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Mol, Bronwyn Ashleigh

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Immunomodulation of the innate immune system: The role of vitamin D in the context of monocytes and macrophages

by

Bronwyn Ashleigh Mol

(1084291)

Thesis

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Supervisor: Dr V. Meyer

Co-supervisor: Dr N.L. Gentle

March 2024

DECLARATION

I declare that this thesis is my own, unaided work. It is being submitted for the Degree of Doctor of Philosophy at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other University.



(Signature of candidate)

_4th_ day of __July____2024____at_Johannesburg____

ABSTRACT

Macrophages are widely distributed cells of the innate immune system with essential roles in homeostasis and disease. Despite concerted efforts, several aspects of macrophage origin, biology, and functionality remain poorly understood. To gain a deeper understanding of these cells, a physiologically relevant, but practical model is required. *In vitro*, macrophages are principally generated from primary monocytes and monocyte-like cell lines through a natural process referred to as monocyte-to-macrophage differentiation. Monocyte-like cell lines have several practical advantages over the use of primary monocytes with the most commonly employed monocyte-like cell lines being THP-1 and U937 cells. Despite their frequent use, no standardised protocol is employed in the differentiation of monocyte-like cell lines to macrophages. Naturally, this results in large discrepancies and a lack of comparability between studies. Furthermore, many of these protocols are not physiologically relevant and produce macrophages that are not responsive to downstream stimuli. 1,25-dihydroxyvitamin D_3 (1,25$(OH)_2D_3$), the biologically active form of vitamin D_3, is a recognised immunomodulator that shows pronounced genomic and non-genomic effects in immune cells. It is also reported as an inducer of monocyte-to-macrophage differentiation, though heavily debated, and a potential macrophage polarisation agent. Despite this, there is relatively little information concerning the role of 1,25$(OH)_2D_3$ in monocyte-to-macrophage differentiation and macrophage biology. This study aimed to develop a more physiologically relevant differentiation protocol for the monocyte-like THP-1 and U937 cell lines. This model was then used to investigate the role of 1,25$(OH)_2D_3$ in monocyte-to-macrophage differentiation and macrophage biology. Assessment of morphological features and the macrophage markers, CD11b and CD14, indicated that in both THP-1 and U937 cells, differentiation induced using a combination of 5 nM of phorbol 12-myristate 13-acetate (PMA) and 10 nM 1,25$(OH)_2D_3$ over 96 hours produced the most mature macrophages. It was observed that 1,25$(OH)_2D_3$ alone was not capable of inducing differentiation, yet when combined with PMA, greatly enhanced macrophage features. THP-1 cells are the most widely employed monocyte-like cell line, and are proposed to be the most reflective of primary monocytes. In this study these cells were shown to be more responsive to the effects of 1,25$(OH)_2D_3$ than their U937 counterparts. As such, RNA-sequencing was used to explore the efficacy of the proposed differentiation protocols and the influence of 1,25$(OH)_2D_3$ on macrophage biology in THP-1 cells. Differential gene expression analysis confirmed that the most effective differentiation protocol was the combination of 5 nM PMA with 10 nM 1,25$(OH)_2D_3$ when considering macrophage associated features including transcription factor usage, adhesion, phagocytosis, and cytokine and cytokine receptor expression. This protocol also produced THP-1-derived macrophages that showed increased expression of genes considered to be primary macrophage markers. These

results also suggested that THP-1 cells differentiated with neither PMA nor PMA with $1,25(OH)_2D_3$ were likely to represent fully polarised macrophages. $1,25(OH)_2D_3$ treatment of THP-1 monocytes and THP-1-derived macrophages produced distinct gene expression profiles with considerably less overlap than expected. Though $1,25(OH)_2D_3$ treatment often affected similar biological processes in both cell types, the genes within these processes found to be differentially expressed in each cell line were often distinct. For example, in THP-1-derived macrophages, but not THP-1 monocytes, $1,25(OH)_2D_3$ treatment resulted in the increased expression of genes encoding numerous antibacterial peptides, several small GTPases and their regulators. Additionally, several type I interferon response related proteins showed decreased expression, while expression of cytokines and cytokine receptors was variable. This, taken together with the morphological work, indicates two potential roles for $1,25(OH)_2D_3$ in macrophages. Firstly, a protective role as it suggests the potential to prime an antibacterial response, while still balancing inflammatory responses and protecting against autoinflammation induced by aberrant type I interferon response. Secondly, a potential role in determining the morphological features, clearly demonstrated through microscopy, and further suggested by the differential expression of a variety of small GTPases and their regulators.

To my husband, for never forsaking me.

And to my parents, for their unwavering love and support.

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LIST OF ABBREVIATIONS

$1,24,25(OH)_3D_3$	1α,24R,25-trihydroxyvitamin D_3
$1,25(OH)_2D_3$	1α, 25-dihydroxyvitamin D_3
$25(OH)D_3$	25-hydroxyvitamin D_3
7-DHC	7-dehydrocholestrol
AKT	serine/threonine kinase
AMPK	AMP-activated protein kinase
ANOVA	Analysis of variance
AP-1	Activator protein-1
BSA	Bovine serum albumin
CAMP	Cathelicidin antimicrobial peptide
CCL	C-C motif chemokine ligand
CCL2	C-C motif chemokine ligand 2
CCR	C-C Motif Chemokine Receptor
cDNA	Complementary DNA
CLR	C-type lectin receptor
C_t	Cycle threshold
CXCL	C-X-C motif chemokine ligand
CXCL10	C-X-C motif chemokine ligand 10
CYP	Cytochrome P450
DMSO	Dimethyl sulfoxide
ECM	Extracellular matrix
ERK	Extracellular signal-regulated kinase
EtOH	Molecular grade ethanol (100%)
ETS	E26 transformation-specific
FBS	Foetal bovine serum
FSC	Forward scatter
g	gravitational force
GAP	GTPase activating protein
GEF	Guanine nucleotide exchange factor
GM-CSF	Granulocyte-macrophage colony stimulating factor
GO	Gene ontology
GRB2	Growth factor receptor-bound protein 2
GTPase	Guanosine triphosphate hydrolase
HLA	Human leukocyte antigen
ICAM	Intercellular adhesion molecules

IFN	Interferon
IFN-γ	Interferon-gamma
IL	Interleukin
IL-10	Interleukin 10 cytokine
IRF	Interferon regulatory factor
ISG15	Ubiquitin-like interferon stimulated gene 15 protein
ITGA4	Integrin-alpha 4 protein
ITGAL	Integrin-alpha L protein
ITGAM	Integrin-alpha M protein
ITGAX	Integrin-alpha X protein
ITGB1	Integrin-beta 1 protein
ITGB2	Integrin-beta 2 protein
JAK2	Janus kinase 2
KDM6B	Lysine demethylase 6B
KEGG	Kyoto Encyclopedia of Genes and Genomes
KLF	Krüppel-like factor
LAMP	Lysosome associated membrane protein
LL-37	Human cathelicidin antimicrobial peptide
log_2FC	log_2 fold change
LPS	Lipopolysaccharide
LSD	Least significant difference
MAF	Musculoaponeurotic fibrosarcoma
MAPK	Mitogen-activated protein kinase
M-CSF	Macrophage colony stimulating factor
MEK	Mitogen-activated protein kinase kinase
MHC	Major histocompatibility complex
MLCLs	Monocyte-like cell lines
mRNA	Messenger RNA
mTOR	Mammalian target of rapamycin
NADPH	Nicotinamide adenine dinucleotide phosphate
NF-κB	Nuclear factor-kappa B
NLR	Nod-like receptor
NLRP3	NLR family pyrin domain containing 3
NOX2	Phagocyte NADPH oxidase
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline

PCA	Principal component analysis
PI3K	Phosphoinositide 3-kinase
PKC	Protein kinase C
PMA	Phorbol 12-myristate 13-acetate
PPAR	Peroxisome proliferator activated receptor
PRR	Pattern recognition receptor
(RT-)qPCR	(Reverse transcription) quantitative PCR
RAB	Ras-associated binding
RAS	Rat sarcoma
RFU	Relative fluorescence units
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute
RXR	Retinoid X receptor
SHC	Src homology 2 domain-containing.
SSC	Side scatter
STAT	Signal transducer and activator of transcription STAT
TBE	Tris-borate-EDTA
TGF	Transforming growth factor
TLR	Toll-like receptor
TNF	Tumour necrosis factor-alpha gene
TNF	Tumour necrosis factor
TNF-α	Tumour necrosis factor-alpha cytokine
TREM	Triggering receptors expressed on myeloid cells
UBC	Ubiquitin C
UVB	Ultraviolet B;
VCAM1	Vascular cell adhesion molecule 1
VD_3	Vitamin D_3
VDR	Vitamin D receptor
VDRE	Vitamin D responsive elements
YWHAZ	Tyrosine 3-monooxygenase/ tryptophan 5-monooxygenase activation protein zeta

1.1 Macrophages in homeostasis and disease

Macrophages are tissue-resident cells of the innate immune system, with ubiquitous distribution, that have distinct roles in homeostasis and disease. Due to their wide distribution, relative abundance, and role in immune surveillance, macrophages and their monocyte precursors are often considered first responders to intrinsic and extrinsic threats (Rua and McGavern, 2015; Funes *et al.,* 2018). Key functions of macrophages include antigen presentation, phagocytosis, initiating, shaping, and resolving the inflammatory immune response and tissue repair (Parisi *et al.,* 2018; Sreejit *et al.,* 2020; Boutilier and Elsawa, 2021). To meet the diverse requirements of the microenvironment in which they reside, macrophages are highly plastic cells by nature (Chen *et al.,* 2023). During pathogenic assault, macrophages are amongst the earliest immune cells to interact with the pathogen. They aid in establishing the immune response through pathogen recognition, initiating the inflammatory response through the recruitment of other immune cells via the production of cytokines and other inflammatory mediators, phagocytosis, and antigen presentation (Kotwal and Chien, 2017; Chen *et al.,* 2023). Likewise, once the pathogenic threat has been neutralised, macrophages have an equally important role in resolving inflammation through the elimination of apoptotic host cells, cell debris and pathogen materials, and mediating tissue repair (Kotwal and Chien, 2017; Boutilier and Elsawa, 2021).

Macrophages also have extensive functions during homeostasis, primarily in the clearance of apoptotic cells, coordinating tissue repair, and angiogenesis (Bassler *et al.,* 2019; Boutilier and Elsawa, 2021; Chaintreuil *et al.,* 2023). Though less well understood, macrophages are also important in maintaining a balanced immune response to the host's microbiome (Kogut *et al.,* 2020). They also have important roles in reproductive success, with distinct roles in implantation, placentation, and establishing maternal-foetal tolerance (Yao *et al.,* 2019; Chambers *et al.,* 2021). Furthermore, as a testament to their importance, individuals with genetic disorders resulting in reduced macrophage numbers (including primary immunodeficiencies such as chronic granulomatous disease) or impaired macrophage function (such as macrophage activating syndrome) often have fatal outcomes due to severe infection or severe inflammation resulting in organ failure (Crayne *et al.,* 2019; Holland and Uzel, 2019; Yu *et al.,* 2021).

Given their importance in both homeostasis and disease, it is not surprising that dysregulation of the macrophage compartment contributes to the pathophysiology of several diseases. Naturally, macrophage dysregulation that results in a consistent and uncontrolled pro-inflammatory state is implicated in autoimmune disorders, including rheumatoid arthritis,

inflammatory bowel disease, allergies, asthma, multiple sclerosis and systemic lupus erythematosus (Funes *et al.,* 2018; Shapouri-Moghaddam *et al.,* 2018; Na *et al.,* 2019; Li *et al.,* 2023). Likewise, this pro-inflammatory state contributes to the chronic inflammation associated with obesity and type 2 diabetes mellitus (Funes *et al.,* 2018; Li *et al.,* 2018; Parisi *et al.,* 2018). Though not well understood, macrophage dysregulation related to inflammatory potential, has also been implicated in several complications during pregnancy, namely preeclampsia, miscarriage, and preterm birth (Yao *et al.,* 2019; Chambers *et al.,* 2021). Conversely, tumour associated macrophages tend to be anti-inflammatory in nature and support tumour growth through immunosuppression, angiogenesis, and increased metastatic potential (Rhee, 2016; Boutilier and Elsawa, 2021; Chaintreuil *et al.,* 2023). Ultimately, there is a requirement for balance and control in macrophages during homeostasis and disease; yet how this is achieved, and the implications if it is not, are not completely understood. Understanding the nature of these cells, in the context of health and disease, requires not only understanding their characteristics and functions, but also having reliable models within which to investigate them.

1.2 The developmental origins of macrophages and their functional diversification

As a part of understanding macrophage plasticity and function in the context of health and disease, it is necessary to address the developmental origins of macrophages and the influence of the tissue microenvironment. Macrophages, along with monocytes and contestably dendritic cells, form part of the mononuclear phagocyte system (Bassler *et al.,* 2019; Hume *et al.,* 2019). Today, thanks to advances in fate-mapping and parabiosis studies in mice, single-cell technologies, and information garnered from human transplants, it is accepted that macrophages have two distinct developmental origins in mice, and in all likelihood in humans as well (Bittmann *et al.,* 2001; Merad *et al.,* 2002; Bittmann *et al.,* 2003; Kanitakis *et al.,* 2004; Ginhoux *et al.,* 2010; Yona *et al.,* 2013; Hashimoto *et al.,* 2013; Nayak *et al.,* 2016; Bajpai *et al.,* 2018; Bian *et al.,* 2020; Byrne *et al.,* 2020). Embryonic macrophages are seeded in tissues during embryogenesis and are foetal yolk-sac- and/or foetal liver monocyte-derived (Yona *et al.,* 2013; Hashimoto *et al.,* 2013; Patel *et al.,* 2021), They are long-lived cells capable of proliferation and are sustained into adulthood, likely forming the basis of all tissue-resident macrophages, at least in early life (Hashimoto *et al.,* 2013; Yona *et al.,* 2013; Sreejit *et al.,* 2020). Alternatively, in response to relevant stimuli macrophages can be derived from monocytes, produced by haematopoietic stem cells in the bone marrow that exit circulation, infiltrate the tissues, and differentiate into macrophages that are incapable of self-renewal but may become long-lived macrophages (Funes *et al.,* 2018; Hume *et al.,* 2019).

1.2.1 Tissue distribution and tissue-specific functions of macrophages

Macrophages are highly heterogeneous and widely distributed throughout mammalian tissues. Depending on their tissue of residence they have distinct names and adopt distinct functions in addition to their roles as phagocytic immune sentinels. For example, the brain has four distinct sets of macrophages including microglia, meningeal, perivascular, and choroid plexus macrophages which in addition to immune surveillance and phagocytosis are involved in synaptic pruning and neuromodulation (Mildenberger *et al.,* 2022). Likewise, splenic red-pulp macrophages and liver-resident Kupffer cells are involved in detoxification and iron recycling in addition to their traditional macrophage functions (Recalcati and Cairo, 2021). Similar diversity in functionality is seen throughout the body with gut macrophages being important in microbiome interactions and intestinal homeostasis, alveolar macrophages partaking in surfactant clearance, osteoclasts in the bone being involved in bone remodelling and so forth (Gordon and Plüddemann, 2017; Sreejit *et al.,* 2020; Nobs and Kopf, 2021). The drivers of this functional diversity are not well understood. However, it is thought that the gene expression profiles of embryonically-derived macrophages are initially tissue independent but gain tissue-specificity following organogenesis and organ maturation (Mass *et al.,* 2016; Bian *et al.,* 2020). The exact transcriptional controls for tissue specification have not been completely elucidated. Nonetheless, it does appear that the tissue microenvironment and biological sex are contributing factors; at least in mice (Bain *et al.,* 2016; Liu *et al.,* 2019).

1.2.2 The contribution of monocyte-derived macrophages to tissue-resident macrophage pools

The degree to which bone marrow monocyte-derived macrophages are recruited to supplement the embryonically-derived macrophage pool in various tissues is highly contentious, and appears to depend on the method used to trace the lineage, the tissue in question, the species, the longevity and age of the organism, and the physiological state, i.e. steady-state or a pathophysiological state (Hume *et al.,* 2019; Locati *et al.,* 2020; Patel *et al.,* 2021; Mass *et al.,* 2023). It has been suggested, based on mice models, that tissue-resident macrophage pools can be separated based on the relative contribution of bone marrow monocyte-derived macrophages to the macrophage pool during steady-state. These are referred to as “open” and “closed” macrophage pools and the degree to which they accept monocyte-derived macrophage contribution is both tissue-specific and temporally controlled. “Closed” macrophage pools include the microglia (brain), Kupffer cells (liver) and Langerhans cells (skin), while “open” macrophage pools are those that are replenished by monocytes at either a slow rate, including kidney and spleen macrophages, and those that require constant macrophage replenishment, including colonic, alveolar and dermal macrophages (Hoeffel and

Ginhoux, 2018; Liu *et al.,* 2019). The degree to which this would be reflected in humans is highly debated. A similarly contentious subject is the longevity of the monocyte-derived macrophages that are recruited and whether they are capable of completely adopting the phenotypic, gene expression and functional attributes that characterise embryonically-derived tissue-resident macrophages present in the pertinent tissue (van de Laar *et al.* 2016; Hume *et al.,* 2019; Locati *et al.,* 2020; Sreejit *et al.,* 2020; Patel *et al.,* 2021). van de Laar *et al*, (2016) indicated that both embryonically-derived macrophages and monocyte-derived macrophages were capable of completely colonizing the alveolar macrophage niche and were almost indiscernible from each other at a gene expression level. Similar observations regarding the replacement of tissue-resident liver macrophages by monocyte-derived macrophages without notable functional alterations have also been observed (Beattie *et al.,* 2016; Scott *et al.,* 2016). Whether this holds true for monocyte-derived macrophages in other macrophage tissue niches remains to be seen, but it does suggest that monocyte-derived macrophages are capable of accurately reflecting embryonically-derived macrophages.

1.2.3 The influence of pathogenesis in macrophage recruitment and functionality

In response to pathogenic assault and inflammation there is a heightened degree of monocyte infiltration and macrophage differentiation in relevant tissues (Luque-Martin *et al.,* 2021; Park *et al.,* 2022). These macrophages may adopt several functional states but primarily function in the production of cytokines, antigen presentation and phagocytosis of both pathogens and cellular debris (Parisi *et al.,* 2018; Sanin *et al.,* 2022). During resolution of inflammation, macrophages may also act as part of the “clean-up crew” removing apoptotic cells, clearing cellular debris and potentially participating in tissue regeneration and angiogenesis (Hesketh *et al.,* 2017; Kotwal and Chien, 2017; Chen *et al.,* 2023). However, once the threat has been eliminated the fate of these recruited macrophages is debatable. It is thought that many will undergo apoptosis due to their limited lifespans (Sreejit *et al.,* 2020). However, some may become long-lived tissue-resident cells taking up the vacant positions left by tissue-resident macrophages that were eliminated during pathogenesis, which likely occurs in a tissue specific manner (Hoeffel and Ginhoux, 2018; Park *et al.,* 2022). There has been some debate as to whether tissue-resident macrophages function principally during steady-state conditions and recruited monocyte-derived macrophages are exclusively required to function during pathogenesis. However, given the highly plastic nature of macrophages, and the capacity to adopt features required of them by the tissue microenvironment, such strict delineation of function is unlikely (Hume *et al.,* 2019; Locati *et al.,* 2020).

1.2.4 Macrophage polarisation

A further layer of complexity in understanding macrophage biology, related to the roles of macrophages during homeostasis and pathogenesis, is polarisation. Polarisation can be broadly defined as the process by which macrophages respond to the milieu of stimuli and signals in a given environment to develop distinct functional attributes (Murray, 2017; Yao *et al.,* 2019). For ease of understanding, macrophage polarisation is generally discussed as a dichotomy between M1 macrophages, widely regarded as pro-inflammatory macrophages and M2 macrophages, thought of as anti-inflammatory, tissue repair macrophages. However, this dual representation does not accurately encapsulate the degree of plasticity and heterogeneity that is reflective of the nature of the macrophages in question. Ultimately, macrophage polarisation is a spectrum bordered by these two extremes, with multiple facets, and functional variations in biology that exist along the spectrum.

Within the broad classification of macrophage polarisation, M1 macrophages are highly pro-inflammatory macrophages that recruit and activate other immune cells and contribute toward cytotoxicity through the production of reactive oxygen species (Funes *et al.,* 2018; Yao *et al.,* 2019; Boutilier and Elsawa, 2021; Luque-Martin *et al.,* 2021). M1 macrophages are generated by exposure to type 1 T-helper cell pro-inflammatory cytokines, particularly interferon-gamma (IFN-γ) and tumour necrosis factor alpha (TNF-α), and through the activation of pathogen recognition receptor signalling following response to a microbial ligand, e.g. lipopolysaccharide (LPS; Genin *et al.,* 2015; Huang *et al.,* 2018; Rao Muvva *et al.,* 2020). In response they produce pro-inflammatory cytokines, including C-X-C motif chemokine ligand 10 (CXCL10), TNF-α, and interleukin-1 beta (IL-1β), and are characterised by surface marker expression of T-cell co-stimulatory proteins (CD80 and CD86), toll-like receptors (TLR2 and TLR4), and major histocompatibility complex (MHC) class II proteins at their cell surface (Vogel *et al.,* 2014; Xue *et al.,* 2014; Boutilier and Elsawa, 2021). It is thought that the M1 macrophage polarisation state is driven by several transcription factors, including signal transducer and activator of transcription (STAT) transcription factors, STAT1 and STAT5, nuclear factor-kappa B (NF-κB), interferon regulatory factors (IRFs), IRF3 and IRF5 and Krüppel-like factor-6 (Qin *et al.,* 2012; Murray, 2017).

Alternatively, M2 macrophages are regarded as highly phagocytic, anti-inflammatory macrophages involved in protecting against helminth infection and capable of promoting angiogenesis and tissue repair (Roszer, 2015; Gerrick *et al.,* 2018; Yao *et al.,* 2019). To reflect their plasticity, M2 macrophages are generally divided into a further four subcategories – M2a-d, although it is accepted that there are numerous other potential M2-like macrophages that cannot be classed herein (Mantovani *et al.,* 2004; Zizzo *et al.,* 2012; Martinez and Gordon,

2014; Chávez-Galán *et al.,* 2015). Broadly speaking, polarisation of M2 macrophages occurs in response to the type 2 T-helper cell cytokines IL-4 and IL-13, though it may be supported by IL-10, transforming growth factor beta (TGF-β) and potentially biologically active vitamin D_3 (Barros *et al.,* 2013; Luque-Martin *et al.,* 2021). Following polarisation, they may produce anti-inflammatory cytokines including IL-10, C-C motif chemokine ligands (CCL), CCL17, CCL18, CCL22, and CCL24 and are characterised by the expression of scavenger receptors (CD163 and MRC1) and the c-type lectin receptor (CD209; Durafourt *et al.,* 2012; Xue *et al.,* 2014; Genin *et al.,* 2015; Atri *et al.,* 2018). The transcriptional programme that drives M2 polarisation is less well-defined than that of M1 macrophages due to the greater degree of heterogeneity exhibited in M2-like macrophages. However, there are some well-known M2 macrophage-associated transcriptional regulators including STAT3, STAT6, peroxisome proliferator activated receptors (PPARs), PPARδ and PPARγ, IRF4, and lysine demethylase 6B (KDM6B; Rutschman *et al.,* 2001; Zimmermann *et al.,* 2003; Satoh *et al.,* 2010; Murray, 2017; Xia *et al.,* 2023). As macrophage polarisation exists on a spectrum, it is important to note that it is not a terminal state and that M1 and M2 polarised macrophages are capable of switching between these polarisation states (Kawanishi *et al.,* 2010; Italiani *et al.,* 2014; Tarique *et al.,* 2015; Rackov *et al.,* 2016; Yu *et al.,* 2019). Additionally, they may express markers for both polarisation states, albeit to different extents (Orecchioni *et al.,* 2019).

1.3 Monocyte-to-macrophage differentiation

Evidently macrophages are highly plastic cells whose behaviour and characteristics are strongly influenced by external cues. In adults, newly recruited macrophages, that may or may not become long-lived tissue-resident macrophages, are derived from haematopoietic stem cell derived monocytes. Several (micro)environmental cues and stimuli trigger this monocyte recruitment and initiate the process of differentiation. Monocyte recruitment is primarily driven by the binding of monocyte C-C motif chemokine receptor 2 (CCR2) to CCL2 and CCL7 (Tsou *et al.,* 2007; Chaintreuil *et al.,* 2023). It is through the process of monocyte extravasation and interactions with specific cytokines that monocytes undergo differentiation into macrophages. The control of monocyte extravasation relies on the protein-protein interaction of several adhesion molecules expressed by monocytes and endothelial cells. Initial monocyte capture and rolling relies on the interaction between E-selectins and P-selectins expressed by endothelial cells and monocyte ligands, including the P-selectin glycoprotein ligand-1 (León and Ardavín, 2008; Yago *et al.,* 2010). Adhesion, locomotion and transendothelial migration is principally achieved through the interaction between endothelial cell surface glycoproteins, intercellular adhesion molecules (ICAMs), ICAM1 and ICAM2, and vascular cell adhesion molecule 1 (VCAM1), as well as the integrin heterodimers expressed on the monocyte cell surface, including integrin-alpha M (ITGAM)/ integrin-beta 2 (ITGB2), integrin-alpha L

(ITGAL)/ITGB2 and integrin-alpha 4 (ITGA4)/ integrin-beta 1 (ITGB1; Schenkel *et al.,* 2004; Rutledge and Muller, 2020). The cytoskeletal alterations brought about by monocyte extravasation have been shown to be important in monocyte-to-macrophage differentiation, however, differentiation is primarily governed by the presence of cytokines in the intercellular space (Matsumoto *et al.,* 1996; Perri *et al.,* 2007; Radley *et al.,* 2019). Key among these cytokines are macrophage colony stimulating factor (M-CSF also known as CSF1), IL-34, and granulocyte-macrophage colony stimulating factor (GM-CSF also known as CSF2; Stanley *et al.,* 1978; Boyette *et al.,* 2017; Boulakirba *et al.,* 2018). Though traditionally used to induce differentiation, these colony stimulating factors are also thought to contribute to the polarisation state adopted by the macrophages generated and show differential expression levels during homeostasis, where M-CSF dominates, and pathogenesis, where GM-CSF dominates (Bender *et al.,* 2004; Hamilton, 2019; Trus *et al.,* 2020; Luque-Martin *et al.,* 2021). While these colony stimulating factors are considered highly important for differentiation it is important to note that *in vivo* many additional factors, not limited to cytokine activity, are likely to contribute to and guide monocyte-to-macrophage differentiation.

1.3.1 M-CSF and GM-CSF as inducers of monocyte-to-macrophage differentiation

Although M-CSF and GM-CSF are both key inducers of monocyte-to-macrophage differentiation, they have been shown to exert their effects through distinct signalling pathways and produce distinct macrophage populations (Lacey *et al.,* 2012). Activation of monocyte-to-macrophage differentiation is most widely reported as acting through the janus kinase 2/ STAT5 (JAK2/STAT5), AMP-activated protein kinase (AMPK), protein kinase C/ mitogen-activated protein kinase/ extracellular signal-regulated kinase (PKC/MAPK/ERK) and phosphoinositide 3-kinase/ serine/threonine kinase (PI3K/AKT) signalling pathways, to control mammalian target of rapamycin (mTOR) signalling and initiate the activation of multiple transcription factors, including STAT5, nuclear factor-kappa B (NF-κB), and activator protein-1 (AP-1; Song *et al.,* 2015; Zeng *et al.,* 2015; Wallner *et al.,* 2016; Sun, 2017; Boulakirba *et al.,* 2018; Y. Kim *et al.,* 2019; M. Zhang *et al.,* 2023). More specifically, M-CSF binds to its homodimeric receptor, CSF1R, in order to activate several signalling pathways involved in differentiation and macrophage function, including the PI3K/AKT and MAPK/ERK pathways (Fig. 1.1; Chaintreuil *et al.,* 2023). Conversely, GM-CSF binds the heterodimeric receptor, CSF2Rα/β, to induce signalling primarily through the JAK2/STAT5 pathway, though it may also act through the MAPK/ERK and PI3K/AKT signalling pathways (Lehtonen *et al.,* 2002; Hansen *et al.,* 2008; Perugini *et al.,* 2010; Hamilton, 2019).

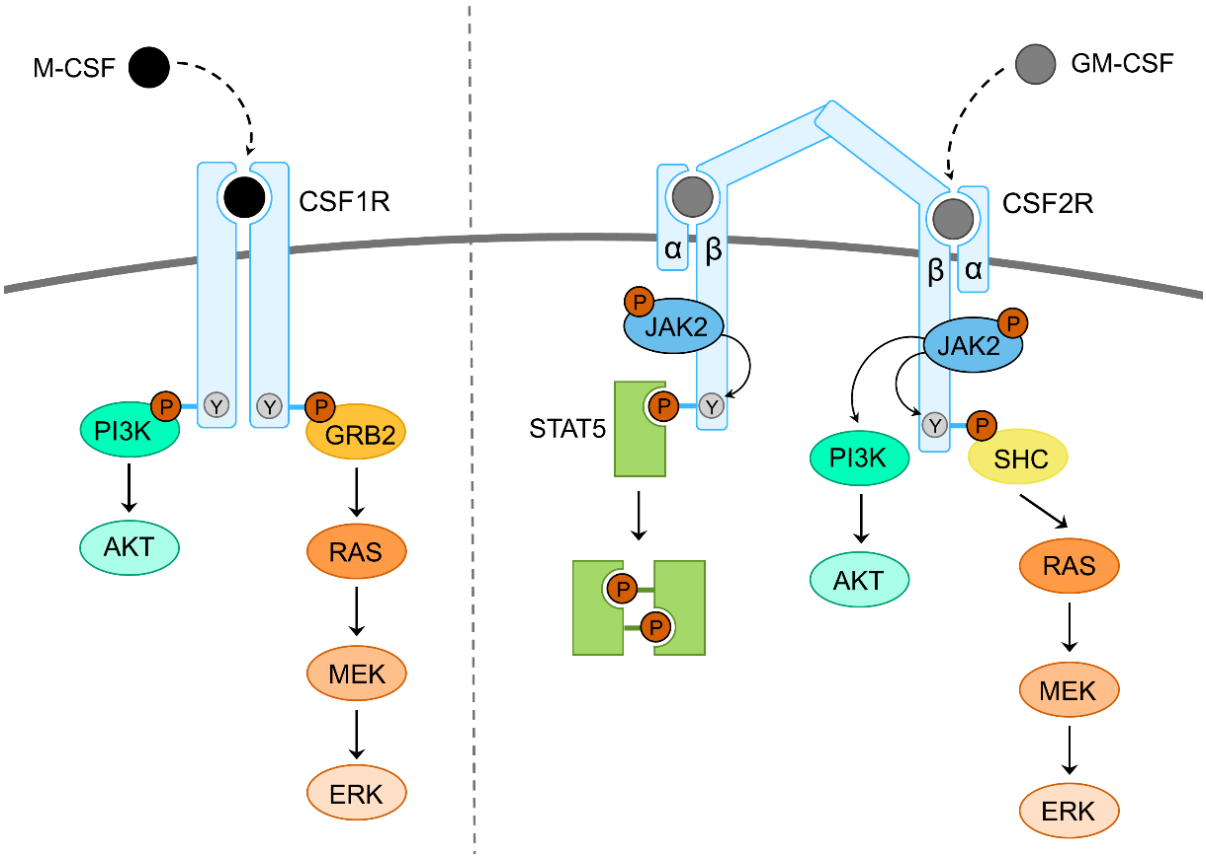


Figure 1.1: Schematic illustrating signalling pathways downstream of M-CSF/CSF1R and GM-CSF/CSFR2 involved in monocyte-to-macrophage differentiation. Abbreviations: M-CSF – macrophage colony stimulating factor; CSF1R – M-CSF receptor; GM-CSF – granulocyte-macrophage colony stimulating factor; CSF2R – GM-CSF receptor; PI3K – phosphoinositide 3-kinase; AKT – serine/threonine kinase; GRB2 – growth factor receptor-bound protein 2; RAS – Rat sarcoma; MEK – Mitogen-activated protein kinase kinase; ERK – Extracellular signal-regulated kinase; JAK2 – janus kinase 2; STAT5 – signal transducer and activator of transcription 5; SHC – Src homology 2 domain-containing. Adapted from Chaintreuil et al., (2023) and Y. Chen et al., (2023).

1.3.2 Phenotypic and functional alterations resulting from differentiation

The process of monocyte-to-macrophage differentiation is accompanied by several notable functional and morphological alterations. These include changes in the expression of cytokines, pathogen recognition receptors and their co-receptors, and an increased capacity for adhesion, phagocytosis, autophagy, antigen presentation, and cell survival (Zeng *et al.,* 2015; Boulakirba *et al.,* 2018; Orekhov *et al.,* 2019). In addition to morphological alterations, the expression of several genes or proteins related to these functions or characteristics are frequently assessed as a method of confirming differentiation (Daigneault *et al.,* 2010; Lund *et al.,* 2016; Pinto *et al.,* 2021). For example, the differentiation-induced activation of both NF-κB and AP-1 is associated with the increased production of inflammatory cytokines such as TNF-α, IL-8, IL-10, CXCL10 and CCL2 (Wolter *et al.,* 2008; Li *et al.,* 2010; Jin *et al.,* 2014; T. Liu *et al.,* 2017; Gschwandtner *et al.,* 2019; Viola *et al.,* 2019). Likewise, differentiation results in increased expression of CD14, a TLR co-receptor, and potentially TLR4, depending

on the differentiating stimulus (Zanoni and Granucci, 2013; Riddy *et al.,* 2018; Tedesco *et al.,* 2018; Ciesielska *et al.,* 2021). The increased expression of integrins associated with migration, adhesion, and phagocytosis – particularly ITGAL (CD11a), ITGAM (CD11b), ITGAX (CD11c), ITGB2 (CD18), and ITGA4 (CD49d) – is among the most frequently assessed metric for confirming differentiation (Forrester *et al.,* 2018; Khan *et al.,* 2018; Riddy *et al.,* 2018; Gažová *et al.,* 2020; Mohd Yasin *et al.,* 2022). Naturally, the expression of many other proteins is assessed, including those related to phagocytosis and autophagy (scavenger receptors: CD163, CD36; lysosome associated membrane proteins (LAMPs): LAMP1 and CD68), human leukocyte antigen (HLA) encoded MHC-II components, proteases required for monocyte extravasation (matrix metalloprotease-1, -2, -9 and -12) and transcription factors involved in regulating differentiation and polarisation (Hesketh *et al.,* 2017; Chistiakov *et al.,* 2018; Forrester *et al.,* 2018; Riddy *et al.,* 2018; Tedesco *et al.,* 2018; Pinto *et al.,* 2021; Mohd Yasin *et al.,* 2022).

1.4 In vitro *models for macrophages*

Given the heterogeneity of macrophages, in developmental origin, tissue specificity, polarisation and, ultimately, their contribution to health and disease, the question of how to study these cells accurately is at the forefront. Ultimately, one single model is not likely to fully encompass the capacities of all macrophages, each model will have its own characteristics and benefits which, while allowing for investigations into macrophage biology, will present their own biases. While animal models allow *in vivo* assessment of macrophage biology at a system-wide level, that is not feasible in humans, there are several disparities which arise based on the differences between species. For example, there are differences in arginase and nitric oxide synthase expression between murine and human macrophages, which are important when considering pathogen clearance (Wculek *et al*., 2021). Similarly, though both species demonstrate macrophage polarisation, the markers that identify these states may be distinct (McWhorter *et al*., 2015).

When studying human macrophages *in vitro* there are several possible approaches. The use of human tissue-resident macrophages is limited as it relies on invasive surgeries, such as biopsies and bronchoscopies, which, even if successful, produce very low macrophage yields that cannot be further expanded nor stored for prolonged periods (Valdés-López and Urcuqui-Inchima, 2018; Duweb *et al.,* 2022). As there is evidence that primary monocytes can differentiate into macrophages that are almost indistinguishable from the tissue-resident macrophages, they are a relevant model for the study of most tissue-resident macrophages (Beattie *et al.,* 2016; Scott *et al.,* 2016; van de Laar *et al.,* 2016). However, the use of primary monocytes has its own limitations. As monocytes only make up 5-10% of leukocytes the yield

from blood donations is not high and there is a risk of cross contamination with other cell types (Qin, 2012; Forrester *et al.,* 2018). Additionally, like primary macrophages, these cells have a finite lifespan, do not proliferate readily and cannot be stored for prolonged periods (Lund *et al.,* 2016; Baxter *et al.,* 2020). Furthermore, studies in primary cells are always associated with extensive donor-based variability (Maeß *et al.,* 2014).

1.4.1 Monocyte-like cell lines as models for monocyte-derived macrophages

An alternative to the use of primary monocytes and macrophages, that can circumvent these difficulties, particularly in basic research is the use of monocyte-like cell lines (MLCLs). While primary monocytes naturally prove a more accurate model for these studies, MLCLs derived from malignant myeloid cells act as a simplified model (Riddy *et al.,* 2018). MLCLs have several advantages over primary monocytes, primarily in their ease of acquisition, storage, and expansion *in vitro,* as well as in displaying genetic homogeneity that removes the inter-individual variability associated with the use of primary monocytes (Qin, 2012; Maeß *et al.,* 2014; Forrester *et al.,* 2018; Noronha *et al.,* 2020; Pinto *et al.,* 2021; Duweb *et al.,* 2022). Several human MLCLs exist, including HL-60, KG-1, ML-2, Mono-Mac-6, THP-1 and U937 (Riddy *et al.,* 2018; Nascimento *et al.,* 2022). Two of the most frequently employed cell lines in the study of monocytes, macrophages and the process of monocyte-to-macrophage differentiation are THP-1 and U937 cells (Song *et al.,* 2015; Lund *et al.,* 2016; Mendoza-Coronel and Castañón-Arreola, 2016; MacKinnon *et al.,* 2020). The THP-1 cell line was established by Tsuchiya *et al.,* (1980), and was derived from the blood of a one-year-old Japanese male patient suffering from acute monocytic leukaemia. The monocytic nature of these cells was confirmed based on their morphology, capacity for phagocytosis, lysozyme production, and mitogenic T-lymphocyte activation (Tsuchiya *et al*., 1980). The U937 cell line was established by Sundström and Nilsson (1976) and was derived from the pleural effusion of a 37-year-old male diagnosed with diffuse histiocytic lymphoma. Immortalised U937 cells were classified as monocytes based on their morphological similarity to monoblastic cells, the presence of eosinophilic granules and the expression of myeloid differentiation markers (Sundström and Nilsson, 1976; MacKinnon *et al.,* 2020). Additionally, neither cell line was Epstein Barr virus transformed (Sundström and Nilsson, 1976; Tsuchiya *et al.,* 1980; Mosialos *et al.,* 1994; Roth *et al.,* 1994; Reischl *et al.,* 1996).

1.4.2 PMA and 1,25(OH)$_2$D$_3$ as inducers of monocyte-to-macrophage differentiation in MLCLs

In practice MLCLs, specifically THP-1 and U937 cells, are differentiated into monocyte-derived macrophages with the aid of phorbol-esters, principally phorbol 12-myristate 13-acetate (PMA) also called 12-O-tetradecanoylphorbol 13-acetate, or retinoic acids such as all-trans retinoic acid or more commonly 1α,25-dihydroxyvitamin D_3 (1,25(OH)$_2$D$_3$; Chanput *et al.,* 2014; Hu and Zuckerman, 2014). Unlike primary monocytes, THP-1 and U937 cells are resistant to M-CSF- and GM-CSF-induced differentiation (Brach *et al.,* 1990; Pession *et al.,* 2003; Kogan *et al.,* 2012; Aldo *et al.,* 2013; Nascimento *et al.,* 2022). PMA is a tumorigenic plant-derived diacylglycerol mimic, which is a potent activator of PKC, that has been shown to induce differentiation in MLCLs through the PKC/MAPK and the PI3K/AKT signalling pathways and mediate NF-κB and AP-1 activation (Song *et al.,* 2015; Zeng *et al.,* 2015). Alternatively, 1,25(OH)$_2$D$_3$, an endogenously synthesised steroid hormone, has also been shown to contribute toward differentiation in THP-1 and U937 cells through the PI3K/AKT and mTOR signalling pathway (Di Rosa *et al.,* 2011; Y. Kim *et al.,* 2019). However, whether 1,25(OH)$_2$D$_3$ alone is able to successfully produce macrophages is debatable, despite its usage as a differentiation agent, with several research groups indicating its inability to successfully differentiate THP-1 and U937 cells (Schwende *et al.,* 1996; Daigneault *et al.,* 2010; Valdés-López and Urcuqui-Inchima, 2018; Rynikova *et al.,* 2023). In spite of this, the contribution of 1,25(OH)$_2$D$_3$ as a well-established immunomodulator means that its role in monocyte-to-macrophage differentiation, macrophage biology, and macrophage polarisation is not so easily dismissed (Sassi *et al.,* 2018; Bishop *et al.,* 2021; Warwick *et al.,* 2021). Though frequently employed in the study of monocyte-to-macrophage differentiation, macrophage polarisation and macrophage biology, the methods employed for differentiation of THP-1 and U937 cells into macrophages is seldomly consistent, nor physiologically relevant.

1.4.3 Inconsistencies in protocols for the generation of macrophages in cell line models

PMA-based differentiation protocols applied to THP-1 and U937 cells are highly variable, with respect to concentration, the period of stimulation, and the inclusion of a rest period (Table 1.1 and 1.2). PMA concentrations used for differentiation range anywhere between 1 nM to 400 nM PMA. Similarly, PMA stimulation periods range from 6–144 hours, which may be followed by a rest period that can range from 3–168 hours. Furthermore, despite its frequent use, it has been shown that high-dose PMA differentiation protocols negatively impact THP-1- and U937-derived macrophage functionality, viability, and responsiveness to M2 polarisation and weaker bacterial stimuli, due to heightened inflammatory responses (Park *et al.,* 2007; Daigneault *et al.,* 2010; Foey and Crean, 2013; Maeß *et al.,* 2014; Lund *et al.,* 2016; Starr *et al.,* 2018). Conversely, low-dose PMA protocols have displayed a phenomenon referred to as de-

differentiation, wherein the cells lose their adherent properties and regain their proliferative capacities, once PMA stimulus has been removed for extended periods (Spano *et al.,* 2013). Differentiation protocols employing $1,25(OH)_2D_3$ are more consistent, generally making use of 100 nM $1,25(OH)_2D_3$ for a minimum of 72 hours, although concentrations of up to 500 nM $1,25(OH)_2D_3$ have been employed (Daigneault *et al.,* 2010; Cervantes *et al.,* 2019). These $1,25(OH)_2D_3$ concentrations far exceed those representative of physiological conditions (Valdés-López and Urcuqui-Inchima, 2018; Rao Muvva *et al.,* 2020). In studies involving primary monocytes the use of 10 nM $1,25(OH)_2D_3$ is generally more common (Ryynänen and Carlberg, 2013; Rao Muvva *et al.,* 2020). Though it may be considered supraphysiological, a 10 nM $1,25(OH)_2D_3$ concentration is achievable, albeit rarely, *in vivo* and thus is considered a more physiologically relevant $1,25(OH)_2D_3$ concentration (Zhang *et al.,* 2013; Rafique *et al.,* 2019). It is worth noting that, like GM-CSF and M-CSF, PMA and $1,25(OH)_2D_3$ are occasionally considered not only as differentiation agents, but as being capable of inducing M1- and M2-polarisation, respectively (Foey and Crean, 2013; Rynikova *et al.,* 2023). The lack of consistency in the differentiation protocols employed leads to a lack of comparability between similar cell line-based studies and difficulties in transferring the conclusions derived therefrom to primary cells. As such, there is a need to develop a differentiation protocol that is both physiologically relevant, receptive to additional stimuli and reflective of the primary macrophages it is meant to represent.

Table 1.1: Reported PMA-based differentiation protocols for THP-1 cells

PMA Concentration (nM)	Cells/ml	Cells/cm^2*	PMA exposure (hours)	PMA rest (hours)	Differentiation assessment	References
1, 2, 4, 8, 16, 40	0.2×10^6	0	48	0	CD11c, CD14	Hoffmann *et al.,* 2019
3	1×10^6	0	24	0	CD11b, Adherence	Dong and Chen, 2015
3	0	3000	24	0	Microscopy, CD54, CD11b, CD11c, CD14, HLA-DR	Pujari *et al.,* 2015.
3, 8, 16, 32, 80, 160	0.25×10^6, 0.5×10^6, 1×10^6	0	24, 48, 72, 96	0	Microscopy, Phagocytosis	Zhou and Ma, 2018.
6, 30, 60	0	100000 - 120000	72	72	Microscopy CD11b, CD14	Spano *et al.,* 2013
8	0.5×10^6	0	24	0	Microscopy	Trinh *et al.,* 2020.
8	0.5×10^6	0	48	0	Microscopy, CD14, TLR2, TLR4	Choudhury *et al.,* 2014.
8	0.5×10^6	0	48	0	Microscopy, CD14, CD163	Mohammad, *et al.,* 2021.
8	1×10^6	0	48	0	CD11b, CD36	Hamamura-Yasuno, *et al.,* 2020.
A: 80	0.2×10^6	0	A: 72	A: 48	Microscopy CD11b, CD14, CD86	Pinto, *et al.,* R.K., 2021.
B: 80	0.2×10^6	0	B: 16	B: 48	Microscopy CD11b, CD14, CD86	Pinto, *et al.,* R.K., 2021.
C: 8	0.2×10^6	0	C: 48	C: 3	Microscopy CD11b, CD14, CD86	Pinto, *et al.,* R.K., 2021.
8, 25, 50, 100, 200	0.2×10^6	0	48	0, 24, 48, 120	Microscopy, Adherence, FSC, SSC	Lund *et al.,* 2016.
8	0.6×10^6	0	48	48	Microscopy	Hassan *et al.,* 2018.
16	0.05×10^6	0	24	0	CD11c	Safar *et al.,* 2019.
16	0	143000	24, 72	0, 120	Microscopy, CD11b mRNA	Okamoto, *et al.,* 2021.
16	0.6×10^6	0	48	0	LC-MS/MS proteomics	Tarasova *et al.,* 2016.
16 then 4	0	286000	48 then 24	0	Microscopy CD11b mRNA, CD68 mRNA	Maeß *et al.,* 2014.
16	0.25×10^6	0	72	0	Microscopy	van den Bogaart *et al.,* 2017.
16	2.0×10^6, 0.25×10^6	0	144	0	CD14	Aldo *et al.,* 2013.
19, 190	0.05×10^6	0	48	0	Microscopy, CD14, CD68	Hoefert *et al.,* 2015.
20, 40, 80, 120, 160	0	421000	48	0	Microscopy, CD14 (mRNA)	Wang *et al.,* 2021.

Table 1.1: Continued…

PMA Concentration (nM)	Cells/ml	Cells/cm^2*	PMA exposure (hours)	PMA rest (hours)	Differentiation assessment	References
20	0	78000	72	24	CD11b, CD11c	Dowal *et al.,* 2017
20, 200	0.3 - 0.5 × 10^6	0	72	120	Microscopy,	Majumdar and Kraft, 2020.
30	1 × 10^6	0	24	0	CD11b	Huang *et al.,* 2015.
30	0	16000	72	0	Microscopy, CD11b, CD18, CD36	Kim *et al.,* 2015
40	0.5 × 10^6	0	24, 72	0	Microscopy CD36	Paland *et al.,* 2013.
40	0	105000	48	0	Microscopy	Je *et al.,* 2016.
40	0	31000	72	0	CD14	Foey and Crean, 2013
40	0	263000	72	0	Microscopy	Notararigo *et al.,* 2014.
40	1 × 10^6	0	72	24	Microscopy, CD11c, CD86, Adhesion	Kao *et al.,* 2020.
50, 100, 200	0.5 × 10^6	0	24, 48, 72	0	Microscopy	Chang *et al.,* 2021.
80	1 × 10^6	0	24	24	Microscopy	Zha *et al.,* 2016.
80	0.5 × 10^6	0	24, 48	0	Microscopy CD80, CD71, CD11b, CD14	Grytting *et al.,* 2019.
80	0.1 × 10^6	0	48	120-168	Microscopy, CD14, CD11b, CD11c, CD80, CD86, CD163, CD68	Padilla *et al.,* 2017.
96	0	47000	48	24	Microscopy	Alqarni *et al.,* 2018.
100	0	6500	24	0	Microscopy	Padberg *et al.,* 2020.
100	1 × 10^6	0	24	48	Microscopy CD14	Mendoza-Coronel and Castañón-Arreola, 2016.
100	1 × 10^6	0	24	48	Microscopy, CD11b, CD36, CD14, CD16 and CD54	Mehta and Dhawan, 2020.
100	0.2 - 0.5 × 10^6	0	24	48	Microscopy	Nguyen *et al.,* 2022
100	0	133000	6, 24, 48	0	CD54	Alidousty *et al.,* 2014
100	0	158000, 62500, 47000	24, 48	0	Microscopy, CD11b	Xu *et al.,* 2015.
100	1 × 10^6	0	48	0	Microscopy	Wang *et al.,* 2016

Table 1.1: Continued…

PMA Concentration (nM)	Cells/ml	Cells/cm^2*	PMA exposure (hours)	PMA rest (hours)	Differentiation assessment	References
100	0.5×10^6	0	72	0	Microscopy	Herdoiza Padilla *et al.,* 2019
100	0.5×10^6	0	96	0	Microscopy CD14 CD11b	Camilli *et al.,* 2016
150	0	53000	24	24	Microscopy, CD14, CD36, CD68, CD71	Genin *et al.,* 2015.
150	0.4×10^6	0	72	0	Microscopy (Liu staining), CD14, CD68	Tsai *et al.,* 2016.
150	0.2×10^6	0	72	24	Microscopy, CD86, CD14, CD68	Krisnawati, *et al.,* 2019.
160	0.5×10^6	0	24	0	Microscopy, CD68	Luo *et al.,* 2016.
160	0.2×10^6	0	24	0	Microscopy, Adherence	Moussa *et al.,* 2018.
160	0	104000	24, 48, 72	0	Adherence, CD11b, CD14,	Li *et al.,* 2017.
160	0	104000	24	48	Microscopy	Jiang *et al.,* 2017.
160	0.2×10^6	0	24	72 - 96	Microscopy	Yen, *et al.,* 2015.
160	0.5×10^6	0	48	0	Microscopy	Schopohl and Melzig, 2014.
160	0.5×10^6	0	48	0	Microscopy, CD36	Xu *et al.,* 2016.
160	0	263000	48	0	Microscopy	Sekhon-Loodu and Rupasinghe, 2015.
160	0	353000	48	0	CD11b, CD14	Park *et al.,* 2018.
160	0.5×10^6	0	48	0	Microscopy	Gatto, *et al.,* 2020.
160	0.2×10^6	0	48	48	Microscopy, CD11b, CD14	Gopinath *et al.,* 2021.
200	0	13000	24	0	Microscopy, CD68	Zhao *et al.,* 2018.
200	0.2×10^6	0	48	0	Microscopy, adherence, CD86, CD11b	Liu *et al.,* 2019.
200	1×10^6	0	72	0	CD36	Kou *et al.,* 2013.
200	0	200000	72	0	CD206, CD163	Neu *et al.,* 2013.
200	0	5400	72	3.5	Microscopy, CD14, CD11b, CD206, CD71	Øya, *et al.,* 2019.
200	0	42000	72	48	Microscopy, HLA-A, HLA-B, HLA-C, HLA-DR, CD11b, CD14, CD33, CD68, CD163, CD206	Wu *et al.,* 2014.

Table 1.1: Continued…

PMA Concentration (nM)	Cells/ml	Cells/cm^2*	PMA exposure (hours)	PMA rest (hours)	Differentiation assessment	References
200	0.15×10^6	0	72	120	Microscopy	Ota *et al.,* 2017.
200	0.1×10^6	0	72	120	Microscopy	Mulvaney *et al.,* 2019.
240	0.2×10^6	0	48	24	Microscopy, CD11b, CD68	Lai, *et al.,* 2021.
300	0	5000	54	72	CD68 (mRNA), CD80, CD206	Tedesco *et al.,* 2018.
320	1×10^6	0	18	0	Microscopy, CD14, CD68, CD86, CD206	Xu *et al.,* 2018.
320	0.2×10^6	0	24	0	Microscopy, CD68, CD14, CD204, CD206	Zeng *et al.,* 2019.
320	0.5×10^6	0	72	0	Microscopy, CD14	Zhang *et al.,* 2014
400	0	35000	6	0	Microscopy CD68, CD204, CD206	Jeong *et al.,* 2014.

*Cells/cm^2 are derived from cells plated per well and rounded up to the nearest 1000

Table 1.2: Reported PMA-based differentiation protocols for U937 cells

PMA Concentration (nM)	Cells/ml	Cells/cm^2*	PMA exposure (hours)	PMA rest (hours)	Differentiation assessment	References
1, 2, 4, 8, 16, 40	0.2 × 10^6	0	48	0	CD11c, CD14	Hoffmann *et al.,* 2019.
2, 4, 8, 16	0	107000	16	0	Microscopy	Debelec-Butuner, *et al.,* 2014.
2, 4, 16	0	83000	24	0	Microscopy	Song, *et al.,* 2015.
10, 25, 50, 100, 200	0.6 × 10^6	0	48	0	Microscopy, CD14	Kuno *et al.,* 2020.
10	1 × 10^6	0	48	0	Microscopy	Chai *et al.,* 2014.
16	1 × 10^6	0	24	0	Microscopy	Ruuska *et al.,* 2013.
16	1 × 10^6	0	24	48	Microscopy CD68, CD163	Nascimento, *et al.,* 2022.
16	0.5 × 10^6	0	48	0	CD11b and CD14	Xu *et al.,* 2018.
16, 80, 160	0.4 × 10^6	0	48	48	Microscopy	Emirbayer, *et al.,* 2017.
20	0	52000	72	24	CD11b/ CD11c	Dowal, *et al.,* 2017.
25	0	143000	48	0, 144	Microscopy and CD14, TLR2, CD11b, CD11c, CD68, CD86	López and Urcuqui-Inchima, 2018.
80	0	52000	24	24	Microscopy, CD86, CD163, CD206,	Yamamichi *et al.,* 2017.
80	0.5 × 10^6	0	48	24	Microscopy	Herrera *et al.,* 2019.
80	0.5 × 10^6	0	96	0	Microscopy CD14 CD11b	Camilli, *et al.,* 2016.
80	0.45 × 10^6	0	120	0	Microscopy	Dong *et al.,* 2020.
96	0.1 × 10^6	0	48	0	Microscopy	Tusiimire *et al.,* 2016.
100	1 × 10^6	0	24	48	Microscopy CD14	Mendoza-Coronel and Castañón-Arreola, 2016.
100	0.5 × 10^6	0	48	18	CD11b	Raghunathan, *et al.,* 2019.
100	0	500000	72	0	CD80, CD86, CD206	Fong, *et al.,* 2015.
150, 250	1 × 10^6	0	48, 72	0	Microscopy	Prasad, *et al.,* 2021.
160	0	52000	12	0	Microscopy, CD68 (mRNA)	Taniguchi, *et al.,* 2015.
160	0.5 × 10^6	0	24, 48	0	Microscopy	Prasad, *et al.,* 2020.
160	0.25 × 10^6	0	120	0	Microscopy, CD11b, CD68	Galindo and Clavijo-Ramírez, 2020.

*Cells/cm^2 are derived from cells plated per well and rounded up to the nearest 1000

1.5 1,25(OH)$_2$D$_3$ synthesis, biological effects, and role as an immunomodulator

1.5.1 1,25(OH)$_2$D$_3$ synthesis

Vitamin D is a steroid hormone, that exists in two predominant forms under physiological conditions, vitamin D_2 and vitamin D_3 (Harrison *et al.,* 2020). Dietary vitamin D may be obtained in small amounts from plants and fungi (vitamin D_2) or animal products such as fish, eggs, butter, and liver (vitamin D_3; Baeke *et al.,* 2010). However, in humans the main source of vitamin D arises from the endogenous production of vitamin D_3 in the skin (Fig. 1.2; Teymoori-Rad *et al.,* 2019). Endogenous production of vitamin D_3 occurs in the dermis and relies on the UVB catalysed conversion of 7-dehydrocholestrol into pre-vitamin D_3, which is subsequently converted to vitamin D_3 through thermal isomerisation (Holick *et al.,* 1977; Holick *et al.,* 1980; Holick, 1981). Before becoming bioactive, vitamin D_3 undergoes two hydroxylation steps catalysed by cytochrome P450 (CYP) members (Baeke *et al.,* 2010). Vitamin D_3 and its hydroxylated metabolites are transported throughout the body by binding to the vitamin D binding protein (Xie *et al.,* 2018). The first hydroxylation of vitamin D_3 to the predominant circulating form of vitamin D, 25-hydroxyvitamin D_3 (25(OH)D_3), is principally catalysed by hepatic CYP2R1, and to a lesser extent, ubiquitously expressed CYP27A1 (Cheng *et al.,* 2004; Pikuleva, 2006; Zhu *et al.,* 2013; Voltan *et al.,* 2023). Hydroxylation of 25(OH)D_3 to bioactive vitamin D_3, 1,25(OH)$_2$D$_3$, is catalysed by CYP27B1 which is expressed in the kidneys, immune cell surfaces and certain epithelial cells (Stoffels *et al.,* 2006; Adams *et al.,* 2014; Bishop *et al.,* 2021). The production of 1,25(OH)$_2$D$_3$ by CYP27B1 is under strict regulation in the kidney, with fibroblast growth factor 23, the parathyroid hormone, calcium concentration and 1,25(OH)$_2$D$_3$ acting as key regulators of CYP27B1 expression and activity (Adams *et al.,* 2014; van der Meijden *et al.,* 2016). This strict regulation of CYP27B1 is not imposed in extra-renal sites, rather it is the bioavailability of 25(OH)D_3 that ultimately determines the production of 1,25(OH)$_2$D$_3$ (Harrison *et al.,* 2020). In addition to the regulation of CYP27B1, the availability of 1,25(OH)$_2$D$_3$ is also determined by the activity of CYP24A1, which further hydroxylates 1,25(OH)$_2$D$_3$ to 1α,24R,25-trihydroxyvitamin D_3 (1,24,25(OH)$_3$D$_3$), and in so doing inactivates it (Jones *et al.,* 2012).

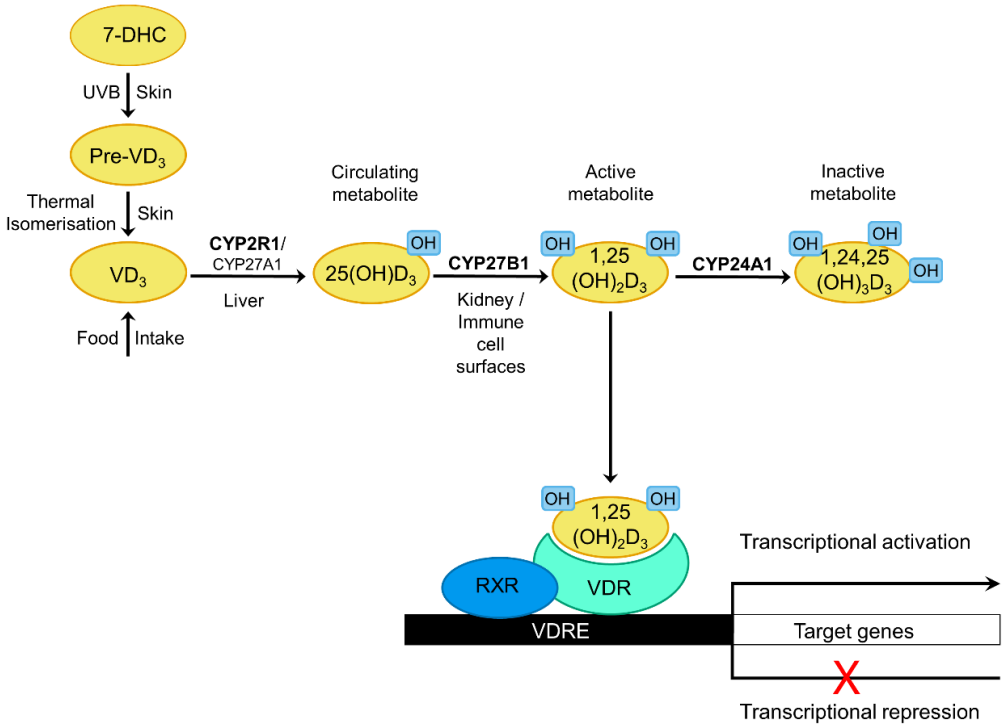


Figure 1.2: Schematic illustrating the synthesis, metabolism and potential genomic actions arising from $1,25(OH)_2D_3$ binding to VDR. Abbreviations: 7-DHC – 7-dehydrocholestrol; UVB – ultraviolet B; VD_3 – vitamin D_3; CYP – cytochrome P450; $25(OH)D_3$ – 25-hydroxyvitamin D_3; $1,25(OH)_2D_3$ – 1α, 25-dihydroxyvitamin D_3; $1,24,25(OH)_3D_3$ – 1α,24R,25-trihydroxyvitamin D_3; VDR – vitamin D receptor; RXR – retinoid X receptor; VDRE – vitamin D responsive elements. Adapted from Lee (2020).

1.5.2 Genomic and non-genomic effects of $1,25(OH)_2D_3$

The effects exerted by biologically active $1,25(OH)_2D_3$ can be separated into genomic and non-genomic effects. The non-genomic actions of $1,25(OH)_2D_3$ are tissue specific, and include the opening of calcium channels, the activation of lipid- and protein- kinases (including PI3K, MAPK and PKC), and the generation of second messengers (Hii and Ferrante, 2016; Dimitrov *et al.,* 2021). The genomic effects of $1,25(OH)_2D_3$ are a result of its high affinity binding with the vitamin D receptor (VDR; Fig. 1.2), a nuclear receptor and transcription factor, following intracellular uptake (Lin, 2016; Carlberg, 2019). The VDR protein is widely expressed across many tissues, with the largest quantities found in the parathyroid and adrenal glands, intestine, skin, and kidneys (Wang *et al.,* 2012; Nurminen *et al.,* 2019). Notably it is also expressed in most cells of the immune system (Szymczak and Pawliczak, 2016). Following $1,25(OH)_2D_3$ ligand binding by VDR, VDR forms a heterodimer with the retinoid x receptor (RXR), together they are capable of binding vitamin D responsive elements (VDRE) in target genes in order to activate or inhibit transcription (Medrano *et al.,* 2018; Warwick *et al.,* 2021). Genes that possess VDREs and show VDR binding, using techniques such as chromatin immunoprecipitation sequencing are considered primary vitamin D targets (Warwick *et al.,* 2020, Warwick *et al.,* 2021).

1.5.3 The role of 1,25(OH)$_2$D$_3$ as an immunomodulator

In addition to its well-established role in calcium homeostasis, 1,25(OH)$_2$D$_3$ has been shown to have potent regulatory effects in cells of both the innate and adaptive immune system, with cell-type specific effects having been observed (Szymczak and Pawliczak, 2016; Medrano *et al.,* 2018; Sassi *et al.,* 2018; Dimitrov *et al.,* 2021; Bishop *et al.,* 2021). There are several lines of clinical and experimental data that show a protective role of 1,25(OH)$_2$D$_3$ during bacterial and viral infections, including: *Mycobacterium tuberculosis*, *Streptococcus pneumoniae*, human immunodeficiency virus, hepatitis B virus, hepatitis C virus, dengue virus, zika virus, rhino virus, respiratory syncytial virus infections, among others (Bergman *et al.,* 2013; Cusato *et al.,* 2016, 2017; Meireles *et al.,* 2016; Alvarez *et al.,* 2019; Cervantes *et al.,* 2019; Teymoori-Rad *et al.,* 2019; Rao Muvva *et al.,* 2020; Zacharioudaki *et al.,* 2021; Castillo *et al.,* 2021; Fernandez *et al.,* 2023). 1,25(OH)$_2$D$_3$ has also been shown to have immunomodulatory and protective roles in several models for auto-inflammatory disorders, though it has seen little clinical success as a therapeutic (Ding *et al.,* 2013; Riek *et al.,* 2013; Carvalho *et al.,* 2017; Bishop *et al.,* 2021). The exact effects of 1,25(OH)$_2$D$_3$ in these contexts are specific to the cell-type, physiological state and the disease in question (Fitch *et al.,* 2016; Anderson *et al.,* 2020; Dimitrov *et al.,* 2021; Bishop *et al.,* 2021). Though not a coverall, some of the immunomodulatory effects of 1,25(OH)$_2$D$_3$ include modulation of cytokine and chemokine expression, regulation of interferon responses, antimicrobial peptide production, regulation of T-cell, B-cell, and monocyte differentiation, M2 macrophage polarisation, altered expression of pattern recognition receptors (PRRs), and altered cholesterol metabolism (Riek *et al.,* 2013; F. Zhang *et al.,* 2015; Cusato *et al.,* 2016; Arababadi *et al.,* 2018; Cervantes *et al.,* 2019; Liang *et al.,* 2019; Castillo *et al.,* 2021; Fernandez *et al.,* 2023).

Though demonstrating numerous functions in other immune cells, including B- and T-cells, 1,25(OH)$_2$D$_3$ is perhaps best understood in monocytes at a transcriptomic, epigenomic, and proteomic level, as monocytes are highly responsive to 1,25(OH)$_2$D$_3$ and modelled with some degree of accuracy using THP-1 cell line (Neme *et al.,* 2016; Carlberg, 2019; Nurminen *et al.,* 2019). Within this context, 1,25(OH)$_2$D$_3$ demonstrates many of the immune related functions described above. However, the degree to which monocyte-derived macrophages reflect these processes is less well understood. Though 1,25(OH)$_2$D$_3$ has been shown to have pronounced effects on aspects of macrophage biology such as polarisation and macrophage-pathogen interactions, less is understood about the influence of 1,25(OH)$_2$D$_3$ on maintaining what may be considered homeostasis (X. Zhang *et al.,* 2015; Liang *et al.,* 2019; Fernandez *et al.,* 2023). Naturally, to understand the role of 1,25(OH)$_2$D$_3$ in macrophages in disease, it is first necessary to understand its role during homeostasis and the degree to which it may influence macrophage functionality.

1.6 Research objectives

Given the complexity of macrophage biology and the importance of macrophages in homeostasis and disease, having a practical, yet physiologically relevant model to study these cells would contribute to a better understanding of their functionality. Furthermore, the role of $1,25(OH)_2D_3$ in innate immunity is relatively well established, yet in the context of macrophage biology is relatively poorly understood with several controversies surrounding its precise role. While it is touted as an inducer of monocyte-to-macrophage differentiation in MLCLs, several bodies of research indicate that this may not be accurate. Additionally, $1,25(OH)_2D_3$ has also been reported to be a potential M2 polarisation agent, though this is disputed. Furthermore, despite the importance of macrophages in health and disease and the potential genomic influence of $1,25(OH)_2D_3$ therein, there is comparatively little work that directly assesses the role of $1,25(OH)_2D_3$ in macrophages when compared to their monocyte precursors.

As such, the objectives of this study were to:

I. Develop a more physiologically relevant protocol for the induction of monocyte-to-macrophage differentiation in two frequently employed monocyte-like cell lines, THP-1 and U937 cells, using two well-reported differentiation agents, PMA and $1,25(OH)_2D_3$.

II. To determine the success of these differentiation protocols in THP-1 and U937 cells, at multiple levels, through the assessment of morphology, protein expression of the macrophage markers CD14 and CD11b (ITGAM) and assess their $1,25(OH)_2D_3$ responsiveness based on the messenger RNA (mRNA) expression of several cytokines and the primary $1,25(OH)_2D_3$ target gene, cathelicidin antimicrobial peptide gene (*CAMP*).

III. To characterise the macrophages generated from these differentiation protocols and determine the influence of $1,25(OH)_2D_3$ in the process of monocyte-to-macrophage differentiation through RNA-sequencing and differential gene expression analysis.

IV. To compare the influence of $1,25(OH)_2D_3$ in THP-1 monocytes and THP-1-derived macrophages using RNA-sequencing and differential gene expression analysis.

2.1 Introduction

In the study of monocyte biology, monocyte-to-macrophage differentiation, macrophage biology, and macrophage polarisation, it is commonplace to use monocyte-like cell lines (MLCLs) as alternatives to primary monocytes and macrophages. While primary cells naturally prove more accurate models for these studies, MLCLs derived from malignant myeloid cells act as simplified models with several practical advantages (Riddy *et al.,* 2018). MLCLs are readily available, proliferative, comparatively easy to culture, and can be stored for years in liquid nitrogen (Forrester *et al.,* 2018). The acquisition of primary monocytes or tissue macrophages, on the other hand, require either blood donations or invasive surgeries such as biopsies or bronchoscopies, respectively (Valdés-López and Urcuqui-Inchima, 2018; Duweb *et al.,* 2022). As monocytes only make up 5-10% of leukocytes, their yield from blood donations is not high and there is a risk of cross contamination with other cell types (Qin, 2012; Forrester *et al.,* 2018). Additionally, primary cells have a finite lifespan, do not proliferate readily, and do not permit prolonged storage (Lund *et al.,* 2016; Baxter *et al.,* 2020). Unlike primary cells, MLCLs offer a system with a genetically homogenous background, removing a large degree of the variability observed with primary cells (Qin, 2012; Maeß *et al.,* 2014; Noronha *et al.,* 2020; Pinto *et al.,* 2021; Duweb *et al.,* 2022). Among the most commonly employed MLCLs in the study of monocyte and macrophage biology, monocyte-to-macrophage differentiation, and macrophage biology are THP-1 and U937 cells (Riddy *et al.,* 2018; Nascimento *et al.,* 2022).

Numerous alterations are induced in the process of monocyte-to-macrophage differentiation, including changes in the expression of chemokines and cytokines, pathogen recognition receptors and co-receptors, proteins involved in phagocytosis, autophagy, migration, adhesion, cell survival, and cellular morphology (Zeng *et al.,* 2015; Boulakirba *et al.,* 2018). As such, the assessment of monocyte-to-macrophage differentiation is often associated with these features. The most frequently employed methods to assess differentiation are microscopy, to characterise morphological alterations, and mRNA or protein quantification of macrophage associated markers (Daigneault *et al.,* 2010; Lund *et al.,* 2016; Riddy *et al.,* 2018; Pinto *et al.,* 2021). The increased expression of CD11b and CD14 represent two of the most common markers used for the assessment of successful macrophage differentiation (Ziegler-Heitbrock and Ulevitch, 1993; Tedesco *et al.,* 2018). ITGAM/CD11b, along with its beta-chain binding partner CD18, have several immunological functions including migration, adhesion, phagocytosis, and the production of anti-inflammatory cytokines, such as, IL-10 and TGF-β (Khan *et al.,* 2018). The GPI-anchored receptor, CD14, is a co-receptor for several TLRs and facilitates TLR binding of pathogen associated molecular patterns (Zanoni and Granucci,

2013; Ciesielska *et al.,* 2021). Additionally, as the initiation of monocyte-to-macrophage differentiation is often associated with an immune response, mRNA or protein quantification of various cytokines and chemokines is standard practice when assessing successful monocyte-to-macrophage differentiation. For example, activation of NF-κB and AP-1, associated with the induction of differentiation, is responsible for the production of inflammatory cytokines and chemokines including TNF-α, IL-8, IL-10, CXCL10 and CCL2 (Li *et al.,* 2010; Jin *et al.,* 2014; T. Liu *et al.,* 2017; Gschwandtner *et al.,* 2019; Viola *et al.,* 2019).

Despite similarities in the assessment of monocyte-to-macrophage differentiation, the protocols employed for the induction of differentiation are highly variable. Induction of monocyte-to-macrophage differentiation in THP-1 and U937 cells is most frequently achieved using diacylglycerol mimic PMA, alternatively several differentiation protocols employ biologically active vitamin D_3, $1,25(OH)_2D_3$ (Chanput *et al.,* 2014; Hu and Zuckerman, 2014). Despite the frequent employment of PMA for differentiation, there is little consistency in its application. These protocols employ highly variable PMA concentrations (1 – 400 nM), stimulation periods (6 – 144 hours), and rest periods (0 – 168 hours; Table 1.1). Furthermore, when used at high concentrations, PMA has been shown to reduce cell viability and interfere with downstream applications of these cells (Park *et al.,* 2007; Maeß *et al.,* 2014; Lund *et al.,* 2016; Starr *et al.,* 2018). Alternatively, protocols using $1,25(OH)_2D_3$ are more standardised and use of 100 nM $1,25(OH)_2D_3$ for a minimum of 72 hours is most common (Daigneault *et al.,* 2010; Valdés-López and Urcuqui-Inchima, 2018). However, this does not represent a physiologically relevant $1,25(OH)_2D_3$ concentration (Valdés-López and Urcuqui-Inchima, 2018; Rao Muvva *et al.,* 2020). In *in vitro* studies of the effect of $1,25(OH)_2D_3$ in MLCLs and primary monocytes and macrophages it is commonplace to use 10 nM 1,25(OH)2D3, which is achievable *in vivo* and considered more physiologically relevant (Ryynänen and Carlberg, 2013; Zhang *et al.,* 2013; Rafique *et al.,* 2019). Thus, 10 nM $1,25(OH)_2D_3$ may represent a more appropriate alternative for monocyte-to-macrophage differentiation protocols. Nevertheless, despite its use and irrespective of the concentration employed, there is some debate as to whether $1,25(OH)_2D_3$ is capable of inducing complete macrophage differentiation in THP-1 and U937 cells (Schwende *et al.,* 1996; Daigneault *et al.,* 2010; Valdés-López and Urcuqui-Inchima, 2018; Rynikova *et al.,* 2023). What is not questioned though, is that the presence of $1,25(OH)_2D_3$ exerts distinct effects on both monocytes and macrophages that alter the morphology, functionality and immune landscape of these cells (Valdés-López and Urcuqui-Inchima, 2018; Nurminen *et al.,* 2019; Fernandez *et al.,* 2022). The lack of consistency in the generation of these THP-1- or U937-derived macrophages has led to discrepancies between studies and a lack of comparability in the results generated.

The development of an optimised and standardised protocol for the differentiation of MLCLs would serve to increase the reproducibility and accuracy of future studies and the conclusions drawn from them. As such, the aim of this study was to develop an optimised differentiation protocol for THP-1 and U937 cells that would generate a mature macrophage using conditions that more closely reflect physiological conditions that may limit interference with downstream applications. To assess the success of the proposed protocols, phenotypic alterations associated with monocyte-to-macrophage differentiation such as, alterations in morphology and the expression of macrophage associated cell surface receptors were assessed. Additionally, to determine if the proposed protocols influenced the inflammatory state of THP-1-derived and U937-derived macrophages, the mRNA expression of several cytokines was also assessed. The results obtained were used to determine differentiation conditions most likely to produce a phenotypically mature macrophage that was not in a heightened inflammatory state and to select a cell line appropriate for the study of the impact of $1,25(OH)_2D_3$ in the context of monocyte-to-macrophage differentiation.

2.2 Materials and Methods

2.2.1 Cell culture and differentiation

2.2.1.1 Cell lines, maintenance, and experimental culturing

To study monocyte-to-macrophage differentiation cell lines THP-1 and U937 were selected. The THP-1 cell line (HIV Reagent program ARP-9942) was a gift from Dr H. Ranchod (NICD, South Africa) and the U937 cell line (ATCC CRL-1593.2) was acquired from Cellonex (South Africa). The THP-1 and U937 cell lines were maintained in tissue culture media comprised of Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 2 mM L-glutamine (Pan Biotech, Aidenbach, Germany), 100 U/ml penicillin and 0.1 mg/ml streptomycin (Sigma Aldrich, St. Louis, MO, USA) supplemented with either 10% or 5% foetal bovine serum (FBS; Pan Biotech, Aidenbach, Germany) for THP-1 and U937 cells, respectively. THP-1 cells were maintained between 0.4×10^6 – 0.8×10^6 cells/ml, whereas U937 cells were maintained between 0.2×10^6 – 1×10^6 cells/ml. All cell counts were conducted using a Countess II FL automated cell counter (Thermo Fisher Scientific, Waltham, MA, USA) and facilitated by Trypan blue exclusion dye (Sigma Aldrich, St. Louis, MO, USA). Cells were incubated, during maintenance and experimental procedures in a humidified (95%) incubator at 37 °C with 5% CO_2. For experimental purposes, cells were cultured in tissue culture media supplemented with 10% FBS and used between passage 5 and 10 from revival, with a maximum cumulative passage number of 26 for THP-1 cells and 28 for U937 cells. Cells were seeded in 60 mm culture plates with a 22.1 cm^2 growth area (TPP, Trasadingen, Switzerland) at a density of 0.5×10^6 cells/ml and 0.2×10^6 cells/ml for THP-1 and U937 cells, respectively. U937 cells were

seeded at a lower density due to their rapid doubling time. To optimise a differentiation protocol that generated a mature macrophage phenotype and select an appropriate cell line for future studies a range of differentiation protocols were applied over the course of 96 hours. THP-1 and U937 cells were differentiated using either $1,25(OH)_2D_3$ (Sigma Aldrich, St. Louis, MO, USA), PMA (Sigma Aldrich, St. Louis, MO, USA) or a combination thereof (Fig. 2.1).

2.2.1.2 Differentiation conditions employing exclusively $1,25(OH)_2D_3$

THP-1 and U937 cells were exposed to 10 nM and 100 nM $1,25(OH)_2D_3$ in order to induce differentiation. 100 nM $1,25(OH)_2D_3$ is widely reported to differentiate THP-1 and U937 cells whereas 10 nM $1,25(OH)_2D_3$ was selected as it better reflects a physiological concentration of $1,25(OH)_2D_3$ (Daigneault *et al.,* 2010; Ryynänen and Carlberg, 2013; Valdés-López and Urcuqui-Inchima, 2018; Rafique *et al.,* 2019). The $1,25(OH)_2D_3$ was reconstituted in 100% molecular grade ethanol (EtOH; Sigma Aldrich, St. Louis, MO, USA) to a final concentration of either 5 µM or 50 µM $1,25(OH)_2D_3$. THP-1 and U937 cells were exposed to 10 nM or 100 nM $1,25(OH)_2D_3$ for 48 hours, after which non-adherent cells and spent media were poured off. These cells were then re-supplemented with fresh media and 10 nM or 100 nM $1,25(OH)_2D_3$ and allowed to differentiate for a further 48 hours. For U937 cells, treatment with 10 nM $1,25(OH)_2D_3$ resulted in minimal adherence. As such, these cells were not poured off, rather, they were supplemented with additional media and a further 10 nM $1,25(OH)_2D_3$.

2.2.1.3 Differentiation conditions employing PMA

THP-1 and U937 cells were exposed to 5 nM PMA and a combination of 5 nM PMA and 10 nM $1,25(OH)_2D_3$ to induce differentiation. The use of 5 nM PMA was selected as a low concentration alternative to the more commonly applied PMA concentrations applied for differentiation. The combination of 5 nM PMA with 10 nM $1,25(OH)_2D_3$ was applied as it has been shown that the addition of $1,25(OH)_2D_3$ enhances macrophage differentiation (Valdés-López and Urcuqui-Inchima, 2018). PMA was reconstituted in dimethyl sulfoxide (DMSO) Hybri-Max (Sigma Aldrich, St. Louis, MO, USA) to a final stock concentration of 1 mM. The 1 mM PMA stock was further diluted in RPMI media to a working concentration of 5 µM. For PMA-based differentiation, the cells were exposed to 5 nM PMA or 5 nM PMA with 10 nM $1,25(OH)_2D_3$ for 24 hours. PMA containing media was then removed and the cells were washed with PBS to remove residual PMA and non-adherent cells. The adherent cells were then provided with fresh media and, where relevant, 10 nM $1,25(OH)_2D_3$ and allowed to rest for 72 hours. Higher concentrations of PMA (100 nM) were initially tested but reduced cell viability and were not used. The vehicle control was initially treated with 2 µl/ml (0.04%) EtOH and 0.02 µl/ml (0.0004%) DMSO. The media was re-supplemented after 48 hours and a further 2 µl/ml EtOH was added, and the cells incubated for a further 48 hours.

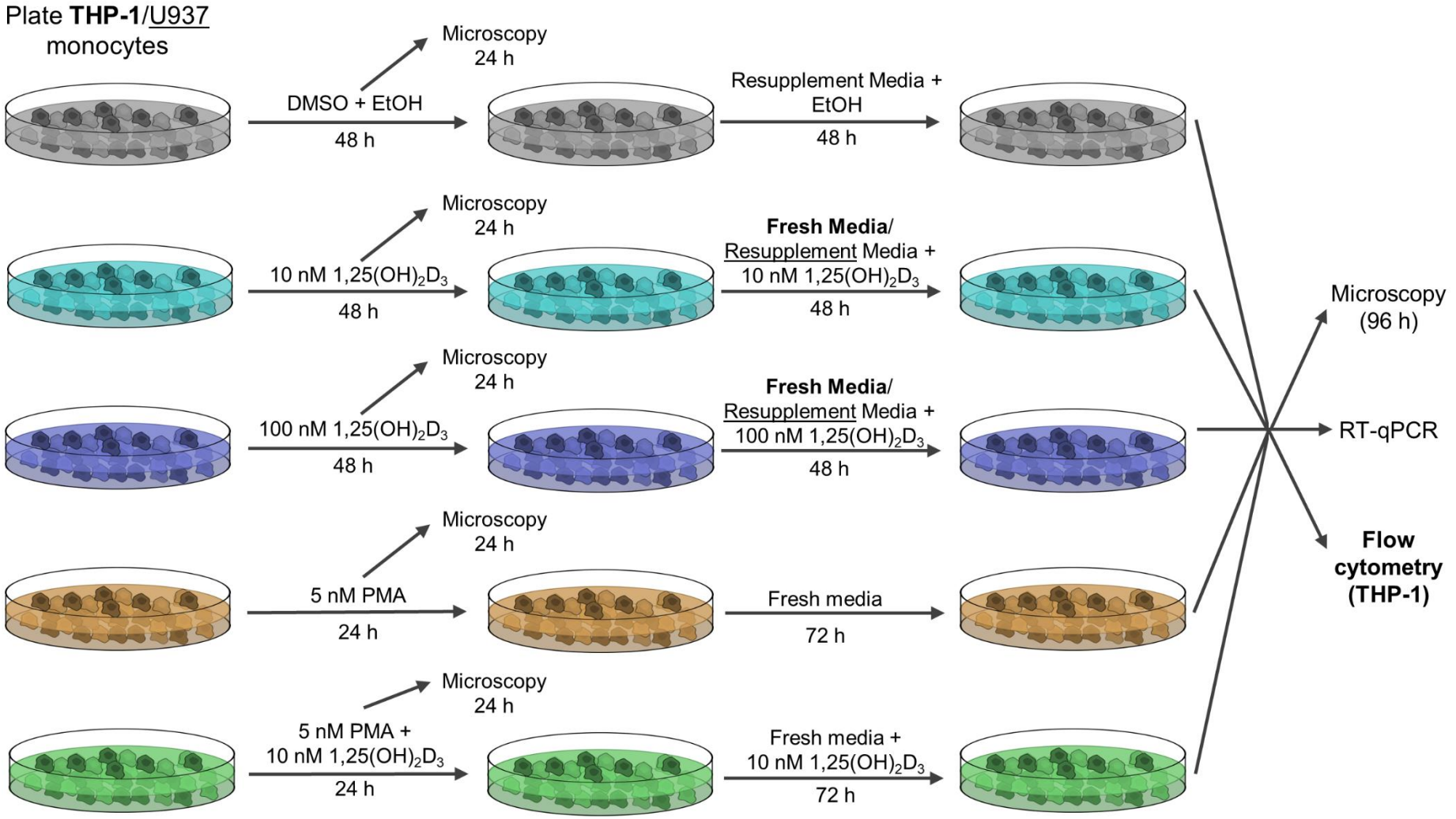


Figure 2.1 Schematic illustrating the differentiation protocols employed over 96 hours for THP-1 and U937 cells and downstream applications. Items in bold pertain exclusively to the differentiation of THP-1 cells. Items underlined pertain exclusively to the differentiation of U937 cells. Abbreviations: DMSO – dimethyl sulfoxide; EtOH – molecular grade ethanol; $1,25(OH)_2D_3$ – 1α, 25-dihydroxyvitamin D_3; PMA – phorbol 12-myristate 13-acetate.

2.2.1.4 Cell harvesting following differentiation

Following 96 hours of differentiation, the cells were detached from the plate using either a cell scraper or 0.25% Trypsin-EDTA (Sigma Aldrich, St. Louis, MO, USA) for 2-3 minutes for treatments with 10 nM and 100 nM $1,25(OH)_2D_3$ or 5 nM PMA and 5 nM PMA with 10 nM $1,25(OH)_2D_3$, respectively. Cells were washed with phosphate buffered saline (PBS; pH 7.4 Sigma Aldrich, St. Louis, MO, USA), counted and assessed for viability using the Trypan-blue exclusion assay. 1×10^6 Cells were stored in 100 µl RNA*later* (Thermo Fisher Scientific, Waltham, MA, USA) at -80 °C for RNA extraction, while 2×10^6 cells were used immediately for the purpose of flow cytometry.

2.2.2 Transmitted light microscopy

Morphological alterations associated with the process of differentiation such as an increase in cell size and adherence were captured by the EVOS FLoid imaging system (Thermo Fisher Scientific, Waltham, MA, USA) with a fixed 20× objective lens (460× magnification) at 24 and 96 hours. Cell sizes were calculated using ImageJ (Schneider *et al.,* 2012), three micrographs for each condition and timepoint were used. From each micrograph the area of a minimum of 25 cells was taken to determine the average area of these cells.

2.2.3 RNA extraction

RNA was extracted from the differentiated cells using a Direct-zol RNA Miniprep kit (Zymo Research, Irvine, CA, USA). Prior to extraction, cells were pelleted at 500 × *g* and the RNA*later* was aspirated. RNA extraction was performed on ice and all centrifugation steps were carried out at 10 000 × *g* for 30 seconds at 4 °C. Each cell pellet was lysed in 300 µl of TRIzol, followed by the addition of 300 µl of 100% (v/v) molecular grade EtOH and mixed thoroughly. The solution was then transferred onto the Zymo-Spin IICR Column and centrifuged and the flow through discarded. Contaminating genomic DNA was removed using a DNase I treatment. First, 400 µl of RNA wash buffer was added to the column and centrifuged, 5 µl of DNase I (6 U/ µl) was combined with 75 µl DNA digestion buffer and added directly to the column matrix and incubated at 25 °C for 15 minutes. Following DNase I treatment, 400 µl of RNA Prewash was added to the column and centrifuged and the flow through discarded, twice. For the final wash step 700 µl of RNA Wash buffer was added to the column and centrifuged for 2 minutes. The column was transferred to a nuclease free microcentrifuge tube and 30 µl of nuclease free water was used to elute the RNA. RNA purity was confirmed if the A260/A280 ratio was ~2.00 and the A230/A260 ratio was between 2.00 and 2.20 when assessed using a Nanodrop One Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

2.2.4 Agarose gel electrophoresis

For each sample extraction RNA integrity was assessed by horizontal gel electrophoresis using a 1% (w/v) agarose gel with tris-borate-EDTA (TBE) buffer. The TBE buffer was prepared using 89 mM Trizma® base, 89 mM of boric acid, and 2 mM of ethylenediaminetetraacetic acid (EDTA) at a pH of 7.94 (Sigma Aldrich, St. Louis, MO, USA). Ethidium bromide (0.6 µg/ml; Sigma Aldrich, St. Louis, MO, USA) was added prior to gel casting to facilitate RNA visualisation following electrophoresis (100V for 30 min). For each sample approximately 500 ng of total RNA was loaded on to the gel. The gels were viewed using a Gel Doc XR+ (Bio-Rad, Hercules, CA, USA). RNA was considered intact if two clear bands representing 28S and 18S rRNA were observed.

2.2.5 cDNA synthesis

A total of 1 µg pure and intact RNA was converted to complementary DNA (cDNA) using a RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA) for each condition. The cDNA reaction was prepared according to the manufacture's guidelines using Oligo $(dT)_{18}$ primers (Table 2.1). The reaction was then incubated for 60 minutes at 42 °C for cDNA synthesis. The reaction was stopped by a 5-minute incubation at 70 °C. The cDNA products were used immediately for quantitative PCR (qPCR) or stored at -80 °C.

Table 2.1: cDNA synthesis reaction components

Component	Volume (µl)	Final conc.
Template RNA (total RNA)	Volume required for 1000 ng	50 ng/ µl
Oligo $(dT)_{18}$ primer	1	-
Nuclease free water	Up to 12	-
5× Reaction buffer	4	1×
RiboLock RNase inhibitor	1	20 U (1 U/ µl)
10 mM dNTP Mix	2	-
RevertAid RT	1	200 U (10 U/ µl)
Final volume	20	-

* Each component was added in the order given

2.2.6 Quantitative PCR

As indicators of $1,25(OH)_2D_3$ activity, differentiation, and inflammation the expression of target genes *VDR*, *CAMP*, *NFKB2*, *CXCL10*, *TNF* (which encodes TNF-α), *CCL2* and *IL10* were measured by qPCR (Table 2.2). Reference genes ubiquitin C (*UBC*) and tyrosine 3-monooxygenase/ tryptophan 5-monooxygenase activation protein zeta (*YWHAZ*) were selected as they are stably expressed in mononuclear cells and macrophages (Vandesompele *et al.,* 2002; Kalagara *et al.,* 2016; Oturai *et al.,* 2016). For each condition evaluated, three biological replicates and two technical replicates were included. Lyophilised primers were

reconstituted in Tris-EDTA buffer (IDT, Coralville, IA, USA) to 100 µM stocks, and further diluted to 10 µM stocks with nuclease free water and stored at -20 °C. Primers were optimised to between 95% and 105% efficiency. Primer specificity was confirmed by horizontal gel electrophoresis using a 3% (w/v) agarose gel with PCR products and visualised as described in section 2.2.4. The qPCR reactions were performed using a Luna Universal qPCR Master Mix (NEB, Ipswich, MA, USA), the gene-specific primers and 25 ng of cDNA per reaction (Table 2.3). Amplification, quantification and melt curve analysis (Table 2.4) was performed using a CFX96 Touch Real Time Detection System (Bio-Rad, Hercules, CA, USA). After qPCR completion, the relative fluorescence units (RFU) baseline threshold was set to 75 for all runs and the cycle threshold (C_t) values were exported from the Bio-Rad CFX Maestro 1.0 software (v. 4.0.2325.0418, Bio-Rad, Hercules, CA, USA) to Microsoft excel (Microsoft Office 11) for normalisation and to calculate relative mRNA expression using the Livak ($2^{-\Delta\Delta Ct}$) method (Livak and Schmittgen, 2001). The ΔC_t values were used to determine statistical significance, the plots show the log_2 fold change in mRNA expression for each gene.

Table 2.2: Primer sequences

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
VDR	Vitamin D receptor	FP: CTGACCCTGGAGACTTTGAC RP: TTCCTCTGCACTTCCTCATC	Vienonen *et al.,* 2003
CAMP	Cathelicidin antimicrobial peptide	FP: GCAGTCACCAGAGGATTGTGAC RP: CACCGCTTCACCAGCCC	Ong *et al.,* 2002
NFKB2	Nuclear factor-kappa B subunit 2	FP:CGGGACAAGAGAAAAGAGGGAGG RP: CTTCCCAGAATTTTAGACGCCCG	
CXCL10	C-X-C motif chemokine ligand 10	FP: TCTAAGTGGCATTCAAGGAGT RP: TCAGACATCTCTTCTCACCC	
TNF	Tumour necrosis factor α	FP: GACCTCTCTCTAATCAGCCC RP: GCTTGAGGGTTTGCTACAAC	
CCL2	C-C motif chemokine ligand 2	FP: CCCAAAGAAGCTGTGATCTTCA RP: TGTGGAGTGAGTGTTCAAGT	
IL10	Interleukin 10	FP: GCCTAACATGCTTCGAGAT RP: GCAACCCAGGTAACCCTTA	

*In the absence of a reference the primers were designed using Primer3 (Untergasser, *et al.,* 2012)

Table 2.3: qPCR reaction components

Component	Gene	Volume (µl)	Final conc.
Luna Universal qPCR Master Mix (2×)		5	1×
Forward Primer	*UBC, YWHAZ, CAMP, NFKB2, CXCL10, TNF, CCL2, IL10*	0.25	250 nM
	VDR	0.5	500 nM
Reverse Primer	*UBC, YWHAZ, CAMP, NFKB2, CXCL10, TNF, CCL2, IL10*	0.25	250 nM
	VDR	0.5	500 nM
Template DNA		0.5	25 ng
Nuclease free water		Up to 10	

Table 2.4: Thermal cycling conditions for amplification of reference and target genes

Step	Cycles	Temperature (°C)	Time (seconds)
Initial denaturation	1	95	60
Denaturation	40	95	15
Annealing and extension (+ plate read)		58	30
Melt Curve	1	60-95 °C in 0.5 °C increments	5 sec / 0.5 °C

2.2.7 Macrophage surface marker quantification

Flow cytometry was employed as a confirmation of differentiation based on the expression of the classical protein markers of differentiation, CD14 and CD11b, cell size analysis through forward scatter (FSC) and cell granularity analysis through side scatter (SSC). For each condition evaluated, three biological replicates and two technical replicates of 1×10^6 cells were included as well as two auto-fluorescent controls per flow cytometry run. The tissue culture media was removed, and the cells were washed with PBS. After the supernatant was discarded the cells were incubated with 50 µl of 10% (v/v) heat-inactivated human serum for 1 minute. The human serum was removed, and the cells were fixed by resuspending the cell pellet in 50 µl of 1.5% (w/v) paraformaldehyde (Sigma Aldrich, St. Louis, MO, USA) in PBS for 30 minutes in the dark. Once fixed the paraformaldehyde solution was removed and the cells pelleted. With exclusion of the auto-fluorescent controls, the cell pellets were stained with 5 µl of FITC conjugated mouse IgG_1 anti-human CD11b clone ICRF44 (0.5 µg/ test) and APC conjugated mouse IgG_1 anti-human CD14 clone 61D3 (0.25 µg/ test) monoclonal antibodies (Invitrogen, Waltham, MA, USA) suspended in 40 µl of 1% (w/v) bovine serum albumin (BSA; Roche, Basel, Switzerland) in PBS and incubated for 30 minutes in the dark. The antibody solution was removed, and the cell pellets were washed twice in 0.1% (v/v) Triton X-100

(Sigma Aldrich, St. Louis, MO, USA) in PBS by centrifugation at 800 × *g* for 8 minutes. Washed cells were resuspended in a final volume of 150 µl 1% (w/v) BSA in PBS. Fluorescence was measured within an hour of sample preparation on a BD Accuri C6 (BD Accuri, Ann Arbor, MI, USA) flow cytometer fitted with a solid-state blue laser (excitation - 488 nm) and a diode red laser (excitation – 640 nm). FITC fluorescent conjugates are excited at 488 nm and emission at 520 nm, whereas APC fluorescent conjugates are excited at 633-647 nm and emission at 660 nm; this allowed for double staining without fluorescent signal crossover and thus no need for compensation. For each sample a total of 75 000 events were captured.

2.2.8 Statistical analysis

Statistical analysis was performed using IBM SPSS statistics (v. 28.0.1.0(142)). Outliers within technical replicates were identified and removed from all datasets. To assess whether the differentiation condition applied significantly altered mRNA expression levels of target genes a one-way analysis of variance (ANOVA) was conducted on the transformed normalised C_t values (ΔC_t). The ΔC_t values were transformed by log_2 (ΔC_t (x)+1), this was done to improve the normality of the distribution and the homogeneity of variance, established using Levene's test, so as to meet the assumptions of the one-way ANOVA. Assuming the one-way ANOVA showed significant difference overall, post-hoc tests in the form of a Fisher's least significant difference (LSD) test and Bonferroni correction for multiple testing were applied. In the case of *VDR* and *IL10* expression in U937, data transformation could not produce a homoscedastic distribution. To determine whether the cell line chosen influenced the mRNA expression of the gene targets a two-way ANOVA was conducted, with both a Fisher's LSD test and Bonferroni correction to assess statistical significance. To assess significant alterations in the expression of CD11b and CD14 as well as changes in FSC and SSC as determined by flow cytometry, a one-way ANOVA was used to determine significant difference overall followed by a Fisher's LSD post-hoc test and Bonferroni correction. The *p* values given in the text or displayed on graphs are those derived from the Fisher's LSD test, those that appear within the text are given with respect to the control unless otherwise specified.

2.3 Results

2.3.1 Morphological characteristics of THP-1 and U937 cells following differentiation

2.3.1.1 1,25(OH)$_2$D$_3$ failed to induce morphological alterations associated with differentiation

Monocyte-to-macrophage differentiation is associated with key morphological alterations, particularly an increase in the cytoplasm to nucleus ratio, which is reflected by an increase in cell size, irregular cell shape which may include cytoplasmic extensions, and cellular adherence which is accompanied by cell flattening (Daigneault *et al.,* 2010; Lund *et al.,* 2016; Rostam *et al.,* 2017; Kuno *et al.,* 2020). In both THP-1 (Fig. 2.2 A) and U937 (Fig. 2.3 A) cells, cells exposed to the vehicle control showed no increase in cell size, no alteration in cell shape, and they did not demonstrate cellular adherence over 96 hours. In THP-1 cells exposure to 10 nM and 100 nM 1,25(OH)$_2$D$_3$ did not increase cell size, however, occasional cytoplasmic extensions were observed and both concentrations resulted in some light adherence in a fair portion of cells (~30-40%; Fig. 2.2 B, C). In U937 cells, neither differentiation with 10 nM 1,25(OH)$_2$D$_3$ nor 100 nM 1,25(OH)$_2$D$_3$ produced an increase in cell size or an alteration in cell shape. Although cellular adherence was induced by 1,25(OH)$_2$D$_3$, this was only seen sparsely (~10%) at 100 nM 1,25(OH)$_2$D$_3$ even after 96 hours (Fig. 2.3 B, C). In these conditions even after 96 hours of exposure, neither the U937 nor THP-1 cells demonstrated mature macrophage morphologies, this was only achieved when the differentiation protocol included PMA.

2.3.1.2. PMA was required to induce morphological alterations associated with differentiation

Exposure of both THP-1 and U937 cells to 5 nM PMA or 5 nM PMA with 10 nM 1,25(OH)$_2$D$_3$ showed an increase in cell size, generation of an irregular cell shape and firm cellular adherence (~80-90%) with cell flattening, albeit to different extents (Fig. 2.2 D, E; Fig. 2.3 D, E). The generation of these prototypical macrophage characteristics was observed within 24 hours of differentiation but was enhanced with the removal of PMA and an additional 72 hours in culture. This was demonstrated in both the U937 and THP-1 cells by the further increase in cell size and cell flattening (Fig. 2.2 D, E; Fig. 2.3 D, E). To wit, in THP-1 cells differentiated with 5 nM PMA the average cell area was 1.67× larger after 24 hours and 1.81× larger after 96 hours when compared to the control (Table 2.5). As mentioned, THP-1 cells differentiated in the presence of 5 nM PMA and 10 nM 1,25(OH)$_2$D$_3$ showed a greater increase in cell size and saw a 2.15× and 2.94× increase in cell area after 24 and 96 hours respectively, when compared to the control. In U937 cells the difference between the 5 nM PMA condition and the 5 nM with 10 nM 1,25(OH)$_2$D$_3$ condition was not particularly noticeable after 24 hours with the increase in cell area being 1.48× and 1.44× respectively. Following the 72-hour rest period, the increase in cell area was considerably larger in both differentiation conditions when

compared to the control and each other with the 5 nM PMA condition showing a 4.41× increase in cell area and the 5 nM PMA with 10 nM 1,25$(OH)_2D_3$ showing a 5.43× increase in cell area. While both of these differentiation conditions appear to produce macrophages, the morphologies exhibited differ quite widely.

2.3.1.3 Differentiation with PMA in the presence or absence of 1,25$(OH)_2D_3$ produced distinct macrophage morphologies

The induction of macrophage differentiation using 5 nM PMA and 5 nM PMA with 10 nM 1,25$(OH)_2D_3$ generated distinct macrophage morphologies, both between cell lines and between differentiation conditions. After 24 hours of exposure to PMA alone, differentiated THP-1 cells produced several morphologies. While some monocyte-like cells were still visible, most cells showed distinct characteristics of differentiation, although, they did not produce a homogenous macrophage-like cell pool. Primarily the cells were large, rounded and adherent, additionally flattened ameboid-like cells and elongated spindle-like cells were also visible, several cells also had distinct cytoplasmic extensions (Fig. 2.2 D). Following the 72-hour rest period there was still a large amount of diversity in the macrophage-like cell morphologies. In general, the cells were large, rounded cells with occasional ameboid and spindle-like cells (Fig. 2.2 D). The 5 nM PMA with 10 nM 1,25$(OH)_2D_3$ differentiation condition produced a larger degree of cell flattening than 5 nM PMA alone. After 24 hours of differentiation there were multiple macrophage morphologies produced, this was predominated by spindle-like and elongated ameboid cell morphologies (Fig. 2.2 E). After the 72-hour rest period the macrophage cells were larger and the macrophage pool was predominated by spindle-like and elongated ameboid cells, but a number of large, rounded macrophages were also observed (Fig. 2.2 E). In U937 cells the macrophage morphology generated by both 5 nM PMA and 5 nM PMA with 10 nM 1,25$(OH)_2D_3$ had a distinct morphology from their THP-1 counterparts (compare Fig. 2.2 D and E with Fig. 2.3 D and E). Upon exposure to 5 nM PMA for 24 hours the U937 cells developed into adherent rounded cells, which with a 72-hour rest period developed predominantly large, rounded cells, with occasional thin spindle-like or ameboid shaped cells (Fig. 2.3 D). Although the U937 cells increased in cell size and adherence with exposure to 5 nM PMA over the 96 hours the resultant macrophage pool was not morphologically homogenous and had a range in cell sizes. Similarly, U937 cells exposed to 5 nM PMA with 10 nM 1,25$(OH)_2D_3$ over 24 hours also produced adherent rounded cells with slight morphological alterations. As with the 5 nM PMA differentiation condition the 72-hour rest period resulted in larger, firmly adherent, rounded and ameboid shaped macrophage-like cells. Unlike the 5 nM PMA differentiation condition however, after 96 hours the resultant cell population produced was more homogenous in appearance and size (Fig. 2.3 E).

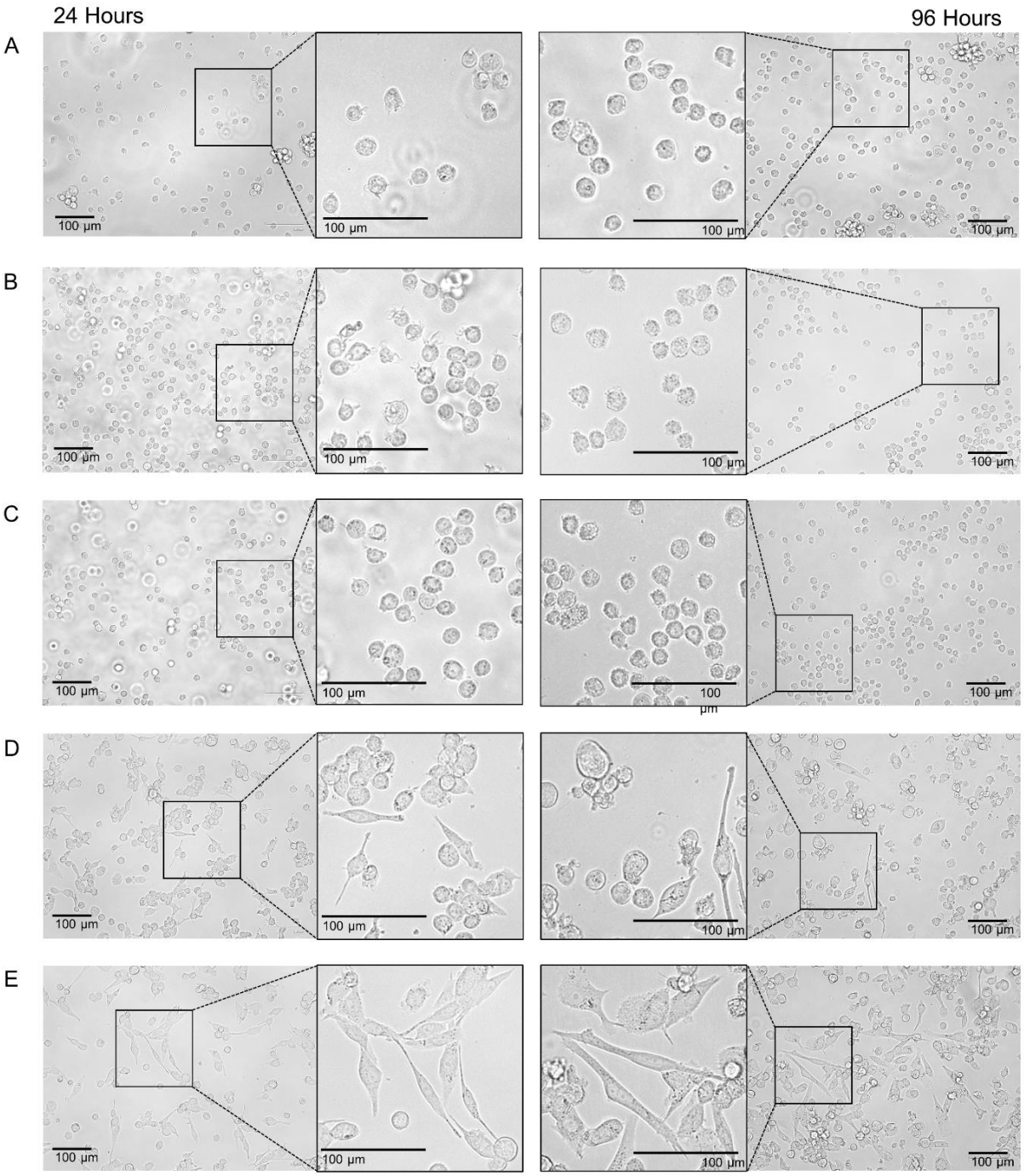


Figure 2.2: THP-1 cells required 5 nM PMA to induce the morphological characteristics of differentiation. The micrographs show THP-1 cells exposed to the vehicle control (A), 10 nM $1,25(OH)_2D_3$ (B), 100 nM $1,25(OH)_2D_3$ (C), 5 nM PMA (D) and 5 nM PMA with 10 nM $1,25(OH)_2D_3$ (E) at 24 and 96 hours. Differentiation conditions employing 5 nM PMA with 10 nM $1,25(OH)_2D_3$ produced an altered morphology compared to 5 nM PMA alone. Incorporating a 72-hour rest period for 5 nM PMA and 5 nM PMA with 10 nM $1,25(OH)_2D_3$ conditions resulted in an increase in cell size compared to 24 hours of differentiation.

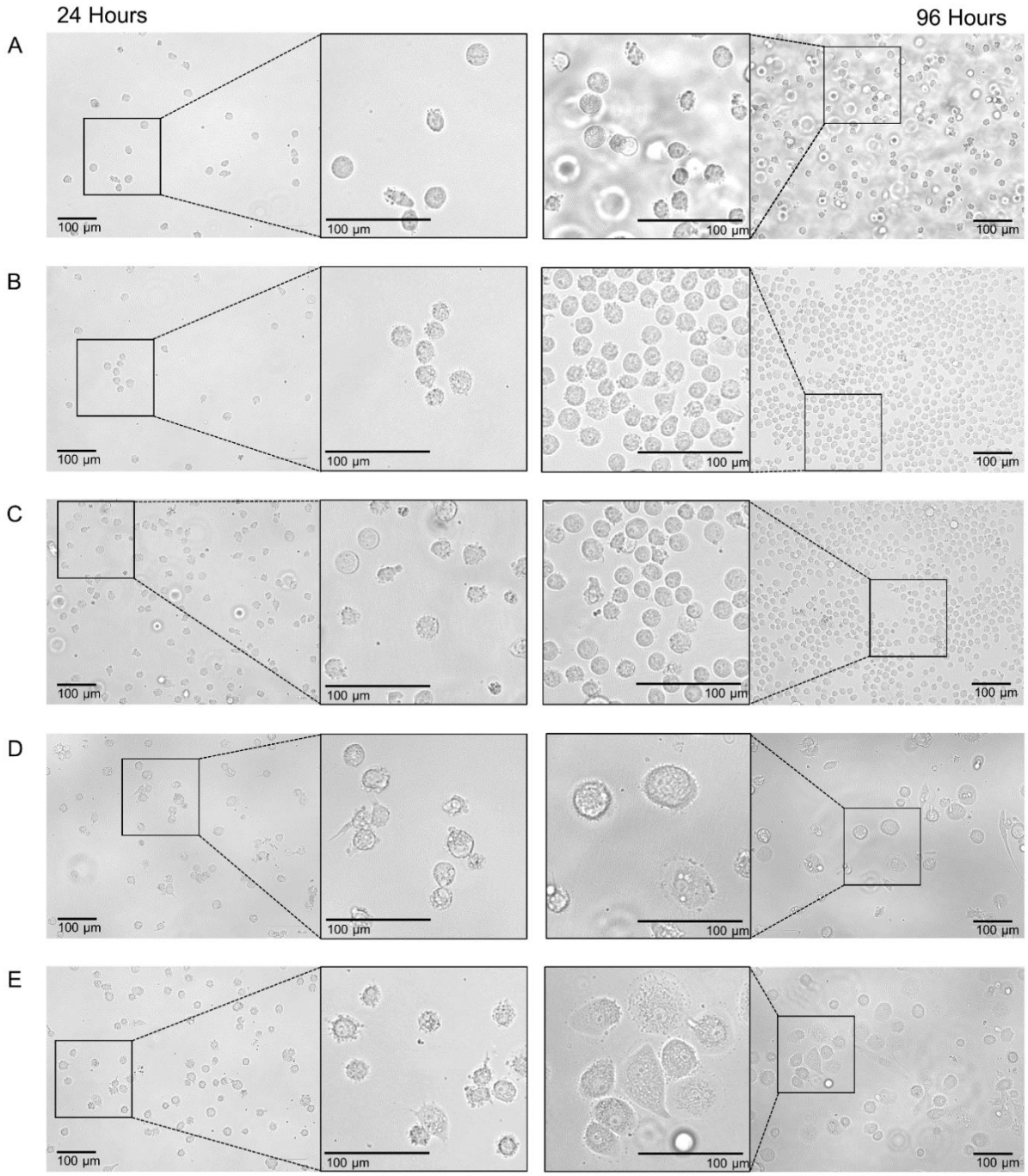


Figure 2.3: U937 cells required 5 nM PMA to induce the morphological characteristics of differentiation. The micrographs show U937 cells exposed to the vehicle control (A), 10 nM $1,25(OH)_2D_3$ (B), 100 nM $1,25(OH)_2D_3$ (C), 5 nM PMA (D) and 5 nM PMA with 10 nM $1,25(OH)_2D_3$ (E) at 24 and 96 hours. Differentiation conditions employing 5 nM PMA with 10 nM $1,25(OH)_2D_3$ produced a more homogenous macrophage pool compared to 5 nM PMA alone. Incorporating a 72-hour rest period for 5 nM PMA and 5 nM PMA with 10 nM $1,25(OH)_2D_3$ conditions resulted in an increase in cell size compared to 24 hours of differentiation.

Table 2.5: Average cell area (μm²) ± 1 SD in THP-1 and U937 cells following differentiation for 24 hours and 96 hours

Differentiation condition	THP-1		U937	
	24 hours (μm²)	96 hours (μm²)	24 hours (μm²)	96 hours (μm²)
Control	279.28 (± 53.46)	286.14 (± 62.93)	360.21 (± 68.60)	307.32 (± 52.83)
10 nM $1,25(OH)_2D_3$	278.06 (± 57.13)	282.43 (± 48.19)	351.61 (± 60.93)	308.40 (± 53.52)
100 nM $1,25(OH)_2D_3$	300.18 (± 52.84)	288.90 (± 50.46)	394.05 (± 65.87)	296.73 (± 56.62)
5 nM PMA	465.40 (± 227.91)	517.64 (± 379.90)	534.07 (± 132.18)	1352.94 (± 863.58)
5 nM PMA + 10 nM $1,25(OH)_2D_3$	600.67 (± 330.65)	842.10 (± 486.75)	519.79 (± 182.76)	1667.03 (± 880.03)

2.3.2 Assessment of the mRNA expression of $1,25(OH)_2D_3$ related genes and inflammatory markers

2.3.2.1 $1,25(OH)_2D_3$ signalling is active in both cell lines

To ensure the activity of $1,25(OH)_2D_3$ in both THP-1 and U937 cells, the mRNA expression of *VDR* and the primary $1,25(OH)_2D_3$ gene target *CAMP* was measured by RT-qPCR. The expression of *VDR* was not significantly altered in any condition in THP-1 cells (Fig. 2.4 A). Exposure to 5 nM PMA alone resulted in a significant decrease in *VDR* mRNA expression in U937 cells ($p<0.001$), which was not observed in any other differentiation condition (Fig. 2.4 B). Upon conducting a two-way ANOVA it was shown that there was a significant interaction between the cell line chosen and the differentiation condition applied ($p=0.018$), with simple main effects analysis indicating that the cell line chosen produced a significant difference ($p<0.001$) in *VDR* expression. As expected, in both THP-1 and U937 cells, exposure to $1,25(OH)_2D_3$ resulted in a significant induction in *CAMP* expression (Fig. 2.4 C and D). Exposure to either 10 nM or 100 nM $1,25(OH)_2D_3$ resulted in an approximately 400-fold increase in *CAMP* expression in both THP-1 cells ($p<0.001$) and U937 cells ($p<0.001$); this effect was not significantly impacted by $1,25(OH)_2D_3$ concentration. While this $1,25(OH)_2D_3$ induction of *CAMP* expression was observed in the 5 nM PMA with 10 nM $1,25(OH)_2D_3$ differentiation condition, it was to a lesser extent, with the increase in *CAMP* expression being 80-fold in THP-1 cells ($p<0.001$) and 130-fold in U937 cells ($p<0.001$). As expected, 5 nM PMA alone showed no significant change in *CAMP* expression in either cell line. Thus, it is evident that $1,25(OH)_2D_3$ signalling is functional in both cell lines.

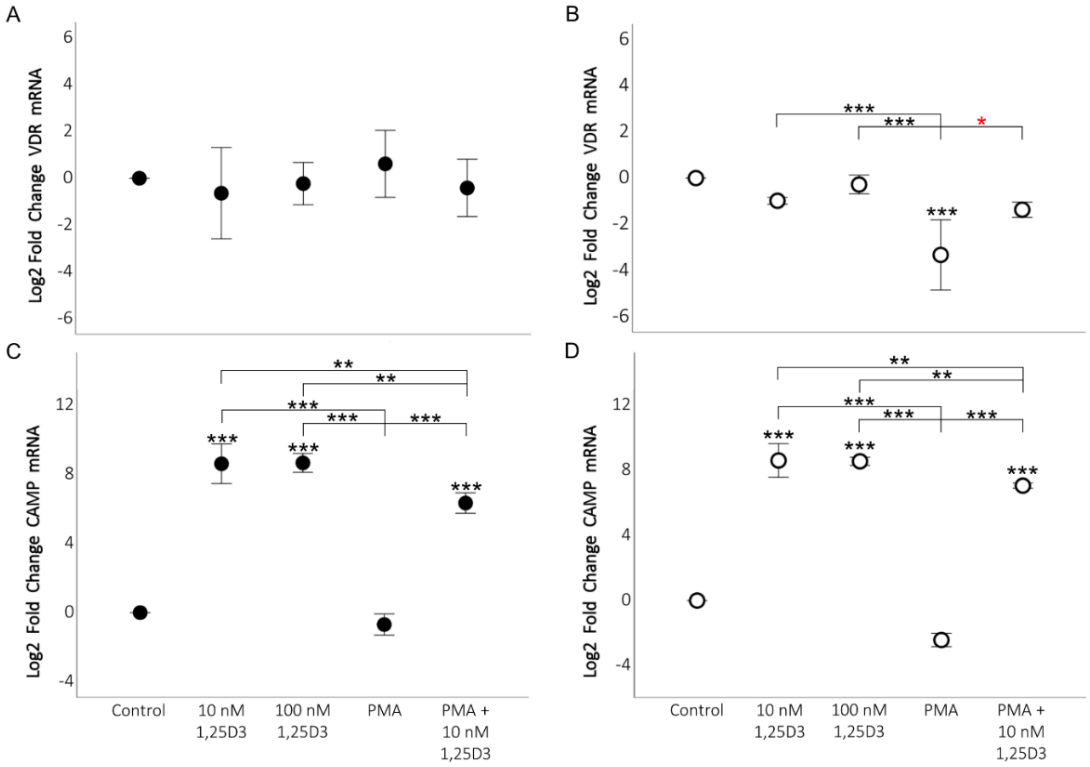


Figure 2.4: $1,25(OH)_2D_3$ signalling through VDR is active in both cell lines as indicated by increased expression of primary VDR gene target *CAMP*. Error bar plots show relative *VDR* (A and B) and *CAMP* (C and D) mRNA expression in THP-1 and U937 cells (n = 3) 96 hours post differentiation. Graphs with solid data points (●) indicate results pertaining to THP-1 cells, hollow data points (○) indicate results pertaining to U937 cells. Overall significance was determined using a one-way ANOVA. Pairwise comparisons were conducted using Fisher's LSD test and significant differentiation effects are given relative to the control (**p*<0.05, ***p*<0.01, ****p*<0.001). Black stars indicate that these differences remained significant after Bonferroni correction. Error bars represent ± 1 standard deviation.

2.3.2.2 PMA induced the mRNA expression of pro-inflammatory cytokine TNF *in U-937 cells*

Monocyte-to-macrophage differentiation and polarisation are accompanied by alterations in inflammatory markers. As such, the mRNA expression of *NFKB2, TNF, CXCL10, CCL2* and *IL10* was assessed after exposure to the various differentiation conditions in THP- 1 and U937 cells. No significant alteration in *NFKB2* expression was observed in either cell line for any condition (Fig. 2.5 A and B). In THP-1 cells, *TNF* expression was not significantly altered by any of the differentiation conditions (Fig. 2.5 C). In contrast, an 18-fold (p<0.001) increase in *TNF* expression was seen in the U937 cells for the 5 nM PMA differentiation condition (Fig. 2.5 D). However, the combination of 5 nM PMA with 10 nM $1,25(OH)_2D_3$ mitigated this effect by reducing the expression of *TNF* by 10-fold (p=0.003) when compared to 5 nM PMA alone. Neither the 10 nM nor the 100 nM $1,25(OH)_2D_3$ differentiation conditions altered *TNF* expression. Following a two-way ANOVA it was shown that there was a significant interaction between the cell line chosen and the differentiation condition applied (p<0.001). The simple main effects analysis indicated that the cell line chosen (p<0.001) and the differentiation condition employed (p<0.001) produced a significant difference in *TNF* expression.

2.3.2.3 The mRNA expression of pro-inflammatory chemokine CXCL10 *was altered by PMA and 1,25(OH)$_2$D$_3$*

The expression of pro-inflammatory chemokine *CXCL10* was significantly increased in the 5 nM PMA conditions in both cell lines (Fig. 2.5 E and F). Exposure to 5 nM PMA alone in THP-1 cells resulted in a 40-fold change (p<0.001) increase in *CXCL10* mRNA expression, in U937 cells this condition resulted in a 16-fold (p<0.001) induction of *CXCL10* expression. This 5 nM PMA induced increased expression of *CXCL10* was offset when 5 nM PMA was combined with 10 nM 1,25(OH)$_2$D$_3$. In THP-1 cells this *CXCL10* induction was completely mitigated, i.e the expression of *CXCL10* given this condition was no longer significantly different from that of the control. A similar effect was observed in the U937 cells in that 5 nM PMA combined with 10 nM 1,25(OH)$_2$D$_3$ showed a 10-fold reduction (p=0.006) in *CXCL10* expression when compared to the 5 nM PMA condition alone. In both cell lines neither the 10 nM nor the 100 nM 1,25(OH)$_2$D$_3$ differentiation conditions produced an alteration to *CXCL10* expression.

2.3.2.4 PMA and 1,25(OH)$_2$D$_3$ altered mRNA expression of pro-inflammatory chemokine CCL2

Monocyte-to-macrophage differentiation and macrophage polarisation is frequently associated with an increased expression of the inflammatory chemokine CCL2 and its' receptor CCR2. The expression of *CCL2* was only significantly induced in the 5 nM PMA conditions in both THP-1 and U937 cells, but to vastly different extents (Fig. 2.5 G and H). The induction of *CCL2* in THP-1 cells when exposed to 5 nM PMA though significant was more moderate than that in U937 cells, with a 10-fold (p=0.002) induction being observed. The combination of 5 nM PMA with 10 nM 1,25(OH)$_2$D$_3$ not only mitigated the 5 nM PMA increase in *CCL2* expression, but rather resulted in a significant 6-fold (p=0.032) reduction in *CCL2* expression when compared to the control. U937 cells showed a 780-fold (p<0.001) increase in *CCL2* expression when exposed to 5 nM PMA, this expression was significantly reduced by 355-fold (p=0.027) when combined with 10 nM 1,25(OH)$_2$D$_3$, however, this significance was not maintained with Bonferroni correction. Upon conducting a two-way ANOVA it was shown that there was a significant interaction between the cell line chosen and the differentiation condition applied (p<0.001), with simple main effects analysis indicating that the cell line chosen (p<0.001) and the differentiation condition employed (p<0.001) produced a significant difference in *CCL2* expression. As with the other inflammatory markers no significant alteration in *CCL2* expression occurred in the 10 nM and 100 nM 1,25(OH)$_2$D$_3$ differentiation conditions in either cell line.

2.3.2.5 PMA and 1,25(OH)$_2$D$_3$ induced the mRNA expression of anti-inflammatory cytokine IL10

As macrophages lend themselves to either pro-inflammatory or anti-inflammatory states depending on the signalling cues of the environment the gene expression of anti-inflammatory cytokine *IL10* was also assessed in both cell lines (Fig. 2.5 I and J). The expression of *IL10* appeared to be induced by exposure to PMA and to a lesser extent 1,25(OH)$_2$D$_3$. THP-1 cells showed a significant increase in *IL10* expression of 20-fold (p<0.001) when exposed to 5 nM PMA. When combining 5 nM PMA and 10 nM 1,25(OH)$_2$D$_3$ the expression of *IL10* was increased to 100-fold (p<0.001). In addition, the 10 nM and 100 nM 1,25(OH)$_2$D$_3$ differentiation conditions also resulted in an increase in *IL10* expression of 8-fold (p=0.004) and 13-fold (p<0.001), respectively. In U937 cells the expression of *IL10* was greatly and significantly induced by differentiation conditions containing 5 nM PMA irrespective of the presence of 1,25(OH)$_2$D$_3$, with a 2000-fold (p<0.001) increase observed for both the 5 nM PMA and the 5 nM PMA with 10 nM 1,25(OH)$_2$D$_3$ differentiation conditions. It must be noted that both 10 nM and 100 nM 1,25(OH)$_2$D$_3$ conditions alone also significantly, though not maintained by Bonferroni correction, induce a moderate increase *IL10* expression of 6-fold (p=0.021; p=0.015). Upon conducting a two-way ANOVA it was shown that there was a significant interaction between the cell line chosen and the differentiation condition applied (p<0.001), with simple main effects analysis indicating that the cell line chosen (p<0.001) and the differentiation condition employed (p<0.001) produced a significant difference in *IL10* expression.

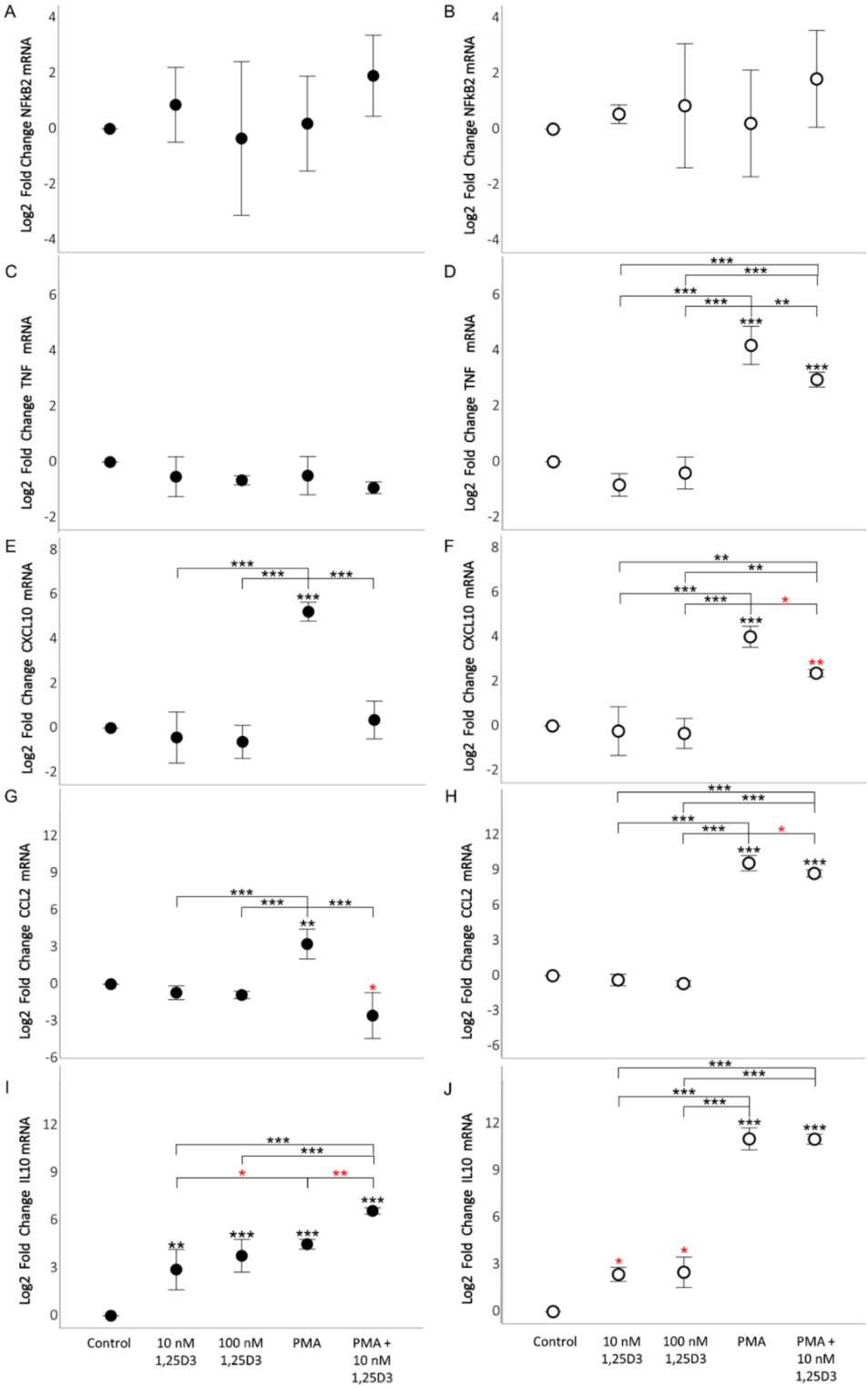


Figure 2.5: The effect of various differentiation conditions on the mRNA expression of inflammatory genes *NFKB2*, *TNF*, *CXCL10*, *CCL2* and *IL10* after 96 hours. The error bar plots show relative *NFKB2* (A and B), *TNF* (C and D), *CXCL10* (E and F), *CCL2* (G and H) and *IL10* (I and J) mRNA expression in THP-1 and U937 cells (n = 3). Graphs with solid data points (●) indicate results pertaining to THP-1 cells, hollow data points (○) indicate results pertaining to U937 cells. Overall significance was determined using a one-way ANOVA. Pairwise comparisons were conducted using Fisher's LSD test and significant differentiation effects are given relative to the control (*p<0.05, **p<0.01, ***p<0.001). Black stars indicate that these differences remained significant after Bonferroni correction. Error bars represent ± 1 standard deviation.

2.3.3 PMA combined with 1,25(OH)$_2$D$_3$ produces the most phenotypically mature macrophages in the conditions examined

Given the microscopy and RT-qPCR results above , THP-1 was selected as the cell line for further analysis using flow cytometry. This was done as the THP-1 cells showed greater responsiveness to 1,25(OH)$_2$D$_3$ than their U937 counterparts in terms of morphology and the modulation of cytokine gene expression. Monocyte-to-macrophage differentiation is accompanied by an increase in cell size and cellular complexity which was confirmed in THP-1 cells using flow cytometry by assessing the FSC and SSC. As was demonstrated with microscopy, only conditions including 5 nM PMA showed a significant increase in FSC (p<0.001) and SSC (p<0.001; Fig. 2.6 A and B). Conditions employing 10 nM or 100 nM 1,25(OH)$_2$D$_3$ alone showed no significant difference from the vehicle control. The increase in cell size and granularity was significantly larger when 5 nM PMA and 10 nM 1,25(OH)$_2$D$_3$ were combined to induce differentiation when compared to the 5 nM PMA condition alone (p<0.001), supporting the microscopy observations. The expression of CD11b and CD14 was significantly induced in all differentiation conditions. The expression of CD11b did not differ significantly between the 5 nM PMA, 10 nM 1,25(OH)$_2$D$_3$ and 100 nM 1,25(OH)$_2$D$_3$ differentiation conditions (Fig. 2.6 C). However, the expression of CD11b was significantly higher than all other conditions in cells exposed to 5 nM PMA with 10 nM 1,25(OH)$_2$D$_3$ (p<0.001). The expression of CD14 is linked to 1,25(OH)$_2$D$_3$ concentration as well as macrophage differentiation (Fig. 2.6 D). Despite showing distinct morphological and phenotypic characteristics of macrophage differentiation the expression of CD14 in the 5 nM PMA condition was significantly lower than the other differentiation conditions. The expression of CD14 was significantly greater in the 100 nM 1,25(OH)$_2$D$_3$ differentiation condition than in the 10 nM 1,25(OH)$_2$D$_3$ condition (p<0.001). Similarly, to CD11b, the expression of CD14 was significantly greater (p<0.001) in the 5 nM PMA with 10 nM 1,25(OH)$_2$D$_3$ condition compared to all other differentiation conditions.

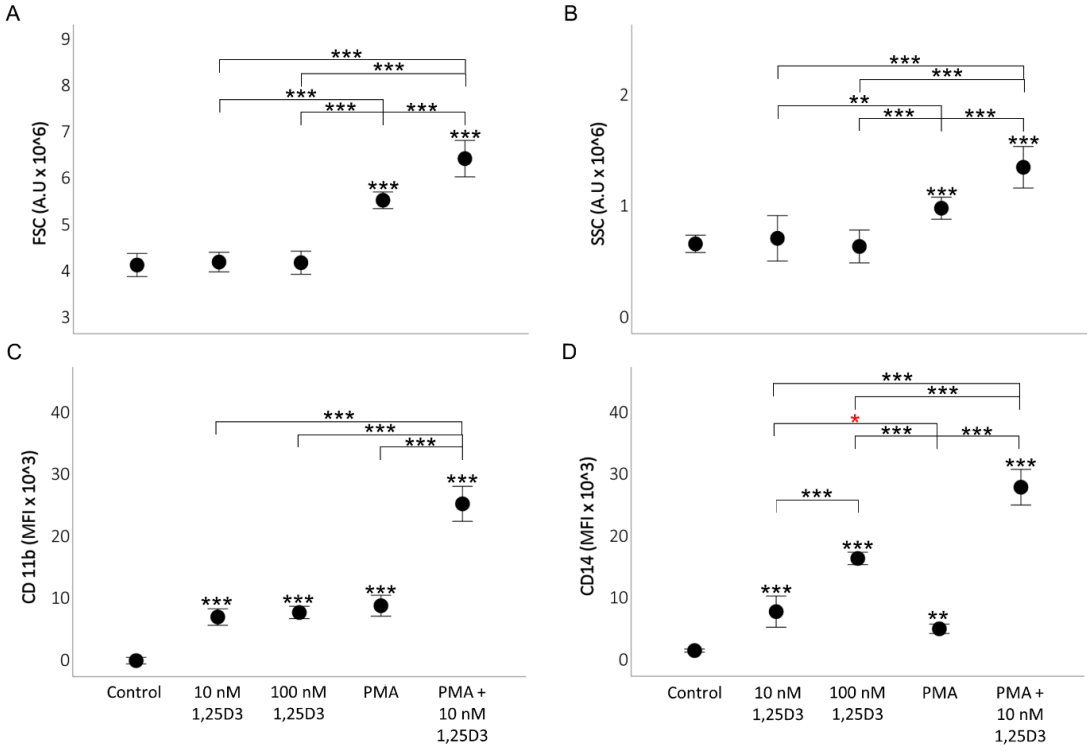


Figure 2.6: Differentiation conditions employing both 5 nM PMA and 10 nM 1,25(OH)$_2$D$_3$ show the greatest increase in cell size and granularity, CD11b and CD14. The error bar plots show the mean FSC (A), SSC (B), CD11b (C) and CD14(D) arbitrary units (A.U) or median fluorescence intensity (MFI) for the various differentiation conditions in THP-1 cells (n = 3), 96 hours post differentiation. Overall significance was determined using a one-way ANOVA. Pairwise comparisons were conducted using Fisher's LSD test and significant differentiation effects are given relative to the control (**p*<0.05, ***p*<0.01, ****p*<0.001). Black stars indicate that these differences remained significant after Bonferroni correction. Error bars represent ± 1 standard deviation.

2.4 Discussion

Monocytes and macrophages have important roles in immunity and homeostasis (Bassler *et al.,* 2019). Studying the process of monocyte-to-macrophage differentiation and subsequent macrophage polarisation is an important aspect of understanding their role in this context (Chistiakov *et al.,* 2018; Orekhov *et al.,* 2019). Monocytes are highly plastic cells, and the process of differentiation is accompanied by vast changes in phenotype (Canè *et al.,* 2019; Pinto *et al.,* 2021). These changes in phenotype, reflected in the change of morphology and functionality, govern the capacity of these cells to perform appropriately in a given context (Boyette *et al.,* 2017; Hesketh *et al.,* 2017; Mohd Yasin *et al.,* 2022). Dysregulation may have several adverse outcomes, be it excessive inflammation during infection, the development of auto-immune disorders or tumour promotion (Murray, 2017; Ma *et al.,* 2019). As the use of primary cells presents its own challenges, the development and application of a biologically representative cell line model is necessary to produce reproducible and reliable results. Consequently, the purpose of this work was to develop a more biologically relevant model using the most commonly employed MLCLs – THP-1 and U937 cells.

Though $1,25(OH)_2D_3$ has been frequently reported as an inducer of differentiation, acting through the mTOR and PI3K signalling pathways (Chung *et al.,* 2007; Hughes *et al.,* 2008; Y. Kim *et al.,* 2019), at both 10 nM and 100 nM concentrations of $1,25(OH)_2D_3$ there was no increase in the cell size, as a result of the increase in cytoplasm. In THP-1 cells, there was moderate adherence, whereas in the U937, even at 100 nM $1,25(OH)_2D_3$ the adherence was negligible. This indicates a slight difference in the two cell lines responsiveness to $1,25(OH)_2D_3$, however, there is no clear reason for this. When considering morphological alterations that represent commitment to macrophage differentiation, PMA appears to be a requirement. When treated with PMA both THP-1 and U937 cells adhered firmly to the culture plate and showed an increase in cell size that is enhanced when a rest period is included. This finding was supported by the work of Daigneault *et al.,* (2010) in THP-1 cells and Valdés-López and Urcuqui-Inchima (2018) in U937 cells. Daigneault *et al.,* (2010) compared primary monocytes and monocyte-derived-macrophages differentiated using human AB serum for 14 days to differentiation protocols in THP-1 cells employing 100 nM $1,25(OH)_2D_3$ for 72 hours, 200 nM PMA for 72 hours and 200 nM PMA for 72 hours followed by 140 hours of rest. They demonstrated that 100 nM $1,25(OH)_2D_3$ failed to produce the phenotypic characteristics of macrophages in terms of morphology, CD14 expression, mitochondrial and lysosome formation and susceptibility to apoptosis. However, PMA could induce these phenotypic alterations and the most biologically representative THP-1 derived macrophages were generated when an initial treatment phase was followed by a rest phase. While this supports the results produced in this study, there are some stark contrasts. Notably, Daigneault *et al.* (2010) reported that the combination of PMA and $1,25(OH)_2D_3$ had no effect on the outcome of differentiation and that the same effects were ultimately achieved using PMA alone. The most likely reason for this is the high concentration of PMA used, as other work has indicated that high concentrations of PMA may reduce the ability of THP-1 cells to respond to alternative stimuli (Park *et al.,* 2007; Maeß *et al.,* 2014).

A similar study was conducted in U937 cells by Valdés-López and Urcuqui-Inchima (2018). They compared the differentiation state of primary monocytes differentiated to macrophages using autologous serum for 6 days with U937 cells exposed to either 20 nM $1,25(OH)_2D_3$, 25 nM PMA, or 25 nM PMA combined with 20 nM $1,25(OH)_2D_3$ for 48 hours initially followed by a 6-day rest period. In accordance with the results of this study, Valdés-López and Urcuqui-Inchima demonstrated that $1,25(OH)_2D_3$ did not induce the morphological changes associated with differentiation, that this was only achieved when the U937 cells were exposed to PMA. As with this study and the study by Daigneault *et al.,* Valdés-López and Urcuqui-Inchima indicated that a rest period greatly enhanced the features of differentiation. This group also found that the combination of PMA and $1,25(OH)_2D_3$ enhanced the morphological features of

differentiation in U937 cells. This adds credence to the idea that this observation may be influenced by the concentration of PMA employed. That being said, the culturing conditions were not identical in these studies, thus, differences may have arisen that are culture dependent and not a result of the differentiation conditions applied.

Several research groups have compared the usage of different PMA concentrations and there is some debate over the efficacy of the use of a low dosage of PMA to induce differentiation in THP-1 and U937 cells. In this study, the use of 5 nM PMA for 24 hours followed by a 72-hour rest period appeared to effectively induce differentiation in both THP-1 and U937 cell lines when considering an increase in cytoplasmic ratio and adherence. There is no consensus on whether or not different concentrations of PMA influence the cell size and shape of the macrophages generated. However, several studies have shown that once a minimum baseline concentration of PMA has been met (indicated at 6 nM, 8 nM, 10 nM or 19 nM) the concentration of PMA no longer effects the morphology of the cells whilst in the presence of PMA, i.e. THP-1 or U937 cells exposed to both high and low concentrations of PMA, will exhibit the similar morphological features with respect to an increase in cytoplasmic ratio (increase in cell size), cellular characteristics and adherence (Hoefert *et al.,* 2015; Lund *et al.,* 2016; Zhou and Ma, 2018; Kuno *et al.,* 2020). Similarly, in U937 cells Kuno *et al.,* (2020) demonstrated that 10 nM PMA not only produced morphologically similar macrophages to those produced in the presence of 200 nM PMA but that the proportion of monocytes to macrophages was comparable, i.e the efficacy of differentiation did not change. This corroborates the finding that a low concentration of PMA can induce differentiation. Kuno *et al.,* (2020) also demonstrated that the cell viability of U937 derived macrophages was significantly lower in those cells exposed to 200 nM PMA, which was observed in the course of this research. When considering cellular adherence, Spano *et al.,* (2013) and Lund *et al.,* (2016) showed that following PMA-induced differentiation and a rest period of 72 hours or 48 and 120 hours, respectively, THP-1 cells that had been exposed to PMA concentrations equal to or below 30 nM showed a significant loss of adherence. This was not an effect observed in this study, however, it is possible that the combination of 5 nM PMA and 10 nM $1,25(OH)_2D_3$ would mitigate this problem, were it to arise as the cells differentiated in the presence of both PMA and $1,25(OH)_2D_3$ showed increased cell spreading and firmer adherence. Though a low concentration of PMA was able to induce differentiation, the macrophages generated when 5 nM PMA and 10 nM $1,25(OH)_2D_3$ were combined appeared to be more mature, especially when considering THP-1 cells. This furthered the assumption that THP-1 and U937 cells respond differently to the presence of $1,25(OH)_2D_3$.

Several lines of evidence have indicated that different macrophage polarisation states are associated with distinct macrophage morphologies (Binnemars-Postma *et al.,* 2016; Malheiro *et al.,* 2016; Rostam *et al.,* 2017; Wheeler *et al.,* 2018). In this study, there was no active induction of polarisation through the use of commonly reported M1-polarising agents such as LPS, IFN-γ and TNF-α or M2-polarising agents such as IL-4 and IL-13 (Chanput *et al.,* 2014; Binnemars-Postma *et al.,* 2016). However, this study demonstrated that the macrophages generated by differentiating THP-1 and U937 cells with 5 nM PMA alone or using a combination of 5 nM PMA and 10 nM $1,25(OH)_2D_3$ produced morphologically distinct macrophages. This was particularly notable in the differentiated THP-1 cells. According to studies in both humans and other mammals, specifically canines and mice, M0 macrophages have an irregular rounded shape, M1 polarised macrophages adopt a large, flat and rounded morphology whereas M2 polarised macrophages are the largest, most flattened (spread/expanded) macrophages that can have heterogeneous morphologies that often include cellular elongation (Vereyken *et al.,* 2011; McWhorter *et al.,* 2013; Binnemars-Postma *et al.,* 2016; Heinrich *et al.,* 2017; Rostam *et al.,* 2017; Wheeler *et al.,* 2018; Xiao *et al.,* 2020). For the U937 cells, the induction of differentiation using either 5 nM PMA or 5 nM PMA with 10 nM $1,25(OH)_2D_3$ over 96 hours, resulted in a large, rounded morphology, the extent of cell spreading was greater in the 5 nM PMA with 10 nM $1,25(OH)_2D_3$ condition but ultimately these cells more closely resemble M0 or M1-like macrophages. Similarly, in THP-1 cells exposure to 5 nM PMA for 24 hours followed by 72 hours rest, produced primarily large, rounded macrophages with occasional cells showing cellular elongation, thus they were more morphologically similar to M0 or M1-like macrophages. However, THP-1 cells exposed to 5 nM PMA with 10 nM $1,25(OH)_2D_3$ for 24 hours followed by 72 hours rest showed a heterogeneous cell population with considerably larger, flatter cells dominated by spindle-like cells and large ameboid like cells, this suggests that they were more representative of M2-like macrophages. The appearance of two distinct morphologies in the differentiated THP-1 cells may not have been anticipated. Zhang *et al*., (2015) showed that PMA differentiated THP-1 cells which were polarised to M1 and M2-like macrophages were morphologically similar and relied on the measurement of cytokines to confirm polarisation. However, it is worth noting that this group employed a high concentration of PMA (160 nM over 48 hours), which has previously been shown to influence downstream polarisation. High concentrations of PMA have been shown to skew THP-1 cells toward a M1-like macrophage state and interfere with or inhibit the generation of M2-like macrophages (Park *et al.,* 2007; Daigneault *et al.,* 2010; Foey and Crean, 2013; Maeß *et al.,* 2014; Lund *et al.,* 2016; Starr *et al.,* 2018). In light of this, it is worth noting that these distinctive morphological features in the PMA and PMA with 10 nM $1,25(OH)_2D_3$ differentiated THP-1 cells were also supported by RT-qPCR based gene expression assessment of cytokine and chemokine profiles.

To further investigate the effects of the various differentiation conditions in THP-1 and U937 cells this study looked at the gene expression of *VDR*, *CAMP* and pertinent pro- and anti-inflammatory cytokines. As there was a notable difference in the response of THP-1 and U937 cells to the presence of $1,25(OH)_2D_3$, the first step was to establish that $1,25(OH)_2D_3$ – VDR signalling axis was functional. In THP-1 cells the expression of *VDR* did not change significantly for any condition. However, in U937 cells the 5 nM PMA condition resulted in a significant decrease in *VDR* expression. This is not entirely unexpected as Kreutz *et al.,* (1993) and Dong *et al.,* (2013) both demonstrated that monocyte-to-macrophage differentiation is accompanied by a decrease in either VDR protein or *VDR* mRNA expression, respectively. Meireles *et al.,* (2016) demonstrated that VDR levels can be restored in the presence of $1,25(OH)_2D_3$, potentially explaining why the 5 nM PMA with 10 nM $1,25(OH)_2D_3$ condition did not demonstrate this decrease in *VDR* expression. Why this downregulation of *VDR* expression was not observed in THP-1 cells may be a result of the large difference in *VDR* baseline expression between the two cell lines. According to information available on the Human Protein Atlas (v.22; https://v22.proteinatlas.org/) the expression of VDR is considerably higher in U937 cells than THP-1 cells (Uhlén *et al.,* 2015; https://www.proteinatlas.org/ENSG00000111424-VDR/cell+line). This does not necessarily reflect a functional difference in VDR signalling, as demonstrated through the quantification of primary $1,25(OH)_2D_3$ gene target, *CAMP*. The expression of *CAMP* was measured to confirm that VDR signalling was active in both cell lines. As expected, conditions containing $1,25(OH)_2D_3$ resulted in a significant increase in *CAMP* expression in both cell lines. The magnitude of this alteration in *CAMP* expression was similar in both cell lines with respect to each condition. It is worth noting that the 10 nM and 100 nM $1,25(OH)_2D_3$ conditions had a greater increase in *CAMP* expression compared to the 5 nM PMA with 10 nM $1,25(OH)_2D_3$ condition, though they did not differ significantly from each other. From this it is plausible to say that the concentration of $1,25(OH)_2D_3$ does not always result in a greater increase in primary $1,25(OH)_2D_3$ target genes, that once a threshold is met the expression will remain consistent. While in U937 cells the difference in magnitude of *CAMP* expression when exposed to $1,25(OH)_2D_3$ after exposure to 5 nM PMA could be attributed to the decreased expression of *VDR*, this does not account for the difference observed in THP-1 cells. It is plausible that this difference may be associated with an alteration in the activity of VDR following differentiation.

When considering the induction of monocyte-to-macrophage differentiation, it has long been shown that the canonical NF-κB signalling pathway, associated with NF-κB1, is activated during this process and functions to prevent apoptotic cell death and facilitate innate immune functions such as Type I interferon production and related cytokines and chemokines (Pagliari *et al.,* 2000; Ma *et al.,* 2005; Vallabhapurapu and Karin, 2009; Busca *et al.,* 2014; Zeng *et al.,*

2015; Orekhov *et al.,* 2019). It has been shown that NF-κB1 is not necessary for PMA induced monocyte-to-macrophage differentiation in THP-1 cells, even though it plays an essential role in macrophage function (Somma *et al.,* 2021). The non-canonical NF-κB signalling pathway, associated with NF-κB2, has also been shown to functionally influence macrophages with roles in polarisation switching, mediating adaptive immune response and modulation of the canonical NF-κB pathway to control inflammation (Li *et al.,* 2010; Jin *et al.,* 2014; Sun, 2017). While the canonical NF-κB pathway is rapidly and transiently activated in monocytes and macrophages, the non-canonical NF-κB pathway is slower to activate but more persistent (T. Liu *et al.,* 2017). As such, this study assessed the mRNA expression of *NFKB2*. No differences were observed in *NFKB2* expression. It is possible that the window in which these alterations would have been visible was missed. Alternatively, *NFKB2* expression does not have to change in order for its activation status to do so. Alteration in the protein expression or activity of upstream members of the *NFKB2* signalling cascade such as the NF-κB-inducing kinase or the IκB kinase α will alter the activation state of NF-κB2 (Sun, 2017). For example, it has been shown that $1,25(OH)_2D_3$ supresses non-canonical NF-κB signalling, either influencing NF-κB2 subunit turnover or modulating its activity through the receptor activator of NF-κB (RANK)/RANK ligand pathway (Fekrmandi *et al.,* 2015; Mazanova *et al.,* 2022). Though the expression of *NF-κB1* was not measured, the mRNA expression of pro-inflammatory cytokines and chemokines that are target genes of NF-κB, specifically *TNF* and *CXCL10*, was assessed (T. Liu *et al.,* 2017).

The (semi-) quantitative assessment of either mRNA or protein expression of pro-inflammatory cytokines, such as *TNF,* and chemokines, such as *CXCL10* and *CCL2,* is frequently employed in the assessment of M1 polarisation of macrophages (Shiratori *et al.,* 2018; Yang *et al.,* 2019; Mohd Yasin *et al.,* 2022). The expression of *TNF* was not significantly altered in any differentiation condition for THP-1 cells. In previous work, TNF-α expression at a gene and protein level is positively correlated with PMA concentration used to induce differentiation (Park *et al.,* 2007; Daigneault *et al.,* 2010; Riddy *et al.,* 2018). However, Lund *et al.* (2016) showed that differentiation conditions employing a lower concentration of PMA do not exhibit any change in *TNF* expression. Additionally, work in primary monocytes does not show increased *TNF* expression following differentiation with macrophage colony stimulating factor, adding support to the use of a lower concentration of PMA (Dong *et al.,* 2013). In contrast, *TNF* expression in U937 cells was significantly increased by the use of 5 nM PMA, however, this increase was mitigated when 5 nM PMA and 10 nM $1,25(OH)_2D_3$ were combined. The increase in *TNF* expression in U937 cells when exposed to PMA has been shown consistently in the past (Song *et al.,* 2015; Riddy *et al.,* 2018; Kuno *et al.,* 2020). The modulation of this increase in the presence of $1,25(OH)_2D_3$ was not unexpected as $1,25(OH)_2D_3$ has been shown

to inhibit TNF-α mRNA and protein expression in monocyte derived macrophages (Greiller and Martineau, 2015; Rafique *et al.,* 2019). This $1,25(OH)_2D_3$ induced modulation of inflammation was also observed in the expression of pro-inflammatory chemokines *CXCL10* and *CCL2*.

Both CXCL10 and CCL2 are important chemokines in monocytes and macrophages, however, their functions extend beyond chemotaxis and thus immune cell recruitment (Zhao *et al.,* 2017; Gschwandtner *et al.,* 2019). Non-chemotactic properties of CXCL10 include promoting the differentiation and polarisation of B cells and T cells and recently, it has been found that CXCL10 can increase the expression of other pro-inflammatory cytokines including IL-12 and IL-23 (Zhao *et al.,* 2017; Karin and Razon, 2018). Expression of *CXCL10* increased significantly in both THP-1 and U937 cells when exposed to the 5 nM PMA condition. This PMA- induced increase of *CXCL10* has been observed in both of these cell lines in the past (Marcuello *et al.,* 2018). When the cells were differentiated using both 5 nM PMA and 10 nM $1,25(OH)_2D_3$ this effect was eliminated, in the case of THP-1 cells, or reduced, in the case of U937 cells. There are several lines of evidence that indicate the ability of $1,25(OH)_2D_3$ to prevent CXCL10 production; in this study, that can be observed at the gene level (Scolletta *et al.,* 2013). However, the exact influence of $1,25(OH)_2D_3$ on *CXCL10* mRNA or protein expression appears to be cell type, context, and stimuli dependent (Hansdottir *et al.,* 2010; Korf *et al.,* 2012; Brockman-Schneider *et al.,* 2014). The various differentiation conditions produced a similar pattern with respect to *CCL2* expression, though to greatly different extents.

In addition to its' chemotactic role, CCL2 has functions in monocytes and macrophages associated with immune cell priming, enhancing phagocytosis, autophagy activation and cell survival (Shachar and Karin, 2013; Gschwandtner *et al.,* 2019). As with *CXCL10* expression, the 5 nM PMA differentiation condition resulted in an increase in *CCL2* expression, although the magnitude of this increase was considerably greater in U937 cells than THP-1 cells. This differentiation induced increase in *CCL2* expression has previously been observed in both cell lines as well as primary monocytes differentiated into macrophages (Dong *et al.,* 2013; Rossetti *et al.,* 2017; Kurynina *et al.,* 2018; Gažová *et al.,* 2020). As indicated herein, other research groups demonstrated a considerably greater PMA-induced increase in *CCL2* expression in U937 cells when compared to THP-1 cells (Riddy *et al.,* 2018). Once again, the combination of 5 nM PMA and 10 nM $1,25(OH)_2D_3$ significantly reduced the expression of *CCL2* expression. The considerable difference in $1,25(OH)_2D_3$ responsiveness between THP-1 and U937 cells was again illustrated as the addition of 10 nM $1,25(OH)_2D_3$ to the 5 nM PMA condition resulted in the elimination of PMA-induced *CCL2* expression. Alternatively, the U937 cells showed down regulation of PMA-induced *CCL2* expression but not it's elimination. This $1,25(OH)_2D_3$-induced effect on *CCL2* expression has been demonstrated before in THP-1

cells, peripheral blood mononuclear cells (PBMCs) and bronchial fibroblasts (Ding *et al.,* 2013; Fitch *et al.,* 2016; Plesa *et al.,* 2020; Heidari *et al.,* 2022). This pro-inflammatory effect illustrated by the increased expression of *TNF*, *CXCL10* and *CCL2* in THP-1 and U937 cells in the presence of 5 nM PMA suggests a proclivity toward M1 polarisation. The anti-inflammatory effect demonstrated by $1,25(OH)_2D_3$ in *TNF*, *CXCL10* and *CCL2* expression was perhaps to be expected, as $1,25(OH)_2D_3$ is generally considered an immunomodulator with anti-inflammatory effects (Ryynänen and Carlberg, 2013; Sassi *et al.,* 2018). As macrophage polarisation exists on a spectrum, the mRNA expression of M2 polarisation marker *IL10* was also assessed.

IL-10 is one of the most potent anti-inflammatory cytokines produced (Mia *et al.,* 2014). It is produced by and has the capacity to regulate the inflammatory activities of the majority of immune cells, both innate and adaptive, including macrophages, dendritic cells, B cells and T cells (Maynard and Weaver, 2008; Carter *et al.,* 2012; Shachar and Karin, 2013; Shouval *et al.,* 2014). The dysregulation of IL-10 expression can lead to autoimmunity, excessive inflammation in response to pathogenic assault or contribute to tumour survival and immune evasion (Trifunović *et al.,* 2015; Saraiva *et al.,* 2020; Chang *et al.,* 2021). As such, it is essential that IL-10 production is balanced. Unlike the mRNA expression of the pro-inflammatory cytokines, the expression of *IL10* was increased as a response to both PMA and $1,25(OH)_2D_3$ in both THP-1 and U937 cells, although $1,25(OH)_2D_3$ -induced effect was once again more pronounced in THP-1 cells. The expression of *IL10* was significantly increased by the 10 nM and 100 nM $1,25(OH)_2D_3$ differentiation conditions in a concentration independent manner in both THP-1 and U937 cells, though THP-1 cells showed a slightly higher fold induction than the U937 cells. When considering the 5 nM PMA differentiation conditions, *IL10* expression was significantly increased in both cell lines, however, U937 cells saw an immense fold induction of *IL10* expression. This finding was supported by the results obtained by Riddy *et al.* (2018) in that while U937 cells saw a dramatic increase in *IL10* expression when exposed to 26 nM PMA, there was no detection of *IL10* in THP-1 cells. When 5 nM PMA and 10 nM $1,25(OH)_2D_3$ were combined there was a significant increase in *IL10* expression when compared to the 5 nM PMA condition alone, in THP-1 cells. However, in U937 cells there was no significant difference between these two conditions. This once again highlights that THP-1 cells and U937 cells appear to be responding differently to the presence of $1,25(OH)_2D_3$. This PMA-induced increase in *IL10* expression has not been frequently examined. However, given that the *IL10* expression was measured 96 hours after initial exposure to PMA it is plausible that the increase in *IL10* expression is a compensatory mechanism for the pro-inflammatory state that appears to occur in response to PMA in both THP-1 and U937 cells (Ma *et al.,* 2016). However, it is worth noting that the strictly anti-inflammatory nature of *IL10* has been called

into question, most recently with respect to its' association with a poor prognosis during COVID-19 pathogenesis (Islam *et al.,* 2021; Lu *et al.,* 2021). In addition to this, clinical trials involving the use of IL-10 did not show the widely purported anti-inflammatory effects as expected and were instead aligned with an increase in inflammation (Mühl, 2013). As such, it cannot be stated that the increase in *IL10* expression is strictly acting to decrease inflammation.

The relationship between $1,25(OH)_2D_3$ and *IL10* expression has not been fully elucidated, with some reports showing increased *IL10* expression and others indicating a decrease in *IL10* expression in the presence of $1,25(OH)_2D_3$ (Guillot *et al.,* 2010; Correale *et al.,* 2011; Niino *et al.,* 2014). Korf *et al.* (2012) proposed that $1,25(OH)_2D_3$-induced anti-inflammatory effects in T cells, measured by a decrease in *TNF*, *NOS2* (encoding inducible nitric oxide synthase), *CXCL9*, *CXL10* and *CXCL11* expression, was driven by an IL-10-dependent mechanism, supporting the traditional anti-inflammatory role of IL-10. In THP-1 cells differentiated with 5 nM PMA with 10 nM $1,25(OH)_2D_3$ this $1,25(OH)_2D_3$ induced IL-10-dependent reduction in inflammation may be plausible. However, in U937 cells differentiated in the presence of 5 nM PMA the expression of *IL10* was not affected by the presence of $1,25(OH)_2D_3$. Therefore, $1,25(OH)_2D_3$-induced mitigation of inflammation cannot be attributed to the activity of IL-10. This is particularly evident given the high expression levels of pro-inflammatory cytokine and chemokine encoding genes observed in these cells, despite the magnitude of *IL10* expression. As the expression of *IL10* is associated with M2 polarisation, it becomes difficult to class THP-1 and U937 cells differentiated with 5 nM PMA alone as being strictly pro-inflammatory, although they have more features indicating toward this. However, with respect to THP-1 cells differentiated using 5 nM PMA combined with 10 nM $1,25(OH)_2D_3$ it is reasonable to state that given that the presence of $1,25(OH)_2D_3$ failed to show increased expression of M1 polarisation markers *CXCL10* and *CCL2* and that there was a significant increase in *IL10* expression, to a greater degree than that induced by PMA alone, these macrophages tend toward an M2 polarised state.

There is little literature regarding the difference in responsiveness to $1,25(OH)_2D_3$ within THP-1 and U937 cells. However, there has been work indicating that acute myeloid leukaemia cells, like most cancers, are inherently different from each other in their transcriptional and signalling networks which could contribute to the differences observed (Assi *et al.,* 2019; Xu *et al.,* 2022). From the results garnered in this study, it appeared that THP-1 cells were more responsive to $1,25(OH)_2D_3$ than their U937 counterparts. This was seen in the morphological alterations, in that THP-1 cells, though they did not display mature macrophage morphologies in the 10 nM and 100 nM conditions, they did show moderate adherence, whereas the U937 cells did not. In addition, when THP-1 cells were differentiated with 5 nM PMA and 10 nM $1,25(OH)_2D_3$ the

resultant macrophages were vastly different in morphology compared to those treated with 5 nM PMA in isolation. While the 5 nM PMA with 10 nM $1,25(OH)_2D_3$ differentiation condition produced slightly larger and flatter macrophages, the general morphology of these macrophage was similar to that of the 5 nM PMA condition alone. Similarly, despite the U937 cells having a higher baseline expression of *VDR*, the expression of *CAMP* did not show a greater fold induction in U937 cells. Likewise, $1,25(OH)_2D_3$ did not induce the same degree of anti-inflammatory properties in U937 cells as it did in THP-1 cells. The implications being that THP-1 cells are more sensitive to $1,25(OH)_2D_3$ when compared to U937 cells. This ought to be taken into consideration when developing studies that look at the influence of $1,25(OH)_2D_3$ in monocyte or macrophage differentiation or function.

From the results discussed above, this work indicates that, should the application require a more neutral macrophage following differentiation, then the use of the 5 nM PMA with 10 nM $1,25(OH)_2D_3$ would provide the best option in both THP-1 and U937 cells. However, these two cell lines, despite both being used as models for monocyte-to-macrophage differentiation and monocyte and macrophage function and polarisation, are not necessarily going to produce the same results. While the patterns of some results are similar this is not the case across the board. As the focus of this body of work was with respect to the impact of $1,25(OH)_2D_3$ signalling in monocytes and macrophages it was decided that THP-1 cell line would, given the aforementioned reasons, act as the best model going forward. This decision was supported by several lines of work that indicate that THP-1 cells represent a more accurate, though simplified, model of primary monocytes and monocyte-derived macrophages (Chanput *et al.,* 2014; Riddy *et al.,* 2018).

Flow cytometry was done for the various differentiation conditions after 96 hours as confirmation for the microscopy results and to confirm macrophage differentiation using CD11b and CD14. As was expected, both the FSC and SSC increased only when THP-1 cells were exposed to 5 nM PMA and the greatest increase in these parameters was seen when 5 nM PMA and 10 nM $1,25(OH)_2D_3$ was used to differentiate THP-1 cells. This increase in FSC and SSC is consistently demonstrated in THP-1 cells differentiated using PMA and confirms the increase in cell size observed during microscopy (Maeß *et al.,* 2014; Forrester *et al.,* 2018; Pinto *et al.,* 2021). THP-1 cells treated with PMA consistently show this increase in SSC which reflects an increase in cellular complexity, this is to be expected as increased cellular granularity is associated with cellular endpoints, such as terminal differentiation, senescence and alterations in cellular metabolism (Haynes *et al.,* 2009; Galván-Peña and O'Neill, 2014).

When studying the process of monocyte-to-macrophage differentiation and subsequent macrophage polarisation, it is commonplace to confirm these states using a selection of cell

surface receptors. In monocyte-to-macrophage differentiation some of the most common cell surface markers include CD11b, CD11c, CD14 and CD68 (Maeß *et al.,* 2014; Forrester *et al.,* 2018; Pinto *et al.,* 2021). M1 polarised macrophages are generally identified through their expression of CD80, CD86, TLR2 and NOS2 whereas M2 polarised macrophages generally express CD163 and CD206 (Hesketh *et al.,* 2017; Takiguchi *et al.,* 2021). While assessment of differentiation and polarisation states of monocytes and macrophages based on a small subset of cell surface markers can in no way capture the complexity and plasticity of monocytes and macrophages, it is one of the most frequently applied techniques (Ginhoux and Jung, 2014; Shakerian *et al.,* 2018).

In this study, THP-1 cells exposed to the various differentiation conditions showed that both PMA and $1,25(OH)_2D_3$ contribute to the increase in CD11b and CD14 expression. THP-1 cells exposed to 10 nM and 100 nM $1,25(OH)_2D_3$ and 5 nM PMA showed an increase in CD11b expression, compared to the control, that did not differ significantly between these conditions. CD11b expression was greatest in THP-1 cells exposed to both 5 nM PMA and 10 nM $1,25(OH)_2D_3$. Taken in isolation, these results agree with the microscopy results in that 5 nM PMA and 10 nM $1,25(OH)_2D_3$ are the most mature macrophages. However, they also imply that 10 nM and 100 nM $1,25(OH)_2D_3$ treatments produce macrophages that are at a similar point in the differentiation pathway as that observed in the 5 nM PMA differentiation condition. This does not agree with the morphological alterations observed in these conditions. However, this pattern has been observed before and thus is not unexpected (Camilli *et al.,* 2016). Had PMA concentration been higher there may have been a greater increase in CD11b expression (Pinto *et al.,* 2021). That being said, as the combination of a low concentration of PMA and $1,25(OH)_2D_3$ saw a significant increase in CD11b expression compared to PMA alone, the use of this combinatorial approach may be advised as it does not come with the associated increase in inflammation and the altered macrophage functionality demonstrated by the use of higher dosages of PMA (Maeß *et al.,* 2014; Lund *et al.,* 2016).

When considering markers of macrophage differentiation, assessment of the expression of CD14 produced results even more contrary than that of the CD11b expression when compared to those observed by microscopy. The results indicated that $1,25(OH)_2D_3$ had a larger contribution to the increase in CD14 expression than PMA, and thus by the traditional line of thought were further in their differentiation towards macrophages. THP-1 cells exposed to 10 nM and 100 nM $1,25(OH)_2D_3$ showed significantly greater CD14 expression than those exposed to 5 nM PMA alone. In addition, the 100 nM $1,25(OH)_2D_3$ condition showed significantly higher CD14 expression than the 10 nM $1,25(OH)_2D_3$ condition, indicating a positive correlation between CD14 expression and $1,25(OH)_2D_3$ concentration. THP-1 cells differentiated with 5 nM PMA and 10 nM $1,25(OH)_2D_3$ showed the greatest increase in CD14

expression, which is in alignment with the results observed by microscopy. This heightened CD14 expression in THP-1 cells exposed to $1,25(OH)_2D_3$ compared to those exposed to PMA alone has been observed before (Daigneault *et al.,* 2010; Camilli *et al.,* 2016). This is to be expected as CD14, like CAMP, has been shown to be a primary $1,25(OH)_2D_3$ target, which would also contribute to $1,25(OH)_2D_3$ concentration effect observed in CD14 expression (Nurminen *et al.,* 2019). However, from this study the implication is that while THP-1 cells exposed to $1,25(OH)_2D_3$ may demonstrate an increase in CD14 expression, this is not necessarily an indication of differentiation. As CD14 is a TLR co-receptor that primarily facilitates TLR-4 binding to LPS it is possible that this increase in CD14 expression is not strictly associated with differentiation and is more closely related to immune cell priming (Guzzo *et al.,* 2012; Zanoni and Granucci, 2013). As such, it may not be wise to use CD14 exclusively as an indication of monocyte-to-macrophage differentiation, especially if the method for differentiation includes $1,25(OH)_2D_3$.

The cell surface expression results indicated that caution should be applied when employing common macrophage markers in isolation as they may generate a false perception of the degree of differentiation. The use of cell surface markers ought to be combined by other methods of assessment to ensure that the results garnered are reflected by other phenotypic changes associated with monocyte-to-macrophage differentiation. As such, when considered in conjunction with the morphological alterations observed, the 5 nM PMA with 10 nM $1,25(OH)_2D_3$ differentiation condition produces the largest, most complex and phenotypically mature macrophages.

2.5 Conclusions

From this study it was found that the combination of 5 nM PMA and 10 nM $1,25(OH)_2D_3$ produced the most morphologically mature macrophages without the pro-inflammatory effects induced by using PMA in isolation. Additionally, it was found that although U937 cells express a higher level of *VDR* they are not as sensitive to the differentiation or immunomodulatory effects of $1,25(OH)_2D_3$ when compared to THP-1 cells. For this reason, THP-1 cells were selected as the cell line to be taken forward for future studies into the effect of $1,25(OH)_2D_3$ in monocyte-to-macrophage differentiation. The assessment of cell size, cell complexity and macrophage markers CD11b and CD14 in THP-1 cells confirmed that 5 nM PMA combined with 10 nM $1,25(OH)_2D_3$ produced the most mature macrophages. That being said, the morphological alterations observed through microscopy and the increased expression of cell surface markers showed some discrepancy. As such, it should be advised that when assessing monocyte-to-macrophage differentiation a multi-faceted approach should be taken to ensure differentiation as the use of only one method may result in a false impression of

differentiation. As differentiation is a complex process, and the contribution of $1,25(OH)_2D_3$ in this process is not fully understood, the next phase of this study was to determine the influence of $1,25(OH)_2D_3$ in this process in THP-1 cells through RNA-sequencing and differential gene expression analysis.

3.1 Introduction

It is now well accepted that many tissues have their own resident pool of self-renewing macrophages that may be supplemented by the recruitment of circulating monocytes, that upon recruitment to tissues, undergo differentiation into macrophages (Bassler *et al.,* 2019). During monocyte recruitment, monocytes leave the circulatory system through leukocyte extravasation and in the process of doing so, go from being proliferative suspension cells to non-proliferative, adherent, tissue-resident cells (Rutledge and Muller, 2020; Medrano-Bosch *et al.,* 2023). In addition to this change in location, key functional aspects that distinguish macrophages from their monocyte precursors include antigen presentation, enhanced phagocytosis and the participation in inflammatory immune responses (Guilliams *et al.,* 2014; Italiani and Boraschi, 2014; Jakubzick *et al.,* 2017; Bassler *et al.,* 2019; Patel *et al.,* 2021).

In more recent years an additional layer of complexity in understanding macrophage biology has emerged, namely the existence of specific macrophage polarisation states. Polarisation is considered a key aspect in understanding the contribution of macrophages to the immune cell landscape, and their contribution to disease pathology (Atri *et al.,* 2018; Boutilier and Elsawa, 2021). M1 polarised macrophages are generally considered to be pro-inflammatory in nature and exhibit increased expression of pro-inflammatory cytokines, MHC-II expression and specific increases in TLR expression (Shapouri-Moghaddam *et al.,* 2018). In contrast, the M2 macrophage polarisation state is not as easily defined, with several subcategories included in nomenclature. Generally, M2 polarised macrophages are associated with the production of anti-inflammatory cytokines, tissue regeneration, angiogenesis, enhanced phagocytic capacity and immunomodulation (Yao *et al.,* 2019). In practice, the differential expression of several markers including integrins, cell adhesion molecules, phagocytic receptors, and specific transcription factors are frequently employed to distinguish monocytes from macrophages (Hesketh *et al.,* 2017; Chistiakov *et al.,* 2018; Orekhov *et al.,* 2019; Mohd Yasin *et al.,* 2022). Likewise, in order to classify macrophages into different polarisation states the expression of key pro-inflammatory or anti-inflammatory cytokines, cell surface markers including TLRs, C-type lectin receptors (CLRs), B7-family ligands, scavenger receptors, MHC-II molecules, and polarisation associated transcription factors are frequently evaluated (Table 3.1).

Table 3.1: Transcription factors associated with macrophage polarisation

Transcription factor family	M1 Polarisation	M2 Polarisation	Inconclusive	References
STAT	*STAT1, STAT2, STAT4, STAT5A, STAT5B*	*STAT3, STAT6*	N/A	Juhas *et al.,* 2015; Murray, 2017; Orekhov *et al.,* 2019
IRF	*IRF1, IRF5, IRF8*	*IRF3, IRF4*	N/A	Murray, 2017; Chistiakov *et al.,* 2018; Orekhov *et al.,* 2019
KLF	*KLF6*	*KLF4*	N/A	Juhas *et al.,* 2015; Murray, 2017; Orekhov *et al.,* 2019
PPAR	N/A	*PPARD, PPARG*	N/A	Juhas *et al.,* 2015; Murray, 2017; Orekhov *et al.,* 2019
NF-κB	*NFKB1, NFKB2, REL, RELA RELB*	N/A	N/A	Juhas *et al.,* 2015; Murray, 2017; Orekhov *et al.,* 2019
AP-1				
JUN	*JUND*	N/A	*JUN, JUNB*	Yang *et al.,* 2014; Fontana *et al.,* 2015a; Garces de Los Fayos Alonso *et al.,* 2018; Fonseca *et al.,* 2019; Orekhov *et al.,* 2019
FOS	*FOS, FOSL1, FOSL2, FOSB*	N/A	N/A	Garces de Los Fayos Alonso *et al.,* 2018; Fonseca *et al.,* 2019; Orekhov *et al.,* 2019
ATF	*ATF2, ATF4, ATF7, BATF, BATF2, BATF3*	*ATF3*	*JDP2*	Sha *et al.,* 2017; Garces de Los Fayos Alonso *et al.,* 2018; Fonseca *et al.,* 2019; Orekhov *et al.,* 2019
MAF	N/A	*MAF, MAFB*	*MAFA, MAFF, MAFG, MAFK*	Kim, 2017; Garces de Los Fayos Alonso *et al.,* 2018; Liu *et al.,* 2020

As monocyte-derived macrophages have clear roles in health and disease, these cells are frequently under investigation. As the use of patient samples or animal models is not always feasible or practical, the use of a reliable cell model for these investigations is necessary. This requirement is most frequently met through the use of the THP-1 cell line (Chanput *et al.,* 2014). THP-1 resembles many characteristics of the human monocytes, including cell morphology, expression of membrane antigens and secretory proteins (Chanput *et al.,* 2015; Riddy *et al.,* 2018). THP-1 cells are used in the study of the monocyte-to-macrophage differentiation process and subsequent polarisation, as well as being an important model for understanding macrophage biology in the context of diseases including infection, cancer, diabetes, and auto-inflammatory diseases among others (Shapouri-Moghaddam *et al.,* 2018; Ma *et al.,* 2019; Wu and Lu, 2019; Grassin-Delyle *et al.,* 2020; Locati *et al.,* 2020).

However, despite their wide usage, no standardised differentiation protocol is applied. This is important as several studies have indicated that the way these cells are differentiated can alter their response to various stimuli. To wit, high concentrations of PMA (~162 nM) have been shown to inhibit responses to weaker stimuli and prevent effective M2 macrophage polarisation (Park *et al.,* 2007; Maeß *et al.,* 2014; Lund *et al.,* 2016). A way to circumvent this would be to reduce the concentration of PMA employed or utilise an alternative agent for the induction of differentiation. However, this brings its own set of complications. The use of lower concentrations of PMA for differentiation have been shown to result in a phenomenon referred to as de-differentiation, wherein the cells regain proliferative capacities and a monocyte-like phenotype (Spano *et al.,* 2013). Similarly, research indicates that alternative differentiation agents, including $1,25(OH)_2D_3$, fail to produce a macrophage like phenotype (Daigneault *et al.,* 2010; Rynikova *et al.,* 2023). A potential route to overcome this is through combining low PMA concentrations with $1,25(OH)_2D_3$, as suggested for the U937 monocyte-like cell line (Valdés-López and Urcuqui-Inchima, 2018).

It is important to note that though the use of PMA and $1,25(OH)_2D_3$ in isolation, is generally employed for differentiation, there has also been work associating their usage with THP-1-derived macrophage polarisation. Polarisation of THP-1-derived macrophages is most frequently achieved *in vitro* using LPS and IFN-γ for M1 macrophage polarisation and IL-4 in the presence or absence of IL-13 for M2 macrophage polarisation (Orecchioni *et al.,* 2019; Mohd Yasin *et al.,* 2022). However, some research groups consider PMA and $1,25(OH)_2D_3$ alone as being capable of resulting in the production of polarised macrophages (Foey and Crean, 2013). There has been evidence to suggest that this does not generate successfully polarised M1 macrophages, and that $1,25(OH)_2D_3$ alone cannot generate macrophages (Rynikova *et al.,* 2023). This begs the question as to whether the differentiation condition employed predisposes the differentiated macrophages towards a particular polarisation state.

Previous results from this body of work explored three potentially more biologically relevant protocols for the differentiation of THP-1 cells into THP-1 derived macrophages, using either 10 nM $1,25(OH)_2D_3$, 5 nM PMA or a combination thereof (which showed the most promising results after 96 hours of differentiation). The success of these protocols was assessed in terms of cellular morphology, the expression of key cell surface markers and the relative mRNA expression of inflammatory genes. Though there was a clear indication that PMA with $1,25(OH)_2D_3$ differentiated THP-1 cells more closely resembled mature macrophages, given the complex and highly plastic nature of these cells, it was deemed necessary to investigate the nature of all three differentiated THP-1 cells more holistically. To do so we employed bulk RNA sequencing to investigate the gene expression profiles of these cells and infer the nature of their differentiated states.

It would be reasonable to suspect that, in addition to that of traditional cell surface markers, THP-1-derived macrophages would have increased mRNA expression of cell adhesion, phagocytosis and inflammation associated genes (Padilla *et al.,* 2017; Shapouri-Moghaddam *et al.,* 2018; Pinto *et al.,* 2021). Cell adhesion forms the backbone of several inherent aspects of macrophage biology, such as interaction with the extracellular matrix (ECM), interaction with immune-, tissue-resident- and apoptotic-cells, and pathogens (Penberthy and Ravichandran, 2016; Harjunpää *et al.,* 2019). This capacity is in many ways associated with other hallmarks of macrophage biology, including enhanced phagocytosis and antigen presentation (Hume *et al.,* 2017; Uribe-Querol and Rosales, 2020; Fu and Harrison, 2021). Likewise, given that macrophages are important innate immune cells, even in the absence of stimulation, it is expected that they are more likely to be primed for an immune response and have altered expression of cytokines, chemokines and their receptors (Murray, 2017; Boutilier and Elsawa, 2021). Given the previous results, it was thus hypothesised that PMA would produce changes in the expression of the genes encoding these molecules, being indicative of THP-1 cells having undergone differentiation, and that the additional presence of $1,25(OH)_2D_3$ would further alter the nature of the THP-1-derived macrophages generated.

3.2 Materials and Methods

3.2.1 Cell culture and differentiation

Based on the experimental findings from Chapter 2, THP-1 cells were selected as the appropriate model to investigate the gene expression profiles of the monocyte-derived macrophages generated from the different differentiation conditions evaluated in this study. THP-1 cells were cultured, maintained, and differentiated as described in section 2.2.1. Following 96 hours of differentiation, the cells were detached from the plate using 0.25% Trypsin-EDTA (Sigma Aldrich, St. Louis, MO, USA) for 3 minutes, assisted by the use of a cell scrapper. Cells were washed with phosphate buffered saline (PBS; pH 7.4 Sigma Aldrich, St. Louis, MO, USA), counted and assessed for viability using the Trypan-blue exclusion assay. Only cells with a viability greater than 80% were considered acceptable for RNA sequencing. THP-1 cell pellets originating from the different differentiation protocols (1×10^6) were stored in 200 µl RNA*later* (Thermo Fisher Scientific, Waltham, MA, USA) at -80 ^{o}C for RNA extraction and sequencing.

3.2.2 RNA sequencing

RNA sequencing was employed to investigate the changes in gene expression observed in THP-1 cells that result from the different differentiation conditions applied. RNA extraction, library preparation and mRNA sequencing services for three biological replicates for each differentiation condition and matching vehicle control were outsourced to Admera Health, LLC (South Plainfield, NJ, USA). Briefly, RNA extraction was performed using the RNeasy Plus 96 kit (Qiagen, Valencia, CA) according to manufacturer's specifications. The RNA quantity was assessed using the Qubit RNA HS assay (Thermo Fisher Scientific, Waltham, MA, USA), with total RNA yields between 468 – 4020 ng. RNA quality was assessed using the Bioanalyzer 2100 Eukaryote Total RNA Nano, with all RIN values found to be > 9.8 (Agilent Technologies, CA, USA). Paired-end, short read RNA sequencing libraries were constructed using the NEBNext® Ultra™ II Directional RNA Library Prep Kit for Illumina (NEB, Ipswich, MA, USA), with poly-A selection, in accordance with manufacturer's guidelines. The adapter sequences applied were AGATCGGAAGAGCACACGTCTGAACTCCAGTCA (read 1) and AGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT (read 2). Sequencing was performed on the NovaSeq 6000 S4 and a minimum of 20 million (2 × 150 bp) reads were obtained for each sample.

3.2.3 Quantification of gene expression

Prior to selective alignment and transcript quantification quality control of the raw RNA sequencing reads was performed using FastQC (v0.11.9; Andrews, 2010). This was followed by selective alignment against the transcriptome and quantification of transcript abundance using Salmon v1.10.0 (Patro *et al.,* 2017). To increase the accuracy and reliability of Salmon based quantification a partial decoy-aware transcriptome file was generated. This was done by aligning the annotated transcriptome to a hard-masked version of the corresponding human genome using MashMap (v2.0.). The sequences generated were extracted and then concatenated to the transcriptome file as decoy sequences and a named list of these decoy sequences was produced. Masking these decoy sequences during quantification restricts ambiguous and spurious mapping, and thus increases the accuracy of transcript quantification (Srivastava *et al.*, 2020). A decoy-aware transcriptome index was generated using the GENCODE v43 annotations of the human reference genome (GRCh38), at a *k*-mer size of 31 (where *k* denotes the minimum sequence length required to generate a valid match during selective alignment and quantification), by including the –decoy argument, together with the file containing the names of the decoy sequences.

Following the construction of the transcriptome index, selective alignment of the paired-end RNA sequencing reads from each biological replicate, for each differentiation protocol, was then performed using the Salmon quant command. As Salmon does not require adapter trimming, the raw RNA sequencing reads were used as input. The library type was automatically inferred by Salmon using the -1 A (-- libType A) option. To specify selective alignment, align and quantify single-end reads that are not paired (i.e. orphan reads), and correct for sequencing and GC content biases, the options –validateMappings, --recoverOrphans, --seqBias and –gcBias were passed to the quant command, respectively. Transcript-level quantifications obtained were then aggregated to the gene level using tximport v1.26.1 (Soneson *et al.,* 2015) to generate the count matrix required for differential gene expression analysis.

3.2.4 Differential expression analysis

Differential expression analysis was conducted using the R package, DESeq2 v1.39.3 (Love *et al.,* 2014) with log fold change shrinkage estimates derived using apeglm v1.22.1 (Zhu, *et al.,* 2019). To summarise this data and assess the overall similarity of the global mRNA expression landscapes observed between the various differentiation conditions and respective controls the gene level estimates underwent variance-stabilising transformation, in preparation for unsupervised principal component analysis (PCA). Pairwise comparisons for changes in gene expression between each differentiation condition (10 nM $1,25(OH)_2D_3$, 5 nM PMA, and

5 nM PMA with 10 nM $1,25(OH)_2D_3$) and its corresponding vehicle control (EtOH, DMSO, and DMSO with EtOH) were evaluated using the Wald test with Benjamini-Hochberg correction. Genes were only considered expressed, in the context of the experiment, if they had a within-group base mean cutoff of > 20. Following differential gene expression analysis, genes were considered to be significantly differentially expressed if they had an Benjamini-Hochberg adjusted *p* value of < 0.05 and an absolute log_2 fold change ($|log_2FC|$) > 1. All *p* values are given with respect to each conditions' respective control.

3.2.5 Over-representation analysis

To gain a broader understanding of the effects of the various differentiation protocols on gene expression, over-representation analysis was conducted using clusterProfiler v4.8.1 (Wu *et al.,* 2021). Over-representation analysis was done for both Gene ontology (GO) terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways using the EnrichGo and EnrichKEGG functions. GO biological process terms with a Benjamini-Hochberg adjusted *p* value of <0.01 and KEGG pathways with a Benjamini-Hochberg adjusted *p* value of <0.05 were considered statistically significantly over-represented within the set of differentially expressed genes. In addition to providing biological insight, the results generated from these enrichment analyses guided the selection of pathways and processes investigated downstream.

3.3. Results

3.3.1. Distinct gene expression profiles result from different differentiation conditions

The quality of the sequencing data produced during this study were evaluated, and as the mean quality score per base was found to be consistently greater than 33, were considered to be of high quality (data not shown). The percentage GC content was similar for all samples, ranging from 50% to 52%, and followed a normal distribution. The per base N score, which indicates an inability of the sequencer to accurately call the base, never exceeded 0.04% for any base in any sample. Similarly, the alignment rate for all replicates, across all differentiation conditions, was found to be high (88.5 – 90.5%), confirming the absence of any contaminants. Clustering analysis achieved with an unsupervised PCA revealed distinct mRNA expression profiles for each differentiation condition employed, illustrated by the formation of distinct clusters (Fig. 3.1). Principal component 1 (PC1) accounted for 77% of the variance, and clearly correlated with the differentiation condition applied, indicating that differentiation produced the greatest alterations in mRNA expression observed. In contrast, the solvents used for the vehicle controls did not result in notable mRNA expression changes when compared to each other, as they clustered together.

When considering the significantly differentially expressed genes identified in THP-1 cells differentiated with 1,25(OH)$_2$D$_3$ (506 genes), PMA (1,161 genes) and PMA with 1,25(OH)$_2$D$_3$ (2,434 genes), the differentiation conditions produced distinct mRNA expression profiles with limited overlap (Fig. 3.2; Data S1 Table S1-S3). Notably, only 133 upregulated and 50 downregulated genes were shared by all three differentiation conditions. There was considerably more overlap observed between PMA and PMA with 1,25(OH)$_2$D$_3$ differentiated THP-1 cells, which shared 500 upregulated and 227 downregulated genes. Furthermore, the combination PMA and 1,25(OH)$_2$D$_3$ produced an even greater alteration in the mRNA expression profile of THP-1 cells. THP-1 cells differentiated using PMA with 1,25(OH)$_2$D$_3$ showed the greatest proportion of uniquely significantly differentially expressed genes (~56%).

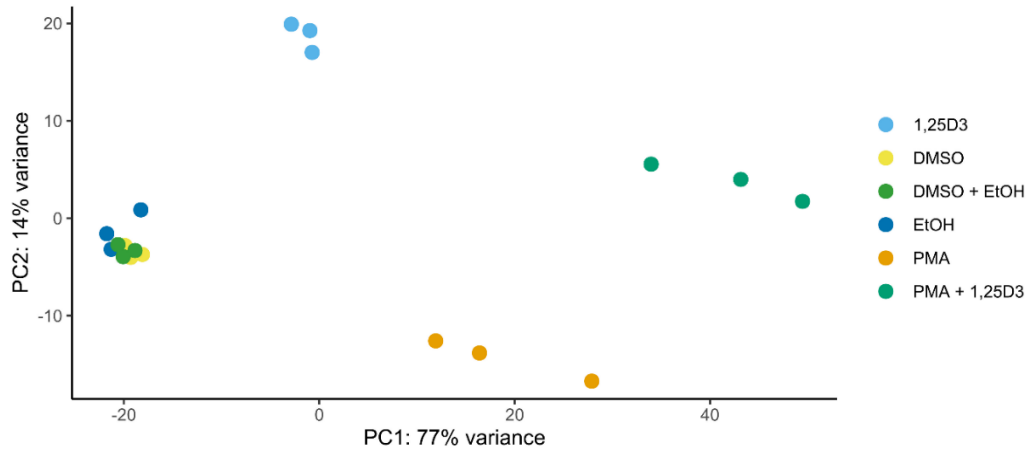


Figure 3.1: Principal component analysis (PCA) of RNA sequencing data indicated distinct gene expression profiles produced by each differentiation condition. An unsupervised PCA plot generated from the variance stabilised gene counts illustrates distinct clusters for THP-1 cells differentiated with 10 nM 1,25(OH)$_2$D$_3$, 5 nM PMA, and 5 nM PMA with 1,25(OH)$_2$D$_3$ over 96 hours (n = 3). The vehicle controls for each differentiation condition did not cluster separately.

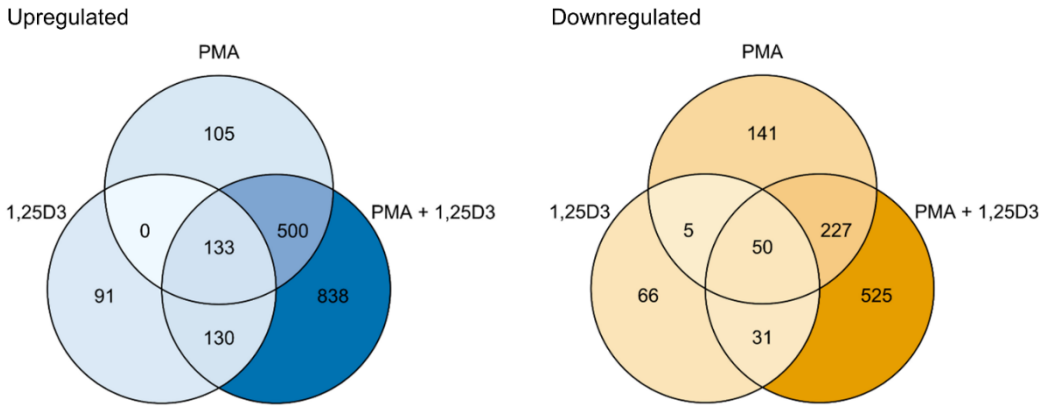


Figure 3.2: THP-1 cells differentiated using PMA with 1,25(OH)$_2$D$_3$ showed the largest alterations in differential gene expression. Venn diagrams illustrating the proportion of upregulated (blue) and downregulated (orange) significantly differentially expressed genes shared and uniquely expressed by THP-1 cells differentiated with 10 nM 1,25(OH)$_2$D$_3$, 5 nM PMA, and 5 nM PMA with 1,25(OH)$_2$D$_3$, with respect to their respective vehicle controls, over 96 hours.

3.3.2. Efficacy of differentiation from the perspective of established macrophage markers

3.3.2.1 Differentiation with PMA and 1,25(OH)$_2$D$_3$ reflects the greatest increase in standard macrophage surface marker mRNA expression

As a confirmation of differentiation, it is commonplace to assess the mRNA or protein expression of specific cell surface markers for macrophages, which commonly include *ITGAM* (CD11b), *ITGAX* (CD11c), *CD14*, *ICAM1* (CD54), *CD36*, and *FCGR1A* (CD64; Hesketh *et al.,* 2017; Mohd Yasin *et al.,* 2022). 1,25(OH)$_2$D$_3$, PMA, and PMA with 1,25(OH)$_2$D$_3$ differentiated THP-1 cells all showed a significant increase in the expression of *ITGAM* (1,25(OH)$_2$D$_3$: log_2FC = 3.49; p<0.001, PMA: log_2FC = 2.38; p<0.001, PMA + 1,25(OH)$_2$D$_3$: log_2FC = 4.39; p<0.001), *CD14* (1,25(OH)$_2$D$_3$: log_2FC = 5.20; p<0.001, PMA: log_2FC = 5.51; p<0.001, PMA + 1,25(OH)$_2$D$_3$: log_2FC = 8.97; p<0.001), and *ICAM1* (1,25(OH)$_2$D$_3$: log_2FC = 2.16; p<0.001, PMA: log_2FC = 3.27; p<0.001, PMA + 1,25(OH)$_2$D$_3$: log_2FC = 5.03; p<0.001) compared to their respective controls (Fig. 3.3). In contrast, the expression of *ITGAX* and *CD36* was only significantly upregulated in PMA (*ITGAX*: log_2FC = 1.64; p<0.001, *CD36*: log_2FC = 1.94; p<0.001) and PMA with 1,25(OH)$_2$D$_3$ (*ITGAX*: log_2FC = 1.95; p<0.001, *CD36*: log_2FC = 2.59; p<0.001) differentiation conditions. The expression of *FCGR1A* was only significantly, but not differentially, upregulated in PMA with 1,25(OH)$_2$D$_3$ differentiated THP-1 cells (log_2FC = 0.45; p=0.002). The magnitude of increase seen for *ITGAM*, *CD14*, *ICAM1*, and *CD36* was greatest in PMA with 1,25(OH)$_2$D$_3$ differentiated THP-1 cells. Notably the expression patterns for *ITGAM* and *CD14* correlated with the respective protein levels (section 2.3.3).

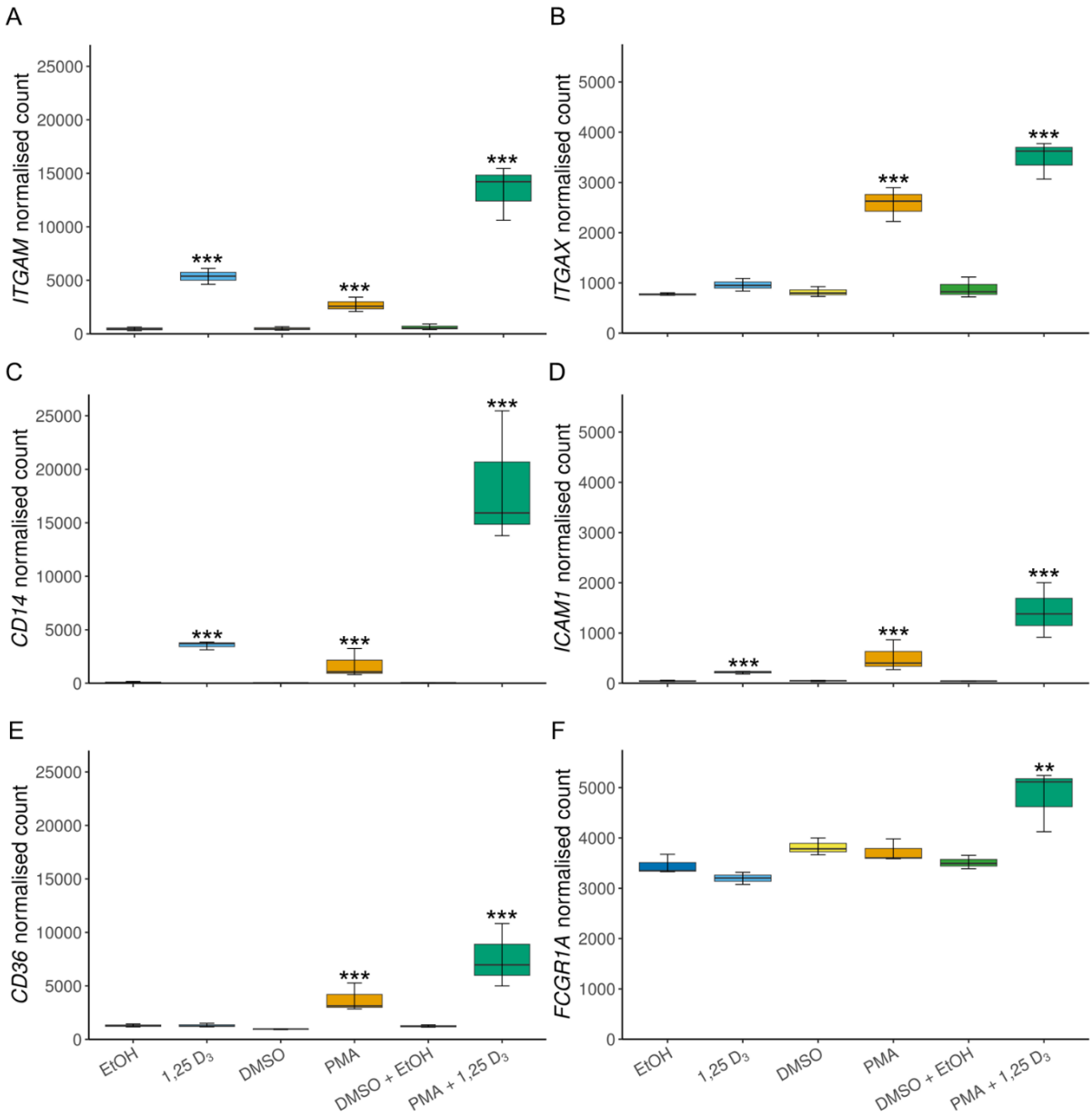


Figure 3.3: THP-1 cells differentiated by PMA with 1,25(OH)$_2$D$_3$ showed the largest increase in macrophage associated cell surface marker gene expression. The boxplots show DESeq2 derived normalised counts for *ITGAM* (A), *ITGAX* (B), *CD14* (C), *ICAM1* (D), *CD36* (E), and *FCGR1A* (F) for the differentiation conditions employed and their respective vehicle controls. Statistically significant differences between differentiation conditions and their respective controls are given based on the Benjamini-Hochberg adjusted *p* values derived from DESeq2 analysis (**p*<0.050, ***p*<0.010, ****p*<0.001).

3.3.2.2 mRNA expression of transcription factors primarily involved in differentiation are principally upregulated in THP-1 cells differentiated with PMA combined with 1,25(OH)$_2$D$_3$

In addition to macrophage surface markers, differentiation state is also assessed based on changes in expression of monocyte and macrophage transcription factors. Perhaps the most well studied example of a macrophage associated transcription factor is PU.1, encoded by *SPI1* (Chistiakov *et al.,* 2018; Orekhov *et al.,* 2019). Additionally, other members of the E26 transformation-specific (ETS) family of transcription factors, including those encoded by *ELF1*, *ETS2*, *EGR2*, and *ETV5*, are implicated in either the restriction of or commitment to macrophage differentiation (Hume *et al.,* 2017). The musculoaponeurotic fibrosarcoma (MAF) family of transcription factors have also been implicated in the control of macrophage differentiation, of which those encoded by *MAF*, *MAFB* and *MITF* have been shown as regulators of this process (Sieweke and Allen, 2013; Tsuchiya, 2015; Hume *et al.,* 2017). Though it is important to note that *MAF* and *MAFB* may also be considered indicators of macrophage polarisation (Kim, 2017; Liu *et al.,* 2020). The expression of *ELF1* and *ETS2* is associated with monocytes and is thought to decrease as differentiation progresses (Sieweke and Allen, 2013; Hume *et al.,* 2017). *ELF1* expression was significantly, but not differentially, reduced in PMA (log_2FC = -0.33; p<0.001) and PMA with 1,25(OH)$_2$D$_3$ (log_2FC = -0.5; p<0.001) differentiated THP-1 cells to a similar extent (Fig. 3.4). In contrast, a significant, though not differential, decrease in *ETS2* was only observed in THP-1 cells exposed to 1,25(OH)$_2$D$_3$ (log_2FC = -0.73; p<0.001) and PMA (log_2FC = -0.43; p<0.001). When considering transcription factors whose expression is associated with macrophages a similar trend was noted to that of the cell surface marker mRNA expression in that PMA with 1,25(OH)$_2$D$_3$ tended to display greater mRNA expression of these genes. THP-1 cells differentiated in the presence of 1,25(OH)$_2$D$_3$ alone resulted in the significantly differentially increased expression of *EGR2* (log_2FC = 2.32; p<0.001) and *MAF* (log_2FC = 2.32; p<0.001), and the significant, but not differential, increase in *MITF* (log_2FC = 0.64; p=0.011). Conversely, THP-1 cells differentiated with PMA and PMA with 1,25(OH)$_2$D$_3$ showed significantly differentially increased expression of *EGR2* (PMA: log_2FC = 4.38; p<0.001, PMA + 1,25(OH)$_2$D$_3$: log_2FC = 6.45; p<0.001), *ETV5* (PMA: log_2FC = 2.37; p<0.001, PMA + 1,25(OH)$_2$D$_3$: log_2FC = 3.21; p<0.001), *MAF* (PMA: log_2FC = 4.02; p<0.001, PMA + 1,25(OH)$_2$D$_3$: log_2FC = 5.82; p<0.001), *MAFB* (PMA: log_2FC = 4.71; p<0.001, PMA + 1,25(OH)$_2$D$_3$: log_2FC = 5.72; p<0.001), *MITF* (PMA: log_2FC = 2.50; p<0.001, PMA + 1,25(OH)$_2$D$_3$: log_2FC = 3.61; p<0.001). Though not differentially expressed, *SPI1* also showed significantly increased expression in PMA (log_2FC = 0.39; p<0.001) and PMA with 1,25(OH)$_2$D$_3$ (log_2FC = 0.72; p<0.001) differentiated THP-1 cells. The magnitude of increased expression for these genes was generally greater for PMA with 1,25(OH)$_2$D$_3$ differentiated THP-1 cells. Collectively, these results indicated that PMA was required to

induce the mRNA expression of many of the well-recognised differentiation associated transcription factors. As with the cell surface markers indicative of macrophage differentiation this PMA induced effect was often heightened by combining PMA with 1,25$(OH)_2D_3$.

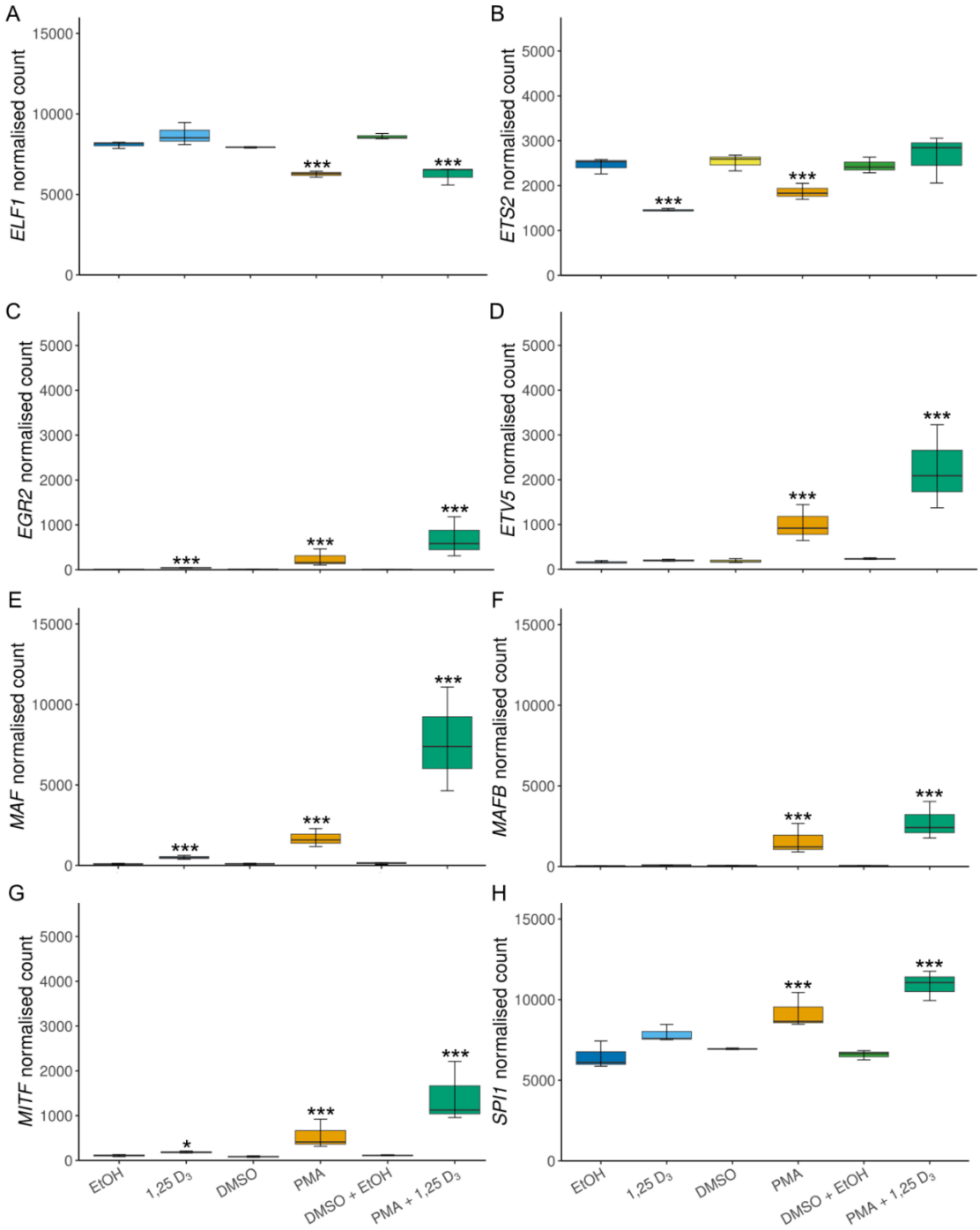


Figure 3.4: THP-1 cells differentiated by PMA with 1,25$(OH)_2D_3$ showed the largest increase in macrophage associated transcription factor encoding genes. The boxplots show DESeq2 derived normalised counts for monocyte associated transcription factor encoding genes *ELF1* (A) and *ETS2* (B), and macrophage associated transcription factor encoding genes *EGR2* (C), *ETV5* (D), *MAF* (E), *MAFB* (F), *MITF* (G), and *SPI1* (H) for the differentiation conditions employed and their respective vehicle controls. Statistically significant differences between differentiation conditions and their respective controls are given based on the Benjamini-Hochberg adjusted *p* values derived from DESeq2 analysis (*p<0.050, **p<0.010, ***p<0.001).

3.3.2.3 The increased mRNA expression of primary macrophage markers is primarily observed in THP-1 cells differentiated with PMA and 1,25(OH)$_2$D$_3$

As a confirmation of differentiation, and as a rudimentary means to establish whether these differentiation conditions produced THP-1-derived macrophages that reflect their primary counterparts, the significantly differentially expressed genes from each differentiation condition were compared to a list of twenty-two primary macrophage marker genes identified by Mulder *et al.* (2021). The expression of these genes was shown to be increased in all macrophages, irrespective of tissue type (Mulder *et al.,* 2021). Of these twenty-two gene markers PMA with 1,25(OH)$_2$D$_3$ differentiated THP-1 cells showed increased significant differential expression of eighteen of these genes (Fig. 3.5). Similarly, PMA showed increased significant differential expression of sixteen of these genes. However, THP-1 cells differentiated with 1,25(OH)$_2$D$_3$ alone only saw the significant differential increase in five of these twenty-two primary macrophage marker genes.

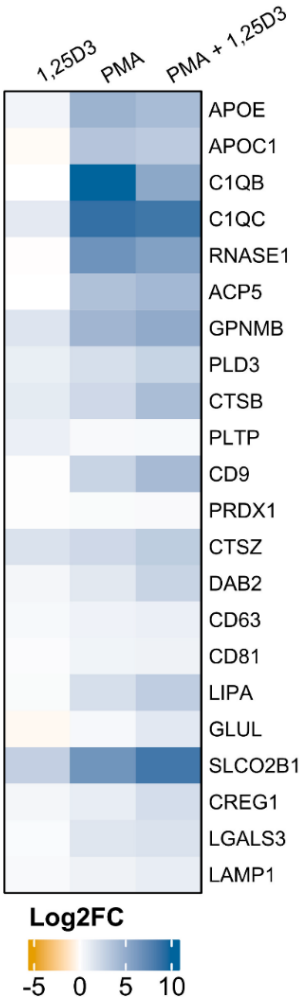


Figure 3.5: THP-1 cells differentiated by PMA with 1,25(OH)$_2$D$_3$ showed the largest increase in the core set of primary macrophage associated genes. Heatmap depicting *log*$_2$FC of selected macrophage marker genes for primary macrophages described by Mulder *et al.* (2021) in each differentiation condition compared to its respective vehicle control.

3.3.3 The influence of differentiation protocols applied on the expression of cell adhesion and phagocytosis related genes

As macrophages represent professional phagocytes, that have left circulation to become tissue-resident cells, they are often characterised by the increased expression of cellular adhesion molecules and as well as those associated with phagocytosis (Padilla *et al.,* 2017; Shapouri-Moghaddam *et al.,* 2018; Pinto *et al.,* 2021). As such, an in-depth analysis of the significantly differentially expressed genes related to these processes was carried out, the *p* values and log_2 fold change values are those generated from the DESeq2 analysis and are given with respect to each differentiation conditions relevant control. It is worth noting that, while this analysis was governed by biological insight, these processes were shown to be significantly over-represented with respect to both GO and KEGG pathway analysis (Fig. S1; Data S1 Table S4-S9).

3.3.3.1 PMA with 1,25(OH)$_2$D$_3$ differentiated THP-1 cells showed the most pronounced increase in B7 family cell adhesion genes

The mRNA expression of the cell adhesion associated B7 ligand family genes, *CD86* and *PDCD1LG2*, were significantly upregulated across all differentiation conditions and the magnitude of this expression appeared to be a function of 1,25(OH)$_2$D$_3$ (Fig. 3.6). The magnitude of increase in *CD86* expression was largest in PMA with 1,25(OH)$_2$D$_3$ differentiated THP-1 cells (log_2FC = 3.44; p<0.001), followed by its expression in PMA (log_2FC = 2.60; p<0.001) and 1,25(OH)$_2$D$_3$ (log_2FC = 1.79; p<0.001) differentiated THP-1 cells. Similarly, the magnitude of increase in *PDCD1LG2* expression was greatest in differentiation conditions employing PMA with 1,25(OH)$_2$D$_3$ (log_2FC = 3.82; p<0.001), followed by 1,25(OH)$_2$D$_3$ (log_2FC = 3.26; p<0.001) and PMA (log_2FC = 1.75; p<0.001). In contrast, *CD274* expression was only significantly upregulated in PMA with 1,25(OH)$_2$D$_3$ (log_2FC = 4.86; p<0.001) and 1,25(OH)$_2$D$_3$ (log_2FC = 4.20; p<0.001) differentiated THP-1 cells, to a similar degree, indicating that its expression was a result of 1,25(OH)$_2$D$_3$ activity.

3.3.3.2 Differential expression of syndecans resulted from differentiation conditions incorporating PMA

The mRNA expression of cell-adhesion related syndecan genes was only significantly differentially expressed in differentiation conditions employing PMA (Fig. 3.6). The expression of *SDC1* was significantly downregulated in PMA with 1,25(OH)$_2$D$_3$ (log_2FC = -1.45; p=0.004) differentiated THP-1 cells. In contrast the expression of *SDC2* was significantly upregulated in both PMA (log_2FC = 2.05; p<0.001) and PMA with 1,25(OH)$_2$D$_3$ (log_2FC = 1.56; p<0.001). Similarly, the expression of *SDC4* was significantly upregulated, to a similar extent, in both

PMA (log_2FC = 1.08; p<0.001) and PMA with 1,25(OH)$_2$D$_3$ (log_2FC = 1.02; p<0.001) differentiated THP-1 cells. Conversely, the expression of *SDC3* was only significantly differentially expressed in PMA with 1,25(OH)$_2$D$_3$ (log_2FC = 1.22; p<0.001) differentiated THP-1 cells. While PMA appears to be required to induce the differential mRNA expression of these genes, the presence of 1,25(OH)$_2$D$_3$ may modulate the effects demonstrated.

3.3.3.3 PMA and 1,25(OH)$_2$D$_3$ shows the most pronounced increase in immunoglobulin superfamily cell adhesion genes

In general, the increased expression of cell-adhesion related immunoglobulin genes was dependent on the presence of PMA, but the magnitude of the difference was influenced by the presence of 1,25(OH)$_2$D$_3$ (Fig. 3.6). The significantly differentially expressed genes in the immunoglobulin superfamily were predominantly upregulated with differentiation, with the notable exception of *NCAM1* expression. As previously indicated the expression of *ICAM1* was significantly differentially expressed in all three differentiation conditions (1,25(OH)$_2$D$_3$: log_2FC = 2.16; p<0.001, PMA: log_2FC = 3.27; p<0.001, PMA + 1,25(OH)$_2$D$_3$: log_2FC = 5.03; p<0.001). *ICAM3* expression was significantly induced in 1,25(OH)$_2$D$_3$ (log_2FC = 1.23; p<0.001) differentiated THP-1 cells alone. *ICAM4* expression on the other hand was only significantly induced in PMA with 1,25(OH)$_2$D$_3$ (log_2FC = 3.52; p<0.001) differentiated THP-1 cells. *CADM1* expression was significantly increased in PMA (log_2FC = 4.53; p<0.001) and PMA with 1,25(OH)$_2$D$_3$ (log_2FC = 3.40; p<0.001) differentiation conditions, with the magnitude of *CADM1* expression greater in PMA differentiated THP-1 cells. Alternatively, a significant decrease in *NCAM1* expression was observed for PMA (log_2FC = -1.27; p<0.001) and PMA with 1,25(OH)$_2$D$_3$ (log_2FC = -2.30; p<0.001) differentiated THP-1 cells. The expression of *TIMD4* was only significantly differentially increased in PMA with 1,25(OH)$_2$D$_3$ (log_2FC = 8.57; p<0.001) differentiated THP-1 cells. The change in expression of *TREM1* appeared to be governed by 1,25(OH)$_2$D$_3$, as its expression was significantly increased in 1,25(OH)$_2$D$_3$ (log_2FC = 4.56; p<0.001) and PMA with 1,25(OH)$_2$D$_3$ (log_2FC = 6.74; p<0.001) differentiated THP-1 cells but not in PMA differentiated THP-1 cells. In contrast, the altered expression of *TREM2* was dependent on PMA, with significant increases observed in 1,25(OH)$_2$D$_3$ (log_2FC = 2.65; p<0.001) and PMA with 1,25(OH)$_2$D$_3$ (log_2FC = 3.34; p<0.001) differentiated THP-1 cells. Genes associated with the siglec family of cell adhesion molecules showed a general increase in expression that was induced primarily by the presence of PMA. *SIGLEC1*, *SIGLEC7*, *SIGLEC9*, *SIGLEC11*, and *SIGLEC16* demonstrated significantly increased expression in PMA and PMA with 1,25(OH)$_2$D$_3$ differentiated THP-1 cells, to a similar degree. In addition to these *SIGLEC14* was only significantly differentially increased in PMA (log_2FC = 1.06; p<0.001) differentiated THP-1 cells. Similarly, in PMA with 1,25(OH)$_2$D$_3$ differentiated THP-1 cells there was a significant increase in *SIGLEC10* (log_2FC = 1.39; p<0.001) and

SIGLEC15 (log_2FC = 1.65; p<0.001) expression. Conversely, the expression of *SIGLEC12* was significantly decreased in PMA (log_2FC = -1.27; p<0.001) and PMA with 1,25$(OH)_2D_3$ (log_2FC = -4.40; p<0.001) differentiated THP-1 cells. Notably, the expression of *SIGLEC6* was only significantly increased in 1,25$(OH)_2D_3$ (log_2FC = 2.44; p<0.001) differentiated THP-1 cells. In general, expression of genes within the immunoglobulin superfamily demonstrated a similar trend that was observed for the other cell adhesion molecules in that PMA was generally required to induce the differential expression of said genes and that by combining PMA with 1,25$(OH)_2D_3$ the expression of these genes generally increased in magnitude.

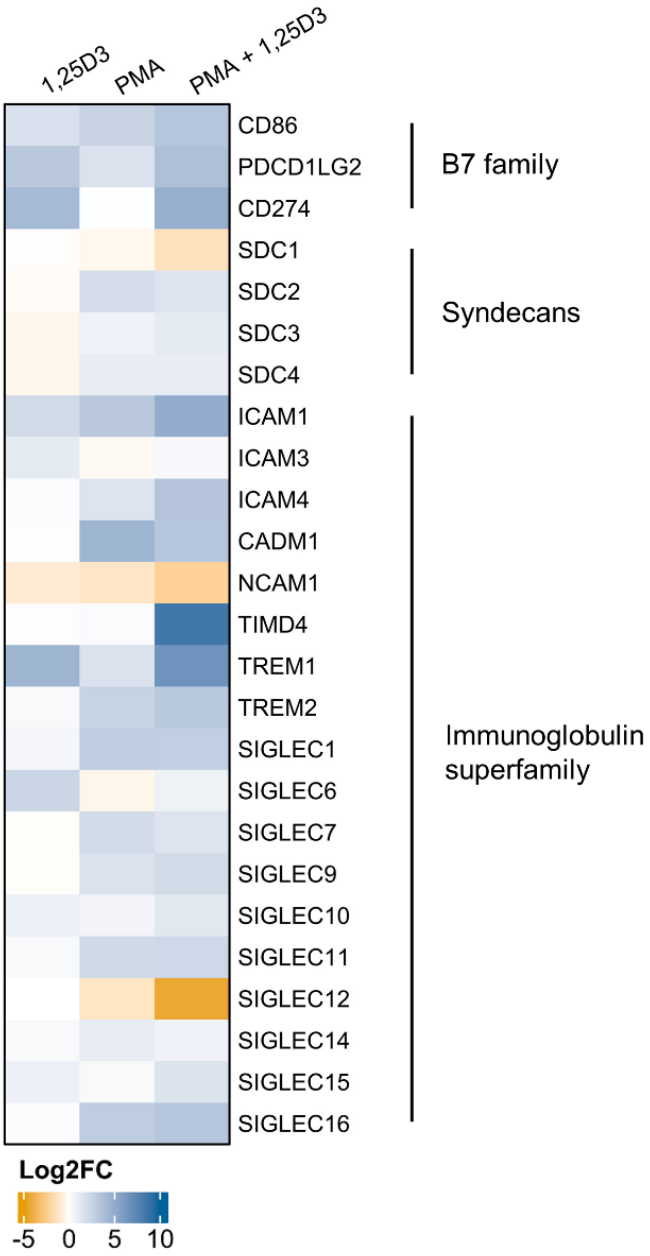


Figure 3.6: PMA with 1,25$(OH)_2D_3$ differentiated THP-1 cells demonstrated the largest increase in mRNA expression of genes encoding cell adhesion proteins. Heatmap depicting log_2FC of selected macrophage cell adhesion associated genes in each differentiation condition compared to its respective vehicle control.

3.3.3.4 The mRNA expression of MHC-I but not MHC-II genes was upregulated in PMA containing differentiation conditions

Of the significantly differentially expressed genes, the expression of classical, non-classical, and pseudo- MHC-I genes were primarily upregulated in PMA and PMA with $1,25(OH)_2D_3$ differentiation conditions (Fig. 3.7). Principally, in PMA and PMA with $1,25(OH)_2D_3$ differentiated THP-1 cells there was significantly differentially increased expression of *HLA-A*, *HLA-B*, *HLA-C*, *HLA-E*, *HLA-F*, and *HLA-H*. $1,25(OH)_2D_3$ differentiated THP-1 cells did not demonstrate this altered MHC-I gene expression except for the significantly increased expression of *HLA-F* (log_2FC = 1.52; p<0.001), though the magnitude was considerably lower than that of the other differentiation conditions. In contrast, the expression of the MHC-II genes, which are frequently used to denote M1 macrophage polarisation, were predominantly unaltered with a few exceptions (Yao *et al.,* 2019). *HLA-DMB* was the only MHC-II gene to have shown significantly increased expression in PMA (log_2FC = 3.26; p<0.001) and PMA with $1,25(OH)_2D_3$ (log_2FC = 4.84; p<0.001) differentiated THP-1 cells. PMA with $1,25(OH)_2D_3$ differentiated THP-1 cells also showed significant reduction in *HLA-DPB1* (log_2FC = -1.10; p<0.001) and *HLA-DQB1* (log_2FC = -1.00; p<0.001) expression. Additionally, the expression of *HLA-DRA* was significantly differentially decreased in $1,25(OH)_2D_3$ (log_2FC = -1.50; p=0.011) differentiated THP-1 cells. These results indicated that PMA containing differentiation conditions demonstrated a notable increase in many of the MHC-I encoding genes but that the same was not observed for the MHC-II encoding genes.

3.3.3.5 The mRNA expression of integrin encoding genes was greatest in PMA containing differentiation conditions

Of the significantly differentially expressed genes related to cell-adhesion and phagocytosis, the gene expression of α-integrins was generally increased, apart from *ITGA2* and *ITGA7*, in PMA and PMA with $1,25(OH)_2D_3$ differentiated THP-1 cells, with the greatest magnitude most frequently observed in the latter (Fig. 3.7). PMA with $1,25(OH)_2D_3$ differentiated THP-1 cells saw a notable decrease in *ITGA2* (log_2FC = -1.20; p<0.001) and *ITGA7* (log_2FC = -1.70; p<0.001) expression. The significantly increased expression of *ITGA6* appeared to be a function of PMA as both PMA (log_2FC = 3.89; p<0.001) and PMA with $1,25(OH)_2D_3$ (log_2FC = 3.87; p<0.001) differentiated THP-1 cells showed a similar magnitude of increase. *ITGA9* expression required PMA but was enhanced by $1,25(OH)_2D_3$, indicated in PMA (log_2FC = 1.24; p<0.001) and PMA with $1,25(OH)_2D_3$ (log_2FC = 2.09; p<0.001) differentiated THP-1 cells. As previously indicated, *ITGAM* was significantly upregulated in all conditions, and the activity of both $1,25(OH)_2D_3$ and PMA appeared to govern this expression ($1,25(OH)_2D_3$: log_2FC = 3.49; p<0.001, PMA: log_2FC = 2.38; p<0.001, PMA + $1,25(OH)_2D_3$: log_2FC = 4.39; p<0.001). In

contrast, *ITGAL* and *ITGAX* expression was primarily driven by PMA. The significantly altered expression of *ITGAL* in PMA (log_2FC = 1.29; p<0.001) and PMA with 1,25(OH)$_2$D$_3$ (log_2FC = 1.65; p<0.001) differentiated THP-1 cells was similar in magnitude. Likewise, and as previously indicated, significantly increased *ITGAX* expression in PMA (log_2FC = 1.64; p<0.001) and PMA with 1,25(OH)$_2$D$_3$ (log_2FC = 1.95; p<0.001) differentiated THP-1 cells was of a comparable magnitude. The significantly differentially expressed β-αgenes, comprised of *ITGB2*, *ITGB4*, *ITGB5* and *ITGB7*. Differentiation conditions that incorporated PMA showed the largest increase in *ITGB2* expression, which was enhanced by 1,25(OH)$_2$D$_3$ (1,25(OH)$_2$D$_3$: log_2FC = 1.20; p<0.001, PMA: log_2FC = 2.09; p<0.001, PMA + 1,25(OH)$_2$D$_3$: log_2FC = 2.95; p<0.001). A similar trend was observed for *ITGB5* expression in that while 1,25(OH)$_2$D$_3$ could induce its expression the magnitude of this expression was greater if PMA was present in the differentiation condition (1,25(OH)$_2$D$_3$: log_2FC = 2.43; p<0.001, PMA: log_2FC = 3.29; p<0.001, PMA + 1,25(OH)$_2$D$_3$: log_2FC = 4.52; p<0.001). Conversely, the expression of *ITGB7* was dependent on the presence of PMA, with the significant increase in its expression in PMA (log_2FC = 3.39; p<0.001) and PMA with 1,25(OH)$_2$D$_3$ (log_2FC = 3.20; p<0.001) differentiated THP-1 cells having a similar magnitude of expression. Notably, the expression of *ITGB4* was significantly, and exclusively, downregulated in PMA (log_2FC = -1.23; P <0.050) differentiated THP-1 cells. These results indicated that the differentiation condition employed affected the expression of key integrins in different ways and to different extents.

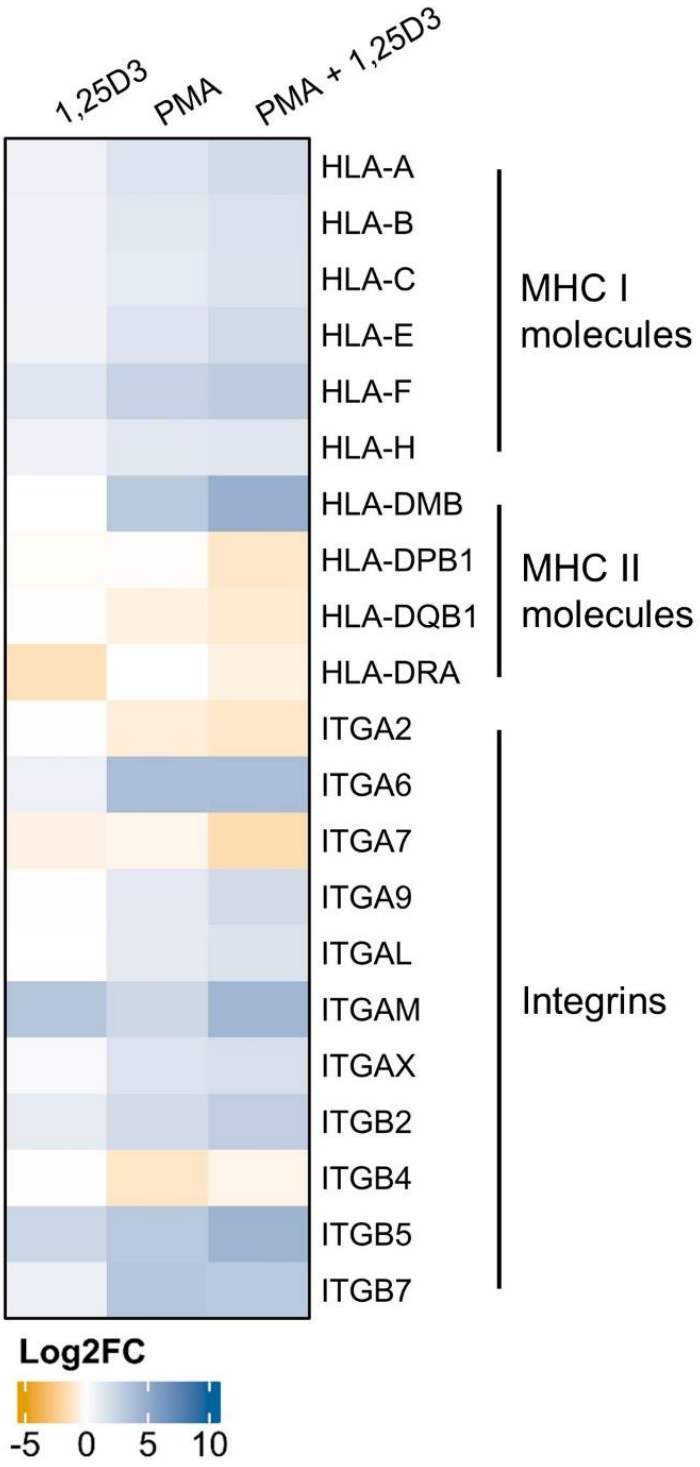


Figure 3.7: PMA with 1,25(OH)$_2$D$_3$ differentiated THP-1 cells demonstrated the largest increase in mRNA expression of genes encoding MHC-I class molecules and integrins. Heatmap depicting *log*$_2$FC of MHC-I and MHC-II class molecules and integrin genes differentially expressed in each differentiation condition compared to its respective vehicle control.

3.3.3.6 PMA with 1,25(OH)$_2$D$_3$ based differentiation showed the greatest mRNA expression of phagocytosis related complement system and receptor genes, as well as Fc receptor genes

There was an overall increase significant differential mRNA expression of phagocytosis promoting receptors in PMA and PMA with 1,25(OH)$_2$D$_3$ differentiated THP-1 cells (Fig. 3.8). PMA with 1,25(OH)$_2$D$_3$ was the only differentiation condition to show a significant differential increase in complement receptor *CR1* (log_2FC = 3.99; p<0.001) mRNA expression. However, the mRNA expression of complement system gene *C2* was significantly upregulated in all three conditions (1,25(OH)$_2$D$_3$: log_2FC = 1.08; p<0.001, PMA: log_2FC = 2.20; p<0.001, PMA + 1,25(OH)$_2$D$_3$: log_2FC = 2.87; p<0.001). The increased mRNA expression of *C3* was also significantly upregulated in PMA with 1,25(OH)$_2$D$_3$ (log_2FC = 1.57; p<0.001) differentiated THP-1 cells. With respect to the Fc receptors, 1,25(OH)$_2$D$_3$ alone had little effect on the differential mRNA expression Fc receptors, apart from *FCGR2B* which was significantly reduced (log_2FC = -1.80; p=0.001). This was not the case in PMA containing differentiation conditions. The expression of *FCAR* and *FCMR* was significantly upregulated in both PMA (*FCAR*: log_2FC = 2.01; p<0.001, *FCMR*: log_2FC = 4.80; p<0.001) and PMA with 1,25(OH)$_2$D$_3$ (*FCAR*: log_2FC = 3.83; p<0.001, *FCMR*: log_2FC = 9.58; p<0.001) differentiation conditions. Likewise, there was a substantial significant increase in *FCGR3A* expression, that was greater in PMA (log_2FC = 6.97; p<0.001) differentiated THP-1 cells when compared to PMA with 1,25(OH)$_2$D$_3$ (log_2FC = 4.68; p<0.001) differentiated THP-1 cells. PMA with 1,25(OH)$_2$D$_3$ differentiated THP-1 cells also saw an increase in *FCGRT* (log_2FC = 1.53; p<0.001) and *FCER1G* (log_2FC = 1.22; p<0.001) expression. However, PMA differentiated THP-1 cells saw a decreased expression of *FCAMR* (log_2FC = -1.09; p=0.015). These results indicated that, by and large, differentiation of THP-1 cells, when in the presence of PMA and enhanced by the presence of 1,25(OH)$_2$D$_3$ therein, resulted in enhanced expression of receptors for opsonised particles.

3.3.3.7 PMA with 1,25(OH)$_2$D$_3$ based differentiation showed the greatest mRNA expression of phagocytosis promoting and inducing TLRS, CLRs and scavenger receptors

Only a subset of PRRs are capable of promoting or inducing phagocytosis, among these are CD14 and the cell surface TLRs, and several CLRs (Iwasaki and Medzhitov, 2015; Fu and Harrison, 2021). Additionally, scavenger receptors and apoptotic cell recognition receptors are capable of inducing phagocytosis (Penberthy and Ravichandran, 2016). As illustrated previously, the expression of *CD14* was significantly increased by all three differentiation conditions, though to different extents (Fig. 3.8). However, 1,25(OH)$_2$D$_3$ differentiated THP-1 cells showed no other differential expression of the cell surface TLRs. Similarly, PMA differentiated THP-1 cells only showed the significantly increased expression of *TLR4* (log_2FC

= 1.28; p<0.001). In contrast, PMA with 1,25$(OH)_2D_3$ differentiated THP-1 cells showed the significantly increased expression of *TLR1* (log_2FC = 2.22; p<0.001), *TLR4* (log_2FC = 1.39; p<0.001), *TLR5* (log_2FC = 1.05; p<0.001), and *TLR6* (log_2FC = 1.3; p<0.001). While the expression of the CLRs was largely dependent on the presence of PMA during differentiation, the magnitude of this expression was largely governed by the presence of 1,25$(OH)_2D_3$ thereafter. In all three differentiation conditions there was a significant induction of *COLEC12* (1,25$(OH)_2D_3$: log_2FC = 2.95; p<0.001, PMA: log_2FC = 5.34; p<0.001, PMA + 1,25$(OH)_2D_3$: log_2FC = 7.09; p<0.001). The expression of *MRC1*, *CD209*, and *CLEC7A* was significantly and substantially induced in PMA differentiated THP-1 cells, furthermore this expression was consistently greater in PMA with 1,25$(OH)_2D_3$ differentiated THP-1 cells. The expression of scavenger receptor *CD163* was significantly induced in all three differentiation conditions (1,25$(OH)_2D_3$: log_2FC = 2.08; p<0.001, PMA: log_2FC = 6.09; p<0.001, PMA + 1,25$(OH)_2D_3$: log_2FC = 6.23; p<0.001). However, the expression of *CD36* was only significantly induced in PMA (log_2FC = 1.94; p<0.001) and PMA with 1,25$(OH)_2D_3$ (log_2FC = 2.59; p<0.001) differentiated THP-1 cells, to a similar magnitude. Likewise, the expression of *MSR1* was significantly increased in PMA (log_2FC = 1.67; p<0.001) and PMA with 1,25$(OH)_2D_3$ (log_2FC = 3.40; p<0.001) differentiated THP-1 cells, although 1,25$(OH)_2D_3$ appeared to enhance this induction of expression. The Tyro3, Axl, and Mer family of receptor-tyrosine kinases is important in the clearance of apoptotic cells and cellular debris and are encoded by *TYRO3*, *AXL*, and *MERTK* (Burstyn-Cohen and Fresia, 2023). *AXL* expression was significantly down regulated in 1,25$(OH)_2D_3$ (log_2FC = -1.30; p<0.001) and PMA with 1,25$(OH)_2D_3$ (log_2FC = -2.30; p<0.001) differentiated THP-1 cells. On the contrary, *MERTK* expression was significantly and substantially induced in PMA (log_2FC = 5.62; p<0.001) and PMA with 1,25$(OH)_2D_3$ (log_2FC = 6.82; p<0.001) differentiated THP-1 cells. It is worth noting that the increased expression of these CLRs and scavenger receptors is particularly associated with M2 polarised macrophages (Binnemars-Postma *et al.,* 2016; Yao *et al.,* 2019; X. Zhang *et al.,* 2023).

3.3.3.8 1,25(OH)$_2$D$_3$ and PMA produced different effects on Rab GTPase mRNA expression

Ras-associated binding **(**RAB) guanosine triphosphate hydrolases (GTPases) regulate vesicle transport and determine vesicle identities, a subset of these Rab GTPases are essential in mediating phagosome formation, phagosome and lysosome fusion (Gutierrez, 2013; Prashar *et al.,* 2017). Of these RAB GTPases *RAB7B* expression required PMA and was significantly increased in PMA (log_2FC = 1.45; p<0.001) and PMA with 1,25(OH)$_2$D$_3$ (log_2FC = 3.05; p<0.001) differentiated THP-1 cells (Fig. 3.8). However, *RAB20* expression was associated with the presence of 1,25(OH)$_2$D$_3$ with significantly increased expression in 1,25(OH)$_2$D$_3$ (log_2FC = 1.04; p<0.001) and PMA with 1,25(OH)$_2$D$_3$ (log_2FC = 2.61; p<0.001) differentiated THP-1 cells. These results indicated that PMA with 1,25(OH)$_2$D$_3$ differentiated THP-1 cells showed the greatest increase in phagosome associated Rab GTPase expression.

3.3.3.9 PMA was required to induce significant differential expression of major lysosomal membrane protein associated genes

The major lysosomal membrane protein encoding genes *CD68*, *LAMP5*, and *SCARB2* were significantly differentially expressed in PMA containing differentiation conditions to a similar degree (Fig. 3.8). The expression of *CD68* was significantly upregulated in PMA (log_2FC = 1.44; p<0.001) and PMA with 1,25(OH)$_2$D$_3$ (log_2FC = 1.76; p<0.001) differentiated THP-1 cells. Likewise, the expression of *SCARB2* was significantly upregulated in PMA (log_2FC = 1.62; p<0.001) and PMA with 1,25(OH)$_2$D$_3$ (log_2FC = 1.66; p<0.001) differentiated THP-1 cells. On the other hand, the expression of *LAMP5* was significantly downregulated in both PMA (log_2FC = -1.60; p<0.001) and PMA with 1,25(OH)$_2$D$_3$ (log_2FC = -2.30; p<0.001) differentiated THP-1 cells. There was also significant differential expression of *LAMP1* in PMA with 1,25(OH)$_2$D$_3$ (log_2FC = 1.04; p<0.001) differentiated THP-1 cells. Alternatively, PMA differentiated THP-1 cells were the only ones that demonstrated significantly increased *LAMP3* expression (log_2FC = 2.14; p<0.001).

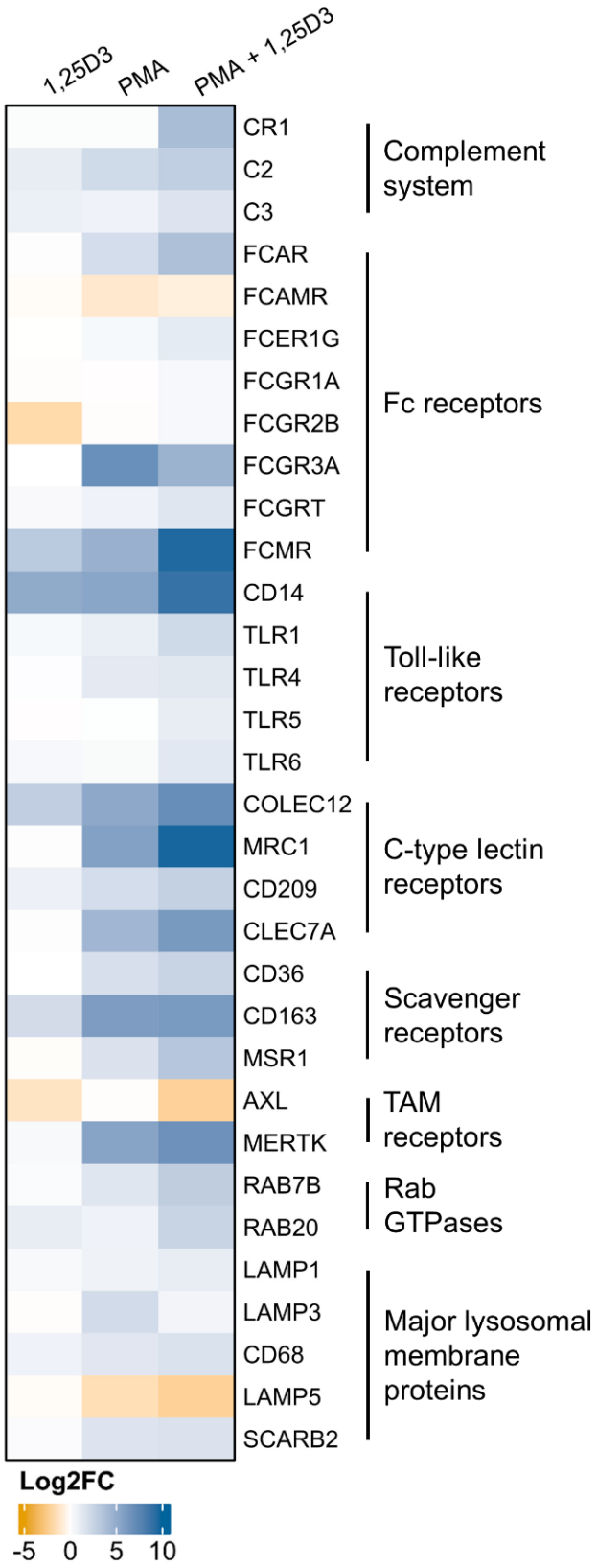


Figure 3.8: PMA with 1,25(OH)$_2$D$_3$ differentiated THP-1 cells demonstrated the largest increase in mRNA expression of genes encoding phagocytosis associated proteins. Heatmap depicting *log*$_2$FC of mRNA expression related to various phagocytosis promoting receptors and phagosome membrane proteins that were differentially expressed in each differentiation condition compared to its respective vehicle control.

3.3.4 Differential mRNA expression of cytokines, chemokines and their receptors

It has been shown that THP-1-derived macrophages tend to develop a propensity toward a particular inflammatory state in accordance with the differentiation protocol employed (Park *et al.,* 2007; Foey and Crean, 2013; Lund *et al.,* 2016; Starr *et al.,* 2018). As such, the differential expression of cytokines, chemokines and their receptors were explored.

3.3.4.1 Differentiation with PMA in the presence or absence of 1,25(OH)$_2$D$_3$ produced alterations in pro-inflammatory and anti-inflammatory cytokine mRNA expression

Overall, there was little alteration in cytokine mRNA expression profiles (Fig. 3.9 A). PMA with 1,25(OH)$_2$D$_3$ differentiated THP-1 cells showed significantly increased *PRL* (log_2FC = 6.09; p<0.001), *IL1B* (log_2FC = 1.92; p<0.001), and *CSF1* (log_2FC = 1.24; p<0.001) expression. The mRNA expression of TNF family cytokines *TNFSF13* and *TNFSF15* were increased in PMA and PMA with 1,25(OH)$_2$D$_3$ differentiated THP-1 cells, the magnitude of *TNFSF15* expression was greater in PMA with 1,25(OH)$_2$D$_3$ differentiated THP-1 cells. In contrast *TNF* was downregulated in PMA (log_2FC = -1.02; p=0.037) differentiated THP-1 cells alone. The expression of the TGF-β superfamily of cytokines did not show a distinct pattern of expression. *TGFB3* expression was upregulated in PMA with 1,25(OH)$_2$D$_3$ (log_2FC = 1.96; p<0.001) differentiated THP-1 cells alone. *GDF11* expression on the other hand was downregulated in PMA with 1,25(OH)$_2$D$_3$ (log_2FC = -1.20; p<0.001) differentiated THP-1 cells alone. Alternatively, *GDF15* expression was upregulated in both PMA (log_2FC = 3.37; p<0.001) and PMA with 1,25(OH)$_2$D$_3$ (log_2FC = 3.77; p<0.001) differentiated THP-1 cells, to a similar degree. The expression of *BMP6* was likewise increased in PMA (log_2FC = 1.32; p=0.019) and PMA with 1,25(OH)$_2$D$_3$ (log_2FC = 4.64; p<0.001) differentiated THP-1 cells, to different degrees. 1,25(OH)$_2$D$_3$ was responsible for the decreased expression of *INHBE* as illustrated in both 1,25(OH)$_2$D$_3$ (log_2FC = -1.40; p=0.004) and PMA with 1,25(OH)$_2$D$_3$ (log_2FC = -2.40; p<0.001) differentiated THP-1 cells. These results indicated that though there was some differential mRNA expression of cytokines in PMA and PMA with 1,25(OH)$_2$D$_3$ differentiated THP-1 cells, no particular class of cytokines was favoured.

3.3.4.2 Differentiation employing 1,25(OH)$_2$D$_3$ combined with PMA produced the largest alteration in cytokine receptor mRNA expression

The significant differential expression of numerous cytokine receptors was primarily observed in PMA with 1,25(OH)$_2$D$_3$ differentiated THP-1 cells, with occasional exceptions (Fig. 3.9 A). For the γ-chain utilising receptors differentiation with PMA with 1,25(OH)$_2$D$_3$ resulted in the significant increase of *IL2RG* (log_2FC = 2.56; p<0.001) expression and significant decrease of *IL2RB* (log_2FC = -4.56; p<0.001), which was also observed for PMA differentiated THP-1 cells

(log_2FC = -2.53; p<0.001). 1,25(OH)$_2$D$_3$ differentiated THP-1 cells saw a significant decrease in *IL7R* (log_2FC = -1.58; p=0.003) expression, not observed in the other two differentiation conditions. Of the IL-4 like cytokine receptors there was a significant decrease in the expression of *IL3RA* in all three conditions (1,25(OH)$_2$D$_3$: log_2FC = -1.44; p<0.001, PMA: log_2FC = -1.24; p<0.001, PMA + 1,25(OH)$_2$D$_3$: log_2FC = -2.98; p<0.001). The mRNA expression of *CSF2RB*, *IL4R*, and *IL13RA1* were significantly differentially induced in PMA with 1,25(OH)$_2$D$_3$ differentiated THP-1 cells alone (*CSF2RB*: log_2FC = 1.35; p<0.001, *IL4R*: log_2FC = 1.81; p<0.001, *IL13RA1*: log_2FC = 1.35; p<0.001). PMA with 1,25(OH)$_2$D$_3$ differentiated THP-1 cells alone showed significant differential mRNA expression of prolactin family receptor genes, wherein *LEPR* expression was increased (log_2FC = 1.31; p<0.001) and *GHR* (log_2FC = -1.52; p=0.002) expression was decreased. When considering the IL-6/12 like cytokine receptors, expression of *IL11RA* was significantly increased and *IL12RB1* was significantly decreased in PMA with 1,25(OH)$_2$D$_3$ differentiated THP-1 cells (*IL11RA*: log_2FC = 1.01; p<0.001, *IL12RB1*: log_2FC = -1.72; p<0.001). Conversely, *IL31RA* expression was only induced in PMA differentiated THP-1 cells (log_2FC = 1.84; p<0.001). *IL10RA* expression was significantly increased in both PMA (log_2FC = 1.69; p<0.001) and PMA with 1,25(OH)$_2$D$_3$ (log_2FC = 3.07; p<0.001) differentiated THP-1 cells, albeit to different extents. The expression of interferon receptors *IFNAR2* and *IFNGR2* were significantly increased in PMA with 1,25(OH)$_2$D$_3$ differentiated THP-1 cells (*IFNAR2:* log_2FC = 1.10; p<0.001, *IFNGR2*: log_2FC = 1.40; p<0.001). *IFNGR1* expression was also increased in both PMA (log_2FC = 2.13; p<0.001) and PMA with 1,25(OH)$_2$D$_3$ (log_2FC = 2.49; p<0.001) differentiated THP-1 cells. The expression of IL-1 like cytokine receptor *IL1R2* was substantially and significantly induced in PMA with 1,25(OH)$_2$D$_3$ (log_2FC = 3.23; p<0.001) differentiated THP-1 cells. The significantly increased expression of *CSF1R* was observed in both PMA (log_2FC = 1.73; p<0.001) and PMA with 1,25(OH)$_2$D$_3$ (log_2FC = 2.67; p<0.001) differentiated THP-1 cells. Of the TNF family of receptors, PMA with 1,25(OH)$_2$D$_3$ differentiated THP-1 cells alone saw the increased expression of *TNFRSF1B* (log_2FC = 1.83; p<0.001), *TNFRSF9* (log_2FC = 3.95; p<0.001*)*, and *TNFRSF10C* (log_2FC = 1.02; p=0.027). The expression of *TNFRSF11A* was significantly increased in PMA (log_2FC = 1.82; p<0.001) and PMA with 1,25(OH)$_2$D$_3$ (log_2FC = 2.35; p<0.001) differentiated THP-1 cells. The mRNA expression of TGF-β receptor *TGFBR3* was downregulated in all three differentiation conditions (1,25(OH)$_2$D$_3$: log_2FC = -1.05; p<0.001, PMA: log_2FC = -1.03; p<0.001, PMA + 1,25(OH)$_2$D$_3$: log_2FC = -1.74; p<0.001). Conversely, the expression of receptors *TGFBR2* and *ACVR1* were increased in PMA with 1,25(OH)$_2$D$_3$ differentiated THP-1 cells (*TGFBR2*: log_2FC = 1.14; p<0.001, *ACVR1*: log_2FC = 1.20; p<0.001). These results indicated that in general differential expression of cytokine receptors resulted from the differentiation of THP-1 cells with PMA combined with 1,25(OH)$_2$D$_3$.

3.3.4.3 The increased mRNA expression of the CC subfamily chemokines and chemokine receptors was primarily observed in PMA with 1,25(OH)$_2$D$_3$ differentiated THP-1 cells

The mRNA expression of CC subfamily chemokines was not differentially expressed in 1,25(OH)$_2$D$_3$ differentiated THP-1 cells (Fig. 3.9 B). An increase in *CCL5* expression was observed for PMA (log_2FC = 1.82; p<0.001) and PMA with 1,25(OH)$_2$D$_3$ (log_2FC = 1.66; p<0.001) differentiated THP-1 cells. Likewise, a notable increase in *CCL24* expression was observed in both PMA (log_2FC = 2.83; p<0.001) and PMA with 1,25(OH)$_2$D$_3$ (log_2FC = 2.49; p<0.001) differentiated THP-1 cells. Though the magnitude of expression differed, the increased expression of *CCL3* was noted in PMA (log_2FC = 2.05; p<0.001) and PMA with 1,25(OH)$_2$D$_3$ (log_2FC = 5.88; p<0.001) differentiated THP-1 cells. However, the significantly differentially increased expression of *CCL3L1* (log_2FC = 2.41; p<0.001), *CCL4* (log_2FC = 6.54; p<0.001), *CCL4L2* (log_2FC = 4.38; p<0.001), and *CCL22* (log_2FC = 1.90; p<0.001) was only observed in PMA with 1,25(OH)$_2$D$_3$ differentiated THP-1 cells. *CCL23* expression on the other hand was significantly reduced in these PMA with 1,25(OH)$_2$D$_3$ (log_2FC = -1.63; p<0.001) differentiated THP-1 cells. There was little alteration in the mRNA expression of the CC subfamily chemokine receptors. *CCR1* expression was significantly induced in all three differentiation conditions, albeit to different extents (1,25(OH)$_2$D$_3$: log_2FC = 1.47; p<0.001, PMA: log_2FC = 1.02; p<0.001, PMA + 1,25(OH)$_2$D$_3$: log_2FC = 2.02; p<0.001). *CCR5* was only significantly differentially increased in PMA differentiated (log_2FC = 2.63; p<0.001) THP-1 cells. These results indicated that the largest contribution to the differential expression of CC subfamily chemokines was seen in PMA with 1,25(OH)$_2$D$_3$ differentiated THP-1 cells.

3.3.4.4 The increased mRNA expression of the CXC subfamily chemokines and chemokine receptors was primarily observed in PMA with 1,25(OH)$_2$D$_3$ differentiated THP-1 cells

The mRNA expression of CXC subfamily chemokines was influenced by both 1,25(OH)$_2$D$_3$ and PMA (Fig. 3.9 B). The increased expression of *CXCL1*, *CXCL6* and *CXCL8* was primarily a function of 1,25(OH)$_2$D$_3$. The increased expression of these genes was of a similar magnitude, for the most part, in both 1,25(OH)$_2$D$_3$ (*CXCL1*: log_2FC = 4.00; p<0.001, *CXCL6*: log_2FC = 3.10; p=0.008, *CXCL8*: log_2FC = 4.77; p<0.001) and PMA with 1,25(OH)$_2$D$_3$ (*CXCL1*: log_2FC = 4.46; p<0.001, *CXCL6*: log_2FC = 4.07; p<0.001, *CXCL8*: log_2FC = 6.06; p<0.001) differentiated THP-1 cells. The expression of *CXCL10* was increased in both PMA (log_2FC = 2.81; p<0.001) and PMA with 1,25(OH)$_2$D$_3$ (log_2FC = 1.38; p=0.007) differentiated THP-1 cells, with the highest expression observed in PMA differentiated THP-1 cells. Increased *CXCL12* expression was observed in both PMA (log_2FC = 2.11; p<0.001) and PMA with 1,25(OH)$_2$D$_3$ (log_2FC = 4.43; p<0.001) differentiated THP-1 cells, with the highest expression observed in PMA + 1,25(OH)$_2$D$_3$ differentiated THP-1 cells. *CXCL16* expression was induced in PMA

(log_2FC = 1.18; p=0.026) and PMA with 1,25(OH)$_2$D$_3$ (log_2FC = 4.11; p<0.001) differentiation conditions and was highest in PMA with 1,25(OH)$_2$D$_3$ differentiated THP-1 cells. There was little alteration in the expression of CXC subfamily chemokine receptors. The expression of *CXCR3* was significantly reduced in all three differentiation conditions (1,25(OH)$_2$D$_3$: log_2FC = -2.04; p<0.001, PMA: log_2FC = -2.05; p<0.001, PMA + 1,25(OH)$_2$D$_3$: log_2FC = -2.98; p<0.001). Conversely, the expression of *ACKR3* was only significantly increased in PMA with 1,25(OH)$_2$D$_3$ differentiated THP-1 cells (log_2FC = 1.63; p=0.015). As with the CC subfamily of chemokines, these results demonstrated that PMA with 1,25(OH)$_2$D$_3$ differentiated THP-1 cells showed the largest alterations in the expression of CXC subfamily chemokines.

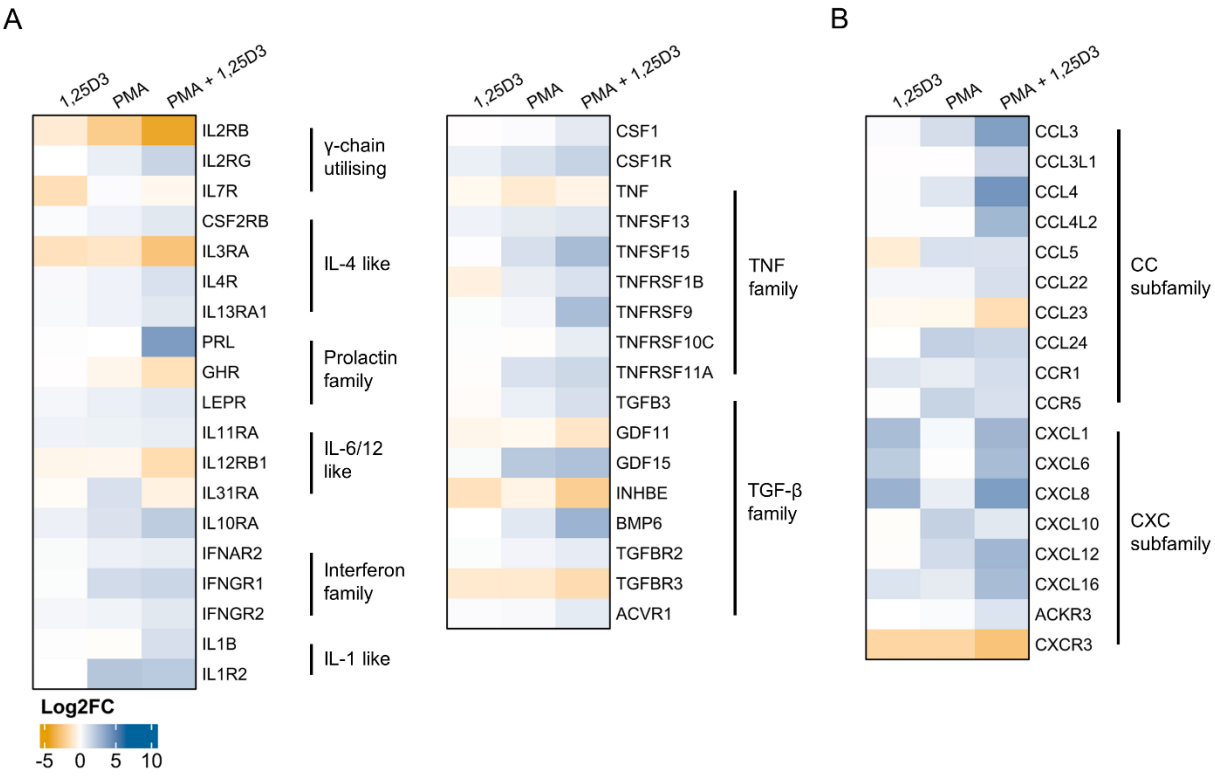


Figure 3.9: PMA with 1,25(OH)$_2$D$_3$ differentiated THP-1 cells showed the largest variation in the mRNA expression of cytokines, chemokines and their receptors. Heatmap depicting the log_2FC of mRNA expression related to the differential expression of various cytokine, chemokines and their receptors in each differentiation condition compared to its respective vehicle control.

3.3.5 Differential expression of transcription factors that drive macrophage polarisation

Previous results indicated significant differential expression of genes frequently associated with macrophage polarisation, particularly those forming part of the B7-family of ligands, toll-like receptors, c-type lectin receptors, scavenger receptors and cytokines. Notably, there was significant differential expression of cell surface receptors and cytokines of M1 macrophage associated *CD86, TLR4*, *IL1B* and *CXCL10* and M2 macrophage associated *MRC1*, *CD163*, *CD209* and *TGFB3* (Murray, 2017; Atri, *et al.,* 2018). As such, it seemed pertinent to assess the expression of transcription factors associated macrophage polarisation (Table 3.1; Chistiakov *et al.,* 2018; Orekhov *et al.,* 2019). When considering the altered expression of M1 macrophage polarisation associated transcription factor encoding genes, there were few significantly differentially expressed genes observed across the different differentiation conditions (Fig. 3.10). In PMA with 1,25$(OH)_2D_3$ differentiated THP-1 cells the expression of *STAT4* was significantly differentially increased (log_2FC = 2.52; p<0.001). Similarly, the increased expression of *BATF2* was seen in both PMA (log_2FC = 1.38; p<0.001) and PMA with 1,25$(OH)_2D_3$ (log_2FC = 1.24; p<0.001) differentiation conditions. In contrast, the expression of *BATF3* was significantly differentially decreased in PMA with 1,25$(OH)_2D_3$ (log_2FC = -2.55; p<0.001) differentiated THP-1 cells. Likewise, *ATF4* expression was significantly differentially reduced in PMA (log_2FC = -1.18; p<0.001) and PMA with 1,25$(OH)_2D_3$ (log_2FC = -1.49; p<0.001) differentiation conditions. In general, when considering the differential expression of M2 polarisation associated transcription factor encoding genes, it could be seen that both PMA and PMA with 1,25$(OH)_2D_3$ differentiated THP-1 cells showed altered expression of these genes. This altered expression tended to be of a greater magnitude in PMA with 1,25$(OH)_2D_3$ differentiated THP-1 cells. The expression of *KLF4* was increased, to a similar extent, across all three differentiation conditions (1,25$(OH)_2D_3$: log_2FC = 1.54; p<0.001, PMA: log_2FC = 1.02; p<0.001, PMA + 1,25$(OH)_2D_3$: log_2FC = 1.7; p<0.001). This was similarly observed for the increased expression of *PPARG* (1,25$(OH)_2D_3$: log_2FC = 1.58; p<0.001, PMA: log_2FC = 1.77; p<0.001, PMA + 1,25$(OH)_2D_3$: log_2FC = 1.74; p<0.001). In PMA and PMA with 1,25$(OH)_2D_3$ differentiated THP-1 cells there was a significant increase in *ATF3* (PMA: log_2FC = 1.52; p<0.001, PMA + 1,25$(OH)_2D_3$: log_2FC = 3.14; p<0.001), and *MAF* and *MAFB* as previously indicated (section 3.3.2.2). The magnitude of this increase was generally larger in PMA with 1,25$(OH)_2D_3$ differentiated THP-1 cells. In PMA with 1,25$(OH)_2D_3$ differentiated THP-1 cells alone there was a significant increase in *PPARD* (log_2FC = 1.06; p<0.001). In addition to these, in PMA with 1,25$(OH)_2D_3$ differentiated THP-1 cells there was also the increased expression of less well defined, in terms of macrophage polarisation, AP-1 family member encoding genes *JUN* (log_2FC = 1.72; p<0.001) and *MAFF* (log_2FC = 3.93; p<0.001).

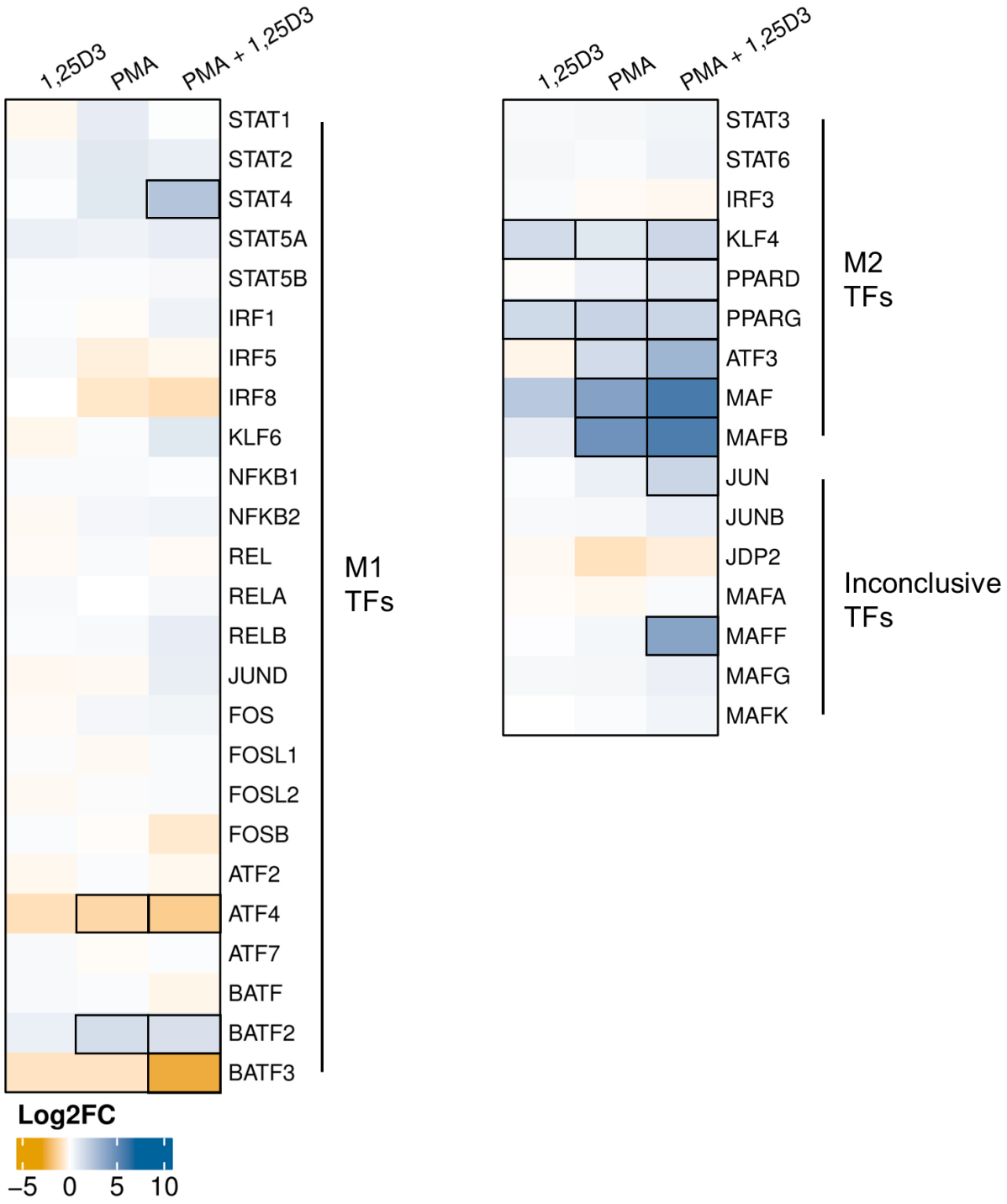


Figure 3.10: PMA and PMA with 1,25(OH)$_2$D$_3$ differentiated THP-1 cells showed increased expression of M2 macrophage polarisation associated transcription factors (TFs). Heatmap depicting the *log*$_2$FC of mRNA expression related to the predominant transcription factor encoding genes associated with M1 and M2 macrophage polarisation in each differentiation condition compared to its respective vehicle control. Genes that were significantly differentially expressed are indicated in with black borders.

3.4. Discussion

THP-1 cells are widely used in the study of monocyte-to-macrophage differentiation, macrophage polarisation and macrophage function. Despite this there is no standard protocol for the differentiation of THP-1 cells to THP-1-derived macrophages. Several groups have indicated that the differentiation protocol applied can produce vastly different THP-1-derived macrophages, with distinct gene expression profiles, resulting in a lack of comparability and reproducibility between studies (Park *et al.,* 2007; Daigneault *et al.,* 2010; Lund *et al.,* 2016; Pinto *et al.,* 2021). Microscopy and flow cytometry (section 2.3.1) indicated that, at least in appearance the use of a low concentration of PMA (5 nM) was able to induce monocyte-to-macrophage differentiation, and that this differentiation could be enhanced by the addition of 10 nM of $1,25(OH)_2D_3$. Herein, RNA sequencing was used to further elucidate the nature of these differentiated THP-1 cells and assess whether based on their gene expression profiles they give clear indication of having undergone differentiation.

It quickly became apparent that though $1,25(OH)_2D_3$ is purported as an inducer of differentiation, there were substantially fewer significantly differentially expressed genes in $1,25(OH)_2D_3$ differentiated THP-1 cells, when compared to their PMA and PMA with $1,25(OH)_2D_3$ counterparts. Furthermore, though distinct from the controls on the PCA, $1,25(OH)_2D_3$ differentiated THP-1 cells clearly lie closer to the monocyte-like THP-1 cells. Similar to what we observed, several studies have shown that phenotypically $1,25(OH)_2D_3$ alone does not produce a macrophage like morphology (Daigneault *et al.,* 2010; Rynikova *et al.,* 2023). Other work has also indicated $1,25(OH)_2D_3$ differentiated THP-1 cells show similar patterns of mRNA expression to undifferentiated THP-1 cells and more closely resemble primary monocytes, rather than monocyte-derived macrophages (H. Liu *et al.,* 2018). Taken together, and in agreement with the morphological analysis (section 2.3.1), it appeared that $1,25(OH)_2D_3$ alone was not capable of producing as pronounced an alteration in THP-1 cells as differentiation conditions employing PMA and that these THP-1 cells are unlikely to represent THP-1-derived macrophages.

Differentiation induced by either PMA or PMA with $1,25(OH)_2D_3$ showed a greater degree of alteration in gene expression, however, they were still distinct from each other. Given the high degree of phenotypic plasticity exhibited by macrophages, and the immunomodulatory nature of $1,25(OH)_2D_3$, it is not surprising that these two differentiation conditions produced macrophages that are not only morphologically distinct, as previously indicated (section 2.3.1), but show large differences in their gene expression profiles. Given that there is evidence that $1,25(OH)_2D_3$ may act to polarise macrophages into a more M2-like state, while PMA is more frequently associated with pro-inflammatory responses, this was not unexpected (Spano *et*

al., 2013; X. Zhang *et al.,* 2015; Lund *et al.,* 2016; Liang *et al.,* 2019). However, the overlap in the gene expression profiles between these two differentiation conditions was still quite large, indicating the maintenance of specific gene expression patterns despite potential duality in induced response.

A panel of well-established monocyte-to-macrophage differentiation associated genes, was assessed to gauge what the presumed effect of these differentiation protocols would be if one were to determine the success of differentiation based on the mRNA expression of a small subset of genes. Regarding the mRNA expression of selected cell surface markers, it could be seen that $1,25(OH)_2D_3$ alone had the lowest potential to induce mRNA expression changes of these markers. PMA was required to induce these changes in most of the selected markers and its efficacy in doing so was enhanced by the addition of $1,25(OH)_2D_3$. This supported the observations made from microscopy and flow cytometry, indicating that these THP-1-derived macrophages are likely the most mature. The requirement of PMA for the induction of differentiation was further supported when considering the increased mRNA expression of key macrophage associated transcription factors *EGR2*, *ETV5*, *MAF*, *MAFB*, *MITF*, and *SPI1*. In general, expression of these genes showed the greatest increase in PMA with $1,25(OH)_2D_3$ differentiated THP-1 cells. Likewise, when considering the short list of primary macrophage marker genes identified by Mulder *et al.* (2021), similar conclusions could be drawn. PMA was required for the induction of the majority of these genes and PMA with $1,25(OH)_2D_3$ differentiated THP-1 cells generally showed the largest increase in expression of these genes. Additionally, this comparison illustrated that, when considering this gene panel, PMA and PMA with $1,25(OH)_2D_3$ differentiated THP-1 cells shared a large degree of resemblance to their primary macrophage counterparts.

From this, albeit limited analysis, it appears that though $1,25(OH)_2D_3$ and PMA differentiated THP-1 cells contribute to the altered mRNA expression of some of these markers, PMA with $1,25(OH)_2D_3$ differentiated THP-1 cells have a more notable increase in all of the appropriate markers. This suggests that should one have selected a similar gene panel for assessment of differentiation, THP-1 cells differentiated using PMA with $1,25(OH)_2D_3$ would likely represent the most mature THP-1-derived macrophages. However, the process of monocyte-to-macrophage differentiation and the resultant nature of the cells produced cannot be so easily encapsulated. A more thorough investigation into the nature of the differentiated THP-1 cells generated from these protocols was assessed based on three of the over-arching alterations that accompany monocyte-to-macrophage differentiation; cell adhesion, phagocytosis and cytokine and cytokine receptor expression (Padilla *et al.,* 2017; Shapouri-Moghaddam *et al.,* 2018; Pinto *et al.,* 2021). In all of these respects, it appeared that PMA was required to induce the bulk of the differential mRNA expression related to these aspects of macrophage biology.

However, despite having little effect by itself, 1,25$(OH)_2D_3$ combined with PMA was generally able to enhance the expression of genes associated with these macrophage characteristics. As such, differential gene expression analysis illustrated that, of the differentiation conditions applied, PMA with 1,25$(OH)_2D_3$ differentiated THP-1 cells had the greatest propensity for cellular adhesion and phagocytosis. This increased expression of cell adhesion molecules, particularly observed in PMA and PMA with 1,25$(OH)_2D_3$ differentiated THP-1 cells, correlated with the observed morphological alterations (section 2.3.1).

With respect to the altered mRNA expression of cell adhesion molecules, it is worth noting that both 1,25$(OH)_2D_3$ and PMA contributed to the increase in B7 family cell adhesion molecules, important in mediating macrophage interactions with T cells, with distinct effects. PMA primarily resulted in the increased gene expression of T-cell co-stimulatory, and thus pro-inflammatory, molecule *CD86*. In contrast, 1,25$(OH)_2D_3$ exposure, in the presence or absence of PMA, resulted in an increase in *CD274* and *PDCD1LG2,* which encode T-cell co-inhibitory molecules, programmed cell death ligand-1 and -2, respectively (Bolandi *et al.,* 2021). This is not unexpected as both genes have been identified as primary 1,25$(OH)_2D_3$ targets and may reflect the role of 1,25$(OH)_2D_3$ as an immunomodulator (Seuter *et al.,* 2013; Neme *et al.,* 2016; Dimitrov *et al.,* 2017). This being said, 1,25$(OH)_2D_3$ in isolation had little effect on the mRNA expression of other cell adhesion molecules. Given that cellular adhesion is a necessary macrophage characteristic to permit leukocyte extravasation and for macrophage interaction within the tissues, this may suggest that these 1,25$(OH)_2D_3$ differentiated THP-1 cells are not reflective of monocyte-derived macrophages.

The pronounced increase in the mRNA expression of syndecan and cell adhesion molecules in PMA and PMA with 1,25$(OH)_2D_3$ differentiated THP-1 cells indicated a proclivity for leukocyte extravasation (Gopal, 2020; Salminen *et al.,* 2020; Lim *et al.,* 2021). Syndecans promote the loose adhesion of leukocytes to the endothelium during leukocyte capture and rolling (Gopal, 2020; Salminen *et al.,* 2020). Additionally, soluble syndecans are also considered important in generating a cytokine gradient and, through a positive feedback loop, in increasing cytokine production (Gopal, 2020). The expression of CAMs, particularly involved in the intravascular crawling during leukocyte migration, was notably higher in PMA and PMA with 1,25$(OH)_2D_3$ differentiated THP-1 cells compared to their respective controls and 1,25$(OH)_2D_3$ differentiated THP-1 cells, except for *ICAM3* and *NCAM1* (Salminen *et al.,* 2020). Increased *ICAM3* expression in 1,25$(OH)_2D_3$ differentiated THP-1 cells again suggest that these cells are more reflective of monocytes, as higher levels of *ICAM3* are detected in monocytes compared to macrophages (Estecha *et al.,* 2012). The decreased expression of *NCAM1* may reflect a reduction in differentiation potential as *NCAM1* expression is associated with monocyte-derived dendritic cells (Milush *et al.,* 2009).

Similar observations were seen in the mRNA expression of siglecs, important immunoglobulin superfamily members involved in cell-cell adhesion as well as cell-ECM adhesion (Lim *et al.,* 2021). Siglecs bind sialic acids which are found on other cells of the body, secreted glycoproteins located within tissues and blood, and in the ECM, in this way the increased expression of many of these siglecs facilitates cell-cell adhesion, cell-matrix adhesion and is involved in the capacity of immune cells to distinguish self vs. non-self (Lübbers *et al.,* 2018; Lim *et al.,* 2021). Generally, monocytes and their derivatives, monocyte-derived macrophages and dendritic cells, express siglec -1, -3, -7, -9, -10, -14 (Lübbers *et al.,* 2018; Gonzalez-Gil and Schnaar, 2021). The expression of siglec -11, -15, -16 is associated with macrophages alone, albeit tissue-resident macrophages (Lübbers *et al.,* 2018). For the most part all the genes encoding these siglecs were upregulated in both PMA containing conditions, implying an enhanced capacity for cell-ECM adhesion, as well as interaction with other immune cells. As *SIGLEC6* expression is associated primarily with cells outside of the myeloid compartment, or dendritic cells, the induction of its expression in $1,25(OH)_2D_3$ differentiated THP-1 cells was unexpected but may lend credence to the notion that they are not committed to macrophage differentiation (Villani *et al.,* 2017). Notably, no THP-1 cells, differentiated or otherwise, showed expression of *SIGLEC3*. The downregulation of *SIGLEC12* observed in both PMA and PMA with $1,25(OH)_2D_3$ differentiated THP-1 cells was an anomaly given that its expression has been noted in macrophages, however, the functionality of SIGLEC12 remains poorly understood (Lim *et al.,* 2021). As such, the influence of the altered expression of this gene cannot be commented upon.

In addition to the increased expression of strictly cell adhesion molecules there was also altered mRNA expression of the apoptotic cell adhesion molecule *TIMD4* and integrators of cell adhesion signalling cascades *TREM1* and *TREM2*, most notably in PMA with $1,25(OH)_2D_3$ differentiated THP-1 cells. The increased mRNA expression of *TIMD4*, which encodes TIM-4, supported the notion that PMA with $1,25(OH)_2D_3$ differentiated THP-1 cells may interact with apoptotic cells, as TIM-4 is responsible for the clearance of apoptotic cells. Its expression is commonly associated with tissue-resident, yolk-sac derived macrophages, specifically Kupffer cells and subcutaneous adipocytes (Lemke, 2019; Zhou *et al.,* 2022). However, there seems to be little to no information on this protein in THP-1 cells, the human protein atlas indicates that it is not expressed in THP-1 cells (Uhlen *et al.,* 2019; https://www.proteinatlas .org/ENSG00000145850-TIMD4/cell+line#leukemia). In agreement, our data showed no reads mapped to the gene in the vehicle control conditions. *TIMD4* expression (i.e. basemean > 20), was only observed for PMA with $1,25(OH)_2D_3$ cells. Notably, in an *in vivo* mouse model, it was shown that for dendritic cells $1,25(OH)_2D_3$ supplementation resulted in decreased *TIMD4* expression at an mRNA level and TIM-4 at a protein level, in contrast to this observation

(Z.Q. Liu *et al.,* 2017). However, this may reflect the purpose of these particular cells rather than the exclusive influence of $1,25(OH)_2D_3$. One of the primary roles of macrophages is the clearance of apoptotic cells and cell debris, as opposed to dendritic cells whose primary function is generally regarded as antigen presentation, contributed to by the phagocytosis of pathogens (Guilliams *et al.,* 2014). Ultimately, this still supports the immunomodulatory function of $1,25(OH)_2D_3$, promoting efferocytosis in macrophages but hobbling antigen presentation to T cells by dendritic cells. The effect of $1,25(OH)_2D_3$ in influencing the immune response was also observed in the altered mRNA expression of triggering receptors expressed on myeloid cells (TREM) encoding genes, *TREM1* and *TREM2*. TREMs are not cell adhesion molecules alone, rather they are important in integrating inflammatory signals with the signals governing leukocyte adhesion (Ford and McVicar, 2009). Evidence suggests that TREM1 and TREM2 have opposite effects in macrophages, with TREM1 shown to support nod-like receptor (NLR) family pyrin domain containing 3 (NLRP3) inflammasome activation whereas TREM2 antagonises this response (Wang *et al.,* 2022; Zhong *et al.,* 2023). The upregulation of *TREM1* seen in both $1,25(OH)_2D_3$ and PMA with $1,25(OH)_2D_3$ differentiated THP-1 cells, attributed to the presence of $1,25(OH)_2D_3$ has been observed before and is associated with the antimicrobial properties of $1,25(OH)_2D_3$ (Kim *et al.,* 2013; Koivisto *et al.,* 2020). In PMA with $1,25(OH)_2D_3$ differentiated THP-1 cells, the concurrent increased expression of *TREM2* could potentially be a compensatory mechanism for this.

Like the immunoglobulin superfamily of genes, the expression of integrins is important not only in facilitating cell adhesion but in guiding immune response particularly observed for phagocytosis. Integrins promote firm cellular adhesion, by linking the cytoskeleton to the ECM, and are important in leukocyte extravasation and are thus key indicators of differentiation (Salminen *et al.,* 2020). The genes encoding several of these integrins were significantly differentially expressed as a result of both $1,25(OH)_2D_3$ and PMA activity, although PMA tended to produce a greater increase in the mRNA expression of integrins. In addition to cell-cell adhesion, integrins are also important in mediating phagocytosis through the attachment of the phagocyte, i.e. macrophage, to the target particle in question (Penberthy and Ravichandran, 2016). The integrin ITGAM/ITGB2 and ITGAX/ITGB2 protein complexes, encoded by *ITGAM*, *ITGAX* and *ITGB2*, which were significantly differentially increased in PMA and PMA with $1,25(OH)_2D_3$ differentiated THP-1 cells, are of particular importance in the phagocytosis of pathogens, forming complement receptor -3 (ITGAM/ITGB2) and -4 (ITGAX/ITGB2) responsible for the recognition of complement molecule iC3b on opsonised targets triggering their uptake by these phagocytes (Ross *et al.,* 1992; Uribe-Querol and Rosales, 2020; Sun *et al.,* 2021). Though there was significant differential expression of *ITGAM* and *ITGB2* in all of the differentiation conditions, and *ITGAX* in both PMA containing

differentiation conditions, the greatest increase in expression was consistently observed in PMA with 1,25$(OH)_2D_3$ differentiated THP-1 cells. This indicates that of the three conditions, with respect to the expression of integrins, these THP-1-derived macrophages may have a greater capacity for cell-ECM adhesion. Additionally, PMA with 1,25$(OH)_2D_3$ differentiated THP-1 cells showed the greatest increased mRNA expression of integrins permitting cell adhesion to an opsonised target particle and support of the phagocytic cascade that follows. While 1,25$(OH)_2D_3$ alone, in many respects fails to induce a range of the features associated with macrophage differentiation, the significant increase in *ITGAM* and *ITGB2* may indicate that 1,25$(OH)_2D_3$ supports the phagocytic role of monocytes more so than the commitment to macrophage differentiation (Rutledge and Muller, 2020).

Following phagocytosis, the presentation of antigens to T cells, which simultaneously relies on cell adhesion, is an inherent function of macrophages. As antigen presentation to T cells is a key aspect of macrophage biology the increased mRNA expression of ubiquitously expressed MHC-I class molecule encoding genes, observed to the greatest extent in PMA with 1,25$(OH)_2D_3$ differentiated THP-1 cells, supports the idea that these cells are primed for antigen presentation (Wieczorek *et al.,* 2017). However, this increased mRNA expression of MHC-I class molecules was not accompanied by the increased mRNA expression of MHC-II class encoding genes. MHC-II expression is particularly associated with professional antigen presenting cells, including M1 polarised macrophages (Wieczorek *et al.,* 2017; Yao *et al.,* 2019). The decreased mRNA expression of MHC-II molecules, *HLA-DRA*, *HLA-DPB1* and *HLA-DQB1* in 1,25$(OH)_2D_3$ and PMA with 1,25$(OH)_2D_3$ differentiated THP-1 cells, indicated a role for 1,25$(OH)_2D_3$ in the reduced expression of these MHC-II molecules, which has been demonstrated before in monocytes (Xu *et al.,* 1993). However, there was increased expression of non-classical MHC-II gene *HLA-DMB* response in PMA and PMA with 1,25$(OH)_2D_3$ differentiated THP-1 cells, which may represent an immune priming response, as the primary function of HLA-DM is to facilitate peptide loading and exchange on the classical MHC-II molecules (Álvaro-Benito *et al.,* 2020). The absence of increased MHC-II class molecule mRNA expression does not reflect a decreased inclination toward phagocytosis, rather a restriction in the presentation of these molecules to T cells. Thus, PMA with 1,25$(OH)_2D_3$ differentiated THP-1 cells showed numerous significantly differentially expressed genes related to the promotion of phagocytosis and phagosome formation.

As professional phagocytes, differentiated macrophages would have an increased expression of receptors capable of detecting opsonised particles, as opsonisation is employed by the immune system to increase the attraction of phagocytes to a molecule in order to enhance phagocytosis (Das *et al.,* 2020). The most well studied opsonins that promote phagocytosis are the complement system and immunoglobulins which are detected by complement receptors and Fc receptors, respectively (Hogarth, 2015; Uribe-Querol and Rosales, 2020). There was an increased mRNA expression of the *C2* complement encoding gene by all three differentiation conditions, illustrating that these cells, though not necessarily displaying the same degree of differentiation, are poised for detection of foreign particles. Given that this increased expression was greatest in PMA with $1,25(OH)_2D_3$ differentiated THP-1 cells, combined with the fact that they were the only cells to show an increased mRNA expression of *C3*, these cells appear to be geared toward phagocytosis to the greatest extent. Furthermore, PMA with $1,25(OH)_2D_3$ differentiated THP-1 cells alone demonstrated a significant differential increase in the mRNA expression of *CR1*, which encodes complement receptor 1, further highlighting the influence of $1,25(OH)_2D_3$ in the increased capacity for phagocytosis for particles opsonised with C1q, C3b and C4b molecules (Uribe-Querol and Rosales, 2020). This increased expression of *CR1* in response to $1,25(OH)_2D_3$ and PMA, albeit separately, has been observed in the promyelocytic cell line HL-60 and is associated with differentiation (Trayner *et al.,* 1998).

In addition to the complement receptors, the increased mRNA expression of opsonic particle binding Fc receptor encoding genes was observed exclusively in the differentiation conditions employing PMA. In both PMA and PMA with $1,25(OH)_2D_3$ differentiated THP-1 cells, the large increases in *FCAR*, *FCMR*, and *FCGR3A* illustrate an enhanced capacity to identify and bind a range of pathogen bound antibodies, containing IgA, IgG and IgM regions (Hogarth, 2015). In addition, PMA with $1,25(OH)_2D_3$ differentiated THP-1 cells also showed significantly differentially expressed *FCGRT* and *FCER1G*. This may illustrate an enhanced capacity for the identification of IgE, which is more frequently associated with the detection of internal parasites (Caraballo and Llinás-Caballero, 2023). Increased expression of *FCGRT* on the other hand may indicate an enhanced capacity for salvaging IgG monomers targeted for degradation during homeostasis and in active infection to enhance both innate and adaptive immunity (Hogarth, 2015; Pyzik *et al.,* 2023). In addition to the enhanced mRNA expression of the opsonic receptors, PMA with $1,25D_3$ differentiated THP-1 cells also showed increased mRNA expression for non-opsonic phagocytosis promoting receptors including TLRs, CLRs and scavenger receptors.

TLRs are not classed as phagocytic receptors, however, certain TLRs can engage with other phagocytic receptors, particularly CD14, in order to stimulate more effective phagocytosis (Kawai and Akira, 2011; Iwasaki and Medzhitov, 2015). As such, the increased mRNA expression of *CD14* in conjunction with *TLR4* expression in PMA differentiated THP-1 cells, and *TLR1*, *TLR4*, *TLR5,* and *TLR6* in PMA with 1,25$(OH)_2D_3$ differentiated THP-1 cells may support the phagocytosis of pathogens, a key function of macrophages (Vidya *et al.,* 2018; Fu and Harrison, 2021). Though 1,25$(OH)_2D_3$ differentiated THP-1 cells showed increased *CD14* expression, as may be expected as *CD14* is a primary 1,25$(OH)_2D_3$ target, this was not accompanied by the increased expression of the TLRs it serves as a co-receptor for (Neme *et al.,* 2019; Nurminen *et al.,* 2019). This indicates that if differentiated, these 1,25$(OH)_2D_3$ differentiated THP-1 cells were unlikely to have the phagocytic potential shown by its PMA and PMA with 1,25$(OH)_2D_3$ counterparts. The substantial increase in C-type lectin associated genes, *COLEC12*, *MRC1*, *CD209*, and *CLEC7A*, seen particularly in the differentiation conditions containing PMA, and to the greatest extent in PMA with 1,25$(OH)_2D_3$ differentiated THP-1 cells, adds credence to the supposed increased phagocytic capacity of these cells which would mark them as more macrophage-like in nature (Fu and Harrison, 2021).

Similarly, the considerable increased mRNA expression of scavenger receptor encoding genes *CD163*, *CD36*, and *MSR1*, seen in PMA and PMA with 1,25$(OH)_2D_3$ differentiated THP-1 cells, indicated a heightened capacity for phagocytosis, more reflective of a macrophage-like state (Mohd Yasin *et al.,* 2022; Taban *et al.,* 2022). Given that phagocytic receptors are frequently used to assess macrophage polarisation, it is worth noting that increased mRNA expression of both M1 macrophage associated *TLR4* and M2 macrophage associated *MRC1*, *CD209* and *CD163* macrophage markers in both PMA and PMA with 1,25$(OH)_2D_3$ differentiated THP-1 cells was observed. This may indicate that these cells were not committed to a particular macrophage polarisation state (Tedesco *et al.,* 2018; Yao *et al.,* 2019; Fernandes *et al.,* 2020; Mohd Yasin *et al.,* 2022).

While pathogen recognition is an important aspect of macrophage biology, the recognition and clearance of apoptotic cells and cell debris is just as relevant, in homeostasis as much as in active infection. The recognition of apoptotic cells relies in part on Axl and Mer tyrosine kinases, encoded by *AXL* and *MERTK* (Lemke, 2019). Differentiation of THP-1 cells with PMA, in the presence or absence of 1,25$(OH)_2D_3$, led to the substantial increase in *MERTK* expression. This once again supported the notion that these cells were more representative of macrophages than THP-1 cells differentiated by 1,25$(OH)_2D_3$ alone. It is worth noting that THP-1 cells exposed to 1,25$(OH)_2D_3$, irrespective of the presence of PMA, showed a

significant reduction in *AXL* expression. This appears to be a yet unreported phenomenon, the purpose of such a reduction may be to balance the rate at which apoptotic cells are cleared and the rate at which anti-inflammatory pathways induced by Tyro3, Axl, and Mer signalling, within phagocytes, are activated (Burstyn-Cohen and Fresia, 2023). This increased mRNA expression of phagocytosis promoting receptors was likewise accompanied by an increased mRNA expression of phagocytic machinery, in the form of the Rab GTPases and the lysosome membrane proteins, in PMA containing differentiation conditions.

Rab GTPases are important mediators of phagosome formation, maturation and phagosome and lysosome fusion (Prashar *et al.,* 2017). The increased mRNA expression of *RAB7B* and *RAB20* in PMA with 1,25$(OH)_2D_3$ differentiated THP-1 cells may suggest a greater capacity for these functions. When considering the mRNA expression of the lysosome associated membrane proteins, the increased expression of *LAMP1*, *CD68*, and *SCARB2* in both PMA and PMA with 1,25$(OH)_2D_3$ differentiated THP-1 cells supports the notion that these cells have a greater predisposition toward phagocytosis (Alessandrini *et al.,* 2017). The exclusive upregulation of *LAMP3* in PMA differentiated THP-1 cells may indicate that they are predisposed toward the colocalization of MHC-II molecules for antigen presentation (Alessandrini *et al.,* 2017). However, this was not accompanied by increased gene expression of classical MHC-II genes. The presence of 1,25$(OH)_2D_3$ appeared to prevent increased expression of *LAMP3*, suggesting that 1,25$(OH)_2D_3$ may prevent MHC-II molecule colocalization, supporting its immunomodulatory role. The decreased expression of *LAMP5* in PMA and PMA with 1,25$(OH)_2D_3$ differentiated THP-1 cells may reflect a limitation in differentiation potential, as *LAMP5* expression, though not well understood, is associated with dendritic cells (Defays *et al.,* 2011; Marongiu *et al.,* 2019). Given that monocyte-derived macrophages are innate immune cells, which have presumably been stimulated in some way to have undergone differentiation, it is not unexpected that following differentiation these cells would have altered cytokine profiles as well as an altered capacity to respond to these cytokines through receptors.

Differential expression analysis clearly indicated that distinct mRNA expression profiles for cytokines and their receptors were generated by the various differentiation conditions. PMA with 1,25$(OH)_2D_3$ differentiated THP-1 cells exclusively exhibited upregulated *IL1B* and *CSF1* expression. Studies have indicated that as part of its antimicrobial function 1,25$(OH)_2D_3$ induces the expression of IL-1β at both a protein and transcript level (Verway *et al.,* 2013; Fernandez *et al.,* 2022). Increased *IL1B* mRNA expression was accompanied by a simultaneous increase in *IL1R2*, whose encoded protein acts as a decoy receptor for IL-1 cytokines as a way of attenuating IL-1 signalling (O'Shea, Gadina and Siegel, 2019). This indicates that even though PMA with 1,25$(OH)_2D_3$ differentiated THP-1 cells showed increased

IL1B expression, they likewise showed increased mRNA expression for a compensatory mechanism. Unlike *IL1B*, the expression of *CSF1* is associated with M2 macrophage polarisation and the generation of an anti-inflammatory environment, reiterating the immunomodulatory effects of $1,25(OH)_2D_3$ (Mia *et al.,* 2014; Orecchioni *et al.,* 2019). Indeed, the increased expression of the CSF-1 receptor encoding gene, *CSFR1*, supports this notion as it is considered an M2 macrophage associated receptor (Roszer, 2015). Additionally, altered mRNA expression of the TNF superfamily in both PMA and PMA with $1,25(OH)_2D_3$ differentiated THP-1 cells likewise showed mRNA expression of both pro- and anti-inflammatory members.

The TNF superfamily of cytokines contain both pro- and anti-inflammatory members. The increased expression of *TNFSF13*, seen particularly in PMA and PMA with $1,25(OH)_2D_3$ differentiated THP-1 cells, is associated with immunosuppression (Chen *et al.,* 2021). In contrast, increased *TNFSF15* expression, also demonstrated by PMA and PMA with $1,25(OH)_2D_3$ differentiated THP-1 cells, is linked to enhanced phagocytosis, anti-microbial functions and pro-apoptotic and anti-angiogenic properties (Sun *et al.,* 2021; Zhao *et al.,* 2022). The significantly decreased expression of *TNF* observed in PMA with $1,25(OH)_2D_3$ differentiated THP-1 cells suggests a reduction of inflammation, as *TNF* encodes a potent pro-inflammatory cytokine, TNF-α (Shapouri-Moghaddam *et al.,* 2018). Higher concentrations of PMA are generally associated with an increase in TNF-α expression, at both an mRNA and protein level (Park *et al.,* 2007; Lund *et al.,* 2016). However, given the low concentration of PMA employed, the presence of a prolonged rest period and the absence of an activating stimulus may have accounted for this decreased *TNF* expression, as it has been indicated that low PMA concentrations may not contribute to increased *TNF* expression (Park *et al.,* 2007; Lund *et al.,* 2016; Giambelluca *et al.,* 2022). The increased expression of genes encoding TNF receptors may suggest a form of immune cell priming, primarily observed in PMA with $1,25(OH)_2D_3$ differentiated THP-1 cells. The increased expression of *TNFRSF11A*, observed in both differentiation conditions employing PMA, may indicate a proclivity of these cells to signal via NF-κB in order to support antiviral immunity, if the activating ligand is bound (Sun, 2017; Dostert *et al.,* 2019). The priming of similar immune supporting functions may be inferred by the increased expression of *TNFRSF1B* and *TNFRSF9*, observed in PMA with $1,25(OH)_2D_3$ differentiated THP-1 cells, as the signalling cascades induced by these receptors are involved in T-cell co-stimulation and the enhanced effectiveness of TNF-α induced apoptosis (Dostert *et al.,* 2019; O'Shea *et al.,* 2019). Similarly, the increased mRNA expression of several other pro- and anti-inflammatory cytokine encoding genes and associated receptors indicated that, although these cells may not illustrate a gene expression profile associated with

an active immune response, there is a large degree of immune cell priming, particularly in PMA with 1,25$(OH)_2D_3$ differentiated THP-1 cells.

The apparent balancing act related to cytokine and cytokine receptor mRNA expression, observed primarily in PMA with 1,25$(OH)_2D_3$ differentiated THP-1 cells, was further reflected in the increased expression of *PRL* and *LEPR*. The expression of prolactin encoding gene, *PRL*, observed exclusively in PMA with 1,25$(OH)_2D_3$ differentiated THP-1 cells, is associated with immunomodulation and tissue regeneration, functions more frequently associated with M2 macrophage polarisation (Costanza *et al.,* 2015). Conversely, in macrophages, leptin interacts with the leptin receptor, encoded by *LEPR*, to promote pro-inflammatory cytokine production and phagocytosis (Mancuso *et al.,* 2018; Kiernan and MacIver, 2021). As such, the concurrent increase in both *PRL* and *LEPR* expression, once again suggests a degree of compensation between pro- and anti-inflammatory pathways, in addition to an enhanced capacity for phagocytosis. This notion was further supported by the altered mRNA expression of TGF-β family cytokines.

The increased mRNA expression of TGF-β family cytokines apart from *GDF11*, principally observed in PMA with 1,25$(OH)_2D_3$ differentiated THP-1 cells, is most frequently associated with immune-modulatory effects, including reduced expression of pro-inflammatory cytokines (Morikawa *et al.,* 2016; Yadin *et al.,* 2016). The decreased expression of *INHBE* in THP-1 cells exposed to 1,25$(OH)_2D_3$ may support these functions. *INHBE* encodes inhibin-beta E, an antagonist for activin A, which inhibits LPS induced cytokine expression, as such, the reduced expression of *INHBE* may contribute to anti-inflammatory effects (Aleman-Muench and Soldevila, 2012). Likewise, the increased expression of *TGFBR2* and *ACVR1,* which encode receptors for TGF-β superfamily members, in PMA with 1,25$(OH)_2D_3$ differentiated THP-1 cells suggests an increased capacity to respond to the TGF-β superfamily cytokines. Additionally, the decreased expression of *TGFBR3* in all three conditions supports this notion as the encoded protein acts as an antagonist to TGF-β signalling and detracts from M2 macrophage polarisation (Yin *et al.,* 2019). *GDF11* mRNA expression was significantly down-regulated in PMA with 1,25$(OH)_2D_3$ differentiated THP-1 cells. The expression of the GDF11 protein, encoded by *GDF11*, has been shown to prevent pro-inflammatory effects and contribute toward M2 polarisation in THP-1 cells differentiated with 162 nM PMA (Duan *et al.,* 2022). Evidence suggests that 1,25$(OH)_2D_3$ does not influence GDF11 protein or mRNA expression (Garcia *et al.,* 2011; Wu *et al.,* 2019). Thus, the reason for this significant reduction in *GDF11* expression cannot be easily accounted for, but it may represent a way in which 1,25$(OH)_2D_3$ balances polarisation states, as has been a general trend observed. Similar to the majority of cytokine expression results, PMA with 1,25$(OH)_2D_3$ differentiated THP-1 cells also showed the largest alterations in the mRNA expression of cytokine receptors.

Though not accompanied by alterations in their related cytokines there was also increased expression of several cytokine receptors, primarily observed in PMA with 1,25$(OH)_2D_3$ differentiated THP-1 cells, suggesting a degree of immune cell priming. The increased mRNA expression of pro-inflammatory related receptors *IL2RG*, *IFNAR2*, *IFNGR1*, and *IFNGR2* indicates that, if presented with the relevant cytokines PMA with 1,25$(OH)_2D_3$ differentiated THP-1 cells may already be primed for a pro-inflammatory response (Garcia-Diaz *et al.,* 2017; Spolski *et al.,* 2018). However, perhaps unsurprisingly considering the immunomodulatory nature of 1,25$(OH)_2D_3$ activity, a concurrent increase in the mRNA expression of the receptors of potent anti-inflammatory cytokines associated with M2 macrophage polarisation, including *IL10RA*, *IL4R*, and *IL13RA1*, was also observed (Roszer, 2015; Suzuki *et al.,* 2015; Junttila, 2018). These general trends were likewise observed in the expression of chemokines and their receptors.

When considering alterations in the mRNA expression of CC subfamily chemokines and their receptors the majority of the differences observed were present in PMA with 1,25D_3 differentiated THP-1 cells. The increased expression of *CCL3*, *CCL3L1*, *CCL4*, *CCL4L2*, and *CCL5* and their receptors *CCR1* and *CCR5*, seen to the greatest extent in PMA with 1,25$(OH)_2D_3$ differentiated THP-1 cells, is associated with pro-inflammatory responses, particularly the recruitment of additional macrophages and natural killer cells, as well as facilitating dendritic cell and T cell interaction (Palomino and Marti, 2015; Poeta *et al.,* 2019; Ma *et al.,* 2021; Lee *et al.,* 2023). Conversely, the increased expression of CCL22 in macrophages is generally considered an anti-inflammatory response and is associated with T cell recruitment and response, particularly that of T-helper 2 and regulatory T cells, and the recruitment of specific natural killer cells, dendritic cells and eosinophils (Ushio *et al.,* 2018; Korobova *et al.,* 2023). As such, the increased *CCL22* mRNA expression observed in PMA with 1,25$(OH)_2D_3$ differentiated THP-1 cells may support these functions and illustrate the immunomodulatory properties of 1,25$(OH)_2D_3$. Likewise, the increased expression of *CCL24* in PMA and PMA with 1,25$(OH)_2D_3$ differentiated THP-1 cells may indicate a proclivity for these cells to engage in the recruitment of eosinophils and in supporting angiogenesis (Palomino and Marti, 2015; Korbecki *et al.,* 2020).

This indication of PMA with 1,25$(OH)_2D_3$ differentiated THP-1 cells demonstrating both pro- and anti-inflammatory chemokine mRNA expression was also seen for the CXC chemokine subfamily. The gene cluster containing *CXCL1*, *CXCL6*, and *CXCL8*, important in neutrophil trafficking, has been shown to be directly regulated by 1,25$(OH)_2D_3$ and may reflect the anti-microbial role of 1,25$(OH)_2D_3$, in addition to potential proangiogenic effects (Ryynänen and

Carlberg, 2013; Cortes *et al.,* 2016). Additionally, increased CXCL12 and CXCL16 expression is associated with proangiogenic effects and guiding the M2 polarisation of recruited macrophages (Sánchez-Martín *et al.,* 2011; M. J. Kim *et al.,* 2019; Jiang *et al.,* 2021; Lee *et al.,* 2021). Their increased mRNA expression, observed in both PMA and PMA with $1,25(OH)_2D_3$ differentiated THP-1 cells, suggests that these conditions may have supported these functions and coincided, at least for PMA with $1,25(OH)_2D_3$ differentiated THP-1 cells, with the functions likely to be induced by the increased expression of the *CXCL1*, *CXCL6*, and *CXCL8* gene cluster. In addition, the increased expression of *ACKR3*, which encodes a receptor for CXCL12, in PMA with $1,25(OH)_2D_3$ differentiated THP-1 cells suggests an increased capacity of these THP-1 derived macrophages to respond to CXCL12 based recruitment and M2 macrophage polarisation (Melo *et al.,* 2018). In contrast, $1,25(OH)_2D_3$ appeared to mitigate the expression of *CXCL10,* otherwise induced by PMA. This downregulation of *CXCL10* by $1,25(OH)_2D_3$ is commonly observed, as *CXCL10* encodes a potent pro-inflammatory chemokine with anti-angiogenic properties, supporting the role of $1,25(OH)_2D_3$ in balancing inflammatory responses while supporting angiogenesis (Korf *et al.,* 2012; Scolletta *et al.,* 2013; Zhao *et al.,* 2017; Karin and Razon, 2018). Notably, there was a significant decrease in the mRNA expression of *CXCR3*, the CXCL10 receptor, in all of the differentiation conditions perhaps indicating a reduced responsiveness to CXCL10 (Palomino and Marti, 2015). In totality, PMA with $1,25(OH)_2D_3$ differentiated THP-1 cells had a diverse cytokine and chemokine profile that is indicative of a degree of priming for anti-microbial response balanced by anti-inflammatory mediators, and chemokines and cytokines that support angiogenesis and wound healing. Notably, PMA differentiated THP-1 cells did not exhibit the excessive pro-inflammatory effects that prohibit responsiveness to weaker stimuli and polarising agents often demonstrated by higher concentrations of PMA (Park *et al.,* 2007; Maeß *et al.,* 2014; Lund *et al.,* 2016; Riddy *et al.,* 2018; Rynikova *et al.,* 2023)

These differentiation conditions produced diverse gene expression profiles, particularly observed in PMA and PMA with $1,25(OH)_2D_3$ differentiated THP-1 cells. Through the analysis of the mRNA expression of genes related to what would be generally considered as hallmarks of macrophage biology, it became apparent that these cells showed features that are used to identify macrophage polarisation states, most notably altered expression of *CD86*, *PDCD1LG2*, *CD274*, *CD163*, *MRC1*, and *CD209*. To explore this further the mRNA expression of the transcription factors identified as guiding and driving macrophage polarisation were assessed. This ultimately illustrated that while PMA and PMA with $1,25(OH)_2D_3$ differentiated THP-1 cells showed alteration in both M1- and M2- polarisation associated transcription factor encoding genes, this effect was more pronounced for the latter.

Certain aspects of transcription factor usage in macrophage polarisation are relatively well defined. Key transcription factors in M1 polarisation include STAT1, STAT2, STAT4, STAT5A, STAT5B, IRF1, IRF5, IRF8, KLF6 and NF-κB (Kaplan, 2005; Tugal *et al.,* 2013; Cunnion *et al.,* 2017; Murray, 2017; Chistiakov *et al.,* 2018; Orecchioni *et al.,* 2019; Orekhov *et al.,* 2019; Emam *et al.,* 2020; Jesser *et al.,* 2021; Chen *et al.,* 2023). Apart from *STAT4*, which exhibited increased expression in PMA with $1,25(OH)_2D_3$ differentiated THP-1 cells, there was little alteration in the expression of these genes. Additionally, the activity of the AP-1 transcription factor is frequently associated with M1 macrophage polarisation, however, several lines of research indicate that this is not the case for all AP-1 family members (Chistiakov *et al.,* 2018; Huang *et al.,* 2018; Orekhov *et al.,* 2019). Of this family, the expression *JUN* was increased in PMA with $1,25(OH)_2D_3$ THP-1 cells, generally suggesting the support of M1 polarisation. However, it is worth noting that *JUN,* and *JUNB,* expression has also been associated with supporting M2 polarisation, as such, its' increased expression may not reflect the support of an M1 polarisation state but rather a balance between polarisation states (Yang *et al.,* 2014; Fontana *et al.,* 2015a; Fontana *et al.,* 2015b).

When considering the ATF family members of the AP-1 transcription factor, there was a fair degree of alteration. The increased expression of *BATF2* in PMA and PMA with $1,25(OH)_2D_3$ differentiation conditions can be associated with M1 polarisation (Roy *et al.,* 2015). However, the significant increase in *ATF3* expression in PMA with $1,25(OH)_2D_3$ differentiated THP-1 cells may suggest the support of M2 polarisation, more so than M1 polarisation (Sha *et al.,* 2017). Likewise, the decreased expression of *ATF4*, observed in both PMA and PMA with $1,25(OH)_2D_3$ differentiation conditions, suggests the support of M2 polarisation as ATF4 has been repeatedly identified as a driver of M1 polarisation (Emam *et al.,* 2020; Gu *et al.,* 2023; Liu *et al.,* 2023). The decreased expression of *BATF3*, observed in PMA with $1,25(OH)_2D_3$ differentiated THP-1 cells, is less likely to act as a predictor for macrophage polarisation in the differentiated THP-1 cells. It is more likely to reflect a decrease in differentiation potential of these cells as *BATF3* expression is principally associated with myeloid-derived dendritic cells (Collin and Bigley, 2018). The MAF family members of the AP-1 transcription factor are less well understood than their counterparts. However, the expression of *MAF* and *MAFB* has been linked to M2 polarisation rather than M1 polarisation (Kim, 2017; Liu *et al.,* 2020). The expression of *MAF* and *MAFB* was increased in both PMA and PMA with $1,25(OH)_2D_3$ differentiated THP-1 cells, though generally higher in the latter.

Of the transcription factors known to regulate M2 macrophage polarisation, STAT3, STAT6, IRF3, IRF4, KLF4, PPAR-δ, and PPAR-γ are among the most well studied (Juhas *et al.,* 2015; Murray, 2017; Chistiakov *et al.,* 2018; Orekhov *et al.,* 2019; Yunna *et al.,* 2020; Boutilier and Elsawa, 2021). While no significant differential gene expression was observed for the M2

polarisation associated STATs and IRFs in any differentiation condition, this was not the case for *KLF4*, and *PPARD* and *PPARG*. The increased expression of *KLF4* and *PPARG*, exhibited in all three differentiation conditions, and *PPARD* in PMA with 1,25$(OH)_2D_3$ differentiated THP-1 cells, illustrates that these cells may be more poised toward M2 polarisation. As the expression of the M2 polarisation associated transcription factors generally exhibited the largest increase in PMA with 1,25$(OH)_2D_3$ differentiated THP-1 cells, this may indicate a role for 1,25$(OH)_2D_3$ in supporting M2 polarisation. Furthermore, given that PMA differentiated THP-1 cells also exhibited the increased expression of M2 polarisation associated transcription factor encoding genes, it is plausible that the use of a lower concentration of PMA would be unlikely to inhibit M2 polarisation, a problem observed with the use of higher concentrations of PMA (Maeß *et al.,* 2014; Lund *et al.,* 2016).

3.5 Conclusions

Overall, 1,25$(OH)_2D_3$ differentiated THP-1 cells shared the potential for several of the cell adhesion and phagocytosis associated macrophage features, demonstrated by PMA and PMA with 1,25$(OH)_2D_3$ differentiated THP-1 cells. However, this was to a considerably lesser extent. Given the gene expression profile of the 1,25$(OH)_2D_3$ differentiated THP-1 cells, in conjunction with the morphological and flow cytometric analysis (section 2.3.1 and section 2.3.3), it was likely that these cells did not accurately represent THP-1-derived macrophages. It is plausible that the THP-1 cells exposed to 10 nM 1,25$(OH)_2D_3$ represent THP-1 monocytes, primed for phagocytosis and differentiation, however, requiring an additional stimulus, such as PMA, to commit to differentiation. While both PMA and PMA with 1,25$(OH)_2D_3$ differentiated THP-1 cells had gene expression profiles that more greatly reflected what may be expected of THP-1-derived macrophages, these features were generally enhanced in PMA with 1,25$(OH)_2D_3$ differentiated THP-1 cells. Likewise, PMA with 1,25$(OH)_2D_3$ differentiated THP-1 cells were the most similar to their primary macrophage counterparts. Markedly, THP-1 cells differentiated with a low concentration of PMA did not exhibit a gene expression profile reflective of a pronounced pro-inflammatory macrophage state, a strong contrast to previous studies that made use of higher PMA concentrations. PMA and PMA with 1,25$(OH)_2D_3$ showed similar potential capacity for phagocytosis and cell adhesion. However, the degree of alteration in mRNA expression of cytokine and chemokine related genes, was generally largest in PMA with 1,25$(OH)_2D_3$ differentiated THP-1 cells and represented an inclination toward a balanced inflammatory state. When considering the mRNA expression of several surface markers and transcription factors associated with macrophage polarisation, both PMA and PMA with 1,25$(OH)_2D_3$ differentiated THP-1 cells showed patterns characteristic of both M1- and M2-polarisation, although more inclined towards M2 polarisation. In summary, PMA and PMA with 1,25$(OH)_2D_3$ differentiated THP-1 cells were most likely to represent THP-1-derived

macrophages, though the latter potentially represented a more mature macrophage. Furthermore, the absence of a pronounced indication of a pro-inflammatory state in PMA and PMA with 1,25$(OH)_2D_3$ differentiated THP-1 cells suggests that these differentiation conditions would be unlikely to interfere with downstream applications, such as, macrophage polarisation or responsiveness to weak stimuli.

4.1 Introduction

The most widely recognised function of bioactive vitamin D_3, $1,25(OH)_2D_3$, is associated with the maintenance of musculoskeletal health, through bone mineralisation, bone remodelling and calcium and phosphorous homeostasis (Latham *et al.,* 2021; Bouillon *et al.,* 2022). $1,25(OH)_2D_3$ has also been shown to exert numerous effects in both innate and adaptive immunity, with $25(OH)D_3$ deficiency being correlated with the pathogenesis of numerous extraskeletal disorders, including increased susceptibility to bacterial, viral, and parasitic infections, as well as a wide range of autoinflammatory disorders (Bellan *et al.,* 2020; Ao *et al.,* 2021; Bishop *et al.,* 2021; Sîrbe *et al.,* 2022). The importance of $1,25(OH)_2D_3$ in immunity is highlighted by the cell-surface expression of CYP27B1, which catalyses the conversion of circulating $25(OH)D_3$ to bioactive $1,25(OH)_2D_3$, on monocytes, macrophages, dendritic cells, T cells and B cells (Bikle *et al.,* 2018). Likewise, these cells all express the VDR, further highlighting the value of $1,25(OH)_2D_3$ in the context of immunity (Häusler and Weber, 2019).

$1,25(OH)_2D_3$ exerts its effects through both genomic and non-genomic actions. When considering the non-genomic effects of $1,25(OH)_2D_3$, the most well understood functions relate to the control of Ca^{2+} and Cl^- channels, the production of secondary messengers, and its interaction with several signalling pathways (Hii and Ferrante, 2016; Gil *et al.,* 2018; Dimitrov *et al.,* 2021). Alternatively, the genomic actions of $1,25(OH)_2D_3$ are primarily mediated through its binding to the transcription factor, VDR (Baker *et al.,* 1988; Kerner *et al.,* 1989; Carlberg, 2022). VDR then favourably, but not exclusively, dimerises with RXR to form an active transcriptional complex, which binds to the regulatory regions of genes referred to as vitamin D response elements (VDREs), altering gene expression (Liao *et al.,* 1990; Haussler *et al.,* 2013; Warwick *et al.,* 2021). If a gene contains a VDRE and shows active binding of VDR, it is generally considered to be a primary $1,25(OH)_2D_3$ target gene.

Monocytes and monocyte-derived macrophages are important innate immune cells with a plethora of functions during both infection and homeostasis. The activity of $1,25(OH)_2D_3$, in relation to the transcriptional alterations wrought by $1,25(OH)_2D_3$, is most well described in monocytes, thanks to the efforts of Carlberg and colleagues (Ryynänen and Carlberg, 2013; Seuter *et al.,* 2013; Neme *et al.,* 2016; Rafique *et al.,* 2018; Nurminen *et al.,* 2019; Koivisto *et al.,* 2020; Warwick *et al.,* 2021). In monocytes, $1,25(OH)_2D_3$ has important functions in antimicrobial defence – through antimicrobial peptide production, iron restriction, altered pattern recognition receptor (PRR) expression, autophagy, and reduced autoinflammation through the antagonism of pro-inflammatory responses (Arababadi *et al.,* 2018; Carlberg, 2019; Dimitrov *et al.,* 2021; Bishop *et al.,* 2021; Ismailova and White, 2022).

Given the close relationship of monocytes and monocyte-derived macrophages, the effects of $1,25(OH)_2D_3$ seen in monocytes are frequently observed in macrophages as well, and the conclusions drawn from monocytes with respect to the influence of $1,25(OH)_2D_3$ are often transferred to macrophages (Sassi *et al.,* 2018; Ao *et al.,* 2021; Ismailova and White, 2022). That being said, it is recognised that $1,25(OH)_2D_3$ produces distinct effects in macrophages, not seen in monocytes, by virtue of the fact that they are ultimately different cell types. This includes the potential influence of $1,25(OH)_2D_3$ on macrophage polarisation, contentiously regarded as an inducer of M2 macrophage polarisation (Foey and Crean, 2013; Nygaard *et al.,* 2022; Rynikova *et al.,* 2023). However, when considering the transcriptional landscape of macrophages in the presence of $1,25(OH)_2D_3$, comparatively little work has been done. Urcuqui-Inchima and colleagues have assessed the influence of $1,25(OH)_2D_3$ on the transcriptome of monocyte-derived macrophages, most frequently in the context of viral infection. This work illustrated that $1,25(OH)_2D_3$ exerts several of its protective effects, through the regulation of pro-inflammatory cytokines, antimicrobial peptide production, and respiratory burst (Fernandez *et al.,* 2022; Valdés-López *et al.,* 2022; Fernandez *et al.,* 2023). Despite considerable interest in the role of $1,25(OH)_2D_3$ in the function of both monocytes and macrophages, as far as could be identified, no direct comparison between these cell types under standardised conditions has been made. Here, we evaluated the influence of $1,25(OH)_2D_3$ on the gene expression profiles of THP-1 monocytes and THP-1-derived macrophages generated under standardised treatment conditions to obtain a more accurate reflection of its activity within these two cell types.

4.2 Materials and Methods

4.2.1 Rationale and study design

Based on the experimental findings presented in Chapter 2 and 3, 10 nM $1,25(OH)_2D_3$ alone is unable to produce THP-1-derived macrophages. As such, using the same cell culture model, it is possible to compare the effects of $1,25(OH)_2D_3$ in THP-1 monocytes and THP-1-derived macrophages. For this analysis, THP-1 cells cultured under vehicle control conditions were thus considered as monocytes in the absence of $1,25(OH)_2D_3$ (monocyte control), while THP-1 cells exposed to 5 nM PMA for 24 hours, followed by a 72-hour rest period, were considered as macrophages in the absence of $1,25(OH)_2D_3$ (macrophage control). THP-1 cells exposed to 10 nM $1,25(OH)_2D_3$ over 96 hours were considered as monocytes in the presence of $1,25(OH)_2D_3$ (monocyte treated), while THP-1-derived macrophages which were cultured in the presence of 10 nM $1,25(OH)_2D_3$ over the entire 96-hour process of differentiation were considered as macrophages in the presence of $1,25(OH)_2D_3$ (macrophage treated).

4.2.2 Analysis of RNA sequencing data and differential gene expression analysis

As this work made use of the RNA sequencing data presented in Chapter 3, the experimental steps outlined in section 3.2.1 and 3.2.2 remained unaltered. Likewise, selective alignment and quantification of transcript abundance (section 3.2.3) was unchanged. Differential expression analyses were also conducted as previously described (section 3.2.4), except that the experimental design was altered, as described in section 4.2.1. Two variance-stabilised unsupervised PCA plots were generated, the first comparing the monocyte control to $1,25(OH)_2D_3$ treated monocytes and the second comparing the macrophage control to $1,25(OH)_2D_3$ treated macrophages. As the work in Chapter 3 indicated that the vehicle controls used for THP-1-derived macrophages in the presence or absence of $1,25(OH)_2D_3$ did not differ from each other, it was not deemed necessary to sequence an additional vehicle control. Differential expression analyses comparing monocytes and macrophages in the presence or absence of $1,25(OH)_2D_3$ were conducted using DESeq2 v1.39.3 (Love *et al.,* 2014), with log fold change shrinkage estimations from apeglm v1.22.1 (Zhu, *et al.,* 2019), as described in section 3.2.4. Pairwise comparisons, which employed the Wald test with Benjamini-Hochberg correction, were conducted between $1,25(OH)_2D_3$ treated and untreated monocytes and between $1,25(OH)_2D_3$ treated and untreated macrophages. Genes were only considered expressed, in the context of the experiment, if they had a within-group base mean cutoff of > 20. Genes were considered to be significantly differentially expressed if they had a Benjamini-Hochberg adjusted p value of < 0.05 and a $|log_2FC| > 1$. p values given are with respect to comparison of the treated and untreated groups for the monocytes and macrophages, respectively. Over-representation analysis of GO biological process terms was done as described in section 3.2.5. This was done for the complete set of differentially expressed genes in monocytes and macrophages treated with $1,25(OH)_2D_3$ those which were significantly up- or down-regulated, and those that were shared between or unique to $1,25(OH)_2D_3$ treated cells.

4.3 Results

4.3.1 1,25(OH)$_2$D$_3$ generated distinct gene expression profiles in monocytes and macrophages

As the RNA sequencing data was shown to be of sufficient quality for subsequent analysis (section 3.3.1), here we focused on the distinct effects of 10 nM 1,25(OH)$_2$D$_3$ on THP-1 monocytes and THP-1-derived macrophages. Clustering analysis, achieved through an unsupervised PCA indicated that 10 nM 1,25(OH)$_2$D$_3$ generated distinct gene expression profiles in the monocytes and the macrophages when compared to their respective controls (Fig. 4.1). In the monocytes, exposure to 10 nM 1,25(OH)$_2$D$_3$ for 96 hours correlated with PC1 and accounted for 86% of the variance observed between the monocytes in the presence and absence of 1,25(OH)$_2$D$_3$ (Fig 4.1 A). Likewise, the presence or absence of 1,25(OH)$_2$D$_3$ in the macrophages correlated with PC1, which accounted for 83% of the variance observed (Fig 4.1B). 1,25(OH)$_2$D$_3$ treatment resulted in the significant differential expression of 506 (354 upregulated, 152 downregulated) and 745 (582 upregulated, 163 downregulated) genes in the monocytes and the macrophages, respectively (Fig 4.2, Data S1 Table S10-S11). Of these only 212 significantly differentially expressed genes were shared between the two cell types, 117 upregulated, 30 downregulated and five genes that showed inverse regulation. When considering differential gene expression unique to 1,25(OH)$_2$D$_3$ treated monocytes and macrophages, 294 and 533 significantly differentially expressed genes were identified, respectively.

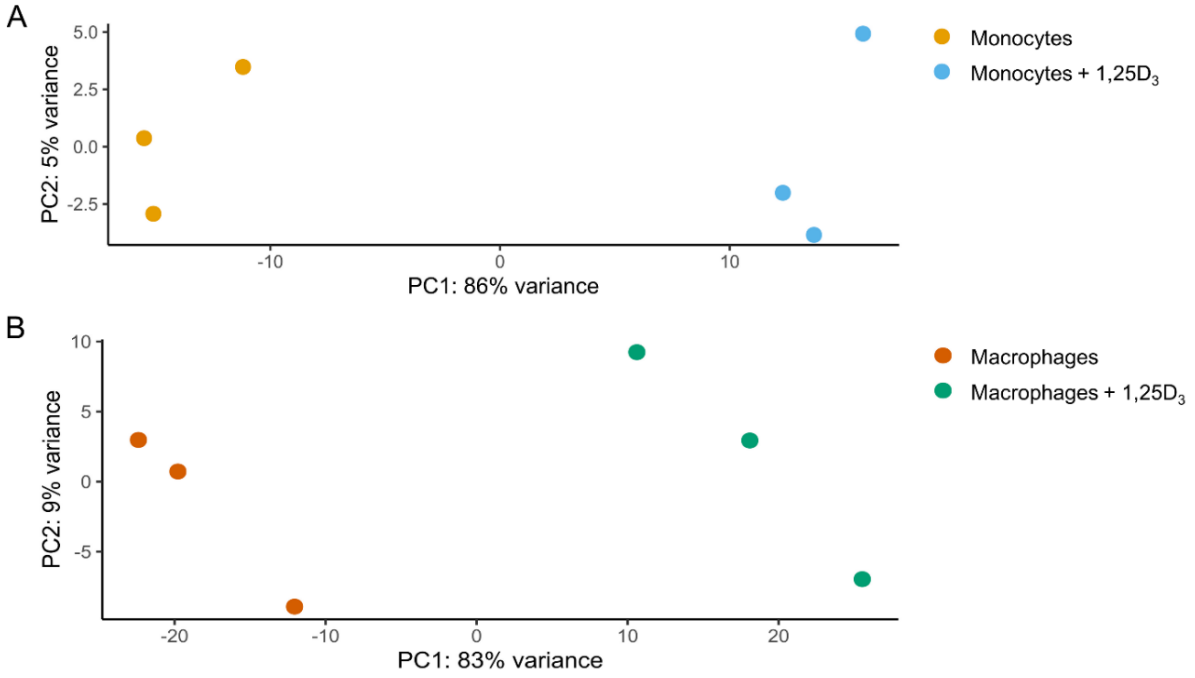


Figure 4.1: Principal component analysis (PCA) for RNA sequencing data indicated distinct clustering for monocytes and macrophages treated with 1,25(OH)$_2$D$_3$. The unsupervised PCA plot generated from the variance stabilised RNA sequencing data illustrates distinct clusters for monocytes (A) and macrophages (B) exposed to 10 nM 1,25(OH)$_2$D$_3$ over 96 hours (n = 3).

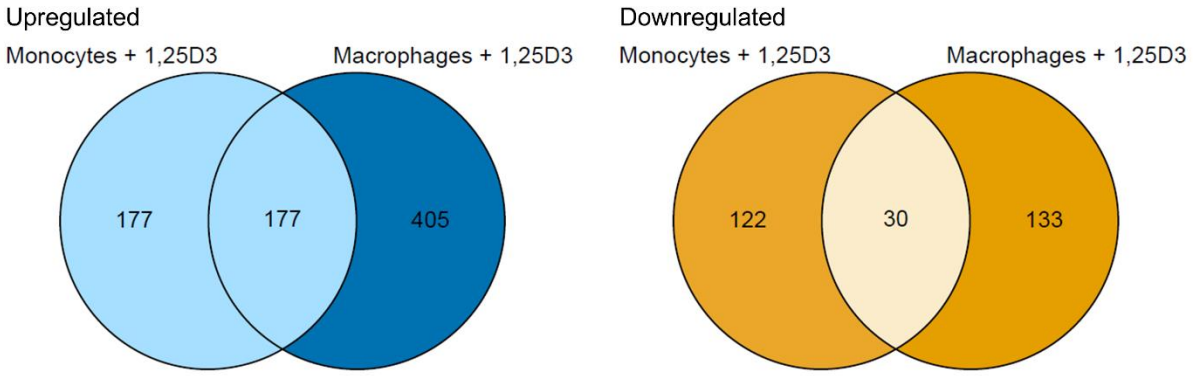


Figure 4.2: $1,25(OH)_2D_3$ treated macrophages showed the largest alterations in differential gene expression. Venn diagrams illustrating the proportion of upregulated (blue) and downregulated (orange) significantly differentially expressed genes shared and uniquely expressed by monocytes and macrophages treated with 10 nM $1,25(OH)_2D_3$ over 96 hours.

4.3.2 $1,25(OH)_2D_3$ treated monocytes and macrophages share numerous primary $1,25(OH)_2D_3$ target genes

To assess the presence of primary $1,25(OH)_2D_3$ targets, genes significantly altered ($P < 0.05$) in response to $1,25(OH)_2D_3$ treatment in the monocytes and macrophages were compared to a curated list of 189 known primary $1,25(OH)_2D_3$ targets in THP-1 cells (Nurminen *et al.*, 2019). In $1,25(OH)_2D_3$ treated monocytes, 166/189 of these were significantly altered, with 108 having shown significant differential (i.e. $|log_2FC| > 1$) expression (Fig 4.3). In $1,25(OH)_2D_3$ treated macrophages, 143/189 were significantly altered, with 94 genes having shown significant differential expression. Of these, 76 were significantly differentially expressed in both $1,25(OH)_2D_3$ treated monocytes and the macrophages, 75 of which were upregulated. Among the shared upregulated genes, were several transcription factor encoding genes including *BCL6*, *NFE2*, and *NR1I2* (Fig. 4.4A). The only exception was the expression of *PNOC,* which was increased in the monocytes ($log_2FC = 1.28$; $p<0.001$) but reduced in the macrophages ($log_2FC = -1.44$; $p<0.001$; Fig. 4.4B). Despite this converse regulation in the expression of *PNOC* compared to the relevant controls, if one considers the normalised count data derived from DESeq2 the expression level of *PNOC* did not appear to be largely different between $1,25(OH)_2D_3$ treated monocytes and $1,25(OH)_2D_3$ treated macrophages. In addition to the 76 shared primary $1,25(OH)_2D_3$ targets, $1,25(OH)_2D_3$ treated monocytes showed the significant differential expression of a further 32 (30 upregulated, 2 downregulated) primary $1,25(OH)_2D_3$ target genes not seen in the macrophages. Likewise, $1,25(OH)_2D_3$ treated macrophages showed the significant differential expression of eighteen primary $1,25(OH)_2D_3$ targets genes (17 upregulated, 1 downregulated) not observed in the monocytes.

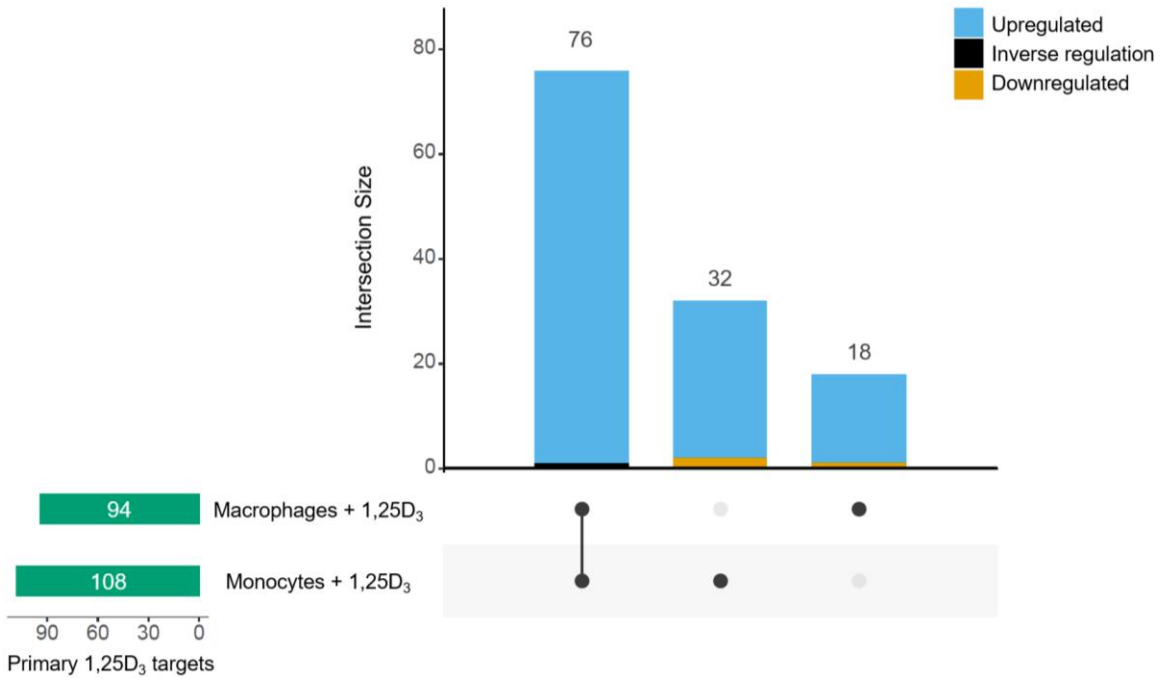


Figure 4.3: The largest proportion of primary $1,25(OH)_2D_3$ targets are shared between $1,25(OH)_2D_3$ treated monocytes and macrophages. The upset plot depicts the proportion of significantly differentially expressed genes that are recognised primary vitamin D_3 targets that are unique to $1,25(OH)_2D_3$ treated monocytes and macrophages and those that are shared between the two.

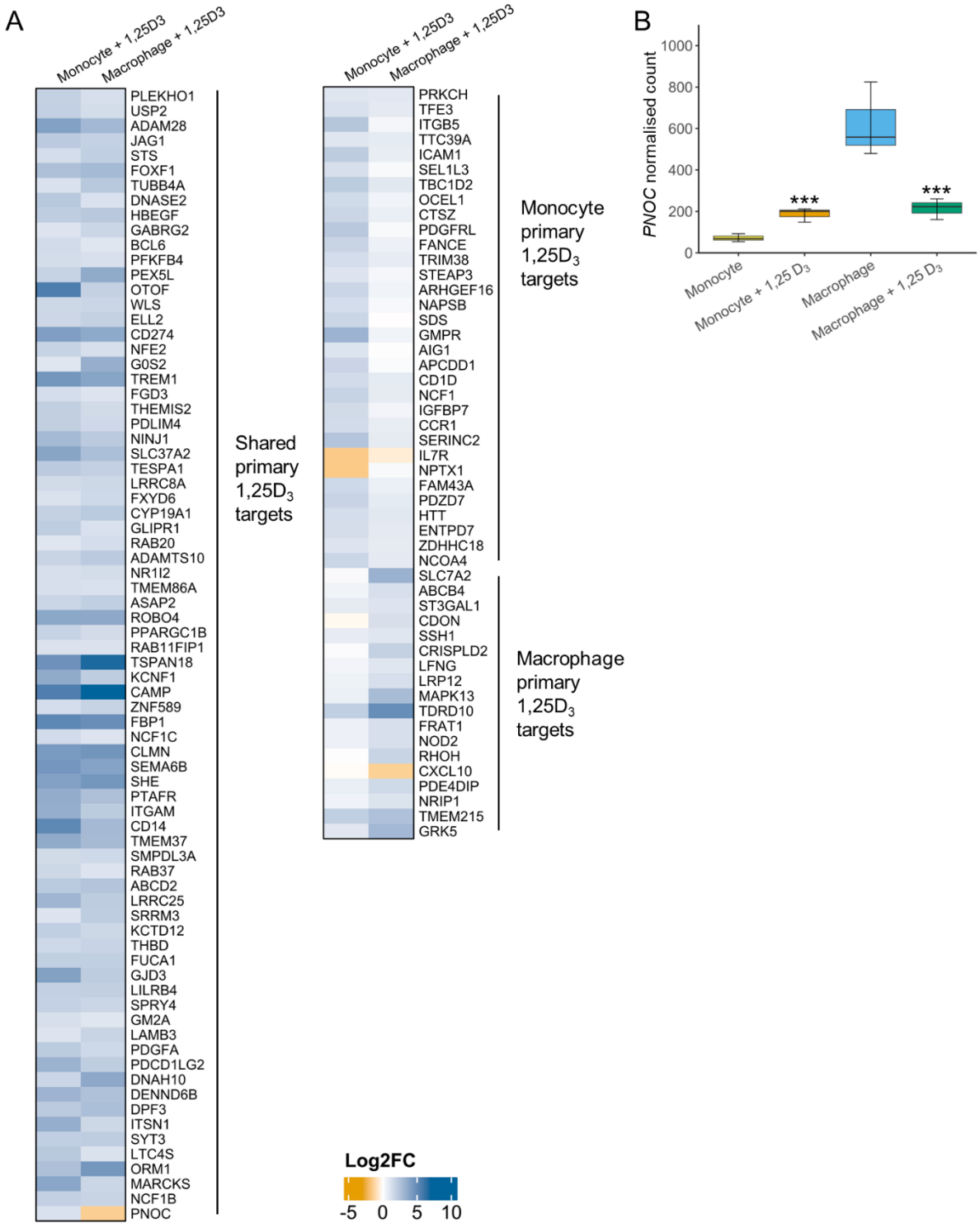


Figure 4.4: Majority of the significantly differentially expressed primary 1,25(OH)$_2$D$_3$ target genes were shared between 1,25(OH)$_2$D$_3$ treated monocytes and macrophages. Heatmap depicting *log*$_2$FC of known primary 1,25(OH)$_2$D$_3$ target genes shared between and unique to 1,25(OH)$_2$D$_3$ treated monocytes and macrophages (A). The boxplot shows DESeq2 derived normalised counts for *PNOC* in the monocytes and macrophages in the presence and absence of 1,25(OH)$_2$D$_3$ (B). Statistically significant differences between 1,25(OH)$_2$D$_3$ treated and untreated monocytes and macrophages, respectively, are given based on the Benjamini-Hochberg adjusted *p* values derived from DESeq2 analysis (*p<0.050, **p<0.010, ***p<0.001).

4.3.3 An overview of the functional potential of 1,25(OH)$_2$D$_3$ responsive genes in monocytes and macrophages

To gain an understanding of the potential physiological implications of 1,25(OH)$_2$D$_3$ activity in monocytes and macrophages, an over-representation analysis for GO biological processes was conducted. This was done for all significantly differentially expressed genes for 1,25(OH)$_2$D$_3$ treated monocytes and macrophages, respectively (Fig. 4.5; Data S1 Table S12-S13) as well as for upregulated and downregulated genes separately (Fig 4.6 A-C; Data S1 Table S14-S16). No significant over-representation for GO biological processes was observed among genes downregulated in 1,25(OH)$_2$D$_3$-treated monocytes. To gain further clarity as to the distinct alterations in gene expression observed for 1,25(OH)$_2$D$_3$ treated monocytes and macrophages, the same analysis was performed for the differentially expressed genes shared between both cell types, as well as those unique to each, respectively (Fig 4.6 D-F; Data S1 Table S17-S19). A large portion of the GO biological processes seen to be over-represented in 1,25(OH)$_2$D$_3$ treated monocytes and macrophages were shared and related to immune functionality. As may be expected from the analyses in Chapter 3, among the over-represented GO biological processes for both the monocytes and macrophages in the presence of 1,25(OH)$_2$D$_3$, were processes related to cell-adhesion and phagocytosis (section 3.3.3). Likewise, among the top over-represented biological processes was the production and regulation of cytokines, chemokines, and their receptors and processes related therein, including, immune cell activation, chemotaxis, and migration (section 3.3.4).

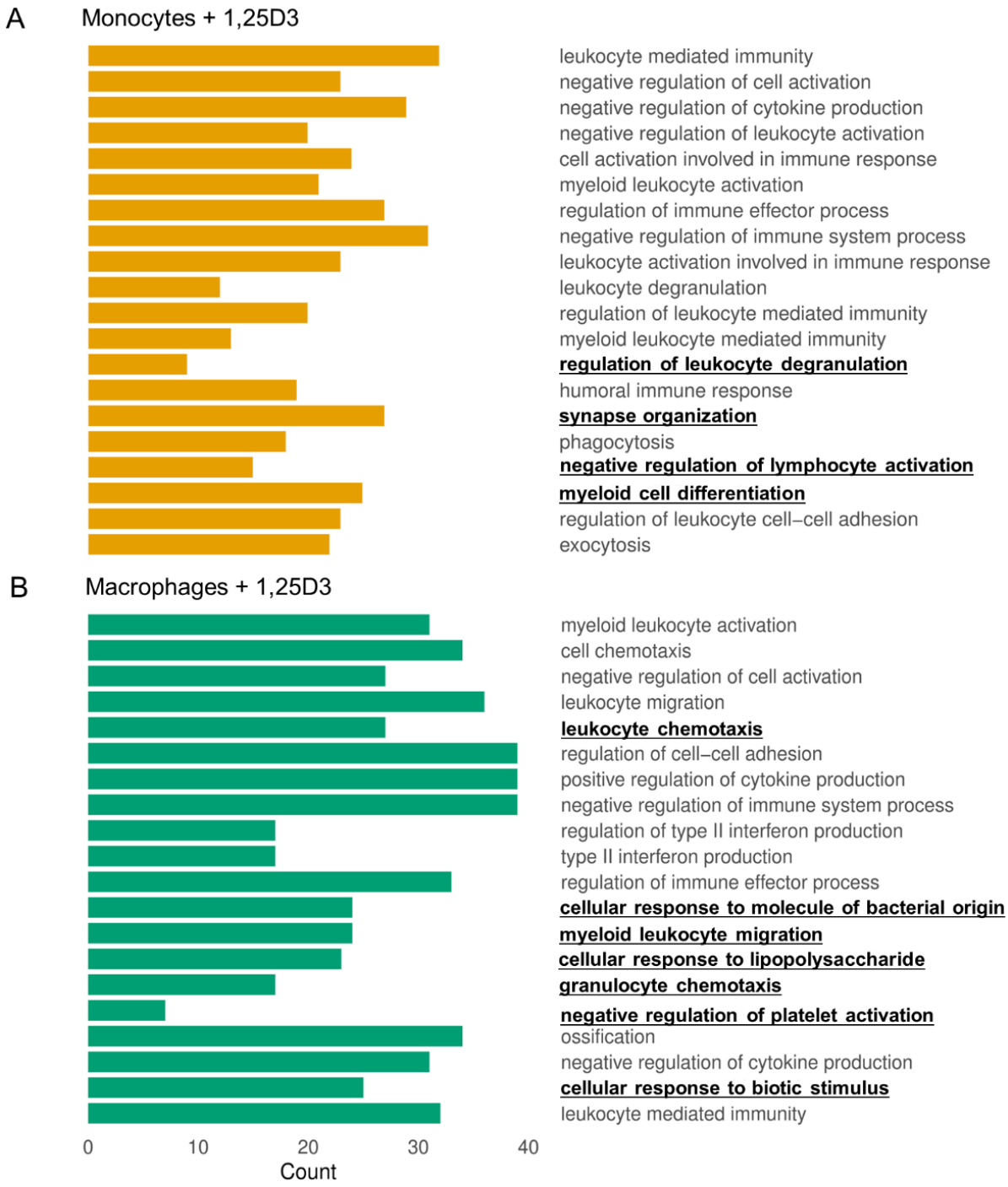


Figure 4.5: Similar biological processes were over-represented among genes differentially expressed in $1,25(OH)_2D_3$ treated monocytes and macrophages. Top 20 significantly over-represented GO biological processes (adj. *p* value < 0.010) in $1,25(OH)_2D_3$ treated monocytes (A) and $1,25(OH)_2D_3$ treated macrophages (B). Biological processes in bold and underlined are those which were significantly over-represented in either $1,25(OH)_2D_3$ treated monocytes or $1,25(OH)_2D_3$ treated macrophages, alone.

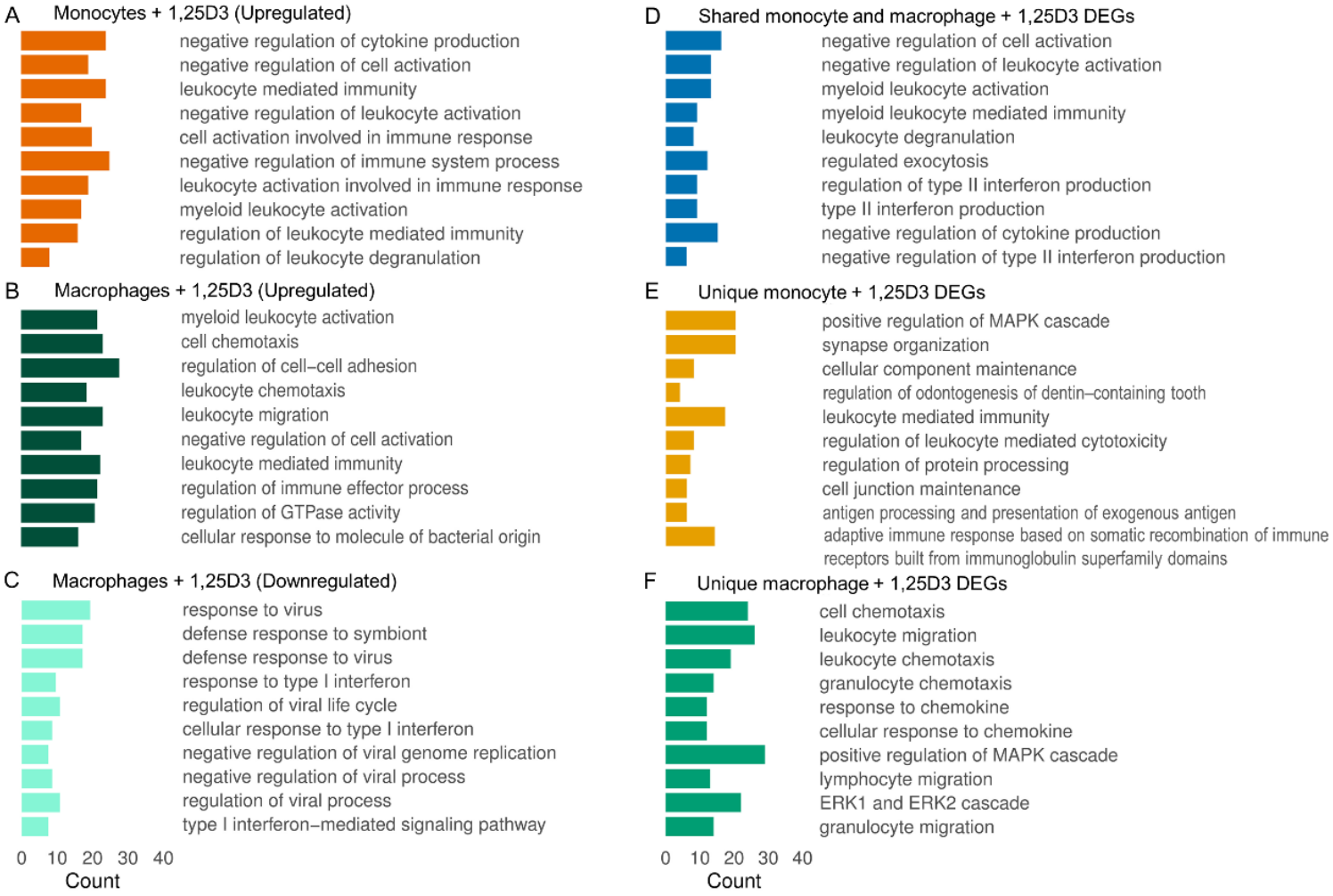


Figure 4.6: Over-representation analysis indicates that 1,25(OH)$_2$D$_3$ treatment in monocytes and macrophages may produce distinct alterations in selected biological processes. Top 10 significantly over-represented GO biological processes (adj. *p* value < 0.010), for differentially expressed genes upregulated (A) in 1,25(OH)$_2$D$_3$ treated monocytes, for genes upregulated (B) and downregulated (C) in 1,25(OH)$_2$D$_3$ treated macrophages, for genes shared between 1,25(OH)$_2$D$_3$ treated monocytes and macrophages (D), and genes uniquely differentially expressed in 1,25(OH)$_2$D$_3$ treated monocytes (E), and 1,25(OH)$_2$D$_3$ treated macrophages (F).

Notably, several other biological processes, that have not been addressed in this work previously, were also found to be significantly over-represented. For example, monocytes and macrophages treated with $1,25(OH)_2D_3$ both showed over-representation of various processes, including those associated with regulation of type II interferon production, exocytosis, secretion, and bacterial defence response. Though both monocytes and the macrophages exposed to $1,25(OH)_2D_3$ showed significant over-representation of similar processes, these were not necessarily a consequence of differential expression of the same genes. When considering the GO biological processes that were exclusively significantly over-represented in $1,25(OH)_2D_3$ treated monocytes, several were associated with differentiation of various cell types, including myeloid cells, osteoclasts, and endodermal cells. Although, for the reasons discussed in Chapter 3, these monocytes are not considered to be differentiated, even though $1,25(OH)_2D_3$ may prime this response. Additional GO biological processes showing exclusive over-representation in $1,25(OH)_2D_3$ treated monocytes were related to the negative regulation of proliferation for numerous cell types, including leukocytes, lymphocytes, mononuclear cells, T cells, and smooth muscle cells. Likewise, several biological processes were exclusively over-represented in the for $1,25(OH)_2D_3$ treated macrophages which related to both structural and immune related processes. Chief among these, were processes related to the regulation of (small) GTPase mediated signalling, determinants of morphology including extracellular matrix organisation, tissue remodelling, and wound healing. Immune related biological processes that were over-represented in $1,25(OH)_2D_3$ treated macrophages included cellular responses to molecules derived from bacteria (lipopolysaccharide), the viral defence response and type I interferon response. Furthermore, for the most part the genes related to viral defence response and type I interferon response were significantly differentially downregulated.

4.3.3.1 $1,25(OH)_2D_3$ supports defence responses to bacteria in both monocytes and macrophages

Antibacterial defence is multifaceted, and the presence of $1,25(OH)_2D_3$ in both the monocytes and macrophages led to the significant differential expression of several genes involved in this process. It is worth noting that the genes that contributed to the over-representation of this GO biological process were often distinct between the two cell types. Both $1,25(OH)_2D_3$ treated monocytes and macrophages demonstrated altered expression of several genes encoding for cytokines and cytokine receptors (*BMP6*, *IL1B*, *CCL3*, *CXCL1*, *CXCL6*, *CXCL8*, *CXCL10*, *IL10RA*, *IL27RA*, *IL7R*, and *GPR15LG*), T-cell co-inhibitors (*CD274* and *PDCD1LG2*) and co-stimulators (*CD86* and *KLRK1*), and PRRs and PRR co-receptors (*CD14*, *NLRC4*, *NLRP1*, *NOD2*, and *TLR1*), among others (Fig. 4.7A). However, this alteration in expression was not necessarily the same between the two cell types. One of the noteworthy differences observed

in the monocytes and macrophages in response to $1,25(OH)_2D_3$ was the significant differential expression of genes encoding antimicrobial peptides, producers of antimicrobial substances, and histones with antibacterial properties (Fig. 4.7B). In both $1,25(OH)_2D_3$ treated monocytes and macrophages, there was a large differential increase in *CAMP* (Monocytes + $1,25(OH)_2D_3$: log_2FC = 5.65; p<0.001, Macrophages + $1,25(OH)_2D_3$: log_2FC = 8.18; p<0.001). In contrast, other antimicrobial peptide encoding genes demonstrated different expression patterns in the monocytes versus macrophages. $1,25(OH)_2D_3$ significantly differentially increased the expression of *MPEG1* (log_2FC = 1.19; p<0.001), *LYZ* (log_2FC = 1.30; p<0.001), *BPI* (log_2FC = 3.20; p<0.001), *PRG2* (log_2FC = 4.02; p<0.001), *H2BC21* (log_2FC = 1.31; p<0.001), and *H2BC4* (log_2FC = 1.10; p<0.001) the macrophages, but not in the monocytes. Conversely, $1,25(OH)_2D_3$ differentially increased the expression of *GNLY* (log_2FC = 5.04; p<0.001) in monocytes, but not in macrophages. While the expression of several antimicrobial peptide encoding genes were increased as a result of the presence of $1,25(OH)_2D_3$ in the monocytes and macrophages, the expression of *DEFB1*, which encodes human β-defensin-1, was differentially decreased in $1,25(OH)_2D_3$ treated monocytes (log_2FC = -1.09; p<0.001) and macrophages (log_2FC = -2.11; p<0.001).

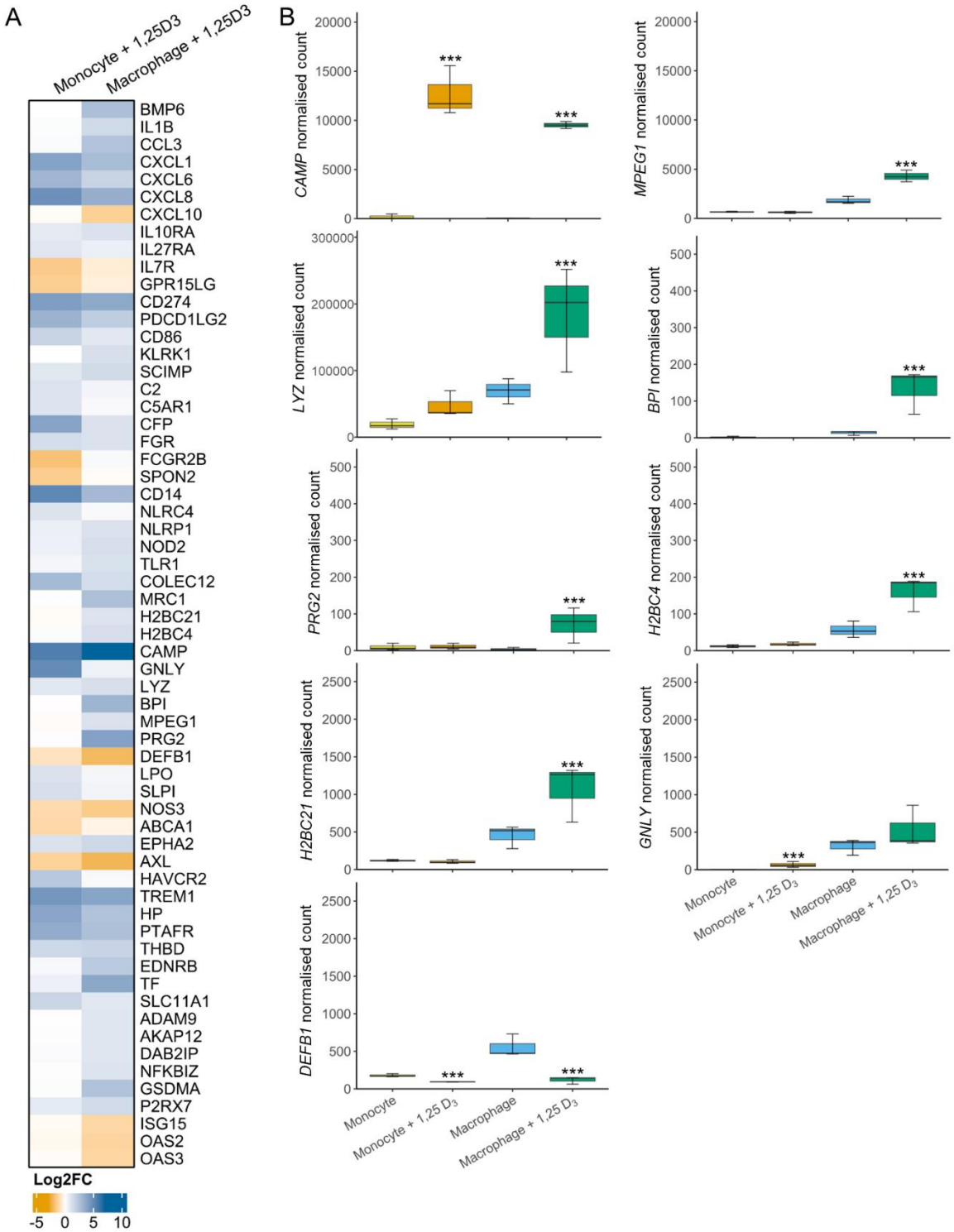


Figure 4.7: 1,25(OH)$_2$D$_3$ treatment in monocytes and macrophages produced distinct alterations in the expression of genes related to antibacterial defence response. Heatmap depicting *log*$_2$FC of genes encoding antibacterial defense related proteins identified as significantly differentially expressed in 1,25(OH)$_2$D$_3$ treated monocytes and macrophages (A). The boxplots show DESeq2 derived normalised counts for antimicrobial peptide encoding genes *CAMP*, *MPEG1*, *LYZ*, *BPI*, *PRG2*, *H2BC4*, *H2BC21*, *GNLY*, and *DEFB1* for the monocytes and macrophages in the presence and absence of 1,25(OH)$_2$D$_3$ (B). Statistically significant differences between 1,25(OH)$_2$D$_3$ treated and untreated monocytes and macrophages are given based on the Benjamini-Hochberg adjusted *p* values derived from DESeq2 analysis (**p*<0 .050, ***p*<0.010, ****p*<0.001).

4.3.3.2 1,25(OH)$_2$D$_3$ contributes to the heightened expression of phagocyte NADPH oxidase subunit encoding genes in monocytes

An important aspect of antimicrobial response is the generation and release of reactive oxygen species (ROS), also referred to as respiratory burst, which is mediated by either the mitochondria or NADPH oxidases (Bikle, 2015; Berridge, 2016; Lu *et al.,* 2018). Though 1,25(OH)$_2$D$_3$ was shown to contribute to the mRNA expression of several genes associated with antimicrobial defence, in both the monocytes and macrophages, only 1,25(OH)$_2$D$_3$ treated monocytes showed significant over-representation of GO biological processes related to respiratory burst. The genes that contributed to the over-representation of this process were glycoprotein encoding gene *CD52*, metal ion transporter encoding gene *SLC11A1*, and phagocyte NADPH oxidase (NOX2) subunit encoding genes *NCF1*, *NCF2* and *CYBB*, as well as pseudogenes *NCF1B* and *NCF1C*. In phagocytes the primary NADPH oxidase involved in the generation of ROS is the phagocyte NADPH oxidase (NOX2). Structurally, NOX2 is comprised of three cytosolic subunits – p47^{phox}, p67^{phox}, and p40^{phox} encoded by *NCF1*, *NCF2*, and *NCF4* respectively – and cytochrome b$_{558}$ – the membrane bound catalytic unit and auxiliary protein encoded by *CYBB* and *CYBA* respectively (Singel and Segal, 2016; Noreng *et al.,* 2022).

The significant, though not always differential, increase in all NOX2 subunit encoding genes was seen for 1,25(OH)$_2$D$_3$ treated monocytes (Fig. 4.8). The significantly differentially increased expression of *NCF1*, *NCF2* and *CYBB* was exclusively observed in 1,25(OH)$_2$D$_3$ treated monocytes (*NCF1*: log_2FC = 1.85; p<0.001, *NCF2*: log_2FC = 1.17; p<0.001, *CYBB*: log_2FC = 1.48; p<0.001). Though not differentially altered, there was a significant increase in the expression of *NCF4* (log_2FC = 0.67; p<0.001) and *CYBA* (log_2FC = 0.45; p<0.001) in 1,25(OH)$_2$D$_3$ in monocytes. While 1,25(OH)$_2$D$_3$ treated macrophages did not show significantly differentially increased expression for any of these genes, when considering the DESeq2 normalised counts, the expression of these components appeared to be greater. Likewise, though not differentially expressed, 1,25(OH)$_2$D$_3$ treated macrophages showed significantly increased expression of *NCF2* (log_2FC = 0.93; p<0.001) and *NCF4* (log_2FC = 0.76; p<0.001). However, it was in 1,25(OH)$_2$D$_3$ treated monocytes alone that there was a significant increase in the expression of all genes encoding subunits of NOX2.

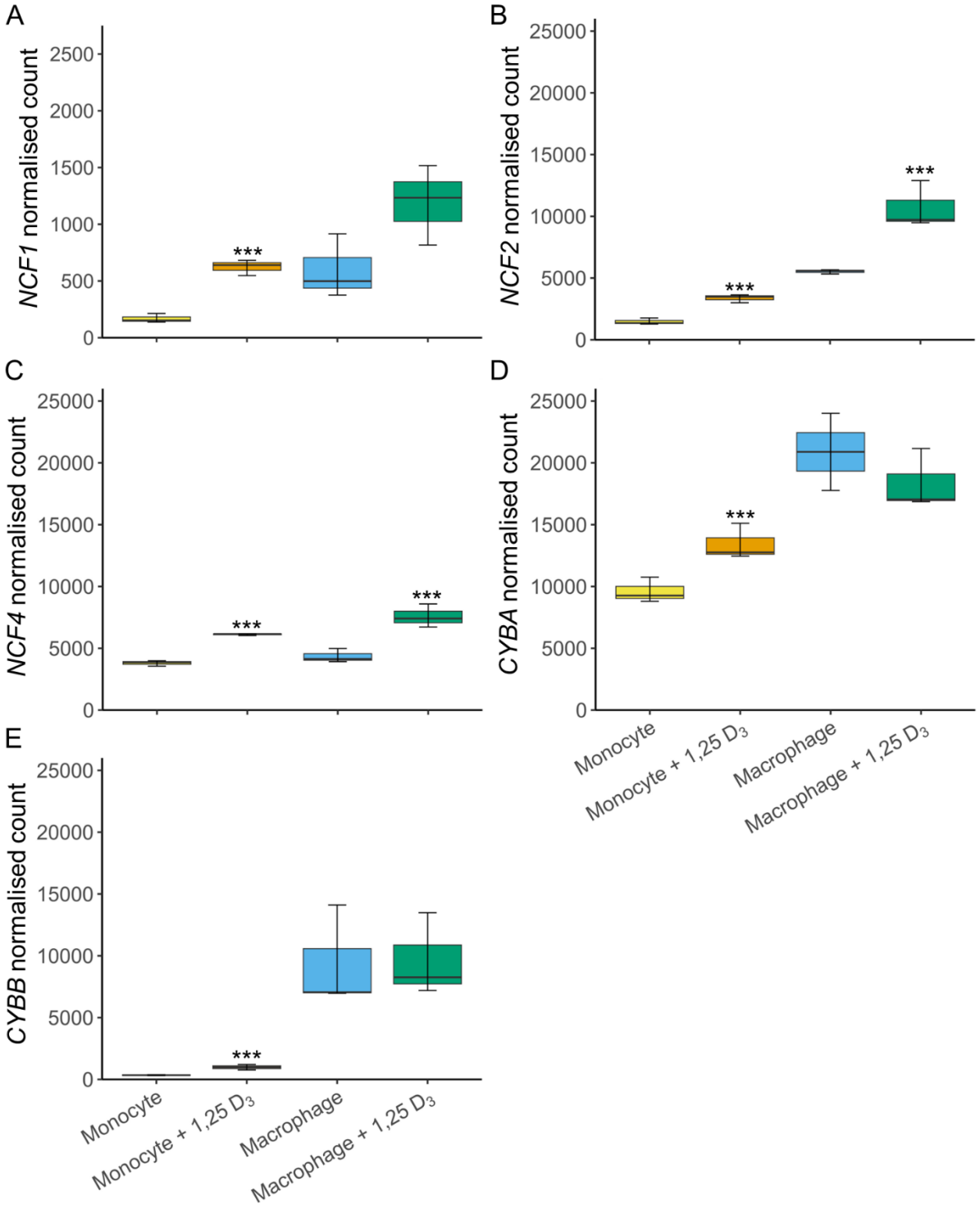


Figure 4.8: 1,25(OH)$_2$D$_3$ treatment in monocytes resulted in the significant increase in the phagocyte NADPH oxidase (NOX2) subunit encoding genes. The boxplots show DESeq2 derived normalised counts for the genes encoding proteins of the phagocyte NADPH oxidase system *NCF1* (A), *NCF2* (B), *NCF4* (C), *CYBA* (D), and *CYBB* (E) for the monocytes and macrophages in the presence and absence of 1,25(OH)$_2$D$_3$. Statistically significant differences between 1,25(OH)$_2$D$_3$ treated and untreated monocytes and macrophages are given based on the Benjamini-Hochberg adjusted *p* values derived from DESeq2 analysis (**p*<0.050, ***p*<0.010, ****p*<0.001).

4.3.3.3 1,25(OH)$_2$D$_3$ alters the expression of type I and type II interferon response related genes in macrophages

Interferons have essential roles in mediating antiviral, and though less well established antibacterial, responses within the host and can be broadly divided into type I, type II, and type III interferons (Schoggins, 2019). 1,25(OH)$_2$D$_3$ treatment in the monocytes and macrophages resulted in the significant differential expression of genes related to the regulation of type II interferon production (Fig. 4.9A), as indicated by GO analysis (Fig. 4.6). This included significant differential expression of genes such as *CD274*, *PDCD1LG2*, *CR1*, and *AXL* as previously illustrated and discussed (section 3.3.3. and 3.4). In contrast, significant over-representation of the GO biological processes related to type I interferon response, was only observed in 1,25(OH)$_2$D$_3$ treated macrophages. The general effect of 1,25(OH)$_2$D$_3$, in the context of type I interferon response, resulted in the decreased expression of several type I interferon response related genes in macrophages (Fig. 4.9. B). 1,25(OH)$_2$D$_3$ treatment resulted in a significant differential decrease in the expression of *ISG15* (log_2FC = -1.18; p<0.001), which encodes ubiquitin-like interferon stimulated gene 15 protein, and the gene encoding the negative regulator of the ISG15 protein, *USP18* (log_2FC = -1.16; p<0.001), in macrophages, but not in monocytes. Additionally, genes encoding enzymes required for ISG15 based ISGylation of target proteins, *HERC5*, *UBA7*, *UBE2L6*, and *TRIM25* (Dzimianski *et al.,* 2019), were also significantly, though not differentially, downregulated in the macrophages but not in monocytes (Fig. S2). Likewise, 1,25(OH)$_2$D$_3$ treatment in the macrophages, but not monocytes, resulted in the significant differential decrease in the mRNA expression of further type I interferon response related genes *IFIT1* (log_2FC = -1.10; p<0.001) and *MX1* (log_2FC = -1.52; p<0.001), *IFI27* (log_2FC = -2.62; p<0.001), *OAS2* (log_2FC = -1.26; p<0.001) and *OAS3* (log_2FC = -1.25; p<0.001), and *IFITM1* (log_2FC = -2.21; p<0.001) and *IFITM3* (log_2FC = -1.19; p<0.001). These genes encode proteins involved in inhibition of viral replication, type I interferon induced apoptosis, RNaseL activation, and prevention of viral entry into the host cytoplasm, respectively. The only exception to this downregulation of type I interferon response related genes was 1,25(OH)$_2$D$_3$ induced significant differential increase of *RBM47*, in both monocytes (log_2FC = 1.24; p<0.001) and macrophages (log_2FC = 1.64; p<0.001).

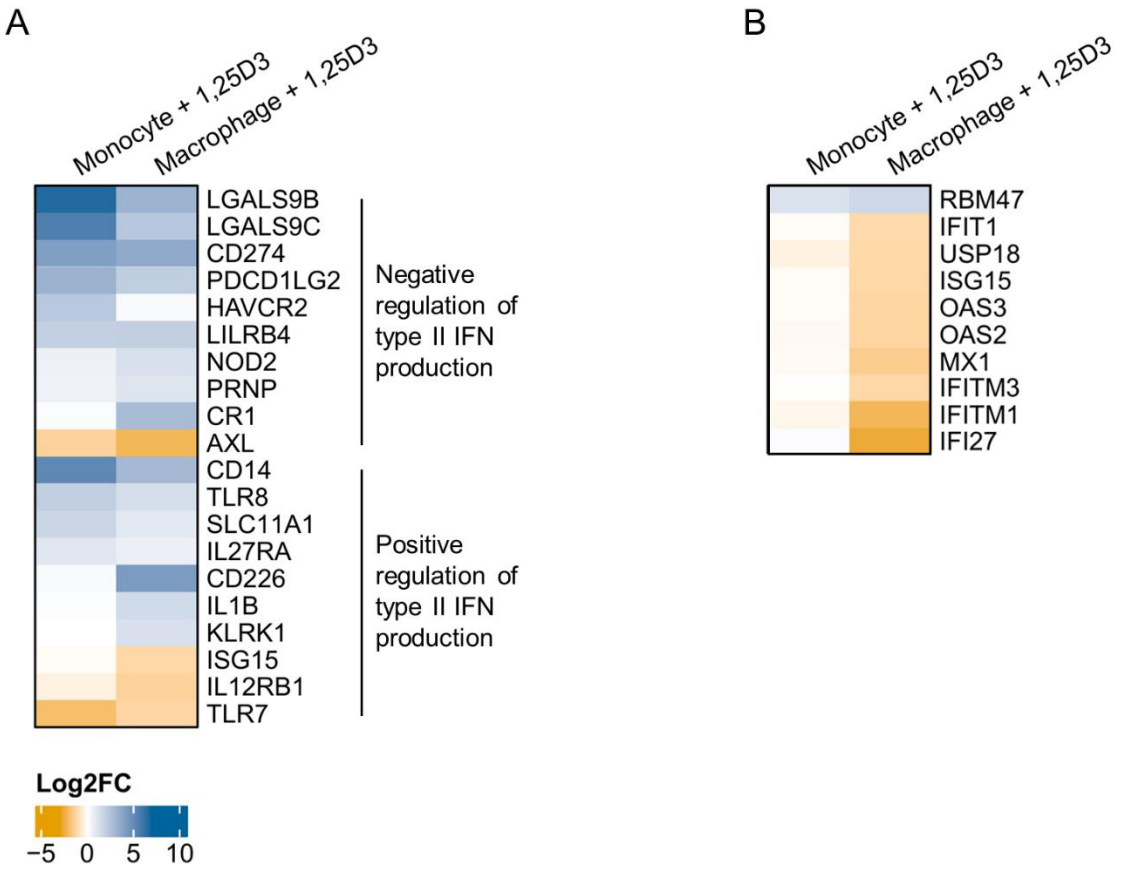


Figure 4.9: 1,25(OH)$_2$D$_3$ treatment in monocytes and macrophages exerted alterations in the expression of genes related to the regulation of type II interferon production, however, alterations related to type I interferon response were primarily observed in 1,25(OH)$_2$D$_3$ treated macrophages. Heatmap depicting *log*$_2$FC of genes encoding regulators of type II interferon production related proteins identified as significantly differentially expressed in 1,25(OH)$_2$D$_3$ treated monocytes and macrophages (A). Heatmap depicting *log*$_2$FC of genes encoding type I interferon response related proteins identified as significantly differentially expressed in 1,25(OH)$_2$D$_3$ treated monocytes and macrophages (B).

4.3.3.4 The altered expression of genes involved in small GTPase mediated signalling were primarily observed in 1,25(OH)$_2$D$_3$ treated macrophages

Small GTPases represent a large family of proteins with numerous roles in the regulation of cellular events including cell signalling - involved in proliferation, differentiation and survival, cell morphology, cell polarity, and vesicle transport (Song *et al.,* 2019; Toma-Fukai and Shimizu, 2019). The activity of these small GTPases is principally regulated by GTP/GDP cycling, wherein the GTP bound form is generally considered active and the GDP bound form inactive. Guanine nucleotide exchange factors (GEFs; GDP→GTP) and GTPase activating proteins (GAPs; GTP → GDP) are largely responsible for the GTP/GDP cycling related to small GTPase protein activity (Cherfils and Zeghouf, 2013). Within the context of small GTPase mediated signalling, of the 41 genes identified as having significant differential expression based on the presence of 1,25(OH)$_2$D$_3$, 18 and 38 were identified in 1,25(OH)$_2$D$_3$

treated monocytes and 1,25(OH)$_2$D$_3$ treated macrophages, respectively. In the macrophages, 1,25(OH)$_2$D$_3$ had a particular influence on the gene expression of small GTPases, GEFs, and GAPs related to the Rho, Ras, and Rab GTPase subfamilies as well as several interacting proteins including effectors and activators (Fig. 4.10).

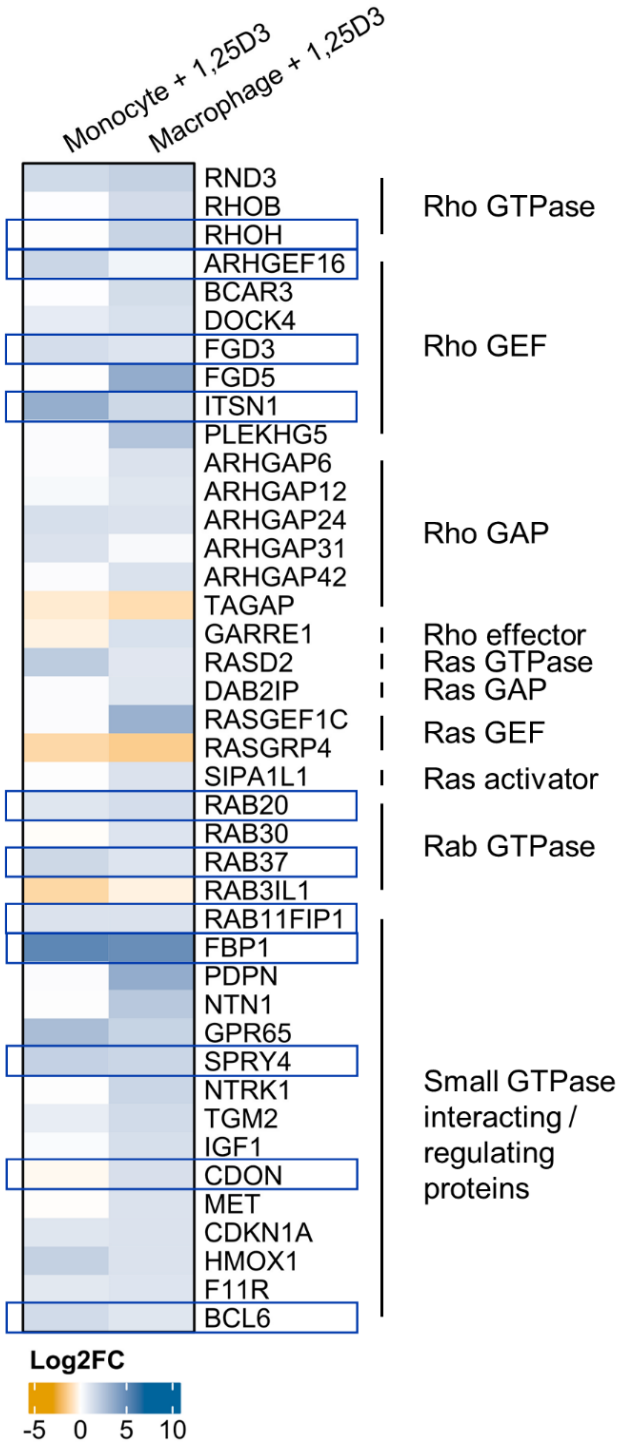


Figure 4.10: 1,25(OH)$_2$D$_3$ treated macrophages showed pronounced alteration in expression of genes involved in small GTPase mediated signalling. Heatmap depicting *log*$_2$FC of genes encoding small GTPases, GAPs, GEFs and additional GTPase regulatory proteins identified as significantly differentially expressed in 1,25(OH)$_2$D$_3$ treated monocytes and macrophages, respectively. The known primary 1,25(OH)$_2$D$_3$ targets are indicated within blue boxes.

The $1,25(OH)_2D_3$ treatment of the macrophages resulted in the significant differential increase in the expression of several genes encoding small GTPases, namely *RND3*, *RHOB*, *RHOH*, *RASD2*, *RAB20*, *RAB30*, and *RAB37*. The expression of *RND3*, *RASD2*, *RAB20*, and *RAB37* was similarly significantly differentially increased in $1,25(OH)_2D_3$ treated monocytes. Of these genes *RHOH*, *RAB20* and *RAB37* are known primary $1,25(OH)_2D_3$ targets. Additionally, $1,25(OH)_2D_3$ treated monocytes showed the significant differential decrease in expression of *RAB3IL1* (log_2FC = -1.19; p<0.001), not observed in their macrophage counterparts. In addition to the altered expression of small GTPase encoding genes, there was also significant differential expression of several GEF and GAP encoding genes in response to $1,25(OH)_2D_3$. Six Rho GEF encoding genes were significantly differentially increased in $1,25(OH)_2D_3$ treated macrophages. Of these, the expression of *FGD3* and *ITSN1* was also significantly differentially increased in $1,25(OH)_2D_3$ treated monocytes. Additionally, the significant differential increase in expression of *ARHGEF16* was only observed in $1,25(OH)_2D_3$ treated monocytes (log_2FC = 1.72; p<0.001). The significant differential increase in expression of Ras GEF encoding gene *RASGEF1C* was only observed in $1,25(OH)_2D_3$ treated macrophages (log_2FC = 3.35; p<0.001). In contrast, the significant differential decrease in Ras GEF encoding gene *RASGRP4* resulting from $1,25(OH)_2D_3$ treatment was observed for both the monocytes (log_2FC = -1.15; p<0.001) and the macrophages (log_2FC = -1.51; p<0.001). Rho GAP encoding *ARHGAP31* and *ARHGAP24* were significantly differentially increased in the monocytes in response to $1,25(OH)_2D_3$ treatment. Likewise, *ARHGAP24* in addition to *ARHGAP6*, *ARHGAP12*, and *ARHGAP42* were significantly differentially increased in $1,25(OH)_2D_3$ treated macrophages. The expression of *TAGAP* was the only Rho GAP encoding gene to show significantly differentially reduced expression in $1,25(OH)_2D_3$ treated macrophages (log_2FC = -1.03; p<0.001). The Ras GAP encoding gene *DAB2IP* showed a significant differential increase in expression in $1,25(OH)_2D_3$ treated macrophages alone (log_2FC = 1.03; p<0.001). When considering the mRNA expression of genes encoding for small GTPase interacting and regulating proteins, $1,25(OH)_2D_3$ resulted in the increased expression of seven and fifteen of these genes in the monocytes and the macrophages, respectively. While there was undeniable overlap in the effect of $1,25(OH)_2D_3$ in the mRNA expression of these small GTPase encoding genes and their associated protein encoding genes, it was clear that this effect was more pronounced in the macrophages than the monocytes.

4.4 Discussion

Despite the purported role of $1,25(OH)_2D_3$ as an immunomodulator, with numerous studies investigating its effects in both the innate and adaptive immune system, the overall effect of $1,25(OH)_2D_3$ in immune cells and its contribution toward immunity is still not fully understood (Sassi *et al.,* 2018; Koivisto *et al.,* 2020; Bishop *et al.,* 2021). Nonetheless, $1,25(OH)_2D_3$ has been well-established to affect the functionality of monocytes and monocyte-derived macrophages (Carlberg, 2019; Ao *et al.,* 2021; Ismailova and White, 2022; Valdés-López *et al.,* 2022). As VDR is a transcription factor, transcriptomic analyses have been employed to understand, in part, the influence of $1,25(OH)_2D_3$ in human monocytes and macrophages (Neme *et al.,* 2016, 2019; Rafique *et al.,* 2018; Nurminen *et al.,* 2019; Castillo *et al.,* 2021; Warwick *et al.,* 2021; Fernandez *et al.,* 2022; Valdés-López *et al.,* 2022). These transcriptomic studies largely consider the effects of $1,25(OH)_2D_3$ in either human monocytes or macrophages in isolation, with differing $1,25(OH)_2D_3$ concentrations and treatment conditions, and no direct comparison made between the two cell types. In this body of work, a direct comparison of the effect of $1,25(OH)_2D_3$ on the transcriptomes of monocytes and macrophages was made.

From the PCA analysis it was evident that $1,25(OH)_2D_3$ had clear effects on both the monocytes and the macrophages. It was also evident, from the offset, that the gene expression profiles generated in the presence of $1,25(OH)_2D_3$ in the monocytes and the macrophages were distinct, with more differentially expressed genes unique to each cell type, than that which was shared between them. In general, the presence of $1,25(OH)_2D_3$ resulted in a larger degree of upregulation, as opposed to downregulation, in gene expression in the monocytes and macrophages. This corresponds with previous studies where THP-1 cells, primary monocytes, primary macrophages, and dendritic cells were treated with vitamin D_3 derivatives (Neme *et al.,* 2016; Clarke *et al.,* 2020; Warwick *et al.,* 2021; Fernandez *et al.,* 2022; Kim *et al.,* 2022). Perhaps unsurprisingly, the largest proportion of previously identified primary $1,25(OH)_2D_3$ targets were found among those differentially expressed genes shared between the monocytes and the macrophages. Given the timeframe of assessment, 96 hours, many of the differentially expressed genes were likely to be secondary $1,25(OH)_2D_3$ targets. This is probable due to the capacity of VDR to directly alter the expression of numerous transcription factors, as demonstrated here by the altered expression of primary $1,25(OH)_2D_3$ targets *BCL6*, *NFE2*, and *NR1I2*, and as previously demonstrated in THP-1 cells by Warwick *et al.* (2021). That being said, the nature of these effects appeared to be cell type specific for the monocytes and macrophages.

While the significantly differentially expressed genes that were shared between the monocytes and macrophages generally showed regulation in the same direction, there were some exceptions to this trend. Notably, the altered expression of primary $1,25(OH)_2D_3$ target gene *PNOC* was observed, with increased expression observed in $1,25(OH)_2D_3$ treated monocytes, but decreased expression shown in their macrophage counterparts. The altered expression of *PNOC*, which encodes prepronociceptin, is likely to reflect, in part, the immunomodulatory effect of $1,25(OH)_2D_3$. The processed proteins derived from prepronociceptin – nociceptin and nocistatin – are important in the perception of pain, with additional functions as regulators of immunity and inflammation, including roles in leukocyte migration and cytokine production (Gavioli *et al.,* 2015; Zhang *et al.,* 2022). The regulation of the expression of this gene, and its encoded proteins is not well understood and appears to be quite complex (Zhang *et al.,* 2019). What has become clear is that PMA, the agent used to induce macrophage differentiation, significantly increases the gene expression of *PNOC* and protein expression of nociceptin in Mono-Mac-6 cells, THP-1 cells and PBMCs (Zhang *et al.,* 2016, 2019, 2022). This increase appears to be, in part, driven by the ERK and p38 pathways, but can be modulated by TLR expression within these cells (Gavioli *et al.,* 2015; Zhang *et al.,* 2016, 2019, 2022). The manner in which $1,25(OH)_2D_3$ contributes to this process has not been elucidated, though the role of $1,25(OH)_2D_3$ in modulating pain perception, in addition to its immunomodulatory effects, has been reported (Habib *et al.,* 2020). Given that *PNOC* is a primary $1,25(OH)_2D_3$ target, as well as the capacity of $1,25(OH)_2D_3$ to influence the mRNA and protein expression of ERK, p38, and several TLRs, its potential contribution to this process in monocytes and macrophages is likely, though it may be cell type specific (Arababadi *et al.,* 2018; W. Liu *et al.,* 2018; Ao *et al.,* 2021). However, confirmation of this would necessitate further study.

In innate immune cells $1,25(OH)_2D_3$ has several relatively well-established effects including the modulation of chemotaxis – in part through its regulation of the production of cytokines and chemokines, bacterial defence, phagocytosis, and cell-adhesion (Sassi *et al.,* 2018; Carlberg, 2019; Koivisto *et al.,* 2020). As expected, GO analysis showed these processes to be significantly over-represented in $1,25(OH)_2D_3$ treated monocytes and macrophages. Despite this, the manner in which $1,25(OH)_2D_3$ contributed to the altered expression of genes involved in these processes was often distinct between $1,25(OH)_2D_3$ treated monocytes and macrophages. For example, in the context of bacterial defence, $1,25(OH)_2D_3$ resulted in a similar degree of increased gene expression of specific chemokines in both monocytes and macrophages, yet showed distinct differences in the gene expression of antimicrobial peptides.

The antimicrobial properties of $1,25(OH)_2D_3$ are well documented (Gil *et al.,* 2018; Sassi *et al.,* 2018; Bishop *et al.,* 2021). In the context of antibacterial defence, the upregulation of genes

encoding the interleukin 8 gene cluster comprised of *CXCL1*, *CXCL6*, and *CXCL8* have previously been shown to be $1,25(OH)_2D_3$ targets in both monocytes and macrophages as was seen here (Ryynänen and Carlberg, 2013). Likewise, the increased expression of *IL1B* in the macrophages has similarly been indicated as a function of $1,25(OH)_2D_3$ (Verway *et al.,* 2013; Bishop *et al.,* 2021; Fernandez *et al.,* 2022). PRRs are essential in detecting and initiating immune responses upon exposure to pathogens, including bacteria, and the expression of numerous PRRs has been shown to be directly and indirectly influenced by $1,25(OH)_2D_3$ (Arababadi *et al.,* 2018; Dimitrov *et al.,* 2021; Li and Wu, 2021). The genes encoding TLR coreceptor, *CD14*, and nod-like receptor 2, *NOD2*, are primary $1,25(OH)_2D_3$ targets whose increased mRNA and protein expression in monocytes and macrophages exposed to $1,25(OH)_2D_3$ has been frequently observed (Wang *et al.,* 2010; Dimitrov and White, 2016; Dimitrov *et al.,* 2021; Bishop *et al.,* 2021; Ismailova and White, 2022). In addition to these attributes one of the most well documented manners in which $1,25(OH)_2D_3$ induces antibacterial functions is through the production of antimicrobial peptides.

Cathelicidin antimicrobial peptide, encoded by *CAMP*, a primary $1,25(OH)_2D_3$ target, was one of the earliest antimicrobial peptides to be linked with $1,25(OH)_2D_3$ activity in innate immune cells (Wang *et al.,* 2004; White, 2010). The human cathelicidin antimicrobial peptide (LL-37) has potent antibacterial properties, with its positive charge permitting the formation of pores in negatively charged bacteria and in the promotion of autophagy and autophagosome-lysosome fusion (Chung *et al.,* 2020; Bhutia, 2022). In keeping with this well-established effect of $1,25(OH)_2D_3$, the expression of *CAMP* was drastically increased in both the monocytes and the macrophages in the presence of $1,25(OH)_2D_3$. While $1,25(OH)_2D_3$ had a significant influence on the mRNA expression of several other antimicrobial peptides, this influence was cell type specific.

When considering the cell type specific effects of $1,25(OH)_2D_3$, $1,25(OH)_2D_3$ treated macrophages exclusively showed upregulation of *H2BC21* and *H2BC4*. These genes encode components of histone H2B which has proven antibacterial properties. These antibacterial effects are multifaceted and include serving as damage associated molecular patterns which interact with several TLRs and by acting as antibacterial peptides with direct cytotoxic effects, through altering bacterial cell membrane permeability, binding LPS, and interacting with bacterial nucleic acids (Weng *et al.,* 2022; Li *et al.,* 2022). Furthermore, increased expression of *H2BC21* in macrophages has been correlated with macrophage extracellular trap formation (Jensen *et al.,* 2023), though further work would be required to determine if a similar effect is induced in this context.

In macrophages alone 1,25$(OH)_2D_3$ induced the differential increase in expression of *LYZ*, *MPEG1*, and *BPI*. Lysozyme, encoded by *LYZ*, displays cytotoxic effects toward Gram-positive bacteria through cleavage of glycosidic bonds, and an increase in its production in response to 1,25$(OH)_2D_3$ has previously been observed (Ardesia *et al.,* 2015; Sassi *et al.,* 2018; Akimbekov *et al.,* 2020). Perforin-2, encoded by *MPEG1*, is a pore-forming transmembrane protein that acts within phagosomes to facilitate the destruction of phagocytosed Gram-positive and Gram-negative bacteria as it permits the passage of phagosomal hydrolases into the bacteria (Bayly-Jones *et al.,* 2020; Merselis *et al.,* 2021). Notably, *MPEG1* has been identified as a 1,25$(OH)_2D_3$ target before, in a transcriptomic analysis in PBMCs, however, in this context the presence of 1,25$(OH)_2D_3$ resulted in the decreased expression of *MPEG1* (Hanel *et al.,* 2020). As PBMCs represent a milieu of different cell types, cell type specific effects may be lost. That said, the monocytes did not show any alteration in *MPEG1* expression in response to 1,25$(OH)_2D_3$. As such, this appears to be a highly specific role of 1,25$(OH)_2D_3$ in the macrophages, which could potentially translate into an enhanced capacity for clearance of phagocytosed bacteria.

The bactericidal/permeability-increasing protein, encoded by *BPI*, has a manifold effect in relation to bacterial pathogenesis. It has been shown to have bactericidal and opsonic properties toward Gram-negative bacteria, as well as exhibiting anti-inflammatory functions (Theprungsirikul *et al.,* 2021). Though the expression of *BPI*, and its encoded protein, is most frequently reported and studied in relation to neutrophils, its mRNA and protein expression have been observed in differentiated U937 cells and primary human macrophages. However, the bactericidal/permeability-increasing protein, while having bactericidal activity, did not appear to act as an opsonin in this instance, but did give an indication of acting as a potential phagocytic receptor (Balakrishnan *et al.,* 2016). The role of 1,25$(OH)_2D_3$ in macrophages thus, may be, in addition to enhancing cytotoxicity toward bacteria, increase the capacity for their detection, as is seen by the contribution of 1,25$(OH)_2D_3$ to the expression of various other PRRs (Arababadi *et al.,* 2018).

Similarly to the aforementioned antimicrobial peptide encoding genes, the expression of *PRG2*, was differentially increased in 1,25$(OH)_2D_3$ treated macrophages. Proteoglycan 2, also called eosinophil major basic protein, is encoded by *PRG2* and the expression of its active protein form is generally a feature of eosinophils granules (Melo and Weller, 2018). While this protein is involved in antimicrobial defence against bacteria, viruses, helminths and protozoa, it generally does so in conjunction with an additional three components, eosinophil-derived neurotoxin encoded by *RNASE2*, eosinophil cationic protein encoded by *RNASE3*, and eosinophil peroxidase encoded by *EPX* (Qiao *et al.,* 2022), none of which were differentially expressed in 1,25$(OH)_2D_3$ treated monocytes or macrophages. While one study indicated that

$1,25(OH)_2D_3$ had the capacity to regulate the expression of *PRG2*, alongside the other three components, this has not been demonstrated in monocytes or macrophages (Lu *et al.,* 2017). A scarcity of information related to the role of this protein in the context of monocytes and macrophages ultimately means no conclusions can be drawn from its altered expression.

In contrast, the differential increased expression of antimicrobial peptide, granulysin, encoding *GNLY* was only observed in $1,25(OH)_2D_3$ treated monocytes. Granulysin acts as an antimicrobial peptide in its 9 kDa form that results in bacterial membrane lysis, as well as exhibiting cytotoxicity toward parasites and fungi (Dotiwala and Lieberman, 2019). In addition, the 15 kDa form of this protein acts as an immune alarmin which stimulates immune cell migration, differentiation and maturation, including the recruitment of monocytes and the support of their differentiation into dendritic cells (Castiello *et al.,* 2011; Clayberger *et al.,* 2012; Sparrow and Bodman-Smith, 2020). The expression of *GNLY*, differential or otherwise, observed in $1,25(OH)_2D_3$ treated monocytes and macrophages in the presence or absence of $1,25(OH)_2D_3$ was unexpected. The expression of *GNLY*, and more specifically granulysin, is associated with natural killer cells and T cells, primarily CD8+ T cells (Dotiwala and Lieberman, 2019; Sparrow and Bodman-Smith, 2020). Information on the expression of *GNLY* in monocytes and macrophages is scarce, although the Monaco dataset on the human protein atlas indicates that it may have some degree of expression in intermediate and non-classical monocytes (https://www.protein atlas.org/ENSG00000115523-GNLY/immune+cell). In THP-1 cells the expression of *GNLY* was shown to be inducible by the presence of *Acholeplasma laidlawii*, a small bacterium lacking a cell wall (Kida *et al.,* 2001; Kida *et al.,* 2002). As such, it appears that $1,25(OH)_2D_3$ may be capable of inducing the expression of *GNLY* in the monocytes, and that macrophages may express this gene even in the absence of an active threat. In this context the influence of $1,25(OH)_2D_3$ in the monocytes appears to promote a bactericidal functionality within these cells and may contribute to immune cell recruitment.

Unlike the other antimicrobial peptides, indicated as being unaltered or increased in response to $1,25(OH)_2D_3$ in the monocytes and macrophages, *DEFB1* was significantly decreased in both the monocytes and macrophages in response to $1,25(OH)_2D_3$. Human β-defensin-1, encoded by *DEFB1*, is involved in membrane lysis of both Gram-positive and Gram-negative bacteria, as well as inducing the expression of potent pro-inflammatory cytokine CCL5 in PBMCs (de Oca, 2010; Álvarez *et al.,* 2018). There is little information related to the effect of $1,25(OH)_2D_3$ on the expression of *DEFB1*, though high $1,25(OH)_2D_3$ concentrations have been shown to reduce its expression in bovine mammary epithelial cells (Téllez-Pérez *et al.,* 2012). Ultimately, the decreased expression of *DEFB1*, especially when considering the relationship of human β-defensin-1 with CCL5, may reflect the anti-inflammatory properties of $1,25(OH)_2D_3$ commonly reported in monocytes and macrophages (Dionne *et al.,* 2017; Önal *et al.,* 2022).

In addition to the expression of antimicrobial peptides, $1,25(OH)_2D_3$ has been shown to have important immunomodulatory effects related to antibacterial activity through the regulation of ROS production, which is mediated by either the mitochondria or NADPH oxidases (Bikle, 2015; Berridge, 2016; Lu *et al.,* 2018). In the monocytes, $1,25(OH)_2D_3$ treatment led to the increased expression of all of the genes that encode the protein subunits of the phagocyte NADPH oxidase (NOX2), namely *CYBA*, *CYBB*, *NCF1*, *NCF2*, and *NCF4*. The increased expression of *NCF1* and *NCF4* is not unexpected, as these are known primary $1,25(OH)_2D_3$ targets in monocytes (Nurminen, Seuter and Carlberg, 2019). Furthermore, the influence of $1,25(OH)_2D_3$ on this NADPH oxidase system and the subsequent increase in ROS production when faced with a pathogen has previously been observed in human monocytes and was shown to participate in the antibacterial activity of $1,25(OH)_2D_3$ toward mycobacteria (Sly *et al.,* 2001). Additionally, a more recent study in the human bone cell line SaOS2, showed that all vitamin D metabolites increased ROS production, contributing toward enhanced proliferation and energy metabolism (Somjen *et al.,* 2010). Overall, this suggests that, in the monocytes at least, $1,25(OH)_2D_3$ may result in the altered expression of NOX2 encoding genes as a potential additional layer of protection against bacterial pathogenesis, among other potential contributions to cellular metabolism.

In keeping with the immunomodulatory properties associated with $1,25(OH)_2D_3$, the presence of $1,25(OH)_2D_3$ in both the monocytes and the macrophages gave an indication of influencing the expression of genes related to the regulation of type II interferon production, particularly IFN-γ. In contrast, the altered expression of type II interferon response related genes, was only shown to be an effect of $1,25(OH)_2D_3$ in the macrophages. $1,25(OH)_2D_3$ has been shown to regulate type II interferon production and response in human monocytes, macrophages, and PBMCs; this activity has been shown to be an aspect of $1,25(OH)_2D_3$ mediated antimicrobial response (Darwich *et al.,* 2009; Fabri *et al.,* 2011; Teles *et al.,* 2013; Kraaij *et al.,* 2014; Ragab *et al.,* 2016). As such, $1,25(OH)_2D_3$ mediated alteration in the expression of genes related to these type II interferon associated processes likely reflects the activity of $1,25(OH)_2D_3$ in the maintenance of a balanced immune state in both the monocytes and macrophages.

Notably, treatment with $1,25(OH)_2D_3$ resulted in the significantly reduced expression of several genes related to the type I interferon response in the macrophages but not in monocytes, with the exception of *RBM47*, whose expression was upregulated in both cell types. Notably, $1,25(OH)_2D_3$ treatment in the macrophages resulted in the significant down regulation of all the genes that encode the proteins and enzymes involved in the ISG15 ISGylation system, namely *ISG15, UBA7*, *UBE2L6*, *HERC5*, *TRIM25* and *USP18*. The expression of ISG15, encoded by *ISG15*, is strongly and swiftly upregulated in response to type I interferons (Perng

and Lenschow, 2018). ISG15 interferes with viral replication through its conjugation to lysine in newly synthesised proteins, a process referred to as ISGylation and aided by three additional conjugating enzymes, namely Ube1L, UbcH8, and Herc5 or Trim25, encoded by *UBA7*, *UBE2L6*, *HERC5*, and *TRIM25* respectively (Dzimianski *et al.,* 2019). This ISGylatoin process is reversed by ubiquitin-specific protease USP18, encoded by *USP18* (Zhang and Zhang, 2011). In addition to ISGylation, ISG15 and USP18 have additional roles in the immune system. In its unconjugated form ISG15 is thought to act as a neutrophil chemokine, that may support dendritic cell maturation, and contribute towards increased IFN-γ production (Perng and Lenschow, 2018; Dzimianski *et al.,* 2019). USP18 competes with Janus kinase 1 on the interferon α/β receptor 2 and by doing so acts to negatively regulate type I interferon signalling (Malakhova *et al.,* 2006).

At face value this would imply that $1,25(OH)_2D_3$ acts to reduce the macrophages responsiveness to type I interferon, which may act to restrict inflammatory response during an active viral infection, in order to prevent excessive inflammation and associated immunopathology, which has been observed previously (Greiller and Martineau, 2015; Ahmed, 2020; Ao *et al.,* 2021). However, the exact influence of $1,25(OH)_2D_3$ on type I interferon responses, and in viral pathogenesis in general, is not well understood, largely dependent on the virus in question, the quantities of $1,25(OH)_2D_3$ employed, and the model being utilised, and has seen a fair degree of controversy (Lee, 2020). As such, in the context of this research and given the absence of a viral threat or synthetic agonist, it is possible that the reduced expression of type I interferon associated genes mediated by $1,25(OH)_2D_3$ in the macrophages may serve as a protective mechanism. To wit, aberrant expression and activation of ISG15 and ISGylation has been implicated in several cancers, neurodegenerative disorders and autoinflammation (Mirzalieva *et al.,* 2022).

It is plausible then that this potential anti-inflammatory, protective role of $1,25(OH)_2D_3$ may account for the differential reduction in expression of additional type I interferon response related genes, *IFIT1*, *MX1*, *IFI27*, *OAS2*, and *OAS3*, exhibited in $1,25(OH)_2D_3$ treated macrophages. For instance, interferon induced protein with tetratricopeptide repeats 1, encoded by *IFIT1*, has been shown to be associated with the pathogenesis of autoimmune disorders systemic sclerosis and Sjogren's syndrome, and its elevated expression is associated with rheumatoid arthritis among other autoimmune disorders (Wu *et al.,* 2023). Furthermore, elevated mRNA expression of the *ISG15*, *HERC5*, *IFIT1*, *MX1*, *IFI27*, and *OAS3* has been associated with dermatomyositis, a rare autoimmune disease associated with muscle weakness and skin irritation (Bilgic *et al.,* 2009; Greenberg *et al.,* 2012; Kim *et al.,* 2020). Similarly, heightened expression of myxovirus resistance protein, encoded by *MX1*, is associated with autoimmune disorder cutaneous lupus erythematosus (Ghoreishi *et al.,* 2012).

Additionally, the pathogenesis of inflammatory bone disorder chronic recurrent multifocal osteomyelitis has recently been shown to be associated with 2'-5'-oligoadenylate synthetase 3, encoded by *OAS3* (Huang *et al.,* 2021). As such, $1,25(OH)_2D_3$ mediated reduction in expression of these genes in the macrophages may reflect an anti-inflammatory mechanism to reduce autoinflammation. This notion is supported by the fact that in all of the aforementioned autoimmune diseases 25(OH)D, circulating vitamin D deficiency is associated with increased disease activity (Azali *et al.,* 2012; Yang *et al.,* 2020; Cutolo *et al.,* 2021; Guo and Nambudiri, 2021; Radić *et al.,* 2023). Likewise, macrophages have shown functional involvement in all of these autoinflammatory disorders, further supporting the protective role $1,25(OH)_2D_3$ in the context of autoimmune disorders specifically as it relates to macrophage involvement, which though needing further validation seems plausible (Udalova *et al.,* 2016; Moreno-Moral *et al.,* 2018; Thompson *et al.,* 2018; Wenzel, 2019; Rizzo *et al.,* 2020; Zhao and Ferguson, 2020).

In support of this notion is the upregulation of *RBM47* seen in response to $1,25(OH)_2D_3$ in both the monocytes and macrophages. The RNA binding motif protein 47, encoded by *RBM47*, is a recently identified RNA binding protein that recognises single stranded RNA and has been shown to have multiple functions including RNA editing, RNA stabilisation and alternative splicing (Guan *et al.,* 2013; Fossat *et al.,* 2014; Shivalingappa *et al.,* 2021). While it has several roles outside of its contribution to immunity, in this context it has been identified as having a complex role in the regulation of type I interferon responses. It has been shown to enhance antiviral activity through delaying the mRNA degradation of *IFNAR1*, which encodes the IFN-α/β receptor (Wang *et al.,* 2021). However, it has also been shown to promote the production of potent anti-inflammatory, immunosuppressive cytokine IL-10, in regulatory B cells, likewise through the stabilisation of its mRNA (Wei *et al.,* 2019). This correlates with $1,25(OH)_2D_3$ induced increase in *IL10* expression previously observed using RT-qPCR for both the monocytes and macrophages (section 2.3.2.5). A further immunosuppressive role of RBM47 was illustrated in zebrafish, in its capacity to inhibit the production of specific IFNs during viral infection through targeting mitochondrial antiviral signalling proteins, which stimulate IFN production, for lysosomal degradation (Lu *et al.,* 2020). In view of this, RBM47 activity appears to regulate type I interferon balance and contribute to immune modulation in this way. Though no direct link between $1,25(OH)_2D_3$ and the expression of *RBM47* has been made, it is possible that this is a further immunomodulatory mechanism imparted by $1,25(OH)_2D_3$ in both the monocytes and the macrophages that may act to protect against autoinflammation.

In addition to the role of 1,25$(OH)_2D_3$ in the regulation of the expression of type I interferon related genes, it was also observed that in the macrophages, the presence of 1,25$(OH)_2D_3$ resulted in the altered expression of a variety of small GTPases and their regulatory components. There are hundreds of small GTPases, and even greater numbers of regulators including GEFs and GAPs, with numerous roles throughout the cell (Cherfils and Zeghouf, 2013; Song *et al.,* 2019). Small GTPases, whose activity is controlled by GDP/GTP switching, are fundamental in the control of cellular dynamics and cell signalling, including functions such as cell cycle control, cell differentiation, cytoskeletal organisation and morphology, cellular adherence, vesicle trafficking and fusion (Cherfils and Zeghouf, 2013; Olson, 2018; Wosik *et al.,* 2018; Song *et al.,* 2019; Su and Zheng, 2021; Travnickova *et al.,* 2021). When considering the various roles of these GTPases and considering the morphological differences observed between the macrophages and 1,25$(OH)_2D_3$ treated macrophages, which were larger, flatter, and more firmly adherent than their counterparts (section 2.3.1.3), it is possible that the influence of 1,25$(OH)_2D_3$ on the expression on several GTPases and various GTPase regulators may have contributed to this altered morphology.

Additionally, several specific small GTPases that showed altered expression were related to specific functions also shown to be influenced by the presence of 1,25$(OH)_2D_3$. For example, 1,25$(OH)_2D_3$ treatment resulted in the significant differential increase in the expression *RAB20* and *RAB37*, in both cell types, as well as *RAB30*, in the macrophages alone. The RAB20 and RAB37 proteins, encoded by primary 1,25$(OH)_2D_3$ target genes *RAB20* and *RAB37*, are involved in phagosome maturation and autophagosome biogenesis and regulation, particularly for bacterial pathogen containing autophagosomes, respectively (Pei *et al.,* 2014; Oda *et al.,* 2016; Nakajima *et al.,* 2019). Similarly, the RAB30 protein, encoded by *RAB30*, has an important role in autophagosome biogenesis and cargo exocytosis promoted by secretory autophagy (Sheng *et al.,* 2017; Song *et al.,* 2018; Wu *et al.,* 2023). 1,25$(OH)_2D_3$ has a well-established relationship in supporting autophagy. This is generally associated with cathelicidin activity, yet it is plausible that 1,25$(OH)_2D_3$ supports this function further through the increased expression of *RAB37*, in both the monocytes and macrophages, and *RAB30*, in the macrophages (Sassi *et al.,* 2018; Ao *et al.,* 2021; Bishop *et al.,* 2021; Bhutia, 2022; Ismailova and White, 2022). Likewise, in macrophages RHOB, which is encoded by *RHOB*, has been shown to be involved in mannose receptor mediated phagocytosis (Zhang *et al.,* 2005). In the macrophages, 1,25$(OH)_2D_3$ resulted in the significant differential increase in the expression of both *RHOB* and mannose receptor encoding genes *MRC1* (log_2FC = 2.67; p<0.001) and *MRC2* (log_2FC = 1.18; p<0.001) indicating a role for 1,25$(OH)_2D_3$ in this process. Unlike RHOB, RHOH is an atypical GTPase, encoded by primary 1,25$(OH)_2D_3$ target *RHOH*. RHOH is involved in the negative regulation of type 17 T-helper T-cell polarisation and its

reduced expression has been associated with defective cross-talk between macrophages and T cells, which results in the presentation of self-antigens and may contribute toward autoimmunity (Tamehiro *et al.,* 2019; Chong *et al.,* 2023). This may represent an additional mechanism through which $1,25(OH)_2D_3$ acts to reduce aberrant immune activation in the macrophages.

$1,25(OH)_2D_3$ also resulted in the decreased expression of *TAGAP* in the macrophages, which likely represents a further protective layer against autoimmunity. The aberrant expression of the T-cell activation GTPase activating protein, encoded by *TAGAP*, is likewise associated with autoimmunity, particularly multiple sclerosis and rheumatoid arthritis, resulting from inappropriate T-cell activation (Berge *et al.,* 2016; Shri Preethi *et al.,* 2023; Xu *et al.,* 2023). This immunomodulatory effect of $1,25(OH)_2D_3$ as it relates to the expression of *TAGAP* appears quite likely as *TAGAP* was recently shown to contain VDRE in its regulatory region, and $1,25(OH)_2D_3$, and its derivative 22-oxa-$1,25(OH)_2D_3$, has been shown to repress TAGAP protein expression in CD4+ T cells (Berge *et al.,* 2016; Shri Preethi *et al.,* 2023). Overall, the contribution of $1,25(OH)_2D_3$ in the regulation of small GTPases may result in several possible effects, which may include alterations to THP-1-derived macrophage morphology, an enhanced capacity for autophagy and phagocytosis, and the restriction of auto-inflammation resulting from the inappropriate activation of T cells. However, this merely represents a range of potential, and any hypothesis drawn here would require further validation.

4.5 Conclusions

Treatment with $1,25(OH)_2D_3$ produced distinct gene expression profiles in monocytes and macrophages. Naturally, numerous differentially expressed genes were shared between these two cell types, a large proportion of which were known primary $1,25(OH)_2D_3$ targets. Though $1,25(OH)_2D_3$ altered the expression of genes that contribute toward the same general function within the monocytes and macrophages, these genes were often distinct between the two cell types. One of the most well recognised roles of $1,25(OH)_2D_3$ in innate immunity, is its contribution toward antibacterial defence, in part through antimicrobial peptide production. Both the monocytes and macrophages showed significant differential expression of *CAMP* in response to $1,25(OH)_2D_3$. However, in addition to this, $1,25(OH)_2D_3$ treated macrophages showed significantly differentially increased expression of several other antimicrobial peptide encoding genes, not observed in their monocyte counterparts. In contrast, $1,25(OH)_2D_3$ treatment in monocytes alone was shown to contribute toward the potential for the generation of reactive oxygen species, particularly through the increased expression of NOX2 subunit encoding genes. Furthermore, in the $1,25(OH)_2D_3$ treated macrophages alone, there was significant differential decrease in numerous type I interferon response encoding genes,

whose aberrant expression is associated with several auto-immune disorders. For both cell types, $1,25(OH)_2D_3$ treatment resulted in altered mRNA expression of small GTPases known to be involved in $1,25(OH)_2D_3$ linked processes, such as autophagy and phagocytosis. Yet, in the macrophages, $1,25(OH)_2D_3$ may have further contributed to macrophage specific responses, through altering cellular morphology, as clearly observed in the morphological assessment (section 2.3.1.3). Consequently, it is likely that $1,25(OH)_2D_3$ participates in several, distinct protective mechanisms in the context of monocytes and macrophages, that in part may be attributed to mechanisms not previously identified as being associated with $1,25(OH)_2D_3$ activity.

5.1 Rationale of the study

Macrophages, and their monocyte precursors, are innate immune cells with central roles in homeostasis and disease. Despite their critical role, many aspects of macrophage biology remain poorly understood. This is influenced by several factors including the highly plastic nature of these cells, difficulties in the isolation of primary macrophages, notable deviation between human and animal models and inconsistencies in the generation of macrophages for experimentation. Inconsistencies in the generation of monocyte-derived macrophages, are frequently observed when using primary monocytes and MLCLs and are known to produce large deviations in results, alter the applicability of these cells in downstream applications and lead to a lack of comparability between studies. MLCLs have several practical advantages in the generation of macrophages and represent a starting point for numerous investigations in basic research. Thus, having a physiologically relevant method for the generation of macrophages from MLCLs is important in the study of differentiation and subsequently macrophage biology. Vitamin D_3, in its biologically active form $1,25(OH)_2D_3$, has been indicated as an important immunomodulator, and a potential, though debated, inducer of monocyte-to-macrophage differentiation and macrophage polarisation agent in human cells. Its influence in monocyte-to-macrophage differentiation and subsequent influence on macrophage biology bares further investigation as there is comparatively little data on this when compared to the role of $1,25(OH)_2D_3$ in monocytes. As such, this study had two interconnected aims. Firstly, to generate a more physiologically relevant differentiation protocol for the differentiation of widely used monocyte-like cell lines, THP-1 and U937, that considered the influence of $1,25(OH)_2D_3$. Secondly, to assess at a transcriptome level, the efficacy of this differentiation protocol, the role of $1,25(OH)_2D_3$ therein, and the potential effect of $1,25(OH)_2D_3$ on macrophages when compared to monocytes.

5.2 Summary of the results

The most effective protocol for the differentiation of THP-1 and U937 cells was a combination of a low dosage of PMA and a more physiologically relevant, 10 nM concentration of $1,25(OH)_2D_3$, over 96 hours. While 5 nM of PMA was capable of inducing differentiation, the presence of $1,25(OH)_2D_3$ greatly enhanced macrophage characteristics, at all levels assessed. However, $1,25(OH)_2D_3$ alone was not considered capable of inducing differentiation in THP-1 or U937 cells. RT-qPCR results indicated that THP-1 cells were more responsive to $1,25(OH)_2D_3$-based immune modulation than their U937 counterparts. Differential gene expression analysis of THP-1 cells exposed to $1,25(OH)_2D_3$, PMA and a combination thereof, confirmed that $1,25(OH)_2D_3$ enhanced the macrophage characteristics produced by PMA, and

reflected those identified in primary macrophages. Furthermore, these analyses indicated that neither THP-1 cells differentiated with PMA or PMA with 1,25$(OH)_2D_3$ were likely to represent fully polarised macrophages. Furthermore, PMA exposed THP-1 cells did not show a particular proclivity toward excessive inflammation, frequently observed with high PMA concentrations. When considering the alterations in gene expression induced by treatment with 1,25$(OH)_2D_3$ in THP-1 monocytes and THP-1-derived macrophages, it was evident that although having demonstrated a notable degree of overlap, these two cell types likewise had distinct responses to 1,25$(OH)_2D_3$. In both THP-1 monocytes and THP-1-derived macrophages gene expression analysis indicated that 1,25$(OH)_2D_3$ contributed toward similar broad biological processes. Despite this, the differential expression of genes related to these processes was often distinct between the two cell types. Notably, 1,25$(OH)_2D_3$ contributed toward the increased expression of several antimicrobial peptide encoding genes, outside of *CAMP*, particularly in THP-1-derived macrophages. Similarly, in these cells 1,25$(OH)_2D_3$ was responsible for the decreased expression of numerous genes associated with type I interferon response, which was not observed in 1,25$(OH)_2D_3$ treated monocytes. Overall, it was evident that 1,25$(OH)_2D_3$ supported the differentiation of THP-1 cells but was not capable of inducing differentiation alone. Despite this, 1,25$(OH)_2D_3$ notably altered the transcriptomic landscape of THP-1-derived macrophages, in a manner that differed from the effects observed in THP-1 monocytes.

5.3 Implications of the findings

The development of an optimised monocyte-to-macrophage differentiation protocol for MLCLs, that is more reflective of physiological conditions, has several applications for future basic research studies. Notably, the use of said protocol would be unlikely to hinder polarisation, nor prevent response to weaker immune stimuli while still representing fully differentiated macrophages. Additionally, the results of this study indicate that while not necessarily being an inducer of differentiation, 1,25$(OH)_2D_3$ has several potential roles in macrophage biology. These include altering macrophage morphology, modulating inflammatory response, enhancing antibacterial defence and phagocytosis. Given the role of macrophages in the innate immune system, their importance in health and disease, this suggests several protective roles of 1,25$(OH)_2D_3$. These would be linked both to protection against bacterial infection, as well as being an immunomodulator with distinct roles in restricting type I interferon responses thus limiting autoinflammation and the potential complications that arise therein.

5.4 Challenges and limitations

Though the success of monocyte-to-macrophage differentiation was assessed at multiple levels in THP-1 and U937 cells, no functional work was undertaken to ensure that these MLCL-derived macrophages had the inherent functional capacities associated with macrophage biology, such as phagocytosis. Additionally, RNA sequencing was only undertaken for the differentiated THP-1 cells, as they appeared more responsive to the immunomodulatory properties of $1,25(OH)_2D_3$. However, for a fully comprehensive analysis of the best model to represent monocyte-derived macrophages the U937 cells exposed to the same conditions ought to have undergone RNA-sequencing as well. However, given the costly nature of RNA-sequencing, it was only deemed feasible to assess the differentiated THP-1 cells for this study. Additionally, while the gene expression profiles indicated that PMA and PMA with $1,25(OH)_2D_3$ differentiation protocols were unlikely to interfere with polarisation, this was not validated in this study. However, M1- and M2-macrophage polarisation have since been attempted following these protocols in the broader group, these results indicated that PMA with $1,25(OH)_2D_3$ is the best protocol to apply prior to polarisation as the presence of $1,25(OH)_2D_3$ aids in commitment to a particular polarisation state, be it M1- or M2-macrophage polarisation in the differentiated THP-1 cells (J. Wasinda, personal communication). As with this, the observations made for the monocyte and macrophage specific alterations in the expression of genes of $1,25(OH)_2D_3$ have not been validated with additional experiments.

Furthermore, while the optimised differentiation protocol for THP-1, and potentially U937, cells more closely represent physiological conditions, MLCLs and any of their derivatives are not primary cells. While they have a valuable role in basic research, any hypothesis generated employing these MLCLs as a representative for either monocytes, macrophages, or dendritic cells requires validation in primary cells before any conclusive statements can be made. Primary cells are highly variable by nature, even more so when considering macrophages which are highly plastic cells and show large degrees of variability between donors. It is not possible to control or account for every aspect of donor variability which may affect the way cells respond to $1,25(OH)_2D_3$. Given the costly nature of RNA sequencing, it was not deemed feasible or necessary for this study to make use of primary cells as this work is in its infancy.

RNA sequencing is a powerful tool because it allows assessment of the complete set of RNA transcripts in the cells in question. However, the transcriptome only represents one level of this process, and a plethora of other factors determine whether these transcripts are actively translated, whether the derived proteins are stable and functional, and so forth. Additionally, it is now well known that the transcriptome has several additional layers of complexity, not accounted for in this study in part due to the use of short-read poly-A enriched RNA

sequencing, in the form of alternative splicing and transcription of non-coding RNAs like small RNAs. Furthermore, though alterations in gene expression were observed, there is no indication as to the mechanism that dictates this change in expression, though this may be inferred from literature for some genes, there are still many that would be unaccounted for.

5.5 Future direction

An optimised protocol for the generation of more physiologically relevant THP-1 macrophages, that reflect their primary counterparts at a transcriptomic level, could see broad application in the field of macrophage biology. However, in light of the results and the limitations of this study, future work ought to include further validation of the MLCL-derived macrophage models. Firstly, through the sequencing of the U937 cells exposed to the same differentiation conditions. Secondly, functional validation of the capacities of these MLCL-derived macrophages and determination of the degree to which they reflect their primary macrophage counterparts. Additional -omics technologies could potentially be undertaken to further validate this model. Transcriptomics, like other -omics technologies, often generates more questions than it answers. While this study indicated that $1,25(OH)_2D_3$ produces distinct gene expression profiles between THP-1 monocytes and THP-1-derived macrophages, and indicated what the altered expression of those genes may contribute toward, several additional studies would be required to validate this. If the aim was to further validate whether treatment with $1,25(OH)_2D_3$ generates distinct effects in monocytes versus macrophages the application of proteomics and metabolomics would add an additional layer of surety. Alternatively, to investigate the manner in which $1,25(OH)_2D_3$ exerts its genomic effects in the macrophages the use of techniques that evaluate protein-genome interactions may be suggested, such as chromatin immunoprecipitation sequencing. A further extension of the work could involve validating at a gene expression level, via RT-qPCR, and at a protein level the altered expression of the various antimicrobial peptides and type I interferon response related proteins shown to be differentially expressed in THP-1-derived macrophages treated with $1,25(OH)_2D_3$. Further functional validation of these findings would also be of use. For example, to assess whether THP-1-derived macrophages treated with $1,25(OH)_2D_3$ demonstrate a heightened capacity for the control of bacterial proliferation and infection.

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Supplementary Figures

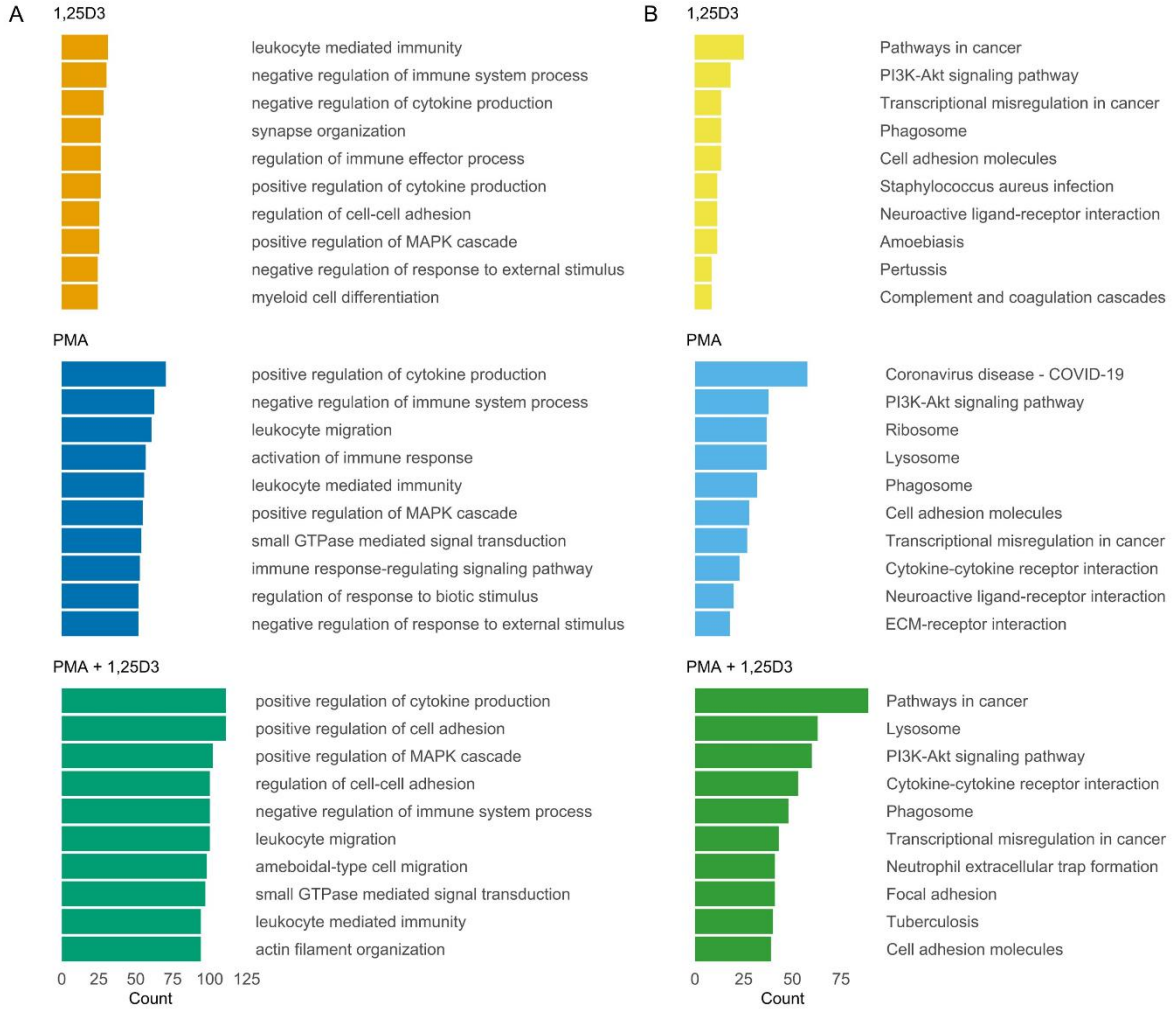


Figure S1: Over-representation analysis shows THP-1 cells differentiated by PMA with $1,25(OH)_2D_3$ had the largest alterations in gene expression associated with macrophage related functions and characteristics. Top 10 significantly over-represented GO biological processes by gene count for each differentiation condition (adjusted *p* value < 0.010; A). Top 10 significantly over-represented KEGG pathways by gene count for each differentiation condition (adjusted *p* value < 0.050; B).

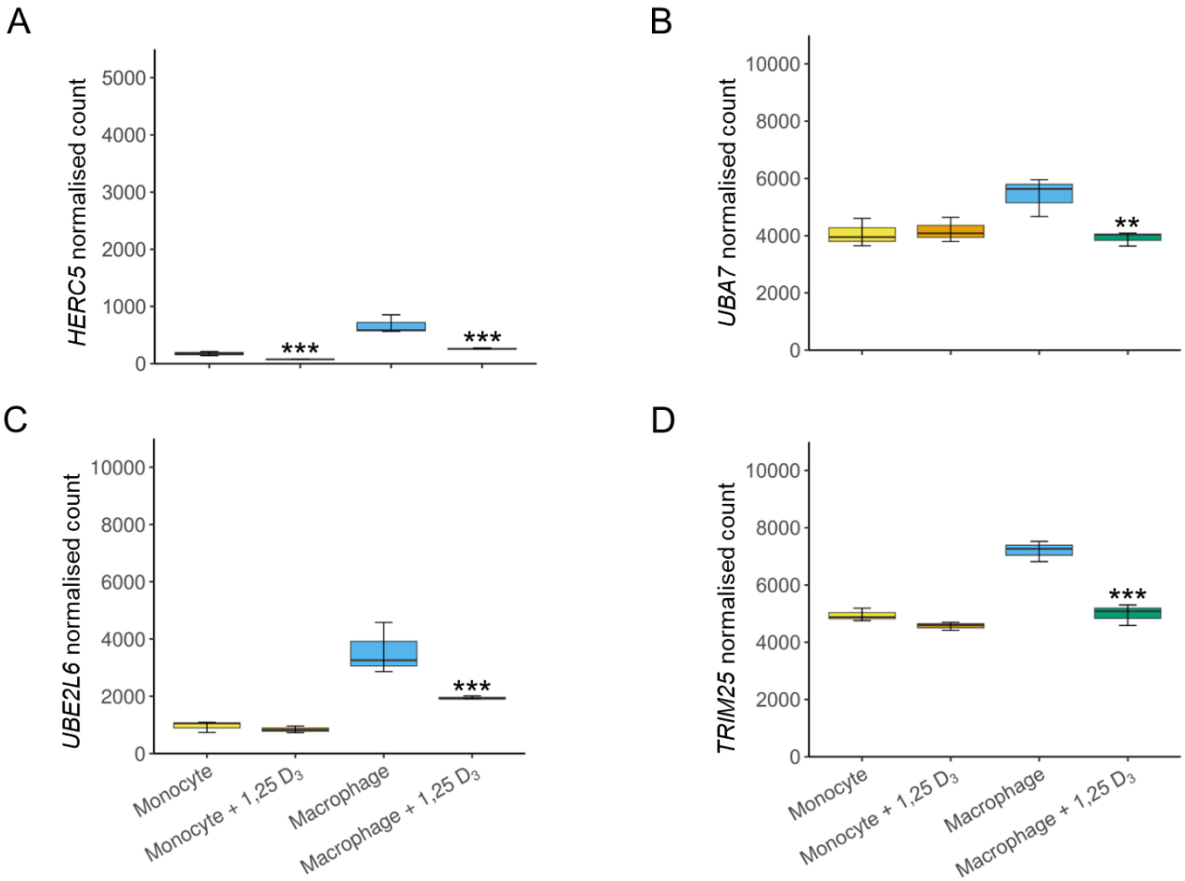


Figure S2: The presence of $1,25(OH)_2D_3$ in THP-1-derived macrophages resulted in the significant decrease in the genes encoding enzymes required for ISGylation. The boxplots show DESeq2 derived normalised counts for the genes related to ISGylation in relation to the type I interferon response *HERC5* (A), *UBA7* (B), *UBE2L6* (C), and *TRIM25* (D) for monocytes and macrophages in the presence and absence of $1,25(OH)_2D_3$. Statistically significant differences between $1,25(OH)_2D_3$ treated and untreated monocytes and macrophages are given based on the Benjamini-Hochberg adjusted *p* values derived from DESeq2 analysis (**p*<0.050, ***p*<0.010, ****p*<0.001).

Supplementary Data Tables

The supplementary data (Data S1; Tables S1-S19) for this work can be accessed via

https://docs.google.com/spreadsheets/d/1arV1wdg7Q9epUCUg5gyR9sQalwGSr1OJ/edit?usp=sharing&ouid=107397644636495421055&rtpof=true&sd=true

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