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Airway Trefoil Factor Expression during Naphthalene Injury and Repair

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While the role of trefoil factors (TFF) in the maintenance of epithelial integrity in the gastrointestinal tract is well known, their involvement in wound healing in the conducting airway is less well understood. We defined the pattern of expression of TFF1, TFF2, and TFF3 in the airways of mice during repair of both severe (300 mg/kg) and moderate (200 mg/kg) naphthalene-induced Clara cell injury. Quantitative real-time PCR for *tff* messenger RNA expression and immunohistochemistry for protein expression were applied to airway samples obtained by microdissection of airway trees or to fixed lung tissue from mice at 6 and 24 h and 4 and 7 days after exposure to either naphthalene or an oil (vehicle) control. All three TFF were expressed in normal whole lung and airways. TFF2 was the most abundant and was enriched in airways. Injury of the airway epithelium by 300 mg/kg naphthalene caused a significant induction of *tff1* gene expression at 24 h, 4 days, and 7 days. In contrast, *tff2* was decreased in the high-dose group at 24 h and 4 days but returned to baseline levels by 7 days. *tff3* gene expression was not significantly changed at any time point. Protein localization via immunohistochemistry did not directly correlate with the gene expression measurements. TFF1 and TFF2 expression was most intense in the degenerating Clara cells in the injury target zone at 6 and 24 h. Following the acute injury phase, TFF1 and TFF2 were localized to the luminal apices of repairing epithelial cells and to the adjacent mesenchyme in focal regions that correlated with bifurcations and the bronchoalveolar duct junction. The temporal pattern of increases in TFF1, TFF2, and TFF3 indicate a role in cell death as well as proliferation, migration, and differentiation phases of airway epithelial repair.

Key Words: **lung; Clara cell; naphthalene; tff1; tff3.**

The conducting airways of the lung occupy a small percentage of the total lung volume, are an injury target zone for air pollutants, and are a primary interface with the environment. Airway narrowing and branching can result in high local deposition of inhaled toxicants in the conducting airway. The epithelium of the airways is also a target of ingested and circulating toxicants. The primary secretory cell of the conducting airways in rodents, the nonciliated bronchiolar (Clara) cell, is uniquely susceptible to injury by substances that require metabolic activation to produce a toxic effect (naphthalene, 4-ipomeanol, and nitronaphthalene), likely due to the abundant cytochrome P450s found in this cell type. In this study, we use naphthalene to create a well-defined injury pattern in the airways of the mouse (Stripp *et al.*, 1995; Van Winkle *et al.*, 1995). Using this well-defined airway wound healing model, coupled with conducting airway-specific approaches to measure gene expression (Baker *et al.*, 2004), we can assess the role of trefoil factors (TFF) in repair in the injury target zone specifically.

The time course of naphthalene-induced Clara cell injury and repair is defined for the strain of mouse used in this study, Swiss mice (Van Winkle *et al.*, 1995), and consists of defined stages of acute injury and Clara cell loss, cell spreading, proliferation, and regeneration (Van Winkle *et al.*, 1997). Naphthalene injury requires metabolism by cytochrome P450, and in the mouse, this is thought to be due to the CYP2F2 isoform, which is found abundantly in mouse Clara cells (Buckpitt *et al.*, 1992, 1995). Clara cells vacuolate as soon as 6 h after exposure and are dead and sloughed at 24 h. The surviving ciliated cells resorb their cilia and become squamated (Lawson *et al.*, 2002; Van Winkle *et al.*, 1996). Surviving cells undergo a proliferation phase that is maximal 2 days after exposure with proliferation occurring throughout the airway, even in airways where there has been no injury (Van Winkle *et al.*, 1995). These proliferated cells begin their migration at 4 days, followed by differentiation 7 days after exposure with a return to steady state at 14 days. The 6-h, 24-h, 4-day, and 7-day time points were examined in this study for both the 200 and the 300 mg/kg doses. At the higher 300 mg/kg dose, Clara cells are still the target, but the lesion extends proximally from the terminal bronchioles and can involve larger airways, such as the lobar bronchus (Plopper *et al.*, 1992). We used two doses in this study to determine if we will see a larger biological response as measured by increased tff expression when more of the airway tree is involved in the injury.

The TFF family contains three proteins (TFF1, TFF2, and TFF3) that are 7–12 kD and contain highly conserved motifs called

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trefoil domains (Taupin and Podolsky, 2003). TFF are intimately associated with mucins and mucous-producing cells during injury and repair in the stomach, intestine, and colon (Goke and Podolsky, 1996; Kindon *et al.*, 1995; Tomasetto *et al.*, 2000; Wright, 2001). TFF are also involved with mucosal epithelial restitution after cell loss through effects that are antiapoptotic, that promote cell migration (Hoffmann, 2005; Kinoshita *et al.*, 2000; Oertel *et al.*, 2001), and that enhance cell proliferation and differentiation (Bossenmeyer-Pourie *et al.*, 2002; Ulaganathan *et al.*, 2001). TFF2 is found more abundantly during repair in areas of proliferation and is thought to be important in gastric mucosal differentiation (Karam *et al.*, 2004; Ribieras *et al.*, 1998). Gene expression for *tff2* increases in areas of ulceration after acute injury but then returns to control levels shortly after (Wong *et al.*, 2000). TFF3 is increased in the colon after ulceration in the goblet cells at the edges of injury where it acts in a paracrine manner to prevent apoptosis (Taupin *et al.*, 2000) and promote migration of surviving cells for mucosal restitution (Kinoshita *et al.*, 2000; Liu *et al.*, 1997). The distribution of these factors has been very well defined in the epithelium of the gastrointestinal (GI) tract but has not been established in the airway epithelium of the lung.

There is some information available about TFF in lung but much of it is derived from end point quantitative PCR (qPCR) from whole-lung homogenates, which dilute the conducting airway epithelial component. All three TFF have been found in whole lung, but their abundance differs depending on the species. TFF1 is expressed in low amounts in both mouse Clara cells (Hertel *et al.*, 2004) and human airway mucous and ciliated cells (dos Santos Silva *et al.*, 2000). TFF1 expression increases in a mouse allergic lung disease model where it is associated with transdifferentiating Clara cells during mucous metaplasia (Kouznetsova *et al.*, 2007). TFF2 is also induced in allergic lung disease (Nikolaidis *et al.*, 2003, 2006). The cellular location of TFF2 in the normal mouse airway epithelium, which rarely contains mucous cells in steady state, has not been determined, likely due to the lack of a specific antibody. TFF3 has its highest expression in the adult human lung, where it is found primarily in goblet cells of the trachea and bronchi in association with MUC5B and in the submucosal glands in association with MUC5AC (dos Santos Silva *et al.*, 2000; Wiede *et al.*, 1999). TFF3 increases ciliated cell differentiation, and this increase is abrogated by inhibition of the epidermal growth factor receptor (EGFr) (LeSimple *et al.*, 2007). Gene expression in whole-lung samples from C57/Bl6 mice showed no *tff3* transcript (Hertel *et al.*, 2004). In the current study, we investigated all three tff on the same samples and endeavored to define both the distribution and the abundance of protein and messenger RNA (mRNA) for TFF in the conducting airways of the mouse.

The goal of the current study was to define the expression of TFF both in normal mouse lung and in the mouse lung exposed to a surrogate toxicant, naphthalene, with the goal of determining if these factors may play a role in conducting airway epithelial wound healing as well as act as markers of epithelial damage. We hypothesized that the expression of all three tff will increase in the airway epithelium during acute injury, squamation, and early proliferation (6 and 24 h), as they do in the gut. During the repair phase, tff1 and tff3 should increase after proliferation has completed and there is migration (4 days) and differentiation of those proliferated cells (7 days) if the role in airways is similar to the GI tract.

METHODS

Reagents. Naphthalene (NA) was purchased from Aldrich Chemical (Milwaukee, WI). The oleic safflower oil (trans-fat-free oil) was manufactured by and a gift of the Adam's Specialty Oil Company (Arbuckle, CA). Naphthalene was dissolved in trans-fat-free oil as a 20 mg/ml (250 mg NA in 12.5 ml oil) or a 30 mg/ml (375 mg NA in 12.5 ml oil) solution 1–2 days before use and kept at −20°C until use.

Animals. Male NIH Swiss mice from Harlan were maintained on a 12-h light/dark cycle with food and water *ad libitum* for 5 days before use. Mice were housed in cages with high efficiency particulate air-filter tops in American Association for Accreditation of Laboratory Animal Care-approved facilities. Animal use protocols were approved by the University of California, Davis, Institutional Animal Care and Use Committee. The mice were injected at the same time of day, between 8:00 and 10:00 A.M. Mice were given either 200 or 300 mg/kg NA or an equivalent volume per body weight of the oil carrier. Animals were killed at 6 h, 24 h, 4 days, or 7 days after naphthalene or vehicle injection by an overdose of pentobarbital ip ($N = 4$–8 per group). The trachea was cannulated and lungs were removed from the chest. Lungs for paraffin embedding and sectioning were fixed at 30 cm of pressure with 1% paraformaldehyde through the tracheal cannula, stored in fixative for < 24 h, and embedded in paraffin. For RNA isolation, the lungs were inflated with RNAlater (Ambion, Austin, TX) and stored in RNAlater for a minimum of 24 h (Baker *et al.*, 2004).

Immunohistochemistry. Care was taken to standardize fixation and processing, so samples from different time points and doses could be compared. Paraffin sections (5 μm) from three mice per treatment group were immunostained. Cross sections of the lung tissue contained three to five terminal bronchioles and one to two larger airways per tissue section per animal, and representative sections were chosen for imaging. Sections from all groups were stained in the same immunochemical run to minimize run-to-run variability and to facilitate cross-group comparisons. A series of dilutions were used to determine optimal antibody concentration. The dilution at which there was moderate positive tissue staining and minimal background staining was chosen. Endogenous peroxidase was blocked with a 10% peroxide solution, and nonspecific binding was blocked with IgG-free bovine serum albumin (Jackson ImmunoResearch Laboratories, West Grove, PA) for 30 min at room temperature. Endogenous biotin was blocked using an Avidin/Biotin blocking kit (Vector Laboratories, Burlingame, CA). TFF1 was detected using a rabbit anti-TFF1 serum (kindly provided by Catherine Tomasetto, PhD) at a dilution of 1:5000 (Kouznetsova *et al.*, 2007). TFF3 was detected using a rabbit anti-TFF3 serum (kindly provided by Daniel Podolsky, MD) at a dilution of 1:5000 (Bergstrom *et al.*, 2008). A biotinylated anti-rabbit antibody cross-absorbed against other species (Jackson ImmunoResearch Laboratories) was used as a secondary, followed by Streptavidin-horseradish peroxidase from the Vectastain Rabbit Kit (Vector Laboratories). The signal was detected with nickel chloride–enhanced 3′,3′-diaminobenzidine tetrahydrochloride. Controls included the substitution of primary antibody with normal sera from rabbits or with PBS and did not have positive staining (see Supplementary fig. 1). Detection of Clara cell secretory protein (CCSP) was accomplished using rabbit anti-CCSP (BioVendor, Candler, NC), and mouse anti-β-tubulin IV (Biogenex, San Ramon, CA) was imaged as previously described using wide band excitation and emission filter sets (Fanucchi *et al.*, 1997), with the exception

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that the secondary antibodies were bound to different fluorochromes. Secondary antibodies (Invitrogen, Carlsbad, CA) were anti-rabbit IgG with Alexa 568 (red) to detect CCSP and anti-mouse IgG with Alexa 488 (green) to detect β-tubulin IV.

Reverse transcriptase-PCR on microdissected airways. To enrich the sample for airway-associated gene expression, the airways were microdissected from the surrounding parenchyma after 24 h in RNAlater at 4°C as previously described (Baker *et al.*, 2004). RNA was isolated with Qiagen RNeasy Plus Mini kit (Catalog # 74134; Qiagen, Valencia, CA) using a protocol slightly modified from the manufacturers instructions. Complementary DNA (cDNA) was made using the TaqMan Reverse Transcription Kit reagents (Applied Biosystems, Foster City, CA). One microgram of RNA was added to RT Buffer (final concentration of 1×), $MgCl_2$ (2.5mM), deoxyribonucleotide triphosphates (0.5mM), random hexamers (2.5μM), placental ribonuclease inhibitor (0.4 U/μl), and Multiscribe reverse transcriptase (1.25 U/μl). The reaction was run for four cycles (25°C for 10 min, 48°C for 30 min, and 95°C for 5 min).

Quantitative PCR. All reagents for the qPCR, except the custom-made primers and probes, were obtained from Applied Biosystems. mRNA expression in dissected airways was measured using cDNA from the previous step and Taqman primers and probes (MWG Biotech, High Point, NA) on the ABI 7500 Fast System. The samples were run in duplicate with glyceraldehyde 3-phosphate dehydrogenase (GAPD) as the internal reference gene. Individual *tff1* and *tff3* PCRs contained 1X TaqMan Fast Mastermix, 5 μl cDNA, 600 nm 5′ and 3′ primers, and 250 nm probe. The *tff2* and *foxj1* PCRs contained 1X TaqMan Fast Mastermix, 5 μl cDNA, and the pre-made gene assay, which contained 900 nm 5′ and 3′ primers and 100 nm probe. *tff1*, *tff3*, and *GAPD* primers and the associated probe tagged with TAMRA (5′) and FAM (3′) were made using the Primer Express Software (Applied Biosystems). A TaqMan gene expression assay for *tff2* (Mm01348265_m1; Applied Biosystems) and *foxj1* (Mm00807215_m1; Applied Biosystems), which contained the primers and probe together, was used to detect the respective mRNA sequences. The *tff2* and *foxj1* probes were tagged with FAM on the 5′ end and minor groove binder on the 3′ end. *tff1*, *tff3*, and *GAPD* probes were tagged with a 5′ fluorescent tag, TAMRA, and a 3′ FAM quencher. *tff1* sequences used were 5′ primer GGGATTCCCGTGGTGCTT, 3′ primer TGGACCTTAGAAGGGACA-TTCTTC, and probe CCCATGGCCATCGAGAACACTCAAGA. *tff3* sequences used were 5′ primer TGTCACATCGGAGCAGTGGTAAC, 3′ primer GCA-CCAGGGCACATTTGG, and probe ACCGTGGCTGCTGCTTTGACTCCA. *GAPD* sequences were 5′ primer TGTGTCCGTCGTGGATCTGA, 3′ primer CCTGCTTCACCACCTTCTTGA, and probe CCGCCTGGAGAAACCTGCC-AAGTATG. The reaction protocol consisted of 1 cycle at 95°C for 20 s and 40 cycles at 95°C for 3 s, followed by 60°C for 30 s.

Statistics and calculations. Fold change gene expression in microdissected airways from three to nine animals per time point was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). The tff and foxj1 cycle threshold (Ct) values were normalized to the Ct values of the endogenous reference (GAPD) to get a ΔCt value. These values were then standardized to the time-matched oil controls or to the *tff1* airway values (for Fig. 1 only), and data for each time point and *tff* were analyzed by ANOVA with Fisher's *post hoc* test to determine treatment effects. Means with SE of the mean are shown. Data were significant at a p value less than 0.05.

RESULTS

Comparison of Airway versus Whole-Lung Expression of tff

To facilitate comparison of these data with previous studies, the degree of enrichment or depletion of various *tff* in whole-lung tissue and microdissected airways from control lung tissue was compared (Fig. 1). *tff1* abundance was equivalent in both the whole-lung sample and the airway sample (Fig. 1A),

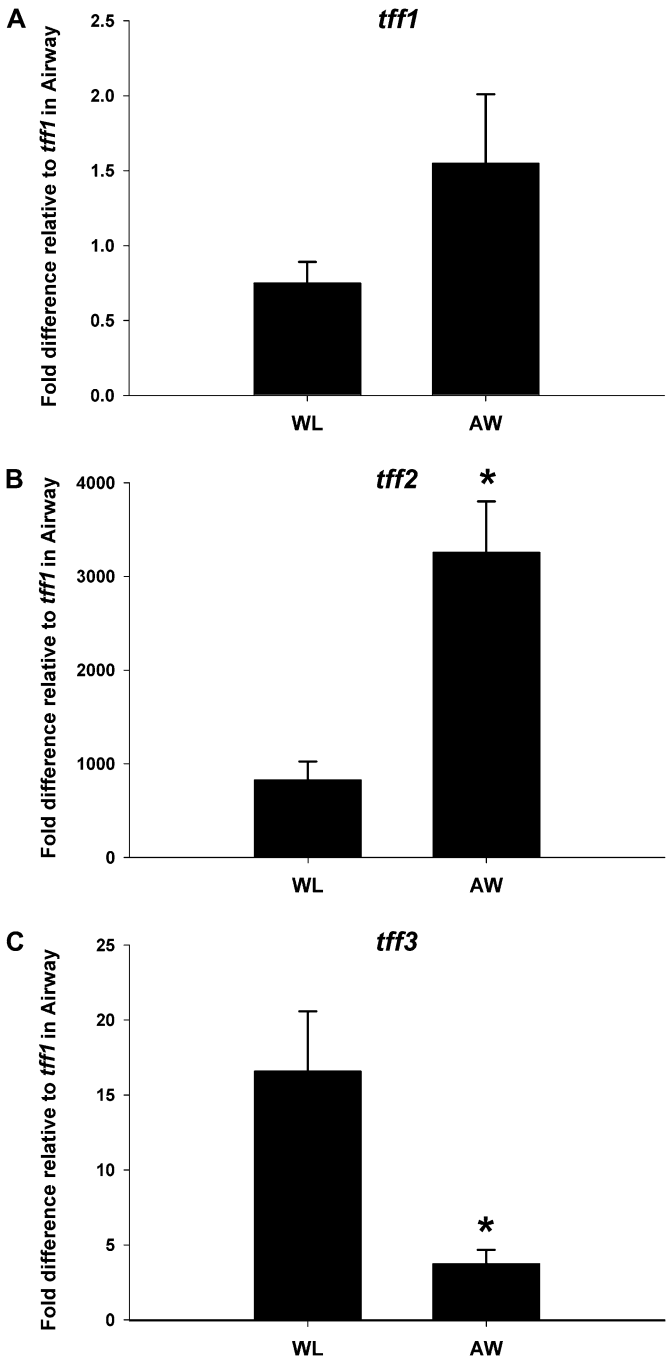

FIG. 1. Comparison of gene expression for all three *tff* in airways (AW) versus whole lung (WL) from 24-h oil control mice. (A) *tff1* was not differentially expressed in airways compared to whole lung. (B) *tff2* was enriched in the airway-specific homogenate threefold. (C) In contrast, *tff3* was significantly less abundant in the conducting airways than in a whole-lung homogenate. Results were calculated using the $\Delta\Delta Ct$ method. GAPD was the reference gene. For ease of comparison of the different tff with each other, all data are expressed as fold difference from *tff1* in the airways. Means and SEs indicate data obtained from eight different animals per end point. *Significantly different from whole lung at $p < 0.05$.

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and *tff3* was more abundant in the parenchyma as the airway sample had significant depletion compared to whole lung (Fig. 1C). Only *tff2* was significantly enriched in the microdissected airway samples (Fig. 1B). In these uninjured tissue samples, the steady-state abundance of *tff2* was the greatest of all three tff analyzed. Both *tff1* and *tff3* were present at much lower levels than *tff2*. The relative abundance of the tff in airways and whole lung was *tff2* > *tff3* > *tff1*.

TFF Expression 6 h after Naphthalene Exposure

Six hours after systemic naphthalene exposure, Clara cells in the terminal bronchioles were swollen and starting to detach from the basement membrane. This change was similar at both doses of naphthalene; however, the lesion was more extensive at the higher dose, extending proximally from the terminal bronchiole at the 300 mg/kg dose. There were no changes in the terminal bronchioles in the control samples from oil-treated mice. The degenerating Clara cells in the terminal bronchioles had abundant expression of both TFF1 and TFF2 proteins. This signal involved the entire cytoplasm and was often intense (Figs. 2B, 2C, 2E, and 2F). Control animals had minimal to moderate TFF1 and TFF3 expression signals in the terminal bronchioles, which was primarily limited to Clara cells and usually in the apical cytoplasm (Figs. 2A and 2D). Cells consistent in location with peribronchiolar-attenuated

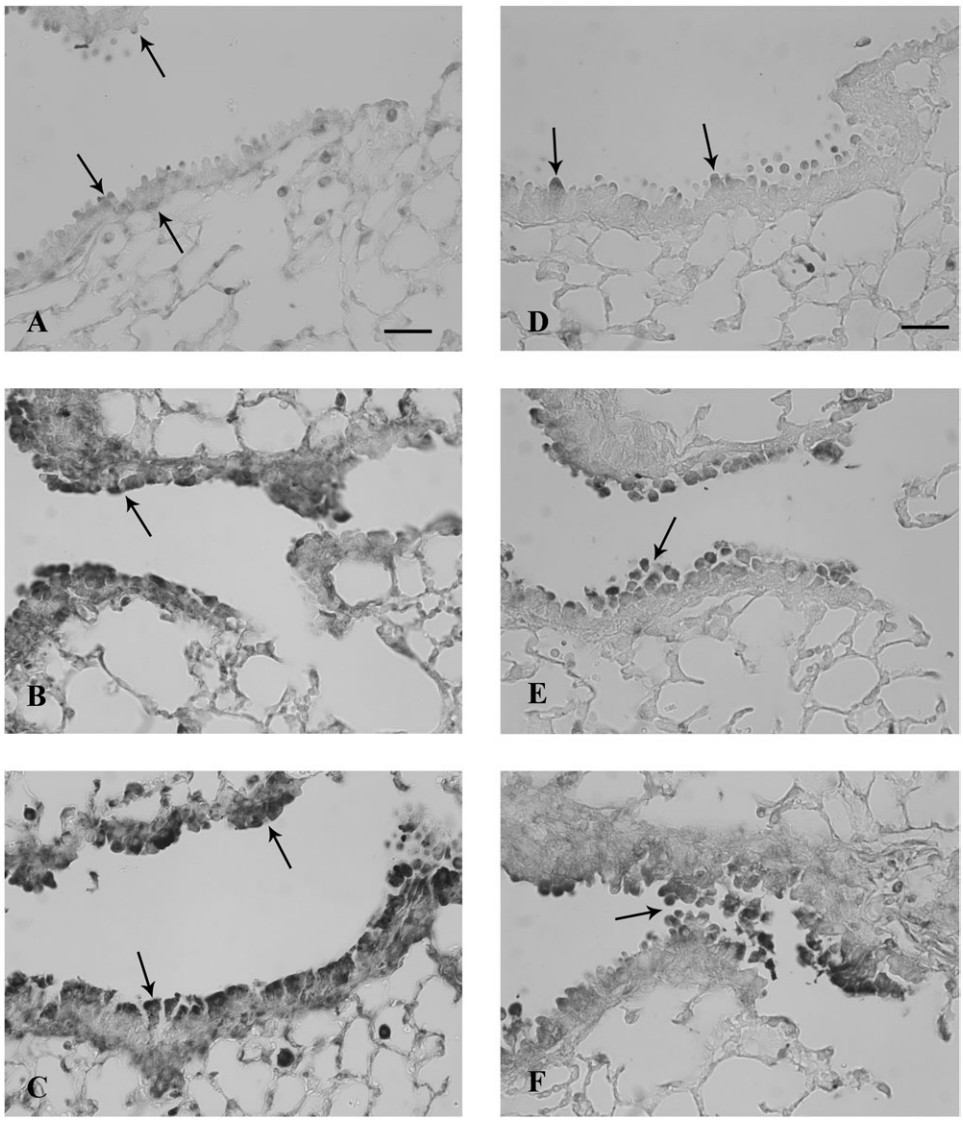

FIG. 2. TFF protein expression in airways 6 h after systemic naphthalene exposure. Immunohistochemical detection (black staining) of TFF1 (A, B, and C) and TFF3 protein (D, E, and F) in terminal bronchioles. Oil controls (A and D) have light staining on the apex of Clara cells and in the peribronchiolar mesenchyme (arrows). Treatment with either 200 mg/kg naphthalene (B and E) or 300 mg/kg (C and F) increased epithelial staining (arrows) in degenerate Clara cells for both TFF1 and TFF2. TFF3 was also expressed in large round cells in alveoli, consistent with macrophages (C). Three mice were analyzed for each group at this time point (200 and 300 mg/kg naphthalene and time-matched oil control). Bar = 50 μm.

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fibroblasts expressed TFF1, in both control and exposed animals at all airway levels (Figs. 2A–C). TFF2 protein expression in the tissue was not assessed at any time point due to the lack of an adequate antibody for the mouse. In contrast to the marked increase in focal protein expression at this time point, the mRNA expression from microdissected conducting airways was not significantly different from the oil control animals for all three *tff*, possibly indicating recruitment of tff protein from other sites (nonairway) for the acute phase of the injury response (Figs. 3A–C). Further the *tff* expression was not significantly different from other *tff* at either dose level.

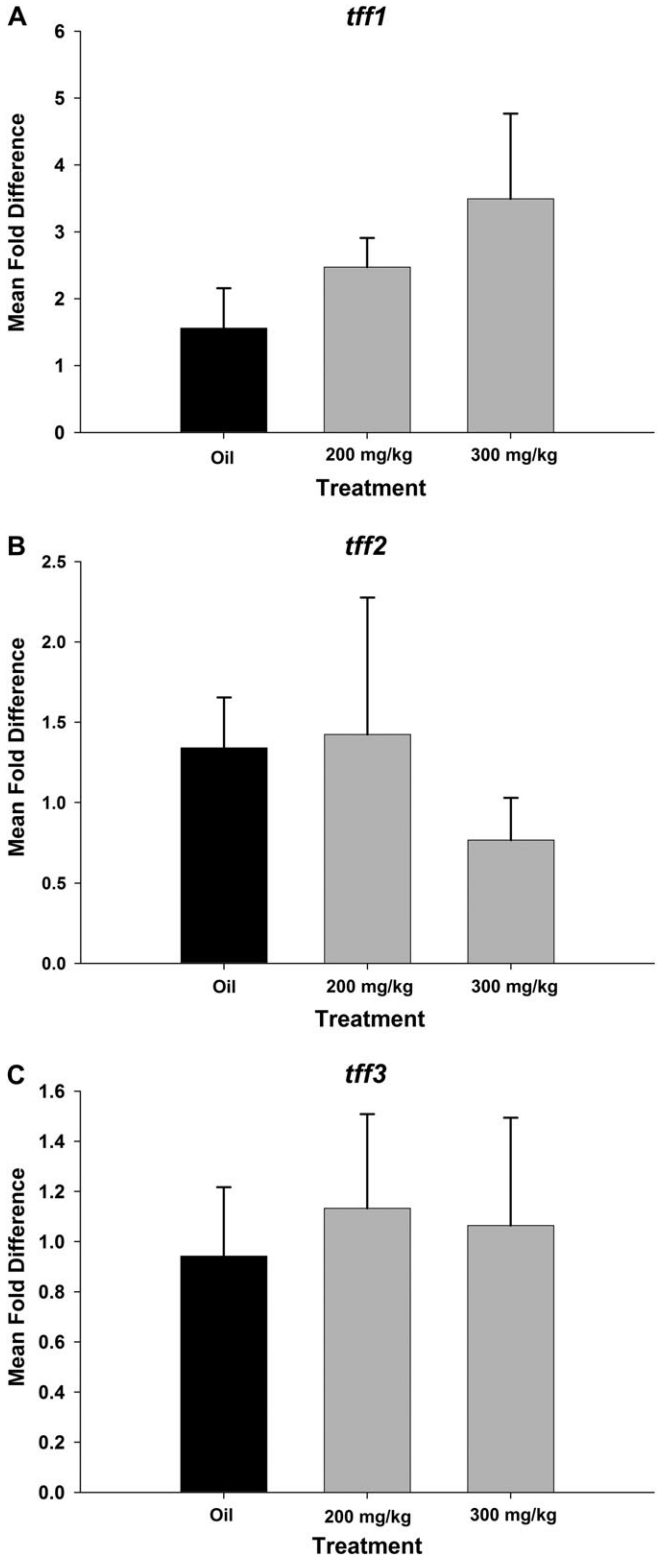

FIG. 3. *tff* mRNA expression in airways 6 h after systemic naphthalene exposure. The fold change in *tff* mRNA expression was calculated by normalizing the raw expression to GAPD (housekeeping gene) and standardizing it to the oil controls. Expression of (A) *tff1*, (B) *tff2*, and (C) *tff3* was not significantly different from the vehicle (oil) control at 6 h after naphthalene injury at either dose (200 or 300 mg/kg). Results were calculated using the $\Delta\Delta Ct$ method. GAPD was the reference gene. Each tff factor is compared to its respective time-matched corn oil control. Means and SEs are from data obtained from a minimum of seven different animals per treatment group.

TFF Expression 24 h after Naphthalene Exposure

Twenty-four hours after systemic naphthalene exposure, the majority of Clara cells in the terminal bronchioles were sloughed from the basement membrane. The terminal bronchioles were lined with a single layer of squamated cells with little to no associated inflammation. This change was similar in both exposure groups with more extensive proximal airway involvement in the 300 mg/kg group. As with the 6-h time point, exfoliating cells had an intense signal for both TFF1 and TFF3, which was more intense in the sections than the TFF1- and TFF3-positive squamated cells (Figs. 4B, 4C, 4E, and 4F). tff1 expression in the squamated cells often involved the entire cytoplasm and adjacent matrix (Figs. 4B and 4C), while the TFF3 signal was restricted to the apical portion of the squamated cells (Figs. 4E and 4F). Abluminal peribronchiolar cells, possibly attenuated fibroblasts as described by Evans *et al.* (1993), continued to express TFF1 (Figs. 4A–C). Along with the TFF1 protein expression, the mean fold change in *tff1* mRNA expression in pulmonary airways was increased (Fig. 5A). The increase at the 300 mg/kg dose was statistically significant from the oil controls as well as the 200 mg/kg dose level ($p < 0.05$) (Fig. 5A). In contrast, *tff2* gene expression was significantly decreased for the high-dose group (Fig. 5B). *tff2* and *tff3* mRNA expression in intrapulmonary airways at both exposures was not significantly different from the oil control group (Fig. 5C).

TFF Expression 4 Days after Naphthalene Exposure

Four days after systemic naphthalene exposure, cells in the terminal bronchioles predominantly were of a low cuboidal morphology for both the 200 and the 300 mg/kg doses. Both TFF1 and TFF3 were expressed in the terminal bronchiolar epithelium of naphthalene-exposed mice (Figs. 6C, 6E, and 6F) at higher levels than oil control mice (Figs. 6A and 6D). In the oil control animals, cells with cytoplasmic expression of TFF1

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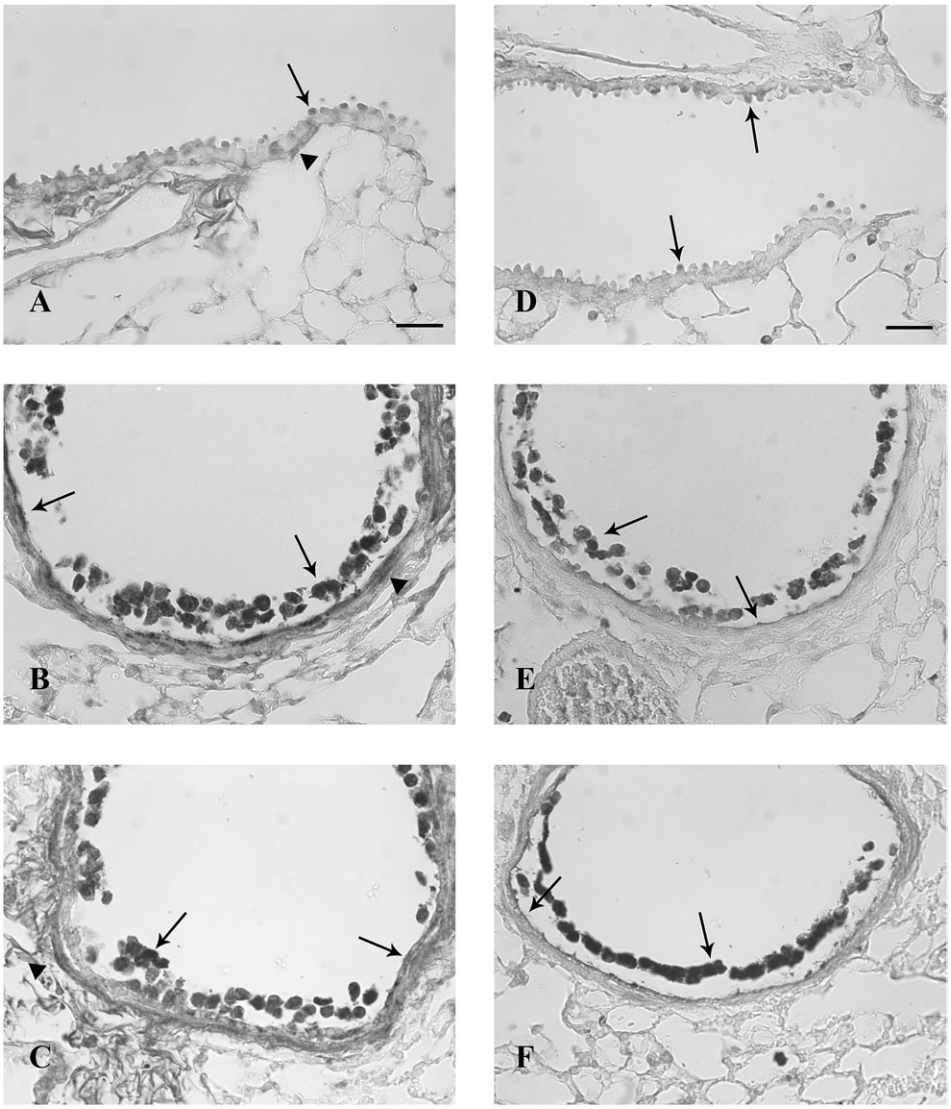

FIG. 4. TFF protein expression in airways 24 h after systemic naphthalene exposure. Immunohistochemical detection (black staining) of TFF1 (A, B, and C) and TFF3 protein (D, E, and F) in distal bronchioles. Oil controls (A and D) have less intense protein expression than the naphthalene exposed (B, C, E, and F). There does not appear to be a difference in level of expression for TFF1 in degenerate Clara cells in the airway lumen when the two doses (200 mg/kg; B) are compared (300 mg/kg; C). However, there is a dose-dependent difference in the intensity of staining for TFF3 with the 300 mg/kg dose having more intense expression (F) than the 200 mg/kg dose (E). Full arrows indicate expression in normal (A and D), dead, and sloughed Clara cells (B, C, E, and F) and newly squamated cells (B, C, E, and F), while arrowheads show TFF1 expression in the peribronchiolar mesenchyme (A–C). Three mice were analyzed for each group at this time point (200 and 300 mg/kg naphthalene and time-matched oil control). Bar = 50 μm.

and TFF3 had the characteristic Clara cell apical protrusions into the airway lumen and peribronchiolar cells again expressed TFF1 in all groups (Figs. 6A–C). In some of these cells, the expression was primarily apical, while the entire cytoplasm of a few cells had a signal for TFF1 and TFF3. tff-positive cells often did not have apical blebs characteristic of differentiated Clara cells. Low cuboidal epithelium that lacks the normal differentiated pattern of expression of Clara cell (CCSP positive) and ciliated cell (β-tubulin IV) markers is common at 4 days following napththalene injury as the epithelium is regenerating and redifferentiating at this time point (Figs. 7A and 7B). The mRNA expression of *tff1* increased in both the 200 and the 300 mg/kg exposure levels, and this increase was dose dependent with mean fold changes of 10.6 in the 200 group and 44.2 in the 300 group as compared to oil controls (Fig. 8A). The 200 mg/kg mean increase was not statistically significant due to variability between animals. The 300 mg/kg dose was statistically significant from the oil group and the 200 mg/kg group as well ($p < 0.05$) (Fig. 8A). There were no significant changes in mean *tff2* and *tff3* fold changes between exposure groups and oil controls (Figs. 8B and 8C).

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TFF Expression 7 Days after Naphthalene Exposure

Seven days after systemic naphthalene, cells in the terminal bronchioles were cuboidal and occasionally had a domed appearance. The cells in the 300 mg/kg group had a low cuboidal appearance. The TFF1 expression was similar to that in oil controls (Figs. 9A and 9D) in that the signal was restricted to the apical portion, especially in the 200 mg/kg group. However, there were a few cells in the terminal bronchiole that had TFF1 and TFF2 signals in the entire cytoplasm. This was more prominent in the 300 mg/kg dose groups (Figs. 9B, 9C, 9E, and 9F). Cells at the branch points nearest the terminal bronchiole had a more intense TFF3 signal in both naphthalene dose groups versus the oil controls (Figs. 9E and 9F). The peribronchiolar cells continued to express TFF1 (Figs. 9A–C). There was a statistically significant increase in *tff1* mRNA expression in the high-dose naphthalene group from the oil control group (Fig. 10A). However, the fold increase was less than at the previous time point (9-fold vs. a previous high of 40-fold increase over controls). There were no significant differences in fold change *tff2* mRNA from oil controls in either exposure group, although the two naphthalene-treated groups were different from each other (Fig. 10B). *tff3* mRNA expression was nearly increased significantly in the 200 mg/kg group versus the oil ($p = 0.058$; Fig. 10B). There was also an increase in *tff3* mRNA in the 300 mg/kg group with a mean fold change of 10 (1–25), but this was not statistically significant. Along with the increase of *tff3* mRNA at 7 days, there was also an increase in ciliated cell marker *foxj1* mRNA expression (Fig. 10D). The *foxj1* increases were statistically significant from the oil controls in all four time points and both dose groups at the 6-, 24-h, and 4-day time points (except for the 200 mg/kg group at 4 days) (data not shown).

Overview of TFF Protein Expression during Naphthalene Injury and Repair

Expressions of TFF1 and TFF3 protein increased at 6 and 24 h after 200 and 300 mg/kg of naphthalene. This protein expression was most evident in the degenerate (6 h) and dead cells (24 h) along with the squamated cells at 24 h (Fig. 10) where TFF1 was punctate and in the entire cytoplasm and TFF3 was in the apical portion of the squamated cells. The TFF1 and TFF3 protein expressions at 4 and 7 days after naphthalene showed slight differences from the oil control animals at the same time points

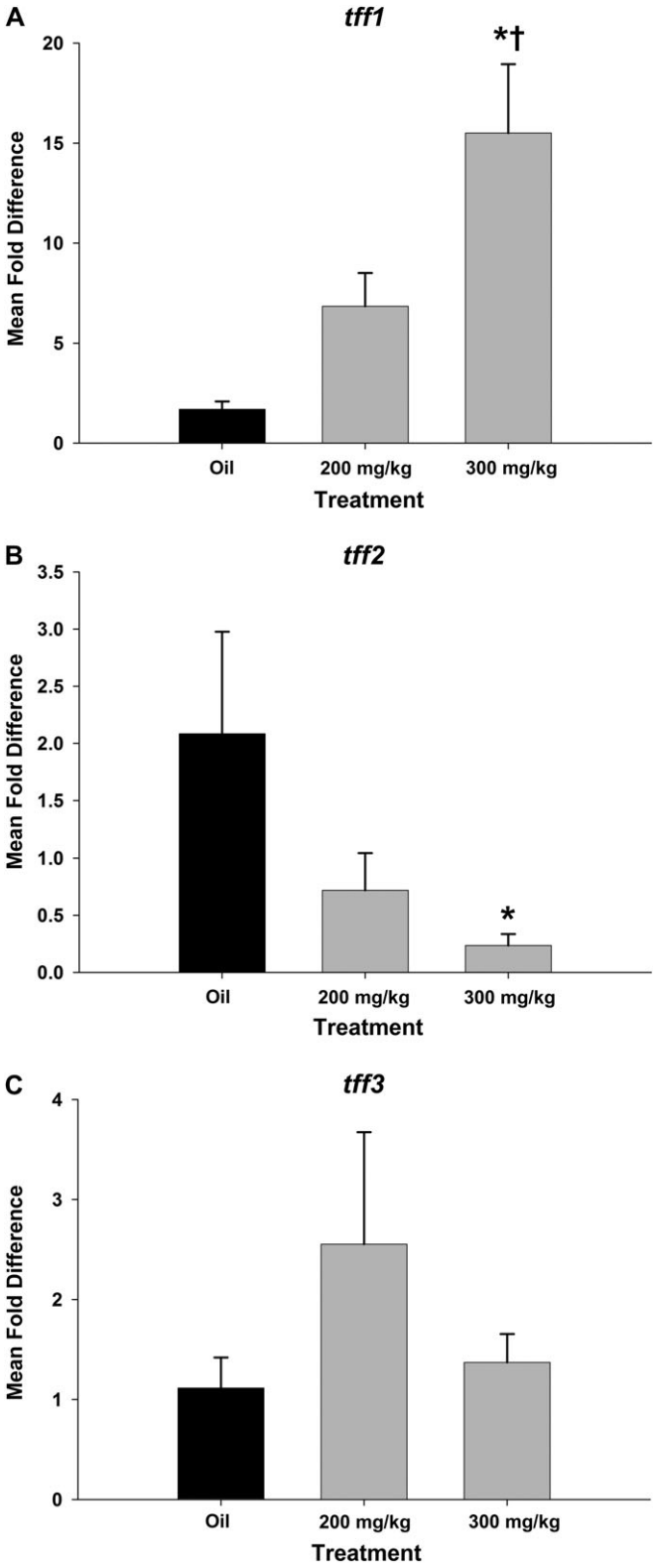

FIG. 5. *tff* mRNA expression in airways 24 h after systemic naphthalene exposure. *tff* Fold change in mRNA expression was calculated by normalizing the raw expression to GAPD (housekeeping gene) and standardizing it to the oil controls. *tff1* expression (A) was significantly increased only in the 300 mg/kg naphthalene exposure group. In contrast, *tff2* (B) expression is significantly decreased at 300 mg/kg. *tff3* (C) did not change significantly. *Significantly different from the oil control group, $p < 0.05$. †Significantly different from 200 mg/kg naphthalene treated, $p < 0.05$. Results were calculated using the $\Delta\Delta Ct$ method. GAPD was the reference gene. Each TFF factor is compared to its respective time-matched corn oil control. Means and SEs are from data obtained from a minimum of six different animals per treatment group.

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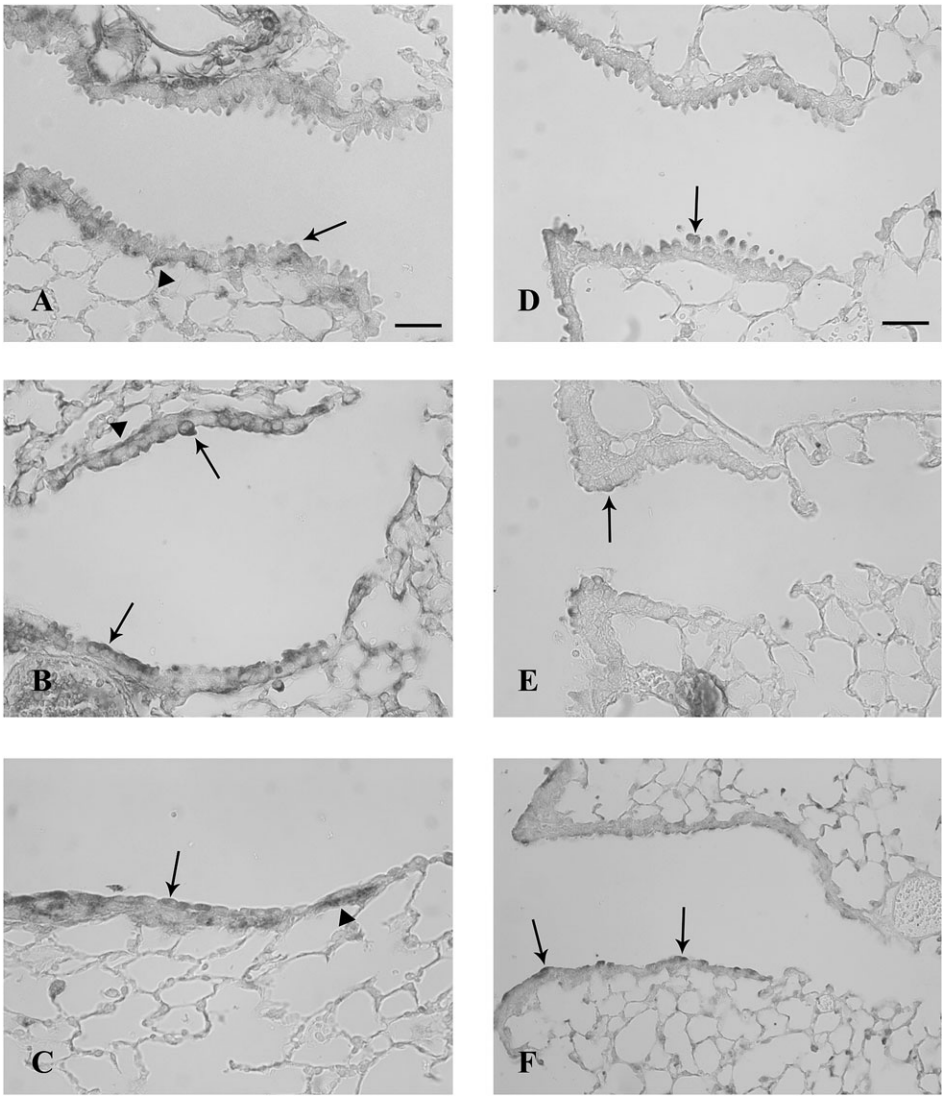

FIG. 6. TFF protein expression in airways 4 days after systemic naphthalene exposure. Immunohistochemical detection (black-gray staining) of TFF1 (A, B, and C) and TFF3 protein (D, E, and F) in terminal bronchioles. Oil controls (A and D) have similar staining to the naphthalene exposed (B, C, E, and F). TFF1 expression was localized to the apex of Clara cells (arrows) and peribronchiolar mesenchyme (arrowheads) in both the 200 mg/kg naphthalene (B) and the 300 mg/kg groups (C). TFF3 was focally expressed in the apex of low cuboidal epithelium most prominently in the 300 mg/kg exposure group (F) and at terminal bronchiole bifurcations in the 200 mg/kg (E) group. Three mice were analyzed for each of the three treatment groups at this time point (200 and 300 mg/kg naphthalene and time-matched oil control). Bar = 50 μm.

with prominent expression at airway branch points, and the cytoplasmic distribution was primarily apical (Fig. 11).

DISCUSSION

This is the first study to document the conducting airway-specific tissue and mRNA expression of all three tff during repair of acute bronchiolar epithelial injury. tff expression in the naphthalene-treated groups was often dose dependent, and greater significance for the gene expression studies was achieved in the high-dose group, likely due to the greater involvement of more proximal airways in the bronchiolar tree than at the low dose. Injury following the 300 mg/kg naphthalene extended to the major daughter bronchioles, while the 200 mg/kg dose affected only distal bronchioles (Plopper *et al.*, 1992). *tff1* mRNA expression increased at all time points after naphthalene exposure with the greatest increases during the squamation and cell migration phases (4 days) of repair (Van Winkle *et al.*, 1997). *tff3* mRNA increased during the early differentiation phase (7 days), while *tff2* was significantly decreased at the end of cell damage and the beginning of the proliferation phase (24 h), possibly correlating with the loss of differentiated Clara cells. As the cells

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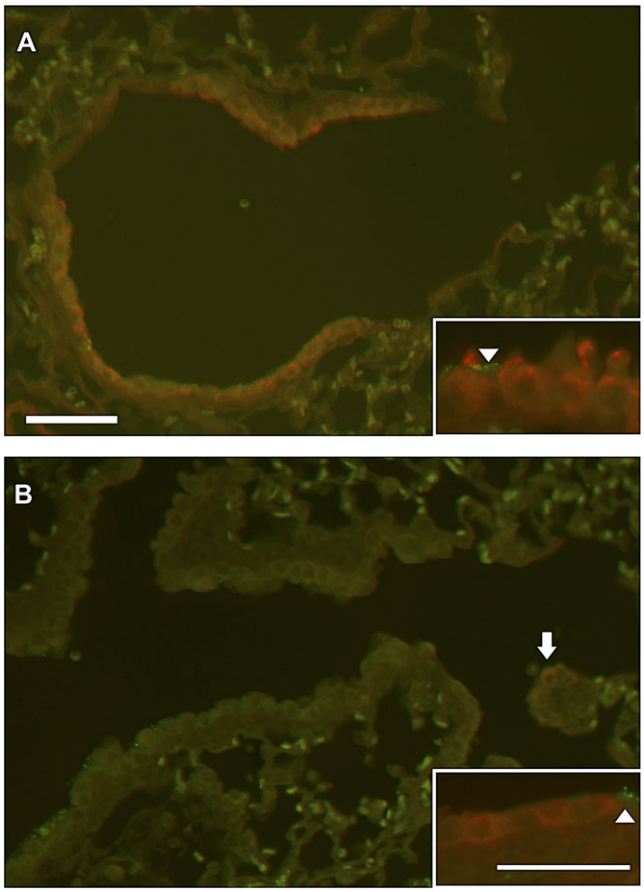

FIG. 7. Cell differentiation in the terminal bronchiole at 4 days after injury from 200 mg/kg naphthalene. CCSP (red staining) for differentiated Clara cells and β-tubulin IV as a marker for cilia (green staining) in the mouse terminal bronchiole of an oil-treated control (A) and a naphthalene injured and repairing airway (B). The control airway contains abundant differentiated Clara cells with characteristic apical domes (inset in A) protruding into the airway and ciliated cells (arrowheads). Terminal bronchiole from the naphthalene-treated animal does not contain abundant CCSP or β-tubulin IV (B). Clara cells that are positive for CCSP (arrow) do not have the domed apical protrusions and are low cuboidal (inset in B). These were found at the terminal bronchiole alveolar duct junction (arrowhead) and in larger more proximal airways including bifurcations. A minimum of three mice were analyzed for each of the treatment groups. Bar = 50 μm.

regenerated, the levels of *tff2* gene expression approached baseline levels (4–7 days). These results indicate that changes in tff expression, in particular the robust increase in *tff1*, are good markers of airway epithelial damage and repair. Figure 11 summarizes the expression pattern of TFF protein during the injury and repair process. In summary, all three TFF were found in the airways and whole lung of Swiss mice. All three tff underwent shifts in cellular expression and relative abundance during the injury repair process: TFF1 and TFF3 increased and TFF2 decreased.

Despite a paucity of information of how TFF cause their effects, tff have been implicated as potential modifiers of allergic lung disease and lung function, possibly through interactions with receptors on cells, although none specific to tff have been identified. TFF3 is thought to regulate ciliated cell differentiation via interactions with the EGFr (LeSimple *et al.*, 2007). EGFr has previously been shown to be increased during naphthalene injury and repair (Van Winkle *et al.*, 1997). However, the role of TFF in the epithelium in response to injury by an environmental toxicant has not previously been described. Mouse models of allergic lung disease have found that both TFF1 (Kouznetsova *et al.*, 2007) and TFF2 (Nikolaidis *et al.*, 2003, 2006) are increased. TFF expression is associated with transdifferentiation of Clara cells to mucous cells in these allergic disease models (Hoffmann, 2005, 2007; Kouznetsova *et al.*, 2007) and TFF1 stabilizes mucins by binding to their cysteine-rich von Willebrand factor C domains (Tomasetto *et al.*, 2000). The naphthalene injury/repair model results in an acute injury that repairs over time to return to steady state (Van Winkle *et al.*, 1997). Mucous cells are not present. What our current study shows is that TFF are involved in restitution of epithelial injury even without mucous cells. The large increases (and low relative background levels) of *tff1* further indicate that expression of *tff1* is an excellent marker of airway epithelial injury from as early as 24 h following exposure out to 7 days. *tff2* has been linked to a QTL on chromosome 17 associated with developmental differences in lung function in inbred mouse strains (Ganguly, 2007). Naphthalene injury has been shown to impair lung function 12 h after injection (Yildirim *et al.*, 2008). There is a significant decrease in *tff* gene expression at the 24-h time point, which might indicate a role for *tff2* in lung function impairment caused by naphthalene. Based on these observations, future investigation of the role of TFF2 in lung development as well as normal lung function appears warranted.

tff are central players in mucosal restitution after injury through their effects on cell proliferation and/or apoptosis. The degenerate epithelium in the terminal bronchioles in this study strongly expressed proteins for both TFF1 and TFF3 6 h after naphthalene exposure. However, *tff* gene expression was not significantly changed for all three tff at this time point. There are several possible explanations for this apparent discrepancy between gene and protein expressions. The increase in local TFF protein may occur post-translationally within the cells, by protein or message stabilization or reduced secretion of the protein, or may be the result of TFF produced at nonairway sites. The latter point is supported by a gradient of TFF1 protein expression between airway vasculature and the airway itself (Fig. 4C). Given the high levels of protein expression in the degenerate Clara cells 6 h after naphthalene, it is also possible that *tff* gene expression, especially that of *tff1* and *tff3*, was increased before the 6-h time point, the first time point at which the message was assessed in this study. The elevated amounts of TFF protein in degenerate Clara cells may be involved in prevention of anoikis (anchorage-dependent apoptosis). TFF1 has been shown to prevent chemical-induced apoptosis and anoikis by reducing the activities of caspases-3, -6, -8, and -9

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(Bossenmeyer-Pourie *et al.*, 2002), whereas TFF3 prevents anoikis by activating nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) (Chen *et al.*, 2000). Following naphthalene injury, Clara cells are lost by necrosis, which is characterized by vacuolization and cell swelling (Plopper *et al.*, 1992, 2001; Van Winkle *et al.*, 1995, 1999). Future assessment of the role of TFF1 and TFF3 in suppression of anoikis would need to include earlier time points.

Following injury, the epithelium must proliferate to repopulate the mucosa after Clara cell loss (Stripp *et al.*, 1995; Van Winkle *et al.*, 1995). Twenty-four hours after naphthalene exposure, squamated cells expressed both tff along with TFF1 and TFF3 in the dead cells. However, TFF1 was more extensively expressed in the entire cytoplasm of the squamated cell, whereas tff3 was just in the apical portion. At 24 h after 200 mg/kg naphthalene exposure, surviving cells within the terminal bronchioles are just starting to proliferate, as are more cuboidal cells in the proximal airways (Lawson *et al.*, 2002; Van Winkle *et al.*, 1995). The increased gene and protein expressions of TFF1 and slight increase of TFF3 protein expression in cells that are beginning to proliferate indicates a possible role of these TFF during proliferation. In the GI tract, TFF1 has been shown to have both antiproliferative and antiapoptotic activities, which are paradoxical (Bossenmeyer-Pourie *et al.*, 2002). Human neoplasms have an increased expression of TFF3, which may indicate its proproliferative activity (Leung *et al.*, 2002; Taupin *et al.*, 2001). The dual expression of TFF1 and TFF3 protein in the surviving squamous cells after naphthalene injury may indicate synergism in promoting proliferation of the squamated cells or they could be counterbalancing each other with antiproliferative and proproliferative activities.

Epidermal growth factor (EGF), EGFr, and transforming growth factor alpha (TGF alpha) are abundant in squamated cells 24 h after naphthalene exposure (Van Winkle *et al.*, 1997). TFF interact with TGF alpha, EGF, and EGFr in the gut and in cell culture systems. TFF3 has also been shown to transactivate the EGFr through an unknown mechanism (Taupin *et al.*, 1999). Mice that overexpress TGF alpha in the pancreas have a marked increase in TFF1 gene and protein expressions in pancreatic ducts, whereas wild-type mice had little to no TFF1 (Ebert *et al.*, 1999). A similar TFF1 increase was seen in the gastric mucosa of mice that generally overexpressed TGF alpha after induction of that gene (Goldenring *et al.*, 1996). Early increases in gene expression of *tff1* along with TGF alpha and EGF (as shown in

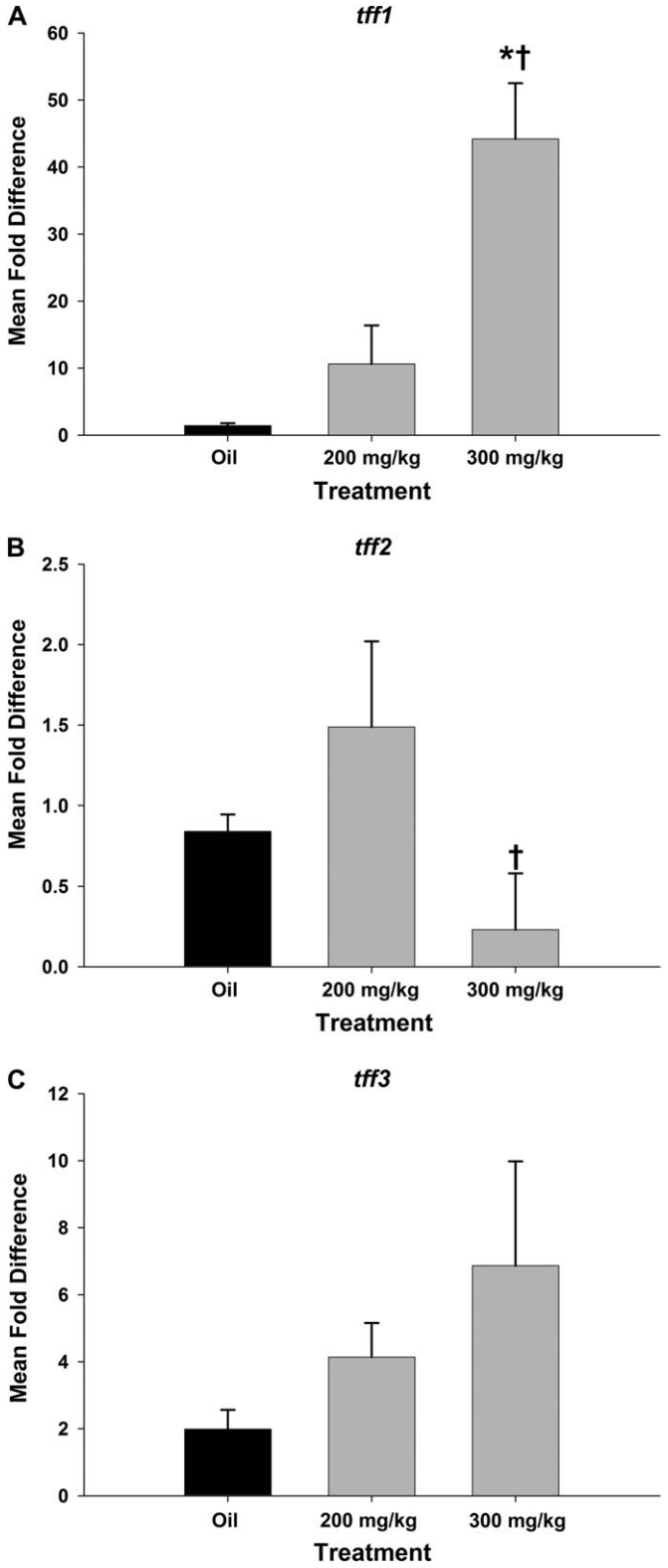

FIG. 8. *tff* mRNA expression in airways 4 days after systemic naphthalene exposure. *tff* fold change in mRNA expression was calculated by normalizing the raw expression to GAPD (housekeeping gene) and standardizing it to the oil controls. *tff1* (A) gene expression significantly increased >40-fold in the group exposed to a single high dose of naphthalene (300 mg/kg) compared to both oil controls and the low-dose group. In contrast, *tff2* gene expression (B) was significantly decreased when the high-dose group was compared to the low-dose group. *tff3* expression (C) is not changed. *Significant from oil control. †Significantly different from 200 mg/kg naphthalene treated ($p < 0.05$). Results were calculated using the $\Delta\Delta Ct$ method. GAPD was the reference gene. Each *tff* factor is compared to its respective time-matched corn oil control. Means and SEs are from data obtained from a minimum of six different animals per treatment group.

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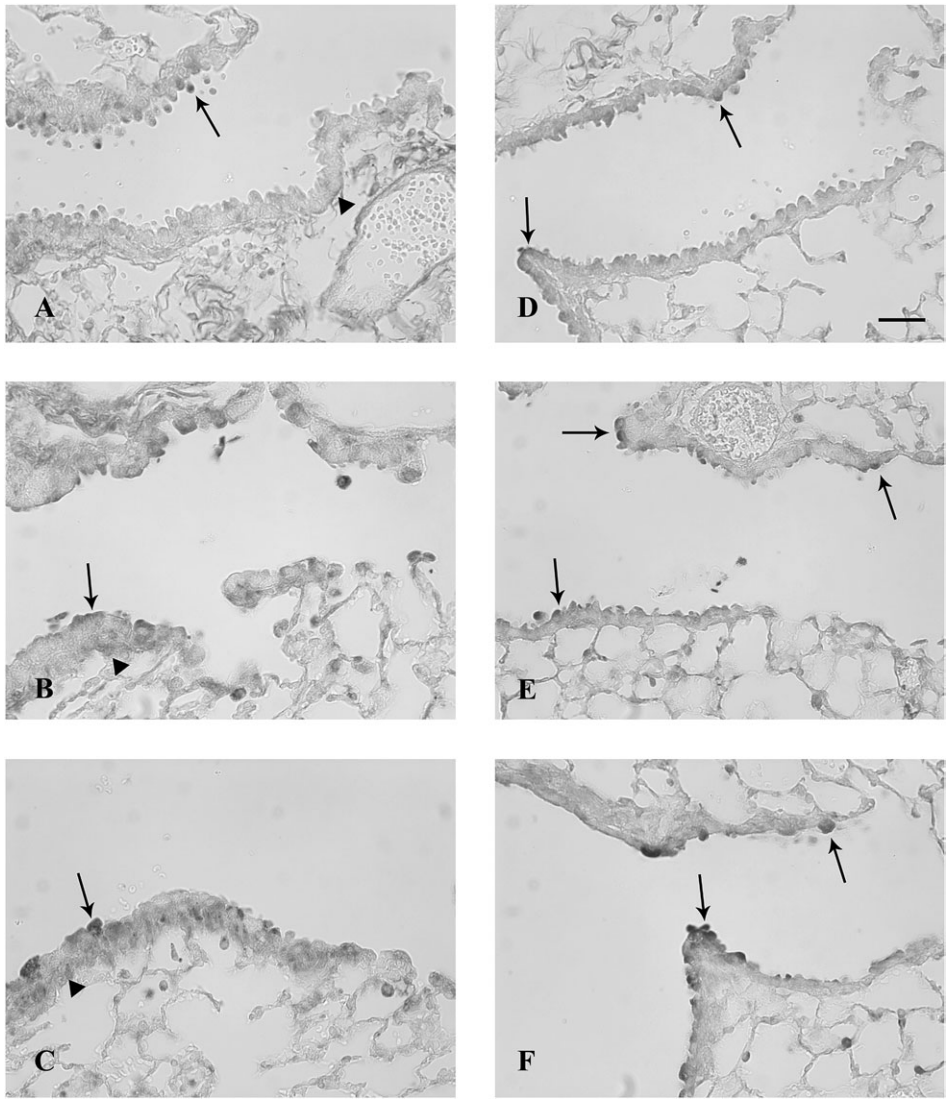

FIG. 9. TFF protein expression in airways 7 days after systemic naphthalene exposure. Immunohistochemical detection (black staining) of TFF1 (A, B, and C) and TFF3 protein (D, E, and F) in terminal bronchioles. Oil controls (A and D) have lighter more diffuse staining than the naphthalene exposed (B, C, E, and F). TFF1-positive cuboidal Clara cells (full arrows) and peribronchiolar mesenchymal cells (arrowheads) were lightly stained but well distributed throughout the terminal bronchiole (B and C) in the naphthalene-exposed groups. TFF3-positive Clara cells (arrows) tended to be more intensely stained and to cluster at airway bifurcations and at the bronchoalveolar duct junction. This was more obvious in the 300 mg/kg naphthalene-treated group (F). Three mice were analyzed for each treatment group at this time point (200 and 300 mg/kg naphthalene and time-matched oil control). Bar = 50 μm.

study Van Winkle *et al*., 1997) may indicate that TGF alpha and EGF induce TFF1 expression. The presence of *tff1* mRNA and protein expressions immediately following squamation and in early proliferation may indicate an important role for TFF1 during airway epithelial proliferation following cell injury and loss.

Four to 7 days after naphthalene exposure, surviving cells have finished proliferating and are beginning their migration/spreading and early differentiation (Van Winkle *et al*., 1997). In the terminal bronchioles, the flattened and low cuboidal cells have few markers of differentiated distal airway cells, cilia, or CCSP expression at 2–4 days following naphthalene injury. TFF1 and TFF3 immunoreactive protein was more intense in subpopulations of cells in the terminal bronchioles after naphthalene when compared to oil controls. This was especially notable for cells at airway bifurcations and at the terminal bronchiole alveolar duct junction, two foci of progenitor cells defined in previous studies (Giangreco *et al*., 2002; Stripp *et al*., 1995). In this model, these cells are the most differentiated at this time point. Possibly, these tff may regulate the involvement of these key sites in the repair response. *tff1* gene expression was very greatly increased in the high-dose naphthalene-exposed group at the 4-day (40-fold) and 7-day time points. TFF1 may either promote migration of cells and/or regulation of differentiation or may

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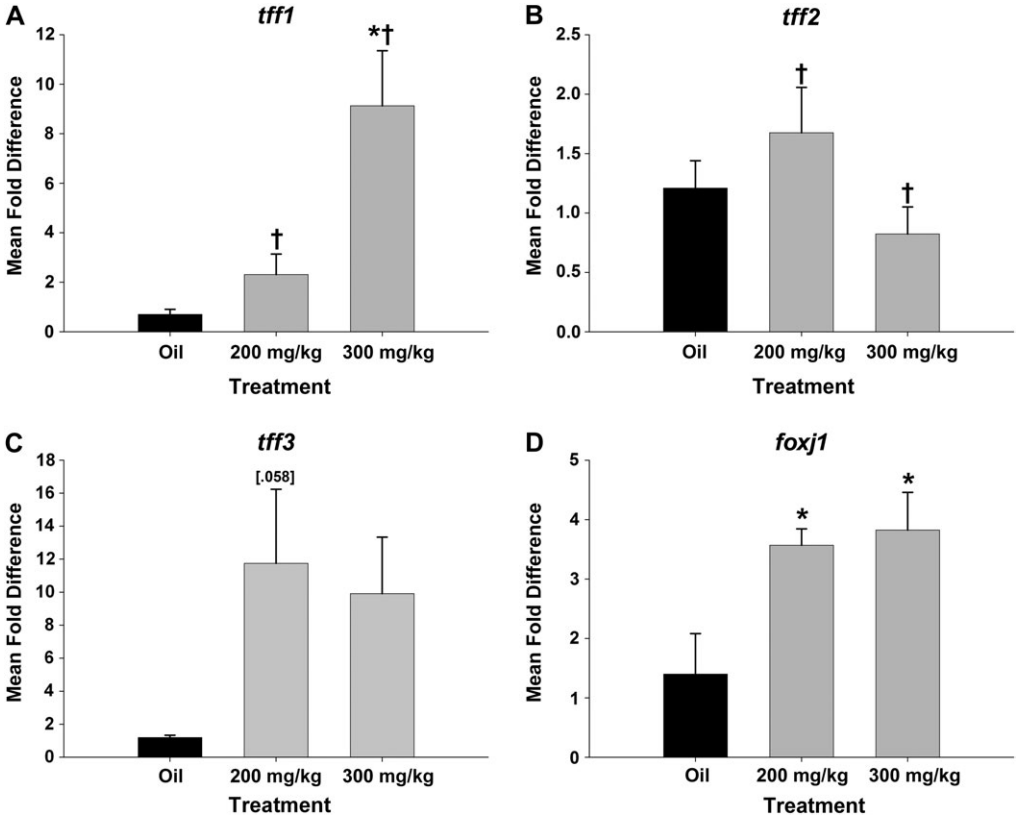

FIG. 10. *tff* mRNA expression in airways 7 days after systemic naphthalene exposure. *tff* fold change in mRNA expression was calculated by normalizing the raw expression to GAPD (housekeeping gene) and standardizing it to the oil controls. *tff1* (A) gene expression remained significantly elevated >eightfold in the group exposed to a single high dose of naphthalene (300 mg/kg) compared to both oil controls and the low-dose group. While *tff2* (B) gene expression was unchanged compared to oil controls for either treatment group. However, the high-dose naphthalene group (300 mg/kg) was significantly different from the low-dose group (200 mg/kg). *tff3* expression was not significantly different from corn oil controls for either treatment group, although the low-dose group just missed significant at $p = 0.06$. *foxj1*, the ciliated cell regeneration marker was significantly elevated at this time point in both groups that had received naphthalene treatment. *Significant from oil control. †Significantly different from 200 mg/kg naphthalene treated ($p < 0.05$). Results were calculated using the $\Delta\Delta Ct$ method. *GAPD* was the reference gene. Each gene is compared to its respective time-matched corn oil control. Means and SEs are from data obtained from a minimum of seven different animals per treatment group for A, B, and C. Means and SEs are from data obtained from three corn oil control animals and four naphthalene-treated animals in D.

be involved in termination of proliferative signals. Given the role that TFF1 plays in the stomach in allowing undifferentiated gastric cells to differentiate to mucous, parietal, or chief cells, TFF1 may have a similar role in the lung.

At 7 days, Clara cells are also redifferentiating, with CCSP and cytochrome P450 starting to be expressed (Van Winkle *et al*., 1996). The cells in both exposure groups were primarily cuboidal or low cuboidal, and the cells were not fully differentiated Clara or ciliated cells. Like the 4-day time point, those cells that expressed TFF1 and TFF3 in the terminal bronchioles after naphthalene had a more intense signal when compared to oil controls. TFF3 expression was more abundant at branch points near the terminal bronchioles. Interestingly, there is a suggestive increase in *tff3* that is mirrored by an increase in expression of *foxj1* (which increases with ciliated cell differentiation). *tff3* has been shown to be involved in ciliated cell differentiation in tracheal xenografts (LeSimple *et al*., 2007), and this early increase in mRNA coincides the onset of cellular differentiation in the epithelium at 7 days (Lawson *et al*., 2002; Van Winkle *et al*., 1995). TFF3 added to EGF-depleted cell cultures resulted in more ciliated cells, and this increased number of cells was abrogated when EGFr was blocked (LeSimple *et al*., 2007). Given the robust cell redifferentiation that is taking place in terminal bronchioles at the 7-day time point, which includes regeneration of both Clara cells and ciliated cells, the increase of *tff3* mRNA is not surprising.

Attenuated fibroblasts are flat thin fibroblasts with long processes that reside just beneath the basal lamina of the entire airway tree (Evans *et al*., 1999). These cells communicate with the basement membrane zone, surrounding nerves and airway stroma. This fibroblast population can produce cytokines and chemokines in response to various insults (Evans *et al*., 1999). Their role after epithelial injury has not been defined. Attenuated fibroblasts have increased cell processes 4 days after naphthalene exposure, but whether this is due to an

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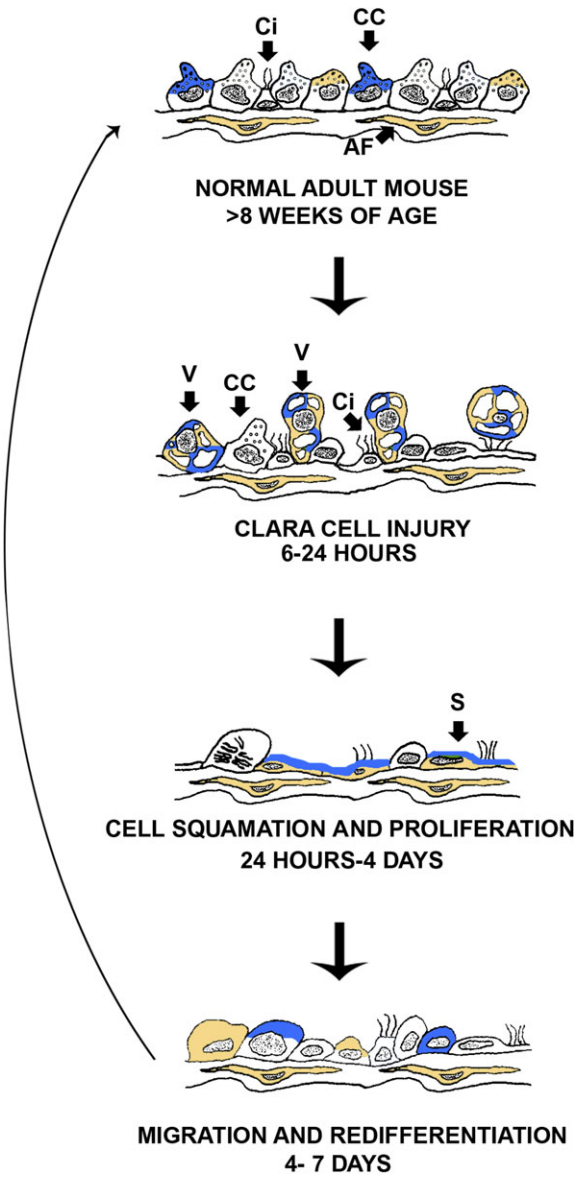

FIG. 11. Temporal expression of TFF1 and TFF3 in terminal bronchioles after systemic naphthalene. This is an illustration of the four time points and controls (steady state) examined. TFF1 is represented by the color gold and TFF3 by the color blue. Both TFF1 and TFF3 are expressed in the apical portion of Clara cells when the terminal bronchiole is in the steady state. At 6–24 h (Clara cell injury), swollen vacuolated Clara cells express both TFF1 and TFF3. At 24 h to 4 days (Ciliated cell squamation and cell proliferation), the dead Clara cells as well as the squamated cells express both TFF1 and TFF3. TFF1 is expressed throughout the squamated cell cytoplasm, while TFF3 is just in the apical portion. At 4–7 days (migration and redifferentiation), TFF1 and TFF3 primarily return to the apical portion of the cells with the occasional cell expressing them throughout the cytoplasm. At all time points, attenuated fibroblasts express tff1. CC, Clara cell; Ci, ciliated cell; AF, attenuated fibroblast.

increase in cell number or just cell processes is not known (Van Winkle *et al.*, 1995). TFF1 expression in peribronchiolar cells with a similar appearance and anatomic location to attenuated fibroblasts was evident at all airway levels, and there was no obvious difference at the different time points and between experimental groups. Confirming evidence would need use high-resolution light microscopy or electron microscopy. Constitutive expression of these factors in the peribronchiolar-associated matrix at all time points of repair may indicate that this area functions as a depot for this growth factor expression.

This study is the first to define the temporal pattern TFF1 and TFF3 mRNA and protein expressions in the mouse during repair of acute airway epithelial injury. *tff2* mRNA was also present and was significantly decreased during acute injury. All three growth factors exhibited temporal patterns in expression following injury. Because TFF1 is of relatively low abundance in whole lung and airways in steady state but shows a pronounced increase with airway epithelial damage that persists for some time, this TFF may represent a good biomarker for airway damage following exposure to toxic pollutants. Further studies are needed to determine the function of all three tff in wound healing in the lung as well as their role in lung function and response to other toxic agents. Future work using knockout mice or selective inhibition of the tff could determine if they work together or act as checks for each other during repair of airway epithelium. In summary, TFF1, TFF2, and TFF3 appear to be involved in both the cellular response to acute airway epithelial injury and subsequent repair in the airway epithelium in a species, the mouse, where mucous cells are rare and the primary secretory cell type is the Clara cell.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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