Modulation of airway epithelial cell function by vitamin D in COPD

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CHAPTER

5

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

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Abstract

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

Introduction

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

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

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

Materials and Methods

Cell culture

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

Cell exposures

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

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

RNA isolation, reverse transcription (RT), and qPCR

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

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

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

SDS-PAGE Western blot

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

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

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

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

Antimicrobial assay

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

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

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

Statistical analysis

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

Results

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

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

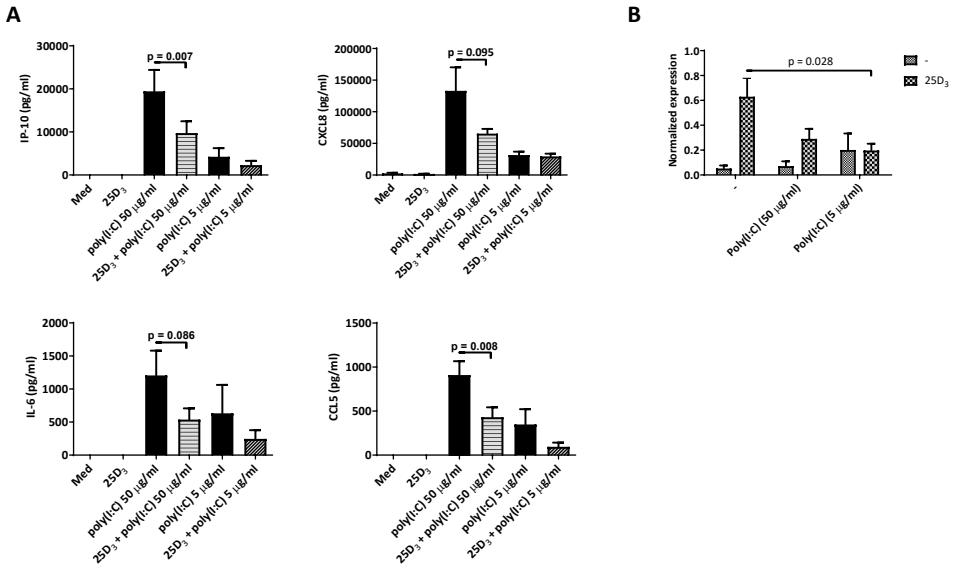

Figure 1. **Effect of 25D3 and poly(I:C) on hCAP18/LL-37 expression and cytokine and chemokine release by bronchial epithelial cells.** Well-differentiated epithelial cells were stimulated with poly(I·C) (at 50 and 5µg/ml) with and without 10^{-7} M $25D_3$ and by $25D_3$ alone for 24 h in duplicate.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

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

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

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

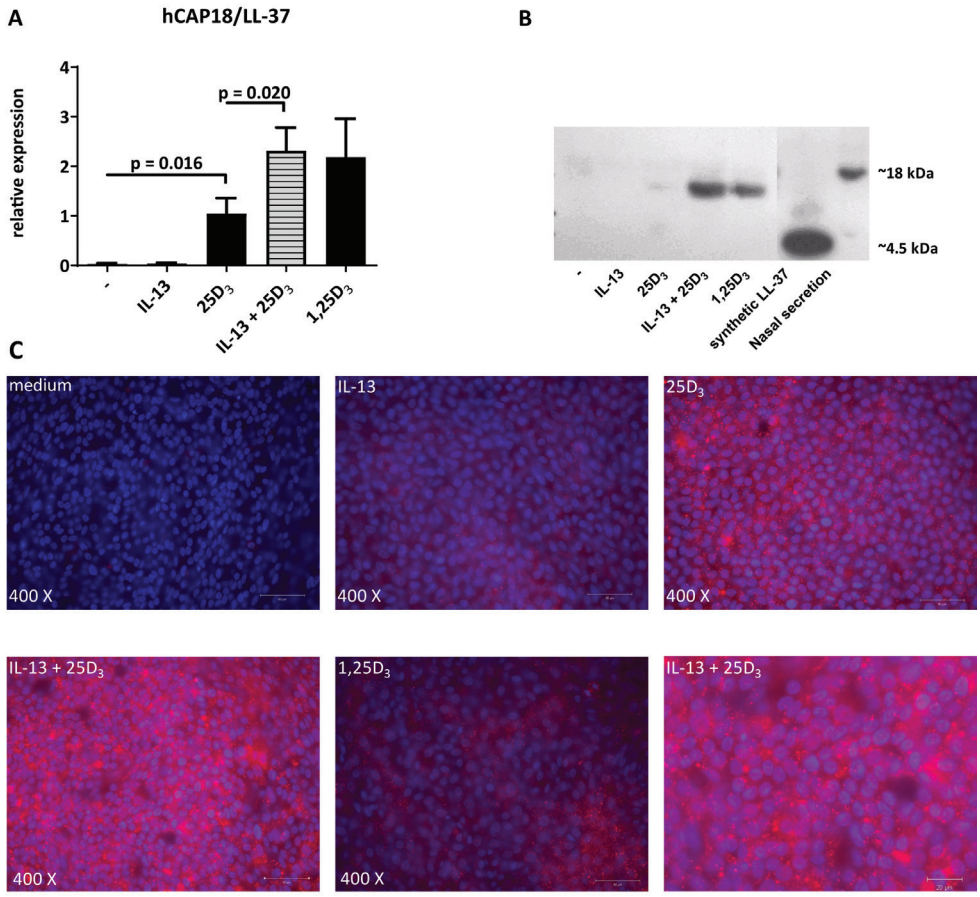

Figure 2. Effect of IL-13 on $25D_3$-mediated induction of hCAP18/LL-37 in bronchial epithelial cells. Cells were pretreated with 100 ng/ml IL-13 for 24 h and then exposed for another 48 h in duplicate to 100 ng/ml IL-13 with and without 10^{-7} M $25D_3$ or 10^{-7} M $1,25D_3$, included as a positive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

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

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

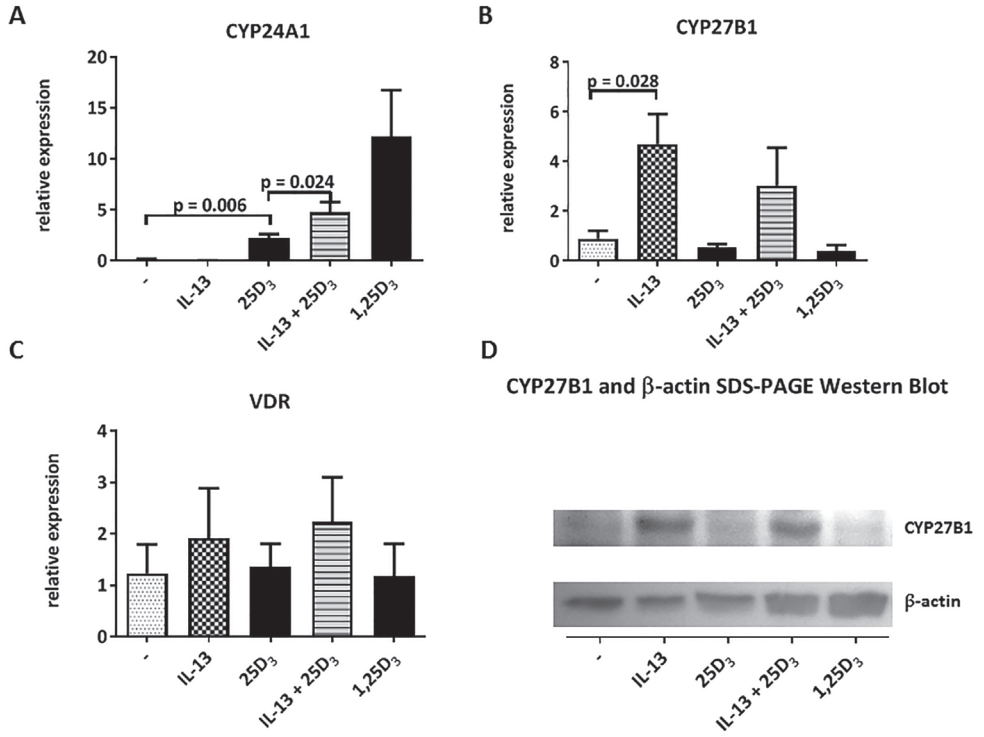

Figure 3. Effect of IL-13 on expression of CYP24A1, CYP27B1, and VDR in bronchial epithelial cells. Cells (n = 5 to 7 donors) were pretreated with 100 ng/ml IL-13 for 24 h and then exposed for another 48 h in duplicate to 100 ng/ml IL-13 with and without 10^{-7} M $25D_3$ or 10^{-7} M $1,25D_3$, included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

IL-13 increases hBD-3 expression

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

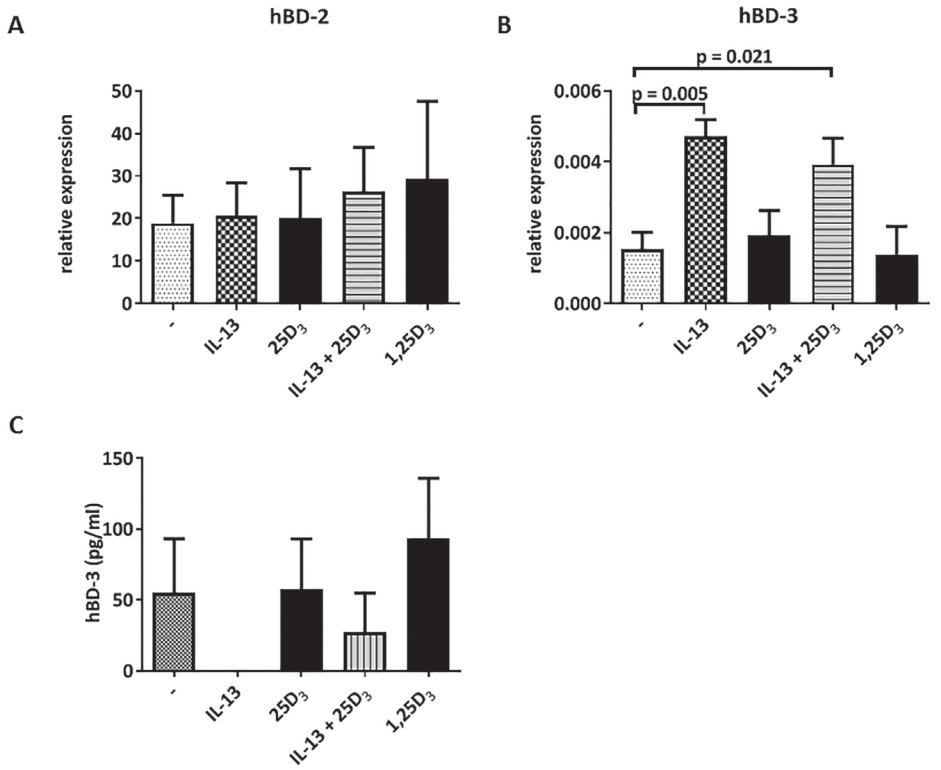

Figure 4. Effect of IL-13 on expression and/or release of hBD-2 and hBD-3 in bronchial epithelial cells. Cells (n = 5 to 7 donors) were pretreated with 100 ng/ml IL-13 for 24 h and then exposed for another 48 h in duplicate to 100 ng/ml IL-13 with and without 10^{-7} M $25D_3$ or 10^{-7} M $1,25D_3$,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

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

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

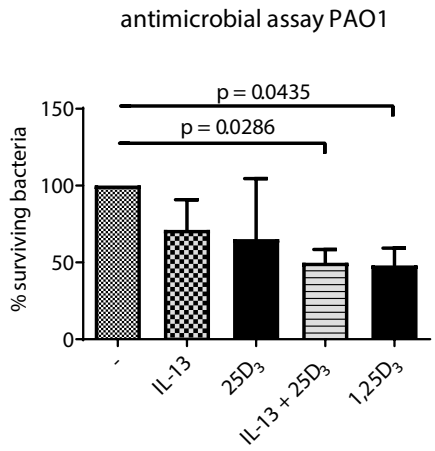

Figure 5. Effect of IL-13 on $25D_3$-mediated antimicrobial activity. Apical surface fluid of cells (n = 3) was pretreated with 100 ng/ml IL-13 for 24 h and then exposed for another 48 h in triplicate to 100 ng/ml IL-13 with and without 10^{-7} M $25D_3$ or 10^{-7} M $1,25D_3$, included as a positive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

Discussion

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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Supplemental data

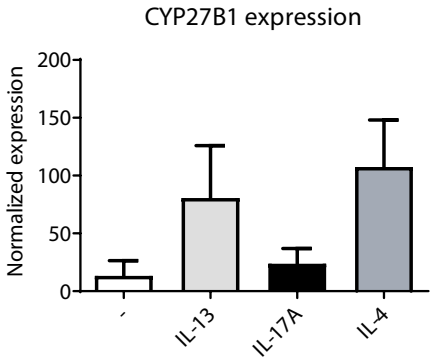

Figure S1. Effect of IL-17A and IL-4 on CYP27B1 expression in bronchial epithelial cells. Bronchial epithelial cells (n= 2 donors) were exposed to 20 ng/ml IL-4 or 10 ng/ml IL-17A for 24 hours in duplicate. RNA isolation, cDNA synthesis, and quantitative PCR were performed as described in the Materials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

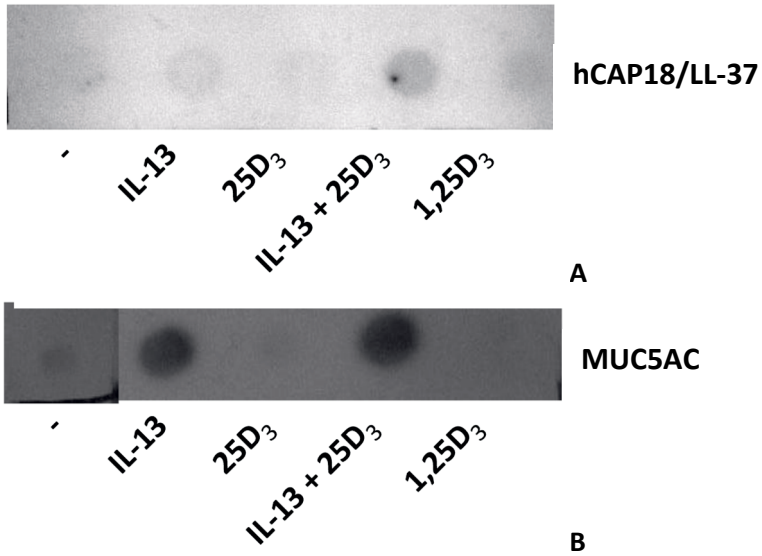

Figure S2 A-B. Effects of IL-13 and 25D3 on apical release of hCAP18/LL-37 and MUC5AC in bronchial epithelial cells. Cells of 2 different donors were pretreated with 100 ng/ml IL-13 for 24 hours and then exposed for another 48 hours in duplicate to 100 ng/ml IL-13 with and without 10^{-7} M $25D_3$ (or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