Vitamin D Induces Global Gene Transcription in Human Corneal Epithelial Cells: Implications for Corneal Inflammation

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Submitted: January 28, 2016
Accepted: April 9, 2016

Citation: Reins RY, Mesmar F, Williams C, McDermott AM. Vitamin D induces global gene transcription in human corneal epithelial cells: implications for corneal inflammation. *Invest Ophthalmol Vis Sci*. 2016;57:2689–2698. DOI:10.1167/iovs.16-19237

Purpose. Our previous studies show that human corneal epithelial cells (HCEC) have a functional vitamin D receptor (VDR) and respond to vitamin D by dampening TLR-induced inflammation. Here, we further examined the timing of the cytokine response to combined vitamin D–TLR treatment and used genome-wide microarray analysis to examine the effect of vitamin D on corneal gene expression.

Methods. Telomerase-immortalized HCEC (hTCEpi) were stimulated with polyinosinic-polycytidylic acid (poly[I:C]) and 1,25-dihydroxyvitamin D_3 ($1,25D_3$) for 2 to 24 hours and interleukin (IL)-8 expression was examined by quantitative (q)PCR and ELISA. Telomerase-immortalized HCEC and SV40-HCEC were treated with $1,25D_3$ and used in genome-wide microarray analysis. Expression of target genes was validated using qPCR in both cell lines and primary HCEC. For confirmation of IκBα protein, hTCEpi were treated with $1,25D_3$ for 24 hours and cell lysates used in an ELISA.

Results. Treatment with $1,25D_3$ increased poly(I:C)-induced IL-8 mRNA and protein expression after 2 to 6 hours. However, when cells were pretreated with $1,25D_3$ for 24 hours, $1,25D_3$ decreased cytokine expression. For microarray analysis, 308 genes were differentially expressed by $1,25D_3$ treatment in hTCEpi, and 69 genes in SV40s. Quantitative (q)PCR confirmed the vitamin D–mediated upregulation of target genes, including nuclear factor of κ light polypeptide gene enhancer in B-cells inhibitor, α (IκBα). In addition to increased transcript levels, IκBα protein was increased by 28% following 24 hours of vitamin D treatment.

Conclusions. Microarray analysis demonstrates that vitamin D regulates numerous genes in HCEC and influences TLR signaling through upregulation of IκBα. These findings are important in dissecting the role of vitamin D at the ocular surface and highlight the need for further research into the functions of vitamin D and its influence on corneal gene expression.

Keywords: vitamin D, cornea, inflammation, toll-like receptors, microarray

The cornea is the anterior covering of the eye that serves to protect the underlying tissues from damage. Along with physical protection from injury, the cornea also defends against infection. Toll-like receptors (TLRs) are pattern-recognition receptors which stimulate an innate immune response upon contact with pathogen-associated molecular patterns.[1] The inflammation resulting from TLR activation leads to recruitment of immune cells, enhanced cytokine, and chemokine production, and initiation of an adaptive immune response to rid the cornea of infection. These inflammatory events triggered by TLRs are dependent on intracellular signaling pathways which lead to the activation of nuclear factor κB (NF-κB), activator protein-1 (AP-1), signal transducer and activator of transcription (STAT), and interferon regulatory factor (IRF) transcription factors.[2,3] In particular, NF-κB is recognized as the key regulator of inflammatory signaling, enhancing proinflammatory cytokine levels and also influencing cell survival.[2,4,5] In addition to acting in a paracrine fashion to influence surrounding cells, proinflammatory cytokines produced by TLR signaling also act in an autocrine feedback loop to further enhance cytokine production and the cellular stress response.[6] Mediators such as interleukin 1 β (IL-1β) and tumor necrosis factor (TNF), for example, are produced by TLR activation and in turn lead to increased NF-κB activation and activation of the mitogen-activated protein kinases (MAPK) pathway.

Inflammatory signals must be regulated and kept in check in order to prevent tissue damage. This is especially true in the cornea, where damage can result in loss of transparency, essential for vision. Toll-like receptors are present at the ocular surface, protecting against infection, but have also been implicated in the pathogenesis of dry eye disease (DED), an inflammatory condition that affects millions of individuals in the United States.[7,8] Patients with DED experience ocular pain, discomfort, burning, and dryness that can result in visual

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Table 1. Primer Sequences Used for qPCR

Gene Name	Forward Primer	Reverse Primer	NCBI Ref. Seq.
GAPDH	5′-GACCACAGTCCATGCCATCA-3′	5′-CATCACGCCACAGTTTCCC-3′	NM_002046
CYP24A1	5′-TTCTCTGGAAAGGGGGTCTC-3′	5′-GTGCACCGACTCAAAGGAAC-3′	NM_000782
DUSP10	5′-ATCTTGCCCTTCCTGTTCCT-3′	5′-ATTGGTCGTTTGCCTTTGAC-3′	NM_007207.5
SERPIN B1	5′-GCCGGATGACATTGAGGACG-3′	5′-AGGTTTAGTCCACTCATGCAAC-3′	NM_030666.3
ILIRL1	5′-ATGGGGTTTTGGATCTTAGCAAT-3′	5′-CACGGTGTAACTAGGTTTTCCTT-3′	NM_001282408
IGFBP3	5′-AGAGCACAGATACCCAGAACT-3′	5′-GGTGATTCAGTGTGTCTTCCATT-3′	NM_000598
IKBA	5′-GACCACACTGCGCCAACAC-3′	5′-CTTCTCCACAACCCTCTGCAC-3′	NM_020529
TRAF4	5′-CAGGAGAGTGTCTACTGTGAGA-3′	5′-CCACACCACATTGGTTGGG-3′	NC_000017.11
CXCL2	5′-CTCAAGAATGGGCAGAAAGC-3′	5′-AAACACATTAGGCGCAATCC-3′	NM_002089
CXCL3	5′-AAAATCATCGAAAAGATACTGAACAAG-3′	5′-GTAAGGGCAGGGACCAC-3′	NM_002090
MAP2K6	5′-TATGGCGCACTGTTTCGGG-3′	5′-CCGAGAGCATTGATGAGTACATT-3′	NM_002758
TGFB2	5′-ATCCTGCATCTGGTCACGGTC-3′	5′-CTTGGCGTAGTACTCTTCGTC-3′	NM_001135599
IL-8	5′-GACCACACTGCGCCAACAC-3′	5′-CTTCTCCACAACCCTCTGCAC-3′	NM_000584
TNFα	5′-TGGAGAAGGGTGACCGACTC-3′	5′-TCCTCACAGGGCAATGATCC-3′	NM_000594.3
CAMP (LL-37)	5′-GGACAGTGACCCTCAACCAG-3′	5′-AGAAGCCTGAGCCAGGGTAG-3′	NM_004345.4
CD14	5′-GAGCTCAGAGGTTCGGAAGAC-3′	5′-GCTGAGGTTCGGAGAAGTTG-3′	NM_000591
DUSP1	5′-AGTACCCCACTCTACGATCAGG-3′	5′-GAAGCGTGATACGCACTGC-3′	NM_004417
DUSP4	5′-GGTCATCGTCTACGACGAGCGCAG-3′	5′-CGGAGGAAAACCTCTCATAGCCGC-3′	NM_001394.6
DUSP5	5′-TGTCGTCCTCACCTCGCTA-3′	5′-GGGCTCTCTCACTCTCAATCTTC-3′	NM_004419

disturbances and disruption of daily activities.[9,10] Therefore, an important area of research is the development of new anti-inflammatory therapeutics that limit aberrant ocular surface inflammation. Vitamin D (1,25-dihydroxyvitamin D_3) has been widely studied for its role in suppressing inflammation in other tissues, and therefore is a good candidate to evaluate at the ocular surface.[11–16] The multifunctional hormone has been implicated in general ocular health and has been shown to be protective during various ocular diseases and pathologies, such as macular degeneration, uveitis, and myopia.[17] However, its transcriptional regulation still needs to be evaluated throughout the eye.

Vitamin D acts through its receptor, the VDR, a member of the nuclear hormone receptor family which acts as a potent transcriptional regulator.[18] The vitamin D receptor is expressed in the human cornea and the enzymes that activate vitamin D are also present in human corneal epithelial cells (HCECs), suggesting that vitamin D has a functional role in the cornea.[19–22] Vitamin D is able to regulate the innate immune response to infection and inflammation through interactions with TLR signaling[23–27] and enhances host protection through antimicrobial peptide production.[28–31] Our previous studies have shown that HCEC respond to vitamin D treatment through decreased production of TLR-induced cytokines and matrix metalloproteinases.[19] Therefore, vitamin D has the potential to be an important regulator of inflammation at the ocular surface.

The goal of the current study was to examine the influence of vitamin D on TLR signaling further and to elucidate its effect on global gene expression in HCEC, adding to the current knowledge of vitamin D function in the cornea. Here, we analyze the timing of the vitamin D–mediated response to TLR activation and use microarray analysis to identify pathways that are involved in this response. This study will yield important information when considering vitamin D as a potential therapeutic for ocular surface inflammation or corneal infection.

Materials and Methods

Cell Culture and Treatment

Immortalized and primary cultures of HCECs were used in these studies. We used KGM-2 (Lonza, Allendale, NJ, USA) and SHEM media to culture telomerase-immortalized human corneal epithelial cells (hTCEpi) and SV40-immortalized (SV40) HCECs, respectively.[32,33] Human donor corneas were received from Saving Sight eye bank (St. Louis, MO, USA) and primary HCEC isolated following overnight corneal digestion with protease (dispase II; Roche Diagnostics, Indianapolis, IN, USA).[34] The optimal range of circulating serum vitamin D ($25D_3$) is between 30 and 80 ng/mL (75–200 nM).[35,36] Therefore, cells were treated with a physiologically relevant concentration of vitamin D for these studies, 10^{-7} M (100 nM, $1,25D_3$; Sigma-Aldrich Corp., St. Louis, MO, USA). In addition, cells were treated with 1 μg/mL TLR3 agonist polyinosinic-polycytidylic acid (poly[I:C]; Invivogen, San Diego, CA, USA) for the indicated times. Polyinosinic-polycytidylic acid is a double-stranded RNA analog, which activates TLR3, as would occur during a viral infection, resulting in NF-κB activation. For the nuclear factor of κ light polypeptide gene enhancer in B-cells inhibitor, α (IκBα) study, hTCEpi were pretreated with 10 μM Bay 11-7082 (Invitrogen, Carlsbad, CA, USA) for 30 minutes before poly(I:C) addition for 6 hours.

RNA Isolation and Quantitative Real-Time PCR

We extracted total RNA from cultured cells using TRIzol reagent (Life Technologies, Grand Island, NY, USA) and a purification and isolation kit (RNeasy kits with DNase I treatment; Qiagen, Valencia, CA, USA). Concentration of RNA was measured with a spectrophotometer (Nanodrop 2000; Thermo Fisher Scientific, Wilmington, DE, USA) and cDNA was transcribed with a cDNA synthesis kit (AffinityScript; Agilent Technologies, Santa Clara, CA, USA). Quantitative PCR was performed using a master mix (Brilliant II SYBR Green QPCR; Agilent Technologies) and intron-spanning primers (Table 1). Untreated samples served as the calibrator for relative quantity determination and all samples were normalized to the housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

Sample Preparation and Microarray Analyses

We treated hTCEpi and SV40 cell lines with $1,25D_3$ (10^{-7} M) or vehicle (0.01% ethanol/PBS) for 6 hours and RNA was collected as above. Biological replicates and dye-swapped samples were used in a genome-wide microarray analysis performed using

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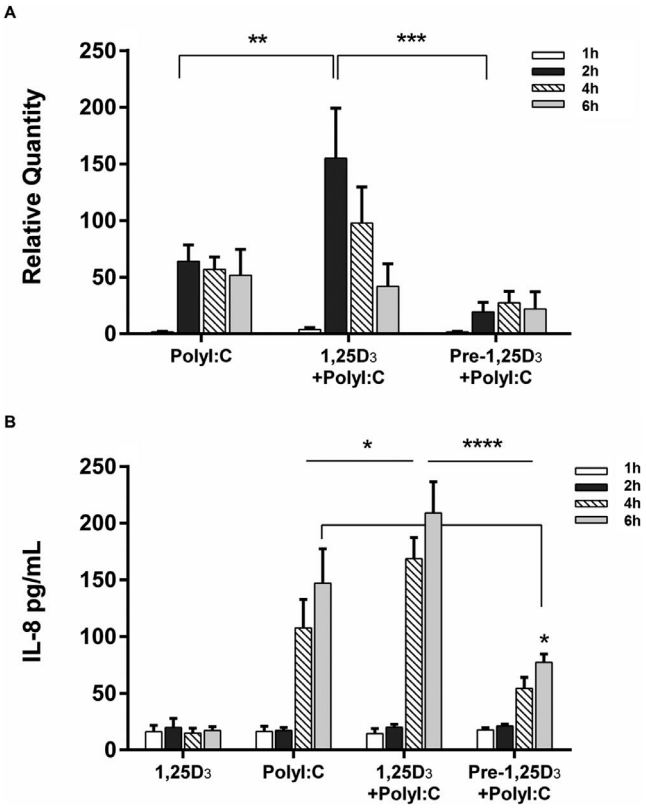

FIGURE 1. Vitamin D affects poly(I:C)-induced IL-8 expression. Telomerase-immortalized HCEC were treated with 10^{-7} M $1,25D_3$ for 2, 4, or 6 hours, or pretreated with $1,25D_3$ for 24 hours prior to poly(I:C) addition (pre-$1,25D_3$). We collected RNA from cell lysates for qPCR analysis of IL-8 expression (**A**) and protein expression was determined in supernatants by ELISA (**B**). Data represent mean ± SEM of four independent experiments. Statistical analysis was by ANOVA with Bonferroni's test for multiple comparisons. $^{*}P < 0.05$. $^{**}P = 0.01$. $^{***}P = 0.001$. $^{****}P = 0.0001$.

microarrays (Human Op-Arrays; Microarrays, Inc., Huntsville, AL, USA). Each array contained 35,035 oligonucleotide probes corresponding to 25,100 unique genes and the analysis was performed as previously described.[37–39] In brief, following RNA isolation, sample quality was assessed with a bioanalyzer (Agilent 2100; Agilent Technologies, Palo Alto, CA, USA). All samples of RNA had integrity values of 10, indicating negligible degradation. Indirect labeling of cDNA was performed using aminoallyl-modified nucleotides (aa-dUTP+dNTP, Sigma-Aldrich Corp.), 15 μg of total RNA, and random hexamer primers (Invitrogen, Life Technologies). After purification (MinElute columns; Qiagen), fluorophores Cy3 and Cy5 (Amersham, GE Healthcare, Pittsburgh, PA, USA) were coupled to cDNA, repurified, and concentrations verified. Dye-swapped samples were pooled and applied to prehybridized microarray slides with coverslips and allowed to hybridize for 38h at 42°C. After washing, dried slides were read on a microarray scanner (GenePix 4000B; Molecular Devices, Sunnyvale, CA, USA) at 10 μm resolution. Tagged image file format images were analyzed with commercial software (GenePix Pro 6.0; Molecular Devices). After flagging incomplete or missing spots, gene pix files were analyzed in R software, version 2.9.1, using the Limma package, to identify differentially expressed genes with vitamin D treatment. Median intensities for Cy3 and Cy5 in each spot were converted to M values ($\log_2$ [$1,25D_3$/control]), and cutoff values established at the absolute value of $M > 0.4$, corresponding to fold change values of greater than 1.3. An additional cutoff was made based on P values < 0.05, which were calculated in the R statistical environment using the empirical Bayes moderate t-test.[40] Microarray data was deposited in National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) data repository under accession numbers GSE72662 and GSE72663.

Gene Ontology Classification

Functional groups and gene annotation clusters in differentially expressed genes were identified using the online Database for Annotation, Visualization, and Integrated Discovery (DAVID; available in the public domain at http://david.abcc.ncifcrf.gov)[41,42] and Protein Analysis Through Evolutionary Relationships (http://www.pantherdb.org),[43] as well as Pathway Studio (Elsevier, Inc., Amsterdam, The Netherlands). Values of $P < 0.05$ were considered significant and were determined using Fisher's exact test. Values measure the probability that the number of differentially regulated genes that fall within a particular gene ontology or functional group is due to chance, based on the distribution of genes in the whole genome.

Protein Detection in Cell Lysates and Supernatants

Interleukin 8 was measured in cell supernatants by ELISA, following stimulation with $1,25D_3$ and poly(I:C) for the indicated times, per the manufacturer's instructions (human IL-8 ELISA MAX; BioLegend, San Diego, CA, USA). For total IκBα and phospho-p38α (T180/Y182) protein determination, cell lysates were collected in lysis buffer #6 (1 mM EDTA, 0.5% Triton X-100, 5 mM NaF, 6 M urea, 1 mM sodium orthovanadate, 2.5 mM sodium pyrophosphate, 10 μg/mL leupeptin, 10 μg/mL, pepstatin, 100 μM PMSF, 3 μg/mL aprotinin in PBS) and assayed with ELISA kits (DuoSet; R&D Systems, Minneapolis, MN, USA). Total protein concentrations from supernatants and lysates were quantified by BCA protein assay (Life Technologies) and all data were normalized to total cellular protein concentration.

RESULTS

Vitamin D Affects Poly(I:C)-Induced IL-8 Expression in a Time-Dependent Manner

Previous studies have indicated that vitamin D plays a role in dampening proinflammatory cytokine production in HCEC.[19] In these studies, treatment with vitamin D for 24 hours significantly decreased the expression of IL-8, IL-1β, IL-6, TNFα, and CCL20 induced by TLR3 agonist poly(I:C), a potent activator of inflammatory signaling. However, early activation of NF-κB (after 2 hours of treatment) was not affected by vitamin D. Therefore, here we further explored the effect of $1,25D_3$ on poly(I:C)-mediated cytokine production, focusing on early time points, to evaluate the initial effect of vitamin D. Costimulation with poly(I:C) and $1,25D_3$ for 2 to 6 hours showed a significant increase in IL-8 production above the expression induced by poly(I:C) alone (Fig. 1). This increase was significant at the mRNA level after 2 hours, with a jump from a 64-fold increase in IL-8 expression with poly(I:C) to a 155-fold increase when $1,25D_3$ was added (Fig. 1A; $P < 0.01$). Concentrations of IL-8 in cell supernatants were also increased at 4 and 6 hours of combined treatment, yielding an increase of approximately 50 pg/mL above poly(I:C) alone (Fig. 1B; $P < 0.05$). However, at the 6-hour time point, IL-8 mRNA expression with combined treatment was no different than with poly(I:C) alone, suggesting that at this time, vitamin D starts to lower IL-8 expression. When we pretreated hTCEpi

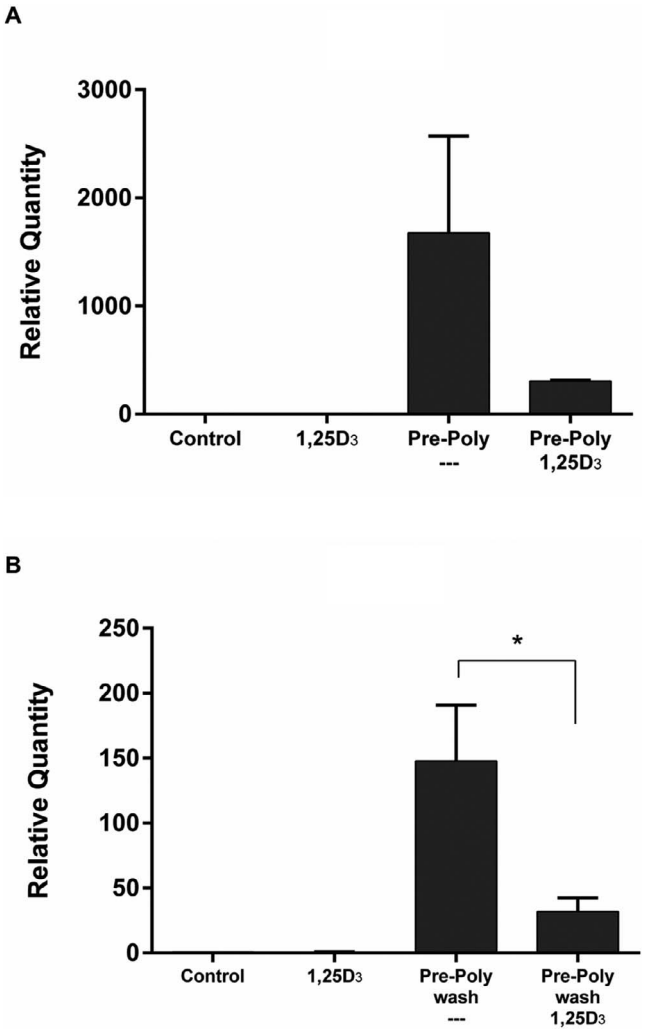

FIGURE 2. Vitamin D reduces IL-8 expression after poly(I:C) pretreatment. We stimulated hTCEpi with 1 μg/mL poly(I:C) for 4 hours and 10^{-7} M $1,25D_3$ was added without washing (**A**) or with washing to remove poly(I:C) (**B**), before culturing for an additional 24 hours. We collected RNA from cell lysates for qPCR analysis of IL-8 expression. Data represent the mean ± SEM of three independent experiments. Statistical analysis was by ANOVA with Bonferroni's test for multiple comparisons. * $P < 0.05$.

with $1,25D_3$ 24 hours before poly(I:C) stimulation, there was a significant reduction in IL-8 expression (50% at 6 hours; $P < 0.05$, Fig. 1).

We next examined the effect of vitamin D when administered after poly(I:C) treatment. When hTCEpi were stimulated with poly(I:C) for 4 hours and then treated with $1,25D_3$ for an additional 24 hours, there was an ~80% decrease in IL-8 expression (Fig. 2A). There was a similar reduction in IL-8 expression when cells were pretreated with poly(I:C) and then washed before $1,25D_3$ addition, indicating that vitamin D does not physically interfere with poly(I:C), disrupting its ability to initiate signaling events (Fig. 2B; $P < 0.05$). These results suggest that vitamin D treatment is able to attenuate IL-8 levels even when poly(I:C) is administered first. However, during the immediate response to poly(I:C), vitamin D cotreatment increases IL-8 expression (Fig. 1).

Vitamin D Regulates Multiple Genes in Corneal Epithelial Cells

In order to further examine the influence of vitamin D on corneal epithelial cell gene expression and its influence on inflammatory events, genome-wide microarray analysis was performed. First, a time course of vitamin D treatment was performed, examining the expression of several genes known to be regulated by $1,25D_3$,[19] to determine an optimal time point for analysis. The hydroxylase CYP24A1, antimicrobial peptide LL-37, and lipopolysaccharide coreceptor CD14 all exhibited maximum transcript levels 6 hours after vitamin D treatment in hTCEpi (Fig. 3). Therefore, for the gene array, cells were treated with $1,25D_3$ for 6 hours and gene expression was compared to vehicle-treated control cells. To determine the optimal concentration, primary HCEC were treated with physiologically relevant concentrations of vitamin D and LL-37 gene expression determined. Based on these data, 10^{-7} M (40 ng/mL) $1,25D_3$ was chosen for this study (Supplementary Fig. S1). Two corneal epithelial cell lines, hTCEpi and SV40 HCEC, were used in the microarray comparison to determine vitamin D–induced gene regulation.

We identified 308 genes in hTCEpi as differentially expressed at 6 hours of vitamin D treatment; while in SV40s, 69 genes were changed. Of these 69 genes, 24 genes (35%) where changed in both cell lines (Fig. 4, Table 2). In both cell lines, the majority of regulated genes were upregulated (77% in hTCEpi and 70% in SV40). Only one gene was downregulated in both hTCEpi and SV40 cells: histone H4 (Table 2). The regulation with the lowest P value in both cell lines was

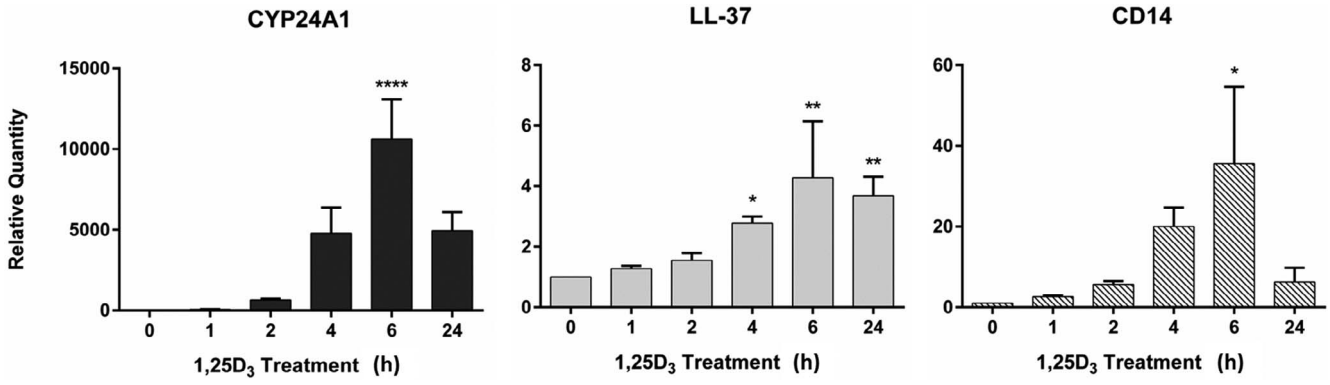

FIGURE 3. Time course analysis of CYP24A1, LL-37, and CD14 expression after vitamin D treatment. We stimulated hTCEpi with 10^{-7}M $1,25D_3$ or vehicle control (0.01% ethanol/PBS) for the indicated times and RNA was collected for qPCR analyses. Data represent the mean ± SEM of three to four independent experiments. Statistical analysis was by ANOVA with Bonferroni's test for multiple comparisons. *$P < 0.05$. **$P = 0.01$. ****$P = 0.0001$.

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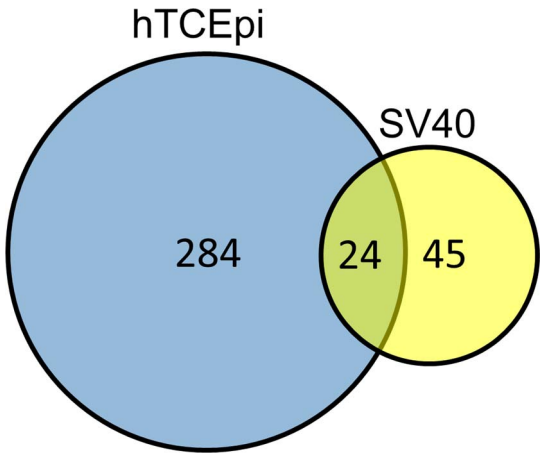

Figure 4. Differential expression of genes between hTCEpi and SV40 cell lines. We found hTCEpi had a greater number of genes regulated by vitamin D treatment than SV40 (308 compared with 69, respectively), with 24 genes in common.

upregulation of the CYP24A1 gene, the cytochrome p450 enzyme that inactivates $25D_3$ and $1,25D_3$ through 24-hydroxylation.

In order to classify the 24 genes that were commonly regulated by $1,25D_3$ in hTCEpi and SV40 cells, enriched biological functions were identified using DAVID software. The largest process influenced by vitamin D was intracellular signaling ($P = 0.0002$), which included genes involved in both protein kinase cascades and GTPase-mediated signal transduction. Interestingly, several genes were identified that play a role in negatively regulating signal transduction, including insulin-

Table 3. Biological Processes Enriched Among Vitamin D–Regulated Genes in hTCEpi

Biological Process	Genes, *n*	*P* Value
Intracellular signaling cascade	47	2.10×10^{-8}
Cell surface receptor linked signal transduction	44	3.80×10^{-3}
Regulation of cell proliferation	36	1.30×10^{-8}
Regulation of apoptosis	33	7.30×10^{-7}
Positive regulation of macromolecule metabolic process	32	8.00×10^{-6}
Response to organic substance	31	6.70×10^{-7}
Positive regulation of biosynthetic process	29	3.10×10^{-6}
Phosphate metabolic process	28	2.20×10^{-3}
Response to wounding	27	1.80×10^{-7}
Regulation of transcription from RNA polymerase II promoter	25	3.60×10^{-4}
Regulation of phosphorylation	24	8.80×10^{-7}
Homeostatic process	24	1.30×10^{-3}
Positive regulation of nucleic acid metabolic process	23	2.60×10^{-4}
Phosphorylation	22	1.20×10^{-2}
Cell cycle	21	1.70×10^{-2}
Response to endogenous stimulus	20	1.70×10^{-5}

The top biological functions influenced by vitamin D in hTCEpi were identified using DAVID software. The list is arranged by descending order by number of genes in each process. Values of $P < 0.05$ were considered significant.

Table 2. Genes That Are Regulated by Vitamin D Treatment in Both hTCEpi and SV40 Cell Lines

Gene Symbol	Gene Name	hTCEpi *P* Value	SV40 *P* Value
Upregulated			
CYP24A1	1,25-dihydroxyvitamin D(3) 24-hydroxylase	2.86×10^{-11}	4.50×10^{-11}
IL1RL1	Interleukin 1 receptor-like 1; ST2	1.65×10^{-8}	2.50×10^{-5}
GEM	GTP-binding protein GEM	8.83×10^{-8}	3.25×10^{-6}
SEMA6D	Semaphorin-6D	9.48×10^{-8}	1.47×10^{-5}
TXNRD1	Thioredoxin reductase 1	5.51×10^{-7}	0.00387
CLCF1	Cardiotrophin-like cytokine factor 1	1.23×10^{-6}	4.66×10^{-5}
SERPINB1	Serpin peptidase inhibitor	2.38×10^{-6}	0.00017
EFTUD1	Elongation factor Tu GTP binding domain 1	3.42×10^{-6}	8.93×10^{-6}
DUSP10	Dual specificity phosphatase 10	7.88×10^{-6}	0.000274
MAFB	V-maf fibrosarcoma oncogene homolog B	8.86×10^{-6}	0.00254
ZNF114	Zinc finger protein 114	9.04×10^{-6}	0.000645
TMEM40	Transmembrane protein 40	1.46×10^{-5}	0.001698
KLK6	Kallikrein-related peptidase 6	4.14×10^{-5}	1.34×10^{-6}
RHOF	Ras homolog family member F	5.87×10^{-5}	0.003357
NET1	Neuroepithelial cell transforming 1	5.95×10^{-5}	0.003533
RNF149	E3 ubiquitin-protein ligase ring finger protein	0.000114	0.001842
IGFBP3	Insulin-like growth factor binding protein 3	0.000423	0.000598
Q8NHV5-2	(uncharacterized)	0.000585	0.001842
G6PD	Glucose-6-phosphate dehydrogenase	0.000785	0.004424
CREG2	Cellular repressor of E1A-stimulated genes 2	0.000823	4.42×10^{-5}
RASD2	GTP-binding protein rhes	0.00086	0.002113
PGM2L1	Phosphoglucomutase 2-like 1	0.001226	0.003013
NFKBIA	Nuclear factor of κ light polypeptide gene enhancer in B-cells inhibitor, α	0.0013760	0.0046
Downregulated			
HIST1H4J	Histone cluster 1, H4j	0.000651	0.004772

Genes are listed in order of increasing *P* value for hTCEpi cells, determined with the empirical Bayes *t*-test using R statistical software.

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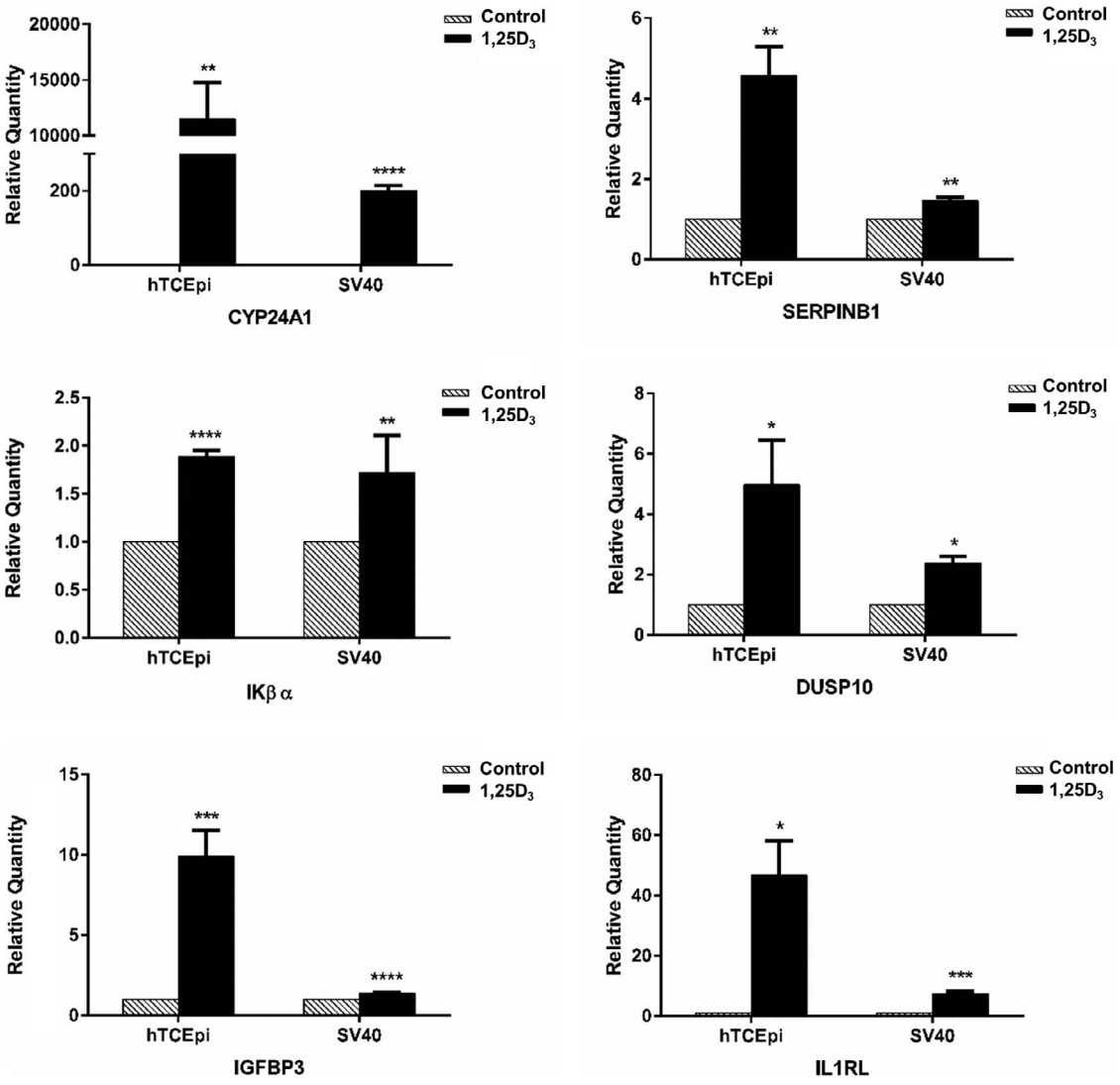

Figure 5. Validation of target genes by qPCR analysis. We treated hTCEpi and SV40 cells with 10^{-7} M $1,25D_3$ for 6 hours and RNA was harvested for qPCR analyses. Data represent the mean ± SEM of three to four independent experiments. Statistical analysis was by Student's *t*-test. $^{*}P < 0.05$. $^{**}P = 0.01$. $^{***}P = 0.001$. $^{****}P = 0.0001$.

like growth factor binding protein 3 (IGFBP3), interleukin 1 receptor-like 1 (IL1RL1 or ST2), and NFKBIA (IκBα). Importantly, when classifying regulated genes by molecular function and cellular compartment, calcium-binding proteins and calcium channel activity were enriched, known areas of vitamin D influence. In addition, the biological function termed "response to vitamin D" was significantly enriched ($P = 0.0002$).

We found hTCEpi cells had a larger number of genes differentially expressed upon vitamin D treatment, indicative of being more responsive. Upon examination of VDR expression, hTCEpi had higher relative receptor expression, possibly explaining the increased responsiveness (data not shown). Thus, this cell line was specifically examined for enriched biological processes among the regulated genes (Table 3). Similar to the gene set in common with SV40s, the largest process influenced by vitamin D was intracellular signaling, which included 47 genes ($P = 2.10 \times 10^{-8}$). Vitamin D–regulated genes were also highly enriched among the processes cell proliferation, apoptosis, wounding, and transcription.

Microarray analyses are useful as a guide to identify changes in the transcriptome based on cell type or treatment. However, changes discovered from arrays must be validated with a complementary technology. Therefore, six genes identified as regulated in both cell lines were examined by real-time quantitative (q)PCR analyses (Fig. 5). These results confirmed the array data. It is also clear that the amplitude of the response, while significant in both cell lines, was higher in hTCEpi cells, explaining the larger number of genes detected by microarray for this cell line. Further, expression of these genes was evaluated in primary HCEC, from two different donors. The pattern of vitamin D regulation was the same as in the cell lines (Fig. 6), confirming the in vivo relevance of the experimental setup.

Vitamin D Attenuates Poly(I:C) Inflammatory Signaling Through Upregulation of NF-κB Inhibitor IκBα

In order to identify how vitamin D could be influencing TLR inflammatory pathways, enriched pathways were examined for genes involved in both TLR signaling and MAPK signaling (Table 4). Engagement of TLR results in an inflammatory

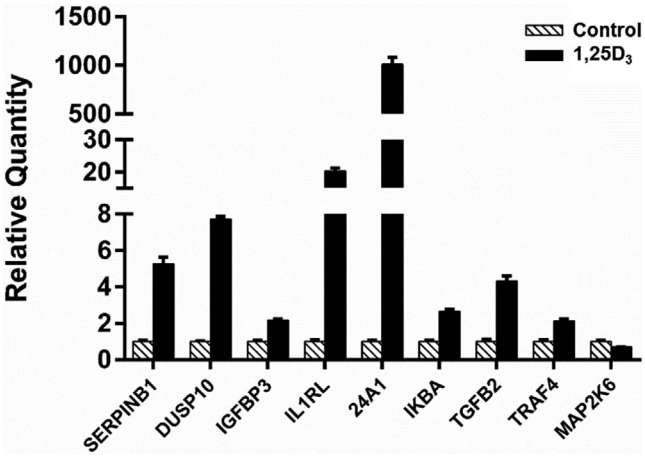

Figure 6. Confirmation of microarray results in primary HCEC. Primary HCECs were isolated from donor corneas and cultures were treated with 10^{-7} M $1,25D_3$ for 6 hours. We collected RNA from cell lysates for qPCR analyses. Graphs reflect data from one of two corneal donors and data are mean ± SEM of triplicate values (24A1, cytochrome P450 hydroxylase CYP24A1).

cascade that activates members of the MAPK and NF-κB pathways, leading to the production of proinflammatory cytokines and proteins involved in the cellular stress response as well as cell survival.

Vitamin D treatment significantly upregulated NFKBIA (IκBα) in both hTCEpi and SV40, as well as in primary HCEC (Table 3, Figs. 5, 6). Nuclear factor of κ light polypeptide gene enhancer in B-cells inhibitor, α is a negative regulator of NF-κB signaling.[5] It sequesters NF-κB in the cytoplasm, preventing nuclear translocation and transcriptional effects. In addition to increased IκBα transcript levels, protein expression of IκBα was also increased by 28% following 24 hours of vitamin D treatment (Fig. 7). Thus, vitamin D treatment increases IκBα, potentially inhibiting NF-κB signaling.

Upon TLR activation, signaling events lead to IκBα phosphorylation, which releases NF-κB and results in IκBα degradation.[2,4] Therefore, we decided to investigate if IκBα was important in poly(I:C)-induced IL-8 expression. When phosphorylation of IκBα was inhibited by an irreversible inhibitor of IκBα phosphorylation—Bay 11-7082—there was a significant 41% decrease in IL-8 production upon poly(I:C) stimulation (Fig. 8A). This demonstrates that the NF-κB pathway is activated through IκBα phosphorylation and this is important for poly(I:C)-mediated cytokine production. Further, 24-hour vitamin D pretreatment significantly increased the expression of total IκBα protein in hTCEpi treated with poly(I:C) (Fig. 8B), suggesting that vitamin D could potentially attenuate poly(I:C) inflammatory signaling via upregulation of the NF-κB inhibitor IκBα.

Discussion

Our prior studies have indicated that vitamin D is able to attenuate TLR-induced inflammatory signals in corneal epithelial cells.[19] In the current study, we explored how vitamin D treatment affects genome-wide gene regulation and TLR3-induced IL-8 expression in these cells, further examining the influence of vitamin D on corneal inflammation. Our findings demonstrate that while direct vitamin D treatment at early time points (6 hours) enhances poly(I:C)-induced IL-8 levels, 24-hour vitamin D pretreatment significantly attenuates this pathway. Microarray findings show that vitamin D regulates multiple genes in HCEC, including genes involved in MAPK and NF-κB pathways, important signaling pathways in TLR-induced inflammation.

Table 4. Genes Regulated by Vitamin D Involved in MAPK Signaling and TLR Signaling Pathways

Gene Symbol	Gene Name
MAPK signaling pathway	
DUSP1	Dual specificity phosphatase 1
DUSP10	Dual specificity phosphatase 10
DUSP4	Dual specificity phosphatase 4
DUSP5	Dual specificity phosphatase 5
GADD45A	Growth arrest and DNA-damage-inducible, α
IL1B	Interleukin 1, β
MAPK13	Mitogen-activated protein kinase 13
MAP2K6	Mitogen-activated protein kinase kinase 6
PDGFA	Platelet-derived growth factor α polypeptide
TGFB2	Transforming growth factor, β 2
FOS	V-fos FBJ murine osteosarcoma viral oncogene homolog
TLR signaling pathways	
IL1B	Interleukin 1, β
MAPK13	Mitogen-activated protein kinase 13
MAP2K6	Mitogen-activated protein kinase kinase 6
NFKBIA	Nuclear factor of κ light polypeptide gene enhancer in B-cells inhibitor, α
PIK3R3	Phosphoinositide-3-kinase, regulatory subunit 3 (gamma)
TLR4	Toll-like receptor 4
FOS	V-fos FBJ murine osteosarcoma viral oncogene homolog

To identify hTCEpi genes, we used DAVID bioinformatics, gene ontology Kyoto Encyclopedia of Genes and Genomes pathway.

The time-course data indicate that the ability of vitamin D to suppress cytokine production is a delayed effect. In the case of inflammation, this could be beneficial: Cytokine production and inflammatory events such as neutrophil infiltration following TLR ligation aid in the resolution of infection or injury. During pathogenic challenge, the inflammatory process is necessary for the ultimate elimination of microbial threat. However, too much inflammation is harmful and a dampening of the response is necessary to restore tissue homeostasis following the insult, preventing chronic inflammation and tissue damage. Therefore, the initial effect of vitamin D to

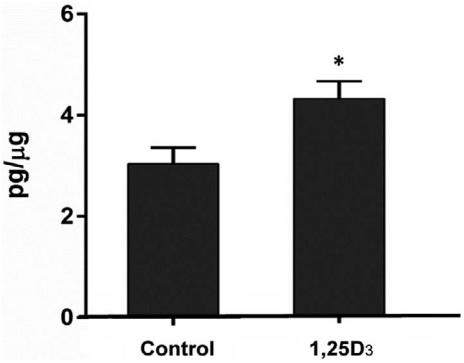

Figure 7. Protein IκBα increases with vitamin D treatment. We treated hTCEpi with 10^{-7} M $1,25D_3$ for 24 hours and total IκBα protein expression was measured in cell lysates by ELISA. Data represent the mean ± SEM of 5 independent experiments. Statistical analysis was by Student's *t*-test. $^*P < 0.05$.

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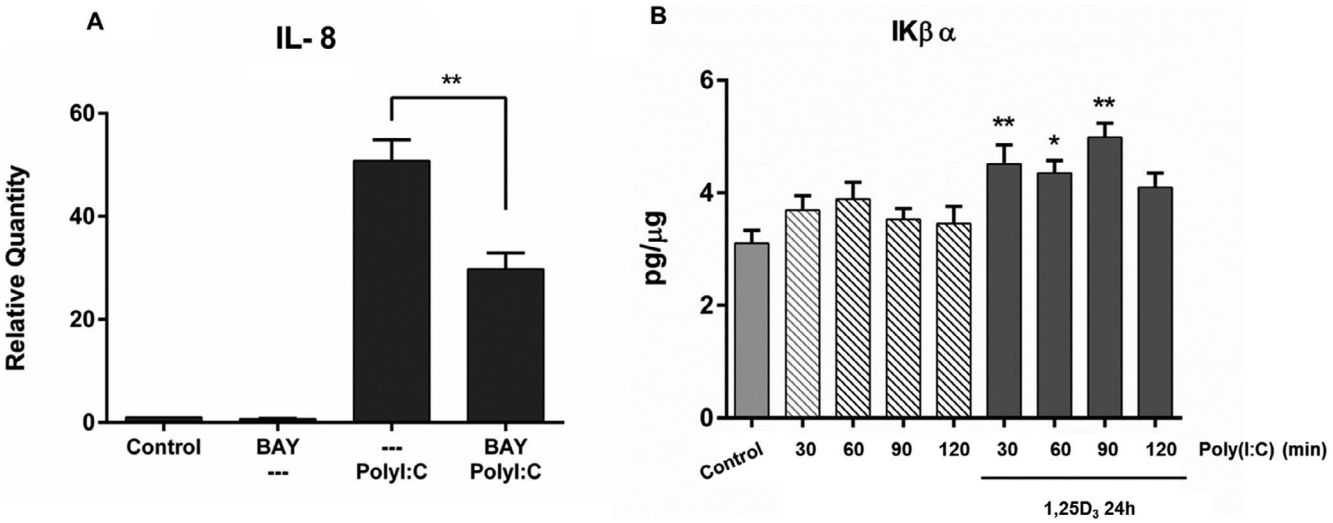

Figure 8. Inactivation of IκBα is important in poly(I:C)-induced IL-8 expression and total IκBα is upregulated by vitamin D during poly(I:C) stimulation. (**A**) We pretreated hTCEpi with Bay 11-7082 for 30 minutes followed by poly(I:C) treatment for 6 hours. Data represent the mean ± SEM of 3 independent experiments. (**B**) We pretreated hTCEpi with 10^{-7} M $1,25D_3$ for 24 hours and stimulated with poly(I:C) for the indicated times. Total protein expression of IκBα was measured in cell lysates by ELISA. The data represent the mean ± SEM of four to six independent experiments. Statistical analysis was by ANOVA with Bonferroni's test for multiple comparisons. $^{*}P < 0.05$. $^{**}P = 0.01$.

increase IL-8 expression could help to jumpstart inflammatory events and then its delayed action may suppress ongoing, potentially harmful cytokine production. Vitamin D was also able to decrease IL-8 levels when the cells were pretreated with poly(I:C), further indicating that it is able to suppress inflammatory signals after their initiation. This is an important finding considering a potential therapeutic use of vitamin D for treatment of ongoing inflammatory conditions.

Nuclear factor κB is a transcription factor that regulates the expression of inflammatory genes. It is activated upon TLR ligation as well as by cytokine signaling. Following TLR activation, signaling events result in phosphorylation of IκBα, which results in its targeting for degradation, hence allowing NF-κB to translocate to the nucleus and activate transcription.[4] Our microarray analysis identified IκBα to be upregulated by vitamin D treatment in both hTCEpi and SV40 cell lines, which was confirmed in primary HCEC as well. During poly(I:C) stimulation, pretreatment with vitamin D also raised IκBα levels, thereby increasing the cell's capacity for inhibiting NF-κB activation during an inflammatory stimulus. Other studies have demonstrated that increasing IκBα transcription and translation decreases NF-κB translocation to the nucleus.[44] In human peritoneal macrophages, vitamin D suppressed NF-κB activation through increasing IκBα expression, resulting in reduced TLR-induced TNFα production.[45] In addition, basal levels of IκBα were lower in cells from mice lacking VDR, increasing the amount of NF-κB in the nucleus of resting cells.[46] Therefore, we suggest that the vitamin D–mediated attenuation of poly(I:C)-induced IL-8 expression in corneal cells is due at least in part from its upregulation of IκBα expression.

Another candidate pathway for the influence of vitamin D on cytokine production is the MAPK family of signaling kinases. Multiple dual specificity protein phosphatase (DUSP) genes were upregulated by vitamin D treatment, as highlighted by the microarray data and confirmed by qPCR (DUSP1, 4, and 5; Table 4, Supplementary Fig. S2A). Dual specificity protein phosphatases negatively regulate signal transduction through dephosphorylating threonine and tyrosine residues on members of the MAPK pathway, rendering them inactive.[47] Dual specificity protein phosphatases preferentially regulate p38 and JNK (c-jun NH2-terminal kinases), the stress-activated MAPKs, leading to downregulation of cytokines and proinflammatory genes.[48] The anti-inflammatory effects of glucocorticoids have been associated with DUSP expression[49] and mice deficient in DUSP have increased levels of cytokines.[50] In particular, we found that DUSP10 expression was increased in hTCEpi, SV40, and primary HCEC (Supplementary Fig. S2A). Dual specificity protein phosphatase 10, also called MKP5, dampens inflammatory signals through its actions on p38, which has been shown to induce cytokine production.[50–53] However, in our study, despite increasing DUSP expression, vitamin D did not decrease p38 phosphorylation. Instead, there was an increase in p-p38 with vitamin D (Supplementary Fig. S2B). Investigating this apparent paradox further, we found evidence that p38 can have dual functions, having both pro- and anti-inflammatory roles depending on the cell type and stimulus involved. Activation of p38 has in fact been shown to increase DUSP expression, limiting the cycle of inflammation.[54] The influence of vitamin D on these pathways needs to be further dissected in order to determine if increased DUSP expression modulates inflammation through p38.

The potential for vitamin D to modulate inflammatory signaling pathways has important clinical relevance in the cornea, not only during infectious keratitis, as previously mentioned, but also for chronic ocular surface inflammation, such as occurs in DED. Dry eye disease is a multifactorial condition that is a significant public health concern, affecting millions of individuals each year in the Unites States alone, causing ocular irritation, burning, pain, and dryness.[7,9,10] Inflammation is involved in the pathogenesis of DED and there have been many studies showing that there is an increase in tear, corneal, and conjunctival proinflammatory cytokines, chemokines, and matrix metalloproteinases during disease.[55–58] Therefore, anti-inflammatory therapeutics have shown promise in the treatment of DED.[59–61] Specifically, MAPK signaling pathways have been shown to be activated during experimental dry eye, with an increase in JNK, ERK, and p38 phosphorylation in the cornea and conjunctiva during desiccating stress.[59,62] The ability of vitamin D to modulate these inflammatory signaling pathways make it a good

therapeutic candidate to evaluate in the treatment of DED and other ocular inflammatory disorders.

In examining the microarray results, we found that the response to vitamin D between the two HCEC cell lines was very different. Baseline levels of gene expression in hTCEpi and SV40 have not been compared and warrant further investigation, based on this differential response. Transcriptome analysis should be performed to compare hTCEpi and SV40 cells with primary HCEC, to determine which cell line most closely resembles nonimmortalized corneal epithelial cells. Our data suggest that gene expression changes in hTCEpi more closely resemble the response of primary cells to vitamin D, based on relative fold changes. However, a separate microarray analysis would be interesting to directly compare similarities and differences among primary cultured HCEC and cell line gene expression. These results would be important to consider when choosing a cell line for in vitro studies.

In summary, we show that vitamin D has a strong influence on gene expression in HCECs. This regulation modulates inflammatory cytokine production, in a time-dependent manner. We propose that the vitamin D–mediated upregulation of IκBα contributes to the dampening of later inflammatory responses. In addition, other genes identified in the microarray could be important in the role of vitamin D in regulating inflammatory signaling, such as *IL1RL*, *GADD45*, *TRAF4*, *TGFB2*, and *MAP2K6*, and will be interesting to investigate further.

Acknowledgments

The authors would like to thank Marisa Simon for her assistance with the GEO submission.

Supported by National Institutes of Health Grant EY13175 (AMM); University of Texas/University of Houston College of Optometry Training Grant T32 07024 (RYR); University of Houston College of Optometry Core Grant EY07551; and Marie Curie Actions FP7-PEOPLE-2011-COFUND (GROWTH 291 795) via the VINNOVA program Mobility for Growth (CW).

Disclosure: **R.Y. Reins**, None; **F. Mesmar**, None; **C. Williams**, None; **A.M. McDermott**, None

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