Modulation of airway epithelial cell function by vitamin D in COPD

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CHAPTER

3

TGF-β1 impairs vitamin D-induced and constitutive airway epithelial host defence mechanisms

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Abstract

The airway epithelium is an important site for local vitamin D (VD) metabolism and this can be negatively affected by inflammatory mediators. VD is an important regulator of respiratory host defence e.g. by increasing expression of hCAP18/LL-37. TGF-β1 is increased in COPD (chronic obstructive pulmonary disease) and known to decrease expression of constitutive host defence mediators such as secretory leukocyte protease inhibitor (SLPI) and polymeric immunoglobulin receptor (pIgR). VD has been shown to affect TGF-β1-signalling by inhibiting TGF-β1-induced epithelial-to-mesenchymal transition. However, interactions between VD and TGF-β1, relevant for understanding host defence in COPD, are incompletely understood. Therefore, the aim of the present study was to investigate combined effects of VD and TGF-β1 on airway epithelial cell host defence mechanisms. Exposure to TGF-β1 reduced both baseline and VD-induced expression of hCAP18/LL-37, partly by increasing expression of the VD-degrading enzyme CYP24A1. TGF-β1 alone decreased the number of secretory club- and goblet cells and reduced expression of the constitutive host defence mediators SLPI, s/lPLUNC and pIgR, effects that were not modulated by VD. These results suggest that TGF-β1 may decrease respiratory host defence both directly by reducing expression of host defence mediators, and indirectly by affecting VD-mediated effects such as expression of hCAP18/LL-37.

Introduction

Patients with chronic obstructive pulmonary disease (COPD) suffer more frequently from respiratory infections than ex- or non-smokers and this may contribute to exacerbations and to further progression of the disease (1, 2). This increased susceptibility to infections can be explained by impaired mucociliary clearance and decreased host defence (3), that may in part result from persistent exposure to cigarette smoke (CS) or to other noxious gases (4-6). The airway epithelium serves as the front line in the lung's host defence by preventing microbes to enter the tissue and bloodstream. Its contribution to this important function is mediated by a combination of mechanisms including (but not limited to) the maintenance of a physical barrier supported by its tight- and adherens junctions, mucociliary clearance, and secretion of both inducible and constitutively expressed host defence peptides and proteins (HDPs), reactive oxygen- and nitrogen species, interferons, chemokines and cytokines (7). In addition to broad-spectrum antimicrobial activity, HDPs also have the ability to modulate immune responses and promote wound repair (8). Under homeostasis, inducible HDPs such as human β-defensin-2 and hCAP18/LL-37 are expressed at low levels and their expression can be increased upon e.g. activation of pattern recognition receptors (9), cytokine and growth factor receptors, and by other mediators such as vitamin D (VD), whereas constitutively expressed HDPs do not require such stimuli for their expression (9-11).

Whereas VD is classically known for its function in the regulation of calcium homeostasis and bone metabolism, multiple studies have shown that it also acts as an important regulator of host defence and immunity, including respiratory host defence (12, 13). This was supported by two clinical trials that showed that VD supplementation reduces the exacerbation rate in VD-deficient COPD patients (14, 15) and a recent meta-analysis that demonstrated that VD supplementation protects against acute respiratory tract infections (16). Various mechanisms may contribute to this protective effect of VD, including direct effects such as VD-mediated increases of hCAP18/LL-37, and/or indirect effects via promotion of CFTR expression or its ability to reduce oxidative stress (11, 17-19). In the airway epithelium, the main circulating form of VD ($25(OH)D_3$) is hydroxylated to generate the active form of VD ($1,25(OH)_2D_3$) by α1-hydroxylase (CYP27B1) (20). Next, $1,25(OH)_2D_3$ binds the nuclear VD receptor (VDR) and heterodimerizes with the

retinoic acid receptor to interact with VD response elements to initiate gene expression of more than 900 genes, including CYP24A1, which converts both $25(OH)D_3$ and $1,25(OH)_2D_3$ into inactive metabolites (12, 21). The airway epithelium is an important site of local VD metabolism and we and others have shown that the expression or the activity of CYP27B1, CYP24A1 and VDR can be modulated by several inflammatory mediators such as TNF-α/IL-1β, IL-17A, nontypeable *Haemophilus influenzae* (NTHi), IL-13, the viral analogue poly(I:C) and CS, which all have been implicated in the pathogenesis of chronic inflammatory lung diseases (5, 20, 22, 23).

Studies in prostate cancer- and stromal cells and hepatocytes suggest that the positive effects of VD might be modulated by TGF-β1 (24, 25). This may be relevant for COPD, since elevated levels of TGF-β1 expression were found in the airways of COPD patients (26-28), although this was not shown by all studies (29). TGF-β1 is a multifunctional cytokine that is produced and activated upon injury, through CS-exposure or by inflammation (30-33). When this injury persists, continued release of TGF-β1 contributes to tissue remodelling, a process that may be driven in part by epithelial-to-mesenchymal transition (EMT) (34). Several studies have shown that VD might counteract TGF-β1-mediated effects on fibrosis, as demonstrated by its ability to inhibit TGF-β1-induced EMT in both mouse models of asthma and fibrosis and in airway epithelial cell lines (17, 35-37). In addition to its role in fibrosis and EMT, TGF-β1 affects respiratory host defence by impairing anti-viral interferon type I and III responses, but also by restricting expression of constitutively expressed host defence mediators such as SLPI and pIgR (31, 32, 38, 39).

Despite this insight into the role of TGF-β1 in the pathogenesis of COPD and other chronic inflammatory lung disease, it is not known whether exposure to TGF-β1 also affects respiratory host defence by affecting VD-metabolism and VD-mediated expression of the HDP hCAP18/LL-37. Moreover, it is currently unknown whether VD modulates TGF-β1-mediated repression of constitutively expressed HDPs. We therefore aimed to study the interaction between VD and TGF-β1 on airway epithelial cell host defence mechanisms. To this end, we first investigated the effects and underlying mechanisms of TGF-β1 on VD-metabolism and on VD-mediated hCAP18/LL-37 expression. Next, we studied effects of VD on TGF-β1-induced changes in epithelial composition and on expression of a group of constitutively expressed host defence mediators, as well as on antibacterial activity.

Materials and Methods

Primary bronchial epithelial cell (PBEC) culture

PBEC were obtained from tumour free bronchial lung tissue from anonymous donors that was collected during lung resection surgery for lung cancer at LUMC. Cells were cultured as previously described with some adaptations (5, 40). Briefly, cultures of bronchial epithelial cells (passage 1) were first expanded in T75 culture flasks, pre-coated with a mixture of 30 µg/ml Purecol (Advanced BioMatrix, San Diego, CA), 5 µg/ml stabilized fibronectin (Alfa Aesar, Thermo Fisher scientific, Landsmeer, The Netherlands) and 10 µg/ml BSA (Sigma Aldrich, Zwijndrecht, The Netherlands). Next, cells were seeded at a density of 5000 cells per well for submerged cultures of (S)-PBEC or 40,000 cells per insert (passage 2) on pre-coated 24-well plates (Corning Costar, Cambridge, MA) and on semi-permeable Transwell inserts respectively (12 mm, 0.4 µm pore-size, Corning Costar). The cells were cultured in BEpiCM-b:DMEM (B/D)-medium (1:1) (ScienCell Research Laboratories, Uden, The Netherlands and STEMCELL technologies, Köln, Germany respectively), supplemented with Bronchial Epithelial Cell Growth Supplement (ScienCell Research Laboratories), and additional 1 nM EC-23 (Tocris, Bio-techne Ltd, Abington, UK) (for the submerged phase of PBEC cultures on inserts only), 25 mM HEPES (Cayman Chemical, Hamburg, Germany), 100 U/ml penicillin and 100 µg/ml streptomycin (ScienCell Research Laboratories). After cells growing on inserts had reached confluence (after 5-7 days), apical medium was removed and the cells were cultured at the air-liquid interface (ALI) and medium was changed 3 times a week with (B/D)-medium supplemented with Bronchial Epithelial Cell Growth Supplement and additional 50 nM EC-23. During refreshment, the apical surface was washed with PBS to remove mucus. After 14 days of air-exposed culture, the cells produced mucus and had developed cilia, and cultures were used for experiments. S-PBEC were cultured until they reached 50-70% confluence after 4-5 days and next cultured for 24 h in B/D-medium supplemented with Bronchial Epithelial Cell Growth Supplement without BSA, BPE, EGF and hydrocortisone before stimulation (B/D-starvation medium).

Experimental design

PBEC that had been differentiated for 14 days at the ALI, were exposed to various concentrations of TGF-β1 (0.2, 1 and 5 ng/ml) and 100 nM 25(OH)D_3 and/or 1,25$(OH)_2D_3$ (Millipore B.V., Amsterdam, The Netherlands) for 24 h to assess

changes in gene expression, or for 48 h to assess SLPI levels in apical washes, airway epithelial antibacterial activity and hCAP18/LL-37 release by Western blot or immunofluorescence. S-PBEC cultures were used to elucidate the mechanism of action of TGF-β1-reduced expression of hCAP18/LL-37. To assess the role of TGF-β1-mediated induction of CYP24A1 or canonical TGF-β1-Smad signalling, S-PBEC were treated with 5 ng/ml TGF-β1 and 100 nM $1,25(OH)_2D_3$ in presence or absence of 10 μM TGF-β1-Smad signalling inhibitor SB431542 (Sigma-Aldrich) or 10 μM of the antifungal ketoconazole (KTZ) that acts as an inhibitor of cytochrome P-450 (CYP) (Sigma-Aldrich) for 24 h.

Silencing of C/EBP-α using siRNA transfection

S-PBEC were used to determine if the TGF-β1-reduced expression of hCAP18/LL-37 was mediated by the transcription factor CCAAT/enhancer-binding protein-α (C/EBP-α). S-PBEC were refreshed with B/D-starvation medium containing 5 ng/ml TGF-β1 and 100 nM $1,25(OH)_2D_3$ and transfected using 3 μl/well RNAiMAX SilentFect tranfection reagent (Thermo Fisher scientific) containing 20 mM *CEBPA*- or negative control- siRNA (Ambion, Thermo Fisher scientific) and incubated for 24 h.

RNA Isolation, reverse transcription (RT) and quantitative (q)PCR

Cells were lysed in RNA lysis buffer (Promega Benelux B.V., Leiden, The Netherlands). Total RNA was robotically extracted using the Maxwell tissue RNA extraction kit (Promega) and quantified using the Nanodrop ND-1000 UV-Vis Spectrophotometer (Nanodrop technologies, Wilmington, DE). For cDNA synthesis, 1 μg of total RNA was reverse transcribed using oligo dT primers (Qiagen Benelux B.V., Venlo, The Netherlands) and M-MLV Polymerase (Thermo Fisher scientific) at 42° C. All qPCR reactions were performed in triplicate on a CFX-384 Real-Time PCR detection system (Bio-Rad Laboratories, Veenendaal, The Netherlands), using primers shown in table I and IQ SYBRGreen supermix (Bio-Rad). The relative standard curve method was used to calculate arbitrary gene expression using CFX-manager software (Bio-Rad). Two reference genes**,** selected using the "Genorm method" (Genorm, Primer design, Southampton, UK), were included to calculate normalized gene expression.

Gene	Encoding Protein	Sequence forward primer	Sequence reverse primer	GenBank accession #
YWHAZ*	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta	ACTTTTGGTACATTGTGGCTTCAA	CCGCCAGGACAAACCAGTAT	NM_001135699.1
RPL27*	ribosomal protein L27	ATCGCCAAGAGATCAAAGATAA	TCTGAAGACATCCTTATTGACG	NM_000988
CAMP	hCAP18/LL-37	TCATTGCCCAGGTCCTCAG	TCCCCATACACCGCTTCAC	NM_004345.3
CYP24A1	24-hydroxylase	TTGGCTCTTTGTTGGATTGTCCGC	TGAAGATGGTGCTGACACAGGTGA	NM_000782.3
CYP27B1	α1-hydroxylase	AACCCTGAACAACGTAGTCTGCGA	ATGGTCAACAGCGTGGACACAAA	NM_000785.3
VDR	vitamin D receptor	ACCTGGTCAGTTACAGCATCC	TGGTGAAGGACTCATTGGAGC	NM_001017535.1
SLPI	secretory leukocyte protease inhibitor	GAGATGTTGTCCTGACACTTGTG	AGGCTTCCTCCTTGTTGGGT	NM_003064
BPIFA1	short palate, lung, and nasal epithelium clone protein	CTTGGCCTTGTGCAGAGC	CAACAGACTTGCACCGACC	NM_016583
BPIFB1	long palate, lung, and nasal epithelium clone protein	CAGTGCCATGCGGGAAAAG	GCTGGAGGATGTTAGCTGTGA	NM_080574
PIGR	Polymeric Immunoglobulin receptor	CTCTCTGGAGGACCACCGT	CAGCCGTGACATTCCCTG	NM_002644
CLCA1	chloride channel accessory 1	ATGGCTATGAAGGCATTGTCG	TGGCACATTGGGGTCGATTG	NM_001285
SCGB1A1	secretoglobin family 1A member 1	ACATGAGGGAGGCAGGGGCTC	ACTCAAAGCATGGCAGCGGCA	NM_003357
FOXJ1	Forkhead box protein J1	GGAGGGGACGTAAATCCCTA	TTGGTCCCAGTAGTTCCAGC	NM_001454

Table I. PCR primers and sequences used for quantitative PCR
*Used as a reference gene, selected using the Genorm method

Western blot

For Western blot analysis of hCAP18/LL-37 release, basal medium was applied to Oasis HLB 1cc extraction cartridges (Waters Chromatography, Etten-Leur, The Netherlands) and the eluate was dried by vacuum centrifugation (CHRIST RVC2-25 Vacuüm system) (41). Lyophilized protein samples were resuspended in 100 µl

reducing SDS-PAGE sample buffer, heated for 5 minutes at 100° C and applied on a 16.5% Tris-Tricine gel as previously described (5). Next, proteins were blotted on a Polyvinylidene fluoride (PVDF) membrane and non-specific binding sites were blocked in PBS containing 5% (v/v) heat-inactivated new born calf serum, 5% (w/v) skimmed milk in PBS. Membranes were probed with 1/200 diluted mouse monoclonal anti-hCAP18/LL-37 (clone 1.1.C12; Hycult Biotech, Uden, The Netherlands) in blocking buffer. Next, the membranes were incubated in 1/1000 diluted rabbit-anti-mouse-HRP (Cell Signaling Technology, Leiden, The Netherlands) in blocking buffer. SuperSignal West Pico ECL Substrate (Thermo Fisher scientific) was used to visualize hCAP18/LL-37 protein using The ChemiDoc™ Touch imager in combination with Image Lab™ software (Biorad).

Immunofluorescence staining of CYP24A1, SLPI, sPLUNC and pIgR

Cells were fixed on Transwell inserts in 1% paraformaldehyde (Millipore B.V.) in PBS for 10 minutes on ice and washed with ice-cold PBS. Next, cells were permeabilized with methanol for 10 minutes at 4° C, washed in PBS and blocked with PBS/1% (w/v) BSA/0.3% (v/v) Triton-X-100 (PBT) for 30 minutes at 4° C. Next, cells were treated for 30 minutes with SFX-signal enhancer (Thermo Fisher scientific) followed by incubation with primary antibodies in PBT for 1 h at RT (table II). After washing in PBS, cells were incubated with an Alexa Fluor 488 labeled secondary antibody (1/200, Alexa Fluor 488 goat anti-rabbit IgG; Thermo Fisher scientific) and Alexa Fluor 568 goat anti-mouse IgG together with DAPI (Sigma Aldrich) in PBT for 30 minutes at RT in the dark. Finally, cells were mounted in ProLong™ Gold Antifade Mountant (Thermo Fisher scientific) and images were acquired using a TCS SP5 Confocal Laser Scanning Microscope (Leica Microsystems B.V., Eindhoven, The Netherlands) and LAS AF Lite software (Leica Microsystems B.V.).

Antibody	Supplier	Catalog #	species	Antibody dilution
CYP24A1	Sigma	HPA022261	rabbit	1/100
pIgR	R&D Systems	MAB27171	mouse	1/50
P63	Abcam	ab124762	rabbit	1/100
P63	Leica	NCL-P63	mouse	1/100
sPLUNC	Hycult Biotech	HM2314	mouse	1/100
SLPI	Hycult Biotech	HM2037	mouse	1/100
Mucin 5AC	Labvision Neomarkers	MS-145-P1	mouse	1/1000
CC16	Hycult Biotech	HM2178	mouse	1/50

Table II. Antibodies used for immunofluorescence staining

SLPI ELISA

Apical washes were obtained by washing the apical surface of the stimulated ALI-PBEC with 200 µl warm PBS for 10 minutes at 37° C. SLPI in apical washes of the treated ALI-PBEC was measured using an ELISA as previously described (42).

Antibacterial activity assay

Antibacterial activity was assessed by applying log-growing cultures of nontypeable *Haemophilus influenzae* (NTHi) on the apical surface of the treated cells as previously described with a few modifications (40). NTHi strain D1 was cultured in Tryptone soya broth containing X and V-factor (TSB XV, Mediaproducts BV, Groningen, the Netherlands) while shaking overnight at 37° C (2). Next, 2 ml of the overnight culture was transferred into fresh 10 ml TSB XV medium and incubated for 4 h at 37° C while shaking to obtain mid log phase growing bacteria. Before applying the washed bacterial suspensions to the apical surface of ALI-PBEC, excess mucus was removed by washing both treated and untreated cells with 200 µl 10 mM sodium phosphate buffer (NaPB) for 15 minutes at 37° C 6 h before the assay. We applied approximately 1 multiplicity of infection (MOI) NTHi in 20 µl NaPB + 1% v/v TSB XV per insert for 2 h. Next, membranes containing the cells with bacteria were dissected from the inserts and placed into tubes containing sterile glass beads and 1% TSB in PBS and cells were disrupted by using a Minilys personal homogenizer (Bertin Instruments, Montigny-le-Bretonneux, France) for 2 times 30 seconds and kept on ice between treatments. Serial dilutions of bacterial suspensions were plated on chocolate agar plates (Biomerieux, Zaltbommel, The Netherlands), and incubated overnight at 37° C to assess surviving bacteria by colony forming unit (CFU) determination.

Statistical analysis

Statistical analysis was conducted using GraphPad Prism 7 (GraphPad Software Inc., La Jolla, CA, U.S.A.). To analyse qPCR results, fold change in gene expression of the stimuli compared to control (CNTRL) was first calculated, followed by log-transformation. All data were analysed using a two-way ANOVA and the Bonferroni post-hoc test. Differences at p values < 0.05 were considered statistically significant.

Results

TGF-β1 impairs baseline and vitamin D-induced expression and release of hCAP18/LL-37 in differentiated primary bronchial epithelial cells

We have previously shown that exposure to pro-inflammatory stimuli impairs VD-induced expression and release of the HDP hCAP18/LL-37 in differentiated ALI-PBEC (43). To investigate if TGF-β1 also affects expression of the VD-responsive HDP hCAP18/LL-37 (*CAMP*), we exposed differentiated ALI-PBEC to various concentrations of TGF-β1 for 24-48 h in presence and absence of $25(OH)D_3$ (this inactive form of VD is converted in PBEC by CYP27B1 into active $1,25(OH)_2D_3$). We first confirmed that both $25(OH)D_3$ and $1,25(OH)_2D_3$ clearly increased expression of *CAMP* mRNA in ALI-PBEC after 24-48 h of incubation (Figure 1A). TGF-β1 dose-dependently limited the VD-increased expression of *CAMP* at both time points, whereas all concentrations of TGF-β1 also decreased baseline expression of *CAMP* after 24 h and - only at the highest dose - after 48 h (Figure 1A). To verify TGF-β1-mediated repression of *CAMP* at the protein level, we exposed ALI-PBEC to the highest dose TGF-β1 (5 ng/ml) in presence and absence of $25(OH)D_3$ and/or $1,25(OH)_2D_3$ for 48 h, and assessed hCAP18/LL-37 secretion in basal medium using Western blot analysis. Using Western blot analysis, both $1,25(OH)_2D_3$ and $25(OH)D_3$ clearly increased release of hCAP18/LL-37 in basal medium, which was reduced by TGF-β1, in line with gene expression data (Figure 1B). These data demonstrate that TGF-β1 interferes with baseline and VD-mediated signalling, resulting in reduced expression and release of the HDP hCAP18/LL-37.

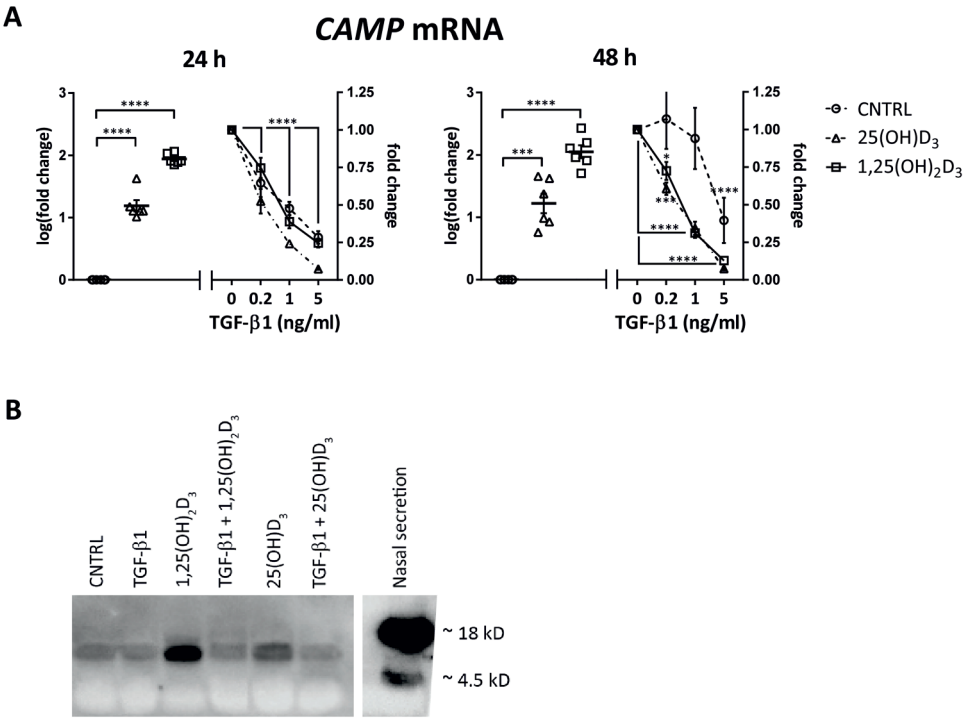

Figure 1. TGF-β1 impairs baseline and vitamin D-induced expression and release of hCAP18/LL-37 in primary bronchial epithelial cells (PBEC). PBEC were differentiated at the air-liquid interface (ALI) followed by 24-48 h stimulation with TGF-β1 with or without inactive 25(OH)D3, active 1,25(OH)2D3 or medium control (CNTRL) to determine mRNA expression of CAMP (hCAP18/LL-37) by qPCR (A), and cells were stimulated for 48 h to assess release of hCAP18/LL-37 by Western blot analysis (B). (A) Relative mRNA expression of CAMP was determined by qPCR. Normalized gene expression was calculated by using the expression of tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta (YWHAZ) and ribosomal protein L27 (RPL27) as reference genes. Fold change in gene expression of $25(OH)D_3$ and $1,25(OH)_2D_3$ compared to CNTRL was first calculated, followed by a log-transformation of the data. To assess effects of different doses of TGF-β1 on CAMP expression in $25(OH)D_3$, $1,25(OH)_2D_3$ and CNTRL-treated cells, fold change for each group was calculated separately. Data are presented as individual values, including means ± SEM and were tested for significance using the two-way ANOVA and the Bonferroni post-hoc test (n = 6 donors). (B) Tris-Tricine gel electrophoresis, followed by Western blot analysis was used to detect hCAP18/LL-37 production in basal medium. Nasal secretion was used as positive control to show both intact hCAP18 peptide and cleaved mature LL-37 peptide at 18 and 4.5 kDa respectively. Western blots are a representative of 4 different donors. The image was cut to include the image of the nasal secretion. *** $p < 0.001$, **** $p < 0.0001$.

TGF-β1 affects the vitamin D-metabolic pathway

To investigate if the effects of TGF-β1 on VD-mediated expression of hCAP18/LL-37 were mediated by changes in the VD-metabolic pathway, we assessed the effects TGF-β1 on the expression of the VD-degrading enzyme CYP24A1, the VD-activating enzyme CYP27B1 and the VD receptor (VDR). ALI-PBEC were exposed to various concentrations of TGF-β1 for 24–48 h in presence and absence of $25(OH)D_3$ and $1,25(OH)_2D_3$. As expected, both forms of VD increased *CYP24A1* expression (Figure 2A left side) compared to control treated cells. In absence of VD, TGF-β1 markedly increased *CYP24A1* expression, and even caused a small further increase in presence of both forms of VD (Figure 2A). Furthermore, we also observed a minor dose-dependent change in *VDR* expression and no effect of TGF-β1 on the expression of *CYP27B1* (Figure 2A). To verify these effects of TGF-β1 on *CYP24A1* at the protein level, we performed immunofluorescence staining using CYP24A1 antibodies and confirmed the ability of TGF-β1 to increase CYP24A1 expression at the protein level in presence and absence of $1,25(OH)_2D_3$ (Figure 2B). Together these data indicate that TGF-β1 affects VD-metabolism by increasing expression of the VD-degrading enzyme CYP24A1.

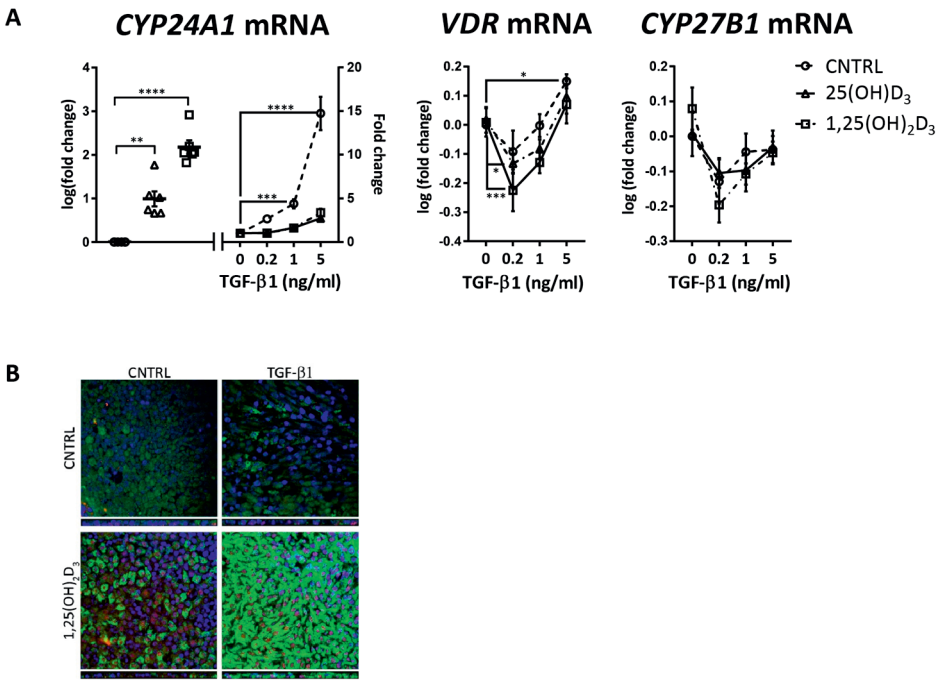

Figure 2. TGF-β1 affects the vitamin D-metabolic pathway in primary bronchial epithelial cells (PBEC). PBEC were differentiated at the air-liquid interface (ALI) followed by 24 h stimulation with TGF-β1 in presence or absence of $25(OH)D_3$, $1,25(OH)_2D_3$ or medium control (CNTRL) to assess mRNA expression of the vitamin D (VD)-degrading enzyme (*CYP24A1*), VD receptor (*VDR*) and the VD-activating enzyme (*CYP27B1*) by qPCR (A). In addition, cells were stimulated with TGF-β1 with or without $1,25(OH)_2D_3$ or medium control (CNTRL) for 48 h to assess expression of CYP24A1 by immunofluorescence (B). (A) Relative mRNA expression of *CYP24A1*, *VDR* and *CYP27B1* was determined by qPCR. Normalized gene expression was calculated by using the expression of tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta (*YWHAZ*) and ribosomal protein L27 (*RPL27*) as reference genes**.** To determine effects of $25(OH)D_3$ and $1,25(OH)_2D_3$ on *CYP24A1* expression, fold change in gene expression was first calculated, followed by a log-transformation of the data. Furthermore, effects of different doses of TGF-β1 on *CYP24A1*, *VDR* and *CYP27B1* expression were determined by calculating the fold change relative to the corresponding control without TGF-β1 (CNTRL, $25(OH)D_3$ or $1,25(OH)_2D_3$). Data are presented as individual values, including means ± SEM and were tested for significance using the two-way ANOVA and the Bonferroni post-hoc test (n = 6 donors). (B) Cells were stimulated for 48 h to assess expression of CYP24A1 and basal cells (P63) by confocal immunofluorescence staining (data of 1 donor are shown, similar findings in 3 other donors). DAPI (blue) was used to stain the nuclei together with antibodies detecting CYP24A1 (green) and P63 (red). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Effects of inhibition of CYP24A1 activity and canonical TGF-β-Smad signalling on the expression of hCAP18/LL-37 (*CAMP*) and *CYP24A1*

We used S-PBEC to further elucidate the underlying mechanisms of TGF-β1-mediated decreases of hCAP18/LL-37 and increases of CYP24A1 (Figure 3A-B), after observing that modulation of *CAMP* and *CYP24A1* expression by TGF-β1 was similar in S-PBEC and ALI-PBEC (data not shown). We first investigated if the TGF-β1-mediated repression of VD-induced hCAP18/LL-37 was fully mediated by increases of CYP24A1 expression. To this end, S-PBEC were exposed for 24 h to TGF-β1 and $1,25(OH)_2D_3$ in presence or absence of the CYP-inhibitor ketoconazole (KTZ) that is known to inhibit CYP24A1 and CYP27B1 activity (44, 45). To circumvent the effect of KTZ-mediated inhibition of the CYP27B1-mediated hydroxylation of inactive $25(OH)D_3$ into active $1,25(OH)_2D_3$, we used only $1,25(OH)_2D_3$ in these experiments. Whereas KTZ significantly increased $1,25(OH)_2D_3$-mediated expression of *CAMP* in both presence and absence of TGF-β1, *CAMP* levels were lower in $1,25(OH)_2D_3$-treated cells in presence of TGF-β1 than in absence of TGF-β1 (Figure 3A). This suggests that the VD-mediated reduction of *CAMP* by TGF-β1 was not fully explained by increased CYP24A1, suggesting involvement of mechanisms other than the VD-metabolic pathway in the observed effects of TGF-β1.

In addition to VD-mediated expression of *CAMP*, the baseline expression of *CAMP* was also reduced by TGF-β1 and this could not be restored by inhibition of CYP24A1 (Figure 3A). TGF-β1 signals through either the canonical-Smad signalling pathway or the non-canonical (MAPK/NF-KB)-pathway (46). To investigate if the canonical TGF-β-Smad signalling pathway was involved in the inhibition of baseline *CAMP* expression as well as the increase in *CYP24A1*, we exposed S-PBEC to TGF-β1 and $1,25(OH)_2D_3$ in presence or absence of SB431542 (an inhibitor of TGF-β type I receptor activin receptor-like kinase (ALK5) and further downstream signalling i.e. via receptor regulated (R)-Smads) (46). We found that treatment with SB431542 fully reversed the effects of TGF-β1 on both *CAMP* and *CYP24A1* expression both in absence or in presence of $1,25(OH)_2D_3$ (Figure 3B). These results indicate that the canonical TGF-β-Smad signalling pathway mediates the reduction of *CAMP* and the promotion of *CYP24A1* expression by TGF-β1.

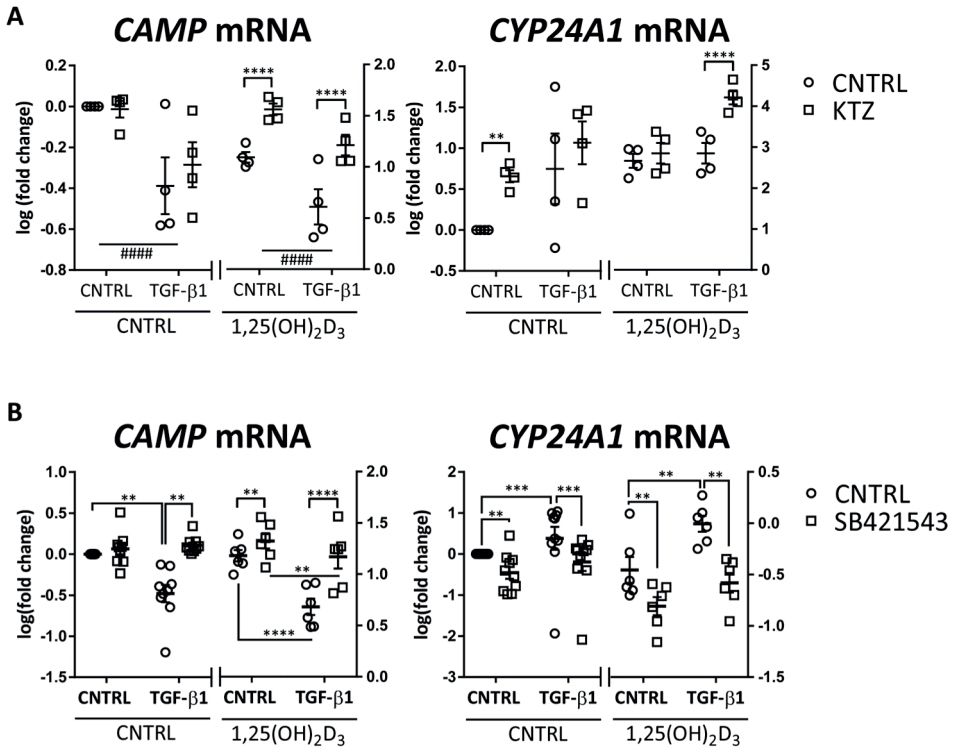

Figure 3. Effects of inhibition of CYP24A1 activity and canonical TGF-β-Smad signaling on expression of hCAP18/LL-37 (CAMP) and CYP24A1 in primary bronchial epithelial cells (PBEC). Semi-confluent layers of submerged (S)-PBEC were cultured in starvation medium overnight and subsequently exposed to the cytochrome P-450 (CYP)-inhibitor ketoconazole (KTZ) (A) or to the canonical TGF-β-Smad signaling-inhibitor SB421543 (B) in presence or absence of TGF-β1 and 1,25(OH)$_2$D$_3$, or medium control (CNTRL) for 24 h to assess mRNA expression of *CAMP* (hCAP18/LL-37) and the vitamin D-degrading enzyme (*CYP24A1*) by qPCR. Relative mRNA expression of *CAMP* and *CYP24A1* was determined by qPCR. Normalized gene expression was calculated by using the expression of tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta (*YWHAZ*) and ribosomal protein L27 (*RPL27*) as reference genes**.** To determine effects of the stimuli, fold change in gene expression compared to CNTRL was first calculated, followed by a log-transformation of the data. Data are presented as individual values, including means ± SEM and were tested for significance using the two-way ANOVA and the Bonferroni post-hoc test (A, n = 4 donors; B, n = 6-10 donors). ** $p < 0.01$, *** $p < 0.001$, ****/#### $p < 0.0001$.

TGF-β1 decreases expression of *CAMP* via the transcription factor CCAAT enhancer binding protein (C/EBP)α

In addition to VD-responsive elements (VDREs) that bind the VDR-RXR-1,25$(OH)_2D_3$-complex, the promotor of *CAMP* also contains a binding site for the transcription factor C/EBPα, which was shown to be required for induction of *CAMP* independent of VD (47). A Study by Li *et al.* showed that TGF-β-Smad signalling inhibits the expression of C/EBPα mRNA in mesenchymal stem cells (48), which suggests involvement of C/EBPα in the suppressive effect of TGF-β1 on *CAMP* expression. We first demonstrated that TGF-β1 also reduced C/EBPα mRNA (*CEBPA*) expression in S-PBEC after 24 h (Figure 4A). To investigate the involvement of C/EBPα in baseline and VD-induced *CAMP* expression we used siRNA. S-PBEC were transfected with *CEBPA*-specific siRNA or negative control siRNA and next cells were exposed to TGF-β1 and 1,25$(OH)_2D_3$ for 24 h. siRNA caused a marked suppression of *CEBPA* expression, and both basal- and 1,25$(OH)_2D_3$-mediated expression of *CAMP* was reduced (Figure 4B). The siRNA induced reductions in both *CEBPA* and *CAMP* expression were more pronounced in TGF-β1-treated cells (Figure 4B). We next measured expression of *CYP24A1*, to exclude possible non-specific effects of *CEBPA* siRNA transfection. Unexpectedly, *CYP24A1*, a gene without any known binding sides for C/EBPα in its promotor, was also decreased (Figure 4B). Since the *CYP24A1*-promotor does contain binding sides for C/EBPβ (*CEBPB*) (49), we considered the possibility that *CEBPA* siRNA also had reduced *CEBPB* mRNA levels. We therefore assessed expression of *CEBPB* and found that this was not affected, suggesting that inhibition of C/EBPα might have indirectly affected expression of CYP24A1 (Figure 4B). Collectively, these data show that TGF-β1-mediated changes in *CAMP* or *CYP24A1* expression are mediated through repression of the transcription factor (C/EBP)α by TGF-β1.

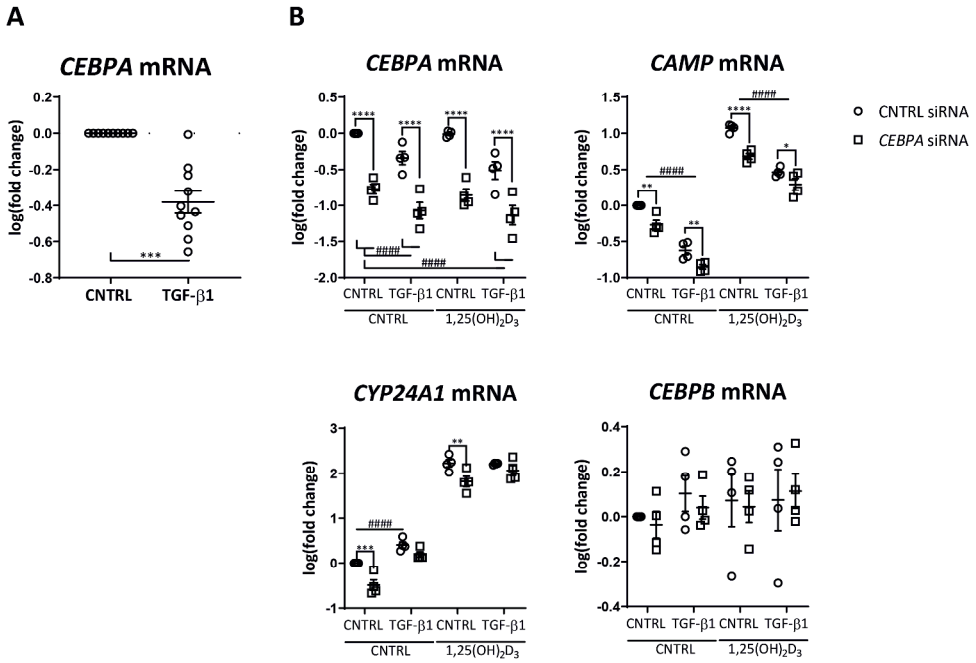

Figure 4. TGF-β1 decreases expression of the *CAMP*-transcription factor CCAAT enhancer binding protein (C/EBP)α in primary bronchial epithelial cells(PBEC). Semi-confluent cultures of submerged (S)-PBEC were cultured in starvation medium overnight and subsequently exposed to TGF-β1 or medium control (CNTRL) for 24 h to assess mRNA expression of C/EBP)α (*CEBPA*) by qPCR (A). PBEC were refreshed with starvation medium containing medium alone (CNTRL) or TGF-β1 with and without $1,25(OH)_2D_3$ and subsequently transfected with CEBPA siRNA for 24 h. Scrambled siRNA was used as a control (CNTRL siRNA). mRNA expression of *CEBPA*, *CAMP* (hCAP18/LL-37), the vitamin D-degrading enzyme (*CYP24A1*) and C/EBP)β (*CEBPB*) was assessed by qPCR (B). Relative mRNA expression was determined by qPCR. Normalized gene expression was calculated by using the expression of tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta (*YWHAZ*) and ribosomal protein L27 (*RPL27*) as reference genes**.** To determine effects of the stimuli, fold change in gene expression compared to CNTRL was first calculated, followed by a log-transformation of the data. Data are presented as individual values, including means ± SEM and were tested for significance using the two-way ANOVA and the Bonferroni post-hoc test (A, n= 10 donors; B, n = 4 donors). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ****/#### $p < 0.0001$.

Effect of TGF-β1 on the expression of constitutively expressed luminal cell-restricted host defence mediators

Since we demonstrated reduced expression levels of the inducible HDP hCAP18/LL-37 in the presence of VD and TGF-β1, we were next interested to investigate whether a similar decrease was also observed in expression of constitutively

expressed host defence mediators such as the HDP SLPI and pIgR, since previous studies showed that were repressed by TGF-β1 alone in ALI-PBEC (31, 50). Investigating effects of the combination of TGF-β1 and VD is especially relevant, since VD reduces the effects of TGF-β1-mediated EMT in airway epithelial cell lines (35). We therefore investigated in ALI-PBEC if VD affected the TGF-β1-induced repression of a selected group of constitutively expressed host defence mediators such as SLPI, s/lPLUNC and pIgR. To this end, differentiated ALI-PBEC were exposed to both TGF-β1 and $1,25(OH)_2D_3$ for 24-48 h and expression of these mediators was assessed. TGF-β1 decreased the expression of all four selected HDPs after 24 h (Figure 5A) and which continued up to 48 h (data not shown). However, $1,25(OH)_2D_3$ did not prevent the TGF-β1-meditiated repression of mRNA expression of these constitutively expressed HDPs (Figure 5A). We additionally verified these effects of TGF-β1 at the protein level using confocal immunofluorescence, and using ELISA to detect SLPI in apical secretions (Figure 5B-C). TGF-β1-treatment reduced both the staining intensity as well as the number of SLPI- and sPLUNC-positive cells, whereas only the number of pIgR-positive cells was reduced upon TGF-β1-treatment. In cultures from some donors, pIgR was relocated from the cell membrane towards the cytoplasm (Figure 5B).

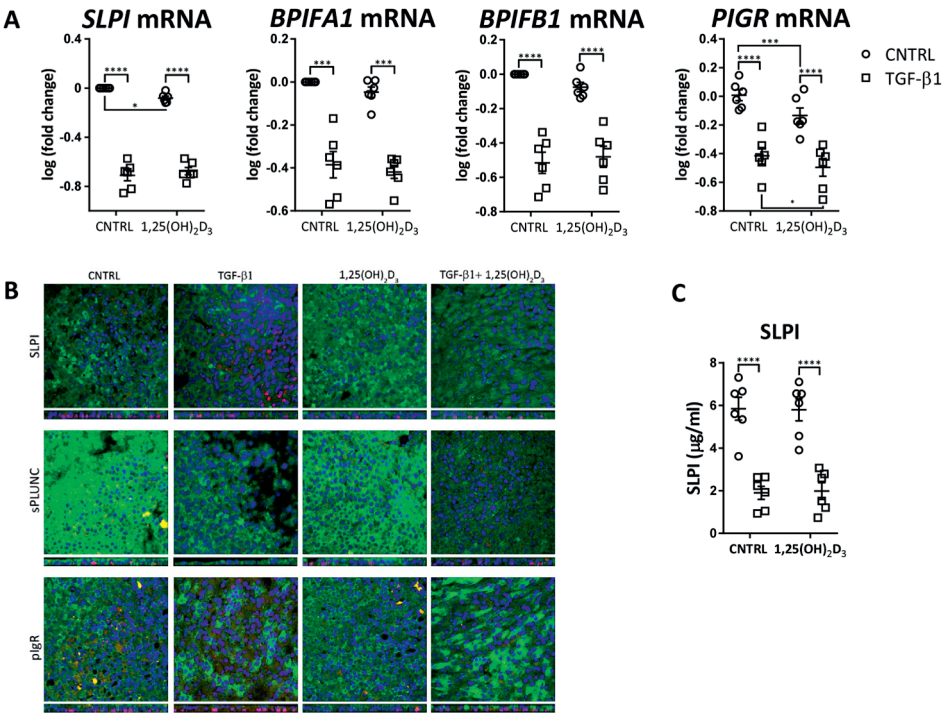

Figure 5. Effect of TGF-β1 on expression of constitutively expressed luminal cell-restricted host defence proteins (HDPs) in primary bronchial epithelial cells (PBEC). PBEC were differentiated at the air-liquid interface (ALI) followed by stimulation for 24 h with TGF-β1 in presence or absence of $1,25(OH)_2D_3$ and medium control (CNTRL) to assess mRNA expression of secretory leukocyte protease inhibitor (*SLPI*), short- and long- Palate, lung, and nasal epithelium clone protein (PLUNC) (*BPIFA1* and *BPIFB1* respectively) and polymeric immunoglobulin receptor (*PIGR*) by qPCR (A). In addition, cells were stimulated for 48 h to assess expression of these HDPs by confocal immunofluorescence (SLPI, sPLUNC and pIgR) and the apical side of the inserts were washed in PBS to assess release of SLPI by ELISA (B). A. Relative mRNA expression of *SLPI*, *BPIFA1*, *BPIFB1* and *PIGR* was determined by qPCR. Normalized gene expression was calculated by using the expression of tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta (*YWHAZ*) and ribosomal protein L27 (*RPL27*) as reference genes. Fold changes in gene expression of the stimuli compared to control (CNTRL) were first calculated, followed by a log-transformation of the data. Data are presented as individual values, including means ± SEM and were tested for significance using the two-way ANOVA and the Bonferroni post-hoc test (n = 6 donors). (B) Confocal immunofluorescence staining of HDPs and basal cells in ALI-PBEC (of 1 donor, which was confirmed in 3-5 other donors), DAPI (blue) was used to stain the nuclei and antibodies (table II) were used for detection of HDPs (green) and basal cells (P63, red) respectively. C. Analysis of SLPI in apical wash by ELISA. Data are presented as individual values, including mean ± SEM and were tested for significance using the two-way ANOVA and the Bonferroni post-hoc test (n = 6 donors). * $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$.

We furthermore investigated functional consequences of the reduced expression of the HDPs by TGF-β1 in presence and absence of VD by studying effects of TGF-β1 and VD on antibacterial activity. Differentiated ALI-PBEC were exposed to TGF-β1 for 48 h in presence and absence of $1,25(OH)_2D_3$ and a killing assay was performed by applying log-growing NTHi on the apical surface of 48 h-exposed cells for 2 h. The cell lysates were next diluted and incubated on agar plates overnight to determine surviving bacteria. In contrast to what we previously observed using a different type of antibacterial assay (5), we found that treatment with $1,25(OH)_2D_3$ did not increase antibacterial activity against NTHi. Although not significant, there was a small trend towards reduction of antibacterial activity by TGF-β1 (Figure S1).

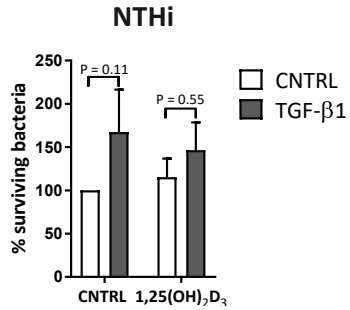

Figure S1. Effects of TGF-β1 on antibacterial activity in differentiated bronchial epithelial cells (PBEC). PBEC were differentiated at the air-liquid interface (ALI) followed by stimulation for 48 h with TGF-β1 with or without $1,25(OH)_2D_3$ or medium control (CNTRL) to assess antibacterial activity by applying log-growing cultures of nontypeable *Haemophilus influenzae* (NTHi) on the apical surface of the treated cells for 2 h. Next, membranes containing the cells with bacteria were dissected from the inserts and mechanically disrupted. Serial dilutions of bacterial suspensions were plated on chocolate agar plates and incubated overnight at 37° C to assess surviving bacteria by CFU determination. Data are presented as means ± SEM % surviving bacteria compared to medium control stimulated cells (CNTRL) and were tested for significance using the two-way ANOVA and the Bonferroni post-hoc test (n = 9 donors).

TGF-β1 modulates epithelial differentiation

To investigate whether the ability of TGF-β1 to reduce expression and release of the constitutively expressed HDPs SLPI, s/lPLUNC and pIgR was explained by TGF-β1-induced changes in epithelial differentiation, effects on differentiation markers were assessed. These experiments were prompted by our recent finding that chronic exposure to CS also impairs expression and release of these HDPs, accompanied by an impairment of end-stage airway epithelial cell differentiation towards club- and goblet cells, being the main source of these HDPs (40). Furthermore it was shown that TGF-β1 directs epithelial cells to dedifferentiate and we therefore investigated if specialized epithelial cells were reduced by TGF-β1 and if VD might counteract this reduction (51). We therefore exposed differentiated ALI-PBEC to TGF-β1 and $1,25(OH)_2D_3$ for 24-48 h and indeed observed that TGF-β1 caused a clear reduction in both mRNA expression of the club- and goblet cell markers *SCGB1A1* and *CLCA1* respectively as well as in the number of CC16 (club cell) and MUC5AC (goblet cell)-positive cells, observed by confocal immunofluorescence staining. mRNA expression of the ciliated cell marker (*FOXJ1*)

as well the number of ciliated cells were unaffected by TGF-β1 (Figure 6 A-B). $1,25(OH)_2D_3$ alone did not affect mRNA or protein expression of these cell markers, and did not prevent the TGF-β1-induced decreases in *SCGB1A1* and *CLCA1* mRNA (Figure 6A; immunofluorescence data not shown). This indicates that TGF-β1 impairs expression of luminal expressed HDPs by reducing the number of secretory cells that express these HDPs, which is not modulated by VD.

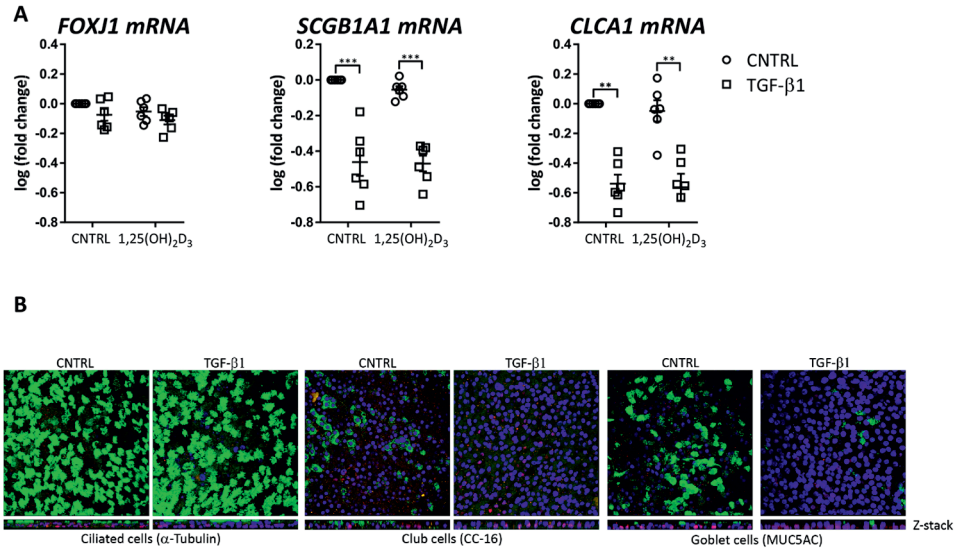

Figure 6. TGF-β1 affects composition of airway epithelium by decreasing the number of secretory epithelial cells in primary bronchial epithelial cells (PBEC). PBEC were differentiated at the air-liquid interface (ALI) followed by stimulation for 24 h with TGF-β1 in presence or absence of $1,25(OH)_2D_3$ or medium alone (CNTRL) to assess mRNA expression of markers related to ciliogenesis (*FOXJ1*), club cells (*SCGB1A1*) and goblet cells (*CLCA1*) by qPCR (A). In addition, cells were stimulated for 48 h to assess the numbers of ciliated-, club- and goblet cells by confocal immunofluorescence (B). (A) Relative mRNA expression of *FOXJ1*, *SCGB1A1* and *CLCA1* was determined by qPCR. Normalized gene expression was calculated by using the expression of tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta (YWHAZ) and ribosomal protein L27 (RPL27) as reference genes. Fold changes in gene expression of the stimuli compared to control (CNTRL) were first calculated, followed by a log-transformation of the data. Data are presented as individual values, including means ± SEM and were tested for significance using the two-way ANOVA and the Bonferroni post-hoc test (n = 6 donors). (B) Confocal immunofluorescence staining of ciliated- (α-tubulin), club- (CC-16), goblet (MUC5AC) and basal cells (P63) in ALI-PBEC (of 1 donor, which was confirmed in 3 other donors), DAPI (blue) was used to stain the nuclei and antibodies (table II) were used for detection of luminal cell markers (green) and basal cells (P63, red) respectively. ** $p < 0.01$, *** $p < 0.001$.

Discussion

Here we demonstrate that TGF-β1 affects VD-metabolism by increasing expression of the VD-degrading enzyme CYP24A1 and reduces VD-mediated expression of the HDP hCAP18/LL37 in both submerged undifferentiated PBEC and in differentiated ALI-PBEC. Moreover, TGF-β1 also reduces baseline expression of hCAP18/LL-37 via its ability to reduce expression of C/EBPα. In fully differentiated ALI-PBEC, TGF-β1 represses expression of constitutively expressed HDPs such as SLPI, s/lPLUNC and pIgR, which might in part be attributed to decreases in the number of secretory club- and goblet cells. Treatment with VD did not counteract these effects of TGF-β1 on HDP expression and epithelial differentiation.

To the best of our knowledge, we are the first to show that TGF-β1 affects vitamin D-metabolism by increasing expression of the VD-degrading enzyme CYP24A1, although an earlier study using hepatic cells did show an association between CYP24A1 and TGF-β1 expression (24). The involvement of CYP24A1 in the TGF- β1 induced decrease in VD-mediated expression of hCAP18/LL-37 was confirmed by inhibiting its activity by KTZ. Since KTZ inhibits also other CYP-enzymes such as CYP27B1, we used $1,25(OH)_2D_3$ to avoid the inhibitory effects of KTZ on the conversion of 25(OH)D3 into $1,25(OH)_2D_3$ by CYP27B1. These TGF-β1-mediated effects on CYP241 help to explain the inhibitory effects of TGF-β1 on VD-induced expression of hCAP18/LL-37 in both undifferentiated and differentiated PBEC. We observed no changes in *CYP27B1* expression and minor changes in *VDR* expression after TGF-β1 treatment, whereas a study in colon cancer cells showed that *VDR* expression was repressed by Snail1 and Snail2 that are known TGF-β1-inducible transcription factors (52). Previous to our findings, Kulkarni *et al.* demonstrated that TGF-β1 reduces baseline expression as well as phenylbutyrate-mediated increases in *CAMP* mRNA expression in a bronchial epithelial cell line, which was reversed by SB421543, an inhibitor of the canonical TGF-β-Smad signalling pathway (53). In line with this, we confirmed that SB421543 also reduced the TGF-β1-mediated increase in *CYP24A1* expression. Furthermore, VD-mediated expression of *CAMP* was even further enhanced when SB421543 was added, suggesting that VD-mediated *CAMP* expression was negatively affected by endogenous TGF-β activity. We examined the underlying mechanisms of TGF-β1-mediated inhibition of baseline *CAMP* expression and demonstrated that TGF-β1 repressed mRNA expression of the transcription factor C/EBPα (*CEBPA*). We furthermore

demonstrated the relevance of this transcription factor by inhibition of *CEBPA* using siRNA, which resulted in a decrease of *CAMP*. TGF-β1 further repressed expression of *CEBPA* and *CAMP* in siRNA treated cells. The importance of C/EBPα in the VD-independent induction of hCAP18/LL-37 (*CAMP*) expression was previously demonstrated by Park and colleagues in keratinocytes (47). Unexpectedly, siRNA-mediated inhibition of *CEBPA* also inhibited expression of *CYP24A1*. By excluding the possibility that *CEBPB* was also targeted by siRNA, we conclude that *CYP24A1* expression may have been indirectly affected by TGF-β1.

In addition to alterations in VD-metabolism and effects, TGF-β1 was also found to reduce the number of luminal secretory cells, possibly via initiation of EMT, and impaired expression of constitutively expressed HDPs SLPI and pIgR, which is in line with previous findings (31, 39, 50). We are however the first to report that TGF-β1 decreases expression of the constitutively expressed HDP s/lPLUNC. We have recently demonstrated that expression of these constitutively expressed host defence mediators was also impaired following chronic CS-exposure, which was accompanied by a selective reduction of differentiation into specialized airway epithelial cells (40). Since CS-exposure is also known to increase expression of TGF-β1 in airway epithelial cells (30, 31), we consider the possibility that the CS-induced repression of constitutive expressed HDPs in airway epithelial cells is in part mediated via the induction of TGF-β1. Interestingly and in line with our finding that TGF-β1 decreases the number of club- and goblet cells, Gohy *et al.* reported correlations between a TGF-β1-mediated decrease of pIgR, epithelial dedifferentiation and increased expression of mesenchymal markers (31). It is important to consider that TGF-β1 only alters differentiation markers at concentrations above 0.5 ng/ml, as shown by Harrop et al (54). Additionally, they demonstrated that expression of MUC5AC and MUC5B in differentiated PBEC was decreased by TGF-β2, which is another TGF-β-isoform and uses the same receptors (54). In contrast to other studies showing inhibition of TGF-β1-mediated effects on EMT by VD (35, 36), we have not observed any ameliorating effects of VD on TGF-β1-mediated effects on expression of SLPI, s/lPLUNC and pIgR, nor on expression of secretory cell markers. This may be explained by the fact that we used differentiated primary airway epithelial cells, where VD-activity may be more efficiently inhibited by TGF-β1, or by the possibility that autocrine expression or processing of secreted immature TGF-β1 might be more efficient in differentiated primary epithelial cells than in bronchial epithelial cell lines. The underlying mechanism for this difference needs to be further elucidated, for example by

comparing CYP24A1 levels, autocrine production of TGF-β1, expression of TGF-β-receptors or extracellular activation of TGF-β1 between cell lines and differentiated primary cells.

One of the strengths of this study is that we used differentiated primary airway epithelial cells that were obtained from multiple donors, instead of tumour-derived or immortalized airway epithelial cell lines, thereby increasing the relevance of our findings. It needs to be noted that PBEC used in this study were not derived from healthy donors, but from healthy parts of lung tissue derived from patients who often have smoked and underwent lung resection surgery for lung cancer. However, we have previously demonstrated that these cells differentiate into ciliated-, club and goblet cells similar to healthy individuals, develop a strong epithelial barrier and that expression of constitutively expressed HDPs and airway epithelial differentiation between donors with and without COPD do not differ in our hands (40). This was in contrast to the findings by Gohy and colleagues, who showed that features of EMT persist in PBEC derived from COPD patients (51), most likely as they used material from patients with more severe stages of COPD.

This study also has a few limitations. First, we were not able to detect hCAP18/LL-37 peptide in apical washes of the stimulated PBEC using Western blot analysis, which is in line with our previous study (5). Therefore, we used pooled and concentrated basal medium and in line with our previous report detected a hCAP18/LL-37 immunoreactive peptide at the size of 14 kDa, and did not detect the 4.5 kDa mature antimicrobial peptide LL-37. Levels of this peptide were increased by VD and reduced by TGF-β1. Another limitation of this study is that we were not able to fully confirm TGF-β1-mediated decreases in HDPs at the functional level by measuring antibacterial activity against NTHi. As hCAP18/LL-37, SLPI and s/lPLUNC also have other activities such as anti-biofilm, immunomodulatory and anti-protease activities in addition to their antibacterial activities (55-57), a more complex culture model using a combination of immune cells and airway epithelial cells might be relevant as an alternative approach to establish the consequences of these changes on host defence.

To extend the relevance of our findings to the situation *in vivo*, further studies are required to compare lung tissue levels of CYP24A1, $1,25(OH)_2D_3$ and expression of these HDPs in healthy donors and donors with chronic inflammatory lung disease or fibrosis. We showed that TGF-β1 reduces the number of club cells, which is in

line with observations in diseases associated with both elevated TGF-β1-levels and reduced numbers of club cells such as COPD, asthma and bronchiolitis obliterans syndrome (46, 58, 59). A contribution of TGF-β1 to the pathogenesis of COPD is supported by findings of two studies showing that TGF-β1 levels correlated with disease severity and airflow limitation in COPD patients (27, 60). In addition, TGF-β1 expression was higher in airway epithelium of smokers with COPD compared to smokers without COPD (26, 27). To date, no treatment is available for COPD patients that selectively targets the harmful effects of TGF-β1, without affecting beneficial effects of TGF-β1. Clinical trials that investigated the use of global inhibitors of TGF-β signalling in oncology or in idiopathic pulmonary fibrosis (IPF) showed that these compounds are frequently associated with adverse (health) effects and limited clinical benefit (61). Drugs such as pirfenidone that block downstream TGF-β pathways without affecting the immune system, might be a better approach and showed promising results in clinical trials in IPF (61, 62). Our study additionally suggests that vitamin D is not a candidate to ameliorate the negative effects TGF-β1 on airway host defence.

In conclusion, we have shown that TGF-β1 reduces host defence of airway epithelial cells by impair ring VD-mediated expression of HDPs as well as constitutively expressed luminal HDPs such as SLPI and s/lPLUNC and pIgR. We have additionally shown that TGF-β1 reduces the number of secretory club- and goblet cells, which might have additional consequences for host defence. We furthermore conclude that TGF-β1 reduces VD-mediated expression of hCAP18/LL-37 via a dual mechanism: directly by reducing expression of an important transcription factor for hCAP18/LL-37 and indirectly via increasing of CYP24A1 that promotes degradation of VD. These findings may have implications our understanding of the role of TGF-β1 in COPD by extending the range of mechanisms affected by TGF-β1.

Acknowledgements

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Statement of Ethics

Use of 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.

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