ANALYSING THE POTENTIAL ANTI-INFLAMMATORY EFFECTS OF VITAMIN D ON HUMAN CORNEAL EPITHELIAL CELLS

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ABSTRACT

Inflammation of the ocular surface is required to remove a range of pathogens which come into contact with the anterior surface of the eye, lowering the risk of further pathological complications, such as diminished sight or blindness. The cornea is responsible for refracting light into the eye and must remain clear to ensure functionality. However, unregulated immune responses are associated with corneal tissue damage in a range of inflammatory ocular diseases, which manifests as a loss of visual capability. Previous studies have shown vitamin D metabolite 1,25-dihydroxyvitamin D3 (1,25D3) suppresses inflammatory pathways by modulating innate immune responses, including the production of pro-inflammatory mediators and antimicrobial peptides.

This research used an *in vitro* model of corneal inflammation comprising of the human telomerase-immortalized corneal epithelial cell line (hTCEpi) together with various agonists to stimulate toll-like receptors (TLR) which are pattern recognition receptors. The TLR agonists produce inflammatory responses similar to the pathogens they represent. The effects of 10^{-7}M 1,25D3 treatment on these responses were investigated. Using qRT-PCR, the study confirmed that 1,25D3 treatment significantly attenuated the expression of hTCEpi cell TLR3 ($p<0.001$), TLR5 ($p<0.001$) and TLR9 ($p<0.001$) stimulated by their agonists as well as expression of associated inflammatory molecules, including IL-6, IL-8 and IL-1β. Interestingly, IL-1β expression was shown to be significantly attenuated by 4h during TLR3 suppression ($p<0.001$), which may have suppressed further inflammation. Although there was an overall attenuation in pro-inflammatory cytokine expression following 1,25D3 treatment, 24h of 1,25D3 treatment alongside TLR3 activation led to a significant increase in the expression of cyclooxygenase-2 (COX-2) ($p<0.01$). This suggested that 1,25D3 is not entirely suppressive towards inflammatory responses, and may exacerbate inflammation.

When hTCEpi cells were stimulated with TLR5 ligands, bacterial flagellin from *Pseudomonas aeruginosa* and *Salmonella typhimurium*, 1,25D3 treatment reduced the expression of pro-inflammatory mediators IL-6, IL-8 and IL-1β ($p<0.001$). The study also used various classes of CpG oligodeoxynucleotide (CpG-ODN), TLR9 ligands which possess different levels of unmethylated CpG motifs, where interestingly, increasing CpG motifs led to a weaker pro-inflammatory response by hTCEpi cells. Addition of 1,25D3 to hTCEpi cells stimulated with either CpG-ODN or *Escherichia coli* (*E. coli*) DNA significantly decreased expression of a range of pro-inflammatory mediators, including IL-6 ($p<0.001$) and IL-8 ($p<0.001$).

Micro-RNA (miR), which regulate inflammatory protein expression, were investigated for their role in the mechanism of action of 1,25D3 during its attenuation of responses to TLR3, TLR5 and TLR9 stimulation. Data showed that inhibition of miR-93-5p and miR-181-3p led to a significant increase in the expression IL-6, IL-8 and IL-1b following all TLR3 and TLR5 stimulations, with some TLR9 stimulations. These data implicate a regulatory role for these miR in ocular inflammation. Finally, the study failed to identify significant cyclic guanosine monophosphate-adenosine monophosphate synthase (cGAS)/stimulator of IFN Gene (STING) activity in hTCEpi cells.

In conclusion, this study provides evidence that 1,25D3 inhibits inflammation caused by a range of pathogens, whilst certain miR may regulate pro-inflammatory mediators following a range of TLR activations. Hence both 1,25D3 and miR are candidates for treatment of ocular inflammatory diseases.

THESIS PRESENTATIONS AND POSTERS

Oral presentations

- Lucy Appleby, Alison McDermott, Stephen Todryk. (2019). Role of miRNA in vitamin D3 suppression of inflammatory cytokines within ocular surface innate immunity. Presented at Immunology North East conference, Northumbria University, 7 June 2019

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- Lucy Appleby, Alison McDermott (2019). The potential role of miRNA in Vitamin D3 suppression of inflammatory cytokines within ocular surface innate immunity. Presented at British Society of Immunology Congress 2019, Arena and Convention Centre, Liverpool, 2 – 5 December 2019

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AUTHOR'S DECLARATION

I declare that the work completed and contained in this thesis has not been submitted for any other award from the work of others, or research degree, and that it is all my own work. I also confirm that this thesis fully acknowledges the work, opinions and contributions of others.

I declare that the word count of this thesis is 40,821 words.

Name: Lucy Elizabeth Appleby

Signature:

Date: 27/06/2022

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LIST OF ABBREVIATIONS

1,25D3	1,25-dihydroxyvitamin D3
25D3	25-hydroxyvitamin D3
AMD	Age-related macular degeneration
AMP	Anti-microbial peptide
BAC	Benzalkonium chloride
CAMP	Cathelicidin antimicrobial peptide
cGAS	Cyclic GMP-AMP synthase
COX	Cyclooxygenase
CpG	Cytosine-guanine
DC	Dendritic cell
pDC	Plasmacytoid dendritic cell
DED	Dry eye disease
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethylsulphoxide solution
DRIP	Vitamin D receptor interacting protein complex
dsDNA	Double stranded DNA
dsRNA	Double-stranded RNA
E. coli	Escherichia coli
ELISA	Enzyme linked immunosorbent assay
FBS	Fetal Bovine Serum
FLA-PA	Flagellin *Pseudomonas aeruginosa*
FLA-ST	Flagellin *S. typhimurium*
HBD	Human beta-defensin
hCAP-18	Human cathelicidin antimicrobial protein-18
HCEC	Human corneal epithelial cells
HSV-1	Herpes simplex virus-1
hTCEpi	Human telomerase-immortalized corneal epithelial cells
IDO	Indolemine 2,3-dioxygenase
IFN-	Interferon
IκBα	nuclear factor of kappa light polypeptide gene enhancer in B cells inhibitor, alpha
IKK	IκB kinase
IL-	Interleukin
IP-	Interferon gamma-induced protein

IRF	Interferon regulatory factor
KC	Keratoconus
KYN	Kynurenine
LPS	Lipopolysaccharide
MDA5	Melanoma differentiation-associated protein 5
miR	Micro-RNA
MMP	Matrix metallopeptidase
mRNA	Messenger RNA
MS	Multiple sclerosis
MTT	3-(4,5-dimethylthiazol-2-yl_-2,5-diphenyl tetrazolium bromide
MUC1	Mucin 1
Muc5AC	Mucin 5
MyD88	Myeloid differentiation response gene 88
NF-ĸB	Nuclear factor kappa b
NLR	Nod like receptor
NLRP3	NOD-like receptor protein 3
NOD1	Nucleotide-binding oligomerization domain-containing protein 1
NOD2	Nucleotide-binding oligomerization domain-containing protein 2
NOTCH1	Notch homolog 1, translocation associated
ODN	Oligodeoxyribonucleotides
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate buffered saline
Poly(I:C)	Polyinosinic:polycytidylic acid
PRR	Pattern recognition receptors
PTH	Parathyroid hormone
RIG1	Retinoic acid-inducible gene I
RIP1	Protein kinase receptor-interacting protein 1
RISC	RNA-induced silencing complex
RLR	Rig like receptor
RXR	Retinoid X receptor
SOCS1	Suppressor of cytokine signaling-1
Src	Proto-oncogene tyrosine-protein kinase Src
ssDNA	Single stranded DNA
STAT	Signal transducer and activator of transcription

STING	Stimulator of interferon genes
TAB2	TAK1 binding protein 2
TAE	Tris-acetate-EDTA
TGF-β	Transforming growth factor β
Th	T helper
TIR	Toll-IL-1R-Resistance
TLR	Toll like receptor
TNF	Tumour necrosis factor
TRIF	TIR-domain-containing adaptor
TRP	Tryptophan
UTR	Untranslated region
UV	Ultra-violet
VEGF	Vascular endothelial growth factor
VDBP	Vitamin D binding protein
VDR	Vitamin D receptor
VDRE	Vitamin D response element

Chapter 1
Introduction

1.1 – THE IMPACT OF OCULAR INFLAMMATION

1.1.1 The impact of inflammation of the ocular surface

Ocular surface diseases can be caused by many events, such as pathogenic infections from viruses, bacteria and fungi, which are detected by the innate immune system, leading to an inflammatory response to remove the microbe and lower the risk of further infection (Ho *et al.,* 2019). However, acute, or often improper, ocular inflammatory responses leads to an influx of immune cells within the cornea, which not only impairs vision by physically 'blocking' the passage of light, but may also cause irreversible inflammatory tissue damage. Clinical symptoms associated with the development of ocular inflammation often include conjunctival swelling, redness, pain and dilation of conjunctival blood vessels.

Although ocular inflammation is remediable, the choices of treatment are often managed in a systemic approach dependable on the severity inflammatory symptoms, with Rathinam *et al.,* (2013) describing diagnosis as 'difficult' due to complex interactions occurring at the ocular surface. Topical steroids are used as a current treatment option for targeting the overall inflammatory process, such as fluorometholone, with prednisolone used for severe ocular inflammation where rapid immunosuppression is required (Pinto-Fraga *et al.,* 2016, Babu *et al.,* 2013). However, Phulke *et al.,* (2017) highlighted the dangers of constant steroid use within the eye, which can manifest as acute alterations in intraocular pressure, alongside further complications such as posterior subcapsular cataracts and glaucoma. Furthermore, steroids can interfere with processes such as ocular wound healing following infection, increasing the risk of developing complications (Petroutsos *et al.,* 1982). Failure to remove exacerbating factors, such as contact lenses infected with bacteria or unnecessary usage of topical medications, may lead to a persistent, robust inflammatory response with subsequent corneal damage.

1.2 – VITAMIN D

1.2.1 Vitamin D

In healthy human subjects, vitamin D is sourced from a diet rich in fatty fish and fortified foods, such as dairy products, whilst also being synthesized in the skin following ultra-violet (UV) B sunlight exposure. Vitamin D is a group

of fat-soluble 'secosteroids', with a similar chemical structure to steroids, except there is a breakage in the B-ring. These secosteroid hormones have a leading role in the absorption of calcium and phosphate. Wang *et al.,* (2005e) also highlighted the role of vitamin D in a range of both cellular and biological functions, reporting over 900 genes that are affected by the hormone, affecting processes such as bone health and immunomodulation (discussed throughout subsection 1.2.5). Consequently, these immunomodulatory effects upon cellular function may lead to protection against overt or improper inflammatory responses, preventing unnecessary tissue damage. For example, Sintzel *et al.,* (2018) characterised vitamin D as an 'anti-inflammatory agent', with a leading role in alleviating disease progression and relieving symptom severity for range of different inflammatory diseases and disorders, including multiple sclerosis (MS). One of the tissues in which vitamin D may prevent overt tissue damage by improper inflammatory responses includes the ocular surface. In 2019, The National Eye Institute reported that ocular conditions affect an estimated 4 million patients in USA and 2 million within the UK; figures that are expected to inflate annually due to both genetic and environmental factors (National Eye Institute, 2019, Fight for Sight, 2020). Many of these diagnoses will lead to further complications, which not only increases the economic pressures of medical care, but includes irreversible damage to vision functionality and subsequently, quality of life.

Reins *et al.,* (2015a) identified not only vitamin D production within corneal epithelial cells, but also significant immunomodulation in regard to innate, inflammatory signaling pathways (discussed further throughout subsection 1.3.4). There are many benefits in ascertaining vitamin D's exact mechanism of action during these suppressive actions, such as identifying any potential increases within pro-inflammatory mediator production. These potential contraindications from vitamin D treatment may affect specific groups of patients, but would also help in identifying a novel target that may increase the efficiency of vitamin D as an anti-inflammatory therapeutic.

1.2.2 Vitamin D and Calcium Homeostasis

The relationship between calcium and vitamin D has been highlighted throughout history, with the first major finding reported in the early 1900s by

Sir Edward Mellanby. Rickets may be caused by a calcium disorder, leading to a weakening of bone composition which subsequently increases the risk of bone breakage and impairment of skeletal function. Subtypes of rickets can affect numerous organs within the body, for example, the kidney impairment seen within a renal rickets diagnosis (Sahay *et al.,* 2013). Sir Edward Mellanby demonstrated the relationship between vitamin D and the development of rickets and associated symptoms, mimicking the pre-disposing risks of rickets in dogs. This included the restriction of outdoor sunlight exposure and implementing a diet purposely deficient of vitamin D sources, which resulted in a vitamin D deficiency and to the development of rickets (DeLuca, 2014). In addition, Elmer McCollum then showed that the vitamin D found within cod liver oil had the ability to not only prevent, but reverse the associated symptoms seen in murine models, highlighting the potential of the hormone in the treatment of the disease (DeLuca, 2014).

From findings such as these, it is now commonly accepted that vitamin D is a vital component of calcium homeostasis. The hormone increases absorption of calcium and phosphate in the intestine, which induces bone resorption to enforce stable, serum calcium levels within the blood, preventing diseases such as rickets. As previously mentioned, vitamin D is sourced from a diet rich in fortified foods and fatty fish, but is also locally produced within the skin following exposure to UVB radiation, stimulating the production of pre-vitamin D and subsequently, vitamin D3, through a process of both thermal and photochemical reactions (Lamberg-Allardt, 2006). Furthermore, studies involving adolescent patients have demonstrated that increased natural and artificial UV light exposure significantly increases serum concentrations of vitamin D, minimizing symptoms associated with rickets, which highlights the importance of vitamin D local production by epithelial cells (DeLuca, 2014).

On a cellular level, vitamin D has the ability to stimulate not only osteoclast activation, but also cell differentiation, which leads to calcium mobilization from bone (Takahashi *et al.,* 2014). Vitamin D also encourages renal reabsorption of calcium when serum levels become low, leading to a natural increase of calcium readily available in the bloodstream from bone stores (Blaine *et al.,* 2015). These findings characterise the role of vitamin D in the active transport of calcium, ensuring ‘super-saturated’ levels of calcium and phosphorus for bone mineralization in healthy individuals (Lamm *et al.,* 1958).

1.2.3 Vitamin D Production

As reviewed by Reins *et al.,* (2015e), there are two main precursors of vitamin D; ergocalciferol (vitamin D2) and cholecalciferol (vitamin D3). As previously discussed, vitamin D2 can be sourced from ingesting products rich in vitamin D, however, vitamin D3 is formed from 7-dehydrocholestrol photo-isomerisation from UVB radiation (290-316 nm) within the epidermis of the skin (Figure 1.1). Vitamin D3 is then removed from the skin's epidermal layer, binding to circulating vitamin D binding protein (VDBP), where it is delivered to the liver to be metabolized (Bikle *et al.,* 2019). Within the liver, vitamin D3 molecules are hydroxylated to 25-hydroxyvitamin D3 (25D3), the primary circulating metabolite form of the hormone within the human body, by cytochrome p450 enzyme CYP2R1; a process occurring within the mitochondrial membrane.

Within the proximal convoluted tubule of the functioning kidney, enzyme CYP27B1 modifies 25D3 further into active metabolite 1,25-hydroxyvitamin D3 (1,25D3) (Bikle *et al.,* 2019). Circulating levels of 1,25D3 are tightly regulated by parathyroid hormone (PTH), which also acts as a major stimulator of not only vitamin D synthesis within the kidneys, but also circulating calcium and phosphate (Khundmiri *et. al* 2016). However, 1,25D3 exerts negative feedback upon PTH, by calcium sensing proteins in the parathyroid recognising hypocalcaemia, which induces PTH secretion. This feedback loop between 1,25D3 and PTH helps prevent the development of bone diseases by ensuring adequate minerals for healthy bone composition and formation by kidney stimulation. Furthermore, 1,25D3 is also negatively regulated by CYP24A1; an enzyme produced by the kidney, which prevents unnecessary toxicity by encouraging catabolism of 25D3 and 1,25D3 metabolites and subsequent excretion of 24-hydroxylated products. For example, catabolism of 25D3 by CYP24A1 leads to the production of 24,25D3, whilst 1,25D3 can be catabolised into 1,24,25D3, both of which may be oxidised into calcitriol acid for excretion (Cappellani *et al.,* 2019).

Although the kidney proximal tubules were assumed to be the primary site of vitamin D production, further tissues and cells which are capable of vitamin D production have been identified. For example, epithelial and immune cells, such as monocytes, often contain the enzyme CYP27B1, and therefore have

the ability to not only source their own supply of 1,25D3, but react to it (Medrano *et al.,* 2018). A further example includes keratinocytes, skin cells which produce vitamin D for crucial cell differentiation. Teichert *et al.,* (2010) implicated a deficiency in this same production in the progression of squamous cell carcinoma, demonstrating a protective effect of the hormone against disease. Furthermore, vitamin D has several important functions outside of calcium and phosphorus regulation, including modulating immune responses.

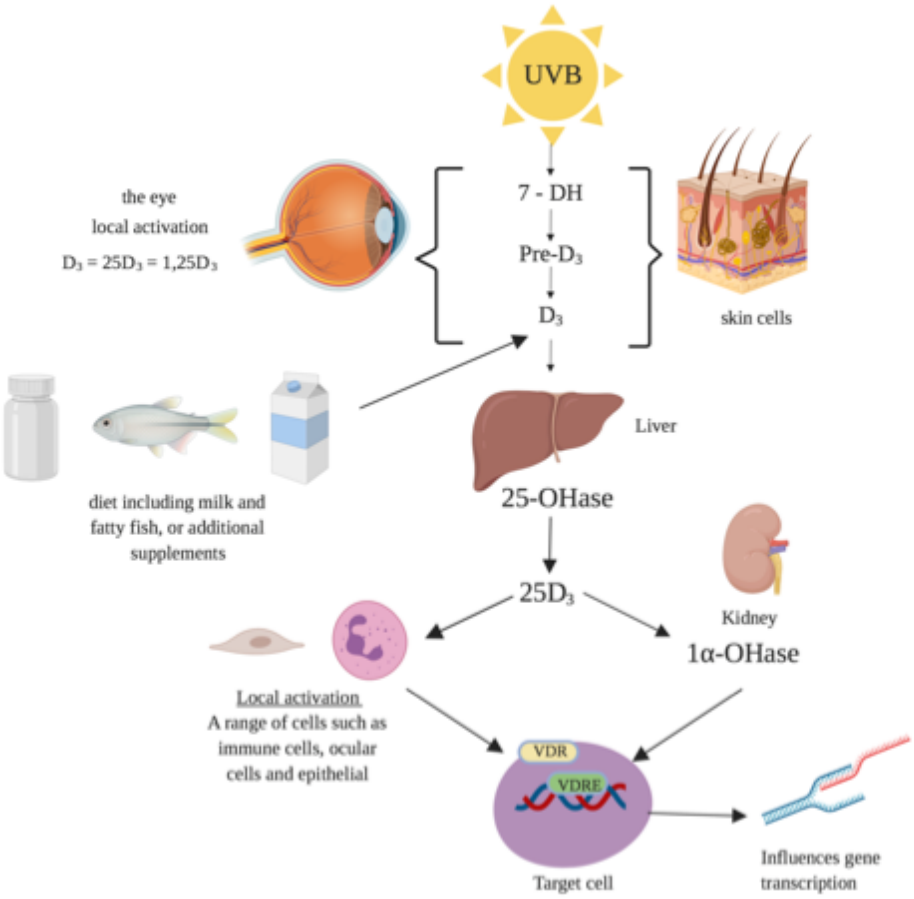

Figure 1.1. – 1,25D3 and 25D3 production and activation. Adapted from Reins, et al. (2015). Ultra-violet B sourced from sunlight penetrates skin cells, which converts 7-dehydrocholesterol (7-DH), to pre-vitamin D3 (pre-D_3). Pre-D_3 then isomerizes to form vitamin D3 (D3). Hydroxylating enzymes then produces 25D3 from D3, with $25D_3$ being the major circulating form of the hormone within the human body. Numerous cells of the body are able to activate 25D3, which is modified further into 1,25D3 at some of these sites, including ocular cells.

1.2.4 Vitamin D and VDR

Once 1,25D3 reaches the target cell, the hormone disassociates from the VDBP and then binds to the vitamin D receptor (VDR), a member of the nuclear hormone receptor family (Khammissa *et al.,* 2018). The VDR is expressed in a range of cells, acting as a transcription factor, which regulates the expression of a wide range of genes, a mechanism which 'affects numerous cellular processes, from proliferation to inflammation' (Janik *et al.,* 2017). Furthermore, the VDR can interact with 1,25D3, meaning the hormone can subsequently modify genetic expression, influencing cellular activity which may be pro-inflammatory or anti-inflammatory. Homology is crucial for VDR function, for example, the N terminal domain is situated close to the DNA-binding domain and is required for binding to the VDRE in target promotors within specific genes. The hinge region then binds to both the DNA-binding domain and the ligand binding domain, which is crucial for not only 1,25D3 binding, but also the site of heterodimerization for the retinoid X receptor (RXR) (Reins *et al.,* 2015e).

Once the VDR is active, it heterodimerizes with RXR, forming a biological complex which is capable of binding with the vitamin D response element (VDRE) of target genes, increasing DNA binding and related transcriptional activity (Peng *et al.,* 2004). RXR binds to the 5' repeat of the VDRE and the 3' repeat of the VDR. The VDRE section of a gene is a promotor region that contains direct hexameric DNA sequence repeats, with the exact motif of A/GGG/TTC/GA, also known as a DR3-type response, allowing DNA polymerase to complete synthesis (Peng *et al.,* 2004).

Cells have various coactivators and corepressors to both activate and inhibit VDR activity. For example, a coactivator such as vitamin D receptor interacting protein complex (DRIP), can bind between VDR to proteins such as RNA polymerase II and transcription start sites of genes (Pike *et al.,* 2012, Oda *et al.,* 2014). DRIP proteins range between a size of 65-250 kD, all capable of targeting the VDR and enhancing transcriptional activity of genes. These proteins function by encouraging the unravelling of chromatin at the start of a gene and allowing subsequent transcription by the recruitment of histone acetyl transferases and demethylases (Bikle *et al.,* 2018). An example of DRIP functioning was shown by Oda *et al.,* (2010) in

keratinocytes, with the DRIP205/MED1 complex binding directly to the VDR which leads to VDR transactivation. Other subunits include MED14 and MED17, each capable of binding to the VDR and effecting the transcription of genes such as cyclin D1, a protein vital for cell cycle development which is often associated with cancer and inflammation (Oda *et al.,* 2010). Further examples of coactivators known to interact with VDR include the steroid receptor coactivators (SRC) 1, 2 and 3 (DeLuca *et al.*, 2021).

In contrast, there are also co-repressors present in cells, which encourage recruitment of histone deacetylases or methyl transferases to the gene, leading to the reversal of the unravelling of chromatin caused by the coactivator (Bikle *et al.,* 2018). RXR is capable of recruiting co-repressors, leading to epigenetic changes that are often associated with genetic repression. Examples of such co-repressors, and arguably the most studied, are the nuclear receptor co-repressor (NCoR) and the silencing mediator for retinoid and thyroid hormone receptors (SMRT), both of which have been shown to effect VDR binding and subsequently downgrades genetic expression (Meyers, 2012). This entire process leads to activation and modulation of both transcription and translation phases for multiple genes, including those involved within the production of pro-inflammatory cytokines by immune cells (Chirumbolo *et al.,* 2017).

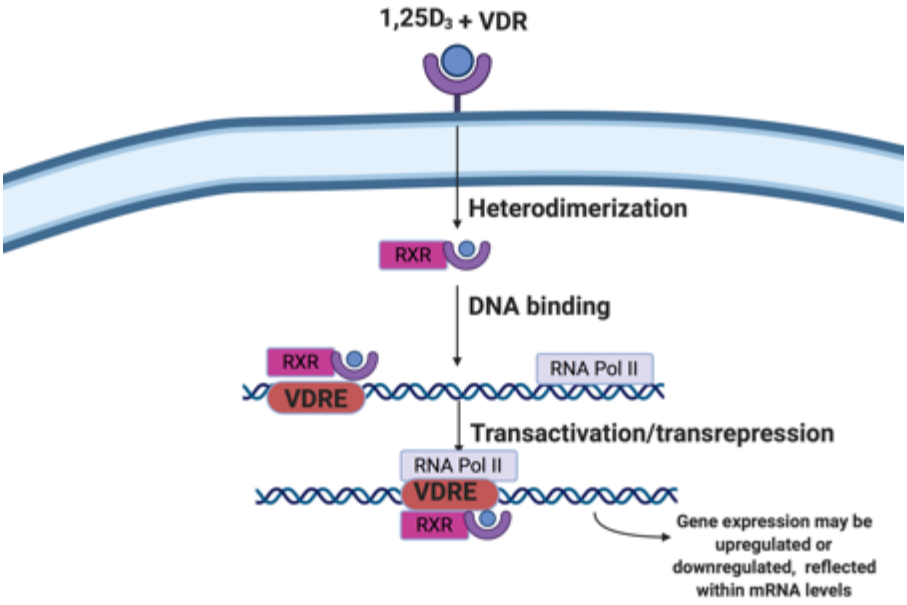

Figure 1.2. – 1,25D3 effects upon VDR and subsequent gene regulation. Ultra-violet B sourced from sunlight penetrates skin cells, which converts 7-dehydrocholesterol (7-DH), to pre-vitamin D3 (pre-D3). Pre-D3 then isomerizes to form vitamin D3 (D3). Hydroxylating enzymes then produce $25D_3$ from D3, with $25D_3$ being the major circulating form of the hormone within the human body. Numerous cells of the body are able to activate $25D_3$, which is modified further into $1,25D_3$ at some of these sites, including ocular cells.

1.2.5 Vitamin D and Immunomodulation

Immune cells of both innate and adaptive origin, such as neutrophils and T cells, work in conjunction to remove the culprit pathogen during the acute inflammatory response, whilst aiding in restorative processes such as wound healing. Inflammation is not only a reaction to infection, but also a response to physical injuries and stresses to cells. The classic physiological changes associated with inflammation at the site of injury include heat, redness and loss of function. Immune cells undergo numerous processes during this inflammatory phase, including cell proliferation, infiltration and subsequent cytokine release. This is followed by a resolution period, during which pro-inflammatory signals are downregulated and the body returns to homeostasis (Figure 1.3). However, overt, sustained inflammatory responses can lead to further tissue damage, causing a potential decrease within organ functionality.

A deficiency in vitamin D is associated with an increased risk of developing many inflammatory diseases, such as rheumatoid arthritis and multiple sclerosis, many of which require pharmaceutical intervention by vitamin D supplementation (Mangin *et al.*, 2014). 1,25D3 treatment has yielded significant results throughout both *in vitro* and *in vivo* studies, showing a beneficial and significant response in protecting against overt inflammatory reactions. For example, on a genetic level, 1,25D3 modulates the activity of NF-κB by increasing the expression of IκBα. This protein inhibits NF-κB activity by sequestering the required proteins in the cellular cytoplasm, leading to a significant decrease within the transcription of pro-inflammatory mediators (Cohen-Lahav *et al.,* 2006). In regard to immune cells, Fiske *et al.,* (2019) showed the expression of major histocompatibility complex-II (MHC-II) and cluster of differentiation 86 (CD86), a powerful co-stimulatory molecule, are decreased in monocytes and macrophages following 1,25D3 treatment, which suppresses the inflammatory response. Furthermore, Saul *et al.,* (2019) conducted an *in vivo* study investigating the effect of 1,25D3 upon CD31+ dendritic cells (DC), with 1,25D3 treatment increasing DC population, modulating DC activity and subsequently reducing the population of priming CD4+ T cells, suppressing the immune response.

However, not all of these studies have shown that 1,25D3 impedes the immune response. For example, on a cellular level, Chandra *et al.,* (2004) showed that the phagocytic activity of macrophages increases following 1,25D3 treatment, which supports the removal of potentially harmful agents, including microbes, during the innate response. Furthermore, 1,25D3 treatment has been shown to significantly increase the transcription and subsequent production of a range of anti-microbial peptides (AMPs), which kill microbes by targeting their cell walls (Szymczak, 2015).

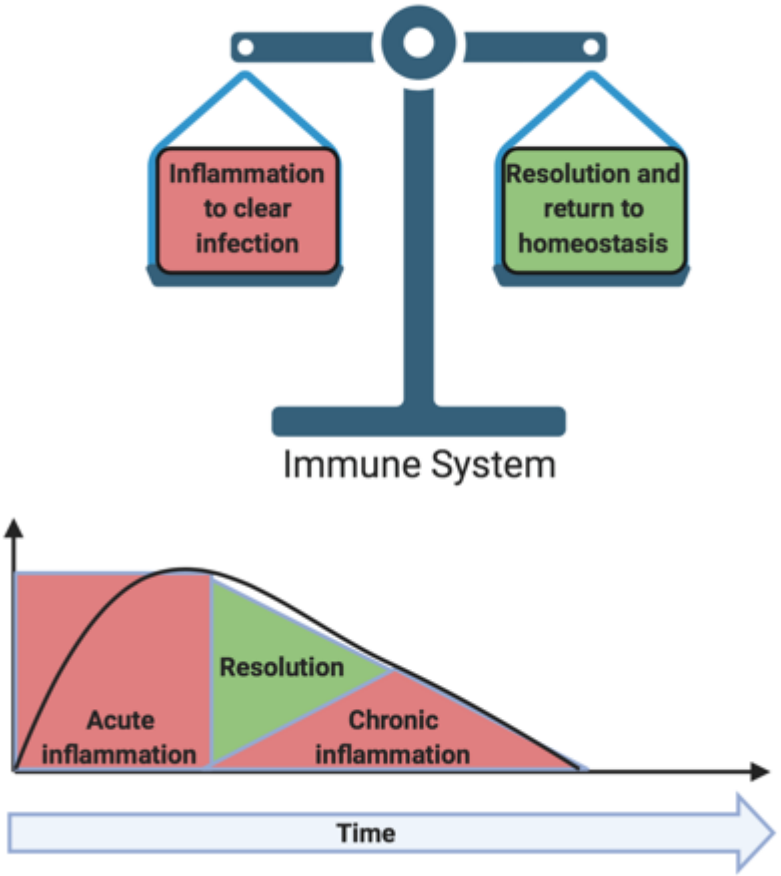

Figure 1.3. Inflammation and Resolution. Adapted from Reins *et al.* (2015a). The acute inflammatory response is required to remove harmful stimuli, such as pathogens or damaged cells, using means such as immune cells and molecular mediators, whilst initiating tissue repair. Once this process is complete, pro-inflammatory signals are suppressed and the body returns to a state of homeostasis during the resolution period. Both periods are required for a healthy, functioning immune system. However, sustained inflammatory responses over a period of time may lead to further damage, causing chronic inflammatory disease.

1.2.6 Vitamin D as an Anti-Inflammatory Therapeutic

Gröber *et al.,* (2013) reported that a serum concentration of >30ng/ml of 25D3 is in healthy range for an adult, with a deficiency classed as <30ng/ml. As previously discussed, a deficiency in vitamin D increases the risk of developing overt immune responses and associated tissue damage (Peterson *et al.,* 2009). Kennel *et al.,* (2010) highlighted the growing trend of individuals presenting low concentrations of vitamin D metabolite 25D3, describing the event as a 'worldwide epidemic', which may be associated with the growing incidence of inflammatory diseases. Studies have shown that

increasing vitamin D serum concentrations leads to a significant decrease in the production of pro-inflammatory cytokines IL-6 and TNF-α, both of which are associated with inflammatory tissue damage (Vita *et al.,* 2014). As vitamin D not only modulates the activation but activity of immune cells, it may impact subsequent symptom severity in a range of inflammatory diseases. *In vivo* studies have shown that the administration of 1,25D3 prevents the development of inflammation associated with diseases such as rheumatoid arthritis and type 1 diabetes, with MS patients often given vitamin D supplements to prevent further nerve damage from improper inflammatory responses (Cantorna *et al.,* 1998, Zella *et al.,* 2003, Hausler *et al.,* 2019).

1.3 - THE OCULAR SURFACE

1.3.1 The eye

The eye is a very complex, and often overlooked, organ which provides a functioning visual system for not only receiving and processing visual details, but also ensures an enjoyable quality of life. Each eye is around one inch wide and deep, filled with a gel-like substance known as vitreous humour (Ghodasra *et al.,* 2016). The main function of the eye is detecting light by photoreceptive cells known as 'rod' and 'cone' cells housed within the retina, which convert light into neural signals for processing by the brain (Ingram *et al.,* 2016). For this process to occur, various components of the eye must work in conjunction to collect as much light as possible. There are three specific layers distinguished within the eye (Figure 1.4). The outer region includes the cornea, used for light refraction by transmitting the light to the lens, whilst the sclera forms a connective tissue coating around the eye to maintain its spherical shape. The conjunctiva, a transparent mucous membrane, covers the sclera, leading into the middle layer of the eye. The ciliary body controls the shape of the lens, whilst the choroid layer provides much needed oxygen and nutrients to ocular cells and tissue, allowing the eye to function efficiently. Here, the iris controls the size of the pupil and subsequently, the volume of light which can pass through the organ to the retina to the photoreceptive cells in the inner layer of the eye.

1.3.2 The cornea, ocular surface and tear film

The cornea is the anterior front of the eye and part of the 'ocular surface', therefore it requires a very robust immune system to ensure microbes such as bacteria and viruses are identified and removed accordingly. The ocular surface includes the cornea epithelium, conjunctival epithelium, the corneoscleral limbus and tear film. These components work together to provide a range of functions, offering physical protection against the entry of micro-organisms whilst ensuring a smooth and clear refractive surface for optical clarity. Cellular constituents of the cornea include epithelial cells, endothelial cells and keratocytes. The corneal epithelium has up to 7 layers of cells, formed from non-keratinized stratified squamous epithelium, which have an average lifespan of around ten days, as these cells undergo events such as apoptosis and desquamation (Sridhar 2018, Notara *et al.,* 2018). Various cells make up the corneal epithelium, including: superficial cells, wing cells and basal cells. The stratified corneal epithelium interacts closely with tear film which is produced by the lacrimal gland, and much like keratinocytes within the skin, are exposed directly to UVB from sunlight.

Tear film is a thin fluid layer which ensures that the epithelial cells remain lubricated and hydrated, whilst also being the first 'layer' of the ocular surface to come into contact with light and microbes (Figure 1.5). Traditionally, it was assumed that tear film was composed of three separate layers. However, research now suggests that tear-film is actually a bi-phasic structure composed of an outer lipid layer with a muco-aqueous phase (McMonnies 2020). The outer lipid layer of tear film is sourced from meibum, which is secreted from the lid margins and is spread into the tear film when the eye blinks. The lipid layer of the tear film is crucial for preventing tears evaporating, which would otherwise leave corneal epithelial cells susceptible to damage and is seen in conditions such as dry eye disease (McMonnies 2020). This lipid layer is composed of varying non-polar and polar lipids, with Willcox *et al.,* (2017) describing the polar lipids as 'vital' for spreading the lipid layer over the muco-aqueous layer. The muco-aqueous layer of tear film is composed of over 1,000 different proteins and peptides (Segev *et al.,* 2020). The mucin component is formed from highly glycosylated proteins which help hydrate the tears, subsequently ensuring that the corneal epithelial cells remain lubricated. Tear film also offers immunological protection to the ocular surface, for example, Schnetler *et al.,* (2012) highlighted the importance of

lactoferrin, which ensures the tears are bacteriolytic, alongside immunoglobulins and defensin antimicrobial peptide production.

The cornea is vital for light refraction and transmission for clear vision, alongside the overlying tear film, which aids in not only light refraction but also the removal of foreign bodies. Siegfried *et al.,* (2015) described a healthy, adult cornea as at least 500μm thick, which allows optimum light refraction. The cornea is vital for protecting the eye against not only microbial and physical trauma, but also UV associated damage. The largest component of the cornea is the stroma, which is composed of dense connective tissue, ensuring functional strength and transparency. The limbus of the cornea produces a supply of stem cells, which Dua *et al.,* (2000) described as 'vital' for healthy cell turnover of the corneal epithelium, as well as in the event of injury. The limbus borders the conjunctiva, which acts as a mucous membrane containing goblet cells which produce the mucins required for tears. A range of disorders can affect the production of tear film, including dry eye disease (discussed in further detail throughout subsection 1.5.2). The corneal endothelium is the final layer and completes the structure of the film. Comprised of a single layer of endothelial cells which ensures hydration of the cornea using a Na^+/K^+ pump, the endothelium ensures a constant pressure within the eye to avoid osmotic swelling (DeLuca, 2014). The corneal endothelium also acts as a physical barrier to the cornea, which is mediated by proteins such as zonula occludens-1, which allow movement of molecules into the corneal stroma from the anterior chamber (Feizi 2018). The corneal epithelial layer is also moderately impermeable to water-soluble substances; however, this can make the application of drugs such as antibiotic eye drops difficult due to poor penetration into areas like the anterior chamber (Agrahari *et al*., 2017). This same epithelial layer also provides an anti-angiogenic function, for example, little to no vascular endothelial growth factor (VEG-F) production prevents the growth of new blood vessels and allow maximum light refraction within the cornea (Le *et al*., 2020).

These ocular structures work in combination to form the ocular surface system, which protects the eye and maintains the function of the cornea. However, some events are unavoidable, for example, during the summer seasonal changes can bring low humidity which equates to less moisture in the air to lubricate the cornea. The transitional months have been described as particularly damaging to the ocular surface, with extreme autumnal wind

capable of drying out the corneal layer, alongside an increase of plant microbes within the environment in the summer, both of which can increase the risk of inflammatory eye diseases such as dry eye disease (DED) (Kumar *et al*., 2016c). Therefore, it is vital that the ocular surface has both physical, anatomical barriers and a functioning immune system to minimise the risk of soft tissue damage caused by foreign pathogens and subsequent inflammation.

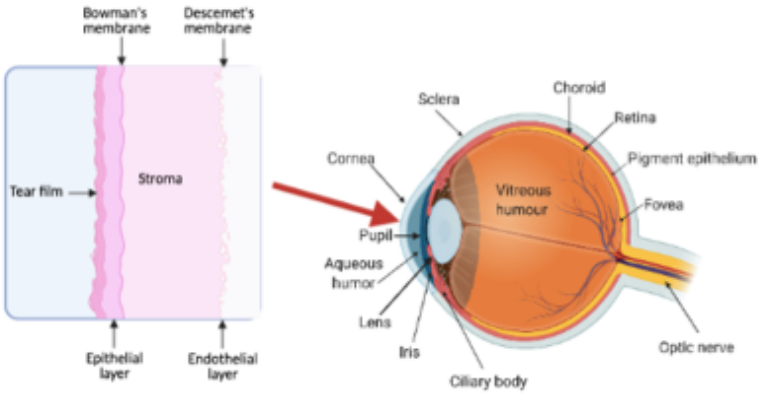

Figure 1.4. The sagittal section of the eye alongside the composition of the ocular surface. The ocular components which form the eye, giving the ability of sight. The ocular surface is composed of the cornea epithelium, conjunctival epithelium, the corneoscleral limbus and tear film.

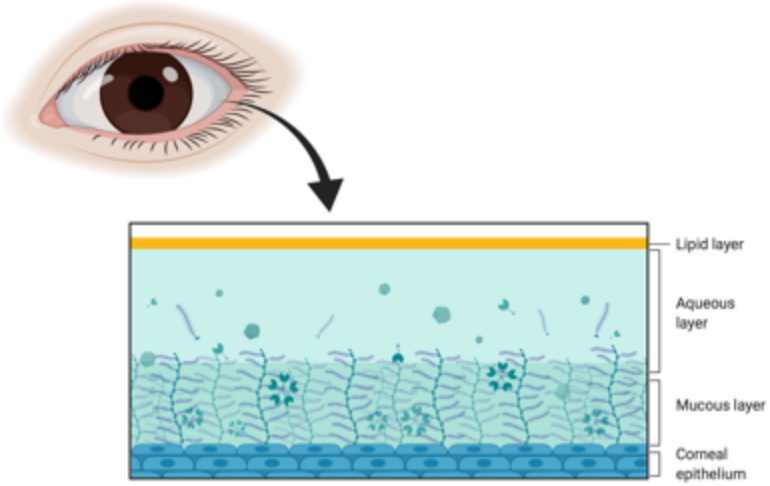

Figure 1.5. Tear Film. The ocular surface receives both physical and immunological protection from tear film, whilst also receiving the oxygen and nutrients required to function. The outer layer of tear film is composed of lipids to ensure limited evaporation, followed by the aqueous layer which contains soluble mucins and proteins for immunological support. The mucous layer includes the glycocalyx, which contains membrane-bound mucins which aid in binding the tear film to the ocular surface.

1.3.3 Vitamin D expression by corneal cells and the ocular surface

As previously discussed, although the main site of active vitamin D production is within the kidneys, there is a growing body of evidence which implicates many cells, including those of the corneal epithelium, are able to produce their own supply of vitamin D. For example, Lu *et al.,* (2017) confirmed that

hydroxylating enzyme CYP24A1 is expressed in human corneal epithelial tissues, accompanying the notion that the cornea can produce its own supply of vitamin D. Furthermore, Elizondo *et al.,* (2014) showed that vitamin D supplementation increased the expression of tight-junction proteins, improving corneal barrier function and increasing transepithelial resistance. This accelerates the wound healing process and minimises risk of infection by opportunistic pathogens. Lu et al., (2020) demonstrated this further whilst highlighting the importance of VDR *in vivo*, showing VDR deficient mice had a thinner epithelial layer, increasing the risk of opportunistic pathogens. However, vitamin D production is not limited to epithelial cells of the ocular surface, with both 25D3 and 1,25D3 metabolites detectable within tear samples from healthy eyes (Lai *et al.,* 2019). Watsky *et al.,* (2011) concluded that a vitamin D deficiency significantly impacts tear production and function, subsequently increasing the risk of ocular infections by weakening corneal support. Likewise, Paz *et al.,* (2003) supported these findings, showing VDR-deficient mice produced lower concentrations of mucin 5 (Muc5AC), a protein which has been ascribed numerous roles, including lubrication and preventing bacterial adhesion to the ocular surface.

1.3.4 The Ocular Immune System and Inflammation

Although the cornea physically protects underlying ocular tissues, the epithelial cells of the cornea also offer anti-microbial protection, minimizing the risk of opportunistic infections. Toll-like receptors (TLR) are pattern recognition receptors (PRR) that play a crucial role during the innate immune response and may be either intracellular or located at the surface of the cell (Table 1.1). Redfern *et al.,* (2006) and (2015) confirmed the expression of nine TLR receptors in corneal epithelial cell line HCEC-SV40 and throughout whole corneal tissue, with most utilizing variants of cytoplasmic TIR-containing adaptor molecules called MyD88 (myeloid differentiation response gene 88) to react and initiate inflammatory pathways (Figure 1.6).

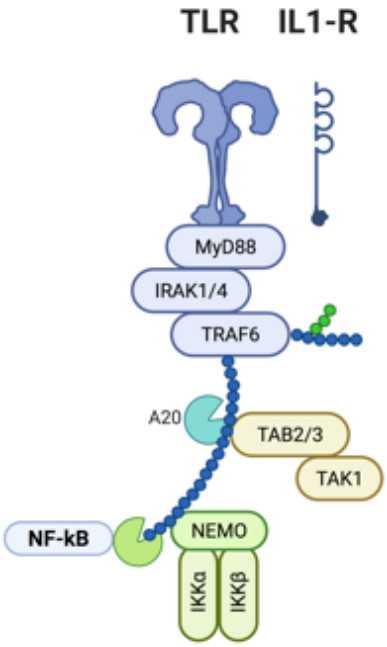

Figure 1.6. Simplified MyD88-dependent signaling. TLR-mediated MyD88-dependent signaling pathway is activated during TLR4, TLR5, TLR9 and TLR7/8 signaling, with similar results seen from the IL1-R pathway. MyD88 binds to the cytoplasmic portions of TIR domains, leading to the recruitment of IRAK-4 phosphorylating IRAK-1, which works in conjunction with TRAF6 to dissociate from the receptor. Interaction with TAK1, TAB1 and TAB2 leads to a larger complex with Ubc12 and Uev1a (not pictured), activating TAK1. TAK1 is then able to phosphorylate IKK complexes, including NEMO, IKKa and IKKb and MAP kinases such as JNK. This leads to transcription induction of NF-ΚB and AP-1 pathways.

However, TLR3 and TLR4 are exceptions, as they can function independently from the MyD88-signalling pathway (Youn *et al.,* 2005). Four of the identified corneal TLR (3, 7, 8 and 9) recognize nucleic acid and are expressed within endosomal membranes, as opposed to the remaining TLR which act within the plasma membrane (1, 2, 4, 5 and 6) (Redfern *et al.,* 2010). Ligand-binding of the intracellular TLR occurs in the lumen of intracellular vesicles, where detection of viruses and bacteria which multiply in the cell are often recognized, triggering a powerful immune response. To date, TLR have been studied extensively at the ocular surface and there is clear evidence that these receptors participate during the innate immune responses to pathogens (Redfern *et al.,* 2010, Lambiase *et al.,* 2011).

Pattern Recognition Receptor	Location	Microbial ligand and example host
TLR1	Plasma membrane - **Forms heterodimer with TLR2 and TLR6**	**Multiple glycolipids, proteolipids –** bacterial peptidoglycans**, lipoteichoic acid –** gram positive bacteria (Choteau *et al.,* 2017)
TLR2	Plasma membrane - **Forms heterodimer with TLR1 and TLR6**	**Multiple glycolipids, proteolipids –** bacterial peptidoglycans**, lipoteichoic acid –** gram positive bacteria (Choteau *et al.,* 2017)
TLR3	Intracellular	**Double-stranded RNA** – viruses (Reins *et al,.* 2015a)
TLR4	Plasma membrane	**Lipopolysaccharide and various host molecules such as HSP60** – gram-negative bacteria (Swaroop *et al,.* 2016)
TLR5	Plasma membrane	**Flagellin** – bacteria (Yang *et al.,* 2017)
TLR6	Plasma membrane - **Forms heterodimer with TLR1 and TLR2**	**Microbial lipoproteins** – mycoplasma (Takeuchi *et al.,* 2001)
TLR7	Intracellular	**Single stranded RNA** – RNA viruses (Crozat *et al.,* 2004)
TLR8	Intracellular	**Single stranded RNA** -viruses (Diebold 2008)
TLR9	Intracellular	**Unmethylated CpG motifs** - bacteria, DNA viruses (Dalpke *et al.,* 2006)
NOD1	Intracellular	**Can cooperate with TLR, also peptidoglycans** - gram negative bacteria (Zhou *et al.,* 2019)
NOD2	Intracellular	**Can cooperate with TLR, also peptidoglycans** – muramyl dipeptide of bacterial cell walls, gram negative bacteria (Zhou *et al.,* 2019)
NLRP3	Intracellular	**Various TLR ligands** – bacterial flagellin and type III secretion systems leading to formation of the protein complex that recruits pro-capase-1 in response to TLR agonists

		(Zhou *et al.,* 2019)
RIG1	Intracellular	**Single or double stranded RNA** – RNA viruses (Saito *et al.,* 2008)
MDA5	Intracellular	**Single or double stranded RNA** – RNA viruses (Saito *et al.,* 2008)
cGAS	Intracellular	**CpG- DNA** – DNA viruses (Bhat *et al.,* 2014)

Table 1.1: Various human innate pattern recognition receptors: listed with their cellular location and preferred ligand type, along with an example host of the ligand. These include Toll-like receptors (TLR) 1-9, Nod-like receptors (NOD) 1 and 2, NOD-like receptor protein 3 (NLRP3), retinoic acid-inducible gene I (RIG1) and melanoma differentiation-associated protein 5 (MDA5).

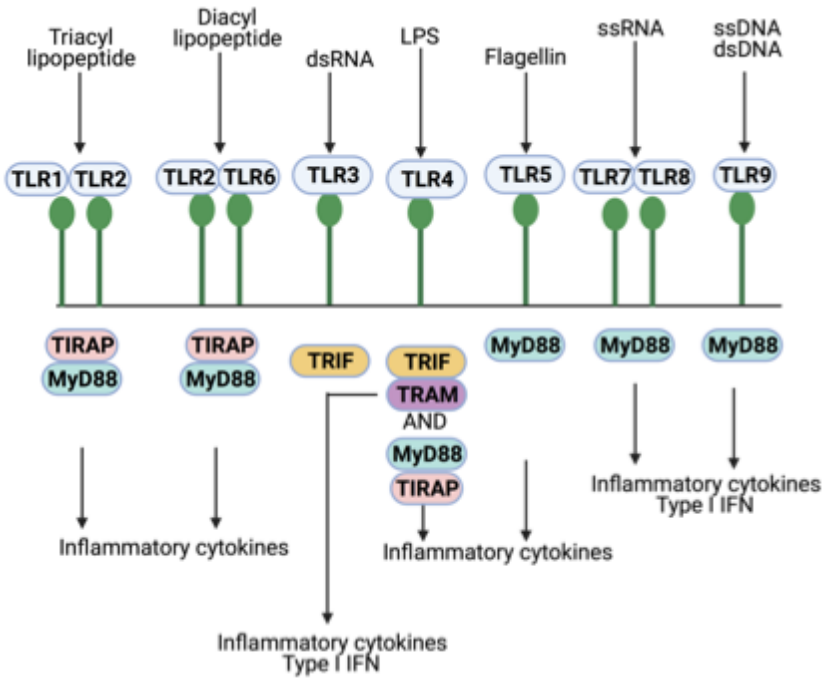

Figure 1.7. Examples of known TLR and their agonists. TLR-mediated immune responses with the required adaptive proteins. TLR1 and TLR2 work in conjunction to detect triacyl lipopeptides, whilst TLR2 can work alongside TLR6 to discriminate patterns associated with diacyl lipopeptides. TLR3 is able to recognise dsRNA, which is associated with replicating viruses. TLR4 recognises bacterial LPS. TLR5 recognises bacterial flagellin components. TLR7 works alongside TLR8 to identify ssRNA. TLR9 is able to recognise ssDNA and dsDNA.

However, TLR are not the only form of PRR which play a critical role throughout innate immunity, with further examples listed in Table 1.1. For example, Oh *et al.,* (2017) and Dominguez *et al,.* (2013) confirmed the expression of nucleotide-binding oligomerization domain-like receptors (NLR) and retinoic acid-inducible gene-I-like receptors (RLR) throughout corneal cells, with these receptors capable of recognizing the same microbes at certain TLR. Whereas TLR are transmembrane proteins with an extracellular liminal binding domain, NLR are intracellular cytosolic proteins which are also capable of triggering an immune response, with activated NOD1 and NOD2 proteins mediating inflammatory responses through NF-ΚB pathways (Carneiro *et al.,* 2008). Although limited, there is evidence of NLR activation during corneal inflammation. For example, studies such as Oh *et al.,* (2017) confirmed an increase in corneal cell NOD2 expression during an immune response to herpes simplex keratitis. However, NLR are not limited to immune responses, but other processes such as neovascularization, which is crucial for wound healing. Kim *et al.,* (2013) demonstrated that NOD1 signaling was significantly active during the process of corneal neovascularization in response to *Chlamydia trachomatis* infection, with the process ending following NOD1 inhibition. Rosenzweig *et al.,* (2011) showed that murine NOD2 deficiency from Crohn's mutation L1007fs, a gene often mutated within patients suffering from Crohn's disease, increases the risk of developing uveitis due to failure of peptidoglycan recognition, similar to the findings reported from colitis patients. Finally, Niu *et al.,* (2015) demonstrated that NLR pyrin domain-containing 3 (NLRP3), a subtype of NLR and intracellular receptor which detects components of damaged cells, was significantly increased during dry eye disease (DED), highlighting the potential involvement of this inflammasome during ocular inflammation.

The RLR receptor family includes melanoma differentiation-associated protein 5 (MDA5) and RIG1, both of which are capable of recognizing viruses. Research into the roles of these receptors during an ocular infection is limited, but studies show an increase in endothelial cell MDA5 expression during a Zika virus infection, suggesting RLR function in an inflammatory capacity (Roach *et al.,* 2017, Singh *et al.,* 2017). In regard to RIG1, Domínguez *et al.,* (2013) showed that this PRR has a role in corneal cells forming a pro-inflammatory response to polyinosinic:polycytidylic acid

(Poly(I:C)), a robust model of double-stranded RNA infections of the ocular surface, which is often associated with viral outbreak.

1.4 - INNATE IMMUNE RECEPTORS

1.4.1 TLR3

TLR are arguably the most commonly researched PRR in regard to innate immunity, not limited to their expression within a wide range of cells, but also their capability of detecting various components of invading pathogens. TLR3 recognizes dsRNA which is derived from viral genomes, typically released from damaged cells or viral particles, launching a powerful immune response which has been implicated throughout ocular inflammatory disease. Although the receptor is commonly studied within the epithelial layer of the cornea, other ocular cells have confirmed TLR3 expression, including corneal fibroblasts, which can also secrete pro-inflammatory cytokines following activation of TLR3 signaling (Liu *et al.,* 2008c).

Herpes simplex virus-1 (HSV-1) can lead to an accumulation of detectable dsRNA which can be detected by TLR3, which, if untreated, can lead to further inflammatory complications such as keratitis (Farhatullah *et al.,* 2004). Keratitis is an ocular infection, which may be caused by both physical traumas and pathogenic microbes, including bacteria and viruses. The condition is described as a 'frequent cause' of visual impairment due to chronic inflammatory damage to corneal tissue. This can lead to irreversible damage which requires corneal transplantation to restore sight (Liesegang 2001). Jin *et al.,* (2007) confirmed that corneal epithelial cells exposed to HSV-1 induces TLR3 signaling and subsequent activation of both the NF-κB and MAPK inflammatory pathways during the first 1-4 hours of infection, increasing production of pro-inflammatory mediators IL-6, IL-8, TNF-α and IFN-β. Dry eye disease (DED) is a multi-factorial disorder driven by tear hyper-osmolarity and film instability, and another ocular example of ocular disease with an overstimulation of TLR3 signaling. Throughout this inflammatory response, a subsequent increase of pro-inflammatory cytokines build a hyper-inflammatory environment, leading to corneal damage (discussed throughout subsection 1.5.2). It is unusual to find dsRNA within the cytosol of healthy cells, with Hartmann *et al.,* (2017) showing that dsRNA must be at least 40bp to induce signaling by TLR3. However, non-viral

mRNA released from necrotic cells undergoing chronic inflammation can lead to improper TLR3 activation and associated tissue damage (Kariko *et al.,* 2004).

The TLR3 receptor is predominately located within the endosomal membrane, however, Matsumoto *et al.,* (2014) showed that TLR3 may be found in other areas of the cell, depending on cell subtype, for example, the cell surface of human fibroblasts. The interaction and identification of dsRNA by TLR3 leads to the dimerization of the two ectodomains of the TLR3 receptor. The molecule TIR-domain-containing adaptor inducing interferon B (TRIF) is then recruited to the cytoplasmic domain of TLR3. This domain of TLR3 is known as Toll-IL-1R-Resistance (TIR) domain, as it shares homology with signaling domains of the IL-1 receptor. TRIF is then recruited after phosphorylation of the Tyr^{759} and Tyr^{858} domains of the TLR3 cytoplasmic domain. Activated TRIF initiates signaling pathways IRF3, AP-1 and NF-κB, each of which induces pro-inflammatory mediators, type 1 interferons and chemokines to attract further immune cells to the site of injury (Sarkar *et al.* 2007, Botos *et al.* 2009). The requirement of TRIF in TLR3 signaling highlights the difference between TLR3 to other TLR signaling pathways, especially those other endosomal TLRs (7-9) which do not require TRIF, but instead require MyD88 (Perales-Linares *et al.,* 2013).

As previously mentioned, numerous inflammatory pathways can be induced following TLR3 activation. For example, IRF3 induction can lead to an antiviral response and type 1 IFN production, whilst protein kinase receptor-interacting protein 1 (RIP1) can lead to cell death via caspase-8 activation through a TLR3-TRIF axis activation (McAllister *et al.,* 2013). Arguably the most discussed inflammatory response is the NF-κB pathway, which may be activated following TLR3 stimulation. This response is mediated by RIP1 and the E3 ubiquitin protein ligase TRAF6, which interact with TRIF and recruit TAK1 binding protein 2 (TAB2) and transforming growth factor-B-activated kinase 1 (TAK1) (Perales-Linares *et al.,* 2013). This activity leads to the formation of NF-κB essential modulators IKKα and IKKβ – which, when combined, form the IKK complex, resulting in the production of both pro-inflammatory cytokines and type 1 interferon. Lan *et al.,* (2012) described the NF-κB pathway as a 'key transcription factor' during important processes

required by the ocular surface, including inflammation, corneal wound healing and angiogenesis.

Highlighting the impact of TLR3 signaling during inflammation of ocular tissue, Johnson *et al.,* (2008) desmonstrated no macrophage infiltration into the corneal stroma of $TLR3^{-/-}$ and $TRIF^{-/-}$ mice following poly(I:C) stimulation, alongside an obliteration of pro-inflammatory cytokine IL-6 and IL-8 production. Although the TLR3-TRIF pathway functions independently from MyD88, studies have shown that poly(I:C) stimulation of TLR3 in $MyD88^{-/-}$ mice leads to an overt inflammatory response, with an amplified level of neutrophil and macrophage infiltration into the corneal stroma, highlighting that other PRR still play a role during overt immune responses (Johnson *et al.,* 2008).

These findings show that not only is TLR3 expressed by corneal epithelial cells, but these TLR3 are fully functional following recognition of dsRNA, activating an efficient MyD88-independent pathway.

1.4.2 TLR9

A further example of a TLR expressed by the ocular surface is TLR9, which Reins *et al.,* (2017b) confirmed in both commercial corneal cell lines and Li *et al.,* (2007c) confirmed within live-donor cornea tissue. Similar to TLR3, TLR9 is an intracellular receptor belonging to the innate immune system, offering protection against both single stranded (ssDNA) and double stranded (dsDNA) DNA (Roers *et al.,* 2016). TLR9 is based within endosomes, with the function of recognizing unmethylated cytosine-guanine (CpG) dinucleotide motifs in bacterial or some viral DNA. The majority of healthy, human DNA contains methylated CpG cytosines, unless the DNA is sourced from mitochondria organelles, implicating this is where methylation occurs (Han *et al.,* 2008).

TLR9 aids in promoting a strong immune response through both innate and adaptive measures. Inactive TLR9 of the endoplasm binds with the microbial ligand through the endocytic pathway, then relocates to the endolysosome of the cell. Once cleaved, TLR9 then gains the ability to recruit MyD88, gaining an active state (Kawasaki *et al.,* 2014). This activation method has been

proposed by Nakad *et al.* (2016) to 'prevent autoimmunity' by only recognising bacterial unmethylated CpG motifs. Pohar *et al.,* (2017) proposed that double CpG motifs found within oligodeoxyribonucleotides (ODN) sequences, ensures appropriate reactions to self and pathogen DNA by TLR9. Phosphorylation of interferon regulatory factor (IRF) 7 is then induced, with inflammatory genes then activated from the NF-KB pathway. These events lead to a potent induction of IFN-α/β proteins, which have the ability to promote production of a range of cytokines, encouraging clearance of foreign material.

TLR9 is therefore an imperative part of any functioning immune system, detecting unmethylated DNA motifs and launching a robust immune response. Guggemoos *et al.,* (2008) highlighted the importance of this receptor *in vivo* using TLR9-deficient mice which had a significant susceptibility to DNA viruses, concluding this was due to an absence of successful DNA recognition. In regard to TLR9 activity of the ocular surface, Jin *et al.,* (2007) demonstrated that TLR9 expression is significantly upregulated during diseases such as HSV-1 keratitis, concluding TLR9 successfully identifies viral dsDNA and promotes pro-inflammatory cytokine release. Sarangi *et al.,* (2007) and Huang *et al.,* (2005c) supported these theories with similar *in vivo* studies, exhibiting that inhibition or silencing of TLR9 in HSV-1 keratitis models lead to a significant decline in visual capabilities, with viral 'overload' in corneal cells inducing heightened inflammatory responses and subsequent tissue damage. Although TLR9 is vital for robust ocular inflammation, the receptor has been shown to promote corneal destruction, which is discussed further throughout chapter 4.

Although ssDNA and dsDNA are generally associated with pathogenic viruses and bacteria, DNA can also be sourced from self, leading to pro-longed chronic inflammation. In a healthy cell, endogenous DNA is housed within the nucleus ready for replication or genetic translation. However, DNA abnormalities may be induced by both exogenous and endogenous sources, including damaging agents such as ultraviolet light and carcinogenic agents. Furthermore, abnormal genetic material, such as viral DNA, may be released from cellular apoptosis (Tubbs *et al.,* 2017). The evolutionary processes of cell-cycle checkpoints and enhancement of DNA damage response pathways prevents overt DNA damage, which would lead to an influx of material for TLR9 activation (Wenzel *et al.,* 2018). However, abnormalities in these

processes may lead to self-DNA present within the cytoplasm; a site associated with the presence of bacterial DNA (Surovstev *et al.,* 2018), leading to improper TLR9 activation and chronic inflammatory responses to self. This is due to the ability of TLR9 sensing self-DNA, leading to an improper inflammatory response (Kaur *et al.,* 2015, Lamphier *et al.,* 2006).

1.4.3 TLR5

Bacterial infections of the ocular surface account for over 70% of conjunctivitis cases. This figure is expected to increase due to everyday procedures such as contact lens application, which introduce bacteria to the ocular surface (Buznach *et al.,* 2005). Therefore, a healthy immune system is required to not only identify bacteria, but induce a robust immune response to prevent further infection. TLR5 is another example of a PRR which functions during both the innate and adaptive immune responses by primarily recognising PAMPs associated with the flagellin component of the bacterial flagella structure. The flagella protein brings motility to bacteria by acting as a moveable appendage, but is also capable of evoking a strong immune response when detected by TLR5 (Hajam *et al.,* 2017). Research has shown that numerous gram-negative bacteria implicated in ocular inflammation express detectable flagellin, including *Pseudomonas aeruginosa* and *Serratia marcescens* (Teweldemedhin *et al.,* 2017, Hozono *et al.,* 2006). These infections cause further inflammatory damage, manifesting as conditions such as conjunctivitis and keratitis

The ocular surface provides strong protection against pathogens in the form of tear production, alongside both the immunological and physical properties of epithelial cells. However, trauma to the corneal epithelium, such as physical abrasion or overt inflammatory damage, increases the risk of developing bacterial infection (Hazlett 2007). Numerous ocular cells express TLR5, including corneal epithelial cells, identifying both gram-negative and gram-positive bacteria breaching the ocular surface (Menon *et al.,* 2015). Ivičak-Kocjan *et al.,* (2013) described TLR5 activation, with the process requiring two bacterial flagellin molecules binding to two TLR5 ectodomains to form an active complex. To initiate NF-κB signalling, the activated TLR5 induces the cytosolic adaptor MyD88, leading to direct interaction with the TIR domain, which allows MyD88 to then recruit phosphorylated IRAK, which in turn, activates TRAF6 and TAK (McNamara *et al.,* 2006, Akira *et al.,* 2004).

TAK will successfully bind TRAF6 to the IKK complex, phosphorylating IκBα, which is then ubiquitinated and degraded, leading to NF-ΚB translocation to the nucleus and transcription of pro-inflammatory genes (Rhee *et al.,* 2008, McNamara *et al.,* 2006). Included within these transcribed pro-inflammatory genes are IL-8 and TNF-α, both of which play a crucial role in the immune response and encourage pathogen removal. However, overt or improper signalling by TLR5 can lead to excessive pro-inflammatory cytokine production, unnecessary tissue damage and potential loss of organ function (Das *et al.,* 2016, Ito *et al.,* 2019).

As discussed within subsection 1.3.2, the corneal epithelium is made up of various cell types. Zhang *et al.,* (2003) characterised TLR5 expression to specific cells of the cornea, with the receptor strategically expressed throughout various layers of the corneal epithelium. This indicates that these corneal epithelial cells are capable of triggering a TLR5 mediated innate response, even when one layer of cells is breached. Reins *et al.,* (2017b) showed that TLR5 of corneal epithelial cells can be stimulated by *Salmonella tryphimurium* flagellin, inducing an inflammatory response of pro-inflammatory IL-6 and IL-8 production. However, Parkunan *et al.,* (2014), concluded that TLR5 had a limited role within protecting the eye following infection by *Bacillus cereus* in $TLR5^{-/-}$ deficient mice, suggesting the that the bacterial flagellin had a greater importance during motility than infection. These findings suggest that it is important to study not only the receptor, but various bacteria which express alternate flagellin.

As previously mentioned, *P. aeruginosa* is the most common cause of bacterial infection in contact lens wearers, and if the infection remains untreated or unresponsive to treatment, overt inflammatory damage can lead to necrosis of the corneal epithelial cells (Kolar *et al.,* 2011). However, *P. aeruginosa* flagella used during bacterial motility is detectable by TLR5 (Garcia *et al.,* 2018, Green *et al,.* 2008, Pachigolla *et al.,* 2007). Cendra *et al.,* (2017) showed that corneal cell TLR5 successfully identified the flagellin of *P. aeruginosa,* promoting a significant increase of pro-inflammatory cytokines IL-1β and IL-18 to aid in the removal of the bacteria.

The same bacterial infections activating ocular cell TLR5 signalling, can also lead to an increase of anti-microbial peptides (AMPs) production, such as human B-defensins (hBD) and cathelicidin (LL-37) (Gordon *et al.,* 2005, Gao

et al., 2013). Not only do AMPs hBD-2 and hBD-3 have efficient antimicrobial activity against *P. aeruginosa,* leading to a successful removal of bacterial colonies, but Redfern *et al.,* (2011) suggested that hBD-2 could work in a negative feedback loop to modulate TLR expression. The human cathelicidin antimicrobial peptide (CAMP) gene encodes for a preproprotein with a 30-amino long N-terminal signal sequence (hCAP-18), a 94 cathelin domain and a 37-amino acid C-terminal cationic AMP domain known as LL-37 (Zanetti *et al*., 1995). Offering protection to mammals, CAMP is secreted in fluids such as saliva and sweat, providing a barrier against pathogens. Therefore, LL-37 is the functional cleavage product of hCAP-18. Historically, hBD-2 is known as an anti-microbial peptide which forms powerful immunomodulatory response towards gram-negative bacteria, aiding in the clearing of bacterial infection by interacting with the bacterial membrane, disrupting the negatively charged composition and forming pores which leads to lysis of the bacteria (Lai *et al.,* 2009). Furthermore, hBD-2 is able to branch between both innate and adaptive immunity, with the AMP capable of recruiting and increasing the activity of dendritic cells, leading to increased TNF and IL-1β release (Auvynet *et al.,* 2009). In regard to the overt-inflammatory responses, Hazlett *et al.,* (2011) described hBD-2 as playing a 'critical role' in the regulation of cytokines and chemokines by suppressing the expression of these inflammatory responses. In regard to ocular inflammation, hBD-2 is inducible in response to both gram positive and gram-negative bacteria (McDermott *et al.,* 2009), with increased expression of hBD-2 in damaged corneal cells associated with DED patients (Narayanan *et al.,* 2003). LL-37 is a member of the cathelicidin family and also has a crucial role in both innate and adaptive immune responses to bacteria through immune cell recruitment and adaptive immune cell stimulation (Yang *et al.,* 2020). In a similar fashion to the mechanism of action used by hBD-2, LL-37 initiates the production of transmembrane pores by disrupting the lipid formation of the bacterial surface (Zeth *et al.,* 2017). Infected epithelial cells are able to recruit wider immune cells, such as neutrophils and monocytes, aiding in the immune response through IL-8 release, in response to LL-37 (Scott *et al.,* 2002). Kumar *et al.,* (2007) showed that injured corneal epithelial cells increased LL-37 expression, boosting the immune response of the ocular surface to clear infection.

However, the findings of the study failed to indicate an important regulatory relationship between TLR5 and hBD-2, with the change in TLR5 expression described as 'modest'. Considering TLR5 has been shown to be crucial for inducing ocular inflammation towards bacteria, the receptor has been shown to promote corneal destruction, which is discussed further during chapter 5.

1.4.4 cyclic GMP-AMP Synthase/Stimulator of interferon Genes pathway

Although TLR9 is widely studied in ocular tissue in response to DNA sensing, and may effectively identify both ssDNA and dsDNA, it is not the only innate DNA sensor present. Baccala *et al.,* (2007) showed that self-DNA obtained from apoptosis led to increased IFN expression and a significant inflammatory response by cells, in the absence of TLR9. Although TLR9 senses cytosolic DNA, other proteins are also capable of this function, with Kondo *et al.,* (2013) showing that the MRE11 protein, which recognises dsDNA within the cytosol but not viral DNA, is capable of activating the **st**imulator of **in**terferon **g**enes (STING) protein, subsequently inducing inflammatory proteins. However, the STING protein can also be produced in response to activation of the cyclic GMP-AMP synthase (cGAS) receptor by DNA. Barber *et al*., (2011) discussed that the cGAS/STING pathway actually has increased expression in host cell populations in comparison to TLR9, concluding that this sensing pathway may induce the most reactive inflammatory response to circulating DNA.

The cGAS receptor identifies CpG-free dsDNA, with the protein binding to DNA via the sugar-phosphate backbone, which is mediated by electrostatic interaction and hydrogen bonding (Cai *et al.* 2014). Cai *et al.,* (2014) demonstrated the importance of cGAS binding to DNA for IFN production by cells, as a mutation in cGAS led to a significant decrease in IFN production and a suppressed inflammatory environment. Following successful binding, Dunphy *et al.,* (2018) described that cGAS then acts as a secondary messenger, activating STING protein through ligand 2' 3'-cGAMP (a cyclic di-nucleotide composed from both GTP and ATP). STING then releases the carboxyl-terminal tail, activating TKB1 and IKb kinase (IKK), relocating to the perinuclear compartments of the cell. Tanaka *et al.,* (2012) described this compositional change as 'crucial', as it allows the now phosphorylated STING protein to bind to IRF3, with TBK1 finally phosphorylating IRF3 and NF-KB.

IRF3 induces IFN expression, whilst NF-KB activates transcription of pro-inflammatory cytokines, including IL-6 and TNF-α (Ahn *et al.,* 2019, Dunphy *et al.,* 2018). Following successful gene transcription, STING is degraded, ensuring that a prolonged immune response is avoided by potential cytokine production.

Although there are obvious differences between the cGAS/STING and TLR9 pathways, both are capable of inducing NF-KB in response to various DNA sources, and therefore a wide range of pro-inflammatory cytokines. Interestingly, the cGAS/STING pathway may also be anti-inflammatory, as during certain conditions it may lead to the induction of the Indolemine 2,3-dioxygenase (IDO) enzyme (Wan *et al.,* 2020). The role of IDO is to catalyse the metabolism of amino acid tryptophan (TRP) into kynurenine (KYN), which in turn, can significantly influence T-cell populations by halting the proliferation of T-cells through functional anergy and increasing the population of regulatory T- lymphocytes (T-regs) $CD4^+$ cells (Aldajani *et al*., 2016, Badawy *et al.,* 2019, Sundrud *et al.,* 2009). This accumulation of T-regs and inhibition of effector T-cells promotes immunosuppression and prevents further chronic inflammation associated with autoimmunity (Huang *et al.,* 2012b). Considering the potential of the cGAS/STING pathway to induce production of pro-inflammatory protein STING, alongside anti-inflammatory enzymes such as IDO, this may be an attractive novel target to examine during ocular inflammation – an area of research which is relatively limited, but investigated further throughout chapter 4.

1.5 – VITAMIN D AND OCULAR INFLAMMATION

1.5.1 Uveitis, Keratitis and Vitamin D

Considering the potential of immunomodulation by vitamin D, it is vital to consider these effects in suppressing overt inflammatory reactions which may cause damage to ocular tissue. 'Uveitis' describes a range of inflammatory ocular diseases which can be caused by foreign microbial molecules and associated with auto-immune diseases. TLR9 has been described as a driving force behind the inflammation associated with uveitis (Allensworth *et al.,* 2011), a condition which has been studied alongside vitamin D as a potential therapeutic. For example, Steinwender *et al.,* (2013) concluded that polymorphisms in the hydroxylating gene CYP27B1, required for vitamin D3 production, led to a significant decrease in circulating metabolite 25D3 in

uveitis patients and an increased risk of severe inflammatory responses. Furthermore, Fang *et al.,* (2014) showed that polymorphisms in 7-dehydrocholestrol reductase (DHCR7), another enzyme required for vitamin D3 producion, is lacking within a majority of patients with Behcet's disease, increasing the risk of developing uveitis. In regard to vitamin D3's immunomodulatory effects during uveitis inflammation, Djeraba *et al.,* (2017) showed that vitamin D3 treatment inhibited nitric oxide and NF-ΚB activity in 33 uveitis patients, suppressing pro-inflammatory cytokine production, which indicated an anti-inflammatory effect of the treatment.

Aside from polymorphisms in vitamin D production, there are numerous factors which increase the risk of developing inflammatory conditions of the ocular surface. For example, the increased population of contact lens wearers, briefly discussed throughout subsection 1.4.3. Although easy to use and relatively safe, contact lens usage increases the risk of ocular infection, with the most common manifestation, keratitis, leading to inflammation within the cornea. This is a relatively common infection, often caused by a lack of hand washing before placing the contact lens on the eyeball, increasing the risk of introducing pathogens to the ocular surface. This risk can be minimised by thorough robust hand washing and storing lenses in a suitable, disinfecting solution to ensure microbes are removed. Clinical symptoms of this infection include eye pain, photophobia and a decline of visual capability.

Ezisi *et al.,* (2018) demonstrated that wearing contact lenses overnight for prolonged periods, significantly dries the ocular surface which can damage the epithelial layer, increasing the risk of developing keratitis. The most common cause of bacterial keratitis is *P. aeruginosa,* with TLR signaling once again implicated as a driving force within the inflammatory responses seen within keratitis development. For example, Sun *et al.,* (2010) showed TLR5 signalling by corneal cells was a significant contributor to the inflammatory response and highlighted the importance of this receptor during this disease. Cong *et al.,* (2015) confirmed that corneal epithelial cell VDR expression significantly increased over 2h during bacterial keratitis, which in turn, led to an increase of cathelicidin AMP expression. Cathelicidin is a 'beneficial' protein which mediates a range of host responses, including bactericidal actions and epithelial wound repair. Vitamin D3's immunomodulatory effects are not limited to the innate immune system, as Tang *et al.,* (2009) showed

that vitamin D treatment significantly eased inflammation within experimental autoimmune uveitis mouse models, with treatment inhibiting Th17 responses. This inhibition influences T cell cytokine production, indicating suppression of the hyper-inflammatory responses associated with the disease progression.

Considering the ability of vitamin D to modulate inflammatory responses, alongside the production of vitamin D by ocular cells, vitamin D should be considered as a therapeutic option to prevent further inflammatory damage associated with both uveitis and keratitis.

1.5.2 Dry Eye Disease and Vitamin D

Dry eye disease (DED) is an example of a multifactorial, inflammatory ocular disease which Wang *et al.,* (2020a) categorized into two main subtypes – aqueous deficient and evaporative. DED affects up to 50% of the adult population, but is difficult to diagnose due to varying diagnostic categories, therefore epidemiologists such as Stapleton *et al.,* (2017) warn that this figure may be much greater, with many patients already self-medicating. Associated risk factors for the development of DED include complications from systemic autoimmune diseases such as rheumatoid arthritis, alongside deficiencies including vitamin A and diminished blinking activity seen in neurological conditions such as Parkinson's disease (Madgula *et al.,* 2017, Findlay *et al.,* 2018). The disease manifests through a range of symptoms, with the most commonly reported including photophobia and general fatigue. Ocular irritation is also reported, stemming from poor tear production and inadequate lubrication – with significant, unmanaged cases leading to a loss of vision (Guo *et al.,* 2010).

Kunert *et al.,* (2000) characterised the role of overt inflammation driving DED progression, implicating improper inflammatory responses from lymphocytes as a key mechanism. Patient studies have shown that during DED development, T-cells infiltrate the eye and upregulate CD4 and CD8 markers, alongside lymphocyte activation marker CD11a, implying that the disease may be driven by T-cell activation and activity (Stern *et al.,* 2002). However, an uncontrolled, innate immune response may lead to ocular tissue damage, whilst an uncontrolled, adaptive immune response may lead to auto-immune disease. For example, whilst T-cells have been implicated as the source of

inflammatory sources associated with DED, epithelial cells of the ocular surface have also been highlighted as a possible perpetrator of soft tissue damage, due to the secretion of potent pro-inflammatory cytokines (Stevenson *et al.,* 2012, Narayanan *et al.,* 2008). Solomon *et al.,* (2001) showed that these pro-inflammatory cytokines include IL-1β, presumed to be from the conjunctival epithelium, alongside IL-6, IL-8 and TNF-α. Interestingly, Reins *et al.,* (2018d) demonstrated that in MyD88-deficient mice displaying DED symptoms, there was a significant loss in pro-inflammatory cytokines production required for ocular surface protection, including IL-1β and TNF-α, highlighting the importance of the MyD88 pathway during ocular inflammation. A change in pro-inflammatory cytokine production, for example TNF-α, can lead to an activated response in mitogen-activate proteins (MAP) kinases. This increases genetic transcription of specific genes in responses to stresses in the eye, including increased MMP-9 production, leading to further ocular surface damage (Pflugfelder *et al.,* 2005, Hessen *et al.,* 2014). Furthermore, Blalock *et al.,* (2008) highlighted that an increase in TNF-α may subsequently increase mucin production, such as mucin 1 (MUC1), altering the formation of tears.

Although numerous cohort studies have analysed the possible predisposing risk factors between serum vitamin D concentrations and DED development, it is the general conclusion that there is no association between the two in regard to disease protection (Jeon *et al.,* 2017, Jee *et al.,* 2016). However, a recent meta-analysis of the relationship between the hormone and DED progression performed by Askari *et al.,* (2020), argued that DED development is associated with a significantly lower level of serum 1,25D3 concentrations, highlighting the 'importance' of vitamin D3 in maintaining ocular health. In regard to treating DED and alleviating associated symptoms, Wang *et al.,* (2015c) showed that vitamin D3 treatment has the ability to significantly suppress MMP-9 production in epithelial cells of patients suffering from chronic rhinosinusitis. As previously mentioned, MMP-9 is often elevated following an increase of TNF-α, indicating that vitamin D treatment may prevent damage associated with MMP-9 during DED.

Currently, research into how vitamin D3 affects mucin production in ocular tissue is scarce, however, Paz *et al.,* (2003) showed that VDR-ablated mice

express a lower concentration of MUC5AC from defunct packaging by goblet cells, highlighting a potential impact of vitamin D upon mucin production. Considering these links and the anti-inflammatory action of vitamin D, the hormone could be utilized as a topical treatment to suppress corneal inflammation in a range of conditions, including DED.

1.5.3 Secondary Eye Disease and Vitamin D

Causes of ocular inflammatory diseases are not limited to pathogens or foreign microbes, but can be linked to vitamin D deficiency and chronic inflammatory diseases, increasing the risk visual impairment. For example; Richer *et al.,* (2013) discussed that both cardiovascular disease and MS diagnosis increase the risk of developing secondary ocular manifestations, due to chronic, systemic inflammatory events. Considering corneal epithelial cells produce and react to their own source of vitamin D, it is important to consider the impact of vitamin D status upon secondary eye disease development. For example, Kaur *et al.,* (2011) demonstrated that in patients with a type 1 diabetes diagnosis, low concentrations of circulating vitamin D were associated with retinopathy development, concluding that the anti-inflammatory effects of vitamin D protect the eye from the development of conditions such as retinopathy and age-related macular degeneration (AMD).

Furthermore, there are a range of ocular manifestations stemming from chronic inflammatory diseases, many of which affect the ocular surface and present as inflammatory ocular disease, for example keratitis. For instance, Clewes *et al.,* (2005) showed that rheumatoid arthritis patients are at increased risk of developing peripheral ulcerative keratitis from systemic inflammation. These persistent, inflammatory responses may lead to the destruction of the corneal stroma in the form of inflowing immune cells and MMP production. Keratitis is also associated with patients diagnosed with auto-immune diseases such as Reiter's syndrome and systemic lupus erythematosus, both of which also show increased, systemic inflammation throughout the body (Sayjal *et al.,* 2002). Interestingly, Arora *et al.,* (2015) demonstrated that a significantly low serum concentration of vitamin D in a case study of child diagnosed with malabsorption was associated with peripheral ulcerative keratitis development. In that study, multi-vitamins and

steroids alleviated symptoms, indicating a potential link between vitamin D status and keratitis development.

1.5.4 Ocular viruses and Vitamin D

Due to the ocular surface being in constant contact with the outside environment, this is often the first site of viral infection. For example, Zhou *et al.*, (2021) confirmed the expression of angiotensin converting enzyme 2 (ACE2) on ocular surface cells, including the cornea. Studies have shown that SARS-Cov-2 enters the host cell by exploiting ACE2 as a potential receptor (Hoffman *et al*., 2020). It has been reported that 4.8% of COVID-19 patients suffer from conjunctivitis as an ocular manifestation of the infection, alongside symptoms such as eye itching and dry eyes from a loss of epithelial cells (Xia *et al*., 2020). Furthermore, human influenza A virus has shown the capacity to exploit the ocular surface for viral entry as the mucosal surface hosts the perfect conditions for replication. However, studies such as that by Creager *et al*., (2018) showed that human tears can significantly inhibit non-ocular H7 isolates of influenza A, however, the virus is still capable of passage through tear film and infecting the ocular surface. These infections can develop into further manifestations such as dry and itching eyes from epithelial cell damage, leaving the virus to enter the body and cause further complications, such as respiratory infections.

However, arguably the most common virus to infect the ocular surface is the herpes simplex virus (HSV). HSV has two serotypes, 1 and 2, both of which are capable of causing ocular disease, however, HSV-1 is more prevalent. Farooq *et al.,* (2011) implicates ocular HSV infections as the 'leading cause' of blindness in the USA, with the virus capable of spreading into both tissue and neurons with ease and an estimated 5-15 per 10,000 annum effected within the UK (College of Optometrists, 2021). The most common viral entry route is through infection of sensory nerves, developing into a latent infection which reactivates at different sites around the body. The herpes virus entry mediator (HVEM) facilitates entry into the conjunctiva of the eyeball, where the virus actively replicates for around 5 days post-infection (Edwards *et al*., 2017). Although TLR9 is capable of detecting HSV-1 unmethylated CpG motifs in the cornea, inducing a powerful NF-KB response which leads to the production of a ray of pro-inflammatory cytokines, HSV-1 can evade

detection, become latent and then remain dormant for years (Takeda *et al.,* 2011). Virus reactivation is associated with physiological stress and inflammation within the cornea, which manifest as events such as epithelial ulceration and herpes stromal keratitis (Edwards *et al*., 2017).

Although relatively unexplored, there are some interesting discussion points regarding HSV infection and vitamin D status. For example, Mowry *et al.,* (2011) presented evidence that showed an increase in circulating vitamin D levels linked to higher titers of HSV-2 in multiple sclerosis patients. In contrast, Kumar *et al.,* (2018a) found vitamin D supplementation downregulated viral titer, indicating that vitamin D treatment prevented viral replication *in vitro*. Both of these studies provide evidence that vitamin D can modulate the viral impact on cells, depending on cellular subtype and inflammatory conditions. As previously discussed, vitamin D is associated with a boost in AMP cathelicidin expression and subsequent production. Lin *et al.,* (2019) discussed studies that showed a decrease of this AMP is associated with a significantly lower viral load from HSV-1, indicating that vitamin D may have an anti-viral potential. Although relatively unexplored, analyzing the potential immunomodulatory effects of vitamin D upon HSV development is vital, due to the growing incidence rate of the virus around the world, in which the majority of the world's population are infected (Edwards *et al.,* 2017).

1.6 – MICRO-RNA – A POTENTIAL VITAMIN D MECHANISM OF ACTION

1.6.1 Micro-RNA

It is clear that vitamin D has immunomodulatory properties against various pathogenic infections which contact the ocular surface, and further systemic inflammatory conditions which affect the eye. Although vitamin D binds to the VDRE of specific genes, it is unknown how this mechanism is regulated during modification of genes, including those involved within the TLR signaling pathways. Previous research has also indicated that vitamin D exposure leads to a suppression in micro-RNA (miR) expression, leading to a weakened inflammatory response (Karkeni *et al.,* 2018). Arboleda *et al.,* (2016) proposed that 'several' mechanisms may be involved during the regulation of miR with vitamin D, including one popular theory that some miR, such as miR-182, have mutliple VDR/RXR binding sites in their primary state,

meaning miR could be regulated by the hormone. Bartel (2004) described MiR as 'short, non-coding RNA molecules' that act as post-transcript regulators on target mRNA, therefore they can increase or repress genetic translation by direct binding of target sites in the 3' untranslated region (UTR) of mRNA transcripts. Baskerville *et al.,* (2005) estimated that miRNA constitute nearly 1% of all predicted genes in mammals, with Cai *et al.,* (2009) adding that the significance of miR expression during disease progression has been 'long over-looked' because of limitations in both technology and methodology. Yet, due to progress in both of these areas, identification of miR individual functions and roles during cellular processes has increased dramatically, with now over 2000 miR identified within the human genome (Hirschberg *et al.,* 2018).

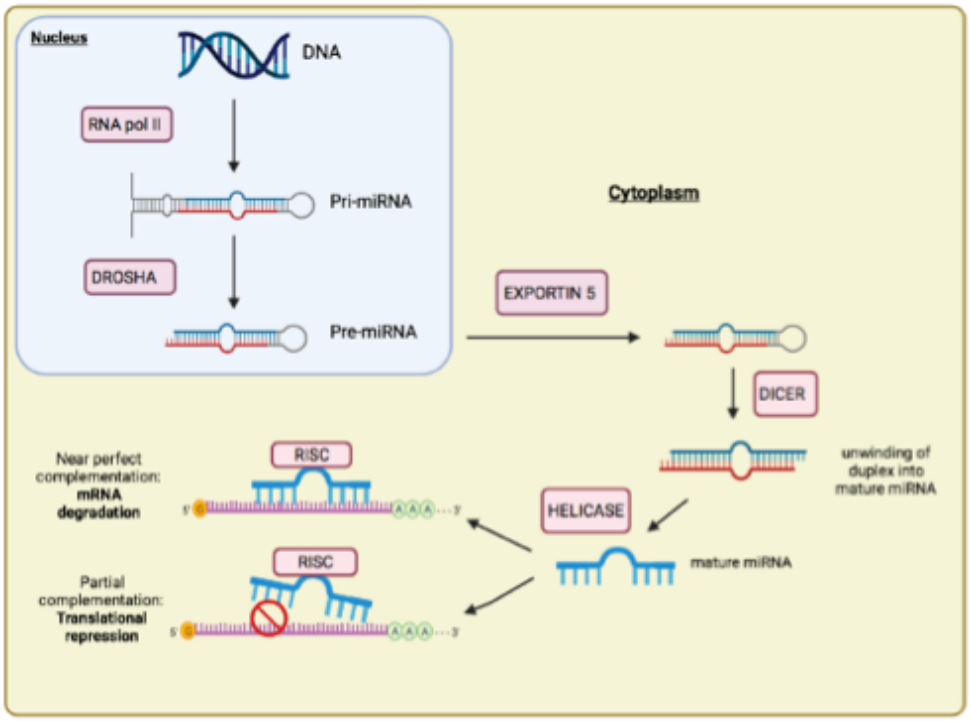

Figure 1.8. miRNA biogenesis. The miRNA genes are usually transcribed by RNA polymerase II, which produces pri-miRNA, which is then cleaved by a type III RNase DROSHA, producing a pre-miRNA. Following transportation out of the nucleus to the cytoplasm by Exportin 5, pre-miRNA is then processed further by another type III RNase – DICER. After the duplex is then unwound, the mature miRNA is incorporated into a protein complex known as RNA-induced silencing complex (RISC). The miRNA-loaded RISC mediates gene silencing via mRNA cleavage and degradation, or translational repression, depending on the complementarity between the miRNA and the targeted mRNA transcript.

The miR mechanism of action involves a precursor miR located in intergenic regions or introns undergoing several processing steps (Figure 1.8), which then leads to translocation to the cytoplasm and the formation of a double stranded, mature miR (Hinske *et al,.* 2017, Rodriguez *et al.,* 2004, Kreth *et al.,* 2018). From here, one single strand of the miR is loaded into the RNA-induced silencing complex (RISC), where it successfully binds to the 5' end to the 3' UTR of the target mRNA and forms a miR-mRNA complex (Neudecker *et al.,* 2016). This complex then has the ability to either repress or degrade the target mRNA, leading to impacts in cell processes such as proliferation, apoptosis and cell differentiation (Iwakawa *et al.,* 2015). As discussed throughout subsection 1.4, vitamin D is able to modulate TLR signaling; the most commonly researched PRR in relation ocular immunity. Vitamin D may be considered a potential therapeutic by suppressing these inflammatory events, however, the exact mechanism of this suppressive action is unknown and therefore it would be beneficial to analyze the potential impact of vitamin D upon miR activity. Doing so would allow identification of any miR which may have a mechanism in the regulation of vitamin D upon a range of inflammatory genes. To aid in this process, the experiments completed in this thesis analysed the effects of vitamin D and various agonists upon the expression of four miR associated with ocular inflammation, which are described in further detail throughout this subsection.

1.6.2 miR-146a - role in inflammation

An example of an miR implicated in inflammatory responses and TLR signaling is miR-146, a family of miR precursors heavily involved within regulation of inflammation. Karrich *et al.,* (2013) demonstrated that during TLR9 activation of pDCs, which are potent immune cells capable of producing type 1 interferons in response to microbial infection, miR-146a is active. When TLR9 of pDC senses microbial associated nucleic acid, this leads to an increase of not only type 1 interferons, but also pro-inflammatory cytokine production and an upregulation of co-stimulatory markers, exacerbating the inflammatory response. An upregulation of miR-146a impaired TLR9-mediated signaling through a decrease in IRAK1 expression, with a subsequent decrease in NF-κB activation and associated pro-inflammatory cytokine production, which actually indicates a regulatory role for miR-146a.

CD11b is a β2 integrin which plays a crucial role during immune responses such as TLR-triggered natural killer cell cytotoxicity and macrophage phagocytosis, and therefore is an important component of the inflammatory response. Furthermore, Bai *et al.,* (2012) characterised the role of miR-146a during TLR9 signaling of DCs, with the miR-146a responsible for delayed NF-KB activation and reduced IL-12p70 production, which TLR9-driven regulated inflammation.

In regard to ocular tissue, Ye *et al.,* (2016) demonstrated that miR-146 had a regulatory role during TLR4/NF-KB signaling by cells in retinal tissue, leading to a decrease in the production of TNF-α. Furthermore, miR-146a works in close connotation with miR-155, an miR which is associated with pro-inflammatory effects, with miR-146a capable of 'switching off' the miR-155 signaling cascade during NF-KB signaling (Testa *et al.,* 2017). However, it is not just inflammatory responses of the ocular surface which have shown miR-146a activity, but other processes such as corneal wound healing. This miR has also been implicated during corneal wound healing during inflammation associated with diabetes. For example, Funari *et al.,* (2013) demonstrated that miR-146a significantly delayed wound healing in diabetic human corneal epithelial cells compared to healthy controls, concluding that dysregulation of miR-146a may lead to abnormal wound healing and increased inflammatory damage (Funari *et al.,* 2013).

These findings indicated a potential negative regulatory role for miR-146a during both TLR signaling and subsequent inflammatory responses, making it a novel target for suppressing overt inflammation. In regard to corneal epithelial cells, potential miR-146a activity has been relatively unexplored during TLR signaling. However, miR-146a has been shown to regulate interleukin-1 receptor-associated kinase 1 (IRAK1) (Liu *et al.*, 2008d), which has been highlighted as a positive regulator of the NF-KB pathway, making it an attractive miR for further analysis.

1.6.3 miR-155 – role in inflammation

Arguably one of the most studied miR in relation to inflammation, the miR-155 family has an extensive role during inflammatory signaling and subsequent

cytokine production. For example, research has shown that the pro-inflammatory cytokine IL-1β is regulated by miR-155, with evidence of such a relationship found in glioma cell lines, with miR-155 regulating IRF3 and therefore impacting upon IL-1β production (Tarassishin *et al.,* 2013). In regard to TLR signaling, Oliveira *et al.,* (2017) conducted a study analyzing the neurological mitral cell layer of cattle following bovine herpes virus 5, finding that miR-155 expression increased following TLR 3 and 9 activation, which consequently led to an increase of inflammatory factors such as pro-inflammatory cytokines. The authors suggested that miR-155 therefore has a mechanism during TLR signaling to BHV5 and may have a role during in inflammatory responses during viral infections. In regard to immune cells, Lind *et al.,* (2015) showed an increase in miR-155 expression following TLR9 stimulation using CpG Oligodeoxynucleotide (ODN) 1826 in DC. This was supported by Du *et al.,* (2014) who showed similar results in macrophages, with a reduction in TNF-α, IL-6 and IL-1β production following TLR9 signaling in miR-155$^{-/-}$ mice, indicating that miR-155 has a regulatory role during TLR signaling. Furthermore, miR-155 does not only affect the innate immune system, but also the adaptive immune system, for example, Huffaker *et al.,* (2012) demonstrated that inhibiting the suppressor of cytokine signaling-1 (SOCS-1), led to miR-155 positively regulates IFN-γ production by CD4^{+} and CD8^{+} T cells. Alongside the regulation of SOCS-1, miR-155 also has the ability to target src homology 2 (SH2) domain containing inositol polyphosphate 5-phosphatase 1 (SHIP1), leading to amplification of the NF-ΚB pathway via a positive feedback loop (Huffaker *et al.,* 2012).

In regard to the role of miR during ocular inflammation, cohort studies of patients diagnosed with secondary uveitis showed decreased miR-155 expression in the DC of Behcet's disease patients compared to healthy subjects (Zhou *et al.,* 2012). The authors concluded that whilst miR-155 does have pro-inflammatory effects, patients showed a decrease in mitogen-activated protein kinase 7-interacting protein 2 (TAB2) with miR-155 upregulation, which is a protein active during TLR signaling, showing that micro-RNA can be both pro-inflammatory and anti-inflammatory depending on exerted effects. Furthermore, miR-155 has been implicated in not only MyD88-dependent TLR signaling (Arenas-Padilla *et al.,* 2018), but also MyD88-independent signaling, such as TLR3, playing a role during

replacement of damaged epithelial cells during corneal wound healing (Wang *et al.,* 2020a).

1.6.4 miR-93 – role in inflammation

This miR remains relatively unexplored in regard to ocular tissue, however, the miR-93 family have been prominently identified in a broad range of inflammatory disorders, including reperfusion injuries and chronic inflammatory diseases (Tian *et al.,* 2017). In regard to the eye, miR-93 has been associated with the prevention of AMD, with an overexpression of miR-93 leading to a significant decrease in angiogenesis and therefore a decline in symptom severity (Wang L *et al.,* 2016). Ha *et al.,* (2014) described glioma as a condition 'driven by chronic inflammation'. There is some evidence that miR-93 is implicated in TLR signaling during ocular tissue, for example, IL-8 decrease is associated with an miR-93 increase during glioma, indicating a suppressive, regulatory role of miR-93 with IL-8 expression and subsequent cytokine production (Fabbri *et al.,* 2015).

1.6.5 miR-181 – role in inflammation

Finally, the miR-181 family has been implicated in regulating numerous pro-inflammatory cytokines in a wide range of disease, with Yingxue *et al.,* (2019) describing miR-181a as an 'essential regulator of inflammation'. Although studied extensively in the retina due its increased expression within this tissue, there is currently limited research regarding the impact of miR-181a expression within the cornea, even though expression has been confirmed. Wang *et al.,* (2018i) associated a significant decrease of miR-181a in corneal epithelial cells with keratoconus (KC) – a thinning of the corneal stroma, which leads to damage of corneal tissue and therefore an increased risk of developing infections. This indicated that miR-181a is vital for the formation of the corneal epithelial layer, which in turn, protects the eye from further infection. Although described as a 'non-inflammatory' condition, Pahuja *et al.,* (2016) showed a significant increase in pro-inflammatory production by these cells, reporting an increase in cytokines such as IL-6, TNF-α and IL-1β. Considering that miR-181a has shown to significantly regulate a range of pro-inflammatory cytokines, including IL-6 and IL-1β through NF-κB suppression in various cells, it would be interesting to observe if miR-181a has a role

during the expression of these cytokines (Galicia *et al.,* 2014). Furthermore, Tian *et al.,* (2020) stated that miR-181a had a role in KC due to the impact of the micro-RNA on SMAD signaling, which can lead to an increase of TGF-β by corneal fibroblasts and increase corneal dystrophy.

1.6.6 miR and vitamin D

Whilst it is known that 1,25D3 has immunomodulatory effects upon TLR signalling, there have also been novel discoveries linking 1,25D3 affecting miR expression and activity during modulatory processes such as inflammation to Dengue virus infection and bone disease progression (Arboleda *et al.,* 2016, Lisse *et al.,* 2013). Not only do miR have an effect on the NF-ΚB signalling pathway and therefore subsequent production of pro-inflammatory mediators, but they can also affect cytokine release following 1,25D3 exposure and may therefore influence miR activity. An example of this is the decrease of IL-1β production by monocytes following exposure to 1,25D3, which led to a significant increase of miR-155 expression (Fitch *et al.,* 2016).

Whilst vitamin D3 has been shown to inhibit inflammation in the ocular surface, there is also evidence of the hormone affecting miR expression in the cells of the ocular surface, although this area is scarcely researched. Merrigan *et al.,* (2017) showed that 1,25D3 exposure has shown to significantly increase expression of miR-21 in the eyes of zebrafish, which is associated with a decline of ocular angiogenesis and VEGF expression in AMD. Chen *et al.,* (2013) showed that the hormone can have a suppressive effect upon TLR2/4 signalling in macrophages, increasing the anti-inflammatory response induced by 1,25D3 by increasing the expression of miR-155 and therefore suppressing related pro-inflammatory cytokine release. As discussed, miR have the ability to actively modulate multiple micro-RNA targets at once, therefore, these proteins may have either pro or anti-inflammatory effects, depending on their target. More specific studies are required into this area to understand which miR are expressed by corneal epithelial cells and how vitamin D affects these miR during cellular function and inflammatory responses, to gauge the effectiveness of this hormone as a therapeutic option to inflammatory ocular disease.

1.7 – RATIONALE AND AIMS

The human telomerase-immortalized corneal epithelial cell line (hTCEpi) is a reliable, robust model for analysing TLR signalling, and demonstrated significant suppression of pro-inflammatory mediators following 1,25D3 treatment (Reins *et al.*, 2015a). These cells possess unlimited growth, whilst maintaining crucial epithelial cell components, for example, tight junction formation from ZO-1 protein (Robertson *et al.*, 2012). Furthermore, they require serum free-medium, making them an excellent candidate for representing native cells of the ocular surface and were used throughout this project.

Reins *et al.,* (2015a) showed 1,25D3 treatment led to a significant suppression of TLR3 signalling and pro-inflammatory mediator production, including IL-8 and IL-6, which are associated with ocular disease. Furthermore, corneal epithelial cells exposed to 1,25D3 produced significant concentrations of AMP LL-37; a further anti-inflammatory measure, discussed further throughout chapter 5. However, these findings must be expanded to understand the wider effects of the hormone, including the effects upon additional TLR receptors, AMPs and pro-inflammatory mediators associated with these inflammatory responses. Although it is known that vitamin D regulates the pro-inflammatory genes associated with TLR ocular inflammation, the exact mechanism of this regulation is not currently understood, therefore, miR is an interesting area to pursue further.

The aim of this project was to investigate immunomodulatory effects on both pro-inflammatory and anti-inflammatory mediators associated with TLR signalling, which may affect inflammatory progression. This was achieved by targeting the following TLR receptors and analysing the effect of 1,25D3 upon a range of pro-inflammatory mediators, including IL-6, IL-8 and IL-1β:

- Chapter 3 – TLR3
- Chapter 4 – TLR9
- Chapter 5 – TLR5

The hypotheses, to be discussed in further detail throughout each chapter, were that 1,25D3 would dampen all pro-inflammatory mediators in response

to various pathogens and TLR signalling. Furthermore, it was predicted that miR-93-5p, miR-146a-5p, miR-155-5p and miR-181a-3p each had a role during these suppressive actions, exploiting models which mimicked features of viral and bacterial infection.

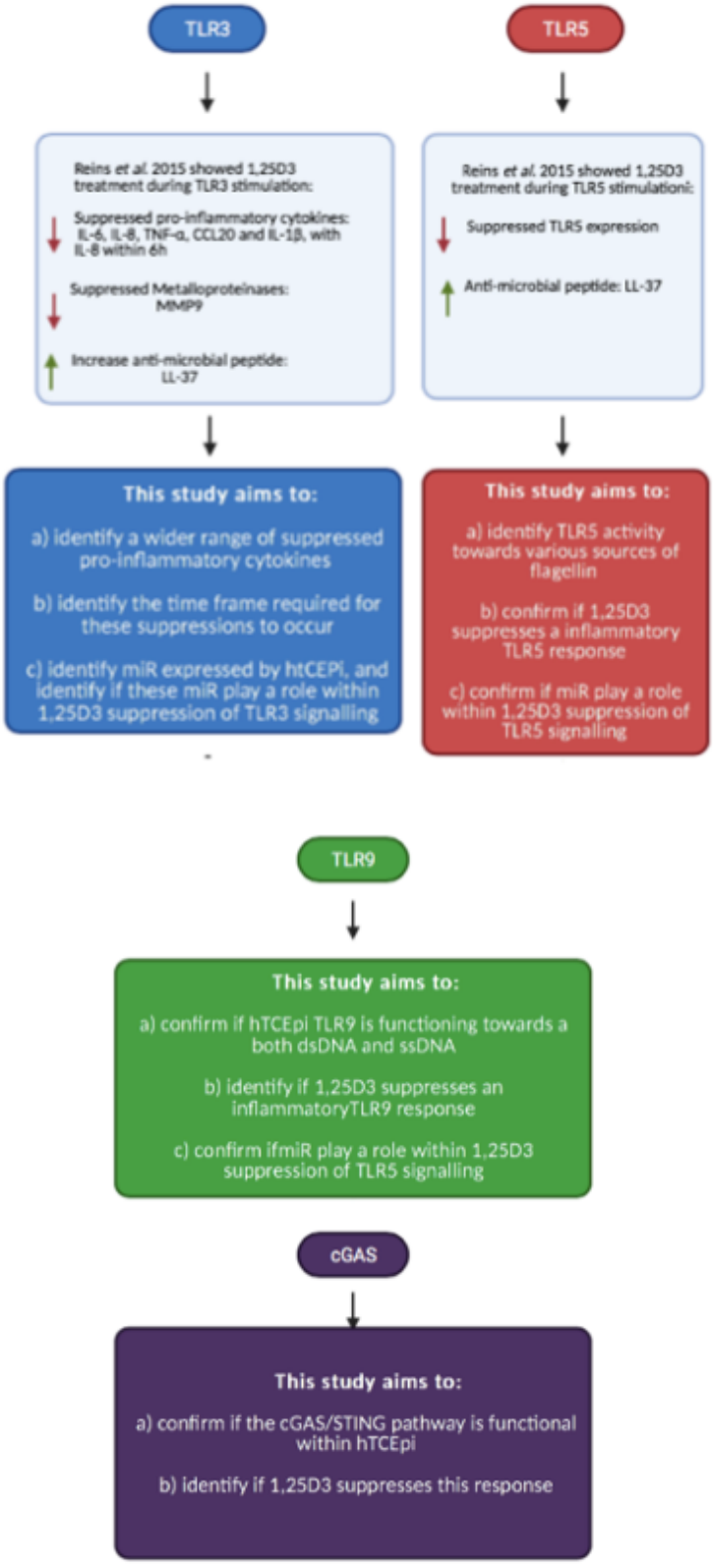

Figure 1.9: the aims of this study. Study aims alongside the previous findings of the Reins *et al.,* (2015a) study, which used 1,25D3 to modulate TLR3 and TLR5 signalling by hTCEpi cells (light blue boxes).

Chapter 2

Materials and Methods

2.1. MATERIALS

The following kits, reagents and chemicals were used throughout the study to achieve the proposed aims (Tables 2.1.1 and 2.1.2).

2.1.1 Kits, reagents and chemicals

Table 2.1: Kits and reagents used throughout the study

Kit, reagent or chemical (stock solution)	Manufacturer
Cell stimulation	
Poly(I:C) (#P1530) (stock: 20mg/ml)	Sigma-Aldrich Corp., St. Louis, MO, USA
S. typhimurium Ultrapure flagellin (FLA-ST) (#tlrl-epstfla-5) (stock: 500ug/ml)	Invivogen, San Diego, USA
Pseudomonas aeruginosa flagellin (FLA-PA) (#trlrl-pafla) (stock: 500ug/ml)	Invivogen, San Diego, USA
ODN2006 (#tlrl-2006) (stock: 500μM)	Invivogen, San Diego, USA
ODN2395 (#tlrl-2395) (stock: 500μM)	Invivogen, San Diego, USA
Double-stranded *E. coli* DNA (#tlrl-ecdna) (stock: 1ml/ml)	Invivogen, San Diego, USA
2'3'cGAMP (#tlrl-nacga23-02) (stock:1ml/ml)	Invivogen, San Diego, USA
Vitamin 1,25D3 (10^{-7}M) (stock: 10^{-4}M)	Invivogen, San Diego, USA
Cell culture	
EpiLife medium	Gibco, New York, USA
Penicillin/Streptomycin (5000U/mL;5000μg/mL)	Gibco, New York, USA
DMEM-Ham's F12	Gibco, New York, USA
Fetal Bovine Serum (FBS) (10%)	Sigma-Aldrich Corp., St. Louis, MO, USA
Gentamicin (3μg/mL)	Sigma-Aldrich Corp., St. Louis, MO, USA
RPMI1406 (R0883)	Sigma-Aldrich Corp., St. Louis, MO, USA
TrypLE removal agent	Gibco, New York, USA
Dimethylsulphoxide solution (DMSO)	Sigma-Aldrich Corp., St. Louis, MO, USA
Trypan Blue Solution (0.4%)	Sigma-Aldrich, UK
10x Cell Lysis Buffer	Qiagen, Valencia, CA, USA
3-(4,5-dimethylthiazol-2-yl_-2,5-diphenyl tetrazolium bromide (MTT) reagent	Sigma-Aldrich Corp., St. Louis, MO, USA

Alkylbenzyldimethylammonium chloride (BAC) solution	Sigma-Aldrich, UK
Molecular biology	
DNase I treatment (DNase, RDD buffer)	Qiagen, Valencia, CA, USA
RNeasy Mini kit (Buffer RLT, Buffer RPE, Buffer RW1)	Qiagen, Valencia, CA, USA
Qiashredder homogenizer kit	Qiagen, Valencia, CA, USA
AffinityScript cDNA synthesis kit	Agilent Technologies, Santa Clara, CA, USA
Brilliant III SYBR Green QPCR master mix	Agilent Technologies, Santa Clara, CA, USA
TaqMan Advanced miRNA Assays	Applied Biosystems, California, USA
Taqman MicroRNA Cells-To-C_T kit	Invitrogen, Massachusetts, USA
miRCURY LNA Power miRNA inhibitor (stock: 10 μM/ml, working: 20 nM/ml)	Qiagen, Valencia, CA
miRCURY scramble inhibitor (stock: 10 μM/ml, working: 20 nM/ml)	Qiagen, Valencia, CA
SYBRSafe reagent	Thermo Fisher Scientific, UK
Hyperladder (50kb)	Bioline, UK
Lipofectamine™ 3000 transfection reagent	Thermo Fisher Scientific, UK
β-mercaptoethanol	Thermo Fisher Scientific, UK
q-PCR primers	Integrated DNA technologies, UK

2.1.2 Buffers

Table 2.2: Buffers used throughout the study for various technique

Buffer	Constituents
Phosphate buffered saline (PBS)	(37mM NaCl, KH_2PO_4 (1.8mM), KCl (2.7nM), Na2HPO_4 (10mM))
TAE buffer	(20nM acetic acid, 1mM EDTA pH 8, 40mM Tris)

2.2. METHODS

2.2.1 Thawing of hTCEpi, SV40-HCEC and THP1 frozen cell lines

The following cell lines were used within this study:

- **hTCEpi cell line**: *in vitro* telomerase immortalized, corneal epithelial cell line, obtained from the Reins *et al.* (2015a) study
- **SV40-HCEC cell line:** *in vitro* corneal epithelial cell line, obtained from the Reins *et al.* (2015a) study
- **THP1 cell line:** *in vitro* immortalized monocyte-like cell line, obtained from the Jaedicke *et al*., (2013) study

All of the cell lines were obtained from frozen stocks stored in 1.5ml ampoules at -140^{o}C. These ampoules were warmed in a 37^{o}C water bath for approximately 5 minutes, pipetted into a 15ml falcon tube containing 5ml of the corresponding medium, using a 5ml pipette. The solution was mixed using a slow pipetting action. Using Trypan Blue, the cell count of the solution was determined to gauge the possible cell population obtained from the ampoule, alongside cell viability (described within subsection 2.2.8). The solution was centrifuged for 2500rpm for 5 minutes, to remove cellular debris and previous cell medium, which was discarded after centrifuging had ended. The cell pellet was re-suspended in the required medium to ensure at least 5 x 10^5 cells within a 75cm^2 (T75) flask, incubated in a horizontal position.

2.2.2 *In vitro* maintenance of hTCEpi, SV40-HCEC and THP1 cells used throughout the study

Short tandem repeat (STR) analysis was completed externally by NuGene (Newcastle upon Tyne, UK) to ensure the human telomerase-immortalized corneal epithelial (hTCEpi) was valid and reliable (Appendix 1). The hTCEpi cells (Robertson *et al.,* 2005) were grown in EpiLife medium (Gibco, USA) with defined growth supplement and Penicillin/Streptomycin (5000U/mL;5000μg/mL) (Gibco, USA). SV-40 transformed human corneal epithelial (SV40-HCEC, Araki-Sasaki *et al.,* 1995) cells were maintained in DMEM-Ham's F12 (Gibco, USA) medium supplemented with 10% FBS (Sigma-Aldrich Corp. USA) and gentamicin (Sigma-Aldrich Corp. USA) (3μg/mL). THP1 monocyte cells used for miR expression confirmation, acting as a positive control for determination of hTCEpi miR expression, were cultured in RPMI1406 with 10% FBS (Sigma-Aldrich Corp. USA). Cells were maintained at 37^{o}C in 5% CO_2.

2.2.3 Propagation of cell lines

After cells had reached a density of 3-5 x 10^6 per flask, cells were passaged. Cells were used between passages 35-50. Cells were detached from T75 ($75cm^2$) growth flasks using TrypLE (Gibco, USA) and transferred into a 15ml falcon tube, followed by a 3-minute centrifuging period at 2500 rpm. The supernatant of each sample was removed, with the pellet re-suspended in 1ml of the corresponding medium. Cells were counted to ensure an appropriate cell population (described within subsection 2.1.8), with a new passage of cells established in a T75 flask with the appropriate medium.

2.1.4 hTCEpi cell stimulation

The hTCEpi cells were detached from T75 flasks using the methods described throughout subsection 2.1.3, re-suspended into six-well culture plates at a population of $2x10^5$ cells per well and maintained at 37°C in 5% CO_2 for 24h to ensure adherence of cells to the plate. After reaching 80% confluence, cells were stimulated under various working concentrations as shown throughout the results sections of chapter 3-5. The agonists used included (manufacturer details included within table 2.1): TLR3 Poly(I:C), TLR5 FLA-ST, TLR5 FLA-PA, TLR9 ODN2006, TLR9 ODN2395, TLR9 *E. coli* DNA, cGAS 2'3'cGAMP and 10^{-7}M vitamin 1,25D3 for up to 24 hours. All TLR ligands agonists were suspended in endotoxin-free physiological water as supplied by the manufacturer to meet required concentrations. 10^{-7}M vitamin 1,25D3 was suspended in 100% ethanol to meet the required concentration. Each stock solution was aliquoted and stored at -20°C, then defrosted to room temperature for each experiment.

Table 2.3: cDNA reaction mix composition

Reagent	Volume
cDNA Synthesis master kit (2x)	10µl
AffinityScript RT/RNase Block Enzyme Mixture	1µl
Oligo(dT) primer	3µl
RNAse-free H_2O	Up to 5µl
Sample RNA (at least 500ng)	1ul
	20µl final

Table 2.4: qPCR reaction mix composition

Reagent	Volume
Syber Green QPCR master mix	10µl
Primer mix (Forward/Reverse primers at 150nM final concentration)	0.5µl
Diluted reference dye (1:500)	0.2µl
RNAse-free H_2O	Up to 5.3µl
cDNA	4µl
	20µl final

2.1.5 RNA isolation and Quantitative real-time PCR (qPCR)

Following the required exposure time for each experiment, supernatant was pipetted from the plate wells, with 350µl of Buffer RLT (Qiagen, USA) containing 2M of β-mercaptoethanol (Thermo Fisher Scientific, UK) pipetted into each well, allowing the solution to incubate with the cells for 2 minutes. The solution was removed by pipette and added into Eppendorf tubes. RNA was isolated from the cells in each well using a combination of Qiashredder, RNeasy, and DNase I treatments (Qiagen, USA). For efficient homogenizing and the avoidance of cross contamination, 350µl of the lysate was pipetted into the Qiashredder homogenizer tubes, placed into 2ml collection tubes. These samples were centrifuged at 5000rpm for 2 minutes, with 70% ethanol added to the homogenized lysate by gentle pipetting. This 700µl final volume was pipetted into a new RNeasy spin column and centrifuged at 10,000rpm for 15 seconds, with the flow-through then removed by pipette. Buffer RW1 (350µl) (Qiagen, USA) was added to each tube by pipette, with the sample centrifuged at 10,000rpm for 15 seconds, with flow-through removed. DNAse digestion was performed using the DNase I treatment, to eliminate genomic DNA contamination. This involved the DNase treatment (10µl of DNase + 70µl Buffer RDD, (Qiagen, USA)) added to each spin column membrane, incubated at room temperature (20-30 ^{o}C) for 20 minutes. More Buffer RW1 (350µl) was added to the RNeasy spin column by pipetting, which was centrifuged at 10,000rpm for 15 seconds, with flow-through removed. Buffer RPE (500µl) (Qiagen, USA) was added to the RNeasy spin column by pipette, which was centrifuged at 5000rpm for 15 seconds, with flow through removed, with this Buffer RPE treatment repeated again. Finally, 20µl of RNAse-free water was pipetted into the RNeasy spin column membrane and

allowed to stand at room temperature (20-30^{o}C) for 10 minutes. The samples were centrifuged for 1 minute at 10,000rpm.

This final solution (1ul) was transferred to the RNA Nanodrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE) to calculate RNA purity. Between the sampling of each solution, electrodes were cleaned with DEPC-treated water following the onscreen prompts. The RNA was used to compose cDNA, which was transcribed with an AffinityScript cDNA synthesis kit (Agilent Technologies, USA) (Table 2.3). Prior to all qPCR experiments, primers were diluted to a final concentration of 10μm forward/reverse, as per the manufacturer's instruction (Integrated DNA technologies, UK). Untreated samples served as the calibrator for relative quantity determination. All samples were normalized to the housekeeping gene, GAPDH, which acted as an internal control gene that was used to validate the values for other genes such as IL-6. Real-time PCR was performed using Eppendorf MasterCycler X50 Thermocycler and Brilliant III SYBR Green QPCR master mix (Agilent Technologies, US) with qPCR reaction mix composition described within table 2.4. Products were amplified using the ABI 7500 Fast analyzer (Thermo Scientific, USA), using the required qPCR cycling parameters (table 2.7) entered into the ABI 7500 SDS v1.4.1 software. Primers were used to detect relative expression receptors and inflammatory mediators of various TLR and associated signaling pathways (described within tables 2.5 and 2.6). Dissociation curve analysis confirmed primer specificity and no template/no RT samples were used for controls.

2.1.6 Quantification of gene expression

GAPDH, the acting housekeeping gene, was used to normalize the data obtained from the ABI 7500 SDS v1.4.1 software using Microsoft Excel, using the Livak and Schmittgen's ($RQ = 2^{-\Delta\Delta Ct}$) method of gene expression analysis. The following calculation was used to calculate mRNA fold change, which was then represented as 'fold change' throughout the study:

$2^{-\Delta\Delta Ct}$ = ΔCt (test samples) = ΔCt (calibrator samples)

Where ΔCt (test samples) = Ct (target gene in test) – Ct (reference gene in tests)
And ΔCt (calibrator samples) = Ct (target gene in control) – Ct (reference gene in control)

Table 2.5: Specifications for primer design

Primer design	Accepted range
Melting temperature	58-61 °C
Primer sequence length	18-25bps
Optimal amplicon length	80-700bps
Max 3' self-complementarity	1

Table 2.6: Primer sequences for qPCR

Gene of interest	Forward/reverse primer sequence	Amplicon length (bps)	Melting temperature (°C)	NCBI Ref. Seq.
GAPDH	5'-GACCACAGTCCATGCCATCA-3' 5'-CATCACGCCACAGTTTCC-3'	71	59	NM_002046
TLR1	5'-GAAGATTTCTTGCCACCCTAC-3' 5'-GAACACAATGTGCAGACTCTC-3'	271	59	NM_003263.4
TLR2	5'-CTGGACAATGCCACATAC-3' 5'-CTAATGTAGGTGATCCTG-3'	210	59	NM_003264.5
TLR3	5'-GCTGCAGTCAGCAACTTCAT-3' 5'-AGGAAAGGCTAGCAGTCATCC-3'	144	59	NM_003265.3
TLR4	5'-GAGCCGCTGGTGTATCTTTGA-3' 5'-CCAGCACGACTGCTCAGAAA-3'	66	59	NM_003266.4
TLR5	5'-TTGCTCAAACACCTGGACAC-3' 5'-CTGCTCACAAGACAAACGAT-3'	149	59	NM_003268.6
TLR6	5'-CATGACGAAGGATATGCCTTCTTTG-3' 5'-TATTGACCTCATCTTCTGGCAGCTC-3'	322	60	NM_006068.5
TLR7	5'-CCTTGAGGCCAACAACATCT-3' 5'-GTAGGGACGGCTGTGACATT-3'	181	59	NM_016562.4
TLR8	5'-TAATAGGCTGAAGCACATCCC-3' 5'-TCCCAGTAAAACAAATGGTGAG-3'	621	60	NM_016610.4
TLR9	5'-CGCCAACGCCCTCAAGACA-3' 5'-GGCGCTTACATCTAGTATTTGC-3'	79	59	NM_017442.4
RIG1	5'-GACTGGACGTGGCAAAACAA-3' 5'-TTGAATGCATCCAATATACACTTCTG-3'	75	59	NM_014314.4

MDA5	5'-ACCAAATACAGGAGCCATGC-3' 5'-CGTTCTTTGCGATTTCCTTC-3'	189	59	NM_ 022168.3
NOD1	5'-CCAAAGCCAAACAGAAACTC-3' 5'-CAGCATCCAGATGAACGTG-3'	180	59	NM_ 006092.4
NOD2	5'-GAAGTACATCCGCACCGAG-3' 5'-GACACCATCCATGAGAAGACAG-3'	174	59	NM_ 022162.3
NLRP3	5'-GATCTTCGCTGCGATCAACA-3' 5'-GGGATTCGAAACACGTGCATTA-3'	94	59	NM_ 1833.95.3
STING	5'-AGCATTACAACAACCTGCTACG-'3 5'-GTTGGGGTCAGCCATACTCAG-'3	104	59	NM_ 198282.4
IL-8	5'-GACCACACTGCGCCAACAC-3' 5'-CTTCTCCACAACCCTCTGCAC-3'	115	59	NM_ 000584
IL-6	5'-GGTACATCCTCGACGGCATCT-3' 5'-GTGCCTCTTTGCTGCTTTCAC-3'	81	59	NM_ 000600.5
IL-1β	5'- GACACATGGGATAACGAGGC-3' 5'- ACGCAGGACAGGTACAGATT-3'	248	59	NM_ 000576.3
IL-12	5'-AAACCTCCCCGTGGCCACTCC-3' 5'-GAAGCATTCAGATAGCTCATCACT-3'	588	60	NM_ 000882.4
COX-2	5'-TGAGCATCTACGGTTTGCTG-3' 5'-TGCTTGTCTGGAACAACTGC-3'	158	59	NM_ 000963.4
TNF-α	5'-TGGAGAAGGGTGACCGACTC-3' 5'-TCCTCACAGGGCAATGATCC-3'	101	59	NM_ 000594.3
IFN-α	5'-GACTCCATCTTGGCTGTGA-'3 5'-TGATTTCTGCTCTGACAACCT-'3	103	59	NM_ 02013.3
IFN-β	5'- CAACTTGCTTGGATTCCTACAAAG-3' 5'- TATTCAAGCCTCCCATTCAATTG-3'	81	59	NM_ 002176.4
hBD-2	5'-GACTCAGCTCCTGGTGAAGC-3' 5'-TTTTGTTCCAGGGAGACCAC-3'	212	59	NM_ 004942.4
hBD-3	5'-CATTATCTTCTGTTTGCTTTGCTC-3' 5'-CGATCTGTTCCTCCTTTGGA-3'	148	59	NM_ 018661.4
LL37	5'-GGACAGTGACCCTCAACCAG-3' 5'-AGAAGCCTGAGCCAGGGTAG-3'	213	59	NM_ 004345.5

Table 2.7: Cycling parameters for qPCR

Step	Temperature	Allocated time
Reverse transcription	45°C	12 minutes
Polymerase activation	95°C	3 minutes
Denaturation	95°C	5 seconds
Annealing/extension	54-60°C	12 seconds
Repeat denaturation and annealing stages for 40 cycles		

2.2.7 Cell viability from MTT Assay

To determine cell viability after exposure to various TLR agonists, MTT assays were used. The hTCEpi cells were seeded at $2x10^5$ per well of a 96 well plate for 24h, using the methods described in subsection 2.2.3. Growth media was removed, cells washed using PBS and serum free media was replenished. Cells were stimulated with various TLR agonists at various concentrations, (manufacturer details described within table 2.1) including: TLR3 Poly(I:C), TLR5 FLA-ST, TLR5 FLA-PA, TLR9 ODN2006, TLR9 ODN2395, TLR9 *E. coli* DNA, cGAS 2'3'cGAMP and vitamin 1,25D3 with 5 mg/ml of 3-(4,5-dimethylthiazol-2-yl_-2,5-diphenyl tetrazolium bromide (MTT) (Sigma-Aldrich, UK) added for 2-4h incubation at 37°C in 5% CO_2. Benzalkonium chloride (BAC) (0.5%) and 100% MTT were used for positive and negative controls, respectively. Dimethyl sylfoxide (DMSO) was added for OD analysis at 570nm and 630nm, with cell viability calculated.

2.2.8 Cell counting with trypan blue

A 10ul aliquot of cell suspension containing 0.4% trypan blue (Sigma, UK) was homogenized using a slow pipetting action and transferred to the C-Chip haemocytometer (LabTech, UK). Both live and dead cells were counted using the Olympus BX61 microscope (Tokyo, Japan), with cells containing trypan blue removed from the counting process due to lack of viability. The following equation was used to confirm cell populations, which was used to confirm the volume of cells within each ml for later experiments:

(Total cells / Number of squares counted) x dilution factor (1:1) x 10^4 = cells/ml

2.2.9 MiR analysis and inhibition

To observe the effect of 1,25D3 upon hTCEpi miR activity, and after cells had reached 80% confluence, hTCEpi were stimulated with TLR agonists and 1,25D3 as described within subsection 2.2.1. Using the Taqman MicroRNA Cells-To-C_T kit (Invitrogen, USA), cells were lysed and reverse transcribed using the corresponding TaqMan Advanced miRNA Assays (Applied Biosystems, USA) within an Eppendorf MasterCycler X50 Thermocycler. Results were analysed, with RNU44 housekeeping miR expression used to standardize results, alongside mRNA expression of pro-inflammatory mediators and receptors, as described throughout subsection 2.1.6). The housekeeping gene RNU44 was used for the miR experiments to normalize the target miRNA expression data and act as an internal control, similar to GAPDH for mRNA expression. Once expression of each miR was observed and a potential change within expression identified, the TLR agonist and 1,25D3 experiments were repeated using a combination treatment of miRCURY LNA Power miRNA inhibitor (Qiagen, USA) and Lipofectamine™ 3000 transfection reagent (Thermo Fisher Scientific, UK) for hsa-miR-93-5p and has-miR-181-3p, alongside an untargeted scramble inhibitor (Qiagen, USA). Inhibitors were then exposed to cells for 24h before experiments began, with qPCR repeated using the methods described throughout subsection 2.2.2 to determine changes in cytokine and receptor expression. Both miRCURY LNA Power miRNA inhibitors and miRCURY scramble inhibitors were re-suspended in sterile TEA buffer to make the stock solutions described within table 2.1, alongside the working solutions used within each experiment.

2.2.10 Storage of cultured cell lines hTCEpi and SV40-HCEC

Cells beyond the required $2x10^5$ cells per well for each experiment, within p35-50, were harvested for storage, ensuring cell populations for future experiments. Using the counting and viability methods described throughout subsections 2.1.6 and 2.1.7, $2x10^6$ cells were suspended in 2.5ml of corresponding medium in a 15ml falcon tube, centrifuged for 2500rpm for 3 minutes. Supernatant was removed using a 5ml pipette, and cells were re-suspended in 10% of DMSO and 90% FBS, which was transferred by 1ml

aliquots into 2ml cryopreservation vials. These vials were then contained within a 'Mr Frosty' freezing container holding Isopropanol (Sigma, UK) for over-night storage at -80^{o}C, removed and stored at -140^{o}C in a designated chest freeze.

2.2.11 Agarose Gel Electrophoresis

To confirm RT-PCR results by specificity of primers, products were analysed using 1.5% agarose gel (0.75g (w/v) of agarose in 50mls of TAE buffer). Using a microwave, the agarose was dissolved into the TAE buffer using 20 second bursts of exposure at half power. SYBRSafe (10μl) (Thermo Fisher Scientific, UK) was added per 100ml of agarose solution, with the gel poured and allow to set for 30 minutes at room temperature. Hyperladder (20μl) (Bioline, UK) was mixed with the PCR products by pipetting in an Eppendorf tube, then transferred to the gel and analysed after 45 minutes at 100v to allow visualization of each band by observation using UV light, with a digital image captured using the Nu:Genius 3+ system (SyngeneUS, USA).

2.2.12 Statistical analysis

Statistical analyses were performed using unpaired (independent) two-tailed, t-tests in experiments comparing two samples. The Shapiro-Wilk ($P>0.05$) was used to confirm normally distributed data, alongside a skewness and kurtosis score of $z = \pm 1.96$ and visual confirmation of histograms and dot box plots. For data that showed non-normally distributed data with a result of $p<0.05$ using the Levene's test, the Mann-Whitney U test was used as this was found to be more appropriate. When more than two samples were analysed with multiple dependent variables, a one-way ANOVA was used, with Bonferroni's test for multiple comparisons. The criterion for statistical significance for these data was a *p* value of <0.05, with all data expressed as a mean with standard error. All statistical tests were performed with GraphPad Prism 6.0 software (GraphPad Software Incorporation, San Diego, CA).

Chapter 3

Vitamin D and Human Corneal Epithelial Cells: analyzing anti-inflammatory effects of 1,25D3 during TLR3 signaling

3.1 Introduction to chapter 3

3.1.1 TLR3 and the Ocular Surface

Infections which affect the ocular surface, including herpes simplex virus-1 (HSV-1), often lead to an increased presence of dsRNA during viral replication (Burgess *et al.,* 2018). The dsRNA then acts as a pathogen associated molecular pattern (PAMP) for detection by multiple receptors, leading to an innate, pro-inflammatory response, removing the pathogen and protecting against further infection (Nguyen *et al.,* 2017). TLR3 is expressed by cells of the ocular surface, including corneal epithelial cells, and may be activated following dsRNA recognition, subsequently inducing the NF-κB pathway and producing pro-inflammatory mediators (Reins *et al.,* 2015a).

Reins *et al.,* (2015a) confirmed that hTCEpi TLR3 activation using poly(:C) led to persistent inflammation, with the subsequent production of pro-inflammatory cytokines and chemokines, causing unnecessary tissue damage after the infection has cleared (Cook *et al.,* 2005). Improper or chronic TLR3 activation by cells of the ocular surface can lead to not only an influx of immune cells into the cornea, but also corneal scarring and vision loss; a phenomenon associated with HSV-1 infections of the cornea (Chen *et al.,* 2019a).

3.1.2 Vitamin D and TLR3

Vitamin D regulates calcium homeostasis (discussed throughout subsection 1.1.2), however, metabolite 1,25D3 is an efficient immunomodulator towards TLR3-driven responses by the cornea following activation of VDR (Reins *et al.,* 2015a). This leads to a significant suppression of not only TLR3, but also a significant suppression of the production of pro-inflammatory mediators, including IL-6, IL-8 and TGF-β, all of which have been implicated in ocular inflammation and associated tissue damage in response to viruses (Li *et al.,* 2006a). The NF-κB pathway activated by TLR3 can be negatively regulated by the IKK family, which are expressed by epithelial cells of the ocular surface (Reins *et al.,* 2015a). The Reins *et al.*, (2015) study concluded that vitamin D regulated NF-κB levels by increasing mRNA stability and decreasing

phosphorylation, impeding NF-κB activity, however, the complete mechanism behind the suppression remains unknown.

3.1.3 miR and TLR3 during inflammation

Taganov *et al.,* (2006) demonstrated the role of miR-146a negatively regulating TLR3/NF-κB signaling, whilst further studies showed that miR-146a significantly suppresses a range of pro-inflammatory cytokines including IL-6 and TNF-α (He *et al,.* 2014). Other miR may also have a role in TLR3 signaling due to their involvement in regulating pro-inflammatory cytokines induced by TLR3 activation, such as miR-93 and miR-181. For example, Xu *et al.,* (2014) highlighted a significant, anti-inflammatory role of miR-93 during uveitis, with miR-93 inhibition leading to a significant increase in IL-1β, IL-6 and TNF-α, increasing cornea damage. Likewise, Wang *et al.,* (2020h) showed miR-181a activity increased TLR4 signaling, exacerbating symptoms associated with inflammation and subsequent pro-inflammatory cytokine expression, whilst Hu *et al.,* (2015) showed a strong correlation between miR-155 expression and macrophage TLR3 signaling, concluding that miR-155 negatively regulated TLR3 expression. Considering the growing evidence of miR regulating pro-inflammatory cytokine and TLR signaling, alongside the effects of vitamin D on miR production discussed throughout subsection 1.6, it is possible that 1,25D3 may be impacting miR activity during the TLR3 immunomodulatory effect identified by Reins *et al.,* (2015a).

3.1.4 Aims and Objectives

Although Reins *et al.,* (2015a) determined that 1,25D3 treatment suppressed TLR3 expression and subsequent production of IL-8 and IL-6, it is important to investigate a wider set of pro-inflammatory mediators associated with dsRNA reactions, such as cyclooxygenase-2 (COX-2) and IL-12.

The COX-2 enzyme is elevated in response to pro-inflammatory mediators such as interleukin-1 (IL-1), leading to an increase in the production of prostaglandins such as prostaglandin E2 (PGE_2), which contributes to the pain and inflammation seen at the ocular surface in conditions such as DED (Shim *et al*., 2012). Although innate immunity is linked heavily to COX-2, including TLR3 signaling, activating enzyme COX-2 can also modulate the

adaptive immune system, leading to a regulatory immune response from neutrophils and macrophages (Randall *et al.,* 2020, Kalinski *et al*., 2012). Increased COX-2 production leads to elevated PGE_2 and an increase in IL-10 production. This increase in IL-10 may encourage IL-12 production, affecting innate and adaptive immune cells, including neutrophils, dendritic cells and T cells (Harizi *et al*., 2002). Considering there are obviously more cells involved within the ocular surface than just corneal epithelial cells, observing the effect of 1,25D3 treatment upon COX-2 expression would be beneficial for future studies, such as those involving wider immune cell populations.

Cytokine IL-12 is produced by corneal epithelial cells, influencing both innate and adaptive immune responses following TLR3 activation (Reins *et al.,* 2017b). Often described as the 'driving force' behind cytotoxic T-cell mechanisms, IL-12 is also capable of increasing IFN-γ production that is associated with T-helper cell activation during glaucoma and ocular scleritis (Palomar *et al.,* 2019, Watford *et al.,* 2003). Nakao *et al.,* (2019) analysed BALB/c mice receiving corneal transplant from C57BL/6 donors, and confirmed a significant increase of corneal IL-12 production in juvenile mice led to increased infiltration of macrophages and a subsequent overt immune response. This is a disadvantage for corneal replacement surgery, which is often required as treatment following complications from ocular inflammation. Although research has begun to analyze the effect of 1,25D3 upon innate immunity and TLR signaling, it is vital to broaden the understanding of cytokines such as IL-12 that have the potential to influence wider immune cells, such as macrophages, that can cause inflammatory damage to the cornea.

The experimental aims were to identify further immunomodulatory effects occurring during 1,25D3 treatment of hTCEpi TLR3 activation. This included investigating a wider range of pro-inflammatory mediators, and the time span required for 1,25D3 to suppress these pro-inflammatory mediators, as an acute inflammatory response is still required to combat an infection. Finally, analyzing miR expression and identifying potential targets will lead to further understanding of the mechanism behind 1,25D3 suppression, potentially identifying a novel target to exploit alongside 1,25D3 treatment, increasing the immunosuppressive effects during TLR3 signaling.

3.2 Results

3.2.1 hTCEpi express a range of PRR

The hTCEpi cell line received third party STR analysis to confirm cell line authenticity (Appendix 1.1). Following this, the results for hTCEpi PRR expression (Figure 3.1A) confirmed expression of TLR1-9, NOD1, NOD2, NLRP3, RIG1 and MDA5, but showed a significantly stronger expression of TLR2, TLR5, TLR6, TLR9, NOD2 ($p<0.01$), NOD1 and MDA5 ($p<0.05$) in comparison to SV40-HCEC. Primer products were confirmed by 1.5% agarose gel electrophoresis analysis (Figure 3.1B).

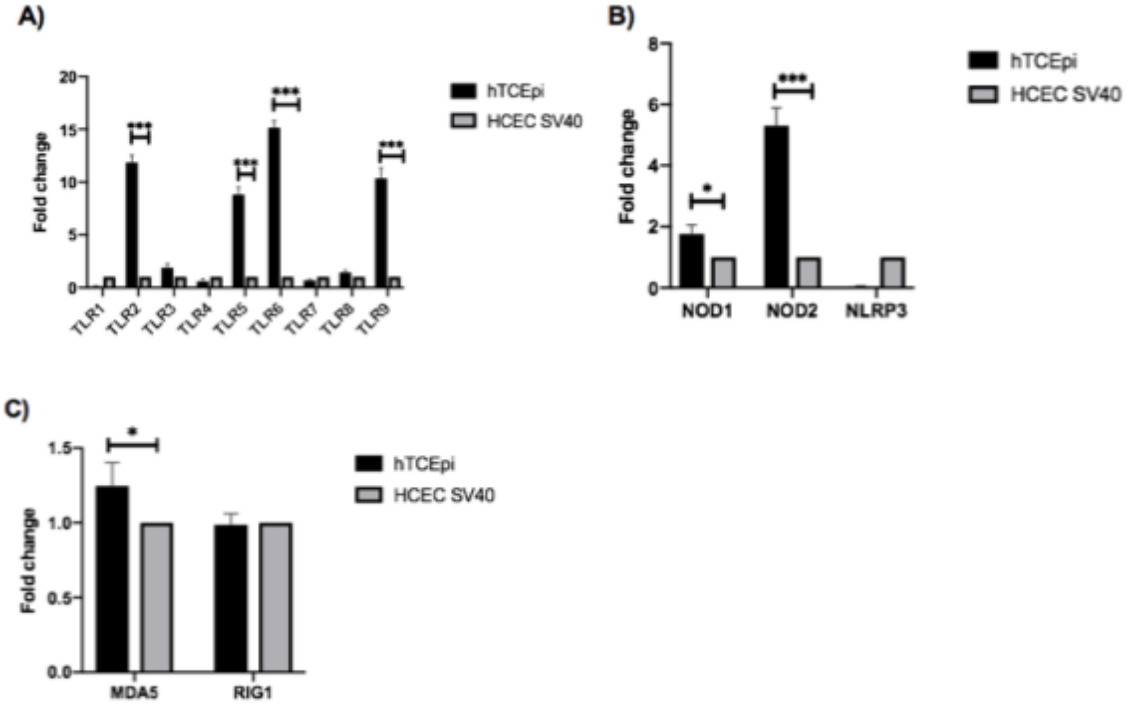

Figure 3.1a: PRR expression profile of hTCEpi cells: PRR expression of (A) TLR, (B) NLR and (C) RLR by hTCEpi were confirmed following mRNA comparison to PRR expression by SV40-HCEC, using RT-PCR. Data represent mean ± SEM of three independent experiments. Statistical analysis was by a one-way ANOVA with Bonferroni test for multiple comparisons (A-C, n=3), p<*0.05, **0.01, ***0.001

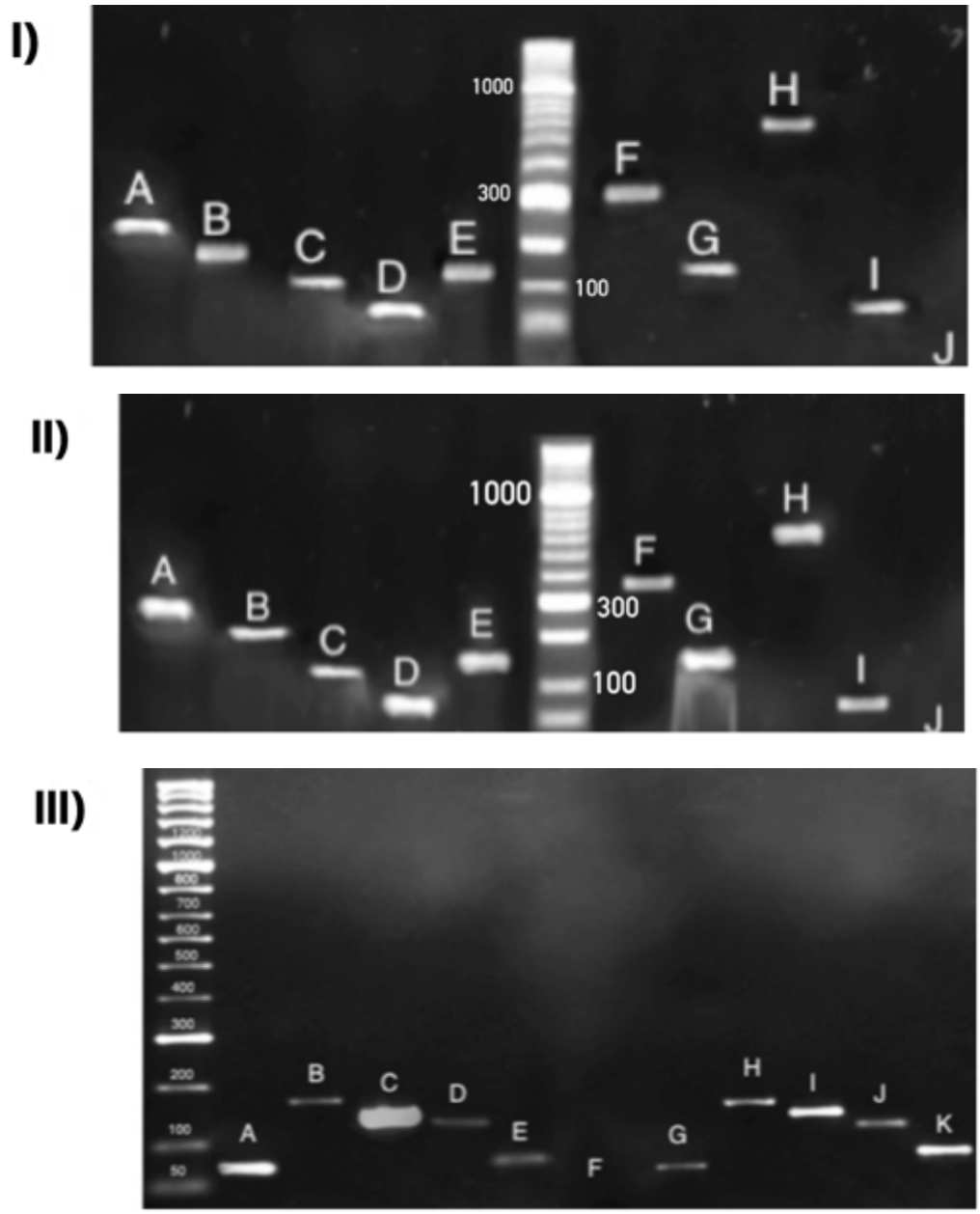

Figure 3.1b: Agarose gel electrophoresis confirmation of qPCR products:

(I) SV40 cell line and (II) hTCEpi cell line, for qPCR product confirmation of: (A) TLR1, (B) TLR2, (C) TLR3, (D) TLR4, (E) TLR5 (F) TLR6, (G) TLR7, (H) TLR8, (I) TLR9 and (J) negative control.

(III) SV40 cell line and hTCEpi cell line expression for various PRR: (A) SV40 RIG1, (B) SV40 MDA5, (C) SV40 NOD1, (D) SV40 NOD2, (E) SV40 NLRP3, (F) negative control, (G) hTCEpi RIG1, (H) hTCEpi MDA5, (I) hTCEpi NOD1, (J) hTCEpi NOD2 and (k) hTCEpi NLRP3.

3.2.2 hTCEpi have a functional TLR3 receptor, which induces IL-8 expression that is suppressed by 1,25D3

Following confirmation of TLR3 mRNA expression by hTCEpi, the aim of the next experiment was to confirm that hTCEpi TLR3 was a functional component of these cells. As Reins *et al.,* (2015a) showed that 0.5μg/ml poly(I:C) induced significant IL-8 expression after 24h, hTCEpi cells were stimulated with 0.5μg/ml poly(I:C) for 6, 24 and 48h to monitor for potential fluctuations of TLR3 mRNA expression. This time course experiment confirmed that 24h exposure to 0.5μg/ml poly(I:C) induced the strongest fold change of hTCEpi TLR3 (Figure 3.2A, $p<0.001$), concluding 24h to be the optimum condition for future experiments. There was a significant decline of TLR3 expression after 48h of poly(I:C) exposure ($p<0.001$), with similar findings identified throughout by Reins *et al.,* (2015a).

Using 0.5μg/ml of poly(I:C), the next aim was to confirm the effect of vitamin D metabolite 1,25D3 upon hTCEpi TLR3 and IL-8 mRNA expression. There was no significant increase in TLR3 or IL-8 expression (Figure 3.2, B-C). However, poly(I:C) exposure significantly increased both TLR3 and IL-8 expression ($p<0.001$). The final experimental condition which contained the dual combination treatment of both poly(I:C) and 1,25D3, demonstrated significant suppression of both TLR3 and IL-8 expression ($p<0.001$).

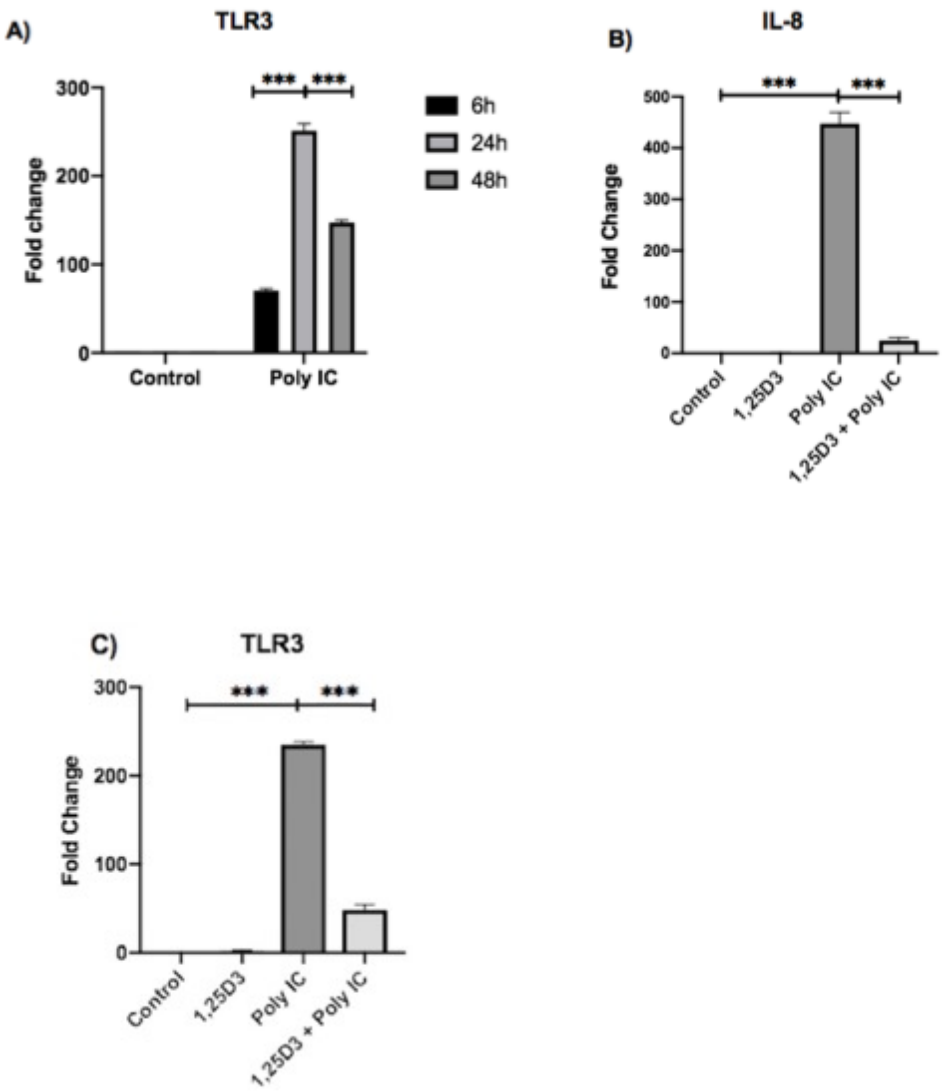

Figure 3.2: Optimised time frame of poly(I:C) exposure for hTCEpi to induce an inflammatory response: (A) TLR3 expression by hTCEpi was confirmed following analysis of samples after 6h, 24h and 48h of poly(I:C) exposure, compared to unstimulated hTCEpi cells. (B) IL-8 mRNA expression by hTCEpi cells with and without poly(I:C) and 1,25D3 treatment for 24h (C) TLR3 mRNA expression by hTCEpi cells with and without poly(I:C) and 1,25D3 treatment for 24h. Data represent mean ± SEM of three independent experiments. Statistical analysis was by one-way ANOVA with Bonferroni test for multiple comparisons (A-C, n=3) p<*0.05, **0.01, ***0.001

3.2.3 1,25D3 suppression of TLR3 signaling did not lead to a decline in cell viability

Cell viability was analysed to confirm that 1,25D3 was actively suppressing TLR3 signaling and IL-8 (Figure 3.2) and the decline in mRNA expression was not due to a decline in the cell population. In regard to 24h of poly(I:C) exposure, 0.2μg/ml and 0.5μg/ml led to no significant alteration in hTCEpi cell viability in comparison to unstimulated cells (Figure 3.3A). However, increasing poly(I:C) concentration to 1μg/ml and 2μg/ml for 24h led to a significant decline in hTCEpi cell viability ($p<0.05$), signifying that these concentrations were toxic to hTCEpi cells.

As 1,25D3 was used throughout the study, it was important to observe the effect of the hormone upon hTCEpi cell viability. Following the same methodology used in the poly(I:C) experiments, the 1,25D3 viability analysis showed that 1,25D3 at 10^{-5}M and 10^{-7}M led to no significant decline of hTCEpi cell viability. Therefore, it was decided that 10^{-7}M would be optimum for future experiments throughout study, as this dose could offer the greatest suppression without impacting cell viability (Figure 3.3B, $p<0.01$). Finally, the dual treatment of poly (I:C) 0.5μg/ml and 1,25D3 10^{-7}M (Figure 3.3C), used throughout subsection 3.2.2, did not lead to a significant change in hTCEpi cell viability, and would be used in future TLR3 experiments.

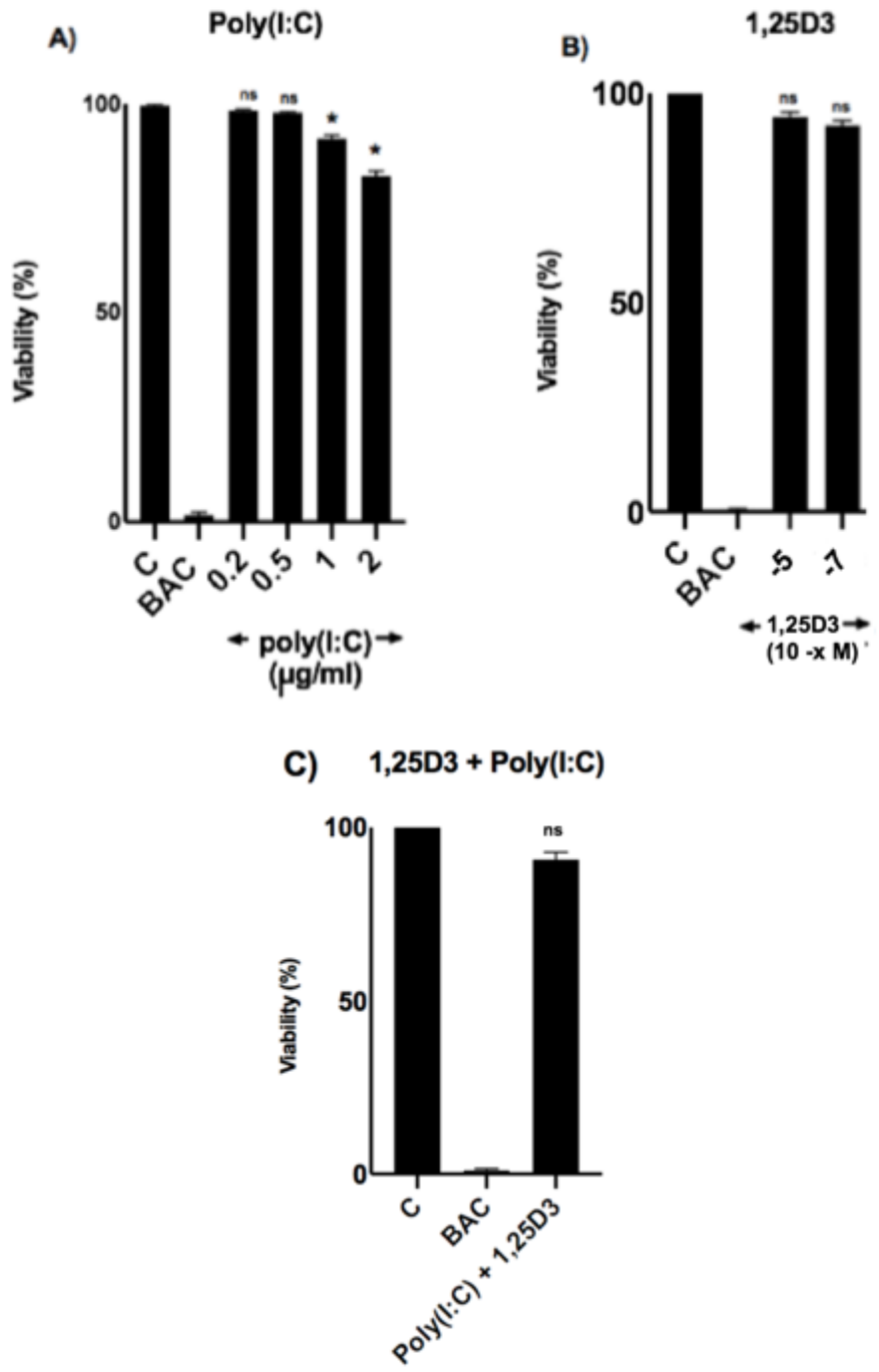

Figure 3.3: Cell viability of hTCEpi cells following various treatment conditions for 24h: (A) poly(I:C), (B) 1,25D3, (C) representing 0.5µg/ml of poly(I:C) and 10^{-7}M of 1,25D3. Negative control utilised unstimulated hTCEpi cells (C) whilst the positive control was generated by hTCEpi with benzalkonium chloride (BAC). BAC was chosen as a suitable positive control as this led to a significant decline in cell viability and lead to easy comparison when analysing the effects of various reagents on hTCEpi viability. Cells are shown as a percentage of viability in comparison to unstimulated cells. Statistical analysis was by one-way ANOVA with Bonferroni test for multiple comparisons against completely unstimulated hTCEpi cells p<*0.05, **0.01, ***0.001

3.2.4 1,25D3 modulates hTCEpi TLR3 response to poly(I:C) by suppressing multiple pro-inflammatory mediators after 24h stimulation

Since confirming that the dual treatment of poly(I:C) 0.5μg/ml and 1,25D3 10^{-7}M was not toxic to hTCEpi cells, the next experimental aim was to confirm a significant suppression within a wider range of pro-inflammatory mediators (Figure 3.4A). Following 24h stimulation of hTCEpi TLR3 using 0.5μg/ml of poly(I:C), the following changes to expression were identified: COX-2 (A, ns), TNF-α (B, $p<0.01$), IL-6 (D, $p<0.001$), IL-1β (E, $p<0.001$) and IL-12 (C, $p<0.01$). Each of these primer products were confirmed by agarose gel electrophoresis (Figure 3.4B). In comparison to 24h of 0.5μg/ml poly(I:C) stimulation alone, the dual combination treatment of poly(I:C) and 1,25D3 led to a decrease of mRNA expression of TNF-α (B, $p<0.01$), IL-6 (D, $p<0.001$), IL-1β (E, $p<0.001$) and IL-12 (C, $p<0.01$).

Interestingly, the combination of poly(I:C) and 1,25D3 treatment didn't indicate a suppressive effect upon COX-2 expression (A, $p<0.01$), but rather enhanced expression in comparison to poly(I:C) stimulation alone. These data provide evidence that 1,25D3 acts in a suppressive manner to a range of pro-inflammatory mediators, but not all.

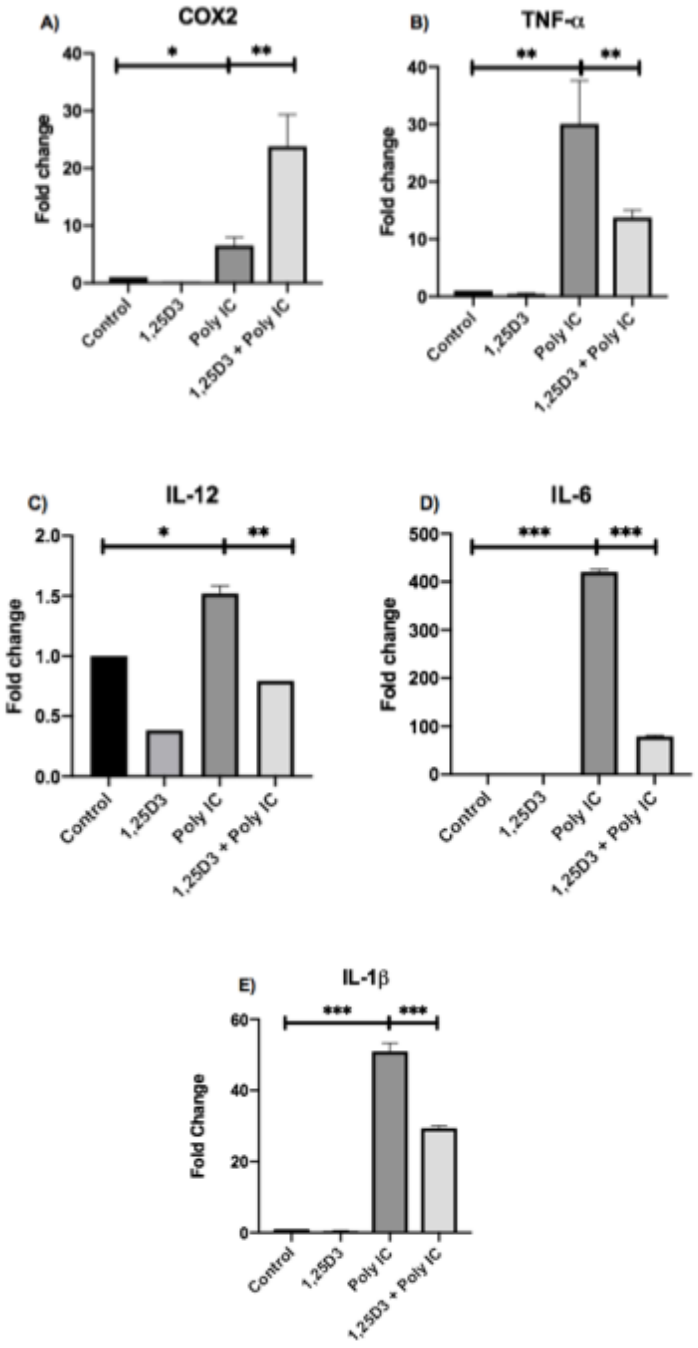

Figure 3.4a: hTCEpi pro-inflammatory mediator expression under various conditions for 24h: Pro-inflammatory mediator mRNA expression by hTCEpi cells with and without 0.5μg/ml poly(I:C) and 10^{-7}M 1,25D3 treatment for 24h. Pro-inflammatory mediators analysed include: (A) COX-2, (B) TNF-α, (C) IL-12, (D) IL-6 and (E) IL-1β. Data represent mean ± SEM of three independent experiments. Statistical analysis was by one-way ANOVA with Bonferroni test for multiple comparison (A-E, n=3) p<*0.05, **0.01, ***0.001

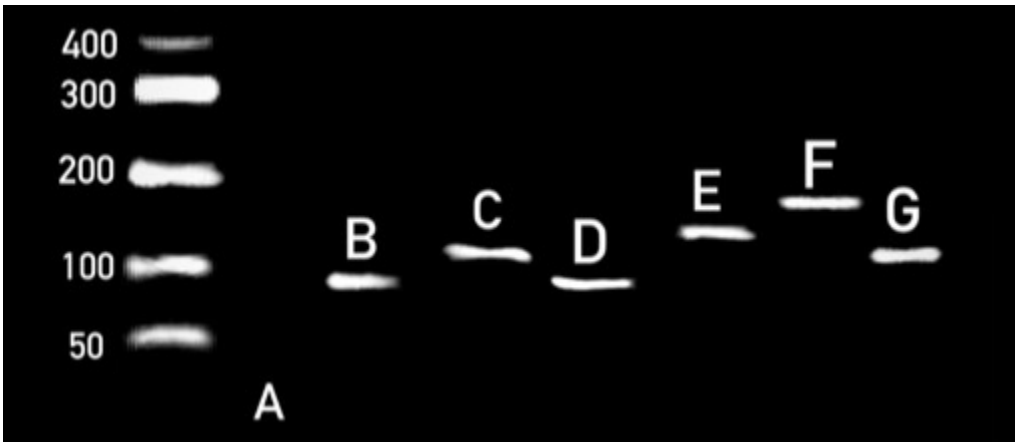

Figure 3.4b: Agarose gel electrophoresis confirmation of qPCR products: products taken from the condition of hTCEpi under 24h of poly(I:C) exposure, for confirmation of final products. GAPDH was included as already confirmed for expression within Figure 3.1b. (A) Negative control, (B) GAPDH, (C) IL-8, (D) IL-6, (E) IL-1β, (F) COX-2 and (G) TNF-α.

3.2.5 1,25D3 treatment suppressed IL-1β expression following 4h of ongoing poly(I:C) stimulation

As the previous data from Figure 3.4a confirmed 10^{-7}M 1,25D3 suppressed a range of hTCEpi pro-inflammatory mediators during ongoing 24h of poly(I:C) TLR3 stimulation, the experiment was repeated using a 4h time-point. It was important to identify the potential effects of 1,25D3 treatment upon the acute inflammatory phase, which would be expected to occur between 4-6h, and how long suppression would take to occur. An influx of pro-inflammatory mediators is vital to remove invading pathogens, however, can lead to significant tissue damage from persistent inflammation. The results showed no significant induction of COX-2, TNF-α or IL-12 expression following 4h of 0.5μg/ml poly(I:C) stimulation (Figure 3.5, A-C). However, there was a significant increase in the expression of IL-6, IL-1β and IL-8 (D, E and F, $p<0.001$) following 4h of poly(I:C) stimulation. Finally, 1,25D3 treatment only significantly suppressed IL-1β expression within 4h (E, $p<0.001$).

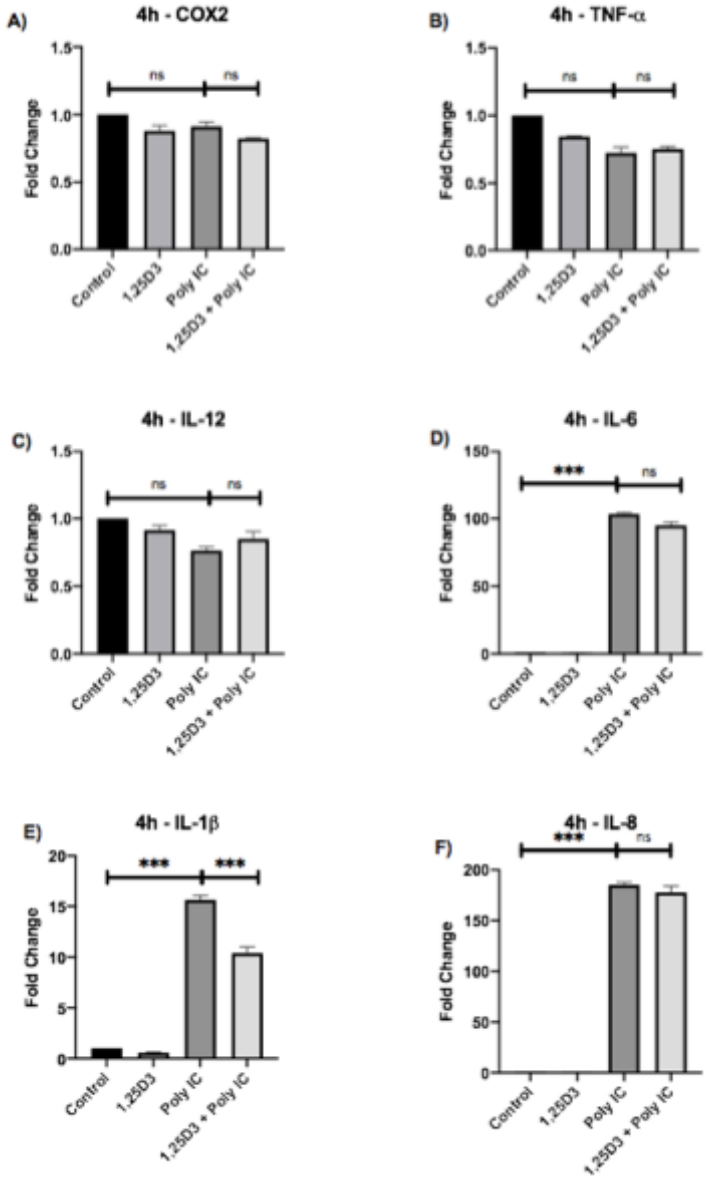

Figure 3.5: hTCEpi pro-inflammatory mediator expression under various conditions for 4h: with and without 0.5μg/ml poly(I:C) and 10^{-7}M 1,25D3 treatment, pro-inflammatory mediators analyse include: (A) COX-2, (B) TNF-α, (C) IL-12, (D) IL-6, (E) IL-1β and (F) IL-8. Data represent mean ± SEM of three independent experiments. Statistical analysis was by one-way ANOVA with Bonferroni test for multiple comparison (A-F, n=3) p<*0.05, **0.01, ***0.001

3.2.6 1,25D3 treatment suppressed IL-8, IL-6, and IL-1β following 6h of poly(I:C) stimulation

The results show that there was no significant induction of COX-2, TNF-α or IL-12 following 6h of 0.5μg/ml poly(I:C) stimulation (Figure 3.6, A-C). However, there was a significant increase in the expression of IL-6, IL-1β and IL-8 (D-F, $p<0.001$) following 6h of poly(I:C) stimulation. Furthermore, 1,25D3 10^{-7}M significantly suppressed the expression of these same mediators by 6h (D-F, $p<0.001$).

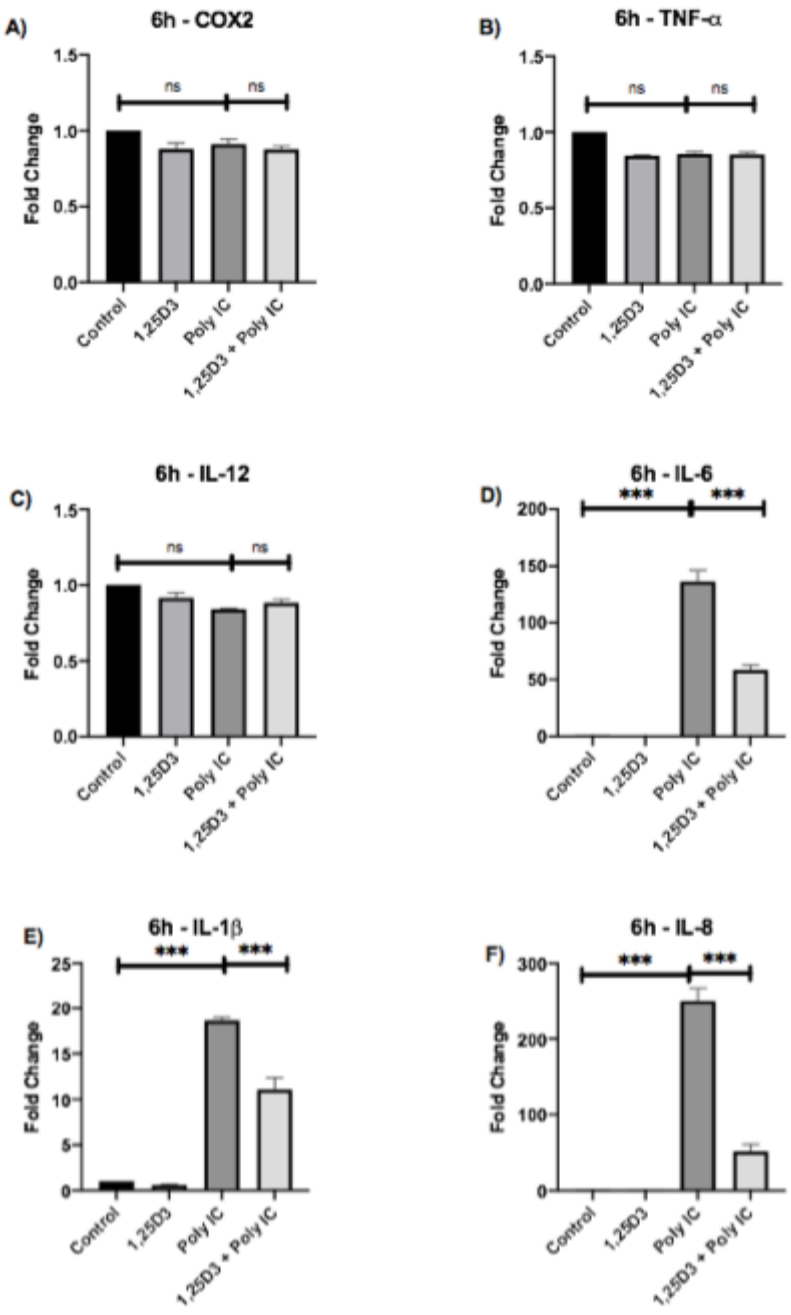

Figure 3.6: hTCEpi pro-inflammatory mediator expression under various conditions for 6h: with and without 0.5μg/ml poly(I:C) and 10^{-7}M 1,25D3 treatment, pro-inflammatory mediators analysed include: (A) COX-2, (B) TNF-α, (C) IL-12, (D) IL-6, (E) IL-1β and (F) IL-8. Data represent mean ± SEM of three independent experiments. Statistical analysis was by one-way ANOVA with Bonferroni test for multiple comparison (A-F, n=3) p<*0.05, **0.01, ***0.001

3.2.7 1,25D3 suppressed IL-6 and IL-1β expression by hTCEpi following 4h of prior poly(I:C) pre-treatment

The previous results seen in Figures 3.5 and 3.6 provided evidence that 1,25D3 suppressed IL-8 and IL-6 expression within 6h, within 4h for IL-1β, during ongoing TLR3 stimulation. The study then aimed to confirm if the expression of these pro-inflammatory mediators would be suppressed by 1,25D3 following prior TLR3 stimulation with poly(I:C). Ongoing TLR3 activation, before 1,25D3 treatment, would closely mimic infection. The results showed a significant decline in the expression of all three mediators and TLR3 following 4h of prior 0.5μg/ml poly(I:C) stimulation followed by 6h of 1,25D3 treatment (Figure 3.7, A-D, $p<0.001$). These results provide evidence that 1,25D3 is able to suppress inflammation caused by prior TLR3 stimulation.

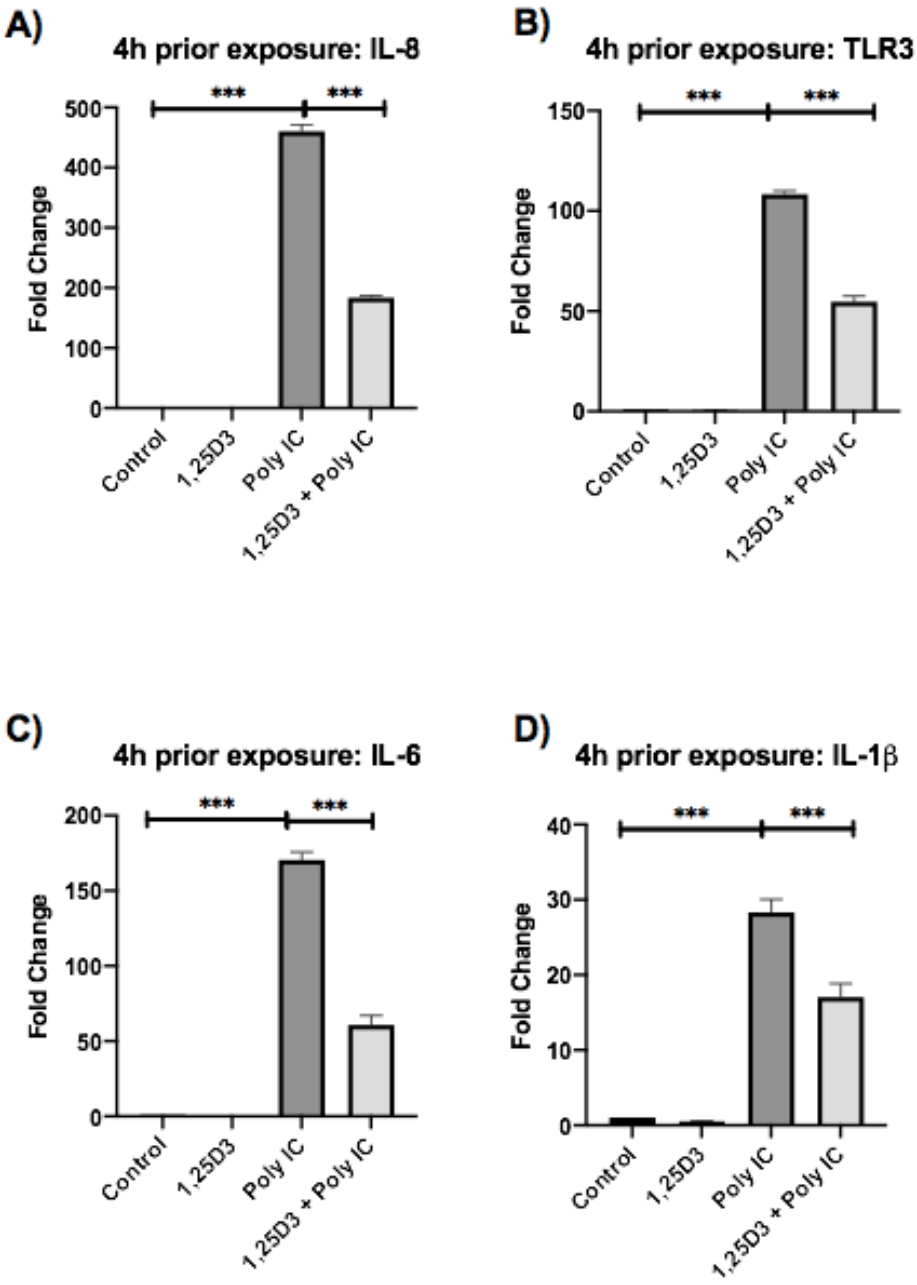

Figure 3.7: hTCEpi pro-inflammatory cytokine expression under various conditions following prior 4h poly(I:C) treatment: Pro-inflammatory mediators and receptor mRNA expression by hTCEpi cells with and without 6h 1,25D3 treatment following 4h of poly(I:C) treatment. The mRNA expression analysis includes: (A) IL-8, (B) TLR3, (C) IL-6 and (D) IL-1β. Data represent mean ± SEM of three independent experiments. Statistical analysis was by one-way ANOVA with Bonferroni test for multiple comparison (A-D, n=3) p<*0.05, **0.01, ***0.001

3.2.8 1,25D3 modulates the expression of miR-93-5p and miR-181-3p during TLR3 stimulation

To confirm the expression of miR-93-5p, miR-146a-5p, miR-155-5p and miR-181a-3p within hTCEpi, expression was compared against the acute monocytic leukemia (THP1) cell line, as there is already evidence of these miR in this cell line (Figure 3.8A). The results showed that hTCEpi cells expressed significantly less of each miR analysed in comparison to THP1 (Figure 3.8A, $p<0.001$. However, as hTCEpi expressed the selected miR, it was decided that these miR would be analysed further in future experiments.

Both treatments of 0.5μg/ml poly(I:C) and 10^{-7}M 1,25D3 led to no significant change within miR-93-5p and miR-181-3p expression. A significant decrease of miR-93-5p expression following treatment with the combination of poly (I:C) and 1,25D3 (C, $p<0.001$) was noted and a decrease for miR-181-3p expression under the same conditions ($p<0.01$). However, neither poly(I:C) nor the dual combination treatment had a significant effect upon miR-146a-5p or miR-155-5p expression, therefore, these miR were not studied further during this chapter.

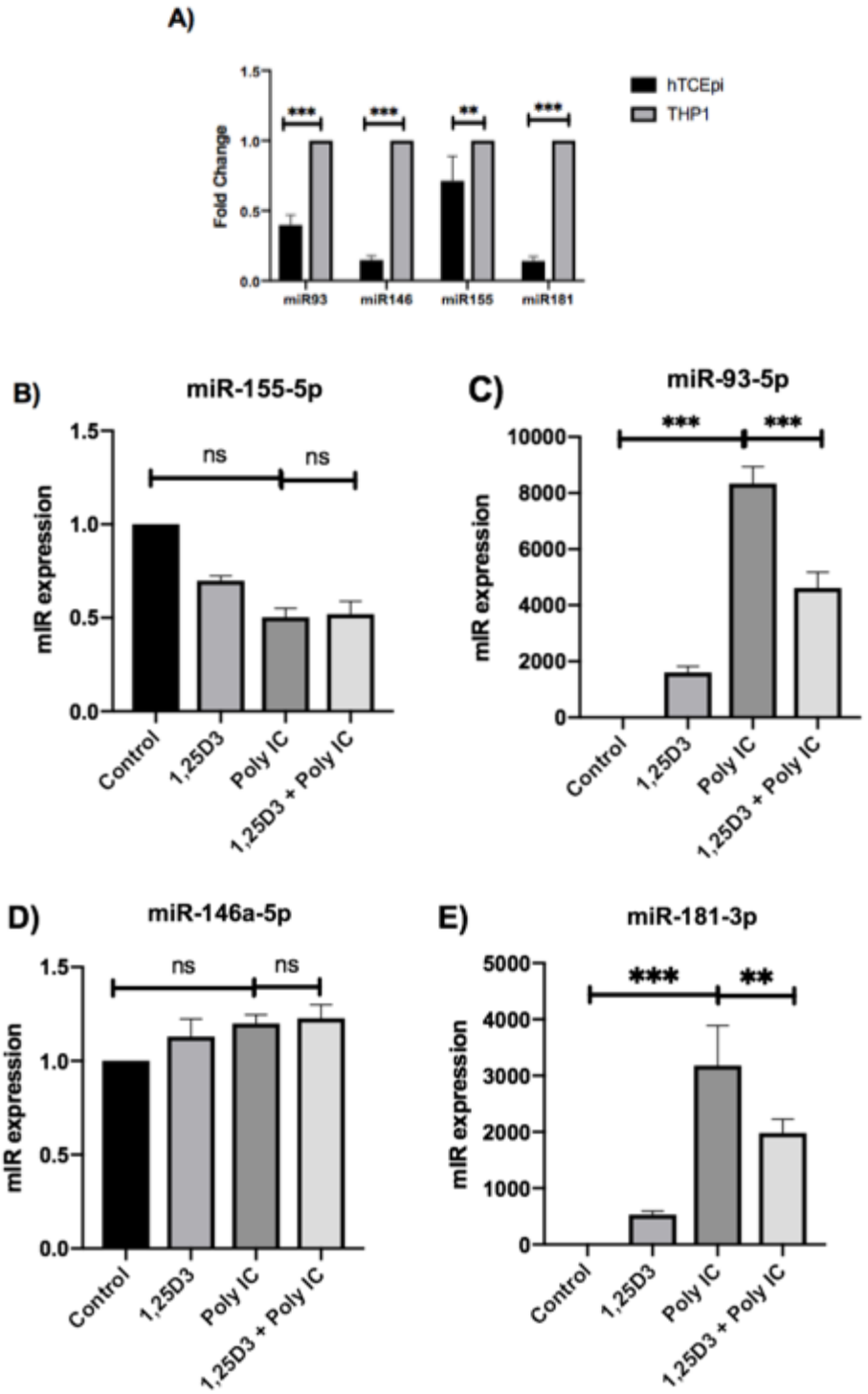

Figure 3.8: miR profile of hTCEpi and THP1 cells, and subsequent miR expression under various conditions of TLR3 activation by hTCEpi: (A) miR expression of both hTCEpi and (B-E) THP1 cells - miR expression by hTCEpi cells following 24h of stimulation with and without poly(I:C) and 1,25D3 treatments. Data represent mean ± SEM of three independent experiments. Statistical analysis was by one-way ANOVA with Bonferroni test for multiple comparisons (A-E, n=3) p<*0.05, **0.01, ***0.001

3.2.9 Inhibiting miR-93-5p led to a significant increase within the expression of IL-6, IL-8 and IL-1β during TLR3 stimulation by poly(I:C)

This experiment used a miR-93-5p power inhibitor to identify the potential role of miR-93 during the 1,25D3 suppression of TLR3 activity and associated mediators. To ensure experiment validity, this was performed alongside a scrambled miR acting as a negative control. The results showed that miR-93-5p had no role during the suppressive action of 1,25D3 against mediators IL-8, IL-6 or IL-1β (Figure 3.9, A-C), as there was still a significant reduction in the cells undergoing dual treatment, compared to those treated with poly(I:C) alone in the presence of a miR-93-5p inhibitor ($p<0.001$). If miR-93-5p had a role within 1,25D3 mechanism of action, it would be expected that inhibiting this miR would prevent suppression of pro-inflammatory mediators.

However, the inhibitor did significantly enhance mediator expression following 24h of 0.5μg/ml poly(I:C) stimulation, particularly IL-8 (A, $p<0.01$), IL-6 (B, $p<0.001$) and IL-1β (C, $p<0.01$). In regard to TLR3 mRNA expression, no significant change in expression was detected, indicating that miR-93-5p has no role during the expression of TLR3 (D), with 1,25D3 treatment still suppressing expression after hTCEpi stimulation, indicating that miR-93-5p had no role during the suppression of TLR3 by 1,25D3.

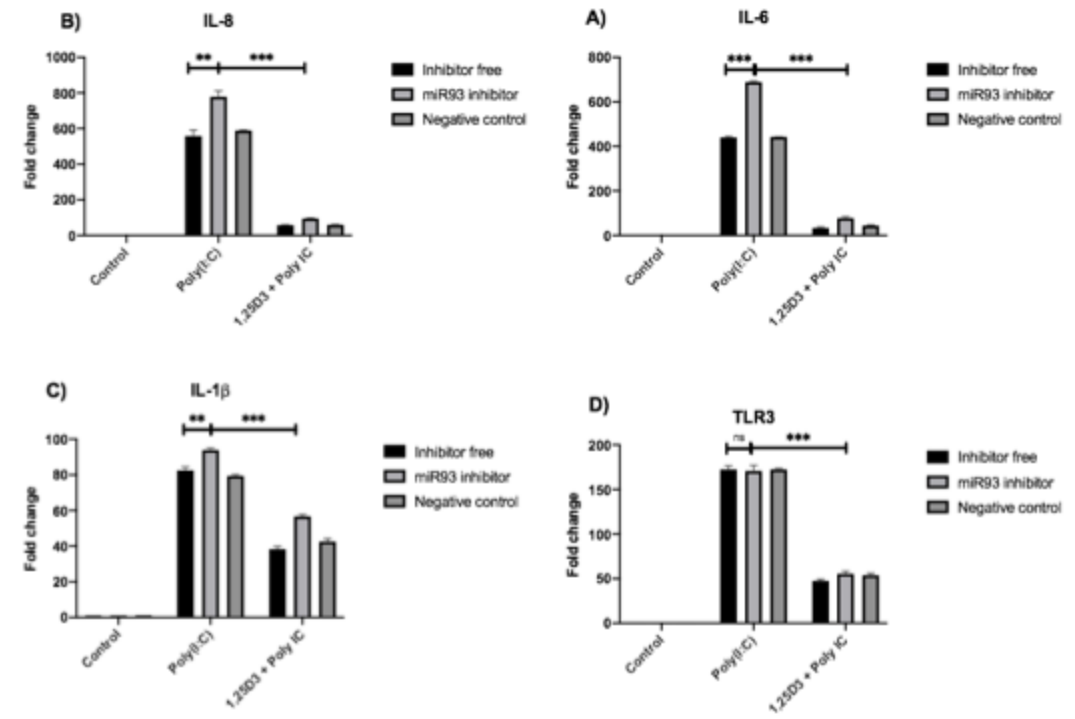

Figure 3.9: Effects of miR-93-5p inhibition upon pro-inflammatory mediator expression in hTCEpi cells under various 24h conditions: with and without 24h 0.5μg/ml poly(I:C) and 10^{-7}M 1,25D3 treatment. The pro-inflammatory mediators having the greatest expression in response to poly(I:C) were chosen from the previous experiments. (A) IL-8, (B) IL-6 (C) IL-1β and receptor (D) TLR3. Data represent mean ± SEM of three independent experiments. Statistical analysis was by one-way ANOVA with Bonferroni test for multiple comparisons (A-D, n=3) p<*0.05, **0.01, ***0.001

3.2.10 Inhibiting miR-181-3p led to a significant increase within the expression of IL-6, IL-8 and IL-1β during TLR3 stimulation by poly(I:C)

The results showed that anti-miR-181-3p enhanced the effect of poly(I:C) upon mRNA expression for IL-8, IL-6 or IL-1β, however, there was no significant change in regard to 1,25D3 suppressive effects (Figure 3.10, A-C). This suggested that miR-181-3p does not have a role in the suppression of TLR3 induced mediator production by 1,25D3. However, inhibiting miR-181-3p showed a significant increase in the expression of IL-8, IL-6 and IL-1β, indicating that miR-181-3p has a regulatory role during the transcription of these mediators following TLR3 stimulation.

In regard to TLR3 mRNA expression, no significant change in expression was detected following miR inhibition, indicating that miR-181-3p has no role in the expression of TLR3 (D). Furthermore, 1,25D3 still suppressed TLR3 expression significantly (D, $p<0.001$), indicating that miR-181-3p had no role during the suppression of TLR3 by 1,25D3.

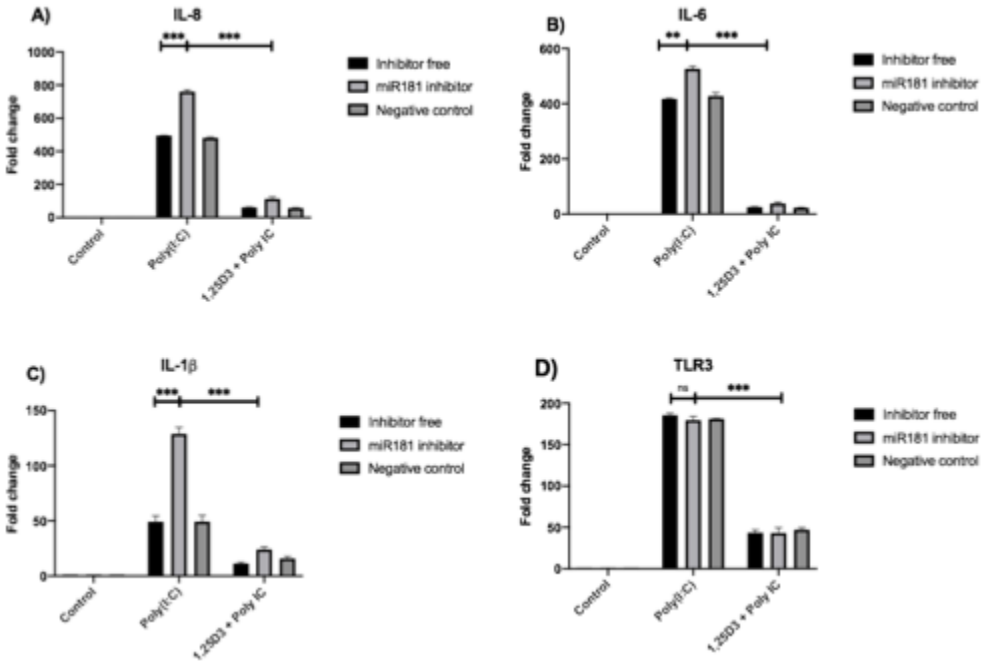

Figure 3.10 – Effects of miR-181-3p inhibition on pro-inflammatory mediator expression by hTCEpi during various TLR3 stimulations for 24h: with and without 0.5μg/ml poly(I:C) and 10^{-7}M 1,25D3 treatment. The pro-inflammatory mediators which had the greatest expression in response to poly(I:C) were chosen from previous experiments; (A) IL-8, (B) IL-6, (C) IL-1β and receptor TLR3 (D). Data represent mean ± SEM of three independent experiments. Statistical analysis was by one-way ANOVA with Bonferroni test for multiple comparison, (A-D, n=3) p<*0.05, **0.01, ***0.001

3.3 Chapter Discussion

3.3.1 hTCEpi cells express multiple PRR, including TLR3

The aims of this chapter were to confirm if 1,25D3 treatment would significantly suppress hTCEpi expression of TLR3 and various pro-inflammatory mediators. The agonist poly(I:C) was used under several conditions of different time points, to mimic viral infection. The results indicated that hTCEpi cells express a range of PRR, including TLR3, which were confirmed with RT-PCR using the positive control of HCEC-SV40 cells (Figure 3.1A). As suggested by the NormFinder software, GAPDH successfully normalized the data throughout the study, a decision which was supported by the similar works of Reins *et al*., (2015). The enzyme is vital in glycolysis for catalyzing the oxidative phosphorylation of glyceraldehyde-3-phosphate, but also regulates other cellular functions including apoptosis and DNA replication (Chuang *et al*., 2005). As these functions are essential for cellular viability, the expression of GAPDH has been described as 'stable and ubiquitous'. Although β-actin, a main component of the cellular actin cytoskeleton, is often incorporated into experiments as a housekeeping gene, previous ocular studies have suggested that β-actin expression can be affected by corneal diseases, such as downregulation in corneal stroma in patients suffering from conditions such as keratoconus (Joseph *et al.,* 2012). As this study was to provide a solid foundation of research for those suffering from ocular disease, it was decided that GAPDH would therefore be the optimum primer.

The RT-PCR data were confirmed by agarose gel electrophoresis analysis (Figure 3.1b), ensuring that the rapidly amplified products obtained from the RT-PCR analysis were indeed the expected amplicon length by visual confirmation upon the gel. However, it would be beneficial to request gel sequencing for slightly degraded bands such as TLR7 (Figure 3.1a, II, G). This smearing could be caused by manual loading of the dye, however, DNA purification of such fragments would ensure the identity of each PRR with a similar primer amplicon length, for example, TLR3 (144bp) and TLR5 (149bp). Systems such as 'Illumina' offer clonal amplification and sequencing of 100-400bp products, which would be optimum for the majority of these TLR (Edinburgh Genomics, 2020).

3.3.2 Poly(I:C) induces an inflammatory response by hTCEpi cells through TLR3 activation

To confirm and expand upon the previous work of Reins *et al*., (2015a), 2ug/ml of poly(I:C) was used to activate hTCEpi TLR3 signaling. Poly(I:C) is a robust, reliable model molecule which mimics the actions of extracellular dsRNA associated with ocular pathogens such as HSV-1, activating hTCEpi TLR3. The NF-ĸB pathway is then induced and produces pro-inflammatory cytokines including IL-6 and chemokines such as IL-8 (CXCL8) (Ueta *et al.,* 2005, Frank-Bertonclj *et al.,* 2018). Treatment with 2ug/ml of poly(I:C) 24h induced a significant increase within a range of pro-inflammatory mediators supporting the findings of Reins *et al*. 2015, including the new, additional data of COX-2 and IL-12 (Figure 3.4A). This model was chosen to be the optimum conditions for future TLR3 experiments within the study, as 48h of the same poly(I:C) exposure led to a weaker inflammatory response. This was most likely due to a limit in the available ligand for TLR3 sampling or poly(I:C) degradation, with the inflammatory process 'resolved'. A strong immune response was required for optimum pro-inflammatory mediator induction, and therefore confirmation of 1,25D3 suppression, hence the decision to continue with a 24h time point.

One obvious limitation throughout this investigation is the possibility that the poly(I:C) stimulation of hTCEpi not only stimulated TLR3, but other intracellular receptors, including MDA5 and RIG1 receptors. These receptors were confirmed to be expressed by hTCEpi (Figure 3.1C) and may be functional during this experiment, aiding in the immune response, which must be considered. The Dominguez *et al.,* (2013) study into corneal cells showed that RIG-1 reacts to poly(I:C), leading to subsequent pro-inflammatory mediator release including IL-8 and IL-6. In regard to hTCEpi, Reins *et al.,* (2015a) showed 1,25D3 suppresses expression of these receptors following poly(I:C) stimulation. Although MDA5 and RIG1 can both identify and react to viral products, it is assumed that RIG-1 has a more prominent role (Loo *et al.,* 2008). Therefore, the results presented from poly(I:C) stimulating hTCEpi may be influenced by RIG1 and MDA5 activation. In future experiments, individual inhibition of these receptors would clarify their role within the

inflammatory response of hTCEpi to poly(I:C) and the subsequent production of pro-inflammatory mediators.

Reins *et al*., (2015) discussed that TLR3-driven inflammatory responses lead to an influx of ocular immune cells into the cornea, with subsequent pro-inflammatory cytokines and chemokines such as IL-6 and IL-8, encouraging an immune response during the acute inflammatory stage which is vital for clearing infection. Although the aim of the study focused within the resolution period of inflammation, 24h, it was important to examine mRNA expression of various pro-inflammatory mediators within the acute inflammatory response of 4h and 6h (Figures 3.5 and 3.6). These experiments gave a wider understanding to potential downfalls in 1,25D3 treatment, for example, dangerously suppressing the initial, required inflammatory response, therefore it was necessary to utilize a wider frame instead of limiting poly(I:C) treatment to 24h.

3.3.3 1,25D3 suppresses TLR3 signaling, which is reflected in the expression of pro-inflammatory mediators IL-1β and IL-10

The 24h time period was used to replicate the resolution period of corneal inflammation, in which it would be expected that the pathogen had been removed as a significant threat and inflammation would lead to unnecessary damage. The results throughout chapter 3 showed that 1,25D3 treatment of hTCEpi did suppress an inflammatory response following TLR3 activation. Supporting the Reins *et al.,* (2015a), 10^{-7}M 1,25D3 suppressed hTCEpi TLR3 signaling by 24h, reflected in a significant decrease in hTCEpi TLR3, IL-6, IL-8, TNF-α and IL-1β mRNA expression (Figures 3.2 and 3.4). This is beneficial, for example, IL-6 and IL-8 production have been heavily implicated in encouraging irreversible tissue damage to the cornea (Ebihara *et al.,* 2011, Sidney *et al.,* 2019).

The dual treatment of poly(I:C) and 1,25D3 led to a significant suppression in IL-1β expression following 4h of prior TLR3 stimulation (Figure 3.6D), which mimics TLR3 activation by pathogens of the ocular surface before treatment. Similar findings were shown during ongoing stimulation, with the dual treatment of poly(I:C) and 1,25D3, leading to suppression of IL-1β expression

by 4h, which was significantly quicker than the 6h required for IL-6 and IL-8 suppression in the same conditions (Figures 3.5 and 3.6). Although this suppression of IL-1β was not immediate, a 4h timeframe may be considered beneficial for a strong enough acute inflammatory response, allowing an influx of immune cells to aid in the removal of viruses, but also in the healing response of the corneal epithelial layer repair.

Furthermore, IL-1β has been implicated in the 'cytokine hierarchy' of ocular disease, with the cytokine playing a vital role during the acute inflammatory response by inducing the expression of other pro-inflammatory mediators, such as chemokine IL-8 (Da Cunha *et al.,* 2018). Da Cunha *et al.,* (2018) performed an *in vivo* study in which adeno-associated virus vectors expressing a range of pro-inflammatory cytokines including IL-1β and IL-6 were delivered to the ocular tissue of C57BL/6 mice. A range of ocular cell processes were then analysed, including cellular infiltration of ocular macrophages, which can result in corneal tissue damage. Worryingly, only a 'marginal' increase of IL-1β expression was required for immune cell infiltration, cytokine production and corneal clouding, leading the authors to conclude that this pro-inflammatory cytokine had a 'highly pathogenic nature' within ocular inflammation and was responsible for many corneal damaging events.

Considering the impact of 1,25D3 treatment upon IL-1β expression, it would be interesting to explore these novel data further. This would show the true impact of 1,25D3 treatment upon TLR3 driven inflammation – is the 1,25D3 suppressive effect significantly reliant on IL-1β suppression influencing other pro-inflammatory mediators by 6h, or is the action of 1,25D3 dependent and purely coincidental that IL-1β is inhibited first? To address this concept, it would interesting to inhibit hTCEpi IL-1β using post-transcriptional silencing by RNA interference (RNAi), similar to the study performed by Ortved *et al*., (2016), who showed silencing the cytokine led to a significant decrease within the expression of other mediators such as TNF-α. This would show the true impact of 1,25D3 treatment upon the expression of the pro-inflammatory mediators – is the 1,25D3 suppressive effect significantly reliant on IL-1β, or is the action of 1,25D3 dependent and purely coincidental that IL-1β is inhibited first?

A further possible theory behind the suppression of IL-1β is that poly(I:C) stimulation of TLR3 leads to IL-1Ra production. IL-1Ra is an anti-inflammatory protein which aids in controlling overt inflammatory responses by competitively binding to IL-1R, a receptor which is expressed by cells of the cornea, is crucial for corneal defense and readily binds to IL-1β (Kennedy *et al.,* 1995, Liu *et al.,* 2020e, Reins *et al.,* 2017b). During the experiments, 1,25D3 could influence the production of IL-1Ra during corneal TLR3 stimulation, with studies such as that of Kong *et al.,* (2006) showing 1,25D3 influencing the expression of IL-1Ra in other human cells, such as skin. Investigating the expression and production of IL-1Ra in future studies in response to the same treatments would confirm this, as this receptor is also implicated in corneal inflammation and driven by IL-1β binding, and may play a significant role during 1,25D3 suppressing TLR3-driven inflammation in the ocular surface.

Similar to IL-1β expression, 1,25D3 significantly suppressed IL-12 expression by hTCEpi after 24h (Figure 3.4). However, following TLR3 activation by poly(I:C), there was no detectable induction of IL-12 expression by 6h, indicating that hTCEpi may induce IL-12 later within the inflammatory response, compared to other pro-inflammatory mediators such as IL-6 and IL-8. Intriguingly, TLR3 activation by poly(I:C) led to no detectable induction in IL-12 expression after 6h, therefore, no suppressive action of 1,25D3 was seen during this timeframe (Figure 3.5C). These data indicated that corneal cells may produce IL-12 later during the acute inflammatory response, in comparison to other pro-inflammatory mediators such as IL-8 and IL-6. This may provide evidence of a potential time delay between the activation of the innate and adaptive immune systems, allowing innate immune cells such as neutrophils to infiltrate the cornea first at 6h. However, further studies would be required to confirm this, beginning with quantification of IL-12 following 1,25D3 treatment to confirm if this increase is biologically relevant. Migration assays using cells such as ocular macrophages would confirm the effects of this IL-12 suppression, with an expectation that cellular migration would correlate with IL-12 suppression.

Interestingly, as IL-12 is closely related to other pro-inflammatory cytokines which are implicated throughout ocular inflammation, this confirms the need to once again broaden the mediators effected by 1,25D3 treatment and which would be strong candidates for *in vivo* analysis. For example, observing IL-10 expression and subsequent production would be beneficial, as this anti-inflammatory protein is not only implicated in suppressing TLR3-driven corneal inflammation in ocular conditions such as HSV-1 (Yan *et al.,* 2001), but may also inhibit IL-12 production through TGF-β activity (Du *et al.,* 1998). Finally, IP-10 and IFN-β are both implicated during TLR3 driven inflammatory conditions of the ocular surface, and both significantly aid in the ocular responses which relate to corneal damage (Ueta *et al.,* 2005).

When studying an intracellular receptor such as TLR3, each of these ligands must enter within the cell to achieve a stimulatory or suppressive effect. Although hTCEpi cells were treated with 10^{-7}M of 125D3, which equates to a working solution of 100nM/ml, and 2ug/ml of poly(I:C), one obvious limitation within this study is the failure to confirm the exact intracellular concentrations of both poly(I:C) and 1,25D3 and subsequent bioavailability. It is important to know the exact dosage required for these 1,25D3 suppressive effects to extend these studies to *in-vivo* models, such as mice, and limit potential toxicity complications. In future replications of the study, liquid chromatography mass spectrophotometry can be used to quantify the intracellular concentration of 1,25D3 within hTCEpi by testing for the concentration of 1,25D3 within the remaining supernatant (Zelzer *et al.,* 2020). It would be expected that the remaining concentration of 1,25D3 would be barely detectable if all hormone was absorbed into the cell. It is also possible to detect the volume of 1,25D3 within the cell by quantifying the fluorescent intensity of bound VDBP, which Kim *et al.,* (2012) demonstrated within RLE-6TN cells, a rat alveolar epithelial cell line.

3.3.4 1,25D3 increases pro-inflammatory COX-2 expression by hTCEpi

Results in Figure 3.4A showed that, unlike IL-1β and IL-10, the dual treatment of poly(I:C) and 1,25D3 led to a significant increase within COX-2 expression. This result is of particular importance, as it highlights that 1,25D3 treatment following TLR3 activation may be a ‘double-edged sword’, and not completely suppressive within the resolution period of inflammation as it exuberates the

expression of pro-inflammatory mediators. Increased COX-2 expression is associated with an upregulation of TLR3, modulating immune responses towards infections usually detected by the receptor (Randall *et al.,* 2020). However, this thesis presents data that shows an alternative result, with 1,25D3 treatment significantly suppressing TLR3, but significantly increasing COX-2.

An increase of COX-2 expression within the resolution period of inflammation could be detrimental, as the enzyme is characterised within the acute inflammatory stage of ocular inflammation in conditions such as DED, alongside being responsible for the formation of a range of prostanoids, including prostaglandins, which have the ability to positively regulate pro-inflammatory cytokines including IL-6 (Chen *et al.,* 2000c, Attiq *et al.,* 2018, Tu *et al.,* 2002, Seo *et al.,* 2014). Following the IL-1β discussion, it is interesting that Xi *et al.,* (2011) demonstrated that IL-1β actually has the ability to suppress cyclooxygenase-2 (COX-2) expression in the cornea. This would be an interesting idea to pursue further within future experiments – could the increase in COX-2 expression seen in the discussed data be influenced by the significant suppression of IL-1β following 1,25D3 treatment, or again, is 1,25D3 single – handedly responsible for this increase? To confirm this, inhibiting hTCEpi IL-1β production, using a method such as the previously discussed RNAi, would aid in identifying a potential a relationship between both the IL-1β and COX-2 in regard to the immunomodulatory effect of 1,25D3.

However, the increase in COX-2 expression following 1,25D3 treatment may be considered as advantageous, with studies showing that COX-2 is beneficial for both corneal wound healing and acute inflammatory responses in the form of cytokines, growth factors and adhesion molecules (Amico *et al.,* 2004, Wilson *et al.,* 2001). Reins *et al.,* (2016c) showed topical 1,25D3 treatment in murine corneal wound models significantly increased neutrophil infiltration into the cornea, but delayed wound healing time by 17% and no increase in vascular endothelial growth factor (VEGF), with the immunosuppressive action of 1,25D3 implicated as the cause. However, that study investigated whole corneal tissue as opposed to epithelial cells alone. The increase of COX-2 from epithelial cells close to the wound following

1,25D3 treatment may increase signaling protein VEGF and anti-inflammatory protein lacritin, both of which are utilized during wound healing with evidence of regulation by COX-2 (Vantaku *et al.,* 2015, Yanni *et al.,* 2010). Liu *et al.,* (2017a) suggested that COX-2 significantly encourages angiogenesis of pterygium – a tissue growth which impairs vision by growth over the cornea whilst encouraging inflammatory damage. The cornea is designated as 'angiogenesis privileged', meaning an influx of VEGF from COX-2 upregulation may lead to a loss of homeostasis and significant corneal neovascularization, losing transparency (Azar 2006). Therefore, it would be useful to analyze the expression of VEG-F produced by these cells following the dual treatment of poly(I:C) and 1,25D3 using techniques such as RT-PCR and ELISA.

The COX-2 results, similar to the various datas regarding IL-1β, IL-10 and IL-8, only demonstrate mRNA expression and subsequent protein expression need to be quantified by means such as enzyme-linked immunosorbent assay (ELISA). This would allow final confirmation regarding the effect of 1,25D3 upon COX-2 expression, deciding if this increase would be biologically relevant. Finally, COX-2 is also associated with immune cell migration during a range of diseases (Guo Z *et al.,* 2015, Lee *et al.,* 2007). To our knowledge, it is currently unknown if COX-2 contributes to the migration of ocular immune cells during TLR3 signaling. Therefore, a scratch assay would be valuable to confirm the effects of this potential increase in COX-2 production upon the wider ocular cell community, as it would be especially significant if COX-2 increased immune cell migration, not only in regard to potential pro-inflammatory damage, but also assessing the potential of this migration upon wound healing of the ocular surface.

3.3.4 1,25D3 suppression of TLR3 signaling does not affect cell viability

The results showed 0.5μg/ml of poly(I:C) did not lead to a significant decline in hTCEpi viability, nor did 10^{-7}M of 1,25D3 during 24h (Figure 3.3, A-C) however, suppression of pro-inflammatory mediators by 1,25D3 was seen throughout the experiments using these concentrations of agonist. These

results indicated that 1,25D3 has a positive immunomodulatory effect, without negatively impacting corneal cell viability.

Harashima *et al.,* (2014) showed that poly(I:C) exposure led to a significant increase TLR3 expression, but also a significant increase in cell death. This observation was supported by Palchetti *et al.,* (2015) who demonstrated an increase of apoptosis in both healthy and dysfunctional cells via the TLR3/Src/STAT1 pathway. Richards *et al.,* (2015) demonstrated 1,25D3 treatment increased cellular apoptosis. Although the process of apoptosis is beneficial to curtail the rate of pathogenic spread by reducing the number of possible number of host cells, consistent cell viability was crucial in these experiments to obtain the highest level of VDR expression and subsequently, an accurate representation of 1,25D3 activity through mRNA expression. Confirming cell viability ensures that the suppression of inflammatory mediator expression following 1,25D3 treatment results from an immunomodulatory reaction, opposed to a decline in the cell population.

3.3.5 miR-155-5p and miR-146a-5p play no direct role within the expression of various pro-inflammatory mediators following TLR3 stimulation, or the suppressive 1,25D3 mechanism, at 24h

The results presented in this thesis showed that neither miR-155-5p or miR-146a-5p were active during 24h of TLR3 activation by poly(I:C) (Figures 3.7B and 3.11). These findings could be influenced by the limitation of timeframes rather than the lack of miR activity – only a 24h window was examined in these experiments, as the focus of the study was observing the effect of 1,25D3 on pro-inflammatory mediators within the resolution period of TLR3 driven inflammation. After 24h, there may have been a decline in miR expression and subsequent activity, therefore, miR would undetectable in these experiments. In the future, smaller time frames mimicking the acute inflammatory response period, such as 4h-6h could be used to detect the possible role of these miR during this response. The reasoning behind this theory is based upon the previous data generated by studies investigating miR-155 during corneal inflammation. Indeed, miR-155 has been shown to be crucial for processes such as corneal wound healing, which links to a reduction of corneal epithelium permeability through the remodeling of tight junctions (Bhela *et al.,* 2015, Wang F *et al.,* 2020). Furthermore, miR-155

inhibition is linked to a significant decrease in macrophage TLR3 expression, reflected by a suppression of immune responses, suggesting that miR-155 negatively regulates TLR3 responses (Hu *et al.,* 2015).

Similar results were obtained in regard to miR-146a-5p expression following TLR3 stimulation (Figure 3.7D). Although this miR is relatively unexplored it is implicated during corneal inflammation (discussed throughout subsection 1.6). Fei *et al.,* (2020) demonstrated miR-146a-5p regulating TLR3 signaling, with an increase of miR-146a-5p leading to an inhibition of NF-κB translocation and subsequent down-regulation of pro-inflammatory cytokine production. Considering the idea that miR-146a-5p is crucial for TLR3 regulation, it would be expected that TLR3 stimulation would lead to an increase of miR-146a-5p expression. However, no significant change in expression miR-146a-5p was detected under any condition after 24h.

As discussed throughout subsection 1.6.2, miR-146a works in close association with miR-155, an miR which is found to give pro-inflammatory effects, with miR-146a capable of 'switching off' the miR-155 signaling cascade during NF-κB signaling (Testa *et al.,* 2017). Therefore, it would be beneficial to inhibit both of these miR at the same time and focus upon the acute inflammatory period, to identify a novel target to lead to significant suppression within TLR3 driven inflammatory responses, such as HSV-1 infections of the cornea.

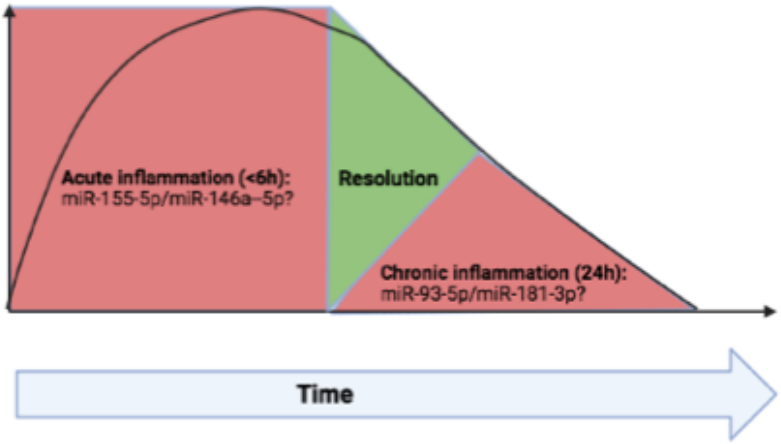

Figure 3.11: The assumed role of the analysed miR during the TLR3 driven inflammatory response: during the acute inflammatory response within 6h, miR-155-5p and miR-146a-5p may be active and influencing the expression of various pro-inflammatory mediators. By 24h, the time period examined within this study and the expected 'chronic inflammatory period', miR-93-5p and miR-181-3p appear to be active and negatively regulating hTCEpi IL-6, IL-8 and IL-1β expression. This should be confirmed by luciferase assays during future studies.

3.3.6 Both miR-93-5p and miR-181-3p may play a role within the expression of various pro-inflammatory mediators following 24h of TLR3 stimulation, but not during the suppressive 1,25D3 mechanism

As there was a significant increase in the expression of miR-93-5p and miR-181-3p of hTCEpi following 24h of TLR3 stimulation using poly(I:C) (Figure 3.7, C and E), this highlights a possible function for both of these miR during TLR3 activation and subsequent IL-8, IL-6 and IL-1β expression. It was hypothesized that there would be a non-suppressive effect from 1,25D3 if either inhibited miR-93-5p or miR-181-3p had a role in the mechanism of action by 1,25D3, which would be reflected in an influx of pro-inflammatory mediators during the dual poly(I:C) and 1,25D3 treatment.

Inhibiting both miR-93-5p and miR-181-3p of hTCEpi led to a significant increase in IL-6, IL-8 and IL-1β mRNA expression following TLR3 activation in comparison to the uninhibited hTCEpi cells (Figure 3.9, A – C and Figure 3.10, A-C), providing novel evidence that these miR negatively regulate pro-inflammatory expression during TLR3 activation of hTCEpi. To our knowledge, this is the first data to provide evidence of this relationship within hTCEpi cells and identifies an interesting novel target. Fabbri *et al.,* (2016)

has characterised miR-93 during the regulation of IL-8 production by neuroblastoma cells, describing IL-8 as 'major molecular target' of miR-93, which targets the 3'UTR of the gene (Fabbri *et al.,* 2016). In regard to IL-6 and the ocular surface, Xu *et al*., (2014) demonstrated the relationship between miR-93 activity and uveitis symptom severity following LPS stimulation, with the miR inhibiting the NF-KB pathway (Xu *et al.,* 2014). Although the experiments mentioned within the Xu *et al.,* (2014) study were conducted using 264.7 monocyte cells, the evidence of miR-93 influencing the NF-KB pathway is of particular importance due to the usage of this pathway during TLR3 signaling, and may support the data within this thesis. In regard to miR-181-3p, previous studies have confirmed that miR-181 targets the binding site of 3'UTR of IL-8 and IL-6 in a range of cells during TLR driven inflammation (Galicia *et al.* 2014), furthermore, in response to viruses (Guo *et al.,* 2013). As this *in vitro* hTCEpi model mimics dsRNA stimulation of TLR3, this is the first evidence that miR-93-5p and miR-181-3p are negatively regulating IL-6, IL-8 and IL-1β during corneal inflammation in response to dsRNA.

There was, however, no change seen within the suppressive action of 1,25D3 following inhibition of these miR, as expression of IL-6, IL-8 and IL-1β still significantly declined by 24h. Interestingly, there is also evidence that miR-181-3p can be regulated by 1,25D3 (Wang *et al.* 2009g). Results of the 2009 study showed 1,25D3 supplementation in acute myeloid leukemia cells led to a decrease in the expression of miR-181, indicating that 1,25D3 has a potential suppressive effect upon miR expression (Wang *et al.,* 2009g). Therefore, it could be assumed that 1,25D3 influences miR-181-3p activity, as opposed to miR-181-3p playing a role during the mechanism of action behind 1,25D3 suppression. This would support the decline in miR-181-3p expression during the suppressive action of 1,25D3, but not the complete abolishment of pro-inflammatory expression during the dual condition treatment.

Although these results indicated that miR-93-5p and miR-181-3p negatively regulate pro-inflammatory mediators, this must be further confirmed by molecular studies, such as a luciferase assay. The luciferase assay would allow quantification of not only the strength, but activity of the promoter region

of the gene by quantifying the level of bioluminescence produced from luciferase activity during luciferin catalyzed into light. This would not only give confirmation of 3'UTR targeting by both miR-93-5p and miR-181-3p in IL-8, IL-6 and IL-1β of hTCEpi, but also demonstrate functionality. More importantly, replicating this experiment and inhibiting both of these miR at the same time would identify a very important relationship of both miR-93-5p and miR-181-3p working in conjunction during the pro-inflammatory response behind TLR3 activation. Furthermore, this would present a very interesting novel target which could be inhibited to prevent overt damage to the cornea. In future studies, it would be beneficial to identify the placement of the miR inhibitors within the hTCEpi cells, as the inhibitor used throughout this thesis is 5' FAM labelled, meaning it is easily identifiable in hTCEpi cells when staining with a nucleic dye, such as DAPI. Obtaining microscopic evidence of the miR-inhibitors would support the inhibitory pro-inflammatory data, confirming inhibitor functionality.

3.3.7 Chapter conclusions

Stimulating TLR3 using poly(I:C), a robust model of dsRNA material associated with pathogenic infections such as HSV-1, led to a significant increase in a range of pro-inflammatory mediators. The study gave clear evidence of 1,25D3 suppressing these pro-inflammatory mediators during both the acute and resolution period of inflammation following TLR3 activation by dsRNA, including novel data of IL-1β and IL-10. Therefore, an assumption could be made that each of these pro-inflammatory mediators' genes express VDRE, which is interacting with the 1,25D3 treatment and leading to the suppression in expression seen by 24h (Figure 3.12). Published research to confirm that these pro-inflammatory mediators express VDRE, including TLR3, is limited. Moving forward, VDRE confirmation by DNA micro-array and high thorough put screening, similar to the work of Lisse *et al.*, (2011) using B-cells, which would confirm expression for a large population of pro-inflammatory genes.

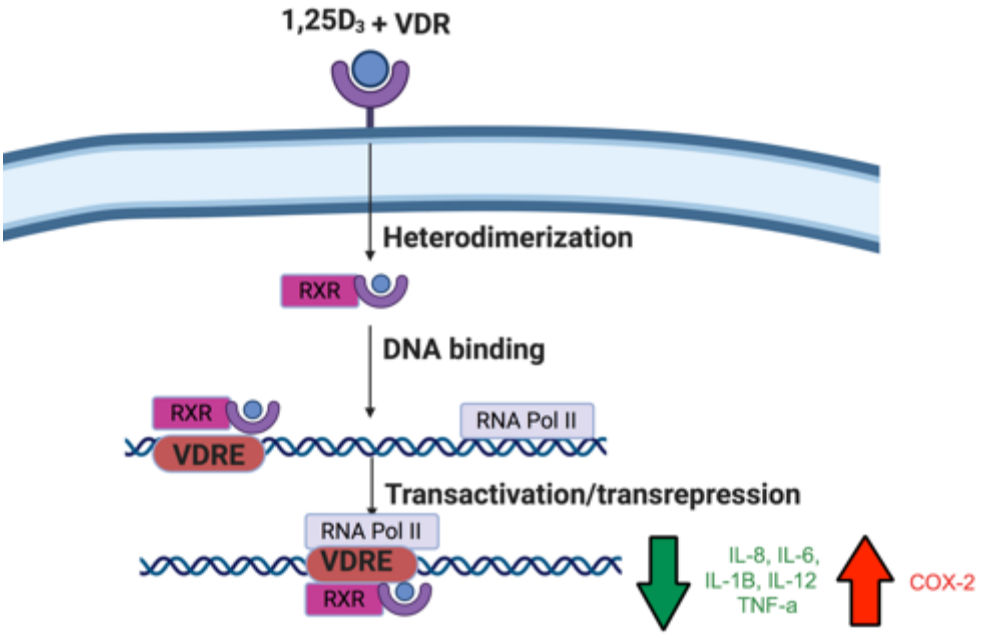

Figure 3.12: The predicted mechanism of action for 1,25D3 treatment following poly(I:C) stimulation of TLR3. TLR3 activation by poly(I:C) leads to activation of the NF-KB pathway, and an accumulation of pro-inflammatory mediators. When 1,25D3 is exposed to the cell, the VDRE of each gene is modulated, which is reflected in a downregulation in the expression of IL-8, IL-6, IL-12, TNF-α and IL-1β, with an upregulation of COX-2 expression.

As discussed, inhibiting miR-155-5p or miR-146a-5p led to no significant effect upon 1,25D3 suppressing TLR3 signaling or TLR3 activation within 24h. Previous *in vivo* studies showed miR-155-KO models left mice susceptible to developing ocular lesions associated with herpes simplex encephalitis (HSE) (Bhela *et al.,* 2014). Data presented in this thesis demonstrated no miR-155 increased expression during 1,25D3 suppression of TLR3 signaling. Furthermore, 24h of 1,25D3 treatment alone did not increase the expression of miR-155-5p. Such an increase may have been advantageous physiologically, as, for example, Wang *et al.,* (2020a) concluded that miR-155 may 'reverse' the breakdown of corneal epithelial cells in response to inflammatory damage.

Inhibiting miR-93-5p and miR-181-3p led to no change during the suppressive mechanism of 1,25D3. These results suggest novel findings that these miR negatively regulate expression of hTCEpi IL-6, IL-8 and IL-1β. Furthermore, it appears that these miR work in conjunction, as inhibiting both miR separately significantly increased the expression of each pro-inflammatory cytokine.

Moving forward, it would be beneficial to confirm if there miR also had a role during the upregulation of COX-2, as recent research has identified that miR-93 actually has the potential to indirectly modify COX-2 expression during glioblastoma following down-regulation of IL-6, IL-8 and IL-1β (Hubner *et al.,* 2020).

Chapter 4

Vitamin D and Human Corneal Epithelial Cells: analyzing potential mechanisms behind anti-inflammatory effects during TLR9 signaling

4.1 Introduction to chapter 4

4.1.1 The Ocular surface, TLR9 and cGAS

DNA sensing pathways are crucial for identifying DNA from microbes which are able to breach the ocular surface, preventing further infectious diseases such as HSV, which can lead to herpetic keratitis and subsequent blindness (Takeda *et al.,* 2011). An example of a DNA sensing tool is TLR9, an intracellular receptor which recognizes both single stranded (ssDNA) and double stranded (dsDNA) DNA, and has been described as a 'crucial component' for corneal immunity (Hayashi *et al.,* 2009, Reins *et al.,* 2017b). Redfern *et al.,* (2015) characterised decreased TLR9 expression within the chronic inflammatory damage associated with DED, demonstrating the importance of TLR9 when detecting microbial DNA abundant with unmethylated-CpG dinucleotides. However, Parthasarathy *et al*., (2018) showed that overt TLR9 signaling led to increased pro-inflammatory responses within dengue virus infection (DENV), which manifests as ocular inflammation by subconjunctival hemorrhage. Furthermore, TLR9 signaling was implicated during the formation of keratic precipitates within the corneal surface and stroma, causing chronic inflammation by further recruitment of macrophages (Chinnery *et al.,* 2015). Therefore, silencing or suppressing of TLR9 may be a potential therapeutic to alleviate hyper-inflammatory tissue damage associated with DNA viruses. For example, Huang *et al.,* (2005c) demonstrated siRNA silencing of TLR9 in mice infected with *Pseudomonas aeruginosa,* suppressed corneal inflammation and subsequent damage.

The cGAS/STING pathway is also activated by dsDNA (discussed throughout subsection 1.3.5), with Wu *et al.,* (2019) implicating a potential role of the pathway during senescence-related AMD inflammation and pathogenesis. Numerous studies have elucidated that type I IFN stimulated from STING is a crucial defence against viral infections caused by dsDNA, inducing an ant-viral response, inhibiting viral replication (Reinert *et al.,* 2016, Aroh *et al.,* 2017). Furthermore, Chen *et al.,* (2018) showed that this pathway actually hinders corneal inflammation in response to *Pseudomonas aeruginosa,* by suppressing inflammatory cytokine responses through NF-κB inhibition. However, the cGAS/STING pathway is relatively unexplored in regard to

ocular surface inflammation, and identifying any potential activity may identify a potential therapeutic target for future studies.

4.1.2 Vitamin D and TLR9

Carvalho *et al*., (2017) demonstrated that vitamin D treatment of dialysis patients led to a significant decrease of lymphocyte TLR9 signaling. However, the study did not state influencing factors such as age and failed to distinguish which vitamin D metabolite was active during the analysis and therefore responsible for the suppressive effects. Nevertheless, *in vitro* studies have supported this theory, for example, one monocyte study demonstrated significant TLR9 and pro-inflammatory IL-6 suppression following 1,25D3 treatment (Dickie *et al.,* 2010), with Ojaimi *et al.,* (2013) showing similar data in monocytes stimulated with unmethylated CpG DNA for 24 hours and then exposed to 1,25D3. Currently, research available into the effects of 1,25D3 upon TLR9 signaling of the ocular surface is limited, however, this area is significant as TLR9 driven inflammation could lead unnecessary ocular surface damage, with pathway suppression preventing this.

4.1.3 miRNA and TLR9 during inflammation

Although research regarding the relationship between miR and TLR9 is limited, the available studies offer opposing arguments. For example, Jayamani *et al.,* (2014) used intestinal epithelial cells to demonstrate no significant change within miR-146 expression following *E. coli* activation of TLR9. However, TLR9 activation of pDC in response to microbial associated nucleic acid upregulated miR-146a expression. This correlated with an increase of pro-inflammatory cytokine production and an up-regulation of co-stimulatory markers, exacerbating inflammation (Karrich *et al.,* 2013). TLR9-associated miR-146a upregulation has also been found to promote CD11b – a β2 integrin which plays a crucial role in inflammation and immune responses, as it is involved in cell activation, cytotoxicity and phagocytosis, exacerbating inflammatory damage (Bai *et al.,* 2012). These findings indicated a potential regulatory role for miR-146a during TLR9 signaling of immune cells, which could be novel in suppressing inflammation. As previously discussed throughout subsection 1.6.2, miR-146a negatively

regulates IRAK1, suppressing NF-KB signaling and subsequent pro-inflammatory mediators (Karrich *et al*., 2013). Although TLR9 activation leads to NF-KB activation, if 1,25D3 influenced the expression of miR-146a, it could be assumed that miR-146a is an example of a miR that is exploited to implicate this suppressive effect. Finally, the absence of miR-155 increases suceptibility towards viral infection in various cells, highlighting the importance of this miR within viral immunity (Rodriguez *et al.,* 2007, O'Connell *et al.,* 2010).

4.1.4 Aims and Objectives

It is important to investigate the potential suppressive effects of 1,25D3 treatment on corneal TLR9 signaling, as both bacteria and viruses are capable of inducing corneal chronic inflammation from TLR9 activation. Viral infections are reported to account for up to 75% of conjunctivitis cases, which manifests as symptoms such as redness, pain and ocular discharge (Keen *et al.,* 2017, Udeh *et al.,* 2008). In subsection 3.2.1, this thesis already confirmed hTCEpi TLR9 expression, therefore, the aim of the experiments in this chapter were to demonstrate that TLR9 was fully functioning; reacting to both ssDNA and dsDNA sources, subsequently producing a range of pro-inflammatory mediators. Throughout the experiments of this thesis, the CpG-ODN detected from various ODN sources will mimic viral, ssDNA, which has been shown to be an efficient model in further studies (Jorgensen *et al.,* 2003). It was appropriate to repeat the same experiments using a source of dsDNA, as numerous viruses in this form are able to infect the eye, presenting as conditions including, but not limited to, conjunctivitis, uveitis and keratitis. Additionally, analysis will confirm that hTCEpi has a functioning cGAS receptor, which, in turn, aids in the inflammatory response and can be suppressed by 1,25D3. Finally, it is hypothesized that 24h 1,25D3 treatment of TLR9 signaling will suppress the expression of these mediators, with either miR-146a-5p, miR-155-5p, miR-93-5p or miR-181-3p involved during this suppressive mechanism.

4.2 Results

4.2.1 hTCEpi expresses fully functioning TLR9 for ssDNA detection, with subsequent induction of pro-inflammatory cytokine expression

As the study described in subsection 3.2.1 previously confirmed unstimulated hTCEpi cells express TLR9, the next experimental aim was to identify if TLR9 was fully functional and verify which CpG oligonucleotide (ODN) subclass would induce the most robust inflammatory response, as each subclass has varying levels of CpG motifs for TLR9 detection. A class B ODN, ODN2006, was used as a ligand for TLR9, with a full phosphorothioate backbone and CpG dinucleotides. ODN2006 has been shown to stimulate TLR9 in a range of cells, including human embryonic kidney cells and lung epithelial cells, inducing a powerful inflammatory response, with significant induction of the NF-κB pathway, with similar data presented throughout this thesis in the form of IL-6, IL-8 and IL-1β (Bauer *et al.,* 2001, Platz *et al.,* 2004, Hanagata 2012). Class A CpG-ODNs are characterised by the traditional phosphodiester (PO) backbone, which occurs naturally within genomic bacterial DNA, an example of which is ODN2336. This subtype of CpG-ODN has been shown to induce IFN-α production by pDC, however, Volpi *et al.,* (2013) showed ODN2336 is a weak inducer of TLR9-dependent NF-κB and subsequently led to little induction of pro-inflammatory mediators. Class C CpG-ODN are a combination of both A and B classes – they contain a complete, natural PO backbone, with increased expression of CpG-ODN motifs (Martinson *et al.,* 2007).

Three different concentrations of ODN2006 (Invivogen, San Diego, USA); 1μg/ml, 2μg/ml and 4μg/ml were used for 24h stimulation of hTCEpi TLR9, with mRNA expression of TLR9 and IL-8 analysed (Figure 4.1, A-B). The results showed 2μg/ml and 4μ/ml of ODN2006 led to significant induction of both IL-8 and TLR9 hTCEpi expression ($p<0.001$). A weaker induction of expression was seen from 1μg/ml ($p<0.01$). The results showed that 2μg/ml ODN2006 exposure for 24h led to the highest induction of IL-8 and TLR9 mRNA expression, therefore this was chosen as the optimum concentration for future ssDNA experiments.

A class C CpG ODN, ODN2395 (Invivogen, San Diego, USA), was used to repeat these experiments, allowing comparison of TLR9 and IL-8 expression using two different forms of ssDNA. The results showed that, in a similar fashion 24h of ODN2395 at 2μg/ml and 4μg/ml induced a significant increase in the expression of TLR9 and IL-8 (Figure 4.1, C-D, $p<0.001$) with a weaker effect seen from 1μg/ml ($p<0.01$). Although 24h of 2μg/ml ODN2395 induced the highest expression of TLR-9 and IL-8, the highest increase of expression overall was obtained TLR9 stimulation with ODN2006. In a potential indication of toxicity, 4μg/ml of ODN2395 produced a lower increase of expression in comparison to 2μg/ml, which may be due to changes in cell viability.

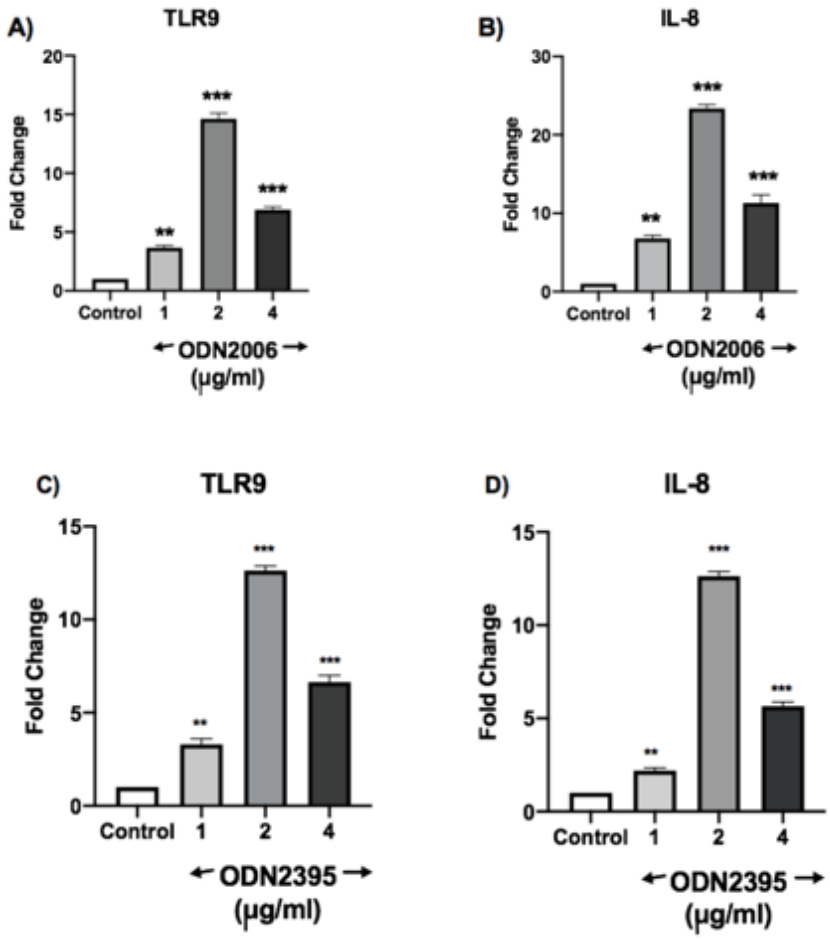

Figure 4.1: Inducing a pro-inflammatory response by TLR9 of hTCEpi with alternate subclasses of ODN: Expression of (A) TLR9 and (B) IL-8 by hTCEpi after exposure to various concentrations of TLR9 agonist ODN2006, alongside expression of (C) TLR9 and (D) IL-8 following exposure to various concentrations of ODN2395. Data represent mean ± SEM of three independent experiments. Statistical analysis was by one-way ANOVA with Bonferroni test for multiple comparisons (A-D, n=3) p<*0.05, **0.01, ***0.001

4.2.2 1,25D3 suppresses IL-8 expression associated with ongoing TLR9 signaling following ssDNA stimulation

As 2μg/ml of ODN2006 was identified as the optimum concentration to induce a pro-inflammatory response through a significant increase in IL-8 expression (Figure 4.1B), the aim of the next experiments was to analyze the expression of wider range of pro-inflammatory mediators and if 1,25D3 modulates expression of TLR9 signaling. Therefore, hTCEpi cells were stimulated using various conditions of agonist ODN2006 and 10^{-7}M 1,25D3, with the mRNA expression of IL-6, IL-1β, IFN-β and IL-8 analysed (Figure 4.2, A-E). The 1,25D3 condition induced an insignificant increase in IL-6, IL-8 and IL-1β expression. However, 24h of ODN2006 stimulation significantly increased the expression of IL-6, IL-8, IL-1β and TLR9 (A-B, $p<0.001$) (C, $p<0.001$) (E, $p<0.001$). Proving the hypothesis, there was a significant decrease seen in: IL-6 (A, $p<0.001$), IL-8 (B, $p<0.001$), IL-1β (C, $p<0.05$) and TLR9 (E, $p<0.001$) during the dual treatment of ODN2006 and 1,25D3.

However, 24h of ODN2006 exposure did not lead to a detectable increase of IFN-β mRNA expression, with 1,25D3 treatment not effecting expression data (D). These results indicated that 1,25D3 treatment had a suppressive effect upon IL-6, IL-8, IL-1β and TLR9 following 24h of hTCEpi TLR9 stimulation using ODN2006.

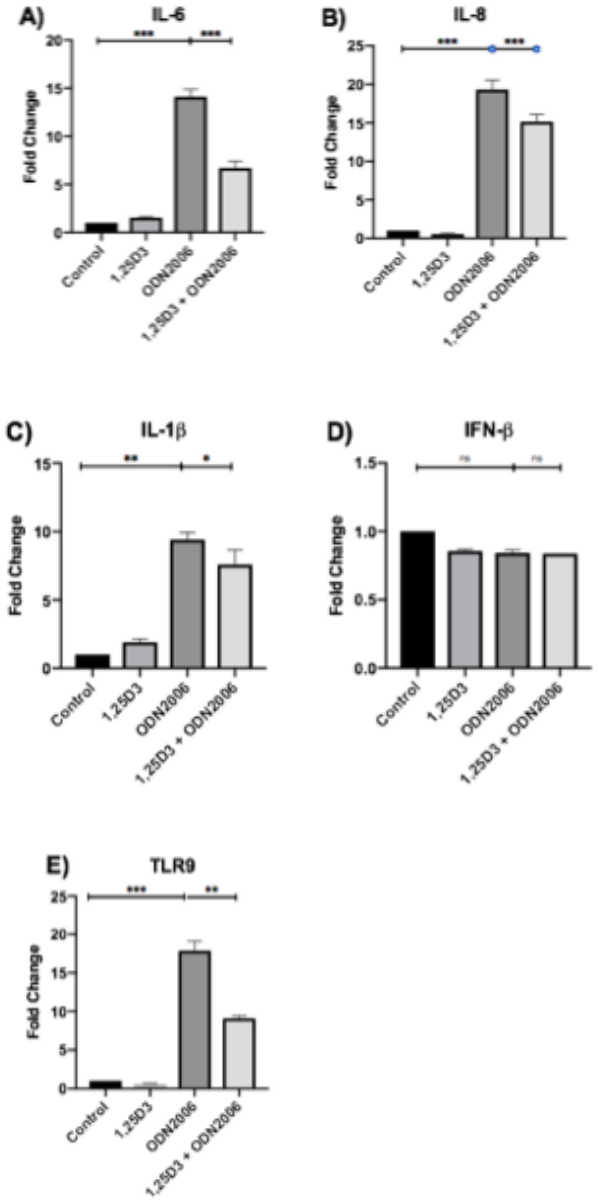

Figure 4.2: Analysing the effect of various ODN2006 and 1,25D3 conditions upon hTCEpi pro-inflammatory mediator expression: hTCEpi pro-inflammatory mediator mRNA expression after exposure to 2μg/ml ODN2006 TLR9 agonist and 10^{-7}M 1,25D3. (A) IL-6 (B) IL-8 (C) IL-1β (D) IFN-β (E) TLR9. Data represent mean ± SEM of three independent experiments. Statistical analysis was by one-way ANOVA with Bonferroni test for multiple comparisons (A- E, n=3), p<*0.05, **0.01, ***0.001

4.2.3 1,25D3 suppresses pro-inflammatory mediators associated with TLR9 signaling following prior ssDNA stimulation

The previous experiments confirmed that 10^{-7}M 1,25D3 significantly suppressed TLR9 signaling, observed by a decrease in genes encoding the pro-inflammatory mediators IL-6, IL-8 and IL-1β (Figure 4.2, A-C). Therefore, the aim of this experiment was to analyze the effect of 1,25D3 treatment upon hTCEpi cells undergoing prior TLR9 stimulation. This models bacterial and viral infections of the eye, which would commonly occur before 1,25D3 treatment. The hTCEpi cells were stimulated for 24h with 2μg/ml ODN2006, then exposed to 10^{-7}M 1,25D3 for 4 hours, in the appropriate dual treatment wells (Figure 4.3, A-E).

The results showed that, treating hTCEpi cells that received prior 24h ODN2006 treatment with 10^{-7}M 1,25D3, led to a significant decline in the expression of IL-6, IL-8, IL-1β and TLR9 (A-C and E, $p<0.001$), in comparison to 24h of ODN2006 exposure alone. Furthermore, 24h of ODN2006 alone led to a significant increase in expression in IL-6, IL-8, TLR9 (A, B and E, $p<0.001$) and IL-1β ($p<0.01$). However, similar to the results seen in subsection 4.2.2, each condition failed to induce a detectable, significant change of IFN-β expression after 24h of ODN2006 treatment.

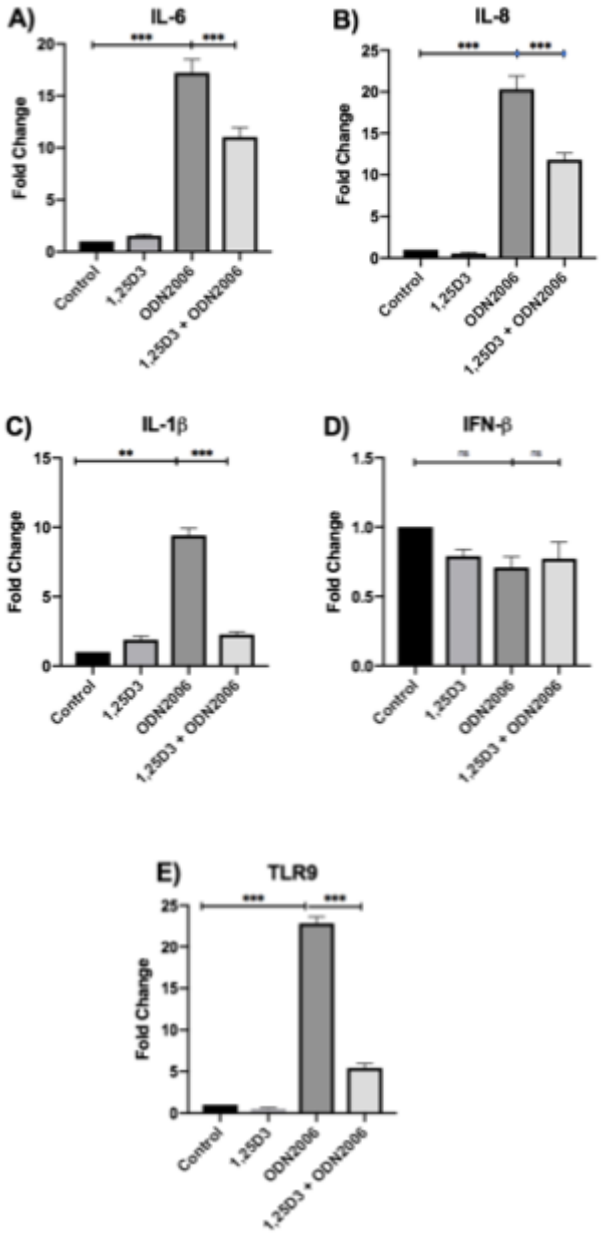

Figure 4.3: Analysing the effect of prior ODN2006 hTCEpi stimulation followed by 4h of 1,25D3 treatment: pro-inflammatory mediator mRNA expression by hTCEpi after exposure to 2μg/ml ODN2006 TLR9 agonist for 24h, then 4h of 10^{-7}M 1,25D3 treatment. (A) IL-6 (B) IL-8 (C) IL-1β (D) IFN-β (E) TLR9. Data represent mean ± SEM of three independent experiments. Statistical analysis was by way one-way ANOVA with Bonferroni test for multiple comparisons (A – E, n=3), p<*0.05, **0.01, ***0.001

4.2.4 The suppressive mechanism of 1,25D3 upon TLR9 signaling does not lead to a significant decline by hTCEpi viability

The previous results from subsection 4.2.1 showed that both 2μg/ml of ODN2006 and 2μg/ml of ODN2395 at 24h induced the highest pro-inflammatory response by hTCEpi in the form of IL-8 expression. Furthermore, 10^{-7}M 1,25D3 treatment significantly suppressed a range of pro-inflammatory mediators, therefore the aim of these experiments was to ensure that gene suppression was occurring, and not 1,25D3 treatment leading to cell death and a lower cell population, which would be reflected in a weaker induction of the pro-inflammatory mediators.

The results showed that both 2μg/ml of both ODN2006 and ODN2395 for 24h during each experiment did significantly lower the viability of hTCEpi cells in comparison to the unstimulated cells (Figure 4.4, A-B, $p<0.05$) which may have contributed to an inflammatory environment from agonist toxicity and subsequent dying cells. Increasing the concentration of both ODN2006 and ODN2396 decreased cell viability significantly, seen at 4μg/ml for both agonist (A and B, $p<0.01$). However, 1,25D3 24h treatment did not significantly affect cell viability (A and B).

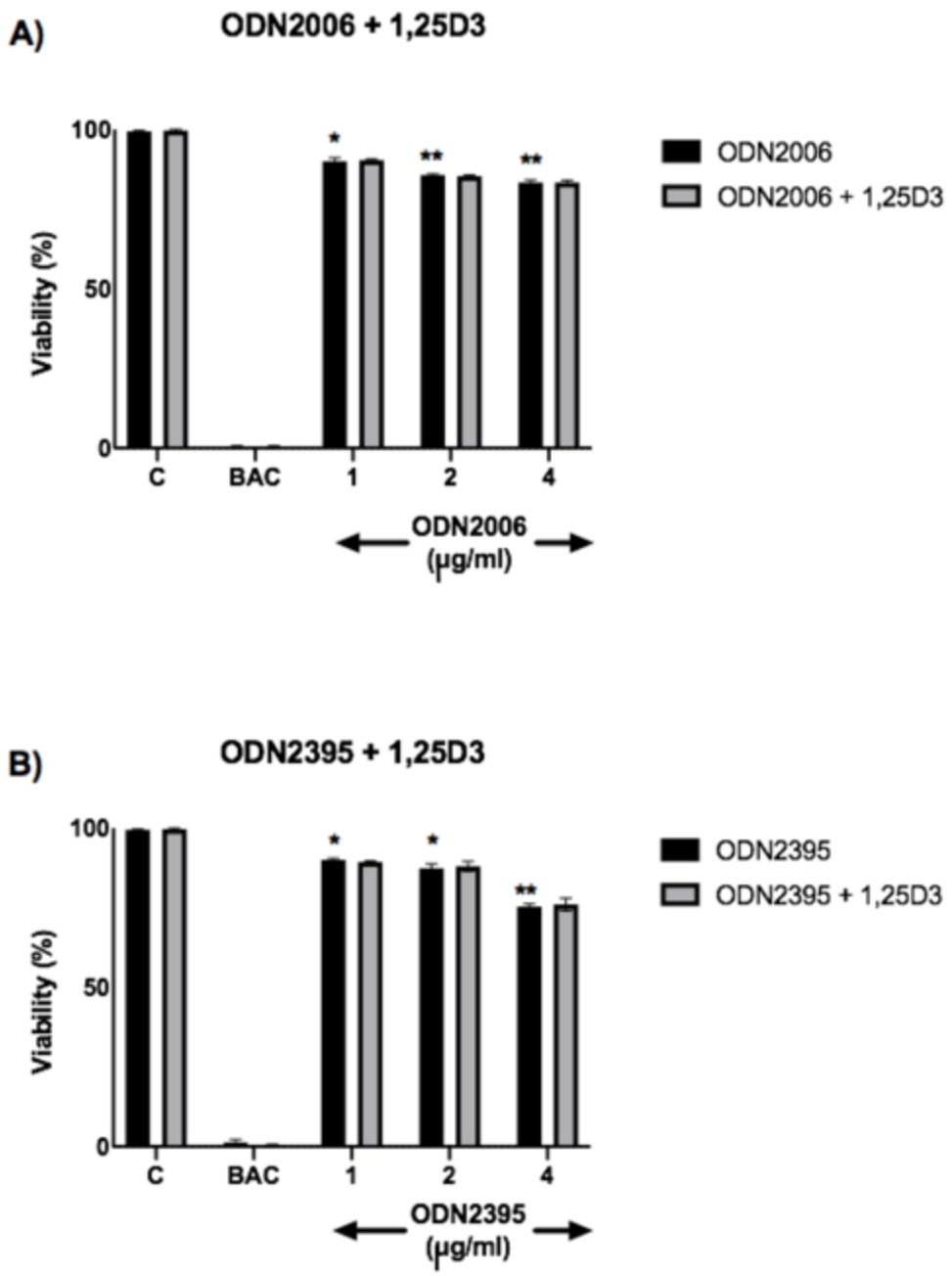

Figure 4.4: Cell viability following stimulation with ODN2006 and ODN2395, alongside 1,25D3 treatment: MTT analysis of hTCEpi cells following 24h exposure to (A) 1, 2 and 4μg/ml of ODN2006 and (B) 1, 2 and 4μg/ml of ODN2395. Negative control utilised unstimulated hTCEpi cells (C) whilst the positive control was generated by hTCEpi with BAC. Cells are shown as a percentage of viability in comparison to unstimulated cells. Statistical analysis was by one-way ANOVA with Bonferroni test for multiple comparisons against completely unstimulated hTCEpi cells (A and B, n=3) p<*0.05, **0.01, ***0.001

4.2.5 hTCEpi expresses fully functioning TLR9 for dsDNA detection and is able to induce pro-inflammatory mediators, which are suppressed by 1,25D3

After confirming that hTCEpi TLR9 receptors were fully functioning towards ssDNA and that 10^{-7}M 1,25D3 treatment led to a significant suppression of TLR9 and associated pro-inflammatory mediators (subsection 4.4.3), the study then aimed to replicate the same experiments using dsDNA from bacterial *E. coli* DNA (Invivogen, San Diego, USA). To identify the optimum concentration of *E. coli* DNA required to stimulate TLR9 of hTCEpi in the future experiments, various concentrations were used, including; 1μg/ml, 2μg/ml and 4μg/ml, as suggested by the manufacturer's instructions. The experiment involved a 24h challenge to ensure maximum TLR9 stimulation and mimicked chronic inflammation in the resolution period of inflammation. Within these experiments, hTCEpi IL-8 and TLR9 expression were analysed. The results showed that 2μg/ml induced the highest expression of both TLR9 and IL-8 following *E. coli* DNA stimulating TLR9 for 24h (Figure 4.5, A-B, $p<0.001$). Therefore, this concentration was selected for future experiments using dsDNA.

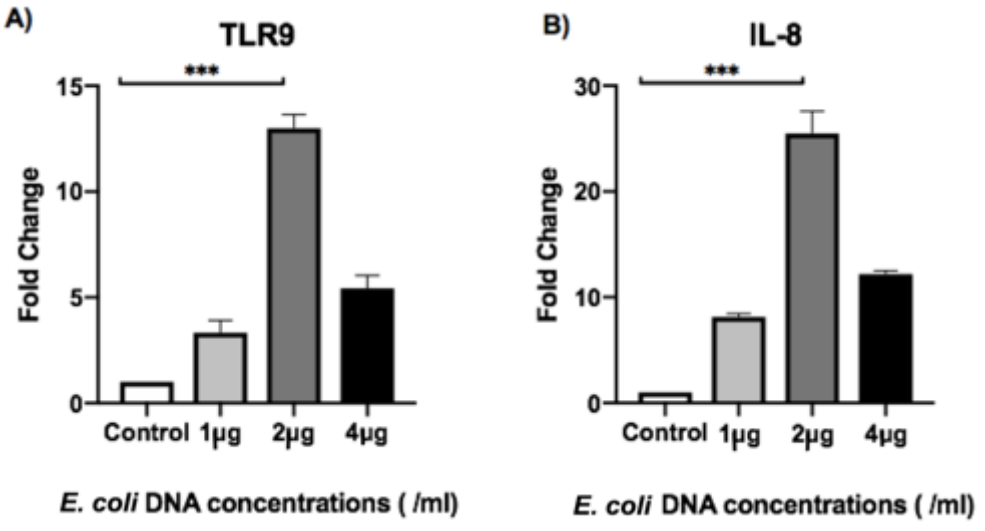

Figure 4.5*: Inducing a pro-inflammatory response by TLR9 in hTCEpi with *E. coli* DNA: expression of (A) TLR9 and (B) IL-8 by hTCEpi after exposure to various concentrations of *E. coli* DNA TLR9 agonist. Data represent mean ± SEM of three independent experiments. Statistical analysis was by one-way ANOVA with Bonferroni test for multiple comparisons (A and B, n=3) p<*0.05, **0.01, ***0.001

4.2.6 Pro-inflammatory mediators induced by dsDNA stimulation of hTCEpi TLR9 are suppressed by 1,25D3

Following confirmation that 2μg/ml of *E. coli DNA* was the optimum concentration to induce the strongest TLR9 and IL-8 mRNA expression (Figure 4.6 A-B), the study then moved onto analyzing IL-1β, IL-6 and IFN-β expression with the aim of identifying 1,25D3 effects upon TLR9 signaling in response to dsDNA. Although *E. coli* is rarely a corneal pathogen, Zhai *et al.,* (2018) highlighted its capability of being an opportunistic infection to the ocular surface, with further research highlighting *E. coli* as a robust option for TLR9 activation due to a rich supply of unmethylated CpG motifs, inducing high production of pro-inflammatory mediators (Dalpke *et al.,* 2006). The results showed that *E. coli* DNA significantly increased the expression of IL-6, IL-8 and IL-1β (Figure 4.6, A-C, $p<0.01$, $p<0.001$), similar to the results seen with ssDNA stimulation (Figure 4.2). This experiment also explored IFN-α expression, as previous research showed that this agonist stimulated IFN-α instead of IFN-β (Krug *et al*., 2001). The data presented in this thesis showed that this treatment also significantly increased IFN-α expression (Figure 3.4D, $p<0.001$).

The dual treatment of 1,25D3 and *E. coli* DNA led to a significant decrease in the expression of IL-6, IL-8, IL-1β and IFN-α (A-D, $p<0.01$, $p<0.001$). These results indicated that *E. coli* DNA did induce a range of different pro-inflammatory mediators at mRNA level, with 1,25D3 treatment significantly suppressing expression. In regard to TLR9 expression (E) and similar to the previous result seen using ssDNA (Figure 4.2), *E. coli* DNA significantly increased TLR9 expression after 24h ($p<0.001$). However, there was a significant decrease of TLR9 expression ($p<0.01$) in comparison to the cells undergoing the dual treatment of 1,25D3 and *E. coli* DNA exposure. These results indicated that 1,25D3 has a suppressive effect upon TLR9 expression following stimulation by dsDNA.

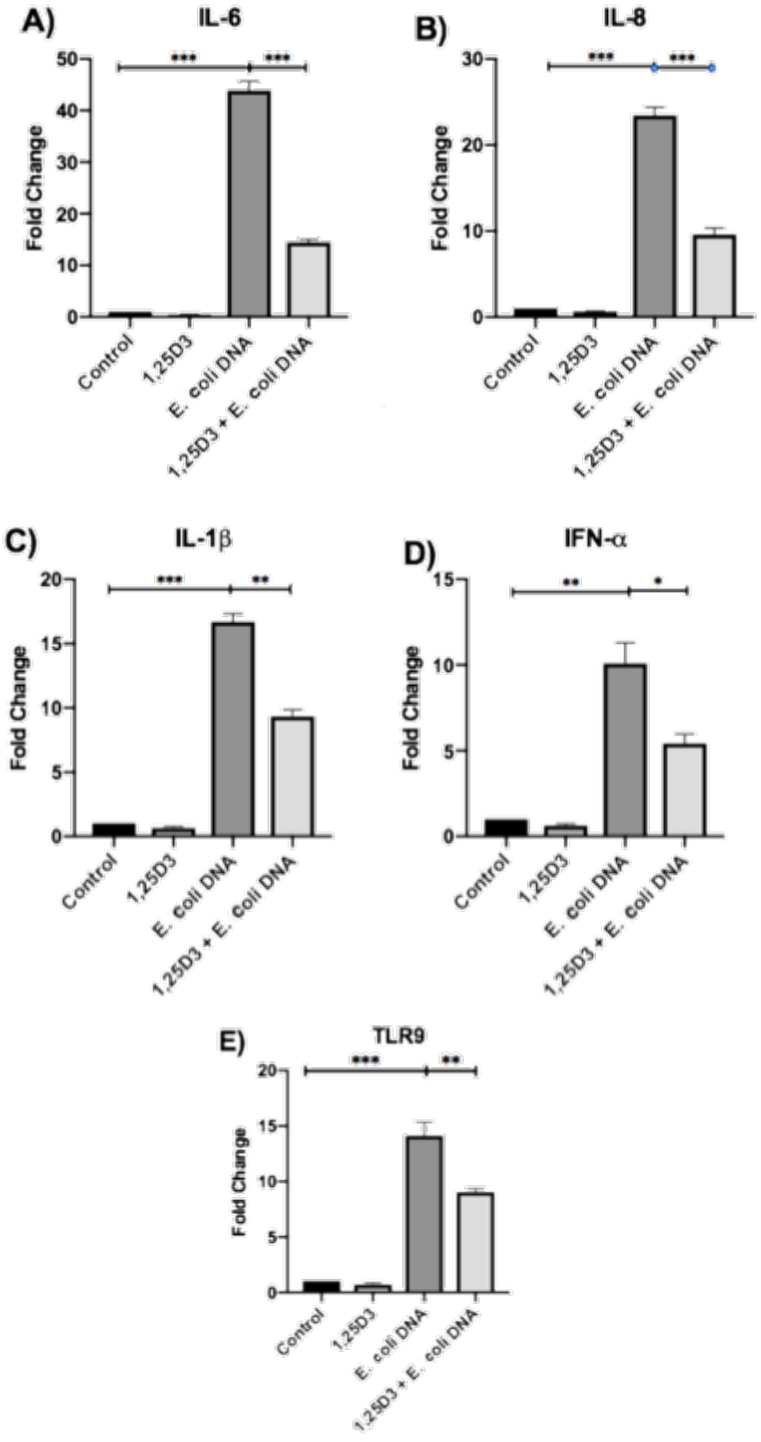

Figure 4.6: Analysing the effect of various *E. coli* DNA and 1,25D3 conditions upon hTCEpi pro-inflammatory mediator expression: pro-inflammatory mediator mRNA expression by hTCEpi after exposure to 2μg/ml *E. coli* DNA TLR9 agonist and 1,25D3. (A) IL-6 (B) IL-8 (C) IL-1β (D) IFN-α (E) TLR 9. Data represent mean ± SEM of three independent experiments. Statistical analysis was by one-way ANOVA with Bonferroni test for multiple comparisons (A- E, n=3), p<*0.05, **0.01, ***0.001

4.2.7 *E. coli* DNA 2μg/ml had a significant impact upon hTCEpi cellular viability

The previous results showed that 2μg/ml of *E. coli* DNA induced the highest expression of TLR9 and IL-8 by hTCEpi (Figure 4.3 A-B), whilst 10^{-7}M 1,25D3 treatment significantly suppressed the pro-inflammatory cytokine expression (Figure 4.6). However, to ensure these results were reliable and not caused due to toxicity issues, cell viability was confirmed (Figure 4.7). The cell viability assay showed that 1μg/ml and 2μg/ml of *E. coli* DNA did significantly decrease cell viability (Figure 4.7, $p<0.01$), with 4μg/ml reducing hTCEpi viability even further ($p<0.001$), in comparison to unstimulated hTCEpi. However, 10^{-7}M 125D3 treatment boosted cell viability in comparison to hTCEpi exposed to 4μg/ml of *E. coli* DNA alone.

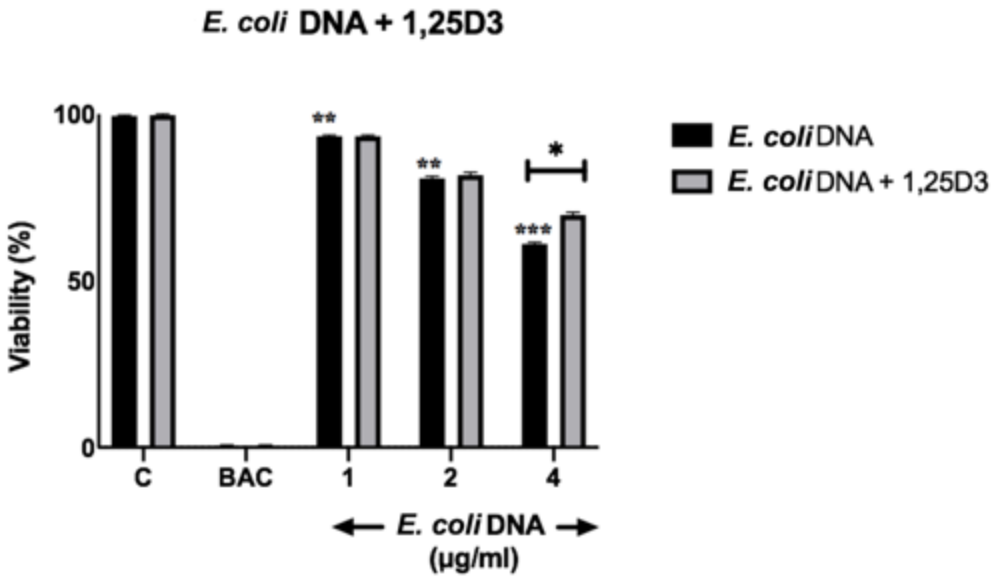

Figure 4.7: Cell viability following stimulation *E. coli* DNA, alongside 1,25D3 treatment: MTT analysis of hTCEpi cells following 24h exposure to 1, 2 and 4μg/ml *E. coli* DNA, alongside 10^{-7}M treatment. Negative control utilised unstimulated hTCEpi cells (C) whilst the positive control was generated by hTCEpi with BAC. Cells are shown as a percentage of viability in comparison to unstimulated cells. Statistical analysis was by one-way ANOVA with Bonferroni test for multiple comparisons against completely unstimulated hTCEpi cells (A, n=3) p<*0.05, **0.01, ***0.001

4.2.8 hTCEpi do not express a fully functioning cGAS receptor and cannot produce an inflammatory response through cGAS/STING activation

Alternate cytosolic DNA sensors may detect self-DNA and therefore exacerbate inflammation alongside TLR9 (discussed throughout subsection 1.3.5). The next aim was to confirm if hTCEpi had one of these functioning DNA receptors, STING, which would then activate the cGAS/STING pathway in response to *E. coli* DNA. Therefore, hTCEpi cells were stimulated for 24h using various concentrations of 2' 3' cGAMP; 50μg/ml and 100μg/ml, then IFN-β induction measured (Figure 4.8, A-B). The results showed that 50μg/ml of 2'3' cGAMP for both 24h and 48h led to no significant induction of either STING or IFN-β (A and B). Increasing the concentration to 100μg/ml, the highest level recommended by the manufacturer, also led to no induction of either STING or IFN-β (ns). These results indicated that the hTCEpi cell line does not have a functioning cGAS/STING pathway following 24h stimulation of 2'3' cGAMP, leading to no significant increase in the expression of either STING and IFN-β.

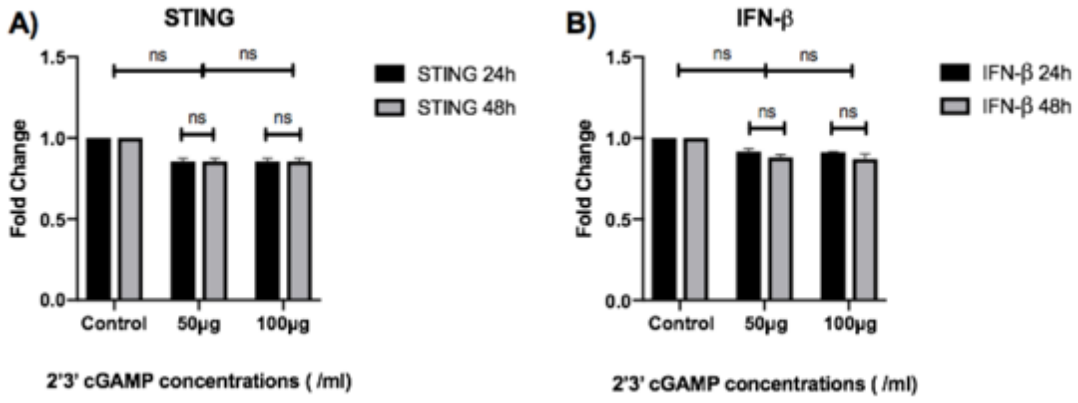

Figure 4.8: Stimulating hTCEpi with various concentrations of 2'3'cGAMP for 24h to induce a pro-inflammatory response: mRNA expression of (A) STING and (B) IFN-β by hTCEpi after exposure to various concentrations of 2'3'cGAMP; 50μg/ml and 100μg/ml. Data represent mean ± SEM of three independent experiments. Statistical analysis was by one-way ANOVA with Bonferroni test for multiple comparisons (A and B, n=3), p<*0.05, **0.01, ***0.001

4.2.9 Both ssDNA and dsDNA stimulation of TLR9 increases the expression of miR-93-5p

Similar to the experiment observing potential changes of miR expression during TLR3 signaling discussed throughout chapter 3, the following experiment was to observe the expression of miR-93-5p, miR-146a-5p, miR-155-5p and miR-181-3p during TLR9 stimulation. The results following 24h stimulation of hTCEpi with *E. coli* DNA showed similar results to those found in TLR3 signaling during stimulation, with a significant increase of expression seen in both miR-93-5p and miR-181-3p (A, $p<0.001$), with similar results seen for the dual treatment of *E. coli* DNA and 1,25D3. However, no significant change was identified in regard to miR-146a-5p and miR-155-5p expression under any experimental condition. Finally, in regard to hTCEpi exposure to 1,25D3 alone, no significant change was seen in the expression of any of the selected miR.

In regard to the ssDNA ODN2006 experiments (Figure 4.9B), the results showed significant increase in the expression of miR-93-5p during ODN2006 stimulation alone and the dual treatment of ODN2006 and 1,25D3 (B, $p<0.001$). However, no significant change was seen in any condition for miR-146a-5p, miR-155-5p or miR-181-3p.

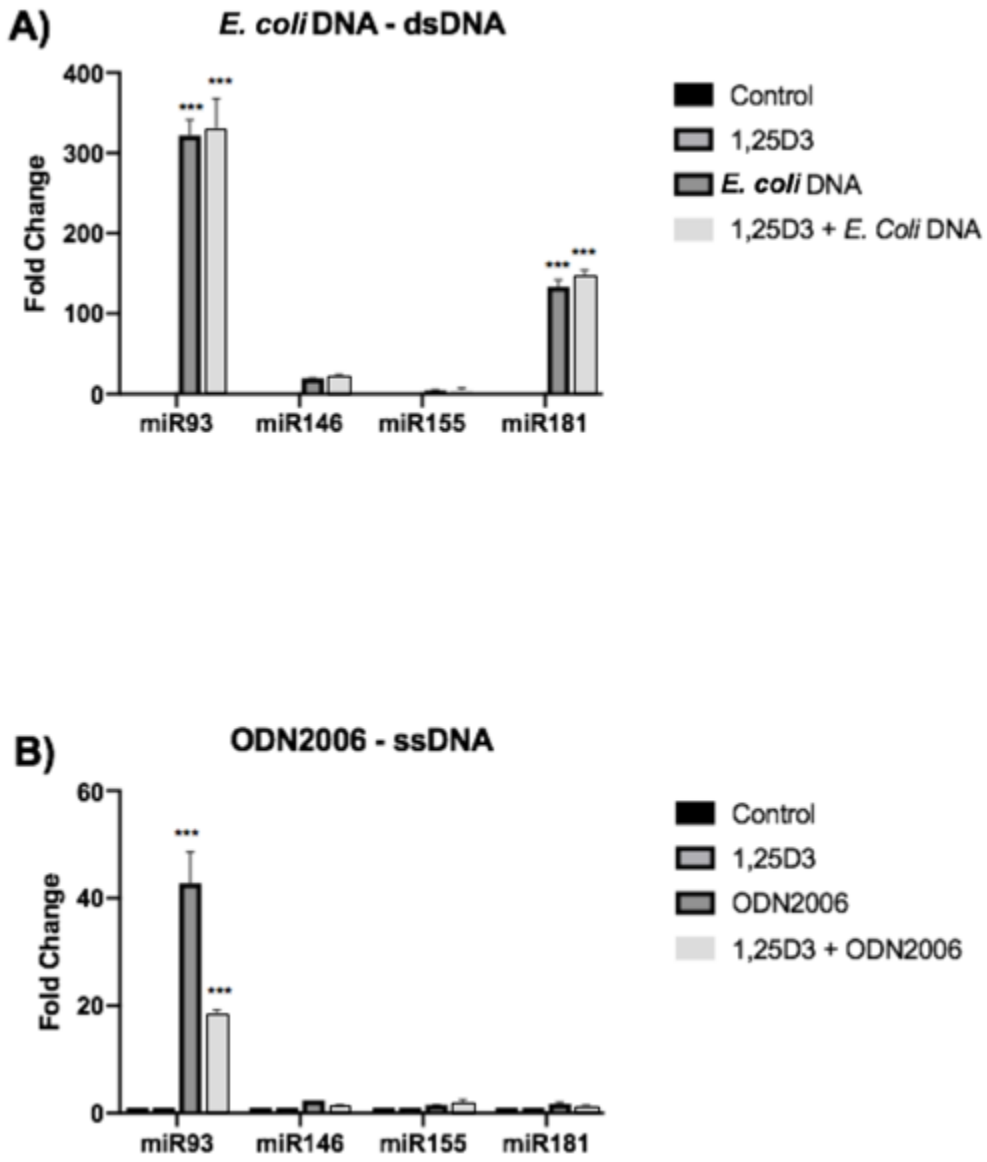

Figure 4.9: Identifying changes within hTCEpi miR expression in response to 24h of *E. coli* DNA or ODN2006 treatment: (A) miR expression by hTCEpi cells following 24h of stimulation with and without *E. coli* DNA and 1,25D3. (B) miR expression by hTCEpi cells following 24h of stimulation with and without ODN2006 and 1,25D3. Data represent mean ± SEM of three independent experiments. Statistical analysis was by one-way ANOVA with Bonferroni test for multiple comparisons (A and B) p<*0.05, **0.01, ***0.001

4.2.10 miR-93-5p does not have a regulatory role in IL-8, IL-6, IL-1β and TLR9 expression following ssDNA stimulation of TLR9

To support the results regarding miR-93-5p regulating IL-6, IL-8 and IL-1β expression following TLR3 activation, an inhibitor specific to miR-93-5p was introduced to hTCEpi cells, with the aim of identifying the potential role of

miR-93-5p during TLR9 signaling or 1,25D3 suppression. It was hypothesized that there would be a non-suppressive effect from 1,25D3 treatment if miR-93-5p had a role in the mechanism of action, as these suppressive actions would be inhibited. To confirm a potential role of miR-93-5p within 1,25D3 suppressive actions, the expression of pro-inflammatory mediators and TLR9 were analysed following miR-93-5p inhibition of hTCEpi stimulated with ODN2006 for 24h. IFN-β was not considered due to the failure of identifying detectable induction throughout the previous ssDNA experiments (Figure 4.2D).

The results showed that inhibiting miR-93-5p had no effect on the suppressive action of 1,25D3 upon expression of IL-6, IL-8, IL-1β or TLR9 following TLR9 stimulation (Figure 4.10, A-D). This suggested that miR-93-5p does not have a role in the mechanism of the suppressive effect of 1,25D3. Nor did this miR have a role in the expression of these mediators during stimulation of TLR9 using ssDNA, as there was no significant change in expression seen in the miR-93-5p inhibited cells in comparison to the normal control cells.

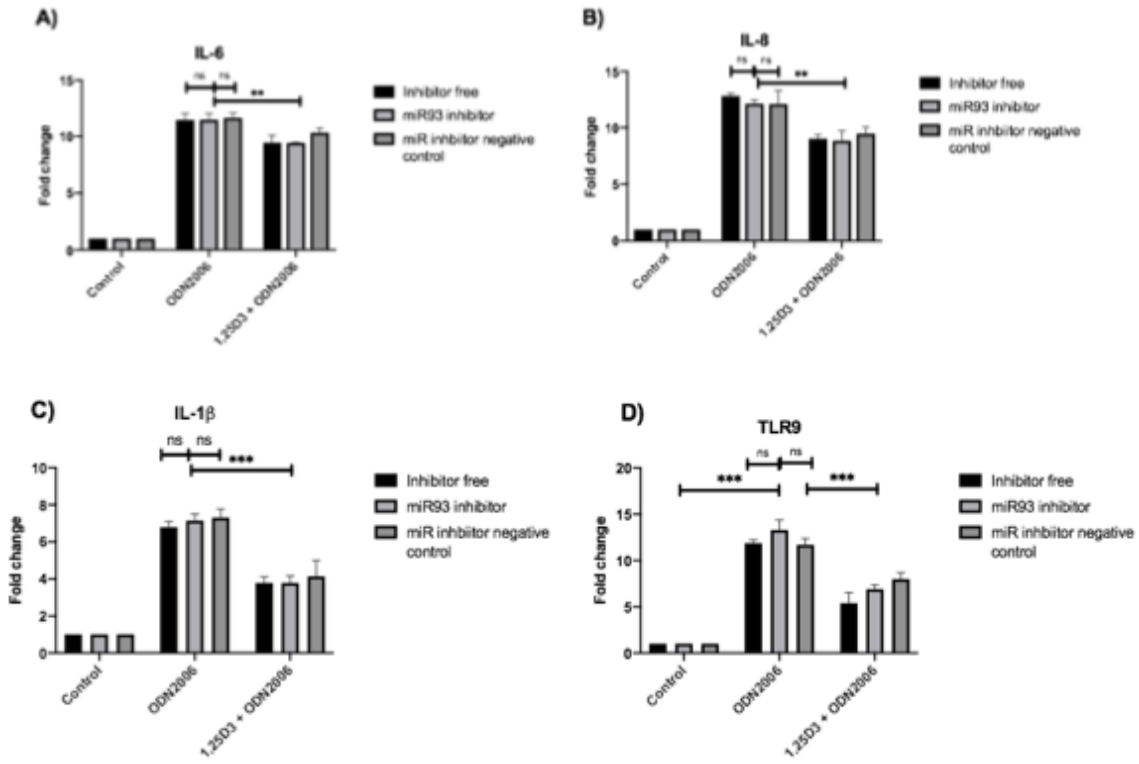

Figure 4.10: Effects of 24h hTCEpi miR-93-5p inhibition upon TLR9 signalling in response to ssDNA and related mediator expression: The pro-inflammatory mediators having the greatest expression in response to ODN2006 were chosen from the previous experiments within this chapter: (A) IL-6, (B) IL-8, (C) IL-1β and (D) TLR9 mRNA expression. Data represent mean ± SEM of three independent experiments. Statistical analysis was by one-way ANOVA with Bonferroni test for multiple comparisons (A-D, n=3) p<*0.05, **0.01, ***0.001

4.2.11 miR-93-5p does have a regulatory role in IL-8, IL-6 and IL-1β expression following dsDNA stimulation of TLR9

The miR-93-5p inhibitor was then used to replicate the experiment from subsection 4.2.10, this time using dsDNA in the form of *E. coli* DNA for 24h of TLR9 stimulation. Again, it was hypothesized that if miR-93-5p had a role during the suppressive effect from 1,25D3, no inhibition would be seen in the expression of pro-inflammatory mediators and TLR9.

The results (Figure 4.11) showed that inhibiting miR-93-5p had no significant effect during the suppressive action of 1,25D3, as IL-6, IL-8, IL-1β, IFN-β and TLR9 (A-E) were all still suppressed. This suggested that miR-93-5p does not have a role in the mechanism of the suppressive effect of 1,25D3 on TLR9 associated expression by hTCEpi. However, during TLR9 stimulation

with *E. coli* DNA in the absence of 1,25D3, inhibiting miR-93-5p did have a significant effect in the expression of the chosen pro-inflammatory mediators, increasing their expression. These significant changes were seen in the following; IL-6 (A) ($p<0.001$), IL-8 (B) ($p<0.001$) and IL-1β (C) ($p<0.001$). However, there was no significant impact upon the expression of IFN-β or TLR9 following dsDNA stimulation (D and E) (ns) following inhibition of miR-93-5p.

Although these results disprove the hypothesis that miR-93-5p has a role during the suppressive action of 1,25D3 upon TLR9 stimulation, these results suggest novel findings that miR-93-5p regulates expression of hTCEpi IL-6, IL-8 and IL-1β following dsDNA stimulation.

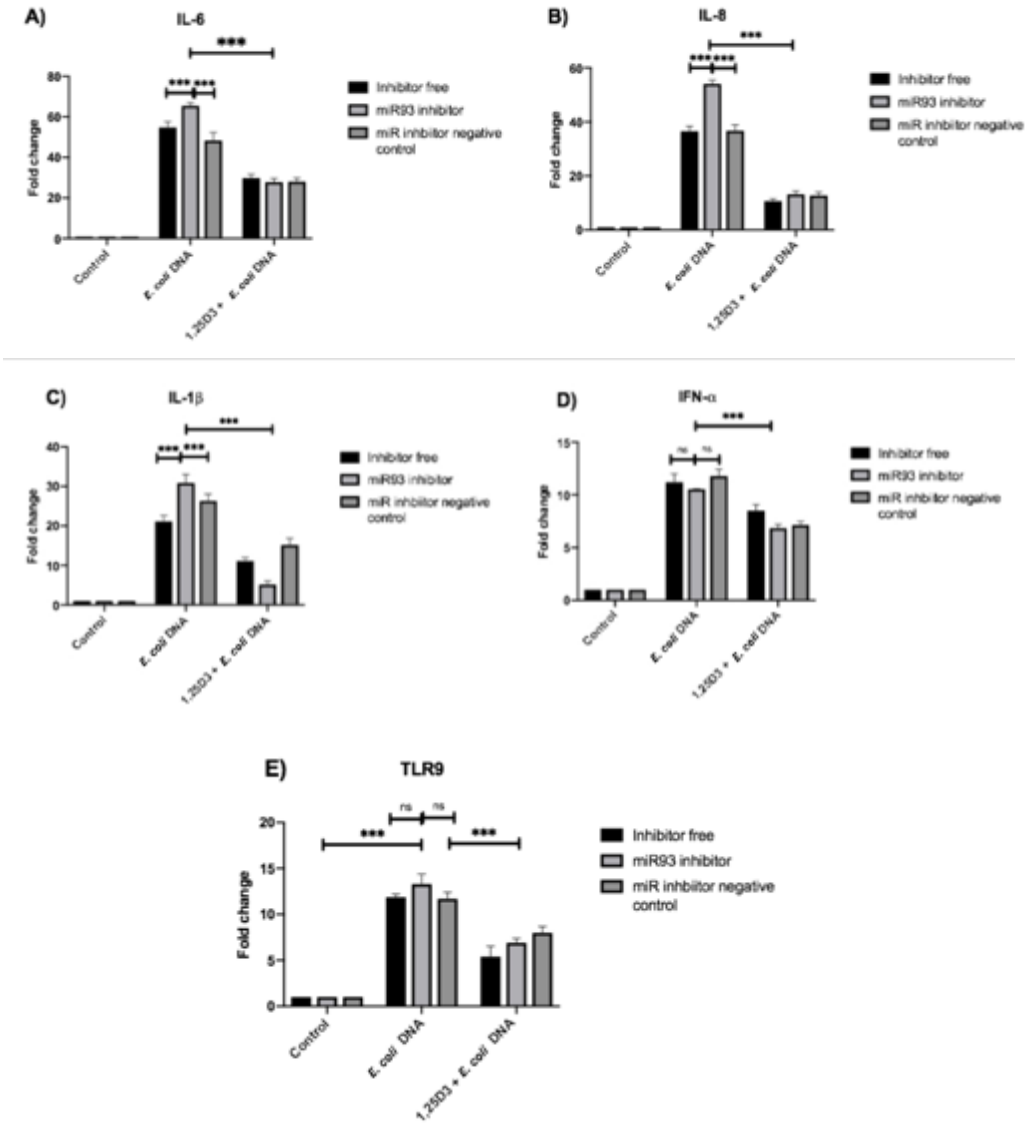

Figure 4.11: Effects of 24h hTCEpi miR-93-5p inhibition upon TLR9 signalling in response to dsDNA and related mediator expression: the pro-inflammatory mediators which showed a significant increase within expression in response to *E. coli* DNA were chosen from the previous experiments: (A) IL-6, (B) IL-8, (C) IL-1β, (D) IFN-α and (E) TLR9 mRNA expression. Data represent mean ± SEM of three independent experiments. Statistical analysis was by one-way ANOVA with Bonferroni test for multiple comparisons (A-E, n=3) p<*0.05, **0.01, ***0.001

4.2.12 miR-181-3p has a regulatory role in IL-8, IL-6 and IL-1β expression following dsDNA stimulation of TLR9

The aim of the following experiments was to confirm if inhibiting miR-181-3p would lead to a significant change in expression of pro-inflammatory mediators in response to *E. coli* DNA. ssDNA was not studied in regard to miR-181-3p as the previous analysis showed no change in miR-181-3p expression during TLR9 stimulation by ODN2006 (Figure 4.9B).

The results from this experiment using *E. coli* DNA as dsDNA to trigger TLR9, showed that inhibiting miR-181-3p also had a significant suppressive effect upon mRNA expression, with each cytokine increasing dramatically: IL-6 (Figure 4.12A, $p<0.001$), IL-8 (B, $p<0.001$) and IL-1β (C, $p<0.001$). However, there was no significant change in the expression of IFN-α or TLR9 (D-E). However, there was no significant change of the suppressive action of 1,25D3, with cytokine suppression still occurring within inhibited cells and the control cells. Although these results disprove the hypothesis that miR-181-3p has a role during the suppressive mechanism of 1,25D3, these results suggest novel findings that miR-181-3p may play a role in regulating the expression of IL-8, IL-6 and IL-1β produced by hTCEpi, following TLR9 stimulation by dsDNA.

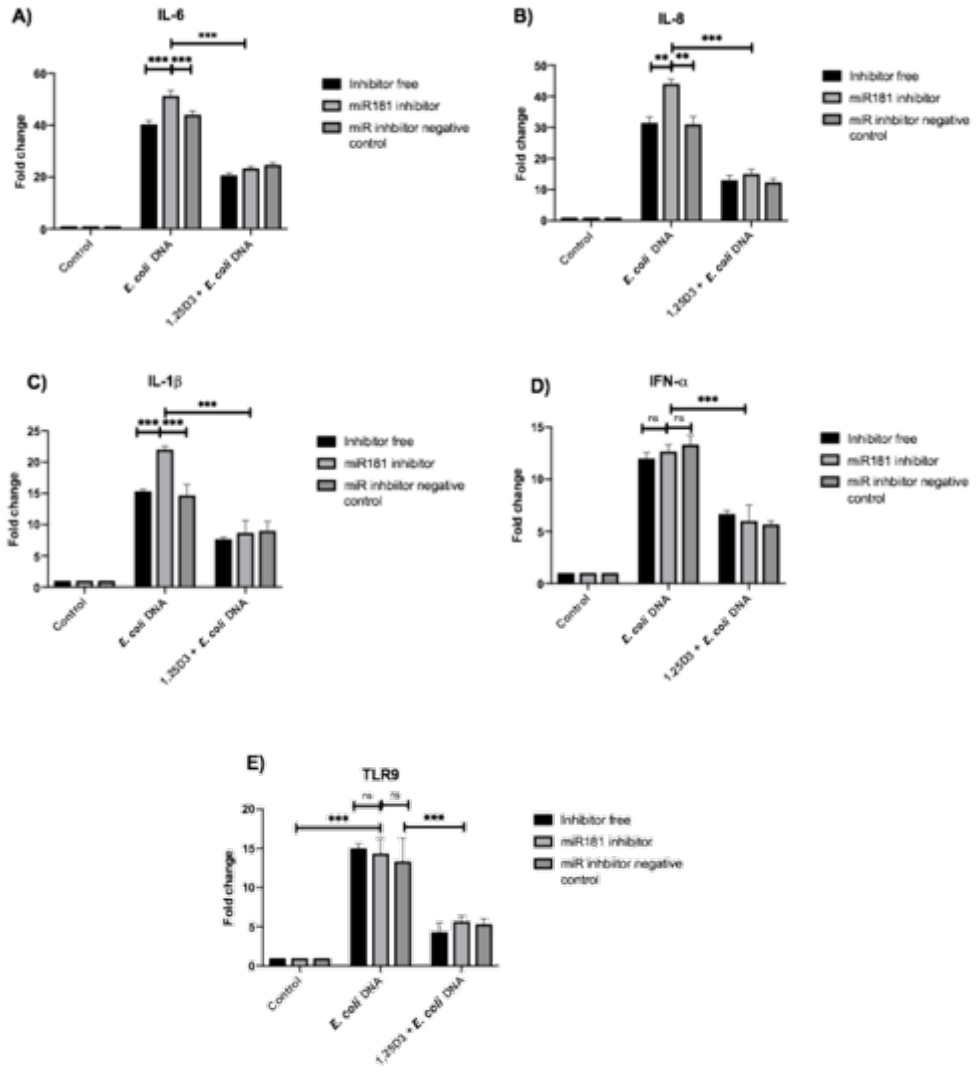

Figure 4.12: Effects of miR-181-3p inhibition upon TLR9 signalling in response to dsDNA and related mediator expression by hTCEpi cells under various 24h conditions: the pro-inflammatory mediators which showed a significant increase in expression in response to *E. coli* were chosen from the previous experiments: (A) IL-6, (B) IL-8, (C) IL-1β, (D) IFN-α and (E) TLR9 mRNA expression. Data represent mean ± SEM of three independent experiments. Statistical analysis was by one-way ANOVA with Bonferroni test for multiple comparisons (A-E, n=3) p<*0.05, **0.01, ***0.001

4.3 Chapter discussion

4.3.1 hTCEpi cells express functioning TLR9

The aim of the experiments performed within this chapter was to confirm if TLR9 was fully functional towards various sources of both ssDNA and dsDNA, producing a range of pro-inflammatory mediators. Additionally, the study aimed to identify an additional DNA sensing pathway known as cGAS/STING. Following this, the effect of 1,25D3 treatment was investigated, aiming to establish if this treatment would have a suppressive effect upon TLR9 signalling. It was hypothesized that the subclass C CpG-ODN, ODN2395 that is specifically recognised by TLR9, would induce a stronger inflammatory effect by 24h. However, the data showed 24h ODN2006 exposure induced a stronger pro-inflammatory response than that seen by ODN2395 with a significant increase in IL-6, IL-8, IL-1β, and TLR9 expression, disproving the hypothesis (Figures 4.1 and 4.2). in the type I interferon response, IFN-β is produced first as opposed IFN-α and was selected for the CpG-ODN experiments, to allow comparison between the cGAS/STING and TLR9 pathways (Kerkmann *et al*., 2003). This was a limitation, as further research has shown that CpG-ODN induce a much more robust IFN-α response in cells such as plasmacytoid dendritic cells (Nehete *et al.,* 2020, Teleshova *et al.,* 2004). Therefore, IFN-α would be beneficial to investigate in future studies using CpG-ODN.

The primary aim of this thesis was to examine preventing overt immune responses within the resolution period of 24h, where pro-inflammatory mediator expression should decline to unstimulated levels. However, CpG-ODN exposure would be expected to be much stronger during the acute inflammatory response at 6h, with more ligand present. Therefore, it would be beneficial to use a smaller range of time points to reflect alternate points of the inflammatory pathway. Tanegashima *et al.,* (2017) showed that CXCL14, combined with ODN2395, induced a significantly higher expression of IL-6, IL-12 and TNF-α in bone marrow derived dendritic cells. CXCL14 is a powerful chemokine, acting as a chemoattractant for wider immune cells, including macrophages and is capable of forming a stable complex with the CpG-ODN as a type of nanodelivery system. Incorporating this complex into future experiments could be an interesting approach, leading to a stronger,

persistant immune response at 24h, similar to that of ODN2006 if there was potential issues with ligand uptake with the C class CpG-ODN.

Hartmann *et al.,* (2000) characterised a significant activation of TLR3 pathway from ODN2006 exposure, supported by Bagchi *et al.,* (2006) demonstrating that this led to a significant production of pro-inflammatory cytokines through the NF-KB pathway. However, it is a point of discussion that CpG-ODN reactions are dependent upon cell type, with Ranjith-Kumar *et al.,* (2008) failing to identify a significant increase within TLR3 expression within bronchial epithelium following ODN2006 treatment. To ensure that hTCEpi TLR9 significantly contributed to the inflammatory response, using $TLR3^{-/-}$ murine models would be an appriopriate method to confirm this, similar to the work of Tabeta *et al*., (2004) who distinguished TLR3 and TLR9 signalling in response to CpG-ODN.

E. coli was used due to previous success of stimulating TLR9 within hTCEpi cells before, however, Coats *et al.,* (2005) showed the *E. coli* LPS component stimulated TLR4 in a range of cells, therefore, the DNA component was selected to avoid improper TLR4 activation. Furthermore, the DNA was sourced from heat-inactivated of *E. coli,* which expresses the same surface proteins as live forms of *E. coli* but minimizes the danger of using live cultures, meaning potential suppressive actions of 1,25D3 could still be identified (Zimmermann *et al.,* 2018, Dalpke *et al.,* 2006). Heterodimers of both TLR2/6 and TLR2/1 recognise diacyl and triacyl lipopeptides (Von-Aulock *et al.,* 2003, Takeda *et al.,* 2002), whilst TLR4 is able to recognise the lipopolysaccharide (LPS) and stimulate an immune response (Kaur *et al.,* 2015). During the experiments mentioned in this thesis, biological assays were conducted by the manufacturer to ensure the absence of LPS and RNA within the *E. Coli DNA*, which could lead to alternate TLR activation (Appendix A2). This is important to ensure the data presented in this thesis demonstrated hTCEpi TLR9 signaling following dsDNA stimulation. In future experiments, flow cytometry would confirm the expression of TLR1, 2 and 6 during hTCEpi stimulation using *P. aeruginosa*, identifying potential, further suppressive effects of 1,25D3 treatment during bacterial infection of the corneal surface. IL-8 was selected as the initial pro-inflammatory cytokine for analysis due to Zimmermann *et al.,* (2018) demonstrating a significant induction of IL-8 by epithelial cells in comparison with other pro-inflammatory

mediators. Successful TLR9 activation was confirmed following 24h of *E. coli* stimulation led to a significant increase in IL-6, IL-8, IL-1β, TLR9 and IFN-α mRNA expression (Figure 4.5).

4.3.2 1,25D3 signficantly suppressed hTCEpi pro-inflammatory mediators following TLR9 activation

Figures 4.2 and 4.3 demonstrated that 1,25D3 significantly suppressed a range of pro-inflammatory mediators, including IL-6 and IL-8, during both ongoing and prior hTCEpi stimulation. There was no significant difference within IL-1β expression following 24h of ODN2006 exposure alone. However, as previously discussed in subsection 3.3.2, IL-1β could be vital within the acute inflammatory response, and may not be detectable by 24h.

In regard to viral infections of the ocular surface, adenovirus infection accounts for an estimated 90% of viral conjunctivitis cases, which leads to further complications such as corneal keratopathy and potential blindness. (Azari *et al.,* 2013). The data presented in this study showed that 1,25D3 treatment significantly suppressed the expression of IL-6, IL-8, IL-1β, TLR9 and IFN-α, during ongoing dsDNA stimulation (Figure 4.6). Suppression of all of these pro-inflammatory mediators is beneficial, as each is significantly upregulated in response to a range of ocular viruses, including HSV-1 and associated corneal nerve degeneration, leading to decreased corneal sensation and damage to sensory fibers (Li *et al.,* 2006b, Chucair-Elliot *et al.,* 2016). Wuest *et al.,* (2006) showed that IFN-α expression is upregulated by cells of the ocular surface in response to TLR9 activation. A decline of IFN-α is also beneficial in treating viral infections of the ocular surface, as this cytokine interferes with the virus's capacity to replicate, whilst increasing the expression of major histocompatibility complex I (MHCI) to stimulate Th1 cells, hence linking the innate and adaptive defence systems (Parham *et al.,* 2009). However, protein quantification is crucial to observe if these changes in mRNA expression are translated to subsequent protein production, and if these changes would be biologically relevant. As with the data generated from the ssDNA, these findings require protein quantification from methods such as ELISA, which would allow confirmation whether these suppressive changes are biologically relevant to the ocular surface.

Inflammation driven by TLR9 signalling may also be stimulated from self-DNA, exacerbating inflammatory damage associated with auto-immune conditions. Although not directly related to the ocular surface, wider research shows that TLR9 dysregulation leads to 'fatal inflammatory responses' for neonates, with type II interferons driving macrophage and natural killer cell activity, exacerbated by self-nucleic acids detected by TLR9 (Stanbery *et al.,* 2020). Pohar *et al.,* (2017) showed even the shortest DNA degradation products, in response to infection or self, can stimulate TLR9 and induce further inflammatory responses. Lee *et al*., (2014) characterised overt TLR9 signalling during murine systemic lupus erythematosus, a chronic ocular inflammatory condition. Therefore, this suppressive action of 1,25D3 of the TLR9 signalling may also be beneficial to ocular conditions related to auto-immune disease, but this should be examined further during future studies.

Although these data showed 1,25D3 suppressing hTCEpi TLR9 signalling, they should be repeated using DNA from known ocular infections. Whilst ocular *E. coli* infections are opportunistic and rare, the gram-negative bacteria *Pseudomonas aeruginosa* has been shown to be a leading cause in bacterial keratitis, with subsequent corneal inflammation and a risk of necrosis, (Teweldemedhin *et al.,* 2017, Borkar *et al.,* 2013, Price *et al.,* 2017). Furthermore, it would be beneficial to observe the hTCEpi cells transfected with the HSV-1 strain, using the methodology of Alekseev *et al.,* (2020).

4.3.3 1,25D3 suppression of TLR9 signaling does not affect hTCEpi viability

To confirm the suppression of TLR9 by 1,25D3 was not caused by a decline in cell population, cell viability was determined after 24h. The results stronger hTCEpi toxicity from ODN2395 than ODN2006, after 24h (Figures 4.4 and 4.5). Lim *et al.,* (2010) demonstrated the effect of ODN treatments upon cell viability, showing that CpG-ODN treatment significantly reduced apoptosis by suppressing the FoxO3a pathway after 12h of exposure, whilst Landrigan *et al.,* (2011) characterised a low toxicity profile for ODN2006. In regard to the dsDNA experiments, 24h of *E. coli* DNA also significantly decreased hTCEpi cell viability (Figure 4.7). However, 1,25D3 treatment did not affect cell viability, and in regard to the high concentrations of *E. coli* DNA, showed a

potential protection by significantly increasing hTCEpi viability, which may be linked to a suppression of the inflammatory environment. These data indicated that 1,25D3 treatment of TLR9 signaling does not significantly impact hTCEpi cell viability. Although these experiments followed the manufacturer's recommendation of working concentrations, it would be beneficial use a range of lower ligand concentrations, as a higher cell viability ensures larger cell population for reporting 1,25D3 suppression of TLR9 signalling.

4.3.4 hTCEpi do not express a functioning cGAS receptor for dsDNA detection

The results showed there was no detectable induction of IFN-β or STING mRNA expression following 24 and 48h of 2'3'cGAMP exposure at varying concentrations, indicating that there no significant activation of the cGAS/STING pathway in hTCEpi cells (Figure 4.8). However, there are obvious limitations present within these experiments, linked to the limited range of concentrations and time points used. Once again, although the experiments in this thesis focused upon the resolution period of inflammation at 24h, it could be that the cGAS/STING pathway was active within the acute inflammatory period at 6h, therefore it would be interesting to investigate this in future replications of the study. Although the concentrations used followed the manufacturer's recommendation, higher concentrations of 2'3'cGAMP could ensure enough available ligand for sampling by the cGAS/STING pathway and therefore a detectable inflammatory response by 24h. Furthermore, there is a lack of hTCEpi viability data, which would eliminate the possibility of potential toxicity to the hTCEpi cells, which could potentially explain the failure to identify an active inflammatory response.

It must be considered that there was a failure of delivery of the ligand to hTCEpi cells, possibly due to ligand size, *in vivo* instability or poor absorption. As discussed within subsection 1.4.4, the cGAS receptor is intracellular and cytosolic, therefore requires successful uptake of ligand into the cell to activate the cGAS/STING pathway. A major limitation of these experiments was the failure to ensure optimum uptake of 2'3'cGAMP into hTCEpi cells. Nano-delivery would be an attractive option for future replications of the study, ensuring that the ligand is delivered in a stable state to the receptor

and optimum concentrations for pathway activation. One example could be using DNA nanoparticles (DNPs), which show evidence of inducing a powerful IDO response, formed from cationic polymers with polyethylenimine (PEI) which aid in the delivery of dsDNA into the cytosol of cells, stimulating the cGAS/STING pathway (Huang *et al.,* 2012b). This method of stimulation could be an alternative option to confirm a functioning cGAS/STING pathway by hTCEpi, whilst analysing IDO expression in the future would identify if this protein was produced within this response. An alternative approach would be using the process of 'fluorination', which involves adding the element fluorine to 2'3'cGAMP at the 2' or 3' nucleotide site and increasing the stability of the ligand in vivo, whilst 'intensifying biological activity and adjuvanticity' (Lioux *et al*., 2016, Smola *et al*., 2021). Finally, replicating the *E. coli* DNA experiments and analysing the expression of STING may confirm involvement of this pathway in the inflammatory response identified throughout Figure 4.6.

4.3.5 miR-155-5p and miR-146a-5p play no direct role within the expression of various pro-inflammatory mediators following TLR9 stimulation, or the suppressive 1,25D3 mechanism, at 24h

Research into miR/TLR9 signalling is scarce, however, significant, as one of these miR may play a role during TLR9 signalling or the suppressive mechanism of 1,25D3 and could be exploited to strengthen the suppressive effect. To compliment those results found in regard to TLR3 signalling (chapter 3), and extend understanding of hTCEpi TLR9 signalling, the same experiments were replicated. There was no detectable miR-146a-5p and miR-155-5p expression following 24h of TLR3 expression, and these data supported the findings from these TLR9 experiments with both 24h exposure to ODN2006 and *E. coli* DNA (Figure 4.9, A-B). Karrich *et al.,* (2013) demonstrated miR-146a regulated the TLR9/NF-KB pathway of human plasmacytoid dendritic cells, leading to a significant decline in the production of pro-inflammatory cytokines, insinuating a relationship between TLR9 and miR. Previous research has argued that this relationship is dependent on cell type and origin, with miR-146 described to be 'despondent' during TLR3, TLR7 and TLR9 signaling, and 'vital' for bacterial stimulation, but not the viral stimulation of cells (Li *et al.,* 2013d, Taganov *et al.,* 2006). Bhela *et al.,* (2015) demonstrated miR-155 is upregulation in response to HSV-1 infection

within a range of cells, including inflammatory cells and T-cells, with miR-155 suppression leading to a diminished pro-inflammatory cytokine response. However, these were whole virus particles, not specifically the DNA component, and therefore may have activated multiple TLR. From these data, it can be concluded that neither miR-146a-5p or miR-155-5p were active following 24h stimulation of hTCEpi TLR9, and therefore, were not studied in context of 1,25D3 suppression.

Similar to the limitations discussed within subsection 3.3.5 in regard to the same miR analysed within the TLR3 experiments, it may be that hTCEpi cells did not have active miR by 24h of TLR9 stimulation. However, these results could be influenced by experimental limitations such as a limited number of time points, with a failure to identify active miR-155-5p or miR-146a-5p within the acute inflammatory period of 6h. This is reflected in the importance of both of these miR during the immune response to pathogenic DNA and the robust production of pro-inflammatory mediators, which would be expected in the beginning of inflammation (Karrich *et al.,* 2013, Rodriguez *et al.,* 2007, O'Connell *et al.,* 2010). As ODN2395 produced a weaker immune response in comparison to ODN2006, it would be interesting to compare the two ligands within the acute inflammatory period, as ODN2395 could generate a much stronger immune response within 6h than ODN2006, demonstrating stronger activity from both miR-155-5p or miR-146a-5p. Although the limitations discussed within subsection 3.3.5 are most likely causes of failure to identify significant miR expression change, other members of the same miR family could be more prominent during the inflammatory response. For example, widening the miR population by micro-array assay would identify a larger population of active miR during TLR9 signalling, for example, miR-146b-5p, which has also been implicated within inflammatory responses (Comer *et al*., 2014).

4.3.6 Both miR-93-5p and miR-181-3p may play a role within the expression of various pro-inflammatory mediators following 24h of TLR9 stimulation, but not during the suppressive 1,25D3 mechanism

It was hypothesized, considering the previous evidence supplied from the miR/TLR3 hTCEpi experiments (Chapter 3), that both miR-93-5p and miR-181-3p would have a negative regulatory role in the expression of IL-6, IL-8

and IL-1β pro-inflammatory mediators during TLR9 signalling. However, following 24h of TLR9 expression, the results showed no significant increase of miR-181-3p expression (Figure 4.9). Therefore, miR-181-3p was not examined further within this thesis in relation to 24h of TLR9 stimulation. Interestingly, similar results were seen when inhibiting miR-93-5p during ODN2006 stimulation, which led to no significant change in IL-6, IL-8, IL-1β or receptor TLR9 mRNA expression, in comparison to the uninhibited control cells or the cells with a negative miR-93-5p inhibitor. These data disproved the hypothesis, indicating that miR-93-5p had no role during 1,25D3 suppressing TLR9 at 24h (Figure 4.10). In response to dsDNA, *E. coli* DNA, miR-181-3p and miR-93-5p expression were significantly increased (Figure 4.9) and inhibition of these miR led to a significant increase within the expression of IL-6, IL-8 and IL-1β after 24h (Figures 4.11 and 4.12). To our knowledge, this is the first evidence of miR-181-3p and miR-93-5p regulation TLR9 signalling within hTCEpi cells, and will be beneficial for future inflammatory studies.

These changes in miR expression to various ligands is probably due to the fact that by 24h in response to CpG-ODN, the activity of these miR was not detectable within hTCEpi cells, but there was still enough ligand present to evoke an immune response. This could suggest that both miR-181-3p and miR-93-5p are active within the initial inflammatory response following ODN2006 exposure, and possibly even the acute inflammatory period, with miR-181-3p undetectable by 24h. Although it was suggested in subsection 3.3.6 that miR-93-5p and miR-181-3p could work in conjunction, these data indicated the requirement of smaller time points to identify this. Ren *et al.,* (2011) demonstrated miR-93-5p downregulation following CpG-DNA exposure and TLR9 activation. This downregulation of miR-93-5p may explain the weaker hTCEpi cell immune response compared to those obtained from dsDNA stimulation, which appeared to induce more robust, persistent inflammation. Inhibiting both miR-181-3p and miR-93-5p did not prevent the suppressive action of 1,25D3 treatment, with a significant decline in the expression of pro-inflammatory mediators, disproving the hypothesis that these miR played a role within 1,25D3 treatment.

4.3.7 Chapter conclusions

Dickie *et al.,* (2010), demonstrated that 1,25D3 had a 'down-regulatory effect' upon monocytic TLR9 signalling and cytokine production. However, the data presented in this thesis gives the first known evidence of 1,25D3 suppressing a range of corneal, TLR9-driven pro-inflammatory mediators in response to both ssDNA and dsDNA. The results also showed that miR-93-5p and miR-181-3p negatively regulate IL-8, IL-6 and IL-1β expression by hTCEpi cells in response to dsDNA. These data may be of particular interest when preventing overt inflammatory responses of the ocular surface in response to the dsDNA found in viruses, such as the previously discussed HSV-1. For example, increasing the expression of both miR-93-5p and miR-181-3p together, may suppress these mediators and overall inflammation. It could be assumed that both of these miR work in conjunction, as inhibiting each miR singularly still led to a significant induction of IL-6, IL-8 and IL-1β by hTCEpi cells. Finally, there was no detectable cGAS/STING pathway activation, however, as discussed this could be either due to failure of ligand delivery to the cell, or a smaller time point required to detect a short, robust immune response.

Chapter 5

Vitamin D and Human Corneal Epithelial Cells: analyzing potential mechanisms behind anti-inflammatory effects during TLR5 signaling

5.1 Introduction to chapter 5

5.1.1 The Ocular surface and TLR5

The ocular surface is constantly exposed to the outside environment, meaning increased risk of developing infections caused by microbes, including bacteria. The cornea induces a robust inflammatory response composed of both pro-inflammatory mediators and reactive anti-microbial peptides (AMPs) to remove such infections, for example, hBD-2 and LL-37 (Faber *et al.,* 2018, Szukiewicz *et al.,* 2020, Zhang *et al.,* 2004). Flagellin is a major component of flagella used for motility and is recognized by TLR5. TLR5 receptor activation induces an inflammatory response involving flagellin-TLR5-MyD88-dependent signaling and NF-κB induction, with subsequent pro-inflammatory cytokine production (Yang *et al.,* 2017). Furthermore, Feuillet *et al.,* (2006) showed $TLR5^{-/-}$ murine models failed to successfully identify bacterial infection, leading to a poor immune response from the innate immune system.

However, overt TLR5 activation may manifest as conjunctivitis, keratitis and cellulitis, all of which can lead to poor vision and potential blindness (Iwalokun *et al.,* 2011). The most common bacterial, corneal infection is caused by *Pseudomonas aeruginosa,* a pathogen associated with visual impairment and blindness through neovascularization and liquefactive necrosis of the cornea (Evans *et al.,* 2013, Kolar *et al.,* 2011). *P. aeruginosa* infection can develop into inflammatory keratitis, with TLR5 characterised as a driving force during the corneal inflammatory response, leading to NF-κB activation and subsequent IL-6, IL-8 and IL-1β production from corneal fibroblasts and macrophages, alongside CXCL1 chemokine (Cendra *et al.,* 2017, Kaur *et al.,* 2015). Furthermore, Sun *et al.,* (2010) used neutralizing anti-TLR5 antibodies to highlight improper TLR5 signaling during inflammatory corneal damage, demonstrating the dangers of overt TLR5 signaling. These overt TLR5 responses have been associated with impairment of epithelial barrier integrity during bacterial infection, which may allow entry for opportunistic pathogens, therefore wound healing is required through re-epithelization (Lopetuso *et al.,* 2017). Anti-microbial peptides (AMPs) are produced to not only aid in bacterial clearance through membrane destruction, but also aid in corneal epithelium healing in response to overt inflammatory damage (Kolar

et al., 2011), highlighting the importance of TLR5 signaling and AMP production working in sync during corneal inflammation.

5.1.2 Vitamin D and TLR5

Vitamin D deficiency is commonly associated with a higher rate of bacterial colonization, including *P. aeruginosa* (Park *et al.,* 2017, Chalmers *et al.,* 2012). Liu *et al.,* (2009d) implicated 1,25D3 in positively regulating AMP hBD-2 production from keratinocytes, which is beneficial for ocular surface wound healing. Furthermore, 1,25D3 has been shown to have immunomodulatory effects during TLR5-associated inflammation, with 1,25D3 pre-treated epithelial cells showing a significant suppression in IL-8 expression and an increase of hBD-2, in response to the gram-negative bacteria *Salmonella* (Huang *et al*., 2016a, Song *et al.,* 2017). Reins *et al.,* (2015a) began to characterise the immunomodulatory effect of 1,25D3 upon inflammatory responses from corneal cells following *P. aeruginosa* exposure, with 1,25D3 treatment significantly increasing antimicrobial activity in the form of AMP LL-37, concluding that 1,25D3 treatment hindered colony growth gram-negative bacteria. Furthermore, 1,25D3 treatment of corneal epithelial cells following *Salmonella typhimurium,* another example of a gram-negative bacteria, led to a significant suppression in pro-inflammatory matrix metallopeptidase 9 (MMP9), a powerful regulator of inflammation associated with ocular surface damage (Reins *et al.,* 2017b, Acera *et al.,* 2013). FLA-ST, flagellin isolated from the gram-negative bacteria *Salmonella typhimurium,* was selected due to Reins *et al.,* (2015a) showing success at inducing an anti-microbial immune response with human corneal cells.

5.1.3 miR and TLR5 inflammation

As discussed throughout subsection 1.5.2, and throughout chapters 3 and 4, miR are regulatory molecules believed to modulate inflammatory responses through genetic transcription. Compared to the roles of miR during inflammatory responses during TLR3 and TLR9 signaling, research analyzing miR activity during TLR5 signaling is scarce. However, Taganov *et al.,* (2006) described miR-146a as a highly 'anti-inflammatory' regulator, suppressing expression TLR5 through inhibiting the NF-κB pathway by IRAK and TRAF6 regulation, both of which are described as 'crucial' for TLR5

signaling (O'Neill *et al.,* 2011). In a similar fashion, miR-155 is a known as an inflammation regulator that has the ability to suppress a range of proteins, including pro-inflammatory signal transducers such as IκBα and MyD88, both of which are required during TLR5 signaling (Ceppi *et al.,* 2009, Tang *et al.,* 2010, Hajam *et al.,* 2017). In regard to *P. aeruginosa*, miR-93 is downregulated in bronchial epithelial cells in response to the infection, with Fabbri *et al.,* (2014) implicating this miR during the upregulation of IL-8 and subsequent tissue damage. Throughout chapters 3 and 4, this thesis has already provided evidence of miR-93-5p and miR-181-3p regulating IL-6, IL-8 and IL-1β pro-inflammatory mediators during TLR3 and TLR9 signaling. Interestingly, Jeon *et al.,* (2015) reported that these same pro-inflammatory mediators are also elevated during TLR5 stimulation.

5.1.4. Chapter aims and hypothesis

It is crucial to investigate therapeutic options for bacterial infections which may breach physical barriers of the ocular surface, with overt TLR5 signalling leading to tissue damage. However, these immune responses can also be beneficial, for example AMPs not only kill bacteria, but functionally, also contribute to epithelial tissue repair. Reins *et al.,* (2015a) began to identify the immunomodulatory role of 1,25D3 enhancing antimicrobial activity against *P. aeruginosa* in corneal cells, which in turn, would be expected to prevent further immune responses, such as pro-inflammatory cytokine production. Within chapter 3, this thesis has already confirmed expression of TLR5 by hTCEpi cells (Figure 3.1A). Therefore, the aim of the experiments within chapter 5 was to investigate the immunomodulatory effect of 1,25D3 upon TLR5 stimulation of hTCEpi cells in response to gram-negative bacteria in further detail. This was completed by analysing the expression of a wider range of pro-inflammatory mediators and antimicrobial peptides. Finally, was hypothesized that at least one of the selected miR would aid in the suppressive 1,25D3 mechanism.

5.2 Results

5.2.1 hTCEpi cells exposed to 24h of FLA-ST do not express a detectable pro-inflammatory cytokine response

The aim of this experiment was to determine if FLA-ST would induce a pro-inflammatory effect in hTCEpi cells. To investigate this, hTCEpi cells were stimulated for 24h using various concentrations of FLA-ST; 10ng/ml, 50ng/ml and 100ng/ml, following the manufacturer's instructions. To confirm TLR5 stimulation, both TLR5 and pro-inflammatory cytokine IL-8 mRNA expression were analysed. The results showed that FLA-ST did not induce an inflammatory response in hTCEpi cells under any concentration after 24h exposure, as indicated with the non-significant change IL-8 (Figure 5.1A), but there was a significant change in TLR5 expression (Figure 5.1B, $p<0.001$).

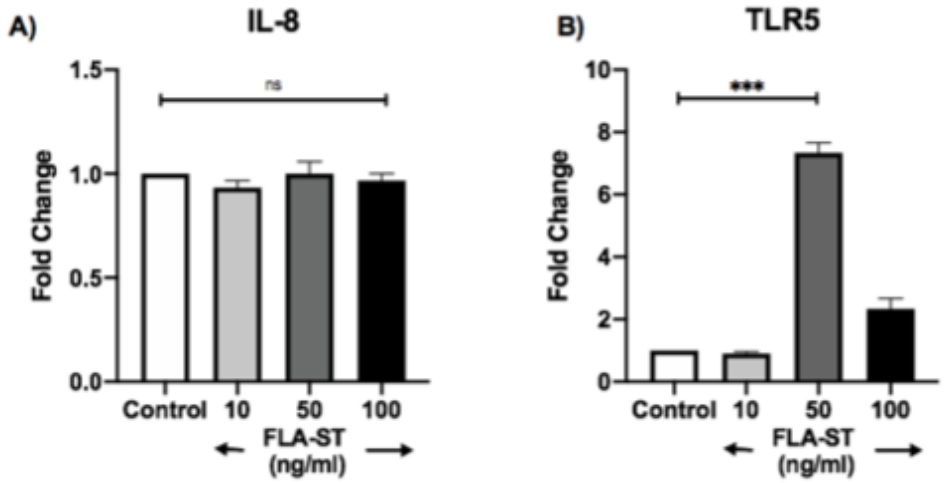

Figure 5.1: Optimising FLA-ST concentration to induce a pro-inflammatory response through TLR5 activation: mRNA expression of (A) IL-8 and (B) TLR5 by hTCEpi cells after exposure to various concentrations of FLA-ST. Data represent mean ± SEM of three independent experiments. Statistical analysis was by one-way ANOVA with Bonferroni test for multiple comparisons (A and B, n=3), p<*0.05, **0.01, ***0.001.

5.2.2 hTCEpi cells exposed to 24h of FLA-ST express a significant increase of anti-microbial peptides

Although 24h of FLA-ST exposure failed to induce detectable levels of IL-8 (Figure 5.1A), the aim of these experiments was to observe if hTCEpi cells would produce AMPs in response to 24h TLR5 stimulation with FLA-ST. To investigate this, hTCEpi cells were stimulated for 24h using various concentrations of FLA-ST; 10ng/ml, 50ng/ml and 100ng/ml, following the manufacturer's instructions. The chosen AMPs for analysis were hBD-2, hBD-3 and LL-37 (Figure 5.2). It was decided that LL-37 would be analysed rather than hCAP-18 due to the previous investigations within the Reins *et al.*, (2015a) study. The results showed that 50ng/ml of FLA-ST for 24h induced a significant increase in the expression of both hBD-2 (A, $p<0.001$) and LL-37 (C, $p<0.001$). 100ng/ml of FLA-ST also produced a significant increase in the mRNA expression hBD-2 (A, $p<0.01$) and LL-37 (C, $p<0.05$), with similar results seen from 10ng/ml (A, $p<0.05$) for hBD-2 and (C, $p<0.01$). However, the results from this experiment showed that no concentration induced a significant increase in expression of hBD-3 (B).

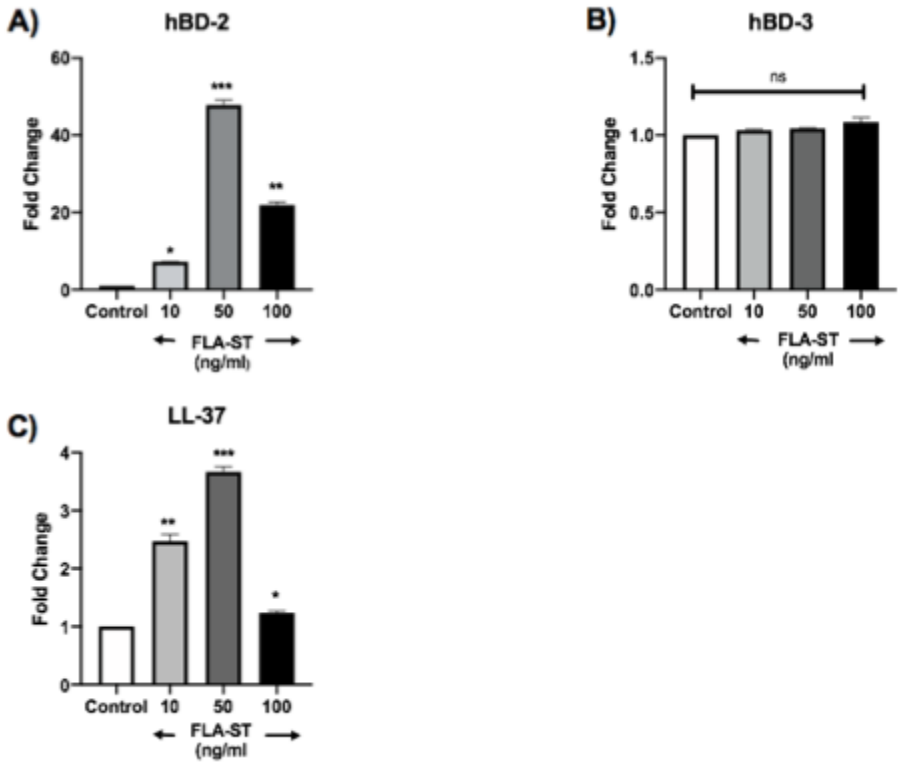

Figure 5.2: Optimising FLA-ST concentration to induce an anti-microbial response from hTCEpi cells: mRNA expression of (A) hBD-2, (B) hBD-3 and (C) LL-37 by hTCEpi cells after exposure to various concentrations of FLA-ST. Data represent mean ± SEM of three independent experiments. Statistical analysis was by one-way ANOVA with Bonferroni test for multiple comparisons (A-C, n=3), p<*0.05, **0.01, ***0.001.

5.2.3 1,25D3 increases LL-37 expression by hTCEpi cells following 24h of FLA-ST stimulation

The aim of this experiment was to observe if 10^{-7}M 1,25D3 exposure would stimulate an increase in the expression of either hBD-2 or LL-37 AMPs, which would be beneficial in clearing a bacterial infection. The AMP hBD-3 was not analysed, due to the lack of significant result seen in Figure 5.2B, concluding that it hBD-3 was not expressed by these cells in response to 24h of FLA-ST. The results showed 24h of FLA-ST stimulation significantly increased hBD-2 expression (Figure 5.3A, p<0.001), and the dual treatment of FLA-ST and 1,25D3 significantly decreasing expression compared to FLA-ST exposure alone (p<0.001). In regard to hTCEpi cell LL-37 expression, FLA-ST treatment for 24h significantly increased expression (Figure 5.3B, p<0.001),

whilst the combination treatment of FLA-ST and 1,25D3 significantly increased LL-37 expression further ($p<0.001$).

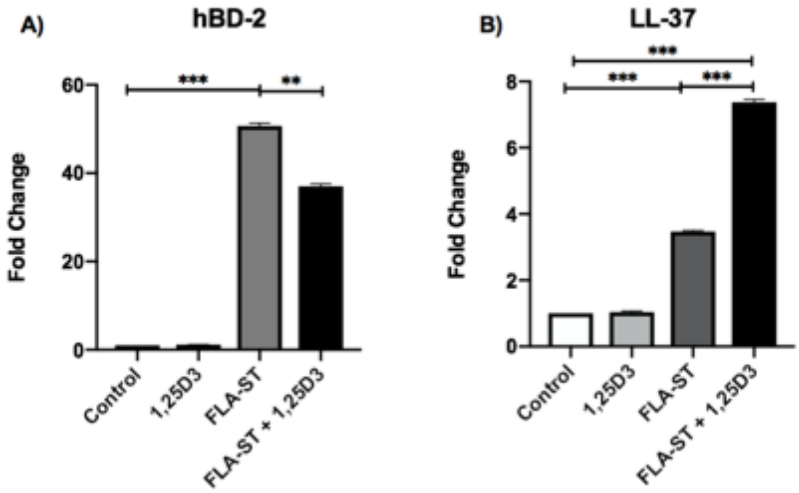

Figure 5.3a: Analysing the effects of 1,25D3 upon induced AMP expression in response to FLA-ST by hTCEpi cells: mRNA expression of (A) hBD-2 and (B) LL-37 by hTCEpi cells after exposure to various experimental conditions. Data represent mean ± SEM of three independent experiments. Statistical analysis was by one-way ANOVA with Bonferroni test for multiple comparisons (A and B, n=3), p<*0.05, **0.01, ***0.001.

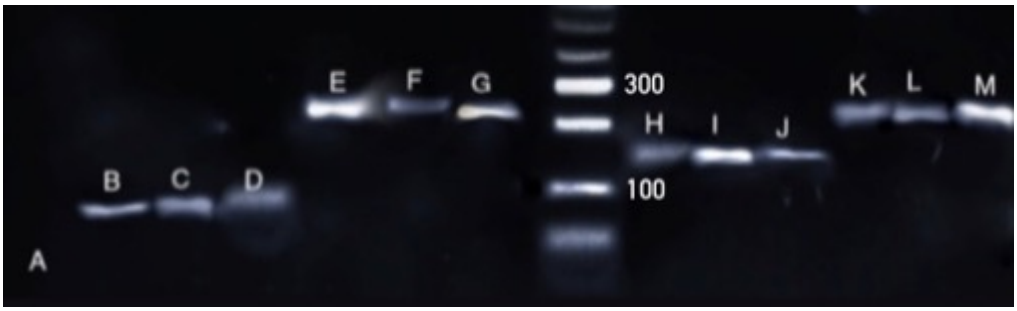

Figure 5.3b: Agarose gel electrophoresis confirmation of qPCR products from figure 5.2a: confirmation of the following qPCR products from each sample to confirm primer efficiency: (A) negative control, (B) control sample – GAPDH, (C) FLA-ST sample – GAPDH, (D) 1,25D3 + FLA-ST, (E) control sample – hBD-2, (F) FLA-ST sample – hBD-2, (G) 1,25D3 + FLA-ST - hBD-2, (H) control sample – hBD-3, (I), FLA-ST – hBD-3, (J) 1,25D3 + FLA-ST – hBD-3, (K) control sample - LL-37, (L) FLA-ST – LL-37 and (M) 1,25D3 + FLA-ST – LL-37.

5.2.4 hTCEpi cells exposed to 24h of FLA-PA express a significant pro-inflammatory response

Data demonstrated that FLA-ST induced a pro-inflammatory response in the acute inflammatory response, which was within 4-6h of flagellin treatment. However, as FLA-ST did not induce a significant increase in IL-8 expression (Figure 5.1A) within the 24h time period, an alternate source of flagellin, at a stronger dose, was used in an attempt to induce a pro-inflammatory response by hTCEpi cells. This would allow confirmation of 1,25D3 suppressing a pro-inflammatory response induced by TLR5 signalling within 24h. Once again, IL-8 and TLR5 expression was measured following 24h of exposure to various FLA-PA concentrations. The results showed that 5μg/ml of FLA-PA induced the highest inflammatory response, reflected in a significant increase of both TLR5 (Figure 5.4B, $p<0.001$) and IL-8 (A, $p<0.001$) expression. Although 6μg/ml of FLA-PA also induced significant increases within the expression of both TLR5 and IL-8 ($p<0.001$) expression, a stronger inflammatory response was obtained from the 5μg/ml conditions, therefore, this concentration of FLA-PA was chosen for future experiments. The condition using 2μg/ml of FLA-PA induced no significant change in expression of either TLR5 or IL-8 (Figure 5.4, A-B).

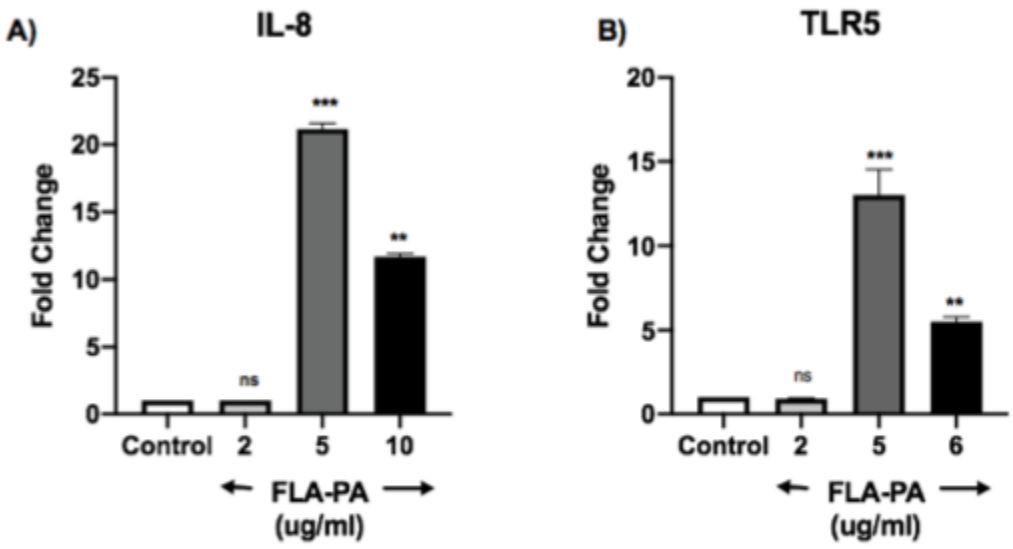

Figure 5.4: Optimising FLA-PA concentration to induce a pro-inflammatory mediator response through TLR5 activation: mRNA expression of (A) IL-8 and (B) TLR5 by hTCEpi cells after exposure to various concentrations of FLA-PA. Data represent mean ± SEM of three independent experiments. Statistical analysis was by one-way ANOVA with Bonferroni test for multiple comparisons (A and B, n=3), p<*0.05, **0.01, ***0.001.

5.2.5 FLA-PA induces expression of various anti-microbial peptides in hTCEpi cells after 24h of exposure

As FLA-ST successfully induced LL-37 and hBD-2 (Figure 5.2, A-B), the aim of this experiment was to observe if hTCEpi cells would produce AMPs in response to 24h stimulation with FLA-PA, another example of a gram-negative bacteria. To investigate this, hTCEpi cells were stimulated for 24h using various concentrations of FLA-PA; 2μg/ml, 5μg/ml and 10μg/ml, following the manufacturer's instructions. The chosen AMPs for analysis were hBD-2, hBD-3 and LL-37 (Figure 5.5, A-C), similar to those investigated in the FLA-ST experiments. The results showed that 5μg/ml of FLA-PA for 24h induced a significant increase in the expression of both hBD-2 (A, $p<0.001$) and LL-37 (C, $p<0.001$). 10μg/ml of FLA-PA also produced a significant increase in the mRNA expression hBD-2 (A, $p<0.05$) and LL-37 (C, $p<0.01$), with similar results seen from 2μg/ml (A, $p<0.01$) for hBD-2 and (C, $p<0.01$) for LL-37. However, all conditions failed to induce significant hBD-3

expression within 24h (B), similar to the results found from the FLA-ST experiments.

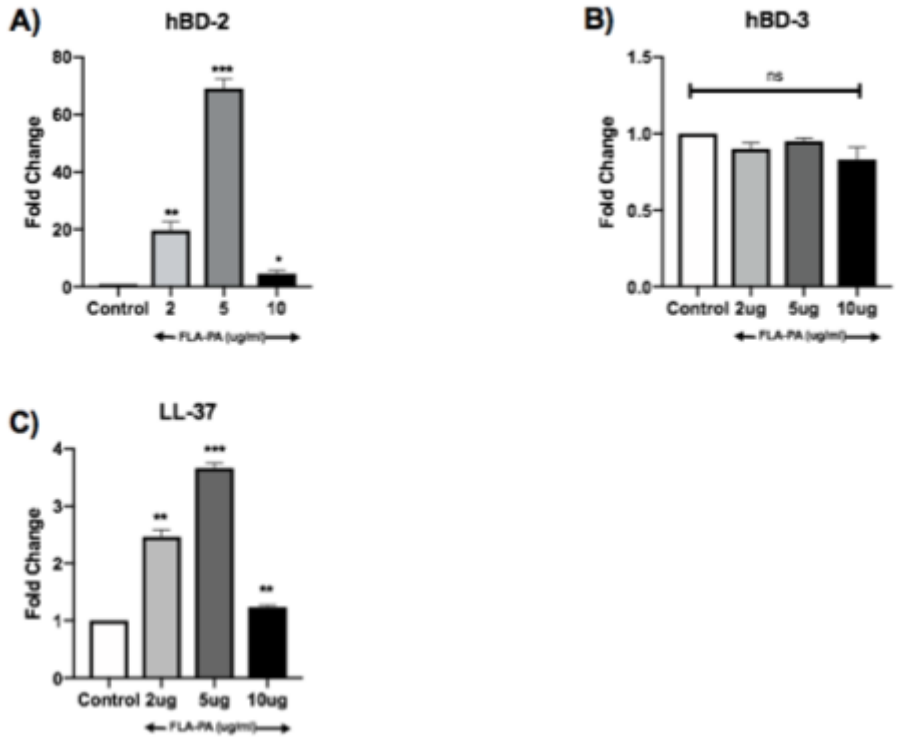

Figure 5.5: Optimising FLA-PA concentration to induce an anti-microbial response from hTCEpi cells: mRNA expression of (A) hBD-2, (B) hBD-3 and (C) LL-37 by hTCEpi after exposure to various concentrations of FLA-PA. Data represent mean ± SEM of three independent experiments. Statistical analysis was by one-way ANOVA with Bonferroni test for multiple comparisons (A- C, n=3), p<*0.05, **0.01, ***0.001.

5.2.6 1,25D3 increases LL-37 expression by hTCEpi cells following 24h of FLA-PA stimulation

The aim of this experiment was to observe if 10^{-7}M 1,25D3 exposure would stimulate an increase in the expression of either hBD-2 or LL-37 AMPs produced in response to FLA-PA, which would be beneficial in clearing a bacterial infection. The AMP hBD-3 was not analysed due to the lack of significant expression seen in Figure 5.5B, concluding that it hBD-3 would not be expressed by these cells after 24h, which would mimic the resolution period of inflammation. The results showed that in regard to hBD-2, 24h of FLA-PA stimulation significantly increased expression (Figure 5.6A, $p<0.001$), and the dual treatment of FLA-ST and 1,25D3 significantly decreased expression compared to FLA-PA exposure alone ($p<0.001$). In regard to LL-37 expression, 24h FLA-PA treatment significantly increased expression (Figure 5.6B, $p<0.001$), whilst the combination treatment of FLA-PA and 1,25D3 significantly increased LL-37 expression further ($p<0.001$).

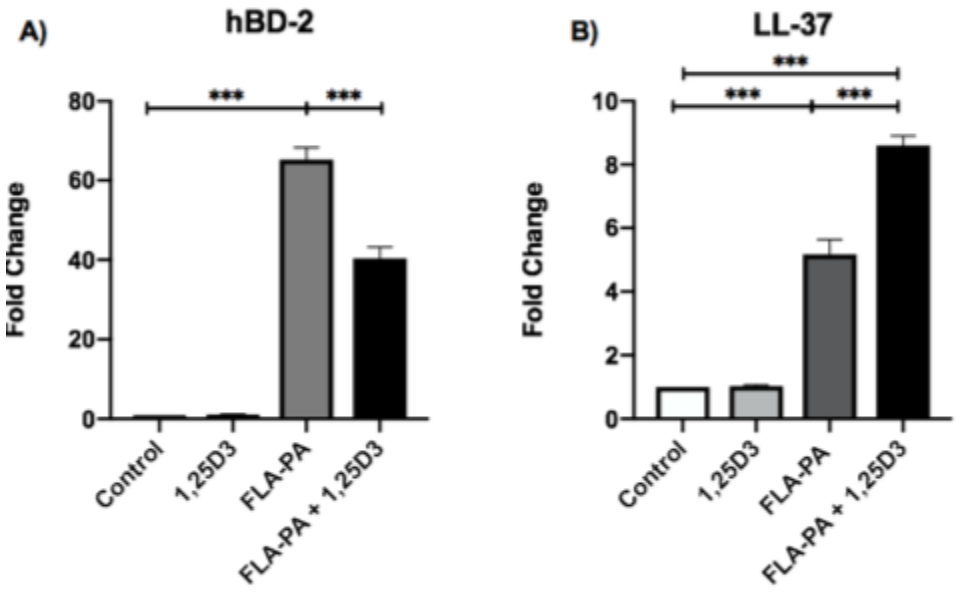

Figure 5.6: Analysing the effects of 1,25D3 upon hTCEpi cells AMP expression induced by FLA-PA: mRNA expression of (A) hBD-2 and (B) LL-37 by hTCEpi cells after exposure to various experimental conditions. Data represent mean ± SEM of three independent experiments. Statistical analysis was by one-way ANOVA with Bonferroni test for multiple comparisons (A and B, n=3), p<*0.05, **0.01, ***0.001.

5.2.7 Pro-inflammatory mediators induced by FLA-PA TLR5 stimulation are suppressed by 1,25D3

Stimulating TLR5 of hTCEpi cells for 24h with 5μg/ml FLA-PA, IL-6, TLR5 and IL-8 expression was analysed in the presence or absence of 10^{-7}M 1,25D3, to identify a potential immunomodulatory response of 1,25D3. The increase in TNF-α expression was found to be insignificant (not pictured). The results showed that FLA-PA exposure increased the expression of IL-6, IL-8, TNF-α and TLR5 significantly (Figure 5.7. A-D, $p<0.001$). In comparison, there was a significant decrease seen in IL-6, IL-8 and TLR5 (C, $p<0.05$) and TLR9 (A-C, $p<0.05$) during the dual treatment of FLA-PA and 1,25D3. There was no significant suppression of TNF-α in this condition (D).

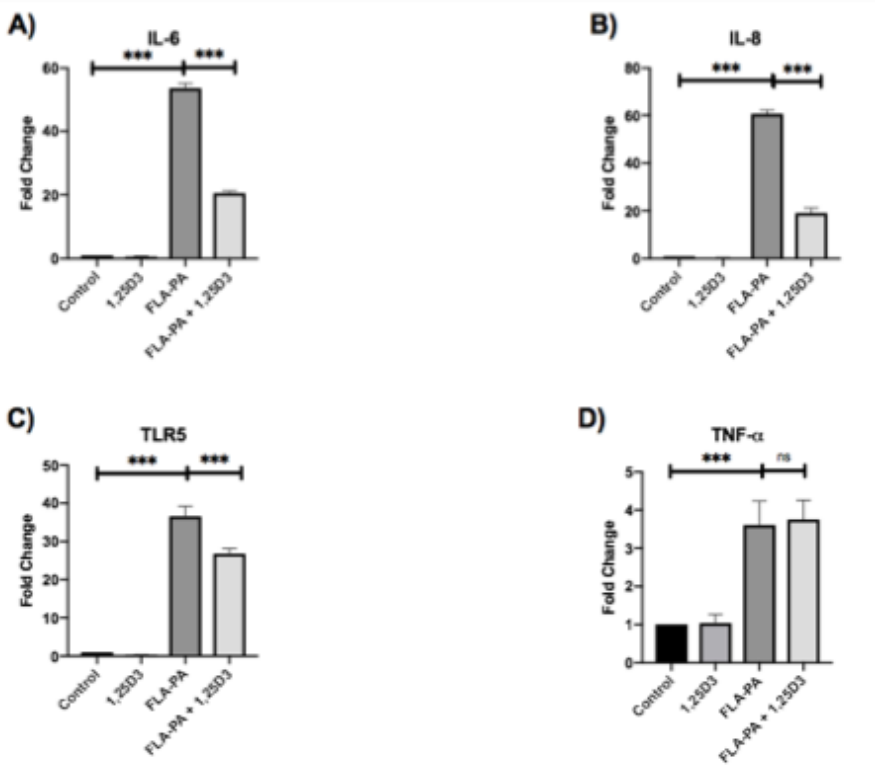

Figure 5.7: Pro-inflammatory mediator expression by hTCEpi cells following 24h TLR5 activation with various conditions of 5μg/ml FLA-PA and 1,25D3: Pro-inflammatory mediator mRNA expression by hTCEpi cells after exposure to 5μg/ml FLA-PA TLR5 agonist and 1,25D3. (A) IL-6 (B) IL-8 (C) TLR5 and TNF-α (D). Data represent mean ± SEM of three independent experiments. Statistical analysis was by one-way ANOVA with Bonferroni test for multiple comparisons (A- E, n=3), p<*0.05, **0.01, ***0.001

5.2.8 1,25D3 began suppressing pro-inflammatory mediators IL-6 and IL-8, alongside TLR5, during ongoing FLA-PA stimulation by 6h

As the previous results from Figure 5.7 showed that 1,25D3 suppressed a range of hTCEpi cell pro-inflammatory mediators during ongoing FLA-PA TLR5 stimulation for 6h, the study aimed to analyze the possible timeframe of this suppressive action to take place. The results showed that there was a significant induction of IL-6, IL-8, TLR5 and TNF-α following 6h FLA-PA stimulation (Figure 5.8, A-D, $p<0.001$). Furthermore, 1,25D3 significantly suppressed the expression of IL-6 (A, $p<0.01$), IL-8 and TLR5 (B and C, $p<0.001$) within 6h. However, 1,25D3 treatment did not significantly suppress TNF-α expression, in fact, there was a significant increase of TNF-α expression in the dual combination of FLA-PA and 1,25D3 (D, $p<0.05$).

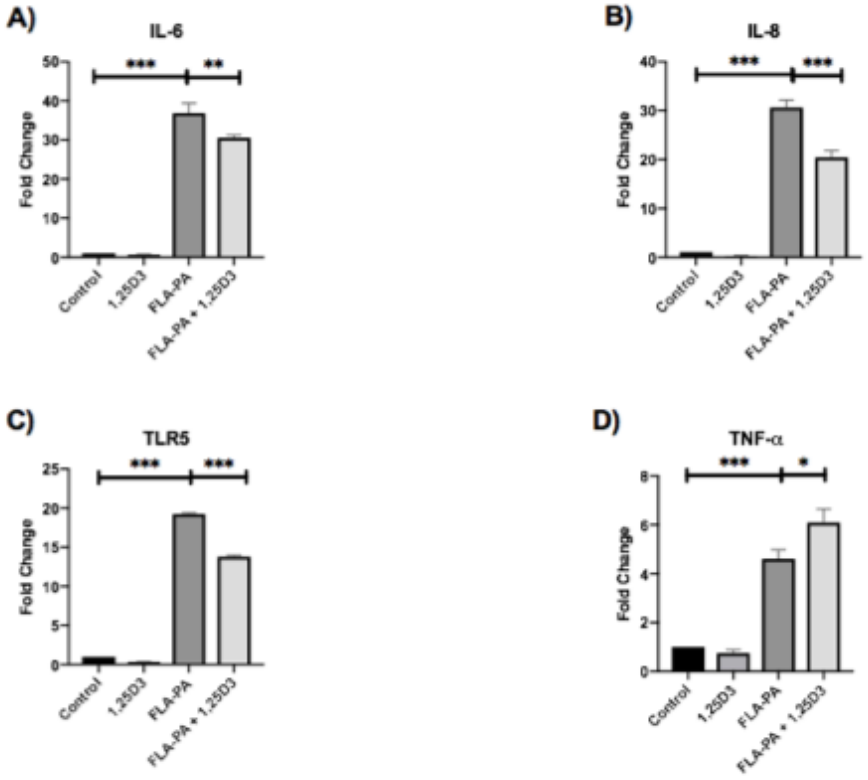

Figure 5.8: Expression of hTCEpi cells pro-inflammatory mediators following 6h of TLR5 activation during various conditions of 5μg/ml FLA-PA and 1,25D3: Pro-inflammatory mediator mRNA expression by hTCEpi cells with FLA-PA and 1,25D3 treatment for 6h. Analyse include: (A) IL-6, (B) IL-8, (C) TLR5 and (D) TNF-α. Data represent mean ± SEM of three independent experiments. Statistical analysis was by one-way ANOVA with Bonferroni test for multiple comparison (A-D, n=3) p<*0.05, **0.01, ***0.001

5.2.9 1,25D3 did not suppress IL-6, IL-8, TLR5 or TNF-α expression by 4h of FLA-PA stimulation

To analyze the potential time-frame required for 1,25D3 to suppress TLR5 signaling further, the previous experiment was replicated, but stopped at 4h. The results (Figure 5.8) showed that there a significant induction of IL-6, IL-8, TLR5 and TNF-α following 4h FLA-PA stimulation (A-D, $p<0.001$). However, 1,25D3 treatment did not significantly suppress the expression of IL-6, IL-8 and TLR5 during this 4h period (A-D).

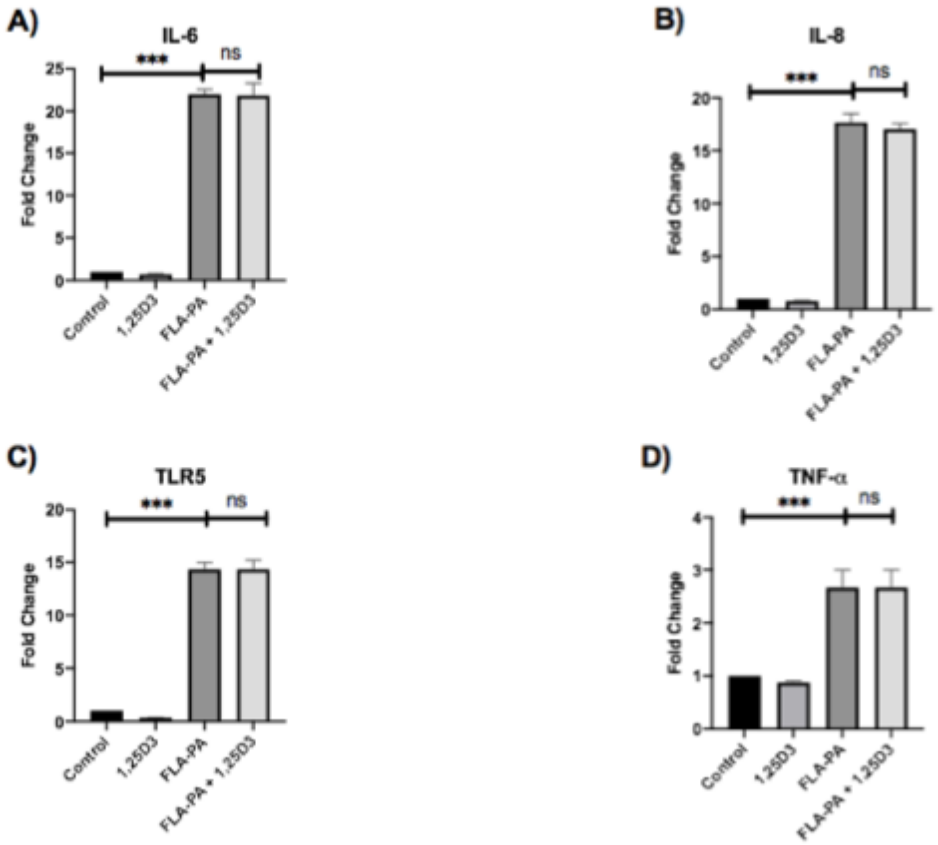

Figure 5.9: Pro-inflammatory mediator expression by hTCEpi cells following 4h TLR5 activation with various conditions of 5μg/ml FLA-PA and 1,25D3: (A) IL-6, (B) IL-8, (C) TLR5 and (D) TNF-α. Data represent mean ± SEM of three independent experiments. Statistical analysis was by one-way ANOVA with Bonferroni test for multiple comparison (A-D, n=3) p<*0.05, **0.01, ***0.001

5.2.10 1,25D3 suppressed IL-6, IL-8 and TLR5 expression by hTCEpi cells following 6h of prior FLA-PA pre-treatment

The previous results in Figure 5.8 provided evidence for 1,25D3 suppression of IL-6, IL-8 and TLR5 by 6h during ongoing TLR5 stimulation. Therefore, additional experiments were performed, which aimed to investigate if increased expression of these pro-inflammatory mediators, resulting from a 4h treatment with FLA-PA and TLR5 activation, could be suppressed by 1,25D3 (Figure 5.10). This would represent TLR5 activation from infections of the ocular surface caused by bacterium possessing flagellin. The results showed a significant decline in the expression of IL-6 (A, $p<0.001$), IL-8 (B, $p<0.001$) and TLR5 (C, $p<0.001$). The combined treatment of FLA-PA and 10^{-7}M 1,25D3 treatment, however, led to no significant effect upon the expression of TNF-α. These results provided evidence that 1,25D3 is able to suppress inflammation related to some pro-inflammatory mediators caused by prior TLR5 stimulation.

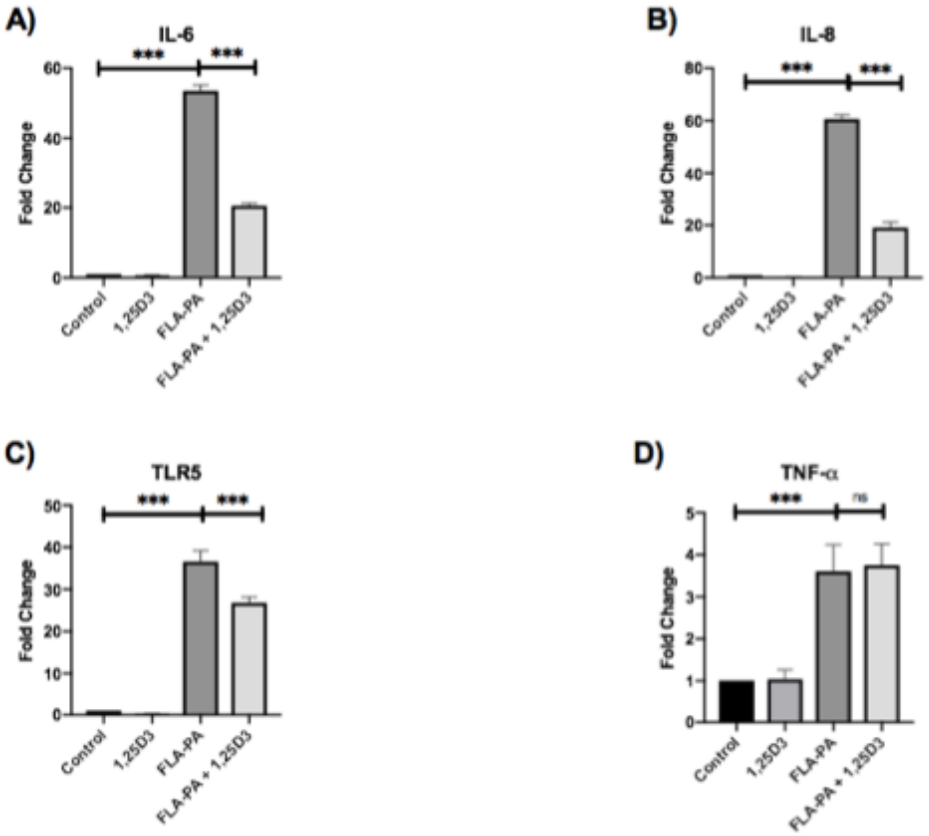

Figure 5.10: Pro-inflammatory mediator expression by hTCEpi cells following TLR5 activation during 4h of prior 5μg/ml FLA-PA exposure, then 1,25D3 treatment: The mRNA expression analysis includes: (A) IL-8, (B) IL-6, (C) TLR5 and (D) TNF-α. Data represent mean ± SEM of three independent experiments. Statistical analysis was by one-way ANOVA with Bonferroni test for multiple comparison (A-D, n=3) p<*0.05, **0.01, ***0.001

5.2.11 FLA-PA (5μg/ml) had a significant impact upon hTCEpi ce;; cellular viability

As the previous experiments showed that 1,25D3 suppressed the pro-inflammatory response of hTCEpi cells to bacteria, the aim of the following experiments was to explore cell viability following the initial exposure to this treatment. The results showed that 5μg/ml of FLA-PA for 24h did significantly lower the viability of hTCEpi cells in comparison to the unstimulated cells (Figure 5.11B, $p<0.05$) which may have contributed to an inflammatory environment resulting from agonist toxicity and subsequent dying cells. The chosen concentration of FLA-ST in the anti-microbial expression experiments, 50ng/ml, led to no significant decreased in cell viability (A). Increasing the concentration of both agonists decreased hTCEpi cell viability significantly, seen at 100ng/ml of FLA-ST and 6μg/ml of FLA-PA (A-B, $p<0.01$). However,

1,25D3 treatment alongside stimulation of both FLA-ST and FLA-PA improved cell viability (A-B).

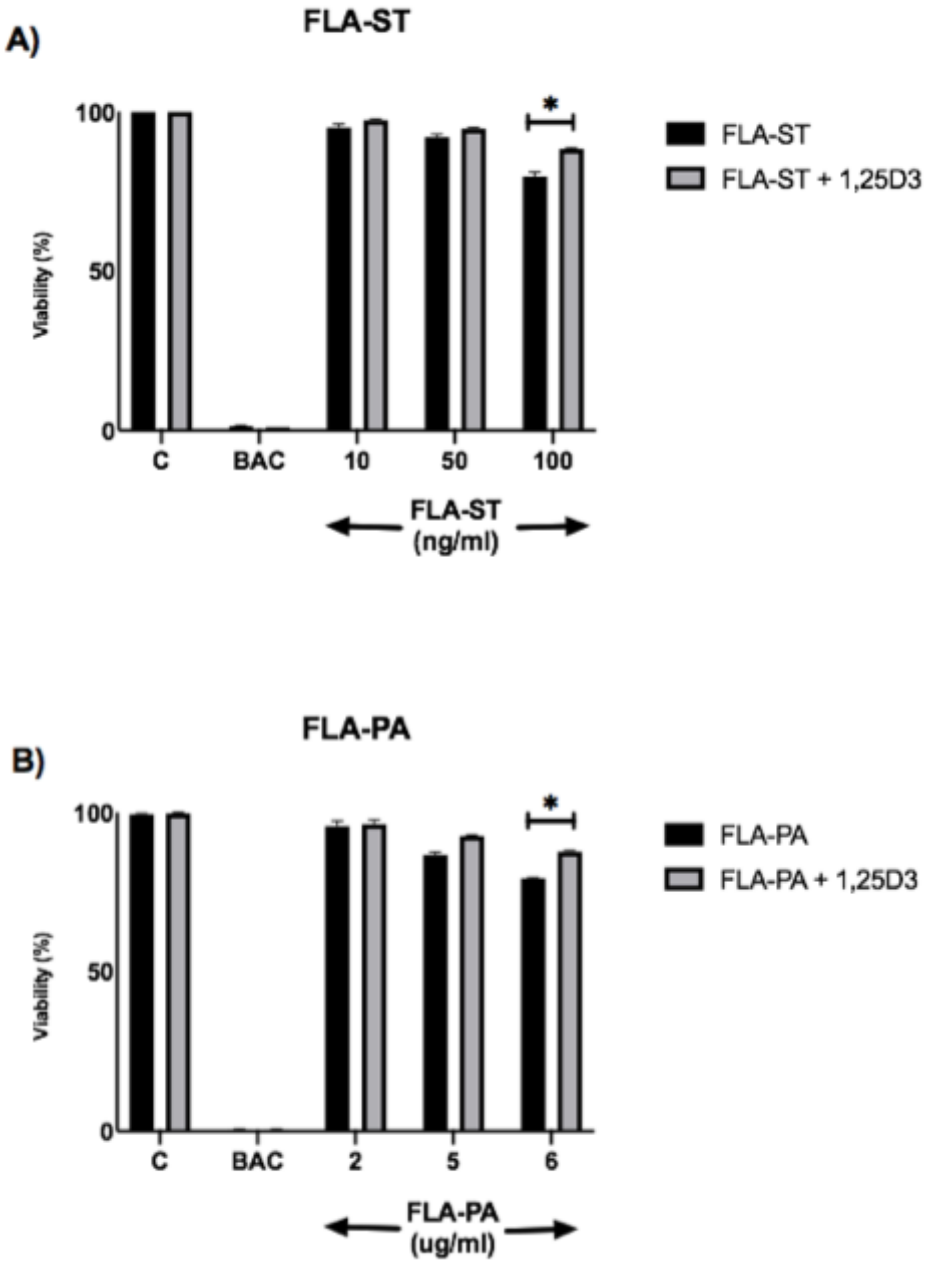

Figure 5.11: hTCEpi cell viability under the various conditions of TLR5 stimulation with FLA-ST, FLA-PA and 1,25D3: MTT analysis of hTCEpi cells following 24h exposure to 10, 50 and 100ng/ml of (A) FLA-ST and 2, 5 and 6μg/ml of (B) FLA-PA, with and without 10^{-7}M 1,25D3. Negative control utilised unstimulated hTCEpi cells (C) whilst the positive control was generated by hTCEpi cells with BAC (BAC). Cells are shown as a percentage of viability in comparison to unstimulated cells. Statistical analysis was by one-way ANOVA with Bonferroni test for multiple comparisons against completely unstimulated hTCEpi cells (A and B, n=3) p<*0.05, **0.01, ***0.001

5.2.12 FLA-PA stimulation of TLR5 increases the expression of miR-93-5p, miR-181-3p and miR-155-5p

Similar to the experiments observing potential changes in miR expression during TLR3 and TLR9 signaling (chapters 3 and 4), the aim of the following experiments was to observe the expression of miR-93-5p, miR-146a-5p, miR-155-5p and miR-181-3p during TLR5 stimulation using FLA-ST and FLA-PA (Figure 5.12, A-B). The experiment began by investigating potential changes in miR expression during hTCEpi cell TLR5 stimulation by FLA-ST, with the results showing no significant change in expression for any of the analysed miR (A). Yet, the results following 24h stimulation of hTCEpi cells with FLA-PA showed significant increase in expression for miR-93-5p, miR-155-5p and miR-181-3p (B, $p<0.001$), with significant results seen for the dual treatment FLA-PA and 1,25D3 ($p<0.05$). However, no significant change was identified in regard to miR-146a-5p under any condition. Finally, in regard to hTCEpi cells exposed to 1,25D3 alone, no significant change was seen in the expression of any of the miR.

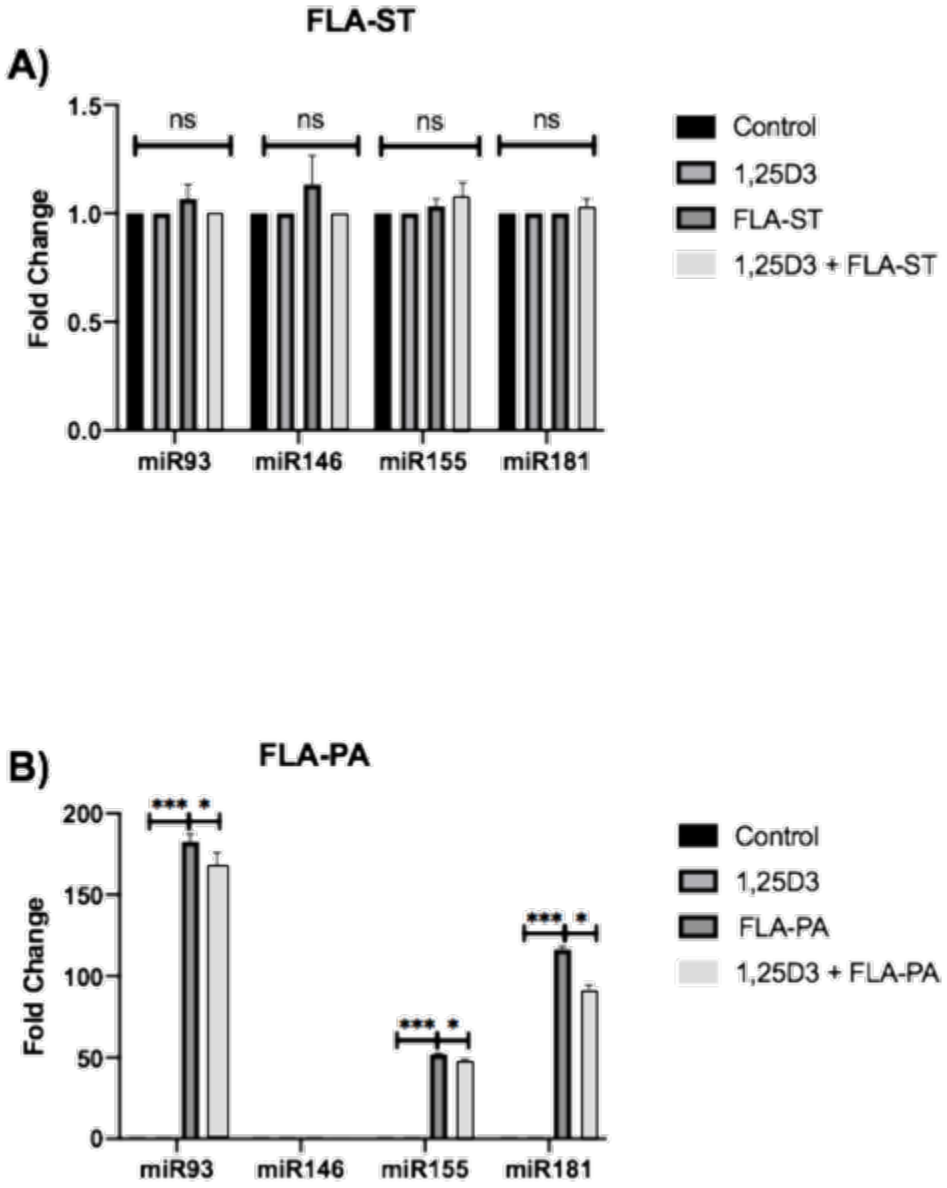

Figure 5.12: miR profile of hTCEpi cells and subsequent miR expression under various conditions of TLR5 activation using FLA-ST (50ng/ml) and FLA-PA (5μg/ml): (A) miR expression by hTCEpi cells following 24h of stimulation with and without FLA-ST and 1,25D3. (B) miR expression by hTCEpi cells following 24h of stimulation with and without FLA-PA and 1,25D3. Data represent mean ± SEM of three independent experiments. Statistical analysis was by one-way ANOVA with Bonferroni test for multiple comparisons (A and B, n=3) p<*0.05, **0.01, ***0.001

5.2.13 miR-93-5p does have a regulatory role in IL-8 and IL-6 expression during FLA-PA stimulation of TLR5

To support the results from subsection 5.2.12, an inhibitor specific to miR-93-5p was introduced to hTCEpi cells, to identify the potential role of miR-93-5p during TLR5 signaling in response to FLA-PA or 1,25D3 suppression of this inflammatory response. It was hypothesized that there would be a non-suppressive effect from 1,25D3 if miR-93-5p had a role in the mechanism of action, as these suppressive actions would be inhibited and reflected in IL-6, IL-8 and TLR5 expression. TNF-α was not considered due to the non-suppressive effect on expression (subsection 5.2.7).

However, inhibiting miR-93-5p led to a significant increase in the expression of IL-6 and IL-8 following stimulation of TLR5 with FLA-PA (Figure 5.13, A-B, $p<0.001$).

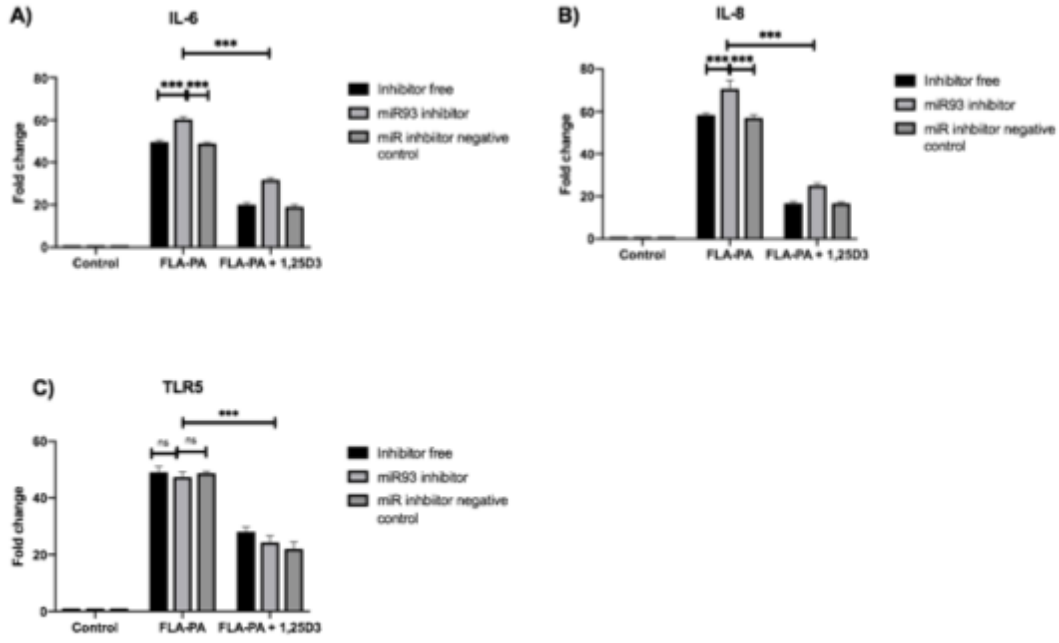

Figure 5.13: Effects of hTCEpi cell miR-93-5p inhibition upon TLR5 signalling and related mediator expression under various 24h conditions: with and without 24h FLA-PA and 1,25D3. The pro-inflammatory mediators having the greatest expression in response to FLA-PA were chosen from the previous experiments. (A) IL-6, (B) IL-8 and (C) receptor TLR5. Data represent mean ± SEM of three independent experiments. Statistical analysis was by one-way ANOVA with Bonferroni test for multiple comparisons (A-C, n=3) p<*0.05, **0.01, ***0.001

5.2.14 miR-181-3p does have a regulatory role in IL-8 and IL-6 expression during FLA-PA stimulation of TLR5

Inhibiting miR-181-3p led to a significant increase of IL-6 (Figure 5.14, A, $p<0.01$) and IL-8 (B, $p<0.001$) expression compared to unstimulated hTCEpi cells, following 24h stimulation with FLA-PA. There was no significant increase of TLR5 expression following inhibition of miR-181-3p. It was hypothesized that there would be a non-suppressive effect from 1,25D3 if miR-181-3p had a role in the mechanism of action, as this miR would not be active. However, 1,25D3 treatment of TLR5 activated cells still significantly suppressed the expression of IL-6, IL-8 and TLR5 following miR-181-3p inhibition (Figure 5.14, A-C, $p<0.001$), indicating that 1,25D3 suppression of TLR5 signaling is not dependent upon miR-181-3p activity.

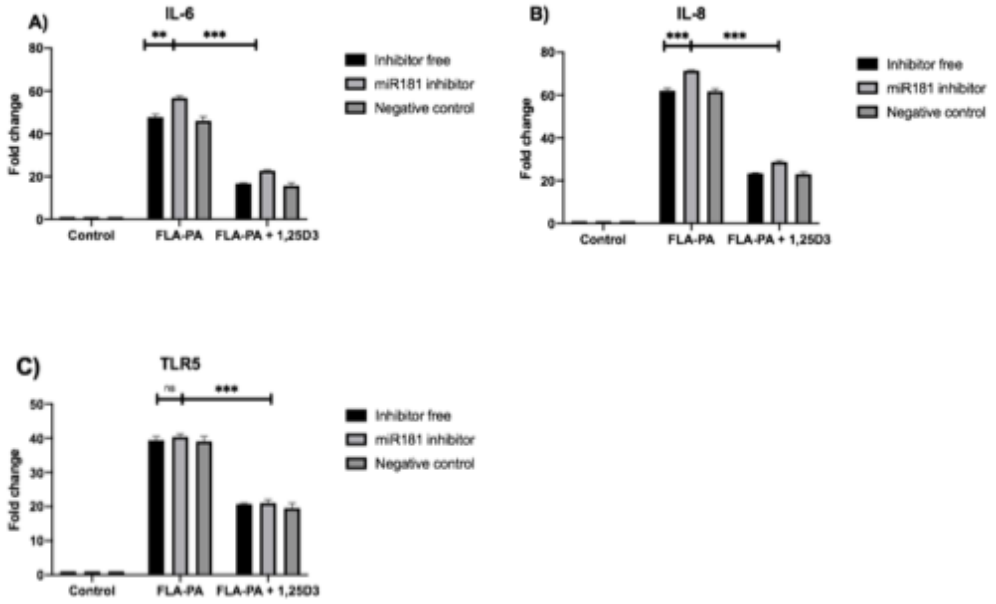

Figure 5.14: Effects of hTCEpi cell miR-181-3p inhibition upon TLR5 signalling and related cytokine expression under various 24h conditions: with and without 24h FLA-PA and 1,25D3. The pro-inflammatory mediators having the greatest expression in response to FLA-PA were chosen from the previous experiments. (A) IL-6, (B) IL-8 and (C) receptor TLR5. Data represent mean ± SEM of three independent experiments. Statistical analysis was by one-way ANOVA with Bonferroni test for multiple comparisons (A-C, n=3) p<*0.05, **0.01, ***0.001

5.3 Chapter discussion

5.3.1 hTCEpi cells form alternate pro-inflammatory responses to the flagellin of various gram-negative bacteria

The aim of the experiments performed within this chapter was to confirm if TLR5 was fully functional towards various sources bacterial flgellin, producing a range of pro-inflammatory mediators at various time points. It was hypothesized that 1,25D3 treatment would significantly suppress the hTCEpi inflammatory response, whilst promoting an increase of AMP expression.

These data showed that 24h of 10ng/ml, 50ng/ml and 100ng/ml FLA-ST exposure failed to induce hTCEpi cell IL-8 expression, but 24h FLA-PA induced a range of pro-inflammatory mediators, including TNF-α and IL-8, which are implicated in the induction of AMPs (Denning *et al.,* 1998) (Figures 5.1A and 5.7). Although numerous factors may influence TLR5 activation, including phase variation of flagellin expression leading to poor bacterial detection, the significant increase within TLR5 expression indicated that TLR5 was active during this stimulation and did detect FLA-ST (Høvding 2008). The major limitation within this experiment was the lack of various time points and the lack of protein quantification data. It would be expected that, by 24h, IL-8 production would be significantly increased and mRNA expression would be more detectable by 6h (Cheng *et al*., 2016), therefore, it would be beneficial to replicate these experiments using shorter time points.

The LPS component of *P. aeruginosa* mediates bacterial adherence by binding to the corneal cell membrane with the complete-outer-core oligosaccharide, leading to bacterial colonisation and infection (Zaidi *et al.*, 2021). Yet, further studies highlight the adhesive properties of *P. aeruginosa* flagellin, with the flagellin binding to mucins in airways (Scharfman *et al*., 2001). However, research into the adhesive properties of FLA-PA with the cornea is limited. Whilst FLA-PA induced a pro-inflammatory effect by the cornea, research concludes human corneal cells only 'respond to flagellin derived from ocular pathogens', (Hozono *et al.,* 2006, Dionne *et al.,* 2014) with ocular *Salmonella typhimurium* infections reportedly rare (Arora *et al.,* 2008), which may explain the weaker immune response to FLA-ST. Bacterial adherence assays such as agar plating would confirm potential bacterial

adherence for both FLA-PA and FLA-ST, or the use of fluorescent microscopy described by Pederson *et al*., (2018) would allow quantification of each colony with hTCEpi cells, with an expectation that FLA-PA is more adherent, reflected in the persistent and robust immune response.

However, both sources of flagellin led to a significant increase in the expression of AMPs hBD-2 and LL-37 (Figures 5.2, A and C, Figures 5.5, A and C), demonstrating an anti-microbial response by hTCEpi cells. Gombart *et al.,* (2018) defined the action of 1,25D3 binding to the VDRE of the cathelicidin gene following TLR stimulation. This increase of CAMP leads to a robust antimicrobial response, with increased phagosome formation and LL-37 activity (Nestel *et al*., 2004). The significant increase of both LL-37 and hBD-2 expression would not only aid in the removal of bacteria through bacterial killing, but would also recruit further immunocytes through chemotaxis. Furthermore, Redfern *et al.,* (2011) highlighted the potential of both of these AMPs downregulating TLR5 expression in a negative feedback loop, which would aid in suppressing an overt inflammatory response. An obvious limitation is the absence of AMP quantification, which could be supported by immunoblotting procedures demonstrating a significant increase in both AMPs. The significant increase of LL-37 expression may of indicated an anti-inflammatory response, as Hemshekhar *et al.,* (2018) demonstrated LL-37 activating the cellular IL-1RA, inducing anti-inflammatory IL-10, which would prevent overt TLR-driven responses. It would be interesting to explore this concept by confirming changes within IL-10 mRNA expression using the methods within this thesis, alongside mediators such as IL-12 and IFN-γ which contribute to corneal damage in response to FLA-PA infection (Hazlett 2005).

By 24h, the expression of hBD-3 may have returned to unstimulated expression following FLA-ST and FLA-PA stimulation (Figures 5.2 and 5.5), however, the absence of epidermal growth factor receptor (EGFR) within the EpiLife medium used to sustain the hTCEpi cells may have been an influencing factor. Exposing the cells to EGFR would activate the EGFR pathway through dimerization of receptor within the membrane, potentially leading to hBD-3 production (Steubesand *et al.*, 2009). In future experiments, the use of EpiLife Defined Growth Supplement (ThermoFisher Scientific,

USA) may support hBD-3 production from hTCEpi cells, which would lead to detectable, significant expression by 24h.

5.3.2 1,25D3 modulated both pro-inflammatory cytokine and anti-microbial expression following TLR5 activation of hTCEpi cells

As FLA-PA induced the strongest immune response at 24h, this model was used for the 1,25D3 experiments to represent the resolution period of corneal inflammation. The resolution period of inflammation represents a significant decline within pro-inflammatory mediators and immune cell migration following the removal of a pathogen, usually occurring by 24h. Data confirmed 1,25D3 treatment significantly suppressed the expression of IL-6 and IL-8 following FLA-PA exposure, which may be considered beneficial as IL-6 is a strong inducer of the corneal acute inflammatory response (Cole *et al.,* 1999, Santacruz *et al.,* 2015). Furthermore, Youker *et al.,* (1992) demonstrated IL-6 upregulating ICAM-1 and neutrophil priming, encouraging neutrophil infiltration. The suppression of IL-8 would expectedly lead to suppression in angiogenesis and inflammatory damage from ocular inflammation (Ghasemi *et al.,* 2011). As both IL-6 and IL-8 suppression occurred within a 6h timeframe, this could prevent overt inflammatory damage, but still allow for the acute inflammatory response (Figure 5.8, A-B). However, 6h of FLA-PA and 1,25D3 treatment increased TNF-α. Increased TNF-α expression in response to FLA-PA infections are associated with corneal ulceration and bacterial keratitis (Thakur *et al.,* 2002). Therefore, this increase of TNF-α is associated with the increased production of tissue-damaging enzymes and polymorphonuclear leukocytes, which aggravates the inflammatory response (Thakur *et al.,* 2002). However, the time-point of 6h represents the acute inflammatory response needed to remove invading pathogens, and this increase of TNF-α could be beneficial to protect the ocular surface.

The same 1,25D3 treatment also significantly increased the expression of LL-37 in response to FLA-ST and FLA-PA, however, there was no change of expression detected for either hBD-2 or hBD-3. Considering that *P. aeruginosa* is the most common cause of bacterial infection within the cornea for contact lens wearers, these results are of significance. The process of

corneal re-epithelization increases the risk of developing bacterial infection due a broken physical barrier of epithelial cells, therefore, increased LL-37 production may lower the risk of bacterial infection. Topical 1,25D3 treatment delays wound healing by 17% *in vivo* and increases neutrophil influx into the cornea (Reins *et al.,* 2015a). In future studies, it would be beneficial to incubate hTCEpi cells treated with 1,25D3 to a live clinical isolate of *P. aeruginosa*, to observe a potential increase in the bacterial killing rate and subsequent decrease of related colonies. The experiments discussed in this thesis used heat-inactivated *E. c*oli DNA, which is easily accessible, safer than live isolates due to less risk of infection and pharmaceutical ease of transport and storage. However, one of the major benefits of using live isolates is that their use exposes hTCEpi cells to other bacterial components, such as LPS for TLR4. Targeting hTCEpi cells with live bacterial isolates can prove difficult when studying individual TLR receptors and characterising their role within inflammation. Therefore, if studying individual TLR receptors similar to the approach seen within this thesis, it is important to use individual components, such as flagellin for TLR5 activation. Furthermore, polymorphisms in flagellin structure are common (Arora *et al.,* 2004). It is also important to stress than there are multiple 'subtype strains' associated with *P. aeruginosa* infection, including invasive and cytotoxic, with the latter presenting more severe symptoms and a subsequent loss of vision (Borkar *et al.,* 2014). Therefore, using various, live strains of *P. aeruginosa* would strengthen the argument that 1,25D3 could be suppressive against full bacterium, and not limited to flagellin.

As previously discussed within subsection 3.3.7, there are VDRE within 5' regions of pro-inflammatory genes (Lisse *et al*., 2011). There are VDRE within hTCEpi cell genes for a range of pro-inflammatory mediators, which are occupied by 1,25D3 within these experiments, suppressing expression and impacting gene expression. However, in regard to AMPs, there is ample research demonstrating active VDRE on these genes. Research shows 1,25D3 treatment leads to the activation of VDRE within the primate-specific retro –transposable element (Alu-SINE) within the promotor are of CAMP (Gombart *et al*., 2009). Reins *et al.,* (2015a) showed that 1,25D3 treatment also increases expression of hBD-2 through VDRE, however, Wang *et al*., (2010j) found that 1,25D3 exposure alone did not induce a significant increase of hBD-2 expression, and additional signalling pathways linked to

TLR activation were required. In the data discussed within this chapter, hBD-2 expression may have been undetectable by 24h, therefore examining a shorter time point, such as 6h, will confirm this theory.

5.3.3 FLA-PA and FLA-ST exposure decreased hTCEpi cell viability, but 1,25D3 treatment did not

High concentrations of FLA-ST (100ng/ml) and FLA-PA (6µg/ml) treatment significantly decreased hTCEpi cell viability (Figure 5.11, A-B). As bacterial colonies increase alongside hTCEpi cells, there is an expected decrease of hTCEpi cell membrane integrity and subsequent rate of membrane rupturing, inducing increased inflammatory responses and rate of cellular apoptosis (Garai *et al.,* 2019). However, 1,25D3 treatment during both FLA-ST and FLA-PA treatments improved hTCEpi cell viability, whilst lowering the pro-inflammatory response. However, as 1,25D3 treatment increased LL-37 expression, this could influence cell viability at 100ng/ml of FLA-ST and 6ug/ml of FLA-PA.

5.3.4 miR-146a-5p has no direct role within the expression of various pro-inflammatory mediators following TLR5 stimulation, or the suppressive 1,25D3 mechanism, at 24h

In regard to FLA-ST, there was no significant change within the expression of any of the miR analysed, alongside no detectable increase of IL-8 by 24h. One possible theory is that FLA-ST induced a detectable level of pro-inflammatory mediators within the acute inflammatory period of 6h, and by 24h, these results were not detectable. To address this further, shorter time points should be analysed, such as 6h. Although TLR5 of the hTCEpi cells was stimulated, leading to an inflammatory in the form of pro-inflammatory mediators, there was no induction of miR-146a-5p expression (Figure 5.12). Funari *et al.,* (2013) characterised miR-146a expression throughout the human cornea and implicated the miR in wound healing, but stated that expression is cell dependent, with corneal limbal cells showing the highest expression of miR-146a (Funari *et al.,* 2013). As discussed throughout chapter 3 and 4, it is probable that miR-146a-5p is active within the acute

inflammatory period during TLR signalling, which would be supported by experiments in smaller time frames, such as 6h.

5.3.5 miR-93-5p and miR-181-3p has a regulatory role in the expression of IL-6 and IL-8 following FLA-PA TLR5 stimulation, but not during 1,25D3 suppression

Similar data to the TLR3 and TLR9 experiments was obtained for the TLR5 experiments, insinuating that miR-93-5p and miR-181-3p regulate IL-6 and IL-8 - MyD88 independent and dependent pathways, during inflammation of the ocular surface (Figures 5.13 and 5.14). Fabbri *et al.,* (2014) established miR-93 regulating IL-8 during *P. aeruginosa* infection in bronchial epithelial cells, with miR-93-5p significantly downregulated in cells accommodating large *P. aeruginosa* colonies. As previously discussed, the experiments in this chapter should be repeated with both miR inhibited at the same time, to identify any potential relationship between the two, alongside luciferase assay confirmation of 3' UTR targeting. Quantifying bacterial colonies present upon hTCEpi cells following miR-93-5p inhibition would extend the TLR5 findings presented within this thesis. It would be expected there would be significant increase of *P. aeruginosa* presence following miR-93-5p inhibition, if this miR aided in bacterial killing. During 1,25D3 treatment of FLA-PA stimulated cells, miR expression was significantly reduced, reflected in Figure 5.12 B, with miR-93-5p ($p<*0.05$), miR-155-5p ($p<*0.05$) and miR-181-3p ($p<*0.05$). However, 1,25D3 still significantly suppressing these mediators following inhibition of miR-181-3p and miR-93-5p (Figures 5.13 and 5.14), therefore, these miR must not play a role during 1,25D3 suppression of TLR5 signaling.

5.3.6 hTCEpi cell miR-155-5p expression increased following 24h of FLA-PA exposure

Interestingly, 24h of FLA-PA stimulation and subsequent 1,25D3 treatment of hTCEpi cells led to a significant increase of miR-155-5p (Figure 5.12B), which was not seen throughout TLR3 and TLR9 signaling. It was assumed that miR-155-5p would be active during the acute inflammatory period, however, during these experiments miR-155-5p expression could be extended due to a prolonged immune response. For example, Wang *et al.,* (2020a) showed

corneal *P. aeruginosa* infection increasing miR-155 expression within 24h, concluding that 'miR-155 promotes host susceptibility to *P. aeruginosa* and prevents macrophage phagocytosis of the bacteria, whilst significantly reducing corneal epithelial permeability of rats.' The fact that miR-155-5p is significantly raised within these experiments is encouraging that this miR is indeed active during the hTCEpi cell inflammatory response and supports investigating the expression of this miR further, during the acute inflammatory response. Future experiments should inhibit miR-155-5p during FLA-PA TLR5 stimulation and 1,25D3 treatment to identify any potential changes within not only pro-inflammatory mediator expression such as IL-6 and IL-8, but also AMPs which were elevated in these experiments, for example, hBD-2.

5.3.7 Chapter conclusions

This chapter confirms 1,25D3 modulating hTCEpi cell TLR5 signaling – an overall suppression of pro-inflammatory mediators after 6h, with an upregulation of AMP LL-37 expression in response to flagellin derived from the most common ocular bacterial infection - *P. aeruginosa.* Both of these aspects are beneficial for removing bacterial infections of the ocular surface, as the acute inflammatory response required in quick response to the bacteria is still present, ensuring maximum bacterial killing and migration of immune cells. The significant increase of LL-37 would aid in not only bacterial killing, therefore preventing further corneal damage associated with infection, but also improve the wound healing response in the corneal epithelium. Once again, miR-93-5p and miR-181-3p both appear to regulate IL-8 and IL-6 expression in response FLA-PA. There was an unexpected result in the increase of miR-155-5p expression, demonstrating that this miR is active during the hTCEpi cell inflammatory response.

Chapter 6
Summary and Future Prospects

Figure 1.9: Study findings: Summary of the new findings made by this study, which used 1,25D3 to modulate TLR signalling in response to various agonists by hTCEpi cells.

6.1.1 1,25D3 treatment: not all suppressive

In regard to wider cystolic DNA sensors, this study suggested that there was no functioning cGAS receptor of hTCEpi cells, as there was no detectable expression by 24h. However, the data within this thesis demonstrated that 1,25D3 treatment significantly suppressed TLR3, 5 and 9 signaling and subsequent expression of pro-inflammatory mediators IL-6, IL-8 and IL-1β (Figure 1.9), extending the work of Reins *et al.,* (2015a). Although Reins *et al.,* (2016c) identified the 8h required for 1,25D3 to suppress IL-8 expression, the data presented throughout chapters 3-5 suggested a phenomenon of pro-inflammatory mediator hierarchy. As discussed within subsection 3.3.3, further research has described the effect of IL-1β promoting the production of further pro-inflammatory cytokines, including IL-8 (Da Cunha *et al.,* 2018). It could be that this same relationship between IL-1β and IL-8 was active throughout the experiments in this study. This would be reflected by the significant increase in IL-1β expression by 6h, before the significant increase in the expression of any other pro-inflammatory mediator. Netto *et al.,* (2005) characterized IL-1β as having a 'key function' in corneal inflammation, playing an important role during apoptosis and MMP production, but also bridging between innate and adaptive immune systems.

Suppression of IL-1β expression by 1,25D3 is particularly interesting, as IL-1β has been described as a 'driving force' behind numerous corneal immune responses, including angiogenesis of epithelial cells, corneal ulcers and associated tissue damage seen in dry eye disease (DED) (Dana 2007, Wilson *et al.,* 2009). Furthermore, Wang *et al.,* (2020b) associated IL-1β production with the 'clouding' of the cornea, driving a cytokine-storm like phenomena, which presents as corneal edema and neovascularization. An inhibition of this cytokine by 4h could limit the production of other pro-inflammatory mediators such as IL-6 and IL-8, which was shown within this thesis. Treatment of TLR3 signaling with 1,25D3 also suppressed IL-12, a powerful chemokine which spans between innate and adaptive immunity of the cornea, highlighting an interesting starting point to investigate the non-direct effects of 1,25D3 on the adaptive ocular immune system.

However, the data reported in this thesis showed that 1,25D3 treatment may not be entirely desirable for inflammatory conditions of the ocular surface, as treatment led to an upregulation of COX-2 expression by hTCEpi cells. Although a suppression of pro-inflammatory mediators in response to dsRNA would be beneficial in cases such as HSV-1, an increase of COX-2 expression is associated with VEGF production. An increase of VEGF could encourage the angiogenesis of inflammatory corneal growths, such as pterygium corneal fibrosis, whilst significantly boosting corneal neovascularization (Sellers *et al.,* 2004). This finding is of specific interest, as it confirms that 1,25D3 does not only suppress inflammatory markers, but may exacerbate them in response to dsRNA.

In regard to AMPs, 1,25D3 treatment increased the expression of anti-microbial peptides hBD-2 and LL-37 in response to the flagellin of both *P. aeruginosa and S. typhimurium.* An increase of these AMPs would be beneficial, as this would aid in bacterial killing and protect the cornea from further infection. However, in future experiments it would be interesting to identify if this upregulation of AMPs occurred during the acute inflammatory response period, which would complement the production and release of pro-inflammatory mediators. It was interesting to note, however, that TLR5 was upregulated in response to both the flagellin of both *P. aeruginosa and S. typhimurium,* yet these responses alternated between pro-inflammatory and antimicrobial production, hinting at a varying response to bacterial flagellin by 24h.

6.1.2 Potential of 1,25D3 treatment in TLR-driven ocular disease

It is important to now consider the potential role of 1,25D3 treatment in relation to ocular disease, especially those diseases in which TLR signaling are implicated. Not only overgrowth of harmful bacterial colonies, but physical contact to the ocular surface during procedures such as the application of contact lenses and ocular surgery, can introduce foreign pathogens to the ocular surface, inducing an inflammatory response through TLR signalling (Galvis *et al.,* 2014). Bacterial infections contribute for up to 70% of reported conjunctivitis cases, the most frequently reported ocular case effecting both children and adults, which bring noticeable economic burden to

healthcare systems (Cervantes *et al.,* 2011, Buznach *et al.,* 2005, Høvding 2008).

P. aeruginosa infection is especially common in contact lens wearers, accomodating for 55% of conjunctivitis cases, as the bacteria successfully adheres and colonizes on the lens during prolonged contact lens use (Teweldemedhim *et al.,* 2017). Furthermore, if these infections are untreated, they are capable of damaging structures of the eye which may lead to complications such as uveitis and keratitis, as discussed within subsection 1.5.1, with a complete loss of vision (Teweldermedhin *et al.,* 2017). Current treatments for *P. aeruginosa* infections include fluoroquinolone antibiotic eye drops, however, numerous studies have shown that these treatments only lead to limited clinical improvement, implicate antibiotic availability, resistance and individual patient allergies (Sinclair *et al.* 1988, Azari *et al.,* 2013). Supplementary steroid prescription is often used alongside antibiotic eye drops to suppress overt corneal inflammation. However, this combination treatment is often discouraged, as prolonged steroid usage weakens the corneal epithelial layer which encourages invasive bacterial infections, but there is no significant improvement in vision when compared to antibiotic treatment alone (Borkar *et al.,* 2014). In comparison, the data in this thesis demonstrated that 1,25D3 treatment significantly improved hTCEpi cell viability during *P. aeruginosa* driven inflammation, indicating that the treatment would not cause cell loss of epithelial layer. Furthermore, the significant decline in pro-inflammatory mediators following TLR5 activation could prevent overt inflammation within the resolution period of inflammation by 24h, whilst the increase in AMP production would aid in the removal of bacteria. The adherence capabilities of *P. aeruginosa* have been described as a 'crucial' component in the bacteria being able to invade corneal cells (Evans *et al*., 2014). It would therefore be interesting to investigate the influence of 1,25D3 topical treatment alongside the bactericidal capacity of antibiotics in relation to bacterial adherence.

Unlike bacterial infections of the corneal surface, treatment options for viral infections are limited to artificial tears and pain relief (Azari *et al.,* 2013). As discussed within subsection 1.5.4, HSV-1 is an example of a virus which can infect corneal epithelial cells which activates TLR9. It is estimated that HSV-1 accounts for 10% of reported uveitis cases, with common treatments

including oral anti-viral agents, such as acyclovir, or topical corticosteroids to control inflammation from extended pro-inflammatory cytokine damage (Rathinam *et al.,* 2007). Similar to the treatment of bacterial infections, there are limitations, such as sustained steroid use thinning the corneal layer, increasing the risk of opportunistic infections circumventing a damaged tissue (Srinivasan *et al.,* 2014).

However, the data presented in this thesis demonstrated 1,25D3 treatment significantly suppressed the expression of pro-inflammatory mediators following TLR9 activation, which may indicate an excellent therapeutic to prevent corneal damage in response to pathogenic DNA, including HSV-1. Furthermore, HSV-1 can lead to associated uveitis, with 1,25D3 potentially alleviating inflammatory symptoms such as swelling and loss of ocular function, which may improve vision (Al-Dujaili *et al.,* 2011). However, it must be emphasized that 1,25D3 treatment alongside steroids could lead to potential danger, as 1,25D3 also suppressed TLR5 signaling, which would not be beneficial with an already damaged corneal surface hosting opportunistic bacterium. This highlights the importance of replicating these experiments using models of ocular disease, such as murine HSV-1 models. Initial HSV-1 diagnosis has been described as 'unhelpful' due to the significantly high incidence rate of the infection and increased latency period, with severe symptoms leading to the patient's initial diagnosis, increasing the risk of complications such as corneal scarring with herpetic keratitis which require corneal allograft (Sugita *et al.,* 2013).

Corneal allograft is not a surgery that is limited to a HSV-1 treatment strategy however, but is also prescribed for a range of secondary eye diseases associated with autoimmune disease, as discussed throughout subsection 1.5.3 (Patel *et al.,* 2002). Originally, TLR9 was thought to respond to DNA by detecting demethylated motifs, however, there is evidence that TLR9 may still react to self-DNA, leading to 'fatal' levels of inflammation, damaging organs and tissue (Marshak-Rothstein *et al.,* 2007, Mouchess *et al.,* 2011). Upregulation of TLR9 expression in rheumatoid patients with peripheral ulcerative keratitis is associated with an increase of systemic inflammatory responses, leading to increased corneal damage in response to self-DNA. Therefore, it could be predicted that localized 1,25D3 treatment at the site of the cornea for conditions of secondary eye disease, may also prevent ocular

surface damage driven by self-DNA. This would ensure not only corneal function, but also decrease the risk of further infections from opportunistic bacteria. In future experiments, it would be useful to investigate this using *in vivo* models of chronic inflammatory disorders to observe the effects of this 1,25D3 suppression of TLR9 signaling by the ocular surface, when inflammation is already systemic and overt.

6.1.3 Potential role of miR

In regard to inflammation of the ocular surface, miR research is limited, however, data from miR analyses within this thesis provided novel evidence of a significant role for both miR-93-5p and miR-181-3p negatively regulating hTCEpi cell expression of IL-8 and IL-6. Although these results indicated that these miR have no role during the suppressive mechanism of 1,25D3, as inhibition of each miR made no significant change in 1,25D3 suppressive capability, these results are beneficial for future studies and potential therapeutics. In regard to IL-1β expression, miR-93-5p and miR-181-3p negatively regulated expression during TLR3 and TLR5 activation, but had no regulative role during 24h of TLR9 signaling.

Arlier *et al.,* (2018) showed that 1,25D3 treatment led to an upregulation of IKBα following TLR stimulation, leading to a suppression of NF-KB. NF-KB is one of the main transcription factors activated by TLR9 stimulation, which can lead to an increase of pro-inflammatory genes and subsequent cytokine release (Tsujimura *et al.,* 2004). Reins *et al.,* (2016c) showed that the 1,25D3 suppression of TLR3 signaling was linked to IκBα upregulation, which led to a significant decline in IL-8 expression. It appears that both miR-93-5p and miR-181-3p play a role in both TLR3 and TLR9 signalling, which are similar TLR pathways in regard to the mediators they produce, but have different MyD88 dependent status. It would be beneficial to observe if that is the same exact mechanism occurring during the suppression of TLR9 signalling within these cells, and if miR-93-5p and miR-181-3p may play a role during this proposed mechanism, by regulating IκBα. This would not only strengthen current understanding of miR during ocular inflammation, but could identify a novel relationship between miR-93-5p and miR-181-3p with IκBα, which may be exploited to suppress ocular inflammation.

In future experiments, it would be beneficial to analyze the effect of pro-inflammatory mediators during the acute inflammatory response, for example, within 6h of TLR9 activation. It is surprising that miR-155-5p showed no role in the expression of these pro-inflammatory mediators, especially as this miR has been implicated in a range of inflammatory events such as the induction of pro-inflammatory cytokine expression, with Mahesh *et al.,* (2019) describing miR-155 family as 'particularly responsive' to IL-1β. Our study mainly focused upon the suppressive action of 1,25D3 after 24h, with the expectation that the acute inflammatory stage of ocular inflammation would have ended, allowing confirmation of the 1,25D3 suppressive effects. However, although this study showed that inhibiting these miR did not affect the suppressive action of 1,25D3, our study did deliver an interesting, novel starting point for future inflammatory work. Therefore, in future studies examining the role of miR during ocular inflammation, it would be beneficial to examine the role of these miR during the acute inflammatory stage, such as 1-6h, where an influx of IL-6 and IL-8 have been identified (Resan *et al.,* 2016).

6.1.4 Future direction

Although the data provided in this thesis provides novel findings in regard to TLR3, TLR5 and TLR9 signaling by hTCEpi cells in response to various agonists associated with ocular infection, such as flagellin and unmethylated CpG DNA motifs, we have also produced novel evidence of miR-93-5p and miR-181-5p negatively regulating the expression of IL-8 and IL-6 during all of these stimulations. Shorter time points of 6h would be beneficial to identify potential miR-155-5p and miR-146a-5p expression within the acute inflammatory period, these findings should be explored further with molecular studies, including using luciferase reporter assays, to confirm 3'UTR targeting. Although these results demonstrated an inhibitory role of miR with cytokine expression, these results should be supported with evidence from methods such as ELISA and western blot, to confirm that these results transpire into biologically relevant diminutions at a protein level.

An interesting future hypothesis to investigate in regard to the acute inflammatory response, would be that miR-155-5p, miR-181-3p and miR-93-

5p each have a role in the regulation of IL-1β during the acute inflammatory process. Studying miR in mice to observe corneal inflammation has been shown to be successful, for example, miR-155-5p activity in uveitis models (Hsu *et al.,* 2015). However, it is important to acknowledge that mice model systems are not without limitation, especially in regard to miR work. Inhibiting miR in mice may impair required homeostasis and organ function, such as impeding a functional immune system and increasing the risk of developing opportunistic infections, leading to a shortening of lifespan in mice used within each study. Furthermore, even if mice models were useful in supporting the hypothesis, it is important to highlight that these findings may not correspond with inflammation of the ocular surface in humans. For example, Hsu *et al.,* (2015) described 'days' for the expression of miR in mice uveitis models to become detectable, and stressed that these miR may be active long before clinical presentation of related symptoms. This identifies a difficulty in using mice models for miR work and the exact timeframe required to identify these active molecules.

The experiments reported in this thesis only analysed the effects of 1,25D3 suppressing epithelial cell TLR5 responses at 6h. A wider range of ocular cells should be studied in future, as well as specific types of immune cells, which could potentially influx into the cornea during the acute inflammatory response. For example, Nourai *et al.,* (2016) showed that 1,25D3 treatment significantly increased macrophage IL-1β production in response to *P. aeruginosa*, highlighting both the potential benefits and harm of 1,25D3 treatment with the latter linked to the exacerbation of inflammatory symptoms associated with bacterial infections of the ocular surface.

Although the data reported in this thesis observed alternate immunomodulatory effects upon hBD-2 and LL-37 expression, Mohammed *et al.,* (2010) showed a significant increase in both hBD-3 and hBD-9 expression during infective keratitis caused by both gram-negative and gram-positive bacteria. Otri *et al.,* (2012) identified a significant increase of hBD-9 expression when the acute inflammatory response had ended, and concluded that bacteria utilize different mechanisms to avoid immune innate responses in the eye. It would be interesting therefore to perform further experiments using gram positive bacteria known to affect the ocular surface, such as *S.*

epidermis (O'Callaghan 2018) and in doing so examine the expression and synthesis of other AMPs including hBD-3 and hBD-9'.

Furthermore, all of these experiments were conducted *in vitro*, specifically, using corneal epithelial cell lines. Although these cells are significant due to their capacity of both physical and anti-microbial protection to the ocular surface, it is important to stress that multiple cells compose the ocular surface, including corneal keratocytes, alongside the infiltrating immune cells, such as neutrophils. Therefore, is it vital to observe the immune-modulatory effects of 1,25D3 treatment *in vivo*, for example, using experimental mice models mimicking uveitis. This ensures that further impacts of 1,25D3 treatment are identified and possible complications. For example, an elevation in AMP LL-37 production may be beneficial for wound healing, however, this may be detrimental during inflammation driven by overt TLR9 signaling. Supporting this, Morizane *et al.,* (2012) showed that LL-37 binding with CpG-DNA, exacerbated IFN release, leading to a further inflammatory response, which may be both beneficial and detrimental.

6.1.5 Conclusion

The data provided in this thesis showed that 1,25D3 modulated hTCEpi cell TLR pro-inflammatory responses following recognition of various sources of both bacterial flagellin and CpG-ODN DNA motifs. The experiments incorporated various forms of CpG-ODN which contained variable levels of DNA methylation, which would be expected in a range of viruses and bacteria. Furthermore, miR activity for both miR-93-5p and miR-181-3p was highlighted in regulating pro-inflammatory mediators IL-6, IL-1β and IL-8 in response to various sources of TLR agonist by the ocular surface. These data also revealed, however, that miR-155-5p, a commonly reported miR during inflammation, was only elevated 24h after bacterial flagellin exposure, indicating a potential of this miR being active during the acute inflammatory response. Finally, following 24h of hTCEpi cells exposed to 2'3' cGAMP, the data presented in this study failed to identify induction of STING and IFN-β. This indicates that, by 24h, there was no detectable activity from a functioning cGAS/STING pathway in hTCEpi cells.

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Appendix

A.1. hTCEpi cell STR analysis

To authenticate the corneal cell line used throughout the study, hTCEpi cells, short tandem repeat (STR) DNA analysis as performed by NorthGene (International Centre for Life, Newcastle upon Tyne, UK). The purpose of this experiment is to evaluate specific STR regions within varying passages of cells, ensuring minimal polymorphism and therefore confirming the accuracy when comparing these data to previous studies. The results were validated against partial, previous hTCEpi cell STR analysis performed by McDermott, *et al.* 2018 to ensure limited genetic drift and potential misidentification. The results showed that results were identical for each loci, expect for FGA, which showed slight antigenic drift, with a result of 17, 25 in comparison to 17, 15 (Table A.1, FGA). These STR results were found to be acceptable and therefore future results from similar experiments, for example, the suppression of TLR3 signaling by 1,25D3, would be comparable.

Loci	L Appleby, 2020	A McDermott, 2018
AMEL	X, Y	X, Y
D3S1358	17	17
D1S1656	11, 13	
D2S441	14	
D10S1248	14	
D1S3S317	11, 12	11, 12
Penta E	12, 15	12, 15
D16S539	9, 11	9, 11
D18S51	12, 15	12 15
D2S1338	17, 24	
CSF1PO	11	11
Penta D	12, 14	12, 14
THO1	7	7
vWA	14, 17	14, 17
D21S11	32.2, 33.2	32.2,33.2
D7S820	11, 12	11, 12
D5S818	11	11
TPOX	8, 10	8, 10
DYS391	10	
D8S1179	12, 13	12, 13
D12S391	19, 24	
D19S433	14.2, 15	
FGA	17, 25	17,15
D22S1045	16	

Table A.1. The STR profile determined for human corneal epithelial cell line hTCEpi (n=2, p=38) by analysis at NewGene, with results compared to the published STR profiles for hTCEpi cells by A McDermott, *et al.* 2018.

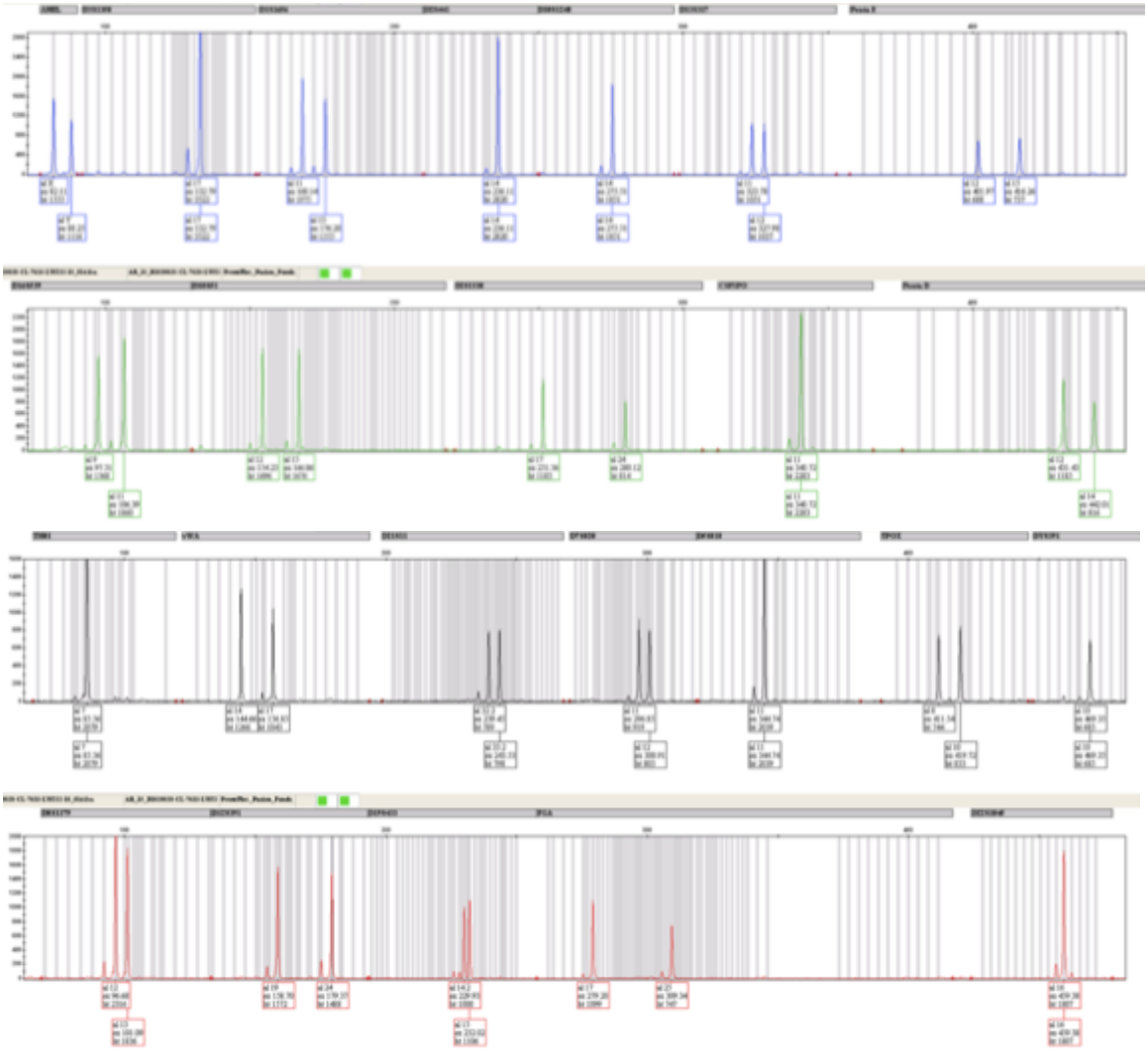

Figure A.1. The STR profile was determined for human corneal epithelial cell line hTCEpi (n=2, p=38) by analysis at NewGene.

A.2 *E. coli* DNA validity

To ensure the only TLR activated following hTCEpi cell exposure to *E. coli* DNA was TLR9, the following biological analysis was completed by the manufacturer Invivogen (Figure A.2). This ensured the inflammatory response seen was not driven by, for example, TLR4 to LPS.

InvivoGen

Certificate of Analysis

PRODUCT NAME dsDNA-EC

PRODUCT INFORMATION

Batch number:	**ECD-41-01**	Quantity:	200 µg
Cat. code:	tlrl-ecdna	Storage temperature:	-20°C
Origin:	DNA from E. *coli K12*	Recommended Retest Date:	Jun. 2021

QUALITY CONTROL

TEST	SPECIFICATION	RESULTS
Physicochemical properties		
Appearance (color)	White	Conform
Appearance (form)	Lyophilized pellet	Conform
Solubility 1 mg/mL, water	Soluble	Conform
Biological assays		
IFN stimulation at 100 ng/mL Performed on THP1-Blue™ ISG cells	Positive	Conform
TLR2 activity at 1 µg/mL Performed on HEK-Blue™ hTLR2 cells	Negative	Conform
TLR4 activity at 1 µg/mL Performed on HEK-Blue™ hTLR4 cells	Negative	Conform
Bacterial endotoxin level		
HEK-Blue™ LPS Detection Kit 2	< 0.01 EU/µg	Conform

Date: 02 Jul. 2020

Reviewed by QA department:

Angélique ZANDONA

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Caution – Not fully tested. Research use only. Not for human use or veterinary use.

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Figure A.2. The quality control testing for bacterial contamination of *E. coli* DNA was determined by manufacturer analysis at Invivogen.