"Identification of a novel method for differentiating human monocytic cell line into macrophages "

Presented by

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STATEMENT OF ORIGINALITY

I certify that this thesis, and the research to which it refers, are the product of my own work conducted during the research years of the MPhil in comparing THP-1 cell differentiation using an established and novel method at London Metropolitan University. Any ideas or quotations from the work of other people, published or otherwise, or from my own previous work are fully acknowledged in accordance with the standard referencing practices of the discipline.

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have always encouraged me and only with their support could I successfully complete this grand and rewarding experience in academic research.

LIST OF FIGURES

ABBREVIATIONS

(PMA) Phorbol 12-myristate-13-acetate

(EVs) Extracellular Vesicles

(THP-1) Tohoku Hospital Pediatrics-1

(LPS) Lipopolysaccharide

(IC) Immune complex

(GC) Glucocorticoid

(SR) Scavenger receptor

(MR) Mannose receptor

(IL-1ra) IL-1 receptor antagonist

(TLR) Toll-like receptor

(MHC) Major histocompatibility complex

(PBMC) Peripheral blood mononuclear cell

(CNS) Central Nervous System

(KC) Kupffer cell

(PS) Phosphatidylserine

(TEM) Transmission electron microscope

(ALI) Acute Lung Injury

ABSTRACT

Introduction: Monocytes and macrophages are essential for optimum function of the natural immune response from recognising and killing infection, to body repair and signal transfer between cells. Macrophages within tissues are specialised macrophages and adapt their structure and function to optimise fighting foreign material and infections. Part of the immune response involves release of microvesicles (MVs) formed by the outward budding of the plasma membrane supporting intercellular communication. This research developed a method for producing macrophage-like cells under conditions that may stimulate *in vivo* using a monocytic cell (THP-1) and a human liver cell (HUH7). The basis for choosing these two cells stemmed from research by Matak *et al* confirming their cellular communication.

Aim: The aim of the research was to compare two different methods of THP-1 monocyte differentiation into M0 macrophages. An *In vitro* protocol was established using phorbol 12-myristate-13-acetate (PMA) and was compared to a conditioned medium model using THP-1 and HUH7 hepatoma cells.

Method: THP-1 cells were incubated with 100nM of PMA for 72 hours to differentiate into M0 macrophage-like cells. The novel method used THP-1 cells incubated with HUH7 conditioned medium and monitored over 120 hours to allow differentiation and confluent cell growth.

Results: THP-1 cells differentiated into M0 macrophage-like cells changing morphology from rounded to elongated cells with the characteristic macrophage phenotype. THP-1 cells with a conditioned medium produced a permanent macrophage like cell line named THP-H.

Conclusion: THP-1 cells differentiated into macrophages using two different methods; an established method of 100nM of PMA over 72 hours and with a novel method of HUH7-conditioned cell medium. However, PMA is a toxic artificial reagent used for differentiating THP-1 cells thus, produced short-lived macrophages. The novel method produced a permanent macrophage cell line, THP-H, with similar physiological conditions for replicating the study *in vivo*. Cell communication, possibly assisted by MVs, occurs between THP-1 and HUH7 cells to produce a novel permanently differentiated macrophage like cell. This novel research has therefore identified a cross-talk between THP-1 and HUH7 cells. Furthermore, these findings can be extended to research the known effects of activated macrophages in inducing hepcidin expression in HUH7 cells, by investigating the signals transferred by EVs between the two cells. Moreover, this research can provide the basis for uncovering mechanisms of communication between the THP-1 and HUH7 cells for cancer diagnosis, cell markers for cell differentiation and a new cell line named THP-H can be fully characterised.

1. INTRODUCTION

Cell differentiation and cell communication is one of the most well researched biological process *in vitro*. Research conducted for this project aims to compare a well-established and novel method of THP-1 monocyte cell differentiation into macrophages. An established method using PMA to induce cell differentiation was used as well as a novel method designed for this research consisting of THP-1 monocytes incubated with a conditioned medium. The following introduction provides a background on the reasons for choosing the THP-1 monocytic cell line and the relevant research published resulting in the initiation of a novel method for THP-1 cell differentiation.

1.1 Monocytes

Monocytes defined as myeloid cells are derived from the bone marrow. They have a major role in immune responses against various infections and injuries (Ceciliani *et al.,* 2020). Monocytes and macrophages are involved in inflammation phases in innate immune responses (Lauvau *et al.,* 2014). Inside the bone marrow the hematopoietic stem cells differentiate into precursor cells and pre-monocytic cells then produce peripheral blood monocytes which develop into resident macrophages after tissue entry (Murray *et al.,* 2011).

1.2 Macrophages

Macrophages are a type of white blood cell first discovered in 1884 by Élie Metchnikoff, a Russian zoologist. They are derived from the bone marrow and differentiate from monocytes into macrophages upon stimulation and entry in specific tissues as seen in Figure 1. Tissue resident macrophages are induced in the first cell populations to detect a tissue injury or pathogen and generate an infiltration of cytokines and chemokines at the site of injury. These macrophages also alter the immune response of invading pathogens and activate immune cells to the inflammation site (Murray *et al.,* 2011). Macrophages play a key role in innate immunity by engulfing and digesting foreign pathogens, microbes, cell debris and cancer cells as seen from their structure in Figure 2. Figure 2 also demonstrates that macrophages consist are the main phagocytic cells of the immune system. Macrophages navigate towards the site of infection and their long, thin pseudopod sticks to the bacteria and engulfs it into a vesicle. The lysosome fuses with the vesicle engulfed bacteria and releases phagocytic enzymes to destroy and digest the foreign bacteria. Any foreign invader to the body with a foreign protein surface is engulfed and digested by macrophages during phagocytosis. These phagocytes are present in all tissues and control entry of pathogens by amoeboid movement.

This project investigates monocytes differentiation into macrophages using a well-established method of PMA treatment as well as treatment with a medium conditioned by HUH7 liver hepatoma cells, which is a novel method to differentiate monocytes. Macrophages are important to study as they are key cells in the immune system and identifying a new method to produce macrophages in the human body could benefit clinical studies to further understand their role in cell communication during an immune response.

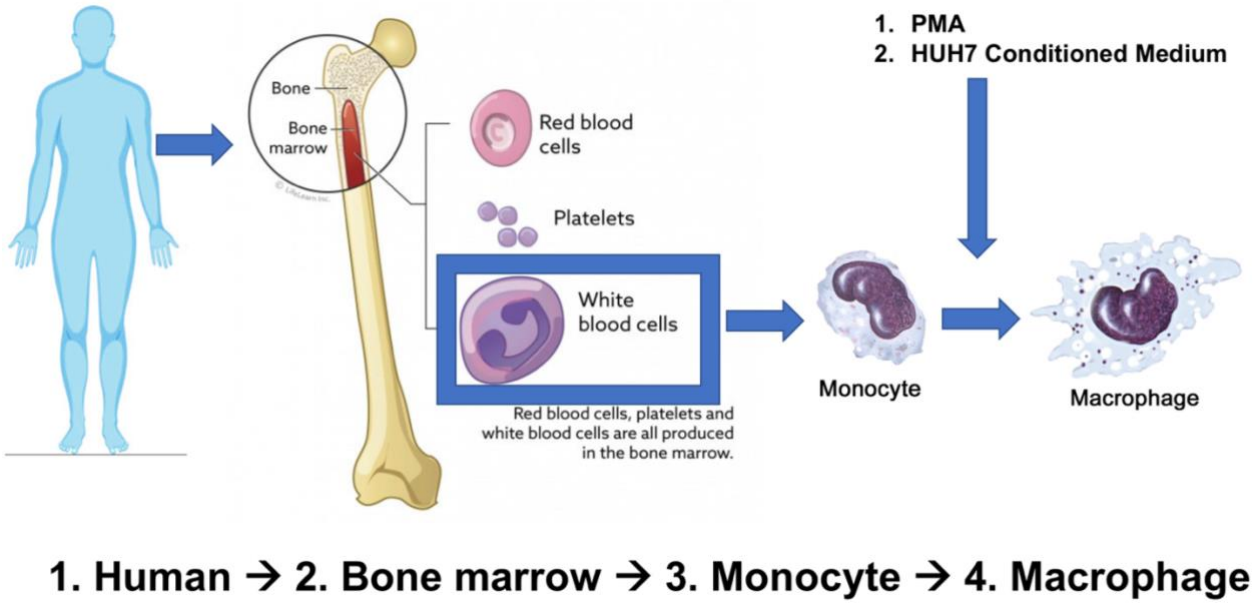

Figure 1: Macrophages originality. Macrophages are derived from the bone marrow white blood cells consisting of monocytes which differentiate into macrophages after moving from the blood stream into tissues within the body they differentiate or are initiated to differentiation with a stimuli such as PMA in a laboratory environment (Adapted from Gordon & Plüddemann, 2017)

Macrophage Structure

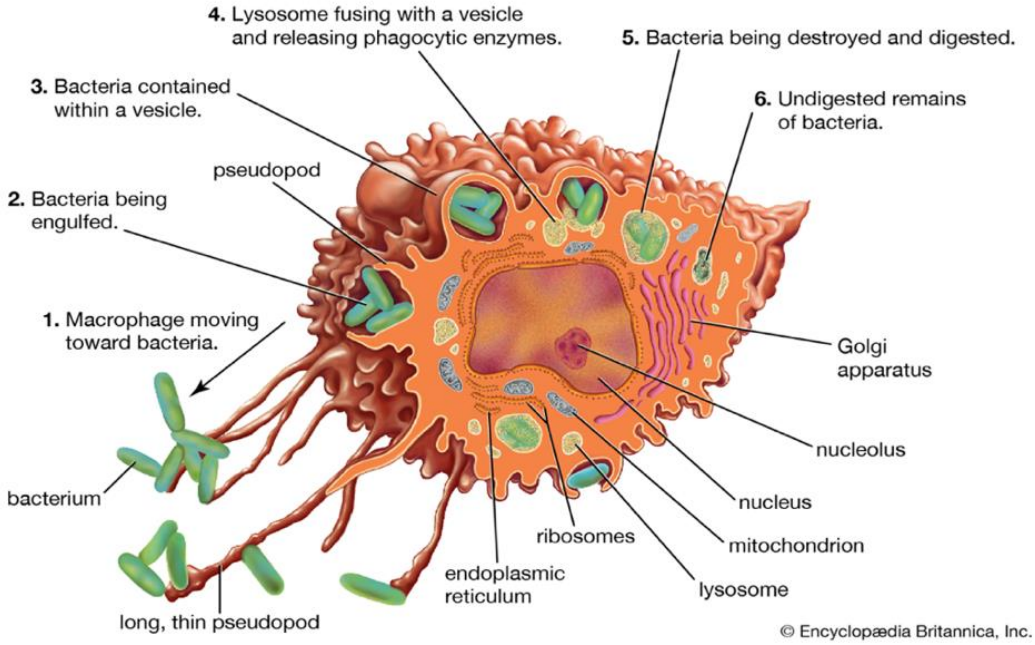

Figure 2: Macrophage structure and function. Macrophages are the principal phagocytic cells of the immune system which ingest, engulf and destroy foreign particles such as bacteria (Encyclopaedia Britannica, 2020).

1.3 Function of Macrophages

Monocytes and macrophages assist the innate immune response. They have multiple roles, with their main roles being recognition of foreign pathogens with various types of pattern recognition receptors (PRRs), production of pro-inflammatory cytokines, as well as phagocytosis to engulf and digest pathogens (Si-Tahar *et al.*, 2009). Monocytes and macrophages are therefore essential in innate immune responses from with key roles in pathogen recognition, phagocytosis, remodelling, tissue repair and signalling using cytokines. Circulating leukocytes are recruited through resident tissue macrophages sensing injury or

infection. Monocytes and macrophages respond to the micro-environmental signals. These signals assist macrophages in maturing into sub-populations which restore tissue homeostasis when promoting healing or destroying microorganisms (Yunna *et al.*, 2020).

1.4 Macrophage polarisation

Changes in the phenotype of macrophages is known by polarisation. Polarisation of macrophages creates major changes in their global transcriptome. Polarised macrophages are identified by molecules they release. In contrast to differentiation, the polarisation process is easily reversible. Macrophage polarisation occurs in reaction to certain eternal stimuli and this encourages polarisation into one of the two main types of macrophages, M1 and M2 macrophages subtypes.

Macrophages are affected by various factors, changing their phenotype and therefore function. Activated macrophages divided into two main types known as M1-like macrophages and M2-like macrophages both of which related to inflammatory responses. M1 macrophages are involved in pro-inflammatory responses while M2 macrophages with anti-inflammatory responses as seen in Figure 3. An effective path for the treatment of diseases is improving the inflammatory cellular environment by modulating the activation state of macrophages.

The microbial component lipopolysaccharide (LPS) the microbial component polarises the macrophage to the M1 phenotype while interleukin 4 (IL-4) induces macrophage polarisation to the M2 phenotype (Mantovani *et al.*, 2015). Pro-inflammatory responses and factors such as

IL-12, IL-6 and tumour necrosis factor (TNF) are produced by M1 macrophages. However, M2 macrophages have anti-inflammatory properties repairing damaged tissues (Murray *et al.*, 2014; Ivashkiv *et al.*, 2013).

1.5 M1 Macrophages

The inflammatory response of the immune system is initiated by the M1 macrophages. M1 polarisation occurs through exposure to lipopolysaccharide or interferon-γ. Th1 T-lymphocytes produce Interferon-γ. The Th1 T-lymphocytes are involved in the immune response, identifying and binding to foreign antigens releasing extracellular interferon-γ which polarises surrounding macrophages into M1 macrophages. The polarisation produces an inflammation cascade initiated by the products of M1. The products consist of pro-inflammatory molecules such as, cytokines and chemokines. The pro-inflammatory species attack the foreign particles, however lack precision and therefore damage surrounding tissues. Inflammation is controlled through polarisation of M2 macrophages and their relevant anti-inflammatory products.

1.6 M2 Macrophages

The degree of the inflammatory response is dependent on M2 macrophages. They release chemokine molecules, cytokines and cytokine mediators that act to reduce inflammation. There are four subtypes of M2 polarised cells and each propose to have a defined anti-inflammatory and tissue repair function. M2a polarisation functions in type 2 immunity and allergies, whereas M2b is involved in immunoregulation and a Th2 T-lymphocyte activator. M2c is

involved in tissue repair, matrix re-modelling and immunoregulation and M2d functions in clearance of apoptotic tissue and angiogenesis. Collectively, the resolution of the inflammatory response and tissue repair is dependent on M2 macrophages.

1.7 Macrophage Subtypes

Studies on macrophage activation states and the stimuli which induce polarisation continue to develop. However, characterisation of two main types of macrophage activation in vitro have been broadly studied; M1 or classical macrophages and M2 or alternative macrophages.

The M1 macrophages are induced by combining IFN-y and pro-inflammatory stimuli (TNF-a or LPS) containing cytotoxic and anti-microbial properties in comparison M2 macrophages which are reparative and anti-inflammatory (Yunna *et al.,* 2020). The diverse stimuli encountered by macrophages after development can change their transcriptional programs, resulting in an activation state. M2 macrophages are induced by immune complexes, parasites, fungal cells, macrophage colony stimulating factor (MCSF), apoptotic cells, tumour growth factor beta (TGF-þ), interleukin-4 (IL-4), (IL-13), (IL-10) and various other signals. Various phenotypic variations are observed in the M2 phenotype as seen in Figure 4 which give rise to the different M2 subsets named M2a, M2b and M2c. These M2 subsets are defined based on their activation stimuli. Research has not identified specific phenotypic markers to characterise macrophage subtypes however research for stimuli used to study macrophage polarisation in vitro has been well established. Therefore, studies identifying changes in groups of markers in experimental mice which may not directly translate to humans. Primary human macrophage

research has disadvantages including difficulty obtaining the primary material, variability between donors and isolation of sufficient quantities for research (Forrester *et al.*, 2018).

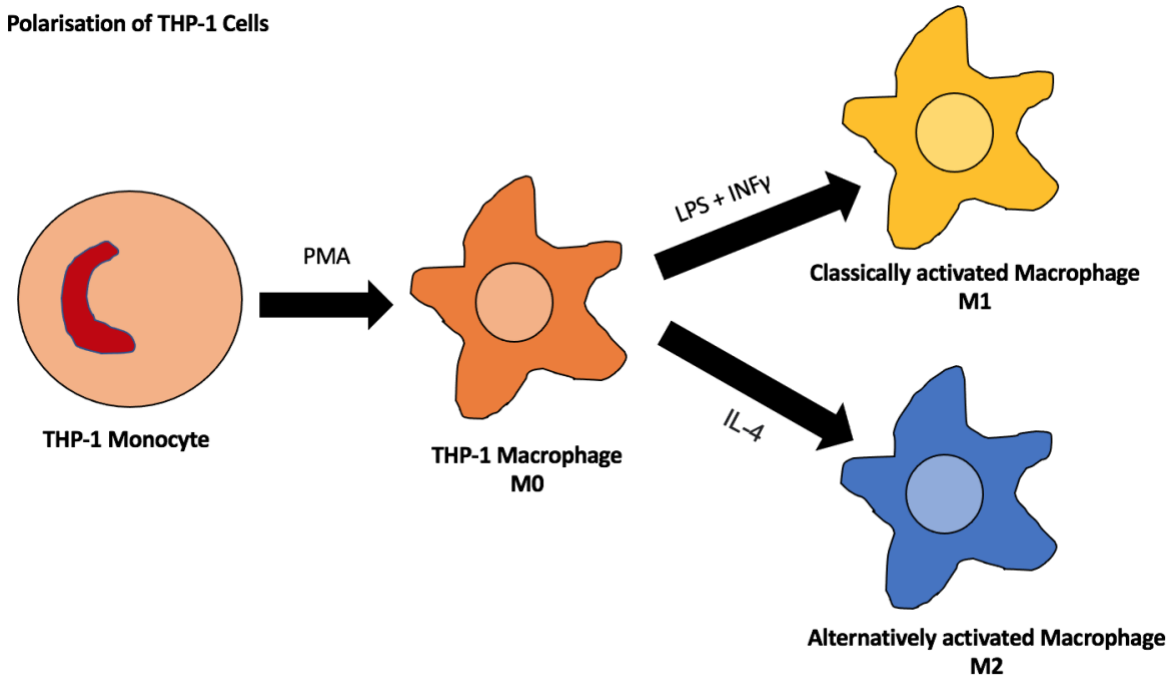

Figure 3: Polarisation of THP-1 Cells. Differentiation of THP-1 monocytes into THP-1 macrophages M0-like cells after treatment with PMA. Addition of LPS and IFN-γ initiating classically activated macrophages M1-like cells (anti-inflammatory). Addition of IL-4 to M0 macrophages stimulates alternatively activated macrophages M2 cells (anti-inflammatory) (Adapted from Chanput *et al.*, 2013).

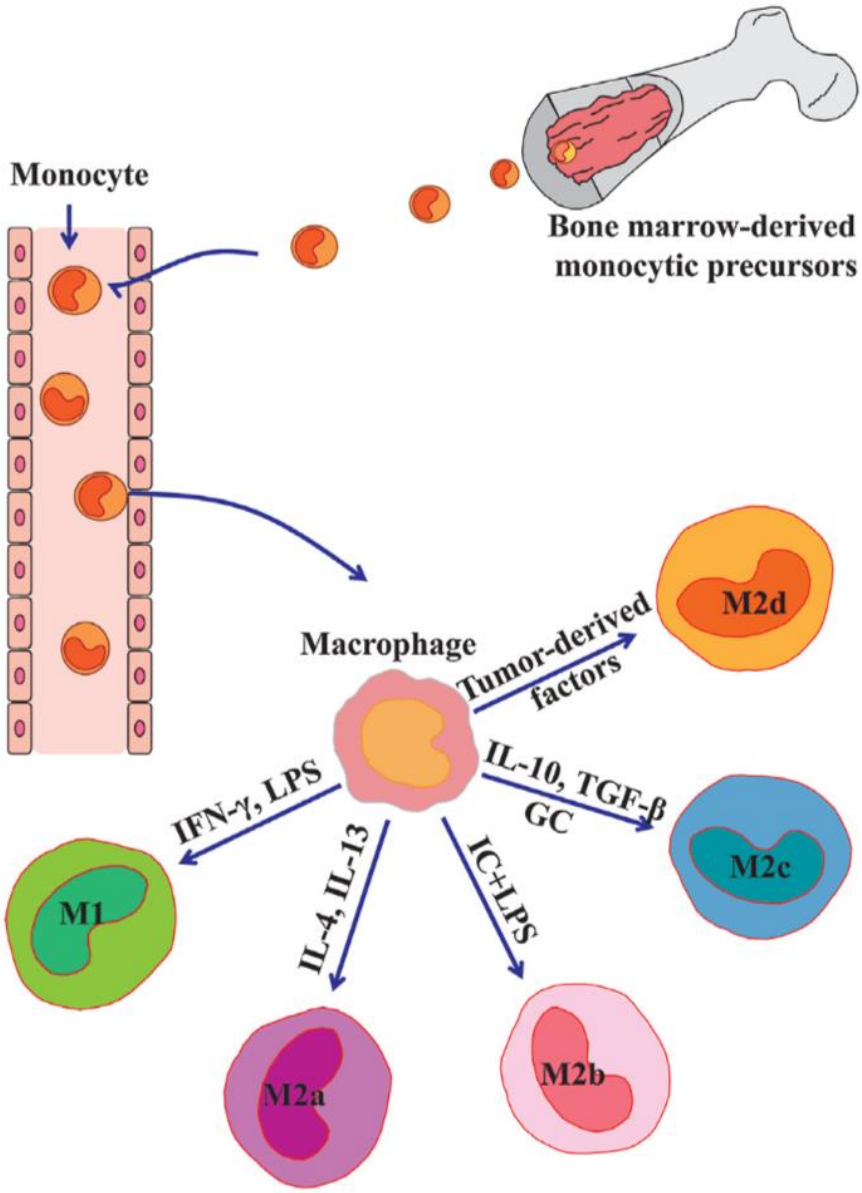

Figure 4: Macrophage Polarisation and Characterisation. Circulating monocytes develop into tissue macrophages that acquire a classical M1 or alternative M2 phenotype depending on external microenvironmental stimuli. The M1 phenotype is stimulated by IFN-γ and LPS that produce high levels of pro-inflammatory cytokines such as TNF-α, IL-6, IL-12 and IL-23. The M2 phenotype is subdivided into M2a, M2b, M2c and M2d depending on the stimuli. Alternative M2 macrophages produce high levels of IL-10 and demonstrated with increased levels of mannose receptor, scavenger receptor, IL-1 receptor antagonist and IL-1 decoy receptor. M1

phenotype assists pro-inflammatory and antitumour responses. M2 phenotype drives angiogenesis, tumour progression and immunosuppression (Chanmee *et al.,* 2014).

1.8 Tissue resident macrophages

Tissue resident macrophages are a heterogenous population of immune cells. They vary according to their activation state, location and homeostasis maintaining functions (Davies *et al.,* 2013). This ranges from homeostatic functions of cell debris clearance, iron processing, infection response, inflammation resolution and the major roles in tissue immune surveillance (Davies *et al.,* 2013). Changes in the cellular environment of macrophages in various tissues leads to their polarisation to a specialised tissue macrophage (Martinez at al., 2014).

In the lungs, alveolar macrophages enhance cellular defence from inhaled antigens cleaning the air space of toxins, infections and allergic particles that have invaded the mechanical defence of the respiratory airways (Chitko-McKown *et al.,* 1992; Arora *et al.,* 2018). Macrophages within the brain known as microglial cells protect the central nervous system (CNS). Microglia cells are vital for synaptic connections during brain development (Herz *et al.,* 2017). In a healthy state, microglia facilitate memory, learning and manage cellular and debris. Macrophages in the lymph nodes identified as soinal or subcapsular sinusoidal function to engulf antigens and present them to B cells (Wynn *et al.,* 2013). The macrophages resident in intestines constitute one of the largest pool of macrophages within the body. They act as sentinels for foreign pathogen recognition and elimination to maintain intestinal homeostasis, regulating gut inflammation and promoting T cell crosstalk (Joeris *et al.,* 2017; Wang *et al.,* 2019). A large tissue containing macrophage population identified as the splenic red pulp

performs various diverse functions, with their main role being engulfing altered and senescent erythrocytes, recovering iron to bone marrow and breaking down haemoglobin, thus contributing to iron homeostasis (Ganz, 2012). Finally, in the liver macrophages as seen in Figure 5 named Kupffer cells (KCs) are a large pool of tissue resident macrophages and function to remove pathogens and toxins (Zannetti *et al.,* 2016).

1.9 Kupffer Cells

Liver macrophages known as Kupffer cells (KCs) seen in Figure 5 have an essential role in the liver for antimicrobial defence. KCs are an effective phagocyte that recognise, ingest, and degrade cellular debris and clear foreign material. KCs originate in the bone marrow as monocytes entering the circulation and move towards the liver where they differentiate into fixed tissue liver macrophages known as KCs. The cells were first discovered by Karl Wilhelm von Kupffer in 1876 and the scientist named them “Sternzellen” (hepatic stellate or star cells) but identified as an integral part of the endothelium of liver blood vessels which they originated from.

KCs are located intravascularly along the hepatic sinusoidal endothelial cells. This optimal location enables their vital role in ensuring liver homeostasis by constantly clearing blood-borne pathogens, associated toxins such as LPS and cellular debris. KCs are equipped with high expression of Fc (CD64), scavenger (CD163), complement, or pattern recognition (TLR4, TLR9) receptors so they exert antimicrobial responses (Triantafyllou et al., 2018).

KCs are specialised liver macrophages which represent the largest population of resident tissue macrophages while present in the lumen of hepatic sinusoids they are identified by expression of CD68. KCs are important in host defence, innate immunity and a major mediator for liver injury and repair. Therefore, they are important in pathogenesis of liver diseases.

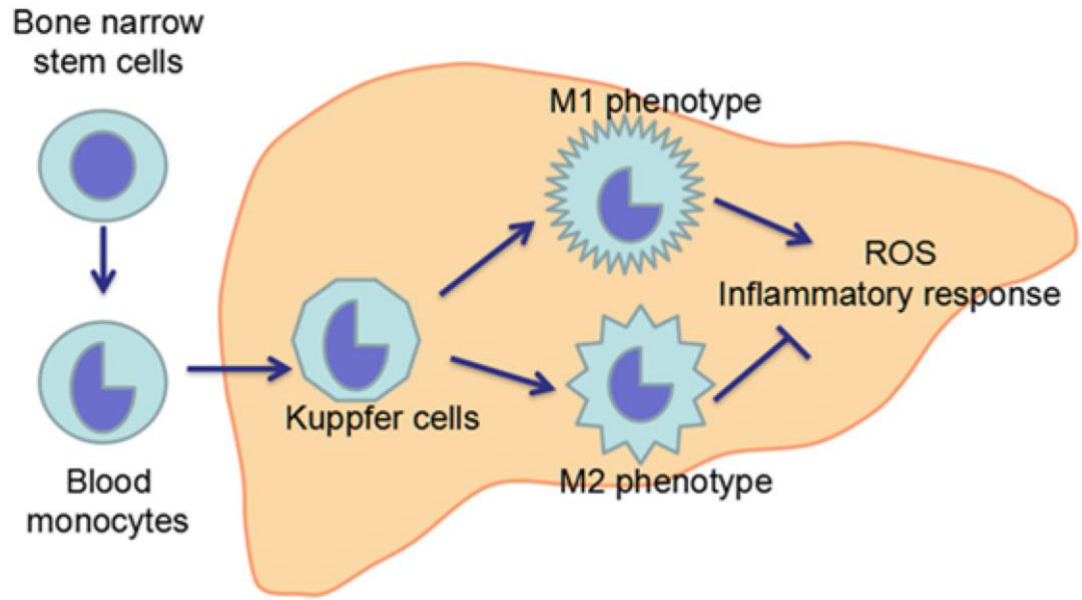

Figure 5: Maturation and polarisation of Kupffer cells. KCs tissue macrophages in the liver originate from the bone marrow giving rise to blood monocytes which migrate to the liver and develop into liver macrophages named KCs. KCs in the liver polarise into two different pathways classical M1 polarisation and alternative M2 polarisation which produce pro-inflammatory and anti-inflammatory effects. Therefore, the imbalance of M1 and M2 polarisation of KCs can progress the pathogenesis of liver diseases (Zeng *et al.*, 2016).

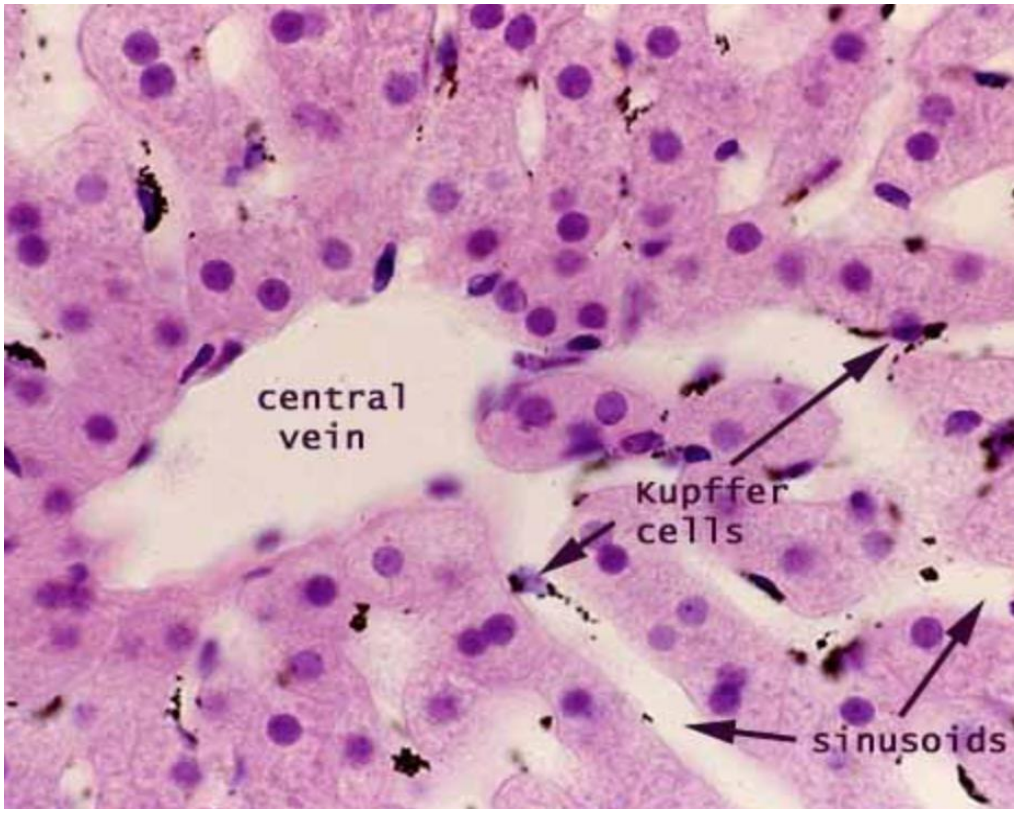

Figure 6: Liver macrophages (KCs) from an animal specimen. Macrophages injected with carbon particles give rise to dark brown appearance. They are derived from the blood monocytes which differentiate into macrophages as they move to the connective tissue. They are scavenger cells for phagocytosis activity in the immune system. KCs can be identified with their oval nuclei associated closely with the sinusoidal spaces (King, 2018).

1.10 Structure of Kupffer cells

The structure of KCs as shown by the image in Figure 6 has an irregular stellate shape with their luminal surface. KCs thicken into the sinusoidal lumen and their body rests on the endothelial lining forming junctional complexes with the endothelial cells. KCs are considered fixed tissue macrophages and are capable of active migration along the sinusoids with and against blood flow such as in areas of liver injury and lymph nodes. These cells contain many phagosomes and lysosomes with the cisternae of their endoplasmic reticulum rich in peroxidase (Zeng *et al.,* 2016).

1.11 Function of Kupffer cells

The KCs cells will proliferate in response to T-helper type 2 inflammatory signals. KCs are present in fetal liver of mice before they are circulating monocytes and research suggest they are derived from prime macrophages first appearing in the yolk sac. This suggests that KCs have a potential dual origin (Sahota *et al.,* 2018). The primary function of these cells is the removal of ingested particles and soluble materials from the blood which are distinguished between particles of 'self' and 'non-self'. They are scavengers for microorganisms, degenerating normal cells, macromolecules and circulating tumour cells. These are non-specific functions, however, KCs also initiate immunological responses. This clearance function is efficient as removal of particulate material is reduced by the hepatic blood flow. These cells have a significant role in clearing gut-derived endotoxins from portal blood resulting without inducing a local inflammatory response (Zeng *et al.,* 2016).

There is a link between release of inflammatory and pro-inflammatory mediators such as tumour necrosis factor (TNFα), interferon- γ (IFN-γ), IL-1, IL-6 and IL-10 mediator supressing macrophage activation and inhibition of cytokine secretion. These chemokines, reactive oxygen species and cytokines released by KCs assist in modulating the local and microvascular effects and functions of stellate cells and hepatocytes. Stellate cells located within the space of Disse (Figure 7) where proteins and plasma components from the sinusoids are absorbed by the hepatocytes, respond to hepatic injury and in healthy liver cells and are the main storage site for vitamin A (Zannetti *et al.*, 2016).

Class II histocompatibility antigens are expressed by KCs and function *in vitro* as an antigen-presenting cell, they are however, are less efficient than macrophages at different sites. In summary, the main role of KCs in the immune system is antigen sequestration using phagocytosis and clearance of immune complexes. These cells have plasticity enabling expression of numerous phenotypes dependent on the local metabolic and immune microenvironment. KCs therefore express a pro-inflammatory M1-like phenotype or different M2 phenotypes involved in resolution of wound healing and inflammation. KCs also play an important protective function with their tolerogenic phenotype as seen in drug and toxin-induced liver injuries while also moving towards a pathologically activated state and contributing to liver diseases with chronic inflammation. Damage in KC activity as scavengers of hepatic blood will introduce pathogens into the systemic circulation and therefore impair activity with systemic infection. Research from liver transplants and bone marrow confirms KCs are derived partly from circulating monocytes. However, these scavenger cells replicate

rapidly and the local proliferation is vital in their expansion after response to liver injury (Zannetti *et al.*, 2016; Zeng *et al.*, 2016).

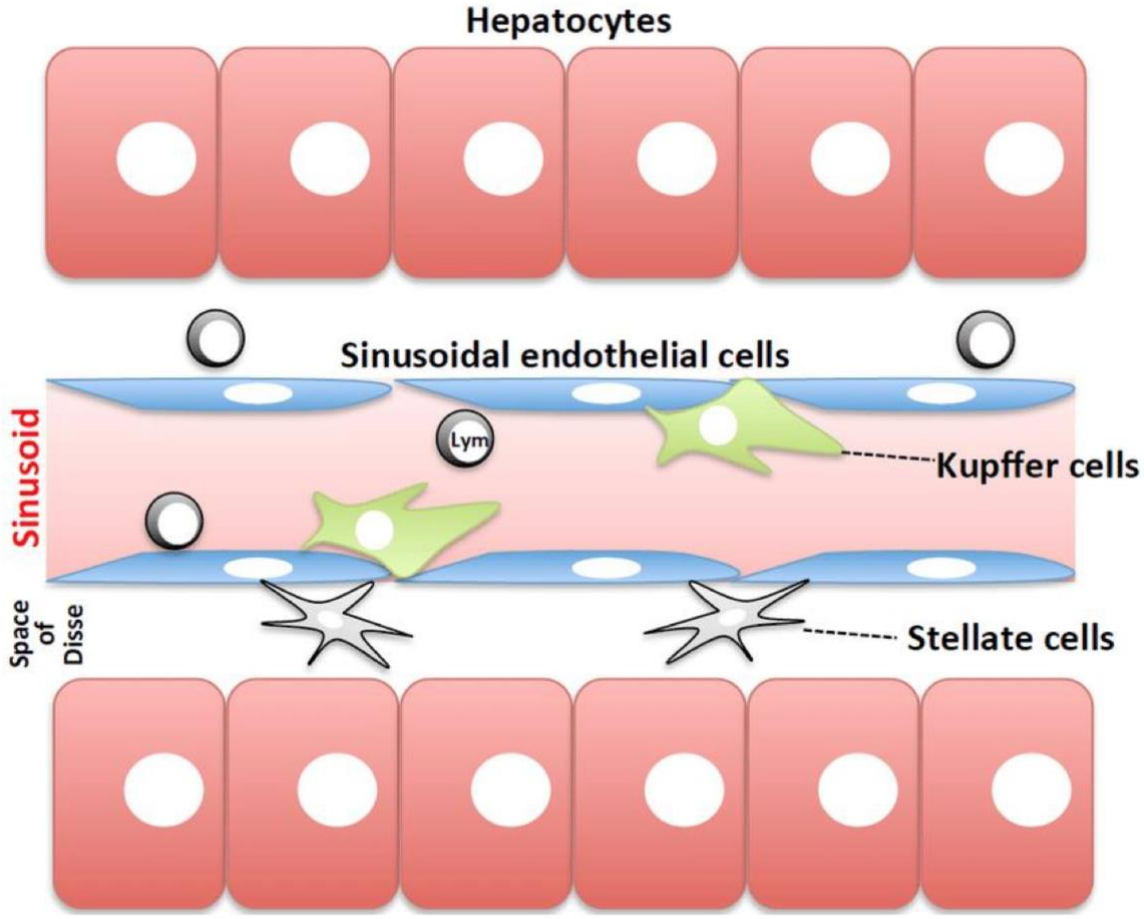

Figure 7: Stellate Cells in the space of Disse. The stellate cells respond to injury and heal liver damage. However, repeated injury and insult to the cells results in long lasting fibrosis, impairing liver function (Ullah Wazir, 2020)

1.12 Hepcidin Iron Regulatory Peptide

The expansion in knowledge of the role of macrophages in homeostasis, development and host defence creates challenging questions regarding how macrophages adapt to the surrounding microenvironment for their various functions. The concept of macrophage activation is key in

explaining and understanding this process. Recent methodological advances have provided opportunities to understand how macrophages integrate and process varying stimuli they are exposed to within the tissues. A thorough understanding of the monocyte and macrophage biology will provide important future therapeutic leads (Rees, 2010).

Therapeutic options include the relevance of hepcidin with THP-1 and HUH7 cells assist in regulating iron homeostasis. Hepcidin is an iron regulatory peptide that blocks the iron ferroportin channels by binding to the gut enterocytes and macrophages thereby inhibiting iron transport into the systemic circulation. In addition, iron is stored in the cells and prevented from being exported. Increased hepcidin results in iron deficiency anaemia, and decreased levels of hepcidin causes hereditary haemochromatosis (HH). Hepcidin is regulated by molecules including hemojuvelin (HJV), HFE (product of high iron gene) and transferrin receptor 2 (TfR2). Two point mutations within the HFE gene lead to HH (Collins *et al.*, 2008).

Hepcidin is predominantly produced by hepatocytes however other cells express hepcidin and facilitate the regulation of hepcidin levels produced by hepatocytes. Macrophages are hypothesised to be key regulators of hepcidin production by the hepatocytes. Macrophages provide large quantities of iron that have been taken from senescent red blood cells. Macrophages collect iron from dying red blood cells which have large amounts of iron stores as well as from the innate and adaptive immune systems. Macrophages in adaptive immunity are attracted and activated by TNF-a. In innate immunity, resident macrophages react to resident cytokines inducing macrophages to play a role in innate immunity (Matak *et al.*, 2009). Studies have shown that activated macrophages induce hepcidin expression in HUH7 hepatoma cells in a co-culture model of THP-1 and HUH7 cells (Matak *et al.*, 2007). Studies have also shown hepatocytes responding to iron changes in isolation, however, macrophages are required for inflammatory regulation of hepcidin.

1.13 THP1 cells used as a model monocytic cell line

THP1 cells are a human leukaemia monocytic cell line which have been widely used to research monocyte and macrophage functions, mechanisms, nutrient, drug transport and signalling pathways (Chanput *et al.,* 2014). There are various methods for differentiating THP-1 cells into macrophages including incubation with PMA and RPMI 1640 cell culture medium. Lipopolysaccharides (LPS) and various other stimuli also differentiate THP-1 cells but this is a short-lived method to produce macrophages. HUH7 cells are a well differentiated hepatoma cell line and were identified as an optimal cell line to investigate the effects of cell differentiation and cell communication with THP-1 cells because a crosstalk exists between hepatocytes and macrophages (Matak *et al.,* 2009). The current study reports a novel method to produce a new permanent macrophage cell line from THP-1 cells which can be grown, characterised and observed for its functions, mechanisms and therapeutic functions (Figure 8).

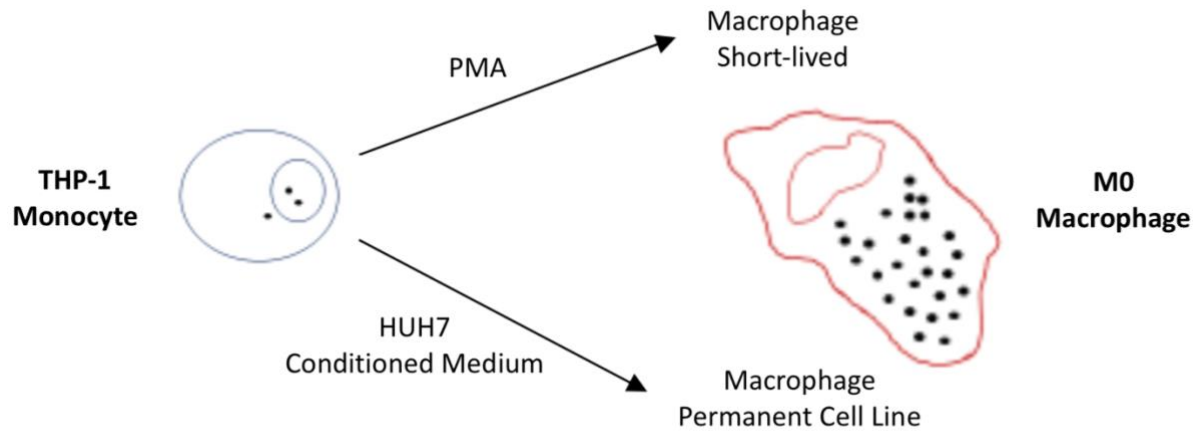

Figure 8: THP-1 monocyte cell differentiation into M0 macrophages using PMA and HUH7 conditioned cell medium. THP-1 cells were differentiated using 100nM of PMA after 3 days into macrophages and this research developed a new method of THP-1 monocyte differentiation into a permanent macrophage cell line following a 24h incubation with HUH7 conditioned medium. This new cell line has similar morphology to PMA-induced differentiated THP-1 cells. The two methodologies were compared, with one being an established method (PMA) and the conditioned medium being novel for this research and THP-1 cell differentiation (Adapted from Matak et al., 2009).

1.14 Characteristics and Versatility of THP-1 cells

Monocytic Leukaemia cell lines, THP-1, U937, HL-60, Mono Mac 6 and ML-2 cells have been studied in various biomedical research settings. The THP-1 cell line was first isolated in 1980 from the peripheral blood of a 1 year old male patient suffering from acute monocytic leukaemia (Tsuchiya *et al.,* 1980). These cells have been researched in immune responses in the monocytic and macrophage states (Daigneault *et al.,* 2010). THP-1 cells resemble primary monocytes and macrophages in differentiation and morphology properties. THP-1 cells present

a large, round single-celled morphology, expressing distinct monocytic markers. Most THP-1 cells adhere to cell culture plates and differentiate into macrophages when exposed to PMA. THP-1 cells have technical advantages over human primary monocytes and macrophages such as their genetic background, which is homogeneous, thus minimising the level of variability in cell phenotype. Also, the genetic modification of THP-1 cells using small interfering RNAs (siRNA) to down-regulate the expression of specific proteins is easily achieved (Chanput *et al.*, 2014). Moreover, monocyte-derived macrophages can polarise into M1, M2a, M2b and M2c cells as seen in Figure 4 (Chanmee *et al.*, 2014). These traits of THP-1 cells collectively suggest that THP-1 cells are a versatile cell line for this study.

The main difference between THP-1 and U937 cells is the maturation stage and their origin. U937 cells originate from tissues and are therefore they are at a mature cellular stage in comparison to THP-1 cells, which originate from blood leukaemia. U937 monocytes exposed to PMA for 72 hours (García *et al.*, 1999) or vitamin D3 for 24 hours (Liu *et al.*, 2005) induced CD14 expression, which is a marker of macrophage expression. Research studies using THP-1 cells as an *in vitro* model are more abundant compared to ML-2 cell line (Qin, 2012). THP-1 cells have been chosen for this project as studies have identified THP-1 cells as a suitable model for primary monocytes and macrophages due to the resemblance in their function, morphology and differentiation markers (Sakamoto *et al.*, 2001). The THP-1 cell line provides a valuable model in studying cell differentiation methods as they provide the closest physiological conditions to primary cells (Auwerx,1991). THP-1 cells are versatile as they are at a less mature stage and therefore growth in cell culture *in vitro* is easily managed. Therefore, THP-1 cells have the desired characteristics for this research to compare THP-1 cell differentiation. (Theus *et al.*, 2004). In addition, PMA-induced macrophage cells mimic native monocyte-derived macrophages in several ways.

Studying the behaviour of macrophages both in normal and pathological conditions has demonstrated versatility of these cells above their well-known immune effector cell functions. Although macrophages were first discovered as active removers of invading foreign pathogens with their phagocytic and antigen presenting activities, these cells are also engaged in regularly clearing aged cells in non-pathologic conditions. Furthermore, macrophages express an array of bioactive molecules promoting tissue remodelling and healing, angiogenesis and support cell proliferation as well as mediating immunosuppression under specific microenvironmental conditions (Stout *et al.,* 2005; Gordon *et al.,* 2014; Pavón *et al.,* 2017).

1.15 Advantages and Disadvantages of THP-1 Cells

THP-1 cells were chosen as a model promyeloid cell as they are a sensitive cell line to external factors and varied culture conditions. THP-1 cells culture conditions alter the monocytic cell line morphology and therefore alter their response to differentiation stimuli (Aldo *et al.,* 2013*).* Confluent THP-1 cells differentiated into a heterogenous population after PMA treatment as confirmed with increased CD14 increased expression. Low confluence caused the THP-1 cells to remain as a homogenous population without an increase in CD14 expression. Moreover, the differences in culturing techniques may therefore account for the discrepancies in the literature for basal THP-1 cell phenotype and their response to PMA stimuli (Aldo *et al.,* 2013). THP-1 cells have many advantages in comparison to PBMC-derived monocytes including their growth rate being quadruple in three and a half days, absence of infectious viruses or toxic by-products and their ease and safety of use. The THP-1 cells are an immortalised cell line and *in vitro* culturing of these cells can be passaged 25 times without changes in activity or cell sensitivity.

Also, the homogeneous genetic background of THP-1 cells reduces their phenotypic variability, thus ensuring reproducible findings. However, the disadvantage is that these effects may be genotype-dependent. Another disadvantage of using THP-1 cells is their malignant background and the limitations of growing the cells under controlled conditions rather than their natural environment which may result in sensitives and responses.

PBMC-derived monocytes have a higher risk of contamination with other blood components such as platelets (Chanput *et al.,* 2014). Commercially THP-1 engineered cells and THP-1 inflammasome test-cells engineered by InvivoGen have facilitated answering specific research questions. Analysis of recent findings in the literature concludes that THP-1 cells have unique characteristics and are a suitable model for investigating and estimating the immune modulating effects of compounds under activated and resting cell conditions (Chanput *et al.,* 2014).

1.16 Differentiation of THP-1 macrophages

THP-1 cells differentiate into macrophage-like cells with PMA exposure, macrophage colony-stimulating factor (M-CSF) or 1α, 25-dihydroxyvitamin D3 (vD3). M-CSF assist in differentiating monocytes into M0 type macrophages (Cao *et al.,* 2019). M-CSF is a secreted cytokine which causes differentiation of hematopoietic stem cells into cell types such as macrophages. Therefore, M-CSF are involved in differentiation, proliferation and survival of monocytes into macrophages.

Different macrophage intermediate phenotypes can develop from THP-1 cells following 2 days of PMA treatment when adhering to cell culture plates therefore differentiating their morphology into elongated amoeboid flat cells with a developed rough endoplasmic reticulum, golgi apparatus and ribosomes within the cytoplasm (Tsuchiya *et al.,* 1982) Daigneault and colleagues (Daigneault *et al.,* 2010) data suggested THP-1 cell differentiation with 200nM of PMA for 3 days followed by 5 days rest in culture medium without PMA which increased macrophage marker expression. The cytoplasmic to nuclear ratio, lysosomal and mitochondrial numbers and differentiation-dependent cell surface markers were included as they changed in a pattern similar to PBMC monocyte-derived macrophages. Collectively, the literature summarises PMA as the most efficient and effective differentiation stimuli for producing THP-1 monocyte-derived macrophages with close similarities to PBMC monocyte-derived macrophages (Mullen *et al.*, 2010; Bastiaan-Net *et al.*, 2013; Chanput *et al.*, 2013).

However, high concentrations of PMA can produce undesirable responses such as an increased rate of apoptosis. Resting differentiated THP-1 cells at least 24 hours in culture medium without PMA increases macrophage markers and decreases NF-κB gene clusters which have upregulated in differentiation. High phagocytic activity, cell adherence, expression of differentiation-dependent cell markers such as CD-14, TLR-2, CD-36, CD11b and CD18 can be analysed to confirm differentiation of THP-1 monocytes to macrophages (Chanput *et al.,* 2014)

1.17 THP-1 research "Daisy Cells"

Another approach for differentiating THP-1 cells can be seen in a study conducted by Sadofsky and colleagues (Sadofsky *et al.*, 2019). The experiment by Sadofsky and colleagues used THP-1 cells to compare the presence and absence of mitogenic stimulation to the newly formed macrophage-like cells named 'Daisy Cells' (Sadofsky *et al.*, 2019). The study used flow cytometry, microscopy, gene microarrays, antigen binding and phagocytosis assays to investigate their aims. Their data established the 'Daisy' cell line which grows mainly in an adherent monolayer. Various antibodies were selected to investigate the THP-1 cell surface phenotypic markers using flow cytometry. The Daisy cells highly expressed CD11c, CD163, CD80, CD169 and CD206 but expressed less CD11b and CD14 in comparison to mitogenic stimulated THP-1 cells. Daisy cells confirmed high levels of immune complex binding whereas stimulated THP-1 cells were barely bound to immune complexes.

The Daisy cell line is suggested to be similar to mature macrophages due to the heightened phagocytic ability of Daisy cells in comparison to mitogen-stimulated THP-1 cells. The authors of the research conducted the experiment as there is currently no true macrophage cell line while *in vitro* experiments required for these cells require mitogenic stimulation of a macrophage precursor THP-1 cell line. The study by Sadofsky and colleagues (Sadofsky et al., 2019) therefore characterised a human macrophage cell line from THP-1 cells comparable to the phenotype of the THP-1 cells.

The analysed phenotype confirms the Daisy cell line is a model for human macrophage cells since it is phenotypically similar to the human alveolar macrophages (Sadofsky et al., 2019). Alveolar macrophage markers CD163, CD11c, CD36 and CD206 and a lack of CX3CR1 and

CD11b are all compatible with the alveolar macrophage phenotype. Macrophages have a fundamental role in inflammation throughout the body as the predominant phagocytes of the innate immune complex. Following activation, the macrophages elicit specific immune responses with their primary function led by the stimuli and environment which macrophages reside.

In vitro studies use human macrophages requiring mitogenic stimulation (e.g. PMA) of macrophage precursor cells (e.g.THP-1). Stimulation with PMA activates protein kinase C causing phosphorylation of downstream signalling molecules. The signalling activation regulates gene expression which is incomparable to human-monocyte derived macrophages. THP-1 cells are from a human monocytic cell line and were initially isolated by Tsuchiya and colleagues (Tsuchiya *et al*., 1980). THP-1 cells grow in suspension and are similar to human monocytes in morphology, membrane antigen expression and secretory products. Studies (Forrester *et al*., 2018) have characterised the cell surface markers of THP-1 monocytes then compared the results to PMA-differentiated THP-1 macrophage cells and observed an increase in macrophage markers such as CD14.

The Daisy cells finding derived a unique sub-clone of THP-1 cells which spontaneously change and differentiate into alveolar macrophage-like cells without PMA addition. The Daisy cell line was derived spontaneously originally from a THP-1 clone which was cultured and produced abnormal cells characterised by a high percentage of adherent cells. This was not typical of THP-1 cells which typically grow in suspension. Therefore, the characterisation of the Daisy cells was conducted to investigate their key phenotypic and transcriptional features

related to mature macrophages, PMA-induced differentiated THP-1 cells and native THP-1 cells (Sadofsky *et al.*, 2019).

1.18 Cell communication and Extracellular vesicles

Eukaryotic cells in multicellular organisms need to communicate with each other in order to maintain tissue homeostasis and to respond to pathogens in the extracellular milieu. Generally, cells exchange information through direct cell–cell contact or by secretion of soluble factors. Mechanisms of intercellular interaction involve the production and release of extracellular vesicles (EVs). Cells interact and influence the extracellular environment and other cells in various ways such as release of different types of EVs, which have various functions depending on their origin and molecular composition. EVs consist of a variety of nanoscale membranous vesicles that are released by many cell types into the extracellular environment which can potentially reach all areas of the body. EVs transfer molecules such as nucleic acids, proteins, and lipids to specific target cells and can be classified according to their size, biogenesis, functions, and composition. There are three main types of EVs: (1) microvesicles (100–1000 nm in diameter); (2) apoptotic blebs (1000–5000 nm in diameter); and (3) exosomes (diameter 20–150 nm). The former two represent heterogeneous populations of vesicles generated by outward budding of the plasma membrane. Exosomes are formed by invagination of endosomal membranes and subsequent production of multivesicular bodies (MVBs). Formally in the literature, the terms exosomes and EVs are used inaccurately as a standardised uniform method for their isolation–characterisation (Caruso *et al.*, 2019).

Recent studies have suggested EVs delivering a variety of molecules including proteins, nucleic acids and lipids to targeted cells to exert pleiotropic effects. Exosomes derived from macrophages have heterogeneity in various cancers and have paradoxical functions in promoting and supressing tumours mainly through post-transcriptional control and regulation of protein phosphorylation in recipient cells. However, EVs secreted from varying phenotypes of macrophages provides leads for therapeutic options (Liu *et al.*, 2020).

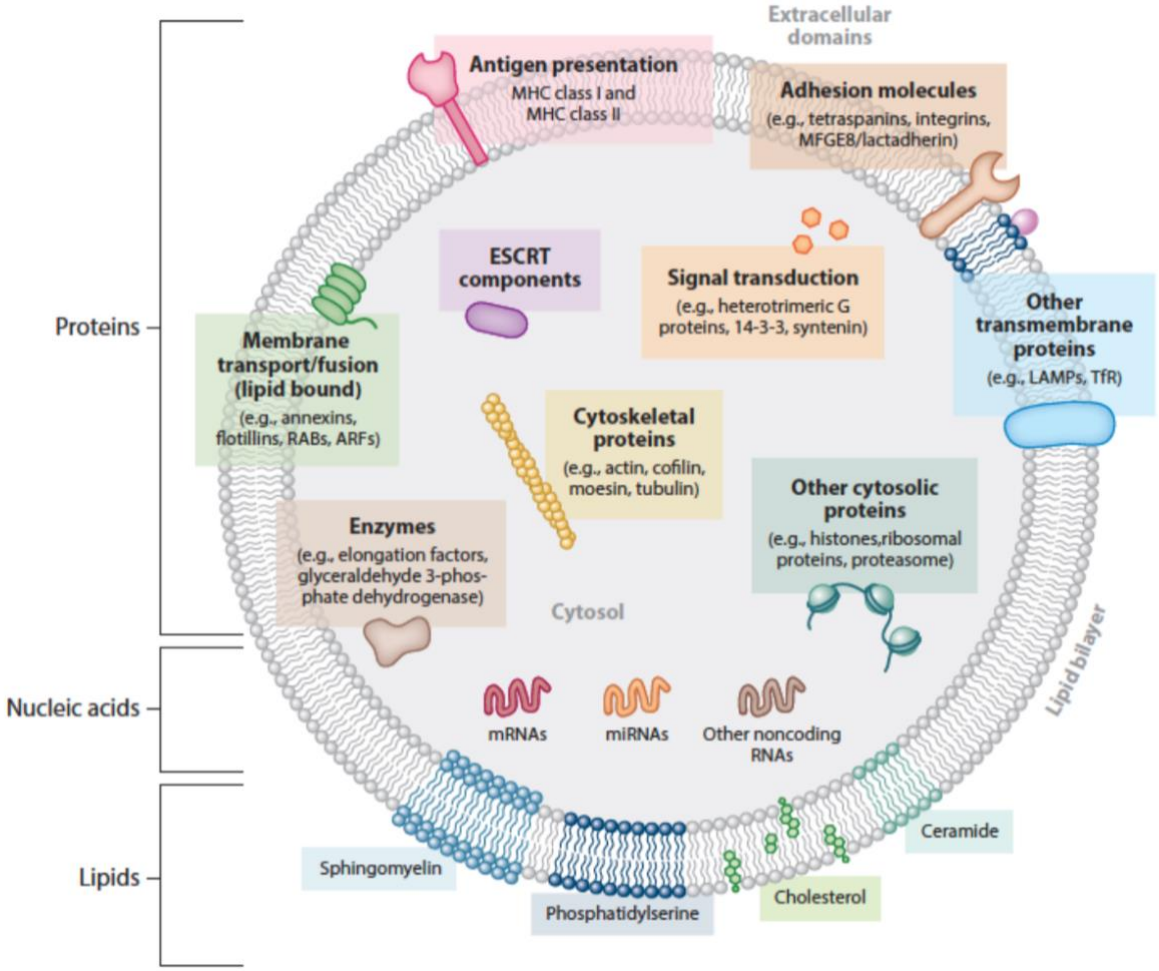

Figure 9: The Overall Composition of Extracellular Vesicles. The composition of lipids, nucleic acids and proteins and the membrane orientation of EVs. Tetraspanins commonly found in EVs include CD81, CD9 and CD63. Listed components may be present in only some subtypes of EVs. Examples include histones, proteasome and ribosome components are secreted in large plasma membrane derived EVs or apoptotic vesicles rather than exosomes. Abbreviations: ESCRT, endosomal sorting complex rewired for transport, LAMP, lysosome-associated membrane protein, MHC, major histocompatibility complex, MFGE8, milk fat globule-epidermal growth factor-factor VIII, ARF, ADP ribosylation factor, RAB, Ras-related proteins in brain and the TfR, transferrin receptor (Colombo *et al.*, 2014).

1.19 MV Production and role in human biology

Microvesicles (MVs) derived from cells are membrane vesicles produced by the outward buddling of the plasma membrane and released in most cell types. The formation of the MVs is dependent on the translocation of the phospholipid phosphatidylserine from the inner to the outer leaflet of the plasma membrane, which is carried by aminophospholipid translocases. Once sufficient phosphatidylserines have been translocated, the shedding of MVs is completed by contraction of the cytoskeletal components initiated by ADP-ribosylation factor 6 (ARF6) (Gudbergsson & Duroux, 2017). MVs carry active molecules including protein, lipids and nucleic acids assisting in mechanisms of intercellular communication. Moreover, they are present in blood, urine and various circulating fluids and are related to the progression of pathophysiological conditions in various diseases. MVs have been revealed in recent studies to have a dual effect of damage and protection of receptor cells, however the mechanism of the active molecules involved in this process remains unclear (Lv *et al.*, 2019). MVs comprise of a varied and heterogenous group of particles released from various cells originating mainly from the plasma membrane. Recent studies have shown MVs contain various proteins, lipids glycoproteins, glycolipids and nucleic acids including DNA, mRNA and noncoding RNAs. Therefore, MVs have the potential for delivering information to surrounding tissues throughout the body. The prevalence of MVs in disease has made them a focus for the development of diagnostics and as a non-invasive tool for detecting pathology in diseases such as cancer, cardiovascular diseases and diabetes. As MVs have also been detected in multiple bodily fluids they are readily available for analysis (Sedgwick and D'Souza-Schorey, 2018).

1.20 Microvesicles impact on monocyte and macrophage function

Monocytes and macrophages assist in regulating angiogenesis via cytokine production and interaction with endothelial cells. Biological effects of macrophage-derived MVs are studied using THP-1 cell line. MVs produced by THP-1 cells express CD18, CD11b, CD54, CD11a, CD29, CD120a, CD120b, VEGFR1, VEGFR2, CD105, CD119 and TDFR2 on the surface (Sokolov *et al.,* 2021). Transfer of an intracellular protein labelled with a fluorescent dye from MVs produced by THP-1 cells has been established in research experiments. The results suggested MVs derived from THP-1 cells inhibit endothelial cell proliferation (Bingle *et al.,* 2006). MVs in high concentrations reduced endothelial cell migration, promote the development of non-branching angiogenesis and decrease the number of vessels formed by endothelial cells. However, in low concentrations, MVs increase endothelial cell migration and increase the number of vessels formed by endothelial cells promoting the development of branching angiogenesis. Therefore, the influence of MVs produced by THP-1 cells on the process of angiogenesis has been established fully. Thus, proteins observed in the MVs composition require further analysis as they may be responsible for the observed effects of MVs on endothelial cells (Sokolov *et al.,* 2021).

Characterisation of macrophage-derived MVs and their role in differentiating naïve monocytes has been researched. This identified the miRNA content of the macrophage-derived MVs. The RNA molecules contained in the macrophage-derived MVs were transported to the targets cells, including monocytes, Epithelial cells, Endothelial cells and fibroblasts. Furthermore, miR-223 can be transported to target cells and become functionally active. MVs therefore can be hypothesised to bind to and activate target cells. MVs induce the differentiation of macrophages and thus defining key components of this response will identify novel targets to regulate host defence and inflammation (Ismail *et al.,* 2013).

Tumour derives microvesicles (TMVs) are key players in tumour progression, modulating biological activity of immune cells such as monocytes, macrophages and lymphocytes. This is correlated in the progression of colon cancer, as macrophages in this tumour are relevant for the recovery process (Baj-Krzyworzeka *et al.,* 2016).

MVs are important mediators for intercellular communication as packaging a variety of important molecular cargo. They have been implicated in the pathophysiology of various inflammatory diseases, however their role in acute lung injury (ALI) remains unknown. The production of MVs from various intra-alveolar precursor cells during the early phase of ALI has been demonstrated. These findings suggest the alveolar macrophage-derived MVs, carrying active TNF may play an important role in initiating ALI (Soni *et al.*, 2016).

MVs are released into the circulation during systemic inflammation. Monocytes are hypothesised to contribute to the uptake of circulating MVs and their increased margination to the pulmonary circulation and functional priming during systemic inflammation produces substantive changes to the systemic MV homing profile. Lung associated monocytes become a dominant target cell population for MVs during systemic inflammation, having significant implications for the function and targeting of endogenous and therapeutically administered MVs, lending novel insights into the pathophysiology of pulmonary vascular inflammation (O'Dea *et al.*, 2020).

Literature suggests MV detection may represent a strategy to gain pathogenic information as mediators of intracellular communication. As new biomarkers of tissue damage, MVs are helpful to examine inaccessible districts. The role of monocyte-derived MVs in multiple sclerosis remains to be investigated. Current drugs used for the treatment of multiple sclerosis appear to interfere with the production of MVs from monocytes (Blonda *et al.,* 2017).

2. AIM AND OBJECTIVES

2.1 Aim

The aim of this study is to compare two different methods of THP-1 monocyte differentiation into macrophages.

2.2 Objectives

1. Differentiating THP-1 cells with phorbol 12-myristate-13-acetate (PMA) to produce M0-type macrophages.

2. Differentiating THP-1 cells with HUH7 in a conditioned cell culture medium to produce macrophage-like cells.

3. MATERIALS AND METHODS

3.1 Cell Lines

THP-1 cells (Human promonocytic cells) were used in most experiments as the source of extracellular vesicles (EVs). The THP-1 cells were purchased from Public Health England (ECACC; Ref No. 88081201) and maintained in a humidified incubator at 37°C with 5% CO_2 in growth medium made of RPMI 1640 containing 25mM glucose, 20% fetal bovine serum and 1% Penicillin Streptomycin (Gibco, Thermo Fisher Scientific, UK) complete growth medium-1 (CGM-1).

HUH7 is a well differentiated hepatocyte derived cellular carcinoma cell line that was originally taken from a liver tumour in a 57-year-old Japanese male in 1982. The line as established by "Nakabayshi and co-workers (1982)". These HUH7 cells were purchased from the Japanese Collection of Research Bioresources Cell Bank and were maintained using RPMI 1640 containing 25mM glucose (Gibco), 10% fetal bovine serum, 1% Kanamycin and 1% Penicillin Streptomycin (Gibco, Thermo Fisher Scientific, UK) complete growth medium-2 (CGM-2) in a humidified incubator at 37^{o}C and 5% CO_2.

3.2 Maintaining cell lines

Non-adherent THP-1 cells were maintained in complete RPMI growth medium and were sub-cultured every 3 to 5 days. The cells were transferred into 50 mL centrifuge tubes and spun at 160 x g for 5 min. The resulting supernatant was discarded and cells were gently loosened in the remaining medium. A total of 5mL of serum-free RPMI was added and cells were

centrifuged as described before. The supernatant was discarded and cell pellets were resuspended complete RPMI growth medium at a concentration of 2-5 x 10^5 cell/mL in a 75 cm^2 flask.

Adherent HUH7 cells were maintained in RPMI 1640 growth medium with 10% fetal bovine serum and 1% kanamycin and 1% Streptomycin at 37°C with 5% CO_2. The adherent cells were sub-cultured depending on confluency every 2 to 4 days. Cells were washed twice with serum-free RPMI and 3mL 0.25% (v/v) trypsin/EDTA in RPMI (Sigma Aldrich) added. After 5 minutes incubation, 5mL growth medium was added to the flask to inactivate the trypsin. The total cell suspension was centrifuged at 160g for 5 minutes followed by one wash in 5mL serum-free RPMI. Cell pellets were resuspended in 20mL CGM-2 and seeded at 2 x10^5 cells/mL into new 75cm^3 culture flasks. Exponentially growing cells visually doubling in cell density every 48 hours with viability of 95% or higher were used in each experiment. The number of cells and viability were determined before the start of every experiment using a haemocytometer with trypan blue dye and light microscopy.

3.3 THP-1 Induced Cell Differentiation with Phorbol 12-Myristate-13-Acetate (PMA)

THP-1 cells were seeded in 6-well plates at 1 x 10^6 cells/mL and differentiated into macrophage-like M0 cells with PMA at 50nM, 100nM and 200nM from a stock solution of 100µM in DMSO. THP-1 cells only and THP-1 cells with DMSO only were used as controls. To confirm the optimum concentration of PMA the THP-1 cells were incubated in 6-well plates with the 50nM, 100nM and 200nM in duplicates and monitored over 72 hours under light microscopy. After several repeats the experiment concluded the majority of cells differentiated form THP-1 monocytes into macrophage-like M0 cells were with 100nM PMA with the most viable cells confirmed using a MTT assay. For extracellular vesicle generation, after 24 hours of differentiation in 25cm^2 flaks (Sigma-Aldrich), cells were washed with sterile PBS and

incubated in 7mL fresh RPMI 1640 serum free media for 48 hours before the supernatant was collected for extracellular vesicle isolation.

3.4 Using Conditioned Medium for Cell Differentiation

HUH7 cells were grown for 48 hours in CGM-2 at 5 x 10^5 cells/mL. The HUH7 cell medium was centrifuged in a 15mL centrifuge tube at 200g for 5 minutes to remove cell debris. Addition of this supernatant was used as conditioned medium for differentiation of THP-1 cells. THP-1 cells were seeded at 2 x 10^5 cells/mL in 2mL in a 6-well cell culture plate (Thermo Fisher Scientific, UK) and then co-cultured with the HUH7 supernatant. THP-1 cells were treated with 1mL and 1.5mL HUH7 conditioned medium in duplicates. Untreated THP-1 cells and THP-1 cells treated with 100nM of PMA were used as negative and positive controls respectively. The co-cultured cells were analysed for cell differentiation every 24 hours using light microscopy over 7 days. After 7 days the differentiated cells (THP-H Cells) adhered to the 6-well plate were trypsinised and subcultured in a 75cm^3 cell culture flask. The new THP-H cells reached full confluency after 7 days in culture and the growth medium changed with CGM-2 every 48 hours.

3.5.1 Light Microscopy of Differentiated THP-1 Cells and Cell length measurement

The morphology of THP-1 and HUH7 cells was observed using an Olympus X81 light microscope. Images were taken using cell^M software (Olympus UK) and a digital monochrome CCD camera.

3.5.2 Image J Cell Length

The untreated and treated THP-1 cells with 50nM of PMA, 100nM of PMA and 200nM of PMA (Figure 11) were taken at 100x magnification. For calculating cell length thirty

cells from each treatment were measured using Image J software. The program is opened and the scale set using the calibration scale image. Select File, 'Open'. The scale needs to be set only once for all the images to be analysed at the same magnification. Straight line icon was selected, line drawn between two points for 200 μm (Figure 11). Plugins was selected from the top bar options and the Cell Magic Wand Tool selected. Then the cell was selected and a round outline appeared around the selected cell. Analyse was selected from the top bar, then set measurements selected and the area selected, standard deviation, minimum and maximum gray value and mean gray value all selected. The decimal places was set to three and OK selected. All the cells measured were selected and the results page opened. This page showed the number of cells analysed, with the cell length, alongside all set measurements selected (mean, standard deviation, minimum and maximum gray value and mean gray value).

3.6 Lysate preparation

3.7 Preparation of Macrophage lysate: A total of 150μL of RIPA lysis buffer consisting of protease inhibitor cocktail (1mM AEBSF, 800nM Aprotinin, 50μM Bestatin 15μM E64 20μM Leupeptin 10μM Pepstatin A 5mM EDTA) (Fisher Scientific. UK) and RIPA buffer (25 mM Tris HCl, pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) (Thermo Scientific, UK) in a 1:100 ratio was added to the macrophages in T25 flasks (ThermoFisher, UK) on ice for 15 minutes to lyse the macrophages. Samples were transferred into 1.5mL eppendorf tubes (Fisher Scientific, UK) and centrifuged at 17,000 x g for 15 minutes. The supernatant was transferred into a new 1.5mL eppendorf tube kept on ice and stored at -20^{O}C and the pellet was discarded.

3.8 Preparation of Extracellular Vesicle (EV) lysate: A total of 40µL of RIPA lysis buffer consisting of protease inhibitor cocktail (1mM AEBSF, 800nM Aprotinin, 50µM Bestatin 15µM E64 20µM Leupeptin 10µM Pepstatin A 5mM EDTA) (Fisher Scientific. UK) and RIPA buffer (25 mM Tris HCl, pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) (Thermo Scientific, UK) in a 1:100 ratio was added to the EV pellets in 1.5mL eppendorf tubes (ThermoFisher, UK) left on ice for 15 minutes to lyse the EVs .Samples were centrifuged sample 17,000 x g for 15 minutes. The supernatant was transferred into a new eppendorf tube kept on ice and stored at -20^{O}C and the pellet was discarded.

3.9 Determination of Protein Concentration of Cell Lysates

Protein concentrations of cell lysates were determined against a standard curve produced using known concentrations of bovine serum albumin (BSA) 2.0 mg/ml in 0.9% saline and 0.05% sodium azide (Pierce, Thermo Scientific, UK) using a BCA assay**.** In a 96 well plate, four replicates of 5µL of cell lysate was diluted in 20µL of sterile PBS, and addition of 200µL working reagent (Reagent A consisting of sodium carbonate, sodium bicarbonate, bicinchoninic acid and sodium tartrate in 0.1 M sodium hydroxide) and (Reagent B containing 4% cupric sulfate) of Pierce Thermo Scientific reagent (Sigma-Aldrich). Protein concentration determination of cell lysates were repeated in replicates to improve accuracy. Protein concentrations were read at 562nm using a FLUOstar Omega Microplate Reader.

3.10 Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

In order to separate proteins based upon their molecular size,10% polyacrylamide gels were made in a total volume of 10mL consisting of 30%(w/w) acrylamide (37:5:1 BIS-acrylamide) (Sigma-Aldrich), 10% (w/v) Sodium Dodecyl Sulfate (SDS) (Fisher Scientific, UK), 1.5M Tris-HCl pH 8.8 for 10% resolving gel or 0.5M Tris-HCl pH 6.8 for the 4% stacking gel

(Sigma-Aldrich), 10% (w/v) ammonium persulphate (APS) (Sigma), 0.8% (v,v) N, N, N', N'-Tetramethylenediamine (TEMED) (Sigma-Aldrich) and deionised water into pre-made gel chambers, and well-forming combs were immediately added and the gel was left to polymerise. After polymerisation, combs were removed and the polyacrylamide gel wells were filled with running buffer pH 8.3 (25 mM Tris, 192 mM glycine, 0.1% SDS (w/v)). Cell lysates with 20µg protein were prepared for running on polyacrylamide gels by addition of equal volume Laemmli sample buffer (4% SDS (w/v), 20% glycerol (v,v), 10% 2-mercaptoethanol (v,v), 0.004% bromphenol blue (w,v) and 0.125 M Tris HCl, pH approx. 6.8) (Sigma-Aldrich). After, samples were heated to 95^{o}C for 10 minutes then centrifuged at 17,000g for 1 min. Samples were loaded into the polyacrylamide gel wells along with a precision protein plus dual colour standard pre-stained Marker (BioRad) ranging from 10 to 250 kD loading 5 µL into standard well.

3.11 Visualisation of Proteins Separated by SDS-PAGE

Coomassie staining was used to detect the presence of proteins in lysate samples. Proteins separated by SDS-PAGE were assessed for protein presence by staining the polyacrylamide gels in 0.1% (w/v) Coomassie brilliant blue R-250 (Sigma) in 25% (v,v) methanol (Sigma), 10% (v,v) acetic acid (Fisher Scientific) for 24 hours. After staining, polyacrylamide gels were de-stained in Coomassie de-stain solution (acetic acid /methanol/ water (1:3:6 v/v)) on an orbital mixer between up to 1 hour and assessed after sufficient de-staining using a UVP ChemiDoc-It imaging system.

3.12 Western Blotting

Following separation of proteins by SDS-PAGE, proteins were transferred onto a Hybond C nitrocellulose membrane (Sigma-Aldrich). Transfer cassettes contained a sponge, Whatman

3MM blotting paper, nitrocellulose membrane (Sigma-Aldrich), SDS- PAGE gel, Whatman blotting paper and a sponge (all equipment was pre-immersed in Transfer buffer containing 25 mM Tris, 192 mM glycine, 20% (v/v) methanol (pH 8.3). The transfer tank was filled with transfer buffer for 40 minutes at 15V using a semidry device (Bio-Rad transfer system). Nitrocellulose membranes were transferred into 5% (w,w) skimmed dried milk (Marvel) in PBS Tween (137mM NaCl, 2.7mM KCl, 10mM Na_2HPO_4, 1.8mM KH_2PO_4, 0.1% (w/v) Tween 20 pH 7.4) for 1 hour in order to block non-specific binding sites. Following blocking, primary antibody rabbit anti-GAPDH (Abcam) was diluted 1:2000 in 5% skimmed dried milk in PBST and incubated with the nitrocellulose membrane overnight at 4°C. Following incubation with primary antibody, nitrocellulose membranes were washed in PBST 3 times for 10 minutes each time. Nitrocellulose membranes were then incubated with secondary antibody goat anti-rabbit IgG (Abcam) conjugated to horse-radish peroxidase (HRP) in 5% skimmed dried milk in PBST for 1.5 hours at room temperature on the orbital shaker. Following incubation, nitrocellulose membranes were again washed in PBST 3 times for 10 minutes each time. Proteins were visualised by washing the nitrocellulose membrane in enhanced chemiluminescence (ECL) reagent (GE Healthcare Amersham) for 2 minutes, sealed in saran wrap and visualised for GAPDH Protein using an iBright imaging system.

3.13 Extracellular Vesicle Isolation

About 7 million human monocytic THP-1 cells were cultured in each T-25 flask (Thermo Fisher Scientific, UK) with CGM-1 and 100nM of PMA (Sigma-Aldrich). After 24 hours of incubation, CGM-1 was discarded, and the T-25 flask was washed with sterile PBS. 7mL of plain RPMI medium was added into each flask for a further 48 hours incubation. The supernatant was then collected and microvesicles (MVs) and exosomes were isolated by differential centrifugation using an optimised method as seen below in Figure 10 (Dozio *et al.*,

2017). No filtration step was included in the isolation method as THP-1 cells release few vesicles under normal conditions therefore filtration would have further decreased the vesicle yield. 28mL of supernatant were collected and cells and apoptotic bodies were removed by centrifugation at 300 x g, 10 mins, 4ºC and 200 x g, 20 mins, 4ºC, respectively. MVs were collected by centrifuging the supernatant at 18,000 x g, 45 mins, 4ºC using a Beckman Coulter ultracentrifuge and SW40 Ti rotor (Beckman Coulter, USA). Finally exosomes were isolated using ultracentrifugation (100,000 x g, 120 mins, 4ºC). MVs and exosomes were resuspended in PBS and all replicate samples of M0 supernatant were collected and pooled together. Pooled extracellular vesicles were re-centrifuged using an SW40Ti rotor for MVs (18,000 x g, 30 mins, 4ºC) as were pooled exosomes (100,000 x g, 70 mins, 4ºC). Isolated EV pellets were lysed and proteins examined using SDS-page and western blotting.

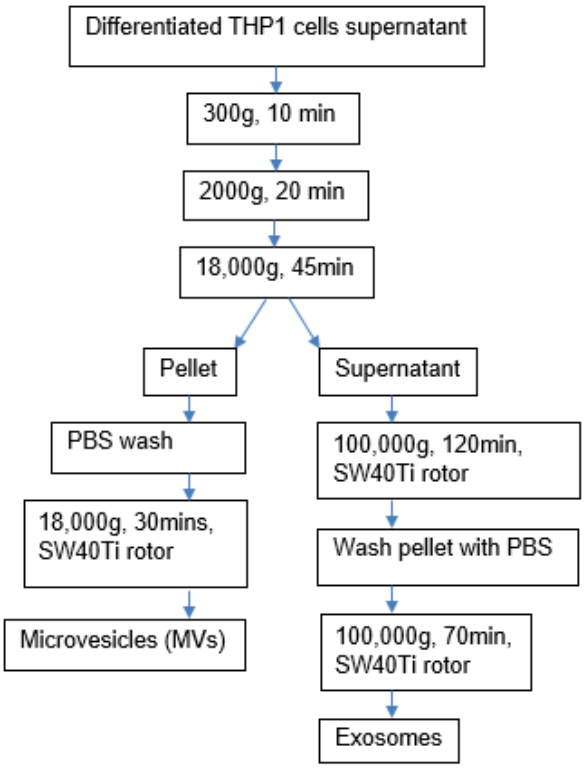

Figure 10: EV isolation optimised protocol. MVs and exosomes were isolated from culture medium supernatants using differential centrifugation (Dozio *et al.*, 2017)

3.14 Flow Cytometry

The Guava Flow cytometer can analyse complex biological studies such as cell viability, cell counting, cytokine detection, cell activation marker, white blood cell phenotyping and various complex molecular analyses can be experimented simultaneously. The flow cytometer is time efficient while generating accurate results which are close to the complex biological responses within the body. This can be used to perform numerous assays however for this research the assay implemented was the Nexin assay for Annexin V labelling of MVs for MV characterisation and reporting apoptosis. Cell viability and numbers can also be confirmed with the ViaCount assay. This distinguishes the viable and non-viable cells based on DNA-binding dyes within the Guava ViaCount reagent. The nucleated cells would be stained with the nuclear dye only while the dying or dead cells stained with the bright viability dye. The combination of dyes enables the Guava ViaCount assay to determine viable, apoptotic and dead cells. Cell debris is also removed from the results due to negative staining with the nuclear dye.

3.15 Annexin Labelling of Microvesicles for Flow Cytometry

Isolated MVs were resuspended in annexin binding buffer (50 mM HEPES, 700 mM NaCl, 12.5 mM $CaCl_2$, pH 7.4) and Annexin V Alexa Fluor 488 conjugated to FITC Solution (25 mM HEPES, 140 mM NaCl, 1 mM EDTA, pH 7.4, 0.1% bovine serum albumin (BSA)) (ThermoFisher Scientific, UK) (1:10 dilution of 5µL annexin V (Sigma-Aldrich)/50µL buffer) wrapped in foil to prevent photo-bleaching of the FITC. Annexin binding buffer and Annexin

V Alexa Fluor 488 conjugated to FITC was added or not as a control in a 200µL final volume. The sample was incubated at room temperature for 30 minutes on a shaker and centrifuged at 25,000 x g for 2 hours to pellet the MVs. Samples were immediately re-suspended in 200µL sterile PBS (137mM NaCl, 2.7mM KCl, 10mM Na_2HPO_4, 1.8mM KH_2PO_4) and analysed using a Guava Millipore flow cytometer, using the ExpressPlus programme, gating against an unlabelled control (Guava EasyCyte, Guava Technologies).

3.16 MTT Assay Format for 24-well tissue culture dish (adherent cells)

THP-1 cells were seeded in 24-well plate, 5 x 10^4 cells / mL, 0.5 mL per well. Cells were allowed to establish growth overnight and treated as required by the experiment. At required time points, 200 µL medium was removed and 50µL MTT solution (Thiazolyl Blue Tetrazolium Bromide (MTT) from Sigma-Aldrich (catalogue number M2128) is stored at 4 ^oC; a stock solution is prepared by dissolving 5 mg MTT per mL of PBS solution and is stored at -20 ^oC. It is light sensitive). This is added to each well while mixing by gentle swirling and incubated at 37 ^oC for 3.5 hours. The MTT and medium were removed and disposed as chemical waste. An optional step of washing the cells gently with 500 µL of PBS for each well and taking care not to dislodge cells. Pipetting the PBS against the side of the well and not directly onto the cells careful to avoid cell loss. Add 500 µL of DMSO (Sigma-Aldrich catalogue number D5879) as a solvent to dissolve (lyse) cells and the purple formazan crystals formed by reduction of MTT by the dehydrogenases; pipette or rotate to complete the dissolution. Read absorbance at 590 nm corrected for light scattering at 700 nm. Data was calculated from adjusting absorbances: $Abs_{590} - Abs_{700}$. MTT assay used to measure cytotoxicity or cell growth

4. RESULTS

The main aim of this research project was to compare different methods of THP-1 monocyte differentiation into macrophages. Firstly, the well-established protocol of using phorbol 12-myristate-13-acetate (PMA) to differentiate THP-1 cells and secondly the use of HUH7 in a conditioned cell culture medium to differentiate THP-1 cells, which is the novel method created in the present study. The respective data confirms that this novel method of using HUH7 differentiates THP-1 cells and establishes a new permanent macrophage cell line.

4.1 Differentiating THP-1 cells with phorbol 12-myristate-13-acetate (PMA) produces M0-type macrophages

4.1.1 PMA concentrations of 50nM, 100nM and 200nM of PMA for THP-1 cell differentiation

THP-1 cells were grown in complete cell culture medium as seen in Figure 11 for several days before cell confluency is achieved. The first step after searching the literature was to confirm the optimum concentration of PMA at either 50nM, 100nM or 200nM and the time interval 24h, 48h or 72h for incubation. After several repeats and monitoring of the cells, THP-1 monocytes differentiated into macrophage-like cells with all three PMA concentrations. THP-1 cells changed morphology from a rounded cell into an elongated macrophage-like cells and the cells were therefore noted as THP-1 cells differentiated to macrophages. Various concentration of PMA were investigated using microscopy, BCA assay, MTT assay, gel

electrophoresis and western blotting to confirm the optimum concentration of PMA to use for differentiating THP-1 cells. Higher concentrations of PMA may exert more chemical harm on the cells as PMA is a tumour promoter chemical and higher concentrations have adverse effects on the lifespan of macrophage-like cells. Thus, further experiments were conducted to determine the optimum time concentration of PMA for differentiating THP-1 cells.

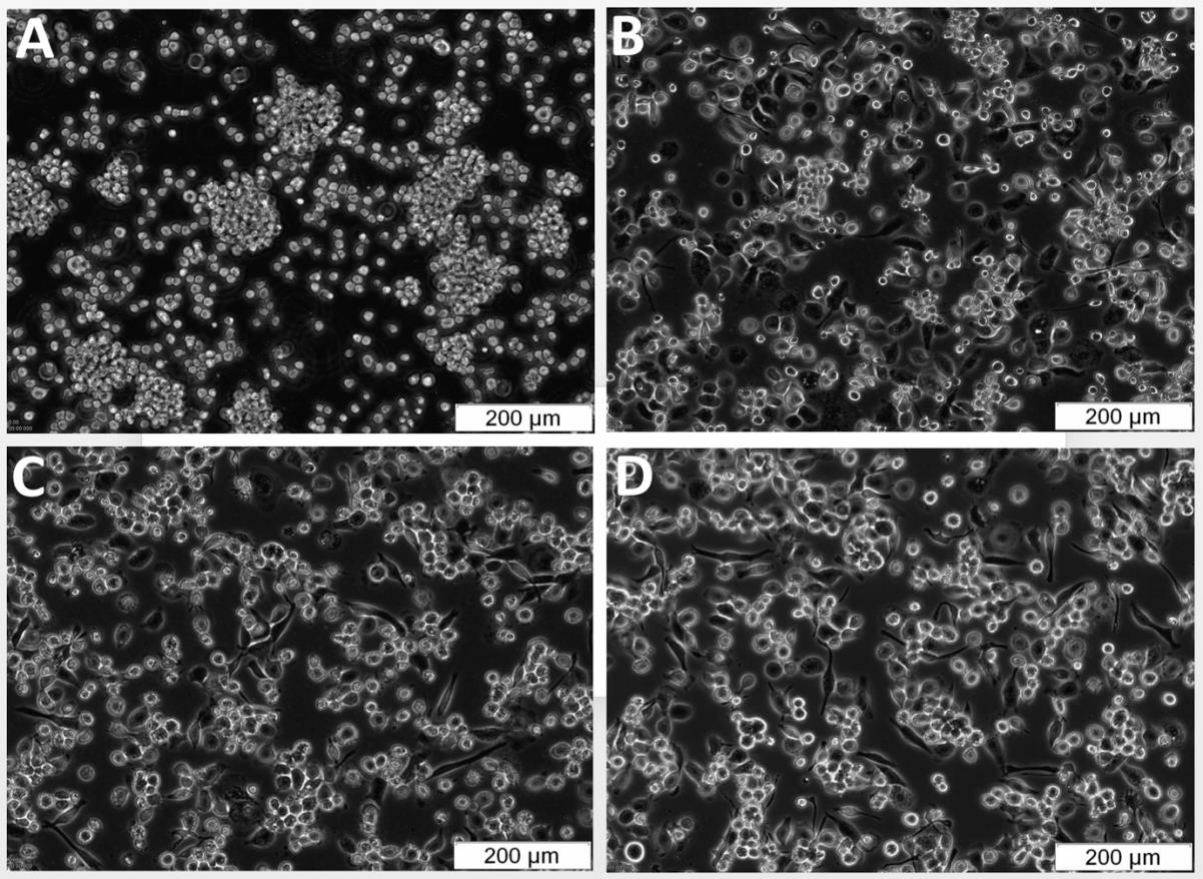

Figure 11: THP1 cells differentiated with 50nM, 100nM and 200nM of PMA into macrophage-like cells after 72h. THP1 monocytic cells were differentiated into macrophages after 72h with 50nM, 100nM and 200nM PMA. Magnification, x100. (A) Control THP1 cell $1x10^6$ cells/mL with DMSO. (B) THP1 cells with 50nM PMA. (C) THP1 cells with 100nM PMA. (D) THP1 cells with 200nM PMA. (A) Control cells mean before treatment 9.5µm, ± SD 2.3, (B) 50nM of PMA treated cells mean 59.7µm, ± SD 18.1 (C) 100nM of PMA treated cells mean 87.3µm, ± SD 41.8 (D) 200nM of PMA treated cells mean 65.3µm, ± SD 34.8. Between untreated cells and 50nM PMA treated cells, 100nM PMA treated cells and 200nM PMA treated cells

± SD 17.7. *n* = 30 cells per well. Statistical analysis by comparing means using 1-way ANOVA showed untreated cells to have a significantly lower mean compared with cells treated with PMA ($p < 0.01$).

4.1.2 Protein Concentrations of Extracellular Vesicles using a BCA Assay

THP-1 cells treated with 50nM, 100nM and 200nM of PMA were isolated for EVs. The protein content of each sample was measured using a BCA assay with a BSA standard curve. Multiple preparations of EV isolates were prepared and the results were relatively consistent as shown in Figures 12 and 13. The three PMA concentrations were analysed and compared for their protein content. THP-1 cells treated with 100nM of PMA had the most consistent protein concentration. Treatment with 100nM of PMA produced cells with the longest length (mean 87.3µm, ± SD 41.8). Therefore, 100nM of PMA treated THP-1 cells would be ideally used for further research. The graph below in Figure 12 shows differentiated THP-1 (M0 macrophage) MVs protein concentration. The results show that 100nM of PMA gives a more consistent protein concentration compared to 200nM of PMA. Also, 50nM of PMA from previous data as seen in Figure 11 does not visually differentiate sufficient THP-1 cells after 72 hours. The data suggests that the optimum concentration of PMA is therefore 100nM of PMA and to optimise the protein concentration for future research, samples can be pooled together when isolating EVs. Proteomics samples would be taken with 100nM of PMA differentiated THP-1 cells if the study was to continue and develop a proteomic profile, however this is part of the future work if this research continued.

Microvesicles Protein Concentration using 50nM, 100nM and 200nM of PMA

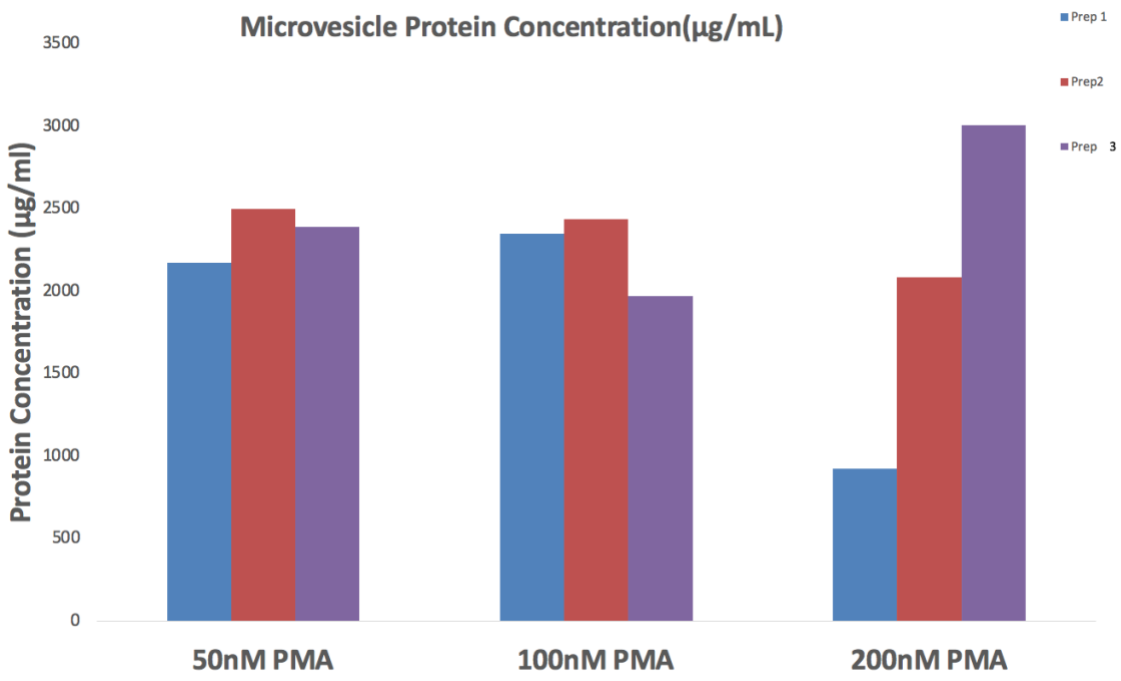

Figure 12: Macrophage Microvesicles Protein Concentration (µg/mL). Microvesicles were isolated from differentiated macrophage supernatants after 72 hours and the protein concentrations of three preparations measure using a BCA assay. Samples were all diluted with PBS in a ratio of 1:5 to make sure the highly concentration samples could be calculated using the standard curve.

Figure 13 below displays the three preparations of exosomes with 50nM, 100nM and 200nM of PMA. The result show 100nM of PMA gives a more consistent protein concentration compared to 50nM and 200nM of PMA. Preparation three for all the PMA concentrations is low compared to preparations one and two and this may be due to the sample being diluted compared to preparation one and two. The graph below in Figure 13 confirms 100nM of PMA is optimum and will produce the highest protein concentration on average when isolating EVs.

Exosomes Protein Concentration using 50nM, 100nM and 200nM of PMA

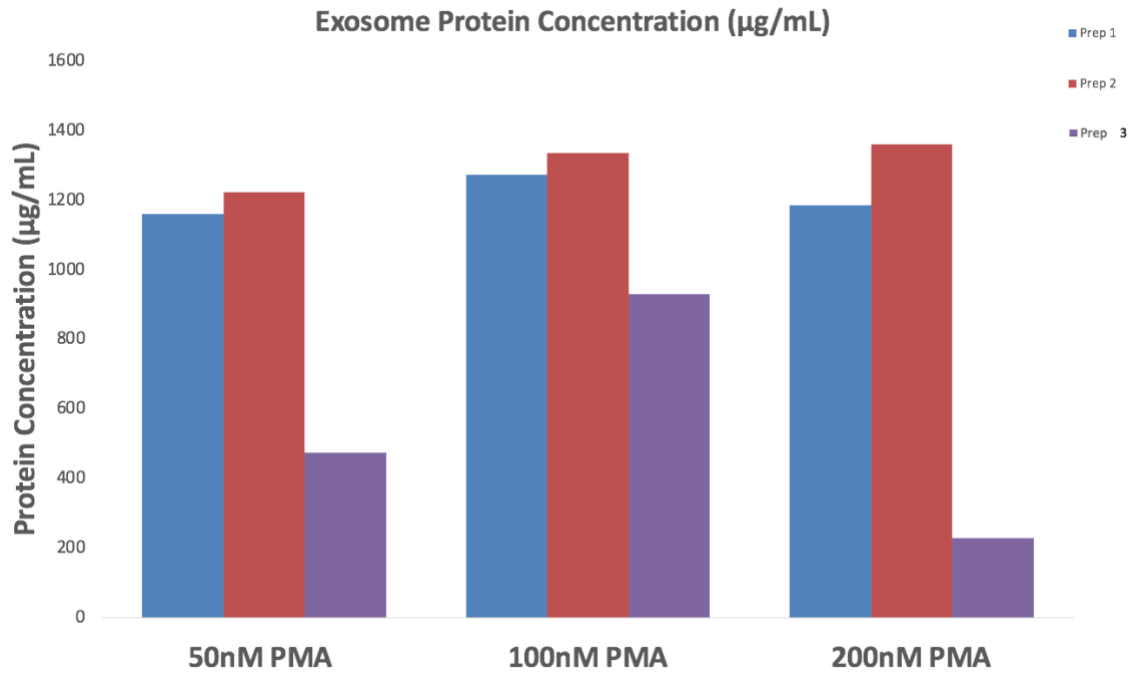

Figure 13: Macrophage Exosome Protein Concentration (µg/mL). Exosomes were isolated from differentiated macrophage supernatants after 72 hours and the protein concentrations of four preparations measured using a BCA assay. Exosome isolates were produced from supernatant collected after 72 hours incubation with PMA.

These results provide data for the optimum concentration of THP-1 differentiation being 100nM of PMA. Further analysis using coomassie blue dye staining using gel electrophoresis and western blotting for proteins can be performed to confirm protein bands and to conclude the optimum PMA concentration to use for differentiating THP-1 cells.

4.1.3 Gel Electrophoresis of Extracellular Vesicles with 50nM, 100nM and 200nM PMA (Preparations 1 and 2)

As seen in Figures 12 and 13 the THP-1 cells were incubated with PMA at concentrations of 50nM, 100nM and 200nM and the figures 14.a and 14.b below are the corresponding coomassie stained gels using the EV samples. Protein concentration of 20-55µg/mL from all EV lysate samples and 500µg THP-1 cell lysate were loaded for protein band quantification. Compared to the protocol used to isolate EVs the samples had fewer ultracentrifuge steps and did not use the SW40Ti rotor for centrifuging in 10mL tubes. After changing from 1mL Eppendorf tubes to 10mL tubes the isolates became concentrated and the total protein concentration for each PMA concentration was consistent in each preparation with the RIPA lysis buffer having no effect on the measurement of protein concentration of EV isolates.

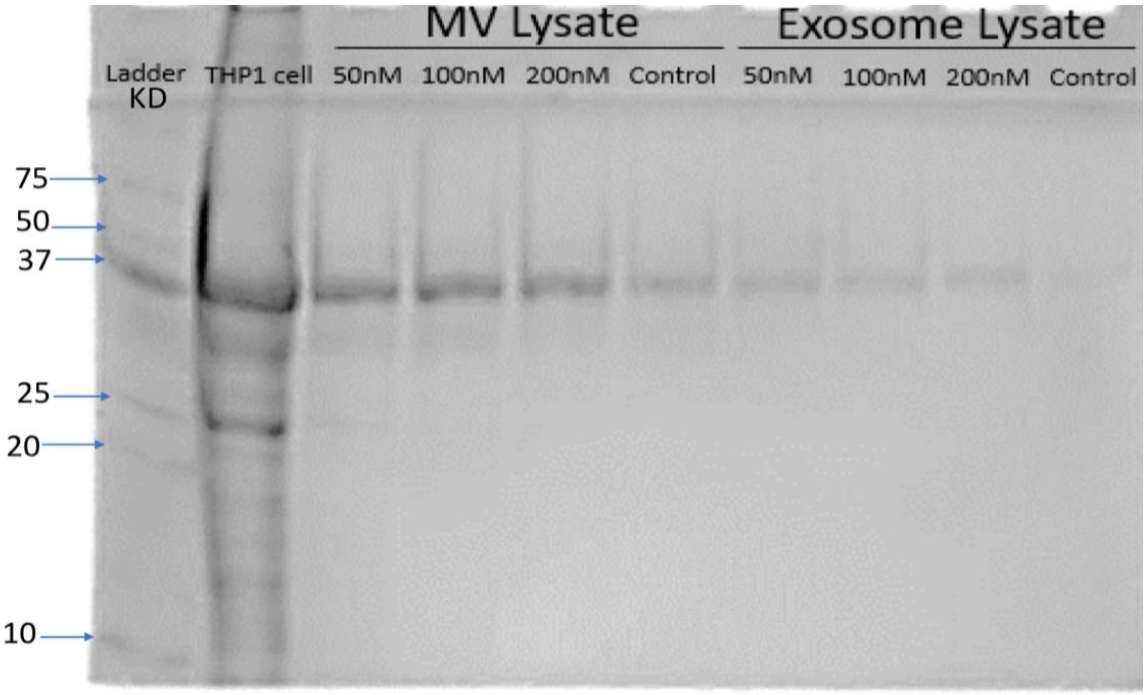

Figure 14.a: Gel Electrophoresis of Extracellular Vesicles with 50nM, 100nM and 200nM PMA (Preparation 1). THP1 cells ($5x10^5$ cell/mL) incubated for 72h with PMA and isolated for extracellular vesicles were prepared for gel electrophoresis. From left, standard ladder, THP-1 cell lysate, MV isolates from 50nM, 100nM and 200nM PMA and control with DMSO. Exosome lysates from 50nM, 100nM and 200nM PMA and control with DMSO. There is a consistent band shown around 37kDa and a faint band around 30kDa in all lysate preparations. THP-1 cell lysates are more concentrated and has clear bands at 37kDa, 30kDa and 25kDa.

Protein bands can be seen in all of the preparations, with a higher intensity in the MV samples. This may be due to the low protein concentration of exosomes compared to the MVs. The samples are however present with protein bands in 50nM, 100nM and 200nM of PMA. Therefore, 100nM can be seen to be present with protein bands here and provides further information for using this concentration for THP-1 cell differentiation.

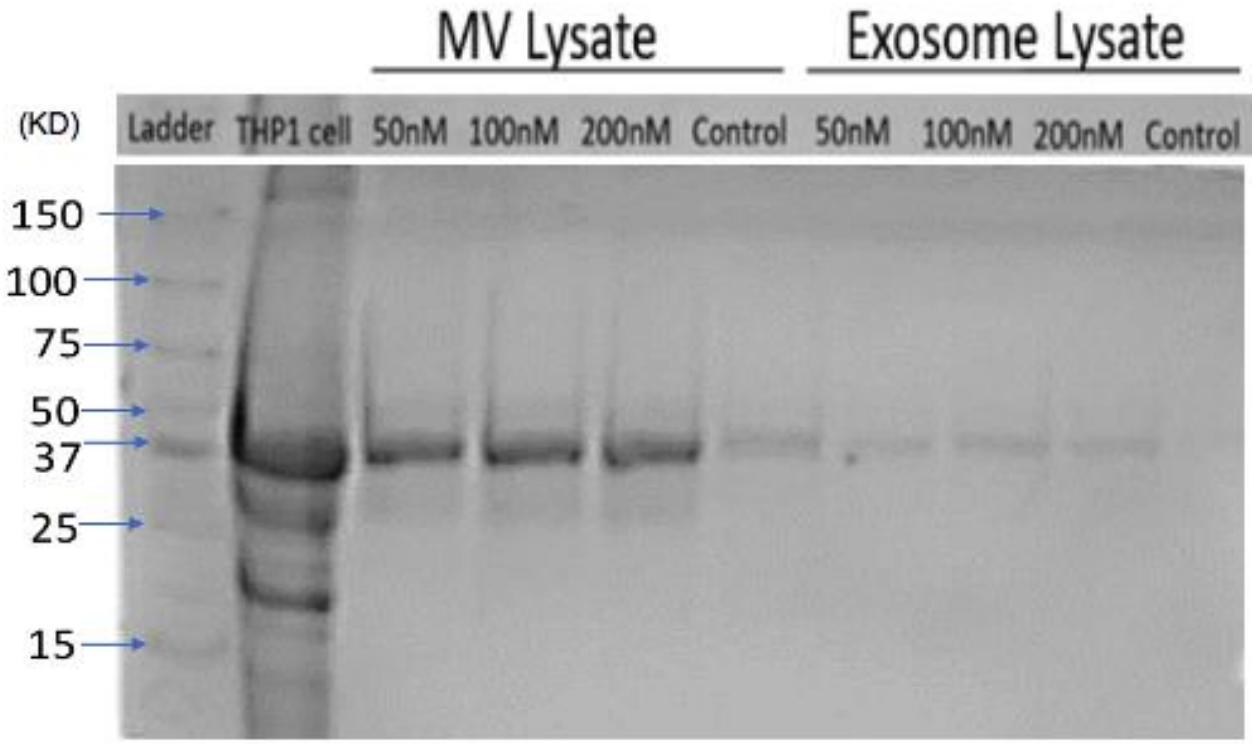

Figure 14.b: Gel Electrophoresis of Extracellular vesicles with 50nM, 100nM and 200nM of PMA (Preparation 2). THP-1 Cells EVs gel electrophoresis with the last ultracentrifuge step repeated from EVs isolated previously. THP-1 cells ($5x10^5$ cell/mL) incubated for 72h with PMA and isolated for EVs were prepared for gel electrophoresis. From left, standard ladder, THP-1 cell lysate, MV isolates from 50nM, 100nM and 200nM of PMA and control with DMSO. Exosome lysates from 50nM, 100nM and 200nM PMA and control with DMSO. THP-1 cell lysate was loaded in a total volume of 10µL and all remaining lysates loaded at 50uL into each well. There is a consistent band shown around 37kDa and a faint band around 30kDa in all lysate preparations. THP-1 cell lysates are more concentration and have clear bands at 37kDa, 30kDa and 25kDa.

Figure 14.a (preparation 1) and 14.b (preparation 2) belong to two different EV isolation preparations and therefore the corresponding protein bands on the gel correspond to two separate preparations for each sample.

The electrophoresis gels were performed for the EV isolates after 72h and 50nM, 100nM and 200nM incubation of PMA. A single consistent band were seen in all EV isolates at around 37kDa in both Figure 14a and 14b.

4.1.4 Expression of GAPDH in THP-1 Extracellular Vesicle Lysates

THP-1 cells were incubated with 50nM, 100nM and 200nM of PMA. EVs were isolated from the supernatant after 72h using differential ultracentrifugation and lysate preparations of each sample produced with RIPA lysis buffer and analysing on the iBright machine. This experiment was conducted to confirm the presence of the housekeeping protein GAPDH which is at 36kDa and the lysates show expression of GAPDH at a similar size in Figure 15.

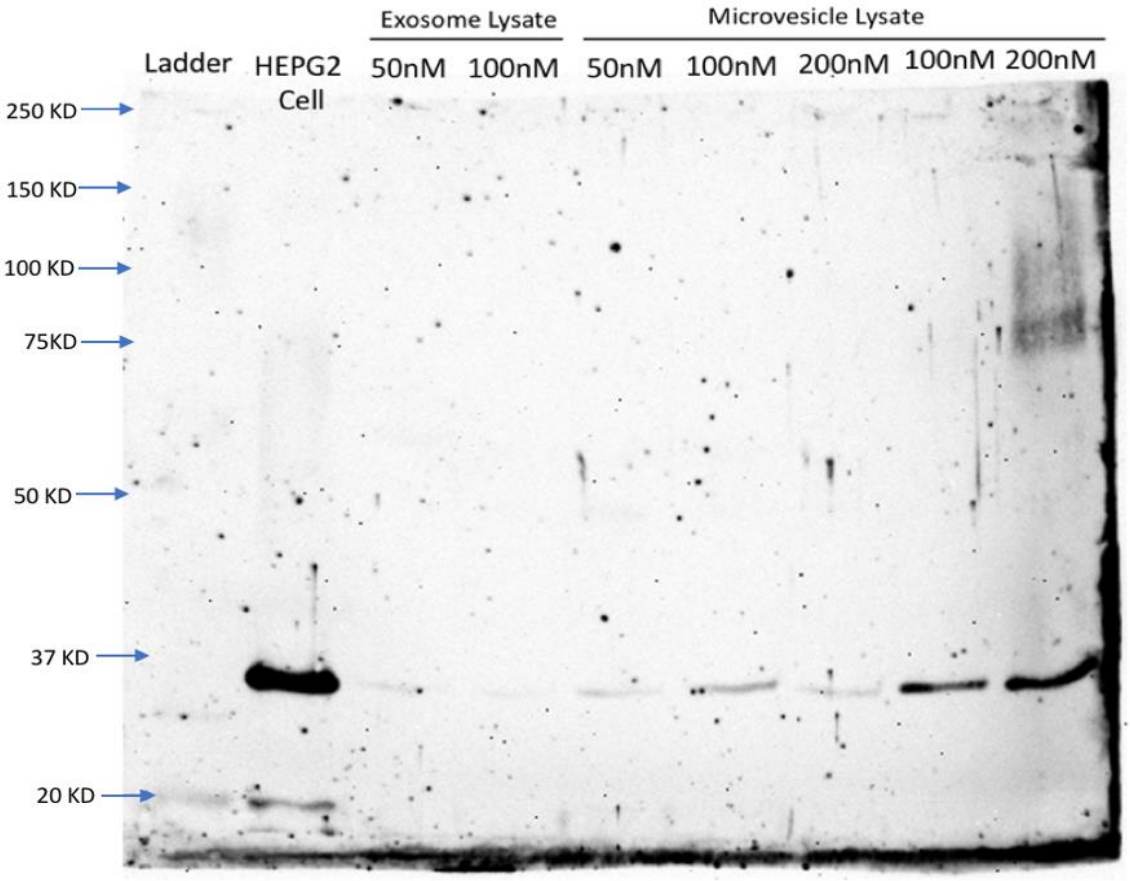

Figure 15: Western blot analysis of extracellular vesicle isolates with anti-GAPDH. Protein expression of GAPDH in extracellular vesicle lysates. GAPDH expressed in all lysate samples. GAPDH expressed in all lysate samples. Lane 1 standard, Lane 2 HEPG2 cell lysate, Lane 3 exosome isolate with 50nM PMA, Lane 4 exosome isolate with 100nM PMA, Lane 5 MV isolate with 50nM PMA, Lane 6 MV isolate with 100nM PMA, Lane 7 MV isolate with 200nM PMA, Lane 8 MV isolate with 100nM PMA, Lane 9 MV isolate with 200nM PMA. GAPDH was highly expressed in MV isolates with 200nM PMA.

4.1.5 Cell Metabolic Activity of 100nM and 200nM of PMA using a MTT Assay

After these experiments the results suggested 100nM and 200nM are both adequate concentrations for differentiating THP-1 cells. The THP-1 cells differentiated with a similar morphology and confluency with both concentrations. An experiment was therefore conducted to measure the cellular metabolic activity as an indicator of cell viability, proliferation and cytotoxicity was performed. The experiment is a MTT assay which uses a colorimetric assay system and the darker the purple solution, the greater the number of viable, metabolic active cells. The experiment took a 3.5 hours of incubation and the assay solution changed colour to a darker shade of purple with higher percentages of cell viability. Therefore, the THP-1 cells with 100nM of PMA had a cell viability of 79.4% with a darker purple solution compared to 63.4% cell viability with 200nM of PMA (lighter purple shade solution) (Figure 16). This confirmed the previous finding of using 100nM of PMA as the optimum concentration for THP-1 cell differentiation.

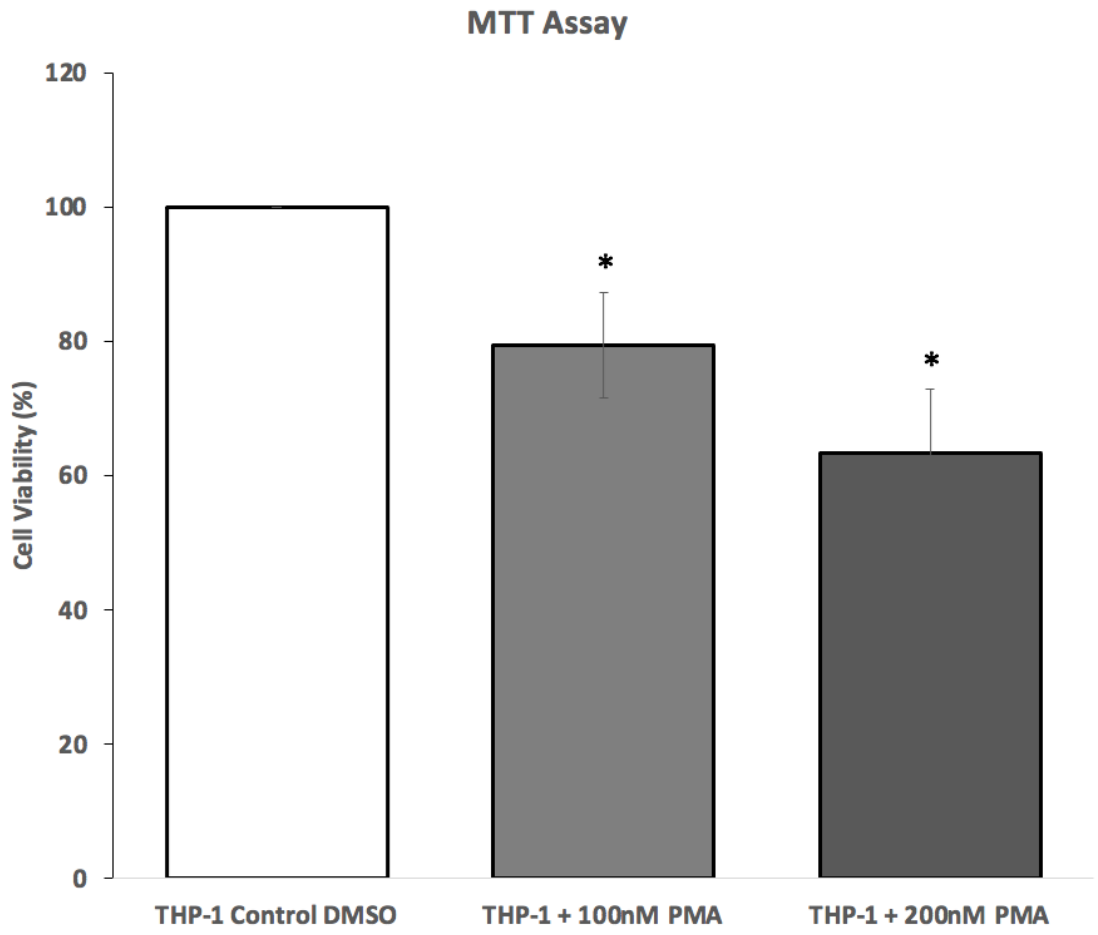

Figure 16: MTT Assay of THP-1 cells with DMSO, 100nM and 200nM of PMA. Cell metabolic activity and viability measured using a colorimetric MTT assay. Five replicates of each experimental condition. (A) Control THP-1 cells with DMSO have 100% viable cells, variance 0 (B) THP-1 cells with 100nM of PMA have 79.4% viable cells, variance 302.3 and (C) THP-1 cells with 200nM of PMA have 63.4% viable cells and variance 441.8. Standard error of mean for control cells is 0, THP-1 cells with 100nM of PMA 7.8 and THP-1 cells with 200nM of PMA 9.4. The asterisks (*) indicate a significant difference (P = 0.0107) between the THP-1 control DMSO cells and THP-1 cells with 100nM and 200nM of PMA.

4.1.6 Optimisation of Incubation Time of 100nM PMA in THP-1 Cells

After confirming 100nM of PMA with THP-1 cells produced the optimum rate of differentiation, time intervals of 24h, 48h and 72h were employed. Studies suggested incubation times varying from 24-72 hours is sufficient for THP-1 cell differentiation (Starr *et al.,* 2018). THP-1 cells only with DMSO were used as a control and THP-1 cells with 100nM of PMA in DMSO used as the experimental cells and observed every 24 hours under a light microscope. At 24 hours the monocytes start changing morphology from rounded to elongated. The THP-1 cells were confirmed to have sufficient cell differentiation with 100nM of PMA after 72h of incubation for this project (Figure 17.a, 17.b, 17.c).

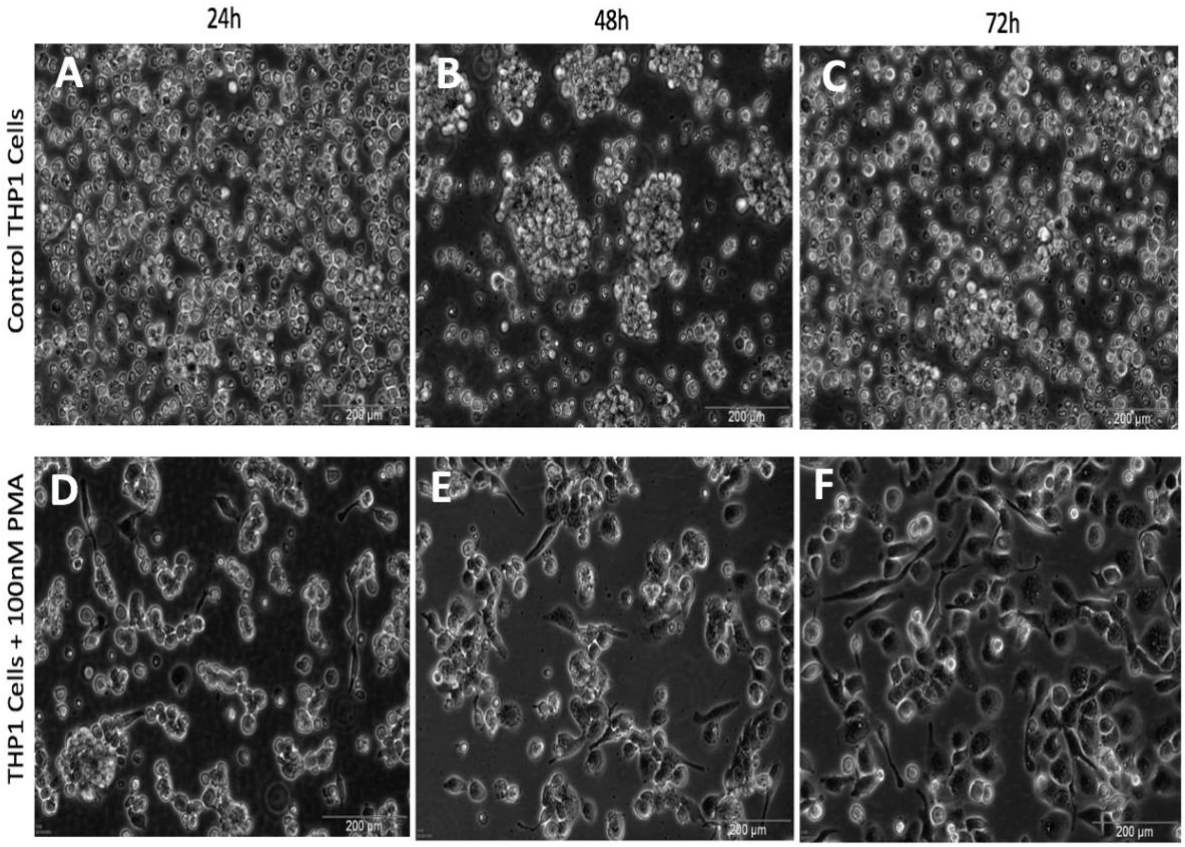

Figure 17.a: Differentiation of THP-1 monocytes into macrophages with 100nM of PMA at 24h, 48h and 72h. THP1 monocytes 1x 10^6 cell/mL differentiating into macrophages with 100nM of PMA. Magnification, x100. Control THP1 cells with DMSO and microscopy images confirm highly differentiated THP1 cells after 72h. At 24h the monocytes start changing morphology from rounded to elongated. After 48h over half the cells have differentiated into macrophages and at 72h nearly all monocytes have differentiated into macrophages with 100nM of PMA.

Optimum Differentiation of THP-1 Cells with 100nM of PMA at 72h

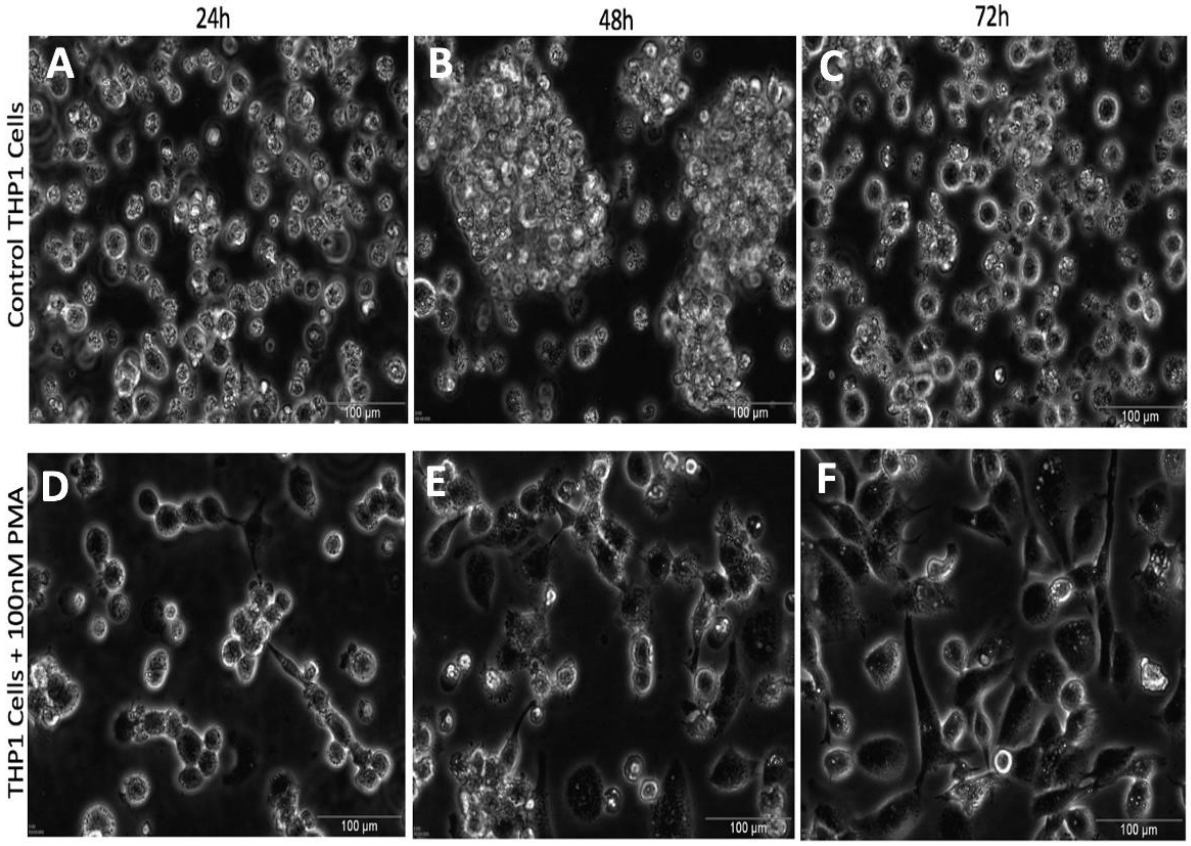

Figure 17.b: Differentiation of THP-1 monocytes into macrophages with PMA at 24, 48 and 72h X200. THP1 monocytes 1x 10^6 cell/mL with light microscopy x200 differentiating into macrophages with 100nM of PMA. THP-1 cells differentiated into macrophage-like cells after 72h. At 24h the THP-1 monocytic cells differentiate morphology from rounded into elongated cells. After 48h over half the cells differentiate into macrophages and at 72h nearly all monocytes have differentiated into macrophages with 100nM of PMA.

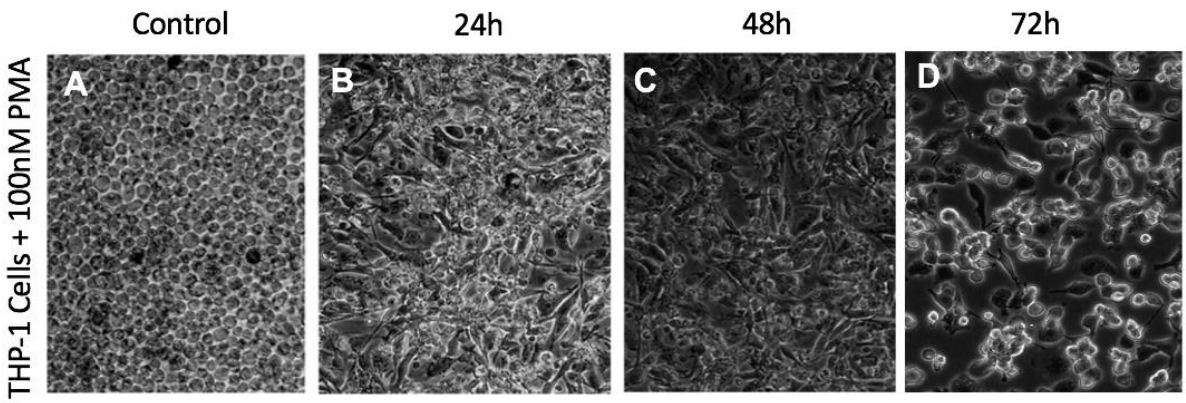

Figure 17.c: Differentiation of THP-1 monocytes into macrophages with PMA at 24, 48 and 72h. Light microscopy x100 of THP-1 monocytes 1x 10^6 cell/mL differentiating into macrophages with 100nM PMA. Control THP1 cells with DMSO and microscopy images confirm differentiated THP-1 cells after 72h nearly all monocytes have differentiated into macrophages with 100nM PMA.

4.1.7 Coomassie Staining THP-1, Macrophage and Microvesicle Lysate Samples

Samples were loaded for gel electrophoresis and coomassie blue staining using THP-1 cells, macrophage and EV lysates. The results show numerous protein bands with THP-1 cell and macrophage lysate. However, the MV lysate only presented a few bands because the sample is not as concentrated as the THP-1 cell and macrophage lysates. Albumin was present in the samples as seen below in Figure 18 as the THP-1 cells require incubation with serum free RPMI 1640 medium before isolating EVs. Therefore, isolated EVs must be pooled together with the absence of albumin to produce a more concentrated sample which displays multiple protein bands throughout the gel.

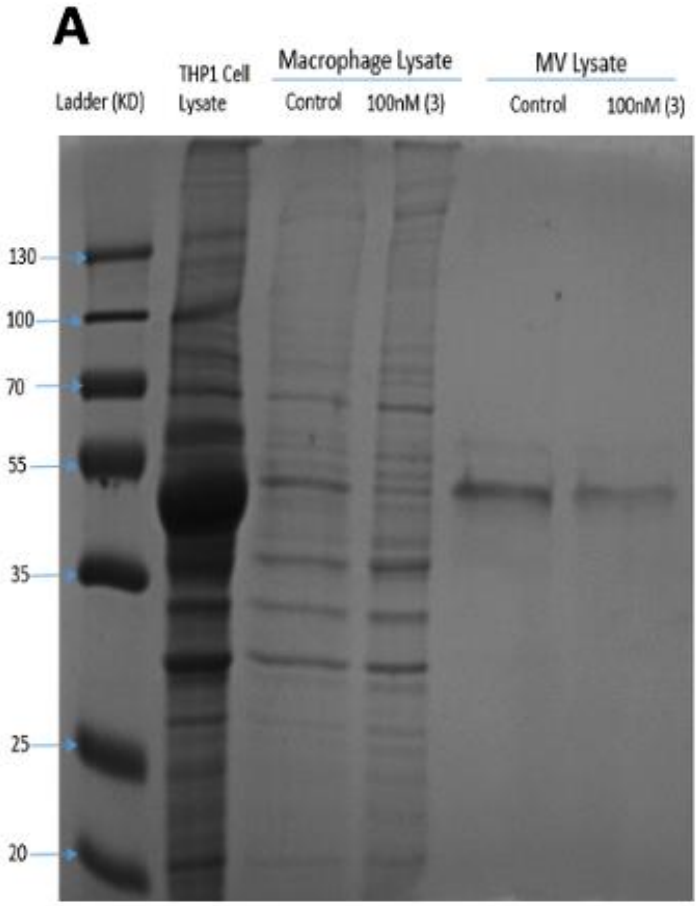

Figure 18: Gel electrophoresis of EV, macrophage and THP-1 lysates. (A) Samples loaded with 50µg of protein concentration per well and THP1 cell lysate 250µg**.**

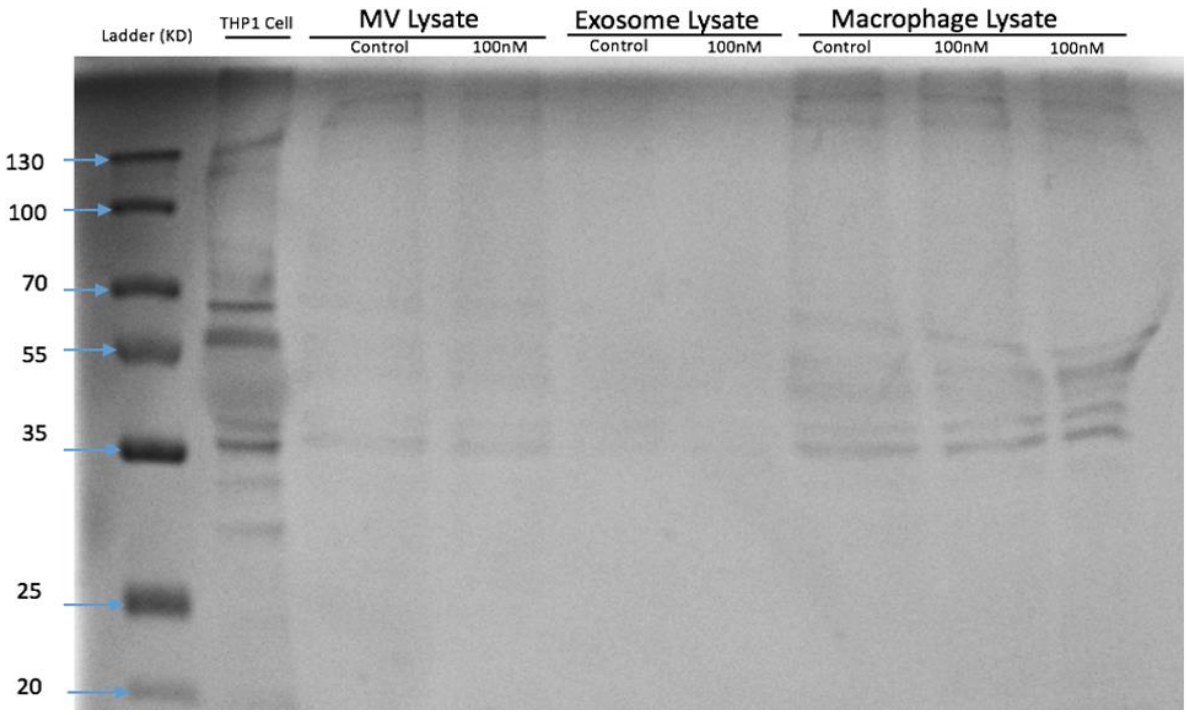

Figure 19: Coomassie stained gel of THP-1 cells, Extracellular Vesicles and Macrophage Lysate. Gels stained in coomassie blue dye overnight and de-stained for protein bands. Ladder range 10-130 kDa. Protein bands for THP1 cell lysate 200µg, Control EVs and EVs with 100nM of PMA 20µg and control macrophages and macrophages with 100nM of PMA loaded with 20µg protein concentration per well. Main protein bands present between 35-130kDa for lysate samples.

The protein concentration loaded per well was 20µg as the volume was not suitable for loading with 50µg per well. However, this was sufficient to give the consistent bands throughout the gel we were expecting. Therefore, the next step would be to isolate more EVs to send for proteomic analysis of the samples.

4.1.8 Potential Next Research Steps of THP-1 Differentiated Cells with PMA

The objective of differentiating THP-1 cells with PMA was achieved using the optimum concentration of 100nM of PMA and 72h incubation. The next step from the results gathered include isolating EVs from the supernatant of these differentiated cells. The aim of the steps is to define the role of EVs in cell differentiation and understand their functions in cell signalling and differentiation by isolating EVs from the differentiated THP-1 cells. This research is novel and will add knowledge to the functions of EVs in cell communication during cell differentiation. This links with the research of cell viability as the differentiated THP-1 cells with 100nM of PMA will be isolated for EV analysis. Several protocols of EV isolation were experimented and an optimised protocol (Figure 10) was produced using an ultracentrifugation method and several isolation processes before producing a pure Microvesicle or exosome pellet. Isolation of these EVs has a disadvantage of being very small (microvesicles) or invisible (exosomes) to the naked eye. Therefore, samples need to be concentrated and pooled together to produce a concentrated EV pellet. This was conducted using the optimised EV protocol diagram as seen in Figure 10 and THP-1 differentiated cells with 100nM of PMA were pooled together for EV isolation as the next step in the process.

4.1.9 Pooling Differentiated THP-1 cells for Isolation of Extracellular Vesicles

The optimised protocol for isolating EVs was established after repeated experiments produced low concentrations of EV isolates. Therefore, THP-1 cells 1 x 10^6 cells/mL were differentiated with 100nM of PMA and the supernatant from these cells was pooled together for EV isolation. As seen in figure 5 below the THP-1 cells were differentiated into macrophage-like cells with 100nM of PMA with the expected morphology change. The samples were pooled together will

7mL in each T25 cell culture flask the supernatant combined to 28mL and this produced a concentrated EV pellet within the range of 30-50μg. This was also analysed on a coomassie gel for protein bands, western blot for specific proteins of EVs such as GAPDH and actin.

To advance this, samples of gel electrophoresis may be sent for further analysis with proteomics for a full protein profile. This reflects the research direction and potential of my results from the current findings.

Pooling Differentiated THP-1 Cells for Extracellular Vesicle Isolation

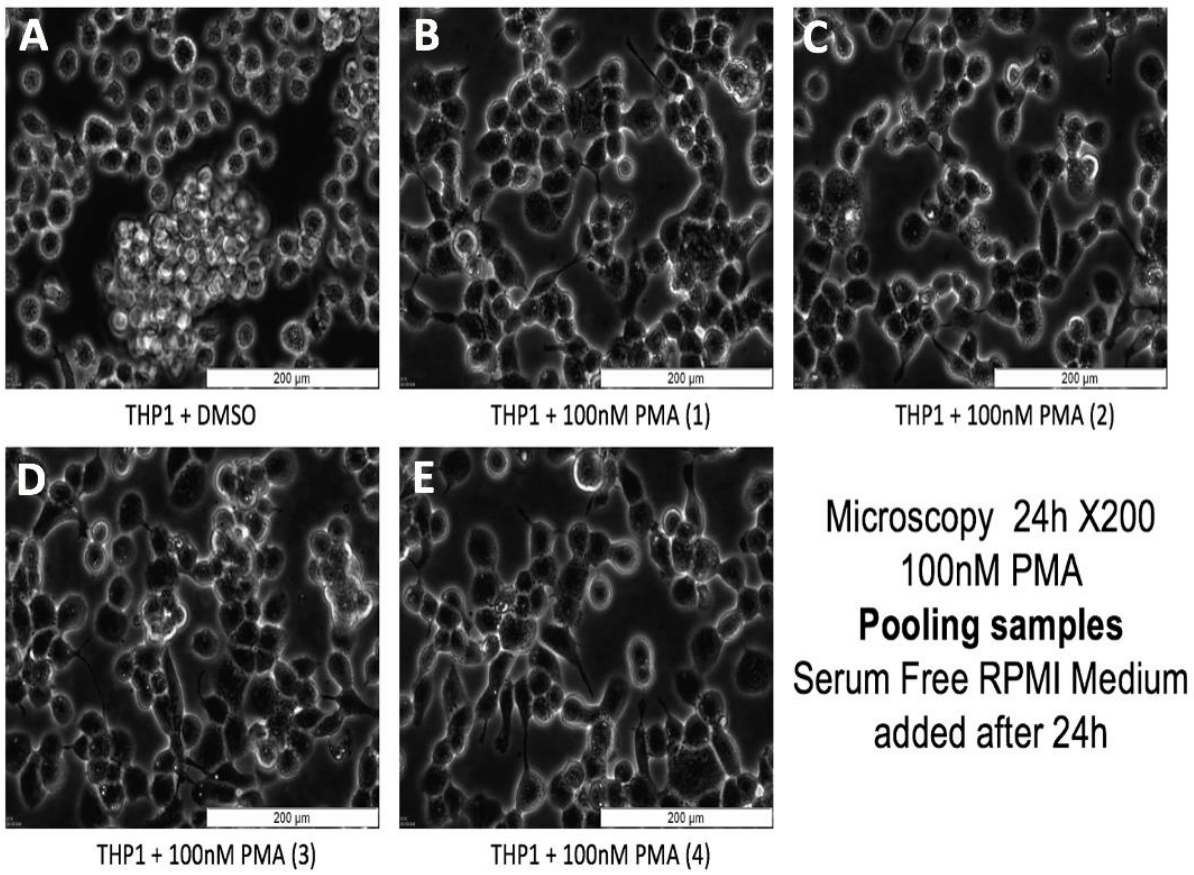

Figure 20: Pooling differentiated THP-1 cells with 100nM of PMA. THP-1 cells 1 x10^6 cell/mL were cultured in 100nM of PMA for 72 hours using four T25 cell culture flasks. The supernatant from all four flasks were combined and the samples pooled together after 72h to produced concentrated EV isolates.

4.1.10 Microvesicle Sizing Characterisation

Flow cytometry is commonly used to confirm the presence of MVs in research. MV isolates from 100nM of PMA were confirmed using sizing beads in the ranges of 0.2µm-2µm to cover the size range of MVs. The elliptical shape seen in Figure 21 shows the presence of MVs between the first sizing bead 0.2 µm and 1.5µm. This is typical of the size of MVs and this confirms characterisation. This finding also confirmed that the optimum method as seen in Figure 10 is reliable for isolating MVs.

Flow cytometry of Microvesicles with Sizing Beads

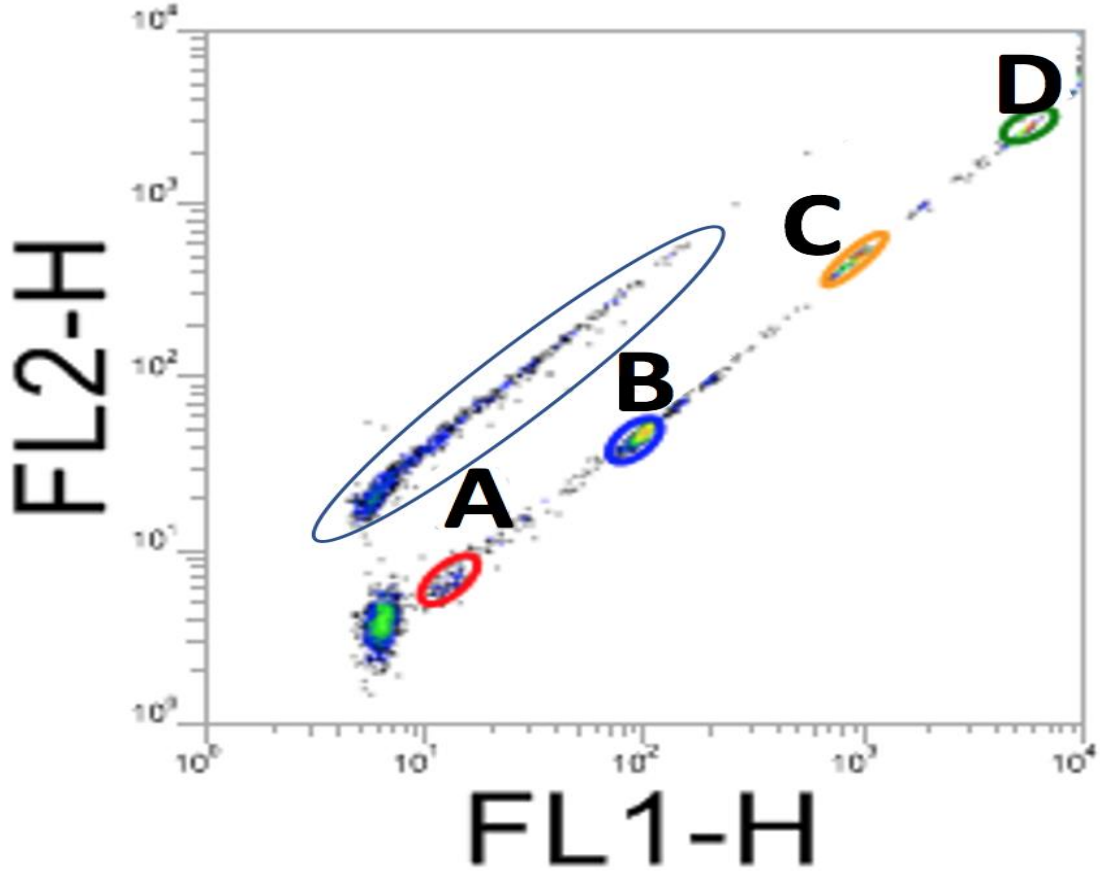

Figure 21: Flow cytometry of Microvesicles. MV isolates from THP-1 cells treated with 100nM of PMA with sizing beads (A) 0.2µm**,** (B) 0.5µm, (C) 1µm, (D) 2µm. The presence of MVs seen between the sizing beads 0.2-1µm within their size range (Circled above).

Figure 21 shows the distribution of MVs for their size and granularity and therefore characterisation of MVs treated with 100nM of PMA. All MVs with 100nM of PMA display the same distribution and the results are consistent within all the samples. The expected size of MVs is between 100nm and 1000nm and Figure 21 represents this range. MVs were only analysed on the flow cytometer as detection of microparticles below 100nm are not detectable

and sensitivity is reduced, Moreover, a Nanosite is necessary for the characterisation of exosomes for the next steps in the project.

4.1.11 Fluorescence in Annexin V Labelled Microvesicles

As seen in Figure 22 and 23 the THP-1 derived MVs labelled with Annexin V bind to the outer surface phosphatidylserine marker of MVs and produce a positive distribution of MV cells. They are further analysed for their change in fluorescence when labelled with Annexin V and as seen below in Figure 22 the THP-1 derived MVs produce a clear green fluorescence when labelled with Annexin V. This confirms the phosphatidylserine on the MVs surface binding to Annexin V.

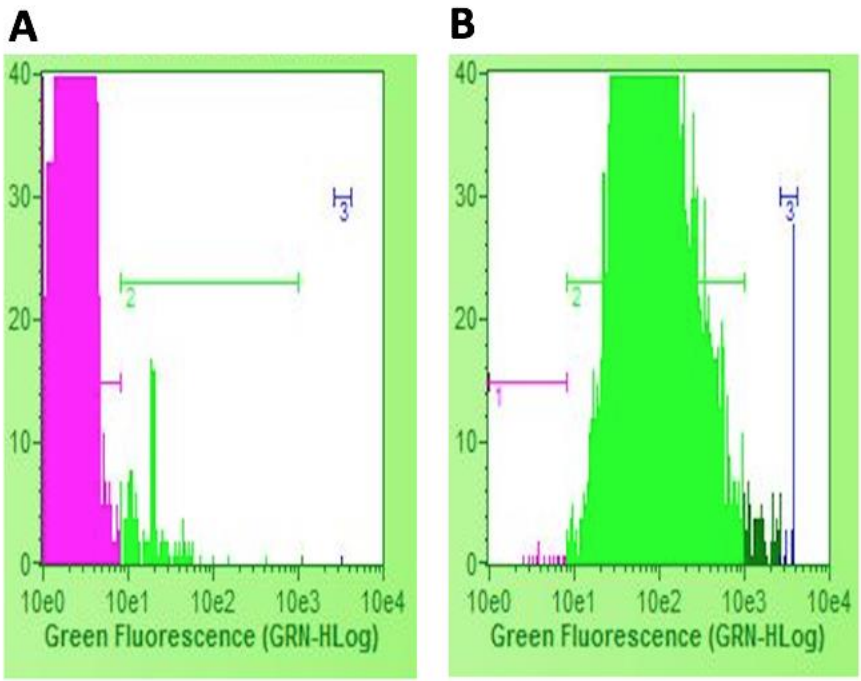

Figure 22: Flow cytometry of THP-1 derived Microvesicles Annexin V fluorescence. (A) MV isolates without Annexin V labelling and not expressing green fluorescence. (B) Graph A unlabelled MV isolates marker 1; 95.98%, marker 2; 3.98% and marker 3; 0.02%. Graph B labelled MV isolates with Annexin V marker 1; 0.32%, marker 2; 96.98%, marker 3; 0.92%. Confirms the presence of Annexin V binding to phosphatidylserine on outer MV surface expressing a green fluorescence with 96.98% of cells expressing a green fluorescence.

4.1.12 Phosphatidylserine Detection with Microvesicles using Annexin V

Intercellular communication is the most researched function identified in MVs. The present study focuses on the cell communication between macrophage EVs and liver hepatoma cells in understanding the cross-talk within a cell culture environment which has clinical implications once further understood. Various markers can be used in western blotting and flow cytometry including tumour susceptibility gene 101 and flotillin being two of the many surface markers used to identify exosomes. Also, phosphatidylserine (PS) is translocated to the

outer leaflet during the membrane shedding process through the inaction of flippase and floppase. Therefore, PS is a marker which can be used in flow cytometry to confirm the presence of MV isolates while labelling MV samples with Annexin V.

4.1.13 Flow Cytometry of Extracellular Vesicles with Annexin V Labelling

Annexin V labelling positive for MV isolates with an elliptical peak as seen in Figure 23 and a shift to green fluorescence as seen in Figure 22. This confirms the presence of MVs and the externalisation of the phosphatidylserine from the inner to the outer leaflet of the THP-1 cell, a positive marker of MVs.

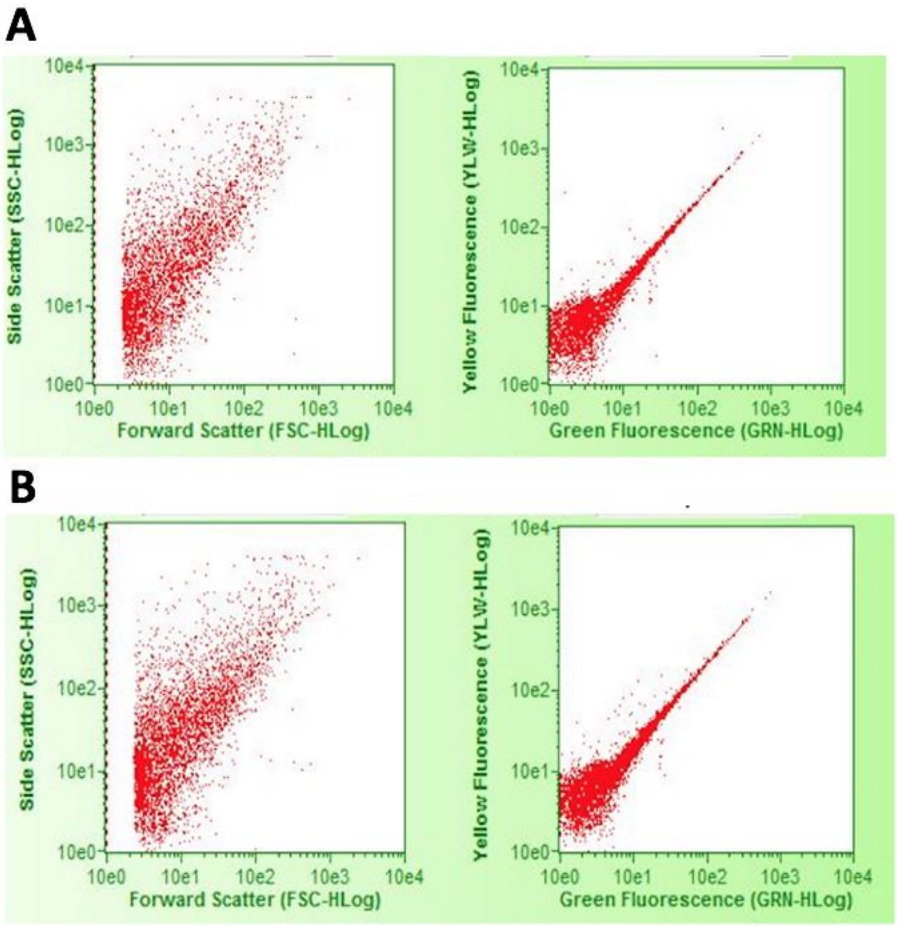

Figure 23 : Annexin V labelling of THP-1 derived Microvesicles. THP-1 derived MVs labelled with Annexin V producing an elliptical positive distribution on the flow cytometer with phosphatidylserine MV marker present in the upper and lower panel. MV samples labelled with Annexin V produced the repeated result and were positively present for Annexin V binding to phosphatidylserine to the outer surface of the MVs

4.2 Differentiating THP-1 cells with a conditioned cell culture medium derived from HUH7 cells produced macrophage-like cells

4.2.1 THP1 cells with HUH7 conditioned medium producing new THP-H cell line

The aim of this experiment was to compare a novel THP-1 cell differentiation method to the traditional well-established PMA-induced macrophages protocol. A method was developed whereby THP-1 cells were cultured in a conditioned medium, HUH7, to observe differentiation of THP-1 cells into macrophage-like cells.

The method consisted of a 6-well plate as seen in Figure 24 to culture THP-1 cells 2 x 10^5/mL incubated with HUH7 supernatant conditioned medium of 1mL and 1.5mL in wells 2 and 5 as seen in Figure 24. The negative control was THP-1 cells only (Well 1 Figure 24) and the positive control was THP-1 cells and 100nM PMA (Figure 24, well 4). A total of 4.5mL final volume was constant in each well, filling the wells with complete growth RPMI medium.

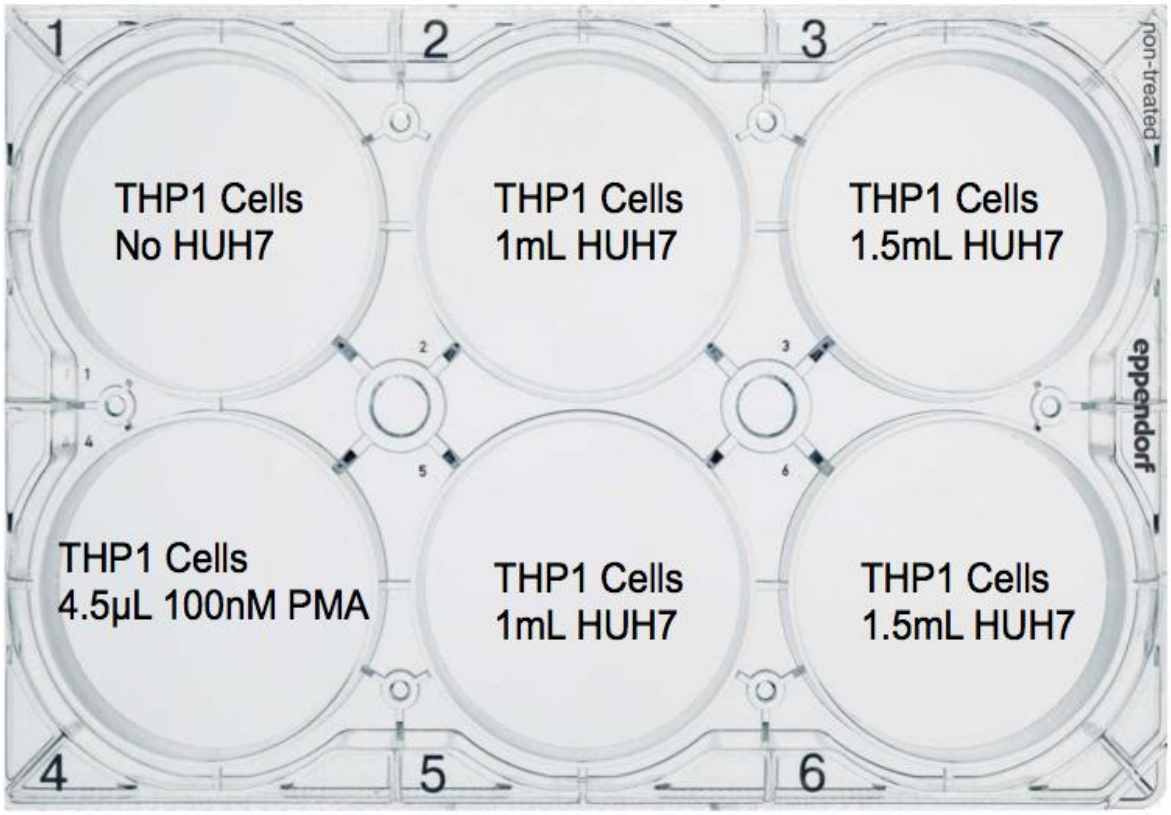

Figure 24: THP-1 cells in HUH7 conditioned medium. A 6-well cell culture plate was used to investigate the THP-1 cell differentiation in a conditioned cell culture medium. Well 1, THP-1 cells (negative control), Well 2 and 5, THP-1 cells with 1mL conditioned medium, Wells 3 and 6, THP-1 cells with 1.5mL conditioned medium and Well 4, THP-1 cells with 100nM of PMA (positive control).

4.2.2 THP-1 Cell Differentiation with a Conditioned Medium

The cells were monitored and visualised under light microscopy every 24 hours. The THP-1 cells started to differentiate after 24 hours as seen in Figure 25a in well 6 with THP-1 cells and 1.5mL conditioned medium. The cells were further monitored every 24 hours to observe the rate of differentiation and confluency.

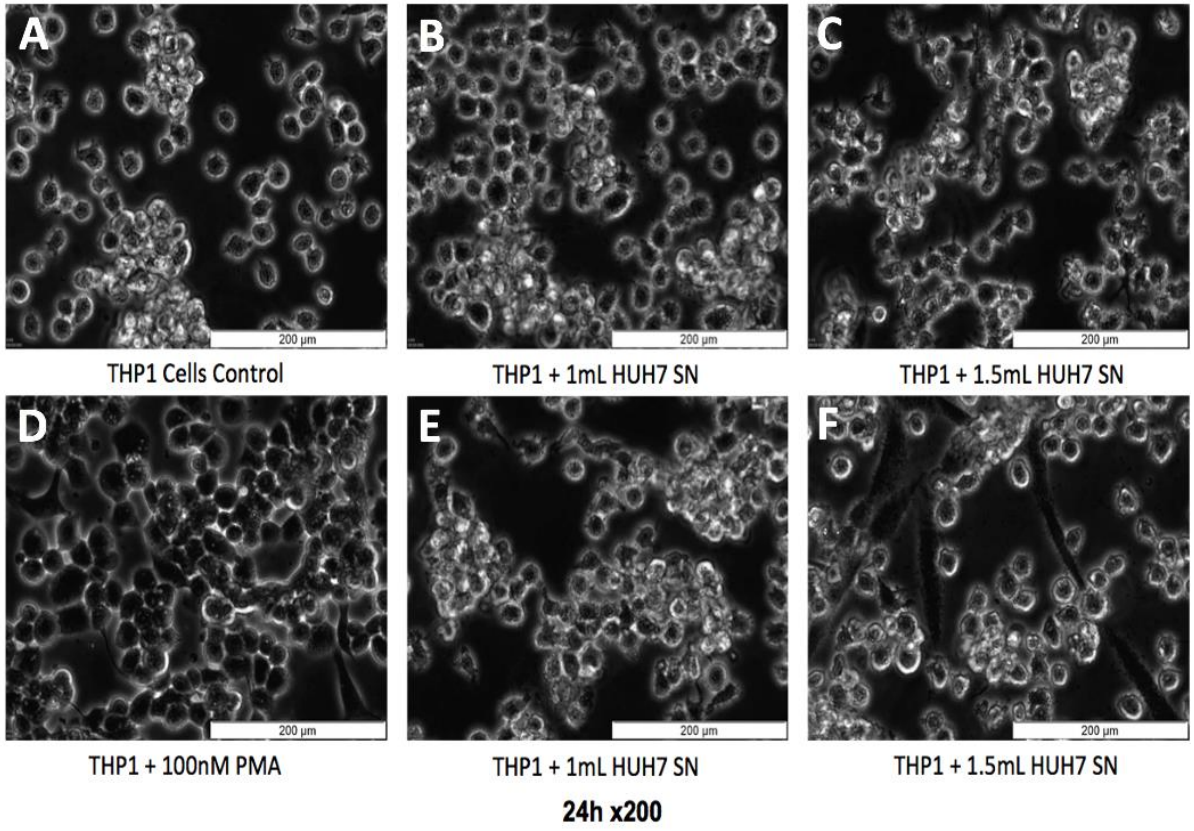

Figure 25.a: THP-1 cells with a conditioned medium after 24h. Magnification, x200. (A) THP-1 cells only (control), (B) THP-1 cells with 1mL conditioned medium, (C) THP-1 cells with 1.5mL conditioned medium, (D) THP-1 cells with 100nM of PMA (positive control), (E) THP-1 cells with 1mL of conditioned medium and (F) THP-1 cells with 1.5mL of conditioned medium. Cells have started differentiation with 1.5mL HUH7 addition and positive control cells with 100nM of PMA have started changing morphology into macrophage-like cells.

After 48 hours, light micrographs as seen in Figure 25b showed significant numbers of cell in all wells. A monolayer of loosely adherent THP-1 cells could be observed at the bottom of the flask, in addition to freely floating cells (Figure 25b (A)), but these were round and not strongly adherent or elongated. The HUH-7- treated THP-1 cells differentiated into macrophage-like cells resembling the THP-1 cells shows a clustered pattern and in a similar morphology to PMA-treated THP-1 cells.

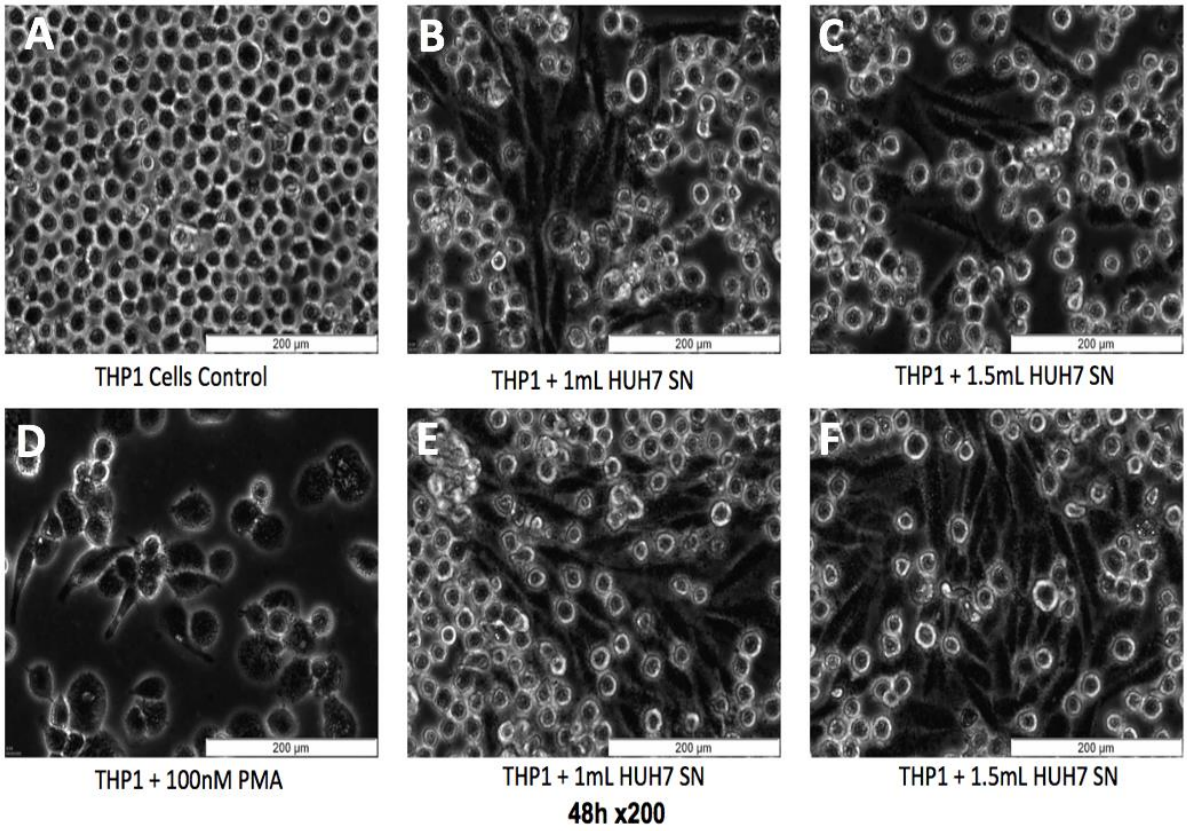

Figure 25.b: THP-1 cells with a conditioned medium after 48h. Magnification, x200. (A) THP-1 cells only (control), (B) THP-1 cells with 1mL conditioned medium, (C) THP-1 cells with 1.5mL conditioned medium, (D) THP-1 cells with 100nM of PMA (positive control), (E) THP-1 cells with 1mL of conditioned medium and (F) THP-1 cells with 1.5mL of conditioned medium. THP-1 cells differentiated in clusters with all conditioned medium. THP-1 cells treated with 100nM of PMA, positive control cells have mostly differentiated into macrophage-like cells.

After incubation for 72 hours later the light micrographs as seen in Figure 25c show the development of new clusters of macrophage-like HUH-7 treated THP-1 cells. Growth of HUH-7-treated THP-1 cells continued on the clustered pattern and a good coherence of cells was visualised in the same wells.

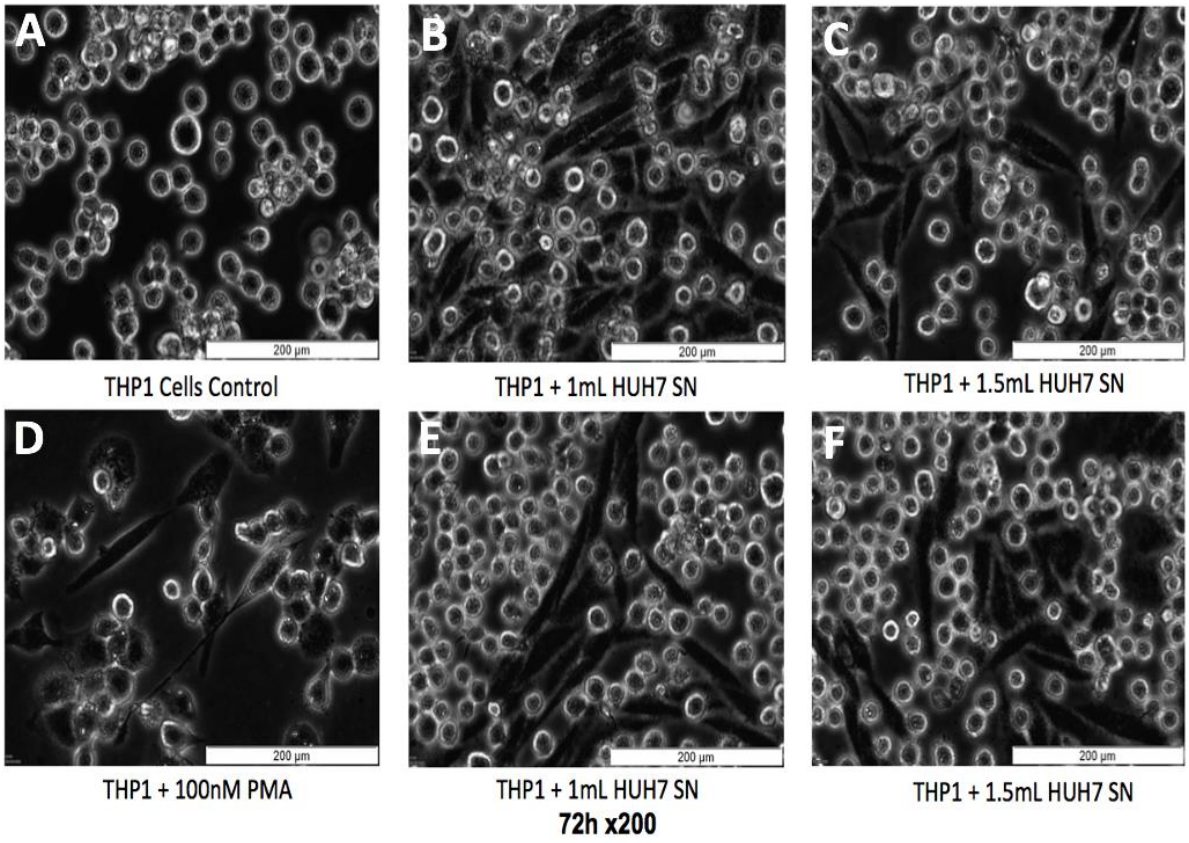

Figure 25.c: HUH-7 treated THP-1 cells after 72h. Magnification, x200. (A) THP-1 cells only (control), (B) THP-1 cells with 1mL conditioned medium, (C) THP-1 cells with 1.5mL conditioned medium, (D) THP-1 cells with 100nM of PMA (positive control), (E) THP-1 cells with 1mL of conditioned medium and (F) THP-1 cells with 1.5mL of conditioned medium. Cells have further differentiated in clusters in all conditioned medium wells. THP-1 cells with 100nM of PMA, positive control cells have differentiated into macrophages and THP-1 cells only are growing and healthy in cell culture.

After 96 hours, the light micrographs images as seen in Figure 25d show the increased confluency of HUH-7-treated THP-1 cells. Growth of these cells is close to full confluency and required another 24 hours incubation before passaging or freezing. The growth continued in clusters with almost all cells changing shape from rounded to elongated cells, characteristic of macrophage-like cells as seen in the positive control THP-1 cells with 100nM of PMA.

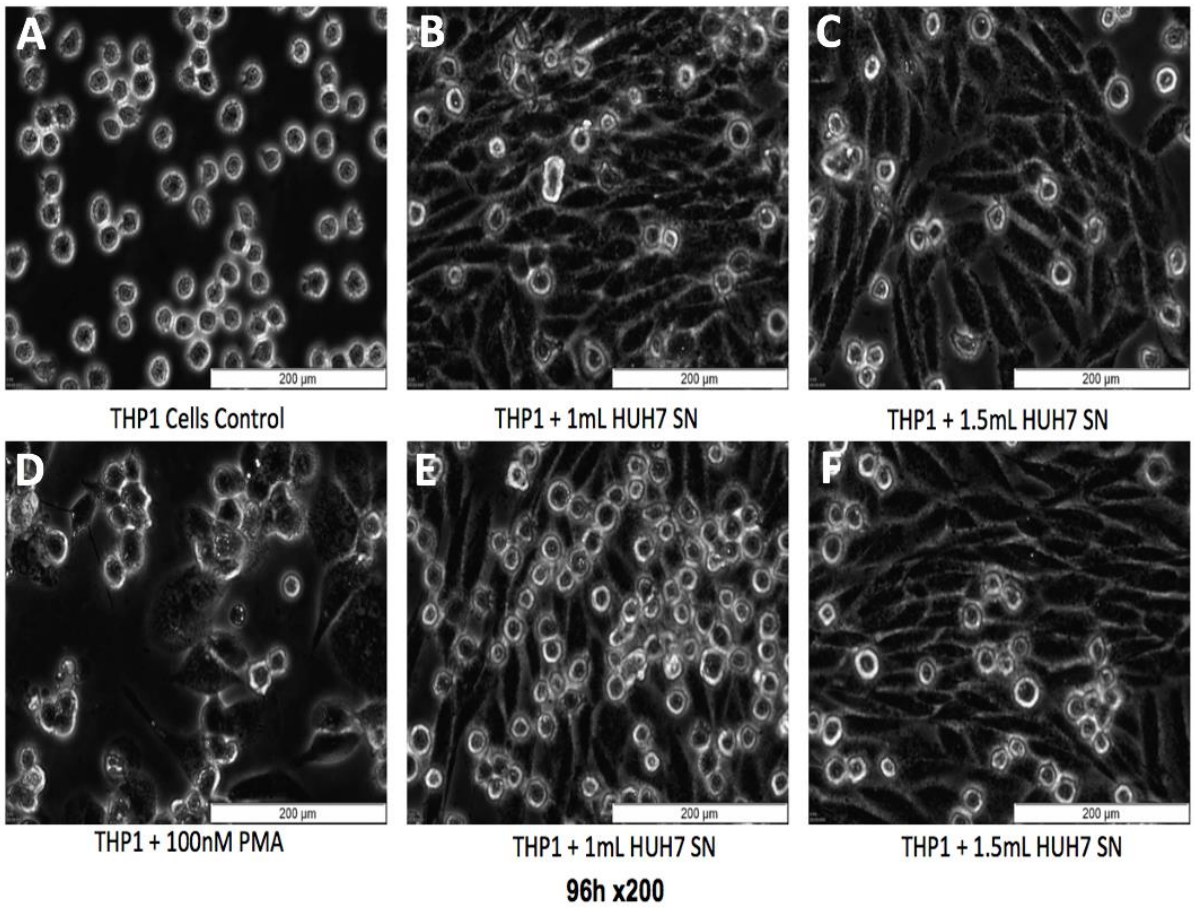

Figure 25.d: THP-1 cells with a conditioned medium after 96h. Magnification, x200. (A) THP-1 cells only (control), (B) THP-1 cells with 1mL conditioned medium, (C) THP-1 cells with 1.5mL conditioned medium, (D) THP-1 cells with 100nM of PMA (positive control), (E) THP-1 cells with 1mL of conditioned medium and (F) THP-1 cells with 1.5mL of conditioned medium. Cells have differentiated further into larger clusters of macrophage-like cells in all conditioned medium wells. THP-1 cells treated with 100nM of PMA, positive control cells, remain differentiated into macrophages and control THP-1 cells only are growing and remain rounded and undifferentiated in morphology in cell culture.

A total of 120 hours after incubation of HUH-7-treated THP-1 cells had fully differentiated into macrophage-like cells and reached approximately 90% confluency. The light micrographs as seen in Figure 25e show HUH-7-treated THP-1 cells which requires passaging into a 75cm^3 cell culture flask for further growth before further analysis or freezing. Growth of the HUH-7-treated THP-1 cells identified a new cell line of THP-1 differentiated cells named THP-H. This is a permanent, potentially macrophage-like, cell line developed during this experiment and further growth and analysis is necessary to identify and characterise the THP-H cell line accurately.

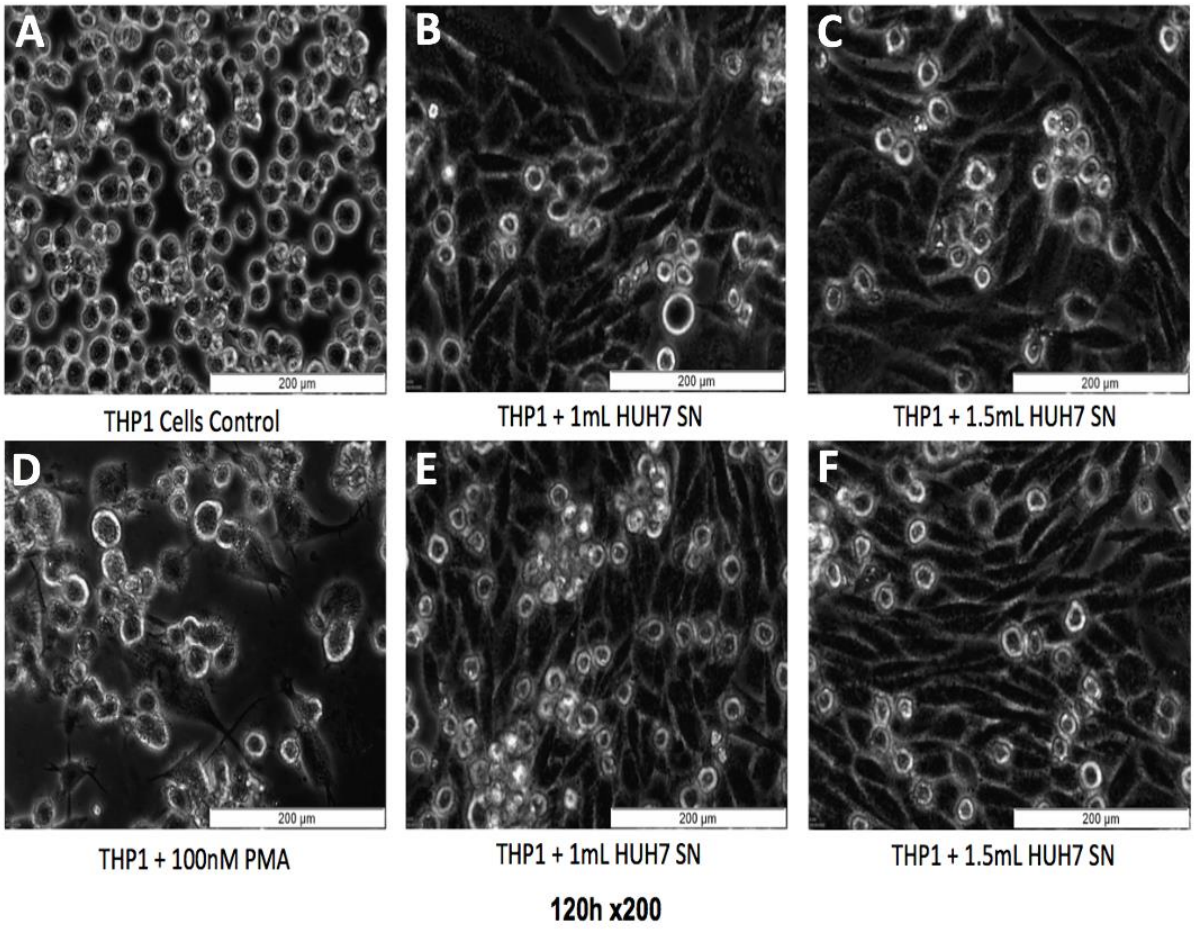

Figure 25.e: THP-1 cells with a conditioned medium after 120h. Magnification, x200. (A) THP-1 cells only (control), (B) THP-1 cells with 1mL conditioned medium, (C) THP-1 cells with 1.5mL conditioned medium, (D) THP-1 cells with 100nM of PMA (positive control), (E) THP-1 cells with 1mL of conditioned medium and (F) THP-1 cells with 1.5mL of conditioned medium. Cells have differentiated fully in the 6 well plate into large clusters of macrophage-like cells in all conditioned medium wells. THP-1 cells with 100nM of PMA, positive control cells have differentiated into characteristic elongated macrophage cells and THP-1 cells only are healthy rounded and growing in bunches in cell culture.

After 120 hours the media of THP-H cells was removed with RPMI 1640 cell culture medium, washed with PBS and trypsinised to transfer the THP-H cells (HUH-7-treated THP-1 cells) into a larger cell culture flask. As seen in Figure 26a, after 24 hours the THP-H cells have adhered to the cell culture flask and novel in morphology however resemble PMA treated THP-1 cells. They were left to grown in complete growth medium for 7 days and the culture medium was changed every 48 hours. After 7 days, as seen in Figure 26b, the THP-H cells were fully confluent in a 75cm^3 cell culture flask and ready for further passaging using trypsin-EDTA or frozen in liquid nitrogen using a freezing liquid onto the cell pellet.

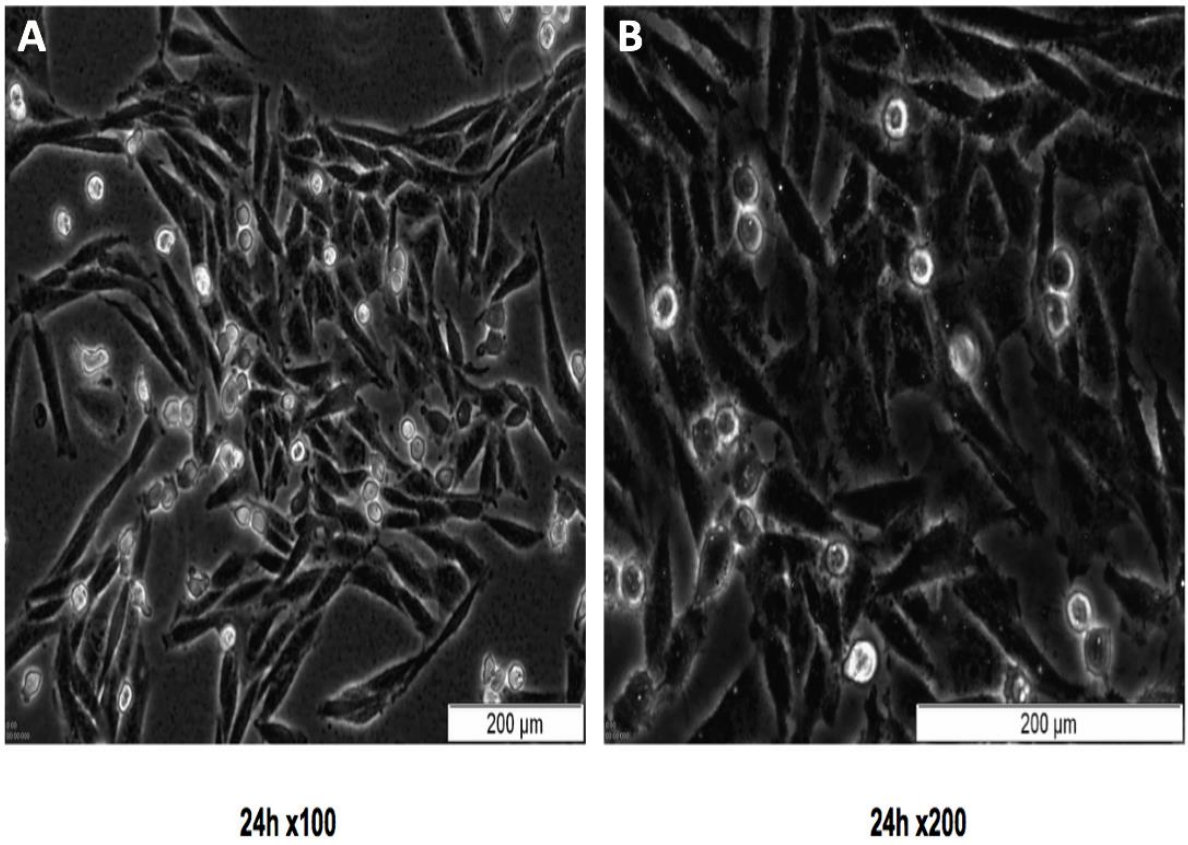

Figure 26.a: THP-H cell line. After 24h in complete growth medium THP-H cells have adhered and growing in cell culture. Magnification, x100 (A), x200 (B). from a 6 well cell culture plate into a 75cm^3 cell culture flask. Cells are healthy and have started growing in culture after 24 hours.

As seen in Figure 26b, after 7 days, the THP-H cells are fully confluent in a 75cm^3 cell culture flask and ready for further analysis or storage. The next steps for this research objective was to fully characterise this novel THP-H cell line in the following ways; using microscopy to assess morphology, using PCR for a genetic profile comparison to THP-1 and HUH-7 cells, creating a proteomic profile using an EV isolation method and using gel electrophoresis with proteomics analysis to identify novel proteins.

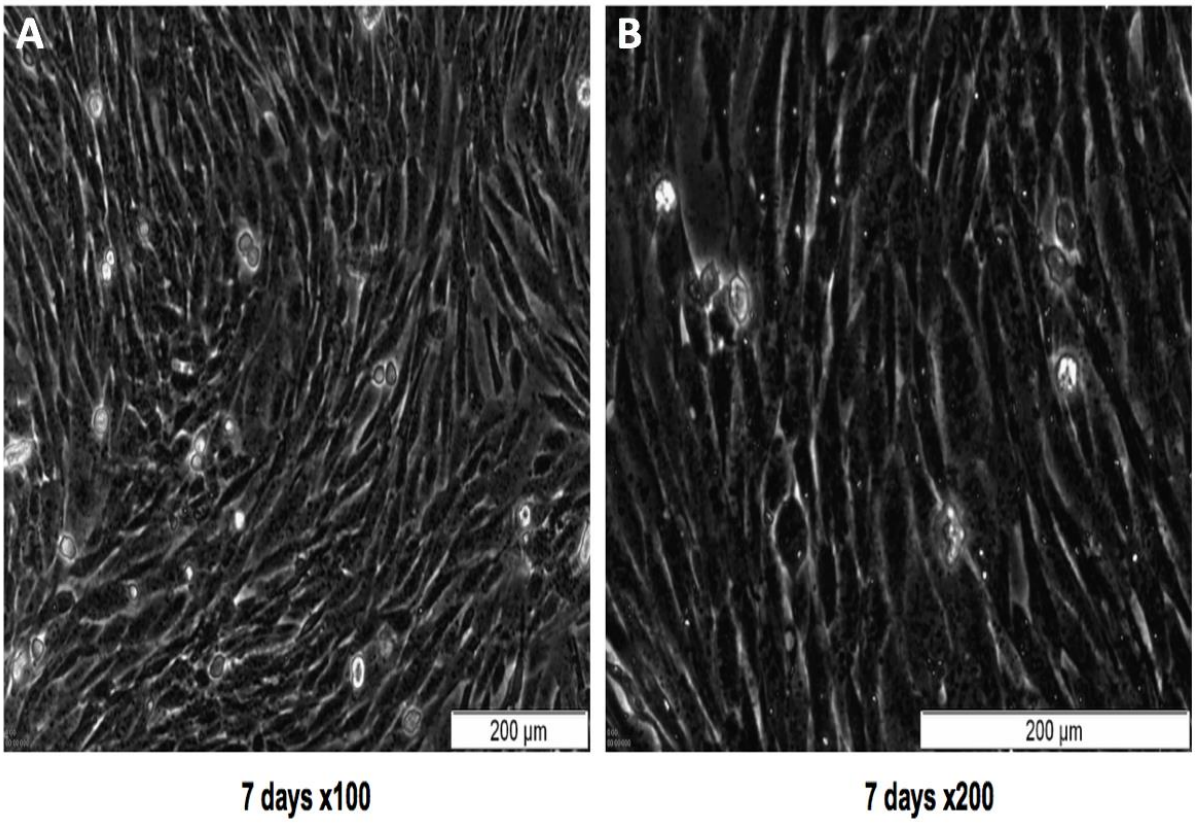

Figure 26.b: THP-H cell line. After 7 days in complete growth in a 75cm^3 cell culture flask. Magnification, x100 (Left), x200 (Right).Cells have reached full confluency in culture and the start of a new permanent cell line established.

The results indicate that THP-1 cells were initiated to differentiate into macrophage-like cells when cultured in a conditioned medium. This produced a permanent macrophage cell line named THP-H, a novel finding of the present study, which has future research prospects such as further elucidating cell communication.

4.3 Summary of results

To summarise the results, the first objective of this study was achieved by differentiating THP-1 cells with 100nM of PMA for 72h incubation to produce M0-type macrophages. This is a well-established methodology for THP-1 cell differentiation and the concentration of 100nM of PMA was used after several preparations and repeats of 50nM and 200nM were compared during microscopy, MTT assay and protein concentrations using a BCA assay. Therefore, accumulation of the data suggested 100nM of PMA with 72h incubation produced sufficient cell differentiation.

The second objective of this study was achieved by differentiating THP-1 cells with a conditioned cell culture medium derived from HUH7 cells to produce macrophage-like cells, which was a novel method proposed by this study.

The hypothesis was that the THP-1 cells may differentiate into macrophage-like cells similar to PMA-treated THP-1 cells when treated with a cultured medium, which was observed after 24h. HUH-7-treated THP-1 cells were named THP-H cells and have an advantage over PMA treated THP-1 cells as the THP-H is a permanent macrophage cell line and PMA treated THP-1 cells are short-lived and produced in an *in vitro* environment, meaning they cannot be replicated or compared to the physiological conditions within the human body. THP-H cell are therefore more comparable and replicate similar physiological conditions within the body.

5. DISCUSSION

THP-1 monocytic cells are widely used to study monocyte and macrophage functions and biology. PMA is a tumour promoter chemical often used to stimulate THP-1 cell differentiation into macrophage-like cells. They mimic the human macrophages in terms of cell morphology, cytokine production and macrophage surface markers (Meijer *et al.,* 2015). However, varying concentrations and incubation times with PMA are used in studies (Lund *et al.,* 2016; Starr *et al.,* 2018). Within this research project the widely used method of differentiation with PMA was studied with 100nM of PMA and an incubation time of 72h after several experiments of optimisation (Park *et al.,* 2007). In agreement with many experiments a reduced PMA concentration enhanced THP-1 cell differentiation (Maeß *et al.,* 2014). This was the first method used to compare different methods of THP-1 monocyte differentiation into macrophages. This project aimed to compare the widely used method of PMA-treated THP-1 cells with a conditioned medium to differentiate THP-1 cells. The latter is a novel method of THP-1 cell differentiation and has been developed to advance the field of cell communication.

5.1 Objective 1: Differentiating THP-1 cells with phorbol 12-myristate-13-acetate (PMA) to produce M0-type macrophages

The first part of the discussion will analyse the first research objective of THP-1 cell differentiation with PMA. After analysing research on using PMA for cell differentiation, varying concentrations were used ranging from 50-200nM of PMA. Therefore, the first

experiment as seen in Figure 11 of the results analyses the THP-1 cells for the optimum concentration of PMA (Lund *et al.,* 2016). After 72h incubation the results produced morphologically distinct macrophage-like cells with all concentrations however the cells with 100nM of PMA had sufficient cell differentiation but further experiments would be necessary to distinguish between 50nM, 100nM and 200nM of PMA as the optimum concentration for differentiating THP-1 cells. Research suggests varying times of incubation of 24-120h (Starr *et al.,* 2018). After several experimental repeats of cell differentiation with all three PMA concentrations the results were consistent and a time interval had to be optimised. Studies use varying PMA concentrations to differentiate THP-1 cells. Concentrations vary from 5ng/ml of PMA (Re, 2017) and 100ng/ml of PMA (Maeß *et al.,* 2014; Zou *et al.,* 2017) as being sufficient for cell differentiation. Therefore, ranges between 5-400ng/ml of PMA are used with varying incubation times between 24-72h (Park *et al.,* 2007) similar to this present study.

Studies with THP-1 cell differentiation used PMA concentration in the range of 10-400ng/mL of PMA and incubated with PMA between 24h-120h (Park *et al.,* 2007). However, high concentrations of PMA are rarely used as they can upregulate the expression of certain genes in differentiated macrophages. This can overwhelm gene expression increases induced by different stimuli. This research focused on designing an experiment to confirm the optimum PMA concentration whilst minimising gene upregulation.

5.2 Extracellular Vesicles

EVs were used in cell communication and intracellular communication. Researching EVs would provide novel data for understanding how EVs assist in the process of cell differentiation and if they play a role of signal transfer or provide assistance through certain proteins and cytokines. Exosomes can promote tumour growth by improving the migration capacity of cancer cells and boosting an immunosuppressive state by inducing mediators such as IL-10 in macrophages (Chung *et al.*, 2007). This makes exosomes potential therapeutic targets for modulating the tumour microenvironment (Bellmunt *et al.,* 2019). EVs have been studied in various literature and cell types for transferring signals, cargo and surface markers from one cell type to another (Tkach & Théry, 2016; Cesi *et al.,* 2016). As suggested by Cesi and colleagues various diseases such as cancer, the levels of EV secretion is altered producing different amounts of vesicle cargo molecules and their profiles can be used to evaluate biomarkers. Transfer of EVs to surrounding cells are involved in the propagation of phenotypic traits. These key properties place EVs into the focus of many recent studies (Cesi *et al.,* 2016).

The Various functional implications proposed for EVs makes it vital to further understand the vesicles themselves. Most studies published analyse mixed EV populations, the next step in this field is to comprehensively compare the different sub-types of EVs and to determine whether some of their key functions are specific or prominent to a given subtype such as exosomes but not present in other EVs. This knowledge is imperative for identifying the necessary EV subtypes for any therapeutic approach (Tkach & Théry, 2016).

Saha and colleagues demonstrated EVs released by cells can function as vehicles for delivering biological material and signals. The data showed alcohol-treated hepatocytes can cross-talk with immune cells with exosomes containing microRNA (miRNAs) (Saha *et al.,* 2016). Therefore, further research of EVs involved in THP-1 cell differentiation can contribute to the knowledge of understanding the cell communication of EVs during cell differentiation.

The ideal method of isolating EVs should be simple, inexpensive, fast and not require equipment which is expensive or complex and allow isolation of EVs from large quantities of samples. The majority of researchers use the ultracentrifugation method with modifications (66.6%), this being the desired method in many EV experiments, resulted in this present study to use the same method for EV isolation (Konoshenko *et al.,* 2018). In order to isolate EVs from the supernatant of these differentiated cells, various methods were researched for the optimum speed and time of EV isolation, and an optimised ultracentrifugation method was developed as seen in Figure 10 (Dozio & Sanchez, 2017). This method produced a more concentrated pellet after isolation with the time and speed of centrifugation researched to produce the characteristics of EVs. The presented results in Figure 12 and 13 display the isolated EVs and their corresponding protein concentrations using a BCA assay.

Gels stained in coomassie blue dye overnight in Figure 18 and 19 consisted of THP-1 cell lysate 200µg and EVs isolated from THP-1 cells differentiated with 100nM of PMA having 20µg of protein. A study used differential centrifugation to produce EV lysates with 10 µg and 20 µg protein (Davis *et al.,* 2019). These samples were run on 12.5% SDS-PAGE electrophoresis gels and coomassie blue stained. The stained gel produced clear prominent

bands throughout the gel, therefore this study agrees with this present study for demonstrating low concentrations of EVs will stain for protein bands (Davis et al., 2019). However as 100nM of PMA would be used due to previous optimisation experiments (Dozio & Sanchez, 2017). The results confirm the supernant of THP-1 differentiated cells should be pooled as seen in Figure 20 to produce one concentrated pellet of EVs.

The present study is the first to produce an optimised EV isolation protocol which can pool samples together for further experimentation of characterising the differentiated THP-1 cells. These results confirm samples as seen in Figure 20 when pooled together and removing serum albumin would produce a more concentrated pellet of EVs and therefore produce multiple bands on a coomassie stained gel. As seen in studies by Davies and colleagues (Davis et al., 2019) and Zhu and colleagues (Zhu et al., 2019), they produced similar EV coomassie stained gels to this present study.

Serum albumin must be removed 24h after cell differentiation and samples loaded with complete growth medium without serum albumin to prevent contaminated albumin samples. Samples pooled together seen in Figure 20 for EV isolation would be the next step for progressing the research objective of THP-1 cells differentiated with PMA. Thus the protein bands for the stained gels can be further analysed for proteomics and a protein profile identified for the EVs.

Characterisation of the EVs was concluded using a Western Blot method as seen in Figure 15 of the results, producing a positive fluorescence with the iBright detection system for GAPDH protein present in EVs. The microvesicles and exosomes both indicated positive for

GAPDH with darker bands at higher concentrations of PMA, which is reflective of higher GAPDH protein content in concentrated samples. GAPDH is a marker for EVs and the presence of the protein band confirms the presence of EVs within the sample. Various researchers using EVs have used GAPDH as a marker for identifying the presence and characterising EVs (Gouin *et al.,* 2017). As an EV tetraspanin, GAPDH is expressed in various studies of EV characterisation including research by, Brzozowski and colleagues which is in agreement to this present study (Brzozowski *et al.,* 2018) and confirms the characterisation of EVs.

Moreover, time intervals of 24h, 48h and 72h were chosen to be incubated with 100nM of PMA as seen in Figures 17.a, 17.b and 17.c of the results. The THP-1 cell morphology changed after 24h incubation with 100nM of PMA, however, after 48h additional cells differentiated and at 72h the cells had reached the optimum confluent level of cell differentiation with the majority of cells elongated in morphology, characteristic of a macrophage-like cell. The optimum cell differentiation concentration of PMA for this experiment was therefore 100nM of PMA incubated for 72h with THP-1 cells. The optimum incubation time of 72h with 100nM of PMA agrees with the majority of published literature in this field (Park *et al.,* 2007; Jin *et al.,* 2018). This corresponds to literature suggesting on average differentiation time of 48-72h with 100nM of PMA (Daigneault *et al.,* 2010).

A MTT colorimetric assay was conducted to confirm cell metabolic activity. The shade of purple derived from this assay was an indicator of cell viability and this was quantitively assessed by measuring the absorbance of the sample using an omega plate reader. The results confirmed 100nM of PMA produced 79.4% viable cells compared to 63.4% viable cells with

200nM of PMA both of which have a significant difference in viable cells compared to the THP-1 cells with DMSO control with 100% of viable cells as seen in Figure 16. A MTT assay is used in studies using EVs to determine cell viability (Fitzgerald *et al.,* 2018) and therefore was also adopted as a method to compare the cell viability between 100nM and 200nM of PMA. After these experiments, it was concluded the optimum concentration of PMA for THP-1 cells was 100nM compared to other experimented concentrations (Starr *et al.,* 2018).

Microvesicles are often characterised by flow cytometry for their granularity and sizing range within 100-1000nm as seen in Figure 21. Sizing beads ranging from 200-2000nm were used and the distribution of the microvesicles were within the characteristic range producing an elliptical shape which can been seen in Figure 21. Various researchers use sizing beads to characterise MVs (Van der Pol *et al.,* 2018). The results from Figure 21 are typical of MVs and MVs from medium of THP-1 cells treated with 100nM of PMA were used to confirm the presence of MVs. These MVs were isolated for the experiments and therefore confirms that the optimised EV isolation protocol as seen in Figure 10 produces the characteristics of MVs.

After EVs are isolated the inner layer of the membrane containing phosphatidylserine (PS) becomes translocated to the outer layer and externalisation of the lipid bilayer occurs. Therefore, PS is used as an EV marker, and Annexin V labelling microvesicles using the flow cytometer allows further characterisation of MVs. As seen in Figure 22, the MV samples were labelled and presented a positive shift towards the green fluorescence using the flow cytometer. This confirms that MVs express PS and therefore confirms another characteristic of MVs. The characteristics of MVs as seen in Figure 23 is in agreement with Figure 21 in terms of its

granularity and elliptical shape. Similar results to this present study have been produced using the flow cytometer for labelling EVs with Annexin V to detect phosphatidylserine as a form of EV characterisation and therefore the method and results are in agreement with previous studies (Arraud *et al.*, 2015; Poon *et al.*, 2019).

5.3 Objective 2: Differentiating THP-1 cells with HUH7 in a conditioned cell culture medium to produce macrophage-like cells

The second objective of the project was to analyse cell differentiation in THP-1 cells using a novel method. The THP-1 cells were cultured in a HUH-7 conditioned medium to assess cell differentiation over several days. Research from Matak and colleagues (Matak *et al.*, 2009) analysed the connection between THP-1 and HUH7 cells and therefore provided a foundation for this research objective. Using a HUH7 conditioned medium with THP-1 cells to initiate THP-1 cell differentiation was a novel method which has future potential after the results were analysed and showed THP-1 cells differentiating. As seen in Figure 24, the cells were experimented in a 6-well cell culture plate with the experimental conditioned medium, THP-1 cells (negative control) and THP-1 cells with 100nM of PMA (positive control). The cells were analysed every 24h and after the first 24h as seen on Figure 25a, the cells differentiated into macrophage-like cells changing morphology from rounded to elongated cells.

This was novel data using a method established in this present study however, requires further analysis to identify the morphology in detail. After 48h of incubation as seen in Figure 25b, the THP-1 cells were more confluent and still rounded in structure. However, the THP-1 cells in the conditioned medium especially with 1.5mL of HUH7 conditioned medium

were differentiating and the majority of the rounded THP-1 cells differentiating into macrophage-like cells. Figure 25c and Figure 25d showed the healthy growth of the cells into macrophage elongated cells with the positive control THP-1 cells treated with 100nM of PMA, which were also similar in morphology to the HUH-7-treated THP-1 cells. After a total of 120h as seen in Figure 25e the cells had reached full confluency in the differentiation state and required a larger area for growth. The cells had adhered to the cell culture flask and required trypsin-EDTA for detachment and transferring into a $75cm^3$ cell culture flask. The growth of the macrophage-like cells after incubation of THP-1 cells in a conditioned medium established the permanent macrophage cell line as seen in Figure 26a. These cells were named THP-H cells as they were a combination of THP-1 and HUH7 cells. The THP-H cells were incubated and grown for a further 7 days before reaching full confluency as seen in Figure 26b. The morphology THP-H was observed to be similar to the PMA induced differentiated THP-1 cells from the light micrographs. However, they have an advantageous effect of being a permanent cell line and not short-lived as seen with PMA-treated THP-1 cells. Matak and colleagues (Matak *et al.,* 2009) investigated the cross-talk between hepatocytes and macrophages in mediating hepcidin regulation. This present study contributes additional knowledge to the suggested cross-talk with the newly developed THP-H cell line. Therefore, the present study results are in agreement with Matak and colleagues with the experimented HUH7 conditioned medium having a positive effect on cell communication and intercellular communication (Maacha *et al.,* 2019) with THP-1 cells and activating cell differentiation (Matak *et al.,* 2009).

This is the first research to date to develop a novel method of THP-1 cell differentiation without addition of a pro-tumour chemical but with a conditioned medium. A novel method was developed and the result reflect a permanent cell line, THP-H, which were made and developed

during the experiment. The cell differentiation requires further research and results from Maacha and colleagues (Maacha *et al.,* 2019) also suggest EVs may assist intracellular communication. Therefore the role of EVs in assisting with cell differentiation correlates as they are one factor which has been identified in affecting cells morphology, functions and responses due to signal transduction and assistance. Further characterisation is required to fully understand the genetics of the THP-H cell line using PCR, protein profile using proteomics and cell surface markers using flow cytometry cell surface labelling. The research potential and future prospects of the results is vast and has many potential benefits in a clinical setting when fully characterised and researched *in vitro*.

Research conducted by Sadofsky and colleagues (Sadofskt *et al.,* 2019) produced findings similar to this project of THP-1 cell differentiation with the HUH-7 conditioned medium. The purpose of their study was to characterise a human macrophage cell line derived from THP-1 cells as there is no true macrophage cell line and *in vitro* experiments require mitogenic stimulation.

The phenotype of the macrophage cell line was compared to the THP-1 cells. THP-1 cells in the presence and absence of a mitogenic stimuli were compared to the macrophage cell line (Daisy) using flow cytometry, microscopy, antigen binding assays, phagocytosis assays and gene microarrays (Sadofsky *et al.*, 2019). The data shows the Daisy cell line grows in an adherent monolayer.

Daisy cells are morphologically adherent in the absence of PMA stimulation compared to THP-1 cells, therefore indicating macrophage maturation. The transmission electron microscope (TEM) revealed Daisy cells have a similar morphology to PMA-treated THP-1 cells. The

PMA-induced differentiated THP-1 had high levels of euchromatin, revealing increased DNA transcription processing. Stimulation with PMA is long lasting and potent which can mask further activity of in vitro experiments. Data from the Daisy cell study showed less loosely coiled chromatin meaning they are in a resting state which has an advantage during an experimental setting (Sadofsky *et al.,* 2019).

The main role of mature macrophages is phagocytosis of foreign material. Daisy and PMA/THP-1 cells function include phagocytosis with more foreign particle uptake with the Daisy cells. Moreover, there was no significant difference seen between the two cell types. The two cell types equally showed capability of lipid accumulation, indicating cell maturity and the binding capacity of the cells was measured using immune complexes. The PMA/THP-1 cells had low binding capability to the particles despite expressing similar levels of all three IgG receptors (CD64, CD32, CD16) to Daisy cells. Expression of a protein is not vital for proving the functional capability of the cell (Sadofsky *et al.,* 2019). This is in contrast to work of Daigneault and colleagues (Daigneault *et al*). Their work observed competent phagocytosis of opsonised latex beads by PMA/THP-1 cells. However, Daisy cells were able to bind to large amounts of immune complexes, therefore indicating a significant difference between the two cell types. This finding also proposes that binding may occur through different mechanism or through functional proteins, such as complement receptors, in these cells.

The results from Sadofsky's research is the only other literature which differentiated THP-1 cells into macrophage-like cells and is a comparative study to the results obtained with the new THP-H cell line in the present study. However, the cells produced in the study by Sadofsky were named Daisy cells with characteristics similar to those of human macrophages whilst expressing a phenotype typical of human alveolar macrophage. The PMA-treated THP-1 cells differentiated into macrophages and the expression of CD14, CD11b and CD36 significantly

increased whilst the expression of CD32 decreased compared with untreated THP-1 cells. However, the Daisy cells showed decreased CD14 and CD11b expression with increased CD80, CD163 and CD206 expression compared with PMA-treated THP-1 cells.

This present study compared two different methods of THP-1 monocyte differentiation into macrophages. Firstly, differentiating THP-1 cells with (PMA), a tumour promoter to produce M0-type macrophages. The second method differentiated THP-1 cells into macrophage-like cells with a HUH-7 based culture. Using PMA to differentiate THP1 cells is an artificial model for researching cell differentiation and is not fully translated into humans, whereas the novel method of HUH-7-treated THP-1 cells developed in this research is a more realistic model for replicating the human body and therefore can better reflect the physiological conditions within the human body for future translation in a clinical setting. This study is the first demonstration of using THP1 cells with conditioned HUH7 medium to produce M0 macrophage cells.

Sadofsky and colleagues (Sadofsky *et al.*, 2019) produced macrophage-like cells in their study with THP-1 cell differentiation, however, this study produces a new methodology to differentiate THP-1 cells to produce a permanent macrophage cell line, THP-H. This is the first experiment confirming production of a new permanent macrophage cell line and therefore additional research is necessary to further characterise the novel THP-H cell line.

6. FUTURE WORK

The future potential of this research can include the characterisation of the developed macrophage cell line, THP-H, using PCR to compare the genetic pattern with THP-1 and HUH7 cells. In addition, flow cytometry should be used to compare the markers involved in PMA differentiated THP-1 cells. A full characterisation of the THP-H cell line can be experimented using various methods such as microscopy, flow cytometry, western blot analysis for novel proteins and a proteomic analysis to develop a protein profile. The protein profile is useful in order to compare the THP-H cell line to established macrophage cell lines and PMA-treated THP-1 cells. The protein profile of macrophage EV isolates from PMA-treated THP-1 cells and THP-H cells can also be compared using proteomics. Furthermore, cell communication and the interaction of THP-1 and HUH-7 cells can be further investigated to elucidate cell communication involved between the two cell lines as well as assess whether EVs assist with this in the context of cell differentiation. This study reports innovative research which may contribute to understanding the mechanisms involved in cell communication as well as identifying novel proteins in the novel THP-H cell line which can be studied with proteomic analyses.

7. CONCLUSION

To conclude, the results obtained from PMA-treated THP-1 cells agree with the current literature which report that THP-1 cells differentiate from monocytes into macrophages upon PMA exposure. A novel method to produce a permanent macrophage cell line, THP-H, is presented in this study which is physiologically similar to human macrophages, and has not been reported in the literature prior to this study. The potential of this research using EVs can add knowledge to the field of THP-1 cell differentiation as we can now further investigate the role of cell signalling of EVs in cell differentiation and cell communication. Proteins and cytokines involved in cell differentiation can be discovered and researched further as future therapeutic targets for clinical treatments once fully researched *in vitro*. Furthermore, this project provides a vital contribution to cell biology and the knowledge of THP-1 cell differentiation, thus leading to a more detailed understanding of cellular communication.

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