# Induced Trefoil Factor Family 1 Expression by Trans-Differentiating Clara Cells in a Murine Asthma Model

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**Asthma is a chronic inflammatory disease of the airways that is accompanied by goblet cell metaplasia and mucus hypersecretion. Trefoil factor family (TFF) peptides represent major secretory products of the respiratory tract and are synthesized together with mucins. In the murine lung, TFF2 is mainly expressed, whereas TFF1 transcripts represent only a minor species. TFF peptides are well known for their motogenic and anti-apoptotic effects, and they modulate the inflammatory response of bronchial epithelial cells. Here, an established mouse model of asthma was investigated (i.e., exposure to *Aspergillus fumigatus* [AF] antigens). RT-PCR analysis of lung tissue showed elevated levels particularly of TFF1 transcripts in AF-sensitized/challenged animals. In contrast, transcripts encoding Clara cell secretory protein (CCSP/CC10) were strongly diminished in these animals. For comparison, the expression of the goblet cell secretory granule marker mCLCA3/Gob-5, the mucins Muc1-Muc6 and Muc19, and the secretoglobins ScgB3A1 and ScgB3A2, as well as the mammalian ependymin-related gene MERP2, were monitored. Immunohistochemistry localized TFF1 mainly in cells with a mixed phenotype (e.g., TFF1-positive cells stain with the lectin wheat germ agglutinin (WGA), which recognizes mucins characteristic of goblet cells). In addition, these cells express CCSP/CC10, a Clara cell marker. When compared with mucins or CCSP/CC10, TFF1 was stored in a different population of secretory granules localized at the more basolateral portion of these cells. Thus, the results presented indicate for the first time that allergen exposure leads to the trans-differentiation of Clara cells toward a TFF1-expressing mucous phenotype.**

**Keywords:** asthma; TFF-domain; trefoil factor; goblet cell metaplasia; mucins

**CLINICAL RELEVANCE**

We show that allergen exposure leads to differentiation of murine Clara cells toward a TFF1-expressing phenotype. This result is a further step in defining the molecular events involved in airway remodeling, which is important in diseases such as asthma.

Trefoil factor family (TFF) peptides (in short: TFFs) are characteristic secretory products of many mucin-producing cells (for reviews, *see* Refs. 1–5) and they are pathologically expressed during many chronic inflammatory diseases, including Crohn's disease, ulcerative colitis and duodenitis, cholecystitis (6), inflammatory bowel disease (7), gastric ulcerations (8), and pancreatitis (9, 10). TFFs play a key role in the maintenance of the surface integrity of mucous epithelia in health and disease by supporting a variety of different mucosal defense and repair mechanisms (3). Besides their function in the formation of the mucus barrier, they typically act as luminal surveillance factors of mucous epithelia by reaching their receptors only when surface integrity is lost—for example, after mucosal damage (for reviews, *see* Refs. 11, 12). TFFs particularly enhance cell migration *in vitro* and protect epithelial cells from induced apoptosis. Both effects are ideally suited to support rapid repair of mucous epithelia by a process termed "restitution" (for review, *see* Ref. 12). TFFs also modulate mucosal differentiation processes. Furthermore, the mucosal immune response is subject to modulation particularly by TFF2 (13).

TFF peptides are major secretory products of the human respiratory tract, where predominantly TFF3 is synthesized in submucosal glands together with the mucin MUC5B, although small amounts of TFF3 are also released by goblet cells together with MUC5AC (14, 15). TFF1 is hardly detectable in the human airways and TFF2 is absent. TFF peptides are motogenic for bronchial epithelial cells and they modulate the inflammatory response of these cells (16–18). Little is known about TFF expression in the murine lung. Generally, there are major differences when compared with the human system (i.e., TFF2 is mainly expressed, TFF1 expression is low, and TFF3 transcripts are not detectable) (19). These species-specific differences might be due to significant distinctions in respiratory anatomy and histology between human and rodents (20, 21): Mice normally exhibit few or no mucus-producing cells in their lower airway epithelium and they lack submucosal glands.

Asthma is a chronic inflammatory disease of the airway caused by an inappropriate immune response. Several lines of evidence support a central role for IL-13 in the development of this disease (22). The pathophysiology involves innate and adaptive immunologic mechanisms triggered by environmental and genetic factors. Asthma is associated with a T-helper type 2 (Th2)-driven acute as well as chronic phase of inflammation. The latter is associated with a disorder of lung physiology diagnosed as airway hyperresponsiveness (AHR). Furthermore, chronic inflammation causes remodeling of the airway wall, which determines the clinical picture of this disease. The structural alterations include smooth muscle hypertrophy, lamina reticularis thickening, mucosal edema, epithelial cell sloughing, cilia cell disruption, goblet cell hyperplasia, and mucus hypersecretion (23–25). Goblet cell hyperplasia is present even in patients with mild asthma (26). Thus, a hallmark feature of patients

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with asthma who die after an acute exacerbation is the almost complete occlusion of airways by excessive mucus.

Based upon the pathologic expression of TFFs during chronic inflammatory diseases and upon the mucus hypersecretion in individuals with asthma, one might also expect the induction of TFF expression during asthma. This view is strengthened by a previous report on TFF2 induction in two murine asthma models (27). Due to the limited accessibility of human specimens from patients with asthma, TFF expression was investigated in an established mouse model of asthma, where the animals develop an asthmatic phenotype after sensitization/challenge with *Aspergillus fumigatus* (AF) antigens (28). To follow up remodeling of the airways, the expression of mucins, the goblet cell secretory granule marker mCLCA3/Gob-5, various secretoglobins including the Clara cell–specific protein CCSP/CC10, as well as the expression of the regeneration-responsive ependymin-related proteins, was investigated for comparison.

## MATERIALS AND METHODS

### Murine Asthma Model

Female C57BL/6 mice (6–8 wk old) were obtained from Charles River (Sulzfeld, Germany) and housed in a specific pathogen–free (SPF) facility. The animals were maintained on laboratory food and tap water *ad libitum* in a regular 12 h dark/light cycle with a temperature of 22°C and were allowed to become acclimatized to their environment for a period of 7 d before the experiment. The following experimental protocol was approved by the Animal Use and Care Committee in Hannover. Each experimental group comprised six animals.

Allergic airway inflammation was induced using a model of experimental asthma described previously (28, 29). Details are illustrated in Figure 1. In brief, mice were sensitized subcutanously and intraperitoneally with equal volumes of 0.1 ml using a mixture of AF extract (lot: XPM3A3; Greer Laboratories Inc., Lenoir, NC) in sterile saline emulsified with incomplete Freund's adjuvant (Sigma-Aldrich Chemie, Taufkirchen, Germany). Sensitization was performed using an allergen dose of 5.4 μg (2.7 μg intraperitoneally and 2.7 μg subcutaneously) per mouse. Control animals received sterile saline. Fourteen days later, animals were challenged with aerosolized AF using a Pari Master system (Pari-GmbH, Starnberg, Germany) under defined flow conditions. Particle size was measured using impactor measurement techniques. Aerosol concentration was measured gravimetrically, and animals were exposed to AF aerosol generated from an AF solution with a concentration of 5.4 mg/ml for 12 min, resulting in a final lung deposited dose of ~ 5 μg per mouse calculated with a respiratory minute volume of 35 ml/min and a deposition factor of 0.15 (30). Control animals received aerosolized saline. The second challenge was performed at Day 21, similar to the first with the exception that control animals received AF as well. Animals were killed 48 h after the second aerosol challenge with an overdose of pentobarbital-Na (Narcoren; Merial, Hallbergmoos, Germany).

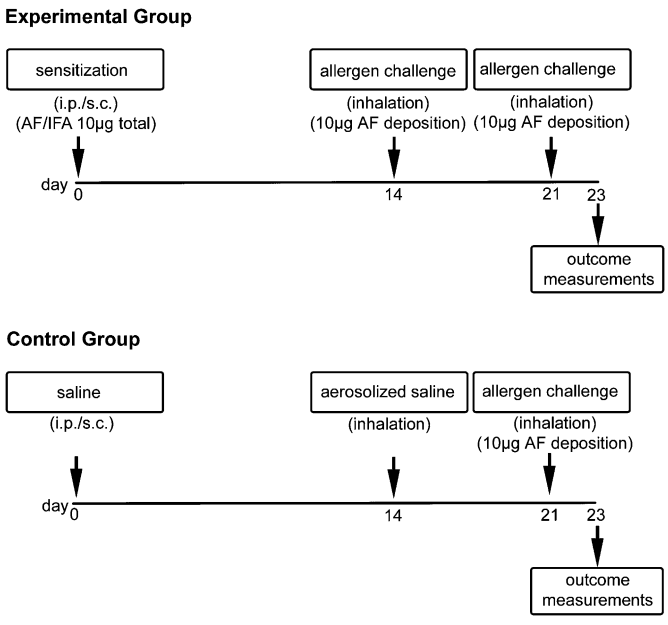


***Figure 1.*** Murine asthma model: sensitization and allergen challenge protocol. Mice of the experimental group were sensitized subcutaneously (s.c.) and intraperitoneally (i.p.) with AF extract on Day 0. Animals were challenged with aerosolized AF on Days 14 and 21. Animals of the control group received sterile saline on Days 0 and 14, and AF on Day 21. All animals were killed on Day 23, BAL was performed, and the lungs were resected for analysis.

Lung function was determined 48 h after the second challenge with AF using head-out body plethysmography as described (31, 32). Methacholine aerosols were generated by a Pari Master aerosol generator. The aerosol concentration in the inhaled atmosphere was continuously determined during the provocation using a clean air mantle aerosol photometer.

### Dissection

Blood was obtained via puncture of the vena cava. Serum was extracted by centrifugation, shock-frozen in liquid nitrogen, and stored at −80°C for further IgE measurements. The trachea was cannulated and bronchoalveolar lavage (BAL) was performed with 0.8 ml ice-cold saline two times. The BAL cells were separated by centrifugation and washed in PBS. Cells were counted using a hemocytometer (Thoma chamber; OmniLab Ltd., Mettmenstetten, Switzerland) and cytospins were performed. The supernatants of the BAL were aliquoted, shock-frozen in liquid nitrogen, and stored at −80°C for further cytokine measurements. For molecular analysis, the right lungs were resected and shock-frozen in liquid nitrogen. The left lungs of the animals were fixed in paraformaldehyde and used for histologic investigations. Proximal and distal regions were selected according to the scheme described in Ref. 33.

### BAL Cell Composition and ELISA

BAL cytospins were stained according to Pappenheim, and 600 cells per animal were differentiated by light microscopy as described previously (29).

Cytokine concentrations in BAL were measured by ELISA techniques (R&D Duoset ELISA-kits; R&D Systems, Minneapolis, MN). Serum immunoglobulin (Ig) E concentrations were measured with Mouse IgE ELISA set (BD Biosciences, Heidelberg, Germany). ELISAs were performed according to the manufacturer's instructions.

### RNA Extraction and RT-PCR Analysis

RNA extraction of murine lung tissue, purification via CsCl ultracentrifugation, and RT-PCR analysis of TFF1, TFF2, and TFF3 expression were as described previously (34). Furthermore, the expression of the following genes was monitored: mucins Muc1, Muc2, Muc3, Muc4, Muc5ac, Muc5b, Muc6, Muc19; mCLCA3/Gob-5; Clara cell secretory protein (CCSP/CC10), secretoglobins B3A1 and B3A2 (ScgB3A1 and ScgB3A2); peroxiredoxin-6 (Prdx6/CC26); mammalian ependymin-related protein 1 and 2 (MERP1 and MERP2). As a control for the integrity of the cDNA preparations, β-actin transcripts were amplified in parallel reactions. The primer pairs used are summarized in Table 1.

### General Histology, Immunofluorescence, and Lectin Histochemistry

Samples of murine tissues were fixed in HEPES-buffered 4% paraformaldehyde (pH 7.4) at 4°C overnight, dehydrated in a series of graded ethanol solutions, and embedded in Technovit 7100 (Heraeus Kulzer GmbH, Wehrheim, Germany), and 2-μm frontal sections were cut as previously described (14, 35).

Immunohistochemistry of the fixed sections was performed as described in detail (36). The following primary antisera were used: affinity-purified polyclonal rabbit Ab502 (1:250 dilution) against the 16 C-terminal amino acid residues of mouse TFF1 (37; kindly provided by Dr. M.-C. Rio and Dr. C. Tomasetto, Illkirch, France). The polyclonal rabbit antiserum against human uteroglobin/urine protein

**TABLE 1. OLIGODEOXYNUCLEOTIDES USED FOR RT-PCR ANALYSIS AND CALCULATED SIZE OF THE PRODUCTS**

| Genes (Accession No.) | Primer Pairs (nucleotide positions) | RT-PCR | Size (*bp*) |
|---|---|---|---|
| TFF1 (NM_009362) | MD7 (97) AAACATGTATCATGGCCC (114)<br>MD8 (419) GAATTCGAGGACTAAAAGTCTG (398) | 60°C, 35 cycles | 323 |
| TFF2 (NM_009363) | MD5 (243) TTCCACCCACTTCCAAAC (260)<br>MD6 (552) AATGCTGTGTCTAGCCACTG (533) | 60°C, 30 cycles | 310 |
| TFF3 (NM_011575) | MD3 (103) CAGATTACGTTGGCCTGTCTCC (124)<br>MD4 (357) ATGCTTGCTACCCTTGGACCAC (336) | 60°C, 35 cycles | 255 |
| Muc1 (NM_013605) | MB370 (1,302) CCCCAGTTGTCTGTTGGGGTC (1,322)<br>MB371 (1,507) GATTCTACCACCACGGAGCC (1,488) | 60°C, 25 cycles | 206 |
| Muc2 (BC034197) | MB495 (640) AACACCAACACCAACACCC (658)<br>MB496 (1,076) TCTCCTCCACCAGCACATAG (1,048) | 60°C, 35 cycles | 428 |
| Muc3 (BC058768) | MB380 (257) TTTCAACTCCACTGCCACC (275)<br>MB381 (682) TCTTCACCCCACTTGTACATC (662) | 56°C, 35 cycles | 426 |
| Muc4 (NM_080457) | MB378 (9,997) AGGCAGAAGAGGAGTGGAGAAG (10,018)<br>MB379 (10,396) AGCAGCCGCAGAAGTGAAAG (10,377) | 60°C, 33 cycles | 400 |
| Muc5ac (L42292) | MB374 (854) GGACCAAGTGGTTTGACACTGAC (876)<br>MB375 (1,111) CCTCATAGTTGAGGCACATCCCAG (1,088) | 60°C, 35 cycles | 258 |
| Muc5b (NM_028801) | MB384 (10,592) CTCCTCAATTCATACAACACCC (10,613)<br>MB385 (10,927) GTTTCCATGTCCCCTTTTACC (10,907) | 60°C, 35 cycles | 336 |
| Muc6 (NM_181729) | MB382 (6,738) CACACAACCAACACCAATTC (6,757)<br>MB383 (7,226) TGAGAAAGGTAGGAAGTAGAGG (7,205) | 60°C, 35 cycles | 489 |
| Muc19 (NM_207243) | MB493 (22,175) GATTATGCGATTGGTTCATCCT (22,196)<br>MB494 (22,523) GTGCAATGTCCCTGAACTCATA (22,502) | 60°C, 35 cycles | 349 |
| mCLCA3/Gob-5 (AB017156) | MB376 (1,861) GCCAAGGAGCCTCGCCTATTCTCAGG (1,886)<br>MB377 (2,371) GAAGCTCTCCCGTGGTCGTAG (2,351) | 60°C, 29 cycles | 511 |
| CCSP/CC10 (X67702) | MB487 (71) CGCCATCACAATCACTGTGGTCA (89)<br>MB488 (269) GAGGGTATCCACCAGTCTCTTCA (247) | 60°C, 15 cycles | 199 |
| Prdx6/CC26 (NM_007453) | MB549 (153) CATTCTCTTTTCCCACCCAC (172)<br>MB550 (608) CTCTCTCCCTTCTTCCAGTC (589) | 60°C, 30 cycles | 456 |
| ScgB3A1 (NM_170727) | MB547 (303) GCCTAGCCTACCATTAAGCC (322)<br>MB548 (561) AGAAAGGTGTGAGCAGCAG (543) | 60°C, 30 cycles | 259 |
| ScgB3A2 (NM_054038) | MB545 (140) TATTCTGCCACTGCCCTTC (158)<br>MB546 (300) TCCACACACTTCTTCAGTCC (281) | 60°C, 30 cycles | 161 |
| MERP1 (AY027861) | MB533 (333) GATTGACCAAGCCACCAAG (351)<br>MB534 (682) AGTCTTCGCTCATCTTCTCC (663) | 60°C, 35 cycles | 350 |
| MERP2 (NM_134065) | MB529 (558) GATTGAACAAGCCACCAAAC (577)<br>MB530 (842) TCCTTAATGCTTAGCTGCAC (823) | 60°C, 28 cycles | 285 |
| β-Actin (NM_007393) | MB555 (723) AAGCTGTGCTATGTTGCTC (741)<br>MB556 (1,029) TCTCCTTCTGCATCCTGTC (1,011) | 60°C, 25 cycles | 307 |

1/Clara cell secretory protein (CCSP; 1:500 dilution) was purchased from DakoCytomation (Glostrup, Denmark), and recognizes also the mouse homolog CC10. Polyclonal goat anti-rat CCSP (1:1,000 dilution; 38) was kindly provided by Dr. B. R. Stripp (Pittsburgh) and polyclonal rabbit anti-mCLCA3/Gob-5 (1:500 dilution; against peptide p3b; 39) was a gift from Dr. A. D. Gruber (Berlin). As secondary antisera we used: Cy3-labeled sheep anti-rabbit IgG F(ab′)$_2$-fragment (1:100 dilution; Sigma-Aldrich Chemie), fluorescein isothiocyanate (FITC)-labeled donkey anti-goat IgG (1:10 dilution; Dianova, Hamburg, Germany), and Cy3-labeled donkey anti-rabbit IgG (1:100 dilution; Dianova).

For lectin histochemistry, the sections were additionally incubated with the FITC-labeled lectin wheat germ agglutinin (WGA, 1:1,000 dilution; Sigma-Aldrich Chemie) in a manner similar to that described previously (40).

Mucins were stained using a combination of Alcian blue 8GX at pH 2.5 and the periodic acid-Schiff (PAS) reaction as described (40). Nuclei were counterstained with Meyer's hematoxylin.

### Statistics

Data are expressed as means with ± SEM as standard deviation (error bars). Data were analyzed concerning Gaussian distribution and variance differences (one-way ANOVA) using Bartlett's test; probability of error was analyzed using Student's *t* test. Significance is indicated in the figures by *one asterisk* ($P \leq 0.05$), *two asterisks* ($P \leq 0.01$), and *three asterisks* ($P \leq 0.001$).

## RESULTS

### Analysis of the BAL Samples and Serum IgE Levels

AHR was determined by body plethysmography 48 h after the second challenge with AF. Animals sensitized and challenged with allergen showed a tendency to increased reactivity toward methacholine as compared with the nonsensitized controls; however, the data were statistically not significant (data not shown).

Leukocytes, macrophages, eosinophils, and lymphocytes were quantified in BAL samples from nonsensitized and AF-sensitized mice 48 h after the last allergen challenge (i.e., at Day 23 according to Figure 1). A compilation of the morphometric analysis is represented in Figure 2. Clearly, inflammatory cells are absent in the control mice, and the number of eosinophils and lymphocytes increased dramatically in the BAL of the sensitized group.

Furthermore, serum IgE concentrations and BAL cytokine levels were measured in nonsensitized and AF-sensitized mice 48 h after the last allergen challenge. Figure 3 summarizes the values for serum IgE as well as for BAL IL-5, IL-13, and eotaxin. The AF-sensitized/challenged animals show elevated serum IgE and BAL IL-5, IL-13, and eotaxin-2 levels.

### RT-PCR Analysis of Lung Tissue

To compare expression of TFF genes in AF-sensitized/challenged and control animals, RNA was isolated from the right lungs of all animals investigated and subjected to RT-PCR analysis. Figure 4 presents the results obtained from one out of three independent experimental series. Generally, all three series gave comparable results. The transcript levels were determined for the three TFF genes (TFF1–3).

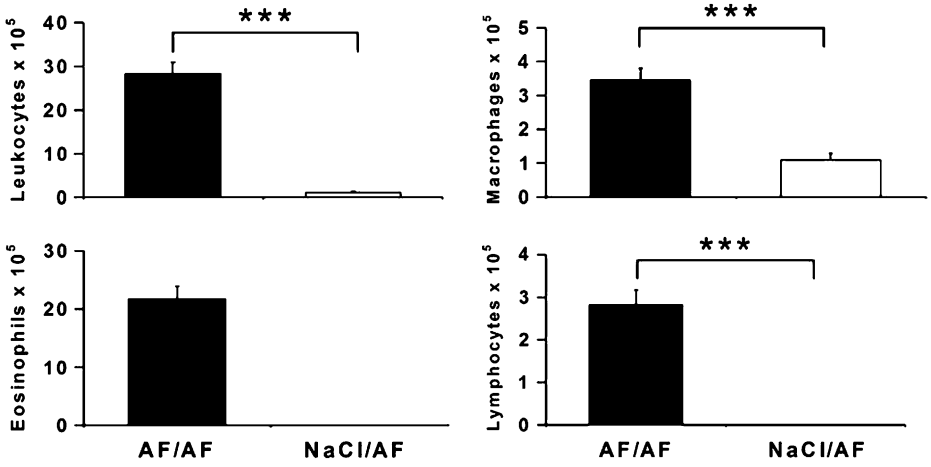


*Figure 2.* Airway inflammation is induced in AF-sensitized/challenged C67BL/6 mice at Day 23. BAL fluid of AF-sensitized/challenged mice (AF/AF, *solid bars*) was compared with controls (NaCl/AF, *open bars*). Shown is the number of leukocytes, macrophages, eosinophils, and lymphocytes. *Error bars* represent ± SEM. Each group consisted of six mice. Statistical analysis of eosinophil numbers could not be performed, due to the lack of values different from zero in the control mice. ***Extremely high significance ($P \leq 0.001$).

Furthermore, the expression of the following genes was also monitored to characterize the expected remodeling of the airways toward a mucous phenotype: mucin genes (Muc1, Muc2, Muc3, Muc4, Muc5ac, Muc5b, Muc6, Muc19) and the goblet cell secretory granule marker gene mCLCA3/Gob-5; the Clara cell-specific genes CCSP/CC10 and Prdx6/CC26 as well as the secretoglobins ScgB3A1 and ScgB3A2; and the mammalian ependymin-related genes MERP1 and MERP2.

Generally, the TFF1 transcript levels were elevated in the AF-sensitized/challenged animals when compared with the controls, the latter group showing some individual variations. In contrast, the TFF2 transcript levels were not significantly altered and TFF3 transcripts were not detectable in the murine lung samples at all.

The mucin genes investigated revealed a diverse pattern. The expression of the transmembrane mucins Muc1 and Muc4 was not altered, whereas the expression of secretory mucins Muc2, Muc5b, and Muc19 were diminished in AF-sensitized/challenged mice and the Muc5ac expression tended to be slightly elevated. Muc3 transcripts were present in some of the control animals only (2/6), and Muc6 expression was rather weak with a tendency to reduced expression in AF-sensitized/challenged mice.

The expression pattern of the mCLCA3/Gob-5 gene encoding a secretory protein associated exclusively with mucin granules showed increased transcript levels in AF-sensitized/challenged animals with individual variations. Interestingly, these variations were congruent with those observed in TFF1 transcript levels.

The expression of the Clara cell–specific gene CCSP/CC10 was clearly reduced in all AF-sensitized/challenged animals. Furthermore, the Prdx6/CC26 and ScgB3A2 showed a tendency toward decreased expression in the AF-sensitized/challenged group, whereas ScgB3A1 transcript levels did not change.

A similar pattern was observed for MERP2, which revealed a reduced expression in AF-sensitized/challenged animals.

### Immunofluorescence Studies

AF-sensitized/challenged animals showed characteristic goblet cell metaplasia (GCM) in the proximal and distal airways as documented by PAS/Alcian blue staining typical of mucins. In contrast, the most distal airways were devoid of GCM (data not illustrated). This mucous phenotype was confirmed by lectin staining with WGA as well as by anti-mCLCA3/Gob-5 immunofluorescence studies, which showed congruent patterns (Figures 5B–5D). In contrast, the control animals were devoid of mucous cells—that is, PAS/Alcian blue staining, lectin staining with WGA, and anti-mCLCA3/Gob-5 immunofluorescence were negative (Figures 5E–5G). Most of the epithelial cells of control animals stained strongly with the Clara cell marker CCSP/CC10 (Figure 5I).

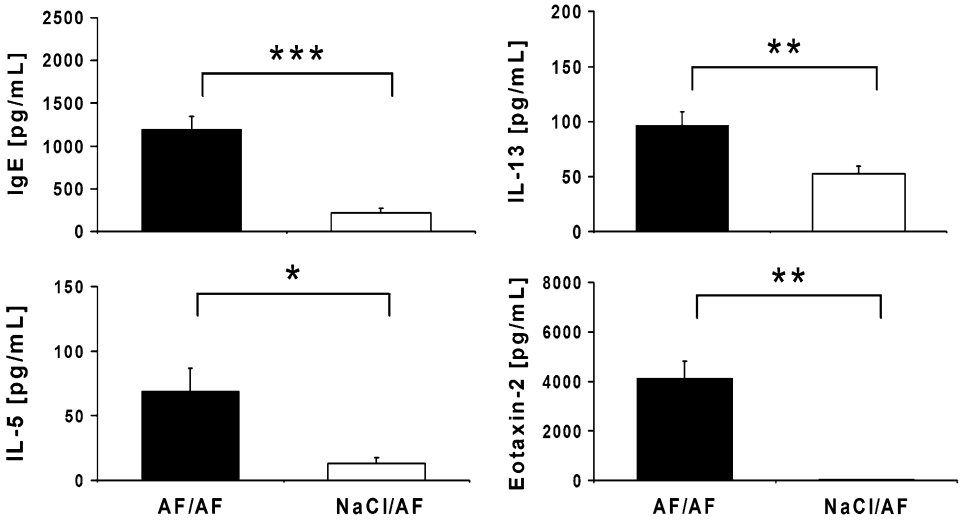


*Figure 3.* Total serum IgE and BAL cytokine concentrations. Shown are the IL-5, IL-13, and eotaxin-2 levels of C67BL/6 mice 48 h after the last allergen challenge. AF-sensitized/challenged mice (AF/AF, *solid bars*) were compared with controls (NaCl/AF, *open bars*). *Error bars* represent ± SEM. Each group consisted of six mice. *Significance ($P \leq 0.05$); **high significance ($P \leq 0.01$); ***extremely high significance ($P \leq 0.001$).

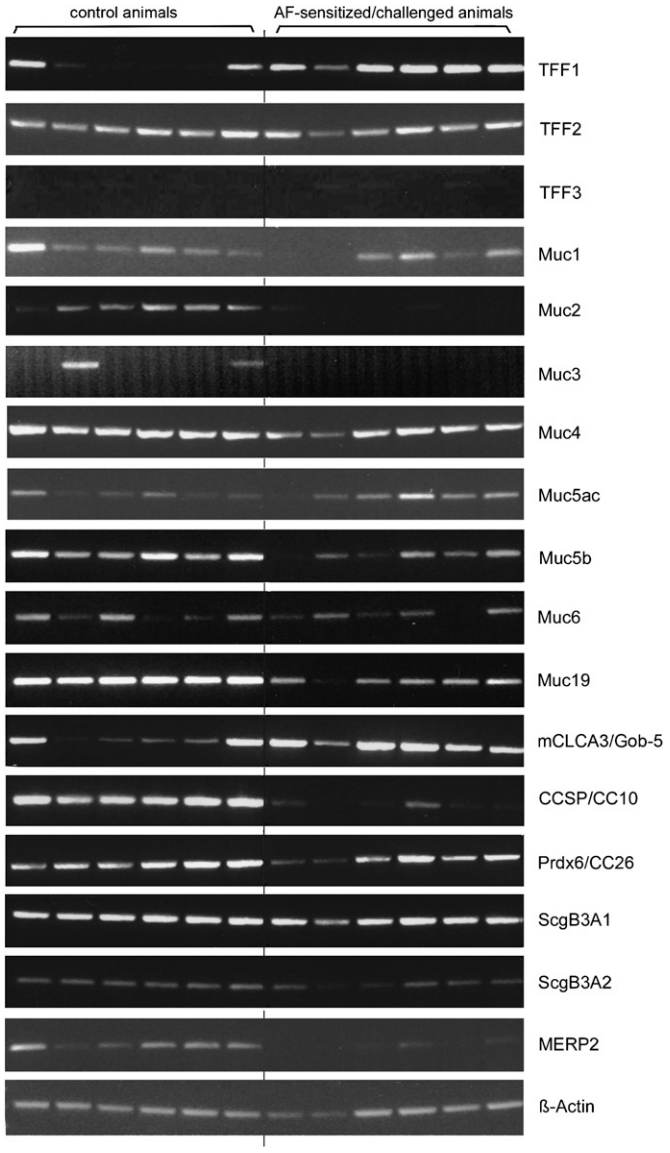


***Figure 4.*** RT-PCR analysis. TFF1, TFF2, TFF3, Muc1, Muc2, Muc3, Muc4, Muc5ac, Muc5b, Muc6, Muc19, mCLCA3/Gob-5, CCSP/CC10, Prdx6/CC26, ScgB3A1, ScgB3A2, and MERP2 expression was monitored in the lungs of the control animals or the AF-sensitized/challenged mice. The integrity of the cDNAs was tested by monitoring the β-actin transcripts.

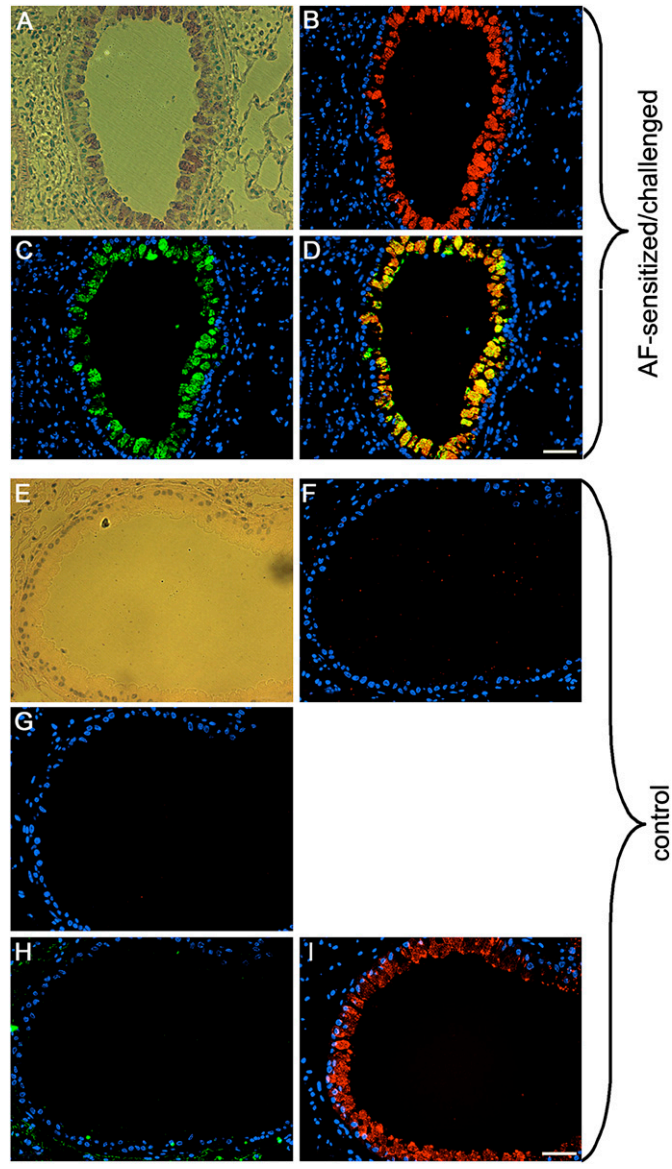


***Figure 5.*** Immunohistochemical characterization of the distal airways from an AF-sensitized/challenged animal (*parallel sections A–D*) or a control animal (*parallel sections E–I*). (*A, E*) Mucin staining with PAS/Alcian blue. (*B, F*) Immunofluorescence with anti-mCLCA3/Gob-5 antiserum/Cy3-label (*red*). (*C, G*) Lectin staining with WGA/FITC-label (*green*). (*D*) Double immunofluorescence with anti-mCLCA3/Gob-5 antiserum/Cy3-label (*red*) and lectin staining with WGA/FITC-label (*green*). (*H*) Immunofluorescence with anti-TFF1 antiserum/Cy3-label (*red*). (*I*) Immunofluorescence with rabbit anti-CCSP/CC10 antiserum/Cy3-label (*red*). Nuclei were counterstained with DAPI (*blue*; *B–D* and *F–I*). *Scale bars*: 50 μm (*A–D*) or 20 μm (*E–I*).

Furthermore, TFF1 immunoreactivity was investigated in dissected airway tissue of control animals and AF-sensitized/challenged mice, respectively (Figures 5H and 6). Typically, proximal and distal regions were analyzed. TFF1 was focally localized only within specific epithelial cells of AF-sensitized/challenged mice (Figures 6A, 6E, and 6G) but not in the control group (Figure 5H). TFF1 staining was always localized at the more basolateral portion of these cells. Furthermore, most of these TFF1-positive cells showed also mucin staining with WGA (Figures 6A, 6E, 6G, and 7), which was exclusively at the apical portion of these cells and was completely separated from TFF1 staining. Only a minority of cells stained either for TFF1 or WGA alone (Figures 7A and 7F). However, neither TFF1 immunoreactivity nor WGA staining was detectable in the most distal airways (data not shown).

Immunohistochemistry was also performed on parallel sections with an antiserum against CCSP/CC10 typical of Clara cells (41). Generally, CCSP/CC10 staining appeared to be much more abundant in the control animals (Figure 5I) when compared with AF-sensitized/challenged animals (Figures 6C, 6F, 6I, and 8). In AF-sensitized/challenged mice, cells that stain with WGA also stain for TFF1 (Figures 6A, 6E, and 6G) and for CCSP/CC10 (Figures 6C, 6F, and 6I), suggesting that CCSP/CC10 and TFF1 are localized mainly in the same cells. However, double immunofluorescence staining of CCSP/CC10 and TFF1 did not reveal

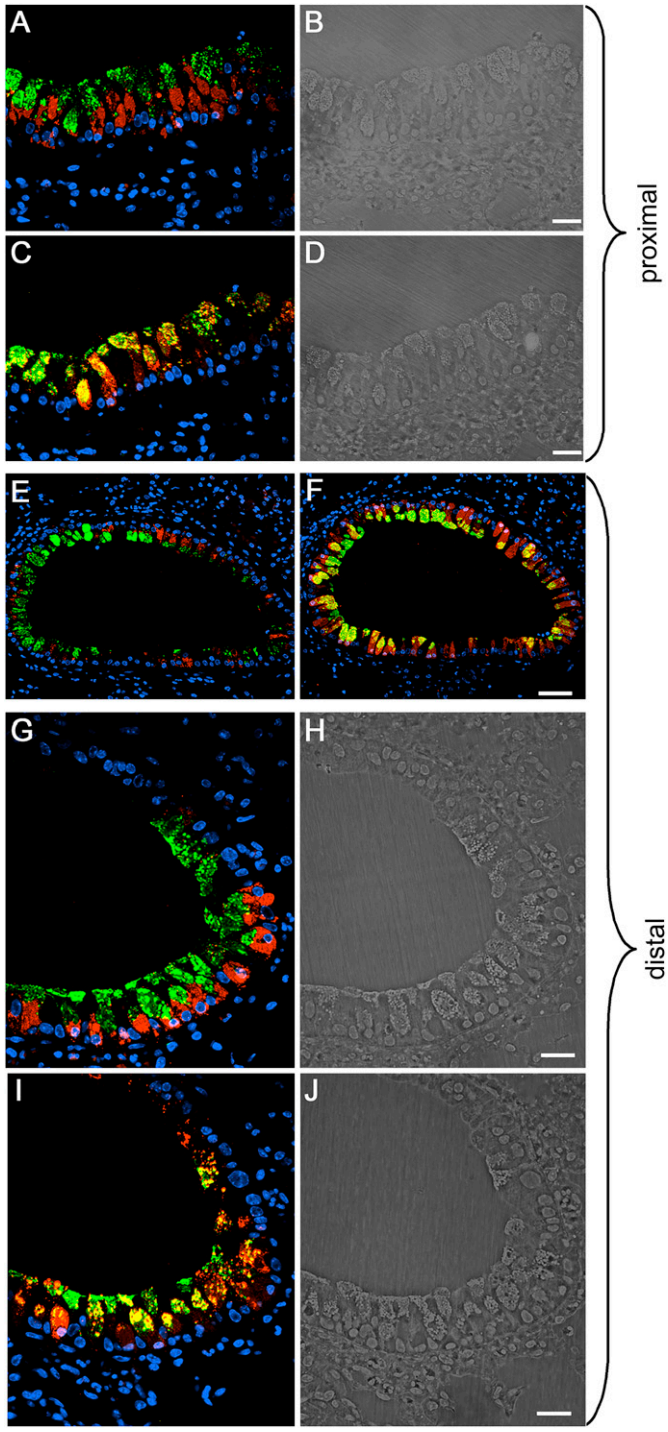


***Figure 6.*** Double localization of mucins and TFF1 or mucins and CCSP/CC10 in the proximal (*A–D*) or distal airways (*E–J*) from an AF-sensitized/challenged animal. (*A*, *E*, *G*) Double immunofluorescence with anti-TFF1 antiserum/Cy3-label (*red*) and lectin staining with WGA/FITC-label (*green*). (*C*, *F*, *I*) Double immunofluorescence with rabbit anti-CCSP/CC10 antiserum/Cy3-label (*red*) and lectin staining with WGA/FITC-label (*green*). Nuclei were counterstained with DAPI (*blue*). (*B*, *D*, *H*, *J*) Phase contrast pictures of *A*, *C*, *G*, and *I*, respectively. *Scale bars*: 20 μm (*A–D*, *G–J*) or 50 μm (*E–F*).

colocalization (Figures 8A and 8C). Whenever localized within the same cell, CCSP/CC10 and TFF1 were located at different portions of these cells, that is, at the apical or the basolateral regions, respectively. Furthermore, there were also some cells in which CCSP/CC10 was distributed all over the cells and not concentrated to the apical portion of these cells. These cells were devoid of TFF1. Consequently, CCSP/CC10 staining was partly overlapping with the WGA staining (Figures 6C, 6F, and 6I) or the mCLCA3/Gob-5 staining (Figures 8B and 8D). Taken together, most of the TFF1-positive cells show characteristics of both Clara cells (CCSP/CC10-positive) as well as of goblet cells (WGA-positive, mCLCA3/Gob-5–positive).

## DISCUSSION

In the model system presented here, the AF-sensitized/challenged animals showed characteristics of an asthmatic phenotype (e.g., GCM, infiltration of the airways with eosinophils and lymphocytes, and increased levels of immune cytokines such as IL-5 and IL-13 typical of the asthmatic lung) (23, 28, 29). The lack of significant AHR in C57BL/6 mice is in line with previous reports (42).

T lymphocytes play a major role in bronchial asthma, producing cytokines such as interleukin (IL)-4, IL-5, and IL-13, which induce IgE production and eosinophil activation. Chemokines, which induce specific types of leukocyte chemotaxis, regulate migration and the accumulation of leucocytes during inflammatory and immune responses (43, 44). In total agreement with the literature, our data clearly showed that in the asthmatic state the Th2-cytokines IL-5 and IL-13, as well as IgE and eotaxin-2, are increased.

### Goblet Cell Metaplasia, Expression of Mucins

The presence of goblet cells in murine airways is recognized as a phenotypic marker in models of allergic asthma. Their appearance reflects GCM as murine airways normally express few, if any, goblet cells (20). Interestingly, the number of mucus-containing cells differs between animals housed under SPF conditions and "stock" animals, the SPF mice having fewer mucous cells (20).

GCM was clearly demonstrated in the proximal as well as the distal airways in this study by the increased expression of mCLCA3/Gob-5 in the asthmatic state (i.e., 48 h after the last allergen challenge). This would be in line with a recent study describing involvement of the distal airways also (45), in spite of previous reports on mucous cell metaplasia predominantly in the proximal airways (33, 46). mCLCA3/Gob-5 (probably a metal-dependent hydrolase) is known as a key molecule in the induction of murine asthma, where it is specifically induced in bronchial goblet cells (47, 48). Furthermore, GCM was visualized in the AF-sensitized/challenged animals by histochemistry (PAS/Alcian blue staining or reactivity with the lectin WGA or mCLCA3/Gob-5 staining). WGA has been shown to recognize Muc5ac as well as an unidentified high–molecular weight glycoconjugate present in the lung of ovalbumin-treated, but not control mice (49).

Surprisingly, most of the mucin genes tested showed decreased expression in the asthmatic state (i.e., 48 h after the last allergen challenge); here, the expression of the secretory mucins Muc2, Muc5b, and Muc19 is diminished in AF-sensitized/challenged animals. Only Muc5ac showed slightly elevated expression levels. The differential regulation of mCLCA3/Gob-5 and mucins is in line with a recent report comparing particularly Muc5ac and Gob-5 expression (50). Thus, GCM in the asthmatic state is accompanied by storage of a WGA-positive high–molecular weight glycoconjugate (49), which seems to differ

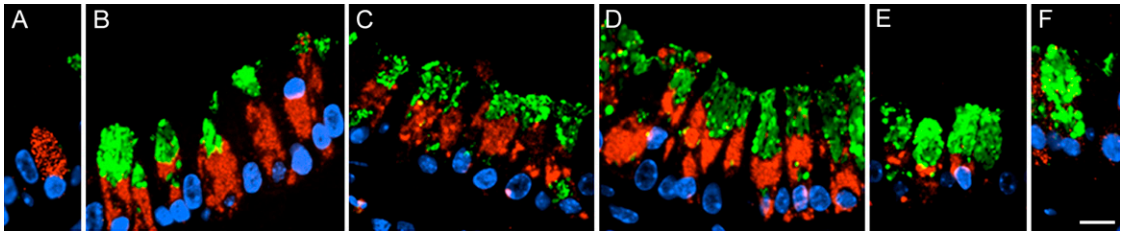


***Figure 7.*** Double localization of TFF1 and mucins in proximal or distal airways from an AF-sensitized/challenged animal. Double immunofluorescence with anti-TFF1 antiserum/Cy3-label (*red*) and lectin staining with WGA/FITC-label (*green*). Nuclei were counterstained with DAPI (*blue*). The cells were arranged in panels A-F according to their increasing mucin content (i.e., their relatively increasing number of WGA-stained secretory granules). *Scale bar*: 10 μm.

from the mucins tested here (i.e., Muc1–6, Muc19). Alternatively, one might propose that the mucin transcript and protein levels do not coincide and mRNA expression occurs well before Day 23, when goblet cells differentiate from precursors, whereas goblet cells in the asthmatic state just store mucins but no longer synthesize them at elevated levels. The latter hypothesis is in total agreement with a previous report which suggested that Muc5ac mRNA is not an accurate indicator of mature goblet cells (51).

### Expression of MERP2

For further characterization of the model system, the expression of the two murine MERP genes was monitored in AF-sensitized/challenged and control animals. MERP1 and MERP2 encode two murine secretory proteins (52, 53) homologous to human UCC1/MERP1, which is up-regulated in colon cancer (54). MERPs share similarity to piscine ependymins, which represent calcium-dependent cell adhesion molecules and are involved in neuroplasticity and regeneration (for review, *see* Ref. 55). A homologous gene has also been detected in echinoderm showing regulated expression during intestinal regeneration (56).

In the course of the studies presented here, a down-regulation of MERP2 in allergic airway inflammation was observed for the first time. In contrast, MERP1 expression could not be detected in murine lung tissue (data not shown). The implications of this unexpected result are far from being understood and future experiments are required to elucidate the role of MERPs during airway inflammation and remodeling.

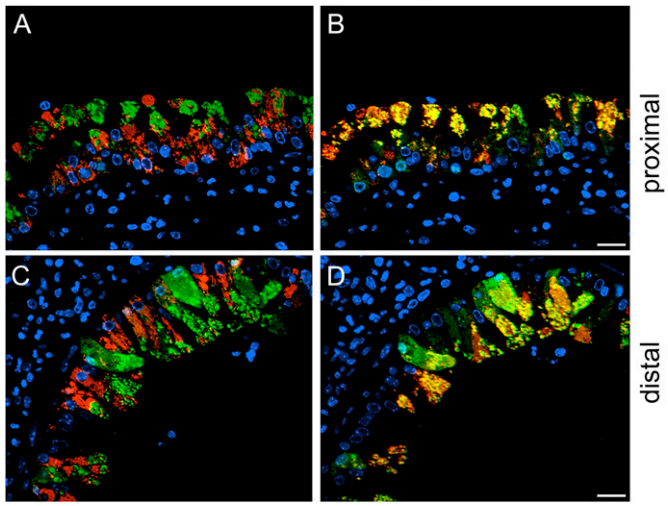


***Figure 8.*** Double localization of CCSP/CC10 and TFF1 or CCSP/CC10 and mCLCA/Gob-5 in proximal (*parallel sections A* and *B*) or distal airways (*parallel sections C* and *D*) from an AF-sensitized/challenged animal. (*A, C*) Double immunofluorescence with goat anti-CCSP/CC10 antiserum/FITC-label (*green*) and anti-TFF1 antiserum/Cy3-label (*red*). (*B, D*) Double immunofluorescence with goat anti-CCSP/CC10 antiserum/FITC-label (*green*) and anti-mCLCA3/Gob-5 antiserum/Cy3-label (*red*). Nuclei were counterstained with DAPI (*blue*). *Scale bars*: 20 μm.

### TFF1 Expression Is Induced in Trans-Differentiating Clara Cells of AF-Sensitized/Challenged Animals

RT-PCR analysis revealed a markedly different expression pattern for the three TFF genes in the murine lung. In control animals, TFF2 is the predominantly expressed TFF gene, whereas TFF1 is only weakly expressed and TFF3 transcripts are not detectable. This situation differs completely from the human system, but is in agreement with previous studies (19). In AF-sensitized/challenged animals, only the TFF1 transcript level was markedly increased; whereas the TFF2 and TFF3 expression did not change. This result is completely in line with a recent report on very strong induction of TFF1, together with mCLCA3/Gob-5, after allergen exposure using a murine asthma model characterized by transgenic overexpression of IL-13 (57). On the other hand, the lack of TFF2 mRNA induction in AF-sensitized/challenged animals seems to contradict previous reports (27, 57, 58). However, the latter models of experimental asthma differ in significant details from the system presented here (e.g., different mouse strains, different experimental allergic induction protocols, different sources of the AF extracts), and this might be the reason why the results are not directly comparable. For example, it is particularly well known that there are considerable differences between mouse strains in terms of airway remodeling (42).

The RT-PCR data for TFF1, CCSP/CC10, and mCLCA3/Gob-5 are in complete agreement with the immunohistochemistry results. TFF1 was not detectable in control animals, whereas this peptide was clearly present in AF-sensitized/challenged animals. TFF1 expression is mainly observed in cells with a mixed phenotype—for example, TFF1-positive cells stain with WGA (Figures 6A, 6E, 6G, and 7), which recognizes mucins (49) characteristic of goblet cells. In addition, these cells express CCSP/CC10 (Figures 6C, 6F, and 6I), a Clara cell marker (41). This mixed phenotype is in line with a previous report describing the trans-differentiation of Clara cells into a mucin-secreting phenotype after antigen challenge (46).

Taken together, the results presented here demonstrate for the first time that AF allergen challenge leads to trans-differentiation of Clara cells toward a TFF1-expressing phenotype accompanied by clearly diminished expression of the secretoglobin CCSP/CC10, whereas the secretoglobins ScgB3A1 and ScgB3A2 (59) show no significant change. This is in contrast to a previous publication reporting on a small but significant increase in CCSP/CC10 after antigen challenge (46). However, other reports are in line with our results describing decreased CCSP levels in individuals with asthma (60).

The TFF1-expressing phenotype of trans-differentiating Clara cells is characterized by a general shift in the secretory phenotype (i.e., from serous toward a more mucous state). Interestingly, both the TFF1-containing granules and the mucin-containing granules are clustered and are located in distinct portions of these cells (i.e., in the basolateral or the apical regions, respectively). This is surprising because in the gastrointestinal tract TFFs are typically co-secreted together with secretory mucins (1, 2). Thus, one might have expected a package of TFF1 and mucins into the same secretory granules also here. The mucous granules at the apical pole are characterized by the storage of mucins (Figure 7), mCLCA3/Gob-5 (Figures 8B and 8D), and CCSP/CC10 (Figures 8A and 8C). This change toward a mucous phenotype is accompanied by the induction of mCLCA3/Gob-5 expression, which is absolutely parallel to TFF1 expression. Remarkably, mCLCA3/Gob-5 and TFF1 represent the two most highly induced genes after antigen sensitization and challenge also in a transgenic asthma model overexpressing IL-13 (57). Recent reports indicate that mCLCA3/Gob-5 exclusively associates with mucin granules and that it is a secreted metal-dependent hydrolase involved in the synthesis, condensation, or secretion of mucins (39, 61, 62).

The observation that TFF1, and not TFF2 or TFF3 expression is induced in trans-differentiating Clara cells could be due to a binding site for the transcription factor FoxA2/HNF-3β (63), which plays a major role in development and inflammation (64). In contrast, there is less binding of FoxA2/HNF-3β to the TFF2 and TFF3 promoters (63). However, the CCSP/CC10 gene, which also contains a binding site for FoxA2/HNF-3β (65), is obviously regulated differently in the experimental model used here.

Currently, the functional role of induced TFF1 expression in trans-differentiating Clara cells is not understood. Recent studies demonstrated that Clara cells show great plasticity in structure and secretory products (59, 66, 67); in particular, they change to a mucous phenotype after antigen challenge (33, 46, 68). Thus, secretion of TFF1 by allergen-induced Clara cells might be only a transient differentiation step toward fully differentiated goblet cells. Remarkably, a comparable induction of TFF1 synthesis has also been observed during differentiation of HT-29 cells toward a mucin-secreting phenotype (69) and during the differentiation of gastric stem cells to pre-pit cells (70).

The trans-differentiation of Clara cells into goblet cells is critically dependent upon EGF and IL-13 (22, 71, 72) and might have similarities to the trans-differentiation of ciliated airway epithelial cells into goblet cells (73). The central role for Clara cell IL-4Rα receptor signaling in mucus production induced by allergen has been recently demonstrated in an elegant study (74). However, TFF1 has also been shown to play a major role as an ERK-regulated differentiation factor for certain gastric cell lineages (70, 75) and the TFF1 promoter is responsive to EGF (76). Based upon the observation that Clara cells trans-differentiate toward a TFF1-expressing phenotype, the hypothesis can be put forward that TFF1 may play an intrinsic role as an autocrine factor for the trans-differentiation of Clara cells into goblet cells. In particular, the well known anti-apoptotic effect of TFF1 (77) together with the synergistic effect of TFFs and EGF (for review, *see* Ref. 12) might be well suited to support this process. Whether TFF1 has indeed such an essential role in the remodeling of the murine lung during the development of asthma could be clarified by future investigations of TFF1-deficient mice (37).

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