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Airway epithelial innate host defence in chronic obstructive pulmonary disease

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CHAPTER 9

Expression of the innate immunity mediator WFDC12 in airway epithelial cells: role of cell differentiation and expression in COPD.

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Submitted

ABSTRACT

Whey acidic protein four-disulfide-core 12 (WFDC12) is an anti-inflammatory mediator that is expressed during airway inflammation. Airway epithelial cells are centrally involved in regulating airway inflammation, however the expression of WFDC12 in these cells, and its role in the chronic inflammatory lung disease COPD, has not been investigated. Therefore, we examined the expression of WFDC12 in (un)differentiated primary bronchial epithelial cells (PBEC) that were exposed to inflammatory stimuli, and compared the expression in COPD and control cell cultures. Exposure to the COPD-related pathogen non-typeable *Haemophilus influenzae* increased mRNA of WFDC12 in undifferentiated PBEC, whereas it only affected WFDC12 secretion in differentiated PBEC. TNFα/IL-1β stimulation increased WFDC12 mRNA in differentiated PBEC, whereas supplemented and endogenously produced TGF-β1 suppressed the expression. Baseline WFDC12 mRNA levels were increased upon differentiation and were mainly observed in luminal cell of differentiated PBEC. This constitutive expression of WFDC12 was furthermore lower in cell cultures from COPD patients when compared to controls. Overall, these findings demonstrate a dynamic regulation of WFDC12 in airway epithelial cells during differentiation. Decreased production as observed in COPD epithelial cell cultures might favor the persistence of inflammation in this disease.

INTRODUCTION

The whey acidic protein four-disulfide-core (WFDC) domain-containing protein family comprises an evolutionary conserved group of proteins, including secretory leukocyte protease inhibitor (SLPI) and elafin (protease inhibitor 3; PI3) (1). These proteins are important regulators of innate immune responses in the lung that display a range of functions including antiprotease and antimicrobial activity, anti-inflammatory effects, and wound healing properties.

Recently, the expression and function of another, previously uncharacterized WFDC domain protein, WFDC12, was described (2). In contrast to the mouse orthologue (3), human WFDC12 did not display antibacterial properties. However, functional studies showed antiprotease activity against neutrophil-derived cathepsin G and reduced IL-8 and MCP-1 secretion *by in vitro* cultured monocytes stimulated with LPS (2). Moreover, LPS increased the expression of WFDC12 in the lungs *in vivo*, and increased expression levels were detected in secretions and tissues derived from the lungs of patients with acute respiratory distress syndrome. Together, these findings suggest a role of WFDC12 in regulating inflammatory responses in the airways.

In addition to immune cells, the airway epithelium plays a central role in regulating airway innate immunity and inflammation (4, 5). Airway epithelial cells are a major source of WFDC proteins in the lung and express several protein family members either constitutively or upon exposure to pro-inflammatory stimuli (6, 7). It remains however unknown if WFDC12 is also expressed by human airway epithelial cells, which mechanisms are involved in its expression, and furthermore whether its expression is affected in chronic inflammatory lung diseases.
Chronic obstructive pulmonary disease (COPD) is a smoking-related lung disorder that is characterized by irreversible, chronic and progressive airflow limitation, bacterial colonization and infection, a persistent inflammatory response in the lung, and remodeling of lung tissue (8-10). It is postulated that alterations in airway epithelial innate immunity contribute to the onset and progression of COPD (5), but the mechanisms that are affected remain incompletely understood. Indeed it has been shown in previous studies that airway epithelial cells in COPD display reduced anti-inflammatory activity, for instance due to reduced expression of club cell secretory protein-16 (11). In addition to this, reduced expression of other anti-inflammatory proteins, such as WFDC12, may also contribute to the reduced anti-inflammatory activity of airway epithelial cells in COPD.

In the present study we examined the expression of WFDC12 in primary bronchial epithelial cells (PBEC) in response to microbial and inflammatory stimuli related to COPD. To this end we examined the effect of the COPD-related respiratory pathogen non-typeable *Haemophilus influenzae* (NTHi), cigarette smoke, the pro-inflammatory cytokines TNFα/IL-1β and the growth factor TGF-β1 on WFDC12 expression. Moreover, expression of WFDC12 was compared between air-liquid interface cultured and mucociliary differentiated PBEC (ALI-PBEC) from COPD patients and non-COPD (ex)smokers.

MATERIALS AND METHODS

Cell culture and stimuli

Primary bronchial epithelial cells (PBEC) were isolated from macroscopically normal lung tissue obtained from patients undergoing resection surgery for lung cancer at the Leiden University Medical Center. Use of such lung tissue that became available for research within the framework of patient care was in line with the "Human Tissue and Medical Research: Code of conduct for responsible use" (2011) (www.federa.org), that describes the no-objection system for coded anonymous further use of such tissue. Therefore, individual written or verbal consent is not applicable. Cells were cultured as previously described (12, 13) either in transwells at the air-liquid interface (ALI) to allow mucociliary differentiation (ALI-PBEC), or cultured in undifferentiated submerged conditions in regular tissue culture plates (referred to as S-PBEC). In Figure 5 ALI-PBEC cultures were derived from COPD patients or non-COPD controls with a smoking history. The disease status of anonymized donors was determined based on lung function data according to the Global Initiative for Chronic Obstructive Lung Disease classification (14) (Table 1). PBEC were stimulated with UV-inactivated non-typeable *Haemophilus influenzae* (NTHi) and exposed to whole cigarette smoke (CS) essentially as previously described (13). The pro-inflammatory cytokines TNFα, IL-1β and the growth factor TGF-β1 (all at 20 ng/ml; PeproTech, Rocky Hill, NJ) were added to the basal culture medium. Neutralization of endogenous TGF-β1 was performed by adding 10 μg/ml anti-TGF-β1 neutralizing antibody to the culture medium, and equal amounts of a monoclonal mouse IgG1 was used as isotype control (both R&D, Minneapolis, MN, USA). In indicated experiments, basal and luminal cell-enriched fractions were isolated from ALI-PBEC cultures based on differential disassociation upon treatment with Ca^{2+} free medium, essentially as previously described (13, 15).

Table 1: Characteristics of COPD and non-COPD patients.

	COPD	non-COPD	p-value
N=	10	8	
Gender (Females/Males)	2/8	2/6	
Age, years	67±7	66±8	
FEV1 , % predicted	64±14	92±22	< 0.02
FEV1/FVC %	57±9	80±9	< 0.0001

Patient characteristics of airway epithelial cell donors. Age and lung function are shown as means ± SD. The mean differences in FEV_1 (% predicted) and FEV_1/FVC (%) were compared using the non-parametric Mann-Whitney test. Abbreviations: COPD = chronic obstructive pulmonary disease, FEV_1= Forced expiratory volume in one second, FVC = Forced vital capacity.

RNA isolation and qPCR

RNA isolation, cDNA synthesis and qPCR analysis was performed as previously described (13) using the primers shown in Table 2. mRNA expression levels were normalized against the reference genes RPL13A and ATP5B, which were selected using the "Genorm method" (Genorm; Primer Design, Southampton, United Kingdom) .

Table 2 Primer sequences

Gene	Forward primer	Reverse primer
WFDC12	5'-AGCCAGGATGGGAGGCCAAGT-3'	5'- CTCAGGGGCAGGTGCCAAGTG-3'
SLPI	5'-CAGGGAAGAAGAGATGTTG-3'	5'-CCTCCATATGGCAGGAATC-3'
PI3	5'-CCGCTGCTTGAAAGATACTG-3'	5'-GAATGGGAGGAAGAATGGAC-3'
IL8	5'-CAGCCTTCCTGATTTCTG-3'	5'-CACTTCTCCACAACCCTCTGC-3'
TGFB1	5'-CTAATGGTGGAAACCCACAACG-3'	5'- TATCGCCAGGAATTGTTGCTG-3'
TP63	5'-CCACCTGGACGTATTCCACTG-3'	5'-TCGAATCAAATGACTAGGAGGGG-3'
KRT5	5'-CCAAGGTTGATGCACTGATGG-3'	5'-TGTCAGACATGCGTCTGC-3'
FOXJ1	5'-GGAGGGGACGTAAATCCCTA-3'	5'-TTGGTCCCAGTAGTTCCAGC -3'
SCGB1A1	5'- ACATGAGGGAGGCAGGGGCTC-3'	5'- ACTCAAAGCATGGCAGCGGCA-3'
RPL13A	5'-AAGGTGGTGGTCGTACGCTGTG-3'	5'- CGGGAAGGGTTGGTGTTCATCC-3'
ATP5B	5'-TCACCCAGGCTGGTTCAGA-3'	5'-AGTGGCCAGGGTAGGCTGAT-3'

ELISA

The secretion of IL-8/CXCL8 (R&D, Minneapolis, MN, USA) was assessed following the manufacturer's protocol. Secretion of WFDC12 by ALI-PBEC was determined using a previously described ELISA (2). To this end, conditioned medium was collected from the basal compartment, and the apical surface samples were collected by washing the cell cultures with 100 µl PBS. Collected samples were directly incubated in a high-binding 96 well plate to allow optimal detection of WFDC12.

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Data analysis

GraphPad PRISM 6.0 (GraphPad Software Inc., La Jolla, Ca) was used for statistical analysis. Analysis of differences was conducted by using a (un)paired Student's t-test, Mann-Witney U test, or one/two-way repeated measurements ANOVA and Bonferroni *post-hoc* test. Differences between groups were considered significant at p-values < 0.05.

RESULTS

NTHi increases WFDC12 protein secretion but not mRNA expression in ALI-PBEC

As monocytes express WFDC12 in response to the microbial component LPS (2), we first studied the effect of microbial stimuli on WFDC12 expression by airway epithelial cells. This was done by stimulating differentiated ALI-PBEC to the COPD-related respiratory pathogen non-typeable *Haemophilus influenzae* (NTHi). Various concentrations of UV-inactivated NTHi were added to the apical surface of ALI-PBEC cultures and after 3 h incubation mRNA expression of *WFDC12* was determined by qPCR. We did not observe an increase in *WFDC12* expression in NTHi stimulated cells (Figure 1A), and therefore assessed whether *WFDC12* expression was induced in a time-dependent manner by NTHi. This was done by exposing ALI-PBEC cultures to NTHi and determining mRNA expression levels after 3, 12, and 24 h of incubation. However, similar to the NTHi-dose response experiments, we did not observe a NTHi-induced expression of *WFDC12* at other time points (Figure 1B). We subsequently

determined whether the inability of ALI-PBEC to increase *WFDC12* gene expression after NTHi stimulation was accompanied by unchanged protein secretion levels. First, we compared the baseline WFDC12 protein release in the basal culture medium and apical surface washes from ALI-PBEC cultures, and observed that the protein was more abundantly secreted in the basal culture medium (Figure 1C). Next, we assessed WFDC12 secretion in

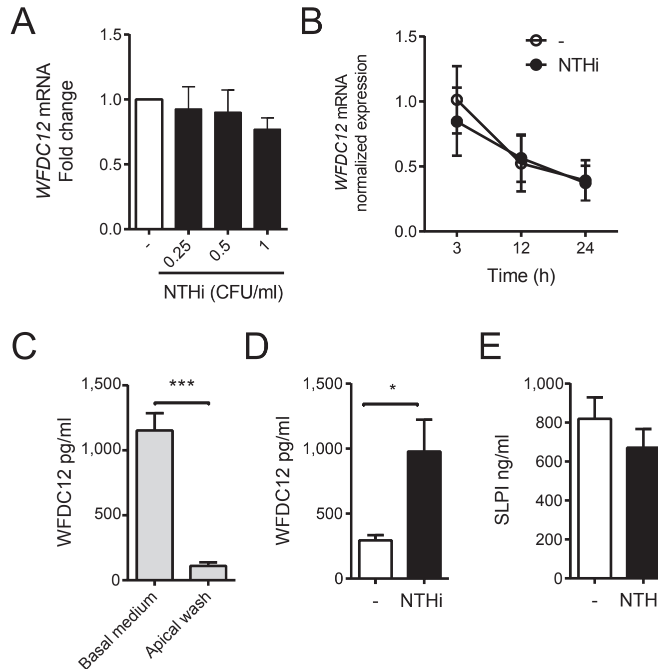

Figure 1. Effect of NTHi on WFDC12 mRNA expression and protein secretion in ALI-PBEC. ALI-PBEC were stimulated with 0.25, 0.5, and 1*10^9 CFU/ml UV-inactivated NTHi and incubated for 3 h (A) or stimulated with 1*10^9 CFU/ml UV-inactivated NTHi, for 3, 12 and 24 h incubation (B), followed by analysis of *WFDC12* expression by qPCR. mRNA expression is shown as normalized values corrected for the reference genes *RPL13A* and *ATP5B*. n=10 independent donors. (C) Baseline WFDC12 protein secretion was assessed in basal culture medium or washings of the apical surface of ALI-PBEC. n=9 independent donors. ALI-PBEC were stimulated with 1*10^9 CFU/ml UV-inactivated NTHi for 24 h. (D) WFDC12 and E) SLPI protein secretion was determined in the basal medium. n=4 independent donors. Results are shown as mean ± SEM. Analysis of differences was conducted with a (A,B) two-way ANOVA with a Bonferroni *post-hoc* test and (C-E) paired t-test. * $p < 0.05$, *** $p < 0.001$.

the basal medium of ALI-PBEC exposed at the apical surface with NTHi. In contrast to the unchanged *WFDC12* mRNA levels, NTHi increased protein secretion in ALI-PBEC cultures after 24 h stimulation (Figure 1D). SLPI secretion was not affected in NTHi-stimulated ALI-PBEC (Figure 1E), suggesting a distinct regulation of WFDC12 protein secretions in response to microbial stimuli. In summary, these findings suggest that NTHi increases the secretion, but not the mRNA expression, of WFDC12 in ALI-PBEC cultures.

NTHi induces mRNA expression of WFDC12 in undifferentiated airway epithelial cells

In a previous study, we observed NTHi-induced expression of the antimicrobial protein RNase 7, which was dependent on the differentiation status of the airway epithelial cells (13). Therefore, we further examined the induction of *WFDC12 i*n undifferentiated submerged PBEC (S-PBEC) and differentiated ALI-PBEC following exposure for 24 hours to UV-inactivated NTHi. Similar to RNase 7, *WFDC12* was induced in S-PBEC but not in ALI-PBEC cultures (Figure 2A). To compare this response with the expression of other WFDC-family members, we also examined *SLPI* and *PI3* mRNA expression levels. *SLPI* was not significantly

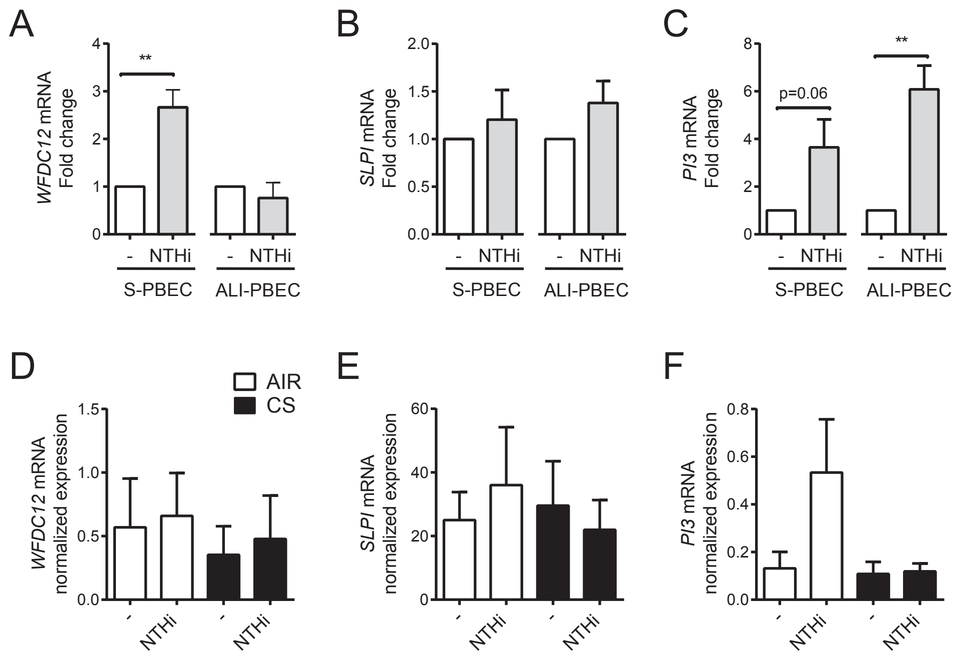

Figure 2. NTHi-induced expression of WFDC12 in S-PBEC and ALI-PBEC and effect of cigarette smoke. S-PBEC and ALI-PBEC were stimulated with $0.5*10^9$ CFU/ml UV-inactivated NTHi, and mRNA expression was determined of (A) *WFDC12*, (B) *SLPI*, and (C) elafin (*PI3*), at 24 h after stimulation. mRNA expression was corrected for the reference genes *RPL13A* and *ATP5B*, and shown as fold change compared to air exposed ALI-PBEC. n=4 independent donors. Results are shown as mean ± SEM. ALI-PBEC were exposed to air or CS and subsequently stimulated with $1*10^9$ CFU/ml UV-inactivated NTHi. mRNA expression of (D) *WFDC12*, (E) *SLPI,* and (F) elafin (*PI3*) was determined by qPCR after 3 h incubation. mRNA expression is shown as normalized values corrected for the reference genes *RPL13A* and *ATP5B.* n=4 independent donors. Results are shown as mean ± SEM. Analysis of differences was conducted with a (A-C) paired t-test and (D-F)one-way ANOVA with a Bonferroni *post-hoc* test. ** $p < 0.01$.

induced in both S- and ALI-PBEC (Figure 2B). In contrast, *PI3* expression was induced by NTHi in both types of PBEC cultures, although its induction in submerged cultures did not reach statistical significance (p=0.06; Figure 2C). We have recently shown that cigarette smoke exposure, in contrast to NTHi, increases RNase 7 expression in ALI-PBEC (13, 16).

Therefore, we questioned if cigarette smoke (CS) exposure, and the combined effect of CS and NTHi, could affect the expression of WFDC12 in ALI-PBEC as well. Cell cultures were first exposed to air or CS during a period of 15 minutes, which in a previous report was shown to cause a transient impairment of the epithelial barrier integrity and induction of innate immune responses (13). Subsequently, cells were stimulated at the apical surface with UV-inactivated NTHi. 3 h after exposure the *WFDC12* expression was determined by qPCR. This post-exposure incubation time was used, because in previous studies we observed that the main effects of CS on mRNA expression of innate immune genes were observed at early time points due to a transient effect (13). Similar to earlier observations, NTHi did not affect *WFDC12* expression, but also CS exposure and the combined exposure of NTHi and CS did not influence the expression (Figure 2D). *SLPI* expression was also not affected by NTHi and CS (Figure 2E). Although not significant, *PI3* induced by NTHi was inhibited by CS (Figure 2F). In summary, these findings suggest that NTHi-induced *WFDC12* expression depends on cell differentiation, but in contrast to RNase 7, is not expressed upon cigarette smoke-induced injury. Moreover, the expression of *WFDC12* is distinct from *SLPI* and *PI3*.

TNFα/IL-1β and TGF-β1 modulate the expression of WFDC12 by ALI-PBEC

Secreted cytokines and growth factors produced by airway epithelial cells or underlying immune and stromal cells, can influence activity of the epithelium (4)(Whitsett and Alenghat, 2014). Therefore, we determined the effect of the pro-inflammatory cytokines TNFα and IL-1β on the expression of *WFDC12* by ALI-PBEC. Combined stimulation with TNFα and IL-1β significantly increased *WFDC12* expression in ALI-PBEC (Figure 3A). As positive control, TNFα and IL-1β also promoted mRNA expression of the pro-inflammatory chemokine IL-8/*CXCL8* (Figure 3B). Previous reports have shown that the growth factor TGF-β1 is increased in COPD (17, 18) and that TGF-β1 can inhibit the expression of SLPI in airway epithelial cells. Therefore, we explored the influence of TGF-β1 on WFDC12 production by determining the effect of recombinant TGF-β1 on ALI-PBEC. TGF-β1 stimulation attenuated the expression of *WFDC12* mRNA in ALI-PBEC (Figure 3C). However, we did not observe a significant suppression of WFDC12 protein secretion into the basal medium of ALI-PBEC (Figure 3D). Interestingly, mRNA expression and protein secretion of IL-8/CXCL8 was also increased by TGF-β1 in ALI-PBEC (Figure 3E,F). This suggests a differential effect of TGF-β1 on WFDC12 and IL-8/CXCL8 expression, which is in contrast to the effects of TNFα/IL-1β stimulation. To further examine whether endogenous TGF-β produced by airway epithelial cells caused suppression of WFDC12, the effect of a neutralizing antibody directed against TGF-β1 was investigated. In line with our findings following addition of TGF-β1, anti-TGF-β1 increased WFDC12 mRNA and protein expression, when compared to an isotype antibody control (Figure 3G,H). Overall, these findings suggest that the pro-inflammatory cytokines TNFα/IL-1β can increase *WFDC12* mRNA expression in airway epithelial cells, whereas exogenously added or endogenously produced TGF-β1 impairs the expression.

TGF-β1 and WFDC12 are differentially expressed in basal and luminal airway epithelial cells

Increased levels of TGF-β1 have been shown to impair the expression of the polymeric immunoglobulin receptor (pIgR) by affecting cell differentiation (18, 19). Therefore, we examined the influence of airway epithelial cell differentiation on the expression of *WFDC12* in ALI-PBEC cultures, and furthermore determined expression levels of TGF-β1 mRNA

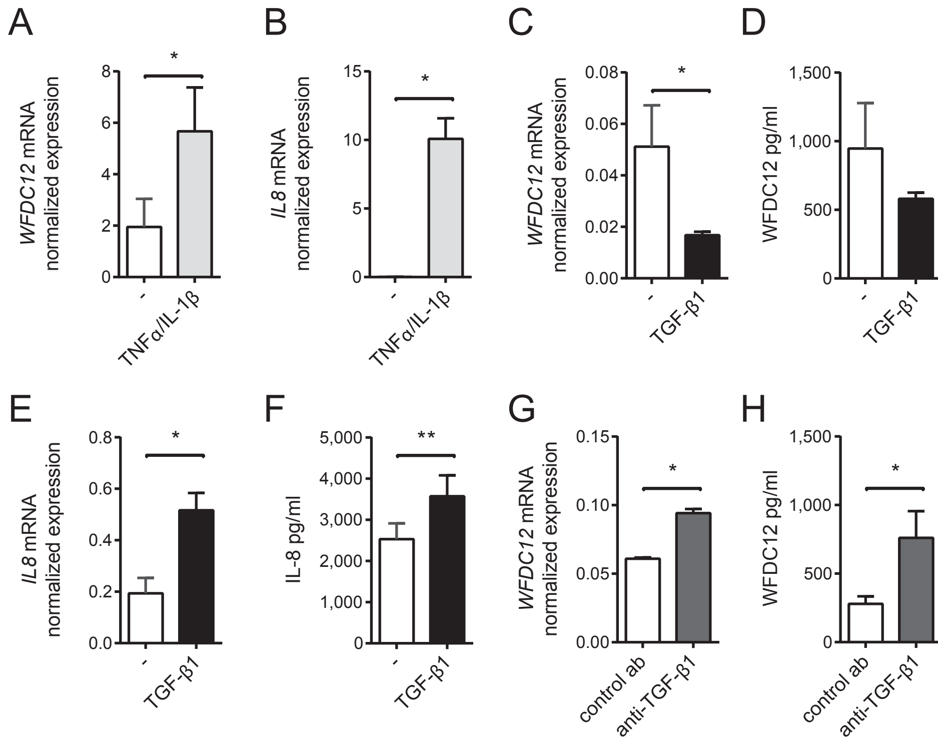

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Figure 3. Effect of TNFα/IL-1β and TGF-β1 on the expression of WFDC12. (A) ALI-PBEC cultures were stimulated with TNFα/IL-1β (both 20 ng/ml) and after 24 h incubation expression of WFDC12 was determined. mRNA expression is shown as normalized values corrected for the reference genes *RPL13A* and *ATP5B*. n=4 independent donors. (B) IL-8/CXCL8 mRNA expression was also determined after TNFα/IL-1β (both 20 ng/ml) stimulation. (C) ALI-PBEC cultures were stimulated with TGF-β1 (20 ng/ml) for 24 h. After incubation *WFDC12* expression was determined. mRNA expression is shown as normalized values corrected for the reference genes *RPL13A* and *ATP5B*. n=4 independent donors. (D) Protein secretion of WFDC12 was assessed in the basal medium of unstimulated or TGF-β1 (20 ng/ml) - stimulated ALI-PBEC, 24 h after stimulation. n=3 independent donors. IL-8/CXCL8 (E) mRNA and (F) protein secretion was also determined after TGF-β1 (20 ng/ml) stimulation. (G) Differentiated ALI-PBEC were incubated for 24 h with an anti-TGF-β1 or isotype control antibody (both 10 μg/ml) that was added to the basal compartment. mRNA expression of WFDC12 was determined by qPCR. (H) Secretion of WFDC12 into the basal medium was assessed by ELISA. n=3-4 independent donors. Results are shown as mean ± SEM. Analysis of differences was conducted with a paired t-test. * $p < 0.05$, ** $p < 0.01$.

(*TGFB1*) during differentiation. First, baseline expression of *WFDC12* and *TGFB1* was determined in undifferentiated ALI-PBEC (i.e. confluent layers of PBEC grown in Transwell inserts), and cells that were differentiated at the ALI for 1 and 2 weeks. *WFDC12* expression levels were significantly increased upon 1 and 2 weeks of airway epithelial cell differentiation at the air-liquid interface (Figure 4A). In contrast, expression of *TGFB1* was significantly reduced after 1 and 2 weeks of cell differentiation (Figure 4B). These findings suggest an inverse relationship between *WFDC12* and *TGFB1* expression, which are respectively increased and decreased during differentiation. Since basal cells predominate in undifferentiated ALI-PBEC,

whereas differentiated cultures are composed of basal and luminal cells, we next isolated enriched luminal and basal cell fractions from fully differentiated ALI-PBEC cultures and determined mRNA expression levels in these fractions. We confirmed successful separation of both fractions by showing higher expression of the basal cell markers *TP63* and *KRT5* in the basal cell fraction, whereas *FOXJ1* (ciliated cells) and *SCGB1A1* (club cells) expression

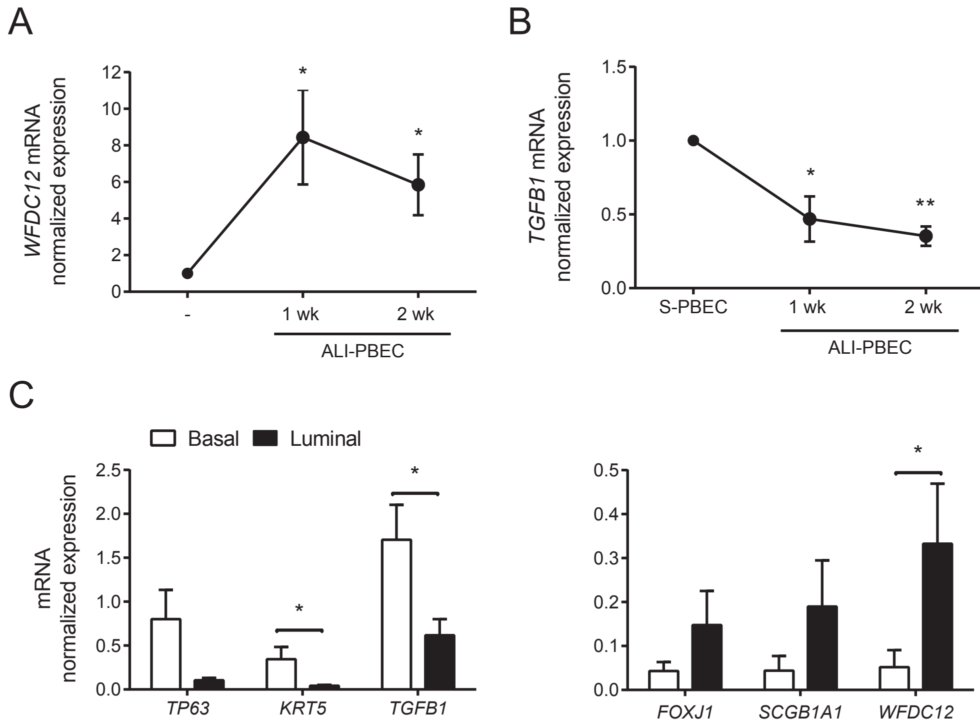

Figure 4. Airway epithelial cell differentiation regulates WFDC12 and TGF-β1 expression. Airway epithelial cell mRNA expression of (A) WFDC12 and (B) *TGFB1* was determined in undifferentiated PBEC (-) and ALI-PBEC differentiated for 1 or 2 weeks. n=6-9 independent donors. (C) Luminal and basal cell fractions were separated in differentiated ALI-PBEC and mRNA expression of *WFDC12* and *TGFB1* was determined. In addition, the expression of *TP63*, *KRT5* (both basal cell markers), *FOXJ1* (ciliated cell marker), and *SCGB1A1* (club cell marker) was determined. n=4 independent donors. Results are shown as mean ± SEM. Analysis of differences was conducted with (A,B) a one-way ANOVA with a Bonferroni *post-hoc* test (compared to (-) undifferentiated) and (C) a paired t-test.* $p < 0.05$.

was more abundant in the luminal cell fraction (Figure 4C). A significantly higher expression of *WFDC12* in the luminal cell enriched fraction was observed, whereas *TGFB1* was more abundant in the basal cell fraction. Overall, these findings suggest that constitutive expression of WFDC12 in airway epithelial cells is increased during differentiation by formation of luminal cells, while TGF-β1 is reduced during differentiation and mainly expressed by basal cells.

Lower expression of WFDC12 in COPD airway epithelial cells

In previous experiments we used epithelial cells that were not selected based on COPD disease status. However, as gene expression may be altered in COPD patients, we further examined the expression levels of *WFDC12* in cultured ALI-PBEC from COPD patients and non-COPD (ex)smokers. Compared to non-COPD cultures, COPD ALI-PBEC displayed a significantly lower expression of *WFDC12* (Figure 5A). Because of the large spread in mRNA expression between individual donors, we further assessed the correlation between lung function and *WFDC12* levels. A significant correlation was observed between WFDC12 and FEV_1 (% predicted) (Figure 5B) and FEV_1/FVC (% predicted) (Figure 5C). These findings suggest lower baseline expression of *WFDC12* in COPD cultures compared to non-COPD, which is correlated to lung function

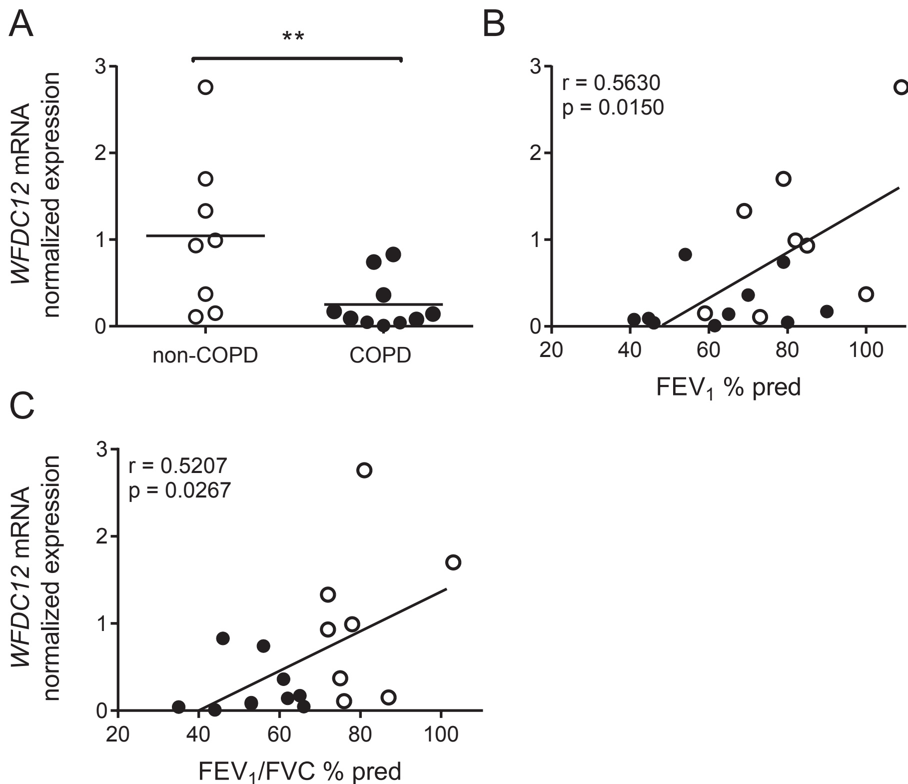

Figure 5. Expression of WFDC12 in COPD and non-COPD ALI-PBEC. (A) Expression of *WFDC12 i*n ALI-PBEC cultures from non-COPD (ex)smokers (open circles, n=8) and COPD patients (black circles, n=11). mRNA expression is shown as normalized values corrected for the reference genes *RPL13A* and *ATP5B*. Individual data points are shown with median and interquartile range. Analysis of differences was conducted with a Mann-Whitney test. ** $p < 0.01$. (B) Correlation between FEV_1 (% predicted) and *WFDC12* expression (r=0.5630, p=0.0150). (C) Correlation between FEV_1/FVC (% predicted) and *WFDC12* expression (r = 0.5207, p = 0.0267).

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DISCUSSION

We report a dynamic regulation of WFDC12 expression in airway epithelial cells, which increases during cell differentiation, and furthermore is distinct from other WFDC-family members. In contrast to the expression of other innate immune mediators (13), *WFDC12* mRNA expression by ALI-PBEC was not affected by stimulation with UV-inactivated NTHi or acute exposures to cigarette smoke. We cannot formally exclude that live bacteria or prolonged exposures to cigarette smoke could have affected WFDC12 expression. Interestingly, UV-inactivated only increased WFDC12 mRNA expression in undifferentiated PBEC, but did increase WFDC12 protein secretion in ALI-PBEC cultures suggesting release of stored WFDC12. In contrast to differentiated ALI-PBEC, *WFDC12* mRNA was further increased in undifferentiated S-PBEC exposed to UV-inactivated NTHi. This finding corresponds with an earlier study examining the expression of the antimicrobial protein RNase 7 (13), and further indicates that differentiated and undifferentiated airway epithelial cells display distinct innate immune properties.

The increase in *WFDC12* in S-PBEC upon stimulation with NTHi suggests that a pro-inflammatory environment may increase the expression of WFDC12 in basal cells. Such a mechanism may contribute to the regulation of airway epithelial innate immunity upon injury, when basal cells may be exposed to respiratory pathogens in the absence of a luminal cell layer (20, 21). Examination of the influence of pro-inflammatory cytokines demonstrated that, in contrast to NTHi, TNFα/IL-1β stimulation did increase *WFDC12* expression in ALI-PBEC. This is likely explained by the fact that these cytokines were added to the basal compartment of the culture and therefore were readily able to stimulate basal cells. In addition to TNFα/IL-1β, we have observed in preliminary studies that IL-4 and IL-17 could also increas *WFDC12* expression in undifferentiated S-PBEC (*unpublished data*). This suggests that a range of other cytokines can induce the expression of WFDC12.

We also observed a decrease in WFDC12 expression by TGF-β1, which is similar to earlier reports demonstrating that TGF-β1 also impaired expression of SLPI (22-24). It becomes more obvious that TGF-β and related growth factor family members play an important role in COPD pathogenesis (25). Besides decreasing the expression of WFDC12, TGF-β1 increased airway epithelial expression of the pro-inflammatory chemokine IL-8/CXCL8. This suggests that TGF-β1 causes imbalances in the expression of anti- and pro-inflammatory factors by the airway epithelium that may contribute to the persistence of airway inflammation. Interestingly, it was reported that TGF-β did not affect the expression of IL-8 in undifferentiated S-PBEC (26). This suggests that airway epithelial responses to TGF-β are also cell differentiation dependent.

Supporting our earlier assumption that differentiated ALI-PBEC express WFDC12 in sufficient quantities, we observed that *WFDC12* mRNA expression became more abundant during cell differentiation. We furthermore detected *WFDC12* mainly in luminal cells of differentiated ALI-PBEC cultures. Indeed, the luminal airway epithelium consists of different cell types, i.e. ciliated, goblet and club cells. Therefore, expression of WFDC12 may be more restricted to a certain luminal cell type, which requires further study. In line with previous studies (27, 28), *TGFB1* expression levels were decreased upon mucociliary differentiation,

and its expression was mainly observed in the basal cell fraction of ALI-PBEC. This suggests an inverse relationship between WFDC12 and TGF-β1 expression during cell differentiation, in which a reduction in TGF-β1 may allow an increase in WFDC12. The distinct expression of WFDC12 in luminal and basal cells of ALI-PBEC, might be explained by a cell differentiation dependent effect of TGF-β. Another explanation for the low expression of WFDC12 by basal cells is the quiescent state of the cells in intact ALI-PBEC cultures, whereas expression in injured conditions can be elevated by inflammatory stimuli.

Previous studies have revealed impaired defense mechanisms in cultured epithelial cells from patients with COPD. This includes a recent report from our group showing that COPD epithelial cell cultures display reduced antibacterial activity and expression of the antimicrobial mediators hBD-2 and S100A7 (29). In the present study we extend these findings by showing that airway epithelial cell expression of *WFDC12* is intrinsically lower in differentiated ALI-PBEC from COPD patients compared to non-COPD controls. Based on the previously described function of the protein as a protease inhibitor and anti-inflammatory mediator (2), it can be speculated that the attenuated expression of WFCD12 by airway epithelial cells in COPD contributes to enhanced airway tissue injury and inflammation. However, the protein may also have additional functions, such as affecting wound repair. It

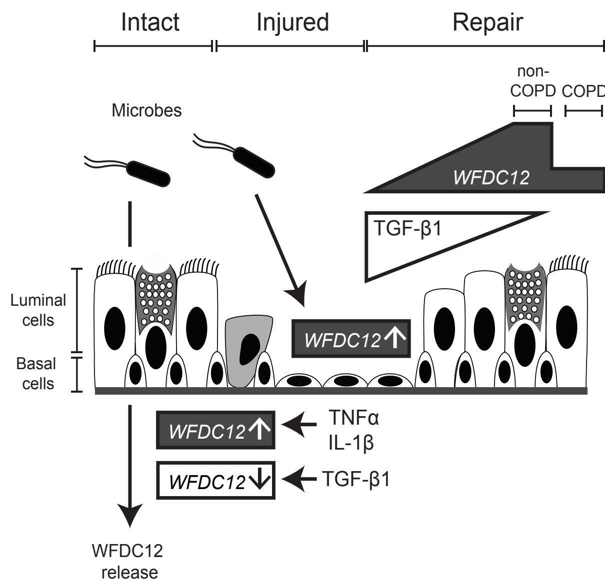

Figure 6. Proposed model for regulation of epithelial WFDC12 expression. Intact airway epithelium consists of luminal cells and basal cells. Release of stored WFDC12 protein by intact epithelium is increased upon exposure to microbes, whereas mRNA expression is increased by TNFα/IL-1β, and inhibited by TGF-β1. Injured epithelium consists of basal cells that restore the injured epithelial layer and that express *WFDC12* upon exposure to microbes. Moreover, basal cells express TGF-β1, which inhibit *WFDC12*. Upon regeneration of the airway epithelium, basal cell numbers decrease, resulting in a reduced level of TGF-β1. Inversely, *WFDC12* expression increases upon differentiation and is high in the luminal cells of restored epithelium. This expression is higher in non-COPD compared to COPD airway epithelial cells.

is furthermore unknown how comparable the levels of WFDC12 are between epithelial cells and macrophages and therefore whether lower levels in COPD epithelium are compensated or overruled by the enhanced numbers of inflammatory macrophages in the lungs of COPD patients. Further research examining both the functional properties of WFDC12 from airway epithelial cells, and studying the expression of this protein in macrophages and other immune cells in COPD is therefore required to investigate this.

Based on our findings, we propose the following model (Figure 6) in which WFDC12 expression is dynamically regulated in the airway epithelium. In intact epithelium, WFDC12 is constitutively expressed in luminal cells, and the protein is released upon exposure to microbes. WFDC12 is furthermore induced by TNFα/IL-1β, and suppressed by TGF-β1, which may be derived from by the epithelium itself, or by immune or stromal cells. Upon epithelial injury, death and/or shedding of luminal cells may result in exposure of the underlying basal cells to invading micro-organisms, which may result in the induction of WFDC12 in these basal cells. Upon epithelial repair, TGF-β1 initially suppresses airway epithelial WFDC12 expression. When repair progresses, TGF-β1 levels decline, whereas WFDC12 expression by luminal cells increases. This constitutive expression of WFDC12 in the luminal cells of restored epithelium is lower in COPD airway epithelial cells when compared to non-COPD smokers.

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