FUNCTION OF VITAMIN D AT THE OCULAR SURFACE AND ITS ROLE DURING CORNEAL INFLAMMATION

A Dissertation Presented to

the Faculty of the Department of Biology and Biochemistry

University of Houston

In Partial Fulfillment

of the Requirements for the Degree

Doctor of Philosophy

By

Rose Y. Reins

August 2015

FUNCTION OF VITAMIN D AT THE OCULAR SURFACE AND ITS ROLE DURING CORNEAL INFLAMMATION

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ABSTRACT

Although first recognized for its role in calcium regulation and bone health, there has been an explosion of research demonstrating that vitamin D is an important modulator of the immune system. In the eye, studies have shown that deficiencies in vitamin D and genetic differences in vitamin D-related genes have a significant impact on the development of various ocular diseases. Vitamin D has been used effectively as a treatment for inflammatory conditions in many tissues, as well as in the eye, attenuating the progression of uveitis, for example. However, up until now, few studies have examined the role of vitamin D at the front part of the eye, the cornea. Maintenance of corneal transparency is essential for vision, and therefore, limiting inflammatory events is beneficial in this tissue.

The work contained in this dissertation demonstrates that vitamin D is activated by corneal epithelial cells and is able to regulate gene expression in these cells, including enhancing antimicrobial peptide production. Vitamin D also modulates the inflammatory response to pattern recognition receptor stimulation, by increasing initial cytokine levels and then acting to dampen pro-inflammatory signals later in the response. *In vivo*, the use of topical vitamin D was explored both during experimental dry eye and in a mouse model of corneal wound healing. Although vitamin D increased tear production in normal mice, a protective effect on the ocular surface, wound healing was delayed and inflammatory signs augmented with vitamin D treatment after epithelial abrasion. These studies fill a gap in knowledge about vitamin D at the ocular surface and highlight the

need for research into the immunomodulatory functions of the hormone in the ultimate goal of using vitamin D therapeutically for corneal inflammation.

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LIST OF ABBREVIATIONS

1,25D$_3$ 1,25-dihydroxyvitamin D_3

25D$_3$ 25-hydroxyvitamin D_3

AAU acute anterior uveitis

AMD age-related macular degeneration

AP-1 activator protein-1

APC antigen presenting cells

CYP24A1 24-hydroxylase

CYP27A1, CYP2R1 25-hydroxylase

CYP27B1 1-alpha-hydroxylase

DAVID Database for Annotation, Visualization, and Integrated Discovery

DED dry eye disease

DHCR7 7-dehydrocholesterol reductase

DR diabetic retinopathy

EAU experimental autoimmune uveitis

EDE experimental dry eye

ELISA enzyme-linked immunosorbent assay

hBD-2 human beta defensin 2

HCEC human corneal epithelial cells

hTCEpi human telomerase-immortalized corneal epithelial cells

HTRA1 high-temperature requirement factor A1

IGFBP3 insulin-like growth factor binding protein 3

IL- interleukin

IL1RL1 interleukin 1 receptor-like 1, ST2

IOP intraocular pressure

IRF interferon response factor

LL-37 human cathelicidin

LPS lipopolysaccharide

MAPK mitogen-activated protein kinases

MMP matrix metalloproteinase

NFKBIA nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor

NFκβ nuclear factor kappa B

P. Aeruginosa *Pseudomonas aeruginosa*

PRR pattern recognition receptor

RIG-1 retinoic acid-inducible gene 1

RXR retinoid X receptor

SNP single-nucleotide polymorphism

TLR Toll-like receptor

UV(B) ultraviolet radiation (B)

VDR vitamin D receptor

VDRE vitamin D response element

VEGF Vascular endothelial growth factor

Chapter 1

Introduction

Chapter includes:

Reins, R.Y., McDermott, A.M., 2015. Vitamin D: Implications for ocular disease and therapeutic potential. Exp. Eye Res. 134, 101–110.

1.1. Vitamin D: An Overview

Although first identified for its role in calcium homeostasis, vitamin D is now recognized to have many diverse functions including effects on immune regulation, proliferation, differentiation, apoptosis, and angiogenesis (Plum and DeLuca, 2010; Prietl et al., 2013). Vitamin D directly or indirectly regulates up to 5% of the human genome, or over 900 different genes (Wang et al., 2005). In addition, the vitamin D receptor (VDR) is almost ubiquitously expressed (Bouillon et al., 2008). The enzyme necessary for conversion of vitamin D to its functional metabolite has been identified in a number of cell types, which are able to utilize circulating vitamin D to form the biologically active hormone. Extrarenal activation, expression, and gene influence suggest that vitamin D function is widespread, with pleiotropic effects within the tissue microenvironment where it is activated.

Vitamin D was initially discovered in the early 1900's as an unknown substance in cod liver oil that was able to cure rickets, a defect in bone mineralization (McCollum et al., 2002; Mellanby, 1989). It was demonstrated that rickets could also be reversed by sunlight exposure or by foods exposed to ultraviolet light (Steenbock, 1924). It was further noted, that specifically, a cholesterol-like molecule in the skin, could produce the anti-rachitic compound through UV irradiation (Hess et al., 1925). This research led to the identification of the structure of vitamin D in the 1930's (Askew et al., 1931; Windaus et al.,1936; Wolf, 2004) and more studies dissecting the role of vitamin D in bone health. Vitamin D is now known to be an important regulator for maintaining

calcium levels, both through increasing absorption of calcium and phosphate in the intestine and inducing bone resorption to keep blood calcium concentrations optimal. In concert with parathyroid hormone, vitamin D stimulates the differentiation and activation of osteoclasts, enhancing calcium mobilization from bone (Bar-Shavit et al., 1983; Suda et al., 2003; van Leeuwen et al., 2001) and increasing renal reabsorption when circulating calcium is low (DeLuca, 2004; Sutton and MacDonald, 2003; Yamamoto et al., 1984).

Vitamin D is obtained from two sources: dietary consumption (fatty fish, fortified foods, supplementation, for example) and local production in the skin (Kamen and Tangpricha, 2010; White, 2008). Exposure to ultraviolet B radiation (UVB 290-315nm) generates pre-vitamin D and then vitamin D (D_3 or cholecalciferol) from 7-dehydrocholesterol through a series of thermal and photochemical reactions in the epidermis. Subsequently, vitamin D undergoes two hydroxylation steps to form the active hormone (Kamen and Tangpricha, 2010; Schauber and Gallo, 2008) (Figure 1.1).

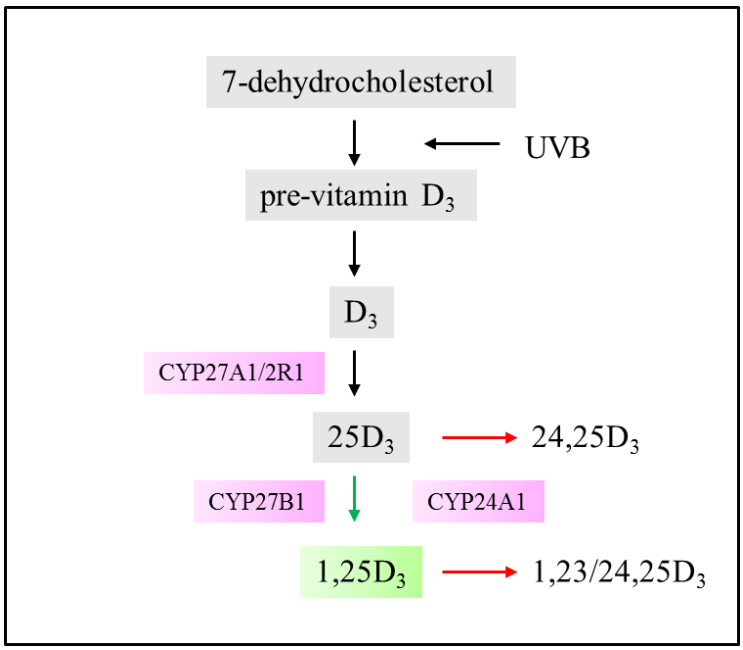


Figure 1.1. Vitamin D activation. Overview of the metabolism of vitamin D_3.

In the liver, vitamin D is hydroxylated to 25-hydroxyvitamin D_3 ($25D_3$ or calcidiol), the primary circulating form, by cytochrome p450 enzymes CYP2R1 and CYP27A1, located in the inner mitochondrial membrane (Bouillon et al., 2008; Jones et al., 1998). Further modification to form the fully functional 1,25-dihydroxyvitamin D_3 ($1,25D_3$ or calcitriol) classically occurs in the kidneys, mediated by 1-alpha-hydroxylase, CYP27B1; however, there are an increasing number of extrarenal tissues that are being identified that are also able to activate vitamin D. 24-hydroxylation, catalyzed by CYP24A1, regulates vitamin D metabolite levels through catabolism of both $25D_3$ and $1,25D_3$, leading to inactivation or the excretion of the 24-hydroxylated products, such as calcitroic acid (Jones et al., 2014; Prosser and Jones, 2004) (Figures 1.1, 1.2).

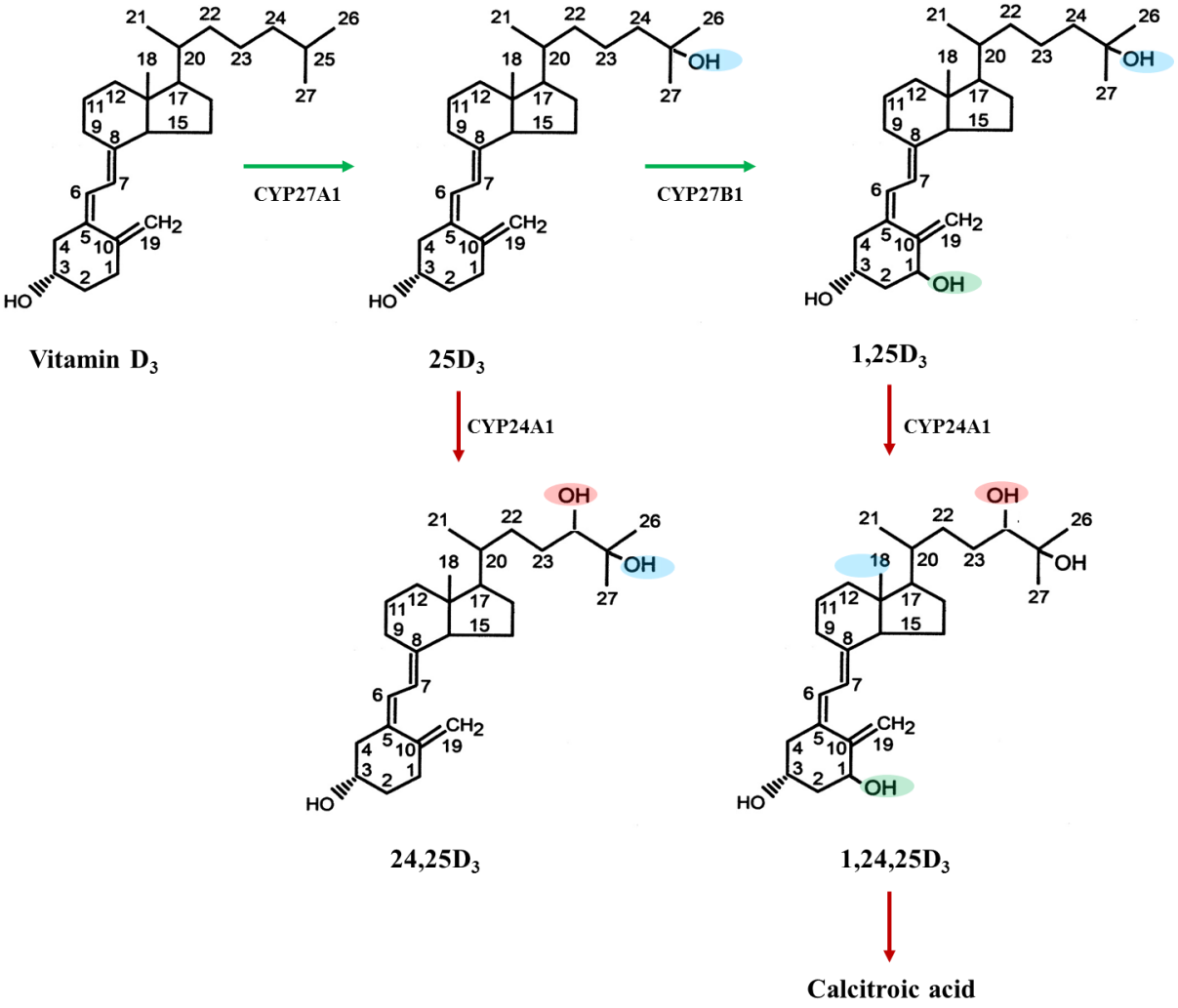


Figure 1.2. Vitamin D metabolism and structures.

After production in the skin or absorption from diet, vitamin D_3 is converted to $25D_3$ through hydroxylation at carbon-25 in the liver by the cytochrome p450 enzyme CYP27A1. $25D_3$ is further hydroxylated by CYP27B1, in the kidney and extrarenal sites, to form the functional $1,25D_3$, which then binds and activates the VDR in many tissues**.** 24-hydroxylation, catalyzed by CYP24A1 results in inactivation, leading to excretion of vitamin products. Original structure modified and permission obtained for use from DeLuca, 2004.

Upon activation, $1,25D_3$ binds to its receptor, the VDR. The VDR is a member of the nuclear hormone receptor family, and shares similar structural and functional domains (Figure 1.3). Following ligand binding, VDR heterodimerizes with retinoid X receptor (RXR) and acts as a transcription factor, binding to both positive and negative vitamin D response elements (VDRE) in target genes. VDREs are located in promoter regions and generally contain direct hexameric repeats separated by a 3 base-pair spacer (Thompson et al., 2002; White, 2008). The VDR, bound to VDRE, then recruits co-activators, mediator complex, and chromatin-modifying enzymes to influence RNA polymerase II binding and gene transcription (Kim et al., 2005; Rachez and Freedman, 2000).

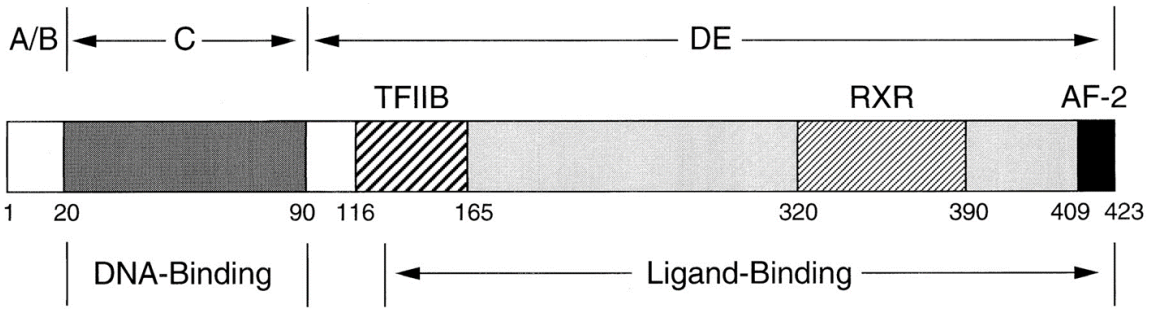


Figure 1.3. Structure of the VDR.

The structure of VDR shares homology to other members of the nuclear receptor family of transcription factors. The N-terminal domain (A/B) is followed by the DNA-binding domain (C), which binds to VDRE in target promoters. A hinge region (D) connects the DNA-binding domain to the ligand-binding domain (E), which, in addition to binding to $1,25D_3$, contains the site for heterodimerization with RXR. At the C-terminus is an activation function (AF-2) domain, which is able to interact with coactivators upon ligand binding. Figure from Jones et al., 1998, with permission.

In addition to regulating transcriptional events related to calcium balance in the kidney proximal tubules, the traditional site of activation, vitamin D is now recognized to affect many cellular functions in a variety of tissues. Extrarenal production was first recognized in patients with sarcoidosis, who had elevated levels of $1,25D_3$, and pulmonary alveolar macrophages were discovered to be capable of producing this active vitamin D (Adams et al., 1983; Barbour et al., 1981). The placenta was also recognized, around this time, to be able to produce $1,25D_3$ (Tanaka et al., 1979). CYP27B1, 1-alpha hydroxylase, was subsequently cloned and discovered in keratinocytes, where vitamin D activation was found to be necessary for normal cell differentiation (Bikle et al., 2004a, 2004b; Fu et al., 1997). Other tissues have since been identified which are also able to activate vitamin D, such as lung, breast, and prostate, where $1,25D_3$ is used locally to influence cellular functions, including proliferation and differentiation (Deeb et al., 2007).

An important function of vitamin D was uncovered with the discovery of the VDR in immune cells. The initial observations of influence on immune function was in macrophages and T cells, which were found to be able to activate vitamin D, using it in an autocrine and paracrine fashion, to affect the local response to inflammation and their own maturation state, leading to increased differentiation of myeloid cells and a reduction of T cell proliferation and cytokine production (Bhalla et al., 1984; Hewison, 2010; Tsoukas et al., 1984, Abe et al., 1981). There has been an explosion of research in the past thirty years surrounding vitamin D's ability to regulate immunity, showing that this is a key function for the hormone. Adams and Hewison (Adams and Hewison, 2012)

even argue that vitamin D's function in mediating inflammation developed before its role in calcium regulation.

Inflammation occurs as a beneficial response to infection, injury, or stressful stimuli. The cardinal signs of inflammation are redness, heat, swelling, pain, and loss of function. Acute inflammatory events, such as immune cell infiltration and cytokine production, are necessary in order to rid the affected tissue of pathogens, in the case of infection, or to aid in the healing response. However, this initial, response must be followed by regulated resolution and dampening of inflammatory signals to prevent chronic inflammation and increased tissue damage (Figure 1.4). Vitamin D has been shown to have a protective effect on the development of chronic inflammation, through suppression of pro-inflammatory cytokines and matrix metalloproteinases, decreasing T-cell proliferation, inhibiting dendritic cell maturation, and enhancement of a T-regulatory response (White, 2008).

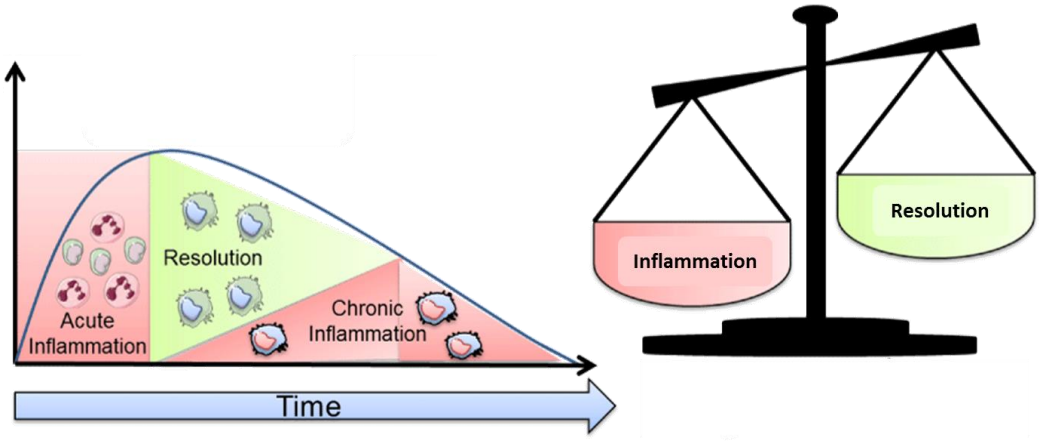


Figure 1.4. Balance of inflammation.

Beneficial acute inflammatory events must be followed by resolution and downregulation of pro-inflammatory signals in order to prevent damaging chronic inflammation (Halade, 2013).

Epidemiological studies highlight vitamin D's importance in maintaining health and point to the now widespread prevalence of vitamin D deficiency (Holick, 2007). Vitamin D status is assessed by measuring circulating $25D_3$ concentration, which has a much longer half-life than $1,25D_3$ (~ 2 weeks versus ~4 hours) and is present at higher circulating concentrations (Holick et al., Endocrine Society, 2011). Although the range of values varies slightly, in general, optimal serum $25D_3$ levels should be between 30-80 ng/mL (75-200 nmol/L), with deficiency/insufficiency recognized to be $25D_3$ <20 ng/mL (50 nmol/L) (Gröber et al., 2013; Holick, 2007; Kennel et al., 2010). The Institute of Medicine, Food and Nutrition Board recommends the adequate intake of vitamin D for most people to be 600IU per day to maintain a healthy status, with an increased intake of 800IU per day for individuals over age 70 (Institute of Medicine (US) Committee to Review Dietary Reference Intakes for Vitamin D and Calcium, 2011).

1.2. Vitamin D and the Eye

There is a growing body of research on locally produced vitamin D and its tissue specific effects (Adams and Hewison, 2012). In the eye, (Figure 1.4) vitamin D target cells were first identified by the presence of vitamin D-dependent calcium binding protein, or calbindin, which was shown to be expressed throughout the human retina (Verstappen et al., 1986). Immunohistochemical staining later identified the presence of VDR in the epithelium of the cornea, lens, ciliary body, and retinal pigment epithelium, as well as the

corneal endothelium, ganglion cell layer, and retinal photoreceptors in the human eye (Johnson et al., 1995). Recently, the presence of vitamin D hydroxylases (CYP27B1, CYP27A1, CYP2R1, and CYP24A1) has been demonstrated in corneal epithelial, endothelial, scleral fibroblasts, nonpigmented ciliary body epithelial, and adult retinal pigment epithelial cell lines (Alsalem et al., 2014; Yin et al., 2011), suggesting that ocular cells have the machinery to activate and regulate vitamin D metabolism. Indeed, most of these cell types were found to be able to convert $25D_3$ into the functionally active $1,25D_3$ (Alsalem et al., 2014). Excitingly, corneal limbal epithelial cells were able to produce vitamin D, *de novo*, in culture when exposed to UVB, similar to cells of the skin (Lin et al., 2012), potentially providing a local source of vitamin D to the ocular surface (Figure 1.5). Other sources of vitamin D in the eye could be the aqueous and vitreous humor and tear film, in which vitamin D metabolites have been shown to be present in rabbits and increase with oral vitamin D supplementation (Lin et al., 2012; Yin et al., 2011).

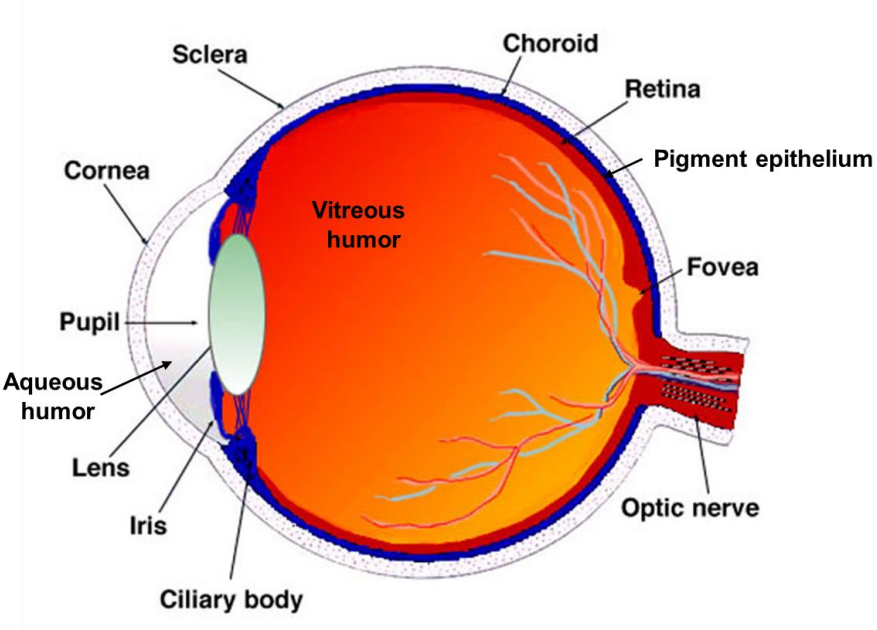


Figure 1.5. Sagittal section of the eye. (Figure from http://webvision.med.utah.edu/)

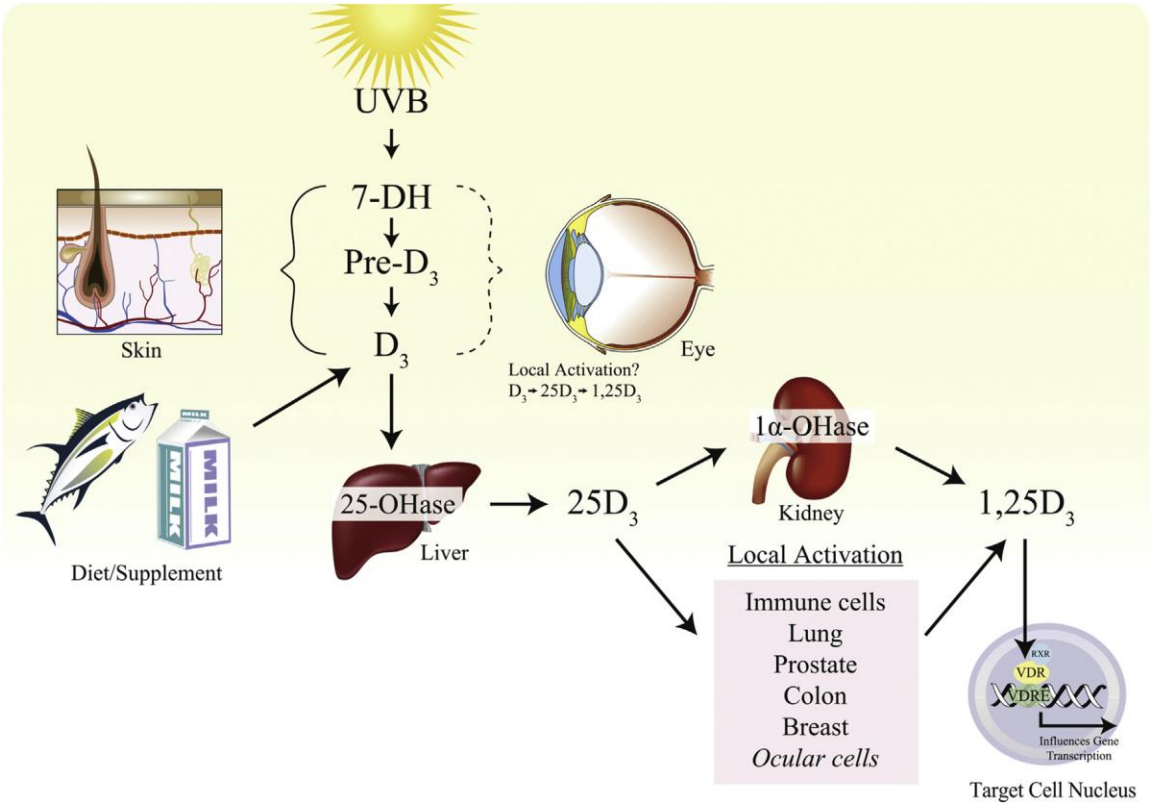


Figure 1.6. Vitamin D production and activation.

UVB from sunlight penetrates the skin, converting 7-dehydrocholesterol (7-DH), to pre-vitamin D_3 (pre-D_3), which then isomerizes to form vitamin D_3 (D_3). Modification through hydroxylation produces $25D_3$, the major circulating form, and subsequently, the active $1,25D_3$. Recent evidence suggests that ocular surface cells also produce vitamin D_3 de novo when exposed to UVB. In addition, many extra-renal tissues and cell types are now recognized to be able to activate $25D_3$, including cells of the eye. (Used with permission from Reins and McDermott, 2015)

With the expression of the receptor and vitamin D regulatory enzymes throughout the eye, studying vitamin D in relation to ocular tissues and pathologies is biologically significant. There is increasing evidence that vitamin D is important in the maintenance of ocular health. The following sections summarize epidemiological and basic science studies on vitamin D (Table 1.1) with a focus on the pathophysiology of various eye diseases and conditions and potential therapeutic roles for this versatile molecule.

Table 1.1.

Summary of studies examining various aspects of vitamin D as it relates to ocular disease, health, and the basic biology of the eye. (Used with permission from Reins and McDermott, 2015)

Vitamin D Status	Low serum $25D_3$ levels associated with disease risk; or high $25D_3$ levels associated with decreased disease prevalence	Myopia[a] Age-related Macular Degeneration[b] Diabetic Retinopathy[c] Dry eye syndrome[d]
Genetic Variations[1]	Gene polymorphisms associated with disease (VDR, CYP24A1, CYP27B1, DHCR7)	Myopia[a] Age-related Macular Degeneration[b] Diabetic Retinopathy[c] Uveitis[f]
Treatment/ Supplementation	Improvement of disease or pathology with either systemic or local vitamin D treatment	Retinoblastoma (mouse)[g] Choroidal Melanoma (mouse)[g] Retinal aging (mouse)[b] Ischemic retinopathy (mouse)[b] Type 2 diabetic retinopathy (rat)[c] Experimental autoimmune uveitis (mouse)[f] Corneal injury (mouse)[d] Corneal transplantation (rat)[d] Corneal neuralgia (human case report)[d] Intraocular pressure (non-human primate)[h]
***In vitro* Cell Studies**	Expression of vitamin D pathway components and/or biological effect of vitamin D treatment	Corneal epithelial cells[d, i] Lens epithelial cells[i] Corneal endothelial cells[i] Scleral fibroblasts[i] Nonpigmented ciliary body epithelial cells[i] Adult retinal pigment epithelial cells[b, i] Ganglion cell layer[i] Retinal photoreceptors[i] Retinoblastoma cells (Y79, Weri-RB1)[g]

a. See 1.2.1. Myopia
b. See 1.2.3. Age-Related Macular Degeneration
c. See 1.2.4. Diabetic Retinopathy
d. See 1.2.7. Ocular Surface Inflammation and Pathology
e. See Table 1.2
f. See 1.2.5. Uveitis
g. See 1.2.2. Retinoblastoma
h. See 1.2.6. Glaucoma
i. See 1.1. Introduction

1.2.1. Myopia

Myopia development is multifactorial, with a combination of genetic and environmental factors playing a role in increased axial elongation. Epidemiological studies have shown that time spent outdoors is protective against myopia development (French et al., 2013; Guggenheim et al., 2012; Rose et al., 2008; Sherwin et al., 2012). Therefore, vitamin D status and pathway genetic variations are being examined in relation to myopia to determine if vitamin D plays a role. In a small multiple regression study, subjects with myopia (<−0.75 diopter) had lower serum $25D_3$ levels compared to non-myopes after adjustment for dietary intakes. While these results suggested vitamin D status could be related to myopia risk, a larger study was needed to confirm this (Mutti and Marks, 2011).

In 2014, a study was published correlating vitamin D levels and myopia in 2038 Korean subjects (Choi et al., 2014). Vitamin D deficiency is very common in the Korean population, particularly adolescents, and myopia rates are increasing (Choi et al., 2011; Yoon et al., 2011). Testing the hypothesis that vitamin D plays a role in myopia risk, Choi et al. (2014) found that spherical equivalent was positively correlated with serum $25D_3$ levels in myopic participants from the Korea National Health and Nutrition Examination Survey (2008-2011), after adjusting for age and sex. This association was particularly significant in the high myopia group. In addition, serum $25D_3$ concentration was also significantly associated with myopia after adjusting for confounding factors such as socioeconomic level, rural versus urban residence, daily milk and calcium intakes, and smoking history. Several variables were not taken into account (time spent

outdoors and sunlight exposure) which have been shown to affect myopia development and vitamin D levels and therefore could have influenced the results of this study. However, although the association is small, this study, as Mutti commented, was important in providing evidence that vitamin D could be a potential therapeutic option to control the increasing rates of myopia (Mutti, 2014).

Another large study examined the association between vitamin D levels and myopia in participants from the Western Australian Pregnancy Cohort (Raine) Study (Yazar et al., 2014). In this study, the authors analyzed potentially confounding variables, such as age, parental myopia, ethnicity, education, time spent outdoors, and ocular sun exposure, measured by conjunctival UV autofluorescence (CUVAF) score. Seasonal variability in serum $25D_3$ concentrations was also taken into account in the analysis. With a total of 946 participants, myopic participants had significantly lower serum $25D_3$ levels than nonmyopic subjects. In addition, the likelihood of being myopic decreased with increasing $25D_3$ levels in multivariable regression models adjusting for time spent outdoors and CUVAF as well as the fully adjusted model. It is important to note that serum $25D_3$ concentrations increased with increasing CUVAF; therefore, as Yazar comments, further studies examining vitamin D levels and sun exposure preceding myopia development would be very helpful in determining vitamin D's importance in protecting against myopia.

Guggenheim et al. performed a large study using prospective data from the Avon Longitudinal Study of Parents and Children (ALSPAC) to determine if the protective effect of time spent outdoors on myopia was mediated by vitamin D (Guggenheim et al.,

2014). They found, as others have reported, that time spent outdoors was associated with increased $25D_3$ levels and reduced incidence of myopia. Also, vitamin D levels were negatively correlated with myopia. However, the protective effect of time spent outdoors was not attenuated when serum $25D_3$ or $25D_2$ was added to the model. This study suggests that vitamin D is not the protective factor in time spent outdoors in regards to myopia development. Several important limitations of this study, such as determination of time spent outdoors using a single questionnaire and refractive error without cycloplegia, should be mentioned here. Further studies that address the causality of protection from time outdoors are definitely warranted.

Genetic polymorphisms in vitamin D pathway genes have been associated with an increased risk for various ocular pathologies, as well as vitamin D status (Table 1.2). Several studies have examined variations in the VDR as potential risk factors for myopia development. The VDR gene is located near loci identified to be associated with myopia (MYP-3) (Annamaneni et al., 2011). In addition, deregulated calcium homeostasis has been implicated in ciliary muscle dysfunction, leading to problems with emmetropization and mechanical stress (Dulhunty et al., 2006). Therefore, in addition to the protective effect of time spent outdoors, vitamin D's ability to regulate calcium levels also suggests that it could be involved in myopia progression. In a multivariate analysis of a Caucasian cohort, a single-nucleotide polymorphism (SNP) in the VDR was found to be significantly associated with the presence of myopia. In a subgroup of myopes from this study (between -0.75 and -4.00 D), three SNPs in the VDR were identified that were significantly linked to myopia (Mutti et al., CLEERE Study Group, 2011). The *Fok1*

VDR polymorphism, however, which has been found to affect calcium homeostasis (Gross et al., 1996; Jurutka et al., 2001), was not found to confer risk for either high or low myopia, although the frequency of the f allele was higher in females, particularly in those subjects with low myopia (Annamaneni et al., 2011).

Table 1.2.

Genetic polymorphisms in vitamin D pathway genes and their known associations with ocular diseases. (Used with permission from Reins and McDermott, 2015)

Pathology	Gene; variation	Association	Publication
Myopia	VDR; *FOK1* VDR; rs2853559 rs2239182 rs3819545 rs2853559	None with high myopia Increased frequency of f allele in myopic females Associated with Caucasian subjects >-0.75 D myopia in both eyes Associated with Caucasian subjects between -0.75 D and -4.00 D	Annamaneni et al., 2011 Mutti et al., 2011
AMD	CYP24A1; rs6127118 rs2762934 rs1570669 rs1570670 rs2274130 rs2296239 rs4809957 VDR; rs2189480	Associated with neo-vascular AMD in family-based cohort Significantly associated with AMD in meta-analysis Associated with neo-vascular AMD in family-based cohort; not significant in all AMD subtypes	Morrison et al., 2011
Diabetic Retinopathy	VDR*; FOK1* VDR; Bsml VDR; Taq I	Decreased incidence of advanced DR in type 1 diabetics Associated with DR in type 1 diabetics Frequency of TT, wildtype genotype, was lower in type 1 patients with severe DR	Taverna et al., 2013 Bucan et el., 2009 Taverna et al., 2002
Uveitis	CYP27B1; rs703842 A>G DHCR7; rs12785878	Increased frequency in HLA-B27-positive AAU patients compared to controls Associated with ocular Behçet's disease patients	Steinwender et el., 2013 Fang et al., 2013

An interesting topic for expanded exploration is the relationship of vitamin D status to the global and ethnic differences seen in myopia rates. Although vitamin D deficiency is a widespread health issue, low serum levels are more frequent in Asian and Middle Eastern populations (Mithal et al., IOF Committee of Scientific Advisors (CSA) Nutrition Working Group, 2009). Darker skin coloration, higher latitudes, low sun exposure, age, and diet all contribute significantly to deficiency (Kift et al., 2013; Lee et al., 2014; Mithal et al., IOF Committee of Scientific Advisors (CSA) Nutrition Working Group, 2009). Similarly, myopia development is associated with a range of both environmental and genetic components (Sherwin and Mackey, 2013) and rates are increasing worldwide, particularly in East and Southeast Asian populations (Choi et al., 2011; Yoon et al., 2011). In total, data suggest a link between vitamin D status and myopia; however the biological significance and mechanism of this protection needs to be addressed and further studies are needed to confirm these findings.

1.2.2. Retinoblastoma

First identified for its ability to regulate calcium absorption, vitamin D is now recognized to have antineoplastic activity against many types of cancers. It has been shown to influence cell differentiation, induce apoptosis, inhibit angiogenesis, and arrest cell growth in various tumors (Frampton et al., 1983; Krishnan et al., 2010; Leyssens et al., 2013; Nagakura et al., 1986; Picotto et al., 2012; Szyszka et al., 2012). In 1966, Verhoeff hypothesized that vitamin D could be an effective treatment for retinoblastoma based on the observation that tumors undergoing spontaneous regression frequently had

calcifications (Verhoeff, 1966). Although it has since been shown that vitamin D treatment does not induce tumor calcification (Albert et al., 1992), vitamin D has been proven effective both *in vitro* and in animal models of retinoblastoma in inhibiting tumor cell growth (Sabet et al., 1999).

Early studies showed the presence of the VDR in Y79 cells, a human retinoblastoma cell line, both in culture and in tumors of Y79 cells injected subcutaneously into athymic nude mice, a xenograft model of the disease (Albert et al., 2002; Saulenas et al., 1988; Wagner et al., 2003). Y79 and Weri-RB1 cells also expressed the CYP24A1 gene (Morrison et al., 2011). Human retinoblastoma tissue and tumors from LH beta-Tag transgenic mice, a model in which the overexpression of the SV40 T antigen induces retinal tumors similar to human disease (Suárez et al., 2007; Windle et al., 1990), also expressed the receptor, suggesting that these tumors would be responsive to vitamin D treatment (Albert et al., 2002; Wagner et al., 2003). $1,25D_3$ treatment did inhibit Y79 cell growth *in vitro*, inducing G0/G1 cell cycle arrest, and apoptosis, or programmed cell death, of the tumor cells. Mechanistically, $1,25D_3$ upregulated Bax, a pro-apoptotic protein, while decreasing the expression of anti-apoptotic Bcl-2, contributing to the increase in cell death (Albert et al., 2002). In both the xenograft and transgenic models of disease, systemic administration of $1,25D_3$ for 5 weeks significantly inhibited tumor growth. $1,25D_3$ treatment increased apoptosis of cancer cells (Audo et al., 2003) and inhibited angiogenesis within tumors (Shokravi et al., 1995). Unfortunately, however, even low doses of $1,25D_3$ (0.05 μg/day) were toxic and

resulted in an increase in mortality and hypercalcemia, as measured by serum calcium and renal calcifications (Albert et al., 2002, 1992; Cohen et al., 1988; Sabet et al., 1999).

An active area of vitamin D research has been the development of VDR agonists which have similar protective actions of $1,25D_3$ but have lower calcemic effects. In mouse models of retinoblastoma, several vitamin D analogues have shown promise in reducing tumor growth while having less toxic systemic effects compared to $1,25D_3$ (Sabet et al., 1999). Specifically, 1,25-dihydroxy-16-ene-23-yne-vitamin D_3 (16,23-D_3) (Albert et al., 2004, 2002; Sabet et al., 1999; Shternfeld et al., 1996; Wilkerson et al., 1998), 1α-hydroxyvitamin D_2 (1α-OH-D_2) (Albert et al., 2004, 2002; Dawson et al., 2003), and 2-methylene-19-nor-(20S)-1α-hydroxybishomopregnacalciferol (2MbisP) (Albert et al., 2005) reduce retinoblastoma tumor size without toxicity. 1α-OH-D_2 has also been shown to inhibit pigmented intraocular tumor growth in a transgenic model mimicking human choroidal melanoma (Albert et al., 2004). Combination therapies are also a possibility in the treatment of retinoblastoma, where a very low concentration of vitamin D could enhance tumor responsiveness to chemotherapy. One such study has shown that $1,25D_3$ administered with Cisplatin significantly reduced tumor growth, while no mortality or toxicity to the kidneys was observed (Kulkarni et al., 2009).

1.2.3 Age-related Macular Degeneration

Because of its role in modulating inflammation, oxidative stress, fibrosis, and angiogenesis, vitamin D has been studied in relation to age-related macular degeneration (AMD). While not classically considered an inflammatory disease, it is now recognized

that inflammatory events, such as complement activation, immune cell recruitment, and proinflammatory cytokine release, play a role in the development of AMD (Coleman et al., 2008; Nussenblatt et al. 2009; Wang et al., 2011). Therefore, Parekh et al. first hypothesized that vitamin D could be protective against AMD progression (Parekh et al., 2007). In an analysis using 7752 individuals from the third National Health and Nutrition Examination Survey (1988-1994), those subjects in the highest quintile of serum $25D_3$ levels had decreased prevalence of early AMD and drusen versus those in the lowest quintile. However, this inverse relationship between vitamin D levels and early AMD was not observed with advanced AMD, possibly accounted for by the much smaller number of subjects with advanced AMD. Examining the association between consumption of foods high in vitamin D and AMD prevalence, this study also found an inverse relationship between milk consumption and early AMD using a food frequency questionnaire, which asked subjects to recall dietary intakes in the month prior to the study (Parekh et al., 2007).

Based on these results, other epidemiological studies have been performed examining the connection between vitamin D levels and AMD. Day et al. (2012) examined the rate of first diagnosis of both neovascular and nonneovascular AMD in Medicare patients based on vitamin D status. Although they did not find a significant association between AMD incidence and vitamin D deficiency, this retrospective study was not able to use patients' laboratory data, and therefore exact $25D_3$ concentrations were not used. In a case study evaluating monozygotic twins with varying AMD phenotypes, twins with less severe AMD and smaller drusen area had higher dietary

vitamin D consumption (Seddon et al., 2011). Although, as noted by Annweiler, dietary vitamin D intake does not necessarily correspond to vitamin D status (Annweiler et al., 2012). In a separate retrospective study analyzing 1045 individuals with an AMD diagnosis, no association was found between serum $25D_3$ levels and disease (Golan et al., 2011). Although this was a large study, several limitations must be pointed out in interpreting the results. AMD was not categorized by severity and clinical data was not available for the analysis; therefore, early and late AMD cases were not separated. In addition, subjects' use of vitamin D supplementation was not known.

In an evaluation of participants in the Carotenoids in Age-Related Eye Disease Study, when separated by age, higher serum $25D_3$ concentration was associated with decreased risk of early AMD in women younger than 75 years old (Millen et al., CAREDS Study Group, 2011). This association, however, was decreased when dietary patterns, BMI, and physical activity were accounted for. Also, most participants were Caucasian, postmenopausal women, and therefore, results were specific to a specialized population. A smaller retrospective study in France found that individuals with low vitamin D serum levels (<50 nmol/L) were also more likely to have AMD, but late-stage AMD in particular (Graffe et al., 2012). A large, cross-sectional study of participants in the Korean National Health and Nutrition Examination Survey (2008-2012), also found an inverse association between $25D_3$ levels and late AMD, however only in men and not with early AMD after adjustment for age, sun exposure, smoking, and heart disease (Kim et al., 2014).

Following up an earlier study, Graffe et al. used optical coherence tomography to measure pathological changes preceding disease and found that vitamin D insufficiency (< 50 nmol/L) was associated with reduced macular thickness (Graffe et al., 2014). Singh et al. (2013) specifically examined subretinal fibrosis in patients with advanced AMD, classified into the Clinical Age-Related Maculopathy Staging (CARMS) group 5. In their single-center study, they found that patients with subretinal fibrosis had significantly lower serum vitamin D levels and were more likely to be vitamin D-insufficient (<50 nmol/L) than patients without fibrosis. This significance was maintained after adjusting for confounding factors such as age, smoking, diet, sex, exercise, and four SNPs known to influence systemic vitamin D concentrations. Interestingly, there was no difference in serum $25D_3$ concentrations between genotypes or between clinical groups 1-5. The authors suggest that this specifically links vitamin D deficiency with subretinal fibrosis. Vitamin D is known to play a role in inhibiting fibrosis in other tissues and low concentrations have been implicated in the pathogenesis of fibrotic diseases, partly through inhibition of transforming growth factor beta (TGF-β) (Artaza and Norris, 2009; Halder et al., 2011; Isik et al., 2012; Petta et al., 2010; Ramirez et al., 2010). Based on these results by Singh et al., studies aimed at determining how vitamin D levels influence tissue changes and fibrosis in AMD would be very interesting. They also warrant an examination of a possible protective role for vitamin D during fibrotic events in other eye diseases as well.

Morrison et al. further explored the relationship between vitamin D and AMD risk (Morrison et al., 2011). They found that in a cohort of 481 sibling pairs, neovascular

AMD risk went down with increasing ultraviolet irradiance, as measured by UV index. In a larger scale study, which included three different cohorts for a total of 2,528 subjects and was controlled for known AMD risk factors (smoking, gender, and age), single point variations, SNPs, in the CYP24A1 gene, but not VDR, correlated with an increased risk for all AMD subtypes in a meta-analysis. Polymorphisms in 24A1 were located chromosomally in a region that was known previously to have AMD susceptibility loci (Iyengar et al., 2004; Seddon et al., 2003). This study importantly suggested a genetic link between AMD prevalence and vitamin D metabolism.

Several genetic variations have been identified as strong risk factors for AMD. In one of these loci, a single-nucleotide polymorphism in the promoter of the *HTRA1* gene (High-temperature requirement factor A1) has been found to significantly increase the likelihood of AMD development (Chen et al., 2009; Dewan et al., 2006; Tong et al., 2010; Yang et al., 2006). The orthologous *HTRA1* promoter region in the rhesus monkey contains 9 VDR binding sites and interestingly, one of these sites is removed by the AMD-associated *HTRA1* SNP (Pahl et al., 2013). *In vitro* studies demonstrated that stimulation with vitamin D lowered the activity of the wild type *HTRA1* in ARPE-19 cells. Further studies are needed to identify if vitamin D signaling influences this promoter region and if disease associated variations affect these pathways. Additionally, more studies are needed to firmly establish that vitamin D status is a risk factor for AMD development and to determine if vitamin D supplementation affects the development of AMD.

Several interesting studies have examined inflammatory events and neovascularization in the mouse retina with vitamin D treatment. In a study using aging mice, Lee et al. demonstrated that subcutaneous treatment with vitamin D significantly reduced signs of retinal inflammation (Lee et al., 2012). Treated mice had fewer macrophages in the subretinal space, less complement (C3d) deposition on Bruch's membrane, and a reduction in retinal amyloid beta accumulation. In addition, vitamin D treatment improved visual function, as measured by the electroretinogram a-wave. In a mouse model of oxygen-induced ischemic retinopathy, intraperitoneal vitamin D treatment inhibited retinal neovascularization in a dose-dependent manner (Albert et al., 2007). These mouse models suggest that vitamin D supplementation could be protective against both dysregulated inflammation and angiogenesis in the retina, providing mechanisms of reduced vitamin D being involved in AMD development.

1.2.4. Diabetic Retinopathy

Vitamin D's ability to inhibit neovascularization also has led researchers to examine the hormone's involvement in diabetic retinopathy (DR) development. In an epidemiological study, Aksoy et al. (2000) found that serum vitamin D concentrations ($25D_3$) were inversely related to the severity of retinopathy in diabetic patients, with the lowest concentrations of the hormone measured in patients with proliferative DR (Aksoy et al., 2000). Patients without associated retinopathy had the highest serum vitamin D concentrations. A similar study classified patients into diabetic groups based on disease severity and also found that patients with proliferative DR had the lowest mean $25D_3$

levels (21.1 ng/mL) (Payne et al., 2012). In addition, vitamin D deficiency was associated with increased risk of retinopathy in an adolescent population with type 1 diabetes (Kaur et al., 2011), however it was not associated with changes in retinal geometric parameters such as vascular branching angle, length-diameter ratio, or tortuosity (Poon et al., 2013). A recent study found that vitamin D insufficiency was not associated with DR severity, seemingly contradicting early reports (Bonakdaran and Shoeibi, 2015). However, patients were grouped based on vitamin D levels of 30ng/mL or lower for insufficiency and then correlations were done with known risk factors for DR and not between diabetic groups.

Genetic variations in the VDR have also been associated with diabetic retinopathy. In a cohort of Caucasian patients with type 1 diabetes, patients with the *Fok*I VDR polymorphism (FF genotype), had a lower incidence of advanced diabetic retinopathy, particularly in those patients whose duration of diabetes was less than 25 years (Taverna et al., 2005). The *Fok*I substitution is a functional polymorphism which has been reported to increase immune cell activity (van Etten et al., 2007) and therefore could have a protective effect on DR development. In other studies, the VDR *Bsml* gene polymorphism was also associated with risk of DR (Bućan et al., 2009) and the Taq I polymorphism with severe DR (Taverna et al., 2002).

Looking to an animal model to study vitamin D's ability to protect against retinopathy, Ren et al. used a rat model of type 2 diabetes (Ren et al., 2012a). They found that animals treated with vitamin D had decreased retinal expression of VEGF and TGF-β1. Histological examination also suggested that vitamin D had a protective effect

in the retinas of these rats. These combined studies suggest that vitamin D status could be important in the prevention of DR, particularly proliferative retinopathy. Further studies are needed to determine the mechanism of vitamin D protection and if it can directly inhibit neovascularization in this sight-threatening condition.

1.2.5. Uveitis

Uveitis is an inflammatory condition that affects the retina and uvea. Inflammation can be caused by an infectious agent or, in the majority of cases, is thought to be autoimmune in nature, driven by retinal antigen-specific T lymphocytes (Caspi, 2010). Because of its ability to dampen inflammation, influence T cell response, and its known ability to have suppressive actions in autoimmune conditions, vitamin D is a good candidate to examine in the context of this sight-threatening disease.

The most common form of uveitis, acute anterior uveitis (AAU), has been strongly associated with the human leukocyte antigen (HLA)-B27 gene (Chang et al., 2005; Suhler et al., 2003; Wakefield et al., 2011). In an interesting retrospective study, the association of a vitamin D hydroxylase gene polymorphism (CYP27B1 rs703842 A>G) with HLA-B27-positive AAU was examined (Steinwender et al., 2013). This study found that individuals with AAU were more likely to have this CYP27B1 variation than HLA-B27-positive controls. Other studies have demonstrated that the rs703842 A>G polymorphism results in lower levels of circulating $25D_3$ (Orton et al., 2008). Therefore, Steinwender et al. (2013) suggested that these HLA-B27 positive individuals

could have an even greater impairment in immune function when vitamin D metabolism is disrupted.

Behçet's disease is an inflammatory disease that involves multiple organs in the body but over half of patients develop uveitis (Yazici et al., 2007). In a prospective study on a Chinese population, polymorphisms in an enzyme necessary for vitamin D production, DHCR7 (7-dehydrocholesterol reductase), were associated with susceptibility to ocular Behçet's disease in particular, demonstrating another genetic variation linking vitamin D metabolism and uveitis (Fang et al., 2014).

Experimental autoimmune uveitis (EAU) is a mouse model of human autoimmune uveitis in which the immune response has been well characterized. In this model, oral vitamin D treatment both prevented the development of disease as well as attenuated retinal autoimmunity once induced (Tang et al., 2009). Importantly, vitamin D inhibited the Th17 response that is responsible for the retinal inflammation in this model, influencing T cell cytokine production and the priming ability of dendritic cells. In similar studies using human samples from patients with Behçet's disease, vitamin D treatment also inhibited Th17 cell differentiation (Tian et al., 2012). These studies provide evidence that vitamin D supplementation could be beneficial not only during the active inflammatory condition but also for prevention of uveitis as well.

1.2.6. Glaucoma

Gene expression studies identified vitamin D as having the potential to modulate genes involved in regulating both aqueous humor outflow and production, as well as the

architecture of the trabecular meshwork, thereby influencing IOP. In both cultured mouse calvarial cells and rat intestinal mucosa, treatment with vitamin D modulated the expression of numerous genes involved in intraocular pressure (IOP) regulation (Kutuzova et al., 2012). Microarray analysis showed that $1,25D_3$ decreased the expression of carbonic anhydrase I (CAI), angiotensin I converting enzyme (ACE), aquaporin 1 channel (AQP1), and various cytoskeletal and extracellular matrix genes such as actin alpha (ACTA1) and fibronectin I. $1,25D_3$ also upregulated matrix metalloproteinases 3, 11, 13, and 14, as well as prostaglandin E receptor 4 for PGE2 (PTGER4), purinergic receptors P2Y and P2RY2, and chemokine (C-C motif) ligand 20 (CCL20).

As a result of these gene expression studies, Kutuzova et al. (2012) examined the effect of $1,25D_3$ or an analog, 2-methylene-19-nor-(20S)-1α,25-dihydroxyvitamin D(3) on IOP in non-human primates. They demonstrated that topical $1,25D_3$, administered twice daily (0.1-15μg $1,25D_3$ or vehicle propylene glycol) reduced IOP in monkeys. Significantly, when $1,25D_3$ was applied to one eye without nasolacrimal duct occlusion, IOP was lowered bilaterally (20% decrease after 3 days, $p<0.05$). However, $1,25D_3$ did not reduce aqueous humor production or effect uveoscleral outflow in this model. Therefore, the mechanism involved in this decrease in IOP still needs to be investigated with further studies.

A very early study suggested that vitamin D, introduced intramuscularly, could decrease IOP in humans (Guist and Steffen, 1953). However, in a case control and randomized controlled study, Krefting et al. examined the association between serum

vitamin D levels, vitamin D supplementation, and IOP and they found no statistical difference in IOP levels between individuals in the lowest serum $25D_3$ group versus the high serum $25D_3$ group (Krefting et al., 2013). Additionally, there was no significant change in IOP in participants who received vitamin D oral supplements (20,000 IU twice per week) at the end of 6 months compared to placebo.

Examining the association between vitamin D status and risk for open-angle glaucoma (OAG), Yoo et al. performed multivariable logistic regression analyses using 6094 participants from the Fifth Korean National Health and Nutrition Examination Survey (Yoo et al., 2014). They found a significant relationship between serum $25D_3$ levels and prevalence of OAG, with increased risk of disease particularly in participants in the lowest quintile of $25D_3$. Additionally, unlike the findings of Krefting et al (2013), IOP was significantly linked to vitamin D levels. This apparent discrepancy could in part be due to differences in participant ethnicity (Caucasian versus South Korean), sample size, or the exclusion of participants with diagnosed disease in the Krefting study. Further epidemiological studies need to be done to determine if vitamin D status is a risk factor for glaucoma.

1.3. The Cornea and Ocular Surface

The cornea is the avascular tissue that forms the anterior surface of the eye, covering the pupil and iris. One of its major functions is to refract light, focusing light back onto the

retina, for photodetection. The cornea is responsible for two-thirds of the eye's total focusing power and tissue transparency is essential for this function. The cornea also functions to protect the rest of the eye from damage, providing a barrier from physical, microbial, UV and other harmful damage. The adult human central cornea is approximately 500 microns thick and can be divided into five layers (Figure 1.6) (Rogers, 2010). The stratified corneal epithelium is the outermost layer and comes into contact with the tear film, which provides hydration and nutrients for the rest of cornea and maintains a smooth corneal surface. Following the epithelial basement membrane and a thin collagenous layer (anterior limiting lamina/Bowman's layer), lies the largest layer of the cornea, the stroma. The stroma is comprised of a regular arrangement of collagen fibrils, or lamellae, which are important for corneal strength and transparency. Keratocytes maintain the architecture of the stroma. Under the stroma is the posterior limiting lamina (Descemet's membrane), followed by the endothelium. The corneal endothelium is a single layer of cells that is adjacent to the anterior chamber and is critically important for maintaining the fluid balance (and hence transparency) in the cornea (Bergmanson, 2010).

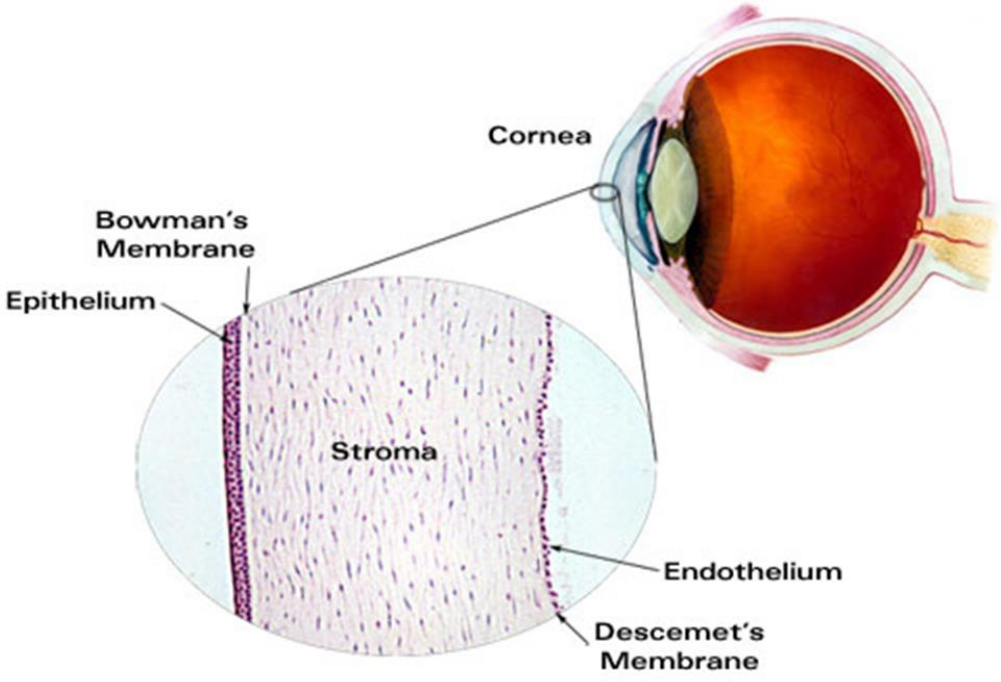


Figure 1.7. The Human Cornea.

The cornea is the transparent tissue at the anterior-most surface of the eye. The structure of the cornea is divided into five distinct layers: the epithelium, Bowman's layer, stroma, Descemet's membrane, and endothelium. (Figure used with permission from http://www.laramyk.com/)

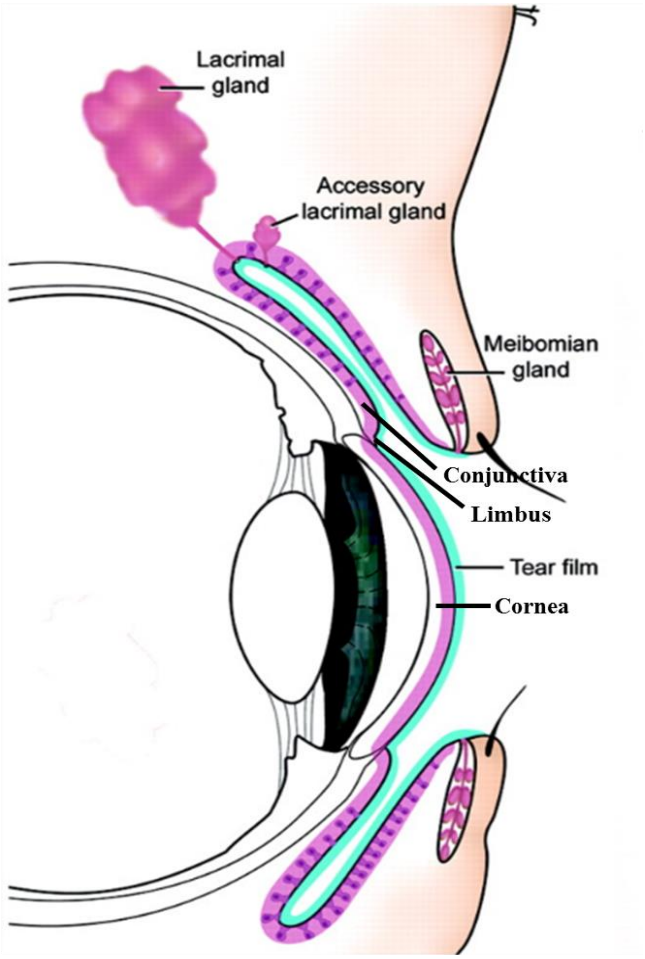


Figure 1.8. The Ocular Surface.

A sagittal view of the ocular surface, which includes the tear film (blue), cornea, conjunctiva, lacrimal glands, and meibomian glands (all highlighted in pink). All components of this ocular surface "system" integrate to help maintain the function of the cornea in protection and light refraction. Figure adapted and used with permission from Gipson, 2007, copyright ARVO.

The structures at the surface of the eye work together to protect and maintain the functions of the cornea and have been termed the "Ocular Surface System" (Gipson, 2007). These include the continuous surface epithelia of the cornea and conjunctiva, meibomian glands, lacrimal gland, and accessory lacrimal glands which all produce various components of the tear film. (DEWS Report, 2007) (Figure 1.7).

At the margin of the cornea is the limbus, a region which is rich in vasculature and contains a stem cell population, important for renewing the epithelium (Dua and Azuara-Blanco, 2000; Pellegrini et al., 2009). The conjunctiva also borders the limbus and is a vascular, mucous membrane which contains mucin-producing goblet cells and extends to cover the inside of the eyelids. Mucins are an important part of the tear film and the glycocalyx which lies in between the corneal epithelium and the tears, tethering the tear film to the ocular surface (Figure 1.8) (Gipson, 2007). The meibomian glands line the lid margins and produce meibum, a specialized sebaceous gland secretion that contributes to the lipid layer in the tear film, important for preventing tear evaporation and spreading across the ocular surface (Millar and Schuett, 2015).

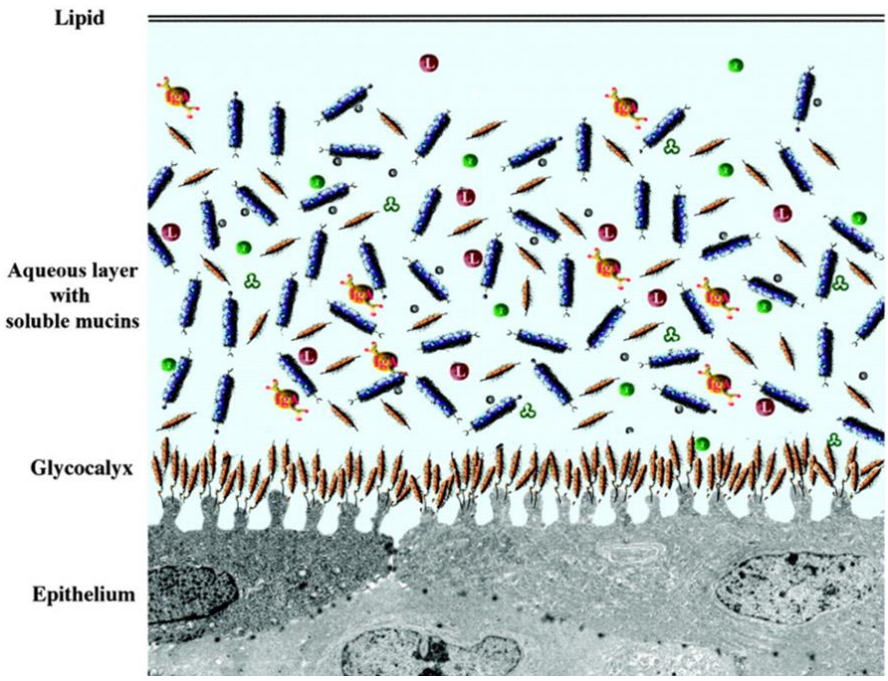


Figure 1.9. The Tear Film.

The tear film is a dynamic fluid layer that provides nutrients, oxygen, and moisture to the ocular surface. It is composed of an outer lipid layer, which protects against evaporation, and an aqueous layer, which contains proteins and soluble mucins. Membrane-bound mucins are a part of the glycocalyx, at the tear film-epithelium interface, and help tether the tear film to the ocular surface. Figure used with permission from Gipson, 2007, copyright ARVO. (Figure not drawn to scale)

1.3.1. Vitamin D and the ocular surface

As previously mentioned in section 1.2., vitamin D-related genes (hydroxylases and VDR) are expressed by cells of the corneal epithelium, stroma, and endothelium (Yin et al., 2011). Further, corneal limbal epithelial cells are able to produce vitamin D, *de novo*, when exposed to UVB, similar to cells of the skin, potentially providing a local source of vitamin D to the ocular surface (Lin et al., 2012). In the cornea, VDR knockout mice had smaller superficial epithelial squamous cell size and decreased total corneal thickness compared to wild-type animals (Lu and Watsky, 2014). Additionally, VDR influenced cell diffusion coefficients within the cornea, possibly playing a role in gap junction communications and development in this ocular tissue (Lu and Watsky, 2014). Interestingly, the rate of epithelial wound healing was decreased in VDR knockout animals (Elizondo et al., 2014). Mucin packaging in conjunctival goblet cells was also altered in VDR-deficient mice, with lower amounts of mucin (Muc5AC) in these animals compared to wild-types (Paz et al., 2003). However, both mucin and wound closure differences seemed to be attributable to vitamin D's influence on calcium homeostasis, as restoring ionized calcium levels in the VDR knockout mice restored normal mucin packaging as well as healing rates in these studies.

Inflammation at the ocular surface must be carefully regulated during infection and injury to prevent loss of corneal opacity and tissue damage. Based on vitamin D's known ability to suppress chronic inflammation and influence the immune response, several groups have studied the immunomodulatory role of vitamin D within the local context of the ocular surface. *In vivo* studies have demonstrated that vitamin D can be

anti-inflammatory at the ocular surface. In a mouse model of injury, topical administration of $1,25D_3$ to sutured mouse corneas inhibited Langerhans cell migration and maturation, and delayed neovascularization in the central cornea (Suzuki et al., 2000a). In a rat keratoplasty model, $1,25D_3$ protected against corneal graft rejection, inhibiting the proinflammatory cytokines interleukin-1 alpha (IL-1α) and tumor necrosis factor alpha (TNFα) (Dang et al., 2004).

In vitro, vitamin D appears to dampen the inflammatory response to infection. Treatment with vitamin D downregulated the expression of IL-1β, IL-6 and IL-8 induced by *Pseudomonas aeruginosa* infection in human corneal epithelial cells (Xue et al., 2002b). In a separate report, $1,25D_3$ also inhibited IL-1α production (Suzuki et al., 2000b) and we have shown that vitamin D reduces Toll-like receptor induced inflammatory cytokines in cultured epithelial cells (section 2.3.5.) (Reins RY, McDermott AM. Vitamin D attenuates Toll-like receptor 3 induced inflammation in human corneal epithelial cells. Invest Ophthalmol Vis Sci 2013; e-abstract 2067). In addition, both $1,25D_3$ and $25D_3$ augmented corneal epithelial barrier function, measured by cell permeability, and upregulated tight junction proteins occludin and ZO-1 (Yin et al., 2011). Vitamin D therefore has the potential to reverse the harmful effects to the corneal epithelial barrier during infection and protect against dysregulated inflammatory conditions.

In a cross-sectional study examining male patients with dry eye syndrome, a condition that is accompanied by ocular surface inflammation (DEWS report, 2007), serum $25D_3$ levels were not found to be associated with severity of disease clinically

(Galor et al., 2014). However, higher serum vitamin D levels were significantly correlated with a decrease in subjective dry eye symptoms, as determined by the Dry Eye Questionnaire 5. In a case report it was observed that a vitamin D-deficient patient with corneal neuralgia had relief from burning pain with vitamin D supplementation, 1,000 IU/day, while topical therapies and lubricants were not effective (Singman et al., 2013). Although this is only a single observation without extensive follow-up, vitamin D's protective effect on ocular surface pain would be interesting to pursue further.

1.4. Dissertation Scope and Significance

Vitamin D is a multifunctional hormone, which not only affects calcium homeostasis, but plays a role in immune system regulation as well as cell growth and survival. Many tissues in the eye are able to both activate and respond to vitamin D, suggesting that vitamin D is a biologically relevant molecule to study throughout the eye. Epidemiological studies demonstrate that vitamin D levels and genetic variations influence the development of a wide range of pathologies, such as myopia, age-related macular degeneration, diabetic retinopathy, and uveitis. In addition, at the cellular level, vitamin D is able to reduce inflammatory mediators, enhance barrier function, and induce cell death of cancerous cells. These studies suggest that vitamin D plays a protective role in ocular health.

The aim of this dissertation is to examine the function of vitamin D in the cornea and its ability to modulate the response to inflammation. Before these studies began, there was not a great deal known about vitamin D and its role at the ocular surface. I became interested in studying vitamin D in the cornea after learning about its role in immunomodulation and antimicrobial peptide production and recognizing that there was a gap in this knowledge in ocular surface tissues. As mentioned, inflammation during infection, disease (such as dry eye syndrome), and wound healing must be carefully regulated to restore corneal integrity important for vision. Therefore, I felt that vitamin D was an exciting area of research, which could potentially be translated into clinical applications. The work presented in this dissertation is based on the assertion that vitamin D is able to modulate gene expression and have a positive influence on inflammation in the cornea. *In vitro* studies examined the ability of human corneal epithelial cells to activate vitamin D and the hormone's influence on TLR-induced inflammation (Chapter 2). Vitamin D's effect on gene expression was also examined in these cells as well as the modulation of various signaling pathways involved in cytokine production (Chapter 3). In addition, vitamin D treatment was used in two mouse models where corneal inflammation is present: dry eye (Chapter 4) and corneal wound healing (Chapter 5). In these *in vivo* models, vitamin D's influence on inflammatory mediators and pathological outcome was examined.

Corneal inflammation must be kept in check in order to prevent tissue damage and loss of transparency. In some instances, a small amount of inflammation is beneficial, such as the contribution of inflammatory cells during the initial stages of

corneal wound healing and infection. But too much inflammation is harmful to the ocular surface. Anti-inflammatory therapeutics are an active area of research in the treatment of conditions in which inflammation contribute to the pathogenesis, such as dry eye syndrome. Vitamin D has a known role in modulating immunity and has been shown to dampen chronic inflammation. Therefore, vitamin D is an exciting molecule to study at the ocular surface. Vitamin D's influence on corneal inflammation needs to be thoroughly evaluated, with a view to the development of novel treatments for inflammatory conditions, such as dry eye syndrome or infection.

Chapter 2

Vitamin D and Human Corneal Epithelial Cells:

Antimicrobial and Anti-inflammatory Effects

2.1. Introduction

Vitamin D is a multifunctional hormone that exerts its effects on many tissues and cell types, in addition to its traditional role in calcium homeostasis. Vitamin D is obtained through diet and supplementation but is also produced in the skin when exposed to sunlight (Kamen and Tangpricha, 2010; White, 2008). Vitamin D must undergo two enzymatic steps to become biologically active (Schauber and Gallo, 2008). The first reaction occurs mainly in the liver, where vitamin D is hydroxylated by the cytochrome p450 enzymes CYP2R1 or CYP27A1, to produce the major circulating form, 25-hydroxyvitamin D_3 ($25D_3$) (Bouillon et al., 2008). $25D_3$ is then further modified through 1-alpha-hydroxylation (CYP27B1) to form the active $1,25D_3$ (Jones et al., 2014; Prosser and Jones, 2004). This activation is now known to occur not only in the kidneys, the site of traditional activation, but also in other tissues, where vitamin D is able to influence a variety of cellular functions (Adams and Hewison, 2012). $1,25D_3$ binds to its nuclear hormone receptor, the vitamin D receptor (VDR) which then interacts with vitamin D response elements (VDRE) to modulate gene transcription (Haussler et al., 1995; Kim et al., 2005). $1,25D_3$ activity is regulated by 24-hydroxylation (carried out by CYP24A1), which inactivates the hormone.

In the eye, as discussed in section 1.2., diverse studies show that vitamin D protects against autoimmune uveitis (Tang et al., 2009), restricts retinoblastoma growth (Albert et al., 1992; Audo et al., 2003) and angiogenesis (Shokravi et al., 1995), stimulates retinal glial cell phagocytosis (Mano and Puro, 1990), and is associated with a

decreased risk of age-related macular degeneration (Graffe et al., 2014, 2012; Millen et al., CAREDS Study Group, 2011; Morrison et al., 2011; Parekh et al., 2007; Singh et al., 2013) and myopia development (Mutti and Marks, 2011; Choi et al., 2014; Yazar et al., 2014). Vitamin D has also been shown to decrease amyloid beta plaques in the aging retina (Lee et al., 2012) and lower intraocular pressure in non-human primates (Kutuzova et al., 2012). However, relatively little is known about vitamin D function or availability at the ocular surface, including the cornea which provides protection and a refractive surface. Recent reports demonstrate that a human limbal epithelial cell line can produce vitamin D_3 *de novo* following UVB exposure and that vitamin D metabolites are present in the aqueous and vitreous humor and tears of rabbits (Lin et al., 2012; Yin et al., 2011a). The widely expressed VDR has been identified in the human corneal epithelium and endothelium, lens, ciliary body, and retinal pigment epithelium, as well as the ganglion cell layer and retinal photoreceptors in the eye (Johnson et al., 1995). In addition, several vitamin D hydroxylases are expressed in cultured human corneal epithelial cells and fibroblasts, retinal pigmented epithelial cells, and non-pigmented ciliary body epithelial cells (Alsalem et al., 2014; Yin et al., 2011), suggesting that the cornea and other ocular tissues have the machinery to activate and regulate vitamin D metabolism on their own and that vitamin D is potentially an important biological mediator in the eye.

One of the key roles of vitamin D is immune system regulation. Epidemiologic studies provide evidence that vitamin D deficiency increases susceptibility to various infections and is associated with a range of chronic diseases, including several

autoimmune conditions (Holick, 2007; Holick and Chen, 2008). Vitamin D modulates innate and adaptive immune responses through the production of anti-microbial peptides (Wang et al., 2004), downregulation of proinflammatory cytokines and chemoattractants, regulation of antigen presenting cell differentiation and maturation (Piemonti et al., 2000), and direct action on T cell function, enhancing development of a Th2 response (Boonstra et al., 2001). In addition, vitamin D influences the expression and activity of various pattern recognition receptors (PRR), including Toll-like receptors (TLR) (Gambhir et al., 2011; Khoo et al., 2011; Sadeghi et al., 2006; White, 2008). TLRs are a family of highly conserved PRRs that are activated in response to microbial and endogenous ligands (Medzhitov et al., 1997; Takeda et al., 2003). Engagement of these innate immune receptors leads to the robust production of cytokines and chemokines that facilitate the recruitment and activation of an adaptive immune response. In other tissues, crosstalk occurs between vitamin D and TLR signaling to regulate responses to inflammation and stress. In both monocytes and keratinocytes, TLR2 ligands increase the expression of CYP27B1, the vitamin D activating hydroxylase, resulting in an upregulation of antimicrobial peptides (Liu et al., 2006; Schauber et al., 2007). In respiratory epithelial cells, viral RNA, a TLR3 agonist, acts similarly to increase the local conversion of $25D_3$ to the active $1,25D_3$ (Hansdottir et al., 2008). Vitamin D also increases CD14 expression, a co-receptor which mediates the response to bacterial antigens in coordination with several other proteins, including TLR2 and TLR4 (Moeenrezakhanlou et al., 2008; Sadeghi et al., 2006; Shin et al., 2010).

An important mechanism for innate immune influence came with the discovery that vitamin D directly induces human cathelicidin antimicrobial peptide (hCAP) expression via a VDRE located in the promoter region of the cathelicidin gene (Wang et al., 2004). While first identified as an antimicrobial peptide, LL-37, the functional cleavage product of hCAP, is now recognized to be a multifunctional peptide, with important immunomodulatory properties, in addition to killing microbes (Huang et al., 2006; Vandamme et al., 2012; Zanetti, 2004). LL-37 neutralizes lipopolysaccharide (LPS) signaling both directly (Larrick et al., 1995; Scott et al., 2000) and indirectly (Mookherjee et al., 2006), dampening inflammation caused by infection. This peptide also influences the response to other TLR ligands (Ganguly et al., 2009; Lande et al., 2007; Mookherjee et al., 2006) and cytokines (Nijnik et al., 2009; Yu et al., 2007), demonstrating its capacity to modulate innate immunity. LL-37 is expressed by human corneal epithelial cells and is up-regulated during inflammation, infection, and injury to the ocular surface (Gordon et al., 2005a; Huang et al., 2006; McDermott, 2004).

The cornea is the transparent tissue covering the eye that functions to protect the rest of the eye from damage and to refract light for vision. Inflammation during infection and injury must be carefully regulated in this tissue to prevent damage and disruption of corneal transparency, vitally important for vision. Because of vitamin D's know role in immunoregulation, in this study, we investigated the functional activity of vitamin D in human corneal epithelial cells (HCEC). Here, we demonstrate that HCEC are able to activate vitamin D, inducing target gene expression through the VDR. We also show that vitamin D is able to enhance antimicrobial peptide production and interact with TLR

stimulation to decrease inflammatory mediators, important aspects in protecting the cornea from infection and damage.

2.2. Materials and Methods

2.2.1. Human Corneal Cells and VDR siRNA Treatment

Human cadaveric donor corneas were obtained from Heartland Lions Eye Bank (St. Louis, MO) within one week of death and primary HCEC were isolated by enzymatic digestion (Dispase II, Roche Diagnostics, Indianapolis, IN) as previously described (McDermott et al., 2003). Cells were maintained in EpiLife medium with defined growth supplement (Invitrogen, Grand Island, NY), 60μM calcium chloride, and penicillin-streptomycin (5000 U/ml; 5000 μg/ml). In addition, scraped epithelial cells and freshly isolated keratocytes were collected from donor corneas and lysed for RNA collection (Pei et al., 2006). Human telomerase-immortalized corneal epithelial cells (hTCEpi) were cultured in KGM-2 medium (Lonza, Allendale, NJ) (Robertson et al., 2005) and SV40-immortalized HCECs were maintained in SHEM media as previously described (Araki-Sasaki et al., 1995). All cells were grown to approximately 80% confluency before use in experiments and were plated at the same density within experiments. HCEC were stimulated with 10^{-7}M vitamin D compounds (D_3 and 1,25D_3, Sigma-Aldrich, St. Louis, MO; 25D_3, EMD Millipore, San Diego, CA) or TLR agonists [1μg/ml Pam3CSK4 (TLR1/2), FSL1 (TLR6/2), Poly(I:C) (TLR3), Flagellin *Salmonella typhimurium* (TLR5), 10^8 cells/ml HKLM (TLR2), or 50μg/ml zymosan (TLR2); Invivogen, San Diego, CA] or

LL-37 (10μg/ml, Peprotech, Rocky Hill, New Jersey) for 24 hours, unless otherwise specified.

To examine the effect of VDR silencing, negative control siRNA and VDR specific siRNA (s14777 and s14779) were purchased from Life Technologies (Grand Island, NY). 10nM siRNAs were transfected into hTCEpi in 12 well plates using Lipofectamine 2000 (Life Technologies) according to the manufacturer's instructions. VDR knock-down was confirmed by RT-PCR and western analysis at 24, 48, and 72 hours post-transfection. Cells were stimulated with $1,25D_3$ 24 hours after transfection.

2.2.2. Quantitative Real-time PCR

Relative expression of vitamin D hydroxylases, VDR, LL-37, and CD14 in cultured cells was determined by real time RT-PCR analysis. Total RNA was extracted from cell samples using RNeasy kits (Qiagen, Valencia, CA) and reverse transcribed using the Optimax First Strand cDNA Synthesis Kit (BioChain, Newark, CA) according to the manufacturer's instructions. Real-time PCR was performed using intron-spanning primers (Table 2.1) and Brilliant II SYBR Green QPCR master mix (Agilent Technologies, Santa Clara, CA). Products were amplified with a Mx3005P QPCR System (Agilent Technologies) with the following program: 10 min at 95^{o}C, followed by 40 cycles of 30s at 95^{o}C, 1 min at 58^{o}C, and 1 min at 72^{o}C. Dissociation curve analysis confirmed primer specificity and no template/no RT samples were used for controls. Samples were analyzed using the ΔΔCt method. Briefly, Ct values for the housekeeping gene, GAPDH, were subtracted from Ct values of the gene of interest (ΔCt). The ΔCt

values for untreated controls were subtracted from the ΔCt for the treated samples (ΔΔCt). mRNA fold change was determined using 2ΔΔCt.

Table 2.1. Primer sequences for real-time PCR.

Gene name	Forward Primer	Reverse Primer	NCBI Reference Sequence
GAPDH	5'-GACCACAGTCCATGCCATCA-3'	5'-CATCACGCCACAGTTTCCC-3'	NM_002046
CYP27A1	5'-CCATCGGGTTAATGTTCCAG-3'	5'-AGTGCAGGTAGCCAGACACC-3'	NM_000784
CYP2R1	5'-AGTGGGTGAACTCATCATTGC-3'	5'-AAGAAGGCTTCCCATTAGGG-3'	NM_024514
CYP24A1	5'-TTCTCTGGAAAGGGGGTCTC-3'	5'-GTGCACCGACTCAAAGGAAC-3'	NM_000782
CYP27B1	5'-AACCCTGAACAACGTAGTCTGCGA-3'	5'-ATGGTCAACAGCGTGGACACAAA-3'	NM_000785
VDR	5'-ACGCCCACCATAAGACCTAC-3'	5'-GCTGGGAGTGTGTCTGGAG-3'	NM_000376
CAMP (LL-37)	5'-GGACAGTGACCCTCAACCAG-3'	5'-AGAAGCCTGAGCCAGGGTAG-3'	NM_004345.4
CD14	5'-GAGCTCAGAGGTTCGGAAGAC-3'	5'-GCTGAGGTTCGGAGAAGTTG-3'	NM_000591
CXCL8 (IL-8)	5'-GACCACACTGCGCCAACAC-3'	5'-CTTCTCCACAACCCTCTGCAC-3'	NM_000584
TNF	5'-TGGAGAAGGGTGACCGACTC-3'	5'-TCCTCACAGGGCAATGATCC-3'	NM_000594.3
IL1B	5'-GCTGAGGAAGATGCTGGTTC-3'	5'-GCTGAGGAAGATGCTGGTTC-3'	NM_000576.2
IL6	5'-TACCCCCAGGAGAAGATTCC-3'	5'-AGTGCCTCTTTGCTGCTTTC-3'	NM_000600.3
CCL20 (MIP3)	5'-CTGGCTGCTTTGATGTCAGTG-3'	5'-GCAGTCAAAGTTGCTTGCTGC-3'	NM_001130046.1
MMP9	5'-CCAACTACGACACCGACGAC-3'	5'-TTGGCCTTGGAAGATGAATG-3'	NM_004994.2
TLR3	5'-GCTGCAGTCAGCAACTTCAT-3'	5'-AGGAAAGGCTAGCAGTCATCC-3'	NM_003265
DDX58 (RIG-1)	5'-GACTGGACGTGGCAAAACAA-3'	5'-TTGAATGCATCCAATATACACTTCTG-3'	NM_014314
IFIH1 (MDA-5)	5'-ACCAAATACAGGAGCCATGC-3'	5'-CGTTCTTTGCGATTTCCTTC-3'	NM_022168.3
IL-23	5'-GTTCCCCATATCCAGTGTGG-3'	5'-CCCTGTGAAAATATCCGATCC-3'	NM_016584.2

2.2.3. Immuno-Blot Analysis for LL-37, CYP24A1, and VDR Expression

LL-37 protein was detected in hTCEpi culture supernatants from control cells and cells stimulated with $25D_3$ or $1,25D_3$ (10-7M) for 24 hours by dot blot as previously reported (Redfern et al., 2011), using a C-terminal rabbit anti-LL37 antibody (1:5000, donated by Dr. Fu Shin Yu Wayne State University, sourced from Panatecs, Heilbronn, Germany). CYP24A1 and VDR expression was detected in whole cell lysates by SDS-PAGE followed by western blot analysis. Briefly, cells were collected in ice-cold RIPA buffer containing a protease inhibitor cocktail (Roche, Nutley, NJ), lysed on ice for 15 minutes, vortexed, and centrifuged for 10 minutes at 14,000rpm. Protein concentration was determined in supernatants using the BCA Protein Assay Kit (Thermo Scientific Pierce, Rockford, IL) and equal amounts of reduced protein were loaded onto 10% SDS-polyacrylamide gels and separated by electrophoresis. After transfer, the nitrocellulose membranes were incubated overnight with primary antibodies (rat anti-VDR 1μg/ml or rabbit anti-CYP24A1 0.7μg/ml, EMD Millipore) diluted in 5% milk/TTBS (Tris-buffered saline/Tween-20), followed by horseradish peroxidase-conjugated goat secondary antibodies (1:5000, R&D Systems, Minneapolis, MN). Chemiluminescence was detected using a Pierce ECL Plus Western Blotting Substrate (Thermo Scientific).

2.2.4. Immunostaining for VDR, RIG-1, LL-37, and hBD2

hTCEpi were grown on Lab-Tek 8-well chamber slides (Sigma) and were stimulated with $1,25D_3$ or left untreated. After 24 hours, cells were fixed in phosphate buffered saline (PBS) containing 4% paraformaldehyde (RIG-1) or methanol (VDR), blocked (PBS

containing 1% BSA/10% goat serum/0.3M glycine/0.1% Tween-20), and incubated with 5μg/ml primary antibodies or IgG controls overnight [rabbit anti-DDX58 RIG-1 (retinoic acid-inducible gene 1), Abcam (Cambridge, MA); rat anti-VDR, Millipore clone 9A7]. This was followed by incubation with Alexa Fluor 488-conjugated goat anti-rat (VDR) or Alexa Fluor 546-goat anti-rabbit (RIG-1) with 1μg/ml DAPI nuclear stain added along with the secondary antibodies.

For NFκβ p65 nuclear translocation, hTCEpi were stimulated for 2 hours with Poly(I:C) and/or $1,25D_3$. After treatment, cells were fixed in 4% paraformaldehyde, permeabilized in methanol, and then blocked with 3% BSA. Cells were incubated with rabbit anti-p65 NFκβ (5μg/ml, Abcam) or rabbit IgG control, followed by Alexa Fluor 488-conjugated donkey anti-rabbit with 1μg/ml DAPI nuclear stain.

For human corneal staining, human donor corneas were incubated, epithelial side up, in Optisol with or without $1,25D_3$ (10^{-7}M) for 24 hours. Following incubation, corneas were fixed in 4% paraformaldehyde for 2 hours, equilibrated in 30% sucrose/PBS overnight, and snap frozen in OCT with liquid nitrogen. 10μm cryosections were blocked for 2 hours at room temperature (2% BSA/.5% Tween-20/1μg/ml Fc-block/PBS) followed by overnight incubation with 2.5μg/ml primary antibodies to LL-37 or hBD-2 (Santa Cruz Biotechnology, Dallas, TX) or control IgG in PBS containing 2% BSA/0.5% Tween-20. Donkey anti-rabbit Alexa Fluor 488 conjugated secondary antibody (4μg/ml; Invitrogen) was applied for 1 hour and coverslips mounted with DAPI counterstain. Fluorescent images of the cells and tissue sections were obtained with a

DeltaVision Core inverted microscope (Applied Precision, Issaquah, WA) and images were deconvolved.

2.2.5. Antimicrobial Assay

To determine if vitamin D was able to influence cell antimicrobial activity, SV40-HCEC were incubated in serum and antibiotic-free media with and without $1,25D_3$ (10^{-7}M) for 24 hours. Supernatants were then collected and tested for antimicrobial activity against *Pseudomonas aeruginosa* ATCC 19660 and *Staphylococcus aure*us ATCC 29213 as previously described (Kumar et al., 2006a). Medium that was not incubated with cells was used as controls in this assay.

2.2.6. Enzyme-linked Immunosorbent Assay (ELISA) and Luminex Assay

hTCEpi were treated with D_3 or $25D_3$ for 24 hours and $1,25D_3$ was quantitated in cell supernatants with a $1,25D_3$ EIA kit according to the manufacturer's instructions (Immunodiagnostic Systems, Scottsdale, AZ). Following stimulation with $1,25D_3$ and Poly(I:C) for 24 hours, IL-8 and MMP-9 were detected in cell supernatants by ELISA, as per the manufacturer's instructions (Human IL-8 ELISA MAX, BioLegend, San Diego, CA; MMP-9 Human ELISA kit, Abcam). IL-1β, IL-6, TNFα, and CCL20 levels in culture supernatants were determined with the MILLIPLEX MAP Human Magnetic Bead Panel (EMD Millipore) using the MAGPIX system.

2.2.7. Flow Cytometry

Intracellular TLR3 expression was determined by flow cytometry, as previously described (Redfern et al., 2011). Briefly, hTCEpi were permeabilized in PBS containing 0.1% Triton X-100/3% BSA for 15 minutes on ice. After blocking in 3% BSA, cells were incubated with 10μg/ml mouse anti-TLR3 (Imgenex, San Diego CA) for 30 minutes followed by Alexa Fluor 488-conjugated goat anti-mouse (Life Technologies). Samples were run on a FACS Canto II (BD Biosciences, San Jose, CA) and data were analyzed with FACS Diva software (BD Biosciences).

2.2.8. Statistical Analyses

Statistical analyses were performed using unpaired, two-tailed, Student's t-tests in experiments comparing two samples. When more than two samples were analyzed, one-way ANOVA was used, with Bonferroni's test for multiple comparisons. All data are representative of a minimum of three independent experiments with $p \leq 0.05$ considered statistically significant. These tests were performed with GraphPad Prism 6.0 software (GraphPad Software Incorporation, San Diego, CA).

2.3. Results

2.3.1. Corneal cells express the machinery for vitamin D metabolism and are able to convert inactive vitamin D compounds to the fully functional $1,25D_3$.

As the cornea is directly exposed to UVB irradiation, we hypothesized that corneal cells also express the machinery to fully activate vitamin D and to produce the functional $1,25D_3$ metabolite. Cultured primary HCEC and the HCEC immortalized cell line, hTCEpi, expressed the activating 25-hydroxylases (CYP27A1 and CYP2R1) and 1-alpha-hydroxylase CYP27B1, and the deactivating enzyme 24-hydroxylase CYP24A1 (Figure 2.1.A). In addition, freshly isolated corneal epithelium, endothelium, and stromal cells from human donor corneas and SV40 HCEC also expressed these genes (see Appendix Figure A.1). We confirmed prior reports that HCEC express the VDR (Alsalem et al., 2014; Johnson et al., 1995; Yin et al., 2011a) and also showed that stimulating these cells with $1,25D_3$ resulted in a nuclear localization of the VDR, as would be expected with ligand-bound receptor (Figure 2.1.B).

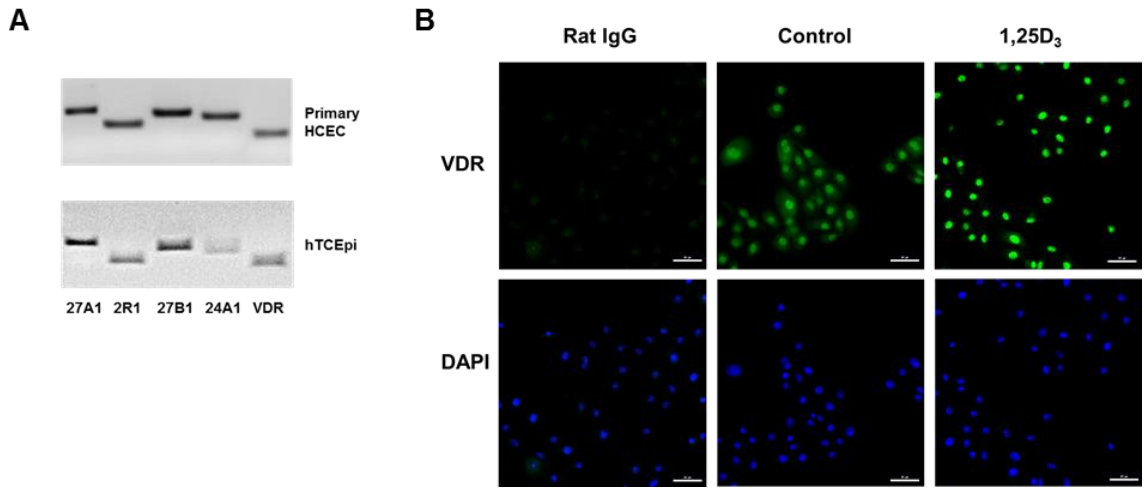


Figure 2.1. HCEC express the enzymes for vitamin D metabolism and a functional VDR.

(A) RT-PCR analysis of hydroxylase and VDR expression in primary HCEC and hTCEpi. (B) hTCEpi were stimulated with $1,25D_3$ (10^{-7}M) or vehicle for 24 hours, then fixed and stained for VDR (green) and DAPI nuclear stain (blue). Images are representative of three independent experiments. Scale bar = 40μm

Importantly, when stimulated with $25D_3$, hTCEpi were able to produce functional $1,25D_3$ that was detectable in culture supernatants (Figure 2.2.A). There was a statistically significant increase ($p<0.05$) in $1,25D_3$ levels with increasing concentrations of $25D_3$ up to a peak of 300 pmol/L with 10^{-7}M $25D_3$ treatment (n=4). Additionally, when cells were treated with 10^{-7}M of the unhydroxylated D_3, there was also a statistically significant ($p<0.05$) increase in $1,25D_3$ above unstimulated control supernatants, although at very low levels (Figure 2.2.B, n=4). This demonstrates that HCEC can directly activate vitamin D metabolites through hydroxylation to produce $1,25D_3$.

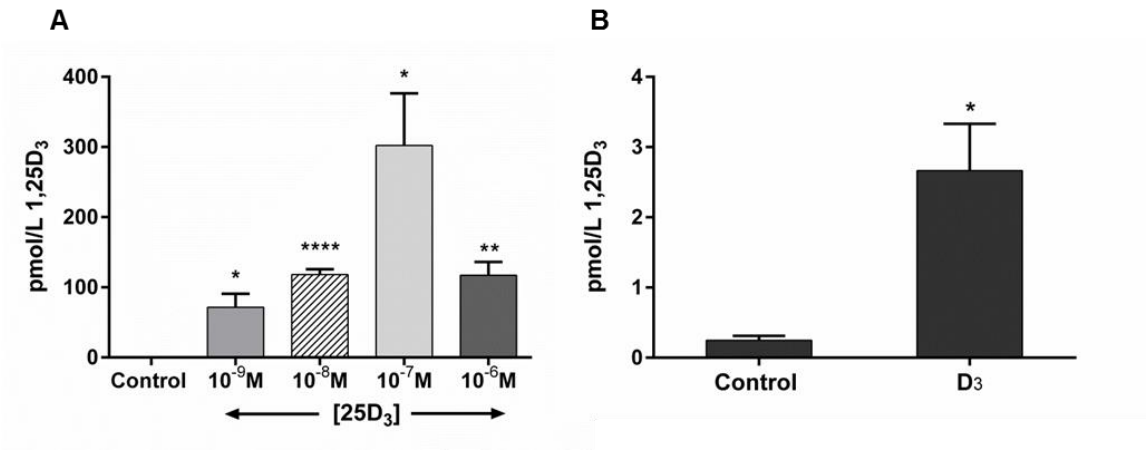


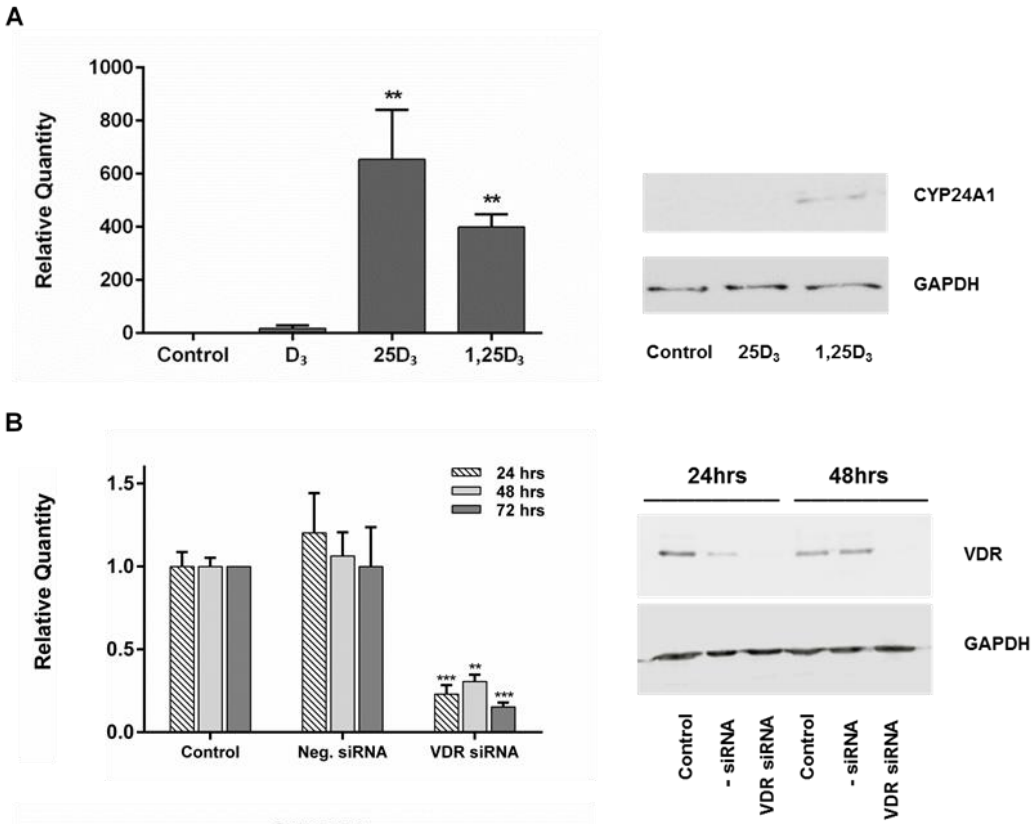
Figure 2.2. HCEC are able to convert inactive vitamin D compounds to the biologically active metabolite, 1,25D_3.

hTCEpi were treated with (A) 25D_3 (10^{-6} M-10^{-9} M) or (B) D_3 (10^{-7}M) for 24 hours and 1,25D_3 was quantitated in cell supernatants by immunoassay. Data represent mean +/- SEM of four independent experiments. Statistical analysis was by ANOVA with Bonferroni's test for multiple comparisons (A) and Student's t-test (B), p<*0.05, **0.01, ****0.0001

2.3.2. HCEC respond to vitamin D through the VDR.

In order to determine if HCEC respond functionally to locally activated vitamin D, primary cultured HCEC were treated with inactive D_3 and 25D_3, or 1,25D_3 for 24 hours. HCEC showed an increase in CYP24A1 gene expression with all vitamin D compounds tested (Figure 2.3.A, n=3). The unhydroxylated D_3 increased CYP24A1 expression 18-fold while 25D_3 stimulation yielded a 655-fold increase relative to untreated control cells. These results demonstrate that HCEC can activate then utilize vitamin D to influence gene transcription.

While most effects of $1,25D_3$ are mediated through the classical activation of its nuclear receptor, the VDR has also been shown to influence cytoplasmic signaling pathways and interact with G-protein coupled receptors at the cell membrane (Larriba et al., 2014). In order to assess if the upregulation of CYP24A1 was dependent on the VDR, we effectively knocked down its expression using siRNA, with decreases in mRNA expression of 80% (+/-5%) after 24 hours of transfection and 85% (+/-3%) after 72 hours (Figure 2.3.B). VDR protein was also knocked down to almost undetectable levels as determined by western blot by 24 hours. When hTCEpi were treated with $1,25D_3$, CYP24A1 mRNA expression increased 3000-fold (+/-279) after 24 hours and VDR silencing completely abolished this response ($p<0.0001$, $n=3$) (Figure 2.3.C), demonstrating not only that vitamin D can act through the VDR in these cells but also that the VDR is functional in corneal epithelial cells.


A
Relative Quantity
1000
800
600
400
200
0
**
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Control
D3
25D3
1,25D3
CYP24A1
GAPDH
Control
25D3
1,25D3
B
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1.5
1.0
0.5
0.0
24 hrs
48 hrs
72 hrs
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Control
Neg. siRNA
VDR siRNA
24hrs
48hrs
VDR
GAPDH
Control
- siRNA
VDR siRNA
Control
- siRNA
VDR siRNA
C
CYP24A1
Relative Quantity
****
3000
2500
2000
1500
100
50
0
Control
1,25D3
VDR siRNA
VDR siRNA+
1,25D3

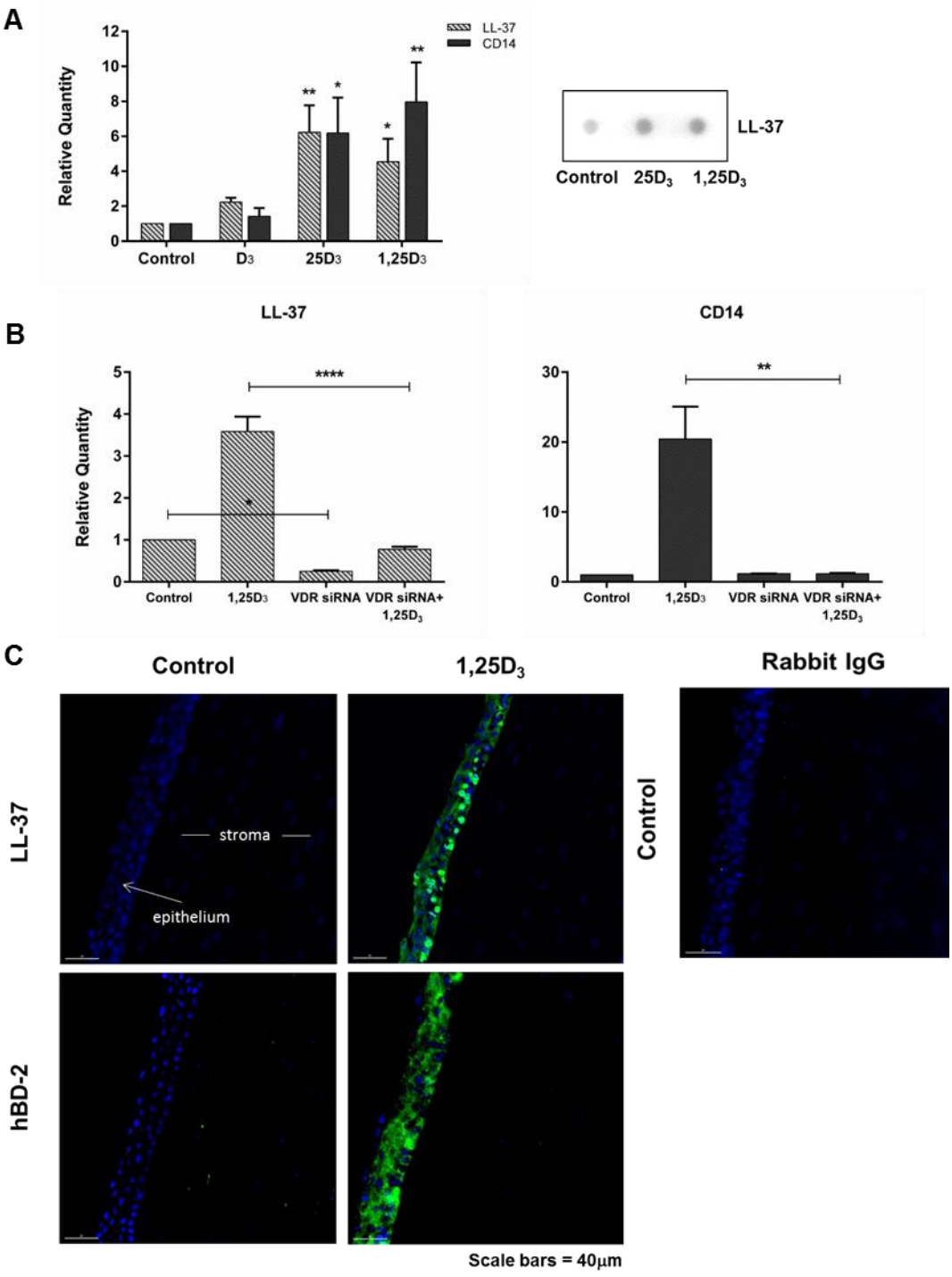
Figure 2.3. HCEC respond to vitamin D by inducing the expression of CYP24A1, regulated by the VDR.

(A) hTCEpi were treated with D_3, $25D_3$, or $1,25D_3$ (10^{-7}M) for 24 hours and CYP24A1 expression was determined by real-time PCR (left) and western blotting (right). Statistical analysis was by Student's t-test, p<**0.01. (B) hTCEpi were left untreated (control), transfected with a non-specific control siRNA (Neg), or VDR siRNA for 24, 48, or 72 hours and analyzed for VDR expression by real-time PCR (left) and Western blot (right). (C) hTCEpi were left untreated (control and $1,25D_3$) or transfected with VDR siRNA for 24 hours and then treated with $1,25D_3$ for 24 hours. CYP24A1 expression was analyzed by real-time PCR. Data represent mean +/- SEM of three independent experiments. Statistical analysis was by two-way ANOVA with Bonferroni's test for multiple comparisons (B) and one-way ANOVA with Bonferroni's test for multiple comparisons (C), p<**0.01, ***0.001, ****0.0001

2.3.3. Vitamin D increases the expression of vitamin D-regulated genes involved in innate immune defense and increases HCEC antimicrobial activity.

We next examined the ability of vitamin D to regulate the expression of several genes important in innate immunity. When hTCEpi were treated with vitamin D metabolites for 24 hours, expression of LL-37, as well as CD14, increased more than 4-fold (Figure 4A, left, n=3). The increase in LL-37 was also detected in supernatants from cells treated with either $25D_3$, or $1,25D_3$, as determined by dot blot analysis (Figure 2.4.A, right). This upregulation was dependent on signaling through the VDR, as knockdown of the receptor significantly inhibited both LL-37 ($p<0.0001$) and CD14 ($p<0.01$) expression following vitamin D treatment (Figure 2.4.B). In addition, VDR silencing significantly lowered the basal transcript levels of LL-37 by 75% ($p<0.05$).

To confirm the upregulation of innate immune response genes in primary tissue, whole human donor corneas were treated with $1,25D_3$ and examined for antimicrobial peptide expression by immunohistochemistry. There was an induction throughout the epithelial layer in LL-37 expression in the vitamin D treated corneas when compared to matched controls (Figure 2.4.C). In addition, human β defensin-2, hBD-2, another antimicrobial peptide that is expressed at the ocular surface, was also increased in the $1,25D_3$ treated corneas (Figure 2.4.C).


A
LL-37
CD14
Relative Quantity
Control
D3
25D3
1,25D3
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B
LL-37
CD14
Relative Quantity
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**
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1,25D3
VDR siRNA
VDR siRNA+ 1,25D3
C
Control
1,25D3
Rabbit IgG
LL-37
hBD-2
stroma
epithelium
Control
Scale bars = 40μm

Figure 2.4. Vitamin D increases the expression of vitamin D-regulated genes involved in innate immune defense.

(A) hTCEpi were treated with 10^{-7}M D_3, $25D_3$, or $1,25D_3$ for 24 hours and expression of LL-37 and CD14 was determined by real-time PCR (left) and LL-37 production was determined by dot blot (right). (B) hTCEpi were transfected with VDR siRNA for 24 hours and then treated with $1,25D_3$ for 24 hours. LL-37 and CD14 expression was analyzed by real-time PCR. Graphs represent mean +/- SEM of three independent experiments. Statistical analysis was by ANOVA with Bonferroni's test for multiple comparisons, p<*0.05, **0.01 compared to control; p<*****0.0001 compared to $1,25D_3$. (C) Human corneas were treated with $1,25D_3$ for 24 hours, fixed in paraformaldehyde, and frozen sections stained for LL-37 or hBD-2 (green) and DAPI nuclear stain (blue). Images are representative of 2-3 independent experiments.

To investigate a possible functional consequence of the modulation of innate immunity related genes, SV40-HCEC were treated with $1,25D_3$ and the supernatants were incubated with a clinical isolate of *P. aeruginosa* (ATCC 19660), a common ocular pathogen, to determine antimicrobial activity. Supernatants treated with $1,25D_3$ (Cells+$1,25D_3$) were more effective at killing *P. aeruginosa* than cells alone, with a decrease in bacterial colonies after incubation (40%, $p<0.01$) (Figure 2.5, left). This suggests that $1,25D_3$ initiated the production of an antimicrobial agent by the SV40-HCEC, leading to a decrease in colony growth. However, the same effect was not seen against the gram-positive organism *S. aureus* (see Appendix Figure A.2). Vitamin D on its own did not increase bacterial killing (Figure 2.5, right).

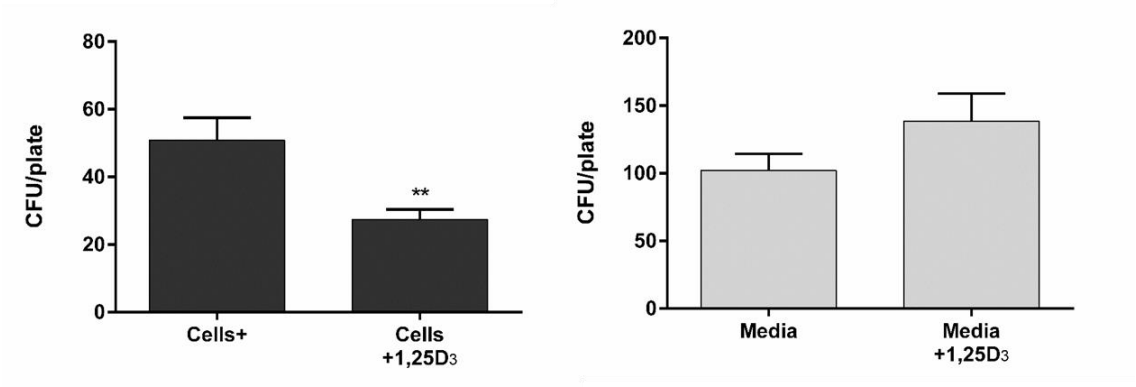

Figure 2.5. Vitamin D increases HCEC antimicrobial activity against *P. aeruginosa*.

SV40-HCEC were left untreated (Cells) or treated with $1,25D_3$ (10^{-7}M) for 24 hours (Cells+$1,25D_3$) and supernatants used in an antimicrobial assay (left). For controls, media only (Media) or $1,25D_3$ in media (Media+$1,25D_3$) were used without cells (right). Data represent mean +/- SEM of three independent experiments. Statistical analysis was by Student's t-test, p<**0.01.

2.3.4. TLR activation increases hydroxylase expression and enhances vitamin D-mediated antimicrobial peptide production.

As TLRs are important sensors of the innate immune system and have been shown to interact with vitamin D's activation and antimicrobial response, we investigated the ability of TLRs to regulate vitamin D hydroxylase expression in HCEC. hTCEpi were stimulated with TLR agonists specific for TLR2 (Pam3CSK4, FSL1, Zymosan), TLR3 (Poly(I:C)), or TLR5 (Flagellin) for 24 hours. Both CYP27B1 and CYP24A1 expression were significantly increased by TLR activation (Figure 2.6.A) ($p<0.05$, n=4-5). Specifically, both TLR2 and TLR3 agonists induced the expression of the activating enzyme, CYP27B1, with the largest increase (21-fold $\pm$5) initiated through Poly(I:C), a double-stranded (ds) RNA analog similar to that of viruses.

We next sought to determine if LL-37 expression could be augmented by combined TLR agonist-vitamin D treatment. Focusing on TLR2 and TLR3 agonists, when $1,25D_3$ was added to cells along with FSL1, Pam3CSK4, Zymosan, or HKLM (TLR2), or with Poly(I:C) (TLR3), there was an increase in LL-37 expression (Figure 2.6.B and Appendix Figure A.3). Poly(I:C) was the most effective in augmenting LL-37 expression in combination with $1,25D_3$, with a 9-fold increase above control (n=4). These results suggest that vitamin D can cooperate with TLRs to enhance innate immunity in HCEC.

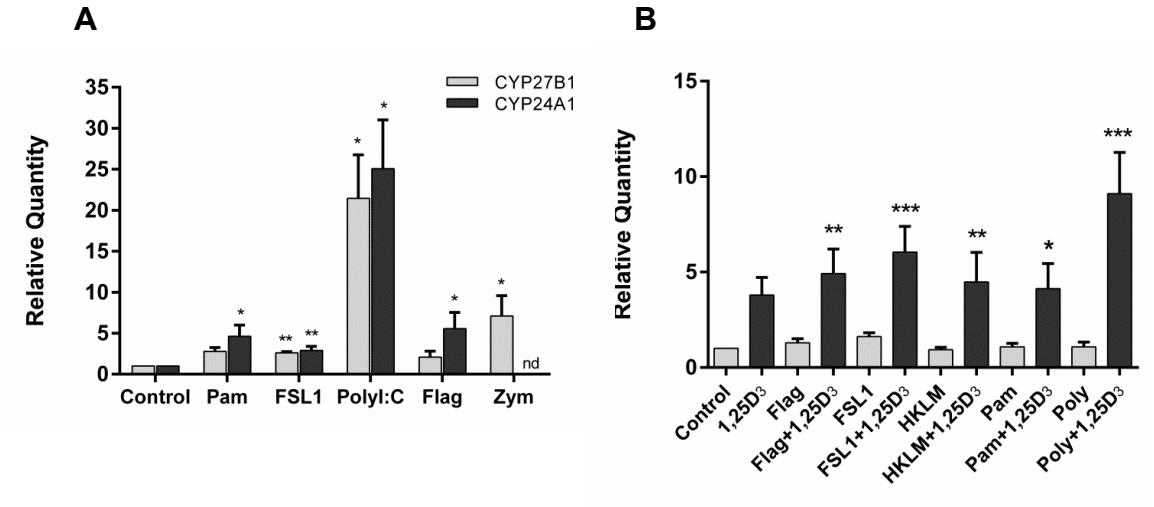

Figure 2.6. TLR activation increases HCEC hydroxylase expression and enhances vitamin D-mediated LL-37 production.

(A) hTCEpi were treated with 1μg/ml TLR agonists for 24 hours (Pam3CSK4 [Pam], FSL1, Poly(I:C), Flagellin [Flag]) or 50μg/ml zymosan (Zym) for 6 hours. CYP27B1 and CYP24A1 expression were determined by real-time PCR. nd = not determined. (B) hTCEpi were treated with TLR agonists [Flag, FSL1, HKLM, Pam, or Poly(I:C)], in combination with $1,25D_3$ (10^{-7}M) for 24 hours and LL-37 expression analyzed by real-time PCR. Data represent mean +/-SEM of 4-5 independent experiments. Statistical analysis was by ANOVA with Bonferroni's test for multiple comparisons, p<*0.05, **0.01, ***0.001

2.3.5. Vitamin D modulates the HCEC response to Poly(I:C) through decreased production of pro-inflammatory cytokines and MMP-9.

Because vitamin D is known to have anti-inflammatory activities in other tissues, we hypothesized that vitamin D treatment could affect cytokine production in HCEC. As shown in Figure 2.7, when hTCEpi were treated with Poly(I:C) for 24 hours, there was a large increase in interleukin (IL)-8 at both mRNA and protein levels (~1000 and ~230

fold increases, respectively). When $1,25D_3$ was added to cells with Poly(I:C), there was a significant decrease in IL-8 production (50% decrease in mRNA and 86% decrease in protein, $p<0.001$, $n=4$). Similarly, IL-1β, IL-6, TNFα, CCL20 (MIP3α), IL-23, and IFNγ levels were attenuated by $1,25D_3$ treatment (Figure 2.7 and Appendix Figure A.4). When $1,25D_3$ was added to the cells four hours after Poly(I:C) stimulation, IL-8 levels were also decreased (38%, $n=4$) (See Chapter 3.3.1, Figure 3.4). The suppression of these inflammatory mediators appears to be dependent on signaling through the VDR, as treatment with VDR siRNA blocked the downregulation of IL-8 by $1,25D_3$ (Appendix Figure A.5). However, LL-37 was not responsible for the decrease in IL-8 expression (Appendix Figure A.5).

In addition to cytokines and chemokines, matrix metalloproteinases (MMPs) are also upregulated by inflammatory stimuli at the ocular surface (Jeong et al., 2012; Li and Pflugfelder, 2005). MMP-9 levels were significantly decreased by 20% when hTCEpi were treated with $1,25D_3$ and Poly(I:C) together ($p<0.05$, $n=4$) (Figure 2.8.A). $1,25D_3$ also reduced basal levels of MMP-9 transcript (42%, $p<0.0001$) and suppressed its expression induced by other TLR agonists, FSL1 (TLR2; $p<0.0001$) and flagellin (TLR5, $p<0.05$) (Figure 2.8.B).

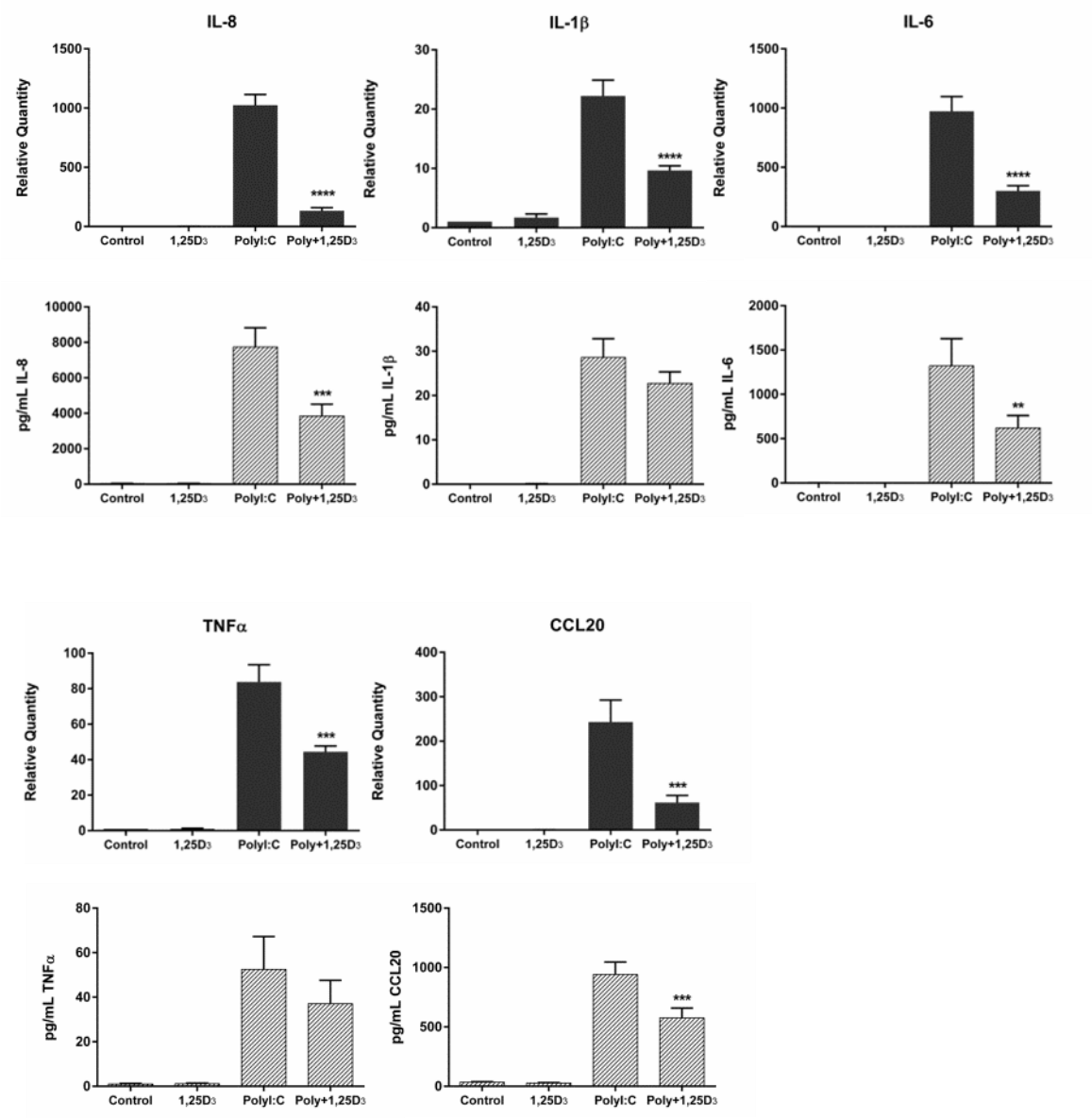

Figure 2.7. Vitamin D modulates HCEC response to Poly(I:C) through decreased production of pro-inflammatory cytokines.

hTCEpi were treated with $1,25D_3$ (10^{-7}M) and/or Poly(I:C) (1μg/ml) for 24 hours. Cell lysates were collected for RNA isolation followed by real-time PCR analysis (top row) and supernatants were used to quantify protein levels (bottom row) of IL-8 (ELISA), and IL-1β, IL-6, TNFα, or CCL20 (Luminex assay). Data represent mean +/-SEM of 4-5 independent experiments. Statistical analysis was by ANOVA with Bonferroni's test for multiple comparisons, with comparison to Poly(I:C) treatment alone, p<**0.01, ***0.001, ****0.0001

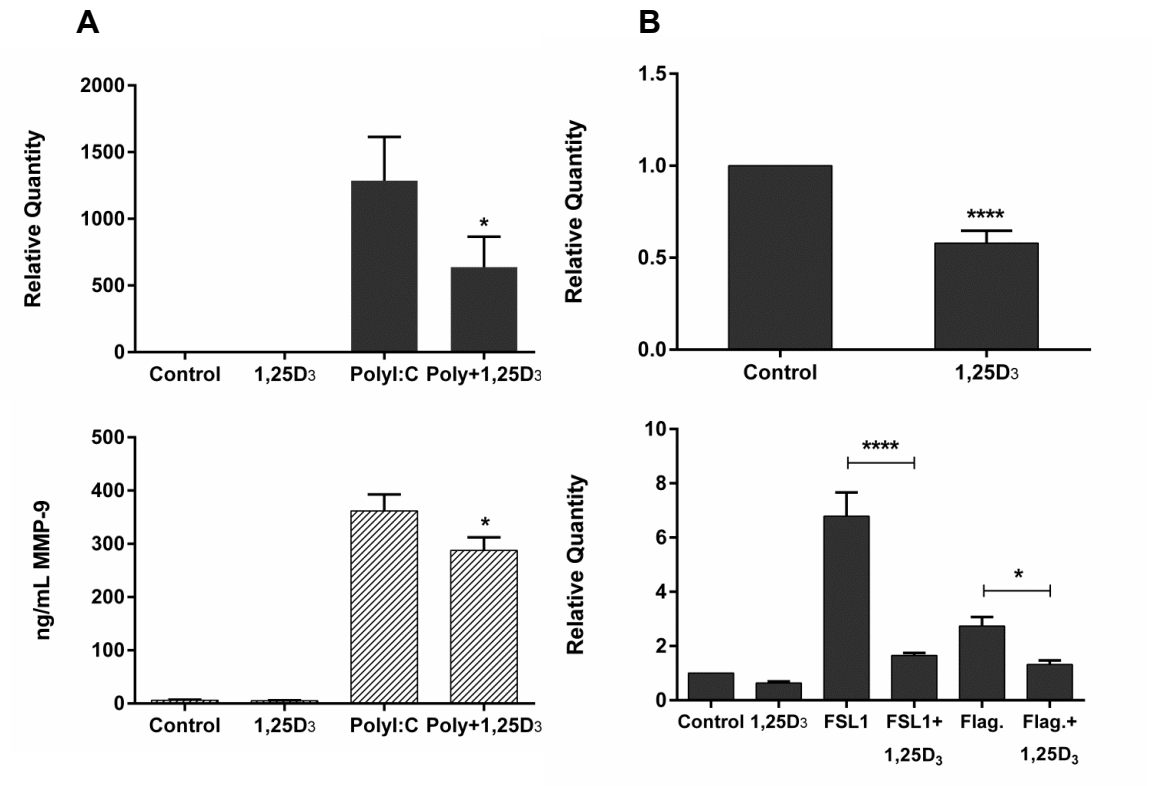

Figure 2.8. Vitamin D treatment decreases both basal levels of MMP-9 and expression induced by TLR activation.

(A) hTCEpi were treated with $1,25D_3$ (10^{-7}M) and/or Poly(I:C) (1μg/ml) for 24 hours. Cell lysates were collected for RNA isolation followed by real-time PCR analysis (top) and supernatants were used to quantify protein levels of MMP-9 by ELISA (bottom). Data represent mean +/-SEM of 4-5 independent experiments. Statistical analysis was by ANOVA with Bonferroni's test for multiple comparisons or Student's t-test, p<*0.05, with comparison between Poly(I:C) and Poly(I:C)+ $1,25D_3$. (B) hTCEpi were treated with $1,25D_3$ (10^{-7}M) for 24 hours (top) or $1,25D_3$ in combination with FSL1 or Flagellin (bottom). Relative quantity of MMP-9 was assessed by real-time PCR. Data represent mean +/-SEM of 4-5 independent experiments. Statistical analysis was by ANOVA with Bonferroni's test for multiple comparisons or Student's t-test, p<*0.05, ****0.0001

2.3.6. Vitamin D decreases the expression of PRRs that are activated in response to Poly(I:C) but activation of downstream NFκβ is not affected.

In order to understand this anti-inflammatory response, specifically to Poly(I:C)-induced inflammation, we investigated if vitamin D can influence PRR expression in HCEC. Following 24 hours of treatment, $1,25D_3$ lowered the RNA expression of TLR3 that was induced by Poly(I:C) 36% ($p<0.05$, n=5) (Figure 2.9.A, left). Using flow cytometry, we observed that $1,25D_3$ also decreased the intracellular expression of TLR3 (Figure 2.9.A, right). Poly(I:C) is a ligand for TLR3 but can also activate the RIG-I (retinoic acid-inducible gene 1)-like receptors RIG-1 and MDA5, cytoplasmic dsRNA helicases that recognize viral RNA. hTCEpi not only expressed these receptors, but the addition of $1,25D_3$ decreased both RIG-1 and MDA5 to ~50% of control levels ($p<0.01$, 0.001, n=5) (Figure 2.9.B and 2.9.C). Similar to TLR3 expression, PolyI:C stimulation increased levels of RIG-1 and MDA5, which were lowered by combined treatment with $1,25D_3$ (see Appendix Figure A.5).

After determining that vitamin D decreased PRR expression, we next wanted to examine downstream activation of NFκβ. When hTCEpi were stimulated with Poly(I:C) for 2 hours, nuclear staining of the NFκβ p65 subunit (green) increased and there was a corresponding decrease in fluorescent staining in the cytoplasm (Figure 2.9.D). However, concurrent treatment or 24 hour pretreatment with $1,25D_3$ did not affect p65 translocation.

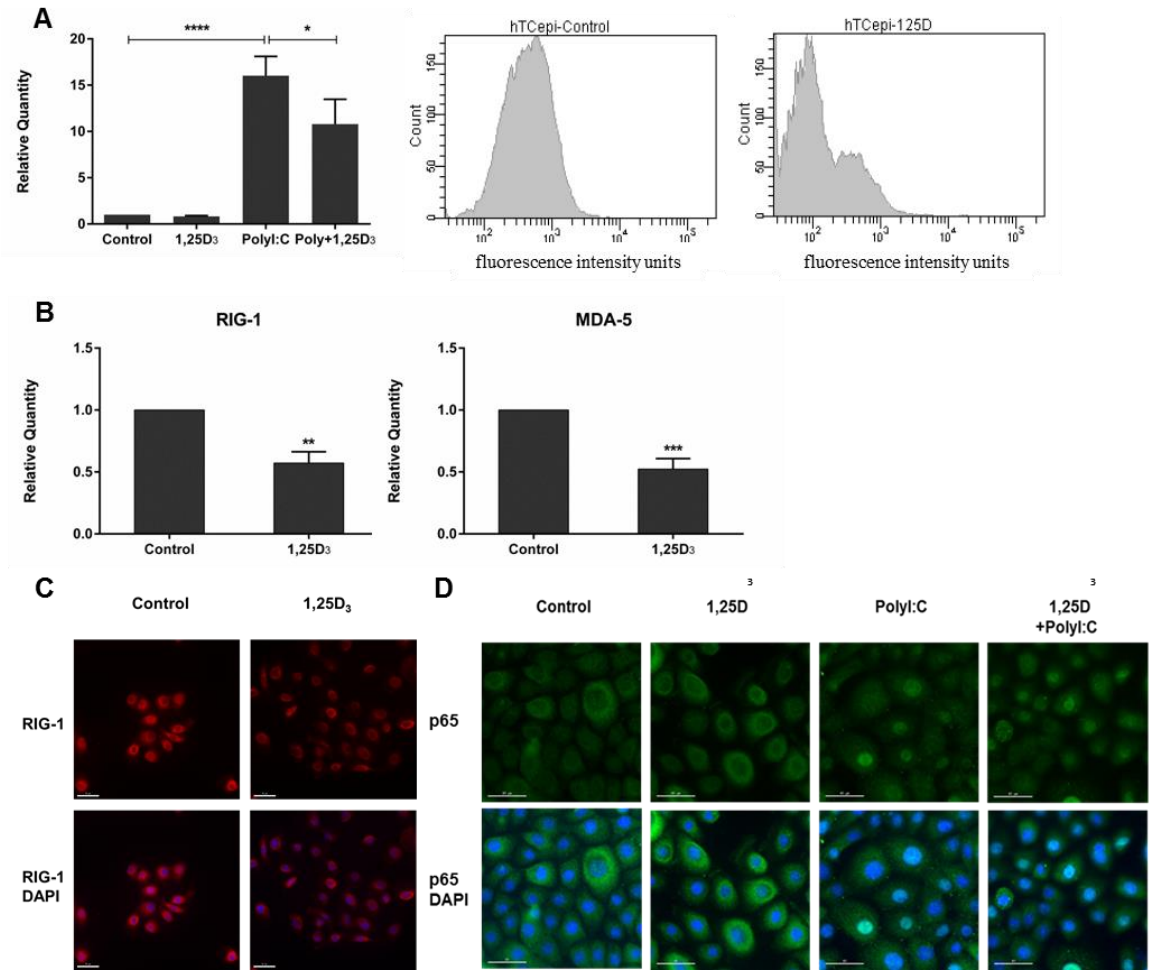

Figure 2.9. Vitamin D lowers the expression of PRRs TLR3 and RIG-1/MDA-5 but does not block NFκβ p65 nuclear translocation following Poly(I:C) stimulation.

(A) hTCEpi were treated with $1,25D_3$ (10^{-7}M) and/or Poly(I:C) (1μg/ml) for 24 hours. TLR3 expression was determined by real-time PCR (left) and flow cytometry analyses (middle and right). (B) RIG-1 (left) and MDA-5 (right) expression was determined by real-time PCR. Data represent mean +/- SEM of 3-4 independent experiments. Statistical analysis was by ANOVA with Bonferroni's test for multiple comparisons (A) or Student's t-test (B), p<*0.05, **0.01, ***0.001, ****0.0001. (C) hTCEpi were stimulated with $1,25D_3$ (10^{-7}M) or vehicle for 24 hours, then fixed and stained for RIG-1 (red) and DAPI nuclear stain (blue). (D) hTCEpi were stimulated with $1,25D_3$ and/or Poly(I:C) for 2 hours and stained with p65 (green) and DAPI nuclear stain (blue). Images are representative of three independent experiments. Scale bar = 40μm

2.4. Discussion

The cornea is the avascular tissue that forms the anterior surface of the eye, covering the pupil and iris. One of its major functions is to refract light, focusing the entry of light back onto the retina, for photodetection. The cornea is responsible for two-thirds of the eye's total focusing power and tissue transparency is essential for this function. The cornea also functions to protect the rest of the eye from damage, providing a barrier from physical, microbial, UV and other insults (Rogers, 2010). Given vitamin D's known role in modulating inflammation and antimicrobial peptide production, we hypothesized that vitamin D could aid in protecting the cornea, both from inflammation and infection, and thus help maintain the function of this tissue. In this study, we examined the ability of HCEC to produce active vitamin D, $1,25D_3$, and to respond to vitamin D treatment through a functional VDR, resulting in altered gene expression of target genes. In addition, we explored the ability of vitamin D to dampen the expression of inflammatory mediators induced by TLR stimulation, while enhancing antimicrobial peptide production.

In order to ascertain the capacity of the cornea to utilize vitamin D and its metabolites and determine the source of vitamin D in the cornea, a better understanding is needed of the production of active vitamin D. Corneal epithelial cells, like keratinocytes of the skin, are directly exposed to UVB from sunlight. Upon exposure, keratinocytes are able to convert 7-dehydrocholesterol into pre-vitamin D, D_3, and then produce the major circulating form of the hormone, $25D_3$ (DeLuca, 2004a). The epidermis is able to then

fully activate $25D_3$ through 1-alpha hydroxylation (CYP27B1) to $1,25D_3$, and use it locally, where vitamin D exerts a range of cellular effects. It has been shown that human limbal epithelial cells are also able to produce D_3 and $25D_3$ following stimulation with UVB (Lin et al., 2012). However, this study did not detect the active $1,25D_3$ in cell supernatants. Our current study directly demonstrates for the first time that human corneal epithelial cells are able to produce the biologically active form of vitamin D, $1,25D_3$, from precursors D_3 and $25D_3$. In addition, these cells express the hydroxylases necessary for full vitamin D activation and inactivation and express a functional receptor. Therefore, it is reasonable to hypothesize that the cornea is able to perform *de novo* synthesis of vitamin D when exposed to sunlight, providing this tissue with a local source of the hormone. Although we attempted to block hydroxylase activity with non-specific cytochrome p450 inhibitors ketoconazole and itraconazole, and specifically with CYP27B1 siRNA, we were not able to block gene induction.

Upon activation and binding to the VDR in other tissues, $1,25D_3$ exerts transcriptional influence through receptor interactions on the promoter regions of genes containing specific response elements, VDREs. Vitamin D regulates its own activity through the production of the CYP24A1 gene, which is strongly induced by $1,25D_3$ binding to the VDR (Kumar et al., 2010; Prosser and Jones, 2004; Schuster, 2011). The resulting enzyme is able to hydroxylate both $25D_3$ and $1,25D_3$, inducing their catabolism, and maintaining vitamin D homeostasis (Jones et al., 2014, 2012). We have shown that HCEC respond to stimulation with vitamin D metabolites by inducing the expression of CYP24A1. This induction is mediated through a functional VDR, as silencing of the

receptor blocks this response. HCEC are therefore able to regulate the activity of vitamin D when it is present.

The cornea is responsible for protecting the rest of eye from not only physical damage, but from infection as well. It is therefore critically important for this tissue to be able to respond quickly and effectively when exposed to pathogens. Vitamin D has been shown to directly induce the production of the antimicrobial peptide, LL-37 (Wang et al., 2004), which has broad spectrum activity against not only bacteria, but viruses and fungi as well (Vandamme et al., 2012; Zanetti, 2004). Like LL-37, hBD-2 is a potent antimicrobial peptide that is expressed at the ocular surface and is known to be increased by inflammatory signals (McDermott, 2004; McDermott et al., 2003). Vitamin D indirectly regulates, both positively and negatively, hBD-2 expression in some cell types, including keratinocytes (Liu et al., 2009; Peric et al., 2009, 2008). In this study, vitamin D treatment induced an increase in LL-37 in HCEC both at the RNA and protein levels. Additionally, $1,25D_3$ increased both LL-37 and hBD-2 in the epithelium of human donor corneas. However no induction of hBD-2 RNA was seen in cultured cells. It would be interesting to further examine the regulation of antimicrobial peptide production, including β-defensins, by vitamin D during infection or pathogenic challenge. Notably, vitamin D increased the bactericidal activity of HCEC against *P. aeruginosa*, as supernatants from vitamin D treated cells killed more bacteria than control. Although not directly tested, we speculate that increased antimicrobial peptide expression likely contributes to this effect. This functional consequence of vitamin D exposure would be

expected to provide a significant benefit to the ocular surface, helping to protect it from infection.

Also important for innate defense in the cornea, TLRs recognize microbial ligands and initiate recruitment of immune cells to the site of infection (Takeda et al., 2003). TLRs are expressed at the ocular surface and contribute to the inflammatory process during infection and wound healing (Redfern et al., 2011). Previous studies have shown that TLR engagement can lead to increased vitamin D activation and enhanced antimicrobial peptide production. For example, TLR2/1 ligands up-regulate the VDR and CYP27B1 in human macrophages, increasing responsiveness to $1,25D_3$, which in turn augments LL-37 production, leading to *Mycobacterium tuberculosis* killing (Liu et al., 2006). Skin injury also results in increased vitamin D activation, causing induction of LL-37, CD14, and TLR2 expression (Schauber et al., 2007). In a positive feedback loop, TLR2 activation further up-regulates LL-37 in the presence of $1,25D_3$. In respiratory epithelial cells, double-stranded RNA, a TLR3 agonist, also stimulates CYP27B1 expression, LL37, and CD14 (Hansdottir et al., 2008). Similarly, here we show that TLR2 and TLR3 agonists increased the expression of both regulatory hydroxylases CYP24A1 and CYP27B1 in HCEC, demonstrating the potential of the cornea to regulate vitamin D activity. These TLR agonists also acted in concert with $1,25D_3$ to further enhance antimicrobial peptide production, increasing the protective ability of HCEC during inflammation.

Engagement of TLRs in the cornea also results in activation of an inflammatory response. While important for defense against infection, this response must be regulated

to protect against tissue damage. dsRNA (PolyI:C agonist) in particular induces a robust response in human corneal epithelial cells (Kumar et al., 2006b), producing potentially harmful levels of pro-inflammatory mediators. In an immunosuppressive role, vitamin D is known to decrease inflammatory mediators in a variety of tissues during disease, infection, and TLR activation (Aranow, 2011; Sadeghi et al., 2006; White, 2008). Several studies demonstrate that vitamin D can also have an anti-inflammatory effect at the ocular surface. Vitamin D decreased inflammatory cytokines and infiltrates in animal models of corneal transplantation and injury (Dang et al., 2004; Suzuki et al., 2000a) and suppressed the expression of IL-1α (Suzuki et al., 2000b), IL-1β, IL-6 and IL-8 (Xue et al., 2002b) in cultured human corneal epithelial cells. In our studies, $1,25D_3$ acted through the VDR to downregulate the expression of IL-1β, IL-6, TNFα, IL-8, and CCL20 (MIP3α) induced by TLR3 agonist Poly(I:C). In addition, $1,25D_3$ lowered MMP-9 expression. Mechanistically, these results could be explained in part by the decrease in PRR expression seen with vitamin D treatment. Interestingly, this anti-inflammatory response was not an immediate response, as vitamin D did not decrease IL-8 levels prior to 6 hours of co-treatment with Poly(I:C) (see Chapter 3.3, Figure 3.3) and did not prevent the NFκβ nuclear translocation that occurs quickly after Poly(I:C) treatment. This pattern of response to vitamin D, allowing the initiation of inflammatory signals, could be helpful, particularly during infection and wound healing, where limited amounts of cytokine and chemokine production are beneficial for the initial inflammatory event. Whereas vitamin D is able to attenuate levels of proinflammatory mediators later in the response, potentially protecting the local tissue environment from damage. In the

cornea it is critically important to minimize inflammation that could disrupt transparency. Therefore, vitamin D's role in immunomodulation and anti-microbial peptide production, as we have shown, will be important to study in the context of ocular surface inflammation, such as that occurs during infection or dry eye syndrome.

Chapter 3

The Effect of Vitamin D on TLR3 activation and Gene Expression in Human Corneal Epithelial Cells

3.1. Introduction

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 microbial ligands, or pathogen-associated molecular patterns (Takeda et al., 2003). 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 kappa β (NFkβ), activator protein-1 (AP-1), signal transducer and activator of transcription (STAT), and interferon response factor (IRF) transcription factors (Chinenov et al., 2013; Takeuchi and Akira, 2010). In particular, NFkβ is recognized as the key regulator of inflammatory signaling, enhancing proinflammatory cytokine levels and also influencing cell survival (Chinenov et al., 2013; Hayden and Ghosh, 2012; Lawrence, 2009). 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 (Blanco et al., 2008). Mediators such as IL-1β and TNFα, for example, are produced by TLR activation and in turn lead to increased NFκβ activation and activation of the MAPK (mitogen-activated protein kinases) 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 induce loss of transparency, essential for vision. TLRs are present at the ocular surface and, in addition to being protective against infection, have also been implicated in the pathogenesis of dry eye syndrome, an inflammatory condition that affects millions of individuals in the United States (Redfern et al., 2013; Schaumberg et al., 2003). Therefore, an important area of research is the development of new anti-inflammatory therapeutics that limit aberrant ocular surface inflammation. Vitamin D has been studied for its role in suppressing inflammation in other tissues. In previous studies, we have demonstrated that vitamin D is able to decrease proinflammatory mediators induced by TLRs in human corneal epithelial cells (HCEC) (see Chapter 2.3). Therefore, the goal of the current study was to examine this mechanism further through timing of the vitamin D response to TLR activation and microarray analysis.

3.2. Materials and Methods

3.2.1. Cell Culture and Treatment

Immortalized and primary cultured human corneal epithelial cells (HCEC) were used in these studies. KGM-2 medium (Lonza, Allendale, NJ) and SHEM media were used to culture telomerase-immortalized human corneal epithelial cells (hTCEpi) and SV40-immortalized HCECs, respectively (Robertson et al., 2005; Araki-Sasaki et al., 1995).

Human donor corneas were received from Saving Sight eye bank (St. Louise, MO) and primary HCEC isolated following an overnight corneal digestion with Dispase II (Roche Diagnostics, Indianapolis, IN) (McDermott et al., 2003). Cells were treated with 10^{-7}M $1,25D_3$ (Sigma-Aldrich, St. Louis, MO) and 1μg/ml TLR3 agonist polyinosinic-polycytidylic acid, Poly(I:C), (Invivogen, San Diego, CA) for the indicated times. For the IKβα inhibitor study, hTCEpi were pretreated with 10μM Bay 11-7082 (Invivogen) for 30 minutes before Poly(I:C) addition for 6 hours.

3.2.3. RNA Isolation and Quantitative Real-time PCR

Total RNA was extracted from cultured cells using TRIzol reagent (Life Technologies, Grand Island, NY) and RNeasy kits with DNase I treatment (Qiagen, Valencia, CA). RNA concentration was measured with a Nanodrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE) and cDNA was transcribed with an AffinityScript cDNA synthesis kit (Agilent Technologies, Santa Clara, CA). Real-time PCR was performed using Brilliant II SYBR Green QPCR master mix (Agilent Technologies) and intron-spanning primers (Table 3.1). Untreated samples served as the calibrator for relative quantity determination and all samples were normalized to the housekeeping gene, GAPDH (See Chapter 2.2.2).

Table 3.1. Primer sequences for real-time PCR.

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

3.2.2. Sample Preparation and Microarray Analyses

hTCEpi and SV40 cell lines were treated with $1,25D_3$ (10^{-7}M) or vehicle (0.01% ethanol/PBS) for 6 hours and RNA collected as above. Biological replicates and dye-swapped samples were used in a genome-wide microarray analysis performed using Human Op-Arrays (Microarrays, Inc., Huntsville, AL) (Figure 3.1). Each array contained 35,035 oligonucleotide probes and 25,100 unique genes and were prepared as previously described (Katchy et al., 2014; Richter et al., 2006; Williams et al., 2007). In brief, following RNA isolation, sample quality was assessed with an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). All RNA samples had integrity values of 10, indicating negligible degradation, with clear bands/peaks for 28S and 18S ribosomal RNA (Figure 3.2). Indirect labeling of cDNA was performed using aminoallyl modified nucleotides (aa-dUTP+dNTP, Sigma-Aldrich), 15μg of starting RNA, and random hexamer primers (Invitrogen, Life Technologies). After purification (MinElute columns, Qiagen), flourophores Cy3 and Cy5 (Amersham, GE Healthcare, Pittsburgh, PA) were coupled to cDNA (Figure 3.2), re-purified, and concentrations verified on a Nanodrop 2000 spectrophotometer. Pooled and dye-swapped samples were applied to pre-hybridized microarray slides with coverslips and allowed to hybridize for 38 hours at 42^{o}C. After washing, dried slides were read on a GenePix 4000B Microarray Scanner (Molecular Devices, Sunnyvale, CA) at 10μm resolution. TIFF images were analyzed with GenePix Pro 6.0 software (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 cut-off 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 (Smyth, 2004).

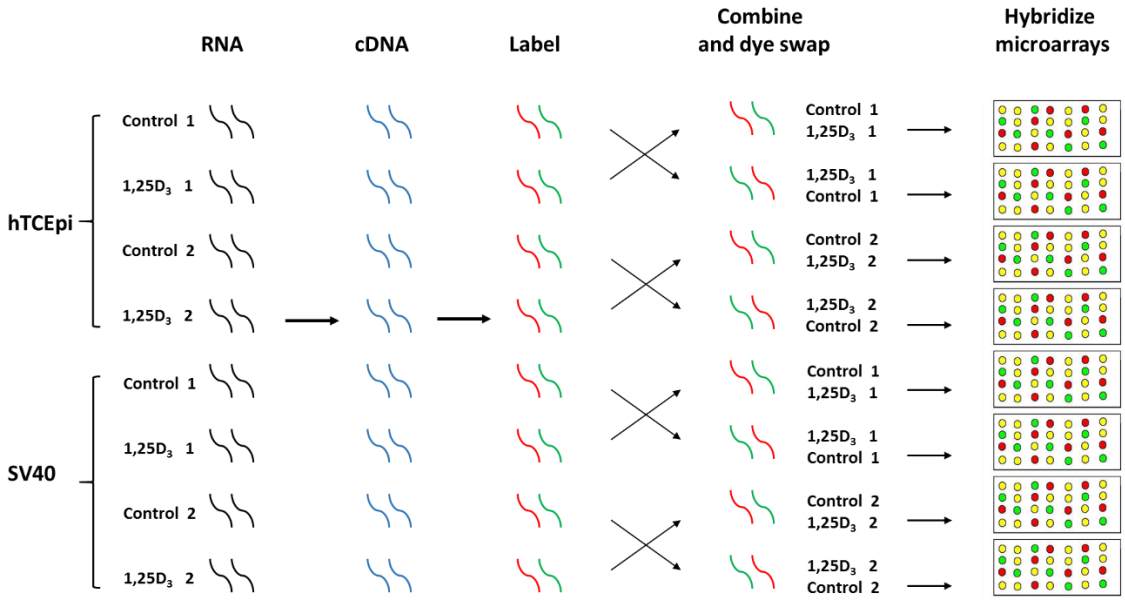

Figure 3.1. Microarray analysis sample preparation scheme.

Analysis was performed with two biological replicates of each cell line treated with 1,25D_3 for 6 hours. Following cDNA synthesis, samples were split into two different tubes and labeled with Cy3 or Cy5 fluorescent dyes. Differentially labeled 1,25D_3 treated and control samples from each replicate were combined and then hydridized onto microarray slides, for a total of 8 arrays.

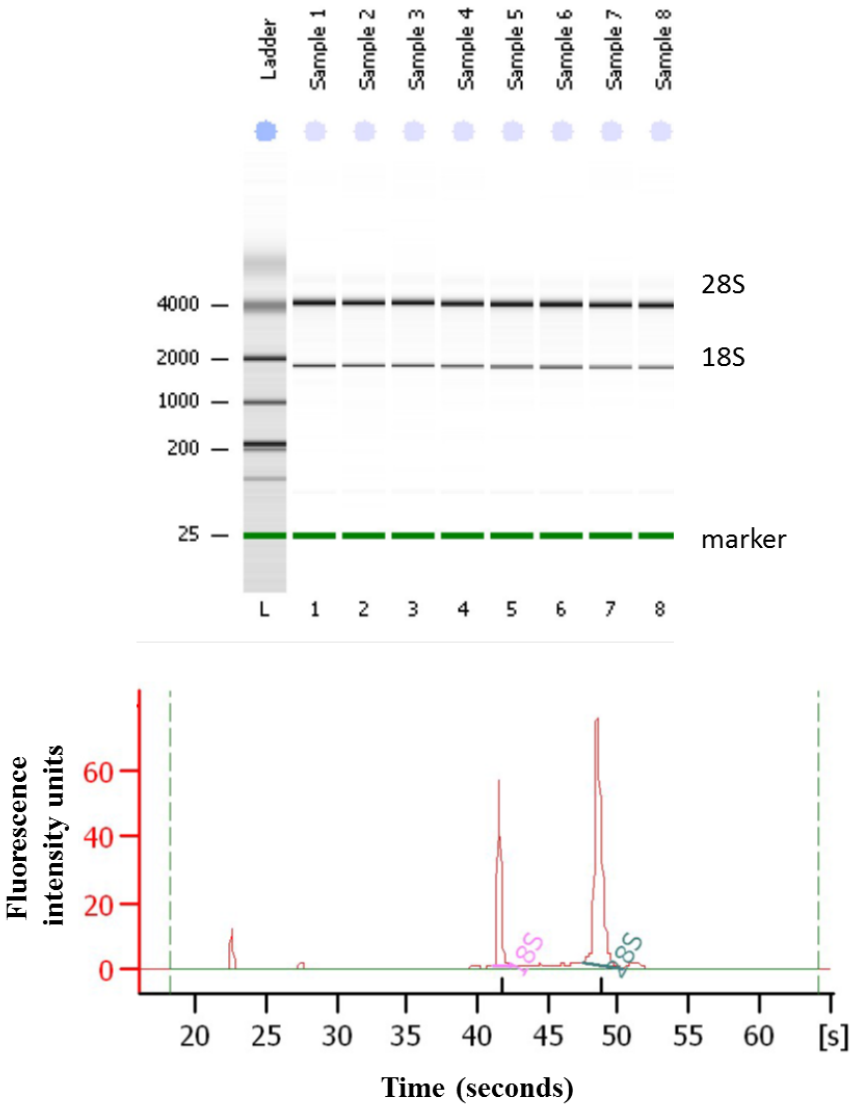

Figure 3.2. BioAnalyzer evaluation of RNA quality.

(Top) Gel image of RNA samples run on a BioAnalyzer chip with intact 28S and 18S ribosomal RNA bands. Lanes 1-4 are hTCEpi and 5-8 are SV40 samples. (Bottom) Example of fluorescence histogram from one RNA sample, showing the 18S and 28S peaks.

3.2.5. Gene Ontology Classification

Functional groups and gene annotation clusters in differentially expressed genes were identified using online software DAVID (Database for Annotation, Visualization, and Integrated Discovery; http://david.abcc.ncifcrf.gov) (D. W. Huang et al., 2009, 2007) and PANTHER (Protein ANalysis THrough Evolutionary Relationships; http://www.pantherdb.org) (Mi et al., 2013), as well as Pathway Studio (Elsevier Inc., Maryland). p-values <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.

3.2.4. Protein Detection in Cell Lysates and Supernatants

IL-8 was measured in cell supernatants by ELISA, following stimulation with $1,25D_3$ and Poly(I:C) for the indicated times, as per the manufacturer's instructions (Human IL-8 ELISA MAX, BioLegend, San Diego, CA). For total IKβα and phospho-p38α (T180/Y182) protein determination, cell lysates were collected in lysis buffer #6 (1mM EDTA, 0.5% Triton X-100, 5mM NaF, 6M urea, 1mM sodium orthovanadate, 2.5mM sodium pyrophosphate, 10μg/mL Leupeptin, 10μg/mL, Pepstatin, 100μM PMSF, 3μg/mL Aprotinin in PBS) and assayed with DuoSet ELISA kits (R&D systems, Minneapolis, MN). For the human phospho-kinase array, total cell lysates were assayed with a Proteome Profiler Antibody Array (R&D systems) (see Appendix Table A.1). 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.

3.3. Results

3.3.1. Vitamin D differentially effects Poly(I:C)-induced IL-8 expression dependent on timing of treatment.

Previous studies have indicated that vitamin D plays a role in dampening inflammatory cytokine production following TLR activation after 24 hours of treatment (see Chapter 2). In the current study, when hTCEpi were co-stimulated with TLR3 agonist Poly(I:C) and $1,25D_3$ for 2-6 hours, there was a significant increase in IL-8 production above the expression induced by Poly(I:C) alone (Figure 3.3). This increase was significant at the RNA 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 (Figure 3.3.A; $p<0.01$). IL-8 concentrations in cell supernatants were also increased at 4 and 6 hours of combined treatment, yielding an increase of about 50pg/ml above Poly(I:C) alone (Figure 3.3.B; $p<0.05$). However, at the 6 hour time point, IL-8 RNA expression with combined treatment was no different than with Poly(I:C) alone, suggesting that after 6 hours, vitamin D starts to lower IL-8 expression. When hTCEpi were pre-treated with $1,25D_3$ for 24 hours before Poly(I:C) stimulation, there was a significant reduction in IL-8 expression (50% at 6 hours; $p<0.05$).

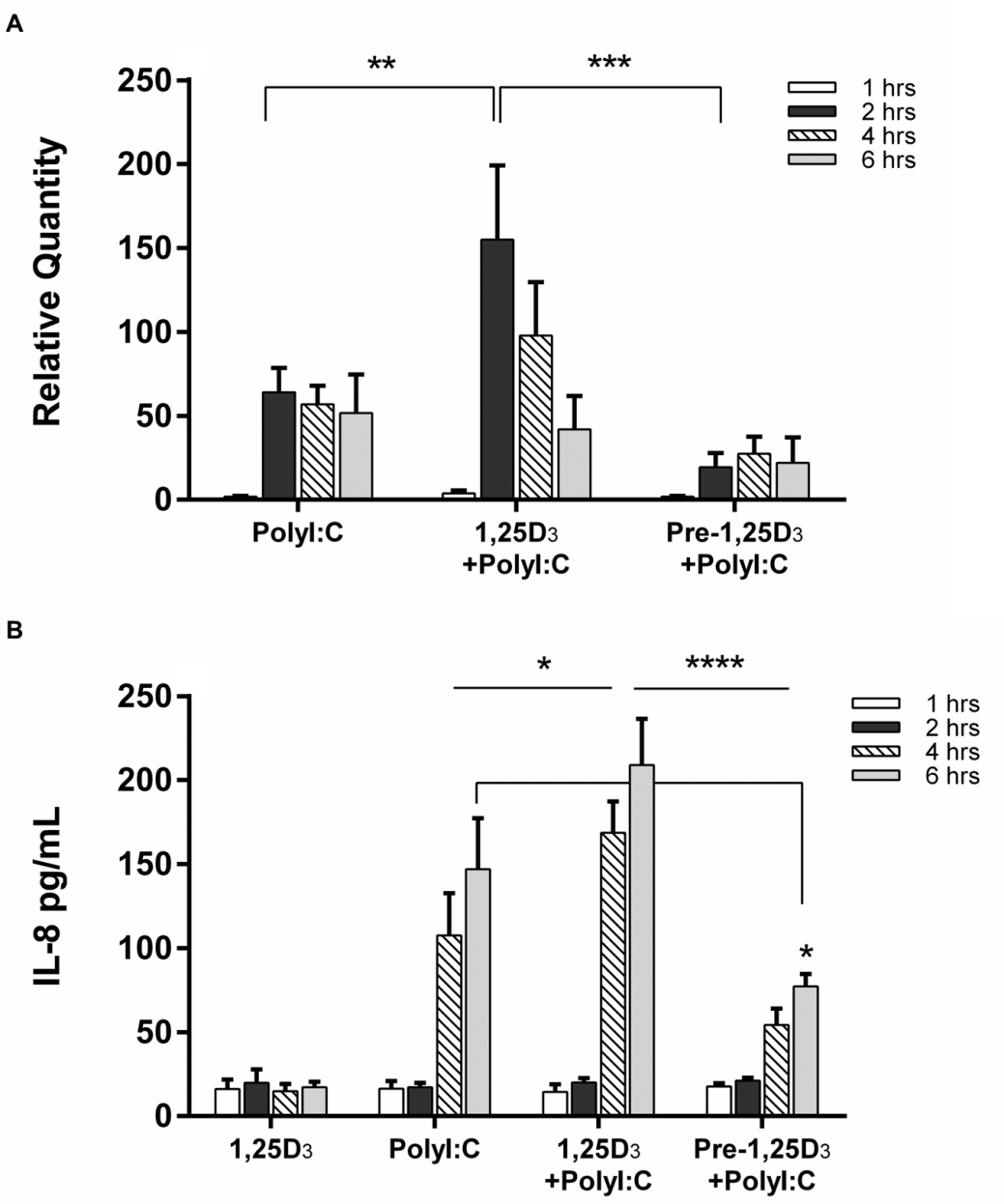

Figure 3.3. Vitamin D's effect on Poly(I:C)-induced IL-8 expression.

hTCEpi were treated with 1μg/ml Poly(I:C) with and without 10^{-7}M $1,25D_3$ for 2, 4, or 6 hours or were pre-treated with $1,25D_3$ for 24 hours prior to Poly(I:C) addition (Pre-$1,25D_3$). RNA was collected from cell lysates for real-time 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, **0.01, ***0.001, ****0.0001.

We next examined the effect of vitamin D when administered after Poly(I:C) treatment. When hTCEpi were pre-stimulated with Poly(I:C) for 4 hours and then treated with $1,25_3$ for an additional 24 hours, there was an ~80% decrease in IL-8 expression (Figure 3.4.A). There was a similar reduction in IL-8 expression when cells were pre-treated 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. (Figure 3.4.B; $p<0.05$). These results suggest that vitamin D treatment is able to attenuate IL-8 levels after 24 hours even when Poly(I:C) is administered first. However, during the immediate response to Poly(I:C), vitamin D co-treatment increases IL-8 expression.

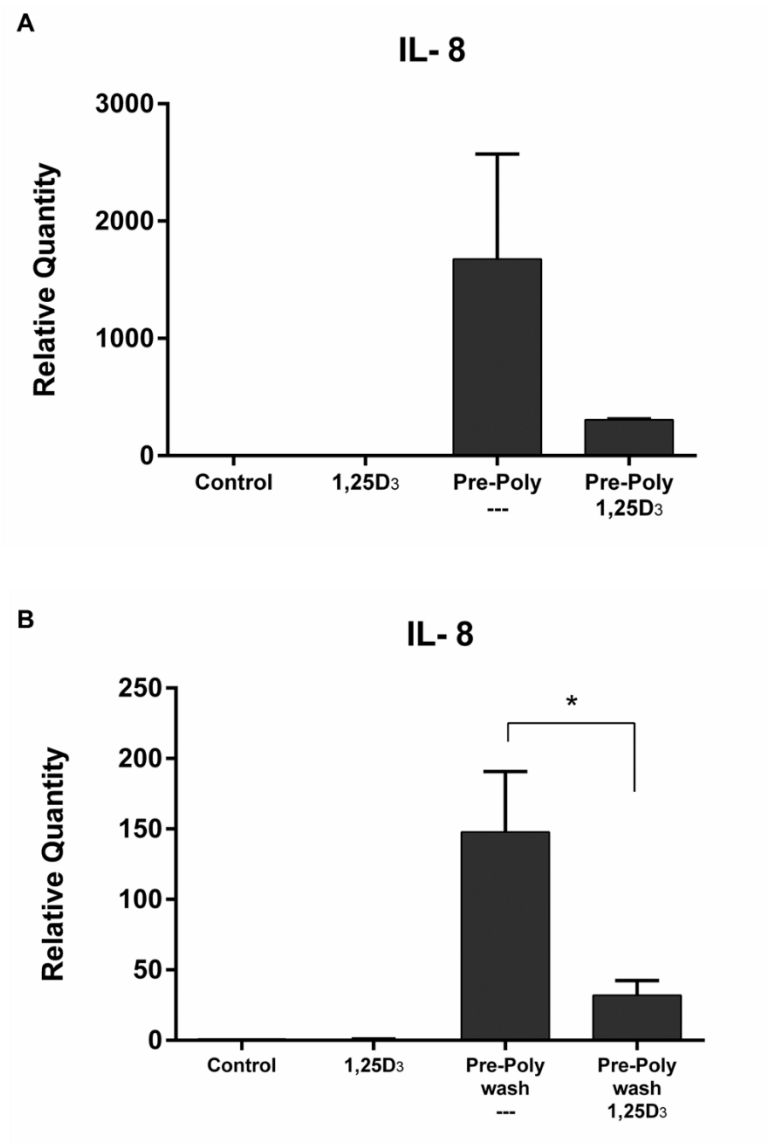

Figure 3.4. The effect of vitamin D on IL-8 expression with Poly(I:C) pre-treatment.

hTCEpi were stimulated with 1μg/ml Poly(I:C) for 4 hours and 10^{-7}M $1,25D_3$ was added without washing (A) or with washing (B), to remove Poly(I:C) before culturing for an additional 24 hours. RNA was collected from cell lysates for real-time 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.

3.3.2. Differential expression of genes after 6 hours of vitamin D treatment.

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 done, examining the expression of several genes known to regulated by $1,25D_3$ (see Chapter 2.3) to determine an optimal time point for analysis. The CYP24A1 hydroxlase, LL-37 antimicrobial peptide, and LPS co-receptor CD14 all had the highest level of expression after 6 hours of vitamin D treatment in hTCEpi (Figure 3.5). Therefore, for the gene array, cells were treated with $1,25D_3$ for 6 hours and gene expression compared to control, vehicle treated cells.

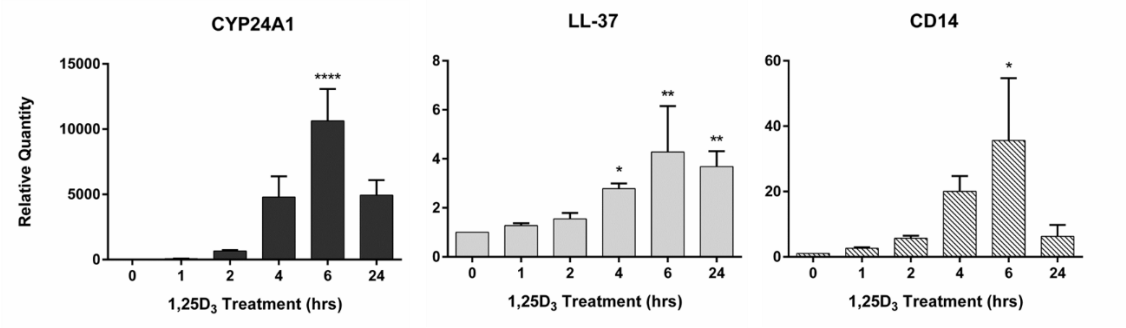

Figure 3.5. Time course of CYP24A1, LL-37, and CD14 expression with vitamin D treatment.

hTCEpi were stimulated with 10^{-7}M 1,25D3 or vehicle control (0.01% ethanol/PBS) for the indicated times and RNA was collected for realtime PCR analyses. Data represent the mean +/-SEM of 3-4 independent experiments. Statistical analysis was by ANOVA with Bonferroni's test for multiple comparisons; p<*0.05, **0.01, ****0.0001.

Both hTCEpi and SV40 HCEC were used in the microarray comparison, to determine the response of the two cell lines to vitamin D. In hTCEpi, 308 genes were identified that were differentially expressed by vitamin D treatment, while in SV40s, only 69 genes were changed (Figure 3.6). In both cell lines, there were more upregulated genes then downregulated (77% in hTCEpi and 70% in SV40). There were only 24 vitamin D regulated genes that both cell lines had in common, and only one of those was downregulated, histone H4 (Table 3.2). The most significant upregulation in both cell lines was seen with the CYP24A1 gene, the cytochrome p450 enzyme that inactivates $25D_3$ and $1,25D_3$ through 24-hydroxylation.

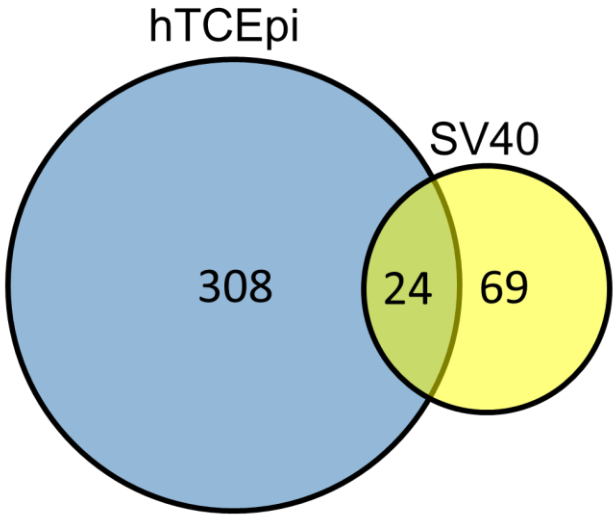

Figure 3.6. Differential expression of genes between hTCEpi and SV40 cell lines.

hTCEpi had a greater number of genes regulated by vitamin D treatment than SV40 (308 compared to 69, respectively), with only 24 genes that were in common.

Table 3.2. Genes that are regulated by vitamin D treatment in both hTCEpi and SV40 cell lines.

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

Gene Symbol	Gene name	hTCEpi p-value	SV40 p-value
	Upregulated		
CYP24A1	1,25-Dihydroxyvitamin D(3) 24-Hydroxylase	2.86E-11	4.50E-11
IL1RL1	Interleukin 1 Receptor-Like 1; ST2	1.65E-08	2.50E-05
GEM	GTP-Binding Protein GEM	8.83E-08	3.25E-06
SEMA6D	Semaphorin-6D	9.48E-08	1.47E-05
TXNRD1	Thioredoxin Reductase 1	5.51E-07	0.00387
CLCF1	Cardiotrophin-Like Cytokine Factor 1	1.23E-06	4.66E-05
SERPINB1	Serpin Peptidase Inhibitor	2.38E-06	0.00017
EFTUD1	Elongation Factor Tu GTP Binding Domain 1	3.42E-06	8.93E-06
DUSP10	Dual Specificity Phosphatase 10	7.88E-06	0.000274
MAFB	V-Maf Fibrosarcoma Oncogene Homolog B	8.86E-06	0.00254
ZNF114	Zinc Finger Protein 114	9.04E-06	0.000645
TMEM40	Transmembrane Protein 40	1.46E-05	0.001698
KLK6	Kallikrein-Related Peptidase 6	4.14E-05	1.34E-06
RHOF	Ras Homolog Family Member F	5.87E-05	0.003357
NET1	Neuroepithelial Cell Transforming 1	5.95E-05	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	4.42E-05
RASD2	GTP-Binding Protein Rhes	0.00086	0.002113
PGM2L1	Phosphoglucomutase 2-Like 1	0.001226	0.003013
NFKBIA	Nuclear Factor Of Kappa Light Polypeptide Gene Enhancer In B-Cells Inhibitor, Alpha	0.0013760	0.0046
	Downregulated		
HIST1H4J	Histone Cluster 1, H4j	0.000651	0.004772

In order to classify the 24 genes in common between hTCEpi and SV40 cells, enriched biological functions were identified using DAVID software (Figure 3.7). The largest process influenced by vitamin D was intracellular signaling, 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, IGFBP3 (insulin-like growth factor binding protein 3), IL1RL1 (interleukin 1 receptor-like 1 or ST2), and NFKBIA (nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha or IKβα). Importantly, when classifying regulated genes by molecular function and cellular compartment, calcium ion 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 also significantly enriched (p= 0.00024672, Pathway Studio Fisher's Exact test).

hTCEpi had a larger number of genes differentially expressed by vitamin D and were used in the TLR stimulation experiments in this study, therefore, we specifically examined biological processes enriched in these cells (Table 3.3). Similar to the gene set in common with SV40s, the largest process influenced by vitamin D was intracellular signaling, which included 47 genes (p=2.10E-08). Vitamin D also regulated genes involved in 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 on target genes of interest. Therefore, six genes that were regulated in both cell lines were examined by real-time PCR analyses (Figure 3.8). These results confirmed the

array data. In addition, expression of these genes was also evaluated in primary HCEC, from two different donors, and the pattern of vitamin D regulation was the same as in cell lines (Figure 3.9).

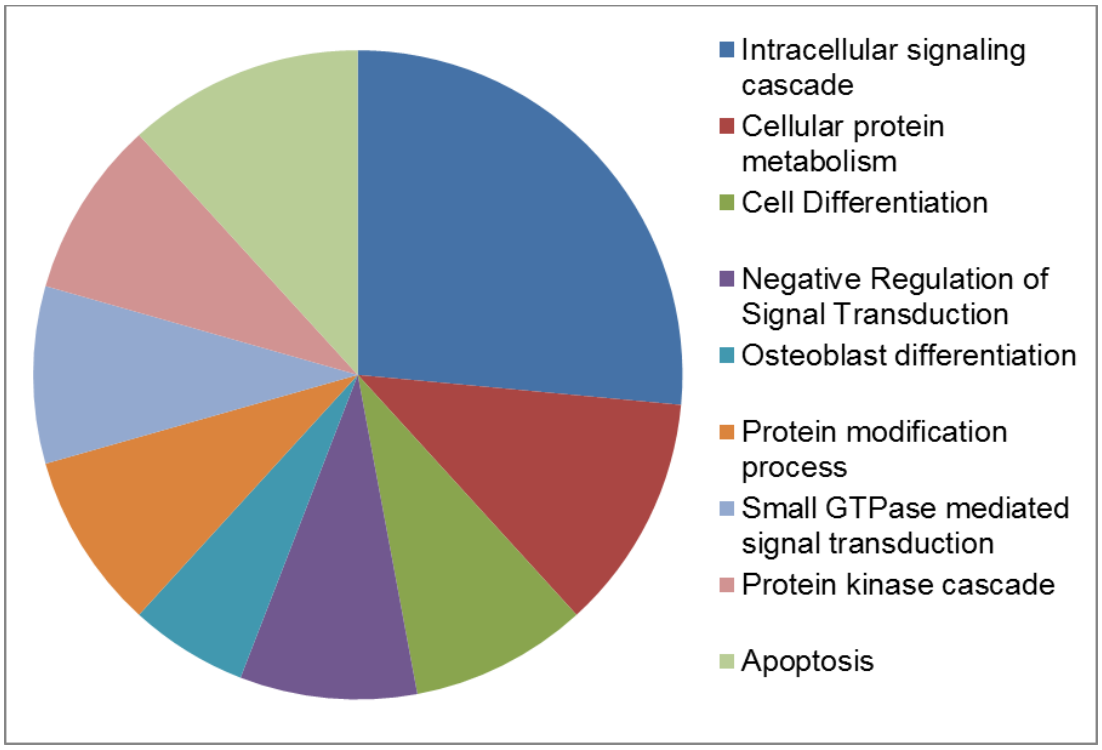

Figure 3.7. Enriched biological processes in vitamin D treated hTCEpi and SV40.

Chart represents categories of gene ontology biological processes that were enriched in vitamin D regulated genes in common between hTCEpi and SV40 cell lines. Analysis was performed using DAVID Bioinformatics online software.

Table 3.3. Biological Processes enriched by vitamin D treatment in hTCEpi.

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. p-values <0.05 were considered significant.

Biological Process	# Genes	p-value
intracellular signaling cascade	47	2.10E-08
cell surface receptor linked signal transduction	44	3.80E-03
regulation of cell proliferation	36	1.30E-08
regulation of apoptosis	33	7.30E-07
positive regulation of macromolecule metabolic process	32	8.00E-06
response to organic substance	31	6.70E-07
positive regulation of biosynthetic process	29	3.10E-06
phosphate metabolic process	28	2.20E-03
response to wounding	27	1.80E-07
regulation of transcription from RNA polymerase II promoter	25	3.60E-04
regulation of phosphorylation	24	8.80E-07
homeostatic process	24	1.30E-03
positive regulation of nucleic acid metabolic process	23	2.60E-04
phosphorylation	22	1.20E-02
cell cycle	21	1.70E-02
response to endogenous stimulus	20	1.70E-05

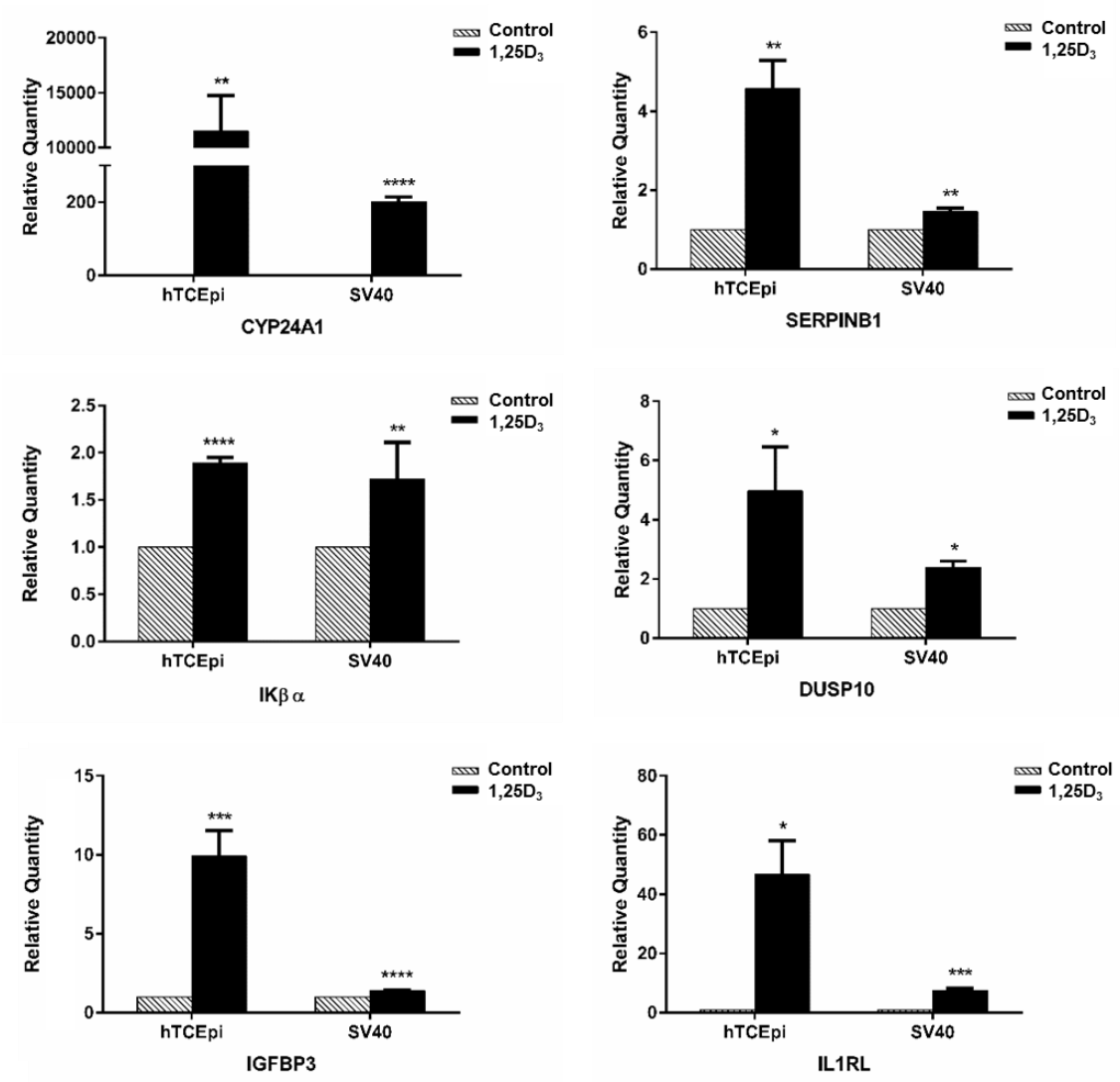

Figure 3.8. Validation of target genes by real time PCR analysis.

hTCEpi and SV40 cells were treated with 10^{-7}M $1,25D_3$ for 6 hours and RNA was harvested for real time PCR analyses. Data represent the mean +/-SEM of 3-4 independent experiments. Statistical analysis was by student's ttest; p<*0.05, **0.01, ***0.001, ****0.0001.

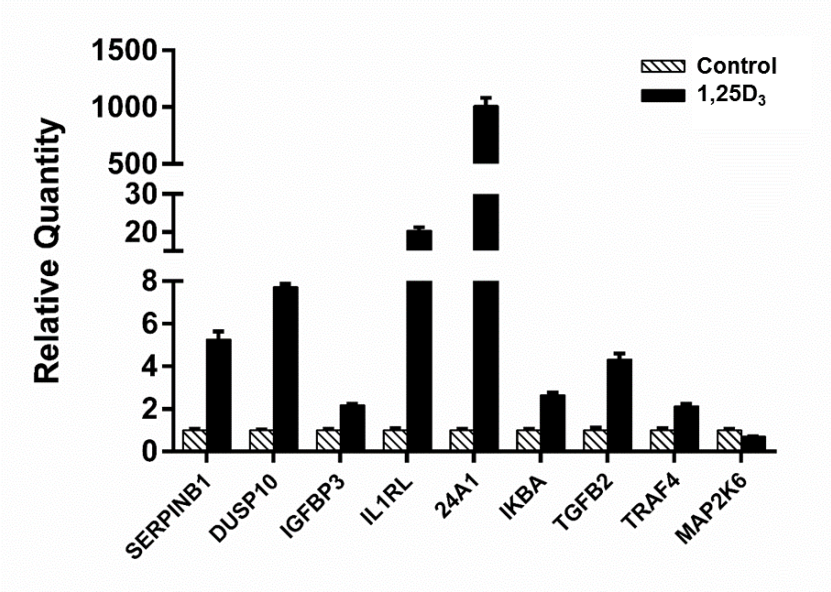

Figure 3.9. Confirmation of microarray results in primary HCEC.

Primary HCEC were isolated from donor corneas and cultures were treated with 10^{-7}M $1,25D_3$ for 6 hours. RNA was collected from cell lystaes for real time PCR analyses. Graphs reflect data from one of two corneal donors and data are mean +/- SEM of triplicate values.

3.3.3. Vitamin D regulates genes involved during inflammation and cellular stress responses in hTCEpi. In order to identify how vitamin D could be influencing TLR inflammatory pathways, enriched pathways were examined and genes involved in both TLR signaling and MAPK signaling cascades were identified (Table 3.4). TLR engagement results in an inflammatory cascade that activates members of the MAPK pathway as well as NFκβ, leading to the production of pro-inflammatory cytokines and proteins involved in the cellular stress response as well as cell survival.

Table 3.4. Genes regulated by vitamin D involved in MAPK signaling and TLR signaling pathways.

hTCEpi genes were identified using DAVID bioinformatics, gene ontology KEGG pathway.

MAPK Signaling Pathway	
Gene symbol	***Gene Name***
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, alpha
IL1B	interleukin 1, beta
MAPK13	mitogen-activated protein kinase 13
MAP2K6	mitogen-activated protein kinase kinase 6
PDGFA	platelet-derived growth factor alpha polypeptide
TGFB2	transforming growth factor, beta 2
FOS	v-fos FBJ murine osteosarcoma viral oncogene homolog
TLR Signaling Pathways	
IL1B	interleukin 1, beta
MAPK13	mitogen-activated protein kinase 13
MAP2K6	mitogen-activated protein kinase kinase 6
NFKBIA	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha
PIK3R3	phosphoinositide-3-kinase, regulatory subunit 3 (gamma)
TLR4	toll-like receptor 4
FOS	v-fos FBJ murine osteosarcoma viral oncogene homolog

NFKBIA (nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha), also known as IKβα, is a negative regulator of NFκβ signaling (Lawrence, 2009). It sequesters NFκβ in the cytoplasm, preventing nuclear translocation and transcriptional effects. Upon TLR activation, signaling events lead to IKβα phosphorylation, which releases NFκβ, and results in IKβα degradation (Chinenov et al., 2013; Hayden and Ghosh, 2012). Vitamin D treatment significantly upregulated IKβα expression in both hTCEpi and SV40 as well as in primary HCEC (Table 3.2 and Figures 3.8, 9). In addition, protein expression of IKβα increased following 24 hours of vitamin D treatment by 28% (Figure 3.10.)

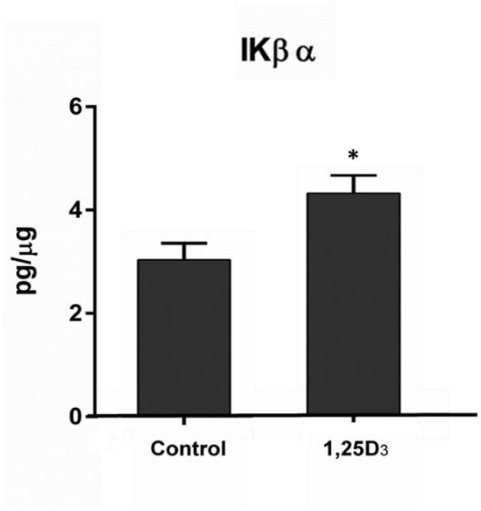

Figure 3.10. Ikβα protein increases with vitamin D treatment.

hTCEpi were treated with 10^{-7}M $1,25D_3$ for 24 hours and total IKβα protein expression 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.

Therefore, we decided to investigate if IKβα was important in Poly(I:C) induced IL-8 expression. When IKβα phosphorylation was inhibited, by an irreversible inhibitor of IKβα phosphorylation, Bay 11-7082, there was a significant decrease in IL-8 production after Poly(I:C) stimulation by 41% ($p<0.01$) (Figure 3.11.A). This demonstrates that the IKβα pathway is activated and is important for Poly(I:C)-mediated cytokine production. Twenty-four hour vitamin D treatment significantly increased the expression of total IKβα in hTCEpi treated with Poly(I:C) (Figure 3.11.B), suggesting that vitamin D could potentially influence Poly(I:C) signaling via upregulation of the NFκβ inhibitor IKβα.

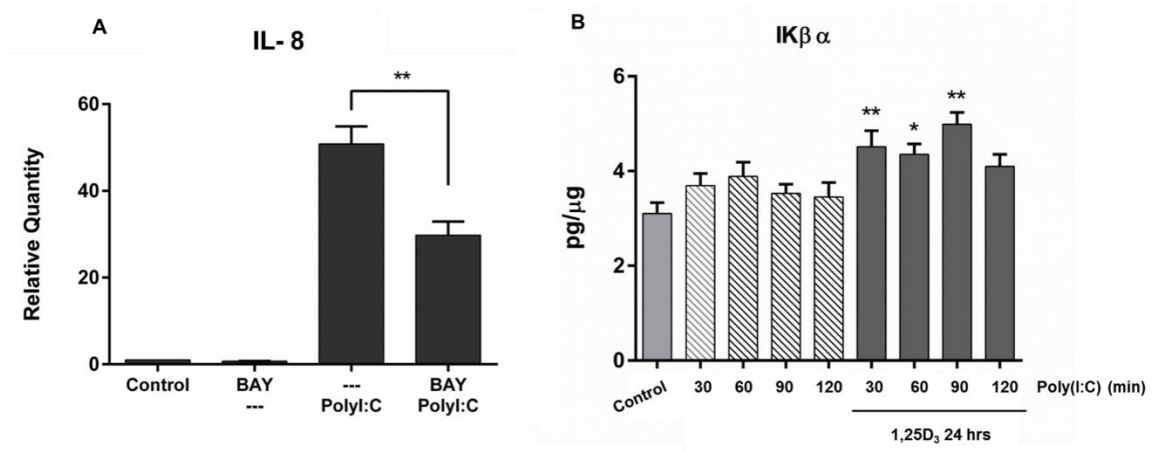

Figure 3.11. Inactivation of IKβα is important in Poly(I:C) induced IL-8 expression and total IKβα is upregulated by vitamin D during Poly(I:C) stimulation.

(A) hTCEpi were pretreated 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) hTCEpi were pretreated with 10^{-7}M $1,25D_3$ for 24 hours and stimulated with Poly(I:C) for the indicated times. Total IKβα protein expression was measured in cell lysates by ELISA. The data represent the mean +/- SEM of 4-6 independent experiments. Statistical analysis was by ANOVA with Bonferroni's test for multiple comparisons; p<*0.05, **0.01.

Another candidate gene for vitamin D's influence on TLR-induced cytokine production was DUSP10 (dual specificity phosphatase 10, MKP5). Like IKβα it was upregulated in hTCEpi, SV40, and primary HCEC and negatively regulates signal transduction. It acts by dephosphorylating members of the MAPK pathway, including p38. Microarray data showed that hTCEpi also increased other dual specificity phosphate family members, DUSP1, 4, and 5, (Table 3.4) and this was confirmed by PCR analysis (Figure 3.12.A). Therefore, we examined p38α phosphorylation, to determine if vitamin D decreased activation of this pathway. Despite the increase in DUSP genes, which are known to block p38 activation, vitamin D treatment actually increased p38a phosphorylation at 24 hours ($p<0.05$) (Figure 3.12.B). In addition, vitamin D did not change Poly(I:C)-induced p38α phosphorylation, which was significantly increased (70%, $p<0.01$) compared to non-treated cells (Figure 3.12.B).

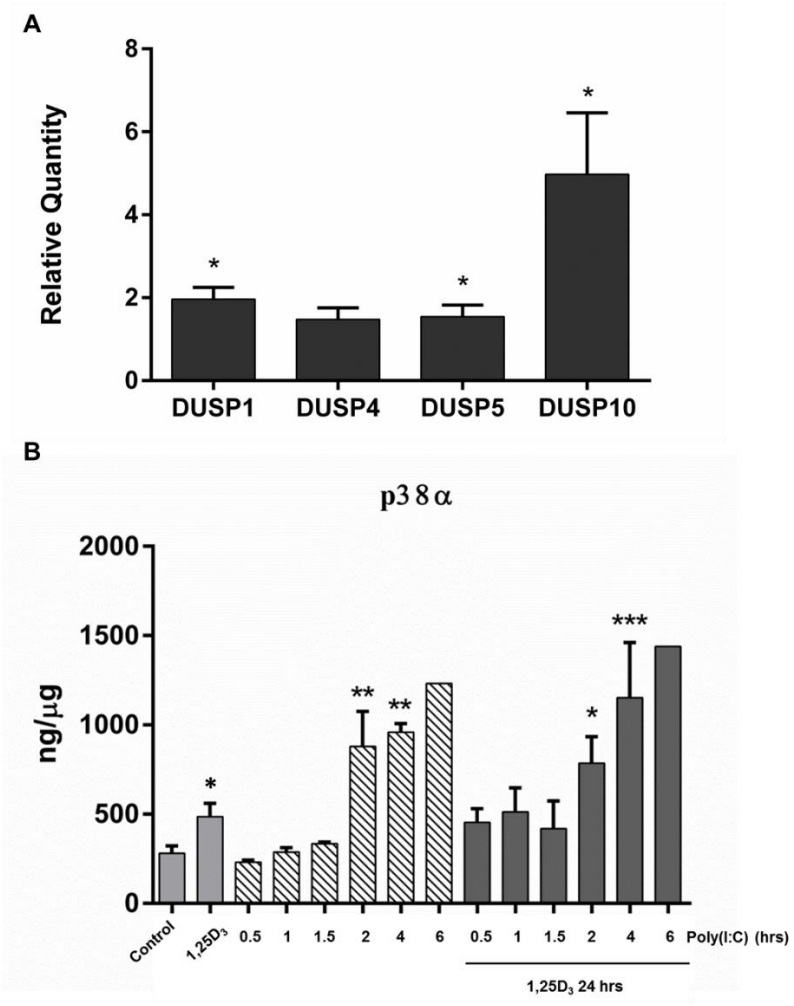

Figure 3.12. Vitamin D upregulates DUSPs however no change is seen in TLR3-induced p38α phosphorylation.

(A) DUSP1, 4, 5, and 10 expression in hTCEpi was evaluated with real-time PCR following 24 hours of treatment with 10^{-7}M $1,25D_3$. Data represent the mean +/-SEM of 3 independent experiments and analysis was by Student's t-test with comparison to control, untreated cells; $p<0.05$. (B) hTCEpi were pretreated with 10^{-7}M $1,25D_3$ for 24 hours and stimulated with Poly(I:C) for the indicated times. Protein expression of phosphorylated p38α (T180/Y182) was measured in cell lysates by ELISA. Data represent the mean +/- SEM of 3 independent experiments. Statistical analysis was by ANOVA with Bonferroni's test for multiple comparisons; p<*0.05, **0.01, ***0.001.

3.4. Discussion

Prior studies indicated that vitamin D was able to attenuate inflammatory signals induced by TLR activation (see Chapter 2.3). In the current study, the effect of vitamin D treatment on TLR3-induced IL-8 expression was examined further. Our findings demonstrated that at earlier time points the effect of vitamin D was contrary to its effect after 24 hours of treatment. Instead of lowering production, IL-8 levels were increased with vitamin D-Poly(I:C) combined treatment through 6 hours. When cells were pretreated with vitamin D for 24 hours, however, IL-8 expression was immediately downregulated upon TLR activation.

The time course data suggests that vitamin D's ability to suppress cytokine production is a later delayed effect. This could be beneficial in the case of inflammation. 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, vitamin D's initial increase in 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 when thinking about using vitamin D therapeutically as a treatment option during inflammatory conditions, suggesting that vitamin D is able to dampen inflammatory events that are ongoing.

Nuclear factor kappa B 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 IKβα, a regulatory protein that binds NFκβ dimers in the cytoplasm, masking the nuclear location signal, thus preventing transcriptional activation. Phosphorylation of IKβα results in its targeting for degradation and subsequent activation of NFκβ, allowing it to translocate to the nucleus (Hayden and Ghosh, 2012). Microarray analysis identified IKβα 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 IKβα levels. After 6 hours of Poly(I:C) treatment, total IKβα expression decreased, as would be expected. Vitamin D increased these levels, increasing the cell's capacity for regulating NFκβ activity. Other studies have demonstrated that increasing IKβα transcription and translation decreases NFκβ translocation to the nucleus (Scheinman et al., 1995). In human peritoneal macrophages, vitamin D suppressed NFκβ activation through increasing IKβα expression, resulting in reduced TLR induced TNFα production (Cohen-Lahav et al., 2006). In addition, basal levels of IKβα were lower in cells from mice lacking the vitamin D receptor, increasing the amount of NFκβ in the nucleus of resting cells (Sun et al., 2006). Therefore, it is

possible that vitamin D's downregulation of Poly(I:C) induced IL-8 expression in HCEC is due in part from its regulation of IKβα expression, which is upregulated at 6 hours after vitamin D treatment.

Another candidate pathway for vitamin D's influence on cytokine production is the MAPK family of signaling kinases, highlighted by the microarray data. Multiple dual specificity protein phosphatase, DUSP, genes were upregulated by vitamin D treatment. DUSPs negatively regulate signaling transduction through dephosphorylating threonine and tyrosine residues on members of the MAPK pathway, rendering them inactive (Liu et al., 2007). DUSPs preferentially regulate p38 and JNK (c-jun NH2-terminal kinases), the stress-activated MAPKs, leading to downregulation of cytokines and pro-inflammatory genes (G. Huang et al., 2009). The anti-inflammatory effects of glucocorticoids have been associated with DUSP expression (Abraham et al., 2006) and mice deficient in DUSP have increased levels of cytokines (Zhang et al., 2015). In particular, DUSP10 expression was increased by hTCEpi, SV40, and primary HCEC. DUSP10, also called MKP5, dampens inflammatory signals through its actions on p38, which has been shown to induce cytokine production (Branger et al., 2002; van den Blink et al., 2001; Wei et al., 2014; Zhang et al., 2015). However, in our study, despite increased DUSP expression, vitamin D did not decrease p38 phosphorylation. Instead, there was an increase in p-p38 with vitamin D. 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. p38 activation has actually been shown to increase DUSP expression, limiting the cycle of inflammation (Kim et al., 2008). Vitamin D's

influence on these pathways need to be further dissected to determine if increased DUSP expression modulates inflammation through p38.

In examining the results for the microarray, it was a significant finding 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 warrants 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 non-immortalized 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 compare and these results should be taken into consideration when choosing a cell line for *in vitro* studies.

Vitamin D clearly has an influence on gene expression in HCEC as would be expected. It also plays a role in modulating inflammatory cytokine production, with a varying response depending on the timing of stimulus. Upregulation of DUSP and IKβα could contribute to the dampening of inflammatory signals later in the response. In addition, other genes identified in the microarray could be important in vitamin D's role in regulating inflammatory signaling, such as IL1RL, GADD45, TRAF4, TGFβ2, and MAP2K6, and will be interesting to investigate further.

Chapter 4

Vitamin D in an *In Vivo* Model of Ocular Surface Inflammation: Experimental Dry Eye

4.1. Introduction

Dry eye disease (DED) is a common ocular surface disease that affects millions of individuals in the United States (Schaumberg et al., 2003). In addition to an impairment in quality of life, work productivity is significantly lost as a result of DED (Patel et al., 2011), demonstrating that DED is an important public health concern. Patients experience feelings of ocular dryness, pain, burning, foreign body sensation, and scratchiness that can be accompanied by visual disturbances and redness (Nichols, 2006). Because symptoms do not always correlate with clinical signs, clinicians use a variety of measures to diagnose DED, including patient questionnaires, tear breakup time, corneal staining, tear osmolality, and tear volume (Smith et al., 2008). Currently, the most common treatment for DED is artificial tears, although cyclosporine, corticosteroids, omega-3 fatty acids, oral tetracycline, and punctual plugs are also prescribed (DEWS, 2007). An active area of research is the development of anti-inflammatory therapies which aim to curb ocular surface inflammation which is an integral part of DED pathology.

DED is defined as "a multifactorial disease of the tears and ocular surface that results in symptoms of discomfort, visual disturbance, and tear film instability with potential damage to the ocular surface. It is accompanied by increased osmolality of the tear film and inflammation of the ocular surface (DEWS, 2007)." The lacrimal and meibomian glands are responsible for producing the aqueous and lipid layers, respectively, of the tear film and disruption of these tissues leads to the two major types

of DED, aqueous-deficient or evaporative dry eye. Tear film insufficiency or instability disrupts the smooth ocular surface (through increased tear breakup and gaps in the tear film across the cornea), creates a hyperosmolar environment, and leads to tissue damage and inflammation of the lacrimal functional unit (ocular surface and tear-secreting apparatus) resulting in dry eye disease (Stern et al., 2010). There is also an autoimmune component to DED. Sjogren's syndrome is a systemic autoimmune disease affecting the lacrimal and salivary glands that causes a severe form of aqueous deficient dry eye. In addition, it has been proposed that there is an autoimmune component in the development of all forms of chronic dry eye (Schaumburg et al., 2011; Stern et al., 2010).

It is well established that inflammation and dysregulation of the immune system play a major role in the pathogenesis of DED. Hyperosmolarity and an insufficient tear film lead to production of pro-inflammatory cytokines and matrix metalloproteinases (MMPs) in the cornea and conjunctiva (Pflugfelder et al., 1999; Solomon et al., 2001; Yoon et al., 2007). Activation of resident antigen-presenting cells (APC) leads to T lymphocyte stimulation and differentiation into effector Th1 and Th17 cells, resulting in infiltration of the ocular surface, propagation of inflammation, and damage to the lacrimal functional unit (Chauhan and Dana, 2009; Schaumburg et al., 2011; Stern et al., 2010).

As previously discussed (see Chapter 1.1), vitamin D is a multifunctional hormone that largely mediates its effects on gene transcription through its receptor, VDR, a member of the nuclear receptor family. In addition to its well-known role in calcium homeostasis, vitamin D has pleiotropic actions on many other tissues, including the

immune system. Importantly for the context of DED, vitamin D has been shown to play a role in modulating inflammation. In general, vitamin D acts to inhibit inflammation, playing a suppressive role during chronic inflammation and inflammatory autoimmune conditions. In various tissues, vitamin D treatment suppresses pro-inflammatory cytokines, chemokines, MMPs, and Th17 differentiation, while augmenting anti-inflammatory cytokines and T regulatory cell differentiation, thus curbing inflammation (see Chapters 1.1, 1.3.1, and 2.1). The active hormone can also induce antimicrobial peptide production and interact with TLR signaling. Both in animal models and in human studies, vitamin D supplementation has been found to produce therapeutic effects in inflammatory conditions. In the eye, vitamin D treatment has been used effectively in mouse models of uveitis (Caspi, 2010), corneal neovascularization (Suzuki et al., 2000b), retinoblastoma (Albert et al., 2002; Audo et al., 2003), ocular melanoma (Albert et al., 2004) and retinal aging (Lee et al., 2012). Topical vitamin D has also been shown to decrease intraocular pressure in a non-human primate model of glaucoma (Kutuzova et al., 2012).

Because vitamin D is known to modulate inflammation in other tissues and there is a significant inflammatory component to DED, the general hypothesis for this study is that topical vitamin D treatment will lead to improved signs of dry eye through modulation of the inflammatory environment at the ocular surface. These experiments utilized a mouse model of EDE to evaluate the effect of topical vitamin D on pro-inflammatory mediator production and disease outcome, measured by tear production and

ocular surface staining to provide new insight into the function of vitamin D at the ocular surface and help determine if vitamin D is a therapeutic option for DED.

4.2. Materials and Methods

4.2.1. Mouse Model of Experimental Dry Eye

To study DED *in vivo*, 6 to 8 week old female C57BL/6 mice (Jackson Laboratories, Bar Harbor, ME) were subjected to EDE conditions as previously described (De Paiva et al., 2009). Mice were housed in an environmentally controlled room, designated for dry eye conditions, with humidity maintained at ~20% and temperature ~70^{o} F. Cages were modified with an open grate on each side to allow for continuous airflow from fans adjacent to each cage (Figure 4.1). In addition to low humidity and air flow, desiccating stress was induced by subcutaneous scopolamine hydrobromide injections (0.5 mg/0.2 ml) (Sigma-Aldrich, Springfield, MO) administered four times a day for five consecutive days at 9:00, 11:30, 2:00, and 4:30. Scopolamine is an anti-muscarinic drug which prevents tear and salivary secretions. Control mice were kept in a normal humidity environment (~65%) without blowing fans or modified cages. Six groups of 5 animals each were examined: control, vehicle-treated, vitamin D-treated control mice, no EDE and control, vehicle-treated, vitamin D-treated EDE mice. All mice were euthanized following five days of treatment. This protocol was approved by the Institutional Animal Care and Use Committee at the University of Houston, and adhered to the standards of

the Association for Research in Vision and Ophthalmology Statement for the use of animals in ophthalmic and visual research.

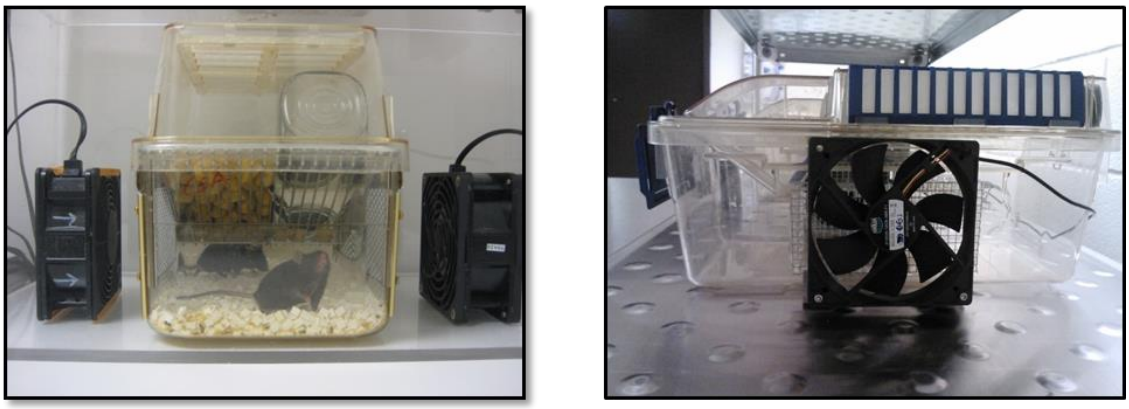

Figure 4.1. Experimental Dry Eye Housing.

Cages were modified on each side to allow for continuous airflow from fans placed adjacent to each cage.

4.2.2. Vitamin D Administration

Both control mice and mice exposed to EDE received topical vitamin D (1α,25-dihydroxyvitamin D(3), 40ng/ml or 100nmol/L; Sigma-Aldrich, St. Louis, MO) or vehicle control (0.1% ethanol/physiological phosphate-buffered saline) twice per day for the 5 day duration of EDE. This concentration has been used previously for evaluating vitamin D in corneal neovascularization (Suzuki et al., 2000a) and corresponds to a physiological level of circulating vitamin D ($25D_3$). For topical application, 5ul of

vitamin D or vehicle control was applied bilaterally to the ocular surface and eyes were held open to avoid blinking for 30 seconds following administration.

4.2.3. Phenol Red Thread Test

Tear volume was measured using phenol red impregnated cotton threads (Zone-Quick; Oasis, Glendora, CA). Mice were held firmly by the scruff of the neck to prevent blinking and the end of a phenol red thread was placed on the palpebral conjunctiva for a count of 15 seconds (Figure 4.2). The amount of tear wicking was determined by measuring the length of red-colored thread. Measurements were taken from five animals, both eyes, per experimental group.

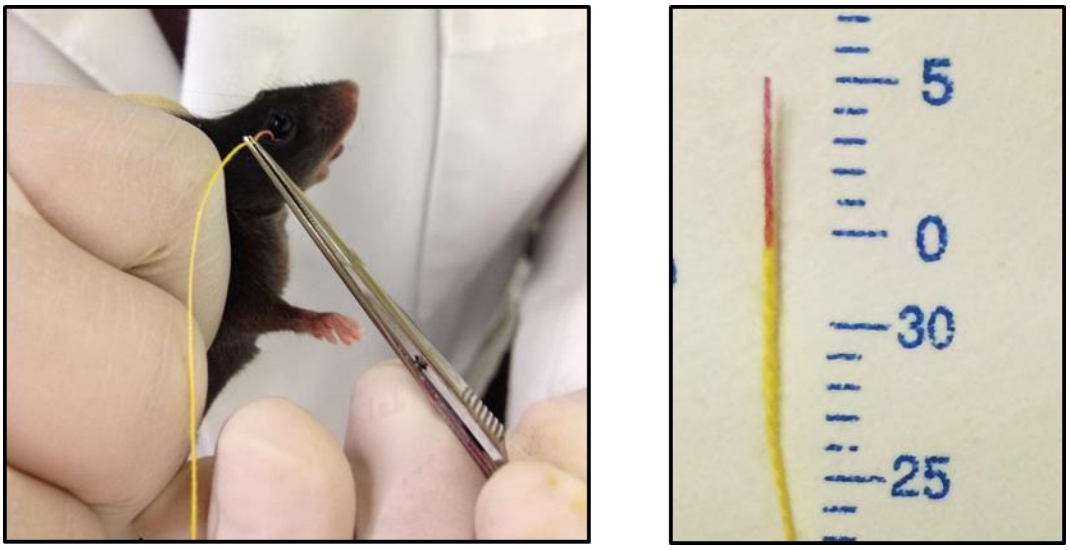

Figure 4.2. Phenol red thread test.

Tear volume was measured with a phenol-impregnated thread placed on the lower conjunctiva (left). Red color change was measured in mm and was recorded as the tear wicking distance (right).

4.2.4. Corneal Fluorescein Staining

Ocular surface damage was assessed by corneal fluorescein staining as previously described (Kodati et al., 2014). Mice were anesthetized with an intraperitoneal injection of ketamine/xylazine (75mg/7.5mg/Kg body weight) (Vedco, Inc., St. Joseph, MO) and 1μL of 1% sodium fluorescein in PBS (Sigma-Aldrich) was instilled into each eye. This was followed by a 400μL PBS wash to remove pooled fluorescein and debris. Eyes were immediately imaged using a slit-lamp microscope with cobalt blue filter or an Olympus stereomicroscope (Model SZX16). For image analysis, pixel intensity was measured in a 1.5mm circular area in the central cornea using Image J software. Each cornea was analyzed three times.

4.2.5. RNA Collection and Quantitative Real-time PCR

Following 5 days of EDE treatment, mice were euthanized and the corneal epithelium was removed by scraping with a number 5 surgical blade. Corneal epithelial tissue from five mice in each group was pooled and RNA extracted for real-time PCR analysis. Total RNA was extracted from scraped epithelium using RNeasy kits (Qiagen, Valencia, CA) followed by cDNA synthesis (Agilent Technologies, Santa Clara, CA). Real-time quantitative PCR was performed using PrimePCR™ SYBR® Green Assays, pre-validated primers for IL-1β, MMP9, CRAMP, mBD3, and HPRT (hypoxanthine-guanine phosphoribosyltransferase) (Bio-Rad, Hercules, California). PCR cycle conditions were as follows: 95^{o} for 2m; 40 cycles of 95^{o} for 5s, 60^{o} for 30s. All samples were normalized

to HPRT and control samples served as the calibrator for relative quantity determination using the ΔΔCT method.

4.3. Results

4.3.1. Vehicle treatment does not induce changes in corneal staining or tear volume.

Before embarking on the EDE model with topical vitamin D, we wanted to examine the effect of the vehicle (0.1% ethanol/PBS) alone on the ocular surface. Three animals per group (control and vehicle) were followed for five days. The vehicle was given topically twice per day over the course of the experiment. After five days, there was no increase in ocular surface staining, as evaluated by corneal fluorescein staining and slit-lamp microscopy (Figure 4.3.A). In addition, there was no statistical difference in tear production between groups, as measured by the phenol red thread test (Figure 4.3.B). Therefore, we felt confident in continuing with this vehicle in the EDE studies.

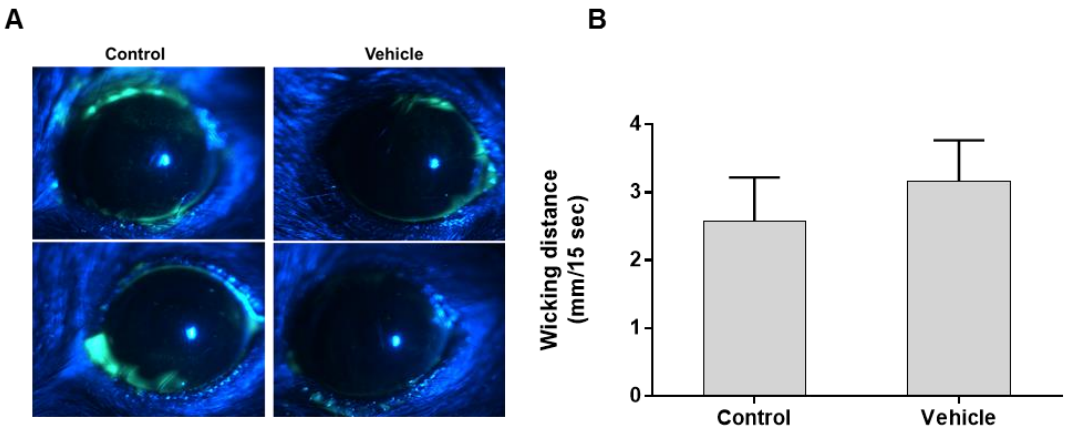

Figure 4.3. Vehicle treatment does not induce changes in corneal staining or tear volume.

8-week old C57BL/6 mice were treated topically with vehicle (0.01% ethanol/PBS) or left untreated for 5 days. (A) Corneal fluorescein staining was performed to examine surface defects. Slit-lamp microscopy images are of 2 corneas, representative of three mice per group. (B) Tear volume was measured by phenol red thread test. Wicking distance of tears was measured over 15 seconds and length measured in millimeters. Data represent mean +/-SEM of 6 corneas per group with analysis by Student's t-test ($p=0.52$).

4.3.2. Vitamin D effect on tear production varies with experimental condition.

In the mouse model of dry eye, like human DES, tear volume is diminished, leading to desiccation and damage. Therefore, we evaluated the effect of topical vitamin D on tear volume, an important parameter in dry eye development. Tear production was quantitated using the phenol red thread test following five days of desiccating stress. In all groups (5 mice each in control, vehicle, and $1,25D_3$ treated), EDE conditions induced a decrease in tear production, as expected, with control values significantly reduced from 3.05 +/- 0.98 mm/15 sec to 0.88 +/- 0.2mm/15 sec ($p<0.01$) (Figure 4.4). In the non-EDE animals, $1,25D_3$ treatment resulted in a significant increase in wicking distance compared to vehicle (2.15 +/- 0.26mm compared to 3.65 +/- 0.4mm/15 sec; $p<0.01$). However, when exposed to EDE, there was no significant difference in tear production with treatment.

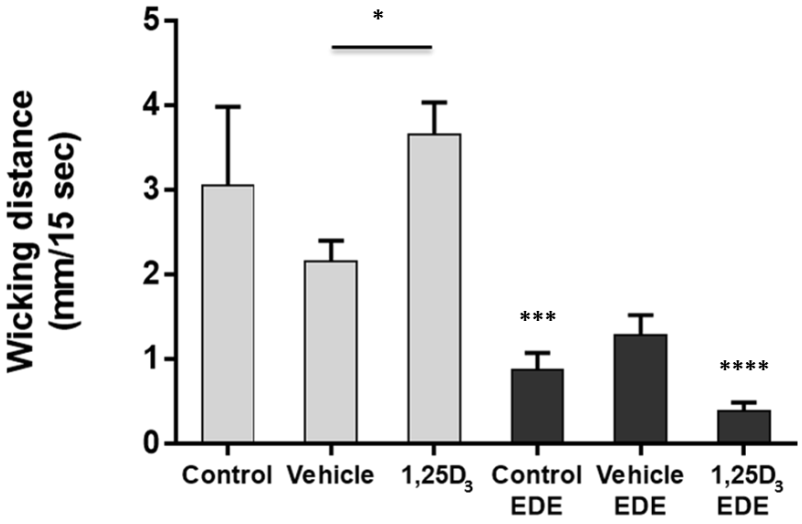

Figure 4.4. Vitamin D effect on tear volume varies between non-EDE and EDE animals.

Tear volume was measured by phenol red thread test after 5 days of treatment. Wicking distance of tears was measured over 15 seconds and length measured in millimeters. Data represent mean +/-SEM of 10 corneas per group. Pairwise comparisons were made between experimental groups (non-EDE vs. EDE) and within experimental groups (vehicle vs. $1,25D_3$) using two way ANOVA with Bonferroni's test for multiple comparisons; p<*0.05, ***0.001, ****0.0001.

4.3.3. Control animals, not exposed to EDE, have a large amount of corneal fluorescein staining.

Another hallmark of EDE, along with a decrease in tear production, is ocular surface damage or defects, visualized by corneal fluorescein staining. After five days of EDE or control conditions, mice were imaged for fluorescein staining. All mice exposed to EDE had extensive ocular surface staining, indicated by diffuse punctate fluorescein staining on the cornea (Figure 4.5A). There was no significant difference in fluorescence intensity between untreated controls, vehicle, or vitamin D treated animals (n=10), although one vitamin D treated mouse had very little corneal staining present (Figure 4.5B).

However, in contrast to findings from the initial control experiment to study vehicle effects (Figure 4.3), control mice from the normal, non-EDE environment had pronounced corneal staining at equal levels with the EDE animals. This surprising result was not reversed with increased washing, suggesting that the control mice had real staining, indicative of damage (Figure 4.6A). However, vehicle treated animals had significantly less staining than controls under the non-EDE conditions (Figure 4.6B). Vitamin D treatment did not result in any significant differences from either control or vehicle.

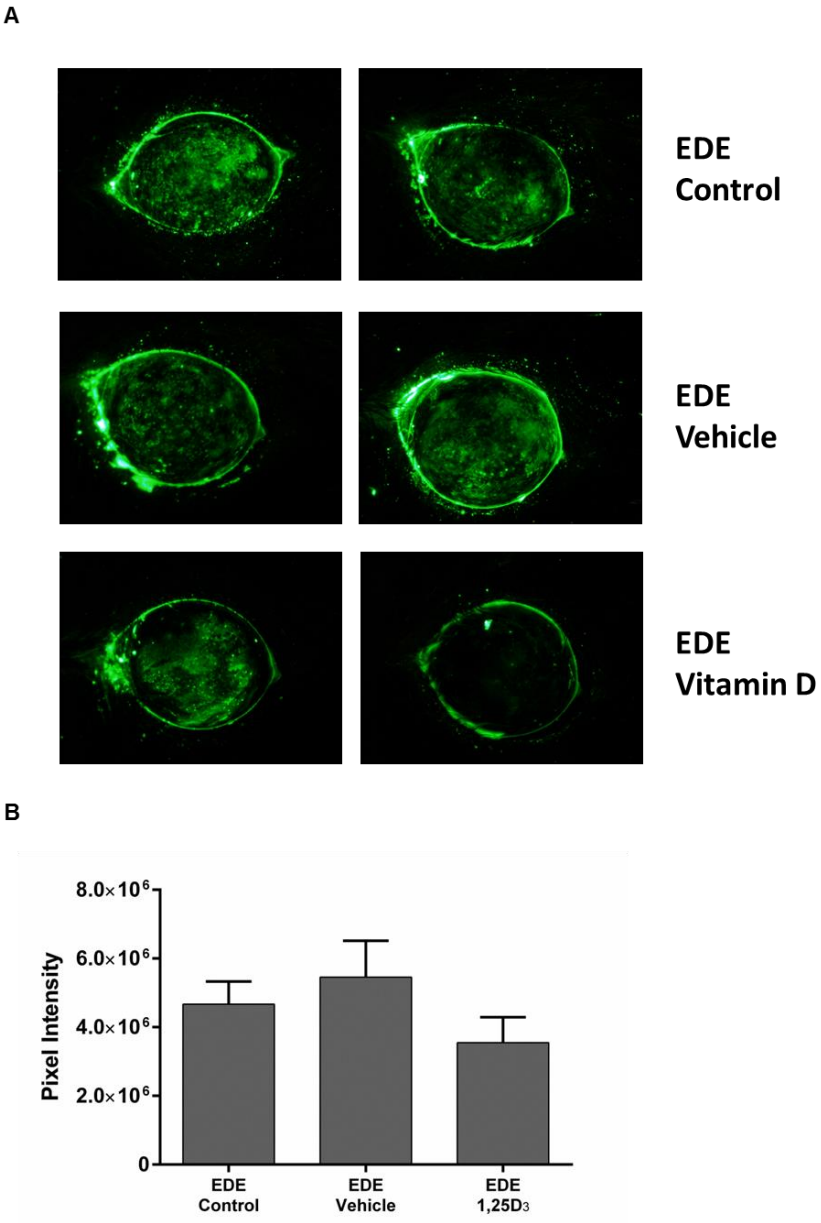

Figure 4.5. No difference in corneal fluorescein staining between EDE groups.

8-week old C57BL/6 mice were exposed to EDE for 5 days and were either untreated or treated topically with vehicle or $1,25D_3$ twice per day. Corneal fluorescein staining was performed to visualize surface damage (A) and fluorescence intensity quantitated (B). Data are mean±SEM (n=10) and images are 3 examples out of 10 eyes per group.

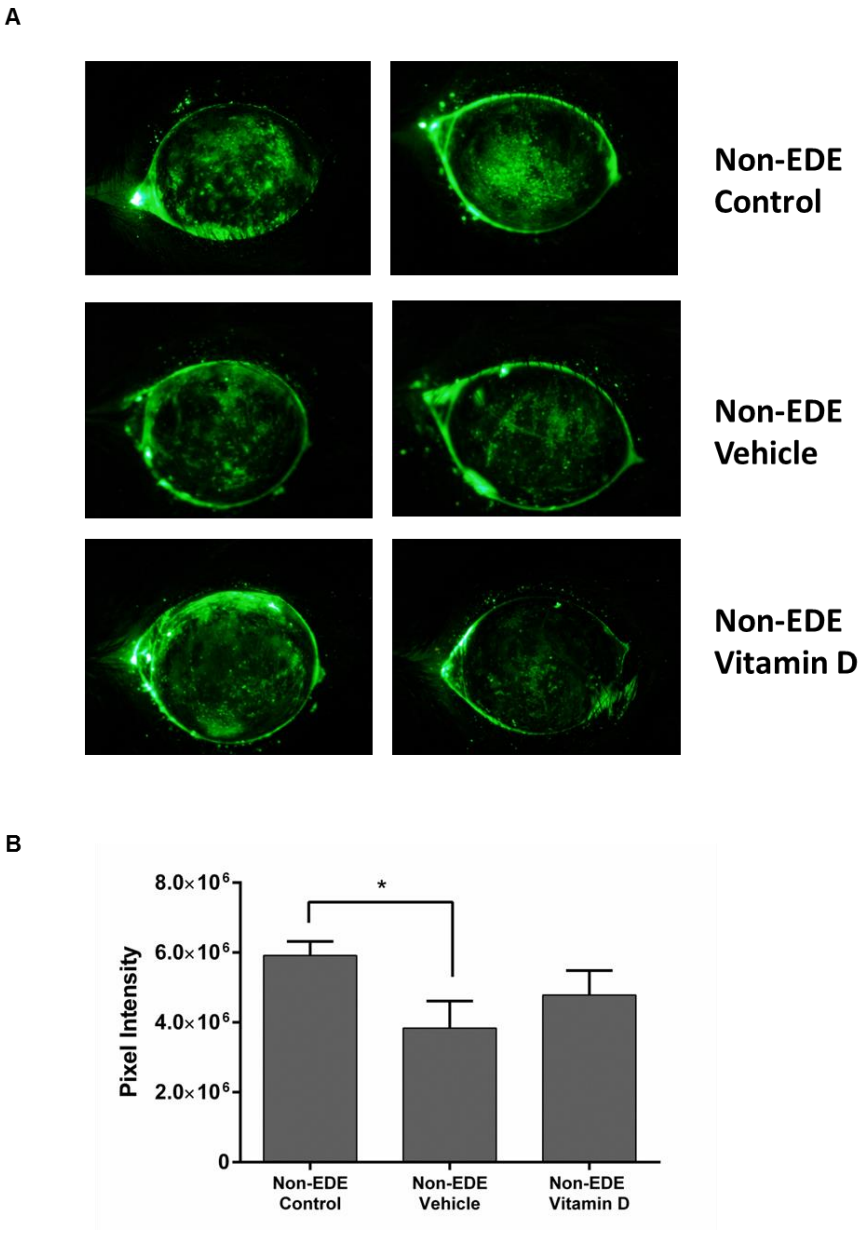

Figure 4.6. Corneal fluorescein staining in non-EDE animals.

8-week old C57BL/6 mice were left in non-EDE conditions for 5 days. Corneal fluorescein staining was performed to visualize surface damage (A) and fluorescence intensity quantitated (B). Data are mean±SEM (n=10) analyzed by the Student's two-tailed t-test ($p<0.05$). Images are 2 examples out of 10 eyes per group.

4.3.4. Pro-inflammatory mediators were not differentially expressed between control and EDE animals.

As fluorescein staining suggested an unexpected baseline level of damage in control non-EDE mice, we next examined the expression of several pro-inflammatory mediators, IL-1β and MMP-9, which are known to be upregulated during EDE in the cornea (Pflugfelder et al., 1999; Solomon et al., 2001; Stern et al., 2010; Yoon et al., 2007; De Paiva et al., 2006; Pflugfelder et al., 2005). In our study, there was no significant increase in either IL-1β or MMP-9 between the untreated non-EDE corneas and EDE animals (Figure 4.7). This result raised our suspicions further as to the status of the control animals.

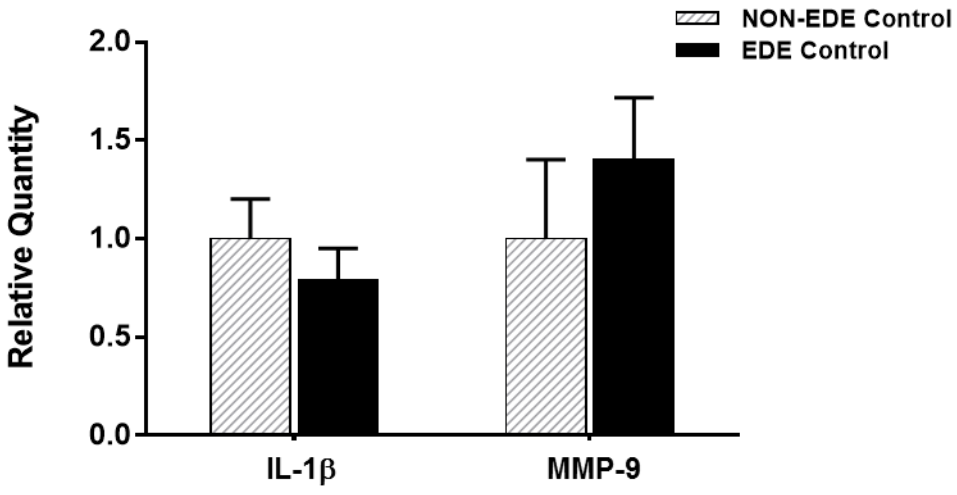

Figure 4.7. IL-1β and MMP9 are not elevated in the corneal epithelium of EDE animals compared to control non-EDE.

8-week old C57BL/6 mice were left in non-EDE conditions or were exposed to EDE for 5 days. The corneal epithelium was removed and RNA collected for RT-PCR analyses. Data represent 10 pooled corneas per group and mean +/-SEM of triplicate values.

4.3.5. Vitamin D lowers the expression of MMP-9 and induces CRAMP during EDE.

We next evaluated the effect of vitamin D treatment on pro-inflammatory proteins as well as on several antimicrobial peptides during EDE. Vitamin D has been shown to decrease MMP-9 expression in various inflammatory conditions. Also, it has been well established that vitamin D induces the expression of the antimicrobial peptide LL-37, in humans, as well as regulating production of the β-defensin, hBD-2 (see Chapter 2.1 and 2.4). Therefore, we measured the expression of IL-1β, MMP-9, CRAMP (the mouse homologue to LL-37), and mBD3 (Bals et al., 1999). While not affecting IL-1β, vitamin D treatment decreased MMP-9 expression by 35% compared to vehicle (Figure 4.8). In addition, vitamin D increased CRAMP expression, a result similar to data from our wounding studies (see Chapter 5.3). However, mBD3 levels remained unchanged between groups.

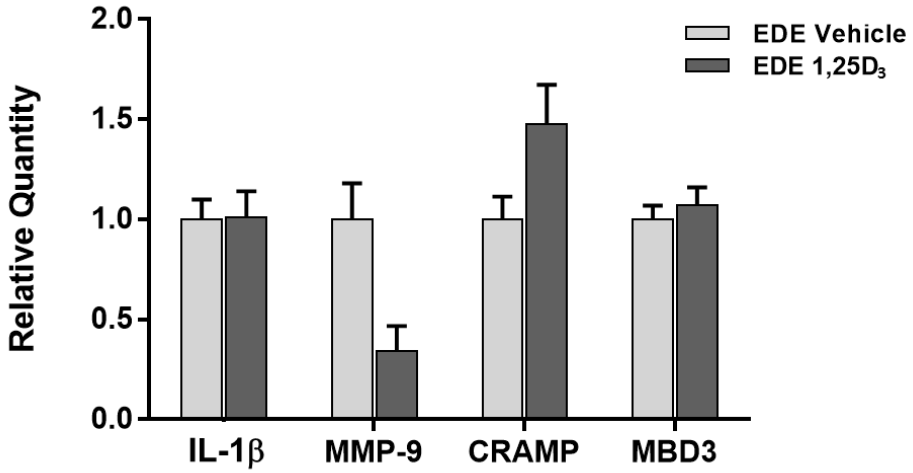

Figure 4.8. Antimicrobial peptide and pro-inflammatory gene relative expression during EDE.

8-week old C57BL/6 mice were exposed to EDE for 5 days and were treated topically with vehicle (0.1% ethanol/PBS) or $1,25D_3$ twice per day. Following experimental conditions, the corneal epithelium was removed and RNA collected for RT-PCR analyses. Data represent 10 pooled corneas per group and mean +/-SEM of triplicate values from one experiment.

4.4. Discussion

Dry eye disease (DED) is a multifactorial disease that has a major inflammatory component. There is a need for better treatment options for this disease and vitamin D may have the potential to fill that role. Vitamin D is able to modulate inflammation and cytokine expression; therefore, it is important to study its effect on EDE, which can be correlated to the human disease. The aim of this study was to evaluate vitamin D treatment on ocular surface damage, tear production, and pro-inflammatory cytokine production during EDE, with the goal of identifying a potential therapeutic option for dry eye syndrome.

In this mouse model of EDE, other studies have demonstrated that desiccating conditions combined with decreased tear production lead to an inflammatory situation similar to human aqueous-deficient dry eye. Others have documented that there is an upregulation of pro-inflammatory cytokines and APC activation at the ocular surface, followed by CD4+ T cell activation in draining lymph nodes, and a subsequent infiltration of Th1 and Th17 cells back at the ocular surface in this model (Chauhan and Dana, 2009; Schaumburg et al., 2011; Stern et al., 2010). The resulting inflammation has been shown to be dependent on CD4+ T cells, as adoptively transferred CD4+ T cells from EDE mice are able to cause disease in the absence of desiccating stress (Niederkorn et al., 2006). Studies in other tissues suggest that vitamin D could potentially dampen this cycle of inflammation, through interactions with T cell and APC activation or through decreasing cytokine and MMP production (Figure 4.9). In cultured primary HCEC, hyperosmolar stress increased vitamin D-related hydroxylase expression, suggesting that

dry eye conditions are able to modulate vitamin D activity at the ocular surface (see Appendix Figure A.7).

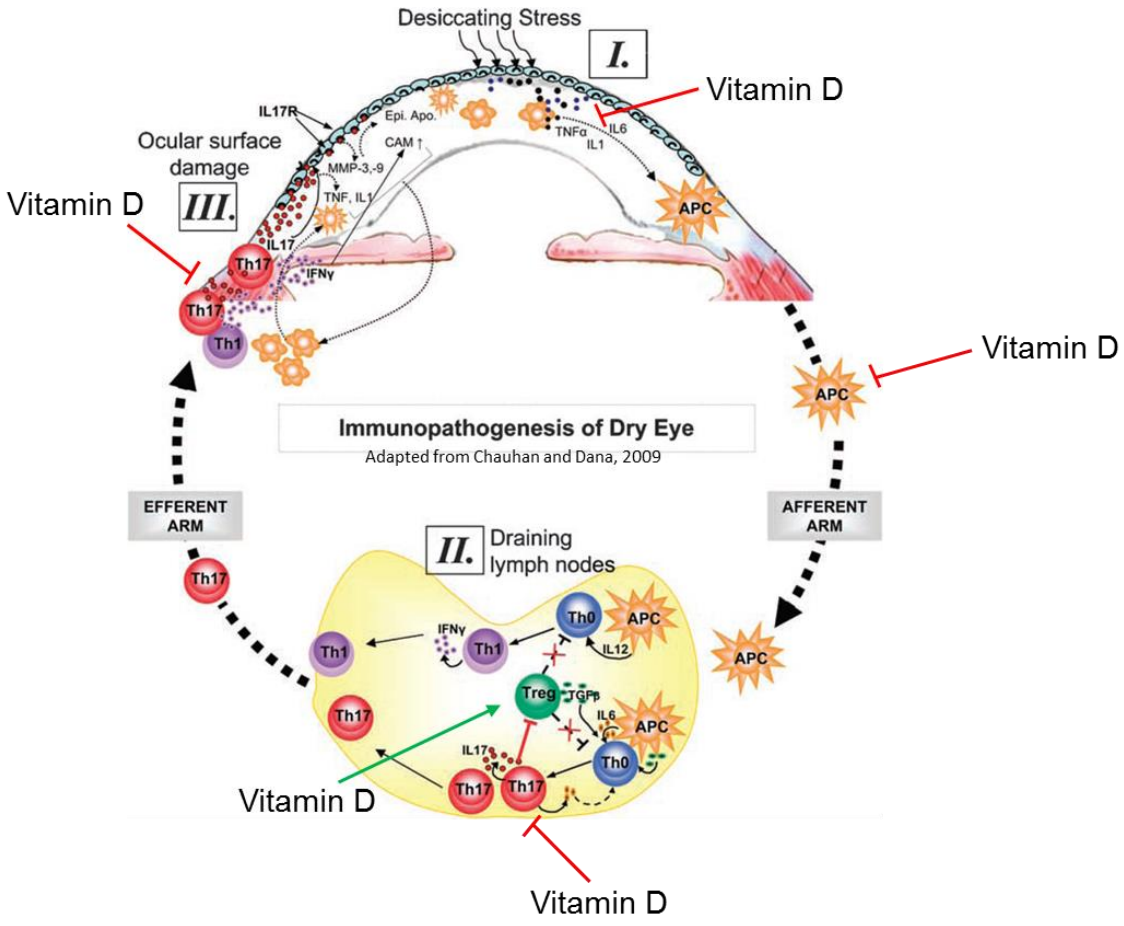

Figure 4.9. Possible points of interactions of vitamin D in the immunopathogenesis of dry eye disease.

Hyperosmolar stress, caused by an increase in desiccation, leads to damage and the production of pro-inflammatory mediators on the ocular surface. Antigen presenting cells (APC) are activated and home to the draining lymph nodes where they prime T cells to expand and differentiate into Th1 and Th17 effector cells. These lymphocytes travel back to the ocular surface where they propagate damaging inflammation. Vitamin D has been shown to influence various aspects of this inflammatory cycle in other tissues and these potential points of interaction are highlighted. Image adapted and used with permission from Chauhan and Dana, 2009.

In previous studies in both the mouse and the monkey, the active form of vitamin D, 1α,25-dihydroxyvitamin D_3 ($1,25D_3$), has been administered safely to the cornea as an eye drop (Kutuzova et al., 2012; Suzuki et al., 2000a). No adverse effects to the ocular surface were reported. In the current study, we first confirmed that the vehicle used to dissolve $1,25D_3$, 0.01% ethanol/PBS, did not increase corneal fluorescein staining or change measurable tear production. After this determination, we evaluated these parameters in vitamin D treated mice. In control animals, vitamin D increased tear production, as measured by the phenol red thread test. These data suggest that in a non-dry eye environment, vitamin D treatment could be beneficial to the ocular surface, preventing desiccation and hyperosmolarity by increasing tear production and/or tear quality. One possible mechanism for this is through maintenance of meibomian gland function. A recent study has shown that topical vitamin D ointment decreases the atrophy of these meibum-producing glands in a mouse model of meibomian gland dysfunction (Jin, 2015). The meibomian glands contribute lipids to the tear film, and blockage or defects in these specialized sebaceous glands lead to increased tear evaporation and evaporative dry eye (Nichols et al., 2011). It is important to note that no histological changes were observed in the corneas of vitamin D treated mice from this meibomian gland study.

In our studies, vitamin D treatment did not significantly decrease ocular surface staining during EDE. There was, however, one mouse that had a reduced amount of fluorescein staining with topical vitamin D. If our sample size was bigger, then perhaps our results would have determined significance. Our original power calculation for

animals needed in this study was based on the phenol red thread test results and number of animals used was also determined from other published studies. However, with the large variability in staining between animals, a larger sample size for this outcome will be needed in future studies. Based on a power of 0.80, 24 eyes should be examined for corneal fluorescein staining to determine a 1.5-fold change in staining with significance of 0.05 (α).

While there was not an observable difference in ocular surface staining between treatment groups, vitamin D did decrease the expression of MMP-9 in the corneal epithelium in a sample of 10 pooled corneas. As this result is a reflection of one experiment, this needs to be repeated for confirmation. MMP-9 is known to be upregulated during EDE and can cause tissue damage through degradation of extracellular matrix components and activation of inflammation mediators, such as IL-1β (Acera et al., 2013). MMP-9 levels have also been used in the diagnosis of ocular surface disease (Kaufman, 2013). Therefore, a decrease in MMP-9 during dry eye development would be a beneficial outcome of vitamin D treatment. Another outcome of benefit to the ocular surface, is the increase in the antimicrobial peptide, CRAMP, in vitamin D treated corneas. We have shown that vitamin D augments LL-37 expression in human corneal epithelial cells, the counterpart of mouse CRAMP (Chapter 2.3.3). CRAMP has been shown to be protective during corneal infection (Gao et al., 2011a; L. C. Huang et al., 2007b). Therefore, these results suggest that vitamin D does offer protection to the cornea during desiccating stress.

However unlike control conditions, in which vitamin D increased tear production, in the dry eye environment there was no change in with treatment. In our EDE model, scopolamine administration blocks cholinergic stimulation of the lacrimal glands, preventing tear secretion. Vitamin D has been to shown to increase expression of muscarinic receptors (M1) in diabetes models (Kumar et al., 2011; Peeyush et al., 2010; Peeyush Kumar et al., 2011). Therefore, our results might be due in part to the use of scopolamine, where vitamin D could be potentiating the effect of scopolamine on M1 receptors. It would be interesting to compare tear production in our current EDE model with that from a model not employing scopolamine, which may be more comparable to human dry eye. Interestingly, a recent study examined a small group of vitamin D deficient patients with dry eye and reported a decrease in tear production compared to patients with normal vitamin D levels, measured by Schirmer test (Kurtul et al., 2015). In addition, tear break up time (TBUT) was decreased (a measure of tear film stability) and dry eye symptoms increased in the deficient cohort. These reports indicate that normal levels of vitamin D are protective against dry eye related signs.

During our experiments, we recognized that our normal mouse housing conditions were not conducive to a healthy ocular surface. This was seen both with diffuse ocular surface fluorescein staining in normal control animals and with levels of IL-1β and MMP-9 expression comparable to that in EDE mice, suggesting the presence of inflammation. In addition, our environmentally controlled room also was not optimized for maintaining constant humidity and temperature levels. An example of this is seen in the graph below (Figure 4.10) where the relative humidity in the EDE room (520) is

variable and spiked above 30%. More concerning is the relative humidity of the normal, non-EDE room (541), which dipped down to similar levels as the EDE room and had an average relative humidity of ~42%, well below desired levels of at least 60%. After many months of trying to optimize staining techniques (washes, dye, microscope) and room conditions (building, dehumidifiers, bedding), we concluded that the EDE model was not ready to be pursued further until environmental conditions could be validated.

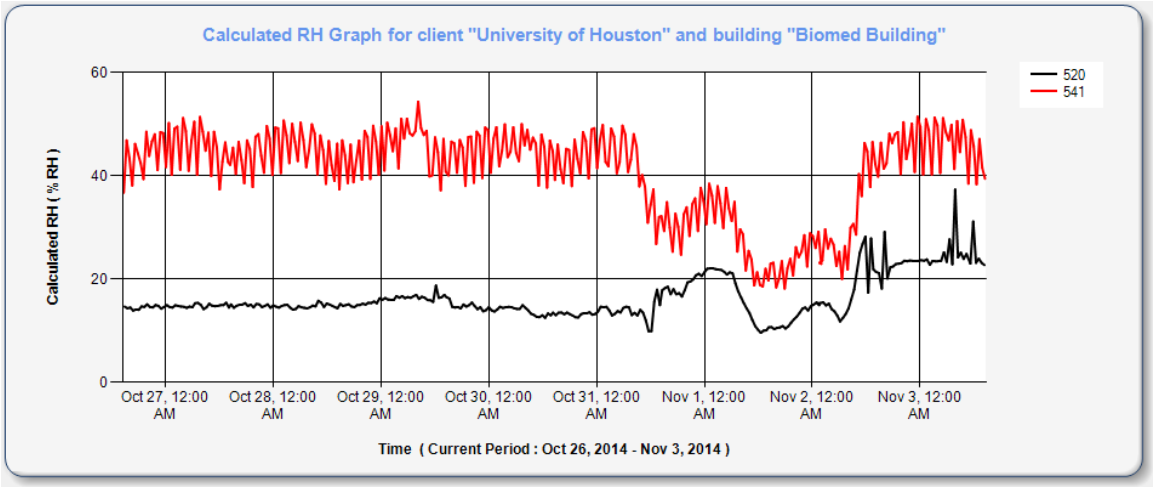

Figure 4.10. Calculated relative humidity (RH) of room 541 (non-EDE room) and 520 (EDE room) over a one-week period.

Our preliminary studies indicate that studying vitamin D in the context of dry eye disease would be worthwhile to repeat once reproducible environmental conditions can be achieved and interesting to expand on. In addition to topical vitamin D, the effect of a vitamin D supplemented diet would also be useful in determining if vitamin D has an effect on ocular surface inflammatory events during dry eye. It has been shown that rabbits fed a high vitamin D diet have an increase in vitamin D compounds in aqueous, vitreous humour, and tears, demonstrating that vitamin D supplementation through diet is an effective method of increasing vitamin D levels in the eye (Lin et al., 2012). Such studies will advance knowledge to provide better therapeutic options for patients with DED in the future.

Chapter 5

Effects of Topical Vitamin D in an *In Vivo* Mouse Model and *Ex vivo* Human Organ Culture Model of Corneal Wound Healing

5.1. Introduction

The cornea is the transparent tissue at the front of the eye that serves both to refract light back onto the retina and to protect the underlying tissues from damage (see Chapter 1.3). Inflammatory events must be tightly regulated in the cornea to maintain these functions. Following a corneal epithelial abrasion, there is a local inflammatory response that is necessary for efficient wound healing and re-epithelialization. As previously discussed, vitamin D has widespread effects on cellular differentiation and proliferation, and can modulate immune responsiveness. $1,25D_3$, the active form of vitamin D, has been widely studied for its immunomodulatory properties and is known to suppress inflammation in a variety of tissues (see Sections 1.1 and 2.1). In addition, vitamin D can induce antimicrobial peptide production, which has been shown to be beneficial during non-sterile corneal wound healing (Huang et al., 2006). Therefore, this study evaluated the effect of vitamin D treatment on the wound healing process in the cornea.

Corneal wound healing involves interactions between epithelial cells, stromal keratocytes, leukocytes, platelets, and nerves which are mediated by growth factors, cytokines, and adhesion molecules (Wilson et al., 2001). After epithelial debridement, there is a coordinated response between all of these factors to ensure efficient wound closure. Basal epithelial cells migrate into the wounded area and undergo cell division to re-epithelize and then re-stratify the injured area. Inflammatory signals from the epithelium induce keratocyte death and injury attracts the infiltration of immune cells into the cornea from the limbal vessels (Wilson et al., 2001, 1996; Yu et al., 2010).

Neutrophils extravasate from the limbal vasculature and migrate into the wounded area using specific adhesion molecules and interactions with underlying stromal keratocytes (Gagen et al., 2010; Li et al., 2006a). This infiltration is necessary for proper wound healing, as defects in neutrophil trafficking result in delayed re-epithelialization (Lam et al., 2011; Li et al., 2007, 2006a, 2006c) In addition to a loss of neutrophil infiltration, too much accumulation also results in delayed wound closure (Liu et al., 2012; Li et al., 2006b), demonstrating the delicate balance of inflammatory events needed during corneal healing.

Other immune cells are also important for effective wound closure. Local dendritic cells, Langerhan's cells, are needed for re-epithelization, migrating with epithelial cells towards the wound center (Gao et al., 2011b). NK (natural killer) cells and γδ T lymphocytes also infiltrate into the cornea and influence recovery. Loss of NK cells results in increased neutrophil infiltration and decreased re-epithelialization and nerve regeneration (Liu et al., 2012). Whereas γδ T cell-deficient mice have delayed wound re-epithelialization and a decrease in corneal neutrophils and limbal platelet accumulation, all necessary for efficient wound repair (Li et al., 2007). Recovery of the keratocyte population, neutrophil infiltration, and wound closure are also dependent on the contribution of platelets, which surround the limbal vessels following wounding (Lam et al., 2015, 2011; Li et al., 2006c).

Corneal nerves also contribute to the wound healing process (Murphy et al., 2001). The cornea is one of the most densely innervated tissues in the body, serving both to protect the cornea from damage and to provide trophic factors necessary for corneal

health and normal maintenance (Kubilus and Linsenmayer, 2010; Marfurt and Ellis, 1993; Müller et al., 2003; Yu et al., 2008). Corneal nerves stem from the ophthalmic lobe of the trigeminal ganglion and thick stromal nerves traverse the anterior limiting lamina to enter the epithelium. These epithelial nerves form a network, the subbasal nerve plexus, of thin, unmyelinated nerve fibers that run parallel to one another (Shaheen et al., 2014). The density of the epithelial nerves increases towards the center of the cornea and they have been shown to respond to chemical, mechanical, and thermal stimulation (He et al., 2010). Upon epithelial debridement, the thin subbasal nerves are destroyed and regeneration begins toward the wound center. Platelets, neutrophils, and γδ T cells have been shown to aid in this process with the release of specific growth factors and cytokines (Li et al., 2011).

In the current study, a mouse model of corneal epithelial debridement was employed to examine the effect of topical vitamin D treatment on wound healing. Both in animal models and in human studies, vitamin D supplementation has been found to produce therapeutic effects on inflammatory conditions. However, in our model, vitamin D delayed wound closure and increased neutrophil infiltration, interfering with the delicate balance of beneficial inflammatory events required for efficient wound closure.

5.2. Materials and Methods

5.2.1. Mouse Model of Corneal Wound Healing

A total of 102 female C57BL/6 mice (8-12 weeks old, Jackson Laboratories, Bar Harbour, ME) were used in these studies. As there is a difference in the rate of wound closure based on sex, only female mice were used in these studies (Wang et al., 2012). Prior to wounding, mice were weighed and anesthetized with an intraperitoneal injection of ketamine/xylazine (75mg/7.5mg/Kg body weight) (Vedco, Inc., St. Joseph, MO). Circular epithelial wounds (~2mm in diameter) were made in the center of the right eye with an Algerbrush II (Alger Equipment Co., Lago Vista, TX) under a dissecting microscope. This method has been used previously to debride the corneal epithelium without disruption of the epithelial basement membrane (Lam et al., 2011; Li et al., 2006a, 2006c). All wounding experiments were started at the same time of day to minimize diurnal variations in inflammatory events.

Immediately following wounding, topical vitamin D ($1,25D_3$; Sigma-Aldrich, St. Louis, MO) (10^{-7}-10^{-9}M) or vehicle (0.02% ethanol/PBS) was applied drop wise (5µl) to wounded corneas. $1,25D_3$ was dissolved in a lower percentage of ethanol vehicle than in previous *in vivo* experiments (see Chapter 4.2). Drops were repeated 5 minutes after the initial application and every 6 hours over the course of the experiments. Eyes were manually held open to avoid blinking for 30 seconds following drop administration. All mice were euthanized by carbon dioxide asphyxiation followed by cervical dislocation at the end of the experiments. This protocol was approved by the Institutional Animal Care

and Use Committee at the University of Houston, and adhered to the standards of the Association for Research in Vision and Ophthalmology Statement for the use of animals in ophthalmic and visual research.

5.2.2. Corneal Wound Imaging

To determine wound size and monitor re-epithelialization, wounded corneas were imaged at the time of wounding and every 6 hours afterwards for 24 hours. Isoflurane anesthesia was administered to each animal before imaging, except at the time of initial imaging. Briefly, mice were placed in an induction box and isoflurane was administered via vaporizer (EZ-Anesthesia, model EZ-SA800) at a dosage of 1-4% inhalant and O_2 flow rate of 0.5-1 liter/minute until there was lack of a reflex to a rear toe pinch. For imaging, 1µl of 1% sodium fluorescein (Sigma-Aldrich) was pipetted onto the central cornea and images were captured with an Olympus stereomicroscope (Model SZX16). For determining percentage of wound closure, wound areas were demarcated and measured using Image J software. The size of epithelial defect was expressed as a percentage of the original wound area. Results were analyzed with a two-way repeated measure ANOVA and Bonferroni's test for multiple comparisons with significance set at $p<0.05$.

5.2.3. Corneal Whole Mount Imaging

At 18 hours post-wounding, a time demonstrated to correlate to peak neutrophil infiltration (Li et al., 2006a), mice were euthanized for corneal whole mount processing. Whole eyes were removed and placed in 2% paraformaldehyde for 15 minutes. Corneas

were then dissected and fixed for an additional 45 minutes. Following blocking and permeabilization (2% BSA/0.01% Triton-X100 in PBS), corneas were incubated overnight at 4^{o}C with 10μg/ml fluorophore-conjugated antibodies: FITC-conjugated anti-Ly6G (to detect neutrophils) (clone 1A8, BD Pharmingen, San Diego, CA), NorthernLights™ NL557-conjugated anti-β-III tubulin (to detect nerves) (R&D Systems, Minneapolis, MN), and DAPI (4',6-diamidino-2-phenylindole, Sigma-Aldrich) to visualize nuclei. Corneas were radially cut to flatten and mounted onto slides with Airvol (Celanese, Dallas, TX, courtesy of Dr. A. Burns). Whole mounts were imaged on a DeltaVision Core microscope (Applied Precision, Issaquah, WA).

Full thickness 20X images were captured from seven fields of view across the diameter of the cornea, centered at evenly spaced intervals, from limbus to limbus on the opposite side (Figure 5.1). x- and y- image coordinates where used to calculate the distance between points. Each image was deconvolved five iterations to improve resolution and reduce blur using SoftWorx software (Applied Precision) (Biggs, 2010). For neutrophil infiltration, nuclei were counted within a square morphometric frame (150 μm in length) (Figure 5.2). Both DAPI and FITC-Ly6G staining were used to identify neutrophils, with distinctive "donut-shaped" nuclei (polymorphonuclear), which were located predominantly in the anterior stroma, as previously reported (Liu et al., 2012) and only those cells which fell within the frame or on the accepted line were counted. A similar method was used to count dividing basal epithelial cells, which were identified by DAPI nuclear staining, enabling visualization of condensed chromosomes that occur during mitosis. For relative nerve-density determination, a 10x10 grid was overlaid on

each image and the presence or absence of subbasal epithelial nerve staining in each square of the grid was recorded to obtain a percentage of squares containing nerves stained with neuron-specific anti-β-III tubulin (Figure 5.3).

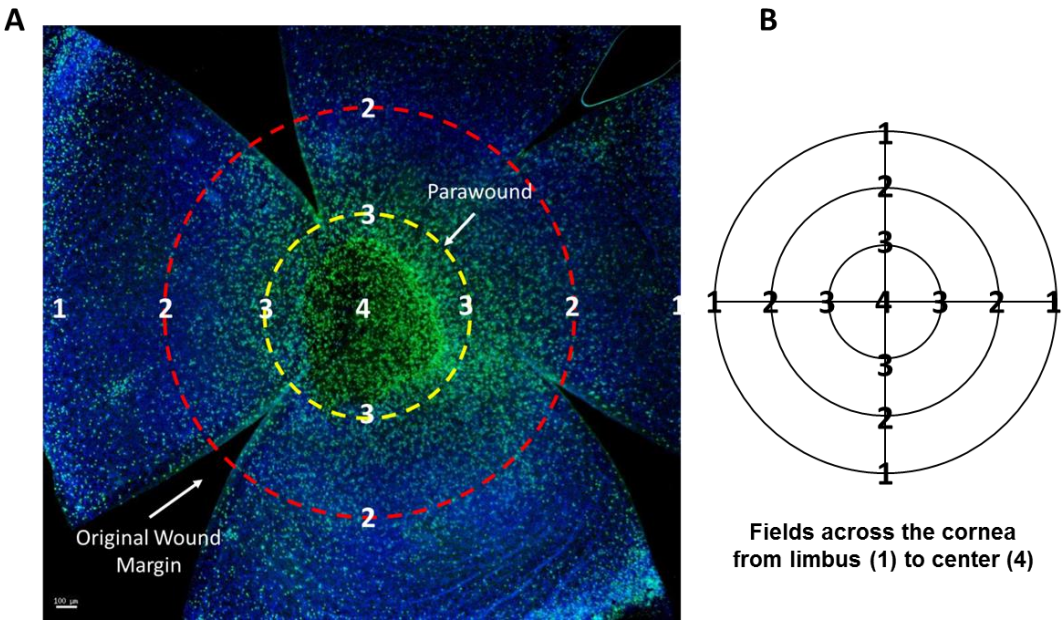

Figure 5.1. Corneal whole mount imaging.

(A) Example of a whole mount, 18 hours post-wound, stained with Ly6G (green) to visualize infiltrating neutrophils and DAPI (blue). Radial cuts divide the cornea into four petals, enabling the cornea to flatten under the coverslip. The wound area is visible in the central cornea surrounded by the parawound region (yellow). The location of the original wound margin is demarcated (red). Scale bar = 100μm (B) Each cornea was measured from the limbus across the center to the opposite limbal region using x- and y- image coordinates and images were taken at even intervals, designated by points 1 through 4.

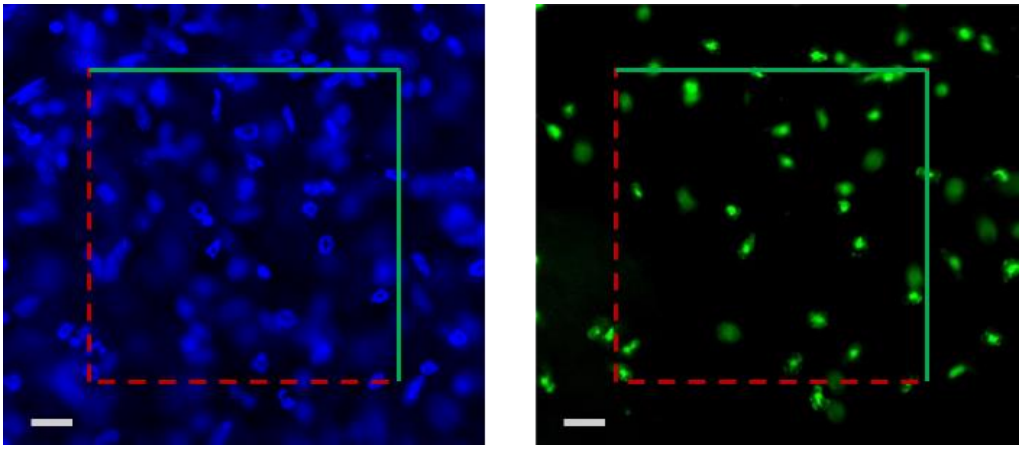

Figure 5.2. Neutrophil counts.

Morphometric counting frame with the accepted line in green and forbidden line in red dashes. Neutrophil "donut-shaped" nuclei were counted using DAPI (blue) and cross-checked with FITC-labeled Ly6G staining (green) throughout the z-stack in each image. Scale bar = 20μm.

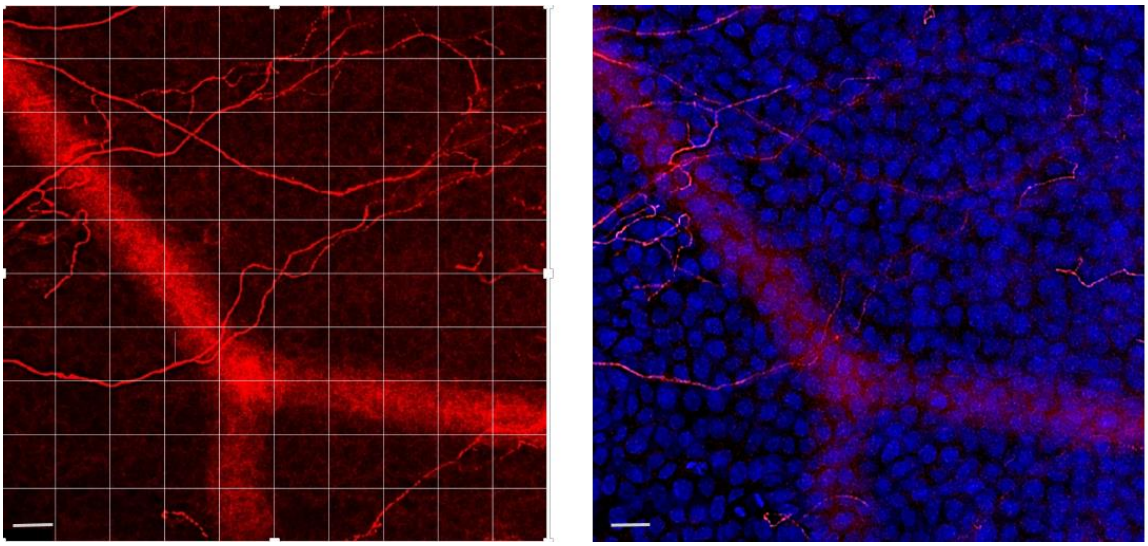

Figure 5.3. Nerve relative density.

On the left, the 10x10 grid used to count subbasal epithelial nerves stained with NorthernLights™ NL557-conjugated anti-β-III tubulin (red). In this image, 57 out of 100 boxes contain a subbasal nerve fiber. A large stromal nerve can be seen beneath the plane of the thin subbasal nerves. On the right, basal epithelial cells are visible, stained with DAPI (blue). Scale bar = 20μm.

5.2.4. Corneal RNA Collection and RT-PCR

Whole corneas were collected for RT-PCR analyses of gene expression 12 and 24 hours post-wounding. Following euthanasia, eyes were removed and corneas harvested with the aid of a dissecting microscope. Eight to ten corneas (from 4-5 mice) were pooled from the same treatment group for each sample. Samples were homogenized on ice and RNA was extracted with an Ambion ToTALLY RNA Total RNA Isolation kit (Life Technologies, Grand Island, NY), using two sequential phenol:chloroform extractions for purification. RNA concentration was quantified with a NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE) and reverse transcribed using an AffinityScript cDNA synthesis kit (Agilent Technologies, Santa Clara, CA). Real-time PCR was performed using intron-spanning primers (Table 5.1) and Brilliant II SYBR Green QPCR master mix (Agilent Technologies). All samples were normalized to GAPDH and control samples served as the calibrator for relative quantity determination using the ΔΔCT method.

Table 5.1. Primer sequences for real-time PCR.

Gene name	Forward Primer	Reverse Primer	NCBI Reference Sequence
CXCL1	5'- TGCACCCAAACCGAAGTC-3'	5'- GTCAGAAGCCAGCGTTCACC-3'	NM_008176.3
CXCL2	5'-TGTCAATGCCTGAAGACCCTGCC-3'	5'-AACTTTTTGACCGCCCTTGAGAGTGG-3'	NM_009140.2
IL-1α	5'- CAGGGCAGAGAGGGAGTCAAC-3'	5'- CAGGAACTTTGGCCATCTTGAT-3'	NM_010554
IL-1β	5'- GCAACTGTTCCTGAACTCAACT-3'	5'- ATCTTTTGGGGTCCGTCAACT-3'	NM_008361
VEGFA	5'- GTCCTGTGTGCCGCTGATG-3'	5'- GCTGGCTTTGGTGAGGTTTG-3'	NM_001025250
TGFβ1	5'-CTTCAATACGTCAGACATTCGGG-3'	5'- GTAACGCCAGGAATTGTTGCTA-3'	NM_011577
TGFβ2	5'- CTTCGACGTGACAGACGCT-3'	5'-TTCGCTTTTATTCGGGATGATG-3'	NM_009367
TNFα	5'- ACTGAACTTCGGGGTGATCG-3'	5'- TGATCTGAGTGTGAGGGTCTGG-3'	NM_013693.3
PDGFa	5'- TGGCTCGAAGTCAGATCCACA-3'	5'- TTCTCGGGCACATGGTTAATG-3'	NM_008808

5.2.5. ELISA

Corneal protein expression of CXCL1, CXCL2, TNFα, IL1β, and VEGF was determined 12 and 24 hours after wounding. Whole corneas were harvested and protein isolated as previously described (Xue et al., 2002a). Eight corneas (from 4 mice) per group were pooled and homogenized in 1ml PBS with protease inhibitors (cOmplete, mini tablets, Roche Diagnostics, Indianapolis, IN). Cells were then lysed through three freeze-thaw cycles and centrifuged at 10,000g. Protein concentrations were determined in supernatants by BCA protein assay (Life Technologies) and protein expression quantified in duplicate using DuoSet ELISA kits (R&D Systems). Results were expressed as amount of target protein per mg total protein from each sample.

5.2.6. Human Organ Culture Model

Human cornea organ culture wounding was performed as previously described (Huang et al., 2006) (Figure 5.4). Briefly, cadaveric corneas, supplied by Saving Sight (St. Louis, MO), were received within five days of donor death and were placed epithelial side up in a culture dish supported by a 0.5% agar/M199 mold. The epithelium was removed from corneas (initial sample), allowed to regrow, and collected again after 48 hours (regrown sample). RNA was isolated and analyzed by RT-PCR for expression of CYP27B1, the hydroxylase that converts vitamin D to its active form, $1,25D_3$. For wound closure studies, $1,25D_3$ (10^{-7}M) (right eye, OD) or vehicle (0.02% ethanol/PBS) (left eye, OS) was applied drop wise to wounded corneas every 6 hours for 24 hours and the wound area determined by fluorescein staining, as in the mouse wounding model (described above). Results were analyzed with a two-way repeated measure ANOVA and Bonferroni's test for multiple comparisons with significance at $p<0.05$.

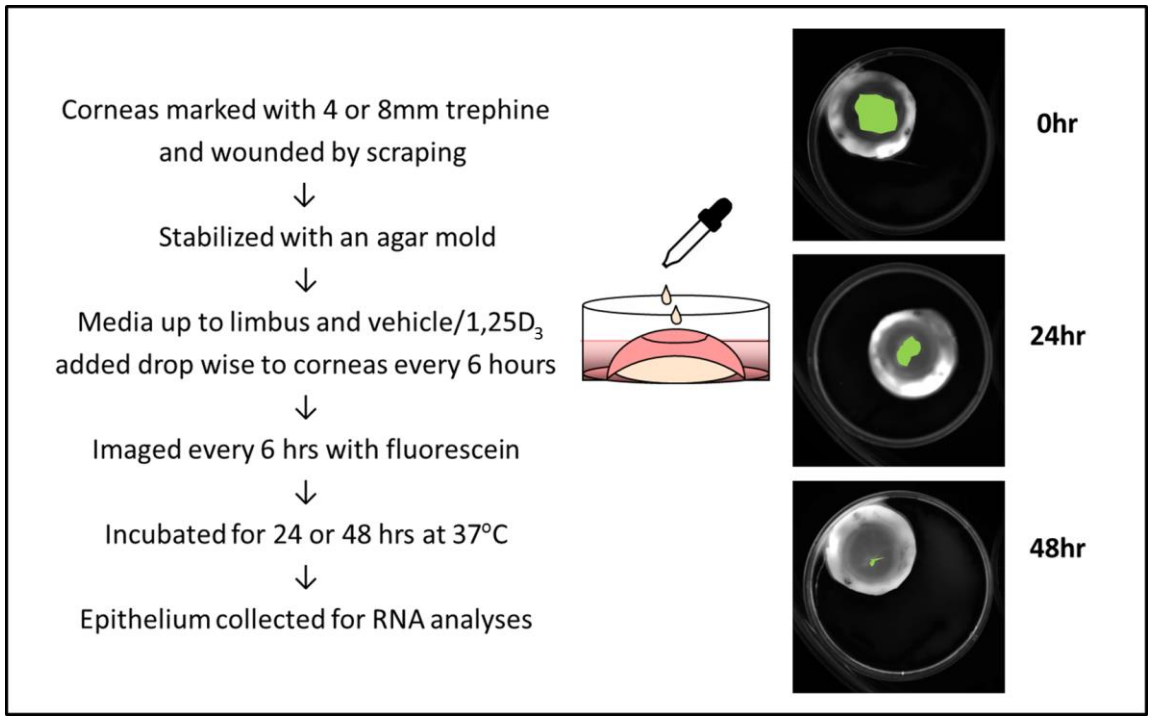

Figure 5.4. **Human donor cornea organ culture model of wounding.**

Donor corneas were wounded by scraping and wound area was visualized with fluorescein staining, as seen on the right in green. Wound closure was monitored every 6 hours for 24 hours, at the same time treatment drops ($1,25D_3$ or vehicle) were administered. For RNA analysis, no treatments were given, and the epithelium was harvested after 48 hours of wounding to determine gene expression following re-epithelialization.

5.3. Results

5.3.1. Topical vitamin D slows corneal wound closure in the initial 18 hours.

In previous studies, both *in vitro* and *in vivo*, vitamin D ($1,25D_3$) has been used to treat human corneal epithelial cells (HCEC) and as a topical application in mouse corneas at a concentration of 10^{-7}M, corresponding to normal levels of circulating vitamin D (~40ng/ml) (See Chapters 2.2.1 and 4.2.2). Although no adverse corneal effects were seen with this concentration, an initial wounding experiment was performed to test the effect of a range of concentrations (10^{-7} to 10^{-9}M) on corneal wound closure. At 18 hours after epithelial debridement, mice treated with $1,25D_3$ had a significantly larger area of percent open wound than vehicle treated animals, at both 10^{-7} and 10^{-9}M ($p<0.05$) (Figure 5.5). As there was not a significant difference in wound closure between 10^{-7} and 10^{-9}M $1,25D_3$, in further wounding studies 10^{-7}M $1,25D_3$ was used, maintaining consistency with previous experiments.

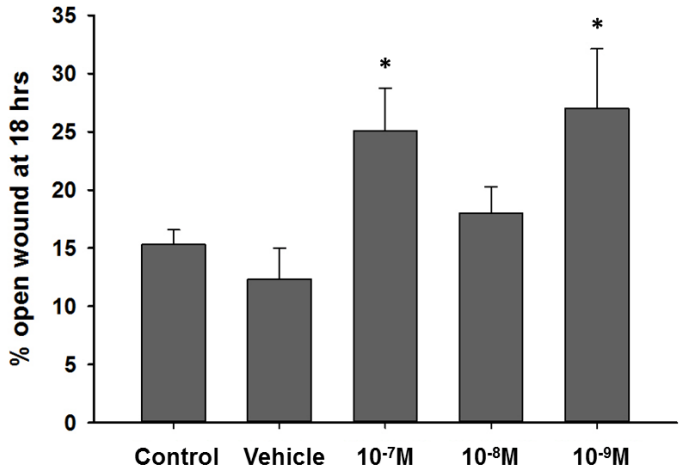

Figure 5.5. Vitamin D increases the percentage of open wound at 18 hours after epithelial debridement.

Mice were imaged immediately after corneal wounding and at 18 hours post-wound with fluorescein staining to visualize wound area. Mice received topical vehicle (0.02% ethanol/PBS) or $1,25_3$ (10^{-7}, 10^{-8}, or 10^{-9}M) twice at the time of wounding and every 6 hours through 18 hours. Wound area remaining open was determined as a percentage of original wound area. Data represent mean $\pm$ SEM and were analyzed with one-way ANOVA and Bonferroni's correction for multiple comparisons, p<*0.05 (n=3 mice/group).

After this initial experiment, wound re-epithelialization was evaluated at 6 hour intervals through 24 hours, to determine the effect of vitamin D on wound closure. $1,25D_3$ treated mice had a delayed rate of wound closure, indicated by a greater percentage of wound remaining open, at both 12 and 18 hours compared to vehicle treatment ($p<0.05$) (Figure 5.6). Following epithelial debridement, wounds from normal mice are nearly completely re-epithelialized within 24 hours (Li et al., 2006a), as was seen with vehicle treatment, having only 2.16% of the wound area remaining open. $1,25D_3$ treated wounds were not completely closed at this time (12.01% open), however this difference did not reach statistical significance.

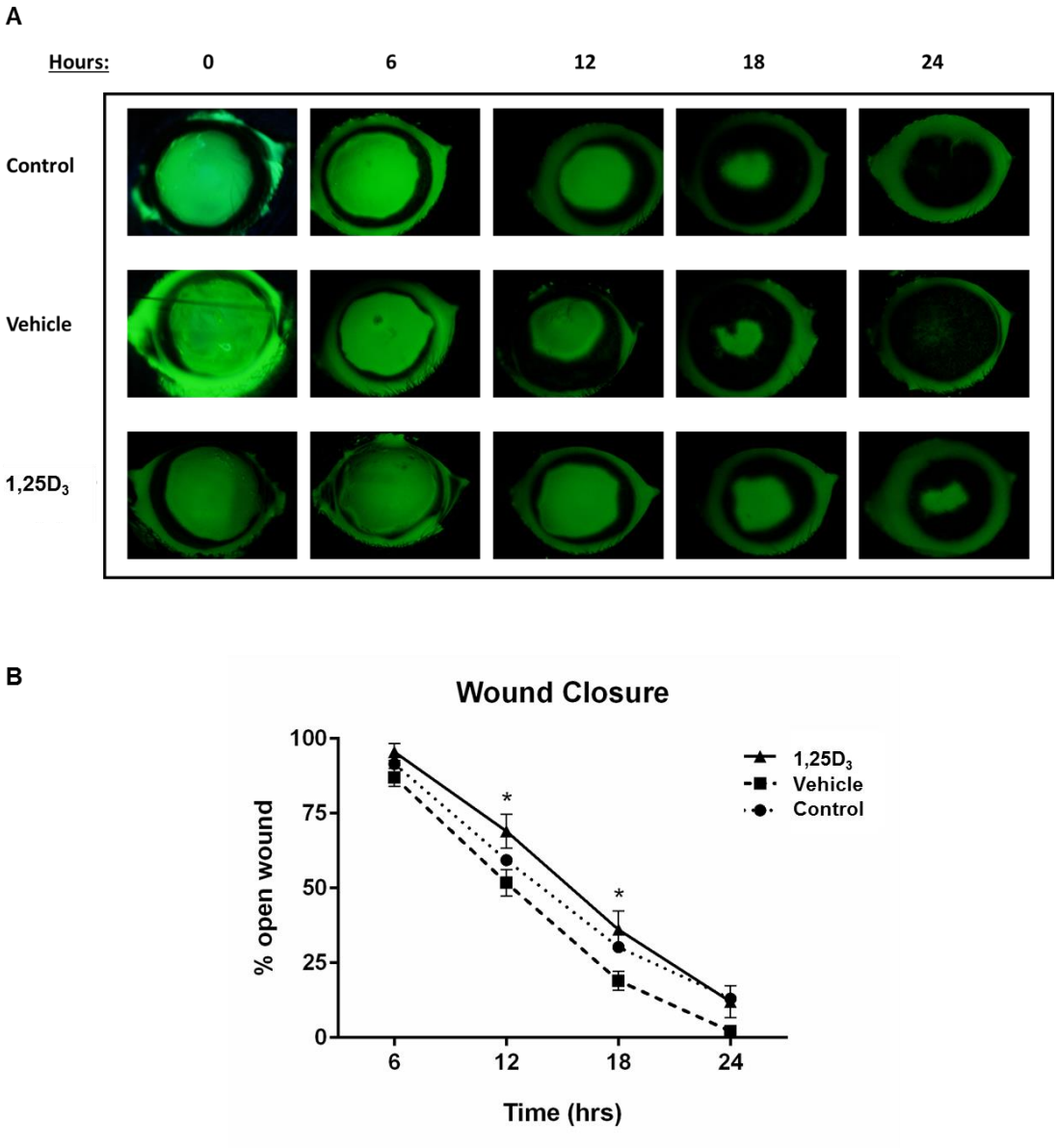

Figure 5.6. *In vivo* wound closure is delayed with vitamin D treatment.

Mice were wounded and treated with vehicle (0.02% ethanol/PBS) or $1,25D_3$ (10^{-7}M) every 6 hours or left untreated (control) for 24 hours. (A) Corneal wound areas were monitored by fluorescein staining every 6 hours. (B) Wound area remaining open was determined as a percentage of original wound area. Data represent mean $\pm$ SEM and were analyzed with two-way repeated measures ANOVA and Bonferroni's correction for multiple comparisons. * = $p<0.05$ (comparison between vehicle and vitamin D treatments; n=9 mice/group).

5.3.2. Vitamin D treatment does not affect basal epithelial cell division.

Re-epithelialization of a corneal wound involves both migration and division of basal epithelial cells to cover the wound. Because vitamin D delayed wound closure, the amount of basal epithelial cell division was quantitated to determine if this process was affected by treatment. At 18 hours after epithelial debridement, there was no significant change in dividing epithelial cell numbers between vehicle and $1,25D_3$ treatment groups across the cornea (Figure 5.7). This suggests that the delay seen in corneal wound healing with vitamin D was not due to reduced basal cell division. However, the power of this experiment was only 0.60, indicating that a difference might arise with an increased sample size.

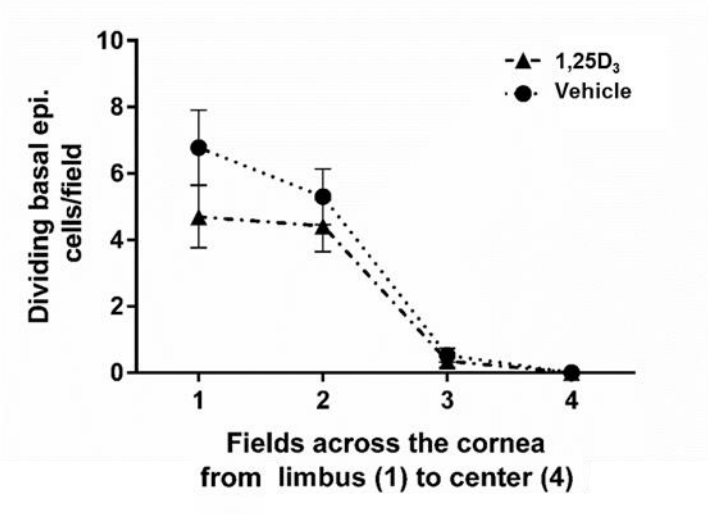

Figure 5.7. Basal epithelial cell division across the wounded cornea at 18 hours after wounding.

Nuclei of basal epithelial cells were identified with DAPI staining. The graph represents the average number of mitotic nuclei counted per field in vehicle or $1,25D_3$ treated corneas 18 hours after wounding. (n=5 corneas/group)

5.3.3. Neutrophil infiltration into the wound area is increased by vitamin D treatment.

Neutrophil extravasation and emigration into the cornea is an important part of the wound healing process and neutrophil depletion causes a delay in wound healing (Li et al., 2006a). Therefore, we next examined neutrophil infiltration in wounded corneas at 18 hours post-wound, the time of peak neutrophil accumulation in corneas under control conditions (Li et al., 2006a). Vitamin D treatment increased the relative number of total neutrophils in the wounded corneas by 40% compared to vehicle (674 vs. 401; $p<0.01$) (Figure 5.8.A). Specifically, there were more neutrophils in the parawound ($p<0.01$) and wound center ($p<0.0001$) regions with $1,25D_3$ treatment (Figure 5.8.B).

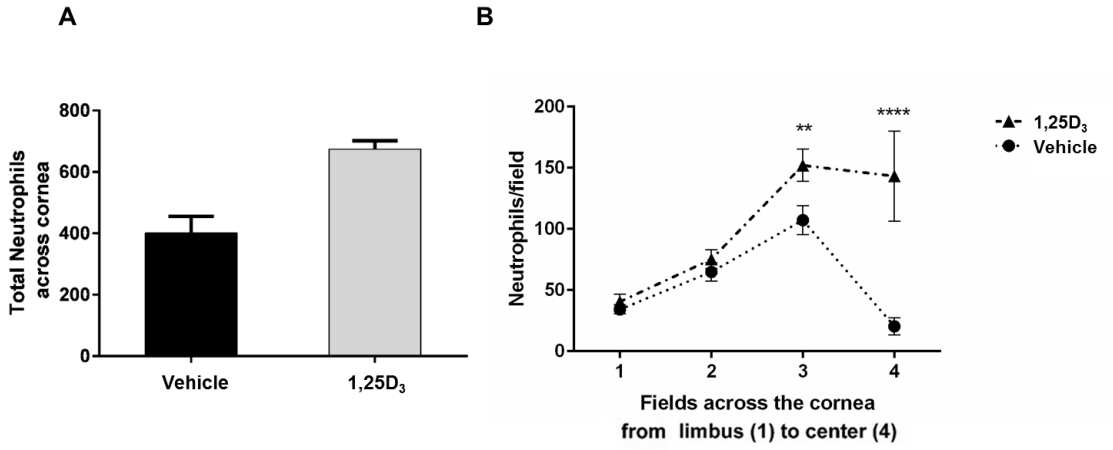

Figure 5.8. Vitamin D treatment increases neutrophil infiltration in wounded corneas.

(A) Total neutrophil counts across the cornea at 18 hours post-wound from fields 1 through 4. Data represent mean ± SEM and were analyzed with Student's two-tailed t-test. $p<0.01$ (n=6) (B) Neutrophil counts per field in vehicle or vitamin D-treated corneas at 18 hours after wounding. Statistical analysis was by two way ANOVA with Bonferroni's test for multiple comparisons. p<**0.01, ****<0.0001 (n=6)

5.3.4. Protein levels of CXCL1 are increased at 12 hours after wounding with vitamin D treatment.

Neutrophils migrate into the cornea from the limbal vessels in response to chemotactic signals resulting from injury. As vitamin D treatment increased this migration into the wounded area at 18 hours after epithelial debridement we examined the expression of CXCL1 and CXCL2, molecules known to be expressed in the cornea and to enhance neutrophil chemotaxis (Carlson et al., 2010, 2007), in wounded corneas. At 12 hours after wounding, prior to the 18 hour peak of neutrophils in the cornea, there was a 38% increase in CXCL1 protein expression with vitamin D treatment, above the increase seen with vehicle (Figure 5.9.A top). However, there was no change at this time point with CXCL2 (Figure 5.9.B top). By 24 hours of wounding, after the peak of neutrophils, there was a decrease in both CXCL1 and CXCL2 protein expression. Examining gene expression, there was no change in CXCL2 relative RNA quantity with treatment at either time point (Figure 5.9.B bottom) but a decrease in CXCL1 at 12 hours (Figure 5.9.A bottom). Wounded corneas, regardless of treatment, had increases in both CXCL1 and CXCL2 relative to unwounded corneas at both time points (bottom).

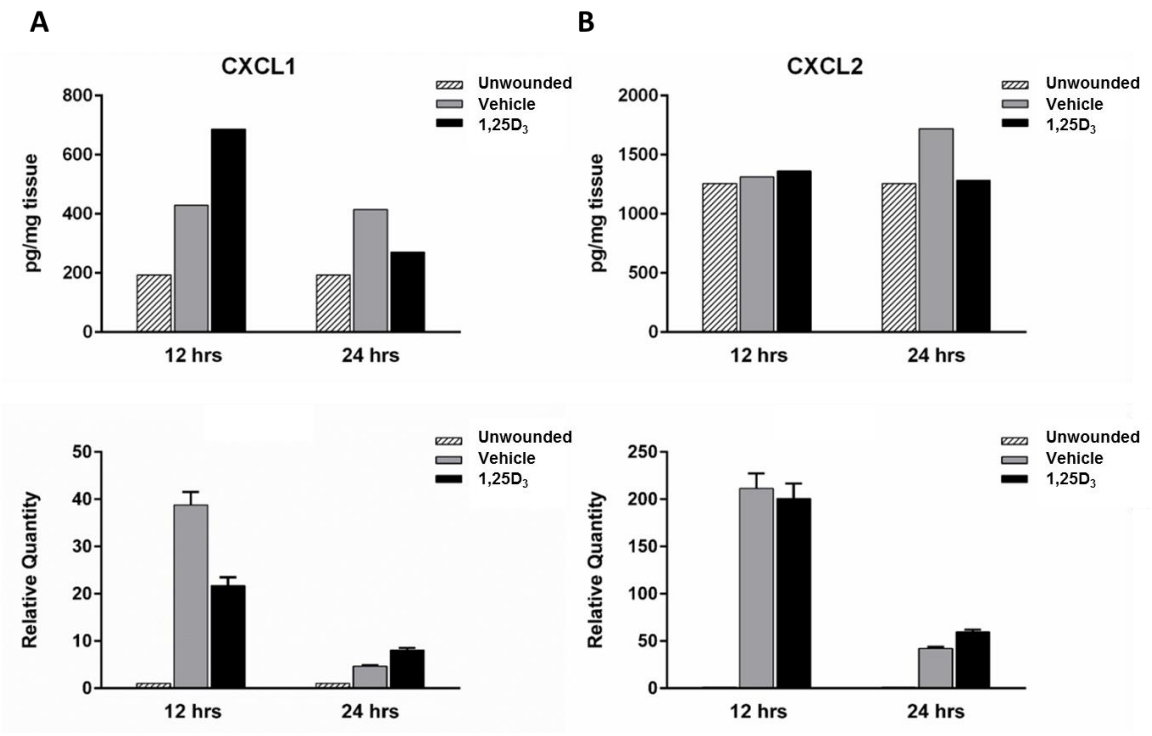

Figure 5.9. Chemokine CXCL1 and CXCL2 expression in wounded corneas.

CXCL1 (A) and CXCL2 (B) expression was determined in corneal homogenates following 12 and 24 hours of wounding. For protein analysis (top row), total corneal protein was collected and ELISAs performed. Samples represent 8 pooled corneas per group at each time point. Graphs are representative experiments (n=2, 24hrs; n=1, 12hrs) of the mean of duplicate values. For RNA analysis (bottom row), relative expression was determined by RT-PCR analysis. Samples represent 10 pooled corneas per group at each time point. Graphs are representative experiments (n=3, 24hrs; n=1, 12hrs) of the mean $\pm$ SEM of triplicate values.

5.3.5. Vitamin D does not affect early nerve regeneration or VEGF expression in the wounded corneas.

Upon epithelial debridement, the thin subbasal nerves are destroyed in the wounded area. These nerves regenerate during wound healing, however, they only reach 65% of their original density by 28 days after wounding (Yu et al., 2008). Vascular endothelial growth factor (VEGF) has been shown to be important in promoting nerve regeneration following corneal epithelial debridement and is upregulated after wounding (Amano et al., 1998; Yu et al., 2008). In other tissues, vitamin D has been shown to modulate VEGF levels, with differing responses dependent on cell type and tissue condition (Jung et al., 2015; Ren et al., 2012b; Yildirim et al., 2014). Therefore, we next examined if there was a change in relative nerve densities and VEGF expression between treatment groups early in the regeneration process.

There was no significant difference in subbasal nerve density with $1,25D_3$ treatment compared to vehicle in any corneal region (Figure 5.10). In addition, there was no change in either VEGF RNA or protein expression 12 and 24 hours after wounding with topical $1,25D_3$. There was however, the expected increase in VEGF when compared to unwounded controls (3-fold increase) (Figure 5.11). These data suggest that vitamin D, while delaying wound closure, does not slow the initial nerve regeneration after epithelial debridement or affect VEGF levels.

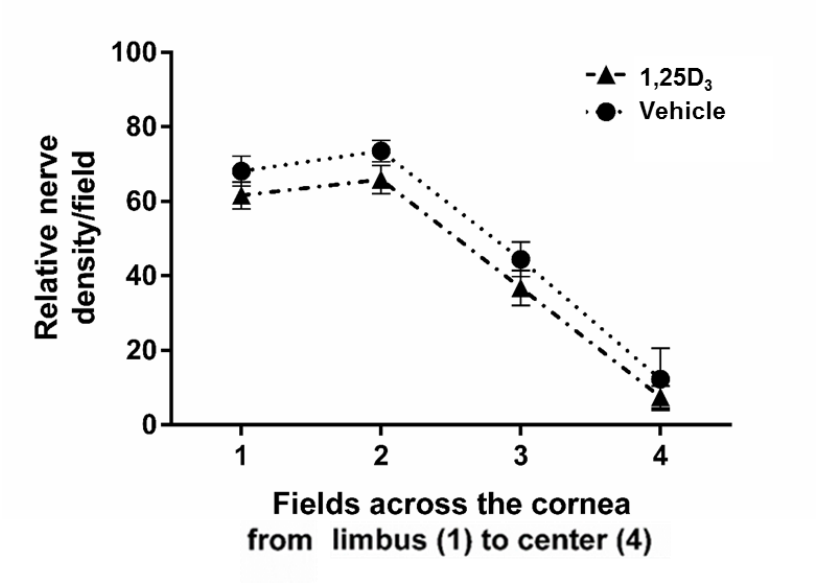

Figure 5.10. Topical vitamin D treatment does not change subbasal nerve density following wounding.

Corneal whole mounts were stained with anti-tubulin β III and subbasal nerves counted in a 10x10 morphometric grid to determine relative nerve densities. The graph represents the average nerve density per field in vehicle or $1,25D_3$ treated corneas 18 hours after wounding. (n=5 corneas/group)

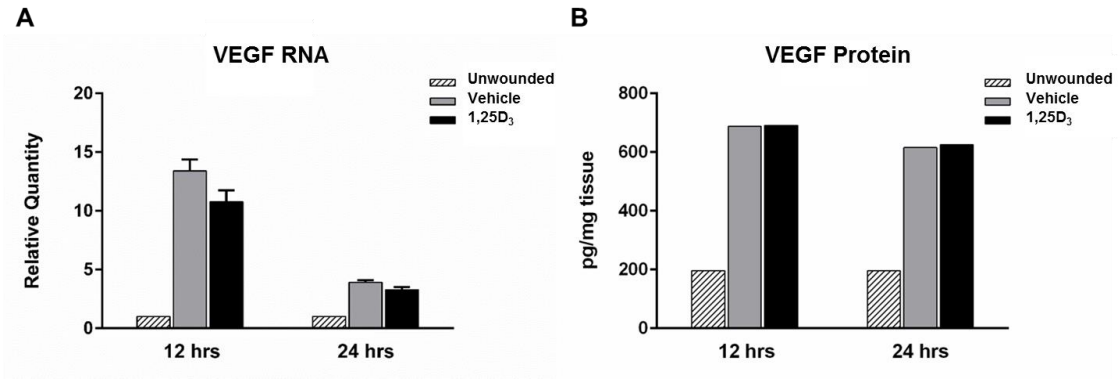

Figure 5.11. VEGF expression in whole corneas at 12 and 24 hours after wounding.

VEGF expression was determined in corneal homogenates following 12 and 24 hours of wounding. For RNA analysis (A), relative expression was determined by RT-PCR analysis. Samples represent 10 pooled corneas per group at each time point. Graphs are representative experiments (n=3, 24hrs; n=1, 12hrs) of the mean $\pm$ SEM of triplicate values. For protein analysis (B), total corneal protein was collected and ELISA performed. Data represent 8 pooled corneas per group at each time point. Graphs are representative experiments (n=2, 24hrs; n=1, 12hrs) of the mean of duplicate values.

5.3.6. Vitamin D treatment does not alter expression of cytokines after wounding.

Cytokines play an important role in integrating varying aspects of the wound healing process. Inflammatory mediators, at controlled levels, aid in advancing re-epithelization and serve as communication bridges, relaying signals between immune cells, epithelial cells, and stromal keratocytes. Vitamin D is able to modulate cytokine expression during inflammation in a number of tissues, including the cornea (Suzuki et al., 2000b; Xue et al., 2002b). Therefore, cytokine expression was compared between treatment groups after wounding. At both 12 and 24 hours post-wound, topical $1,25D_3$ did not change corneal mRNA expression of TNFα, IL-1β, TGFβ1, or TGFβ2 compared to vehicle (Figure 5.12). $1,25D_3$ also did not influence TNFα protein expression at 12 hours, however at 24 hours, there was a decrease in TNFα with $1,25D_3$ (see Appendix Figure A.8).

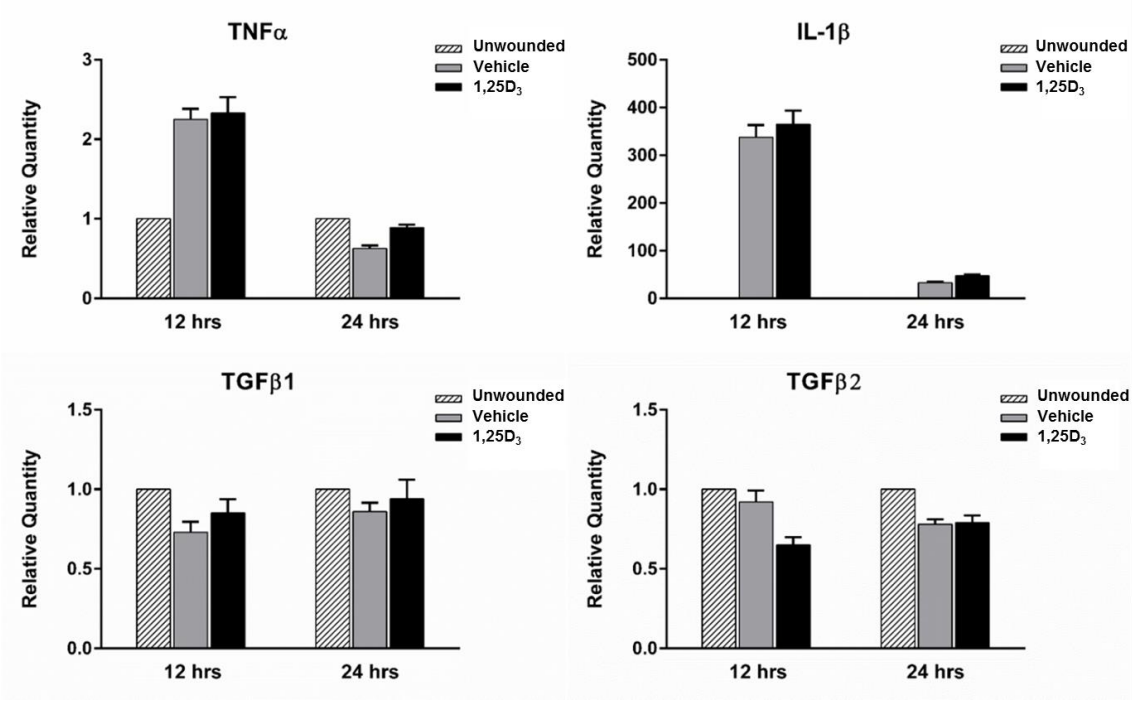

Figure 5.12. Cytokine expression at 12 and 24 hours after corneal wounding.

TNFα, IL-1β, TGFβ1, and TGFβ2 expression was determined in corneal homogenates following 12 and 24 hours of wounding by realtime PCR. Samples represent 10 pooled corneas per group at each time point. Graphs are representative experiments (n=3, 24hrs; n=1, 12hrs) of the mean $\pm$ SEM of triplicate values.

5.3.7. Antimicrobial peptide expression is induced by vitamin D treatment 12 hours after wounding.

Similar to cytokines, the antimicrobial peptide CRAMP has been shown to be important in the cornea during inflammation (L. C. Huang et al., 2007b) and experimental dry eye (see Chapter 4.3.5). The human homologue to CRAMP, LL-37, is important for defense of the cornea against infection and increases migration of human corneal epithelial cells (Huang et al., 2006). At 12 hours after epithelial debridement, $1,25D_3$ treated corneas expressed an increased level of CRAMP (3.58-fold) compared to both unwounded and vehicle treated animals (Figure 5.13). This increase could potentially offer protection to the exposed cornea while the wound is re-epithelializing.

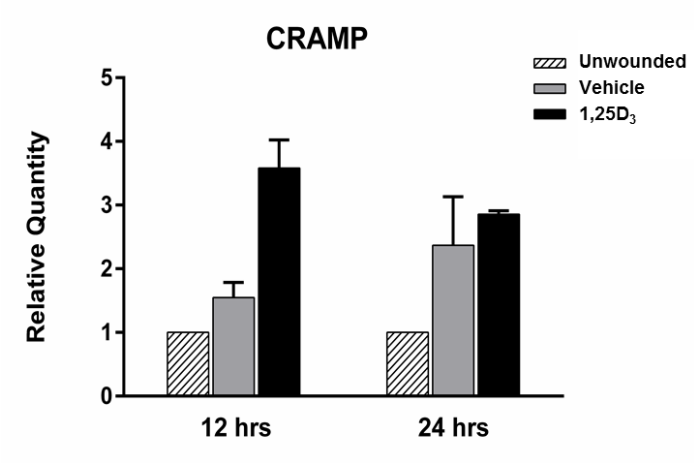

Figure 5.13. Vitamin D increases the expression of CRAMP in wounded corneas.

CRAMP expression was determined in corneal homogenates following 12 and 24 hours of wounding by realtime PCR. Samples represent 10 pooled corneas per group at each time point. Graphs are representative experiments (n=2, 24hrs; n=1, 12hrs) of the mean $\pm$ SEM of triplicate values.

5.3.8. Vitamin D does not change the rate of wound closure in the human organ culture model but does increase CYP27B1 expression.

In addition to a mouse model of wounding, we also wanted to examine vitamin D treatment in human corneal wounding. Donor corneas were wounded by epithelial debridement and wound closure was followed over 24 hours. In this *ex vivo* model, vitamin D treatment did not influence the rate of wound closure (Figure 5.14.A-B). There was no statistical difference in percent open wound at any time point between $1,25D_3$ and vehicle treatment. There was however, a significant increase in CYP27B1 expression, a hydroxylase which converts $25D_3$ to the biologically active $1,25D_3$, after 48 hours of wound healing in the human corneas (1.65-fold increase; $p<0.05$) compared to pre-wounded epithelium. (Figure 5.14.C).

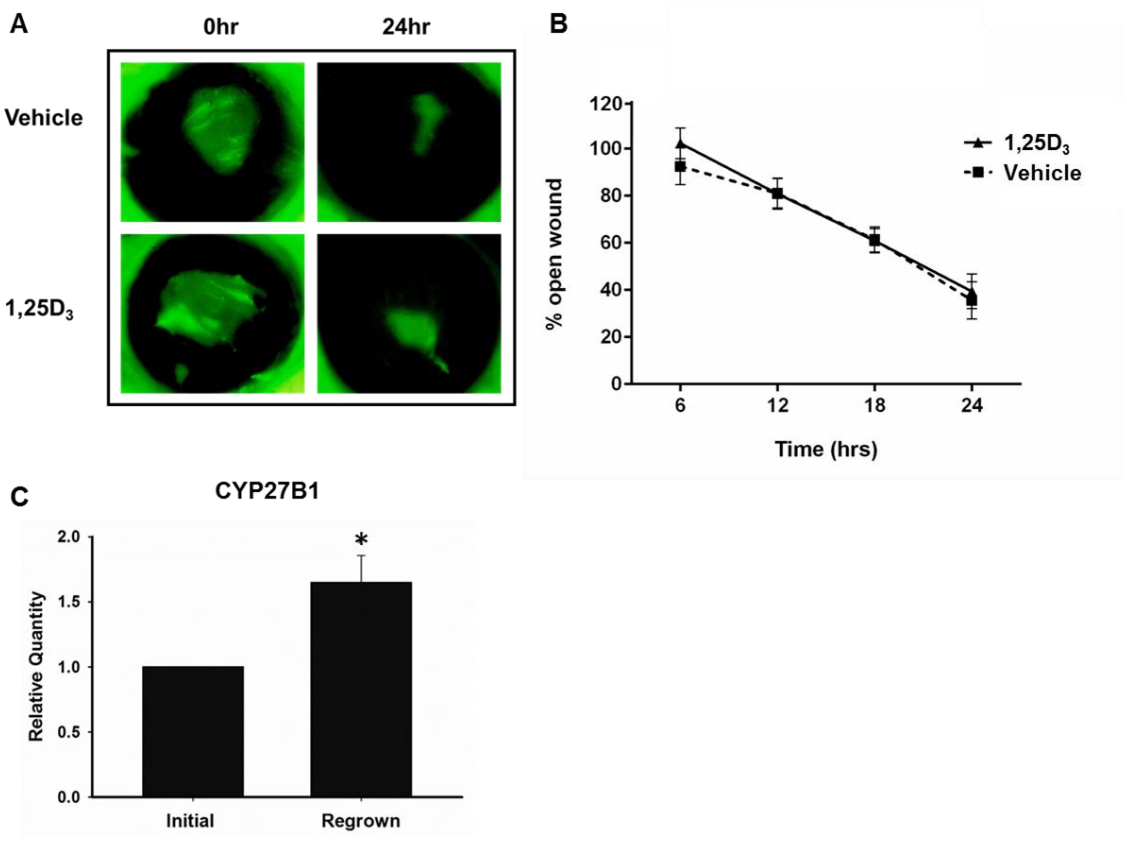

Figure 5.14. Topical vitamin D treatment does not affect wound closure rate in the human organ culture wounding model but wounded corneas express a higher level of the vitamin D activating enzyme, CYP27B1.

(A-B) Corneal wounds were monitored by fluorescein staining every 6 hours for 24 hours. Data were analyzed with repeated measures ANOVA and Bonferroni's correction for multiple comparisons. (n=5) (C) Corneal epithelium was collected at the time of wounding (initial) and scraped after 48 hours of regrowth (regrown) and CYP27B1 expression was determined by RT-PCR. Data were analyzed by student's two-tailed t-test. $p<0.05$ (n=4).

5.4. Discussion

Corneal wound healing is a complex process that involves epithelial cell division and migration, apoptosis of stromal keratocytes, nerve regeneration, and a localized inflammatory response resulting in infiltration of immune cells and platelet extravasation from limbal vessels. All of these components are necessary for efficient wound healing. In the current study, mice treated with topical vitamin D had a delay in wound closure, an increase in neutrophil infiltration, and decreased nerve regeneration in the wound margin.

Following debridement of a 2 mm diameter circular area, re-epithelialization of the murine cornea takes approximately 24 hours, resulting in complete covering of the wounded area with basal epithelial cells (Byeseda et al., 2009; Li et al., 2006c). When wounded mice were treated with topical vitamin D, there was a delay in wound closure at both 12 and 18 hours, with a greater percentage of wound area remaining open. However, there was no significant difference in basal epithelial cell division in these corneas. [In cultured human corneal epithelial cells, vitamin D treatment decreased cell proliferation 27% ($p=0.02$) after 24 hours (see Appendix Figure A.9).] Re-epithelialization involves first the migration of epithelial cells to cover the wound and then a proliferative phase of basal epithelial cells to aid in restratification. Immediately after wounding, cell division is inhibited in the wound area, to allow for efficient migration (Li et al., 2006b; Suzuki et al., 2003; Terai et al., 2011). Our data is in agreement with this model of corneal-wound healing, with almost no basal epithelial cell proliferation in the wounded area at 18 hours post-wound, a time when the wound is still

being re-epithelialized, in either vitamin D or vehicle treated mice. In the human organ culture model of wounding, vitamin D did not change the wound closure rate; however, this *ex vivo* model does not include contributions from infiltrating inflammatory cells. Unfortunately, *in vitro* migration assays monitoring HCEC migration with vitamin D treatment were variable and gave inconclusive results. Cell culture "wounding" by scratch assay did suggest that vitamin D increased migration of HCEC to cover the debrided area and these studies will be interesting to pursue further (see Appendix Figure A.10).

Epithelial debridement results in an inflammatory response that is necessary for efficient wound healing and re-epithelialization. As vitamin D is known to dampen inflammation, our hypothesis was that the delay in wound closure with vitamin D was due in part to an inhibition of the beneficial inflammation in this process. We therefore examined neutrophil infiltration into the corneal stroma and surprisingly, there was an increase in neutrophil accumulation throughout the cornea and specifically in the wounded area at 18 hours. While neutrophils are an essential part of healthy wound healing, too many neutrophils can be detrimental to the process, interfering with basal epithelial cell migration into the wounded area. The increase in neutrophils seen with vitamin D could in part be a result of an increase in chemotactic signals. CXCL1 (C-X-C motif ligand 1, GRO1, KC) is a chemokine that recruits neutrophils to sites of injury and inflammation (Carlson et al., 2010, 2007) and has been shown to be elevated following corneal abrasion (Li et al., 2006a). There was a greater amount of CXCL1 protein in wounded corneas with vitamin D treatment, just prior to the peak in neutrophils,

potentially causing greater numbers of neutrophils in these mice. A major source of CXCL1 is from the stromal keratocytes, which undergo cell death following wounding. As there is an increase in CXCL1 with vitamin D treatment, it would therefore be interesting to ascertain if vitamin D is protective against keratocyte death in this model.

A possible explanation for the increase in neutrophils with topical vitamin D which was not examined in this study, is disruption of neutrophil apoptosis. Neutrophils undergo rapid programmed cell death, or apoptosis, following infiltration to the site of inflammation (Savill, 1997). If this process is inhibited, there would be a greater number of neutrophils remaining in the wounded area. Another interesting situation to further explore would be the effect of vitamin D on NK (natural killer) cell migration into the wounded area. NK cells are immune cells, which are also important for effective wound healing. It has been shown that depleting NK cells in an epithelial abrasion model increases neutrophil infiltration while negatively affecting re-epithelialization and nerve regeneration. In this study, Liu et al. (2012) suggest that NK cells act to limit acute inflammatory events. *In vitro* studies suggest that vitamin D has a negative effect on NK cell development (Weeres et al., 2014). Therefore, investigation into vitamin D's influence of NK cells in wounding is warranted.

In order to further dissect the effect of vitamin D treatment on the wound healing process, nerve regeneration and cytokine expression were also examined in the wounded corneas. Nerves release trophic factors, which help to maintain the health of the corneal epithelium and stroma. Various factors play a role in nerve regeneration, including VEGF, produced by the wounded epithelium and infiltrating neutrophils (Li et al., 2011).

However, there was no difference in either nerve density in the wounded corneas or VEGF expression with topical vitamin D treatment. Cytokines also play an integral role in the localized inflammatory reaction following wounding (Imanishi et al., 2000; Klenkler and Sheardown, 2004; Terai et al., 2011). Disruption in TGFβ signaling delays corneal re-epithelialization (Saika, 2006; Saika et al., 2004; Terai et al., 2011). IL-1 and TNFα also are important mediators of corneal injury induced inflammation, influencing both proliferation and migration of epithelial cells (Imanishi et al., 2000; Klenkler and Sheardown, 2004). Mice deficient in these cytokines have compromised corneal wound healing, with decreased dendritic cell migration in the wounded epithelium (Dekaris et al., 1999; Gao et al., 2011b; Hong et al., 2001). IL-1 is released from the injured epithelium and also induces keratocyte cell death, a coordinated aspect of the wounding process (Wilson et al., 2001, 1996). Interestingly, when NFKβ signaling is disrupted, corneal wound healing is delayed, causing a decrease in pro-inflammatory cytokines and a reduction in cell migration (Chen et al., 2011). However, in addition, levels of IL-1β, TNFα, TGFβ1, and TGFβ2 were not changed with vitamin D treatment in these experiments.

While this study did not detect changes in VEGF/cytokines with vitamin D treatment, it is important to note that whole corneas were used to measure expression. Therefore, the results reflect general corneal expression, with contributions from epithelial, stromal, and immune cells. Subtle differences, for example, in epithelial expression of cytokines or neutrophil expression of VEGF, caused by vitamin D, might have been masked. This especially merits consideration, given the increase in neutrophils

with vitamin D treatment. It would therefore be interesting to examine cytokine expression in the isolated epithelium and compare this to results from whole corneal expression.

Another aspect of wound healing is the protection of injured epithelium from infection during re-epithelialization. Antimicrobial peptides (AMP) are small cationic peptides which have been shown to be expressed during wound healing, providing a line of defense against pathogens (Carretero et al., 2008; Gordon et al., 2005; Huang et al., 2007b; Kai-Larsen and Agerberth, 2008; McDermott, 2004; Steinstraesser et al., 2008). One of these AMPs, CRAMP/LL-37, was first identified in neutrophils, however, now it is known to be expressed in the corneal epithelium (Huang et al., 2007a). In addition to its broad range killing ability, LL-37 is also able to modulate inflammatory signals, bind LPS, and potentiate the wound healing process. It has also been shown to be chemotactic for neutrophils and T lymphocytes (Agerberth et al., 2000) and increases the migration of human corneal epithelial cells in culture (Huang et al., 2006). Vitamin D augments the production of LL-37 in various human tissues, however it is unclear if CRAMP, the murine orthologue, is influenced by vitamin D. In our study, at 12 hours after the initial wound, vitamin D increased CRAMP RNA expression. Therefore, while delaying early wound closure, vitamin D treatment could simultaneously be enhancing protection of the exposed epithelium, preventing infection of the underlying tissue.

In previous studies, vitamin D decreased pro-inflammatory mediators *in vitro* (see Chapter 2) and is known to modulate inflammation in a variety of tissues and diseases. The current study highlights the fact that various factors influence the effects of vitamin

D during inflammation and that its role in inflammation is complex. The response to inflammation can change based on the cell types involved, the source of immune stimulus, the tissue microenvironment, timing of vitamin D administration, vitamin D concentration, and the relative expression of activating and inactivating hydroxylases. The effect of topical vitamin D on corneal wound healing is an important area of further research and should be evaluated when considering vitamin D as a therapeutic option during inflammatory conditions.

Chapter 6

Summary and Conclusions

Vitamin D is a multifunctional hormone that is now known to play a significant role in a variety of biological functions in addition to its traditional role in regulating calcium homeostasis. There are a large number of studies demonstrating that adequate vitamin D levels are important in maintaining health and show that vitamin D is able to be utilized at local tissue sites. In the eye, we have increasing evidence of the association between disease and vitamin D. Epidemiological studies have demonstrated a link between vitamin D deficiency and the development of various ocular pathologies including myopia and age-related degeneration. In addition, genetic polymorphisms in vitamin D-related genes have been implicated in these diseases. Animal models suggest that vitamin D is a potential treatment option for retinoblastoma and uveitis and is protective against inflammatory events related to retinal aging. However, at the beginning of this dissertation, there was a lack of knowledge in the function of vitamin D at the ocular surface. Therefore, the central aim of these studies was to examine vitamin D activity in the cornea, the front part of the eye, in order to ascertain if vitamin D treatment could be beneficial and protective during inflammation.

Vitamin D is an important modulator of the immune system, and in general, acts to protect against harmful levels of inflammation. The cornea is a unique tissue in that it is transparent, which is essential to its primary function of refracting light back onto the retina for vision. Inflammatory events can lead to tissue swelling and damage, and thus, if left unchecked, can be detrimental to corneal transparency and function. For this reason, vitamin D is an interesting molecule to study in this tissue. Increased knowledge

of vitamin D function can be used to determine if it is an appropriate therapeutic option for corneal inflammation.

In these studies, *in vitro* experiments demonstrated that while vitamin D increased initial cytokine levels, it was then able to decrease these inflammatory cytokines and chemokines following TLR activation. TLRs are sensors of the innate immune system which trigger an inflammatory response upon contact with microbial ligands and danger signals from self-tissue. TLRs are activated in the cornea during infection, initiated by various pathogens, and have also been implicated in the pathogenesis of dry eye syndrome, an inflammatory condition of the ocular surface. In addition to dampening high levels of pro-inflammatory mediators, vitamin D was able to augment the production of antimicrobial peptides during TLR stimulation. These results suggest that vitamin D could offer protection during infection and inflammation, guarding against corneal tissue damage.

During dry eye disease, a condition that affects millions of individuals each year, a cycle of aberrant inflammation leads to pain, discomfort, and vision disturbances. Therefore, there is an avid interest in discovering new anti-inflammatory treatment options for DED. Our in vitro studies suggested that vitamin D could dampen inflammatory signals, therefore, we examined the effects of topical vitamin D during an experimental model of DED. Unfortunately, our model system needed improvement due to unstable environmental conditions and further studies will need to be done to validate the model. However, there were several encouraging findings that make the study of vitamin D in the context of DED promising. In control animals, vitamin D increased tear

production, as measured by the phenol red thread test, suggesting that in a non-dry eye environment, vitamin D treatment could be beneficial to the ocular surface, preventing desiccation and hyperosmolarity through increased tears. Vitamin D treatment also increased antimicrobial peptide production, as was found *in vitro*. Antimicrobial peptides protect the cornea through their ability to both kill microbes and dampen inflammation. These small peptides have also been shown to enhance corneal wound healing. In addition to these protective effects, vitamin D also potentially decreased MMP-9 expression, a protease that at high levels leads to extracellular matrix disruption and enhanced inflammation. Further studies will examine vitamin D's ability to influence cytokine production and leukocyte infiltration during DED, as well as the effect of a high vitamin D diet on signs of dry eye disease.

Inflammation also occurs following corneal wound healing; however, the acute inflammatory events immediately after injury are beneficial to the process, at the appropriate level. As vitamin D increased antimicrobial peptides and affected cytokine production *in vitro*, this study also examined the effect of topical vitamin D during corneal epithelial abrasion. Interestingly, the rate of wound closure was delayed and there was an increase in neutrophil infiltration with vitamin D. Experiments in human corneal epithelial cells suggested that vitamin D treatment could initially increase inflammatory events, at the time of injury/insult, but that after initiation, vitamin D could dampen pro-inflammatory signaling, helping to curb the response and aid in resolution of inflammation (Figure 6.1). This could have occurred in the wound healing model, where there was an increase in chemokine production soon after wounding with vitamin D

treatment. It will be important to follow later time points to examine this hypothesis *in vivo* and determine if the wound healing delay is physiologically relevant.

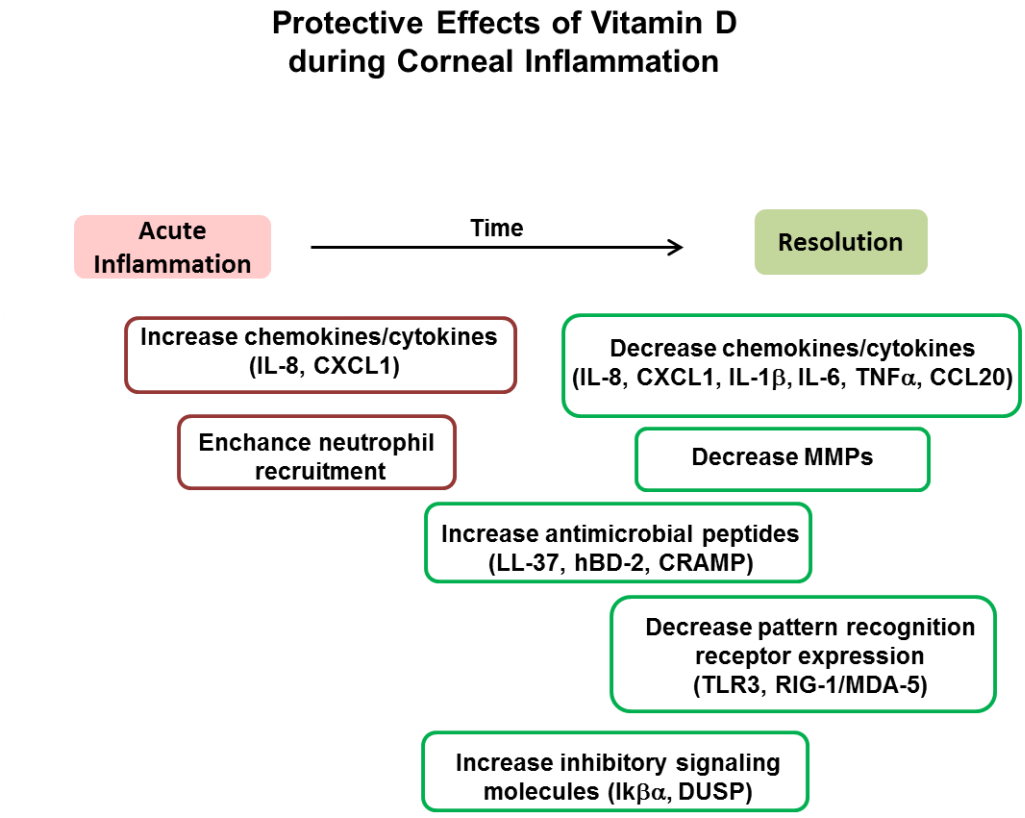

Figure 6.1. The possible protective effects of vitamin D during corneal inflammation.

Both *in vitro* and *in vivo* studies suggest that vitamin D increases initial inflammatory events, while decreasing later inflammation, thus aiding in resolution and the protection of the ocular surface from damage and chronic inflammation.

In total, the work in this dissertation demonstrates that cells in the cornea are able to respond to vitamin D through altered gene expression and modulation of inflammatory mediators. Both *in vitro* and *in vivo* studies suggest that vitamin D can have pro- and anti-inflammatory effects on corneal inflammation, dependent on the timing and type of inflammatory response. It will therefore be exciting to follow further work, examining the benefits of vitamin D therapeutically in the eye and its role in the resolution of inflammation.

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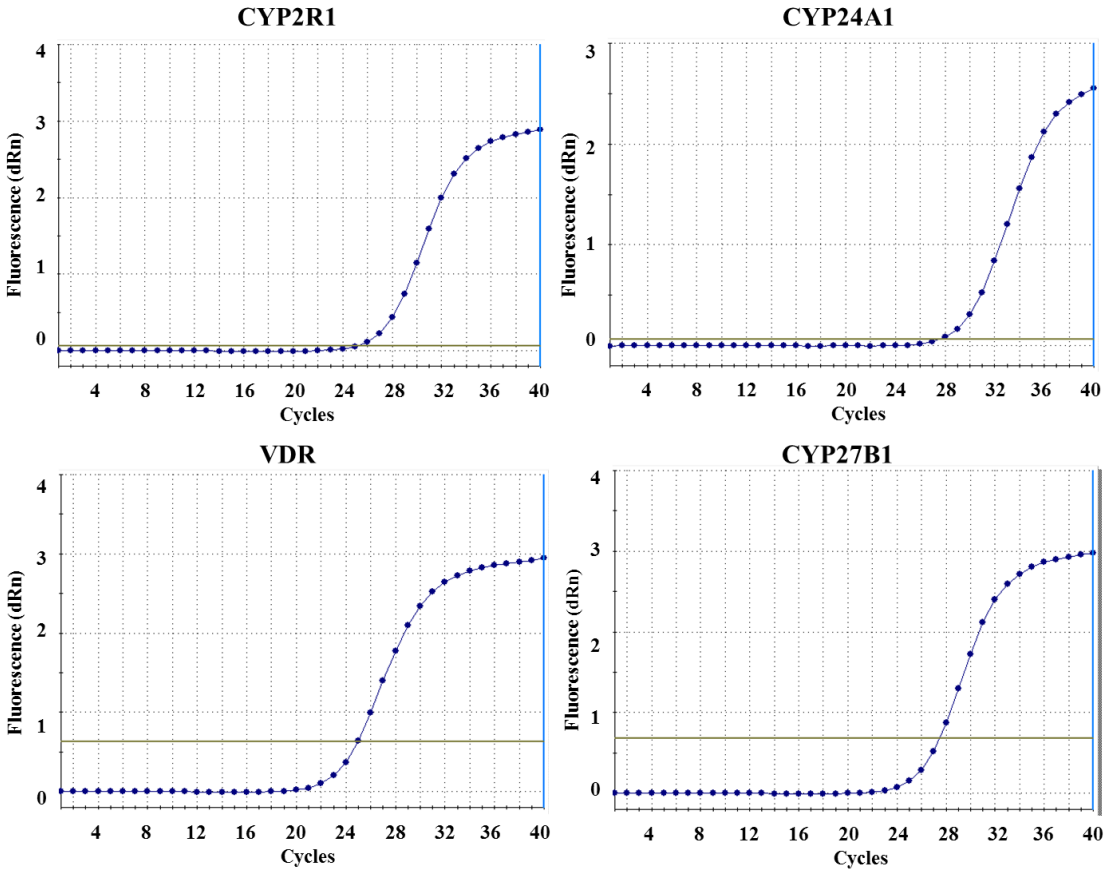
APPENDIX

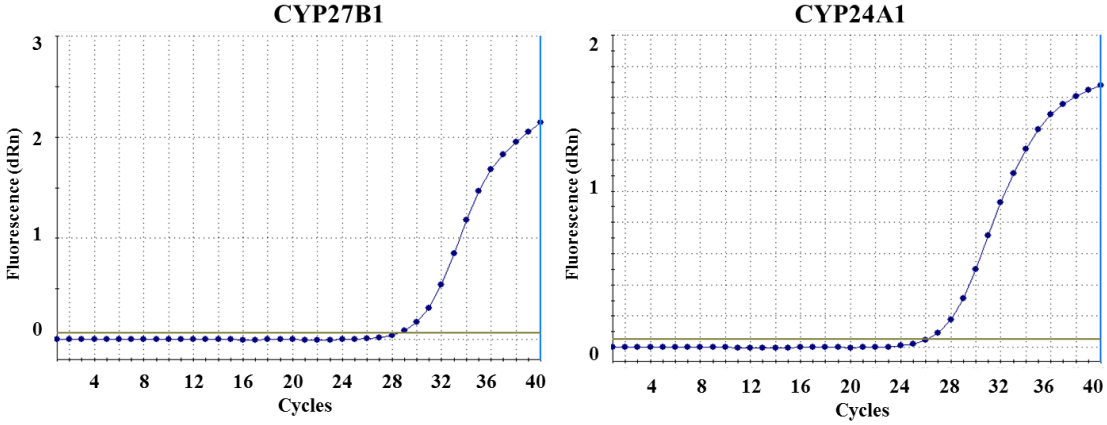
A.1. Expression of VDR-related enzymes and hydroxylases.

The expression of the 25-hydroxylase (CYP2R1), the vitamin D activating 1α-hydroxylase (CYP27B1), the inactivating 24-hydroxylase (CYP24A1), as well as the VDR was confirmed in SV40 cells. In addition, epithelial cells removed from human donor corneas also expressed the activating and inactivating hydroxylases. To examine expression of these enzymes in stromal cells, primary fibroblasts were cultured from human donor corneas, and the expression of CYP2R1, CYP27B1, and the VDR was demonstrated by realtime PCR analysis. (For RNA collection and realtime PCR methods see Chapter 2.2.2; for SV40 cell culture and isolation of primary human corneal cells see Chapter 2.2.1.)

SV40 HCEC



Scraped Epithelial Cells



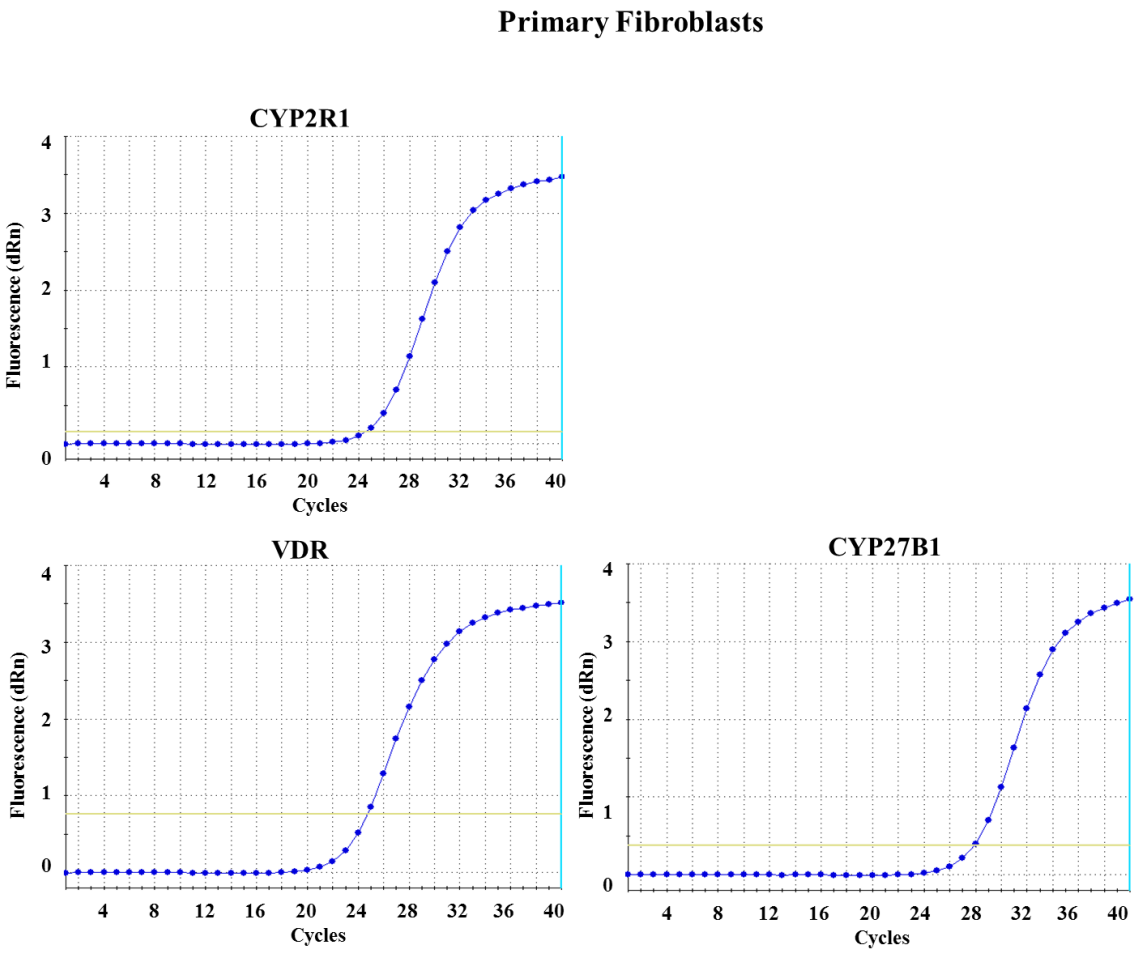


Figure A.1. Human corneal cells express the enzymes for vitamin D metabolism and the VDR.

RT-PCR analysis of hydroxylase and VDR expression in SV40 HCEC, freshly scraped human donor corneal epithelial cells, and fibroblasts cultured from human donor corneas. Amplification plots show gene expression as a measure of fluorescence intensity in each PCR cycle.

A.2. Vitamin D treatment increases *Staphylococcus aureus* colony growth following incubation with cultured supernatants.

To investigate a possible functional consequence of the modulation of innate immunity related genes, SV40-HCEC were treated with $1,25D_3$ and the supernatants were incubated with a clinical isolate of *Staphylococcus aureus*, to determine antimicrobial activity (see Chapter 2.2.3). Supernatants treated with $1,25D_3$ (Cells+$1,25D_3$) were less effective at killing *Staphylococcus aureus* than cells alone, with an increase in bacterial colonies after incubation (5 CFU compared to 32 CFU with vitamin D, $p<0.001$).

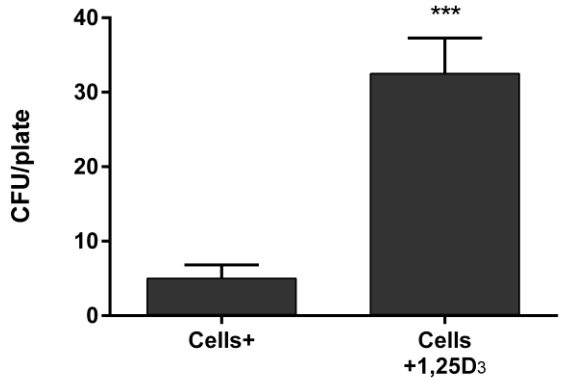


Figure A.2. SV40-HCEC antimicrobial activity against *Staphylococcus aureus.*

SV40-HCEC were left untreated (Cells) or treated with 1,25D3 (10^{-7}M) for 24 hours (Cells+1,25D3) and supernatants used in an antimicrobial assay. Data represent mean +/- SEM of three independent experiments. Statistical analysis was by Student's t-test, p<***0.001.

A.3. Zymosan treatment enhances vitamin D-mediated antimicrobial peptide production.

As TLR agonists increased vitamin-D related enzyme expression, we sought to determine if LL-37 expression could be augmented by combined TLR agonist-vitamin D treatment. Focusing on TLR2 agonists, when $1,25D_3$ was added to cells along with FSL1, Pam3CSK4, HKLM (see Chapter 2.3.4), or Zymosan, there was an increase in LL-37 expression (Figure 2.6.B and Figure A.3). Zymosan treatment augmented LL-37 expression in combination with $1,25D_3$, with a 13-fold increase above control (n=4). These results suggest that vitamin D can cooperate with TLRs to enhance innate immunity in HCEC.

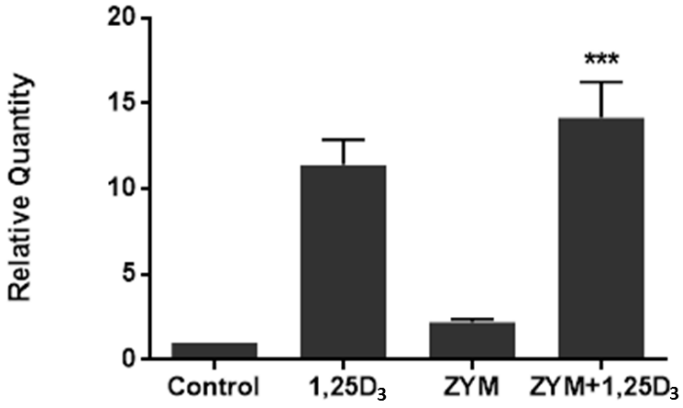


Figure A.3. Zymosan treatment enhances vitamin D-mediated LL-37 production.

hTCEpi were treated with $1,25D_3$ (10^{-7}M) and/or 50μg/ml zymosan (Zym) for 6 hours and LL-37 expression analyzed by real-time PCR (see Chapter 2.2.2). Data represent mean +/-SEM of four independent experiments. Statistical analysis was by ANOVA with Bonferroni's test for multiple comparisons, p<***0.001.

A.4. Vitamin D modulates the HCEC response to Poly(I:C) through decreased production of pro-inflammatory cytokines.

When hTCEpi were treated with Poly(I:C) for 24 hours, there was an increase in both IFNγ and IL-23 gene expression (96 and 104 fold increases, respectively). When $1,25D_3$ was added to cells with Poly(I:C), there was a significant decrease in expression (80% and 74% decreases, $p<0.05$, n=3). This demonstrated that vitamin D treatment could attenuate pro-inflammatory cytokine production (see Chapter 2.3.5).

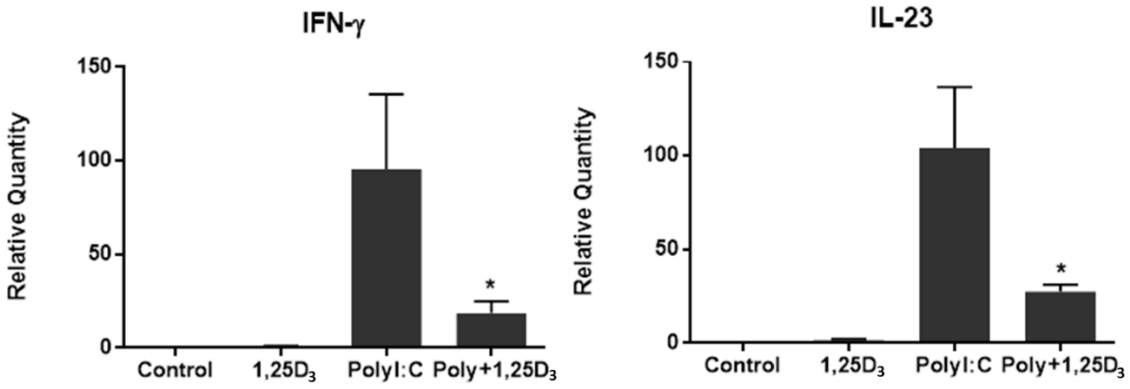


Figure A.4. Vitamin D decreases the Poly(I:C)-induced expression of cytokines IL-23 and IFNγ.

hTCEpi were treated with $1,25D_3$ (10^{-7}M) and/or Poly(I:C) (1μg/ml) for 24 hours. Cell lysates were collected for RNA isolation followed by real-time PCR analysis of IL-23 and IFNγ (See Chapter 2.2.2). Data represent mean +/-SEM of three independent experiments. Statistical analysis was by ANOVA with Bonferroni's test for multiple comparisons, with comparison to Poly(I:C) treatment alone, p<*0.05.

A.5. VDR is necessary for the vitamin D-mediated decrease in cytokine expression; however, LL-37 is not responsible for this effect.

The vitamin D-mediated suppression of inflammatory mediators (see Chapter 2.3.5) appears to be dependent on signaling through the VDR, as treatment with VDR siRNA blocked the downregulation of IL-8 by $1,25D_3$ (Figure A.5.A). However, LL-37 was not responsible for the decrease in IL-8 expression (Figure A.5.B).

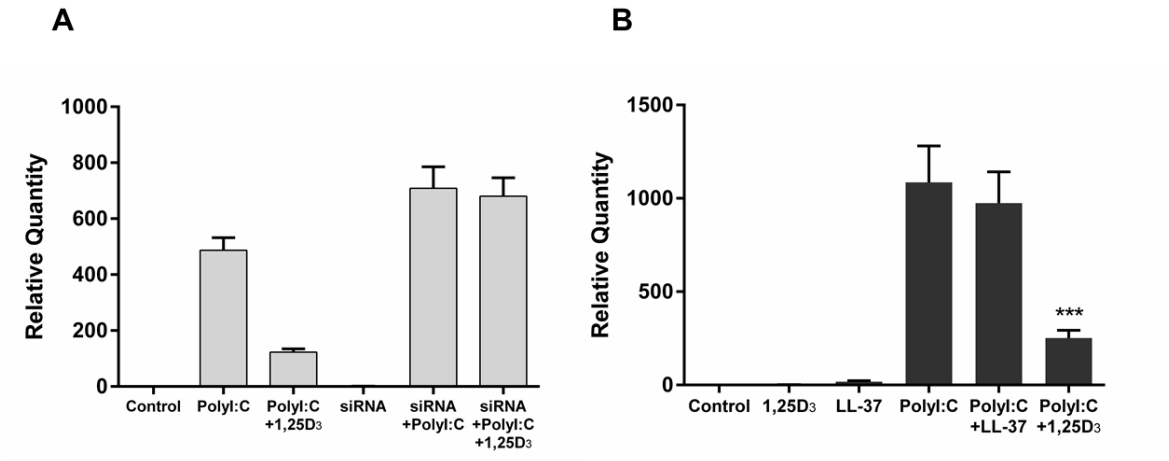


Figure A.5. The downregulation of IL-8 expression following Poly(I:C) treatment is dependent on the VDR and is not mediated by LL-37.

(A) hTCEpi were left untreated or transfected with VDR siRNA for 24 hours and then treated with Poly(I:C) (1μg/ml) and $1,25D_3$ (10^{-7}M) for an additional 24 hours (see Chapter 2.2.1 for siRNA methods). IL-8 expression was analyzed by real-time PCR (Chapter 2.2.2). Graph is a representative of 2 experiments and mean +/- SEM of triplicate values. (B) hTCEpi were treated with Poly(I:C) (1μg/ml), $1,25D_3$ (10^{-7}M), and/or LL-37 (10μg/ml) for 24 hours. Cell lysates were collected for RNA isolation followed by real-time PCR analysis of IL-8 expression. Data represent mean +/- SEM of 4 independent experiments. Statistical analysis was by ANOVA with Bonferroni's test for multiple comparisons, with comparison to Poly(I:C) treatment alone, p<***0.001.

A.6. Vitamin D treatment decreases the Poly(I:C)-induced expression of RIG-1 and MDA-5.

In addition to decreasing TLR3 expression and the basal levels of cytoplasmic pattern recognition receptors RIG-1 and MDA-5 (see Chapter 2.3.6), vitamin D treatment significantly lowered the expression of these receptors following 24 hour Poly(I:C) stimulation (58% decrease in RIG-1 and 66% decrease in MDA-5, $p<0.05$).

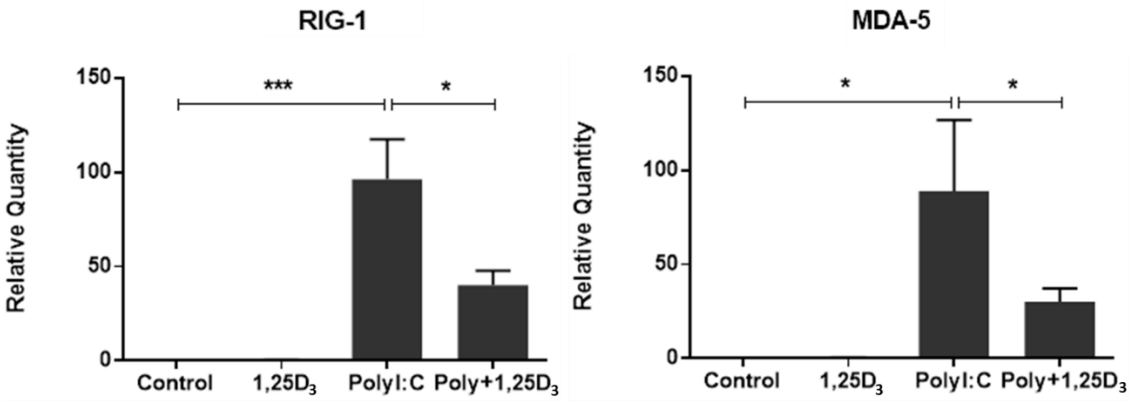


Figure A.6. Vitamin D lowers the expression of intracellular PRRs RIG-1 and MDA-5 following Poly(I:C) treatment.

hTCEpi were treated with $1,25D_3$ (10^{-7}M) and/or Poly(I:C) (1μg/ml) for 24 hours. RIG-1 (left) and MDA-5 (right) expression was determined by real-time PCR. Data represent mean +/- SEM of five independent experiments. Statistical analysis was by ANOVA with Bonferroni's test for multiple comparisons, p<*0.05, ***0.001.

A.7. Vitamin D hydroxylase expression is increased by hyperosmolar stress.

Primary HCEC were cultured in hyperosmolar media to mimic the increased tear film osmolality that occurs during DED. Hyperosmolar stress (450 and 500mOsmM) upregulated the expression of the vitamin D inactivation hydroxylase CYP24A1 (17-fold increase), while the activating enzyme, CYP27B1 was increased at 500mOsmM. These results suggest that dry eye conditions have the potential to regulate vitamin D activation through modulating hydroxylase expression.

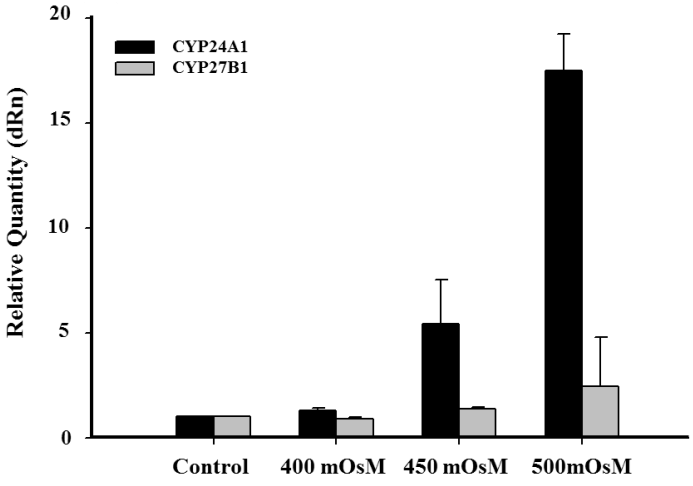


Figure A.7. Hyperosmolar stress influences the expression of vitamin D hydroxylases.

Primary HCEC were treated with SHEMX media with increasing osmolarity (OsM), from 400-500 mOsM, for 24 hours. Cell lysates were collected for RNA isolation followed by real-time PCR analysis of CYP27B1 and CYP24A1 expression (see Chapter 2.2.2). Data represent mean +/- SEM of 2 independent experiments using cells from two different corneal donors.

A.8. Vitamin D treatment decreases TNFα protein expression at 24 hours after corneal debridement.

To determine vitamin D's effect on pro-inflammatory cytokine expression following epithelial wounding (see Chapter 5.2), whole corneas were removed and TNFα levels assessed by ELISA. At 12 hours post-wounding, there was no change in TNFα expression with treatment. However, at 24 hours, vitamin D treatment decreased protein expression in corneal homogenates compared to vehicle (700pg/mg compared to 391pg/mg).

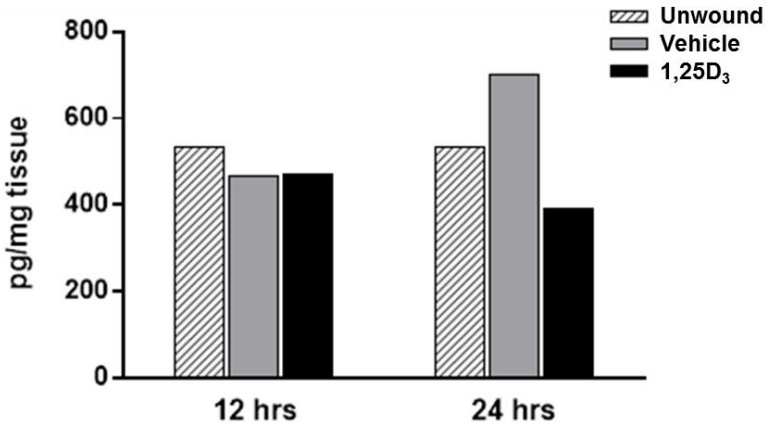


Figure A.8. TNFα expression at 12 and 24 hours after corneal wounding.

TNFα protein expression was determined in corneal homogenates following 12 and 24 hours of wounding by ELISA (see Chapter 5.2). Data represent 8 pooled corneas per group at each time point. Graphs are representative experiments (n=2, 24hrs; n=1, 12hrs) of the mean of duplicate values.

A.9. Vitamin D treatment lowers hTCEpi cell viability after 24 hours of treatment.

In order to determine the effect of $1,25D_3$ on hTCEpi proliferation, an MTT assay was performed (see Figure A.9) following 24 hours of treatment. $1,25D_3$ significantly lowered cell viability compared to control cells, 79% of control levels (p=0.022).

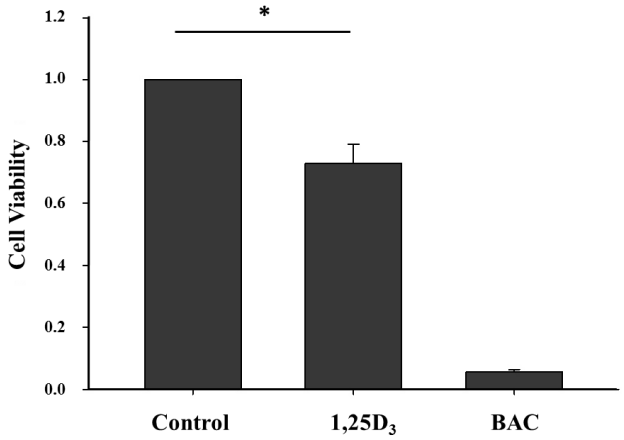


Figure A.9. Cell viability following vitamin D treatment.

hTCEpi were plated in 96 well plates and treated with $1,25D_3$ (10^{-7}M). Following 24 hours, 0.5mg/ml MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) was added to each well and cells were incubated for an additional 2 hours at 37^{o}C. Colorimetric changes were measured at wavelength 590 on a spectrophotometer and OD values normalized to untreated control cells. 0.02% benzalkonium chloride in PBS (BAC) was used as a positive control, indicating loss of cell viability. Data represent mean +/- SEM of 4 independent experiments. Statistical analysis was by Student's t-test with p*=0.022.

A.10. Vitamin D treatment during an in vitro scratch wound.

hTCEpi cells in culture dishes were "wounded" by scraping away a vertical strip of cells and then were stimulated with $1,25D_3$ to examine its effect on wound closure. As early as 6 hours after the scratch, cells were beginning to close the wounded area, as visualized by light microscopy. By 24 hours, the cells treated with $1,25D_3$ were almost completely closed and appeared to close faster than the control treated cells.

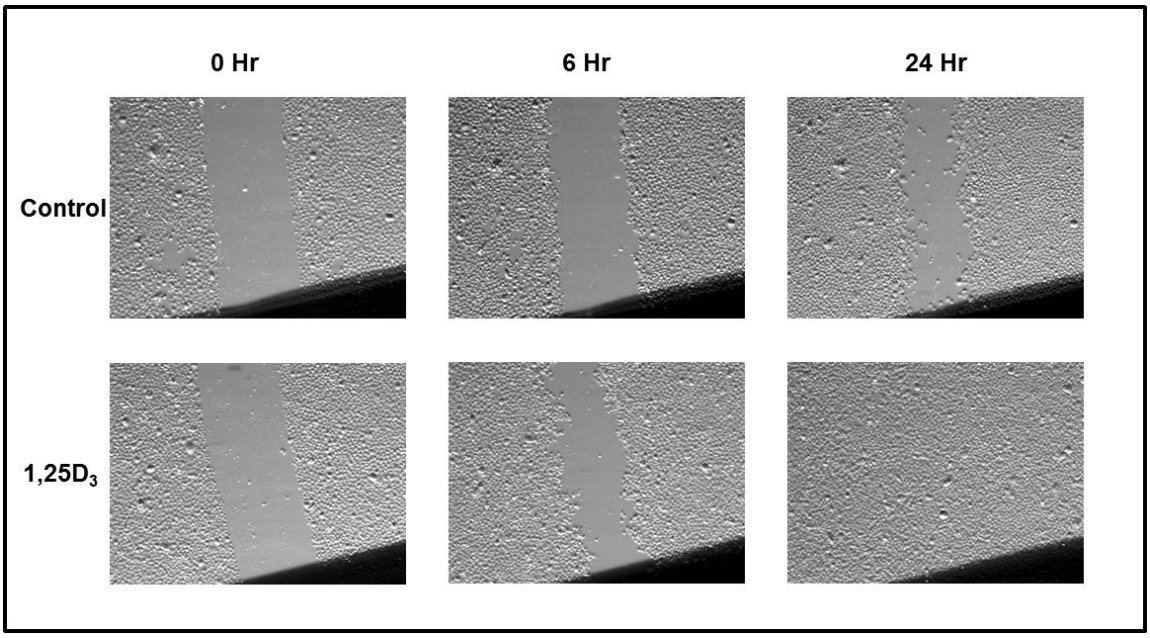


Figure A.10. ***In vitro*** **scratch wound assay.**

hTCEpi were grown to confluency in 6 well plates. A scratch was made manually with a 200µl pipet tip, removing a vertical strip of cells in each well. Wells were treated with $1,25D_3$ (10^{-7}M) or vehicle (0.1% ethanol) and closure of the scratch monitored on an Olympus inverted microscope. Images are representative of 20 wells from 5 independent experiments.

A.11. Vitamin D influences the phosphorylation of multiple kinases involved in intracellular signaling.

In order to pinpoint signaling pathways that are influenced by vitamin D, hTCEpi were treated for 6 hours with $1,25D_3$ and cell lysates were examined by phospho-kinase array (see Chapter 3.2 for methods). Results are from a single experiment, run in duplicate, but indicate that vitamin D is able to affect activation of various signaling kinases through either enhancement or blocking phosphorylation.

Table A.1. Phospho-kinase array results following 6 hour treatment with $1,25D_3$.

	Control		Vitamin D	
Target	**Average**	**SD**	**Average**	**SD**
p38α	1.53	0.88	1.64	0.82
ERK1/2	34.89	2.10	68.82	0.73
JNK 1/2/3	3.80	0.75	2.28	0.66
GSK-3α/β	22.64	8.03	15.28	0.84
EGF R	30.43	3.00	23.82	2.95
MSK1/2	12.51	0.77	6.66	0.24
AMPKα1	12.90	0.74	6.64	1.89
Akt 1/2/3	76.54	6.21	71.92	5.47
TOR	6.42	0.72	6.06	1.50
CREB	10.68	0.34	11.50	0.72
HSP27	4.74	0.91	7.05	0.24
AMPKα2	15.75	1.00	7.74	0.07
β-Catenin	6.20	0.31	5.24	0.59
Src	3.72	0.17	6.74	0.87
Lyn	1.28	1.21	4.27	0.25
STAT2	29.99	1.74	14.58	0.60
STAT5a	5.14	1.20	4.80	1.52
Fyn	2.23	1.39	4.60	0.05
Yes	5.42	0.54	6.39	1.10
Fgr	-0.89	0.64	2.34	0.74
STAT6	10.14	0.11	7.10	0.11
STAT5b	5.87	0.13	4.46	0.64
Hck	1.53	0.13	4.15	0.89
Chk-2	20.64	0.05	25.74	2.00
FAK	2.56	1.17	3.52	0.70
PDGF Rβ	3.72	1.47	1.67	0.26
STAT5a/b	4.29	0.45	1.90	1.07
PRAS40	92.71	0.28	69.25	1.82
p53	54.20	7.42	82.08	5.92
p53	6.48	0.97	13.36	0.31
p53	8.36	0.14	7.54	1.05
eNOS	1.48	0.12	0.58	0.05
p27	2.29	0.14	0.40	0.68
PLC-γ1	1.85	0.23	-0.02	0.03
WNK1	110.47	1.29	102.56	2.30
HSP60	20.17	7.33	5.56	0.95