues were weighed (total lung weight) and separated into individual lobes for hydroxyproline analysis and histological analyses (23–25).

Lung Histopathology

The right lung lobe and trachea were fixed in formalin, embedded in paraffin, and routinely processed (23–25). Sections were stained with Masson trichrome for assessment of epithelial and subepithelial collagen thickness or with Alcian blue–periodic acid-Schiff for assessment of goblet cells.

Morphometric Analysis of Structural Changes

Morphometric evaluation of lung tissue sections was determined as described previously (23, 25, 26). A minimum of five bronchi measuring 150 to 350 μ m luminal diameter were analyzed per mouse.

Hydroxyproline Analysis of Lung Collagen

A portion of each lung sample from TFF2+/+, TFF2–/–, and treated wild-type mice was treated as described previously to determine hydroxyproline content (27). Hydroxyproline values (μ g) were converted to collagen content and concentration (% collagen content/dry weight tissue) as described previously (27).

Immunohistochemistry

Immunohistochemical localization of TFF2 was performed using a rabbit antihuman recombinant TFF2 polyclonal antibody, which cross-reacts with mouse TFF2 (28) using a method described previously (12). To detect growth factors that regulate fibrosis, the following primary antibodies were used: transforming growth factor (TGF)- β polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA), platelet-derived growth factor (PDGF) BB polyclonal antibody (Abcam, Cambridge, MA), and connective tissue growth factor (CTGF) polyclonal antibody (Abcam) according to a previously described method (29). Epithelial cell apoptosis was detected using a monoclonal antibody to Annexin V

(Epitomics, Burlingame, CA) (22). Staining intensity was determined using Image J software with color deconvolution for diaminobenzidine (DAB) and thresholding (29).

Western Blotting

Equal amounts of total lung protein (10–15 μ g) from CO-, NA-, NA⁺DEX-, and NA⁺TFF-treated mice were electrophoresed on 10.5% acrylamide gels as described previously (30). Western blot analyses were then performed with primary polyclonal antibodies to TGF- β 1 (Santa Cruz Biotechnology) or PDGF-BB (Abcam) and the appropriate secondary antibodies. A Coomassie blue-stained protein was assessed to demonstrate equivalent loading of samples. Blots detected with the ECL detection kit (Amersham Pharmacia Biotech, Piscataway, NJ) were quantified by densitometry with a GS710 Calibrated Imaging Densitometer and Quantity-One software (Bio-Rad Laboratories, Richmond, CA). The density of each parameter was corrected for Coomassie blue-stained protein levels and expressed relative to the CO-treated group, which was expressed as 1 in each case.

Statistical Analysis

Lung function studies were analyzed using two-way ANOVA with a Bonferroni post test. Morphometry was expressed as mean with 95% confidence interval and analyzed using the Mann-Whitney test.

Results

Characterization of the Murine NA Model of Airway Epithelial Damage and Repair

NA caused marked airway epithelial denudation, which repairs over time. Haematoxylin and eosin (H&E)-stained lung tissue sections ($n = 10$ for each time point per treatment group) from the NA- and CO-treated groups were examined to assess the impact of NA on airway epithelial denudation. Airway denudation was expressed as the number of epithelial cells lost per 100 μ m of basement membrane.

Although there was no denudation observed in the CO-treated group, NA treatment led to significant epithelial denudation from as early as 12 hours after injection (Figures 1A and 1B), which was

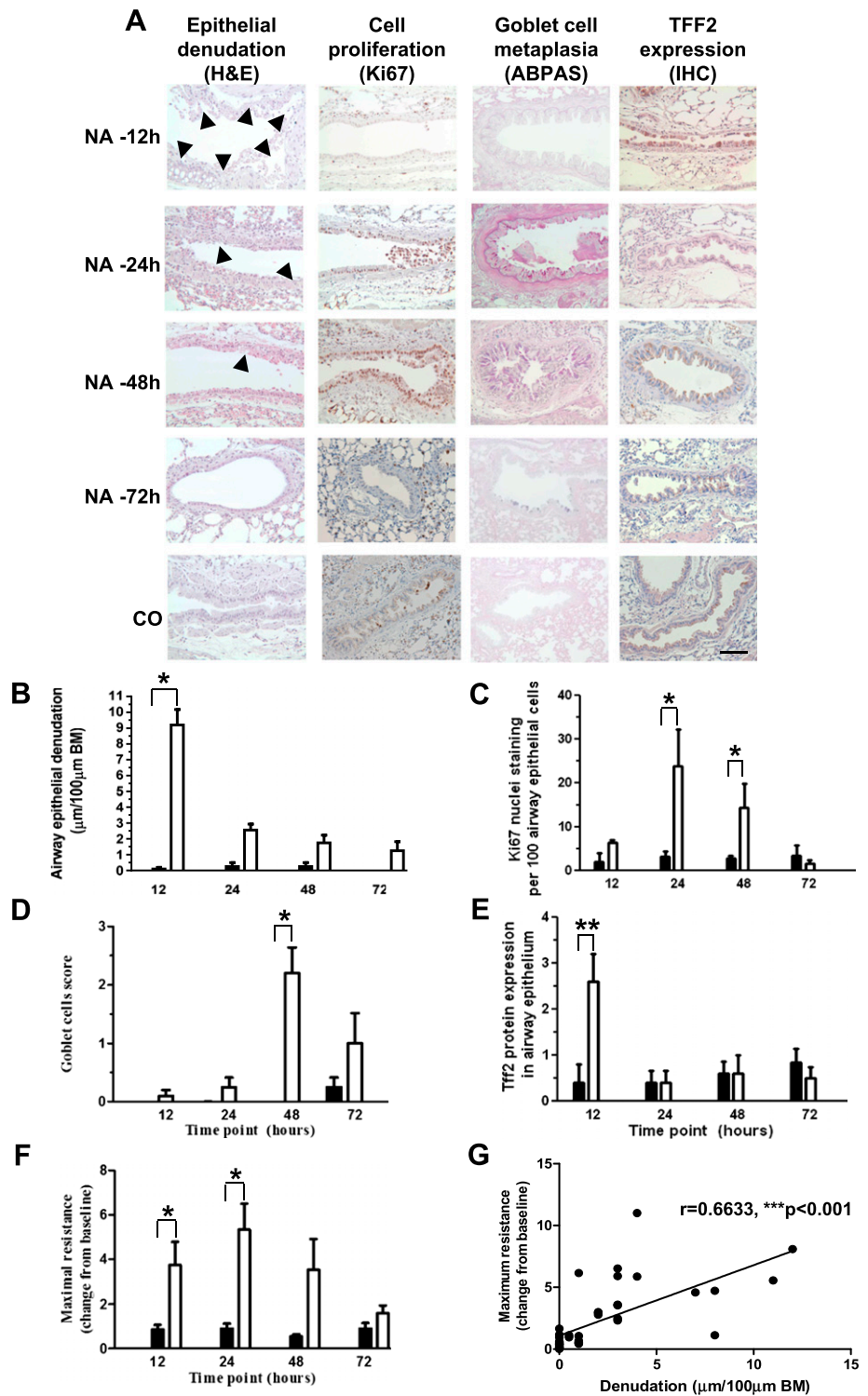


Figure 1. Representative photomicrographs of lung sections (A) stained for epithelial denudation (with hematoxylin and eosin and marked with arrowheads), epithelial cell proliferation (with Ki67), goblet cell metaplasia (with Alcian Blue-periodic acid-Schiff [ABPAS]) and trefoil factor 2 (TFF2) distribution (by immunohistochemistry [IHC]) from mice treated with naphthalene (NA) for 12, 24, 48, and 72 hours and mice treated with corn oil (CO) for 12 hours. Scale bar = 100 μ m. Also shown are the mean $\pm$ SEM values for epithelial denudation (μ m/100 μ m basement membrane [BM]) (B), Ki67 nuclear staining (per 100 airway epithelial cells) (C), goblet cell score (D), and TFF2 protein expression in the airway epithelium (E) from CO-treated (black bars) versus NA-treated (white bars)

most profound at this time point ($P < 0.001$). The degree of loss of integrity gradually reduced thereafter and remained significant for up to 48 hours (24 h, $P = 0.003$; 48 h, $P = 0.03$) when compared with the respective CO-treated groups. At 72 hours, some damage was still observed in the NA-treated group, although this was not significantly different when compared with the control group (Figures 1A and 1B).

NA did not significantly affect airway epithelial cell proliferation. Ki67 is a proliferation marker detected in nuclei when proliferation factors are present. Lung tissue sections were stained for the expression of Ki67 in the NA- and CO-treated groups (Figure 1A). The percentage of proliferating cells among total epithelial cells was determined for each slide.

The percentage of proliferating epithelial cells in the NA-treated group increased at 12 hours, peaked at 24 to 48 hours, and reduced in number at 72 hours (Figure 1C). Proliferation remained low in the CO-treated group throughout the time points measured (12–72 h). There was a significant NA-induced increase in proliferating epithelial cells at 24 and 48 hours (both $P < 0.05$ versus the respective CO-treated measurements) (Figure 1C).

Effects of NA-induced epithelial injury on airway remodeling: goblet cell metaplasia.

Lung tissue sections stained with Alcian-blue periodic acid-Schiff (Figure 1A) from the NA-treated group and CO-treated groups were examined and compared for goblet cell metaplasia, an important feature of airway remodeling. No significant difference between groups was seen at the early time points (12 and 24 h), although a trend toward increased goblet cell number was seen for the NA group. At 48 hours, there was a sharp rise in goblet cell number in the NA-treated group and a significant difference between the NA-treated group versus the CO-treated group ($P = 0.005$). The degree of goblet cell metaplasia reduced in the NA-treated group at 72 hours yet appeared to remain higher than that of the CO-treated group (Figure 1D).

Effects of NA-induced epithelial injury on endogenous TFF2 protein expression.

TFF2 expression was localized in a subset of mucus-secreting goblet cells (Figure 1A) and significantly increased in the NA-treated group at 12 hours ($P = 0.02$) as compared with the control group. TFF2 expression was reduced thereafter (Figure 1E).

Effects of NA-induced epithelial injury on AHR.

Methacholine-induced AHR was measured by invasive plethysmography. There was no significant change in AHR for the CO-treated group throughout the experimental period. In contrast, the NA-treated group showed a marked increase in maximum airway resistance from baseline (saline) at 12 hours, and airway resistance was significantly higher in the NA-treated group as compared with the CO-treated group at this time point ($P = 0.03$). The difference in AHR between the two groups peaked at 24 hours ($P = 0.005$). AHR for the NA-treated group returned to baseline gradually over the subsequent 48- and 72-hour time points (Figure 1F).

Correlation of epithelial denudation with AHR.

The relationship between epithelial denudation and AHR in this mouse model of airway epithelial damage was examined by assessing the correlation between these parameters. There was a strong positive correlation observed between epithelial denudation and AHR ($r = 0.6633$; $***P < 0.001$ [$n = 40$]) (Figure 1G).

Effects of Endogenous TFF2 Deficiency in the Murine NA Model

24 hours after injury. By 24 hours after NA administration, epithelial denudation (Figures 2A and 2B), goblet cell metaplasia (Figures 2A and 2C), epithelial thickness (Figures 2A and 2D), and AHR (Figure 2F) were significantly increased in TFF2 wild-type (+/+) mice ($n = 8$; all $P < 0.05$ versus CO-treated TFF2+/+ mice) compared with that measured in corresponding CO-treated TFF2+/+ animals ($n = 7$). Subepithelial collagen thickness (Figures 2A and 2E) was not statistically different

between NA- versus CO-treated TFF2+/+ mice. Epithelial denudation (Figures 2A and 2B), goblet cell metaplasia (Figures 2A and 2C), and AHR (Figure 2F) were further worsened in NA-treated TFF2 knockout (–/–) mice ($n = 5$), with denudation and AHR being significantly elevated in NA-treated TFF2–/– mice compared with corresponding levels in NA-treated TFF2+/+ mice (both $P < 0.05$ versus NA-treated TFF2+/+ mice). On the other hand, epithelial thickness was equivalently increased in NA-treated TFF2–/– compared with that measured from NA-treated TFF2+/+ mice (Figures 2A and 2D; both $P < 0.05$ versus corresponding levels measured from their respective CO-treated counterparts), whereas no changes in subepithelial collagen thickness (Figures 2A and 2E) were measured between each of the four groups studied.

72 hours after injury. By 72 hours after NA administration, minimal epithelial denudation (Figures 3A and 3B) was measured from each of the groups studied ($n = 7$ mice per group). The results were not statistically different. On the other hand, goblet cell metaplasia (Figures 3A and 3C) continued to be significantly increased in NA-treated TFF2+/+ mice compared with that measured in CO-treated TFF2+/+ animals to a similar extent as that seen at 24 hours after injury (Figure 2C). At this time point, NA-treated TFF2–/– mice did not demonstrate exaggerated goblet cell metaplasia over that measured in the NA-treated TFF2+/+ mice (both $P < 0.05$ versus the respective levels measured from the corresponding CO-treated mice) (Figure 3C). No changes in epithelial thickness (Figures 3A and 3D) or AHR (Figure 3G) were measured between the four groups studied, whereas subepithelial collagen thickness (Figures 3A and 3E) and total lung collagen content (Figure 3F) were now significantly increased in NA-treated TFF2+/+ mice compared with that measured in CO-treated TFF2+/+ mice, which was significantly worsened in NA-treated TFF2–/– mice ($P < 0.05$ versus NA-treated TFF2+/+ mice for both measures).

Up-regulation of TGF- β 1 and CTGF was induced by epithelial damage and repair, independent of TFF2 expression.

TGF- β 1 expression (Figures 4A and 4B) was significantly increased in the airways of NA-treated TFF2+/+ mice after 24 hours compared with the corresponding

Figure 1. (Continued). mice after 12, 24, 48, and 72 hours ($n = 7$ –10 mice per treatment group and time point) as determined from morphometric analysis of these parameters. (F) Mean $\pm$ SEM airway hyperresponsiveness (AHR) measurements (expressed as maximal resistance) from CO- versus NA-treated mice at each of the time points studied ($n = 10$ mice per treatment group and time point). (G) The positive correlation between epithelial denudation and AHR after NA administration ($r^2 = 0.6633$; $n = 40$; $***P < 0.001$). $*P < 0.05$, $**P < 0.01$ versus the CO-treated group at the respective time point studied.

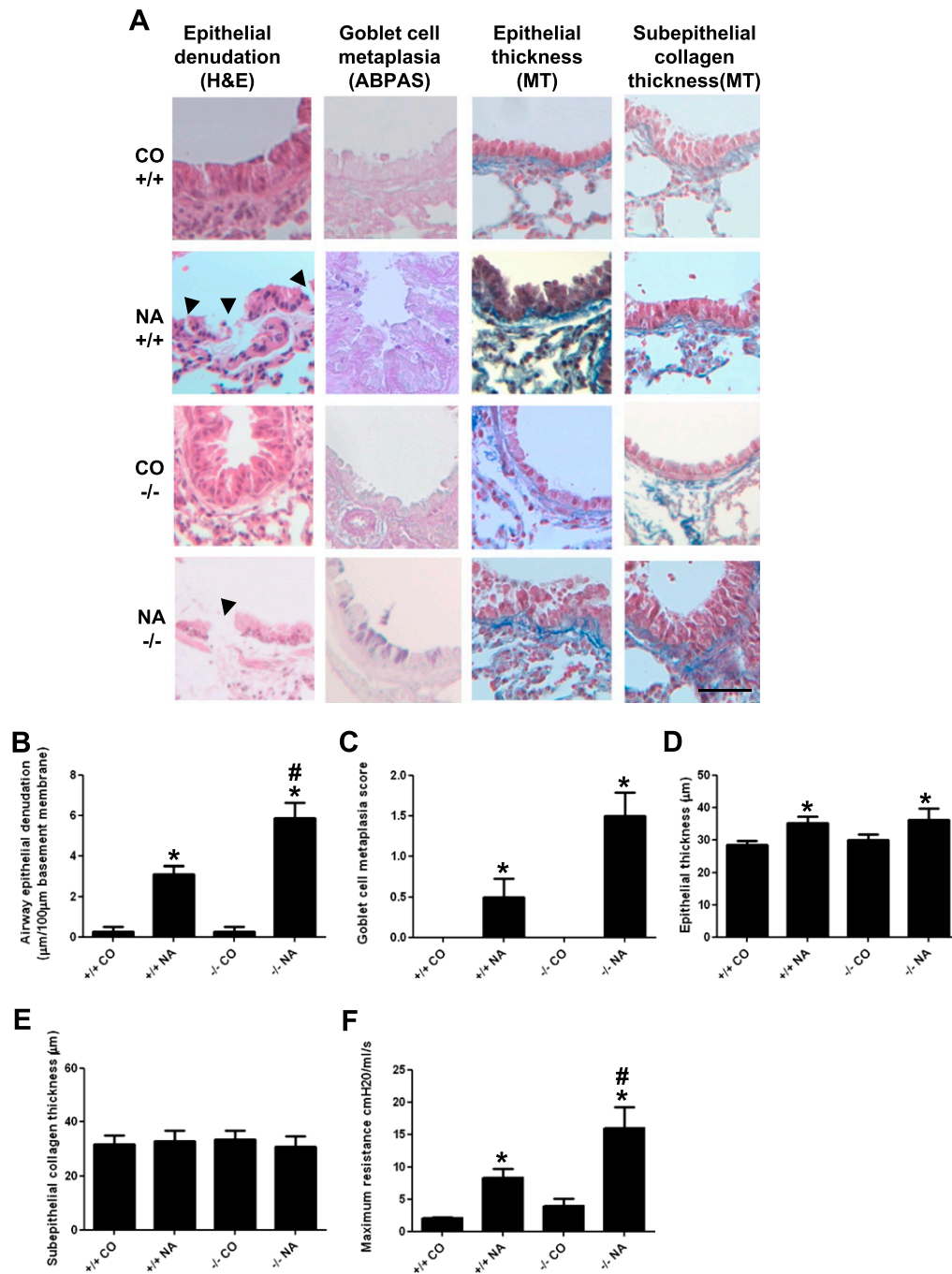


Figure 2. Representative photomicrographs of lung sections (A) stained for epithelial denudation (stained with hematoxylin and eosin and marked with arrowheads), goblet cell metaplasia (stained with ABPAS), epithelial thickness (stained with Masson trichrome [MT]), and subepithelial collagen thickness (stained with MT) from TFF2^{+/+} and TFF2^{-/-} mice treated with CO or NA after 24 hours of treatment. Scale bar = 100 μ m. Also shown are the mean $\pm$ SEM values for epithelial denudation (μ m/100 μ m BM) (B), goblet cell score (C), epithelial thickness (D), subepithelial collagen thickness (E), and AHR (expressed as maximum resistance) (F) from CO- versus NA-treated TFF2^{+/+} and TFF2^{-/-} mice after 24 hours of treatment ($n = 5-8$ mice per treatment group). * $P < 0.05$ versus respective measurements from CO-treated mice; # $P < 0.05$ versus respective measurements from NA-treated TFF2^{+/+} mice.

levels measured from CO-treated TFF2^{+/+} mice ($P = 0.0138$) (Figure 4B). Similarly, the airways of NA-treated TFF2^{-/-} mice had significantly more TGF- β 1 compared with their CO-treated counterparts ($P =$

0.005) (Figure 5B). However, there were no differences in TGF- β 1 immunostaining levels between NA-treated TFF2^{+/+} versus TFF2^{-/-} mice. Similar findings were noted at the 72-hour time point

(Figures 4A and 4C): the airways of NA-treated TFF2^{+/+} and TFF2^{-/-} mice had significantly increased expression of TGF- β 1 as compared with the airways of corresponding CO-treated mice ($P < 0.001$

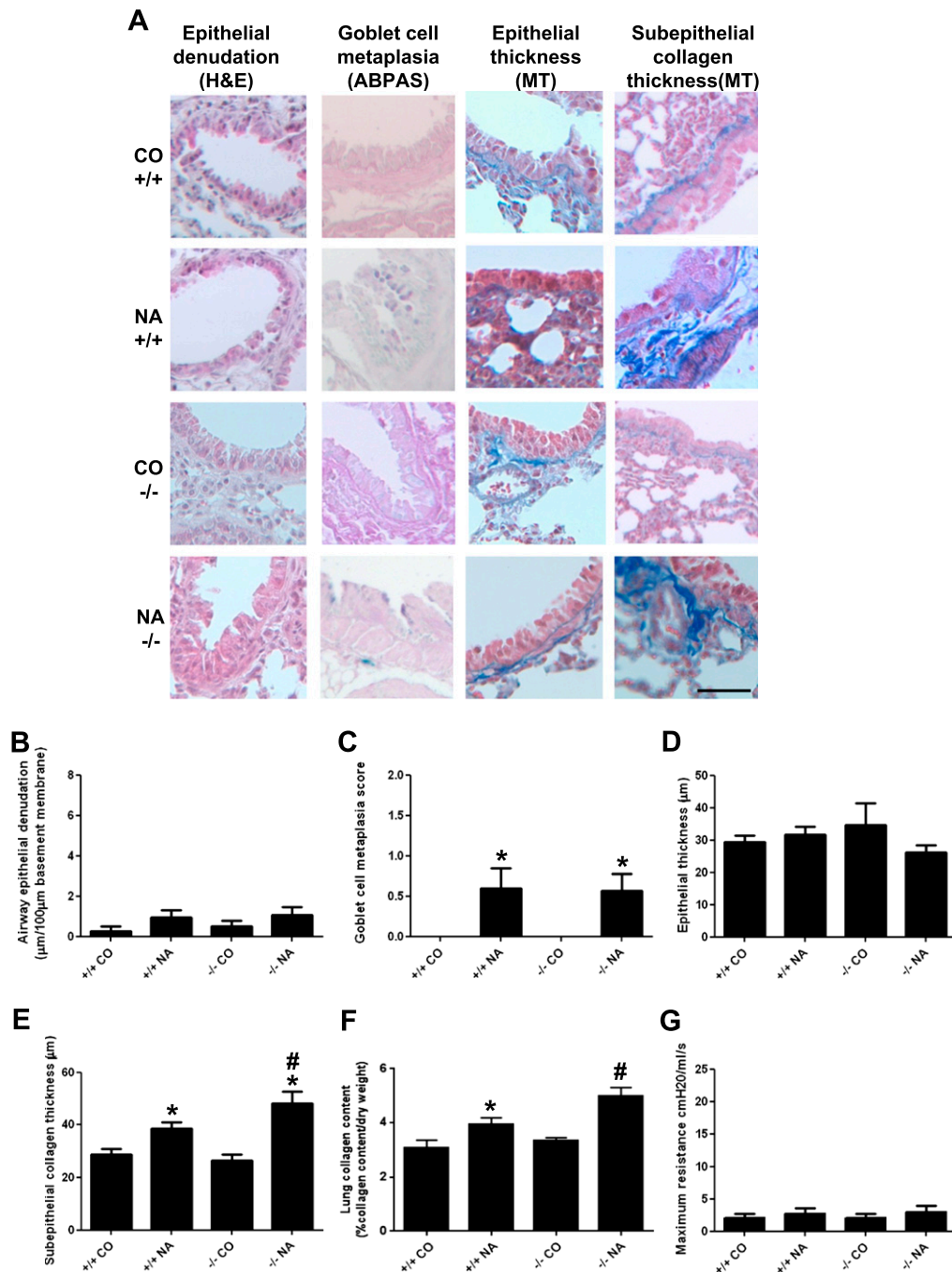


Figure 3. Representative photomicrographs of lung sections (A) stained for epithelial denudation (stained with hematoxylin and eosin and marked with arrowheads), goblet cell metaplasia (stained with ABPAS), epithelial thickness (stained with MT), and subepithelial collagen thickness (stained with MT) from TFF2+/+ and TFF2-/- mice after 72 hours of treatment with CO or NA. Scale bar = 100 μ m. Also shown are the mean $\pm$ SEM values for epithelial denudation (μ m/100 μ m BM) (B), goblet cell score (C), epithelial thickness (D), subepithelial collagen thickness (E), total lung collagen concentration (% collagen content/dry weight lung tissue) (F), and AHR (expressed as maximum resistance) (G) from CO- versus NA-treated TFF2+/+ and TFF2-/- mice after 72 hours of treatment ($n = 5-8$ mice per treatment group). * $P < 0.05$ versus respective measurements from CO-treated mice; # $P < 0.05$ versus respective measurements from NA-treated TFF2+/+ mice.

and $P = 0.0035$, respectively). At this latter time point, TGF- β 1 expression was significantly ($P < 0.001$) increased in CO-treated TFF2-/- mice compared with

that measured in their TFF2+/+ counterparts.

By 24 hours after injury, the airways of NA-treated TFF2+/+ mice had significant

up-regulation of CTGF as compared with CO-treated TFF2+/+ mice ($P = 0.0002$; data not shown). At the same time point, CTGF expression was equivalently

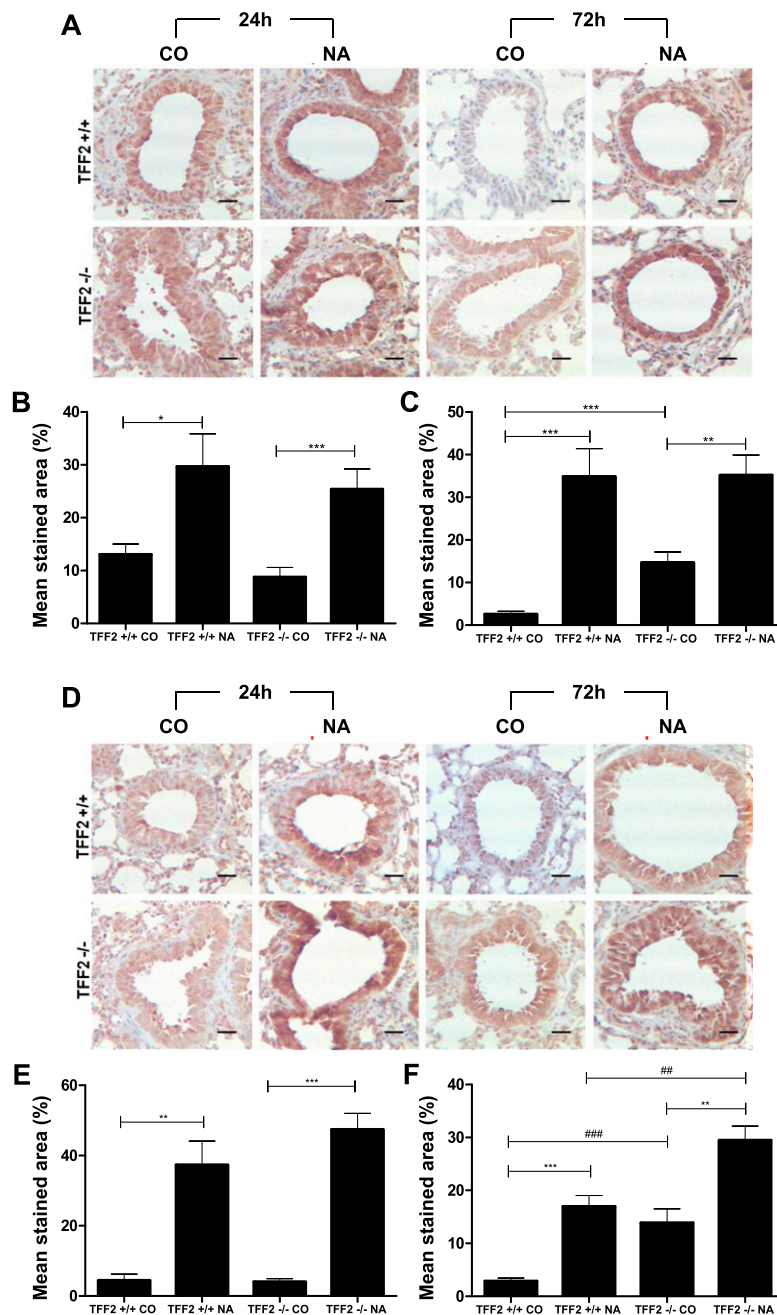


Figure 4. Representative photomicrographs of airways from CO versus NA-treated TFF2^{+/+} and TFF2^{-/-} mice after 24 and 72 hours (A, D) stained for TGF-β1 (A) and PDGF (D). Scale bar = 50 μm. Also shown are the mean ± SEM values of TGF-β1 and PDGF staining in TFF2^{+/+} and TFF2^{-/-} mice after 24 hours (B and E, respectively) and 72 hours (C and F, respectively) as determined from morphometric analysis of these parameters ($n = 5-8$ mice per treatment group and time point). (B) TGF-β1 quantification at the 24-hour time point: * $P = 0.0138$ versus CO-treated TFF2^{+/+} mice; *** $P = 0.001$ versus CO-treated TFF2^{-/-} mice. (C) TGF-β1 quantification at the 72-hour time point: *** $P < 0.001$ versus CO-treated TFF2^{+/+} mice; ** $P < 0.01$ versus CO-treated TFF2^{-/-} mice. (E) PDGF quantification at the 24-hour time point: ** $P < 0.01$ versus CO-treated TFF2^{+/+} mice; *** $P < 0.001$ versus CO-treated TFF2^{-/-} mice. (F) PDGF quantification at the 72-hour time point: *** $P < 0.001$ versus CO-treated TFF2^{+/+} mice; ** $P < 0.01$ versus CO-treated TFF2^{-/-} mice; ## $P < 0.01$ versus NA-treated TFF2^{+/+} mice, ### $P < 0.001$ versus CO-treated TFF2^{+/+} mice.

increased in the airways of NA-treated TFF2^{-/-} as compared with that in CO-treated TFF2^{-/-} animals ($P < 0.001$). However, CTGF expression levels were not significantly different between NA-treated TFF2^{+/+} and TFF2^{-/-} mice (data not shown). By 72 hours after injury, CTGF levels were generally diminished in all four groups of mice studied but were still significantly and similarly increase in NA-treated mice compared with their CO-treated TFF2^{+/+} ($P = 0.0394$) and TFF2^{-/-} ($P < 0.0001$) counterparts (data not shown).

Up-regulation of PDGF was induced by epithelial damage and repair, the latter of which was dependent on TFF2. By 24 hours after NA administration, the airways of TFF2^{+/+} and TFF2^{-/-} mice had significantly more PDGF than their CO-treated counterparts ($P = 0.0011$ and $P < 0.001$ versus CO-treated TFF2^{+/+} and TFF2^{-/-} mice, respectively) (Figures 4D and 4E). However, there were no differences in PDGF levels between NA-treated TFF2^{+/+} and the TFF2^{-/-} mice at this time point. During the epithelial repair process (at 72 h after injury), the airways of NA-treated TFF2^{+/+} mice had a significant up-regulation of PDGF expression in comparison to their CO-treated counterparts ($P < 0.001$) (Figures 4D and 4F), which was further exaggerated in NA-treated TFF2^{-/-} mice ($P = 0.0037$ versus NA-treated TFF2^{+/+} mice; $P = 0.0017$ versus CO-treated TFF2^{-/-} mice) (Figure 4F). PDGF levels were significantly higher in CO-treated TFF2^{-/-} mice compared with CO-treated TFF2^{+/+} mice ($P < 0.001$) (Figure 4F).

Effects of Exogenous Recombinant Treatment in the Murine NA Model

Administration of exogenous TFF2 limits NA-induced epithelial injury. By 24 hours after NA administration, when injury was most pronounced, H&E-stained airway sections from these mice demonstrated significantly more epithelial denudation as compared with that in mice treated with CO ($P = 0.0079$) (Figures 5A and 5B). DEX treatment of mice subjected to NA led to significantly less epithelial denudation than that measured in untreated NA-treated mice ($P = 0.0079$). However, denudation in these DEX-treated mice was still significantly greater than that measured in CO-treated mice ($P = 0.0079$). Similarly, TFF2 treatment resulted in a significant

decrease in NA-induced epithelial damage compared with that measured in NA alone-treated mice ($P = 0.0079$) (Figure 5B). However, denudation in these TFF2-treated mice was still not fully restored to that in CO-treated mice ($P = 0.0159$).

By 72 hours after NA administration, there were no differences in epithelial denudation measurements between either of the groups studied (Figure 5C).

Administration of exogenous TFF2 did not affect goblet cell metaplasia in NA-treated mice. NA treatment appeared to induce a trend toward a time-dependent increase in goblet cell metaplasia, which was noticeable at 24 hours (Figure 5D) and at 72 hours (Figure 5E) after injury, compared with that measured in CO-treated mice. However, this was not significantly different from that measured from CO-treated mice at either time point studied. Furthermore, although DEX and TFF2 treatment of mice exposed to NA appeared to reduce goblet cell metaplasia by 72 hours after injury, this was not significantly different from that measured from NA alone-treated mice.

Administration of exogenous TFF2 limits NA-induced subepithelial collagen deposition but not total lung collagen concentration. As demonstrated in Figure 2E, by 24 hours after NA administration there was no difference in subepithelial collagen thickness between the different groups studied (Figure 6G). By 72 hours after injury, however, NA-treated mice had significantly increased subepithelial collagen deposition ($P = 0.0238$) (Figures 5F and 5H) and total lung collagen concentration ($P < 0.05$) (Figure 5I) as compared with that measured in CO-treated mice. At this latter time point, mice treated with DEX showed no difference in subepithelial collagen thickness (Figure 5H) or total lung collagen concentration (Figure 5I) compared with that measured from NA alone-treated mice, which were both significantly higher than that measured from CO-treated mice. TFF2 treatment provided protection from NA-induced subepithelial fibrosis, which was significantly decreased compared with that measured in NA alone-treated mice ($P = 0.0043$). TFF2 treatment of mice subjected to NA significantly decreased subepithelial collagen deposition compared with DEX treatment ($P = 0.0079$) (Figure 5H). Although TFF2 treatment of mice subjected to NA also induced a trend

toward a decrease in total lung collagen concentration (Figure 5I), this was not statistically different from that measured from NA alone-treated mice.

Administration of exogenous TFF2 inhibits epithelial cell apoptosis and expression of TGF- β 1 and PDGF. To determine the TFF2-induced mechanisms involved with its effects reported above, its ability to regulate epithelial cell apoptosis (annexin V) and expression of profibrotic factors (TGF- β 1, PDGF) was evaluated further. NA induced a significant increase in annexin V staining localized to the cell membrane of airway epithelial cells (Figure 6A) at the 24-hour (Figure 6B) and the 72-hour (Figure 6C) time points and promoted TGF- β 1 (Figures 6D and 6E) and PDGF (Figures 6D and 6F) levels at 72 hours (all $P < 0.01$ versus respective measurements from CO-treated mice). DEX treatment did not influence the NA-induced increase in epithelial cell apoptosis at 24 hours (Figures 6A and 6B) or at 72 hours (Figures 6A and 6C) but significantly reduced the NA-induced up-regulation of TGF- β 1 (Figures 6D and 6E) and PDGF (Figures 6D and 6F) levels at 72 hours (both $P < 0.01$ versus NA treatment alone). On the other hand, TFF2 significantly lowered the NA-mediated elevation of epithelial cell apoptosis at both time points studied ($P < 0.05$ versus NA treatment alone at both time points) (Figures 6A–6C) in addition to the NA-induced up-regulation of TGF- β 1 and PDGF levels (Figures 6D–6F); the latter two returned to levels measured in CO-treated mice (both $P < 0.01$ versus NA treatment alone).

Administration of exogenous TFF2 protects against NA-induced AHR. As demonstrated in Figure 1F, NA administration caused a significant increase in AHR by 24 hours after injury compared with that measured from CO-treated mice (Figures 7A and 7B). DEX treatment modestly, but insignificantly, affected the NA-induced increase in AHR (Figure 7A). On the other hand, TFF2 treatment significantly restored NA-induced AHR to the baseline levels measured from CO-treated mice (Figure 7B).

Discussion

In this study, we used a mouse model of NA-induced epithelial injury to examine the

relationship between epithelial injury and airway remodeling. This model was used to assess the specific role of the epithelium in the progression of remodeling because NA specifically targets Clara cells, the progenitor cells of the airway epithelium (31–34). NA administration led to epithelial denudation, epithelial cell proliferation, epithelial thickness, epithelial cell apoptosis, increased expression of profibrotic factors (TGF- β 1, CTGF, and PDGF) and AHR by 12 to 24 hours after injury, goblet cell metaplasia by 48 hours after injury, and subepithelial collagen deposition and total lung collagen concentration by 72 hours after injury, confirming our previous findings (35) that epithelial injury is a significant contributor to airway remodeling associated with AAD and asthma. Furthermore, we demonstrated for the first time that the epithelial repair factor TFF2 plays an important role in protecting the airways and lung from the airway remodeling and AHR that resulted from NA-induced epithelial injury: as markers of epithelial injury, airway remodeling and AHR itself were worsened by the absence of TFF2, whereas exogenous TFF2 treatment significantly reduced the NA-induced aberrant nature of these parameters.

As previously described, the airways of mice sensitized with OVA to produce a chronic AAD phenotype exhibited a number of important inflammatory and remodeling events (36) characteristic of human disease. However, the airway epithelium had been shown to be left intact in AAD models, with little damage to its structural integrity and barrier function (37). Thus, the results produced in this study supported prior knowledge regarding the limitations of the AAD model and, in part, the requirement to model the epithelial alterations (increased injury and initiation of repair) through the administration of NA to selectively target Clara cells in mice (38).

Our findings extend previously reported *in vitro* studies, which suggested that airway epithelial damage could influence AHR (39). Mechanical stripping of the airway epithelium in tubular segments of airway augmented the magnitude of responsiveness to substances (including methacholine) administered intraluminally. Jeffery and colleagues reported a positive correlation between epithelial loss in endobronchial biopsies

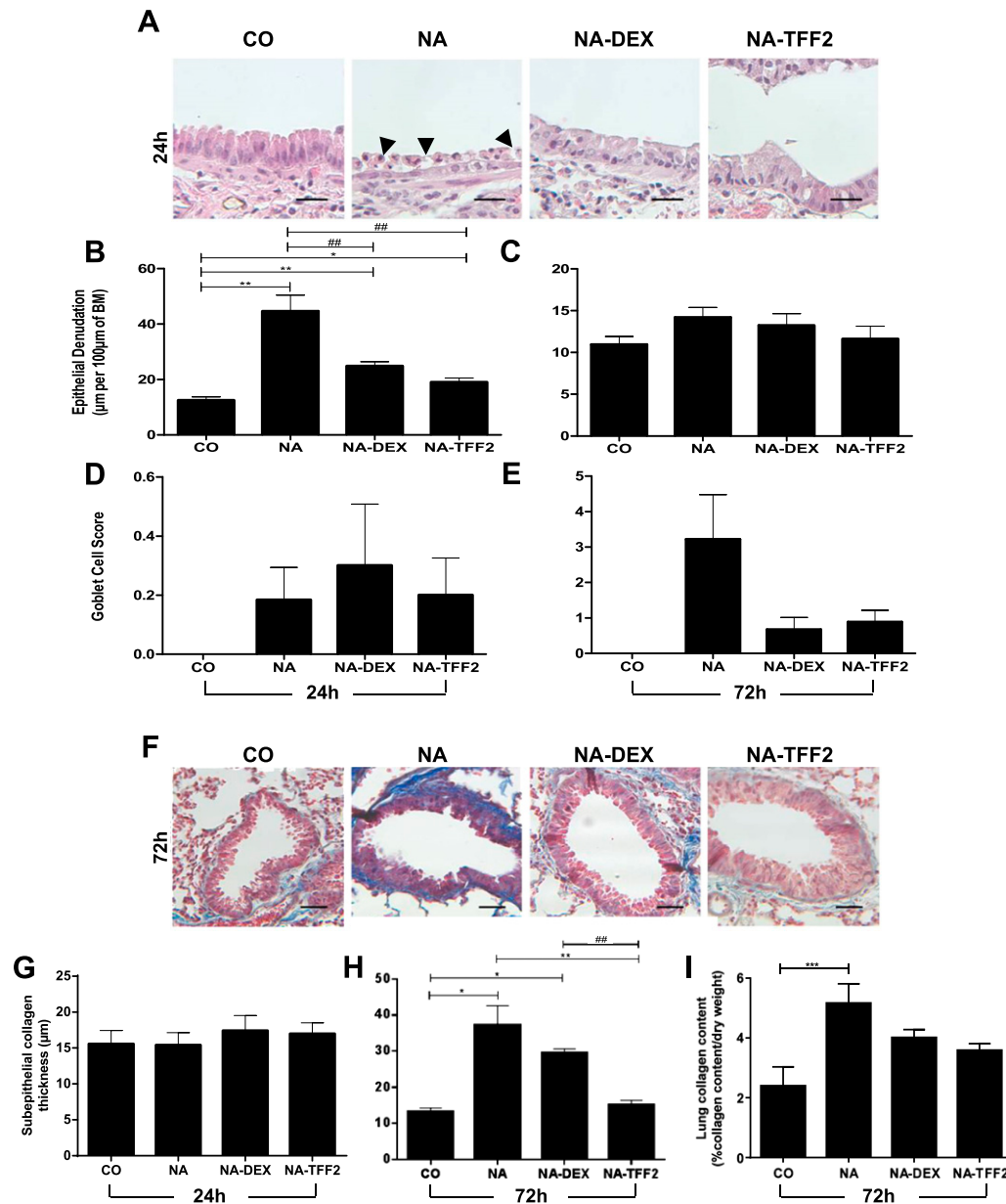


Figure 5. Representative photomicrographs of airways from CO-treated, NA alone-treated, NA⁺ DEX (NA-DEX)-treated, and NA⁺ TFF2 (NA-TFF2)-treated mice after 24 hours stained for epithelial denudation (A) and after 72 hours stained for subepithelial collagen thickness (F). Scale bar = 50 μm. Also shown are the mean ± SEM values for epithelial denudation, goblet cell score, and subepithelial collagen thickness from the four groups of animals after 24 hours (B, D, and G, respectively) and 72 hours (C, E, and H, respectively) as determined from morphometric analysis of these parameters (*n* = 5–8 mice per treatment group and time point). The mean ± SEM total lung collagen concentration (% collagen content/dry weight lung tissue) (I) from each of the four groups analyzed after 72 hours (*n* = 5–10 per treatment group) is included. (B) Epithelial denudation quantification at the 24-hour time point: **P* < 0.05 and ***P* < 0.01 versus CO-treated mice; ##*P* < 0.01 versus NA-alone treated mice. (H) Subepithelial collagen thickness quantification at the 72-hour time point: **P* < 0.05 versus CO-treated mice; ***P* < 0.01, NA alone-treated versus NA-TFF2 mice; ##*P* < 0.01, NA-DEX versus NA-TFF2 mice. (I) collagen concentration quantification: ****P* < 0.001 versus CO-treated mice.

and methacholine responsiveness, suggesting that epithelial damage can directly influence AHR (40). However, it remained uncertain whether there was a causal relationship between epithelial damage and AHR. NA exposure by intraperitoneal injection resulted in

selective Clara cell damage due to cytochrome P450–produced reactive cytotoxic metabolites (41). We showed that NA treatment induced significant epithelial denudation that was most profound at 12 hours with little or no airway inflammation present in H&E-stained lung tissue

sections. This is consistent with previous studies that reported damage of Clara cells at 12 hours and complete epithelial exfoliation at 24 hours after systemic NA exposure (42) with little to no associated inflammation (17, 42). This epithelial damage induced up-regulation of repair

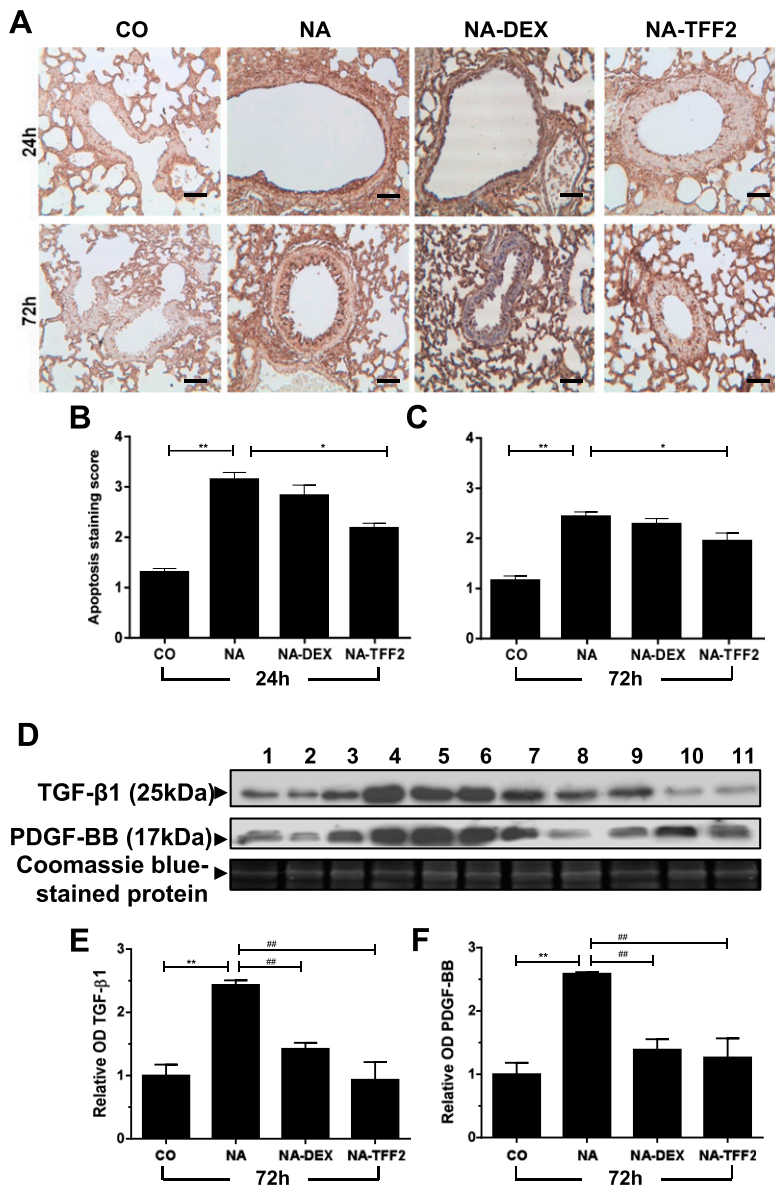


Figure 6. Representative photomicrographs of airways from CO-treated, NA alone-treated, NA+DEX (NA-DEX)-treated, and NA+TFF2 (NA-TFF2)-treated mice after 24 and 72 hours, stained for annexin V (A). Scale bar = 50 μ m. Also shown are the mean $\pm$ SEM values for the apoptosis staining score from the four groups of animals after 24 hours (B) and 72 hours (C) as determined from morphometric analysis of these parameters (n = 5–8 mice per treatment group and time point). (B) Apoptosis staining score at the 24-hour time point: ** P < 0.01 versus CO-treated mice; * P < 0.05 versus NA-alone treated mice. (C) Apoptosis staining score at the 72-hour time point: ** P < 0.01 versus CO-treated mice; * P < 0.05 versus NA alone-treated mice. Also shown are representative Western blots of TGF- β 1 dimer (25 kD) and PDGF-BB (17 kD) expression from CO-treated (lanes 1–3), NA alone-treated (lanes 4–6), NA-DEX-treated (lanes 7–9), and NA-TFF2-treated (lanes 10–11) mice (D) after 72 hours. A Coomassie blue-stained protein was used to demonstrate the quality and equivalent loading of protein samples. The relative optical density (OD) TGF- β 1 (E) and PDGF (F), corrected for Coomassie blue-stained protein density, is included from each of the groups studied, as determined by densitometry scanning (from n = 4–6 mice per treatment group) and is different from that of the untreated group, which is expressed as 1 in each case. (E) Relative OD TGF- β 1: ** P < 0.01 versus CO-treated mice; ## P < 0.01 versus NA alone-treated mice. (F) Relative OD PDGF: ** P < 0.01 versus CO-treated mice; ## P < 0.01 versus NA alone-treated mice.

signals and proliferation of epithelial cells, resulting in epithelial reconstitution by 20 days, also consistent with previous reports (17, 19, 38, 42–44).

The ability of TFF2 to attenuate the progression of key remodeling events, as previously demonstrated in an acute model of AAD (13), provided the rationale to further investigate its role in asthma. In the chronic AAD model, goblet cell metaplasia, mucus hypersecretion, and subepithelial fibrosis were significantly elevated in the absence of endogenous TFF2 expression. These findings suggested that TFF2 had a functional role in the regulation of airway remodeling and provided the rationale to continue establishing the exact mechanisms involved. In addition, through the comparison of saline- versus OVA-treated TFF2 $^{-/-}$ mice, it was found that endogenous TFF2 levels were insufficient to completely protect the airway from the structural alterations caused by chronic allergen exposure and sensitization.

Little is known of the mechanisms regulating epithelial repair in the airway. TFF2 is a motogen that assists the restitution process of the epithelium in the gastrointestinal tract, but its role in the airways remains uncertain (45, 46). We demonstrated that TFF2 protein expression dramatically increased simultaneously with NA-induced epithelial injury, although expression was transient and reduced rapidly over the ensuing 24 hours. This finding is consistent with previous studies where endogenous TFF2 mRNA expression was found to be most intense in the degenerating Clara cells in the injury target zone at 6 to 24 hours (19). TFF2 may thus represent a compensatory/protective factor that is up-regulated in response to epithelial damage.

Consistent with its ability to augment epithelial injury, airway remodeling, and AHR, NA administration to mice caused a significant up-regulation of epithelial cell apoptosis and the profibrotic factors, TGF- β 1, and its down-stream mediators CTGF and PDGF, which were all markedly elevated even after 72 hours after injury. These findings are consistent with previous studies demonstrating that TGF- β 1 levels are significantly increased in asthma (42), whereas PDGF, a potent stimulant of airway smooth muscle cell migration and proliferation, is a growth factor that is thought to play a role in asthma (47). The

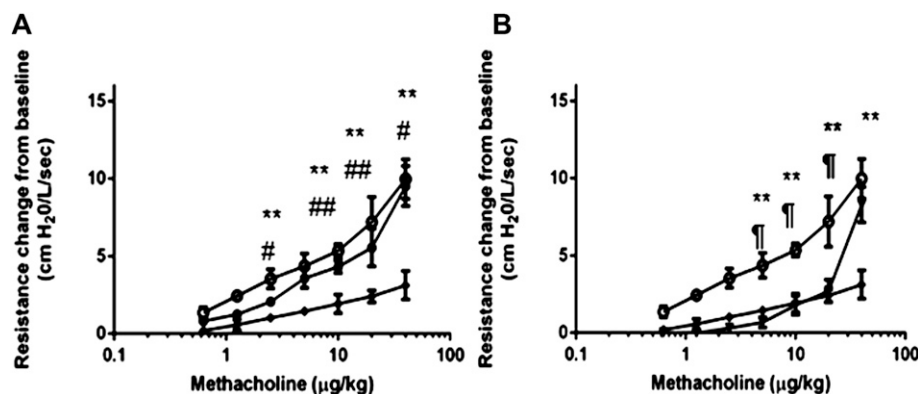


Figure 7. Mean $\pm$ SEM AHR measurements (expressed as maximal resistance) from mice exposed to CO (diamond), NA alone (open circle), and NA+DEX (closed circle) (A) or CO (diamond), NA alone (open circle), and NA+TFF2 (triangle) (B) for 24 hours ($n = 5$ mice per treatment group). ** $P < 0.01$ NA-treated versus CO-treated mice; # $P < 0.05$, ## $P < 0.01$ NA-DEX versus CO-treated mice; ¶ $P < 0.05$ NA-treated versus NA-TFF2 mice.

increased expression of TGF- β 1 and CTGF appeared to occur independently of the presence of endogenous TFF2, whereas the aberrant PDGF levels measured were further elevated in the absence of endogenous TFF2, particularly by 72 hours after injury. However, exogenous TFF2 treatment significantly abrogated the NA-induced up-regulation of TGF- β 1 and PDGF, perhaps suggesting that topical treatment with TFF2 may disrupt the TGF- β 1–PDGF interaction. These findings suggested that TFF2 mediated its protective effects on NA-induced airway remodeling by inhibiting epithelial cell apoptosis (as demonstrated in other models; see References 12 and 48) and by specifically regulating PDGF expression and activity (which were lowered in the presence of endogenous TFF2) in the absence of any effects on CTGF. Furthermore, as an increase in PDGF mRNA expression had previously been associated with increased fibrosis in the rodent lung (49), our findings suggested that TFF2 may have protected from NA-induced subepithelial fibrosis through this PDGF-dependent mechanism. Further studies are warranted to substantiate this hypothesis.

The epithelial protective capacity of TFF2 was comparable to, and in some cases better than, the clinically used corticosteroid DEX. Both treatments, however, failed to completely provide protection from NA, with the epithelial layer of treated mice displaying alterations significantly different from CO-treated mice. In some reports, corticosteroid treatment exacerbates epithelial damage (50),

although this has not been conclusively established. As such, the findings regarding the effects of DEX treatment on the epithelium do not conflict with previous findings.

Thickening as a result of extracellular matrix deposition, caused by the chronic activation of myofibroblasts (51) and the production of various profibrotic mediators (52), is thought to be propagated and maintained by the aberrant repair processes initiated by epithelial injury (53). DEX treatment of mice exposed to NA failed to attenuate subepithelial collagen thickening (54). Current treatment options, including corticosteroids, are unable to reverse the structural alterations characteristic of chronic, severe disease (55). TFF2 treatment was able to significantly decrease the deposition of collagen in the subepithelium to levels of that comparable to CO-treated mice, and thus demonstrated markedly enhanced protection from NA-induced airway remodeling compared with the effects of DEX treatment. Although this was specific to the subepithelial collagen deposition in the absence of any changes on total lung collagen concentration, these findings demonstrated the ability of TFF2 to modulate structural alterations and prevent the remodeling changes thought to be irreversible due to the inadequacies of current treatment options.

AHR, an important feature of asthma, correlates positively with disease severity (56) and is related to the persistent alterations in the airways caused by

chronic inflammation and remodeling events (57). The ability of exogenous TFF2 to ameliorate airway resistance to levels comparable to that observed in CO-treated mice also showed the capabilities of TFF2 treatment in preventing the progression of features of asthma. On the other hand, consistent with previous studies showing that corticosteroid treatment did not possess an ability to affect AHR (58), DEX treatment of mice exposed to NA did not significantly affect AHR.

In conclusion, the current study clearly demonstrated that NA-induced airway epithelial damage was associated with pathophysiological (airway remodeling) changes in the lung that resulted in increased AHR. These findings have important clinical significance because if drugs can be developed that facilitate airway epithelial repair and protection, they may potentially have a therapeutic effect on lung dysfunction. Naturally occurring growth factors may be useful in this regard. Treatment with epidermal growth factor was used successfully in a small study for patients with ulcerative colitis (59). Treatment with epidermal growth factor helped to restore the epithelial barrier and to suppress underlying inflammation. Trefoil peptides may also hold promise in the treatment of acute lung injury and acute respiratory distress syndrome (60). Conversely, the mainstay of conventional asthma therapy is inhaled corticosteroids, which may have detrimental effects on the airway epithelium, including induction of apoptosis and impaired epithelial migration, and hence limited effectiveness on suppressing AHR and other aspects of asthma (61–64). In addition, β_2 -adrenoreceptor agonists have been shown to have no impact on epithelial repair (65). We demonstrate that TFF2 is a promising new treatment option to protect from epithelial damage and the down-stream consequences of this on airway remodeling and AHR, which mediates its actions via the inhibition of PDGF. ■

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

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