ORIGINAL RESEARCH

Proinflammatory 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 to proinflammatory cytokines alters their vitamin D metabolism, antibacterial activity, and expression of hCAP18/LL-37. To investigate this, primary bronchial epithelial cells were differentiated at the air-liquid interface for 14 days in the presence of the proinflammatory 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, and enzymes involved in vitamin D metabolism (CYP24A1 and CYP27B1) was determined using quantitative PCR, Western blot, and immunofluorescence staining. Furthermore, vitamin D–mediated antibacterial activity was assessed using nontypeable *Haemophilus influenzae*. We found that TNF-α/IL-1β treatment reduced vitamin D–induced expression of hCAP18/LL-37 and killing of nontypeable *H. influenzae*. 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 vitamin D receptor expression remained unaffected. Furthermore, we have demonstrated that the TNF-α/IL-1β–mediated induction of CYP24A1 was, at least in part, mediated by the transcription factor specific protein 1, and the epidermal growth factor receptor–mitogen-activated protein kinase 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 suggest that chronic inflammation impairs protective responses induced by vitamin D.

Keywords: vitamin D; airway epithelial cells; host defense; proinflammatory mediators; hCAP18/LL-37

Clinical Relevance

This research points to novel mechanisms whereby inflammation-triggered alterations in vitamin D metabolism impair host defense at the airway epithelial surface, increase the risk of infections, and thus contribute to more (severe) exacerbations and progression of disease. Targeting airway inflammation and/or increasing the dose of vitamin D supplementation will improve the beneficial actions of vitamin D in the airways of patients suffering from chronic respiratory infections.

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

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oxygen and nitrogen species, IFNs, 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 antiinflammatory 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 human cathelicidin antimicrobial peptide 18/leucin leucin 37 (hCAP18/LL-37) the precursor of which, hCAP18, is cleaved by proteases into the 4.5-kD LL-37 mature peptide that displays both antimicrobial and immunomodulatory properties (5–7). This relationship was confirmed by several studies that have revealed an important role for vitamin D in regulating hCAP18/LL-37 expression in, for example, 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 proinflammatory mediators can alter the expression of CYP27B1, CYP24A1, and VDR in extrarenal cells (8, 9, 12–15). Increased local levels of proinflammatory 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 proinflammatory 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 (PBECs), which were differentiated in the presence and absence of the proinflammatory 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 nontypeable *Haemophilus influenzae* (NTHi, a gram-negative bacterium, which is found in the lungs of patients with COPD, CF, and [refractory] asthma [21, 22]). Furthermore, effects of TNF-α/IL-1β, and also of other proinflammatory 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

PBECs 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, TNF-α/IL-1β, or IL-17A, on PBECs, cells were cultured for 14 days in the 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 the presence or absence of 2.5 ng/ml TNF-α/IL-1β or 5 ng/ml IL-17A were exposed to 10^{-7} M $25(OH)D_3$ (Millipore B.V., Amsterdam, the Netherlands), and 10^{-9} M $1,25(OH)_2D_3$ (Millipore B.V.) for 24 hours to assess gene expression, or for 48 hours to assess antibacterial activity, hCAP18/LL-37 release, and 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, Zwijndrecht, the Netherlands), ALI-PBECs were cultured in the presence or absence of TNF-α/IL-1β for 14 days and subsequently stimulated for 24 hours with 3×10^{-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-PBECs were exposed to ultraviolet (UV)-inactivated NTHi for 12 hours.

Submerged, undifferentiated cultures of PBECs were used to elucidate the mechanism of action of TNF-α/IL-1β–induced expression of CYP24A1. Cells were cultured in supplemented bronchial epithelial cell growth medium:Dulbecco's modified eagle medium (BEGM:DMEM) until a confluence of 70% was reached and cultured overnight in starvation medium (BEGM:DMEM, w/o epidermal growth factor [EGF], bovine pituitary extract [BPE], and BSA), preincubated for 1 hour with 2.5×10^{-5} M GM6001 (matrix metalloprotease [MMP] inhibitor; Millipore B.V.), 1×10^{-6} M AG1478 (epidermal growth factor receptor [EGFR] tyrosine kinase inhibitor; Millipore B.V.), 2.5×10^{-5} M U0126 (mitogen-activated protein kinase kinase [MEK1/2] inhibitor; Promega, Leiden, the Netherlands) and stimulated with 10 ng/ml TNF-α/IL-1β for 15 minutes for collection of protein lysates, and 24 hours 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, RT, and Quantitative PCR

Methods for total RNA isolation, reverse transcription and quantitative PCR (qPCR) reactions (primers shown in Table 1) are described in the online supplement.

Table 1. PCR Primers and Sequences Used for Quantitative PCR

Gene	Encoding Protein	Sequence Forward Primer	Sequence Reverse Primer	GenBank Accession No.
*B2M**	β2-microglobulin	GACCACTTACGTTCATTGACTCC	CAGGGTTTCATCATACAGCCAT	NM_004048
*ATP5B**	ATP synthase, H^+ transporting, mitochondrial F1 complex, β polypeptide	TCACCCAGGCTGGTTCAGA	AGTGGCCAGGGTAGGCTGAT	NM_001686
*RPL13A**	Ribosomal protein L13a	AAGGTGGTGGTCGTACGCTGTG	CGGGAAGGGTTGGTGTTCATCC	AB082924
*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
DEFB4	β-defensin 2	ATCAGCCATGAGGGTCTTG	GCAGCATTTTGTTCCAGG	NM_004942
MUC5B	Mucin-5B	GGGCTTTGACAAGAGAGT	AGGATGGTCGTGTTGATGCG	NG_031880
MUC5AC	Mucin-5AC	CCTTCGACGGACAGAGCTAC	TCTCGGTGACAACACGAAAG	NM_001304359
CLCA1	Chloride channel accessory 1	ATGGCTATGAAGGCATTGTCG	TGGCACATTGGGGTCGATTG	NM_001285
FOXJ1	Forkhead box protein J1	GGAGGGGACGTAAATCCCTA	TTGGTCCCAGTAGTTCCAGC	NM_001454
TP63	Tumor protein p63	CCACCTGGACGTATTCCACTG	TCGAATCAAATGACTAGGAGGGG	NM_003722
SCGB1A1	Secretoglobin family 1A member 1	ACATGAGGGAGGCAGGGGCTC	ACTCAAAGCATGGCAGCGGCA	NM_003357

Definition of abbreviations: hCAP18, human cathelicidin antimicrobial peptide 18; LL-37, leucin leucin 37.
*Used as a reference gene, selected using the Genorm method.

Preparation of UV-Inactivated NTHi

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

Bacterial Killing Assay

Killing of NTHi by ALI-PBECs was assessed as described by Pezzulo and colleagues (24) with a few modifications and as described in the online supplement.

Western Blot

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

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 stained as described in the online supplement.

Statistical Analysis

Statistical analysis was conducted as described in the online supplement. Data are shown as median (±min/max) values. Differences at P values less than 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 proinflammatory cytokines affects cellular responses to vitamin D, we exposed PBECs to the proinflammatory cytokines TNF-α/IL-1β during differentiation, followed by 25(OH)D_3 treatment or control (medium) in the 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 PBECs after 24 hours of incubation by 15.1-fold. Moreover, in the presence of TNF-α/IL-1β, expression of *CAMP* was 5.2-fold increased by 25(OH)D_3, which was significantly less compared with control-treated cells ($P < 0.0001$, Figure 1B). We further confirmed these findings at the protein level after 48-hour 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).

TNF-α/IL-1β Impairs 25(OH)D_3-Mediated Killing of NTHi by ALI-PBECs

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-PBECs. First, ALI-PBECs were differentiated in the presence of TNF-α/IL-1β and subsequently stimulated with 25(OH)D_3 or 1,25$(OH)_2D_3$ for 48 hours (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 with 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-PBECs.

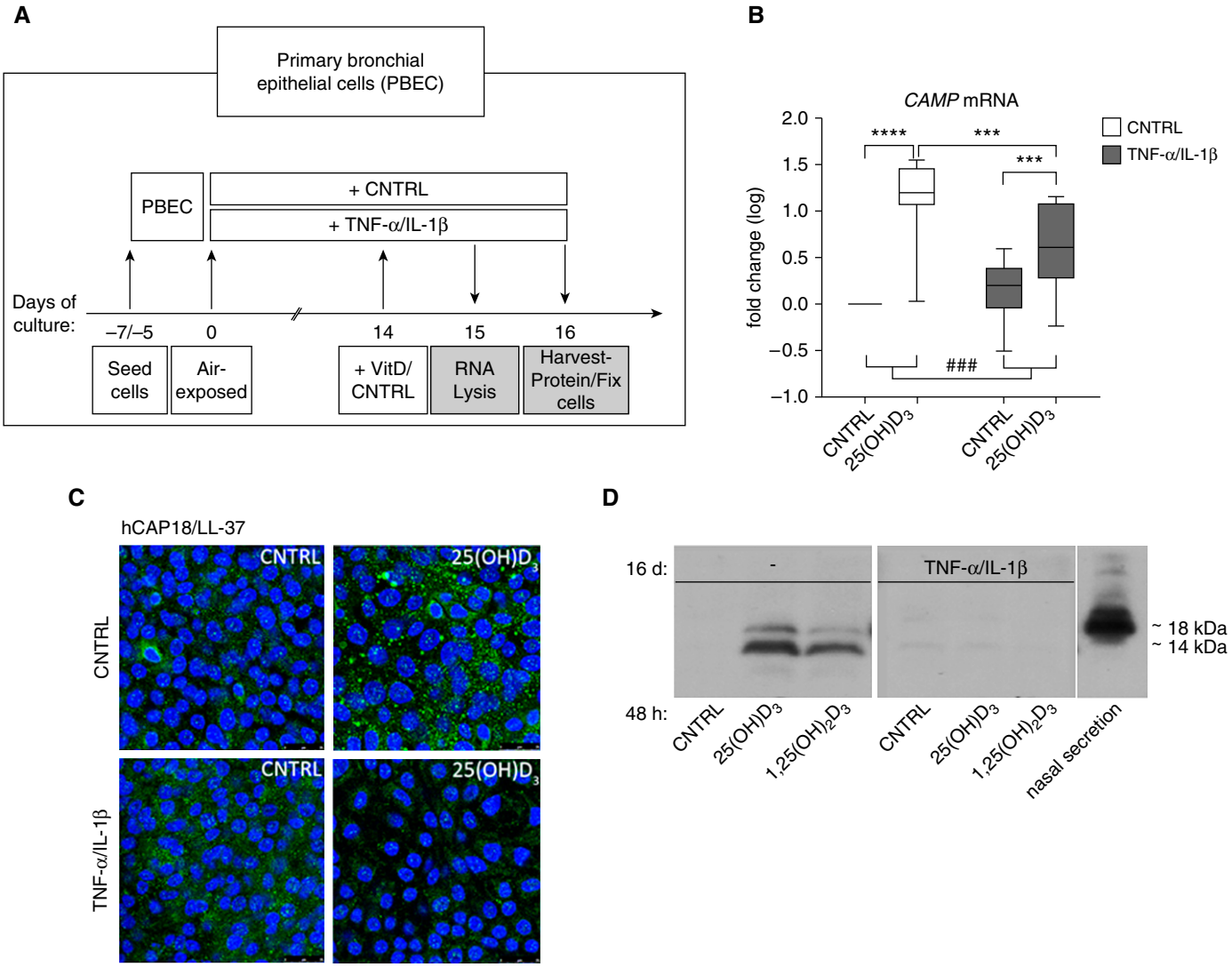

Figure 1. TNF-α and IL-1β (TNF-α/IL-1β) decreases 25(OH)D3-mediated expression and release of human cathelicidin antimicrobial peptide 18/leucin leucin 37 (hCAP18/LL-37) in primary bronchial epithelial cells (PBECs). (*A*) PBECs were seeded on Transwell inserts and cultured for 5–7 days until confluence was reached. Subsequently, PBECs were cultured at the air–liquid interface (ALI) and exposed with and without TNF-α/IL-1β for 14 days followed by 24-hour stimulation with 25(OH)D_3 or medium control (CNTRL) for assessing *CAMP* (hCAP18/LL-37) expression by quantitative PCR (qPCR). In addition, cells were stimulated for 48 hours 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 geometric mean of the expression of the reference genes β2-microglobulin (*B2M*) and ATP synthase, H^+ transporting, mitochondrial F1 complex, β polypeptide (*ATP5B*). Data are presented as median (±min/max) values. Fold change in gene expression of the stimuli compared with 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 one donor, which was confirmed in three other donors), 4′,6-diamidino-2-phenylindole (*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 CNTRLs to show intact hCAP18 peptide at 18 kD. Western blots are a representative of three independent experiments using six different donors. The image was cut to switch sample order for increased consistency. $^{###}P < 0.001$, $^{***}P < 0.001$, $^{****}P < 0.0001$. VitD, vitamin D.

Elevated CYP24A1 Levels Induced by Chronic Exposure of ALI-PBECs 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

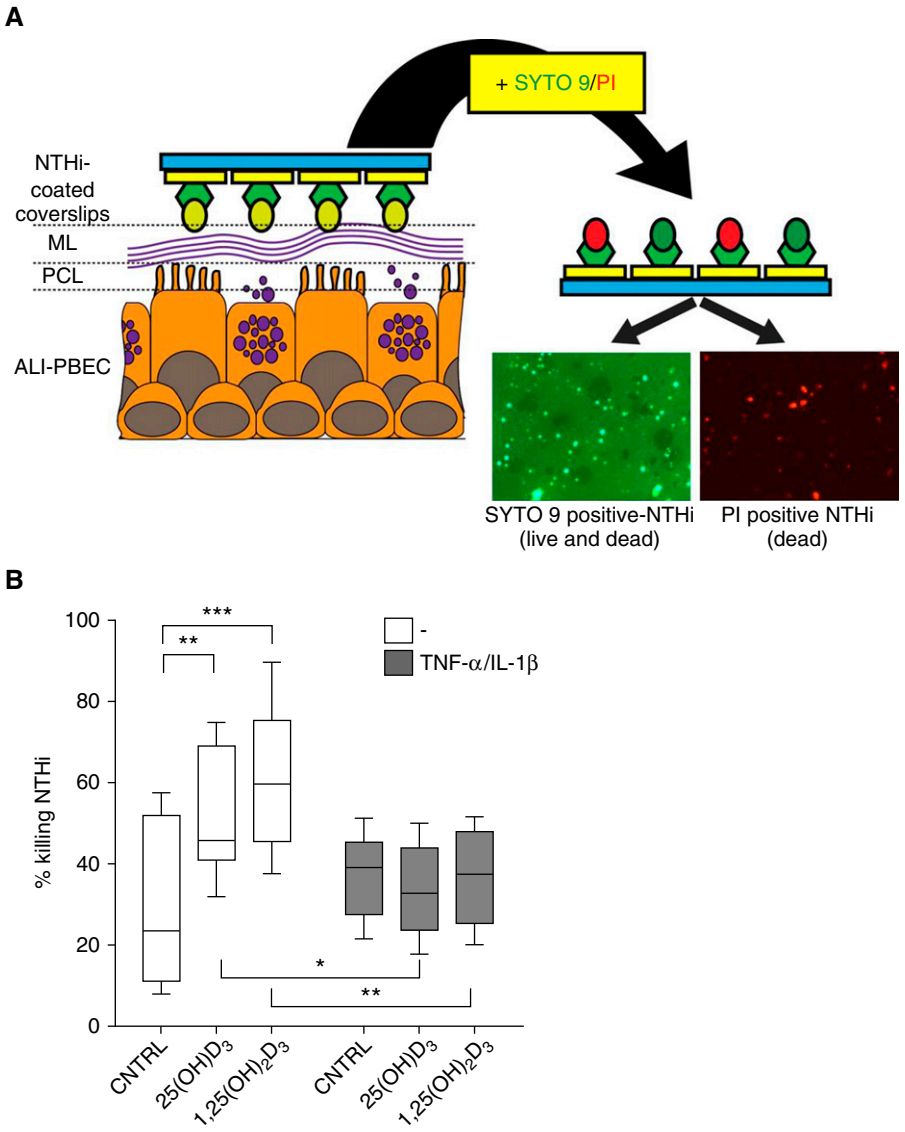

Figure 2. TNF-α/IL-1β impairs vitamin D–mediated killing of nontypeable *Haemophilus influenzae* (NTHi). PBECs ($n = 6$ donors) were differentiated with and without TNF-α/IL-1β for 14 days followed by 48-hour $25(OH)D_3$ and $1,25(OH)_2D_3$ treatment or medium CNTRL in duplicate for assessing antibacterial activity of PBECs. (*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 of the stimulated cells for 1 minute. Next, coverslips were mounted on slides and visualized using SYTO9 (live + dead bacteria) and propidium iodide (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 median (±min/max) values. To analyze the data, a two-way ANOVA and the Bonferroni *post hoc* test was used. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

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 the 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-PBECs were cultured with or without TNF-α/IL-1β for 14 days, followed by 24-hour preincubation in the presence or absence of different doses of ketoconazole before 24-hour treatment with $1,25(OH)_2D_3$ or control. $1,25(OH)_2D_3$ was used for stimulation, because 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 the 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).

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, and 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). In addition, we assessed *CYP24A1* mRNA expression at Days 1, 7, and 14 after starting culture at the ALI in the presence and absence of 2.5 ng/ml TNF-α/IL-1β. In control-treated cells, *CYP24A1* expression was decreased between Days 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

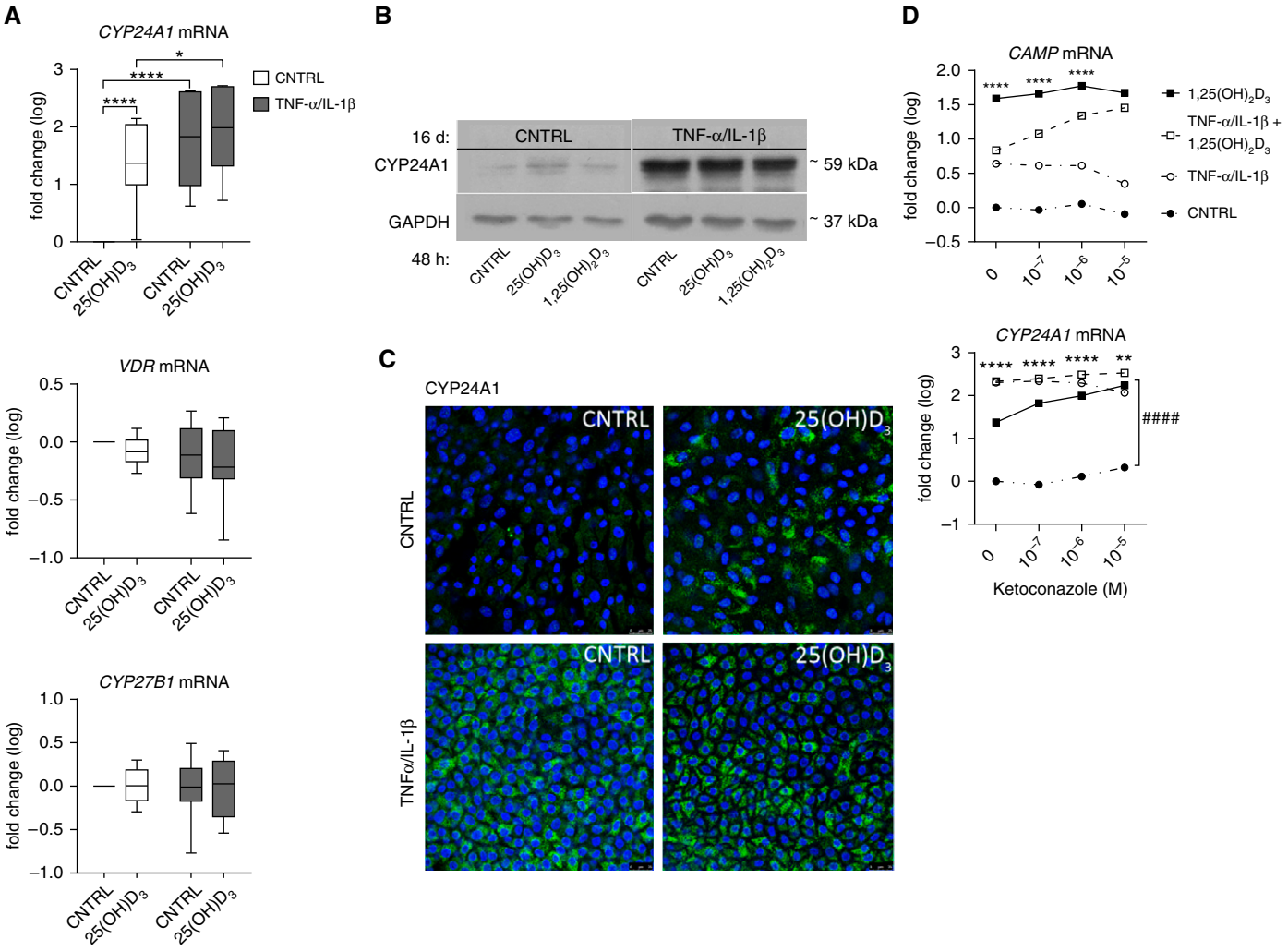

Figure 3. CYP24A1 is induced by chronic exposure to TNF-α/IL-1β and decreases vitamin D–mediated expression of hCAP18/LL-37. PBECs were cultured as described in Figure 1A for assessing *CYP24A1*, vitamin D receptor (*VDR*), and *CYP27B1* mRNA expression and CYP24A1 protein by Western blot analysis and immunofluorescence. (*A*) Relative mRNA expression of *CYP24A1*, *VDR*, and *CYP27B1* was determined by qPCR. Normalized gene expression was calculated by using the geometric mean of expression of the reference genes *B2M* and *ATP5B*. Data are presented as median (±min/max) values. Fold change in gene expression of the stimuli compared with 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 three independent experiments using cells from six different donors. The original image was cut to switch sample order for increased consistency. (*C*) Immunofluorescence staining of CYP24A1 in bronchial epithelial cells (of one donor, which was confirmed in three other donors). 4′,6-diamidino-2-phenylindole (*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, PBECs were cultured with or without TNF-α/IL-1β for 14 days, followed by 24-hour preincubation in the presence or absence of increasing concentrations of ketoconazole (CYP24A1 inhibitor). Afterward, cells were treated for 24 hours with $1,25(OH)_2D_3$ or medium CNTRL in the 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 geometric mean of the expression of *B2M* and *ATP5B*. Fold change in gene expression of the stimuli compared with 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). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{****}P < 0.0001$, $^{\#\#\#\#}P < 0.0001$.

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 Days 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 (Figure 4C).

To further determine the individual contributions 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

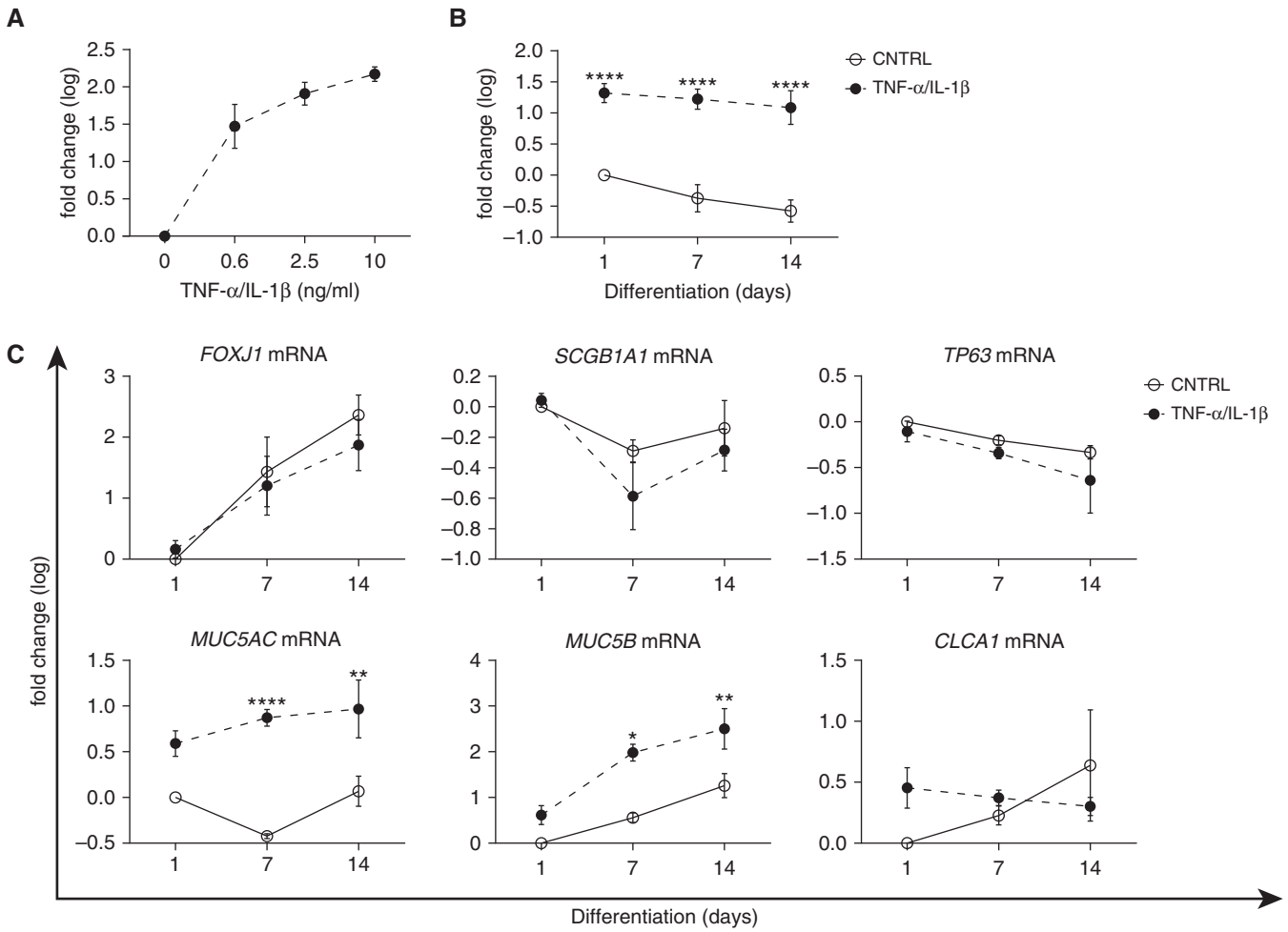

Figure 4. Time- and dose-dependent effects of TNF-α/IL-1β on CYP24A1 expression and epithelial differentiation. (*A*) PBECs were exposed to TNF-α/IL-1β or CNTRL to increasing concentrations of TNF-α/IL-1β for 14 days or (*B*) to TNF-α/IL-1β or CNTRL for 1, 7, and 14 days for determining relative CYP24A1 mRNA expression by qPCR. Normalized gene expression was calculated by using the geometric mean of the expression of *B2M* and *ATP5B*. Data are presented as median (±min/max) values. Fold change in gene expression of the stimuli compared with 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). (*C*) PBECs were exposed to TNF-α/IL-1β or medium 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 (RP) L13A (RPL13A) and RPL27 as reference genes. Data are presented as median (±min/max) values. Fold change in gene expression of the stimuli compared with 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). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{****}P < 0.0001$.

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).

TNF-α/IL-1β–Induced Expression of CYP24A1 Is Mediated by Activation of EGFR, Extracellular Signal–Regulated Kinase 1/2 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-PBECs were cultured for 14 days in the presence and absence of TNF-α/IL-1β and subsequently treated with the Sp1 inhibitor, mithramycin A, for 24 hours. 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 PBECs were used. Cells were pretreated with and without inhibitors of MMP (GM6001), EGFR (AG1478), and

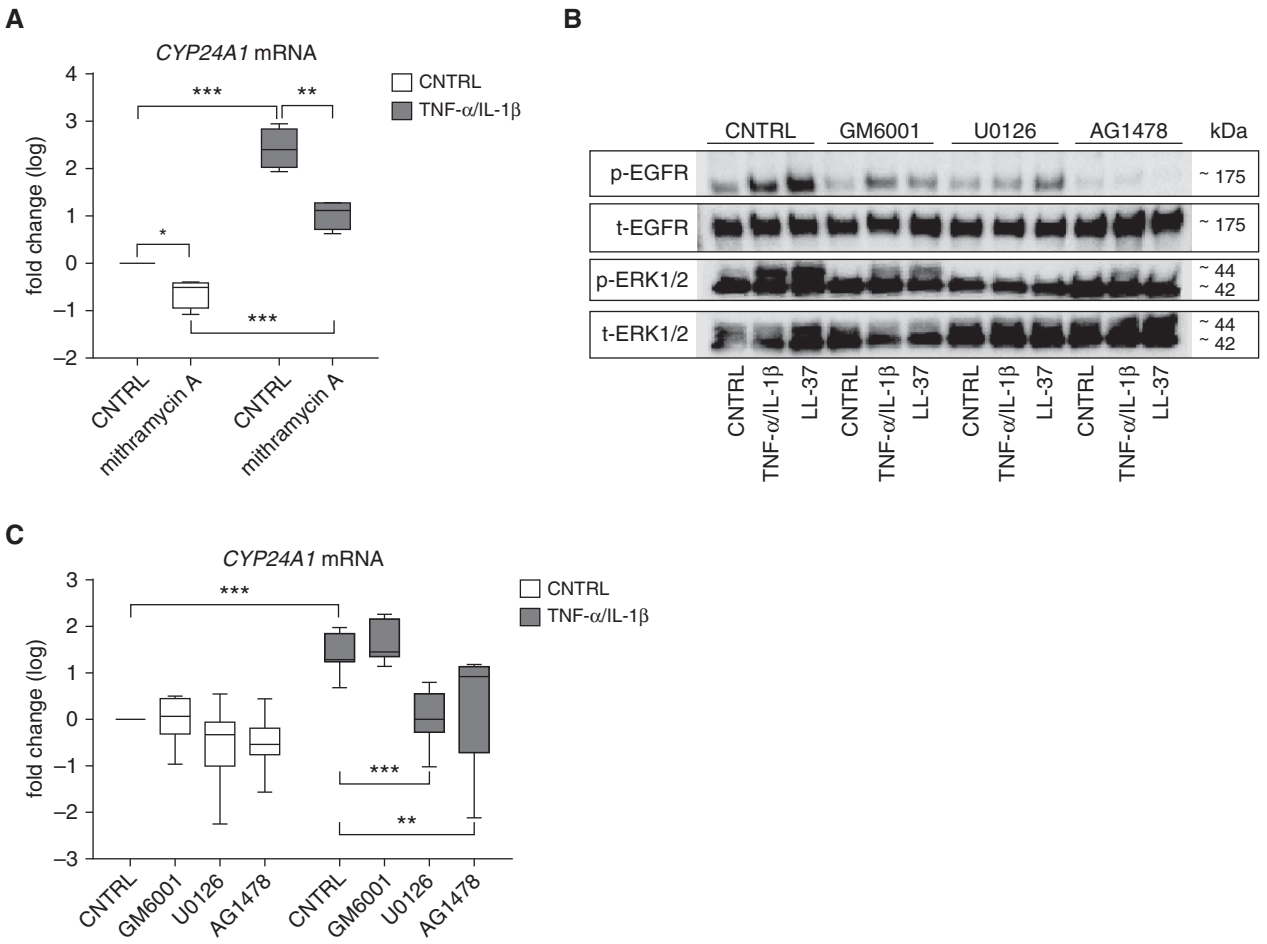

Figure 5. TNF-α/IL-1β–induced expression of CYP24A1 is mediated by activation of epidermal growth factor receptor (EGFR), extracellular signal–regulated kinase (ERK) 1/2, and specificity protein 1 (Sp1). To investigate the role of the transcription factor, Sp1, in TNF-α/IL-1β–mediated expression of CYP24A1, PBECs were differentiated for 14 days in the presence or absence (CNTRL) of TNF-α/IL-1β and subsequently exposed in the presence or absence of TNF-α/IL-1β with mithramycin A or medium CNTRL. (*A*) qPCR was used to assess relative expression of *CYP24A1* mRNA, and normalized gene expression was calculated by using the geometric mean of the expression of *B2M* and *ATP5B*. Data are presented as median (±min/max) values. Fold change in gene expression of the stimuli compared with 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 PBECs ($n = 3$) 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 minutes after 1-hour pretreatment with CNTRL (0.25% vol/vol DMSO in PBS) and matrix metalloprotease (MMP), EGFR, and mitogen-activated protein kinase kinase (MEK1/2) inhibitors. Protein expression of tyrosine phosphorylated (p)-and total (t)-EGFR, p-ERK1/2, and t-ERK1/2 was determined by SDS-PAGE Western blot. Western blots are representative of three independent experiments using three 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 PBECs were preincubated with CNTRL (0.25% vol/vol DMSO) and inhibitors of MMP (GM6001), EGFR (AG1478), and MEK1/2 (U0126), followed by stimulation for 24 hours with TNF-α/IL-1β to assess relative gene expression of *CYP24A1* mRNA by qPCR. Normalized gene expression was calculated by using the geometric mean of the expression of *B2M* and *ATP5B*. Data are presented as median (±min/max) values. Fold change in gene expression of the stimuli compared with 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). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

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 minutes (23). Western blot analysis assessing phosphorylated (p)-EGFR and p–extracellular signal–regulated kinase (ERK) 1/2 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*. PBECs were treated for 24 hours 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 PBECs after 24 hours (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 minutes, it did not affect

TNF-α/IL-1β–induced expression of CYP24A1 after 24 hours. Although no cytotoxic effects were observed microscopically, there was some degree of toxicity, as indicated by an increase in lactate dehydrogenase release in cells that were exposed for 24 hours to AG1478 and GM6001 (Figure E2).

Both Chronic Exposure to IL-17A and Short Exposure to NTHi Increase Expression of CYP24A1

In addition to TNF-α/IL-1β, IL-17A is also 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 patients with poorly controlled asthma (21, 22). To investigate if these epithelial triggers have similar effects on epithelial vitamin D metabolism as TNF-α 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-PBECs were differentiated with or without IL-17A. After 14 days of culture, cells were stimulated for another 24 hours with or without IL-17A and 25(OH)D_3 and, next, expression of *CYP24A1* and *CAMP* mRNA was assessed by qPCR. Similar to TNF-α/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), whereas *VDR* and *CYP27B1* expression remained unaffected by IL-17A (data not shown). To examine if exposure to NTHi affects expression of *CYP24A1*, ALI-PBECs were differentiated for 14 days and subsequently stimulated with 2.5, 5, and 10×10^7 CFU/ml of UV-inactivated NTHi for 12 hours and assessed for CYP24A1 expression by qPCR. We observed that NTHi dose-dependently increased expression of *CYP24A1* compared with 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.

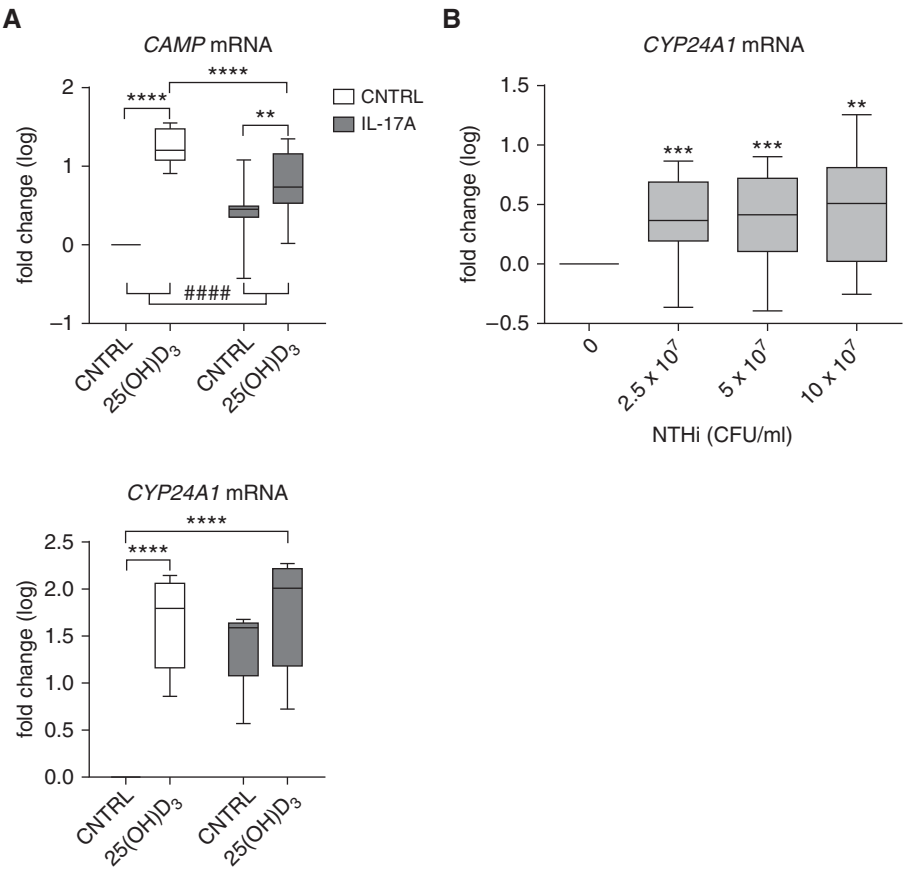

Figure 6. Chronic IL-17A-exposure and short exposure to NTHi increase expression of CYP24A1. (*A*) To investigate effects of IL-17A, PBECs were differentiated with or without IL-17A for 14 days and stimulated for another 24 hours with or without IL-17A and 25(OH)D_3 or medium CNTRL to measure relative mRNA expression of *CAMP* (hCAP18/LL-37) and *CYP24A1* by qPCR. Normalized gene expression was calculated by using the geometric mean of the expression of *B2M* and *ATP5B*. Data are presented as median (±min/max) values. Fold change in gene expression of the stimuli compared with 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 PBECs, cells were differentiated for 14 days and subsequently stimulated with increasing concentrations of ultraviolet-inactivated NTHi for 12 hours before analysis of CYP24A1 expression by qPCR. Normalized gene expression was calculated by using the expression of *RPL13A* and *ATP5B* as reference genes. Data are presented as median (±min/max) values. Fold change in gene expression of the stimuli compared with 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). $^{\#\#\#\#}P < 0.0001$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

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, MEK1/2, and Sp1. These findings are summarized in Figure 7, showing that a local proinflammatory 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

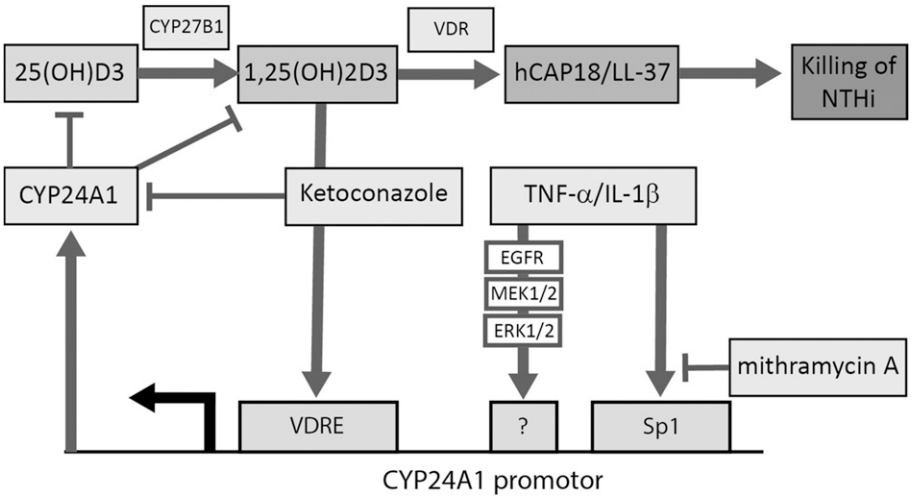

Figure 7. Summary and proposed mechanism for TNF-α/IL-1β–mediated increase of CYP24A1. In PBECs, the circulating metabolite of vitamin D, 25(OH)D_3, is converted by CYP27B1 into the active 1,25(OH)$_2$$D_3$, resulting in expression of the antimicrobial peptide, hCAP18/LL-37, and killing of NTHi via the VDR and subsequent binding of vitamin D response elements (VDREs), which are present on the promoter of *CAMP* and encode for hCAP18/LL-37. Expression of hCAP18/LL-37 and killing of NTHi is impaired by TNF-α/IL-1β exposure via increased expression of CYP24A1, which degrades both 25(OH)D_3 and 1,25(OH)$_2$$D_3$. The loss of hCAP18/LL-37 expression can be restored by ketoconazole, which suppresses CYP24A1 activity. The promoter of CYP24A1 contains, in addition to VDREs, CCAAT-enhancer-binding protein, vitamin D stimulation element and a E26 transformation specific 1 binding site, also of GC-rich boxes, which are binding sites for the transcription factor Sp1. Blocking Sp1-binding sites by mithramycin A decreases TNF-α/IL-1β–mediated expression of CYP24A1. In addition, expression of TNF-α/IL-1β–mediated CYP24A1 can be decreased by inhibiting the mitogen-activated protein kinase pathway using pharmacological inhibitors of MEK1/2.

differentiated bronchial epithelial cells have been reported previously (8, 14, 25). However, to our knowledge, this is the first study that demonstrates how proinflammatory conditions affect these activities. Our observation that CYP24A1 is increased by proinflammatory 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 (28), showing that vitamin D–induced bacterial killing by immortalized human oral keratinocytes was increased by knockdown of CYP24A1 via short interfering RNA (siRNA) transfection. It was also shown in this study that vitamin D–mediated expression of hCAP18/LL-37 correlated with killing of *Fusobacterium 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-β-defensin-2 in oral squamous cells and transformed human bronchial epithelial (16HBE) cells (29, 30). However, in ALI-PBECs, no effects of vitamin D on human-β-defensin-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 two VDREs, other promoter elements, such as three GC-rich boxes, to which the transcription factor, Sp1, can bind. To investigate if TNF-α/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-PBECs. Because *CYP24A1* expression was not fully inhibited by mithramycin A, we cannot exclude other mechanisms via other promoter elements, such as the E26 transformation specific 1 (Ets-1) binding site (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). In addition, it was shown that Sp1-mediated gene expression was facilitated via TNFR1, MEK1/2, and ERK1/2 (34). In line with these findings, we demonstrated that TNF-α/IL-1β increased phosphorylation of ERK1/2 and that blocking MEK1/2 decreased expression of *CYP24A1*. We furthermore showed that TNF-α/IL-1β increased phosphorylation of EGFR, and that inhibition of EGFR phosphorylation decreased phosphorylation of ERK1/2, providing evidence for an additional involvement of 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, as GM6001 did reduce TNF-α/IL-1β–mediated phosphorylation of EGFR and ERK1/2. TNF-α/IL-1β also phosphorylates EGFR via activation of transforming growth factor β-activated kinase 1 (TAK1), independently of MMP-mediated cleavage and release of membrane-bound EGFR ligands, indicating that other pathways might also play a role (37). Furthermore, possible toxic or nonselective effects of these signaling inhibitors upon prolonged incubation (24 h) cannot be fully excluded, because lactate dehydrogenase release was increased after 24-hour exposure to GM6001 and AG1478 (Figure E2). Taken together, one of the mechanisms that results in vitamin D–independent CYP24A1 expression by TNF-α/IL-1β in PBECs likely involves activation of EGFR, MEK1/2, and Sp1. Because studies have shown that IL-17A and NTHi promote mitogen-activated protein kinase/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 proinflammatory 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 coculture 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-day-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-PBECs 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 kD and also weaker expression of a peptide at 18 kD (size of uncleaved hCAP18) was detected, yet expression of a peptide at 4.5 kD (mature antimicrobial active LL-37) was absent. Western blots showing peptides containing LL-37 immunoreactivity at 14 kD were previously reported by us and others (6, 14, 40–42). For example, Sørensen and colleagues detected 14-kD hCAP18 fragments, in addition to 18-kD and 4.5-kD fragments, after 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-kD 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 the presence of proinflammatory cytokines. Furthermore, decreased availability of vitamin D in an inflammatory environment also reduces the impact of vitamin D on inflammation through its antiinflammatory 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 analogs, 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, as oral administration of the drug is reported to be unsafe 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 antiinflammatory 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 patients with COPD and in patients with corticosteroid-resistant asthma, 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 patients with corticosteroid-resistant asthma (51).

In summary, we have demonstrated that epithelial exposure to proinflammatory cytokines reduces the vitamin D–induced antibacterial activity in PBECs. These data suggest that a local proinflammatory 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 contribute to the vitamin D deficiency in such patients (2–4, 52, 53). 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. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

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