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Modulation of airway epithelial cell function by vitamin D in COPD

Jasmijn Adri-Anna Schrumpf

Colophon

Modulation of airway epithelial cell function by vitamin D in COPD
Jasmijn A. Schrumpf
Thesis Leiden University Medical Center

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Modulation of airway epithelial cell function by vitamin D in COPD

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Table of Contents

Chapter 1 **General Introduction and outline of the thesis** **1**

Chapter 2 **Aberrant epithelial differentiation by cigarette smoke dysregulates respiratory host defence** **17**

Eur Respir J. 2018 Apr 26;51(4):1701009

Chapter 3 **TGF-β1 impairs vitamin D-induced and constitutive airway epithelial host defence mechanisms** **57**

J Innate Immun. 2020;12(1):74-89

Chapter 4 **Pro-inflammatory cytokines impair vitamin D-induced host defense in cultured airway epithelial cells** **89**

Am J Respir Cell Mol Biol. 2017 Jun;56(6):749-76

Chapter 5 **Interleukin 13 Exposure Enhances Vitamin D-Mediated Expression of the Human Cathelicidin Antimicrobial Peptide 18/LL-37 in Bronchial Epithelial Cells** **123**

Respir Res. 2011 May 2;12(1):59

Chapter 6 **Prevention of exacerbations in patients with COPD and vitamin D deficiency through vitamin D supplementation (PRECOVID): a study protocol** **151**

BMC Pulm Med. 2015 Sep 23;15:106

Chapter 7 **Impact of the local inflammatory environment on mucosal vitamin D metabolism and signaling in chronic inflammatory lung diseases** **171**

Front Immunol. 2020 Jul 10;11:1433

Chapter 8 **Summary and general discussion** **209**

Addendum **Nederlandse samenvatting** **235**

Curriculum vitae **247**

Publicaties **250**

Dankwoord **253**

CHAPTER

General Introduction and Outline of the Thesis

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Introduction

Vitamin D is a well-recognized for its systemic role in calcium absorption and bone mineralization. In the airways, vitamin D enhances airway epithelial cell homeostasis and is a modulator of both innate and adaptive immune responses (1). The airway epithelium is the front line of the lung's host defense and its main function is to clear and protect the airways from hazardous inhaled substances such pollutants and pathogens (2). These protective airway epithelial cell functions could be affected by for example genetic alterations and environmental insults in early life and/or the long-term exposure to inhaled toxicants, such as caused by cigarette smoking. Consequently, this may increase the susceptibility towards infections and eventually lead to (chronic) inflammation and aberrant immune responses, epithelial remodeling and repair (3, 4). Furthermore, studies have now shown that the airway epithelium from patients with chronic inflammatory lung diseases may differ from healthy subjects, and show signs of altered epithelial differentiation and aberrant repair (4-6). These findings support the hypothesis that the airway epithelium plays a central role in the pathogenesis of chronic inflammatory lung diseases such as asthma and chronic obstructive pulmonary disease (COPD).

Vitamin D deficiency was classically known to be solely associated with bone diseases such as rickets until it gradually became evident that vitamin D deficiency was also associated with many other diseases including chronic inflammatory lung diseases such as asthma and COPD (1, 7-9). Several *in vitro* studies have already indicated that the active form of vitamin D [1,25 dihydroxy-vitamin D ($1,25(OH)_2D$)] might protect the airway epithelium against injury by promoting integrity of the epithelial barrier, dampening immune responses and via the induction of the antimicrobial peptide (AMP) hCAP18/LL-37 (1). However, the precise impact of $1,25(OH)_2D$) on the airway epithelium in a chronic inflammatory environment as observed in chronic lung diseases remains to be defined.

The Airway Epithelium

The lungs are in close contact with the external environment due to the large volumes of air inhaled on a daily basis. The airway epithelium lines the conducting airways and removes and eliminates potentially harmful substances and pathogens to protect the alveoli, where gas exchange occurs, from injury. The epithelium that lines the conducting airways is composed of a pseudostratified cell layer that consists of several epithelial cell types of which the major cell-types are ciliated

cells, secretory and basal cells (10). The basal cells are regarded as the main progenitor cells, that are able to renew and differentiate into intermediate cells followed by end-stage differentiation into secretory- or ciliated cells (5, 10). The ciliated cell is the main cell type of the airway epithelium that transports mucus produced by the submucosal glands and the goblet cells of the surface epithelium out of the airways through highly coordinated ciliary beating, generating a wavelike movement across the epithelial surface (11). Activation of the transcription factor forkhead box J1 (FOXJ1) is involved in the formation of ciliated cells (11), whereas Notch-signaling and activation of SAM pointed domain–containing ETS transcription factor (SPDEF) are the main inducers for secretory cell differentiation (12, 13). The goblet- and club cells are the secretory cells of the conducting airways. Whereas goblet cells are more prominent in the trachea and bronchi, club cells are most the predominant secretory cell type in the terminal and respiratory bronchioles (small airways). Goblet cells predominantly secrete gel-forming mucins such as mucin-5AC (MUC5AC) that trap particles in the gel-matrix to be removed through the mucociliary escalator, which is dependent on proper mucin-hydration and function of ciliated cells (14). Club cells are present throughout the airways and are the main source of club cell protein (CC16), which has been shown to contribute to epithelial homeostasis and to reduce inflammation and might therefore have protective features against development and progression of COPD (15). Furthermore, club cells also secrete antimicrobial compounds, surfactant proteins and mucins such as mucin-5B (MUC5B), which are known regulators of respiratory host defense and they express cytochrome P450 (CYP) enzymes such as CYP2F2 that neutralize xenobiotics (5, 15-17). The fact that CYP2F2 metabolizes some toxins into even more harmful substances might explain why club cells are more sensitive to these toxins than other airway epithelial cell types (Figure 1) (18).

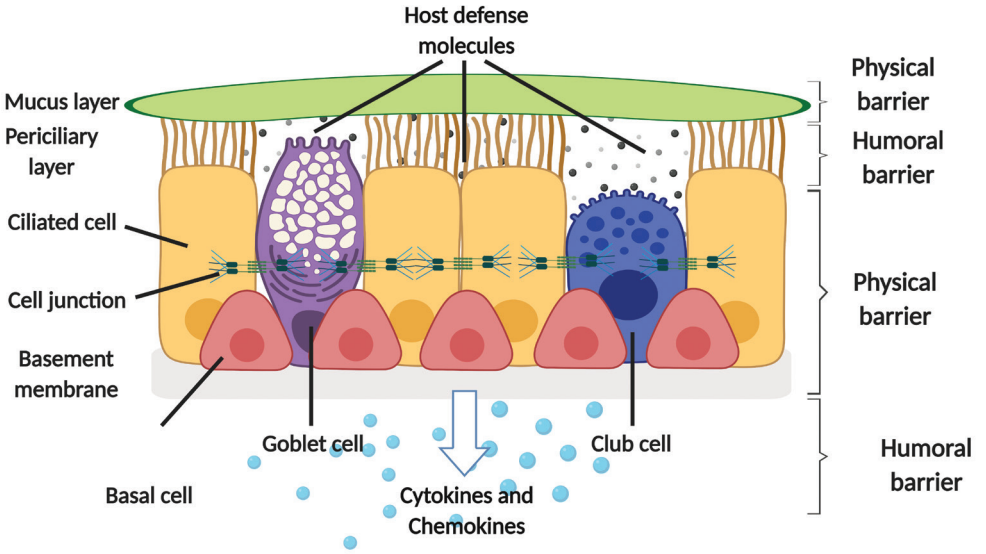

Figure 1. The airway epithelium: cells that line and constitute the physical- and humoral barrier of the conducting airways. Physical defenses are provided by the mucociliary clearance and cell junctions forming the physical epithelial barrier, whereas humoral defenses are provided by the secretion of host defense molecules such as interferons (type I and III), antimicrobial peptides and proteins, cytokines, chemokines and lipid mediators. These mediators are also involved in regulating both innate and adaptive immune responses by attracting and activating immune cells in the submucosa.

Airway epithelial innate host defense involves a physical barrier and a humoral barrier (Figure 1). The physical barrier of the epithelium is maintained by its tight- and adherens junctions and mucociliary clearance of trapped particles and pathogens (3). In chronic inflammatory airway diseases such as asthma and COPD, both of these defenses are compromised, which contributes to increased susceptibility towards infections (19). Furthermore, the ability to repair damage upon infection or exposure to toxicants is also impaired, and it has been hypothesized that aberrant repair results in remodeling of the airway epithelium such as reduced numbers of ciliated cells, goblet cell hyperplasia or epithelial-to mesenchymal transition (EMT) (20). EMT is a process characterized by a transformation of differentiated epithelial cells into mesenchymal cells (21). Furthermore, airway host defense is maintained via the humoral barrier provided by the secretion of host defense molecules such as antimicrobial proteins and peptides (AMPs), secretory immunoglobulin A (sIgA) and reactive oxygen species (ROS) (3). Furthermore upon activation of pattern recognition receptors (PRRs),

interferons, cytokines, chemokines and lipid mediators are released to promote viral clearance and to recruit and activate innate and adaptive immune cells (22, 23). AMPs have, in addition to their broad-spectrum antimicrobial activity, also the ability to modulate immune responses and to promote wound repair (24). AMPs are either constitutively expressed at high levels or their expression can be induced upon activation of PRRs, cytokine and growth factor receptors, and by other mediators such as $1,25(OH)_2D$ (25). In addition to AMPs, ROS also have antimicrobial features and are generated in airway epithelial cells by dual oxidases (DUOX), which are localized on the apical plasma membranes (26, 27). Epithelial clearance of viruses is generally promoted via the expression and release of type I and III interferons, which induces expression of a range of proteins that selectively interfere with virus replication, protein synthesis, or protein trafficking (23). In the chronic inflammatory lung diseases, aberrant secretion of AMPs and impaired expression of interferons contributes to defective clearance of pathogens, despite the increased production of ROS and influx of inflammatory cells (3, 23, 25, 28).

Chronic Obstructive Pulmonary Disease (COPD) and Exacerbations

COPD is a progressive lung disease characterized by chronic obstruction of airflow that interferes with normal breathing and is not fully reversible. The primary cause of COPD is exposure to noxious particles or gases such as tobacco smoking and environmental exposures to biomass fuels or air pollution, but also host factors such as genetics, abnormal lung development and aging may contribute to COPD development (29). COPD is a major cause of chronic morbidity and is currently the 3rd leading cause of death in the world (29, 30). Exacerbations of COPD are episodes of acute worsening of symptoms that require additional therapy, accelerate disease progression and are a substantial burden on health-care systems worldwide through their effects on morbidity and mortality (29, 31). COPD exacerbations are mainly associated with by respiratory viral- and bacterial infections or by air pollution (31). As discussed in the previous paragraph, dysfunctional epithelial innate defense in the lungs of COPD patients likely contributes to the increased susceptibility towards bacterial and viral infections, and thereby to COPD exacerbations. Moreover, multiple studies have now demonstrated that COPD patients generally have lower serum 25(OH)D-levels, which are associated with an increased number and more severe exacerbations (8, 32). Studying the role of vitamin D in COPD exacerbations and airway epithelial innate immune defenses might therefore provide additional insight into the potential benefit of monitoring

vitamin D status of these patients and supplementation of patients with a vitamin D deficiency.

Vitamin D Deficiency

Vitamin D is a pleiotropic hormone that is well known for its role in regulating calcium (Ca^{2+}) and phosphate (PO_4^{2-}) homeostasis and bone mineralization. The receptor for 1,25$(OH)_2$D (VDR) is however expressed in more than 40 tissues and regulates a large number of genes (approximately 0.8–5% of the total genome) (33). As a result, 1,25$(OH)_2$D affects several cellular processes including proliferation, DNA repair, differentiation, apoptosis, membrane transport, metabolism, cell adhesion, and oxidative stress (33, 34). Vitamin D deficiency [serum 25(OH)D < 50 nmol/L (35)] affects more than 30% of the children and adults worldwide and is linked to bone diseases such as rickets and osteoporosis, but also associated with many other diseases including cancer, type 2 diabetes, cardiovascular diseases, Alzheimer's' disease, muscle myopathy, multiple sclerosis, inflammatory bowel disease (IBD), psoriasis, and chronic inflammatory lung diseases including asthma and chronic obstructive pulmonary disease (COPD) (1, 7-9).

Metabolism of Vitamin D

Vitamin D enters the body either via food intake or as a result of its synthesis in the skin under the influence of UV-light (Figure 2). In the intestine, vitamin D_2 or vitamin D_3 enters the circulation actively from the lumen by apical membrane transporters or by passive diffusion through enterocytes (36). In the skin, UVB radiation triggers conversion of the cutaneous reservoir of 7-dehydrocholesterol into pre-vitamin D_3. After isomerization of pre-vitamin D_3 into vitamin D_3, this secosteroid is removed from the skin through binding to the vitamin D binding protein (VDBP) and transported into the circulation (37). This VDBP-bound vitamin D_2 or vitamin D_3, which has a half-life of one day, is transported to the liver where it is converted by vitamin D-25-hydroxylases (CYP2RI and CYP27A1) into 25-hydroxy-vitamin D [25(OH)D]. However, recent studies showed that also other cell types such as airway epithelial cells, keratinocytes, monocytes/macrophages and intestinal epithelial cells express CYP2RI and CYP27A1, and thus are able to (locally) convert vitamin D_3 into 25$(OH)D_3$ (38, 39). 25(OH)D (both 25$(OH)D_2$ and 25$(OH)D_3$) is the main circulating form of vitamin D, which has a half-life of 20 days and its levels are used to assess vitamin D status in the clinic (40). This inactive 25(OH)D needs to be

converted into the active 1,25 dihydroxy-vitamin D ($1,25(OH)_2D$) by 25-hydroxyvitamin D-1α-hydroxylase (CYP27B1) in the kidney, locally in tissues or in several types of immune cells (41-44). $1,25(OH)_2D$ binds the nuclear vitamin D receptor (VDR), which heterodimerizes with the retinoic acid receptor (RXR) to interact with vitamin D response elements (VDREs) that are present on the promoter region of vitamin D target genes (33, 45). The VDR-RXR-complex needs additional corepressors (NCoR, SMRT) and/or co-stimulators (SRC-1, CBP, MED1) to silence or initiate gene transcription (46, 47). In addition to promoting or suppressing gene expression via binding of $1,25(OH)_2D$ to the nuclear (nVDR) and RXR, 1,25(OH)2D furthermore interacts with membrane-bound VDR (mVDR), known as $1,25(OH)_2D$-membrane-associated, rapid response steroid-binding protein (1,25D-MARRS). mVDR is present in the caveolae and induces rapid non-genomic responses through e.g. the generation of second messengers such as Ca^{2+} and/or activation of proteins kinases such as mitogen-activated protein kinases (MAPK) (48). Furthermore, $1,25(OH)_2D$ regulates its own negative feedback by several mechanisms, for example via direct induction of the catabolic enzymes 25-hydroxyvitamin D-24-hydroxylase (CYP24A1) and CYP3A4 (49, 50). CYP24A1 is expressed in most tissues and converts both 25(OH)D and $1,25(OH)_2D$ into $23,25(OH)_2D$ or $24,25(OH)_2D$ and $1,24,25(OH)_3D$ respectively, which are further converted into inactive metabolites and excreted in the bile (49, 51). CYP3A4 is mainly expressed in the liver and small intestines and contributes to the metabolic clearance of 25(OH)D and $1,25(OH)_2D$ by converting 25(OH)D into $4\beta,25(OH)_2D$, and $1,25(OH)_2D$ into $1,23R,25(OH)_2D$ or $1,24S,25(OH)_2D$ (50). Expression of both CYP27B1 and CYP24A1 in the kidneys is tightly regulated to maintain optimal Ca^{2+} and PO_4^{2-}-levels in the circulation. When Ca^{2+}-levels are low, parathyroid hormone (PTH) is secreted by the pituitary glands, which in turn reduces Ca^{2+} excretion and reabsorption of PO_4^{2-} (52). PTH further induces expression of CYP27B1 and represses expression of CYP24A1 in the kidneys (52). This will increase the levels of $1,25(OH)_2D$ in the circulation, which promotes intestinal Ca^{2+} and PO_4^{2-} absorption (52). These elevated circulating Ca^{2+} and PO_4^{2-} levels will subsequently induce expression of fibroblast growth factor 23 (FGF-23) in osteocytes and osteoblasts and impair secretion of parathyroid hormone (PTH) by the parathyroid glands (34). In the kidneys, FGF-23 suppresses expression of CYP27B1 and induces expression of CYP24A1, thereby inhibiting the synthesis and promoting degradation of $1,25(OH)_2D$ (34) (Figure 2).

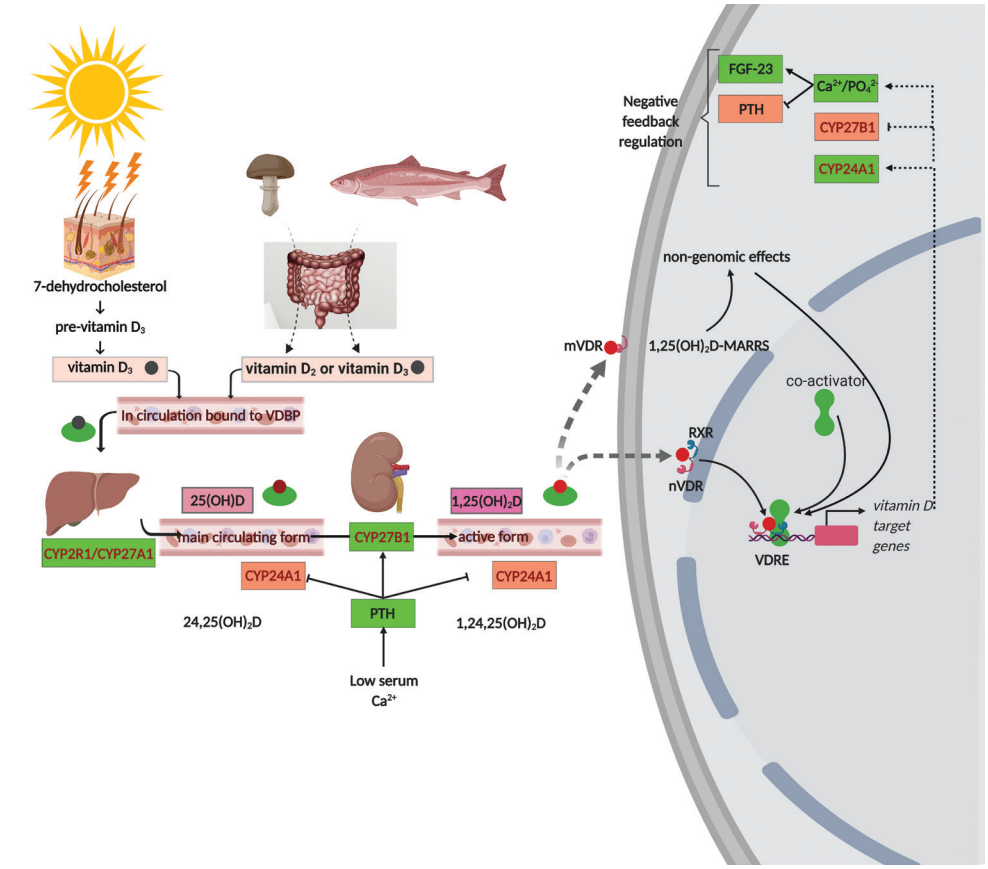

Figure 2. Endocrine vitamin D metabolism. See text for details and references.

Vitamin D and Chronic Inflammatory Lung Diseases

Systemic levels of biologically active $1,25(OH)_2D$ are tightly regulated to achieve sufficient Ca^{2+} and PO_4^{2-} levels levels for optimal bone mineralization, whereas in mucosal tissues local $1,25(OH)_2D$ activation or inactivation can result in $1,25(OH)_2D$ levels that are elevated or decreased (7). The inflamed mucosal tissues of the airways in COPD and asthma patients are constantly exposed to pathogens and to several inflammatory mediators. However, the effects of this exposure on local levels of $1,25(OH)_2D$ in mucosal tissues of the lung and gut are currently unclear. This may however be relevant since many diseases with chronic inflammation of the lung such as asthma and COPD are also associated with vitamin D deficiency (8, 53, 54). These patients have furthermore dysregulated immune responses, altered microbiome composition, impaired epithelial barrier function and aberrant

secretion of host defense molecules thereby increasing their susceptibility towards infections (55-57). Since 1,25$(OH)_2$D is involved in many of these processes, it might provide protection against these features. This may occur via various mechanisms induced by 1,25$(OH)_2$D, including the maintenance of the integrity of the mucosal barrier and the promotion of killing of pathogens (e.g. via the induction of the antimicrobial peptide hCAP18/LL-37) (1). Mechanistic studies have furthermore shown that 1,25$(OH)_2$D is an important mediator of both innate and adaptive immune responses, suggesting the importance of 1,25$(OH)_2$D in various immune-related diseases (58). COPD patients have an increased risk for vitamin D deficiency, which is associated with an increased number and more severe exacerbations (8, 32). Studying the role of 1,25$(OH)_2$D in COPD exacerbations and airway epithelial innate immune defenses might therefore provide additional insight into the potential benefit of monitoring 25(OH)D levels in these patients and supplementation of patients with a vitamin D deficiency.

Outline of the Thesis

The overall aim of the studies described in this thesis is to elucidate the role of inflammation on the protective effects of vitamin D on respiratory host defense in chronic airway diseases with a specific focus on COPD. First, an introduction into the central role of the respiratory epithelium during homeostasis and in the pathogenesis of chronic inflammatory lung diseases such as COPD is provided, followed by a general introduction into vitamin D metabolism and vitamin D deficiency in COPD patients (**Chapter 1**). In the experimental studies that are described in this thesis, the effects of vitamin D (inactive 25(OH)D and active $1,25(OH)_2D$) and inflammation are studied using *in vitro* models of primary bronchial epithelial cells. In these models, we will study the effects of toxic, inflammatory and microbial exposures such as cigarette smoke (**chapter 2**), cytokines (**chapter 3** and **4**), bacteria (**chapter 4**) and viruses (**chapter 5**) on airway epithelial host defense mechanisms with a focus on $1,25(OH)_2D$-mediated epithelial host defense mechanisms, i.e. expression of the AMP hCAP18/LL-37 (Figure 3).

Both smokers and COPD patients are more susceptible for respiratory infections and various studies have shown that cigarette smoke exposure may alter airway epithelial cell composition. We therefore first aimed to investigate in **Chapter 2** if chronic cigarette smoke-exposure during epithelial differentiation alters expression of constitutively expressed host defense molecules and host defense mechanisms through its effect on airway epithelial cell differentiation. Cigarette smoke exposure also induces expression of TGF-β1 in airway epithelial cells. This pleiotropic cytokine is elevated in COPD patients, and in addition to its ability to promote fibrosis, TGF-β1 also impairs airway epithelial host defense mechanisms. Therefore, we next focused in **Chapter 3** on investigating the effects of TGF-β1 on expression of constitutively expressed host defense molecules and on the expression of the $1,25(OH)_2D$-induced hCAP18/LL-37. We furthermore determined mechanisms underlying behind the impaired effects of TGF-β1 on respiratory host defense by investigating direct effects of TGF-β1 on *CAMP* (hCAP18/LL-37) transcription, on vitamin D metabolism and on VDR expression. In **Chapter 4**, we continued to investigate the effects of proinflammatory cytokines (TNF-α, IL-1β, IL-17A), elevated in the airways of COPD and/or in steroid resistant asthma patients, on vitamin D-metabolism and on 25(OH)D and $1,25(OH)_2D$-mediated expression of hCAP18/LL-37. To determine if the 25(OH)D and $1,25(OH)_2D$-mediated respiratory host defense was affected by exposure to proinflammatory cytokines, we assessed epithelial antimicrobial activity against nontypeable *Haemophilus influenzae*.

Chapter 5 describes the immunomodulatory effects 25(OH)D and 1,25(OH)$_2$D on virus-induced (Poly[I:C])-inflammatory responses in airway epithelial cells. In addition, the effects of Th2 inflammation -present in the airways of both allergic asthma and a subset of COPD patients- on vitamin D metabolism and 25(OH)D and 1,25(OH)$_2$D-mediated expression of hCAP18/LL-37 was investigated. To translate our findings to a more clinical level in **Chapter 6,** a study design is provided that describes a multicenter randomized controlled trial that aims to investigate if vitamin D supplementation can indeed protect against COPD exacerbations in a population of vitamin D deficient COPD patients. Finally, in **Chapter 7** a review of all the current knowledge of the effects of disease-associated factors such as inflammation and cigarette smoke exposure on availability and signaling of 1,25(OH)$_2$D in the lungs of patients with COPD and other chronic lung diseases is provided, followed by an general overview and discussion of the results that are presented in this thesis in **Chapter 8**.

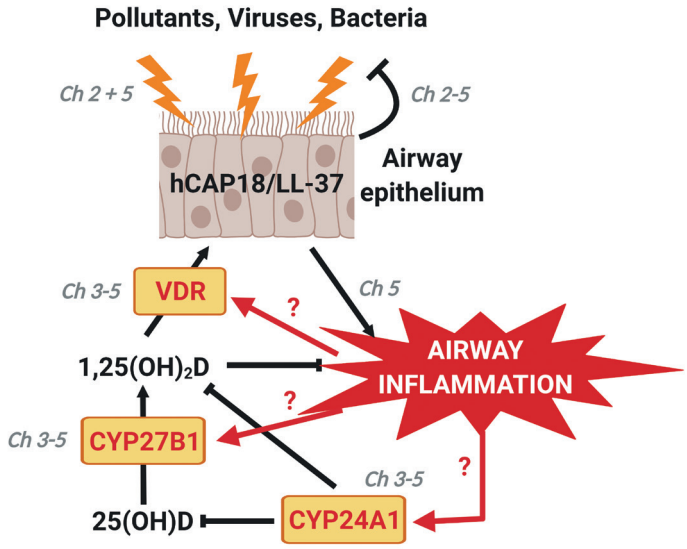

Figure 3. Unknown effects of inflammation on vitamin D metabolism and epithelial host defense in the airways. The separate components of this figure are discussed in the various chapters of this thesis, as indicated by the chapter (Ch) numbers. Vitamin D receptor, VDR; Biologically active vitamin D, 1,25(OH)$_2$D; 25-hydroxyvitamin D-1α-hydroxylase, CYP27B1; circulating inactive vitamin D, 25(OH)D; 25-hydroxyvitamin D-24-hydroxylase, CYP24A1.

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CHAPTER

2

Aberrant epithelial differentiation by cigarette smoke dysregulates respiratory host defence

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Abstract

Research question: It is currently unknown how cigarette smoke-induced airway remodelling affects highly expressed respiratory epithelial defence proteins and thereby mucosal host defence.

Methods: Localization of a selected set of highly expressed respiratory epithelial host defence proteins was assessed in well-differentiated primary bronchial epithelial cell (PBEC) cultures. Next, PBEC were cultured at the air-liquid interface and during differentiation for 2-3 weeks daily exposed to whole cigarette smoke. Gene expression, protein levels and epithelial cell markers were subsequently assessed. In addition, functional activities and persistence of the cigarette smoke-induced effects upon cessation were determined.

Results: Expression of pIgR, SLPI, long and short PLUNC was restricted to luminal cells and exposure of differentiating PBEC to cigarette smoke resulted in a selective reduction of the expression of these luminal cell-restricted respiratory host defence proteins compared to controls. This reduced expression was a consequence of cigarette smoke-impaired end-stage differentiation of epithelial cells, and accompanied by a significant decreased trans-epithelial transport of IgA and bacterial killing.

Conclusions: These findings shed new light on the importance of airway epithelial cell differentiation in respiratory host defence and could provide an additional explanation for the increased susceptibility of smokers and patients with COPD to respiratory infections.

Introduction

Respiratory infections and microbial colonization are a major health burden in smokers, and contribute to exacerbations and to the development and progression of chronic obstructive pulmonary disease (COPD) (reviewed by Sethi(1)). The mechanisms underlying this increased susceptibility of smokers with or without COPD are incompletely understood, but can be attributed in part to epithelial injury and remodelling resulting in a disrupted mucociliary clearance(2). In addition to mucociliary clearance, the airway epithelium contributes to host defence with a wide variety of additional activities(3) that include secretion of antimicrobial peptides that act as endogenous antibiotics or modulate important antimicrobial immune responses via a variety of mechanisms(4). Furthermore, the epithelium produces cytokines and chemokines that initiate an immune response to act against microbial invaders. Finally, transport of polymeric IgA and IgM to the lumen by the polymeric immunoglobulin receptor (pIgR) contributes to adaptive immunity in the lung by inhibiting adherence and facilitating clearance of pathogens, a process called immune exclusion(5). Several of these respiratory host defence proteins (HDPs) in the airways are highly expressed during homeostasis by epithelial cells, suggesting their importance for airway epithelial barrier function. Highly expressed proteins and peptides include -but are not limited to- antimicrobial peptides such as human beta defensin-1 (hBD-1) and lipocalin 2 (LCN2), the secretory leukocyte protease inhibitor (SLPI), pIgR and the epithelial sodium channel regulators short and long palate, lung and nasal epithelium clone protein (s/lPLUNC or *BPIFA1/BPIFB1*)(6-8). Expression of other peptides involved in airway host defence such as Ribonuclease 7 (RNase 7), LL-37 and human beta defensin-2 (hBD-2) is low during homeostasis but can be induced by e.g. inflammatory mediators, microbial products and upon injury of epithelial cells, and thus contribute to clearance of the pathogen and the resulting inflammatory process(4). The pseudostratified airway epithelium is composed of several cell-types, including goblet, club and ciliated cells that reach out toward the lumen of the airways, while basal cells do not reach this lumen in the intact epithelial layer(9). Based on their distinct anatomical positioning, it is not surprising that these different cell-types also produce different types of mediators. For example, expression of pIgR is restricted to the luminal cells of the pseudostratified airway epithelium and is therefore largely regulated by airway epithelial cell differentiation(10), similar to e.g. mucin production by goblet cells. In contrast, expression of the antimicrobial protein RNase 7 is restricted to

basal cells(11). Cigarette smoke is known to induce airway epithelial remodelling in smokers and patients with COPD, characterized by an increase in goblet cells and a reduction in presence of ciliated cells(2). As a result higher levels of mucus are produced by the epithelium, while mucus transport is impaired, thereby compromising mucociliary clearance activity of luminal airway epithelial cells. Currently it is unknown if the expression of proteins that are important for airway epithelial defence is polarized in the epithelium, and if so, how cigarette smoke-induced remodelling of the airway epithelium affects their expression. We hypothesized that cigarette smoke-induced alterations in epithelial cell differentiation result in a decreased expression of proteins that contribute to respiratory host defence (HDPs), which may render the host more susceptible to infection.

Methods

Collection of cells and cell culture

Primary bronchial epithelial cells (PBEC) were obtained from tumor-free resected lung tissue at the Leiden University Medical Center, Leiden, the Netherlands. For this, PBEC were cultured at the air-liquid interface (ALI) for 13 to 19 days (Figure 2A). Apical washes were performed daily; medium was refreshed every other day. Primary bronchial epithelial cells (PBEC) were obtained from tumour-free resected lung tissue at the Leiden University Medical Center, Leiden, the Netherlands. For this, bronchial epithelial cells were isolated from a bronchial ring by enzymatic digestion for 2 h at 37 °C with 0.18% (w/v) proteinase type XIV (Sigma-Aldrich, St. Louis, MO, USA) in Ca^{2+}/Mg^{2+}-free Hank's Balanced Salt Solution (Life Technologies Europe B.V., Bleiswijk, The Netherlands). Next, the obtained cell fraction was expanded in serum-free keratinocyte medium (KSFM, Life Technologies Europe B.V.) supplemented with 0.2 ng/ml epidermal growth factor (Gibco), 25 µg/ml bovine pituitary extract (Life Technologies Europe B.V.), 1 µM isoproterenol (Sigma-Aldrich), 100 U/mL Penicillin (Lonza, Verviers, Belgium), 100 µg/ml Streptomycin (Lonza) and 5 µg/ml Ciproxin. Upon reaching confluence, cells were trypsinized in 0.03% (w/v) trypsin (Difco, Detroit, USA), 0.01% (w/v) EDTA (BDH, Poole, England), 0.1% glucose (BDH) in PBS and stored in liquid nitrogen until further use. For our cultures, cells were thawed in KSFM medium supplemented with the above mentioned supplements until near confluence, seeded on semipermeable transwell inserts with 0.4 µm pore size (Corning Costar, Cambridge, USA) that were coated with a mixture of bovine serum albumin, collagen and fibronectin and cultured as described (11).

Fractionation of the airway epithelial cultures

Luminal and basal cell-enriched fractions were obtained from 3-4 weeks differentiated ALI-PBEC cultures as described previously(11). The luminal cell fraction was spun down and either lysed in RNA lysis buffer or fixed with 1% paraformaldehyde (Millipore B.V., Amsterdam, the Netherlands) in PBS for 10 minutes on ice and washed afterwards in ice-cold PBS. The remaining basal epithelial cell fractions on the transwell inserts were also either lysed in RNA lysis buffer (Promega) or fixed with 1% paraformaldehyde (Millipore B.V.) in PBS for 10 minutes on ice and washed afterwards with ice-cold PBS. Next, cells were stained

as described in the online data supplement with antibodies described in supplemental (s)Table 2.

Chronic cigarette smoke exposure

When confluent, PBECs were air-exposed (day 0) by removal of medium from the apical side of the transwell insert and 4 h later exposed to freshly generated whole cigarette smoke (CS) using 3R4F reference cigarettes (University of Kentucky, Lexington, KY). CS exposure was repeated daily as described in(11), in the figure legends of figure 1 and online supplementary figure S1 and illustrated in figure 1b and online supplementary figure S1. Briefly, cells were exposed in modified hypoxic chambers for 4-5 minutes to either cigarette smoke from 1 cigarette or to room air, after which smoke was removed by ventilation with air during 10 minutes. and cells were subsequently placed back in the incubator overnight. Approximately 18-20 h later, ALI-PBEC were washed apically with PBS and 4 h hereafter exposed to cigarette smoke. This cycle was repeated every day until day 13-19. Cells were harvested for analysis 18-20 h after the last cigarette smoke exposure.

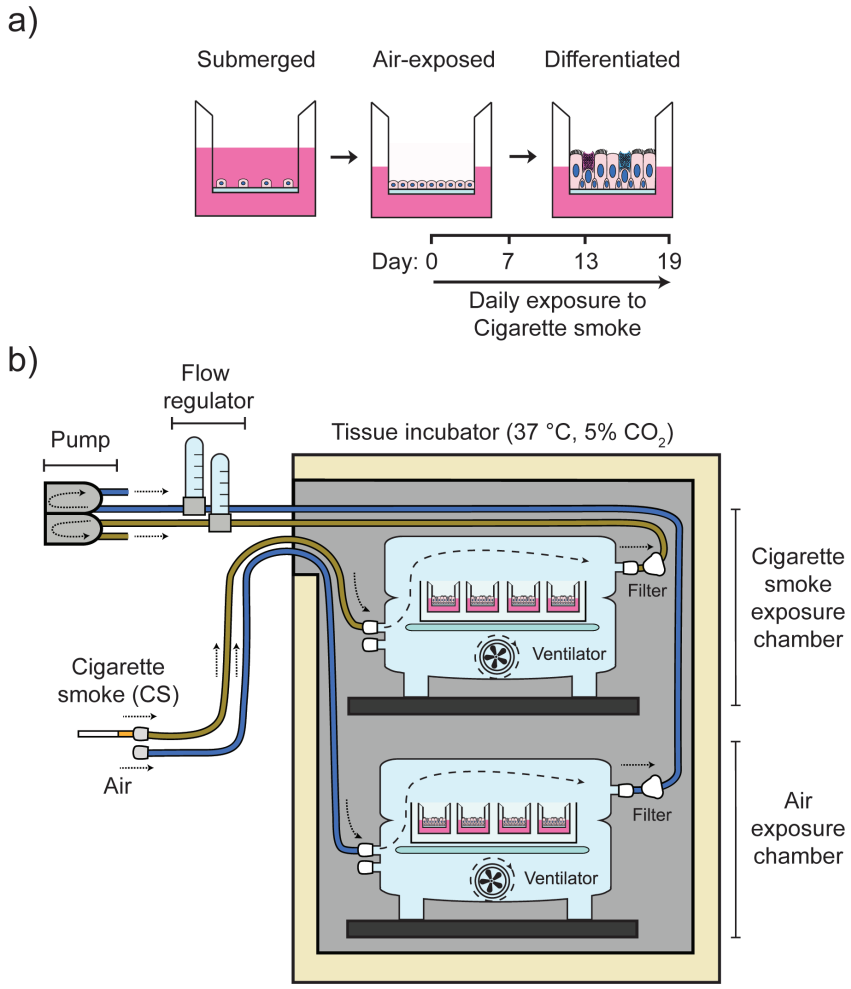

Figure 1. Cell culture set-up and cigarette smoke exposure of primary bronchial epithelial cells differentiated at the air-liquid interface (ALI-PBEC). (A) Primary bronchial epithelial cells (PBEC) were seeded on coated transwells and cultured in submerged conditions until confluent. At day 0, cultures were air-exposed and cultured for additional 13-19 days to allow mucociliary differentiation. (B) Each day, starting at day 0, cultures were exposed to cigarette smoke (CS) by placing them in an exposure chamber that was infused with either cigarette smoke or with air for 4-5 min. Next, residual smoke in the chamber was removed for a period of 10 min. by infusing the chambers with air derived from the incubator. Approximately 4 h before each CS exposure the apical surface of the cultures was washed to remove mucus. Basal medium was changed every other day.

RNA isolation, cDNA synthesis and qPCR

Cells were lysed using lysis buffer from Promega, Leiden, the Netherlands. Next, RNA was extracted using the Maxwell tissue RNA extraction kit (Promega) and quantified using the Nanodrop ND-1000 Spectrophotometer (Nanodrop

technologies, Wilmington, DE). cDNA synthesis was performed using oligo dT primers (Qiagen, Venlo, the Netherlands) and M-MLV Polymerase (Promega) in the presence of RNAsin (Promega). For qPCR analysis, diluted cDNA was mixed with primers (sTable 1) and iQ™ SYBR® Green Supermix (Bio-Rad, Veenendaal, the Netherlands). Reactions were performed in triplicate and results were corrected for the geometric mean of expression of 2-3 reference genes selected using the Genorm method. Expression values were determined by the relative gene expression of a standard curve as determined by CFX manager software (Bio-Rad).

Confocal microscopy

Following fixation with 1% PFA, cell culture inserts and/or cytospins containing luminal epithelial cells were treated with methanol for 10 min at 4 °C, washed with PBS and cells were permeabilized with 1% w/v BSA, 0.3% v/v Triton-X100 in PBS (PBT) for 30 min at 4 °C. After washing with PBS, cells were pre-treated with SFX-signal enhancer (Life Technologies Europe B.V.) followed by incubation with primary antibodies in PBT for 1 h at RT (sTable 2). Next inserts were washed in PBS and incubated with an Alexa Fluor 488 or 568-labeled secondary antibody (Alexa Fluor 488 donkey-anti--mouse IgG; Alexa Fluor 568 donkey--anti-rabbit IgG, Life Technologies Europe B.V.) together with DAPI in PBT for 30 min at RT. 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.).

Transcytosis experiment

Transcytosis capacity of the epithelial cultures was assessed in cultures exposed daily to whole cigarette smoke for 13 days, or air as a control. Dimeric IgA was added to the basal compartment of the cell cultures and 24 h thereafter, apical washes (PBS) were collected and stored at -20°C for further analysis. Apical washes were assessed for secretory (S-)IgA levels by sandwich ELISA(10).

Antibacterial activity assay

Direct antimicrobial activity was assessed in cultures of ALI-PBEC that were exposed daily to whole cigarette smoke or air controls for 13 days, followed by replacement with antibiotics-free cell culture medium for an additional 48 h period. *Moraxella catarrhalis* strain LUH2760 and *Klebsiella pneumoniae* strain LUH2754 were cultured in Tryptic Soy broth (TSB) while shaking overnight at 37^{o} C. Next, the

overnight cultures were transferred into fresh TSB medium (1/50 dilution) and incubated for 4 h at 37°C -while shaking- to obtain mid log-phase-growing bacteria. Bacterial concentrations of log-phase cultures were determined by $OD_{600\ nm}$ measurements, pre-diluted in PBS and final dilution was made in antibiotics-free cell culture medium. Twenty µl of bacterial suspension was added on the apical surface of the cells at a concentration of ~$6x10^5$/ml CFU/ml for *M. catarrhalis* and ~$1x10^4$ CFU/ml for *K. pneumoniae* and incubated at 37°C, 5% CO_2 for 2 h. Hereafter, membranes containing the cells with bacteria were dissected from the inserts and placed into tubes containing sterile glass beads and 1% TSB in PBS. Next cells were disrupted by using a minilys personal homogenizer (Bertin Instruments, Montigny-le-Bretonneux, France) for 2 times 30 s and kept on ice in between. Serial dilutions of both bacterial suspensions were plated on Tryptic Soy Sheep blood (TSS) agar plates (Biomerieux, Zaltbommel, The Netherlands), and incubated overnight at 37°C to assess surviving bacteria by CFU determination.

ELISA

CXCL8/IL-8 production by ALI-PBEC was determined in the basal medium by use of the CXCL8/IL-8 Duoset kit from R&D (MN, U.S.A.). hBD-1 was measured in the apical wash and in the basal medium using the hBD-1 kit from Peprotech (London, U.K.) and SLPI was measured as described(46).

Trans-epithelial electrical resistance

Epithelial barrier integrity of ALI-PBEC cultures was determined during cell differentiation by measuring the trans-epithelial electrical resistance (TEER) using the MilliCell-ERS (Millipore, Bedford, MA). TEER values were shown as $\Omega*cm^2$ and calculated as TEER = (measured value – background value) *surface transwell insert in cm^2.

Inhibition of differentiation by DAPT

At day 0, PBEC were air exposed by removal of the medium in the insert and culture medium of ALI-PBEC was refreshed with medium supplemented with either 5 µM DAPT (Notch signalling inhibitor, Sigma Aldrich, Zwijndrecht, The Netherlands), or solvent control. Every other day, basal medium was changed in a similar fashion up to day 13 when the cells were harvested.

Statistics

Statistical analysis was conducted using GraphPad Prism 7 (GraphPad Software Inc., La Jolla, CA, U.S.A.). Data are shown as mean ± SEM and significance was tested with use of a paired t-test or two-way ANOVA with a Bonferroni corrected post-hoc test. Differences were considered significant at $p < 0.05$.

Results

Respiratory host defence proteins display a polarized distribution in airway epithelial cell cultures

In this study we have focussed on a set of proteins and peptides that are important for respiratory host defence. These host defence proteins (HDPs) were selected based on their constitutive and/or high expression by airway epithelial cells during homeostasis: i.e. SLPI, PLUNC (short/long), pIgR, hBD-1 and LCN2. We first investigated whether expression of these proteins was polarized in the airway epithelial cultures. To this end, we prepared luminal and basal epithelial cell-enriched fractions of well-differentiated primary bronchial epithelial cells (PBEC), cultured at the air-liquid interface (ALI, Figure 2A). We confirmed the successful enrichment of fractions for luminal and basal cells by determining the gene expression of the typical basal cell markers *TP63* and *KRT5* and luminal epithelial cell markers *FOXJ1* (ciliated cells), *SCGB1A1* (club cells), *MUC5AC* and *MUC5B* (both goblet cells) (Figure 1A), and by immunofluorescence staining for p63 (basal cells), CC16 (club cells) and acetylated α-tubulin (ciliated cells) (sFigure 2). Further analysis of these fractions showed that the luminal cell-enriched fraction expressed significantly higher levels of *BPIFA1* (sPLUNC), *BPIFB1* (lPLUNC) and *SLPI* (Figure 2A). In contrast, *LCN2* and *DEFB1* expression did not differ between the luminal and basal cell-enriched fraction (Figure 1A). The luminal cell-specific expression of SLPI and sPLUNC was further confirmed using confocal imaging in which the staining of both proteins did not co-localize with $p63^+$ basal cells, but was highly present at the apical side of the PBEC culture and in the luminal cell-enriched fraction (Figure 2B and online supplementary Figure S2).

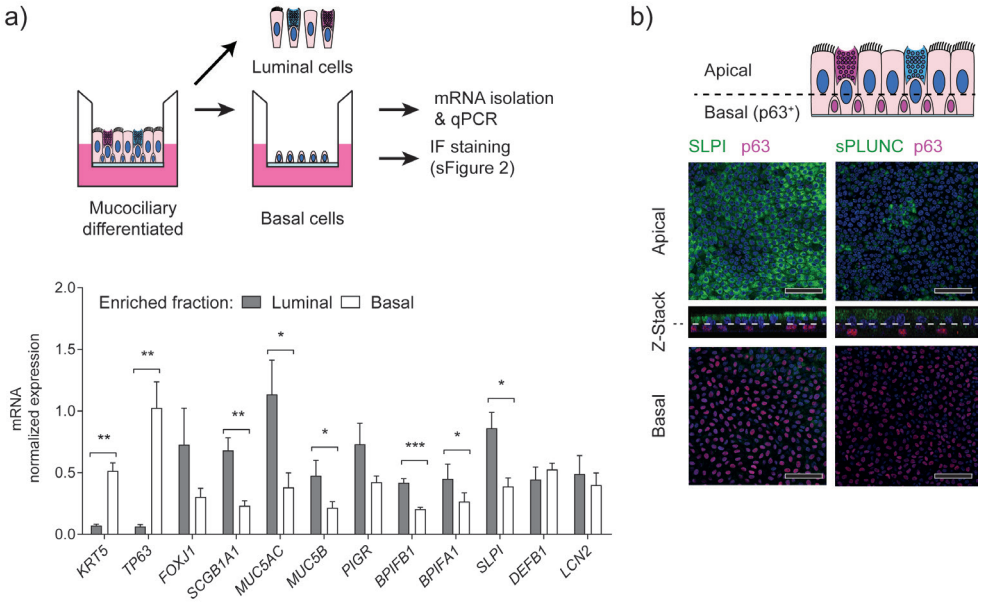

Figure 2. Respiratory host defence proteins display a polarized distribution in air-liquid interface cultures of primary bronchial epithelial cells (ALI-PBEC). (A) PBEC were seeded on coated transwells and cultured in submerged conditions until confluent. At day 0, cultures were air-exposed and cultured at the air-liquid interface. After 3-4 weeks of differentiation luminal and basal cell-enriched fractions were separated followed by RNA isolation, cDNA synthesis and qPCR analysis. Data are shown as target gene expression normalized for the geometric mean expression of the reference genes ATP synthase, H^+ transporting, mitochondrial F1 complex, beta polypeptide (*ATP5B*), β2-microglobulin *(B2M)* and Ribosomal Protein L13a *(RPL13A)*, n=5-7 different donors. Open bars are basal cell-enriched fractions; grey bars are luminal cell-enriched fractions. Statistical significance was tested using a paired t-test. * p<0.05, ** p<0.01, *** p<0.001. (B) Confocal images to visualize polarized distribution of secretory leukocyte protease inhibitor (SLPI) and short palate, lung and nasal epithelium clone protein (sPLUNC) in differentiated ALI-PBEC cultures cells. After 3 weeks of differentiation, cells were fixed in 1% paraformaldehyde and stained using immunofluorescence with primary antibodies against p63 (basal cell marker, red) in combination with primary antibodies against SLPI and/or sPLUNC (both green) and DAPI for nuclear staining (blue). Z-stacks and images of the apical and basal side of stained cells were made by confocal imaging, scale bars equals 50 µm. Images shown are representative for results obtained with cells from 4 different donors.

Chronic cigarette smoke exposure of airway epithelial cell cultures reduces expression of respiratory host defence proteins

We next investigated if cigarette smoke-exposure affected expression of this set of respiratory HDPs. To this end, ALI-PBEC cultures were exposed on a daily basis during 2-3 weeks of differentiation to whole cigarette smoke (CS) (Figure 1 and

supplementary Figure S1). Gene expression analysis showed that *DEFB1* (hBD-1) mRNA levels decreased during differentiation, but were not affected by CS exposure (Figure 3A). On the other hand, expression of *SLPI, BPIFA1* (sPLUNC), *BPIFB1* (lPLUNC) and *PIGR* strongly increased during differentiation, and this increase was significantly prevented by CS (Figure 3A). In contrast, gene expression of *LCN2* (lipocalin 2) was increased by CS exposure during differentiation (Figure 3A). These findings were further confirmed by assessment of hBD-1 and SLPI protein levels in the apical wash and in basal medium from the ALI-PBEC cultures (Figure 3B). Indeed, hBD-1 levels reduced over the time of differentiation in the apical wash, but were not significantly affected by chronic CS-exposure, whereas SLPI levels were significantly lower in chronic CS-exposed cell cultures (Figure 3B). We next performed immunofluorescence staining of the airway epithelial cell cultures and found strongly reduced presence of SLPI-, sPLUNC- and pIgR-positive cells in chronic CS-exposed epithelium compared to air controls (Figure 3C). These results confirmed selective impairment of specific respiratory HDPs by chronic CS exposure during airway epithelial differentiation.

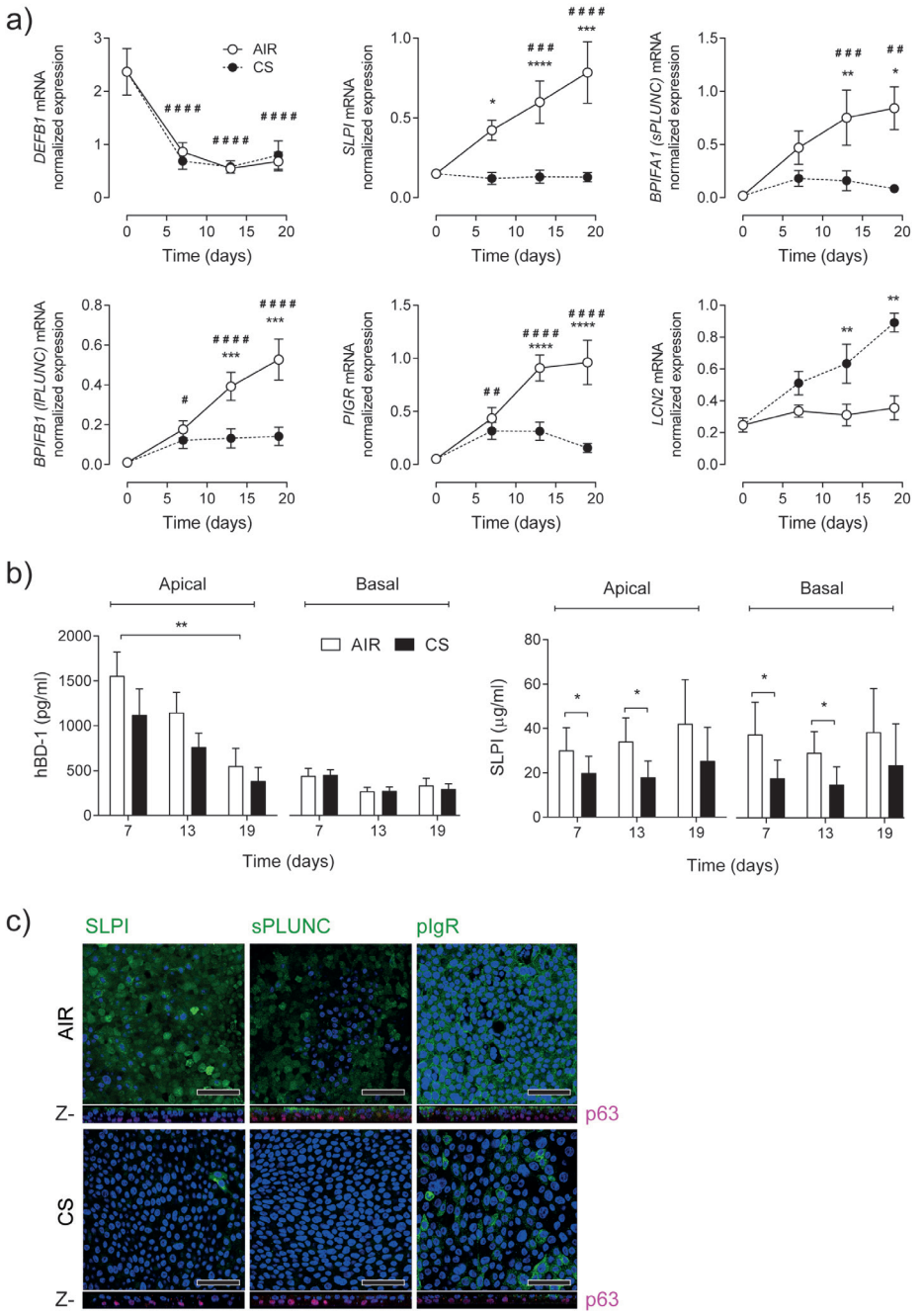

Figure 3. Chronic cigarette smoke exposure of air-liquid interface cultures of primary bronchial epithelial cells (ALI-PBEC) lowers the expression of luminal cell-restricted host defence proteins. (A) ALI-PBEC were daily exposed to whole cigarette smoke (CS) or air as a control (AIR) during differentiation for 13-19 consecutive days. Cells were lysed at several points during this course of time and RNA was isolated followed by cDNA synthesis to assess gene expression of *DEFB1* (human beta

defensin-1), *SLPI* (secretory leukocyte protease inhibitor), *BPIF1A* (short palate, lung and nasal epithelium clone protein), *BPIF1B* (long palate, lung and nasal epithelium clone protein), *PIGR* (polymeric immunoglobulin receptor) and *LCN2* (lipocalin 2). Open circles: air-exposed controls, black circles: CS-exposed cell cultures; data are shown as target gene expression normalized for the geometric mean expression of the reference genes ATP synthase, H^+ transporting, mitochondrial F1 complex, beta polypeptide (*ATP5B*), β2-microglobulin *(B2M)* and Ribosomal Protein L13a *(RPL13A)*; day 0, 7, 13 n=8 different donors and day 19 n=4 different donors. Statistical differences were evaluated using a two-way ANOVA and Bonferroni post-hoc test. * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001 between AIR and CS. # p<0.05, ## p<0.01, ### p<0.001, #### p<0.0001 between AIR at day 7, 13 and 19 and unexposed cultures at day 0. (B) ELISA for hBD-1 and SLPI was performed on the apical wash (Apical) and basal medium (Basal) of these cultures. Open bars: air controls (AIR), black bars: CS-exposed cell cultures (CS); day 7 and 13, n=8 different donors and day 19: n=4 different donors. Statistical differences on T=7 and T=13 (not T=19) was tested using a paired two-way ANOVA to compare AIR and CS. * p<0.05, ** p<0.01. (C) ALI-PBEC were differentiated for 2-3 weeks in which they were daily exposed to CS or air as a control (AIR). Subsequently the cells were fixed in 1% paraformaldehyde and stained using primary antibodies against SLPI, sPLUNC and pIgR (all green staining) in combination with DAPI to stain the nuclei (blue staining). Scale bars are equal to 50 µm. Images shown are representative for results obtained with cell cultures from 4 different donors.

Chronic cigarette smoke exposure reduces host defence of the airway epithelial cell cultures by decreasing apical release of secretory IgA and bacterial killing of *Moraxella catarrhalis* and *Klebsiella pneumoniae*

Next, we assessed if the strong reduction in *SLPI, BPIFA1* (sPLUNC), *BPIFB1* (lPLUNC) and *PIGR* expression levels in the CS-exposed airway epithelial cultures had functional consequences for host defence. We selected pIgR-mediated transfer of dimeric (d)IgA across the epithelium as a proof-of-principle for the consequences on immunomodulatory host defence functions and found this to be significantly reduced in chronic CS-exposed cultures (Figure 4A). We furthermore analysed bacterial killing by chronic CS-exposed cell cultures of the Gram-negative bacteria *Moraxella catarrhalis* and *Klebsiella pneumoniae*, pathogens that are found to be increased in the lungs of patients with stable or acute exacerbations of COPD (12). We observed significantly higher bacterial counts (indicating lower antibacterial activity) in chronic CS-exposed PBEC cultures when compared to air-exposed cultures for both pathogens (Figure 4B). These data indicate that various host defence mechanisms are functionally impaired in CS-exposed epithelial cell cultures, which corresponds with impaired expression of respiratory defence proteins.

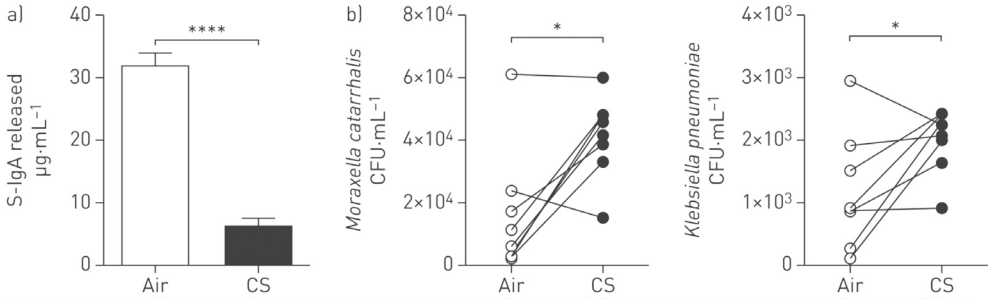

Figure 4. Chronic cigarette smoke exposure of air-liquid interface cultures of primary bronchial epithelial cells (ALI-PBEC) impairs host defence activities. A) ALI-PBEC were daily exposed to whole cigarette smoke (CS) or air as a control (AIR) during differentiation for 13 consecutive days. After 13 days of chronic CS exposure, dimeric (d)IgA transcytosis capacity of the epithelial cultures was assessed by determining secretory (S)-IgA levels in apical washes by ELISA (no S-IgA could be detected in the basal medium, as a control of the assay that does only recognize S-IgA and not d-IgA), n=10 different donors. Open bars: air-exposed cell cultures, black bars: CS-exposed cell cultures. B) After 13 days of chronic CS exposure, ALI-PBEC were cultured for 48 h in antibiotics-free cell culture medium after which they were exposed for 2 h to *Moraxella catarrhalis* or *Klebsiella pneumoniae* at the apical surface of the ALI-PBEC. The surviving bacteria are depicted as colony forming units (CFU)/ml, n=8 different donors. Significance was determined using a paired t-test. *p<0.05, **** p<0.0001.

Cigarette smoke affects end-stage differentiation of airway epithelial cells

Next we assessed if chronic CS exposure affected differentiation of ALI-PBEC by measuring gene expression of epithelial cell-specific markers. Gene expression of the basal cell markers cytokeratin-5 (*KRT5*) and *TP63,* and of cytokeratin-8 (*KRT8*) that is expressed by intermediate/committed progenitor epithelial cells(13), was not affected by CS (Figure 5A). In contrast, expression of the specialized luminal epithelial cell-specific genes *FOXJ1* (ciliated cells), *SCGB1A1* (club cells) and *MUC5B* (goblet cells) increased during differentiation, and this increase was significantly prevented by CS (Figure 5A). Confocal imaging confirmed the aberrant epithelial differentiation in CS-exposed cultures as cells positive for cilia marker acetylated α-tubulin, the club cell marker CC16, and the goblet cell marker MUC5AC were reduced in chronic CS-exposed cultures, while cytokeratin-8 $(CK\text{-}8)^+$ and $p63^+$ cells remained unchanged between air and CS-exposed cultures (Figure 5B).

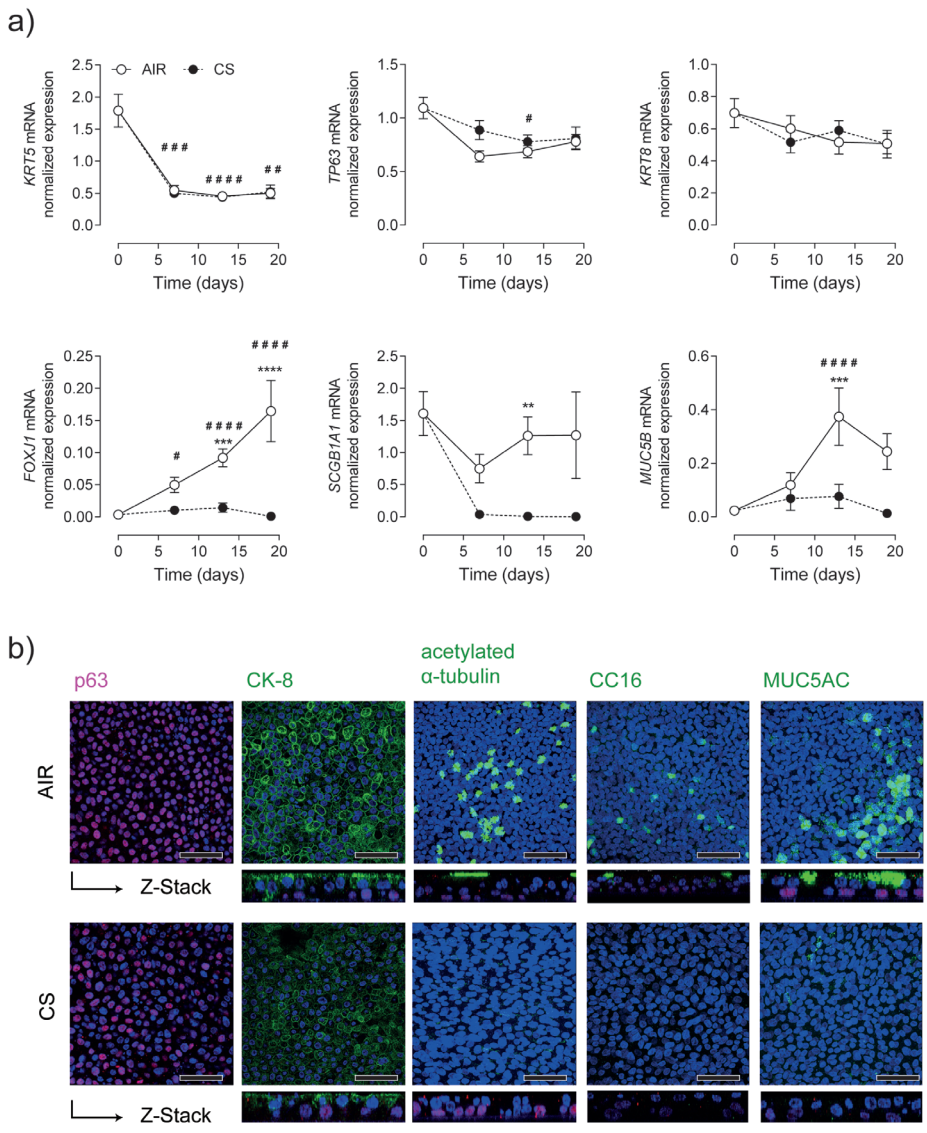

Figure 5. Chronic cigarette smoke exposure of air-liquid interface cultures of primary bronchial epithelial cells (ALI-PBEC) changes cellular composition. (A) ALI-PBEC were exposed during differentiation for 13-19 consecutive days to whole CS. Cells were lysed at several time-points and RNA was isolated followed by cDNA synthesis, to assess gene expression of basal cell markers cytokeratin-5 (*KRT5*) and *TP63*, of early progenitor cell marker cytokeratin-8 (*KRT8*) and of specialized cell markers *FOXJ1* (ciliated cells), *SCGB1A1* (club cells) and *MUC5B* (goblet cells). Open circles: air-exposed controls (AIR), black circles: CS-exposed cell cultures (CS); data are shown as target gene expression normalized for the geometric mean expression of the reference genes ATP synthase, H^+ transporting, mitochondrial F1 complex, beta polypeptide (*ATP5B*), β2-microglobulin *(B2M)* and Ribosomal Protein L13a *(RPL13A)*; day 0, 7, 13 n=8 donors and day 19 n=4 donors. Significance was

determined using a two-way ANOVA and Bonferroni post-hoc test. ** p<0.01, *** p<0.001, **** p<0.0001 between AIR and CS. # p<0.05, ## p<0.01, ### p<0.001, #### p<0.0001 between AIR at day 7, 13 and 19 and unexposed cultures at time 0. (B) ALI-PBEC were differentiated for 2-3 weeks and daily exposed to CS. Subsequently the cells were fixed in 1% paraformaldehyde and stained using primary antibodies against basal cells (p63), presented as a red staining, in combination with primary antibodies against cytokeratin-8 (CK-8), acetylated α-tubulin (ciliated cells), CC16 (club cells) and MUC5AC (goblet cells), which are presented as a green staining; DAPI was used to stain the nuclei (blue staining). Z-stacks and images of the apical and basal side of stained cells were obtained by confocal imaging. Scale bars equals 50 µm. Images shown are representative for results obtained with cells from 4 different donors (CK-8; n=3 different donors).

Reversibility of cigarette smoke-induced effects on host defence protein expression

To assess the persistence of the CS-induced reduction in *SLPI, BPIFA1* (sPLUNC), *BPIFB1* (lPLUNC) and *PIGR* expression levels and of its effect on cellular composition, we allowed the cultures to recover from 13 days of CS exposure by culturing the cells for an additional 6 days without CS exposure. Chronic CS-exposed cultures were able to (partly) recover from the lack of end-stage differentiation, since all specialized luminal cell markers, except for *SCGB1A1* (club cells), significantly increased in expression (Figure 6A). Furthermore, also *SLPI, BPIFA1* (sPLUNC), *BPIFB1* (lPLUNC) and *PIGR* showed enhanced expression compared to day 13 (Figure 6A). In addition, *KRT5* (basal cell marker), and *DEFB1* and *LCN2* increased upon CS cessation, whereas *TP63* and *KRT8* were unaffected (Supplementary Figure S3A). This indicates that the inhibitory effects of cigarette smoke exposure on epithelial differentiation and expression of specific respiratory defence proteins are at least in part reversible. Furthermore, in an attempt to better mimic the *in vivo* situation and establish if the effects observed after chronic CS exposure also can be obtained when exposing an already partly differentiated epithelium to chronic CS, we performed a separate experiment. In this experiment, we first allowed the cultures to differentiate for 1 week, after which we started chronic CS exposure for an additional 12 days. Here we found similar effects of CS exposure on ALI-PBEC cultures regarding cell-type specific markers and HDP expression compared to CS exposure starting from day 0 (Figure 6B and supplementary Figure S3B).

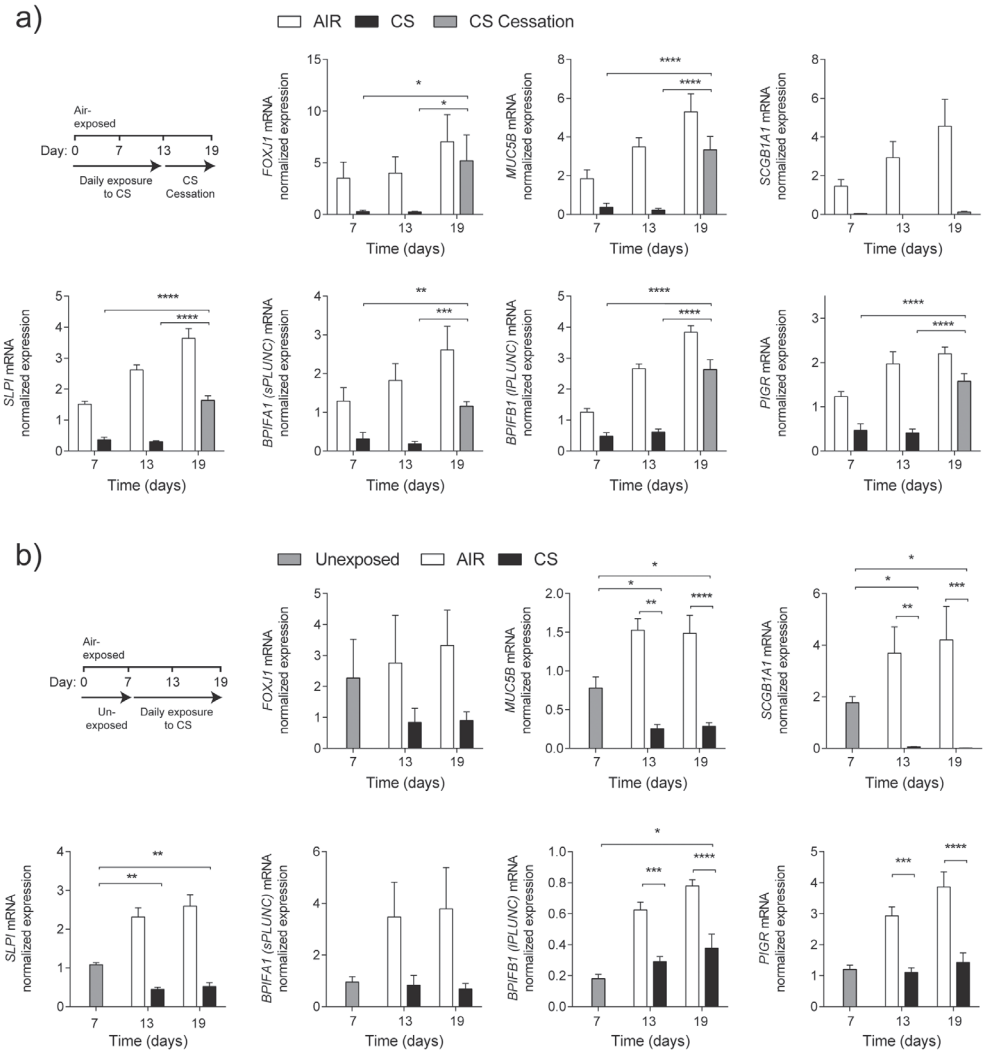

Figure 6. Cigarette smoke-induced impairment of host defence proteins and differentiation markers are partly persistent upon cigarette smoke cessation. (A) Air-liquid interface cultures of primary bronchial epithelial cells (ALI-PBEC) were exposed during differentiation for 13 consecutive days to whole cigarette smoke (CS) after which cultures were continued for another 6 days without CS exposure. Cells were lysed at several points during this course of time and RNA was isolated followed by cDNA synthesis, to assess gene expression of the cell specific markers: FOXJ1 (ciliated cells), MUC5B (goblet cells) and SCGB1A1 (club cells) and of respiratory defence proteins: SLPI, BPIFA1 (sPLUNC), BPIFB1 (lPLUNC) and PIGR. Open bars: air-exposed controls (AIR), black bars: CS-exposed cell cultures (CS), grey bars: CS-exposed cultures that were cultured for an additional week without CS exposure (CS cessation). Data are shown as target gene expression normalized for the geometric mean expression of the reference genes ATP synthase, H+ transporting, mitochondrial F1 complex, beta polypeptide (ATP5B), β2-microglobulin (B2M) and Ribosomal Protein L13a (RPL13A). n=8 different

donors. Statistical differences were evaluated only for the difference between cessation and previous CS expression using a two-way ANOVA and Bonferroni post-hoc test. * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001. (B) ALI-PBEC were air exposed at day 0 and cultured for 7 days under standard conditions. At day 7 cultures were exposed to CS for 12 consecutive days after which the cells were lysed and similar analysed as in (A). Grey bars (start point of culture at day 7): unexposed, open bars: air-exposed controls, black bars: CS-exposed cell cultures. Data are shown as target gene expression normalized for the geometric mean expression of the reference genes ATP5B, B2M and RPL13A; n=6 different donors. Statistical differences were evaluated using a two-way ANOVA and Bonferroni post-hoc test. * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.

Notch signalling inhibition impairs host defence protein expression during differentiation

Our results so far showed an impaired end-stage differentiation into specialized luminal epithelial cells in CS-exposed cultures resulting in reduced levels of SLPI, sPLUNC, lPLUNC and pIgR. Previous studies have shown that Notch signalling is involved in airway epithelial differentiation and that the airway epithelium of smokers displays reduced Notch signalling(14). Therefore, we next examined if Notch signalling was impaired in CS exposed cultures, and if Notch signalling inhibition modulates HDP expression during differentiation. First, we assessed gene expression of components of the Notch signalling cascade and found that chronic CS exposure did not influence gene expression of Notch ligands, receptors or transcriptional co-activators in these cultures (Figure 7A). However, the Notch signalling target genes, *HEY1* and *HEY2*, were significantly reduced by chronic CS exposure, while *HES1* was not (Figure 7B), indicating that chronic CS exposure selectively affects target genes of the Notch signalling pathway.

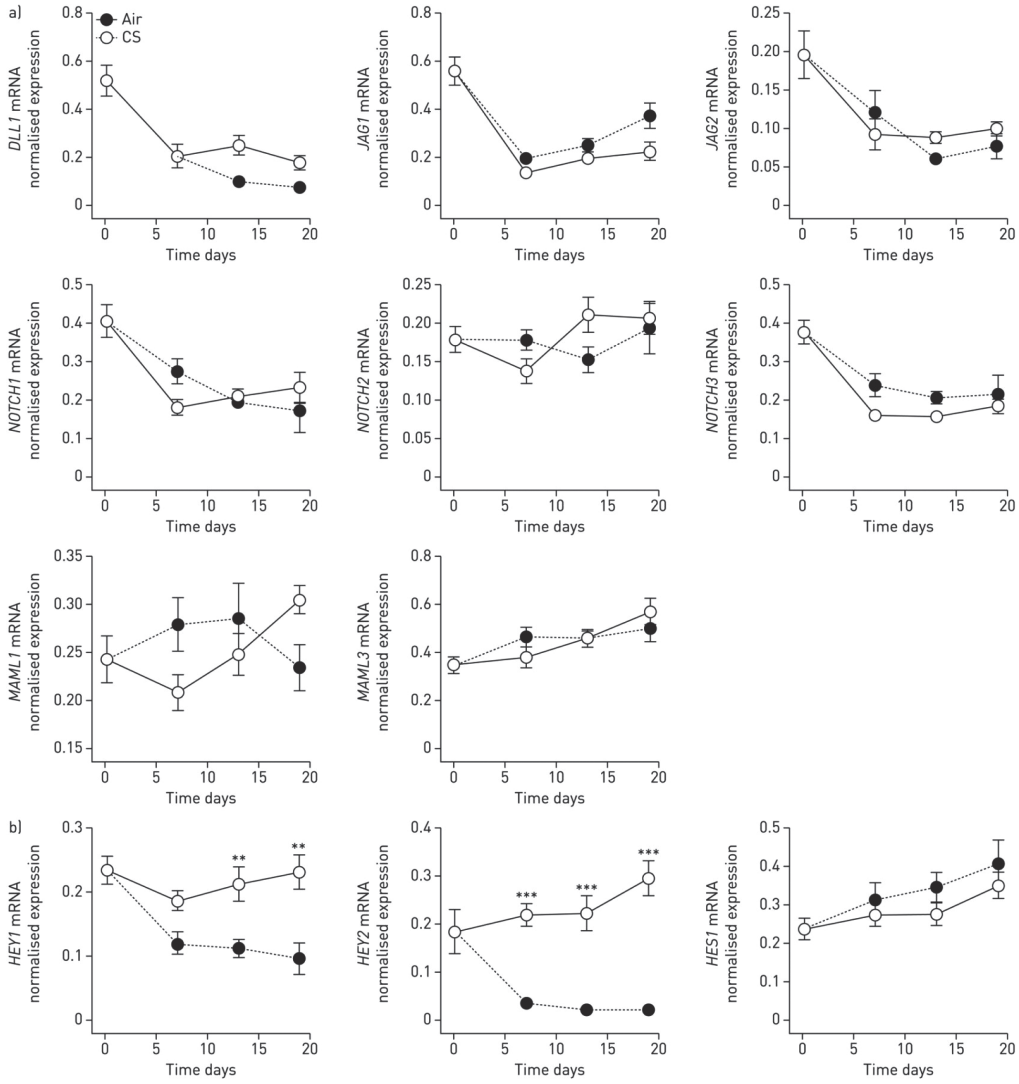

Figure 7. Chronic cigarette smoke exposure of air-liquid interface cultures of primary bronchial epithelial cells (ALI-PBEC) results in selective impairment of Notch signalling. (A) After 2 weeks of differentiation and daily cigarette smoke exposure, ALI-PBEC were lysed, RNA was isolated and cDNA synthesized. Subsequent qPCR analysis was performed on notch signalling ligands *DLL1*, *JAG1* and *JAG2*, on Notch receptors 1-3 and on the transcriptional co-activators *MAML1* and *MAML3*; data are shown as target gene expression normalized for the geometric mean expression of ATP synthase, H^+ transporting, mitochondrial F1 complex, beta polypeptide (*ATP5B*), β2-microglobulin *(B2M)* and Ribosomal Protein L13a *(RPL13A)*. Open circles: air-exposed controls (AIR), black circles: CS-exposed cell cultures (CS); n=8 different donors. (B) Subsequent qPCR analysis was performed on Notch signalling target genes *HEY1*, *HEY2* and *HES1*. Data are shown as target gene expression normalized for the geometric mean expression of the reference genes *ATP5B*, *B2M* and *RPL13A;* n=8 different

donors. Statistical significance was tested using a two-way ANOVA and Bonferroni post-hoc test . ** p<0.01, *** p<0.001 between AIR and CS.

To further investigate the importance of Notch signalling in the expression of host defence proteins, we examined the effect of the γ–secretase inhibitor DAPT, which acts as an inhibitor of Notch signalling (Figure 8A). After 15 days of PBEC differentiation in the presence of DAPT, we measured expression of HDPs. *DEFB1* (hBD-1) and *LCN2* were not affected by DAPT, while gene expression of *SLPI*, *BPIFA1* (sPLUNC), *BPIFB1* (lPLUNC) and *PIGR* were strongly reduced by DAPT incubation (Figure 8B). Furthermore, DAPT-exposed cultures showed a skewing of cell differentiation away from a secretory phenotype towards an increase in ciliated cells (Figure 8C) that was also confirmed by confocal imaging (Figure 8D).

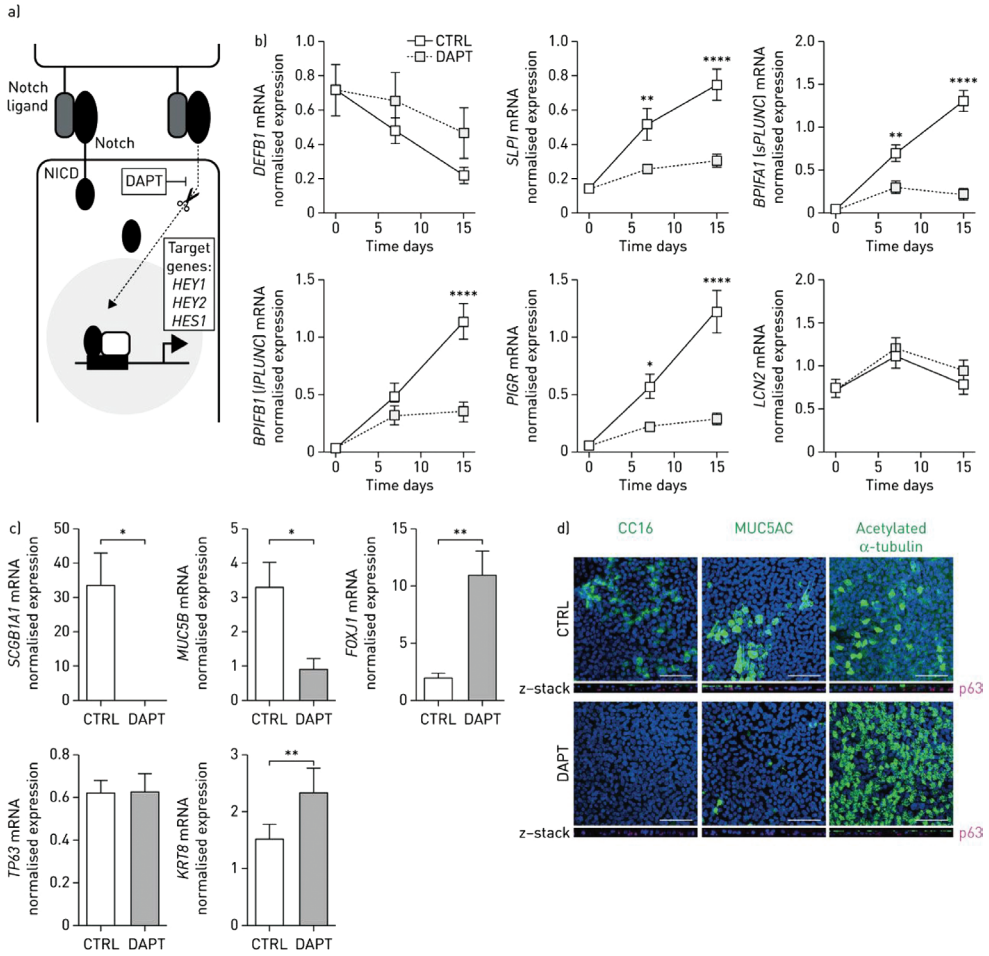

Figure 8. DAPT inhibits host defence protein expression in air-liquid interface cultures of primary bronchial epithelial cells (ALI-PBEC) (A) Mechanism of action of the Notch inhibitor DAPT, a γ-secretase inhibitor that prevents proteolytic cleavage of the Notch intracellular domain (NCID). (B) PBEC were seeded on coated transwells and cultured in submerged conditions until confluent. At day 0, cells were differentiated for an additional 15 days in the presence of 5 µM of the Notch signal transduction inhibitor DAPT in the basal medium or solvent as control. At day 0, 7, and 15 cells were lysed, RNA was isolated and cDNA synthesized. Subsequent qPCR analysis was performed to assess expression of respiratory defence proteins and epithelial cell-specific genes such as: *DEFB1* (human beta defensin-1), *SLPI* (secretory leukocyte protease inhibitor), *BPIFA1* (short palate, lung and nasal epithelium clone protein), *BPIFB1* (long palate, lung and nasal epithelium clone protein), polymeric immunoglobulin receptor (*PIGR*) and *LCN2* (lipocalin 2). Data are shown as target gene expression normalized for the geometric mean expression of the reference genes ATP synthase, H^+ transporting, mitochondrial F1 complex, beta polypeptide (*ATP5B*), β2-microglobulin *(B2M)* and Ribosomal Protein L13a *(RPL13A)*; n=7 different donors. Statistical significance was tested using a two-way ANOVA and Bonferroni post-hoc test . ** p<0.01, *** p<0.001, **** p<0.0001 between CTRL and DAPT. (C) qPCR analysis was performed to assess mRNA expression of the epithelial cell markers *SCGB1A1* (club cells), *MUC5B* (goblet cells), *FOXJ1* (ciliated cells), *TP63* (basal cells), and cytokeratin-8 (*KRT8)* (intermediate cells) after 15 Days of differentiation with DAPT or solvent control, n=6 different donors. Statistical significance was tested using a paired t-test * p<0.05, ** p<0.01 between CTRL and DAPT (D) ALI-PBEC were differentiated for 15 Days with DAPT or solvent control. Subsequently the cells were fixed in 1% paraformaldehyde and stained using primary antibodies against CC16 (club cells), MUC5AC (goblet cells), and acetylated α-tubulin (ciliated cells), which are presented as a green staining; DAPI was used to stain the nuclei (blue staining). Scale bars equals 50 µm. Images shown are representative for results obtained with cells from 3 different donors.

Discussion

Here we demonstrate that cigarette smoke negatively affects expression of the respiratory HDPs: pIgR, SLPI, lPLUNC and sPLUNC. Their expression was significantly reduced in epithelial cells daily exposed to CS during differentiation as a result of an impaired end-stage differentiation of specialized luminal cells. As a consequence, remodelling of the airway epithelium by cigarette smoke has a significant impact on respiratory host defence, underscored by the severely diminished IgA transport across the CS-exposed epithelium and impaired antibacterial defences against *M. catarrhalis* and *K. pneumoniae*. Our data suggest that increasing expression of specific respiratory HDPs (or preventing their decrease) could be of therapeutic interest to improve host defences in the lungs of COPD patients. Furthermore, this (selective) loss may also contribute to changes in lung microbiome composition, which is increasingly recognized as an important contributor to chronic inflammatory lung diseases(15, 16).

The cellular composition of the ALI-PBEC cultures changed drastically upon chronic CS-exposure. While presence of intermediate CK-8^{+} cells (or also called early, intermediate or committed progenitor epithelial cells(13, 17)) was not affected by chronic CS exposure, the specialized luminal cell markers were reduced in chronic CS-exposed cultures. These results indicate that specifically end-stage differentiation seems impaired by CS exposure. The effects of chronic CS exposure were also observed when the cells were first allowed to differentiate for one week in absence of CS. Furthermore, upon cessation of CS exposure, gene expression of most luminal cell markers showed a strong recovery. In contrast, *SCGB1A1* mRNA expression remained absent after almost 1 week of recovery, suggesting an exceptional detrimental effect of CS on club cell differentiation or the regulation of *SCGB1A1* gene expression. This is underscored by a recent study showing that expression of the club cell-protein CC16 (*SCGB1A1*) is reduced in COPD patients and in CS-exposed mice. Loss of CC16 was correlated with increased severity of the disease and CS-induced pulmonary inflammation was lower in mice overexpressing CC16(18).

Cytotoxicity is unlikely to have a major contribution to the CS-induced effects on the ALI-PBEC cultures since we detected no difference in trans-epithelial electrical resistance (TEER) as a measure of barrier function during the course of differentiation between CS and air-exposed controls. We previously observed transient decreases in TEER after acute single CS exposures, normalizing after 24 h

(11). In our chronic CS set-up we measured TEER 18-20h after the previous CS exposure, which may explain why we did not observe significant differences in TEER between air and CS-exposed cultures. Previous studies have shown decreases in TEER by CS, but often use cigarette smoke extract (CSE) and not whole CS (19, 20). CSE has a different composition and concentration than whole CS used in our study. In addition, in some studies CSE was added to the basal medium, resulting in stimulation from the basal side of the transwells (19). This may also contribute to differences found in effects on TEER. Lastly, LDH release was not increased in our CS-exposed cultures, while cellular size appeared larger in the CS-exposed cultures for some donors. Finally, the CS-exposed cultures produced higher amounts of IL-8 and displayed increased mRNA expression of the inducible antimicrobial peptides RNase7 and LL-37 (but not hBD-2). These results are described in the online data supplement and in sFigure 4

Unexpectedly, we did not observe goblet cell hyperplasia in cultures that were exposed to CS, shown previously in smokers (21), guinea pigs (22), rats (23) and in cell lines (23, 24) or PBEC exposed to cigarette smoke extract (CSE)(19). However, Brekman et al. (25) also observed a decline in goblet cells markers in PBEC continuous exposed to CSE. Data are therefore conflicting and dependent on the type of cell culture used. Obviously, in our primary differentiated cultures, whole CS exposure alone is insufficient to induce goblet cell hyperplasia within 19 days. We strongly consider that the findings in patients are more likely explained by a secondary effect of the CS-induced inflammation (which is obviously incompletely represented in our *in vitro* model). For example, neutrophil recruitment as a consequence of the CS-induced inflammation and subsequent release of proteases and other molecules, may help to explain goblet cell formation in patients. In addition, also the presence of other cell types besides neutrophils such as macrophages seem important for goblet cell hyperplasia. This has also been suggested in literature (26-29). Furthermore, several studies suggest that various other factors might have an important role in promoting goblet cell hyperplasia that are involved in COPD pathogenesis, including bacterial and viral infections (30, 31). Whereas previous studies have shown that CS reduces the presence of ciliated cells (19, 25, 32), so far CS-induced airway epithelial remodelling was not yet linked to changes in levels of the highly expressed respiratory HDPs, despite the fact that changes in expression in these proteins have been reported in smokers or patients with COPD. Aarbiou *et al.* showed that expression of SLPI was significantly reduced in damaged bronchial epithelium of COPD patients compared to non-COPD

individuals(33), and Gohy *et al.* showed reduced levels of pIgR in the lungs of patients with COPD, however not in smokers with a normal lung function compared to healthy controls(10). Finally, reduced levels of PLUNC were detected in bronchial brushes performed in current smokers compared to never smokers(34).

We observed impaired anti-bacterial activity of the CS-exposed airway epithelial cultures against *Moraxella catarrhalis* and *Klebsiella pneumoniae*. We also evaluated direct anti-bacterial activity of the chronic CS-exposed cultures using a grid assay with live/dead staining of bacteria (35, 36) and via conventional plating methods against *Pseudomonas aeruginosa* and non-typeable *Haemophilus influenzae,,* but could not detect any differences (data not shown). These data suggest that the observed CS-induced impairment of antimicrobial activity may be pathogen-specific, since it is not observed with all pathogens studied. In addition to impaired antibacterial host defence activities, the loss of HDP expression by the airway epithelium can have further negative effects for the host. For example, loss of SLPI expression (highly expressed in normal lung tissue) can promote inflammation in the lungs of patients with COPD. SLPI acts as an inhibitor of detrimental proteases such as neutrophil elastase, acts as an inhibitor of NFκB activation and it modulates macrophage functions(37-39). sPLUNC is involved in regulation of the epithelial sodium channel (ENaC), thereby regulating airway surface liquid (ASL), and reduced levels could result in lowered ASL volume and impaired mucociliary clearance(40).

Since Notch signalling was previously reported to be impaired in COPD(14), we first analysed the effect of chronic CS exposure on components of the Notch signalling pathway and Notch target genes, and found that CS decreased the expression of selected Notch target genes. When we next used the Notch signalling inhibitor DAPT to inhibit airway epithelial cell differentiation, we found similar effects compared to CS exposure on expression of the selected set of respiratory HDPs. To our knowledge, this is the first study linking Notch signalling to expression of a range of host defence proteins that are increased upon differentiation. Whereas chronic CS exposure resulted in a reduction of all luminal-cell markers, DAPT-exposed cultures showed higher levels of ciliated cells when compared to control-treated cells, in line with previous studies(41, 42). These results suggest that expression of the luminal cell-restricted HDPs is confined to mature secretory epithelial cells, rather that the ciliated epithelium. Further studies using single cell RNA sequencing, may reveal whether the expression is restricted to a certain secretory cell phenotype. The partial similarity between the DAPT-incubated

cultures and the CS-exposed cultures suggests involvement of altered Notch signalling in the CS-induced effects. However, alterations in other signalling pathways might also be involved in the observed effects of CS, such as those involving EGFR(32), TGF-β(25) Wnt(43) and BMP(44). Most likely a more systems/-omics approach has the potential to elucidate in detail all effects of chronic CS exposure on Notch and other signalling pathways(45).

In summary, these findings shed new light on the role of dysregulated host defence in smokers and patients with COPD by highlighting the importance of airway epithelial cell differentiation in the expression of respiratory HDPs. Further investigations into how suppression of epithelial cell differentiation by cigarette smoke contributes to microbial colonization and infections of the airways are warranted in order to develop new therapeutics that restore airway epithelial host defence in patients with COPD.

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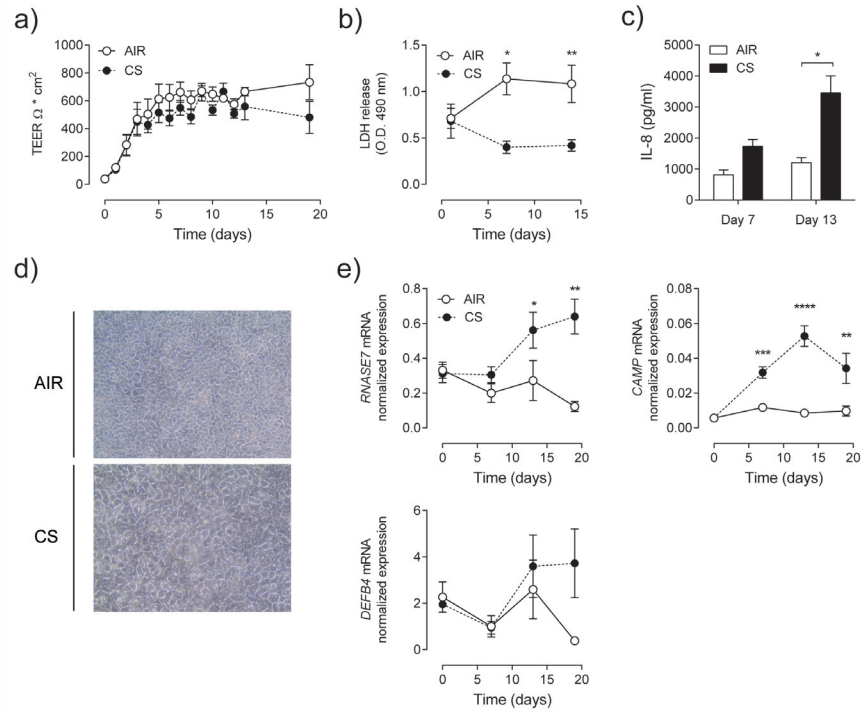
Supplemental results

Reduced respiratory host defence protein levels by chronic CS exposure are not a consequence of toxicity

To exclude that possible toxic effects of the chronic CS exposure affected the observations we made, we performed a selection of additional experiments. We assessed trans-epithelial electrical resistance (TEER) of ALI-PBEC exposed to CS or air as a control: results showed that chronic CS-exposed ALI-PBEC displayed a slight but non-significant decrease of TEER in CS-exposed cultures in the first week of exposure, and a similar TEER as the air-exposed controls in the second week of exposure up until day 19 (sFigure 4A). LDH levels in chronic CS-exposed cell cultures were not increased, but rather reduced compared to air-exposed cultures (sFigure 4B). Indirect evidence for absence of marked cytotoxicity was the observation that chronic CS exposure significantly increased secretion of the neutrophil-attracting chemokine IL-8 at 13 days of differentiation in CS-exposed cells compared to air-exposed controls (sFigure 4C). The cell size in chronic CS-exposed cell cultures seemed bigger in some donors, but not all, compared to air-exposed cultures, but no other morphological changes could be detected by microscopic inspection (an example illustrated in sFigure 4D). Together these data show that chronic CS-exposure-mediated loss of specific HDP expression by ALI-PBEC is unlikely to be a result of toxicity. This conclusion is further supported by measurements on the expression of a selection of inducible HDPs**.** We previously reported induction of *RNASE7* mRNA and protein in ALI-PBEC upon acute exposure to one cigarette (11), in line with these findings, chronic CS exposure also caused a progressive increase in *RNASE7* compared to air-exposed controls (sFigure 4E). In addition also increased *CAMP* gene expression (LL-37-coding gene) was detected in chronic CS-exposed cultures (sFigure 4E). In contrast, we did not observe a significant difference in the expression of *DEFB4* (human β-defensin 2) (sFigure 4E).



sFigure 4. Chronic cigarette smoke exposure of airway epithelial cell cultures does not lead to cell toxicity. Air-liquid interface cultures of primary bronchial epithelial cells (ALI-PBEC) were daily exposed to whole cigarette smoke (CS) or air as a control (AIR) during differentiation for 13-19 consecutive days. (A) Each day trans-epithelial electrical resistance (TEER) measurements were performed ~18 h after the previous CS exposure. Data are expressed as Ω*cm^2. Open circles: air-exposed controls, black circles: CS-exposed cell cultures; n=8 different donors. Significance was determined using a two-way ANOVA and Bonferroni post-hoc test. (B) At several time-points during differentiation apical washes were collected and assessed for LDH content. Open circles: air-exposed controls, black circles: CS-exposed cell cultures; n=6 different donors. Statistical differences were evaluated using a two-way ANOVA and Bonferroni post-hoc test. * p<0.05, ** p<0.01 between AIR and CS. (C) At day 7 and Day 13 (~18 h after the last CS exposure), IL-8 protein levels were assessed by ELISA in the basal medium of the ALI-PBEC cultures. Open bars are air-exposed controls, grey bars are chronic CS-exposed cultures; n=8 different donors. Statistical differences were tested using a paired t-test. * p<0.05. (D) Illustrating phase contrast light microscopy images showing the increasing effects of 13 days of CS exposure (CS) or air as a control (AIR) on cell morphology in some donors. (E) At several time-points during differentiation, cells were lysed and RNA was isolated followed by cDNA synthesis, to assess gene expression of *RNASE7, CAMP* (LL-37) and *DEFB4* (human beta defensin-2). Data are shown as target gene expression normalized for the geometric mean expression of the reference genes ATP synthase, H^+ transporting, mitochondrial F1 complex, beta polypeptide (*ATP5B*), β2-microglobulin *(B2M)* and Ribosomal Protein L13a *(RPL13A)*, n=8 different donors. Statistical differences were evaluated using a two-way ANOVA and Bonferroni post-hoc test. * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001 between AIR and CS.

Supplemental Tables

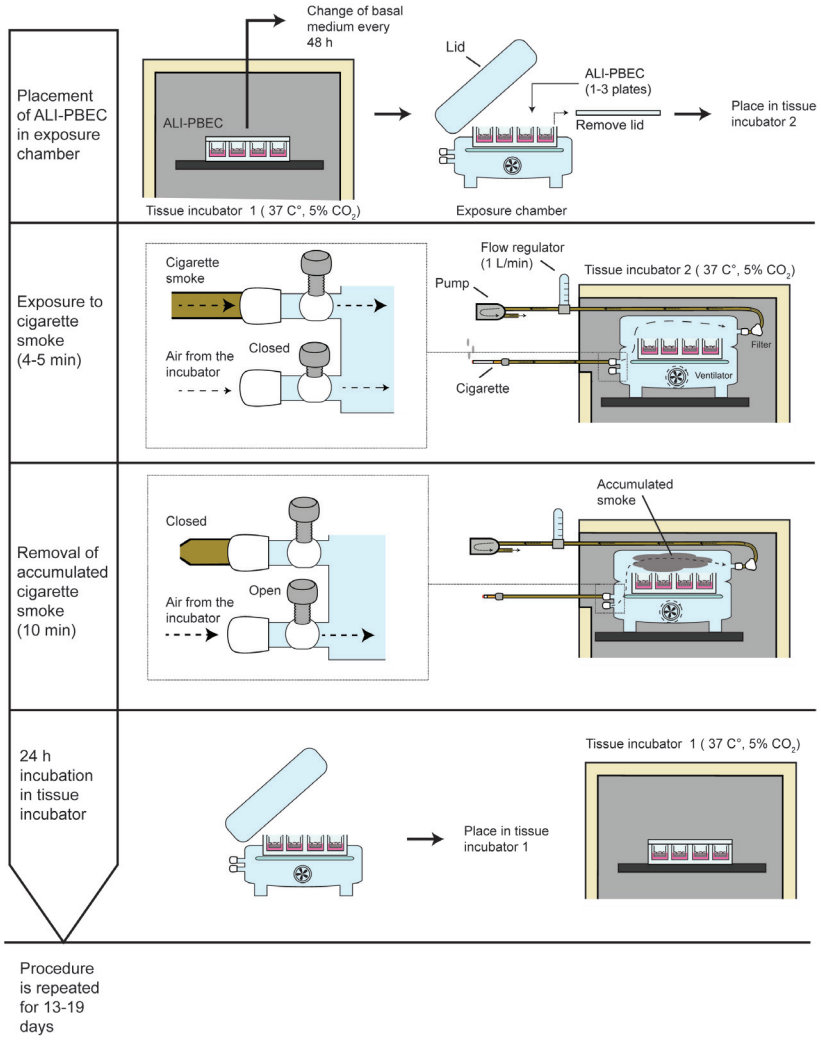
sTable 1. Primer sequences.

Gene	forward sequence (5' to 3')	reverse sequence (5' to 3')
ATP5B	TCACCCAGGCTGGTTCAGA	AGTGGCCAGGGTAGGCTGAT
RPL13A	AAGGTGGTGGTCGTACGCTGTG	CGGGAAGGGTTGGTGTTCATCC
B2M	GACCACTTACGTTCATTGACTCC	CAGGGTTTCATCATACAGCCAT
DEFB1	ATGAGAACTTCCTACCTTCTGCT	TCTGTAACAGGTGCCTTGAATTT
SLPI	CCA GGG AAG AAG AGA TGT TG	CCT CCA TAT GGC AGG AAT C
BPIFA1	CTTGGCCTTGTGCAGAGC	CAACAGACTTGCACCGACC
BPIFB1	CAGTGCCATGCGGGAAAAG	GCTGGAGGATGTTAGCTGTGA
PIGR	CTCTCTGGAGGACCACCGT	CAGCCGTGACATTCCCTG
LCN2	CCTCAGACCTGATCCCAGC	CAGGACGGAGGTGACATTGTA
KRT5	AGGAGTTGGACCAGTCAACAT	TGGAGTAGTAGCTTCCACTGC
TP63	CCACCTGGACGTATTCCACTG	TCGAATCAAATGACTAGGAGGGG
KRT8	TCCTCAGGCAGCTATATGAAGAG	GGTTGGCAATATCCTCGTACTGT
FOXJ1	GGAGGGGACGTAAATCCCTA	TTGGTCCCAGTAGTTCCAGC
SCGB1A1	ACATGAGGGAGGCAGGGGCTC	ACTCAAAGCATGGCAGCGGCA
MUC5B	GGGCTTTGACAAGAGAGT	AGGATGGTCGTGTTGATGCG
MUC5AC	CCTTCGACGGACAGAGCTAC	TCTCGGTGACAACACGAAAG
JAG2	TGGGACTGGGACAACGATAC	AGTGGCGCTGTAGTAGTTCTC
DLL1	GACGAACACTACTACGGAGAGG	AGCCAGGGTTGCACACTTT
NOTCH1	GAGGCGTGGCAGACTATGC	CTTGTACTCCGTCAGCGTGA
NOTCH2	CCTTCCACTGTGAGTGTCTGA	AGGTAGCATCATTCTGGCAGG
NOTCH3	CGTGGCTTCTTTCTACTGTGC	CGTTCACCGGATTTGTGTCAC
NOTCH4	GATGGGCTGGACACCTACAC	CACACGCAGTGAAAGCTACCA
HES1	CCTGTCATCCCCGTCTACAC	CACATGGAGTCCGCCGTAA
HEY1	ATCTGCTAAGCTAGAAAAAGCCG	GTGCGCGTCAAAGTAACCT
JAG1	GCCGAGGTCCTATACGTTGC	CCGAGTGAGAAGCCTTTTCAA
MAML1	CCCCAGTGAGTCATTTCCTCT	GAGGTTGCTTTGCGATATGGA
MAML3	CTTAGGACCTCCCTCTAGTCCA	GTTTTGGTTGTTAAAGGCTTGGG
RNASE7	CCAAGGGCATGACCTCATCAC	ACCGTTTTGTGTGCTTGTTAATG
DEFB4	ATCAGCCATGAGGGTCTTG	GCAGCATTTTGTTCCAGG
CAMP	TCATTGCCCAGGTCCTCAG	TCCCCATACACCGCTTCAC

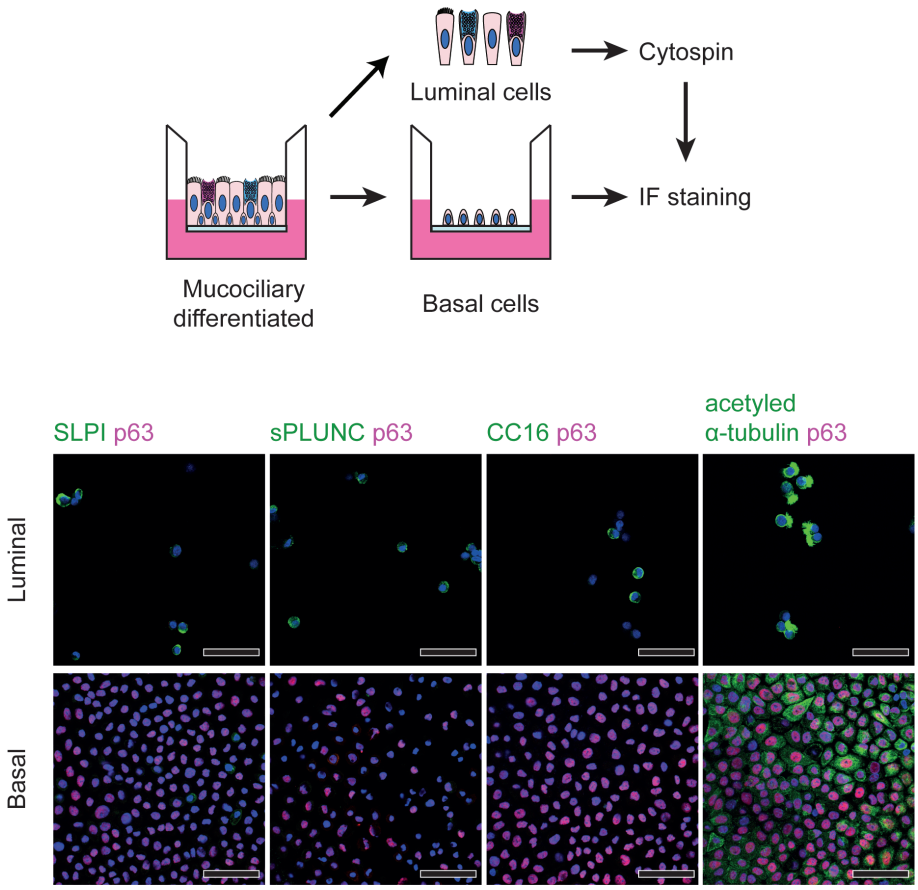
sTable 2. Antibodies used for confocal imaging

Antibody	Supplier	Catalog #	Species	Antibody dilution
CK-8	Novus Biologicals	NBP2-34266	mouse	1/100
pIgR	R&D Systems	MAB27171	mouse	1/100
p63	Abcam	ab124762	rabbit	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
Acetylated α-Tubulin	Sigma Aldrich	T6793	mouse	1/100

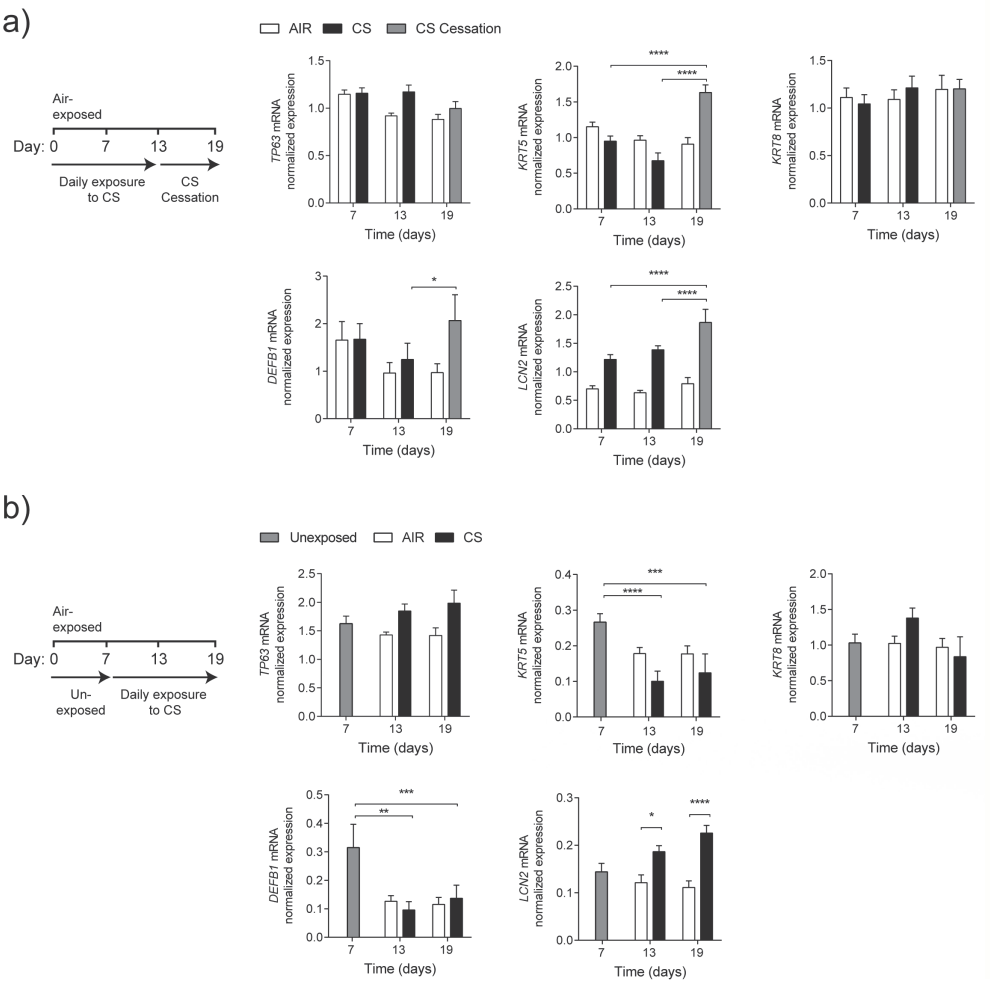
Supplemental Figures



sFigure 1. Details of the cigarette smoke exposure design and procedure. Approximately 4 h before the cigarette smoke exposure, the apical surface of the cell cultures were washed with PBS and every other day the basal medium was replaced. Next, the cells were placed in the exposure chamber and the lid was removed. The closed exposure chamber was then infused with cigarette smoke from 1 cigarette for 4-5 min, or normal air in the control chamber. Hereafter, the tubing from the cigarette is clamped and vents on the exposure chamber connecting to the space in the incubator are opened and the air is refreshed with air from the incubator for an additional 10 min. The smoke-containing air is removed via separate tubing outside the incubator into a fume hood. After the exposure and refreshing, the chamber is opened, the lid placed back on the cells and the cells are placed back in a separate incubator for 20 h when the procedure is repeated.



sFigure 2. Expression of respiratory host defence proteins in the luminal cell fraction of air-liquid interface-differentiated primary bronchial epithelial cells (PBEC). PBEC were seeded on coated transwells and cultured in submerged conditions until confluent. At day 0, cultures were air-exposed and cultured at the air-liquid interface (ALI). After 3 weeks of differentiation, luminal and basal cell fractions were separated. Cells were fixed in 1% paraformaldehyde and cytospins were prepared of the luminal cell enriched fraction. Luminal cell cytospins and the basal cell enriched fraction located on the transwell inserts were subsequently stained using immunofluorescence with primary antibodies against p63 (basal cell marker, red) in combination with primary antibodies against SLPI, sPLUNC, CC16 and acetylated α-tubulin (all green) and DAPI for nuclear staining (blue). Scale bars equal 50 µm. Images shown are representative for results obtained with cells from 3 different donors.



sFigure 3. Persistence of cigarette smoke-induced changes in airway epithelial HDP expression and cellular composition. (A) Primary bronchial epithelial cells (PBEC) were cultured at the air-liquid interface (ALI) and exposed during differentiation for 13 consecutive days to whole CS after which cultures were continued for another 6 days without CS exposure. Cells were lysed at several points during this course of time and RNA was isolated followed by cDNA synthesis, to assess gene expression of the cell specific markers: *TP63*, *KRT5* (basal cells) and *KRT8* (intermediate cells), the HDPs: *DEFB1* (human beta-defensin 1) and *LCN2* (lipocalin 2). Open bars: air-exposed controls (AIR), black bars: CS-exposed cell cultures (CS), grey bars: CS-exposed cultures that were cultured for an additional week without CS exposure (CS cessation). Data are shown as target gene expression normalized for the geometric mean expression of the reference genes ATP synthase, H^+ transporting, mitochondrial F1 complex, beta polypeptide (*ATP5B*), β2-microglobulin *(B2M)* and Ribosomal Protein L13a *(RPL13A)*; n=8 different donors. Statistical differences were evaluated using a two-way ANOVA and Bonferroni post-hoc test. * $p<0.05$, **** $p<0.0001$. (B) ALI-PBEC were air exposed at day 0 and cultured for 7 days under standard conditions. At day 7 cultures were exposed to CS for 12 consecutive days after which

the cells were lysed and similar analyzed as in (A). Grey bars: T=0 (day 7), open bars: air-exposed controls (AIR), black bars: CS-exposed cell cultures (CS). Data are shown as target gene expression normalized for the geometric mean expression of the reference genes ***ATP5B****, B2M* and *RPL13A*. n=6 different donors. Statistical differences were evaluated using a two-way ANOVA and Bonferroni post-hoc test. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

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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.

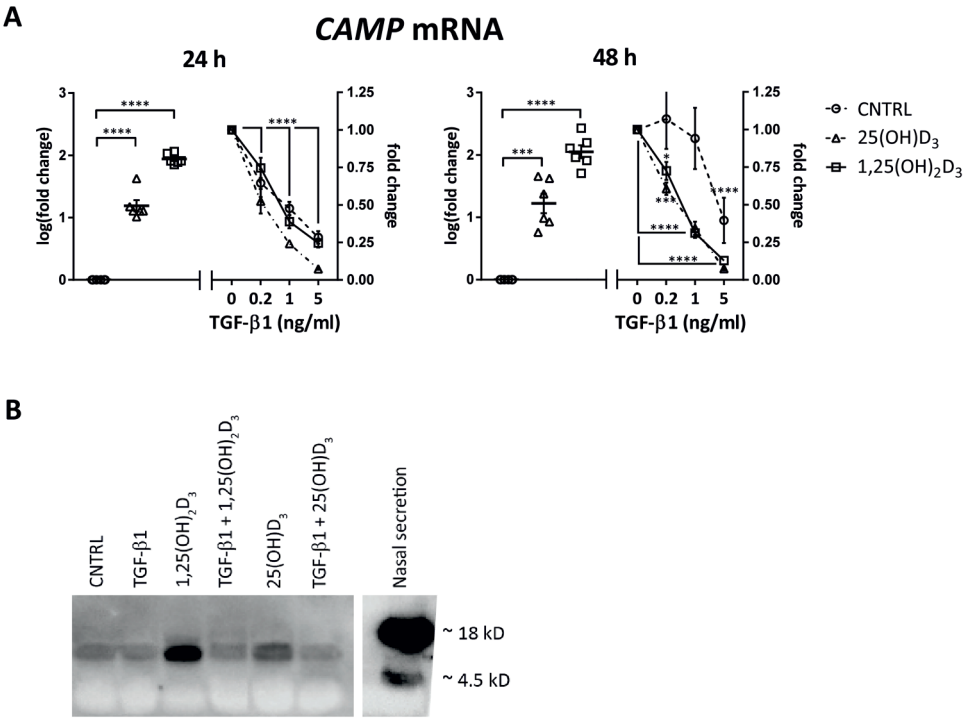

Figure 1. TGF-β1 impairs baseline and vitamin D-induced expression and release of hCAP18/LL-37 in primary bronchial epithelial cells (PBEC). PBEC were differentiated at the air-liquid interface (ALI) followed by 24-48 h stimulation with TGF-β1 with or without inactive 25(OH)D3, active 1,25(OH)2D3 or 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.

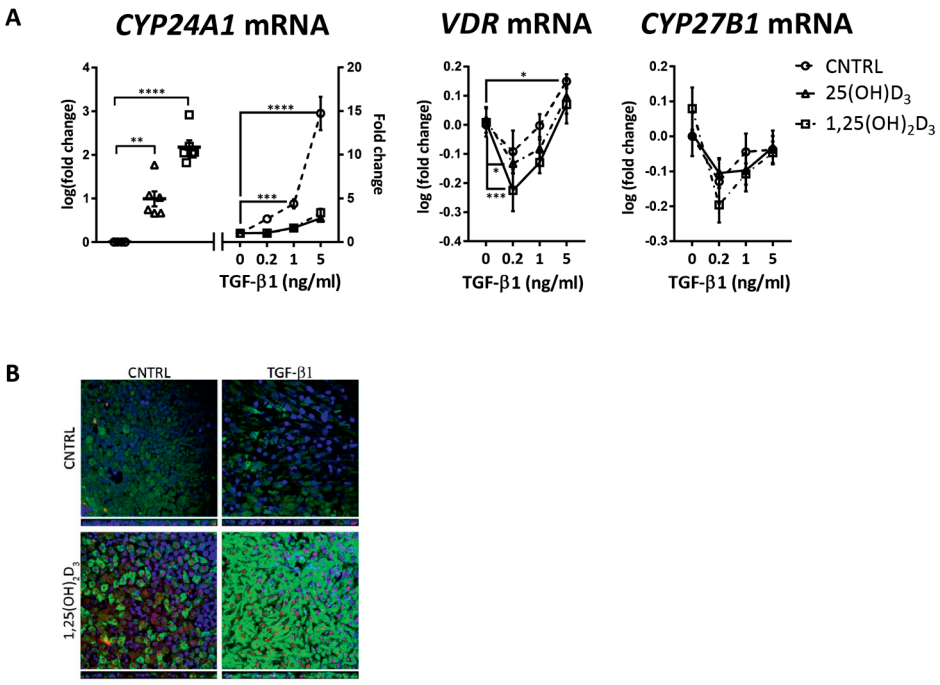

Figure 2. TGF-β1 affects the vitamin D-metabolic pathway in primary bronchial epithelial cells (PBEC). PBEC were differentiated at the air-liquid interface (ALI) followed by 24 h stimulation with TGF-β1 in presence or absence of $25(OH)D_3$, $1,25(OH)_2D_3$ or medium control (CNTRL) to assess mRNA expression 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.

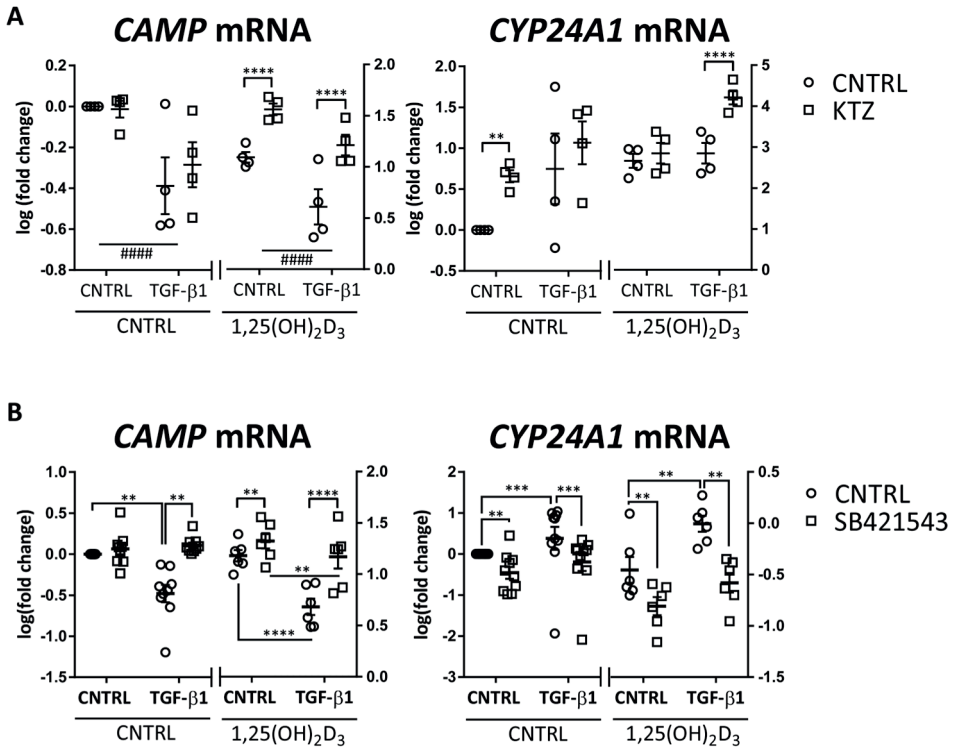

Figure 3. Effects of inhibition of CYP24A1 activity and canonical TGF-β-Smad signaling on expression of hCAP18/LL-37 (CAMP) and CYP24A1 in primary bronchial epithelial cells (PBEC). Semi-confluent layers of submerged (S)-PBEC were cultured in starvation medium overnight and subsequently exposed to the 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.

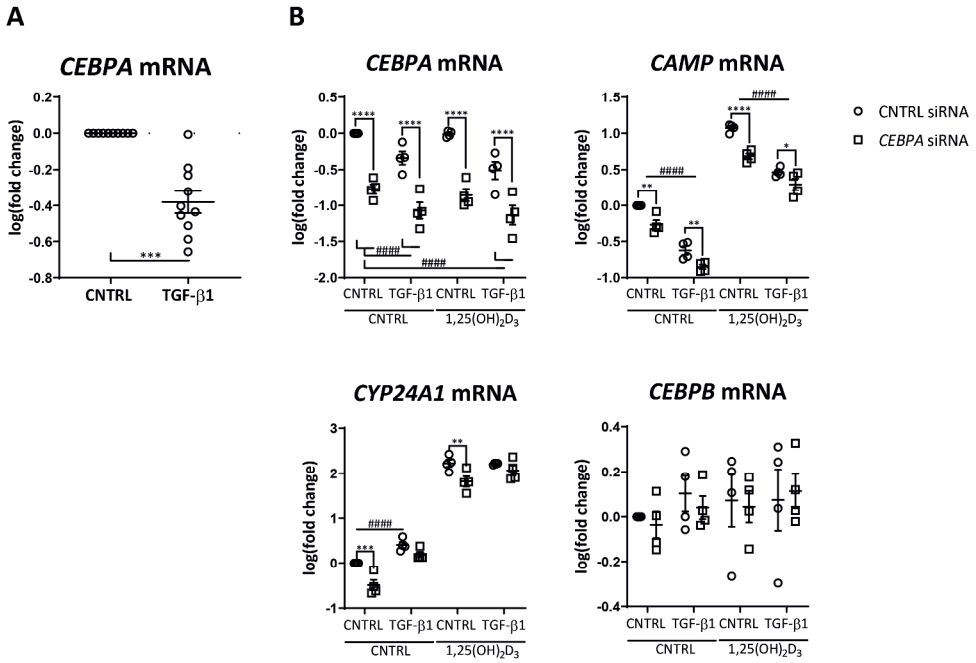

Figure 4. TGF-β1 decreases expression of the *CAMP*-transcription factor CCAAT enhancer binding protein (C/EBP)α in primary bronchial epithelial cells(PBEC). Semi-confluent cultures of submerged (S)-PBEC were cultured in starvation medium overnight and subsequently exposed to TGF-β1 or medium control (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).

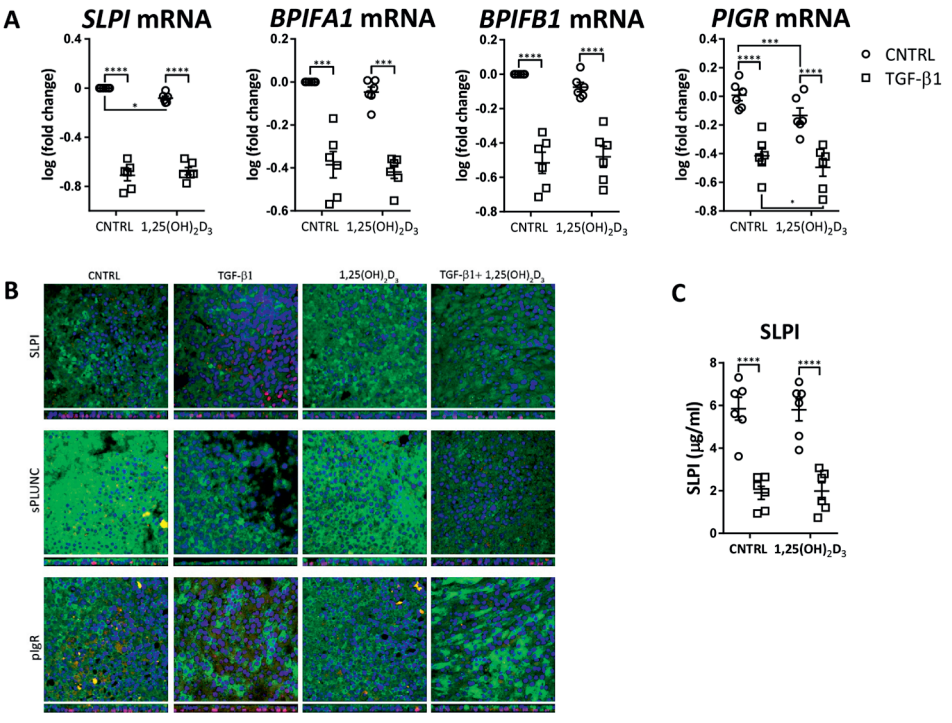

Figure 5. Effect of TGF-β1 on expression of constitutively expressed luminal cell-restricted host defence proteins (HDPs) in primary bronchial epithelial cells (PBEC). PBEC were differentiated at the air-liquid interface (ALI) followed by stimulation for 24 h with TGF-β1 in presence or absence of $1,25(OH)_2D_3$ 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).

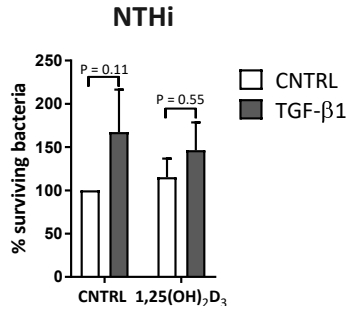

Figure S1. Effects of TGF-β1 on antibacterial activity in differentiated bronchial epithelial cells (PBEC). PBEC were differentiated at the air-liquid interface (ALI) followed by stimulation for 48 h with TGF-β1 with or without $1,25(OH)_2D_3$ or medium control (CNTRL) to assess antibacterial activity 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.

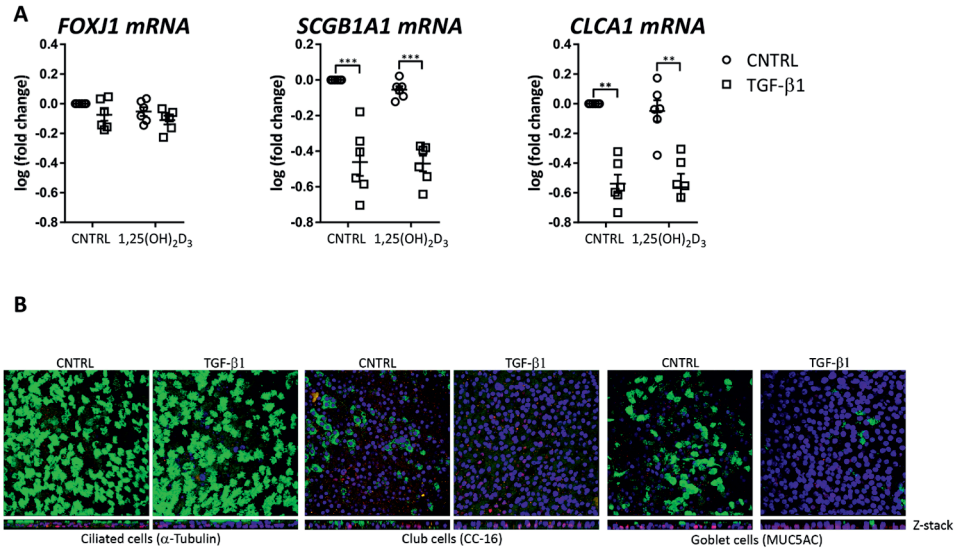

Figure 6. TGF-β1 affects composition of airway epithelium by decreasing the number of secretory epithelial cells in primary bronchial epithelial cells (PBEC). PBEC were differentiated at the air-liquid interface (ALI) followed by stimulation for 24 h with TGF-β1 in presence or absence of $1,25(OH)_2D_3$ 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.

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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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CHAPTER

Pro-inflammatory cytokines impair vitamin D-induced host defense in cultured airway epithelial cells

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Abstract

Vitamin D is a regulator of host defense against infections and induces expression of the antimicrobial peptide hCAP18/LL-37. Vitamin D deficiency is associated with chronic inflammatory lung diseases and respiratory infections. However, it is incompletely understood if and how (chronic) airway inflammation affects vitamin D metabolism and action. We hypothesized that long-term exposure of primary bronchial epithelial cells (PBEC) to pro-inflammatory cytokines alters their vitamin D metabolism, antibacterial activity and expression of hCAP18/LL-37. To investigate this, PBEC were differentiated at the air-liquid interphase for 14 days in presence of the pro-inflammatory cytokines TNF-α and IL-1β (TNF-α/IL-1β), and subsequently exposed to vitamin D (inactive $25(OH)D_3$ and active $1,25(OH)_2D_3$). Expression of hCAP18/LL-37, vitamin D receptor (VDR) and enzymes involved in vitamin D metabolism (CYP24A1 and CYP27B1) was determined using qPCR, Western blot and immunofluorescence staining. Furthermore, vitamin D-mediated antibacterial activity was assessed using non-typeable *Haemophilus influenzae* (NTHi). We found that TNF-α/IL-1β treatment reduced vitamin D-induced expression of hCAP18/LL-37 and killing of NTHi. In addition, CYP24A1 (a vitamin D-degrading enzyme) was increased by TNF-α/IL-1β, whereas CYP27B1 (that converts $25(OH)D_3$ to its active form) and VDR expression remained unaffected. Furthermore, we demonstrated that the TNF-α/IL-1β–mediated induction of CYP24A1 was at least in part mediated by the transcription factor specific protein 1 (Sp1) and the EGFR-MAPK-pathway. These findings indicate that TNF-α/IL-1β decreases vitamin D-mediated antibacterial activity and hCAP18/LL-37 expression via induction of CYP24A1, and suggests that chronic inflammation impairs protective responses induced by vitamin D.

Introduction

Our respiratory tract is continuously exposed to microbes and microbial products. The airway epithelium serves as the front line of host defense in the lung by preventing those microbes to enter the tissue and bloodstream. Airway epithelial host defense is mediated by the physical barrier provided by tight- and adherens junctions, mucociliary clearance and a variety of other mechanisms, including secretion of antimicrobial peptides and proteins (AMPs), reactive oxygen- and nitrogen species, interferons, chemokines and cytokines (1). Impairment of this host defense activity of airway epithelial cells might contribute to chronic inflammatory lung diseases such as asthma, chronic obstructive pulmonary disease (COPD) and cystic fibrosis (CF) (1). Various studies have demonstrated an association between low serum 25(OH)vitamin D_3 ($25(OH)D_3$)-levels and severity and/or prevalence of these chronic inflammatory lung diseases (2-4). These findings suggest a role for vitamin D in chronic lung diseases. Reduced levels of vitamin D could further aggravate host defense and inflammation in these conditions as vitamin D exerts a range of anti-inflammatory activities, including immune modulation, inhibition of oxidative stress, remodeling and enhancement of antimicrobial activity (2-4). In airway epithelial cells, vitamin D promotes host defense in the lung by enhancing the killing of pathogens and by modulation of immune responses (3, 4). This is to some extent mediated via expression of the cathelicidin antimicrobial peptide hCAP18/LL-37, whose precursor hCAP18 is cleaved by proteases into the 4.5 kD LL-37 mature peptide that displays both antimicrobial and immunomodulatory properties (5-7). This relation was confirmed by several studies that have revealed an important role for vitamin D in regulating hCAP18/LL-37 expression in e.g. macrophages and epithelial cells (7-9).

The main circulating form of vitamin D is $25(OH)D_3$, which requires hydroxylation by α1-hydroxylase (CYP27B1) in the kidney or locally in tissues and immune cells for conversion into the active form of vitamin D_3 ($1,25(OH)_2D_3$). This active form binds to the nuclear vitamin D receptor (VDR) which subsequently heterodimerizes with the retinoic acid receptor to interact with vitamin D response elements (VDREs). VDREs are present in the promoter region of more than 900 vitamin D-regulated genes (10), including *CAMP*, which encodes for hCAP18/LL-37. Vitamin D also promotes its own catabolism by inducing CYP24A1, which converts both $25(OH)D_3$ and $1,25(OH)_2D_3$ into inactive $24,25(OH)_2D_3$ and $1,24,25(OH)_2D_3$ (11). The inactivation by CYP24A1 plays a critical role in the availability of active vitamin D,

and overexpression of this enzyme might therefore contribute to impaired vitamin D availability.

It remains however unclear whether modulation of the activity of airway epithelial cells due to airway inflammation contributes to an impairment of vitamin D-induced defense mechanisms in chronic inflammatory lung diseases. It has been shown that pro-inflammatory mediators can alter the expression of CYP27B1, CYP24A1 and VDR in extra-renal cells (8, 9, 12-15). Increased local levels of pro-inflammatory cytokines and other mediators, as well as microbes are indeed present in the airways of patients suffering from chronic inflammatory lung diseases (16-20). We hypothesized that chronic exposure of airway epithelial cells to pro-inflammatory cytokines alters both the metabolism and antimicrobial responses of these cells to vitamin D. To investigate this, we used cultures of primary bronchial epithelial cells (PBEC), which were differentiated in presence and absence of the pro-inflammatory cytokines TNF-α and IL-1β and subsequently treated with vitamin D. Next we assessed expression and release of hCAP18/LL-37 and bactericidal activity against non-typeable *Haemophilus influenzae* [NTHi, a Gram-negative bacterium, which is found in the lungs of COPD, CF and (refractory) asthma patients (21, 22)]. Furthermore, effects of TNF-α and IL-1β and also of other pro-inflammatory mediators such as IL-17A and NTHi on the expression of vitamin D-metabolic enzymes CYP24A1, CYP27B1, and of VDR were also investigated. Finally, we assessed the signaling mechanisms underlying the modified vitamin D metabolism in PBEC cultures.

Materials and Methods

Cell culture

PBEC were obtained from tumor-free bronchial lung tissue from anonymous donors, collected during lung resection surgery for lung cancer. Cells were cultured at the air-liquid interface (ALI) as described in the online data supplement.

Experimental design

To assess the effects of the combination of TNF-α and IL-1β (TNF-α/IL-1β), or IL-17A on PBEC, cells were cultured for 14 days in presence or absence of 2.5 ng/ml TNF-α/IL-1β (Peprotech, Rocky Hill, NJ) or 5 ng/ml IL-17A (R&D Systems, Abingdon, UK)

at the ALI . At day 14, cells in presence or absence of 2.5 ng/ml TNF-α/IL-1β or 5 ng/ml IL-17A were exposed to 10^{-7} M $25(OH)D_3$ (Merck), 10^{-9} M $1,25(OH)_2D_3$ (Merck) for 24 h to assess gene expression, or for 48 h to assess antibacterial activity, hCAP18/LL-37 release, protein expression by Western blot or immunofluorescence. In experiments using the specificity protein 1 (Sp1) inhibitor mithramycin A (R&D Systems) and the CYP24A1 inhibitor ketoconazole (Sigma Aldrich), ALI-PBEC were cultured in presence or absence of TNF-α/IL-1β for 14 days and subsequently stimulated for 24 h with 3 x 10^{-7} M mithramycin A or 10^{-7}, 10^{-6} and 10^{-5} M ketoconazole. To investigate effects of short-term exposures to NTHi, differentiated ALI-PBEC were exposed to UV-inactivated NTHi for 12 h.

Submerged undifferentiated cultures of PBEC were used to elucidate the mechanism of action of TNF-α/IL-1β-induced expression of CYP24A1. Cells were cultured in supplemented-BEGM:DMEM until a confluence of 70% was reached and cultured overnight in starvation medium (BEGM:DMEM, w/o EGF, BPE and BSA), pre-incubated for 1 hour with 2.5 x 10^{-5} M GM6001 (matrix metalloprotease [MMP] inhibitor; Millipore B.V., Amsterdam, The Netherlands), 1 x 10^{-6} M AG1478 (epidermal growth factor receptor [EGFR] tyrosine kinase inhibitor; Millipore B.V.), 2.5 x 10^{-5} M U0126 (MEK inhibitor; Promega, Leiden, The Netherlands) and stimulated with 10 ng/ml TNF-α/IL-1β for 15 min for collection of protein lysates, and 24 h for gene expression analyses. For the assessment of protein lysates by Western blot, 25 μg/ml synthetic LL-37 was included as a positive control (23).

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

Methods for total RNA isolation, reverse transcription and qPCR reactions (primers shown in table I) are described in the online data supplement.

Preparation of UV-inactivated NTHi

NTHi strain D1 was cultured and killed by UV-inactivation as described in the online data supplement.

Bacterial killing assay

Killing of NTHi by ALI-PBEC was assessed as described by Pezzulo et al. (24) with a few modifications as described in the online data supplement.

Western blot

Methods for Western blot analysis are described in the online data supplement.

Immunofluorescence staining of LL-37 and CYP24A1

Cells were fixed on Transwell inserts in 1% paraformaldehyde (Merck) in PBS for 10 min on ice and washed with ice-cold PBS. Next, cells were stained as described in the online data supplement.

Statistical analysis

Statistical analysis was conducted as described in the online data supplement. Data are shown as medians ± min/max values. Differences at p values < 0.05 were considered statistically significant.

Results

TNF-α/IL-1β decreases vitamin D-mediated expression and release of hCAP18/LL-37

To determine if chronic exposure to pro-inflammatory cytokines affects cellular responses to vitamin D, we exposed PBEC to the pro-inflammatory cytokines TNF-α/IL-1β during differentiation, followed by 25(OH)D_3-treatment or control (medium) in presence or absence of TNF-α/IL-1β (Figure 1A). 25(OH)D_3 increased expression of *CAMP* (hCAP18/LL-37) mRNA in well-differentiated PBEC after 24 h of incubation by 15.1-fold. Moreover, in presence of TNF-α/IL-1β, expression of *CAMP* was 5.2-fold increased by 25(OH)D_3, which was significantly less compared to control treated cells ($p < 0.0001$, Figure 1B). We further confirmed these findings at the protein level after 48 h 25(OH)D_3 exposure by immunofluorescence staining, which demonstrated that 25(OH)D_3-induced hCAP18/LL-37 was decreased in TNF-α/IL-1β-treated cells (Figure 1C), and by Western blot analysis showing that both 25(OH)D_3 and 1,25(OH)$_2D_3$-induced hCAP18/LL-37 release in basal medium was virtually absent after TNF-α/IL-1β-treatment (Figure 1D).

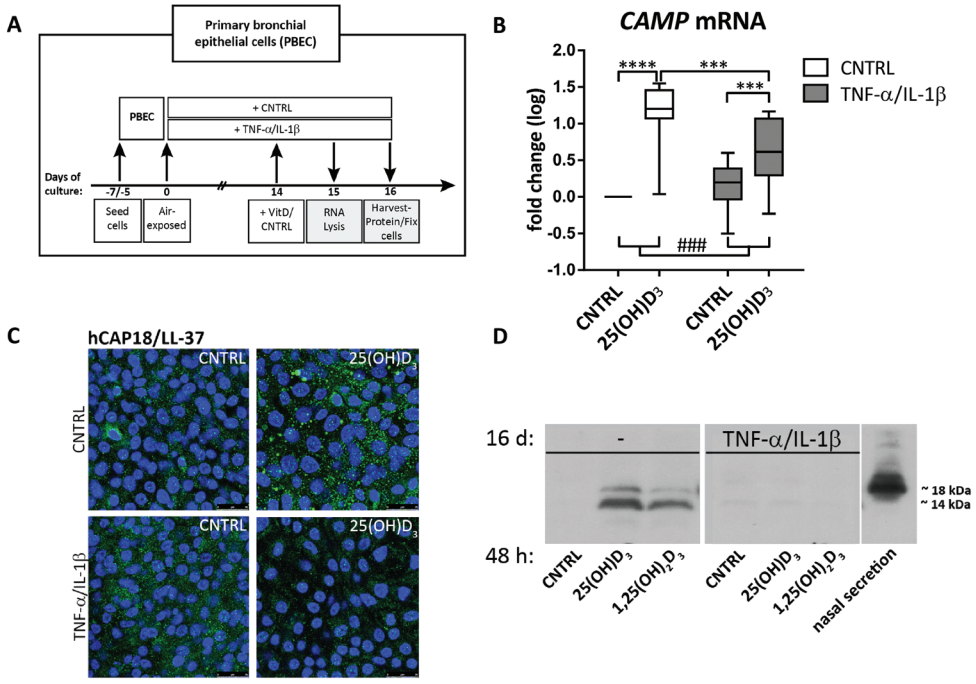

Figure 1. TNF-α and IL-1β (TNF-α/IL-1β) decreases 25(OH)D_3-mediated expression and release of hCAP18/LL-37 in primary bronchial epithelial cells (PBEC) (A) PBEC were seeded on Transwell inserts and cultured for 5-7 days until confluence was reached. Subsequently PBEC were cultured at the air-liquid interface (ALI) and exposed with and without TNF-α/IL-1β for 14 days followed by 24 h stimulation with 25(OH)D_3 or medium control (CNTRL) for assessing *CAMP* (hCAP18/LL-37) expression by qPCR. In addition, cells were stimulated for 48 h to assess release of hCAP18/LL-37 by Western blot analysis (1,25(OH)$_2D_3$ was included as an additional stimulation) and immunofluorescence. (B) Relative mRNA expression of *CAMP* was determined by qPCR. Normalized gene expression was calculated by using the expression of the β2-microglobulin (*B2M*) and ATP synthase, H^+ transporting, mitochondrial F1 complex, beta polypeptide (*ATP5B*) as reference genes. Data are presented as medians ± min/max values**.** Fold change in gene expression of the stimuli compared to control (CNTRL) was first calculated, followed by a log-transformation of the data. Next, data were analyzed using two-way ANOVA and the Bonferroni post-hoc test (n = 11 donors) and differences between CNTRL and 25(OH)D_3 ratios were calculated in CNTRL and TNF-α/IL-1β-treated cells using a paired t-test (C) Immunofluorescence staining of hCAP18/LL-37 in bronchial epithelial cells (of 1 donor, which was confirmed in 3 other donors), DAPI (blue) was used to stain the nuclei and rabbit anti-LL-37 antibody together with Alexa Fluor 488 goat anti-rabbit IgG (green) were used for detection of hCAP18/LL-37. (D) Tris-Tricine gel electrophoresis, followed by Western blot analysis in combination with a LL-37 specific mouse antibody was used to detect hCAP18/LL-37 production in basal medium of the exposed cells. Nasal secretion was used as positive controls to show intact hCAP18 peptide at 18 kDa. Western blots are a representative of 3 independent experiments using 6 different donors. The image was cut to switch sample order for increased consistency. ### $p < 0.001$, *** $p < 0.001$, **** $p < 0.0001$.

TNF-α/IL-1β impairs 25(OH)D_3-mediated killing of NTHi by ALI-PBEC

Previous studies have reported that vitamin D treatment induces antibacterial activity of bronchial epithelial cells (14, 25). We therefore investigated whether TNF-α/IL-1β modulated the vitamin D-induced antibacterial activity of ALI-PBEC. First, ALI-PBEC were differentiated in presence of TNF-α/IL-1β and subsequently stimulated with 25(OH)D_3 or 1,25$(OH)_2D_3$ for 48 h (as indicated in Figure 1A). Bacterial killing activity was determined against the respiratory pathogen NTHi (Figure 2A). We observed that killing of NTHi was higher in cells stimulated with 25(OH)D_3 and 1,25$(OH)_2D_3$ compared to unstimulated controls, and that this effect was decreased by TNF-α/IL-1β (Figure 2B). This suggests that in addition to reducing hCAP18/LL37, chronic exposure to TNF-α/IL-1β also decreases vitamin D-induced antibacterial activity of ALI-PBEC.

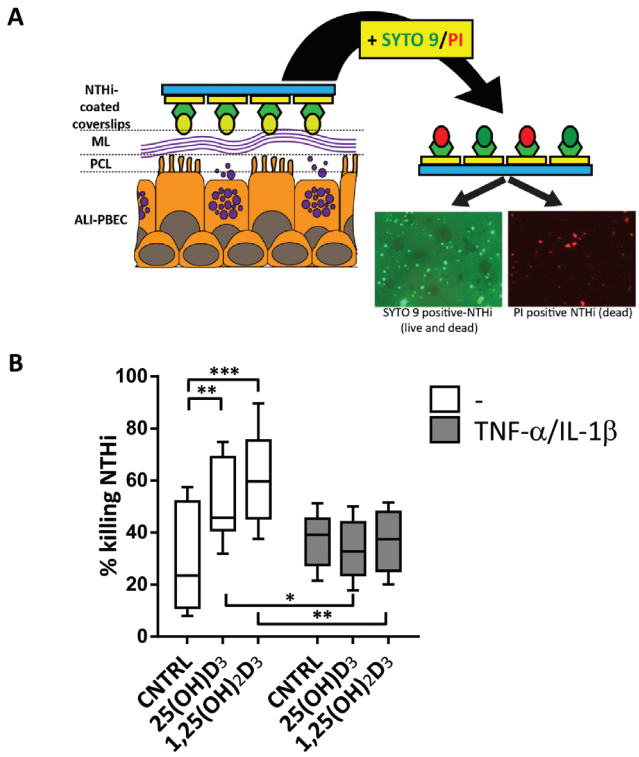

Figure 2. TNF-α and IL-1β (TNF-α/IL-1β) impairs vitamin D-mediated killing of non-typeable *Haemophilus influenzae* (NTHi). Primary bronchial epithelial cells (PBEC, n = 6 donors) were differentiated with and without TNF-α/IL-1β for 14 days followed by 48 h 25(OH)D_3- and 1,25$(OH)_2D_3$ treatment or medium control (CNTRL) in duplicate for assessing antibacterial activity of PBEC. (A) This was performed by binding biotin-linked mid-log phase growing NTHi to streptavidin-linked 6 mm glass

coverslips followed by application on the apical surface for 1 min of the stimulated cells. Next, coverslips were mounted on slides and visualized using SYTO9 (live + dead bacteria) and PI (dead bacteria) and quantified by using fluorescent microscopy. ML = mucus layer, PCL = periciliary liquid layer. (B) The percentage of dead bacteria was assessed by manual counting and data are presented as medians ± min/max values. To analyze the data, a two-way ANOVA and the Bonferroni post-hoc test was used. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Elevated CYP24A1 levels induced by chronic exposure of ALI-PBEC to TNF-α/IL-1β decrease vitamin D-mediated expression of hCAP18/LL-37

We determined whether alterations in $25(OH)D_3$-induced responses by TNF-α/IL-1β were caused by changes in expression of genes important in vitamin D metabolism (*CYP27B1* and *CYP24A1*) or *VDR*. Cells were cultured as previously indicated (Figure 1A) and assessed for mRNA expression of *VDR, CYP27B1* and *CYP24A1*. Expression of the vitamin D degrading enzyme *CYP24A1* was increased after both treatment with TNF-α/IL-1β and $25(OH)D_3$ with no changes in expression of *VDR* and *CYP27B1* (Figure 3A). Western blot analysis showed that CYP24A1 was strongly increased by TNF-α/IL-1β-treatment and only modestly by $25(OH)D_3$ and $1,25(OH)_2D_3$ in absence of TNF-α/IL-1β-treatment (Figure 3B). Immunofluorescence staining of CYP24A1 showed similar effects of TNF-α/IL-1β-treatment as observed by Western blot analysis. CYP24A1 was modestly increased by $25(OH)D_3$ and this effect was also lower than the effect of TNF-α/IL-1β alone. However, this $25(OH)D_3$-mediated effect was more pronounced in immunofluorescence than observed by Western blot analysis (Figure 3C).

Next, we assessed the contribution of CYP24A1 to the vitamin D-mediated reduction of *CAMP* using ketoconazole, which blocks CYP24A1 activity. ALI-PBEC were cultured with or without TNF-α/IL-1β for 14 days, followed by 24 h pre-incubation in presence or absence of different doses of ketoconazole before 24 h-treatment with $1,25(OH)_2D_3$ or control. $1,25(OH)_2D_3$ was used for stimulation since ketoconazole is a broad-spectrum CYP-inhibitor that also blocks CYP27B1, and therefore may affect $25(OH)D_3$ conversion into $1,25(OH)_2D_3$ (26). We demonstrated that ketoconazole partly restored expression of *CAMP* in a dose-dependent manner and did not affect *CYP24A1* expression. However, in cells exposed to $1,25(OH)_2D_3$ in absence of TNF-α/IL-1β, a small ketoconazole-induced increase in *CYP24A1* was observed, which might be explained by inhibition of CYP24A1, increasing the availability of $1,25(OH)_2D_3$ and thus the $1,25(OH)_2D_3$-mediated expression of *CYP24A1* (Figure 3D).

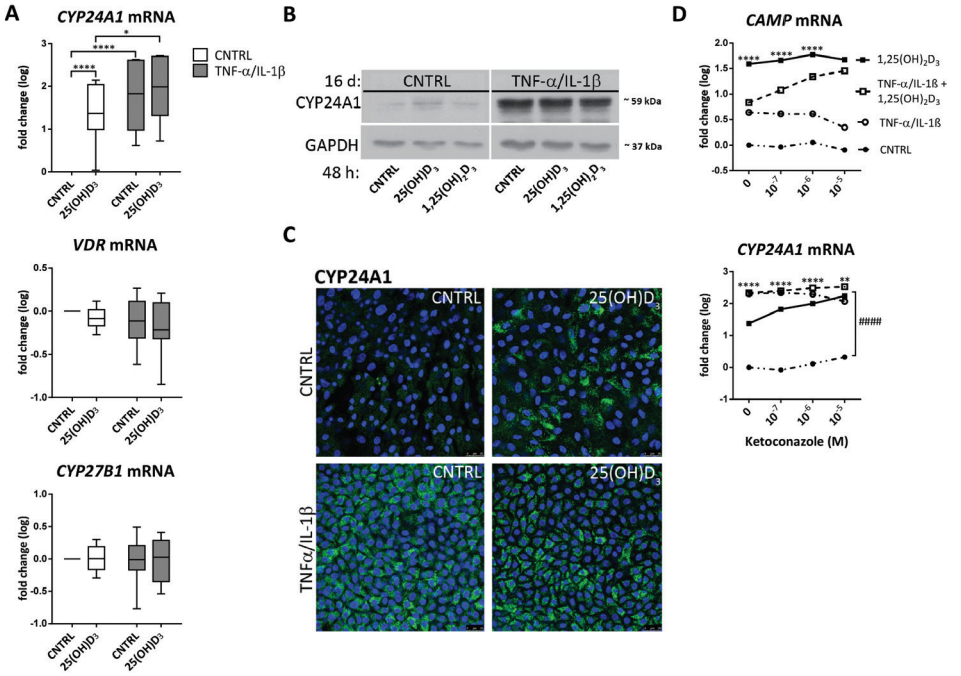

Figure 3. CYP24A1 is induced by chronic exposure to *TNF-α and IL-1ß (***TNF-α/IL-1β) and decreases vitamin D-mediated expression of hCAP18/LL-37.** Primary bronchial epithelial cells (PBEC) were cultured as described in Figure 1A for assessing *CYP24A1*, *VDR* and *CYP27B1* mRNA expression and CYP24A1 protein by Western blot analysis and immunofluorescence. (A) Relative mRNA expression of *CYP24A1*, *VDR* and *CYP27B1* was determined by qPCR. Normalized gene expression was calculated by using the expression of β2-microglobulin (*B2M*) and ATP synthase, H^+ transporting, mitochondrial F1 complex, beta polypeptide (*ATP5B*) as reference genes. Data are presented as medians ± min/max values**.** Fold change in gene expression of the stimuli compared to control (CNTRL) was first calculated, followed by a log-transformation of the data. Next, data were analyzed using two-way ANOVA and the Bonferroni post-hoc test (n = 9-11 donors). (B) Presence of CYP24A1 and GAPDH in protein cell lysates of the exposed cells was assessed by SDS-PAGE followed by Western blot analysis. Western blots are a representative of 3 independent experiments using cells from 6 different donors. The original image was cut to switch sample order for increased consistency. (C) Immunofluorescence staining of CYP24A1 in bronchial epithelial cells (of 1 donor, which was confirmed in 3 other donors). DAPI (blue) was used to stain the nuclei, and rabbit anti-CYP24A1 antibody together with Alexa Fluor 488 goat anti-rabbit IgG antibody (green) were used for detection of CYP24A1. (D) In addition, PBEC were cultured with or without TNF-α/IL-1β for 14 days, followed by 24 h pre-incubation in presence or absence of increasing concentrations ketoconazole (CYP24A1 inhibitor). Afterward, cells were treated for 24 h with $1,25(OH)_2D_3$ or medium control (CNTRL) in presence or absence of both TNF-α/IL-1β and ketoconazole. Relative mRNA expression of *CYP24A1* and *CAMP* (hCAP18/LL37) was determined by qPCR. Normalized gene expression was calculated by using the expression of *B2M* and

ATP5B as reference genes. Fold change in gene expression of the stimuli compared to control (CNTRL) was first calculated, followed by a log-transformation of the data. Next, data were analyzed using two-way ANOVA and the Bonferroni post-hoc test (n = 3 donors). * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$.

Epithelial differentiation reduces expression of *CYP24A1* in time, whereas TNF-α/IL-1β-treatment increases expression of both *CYP24A1* and mucins (*MUC5AC* and *MUC5B*)

We next investigated the effect of duration of exposure and concentration of TNF-α/IL-1β on expression levels of *CYP24A1*. To this end, cells were differentiated in the presence of 0, 0.6, 2.5, 10 ng/ml of TNF-α/IL-1β followed by analysis of *CYP24A1* mRNA expression at day 14. Results showed a dose-dependent increase of *CYP24A1* expression (Figure 4A). Additionally, we assessed *CYP24A1* mRNA expression at day 1, 7 and 14 after starting culture at the ALI in presence and absence of 2.5 ng/ml TNF-α/IL-1β. In control-treated cells, *CYP24A1* expression was decreased between day 1 and 14. In contrast, *CYP24A1* expression was already increased at day 1 in TNF-α/IL-1β-treated cells and remained elevated during 14 days (Figure 4B). This suggests that the enhanced *CYP24A1* expression is not a result of an altered differentiation induced by TNF-α/IL-1β, but a direct effect of this stimulation.

This was also verified by analysis of expression of various markers of epithelial differentiation: whereas TNF-α/IL-1β-treatment caused a significant increase in expression of mucins (*MUC5AC* and *MUC5B*) at day 7 and 14, expression of the goblet cell marker *CLCA1* and markers of ciliated cells (*FOXJ1*), club cells (*SCGB1A1*) and basal cells (*TP63*) were not affected.

To further determine the individual contribution of TNF-α and IL-1β, we compared the effect of both cytokines alone versus the combination on *CYP24A1* and *CAMP* mRNA expression. Therefore, cells were differentiated with and without TNF-α and IL-1β alone or in combination, and subsequently exposed to 25(OH)D_3 or medium control. Both TNF-α and IL-1β alone increased expression of *CYP24A1* and reduced 25(OH)D_3-mediated expression of *CAMP*. The combination of TNF-α/IL-1β gave the strongest effect on both *CYP24A1* and *CAMP* expression, but no synergism between the actions of TNF-α and IL-1β was observed, (Figure E1).

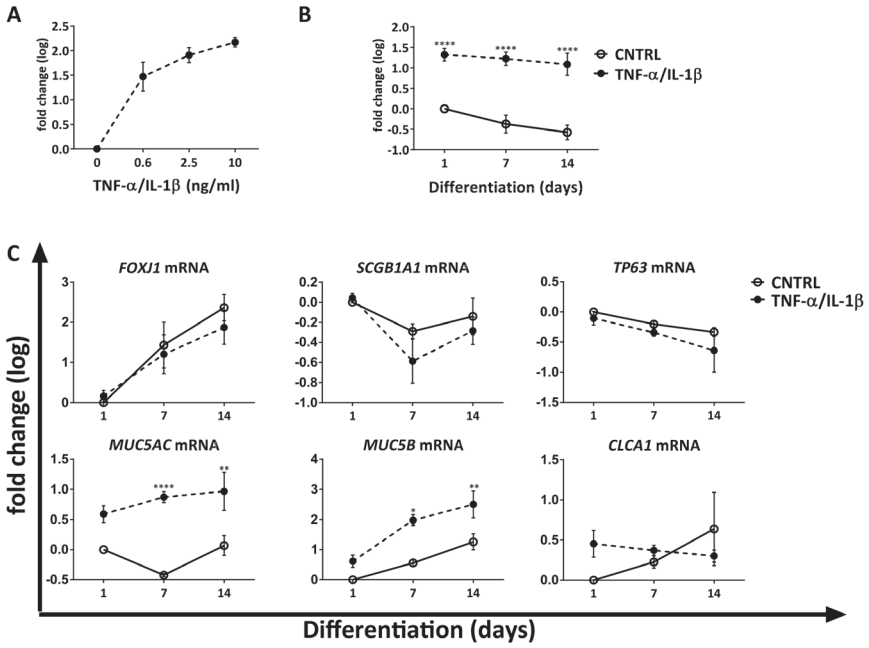

Figure 4. Time- and dose-dependent effects of TNF-α and IL-1β (TNF-α/IL-1β) on CYP24A1 expression and epithelial differentiation. (A) Primary bronchial epithelial cells (PBEC) were exposed to TNF-α/IL-1β or CNTRL for 1, 7 and 14 days or exposed for 14 days to increasing concentrations of TNF-α/IL-1β for determining relative *CYP24A1* mRNA expression by qPCR. Normalized gene expression was calculated by using the expression of β2-microglobulin (*B2M*) and ATP synthase, H^+ transporting, mitochondrial F1 complex, beta polypeptide (*ATP5B*) as reference genes. Data are presented as medians ± min/max values**.** Fold change in gene expression of the stimuli compared to control (CNTRL) was first calculated, followed by a log-transformation of the data. Next, data were analyzed using two-way ANOVA and the Bonferroni post-hoc test (n = 3 donors) (B) PBEC were exposed to TNF-α/IL-1β or medium control (CNTRL) for 1, 7 and 14 days and epithelial differentiation of bronchial epithelial cell was assessed by measuring relative mRNA expression of Forkhead box protein J1 (*FOXJ1*, ciliated cells), secretoglobin family 1A member 1 (*SCGB1A1*, club cells), tumor protein p63 (*TP63*, basal cells), Mucin-5AC (*MUC5AC*), Mucin-5B (*MUC5B*) and chloride channel accessory 1 (*CLCA1*, goblet cells) by qPCR. Normalized gene expression was calculated by using the expression of ribosomal protein L13A (*RPL13A*)- and ribosomal protein L27 (*RPL27*) as reference genes. Data are presented as medians ± min/max values**.** Fold change in gene expression of the stimuli compared to control (CNTRL) was first calculated, followed by a log-transformation of the data. Next, data were analyzed using two-way ANOVA and the Bonferroni post-hoc test (n = 4 donors). ** $p < 0.01$, **** $p < 0.0001$.

TNF-α/IL-1β-induced expression of CYP24A1 is mediated by activation of EGFR, ERK1/2 and Sp1

In addition to two VDREs, the promoter of CYP24A1 also contains three Sp1-binding sites (27). To investigate if the transcription factor Sp1 contributes to the TNF-α/IL-1β-induced expression of *CYP24A1*, ALI-PBEC were cultured for 14 days in presence and absence of TNF-α/IL-1β and subsequently treated with the Sp1 inhibitor mithramycin A for 24 h. Expression of *CYP24A1* mRNA was inhibited by mithramycin A in both untreated as well as TNF-α/IL-1β-treated cells (Figure 5A).

To further explore signaling mechanisms that may contribute to TNF-α/IL-1β-induced expression of *CYP24A1*, submerged cultures of undifferentiated PBEC were used. Cells were pre-treated with and without inhibitors of MMP (GM6001), EGFR (AG1478) and MEK1/2 (U0126) and subsequently exposed to TNF-α/IL-1β and LL-37 (used as a positive control for transactivation of the EGFR for 15 min (23). Western blot analysis assessing p-EGFR and p-ERK1/2 showed that inhibition of MMP, MEK1/2 and EGFR decreased both TNF-α/IL-1β and LL-37-induced phosphorylation of EGFR and ERK1/2 (Figure 5B).

We then investigated if MMP, EGFR and MEK1/2 are involved in TNF-α/IL-1β-mediated expression of *CYP24A1*. PBEC were treated for 24 h with TNF-α/IL-1β, and *CYP24A1* mRNA expression was assessed by qPCR. First, we confirmed that *CYP24A1* mRNA was also increased by TNF-α/IL-1β in undifferentiated PBEC after 24 h (Figure 5C). Furthermore, we showed that inhibition of EGFR and MEK1/2 decreased expression of *CYP24A1* mRNA (Figure 5C). However, whereas inhibition of MMP by GM6001 did inhibit phosphorylation of EGFR and ERK1/2 after 15 min, it did not affect TNF-α/IL-1β-induced expression of CYP24A1 after 24 h. Although no cytotoxic effects were observed microscopically, there was some degree of toxicity as indicated by an increase in LDH release in cells that were exposed for 24 h to AG1478 and GM6001 (Figure E2).

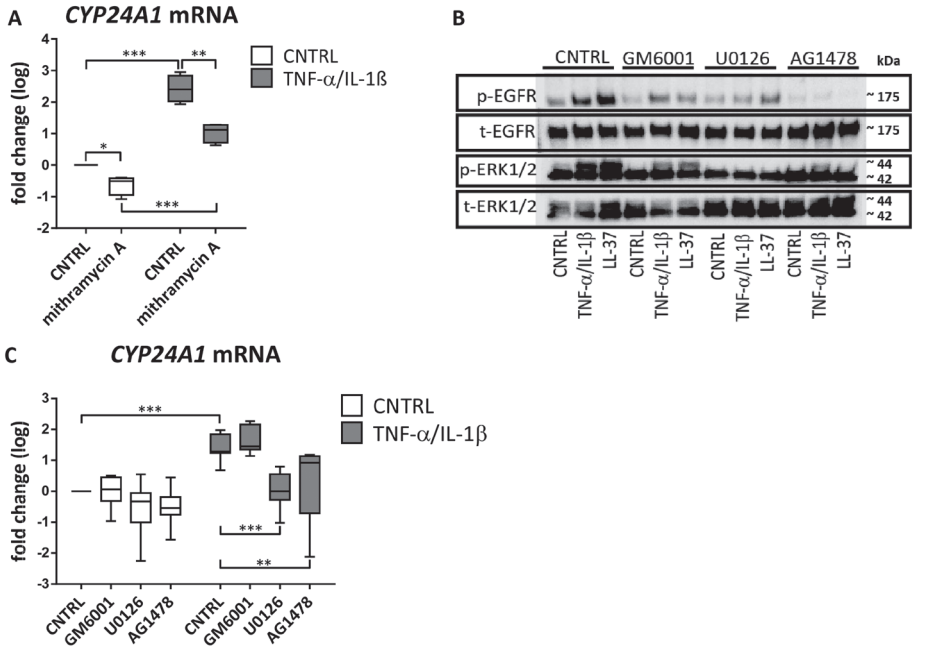

Figure 5. TNF-α and IL-1β (TNF-α/IL-1β)-induced expression of *CYP24A1* is mediated by activation of EGFR, ERK1/2 and Sp1. To investigate the role of the transcription factor Sp1 in TNF-α/IL-1β-mediated expression of *CYP24A1*, Primary bronchial epithelial cells (PBEC) were differentiated for 14 days in presence or absence (CNTRL) of TNF-α/IL-1β and subsequently exposed in presence or absence of TNF-α/IL-1β with mithramycin A or medium control (CNTRL). (A) qPCR was used to assess relative expression of *CYP24A1* mRNA and normalized gene expression was calculated by using the expression of β2-microglobulin (*B2M*) and ATP synthase, H^+ transporting, mitochondrial F1 complex, beta polypeptide (*ATP5B*) as reference genes. Data are presented as medians ± min/max values**.** Fold change in gene expression of the stimuli compared to control (CNTRL) was first calculated, followed by a log-transformation of the data. Next, data were analyzed using two-way ANOVA and the Bonferroni post-hoc test (n = 4 donors) (B) Next, submerged cultures of PBEC (n = 3) were used to investigate the mechanism involved in TNF-α/IL-1β-mediated *CYP24A1* expression: cells were exposed to TNF-α/IL-1β for 15 min after 1 hour pre-treatment with CNTRL (0.25% v/v DMSO in PBS) and MMP, EGFR and MEK-inhibitors. Protein expression of tyrosine phosphorylated (p)-and total (t)-epidermal growth factor receptor (EGFR), p-ERK1/2 and t- ERK1/2 was determined by SDS-PAGE Western blot. Western blots are a representative of 3 independent experiments using 3 different donors. The images were cropped to paste the images of t-EGFR and t-ERK1/2 directly below p-EGFR and t-EGFR (C) In addition, submerged cultures of PBEC were pre-incubated with CNTRL (0.25% v/v DMSO) and inhibitors of MMP (GM6001), EGFR (AG1478) and MEK (U0126) followed by stimulation for 24 h with TNF-α/IL-1β to assess relative gene expression of *CYP24A1* mRNA by qPCR. Normalized gene expression was calculated by using the expression of *B2M* and *ATP5B* as reference genes. Data are presented as medians ± min/max values**.** Fold change in gene expression of the stimuli compared to control (CNTRL) was first calculated, followed by a log-transformation of the data. Next, data were analyzed using two-way ANOVA and the Bonferroni post-hoc test (n = 9 donors). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Both chronic exposure to IL-17A and short exposure to NTHi increase expression of CYP24A1

In addition to TNF-α and IL-1β, also IL-17A is increased in the airways of patients suffering from chronic inflammatory lung diseases (20). Furthermore, the respiratory pathogen NTHi also activates epithelial cells and NTHi infections are found in CF, COPD and poorly controlled asthma patients (21, 22). To investigate if these epithelial triggers have similar effects on epithelial vitamin D metabolism as TNFα and IL-1β, we stimulated epithelial cells with IL-17A and NTHi and assessed their effects. To study the effect of IL-17A exposure, ALI-PBEC were differentiated with or without IL-17A. After 14 days of culture, cells were stimulated for another 24 h with or without IL-17A and $25(OH)D_3$ and next expression of *CYP24A1* and *CAMP* mRNA was assessed by qPCR. Similar to TNFα/IL-1β, IL-17A treatment increased *CYP24A1* expression and impaired $25(OH)D_3$-mediated expression of *CAMP* from a 20.3-fold increase down to a 3.5-fold increase (Figure 6A), while *VDR* and *CYP27B1* expression remained unaffected by IL-17A (data not shown). To examine if exposure to NTHi affects expression of *CYP24A1*, ALI-PBEC were differentiated for 14 days and subsequently stimulated with 2.5 – 5 - 10 x 10^7 CFU/ml of UV-inactivated NTHi for 12 h and assessed for CYP24A1 expression by qPCR. We observed that NTHi dose-dependently increased expression of *CYP24A1* compared to medium control (Figure 6B). These data suggest that inflammatory triggers related to COPD pathology can all affect *CYP24A1* expression and thereby negatively affect vitamin D-mediated effects.

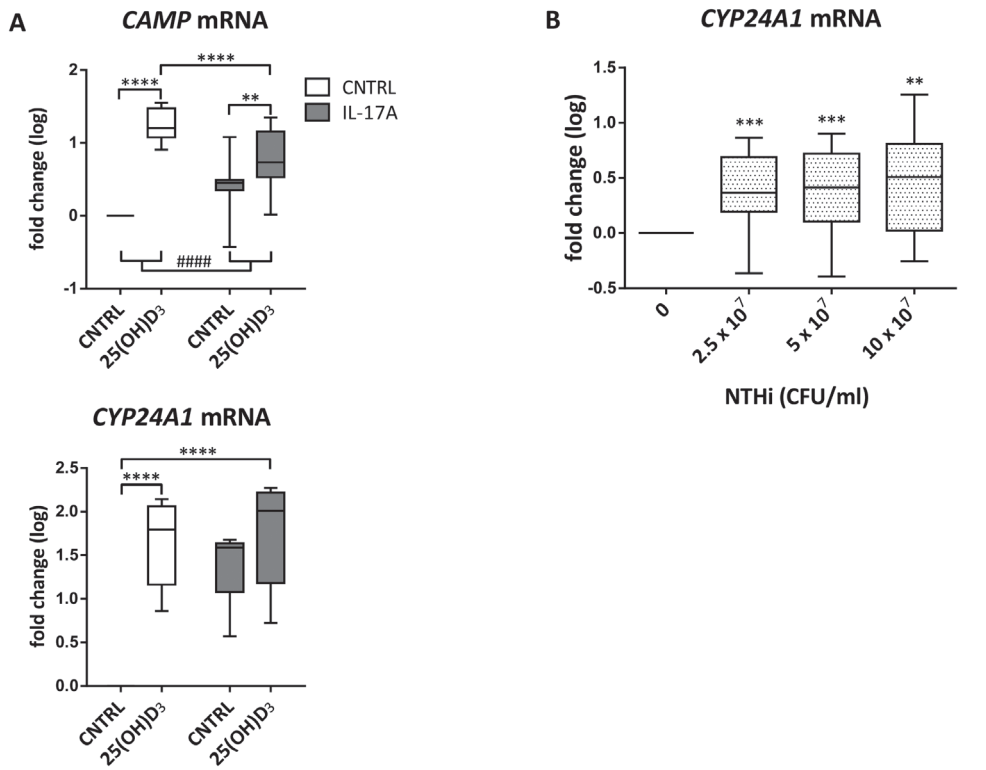

Figure 6. Chronic IL-17A-exposure and short exposure to non-typeable *Haemophilus influenzae* (NTHi) increase expression of *CYP24A1*. (A) To investigate effects of IL-17A, primary bronchial epithelial cells (PBEC) were differentiated with or without IL-17A for 14 days and stimulated for another 24 h with or without IL-17A and 25(OH)D_3 or medium control (CNTRL) to measure relative mRNA expression of *CAMP* (hCAP18/LL-37) and *CYP24A1* by qPCR. Normalized gene expression was calculated by using the expression of β2-microglobulin (*B2M*) and ATP synthase, H^+ transporting, mitochondrial F1 complex, beta polypeptide (*ATP5B*) as reference genes. Data are presented as medians ± min/max values**.** Fold change in gene expression of the stimuli compared to control (CNTRL) was first calculated, followed by a log-transformation of the data. Next, data were analyzed using two-way ANOVA and the Bonferroni post-hoc test (n = 9 donors) and differences between CNTRL and 25(OH)D_3 ratios were calculated in CNTRL and IL-17A treated cells using a paired t-test (B) To examine if NTHi affects expression of *CYP24A1* mRNA in air-liquid interface cultures of PBEC, cells were differentiated for 14 days and subsequently stimulated with increasing concentrations of UV-inactivated NTHi for 12 h before analysis of *CYP24A1* expression by qPCR. Normalized gene expression was calculated by using the expression of Ribosomal Protein L13a (*RPL13A*) and *ATP5B* as reference genes. Data are presented as medians ± min/max values**.** Fold change in gene expression of the stimuli compared to control (CNTRL) was first calculated, followed by a log-transformation of the data. Next, data were analyzed using one-way ANOVA and the Bonferroni post-hoc test (n = 19-21 donors). #### $p < 0.0001$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

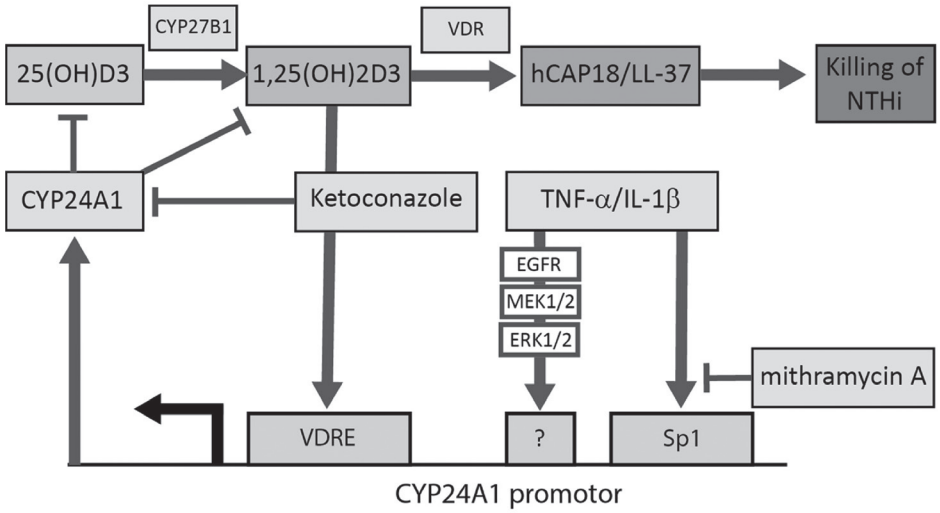

Figure 7. Summary and proposed mechanism for TNF-α/IL-1β-mediated increase of CYP24A1. In primary bronchial epithelial cells, the circulating metabolite of vitamin D, 25(OH)D_3 is converted by CYP27B1 into the active 1,25(OH)$_2D_3$, resulting in expression of the antimicrobial peptide hCAP18/LL-37 and killing of NTHi via the vitamin D receptor (VDR) and subsequent binding of vitamin D response elements (VDREs), which are present on the promoter of *CAMP* and encodes for hCAP18/LL-37. Expression of hCAP18/LL-37 and killing of NTHi is impaired by TNF-α/IL-1β exposure via increased expression of CYP24A1, which degrades both 25(OH)D_3 and 1,25(OH)$_2D_3$. The loss of hCAP18/LL-37 expression can be restored by ketoconazole, which suppresses CYP24A1-activity. The promoter of *CYP24A1* contains in addition of VDREs, CCAAT-enhancer-binding protein (C/EBP), vitamin D stimulation element (VSE), ets-1 binding site (EBS), CCAAT also of GC-rich boxes, which are binding sites for the transcription factor Sp1. Blocking Sp1-binding sites by mithramycin A, decreases TNF-α and IL-1β-mediated expression of CYP24A1. In addition, expression of TNF-α and IL-1β-mediated CYP24A1 can be decreased by inhibiting the MAPK-pathway using pharmacological inhibitors of MEK.

Discussion

Here we show that inflammatory conditions negatively affects vitamin D metabolism in airway epithelial cells resulting in reduced vitamin D-induced expression of hCAP18/LL-37 and antibacterial activity against NTHi. These findings are explained at least in part by the ability of TNF-α/IL-1β and IL-17A to increase expression of the vitamin D-degrading enzyme CYP24A1, whereas expression of VDR and CYP27B1 was not affected. The ability of TNF-α/IL-1β to increase expression of CYP24A1 was in part mediated by EGFR, MEK and Sp1. These findings

are summarized in Figure 7, showing that a local pro-inflammatory environment in the airways might impair the ability of vitamin D to regulate epithelial antibacterial activity.

Effects of vitamin D on hCAP18/LL-37 expression and antibacterial activity in differentiated bronchial epithelial cells have been reported previously (8, 14, 25). However, to our knowledge this is the first study that demonstrates how pro-inflammatory conditions affect these activities. Our observation that CYP24A1 is increased by pro-inflammatory stimuli in primary airway epithelial cells is in line with another study showing that TNF-α, IL-1β, IL-6 and IFN-γ increased expression of CYP24A1 in human trophoblasts (13). Moreover, the importance of CYP24A1 on negatively affecting vitamin D-induced antibacterial defense are supported by a recent study by Wang and colleagues, showing that vitamin D-induced bacterial killing by immortalized human oral keratinocytes was increased by knockdown of CYP24A1 via siRNA transfection (28). It was also shown in this study that vitamin D-mediated expression of hCAP18/LL-37 correlated with killing of *F. nucleatum* (growth of three other bacterial strains were not affected by vitamin D).

In addition to hCAP18/LL-37, previous reports demonstrated that vitamin D also increases other AMPs, such as human-beta-defensin-2 (hBD-2) in oral squamous cells and 16HBE-cells (29, 30). However in ALI-PBEC, no effects of vitamin D on hBD-2 expression were observed (Figure E3). Our finding that inflammatory cytokines affect airway epithelial vitamin D metabolism and thus decrease AMP expression further extends other findings showing alterations in the antibacterial activity of airway epithelial cells due to changes in the local environment, such as alterations in airway surface liquid pH and mucus accumulation (24, 31).

The promoter of *CYP24A1* contains, in addition to 2 VDREs, other promoter elements such as 3 GC-rich boxes, to which the transcription factor Sp1 can bind. To investigate if TNF-α and IL-1β increase transcription of *CYP24A1* via Sp1, we blocked Sp1-binding by using mithramycin A and demonstrated that it reduced expression of *CYP24A1* in ALI-PBEC. Since *CYP24A1* expression was not fully inhibited by mithramycin A, we cannot exclude other mechanisms via other promoter elements such as the ETS-1 binding site (EBS) (32). Moreover, others demonstrated the involvement of SP1 in expression of genes other than *CYP24A1* by TNF-α and/or IL-1β in epithelial cells (33, 34). Additionally, it was shown that Sp1-mediated gene expression was facilitated via TNFR1, MEK1/2, ERK1/2 (34). In line with these findings, we demonstrated that TNF-α and IL-1β increased phosphorylation of ERK1/2 and that blocking MEK1/2 decreased expression of

CYP24A1. We furthermore showed that TNF-α and IL-1β increased phosphorylation of EGFR, and that inhibition of EGFR phosphorylation decreased phosphorylation of ERK1/2, providing evidence for an additional involvement of the EGFR. This is in line with other studies showing that EGFR phosphorylation was increased by TNF-α or IL-1β (35, 36). Although Inhibition of MEK1/2 and EGFR by U0126 and AG1478 respectively decreased TNF-α/IL-1β-mediated *CYP24A1* expression, inhibition of MMP by GM6001 did not show this effect. This was unexpected since GM6001 did reduce TNF-α/IL-1β-mediated phosphorylation of EGFR and ERK1/2. TNF-α/IL-1β also phosphorylates the EGFR via activation of TAK1 independently of MMP-mediated cleavage and release of membrane-bound EGFR-ligands, indicating that also other pathways might play a role (37). Furthermore, possible toxic or non-selective effects of these signaling inhibitors upon prolonged incubation (24 h) cannot be fully excluded, since LDH release was increased after 24 h exposure to GM6001 and AG1478 (Figure E2). Taken together, one of the mechanisms that results in vitamin D-independent CYP24A1 expression by TNF-α and IL-1β in PBEC, likely involves activation of EGFR, MEK1/2 and Sp1. Since studies have shown that IL-17A and NTHi promote MAPK/ERK signaling (38, 39), we speculate that IL-17A and NTHi also mediate *CYP24A1* expression via similar pathways as TNF-α and IL-1β. However, more studies are necessary to elucidate this.

We used differentiated primary airway epithelial cells obtained from multiple donors, instead of tumor-derived or immortalized airway epithelial cell lines, thereby increasing the relevance of our findings. We have used selected recombinant pro-inflammatory cytokines to mimic the inflammatory environment in the lung, whereas in the lung such cytokines are produced by immune and resident cells. Future studies using co-culture systems may provide additional information, because immune cells are also capable of converting vitamin D into its active form. In our study, we confirmed the involvement of CYP24A1 in decreasing vitamin D-mediated expression of hCAP18/LL-37 by inhibiting its activity by ketoconazole. However, we have not verified that 25(OH)D_3-induced killing of NTHi is a consequence of the increased hCAP18/LL-37 by vitamin D. Unfortunately, blocking LL-37-activity by using LL-37 specific blocking antibodies was not successful and neither was transfection using siRNA in 14-days differentiated ALI-PBEC cultures. However, we did demonstrate antibacterial activity and also expression of hCAP18/LL-37, which was mainly located at the apical side of the ALI-PBEC by immunofluorescence, and we detected hCAP18/LL-37 in basal medium by Western blot analysis. Using LL-37 specific monoclonal antibodies, strong

expression of a peptide at 14 kDa and also weaker expression of a peptide at 18 kDa (size of uncleaved hCAP18) was detected, yet expression of a peptide at 4.5 kDa (mature antimicrobial active LL-37) was absent. Western blots showing peptides containing LL-37 immunoreactivity at 14 kDa were previously reported by us and other studies (6, 14, 40-42). For example, Sörensen and colleagues detected 14 kDa hCAP18 fragments, in addition to 18 kDa and 4.5 kDa fragments, following cleavage of hCAP18 by extracts of neutrophil- azurophilic granules, elastase or cathepsin G by Western blot using LL-37-specific antibodies (6). Based on our analysis of basal conditioned medium, we therefore speculate that hCAP18 was mainly cleaved at a different location than in neutrophils, or that the detection of the 4.5 kDa LL-37 peptide was masked.

To further verify that 25(OH)D3-induced killing of NTHi is a consequence of the increased hCAP18/LL-37 by vitamin D, the arrival of new genome editing tools such as CRISPR-Cas9 provides new opportunities to identify the precise contribution of hCAP18/LL-37 or other AMPs to vitamin D-mediated antimicrobial activity in bronchial epithelial cells. To extend the relevance of our findings to the *in vivo* situation, further studies are required to compare lung tissue levels of CYP24A1, CYP27B1, VDR and $1,25(OH)_2D_3$ in healthy donors and donors with chronic inflammatory lung disease.

We have demonstrated in our *in vitro* model that $1,25(OH)_2D_3$ treatment increases killing of NTHi and that this vitamin D-induced killing was impaired in presence of pro-inflammatory cytokines. Furthermore, decreased availability of vitamin D in an inflammatory environment also reduces the impact of vitamin D on inflammation through its anti-inflammatory and immune modulating properties. These observations may help to explain why vitamin D supplementation only prevents exacerbations in severely deficient individuals (43, 44). Our studies suggest that inhibition of CYP24A1, supplementation with CYP24A1-resistant vitamin D analogues, decreasing inflammation and supplementation with higher doses of vitamin D may increase vitamin D-responses in patients suffering from chronic inflammatory lung diseases. Inhibiting CYP24A1 by ketoconazole might not be a good strategy, since oral administration of the drug is reported not to be safe and causes serious side effects, including endocrine dysregulation and interactions with other drugs (45). Other more selective compounds targeting CYP24A1 such as styrylbenzamides and VID400 have not yet been evaluated in clinical trials (46, 47). Another option is the use of $1,25(OH)_2D_3$-analogs that are currently under

investigation in some clinical trials. These substances are less sensitive to degradation by CYP24A1, and have been modified in such a way that the risk of hypercalcemia is reduced (48). Using anti-inflammatory drugs such as glucocorticoids in combination with 25(OH)D_3-supplementation seems an attractive strategy. However, the use of inhaled corticosteroids is largely ineffective in reducing inflammation in most COPD patients and in corticosteroid-resistant asthma patients and long-term use may cause side effects such as pneumonia and osteoporosis (49, 50). Therefore, 25(OH)D_3-supplementation might counteract this by increasing both the host defense and the sensitivity to corticosteroids, as previously shown in corticosteroid-resistant asthma patients (3, 4, 51-53).

In summary, we have demonstrated that epithelial exposure to pro-inflammatory cytokines reduces the vitamin-D induced antibacterial activity in primary bronchial epithelial cells. These data suggest that a local pro-inflammatory environment might impair local vitamin D-mediated host defense in the lung and that circulating vitamin D levels may not always reflect local activity. However, we cannot formally exclude that low-degree systemic inflammation, as observed in patients with (severe) chronic airway inflammation, might affect circulating vitamin D levels and thus contributes to the vitamin D deficiency in such patients (2). These data therefore point to novel mechanisms whereby inflammation-triggered alterations in vitamin D metabolism impair host defense at the airway epithelial surface, increase the risk of infection and thus contribute to more (severe) exacerbations and progression of disease.

Footnotes

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Supplemental figures

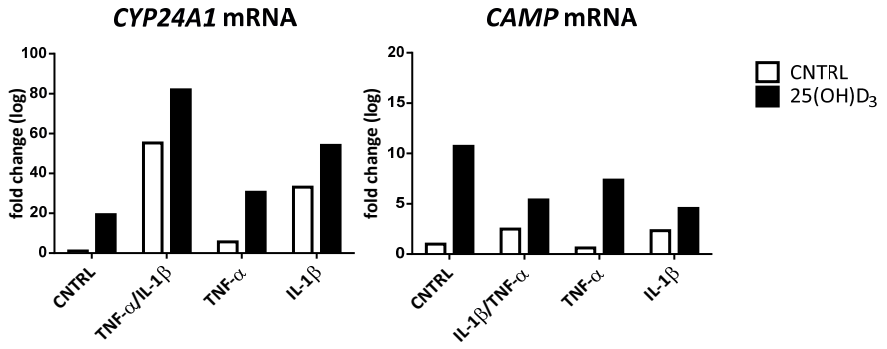

Figure E1. Effect of TNF-α and IL-1β together versus TNF-α and IL-1β alone on expression of CYP24A1 and hCAP18/LL-37 in primary bronchial epithelial cells (PBEC). PBEC (n = 2 donors) were cultured with or without the pro-inflammatory cytokines TNF-α/IL-1β or TNF-α and IL-1β in combination or alone for 14 days followed by 24 h 10^{-7} M 25(OH)D_3-treatment or medium control (CNTRL) to assess *CYP24A1* and *CAMP* (hCAP18/LL-37) mRNA expression by qPCR. Normalized gene expression was calculated by using the expression of the β2-microglobulin (*B2M*) and ATP synthase, H+ transporting, mitochondrial F1 complex, beta polypeptide (*ATP5B*) as reference genes. Fold change in gene expression of the stimuli compared to control (CNTRL) was calculated. Data were represented as means.

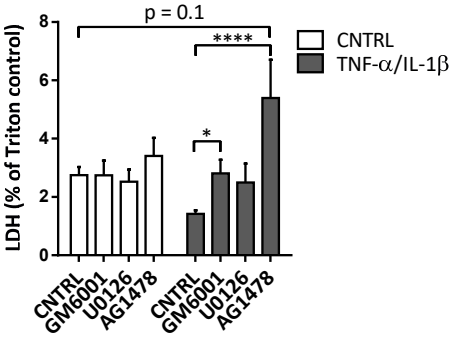

Figure E2. Effect of inhibitors and TNF-α/IL-1β on LDH release in primary bronchial epithelial cells (PBEC). Submerged PBEC cultures (n = 4 donors) were pre-incubated with medium alone (CNTRL; 0.25% v/v DMSO) and inhibitors of MMP (GM6001), EGFR (AG1478) and MEK (U0126) followed by stimulation for 24 h with TNF-α/IL-1β or with 0.1% Triton X-100 (positive control) to assess LDH relaese. Percentage LDH release was calculated as followed: (OD_{490nm} samples x dilutionfactor/OD_{490nm} Triton X-100 controls x dilutionfactor) x 100%. Data were analyzed using a paired t-test and a two-way ANOVA and the Bonferroni post-hoc test. * $p < 0.05$, **** $p < 0.0001$.

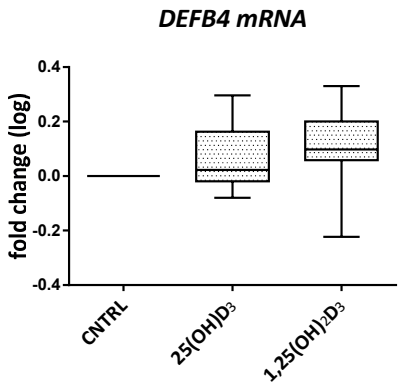

Figure E3. Effect of 25(OH)D_3 and 1,25$(OH)_2D_3$ on hBD-2 (*DEFB4*) expression in primary bronchial epithelial cells (PBEC). PBEC were differentiated for 14 days and subsequently exposed to 10^{-7} M 25(OH)D_3 and 1,25$(OH)_2D_3$ for 24 h in duplicate. *DEFB4* (hBD-2) expression was assessed using qPCR. Normalized gene expression was calculated by using the expression of the β2-microglobulin (*B2M*) and ATP synthase, H^+ transporting, mitochondrial F1 complex, beta polypeptide (*ATP5B*) as reference genes. Data were log-transformed and represented as means ± SEM. Data are presented as medians ± min/max values**.** Fold change in gene expression of the stimuli compared to control (CNTRL) was first calculated, followed by a log-transformation of the data (n = 8-10 donors).

Supplemental methods

Cell culture

PBEC were obtained from tumor free bronchial lung tissue from anonymous donors, collected during lung resection surgery for lung cancer. Cells were cultured as previously described with adaptations (54). Briefly, cultures of bronchial epithelial cells (passage 1) were first expanded in 9 cm culture plates, pre-coated with 10 µg/ml BSA (Sigma Aldrich, Zwijndrecht, The Netherlands), 30 µg/ml Purecol (Advanced BioMatrix, San Diego, CA) and 10 µg/ml fibronectin, before seeding at a density of 40,000 cells per insert (passage 2) on pre-coated semi-permeable Transwell inserts (12 mm, 0.4 µm pore-size, Corning Costar, Cambridge, MA). When confluence was reached (after 5-7 days), apical medium was removed and the cells were cultured at the air-liquid interface (ALI) and refreshed 3 times a week with BEGM:DMEM medium (1:1) (Lonza, Breda, The Netherlands and Invitrogen, Breda, The Netherlands respectively) supplemented with BEGM BulletKit singlequots (bovine pituitary extract (BPE), hydrocortisone, human epidermal growth factor (hEGF), epinephrine, transferrin, insulin, T3, and retinoic acid) (all from Lonza), and additional 15 ng/ml retinoic acid (Sigma-Aldrich), 15 µg/ml BSA (Sigma Aldrich), 1 mM HEPES, 100 U/ml penicillin and 100 µg/ml streptomycin (Lonza). During refreshment the apical surface was washed with PBS to remove excess mucus. After 14 days of air-exposed culture, the cells produced both mucus and had developed cilia (55).

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

Cells were lysed in RNA lysis buffer (Promega). Total RNA was automatically 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 and M-MLV Polymerase (Promega) at 37° C. All qPCR reactions, using primers shown in table I, were performed in triplicate on a CFX-384 Real-Time PCR detection system (Bio-Rad Laboratories, Veenendaal, The Netherlands) with the use of SensiFast supermix (GC-Biotech, Alphen aan de Rijn, The Netherlands). The relative standard curve method was used to calculate arbitrary gene expression using CFX-manager software (Bio-Rad). 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 nr.
B2M*	β2-microglobulin	GACCACTTACGTTCA TTGACTCC	CAGGGTTTCATCA TACAGCCAT	NM_004048
ATP5B*	ATP synthase, H^+ transporting, mitochondrial F1 complex, beta polypeptide	TCACCCAGGCTGGT TCAGA	AGTGGCCAGGGT AGGCTGAT	NM_001686
RPL13A*	Ribosomal Protein L13a	AAGGTGGTGGTCGT ACGCTGTG	CGGGAAGGGTTG GTGTTCATCC	AB082924
RPL27*	Ribosomal protein L27	ATCGCCAAGAGATC AAAGATAA	TCTGAAGACATCC TTATTGACG	NM_000988
CAMP	hCAP18/LL-37	TCATTGCCCAGGTCC TCAG	TCCCCATACACCG CTTCAC	NM_004345.3
CYP24A1	24-hydroxylase	TTGGCTCTTTGTTGG ATTGTCCGC	TGAAGATGGTGCT GACACAGGTGA	NM_000782.3
CYP27B1	α1-hydroxylase	AACCCTGAACAACG TAGTCTGCGA	ATGGTCAACAGCG TGGACACAAA	NM_000785.3
VDR	Vitamin D receptor	ACCTGGTCAGTTAC AGCATCC	TGGTGAAGGACTC ATTGGAGC	NM_0010175 35.1
DEFB4	β-defensin 2	ATCAGCCATGAGGG TCTTG	GCAGCATTTTGTT CCAGG	NM_004942
MUC5B	Mucin-5B	GGGCTTTGACAAGA GAGT	AGGATGGTCGTGT TGATGCG	NG_031880
MUC5AC	Mucin-5AC	CCTTCGACGGACAG AGCTAC	TCTCGGTGACAAC ACGAAAG	NM_0013043 59
CLCA1	Chloride channel accessory 1	ATGGCTATGAAGGC ATTGTCG	TGGCACATTGGG GTCGATTG	NM_001285
FOXJ1	Forkhead box protein J1	GGAGGGGACGTAA ATCCCTA	TTGGTCCCAGTAG TTCCAGC	NM_001454
TP63	Tumor protein p63	CCACCTGGACGTAT TCCACTG	TCGAATCAAATGA CTAGGAGGGG	NM_003722
SCGB1A1	Secretoglobin family 1A member 1	ACATGAGGGAGGCA GGGGCTC	ACTCAAAGCATGG CAGCGGCA	NM_003357

Table 1. PCR primers and sequences used for quantitative PCR

*Used as a reference gene, selected using the Genorm method

Preparation of UV-inactivated NTHi

NTHi strain D1 was cultured on chocolate agar plates (bioMérieux, Zaltbommel, The Netherlands) overnight at 7% CO_2 and 37°C (56). Next, one colony was picked and plated on a second chocolate agar plate and cultured overnight at 7% CO_2 and 37°C. From this culture, multiple colonies were picked and resuspended in 10 ml Tryptone soya broth containing X- and V-factor (TSB XV, Mediaproducts BV, Groningen, the Netherlands) and incubated overnight at 37°C while shaking. Two ml of the overnight culture was transferred into fresh 10 ml TSB XV medium and incubated for 4 hours at 37°C while shaking. Next, the bacteria-containing broth was centrifuged for 10 minutes at 3000 rpm, the pellet was resuspended in PBS and the concentration was adjusted to 1×10^9 CFU/ml based on OD 600 nm measurements. Bacteria were next inactivated by UV-light exposure for 2 hours and killing was confirmed by plating on chocolate plates.

Bacterial killing assay

Killing of NTHi by ALI-PBEC was assessed as described by Pezzulo et al. with a few modifications (24). We used 6 mm glass coverslips instead of gold grids, which were coated with 2% (v/v) 3-aminopropyltriethoxysilane (APTES, $H_2N(CH_2)_3Si(OC_2H_5)_3$; Sigma-Aldrich) solution in acetone for 10 seconds, washed in H_2O and dried at RT. Next, coverslips were immersed in 1 mM 11-mercaptoundecanoic acid (MUA, $HS(CH_2)_{10}COOH$, Sigma-Aldrich) for 30 minutes at room temperature (RT), next in a freshly prepared mixture of 0.1 M N-hydroxysuccinimide (NHS) and 0.1 M 1-ethyl-3-(3-diethylaminopropyl)carbodiimide (EDC) (1:2 molar ratio) (Sigma-Aldrich) for 30 minutes at RT, followed by coupling with 10 ug/mL streptavidin (Sigma-Aldrich) in PBS for 30 minutes. Finally, coverslips were treated with 1 M glycine for 30 minutes, washed in PBS and kept on ice until use.

NTHi strain D1 were cultured as described in TSB XV medium (bioMérieux) while shaking overnight at 37^o C. Next, 2 ml of the overnight culture was transferred into fresh 10 ml TSB XV medium and incubated for 4 hours at 37°C while shaking to obtain mid log phase-growing bacteria. 1 x 10^8 CFU/ml of bacteria (determined at OD 600 nm), were labeled with 0.88 mg/ml EZ-Link sulfo-N-hydroxysuccinimide (NHS)-Biotin (Thermo Scientific) in PBS for 30 minutes on ice. Next, biotin-labeled NTHi were linked to the streptavidin-coated glass coverslips in PBS for 30 minutes and washed two times in PBS and finally in 0.01 M sodium phosphate buffer (pH 7.4), containing 1% TSB XV to remove the unbound bacteria. Expression of *MUC5AC* and *MUC5B* mRNA was elevated by TNF-α and IL-1β-treatment (Figure 4C) and

excessive mucus secretion might interfere with the antibacterial assay by binding of the negatively charged mucins to the cationic AMPs (31). To rule out this possibility, we removed the excess mucus by washing both treated and untreated cells with 200 µl sodium phosphate buffer for 30 minutes at 37° C 6 h before the assay. NTHi-coated coverslips were placed on the apical surface of ALI-PBEC for 1 minute on each side of the coverslip followed by a staining for 30 seconds with ice-cold SYTO 9 and propidium iodide (PI) (Baclight LIVEDEAD staining kit, Life Technologies) and mounting on microscopic slides using Baclight mounting oil (Life Technologies). Digital images were taken with a Zeiss Axio Scope A1 fluorescent microscope and Zeiss Axiocam mRc 5 camera (Carl Zeiss Microscopy, Göttingen, Germany), and live and dead bacteria were counted using Image J software (National Institutes of Health, Bethesda, MD, USA). The antibacterial activity was determined by calculating the percentage of dead bacteria.

Western blot

For Western blot analysis of hCAP18/LL-37 release, basal medium was pooled and purified using Oasis HLB 1cc extraction cartridges (Waters Chromatography, Etten-Leur, The Netherlands) and the eluate was dried by vacuum centrifugation (CHRIST RVC2-25 Vacuüm system) (57). 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 (14). Cells were lysed in ice-cold RIPA buffer (50 mM Tris pH 7.4, 150 mM NaCl, 100 mM Na_3VO_4, 100 mM PMSF, 100 mM EDTA, 1% Triton X-100, 1% sodium dodecyl sulfate [SDS], containing protease inhibitors [Complete Mini, Roche Diagnostics, Almere, The Netherlands]). RIPA lysates were incubated for 30 minutes at 4°C, sonicated and centrifuged at 10,000 × g for 10 minutes. Supernatants were collected, and stored at - 20°C until use. For detection of CYP24A1, phosphorylated (p)-EGFR, total (t)-EGFR, p-ERK1/2 and t-ERK1/2, and GAPDH, RIPA protein lysates were dissolved in reducing SDS sample buffer, heated for 5 minutes at 100° C and applied on a 10% SDS-PAGE gel. 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 (for detection of hCAP18/LL-37 or CYP24A1), or in Tris-buffered saline (TBS) containing 0.01% Tween 20 (v/v) and 1% BSA (w/v) for detection of p-EGFR, t-EGFR, p-ERK1/2, t-ERK1/2, and GAPDH. Membranes were probed with 1/200 diluted mouse monoclonal anti-hCAP18/LL-37 (clone 1.1.C12; Hycult Biotech, Uden, The Netherlands), 1/1000 diluted rabbit-anti-CYP24A1 (Sigma Aldrich), 1/1000 diluted rabbit-anti-p-EGFR (tyrosine), 1/1000

diluted rabbit-anti-t-EGFR, 1/1000 diluted rabbit-anti-p-ERK1/2, 1/500 diluted rabbit-anti-t-ERK1/2 or with 1/10000 diluted rabbit anti-GAPDH (Cell Signaling Technology, Leiden, The Netherlands) in their respective blocking buffers. Next, the membranes were incubated with 1/1000 diluted goat-anti-mouse-HRP conjugated antibody (DAKO, Heverlee, Belgium) for hCAP18/LL-37, or in 1/10.000 diluted goat-anti-rabbit-HRP (Cell Signaling Technology) for CYP24A1, p-EGFR, t-EGFR, p-ERK1/2, t-ERK1/2 and GAPDH in their blocking buffers. ECL (Thermo Scientific) was used to visualize hCAP18/LL-37, CYP24A1, and GAPDH on film (GE Healthcare, Hoevelaken, The Netherlands); SuperSignal West Pico ECL Substrate (Thermo Scientific) was used to visualize p-EGFR, t-EGFR, p-ERK1/2, t-ERK1/2 using The ChemiDoc™ Touch imager in combination with Image Lab™ software (Biorad).

Immunofluorescence staining of LL-37 and CYP24A1

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 and blocked with PBS/1% BSA/0.3% Triton-X-100 (PBT) for 30 minutes at 4 °C. Cells were incubated with 1/100 diluted rabbit-anti-hCAP18/LL-37 (Innovagen, Lund, Sweden) or 1/100 diluted rabbit-anti-CYP24A1 (Sigma Aldrich) in PBT for 1 hour at RT, followed by incubation with an Alexa Fluor 488 labeled secondary antibody (1/200, Alexa Fluor 488 goat anti-rabbit IgG; Invitrogen) together with DAPI in PBT for 30 minutes at RT. 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.).

LDH assay

Submerged PBEC cultures were pre-incubated with medium alone (CNTRL; 0.25% v/v DMSO) and inhibitors of MMP (GM6001), EGFR (AG1478) and MEK (U0126) followed by stimulation for 24 h with TNF-α/IL-1β or with 0.1% Triton X-100 (used as a positive control for LDH release). Samples were 10 times diluted in PBS and the positive control was 50 times diluted in PBS. LDH release was assessed using a Roche Cytotoxicity Detection Kit (Sigma-Aldrich, Zwijndrecht, the Netherlands) according to manufactures' protocol. Percentage of LDH release was calculated as followed: (OD_{490nm} samples x dilution factor/OD_{490nm} positive control x dilution factor) x 100%.

Statistical analysis

Statistical analysis was conducted using GraphPad Prism 6 (GraphPad Software Inc., La Jolla, CA, U.S.A.) To analyze qPCR results, fold change in gene expression of the stimuli compared to control (CNTRL) was first calculated, followed by a log-transformation. Next, data were analyzed using either a one- or two-way ANOVA and the Bonferroni post-hoc test. To analyze data of the bacterial killing assay, the two-way ANOVA test was used followed by a Bonferroni post-hoc test. Data of the LDH assay were analyzed using a paired t-test and a two-way ANOVA followed by a Bonferroni post-hoc test. Differences at p values < 0.05 were considered statistically significant.

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CHAPTER

5

Interleukin 13 Exposure Enhances Vitamin D-Mediated Expression of the Human Cathelicidin Antimicrobial Peptide 18/LL-37 in Bronchial Epithelial Cells

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Abstract

Vitamin D is an important regulator of the expression of antimicrobial peptides, and vitamin D deficiency is associated with respiratory infections. Regulating expression of antimicrobial peptides, such as the human cathelicidin antimicrobial peptide 18(hCAP18)/LL-37, by vitamin D in bronchial cells requires local conversion of 25(OH)-vitamin D_3 ($25D_3$) into its bioactive metabolite, 1,25(OH)2-vitamin D3 ($1,25D_3$), by CYP27B1. Low circulating vitamin D levels in childhood asthma are associated
with more-severe exacerbations, which are often associated with infections. Atopic asthma is accompanied by Th2-driven inflammation mediated by cytokines such as interleukin 4 (IL-4) and IL-13, and the effect of these cytokines on vitamin D metabolism and hCAP18/LL-37 expression is unknown. Therefore, we investigated this with well-differentiated bronchial epithelial cells. To this end, cells were treated with IL-13 with and without $25D_3$, and expression of hCAP18/LL-37, CYP27B1, the $1,25D_3$-inactivating enzyme CYP24A1, and vitamin D receptor was assessed by quantitative PCR. We show that IL-13 enhances the ability of $25D_3$ to increase expression of hCAP18/LL-37 and CYP24A1. In addition, exposure to IL-13 resulted in increased CYP27B1 expression, whereas vitamin D receptor (VDR) expression was not significantly affected. The enhancing effect of IL-13 on $25D_3$-mediated expression of hCAP18/LL-37 was further confirmed using SDS-PAGE Western blotting and immunofluorescence staining. In conclusion, we demonstrate that IL-13 induces vitamin D-dependent hCAP18/LL-37 expression, most likely by increasing CYP27B1. These data suggest that Th2 cytokines regulate the vitamin D metabolic pathway in bronchial epithelial cells.

Introduction

The upper respiratory tract is continuously exposed to pathogens. Epithelial cells function as the front line of host defense in the lung by preventing microbes from entering the bloodstream through the physical barrier provided by the tight junctions (1) and mucociliary clearance (2). Furthermore, epithelial cells secrete antimicrobial peptides and proteins (AMPs), such as lysozyme, lactoferrin and defensins, reactive oxygen and nitrogen species, interferons, and chemokines and cytokines, which eliminate pathogens directly or indirectly via attraction of phagocytic leukocytes (3, 4). In the lung, the AMP human cathelicidin (hCAP18/LL-37) is mainly secreted by neutrophils and epithelial cells, and the active peptide LL-37 displays broad-spectrum antimicrobial activity against bacteria, fungi, and viruses. It also contributes to microbial clearance by other mechanisms, including attraction of phagocytes (5, 6). In epithelial cells, a variety of stimuli, including proinflammatory cytokines (7, 8), microbial products (9), injury (10), endoplasmic reticulum (ER) stress (11), and butyrate, a short-chain fatty acid produced by resident colonic bacteria (12, 13), have been shown to increase expression of hCAP18/LL-37. Recent studies also showed that vitamin D is an important regulator of hCAP18/LL-37 expression in epithelial cells and macrophages (4, 14). Other studies showed that microbial exposures may also increase vitamin D-mediated expression of hCAP18/LL-37 through the induction of 25-hydroxy-vitamin D-1-α-hydroxylase (CYP27B1), which converts 25-hydroxy-vitamin D_3 ($25D_3$) into the active $1,25(OH)_2$-vitaminD3 ($1,25D_3$) (15, 16). One of the well-established functions of vitamin is mediating calcium absorption and bone homeostasis. The major circulating form of vitamin D is the inactive form of vitamin D, $25D_3$, which is bound to the vitamin binding protein (VDBP) and represents the organism‘s vitamin D stock. $25D_3$ is converted into $1,25D_3$ by CYP27B1 in the kidneys. Recent data show that other extrarenal tissues, such as the skin, prostate, lung, and gastrointestinal tract, but also macrophages and monocytes, express CYP27B1 locally (15-17), indicating that vitamin D exerts its actions beyond bone mineralization and could play a role in pathological conditions. In bronchial epithelial cells, $25D_3$ is converted by the locally expressed CYP27B1 into its active metabolite, $1,25D_3$, which binds the vitamin D receptor (VDR) (15, 18). Subsequently, the $1,25D_3$-VDR complex binds to the retinoic X receptor, moves to the nuclei, and binds to vitamin D response elements present in the promoter regions of genes important for recognition and

defense against microbes, such as those encoding hCAP18/LL-37, human β-defensins 2 (hBD-2), NGAL, and CD14 (19-21). In addition, $1,25D_3$ also induces expression of the catabolic enzyme $1,25D_3$ 24-hydroxylase (CYP24A1), resulting in a negative feedback loop (22). In addition to increasing expression of AMPs, such as hCAP18/LL-37, vitamin D exerts various other effects on pulmonary immunity. Vitamin D reduces respiratory syncytial virus (RSV) or double-stranded RNA-induced expression of interleukin 8 (IL-8) in primary tracheal bronchial epithelial cells (23). In addition, vitamin D induces IL-10 secretion by T regulatory cells (Tregs) and therefore may possibly restore defects in IL-10 production by Tregs in steroid-resistant asthma (24). Several epidemiological studies suggest associations between vitamin D status and lung diseases, such as asthma, chronic obstructive pulmonary disease (COPD), and respiratory infections, including tuberculosis (25). In asthma, reduced serum levels of vitamin D are associated not only with lower lung function (24, 26, 27) but also with increased airway hyperresponsiveness, reduced corticosteroid sensitivity (24), and more-severe exacerbations in children (28). The Th2 cytokines IL-13 and IL-4 play an important role in asthma and allergic disorders and are locally produced in the airway wall in atopic asthma (29). These cytokines may modulate susceptibility to bacterial infections, as demonstrated in, e.g., keratinocytes and airway epithelial cells (30-32). This is important, since the bacterial load is higher in asthmatics than in healthy controls and the composition of the bacterial communities in the airways is different from that in healthy subjects, which is associated with the severity of airway hyperresponsiveness (33). In addition, exacerbations of asthma, which are caused mainly by viral infections (34), are also major problem and frequently require hospitalization. Vitamin D may play a protective role in the severity of airway hyperresponsiveness and development of exacerbations, which may be partially explained by its ability to regulate expression of AMPs and reduce airway inflammation. However, the effect of Th2 cytokines on vitamin D responses is not understood. The aim of the present study was to investigate the role of the Th2 cytokine IL-13 on $25D_3$-dependent expression of antimicrobial peptides in well-differentiated bronchial epithelial cells. First, the release of proinflammatory cytokines and chemokines and expression of hCAP18/LL-37 after exposing the cells to poly(I:C) with and without $25D_3$ were determined. Next, the effects of the Th2 cytokine IL-13 on the expression and release of hCAP18/LL-37 in $25D_3$-exposed cells were examined and subsequently expression of genes involved in the vitamin D metabolic pathway, e.g., those encoding CYP24A1, CYP27B1, and VDR, was assessed to examine the mechanisms

of action of IL-13. Finally, antimicrobial activity of the $25D_3$- and/or IL-13-stimulated cells was assessed using an antimicrobial assay.

Materials and Methods

Cell culture

Primary bronchial epithelial cells obtained from tumor-free bronchial lung tissue from anonymous donors, acquired during lung resection surgery for lung cancer, were cultured as described previously (35). Briefly, cultures of bronchial epithelial cells (passage 1) were first expanded and then grown submerged on PureCol (Advanced BioMatrix, San Diego, CA) and fibronectin-coated 24-mm Transwell-clear culture inserts (Corning Costar Corporation, Cambridge, MA) in humidified air supplemented with 5% (vol/vol) CO_2. When the cells reached confluence, they were cultured exposed to air, and 3 times a week the apical side was washed in phosphate-buffered saline (PBS) and the basal side refreshed with fresh Dulbecco's modified Eagle medium (DMEM)-bronchial epithelial cell growth medium (BEGM) (1:1) (Invitrogen, Breda, The Netherlands, and Lonza, Breda, The Netherlands, respectively) supplemented with 15 ng/ml retinoic acid (Lonza). During 14 days of air-exposed culture, the cells started producing mucus and develop cilia (36).

Cell exposures

Differentiated bronchial epithelial cells were exposed at the apical (in 100 µl PBS) and basal sides to poly(I:C) (5 and 50 µg/ml; Invivogen, Toulouse, France) with or without $25D_3$ (100 nM, Merck, Darmstadt, Germany) for 24 h in duplicate. Tumor necrosis factor alpha (TNF-α) and IL-1β (20 ng/ml; Peprotech, Rocky Hill, NJ) were included as positive controls. In experiments to assess the effects of IL-13 on $25D_3$ responses, cells were pretreated with 100 ng/ml IL-13 (Peprotech) to induce the CYP27B1 protein prior to exposing the cells to $25D_3$ for 24 h and then exposed for another 48 h in triplicate to IL-13 with and without 10^{-7} M $25D_3$ or 10^{-7} M $1,25D_3$ (Merck), included as a positive control. Antibiotics-free medium was used when an antimicrobial assay was performed. Basal medium was collected and either used for enzyme-linked immunosorbent assay (ELISA) or for Western blot analysis of hCAP18/LL-37 release. For Western blot analysis of hCAP18/LL-37 release, basal medium was pooled and purified using Oasis HLB 1-ml extraction cartridges (Waters Chromatography, Etten-Leur, The Netherlands), and the eluate was dried

by vacuum centrifugation (Christ Rvc2-25 Vacuüm system) (2). Lyophilized protein samples were resuspended in 100 µl SDS-PAGE sample buffer (12% SDS, 30% glycerol, and 150 mM Tris-HCl, pH 7.0). Apical surface fluid was collected using 100 µl 10 mM sodium phosphate buffer for antimicrobial assays. Cells were either fixed in 1% paraformaldehyde (Merck) for immune fluorescence (IF) or lysed in either RNA lysis buffer RP1 (Macherey-Nagel, Düren, Germany) or in ice-cold RIPA buffer (50 mM Tris [pH 7.4], 150 mM NaCl, 100 mM Na_3VO_4, 100 mM phenylmethylsulfonyl fluoride [PMSF], 100 mM EDTA, 1% Triton X-100, 1% sodium dodecyl sulfate [SDS], containing a protease inhibitor tablet [Complete Mini; Roche Diagnostics, Almere, The Netherlands]). RIPA lysates were incubated for 30 min at 4°C, sonicated, and centrifuged at 10,000 g for 10 min. Supernatants were collected, with protein levels determined using a bicinchoninic acid (BCA) protein assay kit (Thermo Scientific, Etten-leur, The Netherlands), and stored at -20°C until use.

RNA isolation, reverse transcription (RT), and qPCR

Total RNA was extracted using the nucleospin RNA kit (Macherey-Nagel) and quantified using the Nanodrop ND-1000 UV-visibile (UV-Vis) spectrophotometer (Nanodrop Technologies, Wilmington, DE). For cDNA synthesis, 1 µg of total RNA was reverse transcribed using oligo(dT) primers and Moloney murine leukemia virus (M-MLV) polymerase (Promega, Leiden, The Netherlands) at 37°C. All quantitative PCRs (qPCRs), using primers shown in Table 1, were carried out in triplicate on a MyiQ singlecolor real-time PCR detection system or a CFX-384 real-time PCR detection system (Bio-Rad Laboratories, Veenendaal, The Netherlands) with the use of IQ SYBR green supermix (Bio-Rad). The relative standard curve method was used to calculate arbitrary gene expression using the IQ5 software program (Bio-Rad). Three reference genes, encoding β2-micro- globulin (*B2M*), β-actin (*ACTB*), and phospholipase A2 (*YWHAZ*), selected using the "Genorm method" (Genorm; Primer Design, Southampton, United Kingdom), were included to calculate normalized gene expression.

Gene	Tm	Sequence forward primer	Sequence reverse primer	GenBank accession no.
CAMP **(hCAP18/ LL-37)**	64,3	TCATTGCCCAGGTCCTC AG	TCCCCATACACCGCTTCAC	NM_004345.3
CYP24A1	59	TTGGCTCTTTGTTGGATT GTCCGC	TGAAGATGGTGCTGACAC AGGTGA	NM_000782.3
CYP27B1	64,4	AACCCTGAACAACGTAG TCTGCGA	ATGGTCAACAGCGTGGAC ACAAA	NM_000785.3
VDR	58	ACCTGGTCAGTTACAGC ATCC	TGGTGAAGGACTCATTGG AGC	NM_001017535.1
DEFB4 **(hBD-2)**	62	ATCAGCCATGAGGGTCT TG	GCAGCATTTTGTTCCAGG	NM_004942
DEFB3 **(hBD-3)**	62	AGCCTAGCAGCTATGAG GATC	CTTCGGCAGCATTTTGCGC CA	NM_001081551

Table 1. Primer *Tm* and sequences of hCAP18, CYP24A1, CYP27B1, VDR, hBD-2 and hBD-3 genes

SDS-PAGE Western blot

For detection of hCAP18/LL-37, protein samples were dissolved in SDS sample buffer, containing 40 mM 1,4-dithioerythritol (DTE), 6% (wt/vol) SDS, 21% (vol/vol) glycerol, and 160 mM Tris-HCl (pH 7.0), heated for 5 min at 100°C, and applied on a 16.5% Tris-Tricine gel as previously described (37). For detection of CYP27B1 and β-actin, 30 µg protein lysate/lane was dissolved in SDS sample buffer containing 50 mM dithiothreitol (DTT), 4% (wt/vol) SDS, 20% (vol/vol) glycerol, and 200 mM Tris-HCl (pH 7.0), heated for 5 min at 100°C, and applied on a 10% SDS-PAGE gel. Next, proteins were blotted on a polyvinylidene difluoride (PDVF) membrane, and nonspecific binding sites were blocked in PBS containing 5% (vol/vol) heat-inactivated newborn calf serum and 5% (wt/vol) skimmed milk for hCAP18/LL-37 detection, in PBS containing 5% (wt/vol) skimmed milk for detection of CYP27B1, or in PBS containing 0.1% (vol/vol) Tween 20 and 1% (wt/vol) bovine serum albumin (BSA) for detection of β-actin. Membranes were probed with 1/400 diluted monoclonal anti-hCAP18/LL-37, clone 1.1.C12 (Hycult Biotech, Uden, The Netherlands), 1/100-diluted goat-anti-CYP27B1 (Santa Cruz clone C-12; Bio-Connect B.V., Huissen, The Netherlands), or 1/10,000-diluted mouse monoclonal anti-β-actin (clone AC-74; Sigma- Aldrich, Zwijndrecht, The Netherlands) in their blocking buffer. Next, the membranes were incubated with 1/1,000-diluted goat anti-mouse– horseradish peroxidase (HRP)-conjugated antibody (Dako) for hCAP18/LL-37 and β-actin detection or in rabbit anti-goat–HRP-conjugated

antibody (Dako) for detection of CYP27B1. Finally, enhanced chemiluminescence (ECL) was used to visualize hCAP18/LL-37, CYP27B1, and β-actin on film (GE Healthcare, Hoevelaken, The Netherlands).

Measurement of IP-10, IL-6, CCL5, CXCL8, and hBD-3

Protein levels in basal media of gamma interferon-induced protein 10 (IP-10), IL-6 (Hycult Biotech, Uden The Netherlands), chemokine (C-C motif) ligand 5 (CCL5) (R&D Systems, Minneapolis, MN), chemokine (C-X-C motif) ligand 8 (CXCL8) (Sanquin, Amsterdam, The Netherlands) and hBD-3 (Alpha Diagnostics, San Antonio, TX) were assessed using a sandwich ELISA according to the manufacturer's protocols.

Antimicrobial assay

Pseudomonas aeruginosa, clone PAO1 (ATCC, LGC Standards GmbH, Wesel, Germany) (32) was used to investigate the effects of both IL-13 and $25D_3$ on the antibacterial activity of bronchial epithelial cells. A single colony of PAO1 was inoculated into LB broth overnight. One part culture volume was transferred into nine parts of fresh LB broth and incubated for 3 h to obtain bacteria in the mid-log phase of the growth curve. Two hundred fifty CFU of bacteria, determined at a wavelength of 620 nm, in 42.5 µl 10 mM sodium phosphate buffer was mixed with 7.5 µl of apical surface fluid (ASF) and incubated shaking for 3 h at 37°C. The mixtures were next plated on LB plates and incubated overnight at 37°C to assess surviving bacteria by CFU determination.

Immunofluorescence staining of hCAP18/LL-37 in bronchial epithelial cells

Cells that were fixed on Transwell inserts in 1% paraformaldehyde were permeabilized with PBS–1% BSA–0.05% Tween 20 for 30 min at 4°C and next fixed in methanol for 20 min at 4°C. Primary antibodies were incubated for 1 h at room temperature (rabbit-anti-hCAP-18/LL-37, 1/100, (Innovagen, Lund, Sweden) in PBS–1% BSA (PBS-BSA) with the cells, followed by incubation with an Alexa Fluor 647-labeled secondary antibody (1/200, Alexa Fluor 647 goat anti-rabbit IgG; Invitrogen) together with DAPI in PBS-BSA for 30 min at room temperature. Images were acquired using a confocal laser scanning microscope (Zeiss LSM510; Zeiss, Jena, Germany).

Statistical analysis

Results are expressed as means ± standard errors of the means (SEM), and data were analyzed using the paired Student *t* test. Differences at P values of < 0.05 were considered statistically significant.

Results

$25D_3$ suppresses poly(I:C)-induced release of proinflammatory mediators, while $25D_3$-mediated hCAP18/LL-37 expression is partly inhibited by poly(I:C) in well-differentiated bronchial epithelial cells

We first determined whether our bronchial epithelial cells that were cultured at the air-liquid interface responded to the inactive $25D_3$. Previously it was shown that $25D_3$ decreased expression of chemokine CXCL8 induced by poly(I·C) (15). To investigate whether $25D_3$ also reduces chemokine and cytokine expression in our well-differentiated bronchial epithelial cell cultures, cell cultures from 10 different donors were incubated with 50 and 5 μg/ml poly(I·C) in the presence or absence of $25D_3$ for 24 h. Prior to the collection of the supernatants, cells were visually checked for any toxic effects and showed no signs of any toxicity. Poly(I:C) induced release of CXCL8, CCL5, IP-10, and IL-6. Release of IP-10 and CCL5 was suppressed in the presence of $25D_3$, whereas a trend toward suppression was observed in CXCL8 and IL-6 release by $25D_3$ (Figure 1A). These data suggest that in addition to poorly differentiated submerged cultures of tracheobronchial epithelial cells (15), well-differentiated bronchial epithelial cells also respond to $25D_3$ and are most likely to be able to convert inactive $25D_3$ into active $1,25D_3$. Poly(I:C) was previously found to increase $25D_3$-mediated expression of hCAP18/LL-37 in submerged tracheobronchial epithelial cells via induction of the $25D_3$-activating enzyme CYP27B1 (25). In contrast, when we examined this in air-liquid-interface cultured cells, poly(I:C) was found to decrease $25D_3$-induced hCAP18/LL-37 expression (Figure 1B).

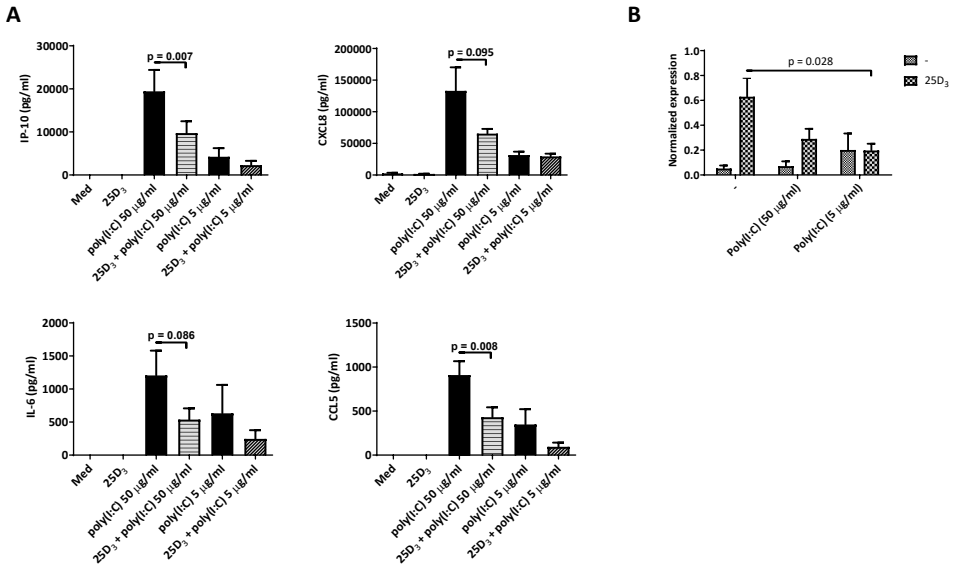

Figure 1. **Effect of 25D3 and poly(I:C) on hCAP18/LL-37 expression and cytokine and chemokine release by bronchial epithelial cells.** Well-differentiated epithelial cells were stimulated with poly(I·C) (at 50 and 5µg/ml) with and without 10^{-7} M $25D_3$ and by $25D_3$ alone for 24 h in duplicate. (A) Inflammatory mediators were measured in basal medium using ELISA. Data are shown as means ± SEM (n = 10 different donors). (B) Quantitative PCR was used to assess relative mRNA expression for hCAP18/LL-37. The expression of the β2-microglobulin, β-actin, and phospholipase A2 reference genes was used to calculate normalized gene expression. Data are represented as means ± SEM.

IL-13 increases 25D3-mediated hCAP18/LL-37 expression and release in bronchial epithelial cells

To examine whether the Th2 cytokine IL-13 affects vitamin D-mediated hCAP18/LL-37 expression in air-liquid-interface cultures, we assessed hCAP18/LL-37 expression after preincubating the cells with IL-13 for 24 h to examine the effect of allergic airway inflammation on responses to vitamin D. $25D_3$ treatment following IL-13 exposure increased hCAP18/LL-37 mRNA compared to the effects of $25D_3$ treatment alone (Figure 2A). This was confirmed at the protein level by SDS PAGE Western blot analysis showing an increased hCAP18/LL-37 protein release in basal medium after exposure to IL-13 and $25D_3$ compared to the effects of $25D_3$ alone (Figure 2B). In addition to Western blotting, the findings were also confirmed using immunofluorescence staining (IF) of hCAP18/LL-37 on bronchial epithelial cells. We observed an intense staining of hCAP18/LL-37, which was increased after exposure to IL-13 and $25D_3$ compared to that with $25D_3$ alone. hCAP18/LL-37 staining was

presented in a granular pattern (Figure 2C), which was also found in keratinocytes by Braff and colleagues (38).

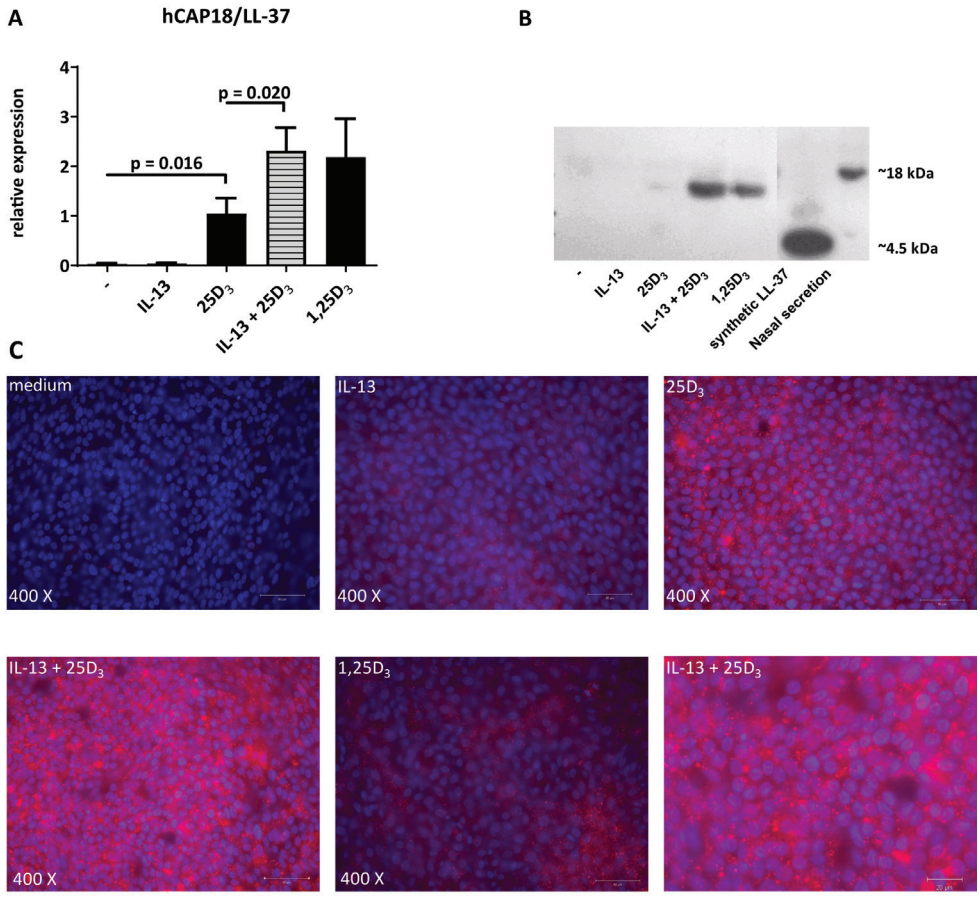

Figure 2. Effect of IL-13 on $25D_3$-mediated induction of hCAP18/LL-37 in bronchial epithelial cells. Cells were pretreated with 100 ng/ml IL-13 for 24 h and then exposed for another 48 h in duplicate to 100 ng/ml IL-13 with and without 10^{-7} M $25D_3$ or 10^{-7} M $1,25D_3$, included as a positive control. (A) Quantitative PCR was used to assess relative mRNA expression for hCAP18/LL-37. The expression of the β2-microglobulin, β-actin, and phospholipase A2 reference genes was used to calculate normalized gene expression. Data are represented as means ± SEM(n = 7). (B) SDS-PAGE, followed by Western blot analysis, was used to assess LL-37 production in basal medium of the exposed cells. This Western blot is illustrative of 5 separate experiments using different donors. Nasal secretion and 25 pg synthetic LL-37 were used as positive controls to show intact hCAP18 and mature LL-37 peptide, respectively. (C) Immune fluorescence staining of hCAP18/LL-37 in bronchial epithelial cells. DAPI (blue) was used to stain the nuclei, and rabbit anti-LL-37 antibody together with Alexa Fluor 647 goat anti-rabbit IgG (red) were used for detection of hCAP18/LL-37. The detailed picture shows hCAP18/LL-37 as a granular staining pattern in the cytoplasm of the cells.

Effect of IL-13 on expression of VDR, CYP24A1, and CYP27B1

To investigate possible mechanisms underlying the effect of IL-13 on $25D_3$-induced hCAP18/LL-37 expression, cells were exposed to IL-13 for 24 h and next incubated for another 48 h in the presence of IL-13 and $25D_3$ to assess expression of VDR, CYP27B1, and CYP24A1. In addition to increasing hCAP18/LL-37, preincubation with IL-13 also increased the effect of $25D_3$ treatment on CYP24A1 mRNA (Figure 3A). Whereas IL-13 caused a significant increase in the expression of CYP27B1, the observed increase in VDR expression did not reach statistical significance (Figure 3B and C). The increase of CYP27B1 mRNA expression by IL-13 was furthermore confirmed by analysis of the CYP27B1 protein in a Western blot (Figure 3D). To verify whether related Th2 cytokines also induce CYP27B1 expression in bronchial epithelial cells, cells from two different donors were exposed to 20 ng/ml of IL-4 for 24 h, resulting in an increased expression of CYP27B1 mRNA (see Figure S1 in the supplemental material). The observation that IL-13 increased $25D_3$-mediated expression of both CYP24A1 and hCAP18/LL-37 suggested that the effect of IL-13 resulted from increased expression of CYP27B1.

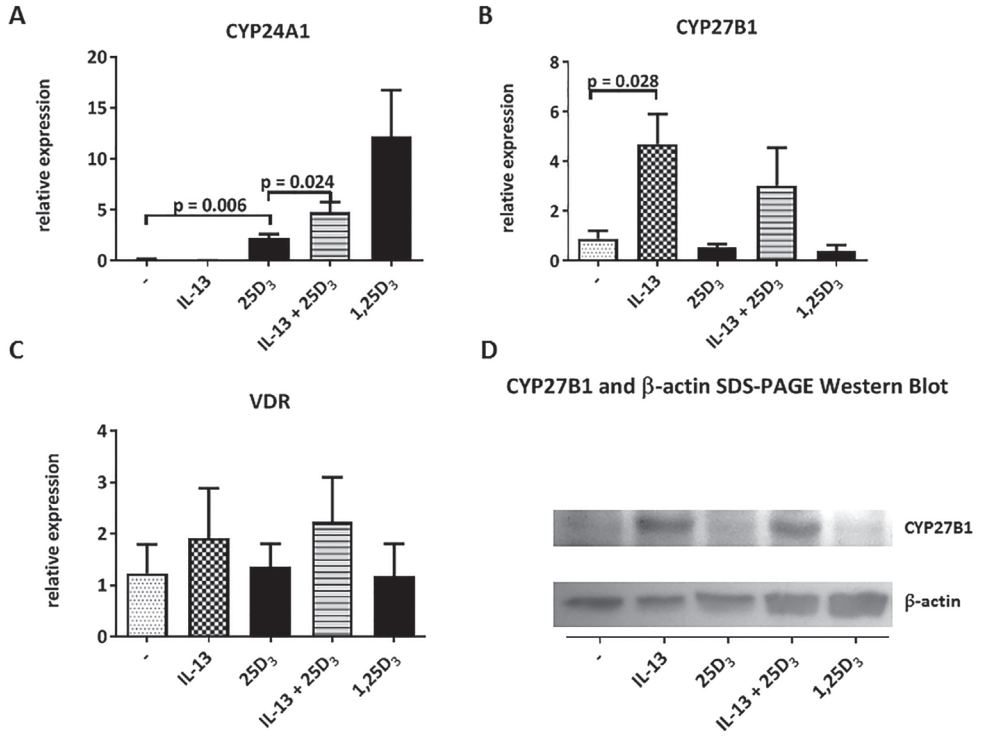

Figure 3. Effect of IL-13 on expression of CYP24A1, CYP27B1, and VDR in bronchial epithelial cells. Cells (n = 5 to 7 donors) were pretreated with 100 ng/ml IL-13 for 24 h and then exposed for another 48 h in duplicate to 100 ng/ml IL-13 with and without 10^{-7} M $25D_3$ or 10^{-7} M $1,25D_3$, included as a positive control. Quantitative PCR was used to assess relative mRNA expression for CYP24A1 (A), CYP27B1 (B), or VDR (C). Data are represented as means ± SEM. SDS-PAGE followed by Western blot analysis was used to assess CYP27B1 and β-actin in protein lysates of the treated cells (D).

IL-13 increases hBD-3 expression

In addition of hCAP18/LL-37, epithelial cells also express a variety of other antimicrobial peptides. To investigate whether expression of other antimicrobial peptides was affected by IL-13 and $25D_3$ or $1,25D_3$, hBD-2 and hBD-3 expression was analyzed using qPCR (Figure 4A and B). Whereas hBD-2 was not affected by these stimuli, hBD-3 expression was increased in IL-13-treated cells. An ELISA was performed, using apical surface fluid (ASF) to confirm increased expression of hBD-3 by IL-13 at the protein level (Figure 4C). In contrast to what was observed for hBD-3 mRNA, IL-13 exposure did not increase hBD-3 peptide in ASF.

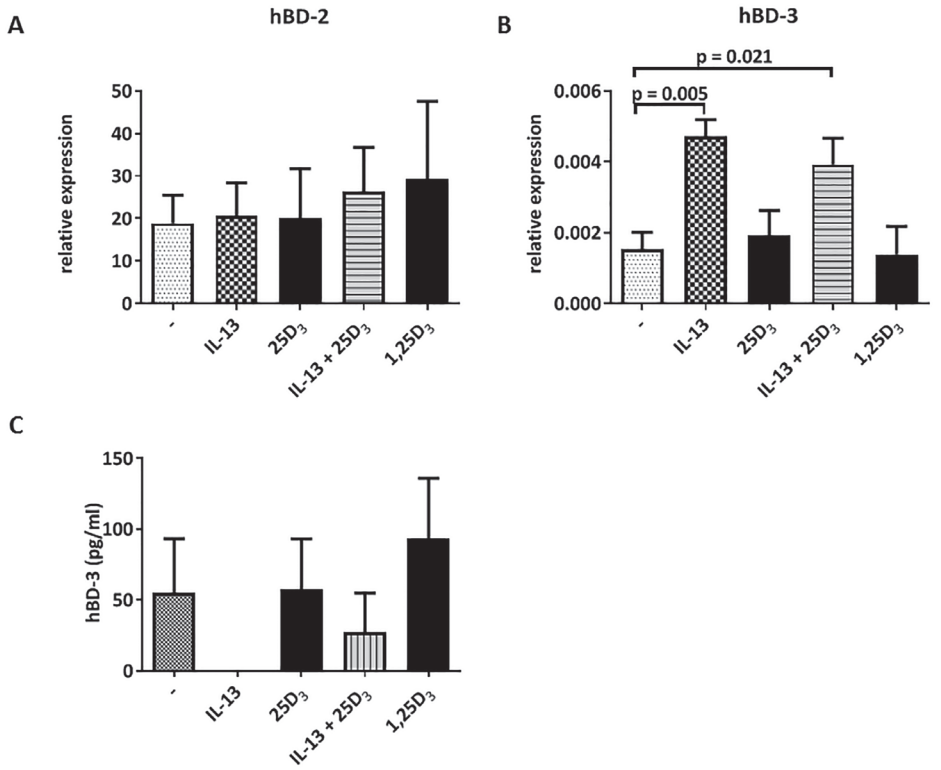

Figure 4. Effect of IL-13 on expression and/or release of hBD-2 and hBD-3 in bronchial epithelial cells. Cells (n = 5 to 7 donors) were pretreated with 100 ng/ml IL-13 for 24 h and then exposed for another 48 h in duplicate to 100 ng/ml IL-13 with and without 10^{-7} M $25D_3$ or 10^{-7} M $1,25D_3$, included as positive control. Quantitative PCR was used to assess relative mRNA expression for hBD-2 and hBD-3. Data are represented as means ± SEM. (A) hBD-2 expression. (B) hBD-3 expression. (C) Apical hBD-3 release was measured in apical surface fluid (ASF) using ELISA. Cells from only 3 out of 6 donors had detectable hBD-3 levels. Data are shown as mean ± SEM.

Both $25D_3$ and $1,25D_3$ increase antimicrobial activity against *Pseudomonas aeruginosa*

To explore whether the increased release of hCAP18/LL-37 in IL-13- and $25D_3$-exposed bronchial epithelial cells was also accompanied by increased antimicrobial activity, an antimicrobial activity assay was performed using ASF incubated with *Pseudomonas aeruginosa* PAO1 (Figure 5). ASF of cells stimulated with $25D_3$ or $1,25D_3$ displayed increased antimicrobial activity against PAO1. However, exposure to IL-13 did not further increase the antimicrobial activity compared to results with $25D_3$ alone.

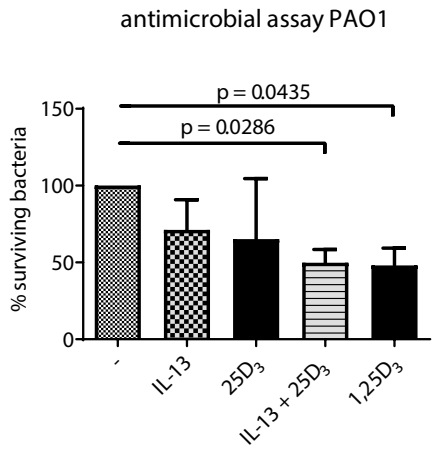

Figure 5. Effect of IL-13 on $25D_3$-mediated antimicrobial activity. Apical surface fluid of cells (n = 3) was pretreated with 100 ng/ml IL-13 for 24 h and then exposed for another 48 h in triplicate to 100 ng/ml IL-13 with and without 10^{-7} M $25D_3$ or 10^{-7} M $1,25D_3$, included as a positive control in antibiotics free medium. After 48 h, apical surface fluid was harvested using phosphate buffer (ASF), and pooled. *P. aeruginosa* (PAO1) was used to determine antimicrobial activity in ASF, which is shown as % surviving bacteria.

Discussion

In the present study, we have shown that exposure to IL-13 increases the ability of $25D_3$ to induce expression of hCAP18/LL-37 and CYP24A1. The induced expression of hCAP18/LL-37 by IL-13 in $25D_3$-treated cells was furthermore confirmed by Western blot analysis and immunofluorescence staining. IL-13 alone did not affect expression of hCAP18/LL-37 and hBD-2 but did increase expression of hBD-3. However, the ELISA data show that hBD-3 was not increased in the apical surface fluid after IL-13 exposure. Secretions from cells treated with $25D_3$ or $1,25D_3$ showed increased antimicrobial activity against *P. aeruginosa* compared to data for control-treated cells. However, the antimicrobial activity was not further increased by IL-13. Analysis of genes involved in vitamin D response and metabolism showed that exposure to IL-13 induced expression of CYP27B1 but did not significantly increase expression of the VDR. However, a small but nonsignificant increase in VDR expression was observed in cells after exposure to IL-13. These results suggest that the effect of IL-13 on $25D_3$-mediated expression of hCAP18/LL-37 and CYP24A1 is

mediated mainly by the enhanced conversion of $25D_3$ into $1,25D_3$ caused by increased expression of CYP27B1.

Results from previous studies in epithelial cells that investigated the effects of vitamin D on hCAP18/LL-37 expression are in line with our results showing that $25D_3$ increases expression and release of hCAP-18/LL-37. However, whereas poly(I·C) increased $25D_3$-mediated hCAP18/LL-37 expression in other studies (15), we observed a decrease. Our study adds to these studies that we used fully mucociliary differentiated air-liquid-interface cultures instead of the poorly differentiated submerged cultures used in the other studies, which may possibly explain the observed difference in effects of poly(I:C) on hCAP18/LL-37 expression. We also demonstrate for the first time that the Th2 cytokine IL-13 increases vitamin D-induced expression of hCAP18/LL-37, most likely by increasing expression of the $25D_3$-activating CYP27B1. A recent study by Liu *et al*. reported increased levels of LL-37, $1,25D_3$, and $25D_3$ in bronchoalveolar lavage (BAL) fluid from allergic subjects after segmental allergen challenge, which is in line with our observations (39). However, their observations could be partly explained by vascular leakage. In addition to the effect of IL-13 on vitamin D-induced expression of hCAP18/LL-37 that was observed in the present study, IL-17A, which is also elevated in asthmatics, also increases vitamin D-mediated expression of hCAP18/ LL-37 in keratinocytes (40). In contrast to what was observed for IL-13 and also for IL-4, we observed that IL-17A did not increase induction of CYP27B1 expression (see Figure S1 in the supplemental material). A MEK-extracellular signal-regulated kinase (ERK)-dependent mechanism was shown to increase vitamin D-induced hCAP18/LL-37 expression by IL-17A (40). In addition to IL-13 in bronchial epithelial cells, transforming growth factor-β (TGF-β) also increases expression of CYP27B1 in keratinocytes (41), resulting in increased vitamin D-dependent hCAP18/LL-37 expression, which is likely important for protection against pathogens during the wound-healing process (42). CYP27B1 expression is also induced by Toll-like receptor 2 (TLR2) agonists in keratinocytes and macrophages (20) and by TLR3 agonists in poorly differentiated bronchial epithelial cells (15). Effects of Th2 cytokines alone on expression of antimicrobial peptides and activity have been investigated previously in epithelial cells, showing inconsistent results. For example, Beisswenger and colleagues reported decreased hBD-2 expression and antimicrobial activity after 4 days' exposure to IL-4 or IL-13 followed by exposure to *P. aeruginosa* and IL-4 or IL-13 (31), while we have previously shown that expression of hBD-2 and hCAP18/LL-37 was increased after 14 days' exposure to IL-

4 and IL-13 (32). In addition to these observations in bronchial epithelial cells, IL-4 and IL-13 were shown to inhibit TNF-α- and IFN-γ- induced hBD-2 and hBD-3 expression in keratinocytes (30), while increased expression of various antimicrobial genes in the gastrointestinal tract of IL-9 transgenic mice was dependent on IL-13 (43).

We observed that $25D_3$ increases both expression and release of hCAP18/LL-37. In addition, we show increased killing of *P. aeruginosa* in apical surface fluid obtained from cells exposed to $1,25D_3$ or the combination of IL-13 and $25D_3$. However, in cells pretreated with IL-13 subsequently exposed to $25D_3$, we did not detect additional killing of *P. aeruginosa* compared to results with $25D_3$ alone, which is in contrast to what was expected based on the findings in our qPCR, Western blot, and immunofluorescence studies. To check if hCAP18/LL-37 was not released only at the basal side, we used the dot blot technique and observed that hCAP18/LL-37 was also increased in the apical airway surface fluid of these cells stimulated with IL-13 and $25D_3$ compared to findings with $25D_3$ alone (see Figure S2A in the supplemental material). This apically secreted hCAP18/LL-37 may have been inhibited by mucins, since IL-13 also increases mucin production (44), which was confirmed in this study by showing increased MUC5AC protein in apical surface fluid from IL-13-treated cells (see Figure S2B). Therefore, the excess anionic mucus present in the apical washes of IL-13-treated cells may have bound the cationic LL-37 in our samples, thereby reducing antimicrobial activity (45). In the lung, the mucus layer is separated from the epithelium by a periciliary layer, both of which are likely mixed during collection of apical surface fluid by washing. A recent study has shown that membrane-bound mucins form a barrier to prevent the mucus from penetrating the periciliary space (46) and thus may protect the AMPs present in this periciliary space from being inhibited by secreted mucins. Furthermore, the membrane-bound mucins that form this barrier may also bind secreted AMPs, which

may therefore not be fully collected by apical washings. In addition, IL-13 might also decrease expression of other AMPs or e.g. affect ion concentrations (47), which may also result in suboptimal

killing of pathogens by hCAP18/LL-37. Analysis of other AMPs, such as hBD-2 and hBD-3, by qPCR did not reveal a decrease in expression of these AMPs by IL-13. Whereas hBD-2 was not affected, hBD-3 expression was significantly higher after IL-13 treatment. However, this was in contrast with the hBD-3 levels in the apical surface fluid and could possibly be explained by the presence of anionic mucins in

the apical surface fluid binding the cationic hBD-3, resulting in lower detection of the hBD-3 peptide.

Nevertheless, since IL-13 exposure may also decrease the expression of other antimicrobial molecules, as has been shown for short palate, lung, and nasal epithelium clone 1 (SPLUNC1) in primary airway epithelial cells (48-50), this possibility cannot be formally ruled out.

Previously it was shown that airway surface fluid has a complex composition of antimicrobial mediators, suggesting that they might act in concert to kill pathogens (51). At 20 µg/ml of LL-37, *P. aeruginosa* survival was reduced by 90% in our experiments (data not shown). However, the amount of the detected hCAP18/LL-37 protein is likely lower than the 25 pg synthetic LL-37 that was used for comparison in our Western blot. It is therefore likely that the observed increase of antimicrobial activity against *P. aeruginosa* by $25D_3$ or $1,25D_3$, cannot be explained only by the induction of hCAP18/LL-37 but may have resulted from the synergistic action with other antimicrobial substances present in the apical surface fluid (52, 53). We demonstrate that exposing the cells to 25D3 with and without IL-13 and $1,25D_3$ induced release of hCAP18/LL-37 in basal medium. Various studies have shown that extracellular hCAP18 is cleaved by proteases releasing the antimicrobial C-terminal cationic 4.5-kDa peptide LL-37 and a 14-kDa N-terminal cathelin protein. Other studies also reported Western blots showing proteins containing LL-37 immunoreactivity at 14 kDa. Edfeldt and colleagues reported the presence of a 14-kDa protein that bound anti-LL-37 antibodies in atherosclerotic lesions (54). In addition, Oren and colleagues reported that dimers or trimers of LL-37 may occur in solution (54, 55). Therefore, it is possible that the 14-kDa anti-LL-37 immunoreactive peptides detected in our experiments were in fact polymers of 4.5-kDa LL-37 peptides or partially cleaved hCAP18.

Effects of Th2 cytokines on expression of other cytochrome P450 (CYP) molecules that are not involved in vitamin D metabolism were demonstrated earlier. Stoilov and colleagues showed that expression of Cyp genes, such as those encoding Cyp4f18, Cyp5a1, and Cyp7b1, were elevated in ovalbumin (OVA)-induced allergic airway disease in mice (56). In addition, IL-4 was shown to increase CYP2E1, most likely via protein kinase C (57). In this study, we report for the first time that IL-13 increases expression of CYP27B1. However, future studies are needed to unravel the pathway by which IL-13 increases expression of CYP27B1 in bronchial epithelial cells.

Our study has some limitations. We were not able to detect increased generation of $1,25D_3$ in IL-13- and $25D_3$-exposed cell cultures, since these levels were below detection limits. Indirect evidence for the generation of increased $1,25D_3$ was the observation that IL-13 also increased $25D_3$-induced expression of CYP24A1. Moreover, whereas the presence of hCAP18/LL-37 in basal medium was readily detected by Western blotting, in the apical surface fluid hCAP18/LL-37 was demonstrated only by dot blot analysis.

What is the clinical relevance importance of our findings? Viral and bacterial infections are a major cause of exacerbations in asthmatics. Viral infections cause release of proinflammatory cytokines and chemokines that mediate recruitment of various other inflammatory cells, which may contribute to inflammation during exacerbation in asthmatics. In the present study, we provide evidence that virus-induced inflammation may be controlled by vitamin D and that vitamin D activation may be increased in atopic asthmatic airways resulting from Th2 inflammation. Importantly, our results show that in the presence of IL-13, vitamin D exposure results in higher expression of hCAP18/LL-37. This cathelicidin displays broad-spectrum antimicrobial activity, affects inflammation and immunity, and stimulates wound repair (42, 58). Therefore, increased cathelicidin expression in asthma may contribute to increased host defense and wound repair. It is important to stress that epithelial cells produce much smaller amounts of hCAP18/LL-37 than neutrophils (59). Therefore, epithelial hCAP18/LL-37 may act initially by preventing the pathogens from disrupting the epithelial barrier. After disruption of the epithelial barrier, epithelial cell-derived hCAP18/LL-37 may serve as an alarmin by attracting neutrophils by its direct chemotactic activity or indirectly by increasing the release of CXCL8 from e.g., bronchial epithelial cells as well as airway smooth muscle cells (60, 61). Thus, recruited neutrophils could contribute to more-effective killing of pathogens and healing of the disrupted epithelium.

In conclusion, our study shows that the Th2 cytokine IL-13 increases expression of the $25D_3$-activating CYP27B1, resulting in conversion of $25D_3$ into its active metabolite $1,25D_3$ and expression of the antimicrobial peptide hCAP18/LL-37 in bronchial epithelial cells. These data suggest that the vitamin D metabolic pathway in these cells, important in modulating the host defense against viral and bacterial infections, might be enhanced by Th2 cytokines.

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Supplemental data

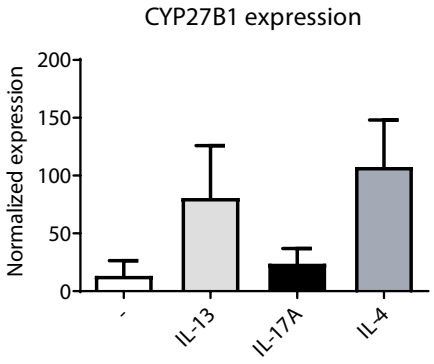

Figure S1. Effect of IL-17A and IL-4 on CYP27B1 expression in bronchial epithelial cells. Bronchial epithelial cells (n= 2 donors) were exposed to 20 ng/ml IL-4 or 10 ng/ml IL-17A for 24 hours in duplicate. RNA isolation, cDNA synthesis, and quantitative PCR were performed as described in the Materials and Methods section. Quantitative PCR was used to assess relative mRNA expression for CYP27B1. Data are represented as means ± SEM, and show that IL17A does not affect expression of CYP27B1.

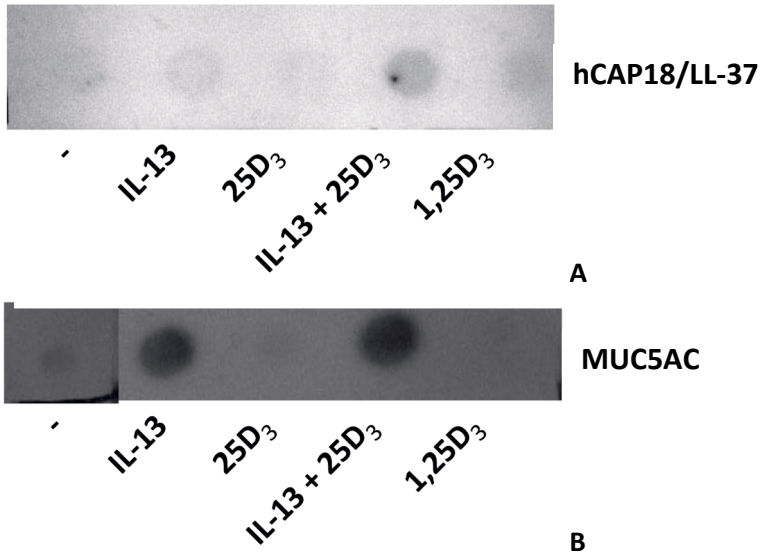

Figure S2 A-B. Effects of IL-13 and 25D3 on apical release of hCAP18/LL-37 and MUC5AC in bronchial epithelial cells. Cells of 2 different donors were pretreated with 100 ng/ml IL-13 for 24 hours and then exposed for another 48 hours in duplicate to 100 ng/ml IL-13 with and without 10^{-7} M $25D_3$ (or 10^{-7} M $1,25D_3$ that was included as positive control). Dot blot analysis was used to assess LL-37 (A) and MUC5AC (B) production in ASF of the exposed cells. To determine apical release of MUC5AC protein and hCAP18/LL-37 protein, ASF of the cultures was diluted and spotted on a methanol pre-incubated PVDF-membrane using a Bio-Dot Microfiltration apparatus (Bio-Rad), and subsequently blocked with PBS/5% skimmed milk for MUC5AC detection or with PBS/5% heat-inactivated new born calf serum/5% skimmed milk overnight at 4°C. For detection 2 µg/ml of mouse-anti-MUC5AC (clone 45M1;Labvision Neomarkers, Fremont, CA) or monoclonal anti-hCAP18/LL-37, clone 1.1.C12 (Hycult Biotech) was used. A HRP-conjugated goat-anti-mouse IgG (subclasses 1+2a+2b+3antibody) (Jackson ImmunoResearch Laboratory, Bar Harbor, ME) was used as a secondary antibody for MUC5AC and a HRP-conjugated goat-anti mouse Ig antibody(Dako) for the LL-37 dot blot and detected using ECL Western blotting substrate (Pierce, Rockford, IL). These Dot blots are both representative of 2 separate experiments using different donors. Medium control (-) of Figure S2B was derived from a different part of the same membrane. Dot blot results show that hCAP18/LL-37 is increased in ASF after treatment with both $25D_3$ and IL-13, and that exposure of the cells to IL-13 resulted in an increase of apical MUC5AC release.

CHAPTER

Prevention of exacerbations in patients with COPD and vitamin D deficiency through vitamin D supplementation (PRECOVID): a study protocol

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Abstract

Background: Vitamin D is well known for its function in calcium homeostasis and bone mineralisation, but is increasingly studied for its potential immunomodulatory properties. Vitamin D deficiency is a common problem in patients with COPD. Previous studies have not demonstrated a beneficial effect of vitamin D on exacerbation rate in COPD patients. However, subgroup analyses suggested protective effects in vitamin D deficient patients. Our objective is to assess the effect of vitamin D supplementation on exacerbation rate specifically in vitamin D deficient COPD patients.

Methods/Design: We will perform a randomised, multi-center, double-blind, placebo-controlled intervention study. The study population consists of 240 COPD patients aged 40 years and older with vitamin D deficiency (25-hydroxyvitamin D concentration < 50 nmol/L). Participants will be recruited after an exacerbation and will be randomly allocated in a 1:1 ratio to receive vitamin D3 16800 IU or placebo orally once a week for 1 year. Participants will receive a diary card to register the incidence of exacerbations and changes in medication during the study period. Visits will be performed at baseline, at 6 months and at 12 months after randomisation. Participants will undergo spirometry, measurement of total lung capacity and assessment of maximal respiratory mouth pressure. Several physical performance and hand grip strength tests will be performed, questionnaires on quality of life and physical activity will be filled in, a nasal secretion sample and swab will be obtained, and blood samples will be taken. The primary outcome will be exacerbation rate.

Discussion: This study will be the first RCT aimed at the effects of vitamin D supplementation on exacerbation rate in vitamin D deficient COPD patients. Also, in contrast to earlier studies that used infrequent dosing regimens, our trial will study effects of a weekly dose of vitamin D supplementation. Second, the immunomodulatory effects of vitamin D on host immune response of COPD patients and underlying mechanisms will be studied. Finally, the effects on physical functioning will be examined.

Introduction

Chronic obstructive pulmonary disease (COPD) is characterized by a persistent airflow limitation and an abnormal inflammatory response of the airways. COPD is predicted to be the third worldwide cause of mortality by 2020 (1). Exacerbations in COPD determine disease-associated morbidity and mortality (2). Patients with frequent exacerbations have a more rapid decline in lung function, worse quality of life and decreased exercise performance. Yet, effective treatment alternatives to prevent exacerbations are still lacking.

Vitamin D deficiency is highly prevalent in patients with COPD (3,4). Traditionally, vitamin D is associated with bone health. The discovery of the presence of vitamin D receptors (VDR) in many other cells, such as monocytes, macrophages, muscle cells and endothelial cells, has elicited hypotheses of direct vitamin D effects on these cells. These hypotheses are further strengthened by local 25-hydroxyvitamin D-1-alpha-hydroxylase activity, which converts the inactive 25-hydroxyvitamin D (25(OH)D) to the active 1,25-dihydroxyvitamin D ($1,25(OH)_2D$), in many of these cells. The presence of vitamin D receptors on immune cells (5) and the high prevalence of vitamin D deficiency among COPD patients has given rise to the hypothesis that vitamin D might have a potential effect in preventing exacerbations in patients with COPD (6).

Vitamin D and the immune system

There is a large body of evidence being generated in vitro and in vivo to demonstrate that vitamin D influences the innate and adaptive immune system. $1,25(OH)_2D$ is the active form of vitamin D that binds to the VDR, thereby influencing the expression of more than 200 genes. VDR is expressed on a range of immune cells such as macrophages, dendritic cells, and CD4-positive T lymphocytes (5). In the innate immune system vitamin D modulates Toll-like receptor (TLR)-induced immune responses through inhibition of the NF-κB-pathway and appears to improve antimicrobial defences in general (7,8). Vitamin D is capable of inducing endogenous expression of the antimicrobial peptides (AMP) such as cathelicidin. This has been reported in monocytes, macrophages, keratinocytes and in lung epithelial cells (9,10). Because AMPs have been found in multiple experimental systems to be essential for defence against a variety of microbial infections, it has been hypothesised that vitamin D can enhance resistance to infections (11). In addition, vitamin D seems capable of modifying the function of cells classically

associated with adaptive immunity whereby activation of VDR downregulates autoimmunity by promoting the differentiation of T-cells into regulatory T-cells (12).

Vitamin D and pulmonary infections

Vitamin D might influence the development and course of tuberculosis. Patients with low 25(OH)D concentrations have a higher risk of active tuberculosis and vitamin D supplementation may shorten the duration of disease (13). It is also known that patients with rickets more frequently suffer from airway infections and pneumonia (14). Several prospective cohort studies in the general population show that lower levels of 25(OH)D are related to increased risk of respiratory infections (15,16). A trial with Japanese schoolchildren during the influenza season demonstrated that, compared to placebo, vitamin D supplementation lowered the incidence of influenza A infections (17). Trials assessing effects of vitamin D supplementation on prevention of respiratory infections in the general adult population have shown conflicting results, which may partly be attributed to differences in prevalence of vitamin D deficiency at baseline and rise of serum 25(OH)D levels during treatments (18,19).

Vitamin D and COPD

Patients with COPD are characterised by an abnormal inflammatory response of the airways. Viral and bacterial infections are important triggers of exacerbations and contribute to its progression. Thus, potential effects of vitamin D on the immune system pose an attractive mechanism for the treatment of COPD. Also, in some (20,21), but not all (22) studies in the general population serum 25(OH)D is positively associated with lung function. Vitamin D deficiency is present in 40 – 80% of patients with COPD and is related to disease severity (3,4). Recent cohort studies, however, did not show a relationship between 25(OH)D levels and exacerbation rate (23,24), although these studies had limited statistical power to rule out effects of vitamin D deficiency.

In addition to exacerbations and lung function, skeletal muscle dysfunction in COPD patients contributes to poor exercise capacity, decreased quality of life and increased mortality (25,26). In COPD patients, vitamin D deficiency is related to impaired physical performance (27). In healthy adults, positive effects of vitamin D supplementation have been demonstrated on muscle function and physical performance in older and frail individuals (28,29).

RCTs vitamin D supplementation in COPD

Few studies have been performed on the effects of vitamin D supplementation in patients with COPD. In the trial performed by Lehouck et al. (30) vitamin D supplementation did not reduce the incidence of exacerbations. However, in a post-hoc analysis of a subgroup of severely vitamin D deficient patients (25(OH)D concentration < 25 nmol/L), vitamin D supplementation decreased the exacerbation rate. In a very recent multi-center trial by Martineau et al. (31) vitamin D protected against moderate to severe exacerbations in a pre-specified subgroup of vitamin D deficient (25(OH)D concentrations < 50 nmol/L) participants, but not in the study population as a whole.

Two studies have been performed assessing the effect of vitamin D supplementation on physical performance in patients with COPD. A pilot RCT did not show effects of vitamin D supplementation on physical performance, but was limited by the small number of 36 participants and short follow-up of 6 weeks (32). Also, the study was not specifically aimed at patients with vitamin D deficiency. In the aforementioned RCT by Lehouck et al. a post-hoc subgroup analysis of 50 participants following a rehabilitation programme during the trial was performed (33). Participants receiving vitamin D supplementation had significantly larger improvements in inspiratory muscle strength and peak exercise tolerance, but not in quadriceps strength and 6-minutes walking distance. However, this study had limited statistical power and is only applicable for patients following a rehabilitation programme. These findings justify a well-designed RCT to study effects of vitamin D supplementation on muscle strength and physical performance.

Little is known about the total dose and dose interval needed for extra-skeletal effects of vitamin D. In the study of Lehouck et al. (30) participants received a monthly dose of 100.000 IU. In the study of Martineau et al. (31) participants received 120.000 IU every two months. A large dose interval improves compliance but might also cause fluctuating levels of vitamin D metabolites (34). In two RCTs assessing the effect of vitamin D supplementation on falls and fractures, an increase of fall and/or fracture incidence were shown using annual high dose supplementation (35,36). While the mechanism by which vitamin D might cause an increase in falls remains uncertain, several authors suggest it is the dosing interval rather than the total dose that determined these outcomes (37). These results emphasize the need for RCT studying a more frequent dosing regimen of vitamin D. In the present study we aim to study the effects of vitamin D supplementation on exacerbation rate in COPD patients with vitamin D deficiency. In addition, we will

also assess the effects of vitamin D on several measures of physical performance. In our trial, we will administer a weekly dosing regimen in contrast to earlier studies, which used larger dosing intervals.

Methods

Study design and participants

The study is designed as a randomised double-blind, multi-center, placebo-controlled trial, with an intervention (n=120) and a control group (n=120). The study population consists of COPD patients with GOLD stages II-IV. Participants will be included if they have a vitamin D deficiency (25(OH)D concentrations <50 nmol/L) and a recent exacerbation of COPD. The eligibility criteria are described in Table 1. Participants will be recruited if they present with an exacerbation of COPD at the outpatient clinic or emergency ward. Screening serum 25(OH)D concentration will be measured during the exacerbation period. Inclusion and randomization will take place 6-8 weeks after screening or at a later time point as soon as the participant has recovered. An exacerbation is defined as sustained worsening of respiratory symptoms during 48 hours requiring oral corticosteroid, antibiotic or combination treatment that was initiated by a physician (38). Convalescence is defined as recovery to the stable state. The recruitment will start in three academic hospitals and five peripheral hospitals in the surroundings of the academic centers. Study visits will be performed at baseline (t=0), 6 months (t=6) and 12 months (t=12) after randomization as depicted in Figure 1. Participants will be contacted by telephone at 2, 4, 8 and 10 months. The study is approved by the Medical Ethics Committee of the VU University Medical Center, Radboud University Medical Center and Leiden University Medical Center. Written consent will be obtained from all participants.

Table 1. Eligibility criteria for the PRECOVID trial

Inclusion criteria	Exclusion criteria
Vitamin D deficiency (serum 25(OH)D < 50 nmol/l)	Severe vitamin D deficiency (serum 25(OH)D <15 nmol/l),
Postbronchodilator FEV_1/FVC < 0.70, FEV_1 < 80% and diagnosis COPD confirmed by a physician	Use of a supplement containing more than 400 IU vitamin D per day
Recent COPD exacerbation	Use of maintenance dose oral corticosteroids
≥ 10 packyears of smoking	Diagnosed asthma
Age ≥ 40 years	Diagnosed osteoporosis
Written informed consent	Self-reported history of hypercalcaemia or nephrolithiasis
Ability to comply with all study requirements	Self-reported presence of sarcoidosis
	Diagnosed chronic kidney disease stage 4 or higher (eGFR ≤ 29 ml/min/1.73 m^2)
	Interfering malignant diseases
	Life expectation of less than 1 year on the basis of concurrent disease
	Current participation in a clinical rehabilitation programme
	Pregnant or lactating women, or subjects who intend to become pregnant within the study period
	Potentially unreliable patients and those judged by the investigator to be unsuitable for the study
	Serious mental impairment i.e. preventing to understand the study protocol or comply with the study aim

Table 1. Eligibility criteria for the PRECOVID trial. *25(OH)D: 25-hydroxyvitamin D; FEV_1: Forced Expiratory Volume in one second; FVC: Forced Vital Capacity; eGFR: estimated Glomerular Filtration Rate with the MDRD formula.*

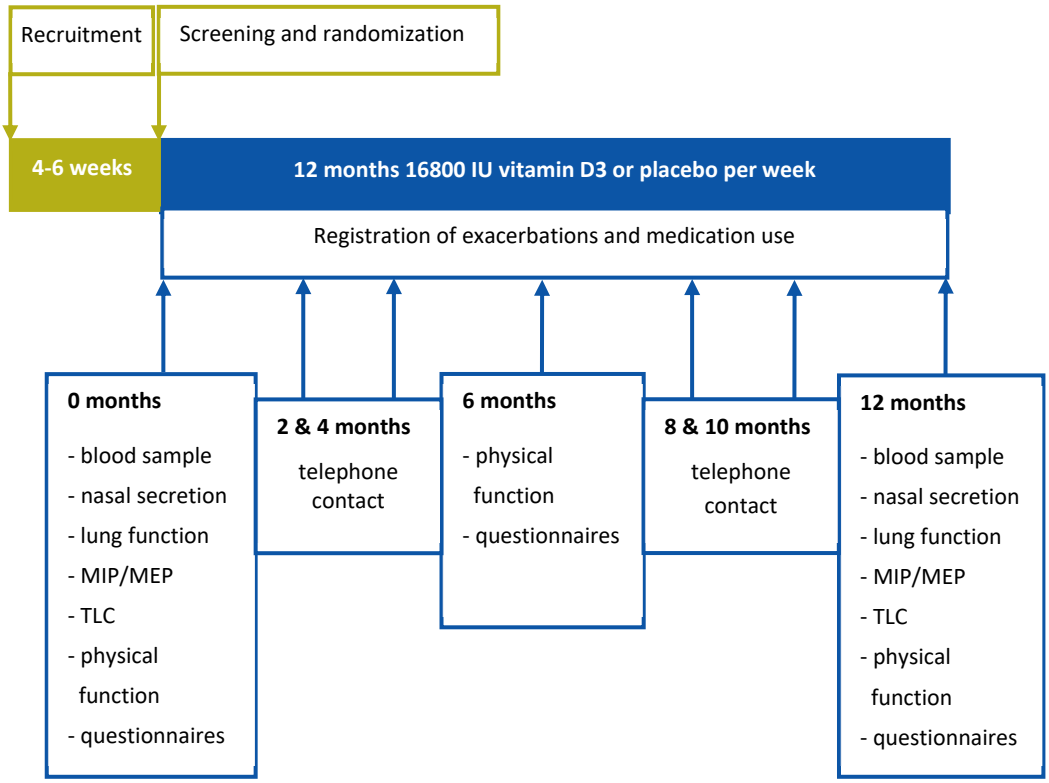

Figure 1. Flowchart of study procedures during the PRECOVID trial. Randomization will take place within 4-6 weeks after an exacerbation. During the study period of one year three study visits and four telephone contacts will take place. *MIP: Maximal inspiratory pressure; MEP: Maximal expiratory pressure; TLC: Total lung capacity.*

Intervention

The participants will receive either vitamin D 16800 IU (3 tablets of 5600 IU) or a matching placebo (3 placebo tablets) orally once a week during 12 months in accordance with the randomisation. The study medication will be prepared under supervision of the clinical pharmacy of the VU University Medical Center. Participants will be asked to return the study medication that is left over, and these tablets will be counted as a measure for compliance. Participants are allowed to use a maximum of 400 IU a day during the study, as this is the recommended daily intake by the Dutch Health Council. Participants will be asked about the usual intake of dairy products. Participants are advised (according to the advice of the Dutch Health Council) to increase their intake to a level corresponding with 1000 mg calcium per day. If this is not feasible, it will be advised to use calcium supplements to a total calcium intake of 1000 mg/day during the study.

Randomization and masking

For treatment allocation in this trial, we will apply the sequential balancing method with study center as the first step in the balancing algorithm, followed by gender, age and current smoking. These variables are (potential) prognostic factors and thus should be balanced over the treatment arms. Pharmacists of the VU University Medical Center, who are independent from the clinical research team, will randomly assign participants by using a computer-generated randomisation list and will prepare the study medication. After the last participant finishes the trial masking will continue until after the data analyses.

Outcomes

Primary outcome

Primary outcome is exacerbation rate. An exacerbation is defined as sustained worsening of respiratory symptoms during 48 hours requiring oral corticosteroid, antibiotic or combination treatment that was initiated by a physician. Respiratory symptoms include at least one of the Anthonisen criteria, which are increased dyspnea, sputum volume or sputum purulence, with or without minor symptoms such as cough, fever, common cold, wheezing or sore throat (38). Participants will register symptoms and changes in medication on a diary card during the whole study period. During each telephone contact and study visit diary cards will be discussed and reviewed. Also questions will be asked regarding hospitalization during the last months. If necessary, information on changes in medication and

COPD exacerbations during the study period will be confirmed through contact with the providing pharmacist, the general practitioner and hospital.

Secondary outcomes

Time to first and second exacerbation and time to fist hospitalisation
Time to first and second exacerbation and time to first hospitalisation will be registered on the basis of the diary card and interviews.

Use of antibiotic and corticosteroids
With the use of earlier mentioned diary card and interviews the use of antibiotics and corticosteroids will be registered.

Spirometry measures
At t=0 and t=12 participants will undergo spirometry. The spirometry will be performed according to American Thoracic Society/European Respiratory Society (ATS/ERS) guidelines (39). Only post-bronchodilator forced expiratory volume in one second (FEV_1) and forced vital capacity (FVC) will be determined. Measurements will take place after inhalation of the sympathomimetic salbutamol and/or the anticholinergic ipratropium bromide. Inspiratory capacity (IC) and expiratory reserve volume (ERV) will also be determined according to ATS/ERS guidelines (39).

Lung volumes
Measurements of absolute lung volume, residual volume (RV), functional residual capacity (FRC) and total lung capacity (TLC) will be assessed by body plethysmography according to guidelines of the ATS/ERS task force at t=0 and t=12 (40) (41).

Maximal respiratory mouth pressures
Respiratory muscle strength will be tested by measurement of the maximal inspiratory and expiratory mouth pressure (MIP and MEP, respectively). Measurement will be made with a mechanical pressure gauge that is connected to a mouthpiece according to the revised ATS guideline (42).

Physical performance
During every visit (t=0, t=6 and t=12), participants will perform physical performance tests.

In the 6-minutes walking test the participant is asked to walk as far as possible during a period of 6 minutes. The participant will walk up and down a hallway. The 6-minutes walking test is performed according to the ATS guidelines (43). In the chair-stands-test the participant will be asked to fold his arms across his chest and to stand up from a sitting position and sit down five times as quickly as possible (44,45). In the 3-meter walking test the participant is asked to walk three meters, turn around 180° and walk back (44,46). In the tandem test the participant is asked to stand with the heel of one foot directly in front of, and touching the toes of, the other foot for at least 20 seconds with his eyes open and closed (44,47).

Muscle strength

Muscle strength will be assessed with measurement of hand grip strength. The participant is seated in a chair and holds the dynamometer in the hand, with the arm at right angles and the elbow by the side of the body. The handle of the dynamometer is adjusted if required. When ready, he squeezes the dynamometer with maximum isometric effort, which is maintained for about 5 seconds. No other body movement is allowed. This measurement will be performed three times with both hands (48).

Quality of life

Quality of life will be assessed by three questionnaires These will be completed at every study visit. The St. George's Respiratory Questionnaire (SGRQ) is designed to measure health impairment in patients with asthma and COPD (49,50) and produces a symptoms score and an activity and impacts score. The Short Form 12 health Survey (SF-12) is a multipurpose short-form generic measure of health status (51). It is a validated subset of the larger SF-36 and monitors health in general and in specific populations. These scores correlate highly with the SF-36 versions (52). The SF-12 has been previously used in patients with COPD (50,53). The Clinical COPD questionnaire (CCQ) is a simple 10-item, health-related quality of life questionnaire with good psychometric properties (54). The questionnaire is responsive to pulmonary rehabilitation and a minimal clinically important difference has recently been defined (55).

Anxiety and depression

Anxiety and depression will be assessed by means of two questionnaires and will be completed at every study visit. The Hospital Anxiety Depression Scale (HADS) is a questionnaire measuring feelings of depression and anxiety (56). Reliability and

validation of the Dutch translation of the HADS has been reported (57). The HADS is frequently applied in research in patients with COPD (58,59). The Center for Epidemiologic Studies Depression Scale (CES-D) is a self-report scale designed to measure depressive symptoms in the general population. The development of the scale has been described in detail elsewhere (60). The scale has been used in several COPD populations (61,62).

Physical activity
Physical activity will be assessed by the Short QUestionnaire to ASses Health enhancing physical activity (SQUASH). SQUASH is a self-report instrument to measure habitual physical activity (63). The questionnaire has been used in COPD populations (64).

Antimicrobial peptides and pro-inflammatory mediators in nasal secretions
Nasal secretions will be collected by vacuum-aided suction to prevent dilution that occurs when using lavage-based methods (65). The nasal secretions will be analyzed for the presence of antimicrobial peptides and pro-inflammatory cytokines (hCAP18/LL-37, HNP1-3, NGAL, IL-1ß, TNF-α, IL-6) using enzyme-linked immunosorbent assays (ELISA).

Typing of bacteria and viruses in nasal secretions
A nasal swab will be obtained using standard procedures. PCR on presence of bacteria and respiratory viruses (non-typeable *Haemophilus influenzae*, *Streptococcus pneumoniae*, *Moraxella catarrhalis* and a panel of respiratory viruses including rhinovirus) will be performed.

Inflammation and host response against infectious agents
In one of the academic centers peripheral blood mononuclear cells (PBMC) and platelets will be derived from 50 participants before and after the intervention. Venous blood will be drawn in EDTA and 3.2% sodium citrate tubes after which PBMC and platelet-rich plasma (PRP)will be isolated, washed and suspended. PBMC will be stimulated with various agonists to TLR (bacterial, fungal and viral) and with microbial stimuli. The following stimuli will be used: RPMI (control), LPS (TLR4), Pam3Cys (TLR2), Poly(I:C) (TLR 3), *Streptococcus pneumoniae,* Non-typeable *Haemophilus influenza, Pseudomonas aeroginosa, Aspergillus fumigatus*, sonicated *Mycobacterium avium* and *Candida albicans*. Pro- and anti-inflammatory cytokines will be measured before and after stimulation: TNF-α, Il-1ß, IL-6, IFN-γ (24 hours incubation) and IL-10, IL-17 and IL-22 (7 days incubation). All cytokines will be

measured in cell culture supernatants using ELISA. PRP will be used for analyses of platelet function in terms of activation and aggregation. Second, phenotyping of circulating immune cells will be done using advanced multiparameter flowcytometry and functional readouts. A whole blood staining will be used to be able to calculate absolute numbers of CD4 (including Treg), CD8 T cells, NK cells, B cells and monocytes. Next, a detailed phenotypic analysis of the T cell compartment will be performed by additional staining of whole blood or isolated PBMC using antibodies. Functional characteristics of the T cell compartment (CD4+, CD8+, CD4+/Foxp3+ (Treg)) with respect to cytokine production (IFN-γ, IL-2, IL-4, IL-5, IL-10 and IL-17A) and their associated transcription factors (Tbet, Gata-3, RORyt) will be analyzed.

Sample size calculation

The study is designed to demonstrate a minimum difference of one exacerbation per patient-year between the vitamin D and the placebo group. We based our assumptions on post-hoc analyses of the RCT by Lehouck et al.(30), which had a similar patient sample, although without selection for vitamin D deficiency. Based on differences in Poisson means, a sample size of 94 participants per group is needed to demonstrate a difference of 1 exacerbation per patient-year with 80% power at 5% significance. This number was based on the assumption of 2.45 exacerbations per patient-year in the intervention group and 3.45 in the placebo group. To account for a dropout rate of 20% 120 participants will be recruited per study group.

Statistical analysis

Primary outcome

The primary outcome of the trial, exacerbation rate, will be analyzed with generalized linear models for a Poisson distribution. Analyses will be adjusted for the variables included in the balancing algorithm for randomization, i.e. study center, gender, age and current smoking. The outcomes will be presented as rate ratios with their 95% confidence interval.

Secondary outcomes

Time to first exacerbation and hospitalisation will be assessed with Kaplan–Meier curves and Cox proportional hazard models. Dichotomous secondary outcomes will be analysed with logistic regression models and continuous secondary outcomes

with analysis of covariance. All analyses will be adjusted for the variables included in the balancing algorithm for randomisation, i.e. study center, gender, age and current smoking. Because of the exploratory nature of the analyses, no correction will be made for the multiple analyses. All analyses of the trial will use the intention-to-treat population, defined as all randomly assigned participants who received at least 1 dose of study medication. A per-protocol analysis will be performed on those participants with a good compliance i.e. obtaining a serum 25(OH)D concentration above the 20th percentile after 12 months. Also, a subgroup analysis will be performed in participants with a baseline serum 25(OH)D concentration of 25 nmol/L or less.

Discussion

This RCT is the first intervention study examining effects of vitamin D supplementation in a cohort of vitamin D deficient COPD patients. With this study we expect to gain more insight in the effects of vitamin D supplementation on exacerbation rate and both pulmonary and physical function. The two previous RCTs (30,31) did not show an effect of vitamin D supplementation in the total study population of COPD patients. They did, however, show a protective role of vitamin D against exacerbations in deficient patients. These are promising results and emphasise the need for an RCT specifically in vitamin D deficient patients considering the high prevalence of vitamin D deficiency in patients with COPD. In our study participants with a severe vitamin D deficiency (25(OH)D <15 nmol/L) will be excluded because of ethical considerations.

Our study differs in several other aspects from previous studies. In addition to the effects of vitamin D on exacerbation rate, present study will focus on potential underlying mechanisms. In nasal secretionthe presence of antimicrobial peptides and pro-inflammatory mediators will be measured and the microbial composition of the nasal mucosa will be analysed. Furthermore, PBMC's from a subgroup of participants will be challenged with TLR-ligands and several pathogens in order to measure cytokine responses. These tests will be performed before and after the intervention to measure effects of vitamin D supplementation on cytokine responses and therefore inflammation and host response. Finally, we will assess physical performance in several different manners. In contrast to earlier studies, we will use a weekly dosing regimen, which leads to more stable 25(OH)D levels

(34), but may influence compliance. This is important since little is known about the total dose and dose interval needed for extra-skeletal effects of vitamin D.
As stated earlier exacerbations have a major impact on somatic and mental health status of COPD patients (2). With the increasing prevalence of COPD, the economic burden will expand significantly (1). Exacerbations constitute the most important direct costs associated with COPD. Treatments directed to reduce incidence and severity of exacerbations improve long-term health status and reduce health care costs (66). Vitamin D might therefore be an attractive treatment alternative, as it is easily applicable, cheap and safe.

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CHAPTER

Impact of the local inflammatory environment on mucosal vitamin D metabolism and signaling in chronic inflammatory lung diseases

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Abstract

Vitamin D plays an active role in the modulation of innate and adaptive immune responses as well as in the protection against respiratory pathogens. Evidence for this immunomodulatory and protective role is derived from observational studies showing an association between vitamin D deficiency, chronic airway diseases and respiratory infections, and is supported by a range of experimental studies using cell culture and animal models. Furthermore, recent intervention studies have now shown that vitamin D supplementation reduces exacerbation rates in vitamin D-deficient patients with chronic obstructive pulmonary disease (COPD) or asthma and decreases the incidence of acute respiratory tract infections. Vitamin D is known to contribute to the integrity of the mucosal barrier, promote killing of pathogens (via the induction of antimicrobial peptides) and to modulate inflammation and immune responses. These mechanisms may partly explain its protective role against infections and exacerbations in COPD and asthma patients. The respiratory mucosa is an important site of local vitamin D metabolism and signaling, a process that can be affected by exposure to inflammatory mediators. As a consequence, mucosal inflammation and other disease-associated factors, as observed in e.g. COPD and asthma, may modulate the protective actions of vitamin D. Here, we discuss the potential consequences of various disease-associated processes such as inflammation and exposure to pathogens and inhaled toxicants on vitamin D metabolism and local responses to vitamin D in both immune- and epithelial cells. We furthermore discuss potential consequences of disturbed vitamin D bioavailability for chronic lung diseases. Additional insight into the relationship between disease-associated mechanisms and local effects of vitamin D is expected to contribute to the design of future strategies aimed at improving local vitamin D bioavailability in chronic inflammatory lung diseases.

Introduction

Vitamin D is a pleiotropic hormone that is well known for its role in the regulation of calcium and phosphate homeostasis and bone mineralization. The receptor for vitamin D (VDR) is however expressed in nearly all tissues and cells and regulates a large number of genes (approximately 0.8-5% of the total genome) (1, 2). As a result, vitamin D affects many additional processes including cell proliferation and differentiation, apoptosis, DNA repair, ion transport, metabolism, cell adhesion, and oxidative stress responses (1, 3). Vitamin D deficiency (serum 25(OH)D < 50 nmol/L; 25(OH)D is the main circulating form of vitamin D and its levels are used to assess vitamin D status in the clinic (4, 5)) affects more than 30% of the children and adults worldwide and is a major cause of bone diseases such as rickets and osteoporosis (6). Increasing evidence has indicated that vitamin D deficiency is also associated with various other diseases such as cancer, cardiovascular disease, Alzheimer's disease and muscle myopathy, as well as several immune-related diseases such as type 1 diabetes, multiple sclerosis, inflammatory bowel disease (IBD), psoriasis and chronic inflammatory lung diseases including asthma, cystic fibrosis (CF) and chronic obstructive pulmonary disease (COPD) (6-9).

Several studies have now shown that vitamin D deficiency is prevalent in COPD patients and inversely correlated with lung function and severity of the disease (8, 10-12). It is currently unknown whether vitamin D deficiency is a cause or consequence of COPD, since many COPD patients have low physical activity levels and spend most time indoors (13). There are however studies suggesting that low vitamin D levels are associated with development of COPD, based on observed associations between polymorphisms in the vitamin D binding protein (VDBP), vitamin D serum levels and COPD severity (8, 10, 11, 14). In addition, one study in mice showed that maternal vitamin D deficiency can impair lung -development, -structure and -function in the offspring and suggests that even before birth, maternal 25(OH)D serum levels are important for a healthy lung development (15). This might be relevant, since associations have been found between lower childhood lung function and development of COPD later in life (16). The link between maternal 25(OH)D status and asthma development is however much clearer, since two recent randomized controlled trials (RCTs) have shown that maternal vitamin D supplementation reduces the risk of childhood asthma/recurrent wheeze (17). This might be explained by the fact that multiple

vitamin D-regulated genes are transcriptionally active during alveolar maturation and a number of these genes are differentially expressed in asthma (18). Additionally, this protective effect was linked to the GG-genotype of the 17q21 functional SNP rs12936231, which is associated with lower expression of *ORMDL3* and increased sphingolipid metabolism (19). Moreover, maternal circulating 25(OH)D levels affect the gut microbiota and can therefore indirectly modulate immune responses in the lung via the gut-lung-axis (20). Also later in life, optimal 25(OH)D levels remain crucial for keeping the lungs healthy. For example, Heulens *et al*. showed that subacute and chronic cigarette smoke (CS) exposure decreased lung function and promoted early signs of emphysema and airway inflammation in vitamin D-deficient mice compared to vitamin D-sufficient animals (21). Similarly in an elastase-induced COPD mouse model, topical administration of vitamin D in the lungs counteracted alveolar damage and improved lung function (22). Yet in humans, it is still unclear whether vitamin D affects COPD development and disease progression. Taken together, these observations suggest an important role for vitamin D during fetal and childhood lung maturation, and indicate that sufficient vitamin D levels might contribute to protection against development of childhood asthma and possibly COPD at older age.

Systemic levels of biologically active vitamin D are tightly regulated to preserve sufficient levels of calcium (Ca^{2+}) and phosphate (PO_4^{2-}) for optimal bone mineralization, whereas in mucosal tissues locally produced (autocrine) active vitamin D levels and signaling can be elevated or decreased upon exposure to inflammatory mediators, pathogens or inhaled toxicants (6). This could be important, since the inflamed airway mucosa of patients suffering from chronic inflammatory lung diseases is constantly exposed to these disease-associated factors (8, 23, 24). Impaired vitamin D bioavailability and signaling might have consequences for disease pathogenesis and progression. Dysregulated host defenses as found in patients with chronic inflammatory airway diseases include aberrant immune responses, altered microbiome composition, impaired epithelial barrier function and aberrant secretion of host defense molecules (25-27). Adequate vitamin D levels may provide protection against these dysregulated processes by maintaining the integrity of the mucosal barrier and promotion of killing of pathogens (e.g. via the induction of the antimicrobial peptide [AMP] hCAP18/LL-37) and via the modulation of both innate and adaptive immune responses (7, 28, 29).

In this review, we first discuss the effects of these disease-associated factors on local bioavailability of vitamin D and vitamin D-induced responses in the lung mucosa. In the second part of the review we will describe the mechanistic links between vitamin D deficiency and the pathogenesis of chronic inflammatory lung diseases such as asthma, CF and COPD, and discuss recent evidence related to the protective effects of vitamin D on COPD and on COPD exacerbations.

Regulation of mucosal vitamin D metabolism in health

Vitamin D enters the circulation either via food intake or as a result of its synthesis in the skin by UVB radiation. It subsequently binds to the VDBP (30, 31), after which this complex is transported to the liver where it is converted by vitamin D-25-hydroxylases (CYP2RI and CYP27A1) into 25-hydroxy-vitamin D [25(OH)D]. However, recent studies showed that also other cell types such as airway epithelial cells, keratinocytes, intestinal epithelial cells, and monocytes/macrophages express CYP2RI and CYP27A1, and thus are able to (locally) convert vitamin D_3 into $25(OH)D_3$ (32, 33). This inactive 25(OH)D needs to be converted into the active 1,25 dihydroxy-vitamin D ($1,25(OH)_2D$) by 25-hydroxyvitamin D-1α-hydroxylase (CYP27B1) in the kidney and in other cells, including several immune- and epithelial cells (34-40). $1,25(OH)_2D$ regulates expression of several genes by binding the nuclear VDR, which heterodimerizes with the retinoic acid receptor (RXR) to interact with vitamin D response elements (VDREs) that are present on the promoter region of these genes (1, 2). VDR is most abundantly expressed in intestinal enterocytes, pancreatic islets, renal distal tubules and osteoblasts, but is also present at lower levels in most other tissues and several other epithelial- and immune cells (41-45). Expression of VDR is classically regulated by $1,25(OH)_2D$, growth factors and hormones such as FGF-23 and PTH respectively, circulating calcium-levels, bile acids, transcriptional co-activators/repressors, and genetic- and epigenetic modifications, which is tissue specific (46-49). $1,25(OH)_2D$ regulates its own negative feedback by several mechanisms, including induction of expression of the catabolic enzymes 25-hydroxyvitamin D-24-hydroxylase (CYP24A1) and CYP3A4 (50, 51). CYP24A1 is expressed in most tissues and converts both 25(OH)D and $1,25(OH)_2D$ into biologically inactive $24,25(OH)_2D$ and $1,24,25(OH)_2D$

respectively (50), whereas CYP3A4, mainly expressed in the liver and small intestines, contributes to the metabolic clearance of 25(OH)D and $1,25(OH)_2D$ by converting 25(OH)D into $4\beta,25(OH)_2D$, and $1,25(OH)_2D$ into $1,23R,25(OH)_2D$ or $1,24S,25(OH)_2D$ (51). Expression of both CYP27B1 and CYP24A1 in the kidneys is tightly regulated to maintain optimal Ca^{2+}- and PO_4^{2-} levels in the circulation, which are important for bone mineralization(52). The complex mechanisms that explain how vitamin D and its metabolic enzymes maintain sufficient Ca^{2+} and PO_4^{2-} levels in the circulation are explained by Quarles *et al.* (52). In summary, it has become increasingly evident that the effects of vitamin D are not limited to homeostasis of Ca^{2+} and PO_4^{2-} and bone mineralization, because several extra-renal cells such as airway epithelial cells and immune cells express the VDR and are capable of converting circulating 25(OH)D into the active $1,25(OH)_2D$ metabolite **(Figure 1)**.

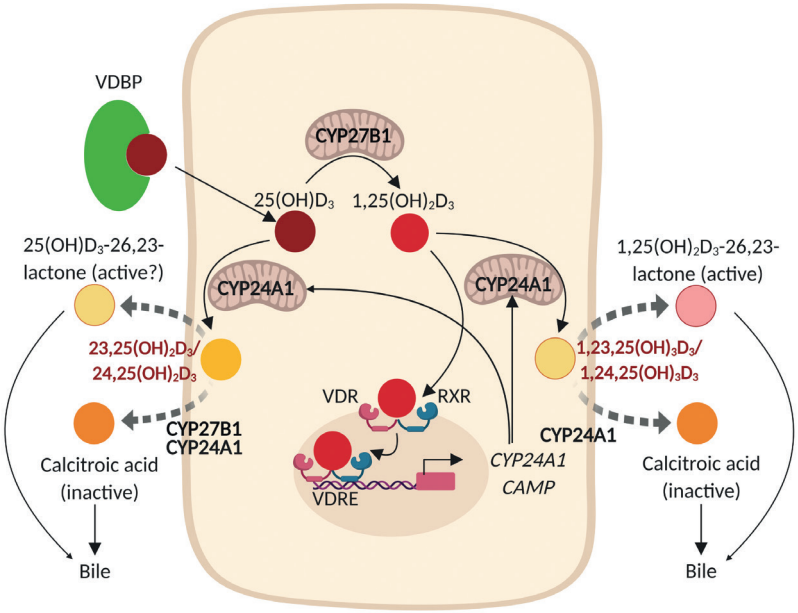

Figure 1. Vitamin D metabolism and expression of hCAP18/LL-37 (*CAMP*) in epithelial cells. The vitamin D binding protein (VDBP)-25(OH)D complex enters the epithelial cell from the circulation and 25(OH)D is subsequently released from the complex. In the cytoplasm, 25(OH)D is hydroxylated by 25-hydroxyvitamin D-1α-hydroxylase (CYP27B1; localized to the inner mitochondrial membrane) into the active metabolite $1,25(OH)_2D$. $1,25(OH)_2D$ subsequently binds to the nuclear vitamin D receptor (VDR) which heterodimerizes with the retinoic acid receptor (RXR) to interact with vitamin D response elements (VDREs) that are present on the promoter region of numerous genes, including *CAMP* (hCAP18/LL-37) and CYP24A1 (25-hydroxyvitamin D-24-hydroxylase). $1,25(OH)_2D$ thereby regulates its own negative feedback via direct induction of CYP24A1 that converts both 25(OH)D and $1,25(OH)_2D$ to biologically inactive $24,25(OH)_2D$ and $1,24,25(OH)_2D$ respectively (53).

Altered regulation of mucosal vitamin D metabolism and vitamin D responsiveness in chronic inflammatory airway diseases

Local bioavailability and activity of $1,25(OH)_2D$ are in part determined by expression of VDR and the equilibrium between the vitamin D metabolic enzymes CYP27B1 and CYP24A1. It is important to realize that mucosal expression of CYP24A1, CYP27B1 and also VDR can be affected by several disease-associated inflammatory mediators, toxicants and pathogens, summarized in **Table I**. As a consequence of this, the local bioavailability of vitamin D in tissues such as the inflamed airways of patients that suffer from chronic inflammatory airway diseases might be reduced.

Cell/tissue type	Stimulus	Effect	References
Primary airway epithelial cells	Poly(I:C); RSV; IL-13; IL-4; PM	CYP27B1 ↑	(38, 54-56)
	TNF-α; IL-1β; IL-17A; TGF-β1; NTHi	CYP24A1 ↑	(57, 58)
	CSE	CYP27B1 ↓	(59, 60)
	A. fumigatus; HRV; RSV	VDR ↓	(56, 61)
BEAS-2B (bronchial epithelial cell line)	HRV; RSV	VDR ↓	(56)
	PM	VDR ↑	(55)
16HBE (bronchial epithelial cell line)	*A. fumigatus*	VDR ↑	(62)
	TGF-β1	CYP27B1 ↑	(54)
	A. fumigatus	CYP27B1 ↑	(62)
A549 (lung carcinoma cell line)	CSE	VDR translocation ↓	(63)
HCT116 (colon cancer epithelial cell line)	LPS; TNF-α	CYP27B1 ↑	(64)
	LPS; TNF-α	VDR ↓	(64, 65)
	LPS	CYP24A1 ↓	(64)
COGA-1A (colon cancer epithelial cell line)	TNF-α ± IL-6	CYP27B1 ↓	(66)

Trophoblasts	TNF-α; IL-1β; IL-6	CYP24A1 ↑	(67)
	IFN-γ	CYP27B1 ↑	(67)
Macrophages	ss-RNA	CYP27B1 ↑ VDR ↑	(68)
Macrophages (derived from THP-1)	CSE	VDR ↑	(69)
Macrophages (derived from THP-1)	BaP	CYP24A1 ↑	(70)
Monocytes	TLR2/1L ± IFN-γ; LPS; IL-15	CYP27B1 ↑ VDR ↑	(39, 71-73)
	IL-4 ± TLR2/1L	CYP24A1 ↑	(39)
Neutrophils	IFN-γ	CYP27B1 ↑	(74)
	S. pneumoniae T4R	VDR ↑	
T cells	T cell activators (anti-CD3/anti-CD28; PHA; PMA/ionomycin)	CYP27B1 ↑ VDR ↑	(75)
B cells	B cell activators (anti-IgM/anti-CD40/IL-21)	CYP27B1 ↑ VDR ↑	(76)

Table 1: Effects of inflammatory mediators on the expression of VDR, CYP24A1 and CYP27B1 in immune cells and epithelial cells.

Abbreviations: Polyinosinic:polycytidylic acid (Poly(I:C); Particulate matter (PM); nontypeable Haemophilus influenzae (NTHi); Aspergillus fumigatus (A. fumigatus); Cigarette smoke extract (CSE); Human rhinovirus (HRV); Respiratory syncytial virus (RSV); Single stranded RNA (ssRNA); Benzo[a]pyrene (BaP); Toll like receptor 2/1 Ligand (TLR2/1L); Phytohemagglutinin (PHA); Phorbol 12-myristate 13-acetate (PMA).

Epithelial cells

Chronic lung diseases are characterized by airway inflammation and impaired respiratory host defense, which is illustrated by the increased susceptibility for respiratory infections and exacerbations (77-79). Furthermore, exposure to inhaled toxicants such as cigarette smoke and air pollutants are associated with disease pathogenesis and exacerbations in COPD, CF and in asthma patients (80-82). It would therefore be of great interest to investigate these effects on vitamin D bioavailability and vitamin D-mediated respiratory host defense in the airway mucosa. Studies in airway epithelial cells have shown that exposure to UV-inactivated nontypeable *Haemophilus influenzae* (NTHi) increased expression of the vitamin D-degrading enzyme CYP241, whereas exposure to viral double stranded-RNA analogue polyinosinic:polycytidylic acid (Poly[I:C]) increased expression of CYP27B1 and conversion of 25(OH)D into the active metabolite (38,

57). On the other hand in the bronchial cell line BEAS-2B, expression of VDR was decreased after infection with respiratory viruses such as human rhinovirus (HRV) and respiratory syncytial virus (RSV) (56). Collectively, these studies have shown in airway epithelial cells that respiratory viral- and bacterial infections can either promote or impair vitamin D activation and responses.

A local airway inflammatory milieu can also exert differential effects on vitamin D bioavailability and signaling, dependent on the type of inflammatory mediators that are predominantly present. We have shown in differentiated primary airway epithelial cells that Th2 cytokines such as IL-4 and IL-13, enhance expression of CYP27B1 and vitamin D-mediated expression of hCAP18/LL-37, which suggests that a Th2-inflammatory environment, as found in allergic airway inflammation increases vitamin D bioavailability (81, 83). The observation that levels of both $1,25(OH)_2D$ and hCAP18/LL-37 were increased in bronchoalveolar lavage (BAL) after allergen challenge is in line with this proposed mechanism (84). This effect of Th2 cytokines was in contrast to the effects (chronic) exposures to the proinflammatory cytokines IL-1β, TNF-α and IL-17A that strongly increased the expression of the vitamin D-degrading CYP24A1, even in absence of vitamin D (57). Furthermore, short-term exposures to TGF-β1, a pleiotropic growth factor which is elevated in the lungs of COPD, CF and asthma patients, also increases the expression of CYP24A1 (85). As a consequence, $1,25(OH)_2D$-mediated expression of the AMP hCAP18/LL-37 was impaired, which was likely the result of the enhanced degradation of both 25(OH)D and $1,25(OH)_2D$ by this enzyme (57, 58). In addition to pathogens and cytokines, exposure to inhaled toxicants such as cigarette smoke (CS) and particulate matter (PM) may also alter expression or activity of VDR and CYP27B1. Studies have demonstrated that cigarette smoking or exposure to CS extract (CSE) decreases expression of CYP27B1 and inhibited membrane bound (m)VDR translocation to the cell membrane in airway epithelial cells and A549 cells (an alveolar tumor cell line) respectively (59, 60, 63) This inhibition reduces the conversion of 25(OH)D to $1,25(OH)_2D$ and $1,25(OH)_2D$-mediated gene expression as well as non-genomic actions of $1,25(OH)_2D$-membrane associated, rapid response steroid-binding (MARRS)- signalling (59, 60, 63). This adverse effect of cigarette smoking on vitamin D bioavailability and effects in airway epithelial cells was recently confirmed *in vivo* by Vargas Buonfiglio *et al.*, who demonstrated that vitamin D supplementation increased antimicrobial activity in apical surface liquid (ASL) in the airway of healthy non-smokers, but not in smokers (59). On the other hand, exposure to PM increases the expression of both CYP27B1 and VDR in airway

epithelial cells, thereby possibly promoting vitamin D bioavailability (55). It is however important to consider that several retrospective and observational studies have demonstrated that air pollution is an independent risk factor for developing vitamin D deficiency (86). In conclusion, exposure to CS, TGF-β1 and presence of a proinflammatory milieu appeared to most strongly decrease vitamin D bioavailability and signaling in airway epithelial cells.

Immune cells

Whereas various studies show that exposure to proinflammatory stimuli affects vitamin D metabolism and reduces the effects of vitamin D in (airway) epithelial cells, the opposite appears to be the case for immune cells. In monocytes, macrophages and neutrophils, effects on vitamin D bioavailability and vitamin D-mediated antimicrobial responses were generally enhanced by these proinflammatory stimuli as illustrated by increased expression of both VDR and CYP27B1 (39, 68, 71-74). It is therefore tempting to speculate that this apparent increase in vitamin D-mediated antimicrobial responses in immune cells in an inflammatory environment may serve as a second line of defense and compensate for the enhanced epithelial degradation of vitamin D during inflammation. Inhaled toxicants may also affect vitamin D responsiveness of immune cells. This is illustrated by two recent studies studying the effects of cigarette smoke on the human monocyte/macrophage-like cell line THP-1. One study showed that treatment with cigarette smoke extract (CSE) increased the expression of VDR without enhancing vitamin D responses (69), while the other study -that focused on the effects of Benzo[a]pyrene (BaP) (a component produced by cigarette combustion)- demonstrated that vitamin D-mediated CYP24A1 expression was induced, which was found to further enhance degradation of $1,25(OH)_2D$ (70). In summary, proinflammatory stimuli generally increased vitamin D responses and bioavailability in immune cells, whereas more studies are needed to fully determine the impact of exposure to cigarette smoke and other inhaled toxicants.

Lung mucosa

Whereas these studies provide evidence that inflammation and inhaled toxicants may affect vitamin D metabolism and responsiveness in epithelial cells and immune cells, it is not clear whether this has an impact on these events in lung tissue of patients with chronic lung diseases. Although evidence is limited, we can speculate that vitamin D bioavailability and responses are also affected by disease-associated factors in mesenchymal cells that are present in the lung mucosa. One study that

showed in a bleomycin fibrosis model and in primary lung mouse fibroblasts that TGF-β1 reduced expression of the VDR might support this assumption (87). It is currently insufficiently studied whether exposures to disease-associated factors promote or impair vitamin D bioavailability and responses in immune-, mesenchymal and epithelial cells combined to give a better reflection of the *in vivo* situation. Interestingly, one study did already show that nasal CYP27B1- and $1,25(OH)_2D$-levels are both reduced in chronic rhinosinusitis (CRS) patients with nasal polyps as compared to CRS-patients without nasal polyps, whereas no difference was found in circulating $1,25(OH)_2D$-levels (88). Since most other studies were performed *in vitro* using monocultures of epithelial cells or immune cells, more complex models are needed to delineate this. Therefore, animal models or preferably more complex animal-free cell culture models using co-cultures or organ-on-a-chip models of primary fully differentiated epithelial cells, airway-derived fibroblasts or smooth muscle cells and immune cells could be considered in future studies.

Protective effects of vitamin D on mucosal homeostasis

After discussing altered vitamin D metabolism and responsiveness in the inflamed airway mucosa, it is important to consider the possible consequences of these inflammation-induced changes in the airway mucosa keeping in mind the pleotropic effects of vitamin D that were introduced earlier. In several cells, tissues and organs, vitamin D regulates multiple cellular processes that affect normal and malignant cell growth and differentiation (89, 90). Vitamin D displays furthermore protective effects on mucosal host defense by maintaining the integrity of the epithelial barrier, inhibition of epithelial-to-mesenchymal transition (EMT), stimulating production of AMPs and modulating both innate- and adaptive immune functions (29, 91, 92). In addition, vitamin D maintains both energetic and survival homeostasis in the mucosal epithelium through the modulation of stress and damage responses, including clearance of disturbing and stressful agents (3, 93) (**Figure 2**).

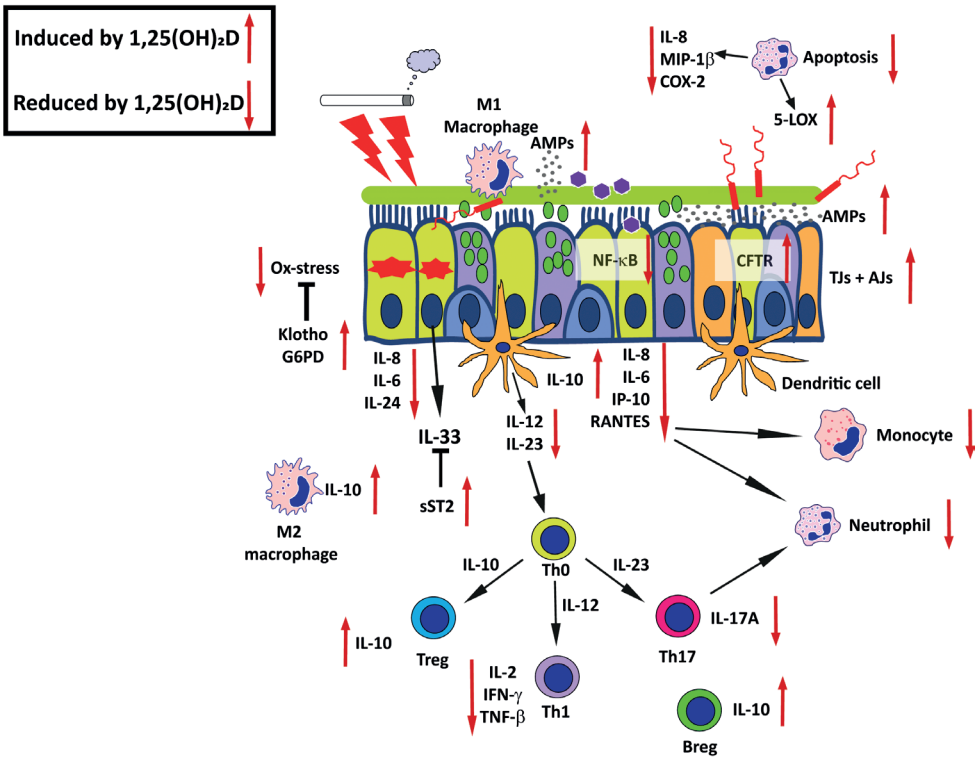

Figure 2. Effect of vitamin D on airway epithelial host defense-mechanisms in chronic airway disease. The promoting or inhibitory effects of vitamin D are indicated by the red arrows.
Abbreviations: Antimicrobial peptides (AMPs); Cystic fibrosis transmembrane conductance regulator (CFTR); Oxidative stress (Ox-stress); Suppressor of cytokine signaling proteins (SOCS); Tight junctions (TJs); Adherens junctions (AJs); Glucose-6-phosphate dehydrogenase (G6PD); Soluble suppression of tumorigenicity 2 (sST2); Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB); Naieve T cell (Th0); Regulatory T cell (Treg); T helper type 1 cell (Th1); T helper type 2 cell (Th2); T helper type 17 cell (Th17); Regulatory B cell (Breg); See text for details and references.

Epithelial barrier function

In chronic inflammatory lung diseases, epithelial barrier function is impaired, and as a consequence the susceptibility towards respiratory infections is increased (94). There is increasing evidence that vitamin D promotes epithelial barrier integrity or protects against epithelial barrier destruction. In cells of the bronchial epithelial cell line 16HBE, vitamin D inhibited CSE-mediated reduction of the epithelial barrier and expression of E-cadherin and β-catenin (95). Recently, two murine studies were published that investigated the effects of vitamin D on pulmonary epithelial barrier function. Shi *et al.* showed that vitamin D-supplementation alleviated lung injury in LPS-treated mice through maintenance of the pulmonary barrier by inducing expression of Zonula occludens (ZO)-1 and occludin in whole lung homogenates (96), whereas Gorman *et al.* showed in healthy mice, fed with a vitamin D-poor diet, that vitamin D supplementation had little effect on epithelial integrity (97). Only the first study that used a more severe mouse model with higher levels of inflammation and edema found an effect on vitamin D on epithelial barrier function. Since inflammation is detrimental for epithelial barrier integrity (98), it cannot be excluded that the main protective effects of vitamin D on the epithelial barrier in the first study by Shi *et al.* were in fact exerted through inhibition of inflammation rather than via direct induction of cell junction proteins. Vitamin D might also promote epithelial barrier function through its ability to increase expression of cystic fibrosis transmembrane conductance regulator (CFTR) in airway epithelial cells (99). CFTR maintains optimal ASL- and mucus hydration, volume and pH that support mucociliary clearance and activity of AMPs (100). Moreover, CFTR is also affected in the airways of smokers and COPD patients (101). In summary, these studies indicate that vitamin D promotes both the integrity and function of the epithelial barrier and might additionally protect against epithelial damage by dampening inflammatory responses.

Anti-fibrotic effects of vitamin D

The loss of epithelial barrier function with a decrease in epithelial polarization and cell-junction proteins and a gain of expression of mesenchymal markers is a hallmark of EMT (94). EMT is primarily involved in development, wound healing and stem cell differentiation, and TGF-β signaling plays a major role in this process (102). Elevated TGF-β1 levels are found in the lungs of patients with chronic inflammatory lung diseases and this was associated with cigarette smoking, inflammation and fibrosis (77, 103). There are indications that vitamin D counteracts various pathways leading to EMT. In mouse models and in airway

epithelial cell lines, vitamin D has been shown to inhibit EMT and fibrosis, in particular when this process is induced by TGF-β1 (87, 104-107).

Effects of vitamin D on epithelial antimicrobial responses

In addition to maintenance of the epithelial barrier and inhibition of fibrosis as discussed in the previous paragraphs, vitamin D is also actively involved in respiratory host defense by a variety of mechanisms (3, 29). Vitamin D is an important inducer of AMPs, which are mostly cationic peptides that have a broad-spectrum antimicrobial activity, the ability to modulate immune responses and to promote epithelial wound repair and angiogenesis (108). hCAP18/LL-37 is likely to be the most prominent AMP that is induced by vitamin D and is expressed in several types of mucosal epithelial cells and immune cells such as monocytes and neutrophils (38, 74, 109). In macrophages and intestinal epithelial cells, vitamin D also increases expression of human β-defensin-2 (hBD-2), whereas in keratinocytes expression of both hBD-2 and human β-defensin-3 (hBD-3) is increased by vitamin D (110-113). Collectively these data show that AMPs are modulated by vitamin D in mucosal tissues, which could have impact on susceptibility to both bacterial and viral infections and on the composition of the microbiota, which will be discussed in the next section .

Effects of vitamin D on innate and adaptive immune responses

Diseases such as COPD and asthma are characterized by chronic inflammation, a low-grade and prolonged inflammation that may result in destruction and aberrant repair of surrounding tissue by growth factors, proteases and cytokines that are released at the site of inflammation (114-116). Cumulative data suggest that vitamin D exerts anti-inflammatory effects via its actions on both innate and adaptive immune responses. Upon viral infection or exposure of pro-inflammatory stimuli such as Poly(I:C) or PM, vitamin D attenuates induced expression of cytokines and chemokines e.g. via inhibition of nuclear factor (NF)-κB or oxidative stress respectively in (airway) epithelial cells (38, 55, 117). Furthermore, vitamin D increases expression of the soluble decoy receptor for IL-33 (sST2) by airway epithelial cells, which in turn inhibits the actions of the type 2 alarmin IL-33 (118). Further effects of vitamin D on local innate and adaptive immune responses in the epithelial mucosa are mediated through its actions on immune and structural cells and have been reviewed by Heulens *et al.*, Vanherwegen *et al.* and Pfeffer *et al.* (29, 119, 120).

Taken together, these findings suggest that on the one hand vitamin D protects against infections by enhancing epithelial barrier function and production of AMPs, and on the other hand vitamin D induces tolerance and dampens proinflammatory responses in various cell types of the airway mucosa. Thereby, vitamin D may prevent exaggerated inflammatory responses and further damage to the mucosal tissue, qualities that are very relevant in the context of chronic inflammatory (lung) diseases (**Figure 2**).

Effects of vitamin D on epithelial oxidative stress and aging

COPD is considered to be a disease of accelerated ageing lungs, underscored by markers of aging being increased in these patients partly as a result of oxidative stress (121). Evidence that vitamin D may protect epithelial cells from oxidative stress was provided by Pfeffer *et al*., who demonstrated that vitamin D increased expression of the antioxidant gene *G6PD* in airway epithelial cells. Furthermore, vitamin D increased the ratio of reduced to oxidized glutathione and decreased the formation of 8-isoprostane after exposure to PM (55). The induction of klotho by vitamin D might be another vitamin D-mediated anti-ageing mechanism (122). Klotho is an anti-ageing protein that is mainly expressed in the kidney, brain and in the lung by airway epithelial cells and exerts its protective effects through the inhibition of inflammation, insulin/IGF-1 signaling and activation of forkhead transcription factor (FoxO) signaling, which enables removal of reactive oxygen species (ROS) (123-125). Expression of klotho is impaired in the airways of smokers and further decreased in the airways of COPD patients and in cultures of the bronchial epithelial cell line 16HBE after CSE exposure (125). These studies suggest that vitamin D may protect against ageing via inhibition of oxidative stress and possibly via its ability to restore klotho expression (**Figure 2**). However, direct evidence showing that vitamin D indeed increases expression of klotho in airway epithelial cells is currently lacking.

Effects of vitamin D on epithelial autophagy and apoptosis

In addition to providing protection against oxidative stress and ageing, data from studies using intestinal epithelial cells suggest that vitamin D may also promote cellular survival via the induction of autophagy and reduction of apoptosis (126, 127). In chronic inflammatory lung diseases, aberrant activation of autophagy plays a role in disease pathogenesis (128). A recent study showed that club cells and autophagy-related proteins were both decreased in COPD patients and that these proteins were important for club cell structure and function in airways (129).

However, the effects of vitamin D on autophagy in the airway mucosa of chronic inflammatory lung diseases are still unclear and need to be further evaluated (128).

Role of vitamin D in the treatment of chronic airway diseases

Clearly vitamin D has pivotal actions in host defense that are relevant in the context of chronic inflammatory lung diseases, in which vitamin D deficiency may be prevalent. Strategies to promote local bioavailability of vitamin D or use it as a treatment itself could be therefore of interest. Here, we will discuss the latest clinical evidence accompanied with functional *in vitro*- and animal studies that may explain the effects of vitamin D treatment on typical hallmarks of chronic airway diseases.

Effect of vitamin D on inhaled corticosteroid responsiveness in chronic airway diseases

Currently, inhaled corticosteroid (ICS)-use with or without long acting bronchodilators is the most frequently used treatment for COPD and asthma patients (130). However, the response to corticosteroids is not always effective in many COPD patients and in patients with steroid resistant (SR)-asthma (131). There are several complex mechanisms that underlie the resistance to corticosteroids in both COPD and SR-asthma that include but are not limited to genetic background, impaired glucocorticoid receptor binding, T helper type 17 cell (Th17)-inflammation and oxidative stress (e.g. from air pollution or smoking) and decreased numbers of IL-10 secreting regulator T cells (Tregs), which normally prevent skewing towards Th17-inflammation (131). Direct evidence of the ability of vitamin D to reverse SR was provided by a study showing that *ex-vivo* stimulation with 25(OH)D promoted generation of IL-10–secreting Tregs which restored sensitivity towards corticosteroids in CD4+ T cells that were derived from SR-asthma patients (132). A further potential treatment role of vitamin D was elegantly illustrated by studies that showed that vitamin D deficiency is associated with decreased steroid responsiveness in asthmatics and by the fact that several potential underlying mechanisms of SR such as oxidative stress and Th17-mediated inflammatory responses could be reversed by vitamin D treatment (55, 133-138). Interestingly,

the corticosteroid dexamethasone was shown to increase expression of the vitamin D degrading enzyme CYP24A1 in renal cells and osteoblasts (139), which suggests a bidirectional interaction between corticosteroids and vitamin D and could further limit its bioavailability for patients. Additional research is needed to determine if vitamin D may also improve corticosteroid responsiveness in COPD.

Vitamin D and exacerbations in COPD

Exacerbations are a major burden for COPD patients, they accelerate decline in lung function and frequently result into hospital admissions (140, 141). Exacerbations are often triggered by pollutants or by bacterial- and/or viral infections (80, 142, 143). COPD patients generally have lower serum 25(OH)D-levels than age- and smoking-matched controls, which is associated with more and more severe exacerbations (8, 10). Several *in vivo* and *in vitro* studies have provided evidence that explain the protective effects of vitamin D on exacerbations in COPD patients and this will be discussed accordingly.

Air pollution

First of all, Pfeffer and colleagues showed that 25(OH)D and 1,25$(OH)_2$D reduce the production of proinflammatory cytokines in part via the ability to enhance antioxidant responses in airway epithelial cells that were exposed to PM (55). This was also demonstrated in human DCs that were matured in presence of PM, where treatment with 1,25$(OH)_2$D counteracted the expansion of proinflammatory IL-17A$^+$ and IFN-γ$^+$ Th17.1 cells (136). In line with this, Bolcas *et al.*, showed that vitamin D supplementation counteracted the development of airway hyperresponsiveness and accumulation of Th2/Th17 cells in mice that had been repeatedly exposed to both diesel exhaust and house dust mite allergens (144). Vitamin D could therefore exert a protective role in air pollution-triggered exacerbations.

Respiratory viral infections

In addition to its protective effects against pollutants, there is also increasing evidence that vitamin D may enhance clearance of respiratory viral infections that account for 30-50% as underlying cause of exacerbations in COPD patients (145). Infections with respiratory viruses such as HRV, coronaviruses and to a lesser extend respiratory syncytial virus (RSV) and (para)influenza virus are present during exacerbations and may predispose the host towards secondary bacterial infections that can eventually lead to uncontrolled bacterial outgrowth, more severe exacerbations and neutrophilic inflammation (145, 146). Two recent *in vitro* studies

showed that acute exposure to relatively high doses (100 - 1000 nM) of 1,25$(OH)_2$D reduced HRV-infection in undifferentiated cultures of airway epithelial cells (56, 147). In those models, vitamin D most likely interfered with viral replication by increasing expression of interferon-stimulated genes and expression of hCAP18/LL-37, which has been shown to have direct antiviral activity (56, 147, 148). In fully differentiated airway epithelial cells, treatment with lower concentrations of 1,25$(OH)_2$D (10 nM) during epithelial differentiation had no effect on acute HRV infection (149). As for other viruses than HRV, both Hansdottir *et al.* and Telcian *et al*. showed that vitamin D did not decrease RSV infection in airway epithelial cells, but did reduce virus-induced inflammatory responses (56, 117). In addition, two other studies reported in influenza (H9N2 and H1N1)-infected A549 cells comparable findings (150, 151). Moreover, inhibitory effects of vitamin D on poly(I:C)-induced inflammatory responses were furthermore confirmed in primary airway epithelial cells Hansdottir *et al*. and by our group (38, 83). Up to now, the afore mentioned studies suggest that higher doses of vitamin D might be protective against HRV-infections in undifferentiated airway epithelial cells only, whereas for other respiratory viral infections vitamin D mainly reduces inflammatory responses without affecting viral clearance. However, more studies are needed, especially in differentiated airway epithelial cells using multiple HRV-serotypes that use different receptors for infection to verify if vitamin D indeed is capable of promoting HRV-clearance. There is more consensus about vitamin D reducing virus-induced inflammatory responses and this may certainly help to alleviate the burden of exacerbations in COPD (38, 83).

Bacterial infections

In addition to viral infections, also bacterial infections are associated with COPD exacerbations and account for approximately 50% of all exacerbations (152). Due to improved study design and sampling techniques from the lower airways using bronchoscopy in recent decades, the causative role of bacteria in COPD-related exacerbations has become clear (152). This was additionally supported by Sethi *et al.*, who found that acquisition of a new strain of pathogenic bacterial species into the airways was linked to COPD exacerbations (153). Recent developments in assessing the airway microbiota using 16S rRNA sequencing techniques further demonstrated that during exacerbations, the relative abundance of Haemophilus, Pseudomonas, and Moraxella was increased and the microbial composition was shifted towards the Proteobacteria phylum (143). The ability of vitamin D to promote antibacterial activity was recently demonstrated in cultures of airway

epithelial cells. In differentiated airway epithelial cells, we have shown that vitamin D treatment enhances epithelial expression of hCAP18/LL-37 and antibacterial activity against NTHi, a Gram-negative bacterium, which is associated with COPD exacerbations (57, 154). In addition, Yim *et al.* demonstrated that vitamin D treatment increased expression of the AMP hCAP18/LL-37 and killing of *Pseudomonas aeruginosa* and *Bordetella bronchiseptica*, which are both Gram-negative bacteria (155). These observed antibacterial effects of vitamin D on airway epithelium *in vitro* were recently confirmed *in vivo* by Vargas Buonfiglio *et al.*. The authors demonstrated that vitamin D supplementation increased antimicrobial activity against the Gram-positive *Staphylococcus aureus* in ASL in healthy non-smokers and was dependent on presence of hCAP18/LL-37 (59).

In murine airways, studies showed no effects of vitamin D on the expression of *Defb4* or *mCramp* (the murine homologue for *CAMP*) (156). This can be explained by the fact that both the promotors of *mCramp* and *Defb4* lack VDREs, suggesting that mice might not be suitable for studying the role of vitamin D in AMP-mediated host defense in infection (157). Indeed, Niederstrasser and colleagues showed no effects of vitamin D deficiency on the susceptibility of mice to pulmonary infection with *Streptococcus pneumoniae* or *Pseudomonas aeruginosa* (158). However, in a recently developed mouse model by Lowry et al., who transfected *mCramp* knockout mice with the human *CAMP* gene, topical vitamin D treatment increased expression of *CAMP* and promoted antibacterial effects on the mucosa of the skin (159). There are also multiple other murine studies that demonstrate protective effects of vitamin D on bacterial infections in the gut, indicating that vitamin D-mediated antibacterial effects are additional modulated by other mechanisms such as via enhancement of epithelial barrier integrity (64, 160). In conclusion, these observations show that vitamin D promotes protection against pollutants and enhances clearance of viral– and bacterial infections (both Gram-positive and negative bacteria) in combination with a dampening effect on exaggerated immune responses and these features might explain why vitamin D (deficiency) is linked to COPD exacerbations.

Modulation of microbiota by vitamin D

There are strong indications that modulation of immune responses and antibacterial activities by vitamin D and/or vitamin D-regulated AMPs as well as autophagy have implications for the composition of the microbiota at the epithelial mucosa of the airways and the gut (161). Evidence for a role of AMPs in regulating

the composition of the microbiota in the gut came from a variety of studies, including those showing that Paneth cell-derived defensins may modulate the composition of the microbiome (162). This notion is further supported by observations showing that many commensal gut bacteria are protected from killing by AMPs such as the vitamin D-inducible hCAP18/LL-37 and hBD-2, whereas pathogens are in general more sensitive (163). Alterations in the gut microbiota have been linked to many diseases of the gut such as IBD but also with diseases affecting the lungs such as COPD and asthma, implicating an important role for the so-called gut–lung axis (164, 165). The mechanisms that explain how gut microbiota affect lung health and disease are complex and include the production of short chain fatty acids (SCFAs). SCFA have a wide range of effects on both immune and structural cells, and the effect of SCFA produced in the intestine on lung immunity may in part be explained by modulation of myeloid cells in the bone marrow, which subsequently migrate to the airways and modulate local immune responses (165). Microbiota that are diverse, rich and contain a higher abundance of SCFA-producing species within these populations are considered to be associated with health (166). In the gut there is strong evidence that both vitamin D deficiency and/or supplementation affect composition of the adult and infant microbiota (166, 167), specifically in relation to disease (168). However, due to the limited number of RCTs and small sample sizes, the precise effects on the microbiota and the mechanisms involved in this are still unclear (166). Alterations in the lung microbiota are also observed in COPD and asthma patients and are likely the result of environmental exposures, airway remodeling, infections and treatments such as the use of antibiotics. This may contribute to disease pathogenesis through altered epithelial innate and adaptive immune responses that damages the airway epithelial barrier and provokes further changes in the lung microbiome that accumulates with increasing disease severity (169, 170). To date only 2 studies describe a possible influence of vitamin D on composition of the microbiota in the airways (171, 172). Toivonen *et al.* showed an association between low serum 25(OH)D-levels and reduced richness of the nasopharyngeal microbiota and bronchiolitis severity in patients with low 25(OH)D-levels (171), whereas in another study vitamin D supplementation decreased the abundance of *Staphylococcus aureus*, *Staphylococcus epidermidis* and Corynebacterium species in sputum samples in vitamin D-deficient CF patients compared to sufficient CF patients (172). In summary, there is evidence that alterations in the airway or gut microbiota can affect chronic airway disease and that these changes could be related to both

vitamin D deficiency and/or supplementation. However, due to the limited number of RCTs and small sample sizes more RCTs are needed in larger patient populations.

Effect of vitamin D supplementation on chronic airway diseases

COPD

The above described protective and therapeutic possibilities of vitamin D, together with observations that many COPD patients are vitamin D deficient, suggest that COPD patients might benefit from vitamin D supplementation. As discussed elsewhere in this review, the link between circulating 25(OH)D-levels and the number of exacerbations has been extensively studied (8). So far however, only 4 RCTs have investigated the effect of vitamin D supplementation in the context of COPD: only 2 out of 4 RCTs showed that vitamin D supplementation reduces the number of exacerbations (173-176). However, in a post-hoc analysis, selecting those patients that were vitamin D deficient, exacerbations were indeed reduced after vitamin D supplementation. Jolliffe *et al.* summarized these 4 RCTs and performed a recent individual participant data meta-analysis and concluded that vitamin D supplementation is only protective against exacerbations in COPD patients with baseline serum 25(OH)D levels < 25 nmol/L (177). These important findings suggest that exacerbations in this specific subset of COPD patients are connected to vitamin D deficiency and this part can be resolved with supplementation. In summary, the protective effects of vitamin D in patients suffering from COPD are most prominent in those with vitamin D deficiency and this would indicate that serum levels 25(OH)D in these patients should always be determined before considering using vitamin D supplementation. Since only 4 RCTs with relatively small patient populations have been conducted in both vitamin D-sufficient and -deficient COPD patients, more RCTs are needed, especially in vitamin D-deficient patients. Currently, a multicenter RCT is being conducted by Rafiq and colleagues in a group of vitamin-deficient COPD patients (25(OH)D < 50 nmol/L), which may reveal whether vitamin D is indeed protective against exacerbations in this group (178).

Vitamin D supplementation in asthma, cystic fibrosis and acute respiratory tract infections

In addition to the effects of vitamin D supplementation in COPD patients, the effects of vitamin D supplementation has also been extensively investigated in other lung diseases (which have associations with vitamin D deficiency) such as asthma, cystic fibrosis, upper respiratory tract infections. Most RCTs that investigated the effects of vitamin D supplementation were performed in acute respiratory tract infections (ARTIs) and asthma. A recent meta-analysis that assessed the effects of vitamin D supplementation in 25 RCTs (11 321 participants) showed that indeed vitamin D supplementation was protective against ATRIs and this effect was again more profound in patients with vitamin D deficiency 25(OH)D < 25 nmol/L at baseline (179). A recent meta-analysis in asthma that included a total of 14 randomized controlled trials (1421 participants), indicated that vitamin D supplementation reduced the rate of asthma exacerbations and increased lung function, especially in patients with vitamin D insufficiency (25(OH)D < 75 nmol/L) (180). Interestingly, in asthma patients that were supplemented with vitamin D, the frequency of respiratory infections was reduced, and this effect was related to the increase of hCAP18/LL-37 (181). CF patients with vitamin D deficiency had a higher rate of exacerbations as compared to patients with sufficient vitamin D levels (182). However only one recent multicenter RCT was conducted and indicated that vitamin D supplementation did not affect the number of exacerbations in CF patients with serum 25(OH)D concentrations between 25 and 137.5 nmol/L (183). In summary, the protective effects of vitamin D supplementation in patients suffering from COPD, asthma or ARTIs are most prominent in those with vitamin D deficiency and this would indicate the importance of establishing serum levels 25(OH)D in these patients as supplementation could reduce unnecessary aggravated disease pathology as a result of this deficiency.

Conclusion and Perspectives

Many drivers of COPD pathogenesis such as chronic exposure to noxious particles and gases, which are present in CS and air pollution, proteolytic enzymes, cytokines and chemokines that are released by infiltrating inflammatory cells, are known to harm the epithelial barrier and cause aberrant remodeling of the airway epithelium with important functional consequences for e.g. host defense. A dysfunctional epithelial barrier increases the susceptibility towards bacterial and viral infections, which are important triggers of COPD exacerbations and these exacerbations contribute importantly to disease progression. Sufficient levels of vitamin D may provide partial protection against these effects by reducing the effects of oxidative stress induced by exposure to inhaled oxidants or those derived from recruited inflammatory cells. Vitamin D furthermore protects against impairment of epithelial barrier function by promoting the integrity of the epithelial barrier, and by modulating both innate and adaptive immune responses. Protection against the detrimental effects of both bacterial and viral infections is provided by the ability of vitamin D to promote of antiviral responses, induce expression of AMPs and modulate of inflammatory responses. Taken together, these activities suggest that vitamin D may provide protection against development and progression of COPD, and against disease exacerbations.

In addition, the local inflammatory milieu as well as the chronic exposure to noxious particles and gases, which are present in CS and air pollution, may negatively affect vitamin D bioavailability and signaling. Here we discussed the recent *in vitro* studies that demonstrated that disease-associated factors such as inflammation and exposure to CS and air pollution could interfere with vitamin D signaling and its degradation and activation by affecting expression of VDR, CYP24A1 and CYP27B1 respectively. These findings indicate that vitamin D bioavailability and the protective effects of vitamin D on the airway mucosa might be impaired especially in patients with COPD with elevated exposures to cigarette smoke and cytokines such as TNF-α, IL-1β, IL-17A and TGF-β1. This suggests that even in patients with sufficient vitamin D serum levels the local activity of vitamin D in the lungs can be improved. We have to start generating more information on both systemic and local bioavailability of vitamin D and gene expression signatures related to vitamin D metabolism or vitamin D responses in COPD (and other chronic inflammatory diseases that are related to vitamin D deficiency), both at baseline and after vitamin

D supplementation. This information could lead to improved treatment strategies that enhance local efficacy of vitamin D, using e.g. specific CYP24A1-inhibitors such as VID400 (184). Alternatively, degradation by CYP24A1 could be prevented by using 1,25$(OH)_2$D analogs that are insensitive to CYP24A1-mediated degradation, such as sulfone and sulfoximine derivatives, that also act as a VDR agonist (185). A third option is to entail the use of combination treatment with vitamin D and anti-inflammatory or certain anti-fibrotic drugs that target cytokines/proteins that are known to potentially decrease bioavailability of vitamin D by inducing expression of CYP24A1 (48, 186, 187). When considering such strategies, it should be noted that these may enhance the calcemic side effects and lead to unwanted inhibition of the immune system. We therefore need to carefully analyze the preclinical *in vivo* and *in vitro* studies and balance the pros and cons of the different strategies. In conclusion, future studies in COPD and but also in other chronic inflammatory diseases that are related to vitamin D deficiency, should be designed with more focus on assessing and improving local bioavailability of vitamin D. These new insights may lead to the development of new treatment strategies, such as those targeting CYP24A1 to enhance local bioavailability of vitamin D resulting in improved homeostasis and protection of the airway mucosa in patients with chronic inflammatory lung diseases.

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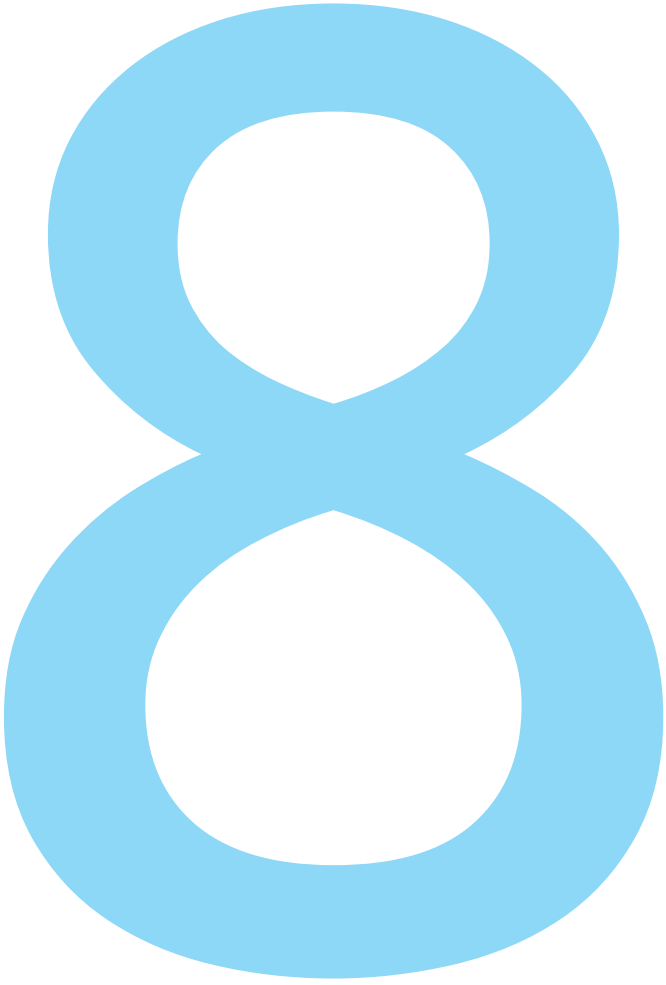
CHAPTER



Summary and General Discussion

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Introduction

In this thesis, a collection of *in vitro* studies is presented that describe the role of vitamin D (biologically inactive 25-hydroxy-vitamin D [25(OH)D] and active 1,25-dihydroxy-vitamin D [1,25$(OH)_2$D]) and inflammatory mediators on respiratory host defense responses and exacerbations, specifically focused on chronic obstructive pulmonary disease (COPD). These *in vitro* studies are followed by a description of a clinical randomized controlled trail [RCT], that aims to investigate in COPD patients whether vitamin D supplementation would indeed be helpful in preventing exacerbations. This thesis is concluded by a review that discusses the latest studies on the effects of disease-associated factors on mucosal vitamin D metabolism and signaling in chronic airway diseases.

The main aim of this thesis is to examine if vitamin D supplementation could be one of the possible strategies to restore the affected host defense responses and could therefore protect against exacerbations in chronic inflammatory lung diseases such as COPD. Before examining the protective role of 1,25$(OH)_2$D in respiratory host defense responses, we first aimed to investigate why respiratory host defense responses are impaired in COPD, by investigating the role of cigarette smoke (CS) and airway epithelial remodeling herein. Furthermore, the presence of disease-associated factors such as inflammation might affect 1,25$(OH)_2$D mediated host defense responses in the lung. We therefore aimed to study the effects of inflammation on the protective role of 1,25$(OH)_2$D in respiratory host defense responses. The aim here was to obtain a better understanding of 1,25$(OH)_2$D signaling and its possible impairment in the lungs of COPD patients, in order to contribute to the discussion on whether (and which) new strategies are needed to enhance local levels of 1,25$(OH)_2$D and protection against exacerbations in COPD.

Overview of the Main Findings in this Thesis

Bacterial- and viral infections are important triggers of COPD exacerbations and the airway epithelium plays a central role in orchestrating immune responses towards invading pathogens (1-4). However, various studies have shown that the physical

and immunological barrier function of the airway epithelium in COPD patients is defective (reviewed in (1, 5)). As a consequence of this airway epithelial dysfunction, the susceptibility towards bacterial- and viral infections is increased and this could further increase exacerbation rate and thereby contribute to disease progression in COPD patients (1, 6, 7). We therefore used *in vitro* culture models of primary airway epithelial cells to study the effects of cigarette smoking and inflammatory mediators on airway epithelial host defense mechanisms, with a specific focus on $1,25(OH)_2D$-mediated epithelial host defense mechanisms. In **Chapter 2,** we first investigated the effects of cigarette smoke (CS) on airway epithelial host defense mechanisms (8). In the studies described in this chapter, we daily exposed airway epithelial cells to whole CS during their differentiation phase to investigate its effects on the expression of constitutively expressed antimicrobial peptides (AMPs) and other host defense mediators such as the polymeric Ig receptor (pIgR). We found that CS exposure reduced the levels of the constitutive expressed host defense mediators SLPI, s/lPLUNC and pIgR and linked this to the inhibitory effects of CS on end-stage epithelial differentiation, i.e. formation of ciliated-, club- and goblet cells. As a result of these inhibitory effects, epithelial antibacterial defenses against *M. catarrhalis* and *K. pneumoniae* were impaired, and also polymeric IgA transport across the airway epithelium was lower after CS exposure. This indicates that CS may have a marked impact on humoral airway epithelial host defense mechanisms through its effect on epithelial remodeling.

In **Chapter 3,** we further explored effects of airway epithelial remodeling on airway epithelial host defense mechanisms, and showed that TGF-β1 caused similar effects as chronic CS exposure on epithelial host defense. Short exposures to TGF-β1 were found to be sufficient to decrease expression levels of the constitutively expressed host defense molecules SLPI, s/lPLUNC and pIgR, as well as numbers of secretory club- and goblet cells (9). We additionally showed that TGF-β1 reduced baseline as well as $1,25(OH)_2D_3$-mediated expression of the AMP hCAP18/LL-37. This inhibitory effect on $1,25(OH)_2D_3$-inducible gene expression could be explained by the observation that TGF-β1 increases degradation of $25(OH)D_3$ and $1,25(OH)_2D_3$ by increasing the expression of the 25(OH)D and $1,25(OH)_2D$-degrading enzyme CYP24A1.

In **Chapter 4**, we further described that long-term exposure of differentiating airway epithelial cells to proinflammatory mediators such as TNF-α, IL-1β and IL-17A, which are elevated in the airways of COPD and refractory asthma patients

(asthma patients, who do not respond to current standard therapy), increased 25(OH)D and 1,25$(OH)_2$D metabolism by enhancing expression of CYP24A1 (10). As a result, the ability of 25$(OH)D_3$ to induce expression of hCAP18/LL-37 and to enhance killing of nontypeable *Haemophilus influenzae* (NTHi) was reduced.

In contrast to **Chapter 3 and 4**, where we showed that 25$(OH)D_3$ and 1,25$(OH)_2D_3$ catabolism was induced and host defense responses in airway epithelial cells were impaired by TGF-β1, TNF-α, IL-1β and IL-17A, in **Chapter 5** we demonstrated that presence of a Th2-inflammatory environment does in fact the opposite (11). We showed that exposure of airway epithelial cells to the Th2 cytokines IL-13 and IL-4 enhanced the expression of CYP27B1, which in turn promoted conversion of the circulating -yet inactive- 25$(OH)D_3$ into the active 1,25$(OH)_2D_3$, thereby inducing expression of hCAP18/LL-37. 1,25$(OH)_2$D treatment was furthermore capable of dampening viral dsRNA-induced inflammatory responses in airway epithelial cells, which was illustrated by a reduction of cytokine- and chemokine release upon Poly(I:C) (a synthetic analog of [viral] dsRNA) stimulation.

To assess whether the afore mentioned protective effects of vitamin D supplementation will indeed reduce exacerbation rate in COPD, a multicenter RCT is designed to study the effects of vitamin D supplementation in a study population that, in contrast to previous RCTs, specifically focusses on vitamin D-deficient patients (serum [25(OH)D] 15 - 50 nmol/L). Study design, protocol and read-outs are described in **Chapter 6** (12).

Finally, in **Chapter 7** we have summarized the current knowledge of the effects of disease-associated factors such as inflammation and cigarette smoke exposure on availability and signaling of vitamin D in the lungs of patients with COPD and other chronic lung diseases. We further aimed to link the possible consequences of an altered 1,25$(OH)_2$D status in the lung to the protective effects of 1,25$(OH)_2$D on mucosal host defense mechanisms, inflammation and exacerbations and explore possible strategies to improve local levels of 1,25$(OH)_2$D.
To answer the question whether vitamin D supplementation could be one of the possible strategies to restore the affected respiratory host defense responses in COPD, we first needed to know how exactly respiratory host defense responses are affected.

Role of Cigarette Smoke and Airway Epithelial Remodeling on Host Defense Responses in COPD

Physical barrier function

Cigarette smoking is an important risk factor for COPD pathogenesis and already in 1957 histological changes in the bronchial epithelium of smokers were described by Chang (13). He described that the bronchial epithelial cell layer was thicker with more stratification and increased numbers of basal and goblet cells, whereas ciliated cell numbers were reduced or had shorter cilia. Our observations in **Chapter 2** showed a marked reduction of cilia development during airway epithelial differentiation when cells were daily exposed to CS (8), and are therefore in line with those of Chang, and the many reports that followed this original observation (14). Moreover, several other studies demonstrated that cilia motility was also impaired in smokers with COPD (14). Expression and function of the cystic fibrosis transmembrane conductance regulator (CFTR) (which maintains optimal apical surface liquid (ASL)- and mucus hydration, volume and pH), is also affected in smokers and in COPD (15). This combination of goblet cell hyperplasia, aberrant expression and function of CFTR and cilia, collectively impairs mucociliary clearance and contributes to airway obstruction and increased susceptibility towards infections (14). Whereas goblet cell hyperplasia is known to be present in smokers and COPD patients, in **Chapter 2** we observed that CS exposure inhibited the formation of goblet cells during epithelial differentiation at the air-liquid interface (ALI) (8). Interestingly, short-term exposure to TGF-β1 was already sufficient to decrease the number of goblet cells in fully differentiated airway epithelial cell cultures (**Chapter 3)** (9). This might indicate that the effect of CS exposure on the expression of mucins and formation of goblet cells could be mediated in part by TGF-β1, which is known to be increased by CS exposure (16). Also, the level of cigarette smoke exposure and the missing immune component could contribute to a lack of goblet cell hyperplasia.

CS exposure furthermore reduces epithelial barrier integrity by causing degradation of its tight and adherens junctions, favoring trans-epithelial invasion of pathogens (7). The loss of expression of tight and adherens junction proteins such as zona occludens (ZO)-1 and E-cadherin, combined with gain of mesenchymal markers, is a hallmark of epithelial-to-mesenchymal transition (EMT) and is present especially in the small airways of (ex-) smokers with and without COPD (17, 18). The effects of chronic CS exposure on airway epithelial barrier integrity were also assessed in

Chapter 2, where only a minor delay was observed in epithelial barrier development during the first week of differentiation, compared to air-exposed cells (8). This was however not significant and could be explained by the fact that decreases in epithelial barrier function (by measuring trans epithelial electrical resistance [TEER]) by CS are transient and normalized at the time of measurement (18-20 h after the previous CS exposure) (19). Altogether, these studies further support the notion that structural epithelial changes may affect physical barrier function in COPD patients and in smokers. Since studies have shown that $1,25(OH)_2D$ may increase epithelial barrier function (see review **Chapter 7**), it would therefore be of interest to investigate if the loss of epithelial junction proteins or epithelial barrier function could be restored by $1,25(OH)_2D$ treatment *in vitro* in CS-exposed airway epithelial cells or by vitamin D supplementation *in vivo* in smokers or COPD patients.

Cellular remodeling

More recent studies have highlighted the importance of club cells in respiratory host defense. Club cells are decreased in COPD patients and by CS exposure *in vitro,* this decrease could negatively impact epithelial barrier function, neutralization of toxins, immunomodulation and epithelial regeneration (20-23). CS furthermore increases production and activation of TGF-β1 in airway epithelial cells and elevated levels of TGF-β1 expression were found in the airways of COPD patients (17, 24-26), although this was not shown in all studies (27). In **Chapters 2 and 3**, we have indeed confirmed that exposure to both CS and TGF-β1 results in a reduction in levels of markers of club cells (8, 9). This could suggest that TGF-β1 might play an important role in the CS-mediated reduction of club cells and club cell-mediated functions in the airway epithelium of COPD patients and smokers. However, to fully determine the role of TGF-β1 in regulating club cell numbers and function (release of CC-10), further studies are needed. This could be achieved by using *in vitro* chronic CS exposure models of airway epithelial cells that are treated with TGF-β1-specific antibodies or TGF-β1-Smad signaling inhibitors followed by assessment of club cell markers. Furthermore, in **Chapter 2** we showed that both ciliated and goblet cells strongly recovered upon cessation of CS exposure, except for club cells that remained absent after almost 1 week of recovery (8). This is in line with studies showing that club cells are more sensitive to damage by CS and other noxious gases than other airway epithelial cell types (28). Elevated TGF-β1-expression levels are known to persist in airway epithelial cell cultures derived from COPD patients, which may help to explain why club cells remain absent after smoking cessation

(29). Future studies could further explore the potential of selective TGF-β1 inhibition in the reduction of club cells and its effects on host defense mediators to consider this as a future treatment strategy in COPD patients.

Humoral respiratory host defense

In addition to the physical barrier, humoral defenses of the airway epithelium are also affected in COPD, and this is at least in part attributable to CS-mediated changes in epithelial composition (30). The humoral barrier is provided by e.g. host defense mediators such as AMPs, reactive oxygen species (ROS), CFTR (which influences pH and therefore activity of AMPs), type I and III interferons and by production of cytokines, chemokines and lipid mediators (31-33). Many of these mediators are directly affected by cigarette smoking or indirectly by CS-induced inflammation or by epithelial remodeling (17, 18, 30, 34). For example, acute CS exposure directly decreases upregulation of inducible AMPs such as human β-defensin-2 (hBD-2) upon triggering by a bacterial component (34), whereas other AMPs such as hCAP18/LL-37 and RNAse7 are increased upon CS exposure (19, 35). Moreover, expression and function of the CFTR is also negatively affected by CS and this process seems to be mediated by TGF-β1 (30, 36). In **Chapter 3** and **4**, we have demonstrated that both chronic CS and short-term TGF-β1 exposures decreased expression of constitutively expressed host defense mediators by affecting airway epithelial cell composition (8, 9). We additionally showed that in particular host defense mediators that are expressed by secretory luminal epithelial cells are reduced upon exposure to CS and TGF-β1 (8, 9). Recent developments in single cell-RNA sequencing confirm that *BPIFB1* (lPLUNC), *SLPI* and *PIGR* are indeed highly expressed by club cells (23, 37). Our results indicate that specifically end-stage differentiation and expression of luminal cell expressed host defense mediators is impaired by chronic CS exposure and, when taking into account the results of **chapter 3**, new research endeavors should focus on investigating whether this is partly regulated by CS-induced expression of TGF-β1. In addition to its effects on airway epithelial antibacterial activity, SLPI and s/lPLUNC are furthermore known to promote respiratory host defense by dampening immune responses, inhibition of proteases and by maintaining apical surface liquid (ASL) hydration (38, 39). Future studies could use more complex culture models, such as an airway-on-chip model with the possibility to expose the cells to airflow and mechanical forces, is a better representation of the *in vivo* situation (40). This model further allows the use of immune cells in combination with well-differentiated airway epithelial cells and

could establish further consequences of both CS exposure and TGF-β1 on other aspects of respiratory host defense such as on immune responses (40, 41).

Vitamin D-mediated host defense responses

Vitamin D ($1,25(OH)_2D$) displays protective effects on host defense responses in the airways, e.g. by maintenance of the of the mucosal barrier integrity, modulation of stress, damage and immune responses and by the promotion of killing of pathogens (e.g. via the induction of the AMP hCAP18/LL-37) (42-44). The VDR is expressed in all epithelial cell types of the lungs with higher expression levels in alveolar epithelial cells and lower expression levels in club cells, compared to other pulmonary epithelial cell types. CYP27B1 is also expressed in all epithelial cell types and higher expression levels are again present in alveolar cells, but lower expression levels are found in both goblet- and ciliated cells. This indicates that all lung epithelial cells, but especially alveolar cells, should be responsive to 25-hydroxy-vitamin D [25(OH)D], the main circulating, yet inactive form of vitamin D (45). Interestingly, expression of CYP24A1 (that converts both 25(OH)D and $1,25(OH)_2D$ into inactive metabolites) is absent in alveolar epithelial cells, but expressed at low levels in club cells and is highly expressed in basal-, ciliated- and goblet cells (45). This suggests that an increase in basal- and goblet cells, as present in the peripheral airways of COPD patients, might increase expression levels of CYP24A1, which degrades $1,25(OH)_2D$ that mitigates its effects on epithelial host defense. Further studies in COPD patients showed that the expression of VDR mRNA and protein in alveolar tissue was gradually reduced in COPD patients with increasing disease severity (46). It would be of interest to determine if changes in expression of genes related to vitamin D metabolism are the result of destruction of alveolar tissue and remodeling of the airway epithelial cell layer, as seen in COPD patients.

In **Chapter 6** we have demonstrated that a pro-inflammatory environment, created by chronic exposure to proinflammatory cytokines such as TNF-α/IL-1β and IL-17A during the differentiation of airway epithelial cells, increased the expression of the $25(OH)_2D$ and $1,25(OH)_2D$-degrading enzyme CYP24A1 without affecting expression of CYP27B1 and VDR (10). We additionally assessed if the changes in CYP24A1 were the result of an altered epithelial differentiation by assessing the effects of TNF-α/IL-1β on the expression of epithelial cell basal, club-, goblet- and ciliated cell markers, and found that expression of these markers was not affected (10). This indicates that TNF-α/IL-1β-mediated increases of CYP24A1 were not explained by

changes in the composition of the epithelial cell layer. Conversely, in **Chapter 4** we found that the TGF-β1-mediated induction of CYP24A1 occurred in concert with a reduction in both club- and goblet cells (47). Despite the fact that similar effects were observed in submerged cultures of undifferentiated airway epithelial cells, we cannot fully exclude that TGF-β1-induced effects on CYP24A1 expression were the result of an altered composition of the epithelial cell layer. To fully elucidate this, airway epithelial cells can be differentiated in presence of stimuli that for example affect end-stage epithelial differentiation, such as CS. Another way is to differentiate cells in presence of a Notch-signaling inhibitor (DAPT), that skews differentiation towards ciliated cells or in presence of IL-13 to increase goblet cell formation. During these processes expression of VDR, CYP27B1 and CYP24A1, and $1,25(OH)_2D$ responses could be monitored at an early/acute phase (after 1 day) and at an later phase of epithelial differentiation (after 3 weeks). In fact, we have already tried this with CS exposure in a pilot study and found that CS inhibited end-stage airway epithelial differentiation as expected and also increased CYP24A1 expression (unpublished observations). Compared to the effects of TNF-α/IL-1β, IL-17A and TGF-β1, the increase in CYP24A1 expression was much less prominent and this might explain why $1,25(OH)_2D$-mediated expression of hCAP18/LL-37 was not affected by CS. In conclusion, both chronic CS exposure and TGF-β1 cause aberrant epithelial remodeling in the conducting airways, thereby disrupting both physical and humoral airway epithelial host defense functions. This might help to explain why smokers with and without COPD have increased susceptibility towards respiratory infections, and how the local environment may determine vitamin D metabolism and action.

Targeting Epithelial Remodeling in COPD

Dysfunctional basal progenitor cells within the airway epithelium are considered to play a fundamental role in the observed aberrant remodeling of the airway epithelium in COPD patients and in smokers and could therefore be a target to reverse this process in COPD (48). Several studies have identified a subset of keratinocyte 5 (KT5)-positive basal cells as basal progenitor cells, and showed that these are characterized by the ability to self-renew and to differentiate into basal-,

secretory-, and ciliated cells (48). COPD patients and smokers with a reduced lung function have lower numbers of $KT5^+$ clone-forming basal progenitor cells than healthy controls, and those cells display signs of exhaustion (49). One of the possible explanations for the observed exhaustion of the basal progenitor cells might be cellular senescence. Senescence is prevalent in both airway- and alveolar epithelial cells in COPD patients and recent studies showed that induction of airway epithelial basal cell senescence results in reduced basal cell proliferation (50-52). Other studies have shown that basal progenitor cells, derived from smokers and COPD patients, have aberrant Notch, TGF-β1, BMP-4 and/or EGFR signaling that also may additionally explain the dysfunctional properties of these progenitor cells (53-56). In our chronic CS-exposure model in **Chapter 3,** we have indeed observed decreases in expression of the Notch-target genes *HEY1* and *HEY2* (but not others) after chronic CS-exposure. In addition, when cells were differentiated in presence of the Notch-signaling inhibitor DAPT, expression of secretory goblet- and club cell markers as well as luminal constitutive host defense mediators was reduced (8), further supporting a role of these pathways in aberrant airway epithelial differentiation. Intervention strategies to treat dysfunctional basal progenitor cells, e.g. by combined targeting of Notch, TGF-β1, BMP-4 or EGFR-signaling pathways, may have the potential to stop or even reverse disease progression. For example, CS-mediated loss of ciliated cells was counteracted through inhibition of EGFR-signaling by Gefinitib during *in vitro* airway epithelial cell differentiation (54). In addition, inhibition of BMP-4 by DMH-1 was able to (partially) reverse CS extract-mediated induction of squamous cell markers and reduction of both ciliated- and secretory cell markers (55), whereas TGF-β1-inhibition during differentiation at the air-liquid interface was able to reverse signs of EMT that were still present in airway epithelial cells, derived from COPD patients (29). It would be interesting to also study effects of such strategies on key enzymes involved in vitamin D metabolism. In **Chapter 3,** we observed that inhibition of TGF-β1-Smad signaling by using SB431542 was sufficient to reverse TGF-β1-mediated reduction of the $1,25(OH)_2D$-inducible hCAP18/LL-37 and this compound also prevented induction of CYP24A1 (9). Perhaps reducing the inflammatory state of the local environment and reducing exposure to inhaled toxicants by smoking cessation could already reverse some features of EMT and other detrimental effects of TGF-β1. It seems that elevated expression levels of EMT-markers could return back to control levels, when COPD-derived airway epithelial cells were cultured at the air-liquid interface for an extended period of time (29). Combining treatment with inhaled corticosteroids

with vitamin D-supplementation could serve to reduce the inflammatory state of the local environment without affecting respiratory host defense responses, which is a well-known side-effect of steroid use (**Chapter 7**, (57)). In conclusion, new approaches to specifically target dysfunctional basal progenitor cells together with improving the local environment could be considered to counteract aberrant airway epithelial remodeling. These new approaches are needed, since remodeling especially of the small airways is currently considered to be a crucial process that precedes further airway obstruction, destruction of alveolar walls and/or development of lung cancer (18, 58).

Modulating of Vitamin D Metabolism in Chronic Inflammatory Lung Diseases

Local availability and action of $1,25(OH)_2D$ depends on expression of VDR and an equilibrium between the vitamin D metabolic enzymes CYP27B1 and CYP24A1, and this can be affected by several inflammatory mediators and exposure to inhaled toxicants, which is discussed in **Chapter 7**. The presence of these inflammatory mediators in chronic inflammatory lung diseases could therefore interfere with expression of VDR, CYP24A1 and CYP27B1, which as a consequence may affect the local levels of $1,25(OH)_2D$. In **Chapter 5** we observed that exposure to Th2 cytokines enhances expression of CYP27B1, thereby promoting 25(OH)D-metabolism in airway epithelial cells, whereas others have shown this effect after exposure to poly(I:C) (11, 60). We have indeed demonstrated in **Chapter 3, 4** and **5** that cytokines that are known to be elevated in both COPD and (refractory) asthma patients, such as IL-4, IL-13, TGF-β1, IL-1β, TNFα and IL-17A, have the ability to alter the expression of genes related to vitamin D metabolism, such as CYP27B1 and CYP24A1 in airway epithelial cells (9, 10, 61). These *in vitro* data suggest that (chronic) airway inflammation can have a large impact on local levels of $1,25(OH)_2D$ through enhanced conversion into $1,25(OH)_2D$ in presence of a Th2-inflammatory environment or enhanced degradation of both 25(OH)D and $1,25(OH)_2D$ in presence of a TGF-β1, Th1-/Th17-inflammatory environment. Nonetheless, airway inflammation in COPD *in vivo* is far more complex and heterogeneous than we have tested so far *in vitro* using monocultures of airway epithelial cells, or using only

immune cells. For that reason, animal models or preferably more complex cell culture models such as organs-on-chips technology in combination with co-cultures of epithelial cells and immune cells that are derived from COPD patients could be used in future studies to fully understand the impact of airway inflammation on $1,25(OH)_2D$ responses and discrepancies between systemic levels of 25(OH)D and local levels of 25(OH)D and $1,25(OH)_2D$. In addition to the demonstrated effects of viruses and cytokines on expression of genes related to airway epithelial vitamin D metabolism (CYP27B1 and CYP24A1), other studies have shown that exposure to inhaled toxicants such as CS and particulate matter (PM) may alter expression or activity of VDR and CYP27B1 (62, 63). This was in contrast to what was observed in most immune cells, such as monocytes and macrophages. In monocytes and macrophages, the effects of $1,25(OH)_2D$ were even enhanced by exposure to most proinflammatory stimuli, apart from Benzo[a]pyrene (BaP), a polycyclic aromatic hydrocarbon produced by cigarette combustion, which increased the expression of CYP24A1 (64-69).

A recent study in COPD patients that assessed both VDR and CYP24A1 expression in parenchymal lung tissue, observed lower expression of both VDR and CYP24A1 compared to non-COPD controls, indicating that also in the lower airways the effects of $1,25(OH)_2D$ might be impaired (70). Importantly, another recent study that investigated vitamin D metabolism in both serum and in lung tissues of both COPD and asthma patients, found evidence to suggest that the conversion of vitamin D_3 into $25(OH)D_3$ was impaired (71). Furthermore, locally in lung tissue and/or in airway epithelial cells derived from both asthma and COPD patients, expression of 25-hydroxylase (CYP27A1), which converts vitamin D_3 into $25(OH)D_3$, was reduced compared to healthy controls, while the opposite was true for VDR. The authors also showed no differences in CYP27B1 expression between groups, which is not in line with our observations *in vitro* of increases in CYP27B1 in presence of Th2 cytokines in **Chapter 5** (11). Furthermore, expression of CYP24A1 was decreased in asthma patients, whereas in lung tissue from COPD patients there was a trend towards a significant increase (log fold change 0.36; $p = 0.06$), which was more in line with our *in vitro* observations in **Chapters 3** and **4** (9, 10). More importantly, another study showed that expression of epithelial $1,25(OH)_2D$-responsive genes was increased in sputum and nasal brushings from asthma patients, whereas this was decreased in lung tissue from COPD patients (72). This study indicates that especially in COPD patients, local levels of $1,25(OH)_2D$ and signaling are indeed impaired and that in addition to CYP24A1, also the expression

of CYP27A1 might play an important role. Collectively, these studies imply that local inflammatory conditions in most cases negatively affect both local levels and activity of $1,25(OH)_2D$ on the airway epithelium.

To enhance local efficacy of $1,25(OH)_2D$, alternative strategies might be considered, such as inhalation with active $1,25(OH)_2D$ or supplementation with $25(OH)D_3$ instead of vitamin D_3, to circumvent the impaired metabolism of vitamin D_3 into $25(OH)D_3$ which is seen in both asthma and COPD patients (71). Also specific CYP24A1 inhibitors, $1,25(OH)_2D$ analogs that are resistant to degradation by CYP24A1, and other VDR agonists can be used, or a combination treatment of vitamin D with anti-inflammatory or certain anti-fibrotic drugs that target TGF-β1 to prevent increases in CYP24A1 (73, 74). It should however be considered that these compounds may enhance the calcemic side effects or impair the immune system. Altogether, we need to fully establish the impact of local inflammation on local $1,25(OH)_2D$ levels and responses by using advanced *in vitro* systems and to some extent animal models, and test these different strategies that may enhance local levels of $1,25(OH)_2D$.

Role of Vitamin D in Airway Epithelial Host Defense Responses

Exacerbations

Exacerbations are a major burden in COPD patients, accelerate decline in lung function and frequently result into hospital admissions and are often triggered by pollutants or by bacterial- and/or viral infections (2-4, 75, 76). COPD patients generally have lower serum 25(OH)D-levels and this is associated with more exacerbations, suggesting that COPD patients might benefit from vitamin D supplementation (77-79). To date, the effects of vitamin D supplementation in the context of COPD exacerbations has been investigated in only 4 RCTs with mixed results: 2 RCTs showed that vitamin D-supplementation reduces the number of exacerbations (80, 81), whereas 2 other RCTs did not find this effect (82, 83). When these 4 RCTs were combined and summarized by using an individual participant data meta-analysis, vitamin D supplementation was shown to be protective against exacerbations in vitamin D deficient COPD patients (baseline serum 25(OH)D levels < 25 nmol/L) (84). This suggests that exacerbations in this group of COPD patients were mainly driven by vitamin D deficiency. To investigate the effects of vitamin D supplementation in vitamin D deficient COPD patients and to gain more insight into

the mechanisms behind the protective effects of vitamin D, the following study was designed: a multicenter RCT that aims to study the effects of vitamin D-supplementation of 6800 IU vitamin D or placebo orally once a week for 1 year on exacerbation rate, systemic and local inflammation, physical performance and quality of life in a study population of vitamin D-deficient patients only (serum [25(OH)D] 15 - 50 nmol/L) (**Chapter 6**) (12). We hypothesize that vitamin D supplementation will improve respiratory host defense responses and will therefore reduce exacerbations and will further improve quality of life in this particular group of COPD patients.

Antibacterial activity

In this thesis, we have identified mechanisms that may underlie the protective effects of vitamin D supplementation *in vitro* in the context of COPD exacerbations, thereby additionally confirming that the airway epithelium is a major target for the effects of locally synthesized $1,25(OH)_2D$. hCAP18/LL-37 is likely to be the most prominent AMP that is induced by $1,25(OH)_2D$ in several types of mucosal epithelial cells and in immune cells, including monocytes and neutrophils (60, 65, 85, 86). The *in vitro* demonstration of $1,25(OH)_2D$-mediated induction of AMPs suggests that vitamin D supplementation could reduce the susceptibility to (respiratory) infections *in vivo.* This was indeed confirmed by several clinical studies, including a recent meta-analysis that showed a protective effect of vitamin D supplementation against acute respiratory tract infections (ARTI) (87). In **Chapter 4,** we have confirmed this ability of $1,25(OH)_2D$ to promote antibacterial activity in differentiated cultures of airway epithelial cells by demonstrating that both 25(OH)D and $1,25(OH)_2D$ increased antibacterial activity against NTHi, a Gram-negative bacterium which is associated with COPD exacerbations (10, 88). We additionally showed that exposure to TNF-α/IL-1β inhibited 25(OH)D and $1,25(OH)_2D$-induced antibacterial effects as well as expression of hCAP18/LL-37, indicating that the 25(OH)D and $1,25(OH)_2D$-mediated antibacterial effects were most likely mediated through the induction of hCAP18/LL-37 (10). Moreover, also other studies have now demonstrated that $1,25(OH)_2D$ treatment increased antibacterial effects against other bacteria such as *E. coli*, *P. aeruginosa* and *B. bronchiseptica* (89, 90). The specific role of hCAP18/LL-37 in the observed antibacterial effects of $1,25(OH)_2D$ on airway epithelium *in vitro* was recently confirmed *in vivo* by Vargas Buonfiglio *et al.*, who demonstrated that the increased antimicrobial activity against the Gram-positive *S. aureus* in ASL after vitamin D supplementation was dependent on presence of hCAP18/LL-37 (63). In addition to

vitamin D, also other strategies of using AMP-inducers such as essential amino acids, sodium butyrate and other (natural) compounds show great potential in their capacity to reduce bacterial infections, especially when some of these compounds are combined (91-93). For example in human colonic epithelial cells (Caco-2), a combination treatment with natural compounds such as andrographolide and isoliquiritigenin synergistically enhanced the AMP human β-defensin-3 (hBD-3) expression and antimicrobial activity, especially against pathogenic bacteria (91). In conclusion, the ability of $1,25(OH)_2D$ to promote bacterial clearance by increasing expression of hCAP18/LL-37 may help to explain why vitamin D deficiency is associated with chronic airway diseases such as asthma and COPD (77, 94).

Inflammation

We have furthermore demonstrated in **Chapter 5** that $1,25(OH)_2D_3$ dampens epithelial release of proinflammatory cytokines and chemokines such as IP-10, RANTES (with a trend towards a decrease in IL-8 and IL-6) that were triggered by stimulation with poly(I:C), a synthetic analog of double-stranded RNA, which is used to mimic viral infections (11). The release of these proinflammatory cytokines and chemokines may further recruit immune cells such as monocytes, eosinophils and neutrophils that secrete proteases and cytotoxic and proinflammatory mediators, which amplifies inflammation, causes alveolar destruction, airway mucus hypersecretion and airway obstruction (95-98). The dampening effect of $1,25(OH)_2D_3$ on the release of cytokines and chemokines in airway epithelial cells was also shown by other studies using other known triggers for COPD exacerbations such as PM, respiratory syncytial virus (RSV), and human rhinovirus (HRV) (99-101). Altogether, these studies indicate that vitamin D supplementation may reduce the severity of exacerbations in COPD by promoting the expression of hCAP18/LL-37 and by dampening the release of proinflammatory cytokines and chemokines in the airways.

Concluding Remarks

Many drivers of COPD pathogenesis such as chronic exposure to noxious particles and gases (including those present in cigarette smoke, biomass fuels or air

pollution), proteolytic enzymes, cytokines and chemokines that are released by inflammatory cells, are known to destruct the epithelial barrier and cause aberrant remodeling of the airway epithelium and as a consequence affect secretion of host defense molecules (7, 102). Therefore, protection and restoration of the epithelial barrier may be a fruitful strategy to halt further disease progression. Firstly, we should consider targeting pathways that restore basal progenitor cell function. Secondly, the local environment should be improved by micronutrients such as vitamin D that protects the airway epithelium from epithelial injury and remodeling. However, we have demonstrated in the studies presented in this thesis that certain airway inflammatory mediators could possibly interfere with vitamin D metabolism by promoting expression of CYP24A1, thereby reducing local levels of $1,25(OH)_2D$ and accompanying protective antimicrobial and anti-inflammatory actions (Figure 1). Therefore, future studies in COPD and but also in other chronic inflammatory diseases that are related to vitamin D deficiency, should be designed with more focus on increasing local levels of $1,25(OH)_2D$ and expression of genes related to vitamin D metabolism, especially CYP24A1. These new insights may yield possible new strategies to target CYP24A1 that enhance local levels and signaling of $1,25(OH)_2D$ to increase protection against exacerbations in COPD patients.

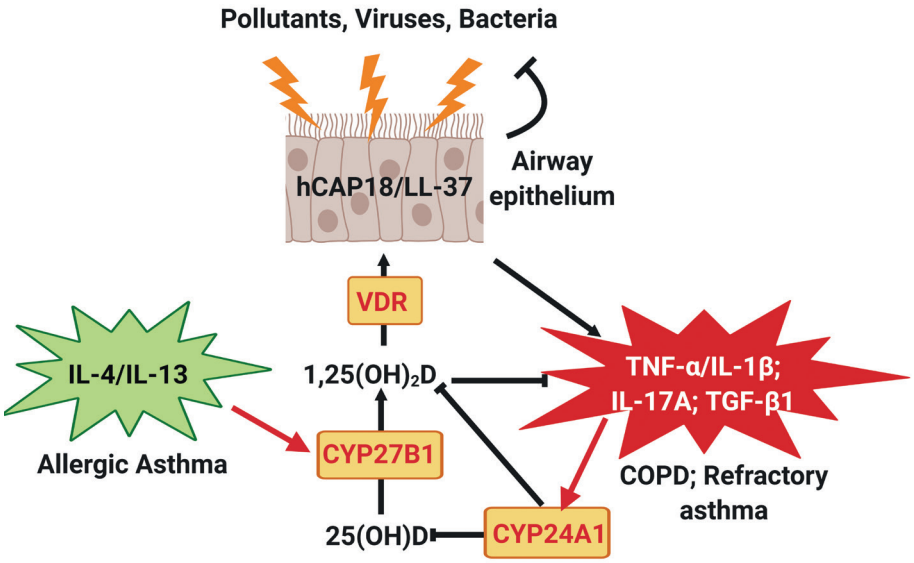

Figure 1. Effects of inflammation on vitamin D-metabolism and epithelial host defense in the airways. Vitamin D receptor, VDR; biologically active vitamin D, $1,25(OH)_2D$; 25-hydroxyvitamin D-1α-hydroxylase, CYP27B1; circulating inactive vitamin D, 25(OH)D; 25-hydroxyvitamin D-24-hydroxylase, CYP24A1

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Addendum

Nederlandse Samenvatting
Dutch summary

Introductie

Vitamine D

Vitamine D staat van oudsher bekend om zijn regulerende rol bij de opname van kalk (calcium) en fosfaat in de darmen en draagt op die manier bij aan het behoud van sterke botten. Vitamine D wordt onder invloed van zonlicht (UV-B straling) in de huid aangemaakt of wordt uit ons voedsel opgenomen. Helaas is de zon in gebieden met hogere breedtegraden, waarin ook Nederland ligt, niet het hele jaar (november – maart) sterk genoeg om ons lichaam van voldoende vitamine D te voorzien. Mede daardoor hebben veel mensen in deze gebieden last van een vitamine D tekort. Daarnaast komt een vitamine D tekort veel voor bij mensen met een donkere huidskleur, die niet veel buiten komen of die in gebieden wonen met veel smog. Een vitamine D tekort werd in het verleden geassocieerd met botaandoeningen zoals rachitis, totdat geleidelijk aan duidelijk werd dat ook meerdere andere aandoeningen geassocieerd zijn met een tekort aan vitamine D, waaronder kanker, diabetes type 2, cardiovasculaire aandoeningen, ziekte van Alzheimer, multiple sclerose, chronische darm ontstekingen, psoriasis en chronische long aandoeningen zoals astma en chronisch obstructieve longziekte (COPD).

Het vitamine D dat in de huid wordt aangemaakt of uit voeding wordt verkregen, is nog niet biologisch actief. Eerst wordt deze inactieve vorm van vitamine D in de lever door de enzymen CYP2RI en CYP27A1 in 25-hydroxy-vitamine D (25(OH)D; ook wel calcidiol of calcifidiol genoemd) omgezet. 25(OH)D is de meest voorkomende vorm van vitamine D in het bloed en een maat voor vitamine D status in het lichaam. In de nieren wordt vervolgens het nog inactieve 25(OH)D door het enzym CYP27B1 omgezet in zijn actieve vorm: 1,25 dihydroxy-vitamine D ($1,25(OH)_2D$; ook wel calcitriol genoemd). Recente onderzoeken hebben echter aangetoond dat ook andere weefsels en celtypen zoals luchtwegepitheelcellen, huidcellen, darmcellen en immuuncellen deze enzymen kunnen aanmaken en dus lokaal calcidiol kunnen omzetten naar de actieve vorm van vitamine D. Als reactie op de aanmaak van actief vitamine D, wordt CYP24A1 aangemaakt in cellen die door $1,25(OH)_2D$ zijn geactiveerd via de vitamine D receptor (VDR). CYP24A1 breekt zowel actief $1,25(OH)_2D$ als inactief 25(OH)D af. Dit “feedback” mechanisme zorgt voor een bescherming tegen te hoge waarden van actief vitamine D, wat bijvoorbeeld tot te

hoge calcium waarden in het bloed zou kunnen leiden en schadelijk is voor organen en botten.

Vitamine D en het luchtwegepitheel

In de luchtwegen is vitamine D een belangrijke factor in de homeostase van het luchtwegepitheel en heeft invloed op zowel het aangeboren als het adaptieve of verworven immuunsysteem. Het luchtwegepitheel is onderdeel van het aangeboren immuunsysteem en fungeert als de frontlinie van de afweer van de longen. Het luchtwegepitheel vormt een fysieke barrière tegen een invasie van pathogenen en inademing van schadelijke deeltjes en gassen afkomstig van sigarettenrook of luchtverontreiniging. Het luchtwegepitheel bestaat uit verschillende celtypen, zoals gobletcellen (slijmbekercellen), trilhaarcellen, clubcellen en basale cellen, die allemaal een unieke rol spelen in het in stand houden van deze barrière. De gobletcellen produceren slijm, die een gelvormige bekleding vormen, dat over de trilharen heen wordt getransporteerd. Onderweg vangt het slijm pathogenen en andere schadelijke deeltjes op, om deze vervolgens door de bewegende trilharen uit de luchtwegen naar de keel te transporteren. Wanneer pathogenen alsnog door de slijmlaag doordringen dan is het lastig om door de epitheliale laag te kunnen, omdat deze cellen sterk aan elkaar verankerd zitten door de aanwezigheid van “tight-“ en “adherens junctions”. Naast deze fysieke barrière, beschermt het epitheel zich ook tegen pathogenen door de uitscheiding van ontstekings- en andere afweermediatoren. Deze ontstekingsmediatoren trekken vervolgens immuuncellen aan en activeren deze om mee te helpen met het klaren van de infectie. Verder scheidt het luchtwegepitheel verschillende afweermediatoren zoals o.a. antimicrobiële peptiden en eiwitten (AMPs) uit. AMPs zijn een natuurlijke vorm van antibiotica en doden virussen, bacteriën en schimmels, maar moduleren ook de immuunrespons en kunnen eventuele weefselschade helpen herstellen. Clubcellen kenmerken zich door het produceren van het CC16 eiwit dat een ontstekingsremmende werking heeft en verder kunnen deze cellen ook bepaalde giftige stoffen neutraliseren. De basale cellen zijn de voorlopercellen van het luchtweg epitheel en spelen een belangrijke rol in het herstel van het epitheel na beschadiging. Deze cellen kunnen zich na beschadiging gaan delen om de wond te sluiten om vervolgens zichzelf te transformeren (differentiëren) tot nieuwe goblet-, trilhaar- en clubcellen.

Omgevingsfactoren zoals langdurige blootstelling aan luchtvervuiling of sigarettenrook, maar ook genetische mutaties kunnen een negatieve invloed

hebben op beschermende epitheelcelfuncties. Dit kan de vatbaarheid voor infecties vergroten en uiteindelijk leiden tot chronische ontsteking en schade die niet meer volledig hersteld kan worden. Bovendien hebben onderzoeken aangetoond dat het luchtwegepitheel van patiënten met chronisch ontstoken luchtwegen verschilt van dat van gezonde mensen, en tekenen vertoont van een afwijkend herstellend vermogen. Deze bevindingen suggereren dat het luchtwegepitheel een centrale rol speelt in het ontstaan en verloop van chronisch inflammatoire longziekten zoals astma en COPD. Verschillende onderzoeken hebben aangetoond dat vitamine D het luchtwegepitheel zou kunnen beschermen tegen luchtweginfecties en schade, door o.a. de epitheliale barrière te versterken, de aanmaak van AMPs (hCAP18/LL-37) te stimuleren en excessieve ontsteking te dempen. Of vitamine D ook zo beschermend is in chronisch ontstoken longweefsel, zoals bij patiënten met chronische longaandoeningen zoals COPD, moet echter nog worden uitgezocht.

COPD

COPD is een progressieve longziekte die wordt gekenmerkt door een chronische obstructie van de luchtwegen en afbraak van de longblaasjes die de normale ademhaling verstoort, hetgeen niet volledig omkeerbaar is. De belangrijkste oorzaak van COPD is langdurige blootstelling aan schadelijke deeltjes en gassen, door bijvoorbeeld het roken van sigaretten, maar ook door luchtvervuiling, wat leidt tot een abnormale ontstekingsreactie in de longen. Daarnaast dragen ook andere factoren, zoals genetische aanleg, abnormale longontwikkeling en veroudering, bij aan de ontwikkeling van COPD. COPD is een belangrijke oorzaak van chronische morbiditeit en is momenteel de derde doodsoorzaak ter wereld. Helaas is er nog geen medicijn dat genezing mogelijk maakt en zijn de huidige behandelingen voornamelijk gericht op het remmen van de ontsteking en het verlichten van benauwdheidsklachten. Een belangrijke oorzaak van morbiditeit en mortaliteit in COPD zijn longaanvallen oftewel exacerbaties. Dit zijn episodes van acute verergering van symptomen (benauwdheid, slijmproductie, hoesten), die aanvullende therapie en mogelijk ziekenhuisopname vereisen. Exacerbaties versnellen de progressie van de ziekte en vormen een aanzienlijke belasting voor gezondheidszorgsystemen wereldwijd, omdat deze patiënten vaak met benauwdheidsklachten in het ziekenhuis terecht komen. Exacerbaties worden voornamelijk veroorzaakt door respiratoire virale en bacteriële infecties, maar ook

door blootstelling aan luchtverontreiniging. Zoals eerder al besproken, verhoogt de verstoorde epitheliale afweer in de luchtwegen van COPD patiënten de kans op infecties, en daarbij dus ook de kans op COPD exacerbaties. Bovendien hebben meerdere studies nu aangetoond dat COPD patiënten over het algemeen lagere vitamine D waarden hebben ten opzichte van gezonde personen, en COPD patiënten met lagere vitamine D waarden hebben vaker en meer ernstigere exacerbaties. Het bestuderen van het beschermende effect van vitamine D op de barrière functie van het luchtwegepitheel en bij exacerbaties in COPD patiënten zou daarom aanvullend inzicht kunnen verschaffen in het belang van monitoren van de vitamine D status en vitamine D suppletie van COPD patiënten met een vitamine D tekort.

Doel proefschrift

Het doel van dit proefschrift is om het effect van ontsteking op de beschermende effecten van vitamine D bij chronische luchtwegaandoeningen te onderzoeken, met name bij COPD. In de experimentele studies die in dit proefschrift worden beschreven, worden de effecten van vitamine D (inactief 25(OH)D en actief $1,25(OH)_2D$) en ontsteking bestudeerd met behulp van celkweek modellen van primaire bronchiale epitheelcellen (afkomstig uit de grotere luchtwegen van de long). In deze modellen worden de effecten bestudeerd van ontsteking en van toxische- en microbiële blootstellingen zoals sigarettenrook **(Hoofdstuk 2)**, cytokinen (**Hoofdstuk 3 en 4)**, bacteriën **(Hoofdstuk 4)** en virussen **(Hoofdstuk 5)** op afweermechanismen van het luchtwegepitheel, zoals de expressie van het AMPs.

Resultaten

Effecten van sigarettenrook en TGF-β1 op afweermechanismen van het luchtwegepitheel

Bacteriële en virale infecties zijn belangrijke veroorzakers van COPD exacerbaties en het luchtwegepitheel speelt een centrale rol bij het orkestreren van afweermechanismen tegen de pathogenen die deze infecties veroorzaken. Eerdere studies hebben echter aangetoond dat de barrièrefunctie van het luchtwegepitheel bij COPD patiënten bepaalde defecten vertoont, zoals een verlaagde doordringbaarheid van de epitheliale cellaag, verhoogde slijmproductie en een afname van de trilhaarfunctie. Als gevolg van deze defecten hoopt het slijm zich op in de luchtwegen en is de gevoeligheid voor bacteriële en virale infecties groter, wat de kans op exacerbaties en verdere ziekte progressie doet toenemen. Een belangrijke oorzaak van het ontstaan van deze defecten is het roken van sigaretten en vaak ziet men dat ook “gezonde” rokers in het algemeen vatbaarder zijn voor luchtweginfecties.

In **Hoofdstuk 2** onderzochten we daarom in een celkweekmodel hoe sigarettenrook de afweermechanismen van het luchtwegepitheel beïnvloedt. In de studie die in dit hoofdstuk wordt beschreven, hebben we luchtwegepitheelcellen dagelijks blootgesteld aan sigarettenrook tijdens de uitrijping (differentiatie) van basale cellen naar gespecialiseerde club-, goblet- of trilhaarepitheel cellen. We ontdekten dat blootstelling aan sigarettenrook de uitrijping van basale epitheliale cellen naar gespecialiseerde club-, goblet- en trilhaarepitheelcellen afremt. Hierdoor werd ook de productie van bepaalde afweermediatoren zoals AMPs, die met name door gespecialiseerde club- en gobletcellen worden aangemaakt, sterk gereduceerd. Als gevolg van de afgenomen specialistische cel populaties, was ook de afweer tegen bepaalde pathogene bacteriën lager. Dit geeft aan dat bloostelling aan sigarettenrook een duidelijke impact kan hebben op antibacteriële afweermechanismen van het luchtwegepitheel door de uitrijping van het epitheel te onderdrukken. Via welke mechanismen sigarettenrook de uitrijping van het luchtwegepitheel beïnvloedt is vooralsnog niet bekend.

Een aanwijzing voor de betrokkenheid van TGF-β1 als een mogelijk mechanisme voor het remmende effect van sigarettenrook op de uitrijping van het luchtwegepitheel wordt gegeven in **Hoofdstuk 3**. TGF-β1 is een cytokine dat o.a.

geassocieerd is met fibrose en is ook in verhoogde mate aanwezig in de luchtwegen van zowel astma als COPD patiënten. Studies in gekweekt luchtwegepitheel hebben tevens laten zien dat de aanmaak van TGF-β1 verhoogd kan worden door blootstelling aan sigarettenrook. In dit hoofdstuk hebben we aangetoond dat TGF-β1 vergelijkbare effecten heeft op de uitrijping en de afweermechanismen van het luchtwegepitheel als sigarettenrook. Korte blootstellingen aan TGF-β1 bleken al voldoende te zijn om niet alleen de aantallen gespecialiseerde club- en gobletcellen te verlagen, maar ook de aanmaak van AMPs en andere afweermediatoren.

Effecten van TGF-β1 op vitamine D-afhankelijke afweermechanismen van het luchtwegepitheel

Eerdere studies hebben aangetoond dat vitamine D effecten van TGF-β1, zoals inductie van fibrose, zou kunnen tegengaan. Om te onderzoeken of vitamine D ook de nadelige effecten van TGF-β1 op zowel uitrijping als aanmaak van afweermediatoren kan tegengaan, hebben we in **Hoofdstuk 3** luchtwegepitheel blootgesteld aan zowel vitamine D als TGF-β1. Helaas kon vitamine D deze nadelige effecten van TGF-β1 op het luchtwegepitheel niet tegengaan. Verder laten we zien dat TGF-β1 de beschermende effecten van vitamine D op het luchtweg epitheel afremt, zoals de inductie van het AMP hCAP18/LL-37. Een verklaring hiervoor kan gevonden worden in de observatie dat TGF-β1 de productie van CYP24A1, het enzym dat vitamine D afbreekt, in het luchtwegepitheel sterk verhoogt. Kort samengevat hebben we in dit hoofdstuk laten zien dat TGF-β1 erg nadelig is voor zowel de vitamine D-afhankelijke als vitamine D-onafhankelijke afweermechanismen van het luchtwegepitheel.

Effecten van ontstekingsmediatoren op vitamine D-metabolisme en vitamine D-afhankelijke afweermechanismen van het luchtwegepitheel

Naast blootstellingen aan sigarettenrook en TGF-β1, zijn de luchtwegen van COPD en astma patiënten in een chronische staat van ontsteking. Dat betekent dat het luchtwegepitheel dus constant wordt blootgesteld aan ontstekingsmediatoren, zoals TNF-α, IL-1β en IL-17A, die vrijkomen uit o.a. immuuncellen. In **Hoofdstuk 4** wilden we daarom de effecten onderzoeken van deze ontstekingsmediatoren op de werking en de omzetting van vitamine D in het luchtwegepitheel.

Luchtwegepitheelcellen werden eerst twee weken lang blootgesteld aan ontstekingsbevorderende mediatoren zoals TNF-α, IL-1β en IL-17A, en vervolgens behandeld met inactief vitamine D (25(OH)D) of actief vitamine D ($1,25(OH)_2D$). We vonden dat blootstelling aan ontstekingsbevorderende mediatoren, net als TGF-β1, de aanmaak van het enzym CYP24A1 verhoogt. Dit kan de afbraak van vitamine D verder verhogen met als gevolg dat het vermogen van vitamine D om de aanmaak van het AMP hCAP18/LL-37 te stimuleren in het luchtwegepitheel sterk wordt verlaagd. Vervolgens hebben we ook m.b.v. een "killing assay" laten zien dat als gevolg van blootstellingen aan ontstekingsbevorderende mediatoren ook de vitamine D-afhankelijke antimicrobiële activiteit tegen een bekend luchtwegpathogeen, het niet-typeerbare *Haemophilus influenzae* (NTHi), was verminderd.

Effecten van type 2 ontsteking op de omzetting van vitamine D

In tegenstelling tot de bevindingen in hoofdstukken 3 en 4, waarin we aantoonden dat TGF-β1 en ontstekingsbevorderende mediatoren de beschermende effecten van vitamine D nadelig beïnvloedden, laten we in **Hoofdstuk 5** laten zien dat de aanwezigheid van een ander type ontsteking in feite het tegenovergestelde doet. Dit type ontsteking, ook wel bekend als type 2 ontsteking, kenmerkt zich door verhoogde waarden van eiwitten afkomstig van een specifieke groep van T cellen, de T-helpercel 2 (Th2), namelijk de cytokinen IL-4 en IL-13. Dit is typerend voor patiënten met allergisch astma, maar daarnaast kunnen ook COPD patiënten verhoogde IL-4 en IL-13 waarden hebben. We toonden in dit hoofdstuk aan dat blootstelling van luchtwegepitheelcellen aan de Th2 cytokinen IL-13 en IL-4, de aanmaak van het enzym CYP27B1 verhoogt. CYP27B1 bevordert de omzetting van het circulerende, maar nog inactieve vitamine D (25(OH)D) naar het actieve vitamine D ($1,25(OH)_2D$). Door deze hogere expressie van CYP27B1 was dus het vermogen van het inactieve vitamine D om de aanmaak van hCAP18/LL-37 in het luchtwegepitheel te induceren juist toegenomen.

Dempende effecten van vitamine D op virus-afhankelijke ontstekingsreacties in het luchtwegepitheel

COPD exacerbaties worden voornamelijk veroorzaakt door respiratoire virale infecties, bacteriële (secundaire) infecties en blootstelling aan luchtverontreiniging. Wanneer het luchtwegepitheel wordt geïnfecteerd, herkennen de receptoren van het luchtwegepitheel bepaalde pathogeen-karakteristieke moleculen, en scheidt het luchtwegepitheel vervolgens als reactie diverse ontstekings- en afweermediatoren uit. Deze mediatoren kunnen enerzijds pathogenen uitschakelen (AMPs, interferonen en vrije zuurstofradicalen) of anderzijds het herstel bevorderen (groeifactoren) of immuuncellen aantrekken vanuit de bloedbaan (cytokinen en chemokinen) om de infectie te helpen klaren. Als gevolg van zowel de infectie zelf en de immuunrespons als reactie op de infectie ontstaat schade aan de longen, die niet altijd meer voldoende kan worden hersteld. Om de gevolgen en de ernst van de exacerbaties in deze patiënten te verlagen, zijn er strategieën nodig die zowel de afweer tegen infecties verhogen en tegelijk de schadelijke immuunreactie dempen. We onderzochten daarom in **Hoofdstuk 5** of vitamine D in het luchtwegepitheel in staat is om, naast het bevorderen van afweer tegen infecties, ook de ontsteking ten gevolge van een virusinfectie kan dempen. We hebben in dit hoofdstuk laten zien dat behandeling van gekweekt luchtwegepitheel met actief vitamine D een door Poly[I:C] (synthetisch dubbelstrengs RNA, wat een virusinfectie nabootst) getriggerde uitscheiding van ontstekingsbevorderende cytokinen en chemokinen kan remmen. Mogelijk remt vitamine D hierdoor ook de influx van immuuncellen, wat de schade na een virale infectie of exacerbatie kan beperken.

Studie naar de effecten van vitamine D-suppletie op exacerbaties in COPD patiënten

Eerdere gerandomiseerde klinische studies (randomized controlled trials; RCTs) in een populatie van zowel vitamine D- sufficiënte en deficiënte patiënten laten zien dat vitamine D suppletie de incidentie van acute luchtweginfecties alleen verlaagt bij patiënten met een vitamine D deficiëntie. Deze bevindingen suggereren dat vitamine D suppletie nuttig kan zijn om ook het aantal exacerbaties in COPD patiënten te verlagen, omdat dit frequent veroorzaakt wordt door zowel virale als bacteriële infecties. Om te beoordelen of suppletie van vitamine D inderdaad het aantal exacerbaties bij COPD kan verminderen bij juist de COPD patiënten, die

vitamine D deficiënt zijn, is een multicenter RCT ontworpen. In deze studie worden de effecten van vitamine D suppletie onderzocht in een studiepopulatie die, in tegenstelling tot eerdere RCT's, bestaat uit vitamine D-deficiënte patiënten (serum [25(OH)D] 15 - 50 nmol/L). De opzet, het protocol en de uitleesparameters van de studie worden beschreven in **Hoofdstuk 6**. Ten slotte hebben we in **Hoofdstuk 7** de huidige kennis over de effecten van o.a. COPD-gerelateerde factoren, zoals ontsteking en blootstelling aan schadelijke deeltjes en gassen (zoals aanwezig in sigarettenrook en luchtvervuiling) op de lokale werking van vitamine D in de luchtwegen gebundeld. Verder wordt in dit hoofdstuk dieper ingegaan op de mogelijke gevolgen van een beperkte beschikbaarheid of activatie van (lokaal) vitamine D in de longen en laat dit hoofdstuk zien dat dit mogelijk consequenties kan hebben op het gebied van gevoeligheid voor infecties, ontstekingen en exacerbaties.

Discussie

Van veel factoren die bijdragen aan de ontwikkeling en verergering van COPD, zoals chronische blootstelling aan sigarettenrook of luchtvervuiling en aanwezigheid van ontstekingsmediatoren (zuurstofradicalen, proteolytische enzymen, cytokinen en chemokinen), is bekend dat ze de epitheliale barrière kunnen verzwakken. Verder kan mede door het chronische karakter van deze blootstellingen ook de schade niet meer volledig worden hersteld. Het versterken van zowel de epitheliale afweer als het vermogen tot herstel van de epitheliale barrière kan daarom een goede strategie zijn om de achteruitgang in het ziektebeloop te stoppen. Ten eerste kan er verder onderzoek worden gedaan naar precieze defecten in de functie van het luchtwegepitheel bij COPD, en met name in de voorlopercellen, zodat deze defecten in de toekomst gerepareerd kunnen worden met medicijnen die daarop aangrijpen. Ten tweede kan verder onderzoek zich richten op een aanpak die het lokale milieu in de longen verbetert m.b.v. micronutriënten zoals vitamine D, zodat het luchtwegepitheel beter beschermd is tegen epitheliale schade als gevolg van ontsteking en infecties.

In de studies die in dit proefschrift worden gepresenteerd, hebben we echter aangetoond dat bepaalde luchtwegontstekingsmediatoren mogelijk het vitamine D

metabolisme zouden kunnen verstoren door de expressie van CYP24A1 te bevorderen, waardoor het vitamine D wordt afgebroken. Daarom moeten toekomstige studies naar COPD, en ook naar andere chronische ontstekingsziekten die verband houden met vitamine D tekort, worden ontworpen met meer aandacht voor het verhogen van lokale waarden van actief vitamine D door bijvoorbeeld de verhoogde expressie van CYP24A1 te verlagen. Een effectieve remming van ontsteking kan hier een belangrijke bijdrage aan leveren. Tot slot zullen deze nieuwe inzichten mogelijke nieuwe strategieën opleveren om de lokale activiteit van vitamine D te verbeteren om zo COPD patiënten beter te beschermen tegen luchtweginfecties en exacerbaties.

Curriculum vitae
Publicaties

Curriculum vitae

Jasmijn A. Schrumpf is geboren op 23 januari 1978 te Roelofarendsveen. Na het behalen van het H.A.V.O. diploma aan het Visser 't Hooft Lyceum te Leiden is zij begonnen aan de studie Medisch Laboratorium onderzoek aan de Hogeschool Leiden. In 2000 behaalde zij haar bachelor diploma na een (onderzoeks)stage van 9 maanden bij de onderzoeksgroep van Dr. A. Gorter op de afdeling pathologie in het Leids Universitair Medisch Centrum. Na het behalen van haar diploma is zij begonnen als research analist onder begeleiding van Prof. dr. Pieter S. Hiemstra en Prof. dr. P.J. Sterk bij de afdeling Longziekten in het Leids Universitair Medisch Centrum. In 2010 is zij begonnen aan een onderzoek naar de rol van vitamine D in het luchtwegepitheel wat in 2012 uitmondde in het publiceren van haar eerste manuscript. Het onderzoek kreeg na deze publicatie een gevolg en in 2014 werd een promotietraject gestart over de modulatie van vitamine D en de functie van het luchtwegepitheel in chronisch obstructieve longziekte (COPD) onder begeleiding van Prof. dr. Pieter S. Hiemstra en Dr. Anne M. van der Does. In 2018 is zij begonnen aan een nieuw onderzoek naar de beschermende effecten van boerderijstof op het luchtwegepitheel in kinderen met astma onder begeleiding van Dr. Hermelijn Smits van de afdeling parasitologie en Prof. dr. Pieter S. Hiemstra bij de afdeling Longziekten in het Leids Universitair Medisch Centrum.

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Dankwoord

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