VITAMIN D_3 PRODUCTION BY OCULAR BARRIER EPITHELIAL CELLS

By

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Abstract

Extra-renal synthesis of vitamin D_3 has been reported in many tissues and cells including barrier sites where it induced immunomodulatory effects. I investigated local synthesis of vitamin D_3 and its role in the induction of host defense peptides (HDPs) in human ocular barrier epithelial cells. I also examined the association between vitamin D receptor (VDR) single nucleotide polymorphism (SNP) with intermediate uveitis (IU) in Caucasians.

Human corneal endothelial (HCEC-12), non-pigmented ciliary body epithelial (ODM-2), and adult retinal pigment epithelial (ARPE-19) cell lines, expressed mRNA and protein for VDR and vitamin D_3 pathway elements and can locally synthesise $1,25(OH)_2D_3$ *in vitro.* These cells upregulated mRNA, but not protein expression of HDPs in response to vitamin D_3. IL-1β and TNF-α did not synergise with vitamin D_3 to upregulate HDPs in ocular barrier epithelial cells.

VDR single nucleotide polymorphisms *BsmI* (rs1544410) is significantly associated with IU. Allele rs1544410-T and genotype rs1544410-CT were higher in IU patients than healthy controls.

The results show that that extra-renal production of active vitamin D_3 occurs in ocular barrier cells and may contribute to immune regulation in the eye. The association with SNP in the vitamin D_3 receptor gene and intermediate uveitis suggests that genetic control of vitamin D_3 may be linked to ocular inflammatory disease.

Dedication

To my amazing parents Abdullah and Haya

for bringing me to the world,

for making me who I am now,

and for giving a special meaning to my life

To my loving husband Ricardo

for believing in me and supporting me

generously and unconditionally

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Abbreviations

$1,25(OH)_2D_3$	1,25-dihydroxyvitamin D_3
11β-HSD	11β- hydroxysteroid dehydrogenase
$25(OH)D_3$	25-hydroxyvitamin D_3
3'UTR	3' untranslated region
7DHC	7-dehydrocholesterol
aa	amino acid
AC	anterior chamber
ACAID	anterior chamber associated immune deviation
AMD	Age-related macular degeneration
AMPs	antimicrobial peptides
APCs	antigen presenting cells
AqH	aqueous humor
ARG1	Arginase1
ARPE-19	adult retinal pigment epithelial cell line
Aβ	amyloid-β
BAB	blood aqueous barrier
BD	Behçet's disease
BL	Bowman's layer
BRB	blood-retinal barrier
CAMP	gene encoding hCAP
CaR	calcium sensing receptor
CARDs	caspase recruiting domains
CBE	ciliary body pigmented epithelium
CCL2	chemokine ligand 2
CCL-20.2	human conjunctival cell line
CCR	chemokines receptor
CGRP	calcitonin gene-related peptide
CMV	cytomegalovirus
CoA	coactivators
COL2A1	collagen II-alpha 1
CoR	corepressors
CTLA-4	cytotoxic T-lymphocyte antigen 4
CXCL12	chemokine ligand 12
CYP450	cytochrome P450
Dab2	phosphoprotein disabled-2
DAPI	4',6-diamidino-2-phenylindole
DBD	DNA binding domain
DBP	vitamin D_3 binding protein

dbSNP	database for single nucleotide polymorphisms
DC	dendritic cells
DEFB4	gene encoding hBD-2
dsRNA	double stranded RNA
DSS	dextran sodium sulfate
EAU	experimental autoimmune uveitis
ECM	extracellular matrix
EIA	enzyme immunoassay
EIU	endotoxin induced uveitis
ELISA	enzyme linked immunosorbent assay
ENaC	epithelial sodium channel
ER	endoplasmic reticulum
FCS	fetal calf serum
FGF-23	fibroblast growth factor 23
FITC	fluorescein isothiocyanate
GB	gall bladder
GM-CSF	granulocyte-macrophage colony-stimulating factor
GR	glucocoricoid receptor
GWAS	genome wide analysis
hBD	human β-defensins
hCAP-18	human cationic antibacterial protein hCAP 18KD
HCD	human kidney cortical collecting duct
HCE	human corneal endothelial
HCEC-12	human corneal endothelial cell line
HD	Human α-defensins
HDP	host defense peptide
HIFCS	heat inactivated fetal calf serum
HKC-8	human kidney cell line
HKF	human corneal fibroblasts
HLA	human leukocytes antigens
HNP	Human neutrophil peptides
HRP	horseradish peroxidase enzyme
HS	human serum
HTLV-1	human T lymphotropic virus Type 1
I	iris
IBD	inflammatory bowel disease
Ig	immunoglobulin
IKK	inhibitor of kappa B kinase
IL	Interleukin
IL-12p40	IL-12 p40 subunit
IL-1Ra	interleukin-1 receptor antagonist
IP-10	interferon-γ-inducible protein 10
IPE	iris pigmented epithelium

IPS-1	interferon-beta promoter stimulator 1
IRAK	interleukin-1 receptor-associated kinase
IRBP	interphotoreceptor retinoid binding protein
IRF-8	IFN regulatory factor 8
IU	intermediate uveitis
KO	knockout
LBD	ligand binding domain
LC-MS/MS	liquid chromatography-tandem mass spectrometry
LCs	langerhans cells
LD	linkage disequilibrium
LNC	lymphonuclear cells
LPS	lipopolysaccharide
LPS-BP	lipopolysaccharide binding protein
LYVE-1	lymphatic vessel endothelial receptor 1
M1	classically activated macrophages
M2	alternatively activated macrophages
M6P	mannose-6-phosphate receptors
MAF	minimum allele frequency
MAVS	mitochondrial antiviral signalling adaptor
MC-1R	melanocortin-1 receptor
MCP-1	monocyte chemotactic protein-1
M-CSF	macrophage colony-stimulating factor
MDA5	melanoma-differentiation-associated gene 5
MHC	major histocompatibility complex
MIF	macrophage migration inhibitory factor
MPR	mannose-6-phosphate (M6P) receptors
MR	mineralocorticoid receptor
MS	multiple sclerosis
Mtb	myobacterium tuberculosis
mVDR	membrane VDR
NCBI	National Center for Biotechnology Information
NFAT	nuclear factor of activated T-cells
NF-κB	nuclear Factor-KappaB
NK	natural killer
NKT	natural killer T
NLRs	nucleotide oligomerisation domain (NOD)-like receptors
NO	nitric oxide
NOD	nucleotide oligomerisation domain
NOS2	nitric oxide synthetase
NPE	non-pigemented ciliary body epithelium
NPY	neuropeptide Y
nVDR	nuclear VDR
ODM-2	non-pigemented ciliary body epithelial cell line

PAMP	pathogen molecular associated pattern
PBMCs	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PD	programmed death
PD-L1	programmed death-1 ligand
PD-L2	programmed death-2 ligand
PE	pigmented epithelium
PEDF	pigment epithelial derived factor
PHCEC	primary human corneal epithelial cells
PHCend	primary human corneal endothelium
PI3K	phosphatidylinositol-3′-kinase
PKC	protein kinase C
PLC	phospholipase C
PRR	Pathogen recognition receptors
PTH	parathyroid hormone
RANKL	receptor activator of nuclear factor-kB ligand
RFLPs	restriction fragment length polymorphisms
RIG-I	retinoic acid inducible gene I
RLRs	retinoic acid inducible gene I like receptors
rMIF	recombinant murine MIF
RNA	ribonucleic acid
RPE	retinal pigment epithelium
RSV	respiratory syncytial virus
RT-PCR	reverse transcription-polymerase chain reaction
RXR	retinoid X receptor
Ser128	serine 128
SGK1	serum and glucocorticoid regulated kinase isoform 1
SLE	systemic lupus erythematosus
SNP	single nucleotide polymorphisms
SOM	somatostatin
SP	substance P
SPF	sun protective factor of 15
SRC	steroid receptor coactivators
ssRNA	single stranded ribonucleic acid
sVEGFR-1	soluble vascular endothelial growth factor receptor-1
T1DM	type 1 diabetes mellitus
TAK	transforming growth factor-beta-activated kinase
TCC	T cell clones
TEER	transepithelial electric resistance
Tg	transgenic
TGF-β	transforming growth factor-β
TGN	trans-Golgi network
Th	T helper

TIMPs-1	tissue inhibitor of metalloproteinases-1
TIR	Toll/interleukin-1 receptor
TJ	tight junction
TLRs	Toll-like receptors
TMB	tetramethylbenzidine
TNF-α	tumour necrosis factor-α
TNF-α R	tumour necrosis factor-α receptor
TNF-β	tumour necrosis factor β
TR	texas red
TRAF	tumour necrosis factor receptor-associated factor
Treg	regulatory T cells
TRIF	TIR-domain-containing adapter-inducing interferon-β
TRPV6	transient receptor potential cation channel, subfamily V, member 6
TSP-1	thrombospondin-1
VDR	vitamin D receptor
VDRE	vitamin D response element
VEGF	vascular endothelial growth factor
VIP	vasoactive intestinal peptide
α-MSH	alpha-Melanocyte-stimulating hormone
β-gal	beta-galactosidase

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1 GENERAL INTRODUCTION

1.1 Vitamin D

1.1.1 Vitamin D metabolism

Vitamin D is a secosteroid hormone required for bone health and mineralisation. Vitamin D can be ingested from diet containing oily fish and fortified food products as well as over the counter dietary supplements. Vitamin D exists as two biological forms, vitamin D_2 (ergocalciferol) which can be obtained from plants and vitamin D_3 which is synthesised in the skin upon exposure to sunlight. After exposure to solar ultraviolet B (UVB) radiation of wavelength 290 to 315 nm, vitamin D_3 precursor 7-dehydrocholesterol (7DHC) in the skin is transformed into previtamin D_3, which is converted into vitamin D_3. Vitamin D_3 is referred to as a secosteroid because it is closely related to steroid hormones, composed of 4 fused rings of cyclopentanoperhydro-phenanthrene (A, B, C, and D).

When 7DHC is exposed to UV radiation, the B-ring is broken at 9,10 carbon-carbon bond to produce previtamin D_3. In a heat independent mechanism, the molecule undergoes further *cis-trans* isomerisation around carbon 6,7 single (s) bond to convert it to vitamin D_3, a process that influences its conformation and stability. The *trans* conformation has been proposed as the preferred ligand for nuclear vitamin D receptor (nVDR) to deliver genomic functions while the *cis* conformation is the ligand required by membrane VDR (mVDR) to induce non-genomic responses (Norman, 2008) as will be explained later in this thesis (Figure 1.1).

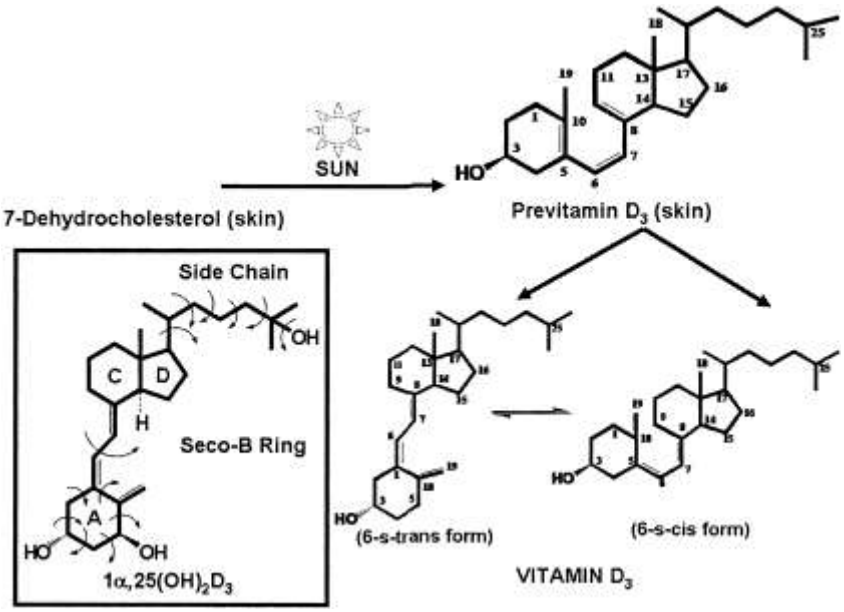

Figure 1.1. The secosetroid structure of vitamin D. The vitamin D precursor 7DHC is formed of 4 4 fused cyclopentanoperhydro-phenanthrene rings (A-D). When exposed to UV radiation the B-ring is broken at 9,10 carbon-carbon bond to produce previtamin D_3. This molecules further isomerises around carbon 6,7 single (s) bond to produce vitamin D_3 of either 6-s-*cis* or 6-s-*trans* conformation. This conformation influences the stability and flexibility of the molecules required for its functional activities (Norman, 2008).

Vitamin D_3 produced in the skin is incorporated is stored in adipocytes while that obtained from diet (along with vitamin D_2) is incorporated into chylomicrons before it is released into the circulation via lymphatic drainage (Holick, 2006;Holick, 2007). Vitamin D intoxication does not occur as a result of prolonged sun exposure. Vitamin D intoxication causes hypercalcaemia which can lead to calcification related complications in the gastrointestinal tract, kidneys, central nervous system, skin, muscles, and eyes (Ozkan et al., 2012). Excess previtamin D is converted by sunlight into lumisterol or tachysterol, components that do not bind DBP and are sloughed off with skin turnover (Chen and Holick, 2003;Shroff et al., 2010).

To produce the active form, vitamin D from skin or diet is subjected to two successive hydroxylations at carbons 25 and 1 (Figure 1.2). It is first hydroxylated to 25-hydroxyvitamin D [25(OH)D] at carbon 25 in the liver by a cytochrome P450 (CYP) enzyme 25-hydroxylase. 25(OH)D is the major circulating form of vitamin D and is used as an indicator of vitamin D status. 25-hydroxylation can be a function of six CYP450 isoforms; one mitochondrial CYP27A1, and five microsomal CYP3A4, CYP2R1, CYP2J2/3, CYP2D25, and CYP2C11 (Zhu and Deluca, 2012). The second hydroxylation step at carbon 1 occurs in the renal tubules by the mitochondrial enzyme 25-hydroxyvitamin D-1-α-hydroxylase; also known as CYP27B1 to produce the biologically active form of vitamin D, 1,25-dihydroxyvitamin D [1,25$(OH)_2$D].

The level of 1,25$(OH)_2$D is downregulated by a renal tubule enzyme known as 25-dihydroxyvitamin-D-24-hydroxylase (24-hydroxylase) that converts 1,25$(OH)_2$D into water-soluble, biologically inactive calcitroic acid (Holick, 2007). In the circulation, vitamin D is transported bound to vitamin D binding protein (DBP). DBP can bind both 25(OH)D or 1,25$(OH)_2$D and it determines their accessibility for cellular uptake through the multi-ligand endocytic receptors cubilin and megalin (Chapter 3). DBP belongs to the albumin superfamily and is structurally related to α-albumin and α-fetoprotein. These proteins have a unique structure with cysteine residues aligned next to each other linked by disulfide bonds that link them to each other and to the other cysteines. DBP has a shorter half-life than 25(OH)D of 2.5 days (Gomme and Bertolini, 2004). The majority of circulating 25(OH)D and 1,25$(OH)_2$D (80-90%) is bound to DBP and to albumin (10-20%), leaving a small free proportion (0.02-0.05%). To measure serum concentrations of

25(OH)D or 1,25$(OH)_2$D, these molecules are either separated from DBP by immunoextraction or detected using antibodies against the metabolite to be measured and DBP (Gomme and Bertolini, 2004;Wootton, 2005;Zerwekh, 2008).

Vitamin D_3 is more potent than vitamin D_2 in delivering the benefits to humans and it is the focus of this project. Higher doses of vitamin D_2 are required to raise serum 25(OH)D_2 levels to those of 25(OH)D_3. Vitamin D_3 is converted to 25(OH)D_3 five times faster than vitamin D_2 by liver 25-hydroxylase and 25(OH)D_3 has a higher affinity to DBP. Microsomal CYP2R1 can convert both forms of vitamin D equally while mitochondrial CYP27A1 performs hydroxylation only on vitamin D_3. The potential of 1,25$(OH)_2D_3$ to bind VDR exceeds that of 1,25$(OH)_2D_2$ by 40% and this allows 1,25$(OH)_2D_3$ to be more efficient in delivering the biological benefits (Armas et al., 2004;Houghton and Vieth, 2006).

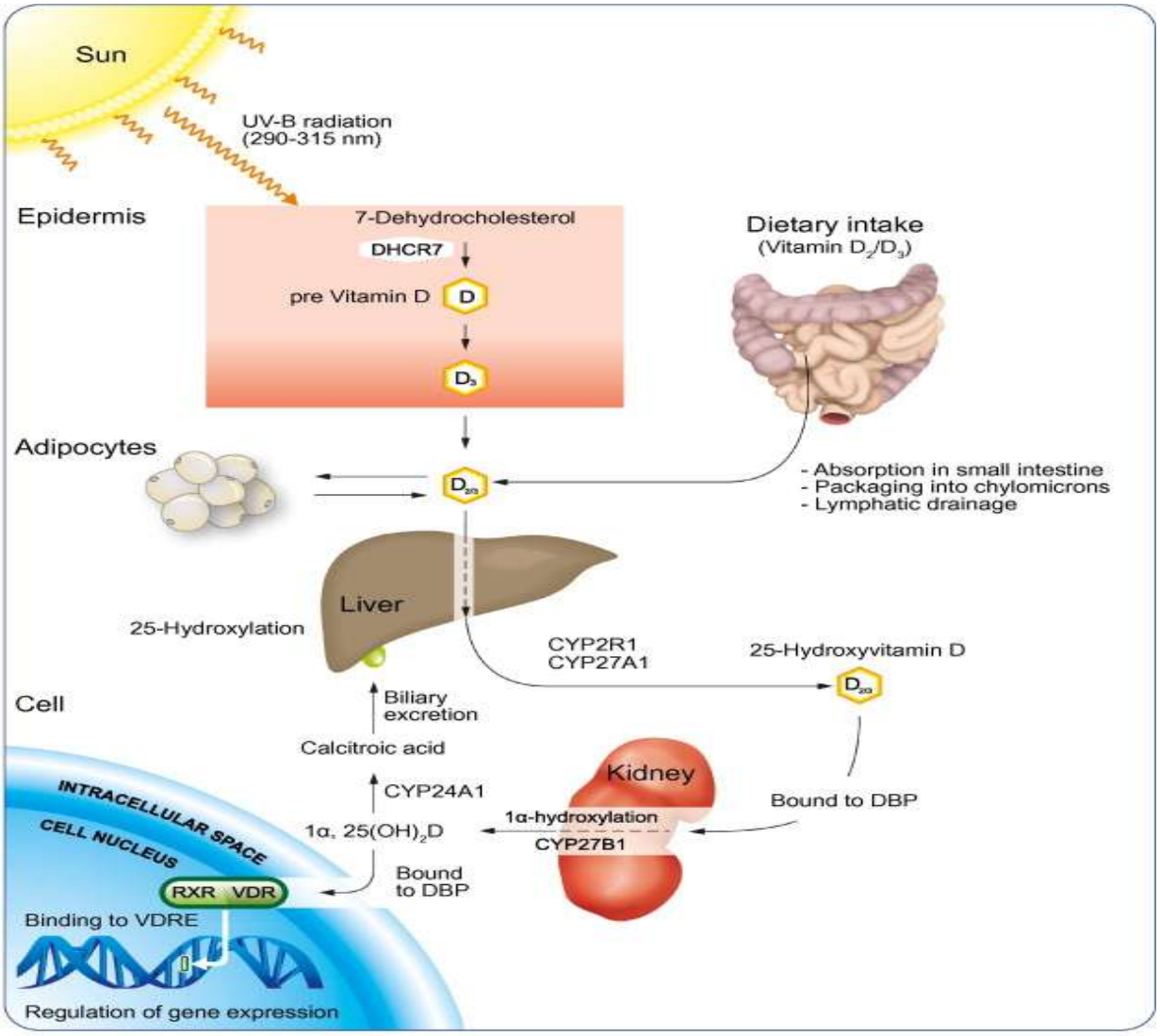

Figure 1.2. Vitamin D metabolism. After exposure to UVB, 7DHC in the epidermis is converted to previtamin D which is further changed by sunlight to vitamin D. Vitamin D can also be obtained from diet and supplementation. Vitamin D from the skin is stored in fat cells and from the diet is incorporated into chylomicrons. It is released into the circulation via lymphatic drainage and circulates bound to DBP. In the liver it is converted by 25-hydroxylase (CYP27A1 or CYP2R1) to the major circulating form 25(OH)D. This is activated in proximal renal tubules by 1-αhydroxylase (CYP27B1) to its active form $1,25(OH)_2D$. 24-hydroxylase (CYP24A1) from proximal renal tubules provides a negative feedback and degrades $1,25(OH)_2D$ into calcitroic acid excreted in bile. $1,25(OH)_2D$ is endocytosed by target cells where it binds to VDR that heterodimerises with RXR. VDR-RXR complex translocates to the nucleus to bind VDRE and modulate gene expression (Kitson and Roberts, 2012).

1.1.2 Vitamin D_3 action

1.1.2.1 Genomic responses

Vitamin D_3 mediates its action via a nuclear/cytoplasmic DNA-binding transcription factor vitamin D_3 receptor (VDR), a member of the nuclear receptor superfamily. Upon binding to $1,25(OH)_2D_3$, VDR heterodimerises with retinoic acid X receptor (RXR) to form a complex. This complex translocates to the nucleus, binds to vitamin D response element (VDRE) in target genes, and recruits regulatory protein coactivators (CoA) to induce gene transcription (Figure 1.3) or corepressors (CoR) to repress transcription (Carlberg and Campbell, 2013;Haussler et al., 2011).

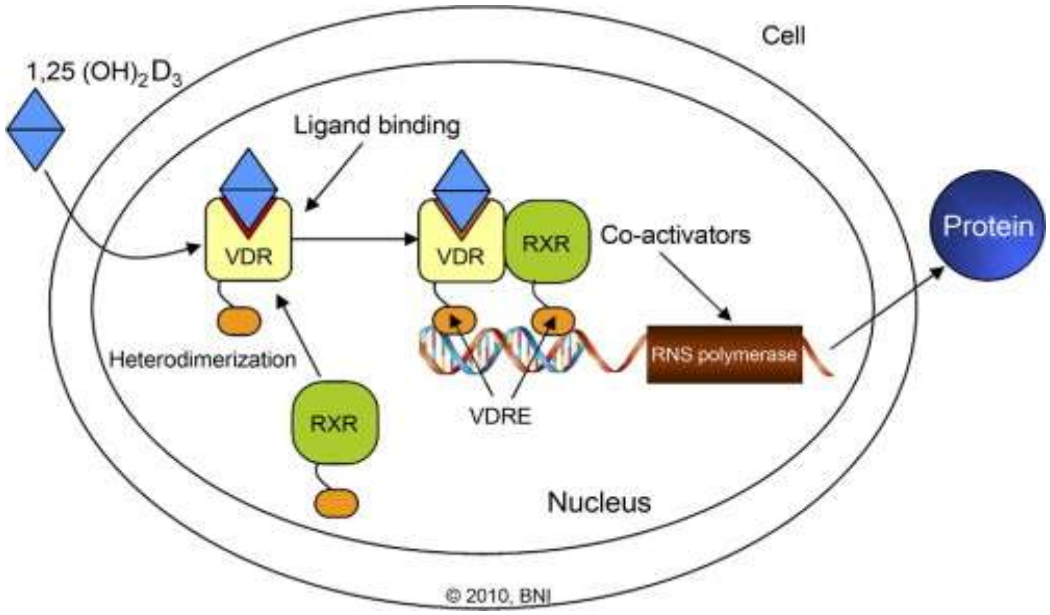

Figure 1.3. VDR activation. When $1,25(OH)_2D_3$ is locally synthesised or supplied from the circulation, it enters the cell and binds to VDR in the ligand-binding pocket. VDR heterodimerises with RXR to form VDR-RXR complex. The complex translocates to the nucleus to bind VDRE and recruits coactivators to stimulate RNA polymerase II and initiate gene transcription (Kalb et al., 2012).

1.1.2.2 Non-genomic responses

While genomic vitamin D_3 actions take place within several hours to days, non-genomic actions are rapid and can be generated within 1–2 min to 15–45 min. It has been reported that VDR can be localised to a lipid-raft caveolae (plasma membrane vesicle involved in signal transduction) in many cell types to which $1,25(OH)_2D_3$ can bind. On binding of $1,25(OH)_2D_3$ to caveolae-associated VDR, one or more second messenger systems can be activated (Figure 1.4).

These second messenger systems include G-protein coupled receptor, phospholipase C (PLC), protein kinase C (PKC), and phosphatidylinositol-3′-kinase (PI3K). This can result in rapid responses like the promotion of calcium influx from extracellular space, enhanced insulin secretion and the generation of cyotosolic kinases required for rapid responses (Campbell et al., 2010;Haussler et al., 2011).

1.1.2.3 Classical vitamin D_3 action

The primary role of vitamin D_3 is calcium and phosphorus homeostasis, through which it maintains a healthy skeleton and ensures sufficient serum levels required for biological functions. Vitamin D_3 maintains serum calcium and phosphorus by increasing intestinal absorption and facilitating bone mineralisation/demineralisation. Both the intestine and bone express VDR. In the intestine, $1,25(OH)_2D_3$ induces the expression of calcium channel [transient receptor potential cation channel, subfamily V, member 6 (TRPV6)] and a calcium binding protein, calbindin 9 K. This causes an increase in intestinal calcium and phosphorus absorption up to 30-40% and 80% respectively (Laird et al., 2010;Shroff et al., 2010).

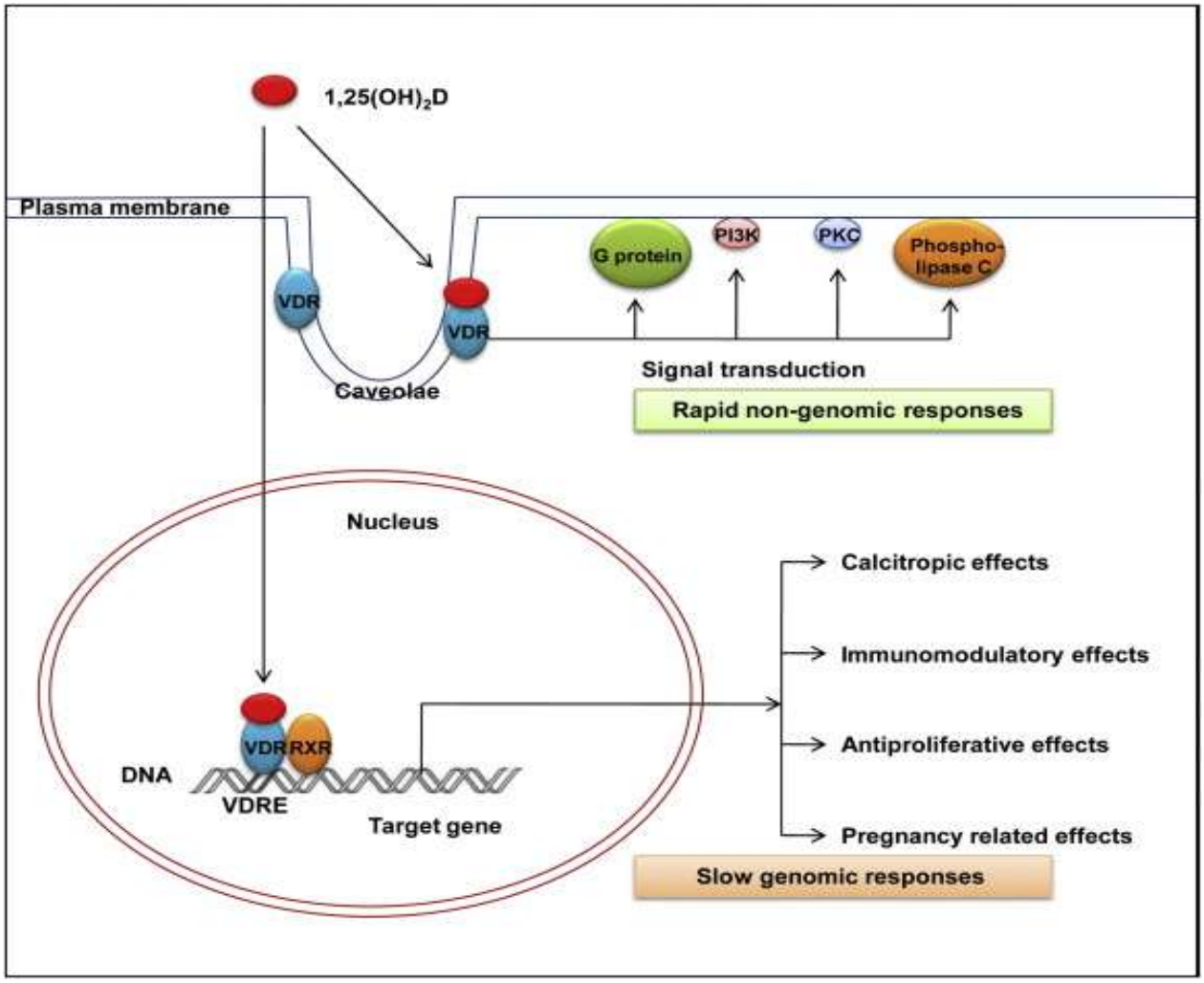

Figure 1.4. Vitamin D_3 genomic and non-genomic actions. Most vitamin D_3 actions are mediated through the classical binding of 1,25(OH)2D_3 to nuclear VDR (nVDR) to induce downstream signalling that will activate or repress gene transcription. Through this signalling pathway, vitamin D_3 plays a role in calcium metabolism, immunomodulation, and tumour suppression. Alternatively, caveolae-associated membrane VDR (mVDR) binds 1,25$(OH)_2D_3$ to activate G-protein coupled receptor, phosphatidylinositol-3′-kinase (PI3K), protein kinase C (PKC), and phospholipase C (PLC) to induce rapid responses (Shin et al., 2010).

Renal production of vitamin D_3 is under tight regulation by several factors. Low serum calcium levels are sensed by the calcium sensing receptor (CaR) in parathyroid gland which triggers the release of parathyroid hormone (PTH). PTH stimulates renal production of $1,25(OH)_2D_3$ and increases intestinal absorption and renal reabsorption of calcium and phosphorus. It increases the expression of the receptor activator of nuclear factor-κB ligand (RANKL) on osteoblasts to bind to its receptor RANK on preosteoclasts. This interaction induces the transformation of osteoblasts into mature osteoclasts which release calcium and phosphorus from bone to stabilise serum levels. The bone secretes fibroblast growth factor 23 (FGF-23) that downregulates $1,25(OH)_2D_3$ to reduce phosphorus and calcium absorption. Finally, $1,25(OH)_2D_3$ provides its own negative feedback by inducing the expression of CYP24A1 (Holick, 2006;Shroff et al., 2010).

1.1.2.4 Non-classical vitamin D_3 action

Many cells and tissues express VDR, CYP27B1, and CYP24A1. This allows these cells not only to respond to VDR, but also to endogenously convert inactive $25(OH)D_3$ into active $1,25(OH)_2D_3$. Vitamin D_3 has a range of functions beyond calcium homeostasis and can regulate more than 200 genes directly or indirectly (Ramagopalan et al., 2010). It can inhibit cell proliferation and induce differentiation, inhibit angiogenesis and enhance apoptosis. Vitamin D_3 controls blood pressure by reducing renin and induces insulin production from β-islets in the pancreas. A major role played by vitamin D_3 is the immunomodulation and induction of antimicrobial peptides (Holick, 2011). Extra-renal vitamin D_3 signalling pathway components expression has been

described in human epithelial cells of the lung, gingiva, urinary bladder, intestine, breast, endometrium, placenta, prostate, and skin (Chen and Holick, 2003;Hansdottir et al., 2008;Hertting et al., 2010;Kemmis and Welsh, 2008;Kong et al., 2008;Liu et al., 2009a;McMahon et al., 2011). Moreover, vitamin D_3 expression has been identified in monocytes, macrophages, dendritic cells (DC), neutrophils and activated T and B cells (Hewison, 2012;Van Belle et al., 2011).

1.1.3 Vitamin D_3 status

Vitamin D_3 status is determined by serum concentration of 25(OH)D_3 as sufficiency (30-100 ng/ml or 75-250 nmol/L), insufficiency (21-29 ng/ml or 52.5-72.5 nmol/L), and deficiency (<20 ng/ml or 50 nmol/L). Vitamin D_3 intoxication only occurs at levels above 100-200 ng/ml (Holick, 2009). Vitamin D_3 production varies with different factors such as geographical location, skin colour, the use of sunscreens, lifestyle, and age. As we move away from the equator (e.g. latitude above 35º), UVB light decreases and so less vitamin D_3 synthesis is expected. Factors that affect the angle at which the sun hits the earth (e.g. cloud cover, ozone, and air pollution) can reduce sunlight available for vitamin D_3 synthesis. A sun protective factor of 15 (SPF) in sunblocks can significantly reduce vitamin D_3. It has been suggested that people with darker skin have more melanin content and need longer UVB exposure to obtain sufficient vitamin D_3 (Kimlin, 2008;Laird et al., 2010). However, recent intriguing results negated the role of skin pigmentation effects on vitamin D_3 synthesis after exposure to UVB. Vitamin D_3 synthesis correlated with baseline serum cholesterol and vitamin D_3 levels but did not correlate with skin pigmentation. There was no difference in 25(OH)D_3 increase between

people with dark skin and people with fair skin when exposed to identical periods of UVB (Bogh et al., 2010).

In Tanzania, people from two tribes, the Maasai and Hazabe had serum 25(OH)D_3 levels of 115 nmol/L which is optimal for bone health and is aimed for by supplementation. These people are moderately covered with clothes and spend most of their time outdoors, but they try to avoid the sun at hot times (Luxwolda et al., 2012). Another study in Johannesburg measuring 25(OH)D_3 in 10-year old black and white boys showed that vitamin D_3 deficiency was not prevalent among the study population. Although the white boys had higher levels of 25(OH)D_3, only 8% of black boys were deficient compared to 1% of white boys (Poopedi et al., 2011). African-Americans have low serum 25(OH)D_3 (Liu et al., 2006), this may be because the amount and angle of sun light near the equator is optimal for sufficient vitamin D_3 synthesis or that these people acquired genetic changes by moving north or by marriage with Western people.

1.1.4 Vitamin D_3 and the immune system

The immune system comprises cells and tissue that protect the body against invading pathogens. The immune defence can be divided into three components, anatomical and physiological barriers, innate immunity, and adaptive immunity. Vitamin D_3 has several effects on cells of both innate and adaptive immune responses. Unlike in the kidneys, in immune cells vitamin D_3 is controlled by signals from the immune system and is not under calcium regulation (White, 2008).

1.1.4.1 Innate immunity

Innate immunity acts as the first line defence and is composed of cellular and humoral components. Cells of the innate immune system include macrophages, dendritic cells (DCs), eosinophils, neutrophils, mast cells, natural killer (NK) cells, and NKT cells. Innate humoral components include complement proteins, antimicrobial peptides (cathelicidin and defensins), C-reactive protein, and lipopolysaccharide (LPS) binding protein (LBP). Innate immune cells do not show specificity to pathogens and do not have memory. They limit pathogen replication and set the scene for the development of specific adaptive immunity. When barriers are breached, innate immune cells rapidly arrive at the site of injury or infection in response to locally produced proinflammatory cytokines such as interleukin-1 (IL-1), tumour necrosis factor-α (TNF-α), and IL-6. Type I interferons (IFN-α and β) function to limit viral spread until more specific response is in place. The function of innate immune cells starts with the recognition of pathogens through the conserved pathogen associated molecular patterns (PAMPs) (Basu and Fenton, 2004;Turvey and Broide, 2010).

1.1.4.1.1 Pathogen recognition receptors (PRR)

PRR are germline encoded receptors expressed by innate immune cells (such as DCs, macrophages, and neutrophils) that sense conserved PAMPs. Engagement of these receptors with their cognate ligands activates the immune response through intracellular signalling pathways that eventually result in the elimination of the assaulting pathogen. Several PRR have been identified and characterised including Toll-like receptors (TLRs), retinoic acid inducible gene I (RIG-I) like receptors (RLRs), nucleotide

oligomerisation domain (NOD)-like receptors (NLRs), and DNA receptors (Kumagai et al., 2008).

1.1.4.1.2 TLRs

To date, 10 TLRs have been identified in humans and 12 in mice. TLRs 1-9 are conserved in both humans and mice. TLR10 is only expressed in humans while TLR11 is limited to mice (Kumar et al., 2011). TLR1, 2, 4, 5, and 6 are expressed on the cell surface and recognise products from bacteria, protozoa and fungi while TLR3, 7, 8, and 9 are contained within endocytic compartments and recognise bacterial and viral nucleic acids. TLRs belong to type I membrane glycoproteins with an extracellular leucine rich domain and intracellular Toll/interleukin-1 receptor (TIR) required for the induction of downstream signalling. TLR2 heterodimerises with TLR1 or TLR6 to form two functionally distinct receptors, TLR2/1 or TLR2/6. TLRs build a signalling cascade by the recruitment of multiple adaptor molecules that will lead to the induction of proinflammatory cytokines (Yang and Seki, 2012) (Figure 1.5).

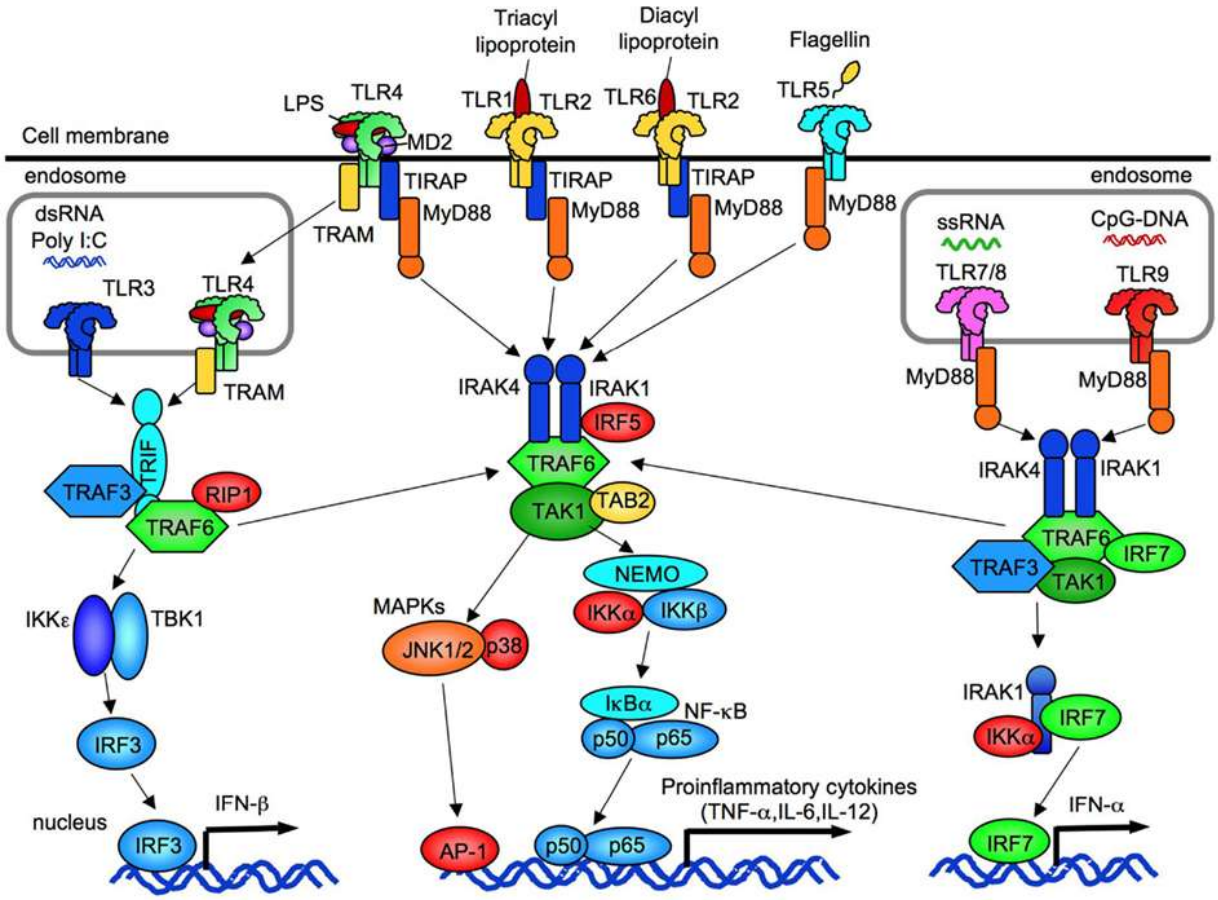

Figure 1.5. TLR ligands and signalling pathway. All TLRs except TLR3 signal through myeloid differential factor 88 (MyD88). Activation of MyD88 by TLR1, 2, 4, 5, and 6 results in the recruitment of other adaptor molecules such as interleukin-1 receptor-associated kinase 1 (IRAK1) and 4, tumour necrosis factor receptor-associated factor 6 (TRAF6), and transforming growth factor-beta-activated kinase 1 (TAK1) that leads to the activation of the inhibitor of kappa B kinase (IKK) complex. IKK activates p38/JNK1/2 and transcription factor nuclear factor-kappa B (NF-κB) to induce the transcription of proinflammatory cytokines (TNF-α, IL-6, and IL-12). TLR7, 8, and 9 recruit MyD88 and the adaptor molecules IRAK1 and 4, TRAF6, and TAK1, which activate interferon regulatory transcription factor 7 (IRF-7) to induce the transcription of IFN-α. TLR3 recruits TIR-domain-containing adapter-inducing interferon-β (TRIF) and TLR4 also recruits an adaptor molecule TRAM to use the TRIF signalling pathway. TRIF signalling pathway recruits TRAF3 and 6 that activate the IKK complex to induce the transcription of IFN-β. TRIF can also induce the transcription of proinflammatory cytokines through the adaptor molecules TRAF6 that induces downstream signalling of the TAK1 and IKK complexes (Yang and Seki, 2012).

1.1.4.1.3 RLRs

RLRs are intracellular sensors of single stranded ribonucleic acid (ssRNA) and double stranded RNA (dsRNA). This group of PRR consists of three members, RIG-I, MDA5 (melanoma-differentiation-associated gene 5) and LPG2. RIG-I and MDA5 contain N-terminal caspase recruiting domains (CARDs), a central helicase/ATPase domain, and a C-terminus regulatory domain (Figure 1.6). LPG2 lacks the CARDs necessary for signalling and plays a regulatory role in the RIG-I/MDA5 signalling pathway (Kumar et al., 2011).

1.1.4.1.4 Antimicrobial peptides

Antimicrobial peptides (AMPs) are positively charged amphipathic small cationic peptides [12-50 amino acids (aa)] rich in cationic and hydrophobic residues. They are produced by cells of the innate immune system and other cell types. They act as natural antibiotics killing many Gram negative as well as Gram positive bacteria, fungi, parasites and viruses. AMPs have roles to play in wound healing, immunomodulation, angiogenesis, and cell growth (Figure 1.7). Because they have multiple actions besides antimicrobial activity, they would be more appropriately called host defence peptides (HDPs) and this term will be used throughout the rest of this thesis. There exist two main classes of HDPs; α-helical structures lacking disulfide bonds (cathelicidin) and β-sheets with disulfide bonds (human α and β defensins). HDPs can be constitutively expressed or induced by microbial products, injury, or inflammation (Lai and Gallo, 2009;Yeung et al., 2011).

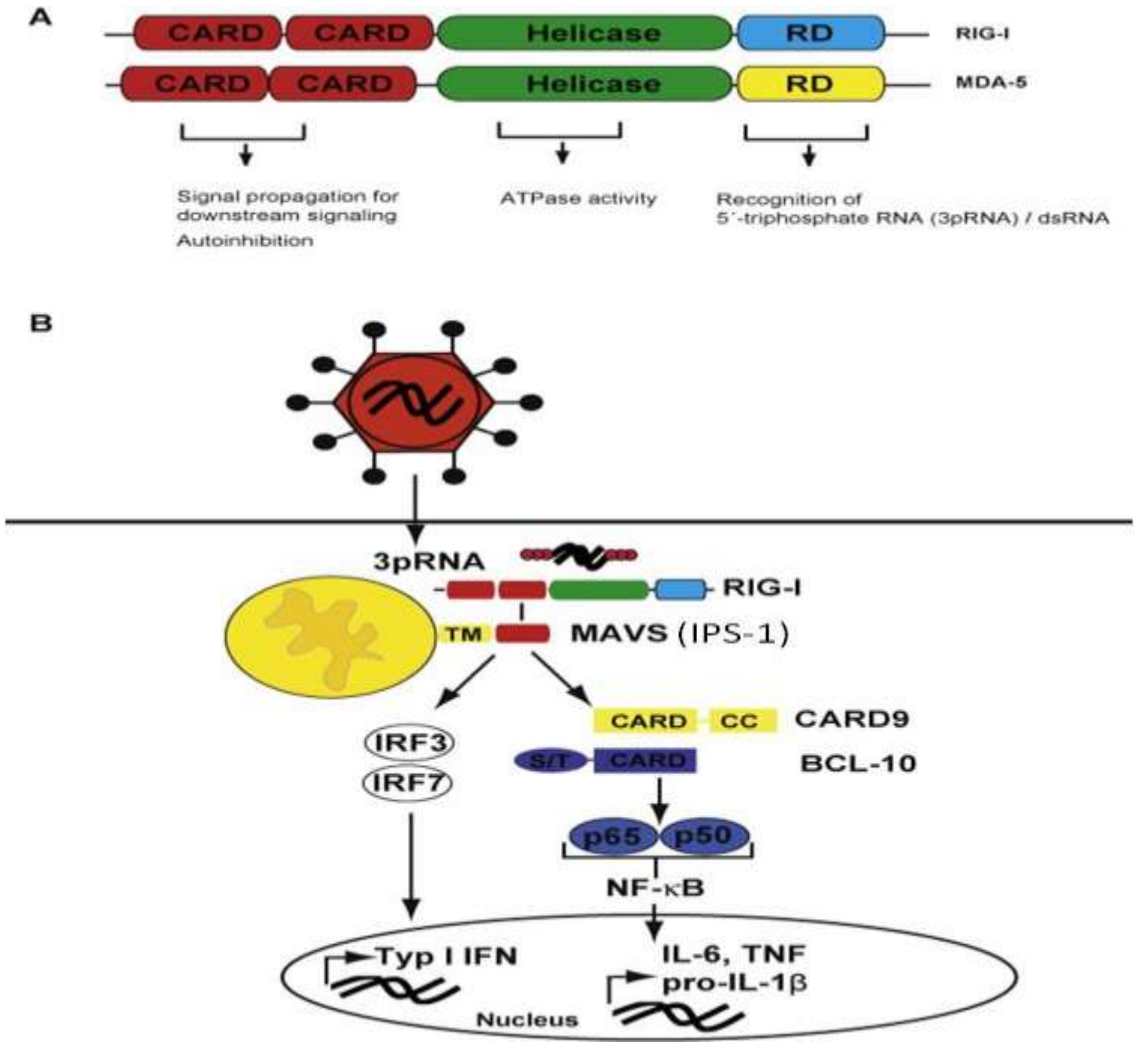

Figure 1.6. RIG-I/MDA5 signalling pathway. Upon activation, CARDs recruit mitochondrial adaptor protein interferon-beta promoter stimulator 1 (IPS-1) (also known as mitochondrial antiviral signalling adaptor [MAVS]) that activates the IKK complex. IKK activates NF-κB and IRF3/7 to induce the transcriptional activation of proinflammatory cytokines and type I interferons (IFN-α and IFN-β) (Poeck and Ruland, 2012).

1.1.4.1.4.1 Cathelicidin

Human cathelicidin (LL-37) is a 37-aa long linear peptide that consists of a conserved N-terminus with two leucines (LL) and a variable cationic C-terminus cathelin domain. The active peptide is located in the C-terminus and is released after cleavage of its inactive precursor human cationic antibacterial protein 18KD (hCAP-18, encoded by the gene *CAMP*) by serine proteases. LL-37 is mainly produced by neutrophils but also by other cells such as monocytes/macrophages, T cells, mast cells, and epithelial cells. LL-37 is also present in human sweat and alveolar lavage (Tjabringa et al., 2005).

Besides its role in antimicrobial killing, LL-37 is a multifunctional molecule that can neutralise LPS, induce chemotaxis for neutrophils, monocyte/macrophages, mast cells, and T lymphocytes, and induce IL-6 and IL-8 production. LL-37 plays a role in DC differentiation and maturation and mast cell degranulation as well as promoting wound healing and modulating angiogenesis (Pistolic et al., 2009;Schiemann et al., 2009;Tjabringa et al., 2005;Yeung et al., 2011). LL-37 is constitutively expressed in some tissues such as gastrointestinal, respiratory, and reproductive tract epithelial cells, salivary glands, and immune cells (such as neutrophil granules, monocytes and macrophages, mast cells, NK cells, and lymohocytes). Expression of LL-37 can be induced or enhanced by inflammatory stimuli, injury, and microbial products (Jager et al., 2010;Steinstraesser et al., 2011). Induction of LL-37 expression is mediated through MEK1/2 and p38 MAPK signalling pathways (Mendez-Samperio et al., 2008;Schauber et al., 2003).

1.1.4.1.4.2 Defensins

Defensins are 29-45 aa long peptides divided into two main classes, α and β defensins classified according to the arrangement of cysteine residues. They function as antimicrobial molecules, selectively inhibiting proinflammatory responses, and inducing chemotaxis and wound healing (Steinstraesser et al., 2011;Yeung et al., 2011). Human α-defensins (HD) are constitutively expressed and are mainly present in neutrophilic granules and Paneth cell granules of the small intestine. They are composed of six members. Human neutrophil peptides (HNP) 1-4 are mainly expressed by neutrophil azurohilic granules and to a lesser extent by monocytes, lymphocytes, and NK cells, while HNP5-6 are expressed by Paneth cells and the genitourinary tract of females.

Human β-defensins (hBD) are predominantly expressed by epithelial cells in the skin and mucosal surfaces and are secreted in a mature form to the outside of the cells (Lehrer and Lu, 2012;Tecle et al., 2010). HBD-1 is consititutively expressed as the gene lacks the regulatory element for NF-κB while hBD-2 and hBD-3 are inducible by bacterial components such as LPS (through TLR stimulation) and proinflammatory cytokines IL-1β and TNF-α through the activation of NF-κB pathway. The expression of hBD-4 is more limited to epithelial cells of testes and epididymis as well as monocyte, macrophages and DCs and can be induced by bacterial products such as bacteria phorbol myristate acetate (Hazlett and Wu, 2011).

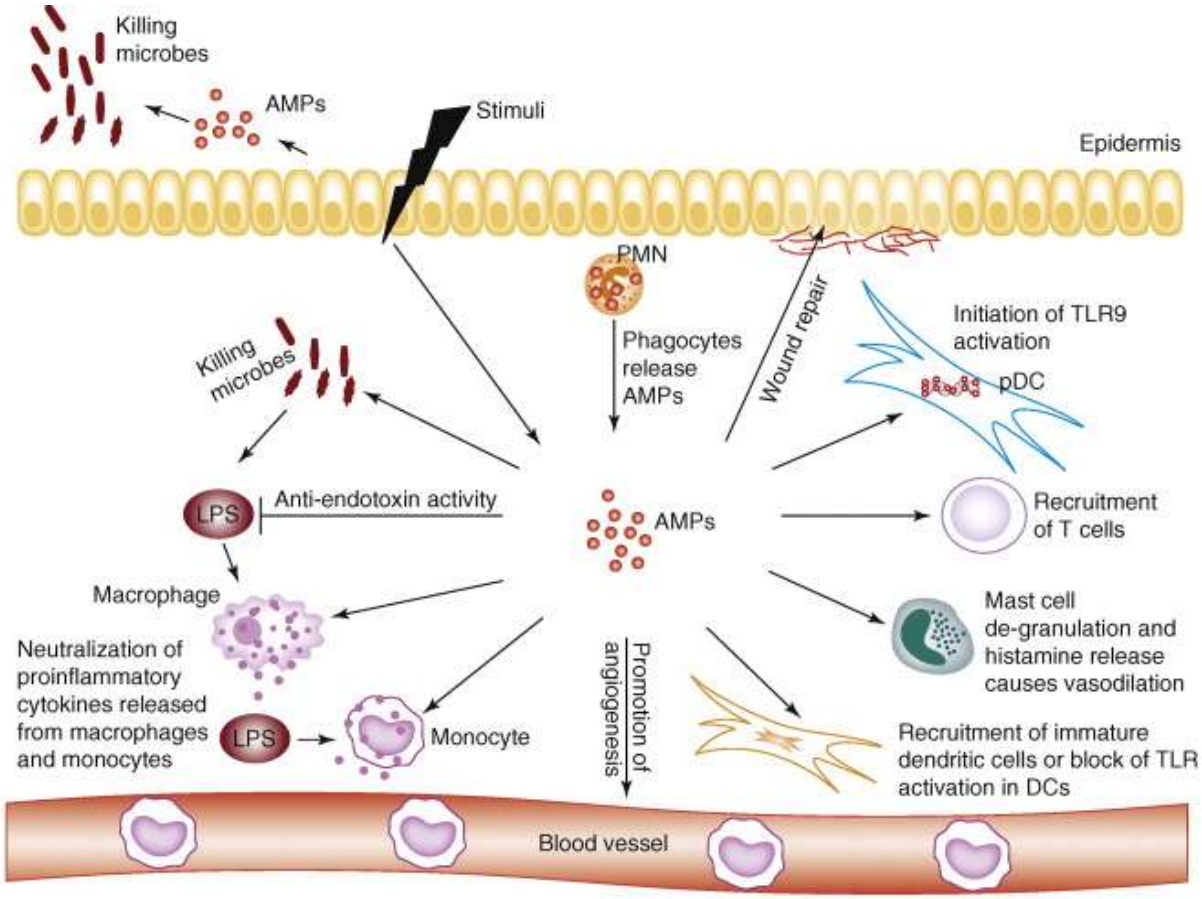

Figure 1.7. Multi-functional HDPs. HDPs show antimicrobial properties and are involved in direct microbial killing. They also have a range of immunomodulatory effects including the recruitment of T cells and DCs, mast cell degranulation, anti-endotoxin activity, neutralisation of proinflammatory cytokines release by macrophages and monocytes, and promotion of wound healing and angiogenesis (Lai and Gallo, 2009).

1.1.4.1.5 Antigen presentation

DCs attracted to sites of inflammation by molecules induced by the innate response process, degrade and present antigens as peptides to naïve T cells of the adaptive response bound to major histocompatibility complex class I and class II (MHCI and MHC II). These are human leukocytes antigens (HLA) class I (A,B, and C) or class II (DR, DP, or DQ) produced by genes located on chromosome 6 in humans. MHC I antigens are found on all nucleated cells of the human body, while MHC II are limited mainly to professional antigen presenting cells (APCs) such as DCs and macrophages (Ryan and Cobb, 2012). Extracellular antigens are presented by MHC class II to $CD4^+$ helper T (Th) cells while intracellular antigens are presented by MHC class I to $CD8^+$ T cells. This induces the activation and differentiation of T cells, thus bridging innate immunity to adaptive immunity.

In addition to DCs and macrophages, B cells and epithelial cells can also act as APCs under certain conditions, but DCs are considered as the primary professional APCs. After activation through TLR, tissue resident DCs mature into professional APCs and migrate from tissue to peripheral lymph nodes to activate T cells. They upregulate the expression of costimulatory molecules (CD40, CD80 and CD86) necessary for T cell activation as well as chemokine receptor (CCR) CCR7 (receptor for homing to lymph node), and downregulate CCR2 and CCR5 (receptors for homing to inflammatory sites) (Toebak et al., 2009;Villadangos and Schnorrer, 2007).

Vitamin D_3 has been shown to increase phagocytosis and chemotaxis in macrophages, decrease their expression of TLR2 and TLR4, and induce the production of HDPs (Baeke et al., 2010). On the contrary, it prevents DCs maturation and reduces the expression of MHC II, co-stimulatory molecules (CD40, CD83 and CD86) turning them into tolerogenic DCs (Figure 1.8). One study has shown that vitamin D_3 inhibited chemotaxis of DCs, reduced IL-12 and TNF-α, but increased IL-6 and IL-1β production. Interestingly, these effects were induced by local conversion of $25(OH)D_3$ into active $1,25(OH)_2D_3$ (Bartels et al., 2010).

1.1.4.2 Adaptive immunity

Adaptive immunity provides a more specific response against an infection, and memory which allows a robust (high-affinity) and rapid response to future infections of the same pathogen. Cells of adaptive immunity are T and B lymphocytes which divide adaptive immunity into cellular and humoral components respectively. The activation of naive $CD4^+$ T helper (Th0) cells causes differentiation into several effector and regulatory subtypes influenced by the microenvironment and cytokines (IL-12 and IL-4 secreted by APCs) present during activation (Figure 1.8). Th1 cells secrete IFN-γ and activate macrophages and NK cells to kill intracellular bacteria, fungi and viruses. Th0 cells differentiate into Th2 type in response to parasitic infections and allergies. Th2 cells stimulate mast cells, neutrophils and B cell production of immunoglobulin (Ig) G1 (IgG1) and IgE.

Th0 cells can also differentiate into Th17 and Th22 subsets in response to extracellular bacteria. Inducible regulatory T cells (Treg) produced from Th cells include Tr1 producing IL-10, Th3 producing transforming growth factor-β (TGF-β), and $CD4^{+}C25^{+}Foxp3^{+}$ Tregs preventing the differentiation of effector T cells through direct cell-cell contact (Hesslein et al., 2011;la Sala A. et al., 2012). For T cell activation, CD28 on T cells bind the costimulatory molecules CD80/CD86 whereas engagement of CD80/CD86 by cytotoxic T lymphocyte antigen-4 (CTLA-4) is inhibitory for T cell activation (Rudd et al., 2009).

Vitamin D_3 prevents B cell differentiation and proliferation and reduces IgG and IgM production. It directs the polarisation of Th cells towards Th2 and Treg phenotypes and not Th1 or Th17 by inhibiting IL-12 and IL-23 production by DCs. It reduces Th1 cytokines IL-2 and IFNγ and Th17 cytokines (IL-17 and IL-21). It induces the development of Tregs expressing of CTLA4 and Foxp3 (Figure 1.9) (Baeke et al., 2010;Jeffery et al., 2009). The promoter region of Foxp3 gene has VDRE through which vitamin D_3 can modulate the immune system towards a regulatory response (Kang et al., 2012).

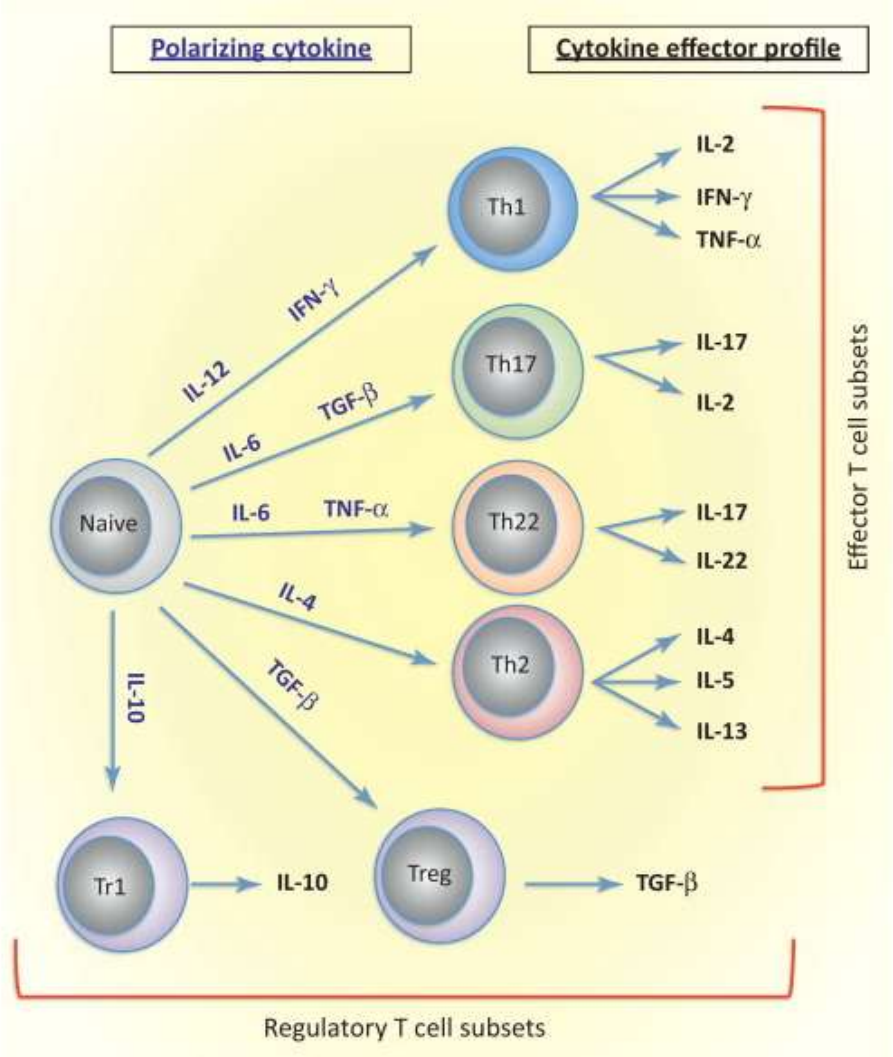

Figure 1.8. Effector and regulatory T cell differentiation. Naïve $CD4^+$ T cells differentiate into different subtypes according to the cytokines present in the microenvironment in which the stimulation occurs. IL-12 and IFN-γ causes the polarisation of T cells into Th1 whereas IL-4 induces Th2 differentiation. IL-6 with TGF-β and IL-6 with TNF-α, lead to the differentiation into Th17 and Th22 respectively. Tregs are IL-10 producing Tregs, TGF-β producing Tregs, and IL-10 producing $CD4^+C25^+Foxp3^+$ Tregs (la Sala A. et al., 2012).

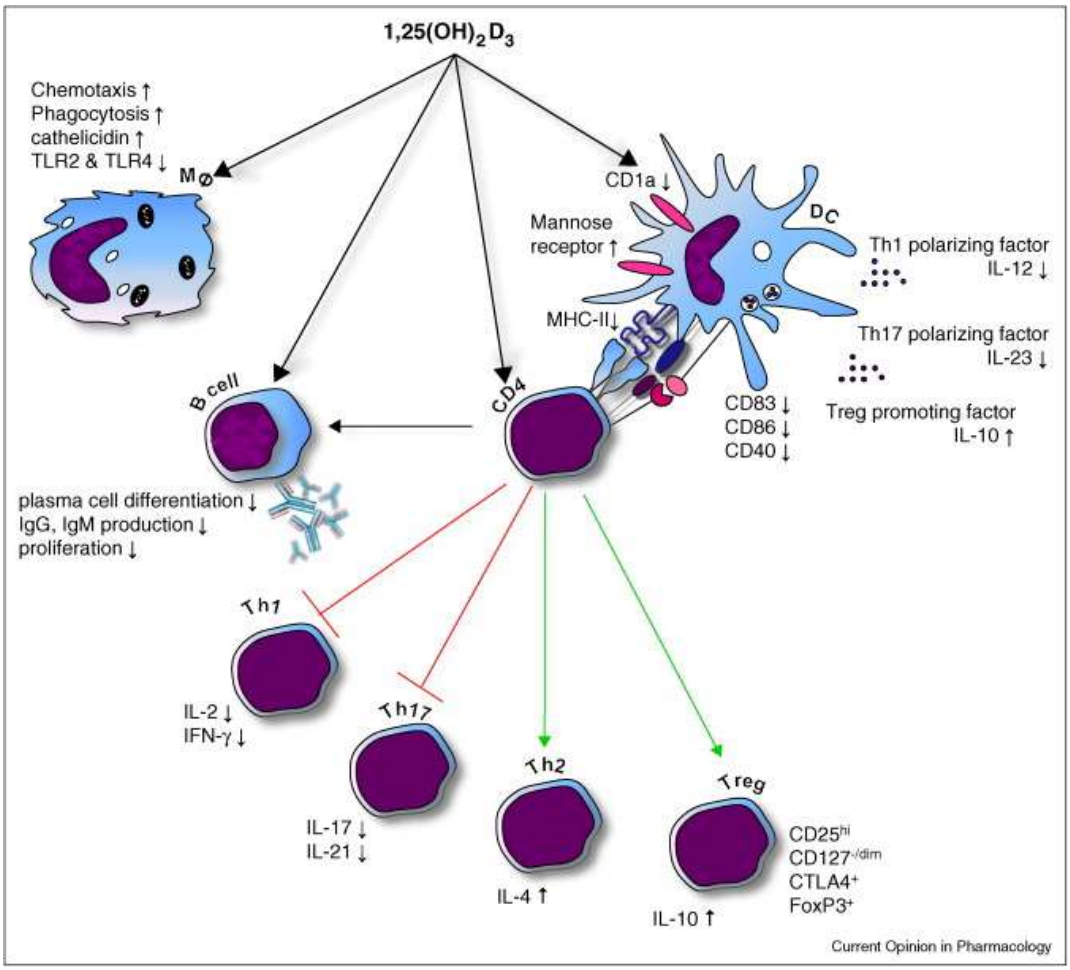

Figure 1.9. Effects of vitamin D_3 on immune cells. Vitamin D_3 increases chemotaxis and phagocytosis of macrophages, induces cathelicidin production and reduces the expression of TLR2 and TLR4. It converts DCs into tolerogenic phenotype by preventing their maturation and reducing the expression of MHC class II and co-stimulatory molecules. It reduces plasma cell differentiation and proliferation. Vitamin D_3 inhibits the differentiation into Th1 and Th17 and directs the polarisation of Th2. It also induces the differentiation of Tregs by upregulating the expression of CTLA4 and Foxp3 (Baeke et al., 2010).

1.1.5 Extra-renal vitamin D_3 synthesis

The interest in the effects of vitamin D_3 on the immune system started with the discovery of extra-renal expression of CYP27B1 in monocytes and macrophages in studies on *Myobacterium tuberculosis* (*Mtb*). Stimulation of TLR2/1 by 19 kDa triacylated lipopeptide of *Mtb* upregulated mRNA expression of VDR and CYP27B1. To understand the downstream signalling of VDR in these cells after TLR2/1 stimulation, monocytes were stimulated with TLR2/1L in the presence or absence of $25(OH)D_3$. $25(OH)D_3$ synergised with TLR2/1 in the upregulation of CYP24A1 and cathelicidin mRNA.

Stimulation of TLR2/1 alone in medium containing human serum (HS) also upregulated CYP24A1 and cathelicidin mRNA without the addition of exogenous $25(OH)D_3$. This induction was not seen when cells were stimulated in the presence of fetal calf serum (FCS) and this could be due to the high levels of $25(OH)D_3$ in HS compared to FCS. VDR, CYP27B1, CYP24A1, and cathelicidin were all upregulated at mRNA level when TLR2/1 stimulation was performed in $25(OH)D_3$ sufficient sera from Caucasians, but not in serum from African-Americans which was found to have low serum $25(OH)D_3$ (Liu et al., 2006).

Further work by the same group has demonstrated that TLR2/1L induced IL-15, a cytokine necessary for CYP27B1 and VDR induction, and cathelicidin production, with the latter two processes due to conversion of $25(OH)D_3$ to active $1,25(OH)_2D_3$. Moreover, macrophages primed by IL-15 produced HDPs activity against *Mtb* when stimulated directly with $25(OH)D_3$ (Krutzik et al., 2008). TLR2/1L also led to the induction of IL-1β from monocytes. TLR2/1L needs IL-1β and VDR signalling to induce hBD-2

(encoded by *DEFB4* gene) (Liu et al., 2009b). Finally, IFN-γ produced by T cell signalling through STAT1 induced IL-15 in monocytes, leading to increased vitamin D_3 metabolism as discussed above, in synergy with signalling via TLR, leading to the uprergulation of cathelicidin and *DEFB4*. Moreover IFN-γ induced an autophagy pathway in *Mtb*-infected monocytes leading to increased phagosome maturation.

Finally, only vitamin D_3 sufficient sera could support IFN-γ induced HDPs production, which has implications for the effect of infection in different ethnic groups (Fabri et al., 2011). In monocytes and macrophages, vitamin D_3 is not regulated by PTH and calcium, but by signals from the immune system. In macrophages, a splice variant of *CYP24A1* was identified that limited the availability of $25(OH)D_3$ and $1,25(OH)_2D_3$ for degradation by CYP24A1. The splice variant lacks a 150 aa sequence at the N-terminal containing mitochondrial targeting domain. This absence of negative feedback allows more synthesis of $1,25(OH)_2D_3$ which may result in hypercalcaemia in inflammatory conditions (Ren et al., 2005).

These interesting findings have encouraged many researchers to investigate the presence of extra-renal vitamin D_3 production in other cells and tissues including those at barrier sites, and whether it affects the induction of HDPs. Local vitamin D_3 synthesis and induction of cathelicidin have been identified in many epithelial cells including respiratory, urinary bladder, gingival, sinonasal, and colon epithelial cells, as well as trophoblasts and skin keratinocytes (Hansdottir et al., 2008;Hertting et al., 2010;Lagishetty et al., 2010;Liu et al., 2009a;McMahon et al., 2011;Schauber et al., 2003;Schrumpf et al., 2012;Sultan et al., 2012;Sultan et al., 2013;Welsh, 2011). In skin,

placental, and respiratory epithelial cells, TLR synergised with VDR to induce HDPs expression whereas in urinary bladder, gingival, sinonasal, and colon epithelial cells local vitamin D_3 synthesis alone induced HDPs.

A genome wide analysis (GWAS) from peripheral blood mononuclear cells (PBMCs) revealed the presence of VDRE in more than 200 genes, including many identified related to autoimmune disease, including type 1 diabetes mellitus (T1DM) and systemic lupus erythematosus (SLE). Genes with VDRE including *IRF8, PTPN2, CTLA-4 and CD40* showed increased expression after vitamin D_3 treatment. Therefore many single nucleotide polymorphisms (SNPs) identified as associated with autoimmune disease are located within VDR binding intervals, suggesting that vitamin D_3 influences several pathways involved in immune responses (Ramagopalan et al., 2010).

Other genes regulated by vitamin D_3 are those of tight junction (TJ) proteins. Upon treatment of colonic carcinoma epithelial cell line (Caco-2) monolayer with $1,25(OH)_2D_3$, there was an upregulation of TJ proteins zo-1, caludin-1 and occludin. $1,25(OH)_2D_3$ increased transepithelial electric resistance (TEER) upon treatment with dextran sodium sulfate (DSS) used to induce acute colitis *in vivo* and reduced their permeability to fluorescein isothiocyanate-D (FITC-D) dye. These findings were replicated in the C57BL/6 mouse model of inflammatory bowel disease (Zhao et al., 2012).

1.1.6 Vitamin D_3 deficiency

The importance of vitamin D_3 starts from early stages in life. In pregnant women, vitamin D_3 deficiency can cause increased risk of gestational diabetes and preeclampsia (gestational hypertension). It can cause a delay in lung and bone development. Vitamin D_3 deficiency can reduce body size and shape at birth and impair bone mineralisation. During childhood vitamin D_3 deficiency can cause rickets (growth failure and bone deformities), growth retardation, increased risk of T1DM, asthma, respiratory tract infections and central nervous system disorders (Principi et al., 2013). In adults vitamin D_3 deficiency has been linked to osteoporosis, osteomalacia, and increased risk of falls and fractures (Lips and van Schoor, 2011).

Low serum vitamin D_3 levels have been linked to many autoimmune conditions such as T1DM, inflammatory bowel disease (IBD), and systemic lupus erythematosus (SLE) as well as infectious diseases such as tuberculosis and seasonal influenza (Van Belle et al., 2011) . There is a geographical association where there is an increase in the incidence of multiple sclerosis (MS) in countries far from the equator. It has been suggested that the decrease of UVB in northern regions leads to less vitamin D_3 synthesis. This was further supported by low serum vitamin D_3 found in MS patients (Summerday et al., 2012). HLA-DRB1*1501 has been associated with susceptibility to MS and recently VDRE have been found in the promoter region which indicates it is regulated by vitamin D_3 (Ramagopalan et al., 2009).

However, if MS is an example of autoimmune disease linked to low UVB exposure and vitamin D_3 levels, it is intriguing that MS prevalence is increasing in Arab countries (although still lower than Western countries) and it remains more frequent in females than in males (Benamer et al., 2009). One possible explanation is that difference in prevalence is related to differences in sex hormones, as autoimmune disease (such as RA and MS) onset appears in females as early as after puberty (18-40), while in males the onset is delayed to the age of 30-40 years and could be as a result of the decline in testosterone (Voskuhl, 2011). It has been noticed that MS symptoms are suppressed during pregnancy and during contraceptive pill usage indicating a protective role for estrogen. Estrogen and vitamin D_3 influence each other, as in murine studies estrogen downregulates CYP24A1 and vitamin D_3 upregulates CYP19, the enzyme required for estrogen synthesis (Disanto et al., 2011;Holmqvist et al., 2010).

An alternative hypothesis proposed by Albert *et al.* suggests that both $25(OH)D_3$ and $1,25(OH)_2D_3$ should be measured in the serum of patients with autoimmune diseases. According to them, low levels of $25(OH)D_3$ are due to increased consumption and that conversely serum $1,25(OH)_2D_3$ would increase, which makes it a better marker of the disease than $25(OH)D_3$. They also believe that the relief of symptoms upon vitamin D_3 supplementation is a temporary silencing of the innate immune response, and that bacteria use VDR to downregulate the immune system and inhibit HDPs, leaving them to grow over time eventually causing autoimmune diseases. Based on an *in silico* model, they suggest the following: (i) bacterial ligands bind to LBP of VDR and disable

CYP24A1 causing a rise $1,25(OH)_2D_3$, (ii) supplementation with $1,25(OH)_2D_3$ will not be able to activate VDR which has been dysregulated by bacterial ligands, (iii) and the high levels of $1,25(OH)_2D_3$ will interfere with other nuclear receptors (such PPAR [the peroxisome proliferator-activated receptors] -γ and α, the GR, and the androgen receptor) involved in the induction of HDPs through binding to their natural receptors (Albert et al., 2009;Marshall, 2008).

The authors suggest that bacterial dysregulation of VDR in women is the reason they are more susceptible to autoimmune diseases. Early during pregnancy, women with MS and RA will have a 40% increase in $1,25(OH)_2D_3$ which will dysregulate VDR and other nuclear receptors and a temporary immunosuppression. After pregnancy when $1,25(OH)_2D_3$ goes back to its previous levels, inflammatory symptoms of autoimmune disease will return (Proal et al., 2009). However it should be stated that the alternative hypothesis has little solid laboratory data to support it and remains highly controversial.

1.1.7 VDR polymorphism

Variations in DNA in the human genome are mostly SNPs. About 7 million common SNPs have been identified with a minimum allele frequency (MAF) of at least 5% across the human population but in common SNPs MAF might vary between different populations (Hinds et al., 2005). SNPs are positions on the genome where there is variation of two alleles expressed by different populations. A person can either be homozygous or heterozygous for a certain SNP as each allele is inherited from one parent. Information on SNPs are collected by organisations such as the International

Haplotype Map Project (Hapmap) Consortium including genomic location and allelic frequencies among different populations (LaFramboise, 2009). A database for SNP (dbSNP) is available for SNP genotyping assays at the National Center for Biotechnology Information (NCBI). The database has reference SNP (rs) numbers to identify each SNP assay (Hinds et al., 2005).

The vitamin D_3 receptor (VDR) gene extends over 100 kb (kilo base pair) located in the long arm of chromosome 12 locus 13.1 (12q13.1) downstream of collagen II-alpha 1(COL2A1) (Uitterlinden et al., 2004b). VDR polymorphism has been associated with susceptibility to or protection from many conditions and diseases and varies among different ethnic backgrounds (Valdivielso and Fernandez, 2006). More than 470 VDR SNPs have been identified in the human VDR gene. Some SNPs can be associated with each other in certain populations and this is called linkage disequilibrium (LD). LD can be inherited as blocks referred to as haplotypes with an average of 10-20kb (Rukin and Strange, 2007). VDR polymorphisms were first identified using restriction fragment length polymorphisms (RFLPs) with the aid of restriction enzymes including EcoRV, BsmI, TaqI, and Tru9I (Uitterlinden et al., 2004b). VDR has been divided into three LD blocks A, B, and C where A is towards the 3' exon 9 and C in the non-coding region (Figure 1.10).

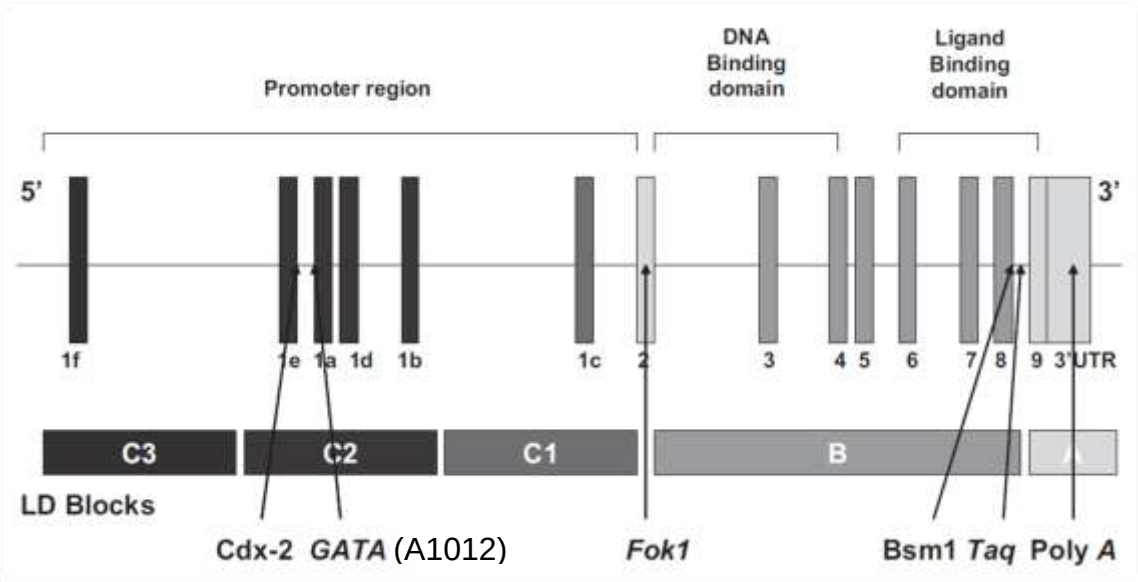

Figure 1.10. VDR gene polymorphism. VDR is located on 12q13.1 and SNPs are shown as LD block A,B, and C with the most commonly studied SNPs. Cdx2 and GATA (A1012G) is in the 5' promoter region, FokI is between blocks B and C while BsmI is closely associated with TaqI and ApaI in block B in the 3'UTR (Rukin and Strange, 2007).

A start codon polymorphism *FokI* (rs2228570) does not have an LD association and is located at the breaking point between LD blocks B and C. It is a T to C substitution (T/C) that alters the start codon resulting in a different size protein. The presence of *FokI* site (T allele) results in a 427 aa protein and the absence of FokI (C allele) results in a shorter 424 aa protein. *FokI* modulates transcription factors in the immune system. The C allele increased transcription by NF-κB, nuclear factor of activated T-cells (NFAT), and promoter for IL-12 p40 subunit (IL-12p40) more than the T allele. Human PBMCs homozygous for the C allele expressed higher mRNA for IL-12

and proliferated stronger upon stimulation with the mitogen phytohemagglutinin (van Etten et al., 2007). Patients with T1DM that were homozygous for *FokI* TT allele or heterozygous for *FokI* CT allele had lower pancreatic β-cell function (Mory et al., 2009). The most commonly studied LD SNPs in block B (intron 8 and exon 9) are the closely linked *BsmI* (C/T; rs1544410), *ApaI* (C/A; rs7975232), and *TaqI* (T/C; rs731236). No functional consequence has been reported for these SNPs but they are located in the 3'untranslated region (3'UTR) that can influence mRNA stability and protein translation efficiency (Arai et al., 2001;Rukin and Strange, 2007;Uitterlinden et al., 2004b). *Cdx2* (G/A; rs11568820) is a promoter region SNP, a known binding site for the *Cdx2*-intestinal transcription factor. It modulates intestinal VDR transcription and calcium absorption. The A allele increases while the G allele reduces VDR transcription and calcium absorption respectively (Arai et al., 2001). *A1012G* (rs4516035) SNP is (A to G) located in 1012 bp close to the exon 1a transcription start site. It is associated with the transcription of GATA3, a transcriptional factor for the differentiation of Th2 cells. The A allele of *A1012G* downregulates Th1 responses (Halsall et al., 2004).

1.2 The human eye

The human eye is a unique structure that receives and transmits light signals to the brain to be translated as images. It is in constant contact with the environment which renders it vulnerable to injury and infection. The eye has specific anatomical and physiological barriers to protect against potential damaging pathology that could lead to loss of visual acuity or even blindness.

1.2.1 Eye structure

The human eye is a spherical structure with a diameter of 2.4 cm. It is composed of a three-layered outer compartment and an interior compartment (Figure 1.11). The three outer layers are the outermost corneosclera (cornea and sclera), the uveal tract (iris, ciliary body, and choroid), and the inner neural retina. The retina is supplied by the central retinal artery and the outer and middle layers are supplied by ciliary vessels. The inner compartment contains aqueous humour (AqH), lens, and vitreous. The AqH is a clear fluid secreted by the ciliary processes. The vitreous comprises two-thirds of the eye volume and it is a transparent avascular inert gel that gives the eye its shape. Vision is reflected by the integrity of a transparent visual axis that starts from the cornea and passes through the anterior chamber (AC), lens, and vitreous, to the retina (Caspi, 2010;Chen et al., 2008).

The cornea forms the outer avascular fibrous coat of the ocular surface acting at the front line to protect the eye against offending environmental and infectious factors. The cornea is made of corneal epithelium, stroma, and corneal endothelium. Corneal epithelium is composed of flattened stratified non-keratinised squamous epithelium. This layer is in continuity with the conjunctiva at the limbus (at corneal edge) from which stem cells arise to replace old or dead corneal epithelial cells. Corneal epithelium is separated from the stroma by Bowman's layer (BL), an avascular basement membrane-like structure rich in collagen fibres. Corneal stroma is made of densely packed transparent regularly oriented collagen bundles. Next to the stroma, exists a thick Descemet's membrane, a basement membrane secreted by the corneal endothelium. The corneal

endothelium consists of a monolayer of hexagonally oriented epithelial cells with a high density of mitochondria and functions to provide nutrients and dehydrates corneal stroma. Corneal endothelial cells do not undergo mitotic division and do not regenerate (Di Girolamo, 2011;Takacs et al., 2009).

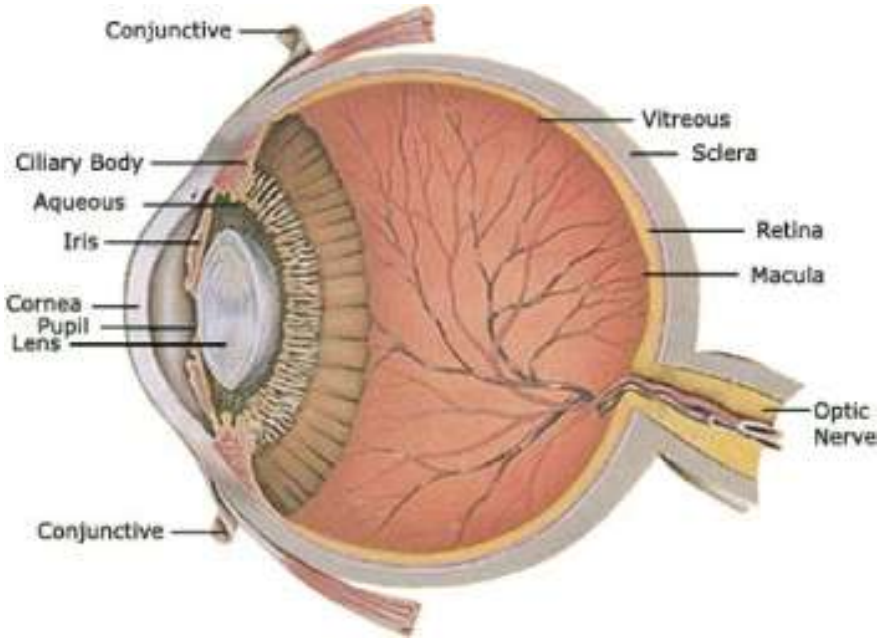

Figure 1.11. A schematic presentation of the human eye. The human eye is composed of an outer corneoscleral layer, uveal tract (iris, ciliary body, choroid), and retina. The AC is filled with aqueous humour produced in the posterior chamber by ciliary processes. The vitreous is located at the posterior of the eye, a gel substance that provides support and maintains the shape of the eye (Asadi et al., 2012).

In the uveal tract, the iris is a circular disk that acts as a diaphragm to regulate the amount of light that passes through to the retina by altering the size of the pupil through contraction of circular sphincter muscles (constricts the pupil) and radial dilator muscles (dilates the pupil). Surrounding the muscles is a stroma, an outer non-pigmented epithelium layer and inner pigment epithelium. The stroma is rich in melanocytes which determine the eye colour (Forrester et al., 2001). The ciliary body is a triangle shaped structure with two regions, anterior pars plicata where the ciliary processes are located and posterior pars plana at which it joins the choroid. The ciliary processes are made of two layers of epithelium that have their apical surfaces opposing each other, an inner non-pigmented ciliary body epithelium (NPE) in contact with AqH in the posterior chamber, and an external pigmented epithelium in contact with ciliary stroma. NPE secretes AqH and is the continuation of iris pigmented epithelium anteriorly and neural retina posteriorly while the pigmented epithelium is the continuation of retinal pigmented epithelium (RPE) (Goel et al., 2010).

The AqH has a unidirectional flow (Figure 1.12) passing from posterior chamber (between the iris and the lens) through the pupil to the AC (between the cornea and the iris) and circulating towards the angle of the AC (trabecular meshwork). The blood supply to the iris and ciliary body is provided by anterior and posterior ciliary arteries as well as choroidal branches (Forrester et al., 2001). The choroid, the outermost region of the uveal tract is a vascular, highly pigmented tissue which provides a network of blood vessels that carry nutrients to the retina (Regatieri et al., 2012).

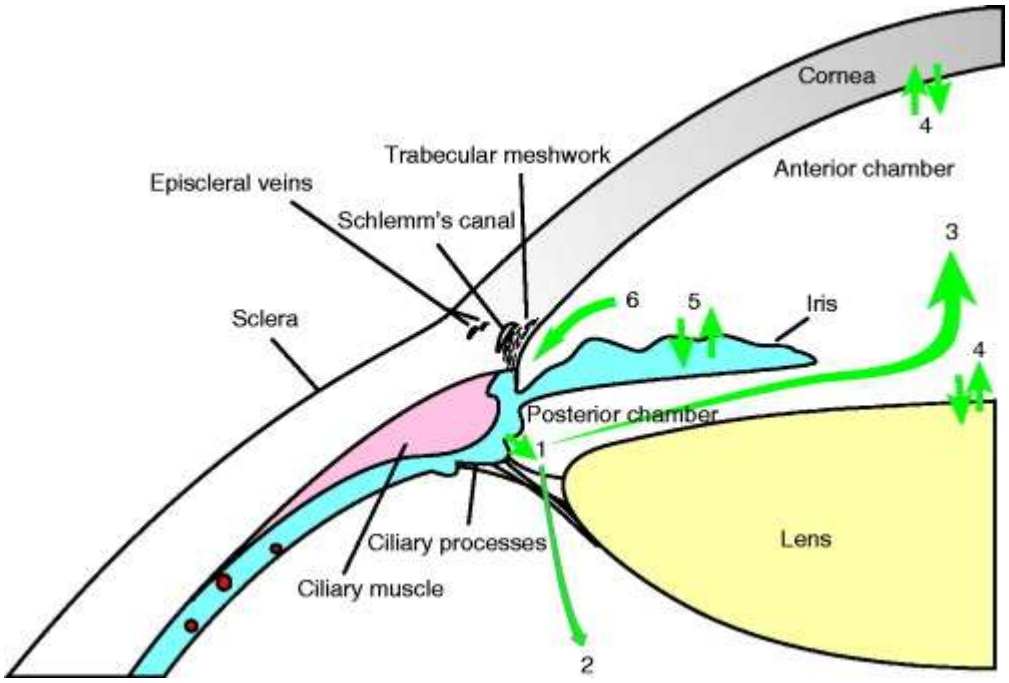

Figure 1.12. Aqueous humour flow. AqH is secreted in the posterior chamber (1) by ciliary processes. Some of it can pass through the vitreous cavity (2) and it is also released to fill the AC through the pupil (3). AqH causes fluid exchange with the cornea (4) and iris (5). The AqH drains into trabecular meshwork into Schlemm's canal and to episcleral veins and blood circulation (6). The figure is taken with permission from (Toris, 2008).

The retina is located at the posterior of the eye and can be divided into outer retina and neuroretina. The outer retina is made of a monolayer of RPE and sits on the multilayered Bruch's membrane, which allows the transfer of nutrients and oxygen from fenestrated choriocapillaries to RPE and waste from RPE to choriocapillaries. The apical surface of RPE faces the outer segment of photoreceptors (Mettu et al., 2012). RPE cells do not regenerate and decrease with age and their dysfunction could threaten

visual acuity such as in age-related macular degeneration (AMD) (Del Priore et al., 2002).

AMD is a chronic disease that occurs with aging and starts with the accumulation of drusen (extracellular deposits). Within drusen are amyloid-β (Aβ) and proinflammatory complement components that contribute to the pathogenesis of AMD. At later stages the degeneration of photoreceptors and RPE cells occurs (Ambati and Fowler, 2012;Anderson et al., 2004). RPE has several functions including transport of nutrients and water to the inner retina, preventing light scatter and photooxidation, reisomerisation of retinoids necessary for vision, and phagocytosis of dead photoreceptors. Neuroretina is formed of photoreceptors (rods and cones) and a ganglion cell layer which transfers the visual signals to the optic nerve. The central retinal artery and choroidal capillaries supply nutrients that diffuse through RPE to the retina (Erickson et al., 2007;Simo et al., 2010).

1.2.2 Antigen presenting cells in the eye

At the external surface of the eye, murine and human studies have shown that bone marrow (BM)-derived cells, mostly immature APCs, are recruited to the cornea from the limbus, the area between the vascularised conjunctiva and avascular cornea. Immature DCs express very low levels of MHC II and lack the expression of co-stimulatory molecules (CD80, CD86, and CD40) which can result in T cell tolerance (Lutz and Schuler, 2002).

In mice, three groups of APCs exist in central corneal stroma; $MHCII^{+}CD11b^{+}CD11c^{-}$ monocytes/macrophages, $MHCII^{-}CD34^{+}$ myeloid precursor cells, and a small group of $B220^{+}CD11c^{lo}$ plasmacytoid DCs. $CD11b^{+}$ cells are suggested to have the potential to form lymphatic vessels during corneal inflammation by differentiating into lymphatic vessel endothelial receptor 1^{+} (LYVE-1^{+}) cells. The corneal stroma expressed $MHCII^{+}CX_3CR1^{+}CD45^{+}$ (CD45 is lymphocyte common antigen) cells with cellular processes that bridge MHC II^{+} cells *in vitro* (Forrester et al., 2010). CX_3CR1 is a receptor for chemokine CX_3CL-1 (fractalkine) and is expressed on myeloid cells such as monocytes, macrophages, DCs, NK cells, and microglia to recruit them to the site of inflammation (Chinnery et al., 2008).

The cornea expresses soluble vascular endothelial growth factor receptor-1 (sVEGFR-1) which neutralises vascular endothelial growth factor-A (VEGF-A), an angiogenesis stimulant. Moreover, the constitutive corneal expression of VEGFR-3 (the receptor for VEGF-C and VEGF-D), thrombospondin-1 (TSP-1), endogenous IL-1 receptor antagonist (IL-1Ra), as well as tissue inhibitor of metalloproteinases-1 (TIMPs-1) in tear film prevent hemangiogenesis and lymphangiogenesis (Barabino et al., 2012). TSP-1 is a potent anti-angiogenic factor and is also produced by mouse iris and cultured RPE (Cursiefen et al., 2004;Zamiri et al., 2005).

$MHCII^{+}CD11c^{+}$Langerin/$CD207^{+}$ langerhans cells (LCs) that also express CD1a (marker for LCs) and CD45 are present in human peripheral and paracentral corneal epithelium while human peripheral and paracentral anterior regions of corneal stroma contain DC-SIGN/$CD209^{+}$ interstitial DCs (Mayer et al., 2007). BM derived DCs

(BMDCs) from CD45 knockout mice ($CD45^{-/-}$) showed decreased cytokine production of IL-12, IL-6 and TNF-α after LPS (TLR4 ligand)-stimulation. In contrast, $CD45^{-/-}$ DCs showed increased IL-12, IL-6 and TNF-α production in response to stimulation of TLR2 and TLR9. The lack of CD45 reduced IFN-γ producing Th1 cells in response to stimulation with LPS-activated DCs (Cross et al., 2008). $MHCII^{+}CD11b^{+}CD11c^{-}CD45^{+}$ monocyte/macrophages are also found in human corneal stroma (Mayer et al., 2007). Another population of APCs that was detected in mice corneal stroma is $MHCII^{+}CD11b^{+}CD45^{+}F4/80^{+}$ macrophages (Forrester et al., 2010). F4/80 is a marker of tolerogenic APCs mainly expressed on macrophages and on a small subset of DCs (Stein-Streilein, 2008).

The uveal tract (iris, ciliary body, and choroid) of all mammalian species contains a similar population of APCs to that of the cornea. The iris contains $CX_3CR1^{+}MHCII^{+}$ DCs and $MHCII^{-}CX_3CR1^{+}$ macrophages. $MHCII^{+}CX_3CR1^{+}$ DCs are present in the ciliary body epithelium. $CD11b^{+}CD11c^{+}$ DCs and macrophages are present in rat choroid as well as $F4/80^{+}$ macrophages. Choroidal $MHCII^{+}CD11c^{+}$ DCs are localised at the basal surface of RPE where shed photoreceptors accumulate, a possible source of autoantigens. The retina in rats contains $CD11c^{+}CD45^{lo}F4/80^{+}$ microglial cells that are immunosuppressive and a small population of $MHCII^{+}$ DCs and macrophages lining the blood vessels in meningeal extension that could play a role in inflammation (Forrester et al., 2010).

Under normal resting conditions, APCs in human and rat eye (e.g. ciliary body and choroid) are tolerogenic, but are motile and can be activated with proinflammatory cytokines during inflammation (Forrester et al., 2005). APCs from mouse retina could present antigen to antigen-experienced T cells, but failed to activate naïve T cells which is in agreement with the tolerogenic ocular environment (Gregerson et al., 2004).

1.2.3 Immune regulation in the eye

The eye has evolved to protect itself against injury including infections. The eye is provided with a set of anatomical and physiological barriers to divert or suppress destructive inflammatory responses. This renders the eye in a state referred to as ocular immune privilege. Other sites of the body that are considered immune privileged are testis, hair follicle, placenta, and the brain (Niederkorn, 2012). Immune privilege of the eye is a concept first introduced by Sir Peter Medawar in 1940 after the survival of a skin allograft in the AC of a rabbit eye. This was explained as immune ignorance that was a result of the lack of draining lymphatics that did not allow the escape of the antigen into peripheral lymph nodes to stimulate an immune response.

More recently, it was revealed that ocular immune privilege is a product of many active immunosuppressive mechanisms in the ocular environment to limit ocular damage by innate and adaptive immunity (Hori, 2008;Taylor, 2009). These mechanisms include the lack of lymphatic drainage and physical barriers, immunosuppressive microenvironment, and regulation of systemic immune response (Zhou and Caspi, 2010).

1.2.3.1 Lack of lymphatic drainage and physical barriers

The cornea is avascular and lacks lymphatic vessels. Lymphatics are also absent in the AC and the retina. Lymphatic drainage is only available for the conjunctiva, sclera, and choroidal capillaries (Barabino et al., 2012;Streilein, 2003;Yucel et al., 2009) . Recently using fluorescent tracers for lymphatic markers such as LYVE-1 in human and sheep eyes, lymphatics were identified in the ciliary body (Yucel et al., 2009). The contribution of this pathway to AqH drainage is still unclear (Kim et al., 2011;Yucel et al., 2009). By injecting a fluorescently labelled antigen into rat AC, Camelo *et al.* demonstrated that soluble antigen can leave the eye to regional lymph nodes of the head and neck, as well as in the marginal zone of the spleen, without being associated with ocular APCs (Camelo et al., 2006).

The eye is designed with external barriers to protect it against traumatic damage such as the bony orbit, eye lids, and eye lashes. The tear film flushing foreign particles and containing antimicrobial substances (such as lysozymes and lactoferrin) as well as IgG and IgA, can neutralise bacteria and viruses to protect the ocular surface (Akpek and Gottsch, 2003). Additional ocular physical barriers are present which include corneal endothelium, blood-aqueous barrier, and blood-retinal barrier. These barriers not only physically prevent passage of immune cells, but also have an active role in suppressing the immune response. Corneal epithelium represents an essential physical barrier with immunomodulatory properties (Akpek and Gottsch, 2003;Hori, 2008) but only structures which are critical to this project will be discussed.

1.2.3.1.1 Corneal endothelium

The role of corneal endothelium is to keep a state of dehydration in the cornea to preserve its transparency. It works as an active fluid pump between the cornea and AqH. Fluid leak to corneal stroma is tightly controlled by the integrity of tight and adherens junctions in corneal endothelium. This integrity might be weakened or lost in inflammatory conditions such as uveitis (Srinivas, 2012). Rauz *et al.* have identified the mRNA expression of apical epithelial sodium channel (ENaC) and the enzyme serum and glucocorticoid regulated kinase isoform 1 (SGK1) in human corneal endothelium. These molecules might support the function of corneal endothelium in sodium transport and maintaining corneal transparency (Rauz et al., 2003).

Moreover, human corneal endothelium expresses the enzyme 11β-hydroxysteroid dehydrogenase 2 (11β-HSD2) as well as glucocorticoid receptor (GR) and mineralocorticoid receptor (MR) (Rauz et al., 2001;Stokes et al., 2000). MR is also known as aldosterone receptor while GR is a receptor for cortisol. These two receptors have 90% aa homology in the DNA binding domain (DBD) and about 50% in the ligand binding domain (LBD). The enzyme 11β-HSD2 acts to prevent binding of glucocorticoids (such as cortisol which is present in high concentrations in plasma) to MR leaving it available for aldosterone (Odermatt and Kratschmar, 2012). Cortisol and aldosterone may induce the expression of SGK1 which in turn activates ENaC to facilitate sodium pumping across the apical surface of corneal endothelium (Rauz et al., 2003).

1.2.3.1.2 Blood-aqueous barrier

Blood aqueous barrier (BAB) is composed of TJs between NPE cells and TJs between vascular endothelial cells of the iris. Leakiness of BAB could allow access of inflammatory cells as well as plasma proteins into the AC (Chen et al., 2008). GR, MR, and 11β-HSD1 are expressed in the non-pigmented ciliary body epithelium (Rauz et al., 2001;Stokes et al., 2000). 11β-HSD1 is the enzyme that catalyses the interconversion between inactive cortisone and active cortisol (Odermatt and Kratschmar, 2012). The production of AqH requires a metabolically active process that involves the transport of water and ions (Kiel et al., 2011). 11β-HSD1 may play a role with GR and MR in sodium transport and AqH production by ciliary epithelium (Rauz et al., 2001).

1.2.3.1.3 Blood-retinal barrier

Blood-retinal barrier (BRB) is made by TJs between retinal pigment epithelial cells and TJs between endothelial cells of retinal vessels. Only small molecules such as glucose and ascorbate are allowed to diffuse through these TJs. A breach of this barrier may occur in conditions such as uveoretinitis, diabetic retinopathy, and AMD (Crane and Liversidge, 2008).

1.2.3.2 Immunosuppressive microenvironment

Breakdown of ocular barriers can occur and can cause the infiltration of immune cells into the eye resulting in a sight damaging inflammatory response. In addition to barriers the eye is rich in soluble and membrane bound molecules that can affect innate and adaptive immune cell activation.

1.2.3.2.1 AqH

AqH contains a range of neuropeptides that diffuse from sensory nerve endings to the eye and cytokines, including alpha-Melanocyte-stimulating hormone (α-MSH), vasoactive intestinal peptide(VIP), somatostatin (SOM), TGF-β2, calcitonin gene-related peptide (CGRP), macrophage migration inhibitory factor (MIF), all can regulate immune cells to suppress inflammation in the AC (Taylor, 2009).

α-MSH is a peptide mainly expressed in the hypothalamus, pituitary gland, and skin with antiinflammatory properties that range from the suppression of proinflammatory (TNF-α and lL-1β) and upregulation of antiinflammatory (lL-10) cytokines (Brzoska et al., 2008). α-MSH binds to melanocortin-1 receptor (MC-1R) on macrophages and dendritic cells differentiated from human monocytic cell lines and neutrophils to mediate the suppression of their production of proinflammatory cytokines, nitric oxide (NO), and reactive oxygen intermediates (Manna et al., 2006). When murine lymph node primed T cells were incubated with α-MSH, there was an 80% reduction of IFN-γ production. APCs treated with α-MSH caused a reduction in IFN-γ by T cells (Taylor et al., 1994a).

In another murine study, AqH inhibited the maturation of LPS-stimulated DCs. AqH downregulated the expression of MHC II, CD80, and CD86 and inhibited their ability to activate T cells. These inhibitory effects on DC maturation were reversed upon blocking TGF-β2 (Wang et al., 2011). Recently, Denniston *et al.* showed that human naïve $CD4^+$T cells failed to proliferate when co-cultured with noninflammatory AqH. AqH treated DCs had a significant reduction in the expression of CD86. Endogenous cortisol and TGF-β both contribute to the suppressive activity of AqH (Denniston et al., 2011).

VIP is a neuropeptide present in the nervous and endocrine systems as well as the lungs and intestine. It posseses immunomodulatory properties as many cells in the body including immune cells such as monocytes and lymphocytes expressed receptors for VIP. In murine studies, VIP has been shown to inhibit macrophage proinflammatory cytokine production (Chorny et al., 2006;Delgado et al., 2004) and in the eye it is produced by human corneal endothelium, iris, ciliary body, and has been detected in AqH (Koh, 2012). VIP in AqH inhibited *in vitro* poliferation and IFN-γ production by antigen stimulated lymphonuclear cells (LNC) in rabbits. Absorbing VIP reversed the inhibitory effect on LNC (Taylor et al., 1994b). The injection of liposome containing VIP into rat vitreous suppressed endotoxin (LPS) induced uveitis (EIU). There was a reduction in the EIU clinical score, the number of infiltrating inflammatory cells to the anterior segment, and mRNA expression of IFN-γ, TNF-α and IL1β (Lajavardi et al., 2007).

The release of some neuropeptides is affected by light/dark conditions which in turn modulate the immune response in the eye. Mice reared in the light (diurnal) showed high levels of VIP in the iris and ciliary body and low levels of another neuropeptide, substance P (SP). In contrast, mice reared in the dark, showed low levels of VIP and increased amounts of SP. This effect seemed to be an adaptation process and could be reversed by putting mice under diurnal conditions (Ferguson et al., 1995). CGRP is present in the AqH of humans and other species and at concentrations present in AqH, inhibited NO production from mouse macrophages (Taylor et al., 1998). MIF is a multifunctional molecule that plays a role as a proinflammatory cytokine and contributes

to the immune privilege of hair follicles by suppressing cytolysis by NK cells (Calandra et al., 2003;Ito et al., 2007). Recombinant murine MIF (rMIF) and rabbit AqH both inhibited macrophage migration *in vitro* and NK-mediated lysis of murine corneal endothelial cell line (Apte et al., 1998).

1.2.3.2.2 Corneal endothelium

Like many cells in the cornea, corneal endothelial cells lack MHC II and only weakly express MHC I. They express CD95L, which upon binding to CD95 on T cells induces T cell apoptosis (Hori, 2008). In mice, corneal endothelial cells constitutively express B7-H1 (programmed death-1 molecule ligand; PD-L1), a member of the CD28/CTLA-4 family and a ligand for programmed death-1 molecules (PD-1) on T cells that downregulates T cell receptor signalling (Hori et al., 2006). When cultured in the presence of human corneal endothelial (HCE) cell line, there was a reduction in the proliferation of all T cells, specifically $CD4^+$ T cells, and Th1 T cells. This inhibition was cell-cell contact dependent. HCE cells suppressed proliferation and IFN-γ production from infiltrating Th1 T cell clones (TCC) prepared from AqH of patients with different inflammatory eye diseases (Behçet's disease [BD] and uveitis). HCE express PD-L1 and PD-L2 and when incubated with Th1 TCC, there was an increase in PD-1 expression on T cells. The ability of HCE to reduce IFN-γ production by Th1 cells was blocked by anti-PD-L1 but not anti-PD-L2. Interestingly, HCE incubated in IFN-γ containing Th1 cultures supernatants upregulated PD-L1 expression indicating the role of inflammation in the expression of these molecules on HCE (Sugita et al., 2009).

When co-cultured in the presence of HCE, human $CD8^+$ T cells had a reduced proliferation. Unlike $CD4^+$ T cells, $CD8^+$ T cells had a poor expression of PD-1 which suggests another molecule is involved. It appeared that the cell-cell contact dependent inhibition of $CD8^+$ T cell proliferation was mediated by membrane bound TGF-β2, highly expressed by HCE. TGF-β2 not only enabled HCE to suppress T cell proliferation, but allowed them to convert both $CD4^+$ and $CD8^+$ T cells into $CD25^+Foxp3^+$ Tregs. $CD4^+$ and $CD8^+$ HCE-induced Tregs inhibited the proliferation of $CD4^+$ and $CD8^+$ effector T cells respectively, via TGFβ1 in both cases (Yamada et al., 2010).

1.2.3.2.3 Intraocular compartments

A suppressive microenvironment is created in the intraocular compartments by pigmented epithelium of the iris, ciliary body, and retina. These tissues although in different locations and functions in the eye, all have immunomodulatory properties that play a role in ocular immune privilege (Zamiri et al., 2007). Murine iris and ciliary body pigmented epithelium (I/CBE PE) inhibited ovalbumin (OVA) stimulated T cell proliferation and reduced production of IL-2, IFN-γ, IL-4, and IL-10 *in vitro*. This inhibition was dependent on direct contact between these cells. This inhibition was not mediated by Fas (CD95)-Fas ligand (FasL; CD95L) interaction and T cell apoptosis was not induced. When cultured with T cells, I/CBE PE produced TGF-β which mediated the inhibitory effect on T cells (Yoshida et al., 2000b;Yoshida et al., 2000a).

Subsequent experiments by Sugita *et al.* have demonstrated the expression of soluble and membrane bound TGF-β (TGF-β1 and TGF-β2) in $B7^+$ (CD80/86) iris PE (IPE). Through membrane bound TGF-β, IPE cells increased the expression of B7,

TGF-β and CTLA-4 on $CD8^+$ T cells and converted them into Tregs (IPE Tregs). IPE Tregs used cell-cell contact and membrane bound TGF-β to inhibit bystander T cell proliferation. By comparison, CB PE cells were not able to convert either $CD4^+$ nor $CD8^+$ T cells into Tregs (Sugita et al., 2006;Sugita et al., 2008)

Murine RPE and ARPE-19 (human adult retinal pigment epithelium cell line) express PD-L1 and PD-L2. In the supernatants of mixed cultures of ARPE-19 and T cells, PD-L1 mediated a reduction in the levels of IFN-γ, IL-8, and monocyte chemotactic protein-1 (MCP-1) produced (Usui et al., 2008). Immortalised murine RPE cells upregulated MHC II expression after treatment with TNFα and IFN-γ and constitutively expressed CD80 but they could not process the antigen β-galactosidase (β-gal) and present it to naive T cells from β-gal-TCR transgenic mice. When these T cells were stimulated with irradiated APCs with β-gal on a monolayer of RPE cells, RPE cells induced T cells to become anergic by inhibiting their proliferation and the production of IFN-γ and IL-2. Anergic T cells recovered from RPE co-cultures and activated with splenic APCs on a monolayer of RPE cells in the presence of β-gal failed to proliferate and showed suppressed IL-2, IFN-γ, and IL-17 production. Although Foxp3 expression was induced on these T cells, they did not become Tregs (Gregerson et al., 2007).

Sugita and colleagues were able to show that RPE cells can induce Tregs. $CD4^+$ T cells cultured in the presence of murine RPE cells showed reduced proliferation, and converted into Tregs. RPE induced Tregs were $CD4^+CD25^+Foxp3^+$ and were suppressive of bystander T cell activation. A novel inhibitory factor CTLA-2α discovered on murine RPE cells was essential for RPE conversion of Tregs *in vitro* (Sugita et al.,

2008). Ocular pigment epithelial cells constitutively express FasL (Sugita, 2009). In ARPE-19 cells, the expression of FasL was increased after treatment with IFN-γ or infection with cytomegalovirus (CMV). ARPE-19 express both Fas ligand in soluble and microvesicle membrane bound forms but it is not clear how FasL is released with microvesicles to induce target cell apoptosis (McKechnie et al., 2006).

Murine primary RPE cultured cells secrete immunomodulatory molecules including TSP-1, TGF-β, SOM, and pigment epithelial derived factor (PEDF). TSP-1 and TGF-β produced in RPE cell supernatants cause the suppression of T cells activation and reduced IFN-γ production. TSP-1 is required for the activation of TGF-β and both are important for the induction of T cell suppression (Zamiri et al., 2005). PEDF suppressed IL-12 and augmented IL-10 production in LPS-stimulated macrophages. PEDF and SOM synergised to suppress NO produced by murine macrophages. When injected simultaneously with LPS into mice ears, PEDF reduced the inflammatory response (Zamiri et al., 2006). PEDF contributes to the prevention of angiogenesis and the survival of retinal stem cells and may contribute to therapeutic treatments of neovascular disease or AMD (Liu et al., 2012a). A murine study (Kawanaka and Taylor, 2011) showed that α-MSH and neuropeptide Y (NPY) produced in supernatants of primary RPE upregulated the expression of Arginase1 (ARG1) and nitric oxide synthetase (NOS2) in primary murine macrophages. These are called alternatively activated macrophages (M2) which are usually induced by Th2 cytokines IL-4 and IL-13 rather than classically activated macrophages (M1) induced by Th1 IFN-γ (Gordon and Martinez, 2010).

1.2.3.3 Regulation of systemic immune response

AC associated immune deviation (ACAID) is a phenomenon characterised by the development of antibody and suppression of cell mediated inflammatory responses after the soluble antigen is injected or shed from tumour in the AC in experimental animals. For ACAID to occur it requires the eye, thymus, spleen, and sympathetic nervous system where removal of any of them within 72 hours after injection inhibits ACAID. It is believed that within 48 hours $F4/80^+$ macrophages in the iris and ciliary body pick up the antigen, transport it to the venous circulation, and lead to the generation of $CD8^+$ T cells that can suppress inflammation. $F4/80^+$ macrophages induce the development of $CD4^-$ $CD8^-NK1.1^+$ thymocytes which migrate to the spleen where they participate with CD1d+ B cells, $CD4^+$ NKT cells, and $CD8^+$ T cells in the generation of ACAID by inducing $CD8^+$ the development of regulatory T cells that inhibit Th1 and Th2 inflammatory responses and the production of antibodies IgM and IgG1 (Camelo et al., 2005;Niederkorn, 2006). $\gamma\delta$ T cells also play a role in the process by producing IL-10 necessary for the induction of ACAID (Ashour and Niederkorn, 2006). As it is vital for the eye to maintain clear vision, ACAID is an example of how the eye can induce systemic immune tolerance.

1.2.4 Uveitis

Uveitis is a group of inflammatory conditions in the eye. Uveitis does not only affect the uveal tract, it may involve the sclera, retina, and optic nerve. It is a cause of 5-20% of legal blindness in the United States and Europe and 25% in the developing world (de Smet et al., 2011). Uveitis is classified anatomically according to the primary site of inflammation as anterior (AC), intermediate (vitreous, pars plana), posterior

(retina or choroid), and pan (AC, vitreous, and retina or choroid) uveitis (Jabs et al., 2005). Patients with uveitis may present with photophobia, red eye, floaters, and blurred vision (Guly and Forrester, 2010).

In intermediate uveitis (IU) inflammation can involve AC, ciliary body, and the retina. It can either be infectious or non-infectious (idiopathic or autoimmune). Infectious IU can occur in toxoplasmosis, tuberculosis, lyme disease, human T lymphotropic virus Type 1 (HTLV-1), and syphilis. Non-infectious IU can be associated with systemic diseases such as sarcoidosis, intraocular lymphoma, and multiple sclerosis. When IU is not associated with an underlying infection or systemic disease but with the presence of snow banks (white exudates) at pars plana, it is referred to as pars planitis (Babu and Rathinam, 2010;de Smet et al., 2011).

Anterior uveitis has been linked to be ankylosing spondylitis and birdshot choroidopathy while posterior uveitis has been linked to sarcoidosis, BD, and Chron's disease. IU has been associated with MS and sarcoidosis (Barisani-Asenbauer et al., 2012). Uveitis has been associated with MHC antigens. Of class I, HLA-B*27 is associated with anterior uveitis, HLA-B*51 with BD, and HLA-A*29.2 with birdshot retinochoroidopathy. With regards to MHC class II, HLA-DRB1*03 has been linked to anterior uveitis whereas HLA-DR2, HLA-DR51, HLA-DR17, and HLA-DRB1*15 have been linked with intermediate uveitis (Du et al., 2009;Tang et al., 1997;Zamecki and Jabs, 2010).

The antigens inducing uveitis have, in most cases, not been identified but experimental autoimmune uveitis (EAU) can be induced in animal models using retinal S antigen or interphotoreceptor retinoid binding protein (IRBP) (Caspi et al., 2008). Humanised models of EAU have been established where mice are made transgenic (Tg) with human MHC II antigens after deleting mouse class II antigens. Several Tg human MHC molecules have been created (e.g. HLA-DR3, HLA-DR4, HLA-DQ6 and HLA-DQ8) and are injected with IRBP or S antigen to induce EAU. This will allow a better human relevant understanding of immune mechanisms and therapeutic targets for the treatment of uveitis (Levy et al., 2011). EAU involves inflammation in the iris, ciliary body, retina, and choroid (Babu and Rathinam, 2010), and involves breakdown in ocular barriers which allows the infiltration of immune cells to the eye. Animal studies and samples from AqH, vitreous, and peripheral blood from patients have allowed an understanding of the cellular types and mechanisms involved in uveitis.

In EAU models, Th1 and Th17 cell mediated responses have been observed but with Th1 IFN-γ being the predominant cytokine locally and in the peripheral lymph nodes (Caspi et al., 2008). In humans, both Th1 IFN-γ and Th2 IL-4, IL-10, and IL-13 cytokines play a role in the pathogenesis of uveitis (Horai and Caspi, 2011). $CD4^+$ $CD25^+$ Tregs induced during the recovery from EAU play an important role in the resolution of intraocular inflammation. They are regulated by IL-10 and other suppressive inhibitory molecules in AqH (Ke et al., 2008). While treatment of PBMCs from patients with BD uveitis with IFN-γ increases NO production, IL-10 reduces NO suggesting its protective role in uveitis (Belguendouz et al., 2011). In humans, myeloid DCs have been isolated

from AqH samples of uveitic patients and showed high MHC I and II expression, but decreased CD86 compared to DCs from peripheral blood of the same patients, a combination which led to reduced activation of naïve T cells (Denniston et al., 2012). Interestingly, this did not involve endogenous cortisol, which was responsible for a similar effect of noninflammatory AqH (Denniston et al., 2011).

TNF-α plays an important role in uveitis and high levels can be found in AqH and serum from patients with uveitis. It is one of the molecules targeted for immunotherapy of uveitis (Khera et al., 2010). TNF-α and its soluble receptors TNF-α receptor I and 2 (TNF-α R1 and TNF-α R2) are significantly elevated in AqH and vitreous fluid from patients with active uveitis compared to controls. Soluble TNF-αRs stimulated increased TNF-α production by infiltrating T cells present in ocular fluids increasing inflammation in this model system (Sugita et al., 2007). Neutralisation of TNF-α reduces retinal inflammation in uveitic patients and the EAU model (Dick et al., 2004;Murphy et al., 2004).

TNF-α represent an important therapeutic target for the treatment of uveitis and ocular inflammation in BD. Monoclonal antibodies against TNF-α such as infliximab and adalimumab have been shown to be effective in treatment of uveitis and BD (Deuter et al., 2008;Gueudry et al., 2012). AqH from patients with idiopathic uveitis had significant increase in the concentrations of IL-6, IL-8, IFN-γ, and CCL2/MCP-1 (chemokine ligand 2 or MCP-1) compared to noninflammatory controls. IL-8 and CCL2/MCP-1 were higher in AqH from BD and herpes-viral uveitis while TGFβ2 and CXCL12 (chemokine ligand 12) were reduced (Curnow et al., 2005). In the AqH of patients with IU, TNF-α, IL-6, IL-8,

IL-1β, IL-12 p70 subunit (IL-2p70), IL-10, and CCL2/MCP-1 are higher than in controls. IL-6 and IL-8 levels in these patients were even higher in AqH samples than in sera (Valentincic et al., 2011).

Cytokine polymorphisms play a role in susceptibility and pathogenesis of uveitis. Polymorphisms in *IL-10* and *TNF-α* have been associated with uveitis in patients in the UK, these SNPs were in loci previously linked to immune mediated inflammation (Atan et al., 2010;Haukim et al., 2002). Another study in Austria has shown an association between a SNP in *IL2RA* and the risk for intermediate uveitis where G allele is protective (Lindner et al., 2011). A meta-analysis study has shown an association of one SNP in *IL10* and a variant between *IL23R* and *IL12R β2* and BD. Monocytes from individuals expressing A allele of *IL-10* variant express less mRNA and produce less IL-10 protein compared to those with the G allele (Remmers et al., 2010).

1.2.5 The role of vitamin D3 in the eye

Very early studies have shown the presence of VDR, calcium-binding protein (vitamin D-dependent), and plasma membrane calcium pump by immunostaining of sections of the different layers of the human eye (Johnson et al., 1995). Treatment with vitamin D_3 reduced the growth of human retinal blastoma cell line Y-79 *in vitro* (Saulenas et al., 1988). Topical treatment of mice cornea with $1,25(OH)_2D_3$ reduced LC migration into the cornea and neovascularisation (Suzuki et al., 2000).

Later research has demonstrated some of the immunomodulatory effects of vitamin D_3 in the eye. When a human corneal epithelial cell line was infected with

Pseudomonas aeruginosa in the presence of $1,25(OH)_2D_3$, the expression of IL-1β, IL-6, and IL-8 was suppressed (Xue et al., 2002). Oral treatment with vitamin D_3 abrogated inflammation and reduced proinflammatory cytokines in lymph node supernatants from mice with EAU. Treatment with vitamin D_3 *in vivo* reduced the expression of RORγt (transcription factor for Th17) and the production of IL-17 when $CD4^+$ T cells from mice lymph nodes were stimulated *in vitro* (Tang et al., 2009).

Similar results were seen in humans where the addition of vitamin D_3 to naïve stimulated $CD4^+$ T cells from patients with BD, upregulated IFN regulatory factor 8 (IRF-8), a transcription factor that negatively regulated Th17 *in vitro*. This was reflected by suppressing the expression of RORγt, IL-17, and CCR6. It reduced the production of IFN-γ by $CD4^+$ T cells cultured in Th17 polarising conditions and to the contrary, it increased IL-10 production. Vitamin D_3 suppressed the production of IL17 and IFN-γ by $CD4^+$ T cells co-cultured with DCs pretreated with Vitamin D_3 compared to those co-cultured with untreated DCs (Tian et al., 2012). Vitamin D_3 genes were recently identified in human, mouse and rabbit corneal epithelial cells. $25(OH)D_3$ and $1,25(OH)_2D_3$ were detected in rabbit tears, AqH and vitreous. $25(OH)D_3$ and $1,25(OH)_2D_3$ increased TEER and upregulated the expression of occludin in human corneal epithelial cell line (Lin et al., 2012;Yin et al., 2011).

Vitamin D_3 rich food and supplement intake correlated inversely with the onset of early AMD in a survey among 40 years and older participant (Parekh et al., 2007). In a recent study, vitamin D_3 intake in women younger than 75 years correlated with

decreased odds of early AMD which suggests a protective role of vitamin D_3 (Millen et al., 2011).

Interestingly, the protective effect of vitamin D_3 has been confirmed *in vivo*. A group of twelve-month old C57BL/6 mice injected with vitamin D_3 emulsified in safflower oil was compared with a group injected with safflower oil alone and were followed for 6 weeks. Vitamin D_3 treatment significantly reduced the number and morphology of inflammatory macrophages and decreased complement deposits in the subretinal space. Vitamin D_3 markedly reduced Aβ along Bruch's membrane and other neurotoxic oligomers and this was reflected by an improved visual function of mice treated with vitamin D_3 (Lee et al., 2012). By comparison, analysis of sib pairs found that while UV exposure was protective for the development of AMD, serum levels of 25(OH)D_3 were not significantly different between patients and controls. After controlling for known risk factors, such as smoking a SNP in *CYP24A1* was associated with onset of disease (Morrison et al 2011).

Low serum 25(OH)D_3 levels were associated with an increase in BD (Karatay et al., 2011). This was associated with decreased Treg and predominating Th1 responses indicating an immunomodulatory role of vitamin D_3 in the eye (Hamzaoui et al., 2010). There was an association between VDR SNP *FokI* and BD in a group of Tunisian patients (Karray et al., 2012;Morrison et al., 2011).

1.2.6 Hypothesis and aims

The eye is constantly exposed to the environment and inflammation as a result of a physical assault or infection can lead to blindness. The eye is protected with epithelial physical barriers (corneal endothelium, BAB, and BRB) at the front and the back to prevent the entry of inflammatory cells to the eye. It is also equipped with physiological molecules to maintain an immunosuppressive environment and clear vision. One potential molecule is vitamin D3. Vitamin D_3 has many immunomodulatory properties and many epidemiological studies suggest an association between low serum vitamin D_3 levels and a range of inflammatory and autoimmune diseases.

A vitamin D_3 extra-renal system has been characterised in monocytes/macrophages and many epithelial cells. It promotes barrier function at different sites in the body and modulates both the innate and adaptive immune responses by suppressing inflammation and inducing antimicrobial peptides. Thus vitamin D_3 appears to provide a mechanism by which the cells/tissues can still defend themselves against pathogens and at the same time limit the damage caused by inflammatory responses. Gene polymorphisms of VDR and related genes have been linked to different inflammatory and autoimmune disorders and they determine with other factors individual responses to inflammation.

Previous studies of vitamin D_3 in the eye have demonstrated extra-renal expression of vitamin D_3 in ocular cells, but did not show the ability of ocular cells to endogenously produce $1,25(OH)_2D_3$. Ocular cells can produce HDPs that can also be detected in ocular fluids where they mediate antimicrobial and immunomodulatory

properties (Garreis et al., 2010a;McDermott, 2009). To date there is no evidence if the ocular HDP production is mediated by endogenous vitamin D_3. VDR SNPs were studied in BD and AMD (Karray et al., 2012;Morrison et al., 2011) but no information on VDR or related genes in IU was available.

It is against this background that I **hypothesise** that extra-renal vitamin D_3 exists in ocular barrier epithelial cells, it induces HDPs production, and that genetic control of this process contributes to uveitis.

Therefore to address the hypothesis I aim to:

1. Investigate and characterise the expression of vitamin D_3 machinery in ocular barrier cells and if they can convert inactive to active vitamin D_3.
2. Study the role of vitamin D_3 in the induction of HDPs in the eye and if vitamin D_3 synergises with Toll-like receptor signalling to upregulate proinflammatory cytokine production.
3. Study the association of genetic variation in *VDR* with intermediate uveitis in Caucasians.

2 MATERIALS AND METHODS

2.1 Cell lines

All cells were grown in a humidified chamber at 37°C with 5% CO_2 and passaged by trypsinisation. Penicillin (100 units/ml) and streptomycin (100 µg/ml) solution were added to all growth media (PAA Laboratories, Yeovil, UK). All cells were grown in 10% heat inactivated fetal calf serum (HIFCS) except human corneal endothelial cells and HKC-8 which were grown in 5% HIFCS (Biosera Ltd, Ringmer, UK).

2.1.1 Human conjunctival cell line (CCL-20.2)

The work described using this cell line was from the project done by a Medical student (Deepali Patel) in our group in 2011, whom I was involved in supervising. CCL-20.2: clone 1-5c-4 previously charachetrised (Chang, 1954) and obtained from American Type Culture Collection, ATCC (Manassas, VA, US) were grown in medium 199 (Invitrogen, Paisley, UK) containing Earle's Balanced Salt Solution (Sigma-Aldrich, Dorset, UK) and 2.2 g/L sodium bicarbonate (Sigma-Aldrich).

2.1.2 Human corneal endothelial cell line (HCEC-12)

HCEC-12 were purchased from the German Resource Centre for Biological Material; DSMZ, Germany. The cell line was established by simian virus (SV) 40 transformation and characterised as immortalised corneal endothelial cells (Bednarz et al., 2000). HCEC-12 were cultured in flasks (Sarstedt,Leicester, UK) precoated with a mixture of laminin and chondroitin sulfate (Sigma-Aldrich, Dorset, UK) in F99 basal medium (Gibco, Invitrogen, Paisley, UK**)** supplemented with 20 µg/ml ascorbic acid, 20

μg/ml human recombinant insulin, and 10 ng/ml basic fibroblast growth factor (bFBF) (all Sigma-Aldrich, Dorset, UK)

2.1.3 Adult retinal pigment epithelial cell line (ARPE-19)

Adult retinal pigment epithelial (ARPE-19) a spontaneously immortalised human RPE cell line, were obtained from American Type Culture Collection (ATCC number: CRL-2302, Middlesex, UK). These were developed and characterised as a model of retinal pigment epithelial cells (Dunn et al., 1996). Cells were cultured in Dulbecco's Modified Eagle Medium:Nutrient Mixture F-12 (DMEM/F12), with glutamax (Gibco, Invitrogen, Paisley, UK).

2.1.4 Non-pigmented ciliary body epithelial cell line (ODM-2)

ODM-2, an SV40 transformed cells originally established and described as a cell line for non-pigmented ciliary body epithelium (Martin-Vasallo et al., 1989), were a kind gift from Dr. Coca Prados (Department of Ophthalmology and Visual Sciences, Yale University, US). Cells were cultured in DMEM, high glucose (4 g/L) (PAA Laboratories, Yeovil, UK).

2.1.5 Human kidney cell line (HKC-8)

HKC-8, an SV40 transformed cell line was established and characterised to be used as a proximal renal tubule cell line (Racusen et al., 1997). The cell line was kindly provided by Dr. Rosemary Bland from the University of Warwick, UK. Cells were grown in DMEM/Hams F12 (PAA Laboratories, Yeovil, UK).

2.2 Macrophage isolation and differentiation

Donation Component cones from healthy donors were obtained from National Blood Services (NBS), Birmingham, UK. Cones containing 10-15 ml of blood were emptied into a 50 ml centrifuge tube (BD, Oxford, UK) and topped up with phosphate buffered saline (PBS) to 50 ml. PBMCs were separated using density-gradient centrifugation. In two 50-ml centrifuge tubes, diluted blood was layered onto 15 ml of density-gradient centrifugation medium (GE Healthcare, Buckinghamshire, UK), and centrifuged for 30 min at 400g. PBMCs were collected and washed a few times with RPMI 1640. Monocytes were isolated using a commercial $CD14^+$ cells separation kit (MACS CD14 MicroBeads; Miltenyi Biotec, Surrey, UK) according to manufacturer's instructions.

Briefly, PBMCs were incubated with anti-human CD14 magnetic microbeads in a column placed on a magnetic separator. $CD14^+$ cells were retained in the column while others were washed through. $CD14^+$ cells were eluted in MACS buffer and washed. Cells were counted and placed in 12-well plates with a density of $1x10^6$ cells per well in RPMI 1640 supplemented with 10% HIFCS. Granulocyte-macrophage colony-stimulating factor (GM-CSF) (10 ng/ml) and macrophage colony-stimulating factor (M-

CSF) (50 ng/ml) were added to monocyte cultures to differentiate M1 and M2 types respectively (Peprotech, London, UK). Medium and cytokines were replaced on day 3 and after 6 days, M1 and M2 differentiated cells were used for experimental purposes.

2.3 Isolation of RNA from primary human corneal endothelial cells (PHCend)

Primary human corneal endothelial cells were generated from redundant donated corneo-scleral tissue following corneal transplant surgery. Donor peripheral corneal rims and central corneal buttons (transplant waste) from penetrating and lamellar keratoplasty surgical procedures, where the donor had given consent for research were used. According to the United Kingdom Guidelines for Organ Donation, only those patients with normal eyes in the absence of absolute exclusion criteria including active transmissible disease or infection, Creuzfeldt-Jakob Disease, intravenous drug abuse, and neurodegenerative disorders, are suitable to donate organs and tissues for transplantation. Additional exclusions include previous ocular surgery, inflammation and tumours such as retinoblastoma. The study was undertaken after formal ethics approval from the Black Country Research Ethics Committee (incorporating the Dudley Research Ethics Committee (LREC 06/Q2702/44)), and all experiments were carried out in accordance with the Tenets of the Declaration of Helsinki. Under a dissecting microscope, Descemet's membrane and corneal endothelial cells were stripped from the posterior surface of the peripheral corneo-scleral region and placed into lysis buffer. Lysate was stored at -20º C until RNA extraction.

2.4 Cell stimulation

Cells were plated in 12-well plates and allowed to grow to confluence. Cells were then washed and placed in fresh serum free medium. Cells were either left untreated (control) or treated with the different TLR1-9 ligands (Axxora Ltd., Exeter,UK), or RIG-I and MDA5 (Invivogen, San Diego, US) or cytokines (Peprotech, London, UK) alone or together with $25(OH)D_3$ or $1,25(OH)_2D_3$ for the required time points (Table 2.1). Cell culture supernatants were collected in 1.5 ml Eppendorf tubes and stored at -20° C until analysis. Adherent cells were washed with PBS and RNA lysis buffer or Trizol were added and stored at -20° C for RNA extraction.

Table 2.1. TLR ligands and concentrations.

TLR Ligand	Receptor	Concentration/ml
Pam3cys	TLR2/1	1 µg
Poly I:C	TLR3	10 or 100 µg
LPS	TLR4	10 µg
Imiquimod	TLR7	1 µg
ODN 2216	TLR9	3 µg
Poly (I:C)/LyoVec	RIG-I	1 µg
Poly (I:C)/LyoVec	MDA5	1 µg

2.5 RNA extraction

RNA extraction was initially performed using RNeasy Mini Kit but Trizol reagent was used subsequently to obtain better RNA yields.

2.5.1 RNeasy Mini Kit

Total RNA was isolated using RNeasy Mini Kit (Qiagen, Crawley, UK) according to manufacturer's instructions. Briefly, RNA was lysed in a guanidine isothiocyanate containing lysis buffer and vortexed. One volume of 70% ethanol was added and mixed carefully by pipetting. The mixture was transferred to an RNeasy Mini spin column with silica-based membrane to ensure better RNA binding to the column. RNA was subjected to several washes to remove contaminants before it was resuspended in RNase free water and stored at -20º C.

2.5.2 Trizol extraction

Cells were lysed in 1 ml Trizol (phenol and guanidine isothiocyanate) reagent (Sigma-Aldrich, Dorset, UK) and stored at -20º C until RNA extraction. Lysates were transferred to 1.5 ml Eppendorf tubes. Chloroform (Sigma-Aldrich, Dorset, UK) was added (200 µl) and vortexed for 15 s. Tubes were allowed to stand for 10 min followed by centrifugation at 12,000g for 30 min at 4º C to separate RNA containing aqueous from organic phase. Aqueous phase was transferred to fresh tubes and 1 µl of Glycoblue (Invitrogen, Paisley, UK) was added to bind and precipitate RNA. Isopropanaol alcohol (Sigma-Aldrich, Dorset, UK) was added (200 µl) to the aqueous phase to facilitate RNA precipitation and stored at -20º C. Tubes were centrifuged at

12,000g for 30 min at 4ºC and supernatants were carefully removed by vacuum suction. RNA was further precipitated by the addition of 70% ethanol (500 µl), vortexted and centrifuged at 12,000g for 5 min at 4º C. Supernatants were removed and RNA was resuspended in 12-20 µl of RNase free water and stored at -20º C.

RNA obtained from both methods was quantified and checked for purity using Nanodrop spectrophotometer (Thermo Fisher Scientific, UK).

2.6 Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA (1 µg) was reverse transcribed to cDNA using Taqman Reverse Transcription Kit (Applied Biosystems, Warrington, UK) following manufacturer's instructions. PCR was performed using GoTaq Flexi DNA polymerase system (Promega, Southampton, UK) in a total volume of 20 µl containing cDNA (1 µl for 18S and 2 µl for all other genes), 0.8 mM $MgCl_2$, 0.25x GoTaq Green Flexi Buffer, GoTaq DNA polymerase (0.01 U/µl), 10 mM dNTP mix ((Promega, Southampton, UK), 1 pmol (0.05 µM) of 18S and 2 pmol (0.1 µM) of other forward and reverse primers (Alta Bioscience, Birmingham, UK). PCR was performed using Gene Amp PCR System 2700 (Applied Biosystems, Warrington, UK) as follows: 5 min at 94° C, followed by three temperature cycles of 1 min at 94° C, annealing for 60 s for vitamin D_3 genes (Table 2.2) and 40 s for TLRs1-10, RIG-I and MDA5 (Table 2.3), and extension at 72° C for 1 min. For cubilin and megalin, pre-amplification was carried out at 95° C for 5 min followed by cycles of denaturation at 94° C for 45 s, annealing for 45 s, and extension at 72° C for

45 s. All PCR reactions ended with a final extension step at 72° C for 7 min. Primer sequences and cycling condition were as shown in Table 2.2 and Table 2.3.

Table 2.2. Vitamin D_3 genes PCR primer sequence and cycle conditions.

Gene*	Forward primer 5'-3'	Reverse primer 5'-3'	Cycles	Annealing Temperature (C°)	Product (bp)
VDR	CGCTCCAATGAGTCCTTCACC	GCTTCATGCTGCACTCAGGC	33	61	421
CYP27B1	CACCTGACCCACTTCCTGTT	TCTGGGACACGAGAATTTCC	35	58	302
CYP24A1	CCCACTAGCCACCTCGTACCAAC	CGTAGCCCTTCTTTGCGGTAGTC	35	60	485
CYP2R1	AGAGACCCAGAAGTGTTCCAT	GTCTTTCAGCACAGATGAGGTA	40	62	259
CYP27A1	GGCAAGTACCCAGTACGG	AGCAAATAGCTTCCAAGG	40	62	292
18S	GTTGGTGGAGCGATTTGTCT	GGCCTCACTAAACCATCCAA	20	55	400
Cubilin	GCGGCTTCACTGCTTCCTA	GAGTGATGGTGTGCCCTTGT	35	53	518
Megalin	TAAGTCAGTGCCCAACCTTT	GCGGTTGTTCCTGGAG	35	53	290

*****VDR and CYP24A1 (Lechner et al., 2007), CYP27B1(Bland et al., 2004), CYP2R1, CYP27A1 (Blomberg et al., 2010), cubilin and megalin (Tsaroucha et al., 2008).

Table 2.3. TLR PCR primer sequence and cycle conditions.

Gene	Forward primer 5'-3'	Reverse primer 5'-3'	Cycles	Annealing Temperature (C°)	Product (bp)
TLR1	ACC AAG TTG TCA GCG ATG TGT T	GAT TGT CCC CTG CTT TTA TTG A	35	61	659
TLR2	GAG TGA GTG GTG CAA GTA TGA	GGG CCA CTC CAG GTA GGT CT	35	61	168
TLR3	TCC CAA GCC TTC AAC GAC TG	TCC TGA AAG CTG GCC CGA AAA	35	61	470
TLR4	TGC GGG TTC TAC ATC AAA	CCA TCC GAA ATT ATA AGA AAA	35	55	412
TLR5	CTC CTT TGA TGG CCG AAT AGC	CCC AAA TGA AGG ATG AAG GTA	35	61	429
TLR6	CAA GGC CCT GCC CAT CTG TAA	TTG GGC CAA AGA AAT TGA AAG	35	61	428
TLR7	CCC CAG CGT CCT TTC ACA GA	CGA GGG CAA TTT CCA CTT AGG	35	61	543
TLR8	ATG CGT GCC TTG TGA TGG TG	GCA ATG CCC GTA GAG ACA AAA	35	61	319
TLR9	CTA CAA CCG CAT CGT CAA AC	ATC GAG TGA GCG GAA GAA GA	35	61	456
TLR10	ACC CCA GCC ACA ACG ACA C	ATC ACG CAA AAG AAC CCA GAA	35	61	488
RIG-I	GAT AGC AAC AGT CAA ACA CAA	TCG GAC ATT GCT GAA GAA GT	35	55	411
MDA5	GCC ACG AAG CAA GCC AAA	GTT CTT TGC GAT TTC CTT CT	35	55	306
18S	GTTGGTGGAGCGATTTGTCT	GGCCTCACTAAACCATCCAA	20	55	400
GAPDH	CCA CCC ATG GCA ATT CCA TGG CA	TCT AGA CGG CAG GTC AGG TCC AC	35	61	350

2.7 Real-time PCR

Reactions were performed in 96-well plates using reagents and primers (Table 2.4) from Applied Biosystems (AB). Each reaction was carried out in a total volume of 20µl containing 10 µl Taqman Universal Master Mix (2x), 1 µl endogenous control 18S primer (VIC/TAMRA 20x), 1 µl specific primers 20x *TaqMan* gene expression assay (Applied Biosystems, Warrington, UK) and 7 µl RNase free water. Relative gene expression was measured and analysed using a LC-480 PCR system (Roche, West Sussex UK). Gene expression was obtained as Ct values (the cycle number at which the log PCR plots intersect with a calculated threshold value) and ΔCt was calculated (Ct of target-Ct of

control gene) and relative gene expression of treated samples compared to untreated controls was calculated as ΔΔCt values ($2^{\wedge\text{-(Ct treated sample-Ct untreated sample)}}$.

Table 2.4. Applied Biosystems gene expression assay ID used for real-time PCR.

Gene	Assay
VDR	Hs01045840_m1
CYP27B1	Hs00168017_m1
CYP24A1	Hs00167999_m1
CAMP	Hs01011707_g1
BD-1	Hs00608345_m1
BD-2	Hs00175474_m1
BD-3	Hs04195435_g1
BD-4	Hs00414476_m1

2.8 Cytokine and HDP enzyme linked immunosorbent assay (ELISA)

ELISA was used to measure cytokines in cell culture supernatants. IL-8 was measured using Human CXCL8/*IL-8* DuoSet ELISA Development Kit (R&D Systems, Abingdon, UK), IL-6 using Human IL-6 ELISA Set OptEIA (BD, Oxford, UK), and HDPs with Standard ELISA Development Kits for hBD-1, 2, and 4 respectively (Peprotech, London, UK). Briefly, 96-well plates were coated with antihuman capture antibody overnight at room temperature (RT) for IL-8 and hBDs and at 4º C for IL-6. Plates were washed several times and blocked with blocking buffer at RT for 1 h. After washing, biotinylated antihuman cytokine was added for IL-8 and hBDs for 2h at RT. This was

followed by several washes and streptavidin conjugated to horseradish peroxidase enzyme (HRP) was added and incubated for 20 min at RT. For IL-6 the detection system (biotinylated antihuman cytokine mixed streptavidin-HRP conjugate) was added for 1 h at RT. At the end of the incubation, the plates were washed and tetramethylbenzidine (TMB) substrate was added for 20 min in the dark. The reaction was stopped with 2N H_2SO_4 and absorbance was measured by spectrophotometry at 450 nm for IL-6 and IL-8 and 405 nm for hBDs. Standard curves were constructed and sample concentrations were extrapolated from a standard curve.

2.9 $1,25(OH)_2D_3$ enzyme immunoassay (EIA)

Cells were grown to confluency in 12-well culture plates. Cells were placed in serum free medium and were either left untreated or treated with a physiological concentration of $25(OH)D_3$ (10^{-7} M) with or without pre-treatment with itraconazole or ketoconazole (10^{-6} M), chemical inhibitors of 1α-hydroxylase (CYP27B1) (both Sigma-Aldrich, Dorset, UK) for 2 h. After 24 h cell culture supernatants were collected for the measurement of $1,25(OH)_2D_3$ and cells were processed for protein extraction and quantification.

$1,25(OH)_2D_3$ concentration was measured by 1,25-Dihydroxy Vitamin D EIA (Immunodiagnostics Systems Limited, Tyne & Wear, UK) according to the manufacturer's instructions. Briefly, supernatants were delipidated and centrifuged at 2000g for 15 min. Immunoextraction of $1,25(OH)_2D_3$ was performed by transferring the delipidated samples to immunocapsules containing gel coated with specific antibody for $1,25(OH)_2D_3$. Samples were placed in a foam rack on a blood tube rotator (5-20

revolutions) at RT for 90 min. After several washes (1000 g for 1 min), $1,25(OH)_2D_3$ was eluted with an elution reagent containing ethanol into glass tubes. Tubes were placed in a heating block at 40º C for 20-30 min to evaporate the elution reagent. Immunopurified $1,25(OH)_2D_3$ residues were dissolved in assay buffer and used for ELISA. Antisheep $1,25(OH)_2D_3$ antibody was added to calibrators and samples and incubated at 4-8º C overnight. Part of the mixture was transferred to a 96-well plate coated with antisheep $1,25(OH)_2D_3$ antibody and incubated at RT for 90 min with shaking. Biotinylated $1,25(OH)_2D_3$ was added to the plate and incubated with shaking for further 60 min. After several washes, HRP-labelled avidin conjugate was added and incubated at RT for 30 min. Unbound conjugate was washed several times and TMB substrate was added and incubated for 30 min. Reaction was stopped with 0.5M HCl and absorbance with measured by spectrophotometry at 450 nm. Concentrations from $1,25(OH)_2D_3$ were obtained from a standard curve of the calibrators.

2.9.1 AqH Samples

AqH (50-150 µl) and serum samples (500 µl) were collected from 7 healthy subjects undergoing cataract surgery at the Birmingham & Midland Eye Centre. All human samples were taken after informed consent approved by the Birmingham East, North and Solihull (BENS) Research Ethics Committee (LREC 08/H1206/165) and from the Black Country Research Ethics Committee (incorporating the Dudley Research Ethics Committee (LREC 06/Q2702/63). Concentrations of $1,25(OH)_2D_3$ in AqH were measure by $1,25(OH)_2D_3$ EIA as described below.

2.10 VDR SNPs

VDR SNPs were selected according to HapMap (www.hapmap.org) and their relevance to the immune system according to epidemiological studies (Arjumand et al., 2012;Orlow et al., 2012;Sanchez-de la Torre et al., 2008).

SNPs FokI, BsmI, and A1012G were selected (Table 2.5). Three VDR SNP genotyping assays: *FokI* (rs2228570) (Poon et al., 2004;Sanchez-de la Torre et al., 2008), *BsmI* (rs1544410) (Al-Daghri et al., 2012), and *A1012G* (rs4516035) (Sanchez-de la Torre et al., 2008) were purchased from Applied Biosystems, Warrington, UK and were tested in 165 patient and 100 control Caucasian samples (Table 2.6). Study population was disease free and was not matched on other criteria. PCR was performed in 384-well plates with a 10 µl total reaction volume containing 20 pg/ml DNA (samples) or water (controls) and 2x LC-480 probe master (Roche, West Sussex UK) followed by endpoint genotyping analysis using the LC-480 system (Roche, West Sussex UK). Genotypes were determined as either homozygous (e.g. AA or GG) or heterozygous (e.g. AG) according to the presence or absence of fluorescence for each genotype.

Table 2.5. Selected VDR SNPs location and functional relevance.

SNP	Location	Function	References
FokI	Start codon	Affects vitamin D protein size Modulates mRNA expression of NFAT, IL-12, and regulates pancreatic function	(Mory et al., 2009;van Etten et al., 2007)
BsmI	3'UTR	mRNA expression and stability	(Arai et al., 2001;Rukin and Strange, 2007;Uitterlinden et al., 2002)
A1012G	Promoter	associated with the transcription of GATA3, transcription factor for Th1	(Halsall et al., 2004)

Table 2.6. VDR SNP location, type, and Applied Biosystem assay ID#.

SNP	**dbSNP**	**Location**	**Polymorphism**	**Assay ID**
FokI*	rs2228570	Coding region	A/G Transition	C__12060045_20
BsmI	rs1544410	3' UTRs	C/T Transition	C___8716062_10
A1012G	rs4516035	promoter 5' UTRs	C/T Transition	C___2880805_10

**FokI* (r2228570) is a C/T transition on the forward strand but the assay has been designed on the reverse strand.

2.11 Immunofluorescence

Ocular barrier cells were cultured on glass chamber slides (BD Bioseciences, Oxford, UK), fixed for 20 min with cold methanol at 4º C and rinsed with PBS. Cells were blocked with 5% BSA in PBS for 1 hour, then treated with mouse-antihuman VDR clone H4537 (R&D Systems, Abingdon, UK), sheep-antimouse CYP27B1 PC290 (The Binding Site Ltd., Birmingham, UK) mouse-antihuman CYP24A1 clone 1E1 (Novus Biologicals, Cambridge, UK), mouse-antihuman mannose-6-phosphate receptors (M6P) (Abcam, Cambridge, UK, clone 2G11), or mouse-antihuman trans-Golgi network 38 (TGN38) clone 2F7.1 (Novus Biologicals, Cambridge, UK) respectively (1:100 in PBS blocking solution) for 1 h at room temperature (RT). Cells were washed several times with PBS then incubated with FITC conjugated antimouse or FITC conjugated antisheep secondary antibody (The Binding Site Ltd., Birmingham, UK), or Texas Red (TR) conjugated antimouse antibody (Abcam, Cambridge, UK) diluted 1:250 in blocking solution for 1 hour at RT. Cells were briefly counter stained with 4',6-diamidino-2-phenylindole (DAPI) (Invitrogen, Paisley, UK) and mounted (Vectashield, Vector Laboratories, Peterborough, UK). Staining was detected using a fluorescent microscope (AxioPlan2 Imaging, Ziess, Cambridge, UK).

2.12 Statistical analysis

Statistical analyses were performed using commercial statistical package software Graphpad Prism 5. Details of statistical calculations are explained in figure legends.

3 CHARACTERISATION OF VITAMIN D_3 PRODUCTION BY HUMAN OCULAR BARRIER CELLS

3.1 Introduction

Extra-renal synthesis of vitamin D_3 has been reported in many tissues and cells including barrier sites. VDR and CYP27B1 have been described in epithelial cells of skin, lung, intestine, prostate, endometrium, and breast as well as cells of the immune system such as macrophages and dendritic cells (Agic et al., 2007;Brozyna et al., 2011;Flanagan et al., 2006;Hansdottir et al., 2008;Hewison et al., 2003;Larriba et al., 2011). Extra-renal vitamin D_3 metabolism extends to the liver 25-hydroxylases involved in the first hydroxylation step in the activation of vitamin D_3. CYP2R1 is suggested to be the major 25-hydroxylase in humans. It has higher affinity than CYP27A1 towards vitamin D_3 and mutations in the *CYP2R1* gene cause disturbance of vitamin D_3 metabolism (Zhu and Deluca, 2012). In the circulation, 25(OH)D_3 is bound to vitamin D_3 binding protein (DBP) which facilitates its cellular uptake by multi-ligand endocytic receptors megalin/cubilin expressed on the apical surface of polarised epithelial cells in many tissues (Christensen and Birn, 2002).

Local conversion of 25(OH)D_3 into 1,25$(OH)_2D_3$ has been shown in respiratory, urinary bladder and colonic epithelial cells, osteoclasts, and macrophages (Gottfried et al., 2006;Hansdottir et al., 2008;Hertting et al., 2010;Kogawa et al., 2010;Lagishetty et al., 2010). Of note, VDR is critical for barrier formation in human skin and the integrity of the mucosal barrier in mouse intestine. In a mouse model of DSS induced colitis, VDR knockout (KO) mice ($VDR^{-/-}$) compared to $VDR^{+/+}$, suffered severe diarrhea and

intestinal bleeding. Their intestines showed reduced TEER that progressed into ulceration and confocal microscopy revealed disrupted TJs. The addition of $1,25(OH)_2D_3$ *in vitro* to human colonic cancer epithelial cell lines SW480 and Caco-2 increased the expression of TJ proteins ZO-1, claudin-1, claudin-2, and E-cadherin. TJ occludin was not induced by $1,25(OH)_2D_3$ but it was completely absent from $VDR^{-/-}$ intestinal mucosa after treatment with DSS. In the presence of $1,25(OH)_2D_3$, a monolayer of Caco-2 cells was protected from damage by the addition of 5% DSS (Kong et al., 2008). Interestingly, silencing of VDR and steroid receptor coactivators (SRC) in primary normal human keratinocytes resulted in a compromised keratinocytes differentiation and hence barrier function. $VDR^{-/-}$ mice also showed a compromised barrier formation as shown by decreased epidermal lamellar body density and reduced lipid secretion (Oda et al., 2009).

Recently, vitamin D_3 has been recognised as an immunomodulatory hormone that regulates both innate and acquired immune responses (Hewison, 2012). One regulatory role of $1,25(OH)_2D_3$ in the immune system (*in vitro)* involves the inhibition of proinflammatory cytokine production from $CD4^+$ T cells and the induction of a regulatory T cell phenotype and a suppressive function (Jeffery et al., 2009). Endogenous conversion of $25(OH)D_3$ has been shown to inhibit DC antigen presentation and chemotaxis (Bartels et al., 2010). The ability to induce the production of HDPs in blood monocytes also supports an interaction between $1,25(OH)_2D_3$ and the immune system. The HDPs LL-37 and hBD1-4 are antimicrobial and immunomodulatory molecules. They

can act as chemotactic agents for T and dendritic cells as well as their ability to induce Th 1 and 2 cytokines (Oppenheim et al., 2003).

As stated in Chapter 1, ocular immune privilege is maintained by a set of anatomical and physiological processes that protect the eye from sight threatening infections and inflammatory responses (Streilein, 2003). The conjunctiva, composed of layers of epithelial cells and a sophisticated stroma forming a mucosal surface that contains a heterogeneous population of lymphoid cells playing an important role in the defense against infections (Fukuda et al., 2006). The sclera is the fibrous support to the global structure of the eye composed of a rich extracellular matrix (ECM) of collagen and elastic fibers together with fibroblasts that are responsible for tissue remodeling and the regeneration of the ECM (Rada et al., 2006). On the posterior surface of the cornea, endothelial cells contribute to barrier function by mediating sodium transport that maintains the cornea in a relatively dehydrated state thereby preserving corneal transparency (Mergler and Pleyer, 2007).

Physical barriers that prevent the entry of immune cells from blood to the eye include BAB and BRB. The BAB is made of TJs between vascular endothelial cells of iris and ciliary body vessels and the non-pigmented ciliary body epithelial cells, while the BRB is composed of TJs between endothelial cells in retinal blood vessels and those between the retinal pigment epithelial cells (Hornof et al., 2005). AqH is produced by the ciliary processes and is secreted into the posterior chamber. This active process is mainly a function of the blood-aqueous barrier which prevents the passage of proteins to

AqH to maintain its transparency (Kiel et al., 2011). AqH flows in a unidirectional movement from the posterior chamber through the pupil to the anterior chamber. It is drained through trabecular meshwork until it ends in episcleral veins (Goel et al., 2010).

To date very few studies have examined the presence of vitamin D_3 synthesising and metabolising pathways and whether human ocular barrier epithelial cells are capable of producing vitamin D_3. This study shows the expression and functionality of the vitamin D_3 system in human ocular barrier epithelial cells. Vitamin D_3 may be important to the ocular barrier function and ocular immune privilege.

3.2 Results

3.2.1 Ocular barrier epithelial cells constitutively express mRNA for vitamin D_3 metabolism

Extra-renal expression of vitamin D_3 synthesising and metabolising components has been described in many organs. To examine the presence of these elements in ocular barrier cells, mRNA expression was examined by RT-PCR. The VDR was strongly expressed in all ocular cells tested except in primary human corneal endothelium (PHCend). This could be due to the little amount of tissue and low RNA obtained from corneal rims (Figure 3.1 A). CYP27B1 was expressed markedly by CCL20.2, HCEC-12, ODM-2, and ARPE-19 cells and weakly by PHCend. The weak expression by PHCend could be due to the scarcity of sample size and low RNA concentration obtained. In contrast, CYP24A1 mRNA was strongly expressed only by CCL20.2 cells and weakly by all other cell types. CYP27A1 was weakly expressed in HCEC-12, ARPE-19 and ODM-2, and undetected in CCL20.2. Furthermore, CCL20.2,

HCEC-12, and ARPE-19 highly expressed CYP2R1 whereas ODM-2 showed weak expression. We also investigated the mRNA expression of cubilin and megalin, which are required for the internalisation of 25(OH)D_3 through DBP. Cubilin was strongly expressed in all cells except CCL20.2 which had a weak expression, while only CCL20.2 weakly expressed megalin (Figure 3.1 B). To summarise, ocular barrier epithelial cells constitutively express mRNA for vitamin D_3 metabolism.

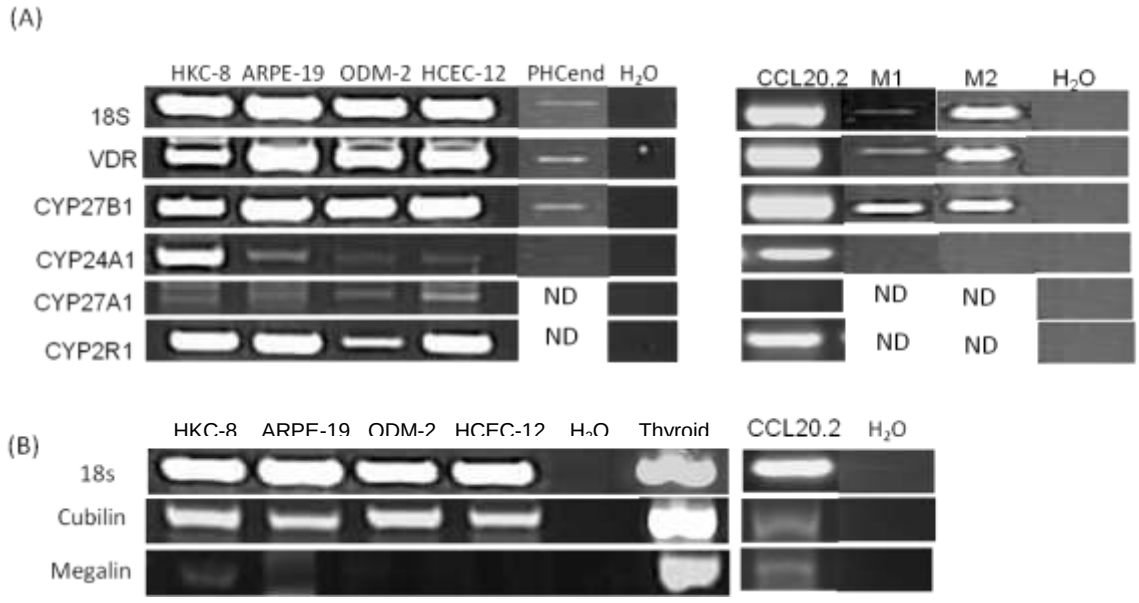

Figure 3.1. Ocular barrier epithelial cells express mRNA for vitamin D_3 elements. Total RNA was isolated from 80-90% confluent ARPE-19 (adult retinal pigment epithelial), ODM-2 (non-pigmented ciliary body epithelial), HCEC-12 (human corneal endothelial) cell lines, PHCend (freshly isolated RNA from primary human corneal endothelium), and CCL20.2 (conjunctival cell line). Conventional RT-PCR was performed for mRNA expression of VDR, CYP27B1, CYP24A1, CYP27A1, CYP2R1 (A), and for cubilin, and megalin (B) compared to 18S as an internal control. HKC-8 (human kidney cell line), M1 (classically activated macrophages), and M2 (alternatively activated [noninflammatory] macrophages) cells were used as positive controls for vitamin D_3 pathway molecules, RNA from primary thyroid cells was used as a positive control for cubilin and megalin, and H_2O was used as a negative control. ND: not done.

3.2.2 Ocular barrier epithelial cells express proteins for vitamin D_3 metabolism

Immunofluorescent staining of all cell types for the different vitamin D_3 elements was performed. The intensity of staining varied between cell types but all cells were positive for all vitamin D_3 proteins. VDR showed a diffuse cytoplasmic and abundant speckled nuclear staining in all cells types. The expression was the strongest in HCEC-12 and ODM-2 and weaker in CCL20.2 and ARPE-19 (Figures 3.2-3.5). CYP24A1 was also present in the cytoplasm where HCEC-12 and ODM-2 showed a strong signal (Figures 3.3 and 3.4), and there was a moderate signal in CCL20.2 (Figure 3.2). CYP27B1 showed an intense cytoplasmic pattern with denser staining in the peri-nuclear area of ODM-2 and ARPE-19 (Figures 3.4 and 3.5), while CCL20.2 (Figure 3.2) showed moderate staining. Interestingly, in HCEC-12 cells, diffuse cytoplasmic staining was present but most of the enzyme appeared within a peri-nuclear vesicle (Figure 3.3).

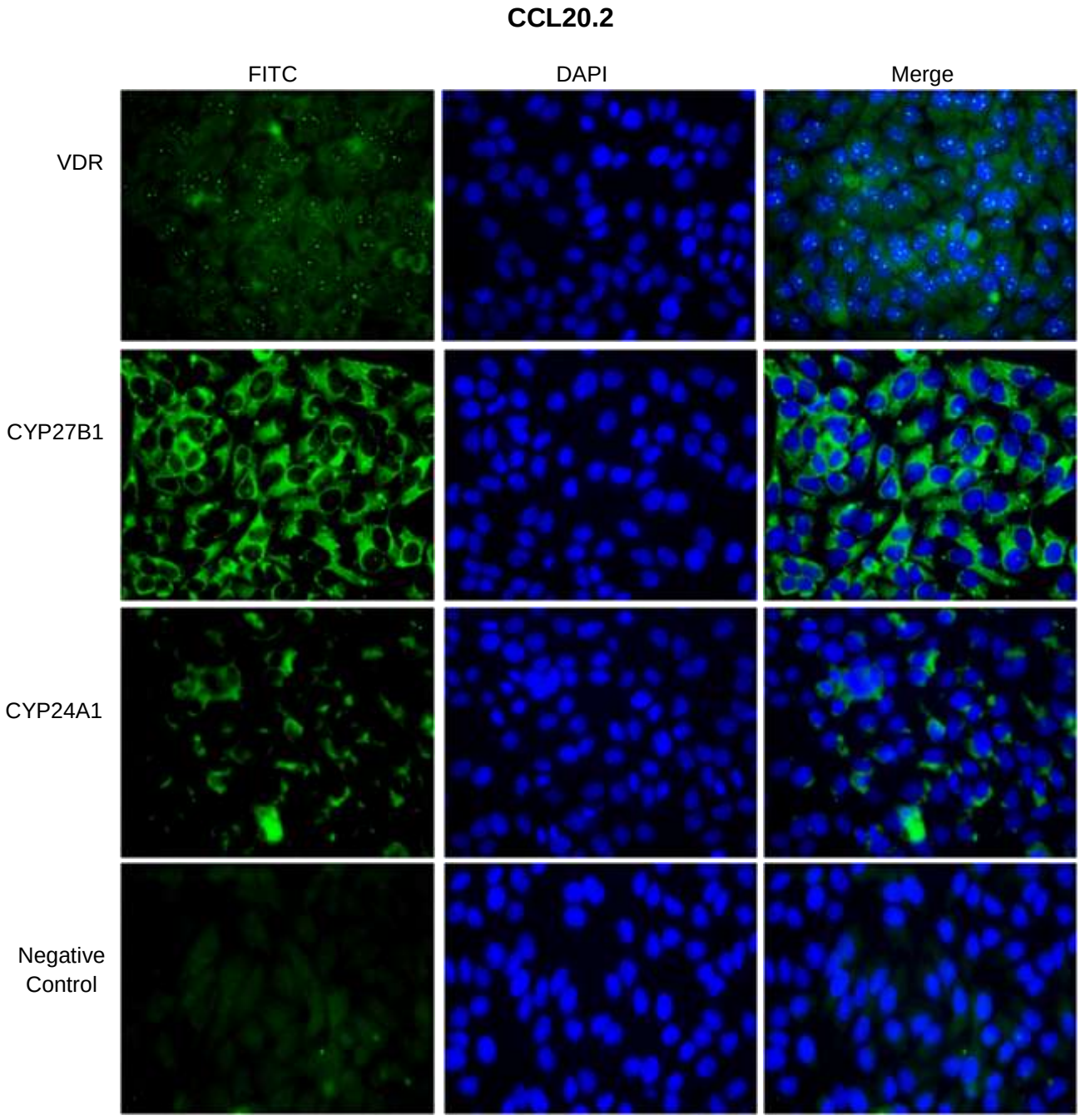

Figure 3.2.CCL20.2 cells express proteins for vitamin D_3 pathway elements. Cells were grown to 40-50% confluency in 8-well chamber slides. Cells were fixed and processed for immunofluorescence staining. Cells were treated with antibodies against VDR, CYP27B1and CYP24A1 and visualised with FITC-conjugated secondary antibodies (green) compared to negative control (primary antibody omitted). DAPI was used as a counter nuclear stain (blue). VDR showed nuclear/cytoplasmic while CYP27B1 and CYP24A1 showed cytoplasmic staining. Pictures are shown as 20x magnification.

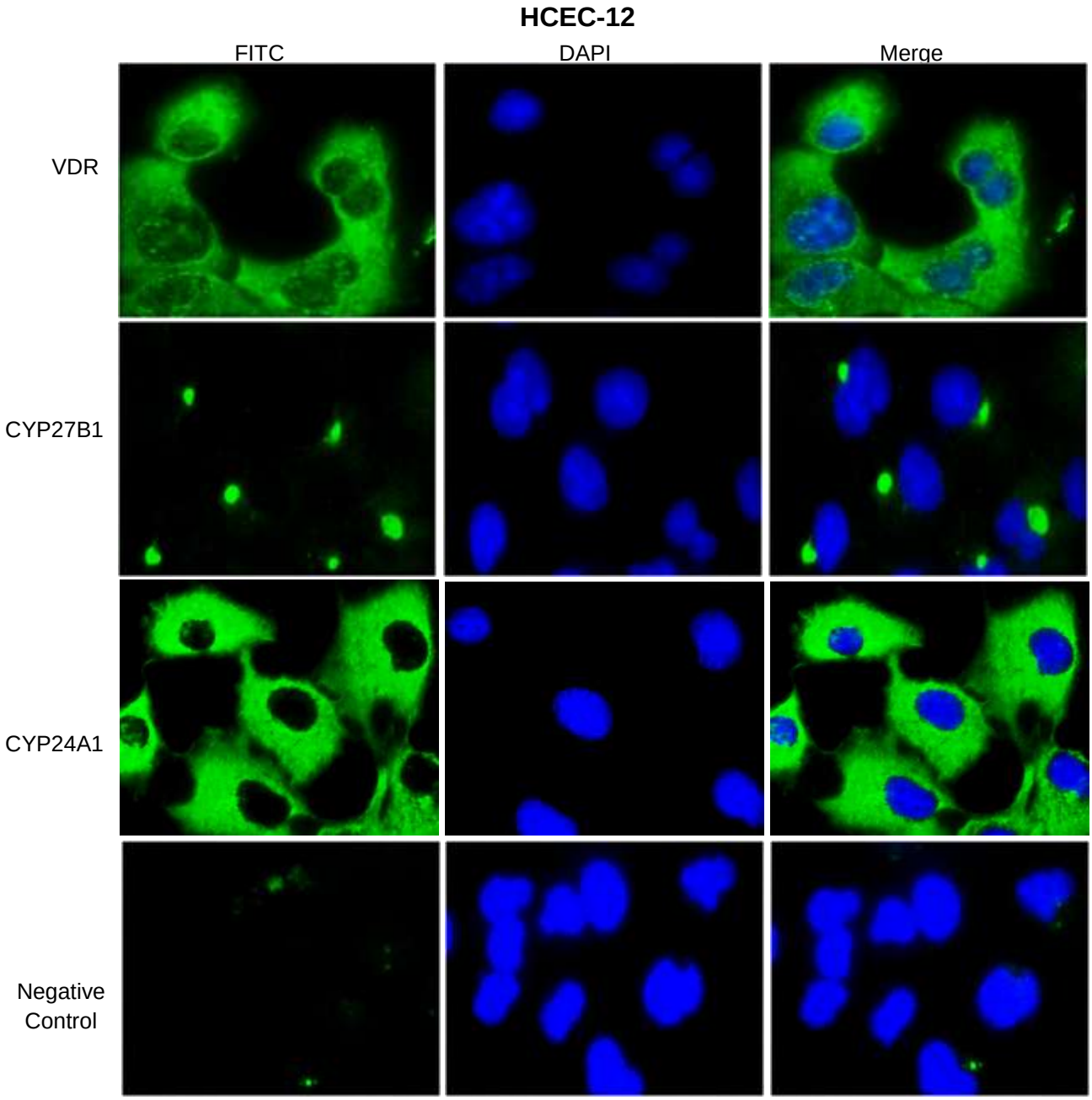

Figure 3.3. HCEC-12 cells express proteins for vitamin D_3 pathway elements. Cells were grown to 40-50% confluency in 8-well chamber slides. Cells were fixed and processed for immunofluorescence staining. Cells were treated with antibodies against VDR, CYP27B1and CYP24A1 and visualised with FITC-conjugated secondary antibodies (green) compared to negative control (primary antibody omitted). DAPI was used as a counter nuclear stain (blue). VDR showed nuclear/cytoplasmic while and CYP24A1 showed cytoplasmic staining. CYP27B1 appeared as a vesicle near the nucleus. Pictures are shown as 40x magnification.

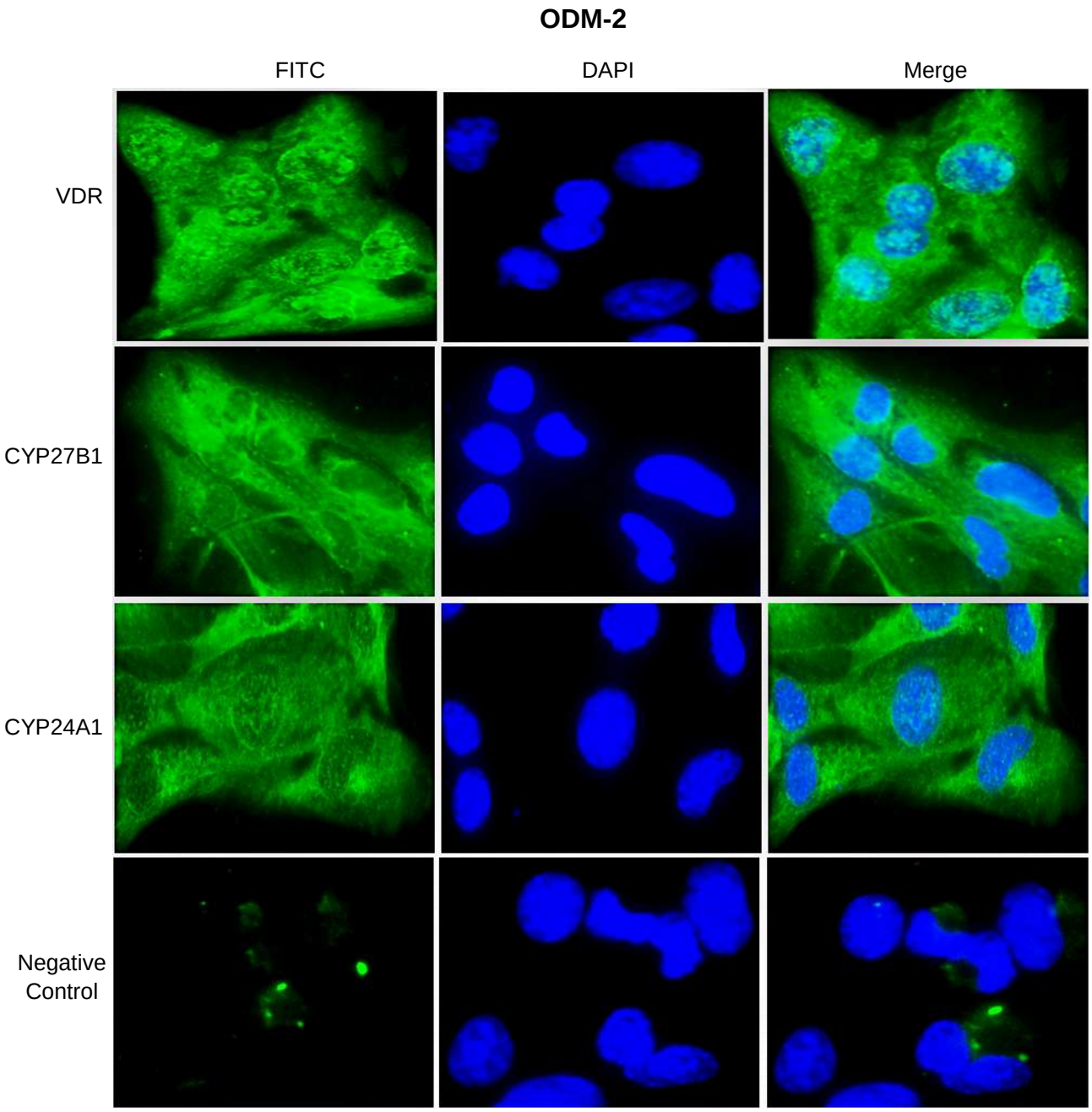

Figure 3.4. ODM-2 cells express proteins for vitamin D_3 pathway elements. Cells were grown to 40-50% confluency in 8-well chamber slides. Cells were fixed and processed for immunofluorescence staining. Cells were treated with antibodies against VDR, CYP27B1and CYP24A1 and visualised with FITC-conjugated secondary antibodies (green) compared to negative control (primary antibody omitted). DAPI was used as a counter nuclear stain (blue). VDR showed nuclear/cytoplasmic while CYP27B1 and CYP24A1 showed cytoplasmic staining. Pictures are shown as 40x magnification.

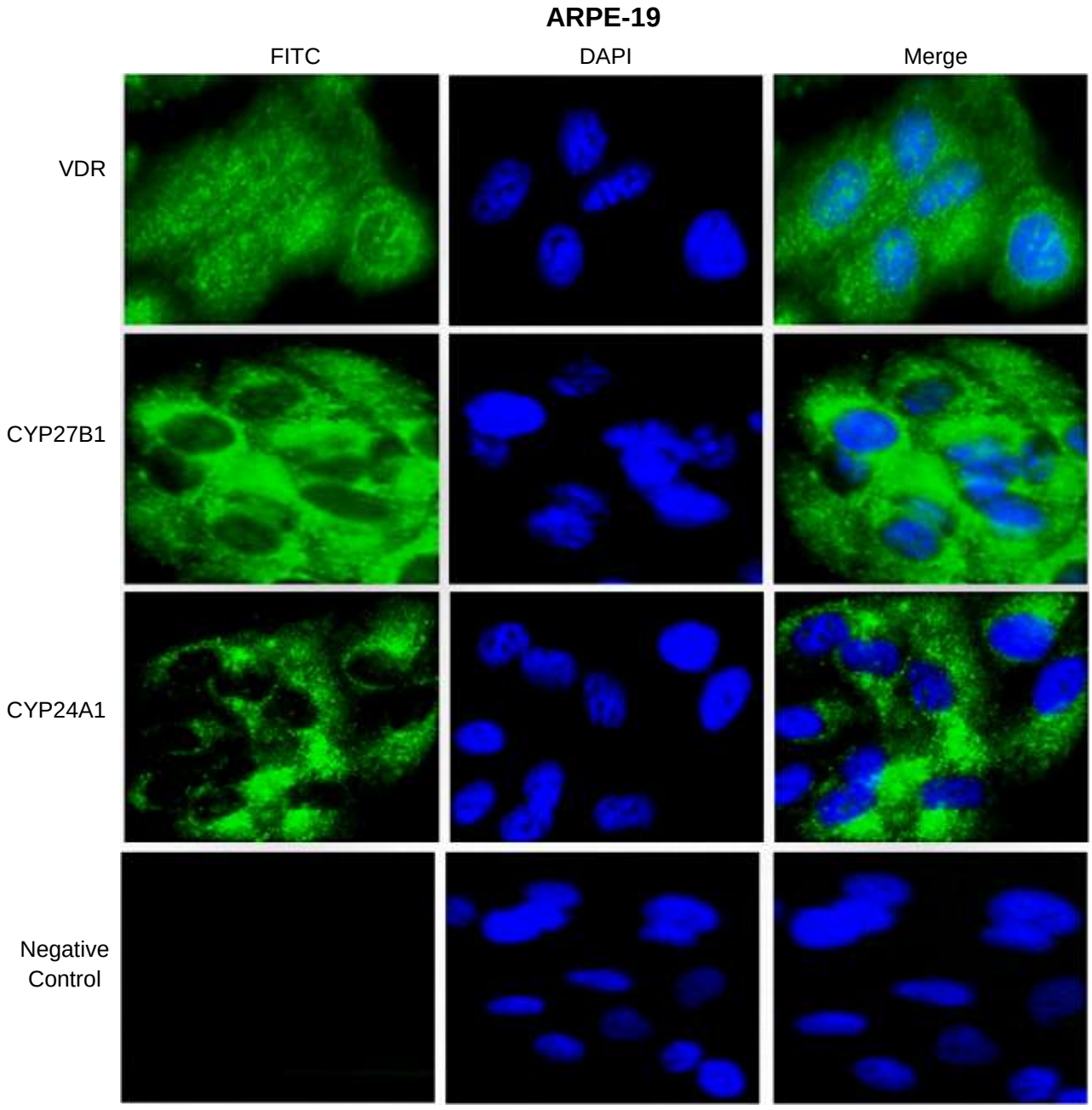

Figure 3.5. ARPE-19 cells express proteins for vitamin D_3 pathway elements. Cells were grown to 40-50% confluency in 8-well chamber slides. Cells were fixed and processed for immunofluorescence staining. Cells were treated with antibodies against VDR, CYP27B1and CYP24A1 and visualised with FITC-conjugated secondary antibodies (green) compared to negative control (primary antibody omitted). DAPI was used as a counter nuclear stain (blue). VDR showed nuclear/cytoplasmic while CYP27B1 and CYP24A1 showed cytoplasmic staining. Pictures are shown as 40x magnification.

3.2.3 CYP27B1 co-localises with mannose phosphate receptors (MPRs) and trans-Golgi network (TGN) in HCEC-12 (human corneal endothelial cells).

CYP27B1 is normally located in the inner membrane of the mitochondria (Adams and Hewison, 2012;Seliskar and Rozman, 2007). All the ocular epithelial cells tested demonstrated a staining pattern consistent with this expression i.e. cytoplasmic. The exception was HCEC-12 cells, which not only expressed CYP27B1 in the cytoplasm, but the majority of the enzyme appeared as a vesicle adjacent to the nucleus. To further identify the nature of this vesicle-like structure, HCEC-12 were stained for Mannose 6 phosphate (M6P) receptors that are known to play a role in the transport of enzymes from the trans-Golgi network (TGN) to lysosomes (Ghosh et al., 2003) and typically shows peri-nuclear localisation. In addition, TGN38 protein, part of the TGN that directs proteins to secretory vesicles, lysosomes, or plasma membrane, was also examined (Gu et al., 2001). CYP27B1 co-localised to both M6P receptors and TGN38 in HCEC-12 (Figure 3.6 A, B, and C). HECE-12 cells stained for CYP27B1 with FITC were also viewed by confocal microscopy which confirmed the peri-nuclear localisation of this enzyme (Figure 3.7). The cytoplasmic staining, together with the peri-nuclear punctuated presence confirms CYP27B1 presence in the TGN in HCEC-12 cells.

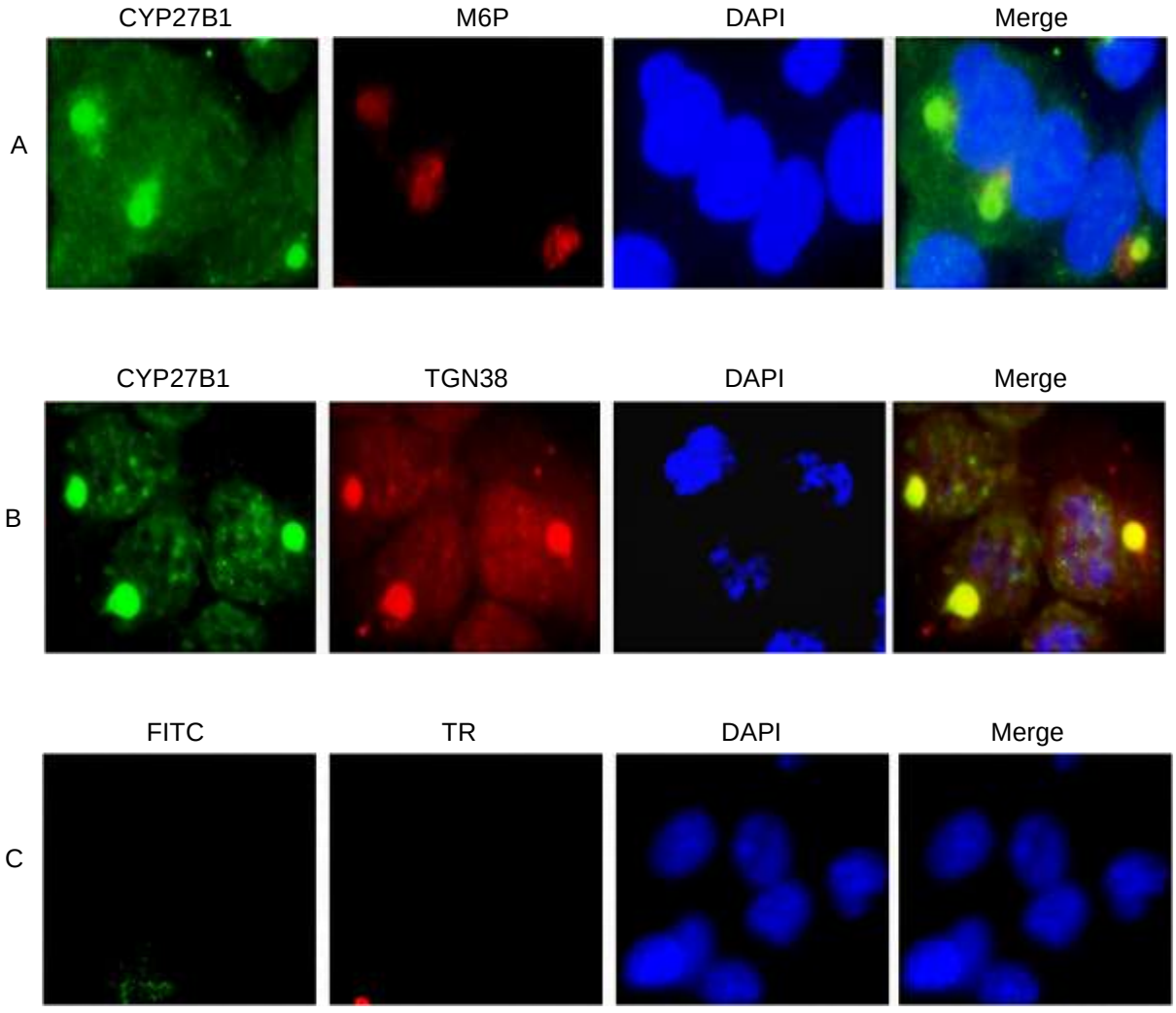

Figure 3.6. CYP27B1 co-localises with Mannose-6-Phosphate (M6P) receptor and trans-Golgi network protein 38 (TGN38) in HCEC-12. HCEC-12 cells were grown to 40-50% confluence in 8-well chamber slides. Cells were fixed with methanol at 4º C for 20 min and processed for immunofluorescence staining. Cells were treated with antibodies against VDR, CYP27B1, and (A) M6P or (B) TGN38 and visualised with FITC (green) or Texas red (TR) (red) conjugated secondary antibodies compared to (C) negative control (primary antibody omitted). DAPI was used as a counter nuclear stain (blue). CYP27B1 co-localised with both M6P and TGN38 in HCEC-12. Pictures are shown as 100X (A and B) and 40X (C) magnification respectively.

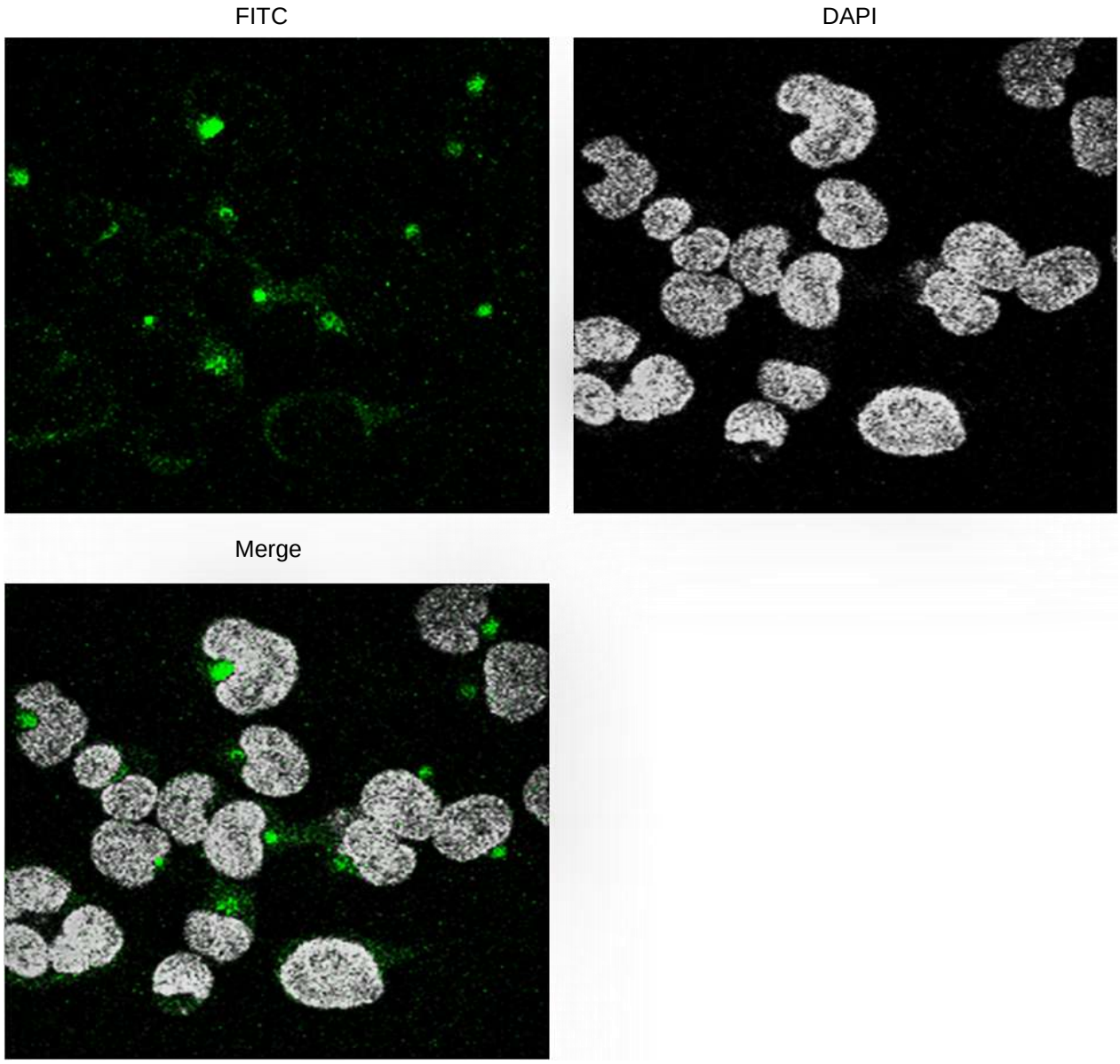

Figure 3.7. CYP27B1 shows a peri-nuclear localisation in HCEC-12. HECE-12 cells were grown to 40-50% confluency in 8-well chamber slides. Cells were fixed and processed for immunofluorescence staining. Cells were treated with antibodies against CYP27B1 and visualised with FITC-conjugated secondary antibodies (green) compared to negative control (primary antibody omitted). DAPI was used as a counter nuclear stain (blue). CYP27B1 co-localised in the peri-nuclear space in HCEC-12. Images were obtained from confocal microscopy and are shown as 40x magnifications.

3.2.4 Ocular barrier epithelial cells can convert inactive vitamin D_3 to the active form $1,25(OH)_2D_3$

Ocular barrier cells have the molecular components necessary to produce $1,25(OH)_2D_3$ locally. We next examined the functional capacity of CYP27B1 and its ability to convert inactive $25(OH)D_3$ (10^{-7} M) into active $1,25(OH)_2D_3$. The rate of conversion was variable amongst the different cells and can be broadly summarised with the highest rates in HCEC-12 and the lowest in ARPE-19 cells (Figure 3.9).

To determine the best time point for $1,25(OH)_2D_3$ measurement in culture supernatants, we started with time course conversion experiments using HKC-8 control cells and ARPE-19. Although ARPE-19 cells did not show significant conversion, the highest levels were at 24 h (Figure 3.8). Likewise, HKC-8 cells showed the highest $1,25(OH)_2D_3$ levels at 24 h, so a 24 h time point was chosen for all the succeeding conversion experiments. Substrate conversion was inhibited by pretreatment with itraconazole (10^{-5} M). Itraconazole did not give consistent inhibition so we changed it to ketokonazole (10^{-6} M). Conversion was inhibited by ketoconazole and the inhibition was statistically significant in cells that produced more than 200 pmol/L/mg protein of $1,25(OH)_2D_3$ (CCL20.2, HCEC-12, and ODM-2).

CCL20.2 (conjunctiva) produced significant amounts of $1,25(OH)_2D_3$ compared to untreated control cells (421.4 pmol/L/mg protein) ($p<0.01$) and double that made by HKC-8 positive control cells (195.4 pmol/L/mg protein) ($p<0.05$) (Figure 3.9). While ARPE-19 had minimal levels of conversion (25.0 pmol/L/mg protein), HCEC-12 were the

most efficient of all cell types at converting the substrate into the active form of $1,25(OH)_2D_3$ (2068.1 pmol/L/mg protein, $p< 0.01$) i.e. 10 times HKC-8 whereas ODM-2 cells produced lower levels (640.1 pmol/L/mg protein).

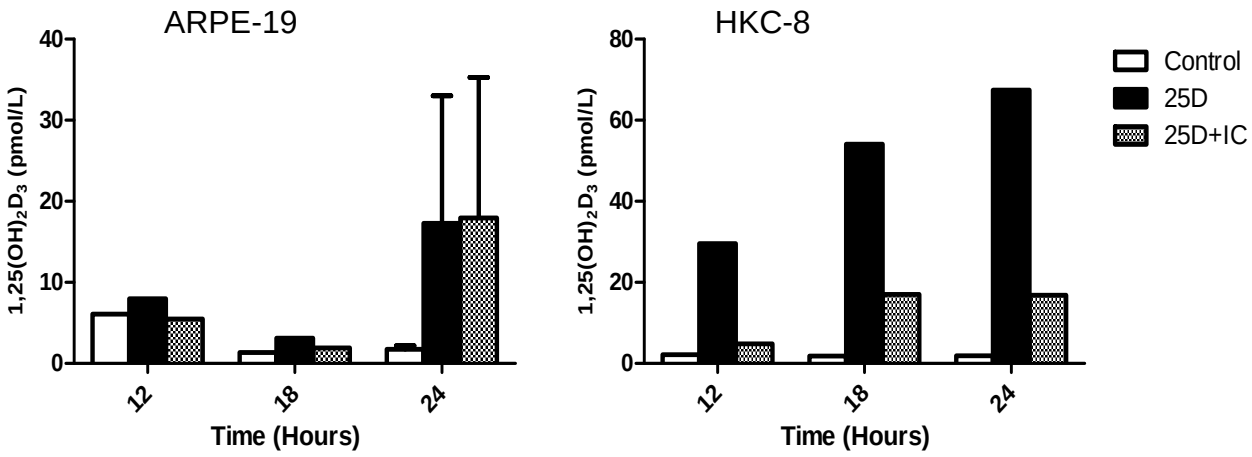

Figure 3.8. ARPE-19 and HKC-8 show the highest 25(OH)D_3 conversion rates at 24 h. ARPE-19 and HKC-8 (control) cells were grown to 80-90% confluence. Cells were either untreated (control) or treated with 25(OH)D_3 (10^{-7} M) for 12, 18, and 24 h with or without pre-treatment with CYP450 inhibitor (10^{-5} M) itraconazole (IC) for 2 h. Cell culture supernatants were collected and stored at -20º C until measurement. $1,25(OH)_2D_3$ was determined by enzyme immunoassay and the results were obtained from a standard curve as pmol/L. HKC-8 showed the highest conversion rate at 24 h. ARPE-19 did not show high conversion rates at all time points but the levels at 24 h were relatively higher and following from HCK-8, 24 h time point was selected for conversion experiments. HKC-8 and ARPE-19 n=1 except ARPE-19 24 h n=3. Graphs show mean $1,25(OH)_2D_3$ concentration pmol/L for each treatment (except ARPE-19 at 24 h shows mean±SD).

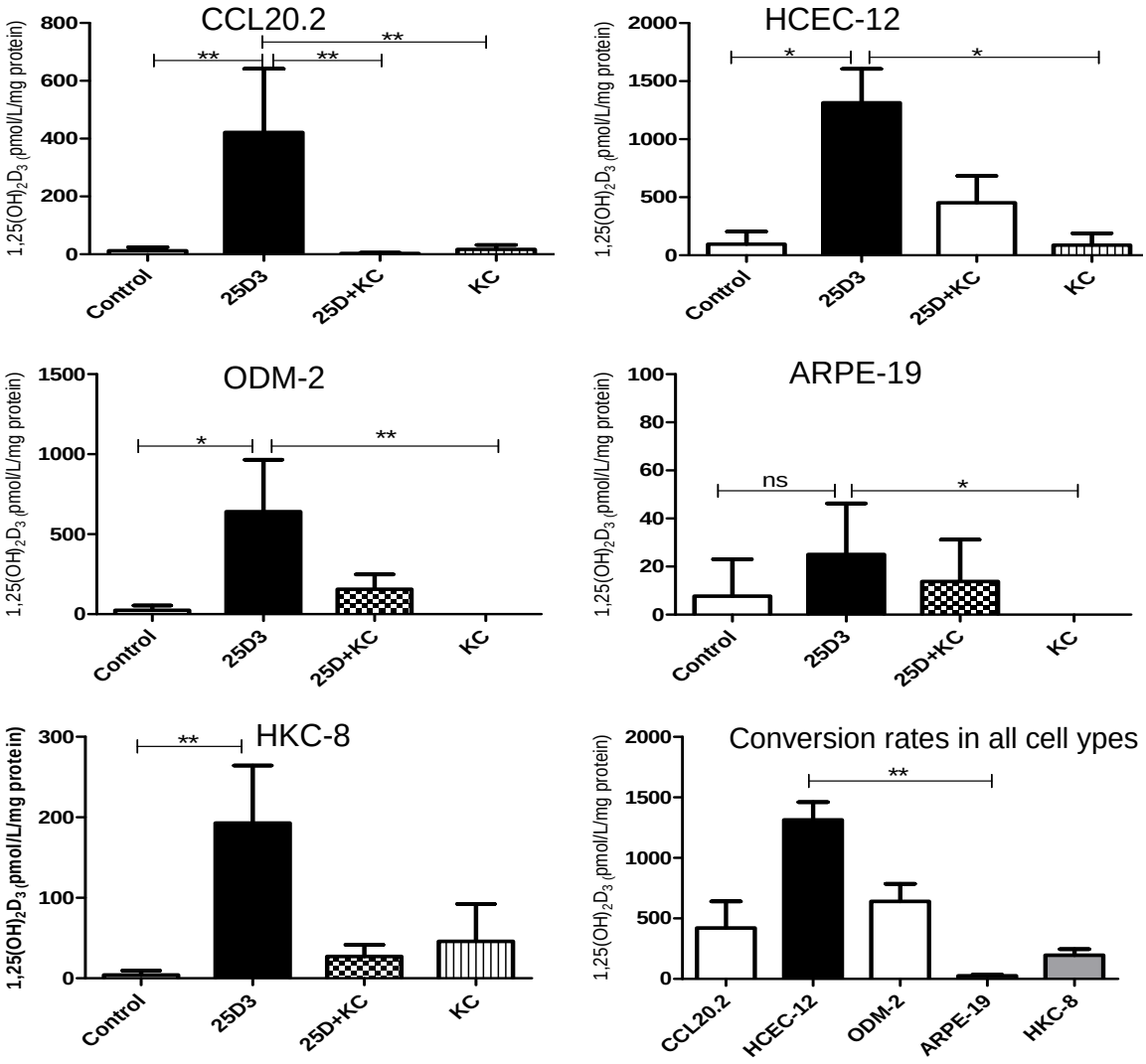

Figure 3.9. Ocular barrier epithelial cells can convert 25(OH)D_3 into active 1,25(OH)$_2D_3$. Cells were grown to 80-90% confluence and were either untreated (control) or treated with 25(OH)D_3 (10^{-7} M) for 24 h in serum free conditions with or without pretreatment with CYP450 inhibitor (10^{-6} M) ketoconazole (KC) for 2 h. Cell culture supernatants were collected and stored at -20º C until measurement. 1,25(OH)$_2D_3$ was determined by enzyme immunoassay and the results were obtained from a standard curve as pmol/L then corrected per mg of protein. HKC-8 cells were used as a positive conversion control. HCEC-12 cells showed the highest conversion rates followed by ODM-2 then CCL20.2, whereas the lowest rates were found in ARPE-19 cells. The bottom right graph compares the conversion rates between cell types. Each experiment was performed at least 3 times. Graphs show mean±SD 1,25(OH)$_2D_3$ per mg protein for each treatment. * p<0.05, **p<0.01 and *** p<0.001 Kruskal-Wallis test.

3.2.5 Macrophages convert inactive vitamin D_3 to the active form $1,25(OH)_2D_3$

Macrophages express VDR and CYP27B1 and have been shown to upregulate genes downstream of $1,25(OH)_2D_3$ activation (Liu et al., 2006). Twenty years earlier, a study (Reichel et al., 1987) showed that activated macrophages were able to convert $25(OH)D_3$ into active $1,25(OH)_2D_3$. Macropahges can also contribute to local $1,25(OH)_2D_3$ production as either infiltrating inflammatory classically activated macrophages (M1) or resident noninflammatory alternatively activated macrophages (M2). To confirm the ability of macrophages to locally convert $25(OH)\ D_3$ into $1,25(OH)_2D_3$, M1 and M2 macrophages were isolated, differentiated (as described in section 2.2) and tested (Beyer et al., 2012;Schwartz and Svistelnik, 2012). Our results show that M1 and M2 cells can both metabolise $25(OH)D_3$ and convert it into $1,25(OH)_2D_3$ (Figure 3.10) although there was no statistically significant difference from control cells. The rates of conversion shown by HCEC-12 and ODM-2 above were even higher than the levels produced by M1 and M2 cells respectively.

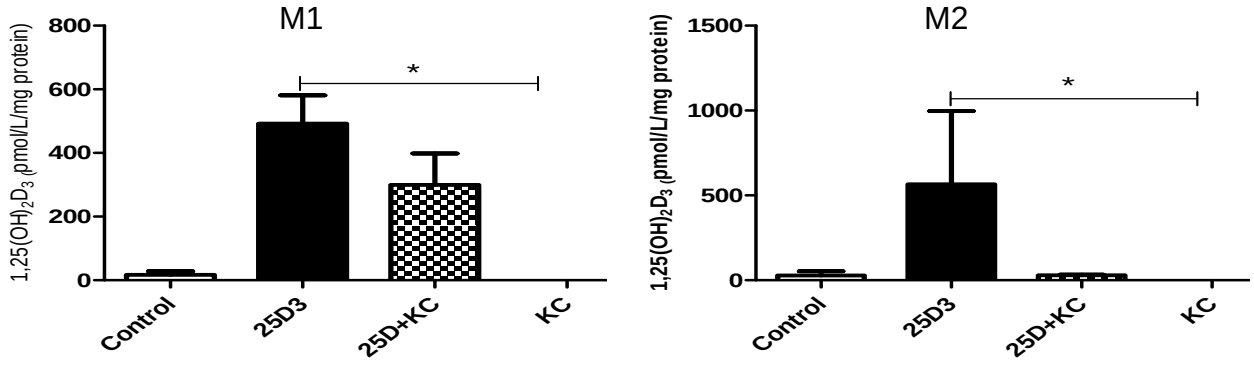

Figure 3.10. Macrophages polarised to M1 and M2 phenotypes convert inactive vitamin D_3 to the active form $1,25(OH)_2D_3$. M1 and M2 macrophages prepared as described in section 2.2 were either untreated (control) or treated with $25(OH)D_3$ (10^{-7} M) for 24 h in serum free conditions with or without pretreatment with CYP450 inhibitor (10^{-6} M) ketoconazole (KC) for 2 h. Cell culture supernatants were collected and stored at -20º C until measurement. $1,25(OH)_2D_3$ was determined by enzyme immunoassay and the results were obtained from a standard curve as pmol/L then corrected per mg of protein. Both M1 and M2 macrophages show similar conversion rates although there was no statistically significant difference from control cells. Each experiment was performed at least 3 times. Graphs show mean±SD $1,25(OH)_2D_3$ per mg protein for each treatment. * $p<0.05$ Kruskal-Wallis test.

3.2.6 $1,25(OH)_2D_3$ can be detected in human AqH and serum samples

To confirm the production of $1,25(OH)_2D_3$ in the eye, 7 AqH samples from normal controls (age 46-84) were tested. AqH samples showed detectable levels of $1,25(OH)_2D_3$ (1.9-4.8 pmol/L). These levels were compared to matched serum samples (Figure 3.11). Serum levels were higher (49.9-89.9 pmol/L) than levels in AqH ($p<0.01$). Serum levels did not show a correlation to AqH i.e. the highest serum level does not necessarily match with the highest levels in AqH. Samples with zero values had a volume of <50µl (Table 3.1).

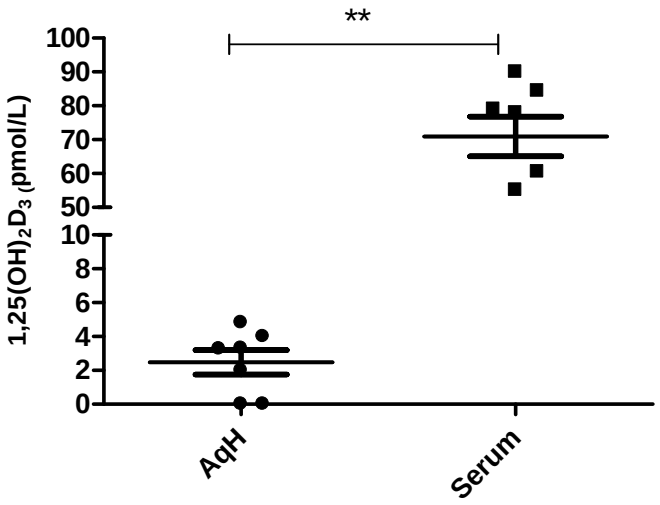

Figure 3.11. $1,25(OH)_2D_3$ can be detected in human AqH. Matched AqH and serum samples from normal controls undergoing cataract surgery were collected, processed, and stored at -80º C until measurement. $1,25(OH)_2D_3$ concentrations (pmol/L) were measured by enzyme immunoassay and the results were obtained from a standard curve as pmol/L. AqH showed detectable levels of $1,25(OH)_2D_3$ but very low compared to those present in serum (**P <0.01 Mann-Whitney test). Graph shows mean±SD.

Table 3.1. Concentrations of $1,25(OH)_2D_3$ in aqueous humour (AqH) do not correlate with age or serum which supports local production. Matched AqH and serum from healthy controls undergoing cataract surgery (five females and two males) of ages between 46 and 84 were collected and tested for $1,25(OH)_2D_3$ by enzyme immunoassay (pmol/L). Concentrations of $1,25(OH)_2D_3$ in AqH did not correlate with age or the levels present in serum.

Gender	Age	$1,25(OH)_2D_3$ (pmol/L)	
		AqH	Serum
F	69	4.81	78.99
F	46	1.99	49.92
F	76	00	89.93
F	84	00	77.85
M	60	3.27	60.47
F	81	3.31	84.35
M	57	3.99	55.06

3.3 Discussion

This study shows that human ocular barrier epithelial cells constitutively express mRNA and protein for the receptor and the metabolic enzymes required in the vitamin D_3 pathway, and can endogenously produce $1,25(OH)_2D_3$ when treated with physiological concentrations of its precursor $25(OH)D_3$ (Table 3.2).

Until recently, vitamin D_3 has not been reported in ocular surfaces. Previous studies have only shown that human primary RPE-choroid expressed mRNA for VDR and CYP27A1 while retinal tissue expressed CYP27B1 and CYP27A1 (Morrison et al.,

2011). Cultured primary rabbit, mouse, and human corneal epithelial cells expressed VDR and CYP27B1. Similar expression was seen in mRNA freshly collected from mouse and rabbit corneal epithelial tissues but there are no data on 25(OH)D_3 conversion (Lin et al., 2012).

In agreement with the speckled cytoplasmic and nuclear VDR staining in ocular barrier cells, VDR has been described in cytoplasm and nucleus of urinary bladder epithelium (Hertting et al., 2010;Midorikawa et al., 1999). The cytoplasmic expression of CYP27B1 in most of the ocular barrier epithelial cell lines is in accordance with similar expression in urinary bladder epithelium (Hertting et al., 2010). Conversely, the punctuated peri-nuclear staining of CYP27B1 in HCEC-12 can be compared to the cytoplasmic localisation in the peri-nuclear space seen in human tumour prostate epithelial cell line (LNCaP) transfected with CYP27B1 (Chen and Holick, 2003). LNCaP cells express CYP27B1 as multiple vesicular structures. The expression of CYP27B1 in TGN could be part of this enzyme's trafficking in these cells which are highly metabolic in nature supplying nutrients to the cornea and dehydrating the stroma (Bonanno, 2012). HCEC-12 were the most efficient in 25(OH)D_3 conversion among ocular barrier epithelial cells tested. It could be that CYP27B1 localisation to TGN is part of its trafficking to get to the cell membrane making it readily available to bind and convert its substrate. Membrane bound CYP450 proteins can recycle through Golgi before reaching their destined locations. Some CYP450 enzymes can exist in the mitochondria and endoplasmic reticulum (ER), an example is rat CYP27B1. At the N-terminus (amino acids 20-36) of rat CYP27B1, there is a targeting signal for mitochondria and

endoplasmic reticulum. Phosphorylation of serine 128 (Ser128) activates a cryptic mitochondrial signal and modulates CYP27B1 destination to the mitochondria or ER (Seliskar and Rozman, 2007). A final possibility is that HCEC-12 cells are not primary and this expression could be an inherent characteristic of this cell line.

Although ocular barrier epithelial cells show constitutive expression of mRNA and protein for CYP24A1, this mitochondrial enzyme is not constitutively expressed in all peripheral organs and tissues. Some cells such as skin keratinocytes, respiratory epithelial cells, and colonic epithelial cell line (Bar et al., 2007;Hansdottir et al., 2008;Peric et al., 2009) only express CYP24A1 in response to $25(OH)D_3$ or $1,25(OH)_2D_3$. Keratinocytes demonstrate a strong upregulation following exposure to UVB light in the presence of 7DHC (Bar et al., 2007). CYP24A1 is also induced after treatment with $25(OH)D_3$ sufficient serum in macrophages (Liu et al., 2006).

Our study also shows that human ocular barrier epithelial cells can convert inactive vitamin D_3 to its active form. It also shows $1,25(OH)_2D_3$ can be measured in human AqH samples. With the exception of ARPE-19, ocular barrier epithelial cells, are able to produce significant levels of active $1,25(OH)_2D_3 > 0.4x10^{-9}$ M at 24 h. This rate of conversion is comparable to that of human primary respiratory epithelial cells, urinary bladder epithelial cell line, and mammary epithelial cells (Hansdottir et al., 2008;Hertting et al., 2010;Kemmis and Welsh, 2008). These rates are much higher than those produced by human kidney cortical collecting duct (HCD) cell line (Bland et al., 2001). Starting from the conjunctiva, ocular barrier conversion is higher in primary human

corneal epithelial cells (PHCEC) (Susarla et al. and Alsalem et al. manuscripts submitted), reaches a peak in corneal endothelium, and starts to decrease in non-pigmented ciliary body epithelium reaching the lowest levels at retinal pigmented epithelium (Figure 3.12).

Ocular barrier epithelial cells appear to be efficient in producing $1,25(OH)_2D_3$ provided they have the substrate available. Despite the requirement of $25(OH)D_3$ for CYP27B1 to convert it to $1,25(OH)_2D_3$, ocular barrier cells can potentially initiate the vitamin D_3 pathway as early as the first 25-hydroxylation step via CYP27A1 and CYP2R1. Extra-hepatic expression of CYP27A1 and CYP2R1 has also been reported previously in other tissues (Blomberg et al., 2010;Ellfolk et al., 2009;Matusiak and Benya, 2007;Shinkyo et al., 2004). CYP27A1 is expressed in human RPE tissue and with its broad substrate specificity, is also involved in cholesterol metabolism. and protects the retina from the toxic 7-ketocholesterol, a metabolite of 7-DHC as a result of photooxidation (Heo et al., 2012). CYP2R1 is expressed in primary human prostate epithelium and thyroid epithelium cell line and tissue, and primary renal kidney epithelial tissue (Bieche et al., 2007;Blomberg Jensen, 2012;Blomberg et al., 2010). Although CYP27A1 was weakly expressed by ocular barrier cells, the expression of microsomal CYP2R1 was considerably greater. Moreover, the 25-hydroxylation activity of CYP2R1 is 26-fold higher than that of CYP27A1 (Shinkyo et al., 2004). This suggests that there is a possibility that ocular cells might be able to synthesise vitamin D_3 directly after exposure to sunlight.

The expression of megalin and cubilin in ocular barrier cells indicate the importance of these receptors in vitamin D_3 metabolism. Megalin has been identified in rat alveolar epithelial cell line, Caco-2 human intestinal epithelial cell line, and co-localised with cubilin in rat epithelial cells of uterus and oviduct (Argraves and Morales, 2004;Oda et al., 2011) but the relevance to the vitamin D_3 pathway has not been investigated in these cells. In mouse gall bladder (GB) epithelium, the expression of megalin and cubilin was distorted in calcuclous GB epithelium. Megalin and cubilin are multi-ligand receptors and may contribute to cholesterol uptake and prevent bile accumulation. Therefore, their disrupted expression or absence may suggest their involvement in gallstone formation (Tsaroucha et al., 2008). Although megalin is more widely expressed in different tissues (Christensen and Birn, 2002), the expression of cubilin is much more pronounced in ocular barrier epithelial cells.

An immune privileged organ such as the eye requires efficient strategies that can protect optical clarity from sight-threatening infections and damaging inflammatory responses. Decreased serum levels in humans of 25(OH)D_3 have been correlated with increased early AMD and low visual acuity in the elderly (Beauchet et al., 2011;Millen et al., 2011). This was further investigated by calculating UVB exposure index which correlated with reduced neovascular AMD. Moreover, SNPs in human *CYP24A1* have been shown to be linked to neovascular AMD (Morrison et al., 2011).

Table 3.2. A summary of gene and protein expression for vitamin D_3 elements by ocular barrier cells as well as the rate of $25D_3$ conversion. Ocular barrier epithelial cell lines are shown with mRNA expression, immunofluorescence staining intensity, and rate of $25D_3$ conversion with the highest as +++ and the lowest as +. HKC-8 cell line was used as a positive control for the expression of vitamin D_3 pathway components and $25(OH)D_3$ conversion. Thyroid mRNA was used a positive control for the expression of cubilin and megalin.

	Cell line	Gene Expression							Immunofluorescence			Rate of $25D_3$ conversion
		VDR	CYP27B1	CYP24A1	CYP27A1	CYP2R1	Cubulin	Megalin	VDR	CYP27B1	CYP24A1	
Ocular Barrier Cells	CCL20-2	++	+++	-	-	+++	+	+	+++	+++	+++	++
	HCEC-12	+++	+++	+	++	+++	+++	-	+++	+++	+	+++
	ODM-2	+++	+++	+	+	++	+++	-	+++	+++	+	++
	ARPE-19	+++	+++	++	+	+++	+++	-	++	+++	++	+
Control Cells	HKC-8	+++	+++	+++	+	+++	+++	+	*ND	ND	ND	++
	Thyroid	ND	ND	ND	ND	ND	+++	+++	ND	ND	ND	ND

* ND: not done

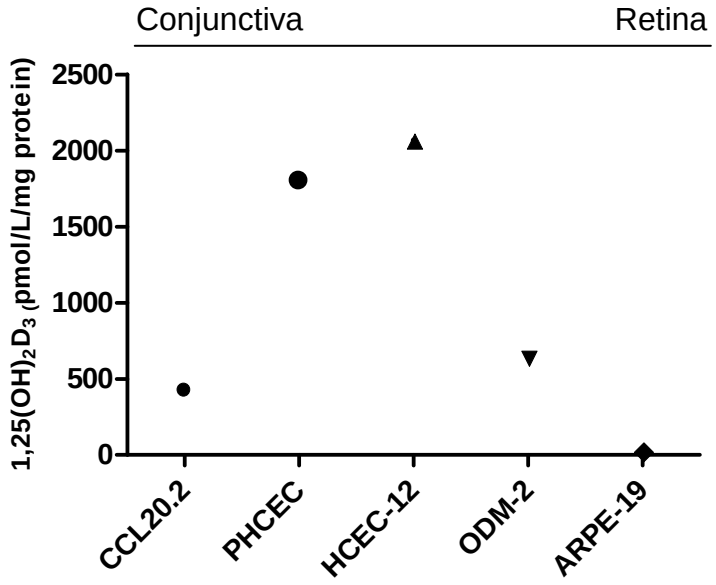

Figure 3.12. The rate of 25(OH)D_3 conversion by ocular barrier epithelial cells according to their anatomical location from the outside to the inside of the eye. A schematic diagram showing 1,25$(OH)_2D_3$ concentrations in the different ocular barrier epithelial cells from the exterior to the interior following their anatomical location. 25(OH)D_3 conversion starts at the conjunctiva, increases at corneal epithelium, reaches a peak at corneal endothelium, decreases at the ciliary body and diminishes at the retina.

To date, there is no mention of the presence of 1,25$(OH)_2D_3$ in human AqH. Yin et al. measured vitamin D_3 metabolites including 25(OH)D_3 and 1,25$(OH)_2D_3$ in rabbit AqH and vitreous samples (Yin et al., 2011). The levels of 1,25$(OH)_2D_3$ obtained in the current study in human AqH (0.004 nM) were lower than those shown in rabbit AqH (0.04 nM). Rabbit AqH levels were measured by liquid chromatography-tandem mass spectrometry (LC-MS/MS), a more sensitive technique suitable for the measurement of vitamin D_3 in a sample size as low as 100 µl (Vogeser, 2010).

There are several reasons why human aqueous levels of 1,25$(OH)_2D_3$ are lower than serum; (i) the recommended sample volume for the enzyme immunoassay is at least 500 µl and the sample volume available from AqH was between 50-150 µl which could have been a limitation for these results. (ii) Aqueous humour is in constant

movement and has a high turnover (2.4 ± 0.6 µl/min) to provide nutrients to and remove waste from the avascular cornea and lens (Goel et al., 2010), therefore the large amounts of $1,25(OH)_2D_3$ produced by corneal endothelium and ciliary body may be used up by the cells or washed away with AqH movement to keep a clear fluid consistency; (iii) as AqH contains less than 1% of proteins in plasma (Freddo, 2001) it may not cross the iris/ciliary body barrier efficiently. Nevertheless, the levels in AqH did not directly match their counterparts in serum which may suggest local synthesis of $1,25(OH)_2D_3$ that is secreted into AqH. Moreover, although vitamin D_3 synthesis is believed to decrease with age (Laird et al., 2010), serum vitamin D_3 levels of patients with ages 69-84 were higher than patients with ages 46-56.

The ability of ocular barrier epithelial cells to locally produce large amounts of $1,25(OH)_2D_3$ may be important for their barrier function. For example, corneal endothelial cells are highly metabolic and represent an important barrier at the anterior segment of the eye and producing large amounts of $1,25(OH)_2D_3$ may help maintain this function. More than 200 genes were significantly upregulated or downregulated in response to stimulation of lymphoblastoid cell line with $1,25(OH)_2D_3$ including several involved in the immune response (Ramagopalan et al., 2010). Similarly the induction of HDPs by $1,25(OH)_2D_3$ may be of importance to ocular barrier function (Liu et al., 2006).

The main caveat in this work is the use of cell lines rather than primary cells. Some attempts were carried out to isolate and grow primary human corneal endothelial cells without success. Several emails were sent to eye banks in Bristol and Manchester to obtain eyes not suitable for transplantation and use them to isolate non-pigmented ciliary body and retinal pigment epithelium without response. However the lines used are

well-established and regarded as good correlates for freshly –isolated ocular cells (Bednarz et al., 2000;Dunn et al., 1996;Martin-Vasallo et al., 1989;Racusen et al., 1997).

In conclusion, this study has demonstrated the expression of vitamin D_3 pathway molecular components by *in vitro* models of ocular barrier epithelial cells and that these cells can convert inactive $25(OH)D_3$ into active $1,25(OH)_2D_3$. This study has also shown that $1,25(OH)_2D_3$ is present in detectable levels in human AqH. Further work will show if these cells can create an environment rich in $1,25(OH)_2D_3$ and if this has an effect on ocular immune protection and immune privilege.

4 THE ROLE OF 1,25(OH)$_2$D$_3$ IN OCULAR EPITHELIAL BARRIER CELLS

4.1 Introduction

Vitamin D$_3$ directly or indirectly influences many immune cells. This is facilitated by the wide expression of VDR in immune cells including neutrophils, macrophages, DCs, as well as activated T and B cells. Vitamin D$_3$ prevents DC maturation, skews T cell development into regulatory phenotypes, and inhibits B cell proliferation (Baeke et al., 2010). Stimulation of $CD4^+CD25^-$ T cells with 1,25(OH)$_2$D$_3$ resulted in an inhibition of proinflammatory cytokines such as IFN-γ and IL-17. At the same time it increased the expression of CTLA4 and FoxP3 (Jeffery et al., 2009). Further investigations showed that endogenous production of 1,25(OH)$_2$D$_3$ caused the conversion of DC into tolerogenic phenotype and enabled them to alter T cell responses (Jeffery et al., 2012). B cell stimulation with 1,25(OH)$_2$D$_3$ prevented plasma cells differentiation and Ig class switching (Chen et al., 2007). This was translated *in vivo* where patients with SLE supplemented with vitamin D$_3$ had decreased Th1 and Th17, restored regulatory T cells, and decreased memory B cells and anti-inflammatory antibodies (Terrier et al., 2012).

As stated in Chapter 1, HDPs are short cationic peptides produced by cells of the immune system and various cell types. They are known for exhibiting antimicrobial as well as immunomodulatory activities (Steinstraesser et al., 2011;Yeung et al., 2011). The immunomodulatory role of vitamin D$_3$ became very clear in studies linking vitamin D$_3$ deficiency to susceptibility to infection with *Mtb* (Gibney et al., 2008;Williams et al.,

2008). When macrophages were stimulated with a TLR2/1 mycobacterial ligand in the presence of vitamin D_3, there was an upregulation of LL-37 genes and increased antimicrobial activity against *Mtb*. African-Americans have higher melanin levels in their skin and thus become less capable of obtaining sufficient vitamin D_3 from UV exposure (Macdonald, 2012). When macrophages were stimulated in the presence of vitamin D_3 insufficient African-American serum, there was a significant reduction in mRNA levels of LL-37 compared to Caucasians (Liu et al., 2006). This was mediated by TLR2/1 induced IL-15 which lead to the induction of CYP27B1 and cathelicidin (Krutzik et al., 2008).

The activation of TLR2/1 also induced IL-1β which together with VDR were required to induce *DEFB4* (Liu et al., 2009b). In contrast to enhanced macrophage antimicrobial response to *Mtb*, vitamin D_3 induced LL-37 increased virulence and promoted resistance to killing of Group A *Streptococcus* by oropharyngeal keratinocytes, macrophages and neutrophils (Love et al., 2012). IFN-γ produced by T cells needs vitamin D_3 to result in subsequent autophagy, autophagosome-lysosome fusion and even the induction of HDPs such as cathelicidin and BD-4 (Fabri et al., 2011).

Skin injury is known to induce HDPs. Injury was induced using sterile incisions in healthy human skin and Streptococcal injections in C57BL/6 mice. Human cathelicidin (LL-37) and mouse cathelicidin-related HDP (CRAMP) proteins were expressed in the extracellular space and granulation tissues. This indicates their synthesis by keratinocytes and granulocytes migrating to the site of injury (Dorschner et al., 2001).

This work was followed further. Injury of human skin induced mRNA expression of TLR2, CD14 (a TLR cofactor), and CYP24A1. Treatment of keratinocytes with TLR2 ligand in the presence of TGF-β released in response to injury induced mRNA expression of CYP27B1. When keratinocytes were treated simultaneously with TGF-β1 and 25(OH)D$_3$, cathelicidin, CD14, and TLR2 were induced. TGF-β1 or 25(OH)D$_3$ alone could not induce the same genes. CYP27B1 deficient mice were used to confirm the results *in vivo*. Mouse CD14 is regulated by 1,25(OH)$_2$D$_3$ and it was absent compared to wild type. In contrast, mouse CRAMP was induced in both CYP27B1 deficient and wild type mice as this gene does not contain VDRE. Keratinocytes transfected with CAMP with disrupted VDRE could not induce cathelicidin in response to 1,25(OH)$_2$D$_3$. TLR2 synegised with 1,25(OH)$_2$D$_3$ to induce mRNA expression of cathelicidin, an effect that diminished with VDR antagonist (Schauber et al., 2007).

The production of HDPs appears to be a key function of vitamin D$_3$ in macrophages and epithelial cells. In respiratory epithelial cells, TLR2/1 has the same role as in macrophages. In addition, stimulation of TLR3 increased endogenous 1,25(OH)$_2$D$_3$ production and both synergised to enhance the expression of LL-37 (Hansdottir et al., 2008). In contrast, in urinary bladder epithelial cells, both exogenous and endogenous 1,25(OH)$_2$D$_3$ were able to upregulate LL-37 in the absence of TLR stimulation.

Vitamin D$_3$ proves to be important for barrier function. In a mouse model of IBD, oral supplementation with vitamin D$_3$ abrogated the inflammatory response in the colon.

Furthermore, vitamin D_3 maintained intestinal barrier integrity by reducing permeability and upregulating the expression of TJ proteins (Zhao et al., 2012). Vitamin D_3 also upregulated TJ proteins in corneal epithelium (Yin et al., 2011) but its role in the induction of HDPs in the eye has not been investigated. HDPs have been detected in ocular cells and fluids (Garreis et al., 2010b;Huang et al., 2007), but it is not clear if vitamin D_3 plays a role in their induction.

In this study, we investigated the role played by vitamin D_3 in the induction of HDPs and if vitamin D_3 synergises with TLR to upregulate proinflammatory cytokines.

4.2 Results

4.2.1 Ocular barrier epithelial cells upregulate vitamin D_3 elements genes in response to 25(OH)D_3 or 1,25(OH)$_2$D$_3$

To confirm that ocular barrier cells have a functional system that can induce the upregulation of vitamin D_3 induced genes such as VDR, CYP27B1, and CYP24A1, cells were treated with physiological concentrations of either inactive 25(OH)D_3 (10^{-7} M) or active 1,25(OH)$_2$D$_3$ (10^{-8} M). HCEC-12, ODM-2, and ARPE-19 cells showed an induction of vitamin D_3 element genes upon treatment with 25(OH)D_3 and 1,25(OH)$_2$D$_3$ (Figures 4.1-4.3) but this induction was not statistically significant except in HCEC-12 at 1 h.

HCEC-12 cells showed a significant 4-fold increase in VDR at 1 h in response to 1,25(OH)$_2$D$_3$ ($p<0.01$) that declined afterwards and about 2-fold increase with 25(OH)D_3 at 8 h. In ODM-2 cells, VDR showed a 2- fold increase at 1 h with 25(OH)D_3. Stimulation with 1,25(OH)$_2$D$_3$ induced a 2-fold increase at 4 and 8h, a peak of 3 fold at 16 h and

back to 2 fold at 24 h. In contrast to HCEC-12 and ODM-2, VDR was not highly upregulated in ARPE-19 cells. There was a minimal increase at all time points with 1,25(OH)$_2$D$_3$ but not with 25(OH)D$_3$ (Figure 4.1).

In HCEC-12 cells, CYP27B1 showed a 4-fold change at 4 h and decreased at 8 and 16 h to 1.5 fold. At 24 h, CYP27B1 expression increased by 2 fold and 1.6 fold with 25(OH)D$_3$ and 1,25(OH)$_2$D$_3$ respectively. CYP27B1 showed a gradual increase in ODM-2 cells until it reached 2 fold and 2.5 fold with 25(OH)D$_3$ and 1,25(OH)$_2$D$_3$ respectively at 8 h. At 16 h there was no induction of CYP27B1 but there was a slight increase of 1.5 fold at with 25(OH)D$_3$ 24 h. CYP27B1 showed a slight 1.5-fold increase in ARPE-19 at 1 h with both 25(OH)D$_3$ and 1,25(OH)$_2$D$_3$. At 4 h there was a 2-fold change with 1,25(OH)$_2$D$_3$ followed by a 1.5 fold change at 8 and 16 h that declined at 24 h. 25(OH)D$_3$ induced an upregulation of 1.5 fold at 1 and 4 h that declined at 8 h. At 8 h there was 2.5 fold change which decreased at 24 h (Figure 4.2).

CYP24A1 was gradually induced in HCEC-12 cells at 4 h (2 fold), 8 h (4 fold), and 16 h (10 fold) in response to both 25(OH)D$_3$ and 1,25(OH)$_2$D$_3$. This dropped at 24 h to 3 and 4 fold with 25(OH)D$_3$ and 1,25(OH)$_2$D$_3$ respectively. In ODM-2 cells, CYP2A1 expressed a 1.5 and 2 fold change at 1 h with 25(OH)D$_3$ and 1,25(OH)$_2$D$_3$ respectively. While at 4 h there was a 10-fold increase with both, stimulation with 25(OH)D$_3$ maintained about 4 fold increase at 8 and 16 h. There was no upregulation caused with 1,25(OH)$_2$D$_3$ at 8 h but at 16 h there was a 4 fold increase at 16 h that declined again at 24 h. ARPE-19 cells showed an induction of CYP24A1, 1.6 fold change, with both

25(OH)D$_3$ and 1,25(OH)$_2$D$_3$ at 4 h. This increased to 3 fold for 1,25(OH)$_2$D$_3$ but remained about 1.6 fold for 25(OH)D$_3$ at 8 h. At 16 and 24 h there was no induction of CYP24A1 with both treatments (Figure 4.3).

In conclusion, ocular barrier epithelial cells showed a trend towards an upregulation of vitamin D$_3$ element genes in response to 25(OH)D$_3$ and 1,25(OH)$_2$D$_3$. HCEC-12 cells appear to be the quickest to upregulate the expression of CYP27B1 at 4 h while CYP24A1 is only induced after 24 h which gives them more time for synthesis of 1,25(OH)$_2$D$_3$. ODM-2 cells showed a slight induction at 4 h increasing its highest expression at 8 h. CYP24A1 is induced as early as 4 h with its highest expression decreasing at 8 h and increasing again at 16 h. This may indicate a tighter regulation of 1,25(OH)$_2$D$_3$ synthesis in these cells. ARPE-19 only showed a slight upregulation of CYP27B1 and a modest induction of CYP24A1 compared to the other cell lines. This fits in with the conversion rates obtained in these cells. We have shown that HCEC-12 cells had the highest 25(OH)D$_3$ conversion rates followed by ODM-2 then ARPE-19 cells. However, due to the lack of statistical significance more repeats are required to provide conclusive results.

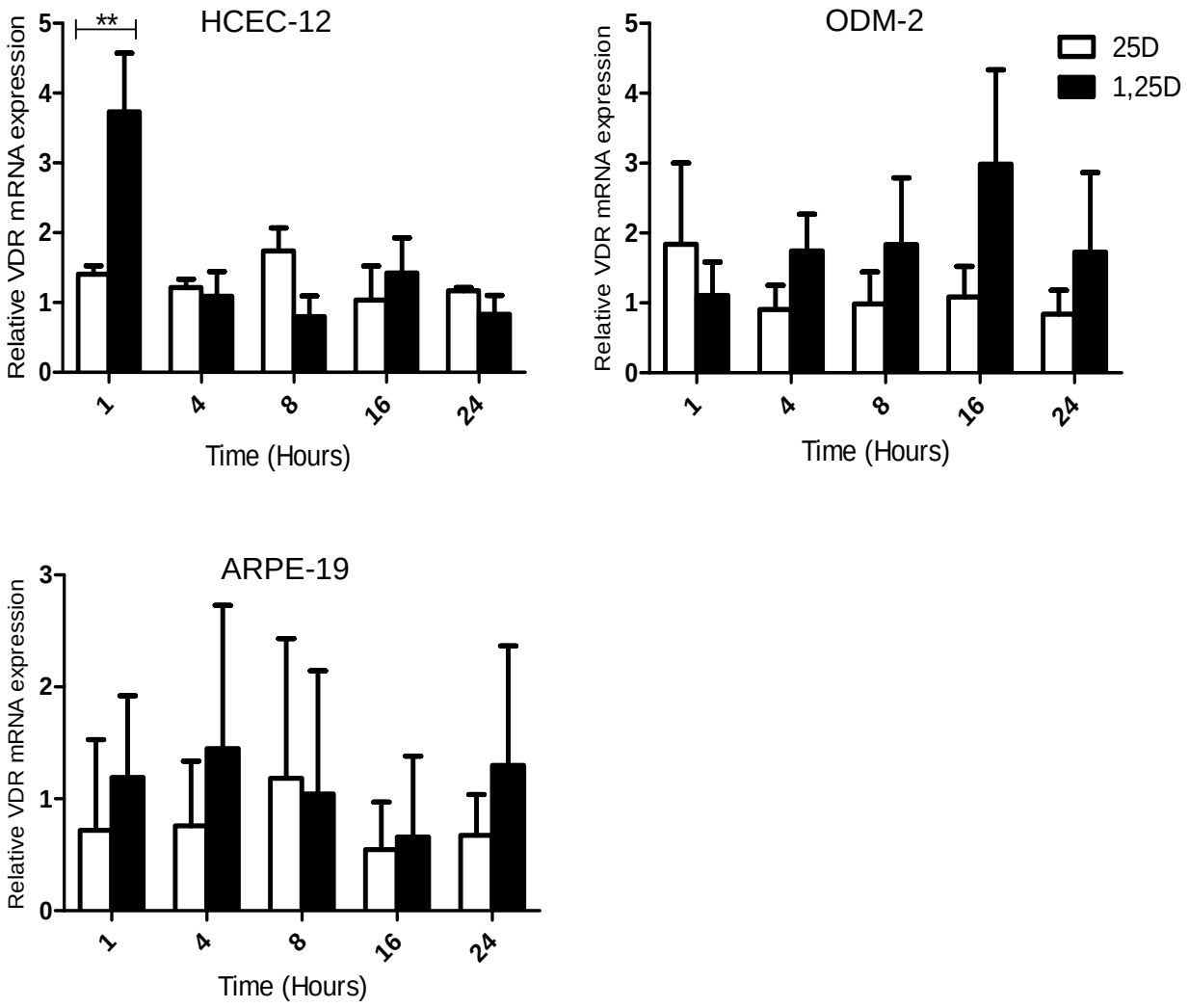

Figure 4.1. Ocular barrier epithelial cells show a higher upregulation of mRNA for VDR in response to 1,25(OH)$_2$D$_3$ than 25(OH)D$_3$. HCEC-12, ODM-2, and ARPE-19 cells were grown to 80-90% confluence and were either left untreated (control) or treated with 25(OH)D$_3$ (10^{-7} M) or 1,25(OH)$_2$D$_3$ (10^{-8} M) for 1, 4, 8, 16, or 24 h in serum free conditions. Total RNA was isolated and real-time PCR was used to measure relative fold change increase in treated samples compared to control (untreated cells) for VDR. VDR showed a significant upregulation only in HCEC-12 cells with 1,25(OH)$_2$D$_3$ at 1 h and a slight upregulation with 25(OH)D$_3$ at 8 h. ODM-2 cells showed a slight upregulation with 25(OH)D$_3$ at 1 h and with 1,25(OH)$_2$D$_3$ 16 h while ARPE-19 cells did not show an upregulation of VDR mRNA expression. This upregulation is not statistically significant. Graphs show mean±SD (at least 3 experimental repeats). Two-way ANOVA with Bonferroni's post test were perfomed, ** $p<0.01$.

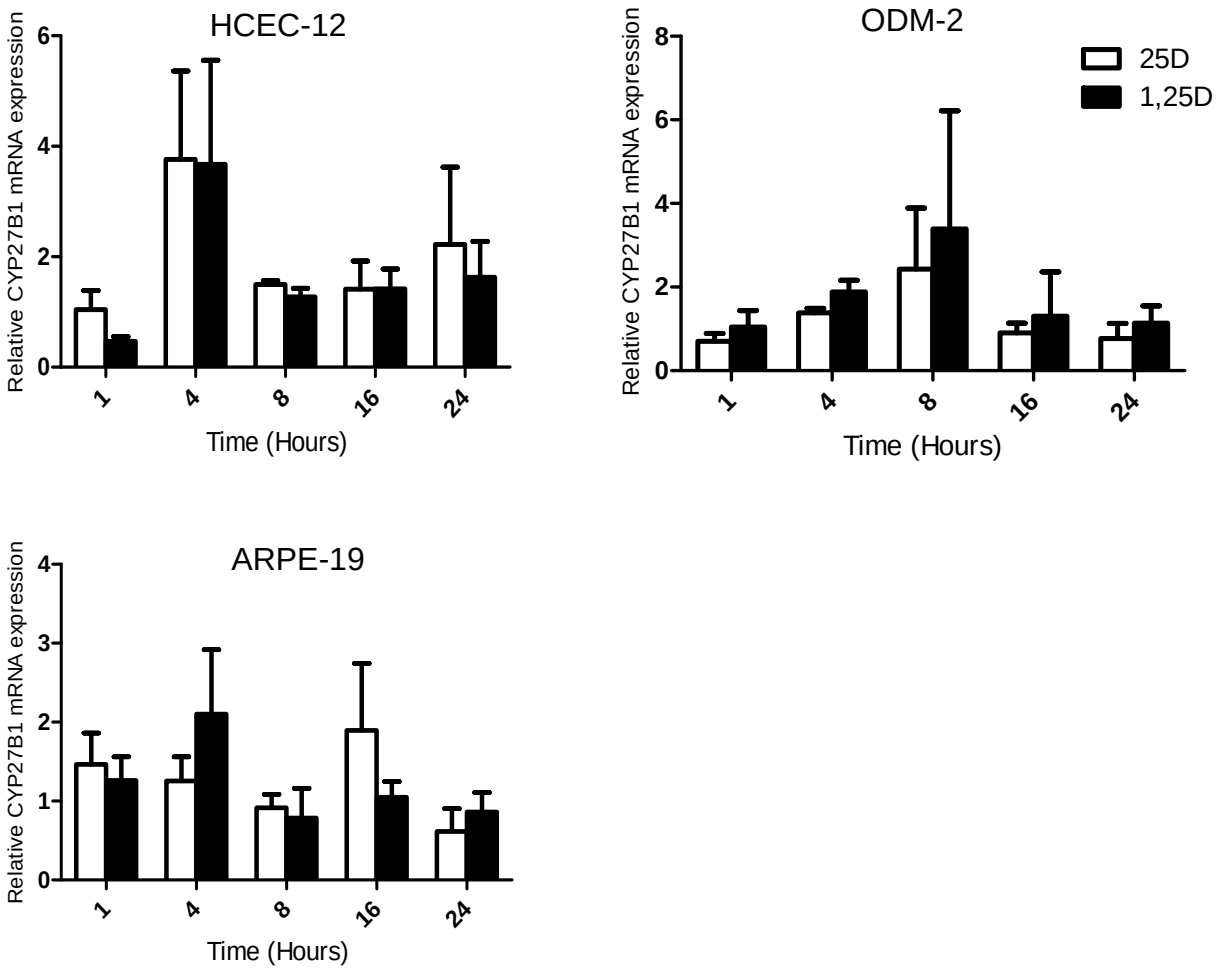

Figure 4.2. Ocular barrier epithelial cells upregulate mRNA for CYP27B1 in response to 25(OH)D$_3$ and 1,25(OH)$_2$D$_3$. HCEC-12, ODM-2, and ARPE-19 cells were grown to 80-90% confluence and were either left untreated (control) or treated with 25(OH)D$_3$ (10^{-7} M) or 1,25(OH)$_2$D$_3$ (10^{-8} M) for 1, 4, 8, 16, or 24 h in serum free conditions. Total RNA was isolated and real-time PCR was used to measure relative fold change increase in treated samples compared to control (untreated cells) for CYP27B1. CYP27B1 was upregulated with both 25(OH)D$_3$ and 1,25(OH)$_2$D$_3$ in HCEC-12 cells at 4 and 24 h. CYP27B1 showed a slight with 1,25(OH)$_2$D$_3$ at 4 h and with both 25(OH)D$_3$ and 1,25(OH)$_2$D$_3$ at 8 h. ARPE-19 cells slightly upregulated CYP27B1 mRNA expression with 1,25(OH)$_2$D$_3$ at 4 h and with 25(OH)D$_3$ at 16 h. However, this upregulation is not statistically significant. Graphs show mean±SD (at least 3 experimental repeats). Two-way ANOVA with Bonferroni's post test were performed.

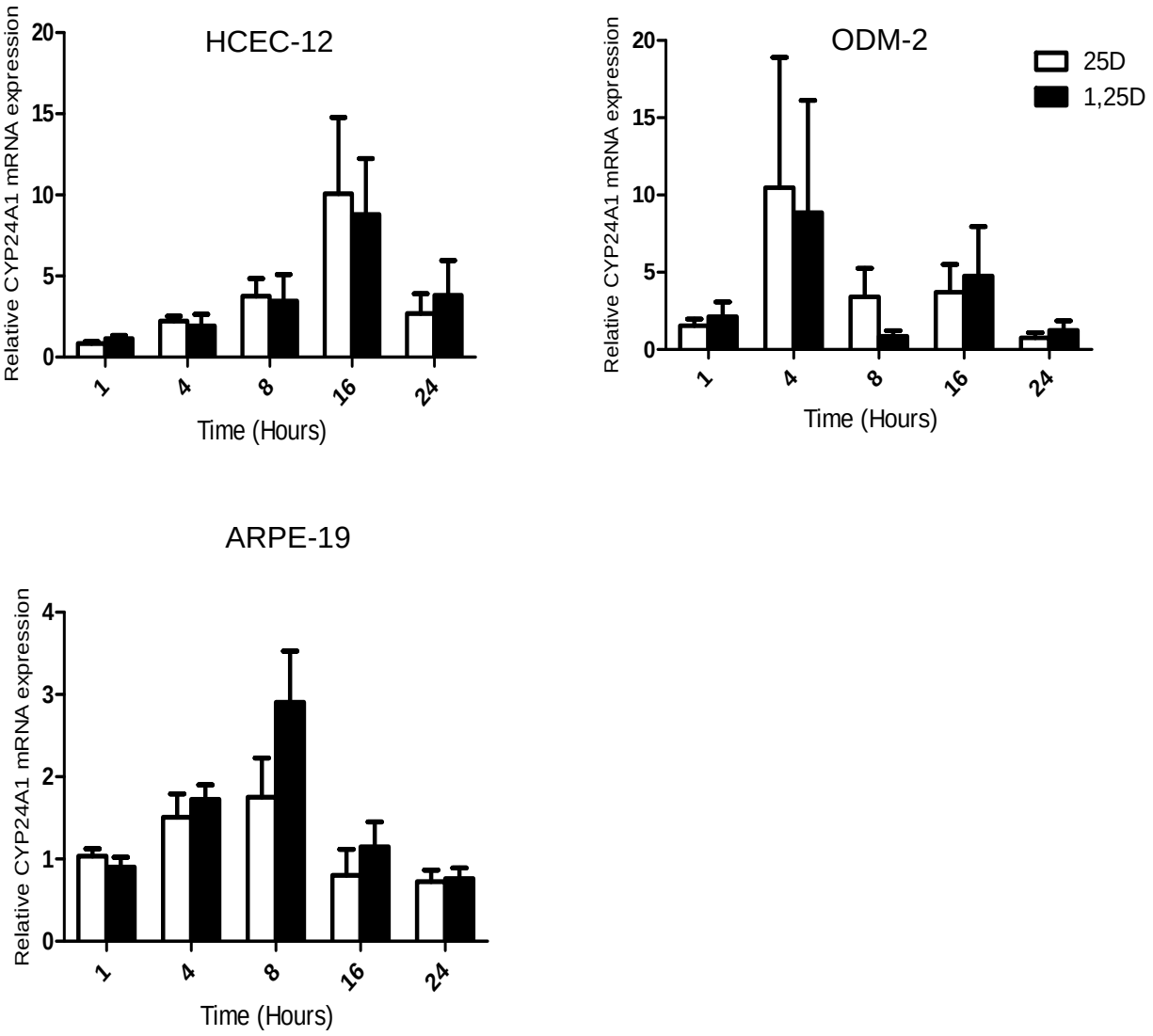

Figure 4.3. Ocular barrier epithelial cells upregulate mRNA for CYP24A1 in response to 25(OH)D$_3$ and 1,25(OH)$_2$D$_3$. HCEC-12, ODM-2, and ARPE-19 cells were grown to 80-90% confluence and were either left untreated (control) or treated with 25(OH)D$_3$ (10^{-7} M) or 1,25(OH)$_2$D$_3$ (10^{-8} M) for 1, 4, 8, 16, or 24 h in serum free conditions. Total RNA was isolated and real-time PCR was used to measure relative fold change increase in treated samples compared to control (untreated cells) for CYP24A1. CYP24A1 showed an upregulation in HCEC-12 with both 25(OH)D$_3$ and 1,25(OH)$_2$D$_3$ at 8 and 16 h and in ODM-2 cells at 4 and 16 h. ARPE-19 cells only upregulated CYP24A1 with 1,25(OH)$_2$D$_3$ at 8. However, this upregulation is not statistically significant. Graphs show mean±SD (at least 3 experimental repeats). Two-way ANOVA with Bonferroni's post test were performed.

4.2.2 Vitamin D_3 does not synergise with TLR in the production of proinflammatory cytokines

It has been shown in macrophages that TLRs synergise with vitamin D_3 to induce downstream effects of VDR activation (Liu et al., 2006). To investigate if VDR behaves similarly in ocular barrier cells, we first screened the cells for the expression of TLR1-10, RIG-I, and MDA5 (Figure 4.4). TLR1, 3, 4, 10, RIG-I, and MDA5 were strongly expressed by all the cell lines. TLR2 was not expressed by HCEC-12 and very weakly in ODM-2 and ARPE-19. TLR5 was absent in HCEC-12 but expressed by ODM-2 and ARPE-19. While TLR6 was expressed in HCEC-12 and ARPE-19, it showed a weak expression in ODM-2. TLR7 and 8 were absent in all cell lines. Likewise, TLR9 was strongly expressed by ODM-2 and ARPE-19 but absent in HCEC-12.

Next the ability of TLR and 25(OH)D_3 to induce proinflammatory cytokines such as IL-6, IL-8, TNF-α, and IFN-γ was measured by ELISA. As 25(OH)D_3 (10^{-7} M) had to be added in serum free conditions (to avoid additional 25(OH)D_3 present in serum), we questioned whether TLRs especially TLR4 would be able to respond in the absence of LPS-binding protein (LPS-LBP) present in serum (Figure 4.5).

When ARPE-19 were treated with different concentrations of Pam3Cys, Poly I:C, and LPS in the presence or absence of serum, ARPE-19 cells responded variably to TLR stimulation through the production of IL-6 and IL-8. There was a 2-fold increase in IL-8 concentrations after treatment with Pam3Cys (1 and 10 µg) and Poly I:C (20 µg) while Poly I:C (100 µg) caused a 4-fold increase in serum free conditions. Stimulation of

ARPE-19 with LPS (10 ng and 1 and 5 µg) caused a 3-fold increase in IL-8 concentrations compared to control cells. The addition of serum increased the background but did not prevent the cells from responding to LPS.

Stimulation of ARPE-19 with Pam3Cys (1 and 10 µg), Poly I:C (20 µg), and LPS (10 ng and 5 µg) doubled IL-6 concentrations while Poly I:C (100 µg) caused a 3-fold increase. Similar to IL-8, the addition of serum increased the background without preventing the cells from responding to LPS. Based on these findings, we decided to carry out the experiments in serum free (SF) conditions.

HCEC-12 cells showed a baseline production of IL-8 but the concentrations were only increased in response to Poly I:C (100 µg). Treatment with 25(OH)D$_3$ does not seem to affect the response of these cells to TLR ligands. ODM-2 cells initially produced high concentrations of IL-8 which were not changed by 25(OH)D$_3$. Untreated ARPE-19 cells produced a higher baseline concentration of IL-8 than HCEC-12 but lower than ODM-2 cells. ARPE-19 cells showed the highest IL-8 production in response to Poly I:C and RIG-I/MDA5 both of which doubled IL-8 concentrations (Figures 4.6).

Also HECE-12 cells produced IL-6 constitutively and there was a slight increase in response to Poly I:C (100 µg) but this was not influenced by 25(OH)D$_3$. There was no increase in IL-6 in ODM-2 cells in response to any of the TLR ligands used and the addition of 25(OH)D$_3$ did not alter this response. ARPE-19 cells produced very low levels of IL-6 compared to HCEC-12 and ODM-2. Consistent with HCEC-12 cells, ARPE-19

cells showed the highest IL-6 amounts upon treatment with Poly I:C. The response to TLR ligands was not affected by the addition of 25(OH)D$_3$ in ARPE-19 (Figure 4.7).

To conclude, ocular barrier cells responded to TLR ligand treatment by the production of IL-8 and IL-6 (except ODM-2) but this response was not affected by the addition of 25(OH)D$_3$. TNF-α and IFN-γ were not detected in ocular barrier cells by ELISA. However, due to the low number of repeats and the lack of statistical significance, these results are inconclusive.

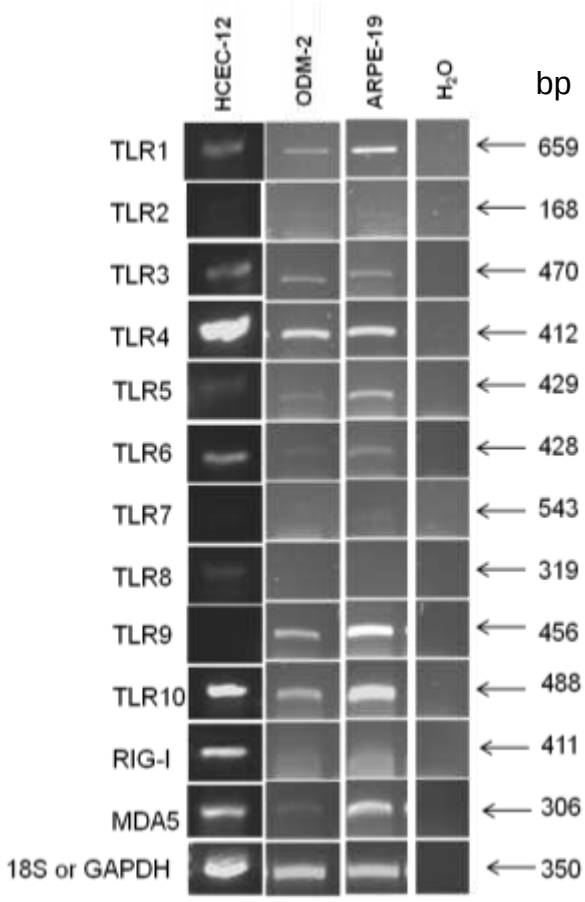

Figure 4.4. Ocular barrier epithelial cells express mRNA for TLRs, RIG-I, and MDA5. HCEC-12, ODM-2, and ARPE-19 cells were grown to 80-90% confluence, and total RNA was extracted. Conventional RT-PCR was performed for mRNA expression of TLR1-10, RIG-I, and MDA5. Expression bands are shown with their corresponding sizes (bp). HCEC-12 cells expressed all TLRs except TLR2, 7, and 9 while ODM-2 and ARPE-19 cells expressed all TLRs except TLR2, 7, and 8. HCEC-12, ODM-2, and ARPE-19 cells expressed RIG-I and MDA5. 18S or GAPDH were used as internal controls and H_2O as a negative control.

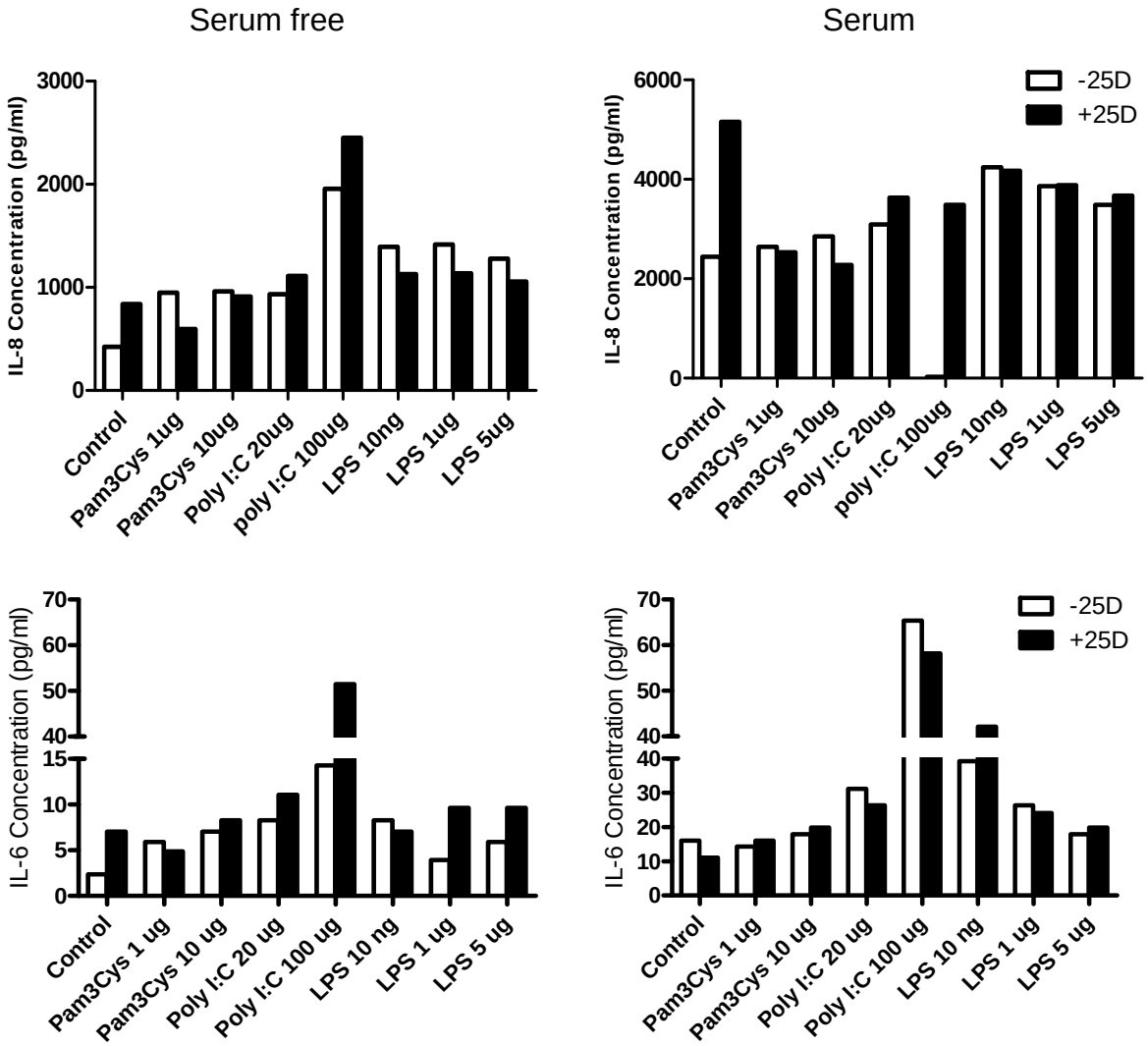

Figure 4.5 ARPE-19 cells produce IL-8 and IL-6 in response to TLR stimulation in the absence and presence of serum by ELISA with or without the addition of 25(OH)D$_3$. ARPE-19 cells were grown 80-90% confluence and were either left untreated (control) or treated with TLR ligands with or without serum in growth media in the presence or absence of 25(OH)D$_3$ (10^{-7} M) for 24 h. Supernatants were collected and cytokine were measured by ELISA. ARPE-19 cells produced IL-8 after treatment with Pam3Cys, Poly I:C, and LPS in serum free medium in the presence or absence of 25(OH)D$_3$. These levels increased in the presence of serum. IL-6 was also produced by ARPE-19 cells after treatment with Poly I:C (100 µg) and LPS (10 ng) in the presence or absence of 25(OH)D$_3$. Similarly, the addition of serum increased IL-6 concentrations but the addition of serum did not alter this response. Graphs show mean cytokine concentrations (n=1).

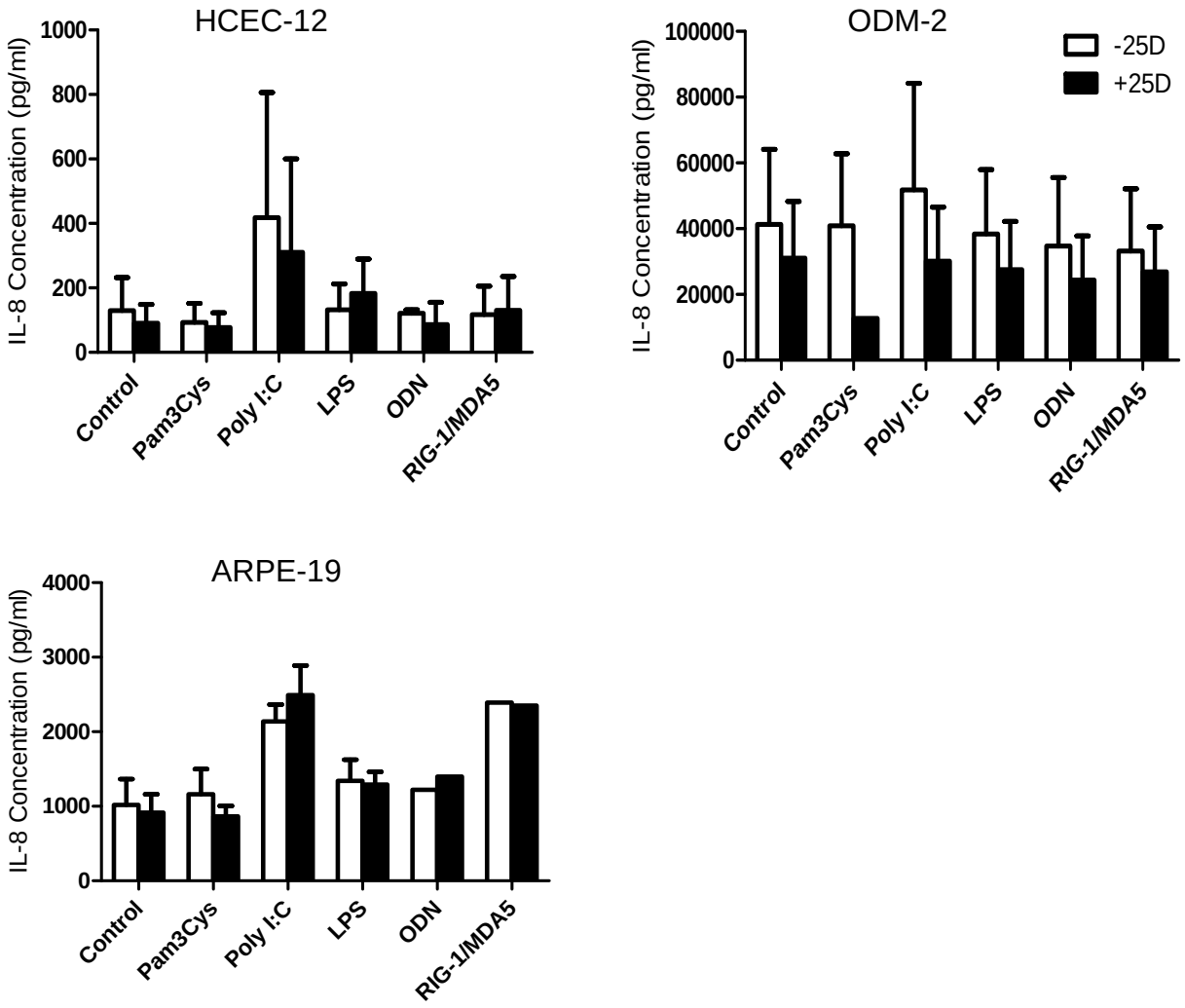

Figure 4.6. Vitamin D$_3$ does not influence IL-8 production after stimulation with TLR and RIG-I/MDA5 ligands in ocular barrier epithelial cells. HCEC-12 (n=2), ODM-2 (n=2), and AREP-19 (n=3 except RIG-I/MDA5 n=1) were grown to 80-89% confluence and were either unstimulated (control) or stimulated with TLR and RIG-I/MDA5 ligands in the absence or presence of 25(OH)D$_3$ (10^{-7} M) for 24 h in serum free conditions. Supernatants were collected and cytokine IL-8 production was measured by ELISA. IL-8 was produced by HECEC-12 in response to Poly I:C and ARPE-19 in response to Poly I:C and RIG-1/MDA5 which was not affected by the addition of 25(OH)D$_3$. ODM-2 showed a constitutive production of IL-8 and that was not influenced by TLR or RIG-I/MDA5 ligands or the presence of 25(OH)D$_3$. Graphs show mean±SD cytokine production compared to untreated control cells (HCEC-12 and ODM-2 n=2 and ARPE-19 n=2 except RIG-I/MDA5 n=1). Two-way ANOVA and Bonferroni's post test were performed.

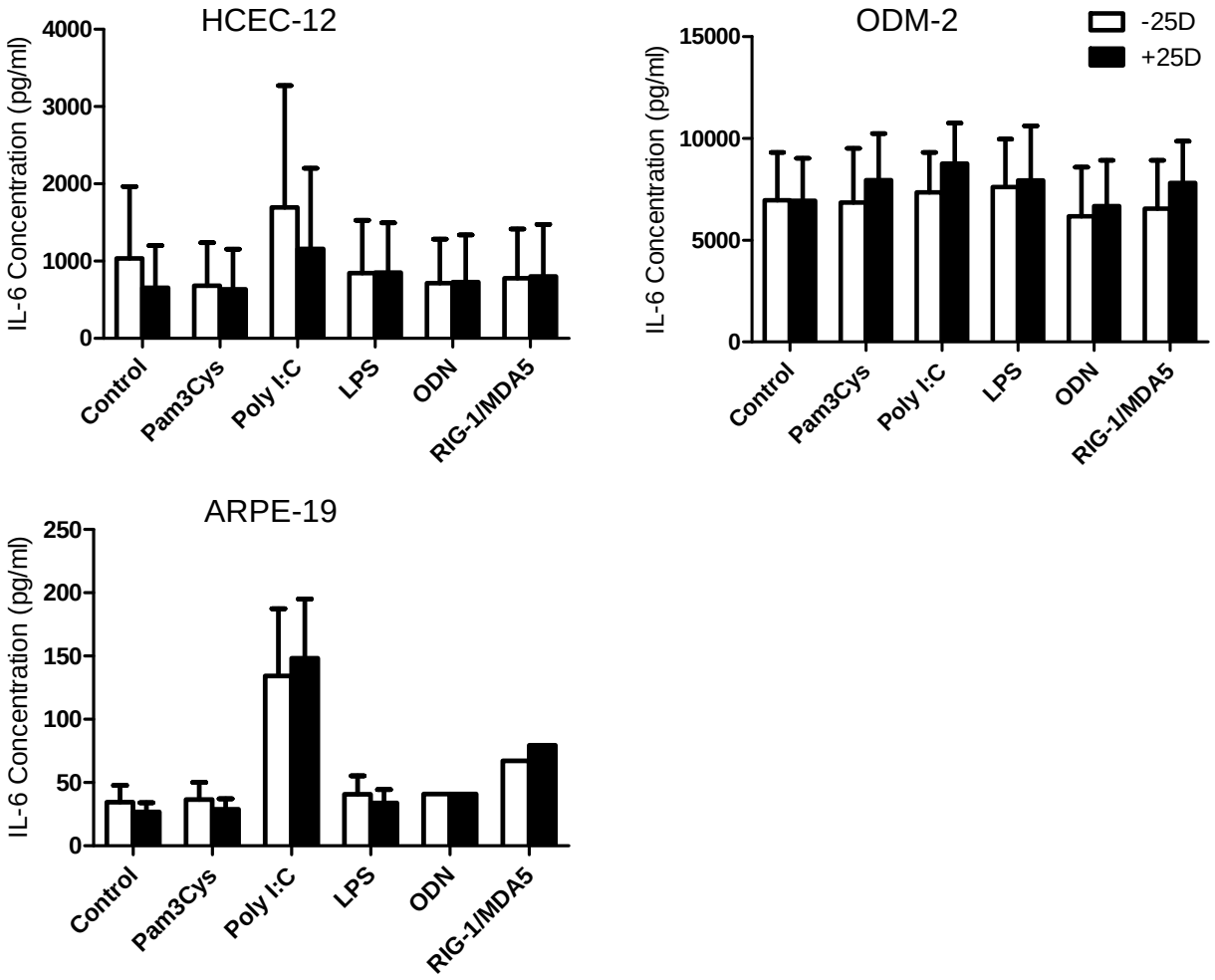

Figure 4.7. Vitamin D$_3$ does not influence IL-6 production after stimulation with TLR and RIG-I/MDA5 ligands in ocular barrier epithelial cells. HCEC-12 (n=2), ODM-2 (n=2), and AREP-19 (n=3 except RIG-I/MDA5 n=1) were grown to 80-89% confluence and were either unstimulated (control) or stimulated with TLR and RIG-I/MDA5 ligands in the absence or presence of 25(OH)D$_3$ (10^{-7} M) for 24 h in serum free conditions. Supernatants were collected and cytokine IL-6 production was measured by ELISA. HCEC-12 cells constitutively produced IL-6 and this was slightly increased in response to Ply I:C. ODM-2 cells initially produced high amounts of IL-6 but these levels were not upregulated by TLR stimulation. ARPE-19 cells produced lower levels of IL-6 and these levels were increased after treatment with Poly I:C. Treatment with 25(OH)D$_3$ did not influence IL-6 levels. Graphs show mean±SD cytokine production compared to untreated control cells (HCEC-12 and ODM-2 n=2 and ARPE-19 n=2 except RIG-I/MDA5 n=1). Two-way ANOVA and Bonferroni's post test were performed.

4.2.3 Ocular barrier epithelial cells upregulate genes for HDPs in response to locally produced as well as exogenous 1,25(OH)$_2$D$_3$ by real-time PCR

To further determine the ability of ocular barrier cells to use downstream signalling of VDR to produce HDPs (CAMP [cathelicidin, LL-37], BD1-4), these cells were tested in the presence of 25(OH)D$_3$ (10^{-7} M) and 1,25(OH)$_2$D$_3$ (10^{-8} M) at 1, 4, 8, 16, and 24 h by real-time PCR.

HCEC-12 showed a gradual increase in the expression of CAMP starting from 4 h reaching a peak at 8 h, then declined at 16 h and low at 24 h. Treatment with 1,25(OH)$_2$D$_3$ induced moderately higher CAMP than 25(OH)D$_3$ at 4 h. ODM-2 did not show any induction of CAMP at 1, 4, 8 h. There was no induction with 25(OH)D$_3$ and a decrease (0.5 fold) with 1,25(OH)$_2$D$_3$ at 16 h and a 1.5 fold-change at 24 h. ARPE-19 showed a slight increase with both 25(OH)D$_3$ and 1,25(OH)$_2$D$_3$ at 1 and 4 h but 4-fold increase induction of CAMP with 25(OH)D$_3$ at 8 h that declined at 16, and 24 h (Figure 4.8).

In HCEC-12 cells, BD-1 was induced by 1,25(OH)$_2$D$_3$ (2.5-fold change) at 4 h, while both 25(OH)D$_3$ and 1,25(OH)$_2$D$_3$ caused about 3-fold increase in its expression at 24 h. ODM-2 cells showed a 2-fold increase by 1,25(OH)$_2$D$_3$ at 1 h, but no change was noticed upon treatment with 25(OH)D$_3$ at all time points. In contrast, at 4 h ARPE-19 cells showed a 3-fold change and 4-fold change in response to 25(OH)D$_3$ and 1,25(OH)$_2$D$_3$ respectively. For all other time points, 25(OH)D$_3$ and 1,25(OH)$_2$D$_3$ showed around 1.5 fold-change (Figure 4.9).

In HCEC-12 cells, BD-2 was increased by 7 fold at 8 h and decreased to 2 fold at 1 h and 5 fold at 16 h in response to 25(OH)D$_3$. At 16 and 24 h, levels declined to 2.5 and 2 fold respectively. Upon treatment with 1,25(OH)$_2$D$_3$, BD-2 was increased 2.5 fold at 4 h and 10 fold at 8 h then decreased to 3 fold at 16 h. ODM-2 cells showed a 2-fold change in BD-2 at 4 h with both 25(OH)D$_3$ and 1,25(OH)$_2$D$_3$, a decline at 8 h, followed by a 3-fold increase by 25(OH)D$_3$ at 16 h and 1,25(OH)$_2$D$_3$ at 24 h. ARPE-19 showed a 1.5 fold change at 4 h with both 25(OH)D$_3$ and 1,25(OH)$_2$D$_3$. At 8 h, treatment with 25(OH)D$_3$ maintained a 1.5 fold change while it decreased with 1,25(OH)$_2$D$_3$. There was a noticeable 10-fold change and 4 fold-change at 16 h in response to 25(OH)D$_3$ and 1,25(OH)$_2$D$_3$ respectively then levels declined at 24 h with both treatments (Figure 4.10).

HCEC-12 cells showed about 1.5-fold and 2-fold change in the expression of BD-3 at 24 h in response to both 25(OH)D$_3$ and 1,25(OH)$_2$D$_3$ respectively while there was no induction at other time points. ODM-2 showed a 1.5 fold change with 25(OH)D$_3$ at 4 and 8 h that declined at 16 and 24 h. BD-3 was upregulated in response to 1,25(OH)$_2$D$_3$ at 8 and 24 h while there was no induction at 16 h. In ARPE-19 cells, BD-3 induction was the highest with 5 fold in response to 25(OH)D$_3$ at 4 h that declined to 3 fold at 8 h. Treatment with 1,25(OH)$_2$D$_3$ caused a 3-fold increase at 4 h that declined to about 1.5 fold at 8, 16, and 24 h (Figure 4.11).

BD-4 showed a 2-fold increase in HCEC-12 cells at 1 and 4 h by 1,25(OH)$_2$D$_3$ that decreased at 8 h then increased again to 3 fold at 16 h and 4 fold at 24 h. BD-4 was not induced in response to 25(OH)D$_3$ at early time points but showed a 3 fold change at 24 h

in HCEC-12 cells . ODM-2 cells also showed a 1.5 fold induction at 8 h that increased to 6 fold at 16 h with 25(OH)D$_3$. Treatment with 1,25(OH)$_2$D$_3$ induced a 3 fold-change at 1 and 4 h followed by a decline at 8 and 16 h, and up to 2.5-fold change at 24 h. In ARPE-19 cells, BD-4 expressed a 5.5 fold-change with 25(OH)D$_3$ and decreased to 3 fold-change at 16 h and declined at 24 h. Treatment with 1,25(OH)$_2$D$_3$ induced a 3 fold-change at 8 h, increased to 5 fold-change at 16 h and declined at 24 h (Figure 4.12).

To conclude, ocular barrier epithelial cells showed a trend towards the upregulation of mRNA expression of hBD1-4 but more repeats are required to draw conclusive results. Raw data are shown in the appendix.

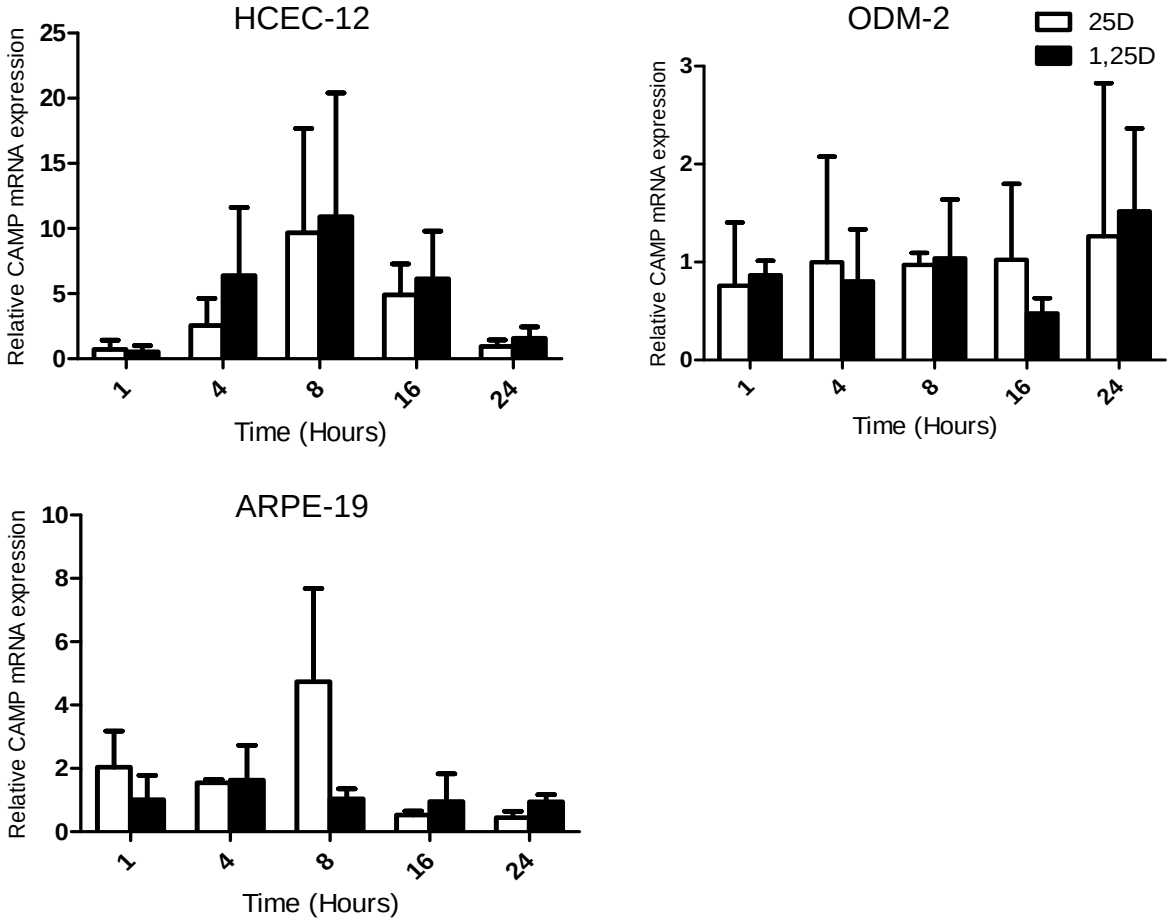

Figure 4.8. Vitamin D$_3$ upregulates mRNA expression of cathelicidin antimicrobial peptide (CAMP) in ocular barrier epithelial cells. HCEC-12, ODM-2, and ARPE-19 cells were grown to 80-90% confluence and they were either left untreated (control) or treated with 25(OH)D$_3$ (10^{-7} M) or 1,25(OH)$_2$D$_3$ (10^{-8} M) for 1, 4, 8, 16, and 24 h in serum free conditions. Total RNA was isolated and real-time PCR was used to measure relative fold change increase in treated samples compared to controls for CAMP. CAMP was only upregulated in HCEC-12 cells in response to both 25(OH)D$_3$ and 1,25(OH)$_2$D$_3$ at 4, 8, and 16 h. ODM-2 cells did not upregulate CAMP but ARPE-19 cells slightly upregulated CAMP with 25(OH)D$_3$ at 8h. This upregulation was not statistically significant (for raw data please see appendix). Graphs show mean±SD (results of at least 3 experiments are shown). Two-way ANOVA and Bonferroni's post test were performed.

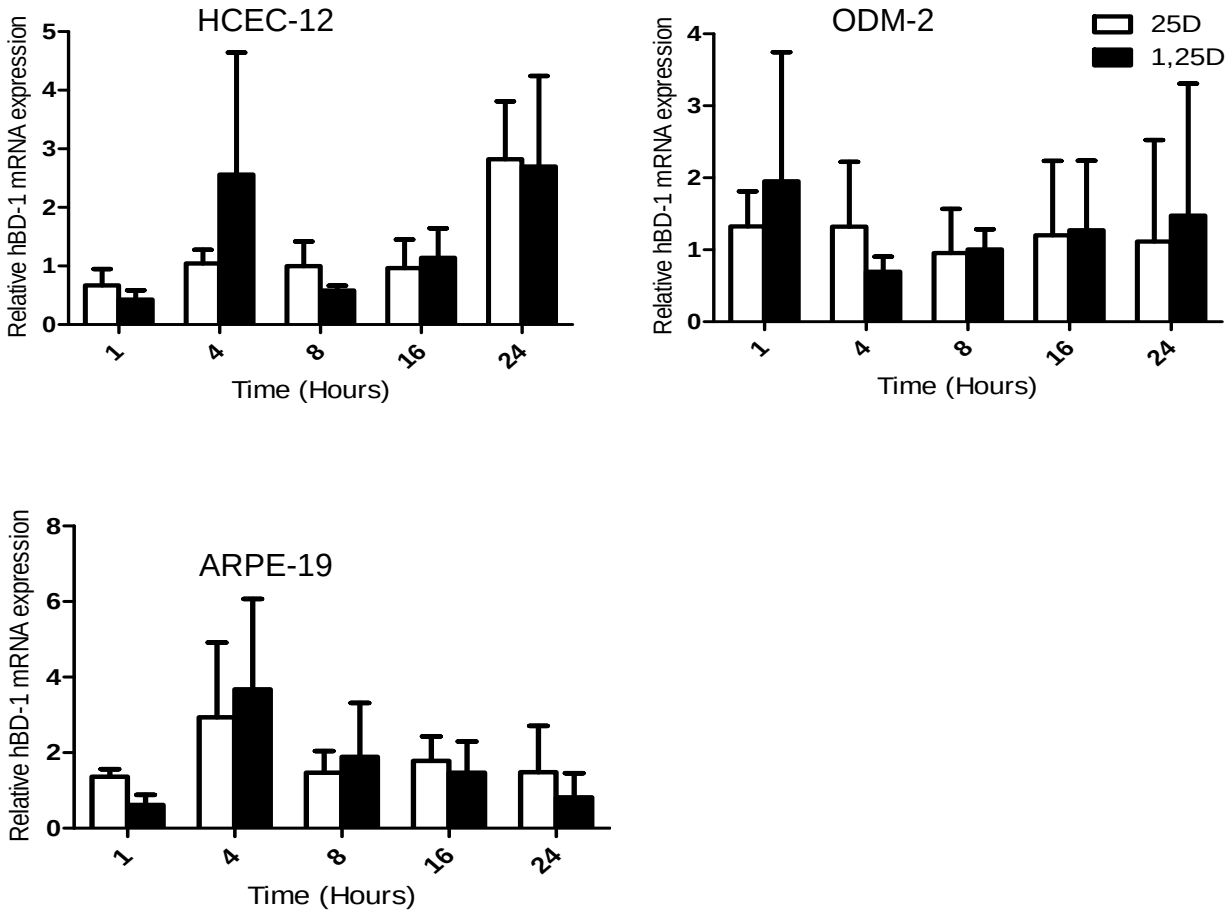

Figure 4.9. Vitamin D$_3$ upregulates mRNA expression of human beta defensin-1 (hBD-1) in ocular barrier epithelial cells. HCEC-12, ODM-2, and ARPE-19 cells were grown to 80-90% confluence and were either left untreated (control) or treated with 25(OH)D$_3$ (10^{-7} M) or 1,25(OH)$_2$D$_3$ (10^{-8} M) for 1, 4, 8, 16, and 24 h in serum free media. Total RNA was isolated and real-time PCR was used to measure relative fold change increase in treated samples compared to controls for hBD-1. HECE-12 cells slightly upregulated hBD-1 with 25(OH)D$_3$ at 4 h and with both 25(OH)D$_3$ and 1,25(OH)$_2$D$_3$ at 24 h. ODM-2 cells did not upregulate hBD-1 whiles ARPE-19 cells showed an upregulation with both 25(OH)D$_3$ and 1,25(OH)$_2$D$_3$ at 4 h. This upregulation was not statistically significant (for raw data please see appendix). Graphs show mean±SD (results of at least 3 experiments are shown). Two-way ANOVA and Bonferroni's post test were performed.

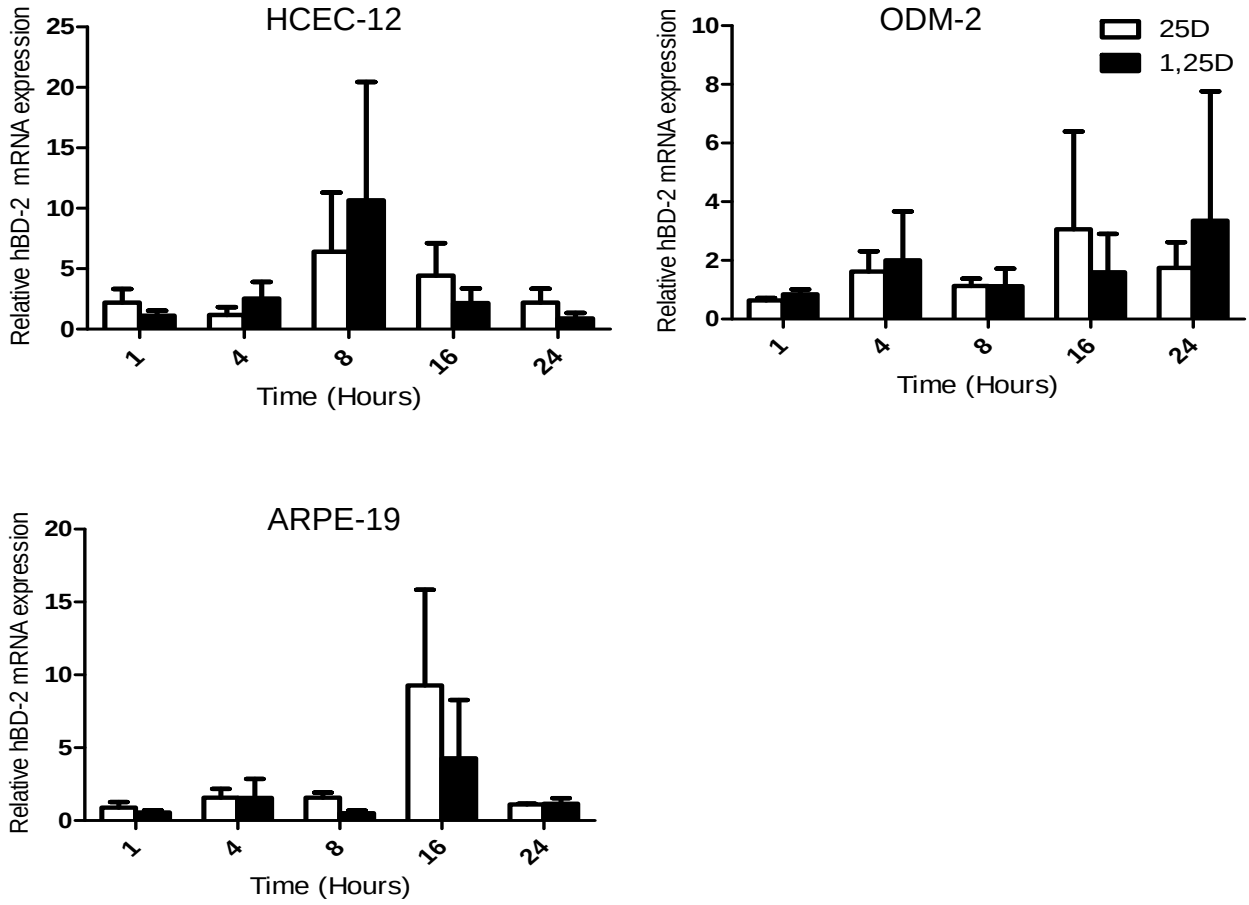

Figure 4.10. Vitamin D$_3$ upregulates mRNA expression of human beta defensin -2 (hBD-2) in ocular barrier epithelial cells. HCEC-12, ODM-2, and ARPE-19 cells were grown to 80-90% confluence and were either left untreated (control) or treated with 25(OH)D$_3$ (10^{-7} M) or 1,25(OH)$_2$D$_3$ (10^{-8} M) for 1, 4, 8, 16, and 24 h in serum free media. Total RNA was isolated and real-time PCR was used to measure relative fold change increase in treated samples compared to controls for hBD-2. HCEC-12 cells showed an upregulation of hBD-2 with both 25(OH)D$_3$ and 1,25(OH)$_2$D$_3$ at 8 h and with 25(OH)D$_3$ at a6 h. ODM-2 did not show an upregulation of hBD-2 but ARPE-19 cells upregulated hBD-2 with both 25(OH)D$_3$ and 1,25(OH)$_2$D$_3$ at 16 h. This upregulation was not statistically significant (for raw data please see appendix). Graphs show mean±SD (results of at least 3 experiments are shown). Two-way ANOVA and Bonferroni's post test were performed.

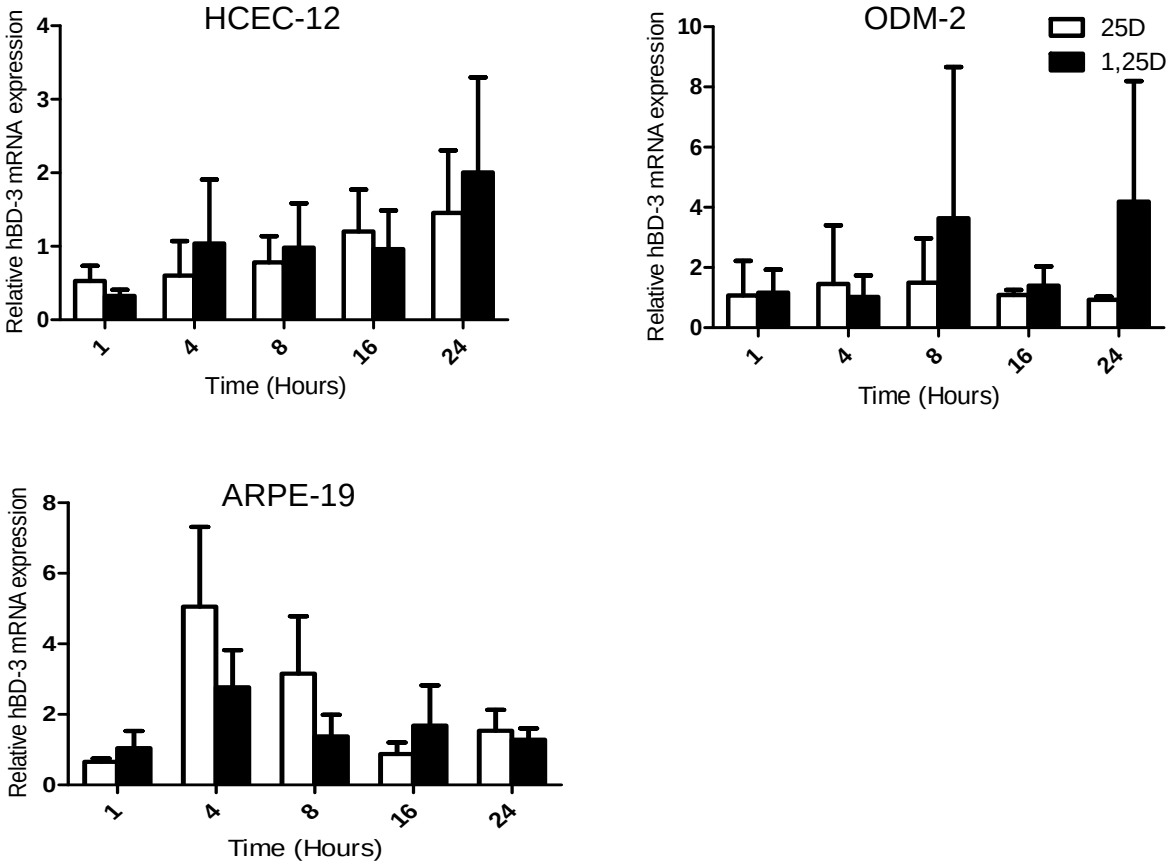

Figure 4.11. Vitamin D$_3$ upregulates mRNA expression of human beta defensin -3 (hBD-3) in ocular barrier epithelial cells. HCEC-12, ODM-2, and ARPE-19 cells were grown to 80-90% confluence and were either left untreated (control) or treated with 25(OH)D$_3$ (10^{-7} M) or 1,25(OH)$_2$D$_3$ (10^{-8} M) for 1, 4, 8, 16, and 24 h in serum free media. Total RNA was isolated and real-time PCR was used to measure relative fold change increase in treated samples compared to controls for hBD-3. HCEC-12 and ODM-2 cells did not upregualte hBD-3 while ARPE-19 cells showed an upregulation with 25(OH)D$_3$ at 4 and 8 h. This upregulation was not statistically significant (for raw data please see appendix). Graphs show mean±SD (results of at least 3 experiments are shown). Two-way ANOVA and Bonferroni's post test were performed.

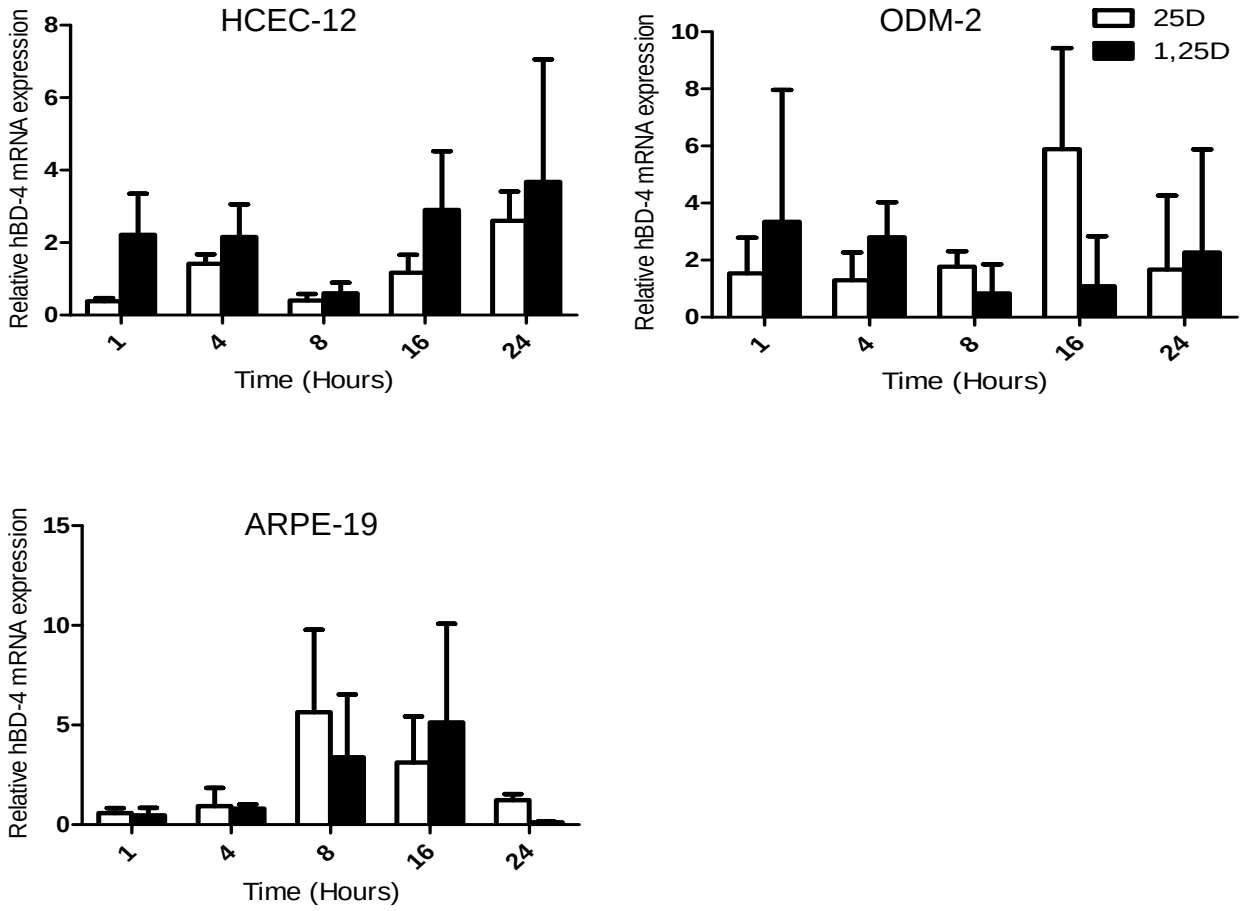

Figure 4.12. Vitamin D$_3$ upregulates mRNA expression of human beta defensin -4 (hBD-4) in ocular barrier epithelial cells. HCEC-12, ODM-2, and ARPE-19 cells were grown to 80-90% confluence and were either left untreated (control) or treated with 25(OH)D$_3$ (10^{-7} M) or 1,25(OH)$_2$D$_3$ (10^{-8} M) for 1, 4, 8, 16, and 24 h in serum free media. Total RNA was isolated and real-time PCR was used to measure relative fold change increase in treated samples compared to controls for hBD-4. HECE-12 and ODM-2 cells did not show any upregulation of hBD-4 while ARPE-19 cells showed an upregulation with 25(OH)D$_3$ at 8 h and 1,25(OH)$_2$D$_3$ at 16 h. This upregulation was not statistically significant (for raw data please see appendix). Graphs show mean±SD (results of at least 3 experiments are shown). Two-way ANOVA and Bonferroni's post test were performed.

4.2.4 Ocular barrier cells do not increase HDP proteins in response to endogenous and exogenous 1,25(OH)$_2$D$_3$ by ELISA.

To investigate whether ocular barrier cells can translate HDP mRNA into protein in response to 25(OH)D$_3$ (10^{-7} M) or 1,25(OH)$_2$D$_3$ (10^{-8} M), we measured HDPs in the culture supernatants after 24 h of treatment.

HCEC-12 showed higher baseline production of BD-1 compared to ODM-2 and ARPE-19 cells but there was no significant difference between control cells or those treated with either 25(OH)D$_3$ or 1,25(OH)$_2$D$_3$ in all cell types. Similar results were observed in M1 and M2 (Figures 4.13 and 4.16). HCEC-12 followed by ODM-2 produced higher amounts of BD-2 compared to ARPE-19 cells. Again there was no significant difference between control and treated cells in all cell types. M1 and M2 cells hardly produced any BD-2 (Figures 4.14 and 4.16). HCEC-12 and ODM-2 cells produced higher BD-4 than ARPE-19 cells. There was a slight increase in BD-4 in response to 1,25(OH)$_2$D$_3$ in ODM-2 cells. Similar to BD-1 and 2, there was no significant difference between control and treated cells in all cell types (Figure 4.15). Macrophages seem to produce higher BD-4 than BD-1 and 2. There was a slight increase in BD-4 produced by M1 and M2 in response to 1,25(OH)$_2$D$_3$ (Figure 4.16).

To conclude, there was a wide-range of protein expression between different experiments which is surprising for cell lines. However the data taken together shows that ocular barrier cells did not upregulate protein for HDPs in response to 25(OH)D$_3$ or 1,25(OH)$_2$D$_3$ respectively.

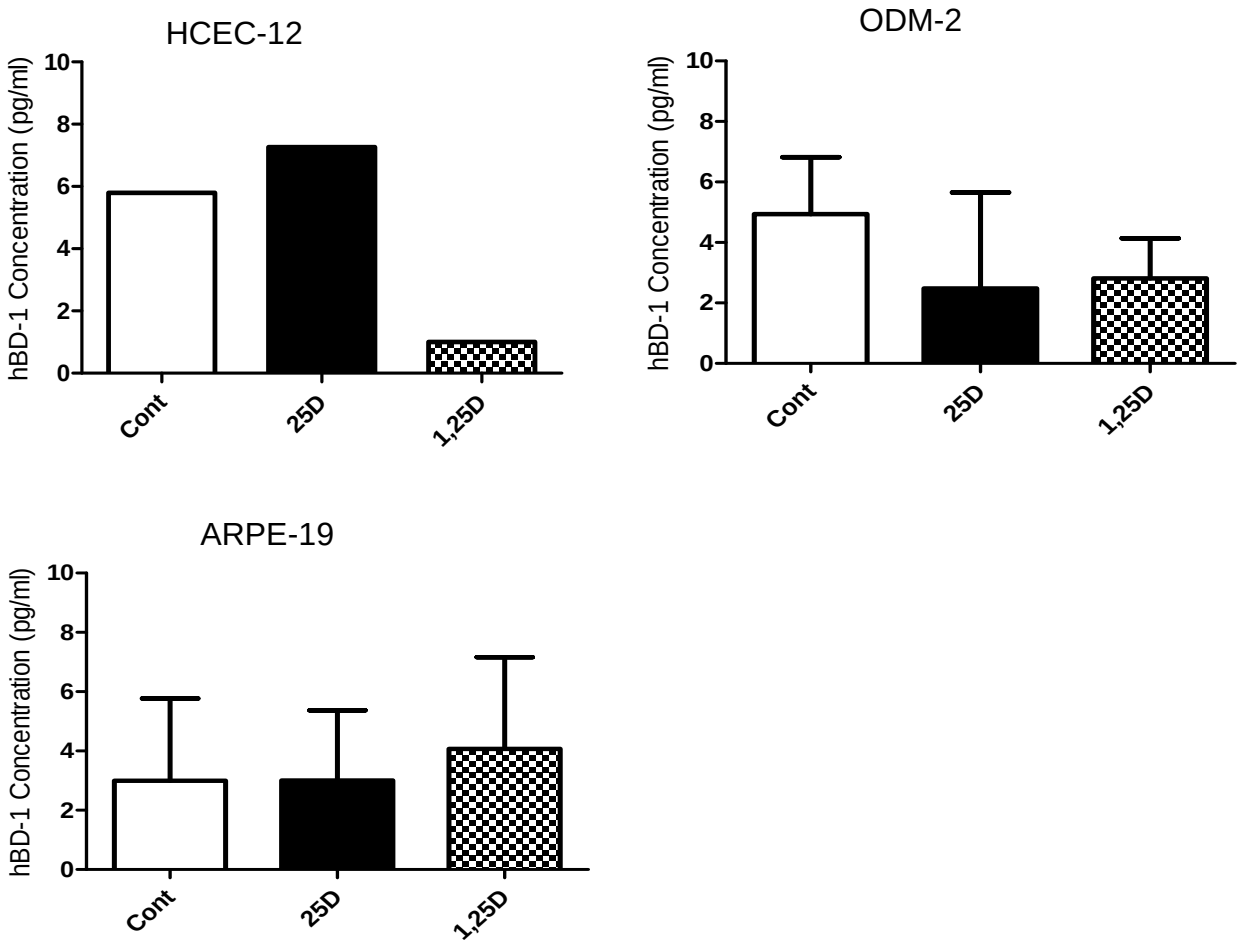

Figure 4.13. Ocular barrier epithelial cells do not upregulate human β definsin-1 (hBD-1) protein in response to 25(OH)D$_3$ or 1,25(OH)$_2$D$_3$. HECE-12, ODM-2, and ARPE-19 cells were grown to confluence and they were wither left untreated (control) or treated with 25(OH)D$_3$ (10^{-7} M) or 1,25(OH)$_2$D$_3$ (10^{-8} M) for 1, 4, 8, 16, and 24 h in serum free media. Supernatants were collected and ELISA was performed to measure the concentrations of hBD-1. Minimum amounts of hBD-1 were produced by ocular barrier epithelial cells and there was no difference in hBD-1 concentrations between control (untreated) cells or those treated with 25(OH)D$_3$ or 1,25(OH)$_2$D$_3$. Graphs show mean±SD (each experiment was performed at least 3 times except HCEC-12 n=1). Kruskall-Wallis test with Dunn's post test were performed.

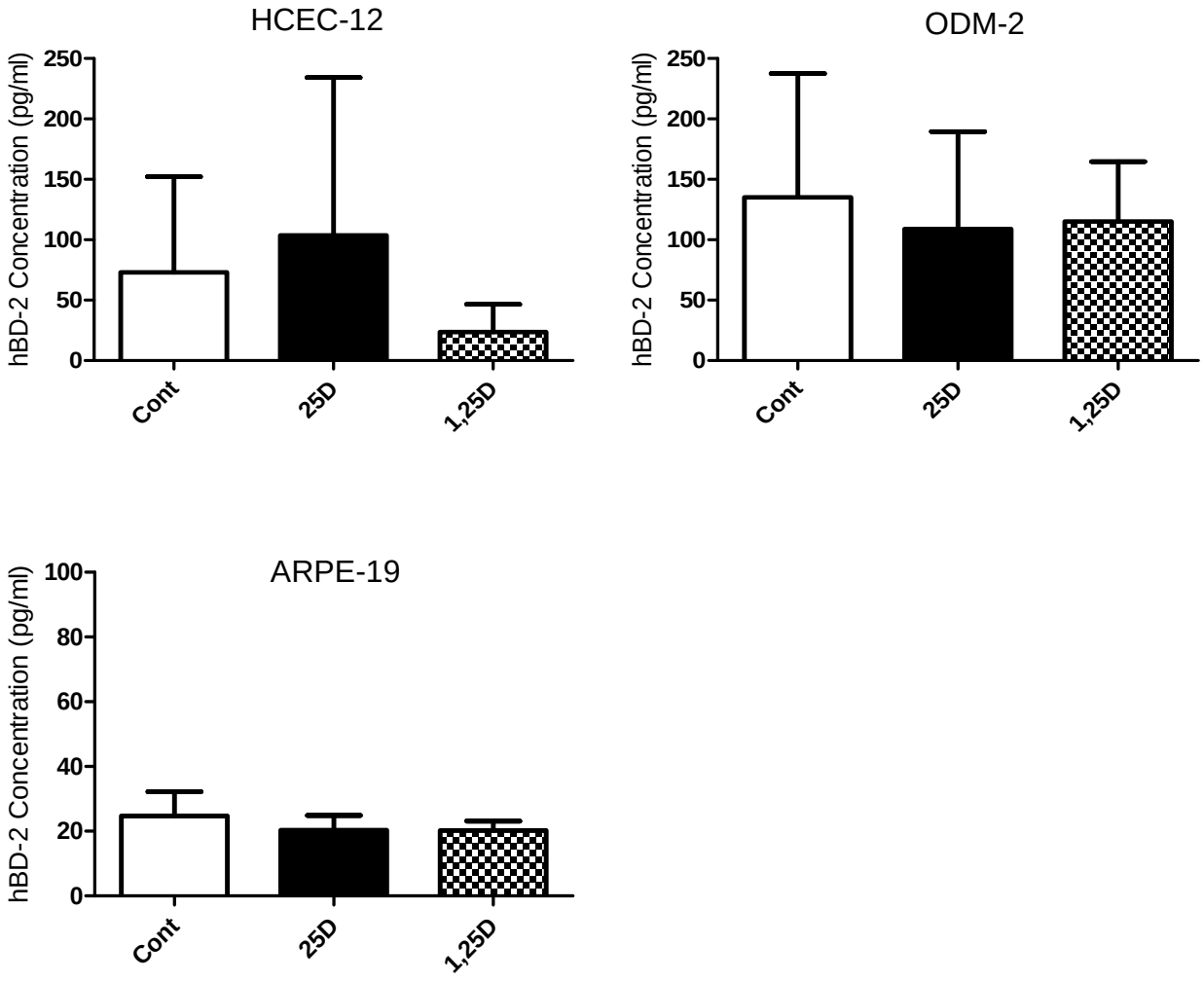

Figure 4.14. Ocular barrier epithelial cells do not upregulate human β definsin-2 (hBD-2) protein in response to 25(OH)D$_3$ or 1,25(OH)$_2$D$_3$. HECE-12, ODM-2, and ARPE-19 cells were grown to confluence and they were wither left untreated (control) or treated with 25(OH)D$_3$ (10^{-7} M) or 1,25(OH)$_2$D$_3$ (10^{-8} M) for 1, 4, 8, 16, and 24 h in serum free media. Supernatants were collected and ELISA was performed to measure the concentrations of hBD-2. There was a constitutive protein expression of hBD-2 in HCEC-12, ODM-2, and ARPE-19 cells but this was not upregulated upon treatment with 25(OH)D$_3$ or 1,25(OH)$_2$D$_3$. Graphs show mean±SD (each experiment was performed at least 3 times). Kruskall-Wallis test with Dunn's post test were performed.

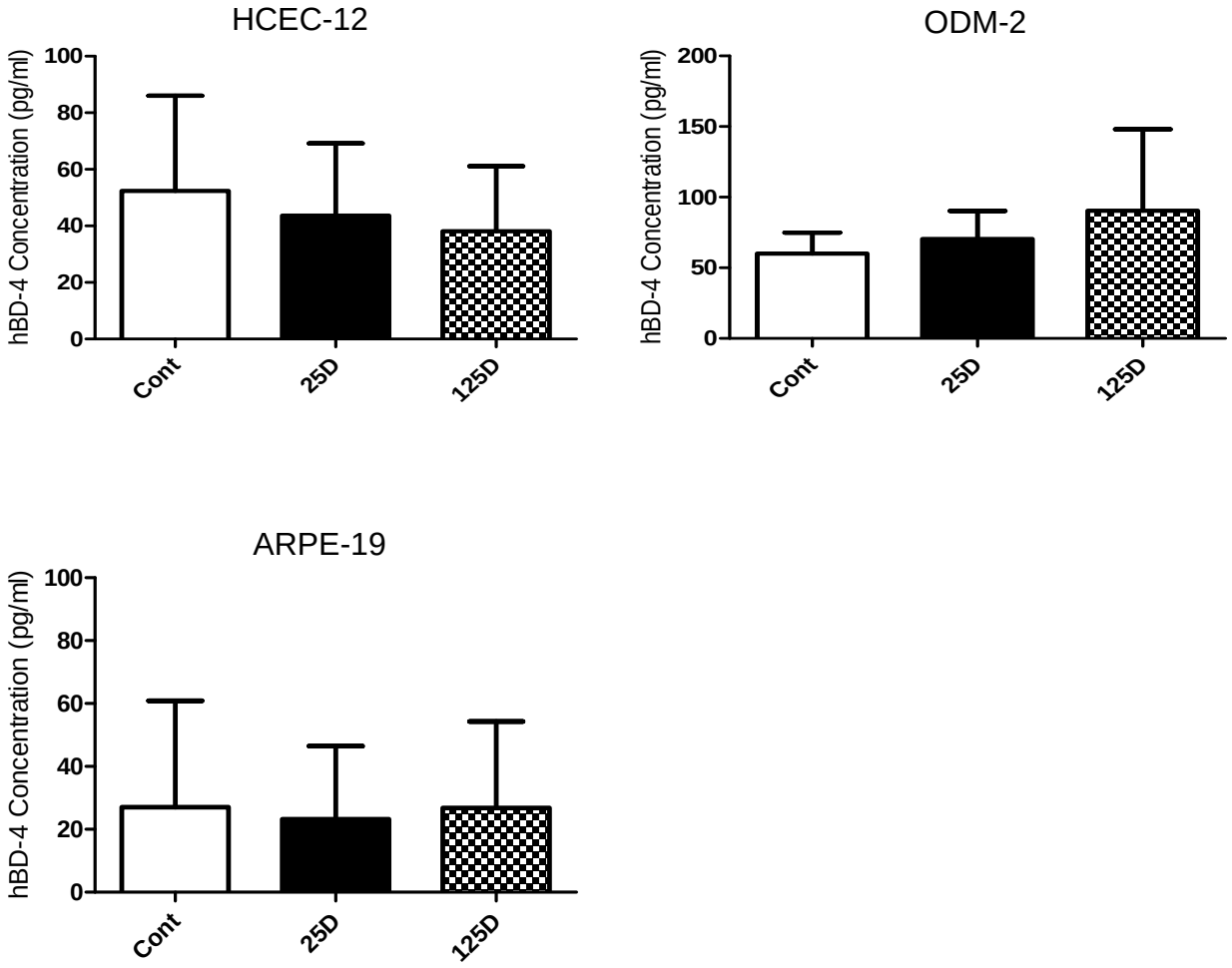

Figure 4.15. Ocular barrier epithelial cells do not upregulate human β definsin-4 (hBD-4) protein in response to 25(OH)D$_3$ or 1,25(OH)$_2$D$_3$. HECE-12, ODM-2, and ARPE-19 cells were grown to confluence and they were wither left untreated (control) or treated with 25(OH)D$_3$ (10^{-7} M) or 1,25(OH)$_2$D$_3$ (10^{-8} M) for 1, 4, 8, 16, and 24 h in serum free media. Supernatants were collected and ELISA was performed to measure the concentrations of hBD-4. There was a constitutive protein expression of hBD-2 in HCEC-12, ODM-2, and ARPE-19 cells but this was not upregulated upon treatment with 25(OH)D$_3$ or 1,25(OH)$_2$D$_3$. Graphs show mean±SD (each experiment was performed at least 3 times). Kruskall-Wallis test with Dunn's post test were performed.

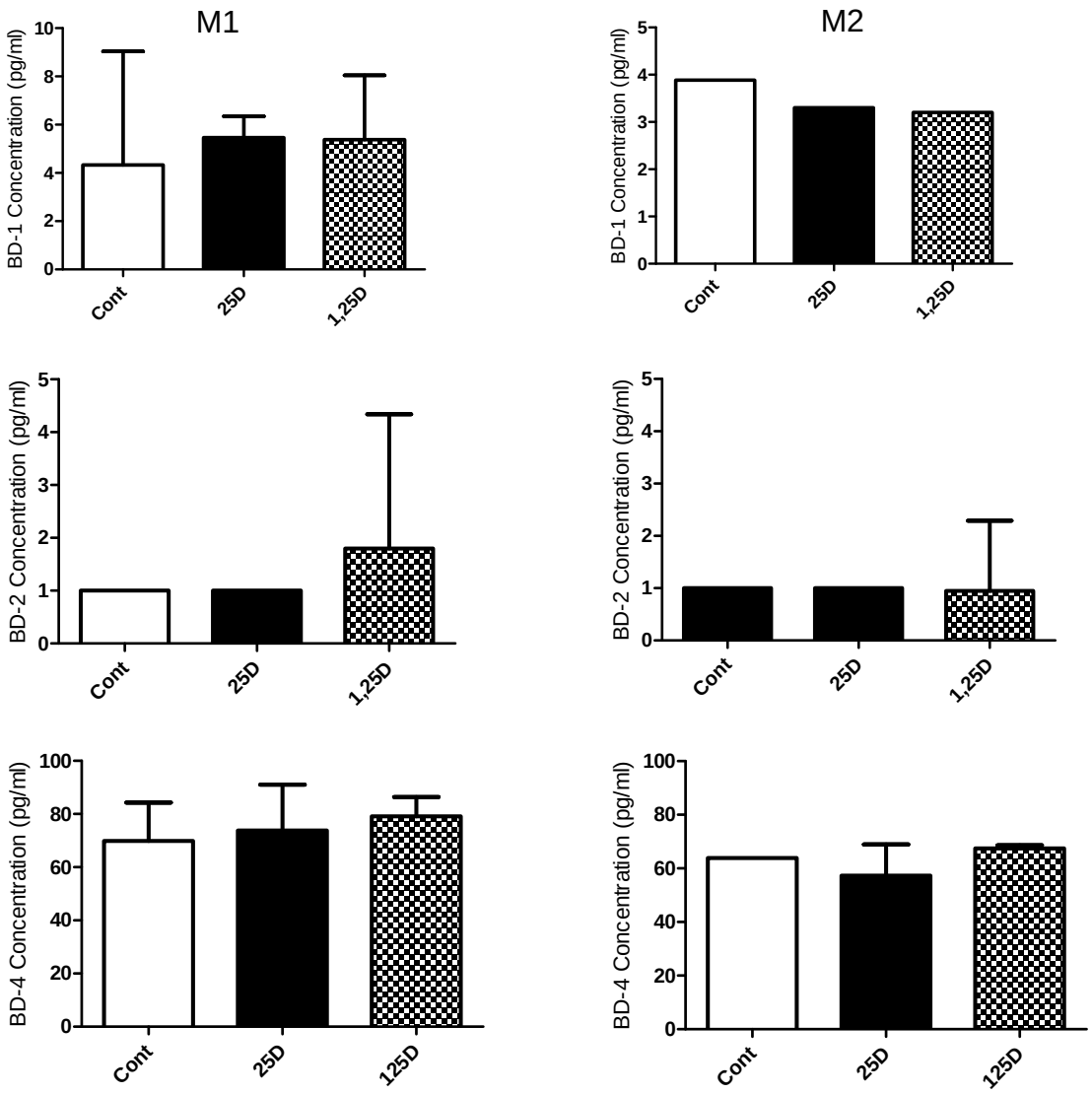

Figure 4.16. Macrophages do not upregulate HDP proteins in response to 25(OH)D$_3$ or 1,25(OH)$_2$D$_3$. M1 and M2 were differentiated from CD14$^+$ monocytes. On day 6 they were wither left untreated or treated with 25(OH)D$_3$ (10^{-7} M) or 1,25(OH)$_2$D$_3$ (10^{-8} M) for 24h in serum free media. Supernatants were collected and ELISA was performed to measure the concentrations of BD-1 (top panel), 2 (middle panel), and 4 (bottom panel). M1 and M2 macrophages did not produce hBD1, 2, and 4 and these levels were not upregulated upon treatment with 25(OH)D$_3$ or 1,25(OH)$_2$D$_3$. Graphs show mean±SD (Experiments with SD bars represents triplicates while the absence of SD indicates a single experiment). Kruskall-Wallis test with Dunn's post test were performed.

4.2.5 Proinflammatory cytokines did not induce HDP proteins in ocular barrier cells.

In macrophages, IL-1β mediated the vitamin D_3 induced upregulation of *DEFB4* (Liu et al., 2009b). Previous work in our lab has also shown the same results (Susarla et al. manuscript submitted). We tested the ability of IL-1β and TNF-α to synergise with 25(OH)D_3 (10^{-7} M) or 1,25(OH)$_2$D$_3$ (10^{-8} M) to upregulate HDP proteins. As shown above, both ODM-2 and ARPE-19 cells did not produce high amounts of BD-1, 2, and 4 in their supernatants.

Untreated HCEC-12 produced higher constitutive levels of BD-1 compared to ODM-2 and ARPE-19 cells. Treatment with IL-1β did not alter this production when added alone or with 25(OH)D_3 or 1,25(OH)$_2$D$_3$ in all cell types. In contrast treatment TNF-α doubled the concentration of BD-1 when added alone and in with 1,25(OH)$_2$D$_3$ respectively and induced a slight increase when added with 25(OH)D_3 in HCEC-12 cells. In ARPE-19 cells, TNF-α showed a slight increase in BD-1 production alone or with 25(OH)D_3 respectively but no change with 1,25(OH)$_2$D$_3$. In ODM-2 cells, there was no change upon the addition of either IL-1β or TNF-α alone or with 25(OH)D_3 or 1,25(OH)$_2$D$_3$ respectively (Figure 4.17).

ODM-2 and ARPE-19 produced a higher baseline production of BD-2 than HCEC-12 cells. BD-2 concentrations increased 2 and 3 fold upon treatment with IL-1β or TNF-α alone in HCEC-12 cells. In the presence of 25(OH)D$_3$, TNF-α doubled the concentration of BD-2 while IL-1β did not show any change. Both cytokines did not alter BD-1 production in the presence of 1,25(OH)$_2$D$_3$ in HCEC-12 cells. When IL-1β or TNF-α were added alone to ODM-2 and ARPE-19 cells, there was a slight decrease in the concentration of BD-2 compared to untreated cells. Both cytokines induced a slight decrease in BD-2 when added in the presence of 25(OH)D$_3$ and a slight increase with IL-1β and IL1,25(OH)$_2$D$_3$ respectively in ODM-2 cells (Figure 1.18).

HCEC-12, ODM-2, and ARPE-19 cells produced similar levels of BD-4 and the treatment with IL-1β or TNF-α in the presence or absence of 25(OH)D$_3$ or 1,25(OH)$_2$D$_3$ did not cause any change in BD-4 concentration (Figure 1.19).

In conclusion, the results show only HCEC-12 upregulated BD-1 and 2 production in response to TNF-α alone and BD-2 in response to IL-1β alone respectively. TNF-α synergised with1,25(OH)$_2$D$_3$ to increase BD-1 levels and with 25(OH)D$_3$ to upregulate BD-2. Due to the low number of repeats and the lack of statistical significance, more experiments are required to confirm these results.

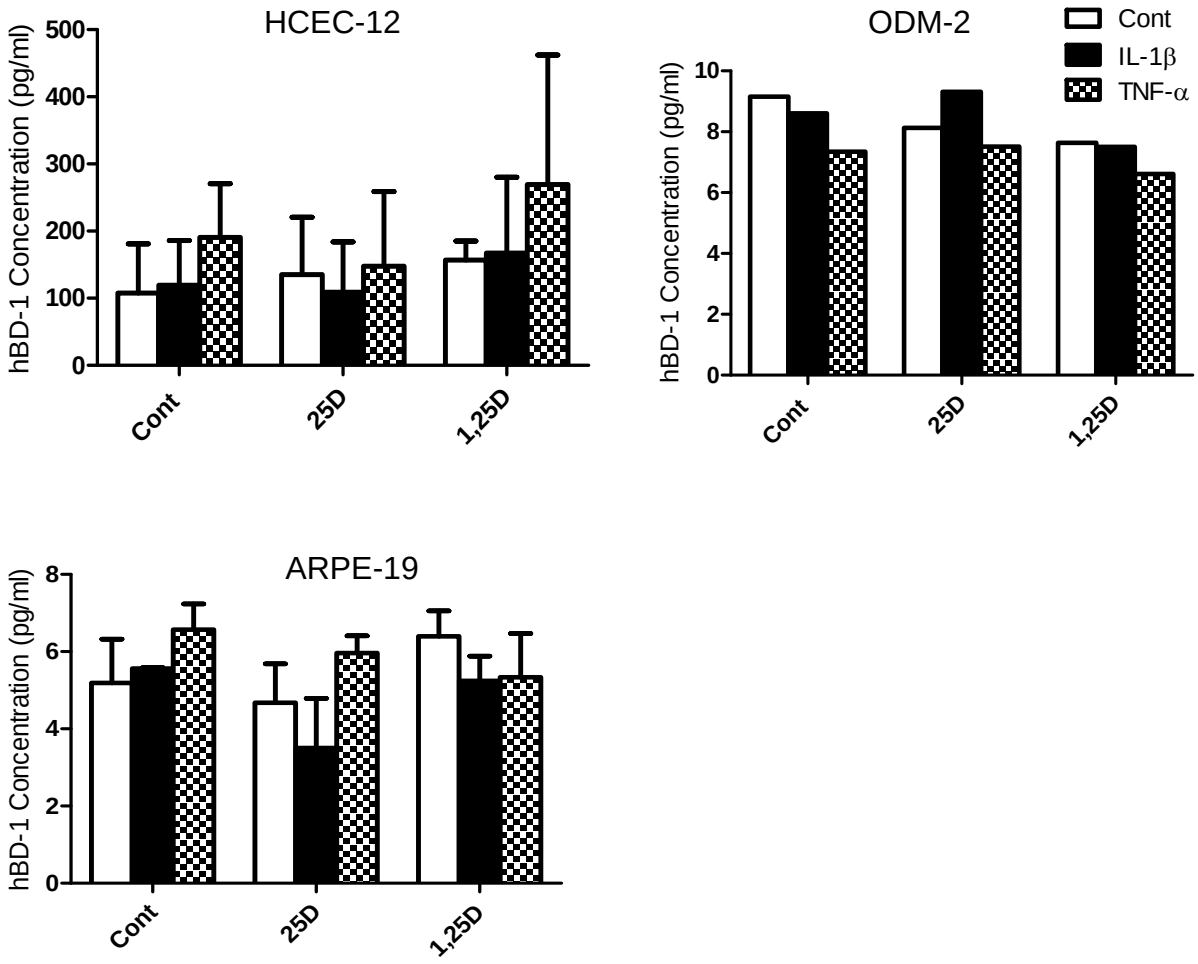

Figure 4.17. IL-1β and TNF-α did not upregulate protein production of human β defensin-1 (hBD-1), in the presence of vitamin D$_3$ in ocular barrier epithelial cells. HCEC-12, ODM-2, and ARPE-19 Cells were grown to confluence and were either treated with IL-1β (1 ng/ml) or TNF-α (5 ng/ml) in the presence or absence of 25(OH)D$_3$ (10^{-7} M) or 1,25(OH)$_2$D$_3$ (10^{-8} M) for 24 h in serum free conditions. Supernatants were collected and hBD-1 concentrations were measured by ELISA. Treatment of ocular barrier epithelial cells with IL-1β or TNF-α did not alter hBD-1 production. Graphs show mean±SD (HCEC-12 n=2, ODM-2 n=1, and ARPE-19 n=2).

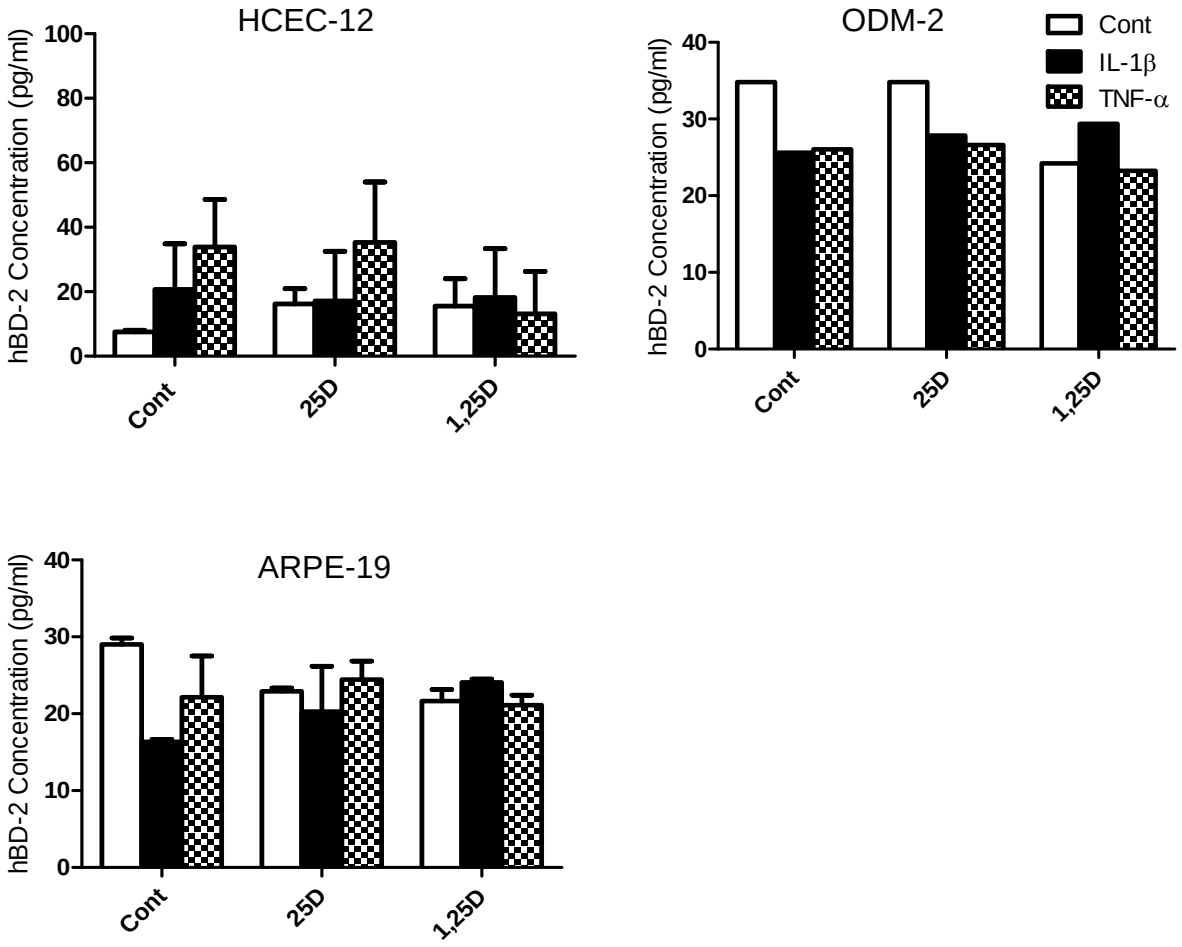

Figure 4.18. IL-1β and TNF-α did not upregulate protein production of human β defensin-2 (hBD-2), in the presence of vitamin D_3 in ocular barrier epithelial cells. HCEC-12, ODM-2, and ARPE-19 Cells were grown to confluence and were either treated with IL-1β (1 ng/ml) or TNF-α (5 ng/ml) in the presence or absence of 25(OH)D_3 (10^{-7} M) or 1,25$(OH)_2D_3$ (10^{-8} M) for 24 h in serum free conditions. Treatment of ocular barrier epithelial cells with IL-1β or TNF-α did not alter hBD-2 production. Supernatants were collected and hBD-2, was measured by ELISA. Graphs show mean±SD (HCEC-12 n=2, ODM-2 n=1, and ARPE-19 n=2).

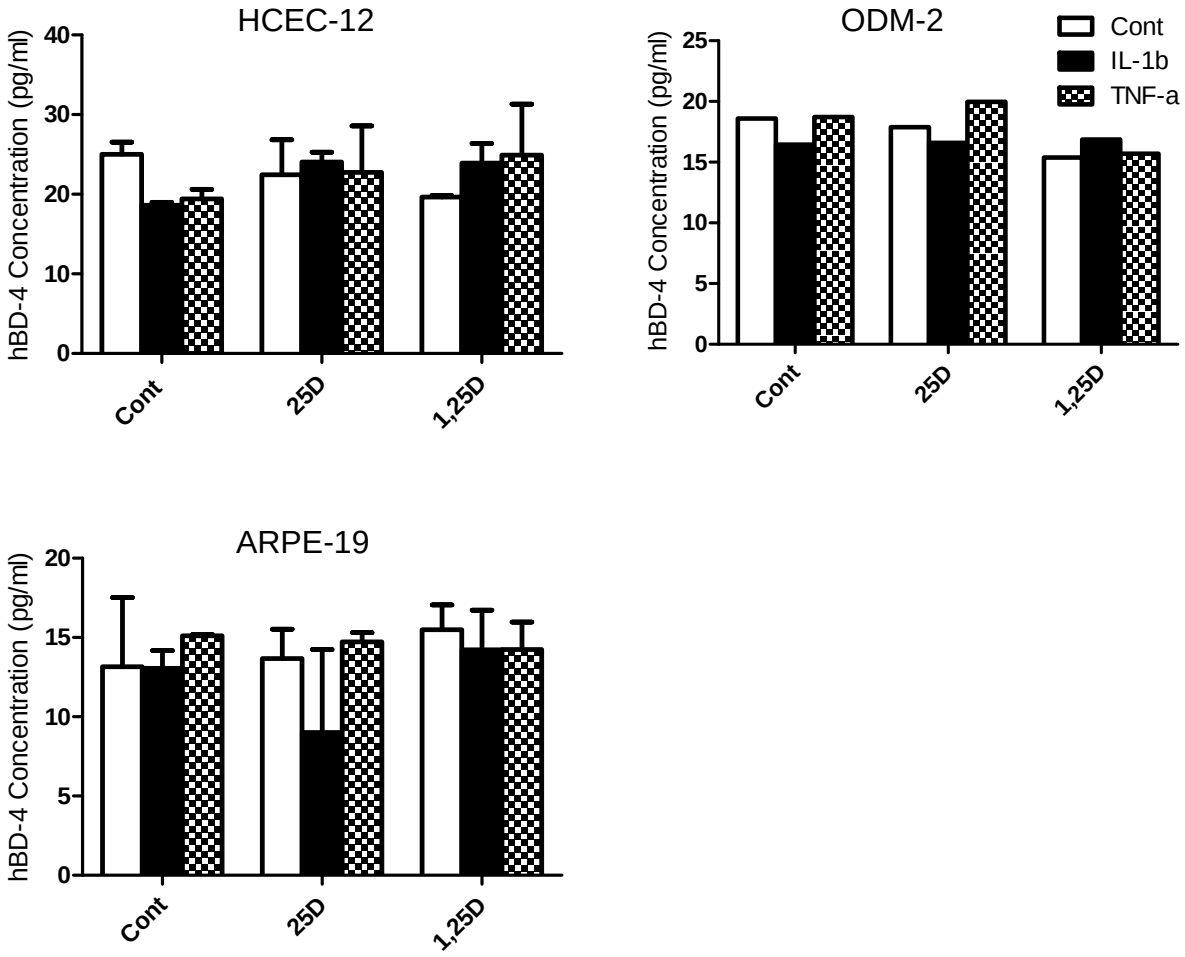

Figure 4.19. IL-1β and TNF-α did not upregulate protein production of human β defensin-4 (hBD-4), in the presence of vitamin D$_3$ in ocular barrier epithelial cells. HCEC-12, ODM-2, and ARPE-19 Cells were grown to confluence and were either treated with IL-1β (1 ng/ml) or TNF-α (5 ng/ml) in the presence or absence of 25(OH)D$_3$ (10^{-7} M) or 1,25(OH)$_2$D$_3$ (10^{-8} M) for 24 h in serum free conditions. Treatment of ocular barrier epithelial cells with IL-1β or TNF-α did not alter hBD-4 production.Supernatants were collected and hBD-4, was measured by ELISA. Graphs show mean±SD (HCEC-12 n=2, ODM-2 n=1, and ARPE-19 n=2).

4.3 Discussion

This study shows that ocular barrier cells can signal through VDR to upregulate the downstream vitamin D_3 elements genes. It also shows that like exogenous 1,25(OH)$_2$D$_3$, local activation of vitamin D_3 by ocular barrier cells showed a trend towards the induction of mRNA of HDPs but not proteins at the time point tested. IL-1β and TNF-α did not significantly upregulate hBD-1, 2 and 4 ocular barrier epithelial cells tested in the presence of vitamin D_3. TLR signalling does not seem to synergise with vitamin D_3 in the induction of proinflammatory cytokines.

Extra-renal expression of vitamin D_3 elements has been described in many epithelial cells such as primary respiratory epithelial cells, urinary bladder intestinal, mammary, endometrium, and prostate epithelial cell lines and skin keratinocytes (Chen and Holick, 2003;Hansdottir et al., 2008;Hertting et al., 2010;Kemmis and Welsh, 2008;Kong et al., 2008). In the kidneys, synthesis of 1,25(OH)$_2$D$_3$ is tightly regulated by serum levels of calcium, phosphorous, and fibroblasts growth factor-23 (FGF-23). Additionally, 1,25(OH)$_2$D$_3$ provides its own negative feedback by decreasing the secretion of parathyroid hormone (PTH) from parathyroid gland (Holick, 2007).

In contrast, extra-renal 1,25(OH)$_2$D$_3$ synthesis is regulated by inflammatory cytokines such as IFN-γ and IL-15 and through the induction of CYP24A1 (Adams and Hewison, 2012). In epithelial cells such keratinocytes, mammary, prostate and respiratory epithelial cells, CYP24A1 was only induced after local conversion of 25(OH)D$_3$ into 1,25(OH)$_2$D$_3$ or stimulation with 1,25(OH)$_2$D$_3$ (Flanagan et al., 2006;Hansdottir et al., 2008;Welsh, 2011;Xie et al., 2002) . Ocular barrier epithelial cells

are no different from these cells where they appear to constitutively express CYP24A1 which was upregulated in response to local synthesis of 1,25(OH)$_2$D$_3$.

A growing body of evidence in literature has confirmed the importance of extra-renal vitamin D$_3$ system for host protection mainly through the induction of HDPs. In macrophages, skin, gingival, placenta, and respiratory epithelial cells, vitamin D$_3$ acted with TLR to induce protective mechanisms (Hansdottir et al., 2008;Liu et al., 2009a;Liu et al., 2006;McMahon et al., 2011;Schauber et al., 2007). We know that ocular cells from the cornea to the retina express a range of PRR including TLR1-10 (Brito et al., 2004;Chang et al., 2006;Kumar and Yu, 2006;Kumar et al., 2004;Ueta and Kinoshita, 2010). RIG-I and MDA5 have been detected in human conjunctival epithelial and RPE cells (Ueta et al., 2010;Ueta et al., 2011;Wornle et al., 2011).

Our results confirm the expression of TLR1-10, RIG-I, and MDA5 in ocular barrier cells and that these receptors are functional upon stimulation. We approached this by the measurement of IL-8 and IL-6 which are produced in response to TLR stimulation in the eye (Cook et al., 2005;Jin et al., 2010;Kumagai et al., 2005;Ueta et al., 2005). Human corneal endothelial cell line increased the production of IL-6 after stimulation of TLR9 (Takeda et al., 2011). Primary human non-pigmented ciliary body epithelial cells produced IL-6 in response to LPS (25 ng) (Brito et al., 2004).

We could show that HCEC-12 and ARPE-19 cells produced the highest levels of IL-8 and IL-6 in response to Poly I:C. In contrast, ODM-2 cells did not upregulate IL-8 or IL-6 after treatment with TLR ligands including LPS. ARPE-19 cells produced high amounts of IL-6 in response to LPS (1ug) (Paimela et al., 2007). We could not replicate

these results with LPS as we used very low concentrations of LPS to try and simulate what can happen in real life. ARPE-19 cells were the only cells that produced high amounts of IL-8 and slightly increased IL-6 in response to RIG-I/MDA5. This has been seen before in ARPE-19 cells that showed a similar increase in IL-8 and IL-6 expression after treatment with Poly I:C that can bind both TLR3 and RIG-I (Wornle et al., 2011). We previously showed that TLR stimulation increased IL-8 and IL-6 but this was not influenced by vitamin D_3 in primary human corneal epithelial cells (PHCEC) and corneal fibroblasts (HKF) (Susarla et al. manuscript submitted). Although some TLR are expressed strongly in ocular barrier cells, we could not detect any synergy between TLR signalling and vitamin D_3 in the production of proinflammatory cytokines.

Ocular barrier cells very poorly express TLR2 which is predominantly expressed in high levels in $CD14^+$ monocytes (Flo et al., 2001;Foster et al., 2007). This may be part of vitamin D_3 role as an immunomodulatory hormone to suppress destructive inflammatory responses to the eye. In PBMC, 1,25(OH)$_2$D$_3$ suppressed the expression of TLR2 and TLR4 and reduced their induction of IL-6 without reducing their ability to induce HDPs (Khoo et al., 2011a). Although one study showed that stimulation of TLR2 in human corneal epithelial cell line increased the production of IL-8 and IL-6 (Kumar et al., 2006), another earlier study showed TLR2 and TLR4 expression was suppressed and stimulation with the corresponding ligands did not increase IL-8 or IL-6 production (Ueta et al., 2004).

HDPs are essential players of innate immunity that imply protective and immunomodulatory properties to affected tissues. TLR2 stimulation induced mRNA and protein of hBD-2 in human corneal epithelial cell line (Kumar et al., 2006). TLR3, 5,and

6/2 stimulation increased mRNA and protein expression of hBD-2 while TLR3 induced LL-37 in primary human corneal epithelial cells (Redfern et al., 2011). Furthermore pre-exposure of human corneal epithelial cell line to TLR5 ligand impaired the signalling pathway downstream of TLR5 stimulation in a dose dependent manner. It reduced IL-8 secretion but supported mRNA expression and secretion of hBD-2 and LL-37 (Kumar et al., 2007).

Based on work from monocytes, TLR synergised with vitamin D_3 in the induction of HDPs. Similar interaction was found in respiratory epithelial cells, colonic epithelial cell lines, and skin keratinocytes (Hansdottir et al., 2008;Lagishetty et al., 2010;Schauber et al., 2007). We have shown previously that 1,25(OH)$_2$D$_3$ could induce HDPs in PHCEC. 1,25(OH)$_2$D$_3$ induced cathelicidin mRNA alone and in synergy with TLR3 and TLR4. It also induced hBD-1 and hBD-2 alone and in synergy with TLR3 and TLR3 and TLR4 respectively (Susarla et al. manuscript submitted). In this study, local synthesis of vitamin D_3 was as good as exogenous supplementation in the induction of HDPs. This did not require TLR activation. This is in concordance with results from trophoblasts, gingival, sinonasal, bronchial, and urinary balder epithelial cells (Hertting et al., 2010;Liu et al., 2009a;McMahon et al., 2011;Schrumpf et al., 2012;Sultan et al., 2013) where vitamin D_3 alone can induce HDPs. Even in the skin, where vitamin D_3 synergised with TLR2/1, vitamin D_3 could induce HDPs without TLR activation (Peric et al., 2010).

In inflammatory conditions, cytokines such as IL-13 and IL-17 were found to enhance vitamin D_3 mediated induction of HDPs such as hBD-2 and LL-37 in skin keratinocytes and bronchial epithelial cells (Peric et al., 2008;Schrumpf et al., 2012).

Conflicting data have been shown in TLR2/1 induced vitamin D_3 dependent *DEFB4* and LL-37 in monocytes. These responses were augmented by IFN-γ and IL-4 while IL-17 had no effect (Edfeldt et al., 2010). In macrophages, it was previously shown that expression of *DEFB4* required IL-1β (Liu et al., 2009b). HBD-1 is constitutively expressed in primary human ciliary body epithelium and ODM-2 while hBD-2 was induced by IL-1 β in ODM-2 and RPE cell line (Haynes et al., 2000). In ODM-2 and ARPE-19 cells we did not see a significant difference in the production of hBDs when we added IL-1β and TNF-α alone or together with 25(OH)D_3 and 1,25(OH)$_2$D$_3$.

The discrepancy in my results between mRNA upregulation and serum concentrations could be due to the short half-life of hBD peptides (Yount et al., 1999) which means that earlier time points should be examined. It may also indicate that these cells produce HDPs constitutively as part of homeostatic function and they do not increase upon stimulation. Although more experiments are required, we can see that IL-1β and TNF-α could show some synergy with vitamin D_3 to upregulate hBD-1 and 2 protein production in HCEC-12 cells which may indicate the importance of vitamin D_3 during inflammation in these barrier cells at the back of the cornea. Cathelicidin has a longer half-life of 3.4 days (Bals et al., 1999), but we did not measure serum concentration in the current study. The mRNA expression of cathelicidin in HCEC-12 at 8 h were equal to or higher than those shown in respiratory, urinary bladder, gingival epithelial cells at 24h (Hansdottir et al., 2008;Hertting et al., 2010;McMahon et al., 2011). Human BD-2 and 3 were induced by 1,25(OH)$_2$D$_3$ in primary human keratinocytes to levels similar to ocular barrier epithelial cells but the expression was boosted by the

simultaneous stimulation of the pathogen recognition receptor NOD2/CARD15 (Wang et al., 2010).

The eye is constantly exposed to sunlight. It has been shown in rabbits cornea exposed to UVB light could locally covert inactive to active vitamin D_3 in the presence of 7DHC. Vitamin D_3 metabolites were also detected in tears, AqH, and vitreous (Lin et al., 2012;Yin et al., 2011). In the Netherlands, vitamin D_3 effects were influenced by seasonal variations. Treatment of PBMCs with 1,25(OH)2D3 *in vitro*, caused a reduction of IL-6 and TNF-α induced by TLR2 and TLR4 stimulation. When tested in the summer, PBMCs showed a pronounced decrease in IL-β, TNF-α, IFN-γ, IL-6, and IL10 after TLR4 stimulation while moderate effects were seen for TLR2. Apparently high vitamin D_3 levels in the summer decreased TLR2 and TLR 4 expression and consequently their downstream effects upon stimulation (Khoo et al., 2011b). Vitamin D_3 interestingly, in hypoxic conditions upregulated hBD-2 in macrophages (Nickel et al., 2012). Hypoxic conditions are created by the immune system in response to inflammation to allow a microenvironment that can resolve the infection and protect local tissue damage (Olender et al., 2003).

In conclusion, although we could not detect an upregulation of HDPs with vitamin D_3 at the protein level, the ability of the eye to locally utilise vitamin D_3 provided the availability of circulating 25(OH)D_3 or even 7DHC and to translate that into the expression of HDPs may indicate the important role of vitamin D_3 in the eye. The constitutive expression of CYP24A1 indicates a tight regulation of this hormone to allow activation just long enough to mediate the required tasks before degradation. With all

the anti-inflammatory properties and the induction of antimicrobial responses, vitamin D_3 may be involved in the immune surveillance and homeostasis in the eye.

5 VDR POLYMORPHISM IN INTERMEDIATE UVEITIS

5.1 Introduction

Uveitis is a wide range of intraocular inflammatory conditions that accounts for about 5-20% of blindness in Western countries and 25% in the developing world (de Smet et al., 2011). Uveitis is classified anatomically by the Standardisation of Uveitis Nomenclature (SUN) uveitis according to the primary site of inflammation (Jabs et al., 2005). Anterior uveitis involves the anterior chamber, while in IU the vitreous is affected. Posterior uveitis is the inflammation of the retina or choroid and panuveitis involves the anterior chamber, vitreous, retina, and choroid. (Chapter 1 Figure 1.11)

IU can affect young and middle age adults which can create an economical and social burden. Patients present with blurry vision, floaters and distorted central vision. IU can occur without a known cause and is known as iodiopathic or pars planitis referring to snowbanks (white exudates) on pars plana in the ciliary body. IU can be associated with a systemic inflammatory diseases such as multiple sclerosis (MS), sarcoidosis, or infectious conditions like human T lymphotropic virus Type 1 (HTLV-1) and Lyme disease (Babu and Rathinam, 2010;Bonfioli et al., 2005;de Smet et al., 2011). In uveitis, IL-12, IFN-γ, TNF-α, and IL-17 are associated with pathogenesis while IL-4 and IL-10 are found to be protective (Atan et al., 2010;Horai and Caspi, 2011).

IU has been linked to HLA-DRB1*15 (Du et al., 2009;Wallace and Niemczyk, 2011). HLA-DRB1*15 is also associated with MS (Gourraud et al., 2012;Zivkovic et al., 2009) and this association has recently been suggested to be female specific, but this will require validation in other disease cohorts (Irizar et al., 2012). Database analysis of

French patients with MS showed that 0.65% had uveitis. In 46% of patients uveitis preceded onset of MS, in 18% it was diagnosed simultaneously and 36% after the onset of MS (Le Scanff et al., 2008).

MS is an inflammatory disease that affects the central nervous system (CNS) and many environmental and genetic factors are believed to be involved (Koch et al., 2013). Uveitis is not regarded as gender specific but studies from different countries have shown a slight increase in females with male to female ratios 1:1.8 in Italy, 1:1.1 in Tunisia, 1.04:1 in Turkey, and 1.2:1 in India (Kazokoglu et al., 2008;Khairallah et al., 2006;Modorati et al., 2004;Parchand et al., 2011). Among MS-associated uveitis, a study in Caucasians have shown that women were mostly affected with a male to female ration 1:2.1 (Le Scanff et al., 2008).

SNPs in different regions of VDR have been linked with autoimmune diseases. As explained in chapter 1, *FokI* (rs2228570) is known as a start codon polymorphism where ATG is changed to ACG and this leads to a different VDR protein size. In the 3' untranslated region (3'UTR), polymorphisms identified were *BsmI (*rs1544410)*, ApaI (*rs7975232), and *TaqI (*rs731236). This is a regulatory region involved in mRNA stability and expression (Fernandes de Abreu et al., 2010;Uitterlinden et al., 2004a;Uitterlinden et al., 2004b;Valdivielso and Fernandez, 2006). Cdx2 and *A1012G* polymorphisms were identified in the 5' promoter region. *Cdx2* (G to A) polymorphisms affects intestinal calcium absorption and body mass density (Arai et al., 2001) while *A1012G* regulates the expression of GATA3, a transcription factor for the polarisation of T helper 1 (Th1) cells (Halsall et al., 2004).

As mentioned in Chapter 1, low serum 25(OH)D_3 levels were associated with an increase in BD (Karray et al., 2012). This was associated with decreased Treg and predominating Th1 responses indicating an immunomodulatory role of vitamin D_3 in the eye (Hamzaoui et al., 2010). VDR SNP *FokI* was associated with BD in a group of Tunisian patients (Karray et al., 2012;Morrison et al., 2011). FokI is a start codon SNP that affects the size of vitamin D protein and has been shown to modulate mRNA expression of NFAT, IL-12, and regulates pancreatic function (Mory et al., 2009;van Etten et al., 2007). BsmI is in the 3'UTR (regulatory region) that influences mRNA stability and expression (Arai et al., 2001;Rukin and Strange, 2007;Uitterlinden et al., 2002). *BsmI*-CT genotype coincided with the presence of higher numbers of IL-12 producing $CD14^+$ cells in T2DM (Al-Daghri et al., 2012). T cells from T1DM patients homozygous for *BsmI* minor allele (TT) produced higher IFN-γ than other genotypes (Shimada et al., 2008). *A1012G* a promoter region SNP that has been linked to the expression of GATA3 and the differentiation of T helper 1 (Th1) cells (Halsall et al., 2004).

In this project we studied VDR SNPs *FokI* (rs2228570), *BsmI (*rs1544410), and *A1012G* (rs4516035) for association with IU in a cohort of patients with idiopathic IU. These SNPs have been chosen based on their functional relevance to the expression and production of vitamin D and effects on the immune response. Controls were disease free and were matched to patients according to ethnicity but not any other criteria.

5.2 Results

5.2.1 Endpoint genotyping

To study VDR SNPs, endpoint genotyping assay was performed. The assay allows an amplification of DNA followed by endpoint genotyping analysis that separates the different genotypes in clusters according to the fluorescent probe in an X-Y plot. Homozygous individuals cluster towards Y or X axis while heterozygotes cluster in the middle for SNPs *FokI* (rs2228570) (Figure 5.1), *BsmI* (rs1544410) (Figure 5.2), *A1012G* (rs4516035) (Figure 5.3).

FokI (rs2228570)

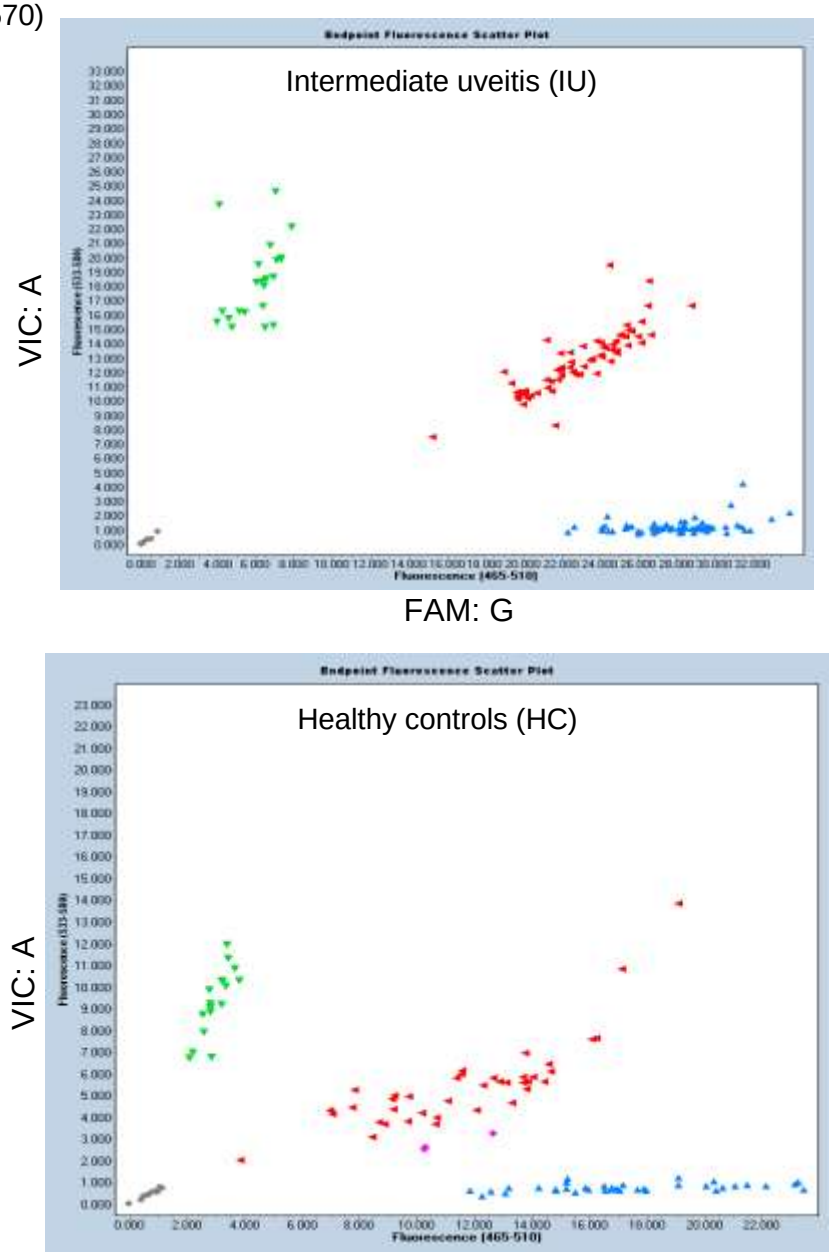

Figure 5.1. Endpoint genotyping allele distribution of VDR SNP FokI (rs2228570) in IU patients vs. healthy controls. The plots show the frequency of FokI (rs2228570) alleles A and G. Each dot represents one sample. Individual samples clustered towards the Y-axis are homozygous for A allele (green), X-axis are homozygous for G allele (blue), and in the middle are heterozygous for AG alleles (red). Gray dots are negative controls and pink dots represent undetected samples by the assay.

BsmI (rs1544410)

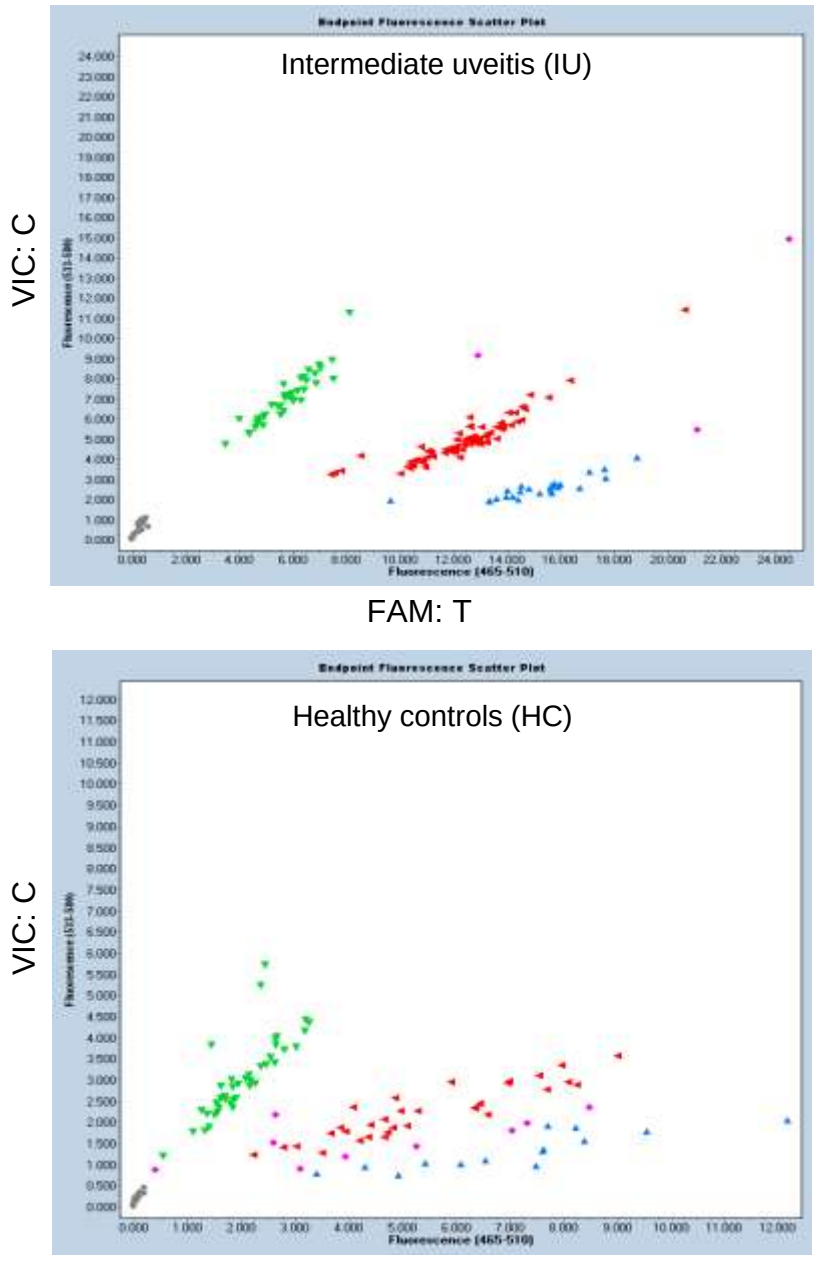

Figure 5.2. Endpoint genotyping allele distribution of VDR SNP BsmI (rs1544410) in IU patients vs. healthy controls. The plots show the frequency of BsmI (rs1544410) alleles C and T. Each dot represents one sample. Individual samples clustered towards the Y-axis are homozygous for C allele (green), X-axis are homozygous for T allele (blue), and in the middle are heterozygous for CT alleles (red). Gray dots are negative control while Pink dots represent undetected samples by the assay.

A1012G (rs4516035)

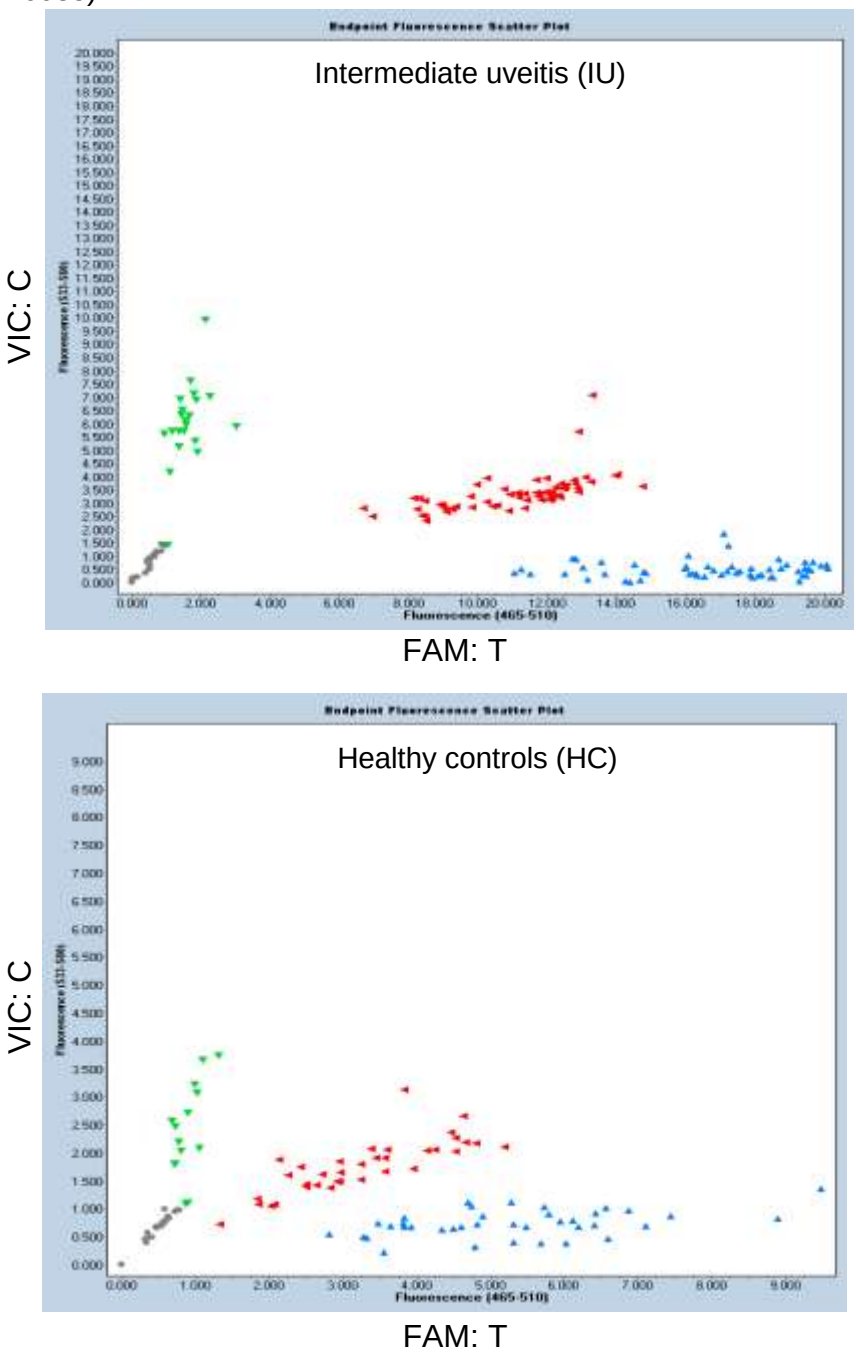

Figure 5.3. Endpoint genotyping allele distribution of VDR SNP A1012G (rs4516035) in IU patients vs. healthy controls. The plots show the frequency of A1012G (rs4516035) alleles C and T. Each dot represents one sample. Individual samples clustered towards the Y-axis are homozygous for C allele (green), X-axis are homozygous for T allele (blue), and in the middle are heterozygous for CT alleles (red). Gray dots are negative controls.

5.2.2 VDR genotype and allele frequencies

The results in Table 5.1 show allele and genotype frequency in patients with IU compared to HC. VDR *BsmI* (rs1544410) is significantly different between patients with IU and HC. Allele rs1544410-T and genotype rs1544410-CT were significantly associated with IU (OR 1.56 95% CI 1.06-2.32, *p*=0.0325 and OR 1.88 95% CI 1.08-3.27, *p*=0.0332 respectively). Conversely, VDR rs1544410-CC genotype was more frequent in healthy controls than IU patients (OR 0.44 95% CI 0.25- 0.78, p=0.007). There was no significant difference found in VDR *FokI* (rs2228570) and *A1012G* (rs4516035). The minor allele in *FokI* (rs2228570) is the A allele, (*BsmI* (rs1544410) is the T allele, and in *A1012G* (rs4516035) is the C allele.

5.2.3 Gender difference between VDR genotypes and alleles

To determine if there are differences between females and males in VDR SNPs, we compared between genotypes and alleles in IU patients and healthy controls (Table 5.2). *FokI* (rs2228570) showed no differences between females and males in IU. In contrast, among healthy controls, genotype rs2228570-AG was significantly more frequent in males (OR 0.24 95% CI (0.01-0.60), *p*=0.0034) whereas genotype rs2228570-GG was significantly more frequent in females (OR 2.82 95% CI (1.09-7.27), *p*=0.0493). Although *BsmI* (rs1544410) was significantly associated with IU, there was no gender difference in both groups. Similarly, there was no difference between females and males in both groups in *A1012G* (rs4516035).

Table 5.1 VDR SNP BsmI (rs1544410) is associated with Intermediate uveitis (IU) in Caucasians. VDR SNPs FokI (rs2228570), BsmI (rs1544410), and A1012G (rs4516035) were analysed using real-time PCR and endpoint genotyping in a study population of Caucasians (only matched for ethnicity but no other criteria). Table shows genotype and allele frequency in IU patients vs. healthy controls (HC).

SNPs Allele/Genotype	IU	HC	OR (95% CI)	p value
FokI (rs2228570)	N=158	N=92		
A	112 (35.4)	73 (39.7)	0.83 (0.57-1.21)	
G	204 (64.5)	111 (60.3)	1.19 (0.82-1.74)	0.395
AA	24 (15.1)	17 (18.5)	0.791 (0.39-1.56)	0.617
AG	64 (40.2)	39 (42.4)	0.92 (0.55-1.6)	0.873
GG	70 (22.0)	36 (39.1)	1.24 (0.73-2.1)	0.506
BsmI (rs1544410)	N=139	N=85		
C	150 (53.9)	110 (64.7)	**0.64 (0.43-0.95)**	
T	128 (46.0)	60 (35.3)	**1.56 (1.06-2.32)**	**0.0325***
CC	38 (27.3)	39 (45.8)	**0.44 (0.25- 0.78)**	**0.007 ****
CT	74 (53.2)	32 (37.65)	**1.88 (1.08-3.27)**	**0.033***
TT	27 (19.4)	14 (16.47)	1.22 (0.600-2.49)	0.706
A1012G (rs4516035)	N=140	N=86		
C	105 (37.5)	63 (36.62)	1.04 (0.70-1.54)	
T	175 (62.5)	109 (63.37)	0.96 (0.65-1.43)	0.9314
CC	21 (15)	14 (16.28)	0.91 (0.43-1.89)	0.945
CT	63 (45)	35 (40.70)	1.19 (0.69-2.05)	0.620
TT	56 (40)	37 (43.02)	0.88 (0.51-1.52)	0.757

Analysis was done using 2x2 tables and Chi-squared test with Yate's correction: * $p<0.05$ and ** $p<0.01$.

Table 5.2 VDR SNP BsmI (rs1544410) shows no difference in between males and females in Caucasian IU patients and healthy controls (HC). VDR SNPs FokI (rs2228570), BsmI (rs1544410), and A1012G (rs4516035) were analysed using real-time PCR and endpoint genotyping in a study population of Caucasians (only matched for ethnicity but no other criteria). Table shows genotype and allele frequency in males and femles of Caucasian IU patients vs. healthy controls (HC).

SNPs Allele/Genotype	IU		OR (95% CI)	*p* value	HC		OR (95% CI)	*p* value
FokI (rs2228570)	**F (N=84)**	**M (N=53)**			**F (N=55)**	**M (N=30)**		
A	65 (36.9)	42 (32.8)	1.18 (0.73-1.91)		44 (37.3)	29 (43.9)	0.76 (0.41-1.40)	
G	111 (63.1)	86 (67.2)	0.85 (0.52-1.37)	0.578	74 (62.7)	37 (56.1)	1.32 (0.71-2.43)	0.4670
AA	15 (17.0)	8 (12.5)	1.44 (0.57-3.63)	0.587	13 (22.0)	4 (12.1)	2.05 (0.61-6.89)	0.371
AG	35 (39.7)	26 (40.6)	0.96 (0.50-1.86)	0.9508	18 (30.5)	21 (63.6)	**0.24 (0.01-0.60)**	**0.0034****
GG	38 (43.2)	30 (46.8)	0.86 (0.45-1.65)	0.774	28 (47.5)	8 (24.2)	**2.82 (1.09-7.27)**	**0.0493***
BsmI (rs1544410)	**F (N=83)**	**M (N=52)**			**F (N=55)**	**M (N=37)**		
C	88 (53.0)	58 (55.7)	0.89 (0.55-1.46)		73 (66.3)	44 (59.4)	1.34 (0.73-2.47)	
T	78 (47.0)	46 (44.2)	1.12 (0.68-1.83)	0.7513	37 (33.6)	30 (40.)	0.74 (0.40-1.37)	0.4248
CC	23 (27.7)	14 (0.26)	1.04 (0.47-2.27)	0.9216	25 (45.4)	14 (37.8)	1.37 (0.58-3.20)	0.6102
CT	42 (50.6)	30 (57.7)	0.75 (0.37-1.51)	0.5311	23 (41.8)	16 (43.2)	0.94 (0.41-2.19)	0.9366
TT	18 (21.7)	8 (15.4)	1.52 (0.61-3.81)	0.4969	7 (12.7)	7 (18.9)	1.60 (0.51- 5.02)	0.6067
A1012G (rs4516035)	**F (N=63)**	**M (N=73)**			**F (N=54)**	**M (N=32)**		
C	54 (42.8)	50 (34.2)	1.44 (0.88-2.35)		36 (57.1)	27 (42.80	0.68 (0.36-1.29)	
T	72 (57.1)	96 (65.7)	0.69 (0.42-1.14)	0.1828	72 (66.1)	37 (33.9)	1.46 (0.77-2.76)	0.3167
CC	10 (15.9)	11 (15.1)	1.06 (0.42-2.70)	0.9136	8 (14.8)	8 (18.8)	0.75 (0.24-2.41)	0.8606
CT	34 (53.9)	28 (38.4)	1.88 (0.95-3.73)	0.0989	20 (37.0)	15 (46.8)	0.66 (0.27-1.62)	0.5025
TT	19 (30.2)	34 (46.5)	0.49 (0.24-1.00)	0.0749	26 (48.1)	11 (34.4)	1.77 (0.72-4.38)	0.3069

Analysis was done using 2x2 tables and Chi-squared test with Yate's correction: * $p<0.05$ and ** $p<0.01$.

5.3 Discussion

Results in this chapter showed an association between *BsmI* (rs1544410) and IU in a cohort of Caucasians. Allele rs1544410-C and genotype rs1544410-CT were significantly increased in patients with IU compared to healthy controls. Conversely, the rs1544410-CC genotype was more frequent in healthy controls. There was no difference in genotype and allele frequency between females and males in *BsmI* (rs1544410).

Extra-renal expression of vitamin D_3 identified a wide range of immunomodulatory functions of this hormone in many organs. In the eye, we and others have shown that many ocular cells express vitamin D_3 pathway components (Lin et al., 2012;Yin et al., 2011) and can locally convert inactive vitamin D_3 to its active form (Alsalem et al. this thesis and manuscript submitted). IU has been linked with MS through HLA-DRB1*15, a molecule that is regulated by vitamin D_3 (Ramagopalan et al., 2009).

FokI (rs2228570) and A1012G (rs4516035) did not show any difference between patients with IU and healthy control group in this thesis although *FokI* (rs2228570)-AG and *FokI* (rs2228570)-GG were significantly more frequent in healthy males and females respectively. *BsmI* is located in intron 8 in the regulatory region of VDR and it may affect mRNA stability (Uitterlinden et al., 2002). SNPs in this region might not have an effect individually but may do as a haplotype with the closely linked SNPs.

Studies on haplotype constructs from 3'UTR of VDR in human osteoblast cell line MG63 showed that minor alleles of *BsmI-ApaI-TaqI* (CCT) haplotype, which is more common in Caucasians, resulted in a 30% faster decay of VDR protein and may result in

lower protein level of VDR available in the cells (Fang et al., 2005;Grundberg et al., 2007). The same haplotype was associated with asthma and atopy in a familial cohort in Canada (Poon et al., 2004).

In PBMCs from a study group in India, CCT haplotype was associated with decreased IL-12p40 and IFN-γ in response to challenge with complete Freund's adjuvant, and $1,25(OH)_2D_3$ *in vitro* and increased IL-10 levels in response to live *Mtb* and $1,25(OH)_2D_3$ in the control group (Selvaraj et al., 2008). SNPs in VDR have been linked to susceptibility to both T1DM and T2DM in different populations. In a recent meta-analysis on papers up to November 2011, there is a significant association between *BsmI* (especially in East Asia) and increased risk to T1DM whereas *BsmI* (only in East Asia) and *FokI* (especially in East Asia) was significantly associated with T2DM (Wang et al., 2012).

In concordance with our results, *BsmI* (rs1544410)-CT genotype was associated with T2DM in Saudi patients, *BsmI*-CT genotype was associated with the presence of higher numbers of IL-12 producing $CD14^+$ cells (Al-Daghri et al., 2012). T cells from T2DM patients with genotype *BsmI*-TT produced higher IFN-γ than other genotypes (Shimada et al., 2008).

Inflammation in uveitis in mouse models is driven by Th1 cells leading to the production of IFN-γ (Horai and Caspi, 2011). This association may indicate a role of VDR polymorphism in the pathogenesis of human uveitis by upregulating inflammatory Th1 response. In contrast to our results, *BsmI* was not associated with BD uveitis in Tunisian patients, but *FokI* was significantly associated (Karray et al., 2012) and this

could be due to the fact that VDR polymorphisms vary with ethnic background . We could not detect any difference in *FokI* and *A1012G* between controls and patients with IU in our patients. *FokI* polymorphism has been found to influence VDR mRNA copy numbers and may regulate CYP27B1 expression to increase active vitamin D_3 production in deficient patients (Ogunkolade et al., 2002). The A allele of *A1012G* increases the expression of GATA3 and directs the polarisation of Th0 cells to Th2 phenotype and it was protective against the inflammatory skin disease psoriasis (Halsall et al., 2004).

Although vitamin D_3 levels have been strongly linked to MS, VDR polymorphisms studies showed no association between SNPs in VDR or other vitamin D_3 related genes such as metabolic enzymes CYP27B1 and CYP2R1, catabolic enzyme CYP24A1, or vitamin D_3 binding protein (DBP) with the aetiology of the disease (Huang and Xie, 2012;Irizar et al., 2012;Simon et al., 2010). Nevertheless, the coexistence of *TaqI* and HLA-DRB1*15 correlated with protection against MS, and only a rare variant of *CYP27B1* was associated with MS (Agliardi et al., 2011;Ramagopalan et al., 2011). It would be worth while investigating the expression of HLA-DRB1*15 in our cohort and try to explain its role with VDR SNPs in the protection against and progression to MS.

Other VDR SNPs and vitamin D_3 related genes polymorphisms may have a role in eye diseases. Polymorphism (G allele at rs4752) in *DBP* increased risk for uveitis in with ankylosing spondylitis in Korean patients (Jung et al., 2011). Another association between SNPs in IL-2 receptor alpha (*IL-2RA*) and IL-7 receptor alpha (*IL-7RA*) and risk of MS (Weber et al., 2008). A SNP in *IL-2RA* has recently been reported to be

associated with IU (Lindner et al., 2011). Several SNPs in *CYP24A1* SNPs were associated with neovascular AMD while *VDR*, *CY27A1*, and *CYP27B1* were not associated (Morrison et al., 2011). In IU, the inflammation extends to ciliary body and peripheral retina (Babu and Rathinam, 2010). We have shown that non-pigmented ciliary body cells can locally convert $25(OH)D_3$ into active $1,25(OH)_2D_3$ (Alsalem et al. manuscript submitted). *BsmI* polymorphisms may affect local production of $1,25(OH)_2D_3$ and increase the risk of developing a stronger Th1 response that can worsen the pathogenesis by the production of IL-12 and IFN-γ.

In conclusion, we have shown that VDR *BsmI* is associated with IU but there was difference among alleles and genotypes between males and females. *FokI* and *A1012G* did not show any association with disease. Following the genetic basis that link uveitis to MS would help get a deeper insight into the pathogenesis and outcome of IU. *BsmI* might have a function in IU alone or in conjunction with closely linked SNPs in VDR or vitamin D_3 related genes.

6 GENERAL DISCUSSION

In this thesis, results have shown (i) that human ocular barrier epithelial cells express the molecular components capable of local conversion of inactive $25(OH)D_3$ into active $1,25(OH)_2D_3$; (ii) local vitamin D_3 synthesis upregulated mRNA expression of HDPs but did not synergise with TLR to induce proinflammatory cytokines; and (iii) a significant association between VDR SNP *BsmI* (rs1544410) and genotype rs1544410-CT was significantly increased in Caucasian patients with intermediate uveitis compared to healthy controls (Figure 6.1)

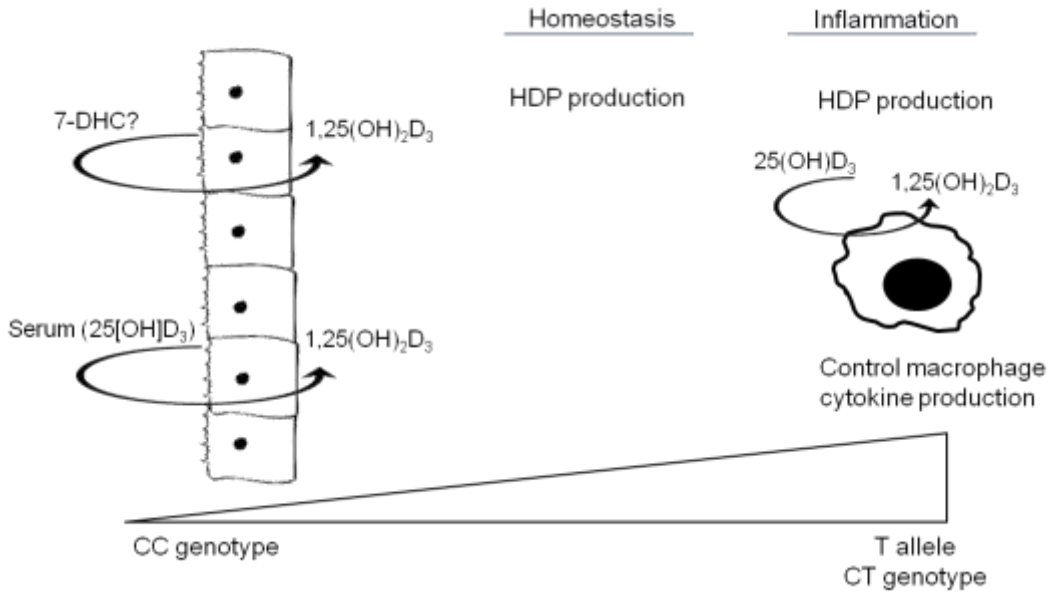

Figure 6.1. A model for the role of $1,25(OH)_2D_3$ in ocular barrier epithelial cells. Local synthesis of $1,25(OH)_2D_3$ (from $25(OH)D_3$ or potentially 7DHC) by ocular barrier epithelial cells controls the production of HDPs during homeostasis to keep a state of immune surveillance. During inflammation, local $1,25(OH)_2D_3$ controls the production of HDPs and cytokine production by infiltrating macrophages. Infiltrating macrophages also contribute to local $1,25(OH)_2D_3$ synthesis and provide a positive feedback to the process.

6.1 Extra-renal vitamin D_3 synthesis by ocular barrier epithelial cells

Consistent with many human epithelial cells, ocular barrier epithelial cells express all the genes for the molecules and enzymes required for the uptake and local synthesis of vitamin D_3. The activity of these genes is reflected by the ability of these cells to take up $25(OH)D_3$ and convert it into $1,25(OH)_2D_3$, whereas the constitutive expression of CYP24A1 suggests a controlled negative feedback mechanism to regulate the levels of $1,25(OH)_2D_3$ produced. As stated earlier, there was a conversion gradient with HCEC-12 cells having the highest rates followed by ODM-2 and ARPE-19.

Corneal endothelium is at the front of the eye and from these data, we can see that endogenous $1,25(OH)_2D_3$ production decreases as we go from the front to the back of the eye. We are not sure if this is related to the amount of UV light available for these cells. It could also be related to the role of corneal endothelium as an important barrier between AqH and corneal stroma followed by ciliary body in BAB and that constitutive $1,25(OH)_2D_3$ production may be required for their protective functions in the eye from front to back.

The localisation of VDR and CYP24A1 was similar between ocular barrier epithelial cells, but CYP27B1 had a different expression pattern in HCEC-12 indicative of its localisation to the TGN. We still have no explanation for this pattern and as we mentioned in Chapter 3, that this enzyme may follow a different trafficking pathway inside these cells. Human breast cancer epithelial cells T-47D internalised Alexa-DBP in the peri-nuclear which colocalised with lysosomes which suggested that DBP traffics through endosomes and lysosomes (Rowling et al., 2006). This may indicate that due to

the presence of CYP27B1 in TGN in HCEC-12, these cells can readily and efficiently convert 25(OH)D_3 delivered by DBP in endocytic vesicles.

The expression of CYP27B1 was detected in untreated cells and it may be helpful to compare it to the expression in cells treated with 25(OH)D_3 or 1,25$(OH)_2D_3$ or even to add labelled 25(OH)D_3 and follow its fate inside the cell. Extra-renal vitamin D_3 may be essential as ocular barrier epithelial cells and many others express CYP2R1 needed for the conversion of vitamin D_3 from the circulation into 25(OH)D_3. A more immediate source of 1,25$(OH)_2D_3$ is needed to resolve infection or inflammation to maintain optical clarity or homeostatic conditions independent of the liver and kidneys.

The main function of 1,25$(OH)_2D_3$ is to support bone growth and health, and extra-renal synthesis of 1,25$(OH)_2D_3$ may also contribute to increase the concentrations of 1,25$(OH)_2D_3$ required for bone health. Serum 1,25$(OH)_2D_3$ concentrations range from 50 to 150 pmol/L (Lips, 2007), however 1,25$(OH)_2D_3$ in culture supernatants from ocular barrier epithelial cells does not reflect *in vivo* levels of vitamin D_3.

To overcome this limitation, we measured 1,25$(OH)_2D_3$ in AqH from normal healthy individuals. The levels that we obtained (1.9-4.8 pmol/L) were much lower than in serum and cell culture supernatants. When 1,25$(OH)_2D_3$ was measured in synovial fluids, the levels were not very different from those in serum (50-100 pmol/L) (Inaba et al., 1997). In the cerebrospinal fluid (CSF) of normal people, levels above 50 pmol/L of 1,25$(OH)_2D_3$ were detected (Balabanova et al., 1984). The levels of 1,25$(OH)_2D_3$ found in pleural fluid from Mtb patients were 100% higher than serum levels (Barnes et al., 1989). It would be interesting to investigate uveitic AqH to identify whether similar

increases in vitamin D_3 are seen in the eye in the context of intraocular inflammation, or tear film in ocular surface disease. This variation between the amounts of $1,25(OH)_2D_3$ in the eye and other biological fluids may be a result of the difference in organ size and volume, turnover of these fluids, and the very limited amount of protein allowed into AqH (Freddo, 2001), as the other tissues are not protected by tissue barriers.

Unlike monocytes/macrophages and epithelial cells, fibroblasts, are unable to convert inactive $25(OH)D_3$ into active $1,25(OH)_2D_3$ although they express vitamin D_3 metabolic and catabolic enzymes. We have previously shown that primary human scleral fibroblasts express CYP27B1 but did not convert $25(OH)D_3$ into $1,25(OH)_2D_3$ (Susarla et al. manuscript submitted). Similarly, dermal fibroblasts express CYP2R1 but not CYP27B1 and can produce $25(OH)D_3$ upon UVB irradiation but not $1,25(OH)_2D_3$ (Vantieghem et al., 2006). Primary synovial fibroblasts also failed to endogenously synthesise $1,25(OH)_2D_3$ *in vitro*. Synovial fibroblasts are thought to regulate synovial levels of $1,25(OH)_2D_3$ with their CYP24A1 activity (Hayes et al., 1992;Smith et al., 1999).

In contrast, primary human gingival fibroblasts express CYP2R1, CYP27A1, and CYP27B1 and can locally synthesise both $25(OH)D_3$ and $1,25(OH)_2D_3$ upon treatment with vitamin D_3 and $25(OH)D_3$ (Liu et al., 2012b;Liu et al., 2012c). With the exception of gingival fibroblasts, it seems that epithelial cells, which act as the first line of defence as barriers in different sites in the body, are designed with the ability to produce $1,25(OH)_2D_3$ that contributes to their protective function against pathogens. This includes epithelial cells in the lung, colon, skin, gingival, breast, placenta, and cornea (Bikle et al., 2004;Halhali et al., 1999;Hansdottir et al., 2010;Kong et al., 2008;Markov et al.,

2012;McMahon et al., 2011;Yin et al., 2011). At least in primary mouse mammary epithelium, mouse intestinal epithelium *in vivo,* Caco-2 cell line, and primary rabbit corneal epithelial cells, vitamin D_3 promotes barrier function by upregulating TJ proteins and barrier formation (Bikle et al., 2004;Kong et al., 2008;Markov et al., 2012;Yin et al., 2011).

6.2 The immunomodulatory role of locally synthesised $1,25(OH)_2D_3$ in ocular barrier epithelial cells

The ability of ocular barrier epithelial cells to convert $25(OH)D_3$ into the active metabolite, $1,25(OH)_2D_3$, prompted us to ask if locally synthesised $1,25(OH)_2D_3$ has any immunomodulatory role in these cells. We first tested the ability of the cells to upregulate vitamin D_3 genes (VDR, CYP27B1, and CYP24A1) in response to local conversion of $25(OH)D_3$ or to $1,25(OH)_2D_3$. Local $25(OH)D_3$ conversion was as efficient in upregulating downstream signalling of VDR activation as direct delivery of $1,25(OH)_2D_3$. In line with the conversion rates, HCEC-12 cells showed the highest fold change increases in vitamin D_3 genes followed by ODM-2 then ARPE-19.

In this project, and due to the difficulties of obtaining and growing primary cells, well-established representative cell lines of ocular barrier epithelial cells were used. The use of cultured cells does not allow a full understanding of the mechanisms used by vitamin D_3 in the eye tissue and does not give information on the interaction between different cell types in the context of local $1,25(OH)_2D_3$ synthesis. However, these cell lines do provide a means of addressing biological pathways that would not be possible with primary cells. A second way to address this point is to use animal models. VDR KO

mice are available commercially and are used to understand the importance of vitamin D_3 for different functions, but they have their own limitations.

In humans, vitamin D_3 is mainly synthesised in the skin through the conversion of 7DHC by UVB exposure. Mice are nocturnal animals that live without sunlight exposure and their skin is covered with fur. To survive, mice need very small amounts of vitamin D_3, which they may have evolved to obtain from diet (particularly chow diet in laboratory conditions) (Holick, 2008;McCann and Ames, 2008). As stated in Chapter 4, mouse CRAMP is not regulated by vitamin D_3 (Dorschner et al., 2001). Homologues of hBDs have been identified in mouse models (mouse BD: mBD) and they contribute to protection and corneal barrier function in mice bacterial keratitis (corneal infection) caused by *Pseudomonas aeruginosa* (*P. aeruginosa*) *in vivo and in vitro* (Augustin et al., 2011;Doss et al., 2010;Wu et al., 2009). It is still unclear if mBD production in mice is vitamin D_3-dependent (Liu et al., 2009b). However, VDR KO mice develop many health and growth problems that mimic those in humans and are still used to understand human disease (Bouillon et al., 2008).

VDR KO mice were smaller in size, developed alopecia, had wrinkled skin, and showed growth arrest when they were compared to wild type. As they grew older, they expressed lower levels of aging-related molecules including NF-κB, FGF-23, insulin like growth factor-I (IGFI), IGFI-receptor (IGFIR), p53, and Klotho. These molecules are required for the immune system, tumour suppression, and determining life span. The decreased expression was only seen in old, but not young, mice suggesting the role of VDR absence in inducing premature aging (Keisala et al., 2009).

The relationship between vitamin D and aging is complex. FGF-23 is induced by $25(OH)D_3$ but in turn inhibits renal phosphate reabsorption and $1,25(OH)_2D_3$ synthesis, and induces CYP24A1 production. FGF-23 knock-out mice show a shortened lifespan, arteriosclerosis, and obstructive pulmonary disease. Therefore while FGF-23 production is almost entirely dependent on $25(OH)D_3$, it's function as a longevity gene is based on control of $1,25(OH)_2D_3$ (Haussler et al., 2010). VDR KO mice were found to develop hypocalcaemia, hyperparathyroidism, and reduced intestinal calcium absorption. Interestingly, VDR KO mice produced more serum $1,25(OH)_2D_3$, expressed higher renal CYP27B1 mRNA, and showed reduced CYP24A1 mRNA expression compared to $VDR^{+/-}$ mice and Tg mice with VDR expression limited to the intestine. VDR KO mice showed skeletal abnormalities with shorter femurs and low bone mass density (Xue and Fleet, 2009). Although VDR KO mice have normal myelopoiesis, they develop several defects in the immune system including reduced T cell proliferation, reduced Th1 cytokines, mature DC phenotype, and decreased T cell homing to inflammatory sites (Bouillon et al., 2008). To date, ocular disease in VDR KO mice has not been investigated.

It is well established that although the eye has a unique structure and immune regulation, innate immune system in the eye plays an important function in immune surveillance and defence against ocular infections (Lambiase et al., 2011). We could show that TLR signalling is functional in ocular barrier epithelial cells, but there was no synergy between vitamin D_3 and TLR activation to affect the proinflammatory cytokines IL-6 and IL-8. In respiratory epithelial cells, the synergy was between vitamin D_3 and

TLR3, rather than TLR2, as in monocytes, to induce cathelicidin (Hansdottir et al., 2008). In ocular barrier epithelial cells we observed that the highest cytokine production was in response to TLR3 and RIG-I/MDA5 activation. As mentioned above, IL-6 and IL-8 are induced through MyD88 signalling and activation of NF-κB (Yang and Seki, 2012). When respiratory epithelial cells were infected with human respiratory syncytial virus (RSV) which interacts with TLR3 or RIG-I/MDA5 *in vitro*, exogenous $1,25(OH)_2D_3$ inhibited NF-κB signalling and reduced IFN-β and interferon-γ-inducible protein 10 (IP-10, CXCL10), a chemokine produced in response to viral infections that attracts Th1 and NK cells. The inhibition of NF-κB and the reduction in IFN-β did not allow viral replication to increase, which indicates that $1,25(OH)_2D_3$ reduces inflammation but maintains an antiviral response.

The induction of HDPs, which are important for innate immunity, by $1,25(OH)_2D_3$ or by local conversion may also contribute to viral infection resolution (Hansdottir et al., 2010;Spurrell et al., 2005). When a human keratinocyte cell line was infected with varicella zoster virus (VZV) and exposed to exogenous LL-37 or hBD-2, viral load was reduced. Moreover, pretreatment of the virus with LL-37 or hBD-2 before infecting the cells resulted in further reduction in viral load. This shows that HDPs have a direct effect on the virus (Crack et al., 2012). VDR KO mice showed increased *in vivo* NF-κB expression and NF-κB translocation to the nucleus in colonic epithelial cells after infection with *Salmonella typhimurium* (*S. typhimurium*) (Wu et al., 2010).

NF-κB is a key molecule in eye infections induced by TLR signalling in bacterial and viral (Herpes simplex-1: HSV-1) keratitis, for the modulation of immune response

(Lan et al., 2012). The fact that local $1,25(OH)_2D_3$ synthesis by ocular barrier cells did not affect IL-6 and IL-8 production in the current thesis after TLR stimulation may explain that the level of $1,25(OH)_2D_3$ induced is inhibitory for inflammatory signals, but permits a certain degree of inflammation to resolve infection.

Although not statistically significant, we can see that local synthesis of $1,25(OH)_2D_3$ induces the expression HDPs in all ocular barrier epithelial cells used in this study. The mRNA expression levels of HDPs were higher in HCEC-12 and ARPE-19 cells than in ODM-2 cells. This may support their role in corneal endothelium and RPE as barriers at the front and back of the eye. Haynes et al. detected hBD-1 in human AqH and vitreous samples in small concentrations (<16 ng/ml) but could not detect hBD-2 (Haynes et al., 2000).

It has been observed in humans that hBDs and LL-37 are sensitive to salt concentrations that might render them ineffective in ocular fluids with a high salt content, such as tears. They can be present in small concentrations but synergistic with each other to overcome the effect of salt. This could be an advantage to the eye as high concentrations can be toxic to ocular cells (Huang et al., 2007;McDermott, 2009). The ability of endogenous $1,25(OH)_2D_3$ to induce HDPs may be beneficial to the eye where there is microbial clearance without inducing local damage by inflammation, thereby maintaining visual clarity.

As mentioned in Chapter 1, HDPs are not only antimicrobial but have immunomodulatory functions. For example, human PBMCs stimulated with IFN-γ and LPS in the presence of LL-37, produced lower levels of TNF-α and IL-12 proteins even

at low concentrations of LL-37 which could resemble physiological conditions. Similar results were seen with lipoteichoic acid (TLR2 ligand), but not with Pam3Csk4 (synthetic TLR2/1 ligand), as there was a reduction of IL-6 production after stimulation of an intracellular PRR NOD2 (nucleotide-binding oligomerisation domain-containing protein 2) with its ligand muramyl dipeptide (MDP). LL-37 downregulated the expression of CD80, CD86, and MHC II on monocytes and DCs after treatment with IFN-γ and LPS and reduced the expression of TNF-α and IL-12 (Nijnik et al., 2009). A similar reduction of proinflammatory cytokine transcription and production was observed when mouse BM derived monocytes and DCs were treated with LPS in the presence of hBD-3. The inhibitory effects of hBD-3 were mediated through the inhibition of MyD88 and TRIFF signalling (Semple et al., 2011).

6.3 Association of VDR SNPs with IU

In this thesis, we have shown an association between *BsmI* and IU in Caucasians but there was no difference between males and females in this association. Although in this project, ocular barrier epithelial cells, in particular the corneal endothelium and ciliary body, are able to efficiently synthesise local vitamin D_3, the eye still gets diseases such as uveitis. In addition, with supplementation there was a reduction in retinal inflammation and accumulation of Aβ associated with aging, indicating that locally synthesised vitamin D_3 does not prevent the effects of aging or is not produced in sufficient levels to prevent aging (Lee et al., 2012). Vitamin D_3 played a protective immunomodulatory role in BD (Tian et al., 2012), but these results are only from *in vitro*

investigations; they do not accurately depict what happens inside the body in eye inflammation in relation to vitamin D_3 levels or supplementation.

Vitamin D_3 metabolism is influenced by many genes including those in the metabolic pathways, DBP, and cubilin and megalin. A systematic review of the different SNPs in relation to the levels of 25(OH)D_3 showed that the minor allele T of *FokI* was associated with higher concentrations of 25(OH)D_3. Only one SNP in *CYP27B1* and one in DBP were associated with 25(OH)D_3 levels. Other SNPs in *CYP24A1*, *CYP27A1*, and *CYP2R1* did not shown any association with 25(OH)D_3 (McGrath et al., 2010).

A genome wide study has shown a significant association between 25(OH)D_3 and several genes involved in, or near genes involved in the vitamin D_3 pathway. A positive association was found with a SNP in *NADSYN1*, a gene encoding nicotinamide adenine dinucleotide (NAD) synthetase was in high LD with one SNP in the skin precursor 7DHC. Another association was found with a SNP in chromosome 10 near *ACADSB* (acyl-coenzyme A dehydrogenase), an enzyme involved in the synthesis of cholesterol and vitamin D_3, in addition to a SNP in *CYP2R1* (Ahn et al., 2010). Although one SNP in cubilin was more frequent among T1DB patients, it was not associated with circulating 25(OH)D_3 or 1,25$(OH)_2D_3$ (Ramos-Lopez et al., 2010).

A very recent study in European women investigating carotenoids in age-related eye disease showed that two SNPs in DBP and four SNPs in *CYP2R1* were associated with serum 25(OH)D_3 levels in women taking supplementation of >400 international units (IU)/day. There was an association only in the blood drawn in the summer but not in the winter (Engelman et al., 2013). 1,25$(OH)_2D_3$ interacts with so

many other genes which may have their own polymorphisms and the influence of these SNPs on vitamin D_3 activity will have wide ranging implications well beyond the mechanism identified in the current study. These complex interactions will require future systems biology approaches to define outcome.

Based on the availability of $25(OH)D_3$, ocular barrier epithelial cells can constitutively convert it into $1,25(OH)_2D_3$. This may indicate an important role for $1,25(OH)_2D_3$ in maintaining homeostatic conditions in the eye. More than 120 gene polymorphisms have been detected in DBP. There are three major known alleles, named after their Gc-globulin (group-specific component or Gc) electrophoretic migration; Gc1f (fast), Gc1s (slow), and Gc2. Gc1 phenotype binds with higher affinity to vitamin D_3 metabolites than Gc2. Gc1f is more frequent in black Africans and black Americans while Gc2 is markedly high in Caucasians (Gomme and Bertolini, 2004;Speeckaert et al., 2006). Liu et al. detected low serum levels of $25(OH)D_3$ in African-Americans compared to Caucasians. When monocytes were activated with TLR2/1L in the presence of sera from African-Americans, cathelicidin mRNA expression was lower than in cells treated with sera from Caucasians (Liu et al., 2006).

The role of DBP in vitamin D function is another area of complexity. Murine studies have shown that DBP null-mice have very reduced levels of serum $1,25(OH)_2D_3$ (less than 1%), but calcium levels were completely normal. Kidney tissue expressed double the mRNA of CYP27B1 compared to wild-type mice, but CYP24A1 was reduced. Although $1,25(OH)_2D_3$ was significantly reduced, tissue accumulation and activation of $1,25(OH)_2D_3$ was not affected by the loss of DBP in null-mice compared to wild type.

These results suggest that DBP is not essential for the delivery of $1,25(OH)_2D_3$ to certain target cells and it can be replaced by low-affinity binding proteins such as albumin (Zella et al., 2008).

When human monocytes were treated with exogenous $25(OH)D_3$ or $1,25(OH)_2D_3$ in the presence of serum from $DBP^{-/-}$ mice there was an increase in the expression of cathelicidin and CYP24A1. This expression was reduced with serum from $DBP^{+/-}$ mice. When $25(OH)D_3$ or $1,25(OH)_2D_3$ were added in serum free medium in the presence of albumin, cathelicidin and CYP24A1 were efficiently induced. Under the same conditions, the addition of as low as 0.1 µM of exogenous DBP significantly reduced the expression of cathelicidin and CYP24A1. Although monocytes lacked the expression of megalin, they were able to internalise DBP, but to a low level compared to control cells expressing megalin and cubilin. This study has also shown that when monocytes were treated with $25(OH)D_3$ in the presence of 5% serum from donors with six combinations of DBP major alleles; Gc1F, Gc1S, and Gc2, cells were more sensitive to $25(OH)D_3$ and induced higher expression of cathelicidin and CYP24A1 when Gc1f was absent. As genetic variants of DBP determine how much of $25(OH)D_3$ or $1,25(OH)_2D_3$ is available for cellular uptake, the authors suggest to determine DBP genotype for individuals in addition to circulating $25(OH)D_3$ measurement (Chun et al., 2010).

Similar findings were reported by Jeffery *et al.* where the addition of DBP to DCs co-cultured with T cells, inhibited the uptake of $25(OH)D_3$ by DCs and suppressed the antiinflammatory effects seen previously on $CD4^+CD25^-$ T cells. The inhibitory effects of DBP were more pronounced in $25(OH)D_3$ than $1,25(OH)_2D_3$ (Jeffery et al., 2012).

Unlike monocytes and DCs, human mammary epithelial cells express cubilin and megalin coreceptors. Human immortalised mammary epithelial (HME) cells were able to upregulate CYP24A1 in response to $25(OH)D_3$ or $1,25(OH)_2D_3$ in the presence or absence of DBP. They were able to internalise DBP by endocytosis and the internalisation was inhibited with the use of a megalin inhibitor. The breast cancer epithelial MCF-7 cell line expresses megalin but lacks the expression of cubilin. Despite the presence of CYP27B1 in these cells, they failed to induce CYP24A1 in response to exogenous $25(OH)D_3$ or $1,25(OH)_2D_3$ (Rowling et al., 2006).

Another molecule at the cytoplasmic tail of megalin, the mitogen-responsive phosphoprotein disabled-2 (Dab2) is believed to be essential for the endocytosis of cubilin and megalin. It has been identified in human primary tubular epithelial cells (PTEC) and in the breast cancer epithelial T-47D cell line after it was induced to differentiate (Chlon et al., 2008;Gallagher et al., 2004). In this context, ocular barrier epithelial cells expressed cubilin mainly, as megalin was expressed but very poorly, indicating that they are capable of internalising DBP-bound $25(OH)D_3$ shown by their efficiency, at least in HCEC-12 and ODM-2, to uptake and metabolise $25(OH)D_3$. The contribution of cubilin and megalin in DBP-$25(OH)D_3$ internalisation by ocular barrier epithelial cells is worthy of further investigation, taking into consideration the lack of evidence on the presence of DBP in AqH or eye tissue.

6.4 Vitamin D_3 in context of inflammation

Vitamin D_3 synthesis is known to be a major advantage of exposure to sunlight but several studies have suggested a protective role of UV light independent of vitamin D_3. Pretreatment of a murine model with UV rays (UVR) did not prevent the onset of experimental autoimmune encephalomyelitis (EAE) induced by subcutaneous injection with myelin oligodendrocyte glycoprotein (MOG) peptide 33-35 (MOG_{33-35}). This pretreatment caused a slight increase in circulating serum $25(OH)D_3$. By comparison, when UVR treatment was continuous before and after immunisation with MOG, there was a significant suppression in EAE clinical score and disease severity. There was a significant increase in $25(OH)D_3$, but it was only transient and did not keep increasing with continuous UVR exposure. Treatment of the animals with increasing doses of $25(OH)D_3$ up to levels causing hypercalcaemia, failed to suppress EAE which suggests a protective role of UVR independent of $25(OH)D_3$ (Becklund et al., 2010).

UVR exposure correlated inversely with risk of developing MS in a Swedish study population and positively with serum $25(OH)D_3$ levels. About 47% of the cases were included in the study within 2 years, and 64% within 4 years of MS onset. Taking the HLA-DRB1*15 genotype into account, there was no association between UVR exposure and HLA-DRB1*15 nor between HLA-DRB1*15 and $25(OH)D_3$ deficiency with risk of developing MS at study inclusion (Baarnhielm et al., 2012).

Murine studies have shown that in addition to interacting with 7DHC in the skin, exposure to UVR interacts with other molecules in the skin such as DNA, lipids and resident APCs to induce immunosuppression. DCs with damaged DNA can migrate to

draining lymph nodes and induce $CD4^+CD25^+Foxp3^+$ Tregs (Hart et al., 2011). In a murine study, male mice from vitamin D_3 deficient mothers kept on a vitamin D_3 deficient diet had significantly lower serum $25(OH)D_3$ than their female littermates. Vitamin D_3 deficient male mice have low levels of the vitamin D_3 precursor 7DHC in their skin, and their kidneys expressed higher levels of CYP27B1 and CYP24A1 mRNA than females.

To study the effect of vitamin D_3 induced by UVR on serum $25(OH)D_3$ and immune response, these mice were exposed to different doses of UVR. Of the vitamin D_3 deficient group, female mice showed an increase in serum $25(OH)D_3$ compared to males, although the levels remained <25 nmol/L. Dietary supplementation of vitamin D_3 increased serum $25(OH)D_3$ in both male and female mice, but male mice still showed levels lower than females, similar to levels in vitamin D_3 sufficient male mice. UVR only increased skin production of $1,25(OH)_2D_3$ in female, but not male mice in the vitamin D_3 deplete group. There was no difference between males and females in UVR's local and systemic immunosuppressive effects upon sensitisation with 2,4-dinitrofluorobenzene (DNFB) in the ear lobes. DCs from UVR irradiated vitamin D_3 deficient male and female mice were isolated and expanded *in vitro*. When injected subcutaneously with DNFB in the ears of naïve mice, they failed to induce a Th1/17 inflammatory response. Furthermore, UVR irradiation suppressed allergic reactions in both male and female vitamin D_3 deficient mice shown by reduced eosinophil numbers and IL-15 in their bronchoalveolar lavage after inhaling ovalbumin (OVA) for three days. If vitamin D_3 protects from autoimmune diseases, the previous study does not explain why females

have more tendencies to autoimmune diseases although they make higher serum 25(OH)D_3 in response to UVR (Gorman et al., 2012).

In sunny countries, such as Saudi Arabia, one would expect women with scarves and veils to be more vitamin D_3 deficient than men who do not need to cover. One study in the east of Saudi showed a high prevalence of vitamin D_3 deficiency in Saudi participants, however there was no difference between men and women although according to the questionnaire >65% and >90% had adequate sunlight exposure and dietary intake respectively (Elsammak et al., 2011). Another study from Jeddah on the west coast of Saudi reported 87.8% deficiency in Saudi men, in particular among obese and older participants with no education (Ardawi et al., 2012). Education can help increase their awareness of the importance of sunlight exposure and vitamin D_3 dietary intake.

However, a study in Jordan has found low circulating 25(OH)D_3 levels among women covering all the body only showing the face and hands (54.8% in summer and 77.6% in winter) and in those that cover all the body including the face and hands (83.3% in summer and 81.8% in winter) compared to ladies with a western dress style (30.8% in summer and 75% in winter). The differences between the three groups in 25(OH)D_3 were not statistically significant, but the two groups of covered women were significantly lower than men as low vitamin D_3 is found in 18% and 45.5.% of Jordanian men in summer and winter respectively (Mishal, 2001). The tendency of people to spend most of their time indoors to avoid the heat, and to watch TV and play video games in

addition to air pollution caused by industry are all factors that contribute to widespread vitamin D_3 insufficiency and deficiency (Mead, 2008).

Based on the results and data presented in this thesis there is a clear case for vitamin D_3 supplementation. Vitamin D_3 is cheap, easily available, and is safe to use, except at very high levels, but there is big debate on how much is enough for an individual and whether it would really help. Vitamin D_3 is also made in the skin and this complicates the decision on the doses of supplementation. The new report from the Institute of Medicine (IOM) recommends 600 IU/day for ages less than 70 years and 800 IU/day for ages older than 70 (Ross et al., 2011). Osteoporosis Canada has also published new guidelines that recommend 400-1000 IU/day for adults below the age of 50 and 800-2000 IU/day for those above 50 years old (Hanley et al., 2010). In a systematic review published recently that included papers through the period from 1973 to 2011, in pathophysiological studies, it was found that vitamin D_3 deficiency in genetically susceptible people can cause the loss of regulatory functions of DCs over Th1 responses.

Cross-sectional studies have shown that serum 25(OH)D_3 levels <30 ng/ml were found in both patients with autoimmune disease as well as healthy patients. Meta-analysis of case-control studies revealed that vitamin D_3 supplementation caused a significant reduction in the risk of developing T1DM. Interestingly, supplementation between the ages of 7-12 months reduced the chances of developing T1DM more than that between 0-6 months. This was explained by the fact that the adaptive immune system develops during that period of 7-12 months (Antico et al., 2012).

Month of birth has been linked to the risk of developing immune mediated diseases such MS, T1DM, Crohn's disease, ulcerative colitis, and SLE where people born in the spring between April and October had more risk which correlated inversely with serum 25(OH)D_3 during the second trimester of pregnancy (Disanto et al., 2012;Kahn et al., 2009;Saastamoinen et al., 2012). In line with these data, we observed in patients with idiopathic IU, the highest incidence was among those born in the spring (Thomas et al. manuscript submitted).

Mice born to mothers kept vitamin D_3 deficient before and during pregnancy had less severe EAE after immunisation with MOG compared to control group. This was accompanied by an upregulation of VDR and heat shock proteins (involved in VDR mediated action in EAE) in the spinal cord up to day 30 after immunisation. Moreover, offspring fed postnatally on a vitamin D_3 rich diet had a delayed and less severe EAE. This supports the concept that the *in utero* vitamin D_3 environment has conditioned the foetus to be more sensitive to vitamin D_3 *ex utero*. Surprisingly, the second generation of these mice had more severe symptoms and the symptoms were similar whether the mother or the father was vitamin D_3 deficient. The study concluded that the mother imprints the foetus, and vitamin D_3 status during pregnancy determines the child's risk for developing an autoimmune disease (Fernandes de Abreu et al., 2010). However, De Luca and Plum have shown, using a different murine strain, that EAE was delayed and less severe in two generations of mice exposed to prolonged vitamin D_3 deficiency (Deluca and Plum, 2011).

It was observed that if people migrate before the age of 15 from an area with a high risk to an area with a low risk of MS, they acquire low susceptibility to MS but if they migrate from a low risk area to a high risk area, they maintain the low risk of their origin (O'Gorman et al., 2012). In contrast, a study on immigrants from the UK and Ireland to Australia showed no effect of age on the prevalence of MS suggesting other environmental factors that play a role before and after the age of 15 (Hammond et al., 2000).

Another study has shown that when people from the French West Indies (known as low MS prevalence) migrated to France and returned to the West Indies, there was a high prevalence among those who migrated to France before the age of 15. Unexpectedly, there was an increase in the prevalence of MS among non-immigrants in the West Indies and the authors suggest that immigrants may have acquired some environmental factors that act before the age of 15 and determine the susceptibility to MS (Cabre et al., 2005). Both studies suggest the involvement of genetic and environmental factors in determining the risk for developing MS. Taken together, we can see there is no clear evidence between vitamin D_3 sufficiency/deficiency or location and autoimmune diseases. The genetic makeup, vitamin D_3 status of the mother during pregnancy, environmental factors, and timing of immune system programming appear to all be involved in how individuals respond to vitamin D_3 and how it can affect them.

6.5 Summary and future studies

In summary, results in this thesis have provided evidence for a complete and functional exra-renal vitamin D_3 system in human ocular barrier epithelial cells capable

of locally converting vitamin D_3 from its inactive to active form. We have also shown that local vitamin D_3 production by these cells can induce the expression of mRNA for the HDPs cathelicidin and hBD1-4. Furthermore, we found an association between the VDR SNP *BsmI* and IU in Caucasians, which supports an immunomodulatory role of vitamin D_3 in autoimmune disease in the human eye. Overall, our results suggest the potential of vitamin D_3 to contribute to immune regulation in the eye during homeostatic as well as inflammatory conditions. Vitamin D_3 might serve as a safe and cheap therapeutic agent to treat or even protect against the onset of an immune mediated inflammatory eye disease.

Based on my results we can suggest that future studies should:

1. Investigate vitamin D_3 metabolism in corneal endothelial cells (primary and cell line) as they express a different staining pattern of CYP27B1 and they seem to be the most efficient in local conversion.

2. Study the effect of vitamin D_3 supplementation on HDP production in ocular fluids and their functional activities.

3. Identify conditions for protein production of HDPs by ocular barrier epithelial cells to see if it matches mRNA expression

4. To test other genetic variants in *VDR* and related genes to provide an insight of the functional relevance of these variants to vitamin D_3 metabolism and association with eye diseases, such as uveitis, and disease outcome. Taking into consideration gender differences, these studies can be used to understand

differences between males and females in vitamin D_3 metabolism and the modulation of immune response.

5. To explore using animal models, how vitamin D_3 can affect EAU both in males and females and how to correlate that to human uveitis.

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8 APPENDIX

Chapter 4

Figure 4.8

Real-time PCR raw data: relative CAMP mRNA expression

HECE-12

Time (h)	**25D**			**1,25D**		
1	2.14	0.03	0.004	1.49	0.03	0.06
4	0.76	6.71	0.16	2.21	16.78	0.17
8	2.05	25.667	1.340	1.784	29.90	1.02
16	0.45	8.582	5.683	0.919	13.228	4.24
24	0.19	0.71	1.93	0.334	1.17	3.23

ODM-2

Time (h)	**25D**			**1,25D**		
1	0.41	0.46	1.40	1.01	0.59	0.99
4	0.60	0.31	2.07	0.85	1.33	0.22
8	0.96	0.86	1.09	0.89	0.58	1.64
16	1.79	0.37	0.90	0.63	0.41	0.39
24	0.16	0.81	2.83	1.41	0.77	2.36

ARPE-19

Time (h)	**25D**				**1,25D**			
1	2.24	0.42	0.28	5.19	3.29	0.27	0.26	0.24
4	1.45	1.64			0.53	2.73		
8	2.15	1.17	2.07	13.53	1.89	0.47	1.13	0.65
16	0.75	0.73	0.36	0.28		2.69	0.16	0.00
24	0.86	0.22	0.68	0.001	1.003	0.36	1.46	0.98

Figure 4.9

Real-time PCR raw data: relative hBD-1 mRNA expression

HCEC-12

Time (h)	**25D**			**1,25D**		
1	0.30	1.22	0.48	0.48	0.25	0.55
4	1.06	0.63	1.44	1.18	1.54	4.96
8	0.76	1.81	0.42	0.49	0.63	0.63
16	0.67	0.30	1.91	1.52	0.57	1.33
24	0.88	4.09	3.48	2.07	1.56	4.46

ODM-2

Time (h)	**25D**			**1,25D**		
1	1.81	0.87	1.29	3.74	0.60	1.51
4	2.22	0.79	0.94	0.91	0.48	0.70
8	1.57	0.59	0.69	1.28	0.77	0.95
16	2.23	0.78	0.59	2.24	0.88	0.69
24	0.65	0.17	2.52	1.02	0.09	3.31

ARPE-19

Time (h)	**25D**			**1,25D**		
1	1.17	1.16	1.76	1.08	0.17	0.59
4	0.96	4.92		1.28	6.07	
8	2.07	0.32	2.01	0.74	0.19	4.73
16	0.55	2.08	2.72	0.65		2.29
24	0.05	0.49	3.92	0.18	1.46	

Figure 4.10

Real-time PCR raw data: relative hBD-2 mRNA expression

HECE-12

Time (h)	25D			1,25D		
1	1.94	0.41	4.27	0.56	0.84	1.94
4	0.54	2.47	0.53	0.42	5.13	2.04
8	1.53	1.51	16.20	0.93	0.78	30.25
16	1.17	9.76	2.36	0.94	0.97	4.57
24	3.88	2.70	0.01	1.62	0.98	0.04

ODM-2

Time (h)	25D			1,25D		
1	0.58	0.72	0.62	0.79	0.74	1.01
4	1.61	2.31	0.94	1.52	3.67	0.84
8	1.19	1.38	0.81	0.87	1.72	0.77
16	1.76	1.04	6.39	0.79	1.10	2.90
24	1.76	0.85	2.62	1.56	0.72	7.76

ARPE-19

Time (h)	25D			1,25D		
1	0.41	0.61	1.66	0.34	0.46	0.86
4	0.99		2.17	0.26		2.85
8	1.01	1.49	2.23	0.50	0.82	0.23
16	2.55	2.85	22.40	8.27	0.27	
24	0.99	1.17	1.14	1.76	0.44	1.25

Figure 4.11

Real-time PCR raw data: relative hBD-3 mRNA expression

HECE-12

Time (h)	**25D**			**1,25D**		
1	0.23	0.77	2.22	0.88	0.67	1.93
4	3.40	0.62	0.35	1.74	0.96	0.37
8	0.24	1.27	2.97	0.45	8.66	1.82
16	1.12	0.87	1.25	1.59	2.04	0.56
24	0.85	1.03	0.89	8.19	0.97	3.40

ODM-2

Time (h)	**25D**			**1,25D**		
1	0.23	0.77	2.22	0.88	0.67	1.93
4	3.40	0.62	0.35	1.74	0.96	0.37
8	0.24	1.27	2.97	0.45	8.66	1.82
16	1.12	0.87	1.25	1.59	2.04	0.56
24	0.85	1.03	0.89	8.19	0.97	3.40

ARPE-19

Time (h)	**25D**			**1,25D**		
1	0.57	0.54	0.85	2.00	0.46	0.68
4	7.32	2.80		3.82	1.71	
8	1.78	1.29	6.39	0.65	0.87	2.60
16	0.59	1.53	0.50		2.82	0.54
24	2.58	0.54	1.49	1.54	0.66	1.66

Figure 4.12

Real-time PCR raw data: relative hBD-4 mRNA expression

HECE-12

Time (h)	25D			1,25D		
1	0.56	0.26	0.32	4.42	1.59	0.63
4	1.53	0.92	1.79	3.70	0.57	2.19
8	0.27	0.191	0.76	0.20	1.17	0.43
16	2.13	0.510	0.88	5.67	0.06	2.98
24	0.981	3.404	3.42	0.27	10.43	0.33

ODM-2

Time (h)	25D			1,25D		
1	2.79	0.38	1.43	7.96	0.55	1.53
4	1.19	2.27	0.43	4.03	3.72	0.65
8	2.31	1.63	1.38	0.47	0.19	1.85
16	9.43	0.03	8.18	0.40	0.01	2.84
24	0.24	0.51	4.26	0.41	0.51	5.88

ARPE-19

Time (h)	25D			1,25D		
1	1.06	0.19	0.50	1.22	0.18	0.01
4	1.85		0.01	1.01		0.59
8	0.38	2.74	13.81	0.01	9.69	0.44
16	7.72	0.55	1.09	10.08	0.17	
24	0.66	1.65	1.38	0.06	0.09	0.19

Publications

1. **Characterisation of Vitamin D Production by Human Ocular Barrier Cells.**
Jawaher A. Alsalem[1], Deepali Patel[1], Radhika Susarla[1], Miguel Coca-Prados[3], Rosemary Bland[4], Elizabeth A. Walker[2], Saaeha Rauz[1], Graham R. Wallace[1] (Submitted).

2. 1,25(OH)2D3 and TLR cross-talk in human ocular cells offers immunoprotection through anti-microbial peptide production.
R. Susarla[1A], G.R. Wallace[1A], K. Oswal[1A] and 3, S. Aroori[1A], **J. Al- Salem[1A]** S.J. Curnow[1A], E.A. Walker[1B], R. Bland[2], S. Rauz[1A and 3*] (Submitted).

3. Pre-receptor Regulation of Glucocorticoids by Immunoregulatory Components in the Human Ocular Surface.
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Association for Research in Vision and Opthalmology, Annual Meeting: Visionary Genomics, Fort Lauderdale, Florida, USA, 2011. "Endogenous production of $1,25(OH)_2D_3$ by ocular barrier cells".

British Scociety for Immunology (BSI congress), Liverpool, UK, 2010. "$1,25(OH)_2D_3$ Production by Ocular Barrier Cells".

Association for Research in Vision and Opthalmology, Annual Meeting "For Sight: The Future of Eye and Vision Research", Fort Lauderdale, Florida, USA, 2010. "A Role for Vitamin D_3 in Ocular Immune Privilege?".