
**Differential Gene Expression Analysis of PMA Treated Pro-monocytic Cell Lines**

by

**Asavela Olona Kama**

**(1614247)**

**Dissertation**

Submitted in fulfilment of the requirements for the degree of

**Master of Science**

in

**Molecular and Cell Biology**

in the faculty of Science , University of the Witwatersrand, Johannesburg, South Africa

Supervisor: Dr Nikki Gentle

Co-supervisor:Dr Vanessa Meyer

August 2023

I, Asavela Olona Kama (1614247), am a student registered for the degree of Master of Science (MSc) by research in the academic year.

__2023___.

I hereby declare the following:

• I am aware that plagiarism (the use of someone else's work without their permission and/or

without acknowledging the original source) is wrong.

• I confirm that the work submitted for assessment for the above degree is my own unaided work except where explicitly indicated otherwise and acknowledged. In this context, I understand that the use of editing services is considered aided work and must be declared.

• I have not submitted this work before for any other degree or examination at this or any other University.

• The information used in the Dissertation HAS NOT been obtained by me while employed by, or working under the aegis of, any person or organisation other than the University.

• I have followed the required conventions in referencing the thoughts and ideas of others.

• I understand that the University of the Witwatersrand may take disciplinary action against me if there is a belief that this is not my own unaided work or that I have failed to acknowledge the source of the ideas or words in my writing.

signature_____13_ ____ day of _ September_____ YEAR 2023

# Acknowledgements

I would like to thank my supervisors, Dr Nikki Gentle and Dr Vanessa Meyer. I appreciate all your hard work, assistance, guidance, wisdom, energy and time. I do not take any of these things for granted. I have learnt a lot from you and I continue to do so. This dissertation would have not been possible without you. I am also grateful for the support I have received from friends and family. To my grandmother, Nambitha Fobo and my mother, Zimkhitha Kama, thank you for the constant encouragement and support. I would like to thank God and my church family for the immense support I have received.

I can not express enough gratitude towards the lab at large. Thank you for all the jokes, the help and the walks on campus. Darisia, Kelda and Yi, you have made this experience better by being there. I appreciate you all so much.

I would also like to acknowledge the National Research foundation for funding this project.

# Research outputs

**Oral presentations:**

- Differential Splicing Analysis of PMA treated promonocytic cell lines (2021). SASBi/SAGS student symposium. Online.

- Differential expression analysis of PMA treated promonocytic cells (2021). MBRT Virtual Postgraduate Research Day, University of the Witwatersrand (Placed 3rd in the Gradflash category)

**Poster presentation:**

- Differential gene expression analysis reveals differential regulation of the NF-kB signalling pathway in PMA-induced monocyte-to-macrophage differentiation of model cell lines (2022). South African Society for Bioinformatics joint conference: BIO22, STIAS Stellenbosch Institute for Advanced Study, Stellenbosch.

# Table of Contents

# List of Tables



# List of Figures

# List of Abbreviations

AK - Adenylate kinase

ATP - Adenosine Triphosphate

CDC - Cell division cycle

CSF - Colony stimulating factor

CTPS - Cytidine Triphosphate Synthase

DAMP - Damage associated molecular pattern

DC- Dendritic cells

DEG - Differentially expressed gene

DNA - Deoxyribonucleic acid

ELISA - Enzyme-linked immunoassay

Fc - Fragment crystallizable

FDR - False discovery rate

G1 - Gap 1

GC - Guanine Cytosine

GM-CSF Granulocyte macrophage-colony stimulating factor

GO- Gene Ontology

GPI - Glycosylphosphatidylinositol

HIV- Human immunodeficiency syndrome

HK- Hexokinase

HMGB- High mobility group box

ICAM - Intracellular adhesion molecule

IL - Interleukin

IRF - Interferon regulatory factor

KEGG - Kyoto encyclopaedia of genes and genomes

LPS - Lipopolysaccharide

MHC - Major Histocompatibility Complex

mRNA - Messenger ribonucleic acid

NFKB - Nuclear factor kappa beta

NGS - Next generation sequencing

NLR - NOD-like receptors

NOD - Nucleotide Oligomerization domain

PAMP - Pathogen associated molecular pattern molecule

PBMC - Peripheral blood mononuclear cells

PCR - Polymerase chain reaction

PKC - Protein kinase C

PMA - Phorbol 12-myristate 13-acetate

PRR - Pathogen recognition receptor

PS - Phosphatidylserine

RAGE - Receptor for advanced glycation endoproducts

RLR - Retinoic acid-inducible gene (RIG-I)-like receptor

RNA - Ribonucleic acid

S1 - Synthesis 1

ssRNA- single-stranded Ribonucleic acid

TLR - Toll-like receptor

TNF - Tumor necrosis factor

VD3 - 1,25-dihydroxy-vitamin D3

VLMM- Variable length markov model

# Abstract

HL-60, THP-1, and U937 are model cell lines that can undergo myeloid differentiation *in vitro*, allowing the study of myeloid cell function in drug metabolism, cytotoxicity, and the aetiology of infections. However, the differentiated end-state of these cells is not well characterised. Moreover, cell line-specific differences in the level of gene expression may confound results obtained from such studies. The aim of this study was thus to compare changes in gene expression between HL-60, THP-1, and U937 cells in response to the differentiation agent, phorbol 12-myristate 13-acetate (PMA), 48 hours after treatment. Gene expression profiles were compared across all three cell lines prior to and post-PMA treatment. Differential gene expression analysis between treated and untreated cells was performed using DESeq2 (v 4.2). Gene over-representation analysis was performed using clusterProfiler (v 4.0). HL-60, THP-1, and U937 cells had similar expression profiles prior to PMA treatment, but different sets of genes were significantly differentially expressed in these cell lines 48 h after treatment with PMA. A total of 475 genes were consistently differentially expressed across all cell lines. These genes were found to be involved in phagosome formation and cell cycle transition. HL-60, THP-1, and U937 cells had 944, 1231, and 624 uniquely differentially expressed genes, respectively. These genes were predominantly involved in energy metabolism and pathogen recognition. Overall, THP-1 cells showed greater potential to detect viruses, while U937 cells showed greater potential to detect bacteria. From this, it can be concluded that while all three cell lines did indeed undergo myeloid differentiation, the macrophage-like cell state produced in each case differed between cell lines.

# 1. Introduction

## 1.1 Innate immunity

The immune system comprises cells that function to regulate cellular activity, maintain cellular homeostasis, and defend against pathogens (Kaufmann, 2019). These cells are divided between two major divisions, namely the innate and adaptive immune responses (Janeway, 1989; Turvey and Broide, 2010). Distinguishing factors between these two responses include the timing of the response and the mechanisms used during the response. Innate cells are activated as a first line of defence against pathogens or any unusual cell activity (Turvey and Broide, 2010). This is achieved through the rapid activation of cells at the site of infection, followed by the migration of cells to the infected area. In both responses, defence mechanisms are enforced against the pathogen, to destroy it and restore tissue homeostasis. Innate cells produce an untargeted response against any foreign element and later activate the adaptive immune response (Turvey and Broide, 2010).

Detection of pathogens by innate immune cells involves the detection of generic or common viral or bacterial elements, hence it is not as specific as the adaptive response. Innate immunity detects pathogen molecules that are important for the survival of the pathogen and virulence in the host. This is effective because these are highly conserved elements, namely, proteins, carbohydrates, nucleic acids, and lipids obtained from the pathogen (Turvey and Broide, 2010). Since these molecules are not typically expressed by healthy human cells, cells that harbour them are identified as foreign (Tang *et al*., 2012). Examples include the peptidoglycan envelope protein found in many bacteria, the zymosan protein detected in fungi, or the single-stranded RNA (ssRNA) found in many viruses. These elements are known as pathogen-associated molecular patterns (PAMPs) and they are recognized by pathogen recognition receptors (PRRs) found on the surface of innate cells (Figure 1.1).

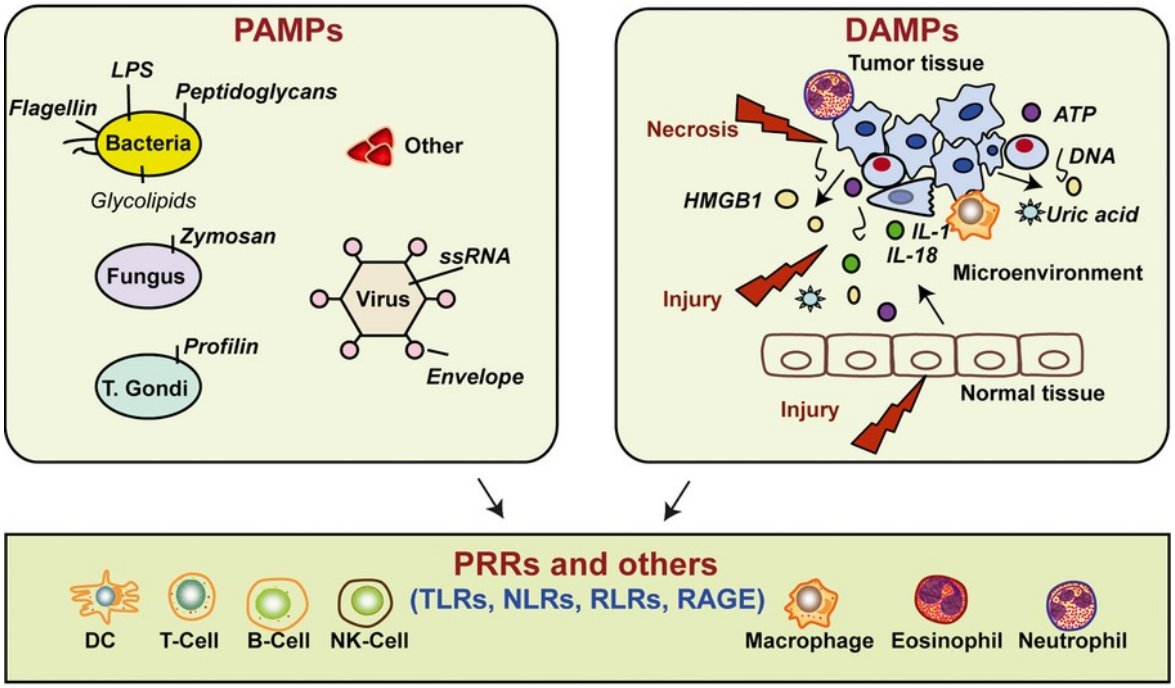



**Figure 1.1: Examples of pathogen-associated molecular patterns (PAMPS) and damage-associated molecular patterns (DAMPS).** Innate immune cells express pathogen recognition receptors (PRR) that detect conserved pathogenic elements (PAMPs) and signals of cellular damage (DAMPs). PRRs include Toll-like receptors (TLR), NOD-like receptors (NLRs), Retinoic acid-inducible gene I (RIG-I)-like receptors (RLR) and Receptor for advanced glycation endoproducts (RAGE). Figure adapted from Tang *et al*. (2012).

PRRs are grouped into various families based on the antigen that they interact with (Takeuchi and Akira, 2010). Toll-like receptors (TLR) are a family of PRRs characterised by receptor dimerization (Takeuchi and Akira, 2010). The structure and location of the dimer determines the type of PAMP that TLRs can interact with. TLRs can be localized on either the extracellular or intracellular region of the cell (Figure 1.2). TLR1 and TLR2 dimerize together to detect bacterial PAMPs (Takeuchi and Akira, 2010). TLRs 2/4/9 interact with a variety of PAMPs from bacteria, fungi, protozoa, and viruses (Takeuchi and Akira, 2010). TLR5 exclusively interacts with bacteria by detecting flagellin. TLR3 interacts with viruses by interacting with double-stranded RNA (Takeuchi and Akira, 2010). TLRs 7 and 8 interact with viruses by detecting single-stranded RNA antigens. In addition to the detection of PAMPs, PRRs detect molecules that are overexpressed in injured cells. Damage-associated molecular patterns (DAMPS) are proteins expressed by injured cells as indicators of alterations in cell metabolism (Tang *et al*., 2012). Examples of cell injury include cell lysis,

which induces the up-regulation of the high mobility group box1 protein (HMGB1), the release of heat shock proteins, and uric acid (Rubartelli and Lotze, 2007).

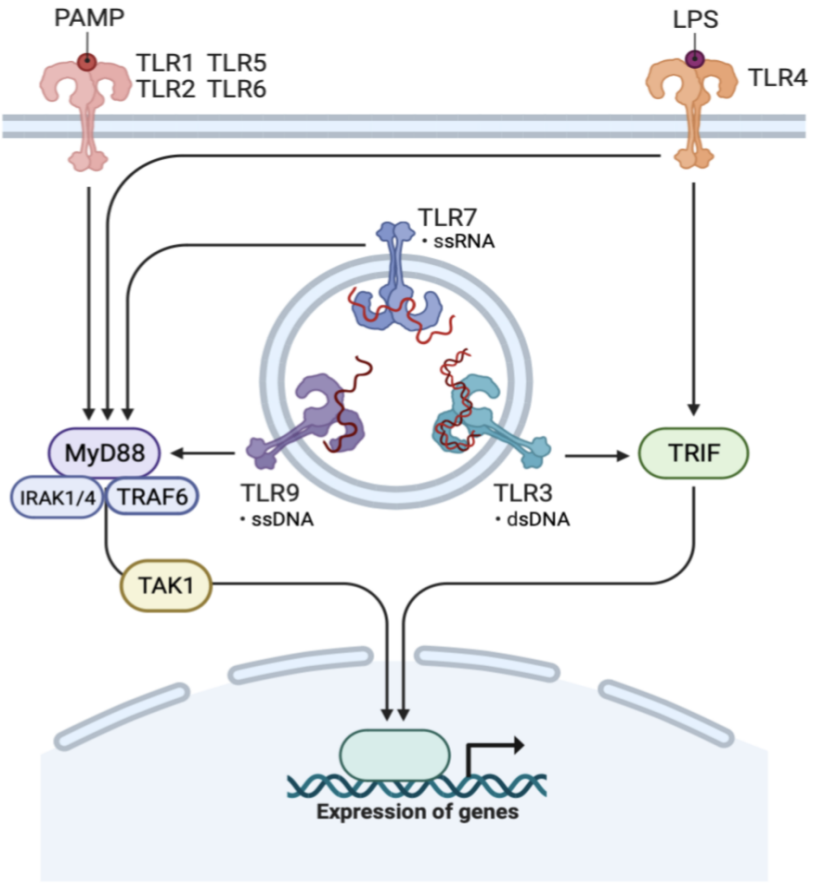



**Figure 1.2 : Extracellular and intracellular interactions between TLRs and PAMPs.** TLRs on the extracellular matrix recognise PAMPs such as LPS, whereas, intracellular TLRs detect nucleic acids such as DNA and RNA. TLR receptors interact with effector molecules such as the myeloid differentiation primary response gene (MyD88), interleukin 1 associated kinase (IRAK1/4) , tumour necrosis factor receptor associated factor 6 (TRAF6), TIR-Domain containing adaptor-inducing interferon $\beta$ (TRIF), and transforming growth factor $\beta$- activated kinase 1 to induce gene expression. Adapted from "TLR signalling pathway (with type I interferon gene expression)", by BioRender.com (2023). Retrieved from https://app.biorender.com/biorender-templates

The binding of a PAMP to a PRR induces an immune response (Takeuchi and Akira, 2010), characterised by the activation of cell signalling cascades that alter gene expression (Figure 1.3). Genes expressing various signalling molecules trigger the cells to initiate self-defence mechanisms against the pathogen and recruit other cells to the site of infection. Among

others, these signalling cascades induce cell proliferation, division, differentiation, and inflammation (Rosales & Uribe-Querol, 2017). Such signalling pathways include the toll-like receptor pathway, the NOD-like receptor pathway, and the NF-κB pathway (Figure 1.2 and Figure 1.3). Cell division is necessary for an effective response to the infection because large quantities of immune cells need to be transported to the region of injury or infection. These viral or bacterial antigens are presented to $CD4^+$ T cells via MHC II receptors. Cytokines and chemokines are released to alter the activity of other cells and initiate an inflammatory response. Inflammation is the ordered recruitment of cells to a site of infection to target foreign pathogens. It is directed by downstream targets of the signalling cascade with transcription factors that are regulators of inflammatory responses. Examples include NF-κB and Interferon regulatory factor (IRF) 3, which regulate the expression of inflammatory and anti-inflammatory cytokines.

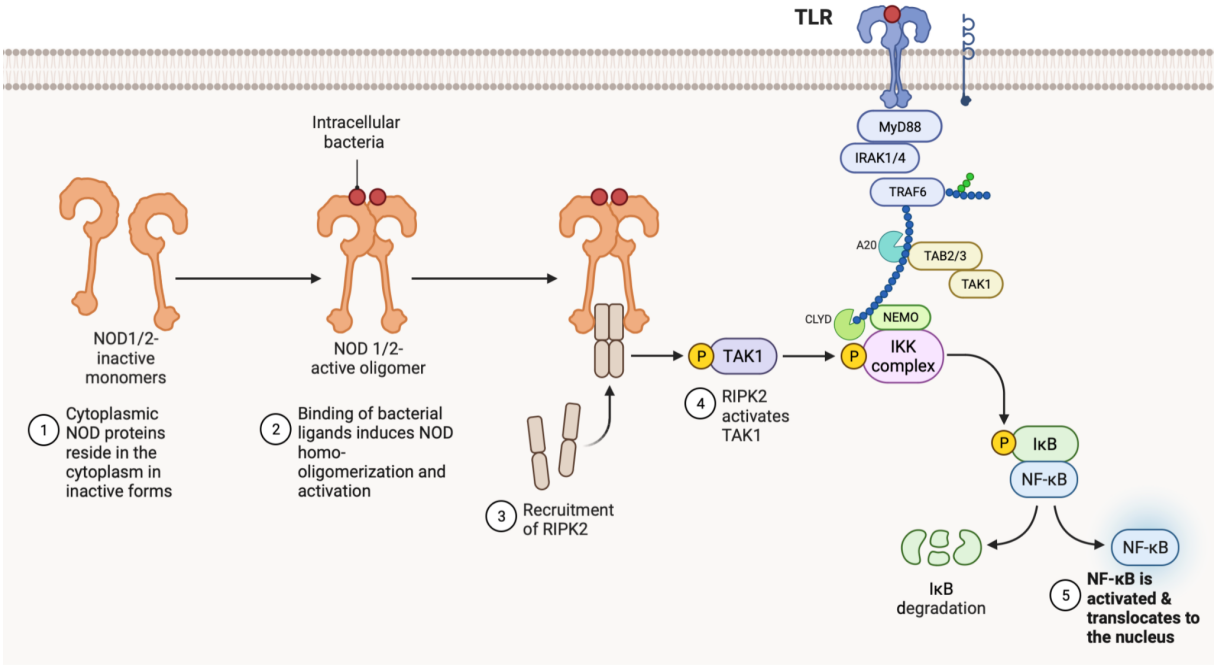



**Figure 1.3 : Interaction between NOD-like receptor, TLR and NF-κB pathways.** Intracellular activity of NOD-like receptors in response to intracellular bacteria (1,2 and 3), leads to the activation of the NF-κB pathway (4 and 5).Translocation of the NF-κB transcription factor is induced by TLR or NOD-like receptor activity (4 and 5). Key proteins involved in this process include the receptor interacting serine/threonine kinase 2 (RIPK2), Inhibitor of nuclear factor kappa-B kinase (IKK), nuclear factor essential modulator (NEMO), and inhibitor of nuclear factor kappa-B (IkB).Adapted from "Detection of Bacterial Peptidoglycan by NOD receptors", by BioRender.com (2023). Retrieved from https://app.biorender.com/biorender-templates

The first evidence of innate immunity was in the discovery of phagocytosis (Janeway, 1989; Kaufmann, 2008), which is the process by which cells detect PAMPs, allowing the ingestion of foreign cell particles into a phagosome. Phagocytic cells are important for removing microbial pathogens and apoptotic cells (Gordon, 2016). Hence, they regulate tissue homeostasis. PRRs detect PAMPs and DAMPs to alert cells of the presence of a threat. PRRs such as the mannose receptor, CD14, and CD36 detect DAMPs from apoptotic cells (Rosales and Uribe-Querol, 2017). Phagocytosis is not only limited to immune cells. Fibroblasts and epithelial cells can also perform phagocytosis, but they are only specialised to ingest apoptotic bodies and not microorganisms (Rabinovitch, 1995). Professional phagocytes engulf a pathogen and destroy it through lysosomal degradation (Rabinovitch, 1995). Such cells include monocytes, neutrophils, dendritic cells, eosinophils, macrophages, and osteoclasts (Rabinovitch, 1995). Following phagocytosis, antigens that are features of the invading pathogen are processed by these cells and presented to the cells of adaptive immunity (Turvey and Broide, 2010).

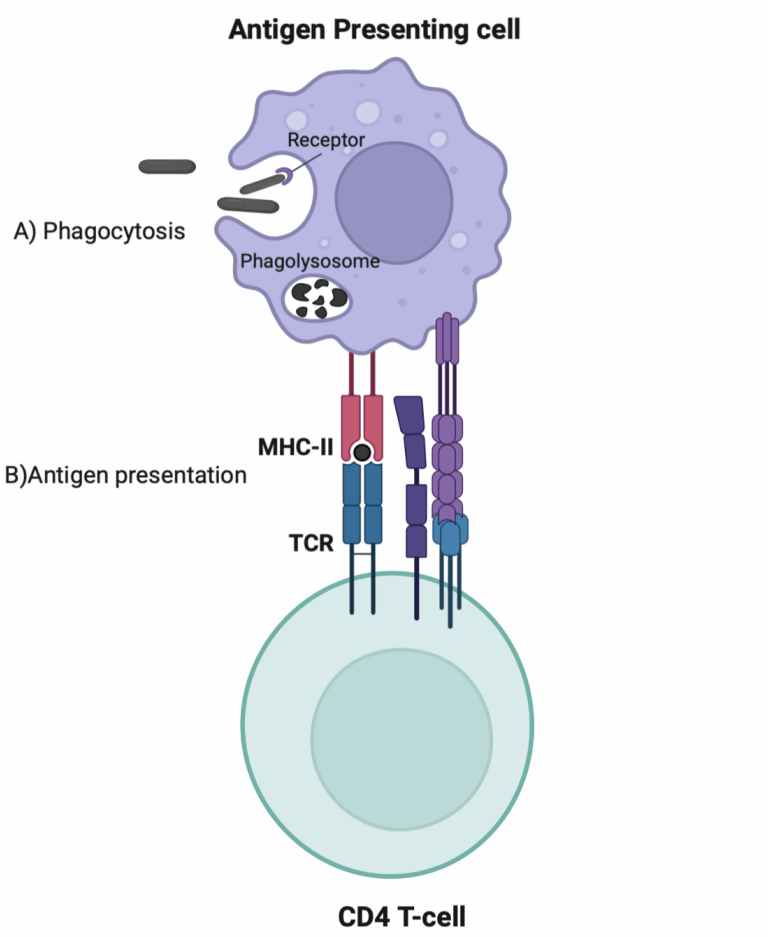



**Figure 1.4: A schematic diagram showing the key processes involved in phagocytosis.** A). Detection of a bacterial PAMP by a PRR on the surface of the phagocytic cell leads to the ingestion of the pathogen into a phagosome. The pathogen is subjected to lysosomal degradation. B) Bacterial antigens are presented to T-cells via the interaction between the major histocompatibility complex (MHC) class II molecules and T-cell receptors (TCR). Adapted from "CD4 T cell and dendritic cell interaction ", by BioRender.com (2023). Retrieved from https://app.biorender.com/biorender-templates

### 1.2. Differentiation of myeloid progenitor cells

Cell differentiation is a process through which cells become specialised for their specific functions. Differentiation of myeloid progenitor cells in the bone marrow results in monocytes, which are found in the circulatory system. This gives rise to a heterogeneous set of cells characterised by the expression of the cell surface receptors, CD14 and CD16 (Passlick *et al.,* 1989). They are grouped into classical (CD14++ and CD16+), intermediate (CD14+ and CD16+), and non-classical monocytes (CD14+ and CD16++) (Kapellos *et al.*, 2019; Ożańska *et al*., 2020). Classical monocytes are associated with the release of

inflammatory cytokines when they enter tissues, hence they are also known as inflammatory monocytes. Intermediate monocytes also release inflammatory cytokines and express interleukin and tumor necrosis factor receptors (Kapellos *et al.*, 2019; Passlick *et al.,* 1989). The release of cytokines leads to the recruitment of cells to the site of infection. Consequently, this results in cells being in close proximity with antigen presenting cells, allowing effective antigen presentation (Kapellos *et al.,* 2019). In contrast, non–classical monocytes produce anti-inflammatory responses to repair damaged tissues during and after inflammation (Passlick *et al.,* 1989). Altogether, monocytes form about 10% of the peripheral blood mononuclear cells (PBMCs) in the circulatory system (Passlick *et al*., 1989). PBMCs are the collection of all the specialised immune cells within the blood (Acosta and Hernandez, 2019). This collection is mostly made up of lymphocytes and monocytes (Sen *et al.*, 2018). The half life of classical monocytes is approximately 48 hours in the circulatory system (Mohri *et al.*, 2001). However, some monocytes migrate into tissues where they may proceed to differentiate into other cell types (Chomarat *et al*., 2000; Jakubzick *et al*., 2013).

In response to cytokine signals, some monocytes travel to sites of infections where they differentiate into either dendritic cells or macrophages. Differentiation begins when the monocytes cross the endothelium, entering the tissue (Chomarat *et al*., 2000). This process is known as transendothelial migration. Various stimuli from the microenvironment drive monocytes toward specific end-states. Granulocyte macrophage-colony stimulating factor (GM-CSF) and interleukin-4 (IL-4) induce a dendritic cell end state (Chomarat *et al*., 2000). IL-6 expression from fibroblasts increases the expression of M-CSF receptors on the surface of monocytes (Chomarat *et al*., 2000). Consequently, the uptake of M-CSF takes place, leading to a macrophage end-state (Chomarat *et al*., 2000). Following differentiation, these cells exhibit a specific set of cell surface markers that indicate their end state.

Tissue-resident monocytes express the CD14 and CD68 markers, and similar genes relative to monocytes found in circulation (Jakubzick *et al.*, 2013). That said, the expression level of some transcripts has been reported to be higher in tissue-resident monocytes compared to circulatory monocytes (Jakubzick *et al*., 2013). Examples of such transcripts include cyclo-oxygenase-2 (COX-2) and MHC-II (Jakubzick *et al*., 2013). Jakubzick *et al.* (2013) suggested that these changes result from microenvironment signals, particularly, the interaction between monocytes and endothelial cells. These differences affect mechanisms

such as the detection of antigens and presentation of antigens using MHC II receptors, as well as inflammatory response involving COX-2 receptors (Jakubzick *et al.*, 2013).

### 1.3 Monocyte-to-macrophage differentiation

In mammalian embryos, the yolk sac gives rise to most adult tissue-resident macrophages (Figure 1.3). As the foetus develops, the liver contributes to the macrophage pool through hematopoiesis (Tan and Krasnow, 2016). However, over time, macrophages may undergo damage or become infected by pathogens (Tan and Krasnow, 2016). The process of monocyte-to-macrophage differentiation thus serves, among others, to replenish damaged or infected embryonically-derived tissue macrophages in adults (Tan and Krasnow, 2016). This process yields various subtypes of macrophages that are specialised to perform their functions in specific environments. They can be broadly grouped into M1 and M2 macrophages based on their involvement in regulating inflammatory responses (Koppensteiner *et al*., 2012). M1 refers to macrophages that are inclined to perform inflammatory responses, whereas M2 is mostly associated with anti-inflammatory responses (Martinez and Gordon, 2014). The microenvironment directs the subtypes of macrophages that can be obtained from monocyte differentiation (Martinez and Gordon, 2014) . High interferon-gamma expression leads to M1 macrophages. In contrast, the expression of interleukin factor-4 induces the M2 macrophage phenotype (Martinez and Gordon, 2014).

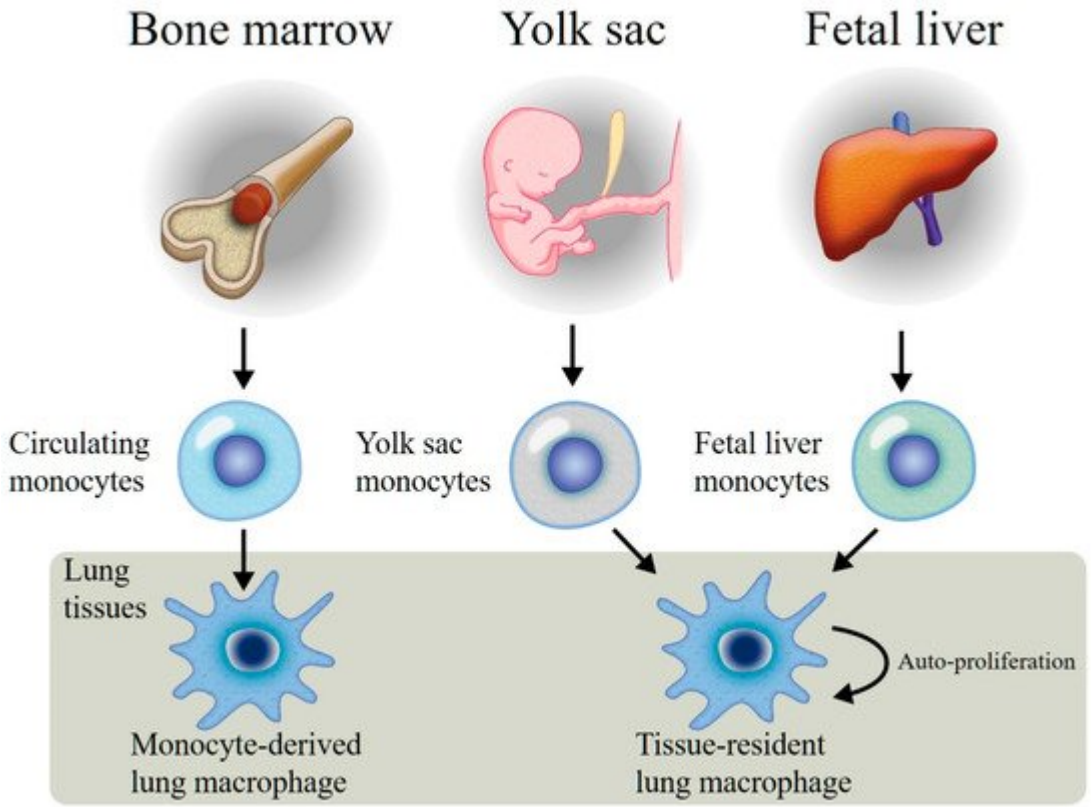



**Figure 1.5: Unique origins of lung macrophages give rise to distinct subsets of macrophages.** Monocyte-derived-macrophages are derived from circulating monocytes. Yolk sac and foetal liver monocytes give rise to tissue-resident macrophages (Yamasaki and Eeden, 2018).

In the lung there are various subpopulations of monocyte-derived macrophages, including alveolar and interstitial macrophages (Tan and Krasnow, 2016). Alveolar macrophages are concentrated in the pulmonary alveolus, where they maintain lung tissue homeostasis (Tan & Krasnow, 2016). Interstitial macrophages are known to be more specialised for antigen presentation and remodelling of the parenchymal tissue in the lungs (Gu *et al.*, 2022; Tan and Krasnow, 2016). Hofbauer cells are placenta macrophages that are essential for the prevention of mother-to-child pathogenic transmissions (Epelman *et al.,* 2014). Microglia in the brain detect the accumulation of altered neurons and prevent infections in the central nervous system (Epelman *et al.*, 2014). Due to the wide variety of tissues and organs that are protected by macrophages, monocyte-to-macrophage differentiation is a crucial process in immunity. The process is central to the understanding of immune responses to microbes such as bacteria, protozoa, fungi, and viruses. Monocyte-to-macrophage differentiation informs the

design of therapeutic strategies targeting a variety of infections or defects such as dysregulated tissue homeostasis and cancer.

### 1.4 Markers denoting monocyte-to-macrophage differentiation

There are notable physical differences in monocyte-derived macrophages relative to monocytes. These include a bigger cell size, with a diameter of 21 micrometres relative to the monocyte cell diameter of 9 micrometres (Koppensteiner *et al*., 2012). This can be associated with increased efficacy of phagocytosis. Macrophages also have a high nuclear to cytoplasmic ratio and exhibit mini pseudopods, granular chromatin irregular nuclei, and cytoplasmic vacuoles (Doshi and Mitragotri, 2010). Differences in cell surface receptor expression are also noted. For example, CCR5 is expressed in significantly greater amounts in macrophages relative to monocytes (Tuttle *et al.*, 1998). This also leads to differences in susceptibility to infection from pathogens that use these receptors. For example, macrophages may be more likely to be infected with human immunodeficiency syndrome (HIV-1) relative to monocytes. Reasons for this have not been clearly elucidated. However, one of the proposed explanations suggests that it is because CCR5 is one of the important receptors for effective HIV-1 infection (Flynn *et al*., 2013; Tuttle *et al.*, 1998).

In addition to CCR5, other receptors are also used to denote macrophage differentiation, namely, CD14, CD36, CD16, CD68, CD11b, and CD64. These are also the most commonly used markers for studying differentiation *in vitro*. CD14 is a GPI-anchored receptor that is involved in the detection of bacterial LPS and the recognition and removal of apoptotic cells (Devitt *et al.*, 1998). It lacks a transmembrane domain, therefore it requires coreceptors in order to induce a downstream cell signalling cascade (Devitt *et al*., 1998). TLR2 and TLR4 are important CD14 co-receptors for microbial ligands (Triantafilou and Triantafilou, 2002). CD36 is a non-collagenous scavenger receptor that is associated with atherogenesis, removal of apoptotic cells, and providing resistance against bacteria that exhibit diacyl lipid PAMPs (Taylor *et al.*, 2005). Integrin receptor, CD11b, is important for the adhesion of macrophages to the endothelium (Taylor *et al.*, 2005).

### 1.5 Studying myeloid differentiation *in vitro*

*In vitro* studies on the differentiation process are essential to enhance our understanding of immune cell activity. Model cell lines are often used instead of primary macrophages because they can be easily substituted in the event of contamination (Gazdar *et al.*, 2010). Some of these cells are myeloid cells whose progression in the stages of myeloid differentiation has been halted (Gocek and Marcinkowska, 2011). HL-60, THP-1, and U937 are examples of commonly used cancer cell lines used to study myeloid differentiation. HL-60 is a cell line that was obtained from an acute promyelocytic leukaemia tumor (Collins *et al.*, 1977). It is considered to be a promyelocytic cell line due to its ability to differentiate into granulocyte-like as well as monocyte/macrophage-like cells in response to various differentiation-inducing agents. Treatment of HL-60 cells with dimethyl sulphoxide, actinomycin D, or retinoic acid results in granulocyte differentiation (Manda-Handzlik *et al.*, 2018). Treatment with phorbol esters induces monocyte-to-macrophage differentiation (Rovera *et al*., 1979).

THP-1 cells were obtained from the blood of a promonocytic myeloid leukaemia patient (Tsuchiya *et al.,* 1980). They are promonocytic, which implies a decreased differentiation potential compared to HL-60. They can only undergo monocyte-to-macrophage and monocyte-to-dendritic cell differentiation. A mixture of ligands, such as IL-4, GM-CSF, and TNFα is used to induce a dendritic-like end state (Berges *et al*., 2005). Tsuchiya *et al.*, (1980) are most cited for their protocol for PMA-induced monocyte-to-macrophage differentiation of THP-1 cells. THP-1 cells are considered the best myeloid cell line, relative to HL-60 and U937 cells, based on the exhibition of a monocytic phenotype even at a steady state (Auwerx, 1991; Baxter *et al*., 2020). U937 is a promonocytic cell line obtained from a histiocytic lymphoma (Sundström and Nilsson, 1976). They are more mature compared to THP-1 cells because they were obtained from tissue cancer (Sundström and Nilsson, 1976). Tissue origin implies further progression in maturation towards potential end-states. A dendritic-like end-state can be induced in U937 cells through exposure to a self-peptide obtained from apolipoprotein, Ep1.B (Stephens *et al*., 2008).

Steady-state HL-60, THP-1, and U937 cells exhibit nuclear and cytoplasmic traits that are between those of monoblasts and monocytes (Swiatecka and Mackie, 2015). Treatment with chemicals such as 1,25-dihydroxy-vitamin $D_3$ ($VD_3$), phorbol esters or sodium butyrate

induces monocyte-to-macrophage differentiation in HL-60 cells (Lotem and Sachs, 1979; Padilla *et al.,* 2000). Phorbol esters such as phorbol 12-myristate 13-acetate (PMA) result in monocyte-to-macrophage differentiation in HL-60 cells (Ramirez *et al.*, 2017). Similarly, treatment of THP-1 and U937 cells with $VD_3$ or PMA results in monocyte-to-macrophage differentiation (Green *et al*., 2020; Rossetti *et al.*, 2017). All three cell lines are suspension cells that differentiate to become adherent cells exhibiting macrophage-like characteristics (Fontana *et al.*, 1981; Tsuchiya *et al.*, 1980). They all adopt an irregular shape and they exhibit phagocytic vacuoles on the cell surface (Harris and Ralph, 1985). The resulting macrophage-like cells have a phagocytic capacity for latex beads and express cytokine profiles that resemble monocyte-derived macrophages (Swiatecka and Mackie, 2015). Thus, they are appropriate models for studying monocyte-to-macrophage differentiation.

### 1.6 PMA as a differentiation-inducing agent

PMA has become a commonly used inducing agent because it was shown to produce a more differentiated phenotype relative to $VD_3$ (Schwende *et al.*, 1996). This was based on a comparison of phagocytic ability, adherence, loss of proliferation, and the expression of marker genes such as GD11b and CD14 in THP-1 cells (Schwende *et al*., 1996). Daigneault *et al*. (2010) showed that macrophage-like cells derived from PMA-treated THP-1 resembled primary macrophages better than $VD_3$-treated cells. This comparison was based on the potency of a TLR2 response, increase in observed lysosomes and mitochondria, as well as resistance to apoptosis in the resulting cells. PMA treatment was shown to produce a proinflammatory macrophage phenotype, which is similar to that induced by GM-CSF (Daigneault *et al*., 2010; Riddy *et al*., 2018).

Notably, the signalling pathways used by PMA and $VD_3$ differ significantly. Inducers such as $VD_3$ induce differentiation through mechanisms that bypass certain pathways observed in myeloid differentiation *in vivo* (Gocek and Marcinkowska, 2011; Hughes *et al.*, 2010). PMA makes use of pathways observed to be crucial for myeloid differentiation in primary macrophages, which makes it a suitable inducer of differentiation (Kim *et al.*, 2014). PMA targets the protein kinase C (PKC) pathway (Daigneault *et al.*, 2010; Kim *et al*., 2014). The activation of PKC leads to a signalling cascade through the use of I Kappa B kinases (IKKs)

(Kim *et al.,* 2014). Kinases phosphorylate their targets and subject them to proteasomal degradation (Ardito *et al.,* 2017). Kinases attach an adenotriphosphate (ATP) molecule on the target protein (Ardito *et al*., 2017). ATP donates a phosphate to the protein, making it recognizable to E3 ubiquitin ligases (Ardito *et al*., 2017). These ligases add ubiquitin molecules to the protein, attracting the proteasomal degradation machinery (Ardito *et al*., 2017). Phosphorylation of the inhibitors of NF-KB, leads to the translocation of the NF-KB transcription factor into the nucleus (Kim *et al*., 2014). This allows the expression of genes that are necessary for monocyte-to-macrophage differentiation. These genes include cytokine genes, inflammatory, and chemokine genes (Kim *et al.*, 2014). There is also expression of genes encoding transcription factors such as PU.1, which is involved in regulating the expression of genes that give rise to macrophage features. Macrophage colony-stimulating factor (M-CSF) is one of the genes regulated by the PU.1 transcription factor and it is necessary to maintain the macrophage phenotype.

### 1.7 Factors affecting PMA-induced myeloid differentiation

Model cell lines differ from primary macrophages in numerous ways and these differences need to be taken into account for the assessment and interpretation of PMA-induced differentiation. Nascimento *et al.* (2022), showed that model cell line differentiation invokes some of the typical cell signalling cascades noted in primary monocyte differentiation differently. They showed that treatment of myeloid model cell lines with M-CSF does not lead to a macrophage-like end state as it would in primary monocytes (Nascimento *et al*., 2022). This indicates differences in the activity of the mitogen-activated protein kinase extracellular signal-regulated kinase (MAPK/ERK) signalling pathway which is typically stimulated by M-CSF to invoke monocyte-to-macrophage differentiation (Richardson *et al.*, 2015). Schildberger *et al.* (2013) reported significantly lower levels of interleukins 6, 8, and 10 in THP-1 cells compared to primary monocytes. Kohro *et al*. (2004) used an oligonucleotide microarray assay and showed that some genes are conversely regulated in primary monocyte-derived macrophages and THP-1-derived macrophages. These genes include IL-1$\beta$ (interleukin 1 b), metalloproteinase 9, and alpha 2 microglobulin (Kohro *et al*., 2004).

There are also baseline gene expression differences between cell lines and they need to be taken into account for assessing myeloid differentiation using cell lines. Sharif *et al.* (2007), showed that there are differences in the responses of macrophages derived from U937 and THP-1 cells to lipopolysaccharide (LPS) treatment. They compared the level of TNF$\alpha$ expression in macrophages obtained from both cell lines, finding that THP-1 macrophages had higher levels of TNF$\alpha$ relative to U937 macrophages. They showed that this resulted from an intact TLR4/TNF pathway in THP-1 cells (Sharif *et al.,* 2007). This implies that there are differences in activated pathways and the level of activity of such pathways can lead to differences in the expression of target genes.

It has been established that among model cell lines, there are genes involved in immune responses that are expressed differently in U937 cells relative to THP-1 and HL-60 (Riddy *et al*., 2018). Following PMA treatment for 48 hours, Riddy and colleagues (Riddy *et al*., 2018) found that ILR1 was not differentially expressed in PMA-treated U937 cells but was in HL-60 and THP-1 cells treated with the same concentration of PMA. Similarly, IL1-$\beta$ was not differentially expressed in PMA-treated U937 cells, but was in HL-60 and THP-1 cells. In contrast, IL1-$\beta$ was up-regulated in PMA-treated THP-1 cells and down-regulated in PMA-treated HL-60 cells. Macrophage-like cells derived from different cell lines are not expected to be the same due to biological differences. Such differences include cell growth and cell differentiation rates. These differences are useful in experimental studies as they account for biological variability. This allows for three biological replicates to be assessed, without the use of three donors. Furthermore, differences need to be taken into consideration for the assessment of myeloid differentiation using these cells.

Using three model cell lines also limits false positive and false negative results that result from assessing cellular functions related to genes that are expressed differently in these cell lines. For example, cells that do not have differential expression of a marker gene that denotes phagocytosis may be falsely considered to not have phagocytic ability if the gene is not differentially expressed across all cell lines. On the contrary, some genes are constantly expressed in abundance in certain cells, as such using them as markers would falsely indicate an increased phagocytic potential. However, this challenge can be overcome by knowing the genes whose relative expression changes across all cell lines and genes that are uniquely differentially expressed in specific cell lines. This will inform marker gene selection, in order

to accurately assess monocyte-to-macrophage differentiation and associated cellular functions when using these three model cell lines.

Differences in microenvironment stimuli affect the resulting differentiated derivatives. Experimental protocols used for differentiation alter the phenotype of the end-state and that needs to be taken into account for the assessment of myeloid differentiation. There is a lack of a defined consensus protocol detailing the appropriate concentration and duration of treatment for PMA induced-differentiation (Daigneault *et al.,* 2010; Baxter *et al.*, 2020). This is an imperative consideration for assessing differentiation *in vitro* because stimuli inducing differentiation also affect marker expression (Mukhopadhyay and Gordon, 2004). Furthermore, the reproducibility of studies and results is strongly influenced by culture conditions, as they can influence differences in gene expression (Lebeyec e*t al*., 2007). Baxter *et al.* (2020) assessed different concentrations of PMA followed by incubation with different cytokines. They showed that different differentiation protocols resulted in THP-1-derived macrophages with unique polarisation states. Treatment with 10 ng/ml of PMA for 24 hours followed by IL-4 incubation resulted in M2 differentiation (Baxter *et al.,* 2020). This was assessed based on receptor expression, such as the up-regulation of CD163 (Baxter *et al.*, 2020). In contrast, when they used a higher concentration of PMA followed by cytokine incubation, effective polarisation was not achieved (Baxter *et al*., 2020) . Daigneault *et al.* (2010) altered a commonly used protocol by incubating PMA-treated THP-1 cells in culture for an additional five days without PMA (Tsuchiya *et al.*, 1980). This alteration resulted in macrophages with increased potency of TLR2 responses and increased numbers of lysosomes and mitochondria.

Despite differences in PMA concentrations, the expression of macrophage differentiation markers remains unaltered in myeloid cell lines (Maeß *et al.*, 2014). Reducing PMA concentration in THP-1 cells does not negatively impact macrophage differentiation. They assessed the expression of macrophage differentiation markers, CD68 and CD11b, in macrophage-like cells obtained from different concentrations (100 ng/ml, 10 ng/ml, and 2.5 ng/ml) and found that the level of expression of macrophage markers and morphology did not significantly change. As a result of the model cell lines being at different maturation stages, concentrations of PMA cannot be standardised across all three cell lines (Fleit and Kobasiuk, 1991; Murao *et al.*, 1983).

### 1.8 Assessment of myeloid differentiation

Markers for macrophage differentiation can be grouped into cell surface receptors and secreted elements. Even though it is evident that primary macrophages are different from myeloid cell-line-derived macrophages, cell surface markers from PBMC-derived macrophages are used to validate the differentiation state of cell-line-derived macrophages. The popularly used markers include CD14, CD16, and CD11b. Techniques such as flow cytometry assess the presence of a marker to validate the differentiation state of a cell (Daigneault *et al.,* 2010). Additionally, an enzyme-linked immunoassay (ELISA) can be used to assess the expression of macrophage cytokines such as TNF$\alpha$, IL1-□, IL-6, and IL-8, while Western blots can be used to detect Mcl-1 and Bax receptors; validating the level of differentiation.

Colbert *et al.*(1983) concluded that the level of expression of steady-state mRNAs was modified during the induction of myeloid differentiation in HL-60 cells. For a transcript-level analysis, RT-PCR is performed. Colbert *et al.* (1983) showed that the morphological changes observed in these cells were a result of changes in mRNA abundances. Using gel electrophoresis, the separation of mRNA products was assessed in undifferentiated HL-60 cells and compared to differentiated cells. This assessment revealed large differences in quantities of mRNA obtained from differentiated and undifferentiated cells (Colbert *et al.*, 1983). Riddy *et al*. (2018) performed qPCR and compared gene expression from primary macrophages and PMA-treated model cell lines (HL-60, THP-1, and U937). Their findings showed that the level of expression of genes that are crucial for immune responses, particularly, the inflammatory response, was significantly different in primary macrophages relative to model cell line macrophages (MCLM). Some of the genes whose expression changed differently in PMA-treated cell lines include IL6, IL1R2, IL10, and CCL4.

Transcript expression analysis has evolved and improved through the use of next-generation sequencing methods (NGS). RNA sequencing (RNA-seq) is a method through which RNA is studied by converting it into cDNA and then sequencing the cDNA library (Wang *et al.,* 2009). This is different from qualitative analyses in that it allows quantification of gene expression instead of merely observing gene expression. Compared to the use of microarrays, this technique requires less prior knowledge of the transcript of the sequence of interest (Casneuf *et al*., 2007). Moreover, errors observed in microarray experiments such as

cross-hybridization are eliminated, while genes that are highly or lowly expressed are distinguished with increased sensitivity (Casneuf *et al.,* 2007). This is achieved through sequencing by synthesis technologies such as the Illumina™ platform. The high-throughput nature of RNA-seq yields a lot of data which can only be interpreted and analysed reliably using advanced statistical and computational methods.

## 1.9 Aims and Objectives

Many of the challenges posed by studying myeloid differentiation using primary cells are overcome through the use of model cell lines. In practice, model cell lines like HL-60, THP-1, and U937 are exposed to specific treatments to induce the activity of molecular pathways of interest. Changes in the expression of genes within these cellular pathways are used to assess drug metabolism, cytotoxicity, the pathogenesis of various infections, and molecular markers of diseases. However, end states derived from different cell lines have been shown to be heterogeneous. This is mostly because the cells have different origins. They require different concentrations of differentiation agents, like PMA, to induce differentiation, and they differentiate at different rates. This makes it difficult to validate experiments and compare experimental results. For example, the use of one cell line might indicate a response to certain stimuli, whereas another cell line does not show a comparable response. As such, there is a need to identify cell line-specific differences in levels of gene expression, which may alter the results obtained from such studies. To improve the interpretation of results, changes in gene expression that occur across all three cell lines and changes that are unique to each cell line, need to be identified.

### Aim

The aim of this study was thus to compare the gene expression profiles of HL-60, THP-1, and U937 cell lines 48 hours after treatment with PMA.

### Objectives

1. To compare gene expression profiles prior to and post-PMA treatment, using Tximport v1.10

2. To identify genes whose average expression changes in response to 48 hours of PMA treatment, using DESeq2 v4.2
3. To determine pathways over-represented by differentially expressed genes, using clusterProfiler v4.0

# 2. Methodology

## 2.1 RNA Sequencing Data Acquisition

In order to examine changes in gene expression induced by PMA treatment, RNA-seq data was obtained from three previously published studies on HL-60, THP-1, and U937 cells, respectively (Table 2.1). These cell lines were selected because they are commonly used to study myeloid differentiation and myeloid cell function. Sequencing data from three biological replicates, for each cell line, obtained at 0- and 48 hours post-treatment, were analysed in this study. All data were obtained as unprocessed FastQ files.

**Table 2.1: An overview of the RNA-seq data included in this study**

| Cell Line | ENA Accession Number | PMA concentration (nM) | Library Preparation Method | Library Configuration | Sequencing Read Length | Reference |
|---|---|---|---|---|---|---|
| HL-60 | PRJNA314802 | 10 000 | Poly-A extraction (Invitrogen cDNA synthesis kit) | Single-end | 86 bp | (Ramirez *et al.*, 2017) |
| THP-1 | PRJNA533591 | 100 | Poly-A extraction (TruSeq Stranded Library Preparation Kit ) | Paired-end | 150 bp | (Green *et al.*, 2020) |
| U937 | PRJNA381911 | 80 | Total RNA extraction (TruSeq Stranded RNA-Seq kit ) | Paired-end | 100 bp | (Rossetti *et al.*, 2017) |

## 2.2 Assessment of RNA Sequencing Read Quality

FastQC v 0.11.9 (Andrews, S, 2010), was used to assess the quality of the sequencing libraries included in this study. It is a commonly used tool for assessing the quality of DNA and/or RNA sequencing data derived from both single-end and paired-end sequencing experiments. FastQC assesses the quality of the data by evaluating it against various quality control metrics (Table 2.2). The data are assigned a “pass”, “warning” or “fail” for each metric, depending on whether or not they meet a specified threshold. These metrics were evaluated in this study in order to identify any potential biases present in the sequencing data that may affect any comparisons between treated and untreated cells, or between cell lines (Table 2).

**Table 2.2 : Quality control metrics assessed using FastQC**

| Quality Control Metric | Pass threshold | Biological Significance |
|---|---|---|
| Per base sequence quality | Phred score >20 | Indicates the accuracy of each base call |
| Sequence length distribution | Uniform length in all sequences | Indicates the accuracy of fragment elongation |
| Number of reads | Uniform number of reads in all files | Indicate sequencing depth in each library |
| Duplicated sequences | Sequence duplication level =0 | Indicates PCR overamplification of DNA fragments |
| Over-represented sequences | First 50 bp < 0.1% of total reads | Detects library contamination from sequences appearing more than expected |
| Per base N content | N < 5% of the sequence | Indicates inability to make a confident base call |
| Adapter content | Adapter sequences detected in < 5% of all reads | Assesses the presence of adapter sequences |
| Per base sequence content | %A, %T, %G, %C | Indicates proportion nucleotide bases at any given position |
| GC-content | Uniform GC% detection in all sequences | Indicates GC distribution of all sequences |
| Per sequence quality score | Average quality per read > 30 | Indicates the quality score distribution over all sequences |

### 2.3. Estimation of Transcript Abundance

Salmon v1.8.0 (Patro *et al*., 2017) was used to estimate the abundance of the mRNA transcripts expressed in each cell line before and after treatment with PMA. Transcript information was based on the Gencode v40 annotation (Frankish *et al.*, 2021) of the GRCh38.p13 human reference genome (GRCh38.P13 Genome Assembly NCBI, 2019). Salmon was used because it employs a quasi-mapping approach that has much lower memory and runtime requirements than traditional splice-aware alignment tools like HISAT2 (Kim *et al*., 2019) and STAR (Dobin and Gingeras, 2015). Quasi-mapping is a technique that enables the quantification of expression, without requiring traditional, direct base-to-base alignment between the reference and sequencing reads (Patro *et al.*, 2017). It also includes additional parameters to correct for biases inherent in RNA-seq sequencing libraries that are not available in other quasi-mapping methods like kallisto (Bray *et al*., 2015). Command line parameters used in this study include `--libraryType`, `--seqBias`, `--gcBias`, `--recoverOrphans`, and `--validateMappings` (Table 2.3).

The `--libraryType` parameter (--A) enables automatic detection of the sequencing library type configuration. This includes the strandedness, directionality, and relative orientation (for paired-end sequencing libraries) of each sequencing read. All sequencing libraries included in this study were stranded, meaning each sequencing read could be quasi-mapped to either the sense or the antisense strand. The HL-60 sequencing libraries were single-ended and thus had no directionality or relative orientation, while the U937 and THP-1 sequencing libraries were paired-end libraries.

Orphan mappings describe events where only one read is successfully quasi-mapped instead of both the forward and reverse reads from a paired-end library (Srivastava *et al.*, 2020). The `--recoverOrphans` parameter was used for the paired-end U937 and THP-1 sequencing libraries, allowing orphan reads to be included in transcript abundance estimates. However, dovetail mappings were not allowed. Dovetail mappings are sequencing reads that map abnormally as a result of a short fragment length inclusion in library preparation (Figure 2.1; Srivastava *et al.*, 2020). They generally occur randomly during library preparation and were therefore excluded. These parameters were not necessary for the HL-60 sequencing libraries as they were single-ended.

**Table 2.3: Command line parameters used in Salmon**

| Parameter | Function |
|---|---|
| **--libraryType** | Automatically detects sequencing library type |
| **--seqBias** | Circumvents overestimation of transcript abundance |
| **--gcBias** | Circumvents underestimation of transcript abundance |
| **--validateMappings** | Selects the most optimal matches between reads and transcripts |
| **--recoverOrphans** | Includes paired-end reads that lack pairs into transcript abundance estimates |

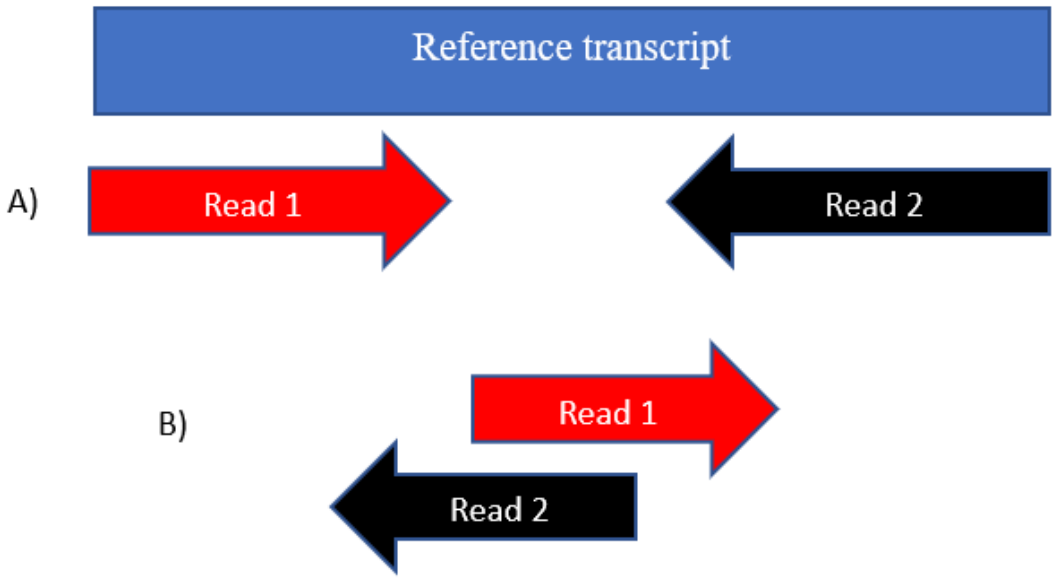



Figure 2.1: **Comparison of expected mapping relative to dovetail mapping in paired-end libraries. A)** Paired-end reads are expected to map on opposite ends of a fragment according to their specified orientation. **B)** Dovetail mapping deviates from this due to unusually short sequences that overlap each other. This unusual mapping is not consistent in all replicates and therefore it was not included in transcript abundance estimates.

Sequence bias is the over-representation of specific RNA-seq reads based on the sequence of bases that occur at the start or end of sequenced fragments (Patro *et al.*, 2017). An

over-representation of these reads can lead to an overestimation of their assigned transcript(s). In order to circumvent the overestimation of transcript abundance, the --seqBias parameter was used. This method uses a variable length Markov model (VLMM) to assess and correct for sample-specific 5′- 3′ bias (Patro *et al*., 2017). The model determines the expected frequencies of bases at the start and end points of RNA-seq reads. The expected frequencies are then compared to the observed sequences resulting in adjusted values obtained are used to improve transcript abundance estimation.

Regions that are GC-rich are less likely to be accurately sequenced (Benjamini and Speed, 2012). This results in an underestimation of transcripts represented by GC-rich reads relative to transcripts that are represented by non-GC-rich reads. The parameter `--gcBias` is used to determine the GC frequency of each sequencing read and compare it to the expected GC frequency (Patro *et al.*, 2017), effectively quantifying the extent of GC bias on each sequencing read. By including this parameter, estimated transcript abundance values are adjusted according to the result of any detected GC-bias.

Finally, in order to ensure that transcript abundance estimates were obtained from the most optimal matches between reads and transcripts, the `--validateMappings` parameter was used. This parameter assesses and scores matches between each sequencing read and all the possible transcripts to which it can be quasi-mapped. Matches with the highest scores are then selected as valid mappings to estimate transcript abundance. The raw total transcript abundance estimates are reported as NumReads in the final output. These raw estimates are also normalised for the effects of transcript length and sequencing depth in each sample and these normalised abundance values are reported as transcripts per million (TPM) values in the final output.

### 2.4 Differential Gene Expression Analysis

In order to identify changes in gene expression (rather than transcript abundance) in response to PMA treatment, transcript abundance values obtained from salmon v1.8.0 (Patro *et al.*, 2017) were converted into gene-level expression counts using tximport v 1.10 (Soneson *et al.*, 2015). In order to account for differences in the composition of sequencing libraries, analyses in this study were limited to include only sequencing reads derived from

protein-coding genes. Genes significantly differentially expressed between PMA-treated and untreated cells were identified using DESeq2 v 4.2 (Love *et al*., 2014). DESeq2 was chosen for this study because of its suitability for studies with small sample sizes, where there is limited variability between biological replicates, such as those conducted in cell lines (Love *et al.*, 2014). As a result of technical biases introduced by genome sequencers and library preparation methods, sequencing depth across samples can sometimes differ due to random sampling, rather than treatment conditions. To minimise the effects of differences in sequencing depth, gene counts were therefore normalised using the median of ratios method implemented in DESeq2 v 4.2 (Love *et al.*, 2014). The median of ratios method determines the geometric mean for each gene across all samples. All gene counts are divided by the geometric mean. The median of these ratios across all conditions is considered a size factor for the specific gene. Gene counts used to assess differential gene expression are then scaled by this size factor to reduce any potential bias in sequencing depth. DESeq2 uses a Wald test to assess the difference in the mean expression level of each gene across treatment conditions. In the case of this study, gene expression in PMA-treated samples was assessed relative to that in untreated samples across all three cell lines. The Benjamini and Hochberg's (BH) procedure was used to adjust for multiple testing, providing an estimate of the false discovery rate (FDR; Benjamini and Hochberg, 1995). An FDR significance threshold of $\alpha = 0.05$ and a $\log_2$ fold change threshold of 2 were used to denote significantly expressed genes.

### 2.5 Gene Over-representation Analysis

The R package, clusterProfiler (Yu *et al.*, 2012), was used to perform gene over-representation analysis (ORA). clusterProfiler performs ORA using a hypergeometric test, in order to assess whether any particular sets of genes are present in a set of differentially expressed genes more often than would be expected by chance. In this study, annotated gene sets obtained from the Kyoto Encyclopedia of genes and genomes (KEGG) database (Kanehisa *et al*., 2010) and Gene Ontology (GO) terms (Ashburner *et al.*, 2000) were used for ORA and assessed against the set of significantly differentially expressed genes obtained from each PMA-treated cell line. The union of all the genes that were expressed in treated and untreated cells was used as the background universe set of genes (a total of 19 357 genes). The `enrichkegg` and `enrichGO` functions were used, with a BH FDR threshold of $\alpha = 0.05$ used to denote significantly over-represented KEGG pathways and GO terms.

# Chapter 3: Results

## 3.1 Assessment of sequencing read quality

Assessment of the quality of sequencing libraries included in this study indicated that the sequence data was of good quality. All phred quality scores were above 20 for both treated and untreated cells (Figure 3.1A). The average number of reads obtained from PMA-treated HL-60, THP-1, and U937 cells, was 12 500 00, 118 600 000, and 126 300 000, respectively. The average number of reads obtained from untreated HL-60, THP-1, and U937 cells was 19 400 000, 113 900 000, and 74 830 000, respectively (Figure 3.1B). The average percentage of duplicate reads detected in PMA-treated HL-60, THP-1, and U937 cells was 67%, 59%, and 39%, respectively, while the average percentage of duplicate reads detected in untreated HL-60, THP-1, and U937 cells was 67%, 59%, and 39%, respectively (Figure 3.1C).

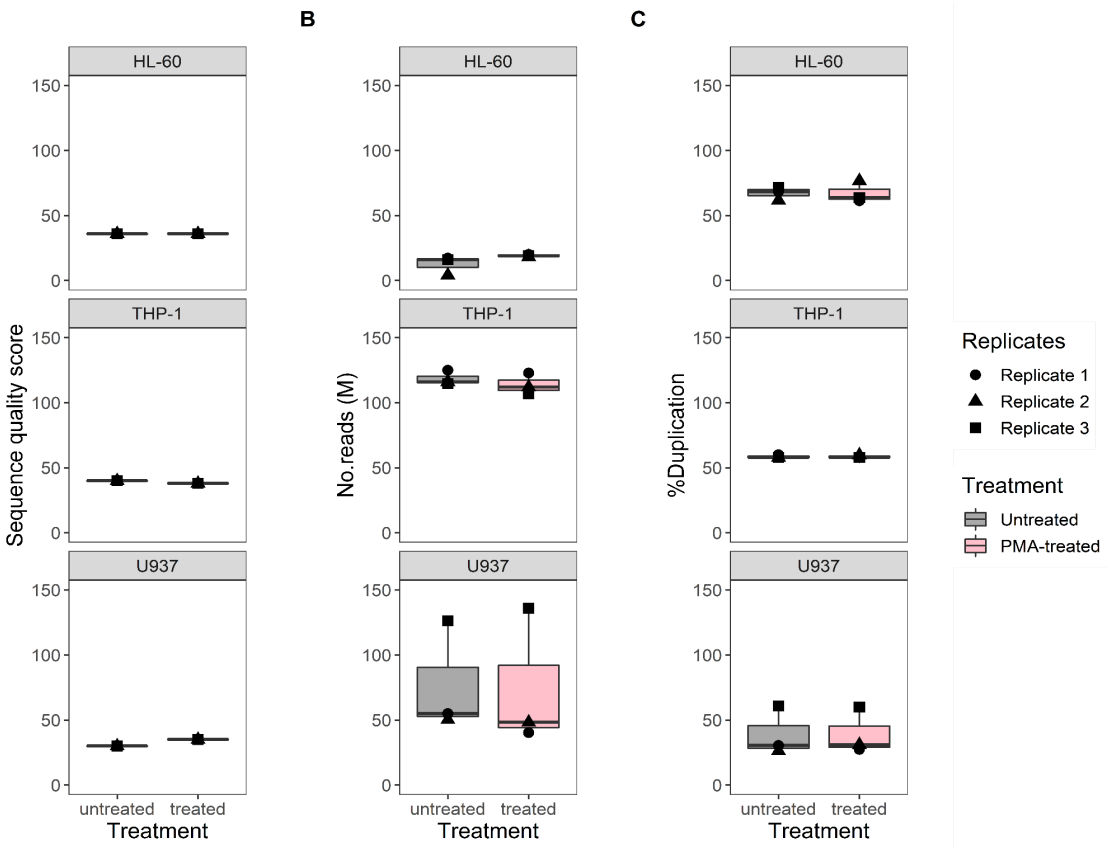



**Figure 3.1: Assessment of sequencing read quality.** A) Sequence quality scores B) Number of sequencing reads and C) Percentage duplication in three replicates. Replicates 1, 2, and 3 are denoted by shapes in untreated (grey) and PMA-treated (pink) cells .

## 3.2 Estimation of transcript abundances

To determine transcript abundances in PMA-treated and untreated HL-60, THP-1, and U937 cells, RNA-seq reads were first quasi-mapped to the transcriptome using Salmon (Patro *et al.*, 2017). On average, 44%, 88%, and 56% of sequencing reads from untreated HL-60, THP-1, and U937 cells were successfully quasi-mapped to the transcriptome, respectively, while 49%, 87%, and 46% of sequencing reads from PMA-treated HL-60, THP-1, and U937 cells were successfully quasi-mapped, respectively (Figure 3.2). The number of orphan reads salvaged, dovetail reads discarded, and reads quasi-mapped to decoy sequences is shown in Figure S1. Reads that were successfully mapped to transcripts were used to estimate the abundance of each transcript. Gene-level aggregation of transcript abundances was performed using the R package, tximport (Soneson *et al.*, 2015). Prior to PMA treatment, 13 651 (more

that 80%) of genes were detected across all cell lines (Figure 3.3A). Only 280 (8%) , 568 (14%) and 844 (16%) of genes detected in the untreated condition were unique to HL-60,THP-1 and U937 cells, respectively (Figure 3.3A). Following PMA treatment, 14 159 genes (more that 90%) were present in all three cell lines (Figure 3.3B). Only 460 (2%), 728 (4%), and 330 (5%) genes were unique to treated HL-60, THP-1, and U937 cells, respectively.

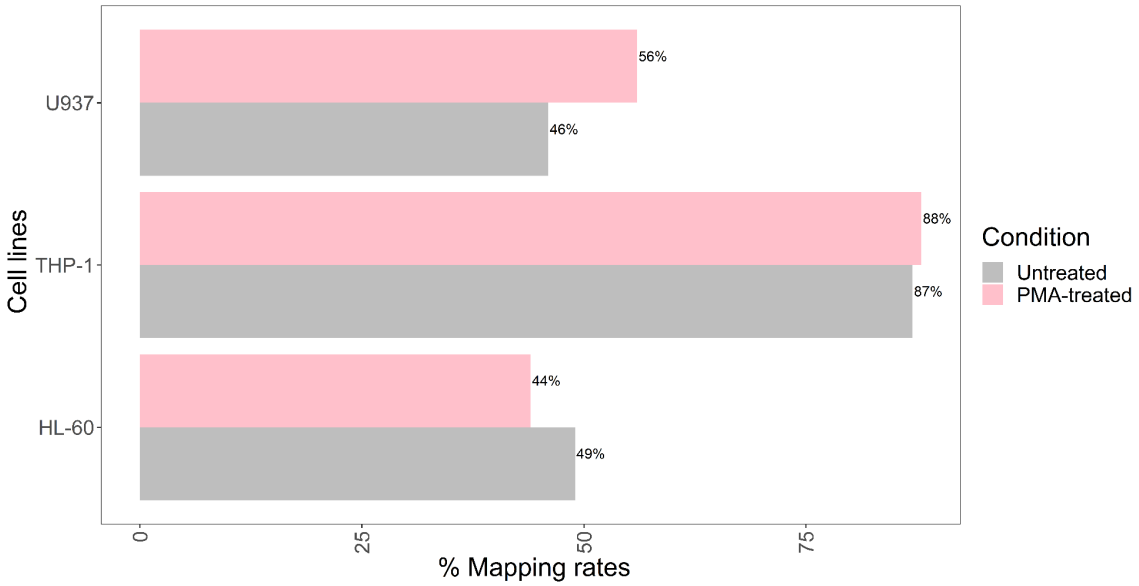



**Figure 3.2: Mapping rates obtained by sequencing reads in HL-60, THP-1, and U937 prior to and post PMA treatment.** Mapping rates for untreated cells are shown in grey, while those for treated cells are shown in pink.

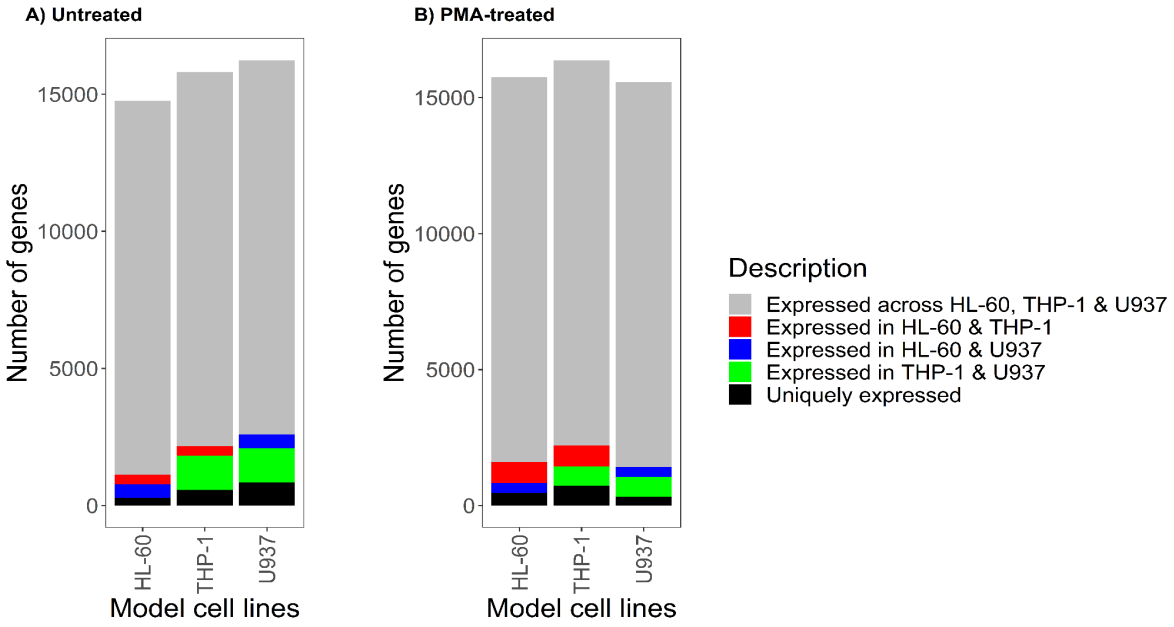



**Figure 3.3: The number of genes detected in HL-60, THP-1, and U937 when A) untreated and B) PMA-treated.** The number of genes present across all cell lines is shown in grey. Uniquely expressed genes are in black.

### 3.3 Differential gene expression analysis

Principal components analysis of normalised gene counts revealed a clear separation of treated and untreated samples, with 80%, 94%, and 99% of the variance captured by the first principal component (PC1) in HL-60, THP-1, and U937 cells, respectively (Figure 3.4). Differential gene expression analysis revealed that 2020, 2498, and 1742 genes were significantly differentially expressed in response to PMA treatment in HL-60, THP-1, and U937 cells, respectively (Figure 3.4). To determine the number of genes that were only differentially expressed in specific cell lines, the set difference across all cell lines was determined. An FDR significance threshold of $\alpha = 0.05$ and a $\log_2$ fold change threshold of 2 were used to denote significantly expressed genes. The number of genes that were significantly differentially expressed in only one of the cell lines included in this study was 944 (15%), 1231 (20%), and 624 (10%) in HL-60, THP-1, and U937 cells, respectively. Conversely, 475 (8%) genes were significantly differentially expressed across all three cell lines (Figure 3.4), of which, 269 (57%) genes were up-regulated in response to PMA

treatment and 206 (43%) genes were down-regulated. Only 9 (2%) of these genes were found to be up-regulated in one cell line and down-regulated in another, or vice versa (Figure 3.5).

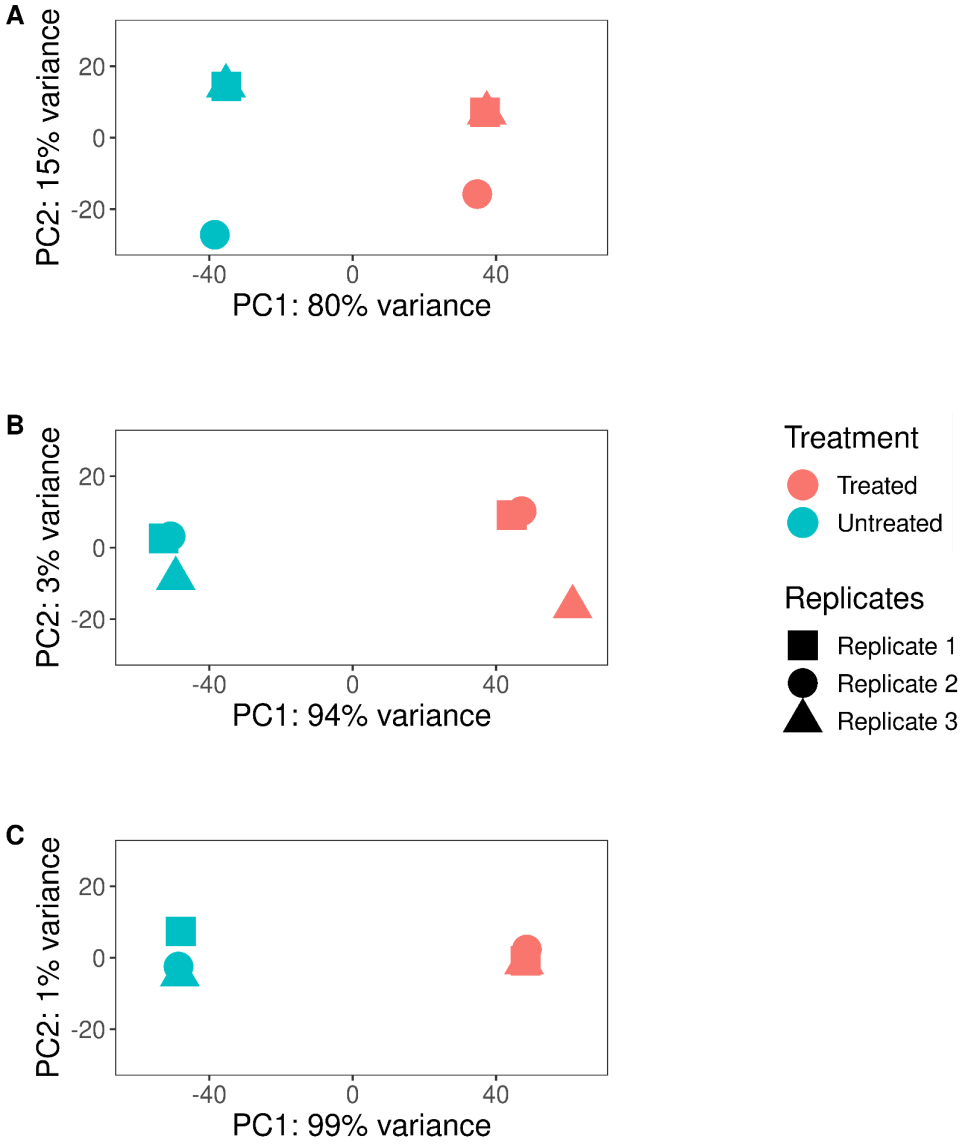



**Figure 3.4: Principal components analysis of untreated and PMA-treated samples.** (A) HL-60 cells (B) THP-1 cells, and (C) U937 cells. Gene counts in three replicates. Replicates 1, 2, and 3 are denoted by shapes in untreated (blue) and PMA-treated (pink) cells.

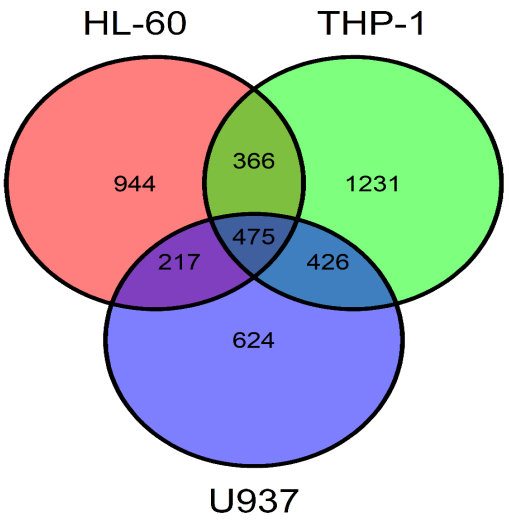



**Figure 3.5: Number of genes significantly differentially expressed in HL-60, THP-1, and U937 cells, 48 hours post-PMA treatment.**

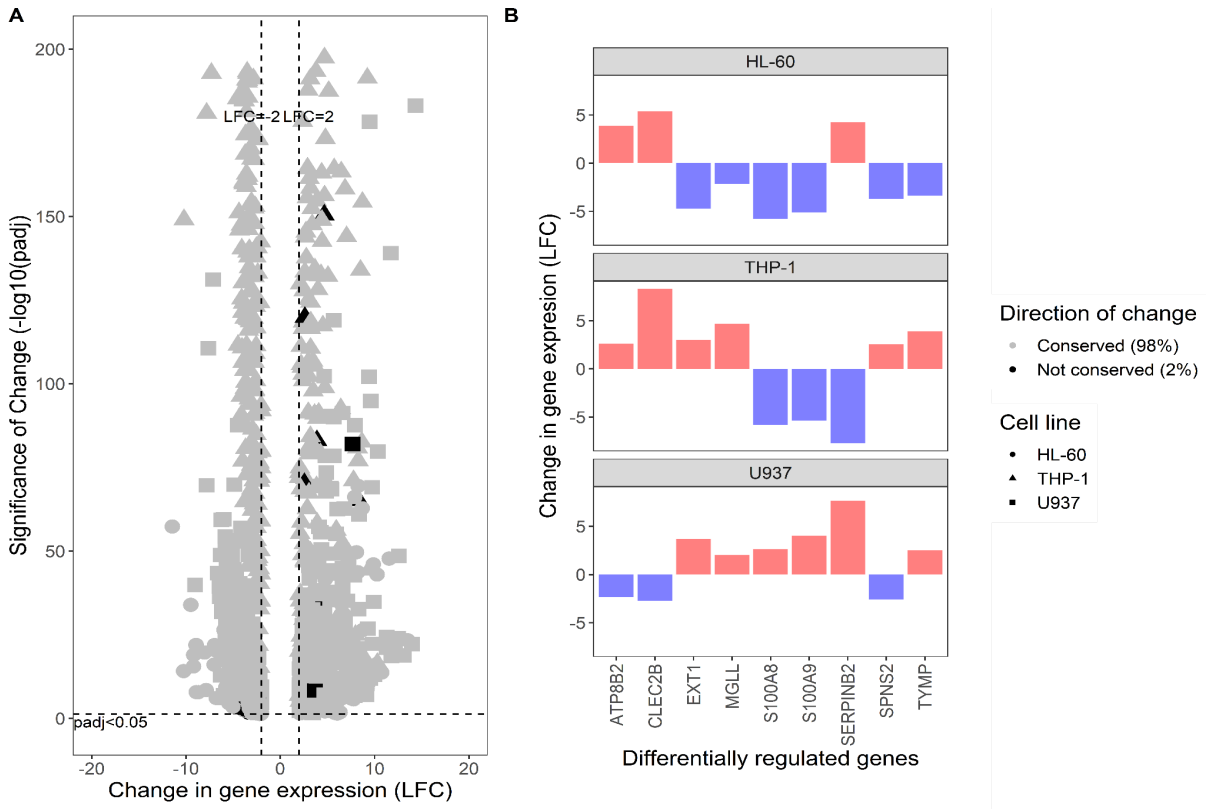



**Figure 3.6: Genes that were significantly up-regulated in one cell line and down-regulated in another, or vice versa.** A) Direction of change of all differentially expressed genes that were detected across all cell lines. B) change in expression of genes differentially expressed across all cell lines, but where the direction of change differs across cell lines. LFC indicates the $\log_2$ fold change in gene expression.

### 3.4 Pathway over-representation analysis

To determine pathways over-represented in genes that were significantly differentially expressed across all three cell lines in response to treatment with PMA, clusterProfiler (Yu *et al.*, 2012) was used. This identified 75 KEGG pathways as being statistically over-represented in genes that were significantly differentially expressed across all three cell lines (Figure 3.7). Such genes were characterised by a $\log_2$ fold change fold of 2 and an FDR significance threshold of $\alpha = 0.05$. The identified pathways included numerous pathways associated with pathogen recognition, for example, the toll-like receptor signalling pathway and NOD-like receptor signalling pathway. Analysis of genes significantly differentially expressed in each specific cell line only, revealed 20, 43, and 36 pathways as being statistically over-represented in HL-60, THP-1, and U937 cells, respectively (Figure S2 and S3). In each case, more than 50% of pathways identified were only statistically over-represented in that cell line and not in either of the others. Pathways that were over-represented in cell-line specific DEGs were pathways detailing metabolic processes that were unique to each cell line.

To assess cell line-specific changes in gene expression that drive metabolic pathways in HL-60, THP-1 and U937 cells, the nucleotide metabolism, protein metabolism and carbohydrate metabolism and absorption pathways were further assessed (Figure 3.8). Different metabolic pathways were assessed in each cell line because unique metabolic processes were found to be over-represented in each cell line. A down-regulation of 81% of genes over-represented in nucleotide metabolism was noted in PMA-treated HL-60 cells. PMA-treated THP-1 cells showed an up-regulation of 83% of the genes over-represented in protein metabolism. An up-regulation of 80% of genes over-represented in carbohydrate metabolism was seen in PMA-treated U937 cells.

To determine cell-line-specific expression patterns in pathogen recognition pathways, genes from the TLR pathway were extracted from each cell line (Figure 3.9). Genes from the NOD-like-receptor pathway were also assessed (Figure S4). Out of 39 genes within the TLR pathway, 19, 24, and 21 were differentially expressed in HL-60, THP-1, and U937 cells. Cell-line-specific DEGs included *TLR3*, *TLR7*, *TLR8,* and *TLR9*, which were only up-regulated in THP-1 cells. There was a unique up-regulation of *TLR2* and *TNF* in U937

cells. There was no differential expression of *CD14* in HL-60 cells. Out of all the DEGs, nine genes had a conserved direction of change, and they were all up-regulated across all cell lines (Figure 3.9B). To assess the monocytic activities of these cells, genes within the phagosome pathway were extracted from each cell line. Out of 56 genes within the pathway, 26, 38, and 28 were differentially expressed in HL-60, THP-1, and U937 cells, respectively (Figure 3.10). Unique up-regulation of *FCGR2A*, *FCGR2B*, and *FCGR31* was noted in U937 cells. A common up-regulation of *CD36* across cell lines was also noted.

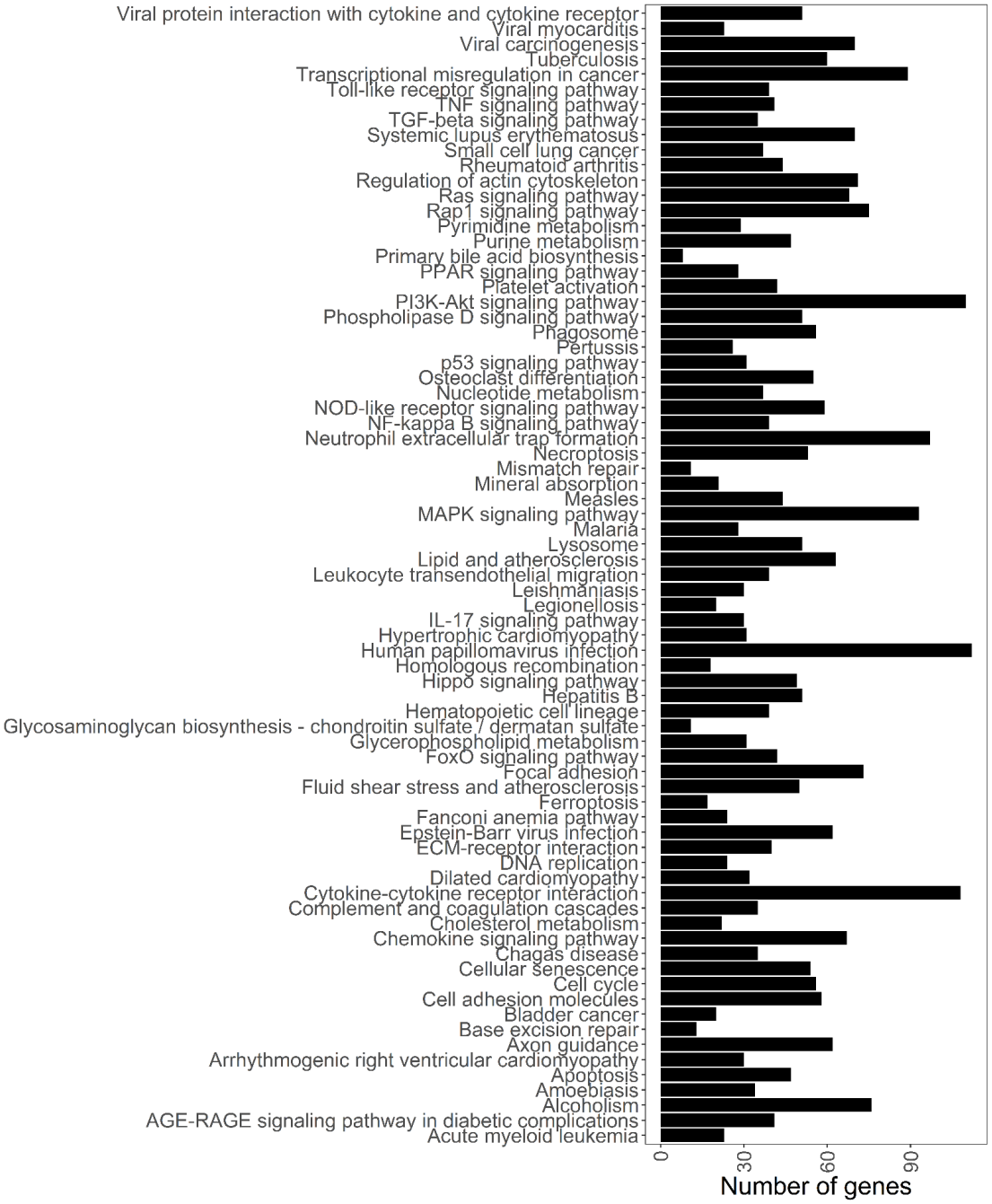



**Figure 3.7: KEGG pathways over-represented in genes that were significantly differentially expressed across all cell lines in response to treatment to PMA.**

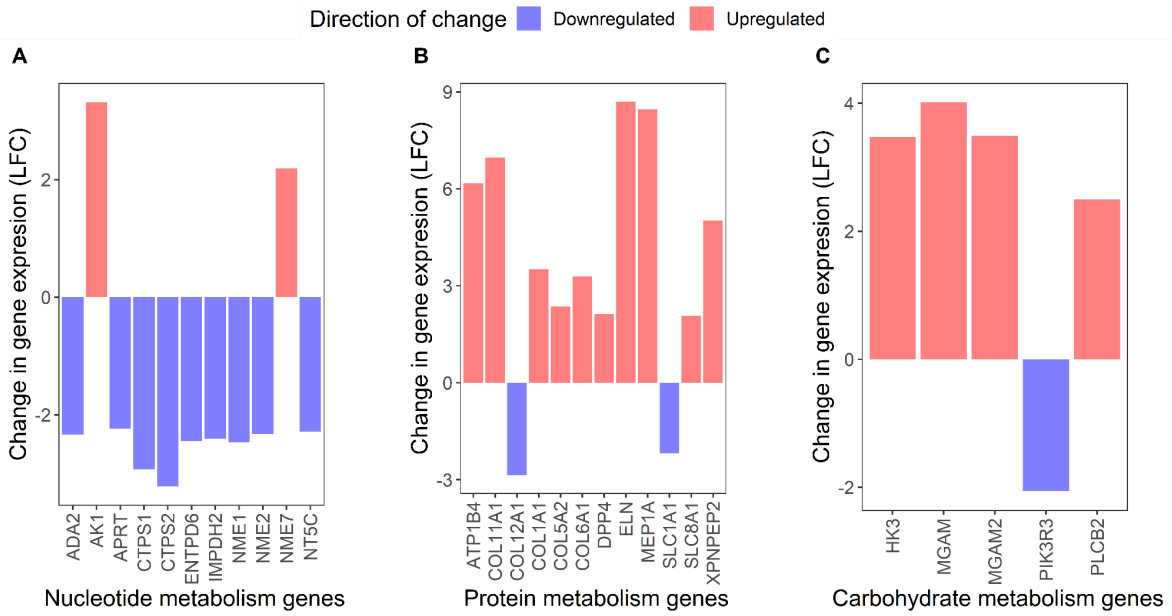
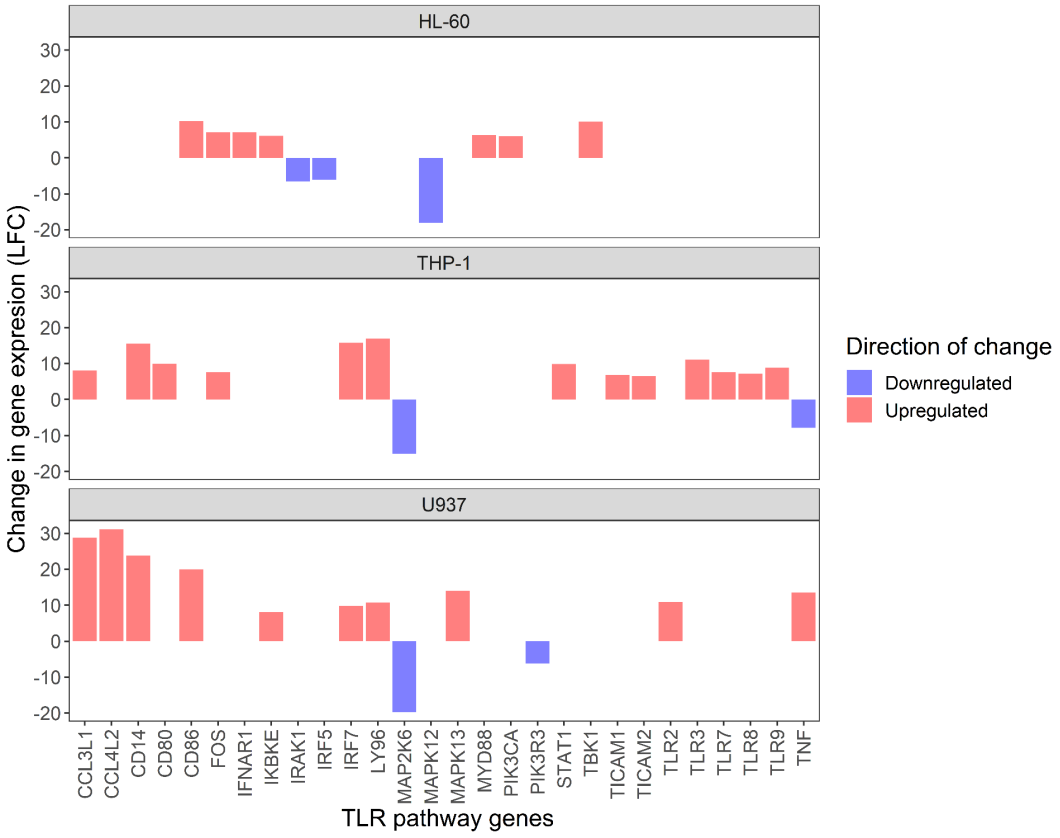



**Figure 3.8: Metabolic pathways that were significantly over-represented in cell-line-specific differentially expressed genes.** (A) HL-60, (B) THP-1 and (C) U937. LFC indicates the $\log_2$ fold change in gene expression.

A)



B)

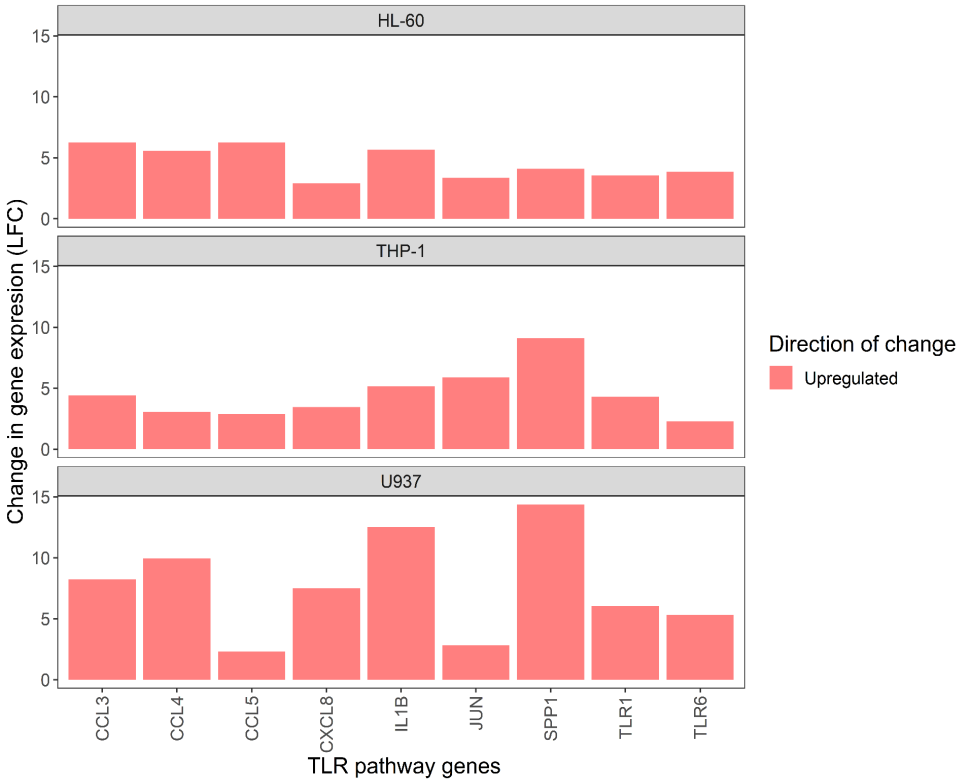



**Figure 3.9 Differentially expressed genes within the TLR pathway. (**A) DEGs that change in a cell-specific manner (B) DEGs that change in the same manner across all cell lines. LFC indicates the $\log_2$ fold change in gene expression.

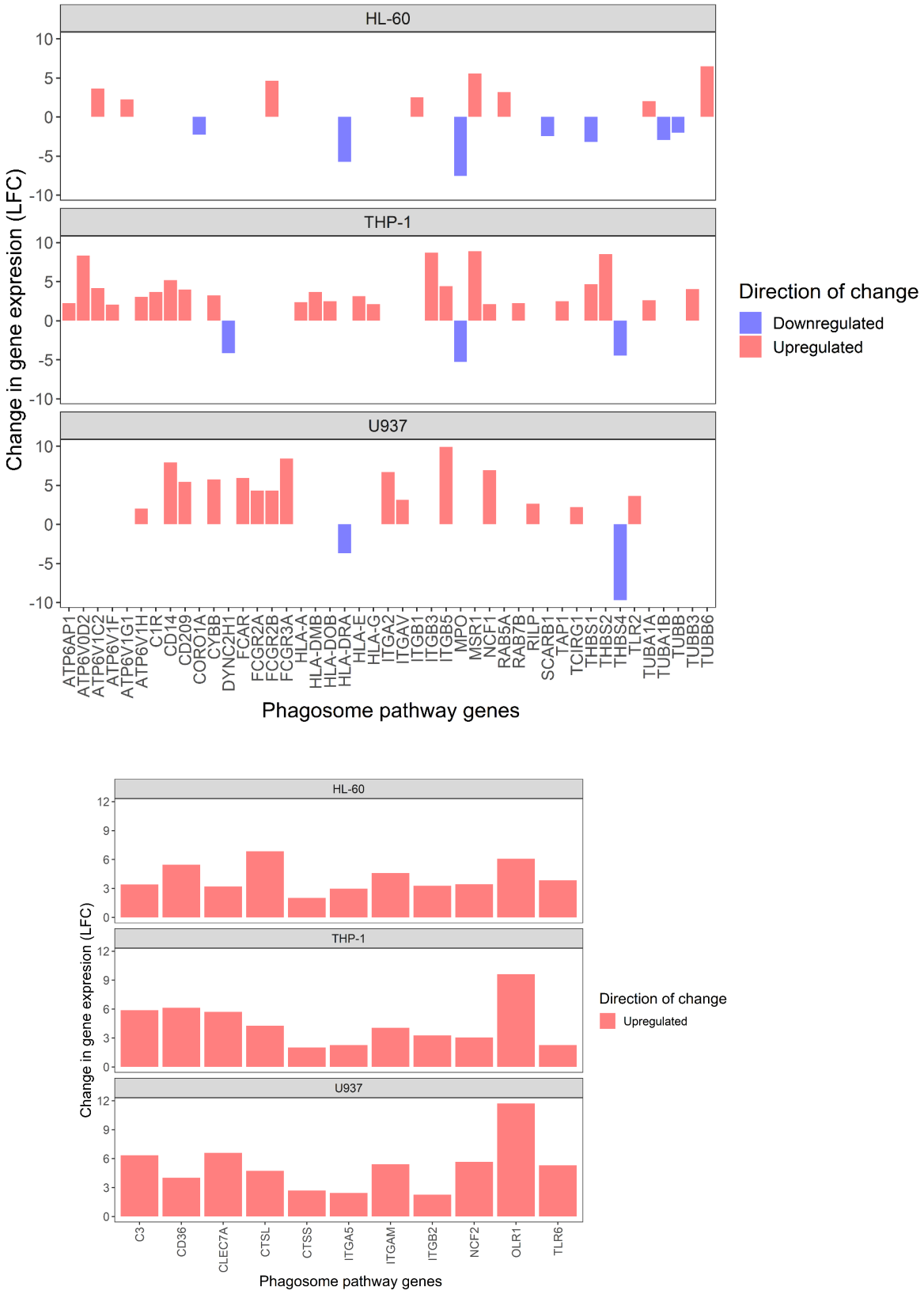



**Figure 3.10: Differentially expressed genes within the phagosome pathway.** (A) DEGs that change in a cell-specific manner (B) DEGs that change in the same manner across all cell lines. LFC indicates the $\log_2$ fold change in gene expression.

### 3.5 Gene ontology over-representation analysis

To determine biological processes that were significantly over-represented in all three cell lines in response to PMA after 48 hours, Gene ontology was used (Ashburner *et al.*, 2000). Genes down-regulated across all cell lines were mostly involved in cell-cycle transition (Figure 3.11), which is the parent term for processes such as G1-S transition, mitosis and chromosome organisation, which were the other over-represented terms. As such, differentially expressed genes that were differentially expressed across all cell lines were assessed further (Figure 3.12). The results showed that 34 genes detected across all three cell lines were involved in cell-cycle regulation. A down-regulation of *E2F,* and key cell division cycle genes such as *CDC25A*, *CDC7* and *CDCA5* was noted.

Up-regulated genes were involved in processes such as myeloid differentiation and leukocyte migration (Figure 3.13). The parent term, myeloid differentiation was assessed further to determine genes whose change in expression was important for the differentiation of all three cell lines. The term consisted of all the genes listed in terms that describe monocyte-to-macrophage differentiation. The results showed that 22 genes that were differentially expressed across all cell lines were involved in myeloid differentiation (Figure 3.14). Key players in myeloid differentiation, including markers such as *CSF1* and *CSF1R* were noted to be up-regulated across all cell lines. The transcription factors encoding the genes, *Bcl-6, CEBPB* and *Jun*, were also up-regulated across all cell lines.

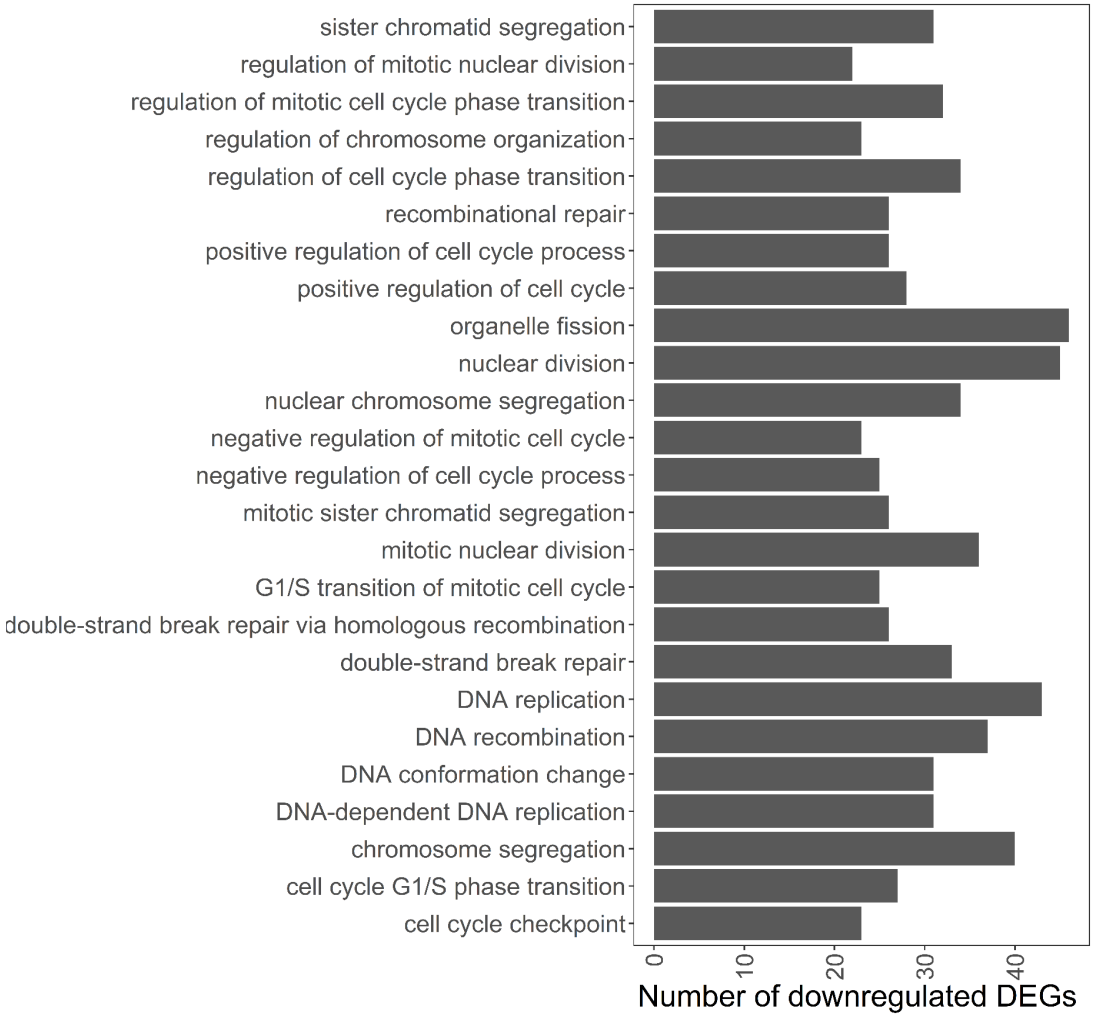



**Figure 3.11 : Gene ontology over-representation analysis of genes involved in biological processes that were down-regulated across all cell lines.**

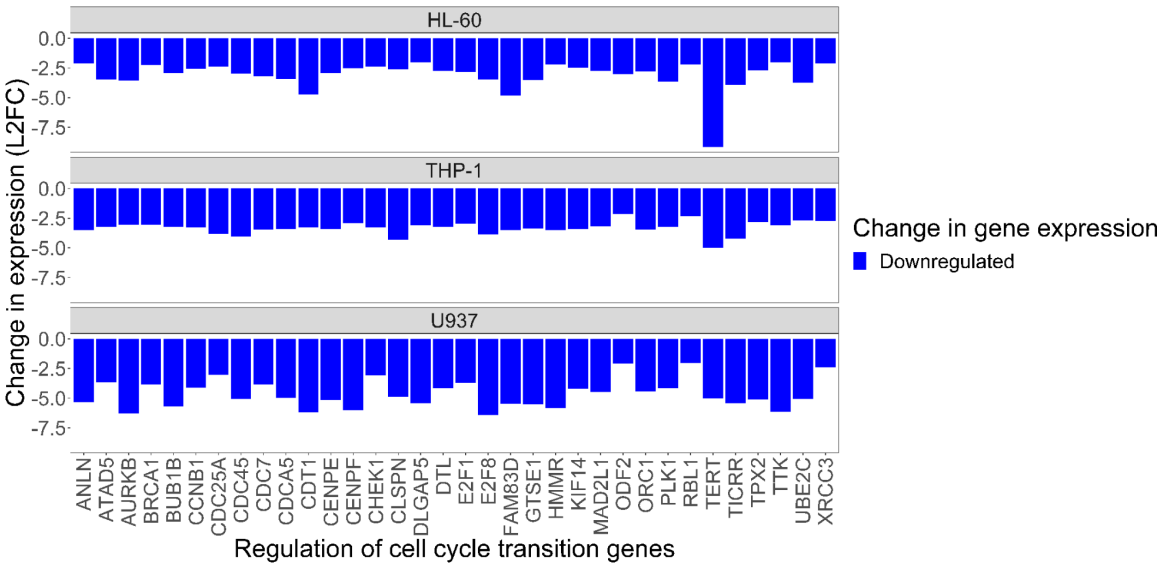



**Figure 3.12: Down-regulated genes involved in the regulation of cell cycle transition across all cell lines.**

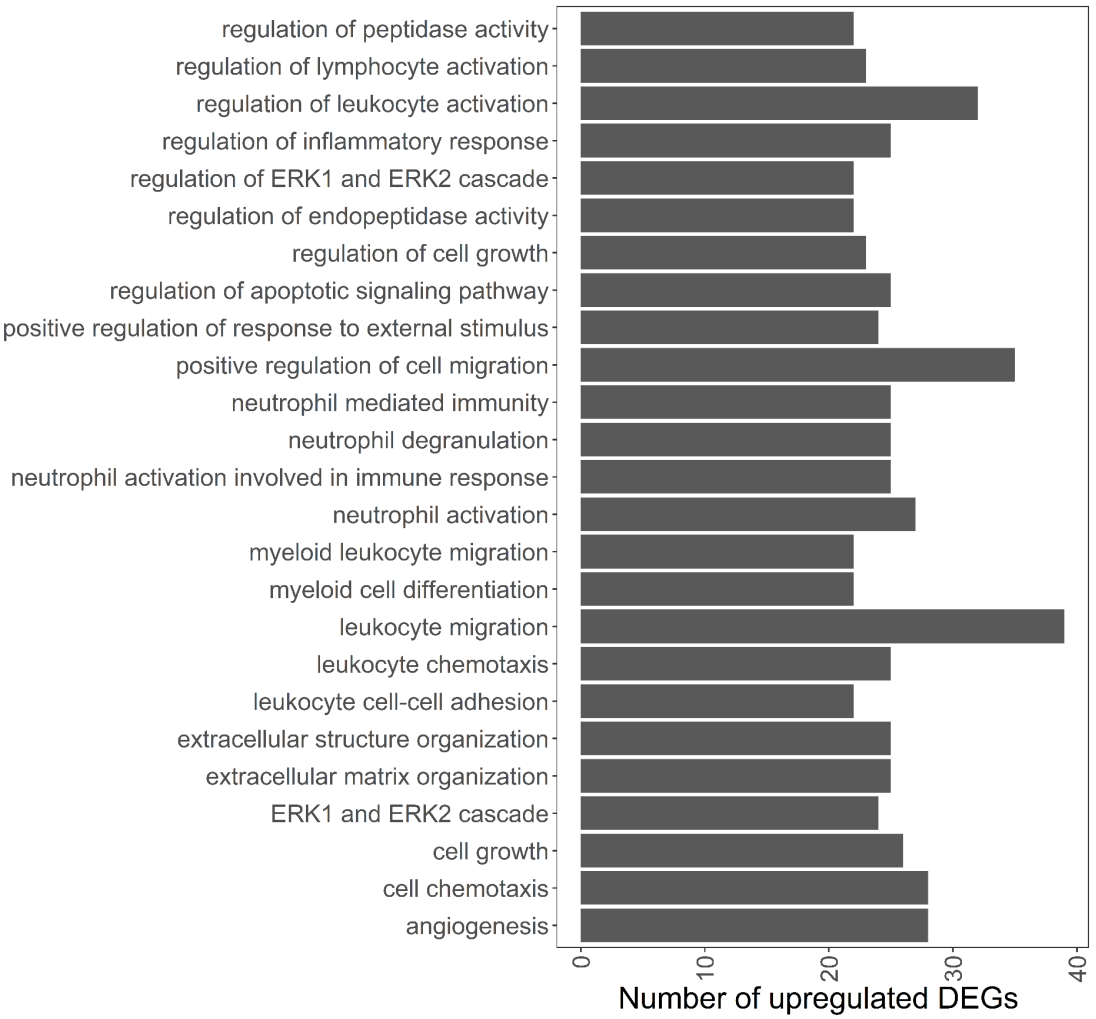



**Figure 3.13: Gene ontology analysis of biological processes over-represented in genes that were down-regulated across all cell lines**

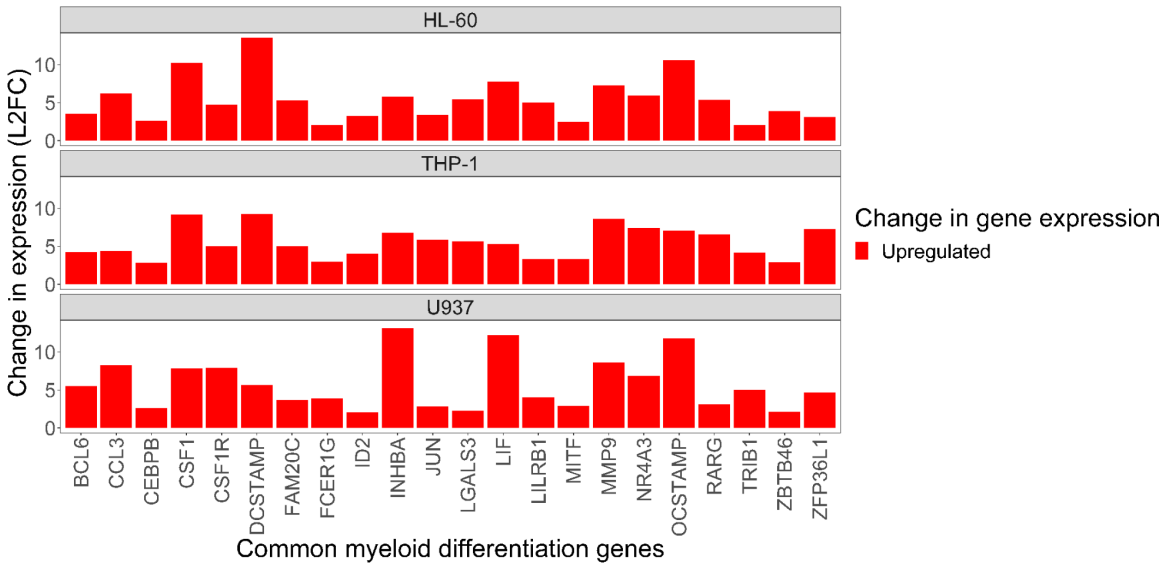



**Figure 3.14: Up-regulated genes involved in myeloid differentiation across all cell lines.**

# 4. Discussion

The model cell lines HL-60, THP-1, and U937 are induced to undergo myeloid differentiation for various immunological studies. However, the differentiated end-state of these cells may vary due to baseline biological differences between the cell lines which requires alterations in vitro differentiation protocols used. As such, this study aimed to compare the gene expression profiles of these cell lines 48 hours after treatment with PMA to induce myeloid differentiation. Results showed that genes involved in phagosome formation, and cell cycle transition were differentially expressed irrespective of the cell line, following PMA-induced differentiation. However, genes within the toll-like-receptor pathway showed cell-line-specific expression patterns, suggesting differences in pathogen detection. Overall, THP-1 cells showed a greater potential to detect viruses , while U937 cells showed a greater potential to detect bacteria.

## 4.1 Quality Control

While all the sequencing data included in this study was deemed to be of high quality based on the metrics evaluated by FastQC, the low mapping rates observed for some samples suggest some level of either genomic DNA or non-human nucleic acid contamination in the sequencing libraries of the HL-60 and U937 cells (Li *et al.*, 2022). Contamination with non-human nucleic acids can occur due to the presence of contaminating mycoplasma in the sequenced cells (Olarerin-George and Hogenesch, 2015), or due to the presence of contaminating nucleic acid material in the sequencer itself (Mortezaei, 2022). While both of these contaminants are problematic, in that they reduce the number of sequencing reads available for analysis in a study, they are not expected to appreciably affect the results of this study, as they cannot be mapped to the human transcriptome and are therefore not included in transcript and gene abundance estimates.

## 4.2 Similarity of baseline gene expression

Baseline gene expression was similar due to the leukemic origins of each cell line (Sawyers, 1999). In all three cell lines, the process of monocyte-to-macrophage differentiation was halted. This occurred in the peripheral blood for HL-60 and THP-1 cells and in a tissue for

U937 cells. This shows that despite differences in sample origins, these cells still maintain the same lineage-specific genes. In support, Wang *et al.* (2013) showed that the transcriptomic profiles of HL-60 and THP-1 cells were more similar, using the ribo-minus RNA-seq method and reads mapped to the genome.

### 4.3 Core response to PMA treatment observed across all cell lines

Since physical changes such as the formation of phagocytic vacuoles and increasing adhesion of cultured cells can be used to monitor the process of differentiation of cell lines *in vitro* (Nichols, *et al.*, 1971), the phagosome pathway was assessed. Irrespective of cell line, DEGs in the phagosome pathways were detected, suggesting that all three cell lines were undergoing transcriptional changes to support the differentiated cells' phagocytic function. This is supported by experimental findings, which indicate that macrophage-like cells have a phagocytic capacity for latex beads (Swiatecka and Mackie, 2015). Phagocytic vacuoles are also seen *in vitro* as these cell lines differentiate (Ueno *et al.*, 2021). These findings suggest shared physical attributes in all three cell lines. Over-representation of genes involved in the phagosome formation suggests that PMA induces an increase in the expression of genes encoding receptors and proteins that facilitate the architecture of such structures in the cell across all three cell lines. Such genes include pattern recognition receptors which are important for detecting threats, opsonic receptors that mark the threat as a target, and receptors for apoptotic cells (Lee *et al.*, 2020).

CD36 was up-regulated across all cell lines, which indicates that they have the potential to remove apoptotic cells through phagocytosis (Taylor *et al.*, 2005). The CD36 receptor is important for the removal of apoptotic cells and bacteria (Taylor *et al.*, 2005). PMA treatment is sometimes associated with the induction of apoptosis (Kumbrink and Kirsch, 2013). This is often seen in conditions where high concentrations of PMA had been used (Starr *et al.*, 2018). Data used in the current study was obtained from studies where 10 000 nM, 100 nM, and 80 nM PMA was added to HL-60, THP-1, and U937 cells, respectively, which would fall within the high range (Park *et al.*, 2007). Assessment of optimal concentrations for THP-1 differentiation showed that 8 nM yielded the best differentiation (Park *et al.*, 2007). It is thus not surprising to note that the transcriptional changes observed here support a potential to respond to apoptotic DAMPs. Apoptotic cells release signals that indicate their cell death to

phagocytic cells. These signals are for detection and clearance. For example, phosphatidylserine (PS) is expressed in apoptotic cells (Lee *et al.,* 2020). Upon detection of such signals, phagocytes exhibit an increase in the level of expression of genes that encode receptors for the detection and clearance of apoptotic cells (Lee *et al.*, 2020). Furthermore, CD36 is also a marker of both monocytes and macrophages (Nicholson, 2016). Therefore, an increase in this gene across all cell lines is indicative of the potential adoption of a macrophage-like phenotype.

A significant down-regulation of genes involved in the regulation of cell cycle phase transition was observed in all three cell lines following PMA treatment. This suggests that PMA-induced differentiation decreases cell proliferation. In support, Gažová *et al.* (2020) showed that PMA induces cell growth arrest by inducing the activity of p53. The observed down-regulation of the gene encoding the E2F1 transcription factor in this study provides further support for the cell cycle arrest, as Kovesdi *et al.* (1986) showed that a decrease in the expression of E2F1 inhibits entry into the S-phase of the cell cycle. There is also down-regulation of DNA repair and synthesis, as well as mitosis (Polager *et al.*, 2002). Furthermore, it allows all the cells to express genes that are important for their final functions after differentiation. Down-regulation of cell-cycle genes leads to an extended G1-S-phase period and delays cell division (Dalton, 2015). The G1 phase is permissive to various signals that influence differentiation and commitment towards specific lineages. This is associated with the decompression of chromatin allowing the reorganisation of nucleic information based on the needs of the cell. Inhibition of cell growth suggests that all three cell lines are committed toward terminal differentiation (Dalton, 2015).

Common up-regulation of genes within the myeloid cell differentiation pathway suggests that all three cell lines are being prepared to take on the myeloid lineage. The *CSF1* gene encodes a cytokine, CSF1, which is important for myeloid cell differentiation and proliferation (Stanley *et al.*, 1978). CSF1 uses the CSF-1R receptor to perform its functions (Yeung *et al.*, 2003). The dimerisation of CSF-1R receptors induces autophosphorylation, which leads to a downstream signalling cascade that leads to the expression of genes that are necessary for myeloid differentiation. These genes lead the cells toward a macrophage lineage (Bartelmez *et al.*, 1989). This is seen in the formation of macrophage colonies in culture. Up-regulation of this gene also implies the activity of myeloid transcription factors such as *ETS* and *PU.1* (Pixley and Stanley, 2004). These transcription factors act as downstream targets of CSF1

during differentiation. CSF1 has also been shown to up-regulate the expression of the gene encoding the transcription factor, Jun (Nakamura *et al.*, 1991). This is consistent with what was seen in this study, which implies that all three of these cells are already transcribing genes for proliferation and regulating immune responses. Furthermore, the up-regulation of key transcription factors for myeloid differentiation is a confirmation that the desired response from PMA-induced myeloid differentiation is achieved in all three cell lines.

### 4.4 Cell line-specific responses to PMA treatment

Differential expression of genes involved in the Toll-like-receptor (TLR) pathway was observed in HL-60, THP-1, and U937 cells, but the changes in expression observed were seen to be cell-line-specific. HL-60 cells showed limited changes in the expression of genes involved in TLR-signalling relative to THP-1 and U937 cells. This may be due to differences in the developmental states of these cells. HL-60 cells are promyelocytes (Collins *et al.*, 1977), whereas THP-1 and U937 cells are promonocytes (Sundström and Nilsson, 1976; Tsuchiya *et al.*, 1980). This is best highlighted by the lack of change in CD14 expression within the HL-60 cell line. CD14 is involved in the detection of bacterial lipopolysaccharides and the removal of apoptotic cells (Devitt *et al.,* 1998). It requires coreceptors in order to induce a downstream cell signalling cascade (Devitt *et al.*, 1998). TLR2 and TLR4 are important CD14 co-receptors for microbial ligands (Triantafilou and Triantafilou, 2002). The lack of a significant change in the expression of CD14 in HL-60 may also be a result of the time at which this analysis was done. This is because the baseline expression of CD14 in THP-1 and U937 is greater relative to HL-60 which does not express CD14 in the untreated condition. These findings are consistent with what has been shown in literature, where prior to treatment, CD14 expression is not detected in HL-60 cells (Li *et al.*, 2002). In contrast, THP-1 cells are noted to have high CD14 expression prior to treatment (Foster *et al.,* 2005; Ciabattini *et al.*, 2006). It was also shown that at 48h post-PMA treatment, THP-1 cells have significantly increased CD14 expression (Park *et al.*, 2007).

THP-1 cells exhibited significant up-regulation of TLRs 3, 7, and 8, which was not observed in HL-60 and U937 cells. Up-regulation of these receptors would suggest that THP-1 cells have increased potential to detect double-stranded RNA (Alexopoulou *et al.*, 2001). In

contrast, U937 cells are best poised for response to bacterial PAMPs, as suggested by the unique up-regulation of TLR2, which is important for the detection of bacterial PAMPs and viral envelope proteins (Okamoto *et al.*, 2009). The up-regulation of both TLR1 and TLR6 in U937 cells implies that U937 cells may be able to detect a wider variety of microbial products from bacteria and fungi relative to HL-60 and THP-1 cells (Okamoto *et al.*, 2009), as dimerization can occur between TLR2 and TLR6 or TLR2 and TLR1, which potentially widens the variety of bacterial, fungal, and protozoal PAMPs that can be detected in U937 cells.

At 48 hours following PMA treatment, only U937 cells exhibited significant up-regulation of genes encoding FC-γ receptors,namely, *FCGR3A,FCGR2A* and *FCGR2B* . This may suggest that U937 cells have an increased potential to opsonize threats and mark them as targets for phagocytosis. *FCGR3A* (CD16) encodes for a late macrophage-specific receptor. It was not expected to be up-regulated in U937 as early as 48h post-PMA treatment. Even so, significant up-regulation of *FCGR3A* in THP-1 and U937 cells is consistent with what has previously been reported in the literature (Callanan *et al.*, 2000; Gil Gonzalez *et al.*, 2022). THP-1 cells did not show significant up-regulation of *FCGR2A* and *FCGR2B* at 48 hours. These genes were seen to be highly expressed in THP-1 cells prior to treatment with PMA. Fleit and Kobasiuk (1991), performed flow cytometry and assessed the expression of Fc receptors, FCγRIIA, and FCγRIIB (encoded by *FCGR2A* and *FCGR2B*), prior to and after PMA differentiation. Their results showed that there is no significant change in the expression of these receptors after treating THP-1 cells with 160 nM PMA for 48 hours (Fleit and Kobasiuk, 1991). Fc receptors are important macrophage receptors for inducing proinflammatory reactions and facilitating cell adhesion (Unkeless, 1989). This may indicate that U937 cells have a potential to drive proinflammatory immune responses.

Most pathways that were over-represented in a cell-line-specific manner were metabolic pathways showing differing metabolic capacities of these cell lines. HL-60 cells had a unique over-representation of nucleotide metabolism, including unique down-regulation of the *CTPS2* gene. This gene encodes a protein that deaminates glutamine to glutamate. Glutamine is important for DNA synthesis (Moffatt and Ashihara 2002). It is an important nitrogen donor for the synthesis of both pyrimidine and purine nucleotides, which are the building units of nucleic acids (Moffatt and Ashihara 2002). This gene is also associated with energy metabolism through the breakdown of ATP (Moffatt and Ashihara 2002). Down-regulation of the gene could mean the depreciation of energy within the cell. However, the unique

up-regulation of the *AK-1* gene in HL-60 cells indicates potential for the expression of adenylate kinase enzyme, which is important for mitochondrial energy metabolism (Janssen, 2000). The increase observed in the expression of this gene might indicate that HL-60 cells rely on energy from mitochondrial energy metabolism undergoing differentiation.

In contrast, THP-1 and U937 use other metabolic pathways for energy production. The unique up-regulation of the *HK3* gene in U937 cells suggests that these cells use energy from glucose metabolism (González *et al.*, 1964). This means that U937 cells have a potential increase in hexokinase, the protein encoded by *HK3* (Gould and Holman, 1993). As a result, there is phosphorylation of glucose to produce glucose-6-phosphate, which is the first step required to initiate glycolysis. Interestingly, glucose metabolism has been associated with M1 macrophages (Kelly and O'Neill, 2015), possibly suggesting that U937 cells have the potential to reach an M1 macrophage end state. However, fully differentiated U937 cells have been reported to lean toward an M2 end state, while THP-1 cells are more inclined to lean toward an M1 end state (Nascimento *et al.*, 2022). Thus this is an interesting finding that warrants further investigation.

### 4.5 Study Limitations

Ideally, in any scientific analysis, one would want the experimental conditions across comparator groups to be as similar as possible to best compare the results. However, in this study, there was an important difference in the methods used to prepare the sequencing libraries obtained from PMA-treated and untreated U937 cells, relative to the other cell lines included in this study. These sequencing libraries were prepared using an rRNA depletion method that results in the extraction of total RNA. (Rossetti *et al.,* 2017), whereas the HL-60 and THP-1 sequencing libraries were prepared using a poly-A selection method that results in the sequencing of predominantly mature mRNAs (Li *et al.*, 2022). In order to account for these differences in the composition of the sequencing libraries, analyses in this study were limited to include only sequencing reads derived from protein-coding genes. While this did not completely eliminate the observed biases in library composition, it did mitigate some of its effects on the results of this study. The analysis would have benefited from the comparison of results from different differentiation agents. Various differentiation agents lead to unique

cellular changes. However, in this study, only one agent was investigated. Even so, the decision to investigate PMA as an activation agent above other agents allowed the assessment of changes in gene expression that are similar to what would be expected in PBMC monocyte differentiation.

# 5. Conclusion

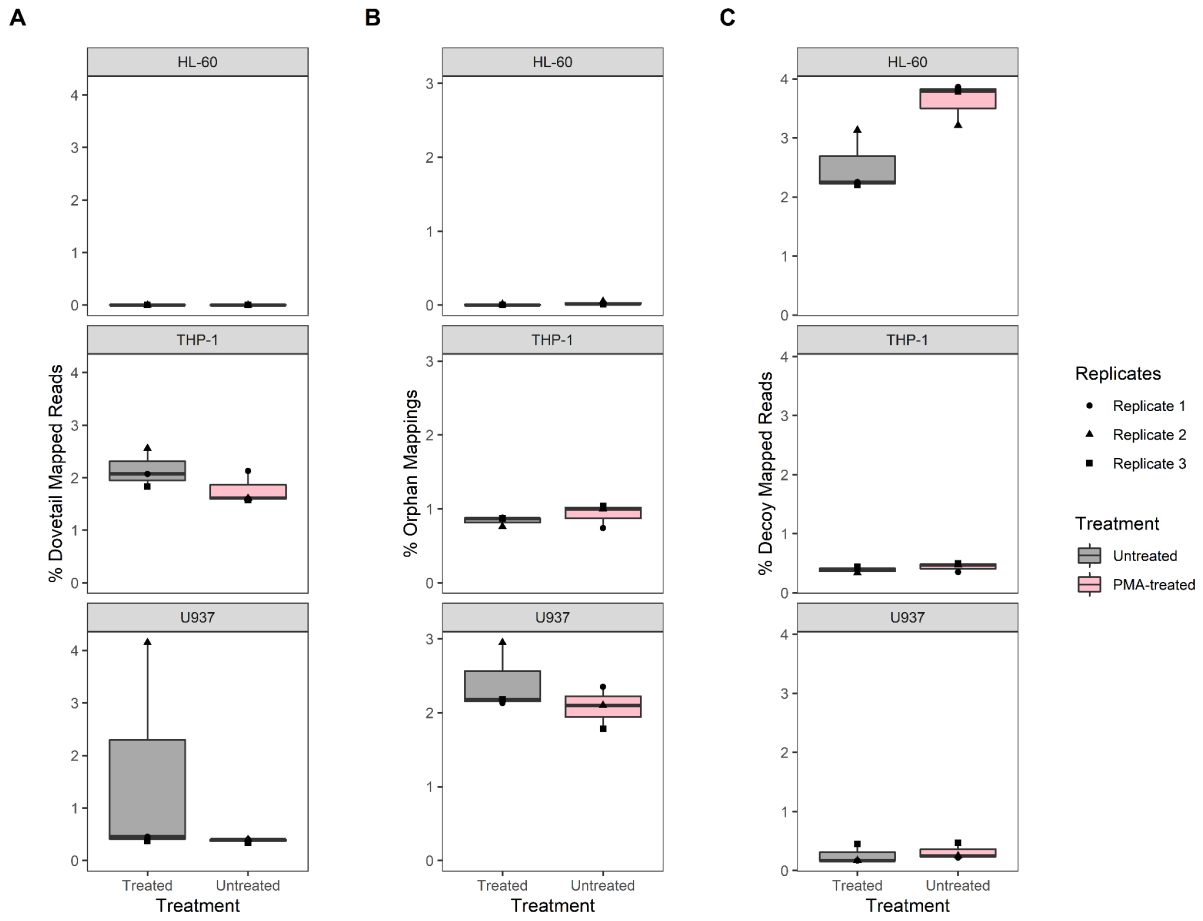
Studying immunological processes using model cell lines is a common practice due to the challenges posed by the use of primary monocytes and macrophages (Gazdar *et al.*, 2010; van Staveren *et al.,* 2009). The HL-60, THP-1, and U937 cell lines are commonly used to study the process of myeloid differentiation (Green *et al*., 2020; Ramirez *et al*., 2017; Rossetti *et al*., 2017). This study has demonstrated that these cell lines express largely the same set of genes at baseline (i.e. when untreated), but that, for the most part, different sets of genes are differentially expressed in HL-60, THP-1, and U937 cells 48 hours after treatment with PMA. Differentially expressed genes that were detected across all cell lines are of relevance to phagocytosis and cell cycle regulation. All cell lines showed the potential for phagocytic activity in response to apoptotic signals. Inhibition of cell cycle progression was noted as a driver of myeloid differentiation. Cell line-specific differences in expression observed involve genes of relevance to energy metabolism and pathogen recognition, and may suggest that these cells have differing capacities to respond to pathogens. All three cell lines were successfully undergoing myeloid differentiation as intended. However, cell-line specific differences need to be considered for the use of these cells in experimental studies. This would improve the reliability of results affected by target genes that show unique differential expression patterns in different cell lines.

**Future work**

All the results obtained in this study were obtained through *in silico* analysis of published experimental data. It would therefore be desirable to further validate these findings through future *in vitro* studies. To validate changes in gene expression, genes identified as being differentially expressed in response to PMA treatment identified in this study could be studied using quantitative reverse transcriptase PCR. Perhaps of even more value would be to

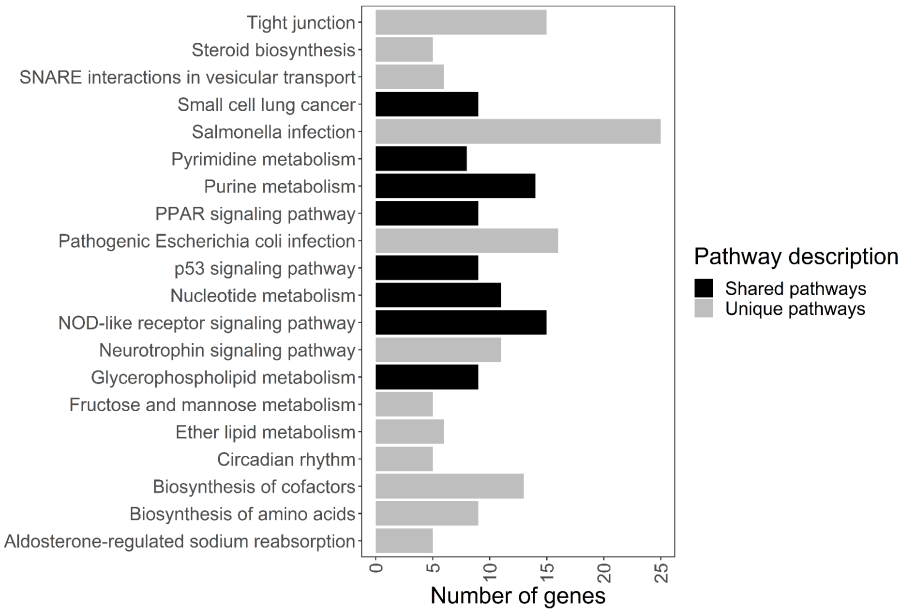
extend this further and assess whether changes at the mRNA level are also reflected at the protein level. To do this, changes in protein abundance could be assessed using flow cytometry and/or western blots. This would confirm which of the differentially expressed genes show significant changes in protein expression.

# Supplementary Data

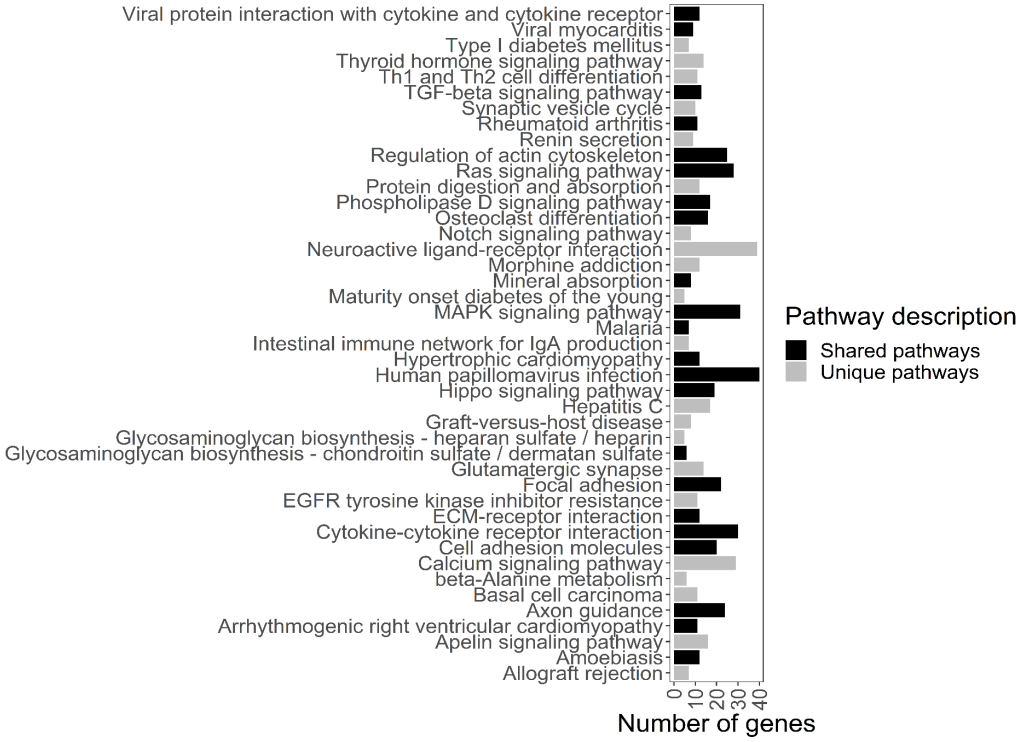



**Supplementary Figure 1: Summary statistics of salmon read mapping parameters.** A) The percentage of dovetail mappings discarded B) The percentage of orphan mappings rescued. C) The percentage of sequencing reads quasi-mapped to decoy sequences

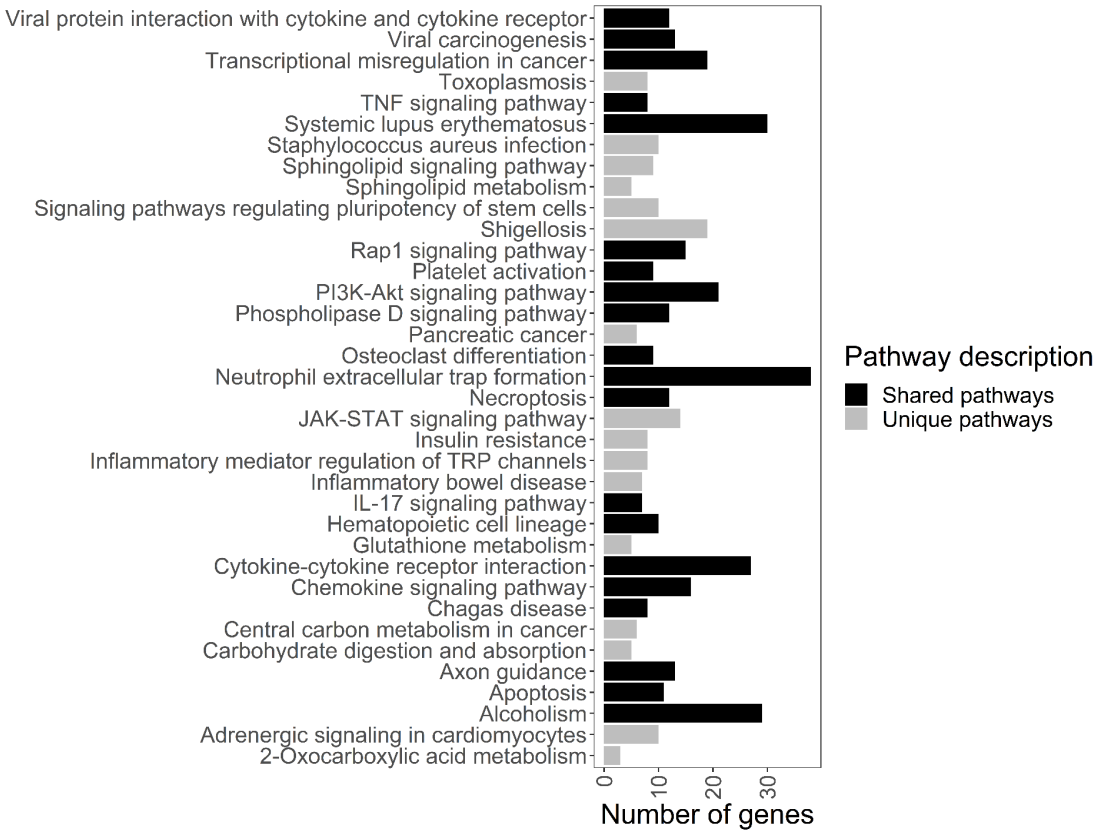
**A)**


Tight junction
Steroid biosynthesis
SNARE interactions in vesicular transport
Small cell lung cancer
Salmonella infection
Pyrimidine metabolism
Purine metabolism
PPAR signaling pathway
Pathogenic Escherichia coli infection
p53 signaling pathway
Nucleotide metabolism
NOD-like receptor signaling pathway
Neurotrophin signaling pathway
Glycerophospholipid metabolism
Fructose and mannose metabolism
Ether lipid metabolism
Circadian rhythm
Biosynthesis of cofactors
Biosynthesis of amino acids
Aldosterone-regulated sodium reabsorption
0
5
10
15
20
25
Number of genes
Pathway description
Shared pathways
Unique pathways

**B)**

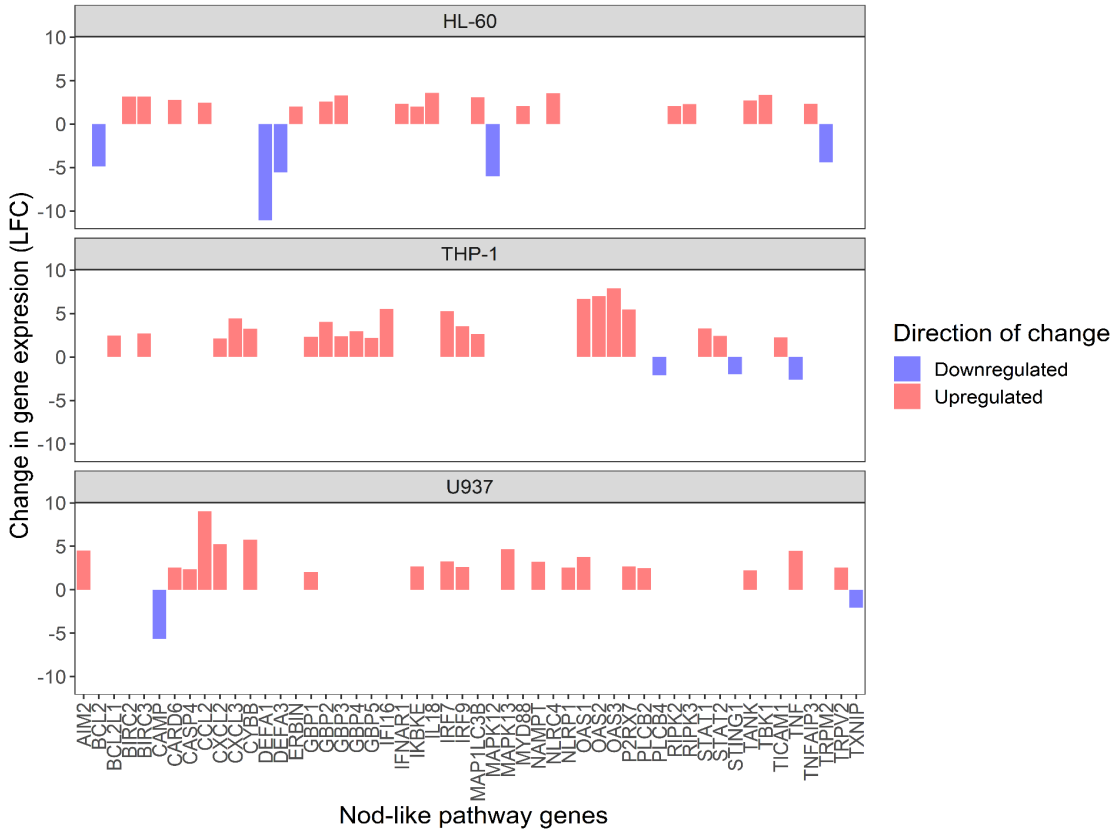

Viral protein interaction with cytokine and cytokine receptor
Viral myocarditis
Type I diabetes mellitus
Thyroid hormone signaling pathway
Th1 and Th2 cell differentiation
TGF-beta signaling pathway
Synaptic vesicle cycle
Rheumatoid arthritis
Renin secretion
Regulation of actin cytoskeleton
Ras signaling pathway
Protein digestion and absorption
Phospholipase D signaling pathway
Osteoclast differentiation
Notch signaling pathway
Neuroactive ligand-receptor interaction
Morphine addiction
Mineral absorption
Maturity onset diabetes of the young
MAPK signaling pathway
Malaria
Intestinal immune network for IgA production
Hypertrophic cardiomyopathy
Human papillomavirus infection
Hippo signaling pathway
Hepatitis C
Graft-versus-host disease
Glycosaminoglycan biosynthesis - heparan sulfate / heparin
Glycosaminoglycan biosynthesis - chondroitin sulfate / dermatan sulfate
Glutamatergic synapse
Focal adhesion
EGFR tyrosine kinase inhibitor resistance
ECM-receptor interaction
Cytokine-cytokine receptor interaction
Cell adhesion molecules
Calcium signaling pathway
beta-Alanine metabolism
Basal cell carcinoma
Axon guidance
Arrhythmogenic right ventricular cardiomyopathy
Apelin signaling pathway
Amoebiasis
Allograft rejection
0
10
20
30
40
Number of genes
Pathway description
Shared pathways
Unique pathways

**Supplementary Figure 2: KEGG pathways over-represented in genes that were significantly differentially expressed in response to treatment to PMA in A) HL-60 cells, and B) THP-1 cells.** Pathways highlighted in grey were uniquely observed in HL-60 and THP-1 cells. Pathways highlighted in black were also seen to be over-represented in the set of genes significantly differentially expressed in all three cell lines.

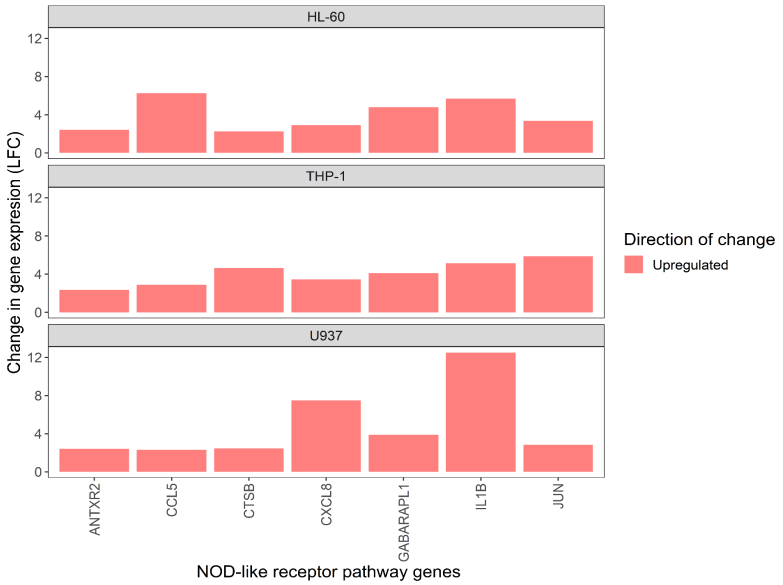



**Supplementary Figure 3: KEGG pathways over-represented in genes that were significantly differentially expressed in response to treatment to PMA in U937 cells.** Pathways highlighted in grey were uniquely observed U937 cells. Pathways highlighted in black were also seen to be over-represented in the set of genes significantly differentially expressed in all three cell lines.

A)




B)




**Supplementary Figure 4: Differentially expressed genes within the NOD-like receptor pathway.** DEGs that change in a cell-line-specific manner B) DEGs that change in the same manner across all cell lines.

# References


Abraham, S.N., St. John, A.L., 2010. Mast cell-orchestrated immunity to pathogens. Nat Rev Immunol 10, 440–452. https://doi.org/10.1038/nri2782

Acosta, J.A., Hernandez, A., 2019. An Overview of Peripheral Blood Mononuclear Cells as a Model for Immunological Research of Toxoplasma gondii and Other Apicomplexan Parasites. Front. Cell. Infect. Microbiol. 9, 24. https://doi.org/10.3389/fcimb.2019.00024

Ahmed, R., Gray, D., 1996. Immunological Memory and Protective Immunity: Understanding Their Relation. Science 272, 54–60. https://doi.org/10.1126/science.272.5258.54

Alexopoulou, L., Holt, A.C., Medzhitov, R., Flavell, R.A., 2001. Recognition of double-stranded RNA and activation of NF-κB by Toll-like receptor 3. Nature 413, 732–738. https://doi.org/10.1038/35099560

Andrews, S, 2010. FastQC: a quality control tool for high throughput sequence data.

Ardito, F., Giuliani, M., Perrone, D., Troiano, G., Muzio, L.L., 2017. The crucial role of protein phosphorylation in cell signaling and its use as targeted therapy (Review). International Journal of Molecular Medicine 40, 271–280. https://doi.org/10.3892/ijmm.2017.3036

Ashburner, M., Ball, C.A., Blake, J.A., Botstein, D., Butler, H., Cherry, J.M., Davis, A.P., Dolinski, K., Dwight, S.S., Eppig, J.T., Harris, M.A., Hill, D.P., Issel-Tarver, L., Kasarskis, A., Lewis, S., Matese, J.C., Richardson, J.E., Ringwald, M., Rubin, G.M., Sherlock, G., 2000. Gene ontology: tool for the unification of biology. The Gene Ontology Consortium. Nat. Genet. 25, 25–29. https://doi.org/10.1038/75556

Auwerx, J., 1991. The human leukemia cell line, THP-1: A multifacetted model for the study of monocyte-macrophage differentiation. Experientia 47, 22–31. https://doi.org/10.1007/BF02041244

Bartelmez, S.H., Bradley, T.R., Bertoncello, I., Mochizuki, D.Y., Tushinski, R.J., Stanley, E.R., Hapel, A.J., Young, I.G., Kriegler, A.B., Hodgson, G.S., 1989. Interleukin 1 plus interleukin 3 plus colony-stimulating factor 1 are essential for clonal proliferation of primitive myeloid bone marrow cells. Exp Hematol 17, 240–245.

Baxter, E.W., Graham, A.E., Re, N.A., Carr, I.M., Robinson, J.I., Mackie, S.L., Morgan, A.W., 2020. Standardized protocols for differentiation of THP-1 cells to macrophages with distinct M(IFNγ+LPS), M(IL-4) and M(IL-10) phenotypes. Journal of Immunological Methods 478, 112721.

https://doi.org/10.1016/j.jim.2019.112721
Benjamini, Y., Hochberg, Y., 1995. Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. Journal of the Royal Statistical Society: Series B (Methodological) 57, 289–300. https://doi.org/10.1111/j.2517-6161.1995.tb02031.x
Benjamini, Y., Speed, T.P., 2012. Summarizing and correcting the GC content bias in high-throughput sequencing. Nucleic Acids Research 40, e72–e72. https://doi.org/10.1093/nar/gks001
Berges, C., Naujokat, C., Tinapp, S., Wieczorek, H., Höh, A., Sadeghi, M., Opelz, G., Daniel, V., 2005. A cell line model for the differentiation of human dendritic cells. Biochemical and Biophysical Research Communications 333, 896–907. https://doi.org/10.1016/j.bbrc.2005.05.171
Bolger, A.M., Lohse, M., Usadel, B., 2014. Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics 30, 2114–2120. https://doi.org/10.1093/bioinformatics/btu170
Bonham, K.S., Kagan, J.C., 2014. Biochemistry of Toll‐Like Receptors, in: John Wiley & Sons, Ltd (Ed.), ELS. Wiley. https://doi.org/10.1002/9780470015902.a0024234
Bray, N.L., Pimentel, H., Melsted, P., Pachter, L., 2016. Near-optimal probabilistic RNA-seq quantification. Nat Biotechnol 34, 525–527. https://doi.org/10.1038/nbt.3519
Callanan, M.B., Le Baccon, P., Mossuz, P., Duley, S., Bastard, C., Hamoudi, R., Dyer, M.J., Klobeck, G., Rimokh, R., Sotto, J.J., Leroux, D., 2000. The IgG Fc receptor, FcγRIIB, is a target for deregulation by chromosomal translocation in malignant lymphoma. Proc. Natl. Acad. Sci. U.S.A. 97, 309–314. https://doi.org/10.1073/pnas.97.1.309
Cassol, E., Alfano, M., Biswas, P., Poli, G., 2006. Monocyte-derived macrophages and myeloid cell lines as targets of HIV-1 replication and persistence. Journal of Leukocyte Biology 80, 1018–1030. https://doi.org/10.1189/jlb.0306150
Chen, S., Huang, T., Zhou, Y., Han, Y., Xu, M., Gu, J., 2017. AfterQC: automatic filtering, trimming, error removing and quality control for fastq data. BMC Bioinformatics 18, 80. https://doi.org/10.1186/s12859-017-1469-3
Chen, S., Liu, M., Zhou, Y., 2018. Bioinformatics Analysis for Cell-Free Tumor DNA Sequencing Data, in: Huang, T. (Ed.), Computational Systems Biology, Methods in Molecular Biology. Springer New York, New York, NY, pp. 67–95. https://doi.org/10.1007/978-1-4939-7717-8_5
Chimal-Ramírez, G.K., Espinoza-Sánchez, N.A., Chávez-Sánchez, L., Arriaga-Pizano, L., Fuentes-Pananá, E.M., 2016. Monocyte Differentiation towards Protumor

Activity Does Not Correlate with M1 or M2 Phenotypes. Journal of Immunology Research 2016, 1–16. https://doi.org/10.1155/2016/6031486

Chomarat, P., Banchereau, J., Davoust, J., Karolina Palucka, A., 2000. IL-6 switches the differentiation of monocytes from dendritic cells to macrophages. Nat Immunol 1, 510–514. https://doi.org/10.1038/82763

Ciossani, G., Overlack, K., Petrovic, A., Huis in 't Veld, P.J., Koerner, C., Wohlgemuth, S., Maffini, S., Musacchio, A., 2018. The kinetochore proteins CENP-E and CENP-F directly and specifically interact with distinct BUB mitotic checkpoint Ser/Thr kinases. Journal of Biological Chemistry 293, 10084–10101. https://doi.org/10.1074/jbc.RA118.003154

Colbert, D.A., Fontana, J.A., Bode, U., Deisseroth, A.B., 1983. Changes in the Translational Activity of Polyadenylated Messenger RNA of HL60 Promyelocytic Leukemia Cells Associated with Myeloid or Macrophage Differentiation1. Cancer Research 43, 229–234.

Collins, S.J., Gallo, R.C., Gallagher, R.E., 1977. Continuous growth and differentiation of human myeloid leukaemic cells in suspension culture. Nature 270, 347–349. https://doi.org/10.1038/270347a0

Crow, M., Lim, N., Ballouz, S., Pavlidis, P., Gillis, J., 2019. Predictability of human differential gene expression. Proc. Natl. Acad. Sci. U.S.A. 116, 6491–6500. https://doi.org/10.1073/pnas.1802973116

Daigneault, M., Preston, J.A., Marriott, H.M., Whyte, M.K.B., Dockrell, D.H., 2010. The Identification of Markers of Macrophage Differentiation in PMA-Stimulated THP-1 Cells and Monocyte-Derived Macrophages. PLoS ONE 5, e8668. https://doi.org/10.1371/journal.pone.0008668

Dalton, S., 2015. Linking the Cell Cycle to Cell Fate Decisions. Trends in Cell Biology 25, 592–600. https://doi.org/10.1016/j.tcb.2015.07.007

de Boer, M., Roos, D., 1986. Metabolic comparison between basophils and other leukocytes from human blood. J Immunol 136, 3447–3454.

Devitt, A., Moffatt, O.D., Raykundalia, C., Capra, J.D., Simmons, D.L., Gregory, C.D., 1998. Human CD14 mediates recognition and phagocytosis of apoptotic cells. Nature 392, 505–509. https://doi.org/10.1038/33169

Dobin, A., Davis, C.A., Schlesinger, F., Drenkow, J., Zaleski, C., Jha, S., Batut, P., Chaisson, M., Gingeras, T.R., 2013. STAR: ultrafast universal RNA-seq aligner. Bioinformatics 29, 15–21. https://doi.org/10.1093/bioinformatics/bts635

Doshi, N., Mitragotri, S., 2010. Macrophages Recognize Size and Shape of Their Targets. PLoS ONE 5, e10051. https://doi.org/10.1371/journal.pone.0010051

Fan, X.D., Stark, G.R., Bloom, B.R., 1989. Molecular cloning of a gene selectively

induced by gamma interferon from human macrophage cell line U937. Mol Cell Biol 9, 1922–1928. https://doi.org/10.1128/mcb.9.5.1922-1928.1989

Fleck, R.A., Romero-Steiner, S., Nahm, M.H., 2005. Use of HL-60 Cell Line To Measure Opsonic Capacity of Pneumococcal Antibodies. Clin Vaccine Immunol 12, 19–27. https://doi.org/10.1128/CDLI.12.1.19-27.2005

Fleit, H.B., Kobasiuk, C.D., 1991. The human monocyte-like cell line THP-1 expresses Fc gamma RI and Fc gamma RII. J Leukoc Biol 49, 556–565. https://doi.org/10.1002/jlb.49.6.556

Flynn, J.K., Paukovics, G., Moore, M.S., Ellett, A., Gray, L.R., Duncan, R., Salimi, H., Jubb, B., Westby, M., Purcell, D.F.J., Lewin, S.R., Lee, B., Churchill, M.J., Gorry, P.R., Roche, M., 2013. The magnitude of HIV-1 resistance to the CCR5 antagonist maraviroc may impart a differential alteration in HIV-1 tropism for macrophages and T-cell subsets. Virology 442, 51–58. https://doi.org/10.1016/j.virol.2013.03.026

Fontana, J.A., Colbert, D.A., Deisseroth, A.B., 1981. Identification of a population of bipotent stem cells in the HL60 human promyelocytic leukemia cell line. Proc. Natl. Acad. Sci. U.S.A. 78, 3863–3866. https://doi.org/10.1073/pnas.78.6.3863

Frankish, A., Diekhans, M., Jungreis, I., Lagarde, J., Loveland, J.E., Mudge, J.M., Sisu, C., Wright, J.C., Armstrong, J., Barnes, I., Berry, A., Bignell, A., Boix, C., Carbonell Sala, S., Cunningham, F., Di Domenico, T., Donaldson, S., Fiddes, I.T., García Girón, C., Gonzalez, J.M., Grego, T., Hardy, M., Hourlier, T., Howe, K.L., Hunt, T., Izuogu, O.G., Johnson, R., Martin, F.J., Martínez, L., Mohanan, S., Muir, P., Navarro, F.C.P., Parker, A., Pei, B., Pozo, F., Riera, F.C., Ruffier, M., Schmitt, B.M., Stapleton, E., Suner, M.-M., Sycheva, I., Uszczynska-Ratajczak, B., Wolf, M.Y., Xu, J., Yang, Y.T., Yates, A., Zerbino, D., Zhang, Y., Choudhary, J.S., Gerstein, M., Guigó, R., Hubbard, T.J.P., Kellis, M., Paten, B., Tress, M.L., Flicek, P., 2021. GENCODE 2021. Nucleic Acids Research 49, D916–D923. https://doi.org/10.1093/nar/gkaa1087

Gazdar, A.F., Girard, L., Lockwood, W.W., Lam, W.L., Minna, J.D., 2010. Lung Cancer Cell Lines as Tools for Biomedical Discovery and Research. JNCI: Journal of the National Cancer Institute 102, 1310–1321. https://doi.org/10.1093/jnci/djq279

Gažová, I., Lefevre, L., Bush, S.J., Clohisey, S., Arner, E., de Hoon, M., Severin, J., van Duin, L., Andersson, R., Lengeling, A., Hume, D.A., Summers, K.M., 2020. The Transcriptional Network That Controls Growth Arrest and Macrophage Differentiation in the Human Myeloid Leukemia Cell Line THP-1. Front. Cell Dev. Biol. 8, 498. https://doi.org/10.3389/fcell.2020.00498

Geneviève, 2018. Understanding how kallisto works. IRIC's Bioinformatics Platform. URL https://bioinfo.iric.ca/understanding-how-kallisto-works/ (accessed 3.25.20).

Gil Gonzalez, L., Fernandez-Marrero, Y., Norris, P.A.A., Tawhidi, Z., Shan, Y., Cruz-Leal, Y., Won, K.D., Frias-Boligan, K., Branch, D.R., Lazarus, A.H., 2022. THP-1 cells transduced with CD16A utilize Fcγ receptor I and III in the phagocytosis of IgG-sensitized human erythrocytes and platelets. PLoS ONE 17, e0278365. https://doi.org/10.1371/journal.pone.0278365

Gocek, E., Marcinkowska, E., 2011. Differentiation Therapy of Acute Myeloid Leukemia. Cancers 3, 2402–2420. https://doi.org/10.3390/cancers3022402

González, C., Ureta, T., Sánchez, R., Niemeyer, H., 1964. Multiple molecular forms of ATP: Hexose 6-phosphotransferase from rat liver. Biochemical and Biophysical Research Communications 16, 347–352. https://doi.org/10.1016/0006-291X(64)90038-5

Gordon, S., 2016. Phagocytosis: An Immunobiologic Process. Immunity 44, 463–475. https://doi.org/10.1016/j.immuni.2016.02.026

Gould, G.W., Holman, G.D., 1993. The glucose transporter family: structure, function and tissue-specific expression. Biochemical Journal 295, 329–341. https://doi.org/10.1042/bj2950329

Green, I.D., Pinello, N., Song, R., Lee, Q., Halstead, J.M., Kwok, C.-T., Wong, A.C.H., Nair, S.S., Clark, S.J., Roediger, B., Schmitz, U., Larance, M., Hayashi, R., Rasko, J.E.J., Wong, J.J.-L., 2020. Macrophage development and activation involve coordinated intron retention in key inflammatory regulators. Nucleic Acids Res 48, 6513–6529. https://doi.org/10.1093/nar/gkaa435

Gu, Y., Lawrence, T., Mohamed, R., Liang, Y., Yahaya, B.H., 2022. The emerging roles of interstitial macrophages in pulmonary fibrosis: A perspective from scRNA-seq analyses. Front. Immunol. 13, 923235. https://doi.org/10.3389/fimmu.2022.923235

Harris, P., Ralph, P., 1985. Human Leukemic Models of Myelomonocytic Development: A Review of the HL-60 and U937 Cell Lines. Journal of Leukocyte Biology 37, 407–422. https://doi.org/10.1002/jlb.37.4.407

Harrow, J., Frankish, A., Gonzalez, J.M., Tapanari, E., Diekhans, M., Kokocinski, F., Aken, B.L., Barrell, D., Zadissa, A., Searle, S., Barnes, I., Bignell, A., Boychenko, V., Hunt, T., Kay, M., Mukherjee, G., Rajan, J., Despacio-Reyes, G., Saunders, G., Steward, C., Harte, R., Lin, M., Howald, C., Tanzer, A., Derrien, T., Chrast, J., Walters, N., Balasubramanian, S., Pei, B., Tress, M., Rodriguez, J.M., Ezkurdia, I., van Baren, J., Brent, M., Haussler, D., Kellis, M., Valencia, A., Reymond, A., Gerstein, M., Guigó, R., Hubbard, T.J., 2012. GENCODE: The reference human genome annotation for The ENCODE Project. Genome Res. 22, 1760–1774. https://doi.org/10.1101/gr.135350.111

Hoffman, W., Lakkis, F.G., Chalasani, G., 2016. B Cells, Antibodies, and More. CJASN

11, 137–154. https://doi.org/10.2215/CJN.09430915

Hogan, R.J., Usherwood, E.J., Zhong, W., Roberts, A.D., Dutton, R.W., Harmsen, A.G., Woodland, D.L., 2001. Activated Antigen-Specific CD8 $^{+}$ T Cells Persist in the Lungs Following Recovery from Respiratory Virus Infections. J Immunol 166, 1813–1822. https://doi.org/10.4049/jimmunol.166.3.1813

Hughes, P.J., Marcinkowska, E., Gocek, E., Studzinski, G.P., Brown, G., 2010. Vitamin D3-driven signals for myeloid cell differentiation—Implications for differentiation therapy. Leukemia Research 34, 553–565. https://doi.org/10.1016/j.leukres.2009.09.010

Jakubzick, C., Gautier, E.L., Gibbings, S.L., Sojka, D.K., Schlitzer, A., Johnson, T.E., Ivanov, S., Duan, Q., Bala, S., Condon, T., van Rooijen, N., Grainger, J.R., Belkaid, Y., Ma'ayan, A., Riches, D.W.H., Yokoyama, W.M., Ginhoux, F., Henson, P.M., Randolph, G.J., 2013. Minimal Differentiation of Classical Monocytes as They Survey Steady-State Tissues and Transport Antigen to Lymph Nodes. Immunity 39, 599–610. https://doi.org/10.1016/j.immuni.2013.08.007

Janeway, C.A., 1989a. Approaching the Asymptote? Evolution and Revolution in Immunology. Cold Spring Harbor Symposia on Quantitative Biology 54, 1–13. https://doi.org/10.1101/SQB.1989.054.01.003

Janeway, C.A., 1989b. Approaching the Asymptote? Evolution and Revolution in Immunology. Cold Spring Harbor Symposia on Quantitative Biology 54, 1–13. https://doi.org/10.1101/SQB.1989.054.01.003

Janeway, C.J., Travers, P., Walport, M., 2001. Immunobiology: The Immune System in Health and Disease, 5th edition. ed. New York: Garland Science.

Janssen, E., 2000. Adenylate kinase 1 gene deletion disrupts muscle energetic economy despite metabolic rearrangement. The EMBO Journal 19, 6371–6381. https://doi.org/10.1093/emboj/19.23.6371

Julien, E., El Omar, R., Tavian, M., 2016. Origin of the hematopoietic system in the human embryo. FEBS Letters 590, 3987–4001. https://doi.org/10.1002/1873-3468.12389

Kanehisa, M., Goto, S., Furumichi, M., Tanabe, M., Hirakawa, M., 2010. KEGG for representation and analysis of molecular networks involving diseases and drugs. Nucleic Acids Res. 38, D355-360. https://doi.org/10.1093/nar/gkp896

Kapellos, T.S., Bonaguro, L., Gemünd, I., Reusch, N., Saglam, A., Hinkley, E.R., Schultze, J.L., 2019. Human Monocyte Subsets and Phenotypes in Major Chronic Inflammatory Diseases. Front. Immunol. 10, 2035. https://doi.org/10.3389/fimmu.2019.02035

Kaufmann, S.H.E., 2019. Immunology's Coming of Age. Front. Immunol. 10, 684.

https://doi.org/10.3389/fimmu.2019.00684
Kaufmann, S.H.E., 2008. Immunology's foundation: the 100-year anniversary of the Nobel Prize to Paul Ehrlich and Elie Metchnikoff. Nat Immunol 9, 705–712. https://doi.org/10.1038/ni0708-705
Kelly, B., O'Neill, L.A., 2015. Metabolic reprogramming in macrophages and dendritic cells in innate immunity. Cell Res 25, 771–784. https://doi.org/10.1038/cr.2015.68
Khatri, P., Sirota, M., Butte, A.J., 2012. Ten Years of Pathway Analysis: Current Approaches and Outstanding Challenges. PLoS Comput Biol 8, e1002375. https://doi.org/10.1371/journal.pcbi.1002375
Kijowski, J., Baj‐Krzyworzeka, M., Majka, M., Reca, R., Marquez, L.A., Christofidou‐Solomidou, M., Janowska‐Wieczorek, A., Ratajczak, M.Z., 2001. The SDF‐1‐CXCR4 Axis Stimulates VEGF Secretion and Activates Integrins but does not Affect Proliferation and Survival in Lymphohematopoietic Cells. STEM CELLS 19, 453–466. https://doi.org/10.1634/stemcells.19-5-453
Kim, D., Paggi, J.M., Park, C., Bennett, C., Salzberg, S.L., 2019. Graph-based genome alignment and genotyping with HISAT2 and HISAT-genotype. Nat Biotechnol 37, 907–915. https://doi.org/10.1038/s41587-019-0201-4
Kohro, T., Tanaka, T., Murakami, T., Wada, Y., Aburatani, H., Hamakubo, T., Kodama, T., 2004. A Comparison of Differences in the Gene Expression Profiles of Phorbol 12-myristate 13-acetate Differentiated THP-1 Cells and Human Monocyte-derived Macrophage. JAT 11, 88–97. https://doi.org/10.5551/jat.11.88
Konopka, K., Pretzer, E., Plowman, B., Düzgünes, N., 1993. Long-Term Noncytopathic Productive Infection of the Human Monocytic Leukemia Cell Line THP-1 by Human Immunedeficiency Virus Type 1 (HIV-1IIIB). Virology 193, 877–887. https://doi.org/10.1006/viro.1993.1197
Kovesdi, I., Reichel, R., Nevins, J.R., 1986. Identification of a cellular transcription factor involved in E1A trans-activation. Cell 45, 219–228. https://doi.org/10.1016/0092-8674(86)90386-7
Kumbrink, J., Kirsch, K.H., 2013. p130Cas acts as survival factor during PMA-induced apoptosis in HL-60 promyelocytic leukemia cells. The International Journal of Biochemistry & Cell Biology 45, 531–535. https://doi.org/10.1016/j.biocel.2012.12.017
Lebeyec, J., Xu, R., Lee, S., Nelson, C., Rizki, A., Alcaraz, J., Bissell, M., 2007. Cell shape regulates global histone acetylation in human mammary epithelial cells. Experimental Cell Research 313, 3066–3075. https://doi.org/10.1016/j.yexcr.2007.04.022
Lee, H.-J., Woo, Y., Hahn, T.-W., Jung, Y.M., Jung, Y.-J., 2020. Formation and

Maturation of the Phagosome: A Key Mechanism in Innate Immunity against Intracellular Bacterial Infection. Microorganisms 8, 1298. https://doi.org/10.3390/microorganisms8091298

Li, C., Wang, Y., Gao, L., Zhang, J., Shao, J., Wang, S., Feng, W., Wang, X., Li, M., Chang, Z., 2002. Expression of toll-like receptors 2 and 4 and CD14 during differentiation of HL-60 cells induced by phorbol 12-myristate 13-acetate and 1 alpha, 25-dihydroxy-vitamin D(3). Cell Growth Differ 13, 27–38.

Li, X., Zhang, P., Wang, H., Yu, Y., 2022. Genes expressed at low levels raise false discovery rates in RNA samples contaminated with genomic DNA. BMC Genomics 23, 554. https://doi.org/10.1186/s12864-022-08785-1

Liu, H., Lorenzini, P.A., Zhang, F., Xu, S., Wong, M.S.M., Zheng, J., Roca, X., 2018. Alternative splicing analysis in human monocytes and macrophages reveals MBNL1 as major regulator. Nucleic Acids Res 46, 6069–6086. https://doi.org/10.1093/nar/gky401

Lotem, J., Sachs, L., 1979. Regulation of normal differentiation in mouse and human myeloid leukemic cells by phorbol esters and the mechanism of tumor promotion. Proc. Natl. Acad. Sci. U.S.A. 76, 5158–5162. https://doi.org/10.1073/pnas.76.10.5158

Love, M.I., Huber, W., Anders, S., 2014. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol 15, 550. https://doi.org/10.1186/s13059-014-0550-8

Love, M.I., Soneson, C., Hickey, P.F., Johnson, L.K., Pierce, N.T., Shepherd, L., Morgan, M., Patro, R., 2019. Tximeta: reference sequence checksums for provenance identification in RNA-seq. bioRxiv 777888. https://doi.org/10.1101/777888

Maeß, M.B., Wittig, B., Cignarella, A., Lorkowski, S., 2014. Reduced PMA enhances the responsiveness of transfected THP-1 macrophages to polarizing stimuli. Journal of Immunological Methods 402, 76–81. https://doi.org/10.1016/j.jim.2013.11.006

Manda-Handzlik, A., Bystrzycka, W., Wachowska, M., Sieczkowska, S., Stelmaszczyk-Emmel, A., Demkow, U., Ciepiela, O., 2018. The influence of agents differentiating HL-60 cells toward granulocyte-like cells on their ability to release neutrophil extracellular traps. Immunol Cell Biol 96, 413–425. https://doi.org/10.1111/imcb.12015

Martin, M., 2011. Cutadapt removes adapter sequences from high-throughput sequencing reads. EMBnet j. 17, 10. https://doi.org/10.14806/ej.17.1.200

Martinez, F.O., Gordon, S., 2014. The M1 and M2 paradigm of macrophage activation: time for reassessment. F1000Prime Rep 6, 13. https://doi.org/10.12703/P6-13

Meylan, P.R.A., Spina, C.A., Richman, D.D., Kornbluth, R.S., 1993. In Vitro

Differentiation of Monocytoid THP-1 Cells Affects Their Permissiveness for HIV Strains: A Model System for Studying the Cellular Basis of HIV Differential Tropism. Virology 193, 256–267. https://doi.org/10.1006/viro.1993.1121

Mitroulis, I., Alexaki, V.I., Kourtzelis, I., Ziogas, A., Hajishengallis, G., Chavakis, T., 2015. Leukocyte integrins: Role in leukocyte recruitment and as therapeutic targets in inflammatory disease. Pharmacology & Therapeutics 147, 123–135. https://doi.org/10.1016/j.pharmthera.2014.11.008

Moffatt, B.A., Ashihara, H., 2002. Purine and Pyrimidine Nucleotide Synthesis and Metabolism. The Arabidopsis Book 1, e0018. https://doi.org/10.1199/tab.0018

Mohri, H., Perelson, A.S., Tung, K., Ribeiro, R.M., Ramratnam, B., Markowitz, M., Kost, R., Hurley, A., Weinberger, L., Cesar, D., Hellerstein, M.K., Ho, D.D., 2001. Increased turnover of T lymphocytes in HIV-1 infection and its reduction by antiretroviral therapy. J Exp Med 194, 1277–1287. https://doi.org/10.1084/jem.194.9.1277

Mortezaei, Z., 2022. Computational methods for analyzing RNA-sequencing contaminated samples and its impact on cancer genome studies. Informatics in Medicine Unlocked 32, 101054. https://doi.org/10.1016/j.imu.2022.101054

Mukhopadhyay, S., Gordon, S., 2004. The role of scavenger receptors in pathogen recognition and innate immunity. Immunobiology 209, 39–49. https://doi.org/10.1016/j.imbio.2004.02.004

Muller, W.A., Weigl, S.A., Deng, X., Phillips, D.M., 1993. PECAM-1 is required for transendothelial migration of leukocytes. Journal of Experimental Medicine 178, 449–460. https://doi.org/10.1084/jem.178.2.449

Murao, S., Gemmell, M.A., Callaham, M.F., Anderson, N.L., Huberman, E., 1983. Control of macrophage cell differentiation in human promyelocytic HL-60 leukemia cells by 1,25-dihydroxyvitamin D3 and phorbol-12-myristate-13-acetate. Cancer Res 43, 4989–4996.

Nakamura, T., Datta, R., Kharbanda, S., Kufe, D., 1991. Regulation of jun and fos gene expression in human monocytes by the macrophage colony-stimulating factor. Cell Growth Differ 2, 267–272.

Nascimento, C.R., Rodrigues Fernandes, N.A., Gonzalez Maldonado, L.A., Rossa Junior, C., 2022. Comparison of monocytic cell lines U937 and THP-1 as macrophage models for in vitro studies. Biochemistry and Biophysics Reports 32, 101383. https://doi.org/10.1016/j.bbrep.2022.101383

Newell, K.A., Asare, A., Kirk, A.D., Gisler, T.D., Bourcier, K., Suthanthiran, M., Burlingham, W.J., Marks, W.H., Sanz, I., Lechler, R.I., Hernandez-Fuentes, M.P., Turka, L.A., Seyfert-Margolis, V.L., 2010. Identification of a B cell signature

associated with renal transplant tolerance in humans. J. Clin. Invest. 120, 1836–1847. https://doi.org/10.1172/JCI39933

Nichols, B.A., Bainton, D.F., Farquhar, M.G., 1971. DIFFERENTIATION OF MONOCYTES. Journal of Cell Biology 50, 498–515. https://doi.org/10.1083/jcb.50.2.498

Nicholson, A.C., Frieda, S., Pearce, A., Silverstein, R.L., 1995. Oxidized LDL binds to CD36 on human monocyte-derived macrophages and transfected cell lines. Evidence implicating the lipid moiety of the lipoprotein as the binding site. Arterioscler Thromb Vasc Biol 15, 269–275. https://doi.org/10.1161/01.atv.15.2.269

Okamoto, M., Hirai, H., Taniguchi, K., Shimura, K., Inaba, T., Shimazaki, C., Taniwaki, M., Imanishi, J., 2009. Toll-like Receptors (TLRs) are expressed by myeloid leukaemia cell lines, but fail to trigger differentiation in response to the respective TLR ligands. British Journal of Haematology 147, 585–587. https://doi.org/10.1111/j.1365-2141.2009.07858.x

Olarerin-George, A.O., Hogenesch, J.B., 2015. Assessing the prevalence of mycoplasma contamination in cell culture via a survey of NCBI's RNA-seq archive. Nucleic Acids Research 43, 2535–2542. https://doi.org/10.1093/nar/gkv136

Ożańska, A., Szymczak, D., Rybka, J., 2020. Pattern of human monocyte subpopulations in health and disease. Scand J Immunol 92. https://doi.org/10.1111/sji.12883

Padilla, P.I., Wada, A., Yahiro, K., Kimura, M., Niidome, T., Aoyagi, H., Kumatori, A., Anami, M., Hayashi, T., Fujisawa, J., Saito, H., Moss, J., Hirayama, T., 2000. Morphologic Differentiation of HL-60 Cells Is Associated with Appearance of RPTPβ and Induction of Helicobacter pyloriVacA Sensitivity. Journal of Biological Chemistry 275, 15200–15206. https://doi.org/10.1074/jbc.275.20.15200

Park, E.K., Jung, H.S., Yang, H.I., Yoo, M.C., Kim, C., Kim, K.S., 2007. Optimized THP-1 differentiation is required for the detection of responses to weak stimuli. Inflamm. res. 56, 45–50. https://doi.org/10.1007/s00011-007-6115-5

Passlick, B., Flieger, D., Ziegler-Heitbrock, H.W., 1989. Identification and characterization of a novel monocyte subpopulation in human peripheral blood. Blood 74, 2527–2534.

Patro, R., Duggal, G., Love, M.I., Irizarry, R.A., Kingsford, C., 2017. Salmon provides fast and bias-aware quantification of transcript expression. Nat Methods 14, 417–419. https://doi.org/10.1038/nmeth.4197

Pesti, R., Kontra, L., Paul, K., Vass, I., Csorba, T., Havelda, Z., Várallyay, É., 2019. Differential gene expression and physiological changes during acute or persistent plant virus interactions may contribute to viral symptom differences. PLoS ONE 14, e0216618. https://doi.org/10.1371/journal.pone.0216618

Pixley, F.J., Stanley, E.R., 2004. CSF-1 regulation of the wandering macrophage: complexity in action. Trends in Cell Biology 14, 628–638. https://doi.org/10.1016/j.tcb.2004.09.016

Polager, S., Kalma, Y., Berkovich, E., Ginsberg, D., 2002. E2Fs up-regulate expression of genes involved in DNA replication, DNA repair and mitosis. Oncogene 21, 437–446. https://doi.org/10.1038/sj.onc.1205102

Qiu, Y., Wang, J., Lei, J., Roeder, K., 2021. Identification of cell-type-specific marker genes from co-expression patterns in tissue samples. Bioinformatics 37, 3228–3234. https://doi.org/10.1093/bioinformatics/btab257

Rabinovitch, M., 1995. Professional and non-professional phagocytes: an introduction. Trends in Cell Biology 5, 85–87. https://doi.org/10.1016/S0962-8924(00)88955-2

Ramirez, R.N., El-Ali, N.C., Mager, M.A., Wyman, D., Conesa, A., Mortazavi, A., 2017. Dynamic Gene Regulatory Networks of Human Myeloid Differentiation. Cell Systems 4, 416-429.e3. https://doi.org/10.1016/j.cels.2017.03.005

Richardson, E.T., Shukla, S., Nagy, N., Boom, W.H., Beck, R.C., Zhou, L., Landreth, G.E., Harding, C.V., 2015. ERK Signaling Is Essential for Macrophage Development. PLoS ONE 10, e0140064. https://doi.org/10.1371/journal.pone.0140064

Riddy, D.M., Goy, E., Delerive, P., Summers, R.J., Sexton, P.M., Langmead, C.J., 2018. Comparative genotypic and phenotypic analysis of human peripheral blood monocytes and surrogate monocyte-like cell lines commonly used in metabolic disease research. PLoS ONE 13, e0197177. https://doi.org/10.1371/journal.pone.0197177

Risso, D., Schwartz, K., Sherlock, G., Dudoit, S., 2011. GC-Content Normalization for RNA-Seq Data. BMC Bioinformatics 12, 480. https://doi.org/10.1186/1471-2105-12-480

Romero-Steiner, S., Frasch, C., Concepcion, N., Goldblatt, D., Käyhty, H., Väkeväinen, M., Laferriere, C., Wauters, D., Nahm, M.H., Schinsky, M.F., Plikaytis, B.D., Carlone, G.M., 2003. Multilaboratory Evaluation of a Viability Assay for Measurement of Opsonophagocytic Antibodies Specific to the Capsular Polysaccharides of *Streptococcus pneumoniae*. Clin Vaccine Immunol 10, 1019–1024. https://doi.org/10.1128/CDLI.10.6.1019-1024.2003

Romero-Steiner, S., Libutti, D., Pais, L.B., Dykes, J., Anderson, P., Whitin, J.C., Keyserling, H.L., Carlone, G.M., 1997. Standardization of an opsonophagocytic assay for the measurement of functional antibody activity against Streptococcus pneumoniae using differentiated HL-60 cells. Clin Diagn Lab Immunol 4, 415–422. https://doi.org/10.1128/cdli.4.4.415-422.1997

Rovera, G., O'Brien, T.G., Diamond, L., 1979. Induction of Differentiation in Human Promyelocytic Leukemia Cells by Tumor Promoters. Science 204, 868–870. https://doi.org/10.1126/science.286421

Rubartelli, A., Lotze, M.T., 2007. Inside, outside, upside down: damage-associated molecular-pattern molecules (DAMPs) and redox. Trends Immunol 28, 429–436. https://doi.org/10.1016/j.it.2007.08.004

Sawyers, C.L., 1999. Chronic Myeloid Leukemia. N Engl J Med 340, 1330–1340. https://doi.org/10.1056/NEJM199904293401706

Schildberger, A., Rossmanith, E., Eichhorn, T., Strassl, K., Weber, V., 2013. Monocytes, Peripheral Blood Mononuclear Cells, and THP-1 Cells Exhibit Different Cytokine Expression Patterns following Stimulation with Lipopolysaccharide. Mediators of Inflammation 2013, 1–10. https://doi.org/10.1155/2013/697972

Schwende, H., Fitzke, E., Ambs, P., Dieter, P., 1996. Differences in the state of differentiation of THP-1 cells induced by phorbol ester and 1,25-dihydroxyvitamin D $_3$. J Leukoc Biol 59, 555–561. https://doi.org/10.1002/jlb.59.4.555

Sen, P., Kemppainen, E., Orešič, M., 2018. Perspectives on Systems Modeling of Human Peripheral Blood Mononuclear Cells. Front. Mol. Biosci. 4, 96. https://doi.org/10.3389/fmolb.2017.00096

Sharif, O., Bolshakov, V.N., Raines, S., Newham, P., Perkins, N.D., 2007. Transcriptional profiling of the LPS induced NF-κB response in macrophages. BMC Immunol 8, 1. https://doi.org/10.1186/1471-2172-8-1

Six, E., Lagresle-Peyrou, C., Susini, S., De Chappedelaine, C., Sigrist, N., Sadek, H., Chouteau, M., Cagnard, N., Fontenay, M., Hermine, O., Chomienne, C., Reynier, P., Fischer, A., André-Schmutz, I., Gueguen, N., Cavazzana, M., 2015. AK2 deficiency compromises the mitochondrial energy metabolism required for differentiation of human neutrophil and lymphoid lineages. Cell Death Dis 6, e1856–e1856. https://doi.org/10.1038/cddis.2015.211

Soneson, C., Love, M.I., Robinson, M.D., 2016. Differential analyses for RNA-seq: transcript-level estimates improve gene-level inferences. F1000Res 4, 1521. https://doi.org/10.12688/f1000research.7563.2

Soneson, C., Love, M.I., Robinson, M.D., 2015. Differential analyses for RNA-seq: transcript-level estimates improve gene-level inferences. F1000Res 4, 1521. https://doi.org/10.12688/f1000research.7563.1

Srivastava, A., Malik, L., Sarkar, H., Zakeri, M., Almodaresi, F., Soneson, C., Love, M.I., Kingsford, C., Patro, R., 2020. Alignment and mapping methodology influence transcript abundance estimation. Genome Biol 21, 239. https://doi.org/10.1186/s13059-020-02151-8

Stanley, E.R., Chen, D.-M., Lin, H.-S., 1978. Induction of macrophage production and proliferation by a purified colony stimulating factor. Nature 274, 168–170. https://doi.org/10.1038/274168a0

Starr, T., Bauler, T.J., Malik-Kale, P., Steele-Mortimer, O., 2018. The phorbol 12-myristate-13-acetate differentiation protocol is critical to the interaction of THP-1 macrophages with Salmonella Typhimurium. PLoS ONE 13, e0193601. https://doi.org/10.1371/journal.pone.0193601

Stephens, T.A., Nikoopour, E., Rider, B.J., Leon-Ponte, M., Chau, T.A., Mikolajczak, S., Chaturvedi, P., Lee-Chan, E., Flavell, R.A., Haeryfar, S.M.M., Madrenas, J., Singh, B., 2008. Dendritic Cell Differentiation Induced by a Self-Peptide Derived from Apolipoprotein E. J Immunol 181, 6859–6871. https://doi.org/10.4049/jimmunol.181.10.6859

Stout, R.D., Jiang, C., Matta, B., Tietzel, I., Watkins, S.K., Suttles, J., 2005. Macrophages Sequentially Change Their Functional Phenotype in Response to Changes in Microenvironmental Influences. The Journal of Immunology 175, 342–349. https://doi.org/10.4049/jimmunol.175.1.342

Sundström, C., Nilsson, K., 1976. Establishment and characterization of a human histiocytic lymphoma cell line (U-937). Int. J. Cancer 17, 565–577. https://doi.org/10.1002/ijc.2910170504

Swiatecka, D., Mackie, A., 2015. The Impact of Food Bioactives on Health: In Vitro and Ex Vivo Models. Springer, Erscheinungsort nicht ermittelbar.

Szilvassy, S.J., Humphries, R.K., Lansdorp, P.M., Eaves, A.C., Eaves, C.J., 1990. Quantitative assay for totipotent reconstituting hematopoietic stem cells by a competitive repopulation strategy. Proceedings of the National Academy of Sciences 87, 8736–8740. https://doi.org/10.1073/pnas.87.22.8736

Tang, D., Kang, R., Coyne, C.B., Zeh, H.J., Lotze, M.T., 2012a. PAMPs and DAMPs: signal 0s that spur autophagy and immunity. Immunol Rev 249, 158–175. https://doi.org/10.1111/j.1600-065X.2012.01146.x

Tang, D., Kang, R., Coyne, C.B., Zeh, H.J., Lotze, M.T., 2012b. PAMPs and DAMPs: signal 0s that spur autophagy and immunity. Immunol Rev 249, 158–175. https://doi.org/10.1111/j.1600-065X.2012.01146.x

Taylor, J.J., Pape, K.A., Steach, H.R., Jenkins, M.K., 2015. Apoptosis and antigen affinity limit effector cell differentiation of a single naïve B cell. Science 347, 784–787. https://doi.org/10.1126/science.aaa1342

Taylor, P.R., Martinez-Pomares, L., Stacey, M., Lin, H.-H., Brown, G.D., Gordon, S., 2005. MACROPHAGE RECEPTORS AND IMMUNE RECOGNITION. Annu. Rev. Immunol. 23, 901–944.

https://doi.org/10.1146/annurev.immunol.23.021704.115816

Triantafilou, M., Triantafilou, K., 2002. Lipopolysaccharide recognition: CD14, TLRs and the LPS-activation cluster. Trends in Immunology 23, 301–304. https://doi.org/10.1016/S1471-4906(02)02233-0

Tsuchiya, S., Kobayashi, Y., Goto, Y., Okumura, H., Nakae, S., Konno, T., Tada, K., 1982. Induction of Maturation in Cultured Human Monocytic Leukemia Cells by a Phorbol Diester1. Cancer Research 42, 1530–1536.

Ueno, T., Yamamoto, Y., Kawasaki, K., 2021. Phagocytosis of microparticles increases responsiveness of macrophage-like cell lines U937 and THP-1 to bacterial lipopolysaccharide and lipopeptide. Sci Rep 11, 6782. https://doi.org/10.1038/s41598-021-86202-5

Unkeless, J.C., 1989. Function and heterogeneity of human Fc receptors for immunoglobulin G. J. Clin. Invest. 83, 355–361. https://doi.org/10.1172/JCI113891

van Staveren, W.C.G., Solís, D.Y.W., Hébrant, A., Detours, V., Dumont, J.E., Maenhaut, C., 2009. Human cancer cell lines: Experimental models for cancer cells in situ? For cancer stem cells? Biochimica et Biophysica Acta (BBA) - Reviews on Cancer 1795, 92–103. https://doi.org/10.1016/j.bbcan.2008.12.004

Wang, H., Hu, H., Zhang, Q., Yang, Yadong, Li, Y., Hu, Y., Ruan, X., Yang, Yaran, Zhang, Z., Shu, C., Yan, J., Wakeland, E.K., Li, Q., Hu, S., Fang, X., 2013. Dynamic transcriptomes of human myeloid leukemia cells. Genomics 102, 250–256. https://doi.org/10.1016/j.ygeno.2013.06.004

Xie, M., Yu, Y., Kang, R., Zhu, S., Yang, L., Zeng, L., Sun, X., Yang, M., Billiar, T.R., Wang, H., Cao, L., Jiang, J., Tang, D., 2016. PKM2-dependent glycolysis promotes NLRP3 and AIM2 inflammasome activation. Nat Commun 7, 13280. https://doi.org/10.1038/ncomms13280

Yeung, Y.G., Jubinsky, P.T., Sengupta, A., Yeung, D.C., Stanley, E.R., 1987. Purification of the colony-stimulating factor 1 receptor and demonstration of its tyrosine kinase activity. Proc. Natl. Acad. Sci. U.S.A. 84, 1268–1271. https://doi.org/10.1073/pnas.84.5.1268

Yu, G., Wang, L.-G., Han, Y., He, Q.-Y., 2012. clusterProfiler: an R Package for Comparing Biological Themes Among Gene Clusters. OMICS 16, 284–287. https://doi.org/10.1089/omi.2011.0118

Zhu, A., Srivastava, A., Ibrahim, J.G., Patro, R., Love, M.I., 2019. Nonparametric expression analysis using inferential replicate counts. Nucleic Acids Research 47, e105–e105. https://doi.org/10.1093/nar/gkz622