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Vitamin D Decreases RSV Induction of NF-κB-linked Chemokines and Cytokines in Airway Epithelium While Maintaining the Antiviral State

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Abstract

Epidemiological studies suggest that low vitamin D levels may increase the risk or severity of respiratory viral infections. In this study, we examined the effect of vitamin D on respiratory syncytial virus (RSV) infected human airway epithelial cells. Airway epithelium converts 25-hydroxyvitamin D_3 (storage form) to 1,25-dihydroxyvitamin D_3 (active form). Active vitamin D, generated locally in tissues, is important for the non-skeletal actions of vitamin D including its effects on immune responses. We found that vitamin D induces IκBα, a NF-κB inhibitor, in airway epithelium and decreases RSV induction of NF-κB driven genes like interferon-β and CXCL10. We also found that exposing airway epithelial cells to vitamin D reduced induction of interferon stimulated proteins with important antiviral activity, e.g., MxA and ISG15. In contrast to RSV-induced gene expression, vitamin D had no effect on interferon signaling and isolated interferon induced gene expression. Inhibiting NF-κB with an adenovirus vector that expressed a non-degradable form of IκBα mimicked the effects of vitamin D. When the vitamin D receptor was silenced with siRNA the vitamin D effects were abolished. Most importantly we found that, in spite of inducing IκBα, and dampening chemokines and interferon-β, there was no increase in viral mRNA or protein or in viral replication. We conclude that vitamin D decreases the inflammatory response to viral infections in airway epithelium without jeopardizing viral clearance. This suggests that adequate vitamin D levels would contribute to reduced inflammation and less severe disease in RSV infected individuals.

Keywords

Human; Lung; Viral; Chemokines; Inflammation

Introduction

Vitamin D is increasingly recognized as a pluripotent hormone with functions that extend beyond its classical role in calcium homeostasis (1). Rapidly growing evidence from epidemiological and basic research studies reveals that vitamin D can modulate immune responses (2–4). Vitamin D deficiency is highly prevalent and has been associated both with increased risk of several inflammatory diseases (5–9) and with susceptibility to infections,

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including viral respiratory infections (10,11). The localized tissue-specific generation of active vitamin D is thought to be a key component of non-classical vitamin D functions. In our previously published data, we showed that normal lung epithelium constitutively converts 25-hydroxyvitamin D_3 (25D = storage form of vitamin D) to 1,25D-dihydroxyvitamin D_3 (1,25D = active form of vitamin D) and that the generation of active vitamin D is increased in the presence of viral infection (12).

The airway epithelium is the first line of defense during respiratory virus infection (13). Respiratory epithelium expresses Toll-like receptors (TLR) and Retinoic acid-inducible gene-I (RIG-I)-like receptors which sense viral RNA. Ligand engagement results in the activation of both nuclear factor κB (NF-κB) and interferon regulatory factors (IRFs) (14–16). The end result is amplification of host defense to microorganisms through secretion of antimicrobial peptides, chemokines and cytokines, and, in particular, type I interferons (13,17). We have shown that vitamin D increases the toll-like receptor, co-receptor CD 14, and the antimicrobial peptide, cathelicidin, in airway epithelium (12). Type I interferons (IFNs) play a crucial role in defense against viruses. They mediate antiviral activity by triggering the expression of >100 IFN stimulated genes (ISGs), the products of which have diverse antiviral activities (18,19).

Chemokines are a family of small molecules that induce chemotaxis of neutrophils, mononuclear cells, or lymphocytes. Chemokines can also stimulate leukocyte degranulation or release of inflammatory mediators (20). Chemokines thus play a pivotal role in orchestrating inflammation and have been found to be produced by an array of cells including epithelial cells (21). Production of chemokines can be either beneficial or detrimental to the host. Inflammation is an essential component of host defense, however, a too vigorous response against microbes or inflammation may be deleterious to the host, leading to impaired organ function. Vitamin D has been shown to inhibit production of inflammatory chemokines in animal models of inflammatory diseases like multiple sclerosis and type-1 diabetes (22–24).

The family of NF-κB transcriptional regulatory factors has a central role in coordinating the expression of a wide variety of genes that control immune responses. NF-κB proteins are present in the cytoplasm in association with inhibitory proteins (IκBs). IκBs are phosphorylated by IκB kinase following cell stimulation, and they are targeted for destruction by the ubiquitin/proteasome degradation pathway (25). The degradation of IκB allows NF-κB proteins to translocate to the nucleus, bind to their DNA binding sites (26), and activate a variety of genes, including cytokines and chemokines (27). Studies in various cell types, including dendritic cells (28–30), fibroblasts (31,32), keratinocytes (33), pancreatic islet cells (23) and kidney cells (34) indicate that vitamin D dampens NF-κB signaling. Several mechanisms have been proposed, including vitamin D induced increase in IκBα (23,32,33), and/or interference with binding of NF-κB subunits, to promoter-regulatory areas (30,31,34).

Epidemiological studies suggest that low vitamin D levels are a risk factor for viral respiratory infections. However, vitamin D has been found to inhibit NF-κB signaling which could result in decreased viral clearance and/or lessened inflammatory responses via inhibition of type I IFN signaling and chemokines. The present study was undertaken to look at the effects of vitamin D on NF-κB signaling, especially as it relates to antiviral immunity in airway epithelium.

Materials and Methods

Reagents

1α 25-Dihydroxy Vitamin D3 was obtained from Calbiochem, San Diego, CA. Lactacystin was obtained from Sigma Aldrich. Interferon-β was purchased from PBL Interferon Source, Piscataway, NJ.

Human Tracheo-Bronchial Epithelial Cells

Human tracheobronchial epithelial (hTBE) cells were obtained from the University of Iowa Cell and Tissue Core under a protocol approved by the University of Iowa Institutional Review Board. Epithelial cells were isolated from tracheal and bronchial mucosa by enzymatic dissociation and cultured in Laboratory of Human Carcinogenesis (LHC)-8e medium on plates coated with collagen for study up to passage 10 as described previously (35). All experiments were conducted using cells from at least three different donors.

Respiratory Syncytial Virus

Cells at 80% confluency were infected with human respiratory syncytial virus (RSV), strain A-2 (MOI 1-2). Viral stocks were obtained from Advanced Biotechnologies Inc (Columbia, MD). The initial stock (3×10^8 PFU/ml) was placed in aliquots and kept frozen at −135°F. The virus was never refrozen. GFP-RSV was a kind gift from Dr. Varga (University of Iowa).

Quantitative RT-PCR

Total RNA was isolated using the RNAqueous – 4PCR kit (Ambion Inc., Austin, TX) following the manufacturer's instructions. RNA quality and quantity were assessed with Experion automated electrophoresis system (Bio-Rad Laboratories, Hercules, CA) using the Experion RNA StdSens Analysis Kit according to the manufacturer's protocol. RNA quality was considered adequate for use if the 28S/18S ratio was >1.2 and the RNA Quality Indicator (RQI) was >7. Total RNA (300 ng) was reverse-transcribed to cDNA using iScript cDNA Synthesis kit (Bio-Rad) following the manufacturer's instructions. PCR reactions were performed using 2 μl cDNA and 48 μl master mix containing iQ SYBR Green Supermix (Bio-Rad), 15 pmol of forward primer and 15 pmol of reverse primer, in a CFX 96 Real Time System as follows: 3 min at 95°C, followed by 40 cycles of 10 s at 95°C and 30 s at 55°C + plate read. The fluorescence signal generated with SYBR Green I DNA dye was measured during annealing steps. Specificity of the amplification was confirmed using melting curve analysis. Data were collected and recorded by CFX Manager Software and expressed as a function of threshold cycle (C_T). The relative quantity of the gene of interest was then normalized to relative quantity of HPRT ($\Delta\Delta C_T$). The sample mRNA abundance was calculated by the formula $2-(^{\Delta\Delta CT})$. Specific primer sets used are as follows (5′ to 3′):

CXCL10 GTCTGAATCCAGAATCGAAGGC (forward), TTGAGGGTTTGCTACAACATGGGC (reverse); *HPRT* GCAGACTTTGCTTTCCTTGG (forward), AAGCAGATGGCCACAGAACT (reverse); *IFNβ* TGGGAGGCTTGAATACTGCCTCAA (forward), TCCTTGGCCTTCAGGTAATGCAGA (reverse); *IκBα* AACCTGCAGCAGACTCCACT (forward), TCCTGAGCATTGACATCAGC (reverse); *ISG15* CTGAGAGGCAGCGAACTCATCTTT (forward), AATCTTCTGGGTGATCTGCGCCTT (reverse); *MXA* GAAGCCCTGCAGAGAGAGAA (forward), AACTCGTGTCGGAGTCTGGT (reverse); *RSV N-gene* CAAGCCCAAAGCTGTGCTAAACCA (forward), AGACAGTGACTATGCGGTCTGCAA (reverse).

Gene specific primers were custom-synthesized and purchased from Integrated DNA Technologies (Iowa City, IA) based on design using gene-specific nucleotide sequences from the National Center for Biotechnology Information sequence databases and PrimerQuest web interface (Integrated DNA Technologies).

Cellular Protein

Whole cell protein extracts were prepared by lysis of cell monolayers in 200 μl of lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% Nonidet P-40), a protease inhibitor cocktail (Roche Bioscience, Palo Alto, CA), and a phosphatase inhibitor cocktail (#524625 Calbiochem). The lysates were sonicated for 20 s and kept at 4 °C for 30 min. After a 5 min centrifugation (14.000 rpm @ 4 °C) the supernatant was saved as whole cell lysate.

Cytosolic and nuclear protein extracts were prepared by lysis of cells in 400 μl of lysis buffer (10mM HEPES, pH 7.8, 10 mM KCl, 2 mM MgCl2, 0.1 mM EDTA). The lysates were incubated on ice for 15 min, 25 μl of 10% Nonidet P-40 was added, and vigorous mixing ensued. After a 30 s centrifugation (14.000 rpm @ 4 °C), the supernatant was saved as the cytosolic fraction. The pelleted nuclei were resuspended in 50 μl of extraction buffer (50mM HEPES, pH 7.8, 50mM KCl, 300mM NaCl, 0.1mM EDTA, 10% glycerol) and incubated on ice for 20 min. After a 5 min centrifugation (14.000 rpm @ 4 °C) the supernatant was saved as nuclear fraction. Protein determinations were made using a protein measurement kit (Bradford Protein Assay, #500-0006) from BioRad. Extracts were stored at −70 °C.

Primary Antibodies

Primary antibodies were obtained from the following: IκBα; Santa Cruz Biotechnology #203 (Santa Cruz, CA), HDAC2; Santa Cruz #7899, ISG15; R&D systems #4845 (Minneapolis, MN), MXA; BacLab #CH-4302 (Muttenz, Switzerland), p65; Santa Cruz #109, total STAT1; Santa Cruz #346, phospho STAT1 tyrosine-701; Cell Signaling Technology #9171 (Beverly, MA), phospho STAT1 serine-727; Cell Signaling #9177, RSV all antigen; Biodesign International #B65860G (Saco ME) VDR; Santa Crus #13133, and IRF-3 Cell Signaling #4962.

Immunoblot Analysis

Protein (25–30 μg of whole cell or 8–15 μg nuclear) was mixed 1:1 with 2× sample buffer (20% glycerol, 4% SDS, 10% β-mercaptoethanol, 0.05% bromophenol blue and 1.25M Tris pH 6.8), loaded onto a 12% or 15% (ISG15) SDS-PAGE gel, and run at 150V for 90 min. Cell proteins were transferred to Immuno-Blot PVDF membrane (Bio-Rad) with a Bio-Rad semidry transfer system, according to the manufacturer's instructions. The PVDF was then incubated with primary antibody (dilutions 1:500 to 1:1000) in 5% milk in TTBS (tris buffered saline with 0.1% Tween 20) overnight. The blots were washed × 3 with TTBS and incubated for 1 h with horseradish-peroxidase (HRP) conjugated secondary anti-IgG antibody (dilution 1:2000 to 1:20.000). The blots were washed again × 3 with TTBS, and immunoreactive bands were developed using a chemiluminescent substrate, ECL Plus (Amersham Biosciences, Piscataway, NJ). An autoradiograph was obtained, with exposure times of 10 s to 2 min. Equal loading of proteins was confirmed with β-actin (Abcam #8226) (primary antibody dilution 1:20.000 and secondary 1:40.000).

Secreted Proteins

Supernatants were collected and frozen at −70 °C. CXCL 10 concentrations were determined using DuoSet ELISA kits from R&D systems (# DY266) following the manufacturer's protocol.

RNA Interference

Control siRNA (Santa Cruz #44230) or target-specific 19–25 nt siRNA designed to knock down VDR gene expression (Santa Cruz #106692) was transfected into hTBE cells at a final concentration of 50 nM using Lipofectamine 2000 (Invitrogen). Transfection mixtures were assembled as previously described (36). Cells were incubated with 200 μl of transfection mixture and 2 ml of antibiotic-free medium for 16–18 h. Transfection efficiency was assessed with using a fluorescent control siRNA (Santa Cruz #36869). Knock down of VDR was confirmed with real time quantitative PCR and Western Blotting.

NF-κB Pathway Inhibition

Inhibition of NF-κB dependent signaling was accomplished by infecting hTBE cells with a recombinant adenoviral vector (MOI=10) that expresses a dominant-negative mutant form (non-degradable) of IκBα or the control vector BglII, as previously described (37). An adenovirus vector expressing GFP was used to assess the level of epithelial cell transgene expression.

Statistical analysis

When two groups were compared, we used two tailed Student's *t* test. To compare three or more groups we used repeated measures analysis of variance (ANOVA) followed by Bonferroni's method to control for multiple comparisons. When treatments were being compared with control but not each other Dunnett's test was used to correct for multiple comparisons. Data is presented as mean +/− SEM. P value of <0.05 was considered statistically significant. These methods were performed using GraphPad Prizm 5 for Windows (GraphPad Software, San Diego, CA).

Results

Vitamin D induces the NF-κB inhibitory protein IκBα in airway epithelium

To determine the effects of vitamin D on NF-κB signaling we examined the effect of vitamin D on the NF-κB inhibitory protein IκBα. In other systems, vitamin D increases the expression of IκBα, therefore, we hypothesized that this was also true for airway epithelium (23,32,33). To test this hypothesis, hTBE cells were treated with 1,25D for 24 h and IκBα mRNA was evaluated with quantitative RT-PCR (qRT-PCR). Treatment with vitamin D led to a statistically significant increase in IκBα mRNA (Fig. 1*A*). There was no change in mRNA half life suggesting an effect of vitamin D on transcription (data not shown).

We proceeded to look at IκBα protein levels and found that vitamin D increases IκBα protein in a dose dependant manner. When we evaluated a viral model, cells infected with respiratory syncytial virus (RSV) for 24 h had decreased IκBα protein levels consistent with RSV induced degradation of IκBα. When cells were pretreated with increasing doses of 1,25D, prior to infection with RSV, we saw a dose dependent increase in IκBα levels in RSV infected and non-infected cells (Fig. 1*B*). To investigate whether the increase in protein levels was due to changes in IκBα phosphorylation and degradation we pretreated airway epithelial cells with 1,25D and infected them with RSV for 24 h with and without the proteasome inhibitor (lactacystin) for the last 3 h of the experiment. We found that, in the presence of a proteasome inhibitor, 1,25D increased both total and phosphorylated IκBα in the presence and absence of RSV infection (Fig. 1*C*). This data, combined, indicates that in airway epithelium vitamin D increases levels of IκBα, a well established inhibitor of NF-κB signaling, by increasing mRNA transcription and protein synthesis. The proteasome inhibitor data suggests that the increase in IκBα with vitamin D exposure is due to increased production and not increased stability.

Vitamin D suppresses IFNβ and interferon stimulated genes in hTBE cells infected with RSV

Infected airway epithelium utilizes TLRs and the RNA helicase, retinoic acid-inducible gene I (RIG-I), to detect and respond to RSV infection (38–42). Activation of those signaling pathways leads to activation of the transcription factors NF-κB and IRFs (43). Following RSV infection, NF-κB translocates into the nucleus and turns on target genes including IFNβ (type I interferon) and several chemokines that are essential for host defense against viral infection (44,45). Having established that vitamin D inhibits NF-κB signaling in airway epithelium, we next asked whether 1,25D limits expression of host defense molecules driven by NF-κB. First, we wanted to establish at which time point RSV-induced gene expression was at maximum. hTBE cells were treated with RSV (MOI 1-2) for 3,6,16 or 24 h and expression of IFNβ, STAT1, interferon stimulated genes (ISGs), and chemokines were examined (Fig. 2*A*). This revealed significant induction at 16 h with maximum at 24 h. We then went on to evaluate the effects of vitamin D on virally induced genes at 24 h. IFNβ is essential to host response against viral infections (46). The gene coding for IFNβ has a NF-κB site in its promoter which, along with ATF-2/c-jun and IRF-3/IRF-7 sites, comprises the enhanceosome, required for transcriptional activation of the IFNβ gene. In turn, IFNβ activates STAT1 and STAT2. hTBE cells were pretreated with 1,25D followed by 24 h of infection with RSV (MOI 1-2). IFNβ mRNA and STAT1 activation were measured with qRT-PCR and Western blotting, respectively. Treatment with 1,25D resulted in significant reduction of IFNβ mRNA and of IFNβ induced STAT1 protein and STAT1 translocation to the nucleus (Fig. 2*B*). Activation of STAT1 and STAT2 leads to the formation of the transcription complex interferon stimulated gene factor 3 (ISGF3). ISGF3 binds to interferon stimulated response elements (ISRE) in ISGs and induces their expression.

We next determined the role of vitamin D in expression of interferon stimulated genes with and without known NF-κB sites in their promoters. CXCL10 (IP-10) is a chemokine that is induced by RSV infection (44,47,48) and has both ISRE and NF-κB sites in its promoter (49). Myxovirus resistance A (MxA) and IFN-stimulated protein of 15kDa (ISG15), on the other hand, are ISGs that directly inhibit viral production (50) and have ISRE sites in their promoters but not functional NF-κB sites (51–53). As before, we pretreated hTBE cells with 1,25D, followed by RSV infection, and looked at gene transcription and protein levels. Treatment with 1,25D resulted in decreased levels of both chemokines and antiviral effectors (Fig. 2*C*). The suppression in transcription was more pronounced for CXCL10 (ISRE and NF-κB sites) with 86% reduction in CXCL10 mRNA, than in the antiviral effectors (ISRE site only), where 56% reduction of MxA and 62% reduction in ISG15 was observed (Fig. 2*C*). This data indicates that 1,25D does modulate IFNβ and ISGs in airway epithelium infected with RSV. These findings raise the question: is vitamin D directly inhibiting both NF-κB and STAT signaling or is it alternatively having a direct effect on only NF-κB with subsequent suppression of NF-κB driven interferon production and STAT activation?

The effects of vitamin D on antiviral effectors occur at the level of NF-κB

We proceeded to determine whether vitamin D could be effecting no only activation of NF-κB but also activation of IRF-3. Recognition of viral RNA by either RIG-I like receptors or TLR results in activation of both NF-κB and IRF-3. If the effects of vitamin D were at the level of dsRNA sensing, one would expect expression of both transcription factors to be decreased. IRF-3 is a latent transcription factor that is activated by phosphorylation by kinases that are activated in response to viral dsRNA (54). This results in the formation of IRF-3 dimers that translocate to the nucleus and bind to promoter areas. IRF-3 activation has been shown to be rapid (< 6 hours) in RSV infection (38). To investigate whether the vitamin D effects could be explained by effects on IRF-3, we looked at translocation of

IRF-3 to the nucleus. hTBE cells were infected with RSV (MOI 1-2) for 1, 3 or 8 h in the presence or absence of vitamin D. Nuclear translocation of IRF-3 was evaluated with Western blot (Fig. 3*A*). Vitamin D did not suppress IRF-3 translocation to the nucleus, but on the contrary, may have even increased it.

Activation of NF-κB leads to induction of IFNβ. The most likely explanation of our data is that suppression of NF-kB by vitamin D leads to production of less IFNβ, which is dependent on this transcription factor for its expression. However, if the effects of vitamin D were downstream of IFNβ, then one would expect vitamin D to have the same effects on STAT1 and ISGs induction by IFNβ itself and RSV. To determine this, hTBE cells were treated with IFNβ (1000 U) or RSV (MOI 1-2) for 24 h in the presence or absence of vitamin D. As expected, both IFNβ and RSV increased expression and activation of STAT1. In contrast to RSV induced STAT1, vitamin D had no effects on IFNβ induced STAT1 (Fig. 3*B*). Furthermore, unlike RSV induced antiviral effectors, vitamin D had no effects on IFNβ induced MxA or ISG15 (Fig. 3*C*). This suggests that vitamin D does not effect interferon signaling downstream of IFNβ.

These results, combined, suggest that the effects of vitamin D are occurring at the level of NFκB.

Overexpressing IkBa mimics vitamin D effects on airway epithelial responses

To further support our hypothesis that 1,25D is modulating airway epithelial response to viral infection by inhibiting NF-κB, we introduced a recombinant adenoviral vector expressing a dominant-negative mutant form (non-degradable) of IκBα (55). We hypothesized that inhibiting NF-κB with the adenovirus vector would mimic the effects of 1,25D. First we assessed the level of epithelial cell transgene expression, by using an adenovirus vector expressing GFP, and found that about 80% of the cells expressed the vector (Fig. 4*A*). hTBE cells were subsequently infected with an adenovirus containing the non-degradable IκBα (AdIκBα S32/36) or an empty vector BglII (AdBglll). Briefly hTBE cells were incubated with adenovirus vector (MOI=10) for 2 h followed by change of media. Cells were infected with RSV (MOI 1-2) 24 h later. We found that inhibiting NF-κB with the AdIκBα mutant resulted in a significant decrease in RSV induction of IFNβ and CXCL10 with a less prominent decrease in MxA and ISG15 (Fig. 4*B*). As expected, the AdIκBα did not affect the expression of MxA or ISG15 when induced by IFNβ (Fig. 4*C*). This data shows that 1,25D has the same effects on IFNβ and interferon driven genes during RSV infection as overexpression of the NF-κB inhibitor IκBα.

Silencing the Vitamin D Receptor abolishes vitamin D effects on airway epithelial responses to RSV

The biological effects of vitamin D are achieved through regulation of gene expression mediated by the vitamin D receptor (VDR) (56). VDR is a transcription factor and it dimerizes with the retinoic X receptor (RXR) upon ligand binding (57). This complex binds to vitamin D responsive elements (VDREs) within the promoter regions of vitamin D responsive genes (58). In order to confirm that our findings are directly due to 1,25D, we next explored whether silencing the VDR would reverse the vitamin D effects. hTBE cells were transfected with control siRNA or VDR siRNA. Transfection efficiency was tested using fluorescent control siRNA. Fluorescent microscopy indicated > 90% transfection efficiency (data not shown). Knock down of the VDR was confirmed with Western blotting (Fig. 5*A*). hTBE cells were pretreated with 1,25D followed by infection with RSV (MOI=1-2) for 24 h. Expression of IFNβ and ISGs was evaluated. When cells were transfected with control siRNA, vitamin D reduced RSV induced expression of IFNβ, chemokines, and antiviral effectors as shown previously in non-transfected cells. When

VDR expression was silenced, vitamin D no longer had immunomodulatory effects (Fig. 5*B*). This data, combined, further supports that vitamin D modulates the inflammatory response to RSV by dampening induction of IFNβ and ISGs.

Vitamin D dose not effect viral replication

Interferon-β and interferon driven genes are central in host defense against viral infection. Based on our findings, vitamin D decreases expression of IFNβ and interferon driven genes by increasing IκBα and, thus, inhibits the canonical NF-κB pathway. This raises the concern that viral replication may be increased in the presence of 1,25D. Consequently, we infected hTBE cells with RSV in the presence or absence of 1,25D and looked at markers of viral quantity and replication. Expression of viral mRNA (N-gene) was measured by RT-PCR. RSV viral proteins were quantified by Western blotting (anti-RSV, all antigen). Viral titers were measured by standard plaque assay, as previously described (59). Lastly, cells were infected with GFP-labeled RSV, fixed with formalin, and viewed with a light and fluorescent microscope. All four modalities suggest that vitamin D does not alter viral replication (Fig. 6). This data shows that in spite of inhibition of IFNβ, and interferon stimulated genes, viral replication is not increased in the presence of 1,25D.

Discussion

We have shown that vitamin D modulates NF-κB signaling in primary airway epithelial cells. Furthermore, we have found that even though vitamin D dampens expression of both chemokines and interferon driven genes, whose role is to limit the spread of a virus, viral load is not increased in the presence of vitamin D. This indicates that vitamin D may dampen the inflammatory response to viruses without negatively affecting viral replication (Fig. 7).

Airway epithelium serves as the first line host defense system against respiratory viral infection. In addition to forming a mechanical barrier, the airway epithelium detects viruses (via pattern recognition receptors) and initiates the host immunological response. Stimulation of NF-κB dependent gene transcription is a central aspect of inflammatory activation by pattern recognition receptors (60). The NF-κB pathway in airway epithelial cells is critical for generation of lung inflammation in response to local or systemic stimuli (61). The epithelial response to viral infections includes the production and secretion of chemokines, type I interferons (IFNβ), and liberation of antimicrobial peptides (17,62,63).

Several epidemiological studies have found an association between low vitamin D levels and respiratory infections (10,11). It is well established that vitamin D modulates both innate and adaptive immune responses (3,64). Vitamin D has been found to induce the antimicrobial peptide cathelicidin in several cell types including airway epithelium (12,65,66). Animal studies suggest that vitamin D may dampen production of chemokines. In a mouse model of multiple sclerosis, vitamin D was found to reverse experimental autoimmune encephalitis by inhibiting synthesis of chemokines (22). Similar effects were found by two different groups using a mouse model for diabetes. Nonobese diabetic mice treated with vitamin D were found to have a decrease in pancreatic islet chemokine expression, which was accompanied by less insulitis and inhibition of type 1 diabetes development (23,24). To our knowledge, this study is the first study to look at the effects of vitamin D on host defense against viral infection.

Vitamin D has been found to regulate NF-κB activity by increasing IκBα levels and/or decreasing DNA binding of NF-κB in keratinocytes, fibroblasts, macrophages, and kidney cells (32–34,67,68). Furthermore, two studies, using mouse models have linked NF-κB inhibition by vitamin D analogs with down-regulation of chemokines and inhibition of

inflammation (23,69). Using a mouse model of type 1 diabetes, Giarratana et al., found that a vitamin D analog up-regulated IκBα, and decreased in vivo and in vitro proinflammatory chemokine production by pancreatic islet cells. This resulted in the inhibition of T cell recruitment into pancreatic islets and type 1 diabetes development. In a mouse model of obstructive nephropathy, Tan et al., showed that paricalcitol, a vitamin D analog, reduced expression of chemokines and inhibited inflammatory cell infiltration in the obstructed kidney via VDR-mediated sequestration of NF-κB signaling. Our study is the first study to investigate the effects of vitamin D on NF-κB signaling in airway epithelium.

The airway epithelium is the major site of RSV infection and plays an important role in initiating antiviral and inflammatory response against the virus. Although a number of cell types secrete chemokines in inflamed tissue, a body of evidence supports a central role for the airway epithelium as an important initiator and modifier of pulmonary inflammation (44,70). This is particularly true for RSV since the virus productively replicates only in the respiratory mucosa. Studies have shown that RSV is among the most potent biologic stimuli to induce chemokine production by respiratory epithelial cells, a process that is largely controlled by activation of NF-κB (71–75). Chemokines can be beneficial or detrimental to the host. Recruitment and activation of inflammatory cells is an essential component of host defense, however, too vigorous response against microbes may be deleterious leading to impaired organ function and potential mortality. Inflammatory chemokines have indeed been implicated as a contributor to the pathogenesis and severity of RSV infection (76–79). Therefore modulating RSV-induced chemokines may have the potential to prevent excessive inflammation. Attempts to find effective antiinflammatory therapies for this condition have been largely unsuccessful thus far. Several clinical trials have looked at using corticosteroids, but the results have not been consistent (80). Haeberle et al., treated RSV infected mice with perflubron (inserting it into the lungs) and found reduction in lung inflammation associated with inhibition of chemokine expression and NF-κB (75). Vitamin D provides a novel treatment option that may reduce lung inflammation and disease severity in RSV infection.

If vitamin D suppresses IFNβ induction and, subsequently, interferon driven genes which have diverse antiviral activities, then how is the antiviral state maintained? One possibility is that the suppression of interferon beta and its products is insignificant and sufficient amounts of antiviral effectors remain to successfully limit viral replication and spread. Another possibility is that induction of antimicrobial peptides, with antiviral activity, by vitamin D counteracts the reduction in interferon driven antiviral genes. We have shown that vitamin D induces cathelicidin in airway epithelium (12). Others have found that vitamin D increases beta defensin-2 (66) which was recently found to have antiviral activity against RSV (81).

Here we show in an in vitro model of primary human airway epithelial cells that even though 1,25D decreases viral (RSV) induction of chemokines and IFNβ, and subsequently interferon driven genes, there is no increase in viral replication. Controlling the inflammatory response to RSV viral infection, while maintaining antiviral activity, may result in decreased disease severity and consequently in decreased morbidity and mortality from this common infection. Vitamin D is safe, cheap, easily available, and could prove to be an effective therapeutic strategy, however, clinical studies are needed.

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The abbreviations used are

1,25D	1,25-dihydroxyvitamin D3
RSV	respiratory syncytial virus
hTBE	human tracheobronchial epithelial
qRT-PCR	quantitative reverse transcriptase polymerase chain reaction
ISG	interferon stimulated gene
IRF	interferon regulatory factor
ISRE	interferon stimulated response element
ISGF3	interferon stimulated gene factor 3
MxA	myxovirus resistance A
VDR	vitamin D receptor

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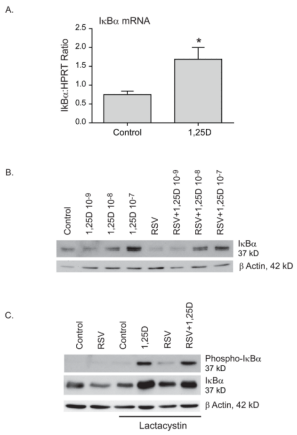

FIGURE 1.

Vitamin D increases expression of the NF-κB inhibitory protein IκBα. *A.* hTBE cells were treated with 1,25D 10^{-7} for 36 h and IκBα mRNA measured by qRT-PCR. Vitamin D increases IκBα mRNA. *B.* hTBE cells pretreated with increasing doses of 1,25D for 16–18 h and then infected with RSV (MOI 1-2) for 24 h. Vitamin D increases IκBα protein in a dose dependent manner. Less IκBα protein is seen in RSV infected cells compared with non-infected cells. *C.* hTBE cells pretreated with 1,25D 10^{-7} followed by infection with RSV (MOI 1-2) for 24 h. The proteasome inhibitor lactacystin (10 μM) was added for the last 3 h of the experiment. 1,25D increased both total and phosphorylated IκBα suggesting that induction of IκBα by 1,25D is due to increased production of IκBα rather than inhibition of its degradation. Paired Student *t*-test. * $p<0.05$ when c/w control.

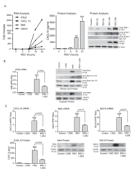

FIGURE 2.

Vitamin D decreases RSV induction of IFNβ and interferon stimulated genes. A. hTBE cells were infected with RSV for 3,6,16 and 24 h. Induction of IFNβ, ISGs, and chemokines was evaluated using qRT-PCR, ELISA and Western blot. Maximum induction was observed at 24 h. *B.* hTBE cells were pretreated with 1,25D 10^{-7} for 16–18 h and then infected with RSV (MOI 1-2) for 24 h. INFβ mRNA was quantified with qRT-PCR. Total STAT1, phosphorylated STAT1 at tyrosine 701 and serine 927 and nuclear STAT1 were observed using Western blot. Vitamin D decreased RSV induced IFNβ mRNA and RSV induction of STAT1 and its nuclear translocation. *C.* hTBE cells were pretreated with vitamin D and infected with RSV as in *B.* CXCL10, MxA and ISG15 mRNA were evaluated with qRT-PCR. CXCL10 protein was measured with ELISA and MxA and ISG15 protein by Western blot. Vitamin D decreased RSV induction of the chemokine CXCL10 and, to a lesser extent, the antiviral effectors MxA and ISG15. ANOVA with Dunnett's test for comparisons with control (*A*) and Bonferroni's test for multiple comparisons (*B,C*). ** $p<0.01$, *** $p<0.001$ when c/w control.

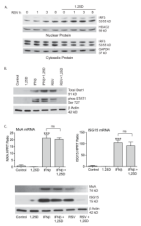

FIGURE 3.

The effects of vitamin D occur at the level of NF-κB. *A.* hTBE cells infected with RSV (MOI 1-2) for 1, 3 or 8 h in the presence or absence of pretreatment with 1,25D 10^{-7}M. Western blot for nuclear and cytosolic IRF-3. Vitamin D does not decrease nuclear localization of IRF-3. *B.* hTBE cells pretreated with 1,25D 10^{-7}M for 16–18 h and then stimulated with IFNβ (1000U) or infected with RSV (MOI 1-2) for 24 h. STAT1 induction was looked at with Western blot. Vitamin D does not effect IFNβ induced STAT1. C. hTBE cells treated as in *B.* and expression of MxA and ISG15 mRNA and protein were evaluated with qRT-PCR and Western blot, respectively. Vitamin D did not influence IFNβ induced MxA or ISG15.

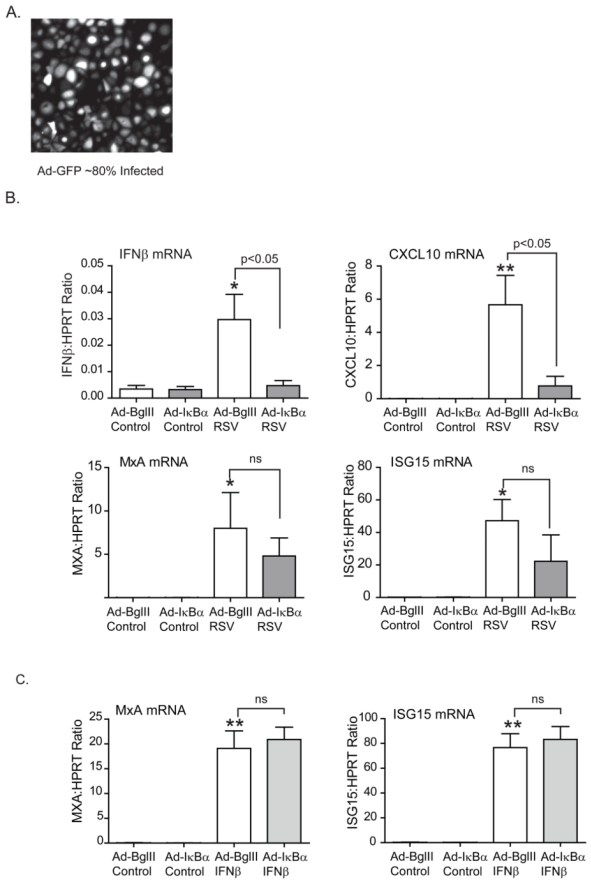

FIGURE 4.

Overexpressing IκBα mimics vitamin D effects. *A.* hTBE cells were infected with GFP labelled adenovirus vector (MOI 10) for 24 h and transgene expression assessed with fluorescent microscopy. About 80% of the cells were found to express the GFP labelled adenovirus vector. *B.* and *C.* hTBE cells were infected with either empty adenovirus vector or an adenovirus vector overexpressing IκBα (MOI 10) for 24 h and then infected with RSV (MOI 1-2) or stimulated with IFNβ (1000U) for 24 h. IFNβ, CXCL10, MxA and ISG15 mRNA was quantified using qRT-PCR. Like 1,25D, the AdIκBα vector significantly reduced RSV induction of IFNβ and CXCL10. There was a trend towards a decrease in the RSV induced antiviral effectors, MxA and ISG15, which did not reach statistical significance. Like vitamin D, the AdIκBα vector had no effects on IFNβ induced MxA and ISG15. ANOVA with Bonferroni's test for multiple comparisons. * $p<0.05$ and ** $p<0.01$ when c/w control.

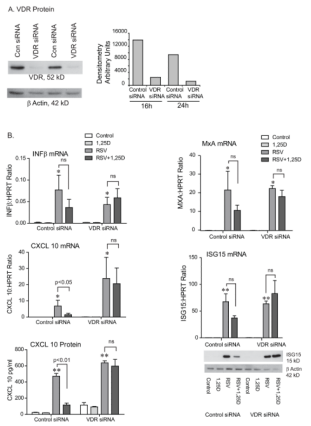

FIGURE 5.

Silencing the vitamin D receptor abolishes the effects of vitamin D on RSV induced IFNβ, chemokines, and interferon stimulated genes. *A*. hTBE cells were transfected with control siRNA or VDR siRNA for 16 or 24 h and VDR protein looked at with Western blot. The VDR siRNA successfully knocked down the VDR. *B*. hTBE cells were transfected with control siRNA or VDR siRNA for 24 h, pretreated with 1,25D for 16–18 h, and then infected with RSV (MOI 1-2) for 24 h. IFNβ, CXCL10, MxA and ISG15 mRNA were evaluated with qRT-PCR, CXCL10 protein with ELISA, and ISG15 protein with Western blot. Vitamin D reduced the expression of IFNβ, CXCL10, MxA and ISG15 in cells transfected with control siRNA, but had no effects in cells transfected with VDR siRNA. ANOVA with Bonferroni's test for multiple comparisons. * $p<0.05$ and ** $p<0.01$ when c/w control.

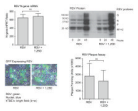

FIGURE 6.

Vitamin D does not jeopardize viral clearance. hTBE cells were infected with RSV (MOI 1-2) with or without pretreatment with 1,25D 10^{-7} M for 16–18 h. Viral quantity and viral replication was analyzed by RT-PCR, Western blot, light microscopy of GFP-tagged RSV (blue=Dapi nuclear stain) and plaque assay. Vitamin D had no effects on the quantity or replication of RSV. Unpaired Student *t* test.

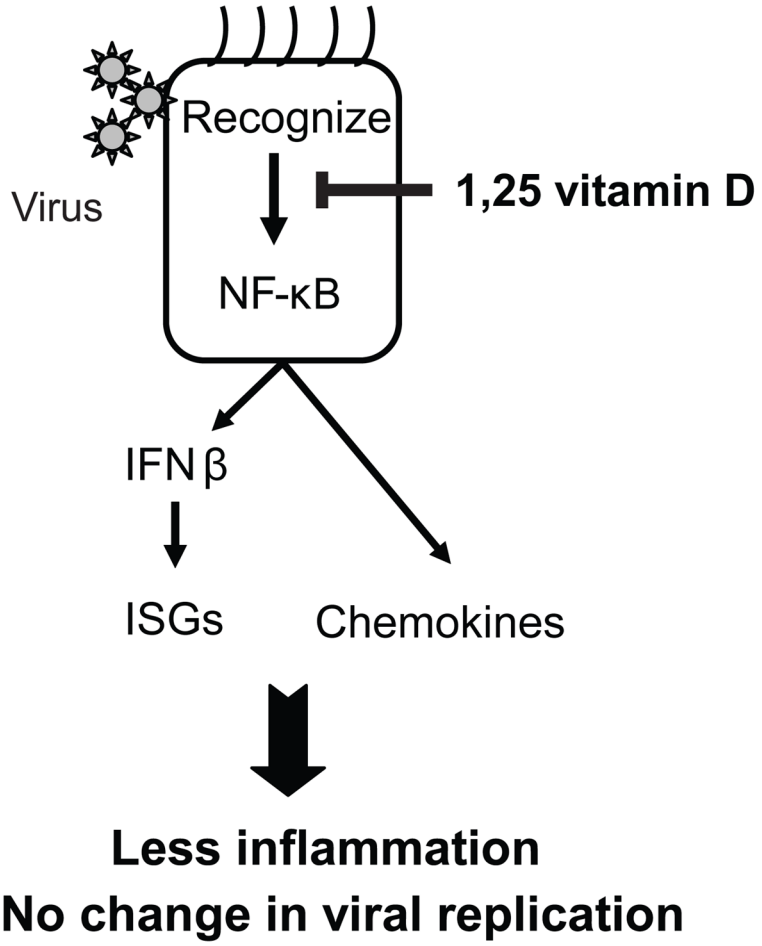

Figure 7.
Vitamin D decreases RSV induction of NF-κB-linked chemokines and cytokines in airway epithelium while maintaining the antiviral state. Respiratory epithelium recognizes viruses via pattern recognition receptors. Ligand engagement results in activation of NF-κB and NF-κB linked genes including IFNβ and chemokines. Vitamin D modulates the airway epithelial responses to RSV by inhibiting NF-κB signaling. In spite of dampening the innate antiviral response, vitamin D does not jeopardize viral clearance.