ASSESSMENT OF CYTOKINE SECRETIONS BY MONOCYTES IN THE PRESENCE OF SYNOVIAL FLUID-DERIVED EXOSOMES

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by

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DECLARATION

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

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Date: 26/1/2025

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LIST OF ABBREVIATIONS

APS	Ammonium persulfate
C5a	Complement component 5a
C5aR1	Complement component 5a receptor 1
C5aR2	Complement component 5a receptor 2
CD9	Cluster of differentiation 9
CD63	Cluster of differentiation 63
CD81	Cluster of differentiation 81
CSF	Cerebrospinal fluid
DC	Dendritic cell
DNA	Deoxyribonucleic acid
DPBS	Dulbecco's phosphate-buffered saline
ECL	Enhanced chemiluminescence
ECM	Extracellular matrix
EDTA	Ethyenediamine tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ESCRT	Endosomal sorting complex required for transport
EV	Extracellular vesicle
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
HCL	Hydrochloric acid
HIV	Human immunodeficiency virus
HRP	Horseradish peroxidase
HSP70	Heat shock protein 70
IL-1β	Interleukin-1 beta

IL-6	Interleukin-6
ILV	Intraluminal vesicle
lncRNA	Long non-coding RNA
MIF	Macrophage migration inhibitory factor
miRNA	Micro RNA
MMP-2	Matrix metalloproteinase-2
mRNA	Messenger RNA
MVB	Multivesicular body
NTA	Nanoparticle tracking
OA	Osteoarthritis
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PRR	Pattern recognition receptor
PVDF	Polyvinylidene difluoride
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SF	Synovial fluid
SHED	Human exfoliated deciduous teeth stem cells
sICAM-1	Soluble intercellular adhesion molecule-1
TBS	Tris-buffered saline
TBS-T20	Tris-buffered saline-Tween 20
TEMED	Tetramethylethylenediamine
TKA	Total knee arthroplasty
TNF-α	Tumour necrosis factor alpha

PENILAIAN REMBESAN SITOKIN OLEH MONOSIT DALAM KEHADIRAN EXOSOME YANG BERASAL DARIPADA BENDALIR SINOVIA

ABSTRAK

Osteoartritis (OA) ialah penyakit sendi degeneratif yang sering berlaku, dicirikan oleh keradangan dan kemerosotan rawan. Exosome yang berasal daripada bendalir sinovia merupakan pengawal selia penting dalam patogenesis OA, yang mempengaruhi tindak balas imun dan rembesan sitokin. Kajian ini bertujuan menilai rembesan sitokin, iaitu molekul pelekat antara soluble intercellular adhesion molecule-1 (sICAM-1), complement component 5a (C5a), dan macrophage migration inhibitory factor (MIF), oleh monosit dalam kehadiran exosome OA. Exosome telah diasingkan daripada bendalir sinovia pesakit OA peringkat akhir melalui ultrasesentrifugasi dan dicirikan menggunakan Western blot serta analisis pengesanan nanopartikel (NTA). Saiz exosome telah disahkan berada antara 30 hingga 150 nm menggunakan NTA. Selain itu, exosome menunjukkan tetraspanin CD9, CD63, CD81, dan HSP70 menggunakan Western blot. Monosit, yang diasingkan daripada darah periferi penderma sihat, telah dikultur bersama exosome pada pelbagai nisbah (1:10, 1:20, dan 1:40) dan tempoh masa (24 dan 48 jam). Tahap sitokin diukur menggunakan ujian imunosorben berkaitan enzim (ELISA). Keputusan telah menunjukkan bahawa interaksi antara monosit dan exosome mempengaruhi rembesan sitokin bergantung kepada masa dan dos. sICAM-1 dan C5a menunjukkan penurunan dengan tempoh inkubasi yang lebih lama, kecuali pada kepekatan exosome yang lebih tinggi di mana rembesan C5a meningkat. Tahap MIF mencapai puncak selepas 48 jam, menunjukkan induksi sitokin yang tertunda. Penemuan ini menonjolkan peranan imunomodulasi exosome dalam OA, sekaligus memberikan pemahaman mengenai proses keradangan yang mendasari perkembangan penyakit.

ASSESMENT OF CYTOKINE SECRETIONS BY MONOCYTES IN THE PRESENCE OF SYNOVIAL FLUID-DERIVED EXOSOMES

ABSTRACT

Osteoarthritis (OA) is a prevalent degenerative joint disease marked by inflammation and cartilage deterioration. Synovial fluid-derived exosomes are emerging as key modulators in OA pathogenesis, influencing immune responses and cytokine secretion. This study aimed to assess cytokine secretions, specifically soluble intercellular adhesion molecule-1 (sICAM-1), complement component 5a (C5a), and macrophage migration inhibitory factor (MIF), by monocytes in the presence of synovial fluid-derived exosomes. Exosomes were isolated from the synovial fluid of late-stage OA patients through ultracentrifugation and characterised by Western blot and nanoparticle tracking analysis (NTA). Exosome size has been confirmed between 30 to 150 nm by NTA. Additionally, exosomes expressed tetraspanin markers CD9, CD63, CD81, and HSP70 as demonstrated by Western blot. Monocytes isolated from healthy donor peripheral blood, were cultured with exosomes at various ratios (1:10, 1:20, and 1:40) and time points (24 and 48 hours). Cytokine levels were quantified using enzyme-linked immunosorbent assay (ELISA). The results of this study showed that monocyte-exosome interactions influenced cytokine secretion in a time- and dose-dependent manner. sICAM-1 and C5a exhibited a declining trend with prolonged incubation, except at higher exosome concentrations, where C5a secretion was increased. MIF levels peaked after 48 hours, suggesting delayed cytokine induction. These findings highlight the immunomodulatory role of exosomes in OA, providing insight into the inflammatory processes underlying disease progression.

CHAPTER 1

INTRODUCTION

1.1 Background of study

Monocytes are circulating white blood cells capable of differentiating into macrophages and dendritic cells (DCs) in various tissues. Monocytes in the circulation originate from precursors in the bone marrow. Monocytes are categorised into subsets that vary in size, receptor expression, and their capacity to differentiate upon stimulation with cytokines and microbial molecules. Monocytes play a crucial role in host antimicrobial defence and are associated with various inflammatory diseases. Moreover, monocytes are recruited to tumour sites and can suppress tumour-specific immune defence mechanisms (Shi & Pamer, 2011). Monocytes circulate in the circulation during inflammation and extravasate into inflamed tissues, following the established leukocyte recruitment cascade, which includes rolling, adhesion, and transmigration (Kratofil et al., 2017). Monocytes play important roles in inflammation as well as in diseases such as osteoarthritis (OA).

OA is classified as a degenerative cartilage disease, marked by a progressive loss of function due to a variety of circumstances, including genetic susceptibility, excessive body weight, ageing, surgical joint treatments, and repetitive joint injuries. Osteoarthritis is the most prevalent chronic articular disease, which can lead to impairment, loss of function, and lower quality of life (Leifer et al., 2022). Osteoarthritis can be classified into two categories which are primary OA and secondary OA.

Primary OA is the deterioration of joints without any identifiable cause, and most often occurs to people of older age. On the other hand, secondary OA results from either

an irregular distribution of force throughout the joint, as seen in post-traumatic cases, or from unusual articular cartilage, and can occur to both young and old people. The severity of clinical symptoms and rate of progression may differ among individuals. Nevertheless, they generally intensify, increase in frequency, and become more impairing over time (Hsu & Siwiec, 2023).

Exosomes are small, extracellular vesicles released by all living cells, serving as key mediators of intercellular communication and tissue crosstalk. They hold significant potential as biomarkers and therapeutic agents for disease diagnosis, prognosis, and treatment monitoring. Exosomes facilitate the transfer of epigenetic information between cells, influencing recipient cell functions and reprogramming their activities (Dilsiz, 2024). They transport diverse biomolecules, including metabolites, DNA fragments, mRNAs, lncRNAs, miRNAs, proteins, and lipids. In OA, exosomes play a pivotal role in pathogenesis, treatment resistance, inflammatory responses, and immune regulation, thereby contributing to disease progression (Dilsiz, 2024).

1.2 Problem statement

Monocytes are one of the immune cells involved in inflammation. In OA, a localised inflammatory response occurs in the joint, which attracts monocytes from the peripheral circulation to the synovium and articular cartilage. Exosomes found in the synovial fluid (SF) of OA patients play a role in the pathogenesis of the disease. Little is known about the interactions between exosomes derived from the synovial fluid and healthy monocytes, and how these interactions affect cytokine production. Therefore, there is a gap in understanding how synovial fluid-derived exosomes influence monocyte cytokine secretions.

1.3 Rationale of study

This study aims to elucidate the immunomodulation of monocytes in the presence of exosomes derived from synovial fluid of OA patients on cytokine production. This aids in identifying potential targets for therapeutic intervention and enhances understanding of the pathogenesis of OA.

1.4 Research objective

1.4.1 General objective

The general objective of this study would be to determine the cytokine secretions, sICAM-1, C5a, and MIF by monocytes in the presence of exosomes derived from synovial fluid of OA patients.

1.4.2 Specific objective

i. To characterise the size of exosomes derived from synovial fluid of OA patients.
ii. To characterise the protein expression of exosomes (CD9, CD63, CD81, HSP70).
iii. To assess cytokine secretions (sICAM-1, C5a, and MIF) by monocytes in the presence of synovial fluid-derived exosomes.

CHAPTER 2

LITERATURE REVIEW

2.1 Immune System

The human immune system is an intricate network of cells, receptors, and molecules that protects the body against infections and tissue damage while also coordinating tissue healing and repair. The system is divided into two categories which are innate and adaptive. Innate immune system is the first line of defence against infectious pathogens depending on specific receptors that identify the pathogens' characteristics. These receptors reside on numerous cells, allowing quick response during an infection. In contrast, the adaptive immune system was thought to be responsible for the development of immunological memory due to its specialised ability to identify individual infections. It frequently provides the body with lifelong immunity towards reinfection from the same pathogen (Austermann et al., 2022).

Innate immunity consists of four types of defensive barriers which are anatomic (skin and mucous membranes), physiologic (temperature, low pH, and chemical mediators), endocytic and phagocytic, and inflammatory responses. Innate immunity to pathogens depends on pattern recognition receptors (PRRs) that enable a select group of immune cells to swiftly identify and respond to various pathogens sharing common structures, referred to as pathogen-associated molecular patterns (PAMPs) (Marshall et al., 2018). The primary role of innate immunity is the quick movement of immune cells to areas of infection and inflammation, facilitated by the secretion of cytokines and chemokines, which are small proteins that mediate cell-cell communication and recruitment. Cytokine production in innate immunity activates various defence mechanisms across the body and stimulates local cellular responses to infection or

injury. The phagocytic function of the innate immune response facilitates the removal of dead cells, antibody complexes, and foreign substances from organs, tissues, blood, and lymph (Marshall et al., 2018).

Furthermore, the adaptive immune system's development is facilitated by the innate immune system's functions and is essential when innate immunity fails to eradicate infectious agents. The adaptive immune response primarily functions to recognise specific “non-self” antigens, distinguishing them from “self” antigens by generating pathogen-specific immunologic effector pathways that eliminate specific pathogens or pathogen-infected cells, and develop immunologic memory that enables rapid elimination of specific pathogens upon subsequent infections (Marshall et al., 2018).

2.2 Monocyte

Monocytes are white blood cells that originate from the bone marrow. With a diameter of 12 to 20 µm, they are the largest white blood cells and roughly twice as large as red blood cells. Due to their relatively large size and convoluted bilobed nuclei, which are frequently referred to as kidney-shaped, monocytes are usually easy to spot in peripheral blood under the microscope. As a component of the innate immune response, monocytes help maintain cellular homeostasis, particularly when inflammation and infection are present. Monocytes play two different roles in which they establish an immune response during infection and inflammation and routinely patrol the body for microbial cells (Espinoza & Emmady, 2023). The surface of monocytes consists of toll-like receptors that interact with pathogen-associated molecular patterns (PAMPs), which are present on the microbial cells. Monocytes migrate from the bone marrow into the

bloodstream and enter tissue sites in 12 to 24 hours in response to these stimuli (Espinoza & Emmady, 2023).

Monocytes initially adhere to the endothelium before gently rolling over the vascular surface to enter the affected areas. Diapedesis is the process by which the monocyte eventually passes through the endothelial cells after adhering strongly to the endothelium (Espinoza & Emmady, 2023). After that, monocytes migrate to the site of inflammation by passing through the endothelium. Both monocyte and endothelial cells interact to initiate the adhesion process. In the peripheral circulation, monocytes function as phagocytes and antigen-presenting cells by ingesting and eliminating foreign pathogen, dead or damaged cells (Espinoza & Emmady, 2023).

2.2.1 Monocytes in inflammation

Monocytes are essential components of the innate immune system and play a crucial role in regulating inflammation. Monocytes are essential in generating inflammatory mediators, regulating innate and adaptive immunity, and facilitating the resolution of inflammation and the restoration of homeostasis. Monocyte dysfunction is often implicated in the pathophysiology of chronic infections as well as in non-infectious inflammatory and autoimmune diseases. Monocytes constitute 5-10% of the total blood cell components with a lifespan of approximately 1 to 3 days in the circulation. During steady state, monocytes play an important role in homeostasis. Following infection, monocytes migrate to the site of inflammation and differentiate into macrophages or dendritic cells (Austermann et al., 2022).

The inflammatory response initiated by infection or tissue damage involves the interplay of numerous cellular and molecular processes. Pathogen recognition is an important step in eliciting an appropriate immune response during infection.

Activation and polarisation of monocytes occur via the recognition of preserved molecular motifs associated with pathogens and tissue damage, mediated by pattern recognition receptors (PRRs). PRRs recognise two primary classes of molecules including pathogen-associated molecular patterns (PAMPs), a highly conserved structural motifs found in pathogens such as viruses, bacteria, fungi, and parasites (Austermann et al., 2022).

2.2.2 Cytokines secreted by monocytes

Cytokines are soluble proteins that are essential for cell signalling during inflammation and immunological processes. Cytokines are secreted by numerous immune and non-immune cells. They act as mediators enabling intercellular communication and guiding the host response to inflammation, infection, and tissue injury. Cytokines are soluble molecules with a molecular weight of less than 30 kDa, encompassing chemokines, interferons, interleukins, lymphokines, and the tumour necrosis factor family of proteins (Megha et al., 2021). These molecules integrate signals from diverse cell types and regulate the growth and activity of target cells. Cytokines influence nearly all biological processes, and their downstream effects are fundamental to diseases including OA, autoimmune diseases, and cancer (Benjamin-Davalos et al., 2021).

Cytokines can either promote or suppress inflammation, depending on their type and the context in which they are secreted either pro-inflammatory cytokines or anti-inflammatory cytokines. Due to their dual role, cytokines can regulate immune functions and therefore, maintaining a balance between defence and tissue repair (Tripathi, 2023). However, persistent inflammation and tissue damage can result from an imbalance in cytokine production in inflammatory diseases such as OA. Excess production of pro-inflammatory cytokines worsens the progression of conditions like

OA by driving joint tissues deterioration and worsen inflammation (Tripathi, 2023).

Pro-inflammatory cytokines possess immune properties that can aid the host in combating invasions by bacteria and other microorganisms in the host environment. The primary function of cytokines is to convey to adjacent tissues the presence of infection or injury. Furthermore, cytokines can enter systemic circulation, leading to immune cell activation and changes in host physiology, including fever and the acute-phase response. On the other hand, anti-inflammatory cytokines are immunoregulatory molecules that suppress the excessive inflammatory response induced by pro-inflammatory cytokines (Duque & Descoteaux, 2014). Under normal physiological conditions, cytokines help control the potentially harmful effects of prolonged or excessive pro-inflammatory responses. Anti-inflammatory cytokines have demonstrated efficacy in various clinical conditions characterised by excessive inflammation. Anti-inflammatory cytokines may serve as therapeutic agents for the treatment of inflammation-related diseases. Nevertheless, cytokine therapy has several limitations when compared to anti-inflammatory biologics, including neutralising antibodies (Liu et al., 2021). Following stimulations, monocytes secrete various cytokines including soluble intercellular adhesion molecule-1 (sICAM-1), complement component 5a (C5a), and macrophage migration inhibitory factor (MIF). These cytokines are crucial for modulating inflammation and recruiting immune cells to sites of injury or infection.

2.2.2.1 Soluble intercellular adhesion molecule-1 (sICAM-1)

Soluble intercellular adhesion molecule-1 (sICAM-1) represents a circulating variant of ICAM-1, a glycoprotein located on the cell surface that plays a role in the adhesion and signalling of immune cells. It is produced through proteolytic cleavage of membrane-bound ICAM-1 or as an alternative splice variant that lacks membrane-binding domains. Increased concentrations of sICAM-1 correlate with inflammation,

modulation of immune responses, and a range of pathological conditions, such as cancer, cardiovascular diseases, and chronic pain (Lüke et al., 2021).

sICAM-1 has a dual function in the regulation of the immune system. It interacts with integrins on leukocytes, enhancing immune cell adhesion to endothelial cells and supporting migration to inflammatory sites. Furthermore, its soluble form functions as a decoy receptor, blocking the interaction between immune cells and tissues, thus influencing inflammation and immune surveillance (Lüke et al., 2021). Under pathological condition, increased sICAM-1 levels are associated with tumour progression, metastasis, and angiogenesis, primarily through the disruption of host immune responses and the creation of a pro-tumorigenic environment (Lüke et al., 2021).

2.2.2.2 Complement Component 5a (C5a)

The complement system is a critical element of innate immunity, comprising multiple pathways that strengthen the host defence against infections. A significant effector molecule in this system is the anaphylatoxin C5a, which serves as a chemoattractant and an inflammatory mediator, engaging with its receptors, C5aR1 and C5aR2, to modulate various immune responses (Li et al. 2019). C5a is produced through the cleavage of complement component C5 and serves as a key regulator of inflammation. It promotes chemotaxis and activation of immune cells such as neutrophils, macrophages, and T cells. C5a promotes the release of pro-inflammatory cytokines, thereby amplifying inflammatory responses. Signalling via C5aR1 is crucial in acute inflammatory processes and tissue damage, while C5aR2 may exhibit regulatory or anti-inflammatory functions contingent on the context (Mrozewski et al., 2024).

2.2.2.3 Macrophage migration inhibitory factor (MIF)

Macrophage migration inhibitory factor (MIF) is a proinflammatory cytokine synthesised by both hematopoietic and non-hematopoietic sources. MIF is a homotrimer with a barrel-like structure and exhibits tautomerase enzymatic activity (Vázquez et al., 2023). It is a multifunctional cytokine that plays a role in immune regulation, inflammation, and cancer progression. MIF, initially recognised as a T-cell product that inhibits macrophage migration, is now recognised to be expressed across various cell types and is essential in immune and inflammatory responses (Valdez et al., 2024).

MIF serves as a crucial upstream mediator of both innate and adaptive immunity, as well as survival pathways that facilitate pathogen clearance, thereby contributing to protection during infectious diseases. MIF, as an immune modulator, worsens harmful inflammation and encourages cancer metastasis and progression, thereby deteriorating disease conditions. Multiple reports have indicated that genetic and physiological factors, such as MIF gene polymorphisms, posttranslational modifications, and receptor binding, regulate the functional activities of MIF (Sumaiya et al., 2022).

2.3 Extracellular vesicles (EV)

Extracellular vesicles (EVs) are tiny particles of lipid bilayer structures secreted by cells, originating from various sources. Extracellular veiscles have been detected in biological samples and cell cultures from human patients, as well as in non-human cells, and across various organisms including plants, bacteria, fungi, and parasites (Du et al.,

2023). Extracellular vesicles transport a diverse array of materials, encompassing proteins like cell surface receptors, signalling proteins, transcription factors, enzymes, and extracellular matrix proteins. Additionally, they also consist of lipids and nucleic acids (including DNA, mRNA, and miRNA) that can be transmitted from parent cells to recipient cells thereby, facilitating molecular transfer and intercellular communication (Kumar et al., 2024). Extracellular vesicles consist of several subtypes categorised by their synthesis and release mechanisms, such as exosomes, apoptotic bodies, and microvesicles. Previous study has shown that EVs mediate a range of physiological and pathological cellular activities (Figure 2.1) by transporting diverse biomolecules and accomplishing intercellular component exchange through a variety of intercellular signalling pathways (Du et al., 2023).

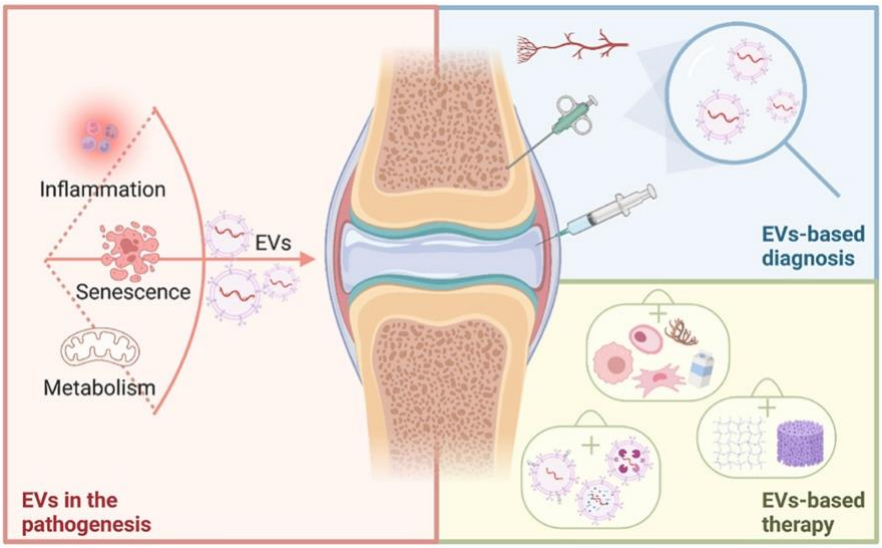

Figure 2.1: The role of extracellular vesicles (EV). EVs contribute to OA pathogenesis by mediating processes such as inflammation, cellular senescence, and metabolic dysregulation. EVs also have a high potential for clinical applications, including their use as diagnostic markers for OA and as therapeutic tools which includes drug delivery, tissue repair, and immune modulation (adapted from Liu et al., 2023).

2.4 Exosome

Exosomes represent a subtype of EV characterised as small membranous vesicles found within the cartilage extracellular matrix (ECM) (Xian et. al., 2022). The size of exosomes is ranged between 30 to 150 nm. They are released by both prokaryotic and eukaryotic cells to facilitate intercellular communication and signalling. Further research has shown that exosomes serve as crucial molecular mediators in cellular communication, facilitating the transport of proteins, metabolites, and various nucleic acids throughout the body (Chen et al., 2024).

Exosomes are secreted by various cell types, including immune cells, cancer cells, and stem cells. Exosomes are essential for physiological regulation, disease progression, immune response, and disease development due to their various functions in intercellular information (Chen et al., 2024). The function and biological properties of exosomes are determined by their cargo, which includes proteins, lipids, and nucleic acids. Exosomes express tetraspanin proteins, such as CD9, CD63, CD81, and HSP70 which are distinguished by a specific molecular structure making it often used as exosome markers (Jankovičová et al., 2020). Under pathological condition, exosomes released by cells can facilitate disease progression, while those released by therapeutic cells may aid in disease treatment. Exosomes originating from therapeutic cells can modulate chondrocyte proliferation, apoptosis, and inflammation, while also enhance ECM synthesis (Xian et al., 2022). Exosomes are also released by biological fluids such as the synovial fluid (SF) and cerebrospinal fluid (CSF).

2.4.1 Biogenesis of exosome

The exosome biogenesis pathway begins with the endocytosis of molecular cargo into the cell. The early endosome, formed by the plasma membrane budding into the cell, serves as the initial vesicle in the endosomal trafficking pathway. Its primary function is

to conduct sorting and determine the fate of endocytosed cargo (Krylova & Feng, 2023). Early endosomes contribute to the endosomal maturation pathway, leading to the multivesicular structure of late endosomes. Exosomes are continuously produced from late endosomes, which develop from the inward budding of the limited multivesicular body (MVB) membrane (Figure 2.2). Late endosomal membrane invagination leads to the generation of intraluminal vesicles (ILVs) within large MVBs. In this process, specific proteins integrate into the invaginating membrane, while cytosolic components are engulfed and stored within the ILVs. Most ILVs are released into the extracellular space through fusion with the plasma membrane, and known as exosomes (Zhang et al., 2019).

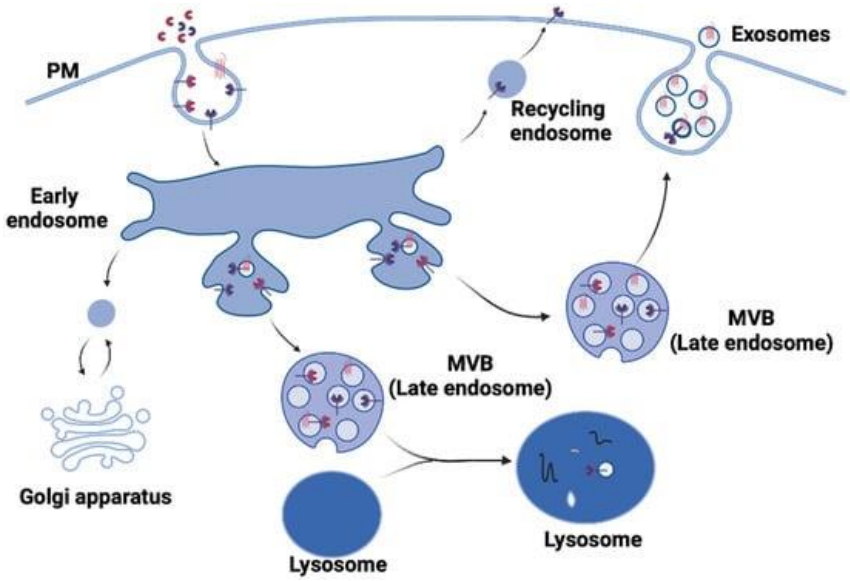

Figure 2.2: Biogenesis of exosomes. Cargo molecules are sorted via endocytosis into early endosomes, which undergo maturation into multivesicular late endosomes (MVBs). MVBs then fuse with the plasma membrane, releasing exosomes (adapted from Krylova & Feng, 2023).

2.5 Osteoarthritis (OA)

Osteoarthritis is a gradually advancing condition of the synovial joint. The joints may become stiff, painful, and inflamed as the cartilage, which serves as a smooth

cushion between the bones, degrades. Although OA can impact any joint, it predominantly affects the knees and hips (Kumar et al., 2023). The key features of OA include cartilage degeneration, bone changes, and joint inflammation (Figure 2.3). Generally, OA manifests as joint pain and functional impairment; nevertheless, the illness demonstrates significant clinical variability, ranging from asymptomatic incidental findings to a very debilitating condition (Sen & Hurley, 2023).

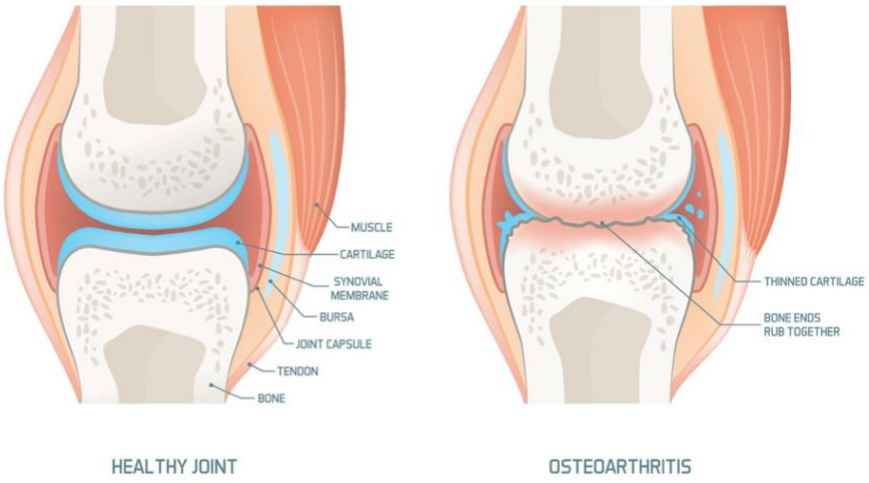

Figure 2.3: Difference between healthy knee and osteoarthritis knee. Healthy joints consist of intact cartilage, providing a smooth protective layer between bones, along with a well-functioning synovial membrane, and joint capsule that ensures proper movement and cushioning. Osteoarthritis-affected knee shows thinned cartilage leading to friction of bone ends, causing pain, stiffness, and impaired joint function and mobility (adapted from https://www.healthdirect.gov.au/osteoarthritis).

The prevalence of osteoarthritis increases as the population ages, and its consequences have a significant impact on society. Osteoarthritis is the biggest contributor of adult impairment, impacting approximately 240 million people globally and contributing to a 20% increase in age-adjusted mortality rates. About 30% of those aged 45 years old and above exhibit radiographic evidence of knee OA, with nearly 50% reporting painful symptoms (Liu et al, 2024). A thorough examination of OA shows that

it is divided into five stages based on the Kellgren and Lawrence grading system, which range from stage 0 to stage 4 (Bandoim, 2024). Stage 0 OA shows no radiographic findings of OA. An individual with stage 1 OA exhibits minimal bone spur development. Bone spurs are osseous growths that frequently form at the junctions of articulating bones within a joint. There may be a slight loss of cartilage, but it is insufficient to jeopardise the joint space. An individual with stage 1 OA typically does not experience pain or discomfort due to the minimal deterioration of the joint components. Stage 2 OA is regarded as a minor stage of the disease. This stage will demonstrate increased bone spur development, although the cartilage typically remains of a healthy dimension. The interstitial space between the bones is normal, and the bones are not in contact or abrading each other. At this stage, SF is generally still available in adequate quantities for normal joint movement. However, individuals may initially encounter symptoms, including pain following prolonged periods of walking or jogging, increased joint stiffness after extended inactivity, and tenderness during kneeling or bending (Bandoim, 2024).

Stage 3 OA is classified as moderate OA. At this stage, the cartilage between the bones exhibits significant deterioration, and the interstitial space between the bones begins to diminish. Individuals with stage 3 OA are prone to recurrent pain when walking, running, bending, or kneeling. They may also encounter joint stiffness following prolonged durations of sitting or upon awakening in the morning. Joint edema may occur during prolonged periods of activity. Stage 4 OA is deemed as severe. Individuals with stage 4 OA endure significant pain and discomfort with ambulation or joint movement. The interstitial space between the bones is significantly diminished. The cartilage is nearly entirely absent, resulting in a stiff and potentially immobile joint. The SF is significantly diminished, impairing its ability to mitigate friction between the

articulating components of a joint (Bandoim, 2024).

Osteoarthritis is a condition that affects all components of the joint, without exception. The aetiology of OA involves a combination of risk factors, mechanical stress, and abnormal joint mechanics. The interaction results in pro-inflammatory markers and proteases that ultimately facilitate joint destruction. The initial alterations in osteoarthritis typically manifest in the articular cartilage, characterised by surface fibrillation, irregularity, and focal erosions. The erosions ultimately reach the bone and progressively enlarge to encompass a greater area of the joint surface. Following cartilage damage, the collagen matrix becomes damaged, leading to the proliferation of chondrocytes and the formation of clusters (Sen & Hurley, 2023). A phenotypic transition to hypertrophic chondrocytes occurs, resulting in cartilage outgrowths that undergo ossification and develop into osteophytes. Increased damage to the collagen matrix leads to chondrocyte apoptosis, and improper mineralisation of collagen leads to thickening of subchondral bone, with bone cysts occurring rarely in advanced stages of the disease. In end-stage OA, the presence of calcium phosphate and calcium pyrophosphate dihydrate crystals is noted, which are thought to play a part in synovial inflammation (Sen & Hurley, 2023).

CHAPTER 3

MATERIALS AND METHODS

3.1 Overview of the study

This study is conducted to assess the cytokine secretions, sICAM-1, C5a, and MIF by monocytes in the presence of exosomes derived from the synovial fluid (SF) of primary osteoarthritis (OA) patients. The flow of the study is summarised as shown in Figure 3.1. First, SF was collected from primary OA patients. Then, the collected SF was processed, and exosomes were isolated from the sample by ultracentrifugation. Next, exosome samples were characterised by Western Blot and nanoparticle tracking analysis (NTA). Cytokine secretions were quantified by Enzyme-Linked Immunosorbent Assay (ELISA).

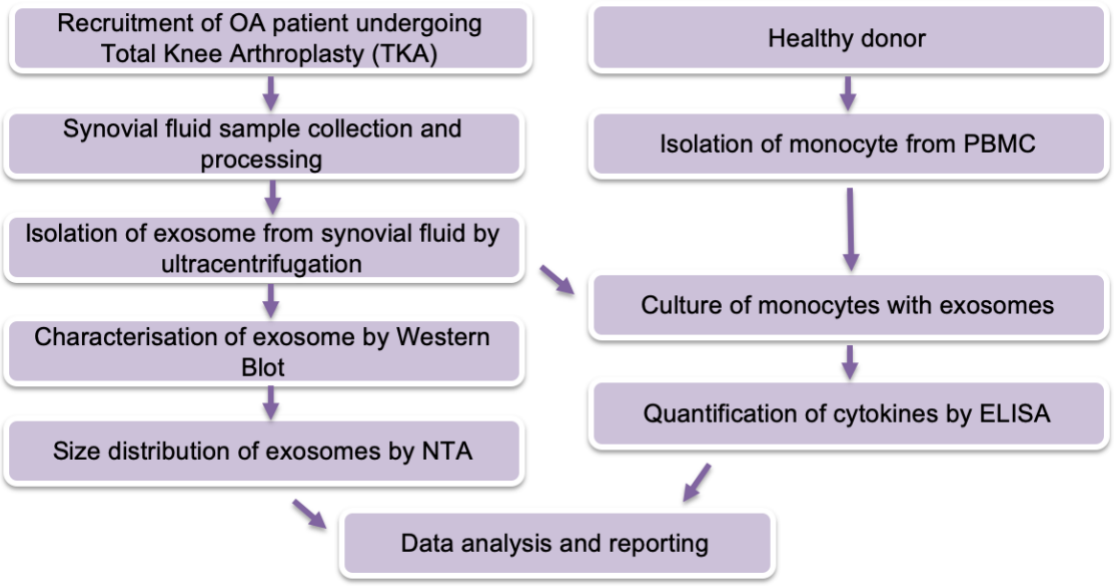

Figure 3.1: Flowchart of study

3.2 List of chemicals, reagents, kits and consumables

The chemicals, reagents, kits, and consumables used in this study are listed in Tables 3.1, 3.2, and 3.3, respectively.

Table 3.1: List of chemicals and reagents

Chemicals and reagents	Manufacturer
10% Sodium dodecyl sulfate (SDS)	Nacalai Tesque, Japan
30% Acrylamide	Nacalai Tesque, Japan
5X MojoSort buffer	BioLegend, USA
Absolute ethanol	HmbG, Germany
Ammonium persulfate (APS)	Bio-Rad, USA
Bleach	Clorox, USA
Calnexin antibody	ABclonal, China
Anti-human CD9 antibody	System Biosciences, USA
Anti-human CD63 antibody	System Biosciences, USA
Anti-human CD81 antibody	System Biosciences, USA
DNase I	Novagen, USA
Dulbecco's phosphate-buffered saline (DPBS)	Corning, USA
Enhanced chemiluminescence (ECL) substrate	Bio-Rad, USA
Ethylenediamine tetraacetic acid (EDTA)	Sigma-Aldrich, USA
GAPDH antibody	System Biosciences, USA
Glycine	Bio-Rad, USA
Goat anti-rabbit HRP	System Biosciences, USA
HSP70 antibody	System Biosciences, USA
Hyaluronidase	Sigma-Aldrich, USA
Isopropanol	Merck, Germany
Laemmli buffer	Sigma-Aldrich, USA
Lymphocyte Separation Medium (LSM)	Corning, USA

Methanol	Supelco, USA
Phosphate-buffered saline (PBS) tablets	Thermo Fisher Scientific, USA
Protein ladder	GeneDireX, Taiwan
Resolving gel buffer	Bio-Rad, USA
RPMI-1640 media	Sigma-Aldrich, USA
Skimmed milk powder	Sunlac, New Zealand
Stacking gel buffer	Bio-Rad, USA
Tetramethylethylenediamine, (TEMED)	Bio-Rad, USA
Tris(hydroxymethyl)aminomethane, Tris	Bio-Rad, USA
Trypan blue	Sigma-Aldrich, USA

Table 3.2: List of kits

Kits	Manufacturer
ExoAB Antibody Kit	System Biosciences, USA
Human C5a ELISA Kit	RayBiotech, USA
Human MIF ELISA Kit	RayBiotech, USA
Human sICAM-1 ELISA Kit	RayBiotech, USA
MojoSort Magentic Cell Separation System	BioLegend, USA
Western ECL Substrate	Bio-Rad, USA

Table 3.3: List of consumables

Consumables	Manufacturer
10 mL vacutainer EDTA tubes	BD Biosciences, USA
6-well plate	SPL Lifesciences, Korea

21-G butterfly needle	Becton, Dickson and Company, USA
Alcohol swabs	Hospitech, Malaysia
Centrifuge tubes (15 mL, 50 mL)	Bio-Rad, USA
Gloves	IRONskin, Malaysia
Microcentrifuge tubes (1.5 mL)	SPL Life Sciences, Korea
Parafilm	Thomas Scientific, USA
Pasteur pipette	Biologix, China
Pipette tips (10 µL, 200 µL,1000 µL)	Labcon, USA
Syringe (50 mL)	Hospitech, Malaysia
Syringe filter (0.22 µL)	Bioflow, Malaysia
Ultracentrifuge tubes (4 mL)	Thermo Scientific, USA

3.3 List of laboratory equipment and apparatus

The laboratory equipment, apparatus, and software used in this study are listed in Tables 3.4 and 3.5, accordingly.

Table 3.4: List of laboratory equipment and apparatus

Laboratory equipment and apparatus	Manufacturer
Beaker	HmbG, Germany
Biosafety cabinet level II	ESCO, Singapore
CO_2 incubator	ESCO, Singapore
Deep freezer (-80°C)	ILSHIN BioBase, South Korea
Duran Schott bottle	Thermo Fisher Scientific, USA
Hemocytometer	Thermo Scientific, USA

Laboratory refrigerator (4°C, -20°C)	Toshiba, Japan
Light microscope	Leica Microsystems, Germany
Microplate reader	Tecan, Switzerland
Multichannel pipette (20-200 µL)	Axygen, USA
Pipettes (10 µL, 200 µL, 1000 µL)	Sartorius, USA
Semi-dry Transfer System	Bio-Rad, USA
Sorvall WX 100+ Ultracentrifuge	Thermo Scientific, USA
Universal 320 & Micro 22R centrifuge	Hettich Zentrifugen, Germany
Vertical Electrophoresis Cell	Bio-Rad, USA
Water bath	Memmert, Germany

Table 3.5: List of softwares

Software	Manufacturer
Fusion X	Vilber Lourmat, France
GraphPad Prism	GraphPad, USA
Microsoft Excel	Microsoft, USA
Tecan i-control	Tecan, Switzerland

3.4 Buffer and reagents

3.4.1 Preparation of 70% ethanol

The preparation of 70% ethanol was prepared by mixing 700 mL absolute ethanol with 300 mL distilled water. The solution was stored at room temperature until used.

3.4.2 Preparation of 1X Phosphate-Buffered Saline (PBS)

One tablet of PBS was dissolved in 1 L distilled water and mixed thoroughly. The solution was stored at room temperature until used.

3.4.3 Preparation of 30 U/mL Hyaluronidase

A 30 U/mL hyaluronidase solution was prepared by diluting 50 µL of 30,000 U/mL hyaluronidase stock solution with 49,5000 µL distilled water and stored at -20°C until further use.

3.4.4 Preparation of 20 U/mL DNase I

A 20 U/mL DNase I was prepared by diluting 3 µL of 10,000 U/mL DNase I stock solution with 150 µL 10X incubation buffer and stored at -20°C until further use.

3.4.5 Preparation of 4.84 mM EDTA/DPBS

Lyophilised ethylenediamine tetraacetic acid (EDTA) buffer powder of 0.73g was dissolved in 50 mL distilled water to produce a 50 mM stock solution. Then, 5,808 mL of the stock solution was diluted with 54,192 mL of DPBS. The diluted solution was aliquoted into 50 mL tubes and stored at -20°C until further use.

3.4.6 Preparation of 1X Running buffer

Running buffer of 1X was prepared by dissolving 3g Tris, 14.4g glycine, and 1g sodium dodecyl sulfate (SDS) in distilled water and the final volume was adjusted to 1 L. The solution was stirred using a magnetic stirrer, and the pH of the solution was adjusted

to 8.2 by using 1M hydrochloric acid (HCL). The solution was then stored at room temperature until further use.

3.4.7 Preparation of 10X Tris-Buffered Saline (TBS)

TBS solution of 10X was prepared by dissolving 24g Tris and 88g NaCl in distilled water and the final volume was adjusted to 1 L. The pH of the solution was adjusted to 7.6 byusing 1M hydrochloric acid (HCL) The solution was stirred overnight using a magnetic stirrer. The solution was then stored at room temperature until further use.

3.4.8 Preparation of 1X Tris-Buffered Saline-Tween 20 (TBS-T20)

TBS-T solution of 1X was prepared by mixing 100 mL 10X TBS, 1 mL Tween-20 and the final volume was adjusted with distilled water to 1 L. The solution was stirred overnight using a magnetic stirrer and then stored at room temperature until further use.

3.4.9 Preparation of Towbin transfer buffer

Towbin transfer buffer was prepared by dissolving 3.03g Tris and 19.9g glycine in 200 mL methanol and the final volume was adjusted with distilled water to 1 L. The solution was stirred overnight using a magnetic stirrer and then stored at room temperature until further use.

3.4.10 Preparation of 10% skimmed milk

Skimmed milk 10% was prepared by dissolving 10g low-fat milk powder in 100 mL 1X TBS-T20 solution and stirred. The solution is freshly prepared prior to use.

3.4.11 Preparation of 10% Ammonium Persulfate (APS)

APS 10% was prepared by dissolving 0.1g APS in distilled water to the final volume of 1 mL. The solution is freshly prepared prior to use.

3.5 Methods

3.5.1 Healthy blood collection

In this study, five healthy donors (n=5) were recruited for blood sample collection. Blood sample was collected from healthy donors with appropriate informed consent as approved by the Human Research Ethics Committee USM (USM/JEPeM/21010038). Approximately, 30 mL blood were collected from each donor and drawn into several EDTA tubes. The inclusion criteria include donors who are healthy with body weight above 45 kg and have an ideal BMI. The donors were male or female between the age of 21 to 50 years old. Meanwhile, the exclusion criteria exclude individuals with chronic diseases such as human immunodeficiency virus (HIV) and cancer, as well as taking antibiotic, aspirin, alcohol, steroid or any other medications in the past three days (Table 3.6).

Table 3.6: The inclusion and exclusion criteria of blood donors

Inclusion Criteria	Exclusion Criteria
Male/female aged 21-50 years old	Have chronic diseases such as HIV and cancer
Healthy and have body weight over 45kg aswell as ideal BMI	Took any medications such as antibiotic, aspirin, alcohol or steroid in the past 3 days

3.5.2 Synovial fluid (SF) sample collection

This study requires five late primary OA patients (n=5) who underwent total knee arthroplasty (TKA). Synovial fluid samples were obtained from patients with proper informed consent as approved by the USM Human Research Ethics Committee (USM/JEPeM/21010038). Approximately 3 mL SF was extracted by a surgeon from the