UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

Immunomodulatory effects of vitamin D on TLR7 and TLR8 signalling in monocytes and monocyte-derived macrophages

by

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Presentations and Awards arising from research

- **KA Naidoo**, BA Mol, NL Gentle, V Meyer. The immunomodulatory effects of vitamin D on cytokine homeostasis in monocytes and monocyte-derived macrophages. SASBMB 2024, Polokwane (7-10 July 2024). Poster presentation by KA Naidoo.

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Contents

List of abbreviations

$1,25D_3$- 1,25-Dihydroxyvitamin D_3

7DHC- 7-Dehydrocholesterol

AIM2- Absent-in-melanoma 2

ANOVA- Analysis of Variance

APCs- Antigen presenting cells

ARDS- Acute respiratory distress syndrome

AU- Adenine

Bp- Base pair

CAMP- Cathelicidin antimicrobial peptide

CCL2- Chemokine (C-C motif) ligand 2

CCR2- CC-chemokine receptor 2

$CD4^+/CD8^+$ - Cluster of differentiation $4^+/8^+$

CD14- Cluster of differentiation 14

CD16- Cluster of differentiation 16

cGAS- Cyclic GMP-AMP synthase

CLRs- C-type lectin receptors

CpG- Cytidine-phosphate-guanosine

CXCL10- C-X-C motif chemokine ligand 10

CYP24- Cytochrome P450 family 24

CYP2R1- Cytochrome P450 family 2 subfamily R member 1

CYP27A1- Cytochrome P450 family 27 subfamily A member 1

CYP27B1- Cytochrome P450 family 27 subfamily B member 1

DAMPs- Damage-associated molecular patterns

DEFβ- Defensin β2

dNTP- Deoxynucleotide triphosphate

DMSO- Dimethyl sulfoxide

dsDNA- Double stranded DNA

ER- Endoplasmic Reticulum

EtOH- Ethanol

FASTA- Fast alignment

FBS- Foetal bovine serum

GM-CSF- Granulocyte-macrophage colony-stimulating factor

GU- Guanine

hCAP18- Human cathelicidin antimicrobial protein

IFN- Interferon

IFN-ϒ - Interferon-gamma

IL10- Interleukin-10

IL13- Interleukin-13

IL1β - Interleukin-1 beta

IL4- Interleukin-4

IL6- Interleukin-6

IP-10- Interferon gamma-induced protein 10

IRAK- Interleukin-1 receptor-associated kinase 1

IRF- Interferon regulatory factor

ISGs- Interferon-stimulated genes

JAK/STAT- Janus kinase/signal transducers and activators of transcription

LPS- Lipopolysaccharide

LRR- Leucine-rich repeat

LSD- Least significant difference

MAPK- Mitogen-activated protein kinase

MCP1- Monocyte chemoattractant protein

M-CSF- Macrophage colony-stimulating factor

MDMs- Monocyte-derived macrophages

MHCII- Major histocompatibility complex class II

MYD88- Myeloid differentiation primary response 88

NK- Natural killer

NLRs- Nucleotide oligomerization domain-like receptors

NO- Nitric oxide

ORNs- Oligoribonucleotides

PAMPs- Pathogen associated molecular patterns

PBMC- Peripheral blood mononuclear cells

PI3K/Akt- Phosphoinositide-3-kinase–protein kinase

PMA- Phorbol 12-myristate 13-acetate

PRRs- Pattern recognition receptors

qPCR- Quantitative polymerase chain reaction

RA- Rheumatoid arthritis

RLRs- Retinoic acid-inducible gene 1 receptors

RNA-seq- RNA sequencing

ROS- Reactive oxygen species

RPMI 1640- Roswell Park Memorial Institute 1640

RT-qPCR- Reverse transcriptase-quantitative polymerase chain reaction

RXR- Retinoid acid receptor

SLE- Systemic lupus erythematosus

SOCS1- Suppression of cytokine signalling 1

ssRNA- Single stranded RNA

THEMIS2- TLR signal transduction modulatory protein

THP-1-Tohoku Hospital Pediatrics-1

TIR- Toll/IL-1R

TLRs- Toll-like receptors

TNFα - Tumour necrosis factor alpha

TRAM- TRIF-related adaptor molecule

TEM1- Triggering receptor expressed on myeloid cells 1

TRIF- TIR-domain-containing adapter-inducing interferon-β

UBC- Ubiquitin C

VDR- Vitamin D receptor

VDREs- Vitamin D responsive elements

YWHAZ- Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta

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ABSTRACT

Monocytes and macrophages are crucial innate immune cells known for their role in antiviral signalling. They respond to viruses via intracellular membrane receptors such as toll-like receptor 7 (TLR7) and toll-like receptor 8 (TLR8). When these receptors are activated, inflammatory cytokines and chemokines are produced downstream which play an important role in the inhibition of viral replication. However, the excessive production of these proinflammatory mediators results in chronic inflammation which can cause multi-organ failure and has been linked to various autoimmune disorders. Thus, there is a need to regulate these pathways. 1,25-dihydroxyvitamin D_3 ($1,25D_3$) is a recognised immune modulator, widely accepted as promoting an anti-inflammatory state. $1,25D_3$ is thought to reduce the production of proinflammatory mediators, thereby playing a role in the maintenance of immune homeostasis. As such, the aim of this research project was to investigate the effect of $1,25D_3$ on TLR7 and TLR8 expression and function in monocytes and monocyte-derived macrophages (MDMs). This was done by assessing the expression of *TLR7* and *TLR8* in Tohoku Hospital Pediatrics-1 cells (THP-1) and THP-1 derived macrophages, and the effect of $1,25D_3$ thereon using RNA-sequencing. Furthermore, *interleukin-1 beta* (*IL1β), chemokine (C-C motif) ligand 2 (CCL2), C-X-C motif chemokine ligand 10* (*CXCL10),* and *Interleukin-10* (*IL10)* expression was measured using RT-qPCR in monocytes and MDMs cultured in the absence or presence of $1,25D_3$. Thereafter, the downstream functional effects were investigated by activating the TLR7 and TLR8 signalling pathways using vesatolimod and motolimod, respectively, in monocytes and MDMs cultured in the absence or presence of $1,25D_3$ and measuring the expression of *IL1β*, *CCL2*, *CXCL10*, and *IL10*. Lastly, to assess the effect of additional $1,25D_3$ supplementation, TLR7 and TLR8-stimulated monocytes and MDMs cultured in a $1,25D_3$ 'sufficient' vs $1,25D_3$ 'insufficient' environment were treated with additional $1,25D_3$, and the expression of *IL1β, CCL2, CXCL10*, and *IL10* were measured using RT-qPCR. Results showed that the expression of *TLR7* was decreased, while the expression of *TLR8* was increased in both monocytes and MDMs cultured in the presence of $1,25D_3$. Additionally, the expression of *IL1β, CCL2,* and *CXCL10* were decreased in the MDMs cultured in the presence of $1,25D_3$. Interestingly, the monocytes responded to TLR8 stimulation, but not TLR7 stimulation, while the MDMs responded to both TLR7 and TLR8 stimulation, regardless of the absence or presence of $1,25D_3$. The expression *IL1β, CCL2,* and *CXCL10* appeared to be enhanced in monocytes cultured in the presence of $1,25D_3$, while only *CCL2* and *CXCL10* expression were enhanced in MDMs cultured in the presence of $1,25D_3$. However, this trend was not observed

with *IL1β* expression, which was decreased in MDMs cultured in the presence of 1,25D_3. Lastly, monocytes and MDMs cultured in a 1,25D_3 'insufficient' environment continued to respond to TLR7 and TLR8 stimulation upon additional 1,25D_3 supplementation, but while monocytes and MDMs cultured in a 1,25D_3 'sufficient' environment continued to respond to TLR8 stimulation, they were no longer responsive to TLR7 stimulation upon additional 1,25D_3 supplementation. Overall, these results highlight the role of 1,25D_3 as an immunomodulator, with its ability to both enhance the production of proinflammatory mediators, while also modulating their production to prevent the risk of creating a hyperinflammatory environment. This research directly demonstrated the contrasting effects of 1,25D_3 on TLR7 and TLR8 signalling which not many studies have highlighted. Additionally, this study aimed to highlight the benefits and importance of maintaining sufficient 1,25D_3 levels.

CHAPTER 1: Literature Review

1.1The immune system

The innate and adaptive systems make up the human immune system, both of which function in conjunction with one another to eradicate pathogen infection. The first line of defence includes the physiological barriers, such as mucous membranes, and the skin, however, pathogens are often able to overcome these barriers, necessitating the actions of cellular immune mechanisms (Michaud and Watson, 2023).

While the innate immune system has the potential to clear pathogen infections, high counts and virulence necessitates the presence of the adaptive immune system. The adaptive immune system responds in a highly specific manner and plays a key role in subsequent infection due to its ability to harbour immunological memory (Lee and Chung, 2021). Adaptive immune cells are grouped into B cells and T cells, both of which recognise and respond to various pathogens. T cells are further classified as either cluster of differentiation 4^+ ($CD4^+$) T helper cells, or cluster of differentiation 8^+ ($CD8^+$) cytotoxic T cells. T helper cells are more associated with the maintenance of immune balance, while cytotoxic T cells are more associated with the targeting and killing of specific cells (Chi *et al.*, 2024). Alternatively, B cells release effector molecules known as antibodies which bind to specific antigens on antimicrobial cells. The binding of antibodies to viral antigens may block the entry of the virus into target cells, thereby inhibiting viral replication and spread (Chi *et al.*, 2024). While the adaptive immune response is critical, the innate immune response plays an indispensable role in activating the adaptive immune response. For example, antigen presenting cells (APCs) such as macrophages, a subset of innate immune cells, display antigens on major histocompatibility complex class I and II (MHCI/II), which are recognised by naïve T helper cells. Furthermore, some APCs can migrate to secondary lymphoid organs where a subset of T helper cells, known as T follicular helper cells, will activate B cells to either produce antibodies or begin the process of B cell memory (Mayer *et al*., 2015; Wieczorek *et al*., 2017)

The innate immune system is made up of natural killer (NK) cells, monocytes, dendritic cells, and macrophages. These cells are crucial upon initial infection as they respond rapidly (Netea *et al.*, 2019). This rapid recognition occurs via pattern recognition receptors (PRRs) which recognise pathogens, including viruses, bacteria, and fungi, thereby initiating crucial antimicrobial signalling pathways (Li and Wu, 2021). As such, these immune cells work in conjunction to eradicate infection via critical immunological processes including phagocytosis,

apoptosis, degranulation, cytokine production, and antigen presentation. There has been debate as to whether innate immune cells harbour immunological memory, however, it has been observed that macrophages, NK cells, and monocytes can distinguish between gram-negative bacteria and gram-positive bacteria but are unable to distinguish between closely related species (Ochando *et al.,* 2023). This has been termed 'trained immunity' and is distinct from adaptive immune memory. Overall, both the adaptive and the innate immune system play a critical role in the effective clearance of various pathogens, and as such the maintenance of immune homeostasis.

1.2 Monocytes and macrophages

Monocytes and macrophages are critical immune cells involved in a variety of immune processes. Monocytes are progenitor cells which originate in the bone marrow (Olingy *et al.,* 2019). As such, they reside in circulation where they can migrate to sites of infection via proinflammatory signals and differentiate into more specialised immune cells such as dendritic cells or macrophages (Shi and Pamer, 2011). Furthermore, monocytes can be classified into various subsets based on their size and cell surface receptors i.e. classical monocytes which highly express cluster of differentiation 14 (CD14), and don't express cluster of differentiation 16 (CD16) ($CD14^{++}/CD16^{-}$), intermediate monocytes which express both CD14 and CD16 ($CD14^{+}/CD16^{+}$), and non-classical monocytes which highly express CD16, and lowly express CD14 ($CD14^{+}/CD16^{++}$; Merah-Mourah *et al*., 2020). In addition, monocytes are known as professional phagocytes, and can recognise a variety of pathogens, thus initiating an inflammatory response. Additionally, monocytes produce effector molecules such as cytokines and chemokines, as well as reactive oxygen species (ROS), such as nitric oxide (NO), which is known to inhibit viral replication thereby preventing systemic spread (Cormican and Griffin, 2020).

One of the main functions of monocytes is their ability to migrate to sites of infection, where they are able differentiate into macrophages upon tissue entry. Granulocyte-macrophage colony-stimulating factor (GM-CSF) and macrophage colony-stimulating factor (M-CSF) are the two major cytokines that drive monocyte-to-macrophage differentiation, where GM-CSF is thought to induce a more proinflammatory macrophage subtype, and M-CSF is thought to induce a more anti-inflammatory macrophage subtype (Orekhov *et al.,* 2019). Macrophages are more specialised immune cells with a wider range of immune functions compared to monocytes. Macrophages are heterogenous in nature, which is further enhanced by their capacity to be polarised as a result of various environmental stimuli, as well as the

microenvironment and localization within the body (Kumar, 2019). Macrophage functions include angiogenesis, wound repair, and apoptosis (Cormican and Griffin, 2020). Macrophages are also able to phagocytose pathogens and present antigens, making them crucial in the activation of cytotoxic T cells and T-helper cells (Lendeckel *et al.*, 2022).

Macrophages exist on a functional spectrum based on the expression patterns on their cell-surface receptors (Ross *et al*., 2021). Which receptors are expressed when is dependent on the context of the local environment, with either end of the spectrum represented by M1-type macrophages and M2-type macrophages (Lendeckel *et al.*, 2022). M1 macrophages are typically described as proinflammatory macrophages and are polarised by stimuli such as interferon-gamma (IFNϒ), or lipopolysaccharide (LPS) (Yang *et al.*, 2014). As expected, these proinflammatory macrophages are associated with the production of proinflammatory mediators such as chemokine (C-C motif) ligand 2 (CCL2), interleukin-6 (IL6), C-X-C motif chemokine ligand 10 (CXCL10), tumour necrosis factor alpha (TNFα), and interleukin-1 beta (IL1β), all of which contribute to further activating surrounding immune cells, thereby contributing to the inhibition of viral replication and promotion of viral clearance (Turner *et al.*, 2014). Additionally, these M1 macrophages also produce ROS, and highly express MHCII, which assists in the efficient presentation of antigens to T helper cells, once again highlighting the link between the innate immune response and the adaptive immune response (Bhattacharya and Aggarwal, 2019).

M2-type macrophages are generally associated with an anti-inflammatory phenotype and are polarised in response to stimuli such as interleukin-4 (IL4) and interleukin-13 (IL13) (Genin *et al.*, 2015). However, these macrophages can be further subdivided into subsets M2a, M2b, M2c, and M2d where M2a macrophages are associated with the clearance of viral debris, while M2b and M2c macrophages are more so associated with maintenance of immune balance via wound repair and tissue remodelling, and lastly M2d macrophages which are most associated with angiogenesis (Bhattacharya and Aggarwal, 2019). As such, the balance between M1 and M2 macrophages is crucial in the maintenance of immune homeostasis, as the overproduction of M1 macrophages might result in the excessive production of proinflammatory mediators, which has been linked to the development of various autoimmune disorders and inflammatory diseases (Lendeckel *et al.*, 2022). On the other hand, the overproduction of M2 macrophages might result in the promotion of tumour growth, as well as immunosuppression (Fig. 1.1).

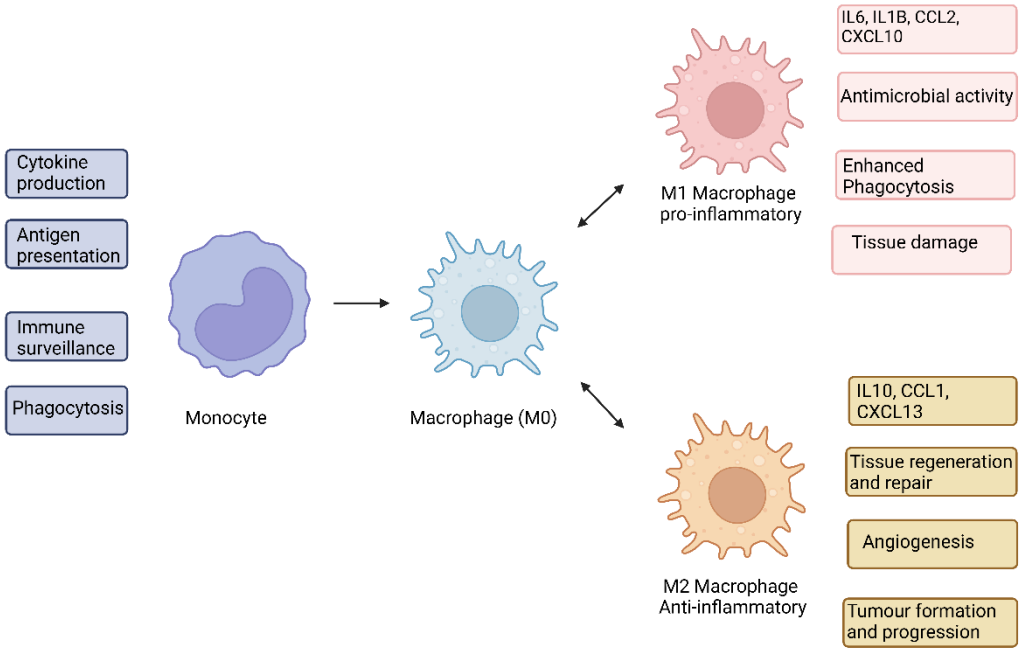

Figure 1.1 Monocyte and macrophage function. Circulatory monocytes differentiate into macrophages via various environmental stimuli. Mature macrophages then play a critical role in the production of inflammatory mediators (M1) and anti-inflammatory mediators (M2), all of which contribute to the antiviral immune response. (Generated using Biorender.com)

Overall, both monocytes and macrophages play a crucial role in the detection of various pathogens, and they are able to do this via PRRs including toll-like receptors (TLRs), retinoic acid-inducible gene 1 receptors (RLRs), nucleotide oligomerization domain-like receptors (NLRs), C-type lectin receptors (CLRs), cyclic GMP-AMP synthase (cGAS) receptors, and lastly absent-in-melanoma 2 receptors (AIM2) (Austermann *et al.*, 2022).

1.3 Pattern recognition receptors

The initiation of acute inflammation requires the recognition of a pathogen by immune cells. This recognition occurs via membrane receptors known as PRRs. PRRs recognise pathogen associated molecular patterns (PAMPs), which are conserved molecular structures shared by a pathogen e.g. β-glucan and lipopolysaccharide (LPS) (Li and Wu, 2021). Additionally, PRRs recognise damage-associated molecular patterns (DAMPs) from cells that are damaged by viral infection or released during cell killing by CD8 T cells. There are a variety of PRRs, classified based on their protein domain homology and the pathogens they recognise. PRRs include TLRs, which recognise a variety of biomolecular PAMPs, RLRs, which respond to viral double stranded DNA (dsDNA), NLRs, which recognise bacterial ligands, CLRs, which respond to fungal PAMPs, and lastly AIM2, which responds to bacterial dsDNA (Austermann *et al.*, 2022; Jang *et al.*, 2015). Furthermore, these PRRs can be present extracellularly on the cell membrane, or intracellularly on the intracellular membranes of lysosomes, endosomes or the

endoplasmic reticulum (ER) (Kawai and Akira, 2010). Alternatively, some PRRs such as RLRs are not membrane bound, but present in the host cytoplasm (Li and Wu, 2021). The binding of both PAMPs and DAMPs, which includes self-RNA, to PRRs activates various downstream signalling cascades which induce the production of antimicrobial defence proteins, cytokines, chemokines, and interferons, all which aid in the clearance of the pathogen.

1.3.1 Toll like receptors

While there are a variety of PRRs present on immune cells, TLRs represent one of the largest varieties. There are 10 known TLRs in humans, all of which recognise specific pathogens (Li and Wu, 2021). TLRs contain an extracellular domain, made up of leucine-rich repeats (LRR) which binds to the ligand, a transmembrane domain, and an intracellular domain, known as the toll/IL-1R (TIR) domain. This TIR domain interacts with various adaptor proteins, such as myeloid differentiation primary response 88 (MYD88), TRIF-related adaptor molecule (TRAM), and TIR-domain-containing-adapter-inducing-interferon-β (TRIF), thereby activating various signal transduction pathways (Kircheis and Planz, 2023). TLR1, 2 ,4, 5, 6, and 10 are extracellular membrane receptors, capable of forming both homodimers and heterodimers upon ligand binding, while TLR3, 7, 8, and 9 are intracellular receptors, expressed on endosomes and lysosomes. Unlike the intracellular TLRs, the extracellular TLRs mainly form homodimers upon ligand binding, thereby inducing a conformational change, allowing for the interaction of the TIR domains which can then interact with the relevant adapter protein to activate downstream signalling.

TLR1, 2, and 6 recognise lipoproteins found on microbial cell walls, while TLR5 recognises flagellin, and TLR4 recognises LPS (Duan *et al.*, 2022). On the other hand, TLR3 is activated by dsRNA, while TLR7 and TLR8 recognise viral single stranded RNA (ssRNA), while TLR9 recognises unmethylated 2'-deoxyribo cytidine-phosphate-guanosine (CpG) DNA motifs commonly found in viruses and bacteria (Martinez-Espinoza and Guerrero-Plata, 2022). Additionally, it is important to note that TLRs do not function alone and are able to crosstalk to ensure viral clearance, while also maintaining immune balance. Overall TLRs play a critical role in the recognition of various pathogens, all which are crucial in mounting an efficient inflammatory response. While there are multiple studies investigating the various TLRs, research on TLR7 and TLR8 expression and function in monocytes and macrophages is limited (Bender *et al.*, 2020).

1.3.2 TLR7 and TLR8

TLR7 and TLR8 are the most closely evolutionarily related TLRs; the genes of which are located next to each other on the X chromosome in humans (Patinote *et al.*, 2020). Both TLR7 and TLR8 are endosomal TLRs that respond to ssRNA viruses with their signalling dependent on the MYD88 signalling protein. As such these receptors play a critical role in antiviral immune signalling. TLR7 and TLR8 have two ligand binding sites that recognise ssRNA degradation products (Tanji *et al.*, 2015). The first site, which is highly conserved for TLR7 and TLR8, binds to guanosine, and uridine, respectively, while the second site, which is not conserved, binds short oligoribonucleotides (ORNs) (Zhang *et al.*, 2018). Importantly, the dimerisation, and as such the activation of either TLR7 or TLR8, is dependent on ORN binding in the second site, as it is this binding that strengthens the binding affinity at the first site. TLR7 and TLR8 are remarkably similar proteins, with only an 8 amino acid difference, which is added to the TLR7 C-terminal domain i.e. TLR7 is made up of 1049 amino acids while TLR8 is made up of 1041 amino acids (Fig. 1.2). Furthermore, both TLR7 and TLR8 have a 26 amino acid signal sequence, which directs the protein to the ER. However, this is cleaved off once the protein reaches the ER, yielding a membrane protein that can then respond to stimulation (Hipp *et al.*, 2015).

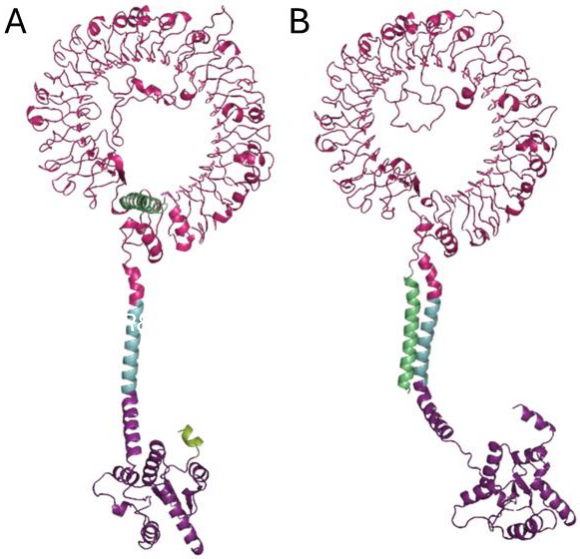

Figure 1.2 TLR7 and TLR8 proteins. Cartoon representation of the TLR7 (A) and TLR8 (B) proteins showing the LRR extracellular domain (pink), the transmembrane domain (blue), and the intracellular TIR-domain (purple). Additionally, both TLR7 and TLR8 contain a 26 amino acid length segment (green) which is cleaved off after translocation to the ER. TLR7 and TLR8 differ by 8 amino acids (yellow). (Structures generated by AlphaFold3 and coloured in PyMOL).

Despite being closely related paralogues, TLR7 and TLR8 are known to have differences. While TLR7 recognises primarily guanine (GU)-rich ssRNA viruses, such as dengue virus, and Zika virus, TLR8 recognises both GU, and adenine (AU)-rich ssRNA viruses, such as hepatitis C virus and influenza A virus (Patinote *et al.*, 2020). However, it is known that various ssRNA viruses can signal through both TLR7 and TLR8 (Martinez-Espinoza and Guerrero-Plata, 2022). In addition, in humans, TLR7 and TLR8 differ in their expression patterns, where TLR7 is mainly expressed on B cells and plasmacytoid dendritic cells, while TLR8 is mainly expressed on monocytes, macrophages, and myeloid dendritic cells (Hamerman and Barton, 2024). Furthermore, TLR7 and TLR8 are also thought to yield distinct cytokine profiles, where TLR7 signalling is more so associated with the type I IFN response, while TLR8 signalling is more so associated with the induction of proinflammatory cytokines such as TNFα, IL6, IL1β etc. (Gorden *et al.*, 2005). Additionally, while differences exist between TLR7 and TLR8 signalling, these receptors can interact with one another, and research has shown a regulatory effect of TLR8 on TLR7, where increased TLR8 expression is associated with a reduced TLR7 expression (Cervantes *et al.*, 2012).

When TLR7 and TLR8 become activated, MYD88 associates with the interleukin-1 receptor-associated kinase 1 (IRAK) complex or TRAF6, which can then associate with the IKKγ/IKβα/IKKβ complex (Fig. 1.3). This results in the translocation of the transcription factors NFkB and interferon regulatory factor 3/7 (IRF3/7) to the nucleus where they can induce the production of several critical inflammatory mediators such as IL1β, IL6, TNFα, CCL2, and CXCL10, as well as type I IFNs including IFNα and IFNβ (Nicolai *et al.*, 2022). TLR7 and TLR8 play a critical role in the initiation of antiviral immune responses, such that viruses target these pathways in an attempt to inhibit these strong antiviral effects. For example, HIV1 produces SNAPIN, a protein that prevents the colocalization of HIV-1 and TLR8, thereby preventing MHC class II expression and inhibiting T cell maturation. (Martinez-Espinoza and Guerrero-Plata, 2022). However, during HIV-1 infection, the production of IFNγ increases TLR7 expression in human macrophages, thereby making these cells more responsive to TLR7 (Petes *et al.*, 2017). Overall, the activation of TLR7 and TLR8 plays a critical role in antiviral immune responses through the induction of proinflammatory mediators which are critical for the inhibition of viral replication and viral clearance (Lind *et al.* 2021).

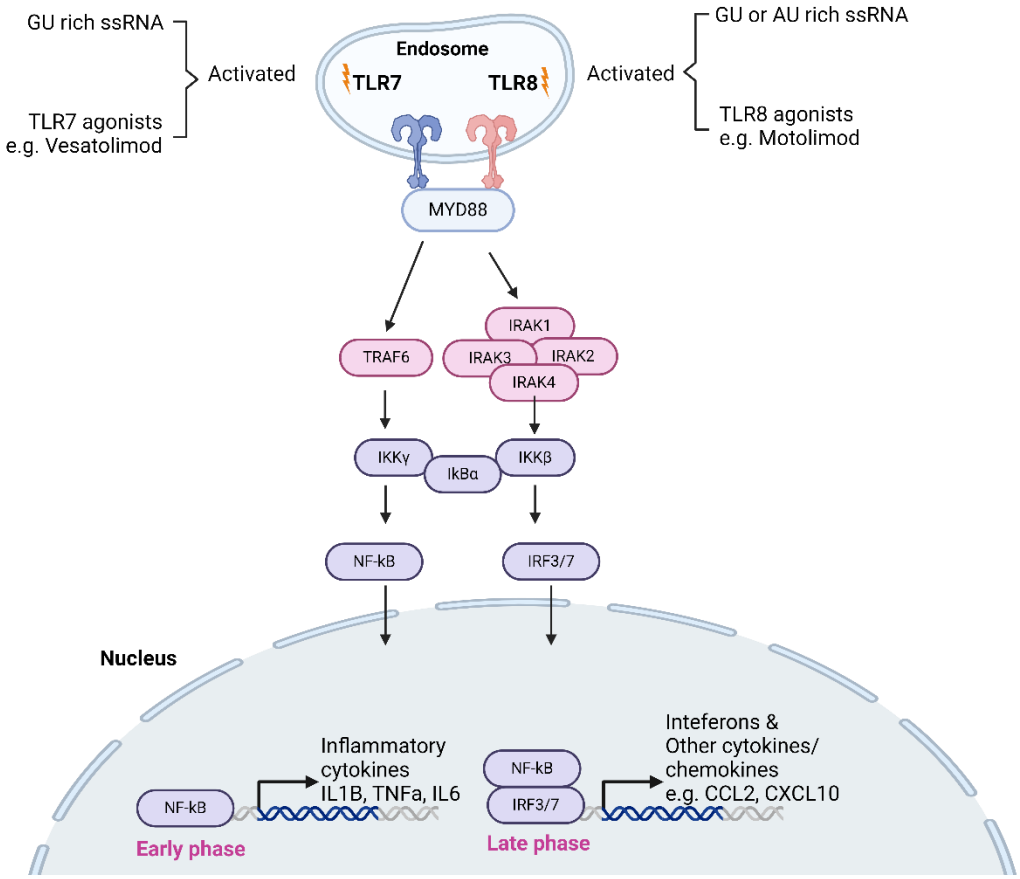

Figure 1.3 TLR7 and TLR8 signalling pathway. TLR7 and TLR8 both interact with the adaptor protein MyD88. MYD88 interacts with TRAF6 and the IRAK complex. This activates IRF3/7 and NF-kB, inducing the transcription of type IFNs and inflammatory cytokines and chemokines (Generated using Biorender.com)

1.4 Inflammatory mediators: Cytokines and chemokines

Proinflammatory mediators, such as inflammatory cytokines and chemokines are induced in response to viral infection. IFNs are produced upon IRF3/IRF7 induction which then induces the transcription of various interferon-stimulated genes (ISGs) (Jafarzadeh *et al.*, 2020). IFNs are thought to inhibit viral replication by preventing the production of polyamines through the acetylation of spermine and spermidine (Li and MacDonald, 2016). Furthermore, IFNγ, a type II IFN, increases the production of NO which results in the nitrosylation of viral molecules, thereby inhibiting viral replication (Garren *et al.*, 2021). On the other hand, inflammatory cytokines and chemokines play a critical role in the inhibition of viral replication through various immunological processes. Examples of these proinflammatory mediators include IL1β, CCL2, and CXCL10 (Turner *et al.*, 2014). IL1β is a potent proinflammatory mediator induced early post TLR7 and TLR8 activation. IL1β exists in its inactive form known as pro-IL1β which

exists in the cytosol (Dinarello, 2017). Upon TLR7/8 activation, pro-IL1β is cleaved by protease-caspase-1 into its active form, IL1β, which is then secreted by the immune cell. IL1β can then act in either an autocrine or paracrine manner by binding to the IL-1R1 receptor present on a variety of immune cells including monocytes and macrophages (Lopez-Castejon and Brough, 2011). When IL1β binds to the IL-1R1 receptor, it forms a trimolecular complex with interleukin-1 receptor accessory protein (IL-1RAP), which then interacts with MYD88 thereby inducing further activation of surrounding immune cells, further inducing cytokine and chemokine production (Weber *et al.*, 2010) (Fig. 1.4).

On the other hand, CCL2 and CXCL10 are known as chemokines, a class of small molecular weight proteins induced downstream of TLR7 and TLR8 signalling. CCL2, also termed monocyte chemoattractant protein (MCP1), is a potent proinflammatory chemokine with a well-known role in monocyte recruitment to sites of infection (Gschwandtner *et al.*, 2019). CCL2 binds to the CC-chemokine receptor 2 (CCR2), which activates various signalling cascades, including the janus kinase/signal transducers and activators of transcription (JAK/STAT), phosphoinositide-3-kinase–protein kinase (PI3K/Akt), and mitogen-activated protein kinase (MAPK) signalling pathways (Fig. 1.4). This then results in cellular migration, apoptosis and the release of chemoattractants which aids in the migration of immune cells to sites of infection. CCL2 plays a role in chemotaxis is via the release of arachidonic acid which increases integrin expression thereby increasing adhesion (Gschwandtner *et al.*, 2019). Additionally, CCL2 is thought to prime immune cells to subsequent infection which is crucial during inflammation (Ansari *et al.*, 2024).

Another potent proinflammatory chemokine is CXCL10, also known for its role in chemoattraction. CXCL10 is also called interferon gamma-induced protein 10 (IP-10) as it is a direct ISG, i.e. CXCL10 contains regulatory elements for NFkB and interferon-stimulated response elements (ISREs), thus, the release of IFNs directly induces CXCL10 expression (Mousaad Elemam *et al.* 2022). CXCL10 binds to the CXCR3a receptor present on a variety of innate immune cells including monocytes and macrophages. The binding of CXCL10 to the CXCR3a receptor activates Gαq and Gαi, which then activates the SRC and PI3K/AKT signalling pathways (Fig. 1.4). This allows CXCL10 to carry out its various functions including cellular migration, and the further activation of surrounding immune cells. CXCL10 is thought to be involved in lymphocyte activation and T cell infiltration (Dufour *et al.*, 2002). Overall, IL1β, CCL2, and CXCL10 play a crucial role in further activating immune cells, thereby aiding in the inhibition of viral replication and as such, the prevention of systemic infection. However,

the excessive production of these proinflammatory mediators is known to cause tissue damage and has been linked to the development of various autoimmune disorders, demonstrating the importance of regulating TLR signalling (Chen *et al.*, 2018).

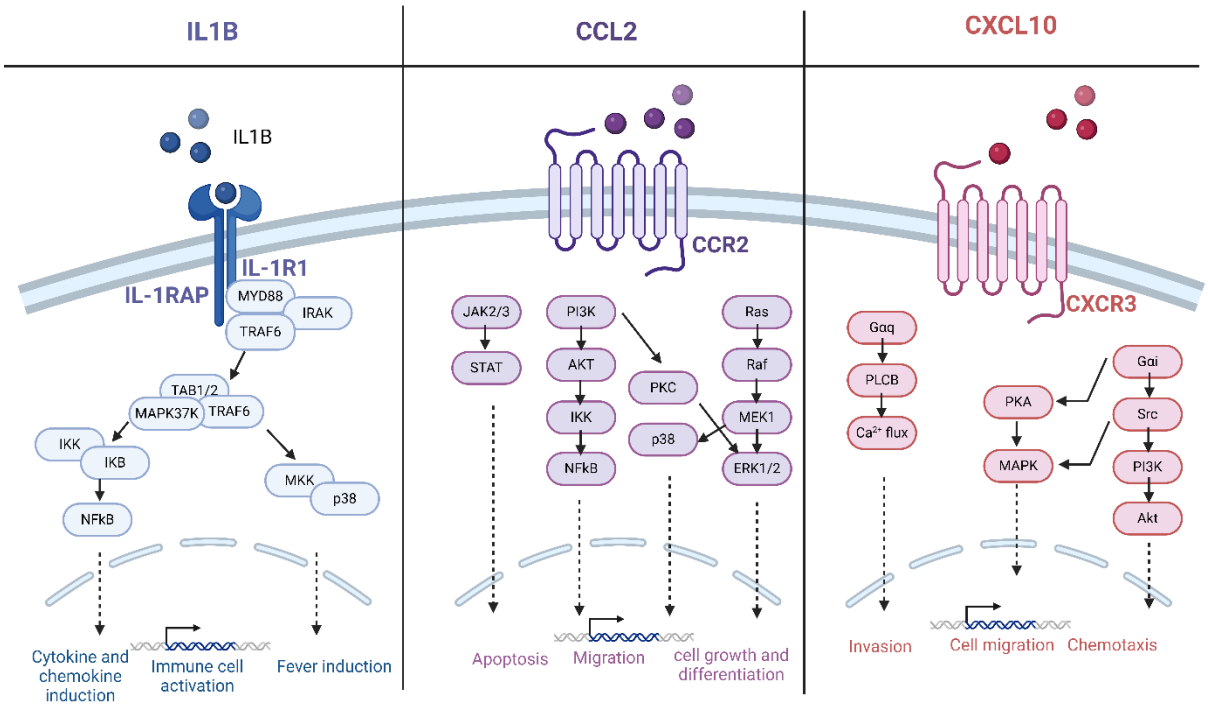

Figure 1.4 Proinflammatory IL1β, CCL2, and CXCL10 signalling pathways. The binding of the proinflammatory mediator to the relevant receptor activates various signalling pathways, thereby allowing the cytokines or chemokines to carry out their functions post TLR7 and TLR8 activation, allowing for an efficient antiviral immune response. (Generated using Biorender.com)

1.4.1 TLR Dysregulation

While the production of proinflammatory mediators is crucial for viral clearance during acute inflammation, the excessive production of these inflammatory mediator's results in persistent inflammation, thereby inducing a hyperinflammatory state. The excessive production of these proinflammatory mediators can be induced by pathogen infection, or as a result of failed TLR autoregulation which has been linked to the development of various autoimmune disorders and inflammatory diseases (Bender *et al.*, 2020). This imbalance leads to an inflammatory state, often termed a "cytokine storm" (Fig. 1.5), and has been observed in patients presenting with severe Covid-19 (Zheng *et al.*, 2022). Additionally, patients in the critical stage of SARS-CoV2 infection present with increased serum levels of IL6, TNFα, CCL2, and CXCL10, often causing acute respiratory distress syndrome, due to tissue damage (Liao *et al.*, 2020). Furthermore,

cytokine storms have also been linked to multi-organ failure and has been linked to the development and severity of autoimmune disorders including rheumatoid arthritis (RA), and systemic lupus erythematosus (SLE), via the increased expression of *TLR7* (Yan *et al.*, 2024; Hawtin *et al.*, 2023; Kircheis and Planz, 2023).

Interestingly, while certain viruses inhibit TLR responses, others, including influenza virus and SARS-CoV2, can cause the excessive release of proinflammatory mediators (Mantovani *et al.*, 2023). Furthermore, certain cytokines, like IL1β, exhibit positive feedback, thereby necessitating the need for negative feedback systems to prevent the overproduction of these cytokines (Weber *et al.*, 2010). The excessive production of CXCL10 during SARS-CoV2 infection was shown to trigger lymphopenia i.e. reduced number of circulating lymphocytes by reducing myeloid progenitor cell proliferation (Guo *et al.*, 2021). Furthermore, high levels of CXCL10 are often used as a predictive biomarker in Covid-19 patients to measure disease severity (Chi *et al.*, 2020). On the other hand, high levels of CCL2 have been associated with lung damage due to increased macrophage migration to the lung, thereby causing acute respiratory distress syndrome (ARDS; Ansari *et al.*, 2024). Additionally, the overexpression of CCL2 post influenza or RSV infection has been linked to disease severity and interestingly, the administration of a CCL2 antagonist in influenza-infected mice has been shown to inhibit pulmonary hyperinflammation (Lin *et al.*, 2010). Overall, the excessive production of proinflammatory mediator's results in a hyperinflammatory environment which exacerbates infection and can even result in death. As such, there is a need to maintain immune homeostasis via the regulation of TLR7 and TLR8 signalling.

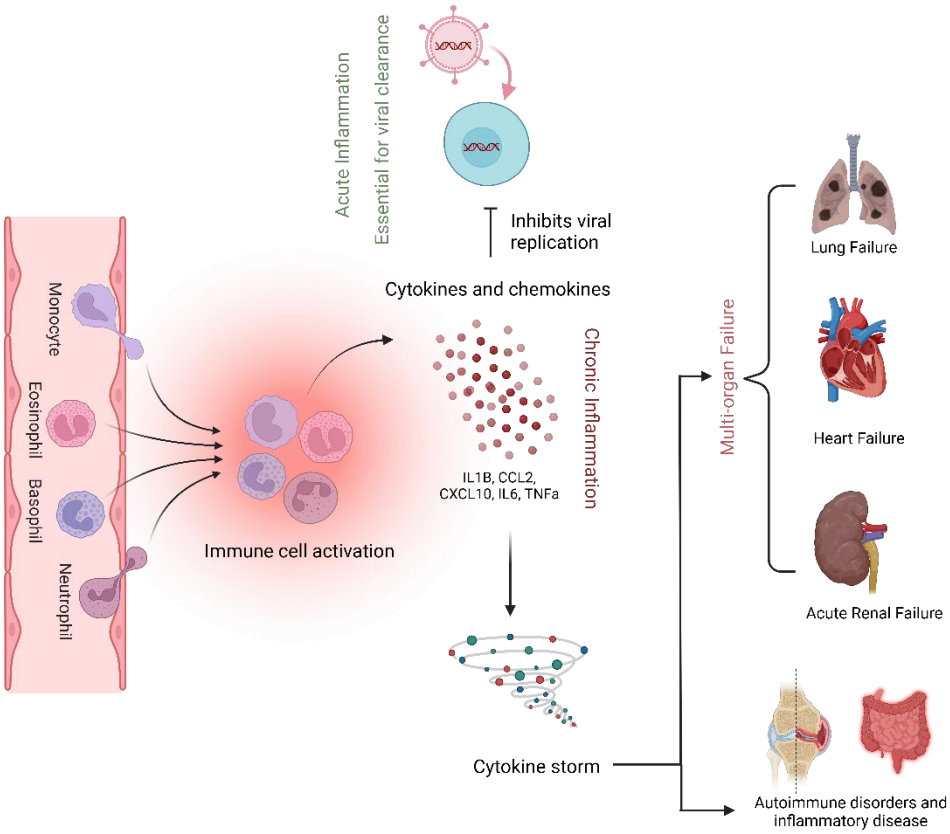

Figure 1.5 The balance between acute inflammation and chronic inflammation during viral infection. During acute inflammation, cytokines and chemokines play a crucial role in the inhibition of viral replication. However, the excessive production of these cytokines and chemokines results in chronic inflammation which results in multi-organ failure, and the development of autoimmune disorders, and inflammatory diseases. (Generated using Biorender.com)

1.5 1,25-dihydroxyvitamin D_3 and the immune system

1,25-dihydroxyvitamin D_3 ($1,25D_3$) is a steroidal hormone known for its role in calcium homeostasis, bone mineralisation, and skeletal health. $1,25D_3$ is primarily obtained via UVB radiation, however, $1,25D_3$ can also be obtained from some dietary sources including egg yolks and cod liver oils (Sadeghi *et al.*, 2014). The biologically active form of $1,25D_3$ is synthesized from 7-dehydrocholesterol (7DHC) via a non-enzymatic reaction in the skin thereby forming cholecalciferol (D_3) (Fig. 1.5). Thereafter, cholecalciferol undergoes 25-hydroxylation via cytochrome P450 family 2 subfamily R member 1 (CYP2R1) and cytochrome P450 family 27 subfamily A member (CYP27A1) to form 25-hydroxyvitamin D_3 ($25D_3$). To become biologically active, $25D_3$ is 1α-hydroxylated by cytochrome P450 family 27 subfamily B member 1 (CYP27B1) to form $1,25D_3$ (Lin *et al.*, 2016). Importantly, both $25D_3$ and $1,25D_3$ are broken down into hydroxylated derivatives by cytochrome P450 family 24 (CYP24), which

are later excreted (Jones *et al.*, 2011). Previously, it was thought that CYP27B1 was only active in the kidneys, thereby limiting $1,25D_3$ activity, however, CYP27B1 has been found to be expressed in other cells including monocytes and macrophages (Aranow, 2011). Additionally, $1,25D_3$ is an immunomodulator known for its role in the maintenance of immune homeostasis and is widely accepted as promoting an anti-inflammatory state (Arababadi *et al.*, 2018).

$1,25D_3$ plays a role in various immunological processes including the suppression of T cell proliferation and differentiation. Additionally, $1,25D_3$ can alter T cell maturation to induce the production of T regulatory cells as opposed to inflammatory Th17 cells (Aranow, 2011). Importantly $1,25D_3$ has been shown to reduce the production of proinflammatory mediators such as TNFα, and IL6 (Roffe-Vasquez *et al.*, 2022). While the way in which $1,25D_3$ alters the expression of immune-related genes has not been fully elucidated, one hypothesis is that $1,25D_3$ binds to the vitamin D receptor (VDR), present in various immune cells, and translocates to the nucleus where it forms a complex with the retinoid acid receptor (RXR). This complex then interacts with vitamin D responsive elements (VDREs) found in the regulatory regions of various target genes (Fig. 1.6) (Arababadi *et al.*, 2018). For example, during bacterial infection, post ligand binding to the relevant TLR receptor, the expression of VDR and 1α-hydroxylase is increased (Ismailova and White, 2021). This results in VDR binding to VDREs in the cathelicidin antimicrobial peptide (CAMP) gene, encoding human cathelicidin antimicrobial protein (hCAP18) and the *DEFB* gene, encoding β2defensin (DEFβ), which have antimicrobial effects (Harten et al, 2018).

During inflammation, $1,25D_3$ is thought to facilitate the switch from proinflammatory M1 macrophages to anti-inflammatory M2 macrophages as a way of maintaining immune homeostasis (Nygaard *et al.*, 2022). While $1,25D_3$ is commonly associated with the resolution of inflammation, $1,25D_3$ can also enhance immune function by increasing the phagocytic and chemotactic ability of monocytes and macrophages, highlighting the ability of $1,25D_3$ to both enhance the immune response, while simultaneously maintaining immune homeostasis to prevent hyperinflammation by reducing the excessive production of proinflammatory mediators (Arababadi *et al.*, 2018).

$1,25D_3$ has been shown to inhibit the production of proinflammatory mediators such as IL6 and TNFα in monocytes which could occur via the modulation of the TLR signalling pathways (Arababadi *et al.*, 2018; Almerighi *et al.*, 2009). There have been previous studies that have investigated the link between $1,25D_3$ and TLRs. For example, Salehi *et al.* (2015) showed that

$1,25D_3$ altered TLR2 and TLR4 expression on monocytes in patients with type II diabetes and autoimmune diabetes. Additionally, colitis patients supplemented with $1,25D_3$ have shown to have reduced TLR9 expression. Furthermore, a $1,25D_3$ insufficiency has been linked to various autoimmune disorders such as SLE, RA, psoriasis, Crohn's disease, and Sjogren's syndrome, all of which have also been linked to the excessive production of proinflammatory cytokines and chemokines (Arababadi *et al.*, 2018). Overall, $1,25D_3$ plays a crucial role in the maintenance of immune homeostasis by preventing hyperinflammation as a result of excessive proinflammatory cytokine and chemokine production.

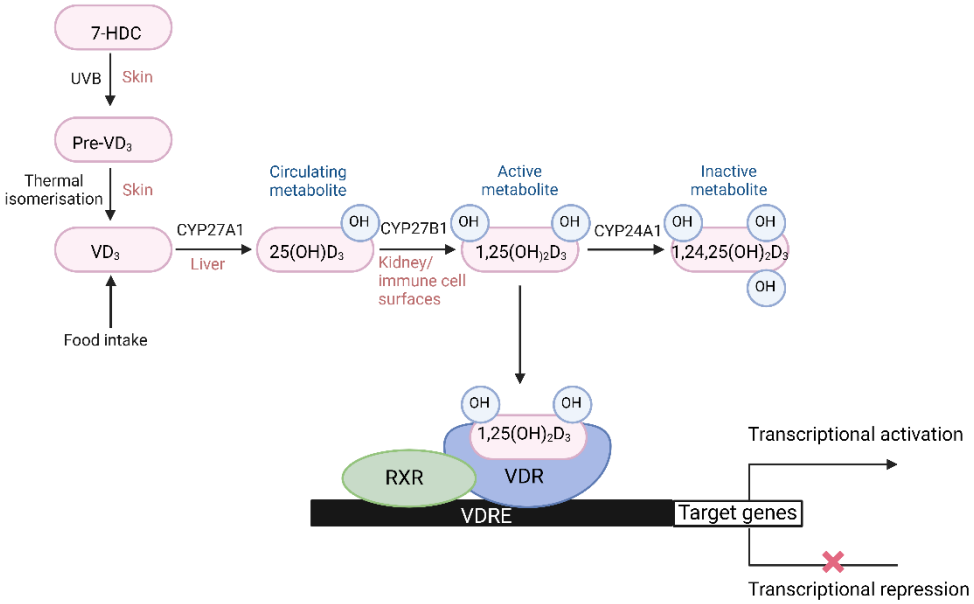

Figure 1.6 Vitamin D metabolic pathway and mechanism of biological action. $1,25(OH)_2D_3$ is produced, followed by the binding to the VDR, and retinoid acid receptor (RXR). This complex will then interact with VDREs, thereby activating or repressing gene expression. (Adapted from Lee, 2020); (Generated using Biorender.com).

1.6 Study Rationale

$1,25D_3$ plays an important role in the maintenance of immune homeostasis which is crucial in the pathogenesis of various autoimmune diseases and inflammatory disorders. While there have been previous studies linking the effect of $1,25D_3$ on TLR signalling, not much research has investigated the impact of $1,25D_3$ on TLR7 and TLR8 expression and signalling in monocytes and macrophages, and the effect of $1,25D_3$ supplementation thereon. As mentioned, TLR7 and TLR8 play a critical role in the recognition of viruses, and subsequently the initiation of antiviral immune responses. However, TLR7 and TLR8 dysregulation has been implicated in the development of various autoimmune diseases and inflammatory disorders, and as such, a better understanding of these signalling pathways, and the effects of $1,25D_3$ thereon, could provide insights into possible therapeutic approaches for hyperinflammation-related disorders arising as a consequence of TLR7 and TLR8 dysregulation.

1.7 Aims and Objectives

The overall aim of this research project was to determine the effect of $1,25D_3$ on TLR7 and TLR8 expression and signalling in monocytes and monocyte derived macrophages (MDMs). This was achieved via the following objectives:

(a) Assess the effect of $1,25D_3$ on the expression of *TLR7* and *TLR8* using RNA sequencing data from monocytes and MDMs cultured in the absence or presence of $1,25D_3$.

(b) Culture and maintain Tohoku Hospital Pediatrics-1 (THP-1) cells in the presence and absence of $1,25D_3$, and differentiation of THP-1s into MDMs in the presence and absence of $1,25D_3$.

(c) Assess the effect of $1,25D_3$ on the expression of inflammatory mediators *IL1β*, *CCL2*, *CXCL10*, and *IL10* using RT-qPCR in monocytes and MDMs cultured in the absence or presence of $1,25D_3$.

(d) Quantifying changes in *IL1β, CCL2, CXCL10,* and *IL10* expression in response to TLR7 and TLR8 stimulation in monocytes and MDMs cultured in the absence or presence of $1,25D_3$.

(e) Assessing the effect of additional $1,25D_3$ supplementation on *IL1β*, *CCL2*, *CXCL10*, and *IL10* expression in TLR7- and TLR8-stimulated monocytes and MDMs cultured in a $1,25D_3$ 'insufficient' environment vs a $1,25D_3$ 'sufficient' environment.

CHAPTER 2: Methodology

2.1 RNA Sequencing

2.1.1 Sequencing, alignment, and transcript quantification

To assess the baseline expression of *TLR7* and *TLR8* in monocytes and monocyte-derived macrophages (MDMs), and the effect of $1,25D_3$ thereon, paired-end short read RNA sequencing (RNA-seq) data was used (PRJEB76891; Mol *et al.*, 2024). For monocytes, RNA-seq data was collected for THP-1 cells, an immortalised human monocytic cell line derived from a patient with acute myeloid leukaemia, cultured in the absence and presence of $1,25D_3$ (Sigma Aldrich, St Louis, MO) for 96 h. For MDMs, THP-1s were differentiated using either 5 nM phorbol 12 myristate-13-acetate (PMA; Sigma Aldrich, St Louis, MO), or 5 nM PMA in combination with 10 nM $1,25D_3$ for 96 h. Prior to transcript quantification, the raw RNA-seq reads were subjected to quality control by FastQC (v0.12.1; Andrews, 2010). FastQC assessed the overall quality of the raw RNA seq reads by assessing factors such as per sequence quality, per sequence GC content, per base N content, as well as the sequence length. These factors are important indicators of confidence in base calling during sequencing. The overall quality across all biological replicates was assessed using MultiQC (v1.24; Ewels *et al.*, 2016).

Thereafter, transcript alignment and quantification were performed using Salmon v1.10.1 (Patro *et al.*, 2017). A decoy-aware transcriptome index was generated by computing a set of decoy sequences by mapping the annotated transcripts against a hard-masked version of the GENCODE v43 annotations of the human reference genome (GRCh38). This increases the accuracy and reliability of Salmon by identifying and masking pseudogenes, thus reducing ambiguous mapping. As Salmon performs partial alignment, the raw RNA seq reads were not trimmed, and were selectively aligned using the Salmon quant command. The library type was automatically inferred by Salmon using the -1A (--libTypeA) option. The –seqBias option was passed through the quant command to correct for sequence-specific biases, and to correct for random hexamer priming bias. Similarly, the –gcBias was passed through the quant command to correct for GC biases. Additionally, the –recoverOrphans option was passed through the quant command to quantify single-end reads that were not paired. Tximport v1.26.1 (Soneson *et al.*, 2015) was then used to aggregate transcript level abundance values to the gene level, generating a count matrix for differential gene expression analysis.

2.1.2 Differential gene expression analysis

To determine the changes in gene expression in response to $1,25D_3$ in monocytes and macrophages, the R package, DESeq2 v1.44.0 (Love *et al.*, 2014) was used. The log fold change shrinkage estimates were derived using apeglm v1.22.1 (Zhou *et al.*, 2018), to filter out the lowly expressed genes that may be falsely identified as being differentially expressed. Pairwise comparisons for differential gene expression were performed between monocytes (THP-1s cultured with 99% molecular grade ethanol) and monocytes + $1,25D_3$ (THP-1s cultured in the presence of 10 nM $1,25D_3$), and between MDMs (THP-1s differentiated into MDMs using 5 nM PMA) and MDMs + $1,25D_3$ (THP-1s differentiated into MDMs using 5 nM PMA in combination with 10 nM $1,25D_3$) using the Wald test with Benjamini-Hochberg correction. Genes were considered expressed if their base mean expression exceeded 20 and were considered significantly differentially expressed at a Benjamini-Hochberg adjusted *p* value of $\alpha < 0.05$, with an absolute $\log_2$ fold change > 1. Using this analysis pipeline, the expression of *TLR7* and *TLR8* in monocytes and MDMs, and the effect of $1,25D_3$ thereon was investigated.

2.2 Cell culture and treatment

To assess whether changes in the expression of *TLR7* and *TLR8* had an impact on the expression of inflammatory mediators downstream of TLR7 and TLR8 signalling, and to assess TLR7 and TLR8 activation, and the impact of $1,25D_3$ thereon, monocyte and MDM cell models had to be established, maintained, and exposed to a TLR7 or TLR8 agonist *in vitro* (Fig. 2.1).

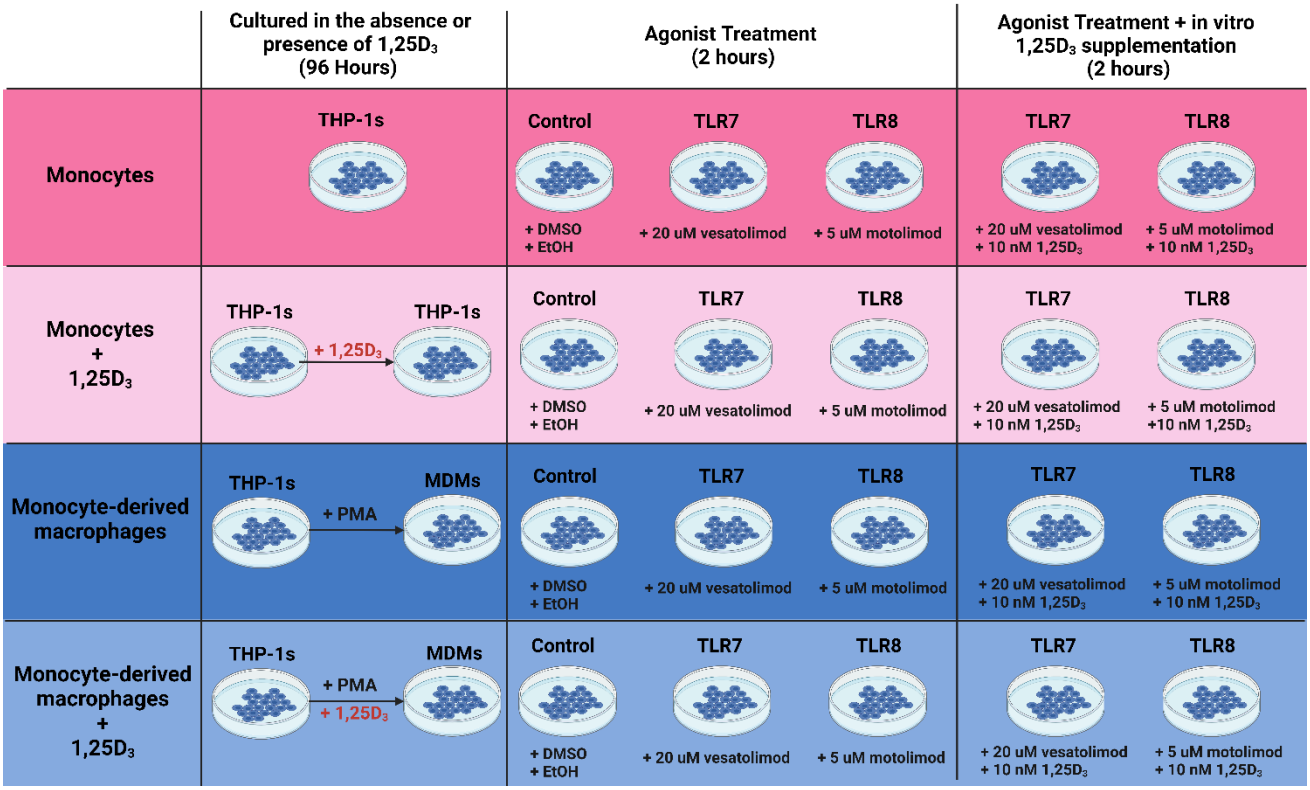

Figure 2.1 Cell Model. Monocytes were cultured in the absence or presence of $1,25D_3$ for 96h. For MDMs, THP-1s were differentiated with either 5 nM phorbol 12 myristate-13-acetate (PMA) or 5 nM PMA in combination with 10 nM $1,25D_3$ for 96h. Monocytes and MDMs were then treated with either vesatolimod (TLR7 agonist) or motolimod (TLR8 agonist) in the presence or absence of 10 nM $1,25D_3$ for 2h. The vehicle control was treated with dimethyl sulfoxide (DMSO) and ethanol (EtOH).

2.2.1 Monocyte culture and treatment

The THP-1 cell line (HIV Reagent program ARP-9942) was a gift from Dr H Ranchod (NICD, South Africa). The THP-1 cells were cultured and maintained in Roswell Park Memorial Institute 1640 (RPMI 1640, Pan Biotech) media, supplemented with 1% stable L-glutamine, which serves as a carbon source for growth and energy supply, 10% (v/v) foetal bovine serum (FBS; PanBiotech) which contains important growth factors and nutrients, and 1% (v/v) penicillin/streptomycin (Sigma Aldrich, St Louis, MO) which prevents bacterial growth. Additionally, for all experimental purposes, cells were used between passage 5 to 10, following revival. Once the cells reached 80% confluency, the cells were plated in 60 mm culture plates with a 22.1 cm2 growth area (TPP, Trasadingen, Switzerland) at a concentration of 5 x 10^5 cells/mL in the presence or absence of 10 nM $1,25D_3$ (Sigma Aldrich, St Louis, MO) previously resuspended in 100% molecular grade ethanol (EtOH; Sigma Aldrich, St Louis, MO) to a final concentration of 5 µM for 96h (Mol *et al.*, 2024). THP-1s were treated with either 20 µM

vesatolimod (Sigma Aldrich, St Louis, MO), or 5 µM motolimod (Sigma Aldrich, St Louis, MO) as determined by the concentration study (Section 2.2.3), and incubated at 37 ℃, 5 % CO_2, and 95 % humidity for 2h. Additionally, the cells were also treated with either 20 µM vesatolimod or 5 µM motolimod in combination with an additional 10 nM 1,25D_3 and incubated at 37 ℃, 5 % CO_2, and 95 % humidity for 2h. The vehicle control contained 0.05% dimethyl sulfoxide (DMSO; Sigma Aldrich, St Louis, MO), accounting for the DMSO the agonists were resuspended in, and 0.002 % molecular grade ethanol (Sigma Aldrich, St Louis, MO), accounting for the ethanol 1,25D_3 was resuspended in. This ensured that the ethanol and DMSO itself did not affect the results and outcome of the experiment (Fig. 2.1). Following incubation, the cell suspension was centrifuged at 400 x *g* for 5 mins, and the supernatant discarded. The cell pellet was then resuspended in phosphate-buffered saline (PBS, pH 7.4; Sigma Aldrich, St Louis, MO) to wash the cells while maintaining osmolarity. Thereafter, a trypan blue exclusion assay (Section 2.3) was performed to determine the cell count and cell viability. Cell aliquots of 1 x 10^6 cells/mL were made by centrifuging cell suspensions at 500 x *g* for 5 mins, discarding the supernatant, and snap freezing the cell pellets in liquid nitrogen. Cell pellets were stored at -80 ℃ for RNA extraction.

2.2.2 Monocyte to macrophage differentiation, culture, and treatment

To differentiate the promonocytic THP-1 cells into macrophages, THP-1 cells were plated at 5 x 10^5 cells/mL in RPMI 1640 (10 % FBS, and 1% p/s) and treated with 5 nM phorbol 12 myristate-13-acetate (PMA; Sigma Aldrich, St Louis, MO) in the presence or absence of 10 nM 1,25D_3 and incubated at 37 ℃, 5 % CO_2, and 95 % humidity for 24 h. After incubation, the media was poured off and the now adherent cells were washed with 2 mL PBS. Thereafter, RPMI 1640 (10 % FBS, 1 % p/s) was added to the plates with or without 10 nM 1,25D_3 and further incubated at 37 ℃, 5 % CO_2, and 95 % humidity for 72 h i.e. the differentiation process occurs for a total of 96 h. The macrophages were then treated with either 20 µM vesatolimod or 5µM motolimod in the presence or absence of an additional 10 nM 1,25D_3 for 2 h, as described for the monocytes (Fig. 2.1, Section 2.2.1).

2.2.3 Concentration Study

To determine the concentration of agonist with which to treat the cells, THP-1s were cultured and maintained as above (Section 2.2.1) and plated in 60 mm culture plates with a 22.1 cm2 growth area at a concentration of 5 x 10^5 cells/mL. THP-1s were then treated with 0 µM (DMSO control to account for what the agonists are suspended in), 3 µM, 5 µM, 10 µM, or 20 µM of either vesatolimod, a TLR7-specific agonist, or motolimod, a TLR8-specific agonist and incubated at 37 ℃, 5 % CO_2, and 95 % humidity for 2 h. Cells were then harvested as above (Section 2.2.1). The expression of *IL1β, CCL2,* and *CXCL10* was then measured by RT-qPCR to assess which concentration of agonist best induced their expression.

2.3 Cell count and viability assay

To assess overall cell health and the effect of treatment on cell viability, the trypan blue assay was routinely used throughout the study. The assay is based on the principle that cells that are healthy and 'alive' possess intact cell membranes, thus preventing the entry of the trypan blue dye and giving a clear appearance when viewed under the microscope, while dead cells with a compromised cell membrane allow the entry of the dye, yielding a blue appearance (Strober, 2015). Using this technique cells can be counted, and viability can be estimated. An equal volume (7 µL) of trypan blue dye (Sigma Aldrich, St Louis, MO) was added to an equal volume of cells suspended in PBS. This solution (10 µl) was then transferred to a haemocytometer and placed into the Countess II FL Automated Cell Counter (Invitrogen, Casrlsbad, California). Throughout the study, cells with only greater than 90% viability were used.

2.4 Screening for mycoplasma contamination

To ensure that cell cultures were free from mycoplasma contamination, the MycoStrip® mycoplasma detection kit was used (Invivogen) according to the manufacturer's instructions. The assay is based on isothermal PCR detection of the 16S rRNA gene for the most commonly found mycoplasma species. Briefly, 1 mL of cell culture supernatant was collected and centrifuged at 16000 x *g* for 5 mins. The supernatant was discarded, and the pellet resuspended in 500 µL sterile PBS forming the 'prepared sample'. Thereafter, 5 µL of the prepared sample was added to 5 µL of the reaction buffer and incubated at 65℃ for 40 mins. After incubation, 200 µL of the migration buffer was added and mixed thoroughly. Thereafter, 100 µL of the mixture was added to the immunochromatographic strip. After 5 mins, the mycoplasma strips displayed a singular line, indicating the absence of mycoplasma contamination (Fig. S1).

2.5 RNA extraction

Total RNA was extracted from frozen cell pellets (1 x 10^6 cells) using the Direct-zol™ RNA Miniprep Kit as per the manufacturer's instructions. To lyse the cells, cell pellets were resuspended in 700 µL Trizol and 700 µL molecular grade ethanol (99%, Sigma Aldrich, St Louis, MO) and mixed thoroughly. This solution was then placed into a Zymo-spin column and centrifuged at 16 000 x *g* for 30 sec at 4 ℃. Following centrifugation, 400 µL of RNA pre-wash was placed into the Zymo-spin column and centrifuged at 16 000 x g for 30 sec at 4 ℃. This ensures the removal of RNases and possible contaminants such as salts, and enzymes. Thereafter, to ensure the removal of all DNA, 5 µL DNase, and 75 µL DNA digestion buffer was added to the Zymo-spin column and incubated at room temperature for 15 mins. After incubation, 400 µL of RNA pre-wash was added directly to the column and centrifuged at 16 000 x g for 30 sec at 4 ℃. This step was repeated followed by the addition of 700 µL of the RNA wash buffer and centrifuged at 16 000 x g for 2 mins at 4 ℃. Lastly, to elute the RNA, 30 µL of DNase/RNase-free water was added to the column and centrifuged at 16 000 x g for 30 sec at 4 ℃.

2.6 Quantity and quality assessment of extracted RNA

To quantify the amount of extracted RNA, spectrophotometry was performed on a Nanodrop™ One microvolume UV-VIS spectrophotometer (ThermoFisher Scientific, Waltham, MA, United States). The extracted RNA (1 µL) was placed onto the spectrophotometer probe and quantified at a wavelength of 260 nm. Readings were further recorded at 230 nm and 280 nm to assess nucleic acid purity and RNA purity, respectively. RNA integrity was assessed by means of agarose gel electrophoresis where a 1.5% (w/v) gel was prepared using 0.375 g of agarose powder (Sigma Aldrich, St Louis, MO) dissolved in 25 mL of a 1X TAE buffer composed of 0.89M boric acid (Merck, Modderfontein, South Africa), 0.89M Tris (Sigma Aldrich,St Louis, MO), and 0.5M ethylenediaminetetraacetic acid (EDTA) disodium salt (Sigma Aldrich, St Louis, MO) at a pH of 8. After the gel had polymerised, 500 ng of RNA was loaded into each well and the gel was allowed to electrophorese at 100 V for 40 min.

2.7 Reverse-transcriptase quantitative polymerase chain reaction (RT-qPCR)

To quantify the changes in gene expression of *IL1β, CCL2, CXCL10,* and *IL10* in monocytes and MDMs treated with and without vesatolimod and motolimod, primers were first designed using NCBI Primer Blast (Ye *et al.*, 2012), in which the target sequence of each gene of interest was entered in fast alignment (FASTA) format. The tool then automatically designed primers

specific to that splice variant (Table 2.1). Thereafter, cDNA synthesis and qPCR were performed using the RevertAid cDNA Synthesis kit (ThermoFisher Scientific, Waltham, MA, United States) and the Luna Universal qPCR master mix (New England Biolabs, Germany), respectively according to the manufacturer's instructions. The cDNA master mixes were prepared using the extracted RNA (Table 2.2) and placed in the Simpli Amp thermocycler (Thermo fisher Scientific, Waltham, MA) set to incubate at 42 ℃ for 60 mins, followed by a heating step at 70 ℃ for 5 mins to terminate the reaction. The cDNA was stored at 4 ℃ prior to use in the qPCR reaction. The cDNA was then added to the qPCR reaction mixture (Table 2.3) and placed in the CFX-96 Thermal Cycler (Bio-Rad, Hercules, CA) to begin the amplification of the target genes. The samples were subjected to PCR at 95 ℃ (initial denaturation) for 15 seconds, followed by a denaturation step at 95 ℃ for 15 seconds, and extension at 60 ℃ for 30 seconds. The denaturation and extension steps were repeated for a further 39 cycles (40 cycles total). Following PCR, a melting step was included, where the temperature was incrementally increased 0.5°C per cycle starting at 60 to 90°C to allow melt-curve analysis. The final results shown are indicative of the relative quantities of the target genes normalised to the quantities of the reference genes ubiquitin C (UBC), and tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta (YWHAZ) using the ΔCT method on Version 1.0 of the CFX Maestro software (v1.0; Bio-Rad Laboratories, CA, USA), according to the formulae below:

$$Normalized\ Expression = \frac{Relative\ quantity\ of\ Gene\ of\ Interest\ sample}{Relative\ quantity(UBC) x Relative\ quantity(YWHAZ)}$$

Where:

$$Relative\ quantity = Efficiency\ of\ primer^{(Cq_{min} - Cq_{max})}$$

Cq represents the quantification cycle obtained from Version 1.0 of the CFX Maestro software (v1.0; Bio-Rad Laboratories, CA, USA).

Table 2.0.1: Primer sequences used in this study

Gene	Protein encoded	Forward Primer (FP; 5' – 3') Reverse Primer (RP; 5' – 3')	Reference*
UBC	Ubiquitin C	FP: ATTTGGGTCGCGGTTCTTG RP: TGCCTTGACATTCTCGATGGT	Vandesompele *et al.*, 2002
YWHAZ	Tyrosine 3-monooxygenase/ tryptophan 5-monooxygenase activation protein zeta	FP: ACTTTTGGTACATTGTGGCTTCAA RP: CCGCCAGGACAAACCAGTAT	Vandesompele *et al.*, 2002
IL1β	Interleukin 1 beta	FP: TCGCCAGTGAAATGATGGCT RP: GGTCGGAGATTCGTAGCTGG	
CCL2	C-C motif chemokine	FP: CCCAAAGAAGCTGTGATCTTCA RP: TGTGGAGTGAGTGTTCAAGT	
CXCL10	C-X-C motif chemokine ligand 10	FP: GTGGCATTCAAGGAGTACCT RP: TTGATGGCCTTCGATTCTGG	
IL10	Interleukin 10	FP: GCCTAACATGCTTCGAGAT RP: GCAACCCAGGTAACCCTTA	

*In the absence of a reference the primers were designed using NCBI primer BLAST

Table 2.2: Reaction components of cDNA synthesis reaction

Component	Volume (µL)
Template RNA	Variable (1 µg)
Oligo (dt) primers	1
Nuclease-free water	Variable (To 12)
5x Reaction buffer	4
Ribolock RNase inhibitor	1
10 mM dNTP mix	2
RevertAid M-MuLV RT	1
Total Volume	20

Table 2.3: Reaction components of qPCR reaction

Component	Volume (µL)	Final Conc
Luna Universal qPCR Master Mix	5	1X
10 µM forward primer	0.25	250 nM
10 µM reverse primer	0.25	250 nM
cDNA	Variable	< 100 ng
Nuclease-free water	Variable (To 10)	
Total volume	10	

2.8 Statistical analysis

Statistical analysis was performed using IBM SPSS statistics v29.0.2.0 (20). To determine whether $1,25D_3$ influenced the expression of *IL1β*, *CCL2*, *CXCL10*, and *IL10* in monocytes and MDMs, a One-way analysis of variance (ANOVA) was performed using the normalized expression values. Assuming the ANOVA showed overall significance, post-hoc tests were performed using the Fisher's least significant difference (LSD) test. A One-way ANOVA with an LSD post-hoc test was also performed to determine whether mRNA expression of *IL1β*, *CCL2*, *CXCL10*, and *IL10* was significantly induced in TLR7- and TLR8-activated monocytes and MDMs cultured in either the presence or absence of $1,25D_3$. A p-value of < 0.050 was considered significant.

CHAPTER 3: Results

3.1 RNA seq data reveals that 1,25D$_3$ impacts TLR7 and TLR8 expression

TLR7 and TLR8 play a critical role in the initiation of the antiviral immune response. However, their individual effects are understudied, and current research is often contradictory, specifically with regards to the expression of TLR7 and TLR8 in monocytes and macrophages. In addition, while the effect of 1,25D$_3$ on various TLRs, such as TLR3 and TLR9, have previously been studied (Dickie *et al.*, 2010), the effect of 1,25D$_3$ on the TLR7 and TLR8 signalling pathways have not been well established. As such, the effect of 1,25D$_3$ on the expression of *TLR7* and *TLR8* in monocytes and MDMs was investigated using RNA seq.

3.1.1 Quality control shows high-quality RNA seq data

FastQC (v0.11.9; Andrews, 2010) was used to determine the quality of the RNA seq data prior to transcript alignment and differential gene expression analysis. All samples had a single length of 151 base pairs. The mean quality score across each base position in the read had a phred score above 30, indicating a 0.1% probability that the base was called incorrectly (Fig.. 3.1 A). The per sequence quality scores showed the peak of the distribution at a phred score of 35, indicating good overall sequencing quality (Fig.. 3.1 B). The per base N content was less than 1%, where N represents the percentage of bases that could not be confidently determined (Fig.. 3.1 C). Lastly, the percentage per sequence GC content was consistently around 50% which is typical of human genomes (Fig.. 3.1 D).

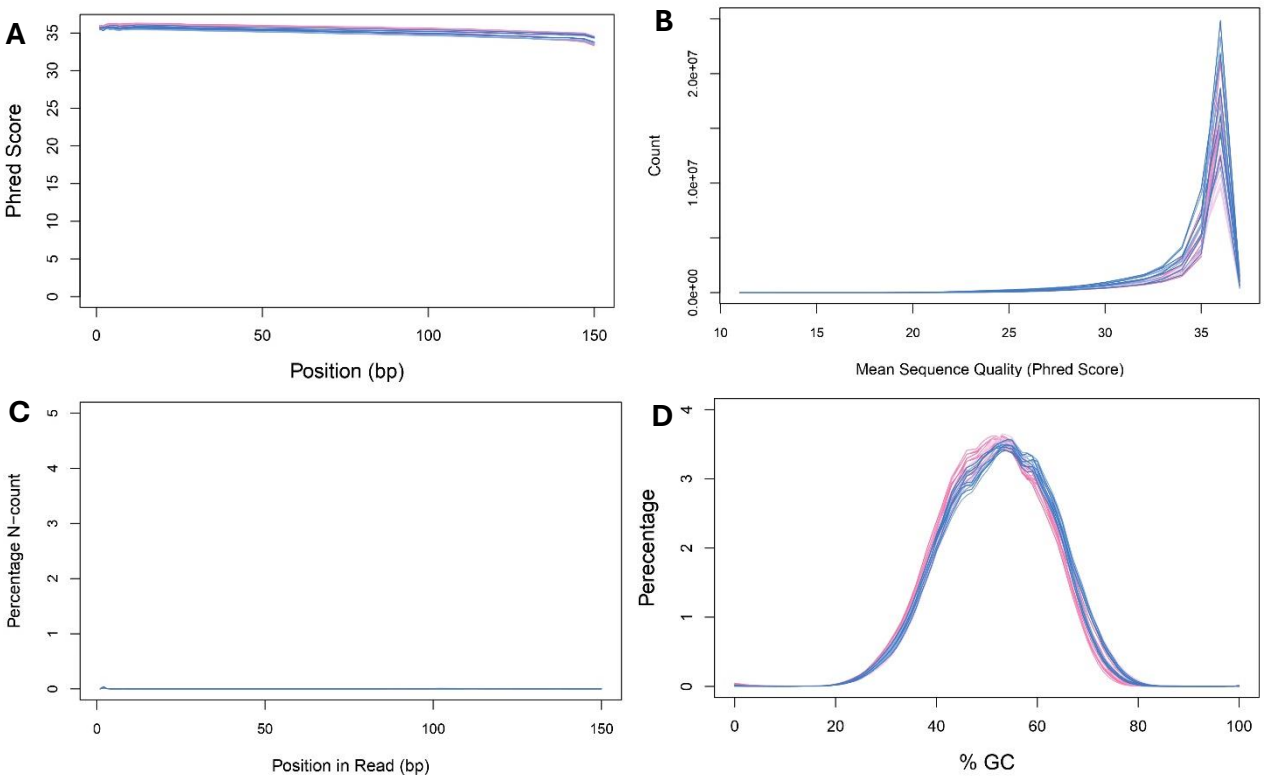

Figure 3.1 The RNA sequencing data was of good quality for alignment to the reference transcriptome and differential gene expression analysis. Using FastQC (v0.11.9; Andrews, 2010) and MultiQC (v1.2.3; Ewels *et al.*, 2016), the per base mean quality score (A), the per sequence quality scores (B), the per base N content (C), and per sequence GC content (D) were all within acceptable ranges.

3.1.2 1,25D$_3$ alters the expression of TLR7 and TLR8 in monocytes and monocyte-derived macrophages

To quantify the expression of *TLR7* and *TLR8* in monocytes and MDMs grown in the presence or absence of 1,25D$_3$, transcripts were first aligned to the GENCODE v43 annotations of the human reference genome (GRCh38) using Salmon v1.10.0 (Patro *et al.*,2017), with the alignment rates for all replicates, for all conditions, seen to be between 88% and 90%. Differential gene expression was then performed using the R package, DESeq2 v1.44.0 (Love *et al.*, 2014), and the DESeq2 derived normalised counts for *TLR7* and *TLR8* were plotted. *TLR7* expression was seen to be significantly lower in monocytes ($p<0.010$), and macrophages ($p<0.001$) cultured in the presence of 1,25D$_3$ (Fig.. 3.2 A). In contrast, *TLR8* expression was seen to be significantly higher in both monocytes and MDMs ($p<0.001$) cultured in the presence of 1,25D$_3$ (Fig. 3.2 B). Notably, the baseline expression of TLR*7* and *TLR8* was lower in the monocytes compared to the macrophages.

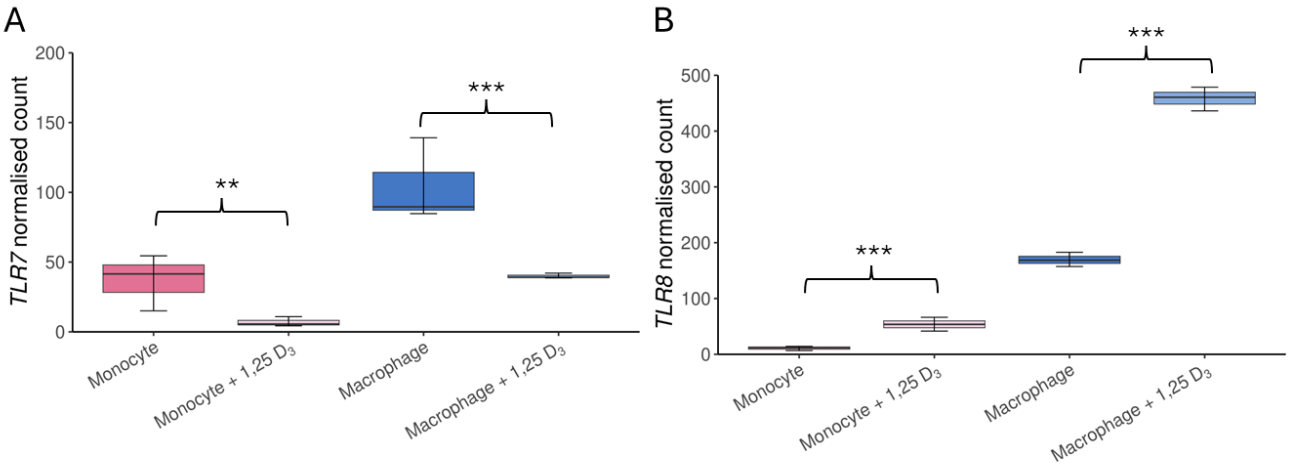

Figure 3.2 The expression of *TLR7* and *TLR8* is altered in the presence of 1,25D$_3$ in THP-1 monocytes and monocyte-derived macrophages. The box plots show DESeq2 derived normalized counts for *TLR7* (A) and *TLR8* (B) for each condition compared to their relevant control. Statistically significant differences between each condition and their respective control are indicated based on the Benjamini-Hochberg adjusted p values derived from DESeq2 analysis (**p<0.010, ***p<0.001).

3.2 1,25D$_3$ alters the expression of downstream pro-inflammatory mediators in monocyte-derived macrophages

Given that culturing cells in the presence of 1,25D$_3$ alters the expression of *TLR7* and *TLR8* in monocytes and MDMs, the expression of various downstream proinflammatory mediators (IL1β, CCL2, and CXCL10), as well as IL10, an anti-inflammatory marker, was determined by RT-qPCR in monocytes and MDMs cultured in the absence or presence of 1,25D$_3$ for 96 h.

3.2.1 THP-1 cells were successfully cultured and differentiated into monocyte-derived macrophages

THP-1 cells were cultured in the absence or presence of 10 nM 1,25D$_3$ for 96 h (Fig. 3.3 A and B). For MDMs, THP-1 cells were treated with either 5 nM PMA, or 5 nM PMA + 10 nM 1,25D$_3$ for 96 h (Fig. 3.3 C and D). The monocytes presented with a characteristic rounded morphology, and remained in suspension regardless of 1,25D$_3$ application (Fig. 3.1 A and B). The MDMs presented with an adherent phenotype and a larger, slightly elongated morphology (Fig. 3.3 C). Notably, MDMs cultured in the presence of 1,25D$_3$ appeared more spindle-like (Fig. 3.3 D).

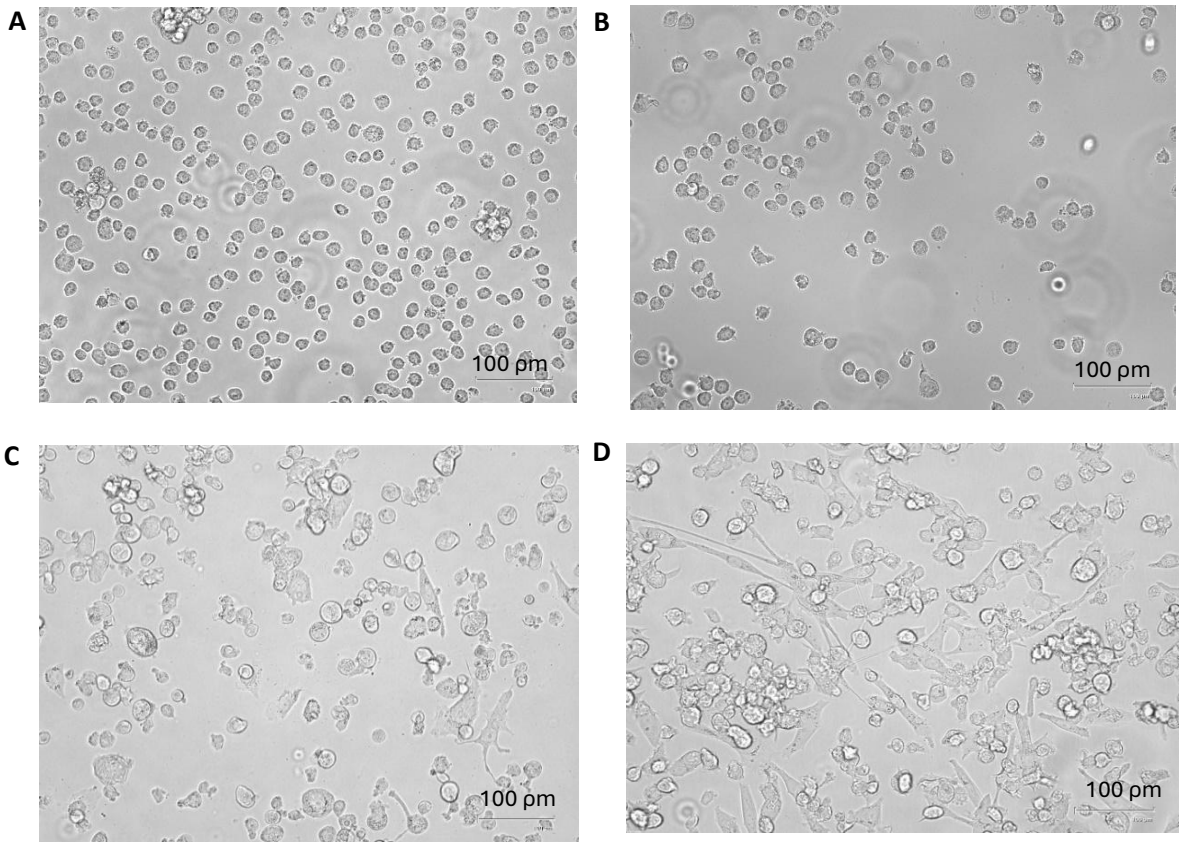

Figure 3.3 THP-1 cells were successfully cultured and differentiated into monocyte-derived macrophages in the presence and absence of 1,25D$_3$. Micrographs show THP-1 cells cultured and maintained in Roswell Park Memorial Institute (RPMI 1640, Pan Biotech) media, supplemented with 1% stable L-glutamine, 10% (v/v) foetal bovine serum (FBS) and 1% (v/v) penicillin/streptomycin. THP-1 cells were then cultured in the absence (A) and presence (B) of 10 nM 1,25D$_3$ for 96 h. For MDMs, THP-1 cells were treated with 5 nM phorbol 12 myristate-13-acetate (PMA) (C) or PMA in combination with 10 nM 1,25D$_3$ (D).

3.2.2 1,25D$_3$ decreases the expression of *IL1β, CCL2,* and *CXCL10* in monocyte-derived macrophages

The expression of *IL1β, CCL2, CXCL10* and *IL10* was quantified by RT-qPCR. A one-way ANOVA was performed to determine the effects 1,25D$_3$ on gene expression in THP-1 monocytes and MDMs cultured in the absence or presence of 10 nM 1,25D$_3$. Pairwise comparisons showed there were no significant differences in *IL1β, CCL2,* and *CXCL10* expression between monocytes cultured in the absence of 1,25D$_3$ compared to those cultured in the presence of 1,25D$_3$. On the other hand, the expression *of IL1β, CCL2*, and *CXC10* were significantly decreased in MDMs cultured in the presence of 1,25D$_3$ (p<0.050; Fig. 3.4). Additionally, the baseline expression of *IL1β, CCL2,* and *CXCL10* was significantly higher in the MDMs compared to the monocytes. However, no significant differences were observed

between monocytes and MDMs cultured in the presence of 1,25D$_3$. Additionally, *IL10* expression was significantly higher in MDMs compared to monocytes, regardless of 1,25D$_3$ treatment. However, while *IL10* expression is higher in MDMs cultured in the presence of 1,25D$_3$, no significant differences in *IL10* expression were observed in either monocytes or MDMs cultured in the presence of 1,25D$_3$. Notably, for the most part, similar patterns of expression were observed using the RNA-seq dataset (Fig. S2).

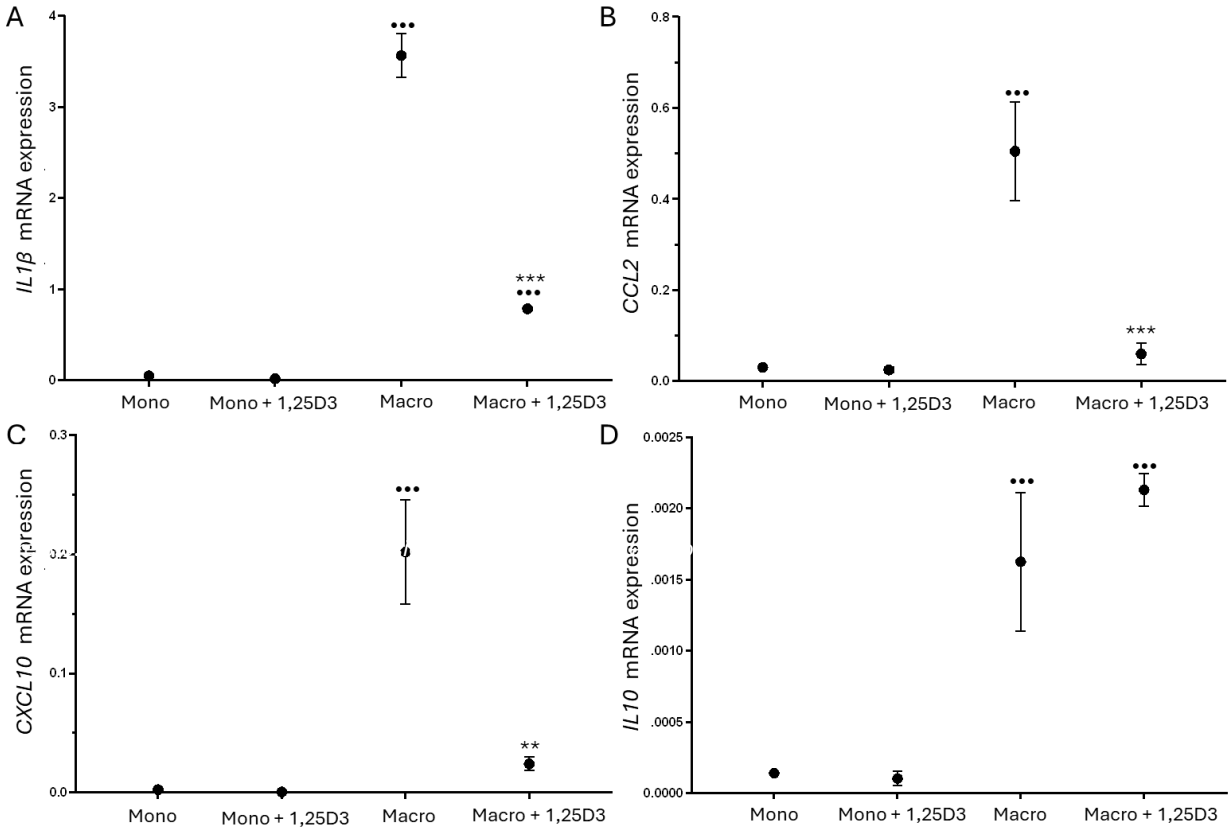

Figure 3.4 1,25D$_3$ decreases the expression of inflammatory mediators in macrophages. Error bar plots show *IL1β* (A), *CCL2* (B), *CXCL10* (C), and *IL10* (D) mRNA expression normalised to *UBC* and *YWHAZ* in monocytes (Mono) and macrophages (Macro) cultured in the absence or presence of 1,25D$_3$ for 96h. Pairwise significant differences in expression between monocytes and macrophages cultured in the presence of 1,25D$_3$ and absence of 1,25D$_3$ are shown (* p<0.050, ** p<0.010). Pairwise significant differences in induction between monocytes and macrophages (monocytes vs macrophages and monocytes+1,25D$_3$ vs macrophages+1,25D$_3$) are shown (••• p<0.001). Error bars show 95% CI (n = 3).

3.3 RT-qPCR reveals 1,25D$_3$ influences TLR7 and TLR8 signalling

Given that 1,25D$_3$ impacted the expression of *TLR7* and *TLR8*, as well as the expression of critical proinflammatory mediators, the functional effects of these changes on downstream signalling was assessed by culturing monocytes and MDMs in the absence or presence of 1,25D$_3$ for 96 h followed by stimulation with either 20 µM vesatolimod, or 5 µM motolimod for 2h. The monocytes and MDMs were also treated with either 20 µM vesatolimod or 5 µM motolimod in combination with an additional 10 nM 1,25D$_3$ for 2h to assess the effect of additional 1,25D$_3$ supplementation. The expression of *IL1β*, *CCL2*, *CXCL10*, and *IL10* were then assessed by means of RT-qPCR. Additionally, cell viability was determined via a trypan blue exclusion assay to ensure cells remained above 90% viability despite treatment conditions.

3.3.1 20µM vesatolimod and 5 µM motolimod were the optimal concentrations with which to treat the cells to induce *IL1β*, *CCL2*, and *CXCL10* expression

The concentration of agonist with which to treat the cells was determined by a concentration study in which THP-1 cells were stimulated with 0 µM, 3 µM, 5 µM, 10 µM, and 20 µM of either vesatolimod or motolimod for 2 hours. Thereafter, the expression of *IL1β*, *CCL2*, and *CXCL10* were measured by RT-qPCR and normalised to *UBC* and *YWHAZ*. The gene expression of *IL1β*, *CCL2*, and *CXCL10* was greatest in the THP-1 cells treated with 20 µM vesatolimod (Fig. 3.5A), whereas the gene expression of *IL1β*, *CCL2*, and *CXCL10* was optimal at 5 µM motolimod, where at 10 µM, and 20 µM motolimod, *CCL2* and *CXCL10* expression began to decrease (Fig. 3.5B). As such, for all experimental purposes, monocytes and MDMs were treated with either 20 µM vesatolimod, or 5 µM motolimod.

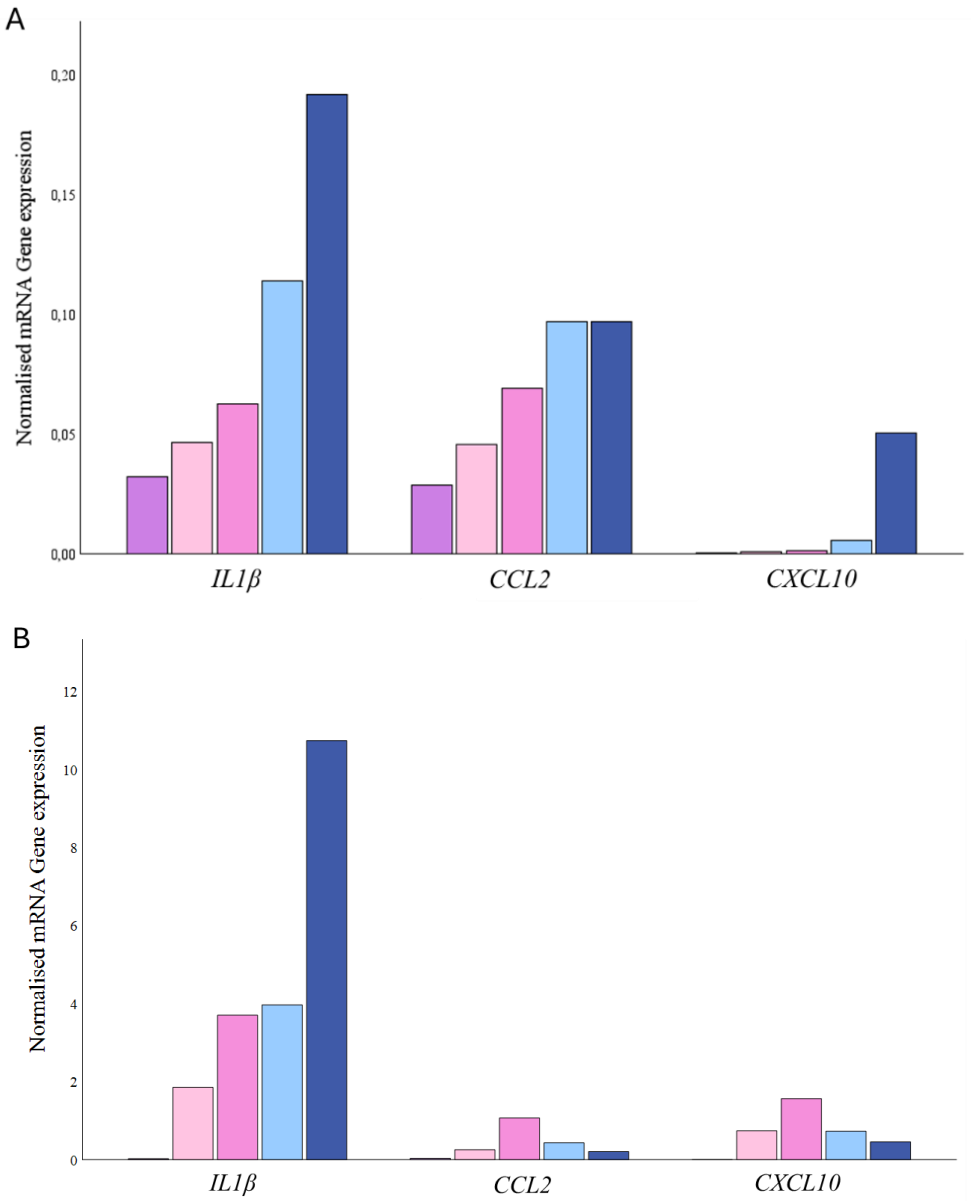

Figure 3.5 Optimal concentration for vesatolimod and motolimod concentration in THP-1 cells. THP-1 cells were treated with 0 µM (purple), 3 µM (light pink), 5 µM (dark pink), 10 µM (light blue), and 20 µM (dark blue) of either vesatolimod (A) or motolimod(B). *IL1β*, *CCL2*, and *CXCL10* mRNA expression was measured and normalised to *UBC* and *YWHAZ*. 20 µM vesatolimod showed the highest expression of *IL1β*, *CCL2*, and *CXCL10*, where 5 µM motolimod showed optimal expression of *IL1β*, *CCL2*, and *CXCL10*.

3.3.2 Cell viability remained above 90% and RNA extracts were intact and pure

The cell viability was determined via a trypan blue exclusion assay which showed that cells remained above 90% viability across all treatment conditions, indicating that the treatments had no adverse effects of the viability of the model (Fig. 3.6). The integrity of the extracted RNA was assessed by means of agarose gel electrophoresis. This was done for all treatment conditions for both monocytes and MDMs. The presence of two distinct bands on the gel represent the 28S and 18S subunits of ribosomal RNA, indicating that the RNA extracted was intact (Fig. 3.7). Additionally, using nanodrop spectrophotometry, the A260/280 and A260/230 ratios were determined to be within the range of 1.8-2.00 and 2.00-2.2, respectively (Table S1). This indicated that the extracted RNA was free from phenol and DNA contamination. Melt curve analysis showed melt curves with single peaks above the baseline threshold, thus, there was amplification of a single product (Fig. 3.8A). The qPCR products of *IL1β*, *CCL2*, *CXCL10*, and *IL10*, as well as the reference genes, *UBC* and *YWHAZ* were allowed to electrophorese on a 3% (w/v) agarose gel, along with a low-range DNA ladder (O'GeneRuler) to ensure the correct gene target was amplified (Fig. 3.9). The expected product sizes determined when designing the primers were the same as those observed for the qPCR products. Specifically, a 133-base pair (bp) product for *UBC*, a 94 bp product for *YWHAZ*, a 143 bp product for *IL1β*, a 137 bp product for *CCL2*, a 199 bp product for *CXCL10*, and a 130 bp product for *IL10* was detected.

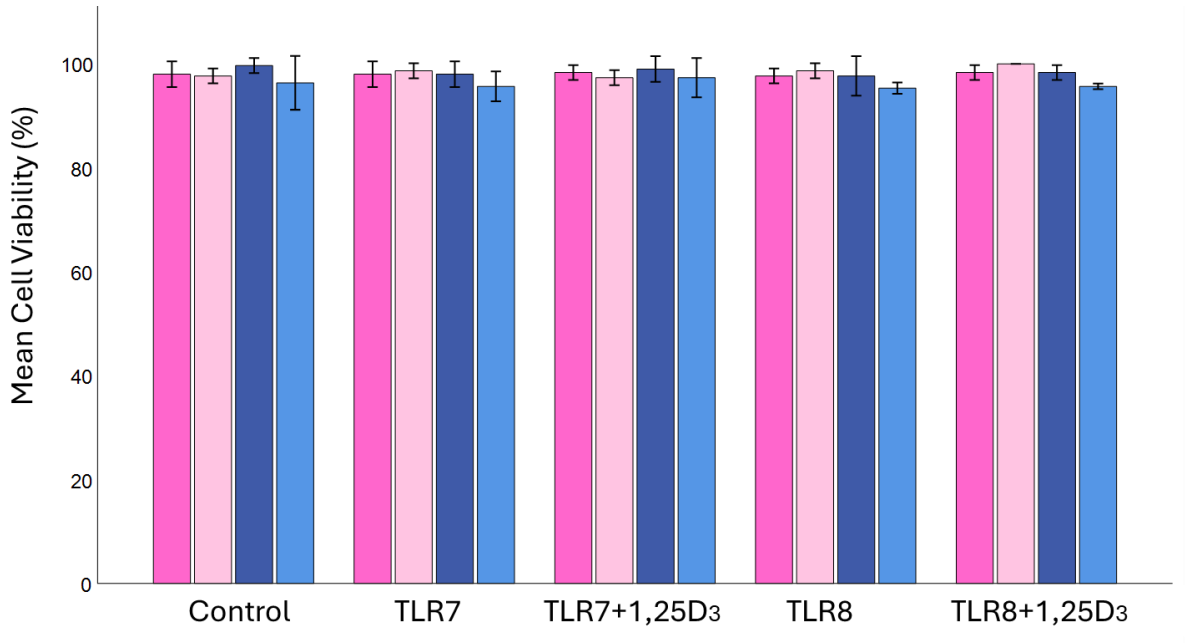

Figure 3.6 Treatments had no effect on cell viability. Cell viability was determined via a trypan blue exclusion assay. Despite the treatment condition, cell viability remained above 90% in monocytes (dark pink), monocytes+1,25D_3 (light pink), MDMs (dark blue), and MDMs+1,25D_3 (light blue).

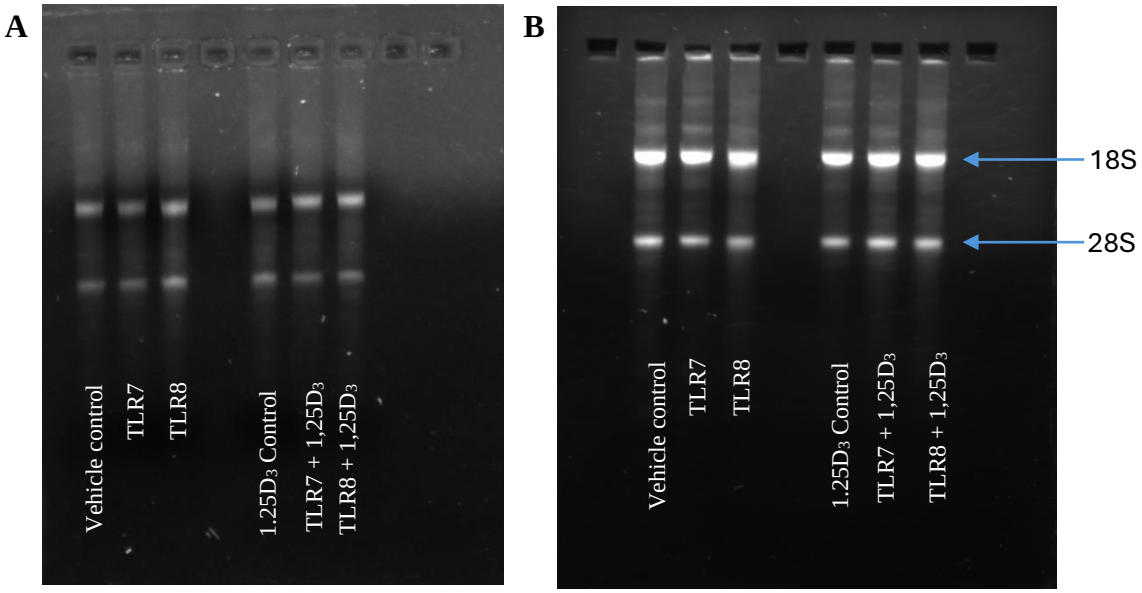

Figure 3.7 Intact RNA was extracted from monocytes and macrophages. A representative RNA 1% agarose gel shows RNA extracted from monocytes (A) and monocyte-derived macrophages (B). Extracted RNA was considered pure and intact, with distinct bands representing the 18S and 18S subunits of ribosomal RNA.

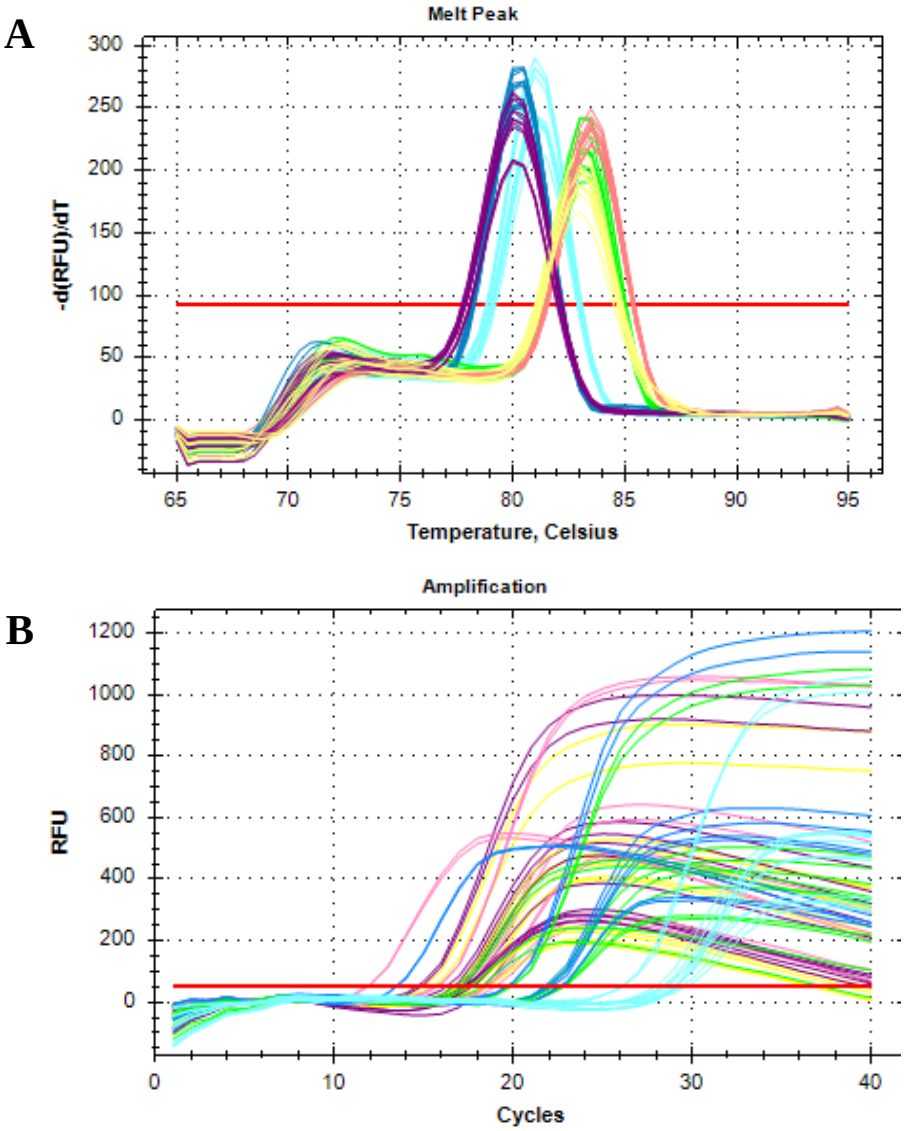

Figure 3.8 Representative melt curve and amplification curve for each gene of interest. Melt curve analysis (A) and amplification curves (B) shown for *UBC* (yellow), *YWHAZ* (purple), *IL1β* (pink), *CCL2* (green), and *CXCL10* (dark blue), and IL10 (light blue). Melt curve analysis showed single peaks for each gene representing the formation of a single product during the qPCR reaction.

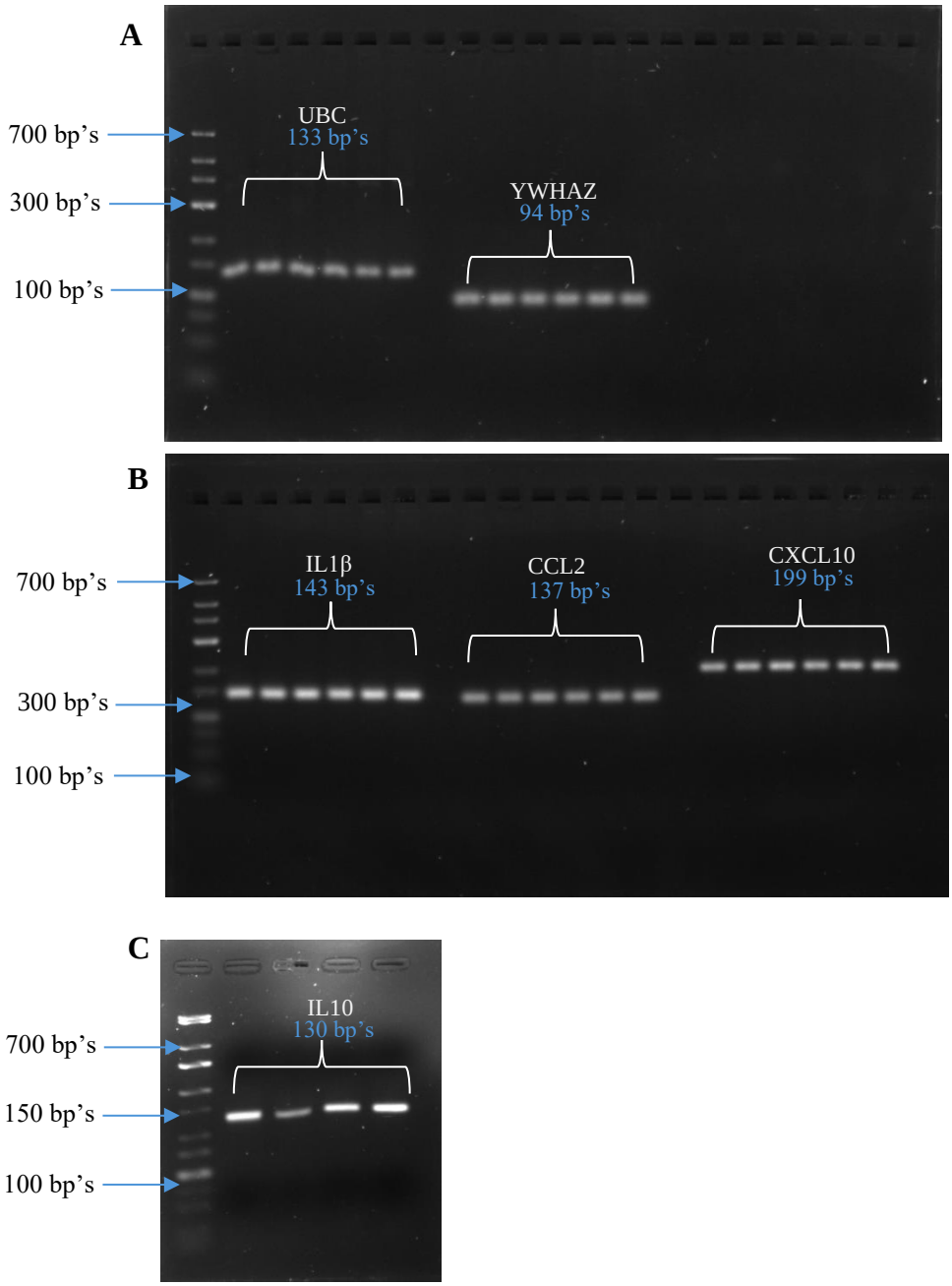

Figure 3.9 The qPCR products amplified were specific to the target genes. Following RT-qPCR amplification, the products were subject to gel electrophoresis on a 3% (w/v) agarose gel. All primers were specific to the target, and matched the designed primers product size, *UBC* (133 bp), *YWHAZ* (94 bp) (A), *IL1β* (143 bp), *CCL2* (137 bp), *CXCL10* (199 bp) (B), and *IL10* (130 bp) (C).

3.3.2 1,25D_3 modulates cytokine and chemokine production in response to TLR7 and TLR8 stimulation in monocytes and monocyte-derived macrophages.

To assess the impact of 1,25D_3 when the TLR7 and TLR8 signalling pathways were activated, THP-1s and MDMs cultured in the absence (Fig. 3.9) and presence of 1,25D_3 (Fig. 3.10) for 96 h were stimulated with either 20 µM vesatolimod, a TLR7-specific agonist, or 5 µM motolimod, a TLR8-specific agonist for 2h. Thereafter, the expression of *IL1β*, *CCL2*, *CXCL10*, and *IL10* were measured by RT-qPCR. A One-way between groups analysis of variance (ANOVA) was performed to determine the effects of TLR7 and TLR8 stimulation on gene expression in monocytes and MDMs. The monocytes and MDMs, cultured in the absence of 1,25D_3, both responded to TLR8 stimulation, with a significant increase in *IL1β*, *CCL2*, and *CXCL10* observed compared to the respective controls. However, *IL1β*, *CCL2*, and *CXCL10* expression remained unchanged in response to TLR7 stimulation in monocytes, while MDMs exhibited increased *IL1β*, *CCL2*, and *CXCL10* expression (Fig. 3.9). Interestingly, *IL10* expression was significantly reduced in TLR7 and TLR8-stimulated MDMs cultured in the absence of 1,25D_3 compared to their respective controls. The same trend was observed in monocytes and MDMs cultured in the presence of 1,25D_3, where MDMs responded to both TLR7 and TLR8 stimulation, while the monocytes responded to TLR8 stimulation, but not TLR7 stimulation (Fig. 3.10). Generally, *IL1β*, *CCL2*, and *CXCL10* expression was reduced in MDMs cultured in the presence of 1,25D_3 compared to MDMs cultured in the absence of 1,25D_3. However, aside from *IL1β*, the fold change in the expression of *CCL2* and *CXCL10* relative to their respective controls was greater in MDMs cultured in the presence of 1,25D_3 compared to MDMs cultured in the absence of 1,25D_3. Whereas the fold changes of *IL1β*, *CCL2*, and *CXCL10* expression compared to their respective controls were all greater in TLR8-stimulated monocytes cultured in the presence of 1,25D_3 compared to TLR8-stimulated monocytes cultured in the absence of 1,25D_3. While the presence of 1,25D_3 significantly reduced the expression of *IL1β* in MDMs, *IL1β* expression remained significantly higher than in its control.

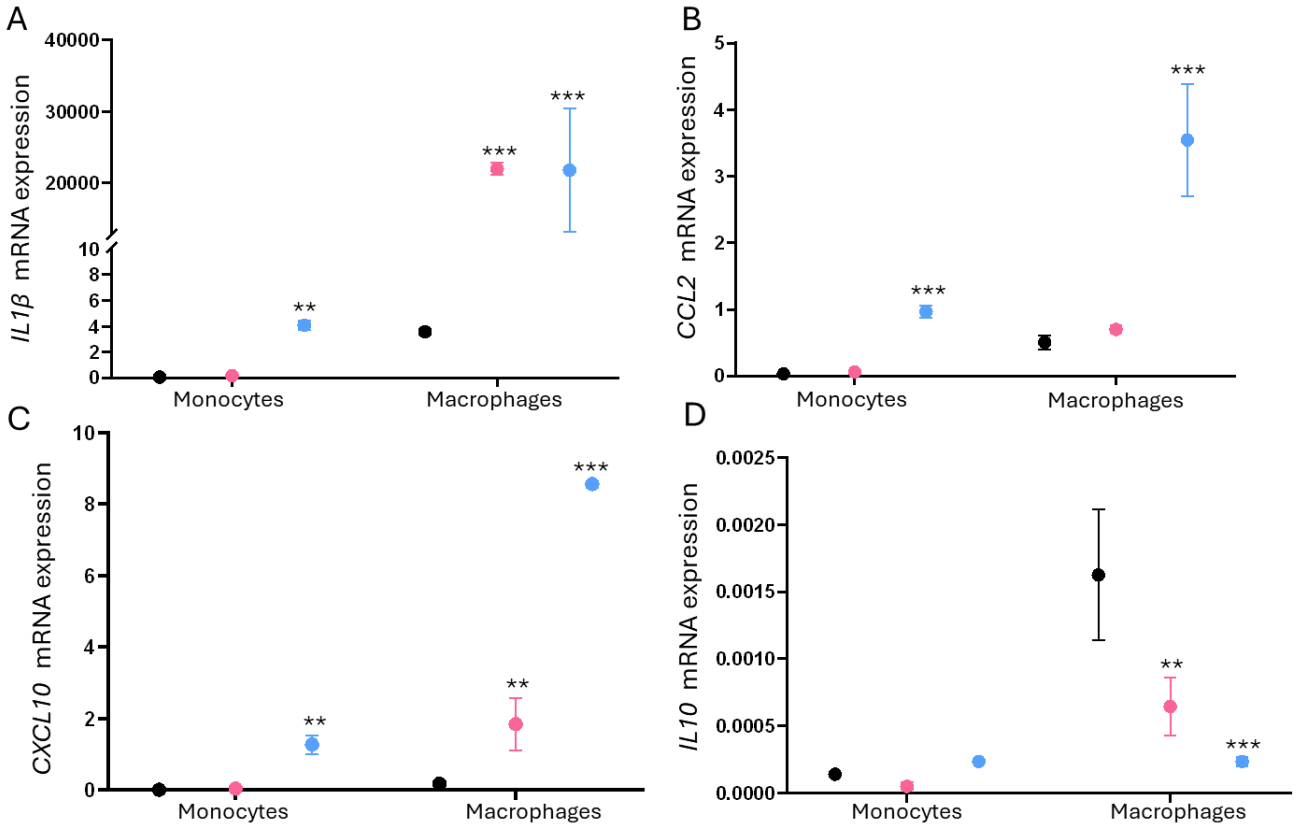

Figure 3.10 Monocyte-derived macrophages respond to TLR7 and TLR8 stimulation to a greater extent than monocytes. The error bar plots show changes in *IL1β* (A), *CCL2* (B), *CXCL10* (C), and *IL10* (D) mRNA expression normalised to *UBC* and *YWHAZ*, in response to TLR7 (pink) and TLR8 (blue) stimulation by 20 µM vesatolimod and 5 µM motolimod relative to the respective controls (black), respectively. Cytokine expression was significantly increased in MDMs only in response to TLR7 stimulation, but in both monocytes and MDMs in response to TLR8 stimulation (*p<0.050 ** p<0.01 *** p<0.001). Error bars show 95% CI (n = 3).

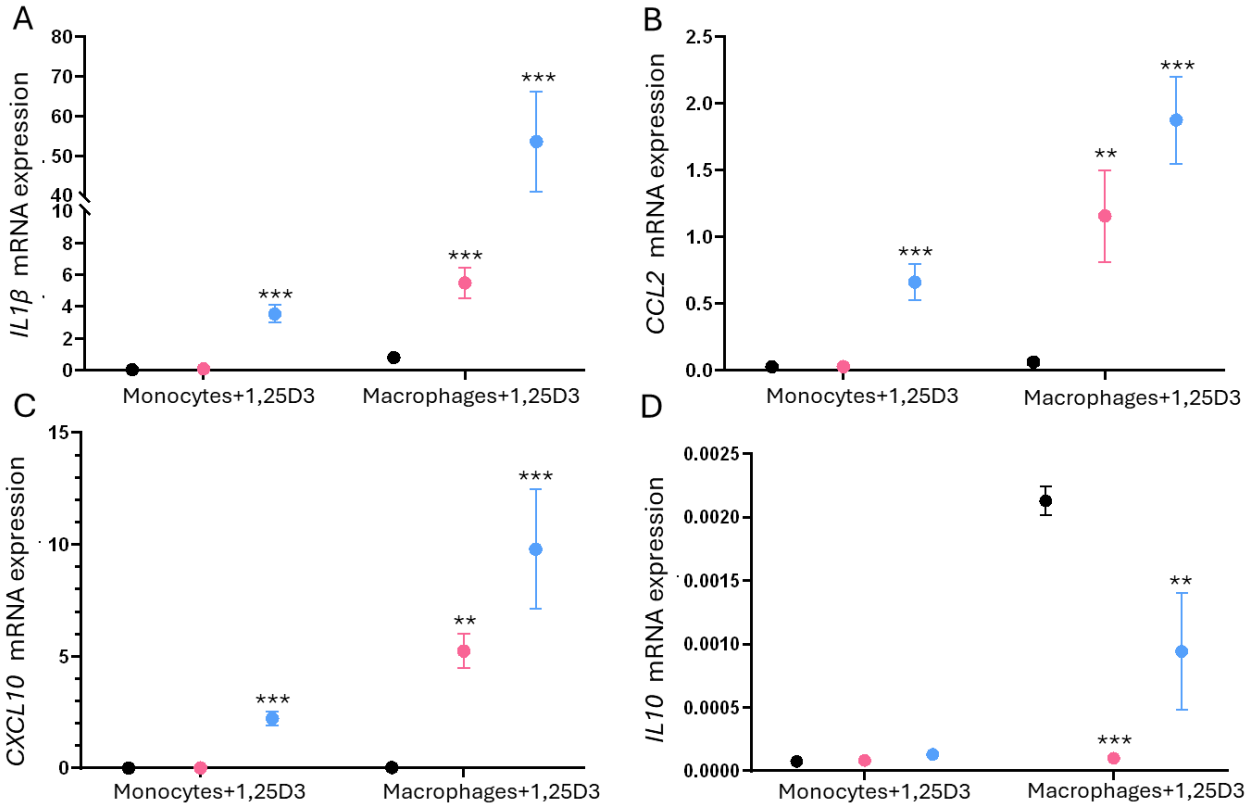

Figure 3.11 Cytokine expression increases to a lower extent in the presence of 1,25D$_3$ in monocytes and monocyte-derived macrophages. The error bar plots show changes in *IL1β* (A), *CCL2* (B), *CXCL10* (C), and *IL10* (D)mRNA expression normalised to *UBC* and *YWHAZ*, in response to TLR7 (pink) and TLR8 (blue) stimulation by 20 µM vesatolimod and 5 µM motolimod relative to the respective controls (black), respectively. Cytokine expression increased significantly in MDMs only in response to TLR7 stimulation, but in both monocytes and MDMs in response to TLR8 stimulation (*p<0.050 ** p<0.01 *** p<0.001). Error bars show 95% CI (n = 3).

3.3.3 Acute 1,25D$_3$ supplementation hinders TLR7 signalling in MDMs previously cultured in the presence of 1,25D$_3$.

The effect of acute 1,25D$_3$ supplementation on TLR7 or TLR8- stimulated monocytes and MDMs cultured in either the absence or presence of 1,25D$_3$ was assessed by culturing monocytes and MDMs in the presence and absence of 1,25D$_3$ for 96 h, followed by stimulation by either 20 µM vesatolimod or 5 µM motolimod in combination with an additional 10 nM 1,25D$_3$ for 2h. The gene expression of *IL1β*, *CCL2*, *CXCL10*, and *IL10* was measured by RT-qPCR and an ANOVA was performed to determine the effects of *in vitro* 1,25D$_3$ supplementation on gene expression in TLR7 and TLR8-stimulated monocytes and MDMs cultured in either the absence or presence of 1,25D$_3$. *IL1β, CCL2,* and *CXCL10* remained significantly induced in TLR8-stimulated monocytes cultured in the absence of 1,25D$_3$, while *IL1β, CCL2*, and *CXCL10* remained significantly induced in response to both TLR7 and TLR8 stimulation in MDMs cultured in the absence of 1,25D$_3$ and further supplemented with 1,25D$_3$. IL10 remained significantly reduced compared to the control in TLR7 and TLR8-stimulated MDMs cultured in the absence of 1,25D$_3$ and further supplemented with 1,25D$_3$ (Fig. 3.11). Interestingly, monocytes and MDMs cultured in the presence of 1,25D$_3$ and further supplemented with 1,25D$_3$ remained responsive to TLR8 stimulation, however, these MDMs were no longer responsive to TLR7 stimulation, with the expression *of IL1β, CCL2*, and *CXCL10* returning to baseline. Additionally, IL10 expression was no longer significantly reduced compared to the control in TLR7-stimulated MDMs cultured in the presence of 1,25D$_3$ and further supplemented with 1,25D$_3$ while *IL10* expression remained significantly reduced in TLR8-stimulated MDMs cultured in the presence of 1,25D$_3$ and further supplemented with 1,25D$_3$ (Fig. 3.12).

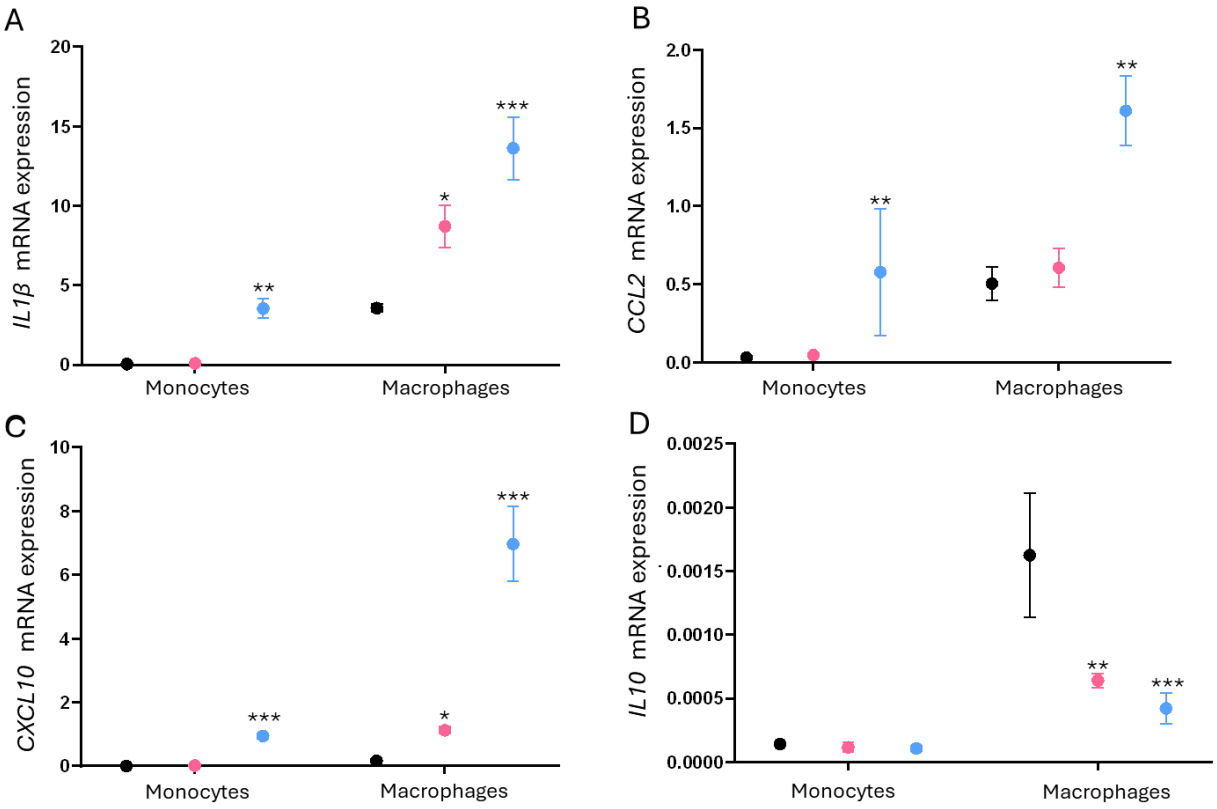

Figure 3.12 Monocytes and monocyte-derived macrophages cultured in the absence of 1,25D$_3$ and then further supplemented with 1,25D$_3$ continue to respond to TLR7 and TLR8 stimulation. The error bar plots show changes in *IL1β* (A), *CCL2* (B), *CXCL10* (C), and *IL10* (D)mRNA expression normalised to *UBC* and *YWHAZ*, in response to TLR7 (pink) and TLR8 (blue) stimulation by 20 µM vesatolimod and 5 µM motolimod in combination with additional 10 nM 1,25D$_3$ for 2h relative to the respective controls (black). Cytokine expression was significantly increased in MDMs in response to TLR7 stimulation, whereas cytokine expression was significantly increased in both monocytes and MDMs in response to TLR8 stimulation (*p<0.050 ** p<0.01 *** p<0.001). Error bars show 95% CI (n = 3).

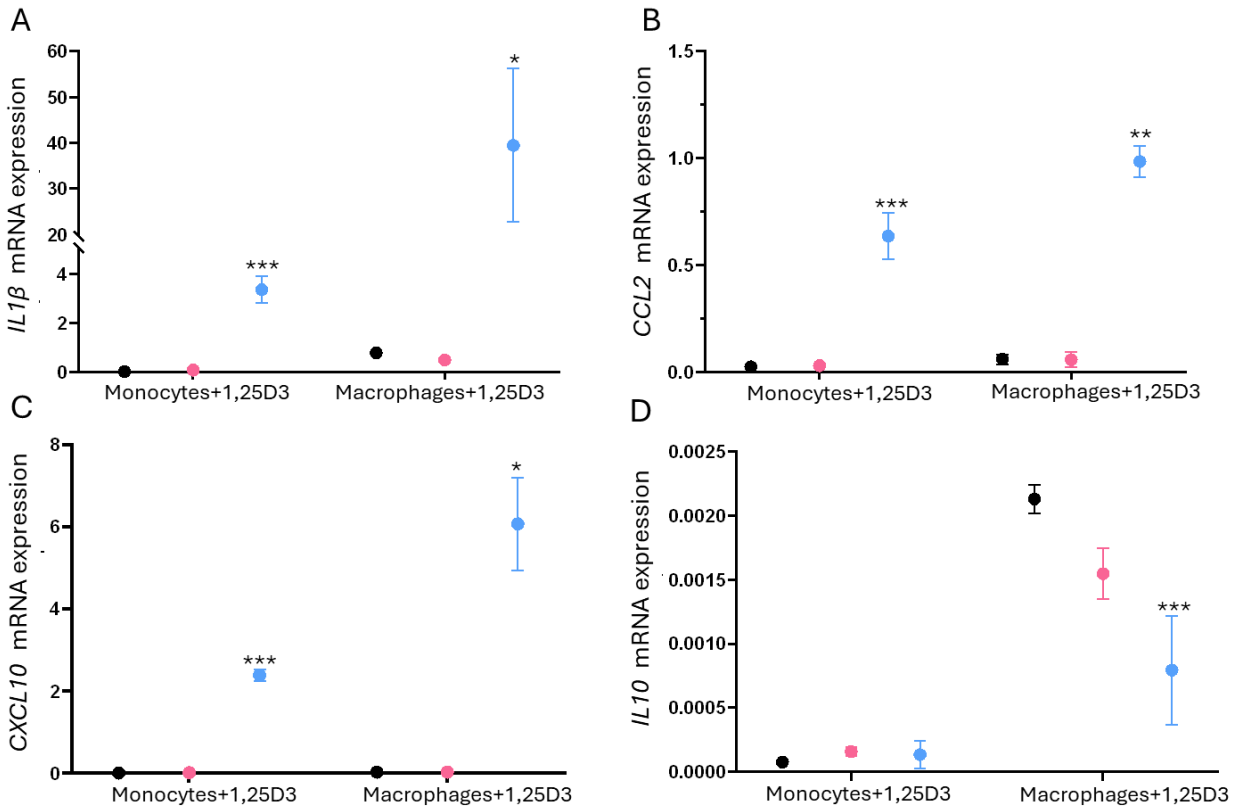

Figure 3.13 Monocyte-derived macrophages cultured in the presence of 1,25D$_3$ and then further supplemented with 1,25D$_3$ no longer respond to TLR7 stimulation. The graphs show changes in *IL1β* (A), *CCL2* (B), *CXCL10* (C), and *IL10* (D)mRNA expression normalised to *UBC* and *YWHAZ*, in response to TLR7 (pink) and TLR8 (blue) stimulation by 20 µM vesatolimod and 5 µM motolimod in combination with additional 10 nM 1,25D$_3$ for 2h relative to the respective controls (black). Cytokine expression was significantly increased in monocytes and MDMs in response to only TLR8 stimulation (*p<0.050 ** p<0.01 *** p<0.001). Error bars show 95% CI (n = 3).

CHAPTER 4: Discussion

4.1 1,25D$_3$ decreased *TLR7* expression and increased *TLR8* expression in monocytes and monocyte-derived macrophages

In this study, *TLR7* and *TLR8* expression was higher in macrophages compared to monocytes. This suggests that similar to the observed increase in the expression of various cell surface markers during monocyte-to-macrophage differentiation, the increased levels of TLR7 and TLR8 may support macrophage function, thereby enabling a wider range of immune functions (Dash *et al.*, 2024). In agreement with the findings of this study, Eng *et al.* (2018) showed that *TLR7* and *TLR8* mRNA levels were increased in PMA-differentiated THP-1s compared to undifferentiated THP-1 cells. A similar trend has been reported from primary monocytes and MDMs (Eng *et al.*, 2018).

Notably, *TLR7* expression was decreased in response to 1,25D$_3$, while *TLR8* expression was increased in response to 1,25D$_3$ in both monocytes and macrophages. However, multiple studies that have identified primary 1,25D$_3$ target genes have not observed any VDR binding sites in the promoter or enhancer regions of either *TLR7* or *TLR8* (Nurminen *et al.*, 2019; Carlberg, 2022; Seuter *et al.*, 2016). Additionally, this was confirmed using JASAPAR, in which no putative VDR binding sites were detected in either the *TLR7* or *TLR8* regulatory regions. As such it is likely that 1,25D$_3$ alters *TLR7* and *TLR8* expression via secondary mechanisms i.e. through physical interactions mediated through unknown proteins that are regulated by 1,25D$_3$ (Voltan *et al.*, 2023). Possible alternative secondary mechanisms by which 1,25D$_3$ may alter *TLR7* and *TLR8* expression include via chromatin modifiers or chromatin repressors, or via co-activators or co-repressors. A study by Voltan *et al.* (2023) identified key 1,25D$_3$ target genes involved in immune processes in THP-1 cells and PBMCs treated with 1,25D$_3$. Some of these 1,25D$_3$ target genes included triggering receptor expressed on myeloid cells 1 (TREM1), which is involved in TLR4 signalling in response to bacterial infection and was increased in response to 1,25D$_3$. Additionally, the TLR signal transduction modulatory protein (THEMIS2), predominantly expressed on macrophages and B cells, is directly increased by 1,25D$_3$. THEMIS2 is responsible for the production of TNFα induced by LPS in response to TLR4 signalling.

Additionally, because viruses can activate both TLR7 and TLR8 signalling, it has been reported that TLR7 and TLR8 regulate one another as a means of maintaining immune balance, thus, it is not completely surprising that 1,25D$_3$ has an opposite effect on their expression (Eng *et al.*,

2018). In addition to their varied expression in immune cells, TLR7 and TLR8 signalling also have distinct cytokine induction profiles, where TLR7 signalling is associated with the type I IFN response, and TLR8 is associated with the induction of pro-inflammatory cytokines (Eng *et al.*, 2018). As such $1,25D_3$ might alter *TLR7* and *TLR8* expression to reduce the type I IFN response, whose dysregulation is associated with the development of autoimmune disorders such as SLE and RA (Choubey and Moudgil, 2011). While both TLR7 and TLR8 are associated with the development of autoimmune diseases, it is more so TLR7 that is associated with the progression of these disorders including systemic lupus, Bechet's disease, and Sjogren's syndrome (Yan *et al.*, 2024). Eng *et al.* (2018) showed that *TLR8* deficiency exacerbated SLE symptoms due to *TLR7* overexpression, while *TLR7* deficiency reduced SLE symptoms. As such, it is possible that $1,25D_3$ plays a protective role by increasing *TLR8* expression to prevent the risk of disrupting immune homeostasis. Further supporting the findings in this study, Alvarez-Rodriguez *et al.* (2012) showed that increased $25(OH)D_3$ serum levels were associated with reduced *TLR7* expression in monocytes, T cells, and B cells, while Massari *et al.* (2013) showed an increased *TLR8* expression in various immune cells with $1,25D_3$ treatment. Additionally, a transcriptomic analysis by Valdes-Lopez *et al.* (2022) of monocytes and macrophages treated with and without $1,25D_3$, showed that macrophages displayed a greater expression of both *TLR7* and *TLR8* compared to monocytes, with reduced *TLR7* expression in the macrophages treated with $1,25D_3$. Taken together, this suggests that $1,25D_3$ alters the expression of *TLR7* and *TLR8* via secondary mechanisms, implying that $1,25D_3$ might play an important role in the maintenance of immune homeostasis.

4.2 $1,25D_3$ decreased the expression of pro-inflammatory mediators in monocyte-derived macrophages

In this study, the baseline expression of *IL1β*, *CCL2*, and *CXCL10* were higher in the macrophages compared to the monocytes. This may be due to the increased expression of cell surface receptors that occurs during monocyte-to-macrophage differentiation (Tuttle *et al.*, 1998). These cell surface receptors might include IL-1R, the receptor for IL1β, CCR2, the receptor for CCL2, and CXCR3, the receptor for CXCL10 (Murray *et al.*, 2015; Gschwandtner *et al.*, 2019; Mousaad Elemam *et al.*, 2022). The increased expression of these proinflammatory mediators in macrophages might also be because macrophages are more specialised immune cells, with more features including enhanced cytokine and chemokine production (Bassler *et al.*, 2019).

While $1,25D_3$ had no effect on the expression of *IL1β*, *CCL2*, and *CXCL10* in monocytes, $1,25D_3$ decreased the expression of these proinflammatory mediators in macrophages. Similarly, Wicinski *et al.* (2021) showed a link between increased $1,25D_3$ and reduced inflammatory markers in obese patients. While the way in which $1,25D_3$ alters the expression of these proinflammatory markers remains unknown, there have been previous studies that show that $1,25D_3$ treatment increases the expression of suppression of cytokine signalling 1 (SOCS1) (Chen *et al.*, 2013; Liu, 2006). Overall, as $1,25D_3$ is thought of as an immunomodulator, it is not unsurprising that $1,25D_3$ would reduce the expression of proinflammatory mediators, with multiple studies supporting this finding (Martens *et al.*, 2020; Tang *et al.*, 2009; Olsen *et al.*, 2022).

Alternatively, the $1,25D_3$ induced decrease in *IL1B*, *CCL2*, and *CXCL10* seen in macrophages could be due to the differentiation protocol applied. In this study, monocytes were differentiated into macrophages using either 5 nM PMA alone, or 5 nM PMA in combination with 10 nM $1,25D_3$. The use of PMA alone, can result in a macrophage skewed towards the M1 pro-inflammatory phenotype (Nascimento *et al*., 2022), however, the incorporation of $1,25D_3$ may play a role in reducing the inflammatory effects of PMA. Decreased expression of *IL1β*, *CCL2*, and *CXCL10* by $1,25D_3$ may be beneficial in reducing the risk of hyperinflammation. Further supporting the findings in this study, Mol *et al.* (2024) showed that PMA-differentiated THP-1s had increased expression of proinflammatory mediators that were ameliorated in the presence of $1,25D_3$, once again demonstrating the immunomodulatory properties of $1,25D_3$ (Mol *et al.*, 2024).

4.3 Monocytes responded to TLR8 stimulation, where macrophages responded to both TLR7 and TLR8 stimulation

In this study, monocytes responded to TLR8 stimulation with 5 µM motolimod, but did not respond to TLR7 stimulation with 20 µM vesatolimod. On the other hand, macrophages responded to both TLR7 and TLR8 stimulation with 20 µM vesatolimod and 5 µM motolimod, respectively. This may be due to the higher *TLR7* expression observed in macrophages compared to monocytes. Furthermore, research has shown that TLR7 and TLR8 have functional differences in terms of their cellular expression and their cytokine induction profile (Gantier *et al.*, 2008). While *TLR7* is expressed to a greater extent in B cells and plasmacytoid dendritic cells, *TLR8* expression is associated more with monocytes, macrophages, and myeloid dendritic cells (Eng *et al.*, 2018; Tanji *et al.*, 2015). Additionally, it should be noted that the agonistic abilities of vesatolimod are best described for TLR7 activation in B cells and

dendritic cells, with its effect being concentration dependent (Tanji *et al.*, 2015). However, even at the highest tested concentration of 20 µM, TLR7 was not activated in monocytes in this study. Given that TLR7 stimulation is a more potent inducer of IFN-mediated responses, whereas TLR8 stimulation is more so associated with the induction of pro-inflammatory mediators (Eng *et al.*, 2018), it is possible that TLR7 activation was not detected due to the choice of genes assessed in response to activation in this study. Nonetheless, Eng *et al.* (2018) reported similar findings from their study - showing that while THP-1s responded to the TLR8-specific agonist ssRNA40, they were unresponsive to the TLR7-specific agonist loxoribine. Furthermore, Eng *et al.* (2018) showed that TLR7 only became active in THP-1s when *TLR8* was silenced, once again highlighting the regulatory role of *TLR8* on *TLR7*. Additionally, Wang *et al.* (2006) showed that while monocytes expressed both *TLR7* and *TLR8*, they were only responsive to TLR8 stimulation by 3M-002, a TLR8-specific agonist. As such, multiple studies have supported the findings in this study demonstrating that monocytes respond to TLR8 stimulation but not TLR7 stimulation, which corresponds to cell-specific *TLR7* and *TLR8* expression.

In contrast, a study by Wang *et al.* (2006) showed that PMA-differentiated THP-1s did not respond to TLR7 stimulation either. However, this is likely a concentration dependent effect, given that in the current study two times more agonist was used to activate TLR7 signalling (10 µM (Wang *et al.*, 20006) vs 20 µM (current study)). Notably, the concentration of the TLR7 agonist used in our study was four times higher than that of the TLR8 agonist (20 µM vs 5 µM, respectively), suggesting that TLR8 is more responsive to activation by synthetic agonists than TLR7. Nonetheless, at the concentrations used in this study, TLR7 and TLR8 stimulation resulted in a similar level of pro-inflammatory cytokine and chemokine induction in macrophages. Further illustrating this point, *IL1β*, a potent proinflammatory mediator, was induced to a similar extent with a 4000-fold change vs 6000-fold change relative to the control in the TLR7-stimulated and TLR8-stimulated macrophages, respectively. *IL1β* is likely induced to a great extent as it plays a crucial role in the activation of other immune cells and contributes to the further induction of various cytokines and chemokines downstream (Dinarello, 2017).

Monocytes and macrophages are two of the major sources of IL1β upon TLR activation (Weber *et al.*, 2010). Previous research by Schindler *et al.* (1990) showed that *IL1β* expression was increased as early as 15 minutes post LPS stimulation. *IL1β* reaches its peak level at 4 hours, which also explains the high level of induction observed in the TLR7 and TLR8-stimulated

macrophages in this study. Furthermore, *IL1β* could be expressed to the extent that it is, as motolimod, the TLR8-specific agonist used in this study, directly activates the NLRP3 inflammasome (Tanji *et al.*, 2013). NLRP3 is a multimeric protein complex known as an inflammasome which causes the induction of protease-caspase1, which in turn converts pro-IL1β (inactive) into the active IL1β (Dash *et al.*, 2024). Additionally, *CCL2* and *CXCL10* were also induced in response to TLR7 and TLR8 activation, as both these chemokines play a critical role in not only the release of chemoattractants but also contributes to immune cell activation (Mousaad Elemam *et al.*, 2002).

Interestingly, *IL1β*, *CCL2*, and *CXCL10* were induced to such an extent that the anti-inflammatory mediator *IL10*, was significantly reduced in TLR7- and TLR8-stimulated macrophages. This is likely due to changes in the microenvironment due to the release of pro-inflammatory mediators. Macrophages exist on a spectrum, where they can be further classified as an M0 macrophage, otherwise known as a naïve macrophage, an M1 macrophage associated with a pro-inflammatory profile, or an M2 macrophage associated with an anti-inflammatory profile. M0 macrophages can be skewed towards the M1 phenotype or the M2 phenotype depending on the microenvironment. In this study, naïve macrophages with the propensity to become either an M1 macrophage or an M2 macrophage (Mol *et al.*, 2024), were used. However, due to the inflammatory environment created, it is likely that TLR7 and TLR8 activation skewed the M0 macrophage toward an M1 pro-inflammatory state. This is supported by the reduced levels of *IL10* observed in these cells (Dash *et al.*, 2024).

4.4 1,25D$_3$ modulated cytokine and chemokine production in macrophages

While 1,25D$_3$ decreased the expression of *IL1β*, the expression of *CCL2* and *CXCL10* appeared to be enhanced in response to 1,25D$_3$ in macrophages. This suggests that 1,25D$_3$ modulates IL1β production to reduce the risk of creating a hyperinflammatory environment as *IL1β* was induced to a much greater extent than *CCL2* and *CXCL10*. Furthermore, as *IL1β* is known to be induced early post-infection, it is possible that 1,25D$_3$ may enhance the initial production of IL1β, however, to prevent the risk of creating a hyperinflammatory environment, 1,25D$_3$ begins to modulate *IL1β* expression to maintain immune homeostasis. Additionally, it is important to note that while 1,25D$_3$ reduced *IL1β* expression, *IL1β* was still significantly induced compared to the control post TLR7 and TLR8 activation i.e. there was still an inflammatory response, just to a lower extent, preventing a possible disruption of immune homeostasis.

While the way in which 1,25D_3 exerts this effect cannot be confidently determined, the 1,25D_3-mediated reduction in *IL1β* could be through increased expression of IL1β antagonists such as the IL-1 receptor antagonist (IL-1RA), which blocks IL1β from binding to the IL1 receptor or alternatively, 1,25D_3 could also increase the expression of IL-1 receptor type II (IL-1RII) expressed on macrophages (Lopez-Castejon and Brough, 2011; Dinarello, 2017). The binding of IL1β to IL-1RII results in no signal due to the absence of a cytoplasmic domain, thereby blocking the IL1β mediated response. Modulation of IL1β is important as the excessive production of *IL1β* has been associated with a variety of autoimmune diseases and inflammatory disorders such as RA, type I diabetes, and periodic fever syndromes (Lopez-Castejon and Brough, 2011). Additionally, the excessive production of IL1β is linked to severe Covid-19 and pathogenesis of disease and has become the target of potential therapeutic intervention (Mardi *et al.*, 2021).

The same trend was not observed in *CCL2* and *CXCL10* expression, where 1,25D_3 enhanced the expression of these chemokines. This is likely because there is no need for 1,25D_3 to modulate their production, as these chemokines were not induced to as great an extent as *IL1β*. This is further supported by the enhanced *IL1β*, *CCL2*, and *CXCL10* expression observed in TLR8-stimulated monocytes in response to 1,25D_3, where 1,25D_3 appears to push monocytes to their 'macrophage potential'. Overall, 1,25D_3 plays its role as an immunomodulator, with its ability to both enhance the production of pro-inflammatory mediators, such as CCL2 and CXCL10, while also modulating the production of pro-inflammatory mediators, such as IL1β, to prevent the risk of creating a hyper-inflammatory environment, highlighting the importance of 1,25D_3 in the maintenance of immune homeostasis.

4.5 Additional 1,25D_3 supplementation does not always result in positive outcome

The idea of acute 1,25D_3 supplementation was investigated in this study by activating TLR7 and TLR8 signalling in the presence of an additional 10 nM 1,25D_3 for 2 h in monocytes and MDMs cultured in either the presence or absence of 1,25D_3 for 96 h, representing a 1,25D_3 'sufficient' and 1,25D_3 'insufficient' environment, respectively. Monocytes and macrophages cultured in the 1,25D_3 'insufficient' environment and further supplemented with 1,25D_3 continued to respond in the same manner, where monocytes responded to only TLR8 stimulation, while macrophages responded to both TLR7 and TLR8 stimulation. However, while the monocytes and macrophages cultured in the 1,25D_3 'sufficient' environment and further supplemented with additional 1,25D_3 continued to respond to TLR8 stimulation, these macrophages were no longer responsive to TLR7 stimulation. Furthermore, in these TLR7-

stimulated macrophages cultured in the $1,25D_3$ 'sufficient' environment and further supplemented with additional $1,25D_3$, *IL10* was no longer significantly reduced indicating the absence of an inflammatory response i.e. these macrophages were no longer skewed toward an M1 phenotype. These results are likely due to the fact that $1,25D_3$ is associated with an increase in *TLR8* expression and decrease in *TLR7* expression in both monocytes and macrophages. This explains why the monocytes and macrophages continued to respond to TLR8 stimulation upon additional $1,25D_3$ supplementation. Interestingly, despite the expected increase in *TLR8* expression upon additional $1,25D_3$ supplementation, there was not an excessive production of the proinflammatory mediators upon stimulation, once again highlighting the protective effects of $1,25D_3$ and its ability to balance inflammation.

$1,25D_3$ supplementation can be beneficial if properly regulated. Smith *et al.* (2018) highlighted the benefits of using $1,25D_3$ in mechanically ventilated, critically ill patients, in which $1,25D_3$ supplementation was found to increase haemoglobin concentrations, thereby improving oxygen transport. Additionally, CRP levels, often associated with severe Covid-19, were decreased in $1,25D_3$ sufficient patients, highlighting the benefits of $1,25D_3$ during infection. While there are multiple studies on the benefits of $1,25D_3$ supplementation, supplementation is most beneficial in patients who are $1,25D_3$ deficient (Jeyakumar *et al.*, 2024). On the other hand, excessive $1,25D_3$ supplementation results in hypercalcemia due to increased calcium uptake (Taylor and Davies, 2018). Excessive $1,25D_3$ has negative effects on multiple systems including the central nervous system, the gastrointestinal system, the cardiovascular system, and the renal system (Razzaque, 2018). $1,25D_3$ build-up is mainly because of irresponsible use or incorrect dosage of $1,25D_3$ supplements, as the $1,25D_3$ obtained via UV-B radiation is a tightly regulated process, and $1,25D_3$ obtained from diet only accounts for about 10-20% of biologically active $1,25D_3$ (Ozkan *et al.*, 2012). All in all, this highlights the importance of maintaining sufficient $1,25D_3$ levels for $1,25D_3$ to exert its effects optimally to maintain immune homeostasis.

CHAPTER 5: Concluding remarks

5.1 Summary of findings

In this study, a decrease in the expression of *TLR7* and an increase in the expression of *TLR8*, relative to baseline, was observed in response to $1,25D_3$ in both monocytes and macrophages. Additionally, the expression of the proinflammatory mediators *IL1β*, *CCL2*, and *CXCL10* was decreased in response to $1,25D_3$ in macrophages. The downstream implications of these changes were investigated by activating the TLR7 and TLR8 signalling pathway in monocytes and macrophages in the presence or absence of $1,25D_3$. Results showed that monocytes responded to only TLR8 stimulation, while macrophages responded to both TLR7 and TLR8 stimulation. Additionally, $1,25D_3$ appeared to enhance the expression of *IL1β*, *CCL2*, and *CXCL10* in the TLR8-stimulated monocytes. Furthermore, while $1,25D_3$ enhanced the expression of *CCL2* and *CXCL10* in the TLR7- and TLR8-stimulated macrophages, *IL1β* expression, while still significantly induced, was reduced in response to $1,25D_3$ highlighting the immunomodulatory effects of $1,25D_3$ on cytokine homeostasis. Finally, the effect of additional $1,25D_3$ supplementation in a $1,25D_3$ 'sufficient' environment vs a $1,25D_3$ 'insufficient' environment was investigated. The monocytes and macrophages cultured in the $1,25D_3$ 'insufficient' environment remained responsive to TLR7 and TLR8 stimulation upon additional $1,25D_3$ supplementation. However, while the monocytes and macrophages cultured in the $1,25D_3$ 'sufficient' environment continued to respond to TLR8 stimulation, the macrophages were no longer responsive to TLR7 stimulation upon additional $1,25D_3$ supplementation, once again highlighting the effect of $1,25D_3$ on TLR7 and TLR8 expression.

5.2 Implications of research

The dysregulation of TLR pathways including the TLR7 and TLR8 signalling pathways is associated with the excessive production of proinflammatory mediators. The excessive production of these mediators' results in chronic inflammation which is associated with tissue damage and multi-organ failure. These cytokine storms have played a role in predicting severe cases of infections, as observed in SARS-CoV2 and influenza patients. Furthermore, the dysregulation of *TLR7* and *TLR8* expression has been implicated in the development of several autoimmune disorders including SLE, RA, Sjogren's syndrome, and psoriasis, all of which are exacerbated in the presence of a $1,25D_3$ deficiency. As such, investigating the impact of $1,25D_3$ on the TLR7 and TLR8 signalling pathways provides insights into a possible, cost-effective therapeutic which may not only assist in reducing the symptoms of these autoimmune disorders, but also assist patients with chronic inflammation as a result of viral infection.

Furthermore, the effect of additional $1,25D_3$ supplementation showed that $1,25D_3$ supplementation is not always beneficial. Instead, this study aimed to highlight the importance of maintaining sufficient $1,25D_3$ levels for $1,25D_3$ to exert a positive effect on the maintenance of immune homeostasis.

5.3 Study limitations and future direction

While $1,25D_3$ had an impact on TLR7 and TLR8 signalling, the way in which $1,25D_3$ exerts this effect could not be determined. As such, to obtain a full understanding, the TLR7 and TLR8 stimulated monocytes and macrophages could be sequenced, to get a transcriptome-wide view during activation and the potential mechanism by which $1,25D_3$ impacts signalling. Furthermore, ATAC-seq could also be used to determine whether $1,25D_3$ exerts its effect via the alteration of chromatin accessibility. Additionally, these studies could incorporate *TLR7* or *TLR8* silencing to further assess the difference in TLR7 and TLR8 signalling in monocytes and macrophages.

In addition, the RT-qPCR genes investigated downstream of TLR7 and TLR8 signalling were limited to *IL1β*, *CCL2*, *CXCL10*, and *IL10*, however, future studies could incorporate a wider range of proinflammatory mediators including type I IFNs such as IFNα and IFNβ. This would be beneficial in confirming the distinct cytokine profiles associated with TLR7 and TLR8 activation. Furthermore, as TLR7 is more so associated with B cells and plasmacytoid dendritic cells, its effect and the effect of $1,25D_3$ thereon could be investigated in these cell types.

Lastly given that the research was conducted using immortalized cell lines, and that it is possible that the process of immortalisation can affect innate immunity, it remains to be seen how these findings translate to freshly isolated monocytes and macrophages.

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Supplementary Figures

Figure S1 Cell cultures were free from mycoplasma contamination. To ensure cell cultures were free from mycoplasma contamination, the MycoStrip® mycoplasma detection kit (Invivogen) was used according to the manufacturer's instructions. P = positive control, T = THP-1 sample, N = negative control.

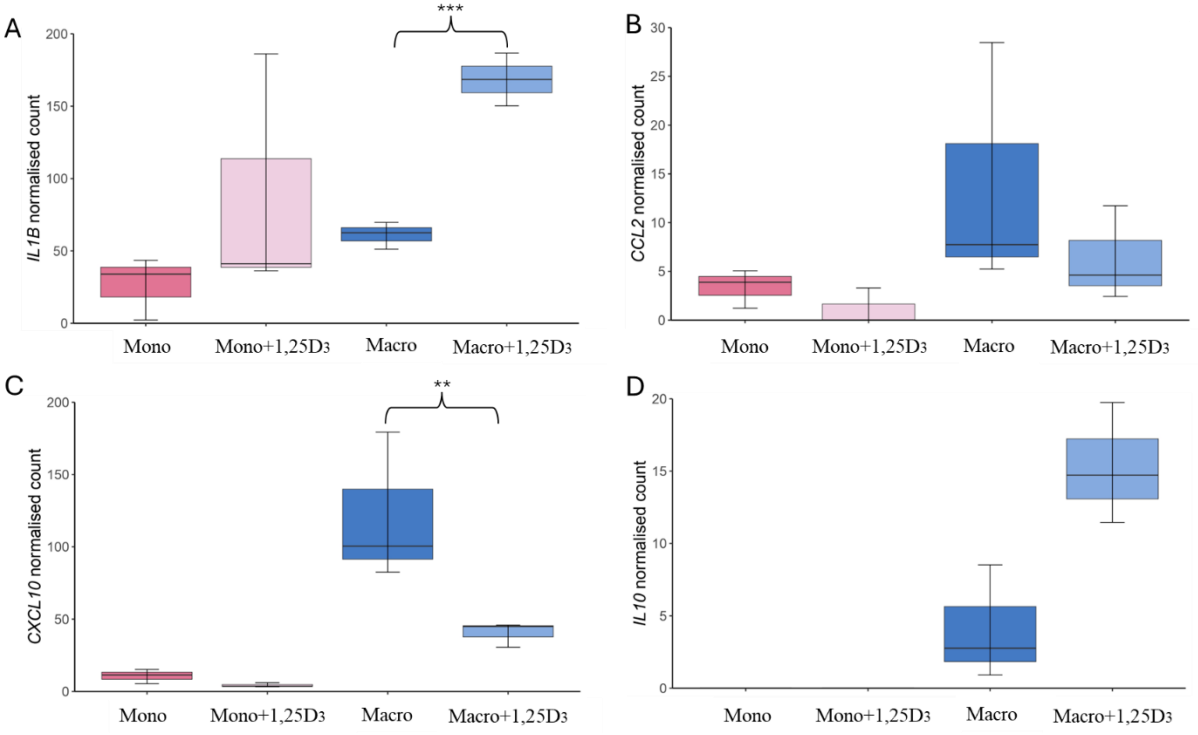

Figure S2 The expression of *IL1β, CCL2, CXCL10, and IL10* in THP-1 monocytes and monocyte-derived macrophages. The box plots show DESeq2 derived normalized counts for *IL1β* (A), *CCL2* (B), *CXCL10* (C), and *IL10* (D) for each condition compared to their relevant control. For the most part, the RNA-seq data corresponds to the RT-qPCR data. Statistically significant differences between each condition and their respective control are indicated based on the Benjamini-Hochberg adjusted p values derived from DESeq2 analysis (**p<0.010, ***p<0.001).

Table S1 RNA concentration, purity, and nucleic acid purity

Replicate	Sample	Concentration (ng/µL)	A260/280	A260/230
Monocyte Rep 1	Control	138.3	2.23	2.12
	TLR7 treated	101.1	2.00	2.0
	TLR8 treated	124.8	2.10	2.03
	TLR7 + 1,25D_3	98.2	2.06	2.0
	TLR8 + 1,25D_3	111.3	2.02	2.01
Monocyte Rep 2	Control	88.5	2.03	2.12
	TLR7 treated	89.7	2.01	1.99
	TLR8 treated	97.53	1.99	2.02
	TLR7 + 1,25D_3	118.1	2.17	2.17
	TLR8 + 1,25D_3	92.3	2.05	2.03
Monocyte Rep 3	Control	86.3	1.99	2.12
	TLR7 treated	98.5	2.01	2.03
	TLR8 treated	97.3	2.03	2.1
	TLR7 + 1,25D_3	89.9	1.93	2.05
	TLR8 + 1,25D_3	101.3	2.04	2.17
Monocyte + 1,25D_3 Rep 1	Control	127.6	2.04	2.12
	TLR7 treated	98.4	2.03	2.13
	TLR8 treated	101.3	2.1	2.1
	TLR7 + 1,25D_3	150.9	2.12	2.19
	TLR8 + 1,25D_3	107.6	2.02	2.1
Monocyte + 1,25D_3 Rep 2	Control	127.6	2.03	2.05
	TLR7 treated	123.7	2.01	2.03
	TLR8 treated	95.5	1.9	2.00
	TLR7 + 1,25D_3	261.5	2.01	2.01
	TLR8 + 1,25D_3	92.6	2.02	2.08
Monocyte + 1,25D_3 Rep 3	Control	174.6	2.00	2.01
	TLR7 treated	181.9	2.02	2.01
	TLR8 treated	159.5	2.02	1.99
	TLR7 + 1,25D_3	189.6	1.99	2.0
	TLR8 + 1,25D_3	309.7	2.05	2.04

Macrophage	Control	146.8	2.01	2.17
Rep 1	TLR7 treated	166.7	1.99	2.18
	TLR8 treated	127.5	2.00	2.2
	TLR7 + 1,25D_3	160.8	2.02	2.17
	TLR8 + 1,25D_3	141.4	2.00	2.1
Macrophage	Control	146.8	2.0	2.2
Rep 2	TLR7 treated	142.6	2.06	2.13
	TLR8 treated	154.6	2.19	2.18
	TLR7 + 1,25D_3	105.6	2.02	2.06
	TLR8 + 1,25D_3	166.2	1.99	2.03
Macrophage	Control	95.4	2.0	2.1
Rep 3	TLR7 treated	154.5	2.1	2.2
	TLR8 treated	116.6	2.2	2.12
	TLR7 + 1,25D_3	154.2	2.12	2.03
	TLR8 + 1,25D_3	163.9	2.00	2.05
Macrophage	Control	108.5	2.09	2.12
+ 1,25D_3	TLR7 treated	122.1	2.03	2.16
Rep 1	TLR8 treated	201.2	2.02	2.14
	TLR7 + 1,25D_3	282.0	2.06	2.05
	TLR8 + 1,25D_3	155.4	2.03	2.1
Macrophage	Control	312.3	2.1	2.12
+ 1,25D_3	TLR7 treated	305.7	2.02	2.19
Rep 2	TLR8 treated	343.5	2.08	2.01
	TLR7 + 1,25D_3	330.4	2.0	2.05
	TLR8 + 1,25D_3	314.0	1.9	2.05
Macrophage	Control	286.1	2.03	2.03
+ 1,25D_3	TLR7 treated	346.5	2.08	2.00
Rep 3	TLR8 treated	396.1	2.01	2.04
	TLR7 + 1,25D_3	262.7	2.04	2.01
	TLR8 + 1,25D_3	241.9	2.06	2.00

The extracted RNA (1 µL) was placed onto the spectrophotometer probe and quantified at a wavelength of 260 nm. Readings were further recorded at 230 nm and 280 nm to assess nucleic acid purity and RNA purity, respectively.