Induced Pluripotent Stem Cells Derived Mesenchymal Stem Cells and Macrophages Interplay to Promote Tissue Repair

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Induced Pluripotent Stem Cells Derived Mesenchymal Stem Cells and Macrophages Interplay to Promote Tissue Repair

A thesis submitted in partial fulfilment of the requirements of Manchester Metropolitan University for the degree of Doctor of Philosophy

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List of Abbreviations

AB	AlammarBlue
HEPES	(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)
DMEM	Dulbecco’s modified eagle’s medium
APS	Ammonium persulphate
ANOVA	Analysis of variance
APC	Antigen presenting cell
ATF3	Activating transcription factor 3
AREG	Amphiregulin
Arg1	Arginase-1
BSA	Bovine Serum Albumin
CD4	Cluster of differentiation 4
CD8	Cluster of differentiation 8
cDNA	Complimentary deoxyribonucleic
CM	Classical monocytes
CXCL11	The chemokine (C-X-C motif) ligand 11
DAMP	Damage-associated molecular pattern
DNA	Developmental pluripotency associated
DFUs	Diabetic foot ulcers
DMSO	Dimethyl sulphoxide
PBS	Dulbecco's phosphate-buffered saline
ESCs	Embryonic stem cells

EGF	Epidermal growth factor
EDTA	Ethylenediaminetetraacetic acid
ECM	Extra cellular matrix
FGF-2	Fibroblast growth factor-2
FITC	Fluorescein isothiocyanate
FBS	Foetal bovine serum
FSC	Forward scattered light
GAL-3	Galectin-3
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GR	Glucocorticoid receptor
HDMEC	Human Dermal Microvascular Endothelial Cells
IC	Immune complex
IM	Intermediate monocytes
iPSCs	Induced pluripotent stem cells
IFN-y	Interferon-gamma
IRF4	Interferon regulatory factor 4
IRF5	Interferon regulatory factor 5
IL-4	Interleukin-4
IL-6	Interleukin-6
IL-10	Interleukin-10
iPSCs	Induced pluripotent stem cells
LPS	Lipopolysaccharides

LXR	Liver X receptor
M0	Macrophage type 0
M1	Macrophage type 1
M2	Macrophage type 2
MSCs	Mesenchymal stem cells
MEA	Microelectrode array
MPLA	Monophosphoryl lipid A
µg	Microgram
µl	Microliter
µm	Micrometre
ml	Millilitre
mM	Millimolar
NCM	Non-classical monocytes
ng/ml	Nanogram per millilitre
ng	Nanograms
NOS2	Nitric oxide synthase 2
NHS	National Health Service
NEAA	Non-essential amino acids
PMA	Phorbol 12-Myristate 13-Acetate
PBS	Phosphate buffered saline
PDGF	Platelet-derived growth factor
PAGE	Polyacrylamide gel electrophoresis

PAMP	Pathogen-associated molecular pattern
PCR	Polymerase chain reaction
PI	Propidium iodide
PMSF	Phenylmethylsulfonyl fluoride
PPARγ	Peroxisome proliferator-activated receptor γ
RIPA	Radio immunoprecipitation assay
RIPA	Radioimmunoprecipitation assay
ROS	Reactive oxygen species
qPCR	Real-time polymerase chain reaction
RT-q-PCR	Real-time Quantitative PCR
rpm	Revolutions per minute
RNA	Ribonucleic acid
RNAseq	RNA sequencing
RT	Room temperature
RPKM	Reads Per Kilobase per Million mapped reads
RPMI	Roswell Park Memorial Institute
SSC	Side scattered light
SOCS	Suppressor of cytokine signalling
NaCl	Sodium chloride
SDS	Sodium dodecyl sulfate
TEMED	Tetramethylethylenediamine
TGF	Transforming growth factor

TGF-β1	Transforming growth factor-beta1
TGF-β3	Transforming growth factor-beta3
TGF-β	Transforming growth factors β
Treg cell	Regulatory T cell
Tris-HCl	Tris-hydrochloride
TNFα	Tumour necrosis factor a
VEGF	Vascular endothelial growth factor
°C	Degree Celsius
γδ	Gamma delta

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Abstract

Macrophage polarisation into the pro-inflammatory M1 phenotype and the anti-inflammatory M2 phenotype plays a crucial role in inflammation, resolution, and tissue regeneration. It was well established that lipopolysaccharide (LPS) directed macrophages toward M1 polarisation. It is well established that lipopolysaccharide (LPS) directs macrophages toward M1 polarization. In contrast, interleukin-4 (IL-4) and interleukin-13 (IL-13) promote M2 polarization; however, the underlying mechanisms remain incompletely understood. Mesenchymal stem cells (MSCs) were known to modulate macrophage function and promote an anti-inflammatory phenotype, but how they influenced polarisation at the molecular level had yet to be fully elucidated.

This study initially carried out RNA deep sequencing (RNA-Seq) on macrophages and, for the first time, discovered that LPS suppressed the expression of the Mer proto-oncogene tyrosine kinase (MerTK) gene in macrophages. This finding was validated at the transcriptional level using RT-qPCR and at the protein level through western blotting and confocal microscopy. Furthermore, inhibition of NF-κB restored MerTK expression in response to LPS, as confirmed by RT-qPCR. Promoter analysis identified an E-box motif within the MerTK promoter, which is essential for activation by enhancers such as the circadian clock genes BMAL1 and CLOCK. LPS-mediated NF-κB signalling interfered with the binding of these enhancers to the E-box, resulting in transcriptional repression. Conversely, treatment of macrophages with IL-4 and IL-13 led to an upregulation of MerTK protein expression, as demonstrated by RT-qPCR and western blotting. These findings suggest MerTK is a critical molecular switch in macrophage polarisation.

Extending this investigation to MSCs-mediated effects, MSCs were generated from induced pluripotent stem cells (iPSCs), which offer a scalable and less invasive alternative to traditional MSCs sources limited by donor variability and invasive harvesting procedures. iMSCs were initially generated via directed differentiation of iPSCs in the presence of the transforming growth factor beta (TGFβ) inhibitor SB431542. The resulting iPSCs-derived MSCs (iMSCs) displayed a typical spindle-shaped morphology and expressed characteristic MSCs surface markers (CD73, CD90, CD105) with minimal CD45 expression, as confirmed by flow cytometry. Notably, the condition medium of iMSCs exhibited strong anti-inflammatory activity and upregulated MerTK expression, while exosomes isolated using Exo-spin™

mini-HD columns from iMSCs enhanced STAT3 phosphorylation, as evidenced by western blot analysis. These results suggested a paracrine mechanism through which MSCs promoted an anti-inflammatory macrophage phenotype.

Collectively, these findings identified MerTK in macrophages as a key regulator of MSCs–macrophages crosstalk, modulated by both inflammatory signalling and circadian rhythms. They offer novel mechanistic insights into how iMSCs modulate the immune response and promote tissue regeneration through their interaction with macrophages, highlighting promising therapeutic potential for treating inflammatory disorders and supporting tissue repair.

Declaration and Copyright Statements

Declaration

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Chapter 1: Introduction

1.1 Background

Tissue repair is a sophisticated healing process in which the body utilises a structured approach to restore or replace damaged tissue (Eming, Martin and Tomic-Canic, 2014). Similar to wound healing, this process involves the coordinated interaction of various cell types, from pluripotent stem cells to tissue-specific and dedifferentiated cells. These interactions integrate complex cellular mechanisms, including cell signalling, migration, proliferation, and extracellular matrix (ECM) formation (El Mohtadi *et al.*, 2021). Tissues comprise multiple cell types with distinct functions, transcriptional programmes, and division capacities. Precise injury signals are essential for regulating the extent and timing of the repair process, while cellular plasticity plays a critical role in ensuring efficient tissue regeneration (Gurley and Sanchez Alvarado, 2008; Khan, Neumann and Sinha, 2020). The tissue microenvironment, where cells interact to form functional organs, is crucial for this process, with the ECM providing essential structural support.

During tissue repair, various cell types, including stem cells, macrophages, T cells, B cells, and fibroblasts, contribute by undergoing proliferation, differentiation, and transdifferentiation to restore damaged tissue (Plikus *et al.*, 2021). Among these, mesenchymal stem cells (MSCs) have emerged as key players due to their capacity to differentiate into various cell types, secrete paracrine factors, and modulate the immune response (Dimarino, Caplan and Bonfield, 2013).

MSCs, derived from bone marrow, adipose tissue, and umbilical cord tissues, are critical for maintaining tissue homeostasis and facilitating repair. They achieve this by promoting anti-inflammatory responses, enhancing angiogenesis, and supporting the remodelling of the extracellular matrix (Fu and Li, 2009). However, conventional MSCs sources face several limitations, including donor variability, restricted proliferative capacity, and ethical concerns. These challenges have led to exploring alternative sources, such as MSCs derived from induced pluripotent stem cells (iPSCs-MSCs).

iPSCs-MSCs provide a scalable and renewable stem cell source with consistent characteristics, making them an attractive option for regenerative medicine (Spitzhorn *et al.*, 2019). These cells exhibit classical MSCs properties while demonstrating enhanced immunomodulatory functions, allowing them to influence key aspects of tissue repair (Sun *et al.*, 2022). A crucial aspect of this process is their interaction with macrophages, which play a dual function in inflammation and healing.

Macrophages exist along a dynamic spectrum, transitioning between pro-inflammatory (M1) and anti-inflammatory (M2) phenotypes (Wynn and Vannella, 2016). This transition is essential for resolving inflammation and initiating tissue regeneration (Mantovani *et al.*, 2013). Dysregulated macrophage polarisation, characterised by prolonged activation of M1 macrophages and inadequate transition to M2 macrophages, is associated with chronic inflammatory diseases and impaired wound healing.

Recent studies indicate that iMSCs regulate macrophage behaviour by secreting bioactive factors that encourage M2 polarisation (Arabpour, Saghazadeh and Rezaei, 2021; Francois *et al.*, 2012). However, the precise mechanisms by which MSCs educate macrophages to transition from M1 to M2 remain unclear. Understanding the molecular mechanisms that govern these interactions is essential for developing targeted therapies to augment MSCs-mediated tissue regeneration and repair.

In this context, elucidating the mechanisms underlying macrophage polarisation and harnessing the regenerative potential of iMSCs represent promising avenues for advancing therapies for chronic wounds, inflammatory disorders, and degenerative diseases. By leveraging the immunomodulatory properties of iMSCs, this study aims to identify key molecular targets and develop clinically translatable strategies to optimise their therapeutic application in tissue repair.

1.2 Inflammation in Tissue Repair

Inflammation is an evolutionarily conserved process that supports the host's tissue injury response. It is essential for restoring tissue homeostasis, promoting tissue repair, and facilitating tissue regeneration (Broughton, Janis and Attinger, 2006). The innate immune system orchestrates this dynamic process, coordinating the activities of neutrophils, macrophages, mast cells, and leukocytes (van Tuijl *et al.*, 2019). The tightly regulated nature of inflammation ensures an optimal trajectory for wound healing, ultimately leading to effective tissue repair with minimal fibrosis (Cowin *et al.*, 2021). Thus, Inflammation represents a critical biological response central to tissue repair.

With the emergence of novel strategies to modulate inflammation, there is an increasing impetus to comprehensively evaluate this fundamental biological process. In recent decades, scientific advancements have significantly enhanced our understanding of the complex molecular and cellular pathways governing wound inflammation (Guo and Dipietro, 2010; Zhang *et al.*, 2021a). Studies employing *in vitro* models and animal systems have clarified the complex interactions between inflammatory pathways and immune cells, particularly macrophages, that govern wound resolution and tissue regeneration (Hong and Tontonoz, 2008; Oishi *et al.*, 2017). Chronic pathological conditions can dysregulate the inflammatory cascade, leading to persistent inflammation and impaired wound resolution (Eming, Krieg and Davidson, 2007). Risks such as bacterial infections, recurrent ischemic insults, and systemic inflammatory states exacerbate dysregulated immune responses, ultimately promoting fibrosis and pathological scarring (Las Heras *et al.*, 2020; Wilgus *et al.*, 2008). Therefore, fine-tuning the inflammatory response is imperative for facilitating the transition from the exudative phase to granulation tissue formation, ensuring appropriate tissue repair (Cowin *et al.*, 2021).

Chronic inflammation is implicated in various human diseases, including cerebrovascular, cardiovascular, arthritic, dermatologic, pulmonary, hematologic, hepatic, and gastrointestinal diseases, as well as diabetes mellitus (Fig.1.1) (Pan *et al.*, 2011). The inflammatory response triggers the up-regulation of multiple pro-inflammatory enzymes, cytokines, reactive oxygen and nitrogen species (RO/NS), alongside signalling proteins within affected tissues and cells (Forrester and Bick-Forrester, 2005). These mediators elevated systemic and tissue-specific levels correlate with increased disease susceptibility and progression. Consequently, targeting inflammatory signalling pathways is widely recognised as a promising therapeutic approach for tissue repair, disease mitigation and chemoprevention.

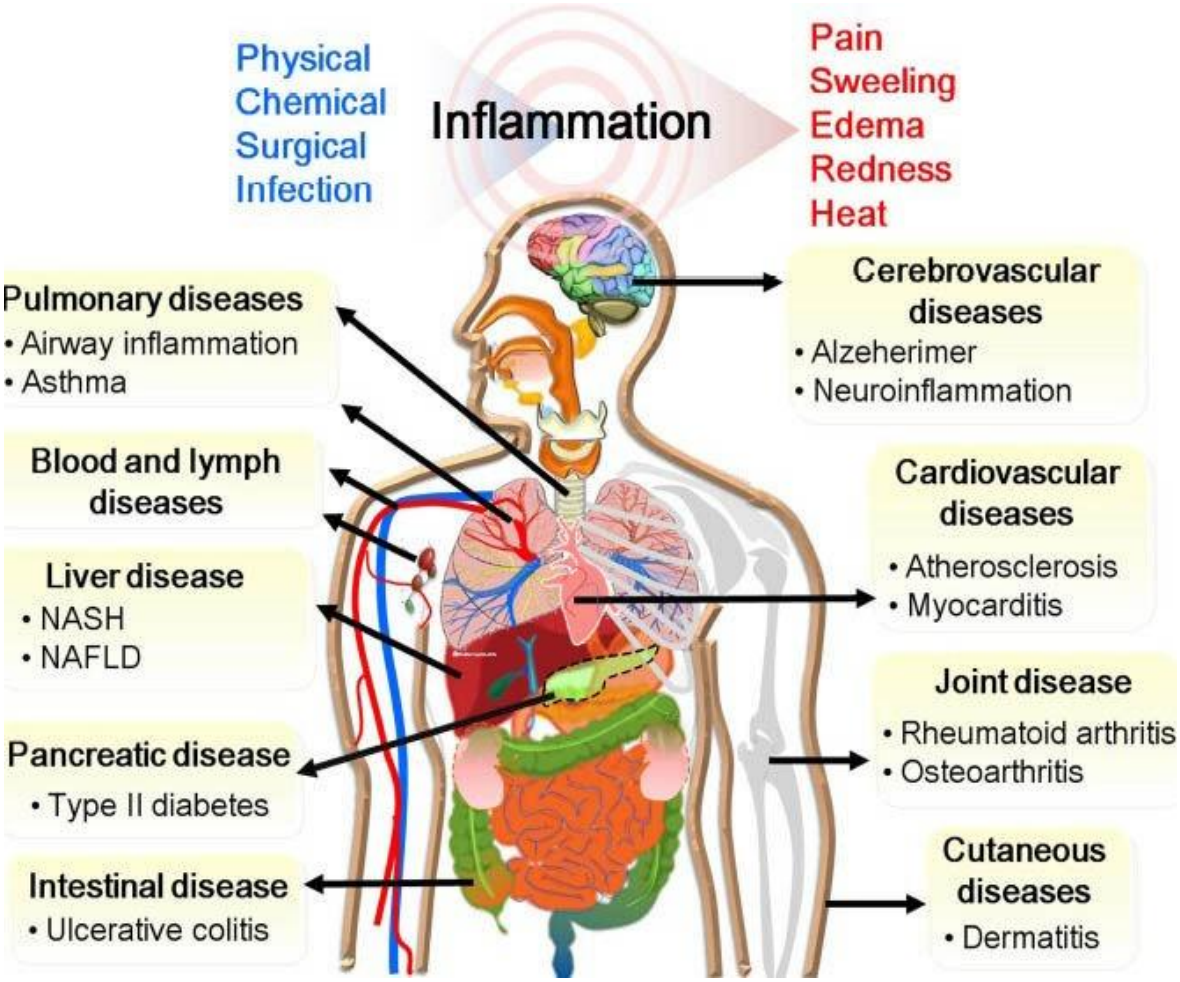


Fig.1.1 Chronic inflammation is associated with a range of human diseases. Inflammation, triggered by various stimuli such as physical, chemical, surgical, or infectious insults, underlies a wide spectrum of chronic diseases across multiple organs. The figure illustrates inflammation-associated pathologies, including pulmonary diseases such as airway inflammation and asthma, blood and lymph disorders, liver conditions such as NASH and NAFLD, pancreatic dysfunction such as type II diabetes, and intestinal diseases like ulcerative colitis. Inflammation also contributes to cerebrovascular diseases such as Alzheimer's and neuroinflammation, cardiovascular diseases including atherosclerosis and myocarditis, joint diseases such as rheumatoid arthritis and osteoarthritis, and cutaneous conditions like dermatitis. Classical signs of inflammation, including pain, swelling, edema, redness, and heat, are highlighted to emphasise the systemic consequences of chronic inflammatory responses (Pan *et al.*, 2011).

1.3 The Role of MSCs and Macrophages in Anti-Inflammation

As mentioned above, inflammation is a vital cellular process that initiates tissue repair by activating a cascade of cellular responses following injury. It begins with releasing signalling molecules, including cytokines and chemokines, from damaged cells, which attract immune cells to the affected area (Schett and Neurath, 2018). This initial response increases blood flow and vascular permeability, facilitating the migration of key immune cells such as neutrophils, monocytes and macrophages (Forbes and Rosenthal, 2014). These immune cells remove debris and pathogens through phagocytosis, creating an environment conducive to tissue repair (Wynn and Vannella, 2016).

Macrophages are key to tissue repair and regeneration, demonstrating their ability to adapt to damaged tissues (Mosser, Hamidzadeh and Goncalves, 2021; Wynn and Vannella, 2016). Subsequent to an injury, these cells are rapidly recruited to the affected area to eliminate debris, release cytokines, and orchestrate the healing responses (Mosser, Hamidzadeh and Goncalves, 2021). As the repair process advances, macrophages transition from a pro-inflammatory state (M1) to an anti-inflammatory state (M2), during which they secrete growth factors such as TGF-β1, IGF-1, and VEGF, all of which contribute to angiogenesis and tissue regeneration phase (Das *et al.*, 2015; Owen and Mohamadzadeh, 2013).

Dysregulated macrophages can cause chronic inflammation and fibrosis by producing excessive pro-fibrotic mediators such as TGF-β1, which subsequently leads to tissue scarring and organ dysfunction (Brancewicz *et al.*, 2025; Wynn and Ramalingam, 2012). Nonetheless, these cells also present therapeutic potential owing to their regenerative properties. Contemporary strategies that target macrophage behaviour—through cytokine modulation or selective depletion—are currently under investigation for the treatment of fibrotic diseases. Therefore, maintaining a precise balance between M1 and M2 macrophage polarization is critical for effective tissue repair, as dysregulation of this dynamic can impede the resolution of inflammation or promote pathological fibrosis.

Another critical aspect of anti-inflammation involves mesenchymal stem cells (MSCs), multipotent stromal cells capable of differentiating into various cell types, including osteoblasts, chondrocytes, and fibroblasts (Kode *et al.*, 2009). MSCs are recruited to the injury site in response to inflammatory signals, particularly interleukin-6 (IL-6) and tumour necrosis factor-alpha (TNF-α) (Mackenzie and Flake, 2001; Maxson *et al.*, 2012). Upon arrival, MSCs secrete a range of paracrine factors,

including growth factors such as vascular endothelial growth factor (VEGF) and transforming growth factor-beta (TGF-β), which facilitate angiogenesis and stimulate fibroblast activity (Song, Scholtemeijer and Shah, 2020; Sun *et al.*, 2022; Wong *et al.*, 2015). MSCs play a pivotal role in extracellular matrix reconstruction and tissue regeneration through these cellular interactions.

Beyond their regenerative potential, MSCs exhibit crucial immunomodulatory functions (Kode *et al.*, 2009). They interact with immune cells, including macrophages, T cells, and natural killer (NK) cells, to regulate the inflammatory response. MSCs release factors such as prostaglandin E2 (PGE2) and indoleamine 2,3-dioxygenase (IDO), which suppress excessive immune activation and promote a shift from pro-inflammatory M1 macrophages to anti-inflammatory M2 macrophages (Braza *et al.*, 2016; Philippidis *et al.*, 2004; Zhou *et al.*, 2019). This transition is essential for mitigating inflammation and advancing the repair process from the inflammatory to the proliferative phases. MSCs also influence gene expression in adjacent cells, improving their survival and promoting wound healing by secretion of exosomes containing microRNAs (Ismail *et al.*, 2013; Liu *et al.*, 2018).

In conclusion, MSCs and macrophages play central roles in inflammation and tissue repair cellular dynamics. They contribute to the reconstruction of damaged tissues and regulate immune responses to prevent chronic inflammation. This dual function underscores their potential as therapeutic agents in regenerative medicine and inflammatory diseases.

1.4 Monocytes

Monocytes constitute approximately 2% to 8% of human leukocytes. Beyond their essential functions in development, homeostasis, and inflammation, monocytes also remove apoptotic and necrotic cells (Serbina *et al.*, 2008). These cells derive from hematopoietic stem cell (HSC) monoblasts, which subsequently differentiate into promonocytes and mature into monocytes (Gordon and Taylor, 2005). While the precise nature of monocyte heterogeneity remains incompletely understood, evidence suggests that these cells undergo maturation within the bloodstream before being recruited to sites of tissue injury. Notably, the point at which monocytes exit the circulation may critically influence their subsequent functions and phenotypic specialisation (Sunderkotter *et al.*, 2004).

In mice, two populations of monocytes have been identified: inflammatory and patrolling monocytes, based on the duration they remain in the blood before migrating to tissues (Geissmann, Jung and Littman, 2003). The Nomenclature Committee of the International Union of Immunologic Societies in Berlin, Germany, officially classified human monocytes. This system divides human monocytes into three subsets: 1. The significant or classical population (90%) has high CD14 but no CD16 expression ($CD14^{+}CD16^{-}$). 2. The intermediate subset with low CD16 and high CD14 ($CD14^{+}CD16^{+}$). 3. The subset with low CD14 but high CD16 expression, or nonclassical subset ($CD14^{dim}CD16^{+}$) (Fig.1.2) (Das *et al.*, 2015). Human classical and intermediate monocytes exhibit inflammatory properties and are considered inflammatory monocytes. In contrast, the nonclassical subset shows a patrolling or crawling behaviour along blood vessel walls and responds to virus-related infections. Therefore, inflammatory monocytes demonstrate plasticity, allowing them to change their phenotype based on environmental signals and immune responses following pathogen exposure (Mitchell, Roediger and Weninger, 2014).

Despite the controversy, it has been noted that inflammatory monocytes generate patrolling monocytes in the bone marrow or blood (Mitchell, Roediger and Weninger, 2014). For instance, a rare subset of monocytes expresses tyrosine kinase with Ig and EGF homology domains-2 (TIE2), the angiopoietin receptor. This subset is called TIE2-expressing monocytes, and both the TIE2-expressing and intermediate ($CD14^{+}CD16^{+}$) monocytes, which possess high angiogenic potential, have been associated with liver regeneration (Schauer *et al.*, 2014). Furthermore, monocytes play a role in tissue regeneration, as evidenced by the osteoclasts in regenerating salamander limbs, which develop through the fusion of monocytes (Fischman and Hay, 1962).

It is acknowledged that some macrophages derived from circulating monocytes and are continually replenished both under normal conditions and during inflammation by monocytes from the bloodstream (Ogle *et al.*, 2016; van Tuijl *et al.*, 2019). The transition from monocytes to macrophages, encompassing the distinct phenotypes generated by various monocyte subsets and the conditions that facilitate this process, has been the focus of recent research. Some studies propose that monocytes enter tissues as inflammatory monocytes and then transition into anti-inflammatory monocytes before differentiating into macrophages (Arnold *et al.*, 2007). Others suggest anti-inflammatory macrophages are directly recruited from the blood to generate monocyte-derived macrophages (Arnold *et al.*, 2007; Awojoodu *et al.*, 2013). These differing perspectives may be due to variations in tissue injury contexts, though direct experimental validation is lacking (Ginhoux *et al.*, 2016). Moreover, substantial evidence supports a model in which macrophages derived from various sources. Some macrophages originate from embryonic progenitors and are sustained locally in tissues throughout adulthood (Ogle *et al.*, 2016). Specifically, macrophages in the heart, lungs, brain, skin, and liver are derived prenatally from yolk-sac endothelium, foetal liver monocytes, and early erythroid-myeloid progenitors rather than from adult monocytes (Ogle *et al.*, 2016; Yona *et al.*, 2013). In some injury contexts, such as Th2-associated infections, local tissue macrophage populations can expand primarily through proliferation rather than monocyte recruitment (Jenkins *et al.*, 2011). Overall, these insights underscore the roles of monocytes in tissue homeostasis and injury response, highlighting their capacity to differentiate into macrophages by tissue context and inflammatory conditions.

In conclusion, monocytes represent a dynamic and versatile immune system component, bridging innate immunity, tissue repair, and regeneration. Their functional diversity, exemplified by distinct phenotypic subsets and adaptive plasticity, highlights their critical role in inflammatory and homeostatic processes. Identifying angiogenic monocyte subsets, such as TIE2-expressing monocytes, underscores their potential therapeutic applications in tissue regeneration and angiogenesis. However, further research is required to elucidate the mechanisms underlying their heterogeneity, maturation pathways, and functional transitions. Advancing this understanding could pave the way for innovative strategies to harness monocyte functions in regenerative medicine and inflammatory disease management.

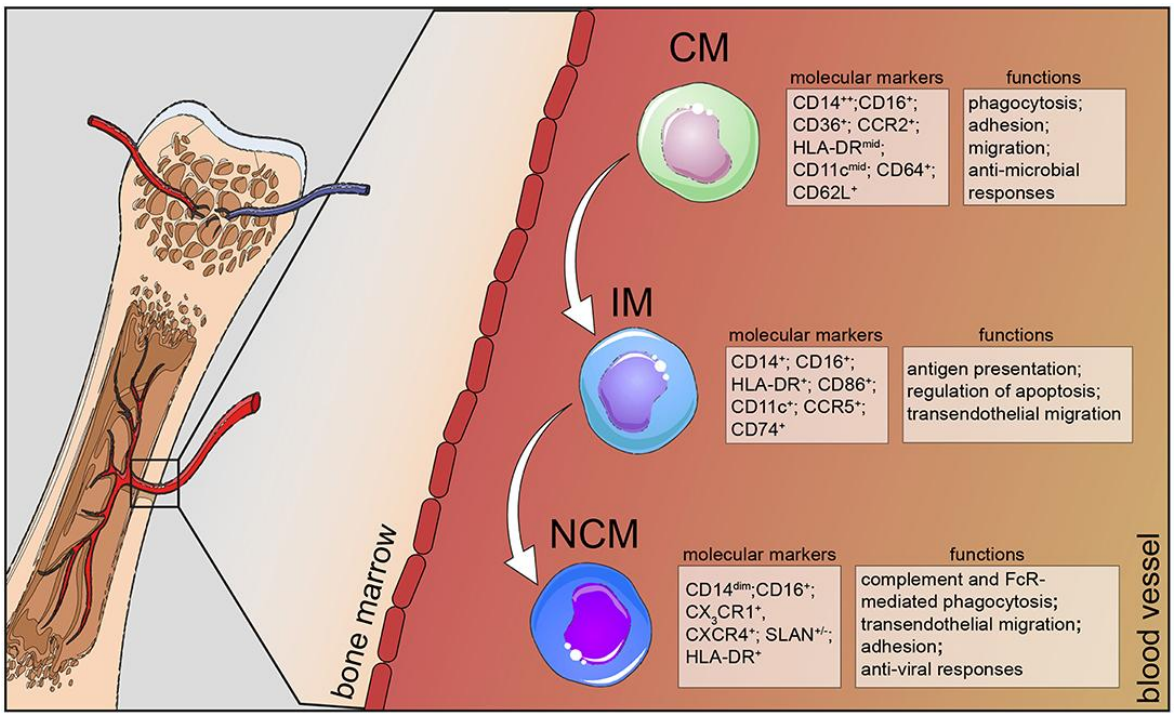


Fig.1.2 Human Monocyte Subsets. Human monocytes mature in the bone marrow and enter circulation as $CD14^+$ classical monocytes ($CD14^+CD16^-$). They transition through an intermediate stage ($CD14^+CD16^+$) into non-classical monocytes ($CD14dimCD16^+$). Classical monocytes express CD36, CCR2, and CD64, aiding in antimicrobial defense, adhesion, and phagocytosis. Intermediate monocytes, marked by CCR5 and HLA-DR, contribute to antigen presentation and migration. Non-classical monocytes, including $SLAN^+$ and $SLAN^-$ subsets, express CX3CR1 and specialise in FcR-mediated phagocytosis, transendothelial migration, and antiviral responses (Kapellos et al., 2019). Abbreviations: CM, classical monocytes; IM, intermediate monocytes; NCM, non-classical monocytes; CCR2, C-C chemokine receptor type 2; HLA-DR, Human Leukocyte Antigen – DR isotype; CCR5, C-C chemokine receptor type 5; CX3CR1, C-X3-C chemokine receptor 1; CXCR4, C-X-C chemokine receptor type 4; SLAN, 6-sulfo LacNAc.

1.5 Macrophages

Macrophages are a vital part of the human immune system. They are recognised for their capacity to engulf and remove foreign invaders such as bacteria, intracellular parasites, and tumour cells. They also play a key role in immune defence, self-stabilisation, and surveillance. Nevertheless, their functions go beyond merely acting as immune cells; they are also critical in development, tissue remodelling, tissue repair, angiogenesis, and metabolism.

1.5.1 Discovery of Macrophages

Macrophages, first characterised by Metchnikoff in his research on primitive organisms lacking adaptive immune systems, were designated as phagocytes, deriving from the Greek terms phagein (meaning to eat) and cytes (meaning cells) (Gordon, 2008). The process of phagocytosis was thought to be connected to the internal balance of tissue absorption and nutrient uptake. Later, Metchnikoff suggested that it could also shield the body from invaders. This observation formed the groundwork for our current understanding of how cells defend against microorganisms. As a result, the original idea of macrophages fostering "balance" in the host (homeostasis) was primarily neglected and overshadowed by their role in cellular immune responses (Mosser, Hamidzadeh and Goncalves, 2021).

Macrophages are present in nearly all body tissues to support proper organ function. Their phagocytic capabilities enable them to eliminate and recycle many dead cells and tissue fragments, vital for organisms lacking adaptive immune responses or blood. Metchnikoff first demonstrated the role of tissue macrophages in healing in invertebrates without a blood system (Gordon, 2008). However, in early embryos of higher vertebrates, tissue macrophages can promote healing and tissue regeneration even before blood vessels develop. Under physiological conditions, macrophages are usually the only immune cells present in specific tissues, such as the eyes, cartilage joints, breasts, brain parenchyma, and ovaries, where they maintain tissue integrity by integrating input signals from the tissue and communicating instructions to adjacent stromal cells (Chinnery, McMenamin and Dando, 2017; Chua *et al.*, 2010; Kurowska-Stolarska and Alivernini, 2017; Wu *et al.*, 2004). The role of macrophages in maintaining bodily balance is so crucial that they are even present in human breast milk, potentially aiding in the digestive balance of infants (Blau *et al.*, 1983). Macrophages are essential for newborns' inflammatory control and healing response, and they are present in every organ, including the epidermis, cornea, and cartilage joints that lack blood vessels (Panahipour *et al.*,

2019). In these cases, macrophages function as sensors, gathering information from the tissue and transforming it into an induced response related to the physiological functions necessary for the organ's daily operation.

Macrophages are recognised for their ability to sense the microenvironment and respond to the needs of organs. Metchnikoff describes how macrophages constantly strive for balance and assist in the unique functions of different organs. For instance, during pregnancy, macrophages receive signals from hormones and are redirected to establish an immune-tolerant environment where the embryo can develop. Additionally, they play a role in forming the placenta, which is vital for fetal growth (Renaud and Graham, 2008). In the heart, macrophages interact with cardiomyocytes, sense their needs, and enhance their repolarisation, thereby maintaining cardiac conduction (Hulsmans *et al.*, 2017). Recent research also suggests macrophages can transmit osmotic signals from tissues and regulate blood pressure in mice and rats on a high-salt diet (Machnik *et al.*, 2009). Therefore, maintaining body balance is the primary role of macrophages. They interpret their microenvironment as sensors and instruct neighbouring cells to achieve equilibrium. These "outputs" are significantly more intricate than the inflammatory cytokines usually linked to macrophage activation. They comprise matrix metalloproteinases that modify the extracellular matrix, vascular endothelial growth factor (VEGF), and thrombospondin, which support angiogenesis, along with growth factors that aid tissue repair. Therefore, viewing macrophages solely as "immune" cells is an oversimplification.

1.5.2 Origin of Macrophages

Macrophages are innate immune cells of the myeloid lineage, originating from precursor cells in the yolk sac (YS) and later in the foetal liver and bone marrow (Epelman, Lavine and Randolph, 2014). Early macrophages migrate to various tissues, establishing specialised populations referred to as resident tissue macrophages (RTMs), which include microglia in the brain, Kupffer cells in the liver, and alveolar macrophages in the lungs (Bleriot, Chakarov and Ginhoux, 2020; Lee and Ginhoux, 2022). Furthermore, monocytes generated in the foetal liver colonise diverse organs before birth, resulting in the formation of long-lived macrophages capable of self-renewal independently of contributions from bone marrow-derived cells (Hoeffel and Ginhoux, 2018). This embryonic lineage lays the groundwork for tissue-specific macrophage populations that endure throughout the lifespan.

In adulthood, macrophages predominantly originate from haemopoietic stem cells (HSCs) in the bone marrow. These cells differentiate into monocytes and enter the bloodstream (Pittet, Nahrendorf and Swirski, 2014). Upon encountering infection, injury, or inflammation sites, these monocytes migrate into tissues, transforming into macrophages or dendritic cells (Yang *et al.*, 2014). These adult-derived macrophages are typically involved in inflammatory responses and are continuously replenished from circulating monocytes. Unlike their embryonic counterparts, these inflammatory macrophages are shorter-lived and primarily function in immune defence and tissue repair during acute events (Elhag *et al.*, 2021).

Tissue-resident macrophages primarily originate from embryonic sources and maintain their populations through self-renewal. Inflammatory macrophages, on the other hand, are derived from bone marrow monocytes and respond swiftly to infection or injury (Mosser, Hamidzadeh and Goncalves, 2021). This dual origin underscores macrophages' adaptability and essential role in immunity and tissue health homeostasis.

1.5.3 Resident Tissue Macrophages (RTMs)

1.5.3.1 The function of RTMs

In various organs, RTMs perform numerous vital functions that help maintain balanced organ function (Schyns *et al.*, 2019). These functions include removing damaged cells or foreign substances, protecting neuronal synapses, trimming unnecessary neuronal connections, safeguarding the vascular system, and acting as the first line of defence against invasive pathogens (Fig.1.3) (Chakarov *et al.*, 2019). RTMs in the lungs remove excess surfactants and eosinophilic substances, while those in the skeleton (osteoclasts) absorb excess bone (Roodman, 1996; Spix *et al.*, 2022). RTMs also contribute to developing specific tissues, such as the hippocampus, kidneys, bone marrow, liver, and spleen, by eliminating nuclei and fragments (Bleriot, Chakarov and Ginhoux, 2020). In the nervous system, macrophages interact with neurons, which is essential for the overall health of the system. In the central nervous system, these cells are called small glial cells and aid in removing dead neurons and regulating blood flow and nutrition to other cells (Sun and Jiang, 2024). In the peripheral nervous system, macrophages help protect and repair damaged neurons (Liu *et al.*, 2019). RTMs in the liver are known as Kupffer cells and play a role in removing bacteria and other toxins from circulation (Rasheed and Rayner, 2021). In the skin, RTMs assist in protecting against infections and maintaining the integrity of the skin barrier (Lee and Ginhoux, 2022).

RTMs in the heart facilitate the removal of damaged cells and promote tissue repair following injury (Poulis *et al.*, 2022). Additionally, RTMs regulate the immune response by phagocytosing pathogens and releasing pro-inflammatory signals. They also contribute to angiogenesis by interacting with endothelial cells and forming new blood vessels (Bleriot, Chakarov and Ginhoux, 2020; Lee and Ginhoux, 2022).

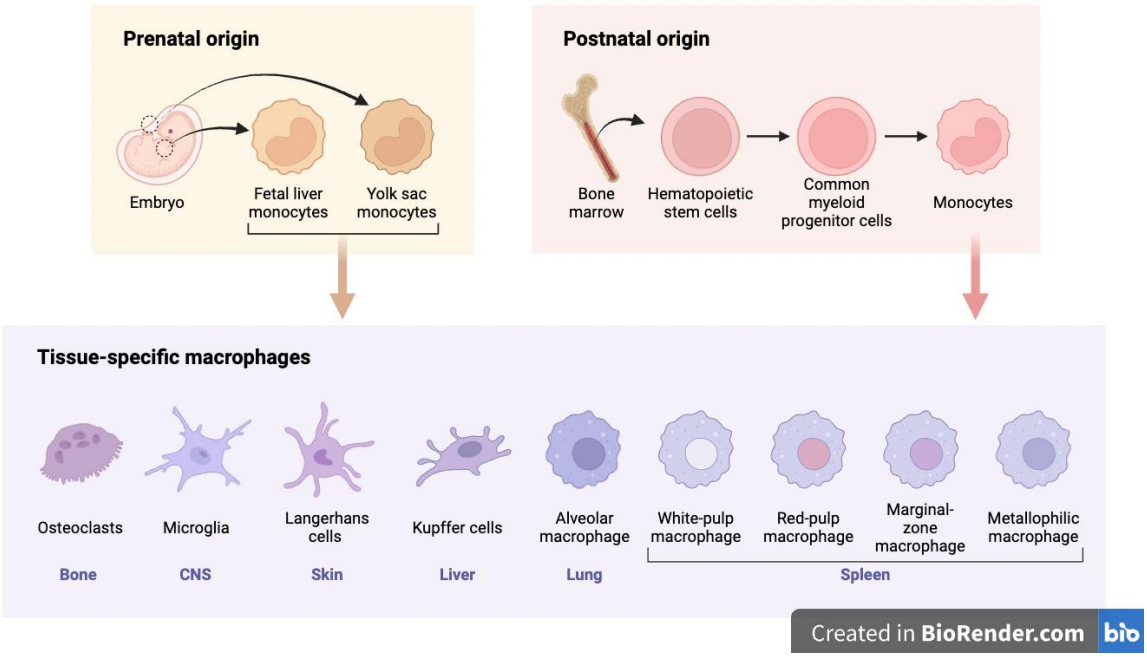


Fig.1.3 The Sources of tissue-resident macrophages are monocytes originating from the bone marrow (the most well-known), monocytes derived from the foetal liver, and macrophages from the yolk sac. Circulating monocytes differentiate into macrophages based on environmental signals. In contrast, tissue-resident macrophages can originate from embryonic precursors, such as microglia from the yolk sac and Langerhans cells from the foetal liver, maintaining themselves through self-renewal. Depending on their location, these macrophages perform specialised functions, such as Kupffer cells clearing debris in the liver, alveolar macrophages defending the lungs, and red pulp macrophages regulating iron in the spleen (Pei and Yeo, 2016). Created in BioRender.com.

1.5.3.2 Inducers of RTM Phenotypes

It has been reported that two main factors can induce resident tissue macrophages to adopt the phenotype of immune cells (Mosser, Hamidzadeh and Goncalves, 2021). In a stable state, the resident macrophages in the tissue may be exposed to regulatory molecules derived from the tissue, such as adenosine, PGE2, resolvins, and lipoxins, for an extended period. This exposure boosts cell growth and proliferation, helping to maintain normal tissue development. These regulatory factors also affect the phenotype of apoptotic cells (Fadok *et al.*, 1998). The second factor is that macrophage growth factors, particularly M-CSF (Macrophage Colony-

Stimulating Factor), produced by various cells, can drive macrophages to polarise into M2 phenotypes that promote growth and angiogenesis (Foucher *et al.*, 2013; Hamilton *et al.*, 2014). During a physiological condition, M-CSF predominates as it is continually secreted by cells in the tissue microenvironment to support tissue homeostasis. Simultaneously, inflammatory granulocytes temporarily produce M-CSF during inflammation to enhance the inflammatory response of all exposed macrophages (Hamilton *et al.*, 2014).

1.5.4 M1 and M2 Macrophages

Macrophages exhibit remarkable plasticity, with their functional states broadly classified into classically activated (M1) and alternatively activated (M2) phenotypes (Fig.1.4) (Patel *et al.*, 2017). These subsets are distinguished by distinct surface markers, cytokine profiles, and biological roles (Mantovani *et al.*, 2004). However, emerging evidence suggests that macrophage polarisation is governed by a complex and dynamic regulatory network rather than a linear binary model (Atri, Guerfali and Laouini, 2018). A key metabolic determinant of M1/M2 polarisation is arginine metabolism, which operates through two opposing pathways: M1 macrophages rely on the inducible nitric oxide synthase (iNOS) pathway, converting arginine into citrulline and nitric oxide (NO), whereas M2 macrophages utilise the arginase pathway, which metabolises arginine into ornithine and urea (Lampiasi, Russo and Zito, 2016).

Upon stimulation with lipopolysaccharide (LPS) and Th1 cytokines such as interferon-gamma (IFN-γ) and tumour necrosis factor-alpha (TNF-α), macrophages undergo M1 polarisation, acquiring a pro-inflammatory phenotype characterised by the expression of Toll-like receptors (TLR2, TLR4), CD80, CD86, iNOS, and major histocompatibility complex class II (MHC-II) (Murray, 2017). These cells secrete a range of pro-inflammatory cytokines and chemokines, including TNF-α, IL-1α, IL-1β, IL-6, IL-12, CXCL9, and CXCL10, which amplify M1 polarisation through a positive feedback loop, recruiting additional macrophages into the inflammatory state (Yao, Xu and Jin, 2019). Transcription factors such as nuclear factor-kappa B (NF-κB), signal transducer and activator of transcription 1 (STAT1), STAT5, interferon regulatory factor 3 (IRF3), and IRF5 are central regulators of M1-associated gene expression (Yao, Xu and Jin, 2019). NF-κB and STAT1 are the primary pathways driving M1 polarisation, leading to enhanced antimicrobial and tumoricidal activity.

Conversely, M2 polarisation is induced by Th2 cytokines, primarily interleukin-4 (IL-4) and interleukin-13 (IL-13), while other cytokines, including IL-10, IL-33, and

transforming growth factor-beta (TGF-β), serve to reinforce the M2 phenotype by promoting Th2-mediated responses (Porta *et al.*, 2015). M2 macrophages are characterised by the expression of specific surface markers, including CD206, CD163, CD209, FIZZ1, and Ym1/2. Their secretory profile includes anti-inflammatory cytokines and chemokines such as IL-10, TGF-β, CCL1, CCL17, CCL18, CCL22, and CCL24, which promote tissue repair and immune resolution and facilitate M2 polarisation (Wang, Liang and Zen, 2014). The transcriptional landscape of M2 macrophages is regulated by STAT6, IRF4, JMJD3, peroxisome proliferator-activated receptor delta (PPARδ), and PPARγ, with STAT6 emerging as the central driver of M2 activation (Murray, 2017; Yao, Xu and Jin, 2019).

In summary, macrophages are pivotal in orchestrating immune responses, preventing infections, promoting tissue repair, supporting angiogenesis, and modulating immune homeostasis. Their phenotypic plasticity enables them to dynamically adapt to environmental cues, balancing inflammation and repair processes. Understanding the molecular mechanisms governing M1/M2 transitions is crucial for developing targeted therapeutic strategies to modulate macrophage function in inflammatory diseases, tissue regeneration, and cancer immunotherapy.

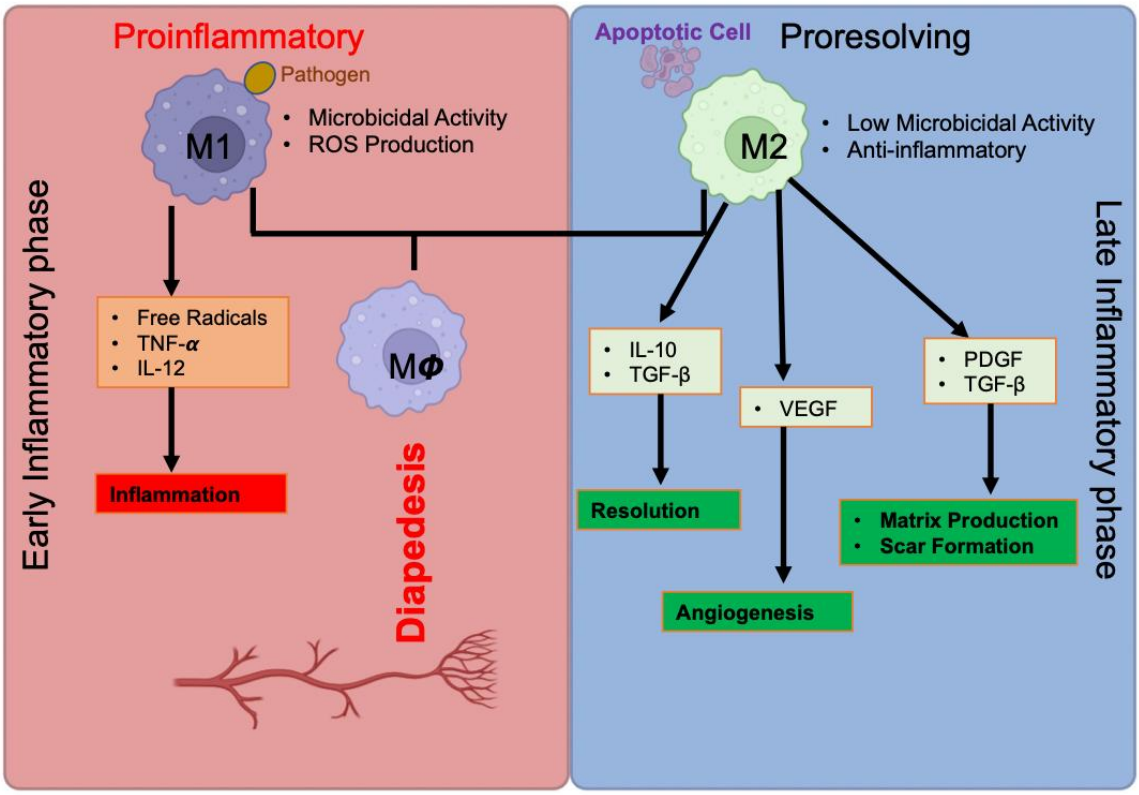


Fig.1.4 Schematic Representation of M1/M2 macrophage Phenotype Pathways. The transition from the early to late phases of inflammation through the roles of M1 and M2 macrophages. In the early inflammatory phase, M1 macrophages respond to pathogens with high microbicidal activity and production of reactive oxygen species (ROS), releasing proinflammatory mediators like free radicals, TNF-α, and IL-12, leading to inflammation. Diapedesis occurs as immune cells migrate from the blood into tissues. In the late inflammatory phase, M2 macrophages are activated by apoptotic cells and exhibit low microbicidal activity with anti-inflammatory effects. They release IL-10 and TGF-β to promote resolution, VEGF to support angiogenesis, and PDGF and TGF-β to drive matrix production and scar formation, thereby supporting tissue repair and healing. PDGF (platelet-derived growth factor), TGF (transforming growth factor), TNF (tumour necrosis factor), and VEGF (vascular endothelial growth factor) (Das *et al.*, 2015).

1.5.5 The Subsets of M2 Macrophages

The plasticity of macrophages in tissue repair is a crucial process. Upon injury, macrophages migrate to the injury site through diapedesis from the systemic circulation (Wynn and Vannella, 2016). Initially, the inflammatory environment leads to the polarisation of M1 macrophages, which possess microbicidal properties and support type 1 helper T-cell responses mediated by IL-12 (Wynn, Chawla and Pollard, 2013). These responses are necessary for the initial stages of tissue repair. Later, as the wound microenvironment changes, efferocytosis (the clearance of apoptotic cells) causes M1 macrophages to transition towards M2 polarisation (Wynn and Vannella, 2016). M2 macrophages support type 2 helper T-cell-related effector functions and play a more reparative role in the later stages of tissue repair (Chen *et al.*, 2023).

As mentioned above, M1-like macrophages primarily trigger proinflammatory responses and are typically linked to the Th1 response, whereas M2-like macrophages promote trophic functions and support tissue tolerance (Wang *et al.*, 2020; Yao, Xu and Jin, 2019). M2 macrophages are categorised into four subsets: M2a, M2b, M2c, and M2d (Fig.1.5). M2a macrophages are activated by IL-4 or IL-13, leading to the elevated expression of IL-10, TGF-β, CCL17, CCL18, and CCL22, which enhance endocytic activity, promote cell growth, and facilitate tissue repair. M2b macrophages are stimulated by immune complexes, Toll-like receptor (TLR) ligands, and IL-1β, resulting in the release of both pro- and anti-inflammatory cytokines, including TNF-α, IL-1β, IL-6, and IL-10, thereby modulating immune responses and inflammatory reactions. M2c macrophages, also known as inactivated macrophages, are induced by glucocorticoids, IL-10, and TGF-β, leading to the secretion of IL-10, TGF-β, CCL16, and CCL18 and playing a crucial role in the phagocytosis of apoptotic cells. M2d macrophages, induced by TLR antagonists, promote angiogenesis and tumour progression by releasing IL-10 and vascular endothelial growth factor (VEGF).

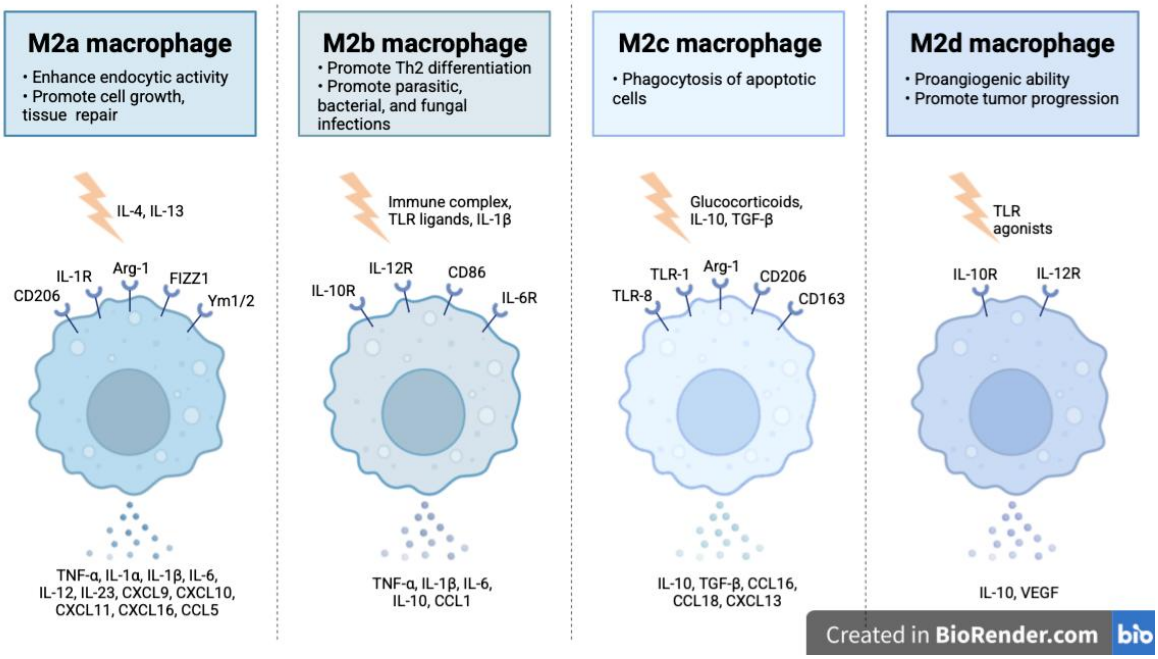


Fig.1.5 Characteristics of M2 Macrophage Subsets: Stimuli, Surface Markers, Secreted Cytokines, and Biological Functions. M2-like macrophages can be subdivided into M2a, M2b, M2c, and M2d phenotypes based on distinct microenvironmental stimuli. Each phenotype exhibits unique expression patterns of cytokines, chemokines, and receptors, which determine their specific functions. Created in BioRender.com.

1.5.6 Tumour-Associated Macrophages (TAMs)

Tumour-associated macrophages (TAMs) are specialised macrophages located within tumours or environments enriched with tumour cells. They play a vital role in the progression of cancer and the outcomes of treatment (Pei and Yeo, 2016). TAMs constitute a significant portion of solid tumour cellularity, often forming a major component of the tumour mass (Siveen and Kuttan, 2009). They originate from monocytes recruited by tumour cells through soluble mediators such as chemokine (C–C) ligand 2 (CCL2) (Jinushi and Komohara, 2015). Upon exposure to anti-inflammatory signals, including IL-4, IL-10, TGF-β, and prostaglandin E2, these monocytes differentiate into M2-like macrophages, suppressing anti-tumor immunity due to their reduced capabilities in antigen presentation (Jinushi and Komohara, 2015; Siveen and Kuttan, 2009).

M2-like TAMs hinder the immune response by suppressing IL-12 production, thereby impairing the activation of natural killer (NK) cells, T helper 1 (Th1) cells, and CD4+ T cells, which are crucial for anti-tumour defence (Gabrilovich, Ostrand-Rosenberg and Bronte, 2012). Additionally, they secrete chemokine CCL22, which attracts regulatory T cells (Tregs) to the tumour site, further dampening tumour-specific T cell responses (Curiel *et al.*, 2004). Beyond immune modulation, TAMs facilitate tumour progression through non-immune mechanisms, such as promoting vasculogenesis and angiogenesis by releasing vascular endothelial growth factor (VEGF), which supports the formation of new blood vessels to sustain tumour growth (Kitamura, Qian and Pollard, 2015).

1.5.7 Macrophages in Tissue Repair

Macrophages play a vital role in tissue repair, regeneration, and fibrosis, dynamically adapting to the changing needs of damaged areas (Fig.1.6). In the immediate aftermath of injury, inflammatory monocytes and tissue-resident macrophages are rapidly recruited to the site, releasing a cascade of cytokines and growth factors that initiate the healing process. These cells orchestrate the inflammatory response, clearing debris and pathogens while signalling other immune and structural cells to mobilise for repair (Wynn and Vannella, 2016). Their activity is critical in shaping the initial phases of wound healing, yet their influence extends far beyond the inflammatory stage.

As healing progresses, macrophages experience a pivotal transformation, transitioning from a pro-inflammatory state (M1) to an anti-inflammatory (M2) and regenerative phenotype (Bi *et al.*, 2019). This change is characterised by the secretion of factors such as TGF-β1, IGF-1, and VEGF, which facilitate the proliferation of fibroblasts and endothelial cells, encourage angiogenesis, and enhance the survival of tissue progenitor cells (Wynn and Vannella, 2016). Moreover, their interactions with the extracellular matrix and adjacent stromal cells cultivate an environment conducive to regeneration, thereby ensuring the restoration of functionality in damaged tissue structure.

The delicate balance of macrophage activity is crucial in determining whether tissue healing proceeds efficiently or veers toward pathological outcomes (Wynn, Chawla and Pollard, 2013). When the inflammatory response is excessive or unresolved, macrophages can drive chronic injury, leading to persistent tissue damage and fibrosis. The overproduction of pro-fibrotic mediators, particularly TGF-β1, induces fibroblast activation and excessive extracellular matrix deposition, resulting in scarring that disrupts normal organ function (Oliver, Davis and Bohannon, 2024). In various chronic diseases, such as liver cirrhosis, pulmonary fibrosis, and cardiac remodelling, macrophages are essential in progressing from acute injury to chronic fibrotic disease (Jiang *et al.*, 2024).

Despite their potential to exacerbate fibrosis, macrophages also present significant promise as therapeutic targets within regenerative medicine (Spiller and Koh, 2017). Their capacity to facilitate resolution and promote tissue remodelling has prompted extensive research into strategies to modulate their functional activity. Furthermore, experimental methodologies involving colony-stimulating factors, cytokine modulation, and the targeted depletion of specific macrophage subsets suggest that

altering macrophage behaviour could be crucial in treating fibrotic and degenerative conditions (Fadok *et al.*, 1998; Lee *et al.*, 2020; Yosef *et al.*, 2018).

Macrophages are also essential in tissue regeneration beyond merely repairing damage (Das *et al.*, 2015). Studies in neonatal heart repair and limb regeneration in amphibians have demonstrated that macrophages create a pro-regenerative microenvironment that fosters the survival and differentiation of stem cells (Aurora *et al.*, 2014; Gurley and Sanchez Alvarado, 2008). By secreting growth factors and influencing cellular signalling pathways, they enable tissue renewal in ways that go beyond simple wound closure (Eming, Martin and Tomic-Canic, 2014; Forbes and Rosenthal, 2014). They coordinate regeneration, including inflammation, stem cell activation, and matrix remodelling, making them essential for natural and therapeutic repair processes.

While their inflammatory functions are critical for immediate defence and resolving injury-related debris, sustained activation may result in detrimental fibrosis. Concurrently, their regenerative capabilities present opportunities for therapeutic interventions promoting tissue repair. The challenge resides in effectively utilising their advantageous functions while mitigating their potential for adverse effects. This pursuit drives research in immunology, regenerative medicine, and fibrosis treatment.

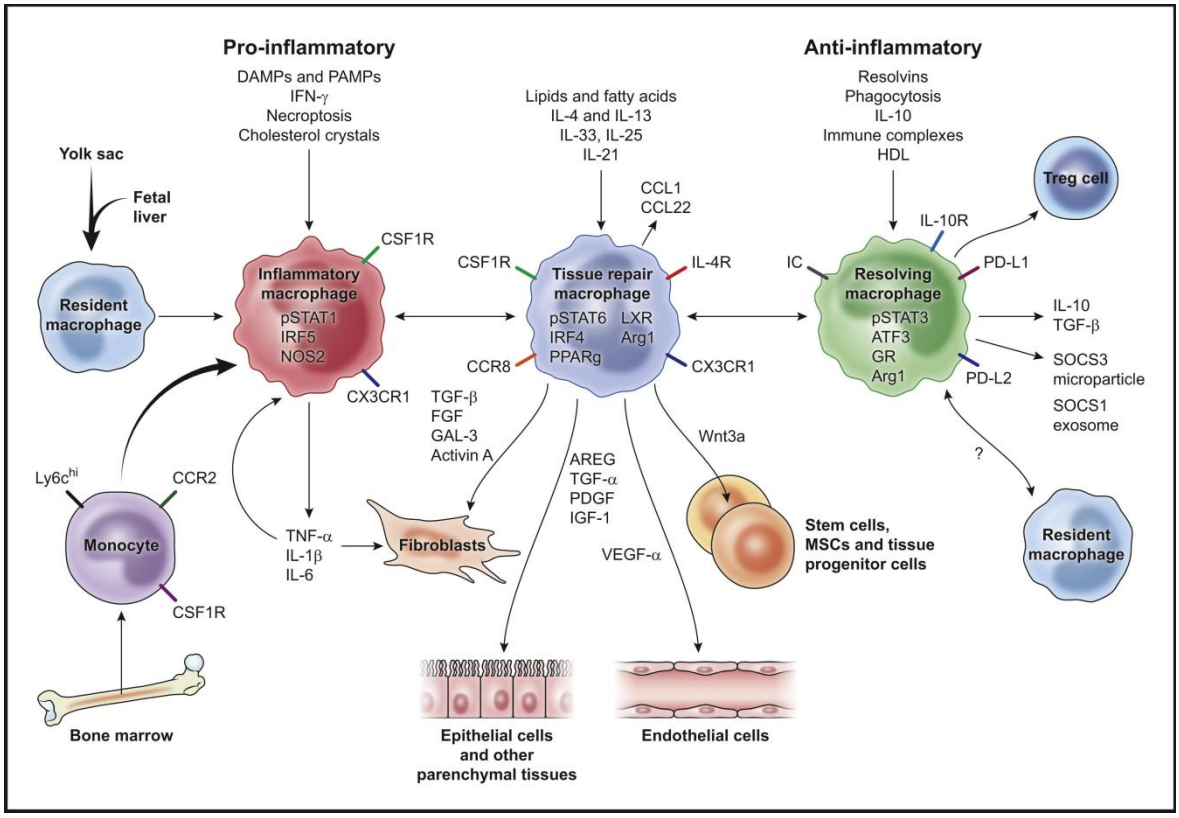


Fig.1.6 Mechanisms Regulating Key Macrophage Activation States in Tissue Repair, Regeneration, and Fibrosis. Resident macrophages originate from the yolk sac and foetal liver, while recruited monocytes from the bone marrow supplement them after injury. Both types respond to DAMPs, PAMPs, cytokines, and growth factors, undergoing phenotypic changes to regulate inflammation, repair tissue, and promote regeneration. They stimulate fibroblasts, epithelial, endothelial, and progenitor cells for healing. In later recovery, macrophages shift to a pro-resolving state to suppress inflammation and restore tissue. Dysregulation can lead to fibrosis and damage. Some recruited monocytes become resident-like, but mechanisms of homeostasis remain under study. Abbreviations: DAMP, damage-associated molecular pattern; PAMP, pathogen-associated molecular pattern; Treg cell, regulatory T cell; IRF5, interferon regulatory factor 5; NOS2, nitric oxide synthase 2; LXR, liver X receptor; AREG, amphiregulin; Arg1, arginase-1; IRF4, interferon regulatory factor 4; PPARγ, peroxisome proliferator-activated receptor γ; FGF, fibroblast growth factor; GAL-3, galectin-3; TGF, transforming growth factor; IC, immune complex; GR, glucocorticoid receptor; ATF3, activating transcription factor 3; SOCS, suppressor of cytokine signalling (Wynn and Vannella, 2016).

1.5.8 Mer Proto-oncogene Tyrosine Kinase

Mer proto-oncogene Tyrosine kinase (MerTK), identified in 1992 by Hanafusa and his colleagues, was first found as a truncated version of a tyrosine kinase (Env-Ryk, v-Ryk, or v-Eyk) derived from the RPL30 chicken retrovirus transforming gene. (Jia *et al.*, 1992). MerTK is a member of the MER/AXL/TYRO3 (TAM) receptor kinase family and encodes a transmembrane protein with two fibronectin type-III domains, two Ig-like C2-type (immunoglobulin-like) domains, and one tyrosine kinase domain. It is universally acknowledged that LPS and IFN-γ are commonly employed as standard inducers for M1 macrophage polarisation, whereas IL-4 and IL-13 serve as the standard agents for M2 macrophage polarisation *in vitro*. Nevertheless, several other proteins and stimulants can affect macrophage polarisation, with TAM receptor signalling notably playing a role by suppressing M1 polarisation and promoting M2 polarisation (Fig.1.7) (Myers, Amend and Pienta, 2019). This PhD research investigates the role of MerTk in macrophage regulation.

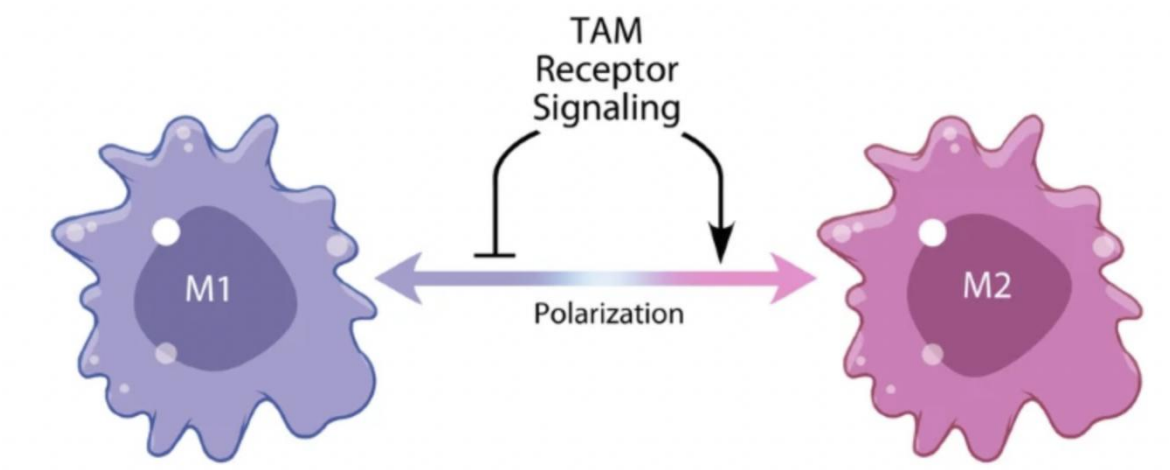


Fig.1.7 The TAM Receptors (Tyro3, Axl and MerTK) Signalling Skews Macrophage Polarisation. TAM receptor binding and downstream signalling dampen M1 polarisation and promote M2 polarisation (Myers, Amend and Pienta, 2019).

1.5.8.1 Regulation of MerTK

MerTK plays a crucial role in regulating immune homeostasis, especially in the phagocytosis of apoptotic cells and the suppression of inflammatory responses (Liu *et al.*, 2024b). Its activity is mainly mediated through the binding of ligands such as Gas6 (Growth arrest-specific 6) and Protein S, which connect MerTK to phosphatidylserine on apoptotic cells (Malawista *et al.*, 2016). This interaction activates downstream signalling pathways, such as PI3K-Akt and MAPK, facilitating the engulfment of apoptotic debris and the resolution of inflammation (Nishi *et al.*,

2019; Zhang, Earp and Liu, 2019). Regulation of MerTK is tightly controlled through mechanisms such as proteolytic cleavage, which results in the release of a soluble Mer ectodomain that acts as a decoy receptor alongside transcriptional modulation influenced by inflammatory cytokines (Lahey *et al.*, 2024). Thus, dysregulation of MerTK activity has been linked to various pathologies, including chronic inflammation, autoimmunity, and cancer, highlighting its significance as a therapeutic target for restoring immune balance.

Moreover, MerTK is essential in the circadian regulation of retinal pigment epithelial (RPE) cells, specifically in the phagocytosis of the photoreceptor outer segments (POS) (Finnemann and Nandrot, 2006). This process is vital for sustaining photoreceptor health and is intricately regulated according to a daily rhythm. Research has demonstrated that the function of MerTK in RPE cells exhibits rhythmicity, with ligand availability fluctuating throughout the circadian cycle, thereby indicating that MerTK is instrumental in the precise regulation of phagocytosis timing (Casanova-Acebes *et al.*, 2013; Parinot *et al.*, 2024).

1.5.8.2 Function of MerTK

MerTK signalling is essential for transmitting signals from the extracellular matrix to the cytoplasm by forming connections with ligands such as LGALS3, TUB, TULP1, or GAS6. This process is initiated when these ligands bind to MerTK, located at the cell surface, subsequently triggering the autophosphorylation of MerTK's intracellular domain. As a result, this mechanism generates docking sites for downstream signalling molecules, thereby initiating critical signalling cascades. Various reports indicate that MerTK regulates many processes, including the clearance of apoptotic cells by macrophages, platelet aggregation, cytoskeleton reorganisation, and the engulfment of cellular debris (Moon *et al.*, 2020; Sather *et al.*, 2007).

MerTK is pivotal in macrophage-mediated processes, contributing to immune regulation and tissue repair. First, MerTK facilitates the recognition of apoptotic cells by macrophages through binding to the "eat me" signal phosphatidylserine (PtdSer) (PS) on apoptotic cell membranes (Zhou *et al.*, 2020). As a PS receptor, MerTK engages with externalised apoptotic cells by utilising bridging molecules such as Gas6 and Protein S, thereby facilitating efferocytosis and inhibiting secondary necrosis (Penberthy *et al.*, 2017). This interaction, mediated via the PtdSer-GAS6-MerTK axis, promotes the phagocytosis of apoptotic cells, a crucial mechanism for maintaining tissue homeostasis and preventing chronic inflammation. Knockout

studies in mice have shown that the absence of MerTK leads to defective apoptotic cell clearance and an increase in pro-inflammatory cytokines like IL-1β and TNF-α, contributing to an immunogenic environment (Fig.1.8) (Birge *et al.*, 2016). Beyond its role in efferocytosis, MerTK also functions as an inhibitory receptor that suppresses NF-κB activation and inflammatory cytokine production, thereby promoting immune tolerance (Birge *et al.*, 2016; Cvetanovic and Ucker, 2004; Tibrewal *et al.*, 2008). This inhibitory signalling is independent of efferocytosis, suggesting that MerTK can modulate immune responses simply through apoptotic cell binding.

Second, the activation of MerTK signalling pathways elicits anti-inflammatory responses in macrophages, driving the resolution of inflammation (Zhou *et al.*, 2020; Zizzo *et al.*, 2012). This function underscores the importance of MerTK in mitigating excessive inflammatory damage and fostering a return to tissue equilibrium. Third, increased MerTK expression in macrophages infiltrating injured tissues, such as those affected by cardiac injury, extends beyond phagocytosis (Cai *et al.*, 2017; DeBerge *et al.*, 2017b). These macrophages contribute to tissue regeneration through enhanced angiogenesis, mediated by the upregulation of vascular endothelial growth factor (VEGF) and the production of anti-inflammatory cytokines such as IL-10 and IL-1 receptor antagonist (IL-1RN). However, this regenerative capability is a double-edged sword, as elevated MerTK expression in tumour-infiltrated macrophages mirrors its role in tissue repair by promoting cancer metastasis by releasing regenerative factors. Thus, MerTK exemplifies a key regulatory axis in macrophage function, with implications ranging from inflammation resolution to tissue regeneration and disease progression.

It also plays a vital role in inhibiting Toll-like receptors (TLRs)-mediated innate immune response by activating STAT1, which selectively induces the production of cytokine signalling SOCS1 and SOCS3 suppressors (Lee *et al.*, 2012b). In current research, many papers show that the upregulation of MerTK signalling plays a vital role in anti-inflammation (Zhang *et al.*, 2019; Shirakawa *et al.*, 2020; Choi *et al.*, 2013). Research shows that clinical reperfusion following a myocardial infarction significantly increases the soluble form of MerTK (DeBerge *et al.*, 2017a). The expressions of galectin-3 and MerTK lead to the functional maturation of osteopontin-producing macrophages via the activation of STAT3 and ERK in cardiac macrophages after myocardial infarction (Shirakawa *et al.*, 2020).

Additionally, the activation of MerTK and the induction of M2c polarisation are

necessary for the efficient clearance of early apoptotic cells (EACs) by human macrophages. This suggests that the activation of MerTK and the polarisation of macrophages into the M2c phenotype may be necessary to regulate immune responses and tissue repair (Zizzo *et al.*, 2012).

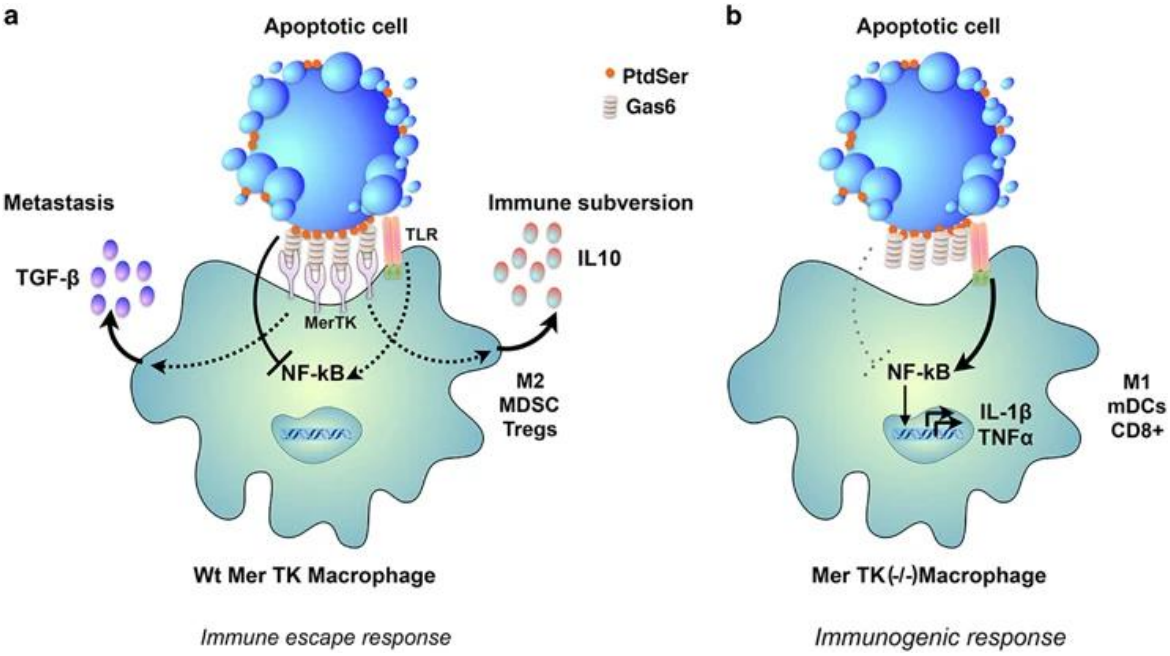


Fig.1.8 The Phosphatidylserine Receptor MerTk Functions as an Inhibitory Receptor, Maintaining Homeostasis and Tissue Tolerance. It facilitates efferocytosis by recognising externalised PtdSer on apoptotic cells through its bridging molecule, Gas6. Upon activation, MerTk transmits inhibitory signals that suppress NF-kB and limit the production of inflammatory cytokines from TLRs. Furthermore, effective efferocytosis promotes the release of tolerogenic factors such as IL-10) and TGF-β), which shape the local microenvironment to support M2 macrophages, immature dendritic cells (DCs), and regulatory T cells (Tregs). In circumstances wherein MerTk is either knocked out or pharmacologically inhibited, the activation of TLR-induced inflammatory cytokines continues unabated, thereby establishing an immunogenic environment conducive to M1 macrophages, mature antigen-presenting dendritic cells, and CD8+ T lymphocytes (Birge *et al.*, 2016).

1.5.8.3 Expression of MerTK Gene in Human Cells/Tissue

MerTK is primarily found on macrophages and dendritic cells, with reduced levels observed in vascular endothelial cells (ECs) and vascular smooth muscle cells (SMCs) (Lee *et al.*, 2023b; Liu *et al.*, 2023). Physiologically, this expression is observed in various other cell types, including microglia, monocytes, natural killer (NK) cells, epithelial cells, and platelets (Huelse *et al.*, 2020a). While absent in normal B and T lymphocytes, MerTK is significantly upregulated in activated B and T lymphocytes in the tumour microenvironment (Cook *et al.*, 2013). In addition, high levels of MerTK are observed in the central nervous system (CNS), lung, kidney, prostate cancer, and in diseased tissues, including metastatic prostate cancer and

liver fibrosis (Liu *et al.*, 2024b). Moreover, MerTK is found in minimal amounts in the aorta, heart, and skeletal muscle (Al-Zaeed *et al.*, 2021; Liu *et al.*, 2023).

1.5.8.4 MerTK and Human Tissue-Specific Diseases

It has been reported that MerTK is involved in a diverse array of pathological conditions, including cancer, cardiovascular disease, and neurological disorders (Cai *et al.*, 2017; Thorp *et al.*, 2011). Elevated expression of MerTK in disease states may serve as a compensatory response to promote tissue protection and functional preservation, whereas MerTK deficiency has been correlated with exacerbated disease outcomes (Cai *et al.*, 2017; Thorp *et al.*, 2011). Aberrant overexpression of MerTK has been observed across multiple human cancers, such as melanoma, leukaemia, and cancers of the prostate, lung, breast, and liver (Cummings *et al.*, 2013). Due to its role in regulating the innate immune response, maintaining tissue homeostasis, supporting reparative mechanisms, and influencing platelet aggregation, MerTK is regarded as a promising therapeutic target in oncology (Huelse *et al.*, 2020a). Within the central nervous system (CNS), MerTK is primarily expressed by microglia, infiltrating macrophages, and astrocytes (Konishi *et al.*, 2020). Its expression is notably upregulated in response to neurological disorders, including traumatic brain injury (TBI), stroke, and primary brain tumours (Liu *et al.*, 2024b). In contrast, advanced atherosclerosis is associated with MerTK cleavage, with MerTK deficiency linked to increased infarct size and impaired cardiac function (DeBerge *et al.*, 2017b; Thorp *et al.*, 2011). Mouse models engineered to resist MerTK cleavage have demonstrated reduced infarct size and improved cardiac function, highlighting MerTK's potential therapeutic relevance in cardiovascular pathology (DeBerge *et al.*, 2017b).

This study investigated the effects of LPS-induced NF-κB activation on MerTK expression and inflammatory cytokine production in human U937-derived macrophages.. The study further explored how modulation of this pathway by an NF-κB inhibitor, as well as treatment with iPSCs-derived mesenchymal stem cell-conditioned medium and exosomes, may influence the inflammatory response. These experiments were designed to explore the mechanisms regulating MerTK expression and modulating inflammation, with the goal of identifying potential therapeutic strategies for chronic inflammatory diseases.

1.5.9 Nuclear factor κ B (NF-κB) in Macrophages

The nuclear factor kappa B (NF-κB) signalling pathway, discovered in 1986, is an important regulator of cellular responses to a wide range of external stimuli,

including inflammation, stress, and infection (Sen and Baltimore, 1986; Singh *et al.*, 1986). Initially characterised as a transcription factor involved in the immune response of B lymphocytes, subsequent research has unveiled its central role in numerous biological processes, including cell survival, proliferation, differentiation, and apoptosis (Guo *et al.*, 2024). The NF-κB family comprises five proteins: NF-κB1 (p105/p50), NF-κB2 (p100/p52), RelA (p65), RelB, and c-Rel, which form various homo- or heterodimeric complexes to exert their effects (Ahmad *et al.*, 2022). These complexes are primarily regulated through two pathways—canonical and non-canonical—activated by different stimuli and employing distinct molecular mechanisms.

In its canonical form, NF-κB signalling is activated by inflammatory cytokines, such as tumour necrosis factor-alpha (TNF-α) and interleukin-1 beta (IL-1β), as well as bacterial components like lipopolysaccharide (LPS). LPS, a component of Gram-negative bacterial cell walls, binds to Toll-like receptor 4 (TLR4) on macrophages, initiating a signalling cascade that activates NF-κB (Krappmann *et al.*, 2004; Sharif *et al.*, 2007). Similarly, IL-1β stimulates the pathway in an autocrine and paracrine manner, perpetuating cytokine production and sustaining inflammation (Jang *et al.*, 2006). This rapid activation induces the expression of pro-inflammatory mediators, including cytokines and chemokines, that are essential for immune defence and tissue repair. Conversely, the non-canonical pathway, triggered by signals such as CD40 ligand and B-cell activating factor, is slower and sustains activation for lymphoid organ development and immune cell maturation (Fig.1.9) (Elgueta *et al.*, 2009).

Macrophages, as central effectors of the innate immune system, play a crucial role in inflammation and tissue repair through their interaction with the NF-κB pathway (Park *et al.*, 2018). NF-κB activation influences macrophage polarisation into pro-inflammatory states (Liu *et al.*, 2017). M1 macrophages, induced by stimuli such as LPS, IFN-γ and IL-1β, secrete high levels of inflammatory mediators that facilitate pathogen clearance and initiate tissue repair (Wynn and Vannella, 2016). Conversely, M2 macrophages, influenced by anti-inflammatory signals, promote the resolution of inflammation and contribute to tissue remodelling by producing angiogenic factors and extracellular matrix components such as IL10, TGFβ and VEGF (Mantovani *et al.*, 2013). Transitioning from M1 to M2 macrophages is essential for resolving inflammation and ensuring proper healing. Therefore, dysregulation of NF-κB signalling can disrupt this balance, leading to chronic inflammation or impaired repair.

As NF-κB signalling intricately interacts with other pathways, such as PI3K/AKT, MAPK, and JAK-STAT, its role in orchestrating cellular processes is embedded in a more extensive network of molecular crosstalk (Beinke and Ley, 2004; Guo *et al.*, 2024; Hayden and Ghosh, 2008). These interactions allow it to modulate diverse biological processes, including immunity, inflammation, metabolism, and tumorigenesis. Given the critical role of NF-κB in physiological and pathological settings, this pathway remains a focal point for translational research to develop therapeutic interventions. A deeper understanding of NF-κB signalling mechanisms and its interactions with other cellular pathways is essential for designing precise, context-specific therapies that harness its regulatory potential while mitigating its pathological effects.

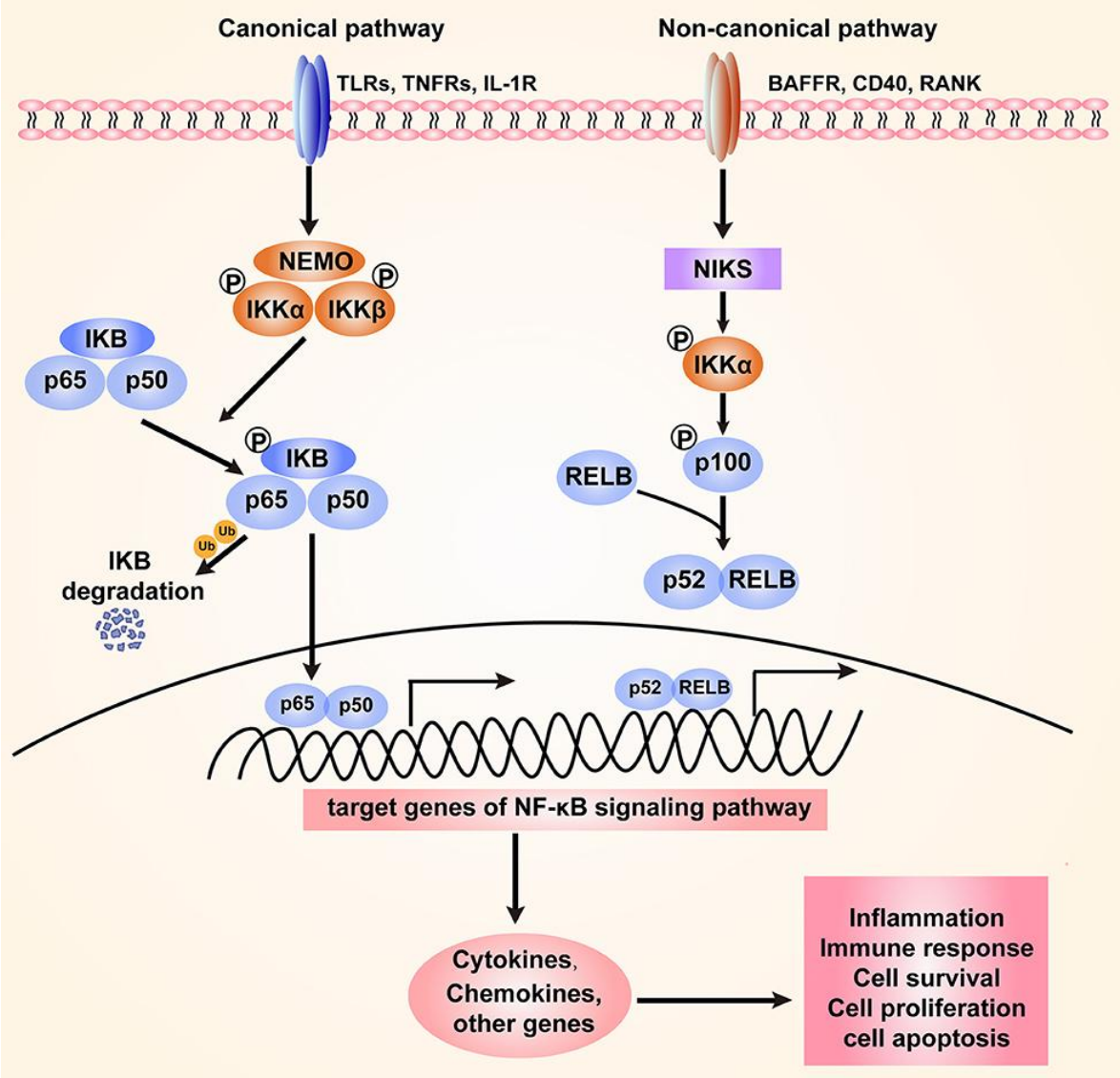


Fig.1.9 The canonical and non-canonical NF-κB signalling pathway. The canonical NF-κB pathway is triggered by receptors such as TLRs, TNFRs, and IL-1R. This activation results in the phosphorylation and subsequent degradation of the inhibitory protein IκB, allowing NF-κB to dissociate from the IκB complex and translocate into the nucleus. In contrast, the non-canonical pathway relies on the activation of the NF-κB2 (p100)/RelB complex by BAFFR, CD40, and RANK. This pathway involves the phosphorylation of NIK, which then activates IKKα, leading to the formation of the p52-RelB heterodimer that translocates to the nucleus. Both pathways play a crucial role in regulating diverse cellular processes by controlling the expression of cytokines, chemokines, and other target genes (Peng *et al.*, 2020).

1.5.10 Immune Rhythm

The circadian clock regulates an approximately 24-hour rhythm, optimising the organism's ability to anticipate and respond to regular, recurring environmental events (Vitaterna, Takahashi and Turek, 2001). This circadian regulation similarly governs the immune system, which exhibits pronounced 24-hour rhythmicity in its basal state and during activation (Zeng *et al.*, 2024). The circadian clock regulates immune function and tissue repair by modulating inflammatory responses, cellular proliferation, and healing efficiency. Disruptions in this system result in diminished recovery and an elevated disease risk (Dierickx, Van Laake and Geijsen, 2017; Garbarino *et al.*, 2021; Keller *et al.*, 2009).

1.5.10.1 Discovery of Immune Rhythms

Immune rhythm was first elucidated in the innate immune system in 1960, with the identified diurnal variations in immune activity (Halberg *et al.*, 1960). This discovery marked a crucial moment in immunological research, underscoring the influence of temporal factors on immune function. A decade later, similar time-dependent fluctuations were observed in the adaptive immune system, demonstrating that circadian rhythms govern both branches of the immune response (Fernandes *et al.*, 1976). These early studies established the foundation for our current understanding of circadian regulation in immunology. It is now recognised that the circadian clock profoundly influences immune cell migration, activation, and functional responses. The circadian regulation of the immune system is indispensable for optimising the timing of immune responses, enhancing the organism's ability to defend against pathogens, and maintaining immune homeostasis (Wang *et al.*, 2022).

1.5.10.2 E-box

In 1985, the E-box was identified as a regulatory element within the immunoglobulin heavy-chain enhancer through a collaborative study by Susumu Tonegawa and Walter Gilbert, highlighting its role in transcriptional regulation (Church *et al.*, 1985; Ephrussi *et al.*, 1985). In 1997, scientists first identified an E-box-regulated circadian transcriptional enhancer upstream of the period (per) gene in Drosophila melanogaster, demonstrating its role in high-level mRNA transcription independent of rhythmicity (Hao, Allen and Hardin, 1997). E-box is also a DNA response element observed in certain eukaryotic organisms (Massari and Murre, 2000). It is a crucial regulatory site for gene expression in various tissues, including neuronal and muscular systems. This sequence is commonly denoted as CANNTG, where "N" can be any nucleotide (Malik, Huang and Schmidt, 1995). It encompasses a

conserved palindromic motif, CACGTG, which acts as a recognition site for transcription factors (Chaudhary and Skinner, 1999). Following their binding to the E-box within gene promoter regions, these transcription factors facilitate the recruitment of additional regulatory proteins and enzymatic complexes that initiate transcription, thereby enabling the conversion of DNA into messenger RNA (mRNA).

1.5.10.3 Circadian Rhythm Mechanism

The circadian rhythm is predominantly regulated by the suprachiasmatic nucleus (SCN), a diminutive region within the hypothalamus that functions as the principal biological clock (Gillette and Tischkau, 1999). The SCN receives direct input from the retina via the retinohypothalamic tract, thereby enabling synchronisation with the external light-dark cycle (Husse, Eichele and Oster, 2015). Exposure to light during the morning hours signals the onset of wakefulness, whereas the absence of light facilitates the secretion of melatonin, a hormone that assists in inducing sleep (Gillette and Tischkau, 1999; Husse, Eichele and Oster, 2015; Masters *et al.*, 2014). This primary biological clock orchestrates peripheral clocks in various organs, including the liver, heart, and kidneys, ensuring that the body's internal processes remain synchronised (Shimizu, Yoshida and Minamino, 2016; Vitaterna, Takahashi and Turek, 2001).

At the molecular level, circadian rhythms are regulated by a transcription-translation feedback loop (TTFL), constituting a cycle of gene expression that maintains a consistent 24-hour oscillation (Fig.1.10) (Minegishi *et al.*, 2018). The primary components of this loop are the CLOCK and BMAL1 proteins, which form a heterodimer that binds to E-box elements in DNA to activate the transcription of PER (PER1, PER2) and CRY (CRY1, CRY2) genes (Scheiermann *et al.*, 2018). These proteins accumulate in the cytoplasm, dimerise, and ultimately translocate to the nucleus, where they inhibit the activity of CLOCK and BMAL1 (Chiou *et al.*, 2016). This suppression effectively prevents the further production of PER and CRY, thus closing the feedback loop (Chiou *et al.*, 2016; Lee *et al.*, 2015). Over time, PER and CRY proteins undergo phosphorylation by kinases such as CK1δ, CK1ε, and CK2, marking them for degradation via the ubiquitin-proteasome pathway (Cao *et al.*, 2021; Knippschild *et al.*, 2014). Upon PER/CRY degradation, CLOCK and BMAL1 regain activity, restarting the cycle (Cao *et al.*, 2021; Chiou *et al.*, 2016; Knippschild *et al.*, 2014).

In addition to PER and CRY, various regulatory elements modulate the circadian rhythm. REV-ERBα and REV-ERBβ inhibit BMAL1 transcription, whereas RORα

and RORγ counteract this inhibition by promoting BMAL1 expression (Ikeda *et al.*, 2019; Jetten, 2009). These molecular mechanisms orchestrate critical physiological functions, including metabolism, hormone secretion, and immune responses, ensuring synchronisation with the day-night cycle.

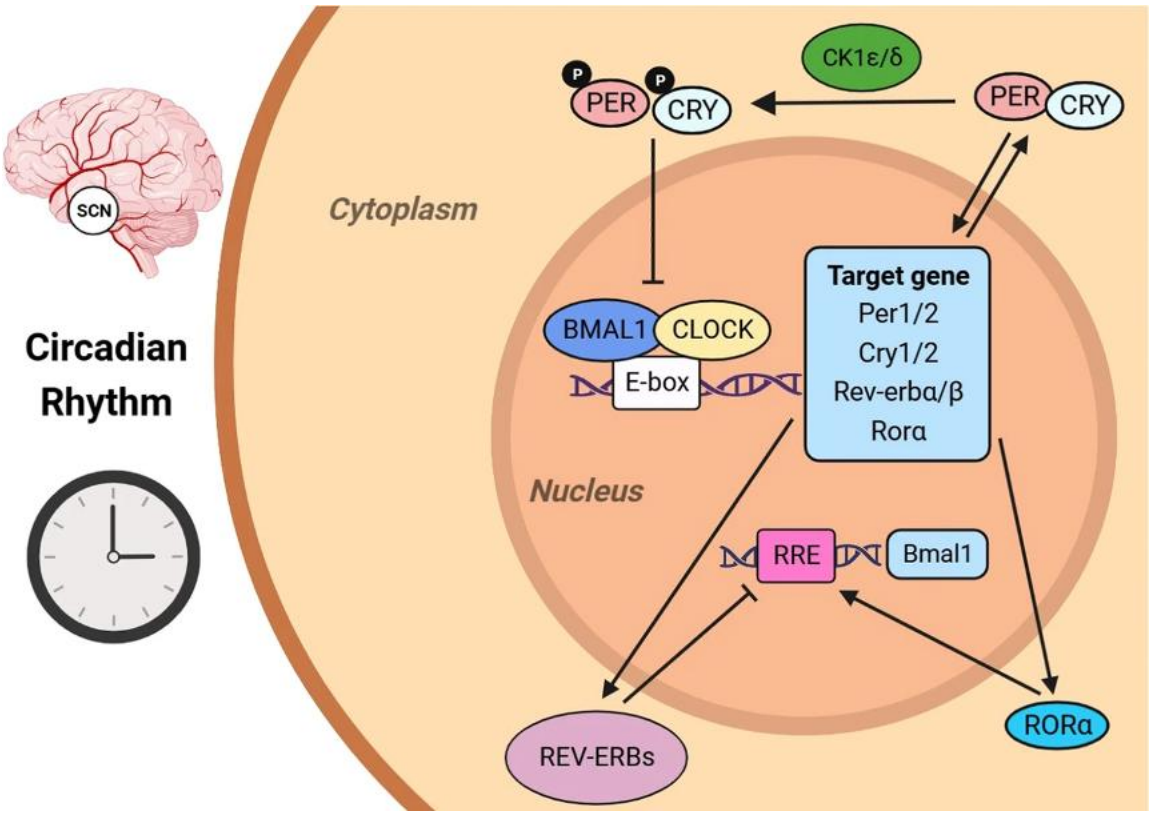


Fig.1.10 Circadian Rhythm Mechanism. The suprachiasmatic nucleus (SCN) in the hypothalamus regulates the body's circadian rhythm and synchronises peripheral clocks. The CLOCK/BMAL1 complex binds to E-box elements to activate target genes, while PER/CRY proteins accumulate, dimerise, and inhibit CLOCK/BMAL1 after phosphorylation by CK1δ/CK1ε. Additionally, REV-ERBα suppresses Bmal1 expression via the RRE, whereas RORα promotes it (Zeng *et al.*, 2024).

Circadian rhythms can be disrupted due to genetic mutations, shift work, jet lag, or irregular sleep schedules, resulting in the development of Circadian Rhythm Sleep-Wake Disorders (CRSWDs) (Ingram, 2020). Such disorders arise when the internal biological clock is misaligned with external temporal cues, leading to sleep disturbances and metabolic issues (Kim, Lee and Duffy, 2013). Chronic misalignment of circadian rhythms has been associated with various health conditions, including obesity, cardiovascular diseases, mood disorders, and cognitive decline (Kim, Lee and Duffy, 2013; Scheer *et al.*, 2009; Walker *et al.*, 2020). Recent research indicates that targeting specific molecular components of the circadian clock, such as REV-ERBs, RORs, and melatonin pathways, may provide potential therapeutic approaches for circadian-related disorders, including insomnia

and neurodegenerative diseases (Ikeda *et al.*, 2019; Jetten, 2009; Ruan, Yuan and Eltzschig, 2021).

1.5.10.4 Circadian Regulation of Macrophages

Circadian rhythms regulate macrophage function by modulating TLR signalling, metabolism, and inflammatory responses (Zeng *et al.*, 2024). TLR4, TLR9, TLR2, and TLR6 expression follows a rhythmic pattern, influencing immune activation and sepsis severity (Halberg *et al.*, 1960; Silver *et al.*, 2018; Vidya *et al.*, 2018). Studies show TLR4 signalling is under circadian control, with endotoxin-induced lethality varying based on exposure time (Halberg *et al.*, 1960). Similarly, macrophage polarisation is influenced by the circadian clock, where BMAL1 inhibits excessive inflammation by regulating glycolysis (Zeng *et al.*, 2024). Loss of BMAL1 leads to uncontrolled metabolic shifts, increasing susceptibility to septic shock through PD-L1/T-cell interactions (Timmons *et al.*, 2020). Additionally, PER1 and PER2 deficiencies drive a pro-inflammatory M1-like state (Xu *et al.*, 2014), while melatonin induces M2 polarisation via RORα and metabolite signals (Ding *et al.*, 2019; Zeng *et al.*, 2024).

Macrophages contribute to inflammation by secreting cytokines and chemokines, with REV-ERBα playing a crucial role in suppressing inflammatory responses through multiple mechanisms (Timmons *et al.*, 2020). The absence of REV-ERBα leads to heightened inflammation in alveolar macrophages (Pariollaud *et al.*, 2018), whereas its agonists effectively reduce inflammatory gene expression (Gibbs *et al.*, 2012a). Additionally, REV-ERBα regulates inflammatory markers in microglia and colitis models and directly controls Nlrp3 mRNA levels in hepatitis (Zeng *et al.*, 2024).

Another key regulator of macrophage inflammation is the PER/CRY complex, which inhibits inflammatory mediators. The loss of PER1 leads to an increase in CCR2 expression and the migration of macrophages (Wang *et al.*, 2016), while the deletion of PER2 results in an enhancement of TNFα and IL-12 production (Silver *et al.*, 2012). CRY proteins exert a negative regulatory effect on the cAMP-PKA-NF-κB signalling pathway, and their deficiency yields elevated expression levels of IL-6, TNFα, and iNOS (Narasimamurthy *et al.*, 2012; Zeng *et al.*, 2024). Targeting REV-ERBα with compounds such as GSK4112 has demonstrated the capability to suppress NLRP3 and IL-1β expression in instances of LPS-induced inflammation (Yu *et al.*, 2019). Additionally, disruptions to the circadian rhythm and the downregulation of KLF4 negatively affect macrophage function during ageing

(McRae and Hargreaves, 2022), emphasising circadian regulation's significance in maintaining immune balance.

1.5.11 CD33 as a Dual Regulator of Myeloid Cell Function and Inflammation in LPS-Stimulated Macrophages

CD33 is a sialic acid-binding immunoglobulin-type lectin (Siglec) receptor expressed on various phagocytic and myeloid lineage cells, including macrophages, monocytes, and dendritic cells. It exists in two isoforms: CD33M (long form), which retains the sialic acid-binding site and generally inhibits phagocytic activity, and CD33m (short form), which may promote phagocytosis, migration, and proliferation (Bhattacherjee *et al.*, 2021; Butler, Thornton and Brown, 2021).

Structurally, CD33 contains an extracellular sialic acid-binding domain, a transmembrane region, and intracellular inhibitory motifs—specifically ITIM and ITIM-like domains—which suppress activation signals downstream of immune receptors (Crocker, Paulson and Varki, 2007). CD33 modulates immune cell function by engaging with sialic acid ligands, leading to ITIM phosphorylation and recruitment of SHP-1/2 phosphatases. This suppresses ITAM-mediated activation and may downregulate the production of pro-inflammatory cytokines such as IL-1β (Butler, Thornton and Brown, 2021). As IL-1β enhances phagocytic activity in macrophages, CD33 may function as a negative regulator of this process by modulating IL-1β expression through ITIM-mediated signalling. Investigating this regulatory axis is particularly relevant in the context of LPS-induced inflammation and septic conditions, where excessive immune activation can exacerbate tissue damage. Therefore, CD33 was examined in this study not only as a myeloid lineage marker but also as a potential modulator of MerTK expression and inflammatory gene regulation in LPS-stimulated macrophages.

1.6 Mesenchymal stem cells (MSCs)

Mesenchymal stem cells (MSCs) are multipotent cells that can be isolated from various adult tissues, including bone marrow, umbilical cord, adipose tissue, liver, tooth root and peripheral blood (Maxson *et al.*, 2012). These cells can be induced to differentiate into mesodermal lineage cells, such as adipocytes, chondrocytes, and osteoblasts (Dimarino, Caplan and Bonfield, 2013). It is noteworthy that they also have the capability to differentiate into ectodermal or endodermal cell lineages (Zhang *et al.*, 2023). Phenotypically, it is customary for mesenchymal stem cells (MSCs) to express cell surface antigens, including CD90, CD73, CD105, and stem cell antigen-1 (Sca-1). In contrast, they do not express markers of hematopoietic

cells such as the endothelial marker CD31, major histocompatibility complex class II (MHC II), CD11b, CD45, and CD34 (Li and Hua, 2017b).

MSCs are believed to originate from pericytes, which are perivascular cells with diverse functional roles (Caplan, 2008). The interaction between pericytes and endothelial cells is thought to play a crucial role in MSC differentiation by influencing their lineage commitment and function (Crisan *et al.*, 2008). Following injury, vascular alterations occur even at the microvascular level, triggering pericyte differentiation into MSCs (Dimarino, Caplan and Bonfield, 2013). These MSCs migrate to the injury site, contributing to tissue repair while modulating the surrounding *in vivo* environment (Fu and Li, 2009). Given their multipotent nature, pericytes have the capacity to direct MSC function toward specific regenerative outcomes (Fig.1.11) (Dimarino, Caplan and Bonfield, 2013). Ultimately, the *in vivo* microenvironment defines MSCs differentiation and determines the final tissue outcome.

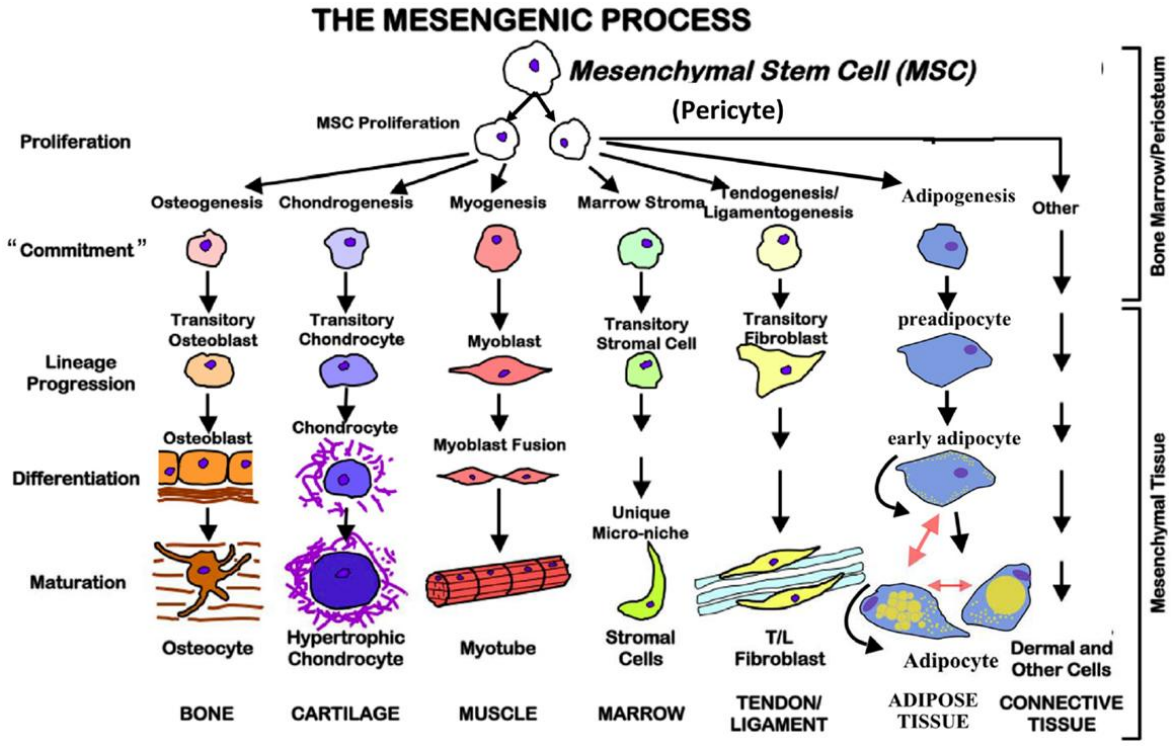


Fig.1.11 The Mesengenic Process. MSCs are multipotent and have the capacity to proliferate and differentiate into various cell types depending on environmental cues. Additionally, they can transition between different lineages in response to specific signals (Dimarino, Caplan and Bonfield, 2013).

1.6.1 MSCs: from Bench to Bedside

MSCs are regarded as some of the most versatile cell types utilised in cellular therapy. First identified and characterised by Arnold Caplan in 1991, MSCs have undergone considerable research regarding their therapeutic potential across multiple diseases, such as inflammatory disorders, graft-versus-host disease (GVHD), and degenerative conditions (Caplan, 1991; Kelly and Rasko, 2021). Familiar sources of MSCs include bone marrow (BM), adipose tissue, umbilical cord, and placenta-derived decidua stromal cells (DSCs). These somatic progenitors demonstrate the ability to differentiate into three lineages of paraxial mesoderm—adipogenesis, chondrogenesis, and osteogenesis. Moreover, they show extensive immunomodulatory characteristics that make them suitable for treating immune and inflammatory diseases, enhancing tissue regeneration, and facilitating third-party applications (Pittenger *et al.*, 1999).

Several clinical trials of MSCs transplantation for treating various diseases have recently been conducted, demonstrating that pre-transplant systemic administration of MSCs extends allograft survival in solid organs prior to transplantation (Kode *et al.*, 2009; Mackenzie and Flake, 2001; Sun *et al.*, 2022). The first demonstration that MSCs could extend skin graft survival in vivo occurred in 2002 (Bartholomew *et al.*, 2002). In 2004, a significant study showed that haploidentical MSCs were successfully transplanted into a patient with severe treatment-resistant grade IV acute graft-versus-host disease affecting the gastrointestinal tract and liver. The patient exhibited significant clinical improvement and maintained a stable health condition after one year, indicating that MSCs may possess a substantial immunosuppressive effect in vivo (Le Blanc *et al.*, 2004). Further research consistently emphasized MSC's ability to modulate the immune response, suppressing diverse immune cell functions, aiding in haematopoietic stem cell engraftment, and showing promise for preventing and treating GVHD in haematopoietic stem cell transplantation (HSCT) (Grégoire *et al.*, 2019; Kelly and Rasko, 2021; Wu *et al.*, 2013). However, some researchers argue that the mechanisms of MSCs in the wound-healing process remain inadequately understood (Guillamat-Prats, 2021; Madani *et al.*, 2022). This thesis focuses on a concise summary of current knowledge of MSCs for tissue repair.

Another impressive study showed intravenous MSCs confer long-term immune tolerance despite transient engraftment by modulating lung monocytes and macrophages through TSG-6 (Ko *et al.*, 2016). These primed cells exhibit regulatory

properties, suppress T-cell responses, and protect against ocular inflammation in allo- and autoimmune models. Adoptive transfer of MSCs-induced monocytes/macrophages prevents disease, while their depletion or TSG-6 knockdown abrogates tolerance, highlighting a key mechanism of MSCs-mediated immunomodulation through innate immune reprogramming (Romano *et al.*, 2019).

Additionally, previous research has shown that liposuction micro-fragmented adipose tissue (MFAT) exhibits therapeutic potential in osteoarthritis by reducing pain and promoting tissue repair regeneration (Guo *et al.*, 2021). Analysis of MFAT from eight donors revealed variability in cluster size, affecting MSCs colony formation. Isolated cells predominantly expressed stemness markers (CD105/CD90/CD45+). MFAT secreted cytokines with anti-inflammatory, pro-regenerative, anti-scarring, pro-angiogenic, and antibacterial properties. Conditioned medium enhanced angiogenesis and suppressed inflammation. These findings highlight the biological mechanisms underlying MFAT's long-lasting therapeutic effects in osteoarthritic joints.

1.6.2 Immunomodulatory Properties of MSCs

One of the most crucial therapeutic attributes of MSCs is their capacity to modulate immune responses (Kode *et al.*, 2009). MSCs engage with various immune cells, such as T cells, B cells, natural killer (NK) cells, and macrophages, via direct cell-to-cell interactions and the secretion of bioactive molecules (English, 2013). These interactions suppress pro-inflammatory responses and promote anti-inflammatory pathways.

Macrophages, in particular, are vital in maintaining tissue homeostasis and mediating immune responses. Depending on the microenvironment, macrophages can assume diverse phenotypes: the pro-inflammatory M1 phenotype and the anti-inflammatory, tissue-repairing M2 phenotype. MSCs are recognised for their ability to educate macrophages towards the M2 phenotype through the secretion of cytokines, growth factors, and extracellular vesicles (Eggenhofer and Hoogduijn, 2012). This polarization is associated with increased production of anti-inflammatory mediators, including interleukin-10 (IL-10) and transforming growth factor-β (TGF-β), alongside an increased phagocytic activity facilitated by the expression of MerTK, a receptor associated with efferocytosis.

The capability of iPSCs-derived MSCs to affect macrophage polarisation has significant implications for treating chronic inflammatory diseases, autoimmune

disorders, and tissue injuries. Understanding the mechanisms behind this process is crucial to maximize the therapeutic benefits of MSCs.

1.6.3 MSCs in Tissue Repair

It is universally acknowledged that MSCs in normal skin play an essential role in tissue repair, suggesting that the application of exogenous MSCs is a promising solution for treating nonhealing wounds (Maxson *et al.*, 2012). One reason that MSCs are capable of repairing tissue is their inherent potential to differentiate into mesodermal cell lineages, which encompass chondrogenesis and adipogenesis (Luzzani *et al.*, 2015). Additionally, MSCs play crucial roles in the immunoregulatory environment and exhibit immunosuppressive properties due to their secretion of extracellular vesicles, cytokines, growth factors, and chemokines, as well as the promotion of indoleamine 2, 3-dioxygenase (IDO) production in recipient macrophages during apoptosis (Bloor *et al.*, 2020). Furthermore, MSCs facilitate the transition of monocytes to macrophages and enhance the efficacy of macrophages in eliminating pathogens, thereby contributing to tissue regeneration (Vasandan *et al.*, 2016). MSCs have the capacity to facilitate the conversion of macrophages from the M1 phenotype to the M2 phenotype (Stevens *et al.*, 2020). Several mechanisms have been proposed to explain these effects, including the secretion of bioactive molecules, modulation of immune cell subsets, and paracrine communication. For instance, MSCs promote tissue healing in inflammatory bowel disease (IBD) via TNF-stimulated gene-6 (Yang *et al.*, 2019). Despite these insights, the precise molecular pathways and signalling cascades by which MSCs exert their immunoregulatory effects remain incompletely characterised. Continued research is therefore essential to fully elucidate the mechanisms underpinning MSCs-mediated tissue regeneration and immunomodulation.

Another advantageous effect of MSCs techniques in mitigating inflammation is the production of various anti-inflammatory molecules, including hepatocyte growth factor (HGF), prostaglandin E2 (PGE2), and nitric oxide (NO) (Kode *et al.*, 2009). These factors can regulate immune cells, such as macrophages, monocytes, B-cells, dendritic cells, T-cells, and natural killer (NK) cells. Furthermore, exosomes derived from MSCs, which influence the functionality of target cells, have been utilised in clinical trials to demonstrate the efficacy of treatments for numerous conditions, including bronchopulmonary dysplasia and various autoimmune disorders, in addition to facilitating angiogenesis and tissue repair (Marote *et al.*, 2016; Zhang *et al.*, 2015).

Evidence indicates that MSCs possess the capability to inhibit pro-inflammatory cytokines produced by macrophages. It is reported that MSCs can educate the phenotype of tissue macrophages, which have characteristics such as programmed death ligands 1 and 2, transforming growth factor β1 (TGF-β1) and high levels of IL-10 (Eggenhofer and Hoogduijn, 2012). The importance of reduced T cell proliferation and augmentation of fibroblast proliferation can be proven to suppress inflammatory responses (Lu *et al.*, 2021). In addition, MSCs-educated macrophages have the ability to promote the production of fibrotic growth and tissue repair factors, including the immune-regulating cytokine IL-6 and TGF-β1. TGF-β1 is the key to the proliferation phase of tissue repair via accelerating angiogenesis, fibroblast proliferation and re-epithelization (Stevens *et al.*, 2020). Consequently, the synergistic effect of MSCs and macrophages on tissue repair holds substantial value for exploration.

While MSCs have demonstrated advantages, several challenges and issues may impact their clinical application. Firstly, the capacity of MSCs to differentiate and proliferate diminishes with age, especially among those sourced from bone marrow. Although bone marrow is among the most commonly utilised and readily available sources of MSCs, the process of organismal ageing decreases the density of MSCs within the bone marrow and undermines their osteogenic potential (Lin *et al.*, 2019). Moreover, it has been reported that functional heterogeneity exists among MSCs, which may be attributed to variations in donor sources, culture protocols, and expansion levels. Furthermore, the limited expansion capacity of MSCs in culture restricts the generation of the substantial quantities of cells necessary for clinical therapies, as they typically experience culture senescence after approximately 8 to 10 passages (Hynes *et al.*, 2014).

Furthermore, isolating MSCs is invasive to adult tissue, and the sources available are exceedingly limited, such as umbilical cord blood and bone marrow (Bloor *et al.*, 2020). Consequently, it is plausible that the conventional methods employed in harvesting mesenchymal stem cells are not optimal for clinical therapies. It is imperative to identify an alternative source of MSCs to enhance the production of functional MSCs for tissue regeneration properties.

In conclusion, the preceding content has examined the beneficial impact of mesenchymal stem cells on biomedical applications, highlighting several well-documented potential therapeutic avenues for clinical diseases demonstrated

through MSC techniques. However, it is imperative that alternative sources of MSCs be extensively developed to optimise their functionality in tissue repair.

1.6.4 Mesenchymal Stem Cells Influence Macrophage Phenotype.

Macrophages can phagocytose MSCs under various physiological conditions. In addition to this, MSCs exert paracrine effects and can transfer mitochondria to macrophages, collectively promoting the polarization of macrophages toward anti-inflammatory phenotypes (Lu *et al.*, 2023).

1.6.4.1 Phagocytosis

The first mechanism involves phagocytosis, where both living and apoptotic MSCs and their cellular fragments are engulfed by macrophages. This process triggers a phenotypic shift from M1 to M2 macrophages, enhancing their ability to release anti-inflammatory mediators and promote tissue healing (Fig.1.12).

Intravenous infusion of mesenchymal stem cells (MSCs) triggers immune regulation by promoting their phagocytosis by monocytes and macrophages, leading to a shift toward an anti-inflammatory M2 phenotype (Braza *et al.*, 2016; Weiss and Dahlke, 2019). Once in the body, MSCs are quickly captured in the lung's microvessels, where monocytes engulf them, activating the monocytes and directing their migration to other tissues (de Witte *et al.*, 2018). In co-culture studies, macrophages that phagocytose MSCs show heightened phagocytic capacity and express more anti-inflammatory markers (Kim and Hematti, 2009). In asthma models, alveolar macrophages promptly phagocytose mesenchymal stem cells (MSCs), which facilitates their differentiation into the M2 phenotype and mitigates inflammation (Braza *et al.*, 2016). Interestingly, even dead or heat-inactivated MSCs retain their ability to induce anti-inflammatory macrophage responses, demonstrating that it is their components, not just their viability, that drive this effect (Luk *et al.*, 2016). In graft-versus-host disease models, MSC apoptosis proves crucial for their immunomodulatory function, as macrophages that phagocytose apoptotic MSCs release factors that suppress inflammation (Galleu *et al.*, 2017). Collectively, MSCs and MSC apoptosis consistently promote the transformation of monocytes and macrophages into anti-inflammatory cells.

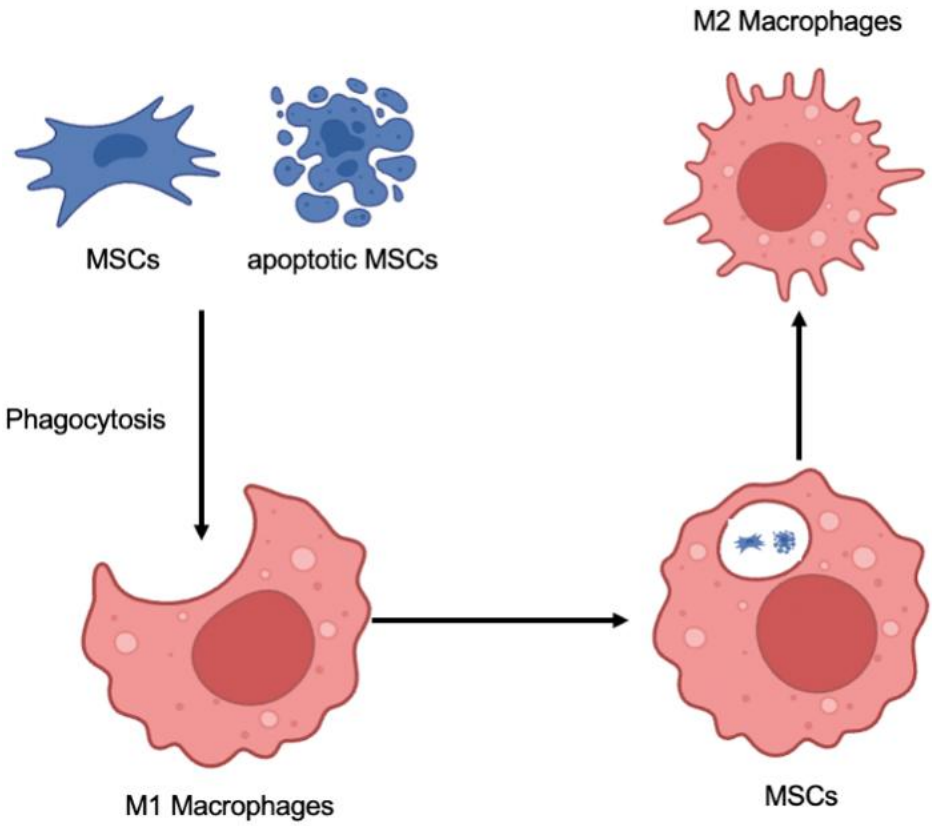


Fig.1.12 MSCs Facilitate the Transformation of Monocytes and Macrophages through Phagocytosis. At the injury site, M1 macrophages trigger a pro-inflammatory response by releasing inflammatory factors and attracting monocytes. Following MSC injection, both live and apoptotic MSCs are engulfed by monocytes and macrophages. This process alters their inflammatory profile, enhances the release of anti-inflammatory factors, and initiates tissue repair (Lu *et al.*, 2023).

1.6.4.2 Paracrine Actions

Another central pathway is the paracrine effect, where MSCs secrete extracellular vesicles (EVs) that are rich in bioactive molecules such as microRNAs, mRNAs, and proteins (Zhou *et al.*, 2019). These vesicles interact with macrophages, inducing their transformation into the M2 phenotype and reducing inflammatory responses (Li and Hua, 2017a). EV-mediated transfer of specific microRNAs disrupts pro-inflammatory signalling pathways within macrophages, thereby amplifying their reparative functions (Lu *et al.*, 2023).

1.6.4.2.2 Extracellular Vesicles (EVs)

Numerous studies indicate that exosomes derived from MSCs possess considerable potential in the field of regenerative medicine (Marote *et al.*, 2016; Oh *et al.*, 2018; Zhang *et al.*, 2015; Zhou *et al.*, 2023). It has been reviewed that MSCs-derived exosome promotes the polarisation of M2 macrophages, which impacts multiple cellular processes and diseases, including skin injury, cardiomyopathy, liver injury, and autoimmune diseases (Fig.1.13) (Arabpour, Saghazadeh and Rezaei, 2021).

MSCs-derived extracellular vesicles (EVs) carry non-coding RNAs, mRNAs, and proteins, which drive their immunomodulatory effects on monocytes and macrophages (Arabpour, Saghazadeh and Rezaei, 2021). MicroRNAs, key components of MSC-exosomes, regulate immune responses (O'Brien *et al.*, 2018). For example, microRNA-182 targets TLR4, inhibiting the TLR4/NF-κB pathway and promoting M2 polarisation (Zhao *et al.*, 2019). MicroRNA-223 from MSC-EVs reduces LPS-induced inflammation in sepsis models, while microRNA-17 inhibits NLRP3 inflammasome activation by suppressing thioredoxin-interacting protein expression (Ismail *et al.*, 2013; Liu *et al.*, 2018). MSC-EVs also transfer mRNAs, such as angiopoietin 1, which increases IL-10 and decreases TNF-α expression in macrophages (Tang *et al.*, 2017). Additionally, MSC-EVs deliver proteins like CCR2, which binds CCL2, blocking M1 recruitment and reducing pro-inflammatory cytokines (Shen *et al.*, 2016). CD73-enriched MSC-EVs activate adenosine signalling, shifting macrophages from M1 to M2, lowering TNF-α, and increasing IL-10 levels (Zhai *et al.*, 2021; Zhou *et al.*, 2019).

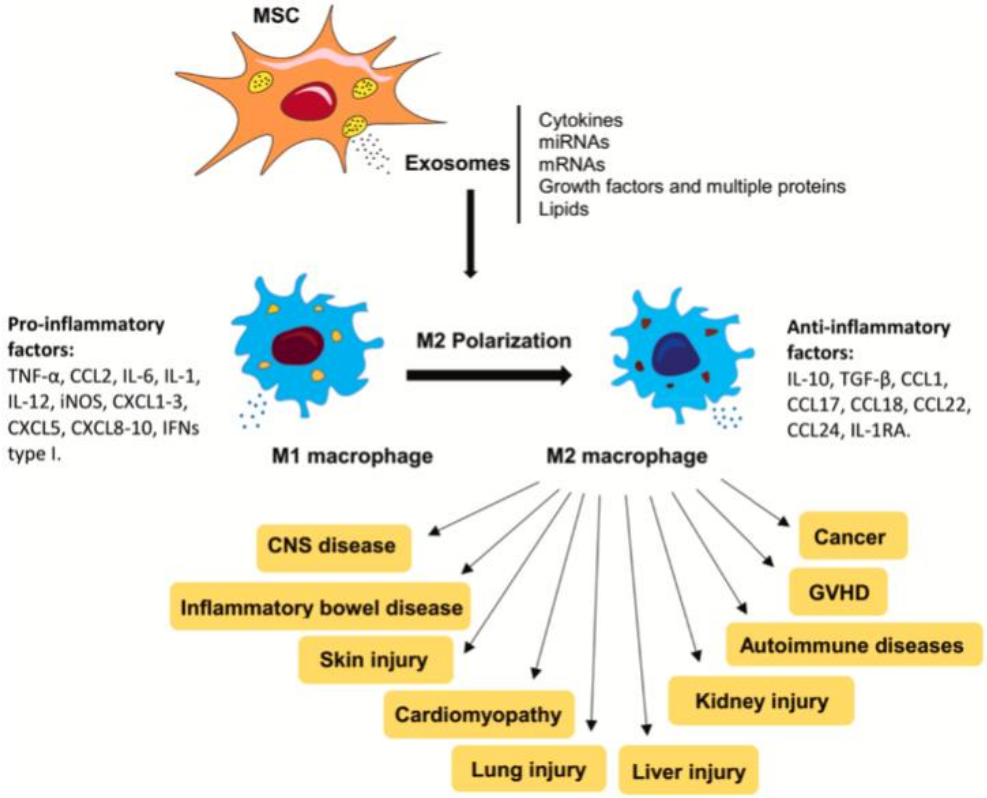


Fig.1.13 Anti-Inflammatory and M2 Macrophage Polarisation-Promoting Effect of MSCs-Derived Exosomes in Inflammatory Diseases. Mesenchymal stem cell (MSCs)-derived exosomes influence macrophage polarisation. MSC exosomes carry cytokines, miRNAs, mRNAs, proteins, and lipids that help shift macrophages from a pro-inflammatory M1 state to an anti-inflammatory M2 phenotype. M1 macrophages produce inflammatory mediators (e.g., TNF-α, IL-6, IL-1), while M2 macrophages secrete anti-inflammatory factors (e.g., IL-10, TGF-β). The resulting M2 polarisation is associated with therapeutic benefits across various inflammatory and injury-related diseases (Arabpour, Saghazadeh and Rezaei, 2021).

1.6.4.3 Mitochondrial Transfer

The third mechanism discussed is the transfer of mitochondria from MSCs to macrophages. This transfer, facilitated by tunnelling nanotubes (TNTs) and macrovesicles (MVs), enhances mitochondrial function in macrophages, boosting their metabolic activity and further promoting the shift to an anti-inflammatory state (Fig.1.14) (Zhou *et al.*, 2019). This metabolic reprogramming is crucial as it alters macrophage energy production from glycolysis to oxidative phosphorylation, a hallmark of the M2 phenotype.

Under oxidative stress, MSCs activate the PINK1/Parkin mitophagy pathway, which releases mitochondria into MVs for transfer to macrophages (Kane and Youle, 2011; Phinney *et al.*, 2015). This enhances macrophage bioenergetics and promotes their shift to the anti-inflammatory M2 phenotype through increased oxidative phosphorylation (Zhou *et al.*, 2019). Additionally, TNTs facilitate direct mitochondrial transfer between MSCs and macrophages, thus increasing their phagocytic activity and further supporting M2 polarisation (Morrison *et al.*, 2017). Blocking TNTs reduces both mitochondrial transfer and macrophage phagocytosis, highlighting the role of mitochondrial exchange in MSC-driven immune modulation (Yuan *et al.*, 2021). These insights emphasise mitochondrial transfer as a key mechanism in MSC therapies for inflammatory diseases and tissue repair.

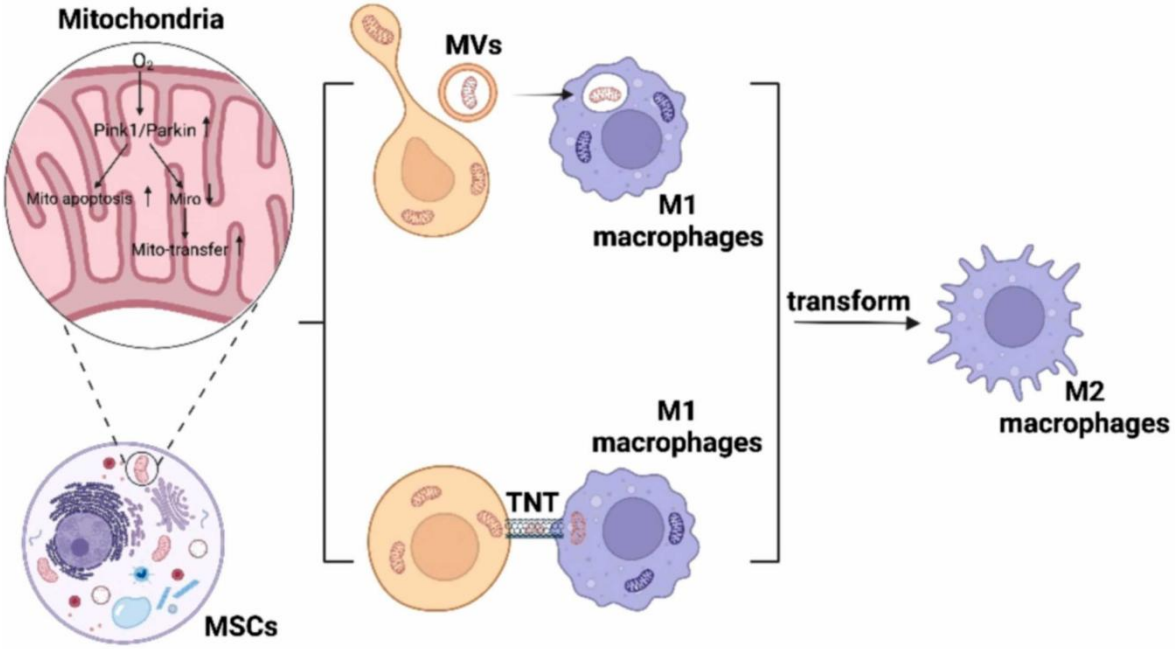


Fig.1.14 MSCs Induce Phenotypic Changes in Monocytes and Macrophages through Mitochondrial Transfer. The mechanism by which mesenchymal stem cells (MSCs) facilitate the phenotypic transition of pro-inflammatory M1 macrophages to anti-inflammatory M2 macrophages occurs through the process of mitochondrial transfer. In response to stress signals, such as hypoxia and mitochondrial damage, MSCs initiate the transfer of mitochondria via the Pink1/Parkin and Miro pathways. Mitochondria are conveyed to M1 macrophages through either microvesicles (MVs) or tunneling nanotubes (TNTs). The incorporation of healthy mitochondria by M1 macrophages promotes their transformation into M2 macrophages, thus contributing to the resolution of inflammation and enhancing tissue regeneration (Zhou *et al.*, 2019).

1.7 Induced Pluripotent Stem Cells (iPSCs)

Induced pluripotent stem cells (iPSCs) represent a ground-breaking advancement in regenerative medicine and stem cell biology. First described by Takahashi and Yamanaka in 2006, iPSCs are derived from reprogramming somatic cells to a pluripotent state through the enforced expression of specific transcription factors, including Oct4, Sox2, Klf4, and c-Myc (Takahashi *et al.*, 2007). This technology circumvents the ethical concerns associated with embryonic stem cells (ESCs) and provides an almost limitless supply of patient-specific cells for various therapeutic applications (Moradi *et al.*, 2019). iPSCs exhibit properties analogous to those of ESCs, such as self-renewal and the ability to differentiate into all three germ layers, rendering them a versatile tool for investigating disease mechanisms, drug discovery, and regenerative therapies (Zakrzewski *et al.*, 2019).

1.7.1 Cell Reprogramming

iPSCs are artificially derived stem cells that originate from specialised (differentiated) cells, such as skin or blood cells. The reprogramming process involves introducing four key transcription factors—Oct4, Sox2, Klf4, and c-Myc—known as Yamanaka factors (Fig.1.15) (Takahashi *et al.*, 2007; Takahashi and Yamanaka, 2006). These factors reset the mature cells to an embryonic-like state, granting them the ability to develop into any cell type in the body. This discovery revolutionised regenerative medicine by providing a patient-specific, ethical alternative to embryonic stem cells (ESCs). iPSCs are widely used in disease modelling, drug testing, and potential cell-based therapies for conditions such as neurodegenerative diseases, heart disease, and diabetes (Cerneckis, Cai and Shi, 2024; Moradi *et al.*, 2019).

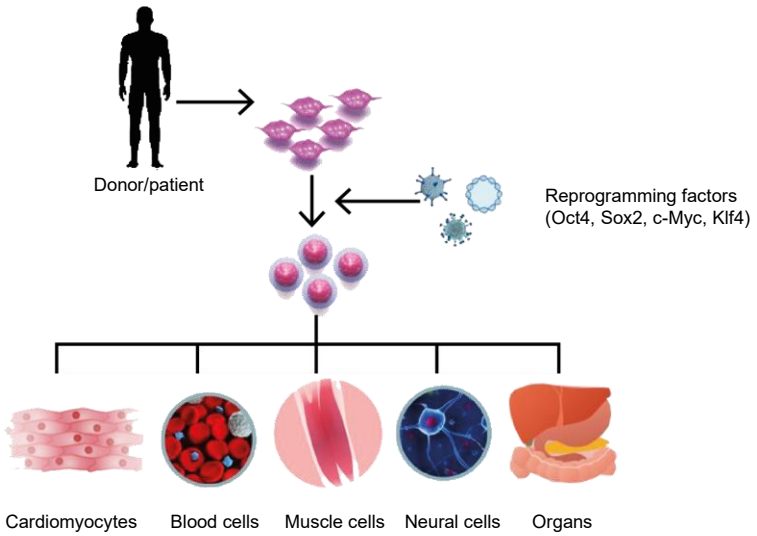


Fig.1.15 Directed Differentiation of iPS Cells. The generation and differentiation potential of induced pluripotent stem cells. Adult somatic cells, such as skin fibroblasts, are reprogrammed into iPSCs using transcription factors (Oct4, Sox2, Klf4, and c-Myc). iPSCs possess pluripotency and can differentiate into a wide range of specialised cell types derived from all three germ layers, including neurons (ectoderm), blood and muscle cells (mesoderm), and hepatocytes (endoderm). This highlights their potential applications in regenerative medicine, disease modelling, and drug screening (Sugimoto and Eto, 2021).

1.7.2 Application of iPSCs

1.7.2.1 Disease Model

The versatility of iPSCs renders them an indispensable instrument for investigating a broad spectrum of diseases, as they empower researchers to create patient-specific models (Rowe and Daley, 2019). Given that iPSCs preserve the genetic identity of the donor, they can be differentiated into specialised cell types pertinent to various disorders (Ebert, Liang and Wu, 2012). This capability enables scientists to examine disease progression at a cellular level and to investigate potential therapeutic interventions.

Genetic modification techniques, such as CRISPR, significantly enhance the value of iPSCs in biomedical research by facilitating precise modifications to the genome (Li *et al.*, 2023a). Researchers can investigate their implications on disease mechanisms by introducing or correcting specific mutations and identifying potential therapeutic targets. For instance, iPSCs can be differentiated into dopaminergic neurons, enabling scientists to introduce disease-related mutations pertinent to Alzheimer's disease and examine their effects (Barman *et al.*, 2020). Likewise, these cells can be converted into cardiomyocytes to investigate cardiovascular diseases, where mutations in ion channel genes elucidate the underlying causes of cardiac condition disorders (Musunuru *et al.*, 2018).

Utilising iPSCs-based models, researchers are able to gain comprehensive insights into the manner in which genetic variations impact disease progression and to develop targeted therapies (Ebert, Liang and Wu, 2012; Rowe and Daley, 2019). This methodology not only enhances our understanding of complex diseases but also promotes personalised medicine by allowing for drug testing on patient-derived cells, thereby resulting in more effective and customised treatment strategies.

1.7.2.2 Drug Research and Development

Induced pluripotent stem cells combined with gene editing technologies like CRISPR have significantly improved drug research and development by enabling the creation of precise disease models (Ebert, Liang and Wu, 2012; Rowe and Daley, 2019). Traditional drug testing often relies on animal models or generic cell lines, which may not accurately replicate human disease conditions. iPSCs can be derived from patients and genetically modified to mimic disease-specific mutations, allowing for more relevant and personalised drug screening (Elitt, Barbar and Tesar, 2018).

One significant advantage of this approach is the ability to study drug resistance.

Researchers can create highly specialised models to test new compounds that may overcome these resistance mechanisms by introducing genetic mutations that make cells resistant to specific treatments (Ebert, Liang and Wu, 2012; Okano and Morimoto, 2022). For example, in cancer research, iPSCs can be modified to express mutations that make tumour cells unresponsive to chemotherapy (Sharkis *et al.*, 2012). This allows scientists to identify new drugs or combinations that bypass resistance and restore treatment effectiveness.

Beyond drug resistance, iPSCs-derived models also help predict drug toxicity and efficacy, reducing the reliance on animal testing and improving the likelihood of success in clinical trials (Silva and Haggarty, 2020). By providing a more accurate representation of human disease, iPSC-based research accelerates drug discovery and paves the way for more effective and personalised treatments.

1.7.2.3 Personalized Medical Care

In personalised medicine, iPSCs-derived allogeneic cell therapy presents significant therapeutic potential by offering a versatile and scalable solution for tissue regeneration (Ortiz-Vitali and Darabi, 2019; Park *et al.*, 2023). One of the key advantages of this approach is the ability to modify these cells to reduce the risk of immune rejection, which is a major challenge in transplantation and cell-based therapies.

The ability to customise iPSCs for individual patients makes them particularly valuable in regenerative medicine. By generating patient-specific or immune-compatible cells, iPSC-based therapies can be tailored to replace damaged or lost tissues (Chun, Byun and Lee, 2011). For instance, in cardiovascular diseases, iPSCs can be differentiated into myocardial cells to restore heart function, while in diabetes, they can be transformed into pancreatic beta cells to help regulate insulin production (Musunuru *et al.*, 2018; Park *et al.*, 2023; Silva *et al.*, 2022). This ability to create patient-matched or universal donor cells not only enhances treatment effectiveness but also minimises the need for immunosuppressive drugs, making regenerative therapies safer and more accessible.

Overall, iPSCs-derived allogeneic therapy represents a major advancement in personalised medical care, offering a promising pathway for treating degenerative diseases and improving long-term patient outcomes.

1.7.3 Advantages of iPSCs-Derived Allogocytic Cell Therapy

iPSCs have unique biological characteristics that make them extensively applicable in regenerative medicine, disease modelling, drug screening, and personalised medicine. The benefits of iPSCs can be summarised as follows:

1.7.3.1 Pluripotency and Differentiation Potential

iPSCs possess pluripotency, similar to embryonic stem cells, enabling their differentiation into nearly any cell type within the human body (Takahashi *et al.*, 2007). This makes them extremely valuable for tissue regeneration, encompassing neuronal, cardiac, and pancreatic cells, which are essential for addressing conditions such as neurodegenerative diseases, heart disease, and diabetes (Barman *et al.*, 2020; Ebert, Liang and Wu, 2012; Okano and Morimoto, 2022).

1.7.3.2 Immune Compatibility

Since iPSCs can be derived from a patient's own cellular material, they present the opportunity for entirely personalised therapeutic approaches, thereby significantly minimising the risk of immune rejection (Chun, Byun and Lee, 2011). Moreover, advancements in gene editing technologies, such as CRISPR, facilitate modifications that can augment immune tolerance, rendering iPSCs-derived cells even more suitable for transplantation procedures (Barman *et al.*, 2020; Li *et al.*, 2023a).

1.7.3.3 Ethical Advantages

In contrast to ESCs, which present ethical concerns associated with the destruction of embryos, induced pluripotent stem cells (iPSCs) are derived from adult somatic cells, thereby circumventing these controversies (Zakrzewski *et al.*, 2019). Consequently, iPSCs are more broadly accepted in both research and clinical applications.

1.7.3.4 Scalability and Standardization

Compared to autologous therapies, iPSCs-derived allogeneic cell therapy is more resource- and time-efficient because autologous treatments require a separate manufacturing process for each patient (Moradi *et al.*, 2019; Zakrzewski *et al.*, 2019). On the other hand, allogeneic iPSC-derived cell therapy allows for the large-scale production of standardised cell lines, ensuring consistent quality, cost-effectiveness, and regulatory compliance (Wagner and Welch, 2010; Zakrzewski *et al.*, 2019).

As previously mentioned, for instance, mesenchymal stromal cells (MSCs) are

extensively utilised in cell therapy because of their self-renewal capacity, immunomodulatory properties, and potential for tissue repair. However, MSCs are highly heterogeneous, with variations arising from different tissue sources, donor differences, and long-term expansion, all of which can negatively impact their therapeutic effectiveness (Maličev and Jazbec, 2024). The ability to generate MSCs from iPSCs offers a promising alternative, providing a standardised and potentially unlimited cell supply (Bloor *et al.*, 2020; Sun *et al.*, 2022). This approach overcomes the heterogeneity of primary MSCs, ensuring reproducibility and scalability for large-scale cell manufacturing.

1.7.4 iMSCs

As previously described, mesenchymal stem cells (MSCs) possess considerable potential for the treatment of various diseases; however, the inconsistency observed in the outcomes of adult MSC clinical trials, frequently ascribed to cellular heterogeneity, continues to present a significant challenge (Zhou *et al.*, 2021).

First, the differentiation and proliferation capacities of MSCs diminish with age, particularly in MSCs derived from bone marrow, limiting their efficacy in older patients (Frobel *et al.*, 2014). Second, MSCs exhibit restricted pluripotency, and their effectiveness declines significantly after several passages, which poses a challenge for large-scale cell expansion and consistent therapeutic outcomes (Hynes *et al.*, 2014). Third, sourcing MSCs is inherently difficult due to the minimal availability of suitable tissues, such as cord blood, umbilical tissue, adipose tissue, and bone marrow, making the process resource-intensive (Devito *et al.*, 2019). Lastly, isolating MSCs from adult tissues often involves invasive procedures, further complicating their accessibility and raising ethical and practical concerns. Addressing these limitations is essential for optimising MSCs-based therapies and realising their full clinical potential.

Therefore, MSCs, derived from bone marrow, adipose tissue, or the umbilical cord, are constrained by limited availability, unstable quality, inherited heterogeneity, and a relatively low cost-benefit ratio, making them less practical for scalable or "off-the-shelf" applications. To address this issue, it is imperative to establish a standardised methodology for the production of MSCs, especially for their effective application in clinical settings. The production of induced pluripotent stem cell-derived mesenchymal stem cells (iMSCs) emerges as an exceptional option, facilitating the generation of an unlimited quantity of cells from genetically identical induced pluripotent stem cell lines (Bloor *et al.*, 2020). Furthermore, induced mesenchymal stem cells (iMSCs) provide a homogeneous cell population, thereby eliminating the issue of donor-to-donor variability often observed in adult mesenchymal stem cells (MSCs). This standardisation effectively addresses the challenges related to cellular heterogeneity and guarantees consistent therapeutic outcomes in clinical applications (Lee *et al.*, 2023a).

iMSCs, derived from Induced Pluripotent Stem Cells (iPSCs), address these limitations by offering unlimited sources, stable quality, high homogeneity, and better controllability (Fig.1.16). Their scalable production makes them more suitable

for commercial applications, highlighting their potential superiority over traditional MSCs.

iPSCs-derived MSCs can potentially be used in tissue regeneration and tissue repair because of their expandability (Yang *et al.*, 2019). More standardised cell preparations can be generated due to the capacity of iMSCs to assimilate into the ground state. The ageing of DNA methylation patterns differs between iPSC-MSCs and MSCs. Furthermore, the DNA methylation patterns of iPSC-MSCs are more critical to their rejuvenation potential than that of MSCs (Frobel *et al.*, 2014). In addition, iPSCs-MSCs can also reduce the possibility of tumorigenesis (Shao *et al.*, 2013). Consequently, iMSCs play a pivotal role in tissue regeneration within this project. This innovative method mitigates previous drawbacks associated with donor inconsistency and scalability, thereby enabling large-scale production of iPSC-derived mesenchymal stem cells for clinical applications.

Several studies have shown that iPSCs-derived MSCs can replicate the phenotypic and functional characteristics of primary MSCs (Bloor *et al.*, 2020; Devito *et al.*, 2019; Frobel *et al.*, 2014). Functionally, they can differentiate into mesodermal lineages and demonstrate significant immunomodulatory effects. Despite these promising characteristics, the differentiation process requires careful optimisation to guarantee the production of high-quality MSCs with therapeutic efficacy.

Consequently, iPSCs-MSCs play a significant role in tissue regeneration throughout this project. This novel methodology addresses previous issues of inter-donor inconsistency and scalability, facilitating the large-scale production of iPSCs-derived mesenchymal cells for clinical use application.

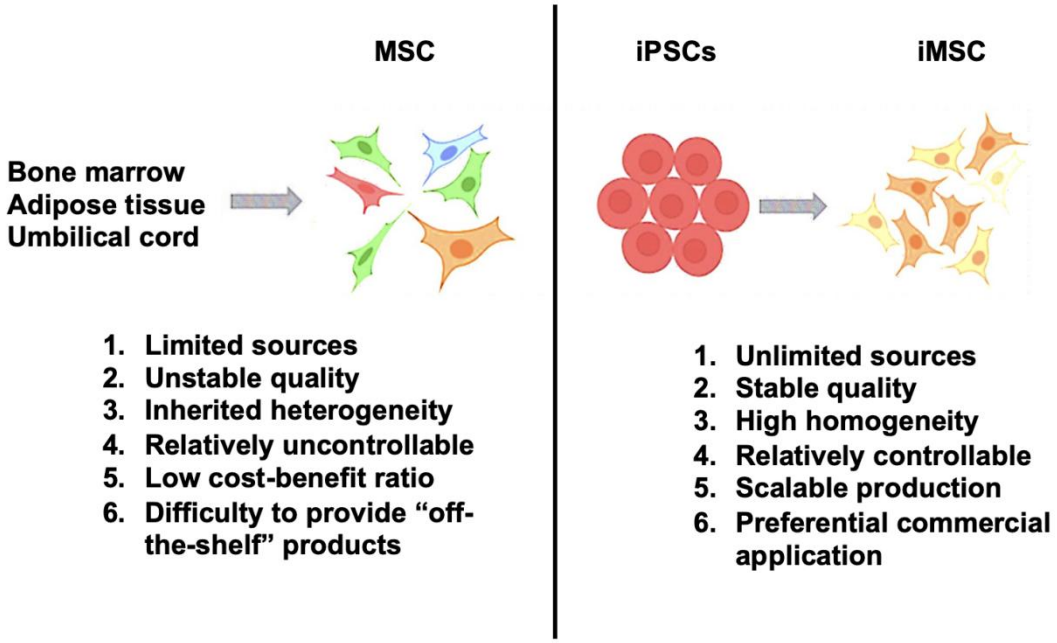


Fig. 1.16 The Difference between iPSCs-Derived MSCs and other Tissue-Derived MSCs. MSCs derived from primary tissues (e.g., bone marrow, adipose tissue, and umbilical cord) with induced mesenchymal stem cells (iMSCs) generated from induced pluripotent stem cells (iPSCs). While traditional MSCs face limitations such as donor-dependent variability, heterogeneity, and difficulty in standardization, iMSCs offer advantages including unlimited source availability, stable quality, high homogeneity, and scalable production, making them more suitable for commercial and therapeutic applications.

1.8 LPS: A Widely Recognized Module in Triggering the Inflammatory Response

Lipopolysaccharide (LPS), a component of microbial cell walls and a specific ligand for Toll-like receptor 4 (TLR4), has been widely used to mimic systemic and local infection across various tissues (Crompton *et al.*, 2016). The host responds to bacteria and other pathogens via pattern-recognition receptors (PRRs) that detect specific microbial components and metabolites, known as pathogen-associated molecular patterns (PAMPs). PAMPs signal microbial invasion, alerting the immune system to potentially harmful pathogens. In addition to detecting PAMPs, PRRs like TLR4 can also recognise damage-associated molecular patterns (DAMPs). Unlike PAMPs, DAMPs are endogenous molecules released by stressed, injured, or dying host cells. These molecules signal cellular damage or distress rather than microbial invasion, yet they similarly activate the immune response (Kumar *et al.*, 2013). The recognition of DAMPs by PRRs is vital in sterile inflammation, where tissue injury occurs without an infection, such as in trauma or ischemia-reperfusion injury.

Indeed, Inflammatory markers and tissue repair have an intricate and complex relationship. When LPS binds to TLR4, it forms a complex with co-receptors MD-2 and CD14, initiating a signalling cascade through MyD88-dependent and MyD88-independent (TRIF) pathways (Liu *et al.*, 2017; Sharif *et al.*, 2007). This cascade results in the activation of transcription factors such as nuclear factor kappa B (NF-κB), mitogen-activated protein kinases (MAPKs), and interferon regulatory factors (IRFs), leading to the production of pro-inflammatory cytokines, including tumour necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β), and interleukin-6 (IL-6), as well as type I interferons. These cytokines and interferons play a critical role in the immune response by promoting inflammation, recruiting immune cells to the site of infection or injury, and enhancing the body's ability to clear pathogens (Cowin *et al.*, 2021; Eming, Krieg and Davidson, 2007).

However, while the activation of TLR4 by LPS and the subsequent recognition of DAMPs are essential for an effective immune response, their persistent stimulation can lead to chronic inflammation (Piccinini and Midwood, 2010). This is particularly evident in non-healing wounds, such as venous leg ulcers and diabetic foot ulcers, where continuous TLR4 activation by LPS or DAMPs leads to prolonged cytokine production, excessive inflammation, and impaired healing (Landén, Li and Ståhle, 2016; Li and Wu, 2021). The result is often fibrosis and tissue damage, complicating the wound-healing process. Furthermore, mutations in TLR4 have been associated with an increased risk of developing chronic wounds, underscoring the receptor's

crucial role in maintaining the balance between necessary immune activation and the prevention of chronic inflammation (Chen *et al.*, 2013).

It is noteworthy that LPS induce a transition from M2- to M1-like macrophages, which holds significant implications for cancer therapy through the targeting of tumour-associated macrophages (TAMs). While M2 TAMs support tumour progression by facilitating immune suppression, M1 macrophages play a pivotal role in enhancing anti-tumour responses (Lin *et al.*, 2022; Wang *et al.*, 2024a). LPS was initially documented to induce tumour regression in murine models and was subsequently discovered to exert its effects indirectly by stimulating host macrophages to release TNFa responsible for the anti-tumour effect. Although LPS is not clinically viable due to its associated toxicity, safer derivatives such as monophosphoryl lipid A (MPLA) may provide analogous benefits (Zhang *et al.*, 2021b). Consequently, targeting MerTK or employing non-toxic TLR agonists could represent promising strategies for reprogramming tumour-associated macrophages and augmenting anti-cancer immunity.

In conclusion, LPS, as a potent ligand for TLR4, plays a critical role in simulating microbial invasion and sterile inflammation by activating innate immune responses through the recognition of PAMPs and DAMPs. While this activation is essential for initiating protective inflammatory responses and recruiting immune cells to sites of infection or injury, its sustained stimulation can lead to chronic inflammation, impaired wound healing, and tissue damage, as observed in conditions like diabetic ulcers and venous leg ulcers. Moreover, the LPS-induced shift from M2- to M1-like macrophage phenotypes underscores its potential in reprogramming tumour-associated macrophages (TAMs), thereby enhancing anti-tumour immunity. Despite LPS's therapeutic promise, its toxicity limits clinical application; however, safer derivatives such as MPLA offer viable alternatives. These insights collectively highlight the dual-edged nature of LPS-TLR4 signalling and its translational relevance in immune modulation and regenerative medicine.

1.9 Anti-inflammatory Compound

1.9.1 Bay 11-7082

BAY 11-7082 (BAY) is an inhibitor of κB kinase (IKK) known for its pharmacological effects, including anticancer, neuroprotective, and anti-inflammatory properties (Krishnan *et al.*, 2013). Previous studies explored the BAY significantly inhibited the production of nitric oxide, prostaglandin E_2, and tumour necrosis factor-α. It also reduced the translocation of p65, the major subunit of nuclear factor-κB, along with its upstream signalling events, including the phosphorylation of IκBα, IKK, and Akt (Lee *et al.*, 2012a). Moreover, BAY suppressed the translocation and activation of activator protein-1, interferon regulatory factor-3, and signal transducer and activator of transcription-1 by inhibiting the phosphorylation or activation of extracellular signal-regulated kinase, p38, TANK-binding protein, and Janus kinase-2 (Lee *et al.*, 2012a; Zanotto-Filho *et al.*, 2010). These findings suggest that BAY is a multitarget inhibitor with potential as a lead compound for developing potent anti-inflammatory drugs targeting multiple inflammatory pathways.

1.9.2 CBL0137

CBL0137 hydrochloride, also known as Curaxin-137 hydrochloride, is an inhibitor of the histone chaperone FACT (facilitates chromatin transcription). It activates the tumour suppressor protein p53 and inhibits NF-κB, with EC_{50} values of 0.37 μM and 0.47 μM, respectively (Burkhart *et al.*, 2014; Gasparian *et al.*, 2011). By targeting the histone chaperone FACT, CBL0137 disrupts chromatin dynamics, indirectly suppressing NF-κB-dependent gene expression. Additionally, CBL0137's suppression of NF-κB activity confers significant anti-inflammatory properties, making it a promising therapeutic candidate for both cancer and inflammation-driven diseases. Its dual ability to modulate multiple pathways, including NF-κB and p53, underscores its potential as a lead compound in drug development.

1.9.3 Dexamethasone

Dexamethasone, a synthetic glucocorticoid, is a potent anti-inflammatory and immunosuppressive agent that indirectly inhibits NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells). Dexamethasone suppresses NF-κB activity primarily by activating glucocorticoid receptors (GRs), which translocate to the nucleus and interfere with NF-κB signalling (Chang, Llanes and Schumer, 1997). This occurs via multiple mechanisms: GRs physically interact with NF-κB subunits (e.g., p65), inhibiting their transcriptional activity, and inducing the expression of IκBα, an inhibitor that sequesters NF-κB in the cytoplasm, preventing its activation.

By attenuating NF-κB-mediated gene expression, dexamethasone effectively reduces the production of pro-inflammatory cytokines and mediators, making it a key therapeutic agent for managing inflammatory and autoimmune conditions (Yao *et al.*, 2015).

1.9.4 SR9009

SR9009, a synthetic pyrrolidinecarbamate compound, is a selective REV-ERBα/β agonist with potent pharmacokinetic properties, including blood-brain barrier penetration. By enhancing REV-ERBα activity, SR9009 suppresses BMAL1 expression, reducing pro-inflammatory cytokine production (e.g., IL-1β and IL-18) via NLRP3 inflammasome inhibition (Hong *et al.*, 2021). In bone marrow-derived mouse macrophages, SR9009 shifts polarization toward the anti-inflammatory M2 phenotype, promoting tissue repair and immune resolution (Sitaula *et al.*, 2015). Beyond inflammation, SR9009 disrupts tumour metabolism and viability in cancer models, demonstrating its multifaceted role in regulating circadian-driven processes (Wagner, Monjes and Guido, 2019). These findings highlight SR9009's therapeutic potential for managing inflammatory, metabolic, and tumour-related disorders.

1.10 Transcription Factor

1.10.1 Glucocorticoid Receptor

Glucocorticoid receptor (GR) is a nuclear receptor that plays a central role in macrophage polarisation and the regulation of inflammation through its impact on cellular signalling pathways and gene expression. Upon binding to glucocorticoids, the GR translocates to the nucleus, where it interacts with glucocorticoid response elements (GREs) in the promoter regions of target genes, activating anti-inflammatory gene expression while repressing pro-inflammatory transcription factors such as NF-κB and AP-1 (Diaz-Jimenez, Kolb and Cidlowski, 2021). In macrophages, GR signalling promotes polarization toward the M2 phenotype, characterised by enhanced expression of anti-inflammatory mediators like IL-10 and arginase-1 while downregulating pro-inflammatory cytokines like TNF-α, IL-1β, and IL-6 (Deochand *et al.*, 2024; Xie *et al.*, 2019). Mechanistically, the GR also interferes with intracellular signalling pathways such as MAPK and JAK-STAT, dampening inflammatory responses and promoting tissue repair. This dual action of transcriptional regulation and signalling pathway modulation underscores the GR's critical role in maintaining immune homeostasis and resolving inflammation, particularly in chronic inflammatory and autoimmune contexts.

1.10.2 NR1D1

Nuclear receptor Rev-Erbα (NR1D1) are members of the nuclear receptor superfamily and are essential components of the regulation expression of the circadian clock. At the fundamental of the circadian clock regulation are the transcriptional proteins BMAL1 and CLOCK, which form a heterodimer. This complex promotes the transcription of other factors including PER1/2/3, CRY1/2, and REV-ERBα/β, which further regulate the expression and activity of BMAL1/CLOCK (Scheiermann, Kunisaki and Frenette, 2013). One key aspect of the circadian clock's regulation is its interaction with the nuclear receptors RORα (retinoic acid-related orphan receptor α) and Rev-Erbα. These receptors are transactivated by CLOCK/BMAL1 and provide a stabilising loop within the clockwork. They regulate BMAL1 transcription by competing for shared RORE promoter elements, either activating (RORα) or repressing (Rev-Erbα) (Alenghat *et al.*, 2008). REV-ERBα has also been implicated in various physiological processes beyond the circadian clock by recruiting nuclear receptor corepressor-1(NCoR) and histone deacetylase 3 (HDAC3), resulting in target gene repression (Yin and Lazar, 2005).

It is also noted that disruptions in circadian timing, such as those caused by chronic shift work, have been associated with an increased risk of diseases, including cardiovascular and cerebrovascular diseases, metabolic disorders, and cancer (Harrington, 2010). Macrophages, crucial facilitators of innate immune responses, display strong circadian rhythms in gene expression, encompassing genes that govern pathogen detection and cytokine release (Keller *et al.*, 2009). However, the molecular mechanisms that link immune function to the circadian clock remain unknown. Rev-Erbα has been identified as a critical intermediary between the core clockwork and inflammatory pathways, playing an essential role in the temporal gating of pro-inflammatory cytokine responses (Gibbs *et al.*, 2012b).

1.10.3 CCAAT-Enhancer-Binding Proteins (C/EBPs)

C/EBP proteins bind to the CCAAT box motif in gene promoters and are characterised by a conserved basic-leucine zipper (bZIP) domain at the C-terminus, essential for dimerisation and DNA binding (Wedel and Ziegler-Heitbrock, 1995). They form homo- or heterodimers with other C/EBPs or transcription factors, enabling specific DNA binding. Knockout studies have demonstrated that the deficiency of C/EBP-α results in lipodystrophy, diminished granulocyte count, and fatal perinatal hypoglycemia because of impaired hepatic glucose efflux, while C/EBP-β knockout mice maintain viability but they exhibit female infertility, defective

mammary epithelial differentiation, and compromised immune function (Nerlov, 2007; Ramji and Foka, 2002). In addition, C/EBPϵ induces growth arrest by upregulating p27 and downregulating cyclins/cdks, promotes apoptosis by suppressing Bcl-2 and Bcl-x, and regulates secondary granule protein gene expression, all mediated by its N-terminal activation domain, with c-Myc inhibiting but Bcl-x having no effect on differentiation (Nakajima *et al.*, 2006). Therefore, these proteins are widely expressed in various tissues, including the liver, adipose tissue, hematopoietic cells, brain, and kidneys, regulating cell proliferation, differentiation, metabolism, and immunity.

1.11 Hypothesis

Modulation of macrophage polarisation through inflammatory signalling pathways and therapeutic interventions—including cytokines, small molecules, and iPSC-derived MSCs—can enhance MerTK expression, shift macrophages toward an anti-inflammatory phenotype, and improve tissue regeneration.

1.12 Aim and Objectives

1.12.1 Aim

This study aims to investigate the molecular and cellular interplay between mesenchymal stromal cells (MSCs) and macrophages during inflammation and tissue repair, focusing on mechanisms of macrophage polarisation and identifying therapeutic strategies—including small molecules, cytokines, and iPSC-derived MSCs—that can enhance anti-inflammatory macrophage function and promote tissue regeneration.

1.12.2 Objectives

1. To elucidate the molecular mechanisms by which LPS-induced inflammation affects macrophage function:

a. Characterise transcriptional changes in macrophages following LPS stimulation using RNA-seq.

b. Analyse the role of NF-κB activation in suppressing MerTK and circadian-regulated genes, delaying resolution and tissue repair.

2. To evaluate the immunomodulatory and reparative properties of iMSCs:

a. Assess the ability of iMSCs-conditioned medium to modulate macrophage polarisation toward an anti-inflammatory (M2) phenotype.

b. Explore the effects of feeder-free and feeder-supported conditions and the role of growth factors like TGF-β and FGF-β in enhancing iMSCs functionality.

3. To investigate therapeutic interventions to restore macrophage function:

a. Evaluate the role of IL4/IL13 and NF-κB inhibitors in restoring MerTK expression and promoting inflammation resolution.

b. Explore the synergistic potential of iMSCs-based therapies combined with cytokine treatments for enhancing tissue repair.

Chapter 2: Materials and Methods

2.1 Materials

Table 1 List of Cell line

Description	Vendor
Human haematopoietic cell line - U937	ATCC®CRL-1593.2
CD33 - KO - U397 cell line	University of Alberta
Human haematopoietic cell line - THP1	ATCC®TIB-202
Human microglial cell line - HMC-3	ATCC®CRL-3304
CF1 Mouse Embryonic Fibroblasts, irradiated	Gibco / A34181
EmbryoMax® Primary Mouse Embryonic Fibroblasts	Sigma-Aldrich / PMEF-CFL-P1
Induced Pluripotent Stem Cell line	Department of Pediatrics Icahn School of Medicine at Mount Sinai, New York, NY 10029, USA
Adipose tissue derived mesenchymal stem cell line	Manchester Metropolitan University
Mouse microglial cell line – BV2	Cytion/305156

Table 2 List of bioreagents, kits and chemicals

Description	Vendor / Cat # or Ref #
Roswell Park Memorial Institute Medium (RPMI1640)	Lonza / BE12-115F
	Corning / 10-041-CV
Dulbecco's Modified Eagle Medium (DMEM) Low Glucose (1 g/l), with L-Glutamine, with Sodium Pyruvate	Capricorn Scientific /DMEM-LPA
Dulbecco's Modified Eagle's Medium (DMEM) High Glucose (4.5 g/L) and sodium pyruvate without L-glutamine	Corning / 15-013-CV
Dulbecco's Phosphate Buffered Saline without Calcium or Magnesium, 500mL	Lonza / BE17-512F
	Corning / 21-031-CV
Value Heat Inactivated Fetal bovine Serum (FBS)	Gibco / A5256801
L-Glutamine	Gibco™ / A2916801
GLUTAMAX	Invitrogen / 35050-061
Penicillin-Streptomycin (10,000 U/mL)	Gibco™ 15140122
ACTIN TaqMan® Gene Expression Assays (Hs01060665_g1)	Thermo-Fisher scientific / 4331182
TNF TaqMan® Gene Expression Assays (Hs00174128_m1)	Thermo-Fisher scientific / 4331182
IL-1B TaqMan® Gene Expression Assays (Hs01555410_m1)	Thermo-Fisher scientific / 4331182

ABCA1 TaqMan® Gene Expression Assays (Hs01059137_m1)	Thermo-Fisher scientific / 4331182
IL-6 TaqMan® Gene Expression Assays (Hs00174131_m1)	Thermo-Fisher scientific / 4331182
MerTK TaqMan® Gene Expression Assays (Hs01031979_m1)	Thermo-Fisher scientific / 4331182
IL-10 TaqMan® Gene Expression Assays (Hs00961622_m1)	Thermo-Fisher scientific /4331182
MSR1 TaqMan® Gene Expression Assays (Hs00234007_m1)	Thermo-Fisher scientific / 4331182
IL-1RN TaqMan® Gene Expression Assays (Hs00893626_m1)	Thermo-Fisher scientific / 4331182
Arg1 TaqMan® Gene Expression Assays (Hs00163660_m1)	Thermo-Fisher scientific / 4331182
18s TaqMan® Gene Expression Assays (Hs03003631_g1)	Thermo-Fisher scientific / 4331182
GAPDH TaqMan® Gene Expression Assays (Hs02786624_g1)	Thermo-Fisher scientific / 4331182
Total RNA Purification Plus Kit	Norgen / P4-0016
Applied Biosystems™ High-Capacity cDNA Reverse Transcription Kit	Applied Biosystems™ / 4368814
Rnase inhibitor	Thermo Scientific™/ N8080119
TaqMan™ Fast Advanced Master Mix	Applied Biosystems™ / 4444963

QuantiNova PCR Kits	QIAGEN / 208256
DEPC-Treated Water	Invitrogen™ / AM9920
Human Mer APC-conjugated Antibody	Bio-techne / FAB8912A
Mouse IgG2B APC-conjugated Antibody	Bio-techne / IC0041A
Mouse IgM Isotype Control, PE	Invitrogen™ / MGM04
SSEA4 Monoclonal Antibody (eBioMC-813-70(MC-813-70)), Alexa FluorTM488	Invitrogen™ / 55-8843-42
Mouse IgG3 Isotype Control, FITC	Invitrogen™ / 11-4742-42
TRA-1-60 (Podocalyxin) Mouse anti-Human, PE, Clone: TRA-1-60	eBioscience / 12-8863-82
Mouse IgG1k Isotype	Affymetrix eBioscience / 12-4714-81
CD73 Monoclonal Antibody (AD2), PE	Invitrogen™ / 12-0739-42
CD105 (Endoglin) Monoclonal Antibody (SN6), PE	Invitrogen™ / 12-1057-42
CD90 (Thy-1) Monoclonal Antibody (eBio5E10 (5E10)), PE	Invitrogen™ / 12-0909-42
CD45 Monoclonal Antibody (HI30), PE	Invitrogen™ / 12-0459-42
Annexin V Apoptosis Detection Kits	Invitrogen™ / 88-8005-74
AlamarBlue™ Cell Viability Reagent	Invitrogen™ / DAL1025
Radioimmunoprecipitation buffer	Sigma / R0278
Protease Inhibitor Cocktail	Sigma / P8340-1ML
Phosphatase Inhibitor Cocktail 2	Sigma-Aldrich / P5726-1ML
Pierce™ BCA Protein Assay Kit	Thermo Scientific™ / 23227

Acrylamide/Bis-acrylamide, 30% solution	Sigma / A3699
PageRuler™ Plus Prestained Protein Ladder, 10 to 250 kDa	Thermo Scientific™ / 26619
Immobilon® -PSQ PVDF Membrane	Millipore / ISEQ00010
ECL Western Blotting Substrate Chemiluminescent Western ECL HRP	Thermo Scientific™Pierce™ / 32106
Bovine Serum Albumin	Sigma Aldrich / A2153
Tween®	Sigma / BCBF5959V
SuperSignal™ West Femto Maximum Sensitivity Substrate	Thermo Scientific™ / 34094
Stripping buffer	Thermo Scientific™ / 46430
NuPAGE™ LDS Sample Buffer (4X)	Invitrogen™ / NP0007
Phorbol myristate acetate	Sigma Aldrich/ P8139
Dimethyyl sulfoxide	Sigma / 276855
Trypan Blue solution	Corning / 17416001
Trypsin-EDTA solution Trypsin-EDTA (0.25%)	Thermo-Scientific Gibco™ / 25200056
Tryple express	Gibco™ / 12604021
DAPI 4',6-Diamidine-2'-phenylindole dihydroch	Sigma / D9542-1MG
Paraformaldehyde	Sigma / P6148
N.N.N',N' Tetramethyyl-Ethylenediamine - 100 ml	Sigma / LOT71K1515 T8133
Ammonium Persulfate	Sigma / A3678-25

β-mercaptoethanol	Sigma / M3148
Tris-Base	Fisher Bioreagents / BNP152-1
Glycine	Sigma-Aldrich / G8898-500G
Triton-X 100	Sigma / 2315025
Methylated Spirit (IDA 99), 99% (v/v), Pure, (Industrial Methylated Spirit, 74 0.P.)	Fischer Chemical / 10552904
Ethanolo (Ethanol 99%+, Absolute, Extra Pure, SLR, Fisher Chemical)	Fischer Chemical / 10680993
Acetylcholine chloride (-ACh-2acetyloxyethyl(trimethyl) azanium; chloride	Sigma-Aldrich / A2661
Lipopolysaccharides	Sigma-Aldrich / L2637
Amersham™ Protran® Western blotting membranes, nitrocellulose	Cytiva™ / 10600007
T0901317	Sigma / T2320
REV-ERB Agonist II, SR9009	Sigma / 554726-25MG
Ponceau S solution	SIAL / P7170
Geltrex™ LDEV-Free, hESC-Qualified, Reduced Growth Factor Basement Membrane Matrix	Gibco / A1413301
Corning® Matrigel® Basement Membrane Matrix	Corning / CLS356237
Fibronectin	Sigma / FC010
Porcine Gelatin	Sigma Aldrich / G1890
500ML 0.1% Gelatin in Water	Stem Cell Technologies / 07903
DMEM/F12 Base Medium	Invitrogen / A1516901

E8 supplement	Invitrogen / A1517101
Vitronectin	Life Technologies / A14700
Collagen IV	Sigma / C5533
H-1152 ROCK Inhibitor	EMD Millipore / 555550
Y27632 ROCK Inhibitor	Tocris / 1254
Human FGF-Basic (146aa) 50µG	Peprotech / AF-100-18C
Stemline II hematopoietic stem cell expansion medium	Sigma / S0192
Insulin	Sigma / I9278
Lithium chloride (LiCl)	Sigma / L4408
Collagen I solution	Sigma / C2249
DMEM/F12	Invitrogen / 11330032
Human IL-4 20µG	Peprotech / 200-04
Human IL-13 10µG	Peprotech / 200-13
Recombinant human BMP4 50µG	Peprotech / 120-05
Human/Murine/Rat Activin A (CHO Cell) 10µG	Peprotech / 120-14P
Human IL-1 beta Recombinant Protein	Peprotech / 200-01B
Iscove's modified Dulbecco's medium (IMDM)	Invitrogen / 12200036
Ham’s F12 nutrient mix	Invitrogen / 21700075
Sodium bicarbonate	Sigma / S5761
L-ascorbic acid 2-phosphate Mg2+	Sigma / A8960

1-thioglycerol	Sigma / M6145
Sodium selenite	Sigma / S5261
Non essential amino acids	HyClone / SH30853.01
Chemically defined lipid concentrate	Invitrogen / 11905031
Embryo transfer grade water	Sigma / W1503
Polyvinyl alcohol	Parteck® / 1415171000
Holo-transferrin	Sigma / T0665
ES-CULT M3120	Stem Cell Technologies / 03120
STEMSPAN serum-free expansion medium (SFEM)	Stem Cell Technologies / 09650
L-ascorbic acid	Sigma / A4544
PDGF-BB	Peprotech / 100-14B
KnockOut™ Serum Replacement	Gibco™ / 10828010
Mitomycin C from Streptomyces caespitosus	Sigma Aldrich / M0503
Goat anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor™ 488	Invitrogen / A11034
Goat Serum	GibcoTM / 16210064
Alexa Fluor 568 Phalloidin	Thermo Scientific™ / A12380
4′,6-Diamidino-2-phenylindole dihydrochloride (DAPI)	Sigma-Aldrich / D9542
VECTASHIELD® HardSet™ Mounting Medium	Vector / H-1400
Mer (D21F11) XP® Rabbit mAb	Cell Signaling Technology / 4319S

GAPDH (D16H11) XP® Rabbit mAb	Cell Signaling Technology / 5174S
Mouse anti-GAPDH antibody 6C5	Abcam / ab8245
β-Actin (13E5) Rabbit mAb	Cell Signaling Technology / 4970S
Cleaved Caspase-3 (Asp175) Antibody #9661	Cell Signaling Technology / 9661
Anti-alpha Tubulin antibody [DM1A]	Abcam / ab7291
Anti-STAT3 antibody [EPR787Y]	Abcam / ab68153
Phospho-Stat3 (Tyr705) (D3A7) XP® Rabbit mAb	Cell Signaling Technology / 9145
Anti-Rabbit IgG (whole molecule)–Peroxidase antibody produced in goat	Sigma Aldrich / A6154
Anti-Mouse IgG (whole molecule)–Peroxidase antibody produced in rabbit	Sigma Aldrich / A9044
Exo-spin mini-HD columns	CellGS / EX05-5
Exo-spinTM Buffer	CellGS / EX06-250
LB Agar	Invitrogen / 22700025
LB Broth base	Invitrogen / 12780-052
Ampicillin Sodium Salt	Sigma-Aldrich / A0166
Subcloning Efficiency™ DH5α Competent Cells	Invitrogen™/18265017
QIAprep Spin Miniprep Kit	Qiagen / 27104
QIAGEN Plasmid Midi Kit	Qiagen / 12143
Pierce™ Firefly Luciferase Flash Assay Kit	Thermo Scientific™ / 16174

Table 3 List of equipment

Description	Vendor / Cat # or Ref #
Nunc EasYFlask 25cm^2 T-25	Thermo-Fiscer scientific / 156340
Nunc EasYFlask 75cm^2 T-75	Thermo-Fisher scientific / 156499 ·
0.2uM filter	Merck
6 well plate Nunclon™ Delta Surface	Thermo-Fisher scientific / 140675
12 well plate	Starlabs
24 well plate	ibidi
96 well Tissue culture treated plate, with lid, black, sterile	Greiner Bio-One Cryos™ / 655086
µ-Plate 96 Well Black, Square, ibiTreat, Sterile	Ibidi / IB89626
Thermo Scientific™ Nunc™ Lab-Tek™ II Chambered Coverglass	Thermo Scientific™ / 155409
Tipone® 1000 µL	StarLab / S1122-1730
Tipone® 200 µL 1120-8710	StarLab / S1120-8710
Tipone®20/10 µL	StarLab / S1120-3710
Pipette	Gibson
Falcon Tube for Flow cytometry	Falcon™ / 352058
Eppendorf tube	Greiner Bio-One Cryos™ / 123278
Tipone®1.5 mL natural flat cap microcentrifuge tubes	StarLab / S1615-5500
Tipone®2 mL natural flat cap microcentrifuge tubes	StarLab / E1420-2000

PCR Eppendorf, 0.2 ml PCR Tube	StarLab / I1402-8100
SimpliAmp Thermal Cycler	Thermo Scientific / A24812
Blue-Ray Biotech TurboCycler 2 Thermal Cycler	Scientific Laboratory Supplies / MOL2702
Bio-Rad CFX96 Touch Real-Time qPCR System	Bio-Rad / 55448
Trans-blot SD Semydry Transfer Cell	BioRad / 1703940
dH2O Elix® Progard TS2	Merck Millipore LtD / PR0G0T0S2
Primo Vert Tissue Culture Microscope	ZEISS
Mini Vortex Mixer Fisher Scientific	Fischer Scientifc / 14-955-151
NanoDrop™ One/OneC Microvolume UV-Vis Spectrophotometer	Thermo Scientific™ / 13-400-525
Thermal cycler StepOnePlus™ Real-Time PCR System	Thermo Scientific™ / 4376600
CFX Connect Real-Time PCR Detection System	BioRad / 1855201
PowerPac™ Basic	BioRad / 1645050
Slide dual chamber for cell count	BioRad / 145-0011
Centrifuge tube 15 ml (15ml, 120x17mm, PP)	Sarstedt Tube / NC9531248
Centrifuge tube 50 ml (Screw Cap Tube 50ml, 28X114mm, PP, conical base with skirted bottom)	Sarstedt Tube / NC9874179
96-Well PCR Plate, Semi-Skirted, Raised Rim, Low Profile (for FAST® Systems), natural	Starlab / E1403-7700

Mini-PROTEAN® Spacer Plates with 1.0 mm Integrated Spacers	BioRad / 1653311
Mini-PROTEAN® Short Plates	BioRad / 1653308
Mini-PROTEAN Tetra Cell	BioRad / 1658004EDU
Eppendorf centrifuge	Sigma® / Z604062
Odyssey® XF Imaging System	LI-COR
ChemiDoc Touch Systems	BioRad / 1708370
Centrifuge Sigma 3-16K	DJB Lab care / 10280
Spectrafuge™ 24D Digital Lab Microcentrifuge	Labnet international / C2400
-80 freezer (-86°C UltraFlow Freezer)	Nuaire
Mr. Frosty	Thermo Scientific™ / 5100-0001
Water bath	Grant JB Nova / JBN12
Incubator (Nuaire ISO class 5 HEPA)	Nuaire
BioRad TC10™ automatic cell count	BioRad / 1450010
Live cell imaging microscope	Leica / CTR 6000
MACSQuant® Analyzer 16 Flow Cytometer	Miltenyi Biotec
BD FACSCelesta™	BD Biosciences
FLUOstar spectrophotometer	BMG-Labtech FluOstar Omega
GloMax Explorer	Promega
Inc Accublock™ Digital Dry Bath	Labnet international
Class II Cabinet	

Timer	Fisher Scientific / 11799795
STELLARIS 5 Confocal Microscope	Leica

Table 4 List of software

Description	**Vendor**
Microsoft Office	Microsoft
OriginPro	OriginLab
Adobe Illustrator	Adobe
Zeiss ZEN Lite Software	Zeiss
Imagine Studio™ Lite	LI-COR®
BDFacs suit V1.0.6	BD Bioscience
Leica Application Suite X (LAS X)	Leica
Fiji	ImageJ
StepOnePlus™ Software v2.3	Applied Biosystems
Prism version 9	GraphPad
FlowJo version 10.8.1	BD Bioscience
CFX Maestro Software	BioRad
SnapGene	Dotmatics

2.2 Methods

2.2.1 Cell Culture

Cell culture and treatment were conducted using aseptic techniques within a Class II tissue culture safety cabinet. The cells were maintained or incubated at 37 degrees Celsius in a humidified environment with 5% CO_2 unless otherwise specified. All protocols were approved by the Science and Engineering Research Ethics and Governance Committee and, on 29 March 2022, were granted a favourable ethical opinion.

2.2.1.1 Complete Media Preparations

The medium was pre-warmed to 37 degrees Celsius and then supplemented with various solutions (as detailed in the provided table). 1 mL aliquots of the medium were regularly added to 12-well tissue culture plates, which were subsequently inspected using an inverted light microscope for any signs of infection and to assess the integrity of the cell culture media.

Table 5 RPMI-1640 Complete Medium for U937/THP-1 monocyte cells

Components	**Volume**
Roswell Park Memorial Institute Medium (RPMI) (Lonza, UK)	440 ml
Heat-inactivated FBS from Thermo Fisher Scientific (Waltham, MA, USA)	50ml
L-glutamine (Lonza, Switzerland)	5 ml
Penicillin/streptomycin (Sigma-Aldrich, St louis, MO, USA)	5 ml

Table 6 DMEM Complete Medium for HMC-3 Microglia

Components	Volume
Dulbecco's Modified Eagle's Medium (DMEM) with high glucose (Lonza, Switzerland)	390 ml
Heat-inactivated FBS from Thermo Fisher Scientific (Waltham, MA, USA)	100ml
L-glutamine (Lonza, Switzerland)	5 ml
Penicillin/streptomycin (Sigma-Aldrich, St louis, MO, USA)	5 ml

Table 7 DMEM Complete Medium for Mesenchymal Stem Cells/Mouse Embryonic Fibroblast Cells/BV2 Microglia

Components	Volume
Dulbecco's Modified Eagle's Medium (DMEM) with high glucose (Lonza, Switzerland)	440 ml
Heat-inactivated FBS from Thermo Fisher Scientific (Waltham, MA, USA)	50ml
L-glutamine (Lonza, Switzerland)	5 ml
Penicillin/streptomycin (Sigma-Aldrich, St louis, MO, USA)	5 ml

Table 8 hiPSc Media

Components	Volume
DMEM/F12 Base medium (Gibco, USA)	365 ml
Knockout serum (Gibco, USA)	100 ml
Conditioned media from MEFs	20 ml
Mem non-essential amino acids solution (Gibco, USA)	5 ml
L-glutamine (Gibco, USA)	5 ml
Penicillin/streptomycin (Gibco, USA)	5 ml
β-Mercaptoethanol (Sigma-Aldrich, St louis, MO, USA) (NOTE: Prepare a solution 1:100 (10ul+990ul PBS)	350 µl
Human FGF-Basic (PeproTech, USA)	500 µl

2.2.1.2 Thawing of Frozen Cell Line

One vial containing cells was retrieved from the liquid nitrogen tank and defrosted in a water bath maintained at 37°C to facilitate rapid thawing. This procedure was executed utilising aseptic techniques, which included the application of 70% industrial methylated spirit (IMS) to effective surfaces before opening the tube. The cells were subsequently transferred into a 15 ml tube containing 10 ml of complete medium and centrifuged at a speed of 300 g for a duration of 5 minutes. Following centrifugation, the cells were relocated into a T75 flask and incubated at 37°C in a 5% CO_2 atmosphere. The growth of the cells was monitored for viability utilising an inverted light microscope.

2.2.1.3 Passaging Suspension Cells

Diluting the cells directly in the T75 flask while continuing to expand them allows for maintaining the cell culture without the need for centrifugation and resuspension. Withdrawing a portion of the cells and diluting the remaining cells with fresh growth media to an appropriate seeding density for the cell line is necessary when adjusting the cell density before passaging. Both methods can be employed to split

suspension cells depending on the cell culture conditions and the experiment requirements.

2.2.1.4 Passaging Adherent Cells

The cells were removed from the medium in T75 flasks and washed 2-3 times with Dulbecco's phosphate-buffered saline (DPBS). A small volume of pre-warmed Trypsin was added to the cultured cells for 3 minutes at 37 °C and 5% CO_2 until they detached. The trypsin was neutralised by adding routine cell culture media. The cells were centrifuged at 300G for 5 minutes to form a pellet, re-suspended in culture media, and seeded at the appropriate density in a new tissue culture flask for further growth and expansion.

2.2.1.5 Cell Counting

Cell counting was performed using the trypan blue exclusion method, in which the non-permeant dye trypan blue was used to assess cell viability based on membrane integrity. Viable cells excluded the dye, while non-viable (necrotic) cells absorbed it due to compromised membranes. A 1:1 mixture of 50 µl of cell suspension and 50 µl of trypan blue was prepared. Then, 10 µl of the mixture was loaded onto each side of a Neubauer haemocytometer. Cell counts were performed under a light microscope by counting the cells in the central squares of the grid. Counts were conducted in duplicate, and results were accepted only when the two counts differed by no more than 10%.

2.2.1.6 Cryopreservation Cells

After measuring the cell count, the cells were typically adjusted to a specific concentration at a density of $1x10^6$/mL in a freezing medium comprising 90% FBS and 10% DMSO. The cells were transferred to 1 ml cryogenic vials and stored in a container known as Mr Frosty, which enabled gradual freezing in a -80 ℃ freezer overnight. The following day, the cells were preserved in liquid nitrogen for long-term storage.

2.2.1.7 Feeder-dependent (MEF) Culture of iPSCs

2.2.1.7.1 Inactivation of Murine Embryonic Fibroblast Feeders

One vial of P0 Murine Embryonic Fibroblast (MEF) feeder was thawed rapidly in a 37 ℃ water bath using aseptic technique, wiping with 70% industrial methylated spirit (IMS) before opening. The cells were transferred to a 15 ml tube containing 6 ml of complete DMEM medium and centrifuged at 300G for 5 minutes. The cells were transferred into a T75 flask and incubated at 37 ℃ for 3 to 5 days. One flask

was split into 15 T75 flasks and incubated for another 3 to 5 days. Afterwards, the medium was removed, and 20 ml of complete DMEM medium containing 10 µg/ml of mitomycin C (Bio-Techne Ltd 3258-10mg) was added to each flask, which was then incubated for 3 hours. The medium was removed, and 20 ml of PBS was added to wash away the mitomycin C. The MEF cells were dissociated with TrypLE and incubated for 5 minutes. Afterwards, cells were transferred to a 50ml tube containing 5 ml of FCS and 10 ml of PBS to wash the flask. After centrifugation, the supernatant was removed, and the freezing medium (90% FCS + 10% DMSO) was added according to the cell concentration (1.2 x 10^6/ml). The cells were then placed in a methanol freezing box in a -80 ℃ freezer overnight and stored in liquid nitrogen the following day.

2.2.1.7.2 Preparation of ROCK Inhibitor

Ten milligrams of Y27632 were dissolved in 3 mL of sterile distilled water (dH_2O) to prepare a 10 mM concentration of the ROCK inhibitor. The solution was dispensed into 20 µL aliquots and stored at -80°C. For use, 1 µL of the 10 mM ROCK inhibitor solution was added to 1 mL of stem cell media.

2.2.1.7.3 Feeder-Dependent Culture of iPSCs Maintenance

The procedure commenced with the plating of 1 ml of sterile 0.1% gelatin in each well of a 6-well plate, followed by an overnight incubation period. Subsequently, inactivated mouse embryonic fibroblasts (MEFs) were plated onto the gelatin at a density of 0.75 x 10^5 cells/ml. The plate was gently shaken to ensure an even distribution of the cells and was then allowed to rest overnight. The induced Pluripotent Stem Cells (iPSCs) were subsequently thawed, centrifuged, and resuspended in a 10 µM ROCK inhibitor medium prior to being plated onto the MEF feeder layer. The plate was then incubated in a 37°C, 5% CO_2 environment, with the medium being changed daily, excluding the ROCK inhibitor.

2.2.1.8 Feeder-Free Culture of iPSCs

2.2.1.8.1 Matrigel-Coated Culture Plates

Following the manufacturer's guidelines, proper handling and thawing of Matrigel (Corning, US) were considered of paramount importance. Specifically, Matrigel was thawed on ice, and all associated components were maintained at a chilled temperature prior to aliquoting or dilution. Aliquots of 300 µl were typically prepared, although the volume varied depending on the protein concentration specified in the analysis certificate for each production lot. These aliquots were stored at -80°C.

To prepare the Matrigel solution, 300 µl of Matrigel was diluted in 6 ml of ice-cold DMEM/F-12 medium and mixed thoroughly by pipetting up and down. Subsequently, 1 ml of the Matrigel solution was transferred into each well of a six-well plate and gently swirled to ensure even surface coating. The plates were then incubated at 37°C for 1 hour or overnight to allow for gelling and surface coating. Matrigel-coated dishes were stored at 37°C for up to three days or wrapped in Parafilm and kept at 4°C for up to two weeks. Prior to use, plates stored at 4°C were warmed to 37°C for at least 30 minutes.

In preparation for cell culture, any excess Matrigel solution was aspirated, and an appropriate volume of fresh medium was added to fully cover the surface. The plate or dish was then returned to the incubator to warm and equilibrate with CO_2. After 10 minutes to several hours of equilibration, cells were transferred onto the prepared surface.

2.2.1.8.2 Geltrex-Coated Culture Plates

To optimally thaw the Geltrex™ Basement Membrane Matrix (Gibco, US), it was advisable to place it at a temperature between 2–8°C overnight. However, considering potential fluctuations in refrigerator temperature, the extract was thawed on ice in the refrigerator as a precaution. It was important to note that the basement membrane matrix gelled within 5–10 minutes when the temperature exceeded 15°C. Therefore, it was not necessary to keep it on ice if used within five minutes and if the ambient temperature remained below 25°C. Pre-chilling pipette tips, tubes, plates, or other tools that came into contact with the extract were not essential. Nevertheless, partial tubes and aliquots were kept on ice to prevent premature gelling, as smaller volumes tended to warm more rapidly.

To prepare the Geltrex™ solution, the matrix was gently pipetted vertically to avoid introducing air bubbles. Subsequently, 1 mL of Geltrex™ was diluted into 29 mL of DMEM/F-12 medium, with adjustments made as needed to achieve the optimal coating concentration for the intended application. The diluted solution was then applied generously to cover the growth surface and coated objects fully, and the mixture was left to incubate for 60 minutes at room temperature. For long-term storage, each dish was sealed with Parafilm to minimise dehydration. The coated dish remained stable for up to two weeks when stored at 2–8°C. Care was taken to prevent the surface from drying out, and the storage temperature was maintained within the 2–8°C range to avoid premature gelling. At the time of use, the Geltrex™

coating was aspirated, and cells were immediately plated in the pre-equilibrated culture medium.

2.2.2 Immunofluorescence Microscopy

Cells were subjected to a washing procedure using phosphate-buffered saline (PBS) and subsequently fixed in 4% paraformaldehyde for a duration of 30 minutes. Following fixation, the cells were permeabilised using 0.1% Triton X-100 in PBS for a period of 10 minutes. Notably, permeabilisation was omitted during the staining process for cell surface proteins. The cells were then subjected to an additional wash with PBS and incubated in a blocking buffer for 60 minutes. This blocking buffer comprised 5% bovine serum albumin (BSA) and 0.1% Tween 20 in PBS. The cells were allowed to incubate overnight at 4°C in the blocking buffer containing primary antibodies. Upon completion of the primary antibody incubation, the cells were washed three times with PBS and subsequently incubated in blocking buffer supplemented with the appropriate Alexa Fluor-conjugated secondary antibodies for 1 hour in a dark environment at room temperature (See table 9). Following secondary antibody incubation, the cells were counterstained with DAPI (1 µg/mL) to label nuclei and Phalloidin (1:400 dilution from a 66 µM stock) to stain F-actin. Fluorescence was detected using fluorescence microscopy, specifically employing the Leica STELLARIS 5 Confocal Microscope.

Table 9 Antibodies and dilution used for immunofluorescence

Antibodies	**Dilution**
Mer (D21F11) XP® Rabbit mAb	1:200
Goat anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor™ 488	1:400

2.2.3 Flow Cytometry

2.2.3.1 Biomarker Analysis

Cell pellets were washed twice with FACS buffer, consisting of PBS, 0.1% BSA, and 0.04% NaN3, followed by incubation with antibody (see table 10) sourced from eBioscience, USA, for a duration of one hour on ice. Subsequently, a 30-minute incubation with an Isotype antibody, also obtained from eBioscience, USA, at the corresponding concentration was used as a control. The analysis was conducted using a MACSQuant® Analyzer 16 (Miltenyi Biotec) and FlowJo version 10.8.1 software.

Table 10 Antibodies and Dilution Used for Flow Cytometry

Antibodies	**Dilution**
Human Mer APC-conjugated Antibody	1:100
Mouse IgG2B APC-conjugated Antibody	1:200
Mouse IgM Isotype Control, PE	1:200
SSEA4 Monoclonal Antibody (eBioMC-813-70(MC-813-70)), FITC	1:50
Mouse IgG3 Isotype Control, FITC	1:200
TRA-1-60 (Podocalyxin) Mouse anti-Human, PE, Clone: TRA-1-60	1:50
Mouse IgG1k Isotype	1:200
CD73 Monoclonal Antibody (AD2), PE	1:100
CD105 (Endoglin) Monoclonal Antibody (SN6), PE	1:100
CD90 (Thy-1) Monoclonal Antibody (eBio5E10 (5E10)), PE	1:100
CD45 Monoclonal Antibody (HI30), PE	1:100

2.2.3.2 Cell Death and Apoptosis Studies

The analysis employed FITC-conjugated annexin-V (Annexin V-FITC Apoptosis Kit, Invitrogen USA). Initially, the cells were subjected to a wash with phosphate-buffered saline (PBS), followed by a subsequent wash with 1X binding buffer, after which the cells were resuspended in 1X binding buffer at a concentration of $2X10^6$/ml. Subsequently, 100µl of the cell suspension was combined with 5 µl of fluorochrome-conjugated Annexin V and incubated for 10-15 minutes at room temperature. Following this incubation, the cells were washed with 1X binding buffer and resuspended in 200 µl of 1X binding buffer. This 200 µl cell suspension was then mixed with 5 µl of Propidium Iodide Staining Solution and analysed using the MACSQuant® Analyzer 16 (Miltenyi Biotec) within four hours, ensuring storage at 2-8 degrees Celsius and kept in the dark. All data were analysed utilising FlowJo software, version 10.8.1 (BD Bioscience). The resulting plots were categorised into four distinct sections: 1) viable cells, which are negative for both probes (PI/FITC -/-; Q4); 2) apoptotic cells, which are PI negative and Annexin positive (PI/FITC -/+; Q3); 3) late apoptotic cells, which are positive for both PI and Annexin (PI/FITC +/+; Q2); 4) necrotic cells, which are PI positive and Annexin negative (PI/FITC +/-; Q1).

2.2.4 Proliferation Assay

The AlamarBlue (AB) reagent is widely utilised to assess cell viability in cell culture experiments. A volume of 100 µl of the cell culture was dispensed into each well of a 96-well plate, ensuring the appropriate cell density. Subsequently, the cells were treated with the relevant reagents for the designated duration. Following this, 20 µl of AB reagent was introduced into each well prior to incubation at 37 °C for a period of 2 hours. Assays were conducted in 96 microplate wells, and the fluorescence was measured utilising a FLUOstar Omega spectrophotometer (BMG Labtech).

2.2.5 Real-time quantitative PCR (RT-qPCR)

2.2.5.1 RNA Extraction

RNA extraction was conducted utilising the Total RNA Purification Plus Kit (Geneflow, UK) in accordance with the manufacturer's guidelines. The quantified RNA was assessed using a Nanodrop 2000 instrument (Thermo Fisher Scientific, USA). The instrument settings were adjusted to the nucleic acid tab, with the RNA option duly selected from the drop-down menu. A blank reading was established by placing 1 microliter of nuclease-free water on the pedestal and selecting the blank option. After cleaning the pedestal with lens tissue, 1.5 µL of each sample was introduced, and the measure button was activated. Absorbance readings at 260 and 280 nm wavelengths were recorded, and the absorbance ratio (260:280) was subsequently calculated. The RNA was subsequently diluted to a concentration of 400 ng/microliter to facilitate cDNA synthesis.

2.2.5.2 Reverse Transcription

To synthesise single-stranded complementary DNA (cDNA) from total RNA, the High-Capacity cDNA Reverse Transcription Kits with RNase Inhibitor (Applied Biosystems, USA) were employed. To prepare the 2X reverse transcription mixture, the requisite reagents and enzymes were introduced into a reaction vessel (refer to Table 11) while maintained on ice and mixed gently. Subsequently, total RNA was incorporated into the 2X reverse transcription master mix to generate a 1X mixture. The reverse transcription reaction was conducted using a thermal cycler (see Table 12). Upon completion of the reaction, the resultant cDNA was preserved in an appropriate container at a temperature of -80 °C for subsequent utilisation.

Table 11 Prepare the 2× RT Master Mix (per 20-μL reaction):

Component	Volume/Reaction(μL)
10× RT Buffer	2.0
25× dNTP Mix (100 mM)	0.8
10× RT Random Primers	2.0
MultiScribeTM Reverse Transcriptase	1.0
RNase Inhibitor	1.0
Nuclease-free H2O	3.2
Total per Reaction	10.0

Table 12 Thermal Cycler

	Step 1	Step 2	Step 3	Step 4
Temperature (°C)	25	37	85	4
Time	10 min	120 min	5 min	∞

2.2.5.3 Quantitative PCR

2.2.5.3.1 TaqMan

To initiate the PCR reaction, the TaqMan® Fast Advanced Master Mix was thoroughly combined. Subsequently, the frozen samples and the TaqMan® Assays primer were thawed on ice and resuspended by vortexing, followed by a brief centrifugation. The total volume required for each component was determined by multiplying the volume designated for one reaction by the total number of samples (see Table 13). The components were mixed gently to prevent excessive vortexing, followed by brief centrifugation prior to the assembly of the PCR reaction plate. Following this, all samples underwent indexed polymerase enzyme sequencing on a CFX Connect RT PCR Thermocycler (Biorad, UK). The cycling conditions, excluding the annealing temperature/time and the number of cycles, remained

consistent across all primer sets (refer to Table 14). Data were analysed utilising the $2^{-\Delta\Delta CT}$ method, a mathematical approach employed to ascertain the relative expression of a target gene in comparison to a reference or housekeeping gene. The ΔΔCT (Delta Delta CT) is derived by subtracting the CT value of the target gene from the CT value of the reference gene and subsequently subtracting this ΔCT value from the ΔCT of the sample of interest. The resultant value is subsequently employed to calculate the relative expression of the target gene using the $2^{-\Delta\Delta CT}$ methodology.

Table 13 Prepare the PCR Reaction Mix (per 20-µL reaction)

Component	**Volume (µL) for 1 reaction**
TaqMan® Fast Advanced Master Mix (2X)	10.0
TaqMan® Assay primer/probe (20X)	1.0
cDNA template	2.0
Nuclease-free water	7.0
Total volume per reaction	20.0

Table 14 Cycling Conditions for RT-qPCR.

Bio-Rad CFX96™ System	**UNG activation**	**Polymerase activation**	**PCR (40 cycles)**	
	Hold	Hold	Denature	Anneal/extend
Temperature	50°C	95°C	95°C	60°C
Time (min:sec)	02:00	00:20	00:03	00:30

2.2.5.3.2 SYBR Green

The master mix consisted of 2 µl of cDNA, 5.2 µl of RNase-free water, 10 µl of SYBR Green master mix, 1.4 µl of forward primer (0.7 µM), and 1.4 µl of reverse primer (0.7 µM). The prepared solutions were placed in the thermocycler and subjected to the following cycling conditions: an initial denaturation at 95°C for 3 minutes,

followed by 40 cycles of denaturation at 95°C for 10 seconds and annealing/extension at 55°C for 30 seconds.

The forward and reverse primers for qPCR were designed based on sequences obtained from a published study (Table 15) (Bao *et al.*, 2022; Cai *et al.*, 2020; Ouyang *et al.*, 2023; Tha *et al.*, 2000; Tian *et al.*, 2019; Wolde *et al.*, 2020). Prior to use, the primer specificity was validated using the NCBI BLAST tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome). This verification process ensured that the selected primers were specific to the target gene and did not exhibit significant homology to off-target sequences, thereby confirming their suitability for quantitative PCR analysis.

Table 15 qPCR Primers

Gene	**Forward Primer Sequence 5-3'**	**Reverse Primer Sequence 5-3'**
mIL-1B	CACAGCAGCACATCAACAAG	GTGCTCATGTCCTCATCCTG
mTNF	CCTGTAGCCCACGTCGTAGC	AGCAATGACTCCAAAGTAGACC
mIL-6	GAGGATACCACTCCCAACAGACC	AAGTGCATCATCGTTGTTCATACA
mMerTK	GAAGGAGAGTTTGGGTCTGTAA	GTTGTCCAACTTCATGGTCTTC
mACTIN	CCACACCCGCCACCAGTTCG	CCCATTCCCACCATCACACC
mGAPDH	GTTTGTGATGGGTGTGAACCA	CCTTCCACAATGCCAAAGTTGT
hIL-1B	ACAGATGAAGTGCTCCTTCCA	GTCGGAGATTCGTAGCTGGAT
hIL-6	ACTCACCTCTTCAGAACGAATTG	CCATCTTTGGAAGGTTCAGGTTG
hIL-10	AAGGCGCATGTGAACTCCCT	CCACGGCCTTGCTCTTGTTTT
hTNF	CAGGTCCTCTTCAAGGGCCAA	GGGGCTCTTGATGGCAGAGA
h18S	CGCCGCTAGAGGTGAAATTC	CGAACCTCCGACTTTCGTTCT
hGAPDH	CCACATCGCTCAGACACCAT	AGTTAACAGCCCTGGTGA

2.2.6 Immunoblotting

2.2.6.1 Protein Extraction

The protein was extracted utilizing an ice-cold radioimmunoprecipitation assay (RIPA) buffer, which constitutes a lysis buffer comprised of 25 mM Tris-HCl, 150 mM NaCl, 0.5% sodium deoxycholate, 0.5% SDS, 1 mM EDTA, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 1% Triton X-100. Prior to utilization, a protease inhibitor cocktail (10:1000) was incorporated. The media was removed from cells in six-well plates, and the cells were scraped and lysed using RIPA buffer. Subsequently, the cells were subjected to vigorous mixing with a vortex mixer and centrifuged at 13,500 g for 15 minutes at 4°C. The supernatant was collected and utilized for SDS-PAGE analysis.

2.2.6.2 Protein Quantification

The protein concentration of the samples was quantified utilizing the BCA Protein Assay Kit (ThermoFisher, USA). A BCA working reagent was prepared through the combination of equal volumes of BCA reagents A and B. Subsequently, a series of protein standards was established by diluting a known concentration of a protein standard, such as bovine serum albumin, in an appropriate buffer for the samples. The samples were arranged to ensure they were in the same buffer as the standards and maintained at an equivalent pH. A volume ranging from 50 to 100 µl of the BCA working reagent was dispensed into each well of a 96-well plate, followed by the addition of 50 to 100 µl of samples and standards to their corresponding wells in the plate. The plate should be incubated at 37°C for a duration of 30 to 60 minutes. Subsequently, the absorbance of each well was measured at 562 nm using the GloMax Explorer microplate reader (Promega, USA). A standard curve was constructed by plotting the absorbance values of the standards against their known protein concentrations. This standard curve will be utilized to ascertain the protein concentration of the samples.

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

All samples were standardised to ensure an equivalent amount of protein was present across all specimens. Following the standardisation process, the samples were diluted in a ratio of 3:1 with LDS sample buffer (4X) (Invitrogen, USA) and subjected to heating at 95°C for a duration of 10 minutes to denature the proteins. Subsequently, the samples were permitted to cool for 10 minutes prior to being loaded onto the stacking gel. Please refer to Table 13 for the composition of

separating buffers with varying concentrations. A total of 4.5 ml of buffer was pipetted into a gel caster (Bio-Rad, USA). The gel was allowed to polymerise at room temperature before adding the stacking gel.

The stacking gel was prepared by combining 0.33 mL of cold 30% acrylamide with 0.8% bisacrylamide, 0.4 mL of 0.625M Tris-HCl at pH 6.6 (75.7 g/L), 0.4 mL of 5% SDS, 0.87 mL of dH_2O, 10 µL of 10% APS, and 2 µL of TEMED in a universal tube. The tube was inverted 2-3 times to ensure thorough mixing. Subsequently, the stacking gel was carefully pipetted on top of the polymerised separating gel. A comb was inserted into the gel, and the gel was permitted to solidify at room temperature.

Two gel casting devices were positioned within an electrophoresis tank filled to capacity with 1x electrophoresis buffer, as illustrated in Table 17. The combs were subsequently removed, and 20 µg of protein was carefully introduced into each well, while 5 µl of a protein size ladder was placed in the outermost wells. The electrophoresis tank was then connected to a power supply, which was set to a minimal voltage and maximal current before being activated. The initial voltage was calibrated to 60V and was increased to 200V upon the dye front reaching the interface between the stacking and resolving gel. The electrophoresis process was continued until distinct separations of the bands within the protein ladder were clearly observed.

Table 16 Composition of Separating Buffer

	7.5%	**10%**	**12.5%**	**15%**
Acr/Bis (ml)	1.5 (2.5)	2.0 (3.3)	2.5 (4.2)	3 (5)
1.88M Tris (ml)	1.2 (2.0)	1.2 (2.0)	1.2 (2.0)	1.2 (2.0)
0.5% SDS (ml)	1.2 (2.0)	1.2 (2.0)	1.2 (2.0)	1.2 (2.0)
dH_2O (ml)	2.1 (3.5)	1.6 (2.7)	1.1 (1.8)	0.6 (1)
TEMED (µl)	5.0 (8.3)	5.0 (8.3)	5.0 (8.3)	5.0 (8.3)
10% APS (µl)	30 (50)	30 (50)	30 (50)	30 (50)

Table 17 Electrophoresis Buffer

Component	Volume
Tris-Base	30.2g
Glycine	144g
dH_2O	Make up to 1 litre
Prior to use dilute 1:10 with dH2O and add 0.1% SDS, eg 10 ml of 10% SDS /litre	

2.2.6.4 Protein Transfer

The transfer apparatus was assembled by incorporating a transfer cassette or tank, a power supply, and a gel-membrane sandwich comprised of the gel, a piece of blotting paper, and either a nitrocellulose or polyvinylidene fluoride (PVDF) membrane. Subsequently, the transfer buffer was prepared utilizing a 1X running buffer containing 20% methanol, suitable for medium-sized proteins. The gel-membrane sandwich was formed by positioning the gel between two sheets of wet blotting paper and the PVDF membrane. This sandwich was then placed into the transfer cassette or tank, ensuring adequate contact between the gel and the membrane. The apparatus was connected to the power supply, which was adjusted to deliver maximum voltage and minimal current; the voltage was set to 100V following the initiation of power. The transfer process was conducted for a duration of one hour to ensure completion. Finally, the successful transfer was verified by removing the membrane and assessing protein transfer by applying Ponceau S solution (Sigma-Aldrich, USA) as a staining agent.

2.2.6.5 Chemiluminescent Western Blots Detection

Primary antibodies were diluted in a blocking solution following the manufacturer's specifications (Table 18). The membranes were subsequently incubated at room temperature overnight with gentle agitation. Following this, the membranes underwent three washes of five minutes each in PBS-Tween before incubation with a secondary antibody (Table 19), which was diluted in a 5% (w/v) solution of milk powder in PBS-Tween, as per the manufacturer's recommendations. The membranes were then incubated for one hour at room temperature and subjected

to four additional washes of five minutes each in PBS-Tween. The developing solution was prepared by mixing it in equal parts with a horseradish peroxidase solution (Thermo-Fisher Scientific). An aliquot of 1 ml of this mixture was evenly applied to each membrane. After one minute, the excess solution was removed, and the membranes were subsequently visualised using a Chemidoc Touch transilluminator (Bio-Rad).

Table 18 Primary Antibodies and Dilution Used for Immunoblots

Primary antibodies	**Dilution**
Mer (D21F11) XP® Rabbit mAb	1:2000
GAPDH (D16H11) XP® Rabbit mAb	1:5000
Phospho-Stat3 (Tyr705) (D3A7) XP® Rabbit mAb	1:2000
Anti-STAT3 antibody [EPR787Y]	1:2000
Anti-alpha Tubulin antibody [DM1A]	1:5000

Table 19 Secondary Antibodies and Dilution Used for Immunoblots

Secondary antibodies	**Dilution**
Anti-Rabbit IgG (whole molecule)–Peroxidase antibody produced in goat	1:10000
Anti-Mouse IgG (whole molecule)–Peroxidase antibody produced in rabbit	1:10000

2.2.7 Statistical Analysis

Data throughout this thesis were expressed as mean ± SD. Statistical differences were calculated using unpaired two-tailed Student's t-test or one-way ANOVA with Bonferroni correction for multiple comparisons. A probability of $p < 0.05$ was considered statistically significant. Ns not significant; *$p < 0.05$; **$p < 0.01$; ***$p <$

0.001; ****p < 0.0001. Prism9 GraphPad software, FlowJo, ImageJ and R studio were used to illustrate differentially expressed genes.

Chapter 3: Mer Receptor Tyrosine Kinase as an Essential Player in Tissue Repair Related to Macrophage Polarisation

3.1 Introduction:

Macrophages are key regulators of the innate immune system and exhibit remarkable plasticity. Depending on microenvironmental signals, they polarise into either pro-inflammatory M1 or anti-inflammatory M2 phenotypes (Das *et al.*, 2015; Mosser, Hamidzadeh and Goncalves, 2021). M1 polarisation is typically triggered by stimuli such as LPS, leading to the production of cytokines like IL-1β and TNF-α, as well as reactive oxygen and nitrogen species (Andersson *et al.*, 1992; Chen *et al.*, 2023). Conversely, M2 macrophages—induced by IL-4 and IL-13—secrete IL-10 and promote tissue remodelling, debris clearance, and angiogenesis, contributing to inflammation resolution (Scott *et al.*, 2023). The balance between these states influences whether tissue damage progresses or repair occurs.

LPS, a component of Gram-negative bacterial cell walls, activates Toll-like receptor 4 (TLR4), which in turn stimulates transcription factors such as NF-κB, AP-1, and STAT1. This cascade initiates classical M1 polarisation and enhances antimicrobial functions, but sustained activation can contribute to chronic inflammation (Feng *et al.*, 2011; Sharif *et al.*, 2007). Consequently, LPS is widely utilised as a standardised tool for studying innate immune responses and macrophage activation in research models.

MerTK, a receptor tyrosine kinase, plays a pivotal role in efferocytosis, the clearance of apoptotic cells—an essential process for maintaining tissue homeostasis and resolving inflammation (Liu *et al.*, 2024b). Beyond its role in clearing dying cells, MerTK signalling actively promotes the resolution of inflammation, facilitates tissue regeneration, and prevents secondary necrosis, which otherwise exacerbates tissue damage (Nishi *et al.*, 2019; Zhang, Earp and Liu, 2019). This dual functionality positions MerTK as a key mediator in the transition from inflammation to tissue repair, ensuring the immune response concludes without excessive collateral damage. Studies indicate that MerTK deficiency impairs tissue repair, prolongs inflammation, and delays cardiac recovery following injury (DeBerge *et al.*, 2017a). These findings highlight MerTK as a potential therapeutic target in conditions where unresolved inflammation and defective tissue repair contribute to disease progression.

Inflammatory and circadian rhythms tightly regulate the transcriptional programs that coordinate immune responses (Zeng *et al.*, 2024). Macrophages are central to these processes, responding dynamically to environmental signals through changes in gene expression (Sun, Jiang and Horng, 2022). The circadian clock, governed by

core transcription factors such as BMAL1 and CLOCK, regulates gene expression through E-box motifs, which drive rhythmic patterns in key physiological pathways (Ripperger and Schibler, 2006). Emerging evidence suggests that these circadian mechanisms intersect with inflammatory signalling, yet the extent and functional implications of this crosstalk remain poorly understood (Fatima, Sonkar and Singh, 2022; Yoshida *et al.*, 2014). In particular, how inflammation impacts the expression of E-box–regulated genes involved in macrophage-mediated tissue repair is not well characterised. Understanding this interface is critical for uncovering how disruptions in circadian regulation may contribute to chronic inflammation and impaired tissue regeneration. Circadian rhythms regulate diverse biological processes, including immune function, metabolism, and tissue repair (Ruby, Major and Hinrichsen, 2021). Disruption in circadian gene expression has been linked to chronic inflammatory diseases, delayed tissue repair, and metabolic disorders (Paatela, Munson and Kikyo, 2019). A key mechanistic link between inflammation and circadian disruption lies in the interplay between NF-κB and BMAL1/CLOCK (Guo *et al.*, 2015). NF-κB, a central transcription factor in inflammation, is rapidly activated by LPS (Liu *et al.*, 2017) and has been shown to inhibit BMAL1/CLOCK activity either by competitively binding to E-box motifs or through direct transcriptional repression (Shen *et al.*, 2021b; Srinivasan and Walker, 2022). This crosstalk may explain the observed suppression of MerTK and other E-box-regulated genes in inflammatory conditions. By inhibiting BMAL1/CLOCK activity, NF-κB disrupts circadian regulation, impairing tissue repair and immune resolution processes. The findings elucidate the manner in which inflammatory signalling pathways intersect with circadian regulatory mechanisms, thereby exacerbating tissue damage and hindering recovery.

IL-4 and IL-13, in addition to driving M2 polarisation, promote efferocytosis and repair, counteracting LPS-induced suppression of reparative genes like MerTK (Allen, 2023; Korns *et al.*, 2011). These cytokines offer therapeutic potential for restoring homeostasis during inflammation. Similarly, pharmacological NF-κB inhibitors have been explored for their ability to suppress pro-inflammatory signalling and promote tissue repair. However, the extent to which NF-κB directly regulates MerTK remains unclear and is a focus of this thesis.

This study aims to investigate how inflammatory and circadian signalling intersect to regulate macrophage function and tissue repair, with a focus on MerTK modulation. By examining the transcriptional and functional responses of macrophages to LPS, cytokines, and small-molecule inhibitors, this work identifies

mechanisms and therapeutic strategies that may enhance inflammation resolution and regenerative outcomes.

3.1.1 Aims and Objectives

Aims:

This study seeked to provide a comprehensive understanding of the molecular mechanisms governing macrophage activation and polarisation in response to inflammation. Specifically, it focused on characterising the transcriptional changes induced by LPS stimulation, including the suppression of MerTK and circadian-regulated genes, and uncovering the mechanistic role of NF-κB activation in these processes. Additionally, the study investigated the therapeutic potential of IL-4 and IL-13 in restoring MerTK expression and promoting tissue repair, alongside evaluating the effects of NF-κB inhibitors in modulating MerTK and resolving inflammation. By elucidating the intricate crosstalk between NF-κB activation and circadian regulation, this research aimed to identify actionable targets for mitigating inflammation and promoting tissue regeneration, contributing valuable insights into the resolution of chronic inflammatory diseases and tissue injury.

Objectives:

1. Characterising the transcriptional changes in macrophages following LPS stimulation using RNA-seq.

2. Investigating the suppression of MerTK and circadian-regulated genes and the underlying mechanisms.

3. Exploring the role of IL-4/IL-13 in restoring MerTK expression and promoting tissue repair.

4. Examining the recruitment effect of NF-κB inhibitors on MerTK and their potential to modulate inflammatory responses.

5. Elucidating the crosstalk between NF-κB activation and circadian regulation in macrophage polarisation.

3.2 Methods:

3.2.1 Cell Culture

U937/THP-1 monocyte cells were cultured in RPMI-1640 medium supplemented with 10% foetal bovine serum (FBS), 1% penicillin-streptomycin, and 1X L-glutamine at a final concentration of 2mM. BV2 microglia cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS, 1% penicillin-streptomycin, and 1X L-glutamine at a final concentration of 2mM. All cell lines were maintained in a humidified atmosphere at 37°C with 5% CO_2.

3.2.2 Differentiation of Monocytes into M0-like Macrophages

CD33 knock-out U937 cells used in this study were kindly provided by Dr. Abhishek Bhattacherjee (University of Toronto) (Bhattacherjee *et al.*, 2021). The gene editing was performed by the collaborating group and not generated in-house. In this study, these cells were used for downstream differentiation, treatment, and phenotypic analysis to investigate the role of CD33 in macrophage function under inflammatory conditions. All subsequent experiments involving these knock-out cells, including PMA-induced differentiation, LPS stimulation, and flow cytometry, were conducted as part of this thesis work.

3.2.3 CRISPR-Cas9 Gene editing for CD33 Knockout

A CD33 CRISPR/Cas9 knockout plasmid designed to disrupt gene expression by creating a double-strand break (DSB) in a 5' constitutive exon of the human CD33 genome was employed in experiments. The CD33 CRISPR/Cas9 knockout plasmid is composed of a pool of three plasmids, each encoding the Cas9 nuclease and a target-specific 20 nt guide RNA (gRNA) aimed at achieving maximum knockout efficiency (Bhattacherjee *et al.*, 2019).

3.2.4 LPS Stimulation

To induce an inflammatory response, macrophage-like cells were treated with lipopolysaccharide (LPS) at a final concentration of 100 ng/mL for 6 hours. Four experimental groups were established: CD33WT, CD33KO, WT_LPS, and KO_LPS.

3.2.5 RNA Sequencing (RNA-seq)

3.2.5.1 Total RNA Preparation

Total RNA was prepared from U937-derived macrophages treated with PBS and 100 ng/ml of LPS using the Total RNA Purification Plus Kit (Geneflow, UK) according to the manufacturer’s protocol. RNA concentration and quality were

assessed using the Nanodrop 2000 instrument (Thermo Fisher Scientific, USA). All RNA samples were extracted at 1000 ng and transferred into 1.5 ml EP tubes for delivery to BGI Genomics Co., Ltd (Project ID: F22FTSECKF9003_HUMkdsgR).

3.2.5.2 Library Construction

To begin this process, a certain amount of total RNA samples was taken, and oligo dT beads were used to enrich mRNA with poly A tail. The mRNA molecules were then fragmented into small pieces and synthesised into first-strand cDNA using random primers. The second strand cDNA was synthesised with dUTP instead of dTTP, and the synthesised cDNA was subjected to end-repair and 3' adenylation. Adaptors were ligated to the ends of these 3' adenylated cDNA fragments. The U-labeled second-strand template was then digested with Uracil-DNA-Glycosylase (UDG), and PCR amplification was performed. Library quality control was performed, followed by library circularisation. The library was then amplified to make DNA nanoball (DNB), and sequencing was performed on the DNBSEQ (DNBSEQ Technology) platform. After sequencing, the raw reads were filtered. Data filtering includes removing adaptor sequences, contamination, and low-quality reads from raw reads (Chen *et al.*, 2018).

3.2.5.3 Parameters for Data Filtering

Raw data with adapter sequences or low-quality sequences was filtered using a series of data processing steps completed by SOAPnuke software developed by BGI. The filter parameters were: '-n 0.01 -l 20 -q 0.4 --adaMR 0.25 --ada_trim --polyX 50 --minReadLen 150'. These steps included filtering adapter sequences by cutting reads that matched 25.0% or more of the adapter sequence (with a maximum of 2 base mismatches allowed), filtering read length by discarding reads less than 150 bp, removing N's if they accounted for 1.0% or more of the read, removing polyX if it exceeded 50 bp, and filtering low-quality data by discarding reads where bases with a quality value less than 20 accounted for 40.0% or more of the read. The final output was clean reads with a quality value system set to Phred+33 (Chen *et al.*, 2018).

3.2.5.4 RNA-seq Analysis

Differential gene expression analysis was conducted using DESeq2, and genes with $|\log_2$ fold change$| > 1$ and adjusted p-value < 0.05 were considered significant (Robinson, McCarthy and Smyth, 2010).

3.2.5.5 Clustering and Functional Enrichment Analyses

Heatmaps were generated using the pheatmap package in R (version 3.6.1) (Kolde, 2015). The package provides functionality for hierarchical clustering, annotation integration, and visualisation customisation. The biological functions of differentially expressed genes (DEGs) were identified through Gene Ontology (GO) enrichment analysis. The data was then visualised using the DAVID4 online tool. The GO functional analysis of DEGs was divided into three categories: biological process (BP), molecular function (MF), and cellular component (CC). A cutoff of a P-value of less than 0.05 and a gene count more significant than five was set. Additionally, KEGG pathway analysis was conducted separately on up-regulated and down-regulated DEGs using the clusterProfiler package in R-Studio to identify crucial pathways with a P-value of less than 0.05 (Yu *et al.*, 2012).

3.2.6 Real-Time PCR Analysis for the Validation of RNA-Seq Results

To validate the differentially expressed genes (DEGs) obtained from RNA-Seq analysis, real-time quantitative reverse transcription PCR (RT-qPCR) was performed. Total RNA (800 ng) prepared for RNA-Seq library was reverse transcribed using the High-Capacity cDNA Reverse Transcription Kits with RNase Inhibitor (Applied Biosystems, USA). The RT-qPCR was done using TaqMan® Fast Advanced Master Mix in a final volume of 20 µL. The PCR reactions were triplicated on CFX Connect RT PCR Thermocycler (Biorad, UK). The cycle threshold (Ct) values were normalised to β-Actin as a reference gene, and relative quantification of transcripts was done using the comparative $2^{-\Delta\Delta CT}$ method (See section 2.2.5).

3.2.7 Real-time Quantitative Polymerase Chain Reaction (RT-qPCR)

For subsequent experiments, U937/THP-1 cells were seeded at 1x10^6 cells/well in 6-well plates, whereas BV2 cells were seeded at a density of 4x10^5 cells/well in 6-well plates. RNA was isolated using the Total RNA Purification Plus Kit (Geneflow, UK) according to the manufacturer's protocol, RT-qPCR was performed using standard protocols, and data was analysed using the $2^{-\Delta\Delta CT}$ method, as previously described (See section 2.2.5).

3.2.8 Western Blot

Cells were lysed with an ice-cold radioimmunoprecipitation (RIPA) buffer containing a protease inhibitor cocktail (10:1000) was added to the cell pellets to lyse the cells. Samples were centrifuged at 13,000 × g for 15 min at 4 ℃. The protein from cells was mixed with a 4 × Loading buffer boiled in a water bath for 10 min and then

centrifuged. Total protein samples were separated by SDS-PAGE and were transferred to a PVDF membrane. Membranes were incubated in primary antibodies MerTK (No.# 4319S, Cell Signaling Technology) overnight at 4℃ and then incubated with an HRP IgG secondary. After using bound HRP IgG, the membrane was exposed to the ChemiDoc Touch Imaging System (Bio-rad, UK) (See section 2.2.6).

3.2.9 Flow Cytometry

U937-derived macrophages were stained with a fluorescein APC-conjugated anti-MerTK antibody or isotype as control (Bio-techne). Flow cytometry was performed using a MACSQuant® Analyzer 16 (Miltenyi Biotec), and data were analysed using FlowJo version 10.8.1 software. Mean fluorescence intensity (MFI) was quantified to evaluate MerTK expression (See section 2.2.3).

3.2.10 Immunofluorescence Staining

Cells were cultured on glass coverslips and treated with different conditions, fixed with 4% paraformaldehyde and permeabilised with 0.1% Triton X-100. Cells were blocked with 5% BSA and incubated with the following primary antibodies. MerTK was detected using a primary antibody (Cell Signaling Technology) and a secondary antibody conjugated to Alexa Fluor 488 goat anti-rabbit IgG (Invitrogen). Phalloidin (Alexa Fluor 568, Invitrogen) was used to stain F-actin, and DAPI (blue; Thermo Fisher) was applied for nuclear staining. Stained cells were imaged using a Leica STELLARIS 5 Confocal Microscope. Merged images were analysed for MerTK localisation and expression changes under various treatments (See section 2.2.2). Image analysis was performed using ImageJ for quantification and Imaris (Bitplane) for 3D visualisation and detailed assessment of changes in MerTK localisation and expression under different treatment conditions.

3.2.11 In Silico Promoter Analysis

The human MerTK promoter sequence (2 kb upstream of the transcription start site) was retrieved from the website of UCSC (https://genome.ucsc.edu/cgi-bin/hgTracks?db=hg38&lastVirtModeType=default&lastVirtModeExtraState=&virtModeType=default&virtMode=0&nonVirtPosition=&position=chr2%3A111898607%2D112029561&hgsid=2403461629_jSptGi3A3AfqrMp902P2RmFSp5uS). The presence of E-box motifs (CACGTG) was identified using the JASPAR database for transcription factor binding site prediction.

3.2.12 Circadian Synchronisation Assay

Macrophages were synchronised using Dexamethasone (100 nM) (Matsumura *et al.*, 2014) or 50% serum (Balsalobre, Damiola and Schibler, 1998) for 2 hours. After that, the completed medium with 10% serum was replaced, and cells were harvested at specified time points for RNA and protein extraction. RT-qPCR and Western blot analyses were performed to assess the circadian expression of MerTK.

3.2.13 Statistical Analysis

Data throughout this chapter were expressed as mean ± SD. Statistical differences were calculated using unpaired two-tailed Student's t-test or one-way ANOVA with Bonferroni correction for multiple comparisons. A probability of $p < 0.05$ was considered statistically significant. Ns not significant; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$. Prism9 GraphPad software and Rstudio were used to illustrate differentially expressed genes. R packages including "limma", "tidyverse", "pheatmap", "DESeq2", "clusterProfiler", and "rms" were performed to explore the association between stimulated with PBS and stimulated with LPS in M0-like macrophages.

3.3 Results

3.3.1 Identification of LPS-Induced Signalling Changes in U937-Derived Macrophages

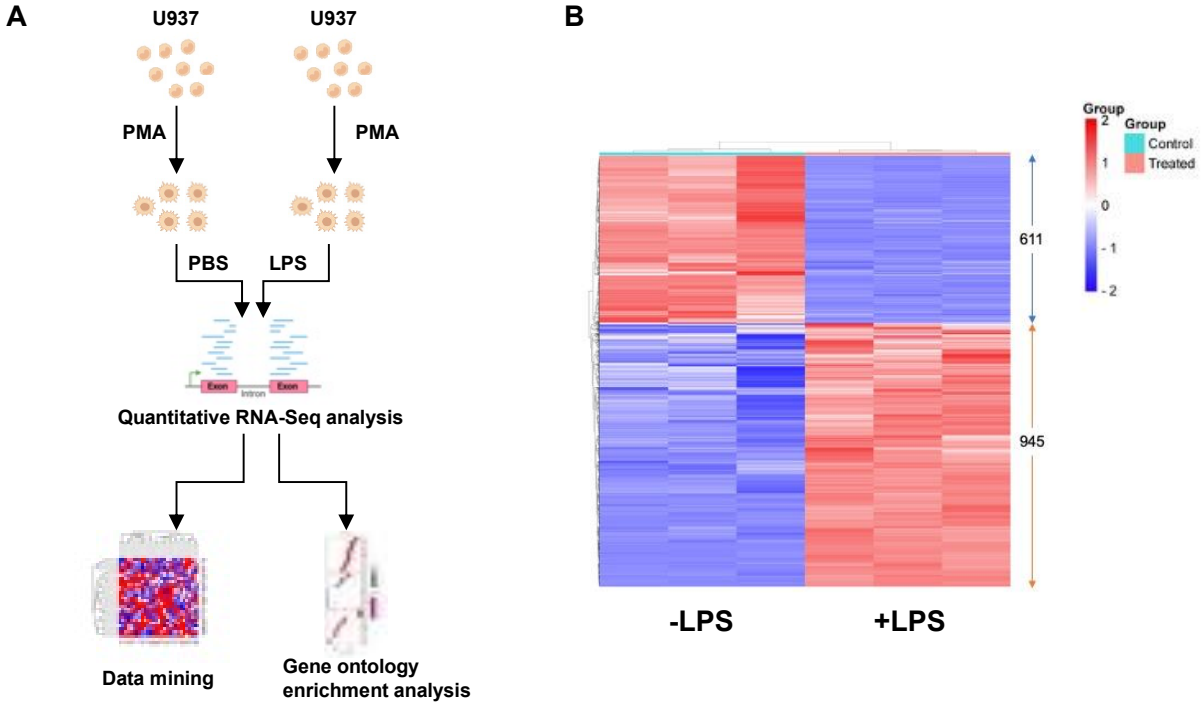


Fig.3.1 RNA-seq Analysis of U937-Derived Macrophage Polarisation Induced by LPS Treatment. (A) Workflow for differentiation and RNA-seq analysis. U937 monocyte cells were first treated with 50 ng/ml phorbol-12-myristate-13-acetate (PMA) for 48 hours to facilitate their differentiation into M0-like macrophages. After the differentiation process, the M0-like macrophages were incubated for a period of 6 hours under two distinct conditions: a control group treated with phosphate-buffered saline (PBS) and an experimental group exposed to lipopolysaccharide (LPS) at a concentration of 100 ng/ml. Upon completion of the treatment period, total RNA was extracted, and RNA sequencing (RNA-seq) was conducted to evaluate gene expression changes associated with macrophage function polarisation (n = 3 per group). (B) The heatmap clearly depicts the differential gene expression observed in the RNA-seq analysis, showcasing 945 significantly upregulated genes and 611 downregulated genes. This visual comparison between the PBS-treated control group and LPS-stimulated macrophages underscores the profound transcriptional changes induced by LPS. Genes with increased expression in response to LPS, represented in red, align with the characteristics of pro-inflammatory macrophage polarisation. Conversely, those with reduced expression, indicated in blue, reflect the suppression of specific pathways during this transition. These findings emphasise the dynamic shifts in gene expression that occur as macrophages move from a resting M0-like state to a highly activated pro-inflammatory phenotype, revealing key molecular drivers of this polarisation process.

To investigate the genomic alterations associated with macrophage polarisation, RNA sequencing (RNA-seq) was utilised to analyse differential gene expression profiles under specified treatment conditions. Monocytes U937 were cultured at a density of 0.5×10^6 viable cells per millilitre and seeded into 6-well plates. To induce differentiation into macrophage-like cells, the monocytes were treated with 50 ng/mL phorbol 12-myristate 13-acetate (PMA) in RPMI complete medium and incubated for 48 hours at 37°C in a 5% CO_2. Successful differentiation of U937 monocytes into macrophage-like cells was confirmed by morphological changes and adherence to the culture surface following 48 hours of PMA treatment. Following differentiation, the cells were thoroughly washed twice with fresh complete medium to remove residual PMA. Subsequently, they were incubated for an additional 24 hours in PMA-free complete medium to facilitate maturation into resting M0-like macrophages (Nejati Moharrami *et al.*, 2018; Xu *et al.*, 2018a). After the initial differentiation phase, the M0-like macrophages underwent two distinct treatment conditions for 6 hours. One group received treatment with phosphate-buffered saline (PBS) as a control, while the experimental group was subjected to stimulation with lipopolysaccharide (LPS) at a concentration of 100 ng/ml to induce a pro-inflammatory response (refer to Fig.3.1. A). The RNA-seq analysis identified 1,556 genes that displayed statistically significant modifications in their expression levels. Among these, 945 genes were found to be upregulated in reaction to LPS treatment, which signifies their activation or enhanced expression under inflammatory circumstances. On the other hand, 611 genes exhibited downregulation, indicating reduced expression levels. These findings provide insights into the transcriptomic changes that occur during macrophage polarisation, particularly in response to LPS stimulation, and highlight the dynamic regulation of gene expression associated with the transition from a baseline macrophage state to a pro-inflammatory phenotype (Fig.3.1. B).

3.3.2 Gene ontology (GO) enrichment and KEGG pathway analysis of downregulated expressed genes in LPS-stimulated macrophages.

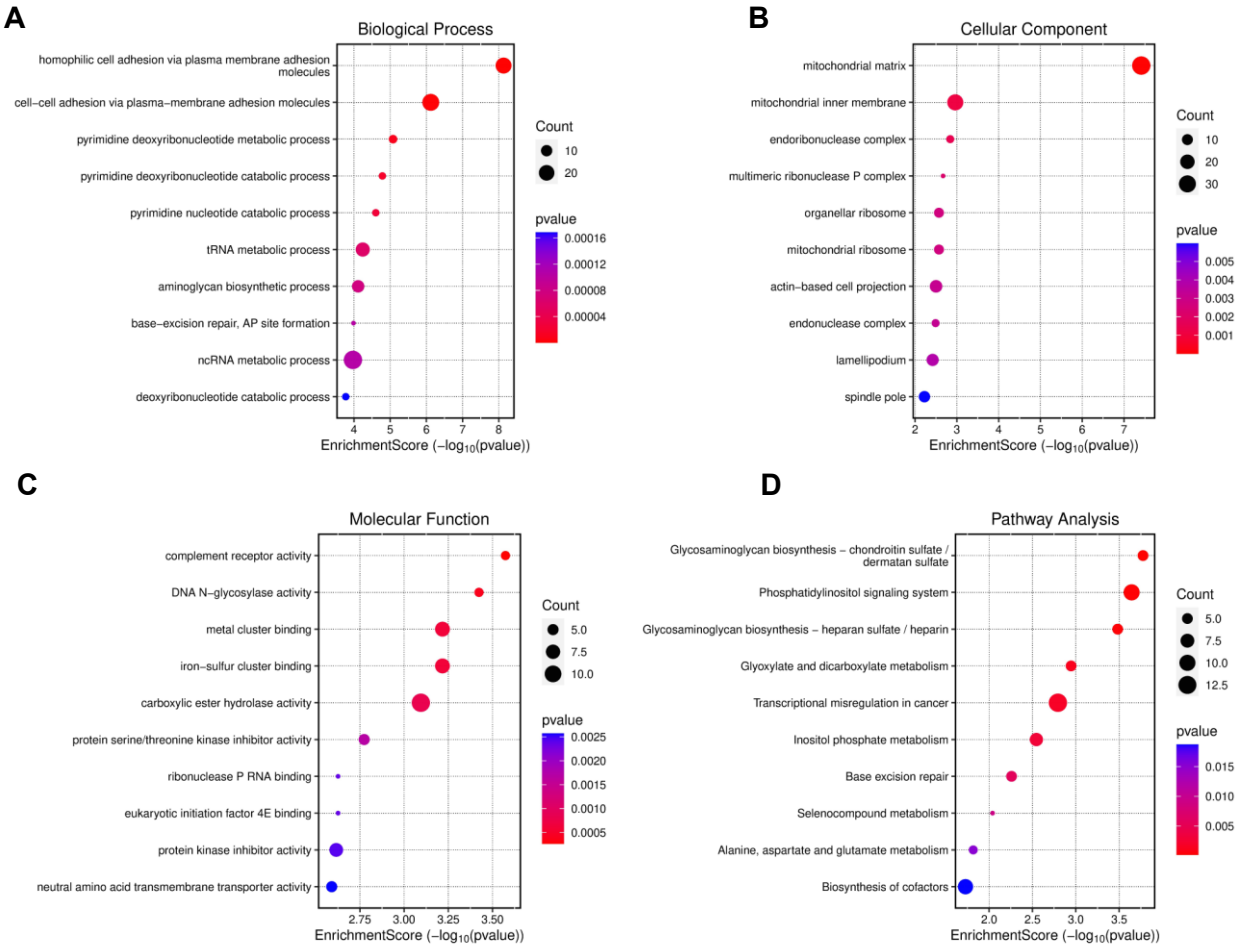


Fig.3.2 Gene ontology enrichment and Pathway Analysis of Downregulated Genes Following LPS Treatment. (A) Biological Process: The enrichment analysis highlights significantly enriched biological processes, including cell adhesion via plasma membrane adhesion molecules, pyrimidine metabolism, tRNA metabolic processes, and base excision repair. (B) Cellular Component: The enriched cellular components include the mitochondrial matrix, inner membrane, organellar ribosomes, endonuclease complexes, and lamellipodia, indicating the involvement of mitochondrial and ribosomal structures in response to LPS. (C) Molecular Function: Functional analysis shows significant enrichment in complement receptor activity, DNA N-glycosylase activity, metal cluster binding, and protein kinase inhibitor activity, suggesting the role of enzymatic and receptor activities in macrophage polarisation. (D) KEGG Pathway Analysis: The pathway analysis identifies key enriched pathways such as glycosaminoglycan biosynthesis, phosphatidylinositol signalling, base excision repair, and amino acid metabolism, reflecting the extensive functional and metabolic reprogramming induced by LPS stimulation. Enrichment scores are displayed on the x-axis, with dot size representing gene count and colour indicating the significance level (p-value).

RNA sequencing analysis identified 611 genes that exhibited significant downregulation after lipopolysaccharide (LPS) treatment. Functional enrichment analysis indicated that these genes are linked to processes critical for cellular maintenance, encompassing nucleotide metabolism, transfer RNA (tRNA) processing, and base excision DNA repair (Fig.3.2. A). This suppression indicates a shift in cellular priorities, diverting resources from routine maintenance to enhance inflammatory and immune functions during macrophage activation. At the cellular level, the downregulated genes were primarily associated with mitochondrial components, including the mitochondrial matrix, inner membrane, and ribosomal structures (Fig.3.2. B). This observation signifies a decline in mitochondrial activity and protein synthesis, which are likely consequences of the metabolic reprogramming induced by LPS. From a molecular standpoint, functions such as DNA N-glycosylase activity, metal cluster binding, and complement receptor activity were notably downregulated, suggesting a reduction in enzymatic activity and receptor-mediated signalling (Fig.3.2. C). These alterations reflect decreased cellular processes associated with DNA repair and metabolic regulation, consistent with the macrophage's transition towards immune activation. Pathway analysis further revealed suppression of essential metabolic and biosynthetic pathways, such as glycosaminoglycan biosynthesis, phosphatidylinositol signalling, and amino acid metabolism (Fig.3.2. D). The downregulation of these pathways implies a diminishment in energy-intensive biosynthetic activities and a potential deterioration of cellular repair capabilities.

Collectively, these findings illustrate that LPS treatment prompts extensive downregulation of genes implicated in cellular maintenance, metabolism, and repair, indicating a coordinated shift in macrophage function to prioritise inflammatory and immune responses. This transcriptional reprogramming constitutes a hallmark of macrophage polarisation during immune activation.

3.3.3 Gene ontology (GO) enrichment and KEGG pathway analysis of upregulated expressed genes in LPS-stimulated macrophages.

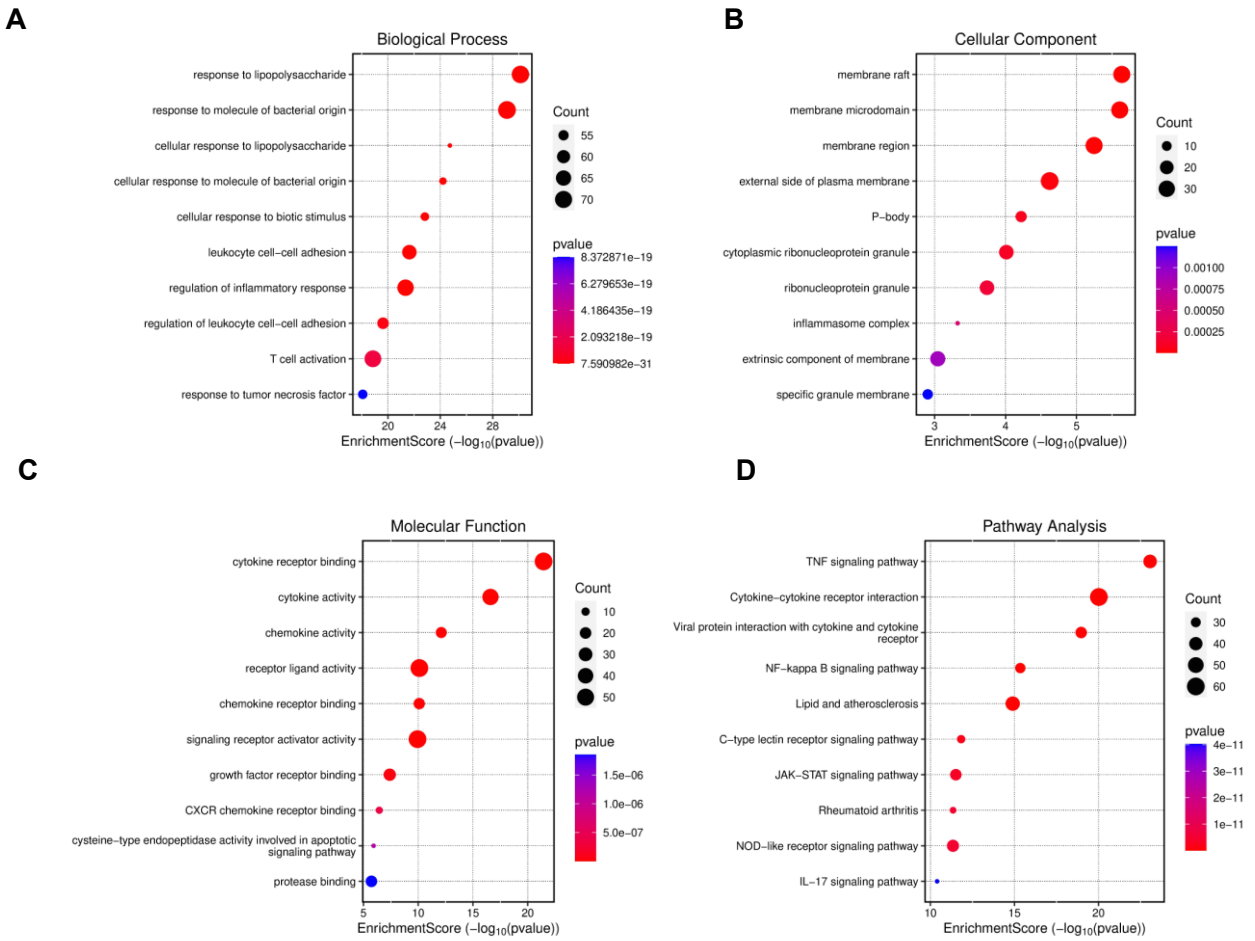


Fig.3.3 Gene ontology enrichment and Pathway Analysis of Upregulated Genes Following LPS Treatment. (A) Biological Process: The biological processes enriched in upregulated genes include responses to lipopolysaccharide, reactions to molecules originating from bacteria, cellular responses to biotic stimuli, adhesion among leukocyte cells, and the regulation of inflammatory responses. This indicates an enhanced inflammatory and immune response triggered by LPS stimulation. (B) Cellular Component: Upregulated genes were enriched in cellular components such as membrane raft, membrane microdomain, external side of plasma membrane, inflammasome complex, and specific granule membrane. These findings suggest an increased involvement of membrane-associated structures and immune-related complexes in macrophage activation. (C) Molecular Function: Enrichment analysis of upregulated genes revealed significant molecular functions, including cytokine receptor binding, cytokine activity, chemokine activity, and receptor-ligand activity. These findings imply that the upregulated genes contribute to processes integral to immune activation, inflammation, and potentially the transition to a pro-inflammatory macrophage phenotype. (D) KEGG Pathway Analysis: Enriched KEGG pathways for upregulated genes, including TNF signalling, NF-κB signalling, and cytokine-cytokine receptor interactions, demonstrating the activation of pro-inflammatory cascades essential for macrophage responses to LPS stimulation. Collectively, these findings emphasise the activation of pro-inflammatory molecular mechanisms, immune signalling pathways, and cellular processes that characterise the transition to a pro-inflammatory macrophage phenotype. The dot size represents gene count, while the colour gradient indicates statistical significance (p-value).

The RNA sequencing analysis identified 945 genes that exhibited significant upregulation following LPS treatment, prompting further examination of their functional roles through Gene Ontology (GO) enrichment and KEGG pathway analysis. The findings indicated a strong association of upregulated genes with processes related to immune activation and inflammatory responses. Notably, genes implicated in the reaction to molecules of bacterial origin and lipopolysaccharides were enriched, reflecting the activation of macrophages towards a pro-inflammatory state. Furthermore, processes such as leukocyte adhesion, regulation of inflammation, and T-cell activation suggested enhanced immune communication and pathways of activation pathways (Fig.3.3. A). At the cellular level, the upregulated genes were found to be enriched in membrane-associated components, including membrane rafts, microdomains, and the external plasma membrane, which are essential for signal transduction and the localisation of immune receptors. Upregulated genes within inflammasome complexes and granule membranes underscore the activation of intracellular immune structures, indicating increased inflammasome activity and the secretion of inflammatory mediators (Fig.3.3. B). Molecular function analysis revealed enrichment in cytokine receptor binding, chemokine activity, and receptor-ligand interactions, thereby reflecting the functional role of these genes in promoting inflammation (Fig.3.3. C). Enrichment in pathways such as TNF signalling, NF-κB signalling, and cytokine–cytokine receptor interaction suggests activation of pro-inflammatory programmes in LPS-stimulated macrophages, consistent with known M1 polarisation markers (Fig.3.3. D) (Chen *et al.*, 2023; Das *et al.*, 2015). Overall, the upregulated genes demonstrate a transcriptional program prioritising immune defence, pro-inflammatory signalling, and cellular adaptation in response to LPS stimulation. These findings highlight the dynamic molecular alterations that drive macrophages towards a pro-inflammatory phenotype during LPS stimulation.

3.3.4 Gene Expression Profiling in Response to LPS Stimulation

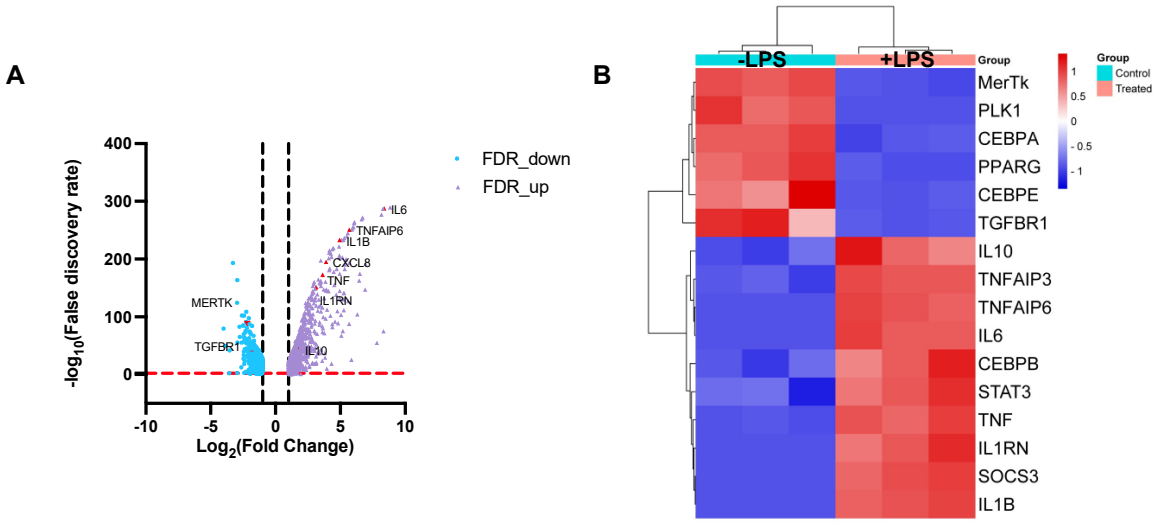


Fig.3.4 Differential Expression of Genes in Response to LPS Treatment. (A) Volcano plot illustrating the distribution of differentially expressed genes in U937-derived macrophages following LPS stimulation. The x-axis indicates the log2 fold change in gene expression, whereas the y-axis represents the -log10 false discovery rate (FDR). Genes that are significantly downregulated (FDR_down) are displayed in blue, while upregulated genes (FDR_up) are shown in purple. Key genes like IL6, TNF, IL1B, IL10, and MerTK are highlighted. (B) Heatmap of selected genes showing expression patterns between control (-LPS) and LPS-treated (+LPS) groups. Rows depict individual genes, and columns correspond to sample groups. The colour scale reflects normalised expression values, with red indicating upregulation and blue representing downregulation. Data shown represent three independent biological replicates (n = 3).

In response to LPS stimulation, RNA sequencing analysis revealed significant transcriptional alterations indicative of a complex inflammatory response in macrophages. Notably, the pro-inflammatory cytokines TNF, IL-1B, and IL-6 were significantly upregulated, thereby promoting the classical inflammatory response. Furthermore, the concurrent upregulation of anti-inflammatory factors, including IL-10 and IL-1RN, underscores a critical regulatory mechanism. This dual response suggests that macrophages initiate inflammation while activating pathways to mitigate excessive inflammatory damage, thereby maintaining a balance between immune activation and resolution. The volcano plot (Fig.3.4. A) clearly illustrates this dichotomy, where TNF, IL-1B, and IL-6—key drivers of inflammation—are significantly upregulated. Concurrently, IL-10 and IL1-RN demonstrate substantial

increases, emphasising their roles in modulating and containing the inflammatory response.

Conversely, the downregulation of genes such as MerTK and TGFBR1, which are associated with anti-inflammatory and tissue-repair functions, further underscores the transition of macrophages toward a predominantly pro-inflammatory phenotype. The heatmap (Fig.3.4. B) corroborates these findings, demonstrating a distinct clustering of gene expression between LPS-treated and control groups. Pro-inflammatory genes, including TNFAIP3, TNFAIP6, STAT3, and SOCS3, were significantly upregulated, supporting the activation of inflammatory and feedback regulatory pathways. Nevertheless, the upregulation of IL-10 and IL-1RN in this context reflects an early anti-inflammatory response, likely aimed at preventing uncontrolled inflammation and subsequent tissue damage. Importantly, these findings suggest that LPS-induced macrophage polarisation involves a dynamic and tightly regulated process. The simultaneous induction of both pro-inflammatory and anti-inflammatory factors reveals the complexity of the macrophage response, where anti-inflammatory mediators swiftly accompany initial inflammation to ensure immune homeostasis. This discovery underscores the nuanced role of macrophages in managing the balance between inflammation and resolution during immune activation.

3.3.5 Validation of RNA-seq Results Using RT-qPCR

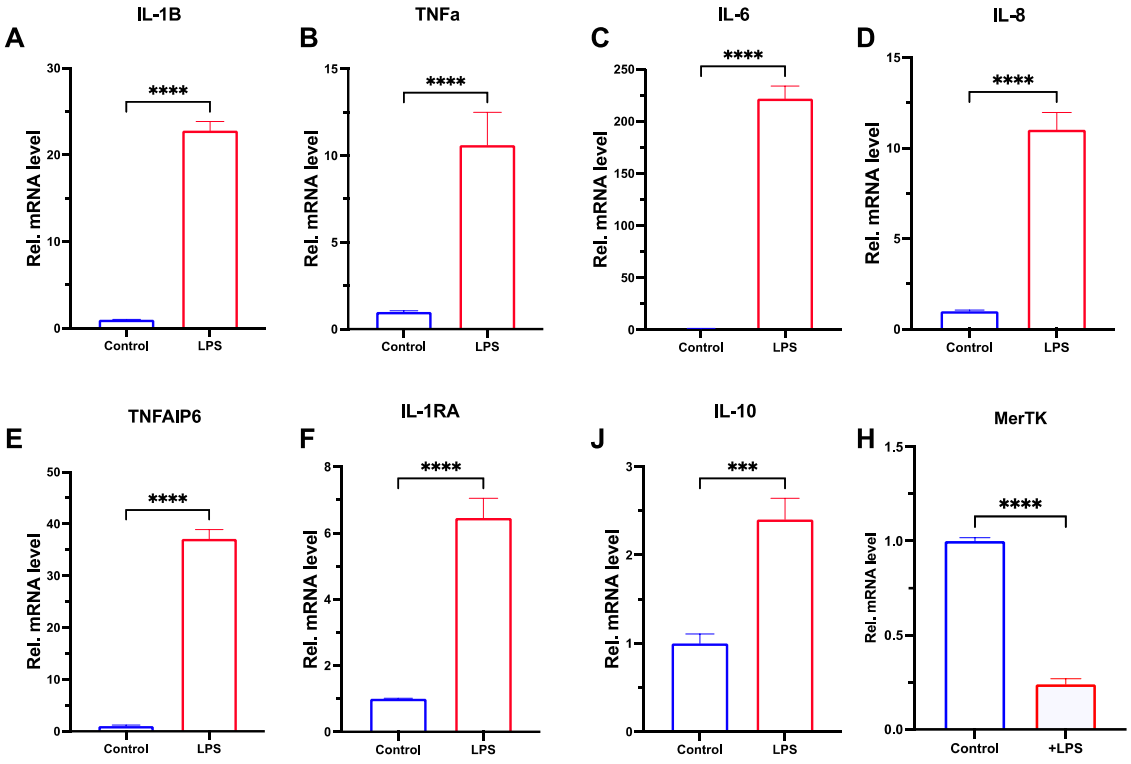


Fig.3.5 RT-qPCR Quantified Relative mRNA Expression Levels of Selected Genes to Validate RNA-seq Findings in U937 derived macrophages. Expression of pro-inflammatory genes (IL-1B, TNFα, IL-6, IL-8, and TNFAIP6) and anti-inflammatory genes (IL-1RA and IL-10) exhibited a significant increase in response to LPS) treatment when compared to the control group. Conversely, MerTK, a gene known for its association with anti-inflammatory and homeostatic functions, demonstrated notable downregulation following LPS stimulation. The data are presented as the mean ± standard error of the mean (SEM), and statistical significance is indicated (***p<0.001, ****p<0.0001). Data shown represent three independent biological replicates (n = 3).

To validate the RNA-seq results, RT-qPCR was conducted to assess the relative expression levels of mRNA from selected pro-inflammatory and anti-inflammatory genes in control and LPS-treated macrophages. The results indicated a significant increase in the expression of pro-inflammatory genes, notably IL-1B, TNFα, IL-6 and IL-8, in response to LPS stimulation (Fig.3.5. A-D). These findings are consistent with the RNA-seq data, thereby confirming the substantial upregulation of key cytokines and inflammatory mediators during macrophage activation.

Furthermore, along with pro-inflammatory factors, anti-inflammatory mediators such as TNFAIP6, IL-1RA, and IL-10 also exhibited significant upregulation following LPS

treatment, suggesting a concurrent activation of regulatory pathways to modulate the inflammatory response (Fig.3.5. E-J). This observation supports the premise of a balanced immune response, whereby macrophages initiate inflammation while simultaneously activating mechanisms to prevent excessive tissue damage.

Notably, MerTK, a gene associated with anti-inflammatory and tissue-repair functions, was significantly downregulated following LPS stimulation, which aligns with the RNA-seq findings (Fig.3.5. H). This suppression further underscores the polarisation of macrophages towards a pro-inflammatory phenotype, as MerTK is frequently associated with immune resolution and macrophage-mediated clearance of apoptotic cells.

In summary, the RT-qPCR results strongly corroborate the RNA-seq findings, demonstrating the accuracy and reproducibility of the transcriptomic analysis. The upregulation of pro-inflammatory cytokines and anti-inflammatory factors, in conjunction with the downregulation of MerTK, illuminates the dynamic transcriptional changes in macrophages in response to LPS, reflecting their functional shift towards immune activation and inflammation regulation.

3.3.6 Validation of MerTK Downregulation in LPS-Treated Macrophages

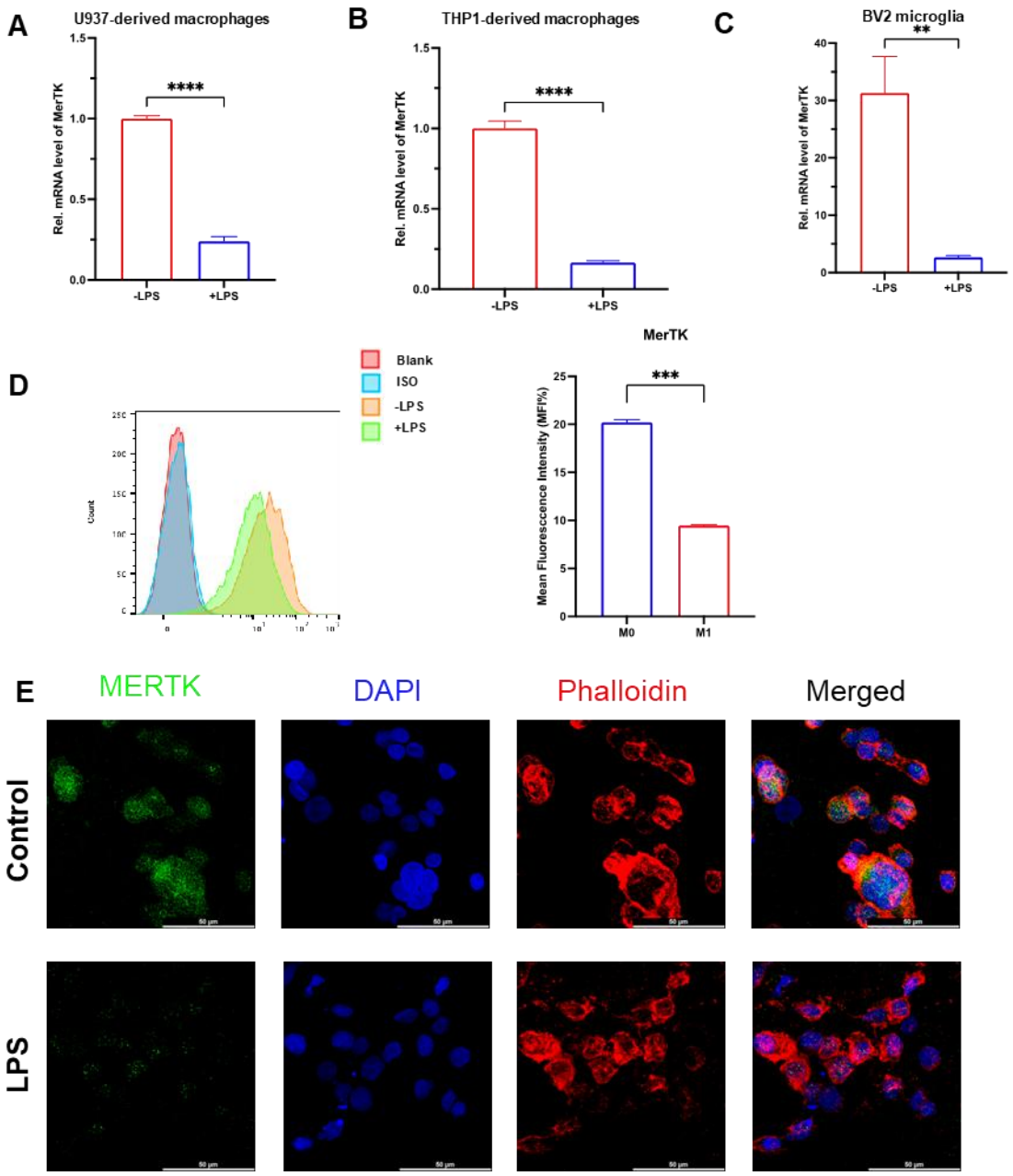


Fig.3.6 Validation of MerTK Downregulation in LPS-Treated Macrophages. (A–C) RT-qPCR analysis of MerTK expression in U937-derived macrophages (A), THP1-derived macrophages (B), and BV2 microglia (C) following LPS stimulation. LPS treatment significantly decreased MerTK mRNA expression in all tested macrophage models. (D) Flow cytometry analysis of MerTK protein levels in U937-derived macrophages. Left panel: Representative flow cytometry histograms for control and LPS-treated cells. Right panel: Quantifying mean fluorescence intensity (MFI) indicates a significant reduction in MerTK protein expression following LPS treatment. (E) Immunofluorescence staining for MerTK (green), F-actin (Phalloidin, red), and nuclei (DAPI, blue) in control (-LPS) and LPS-treated (+LPS) macrophages. Merged images illustrate diminished MerTK staining in LPS-treated cells, confirming its downregulation at the protein level. Scale bars = 50 μm. The data are presented as the mean ± standard error of the mean (SEM), and statistical significance is indicated (**p<0.01, ***p<0.001, ****p<0.0001). Data represent three independent biological replicates (n = 3).

To further validate the observed downregulation of MerTK from the RNA-seq analysis, RT-qPCR, flow cytometry, and immunofluorescence staining were conducted across various macrophage models. The RT-qPCR findings demonstrated a significant reduction in MerTK mRNA expression subsequent to LPS treatment in macrophages derived from U937 (Fig.3.6. A), THP1-derived macrophages (Fig.3.6. B), and BV2 microglia (Fig.3.6. C). This consistent downregulation across different macrophage cell types indicates that the suppression of MerTK is a prominent characteristic of the LPS-induced inflammatory response.

Flow cytometry analysis of MerTK protein levels (Fig.3.6. D) revealed a considerable decrease in mean fluorescence intensity (MFI) following LPS treatment, further confirming the protein level reduction. The histograms from the flow cytometry illustrate a notable shift in MerTK expression, with LPS-treated cells exhibiting a diminished fluorescence intensity compared to control samples.

Immunofluorescence staining (Fig.3.6. E) provided spatial confirmation of the MerTK downregulation. In control cells (-LPS), intense MerTK staining (green) was identified and localised across the macrophage membranes and cytoplasmic regions. However, following LPS stimulation (+LPS), MerTK staining was significantly attenuated, as evidenced by a reduction in green fluorescence. The merged images, which include staining for F-actin (red) and nuclei (DAPI, blue), illustrate that LPS-treated macrophages display diminished MerTK protein levels while maintaining their structural integrity.

These findings collectively substantiate the downregulation of MerTK at both the transcriptional and protein levels in macrophages following LPS treatment. The suppression of MerTK, a receptor tyrosine kinase integral to anti-inflammatory signalling and efferocytosis, signifies the polarisation of macrophages towards a pro-inflammatory state. This downregulation may contribute to the diminished capacity for immune resolution and tissue repair during the LPS-induced inflammatory response.

3.3.7 NF-κB Signalling Mediates LPS-Induced MerTK Reduction

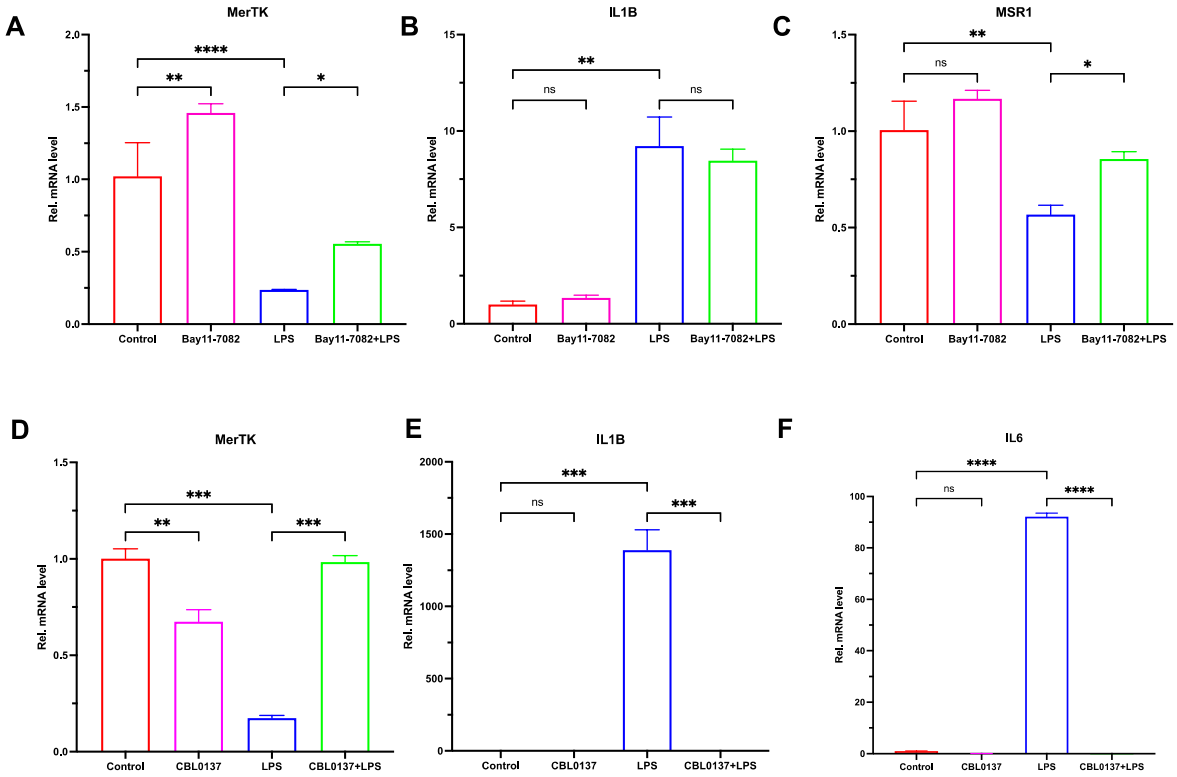


Fig.3.7 Investigating the Role of NF-κB Pathway in LPS-Mediated MerTK Downregulation in U937-derived macrophages. Relative mRNA levels of MerTK (A), IL1B (B), and MSR1 (C) in macrophages treated with the NF-κB inhibitor Bay11-7082 alone, LPS alone, or both. Bay11-7082 significantly restored MerTK and MSR1 expression while reducing the inflammatory response induced by LPS, as indicated by IL1B levels. Relative mRNA levels of MerTK (D), IL1B (E), and IL6 (F) in macrophages treated with the NF-κB inhibitor CBL0137 alone, LPS alone, or both. Similar to Bay11-7082, CBL0137 significantly restored MerTK expression and reduced the inflammatory cytokines IL1B and IL6 induced by LPS. Data are presented as mean ± SEM. Statistical significance: ns (not significant), *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Data represent three independent biological replicates (n = 3).

To determine whether the downregulation of MerTK induced by LPS is mediated through the NF-κB pathway, macrophages were treated with two NF-κB inhibitors, Bay11-7082 and CBL0137, alongside LPS stimulation. RT-qPCR analysis was conducted to evaluate the expression levels of MerTK, the pro-inflammatory cytokines IL1B and IL6, and the anti-inflammatory related gene MSR1.

Treatment with the NF-κB inhibitor Bay11-7082 markedly restored MerTK expression, which had been otherwise downregulated by LPS (Fig.3.7. A). Correspondingly, MSR1, which mediates M2 macrophage polarisation, partially

recovered after Bay11-7082 treatment (Fig.3.7. C). Conversely, Bay11-7082 significantly decreased the LPS-induced upregulation of IL-1B (Fig.3.7. B), effectively suppressing the inflammatory response driven by NF-κB.

A similar pattern was observed with the second NF-κB inhibitor, CBL0137. Administration of CBL0137 reinstated MerTK mRNA levels, which had been suppressed by LPS (Fig.3.7. D). Additionally, CBL0137 significantly downregulated the expression of the pro-inflammatory cytokines IL-1B (Fig.3.7. E) and IL-6 (Fig.3.7. F) induced by LPS stimulation. These findings strongly indicate that NF-κB activation is essential for the LPS-induced suppression of MerTK and the concomitant upregulation of inflammatory mediators.

The results demonstrate that the NF-κB pathway partially mediates the downregulation of MerTK induced by LPS. Inhibition of NF-κB restores MerTK expression and mitigates the inflammatory response, as evidenced by the reduced levels of IL-1B and IL-6. These findings yield mechanistic insights into the regulation of MerTK during LPS-induced macrophage polarisation and suggest that targeting NF-κB could facilitate the modulation of the balance between inflammation and immune resolution.

3.3.8 CEBPE as a Key Transcriptional Regulator of LPS-Induced MerTK Downregulation in CD33 Knockout U937 Cell Line-Derived Macrophages

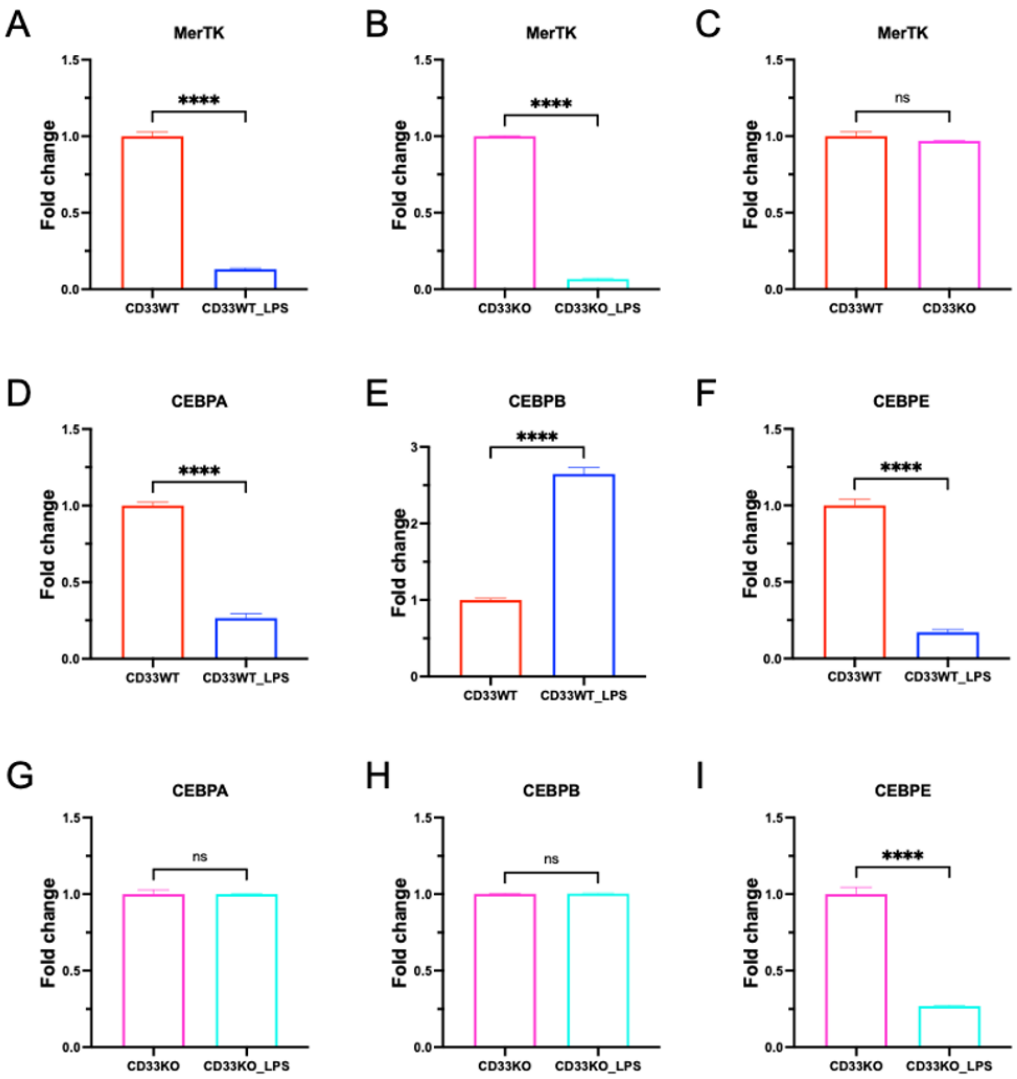


Fig.3.8 The Expression Levels of MerTK and CEBP Family Transcription Factors in CD33 Wild-type (CD33WT) and Knockout (CD33KO) derived Macrophages upon LPS Stimulation. (A-C) Fold change of MerTK expression in CD33WT and CD33KO macrophages with or without LPS treatment. MerTK was significantly downregulated in CD33WT macrophages upon LPS stimulation (A) and similarly reduced in CD33KO macrophages following LPS treatment (B). No significant difference was observed in MerTK expression between untreated CD33WT and CD33KO macrophages (C). (D-F) Expression of CEBPA, CEBPB, and CEBPE in CD33WT macrophages with or without LPS treatment. CEBPA and CEBPE were significantly downregulated following LPS stimulation (D, F), whereas CEBPB was significantly upregulated upon LPS treatment (E). (G-I) Expression of CEBPA, CEBPB, and CEBPE in CD33KO macrophages with or without LPS treatment. No significant difference was observed in CEBPA and CEBPB expression (G, H), while CEBPE was significantly downregulated upon LPS treatment in CD33KO macrophages (I). Data are presented as mean ± SEM, and statistical significance is indicated as **** $p < 0.0001$; ns = insignificant. Data represent three independent biological replicates (n = 3).

This study examined the expression of MerTK and CEBP family transcription factors in CD33 wild-type (CD33WT) and knockout (CD33KO) macrophage-derived macrophages upon LPS stimulation as demonstrated by the RNA-seq results. MerTK expression was significantly downregulated in CD33WT macrophages following LPS treatment (Fig.3.8.A). A comparable trend was noted in CD33KO macrophages, where LPS also induced a substantial decrease in MerTK expression (Fig.3.8.B). However, upon comparison of untreated CD33WT and CD33KO macrophages, no significant difference in MerTK expression was identified (Fig.3.8.C), thereby indicating that CD33 does not regulate MerTK under baseline conditions.

Concerning the C/EBP transcription factors, both CEBPA and CEBPE exhibited significant downregulation in CD33WT macrophages in response to LPS stimulation (Fig.3.8 D, F). Conversely, the expression of CEBPB demonstrated a significant upregulation post-LPS treatment (Fig.3.8 E), indicating a potential involvement in the inflammatory response.

In CD33 knockout macrophages, LPS treatment did not significantly affect the expression of CEBPA and CEBPB expression (Fig.3.8. G, H), indicating that the changes in CEBPB and CEBPA induced by LPS were effectively inhibited by CD33 knockout. However, CEBPE was significantly downregulated in CD33 knockout macrophages following LPS stimulation (Fig.3.8. I), mirroring the trend observed in CD33 wild-type macrophages.

3.3.9 E-Box Regulation of MerTK

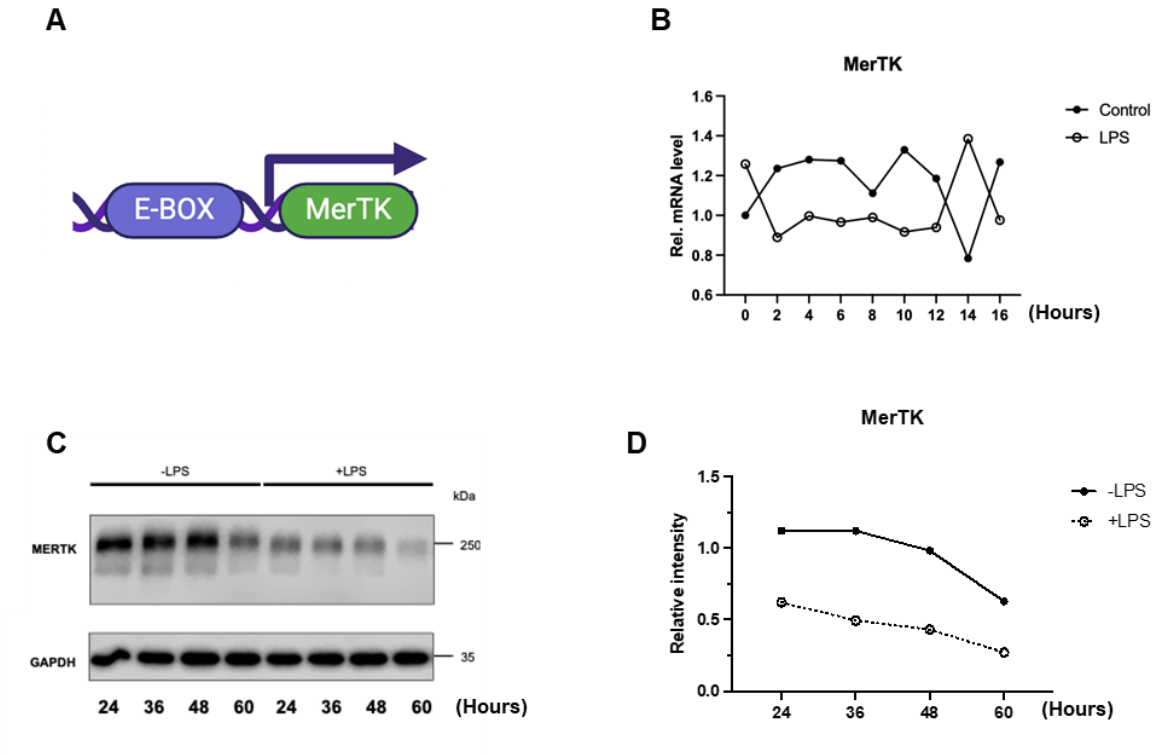


Fig.3.9 Investigating the Circadian Regulation of MerTK Expression in U937-derived macrophages. (A) In silico analysis of the MerTK promoter sequence identified an E-box (CACGTG) motif, a known binding site for circadian clock regulators, suggesting potential circadian control of MerTK expression. (B) MerTK mRNA expression levels in macrophages synchronised with 100 nM dexamethasone were measured at different time points using RT-qPCR. The results demonstrate oscillatory MerTK expression in control conditions (-LPS) but show a disrupted pattern in LPS-treated macrophages (+LPS). (C) Western blot analysis evaluated MerTK protein levels over time in macrophages following synchronisation with dexamethasone. LPS treatment led to a pronounced reduction in MerTK protein expression compared to controls. (D) Quantification of MerTK protein levels from the Western blot (panel C). Relative intensity measurements confirm that LPS disrupts the rhythmic expression of MerTK, resulting in progressively lower protein levels compared to control conditions. Data represent three independent biological replicates (n = 3).

An in-silico analysis of promoter sequences was conducted to investigate whether circadian rhythms influence the regulation of MerTK expression. The findings indicated the presence of an E-box motif (Fig.3.9. A), which serves as a canonical binding site for circadian clock regulators, namely BMAL1 and CLOCK. Consequently, it is plausible that circadian transcriptional control governs MerTK expression.

To experimentally evaluate this hypothesis, macrophages were synchronised using

100 nM dexamethasone and subsequently harvested at various time intervals for RT-qPCR analysis to assess MerTK mRNA expression. Under control conditions without LPS treatment, MerTK expression exhibited rhythmic oscillations that align with circadian regulation (Fig.3.9. B). In contrast, LPS treatment disrupted this rhythmic expression, resulting in MerTK mRNA levels that remained consistently suppressed compared to control macrophages.

Western blot analysis confirmed the expression of MerTK protein across different time points (Fig.3.9. C). Additionally, LPS treatment abolished this rhythmicity, leading to a gradual reduction in MerTK protein levels. The quantification of band intensities (Fig.3.9. D) substantiated a significant decrease in MerTK protein expression in LPS-treated cells relative to controls, particularly at the later time points.

These results demonstrate that MerTK expression is regulated in a circadian manner under normal conditions, likely mediated through the E-box motif present in its promoter. Conversely, LPS treatment disrupts this circadian rhythm, resulting in sustained suppression of MerTK at both the mRNA and protein levels. This disruption may contribute to the pro-inflammatory phenotype observed in LPS-stimulated macrophages, as MerTK is a critical mediator of anti-inflammatory and homeostatic processes.

3.3.10 Changes of Clock Genes and Catabolic Pathway-Related Genes upon LPS Treatment in U937-Derived Macrophages.

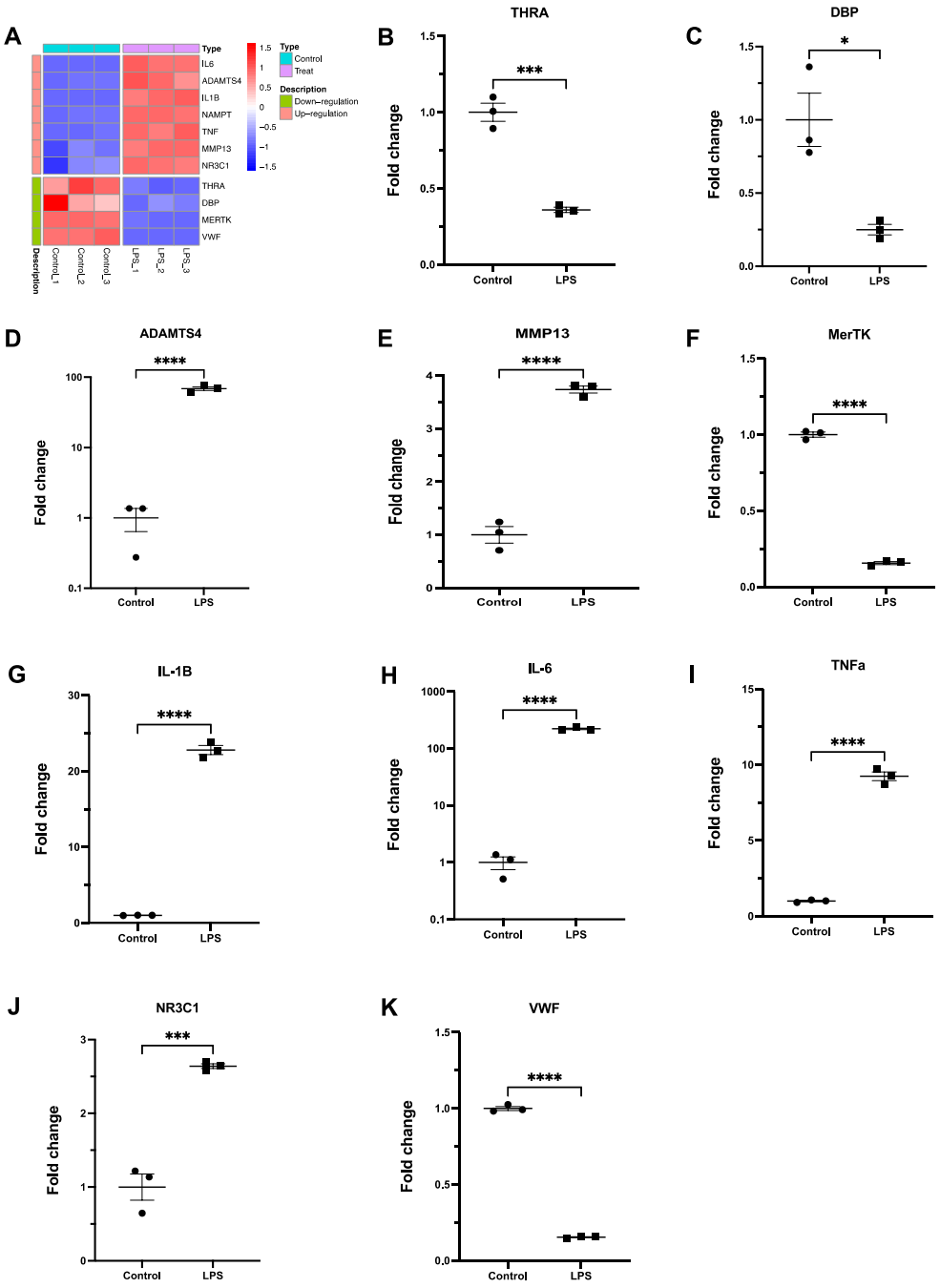


Fig.3.10 Changes of clock genes and catabolic pathway-related genes upon LPS treatment in U937-derived macrophages. (A) Heatmap displaying RNA-seq results of the clock and catabolic pathway-related genes in control and LPS-treated macrophages. Genes such as THRA, DBP, ADAMTS4, MMP13, and MerTK were downregulated following LPS treatment, whereas inflammatory markers such as IL1B, IL6, and TNFA were upregulated. THRA (B), DBP (C), ADAMTS4 (D), MMP13 (E), and MerTK (F) demonstrated significant downregulation after LPS treatment. Pro-inflammatory cytokines IL-1B (G), IL-6 (H), and TNFA (I) were significantly upregulated, confirming the inflammatory response. NR3C1 (J) and VWF (K) were also markedly suppressed upon LPS treatment. Data are presented as mean ± SEM, with statistical significance indicated (*p<0.05, ***p<0.001, ****p<0.0001). Data represent three independent biological replicates (n = 3).

LPS treatment triggered significant changes in gene expression, consistent with an activated inflammatory response and accompanying shifts in regulatory pathways. Circadian- and transcription-related genes, such as THRA (Fig.3.10. B) and DBP (Fig.3.10. C), were significantly downregulated, suggesting suppression of rhythmic and nuclear signalling pathways in response to immune activation.

Conversely, ADAMTS4 and MMP13, both involved in extracellular matrix degradation, were upregulated following LPS stimulation, indicating enhanced matrix remodelling activity that often accompanies inflammation and tissue injury. In line with this, classical pro-inflammatory cytokines IL1B (Fig.3.10. G), IL6 (Fig.3.10. H),, and TNFa (Fig.3.10. I), were markedly upregulated, confirming effective activation of innate immune signalling pathways.

Interestingly, NR3C1 (Fig.3.10. J), which encodes the glucocorticoid receptor and serves as a key regulator of inflammation and circadian rhythms, was also significantly upregulated, possibly reflecting a feedback mechanism aimed at tempering the inflammatory response. MerTK (Fig.3.10. F), a gene associated with efferocytosis and immune tolerance, was downregulated, suggesting impaired resolution of inflammation. VWF (Fig.3.10. K), involved in endothelial function and vascular homeostasis, was also reduced following LPS exposure.

These findings imply that LPS induces a pro-inflammatory response while simultaneously disrupting genes regulated by circadian mechanisms, particularly those featuring E-box motifs. It is plausible that the NF-κB pathway, which is activated by LPS, interferes with the machinery of circadian transcription, thereby suppressing E-box-mediated gene expression. This alteration in circadian regulation may contribute to the prolonged inflammatory state observed in macrophages treated with LPS, offering new insights into the intricate relationship between inflammation and circadian.

3.3.11 Dexamethasone's Ability to Restore MerTK Expression while Reducing Inflammatory Cytokine Production.

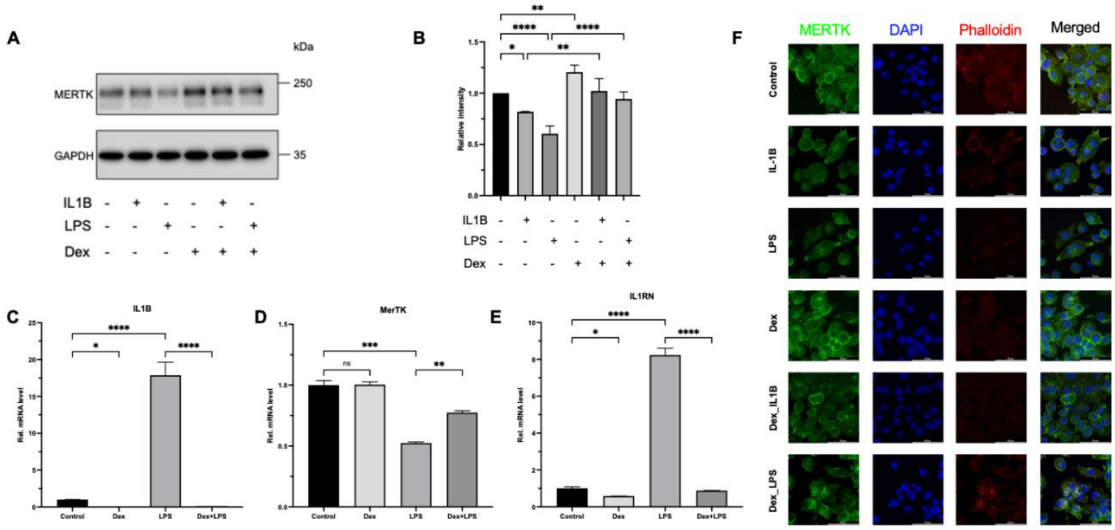


Fig.3.11 Effect of Dexamethasone on MerTK Expression During LPS/IL1B Stimulation in U937-derived macrophages. (A) Western blot analysis of MerTK protein levels across various treatment conditions: control, IL-1β, LPS, Dexamethasone (Dex), and their combinations. GAPDH acted as a loading control. The blot shown is representative and was selected for clarity and consistent loading. (B) Quantification of MerTK relative intensity based on Western blot analysis. LPS treatment significantly reduced MerTK protein levels, while dexamethasone treatment partially restored MerTK expression. (C–D) RT-qPCR analysis of the pro-inflammatory cytokine IL-1B (C), the anti-inflammatory marker MerTK (D), and IL1RN levels showed a decrease in MerTK and an increase in IL-1RN. Dexamethasone treatment mitigated these effects. (E) The level of mRNA was assessed across different treatment groups. The administration of LPS led to a significant increase in IL-1β levels, a decrease in MerTK, and an upregulation of IL-1RN. Treatment with dexamethasone effectively mitigated these effects. (F) Immunofluorescence staining for MerTK (green), F-actin (Phalloidin, red), and nuclei (DAPI, blue) was conducted under control and various treatment conditions. The merged images reveal reduced expression of MerTK in macrophages treated with LPS, which was partially restored after dexamethasone treatment. Scale bars = 50 μm. The data are presented as the mean ± SEM, and statistical significance is indicated (ns: not significant, *p<0.05, ****p<0.01, ***p<0.001, ****p<0.0001). Data represent three independent biological replicates (n = 3).

To determine whether dexamethasone (Dex) possesses the ability to counteract the downregulation of MerTK induced by lipopolysaccharide (LPS), macrophages were subjected to treatments with LPS, IL1B, and Dex, either separately or in combination. Subsequently, an assessment of MerTK expression was conducted at both the protein and mRNA levels.

Western blot analysis (Fig.3.11. A) exhibited a significant reduction in MerTK protein levels following exposure to LPS, which aligns with previously documented findings. The administration of dexamethasone led to a partial restoration of MerTK protein levels despite the persistent presence of LPS. Quantitative analysis of Western blot band intensities supported this observation, revealing a statistically significant recovery of MerTK expression in the Dex+LPS group compared to the LPS alone group (Fig.3.11. B).

RT-qPCR analysis further confirmed these findings. LPS treatment significantly increased the levels of the pro-inflammatory cytokine IL1B (Fig.3.11. C) and upregulated the production of the anti-inflammatory cytokine IL1RN (Fig.3.11. E). Conversely, LPS treatment markedly decreased MerTK mRNA expression (Fig.3.11. D). The application of dexamethasone mitigated these inflammatory responses, reinstating MerTK mRNA levels while concurrently reducing IL1B expression, thereby indicating the potential regulatory role of glucocorticoids in alleviating LPS-mediated inflammation.

Immunofluorescence staining provided additional spatial confirmation of these results (Fig.3.11. F). LPS treatment resulted in a significant reduction in the localisation of MerTK protein (green) within macrophages; however, treatment with dexamethasone restored MerTK expression to levels comparable to those observed under control conditions. Merged images further illustrated that the localisation of MerTK coincided with cellular structures (F-actin, red) and nuclei (DAPI, blue), emphasising the preservation of macrophage morphology across the various treatment conditions.

These results demonstrate that dexamethasone effectively mitigates the LPS-induced suppression of MerTK at both mRNA and protein levels. Dexamethasone's ability to restore MerTK expression while diminishing the production of inflammatory cytokines suggests a pivotal role in regulating inflammation and the modulation of macrophage activation. This finding highlights the therapeutic potential of glucocorticoids in influencing MerTK expression during inflammatory responses induced by LPS.

3.3.12 REV-ERB Agonist SR9009 Counteracts LPS-Induced Suppression of MerTk Expression at Transcriptional and Translational Level

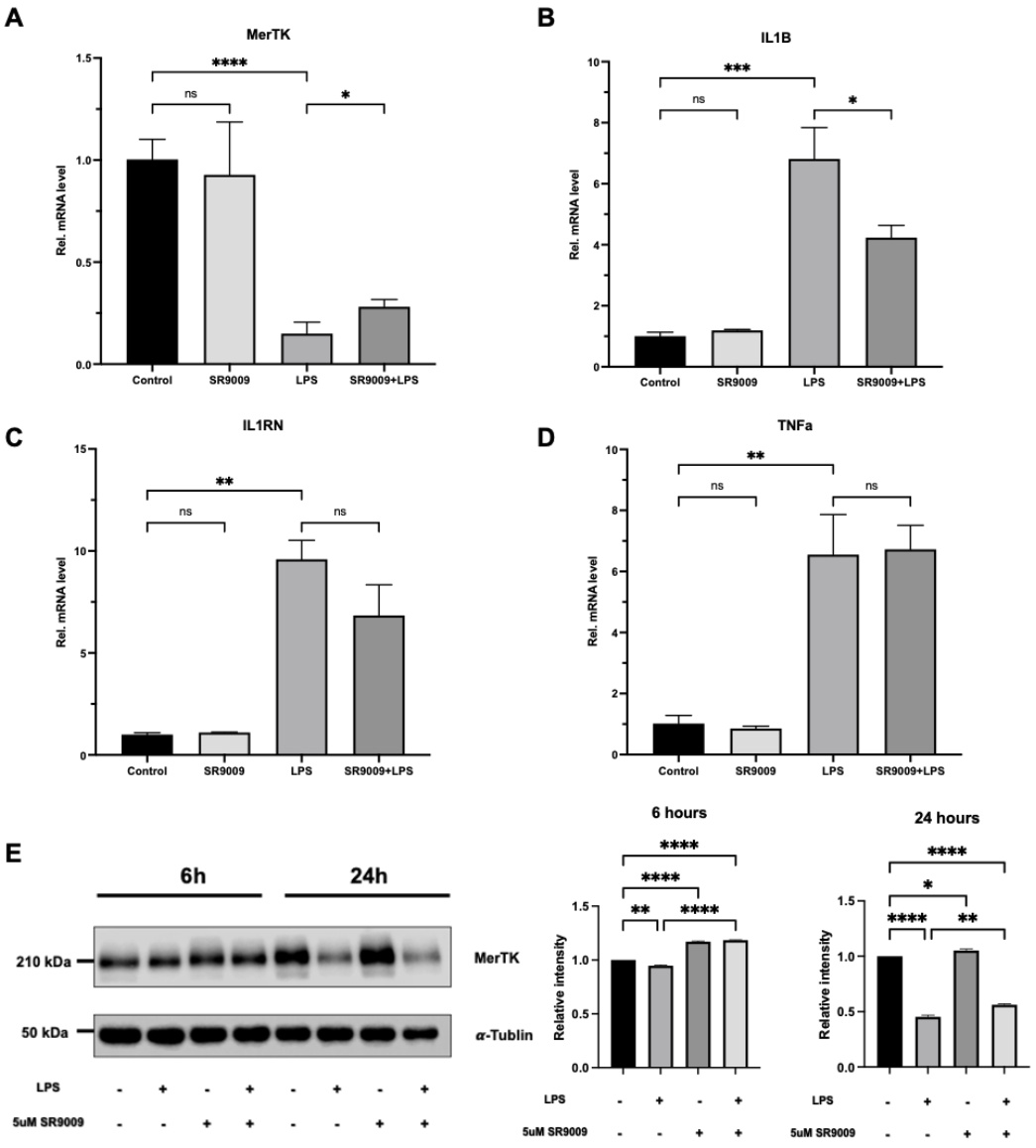


Fig.3.12 SR9009 regulates MerTK expression at both the transcriptional and translational levels, counteracting the inflammatory effects of LPS in U937-derived macrophages. (A–D) RT-qPCR analysis of mRNA expression levels for MerTK (A), IL1B (B), IL1RN (C), and TNFα (D) under various treatment conditions (Control, LPS, SR9009, and LPS + SR9009) after 6 hours. LPS significantly decreased the expression of MERTK while elevating the expression of pro-inflammatory cytokines (IL1B and TNFα) and the anti-inflammatory cytokine IL1RN. Treatment with SR9009 restored MerTK expression, lowered IL1B and TNFα levels, and further boosted IL1RN expression. Data are normalised to a housekeeping gene (e.g., GAPDH) and presented as mean ± SEM from independent experiments. (E) Western blot analysis of MerTK protein levels under the same experimental conditions, analysed at 6 hours and 24 hours. α-tubulin served as a loading control.The blot shown is representative and was selected for clarity and consistent loading. The data are presented as the mean ± SEM, and statistical significance is indicated (ns: not significant, *p<0.05, ****p<0.01, ***p<0.001, ****p<0.0001). Data represent three independent biological replicates (n = 3).

To evaluate the effects of LPS and SR9009 on MerTK expression and inflammatory signalling, RT-qPCR analysis conducted six hours post-treatment indicated that LPS significantly suppressed MerTK mRNA expression, consistent with its inhibitory effect on macrophage regulation. Additionally, LPS markedly elevated the pro-inflammatory cytokines IL1B and TNFα expression alongside the anti-inflammatory cytokine IL1RN (Fig. 3.12 A-D). Treatment with SR9009 effectively mitigated these alterations by restoring MerTK expression, decreasing the elevated levels of IL1B, and exerting a moderate effect on the expression of TNFα and IL-1RN.

Western blot analysis provided further insights into MerTK protein dynamics at 6 and 24 hours (Fig. 3.12 E). LPS treatment resulted in a pronounced reduction in MerTK protein expression, particularly at the 24-hour mark, suggesting a sustained suppressive effect. Quantification of band intensities confirmed this observation, showing a clear downregulation of MerTK following LPS exposure. Importantly, treatment with SR9009 led to a partial restoration of MerTK protein levels. While the increase in MerTK expression was not fully sufficient to reverse the LPS-induced suppression, especially at the 24-hour time point, a modest recovery was observed in both time frames. This suggests that SR9009 exerts a regulatory effect on MerTK protein expression, although its efficacy may depend on factors such as concentration and LPS-induced inflammatory burden.

These findings elucidate SR9009 demonstrates therapeutic potential by restoring MerTK expression and promoting a shift in the cytokine profile toward a more anti-inflammatory state. These results underscore the promise of SR9009 as a therapeutic agent capable of modulating inflammatory signalling and promoting immune resolution.

3.3.13 IL-4/IL-13 increase the M2 marker CD206

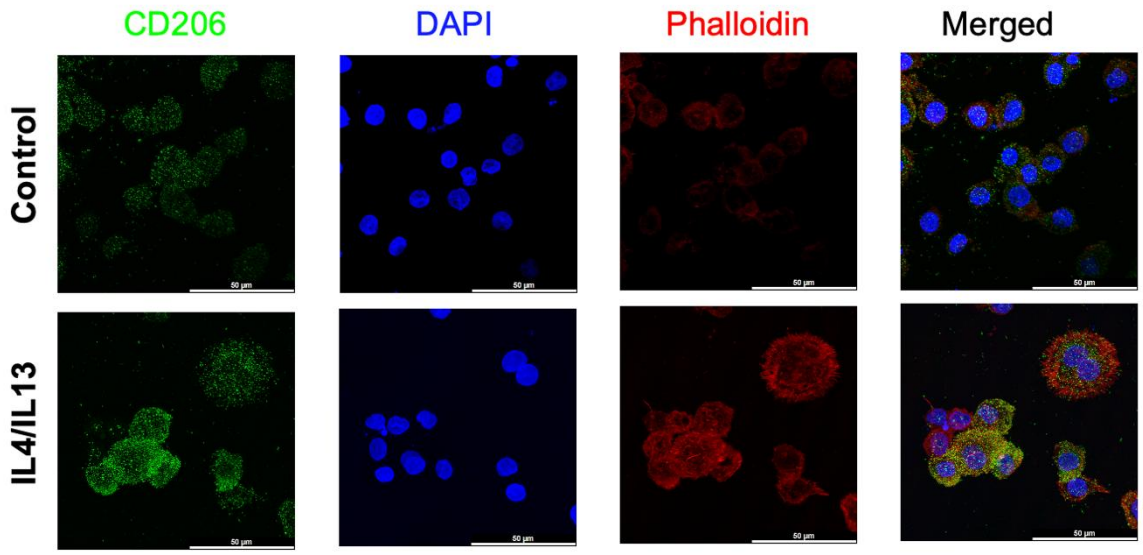


Fig.3.13 IL-4/IL-13 upregulates the M2 macrophage marker CD206 expression in U937-derived macrophages. Immunofluorescence staining for CD206 (green), F-actin (Phalloidin, red), and nuclei (DAPI, blue) was conducted under control and various treatment conditions. The merged images reveal an increased expression of CD206 in macrophages treated with IL-4 and IL-13. Scale bars = 50 µm. The experiment was performed in three independent biological replicates (n = 3). The image shown is representative and was selected based on clarity and consistent staining.

This experiment demonstrated that treatment with IL-4 and IL-13 enhances the expression of the M2 macrophage marker CD206 (Fig. 3.13). In the control group, baseline CD206 expression is observed, with minimal overlap between CD206 (green), nuclei (DAPI, blue), and the cytoskeleton (Phalloidin, red). In contrast, the IL-4/IL-13-treated group shows a marked increase in CD206 expression, as evidenced by stronger green fluorescence and co-localisation with the cytoskeleton in the merged image. These results indicate that IL-4 and IL-13 effectively promote M2 macrophage polarisation through upregulation of CD206.

3.3.14 IL-4/IL-13 Enhance MerTK Expression and Counteract LPS-Induced Suppression in U937-derived macrophages.

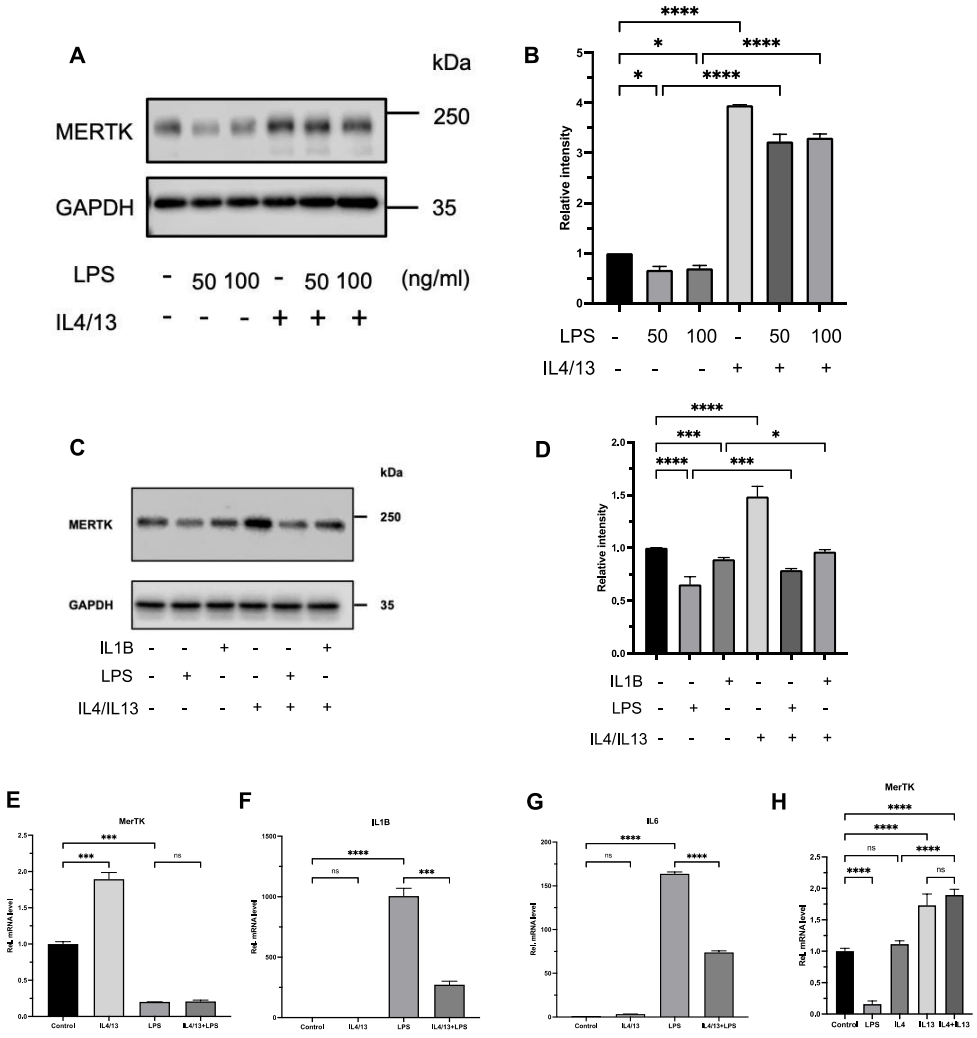


Fig.3.14 IL-4/IL-13 Enhance MerTK Expression and Counteract LPS-Induced Suppression in U937-derived macrophages. (A) Western blot analysis of MerTK protein levels in macrophages treated with LPS (50 or 100 ng/ml), with or without IL-4/IL-13 stimulation. GAPDH served as a loading control. (B) The quantification of Western blot band intensities confirms that IL-4/IL-13 significantly increases MerTK protein levels, even in the presence of LPS, compared to LPS treatment alone. (C) Western blot analysis of MerTK protein expression under IL-1B, LPS, and IL-4/IL-13 treatment conditions. GAPDH was used as a loading control. (D) Quantifying relative MerTK protein intensities from panel C demonstrates that IL-4/IL1-3 restore MerTK levels despite IL1B and LPS stimulation. (E–H) RT-qPCR analysis of MerTK (E), IL1B (F), and IL6 (G) mRNA expression levels across control, IL-4/IL-13, LPS, and combined treatment conditions. IL-4/IL-13 significantly increase MerTK mRNA levels and partially restore them under LPS treatment (E). LPS upregulates the pro-inflammatory cytokines IL-1B (F) and IL-6 (G), while IL-4/IL-13 attenuate these effects. H further confirms the upregulation of MerTK at the transcriptional level following IL-4/IL-13 treatment. All experiment was performed in three independent biological replicates (n = 3). The blot image shown is representative and was selected based on clarity and consistent staining. The data are presented as the mean ± SEM, and statistical significance is indicated (ns : not significant, *p<0.05, ****p<0.01, ***p<0.001, ****p<0.0001). Data represent three independent biological replicates (n = 3).

This study aims to examine whether interleukins IL-4 and IL-13 can restore the expression of MerTK and mitigate the suppression induced by LPS. For this purpose, macrophages were treated with LPS alone or in conjunction with IL-4 and IL-13.

Western blot analysis (Fig.3.14. A) indicated that treatment with LPS (50–100 ng/ml) substantially reduced MerTK protein levels. In contrast, co-treatment with IL-4 and IL-13 effectively restored MerTK expression to levels comparable to those of untreated controls. Further quantifying band intensities (Fig.3.14. B) confirmed a significant increase in MerTK protein levels in the presence of IL-4 and IL-13, even amidst LPS stimulation.

Further evaluation (Fig.3.14. C) explored whether IL-4 and IL-13 could counteract the effects of IL-1B, another pro-inflammatory cytokine. The western blot results demonstrated that IL-4 and IL-13 restored the MerTK protein expression that both LPS and IL1B suppressed. The quantification of relative band intensities (Fig.3.14. D) illustrated that IL-4 and IL-13 significantly elevated MerTK levels under inflammatory conditions, thereby affirming their role in reversing the downregulation of MerTK.

RT-qPCR analysis further validated these findings at the transcriptional level, as IL-4 and IL-13 treatment significantly increased MerTK mRNA expression (Fig.3.14. E), even in the presence of LPS, confirming their regulatory effect. In contrast, LPS treatment markedly elevated the mRNA levels of pro-inflammatory cytokines, including IL-1B (Fig.3.14. F) and I-L6 (Fig.3.14. G). Notably, co-treatment with IL-4 and IL-13 reduced IL-1B and IL-6 expression, which further substantiated their anti-inflammatory role. Panel H corroborated the overall increase in MerTK mRNA expression following treatment with IL4 and IL13, underscoring its consistent upregulation under these specified conditions (Fig.3.14. H).

These results demonstrate that IL-4 and IL-13 are critical in restoring MerTK expression under inflammatory conditions induced by LPS and IL-1B. Given the established role of MerTK in tissue repair and immune resolution, these findings advocate for the potential therapeutic application of IL-4 and IL-13 in promoting MerTK-dependent pathways that facilitate inflammation resolution and tissue repair. The capability of IL-4 and IL-13 to enhance MerTK expression underscores their therapeutic relevance, particularly in scenarios such as cardiac repair, where MerTK has been implicated in fostering recovery and resolving inflammation.

3.4 Discussion

In this study, RNA-seq analysis was employed to elucidate the transcriptional alterations in macrophages derived from U937 in response to LPS stimulation, a well-established model for pro-inflammatory activation (Lebaudy *et al.*, 2020; Orecchioni *et al.*, 2019). LPS demonstrates dual functionality by promoting pro-inflammatory cytokines to stimulate immune activation and anti-inflammatory mediators to modulate inflammation (Andersson *et al.*, 1992; Marie *et al.*, 1996). This balance ensures an effective defence against pathogens while mitigating excessive immune responses that could result in tissue damage. Our findings also indicate a significant reprogramming of the macrophage transcriptome, which includes the upregulation of pro-inflammatory mediators (IL-1B, TNF, IL-6, IL-8) and the concurrent activation of anti-inflammatory factors such as IL-10 and IL-1RN. This dual response highlights macrophages' intricate and dynamic role in balancing pro-inflammatory activation for effective pathogen defence with immune regulation to avert excessive responses that may lead to tissue damage. However, alongside these canonical inflammatory pathways, we identified a notable suppression of genes associated with anti-inflammatory processes, tissue repair, and circadian regulation, particularly those regulated through E-box motifs, including MerTK, THRA, and DBP. These results offer new insights into the complex molecular mechanisms underlying LPS-induced macrophage polarisation and suggest novel regulatory networks mediated through the interplay of NF-κB and circadian transcription factors.

3.4.1 LPS Drives a Coordinated Pro-Inflammatory and Anti-Inflammatory Response

The significant upregulation of TNF, IL-1B, and IL-6 following LPS treatment aligns with their well-established roles as key players in the pro-inflammatory M1 macrophage phenotype. These cytokines function as pivotal regulators of immune activation, thereby facilitating pathogen clearance and the recruitment of immune cells (Jang *et al.*, 2006). Notably, the upregulation of IL-10 and IL-1RN in this context indicates the presence of an intrinsic feedback mechanism intended to mitigate excessive inflammation. Previous studies also proved that LPS regulates pro- and anti-inflammatory cytokines (Ferreira *et al.*, 2021). This dichotomous response highlights the importance of macrophage plasticity, allowing macrophages to modify their functional outputs in accordance with the inflammatory environment.

Interestingly, these findings resonate with previous studies showing that IL10 upregulation is essential for countering uncontrolled NF-κB activity during inflammation (Marie *et al.*, 1996; Ouyang and O'Garra, 2019). IL10, a potent anti-inflammatory cytokine, partially exerts its function by inhibiting the NF-κB signalling pathway, thereby offering negative feedback to terminate inflammatory responses (Iyer and Cheng, 2012; Saraiva and O'Garra, 2010). However, despite this regulatory feedback, MerTK, a crucial gene implicated in tissue repair and regeneration, was markedly suppressed under LPS stimulation. This observation implies that macrophages tend to prioritise inflammatory signalling over tissue repair during acute immune response activation.

The imbalance between pro-inflammatory activation and tissue-repair mechanisms may significantly affect chronic inflammation and protracted tissue repair. Continued NF-κB activation in macrophages has been associated with suboptimal outcomes in tissue repair, as sustained inflammation hinders the transition to the reparative phase (Eming, Wynn and Martin, 2017; Guo *et al.*, 2024). The results of our investigation indicate that the inhibition of MerTK may play a role in this impairment, as its absence has been demonstrated to impede the clearance of apoptotic cells, which is an essential process for the resolution of inflammation and the facilitation of tissue repair (Bosurgi *et al.*, 2017; Nishi *et al.*, 2019).

3.4.2 Role of NF-κB in LPS-Induced Suppression of MerTK and Pro-Inflammatory Macrophage Polarization

The findings of this study present compelling evidence that the NF-κB pathway plays a central role in mediating the downregulation of MerTK induced by LPS, alongside the associated upregulation of pro-inflammatory cytokines. Bay117082 and CBL00137 have been used as inhibitors of NF-KB (Burkhart *et al.*, 2014; Lee *et al.*, 2012a). Treatment with the NF-κB inhibitors Bay11-7082 and CBL0137 effectively reversed the suppression of MerTK expression, highlighting the crucial role of NF-κB in this regulatory mechanism. The restoration of MerTK through both inhibitors highlights their potential to reinstate essential anti-inflammatory pathways necessary for the resolution of macrophage-mediated immune responses. Notably, the partial recovery of MSR1 expression with Bay11-7082 further supports the idea that NF-κB inhibition can re-establish broader reparative functions disrupted by LPS stimulation.

In addition to restoring MerTK expression, both inhibitors markedly decreased the

pro-inflammatory cytokines IL-1B and IL-6 expression. The differing effects observed between Bay11-7082 and CBL0137, with CBL0137 more markedly reducing both IL-1B and IL-6 expression, suggest that these compounds may modulate distinct components of the NF-κB signalling pathway or exert differential off-target effects. However, further investigation is required to determine whether these differences reflect different mechanisms of action or variations in potency. The results highlight the dual role of NF-κB in simultaneously promoting inflammatory mediator expression and partially suppressing reparative pathways such as MerTK signalling. This interplay provides a mechanistic explanation for the sustained inflammatory state observed during LPS-induced macrophage polarisation. By suppressing NF-κB activation, these inhibitors mitigate the inflammatory response and restore pathways essential for immune resolution, thereby rebalancing the inflammatory and anti-inflammatory functions of macrophages.

The ability of NF-κB inhibitors to restore MerTK expression and reduce levels of inflammatory cytokines suggests that targeting this pathway could be a viable strategy for modulating macrophage polarisation in conditions characterised by excessive inflammation. This is particularly relevant for chronic inflammatory diseases, where the dysregulation of macrophage function contributes to tissue damage and delayed resolution. Future studies should explore the potential of combining NF-κB inhibitors with other modulators, such as IL-4 or IL-13, to further enhance the resolution of inflammation and promote tissue health repair.

3.4.3 The Role of CEBPE in LPS-Induced MerTK Downregulation and the Influence of CD33 on CEBP Transcriptional Regulation

MerTK expression is transcriptionally regulated, with a CEBP binding response element in its promoter region that plays a crucial role in its transcription, as confirmed by our research collaborators (Walsh *et al.*, 2021). Given the importance of MerTK in macrophage function, understanding the upstream regulators of its expression is essential for elucidating the mechanisms underlying macrophage polarisation.

In our CD33 knockout U937-derived macrophage model, the loss of CD33 did not affect the LPS-induced reduction in MerTK expression, indicating that CD33 is not directly involved in this regulatory process. Instead, the data suggest that the transcriptional suppression of MerTK upon LPS stimulation follows a distinct regulatory pathway.

LPS treatment resulted in an increase in CEBPB and a reduction in both CEBPA and CEBPE gene expression in CD33WT macrophages; however, LPS caused CEBPB and CEBPA changes were blocked by CD33KO, but CEBPE gene expression was reduced drastically as in CD33WT macrophages. Therefore, LPS-mediated MerTK gene expression reduction was only paralleled with LPS-caused CEBPE gene expression reduction, implying that CEBPE is the key possible early transcription factor. The direct association between CEBPE downregulation and MerTK suppression highlights a potential mechanism by which LPS-induced inflammatory signalling disrupts macrophage homeostasis. Further studies are needed to confirm whether CEBPE directly binds to the MerTK promoter to regulate its transcription and to explore the broader implications of this regulatory axis in inflammatory responses and immune modulation.

3.4.4 LPS Disrupts E-Box-Driven Circadian Gene Expression

A significant observation in our study is the downregulation of an E-box-containing gene, MerTK, following stimulation with LPS, consistent with prior evidence showing that acute inflammation can suppress efferocytosis and anti-inflammatory signalling. Importantly, MerTK's expression pattern parallels that of classical circadian regulators such as THRA and DBP, both of which were also downregulated in response to LPS. While MerTK has not traditionally been classified as a core clock gene, recent studies have hinted at rhythmic patterns of its expression in macrophages, particularly in tissues with high phagocytic activity (Casanova-Acebes *et al.*, 2013; Parinot *et al.*, 2024). Our findings provide further support for this emerging view and point to the possibility that MerTK is regulated, directly or indirectly, by the molecular circadian machinery.

Considering the essential role of MerTK in efferocytosis and tissue repair, it is particularly compelling to investigate the mechanisms through which LPS suppresses MerTK expression. MerTK plays a crucial role in the clearance of apoptotic cells, thus facilitating the resolution of inflammation and promoting tissue repair (DeBerge *et al.*, 2017b; Zizzo *et al.*, 2012). Under normal conditions, the rhythmic expression of MerTK indicates that the tissue repair processes regulated by circadian rhythms correspond with physiological demands (Casanova-Acebes *et al.*, 2013; Parinot *et al.*, 2024). However, LPS-induced NF-κB activation disrupts this rhythmicity, resulting in prolonged suppression of MerTK (Guo *et al.*, 2015). This disruption may impair the transition of macrophages into a reparative state, consequently delaying tissue repair and regeneration.

E-box motifs constitute canonical DNA sequences bound by the circadian transcription factors BMAL1 and CLOCK, which regulate the transcription of core circadian genes and their downstream effectors. Recent research has elucidated a direct interaction between NF-κB and the circadian machinery, wherein the activation of NF-κB results in the suppression of BMAL1/CLOCK functionality, thereby disrupting pathways regulated by circadian rhythms (Guo *et al.*, 2015; Shen *et al.*, 2020). The robust upregulation of pro-inflammatory cytokines such as IL1B, IL6, and TNFA was expected and serves to validate the inflammatory state induced by LPS. The simultaneous induction of ADAMTS4 and MMP13 indicates that matrix remodelling processes are also engaged, which often occurs during tissue injury and immune infiltration. Interestingly, while most inflammatory genes were induced, VWF—typically linked to endothelial activation—was suppressed, possibly reflecting endothelial dysfunction or a regulatory attempt to limit thrombosis.

The upregulation of NR3C1 following LPS exposure adds an additional layer of complexity. As the glucocorticoid receptor, NR3C1 is both a mediator and a target of circadian rhythms (Timmermans, Souffriau and Libert, 2019). Its induction could reflect a compensatory response aimed at dampening inflammation, although it may also influence MerTK expression in a context-dependent manner. Notably, the temporal relationship between glucocorticoid signalling and MerTK expression has yet to be fully elucidated, and this may represent a regulatory axis worth exploring further.

Overall, the downregulation of E-box-containing genes highlights the implications of NF-κB activation in disrupting circadian transcriptional networks, which may lead to prolonged inflammatory states and impaired tissue regeneration. MerTK is not only a key effector of immune resolution but also potentially synchronised with circadian rhythms. Understanding how MerTK fits into the larger circadian-immune axis could open the door to novel therapeutic strategies aimed at restoring temporal balance in chronic inflammatory diseases.

3.4.5 Dexamethasone Partly Restores MerTK Expression and Modulates the Inflammatory Response in LPS-Stimulated Macrophages

The results demonstrate that Dex effectively partly restores MerTK expression and mitigates the inflammatory response in macrophages subjected to LPS stimulation. This restoration of MerTK suggests that Dex may counteract the inhibitory effects of LPS on anti-inflammatory signalling pathways, thereby fostering a phenotypic shift

in macrophages toward pro-resolving functions. However, while Dex enhances the protein levels of MerTK, it does not appear to directly upregulate MerTK mRNA expression. Furthermore, it has been studied that an extensive analysis of the 5-kb putative promoter region of MerTK failed to identify any DNA sequences directly responsive to Dex treatment (Walsh *et al.*, 2021). These observations suggest that Dex may exert its regulatory effects primarily through post-transcriptional mechanisms, potentially stabilising MerTK protein rather than directly modulating MerTK gene transcription. However, although Dex exhibited partial antagonism of LPS-mediated suppression of MerTK, its inability to fully restore MerTK expression suggests a mechanistic limitation in its modulation of NF-κB signalling. While Dex is known to inhibit NF-κB activation, evidence indicates that it does not prevent NF-κB nuclear translocation (Castro-Caldas *et al.*, 2003; Machuca *et al.*, 2006). Consequently, NF-κB remains transcriptionally active within the nucleus, where it can interfere with E-box-mediated transcription by obstructing the recruitment of essential transactivators, such as those required for BMAL1 binding (Shen *et al.*, 2021a). This residual NF-κB activity may therefore sustain transcriptional repression of MerTK, even in the presence of glucocorticoid treatment, highlighting a potential disconnect between upstream inhibition and downstream transcriptional outcomes in inflammatory contexts.

Additionally, LPS significantly increases the expression of the cytokine IL-1B, a hallmark of pro-inflammatory macrophage activation. Dex treatment reduces IL-1B expression, demonstrating its efficacy in suppressing the inflammatory cascade triggered by LPS. Interestingly, while LPS also upregulates the anti-inflammatory cytokine IL-1RN, Dex exerts a more moderate effect on its expression. This nuanced modulation implies that Dex does not suppress the entire cytokine network but rather rebalances the inflammatory microenvironment, fostering a controlled resolution of inflammation.

These findings underscore the dual action of Dex in both restoring MerTK expression and selectively modulating the inflammatory cytokine profile. By enabling a balance between inflammation and repair, Dex addresses the key macrophage polarisation dysfunction often observed in inflammatory conditions. This ability to fine-tune macrophage responses, rather than indiscriminately dampening immune activity, highlights Dex's therapeutic potential in inflammatory diseases characterised by macrophage dysregulation.

3.4.6 SR9009 Restores MerTK Expression in LPS-Induced Inflammatory Macrophages

This study highlights the role of SR9009 in regulating MerTK expression during LPS-induced macrophage activation. LPS exposure significantly suppressed MerTK protein levels while concurrently inducing pro-inflammatory cytokines such as IL-1β and IL-6 (Fig. 3.12). This pattern supports the interpretation that LPS promotes a pro-inflammatory macrophage phenotype by downregulating reparative pathways, such as MerTK, while sustaining inflammatory signalling.

SR9009, REV-ERB Agonist, has demonstrated significant anti-inflammatory properties in various studies (Cui *et al.*, 2021; Dierickx *et al.*, 2019; Hong *et al.*, 2021; Wagner, Monjes and Guido, 2019). In the current study, SR9009 treatment did not fully reverse the suppression of MerTK, but a partial restoration was observed, suggesting therapeutic potential. This limited recovery may reflect the concentration of SR9009 used, which might not have been sufficient to counter the potent inhibitory effects of LPS, particularly at the 24-hour time point. Alternatively, the strong inflammatory environment generated by LPS could inherently restrict the re-expression of MerTK.

Overall, the findings suggest that although LPS effectively suppresses MerTK expression, SR9009 may partially attenuate this effect, highlighting its potential immunomodulatory role. It is possible that the restoration of MerTK expression is mediated by the anti-inflammatory actions of SR9009. Further investigation, particularly with optimized dosing and time-course studies, is warranted to better define the capacity of SR9009 to restore MerTK expression and support the resolution of inflammation.

3.4.7 IL-4/IL-13 Promote MerTK-Mediated Tissue Repair

To address the suppression of MerTK, we investigated the effects of IL4/IL13, cytokines known to polarise macrophages toward the M2-like reparative phenotype (Scott *et al.*, 2023). Previous studies have shown that MerTK-mediated efferocytosis not only aids in removing apoptotic debris but also triggers downstream anti-inflammatory signalling pathways (DeBerge *et al.*, 2017a; Shirakawa *et al.*, 2020). Extensive reviews have highlighted the role of IL-4 and IL-13 in tissue repair, particularly in cardiac repair (Allen, 2023; Bakhshian Nik, Alvarez-Argote and O'Meara, 2022) However, the underlying mechanisms remain inadequately defined. Our findings further confirm that IL4/IL13 mitigate LPS-induced suppression of

MerTK and reduces inflammatory cytokine production (IL1B and IL6), highlighting their dual role in suppressing inflammation and activating tissue repair programs.

Additionally, it is noted that growth arrest-specific protein 6 (GAS6) is the ligand for MerTK, and its role in upregulating CD206 has been well established. For example, in THP-1-induced M1 macrophages treated with Gas6, there is a significant increase in the protein levels of the M2 marker CD206 and elevated mRNA expression of M2-associated genes CD206 and IL10 (Nam *et al.*, 2018). Similarly, IL-4/IL-13 have been shown to enhance CD206 expression, thereby driving the polarisation of macrophages toward the M2 phenotype (Scott *et al.*, 2023). Notably, IL-4 and IL-13 also upregulate MerTK expression, suggesting that GAS6 and IL-4/IL-13 promote CD206 expression, at least in part, through their ability to increase MerTK levels.

Nonetheless, the therapeutic efficacy of IL4/IL13 may depend on the timing and context of their administration. Inflammatory macrophages activated by lipopolysaccharide (LPS) may show resistance to the reprogramming effects of IL4 and IL13 due to sustained NF-κB activity. Therefore, strategies aimed at targeting NF-κB, whether through pharmacological inhibitors or glucocorticoids like dexamethasone, may be crucial in sensitising macrophages to IL4 and IL13 signalling and in restoring MerTK-dependent repair pathways.

3.5 Conclusion

This study reveals a complex interplay between LPS-induced inflammation, circadian regulation, and tissue-repair pathways in macrophages, which provides novel insights into the complex regulatory network that governs macrophage activation during inflammation. LPS drives a robust inflammatory response while suppressing MerTK-dependent tissue-repair programs and circadian E-box-driven genes. This suppression, mediated by NF-κB, highlights the critical role of inflammation in disrupting macrophage homeostasis and delaying tissue repair. Identifying NF-κB as a central node linking inflammatory signalling to circadian disruption and tissue repair suppression offers new therapeutic targets. Importantly, we demonstrate that IL-4/IL-13 restore MerTK expression and promotes reparative macrophage functions, providing a promising strategy to enhance tissue repair and resolve inflammation. These findings have significant implications for therapeutic applications in tissue repair, cardiac recovery, and chronic inflammatory conditions where macrophage dysregulation impairs tissue regeneration.

Chapter 4: M2 Macrophage Polarization-Promoting Effect of Induced Pluripotent Stem Cell-derived Mesenchymal Stem Cells in Tissue Regeneration

4.1 Introduction

4.1.1 Interaction between MSCs and Macrophages in Tissue Repair

iPSCs-derived mesenchymal stem cells (MSCs) and macrophages represent a promising convergence of regenerative medicine and immunomodulation, offering new avenues for enhancing tissue repair. MSCs play a key role in immune regulation and immunosuppressive effects through the secretion of extracellular vesicles, cytokines, growth factors, chemokines, and by promotion of indoleamine 2, 3-dioxygenase (IDO) production in recipient macrophages during apoptosis (Bloor *et al.*, 2020). Moreover, MSCs facilitate the monocyte-to-macrophage transition and enhance macrophage function to eliminate pathogens, thereby promoting tissue regeneration (Vasandan *et al.*, 2016). Notably, MSCs contribute to the M1 to M2 macrophage transition (Stevens *et al.*, 2020). However, the precise mechanisms by which MSCs repair tissue injuries and modulate inflammatory diseases remain largely undefined. As orchestrators between adaptive and innate immune cell subsets within the tissue niche, MSCs play a fundamental role in tissue regeneration and wound healing. In inflammatory bowel diseases (IBD), for instance, MSCs promote tissue repair through TNF-α-stimulated gene-6 (TSG-6) (Yang *et al.*, 2019). Thus, MSCs contribute to tissue regeneration by secreting bioactive molecules that mediate intercellular communication.

MSCs have been reported to exert synergistic effects with macrophages in tissue repair. Specifically, MSCs educate tissue macrophages, inducing a phenotype characterised by programmed death ligands 1 and 2 (PD-L1, PD-L2), transforming growth factor-β1 (TGF-β1), and high levels of IL-10. These immunomodulatory properties contribute to T-cell suppression and fibroblast proliferation, thereby dampening the inflammatory response (Lu *et al.*, 2021). Additionally, MSCs-educated macrophages enhance the production of fibrotic growth factors, wound-healing mediators, and immune-regulating cytokines, including IL-6 and TGF-β1. TGF-β1 is particularly critical in wound healing, as it accelerates angiogenesis, fibroblast proliferation, and re-epithelialization (Stevens *et al.*, 2020). These findings underscore the significant therapeutic potential of MSC–macrophage interactions in regenerative medicine.

4.1.2 TGF-β Signalling Inhibition for iPSC-Derived Mesenchymal Stem Cell Differentiation

The transforming growth factor-beta (TGF-β) signalling pathway plays a pivotal role in maintaining pluripotency and directing lineage commitment in pluripotent stem cells (Xu *et al.*, 2018b). In iPSCs, TGF-β signalling, mediated through SMAD2 and SMAD3 activation, regulates the expression of key pluripotency factors such as NANOG, OCT4, and SOX2 (Liu *et al.*, 2024a; Xu *et al.*, 2018b). These transcription factors collectively preserve stem cell identity and inhibit differentiation programs (Swain *et al.*, 2020). Conversely, inhibiting TGF-β signalling disrupts this pluripotency network, thereby promoting differentiation into mesodermal lineages, including MSCs (Leyendecker Junior, 2018).

One widely utilised method for differentiating iPSCs into MSCs involves treatment with SB431542, a selective small-molecule inhibitor of TGF-β signalling. Several studies have demonstrated that SB431542 treatment effectively generates MSC-like cells without requiring embryoid body (EB) formation or additional selection steps (Chen *et al.*, 2012; Leyendecker Junior, 2018; Zhao *et al.*, 2015). SB431542 specifically targets activin receptor-like kinase (ALK) receptors 4, 5, and 7, thereby inhibiting SMAD2/3 phosphorylation, a key event in TGF-β-mediated transcriptional regulation (Inman *et al.*, 2002; Levolger *et al.*, 2019). This inhibition downregulates NANOG expression, disrupting the pluripotency-associated transcriptional circuitry and directing iPSCs toward a mesenchymal lineage (Leyendecker Junior, 2018; Xu *et al.*, 2008). Additionally, TGF-β inhibition has been recognized as a key driver of epithelial-to-mesenchymal transition (EMT), a process that prepares iPSCs for mesodermal differentiation and facilitates the derivation of mesenchymal stem-like cells (Chen *et al.*, 2012; Katsuno, Lamouille and Derynck, 2013; Wendt, Allington and Schiemann, 2009). This strategy capitalizes on the dual function of TGF-β inhibition, which both suppresses pluripotency and induces EMT, thereby enriching a cell population that exhibits classic MSC markers such as CD73 and CD90, as well as multilineage differentiation potential (Leyendecker Junior, 2018; Zhao *et al.*, 2015).

This methodology presents several advantages, notably its provision of a more direct and efficient differentiation pathway in comparison to conventional EB-based methods. Furthermore, it offers a platform to examine the molecular mechanisms by which TGF-β signalling regulates lineage commitment and mesenchymal identity within pluripotent cells. A thorough understanding of these mechanisms not only

enhances our capacity to generate clinically significant MSC populations but also provides valuable insights into the overarching regulatory networks that govern stem cell behaviour fate. Therefore, the use of SB431542 to inhibit TGF-β signalling in iPSCs represents a powerful strategy for deriving MSC-like cells (Chen *et al.*, 2012). By targeting a critical pathway that maintains pluripotency, this approach exploits the natural lineage plasticity of iPSCs and efficiently channels them toward MSCs (Leyendecker Junior, 2018; Zhao *et al.*, 2015).

4.1.3 Potential Role of FGF-β in Enhancing Characteristics of iPSCs-Derived MSCs

Fibroblast growth factor beta (FGF-β) plays a key role in regulating stem cell biology, including maintenance, proliferation, and differentiation. In MSCs, FGF-β has been shown to delay replicative senescence, promote cytoskeletal organisation, and support the expression of characteristic surface markers (Coutu and Galipeau, 2011; Mossahebi-Mohammadi *et al.*, 2020). These effects are thought to occur through activation of downstream pathways such as MAPK/ERK, which are involved in cell growth and fate determination. Given its broad influence on cellular behaviour, FGF-β is increasingly investigated for its potential to enhance the phenotypic and functional properties of iPSC-derived MSCs, though its precise role in the maturation process remains to be fully elucidated.

4.1.4 The Role of MSCs-Conditioned Medium

Given their significant potential in clinical applications, MSCs have been extensively investigated in regenerative medicine. However, several challenges related to MSC-based therapies have emerged in recent years (Peshkova *et al.*, 2023). Apart from concerns regarding tumorigenicity, MSCs often exhibit low survival rates post-transplantation (Huang *et al.*, 2010). Furthermore, their in vivo behaviour, including surface molecule expression and paracrine activity, is highly unpredictable and subject to microenvironmental influences (Jin *et al.*, 2022; Levy *et al.*, 2020; Peshkova *et al.*, 2023). In this context, utilising the secretome of MSCs rather than the MSCs themselves appears to be an appealing option, as it has long been posited that the paracrine activity of MSCs, which underpins their immunomodulatory properties, contributes even more significantly to their therapeutic potential than their differentiation capabilities.

MSCs-conditioned medium, which contains soluble factors, cytokines, growth factors, and extracellular vesicles, has demonstrated therapeutic efficacy in various

pathological conditions (Kim and Hematti, 2009; Morrison *et al.*, 2017; Zhou *et al.*, 2019). This cell-free approach offers several advantages, including a reduced risk of immune rejection and tumorigenicity(Jin *et al.*, 2022; Peshkova *et al.*, 2023). Thus, MSCs-conditioned medium serves as a potent alternative for achieving anti-inflammatory effects, promoting tissue repair, and modulating immune cell function.

4.1.5 Aims and Objectives

Aims:

This chapter focuses on the generation of large quantities of high-quality mesenchymal stem cells from induced pluripotent stem cells using cost-effective TGF-β inhibitors. By employing this approach, I investigated the ability of these MSCs to modulate inflammatory responses and promote macrophage polarisation, which are critical processes for tissue repair and immune regulation. This research seeked to contribute to the development of scalable and efficient MSCs production methods, ultimately advancing their therapeutic potential in regenerative medicine and inflammatory disease treatment

Objectives:

1. Investigating the influence of feeder-free and feeder-supported culture conditions on iPSCs growth and pluripotency.

2. Evaluating the efficacy of TGF-β inhibitors in directing iPSCs differentiation into MSC-like cells.

3. Defining the role of FGF-β in the maturation and functional enhancement of iPSC-derived MSCs.

4. Examining the ability of iPSCs-derived MSC-conditioned medium/exosome to modulate macrophage polarisation toward an anti-inflammatory M2 macrophages phenotype.

4.2 Methods

4.2.1 Expansion of iPSCs

iPSCs were generated and characterized as previously described (Mulero-Navarro *et al.*, 2015). The cells were cultured on irradiated mouse embryonic feeder cells in a medium comprising Dulbecco's Modified Eagle's Medium, 20% Knock-Out Serum Replacement, 100 µM nonessential amino acids, 2 mM glutamine, 100 µM mercaptoethanol, and 10 ng/mL of basic Fibroblast Growth Factor (bFGF). Following dissociation with TrypLE Express, they were passaged to new feeder layers as single-cell suspensions (See section 2.2.1.7).

4.2.2 A Simple Method for Deriving Functional MSCs

Human iPSCs were cultured in 6-well plates according to a feeder-free protocol (See section 2.2.1.8) until they attained approximately 80% confluence. Subsequently, the iPSC medium was substituted with MSC medium, comprising DMEM with low glucose, 10% fetal bovine serum (FBS), 2 mM L-Glutamine and 1% penicillin/streptomycin. The MSC medium was refreshed every three days. After 14 days of culture, the cells were dissociated utilizing TrypLE and subsequently expanded in MSC medium on dishes coated with 0.1% gelatin. Upon reaching confluence (within 3-5 days), the cells were harvested with TrypLE and regularly passaged at a 1:3 ratio (Zou *et al.*, 2013).

4.2.3 SB431542 Inhibitor Differentiation Method

To initiate differentiation, bulk cultures of iPSCs were inoculated into Geltrex-coated vessels utilizing E8 medium in accordance with the manufacturer's guidelines. For optimal outcomes, the cells were seeded in substantial colonies at a high confluence, with some cell lines necessitating a one-passage adaptation to E8/Geltrex conditions prior to MSC differentiation. Upon reaching confluence, the medium was transitioned to DMEM-Ham’s F-12 basal medium, supplemented with 20% knockout serum replacement (KOSR), 1 mM L-glutamine, and 10 mM nonessential amino acids, excluding bFGF, and further supplemented with 10 µM SB431542 (SB) in DMSO. Following a ten-day period of daily alterations to KOSR+SB medium, the cells were passage into a single-cell suspension using TrypLE. At the initial mesenchymal passage (MP0), single cells were reseeded at a density of 40,000 cells per cm² into MSC medium which included DMEM with high glucose, 10% of FBS, 2 mM L-glutamine, and 1% penicillin/streptomycin. Subsequently, cells were

seeded at a density of 20,000 cells per cm² (MP1) in the MSC medium, with later passages at 10,000 cells per cm² (MP2, MP3, etc.) (Chen *et al.*, 2012).

4.2.4 Verification of iMSCs by Detecting Surface Markers via Flow Cytometry

Expanded iMSCs were harvested utilizing TrypLE solution. The cells were centrifuged at 300 × g for a duration of 5 minutes, subsequently resuspended in 2 milliliters of phosphate-buffered saline (PBS) through a 35-micron nylon mesh filter (Thermo Fisher Scientific). After a second centrifugation at 300 × g for 5 minutes, the cells were resuspended to achieve a final volume of 1 milliliter of PBS. The filtered cells were dispensed into 100-microliter aliquots and incubated with the antibodies CD45, CD73, CD90, and CD105 for 30 minutes at room temperature, protected from light, before running on the flow cytometer.

4.2.5 Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR)

U937 monocytes were differentiated into macrophage-like cells through incubation with 50 ng/mL phorbol 12-myristate 13-acetate (PMA) in RPMI complete medium (See section 3.4.2.1). The serum-free conditioned medium (CM) was derived from iPSCs-derived MSCs. M0-like macrophages were treated with LPS, 50% of CM, and both for 6 hours. Afterwards, RNA was isolated using the Total RNA Purification Plus Kit (Geneflow, UK) following the manufacturer's protocol. RT-qPCR was performed using standard protocols, and data were analysed using the $2^{-\Delta\Delta CT}$ method, as previously described (See section 2.2.5).

4.2.6 Conditioned Medium from iPSC-MSCs

Healthy iPSC-derived mesenchymal stem cells (MSCs) were cultured in T75 flasks until reaching the appropriate confluence. The culture medium was then replaced with a serum-free formulation, and cells were incubated for 24 hours. The conditioned medium was subsequently harvested for downstream applications, including treating macrophages or microglia and exosome isolation.

4.2.7 Isolation of EVs from iMSCs

The procedure for isolating exosomes commenced with transferring 30 mL of conditioned medium derived from iMSCs into a microcentrifuge tube. This was followed by centrifugation at 300 × g for a duration of 10 minutes to segregate cellular constituents from the supernatant. Subsequently, the supernatant was carefully decanted into a new microcentrifuge tube and subjected to centrifugation at 9000 × g for 50 minutes, eliminating residual cellular debris. The resultant

supernatant was subsequently combined with Exo-spin™ Buffer (Cell Guidance Systems Ltd, UK) in a 2:1 ratio, wherein 15 mL of the buffer was added to 30 mL of the supernatant, mixed thoroughly by inversion, and allowed to incubate at 4°C for a minimum of one hour. Following this incubation, the mixture underwent centrifugation at 9000 × g for two hours. The ensuing supernatant was meticulously aspirated and discarded, ensuring the preservation of the exosome-containing pellet. This pellet was then resuspended in 100 µL of PBS. The Exo-spinTM mini-HD column purification procedure entailed the elimination of the preservative buffer and the outlet plug, followed by the immediate equilibration of the column through the sequential addition of 3 × 2.5 mL of phosphate-buffered saline (PBS), facilitating passive drainage without the necessity for centrifugation. Following equilibration, 100 µL of the exosome-containing sample with 900 µL of PBS was meticulously applied to the column, allowing for gravitational drainage. Ultimately, a new 1.5 mL microcentrifuge collection tube was utilised, which received an extra 400 µL of PBS to collect the high purity exosome-containing eluate (Welton *et al.*, 2015; Witwer *et al.*, 2013).

4.2.8 Characterize Exosome Morphology by Scanning Electron Microscopy (SEM)

Isolated exosome was visualised using Zeiss Crossbeam 350 FIB-SEM. Fix the exosome sample with 2.5% glutaraldehyde in phosphate-buffered saline (PBS) for 1–2 hours at 4°C to preserve structural integrity. After fixation, wash the sample with PBS and dehydrate it through a graded ethanol series (30%, 50%, 70%, 90%, and 100%) to remove water content. Deposit the dried exosomes onto a conductive surface, such as a silicon wafer or gold-coated cover slip, and sputter-coat them with a thin layer (5–10 nm) of gold or platinum to enhance conductivity. Finally, image the exosomes using a field emission SEM (FE-SEM) at an appropriate accelerating voltage (typically 5–15 kV).

4.2.9 Statistical Analysis

Data throughout this chapter were expressed as mean ± SD. Statistical differences were calculated using unpaired two-tailed Student's t-test or one-way ANOVA with Bonferroni correction for multiple comparisons. A probability of $p < 0.05$ was considered statistically significant. Ns not significant; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$. Prism9 GraphPad software was used to illustrate differentially expressed genes.

4.3 Results

4.3.1 iPSCs Culture Under Feeder-Free and Feeder Conditions

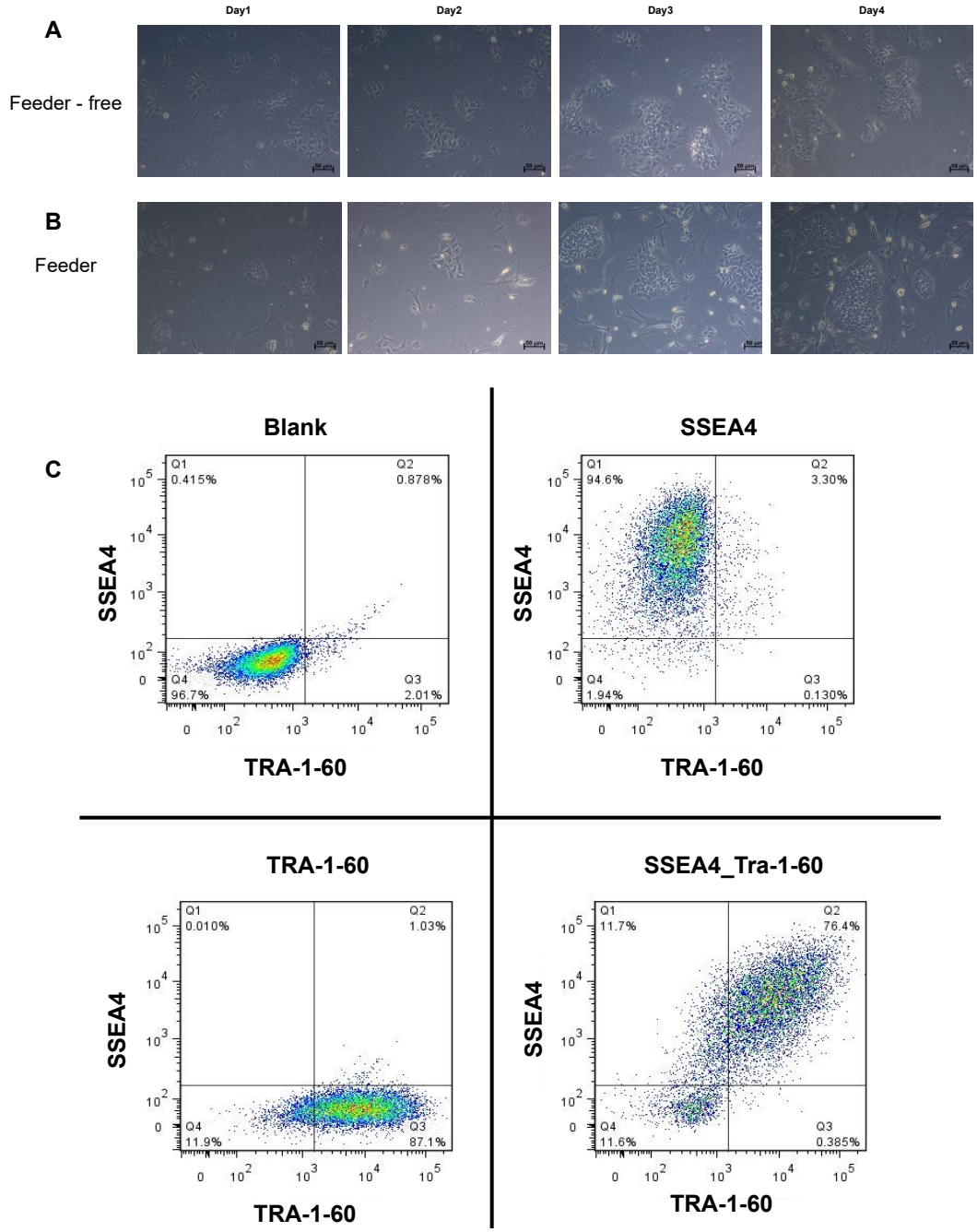


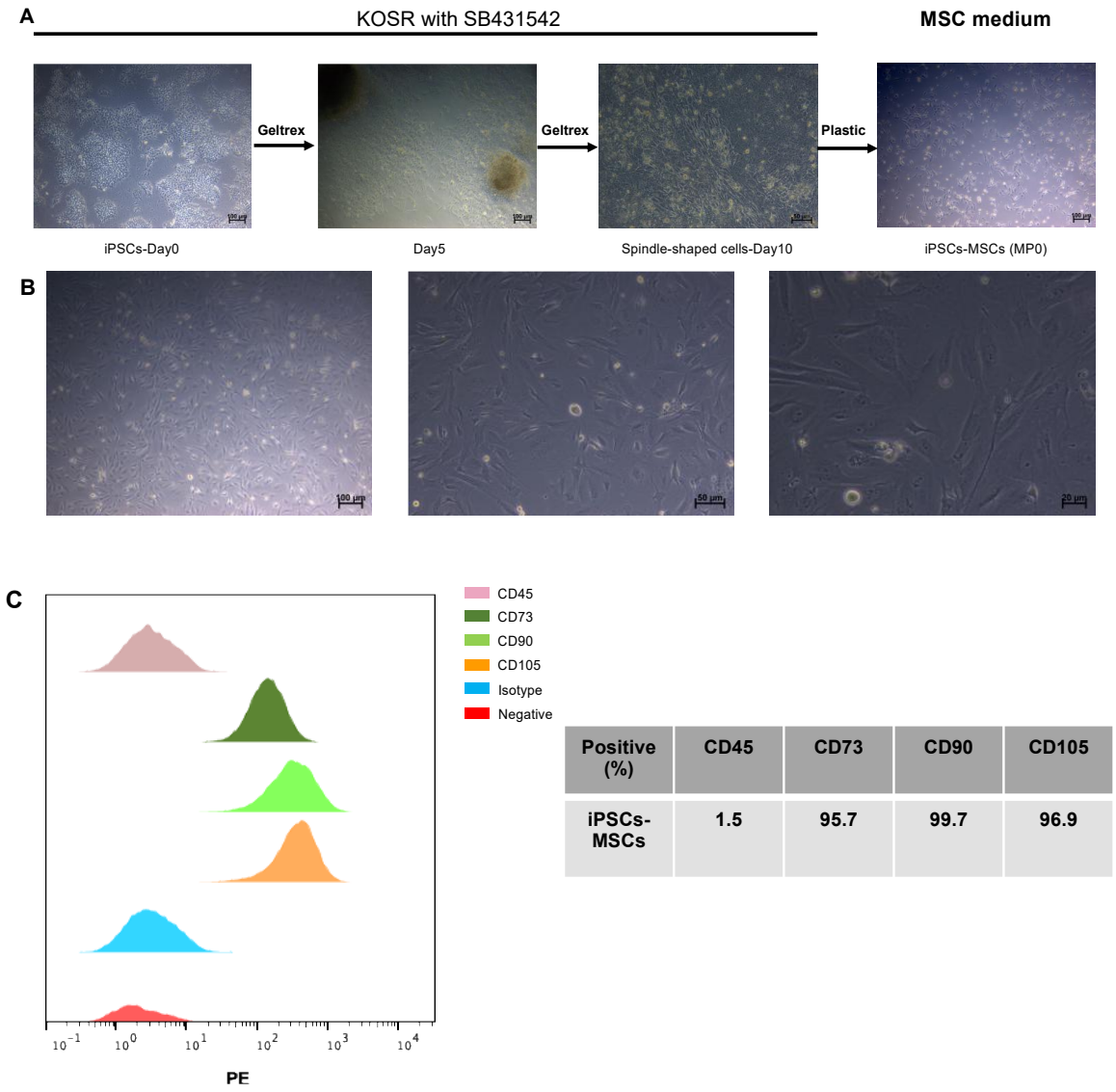
Fig.4.1 Comparative Culture Conditions and Surface Marker Analysis of iPSCs. (A) Morphological assessment of iPSCs cultured in a feeder-free system over four days demonstrates colony formation and growth without feeder cells. (B) Morphological evaluation of iPSCs cultured with MEF feeder cells reveals enhanced colony morphology over the same period. (C) Flow cytometric analysis confirms the expression of iPSC-specific surface markers TRA-1-60 and SSEA4. The figure presents four staining conditions for the same iPSC line: unstained, TRA-1-60-stained, SSEA4-stained, and dual-stained with both TRA-1-60 and SSEA4. The dual-stained group demonstrates high co-expression of these pluripotency markers, confirming the iPSC identity. The experiments were performed in three independent biological replicates (n = 3).

The comparison of the morphology of iPSCs cultured on feeder-free (Fig.4.1.A) and feeder-dependent (Fig.4.1.B) systems indicates that the two culture systems have no significant differences in their effects on cell morphology. In the feeder-free system, iPSCs are cultured on matrices coated with Geltrex, providing an extracellular matrix-like substrate for the cells to attach (Wagner and Welch, 2010). This system facilitates the growth of iPSCs without the need for feeder cells, simplifying the culture process and lowering the risk of contamination. In the feeder-dependent system, iPSCs are cultured on a layer of MEFs, offering a feeder layer for the cells to grow (Amit *et al.*, 2003). This system offers a more intricate environment for the cells, with feeder cells supplying additional signalling molecules and support for iPSCs growth.

Regarding morphology, iPSCs cultured on feeder-free matrices exhibited a growth rate and morphology similar to those grown on MEFs. This indicates that the matrix coated with Geltrex provides a suitable substrate for iPSCs to grow and maintain their undifferentiated state. However, there is a slight difference in the shape of the feeder cells between the two systems. The feeder cells in the feeder-based system appear more rounded than those in the feeder-free system, which may reflect variations in the microenvironment surrounding the cells. Overall, the choice between feeder-based and feeder-free systems may depend on the specific requirements of the experiment or application, but both systems can support the growth and maintenance of iPSCs in an undifferentiated state.

Flow cytometric analysis validated the pluripotency of the cultured iPSCs (Fig.4.1.C). The cells displayed robust co-expression of TRA-1-60 and SSEA4, significant markers indicative of pluripotency (Fong *et al.*, 2009). The blank control exhibited minimal marker expression, whereas the experimental groups consistently demonstrated a high percentage of TRA-1-60 and SSEA4-positive cells, confirming the iPSCs populations' pluripotent status within both feeder-free and feeder-dependent culture systems.

4.3.2 SB431542-Treated iPSCs Passaged in MSC Media Rapidly Differentiate into MSCs-like Cells



Positive (%)	CD45	CD73	CD90	CD105
iPSCs-MSCs	1.5	95.7	99.7	96.9

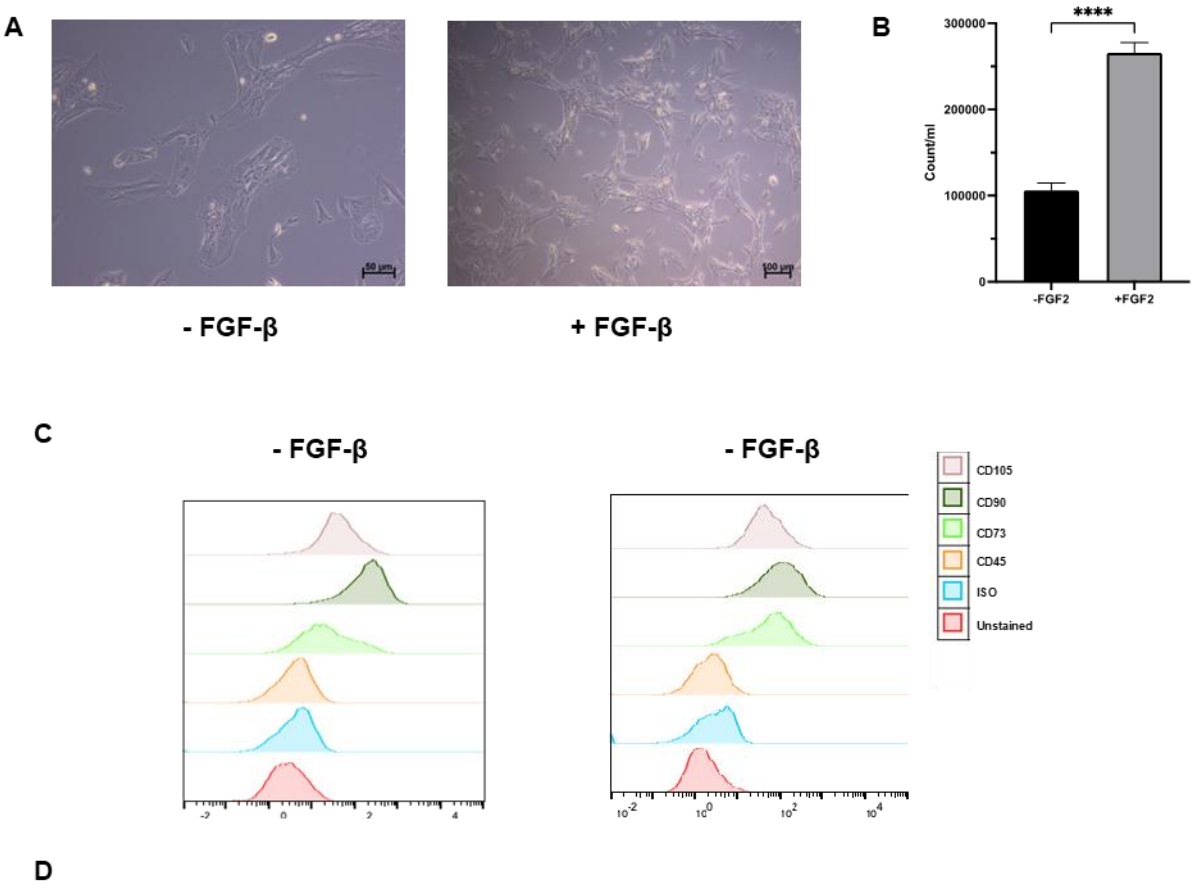
Fig.4.2 Differentiation of iPSCs into MSCs-Like Cells using TGF-β inhibitor. (A) Morphological alterations observed during the differentiation of iPSCs into MSC-like cells throughout the differentiation process. The images illustrate the transition from compact iPSC colonies to spindle-shaped, fibroblast-like cells characteristic of MSCs. (B) Representative phase contrast images of MP3, with magnifications at X4, X10, and X20. (C) Flow cytometric analysis verifies the expression of MSC surface markers (CD73, CD90, CD105) and the absence of the hematopoietic marker CD45 in iPSCs-derived MSCs. The histogram overlays demonstrate clear positivity for MSCs-specific markers, alongside corresponding quantifications in the adjacent table. All experiments were performed in three independent biological replicates (n = 3).

The differentiation of iPSCs into mesenchymal stem cell (MSC)-like cells utilising TGF-β inhibitors was clearly evidenced through distinct morphological changes

(Fig.4.2.A). Initially, the compact colonies characteristic of iPSCs transitioned into spindle-shaped, fibroblast-like cells. This morphological progression, observed over several stages, aligns with the established characteristics of MSCs, thereby indicating successful differentiation (Fig.4.2.B).

Flow cytometric analysis further confirmed the mesenchymal phenotype of the differentiated cells (Fig.4.2.C). Notably, high expression levels of MSC markers CD73 (95.7%), CD90 (99.7%), and CD105 (96.9%) were recorded, coupled with minimal expression of the hematopoietic marker CD45 (1.5%). These findings substantiate the identity of the differentiated cells as MSCs-like, thereby demonstrating effective differentiation from iPSCs under TGF-β inhibition.

4.3.3 FGF-β Serves as a Requisite for the Maturation of Mesenchymal Stem Cells.



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	CD45	CD73	CD90	CD105
- **FGF-β**	0.40%	37.80%	93.30%	59.70%
+ **FGF-β**	0.30%	82.50%	96.30%	91.10%

Fig.4.3 Role of FGF-β in the Maturation of Mesenchymal Stem Cells (MSCs). (A) Morphological observation of iPSCs-derived MSCs cultured with and without FGF-β supplementation. Images show improved elongation and maturation of MSCs with FGF-β treatment compared to untreated controls. (B) Flow cytometric analysis comparing the expression of MSC surface markers (CD73, CD90, CD105) and the hematopoietic marker CD45 under FGF-β-treated and untreated conditions. Histograms illustrate higher expression levels of MSCs-specific markers in the presence of FGF-β, demonstrating its role in promoting MSCs maturation. All experiments were performed in three independent biological replicates (n = 3).

Supplementation with FGF-β has been shown to significantly enhance the maturation of iPSC-derived MSCs, as evidenced by observable morphological transformations (Fig.4.3.A). FGF-β-treated cells displayed improved elongation and alignment, traits characteristic of mature MSCs, in contrast to the less structured morphology noted in untreated control cells.

Flow cytometric analysis further validated that FGF-β enhances the expression of MSC-specific markers, namely CD73, CD90, and CD105 (Fig.4.3.D). Cells cultured in the presence of FGF-β exhibited elevated expression of these markers, indicating an improvement in mesenchymal characteristics. Conversely, the absence of FGF-β resulted in diminished marker expression, thereby reaffirming the critical role of FGF-β in the maturation of MSCs. It is also noteworthy that the observed differences in marker expression between Fig.4.2.C and Fig.4.3.D may reflect biological variation associated with different MSC passages or culture conditions. Marker expression in MSCs is known to fluctuate slightly over serial passages, particularly for CD105 and CD90, without altering their multipotent phenotype.

4.3.4 Anti-Inflammatory Effects of iPSC-Derived MSCs-Conditioned Medium

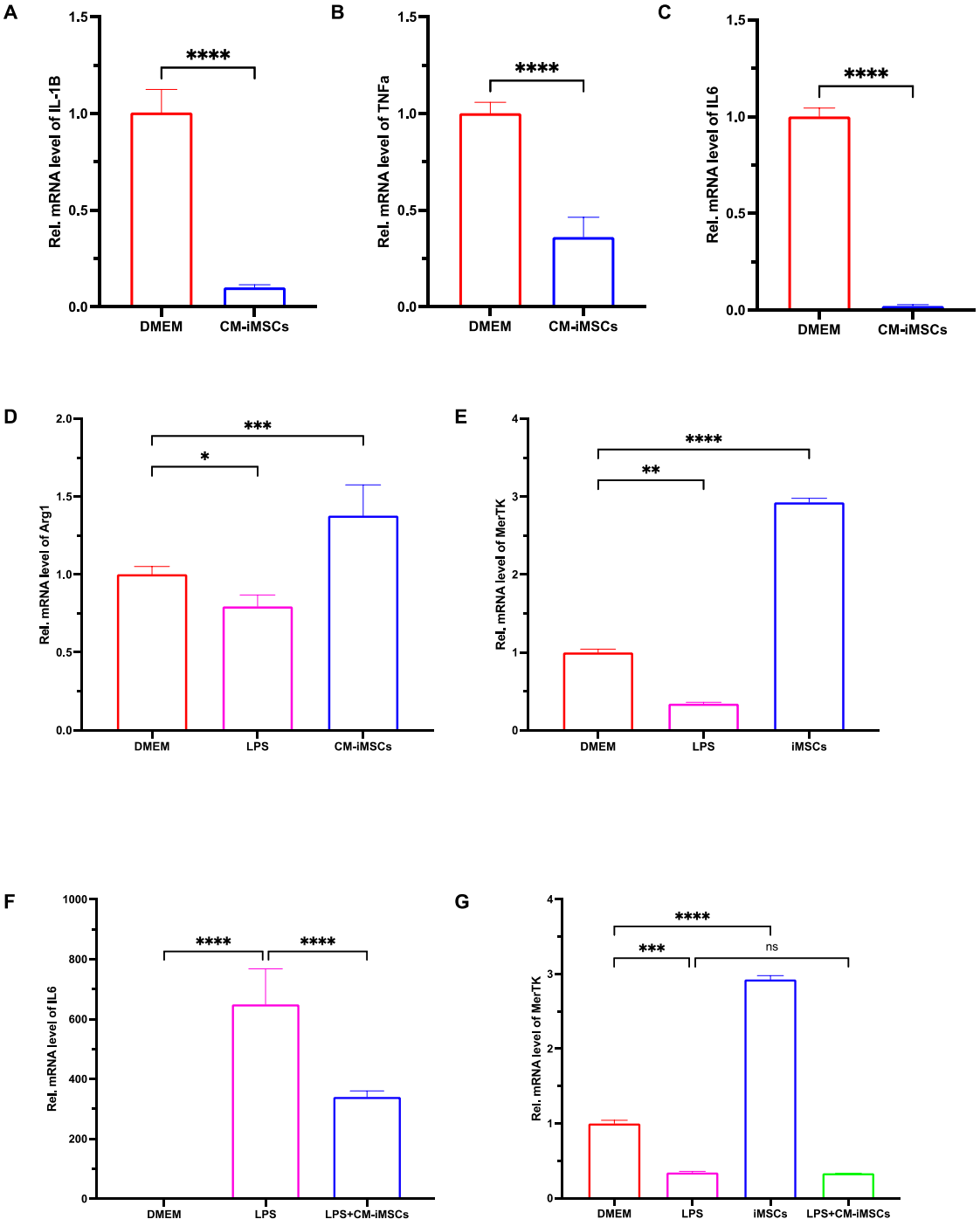

Fig.4.4. PCR Analysis of iPSCs-Derived MSCs-Conditioned Medium Effects on U937-derived Macrophage Polarisation. Relative mRNA expression levels of anti-inflammatory markers (Arg1), M2 macrophage markers (MERTK), and pro-inflammatory markers (IL-6, IL1B and TNF-α) in macrophages cultured with iPSC-derived MSC-conditioned medium. The data demonstrate a significant increase in anti-inflammatory and M2 markers (Arg1, MERTK) alongside a reduction in pro-inflammatory markers (IL-6, IL-1B and TNF-α), indicating that the conditioned medium educates macrophages toward an M2 phenotype. The data are presented as the mean ± SEM, and statistical significance is indicated (ns: not significant, *p<0.05, ****p<0.01, ***p<0.001, ****p<0.0001). Data represent three independent biological replicates (n = 3).

The conditioned medium derived from iPSCs-derived MSCs resulted in a significant enhancement of the expression of the anti-inflammatory cytokine Arg1 in macrophages when compared to the control groups, as demonstrated by the polymerase chain reaction (PCR) analysis (Fig. 4.4). The observed upregulation signifies that the conditioned medium fosters an anti-inflammatory environment conducive to tissue repair and the modulation of immune functions.

A noteworthy increase in MerTK expression, indicative of M2 macrophage polarisation, was observed in macrophages cultured with the medium conditioned by iPSC-derived mesenchymal stem cells. This finding suggests that the conditioned medium effectively guides macrophages to adopt an M2 phenotype associated with immunoregulation and tissue regeneration.

Conversely, the expression of pro-inflammatory cytokines IL-1β, IL-6, and TNF-α was significantly reduced in macrophages exposed to the conditioned medium. These findings further substantiate the anti-inflammatory potential of iPSC-derived MSCs-conditioned medium in the modulation of macrophage functionality.

4.3.5 Exosomes isolated from mesenchymal stem cells derived from induced pluripotent stem cells enhance STAT3 signalling in U937-derived macrophages.

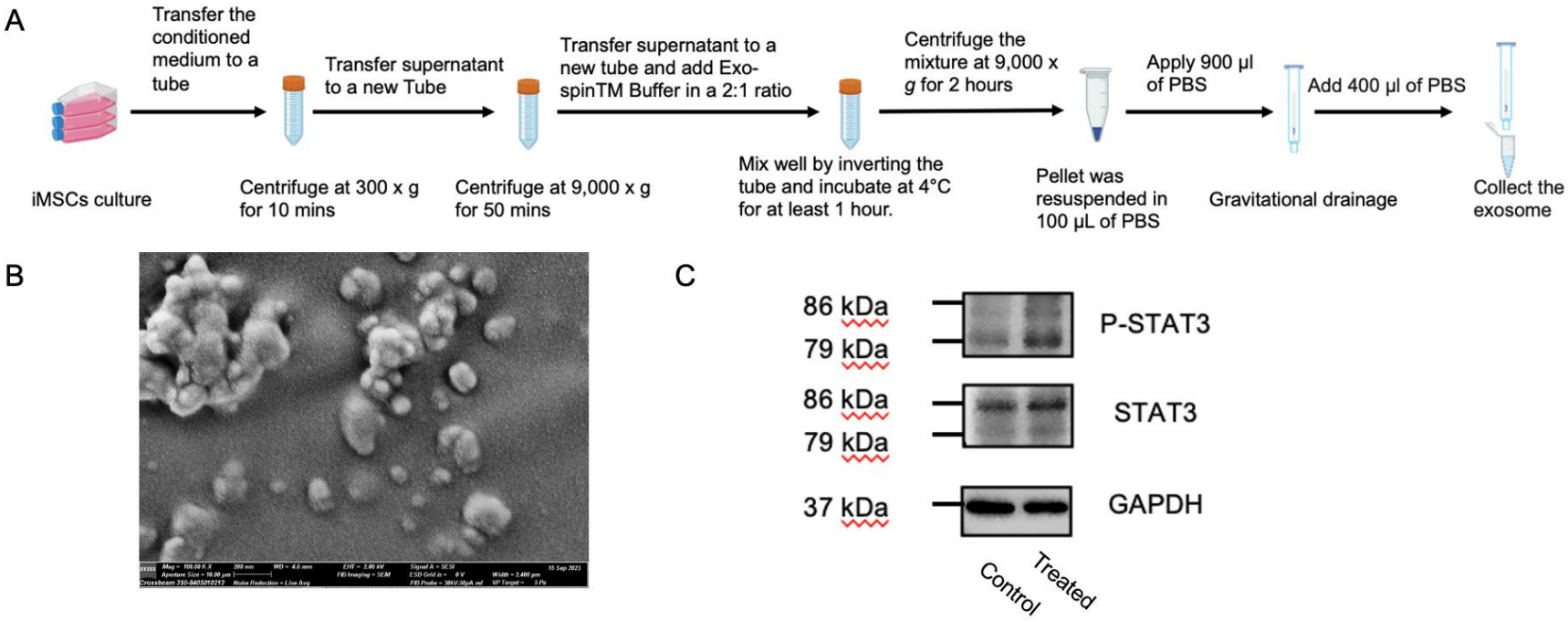

Fig.4.5 Isolation, Characterisation, and Functional Analysis of iMSC-Derived Exosomes on U937-Derived Macrophages. (A) Schematic workflow for isolating exosomes from induced mesenchymal stem cells (iMSCs). The process involves sequential centrifugation steps to remove cell debris and concentrate exosomes using Exo-spin™ Buffer, followed by resuspension in PBS for further analysis. (B) Scanning electron microscopy (SEM) image of isolated exosomes, showing their characteristic morphology and nanoscale size. (C) Western Blot Analysis of STAT3 and P-STAT3 in Control and Treated U937-derived macrophages. Protein lysates were collected from control and treated cells and analysed via western blot using antibodies against phosphorylated STAT3 (P-STAT3), total STAT3, and GAPDH (loading control). Both P-STAT3 and STAT3 blots show two bands corresponding to the known isoforms: STAT3α (~86 kDa) and STAT3β (~79 kDa). GAPDH (~37 kDa) was used as a loading control. Blot shown is representative of three independent experiments (n = 3).

The supernatant has been demonstrated to exhibit significant anti-inflammatory effects. To further clarify the role of extracellular vesicles isolated from mesenchymal stem cells and to extract exosomes from induced mesenchymal stem cells (iMSCs), a sequential centrifugation and precipitation method using Exo-spin™ Buffer was employed (Fig.4.5.A). The isolated exosomes were characterised through SEM which corroborated their anticipated morphology and size distribution (Fig. 4.5.B). The SEM imagery denotes a heterogeneous population of vesicles exhibiting a characteristic spherical shape, in alignment with prior reports pertaining to exosome morphology. The identification of nanosized vesicles substantiates the successful isolation of exosomes from iMSCs-conditioned medium.

To explore the functional impact of iMSCs-derived exosomes on macrophage signalling, macrophages derived from U937 were treated with exosomes or phosphate-buffered saline (PBS) as a control. Western blot analysis was conducted to evaluate the expression levels of phosphorylated STAT3 (P-STAT3), total STAT3, and GAPDH as a loading control (Fig. 4.5.C). The results demonstrate an increase in P-STAT3 levels in macrophages treated with exosomes compared to the control group, while total STAT3 levels exhibited no significant change. This finding suggests that exosomes derived from iMSCs may activate STAT3 signalling in macrophages, potentially influencing their functional phenotype.

4.4 Discussion

This study comprehensively analysed the potential contributions of iPSCs-derived MSCs to tissue regeneration and immune modulation. By integrating differentiation protocols, morphological observations, molecular characterisation, and functional assays, this research underscored the critical aspects of iPSCs-derived MSCs biology and their therapeutic relevance. The subsequent discussion synthesises these findings with existing literature, explores their broader implications in regenerative medicine, and identifies limitations along with potential future directions.

4.4.1 Optimisation of iPSCs Culture Conditions

The preliminary findings contrasting feeder-free and feeder-supported culture systems for iPSCs highlight the significance of optimising growth conditions to preserve pluripotency and facilitate robust cellular proliferation. The morphological distinctions observed—wherein feeder-supported iPSCs exhibit compact, well-defined colonies in comparison to the slightly irregular colonies characteristic of feeder-free systems—correlate with previous research that underscores the importance of feeder layers, such as mouse embryonic fibroblasts (MEFs), in delivering crucial growth factors and providing extracellular matrix support (Amit *et al.*, 2003).

Feeder-free systems provide significant advantages for clinical applications, particularly in creating Xeno-free conditions suitable for therapeutic use. However, the reduced structural integrity and colony compactness observed in this study are in accordance with previous reports indicating that feeder-free systems may lack certain secreted factors essential for maintaining the optimal iPSC state (Wagner and Welch, 2010). Recent advances in chemically defined, feeder-free media and substrates may help address these challenges. For future work, a direct comparative analysis of signalling pathways activated under feeder-free and feeder-supported conditions could clarify the specific molecular mechanisms responsible for these differences.

4.4.2 Differentiation of iPSCs into MSCs

The effective differentiation of iPSCs into MSCs-like cells through TGF-β inhibition represents a robust and reproducible method for generating MSCs with consistent mesenchymal characteristics (Chen *et al.*, 2012; Zhao *et al.*, 2015). Morphologically, the transition from compact iPSC colonies to spindle-shaped, fibroblast-like cells

closely resembles the established differentiation trajectory of MSCs. This process underscores the potential of iPSCs as a scalable source for MSCs, effectively addressing the limitations associated with primary MSCs, which include donor variability, cellular senescence, and constrained proliferation capacity.

Flow cytometric analysis corroborates the expression of MSCs-specific markers, namely CD73, CD90, and CD105, while demonstrating a minimal presence of the hematopoietic marker CD45. This reinforces the mesenchymal phenotype of the differentiated cells. These findings are in accordance with the criteria established by the International Society for Cellular Therapy for defining MSCs (Bloor *et al.*, 2020). Furthermore, the elevated expression levels of these markers indicate that the differentiation protocol successfully replicates the molecular signature of primary MSCs.

A notable advantage of using iPSCs is the capacity to derive MSCs from genetically diverse populations, thereby facilitating patient-specific applications. Moreover, the application of TGF-β inhibitors corresponds with recent advancements in small-molecule-driven differentiation, presenting a more controlled and efficient methodology than traditional growth factor-based techniques. Future investigations may examine the temporal dynamics of gene expression throughout the differentiation process to elucidate the critical regulatory networks involved in mesenchymal lineage commitment.

4.4.3 FGF-β as a Maturation Agent for MSCs

Adding FGF-β during the maturation phase significantly improved the morphological and molecular characteristics of iPSC-derived MSCs. Elongating and aligning cells treated with FGF-β indicate enhanced cytoskeletal organisation and structural maturity. Moreover, flow cytometric analysis indicated an increased expression of MSC-specific markers, suggesting that FGF-β facilitates phenotypic and functional enhancements maturation.

FGF-β has been widely recognised for its role in stem cell maintenance and differentiation (Coutu and Galipeau, 2011; Mossahebi-Mohammadi *et al.*, 2020). The capacity of FGF-β to enhance the characteristics is likely attributed to the activation of downstream signalling pathways, such as the MAPK/ERK pathway, which is crucial for regulating cellular proliferation, survival, and differentiation. This study represents the first report indicating that FGF-β supplementation can alleviate replicative senescence and augment the therapeutic potential of MSCs. Future

research endeavours may concentrate on elucidating the specific molecular pathways activated by FGF-β in iPSCs-derived MSCs, thereby providing valuable insights into its function as a maturation agent.

It is imperative to note that utilising FGF-β may hold significant clinical implications for augmenting the therapeutic efficacy of MSCs. By facilitating a more robust mesenchymal phenotype, MSCs treated with FGF-β may demonstrate enhanced engraftment and functionality in vivo. Nonetheless, further research is required to assess the long-term stability and functionality of these cells, particularly in preclinical disease models.

4.4.4 Anti-Inflammatory Effects of MSCs-Conditioned Medium

One of the most significant findings of this study is the ability of iPSC-derived MSCs-conditioned medium to polarise macrophages towards an anti-inflammatory M2 phenotype. The upregulation of M2-specific markers (Arg1, MerTK) and the downregulation of pro-inflammatory markers (IL-1B, IL-6, and TNFα) highlights the potential of MSC-conditioned medium to modulate immune responses. These results are consistent with previous studies demonstrating the immunomodulatory effects of MSC secretomes, which are believed to play a critical role in tissue repair and immunity regulation.

MerTK, a key marker of M2 macrophages, has been implicated in clearing apoptotic cells and resolving inflammation (Cai *et al.*, 2017; Zizzo *et al.*, 2012). The significant increase in MerTK expression observed in this study indicates that MSCs-conditioned medium suppresses pro-inflammatory pathways and actively promotes tissue repair mechanisms. This dual role of modulating inflammation and facilitating repair positions MSC-conditioned medium as a promising therapeutic tool for inflammatory and autoimmune diseases.

The ability of MSC-conditioned medium to influence macrophage polarisation underscores the importance of the MSCs secretome, which includes a complex array of cytokines, growth factors, and extracellular vesicles. Notably, the absence of cellular components in the conditioned medium reduces the risk of potential tumorigenicity or immune rejection, making it an attractive alternative to whole-cell therapies. Future studies could aim to identify specific components within the conditioned medium responsible for macrophage polarisation, enabling the development of targeted therapies.

4.4.5 iMSCs-Derived Exosomes as Modulators of STAT3 Signalling in Macrophages

Exosomes are small extracellular vesicles secreted by various cell types, including mesenchymal stromal cells (MSCs), and are increasingly recognised as key mediators of intercellular communication (Marote *et al.*, 2016; Zhou *et al.*, 2023). They carry a bioactive cargo of proteins, lipids, and nucleic acids that can modulate immune responses, influence macrophage polarisation, and promote tissue repair. Given their ability to mimic many therapeutic effects of parent MSCs, exosomes are considered promising cell-free alternatives for regenerative medicine. In this study, we successfully isolated and characterised exosomes derived from iPSC-derived MSCs (iMSCs) using a rigorously established isolation protocol (Welton *et al.*, 2015; Witwer *et al.*, 2013). Scanning electron microscopy (SEM) confirmed the presence of nanosized vesicles, validating their identity as exosomes. Investigating these iMSC-derived exosomes provides valuable insight into the mechanisms by which iMSCs exert their immunomodulatory and reparative functions.

The activation of the STAT3 signalling pathway in macrophages derived from U937 following treatment with exosomes indicates that exosomes derived from iMSCs have the capacity to modulate macrophage function (Tian *et al.*, 2022). STAT3 serves as a crucial transcription factor involved in the polarisation of macrophages, particularly in promoting the anti-inflammatory M2 phenotype (Fan *et al.*, 2022; Xia *et al.*, 2023). This finding is consistent with previous research indicating that exosomes derived from iMSCs can elicit immunosuppressive and tissue-regenerative effects by influencing macrophage behaviour. The observed elevation in phosphorylated STAT3 levels implies that the cargo carried by the exosomes, which may encompass cytokines, microRNAs, or other bioactive molecules, could activate the STAT3 pathway, thereby precipitating downstream transcriptional modifications in macrophages.

The findings contribute to the growing body of evidence that substantiates the therapeutic potential of iMSC-derived exosomes in the modulation of immune responses. It is imperative that forthcoming studies endeavour to delineate the specific molecular constituents that mediate STAT3 activation, as well as evaluate the functional ramifications of this activation on macrophage polarisation and inflammatory responses utilising models that more accurately reflect physiological conditions.

4.5 Conclusion

Overall, the findings of this chapter have significant implications for the field of regenerative medicine. The ability to generate iMSCs from iPSCs provides a consistent and scalable source of cells, overcoming the challenges associated with primary MSCs. The immunomodulatory effects of MSCs-conditioned medium further expand the therapeutic potential of MSCs, offering a cell-free approach to modulate immune responses and promote tissue repair. iPSCs-derived MSCs could be particularly valuable in treating chronic inflammatory diseases, such as rheumatoid arthritis, inflammatory bowel disease, and systemic lupus erythematosus. Additionally, their potential to promote M2 macrophage polarisation may have applications in tissue repair, organ transplantation, and fibrosis resolution. The use of small molecules such as TGF-β inhibitors and FGF-β to enhance differentiation and maturation represents a significant advancement in the field. These approaches improve the quality of iPSCs-derived MSCs and streamline the manufacturing process, making it more feasible for clinical translation. However, further optimisation is needed to ensure consistency and reproducibility across different iPSC lines and differentiation protocols.

While this study provides valuable insights into the potential of iPSCs-derived MSCs, several limitations warrant consideration. First, the functional assays in this study were primarily conducted *in vitro*. While the results demonstrate the immunomodulatory and differentiation capacity of iPSCs-derived MSCs, in vivo studies are needed to validate their therapeutic potential in relevant disease models. Second, the heterogeneity of iPSCs and their derivatives remains a challenge. Although the differentiation protocols used in this study yielded MSCs with consistent marker expression, variability between different iPSC lines could impact the reproducibility of these findings. Future studies should explore single-cell RNA sequencing to characterise the heterogeneity of iPSC-derived MSCs and identify subpopulations with enhanced therapeutic potential. Third, while the MSC-conditioned medium demonstrated significant immunomodulatory effects, the specific components responsible for these effects were not identified. Proteomic and metabolomic analyses of the conditioned medium could provide insights into the key factors driving macrophage polarisation.

Additionally, engineering MSCs to enhance the secretion of these factors could further improve their therapeutic efficacy. Finally, the long-term stability and safety of iPSCs-derived MSCs need to be assessed. While iPSCs offer a renewable source

of cells, their propensity for genomic instability and potential for tumorigenicity remain concerns. Rigorous quality control measures, including karyotyping and genomic sequencing, should be implemented to ensure the safety of iPSCs-derived MSCs for clinical use.

Chapter 5: Conclusion and Future Work

5.1 Summary of Key Findings

1. LPS-induced inflammation in macrophages resulted in significant transcriptional changes, characterised by upregulation of pro-inflammatory cytokines (e.g., IL-1β, IL-6) and downregulation of MerTK and circadian-regulated genes.

2. Small molecule inhibitors of NF-κB (e.g., Bay11-7082, CBL0137) and cytokines IL-4/IL-13 were partially effective in restoring MerTK expression and reducing pro-inflammatory gene expression.

3. MerTK contains an E-box element in its promoter region, suggesting circadian transcription factors regulate it and may be suppressed during inflammation via NF-κB-mediated inhibition of E-box activity.

4. CEBPE and MerTK were both downregulated in LPS-stimulated macrophages, suggesting a coordinated role in regulating inflammation resolution and highlighting their potential as therapeutic targets in restoring macrophage reparative function.

5. FGF-β supplementation during iMSC maturation improved morphological features and expression of MSC markers, suggesting potential benefits in stem cell preparation for therapeutic use.

6. iPSCs-derived MSCs (iMSCs) demonstrated immunomodulatory capacity, enhancing macrophage polarisation towards the M2 phenotype and increasing MerTK expression under inflammatory conditions.

5.2 General Discussion

Macrophages, as central mediators of immune responses, exhibit a temporal phenotypic shift from a pro-inflammatory (M1) to a reparative (M2) state (Mantovani *et al.*, 2013; Wynn and Vannella, 2016). Mantovani et al. described the molecular drivers of M1/M2 polarisation, highlighting how macrophage plasticity underlies immune homeostasis, while Wynn and Vannella demonstrated the importance of this transition in tissue repair across multiple inflammatory disease models. These studies provide a foundational framework for investigating how external factors, such as NF-κB activation or iMSC-derived signals, may influence macrophage phenotype and function in the context of inflammation and regeneration. A significant finding of this research is the dual role of macrophages in the inflammation and resolution phases. In the context of the LPS-induced inflammatory state, macrophages initially exhibit a pro-inflammatory phenotype characterised by the secretion of cytokines such as TNF-α, IL-1β, and IL-6 (See section 3.3). These cytokines enhance inflammation through positive feedback loops and accelerate signalling via pathways such as NF-κB. While anti-inflammatory factors, including IL-10 and TGF-β, are subsequently produced, their emergence occurs later due to the time required to transition to an anti-inflammatory phenotype. It is possible that the organism prioritises the clearance of pathogens and the protection of tissues, thereby sustaining a predominantly pro-inflammatory environment. A previous study highlights the immune system's delicate balance between pathogen eradication and tissue preservation, with an early pro-inflammatory response reflecting the body's prioritisation of infection control and tissue protection (Rouse and Sehrawat, 2010). This pro-inflammatory milieu, although essential for pathogen clearance, delays the transition to a reparative phenotype due to the metabolic and transcriptional demands required for M2 polarisation. Notably, this metabolic bifurcation—glycolysis for M1 versus oxidative phosphorylation for M2—adds another layer of complexity to the timing of inflammation resolution.

The multifaceted role of MerTK in tissue repair emphasises its regulatory function in the polarisation of macrophages and its extensive implications across various domains, including inflammation, ageing, cancer, and regeneration medicine (DeBerge *et al.*, 2017a; Huelse *et al.*, 2020b; Walsh *et al.*, 2021; Zizzo *et al.*, 2012). Central to macrophage transition is the MerTK receptor tyrosine kinase, which plays a pivotal role in facilitating efferocytosis and modulating excessive inflammation (DeBerge *et al.*, 2017a; Moon *et al.*, 2020; Shirakawa *et al.*, 2020). The loss of

MerTK function prolongs inflammation, promotes tissue damage, and prevents the efficient clearance of apoptotic cells, which can lead to chronic inflammation or autoimmune responses (Jhang *et al.*, 2018; Walsh *et al.*, 2021). This study found that the suppression of MerTK through NF-κB activation highlights the fragile interplay between inflammation and tissue repair. Therefore, enhancing MerTK expression may offer a novel approach to identifying drugs to treat chronic inflammatory diseases and facilitate tissue regeneration.

The suppression of MerTK mediated by NF-κB represents a crucial checkpoint wherein unresolved inflammation may escalate to chronic pathology. This assertion is further substantiated by data indicating compromised clearance of apoptotic cells and the potential emergence of autoimmune responses under conditions of MerTK dysfunction downregulation (Cheong *et al.*, 2006; Sather *et al.*, 2007). The study convincingly illustrates how inflammatory stimuli disrupt MerTK expression, consequently prolonging the inflammatory state and hindering tissue repair processes.

It is also noteworthy that MerTK plays a crucial role in the microglial phagocytosis of myelin and oligodendrocytes (Healy *et al.*, 2016). MerTK has been found to be downregulated, and CEBPβ increased in aged microglia (Li *et al.*, 2023b). Our RNA deep-sequencing analysis of an LPS-stimulated human macrophage cell line revealed similar trends, showing a significant reduction in MerTK and an increase in CEBPβ. These findings suggest a link between chronic inflammation and ageing in microglia/macrophages. Notably, low-grade systemic inflammation has been shown to stimulate microglial turnover and accelerate the onset of Alzheimer 's-like pathology, further emphasising the impact of inflammatory processes on neurodegeneration (Guerrero-Carrasco *et al.*, 2024). Therefore, our LPS-macrophage model is valuable in vitro for studying cellular ageing and inflammation-related neurodegeneration.

Another notably innovative aspect of this research pertains to the transcriptional regulation of MerTK. The identification of CEBPE as a significant driver of MerTK expression—and its selective downregulation in response to LPS—affords a mechanistic explanation for the suppression of MerTK. CEBPE was first confirmed as a key regulator of MerTK expression, as supported by identifying a CEBP binding response element in the MerTK promoter. Upon LPS stimulation in CD33WT macrophages, there was an increase in CEBPB, a transcription factor associated with inflammation, while both CEBPA and CEBPE were significantly reduced,

indicating their potential roles in maintaining macrophage homeostasis. Notably, in CD33 knockout macrophages, the LPS-induced changes in CEBPA and CEBPB were blocked, suggesting that CD33 influences these transcription factors under inflammatory conditions. However, CEBPE suppression remained unaffected by CD33 knockout, indicating that its downregulation occurs independently of CD33 signalling. Notably, the LPS-induced reduction in MerTK expression closely mirrored the decrease in CEBPE, suggesting that CEBPE is a key transcription factor driving MerTK expression, and its suppression under inflammatory conditions may contribute to the loss of MerTK and a shift toward a pro-inflammatory macrophage phenotype. This observation also indicates a bifurcation in the transcriptional pathways that govern MerTK, suggesting that targeting CEBPE directly may provide novel therapeutic avenues to restore MerTK expression during treatment inflammation.

Circadian regulation represents an additional crucial layer in the modulation of MerTK. Some studies have shown that the expression of MerTK is regulated by circadian rhythm (Casanova-Acebes *et al.*, 2013; Parinot *et al.*, 2024). The disruption of circadian rhythms by lipopolysaccharide (LPS) and its ensuing effects on MerTK expression introduce a significant dimension to our comprehension of immune dysregulation (Fig.5.1). Previous studies have revealed a direct link between NF-κB and the circadian clock, showing that NF-κB activation suppresses BMAL1/CLOCK activity, leading to disruption of circadian-regulated pathways (Guo *et al.*, 2015; Shen *et al.*, 2020). Additionally, biochemical, biophysical, and chromatin immunoprecipitation analyses demonstrate that NF-κB binds to the transactivation domain of BMAL1, competes with CRY1 and CBP/p300 for BMAL1 interaction, and co-occupies E-box elements with BMAL1 and CLOCK at core clock gene promoters, collectively supporting a model in which NF-κB directly modulates circadian transcription and mediates bidirectional crosstalk between inflammatory and circadian pathways (Shen *et al.*, 2021a). Considering that circadian misalignment is prevalent in chronic diseases, this association underscores the necessity of incorporating temporal biology into therapeutic strategies to modulate macrophage function and tissue repair.

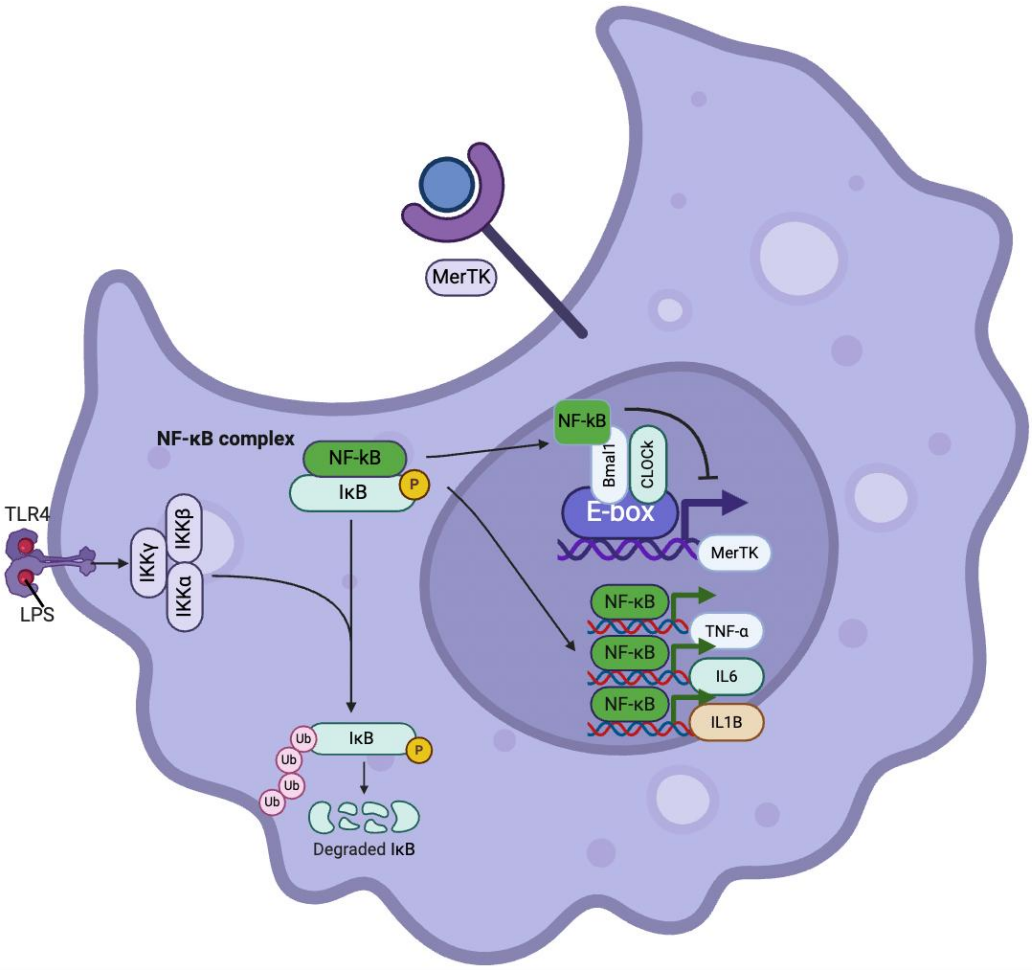

Fig.5.1 NF-κB–Mediated Disruption of Circadian Gene Expression in Macrophages. LPS stimulation of macrophages via TLR4 activates NF-κB, which translocates to the nucleus to drive transcription of pro-inflammatory cytokines (TNF-α, IL-6, IL-1β). In parallel, NF-κB binds to the Bmal1 activation domain, repressing the BMAL1 and inhibiting E-box–driven transcription of MerTK. Created in BioRender.com.

From a therapeutic perspective, this study establishes that cytokines, specifically IL-4 and IL-13, can mitigate the inhibitory effects of LPS on MerTK expression. This action facilitates the restoration of M2 polarisation and promotes tissue regeneration. These findings are consistent with earlier evidence derived from myocardial infarction models and further reinforce MerTK as a promising target for regenerative therapies (Allen, 2023; Bakhshian Nik, Alvarez-Argote and O'Meara, 2022). Additionally, the drug that enhances MerTk expression has significant research potential for clinical applications. For example, it has been proved that MerTK-dependent efferocytosis by monocytic myeloid-derived suppressor cells (M-MDSCs) has been shown to contribute to the resolution of post-lung transplant ischemia-reperfusion injury, highlighting its therapeutic potential in promoting tissue protection and repair (Leroy *et al.*, 2024).

LPS-induced downregulation of MerTK and the shift from M2- to M1-like macrophages have significant implications for cancer therapy by targeting tumour-associated macrophages (TAMs). While M2 TAMs support tumour progression through immune suppression, M1 macrophages enhance anti-tumour responses (Wang *et al.*, 2024a; Yang *et al.*, 2020). MerTK-driven efferocytosis fosters immune tolerance and advances tumour progression in osteosarcoma by promoting M2 macrophage polarisation and upregulating PD-L1 expression (Lin *et al.*, 2022; Wang *et al.*, 2024b). These findings suggest that MerTK inhibition contributes to M1 macrophage repolarization and could improve the tumour's immune environment. As MerTK inhibitors are being explored in oncology, their combination with immunotherapies such as checkpoint inhibitors or CAR-T cell therapy may enhance treatment efficacy. It is interesting to note that LPS, initially observed to induce tumour regression in mice, was later found to act indirectly by stimulating host macrophages to release TNFa responsible for the anti-tumour effect (Shear and Perrault, 1944; van Loo and Bertrand, 2023). Although LPS itself is not clinically viable due to toxicity, safer derivatives like MPLA may offer similar benefits (Sun *et al.*, 2021; Zhang *et al.*, 2021b). Overall, targeting MerTK or using non-toxic TLR agonists could be promising strategies to reprogram TAMs and boost anti-cancer immunity.

The ability to produce economical and high-quality induced mesenchymal stem cells is crucial for advancing regenerative medicine, as it enables scalable, reproducible, and ethically sustainable sources of therapeutic cells for widespread clinical application (Bloor *et al.*, 2020; Fu and Li, 2009). In Chapter 4, economical and high-quality induced mesenchymal stem cells can be synthesised by iPSCs with TGF inhibitor incorporating FGF, representing a significant advancement and offering a renewable and scalable source of therapeutic cells for regeneration medicine.

The incorporation of iMSCs into the repair therapeutic paradigm presents compelling opportunities. Through their conditioned medium, iPSCs-MSCs were shown to promote M2 polarisation in macrophages, reduce pro-inflammatory markers, and enhance tissue regeneration processes via increasing MerTK. Additionally, it has been demonstrated that exosomes promote anti-inflammatory macrophage polarisation through activation of the STAT3 pathway (Tian *et al.*, 2022), a finding that aligns with the results of this study. It is noteworthy that prior research has demonstrated that the activation of MerTK results in the phosphorylation of STAT3 (Eom *et al.*, 2018) (Fig.5.2). Therefore, it is possible that

iMSCs educate the polarisation of macrophage through the MerTK-STAT3 signalling axis.

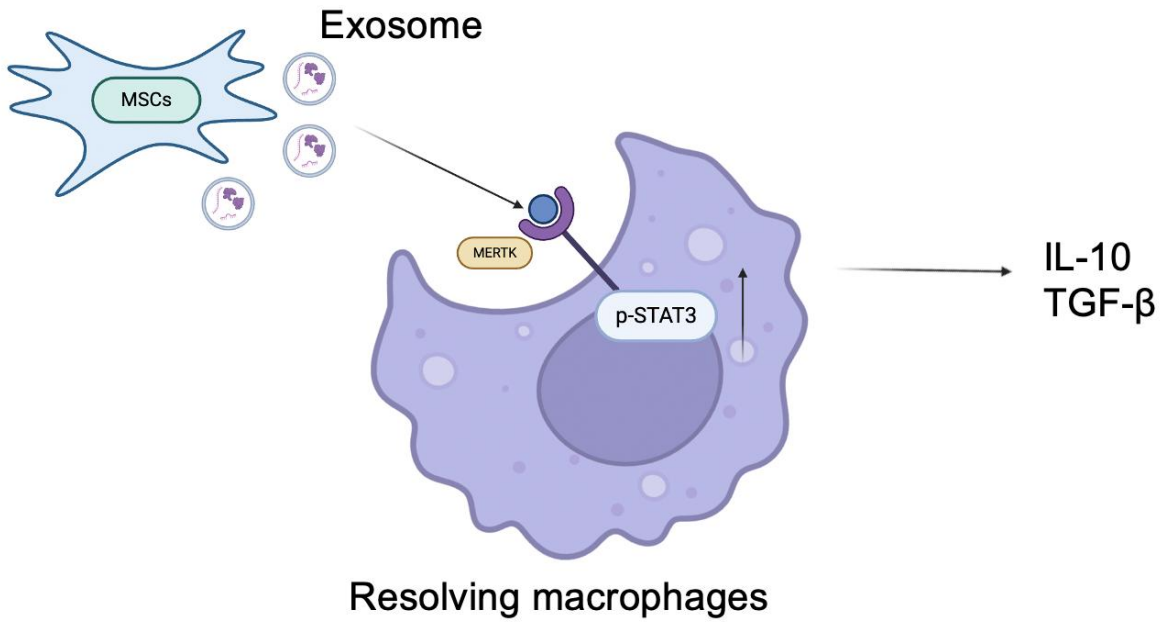

Fig.5.2 Mesenchymal Stem Cells (MSCs) Promote the Anti-inflammatory Activity of Resolving Macrophages Through Exosome-Mediated Signalling. MSCs release exosomes that interact with the MERTK receptor on macrophages, triggering downstream phosphorylation of STAT3 (p-STAT3). This signalling pathway enhances the expression of anti-inflammatory cytokines such as IL-10 and TGF-β, supporting the transition of macrophages into a resolving phenotype that contributes to inflammation resolution and tissue repair. Created in BioRender.com.

5.3 Limitations and Future Work

While the findings of this thesis provide valuable insights into the immunomodulatory effects of iPSCs-derived mesenchymal stem cells (iMSCs) on macrophage polarisation, several critical limitations must be acknowledged before these results can be confidently translated into clinical settings. A significant gap lies in the circadian regulation of MerTK, a receptor tyrosine kinase implicated in efferocytosis and macrophage phenotype modulation. Although previous studies have identified E-box elements in the MerTK promoter, suggesting a role for circadian transcriptional control, in vitro confirmation of human MerTK rhythmicity remains absent (Casanova-Acebes *et al.*, 2013; Parinot *et al.*, 2024). Real-time bioluminescence tracking using MerTK::LUC reporter constructs, ideally in conjunction with macrophage co-culture systems, could provide the temporal resolution necessary to delineate its dynamic expression patterns under physiologically relevant conditions.

The spatial relationship between CD206 and MerTK was also left unresolved. Although both markers were evaluated under comparable treatment protocols, the lack of co-localization studies leaves the cellular context of their expression uncertain, making it unclear whether they identify the same subset of macrophages or function independently. This is a crucial oversight, given that spatial coordination may underlie functional synergy during macrophage polarisation. Employing high-resolution imaging or flow cytometric co-staining strategies would clarify this relationship and strengthen conclusions drawn about macrophage subset identity and behaviour.

Additionally, the regulatory relationship between phosphorylated STAT3 (p-STAT3) and MerTK remains insufficiently characterised. Given that STAT3 is a well-established transcriptional regulator involved in anti-inflammatory macrophage programming and that MerTK is often associated with resolution-phase macrophages, their potential interaction warrants further investigation. Whether p-STAT3 directly modulates MerTK expression or whether their signalling pathways converge in shaping macrophage function is unknown. Advanced confocal microscopy to assess subcellular localisation and potential co-expression, combined with chromatin immunoprecipitation (ChIP) assays or loss-of-function experiments, could uncover previously unexplored regulatory mechanisms linking these two pathways.

Another underexplored axis of interaction is the bidirectional communication between macrophages and mesenchymal stromal cells. It is well established that mesenchymal stem cells facilitate the polarisation of macrophages towards a reparative M2 phenotype (Arabpour, Saghazadeh and Rezaei, 2021; Francois *et al.*, 2012; Zhang *et al.*, 2010). However, the reciprocal effects, especially the way macrophages influence the functionality of mesenchymal stem cells, have remained largely uncleared. A comprehensive understanding of how activated macrophages modify the transcriptomic or proteomic landscape of mesenchymal stem cells may reveal novel feedback mechanisms that are critical to the resolution of inflammation and tissue regeneration. Thus, integrating transcriptomic profiling of mesenchymal stem cells following exposure to macrophages could illuminate these potentially reciprocal dynamics.

A broader challenge concerns the experimental models used in macrophage differentiation. The use of monocytic cell lines such as U937, THP1, or BV2 microglia, while experimentally convenient, does not recapitulate the functional phenotype of primary human macrophages. These cell lines often exhibit aberrant responses and epigenetic profiles, raising concerns about the physiological relevance of the results. Moving toward primary human monocytes or monocyte-derived macrophages from peripheral blood would help mitigate this translational disconnect.

Equally critical is the intrinsic heterogeneity of iPSCs and their derivatives. Variability in differentiation efficiency, epigenetic state, and secretory profiles among iMSCs batches compromises both the reproducibility and therapeutic reliability of experimental findings. This variability may confound attempts to identify specific bioactive factors within the conditioned medium that drive M2 polarisation. Certainly, the study does not fully elucidate the specific molecular mediators involved in macrophage modulation, resulting in a lack of mechanistic clarity. The application of proteomic, metabolomic, and transcriptomic methodologies, including RNA sequencing and phosphoproteomic pathway mapping, has the potential to provide valuable insights into the signalling pathways activated by these secreted molecules.

Finally, the absence of *in vivo* validation limits the generalizability of the findings. While *in vitro* systems afford mechanistic clarity, they lack the spatial, temporal, and systemic complexity of living organisms. Future work must extend these investigations to appropriate animal models to assess the durability, efficacy, and safety of macrophage-modulating strategies, particularly those involving circadian

regulators or iMSCs-derived factors. These preclinical studies will be essential to determine therapeutic feasibility in chronic and acute inflammatory contexts.

In summary, although this thesis establishes a foundational understanding of iMSCs-macrophage interactions, its translational potential is contingent upon addressing limitations in spatial analysis, cellular models, and mechanistic clarity, particularly the unresolved connection between p-STAT3 and MerTK, along with *in vivo* validation. A systematic resolution of these challenges will not only strengthen the reliability of future findings but may also facilitate the advancement of next-generation immunomodulatory therapies.

5.4 Conclusion

Tissue repair is a fundamental biological process maintaining the body's integrity following injury. Despite the human body's remarkable ability to repair itself, certain conditions, including chronic wounds, ageing, diabetes, and inflammatory disorders, disrupt these processes, leading to persistent tissue damage and poor healing outcomes. This thesis explores the complex interplay between macrophages, circadian regulation, and induced pluripotent stem cell-derived mesenchymal stem cells (iPSCs-MSCs), providing new insights into the cellular and molecular mechanisms that underpin tissue repair and regeneration.

Macrophages play a central role in orchestrating the phases of tissue repair: inflammation, proliferation, and remodelling. These immune cells are distinguished by their remarkable plasticity, transitioning from an M1 pro-inflammatory phenotype in the early stages of healing to an M2 reparative phenotype during tissue regeneration. Dysregulation in this transition, often caused by sustained inflammation or an adverse microenvironment, can hinder healing and contribute to chronic wounds. Recent studies have identified key molecular pathways involved in macrophage function, including the Mer proto-oncogene tyrosine kinase (MerTK) and sialic acid-binding immunoglobulin-like lectin 3 (CD33), both of which regulate macrophage polarisation, inflammation resolution, and tissue repair. This study demonstrates that LPS inhibit the mRNA expression of the MerTK via RNA sequencing.

First, activating NF-kB inhibited the expression of MerTK. NF-kB inhibitors, including Dex, CBL01037, Bay11-7082, and SR9009, restore the LPS-inhibited MerTK but do not completely recover it. Therefore, it is partly inhibited via the NF-KB pathway. Additionally, IL-4/IL-13 was first found to promote the expression of MerTK in this study. Both increase the M2 macrophage marker CD206. So, it is possible that IL-4/IL-13 induces M2 by increasing MerTK expression.

Next, LPS treatment increased CEBPB and reduced CEBPA and CEBPE gene expression in CD33 WT macrophages. However, LPS-induced changes in CEBPB and CEBPA were blocked by CD33 knockout, but CEBPE gene expression was drastically reduced, as in CD33 WT macrophages. LPS-mediated reduction of MerTK gene expression was only paralleled with LPS-caused reduction of CEBPE gene expression, implying that CEBPE is the key possible early transcription factor.

Additionally, an E-box element (CACGTG) was identified in the promoter region of

MerTK. Consequently, a circadian experiment was conducted to observe the temporal variations in the expression of different MerTK protein isoforms. This research underscores the significance of circadian rhythms in macrophage-mediated healing. Circadian regulation governs the timing of immune responses and tissue regeneration, yet inflammatory stimuli, such as lipopolysaccharides (LPS), can disrupt circadian gene expression. This study provides compelling evidence that NF-κB-mediated inflammation suppresses MerTK expression, impairing macrophage homeostasis and prolonging the inflammatory phase. Such disruptions create a feedback loop that exacerbates tissue damage and delays the transition to the regenerative phase.

Induced pluripotent stem cells (iPSCs) have emerged as a transformative tool in regenerative medicine, offering a renewable source of mesenchymal stem cells (MSCs). iPSCs-derived MSCs possess significant immunomodulatory and reparative properties, making them ideal candidates for therapeutic applications. These cells have the capacity to modulate macrophage behaviour, facilitating a transition from an inflammatory to a reparative phenotype, thus enhancing tissue regeneration. In Chapter 5, the optimisation of iMSC production was achieved by incorporating a modest dose of FGF, contributing to cost reduction and generating high-quality MSCs. Subsequently, the supernatant derived from the produced cells was employed to treat the model of inflammatory-activated macrophages, exhibiting a pronounced inhibitory effect on inflammation. Concurrently, the expression of MerTK was observed to increase following treatment with the conditioned medium of MSCs, indicating that mesenchymal stem cells may elevate the expression of MerTK in macrophages, thereby inhibiting the polarization of pro-inflammatory macrophages. Additionally, iMSCs-derived exosomes regulate macrophages through the activation of the STAT3 pathway.

In conclusion, this thesis investigates the intricate mechanisms underpinning tissue repair, focusing on the roles of macrophage plasticity, circadian regulation, and iPSCs-derived mesenchymal stem cells (iMSCs). It demonstrates that macrophage-mediated inflammation and resolution are tightly regulated by molecular pathways, including MerTK. Inflammatory stimuli such as LPS were shown to suppress MerTK expression, partly through NF-κB activation and associated transcriptional changes, particularly the downregulation of CEBPE. Disruption of circadian rhythms by LPS further impairs MerTK expression, extending the inflammatory phase and delaying tissue regeneration. Additionally, this work highlights the potential of iMSCs to

modulate immune responses by promoting M2 macrophage polarisation, largely through increased MerTK expression and STAT3 activation. Optimisation of iMSCs production and application of their conditioned medium reveal promising strategies to enhance tissue repair by restoring macrophage balance and resolving inflammation.

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