MORPHOLOGICAL EVALUATION OF M1 AND M2 MACROPHAGES DERIVED FROM THP-1 CELL UNDER HYPOXIC MICROENVIRONMENT

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MORPHOLOGICAL EVALUATION OF M1 AND M2 MACROPHAGES DERIVED FROM THP-1 CELL UNDER HYPOXIC MICROENVIRONMENT

by

CHIN WEI SIANG

Dissertation submitted in partial fulfilment of the requirements for the degree of Bachelor of Health Science (Honours)

(Biomedicine)

January 2025

CERTIFICATE

This is to certify that the dissertation entitled “Morphological Evaluation of M1 and M2 Macrophages Derived from THP-1 Cell under Hypoxic Microenvironment.” is the bona fide record of research work done by Mr. Chin Wei Siang during the period from August 2024 to January 2025 under my supervision. I have read this dissertation and, in my opinion, it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation to be submitted in partial fulfilment for the degree of Bachelor of Health Science (Honours) (Biomedicine).

Main supervisor,

……..

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DECLARATION

I hereby declare that this dissertation is the result of my investigations, except where otherwise stated and duly acknowledged. I also declare that it has not been previously or concurrently submitted as a whole for any other degrees at Universiti Sains Malaysia or other institutions. I grant Universiti Sains Malaysia the right to use the dissertation for teaching, research and promotional purposes.

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(CHIN WEI SIANG)

Date: 31 January 2025

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LIST OF SYMBOLS AND ABBREVIATIONS

℃	Degree Celsius
α	Alpha
β	Beta
IL-1 β	interleukin-1β
TNF- α	tumour necrosis factor-α
TGF-β	transforming growth factor-beta
IL-4	interleukin-4
IL-6	interleukin-6
IL-10	interleukin-10
IL-12	interleukin-12
IL-13	interleukin-13
$\boldsymbol{CoCl_2}$	cobalt (II) chloride
PAMPs	pathogen-associated molecular patterns
DAMPs	damage-associated molecular patterns
LPS	lipopolysaccharides
IFN-γ	interferon-gamma
GM-CSF	granulocyte-macrophage colony-stimulating factor
STAT1	signal transducer and activator of transcription 1

NF-κB	nuclear factor-kappa B
IRF-5	interferon regulatory factor 5
NADPH	nicotinamide adenine dinucleotide phosphate
Arg-1	arginase-1
PPAR-γ	peroxisome proliferator-activated receptor gamma
HIF-1α	Hypoxia-Inducible Factor-1 alpha
FBS	Fetal Bovine Serum
Pen-Strep	Penicillin Streptomycin
PMA	Phorbol 12-myristate 13-acetate
PBS	Phosphate-Buffered Saline
DMOG	Dimethyloxalylglycine
DFO	Deferoxamine
TBS-T	Tris-buffered saline with 0.1% Tween-20

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ABSTRAK

Makrofaj memainkan peranan penting dalam sistem imun, di mana makrofaj M1 bertindak sebagai pemangkin tindak balas pro-radang, manakala makrofaj M2 membantu dalam tindak balas anti-radang dan pembaikan tisu. Kajian ini meneliti perubahan morfologi makrofaj M1 dan M2 yang berasal daripada sel THP-1 dalam persekitaran mikro hipoksia. Hipoksia, satu keadaan biasa dalam pelbagai senario patologi, memberi kesan yang ketara terhadap tingkah laku dan morfologi makrofaj. Polarisasi sel THP-1 kepada fenotip M1 dan M2 dilakukan menggunakan rangsangan tertentu, dan perubahan morfologi mereka dianalisis melalui pencitraan mikroskopik. Analisis western blot turut dijalankan untuk mengesahkan kejayaan induksi persekitaran mikro hipoksia, yang ditunjukkan melalui peningkatan faktor hipoksia-inducible factor-1 alpha (HIF-1α), iaitu penanda utama bagi hipoksia. Hasil kajian menunjukkan perubahan ketara dalam bentuk dan struktur sel antara makrofaj M1 dan M2 di bawah persekitaran mikro hipoksia, yang menonjolkan kesan kekurangan oksigen terhadap polarisasi dan morfologi makrofaj. Analisis lanjut mendapati bahawa persekitaran mikro hipoksia mempercepatkan peralihan fenotip makrofaj, di mana makrofaj M1 mengalami perubahan morfologi yang minimum. Sebaliknya, makrofaj M2 menunjukkan peralihan ketara ke arah struktur seperti M1, yang ditandai dengan pengurangan pemanjangan sel dan penonjolan sitoplasma. Penemuan ini memberikan pandangan yang lebih mendalam tentang tingkah laku makrofaj dalam persekitaran mikro hipoksia, serta peranan mereka dalam perkembangan penyakit seperti kanser, iskemia, dan keradangan kronik. Kajian ini menekankan kepentingan meneroka tingkah laku makrofaj untuk mengenal pasti sasaran terapeutik baharu bagi penyakit berkaitan hipoksia dan mengkaji potensinya dalam bidang imunoterapi serta perubatan regeneratif.

MORPHOLOGICAL EVALUATION OF M1 AND M2 MACROPHAGES DERIVED FROM THP-1 CELL UNDER HYPOXIC MICROENVIRONMENT

ABSTRACT

Macrophages play a crucial role in the immune system, with M1 Macrophages promoting pro-inflammatory responses and M2 Macrophages facilitating anti-inflammatory and tissue repair mechanisms. This study investigates the morphological changes of M1 and M2 macrophages derived from the THP-1 cell under hypoxic microenvironment. Hypoxia, a common feature in various pathological environments, significantly influences morphological behaviour Polarization of THP-1 cells into M1 and M2 phenotypes was achieved using specific stimuli, and their morphological adaptations were analysed through microscopic imaging. Western blot analysis was employed to validate the successful induction of hypoxic microenvironment, confirming the upregulation of hypoxia-inducible factor-1 alpha (HIF-1α), a key marker of hypoxia. The results reveal distinct changes in cell shape, and structure between M1 and M2 macrophages under hypoxic microenvironment, highlighting the impact of oxygen deprivation on macrophage polarization and morphology. Further analysis demonstrated that hypoxic microenvironment enhances the phenotypic shift in macrophage, with minimal morphological changes observed in M1 macrophages. In contrast, M2 macrophages exhibited a significant transition toward an M1-like structure, characterized by decreased cell elongation and cytoplasmic protrusions. These findings contribute to a better understanding of macrophage behaviour in hypoxic microenvironments, offering insights into their roles in disease progression, including cancer, ischemia, and chronic inflammation. This study underscores the importance of exploring macrophage behaviour to identify potential therapeutic targets for hypoxia-associated diseases and their implications in immunotherapy and regenerative medicine.

CHAPTER 1: INTRODUCTION

1.1 Research Background

Macrophages, specialized immune cells derived from monocytes, play a key role in immune defence (Gordon, 2007). These cells are widely distributed across various tissues, serving as critical components of the innate immune system. Their primary functions include detecting, engulfing, and destroying pathogens, cellular debris, and other harmful entities. Besides that, macrophages are essential in coordinating immune responses by releasing cytokines and chemokines that recruit and activate other immune cells (Arango Duque and Descoteaux, 2014). Beyond immune defence, they also contribute significantly to tissue repair, remodelling and homeostasis, making them indispensable for maintaining overall physiological balance.

One of the defining features of macrophages is their remarkable plasticity, enabling them to polarize into distinct phenotypes to adapt to diverse microenvironmental signals (Shapouri-Moghaddam et al., 2018). This polarization is categorized into two primary states, which are M1 and M2 states. M1 macrophages, which exhibit pro-inflammatory properties, secrete pro-inflammatory cytokines such as interleukin-1β (Il-1 β), tumour necrosis factor-α (TNF- α), interleukin-6 (IL-6), and interleukin-12 (IL-12), which exacerbate local inflammation and contribute to plaque instability (Gerhardt et al., 2022). In contrast, M2 macrophage exhibit anti-inflammatory and contribute to tissue repair by releasing mediators such as interleukin-10 (IL-10), transforming growth factor-beta (TGF-β), interleukin-4 (IL-4), and interleukin-13(IL-13) (Kawai et al., 2024).

Hypoxia, a condition of reduced oxygen availability, is a significant microenvironmental factor that influences macrophage behaviour (Henze and Mazzone, 2016). This condition is commonly observed in ischemic tissues and the tumour microenvironment. Hypoxia

profoundly impacts cellular metabolism, signalling pathways, and functional states (Brahimi-Horn et al., 2007). Hypoxia-inducible factors (HIFs) play a pivotal role in regulating macrophage adaptation under low oxygen conditions. These factors drive metabolic reprogramming and regulate cytokine production, further shaping M1 and M2 polarization dynamics. Under hypoxia conditions, macrophages may exhibit altered functional profiles, contributing to either the exacerbation of inflammation or enhanced tissue repair, depending on the context.

While the plasticity of macrophages is critical for maintaining immune equilibrium and responding to microenvironmental changes, dysregulated macrophage polarization under hypoxia has been linked to the pathogenesis of chronic inflammation, fibrosis, and cancer. For example, an imbalance favouring M1 polarization may exacerbate inflammation, whereas excessive M2 polarization may promote tumour progression and fibrosis. Understanding the mechanisms underlying macrophage adaptability under hypoxic conditions is crucial. Such insights will advance the understanding of macrophage plasticity and its implications in inflammation, tissue repair, and disease progression, potentially paving the way for novel immunological and regenerative therapies.

1.2 **Problem Statement**

Macrophages are versatile immune cells that play a critical role in inflammation, tissue repair, and disease progression. Depending on environment cues, macrophages polarize into two distinct phenotypes, which are pro-inflammatory M1 macrophages and anti-inflammatory M2 macrophages. These polarization states are associated with specific functional roles and morphological characteristics. Hypoxia, a condition characterized by reduced oxygen levels, is a common feature of numerous pathological environments, including cardiovascular diseases, cancer, and chronic inflammatory conditions. This low-oxygen microenvironment profoundly alters macrophage behaviour, influencing both their polarization and their morphology.

While most studies focus primarily on the functional or biochemical analyses of macrophages under hypoxic conditions, limited attention has been given to understanding the specific morphological adaptations induced by hypoxia. Morphological changes are not merely structural. They reflect underlying cellular processes and play a crucial role in macrophage functions, including migration, adhesion, and interaction with other cells in the microenvironment. Given that macrophage morphology can impact their role in disease progression and tissue homeostasis,

Despite the importance of macrophage in hypoxic conditions, there remains a lack of morphological study of how M1 and M2 macrophages derived from THP-1 cells adapt structurally under such stress. Addressing this gap is critical for advancing our understanding of macrophage dynamics in hypoxic microenvironments and their implications for disease mechanisms. This study seeks to bridge this knowledge gap by focusing on the detailed morphological evaluation of M1 and M2 macrophages under hypoxic conditions induced by cobalt (II) chloride ($CoCl_2$).

1.3 **Objectives**

1.3.1 **Main Objectives**

To study the morphological changes in M1 and M2 macrophages derived from THP-1 cells under hypoxic microenvironment.

1.3.2 **Specific Objectives**

I. To induce polarization of THP-1 cells into M1 and M2 macrophage phenotypes.

II. To induce hypoxic conditions using $CoCl_2$ on polarized M1 and M2 macrophages.

III. To observe the morphological changes of M1 and M2 macrophages under normoxic and hypoxic microenvironment using microscopic analysis.

1.4 **Hypothesis**

I. Hypoxic microenvironment will have minimal effects on the morphology of M1 macrophages, with most retaining their pre-hypoxic characteristics.

II. Hypoxic microenvironment will induce significant morphological changes in M2 macrophages, causing them to adopt a shape similar to that observed in M1 macrophages.

III. Morphological adaptations under hypoxic conditions will differ between M1 and M2 macrophages, reflecting the polarization-specific responses to the hypoxic microenvironment.

1.5 **Rationale of Study**

The study of macrophages, particularly their polarization into M1 (pro-inflammatory) and M2 (anti-inflammatory) phenotypes, has garnered significant attention due to their critical roles in immunity and tissue homeostasis. Hypoxia, a condition characterized by low oxygen levels, is a common feature in many pathological states such as cancer, chronic wounds, and ischemic diseases. While existing research has focused extensively on the functional and molecular changes in macrophages under hypoxic conditions, less attention has been paid to their morphological alterations.

Understanding the morphological changes in M1 and M2 macrophages derived from the THP-1 cell line under hypoxic microenvironment is essential for elucidating how cellular structure correlates with function in adverse environments. For instance, changes in cell shape and surface area can directly affect the macrophages' phagocytic ability, as these adaptations influence how effectively they can engulf pathogens or debris (Oya et al., 2013). This study addresses a critical gap in the literature by exploring these changes using a well-established in-vitro model. The findings may enhance our understanding of hypoxia-driven macrophage behaviour and provide insights into therapeutic strategies targeting hypoxia-induced diseases.

By contributing to the growing body of knowledge on macrophage biology, this research may offer valuable implications for both basic science and clinical applications, including drug development and disease management.

CHAPTER 2: LITERATURE REVIEW

2.1 Macrophages

Macrophages are immune cells that either reside within tissues or infiltrate them in response to various stimuli. They constitute 20–30% of all leukocytes and are widely distributed across both lymphoid and non-lymphoid tissues throughout the body (Zhang et al., 2021). Historically, macrophages were thought to arise solely from circulating monocytes that migrate into tissues and differentiate upon arrival. However, this view has evolved. It is now well established that macrophages originate from diverse progenitor sources, including both embryonic precursors and monocyte-derived progenitors (Shapouri-Moghaddam et al., 2018).

Macrophages are present in every tissue of the body and exhibit remarkable functional diversity. They are essential in tissue development, immune surveillance, and the maintenance of tissue homeostasis (Austermann et al., 2022). Tissue-resident macrophages, such as osteoclasts in bone, alveolar macrophages in the lungs, microglial cells in the central nervous system (CNS), histiocytes in connective tissue, Kupffer cells in the liver, and Langerhans cells in the skin, are specialized for their respective microenvironments. Despite these tissue-specific specializations, the core functions of macrophages remain consistent across different tissues (Arabpour et al., 2021). These core functions include phagocytosis, where macrophages engulf and degrade pathogens, dead cells, and debris; immune surveillance, acting as sentinels to detect potential threats; and antigen presentation, bridging innate and adaptive immunity by processing and presenting antigens to T-cells (Epelman et al., 2014).

One of the defining features of monocytes and macrophages is their plasticity, enabling them to adapt to various functional states in response to environmental cues. These cells can transition from a basal, homeostatic state to a pro-inflammatory phenotype, which is critical for pathogen elimination and the management of inflammation. During the resolution phase of inflammation, macrophages undergo a phenotypic switch from a pro-inflammatory state to an

anti-inflammatory or pro-resolving state, facilitating tissue repair and the restoration of homeostasis. This dynamic ability to modulate their function highlights the dual role of macrophages in both promoting and resolving inflammation.

The process of macrophage polarization is driven by distinct environmental signals and pathological conditions. Depending on the cues they receive, macrophages can adopt various phenotypes that are broadly categorized as either pro-inflammatory (often referred to as M1) or anti-inflammatory and tissue-repairing (M2). This phenotypic flexibility allows macrophages to fulfil diverse roles in immune responses, ranging from initiating and sustaining inflammation to promoting its resolution and supporting tissue repair (Austermann et al., 2022).

2.2 M0 Macrophage

M0 macrophages are considered a precursor or baseline phenotype that can be activated and polarized into distinct functional states, such as pro-inflammatory M1 macrophages or anti-inflammatory M2 macrophages, in response to specific microenvironmental stimuli (Hickman et al., 2023). It originates from monocytes, these cells exhibit remarkable plasticity, enabling them to respond to environment and adapt their function according to the body's needs (Chaintreuil et al., 2023). In their resting state, M0 macrophages are undifferentiated, meaning they do not display specific functional roles. However, this state also makes them highly versatile, allowing them to polarize into either M1 or M2 macrophages depending on the stimuli they encounter (Bajgar and Krejčová, 2023).

Morphologically, M0 macrophages are generally round or oval in shape, and may exhibit slight clustering due to cell-to-cell adhesion (McWhorter et al., 2013). Functionally, they express a balanced profile of surface markers and cytokines, reflecting their readiness to adapt to a wide range of signals (Tarique et al., 2015). This state is crucial for maintaining tissue homeostasis and providing a reservoir for rapid activation when immune challenges arise.

M0 macrophages play a significant role in other pathological and physiological processes. During inflammation, these cells serve as first responders, ready to polarize into functional macrophages that can either escalate the immune response (M1) or help resolve it (M2) (McWhorter et al., 2013). Their adaptability makes them central to the balance between immunity and repair. However, this same plasticity can also contribute to chronic inflammation or immune evasion when dysregulated, as observed in autoimmune diseases and infections.

2.3 M1 Macrophage

Macrophages are highly plastic immune cells capable of adopting diverse functional phenotypes in response to various microenvironmental stimuli, such as pathogen-associated molecular patterns (PAMPs), damage-associated molecular patterns (DAMPs), and cytokines released by neighbouring cells (Austermann et al., 2022). These stimuli include bacterial components like lipopolysaccharides (LPS), viral RNA, apoptotic cell debris, and inflammatory mediators such as interferons and interleukins, which collectively shape macrophage behaviour to meet the specific demands of their tissue environment. This dynamic process, known as macrophage polarization, enables macrophages to adapt their behaviour based on the signals encountered within specific tissues (Lee, 2019). Among the diverse macrophage subpopulations, two major types with opposing functions have been extensively studied: classically activated (M1) macrophages and alternatively activated (M2) macrophages. The phenomenon of macrophage polarization refers to the ability of macrophages to adopt these different phenotypes in response to environmental cues.

In various tissues, macrophages undergo polarization based on environmental changes, giving rise to different functional subtypes. During infection, macrophages initially polarize into the pro-inflammatory M1 phenotype to combat pathogens. Following the resolution of the

infection, a shift toward the anti-inflammatory M2 phenotype occurs, promoting tissue repair and homeostasis.

M1-polarized macrophages, also referred to as classically activated macrophages, are primarily activated by microbial components such as lipopolysaccharide (LPS), which is a potent inducer of pro-inflammatory responses. In addition to LPS, Th1 cytokines such as interferon-gamma (IFN-γ) and granulocyte-macrophage colony-stimulating factor (GM-CSF) further enhance M1 polarization, amplifying their pro-inflammatory and antimicrobial functions (Zhang et al., 2021). These cells play a crucial role in mounting a Th1 immune response against infections. M1 macrophages are characterized by the production of pro-inflammatory cytokines, including tumour necrosis factor-alpha (TNF-α), interleukins IL-1, IL-6, IL-12, and IL-23 through the activation of different transcription factors, such as signal transducer and activator of transcription 1 (STAT1), nuclear factor-kappa B (NF-κB), and interferon regulatory factor 5 (IRF-5) (Pérez and Rius-Pérez, 2022). Functionally, they are involved in the defence against viruses, intracellular bacteria, and tumours.

M1 macrophages express surface markers such as CD80, CD86, and CD16/32, which are essential for antigen presentation and immune activation. CD80 and CD86 act as co-stimulatory molecules that bind to CD28 on T cells, providing necessary signals for T cell activation. CD16/32, as Fc receptors, facilitate the binding and internalization of antigen-antibody complexes, further enhancing the macrophage's role in adaptive immunity (Yunna et al., 2020). Additionally, M1 macrophages generate reactive oxygen species (ROS) via the activation of the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase system, thereby exhibiting robust antimicrobial and antitumoral activities. However, their persistent activation can result in ROS-induced tissue damage, impair tissue regeneration, and hinder wound healing. To counteract such tissue damage, regulatory mechanisms involving M2 macrophages are activated to resolve chronic inflammation (Lee, 2019).

2.4 M2 Macrophage

M2-polarized macrophages, also known as alternatively activated macrophages, are induced by Th2 cytokines such as interleukin-4 (IL-4) and interleukin-13 (IL-13) and cell exposure to immune complexes, glucocorticoids, or secosteroids (vitamin D3) (Arabpour et al., 2021). These macrophages exhibit anti-inflammatory properties and play essential roles in tissue repair and remodelling. M2 macrophages are associated with immune regulation, matrix deposition, tissue remodelling, and graft acceptance.

They express markers such as arginase-1 (Arg-1), the mannose receptor (CD206), anti-inflammatory factor interleukin-10 (IL-10), and chemokines CCL17 and CCL22 (Yunna et al., 2020). M2 macrophages produce anti-inflammatory cytokines, including IL-10 and transforming growth factor-beta (TGF-β), through the activation of several transcription factors, including STAT3, STAT6, IRF-4, and peroxisome proliferator-activated receptor gamma (PPAR-γ) (Pérez and Rius-Pérez, 2022). M2 macrophages also secrete chemokines such as CCL17 and CCL22, contributing to their regulatory functions.

Functionally, M2 macrophages aids in the clearance of debris and apoptotic cells. They promote angiogenesis, wound healing, and fibrosis, thus contributing to tissue regeneration. M2 macrophages are predominantly involved in Th2 responses, parasite clearance, immunoregulation, and, in certain contexts, tumour progression (Lendeckel et al., 2022).

Overall, the balance between M1 and M2 macrophage polarization is critical for maintaining immune homeostasis. While M1 macrophages provide essential defence against pathogens and tumours, excessive M1 activation can result in chronic inflammation and tissue damage. Conversely, M2 macrophages support tissue repair and regeneration but may also contribute to immunosuppression and tumour progression under specific conditions (Wang et al., 2014). Understanding the mechanisms that govern macrophage polarization offers valuable

insights into the development of therapeutic strategies targeting macrophage-driven pathologies.

2.5 THP-1 cells

THP-1 cells are a human monocytic cell line derived from the blood of a patient with acute monocytic leukemia (Tsuchiya et al., 1980). These cells are a widely used human monocytic cell line in immunological and biomedical research. Early research showed that THP-1 cells share morphological and functional characteristics with primary monocytes and macrophages, including similar differentiation markers (Hjort et al., 2003).

Due to either financial and ethical limits of animal and human in vivo studies make in vitro experiments more important for gaining specific insights and developing applications. Therefore, THP-1 cell line would be a better choice over PBMC-derived monocytes or macrophages. In fact, there are several advantages of using THP-1 cells. First, the average doubling time for monocytes ranges from 35 to 50 hours, whereas under optimal conditions using RPMI 1640 medium with 10% FBS, THP-1 cells can multiply fourfold within three and a half days. This growing rate is much higher compared to that of PBMC-derived monocytes (Chanput et al., 2014a).

Additionally, THP-1 cells can be cultured in vitro for up to 25 passages (approximately three months) without losing sensitivity or functional activity. Unlike PBMC-derived monocytes, which depend on specific cytokines such as IL-1β or TNF-α for survival and to prevent apoptosis, THP-1 cells do not require these survival factors. However, it is worth noting that while LPS is commonly used to activate macrophages, it has been shown to induce apoptosis at high concentrations or prolonged exposure, underscoring the importance of carefully optimizing its dosage in macrophage culture systems (Mangan et al., 1991).

Moreover, the availability of PBMC-derived monocytes is often limited, and these cells cannot be cryopreserved in liquid nitrogen. On the other hand, THP-1 cells can be stored for several years and, when recovered following proper protocols, retain their monocyte-macrophage characteristics and viability without significant changes (Chanput et al., 2014a). These factors make THP-1 cells a reliable and efficient alternative for in vitro studies.

THP-1 cells can be induced to differentiate into a macrophage-like phenotype by using phorbol-12-myristate-13-acetate (PMA), 1α, 25-dihydroxyvitamin D3 (vD3), or macrophage colony-stimulating factor (M-CSF) (Chanput et al., 2014a). Based on literature, it can be summarized that PMA is the most effective differentiation agent to obtain mature THP-1 monocyte-derived macrophage with similarities to PBMC monocyte-derived macrophages (Chanput et al., 2013). Phorbol 12-myristate 13-acetate (PMA) is a phorbol compound derived from *Croton tiglium*, a plant belonging to the Euphorbiaceae family. Initially identified as a tumour promoter in mouse skin, subsequent research linked its carcinogenic effects to its proinflammatory properties and activation of protein kinase C (PKC), resulting in a wide range of biological effects (Damascena et al., 2022). Today, PMA is commonly used in research to induce tumor development and activate leukocytes (Goel et al., 2007). THP-1 monocytes can be fully differentiated into macrophages after at least 48h of incubation at a minimal concentration of 10 ng/mL PMA (162 nM) (Genin et al., 2015). It should take into account that excessive PMA concentrations can trigger unwanted responses, such as cell detachment and cell death. Therefore, it is essential to allow differentiated THP-1 macrophages to rest for at least 24 hours in culture media without PMA to enhance macrophage marker expression (Chanput et al., 2014a).

2.7 M1/M2 polarization

Macrophages are versatile immune cells capable of adopting different activation states depending on the surrounding microenvironment. These states are commonly classified as M1 (classically activated) and M2 (alternatively activated) macrophages. This polarization is critical for regulating immune responses, tissue repair, and disease progression. Understanding the mechanisms underlying M1/M2 polarization is vital, particularly in the context of pathological conditions such as cancer, chronic inflammation, and hypoxia.

M1 macrophages are typically induced by pro-inflammatory stimuli, including interferon-gamma (IFN-γ), and lipopolysaccharides (LPS). These stimuli activate several intracellular pathways that drive the classical activation of macrophages. The binding of LPS to Toll-like receptor 4 (TLR4) on the macrophage surface triggers the NF-κB signaling pathway (Chistiakov et al., 2018). This leads to the transcription of pro-inflammatory cytokines, such as TNF-α, IL-1β, and IL-6, and other markers of inflammation. NF-κB activation is a crucial driver of M1 polarization, enabling macrophages to mount an effective immune response (Biswas et al., 2006). IFN-γ activates the JAK/STAT pathway, specifically STAT1, which induces the transcription of pro-inflammatory genes (Xia et al., 2023). These include genes involved in the production of iNOS (inducible nitric oxide synthase), which produces nitric oxide (NO)—a molecule essential for microbial killing. NO and ROS (reactive oxygen species) production further enhance the macrophage's ability to eliminate pathogens.

M2 macrophages are typically induced by anti-inflammatory cytokines such as IL-4, and IL-13. These signals activate alternative pathways that promote tissue repair, immune suppression, and resolution of inflammation. IL-4 and IL-13 bind to their respective receptors on macrophages, activating the JAK/STAT6 signalling pathway (Han et al., 2022). This activation drives the expression of genes associated with M2 polarization, including Arginase-

1 (ARG1), mannose receptor (CD206), and resolvin D1. These markers are involved in tissue repair, immune modulation, and the resolution of inflammation.

2.8 Hypoxia

Hypoxia is a condition characterized by reduced oxygen levels (pO2, 0–20mmHg) which arises as a result of a compromised or dysfunctional vascular system, which limits blood flow and oxygen supply. Hypoxia is commonly associated with several pathological conditions, such as inflammation, hepatic ischemia, organ transplantation, cerebral stroke, myocardial infarction (MI), arthritic joints, atherosclerotic plaques, and malignant tumours (Yakupova et al., 2022). Under hypoxic conditions, cells activate adaptive mechanisms to cope with the reduced oxygen availability.

The study of hypoxia in laboratory settings relies on experimental methods that mimic reduced oxygen availability in cells and tissues. These methods can be broadly categorized into physical and chemical approaches, both of which are widely utilized depending on the research objectives and experimental constraints. Physical induction of hypoxia is typically achieved by culturing cells in hypoxic chambers where the oxygen concentration is tightly regulated. These chambers allow researchers to simulate physiological oxygen levels, ranging from mild hypoxia (5–10% O_2) to severe hypoxia (<1% O_2) (Wu and Yotnda, 2011). This method closely mimics in vivo conditions and is ideal for long-term studies where gradual changes in oxygen levels are necessary. However, it requires specialized equipment and can be challenging to maintain for high-throughput experiments.

Chemical agents offer a simpler and more accessible means of inducing hypoxia by directly stabilizing Hypoxia-Inducible Factor-1 alpha (HIF-1α), a key mediator of hypoxic responses. There are several types of hypoxia mimetics, including Dimethyloxalylglycine (DMOG), Cobalt (II) Chloride, Deferoxamine (DFO) and so forth (Davis et al., 2019). Cobalt

(II) chloride is among the most commonly used hypoxia mimetics. Cobalt (II) chloride mimics hypoxia by inhibiting prolyl hydroxylases, the enzymes responsible for HIF-1α degradation under normoxic conditions (Kumanto et al., 2017). This results in the accumulation of HIF-1α and the activation of downstream hypoxia-responsive genes. Chemical methods have several advantages, including simplicity, cost-effectiveness, and the ability to uniformly induce hypoxia in large cell populations.

$CoCl_2$ works by stabilizing hypoxia-inducible factor alpha (HIF-α) subunits through the inhibition of prolyl hydroxylase domain (PHD) enzymes (Appelhoff et al., 2004). This inhibition occurs as cobalt ions substitute for iron in the active site of PHD enzymes, which are essential for hydroxylating HIF-α under normoxic conditions. Consequently, HIF-1α accumulates, translocates to the nucleus, and activates downstream hypoxia-responsive genes, such as VEGF and GLUT1, which regulate processes like angiogenesis and glycolysis (Muñoz-Sánchez and Chánez-Cárdenas, 2019). This mechanism closely mimics natural cellular responses to hypoxia, making $CoCl_2$ a robust tool for inducing hypoxic effects in macrophage polarization studies.

Thus, to validate the hypoxic conditions induced by cobalt chloride ($CoCl_2$), we utilized HIF-1α and beta-actin as markers. HIF-1α is a well-established indicator of cellular hypoxia, as its stabilization and nuclear translocation are hallmark responses to hypoxic conditions. Measuring the expression of HIF-1α allowed us to confirm the activation of hypoxia-responsive pathways. Beta-actin, on the other hand, was used as a loading control to ensure the accuracy and consistency of protein expression analysis. The combination of these markers provided a reliable means to validate the efficacy of $CoCl_2$ in mimicking hypoxic conditions in our macrophage polarization studies (Park et al., 2014).

CHAPTER 3: MATERIALS AND METHODOLOGY

3.1 Study flow chart

The flow chart of the study is shown in Figure 3.1

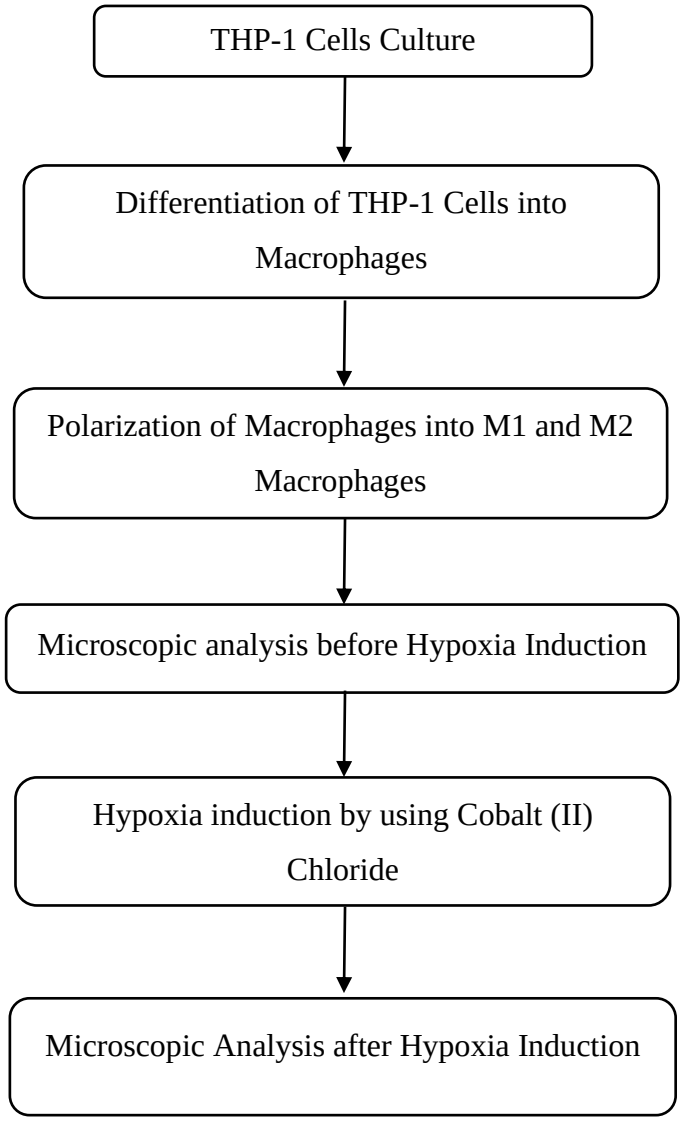

***Figure 3.1**: Flow chart of study*

3.2 Materials

3.2.1 Chemicals and Reagents

The consumables, chemicals, and reagents used in this study are listed in Table 3.1

Chemicals and Reagents	Suppliers
RPMI-1640 medium	Thermo Fisher Scientific, US
Fetal Bovine Serum (FBS)	Sigma-Aldrich, US
Penicillin Streptomycin (Pen-Strep)	Thermo Fisher Scientific, US
Phorbol 12-myristate 13-acetate (PMA)	Sigma-Aldrich, US
Interleukin-4 (IL-4)	Sigma-Aldrich, US
Interleukin-13 (IL-13)	Sigma-Aldrich, US
Interferon-γ (IFN- γ)	Sigma-Aldrich, US
Lipopolysaccharide (LPS)	Sigma-Aldrich, US
Cobalt (II) Chloride ($CoCl_2$)	Sigma-Aldrich, US
Phosphate-Buffered Saline (PBS)	Thermo Fisher Scientific, US
Radioimmunoprecipitation assay buffer	Sigma-Aldrich, US
SDS-PAGE gel	Sigma-Aldrich, US
Electrophoresis buffer (Tris-Glycine-SDS buffer)	Sigma-Aldrich, US
Laemmli buffer	Sigma-Aldrich, US
Blocking solution (5% non-fat milk or 5% BSA in TBS-T)	Sigma-Aldrich, US
TBS-T (Tris-buffered saline with 0.1% Tween-20)	Sigma-Aldrich, US
Anti-HIF-1α antibody	Sigma-Aldrich, US

Anti-β-actin antibody	Sigma-Aldrich, US
HRP-conjugated secondary antibody	Sigma-Aldrich, US
ECL substrate (chemiluminescence reagent)	Sigma-Aldrich, US
Distilled water	PPSK, USMKK
70% alcohol	HmbG Chemicals, Malaysia

Table 3.1*: List of Chemicals and Reagents*

3.2.2 Apparatus and Equipment

The laboratory equipment used in this study is listed in Table 3.2

Laboratory apparatus	Suppliers
Biosafety cabinet	Esco, Singapore
CO_2 incubator	Esco, Singapore
Inverted Microscope	Leica Microsystems, German
Centrifuge	Hettich, German
Analytical Balance	Gaia Science, Malaysia
Pipettes	Eppendorf, German
Pipette Tips	Axygen Scientific, US
T25 Cell Culture Flasks	Fisher Scientific, Singapore
6-well plates	Biologix, US
Sterile 15mL Falcon Tubes	Biologix, US
Sterile 50mL Falcon Tubes	Biologix, US
Sterile Filters (0.22µm)	Bioflow Lifescience, Malaysia
50mL syringe without needle	Hospitec Manufacturing, Malaysia

***Table 3.2**: List of Apparatus and Equipment*

3.2.1 Preparation of Reagent

3.2.1.1 Preparation of 70% ethanol solution

A 70% ethanol was prepared using a 1-liter measuring cylinder by thoroughly mixing 700mL of ethanol with 300mL of distilled water. The solution was thoroughly mixed to ensure accuracy and homogeneity, making it suitable for laboratory use.

3.2.1.2 Preparation of complete media

The complete media was prepared by pre-warming all the components, including the RPMI-1640 basal medium, foetal bovine serum (FBS), and penicillin-streptomycin solution at 37℃ for 5 minutes to thaw and ensure optimal mixing. A total of 45mL of RPMI-1640 basal medium was combined with 5mL of FBS to achieve a final concentration of 10% FBS, promoting enhanced cell growth and viability. Additionally, 0.5mL of penicillin-streptomycin solution was added to prevent bacterial contamination. The mixture was then thoroughly mixed to ensure uniform distribution and subsequently filtered using a 0.22µm membrane filter to remove any potential contaminants. The prepared medium was then stored at 4℃ for use in cell culture experiments.

3.2.1.3 Preparation of RIPA Buffer

To prepare the RIPA buffer, combine 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% NP-40 (Nonidet P-40), 0.5% Sodium Deoxycholate, and 0.1% SDS (Sodium Dodecyl Sulfate). Additionally, add protease inhibitors such as 1 mM PMSF, 1 µg/mL aprotinin, and 1 µg/mL leupeptin, along with phosphatase inhibitors like 1 mM sodium orthovanadate to protect proteins from degradation. This buffer is used to lyse cells and extract total protein. After mixing, store the RIPA buffer at 4°C and use fresh on the day of sample preparation to ensure the optimal extraction of proteins.

3.3 Methodology

3.3.1 THP-1 Culture

THP-1 cells were cultured in complete media containing RPMI-1640 medium and supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin to support optimal growth and viability. The cells were maintained in a humidified incubator at 37℃ with 5% CO_2 to mimic physiological conditions. Upon arrival from the cryopreservation store, THP-1 cells with passage 15 were thawed rapidly in a 37℃ water bath for 2-3 minutes and immediately transferred into 10mL of pre-warmed complete RPMI-1640 medium in a T25 flask. The cells were gently mixed and allowed to recover for 24 hours, after which the medium was changed to remove residual DMSO from the cryopreservation. Cell density was carefully monitored and kept in the density of $1 - 2 \times 10^6\ cells/m\ell$ to prevent overgrowth, which could compromise the quality and consistency of subsequent experiments. The culture medium was replenished with fresh complete RPMI-1640 every 3-4 days to ensure an adequate supply of nutrients and maintain a stable environment for cell growth. Regular observation under a inverted microscope was performed to assess cell morphology, confluency, and any signs of contamination.

Once the cells were actively proliferating and in the exponential growth phase, they were passaged. To passage the cells, the culture medium was aspirated into a sterile $15mL$ centrifuge tube and centrifuged at 80.5g for 5 minutes at room temperature. The supernatant was discarded, and the cell pellet was resuspended in $1mL$ of fresh pre-warmed complete media. From the resuspended cells, $0.5mL$ of the suspension was transferred into each of two new culture flasks. Fresh medium was added to each flask to bring the total volume to $5mL$, ensuring an optimal environment for cell growth. When the cells reached confluence, cell viability was assessed using a trypan blue exclusion assay to ensure that the healthy and viable cells were used for downstream experiments.

3.3.2 THP-1 Differentiation into M1 and M2 Macrophages

THP-1 cells were first seeded into 6-well plates at a density of $1 \times 10^6\ cells/mL$ in complete media to ensure the cells were evenly distributed and in optimal numbers to undergo differentiation. Following seeding, differentiation into macrophage-like cells, M0 was initiated by treating the cells with $10\ ng/mL$ of Phorbol 12-myristate 13-acetate (PMA) (Damascena et al., 2022). The PMA stock solution was prepared at a concentration of $10\ \mu g/mL$, and $2\ \mu L$ of the stock solution was added to each well containing $2\ mL$ of medium, resulting in a final concentration of $10\ ng/mL$. PMA promotes cell adherence and differentiation. The cells were incubated with PMA for 3 days, during which adherence and differentiation were monitored under an inverted microscope. After the treatment period, the cells were gently washed with sterile PBS to remove residual PMA and any non-adherent cells. Following PMA treatment, the PMA-containing medium was carefully removed and replaced with fresh RPMI-1640 medium without PMA. The cells were then allowed to rest for 24 hours in the fresh medium to recover from PMA-induced activation and reach a stable state suitable for polarization.

For M1 macrophage polarization, the cells were treated with $5\ ng/mL$ interferon-gamma ($IFN-\gamma$) and $10\ ng/mL$ lipopolysaccharide (LPS) for 72 hours. This combination activates the classical pro-inflammatory pathway, resulting in M1 macrophage polarization. Both $IFN-\gamma$ and LPS were prepared from $10\ \mu g/m\ell$ stock solution, with $1\ \mu L$ of the $IFN-\gamma$ stock solution and $2\ \mu L$ of LPS stock solution added to each well. For M2 macrophage polarization, the cells were treated with $25\ ng/mL$ interleukin-4 (*IL-4*) and $25\ ng/m\ell$ interleukin-13 (*IL-13*) for 72 hours to activate the alternative anti-inflammatory pathway. The required concentrations were achieved by adding $5\ \mu L$ of the *IL-4* stock solution and $5\ \mu L$ of the *IL-13* stock solution, both prepared from $10\ \mu g/mL$ stock solutions, to each well.

3.3.3 Induction of Hypoxia

After the differentiation is successful, 100µM cobalt chloride ($CoCl_2$) was added to the medium in each well to induce hypoxia. To prepare the cobalt chloride solution, 5.9 mg of cobalt chloride (25 mM) was accurately weighed using an analytical balance. The weighed cobalt chloride was then dissolved in 1 mL of distilled water to create the stock solution, ensuring thorough mixing until completely dissolved. The solution was sterilized by passing it through a 0.22 µm syringe filter to remove any particulates and ensure sterility. From the prepared stock solution, 12 µL was added to each well of the cell culture plate to achieve a final concentration of 100 µM of $CoCl_2$. The cells were then incubated under normal culture conditions (37℃, 5% CO_2) for 24 hours to mimic hypoxic conditions, enabling the study of hypoxia-induced changes in M1 and M2 macrophages.

3.3.4 Validation of Hypoxia Induction

Western blot was performed to assess hypoxia induction by detecting the expression of HIF-1α. Following hypoxia treatment, cells were washed twice with ice-cold PBS and lysed using RIPA buffer supplemented with protease and phosphatase inhibitors. The lysates were incubated on ice for 30 minutes, vortexed intermittently, and centrifuged at 12,000 g for 15 minutes at 4°C to remove debris. Protein samples were mixed with 2x Laemmli buffer, boiled at 95°C for 5 minutes, and separated on a 12% SDS-PAGE gel. Proteins were transferred to a PVDF membrane using a wet transfer system at 100 V for 90 minutes at 4°C. The membrane was blocked with 5% non-fat milk in TBS-T for 1 hour at room temperature, followed by overnight incubation at 4°C with a primary antibody against HIF-1α diluted in TBS-T with 1% BSA. After three washes with TBS-T, the membrane was incubated with an HRP-conjugated secondary antibody for 1 hour at room temperature and washed again. Protein bands were visualized using ECL substrate, and the appearance of the HIF-1α band in the hypoxia-treated samples confirmed successful induction of hypoxia.

3.3.5 Microscopic Morphological Analysis

The morphology of the polarized macrophages under both hypoxic and normoxic conditions are observed using an inverted microscope. The microscopic images will be captured at various magnifications, such as $40\times$, $100\times$, $200\times$, and $400\times$ to assess cell shape, size, and morphological features such as cell spreading, aggregation, or structural alterations. The observation in microscopic will enable comparisons between different macrophage polarization states and their responses to hypoxic conditions.

CHAPTER 4: RESULTS

4.1 THP-1 Cell Culturing Morphology

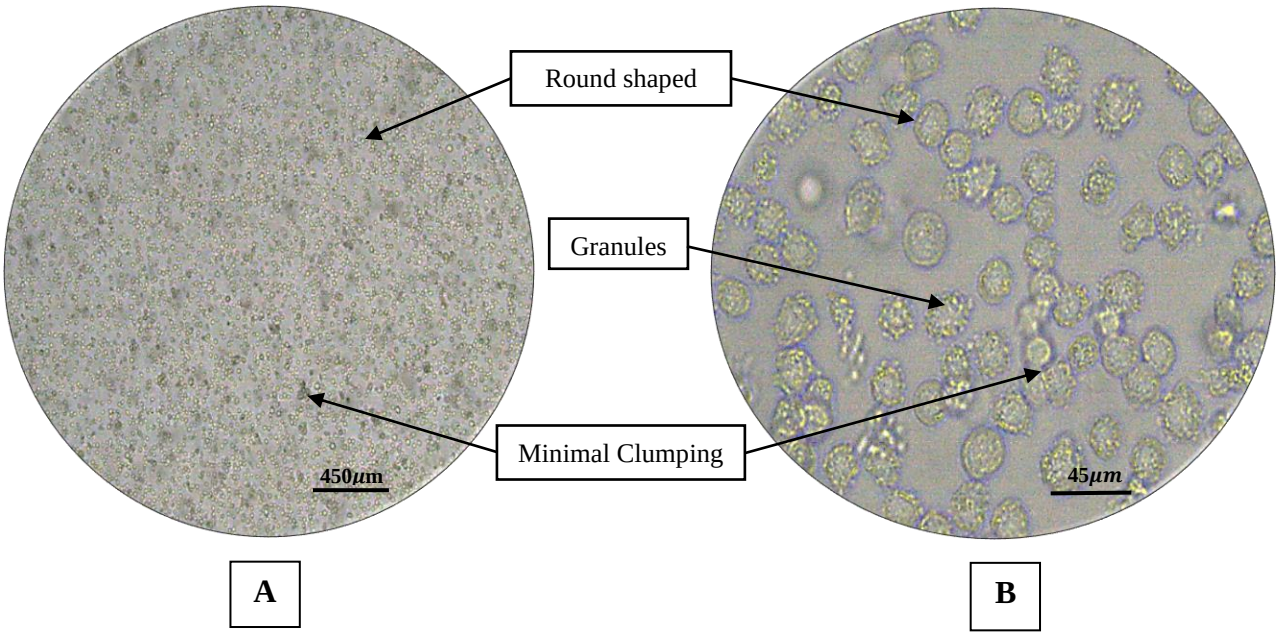

Fig 4.1. *(A) Under 40x magnification. (B) Under 400x magnification*

Figure 4.1 shows the morphology of THP-1 cells observed under two magnifications: 40× (Panel A) and 400× (Panel B). In Panel A, the cells appear as uniformly distributed, round-shaped structures with minimal clumping, which are the characteristics of undifferentiated THP-1 cells in suspension culture.

In Panel B, the spherical morphology of the cells is clearly visible, with distinct cellular boundaries and smooth surfaces characteristic of undifferentiated THP-1 cells. At this magnification, granular cytoplasmic patterns are observable, reflecting active cellular metabolism. Regions with consistent cell size and distribution further demonstrate the homogeneity of the culture. Notably, no signs of contamination, such as small dots, spiral-shaped artifacts, or debris, are observed, ensuring the purity and reliability of the culture. These observations confirm the success of the THP-1 cell culturing process, providing a strong basis for subsequent differentiation into M0 macrophages.