Modulation of airway epithelial cell function by vitamin D in COPD

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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).

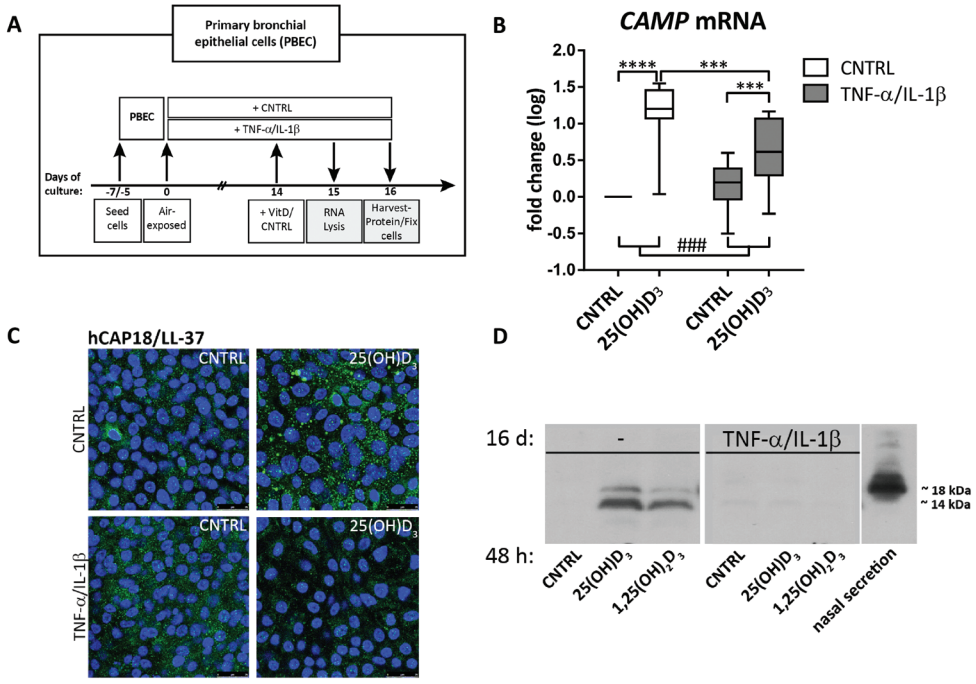

Figure 1. TNF-α and IL-1β (TNF-α/IL-1β) decreases 25(OH)D_3-mediated expression and release of hCAP18/LL-37 in primary bronchial epithelial cells (PBEC) (A) PBEC were seeded on Transwell inserts and cultured for 5-7 days until confluence was reached. Subsequently PBEC were cultured at the air-liquid 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.

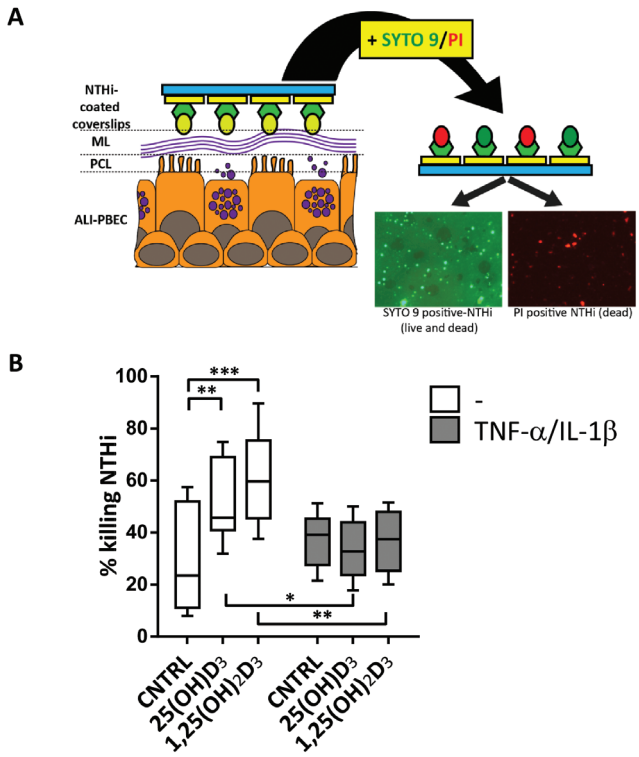

Figure 2. TNF-α and IL-1β (TNF-α/IL-1β) impairs vitamin D-mediated killing of non-typeable *Haemophilus influenzae* (NTHi). Primary bronchial epithelial cells (PBEC, n = 6 donors) were differentiated with and without TNF-α/IL-1β for 14 days followed by 48 h 25(OH)D_3- and 1,25$(OH)_2D_3$ treatment or 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).

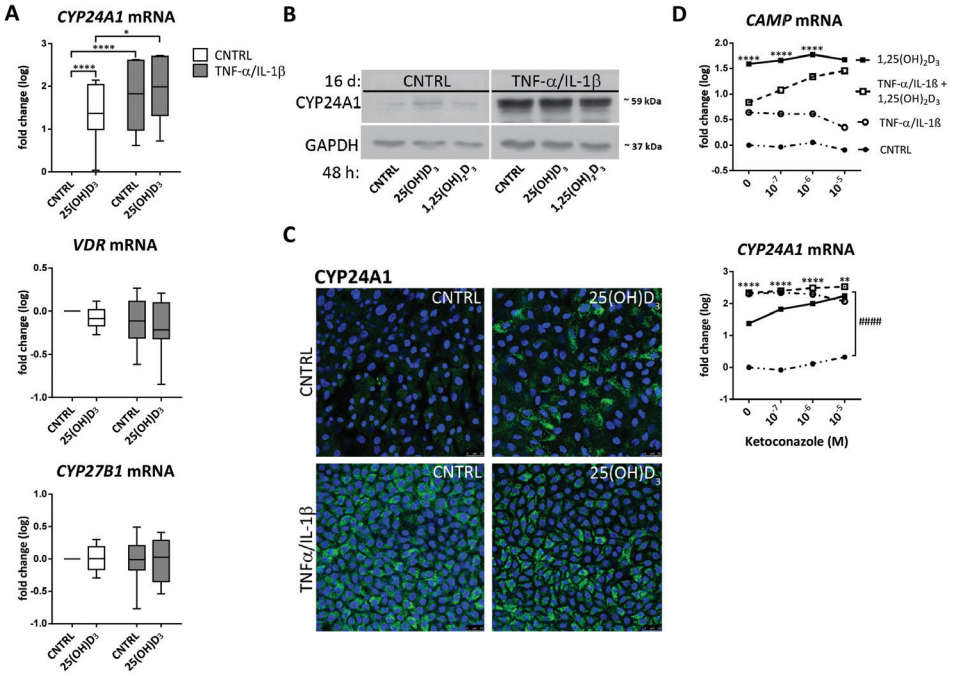

Figure 3. CYP24A1 is induced by chronic exposure to *TNF-α and IL-1ß (***TNF-α/IL-1β) and decreases vitamin D-mediated expression of hCAP18/LL-37.** Primary bronchial epithelial cells (PBEC) were cultured as described in Figure 1A for assessing *CYP24A1*, *VDR* and *CYP27B1* mRNA expression and CYP24A1 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).

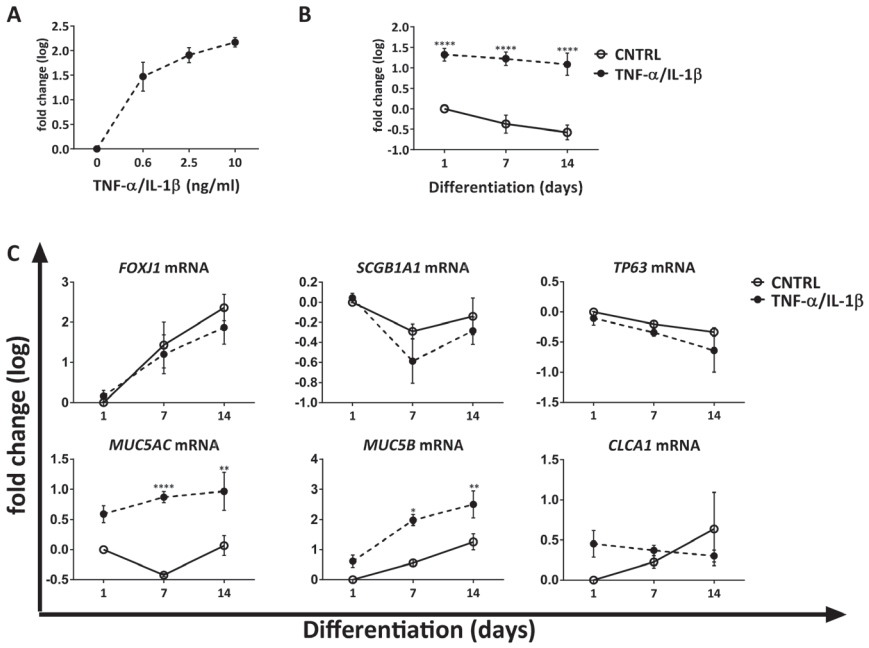

Figure 4. Time- and dose-dependent effects of TNF-α and IL-1β (TNF-α/IL-1β) on CYP24A1 expression and epithelial differentiation. (A) Primary bronchial epithelial cells (PBEC) were exposed to TNF-α/IL-1β or CNTRL for 1, 7 and 14 days or exposed for 14 days to increasing concentrations of TNF-α/IL-1β 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).

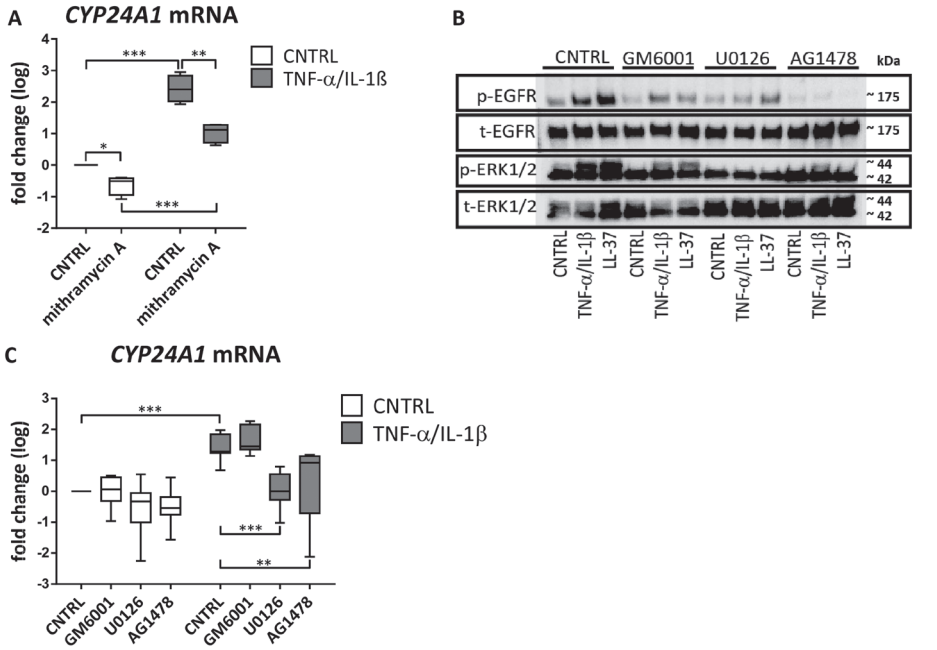

Figure 5. TNF-α and IL-1β (TNF-α/IL-1β)-induced expression of *CYP24A1* is mediated by activation of EGFR, ERK1/2 and Sp1. To investigate the role of the transcription factor Sp1 in TNF-α/IL-1β-mediated expression of *CYP24A1*, Primary bronchial epithelial cells (PBEC) were differentiated for 14 days 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.

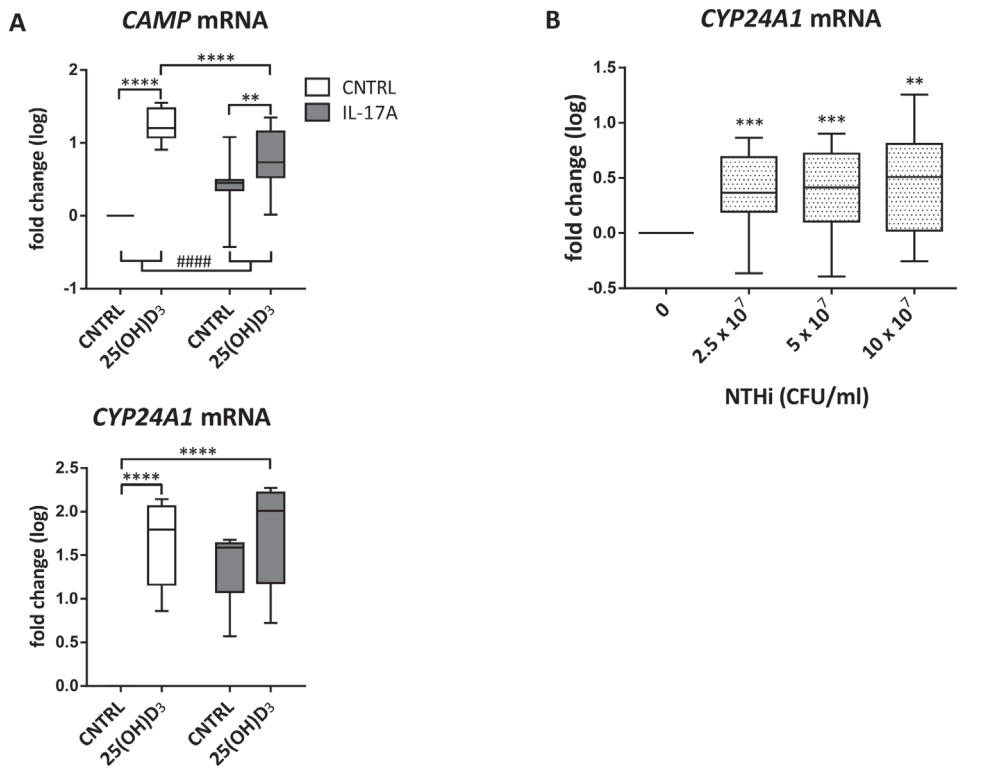

Figure 6. Chronic IL-17A-exposure and short exposure to non-typeable *Haemophilus influenzae* (NTHi) increase expression of *CYP24A1*. (A) To investigate effects of IL-17A, primary bronchial epithelial cells (PBEC) were differentiated with or without IL-17A for 14 days and stimulated for another 24 h 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). #### $p < 0.0001$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

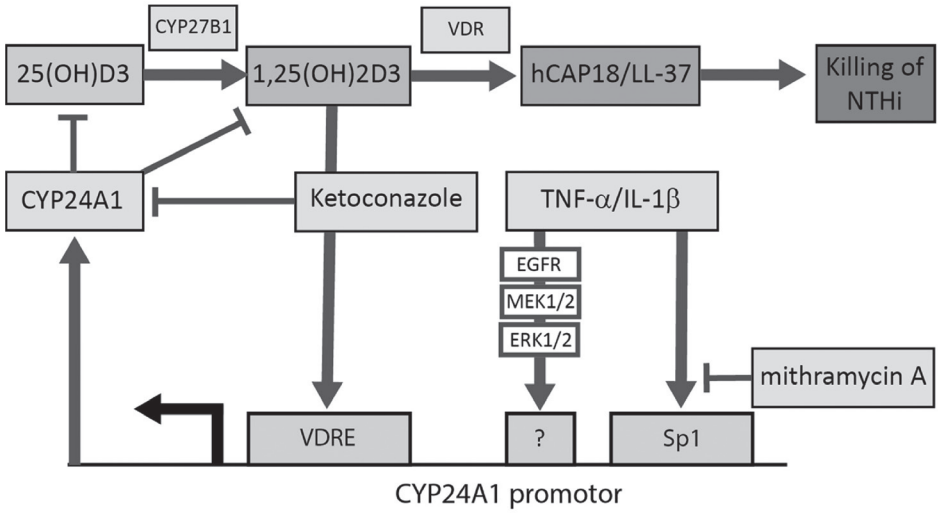

Figure 7. Summary and proposed mechanism for TNF-α/IL-1β-mediated increase of CYP24A1. In primary bronchial epithelial cells, the circulating metabolite of vitamin D, 25(OH)D_3 is converted by CYP27B1 into the active 1,25(OH)$_2D_3$, resulting in expression of the antimicrobial peptide hCAP18/LL-37 and 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

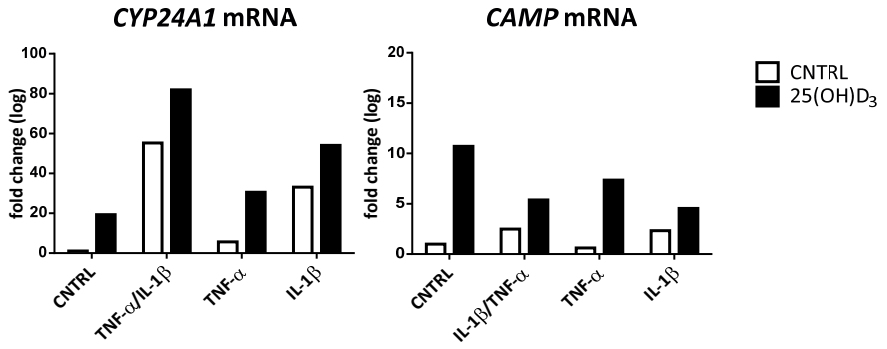

Figure E1. Effect of TNF-α and IL-1β together versus TNF-α and IL-1β alone on expression of CYP24A1 and hCAP18/LL-37 in primary bronchial epithelial cells (PBEC). PBEC (n = 2 donors) were cultured with or without the pro-inflammatory cytokines TNF-α/IL-1β or TNF-α and IL-1β in combination or alone for 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.

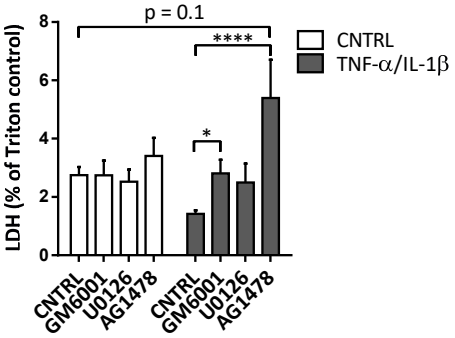

Figure E2. Effect of inhibitors and TNF-α/IL-1β on LDH release in primary bronchial epithelial cells (PBEC). Submerged PBEC cultures (n = 4 donors) were pre-incubated with medium alone (CNTRL; 0.25% v/v DMSO) and inhibitors of MMP (GM6001), EGFR (AG1478) and MEK (U0126) followed by stimulation for 24 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. * $p < 0.05$, **** $p < 0.0001$.

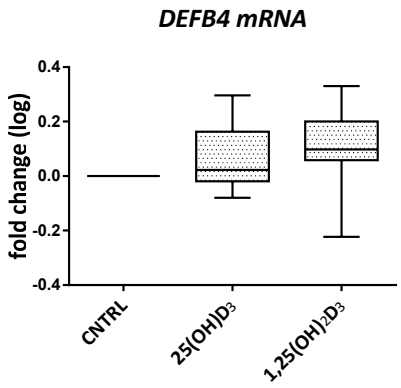

Figure E3. Effect of 25(OH)D_3 and 1,25$(OH)_2D_3$ on hBD-2 (*DEFB4*) expression in primary bronchial epithelial cells (PBEC). PBEC were differentiated for 14 days and subsequently exposed to 10^{-7} M 25(OH)D_3 and 1,25$(OH)_2D_3$ for 24 h in duplicate. *DEFB4* (hBD-2) expression was assessed using 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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