IMMUNOPHENOTYPIC PROFILES OF MONOCYTIC MICROPARTICLES DERIVED FROM HUMAN BLOOD MONOCYTES AND THEIR POTENTIAL ROLE IN COAGULATION AND ENDOTHELIAL CELL ACTIVATION

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by

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LIST OF SYMBOLS AND ABBREVIATIONS

%	Percentage
°C	Degree Celsius
µg	Microgram
µL	Microliter
µm	Micro metre
µM	Micro molar
APC	Antigen-presenting cell
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
CCR	C-C chemokine receptor
CD	Cluster of differentiation
CLP	Common lymphoid progenitor cells
CMP	Common myeloid progenitors
CO_2	Carbon dioxide
CSF	Colony-stimulating factors
CX3CR1	Fractalkine receptor
CXCR	Chemokine receptor CXCR
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
eMP	Endothelial microparticles
ERK1/2	Extracellular signal-regulated kinase 1/2

FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
FSC	Forward scatter
g	Gram
GMP	Granulocytes-macrophages progenitor cells
HCl	Hydrochloric acid
HEPES	Hydroxyethyl piperazineethanesulfonic acid
HLA-DR	Human leukocyte antigen DR
HSC	Hematopoietic stem cells
HUVEC	Human umbilical vein endothelial cells
ICAM-1	Intercellular adhesion molecules 1
IgG	Immunoglobulin G
IL	Interleukins
L	Litre
LAIR-1	Leukocyte-associated immunoglobulin-like-receptor 1
LFA-1	Lymphocytes function-associated antigen 1
LPS	Lipopolysaccharides
MACS	Magnetic-activated cell sorting
MCP-1	Monocyte chemoattractant protein 1
MEP	Megakaryocytes-erythrocytes progenitor cells
MFI	Mean fluorescence intensity
mg	Milligram
MHC	Major histocompatibility complex
MISEV2018	Minimal information for studies of extracellular vesicles 2018

mL	Millilitre
mM	Millimolar
mMP	Monocytic microparticles
MP	Microparticles
mRNA	Messenger RNA
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
ng	Nanogram
O_2^-	Superoxide anion
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PE	Phycoerythrin
PerCP/Cy	Peridinin chlorophyll A protein
PIP5K2A	Phosphatidylinositol-5-phosphate 4-kinase, type II, alpha
PPAR-γ	Peroxisome proliferator-activated receptor γ
PS	Phosphatidylserine
PSG	Penicillin, streptomycin with glutamine
PSGL-1	P-selectin glycoprotein ligand 1
PT	Prothrombin time
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SSC	Side scatter
TAE	Tris acetate-EDTA
TF	Tissue factor
TFPI	Tissue factor pathway inhibitor

TGF-β1	Transforming growth factor beta 1
TLR	Toll-like receptor
TM	Thrombomodulin
TNF-α	Tumor necrosis factor alpha
VCAM-1	Vascular cell adhesion molecules 1
VEGF	Vascular endothelial growth factors

PROFIL IMUNOFENOTIPIK MONOSITIK MIKROPARTIKAL YANG DIREMBES DARIPADA SEL DARAH MONOSIT MANUSIA DAN PERANANNYA DALAM KOAGULASI DAN PENGAKTIFAN SEL ENDOTELIA

ABSTRAK

Monositik mikropartikal (mMP) adalah mikrovesikal heterogen kecil yang dirembes oleh sel monosit yang terhasil daripada proses pengaktifan atau apoptosis. Peningkatan paras edaran mMP yang ketara dalam pelbagai jenis penyakit menunjukkan potensinya sebagai biopenanda. Walau bagaimanapun, peranan fenotipik dan fungsi mMP yang terhasil daripada sel darah monosit manusia tidak diketahui. Kajian ini bertujuan untuk mengenal pasti ciri-ciri mMP yang dirembes daripada sel monosit, $CD14^+$ monosit, dan $CD16^+$ monosit melalui penilaian terhadap pengekspresan antigen permukaan dan mengenal pasti peranannya dalam penggumpalan dan fungsi sel endotelia. Kesemua subjenis sel monosit telah dikultur dengan kehadiran lipopolisakarida (LPS) selama 18 jam. Monositik MP seterusnya diasingkan daripada supernatan yang telah dikultur melalui kaedah ultrapengemparan sebelum dianalisa menggunakan teknik sitometri aliran. Sementara itu, penilaian masa protrombin (PT) telah dijalankan untuk menilai potensi mMP dalam penggumpalan. Untuk mengkaji peranan mMP terhadap fungsi sel endotelial, mMP telah dikultur bersama dengan sel endotelial vena umbilikus manusia (HUVEC). Kadar ekspresi intercellular adhesion molecule 1' (ICAM-1) dan 'vascular cell adhesion molecule 1' (VCAM-1) pada sel endotelia serta perembesan mikropartikal endotelial (eMP) selepas pengkulturan bersama mMP-sel endotelial telah ditentukan menggunakan

kaedah ‘real-time PCR’ dan teknik sitometri aliran. Kajian ini telah menunjukkan bahawa CD14 dan CD16 telah diekspres pada permukaan mMP daripada kesemua subjenis sel monosit pada corak yang sama seperti sel induk mereka. Tambahan pula, mMP mengekspres CD142 dan masa penggumpalan yang lebih singkat dengan kehadiran mMP yang terhasil daripada sel monosit yang dirangsang dengan LPS. Sementara itu, tahap pengekspresan ‘intercellular adhesion molecule 1’ (ICAM-1), ‘vascular cell adhesion molecule 1’ (VCAM-1) dan kadar mikropartikal endotelial (eMP) telah meningkat dengan kehadiran mMP daripada sel monosit. Hasil kajian ini mencadangkan bahawa kombinasi ‘Annexin-V’ dengan CD14 dan CD16 adalah penanda permukaan sel yang berpotensi untuk pengesanan mMP. Tambahan pula, mMP berpotensi untuk memiliki ciri pro-penggumpalan yang mana CD142 pada permukaannya berkemungkinan memainkan peranan utama dalam penggumpalan dan mMP mampu untuk mengaktifkan sel endotelial sekaligus mencadangkan potensi peranannya dalam fungsi sel endotelial.

IMMUNOPHENOTYPIC PROFILES OF MONOCYTIC MICROPARTICLES DERIVED FROM HUMAN BLOOD MONOCYTES AND THEIR POTENTIAL ROLE IN COAGULATION AND ENDOTHELIAL CELL ACTIVATION

ABSTRACT

Monocytic microparticles (mMP) are small heterogeneous microvesicles derived from monocytes following cellular activation or apoptosis. Significant elevation of circulating mMP in various diseases increases its potential as a biomarker. However, the phenotypic and functional role of mMP derived from human blood monocytes is unknown. This study intended to characterise mMP derived from whole monocytes, $CD14^+$ monocytes, and $CD16^+$ monocytes by assessing surface antigen expressions and identify their role in coagulation and endothelial cell function. All monocyte subtypes were cultured in the presence of lipopolysaccharides (LPS) for 18 hours. Monocytic MP were purified from culture supernatants by ultracentrifugation before being analysed using flow cytometry. Meanwhile, prothrombin time (PT) assay was performed to assess the coagulation potential of mMP. To assess the role mMP in endothelial cells function, mMP were co-cultured with human umbilical vein endothelial cells (HUVEC). The expression level of ICAM-1 and VCAM-1 on HUVEC as well as the release of endothelial MP (eMP) upon mMP-HUVEC co-culture were then determined by using real time PCR and flow cytometry respectively. This study has shown that CD14 and CD16 were expressed on all monocyte subtypes-derived mMP similar to their origin cells. Additionally, CD142 were expressed on mMP and coagulation time was shortened in the presence of LPS-stimulated whole

monocyte-derived mMP. Meanwhile, the expression of intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) were increased in the presence of mMP derived from whole monocytes and mMP increased the level of endothelial microparticles (eMP). These findings suggest that Annexin-V in combination with CD14 and CD16 could be possible surface markers for mMP phenotyping. Furthermore, mMP may possess procoagulant properties as CD142 on their surface may be the major player in coagulation and they were able to activate endothelial cells, suggesting a potential role in endothelial cell function.

CHAPTER 1

INTRODUCTION

1.1 Overview

Microparticles (MP) are a population of submicron vesicles released from various cell types following activation or apoptosis. As MP are derived from different cell types, different MP exhibit different identities and play different roles (Rousseau *et al.*, 2015). Microparticles carry their original cell's characteristics including membrane proteins, cytosolic contents and genetic information (Diehl *et al.*, 2012). During MP formation, the cytoskeleton membrane of activated cells undergoes phospholipids rearrangement which results in the expression of phosphatidylserine (PS) on the outer membrane layer. The detection of MP is performed by the identification of origin cell antigens in combination with PS, which is directly targeted by Annexin-V (Voudoukis *et al.*, 2016). For example, monocyte-derived MP can be detected by using anti-CD14 (Bardelli *et al.*, 2012), platelet-derived MP can be identified using anti-CD41 (Flaumenhaft *et al.*, 2009), and anti-CD31 is used for identifying endothelial cell-derived MP (Shantsila, 2008).

Microparticles participate in physiological and pathological processes at the molecular level (Lu *et al.*, 2017). They serve as important mediators in intercellular interaction particularly in the signalling pathway in an autocrine or paracrine manner (Benameur *et al.*, 2019). Under normal conditions, MP act as cell signalling molecules by expressing protein ligands and transferring mRNA between cells (Ratajczak *et al.*, 2006). Consequently, genetic information exchange activates recipient cells and alters

their functions in terms of surface proteins and gene expression as well as cytosolic molecule activities (Barteneva *et al.*, 2013). For instance, MP are capable of activating monocytes and endothelial cells via interleukin 1β (IL-1β) activity (Wang *et al.*, 2011) as well as inducing proliferation and angiogenic activity in endothelial cells by the transfer of mRNA (Barteneva *et al.*, 2013). Microparticles are also able to induce expression of tissue factor (TF) and cytokine secretion by endothelial cells (Wen *et al.*, 2014). Elevated level of MP has been observed in various disease states suggesting their crucial role as biomarkers. Previous studies have reported the association of increased MP number with cardiovascular disease (Batool, 2013), autoimmune disease (Halim *et al.*, 2016), and cancer (Wu *et al.*, 2013).

Monocytes are one of the sources of circulating MP in peripheral blood. During inflammation, monocytes participate in innate immunity as phagocytic cells to destroy invading pathogens. Monocytes consist of three subsets, which are distinguished based on the expression of CD14 and CD16. The three monocyte subsets are the classical $CD14^{++}CD16^{-}$ monocytes, non-classical $CD14^{+}CD16^{++}$ monocytes, and intermediate $CD14^{++}CD16^{+}$ monocytes (Stansfield and Ingram, 2015; Ziegler-Heitbrock *et al.*, 2010). The different distribution of membrane CD14 and CD16 expression remarks their different functional properties in immune functions, phagocytic activity, and cytokine profiles. Activated monocytes subsets may release MP known as monocytic MP (mMP) in response to stimuli.

Although circulating MP are mainly derived from platelets during normal condition, the release of mMP at high level has been observed during endotoxemia and disseminated intravascular coagulation (Wang *et al.*, 2011). The expression of PS and

TF on mMP membrane are thought to define their potential role in haemostasis (Aleman *et al.*, 2011). A previous report has demonstrated that mMP may promote downstream thrombotic events by inducing the expression of TF and von Willebrand factors on endothelial cells (Essayagh *et al.*, 2007). Apart from that, mMP have been shown to amplify inflammation by IL-1β activity as well as inducing intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) expressions by endothelial cells (Wang *et al.*, 2011). However, the exact role of mMP in coagulation and inflammation particularly their interaction with endothelial cells remains unclear. Most mMP studies have been carried out using monocytic cell lines or primary monocytes without distinguishing their subsets. Therefore, this study intended to characterise the phenotypic properties of mMP derived from human monocyte subsets as well as their potential roles in coagulation and endothelial cell activation.

1.2 Human peripheral blood mononuclear cells (PBMC)

1.2.1 The origin and components of PBMC

Human peripheral blood mononuclear cells (PBMC) are a population of peripheral immune cells that consist of a single round nucleus. In humans, the components of PBMC include lymphocytes, monocytes, natural killer cells, and dendritic cells. The composition of each cell type varies across individuals, with T cells are between 70% to 85%, B cells and natural killer cells are between 5% to 10% and 5% to 20% respectively as well as monocytes between 2% to 10% present as main constituents, and dendritic cells are only constitute 1% to 2% of circulating PBMC (Kleiveland,

2015). These circulating immune players are crucial in controlling immune activity in healthy individuals and are capable of responding to any intruders or pathogens in an inflammatory manner (Haudek-Prinz *et al.*, 2012).

1.2.2 PBMC isolation

Peripheral blood and umbilical cord blood are the primary sources of PBMC. Usually, whole blood is collected through venepuncture which is easy and less invasive, allowing for repeated sampling (Arosio *et al.*, 2014). Isolation of PBMC from whole blood is commonly facilitated by density gradient centrifugation method using Ficoll-Hypaque solution (Chan *et al.*, 2013). A gradient medium permits the separation of blood cells in whole blood in which they are distributed in different layers based on their size and density.

Ficoll-Hypaque solution contains sodium diatrizoate and Ficoll, a sucrose polymer with a high molecular weight allowing rouleaux formation of erythrocytes (Fuss *et al.*, 2009). With a specific gravity of 1.077 at room temperature, Ficoll-Hypaque solution successfully separates PBMC from granulocytes and erythrocytes as they are denser than lymphocytes, monocytes, and platelets, but less dense than granulocytes and erythrocytes (Kleiveland, 2015). Hence, mononuclear cells are collected on the top of the Ficoll-Hypaque layer, while erythrocytes and granulocytes are isolated at the bottom of the Ficoll-Hypaque layer (Figure 1.1).

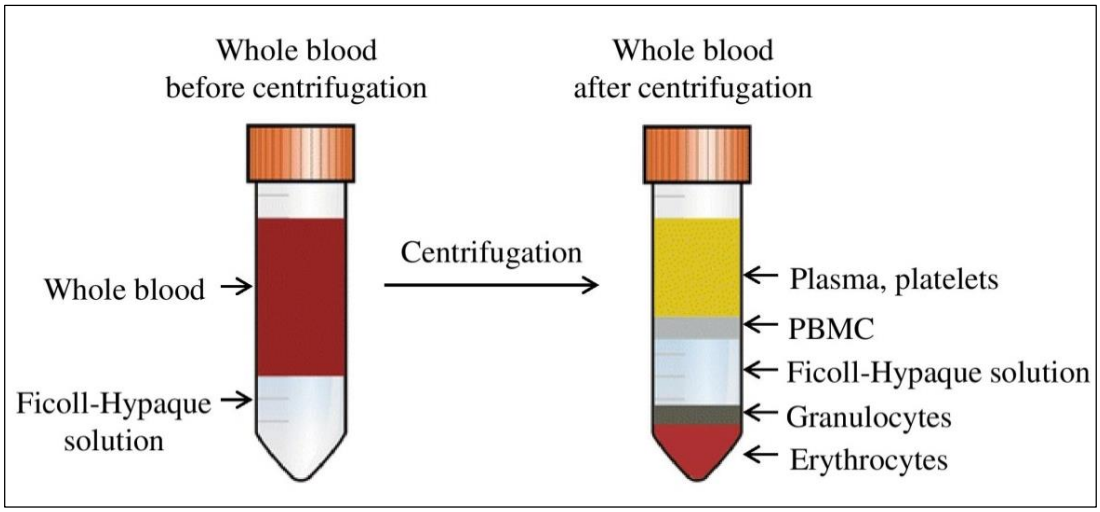


Figure 1.1: Isolation of PBMC from whole blood. Ficoll-Hypaque solution is layered at the bottom of the whole blood followed by centrifugation at 544 × *g* for 20 minutes. After centrifugation, blood components are separated in different layers based on their density. (Modified from Janetzki, 2016)

1.3 Human blood monocytes

Monocytes are agranular leucocytes of myeloid lineage which circulate in the blood and act as the main mononuclear phagocytes. In healthy humans, monocytes account for approximately 2% to 10% of circulating leucocytes (Appleby *et al.*, 2013; Yona and Jung, 2010), which is approximately 450 monocytes/L blood (Robbins and Swirski, 2010). The number of monocytes can rapidly elevate due to stress and disease conditions (Ziegler-Heitbrock, 2015). An increase in monocyte count or monocytosis suggests the presence of an infection or inflammation (Yang *et al.*, 2014).

1.3.1 The biology of monocytes

Monocytes originate from haematopoietic stem cells (HSC) in the bone marrow through several progenitor stages (Figure 1.2). The HSC subsequently differentiate into multipotent progenitors (MPP) before producing two further progenitor cells which are $CD34^+$ common myeloid progenitor cells (CMP) (Akashi *et al.*, 2000) and common lymphoid progenitor cells (CLP) (Tortora and Derrickson, 2011). The CMP develop into erythrocytes, platelets, and leucocytes, while CLP develop into lymphocytes. Some of the CMP then differentiate into either megakaryocytes–erythrocytes progenitor cells (MEP) or granulocytes–macrophages progenitor cells (GMP) (Iwasaki and Akashi, 2007), which are important for erythroid-lineage cells and myeloid-lineage cells respectively. Subsequently, GMP further differentiate into monocytes and granulocytes.

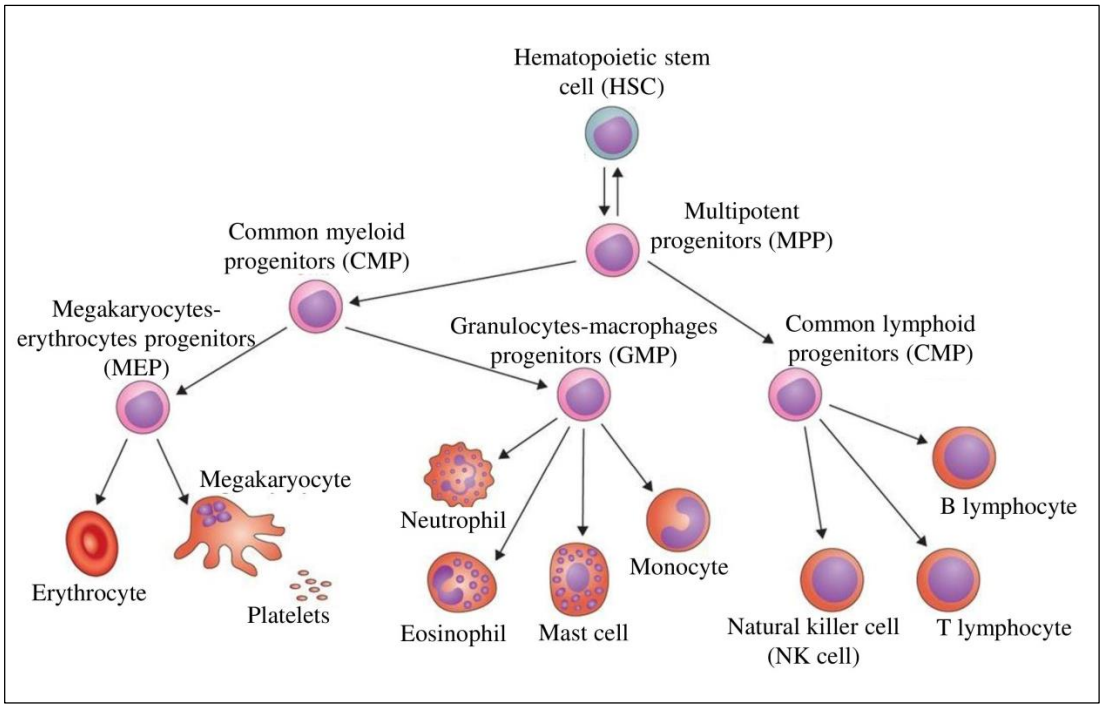


Figure 1.2: Formation of human blood cells. Blood cells originate from haematopoietic stem cell (HSC) in the bone marrow through several committed progenitor stages including multipotent progenitor (MPP), common myeloid progenitors (CMP), and common lymphoid progenitors (CMP). Granulocytes–macrophages progenitors (GMP) and megakaryocytes–erythrocytes progenitors (MEP) further differentiated from CMP. MEP develop into erythrocytes and megakaryocytes. GMP develop into granulocytes and monocytes. CMP further develop into natural killer cell, T lymphocytes, and B lymphocytes. (Modified from Wahlster and Daley, 2016).

The development of blood cells from HSC involves cytokines and growth factors that actively regulate the development and proliferation of specific progenitor cells. Growth factors such as colony-stimulating factors (CSF) and interleukins (IL) trigger the differentiation of progenitor cells in the bone marrow (Tortora and Derrickson, 2011). When the development process is complete, monocytes subsequently migrate to the circulation as inert cells. Monocytes migrate and mature at the site of infection following stimulation by pathogenic invaders such as bacteria and viruses. The lifespan of circulating monocytes in humans is one to three days before undergoing programmed-cell death (Yang *et al.*, 2014). In fact, the short lifespan of monocytes explains that blood acts as an extensive myeloid progenitor's reservoir, which allows for the continuous reformation of monocytes (Serbina and Pamer, 2006).

1.3.2 General functions of monocytes

Monocytes play an important role in both pro-inflammatory and anti-inflammatory immune responses. During inflammation, monocytes migrate to the site of inflammation and differentiate into M1 pro-inflammatory macrophages prior to the secretion of pro-inflammatory cytokines, including IL-6, IL-1β, and tumour necrosis factor α (TNF-α) (Murdock *et al.*, 2015). On the other hand, anti-inflammatory monocytes differentiate into M2 anti-inflammatory macrophages, which are capable of producing anti-inflammatory cytokines, such as IL-10 and transforming growth factor beta 1 (TGF-β1), and restoring damaged cells (Thomas *et al.*, 2015).

The production of pro-inflammatory cytokines such as TNF-α, IL-1β, IL-6, and IL-8 by monocytes further results in oxidative and non-oxidative antimicrobial activities in an autocrine as well as paracrine manner, which lead to local and systemic inflammation. For example, TNF-α enhances the inflammatory response by monocytes in an autocrine manner through binding with TNF-α receptor 1 (TNFR1), which is expressed on monocytes, thus up regulating the release of pro-inflammatory cytokines (Gane *et al.*, 2016). Meanwhile, the paracrine effect of IL-1β secreted by monocytes includes stimulating $CD4^+$ T cell differentiation into T helper cell lineages (Santarlasci *et al.*, 2013) as well as inducing the production of histamine by mast cells to promote vasodilation during inflammation (Arango Duque and Descoteaux, 2014). However, excessive monocyte activation and cytokine imbalance may result in exaggeration of inflammation (Peraçoli *et al.*, 2003).

Resolution of inflammation by monocytes is critical to decelerate inflammatory process and restore homeostasis. Monocytes synthesise and secrete anti-inflammatory cytokines such as IL-6, IL-10, and TGF-β1, which generate inhibitory effects to control the inflammation process. Endogenous IL-6 actively participates in neutralising toxic effects produced by elevated TNF-α secretion, therefore down regulating the activity of TNF-α (Peraçoli *et al.*, 2003). Similarly, IL-10 secreted by monocytes supress various pro-inflammatory cytokines production and decrease scavenger function and antigen presentation activity by immune cells (Iyer and Cheng, 2012). Meanwhile, monocytes may inhibit proliferation of T cells and exert immunosuppression effect on macrophage and leucocytes via TGF-β1 activity (Travis and Sheppard, 2014).

1.3.3 Blood monocyte heterogeneity

Monocytes were originally thought as a single population in the blood circulation until the discovery of two different phenotypes in the late 1980s (Passlick *et al.*, 1989). In 2010, the nomenclature committee of the International Union of Immunological Societies (IUIS) classified monocytes into three functional subsets based on the expression of CD14 and CD16 antigens (Ziegler-Heitbrock *et al.*, 2010). CD14 is identified as a co-receptor for lipopolysaccharides (LPS) and Toll-like receptor 4 (TLR 4), while CD16 acts as Fcγ receptor III (FcγRIII) and engages in innate immune activity (Shantsila *et al.*, 2011). The three monocyte subsets are the classical $CD14^{++}CD16^{-}$ monocytes, non-classical $CD14^{+}CD16^{++}$ monocytes, and intermediate $CD14^{++}CD16^{+}$ monocytes. Previous studies have reported that different monocyte subsets have different biological functions, antigen-presenting activity, and phagocytic capacity (Table 1.1) (Gordon and Taylor, 2005; Wong *et al.*, 2011; Zhao *et al.*, 2009). However, when the non-classical $CD14^{+}CD16^{++}$ monocytes and intermediate $CD14^{++}CD16^{+}$ monocytes are unable to be separated *in vitro*, they are referred to as $CD16^{+}$ monocytes (Ziegler-Heitbrock *et al.*, 2010).

Table 1.1: Classification of human monocyte subsets.

Monocyte subsets	Surface receptor	% in monocytes	Functions
Classical	$CD14^{++}CD16^{-}$	85% to 90%	Phagocytosis
Non-classical	$CD14^{+}CD16^{++}$	7% to 8%	Patrolling
Intermediate	$CD14^{++}CD16^{+}$	2% to 3%	Antigen presentation, pro-inflammatory

(Modified from Yang *et al.*, 2014)

1.3.3(a) Classical $CD14^{++}CD16^{-}$ monocytes

The classical $CD14^{++}CD16^{-}$ monocytes are the major subset of monocytes, which exist approximately 85% to 90% in the circulation. The classical $CD14^{++}CD16^{-}$ monocytes display high level of CD14 and lack CD16 expression on their surface (Stansfield and Ingram, 2015; Wong *et al.*, 2011). The classical $CD14^{++}CD16^{-}$ monocytes participate in the innate immune system by fighting against foreign organisms (Idzkowska *et al.*, 2015). They exhibit premature phenotypes and migrate to sites of infection via extravasation before differentiating into macrophages (Patel *et al.*, 2017; Reynolds and Haniffa, 2015). Monocyte extravasation is the movement of monocytes through the vascular endothelium to the infected site, which involves monocyte adhesion to the adhesion molecules ICAM-1 and VCAM-1, monocyte rolling, and monocyte transmigration through endothelial cells (Gerhardt and Ley, 2015).

The classical $CD14^{++}CD16^{-}$ monocytes possess high phagocytic capacity as they express scavenger receptor type B class 1 (CD36 and CD163) (Mukherjee *et al.*, 2015), receptor for complement component C1q1 (CD93), opsonin receptor FcγRI (CD64), and FcγRII (CD32) (Cros *et al.*, 2010). These molecules are important for phagocytosis and destruction of pathogens by the classical $CD14^{++}CD16^{-}$ monocytes. The classical $CD14^{++}CD16^{-}$ monocytes also express CD11a/CD18 (Stec *et al.*, 2012) and complement receptor 3 (CR3) (CD11b/CD18) (Wong *et al.*, 2011), which are important in facilitating phagocytosis, intracellular signalling, intracellular adhesion, and monocyte migration (Lukácsi *et al.*, 2017). This monocyte subset also presents with high myeloperoxidase (MPO) activity and produces reactive oxygen species

(ROS), which are crucial in antimicrobial activity (Anbazhagan *et al.*, 2014; Cros *et al.*, 2010). This suggests that most of the classical $CD14^{++}CD16^{-}$ monocytes are active scavenger cells in nature.

Apart from that, the classical $CD14^{++}CD16^{-}$ monocytes express high C-C chemokine receptor type 2 (CCR2), chemokine receptor type 1 (CXCR1), CXCR2, and CXCR4 as well as adhesion molecule CD62L (L-selectin) (Ancuta *et al.*, 2003). CCR2, a receptor for monocyte chemoattractant protein-1 (MCP-1/CCL) as well as CXCR1, CXCR2, CXCR4, and CD62L are important in assisting chemotaxis of monocytes in response to stimuli, particularly during inflammation (Kim *et al.*, 2010). However, the expression of fractalkine receptor (CX3CR1) and CCR5 are low on the classical $CD14^{++}CD16^{-}$ monocytes (Stec *et al.*, 2012).

Nevertheless, the classical $CD14^{++}CD16^{-}$ monocytes are characterised by the capability to produce cytokines in response to stimulations such as LPS. The classical $CD14^{++}CD16^{-}$ monocytes secrete various pro-inflammatory cytokines such as IL-10, IL-6, IL-8, IL-1β, and TNF-α (Cros *et al.*, 2010), indicating their role in inflammation. The classical $CD14^{++}CD16^{-}$ monocytes are lack of TLR 2, TLR 4, and TLR 5, as well as co-stimulatory molecules CD80, CD86, and major histocompatibility complex (MHC) class II molecules (Mukherjee *et al.*, 2015). This suggests that the classical $CD14^{++}CD16^{-}$ monocytes may have less role in antigen presentation. Antigen presentation is the process where peptides or antigens of foreign organisms are presented to lymphocytes by antigen-presenting cells such as monocytes. Antigen

presentation is mediated by MHC class I and MHC class II molecules expressed on cells, which are recognised by $CD8^+$ and $CD4^+$ T cells, respectively (Roche and Cresswell, 2016).

1.3.3(b) Non-classical $CD14^+CD16^{++}$ monocytes

The non-classical $CD14^+CD16^{++}$ monocytes make up approximately 7% to 8% of total monocytes (Mukherjee *et al.*, 2015). This monocyte subset expresses high CD16 and relatively low CD14. They are considered as the mature stage of monocytes compared to other subsets as they display similar characteristics with tissue macrophages in terms of low cytokine productions (Merino *et al.*, 2011).

The non-classical $CD14^+CD16^{++}$ monocytes are involved in inflammation and they are referred to as the pro-inflammatory subset due to their mobilisation during inflammation (Zawada *et al.*, 2012). This monocyte subset exhibits high level of proteins RhoC, RhoF, and Rho GTPase; their activators guanine nucleotide exchange factors such as VAV2 and ARHGEF18; as well as downstream effector phosphatidylinositol-5-phosphate 4-kinase, type II, alpha (PIP5K2A), and protein kinase N1 (PKN1) (Wong *et al.*, 2011). During cell movement, proteins RhoC, RhoF, and Rho GTPase coordinate actin cytoskeletal reorganisation and thus provide the driving force for cell chemotaxis (Wong *et al.*, 2012). The presence of these proteins, which are particularly involved in cytoskeleton reorganisation represent the molecular basis of the non-classical $CD14^+CD16^{++}$ monocytes' patrolling behaviour (Cros *et al.*, 2010). This patrolling property of the non-classical $CD14^+CD16^{++}$ monocytes is important for innate surveillance of tissues (Idzkowska *et al.*, 2015). The non-classical

$CD14^{+}CD16^{++}$ monocytes patrol the resting endothelium layer for vasculature surveillance and subsequently detect infected cells and remove debris (Carlin *et al.*, 2013; Idzkowska *et al.*, 2015). Additionally, the expression of chemokine receptor CX3CR1 is highly abundant on the non-classical $CD14^{+}CD16^{++}$ monocytes, although CCR2 and CD62L are not expressed on them (Carlin *et al.*, 2013). Chemokine receptor CX3CR1 is important in mediating the accumulation of monocytes (Geissmann *et al.*, 2003), thus enhancing the patrolling capability of this monocyte subset.

The non-classical $CD14^{+}CD16^{++}$ monocytes are less responsive to LPS (Skrzeczyńska-Moncznik *et al.*, 2008) but respond strongly to nucleic acids and viruses via TLR 7 and TLR 8 (Cros *et al.*, 2010). Upon activation by nucleic acids and viruses, the non-classical $CD14^{+}CD16^{++}$ monocytes secrete high level of pro-inflammatory cytokines such as IL-1β and TNF-α (Mukherjee *et al.*, 2015) through the MyD88-MEK pathway (Cros *et al.*, 2010), while LPS stimulation led to low level of IL-1β and TNF-α secretion (Wong *et al.*, 2011). However, another study has shown that exposure of the non-classical $CD14^{+}CD16^{++}$ monocytes to LPS resulted in high production of IL-1β and TNF-α (Dutertre *et al.*, 2012). This discrepancy may be due to the use of different anti-CD14 antibodies clone, since few anti-CD14 clones such as M5E2 has shown to inhibit LPS activity (Power *et al.*, 2004).

In contrast to the classical $CD14^{++}CD16^{-}$ monocytes, the non-classical $CD14^{+}CD16^{++}$ monocytes express notably high level of co-stimulatory molecules CD80, CD86, and human leukocyte antigen DR (HLA-DR) as well as TLR 2, TLR 4, and TLR 5, indicating their role in antigen presentation (Mukherjee *et al.*, 2015). The non-classical $CD14^{+}CD16^{++}$ monocytes exhibit poor phagocytic capacity. In contrast to the classical

$CD14^{++}CD16^{-}$ monocytes, the production of ROS, MPO, and lysozyme as well as the expression of scavenger receptor CD36 is low on the non-classical $CD14^{+}CD16^{++}$ monocytes (Idzkowska *et al.*, 2015; Tallone *et al.*, 2011). These properties are correlated to the low expression of CR3 and intermediate level of CR4 (CD11c/CD18) (Moniuszko *et al.*, 2015), thus limiting their role in phagocytosis.

1.3.3(c) Intermediate $CD14^{++}CD16^{+}$ monocytes

The intermediate $CD14^{++}CD16^{+}$ monocytes are a minor population, constituting a small percentage with only 2% to 3% of total monocytes. This discrete subset expresses both CD14 and CD16 antigens on their surface (Ziegler-Heitbrock and Hofer, 2013). It has been reported that, monocyte maturation involves the transitional process of the classical monocytes to the intermediate monocytes before differentiating into mature non-classical monocytes (Idzkowska *et al.*, 2015; Ziegler-Heitbrock *et al.*, 2010).

The intermediate $CD14^{++}CD16^{+}$ monocytes have high phagocytic function and produce high level of ROS than other monocyte subsets (Rossol *et al.*, 2012). Furthermore, the expression of both opsonin receptors FcγRIII and FcγRI as well as CD36 scavenger receptor are highly expressed on the intermediate $CD14^{++}CD16^{+}$ monocytes (Tallone *et al.*, 2011), thus facilitating phagocytosis by this monocyte subset. The phagocytic capacity of the intermediate $CD14^{++}CD16^{+}$ monocytes is further enhanced with the high expression of CD11a/CD18, CR3 (Skrzeczyńska-Moncznik *et al.*, 2008), and CR4 (Sulicka *et al.*, 2013). These three types of β_2 integrin are important adhesion and signalling molecules in which CD11a/CD18 integrin binds

to ICAM-1, ICAM-2, ICAM-3, and ICAM-5; CR3 integrin binds to complement proteins iC3b and C4b; and CR4 integrin binds to iC3b, ICAM-1, and fibrinogen (Podolnikova *et al.*, 2015). The binding of these integrins with their main ligands subsequently mediates monocyte recruitment to the site of inflammation and phagocytosis via complement cascades (Schittenhelm *et al.*, 2017).

Additionally, the intermediate $CD14^{++}CD16^{+}$ monocytes play a role as antigen-presenting cells in enhancing immune response towards infections. The intermediate $CD14^{++}CD16^{+}$ monocytes display higher TLR 2, TLR 4, and TLR 5 compared to the other two monocyte subsets, as well as CD80 and CD86 co-stimulatory molecules, suggesting their significant role in pro-inflammatory function and antigen presentations (Mukherjee *et al.*, 2015). Toll-like receptors 2, TLR 4 and TLR 5 are the co-receptors for CD14 expressed on monocytes, which are responsible in the recognition of bacterial lipoprotein, LPS, and bacterial flagellin respectively (Sabroe *et al.*, 2003). Thus, they may elicit pro-inflammatory cytokine production, antigen presentation function, and secretion of multiple specific antibodies (Tadema *et al.*, 2011). The intermediate $CD14^{++}CD16^{+}$ monocytes have high expression of HLA-DR and enhance the proliferation of $CD4^{+}$ T lymphocytes (Zawada *et al.*, 2012). This further facilitates the activity of antigen presentation, which is important for initiating effector and memory T cell activation during infection.

Some of the phenotypic and functional features of the intermediate $CD14^{++}CD16^{+}$ monocytes resemble both the classical $CD14^{++}CD16^{-}$ and non-classical $CD14^{+}CD16^{++}$ monocytes, designating them as a translational population. The intermediate $CD14^{++}CD16^{+}$ monocytes express CCR1, CCR2, and CXCR2, which are

present on the classical $CD14^{++}CD16^{-}$ monocytes, as well as CX3CR1, which is frequently expressed on the non-classical $CD14^{+}CD16^{++}$ monocytes (Idzkowska *et al.*, 2015). The interaction between CX3CR1 with its ligand CXL1 promotes leucocyte recruitment and migration via endothelial cells, thus suggesting their function in transendothelial migration and leucocyte infiltration, particularly during inflammation (Hettinger *et al.*, 2013). In addition, this less abundant subset can be recognised by the expression of CCR5, which is a chemokine receptor of CCL5, macrophage inflammatory proteins 1α (MIP-1α), and MIP-1β as well as a viral co-receptor for human immunodeficiency virus (HIV) (Waller and Sampson, 2018). Additionally, the intermediate $CD14^{++}CD16^{+}$ monocytes has been reported to be involved in atherosclerotic lesions via MCP-1, by attracting MCP-1 to atherosclerotic lesions in a CCR5-dependent manner (Rogacev *et al.*, 2011).

The intermediate $CD14^{++}CD16^{+}$ monocytes are able to mobilise to the inflammatory site as well as to expand their number in numerous disease denotes their pro-inflammatory functions (Mukherjee *et al.*, 2015; Yang *et al.*, 2014). The intermediate $CD14^{++}CD16^{+}$ monocytes release several inflammatory cytokines to the extracellular matrix such as IL-6, IL-8 (Cros *et al.*, 2010), IL-1β, and TNF-α in a significantly higher level compared to the classical and non-classical monocytes (Wong *et al.*, 2011).

1.4 Extracellular vesicles

Extracellular vesicles (EV) are a heterogeneous population of vesicles with different sizes and contents which are released by cells under normal or disease conditions. They serve as important mediators of physiological process in normal and pathological states. Extracellular vesicles consist of exosomes and microparticles (MP), in which they can be distinguished mainly based on size, composition, and mechanism of formation (György *et al.*, 2011) (Table 1.2). Exosomes are the smallest vesicles ranging between 30 and 100 nm in diameter. They consist of endocytic markers including tetraspanins and HSP73 (Chuo *et al.*, 2018; Mathivanan *et al.*, 2010) and have a low density approximately 1.13 to 1.19 g/mL (van der Pol *et al.*, 2012). Upon fusion of multivesicular bodies containing intraluminal vesicles with the plasma membrane, exosomes are released from multivesicular bodies through exocytosis (Ståhl *et al.*, 2019). Lipid compositions of exosome consist of cholesterol and phosphatidylserine (PS), and their cytosolic contents include proteins, mRNA, miRNA, and lipids (Burger *et al.*, 2013).

Table 1.2: Characteristics of extracellular vesicles.

	Exosomes	**Microparticles**
Size	30 – 100 nm	100 – 1000 nm
Origin	Intraluminal vesicles within multivesicular bodies	Plasma membrane
Isolation	100,000 × *g*	20,000 × *g*
Mechanism of formation	Fusion of multivesicular bodies with the plasma membrane	Outward blebbing of the plasma membrane
Release	Constitutive or cellular activation	Constitutive or cellular activation
Lipid membrane composition	Enriched in cholesterol and ceramide, exposed phosphatidylserine, contain lipid raft	Exposed phosphatidylserine, enriched in cholesterol and diacylglycerol, contain lipid raft
Cytosolic content	Proteins, mRNA, miRNA, lipids	Proteins, mRNA, miRNA, lipids
Protein markers	Tetraspanin protein CD63, HSP73	Integrins, selectins, parent cell antigens

(Adapted from Mause and Weber, 2010)

Meanwhile, MP are small heterogeneous vesicles ranging from 100 to 1000 nm which exhibit irregularity in terms of their shape due to different cellular origins (Morel *et al.*, 2011b). Besides displaying several antigens of their origin cell and PS on their surface, MP contain cytosolic proteins, mRNA, miRNA, and lipids (Mause and Weber, 2010). Microparticles are shed directly from cells by outward blebbing of the plasma membrane (Zaborowski *et al.*, 2015). Although MP possess several characteristics that are similar to exosomes, MP can be separated from exosomes by centrifugation at 20,000 × *g* (Mause and Weber, 2010).

1.4.1 Microparticles

Microparticles, formerly known as 'platelet dust' were discovered by Wolf in 1967 (Wolf, 1967). Over the past decades, MP have been previously considered only as an innate cell residues or a cell by-products of activated cells (Distler *et al.*, 2005). Physiologically, they are virtually released from almost all cell types into the extracellular space during cell growth, cell activation, or apoptosis (Spencer *et al.*, 2018). Microparticles are also released by cells in response to pathological or stress conditions such as oxidative stress, sheer stress (Burnouf *et al.*, 2015), and hypoxia (Chen *et al.*, 2013). Shedding of MP *in vitro* may be enhanced by certain stimuli including calcium ionophore, histamine (Cerri *et al.*, 2006), endotoxin (Li *et al.*, 2010), and cytokines (Liu *et al.*, 2007).

In normal individuals, MP are found at a very low level (Albert *et al.*, 2018), reflecting a normal physiological process (Zhou *et al.*, 2015). Recently, numerous studies have recognised the importance of MP as biomarkers in biological processes such as in

haemostasis and inflammation (Mooberry and Key, 2016; Suades *et al.*, 2015). Elevated number of circulating MP has been observed in many disease states particularly in autoimmune disease, cardiovascular disease, and thrombosis (Piccin *et al.*, 2007). For instance, $CD11a^+$ MP derived from leucocytes have been detected in the early stage of acute coronary syndrome (ACS) at increasing level (Chironi *et al.*, 2006), while increased level of platelet-derived MP has been correlated with stroke, sepsis, and deep vein thrombosis (Hoyer *et al.*, 2010).

1.4.2 Origin, features, and formation of microparticles

Microparticles are originated from various types of eukaryotic cells including blood cells such as erythrocytes and leucocytes; platelets, vascular lining cells, cells of the tissue and organs (Wang *et al.*, 2014) as well as tumour cells (Distler *et al.*, 2005). Thus, MP are heterogeneous in terms of protein composition, size, and density depending on their cellular origin (Zubairova *et al.*, 2015). As they can be derived from almost all cell types, they can be easily identified in human body fluid such as in blood, urine, plasma, and saliva (Street *et al.*, 2012). In healthy humans, the average concentration of MP circulating in peripheral blood is within 5 to 50 μg/mL (Hoyer *et al.*, 2010). Proportionally, circulating MP are mainly constituted of platelet- and megakaryocyte-derived MP, which are 80% of total blood MP (Flaumenhaft *et al.*, 2009), while MP derived from endothelial cells and leucocytes are approximately 10% each (Caby *et al.*, 2005).

The structure of MP is less homogeneous, and their membrane consists of phospholipids and numerous markers (Figure 1.3). The phenotypic and cytosolic characteristics of MP predominantly resemble their origin cell's identity (Żmigrodzka *et al.*, 2016). For example, MP derived from platelets display CD36, CD62P (Alkhatatbeh *et al.*, 2011), and CD42b (Flaumenhaft *et al.*, 2009), while MP derived from monocytes express CD14 (Bardelli *et al.*, 2012), and CD31 and CD144 for MP derived from endothelial cells (Shantsila, 2008). Microparticles also express other membrane proteins including PS, tissue factor (TF), and P-selectin glycoprotein ligand-1 (PSGL-1) (Halim *et al.*, 2016) on their external membrane.

In addition, MP carry essential cytoplasmic proteins (Choi *et al.*, 2013), nucleic acids including DNA, RNA, mRNA , microRNA, and long noncoding RNA (Morello *et al.*, 2013), as well as lipids and organelles (Mause and Weber, 2010). The genetic contents of MP allow them to act as messengers and mediate communication between cells. Microparticles convey biomolecules and transmit signal to the surface receptors of recipient cells, thus trigger the alteration in their phenotypic expression and cellular functions (Valadi *et al.*, 2007). Previously, it has been reported that different mechanisms such as cell-to-cell contact or release of signalling mediators permit an effective information transmission by MP from the parent cell to the target cells (Mause and Weber, 2010). For example, platelet-derived MP may fuse with haematopoietic cells and subsequently transfer CD41 antigen expressed on blood platelets to haematopoietic cells (Janowska-Wieczorek *et al.*, 2001).

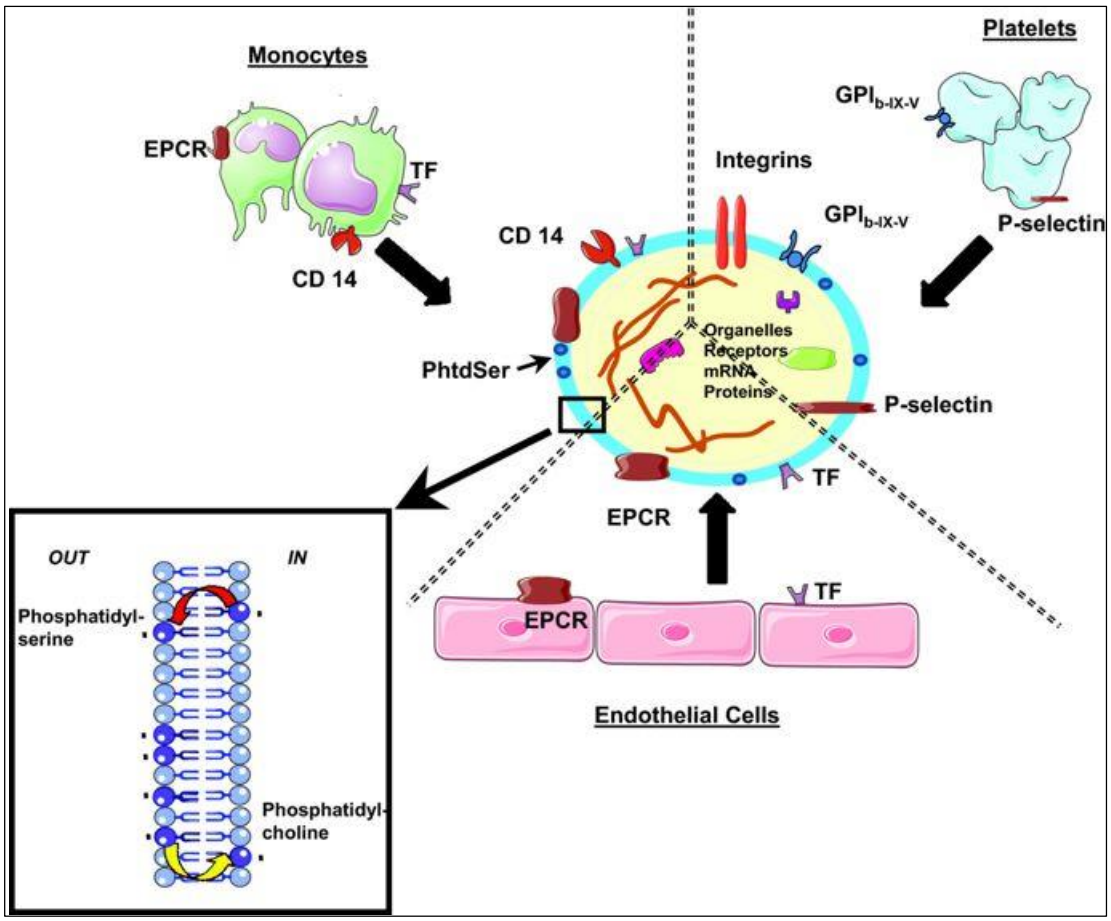


Figure 1.3: Membrane structure of microparticles derived from monocytes, platelets and endothelial cells. The membrane proteins and cytosolic contents of MP tend to mirror their origin cells. Monocyte-derived MP express CD14, platelet-derived MP express P-selectin, and endothelial cell-derived MP express endothelial cell protein C receptor (EPCR). (Adapted from Meziani *et al*., 2010)

The formation of MP involves two main steps, which are the rearrangement of cytoskeleton and externalisation of PS (Said *et al.*, 2018). Usually, the distribution of phospholipids on cell membrane is asymmetrical under resting condition. The positively charged phospholipids including phosphatidylcholine and sphingomyelin are exposed on the lipid bilayer membrane, while the negatively charged phospholipids such as PS and phosphatidylethanolamine are located inside the membrane (Piccin *et al.*, 2007).

Upon cellular activation, intracellular calcium increases in response to stimuli, thus resulting in the activation of calcium-dependent enzymes such as kinase, calpain, and gelsolin as well inhibition of phosphatase (Morel *et al.*, 2011a) (Figure 1.4). The activation of these enzymes further facilitates the cleavage of cytoskeleton proteins (Cohen *et al.*, 2002) including filament, talin, and α-actinin (Nolan *et al.*, 2008). In addition, the calcium influx consequently alters the function of the three important cytosolic enzymes, namely flippase, floppase, and scramblase (Burger *et al.*, 2013). The activation of floppase and scramblase by calcium influx depends on adenosine triphosphate (ATP). Floppase governs the translocation of PS and phosphatidylethanolamine rapidly to the outer leaflet and scramblase mediates phospholipid randomisation down the concentration gradient, while internalising phosphatidylcholine and sphingomyelin (Bevers and Williamson, 2010). These enzymatic actions subsequently disrupt the membrane asymmetry. As the action of flippase and aminophospholipid translocase to maintain the normal asymmetric phospholipid distribution is inhibited by calcium influx, the back-transition of PS to

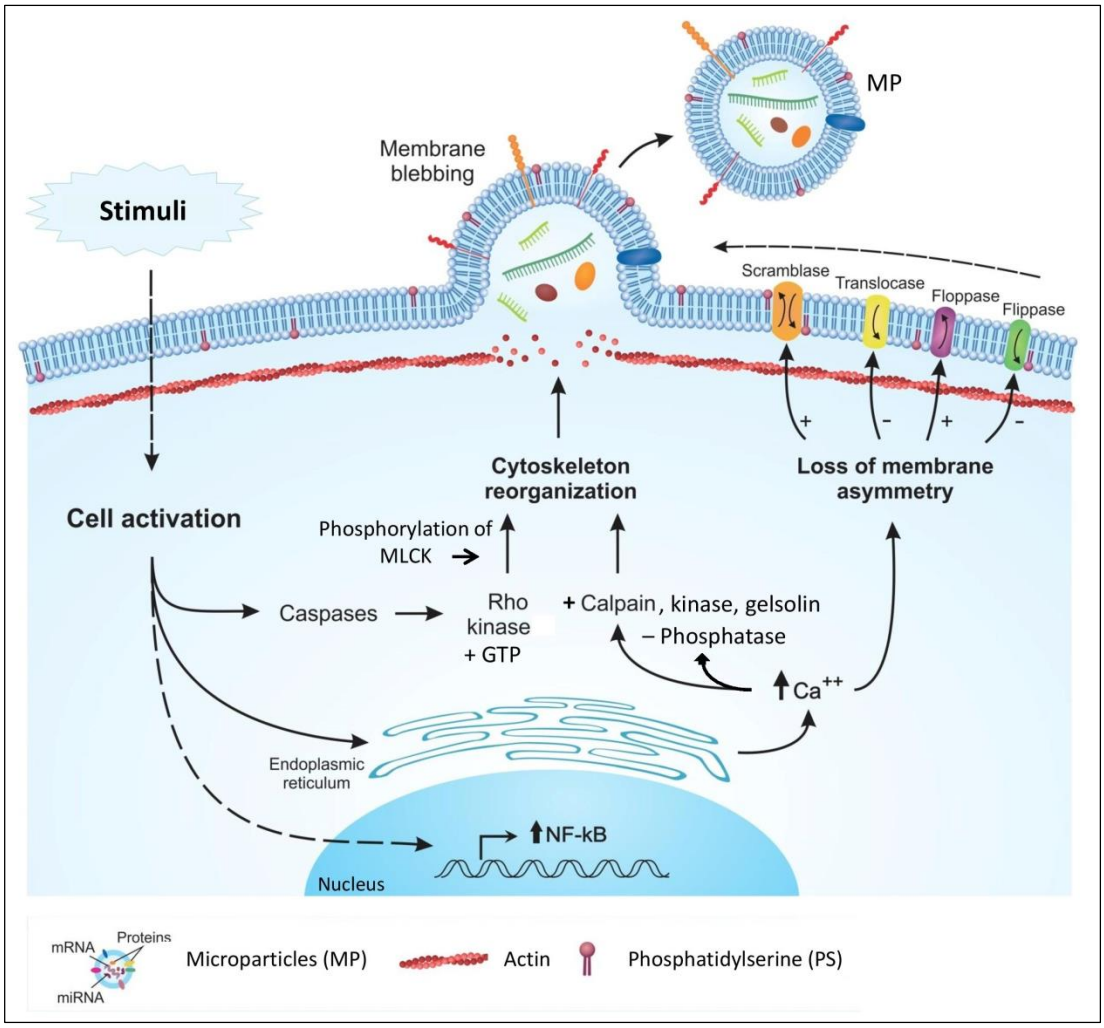


Figure 1.4: The formation of microparticles. Cell activation by stimuli results in activation of caspases, and binding of GTP to Rho kinase, which leading to phosphorylation of myosin light-chain kinase (MLCK). Cell activation also results in increased calcium influx within cells, leading to activation of kinase, calpain, and gelsolin and inhibits phosphatase, thus resulting in cytoskeleton reorganisation. Calcium influx further activates scramblase and floppase, as well as inhibits flippase and translocase, and consequently facilitating the externalisation of PS. (Modified from Favretto *et al.*, 2019)

the inner leaflet is prevented (Daleke, 2003; Herring *et al.*, 2013). Thus, phospholipid imbalance, cytoskeleton proteolysis, and weakening of protein fibrils favour the cellular blebbing, which ultimately leads to the shedding of MP (Burnier *et al.*, 2009).

Cell activation may subsequently induce the activation of Rho by caspase-2 (Sapet *et al.*, 2006) and caspase-1 cleavage (Coleman *et al.*, 2001) which further initiates the conversion of guanosine diphosphate (GDP) to guanosine triphosphate (GTP). Subsequently, the binding of GTP to Rho promotes the C-terminal cleavage of Rho-associated kinase I (ROCK-I) (Coleman *et al.*, 2001) and ROCK-II (Sapet *et al.*, 2006), thus leading to an increase in the phosphorylation of myosin light-chain kinase (MLCK). The phosphorylation activity in turns facilitates the detachment of cytoskeleton from the membrane and release of MP (Distler *et al.*, 2005).

1.4.3 Methods of microparticle detections

Microparticles express PS and other surface antigens on their membrane. Their mother cell–mimicking property allows for the subpopulation identification as well as determination of cellular origin of MP (Barteneva *et al.*, 2013). Minimal information for studies of extracellular vesicles 2018 (MISEV2018) has provided a basic guideline in characterising MP including characterisation of protein membrane, cytosolic components such as protein and genetic materials, soluble extracellular proteins including cytokines and growth factors, as well as structural details of MP (Théry *et al.*, 2018).

Flow cytometry is the most widely used method to identify MP population. Flow cytometry is a fast method and provides high-resolution of quantitative and qualitative data based on single MP. This convenient method permits the analysis of large numbers of sample in a short time (Burger *et al.*, 2013). The use of flow cytometry allows for detection of MP population by double staining with Annexin-V and antigen of interest (Nomura *et al.*, 2008), thus defining the origin of MP. Annexin-V, which binds to PS, enables the detection of MP. In addition, assessment of light scattering intensity assists in size determination of MP (Gradziuk and Radziwon, 2017), while total amount of MP can be counted using commercial beads (Lacroix *et al.*, 2010). Flow cytometry is also able to assess the protein content of MP, which is expressed as molecular mass units (Jy *et al.*, 2004).

Currently, the detection of MP is ascertained by Western blotting, which mainly assesses the total amount of protein content in MP. Western blotting can identify the molecular weight of MP proteins since they are separated based on their molecular weight on a gel electrophoresis (Coumans *et al.*, 2017a). Western blotting are also capable of specifying the origin of MP as it provides information regarding the specific antigen expressions on MP (Barteneva *et al.*, 2013; Berezin, 2015). By using specific primary antibodies that directly target the antigens expressed on MP, Western blotting is useful in characterising MP. However, the use of Western blotting in translational studies is limited as it requires MP in a large quantity (Street *et al.*, 2012). This method is also unable to measure the size of MP (Barteneva *et al.*, 2013).

Electron microscopy techniques such as scanning electron microscopy (SEM), transmission electron microscopy (TEM) and as well as confocal laser scan

microscopy were used to assess the morphology and size of individual MP (Théry *et al.*, 2018). On ultrathin sections, MP appear as single vesicles displaying heterogeneous internal content (Barteneva *et al.*, 2013), with the diameter ranging from 0.02 to 0.04 µm (Duarte *et al.*, 2012) and 0.3 to 0.7 µm (Burger *et al.*, 2011), while larger MP of 1 µm in size are characterised using freeze-fracture and SEM (Rood *et al.*, 2010). Both SEM and TEM are unable to provide data on protein content and genetic information of MP. In addition, the cellular origin of MP could not be determined as well by microscopy (Barteneva *et al.*, 2013).

Another detection method that can be used to characterise MP is by ELISA, a simple and reproducible method which relies on the basis of MP binding to monoclonal antibodies conjugated with fluorescein. ELISA provides quantitative assessment of specific molecules of MP such as membrane and cytosolic proteins as well as cytokine production (Théry *et al.*, 2018). This method also permits the processing of a large number of samples at one time (Gradziuk and Radziwon, 2017), allowing for simultaneous repetition of experiments. MP detection using ELISA usually involves the recognition and measurement of phospholipids on MP. Nonspecific binding of Annexin-V to other antigens may lead to unreliable results, thus limiting the efficiency of this method (Lacroix *et al.*, 2010). However, ELISA is unable to measure the size of MP and the presence of insoluble antigens, as the antibodies only bind to soluble antigens (Nomura and Shimizu, 2015).

1.4.4 The functions of microparticles

Microparticles play a significant role in cell communication *in vivo* by transmitting and exchanging information between cells (Hoyer *et al.*, 2010). Microparticles act as signalling molecules since they possess membrane signalling proteins and lipids (Figure 1.5). Surface ligands expressed on MP allow direct stimulation of target cells. For example, platelet-derived MP express CD41 and CD62P (P-selectin) on their surface, which permit their attachment on endothelial cells, thus transmitting the signals and resulting in endothelial cell activation (Distler *et al.*, 2005). Microparticles also contain growth factors such as vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF) as well as express bioactive lipids such as sphingosine-1-phosphate, which promotes proliferation of endothelial cells and tissue regeneration, particularly during morphogenesis (Varon and Shai, 2015). Meanwhile, proteins on MP originated from lymphocytes facilitate the differentiation of haematopoietic cells to megakaryocytes (Hugel *et al.*, 2005), while glycoprotein IIb/IIIa expressed on MP derived from platelets enhance haematopoietic stem and progenitor cells engraftment (Janowska-Wieczorek *et al.*, 2001).

Additionally, MP are involved in cell interaction by transferring receptor protein from a cell to other cells. Platelet-derived MP transfer adhesion molecules from platelets to endothelial cells as well as tumour cells, thus promoting the adhesion of leucocytes to endothelial cells (Janowska-Wieczorek *et al.*, 2005). A previous study has reported that the transfer of CCR5 by monocyte-derived MP increases the susceptibility of cells to human immunodeficiency virus-1 (HIV) (Mack *et al.*, 2000).

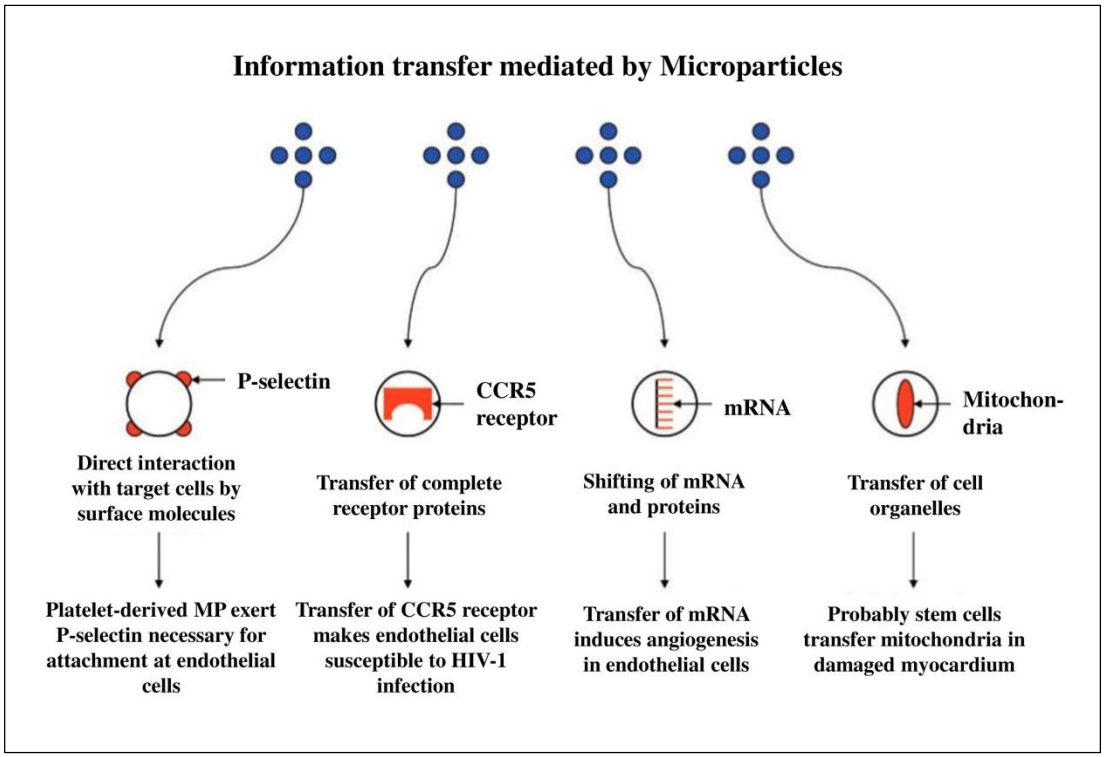


Figure 1.5: The function of microparticles. Microparticles are involved in cellular interaction by transferring information via surface molecules, transfer of protein receptors, transfer of mRNA or transfer of cell organelles. (Adapted from Hoyer *et al.*, 2010)

It has been previously demonstrated that the transfer of CCR5 receptor by MP to cells that lack of this chemokine co-receptor, including endothelial cells and cardiomyocytes, results in increased cell vulnerability towards virus infection (Mack *et al.*, 2000). Therefore, this suggests that MP are actively participate in pathological conditions.

Microparticles also play a role in RNA exchange between cells. For example, MP derived from endothelial progenitor cells activate angiogenic programme within endothelial cell *in vitro* by transferring mRNA (Deregibus *et al.*, 2007). Subsequently, angiogenic activity promotes the proliferation and survival of endothelial cells as well as formation of new capillary (Hoyer *et al.*, 2010). The interaction between endothelial cells with α_4 and β_1 integrins expressed on MP that are involved during MP incorporation into endothelial cells may be inhibited by the addition of RNase (Deregibus *et al.*, 2007), thus confirming the role of MP as exchange vectors of mRNA. Termination of MP incorporation into endothelial cells results in the failure of MP-mediated RNA transfer and subsequently attenuates the process of angiogenesis both *in vitro* and *in vivo*.

Previously, recovery of hypoxia-injured myocardial tissue has been observed after stem cell infusion, in which MP have been suggested to transfer healthy mitochondria to hypoxia-injured myocardial tissues (Mack, 2006), thus improving tissue regeneration. Meanwhile, it has been reported that the ability of mitochondria-depleted MP to induce IL-8, ICAM-1, and VCAM-1 expression in endothelial cells was significantly reduced compared to control (Puhm *et al.*, 2019). Thus, this finding suggests that MP may activate endothelial cells by transferring mitochondria.

1.5 Monocytic microparticles (mMP)

Monocytic microparticles (mMP) are shed from the plasma membrane of monocytes in response to stimulation. Similar to other MP, mMP display similar surface antigens to monocytes. Other than CD14, mMP also express other myeloid markers including CD11a, CD11b, and HLA-DR (Takeshita *et al.*, 2014). Monocytic MP also express TF and PSGL-1 on their surface. As mMP are released from monocytes, the population of monocytes in peripheral blood itself reflects the quantity of circulating mMP (Halim *et al.*, 2016).

Monocytic MP may carry different characteristics *in vivo* as well as *in vitro* depending on the type of stimuli. For instance, mMP released upon LPS stimulation express higher PS compared to mMP derived from stimulation by P-selectin–Ig chimera (Bernimoulin *et al.*, 2009). Monocytic MP derived from LPS-stimulated monocytes consist of nuclear proteins and mitochondria, which are essential for energy pathways and metabolism. In addition, leukocyte-associated immunoglobulin-like-receptor-1 (LAIR-1) is only expressed on mMP following stimulation with P-selectin–Ig chimera (Schindler *et al.*, 2016). These differences in terms of membrane protein composition and content may contribute to their different biological activities.

1.5.1 The role of mMP in inflammation

The association of mMP with inflammation has been reported previously (Hugel *et al.*, 2005). Apart from participating in cellular interaction, mMP may contribute in inflammation by enhancing the release of numerous inflammatory cytokines and chemokines by immune cells (van Hezel *et al.*, 2017) as well as up regulating the expression of adhesion molecules by endothelial cells (Cloutier *et al.*, 2013).

Vascular endothelial cells of the blood vessels are one of the effector cells of mMP (Lovren and Verma, 2013). During inflammation, the pro-inflammatory properties of mMP are mainly exerted on endothelial cells. Monocytic MP regulate inflammatory response in endothelial cells via activation of extracellular signal-regulated kinase 1/2 (ERK1/2) and phosphorylation of NF-κB pathway (Cerri *et al.*, 2006; Puddu *et al.*, 2010). *In vitro*, mMP may promote pro-inflammatory activity of endothelial cells by fusion and internalisation of mMP containing IL-1β and inflammasome via PS, which are expressed on mMP (Wang *et al.*, 2011). Protein ligands such as PSGL-1 and P-selectin that are expressed on mMP permit and increase their adhesiveness to adhesion molecules on endothelial cells (Bernimoulin *et al.*, 2009). Subsequently, binding of mMP to endothelial cells induces the release of TNF-α, IL-1β, IL-6, IL-8, and MCP-1 by endothelial cells (Neri *et al.*, 2011), thus further enhancing inflammation. Together with pro-inflammatory cytokines, mMP enhance ICAM-1, VCAM-1, and E-selectin expression by endothelial cells, which are important for leucocyte chemotaxis (Lovren and Verma, 2013). Additionally, mMP release caspase-1 in response to sepsis, thus leads to increased nitrosative stress through PI3 kinase and ERK1/2 pathway (Mastronardi *et al.*, 2011), as well as induced apoptosis in endothelial cells (Mitra *et al.*, 2016; Smit *et al.*, 2015). Nitrosative stress is an imbalanced condition between the

production and elimination of reactive nitrogen species (RNS), and mMP may increase the production of nitrogen oxide (NO), thus inducing nitration of proteins and cell damage (Mastronardi *et al.*, 2011).

Additionally, the interaction between mMP and endothelial cells consequently induces endothelial vesiculation, resulting in the release of endothelial microparticles (eMP). The shedding of eMP results from the activity of pro-inflammatory factors, including growth factors such as vascular endothelial growth factors (VEGF) (Neves *et al.*, 2019) and cytokines such as IL-6 and TNF-α, which are released following mMP–endothelial cell interaction (Deng *et al.*, 2017). In turn, eMP elicit a pro-inflammatory response in endothelial cells by enhancing adhesion molecule expression on endothelial cells, thus resulting in monocyte adhesion (Jansen *et al.*, 2017).

Monocytic MP may exert an autocrine effect on their origin monocytes. The inflammatory response of monocytes contributed by mMP is mainly through the lipid fraction on mMP membrane, which binds to and activates TLR 4 on monocytes (Thomas and Salter, 2010). The interaction of mMP with monocytes subsequently induces the release of pro-inflammatory cytokines IL-1β (Wang *et al.*, 2011), superoxide anion (O_2^-) production, and activation of NF-κB pathway in monocytes (Bardelli *et al.*, 2012), which may exacerbate the inflammatory condition. Monocytic MP also facilitate monocyte–endothelial cell interaction, which is the initial step in vascular inflammation. During inflammation, mMP support the attachment of monocytes to ICAM-1 expressed on activated endothelial cells by transferring oxidised phospholipids, RANTES, or caspase-3 to endothelial cells (Edrissi *et al.*, 2016), as well as activating lymphocyte function–associated antigen 1 (LFA-1) and

macrophage antigen 1 (MAC-1) in monocytes (Batool, 2013). These processes favour transmigration and recruitment of activated leucocytes in the vascular intima during inflammation. Subsequent leucocyte infiltration at the inflammatory site eventually leads to the development of inflammatory condition (Puddu *et al.,* 2010).

On the other hand, the anti-inflammatory effect of mMP may be seen through the down regulation of pro-inflammatory activity by cytokines at the early inflammatory stage. For instance, mMP hamper the activation of monocytes by inhibiting TNF-α, IL-8, and IL-6 secretion, as well as induce the release of transforming growth factor-β1 (TGF-β1) and IL-10 (Gasser and Schifferli, 2004). Monocytic MP also amplify the expression of peroxisome proliferator-activated receptor γ (PPAR-γ) protein in monocytes (Neri *et al.*, 2011) to reduce inflammatory activity. PPAR-γ protein attenuates the signalling pathway through the restriction of NF-κB activity, leading to down regulation of adhesion molecule expression on the target cells (Halim *et al.*, 2016; Sahler *et al.*, 2014). Besides that, Annexin A1, an anti-inflammatory glucocorticoid-regulated protein that is encapsulated within mMP, contributes to the immunosuppressive effect of mMP (Dalli *et al.*, 2008). The resolution mechanisms of inflammation modulated by Annexin A1 involve the suppression of pro-inflammatory molecules release, limiting leucocyte diapedesis, and initiation of the conversion of monocytes and macrophages into a pro-resolving phenotype (Sugimoto *et al.*, 2016) such as CD206 (Lee *et al.*, 2002) to restore tissue homeostasis. Overall, mMP exert a negative feedback loop of anti-inflammatory effect through the inhibition of signalling pathway to preserve vascular integrity.

1.5.2 The role of mMP in coagulation

The close interaction of inflammation and coagulation is crucial for the immune system. Blood clotting activity at the site of inflammation is crucial in preventing dissemination of infection through the bloodstream. One of the possible mechanisms of the coagulation cascade activation by inflammation is via the involvement of surrounding MP (Foley and Conway, 2016). It has been reported that mMP may exert dual function in coagulation either as a procoagulant or as an anticoagulant (Khan *et al.*, 2016; Shustova *et al.*, 2017).

The potential procoagulant properties of mMP are mainly contributed by TF and PS, which are abundantly expressed on mMP. TF is an initiator of coagulation process since it acts as a receptor for FVII/VIIa (Mooberry and Key, 2016). *In vivo*, the binding of TF with FVII/VIIa forms a TF–FVIIa complex, inducing the activation of FX and FIX (Mackman *et al.*, 2007) (Figure 1.6). Activated FX and FIX further trigger the activation of downstream coagulation cascade, which results in generation of thrombin (FIIa). Subsequently, thrombin promotes the conversion of fibrinogen into fibrin and initiates the aggregation of platelets for blood clot formation (Choi and Levi, 2006). Accumulation of fibrin also increases the density and strengthens the fibrin network, thus resulting in resistance to fibrinolysis (Aleman *et al.*, 2011). Additionally, the interaction between PSGL-1 on mMP with P-selectin on platelets may cause mMP to bind to and transfer TF to platelets. It has been previously demonstrated that TF-expressing mMP contribute to the propagation of blood clot during thrombus

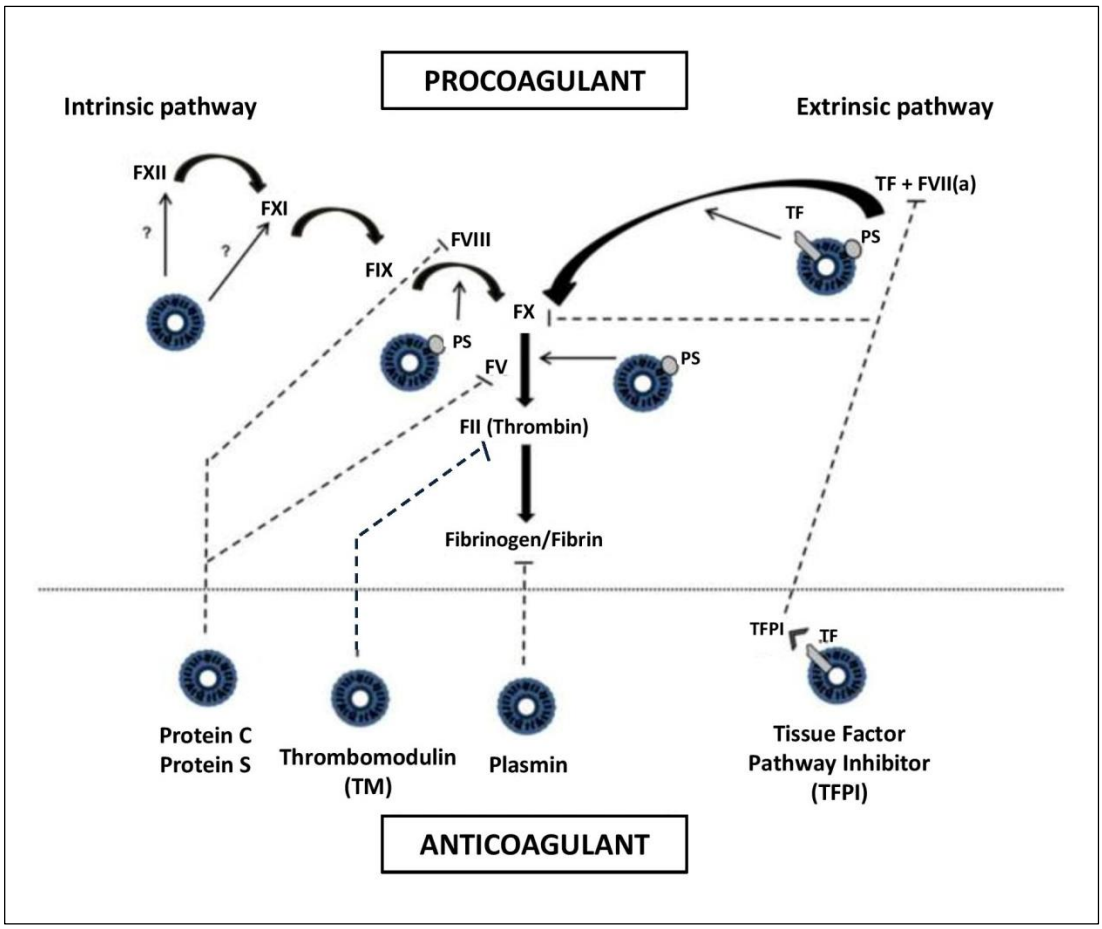


Figure 1.6: The role of mMP in coagulation. Monocytic MP play a role in coagulation by acting as a procoagulant via tissue factor (TF) activity or as an anticoagulant via tissue factor pathway inhibitor (TFPI), protein C, or protein S. (Modified from Mooberry and Key, 2016)

development in preclinical models (Engelmann and Massberg, 2013; Muller *et al.*, 2003). Elevated number of circulating TF-expressing mMP were reported in sickle cell disease, thromboembolism, malignancy, sepsis, and atherosclerosis (Ye *et al.*, 2012).

Meanwhile, PS increases the procoagulant activity of mMP and aids in the formation of blood clot. Negatively charged PS on mMP may interact with γ-carboxyglutamic acid (GLA) domain in the clotting protein (Owens and Mackman, 2011). This interaction facilitates the recruitment of protein cascade FVII, FIX, FX, and prothrombin. A previous study has shown that defect in the production of PS-expressing MP in patients with Scott syndrome results in high bleeding tendency. A high level of PS-expressing MP has been observed in patients with IgA nephropathy and associated with intraglomerular coagulation (He *et al.*, 2015). In addition, PS-expressing MP have been related with hypercoagulable state in patients suffering from colon cancer (Zhao *et al.*, 2016). Meanwhile, inhibition of PS with lactadherin results in prolonged clotting formation, reduced formation of fibrin, and inhibition of thrombin production (Guo *et al.*, 2018), thus indicating the direct involvement of PS in coagulation.

Besides possessing procoagulant properties, mMP induce a negative feedback loop in coagulation through fibrinolytic mechanism or anticoagulation (Owens and Mackman, 2011) through tissue factor pathway inhibitor (TFPI), thrombomodulin (TM), activated protein C, and protein S. Briefly, TFPI is a primary inhibitor of coagulation cascade that prevents procoagulant response at the early phases of the extrinsic pathway. TFPI blocks the activity of FXa by forming TFPI–FXa complex *in vivo* (Dahm *et al.*, 2008). The TFPI–FXa complex further inhibits the catalytic activity of

TF–VIIa complex and prothrombinase in an FXa-dependent manner (Wood *et al.*, 2014). Consequently, this activity modulates the downstream pathway of coagulation cascade and thus prevents blood clot formation. During FXa inhibition by TFPI, protein S acts as a cofactor of TFPI that enhances the binding of TFPI to FXa (Hackeng *et al.*, 2006).

In addition, TM, a transmembrane molecule regulates anticoagulant response by binding to thrombin, forming thrombin–TM complex. This complex simultaneously activates protein C to form activated protein C (APC) (Dahlbäck and Villoutreix, 2005). Activated APC further cleaves and transforms FVIIa and FVa into inactive forms (Ezihe-Ejiofor and Hutchinson, 2013). With the aid of protein S, TM and APC further limit the formation of thrombin by directly inducing prothrombinase inhibition, thus preventing the formation of blood clot.

1.6 The role of mMP in disease

Monocytic MP contain various cellular proteins and genetic molecules that allow them to participate in pathological settings. A high number of circulating mMP is often associated with disease pathogenesis and severity (Souza *et al.*, 2015). Particularly, mMP actively play a role in inflammation and thrombotic events such as in cardiovascular disease, autoimmune disease, and metabolic disorders.

Monocytic MP are involved in the progression of autoimmune diseases such as rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE). The presentation of autoantigens to antigen–presenting cells by mMP may occur under favourable

conditions such as pronounced systemic inflammation or infection (Shao, 2016). In SLE patients, elevation of type 1-interferon (IFN) production and interferon-inducible gene expression have been observed, in which the activation of IFN pathway may increase the severity of SLE (Crow *et al.*, 2015). Monocytic MP interact with IFN-α, thus leading to monocyte activation as well as IL-6, IL-8, and TNF-α release (Nielsen *et al.*, 2014). This interaction consequently results in worsening of inflammation in SLE patients. In RA patients, mMP in the synovial fluid induces the release of IL-1, IL-6, IL-8, and MCP-1; and synoviocyte activation which may cause synovitis (Viñuela-Berni *et al.*, 2015). Activation of synovial fibroblasts by mMP also leads to the production of matrix metalloproteinases (Distler *et al.*, 2005) that degrade extracellular matrix protein, thus causing cartilage and joint destructions (Araki and Mimura, 2017).

Elevated levels of mMP and changes in their phenotypes have been observed in metabolic disorders such as atherosclerosis and familial hypercholesterolaemia (FH). Monocytic MP facilitate plaque formation and immune cell accumulation at the blood vessel of murine models (Hoyer *et al.*, 2012), thus increasing the risk of thrombosis. Patients suffering from FH, a disease associated with high level of low-density lipoprotein (LDL), are at higher risk of atherosclerosis development (Halim *et al.*, 2016). The increase of mMP in FH patients is related to LDL cholesterol oxidisation via CD36-dependent mechanism (Hjuler Nielsen *et al.*, 2015). Thus, mMP may contribute to acceleration of atherosclerotic event in FH.

1.7 Rationale of the study

Microparticles have gained interest as they have been reported as important player in inflammation, coagulation, and alteration of endothelial cell functions. Previous studies on MP were mainly performed on MP derived from erythrocytes (Said *et al.*, 2018), platelets (Zhao *et al.*, 2016), lymphocytes (Tahiri *et al.*, 2016), and endothelial cells (Deng *et al.*, 2017; Herring *et al.*, 2013). The level of MP has been reported to increase in number during pathological conditions particularly during inflammation, reflecting their function as potential biomarkers. However, information regarding the interaction between MP derived from monocytes and endothelial cells is limited. Although several studies have been performed on mMP, most of them used monocytic cell lines instead of primary monocytes (Wang *et al.*, 2011; Wen *et al.*, 2014). As MP with different characteristics may arise from different cell types, theoretically, mMP derived from human blood may exhibit different phenotypic and functional properties compared to those derived from monocytic cell lines (Halim *et al.*, 2016). Therefore, this study was carried out to characterise surface antigen expression of mMP derived from human monocyte subsets and their potential functions in blood coagulation as well as in endothelial cell activations.

This study provides preliminary data on mMP surface phenotypes, coagulation potential, and activation of endothelial cells by mMP derived from human monocytes. Phenotypic information of mMP may be useful as a potential biomarker in diagnosis and prognosis of inflammatory conditions. Additionally, a better understanding on how mMP regulate the coagulation pathway and endothelial cell activation permits their manipulation in developing therapeutic agents and better treatment for various

diseases. We hypothesise that mMP derived from LPS-stimulated human monocytes display their origin cell's antigens, promote coagulation activity, and activate endothelial cells.

1.8 Objectives

1.8.1 General objective

To study phenotypic profiles of mMP and their role in coagulation and endothelial cell activation.

1.8.2 Specific objectives

1. To characterise cell surface phenotypes of mMP derived from LPS-stimulated whole monocytes, $CD14^+$ monocytes, and $CD16^+$ monocytes.
2. To assess coagulation potential of mMP derived from LPS-stimulated monocytes.
3. To measure the expression of adhesion molecules ICAM-1 and VCAM-1 by endothelial cells following culture with mMP.
4. To assess cell surface phenotypes of endothelial microparticles (eMP) derived from endothelial cells cultured with mMP derived from monocytes.

CHAPTER 2

MATERIALS AND METHODS

2.1 Experimental design

This study was performed as shown in Figure 2.1. Briefly, blood were collected from healthy donors followed by PBMC isolation. Then, whole monocytes were further isolated from PBMC using Pan Monocytes Isolation Kit. $CD14^+$ monocytes and $CD16^+$ monocytes were then purified from the whole monocytes using CD16 Isolation Kit. Whole monocytes, $CD14^+$ monocytes and $CD16^+$ monocytes were further cultured in the presence or absence of LPS for 18 hours. Monocytic MP were isolated from the culture supernatants by ultracentrifugation before being assessed for their surface phenotypes by flow cytometry and coagulation property by Start4 coagulometer. Monocytic MP were also cultured with HUVEC. The level of ICAM-1 and VCAM-1 expressions by endothelial cells upon culture with mMP was measured by real-time PCR. Meanwhile, the expression of CD31 on HUVEC and eMP following culture with mMP was assessed by flow cytometry

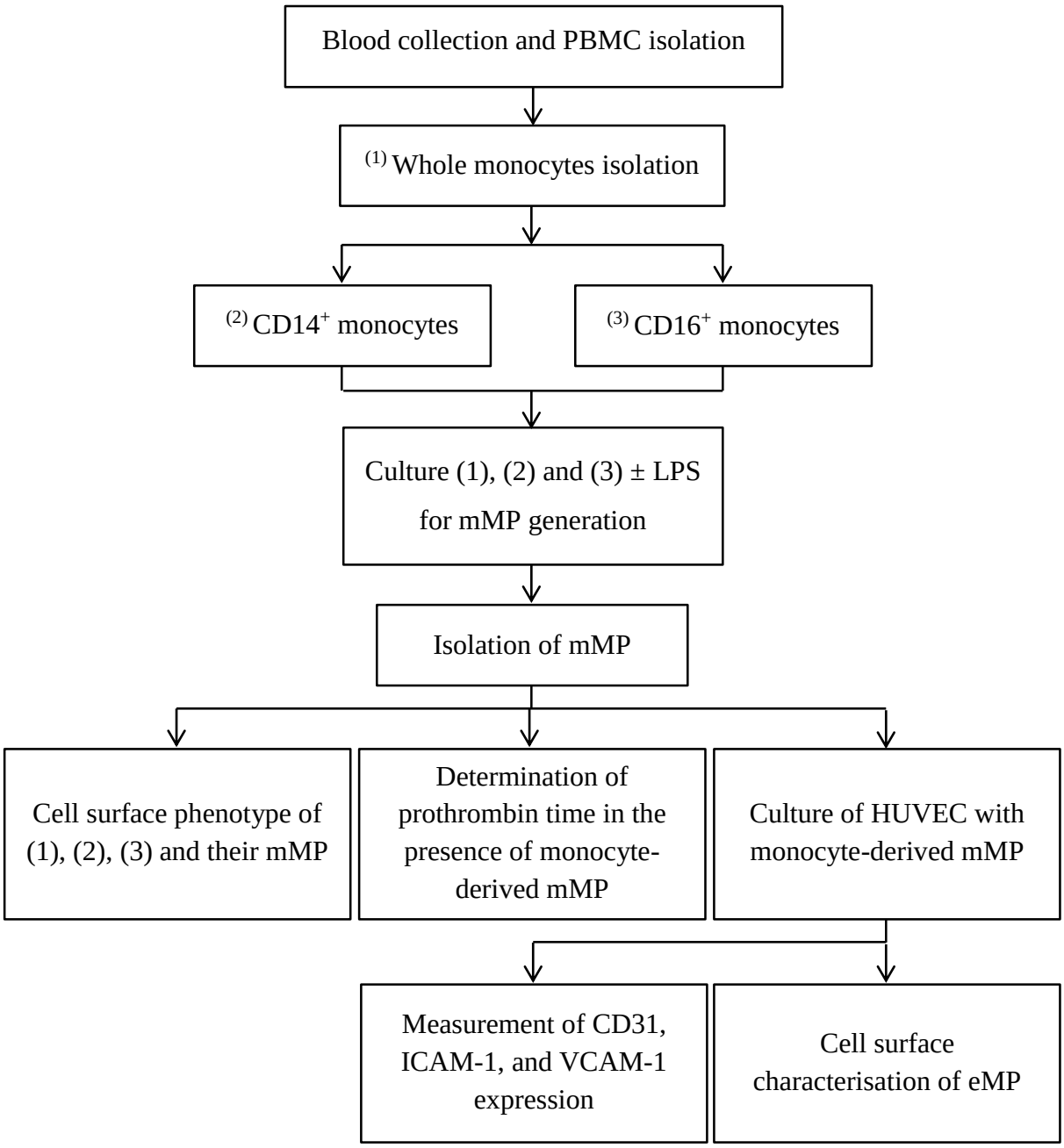


Figure 2.1: Flow chart of the study. Blood was collected from healthy donors. (1) Whole monocytes, (2) $CD14^+$ monocytes, and (3) $CD16^+$ monocytes were cultured in the presence or absence of LPS for 18 hours. Cell surface phenotypes of monocyte subsets and mMP were assessed. Prothrombin time and endothelial cell activation were also determined.

2.2 Materials

2.2.1 List of chemicals and reagents

All reagents used in this study were listed in Table 2.1.

Table 2.1: List of chemicals and reagents

Reagents	Manufacturer
0.25% Trypsin-1 mM EDTA	Nacalai Tesque, Japan
0.4% Trypan blue solution	Sigma Aldrich, UK
10X Annexin-V binding buffer	Becton Dickinson, USA
2% Gelatine Type B solution	Sigma Aldrich, UK
2-mercaptoethanol	GIBCO, USA
6X RNA loading dye	ThermoFisher Scientific, USA
Acetic acid	Sigma Aldrich, UK
Agarose powder	Vivantis, Malaysia
Bovine serum albumin (BSA)	Amresco, USA
Dimethyl Sulfoxide (DMSO)	Fisher Scientific, USA
Endothelium cell growth medium 2	PromoCell, Germany
Ethylenediaminetetraacetic acid (EDTA)	Sigma Aldrich, UK
Fetal bovine serum (FBS)	Sigma Aldrich, UK
Ficoll-Paque PLUS solution	GE HealthCare, USA
Human AB serum	Sigma Aldrich, UK
Hydroxyethyl piperazineethanesulfonic acid (HEPES)	GIBCO, USA
Neoplastine	Diagnostica Stago, USA

Non-essential amino acid	GIBCO, USA
Normal pooled plasma	Diagnostica Stago, USA
Penicillin-streptomycin with glutamine (PSG)	GIBCO, USA
Phosphate-buffered saline (PBS)	Amresco, USA
Primers (GAPDH, ICAM-1, VCAM-1)	Integrated DNA Technologies, USA
RiboRuler High Range RNA ladder	ThermoFisher Scientific, USA
Rosewell Park Memorial Institute-1640 (RPMI-1640)	Sigma Aldrich, UK
Sodium azide	Sigma Aldrich, UK
Sodium Pyruvate	GIBCO, USA
Tris-base	Sigma Aldrich, UK

2.2.2 List of antibodies

All antibodies used in this study are listed in Table 2.2.

Table 2. 2: List of antibodies

Antibodies	Manufacturer
Allophycocyanin (APC) Mouse Anti-Human CD16 (Clone: B73.1)	BD Bioscience, USA
Fluorescein isothiocyanate (FITC) Annexin-V	BD Bioscience, USA
Phycoerythrin (PE) Mouse Anti-Human CD14 (Clone: MϕP9)	BD Bioscience, USA
PE Mouse Anti-Human CD142 (Clone: HTF-1)	Miltenyi Biotech, Germany
PE Mouse Anti-Human IgG_1 (Clone: X40)	BD Bioscience, USA
Peridinin chlorophyll A protein (PerCP/Cy5.5) Anti-Human CD31 (Clone: WM59)	BioLegend, CA

2.2.3 List of commercial kits

All commercial kits used in this study are listed in Table 2.3.

Table 2. 3: List of commercial kits

Commercial kits	Manufacturer
BD CompBeads Anti-Mouse Ig, κ/ Negative Control Compensation Particles Set	BD Bioscience, USA
BD TruCount beads	BD Bioscience, USA
LUNA® Universal One-Step RT-qPCR Kit	New England BioLabs, USA
MACS CD16 Isolation Kit, Human	Miltenyi Biotec, USA
MACS Pan Monocytes Isolation Kit, Human	Miltenyi Biotec, USA
Mini & MidiMACS™ Starting Kit	Miltenyi Biotec, USA
RNeasy Mini Kit	Qiagen, Germany
ToxinSensor Chromogenic LAL Endotoxin Assay Kit	GenScript, USA

2.2.4 List of equipment

All equipment used in this study are listed in Table 2.4.

Table 2. 4: List of equipment

Equipment	Manufacturer
Applied Biosystems 7500 RT-PCR machine	Applied Biosystem, USA
Autoclave sterilizer	Amerex Instruments, USA
BD FACS Canto II Flow Cytometer	BD Bioscience, USA
CO_2 incubator	Binder, Germany
Gel Doc™ XR+ imaging system	Bio-Rad, USA
Inverted microscope	Leica, Germany
Mechanical pipette	Sartorius, Germany
Mikro 22 R Centrifuge	Hettich Zentrifugation, Germany
NanoDrop ND-2000 Spectrophotometer	Thermo Scientific, USA
Neubauer counting chamber	Marienfeld, Germany
Start® 4 semi-automated coagulometer	Stago, USA
Universal 320 centrifuge	Hettich Zentrifugation, Germany
Vortex mixer	ERLA Technologies (M) Sdn Bhd, Malaysia
Waterbath	Memmert, Germany

2.2.5 List of softwares

All softwares used in this study are listed in Table 2.5.

Table 2. 5: List of softwares

Softwares	Manufacturer
ABi 7500 Real Time-PCR software	AB system, USA
FCS Express 5 Flow Research Edition	De Novo, USA
Prism 7, Academic use	Graphpad, USA
Image Lab™ software	Bio-Rad, USA
BD FACS DIVA software V7.0.1	Becton Dickinson, USA

2.3 Media

2.3.1 Preparation of sera

One bottle of frozen fetal bovine serum (FBS) and heat-inactivated frozen human AB serum were thawed by heat-inactivated at 56°C for 30 minutes with gentle agitation. Aliquots of 50 mL were stored at -20°C until used.

2.3.2 Complete AB medium

Rosewell Park Memorial Institute (RPMI)-1640 was mixed with 10% human AB serum, 1X penicillin-streptomycin-glutamine, 1 mM sodium pyruvate, 0.1 mM non-essential amino acids, 10 mM HEPES buffer, and 50 µM 2-mercaptoethanol. The media was thoroughly mixed and used immediately.

2.3.3 Endothelial Cell Growth Medium 2

Endothelial Cell Growth Medium 2 contains 2% fetal calf serum (FCS), 5 ng/mL epidermal growth factor, 10 ng/mL fibroblast growth factor, 20 ng/mL insulin-like growth factor (Long R3 IGF), 0.5 ng/mL vascular endothelial growth factor 165, 1 µg/mL ascorbic acid, 22.5 µg/mL heparin, 0.2 µg/mL hydrocortisone, and 1% penicillin-streptomycin. The medium was used immediately for cell maintenance.

2.4 Buffers and reagents

2.4.1 Preparation of 1X phosphate-buffered saline (PBS)

PBS tablet was dissolved in distilled water in a ratio of 1:100 mL. The pH of the buffer was adjusted to 7.4 using 1 M HCl and autoclaved at 121°C at 15 psi (100 kPa) pressure.

2.4.2 Preparation of 70% ethanol

Ethanol 70% was prepared by mixing absolute ethanol in a ratio of 3:1 with distilled water. The solution was stored at room temperature until used.

2.4.3 Preparation of magnetic-activated cell sorting (MACS) buffer

Magnetic-activated cell sorting (MACS) buffer was prepared by mixing 1X PBS with 0.5% bovine serum albumin (BSA), and 2 mM EDTA. The pH of the buffer was adjusted to 7.2 using 1 M HCl and filtered using 0.22 µm filter. The MACS buffer was stored at 2 - 8°C until used.

2.4.4 Preparation of fluorescence-activated cell sorting (FACS) buffer

Fluorescence-activated cell sorting (FACS) buffer was prepared by adding 0.5% BSA and 0.05% sodium azide into 1X PBS. The solution was filtered using 0.22 µm filter and stored at 2 - 8°C until used.

2.4.5 Preparation of 1X binding buffer

Binding buffer (10X) contains sterile 0.1 M HEPES (pH 7.4), 1.4 M NaCl, and 25 mM CaCl2 solution. To prepare 1X binding buffer, binding buffer stock of 10X concentration was diluted in a ratio of 1:10 with distilled water. The solution was filtered using 0.22 µm filter and stored at 2 - 8°C until used.

2.4.6 Preparation of 1X Tris Acetate-EDTA (TAE) buffer

Tris acetate-EDTA (TAE) buffer was prepared by dissolving 20 mM Tris-base, 1mM EDTA, and 40 mM acetic acid in distilled water. The pH of the buffer was adjusted to 8.3 using 1 M HCl and stored at room temperature.

2.4.7 Dilution of lipopolysaccharides (LPS)

One mg of lyphophilised lipopolysaccharides (LPS) powder from *Escherichia coli* O26:B6 was initially reconstituted in 1 mL PBS as recommended by the manufacturer to make up 1 mg/mL LPS of stock solution. LPS was then diluted in 1X PBS to the final concentration of 10 µg/mL. Reconstituted stock solutions were dispensed at 500 µL into microcentrifuge tubes and stored at -20°C until used.

2.5 Methods

2.5.1 General cellular methods

2.5.1(a) Autoclaving

Unless otherwise stated, all heat-resistance apparatus, equipment, appropriate materials, and reagents were autoclaved at 121°C, 15 psi (100 kPa) for 30 minutes.

2.5.1(b) Centrifugation

Unless otherwise indicated, centrifugation of blood sample was performed at 544 × *g* for 20 minutes at 25°C for the purpose of PBMC isolation. All washing steps for PBMC, whole monocytes, $CD14^+$ monocytes, and $CD16^+$ monocytes were performed at 544 × *g* for 5 minutes at 25°C. All washing steps for HUVEC were performed at 220 × *g*, 25°C for 3 minutes. Samples for flow cytometry analysis were centrifuged at 1,200 × *g* for 5 minutes at 25°C followed by ultracentrifugation of supernatants at 20,000 × *g* for 60 minutes at 4°C for MP recovery.

2.5.1(c) Assessment of cell viability

Cell viability of PBMC, monocytes, $CD14^+$ monocytes, $CD16^+$ monocytes and HUVEC were assessed using haemocytometer. Cells were mixed with 0.4% Trypan blue in a ratio of 1:1. Ten µL of the mixture was loaded onto a hemocytometer counting chamber and live cells were counted by Trypan blue exclusion. Cell concentration and percentage of cell viability were calculated using the following formula:

$$\text{Concentration of cells (cell/ mL)} = \left(\frac{\text{Total number of viable cells}}{\text{Number of grid squares}}\right) \text{x (Dilution factor x } 10^4)$$

$$\text{Cell viability (\%)} = \frac{\text{Total number of viable cell}}{\text{Total cell number}} \text{ x } 100$$

2.5.1(d) Assessment of cell morphology

The morphology of all cells during cell maintenance as well as before and after culture were assessed using inverted microscope from lower (10×) to higher magnification (40×).

2.5.1(e) Determination of cell yield

Cell yield was calculated to determine the efficacy of cell separation of monocytes, $CD14^+$ monocytes, and $CD16^+$ monocytes. The isolation yield was counted by using the following formula:

$$\text{Isolation yield} = \frac{\text{Number of cells after isolation}}{\text{Number of cells before isolation in PBMC}} \times 100$$

2.5.1(f) Determination of cell purity

The purity of isolated whole monocytes, $CD14^+$ monocytes, and $CD16^+$ monocytes was evaluated by staining with appropriate antibodies. The cell populations were then assessed using flow cytometry and the percentages of purified cell populations were identified.

2.5.1(g) Determination of cell recovery rate

Cell recovery rate was assessed after cell thawing and cell isolation process. For cell recovery rate after cell thawing, the number of viable cells before freezing and after thawing was counted and cell recovery rate was calculated using the following formula:

$$\text{Cell recovery} = \frac{\text{Number of viable cells after thawing}}{\text{Number of viable cells before freezing}} \times 100$$

To calculate cell recovery rate after cell isolation process, PBMC, whole monocytes, $CD14^+$ monocytes and $CD16^+$ monocytes were stained with appropriate antibodies before being analysed using flow cytometry. Cell recovery rate after cell isolation process was calculated using the following formula:

$$\text{Cell recovery (\%)} = \frac{\text{Percentage of positively stained cells after isolation}}{\text{Percentage of positively stained cells before isolation (in PBMC)}} \times 100$$

2.5.2 Cell isolation

2.5.2(a) Isolation of peripheral blood mononuclear cell (PBMC)

Blood was collected from healthy donors with appropriate informed consent as approved by the Research Ethics Committee (Human) USM (USM/JEPeM/15040128). Blood was collected into EDTA anticoagulant tubes via standard venipuncture in 30 – 50 mL. Ten ml blood was transferred into a 50 mL tube and was diluted in a ratio of 1:2 with 1X PBS. Blood was gently mixed and slowly underlaid with 10 mL Ficoll-Paque PLUS solution into the bottom of the blood mixture. The blood mixture was centrifuged at 25°C, 544 × *g* without brakes for 20 minutes. PBMC were carefully collected from the buffy coat by using pasteur pipette into a 50 mL tube. PBS was added into the tube containing PBMC up to 50 mL. Cell count was performed using hemocytometer and cells were centrifuged at 25°C, for 10 minutes at 544 × *g*. Supernatants were discarded and cell pellets were resuspended in complete AB medium or MACS buffer.

2.5.2(b) Isolation of whole monocytes

Unless otherwise stated, whole monocytes will be referred to as monocytes hereafter. Isolation of human whole monocytes from PBMC was performed using Pan Monocyte Isolation Kit as indicated by the manufacturer. PBMC were resuspended in 40 µL of MACS buffer per 10^7 total cells. Then, PBMC suspensions were stained immediately with 10 µL of FcR Blocking reagent and Biotin-Antibody Cocktail per 10^7 total cells followed by incubation for 5 minutes at 2 – 8°C. Subsequently, 30 µL of MACS buffer per 10^7 total cells was added into the PBMC suspensions followed by the addition of

20 µL Anti-Biotin Microbeads per 10^7 total cells. PBMC suspensions were incubated for another 10 minutes at 2 – 8°C before whole monocytes being immunomagnetically isolated by using MS or LS column, depending on cell concentration. The MS or LS column were initially rinsed with 500 µL or 1000 µL MACS buffer respectively. PBMC suspensions were then loaded onto MS or LS column and unlabelled monocytes were collected from the negative fraction that passed through the column. The column was washed for three times with 500 µL MACS buffer for MS column or 1000 µL MACS buffer for LS column.

2.5.2(c) Isolation of $CD14^+$ monocytes and $CD16^+$ monocytes

The $CD14^+$ monocyte and $CD16^+$ monocyte subpopulations were further isolated from monocytes using CD16 Isolation Kit according to the manufacturer's instructions (Figure 2.2). Monocyte suspensions were centrifuged at 300 × *g* for 10 minutes at 25°C and supernatants were discarded. Cell pellets which consist of monocytes were resuspended in 50 µL of MACS buffer per 5 x 10^7 total cells. Monocyte suspensions were subsequently stained with 50 µL of CD16 Microbeads per 5 x 10^7 total cells prior to incubation at 2 – 8°C for 30 minutes. Monocyte suspensions were washed with 1 – 2 mL MACS buffer per 10^7 total cells following centrifugation at 300 × *g* for 10 minutes at 25°C. Supernatants were discarded and cell pellets were resuspended in 500 µL MACS buffer up to 10^8 total cells. To isolate $CD14^+$ monocytes and $CD16^+$ monocytes, monocyte suspensions were subjected to magnetic separation method using MS or LS column, depending on cell concentration. The MS or LS column were initially rinsed with 500µL or 1000µL MACS buffer respectively prior to monocyte

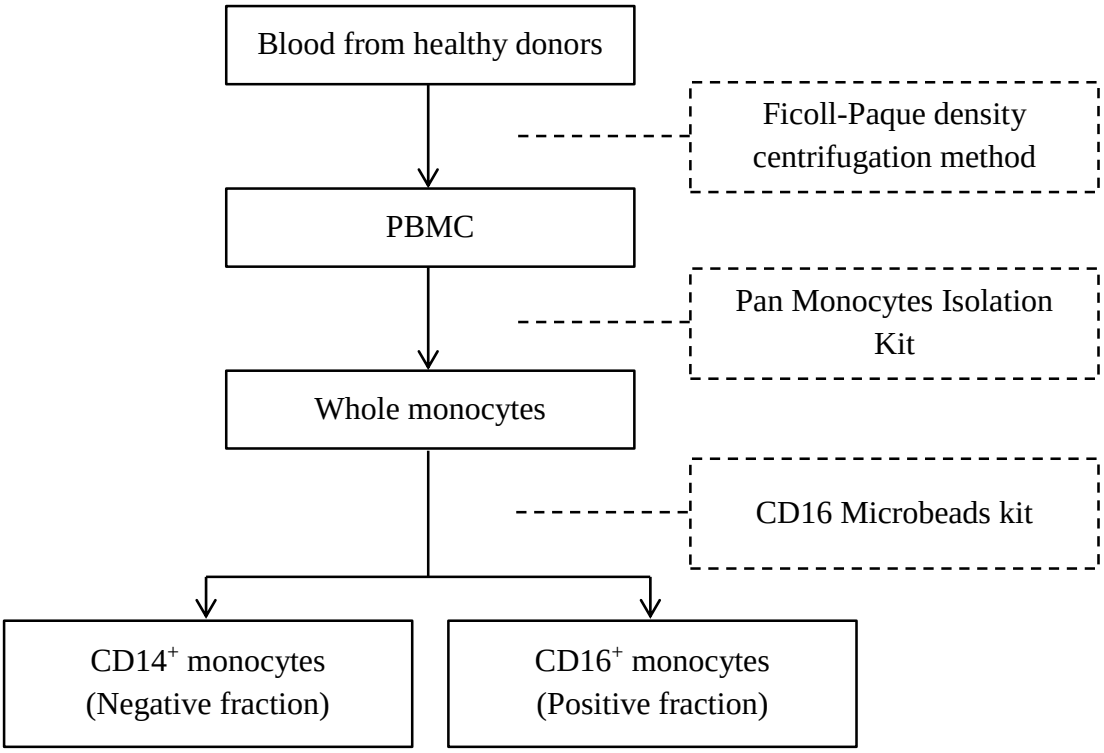


Figure 2.2: Isolation of PBMC, whole monocytes, $CD14^+$ monocytes, and $CD16^+$ monocytes. PBMC were isolated from human blood followed by isolation of whole monocytes. Both $CD14^+$ monocytes, and $CD16^+$ monocytes were further isolated from purified monocytes and collected from the negative and positive fractions respectively.

suspensions being loaded onto MS or LS column. Then, unlabelled $CD14^+$ monocytes were collected into a new tube from the negative fraction, which have passed through the column. The washing steps with 500 µL MACS buffer for MS column or 1000 µL MACS buffer for LS column were performed for three times. The column was then placed onto a new tube. Magnetically labelled $CD16^+$ monocytes were then eluted immediately from the positive fraction using a provided plunger.

2.5.2(d) Culture of human umbilical vein endothelial cells (HUVEC)

Human umbilical vein endothelial cells (PromoCell, Germany) were cultured in a T25 or T75 flask coated with 0.2% gelatine type B solution and maintained in complete Endothelial Cell Growth Medium 2. The medium was replaced every two to three days. All cell washing steps were performed using 1X PBS. Cells were allowed to grow in a 37°C, 5% CO_2/air atmosphere incubator until 70 – 80% confluent. Trypsinisation was performed using 0.25% Trypsin-EDTA for 5 minutes at room temperature followed by the addition of an equal volume of medium to neutralise the trypsin. Detached cells were centrifuged at 220 × *g* for 3 minutes at 25°C to pellet the cells. Cells were routinely passaged every two to three days and were used between the 5^{th} and 10^{th} passage (Liao *et al.*, 2014).

Confluent monolayer of HUVEC in a treated 6-well plate were serum starved for four hours prior to culture with 300 µg/mL LPS-stimulated or unstimulated mMP derived from whole monocytes for two hours in serum free condition (Wang *et al.*, 2011). HUVEC were subsequently trypsinised and centrifuged at 220 × *g* for 3 minutes at 25°C to pellet the cells. The washing steps were further performed for three times using

1 mL of 1X PBS and centrifuged at 220 × *g* for 3 minutes at 25°C. Cell pellets were collected and resuspended in an appropriate buffer prior to flow cytometry or real-time PCR analyses.

2.5.2(e) HUVEC cryopreservation and thawing

Confluent HUVEC were trypsinised before being centrifuged at 220 × *g* for 3 minutes at 25°C. A cryopreservation solution was prepared by slowly adding 10% dimethyl sulfoxide (DMSO) into 90% heat-inactivated FBS. Cell pellets were resuspended gently in a cryopreservation solution to the final concentration of $2 \times 10^5 – 5 \times 10^5$ cells/mL. The cell suspensions were dispensed into 1.5 mL cryogenic vials and immediately transferred to a -20°C freezer. After 20-30 minutes, the vials were transferred to a -80°C freezer for 24 hours before being transferred to a liquid nitrogen container (-196°C).

Frozen HUVEC were thawed rapidly in a 37°C water bath for 1 minute. Cells were then transferred into 0.2% gelatine type B solution coated cell culture flask containing pre-warmed culture medium. The cells were then allowed to attach on a culture flask by incubating in a 37°C, 5% CO_2/air atmosphere incubator for 24 hours. The medium was replaced after 24 hours of initial culture to remove DMSO.

2.5.3 Generation and isolation of microparticles (MP)

2.5.3(a) Monocytic MP (mMP)

Whole monocytes, $CD14^+$ monocytes and $CD16^+$ monocytes were cultured in a 96-well plate at a concentration of 2×10^5 cells/mL in complete AB medium. Cells were stimulated with 1 µg/mL LPS from *Escherichia coli* O26:B6 based on optimization and were allowed to grow in an incubator at 37ºC in 5% CO_2 atmosphere. Following 18 hours incubation (Wen *et al.*, 2014), cell viability was assessed using Trypan blue exclusion. Cultured supernatants were centrifuged at 500 × *g*, 25°C for 5 minutes. Supernatants were kept for mMP isolation while cell pellets were further washed once with 1 mL FACS buffer before being centrifuged at 500 × *g*, 25°C for 5 minutes. Cell pellets were collected and used directly for flow cytometry and clot time analysis.

Monocytic MP isolation was performed by subjecting culture supernatants to another centrifugation at 1,200 × *g*, for 5 minutes, at 25°C followed by ultracentrifugation at 20,000 × *g*, for an hour, at 4°C. The pellets containing mMP were collected and used directly for flow cytometry and clot time analysis, and culture with HUVEC.

2.5.3(b) Endothelial MP (eMP)

Confluent HUVEC were seeded onto a treated 6-well plate at 1×10^5 cells/mL complete Endothelial Growth Medium 2. Cells were grown for 24 hours until they reach 70 - 80% confluence in a 37°C, 5% CO_2/air atmosphere incubator. To mimic inflammatory condition, confluent endothelial monolayer was stimulated with 1 µg/mL LPS from *Escherichia coli* O26:B6 or incubated with 300 µg/mL mMP derived

from LPS-stimulated or unstimulated monocytes for 18 hours in a 37°C, 5% CO_2/air atmosphere incubator (Wang *et al.,* 2011). Following incubation, culture supernatants were collected into a new tube. Attached HUVEC were trypsinised and centrifuged at 220 × *g* for 3 minutes at 25°C. Cell pellets were washed once with 1 mL FACS buffer before being centrifuged at 500 × *g*, 25°C for 5 minutes. Cell pellets were resuspended in FACS buffer for flow cytometry analysis.

Cultured supernatants containing eMP were centrifuged at 500 × *g*, 25°C for 5 minutes. Then, supernatants were collected and subjected to another centrifugation at 1,200 × *g*, for 5 minutes, at 25°C followed by ultracentrifugation at 20,000 × *g*, for an hour, at 4°C. The pellets were collected and resuspended in 1X Binding buffer for flow cytometry analysis.

2.5.4 Endotoxin detection test

Isolated mMP were assessed for the interference of endotoxin using a ToxinSensor Chromogenic Limulus Amebocyte Lysate Endotoxin Kit according to manufacturer's instructions. Briefly, 100 µl of mMP were dispensed into endotoxin-free vials and mixed thoroughly for 30 seconds. Then, 100 µl of reconstituted Limulus Amebocyte Lysate (LAL) was added to each vial and mixed well by swirling gently followed by incubation at 37°C in water bath for 45 minutes. After incubation, 100 µl of reconstituted chromogenic substrate solution was added to each vials before being swirl gently. The solution were then incubated at 37°C in water bath for 6 minutes. Subsequently, 500 µl of reconstituted Color-stabilizer #1 (stop solution) was added to each vials and mixed well by swirling gently followed by the addition of 500 µl of

reconstituted Color-stabilizer #2. The solution was mixed before 500 µl of reconstituted color-stabilizer #1 was added to each vials. Lastly, the solution was mixed gently and the absorbance of each reaction was read at 545 nm using a photometer. Distilled water was used a blank to adjust the photometer to zero absorbance.

2.5.5 Monocytic MP quantification by spectrophotometer

Isolated mMP derived from monocytes were counted based on protein concentration using a NanoDrop ND-2000 spectrophotometer (Sahler *et al.*, 2014). Two µL of mMP sample was loaded onto the lower pedestal of the spectophotometer and were measured at 280 nm.

2.5.6 Monocytic MP quantification using BD TruCount Tubes

BD TruCount tubes were added with 20 µL of anti-CD14 and anti-CD16 antibodies. Then, 50 µL of mMP samples were added and mixed by using a vortex. Samples were incubated for 15 minutes in the dark at room temperature. After incubation, 350 µL 1X binding buffer were added and samples were mixed using a vortex. Samples were then incubated for another 15 minutes in the dark at room temperature before being analysed using a flow cytometer. Absolute count of mMP was quantified based on the following formula as provided by the manufacturer:

$$\text{mMP absolute count (mMP/ µL)} = \frac{X}{Y} \times \frac{N}{V}$$

X= Number of positive cell events
Y= number of bead events
N= Number of beads per test (50600)
V= Volume per test

2.5.7 Cell and MP staining for flow cytometry

Following 18 hours culture, whole monocytes, $CD14^+$ monocytes and $CD16^+$ monocytes at 2×10^5 cells/mL were resuspended in 50 µL FACS buffer and subsequently incubated with 0.1 µg/mL of human phycoerythrin (PE)-conjugated anti-human CD14, and allophycocyanin (APC)-conjugated anti-human CD16. To identify surface antigen expression on HUVEC, 1×10^6 cells/mL HUVEC were resuspended in 50 µL FACS buffer and directly stained with 0.1 µg/mL peridinin chlorophyll A protein (PerCP/Cy5.5)-conjugated anti-human CD31 and Annexin-V-fluorescein isothiocyanate (FITC). All cells were incubated for 20 minutes in the dark at 2 – 8°C. Labelled cells were centrifuged at 500 × *g* for 5 minutes at 25°C and pellets were resuspended in 400 µL FACS buffer followed by flow cytometry analysis.

Following MP isolation, mMP and eMP pellets were resuspended in 100 µL 1X Binding buffer. Monocytic MP were subsequently incubated with Annexin-V-FITC, 0.1 µg/mL PE-conjugated anti-human CD14, and APC-conjugated anti-human CD16. Meanwhile, eMP were incubated with Annexin-V-FITC and 0.1 µg/mL PerCP/Cy5.5-conjugated anti-human CD31. All MP were incubated for 20 minutes in the dark at room temperature. Then, 1X binding buffer was added up to 400 µL before being analysed by flow cytometry.

2.5.8 Data acquisition and flow cytometry analyses

Stained samples were acquired using FACS Canto II flow cytometer for both cells and MP to determine surface antigen expressions. Appropriate flow cytometric colour compensation was performed using CompBeads. Cells were gated based on forward scatter (FSC) and size scatter (SSC) on linear scale. Approximately, 10,000 cell events were acquired at a medium flow rate. Monocytic MP and eMP were assessed based on logarithmic scale of FSC and SSC profiles. The lower minimum threshold was determined on FITC at 200 above the background noise. Monocytic MP and eMP were acquired at 50,000 events within the MP gate at a low flow rate.

Flow cytometry data analyses were performed using FCS Express 5 software. The expression of surface antigen on MP was determined based on double positive staining for Annexin-V and appropriate antigens of interest. Meanwhile, the expression of surface antigens on monocyte subsets or HUVEC were determined based on the mean fluorescence intensity (MFI). The MFI of the sample was determined by fold change over control, which was defined by using the following formula:

MFI (fold change) = MFI sample / MFI control

2.5.9 Prothrombin time (PT) assay

Isolated monocytes and their mMP were resuspended in 50 µL complete AB medium. Samples were loaded into a cuvette and incubated with 50 µL pre-warmed normal pooled plasma for 180 seconds at 37°C. Then, 50 µL neoplastine was added to the sample and clot time was measured immediately using diagnostic Start® 4 semi-automated coagulometer.

2.5.10 General molecular methods

2.5.10(a) Total RNA extraction

The total RNA of human umbilical vein endothelial cells (HUVEC) was extracted using RNeasy Mini Kit according to the manufacturer's instructions. Following HUVEC isolation, 600 µL of Buffer RLT was added to the cell before being mixed using a vortex for one minute. Then, 600 µL of 70% ethanol was added to the lysate and mixed by pipetting. The sample was transferred to an RNeasy Mini spin column in a 2 ml collection tube. The sample was centrifuged at 9,280 × *g* for 15 seconds. The flow through was discarded followed by the addition of 700 µL of Buffer RW1 before being centrifuged at 9,280 × *g* for another 15 seconds. The steps were repeated twice with the addition of 500 µL of Buffer RPE and the second centrifugation was two minutes at the same speed. Then, the mixture was further centrifuged at maximum speed (31,514 × *g*) for one minute. The RNeasy spin column was placed in a new 1.5 mL collection tube and 40 µL of RNase-free water was directly added to the spin column membrane. The sample was then centrifuged at 9,280 × *g* for one minute to

elute the RNA. Isolated total RNA was collected and used immediately for real-time PCR analysis.

2.5.10(b) Determination of RNA concentration and purity

The total RNA concentration was quantified using a NanoDrop ND-2000 spectrophotometer. Two µL of RNA was loaded onto the lower pedestal of the spectrophotometer and the sample was measured at 450 nm. The concentration of RNA was determined by OD_{280} absorbance and presented as µg/mL. The RNA purity was then assessed based on the ratio of A_{260nm}/A_{280nm}.

2.5.10(c) RNA integrity test

The extracted total RNA of HUVEC was assessed for RNA integrity by gel electrophoresis. Agarose gel was prepared by mixing 0.8% agarose powder in 1X Tris-Acetate-EDTA (TAE) buffer. The mixture was then loaded into the gel cassette and allowed to solidify for 30 minutes. Then, solidified agarose gel was transferred into the casting tray and TAE buffer was loaded into the casting tray until the agarose gel was covered. The total RNA samples were mixed with 6X RNA loading dye in a ratio of 1:5, whereas the RiboRuler High Range RNA ladder was mixed with loading dye in a ratio of 1:1 before being loaded into the gel. Gel electrophoresis was conducted at 70 V for 50 minutes. Then, denaturing agarose gel was stained with ethidium bromide (EtBr) for 5 minutes followed by observation of total RNA under ultraviolet (UV) light using Gel Doc™ XR+ imaging system.

2.5.10(d) Dilution of GAPDH, intercellular adhesion molecule 1 (ICAM-1), and vascular cell adhesion molecule 1 primers (VCAM-1)

Lyphophilised GAPDH, intercellular adhesion molecule 1 (ICAM-1), and vascular cell adhesion molecule 1 (VCAM-1) primers were dissolved in appropriate volume of RNase-Free water as recommended by the manufacturer to make up 100 µM primer stocks. All primers were further diluted in RNase-Free water to the final concentration of 10 µM. Reconstituted stock solutions were dispensed at 100 µL into microcentrifuge tubes and stored at -20°C until used.

2.5.10(e) Determination of amplification efficiency

The amplification efficiency (*E*) of real-time PCR was assessed using a serial dilution method. The RNA template was diluted at 5 points of 1:2 serial dilution factors which were 10, 5, 2.5, 1.25 and 0.625 ng of RNA prior to real-time PCR assay. Subsequently, standard curves were generated based on the Ct value and RNA concentrations. The linearity of the standard curves was further assessed based on Pearson's coefficient (*r*) test. The amplification efficiency of real-time PCR assay was determined based on the slope of the standard curves and calculated using the following formula:

$$E\ (\%) = \left[\frac{-1}{10^{\text{slope}} - 1} \right] \text{X } 100$$

E= amplification efficiency

2.5.11 Detection of ICAM-1 and VCAM-1 expression

Prior to real-time PCR assay, samples were prepared using LUNA® Universal One-Step RT-qPCR Kit as the manufacturer's instructions (Table 2.6). Briefly, mastermix solution was prepared by mixing the reagents to a final concentration of 1X Luna Universal One-Step reaction Mix, 1X Luna WartStart RT Enzyme Mix, 0.4 µM forward and reverse primers and 2.7 µL of Nuclease-free water. Subsequently, 9µL of the mastermix solution was dispensed into the 0.2 mL real-time PCR microcentrifuge tubes. The RNA template with a final concentration of 0.1µg/mL was further added into the mastermix solution to the final volume of 10 µL followed by real-time PCR assay immediately.

Detection of adhesion molecules ICAM-1 and VCAM-1 by real-time PCR was performed using a SYBR Green approach. Primer sequences were adapted from previous studies (Baek *et al.*, 2018; Chu *et al.*, 2017) and synthesised by Apical Scientific (Table 2.7). GAPDH was used as a reference gene and non-template sample was used as a negative control. The thermal cycling conditions were listed as in Table 2.8. Dissociation stage was performed based on the recommendation by the real-time PCR machine's manufacturer.

Table 2.6: Preparation of samples for real-time PCR assay.

Components	10 µl reaction	Final concentration
Luna Universal One-Step Reaction Mix (2X)	5.0 µL	1X
Luna WarmStart RT Enzyme Mix (20X)	0.5 µL	1X
Forward primer (10 µM)	0.4 µL	0.4 µM
Reverse primer (10 µM)	0.4 µL	0.4 µM
Template RNA	1.0 µL	≤ 1 µg (total RNA)
Nuclease-free water	2.7 µL	-

Table 2.7: The primer sequence for real-time PCR

Gene	Forward primer (5'-3')	Reverse primer (3'-5')
ICAM-1 (NM_000201.2, 3249 bp)	GGC CGG CCA GCT TAT ACA C	TAG ACA CTT GAG CTC GGG CA
VCAM-1 (NM_001078.3, 3220 bp)	TCA GAT TGG AGA CTC AGT CAT GT	ACT CCT CAC CTT CCC GCT C
GAPDH (NM_002046.5, 1421 bp)	CCT GCA CCA CCA ACT GCT TA	GGC CAT CCA CAG TCT TCT GAG

(Adapted from Baek *et al.*, 2018; Chu *et al.*, 2017)

Table 2.8: The thermal cycling condition for ICAM-1 and VCAM-1 expression using real-time PCR

Cycle steps	Temperature	Time	Cycles	Stage
Reverse transcription	55°C	10 minutes	1	1
Initial denaturation	95°C	1 minutes	1	2
Denaturation	95°C	10 seconds	40	3
Extension	60°C	60 seconds	1	3
Dissociation stage			1	4

2.5.12 Data acquisition by real-time PCR

Data acquisition of real-time PCR was performed using Applied Biosystem 7500 real-time PCR machine and the cycle threshold were obtained by ABi 7500 real-time PCR software.

The relative expression level of ICAM-1 and VCAM-1 was further calculated in a fold change over control by using double delta Ct analysis ($2^{-\Delta\Delta Ct}$) method (Rao *et al.*, 2013) as the following formula:

TE = Tested experimental gene

HE = Housekeeping gene experimental

TC = Tested control gene

HC = Housekeeping gene control

Step 1: Calculate ΔCTE and ΔCTC

$\Delta CTE = TE - HE$

$\Delta CTC = TC - HC$

Step 2: Calculate ΔΔCt

$\Delta\Delta Ct = \Delta CTE - \Delta CTC$

Step 3: Calculate the value of $2^{-\Delta\Delta Ct}$

2.5.13 Statistical analyses

Statistical analyses were performed using paired Student's t-test by Graphpad Prism 7.0 software for all experimental data. Data were presented as means ± standard error of mean (SEM). Values of $P<0.05$ (*), $P<0.01$ (**) and $P<0.001$ (***) were considered statistically significant. Experiment was repeated for at least three biological replicates.

CHAPTER 3

RESULTS

3.1 Identification of monocyte populations in peripheral blood mononuclear cells (PBMC)

Monocyte populations were identified on PBMC by the expression of CD14 and CD16. PBMC populations were detected based on SSC/FSC profiles by flow cytometry (Figure 3.1A) before being further gated based on the expression of CD14 and CD16. The percentage of monocytes that express $CD14^+$ and $CD16^+$ was 41.10% and 19.79% respectively (Figure 3.1B).

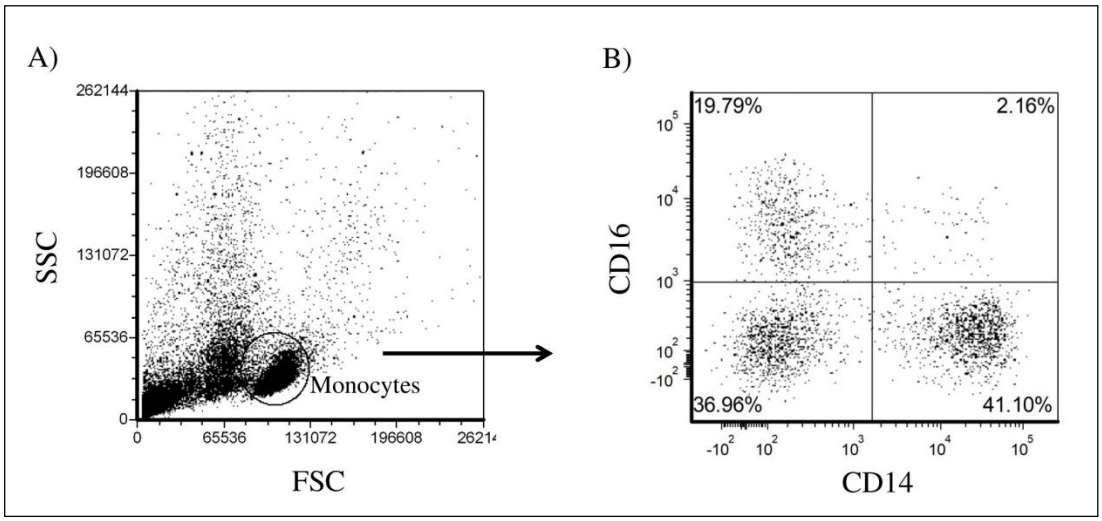


Figure 3.1: Identification of monocyte populations in PBMC. PBMC were stained with anti-CD14 and anti-CD16 followed by flow cytometry analyses. A) Dot plot shows live monocytes in PBMC based on SSC/FSC profiles. B) Dot plot shows the expression of CD14 and CD16 on gated monocytes. Data are representative from four independent experiments (n=4).

3.2 Cell surface phenotypes of monocytes

3.2.1 Purity of whole monocytes

Whole monocytes were isolated from PBMC and were collected from the negative fraction using Pan Monocyte Isolation Kit as described previously in Section 2.5.3(b). Following isolation, whole monocytes were identified based on SSC/FSC profiles (Figure 3.2A) before being further gated based on CD14 and CD16 expressions (Figure 3.2B). The purity of whole monocytes, $CD14^+$ monocytes (Figure 3.2C), and $CD16^+$ monocytes (Figure 3.2D) after isolation was >90%, >85%, and >70% respectively.

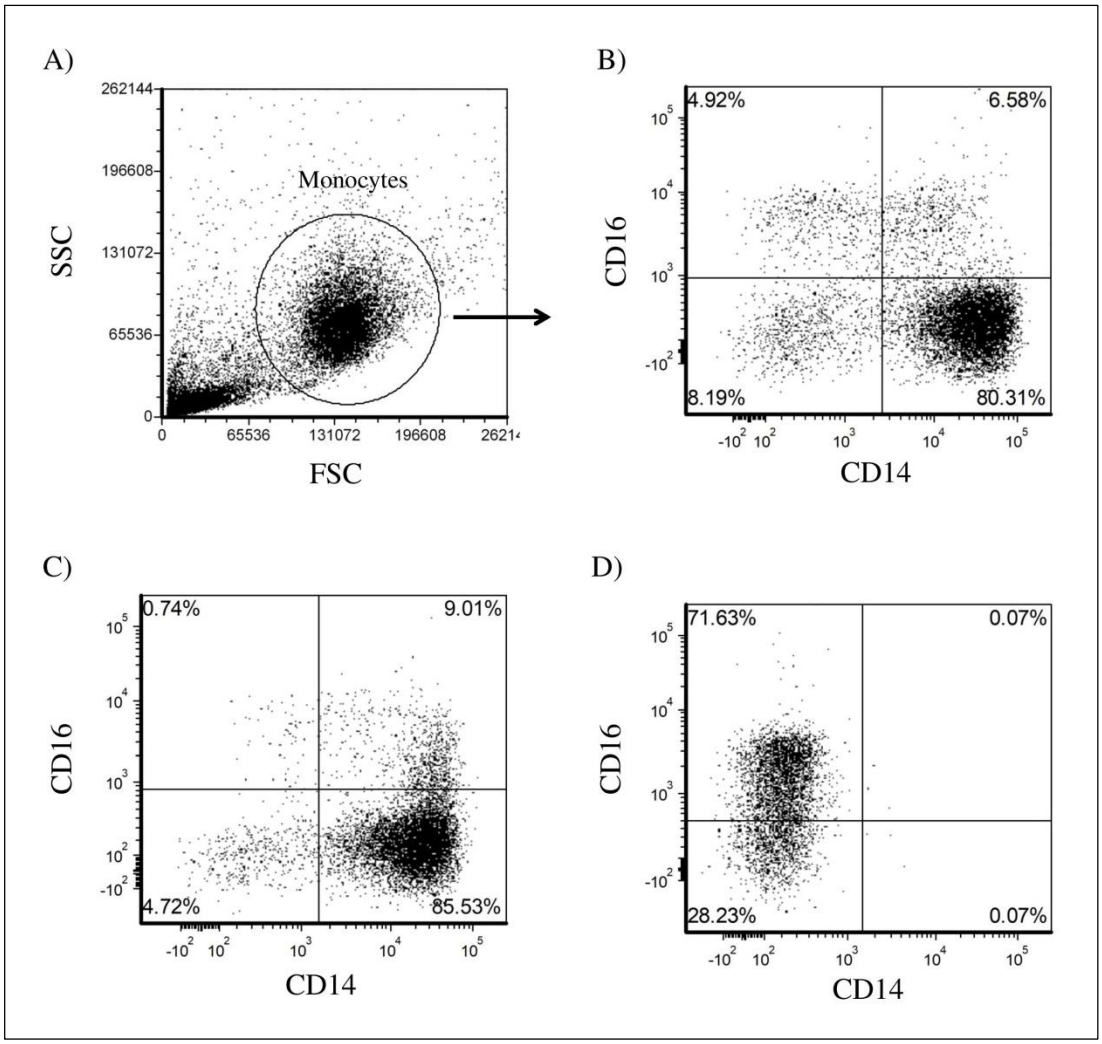


Figure 3.2: Purity of whole monocytes, CD14$^+$ monocytes, and CD16$^+$ monocytes following immunomagnetic isolation. Whole monocytes, CD14$^+$ monocytes, and CD16$^+$ monocytes were stained with anti-CD14 and anti-CD16 prior to flow cytometry analyses. A) Purified whole monocytes were gated on SSC/FSC profile and further gated on B) CD14/CD16. Dot plots show the positive population of C) CD14$^+$ monocytes and D) CD16$^+$ monocytes were assessed based on CD14 and CD16 expressions. Data are representative from four independent experiments (n=4).

3.2.2 Assessment of CD14 and CD16 expressions on whole monocytes, $CD14^+$ monocytes, and $CD16^+$ monocytes

The expression of CD14 and CD16 on whole monocytes, $CD14^+$ monocytes, and $CD16^+$ monocytes was assessed by flow cytometry following 18 hours culture in the presence or absence of LPS. The intensity of CD14 and CD16 expressions on all monocyte subtypes were determined based on the mean fluorescence intensity (MFI).

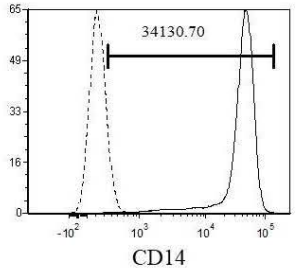
The expression of CD14 and CD16 on whole monocytes were 34130.70 and 745.92 at 0 hour respectively (Figure 3.3A). Following 18 hours culture, the expression of CD14 on unstimulated whole monocytes was 27407.70, which was higher compared to their LPS-stimulated counterparts which was 19024.20. The expression of CD16 on unstimulated and LPS-stimulated whole monocytes was albeit low, which was approximately 440.36 and 368.42 respectively.

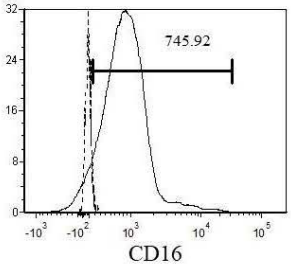
On $CD14^+$ monocytes, the intensity of CD14 and CD16 expressions at 0 hour were 34570.13 and 687.36 respectively (Figure 3.3B). In the presence of LPS, the intensity of CD14 expression on $CD14^+$ monocytes was 16852.05, which was lower compared to 21199.73 on unstimulated $CD14^+$ monocytes. Similarly, the expression of CD16 on $CD14^+$ monocytes was low where the expression intensity was 244.80 on unstimulated $CD14^+$ monocytes and 194.88 on LPS-stimulated $CD14^+$ monocytes.

Meanwhile, the expression of CD14 and CD16 on $CD16^+$ monocytes at 0 hour were 646.68 and 2693.39 respectively (Figure 3.3C). The expression of CD14 on $CD16^+$ monocytes following 18 hours culture was low. The expression of CD14 on

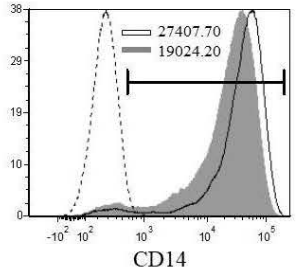
A) Whole monocytes

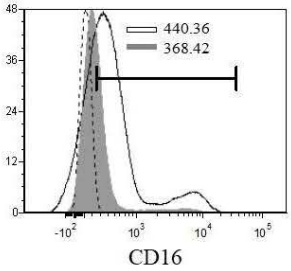
0 hour





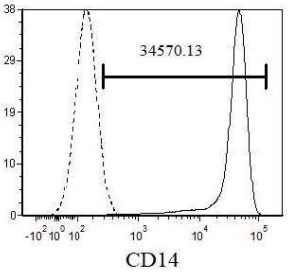
18 hours

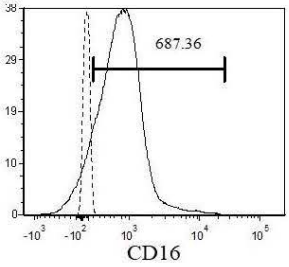




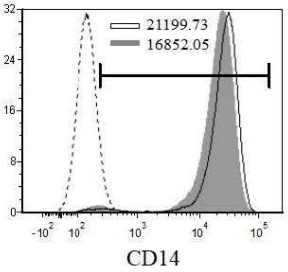
B) CD14^{+} monocytes

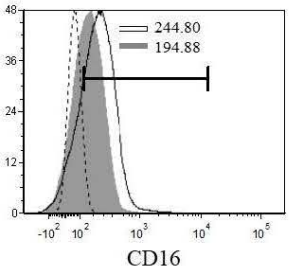
0 hour





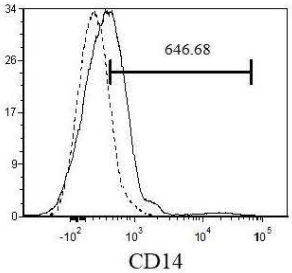
18 hours

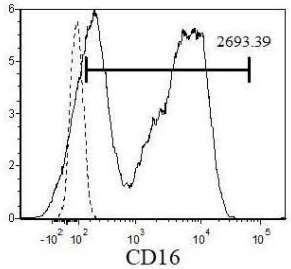




C) CD16^{+} monocytes

0 hour





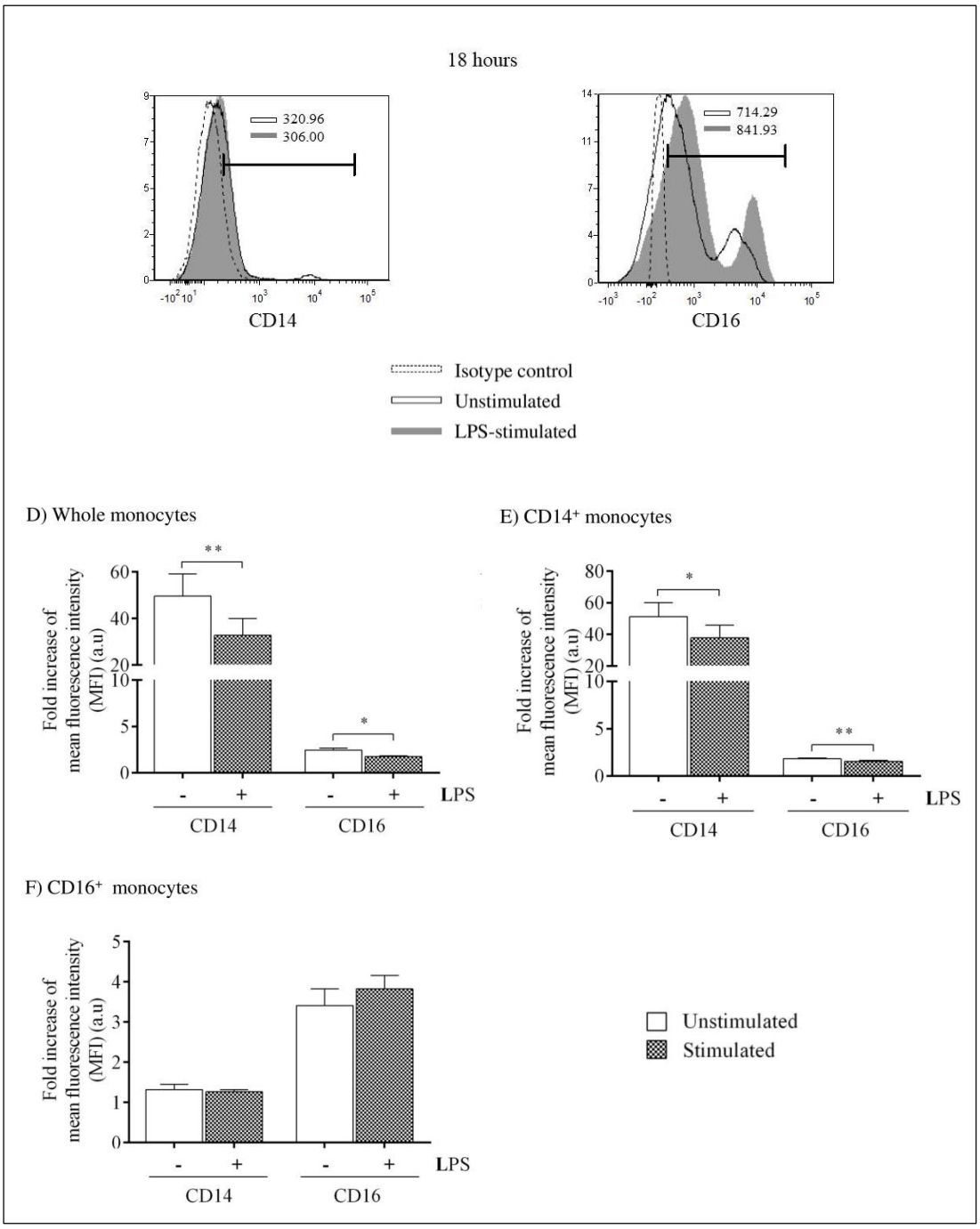


Figure 3.3: The expression of CD14 and CD16 on monocyte subsets. Whole monocytes, $CD14^+$ monocytes, and $CD16^+$ monocytes were labelled with anti-CD14 and anti-CD16 antibodies prior to flow cytometry analysis. Histograms show the expression of CD14 and CD16 on unstimulated (black line) and stimulated (grey-filled) A) whole monocytes, B) $CD14^+$ monocytes and C) $CD16^+$ monocytes at 0 and 18 hours of culture in the presence or absence of LPS. Isotype controls (dotted line) were used as negative controls. D) Fold change in mean fluorescence intensity (MFI) of CD14 and CD16 expressions on monocyte subsets over control of all monocyte subsets following 18 hours culture. Data shown are representative from four independent experiments. Error bars represent mean ± standard error of the mean (SEM), *p<0.05, **p<0.01 (n=4).

unstimulated $CD16^+$ monocytes was 320.96, while on LPS-stimulated $CD16^+$ monocytes was 306. In contrast, the MFI intensity of CD16 expression on unstimulated $CD16^+$ monocytes was 714.29 compared to LPS-stimulated $CD16^+$ monocytes, which was 841.93.

The changes in MFI of CD14 and CD16 expressions on all unstimulated and LPS-stimulated monocyte subsets following 18 hours culture were statistically analysed (n=4) (Figure 3.3D–F). The changes in MFI were calculated as fold change over control, which was defined by MFI of sample divided by the MFI of isotype control. The expression of CD14 and CD16 on all monocyte subtypes was reduced following LPS stimulation with the exception of CD16 expression on $CD16^+$ monocytes.

The expression of CD14 on unstimulated whole monocytes was 49.56 fold higher than isotype control. In the presence of LPS, CD14 expression on whole monocytes decreased to 32.72 fold (p=0.008) than on unstimulated monocytes. A similar expression pattern was observed for CD16 expression on whole monocytes. The expression of CD16 on unstimulated whole monocytes was 2.45 fold and significantly decreased to 1.75 fold (p=0.03) on LPS-stimulated whole monocytes.

A significant decrease of CD14 expression was observed on LPS-stimulated $CD14^+$ monocytes, which was 37.73 fold compared to 51.17 fold (p=0.026) on unstimulated $CD14^+$ monocytes (Figure 3.3.E). Despite low MFI, CD16 expression on LPS-stimulated $CD14^+$ monocytes was 1.56 fold which was significantly lower compared to unstimulated $CD14^+$ monocytes, which was 1.83 fold (p=0.004).

In the presence of LPS, CD14 expression on $CD16^+$ monocytes was 1.32 fold higher than isotype control and decreased to 1.26 fold on LPS-stimulated $CD16^+$ monocytes (Figure 3.3F). In contrast, CD16 expression on unstimulated $CD16^+$ monocytes was 3.41 fold and increased to 3.82 fold on LPS-stimulated $CD16^+$ monocytes, although not significant.

3.3 Quantification of monocytic microparticles (mMP)

Monocytic MP derived from the whole monocytes, $CD14^+$ monocytes, and $CD16^+$ monocytes were quantified by using TruCount beads to obtain the absolute amount of mMP (mMP/µL) released in culture supernatants. The absolute amount of mMP was defined by (events of mMP/beads events) × (total number of beads/total volume). Bead events were obtained from Trucount beads region gated on SSC/FSC profiles (Figure 3.4A), while the number of Annexin-V^+/$antigen^+$ mMP events were obtained from the region gated on Annexin-V^+/$CD14^+$ or Annexin-V^+/$CD16^+$.

The absolute number of Annexin-V^+/$CD14^+$ mMP derived from unstimulated whole monocytes was 288.1 ± 114.1 mMP/µL and significantly increased on LPS-stimulated whole monocytes, which was 564.1 ± 100.4 mMP/µL (p=0.014) (Figure 3.4B). Likewise, the absolute count of Annexin-V^+/$CD16^+$ mMP from unstimulated whole monocytes was 258.1 ± 27.78 mMP/µL, which was significantly lower compared to LPS-stimulated whole monocytes, which was 443.4 ± 47.06 mMP/µL (p=0.018).

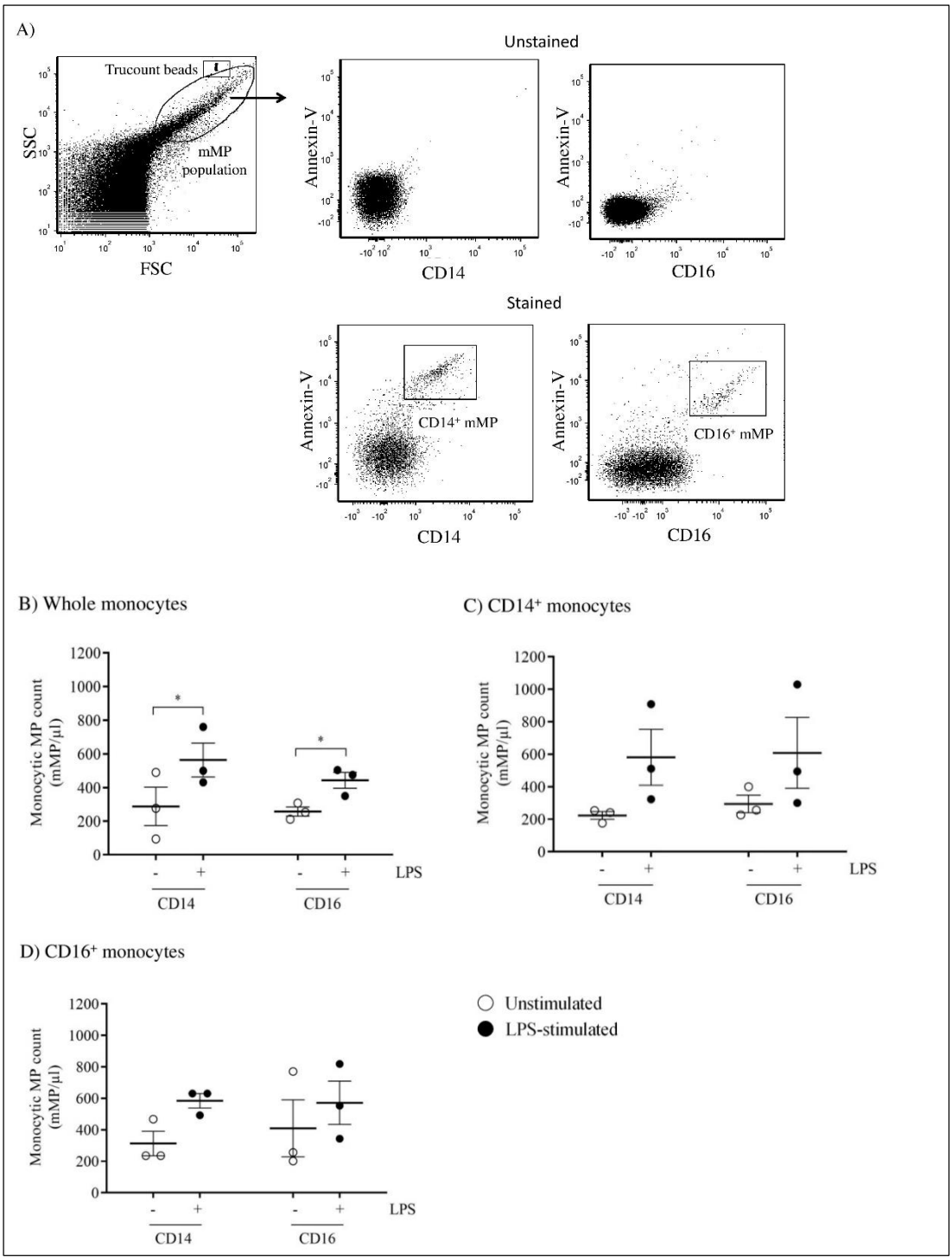


Figure 3.4: Quantification of mMP derived from monocyte subsets by TruCount beads. A) Monocytic MP population and TruCount beads were assessed on SSC/FSC profiles. Unstained and stained mMP were further gated on Annexin-V$^+$/CD14$^+$ and Annexin-V$^+$/CD16$^+$ populations. Absolute number of mMP derived from B) whole monocytes, C) CD14$^+$ monocytes, and D) CD16$^+$ monocytes were quantified in the presence or absence of LPS. Data shown are representative from three independent experiments. Error bars represent mean ± standard error of the mean (SEM), *p<0.05 (n=3).

In the absence of LPS, the absolute count of Annexin-V^+/$CD14^+$ mMP derived from unstimulated $CD14^+$ monocytes was 223.6 ± 23.87 mMP/µL and increased to 581.4 ± 172.3 mMP/µL following LPS stimulation, although not significant (Figure 3.4C). Meanwhile, the absolute number of LPS-stimulated $CD14^+$ monocyte-derived mMP expressing Annexin-V^+/$CD16^+$ increased to 608.2 ± 217.8 mMP/µL compared to those derived from unstimulated $CD14^+$ monocytes which was 294.5 ± 53.76 mMP/µL.

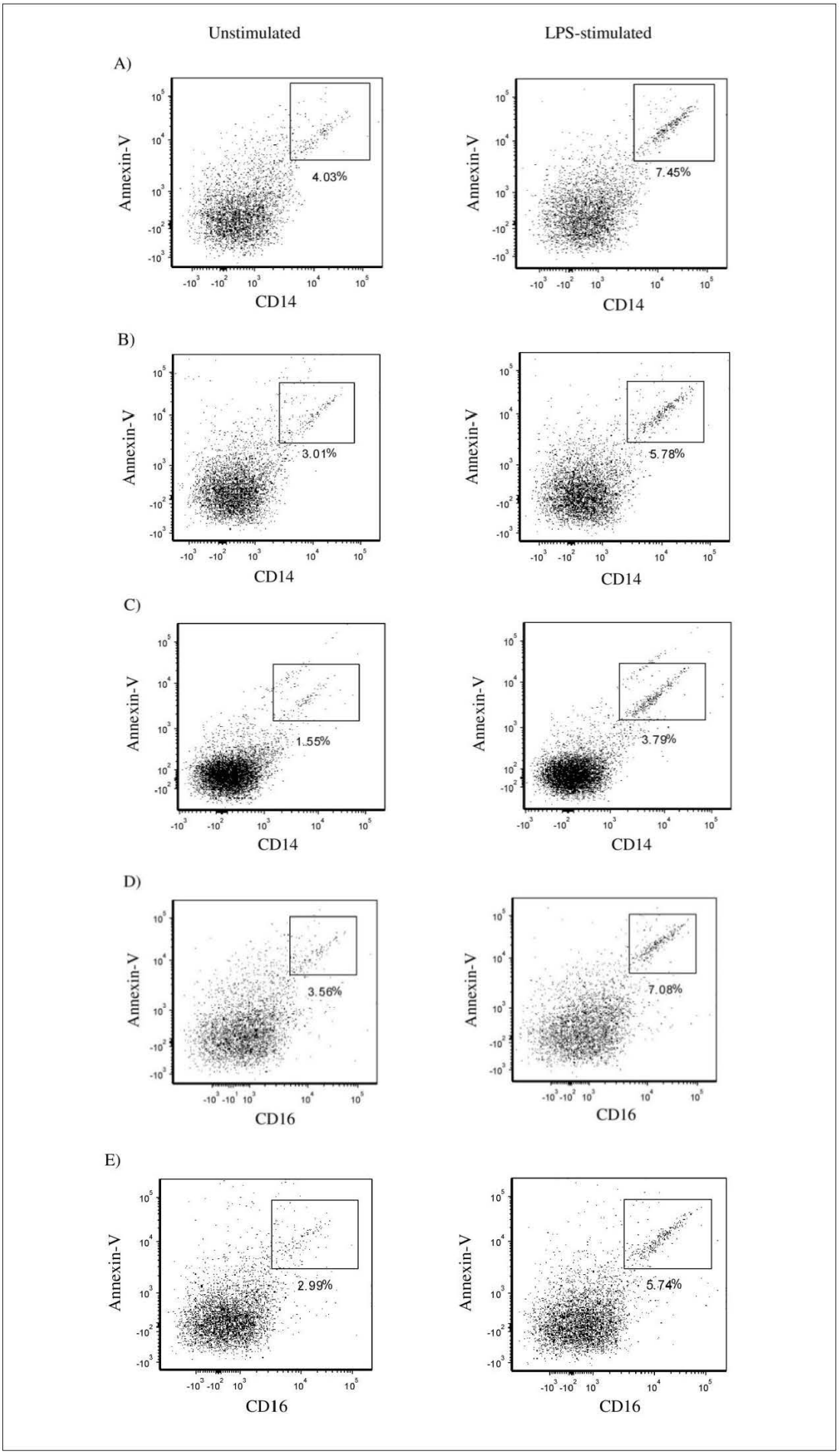
Meanwhile, the number of mMP expressing Annexin-V^+/$CD14^+$ derived from $CD16^+$ monocytes decreased in the presence of LPS. The number of Annexin-V^+/$CD14^+$ mMP derived from unstimulated $CD16^+$ monocytes was 313.1 ± 77.31 mMP/µl compared to their LPS-stimulated counterparts which was 584.6 ± 46 mMP/µL (Figure 3.4D). Similarly, the count of Annexin-V^+/$CD16^+$ derived from unstimulated $CD16^+$ monocytes was 409.5 ± 181.2 mMP/µL and the expression of Annexin-V^+/$CD16^+$ on $CD16^+$ monocyte-derived mMP increased to 571.8 ± 137.5 mMP/µL, although not significant.

3.4 Assessment of CD14 and CD16 expressions on mMP derived from whole monocytes, $CD14^+$ monocytes, and $CD16^+$ monocytes

The expression of CD14 and CD16 in combination with Annexin-V was assessed on mMP derived from the whole monocytes, $CD14^+$ monocytes, and $CD16^+$ monocytes. Monocytic MP were isolated from culture supernatants as described in Section 2.5.4(a). The detection of endotoxin level in mMP derived from all monocyte subsets was initially performed using ToxinSensor™ Chromogenic Limulus Amebocyte Lysate (LAL) Endotoxin Assay Kit. All mMP cultures in this study were free from endotoxin contamination as determined by the endotoxin detection assay (~0.7 EU/mL). By using forward and side scatter on logarithmic scale of a flow cytometer, mMP were identified based on the expression of PS indicated by Annexin-V and CD14 or CD16 (Figure 3.5).

CD14 and CD16 in combination with Annexin-V were expressed on all mMP derived from unstimulated and LPS-stimulated whole monocytes, $CD14^+$ monocytes, and $CD16^+$ monocytes at different intensities. The expression of these antigens were higher on mMP derived from stimulated whole monocytes, $CD14^+$ monocytes, and $CD16^+$ monocytes compared to those from unstimulated mMP (Figure 3.4A–F).

Monocytic MP derived from unstimulated whole monocytes express 4.03% Annexin-$V^+/CD14^+$ and increased to 7.45% following LPS stimulation (Figure 3.5A). Likewise, the expression of Annexin-$V^+/CD14^+$ on mMP derived from unstimulated $CD14^+$ monocytes was 3.01%, while on mMP derived from LPS-stimulated $CD14^+$


Unstimulated
LPS-stimulated
A)
Annexin-V
CD14
4.03%
7.45%
B)
3.01%
5.78%
C)
1.55%
3.79%
D)
CD16
3.56%
7.08%
E)
2.99%
5.74%

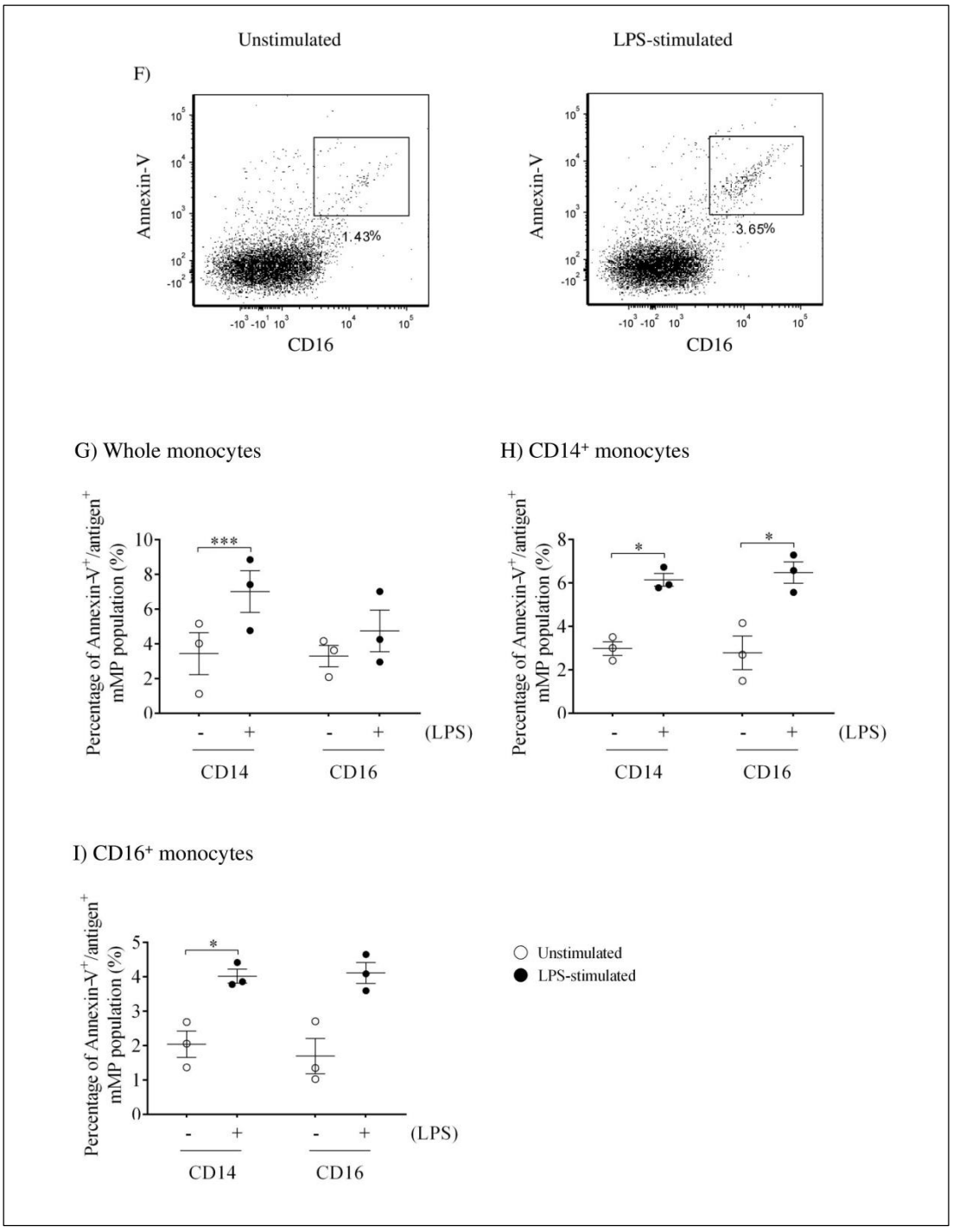


Figure 3.5: The expression of CD14 and CD16 on mMP derived from whole monocytes, CD14$^+$ monocytes, and CD16$^+$ monocytes. Monocytic MP derived from all monocyte subtypes were stained with Annexin-V in combination with anti- CD14 or anti-CD16. A–C) Dot plots show mMP population derived from unstimulated and LPS-stimulated cells gated on Annexin-V$^+$/CD14$^+$ and D–F) Annexin-V$^+$/CD16$^+$ population. Isotype control was used as a negative control. Percentage of double positive mMP population derived from G) whole monocytes, H) CD14$^+$ monocytes, and I) CD16$^+$ monocytes were calculated. Data shown are representative from three independent experiments. Error bars represent mean ± standard error of the mean (SEM), *p<0.05, **p<0.01, ***p<0.001 (n=3).

monocytes was 5.78% (Figure 3.5B). Meanwhile, the expression of Annexin-$V^+/CD14^+$ on mMP derived from unstimulated and LPS-stimulated $CD16^+$ monocytes was relatively low, which was only 1.55% and 3.79% respectively (Figure 3.5C).

Meanwhile, the expression of Annexin-$V^+/CD16^+$ was 3.56% on mMP derived from unstimulated whole monocytes (Figure 3.5D). This expression was elevated to 7.08% on mMP derived from LPS-stimulated whole monocytes. Similarly, the expression of Annexin-$V^+/CD16^+$ on mMP derived from unstimulated $CD14^+$ monocytes was 2.99% and increased to 5.74% on those derived from LPS-stimulated $CD14^+$ monocytes (Figure 3.5E). On mMP derived from unstimulated $CD16^+$ monocytes, the expression of Annexin-$V^+/CD16^+$ was only 1.43% (Figure 3.5F). However, the expression of Annexin-$V^+/CD16^+$ was higher on LPS-stimulated $CD16^+$ monocyte-derived mMP which was 3.65%.

Overall, the expression of Annexin-V in combination with CD14 or CD16 on mMP from three independent experiments were increased on LPS-stimulated monocyte-derived mMP compared to their unstimulated counterparts (Figure 3.5G–I). The percentage of Annexin-$V^+/CD14^+$ on LPS-stimulated whole monocyte-derived mMP was significantly higher by 7.01 ± 1.19% compared to on mMP derived from unstimulated whole monocytes which was 3.44 ± 1.2% (p=0.006) (Figure 3.5G). The expression of Annexin-$V^+/CD16^+$ on LPS-stimulated whole monocyte-derived mMP was 4.747 ± 1.19% compared to on whole monocyte-derived mMP in the absence of LPS which was 3.30 ± 0.62%.

Similarly, a significant increase of Annexin-V^+/$CD14^+$ and Annexin-V^+/$CD16^+$ expressions was observed for $CD14^+$ monocytes-derived mMP (Figure 3.5H). The expression of Annexin-V^+/$CD14^+$ on LPS-stimulated $CD14^+$ monocyte-derived mMP was 6.14 ± 0.29% compared to their unstimulated counterparts, which was 2.98 ± 0.31% (p=0.032). The percentage of Annexin-V^+/$CD16^+$ expression on unstimulated $CD14^+$ monocyte-derived mMP was 0.27 ± 0.72%. In the presence of LPS, these antigen expression on $CD14^+$ monocyte-derived mMP was significantly increased to 6.48 ± 0.49% (p=0.033).

The percentage of Annexin-V^+/$CD14^+$ expression was significantly increased from 0.20 ± 0.38% on mMP derived from unstimulated $CD16^+$ monocytes to 4.02 ± 0.20% (p=0.039) on those from LPS-stimulated $CD16^+$ monocytes (Figure 3.5I). A similar expression pattern was observed for Annexin-V^+/$CD16^+$ on $CD16^+$ monocytes. Approximately 4.11 ± 0.3% of Annexin-V^+/$CD16^+$ was expressed on LPS-stimulated $CD16^+$ monocyte-derived mMP compared to 1.69 ±0.51% on mMP derived from unstimulated $CD16^+$ monocytes, although not significant.

3.5 Assessment of mMP role in coagulation

3.5.1 CD142 expression on monocyte subsets and their derived mMP

The expression of CD142 or tissue factor (TF) was assessed on LPS-stimulated whole monocytes (Figure 3.6A), $CD14^+$ monocytes (Figure 3.6B), and $CD16^+$ monocytes (Figure 3.6C) as well as their derived mMP. The MFI of CD142 expression was calculated as fold change over control.

The intensity of CD142 expression on unstimulated whole monocytes was 1.951 fold compared to isotype control and significantly increased to 2.77 fold (p=0.005) upon LPS stimulation (Figure 3.6D). Likewise, CD142 expression was higher on LPS-stimulated $CD14^+$ monocytes which was 3.24 fold compared to 2.71 fold on unstimulated $CD14^+$ monocytes although not significant. Meanwhile, the expression intensity of CD142 was significantly increased from 1.14 fold on unstimulated $CD16^+$ monocytes to 1.52 fold (p=0.018) on LPS-stimulated $CD16^+$ monocytes.

On the other hand, the percentage of Annexin-V^+/$CD142^+$ expressed on mMP derived from unstimulated whole monocytes was 7.56 ± 1.09% (Figure 3.6E). The percentage of Annexin-V^+/$CD142^+$ on whole monocytes-derived mMP was significantly increased to 11.16 ± 0.84% (p=0.015) in the presence of LPS. The percentage of monocytic MP derived from unstimulated $CD14^+$ monocytes which expressed Annexin-V^+/$CD142^+$ was 1.43 ± 0.1% and increased to 3.01 ± 0.46% on mMP derived from stimulated $CD14^+$ monocytes, however not significant. Likewise, Annexin-V^+/$CD142^+$ expressed on mMP derived from unstimulated $CD16^+$ monocytes was 0.27 ± 0.7%, which was lower compared to 3.32 ± 0.19% on LPS-stimulated $CD16^+$ monocytes, although not significant.

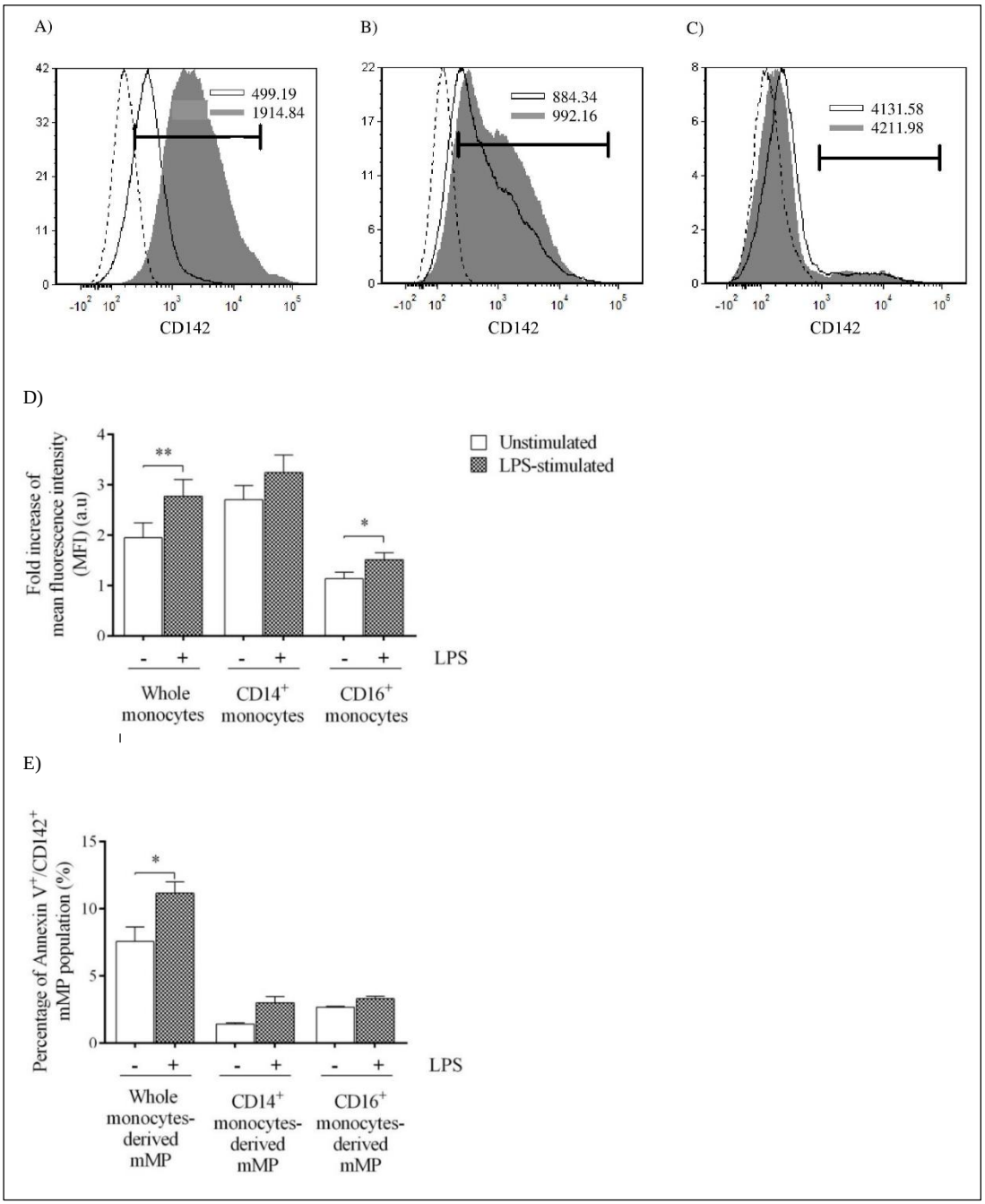


Figure 3.6: The expression of CD142 on monocyte subsets and their derived mMP. Histogram show the expression of CD142 on A) whole monocytes, B) $CD14^+$ monocytes, and C) $CD16^+$ monocytes. D) Bar chart shows the fold change of CD142 expression on whole monocytes, $CD14^+$ monocytes, and $CD16^+$ monocytes. E) Bar chart shows the percentage of CD142 expression in combination with Annexin-V on mMP derived from whole monocytes, $CD14^+$ monocytes, and $CD16^+$ monocytes. Data shown are representative from three independent experiments. Error bars represent mean ± standard error of the mean (SEM), *p<0.05, **p<0.01 (n=3).

3.5.2 Analysis of prothrombin time (PT) in the presence of mMP

The increased expression of CD142 on mMP derived from whole monocytes led us to assess clotting time in the presence of mMP by measuring prothrombin time. The clotting time was first assessed on whole monocytes and PBMC as a control. Normal pooled plasma was used as an experimental control to determine the normal range of clotting time in a healthy physiological state.

This study has shown that plasma clotting time were unaffected by the addition of both unstimulated and stimulated monocytes regardless the concentration of the cells used (Figure 3.7). The plasma clotting time for unstimulated monocytes at 1×10^6 cells/mL was 12.70 ± 0.21 seconds. The time taken for clot formation was slightly reduced after the addition of LPS-stimulated monocytes at 1×10^6 cells/mL, which was 12.97 ± 0.09. Similarly, the plasma clotting time for unstimulated monocytes at 5×10^6 cells/mL was 16.9 ± 3.95 seconds and slightly reduced to 16.2 ± 3.6 seconds in the presence of a similar cell concentration of LPS-stimulated monocytes.

In contrast, plasma clotting time was reduced in the presence of mMP derived from LPS-stimulated monocytes. A significant reduction in time taken for clot formation was observed after a higher concentration of mMP derived from stimulated monocytes was added. Prothrombin time of mMP derived from unstimulated monocytes at 1×10^6 cells/mL was 18.47 ± 5.72 seconds and decreased to 17.73 ± 5.19 seconds in the presence mMP derived from stimulated monocytes. The addition of higher mMP concentration derived from stimulated monocytes at 5×10^6 cells/mL resulted in a significant reduction of clotting time, which was 12.43 ± 0.89 seconds compared to unstimulated counterparts, which was 12.8 ± 0.85 seconds (p=0.008).

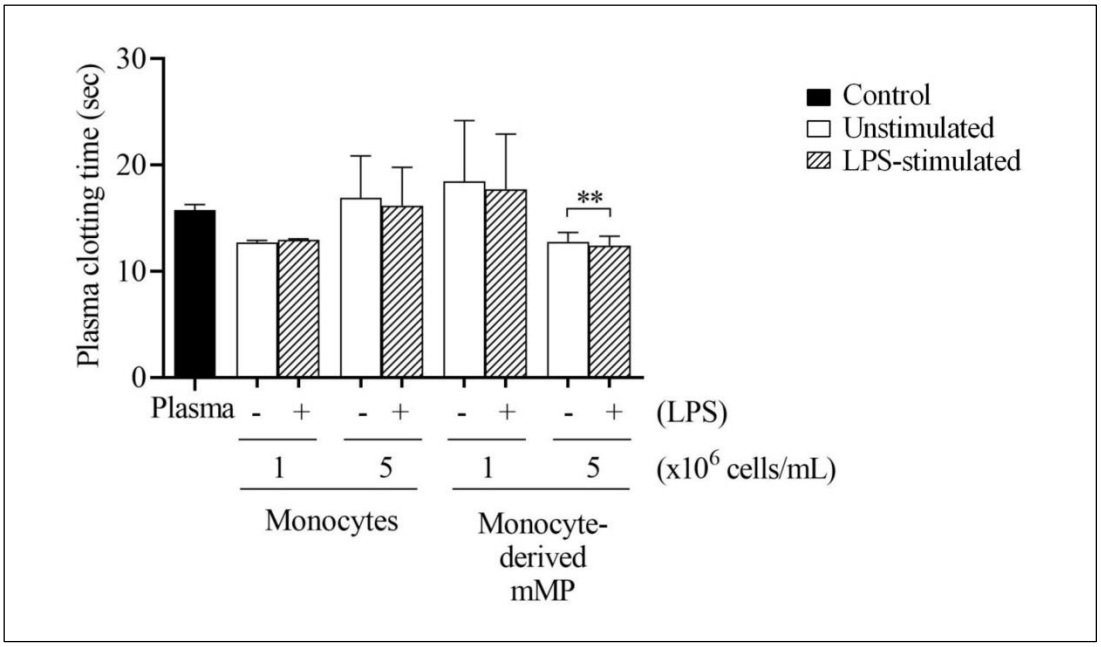


Figure 3.7: Plasma clotting time by monocytes and their derived mMP. Prothrombin time (PT) assay was performed using semi-automated Start 4 coagulometer. Bar charts represent the plasma clotting time taken for monocytes and their derived mMP after incubation with normal pooled plasma for 180 seconds followed by the addition of neoplastin. Normal pooled plasma was used as a control. Results are from three independent experiments. Error bars represent mean ± standard error of the mean (SEM), $^{**}p<0.01$ (n=3).

3.6 Assessment of endothelial cell activation in the presence of mMP

3.6.1 Expression of endothelial cell markers following culture with mMP

The potential role of mMP in altering endothelial cell phenotype was examined. The expression of CD31 on endothelial cells was first identified by flow cytometry. Confluent monolayers of HUVEC were successfully cultured in the presence or absence of mMP. HUVEC expressed CD31 at various intensities depending on the type of stimulation (Figure 3.8A). The expression of CD31 on HUVEC without stimulation was 19.28 fold higher than isotype control and significantly increased to 39.83 fold (p=0.005) in the presence of mMP derived from unstimulated monocytes. In the presence of mMP derived from LPS-stimulated monocytes, CD31 expression on HUVEC was 46.15 fold, which was significantly higher compared to 36 fold (p=0.033) when modulated by LPS alone.

Meanwhile, the level of ICAM-1 and VCAM-1 expression was conducted by serum-starving HUVEC followed by incubation with or without mMP. The integrity of HUVEC total RNA was first assessed using gel electrophoresis, which was highly integrated (Appendix A). The expression level of ICAM-1 and VCAM-1 by endothelial cells were analysed by real-time PCR. GAPDH was chosen as a reference gene while unstimulated HUVEC was used as a negative control. High linearity for slope of standard curve was obtained for GAPDH, ICAM-1, and VCAM-1 (Appendix B). Meanwhile, the amplification efficiency of real-time PCR assay was 105.12% for GAPDH, 101.74% for ICAM-1, and 102.96% for VCAM-1.

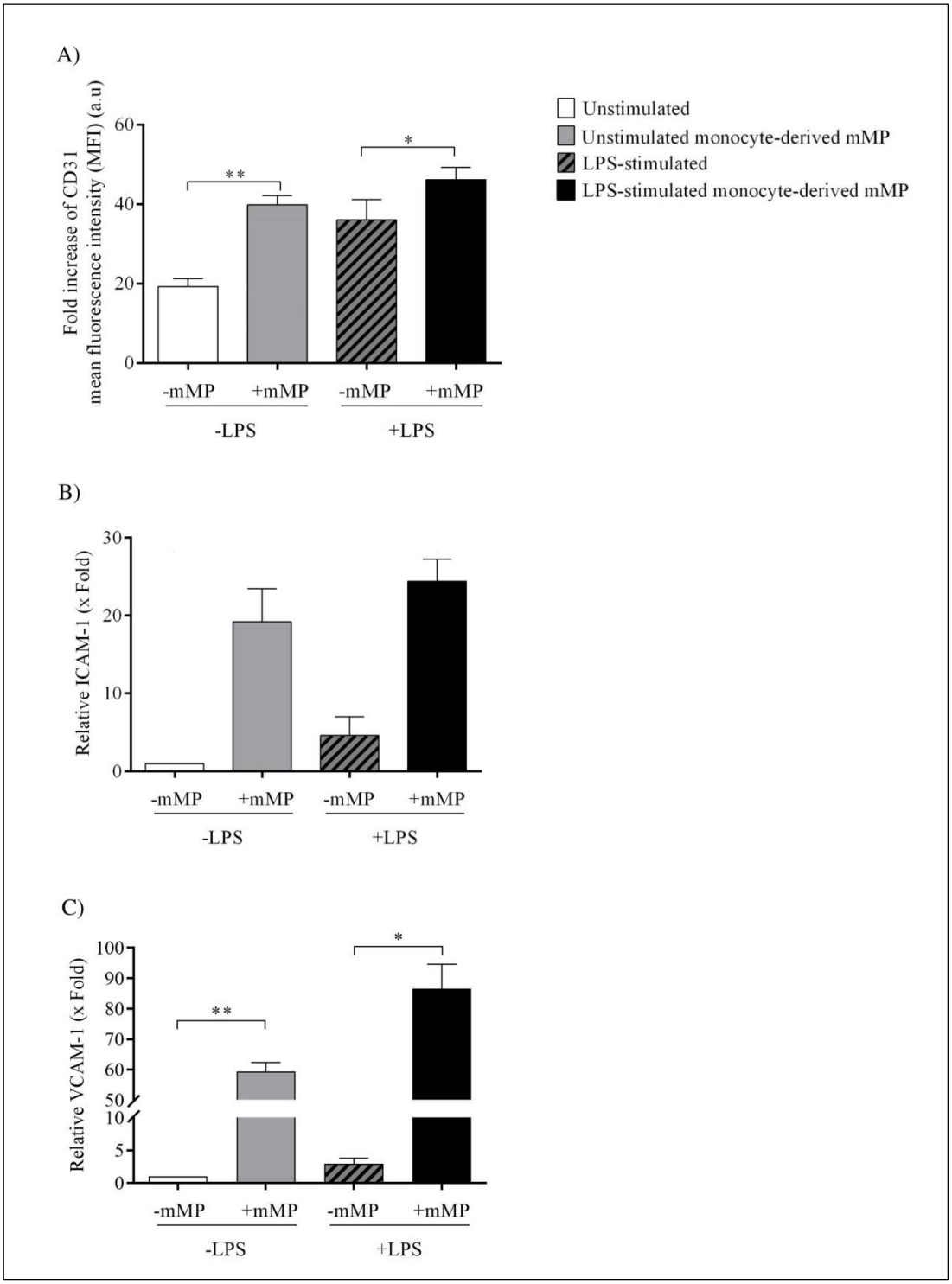


Figure 3.8: The expression of endothelial cell markers on HUVEC in the presence of mMP. HUVEC were stimulated with LPS, unstimulated monocyte-derived mMP, or LPS-stimulated monocyte-derived mMP. The expression of A) CD31, B) ICAM-1, and C) VCAM-1 were calculated in a relative fold change. Unstimulated HUVEC were used as an experimental negative control and GAPDH was used as a reference gene. Results are representative from three independent experiments. Error bars represent mean ± standard error of the mean (SEM), *p<0.05, **p<0.01 (n=3).

Under serum-free condition, mMP were able to govern the expression of adhesion molecules on HUVEC. Monocytic MP from both unstimulated and LPS-stimulated monocytes induced the expression of ICAM-1 (Figure 3.8B) and VCAM-1 (Figure 3.8C). In the presence of unstimulated monocyte-derived mMP, the expression of ICAM-1 on HUVEC was increased 19.22 fold compared to unstimulated HUVEC. Meanwhile, the expression of ICAM-1 on HUVEC in the presence of LPS-stimulated monocyte-derived mMP was increased 24.42 fold compared to HUVEC stimulated with LPS alone which was 4.58 fold.

Similarly, the expression of VCAM-1 was significantly higher on HUVEC stimulated with unstimulated monocyte-derived mMP, which was 59.30 fold (p=0.002) compared to unstimulated HUVEC. In the presence of mMP derived from LPS-stimulated monocytes, the expression of VCAM-1 was 86.49 fold, which was significantly higher compared to 2.99 fold (p=0.01) on those stimulated with LPS alone.

3.6.2 CD31 expression on endothelial microparticles (eMP) in the presence of mMP

After demonstrating the capability of mMP from monocytes in enhancing the expression of CD31 and adhesion molecules by HUVEC, we further assessed the eMP production upon culture with mMP. Endothelial MP population was identified based on the SSC/FSC profiles of a flow cytometer, followed by gating on Annexin-V^+/$CD31^+$ population. Culture supernatants from unstimulated HUVEC was analysed for the presence of eMP to determine the baseline production of eMP.

In the absence of LPS, the percentage of Annexin-V^+/$CD31^+$ on eMP was 15.23% (Figure 3.9A). The percentage of these antigens on eMP was increased to 24.01% following LPS stimulation (Figure 3.9B). In the presence of mMP from unstimulated whole monocytes, the percentage of Annexin-V^+/$CD31^+$ expression on eMP was 17.96% (Figure 3.9C), which was lower compared to 30.94% with the presence of LPS-stimulated whole monocyte-derived mMP (Figure 3.9D).

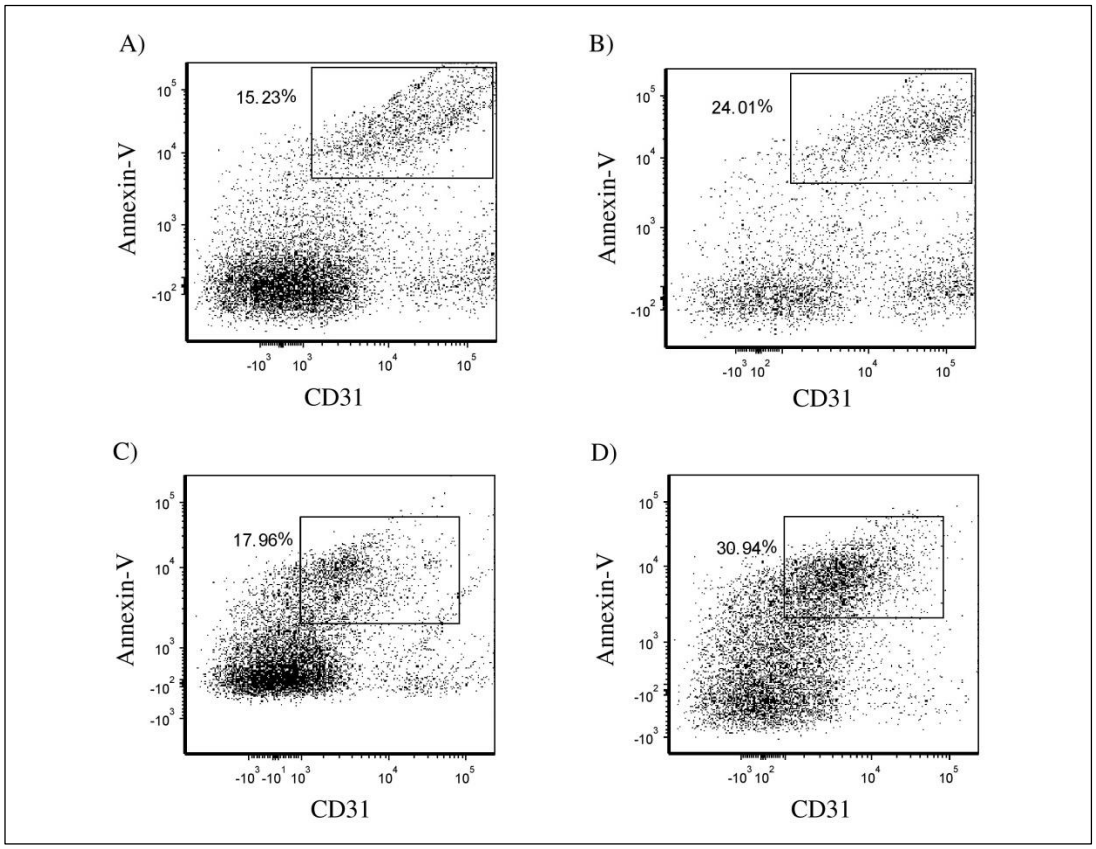


Figure 3.9: Analysis of CD31 expression on endothelial microparticles (eMP). Microparticles derived from endothelial cells were identified as Annexin-V^+/$CD31^+$ population on CD31/Annexin-V profile. Dot plots show the gated eMP population derived from A) unstimulated HUVEC, B) LPS-stimulated HUVEC, C) HUVEC stimulated with mMP from unstimulated monocytes, and D) HUVEC stimulated with mMP derived from LPS-stimulated monocytes. Results are representative from three independent experiments (n=3).

CHAPTER 4

DISCUSSION

Microparticles (MP) have gained interest due to their important role in various biological activities particularly in inflammation (Batool, 2013), thrombosis (Mooberry and Key, 2016), and endothelial dysfunction (Helbing *et al.*, 2014). However, phenotypic characteristics and functional roles of MP derived from human blood monocytes remain unknown. Most *in vitro* studies on mMP were conducted by using cell lines or without distinguishing the blood monocyte subsets. Thus, this study intended to investigate the phenotypic profiles of mMP derived from different blood monocyte subsets such as whole monocytes, $CD14^+$ monocytes, and $CD16^+$ monocytes as well as their potential role in coagulation and endothelial cell functions.

4.1 Expression of CD14 and CD16 on monocyte subsets

Cell surface expression of whole monocytes, $CD14^+$ monocytes and $CD16^+$ monocytes as well as their derived mMP was examined. There are several types of stimuli used to generate mMP *in vitro* such as LPS, calcium ionophore, histamine (Cerri *et al.*, 2006), and P-selectin–Ig chimera (Bernimoulin *et al.*, 2009). Lipopolysaccharides (LPS) have been extensively used for immune cell activation due to its high binding capacity to CD14, a co-receptor for LPS. Additionally, LPS is able to elicit strong immune response in humans and activate immune cells via TLR 4 (Soop *et al.*, 2013). The mechanism of TLR 4 activation by LPS involves the binding of LPS with serum LPS-binding protein (LBP), in which LBP facilitates in transferring LPS molecule to CD14 (Harris *et al.*, 2001). The interaction between LPS and CD14 forming CD14–LPS

complex is essential to initiate cell activation efficiently through TLR 4 and MD-2 molecule (Latz *et al.*, 2002). MD-2 molecule is a protein that associates with TLR 4 on the cell surface, forming a receptor complex for LPS recognition (Ohnishi *et al.*, 2003). TLR 4/MD-2 complex is a potent receptor for LPS recognition and evokes multimolecular complex on the plasma membrane (Borzęcka *et al.*, 2013). Therefore, LPS was used in this study to induce mMP production by monocyte subsets. Optimisation of LPS concentration was initially performed to determine the optimum working concentration for mMP generation. The working concentration of 1 µg/mL LPS was chosen as it enhanced the release of mMP without compromising cell viability (Appendix C).

The permeability of cell membrane is a vital indicator for cell function in *in vitro* studies (Aysun *et al.*, 2016). A routine cell viability measurement after cell isolation and culture is critical to ensure the quality and reliability of cells (Mallone *et al.*, 2011). In this study, cell viability assessment was performed using Trypan blue exclusion for all cell types to ensure that mMP were primarily released following LPS stimulation and not due to cell death. Only cells with 95% viability or higher were used for mMP assessment by flow cytometry as recommended previously (Bernimoulin *et al.*, 2009). Apart from that, the contamination of endotoxin in mMP preparation and culture may severely affect the reliability of the experimental data. Endotoxin contamination may arise during cell culture preparations including from media, sera, consumables, and growth additives (Magalhães *et al.*, 2007). In this study, no endotoxin contamination was detected in mMP.

Upon cell activation by LPS, mMP were released into culture supernatants. Numerous protocols for mMP isolation were listed in MISEV 2018 including immunoisolation,

precipitation, density gradient, and filtration. However, the choice of mMP separation method as well as the purity of mMP is crucial as contaminants may attribute to alteration of mMP function (Théry *et al.*, 2018). Among all, the differential ultracentrifugation technique is widely used to isolate mMP (Gardiner *et al.*, 2016). This method is able to isolate mMP with high recovery rate (Mateescu *et al.*, 2017). In this study, culture supernatants were subjected to two-steps centrifugation followed by ultracentrifugation to isolate mMP as recommended previously (Crompot *et al.*, 2015). Culture supernatants were first centrifuged at 500 × *g* to facilitate the separation and removal of cells from mMP in the supernatants. It has been reported that an initial centrifugation with lower speed between 300 and 500 × *g* for 5 to 20 minutes is a crucial step in obtaining cell-free supernatants (Orozco and Lewis, 2010). The second centrifugation at 1,200 × *g* was performed to remove remaining cells or debris in the supernatants (Coumans *et al.*, 2017b), thus assisting in successful separation of true events from background noise during flow cytometry analysis (Dey-Hazra *et al.*, 2010). Low centrifugation speed may result in incomplete removal of cells and subsequently leads to flow cytometry analysis disturbances due to debris and unwanted cellular events (Dey-Hazra *et al.*, 2010). Supernatants containing mMP were further ultracentrifuged at 20,000 × *g* as higher speed centrifugation is able to avoid exosome contamination during mMP isolation since mMP are pelleted and exosomes remain in the supernatant (Menck *et al.*, 2017).

It should be considered that some mMP might be lost during centrifugation. Therefore, mMP was isolated at 4°C to ensure their stability since ultracentrifugation may produce heat which leads to mMP degradation (Dey-Hazra *et al.*, 2010). Additionally, mMP was freshly isolated to ensure high yield of mMP (Lacroix and Dignat-George,

2012) as delay in isolation may result in mMP loss and changes in mMP characteristics as well as their morphology (Dey-Hazra *et al.*, 2010; Poncelet *et al.*, 2015).

In this study, flow cytometry was used to assess surface antigen expression by mMP (Crompot *et al.*, 2015). During flow cytometry analysis, mMP was gated based on the positive expression for selected markers in combination with Annexin-V. A defined size beads have been previously used to facilitate detection of mMP on flow cytometry (Rousseau *et al.*, 2015). In this study, particle-size beads were not used since they may generate non-specific staining; in addition, the refractive index of particle-size beads and mMP are different (van der Pol *et al.*, 2012). This may result in underestimation of the size of mMP and therefore contribute to false-positive results (Crompot *et al.*, 2015).

Nevertheless, the limitation of mMP analysis using conventional flow cytometry has been persistently debated due to the smaller size of mMP compared to normal blood cells. Firstly, the minimal detection limit for light scattering of conventional flow cytometry is approximately 0.5 μm (van der Pol *et al.*, 2010). However, only a small fraction of mMP with size 0.5 μm or larger can be efficiently resolved, which affects the analysis of smaller mMP population (Chandler *et al.*, 2011). The flow cytometric threshold has been recommended to be extended down to as low as possible to determine mMP of 1 μm or less in diameter (Menck *et al.*, 2017). Therefore, in this study, a lower minimum threshold at 200 was defined on FITC parameter just above the background noise, which theoretically the defined threshold discriminates the high background noise and excludes debris (Inglis *et al.*, 2015). Secondly, overlapping noise acquired from the electronic part and buffers used may severely compromise

mMP detection, which is below 0.2–0.3 μm by conventional flow cytometer (Pospichalova *et al.*, 2015). A significant amount of background noise due to cell debris and precipitates is generated from unfiltered buffers, leading to false-positive microparticle analysis (Dey-Hazra *et al.*, 2010; Nielsen *et al.*, 2014). As it is crucial to eliminate contaminating particles in buffers or fluidic system prior to flow cytometry analysis, therefore, all buffer used for flow cytometry analysis were initially filtered using 0.2 μm filter in this study as previously recommended (Rousseau *et al.*, 2015). Additionally, mMP were assessed based on forward scatter (FSC) and side scatter (SSC) on the logarithmic scale in this study. As mMP may generate dim signals close to the background noise, the use of logarithmic scale assists in visualising small particles and separation of actual events from scatter noise on the dot plots (Nielsen *et al.*, 2014). Thirdly, occurrences of swarm or coincidence detection of mMP due to small size could lead to misinterpretation of results (Rousseau *et al.*, 2015). Swarm or coincidence detection may occur when multiple smaller mMP are detected and therefore are considered as a large single mMP as the fluorescence merges into a single electronic event (Chandler, 2016). To best overcome this limitation, accurate discrimination between coincident event and fluorescent event as well as good resolution of mMP may be achieved with low acquisition rate (Nolan and Jones, 2017; Pospichalova *et al.*, 2015). Therefore, mMP enumeration in this study was conducted at low flow rate, which was 10 μL/min. Sample acquisition at low flow rate also maintains the single stream of mMP that passes through the fluidic system and prevents doublet detection.

Monocytic MP staining with antibodies directed towards antigens of origin cells is the most frequently used method to characterise the cellular origin of mMP in peripheral

blood (Barteneva *et al.*, 2013). Meanwhile, Annexin-V, a family of calcium-dependent phospholipid binding proteins was used as a common marker for mMP detection (Fink *et al.*, 2011). Staining of mMP with Annexin-V in combination with other surface antibodies allows for targeting of monocyte-derived MP and prevents acquisition of false events (Dey-Hazra *et al.*, 2010). In this study, Annexin-V was used in combination with anti-CD14 or anti-CD16 to detect mMP derived primarily from monocyte subtypes. As monocytes express CD14 and CD16 on their surface (Mandl *et al.*, 2014), mMP were identified by the positive expression of PS with CD14 or CD16. To the best of our knowledge, this is the first study that characterise the surface phenotypes of mMP derived from $CD14^+$ monocytes and $CD16^+$ monocytes.

Based on flow cytometry analysis, CD14 and CD16 were expressed on all unstimulated and LPS-stimulated monocyte subtypes, which is similar to previous report (Yona and Jung, 2010). However, the expression intensity of these antigens differed across cell types. CD14 was highly expressed on unstimulated and LPS-stimulated whole monocytes and $CD14^+$ monocytes but not on $CD16^+$ monocytes. This finding supports the previous report that CD14 is abundantly expressed on whole monocytes (Naeim *et al.*, 2018), but low on $CD16^+$ monocytes (Hofer *et al.*, 2015). Meanwhile, CD16 expression was low on all unstimulated and LPS-stimulated monocyte subtypes, which is possibly due to low frequency of $CD16^+$ monocytes in the circulation. Previous studies have reported that $CD14^+$ monocytes account for 90% and $CD16^+$ monocytes constitute 10% of total monocytes in healthy individuals (Mukherjee *et al.*, 2015; Sánchez-Torres *et al.*, 2001). Taken together, these findings suggest that different monocyte subsets display different levels of CD14 and CD16 expression on their surface.

LPS stimulation resulted in down regulation of CD14 and CD16 expressions on all stimulated cells, with the exception for CD16 on $CD16^+$ monocytes. The type of stimulants used may alter the intensity of surface antigen expressions on the cell (van der Vlist *et al.*, 2012). In line with previous results, the loss of CD14 (Thaler *et al.*, 2016) as well as CD16 expression on whole blood (Poehlmann *et al.*, 2009) and monocytes (Belge *et al.*, 2002) following LPS stimulation has been observed. During inflammation, the loss of CD14 may be correlated with internalisation of CD14 by the cells (Buckner *et al.*, 2011) or shedding from the cells in soluble form, known as soluble CD14 (sCD14) (Bazil and Strominger, 1991; Buckner *et al.*, 2011). Thus, we suggest that reduced CD14 on all stimulated monocyte subtypes may be due to the activation effect of LPS on the cells, initiating rearrangement of cytoskeletons for mMP budding.

$CD16^+$ monocytes have been previously reported to be elevated up to 40% in pathological state (Sánchez-Torres *et al.*, 2001) such as during trauma, sepsis, (Kratofil *et al.*, 2017) and cardiovascular diseases (Libby *et al.*, 2013). The increased expression of CD16 on $CD16^+$ monocytes by LPS in this study indicates that $CD16^+$ monocytes may play a significant role in inflammation as previously reported (Ong *et al.*, 2018). The increased CD16 expression may have resulted from the transition process of monocytes from classical monocytes to non-classical $CD16^+$ monocytes following stimulation (Ziegler-Heitbrock *et al.*, 2010).

On the other hand, a previous study has shown that CD14 expression on MM6 and THP-1 cell lines was increased upon LPS treatment (Wen *et al.*, 2014). The different findings between ours and the previous study may be due to differences in cell type

used, in which this study used isolated human monocyte subtypes instead of monocytic cell lines which were used in other study. This important difference explains that the same type of stimuli may give rise to distinct phenotypic expression pattern by different cell types (Barteneva *et al.*, 2013).

4.2 Expression of CD14 and CD16 on monocyte subsets-derived mMP

The absolute count of mMP released was further determined using Trucount beads (Orozco and Lewis, 2010). Our results have shown that LPS-stimulated monocytes resulted in higher mMP count compared to unstimulated monocytes. This result indicates that LPS enhanced the release of mMP from stimulated whole monocytes, $CD14^+$ monocytes and $CD16^+$ monocytes. Similar to our result, previous study has shown that LPS initiates cellular changes in THP-1 and MM6 monocytic cells line, thus lead to mMP shedding (Wen *et al.*, 2014). Additionally, LPS stimulation which mimics infection suggests that the level of mMP may be increased during disease condition as previously reported (Kornek *et al.*, 2012). Elevation of mMP level has been observed in various clinical state including acute coronary syndrome (ACS) (Suades *et al.*, 2016), diabetes, hypertension (Ogata *et al.*, 2006), and atherosclerosis (Paudel *et al.*, 2016).

The expression of CD14 and CD16 on mMP was further assessed on mMP. In this study, low levels of mMP derived from unstimulated whole monocytes, $CD14^+$ monocytes, and $CD16^+$ monocytes were detected, indicating ongoing vesiculation. A previous study has reported that low level of MP has been detected in healthy individuals (Albert *et al.*, 2018), which may have resulted from normal biological

events such as programmed cell death (Meziani *et al.*, 2010) or cell growth (Camussi *et al.*, 2010). In an *in vitro* study, pre-analytical processes including blood sampling, centrifugation and cell culture processes may induce the release of some mMP (Lacroix *et al.*, 2011). This study has also shown that mMP express CD14 and CD16 similar to their origin cells. The expression of similar surface marker to origin cells on mMP has been widely described (Halim *et al.*, 2016; Nomura *et al.*, 2008). For instance, platelet-derived MP express CD41 and CD62P (Yari *et al.*, 2018), while endothelial-derived MP express CD31 (Deng *et al.*, 2017).

4.3 Monocytic microparticles in coagulation

Blood coagulation is one of the vital activities during inflammation, which reduces bacterial dissemination via bloodstream by the formation of blood clot at the site of inflammation (Halim *et al.*, 2016). Tissue factor (TF) or CD142 is a primary activator of the extrinsic coagulation cascade (Chiva-Blanch *et al.*, 2017). Tissue factor initiates the coagulation pathway by acting as an allosteric cofactor for plasma clotting protease factor VIIa (FVIIa), forming the enzymatic complex TF–FVIIa that triggers clotting protein factors IX and X (Rao and Pendurthi, 2012). Meanwhile, phosphatidylserine (PS) is an anionic phospholipid which recruits the components of the clotting cascade, triggered by electrostatic interaction of PS and positively charged γ-carboxyglutamic acid (GLA) domains in the clotting proteins (Owens and Mackman, 2011). Both TF and PS play a significant role in the coagulation process.

Monocytic MP express TF and PS on their surface, which suggests their function in blood clotting activity (Aleman *et al.*, 2011). In this study, TF expression was increased on whole monocytes, $CD14^+$ monocytes, and $CD16^+$ monocytes in the presence of LPS, which is similar to a previous report (Egorina *et al.*, 2005). As TF involves in coagulation, increased TF expression on monocytes may be a part of the innate immune response to prevent the spread of pathogenic agents (Bode and Mackman, 2014), thus containing the infection locally. In this study, TF expression was increased on LPS-stimulated monocyte-derived mMP which was similar to previous report (Liu *et al.*, 2007).

Monocytic MP are involved in coagulation and may act as a procoagulant (Aleman *et al.*, 2011) or anticoagulant (Mooberry and Key, 2016). As TF was expressed on mMP, the coagulation potential of mMP was further assessed using prothrombin (PT) time assay. Under normal physiological state, the coagulation process occurs naturally between 11 and 15 seconds when assessed using PT assay (Barmore and Burns, 2018).

This study demonstrated that plasma clotting time measurement was unaffected by the presence of both unstimulated and stimulated monocytes despite an up regulation of TF on stimulated monocytes. It was previously reported that TF may be expressed on activated monocytes but does not trigger coagulation cascade, which may be due to TF encryption (Bach, 2006). TF encryption is a post-translational suppression of TF procoagulant activity (PCA), whereby TF may bind to FVII or FVIIa but unable to fully activate FIX or FX and thus fails to trigger coagulation cascade (Rao *et al.*, 2012). Tissue factor encryption may be due to sequestering of TF in the lipid raft, which is rich in cholesterol (Chen and Hogg, 2013), dimerisation and oligomerisation of TF (Bach, 2006) as well as presence of anticoagulant phospholipids (Rao and Pendurthi, 2012), thus preventing the interaction and activation of TF. However, another study has demonstrated that LPS-activated monocytes significantly induced coagulation (Øvstebø *et al.*, 2012). This discrepancy may be due to differences between the stimuli used. In this study, LPS from *E.coli* strain O26:B6 was used, which was different from other study. In addition, different LPS forms may result in different effects on monocytes. Exposure of human monocytes to LPS from wild-type *N. meningitidis* resulted in higher TF activity compared to LPS-deficient *N. meningitidis,* although both forms of LPS enhanced the expression of TF on monocytes (Øvstebø *et al.*, 2012).

Monocytic MP derived from LPS-stimulated whole monocytes results in significant plasma clotting time compared to their unstimulated counterparts, emphasising their potential procoagulant property. This data are similar to previous study, where mMP derived from monocytes reduced plasma clotting time, thus acting as a procoagulant (Wen *et al.*, 2014). As a higher concentration of mMP may promote faster coagulation formation, they possibly contribute in thrombosis progression (Owens and Mackman, 2011). Tissue factor-bearing MP are highly procoagulant and have been attributed to thrombosis in many diseases including sepsis and cancer (Feng *et al.*, 2010). However, whether this procoagulant potential is mainly contributed by TF, PS, or the interaction between TF and PS remains unknown. Previously, the procoagulant properties of mMP were attributed to TF activity, in which mMP derived from monocytes and THP-1 cells supported thrombin generation and fibrin formation and increased fibrin network density in a TF-dependent manner (Aleman *et al.*, 2011). On the other hand, another study has reported that mMP derived from monocytes initiate blood coagulation in a TF-independent manner and are more reliant on PS on their surface (Wen *et al.*, 2014). The increased TF expression on mMP as well as altered prothrombin time in this study suggests that the procoagulant properties of mMP are mainly TF-dependent and regulated through the extrinsic pathway. Hence, further study should identify the potential mechanism of mMP-induced coagulation.

4.4 Monocytic microparticles in endothelial cell activation

As inflammation and coagulation are closely related, mMP could possibly play an important role in inflammation. Monocytic MP have been reported to promote inflammatory activities in response to infection (Halim *et al.*, 2016). The interaction between MP and endothelial cells during inflammation has been shown in a previous *in vitro* study (Wen *et al.*, 2014). However, information regarding the ability of mMP to interact with endothelial cells is limited. In this study, the role of mMP in activating endothelial cells was further assessed in a culture condition.

A standard curve is used to determine the dynamic range of an assay, detection limit, and quantification limit (Svec *et al.*, 2015). In this study, the standard curve showed that real-time PCR assays provided reliable and accurate results as high linearity of slope ($r^2 \geq 0.98$) over five orders of RNA concentrations of 10, 5, 2.5, 1.25 and 0.625 ng as well as high efficiency for all genes used were obtained (Appendix B). A starting RNA concentration of 10 ng was chosen as the optimal RNA concentration as it generated the highest output of targeted gene amplification at the lowest cycle compared to other RNA concentrations. Dissociation curves of all genes were also assessed to ensure the specificity of the primer used. The dissociation curves showed that a single peak was plotted for all primers used, which indicates that the primer specifically targeted the genes of interest (Appendix D).

We have shown that the number of mMP derived from whole monocytes, $CD14^+$ monocytes, and $CD16^+$ monocytes was increased following LPS stimulation, indicating the elevation of mMP in inflammatory condition. During inflammation, the interaction between two major cellular players, which are monocytes and endothelial

cells, is pivotal in initiating transendothelial migration of monocytes to the site of infection (Wang *et al.*, 2014), thus endothelial cells are potentially the main effector cells of mMP (Lovren and Verma, 2013). Monocytic MP are involved in endothelial dysfunction, vascular inflammation, and sepsis (Hoyer *et al.*, 2012). To date, the exact mechanism carried out by mMP in regulating endothelial activation and function is unclear. Therefore, the interaction between mMP derived from monocytes and endothelial cells were further assessed by measuring the level of CD31 as well as adhesion molecules including ICAM-1 and VCAM-1 expressed by endothelial cells.

Monocytic MP derived from unstimulated and stimulated monocytes increased the expression of CD31, ICAM-1, and VCAM-1 on endothelial cells at various intensities. Monocytic MP derived from stimulated monocytes up regulated CD31 and adhesion molecules higher than those activated by LPS, which suggests that mMP activate endothelial cells. Previously, 300 µg/mL of mMP derived from stimulated THP-1 was able to activate endothelial cells, resulting in the expression of ICAM-1, VCAM-1, and E-selectin after 2 hours culture (Wang *et al.*, 2011). It has been reported that mMP mediate the activation of endothelial cells via IL-1β-dependent pathway (Wang *et al.*, 2011). Monocytic MP regulation involves the activation of intracellular signalling pathway by ERK1/2 phosphorylation and degradation of IκB-α, which results in NF-κB translocation, thus leading to the expression of ICAM-1, VCAM-1 and E-selectin by endothelial cells (Wang *et al.*, 2011). Subsequently, the expression of ICAM-1, VCAM-1, and E-selectin facilitate monocyte extravasation through the endothelial cells of the blood vessels to the sites of inflammation (Halim *et al.*, 2016). Thus, these data suggest that elevated expression of CD31, ICAM-1, and VCAM-1 may lead to

amplification of inflammation by enhancing adhesion molecule expressions and facilitating transmigration of monocytes (Lee *et al.*, 2014).

In this study, endothelial cell activation was mainly regulated by mMP since endothelial cells were initially cultured in a serum-starved condition prior to culture with mMP as previously recommended (Chowdhury *et al.*, 2006). Serum components such as growth factors may stimulate and activate cells, thus generating false positive results (Antypas *et al.*, 2014). Additionally, as eMP are also able to enhance adhesion molecule expressions, culture of mMP with endothelial cells for 2 hours was able to up regulate adhesion molecule expression without inducing the vesiculation of endothelial cells. A previous study has demonstrated that the level of eMP release following TNF-α stimulation for 1 and 2 hours were consistent with the basal level at 0 hour (Lee *et al.*, 2014). The increased endothelial cell vesiculation was only observed at 4 hours of stimulation and eMP was increased over time.

Additionally, as mMP are also released constitutively under physiological condition especially during cell growth (Camussi *et al.*, 2010; Ratajczak *et al.*, 2006), their role in physiology during healthy state should not be ignored. A previous report has suggested that MP derived from various cells under normal haemostasis condition are involved in cell signalling and transfer of membrane proteins (Ståhl *et al.*, 2019). For example, MP derived from platelets mediate the transfer of adhesion molecules from platelets to haematopoietic cell and endothelial cells, thus enhancing their adhesion capacity and engraftment (Baj-Krzyworzeka *et al.*, 2002). As CD31, ICAM-1, and VCAM-1 expressions by endothelial cells can be enhanced by mMP derived from unstimulated monocytes, mMP may possibly aid in adhesion activity of immune cells

to endothelial cells. Taken together, mMP are beyond than inert cell fragments but are capable of altering the response of their effector cells and involve in cell–cell interactions as biological active communicators during physiological and pathological conditions (Wen *et al.*, 2014). To our knowledge, this is the first study that demonstrates the potential role of mMP derived from human monocytes.

In addition to endothelial cell marker expressions, the ability of mMP in inducing vesiculation of eMP was also assessed. A previous report has shown that elevation of eMP is a hallmark of endothelial cell activation (Wassmer *et al.*, 2006). The finding from other study has demonstrated that mMP were able to induce the release of eMP in an *in vitro* brain inflammation model (Wang *et al.*, 2011). Consistent with this study, the increased eMP release upon culture with mMP derived from stimulated monocytes was observed. A positive correlation between elevated number of eMP and coronary endothelial function impairment was observed in patients with coronary artery disease (Wang *et al.*, 2014). Additionally, worsening of pulmonary and capillary leak reflected by the high number of eMP release was previously demonstrated *in vivo* (Densmore *et al.*, 2006). Therefore, these findings suggest that mMP are able to enhance the vesiculation of eMP and they may play important role in exacerbation of vascular injury as previously reported (Reid and Webster, 2012).

CHAPTER 5

CONCLUSION

In conclusion, characterisation and assessment of the potential role of mMP is challenging. In this study, cell surface phenotypes of mMP was characterised. This study shows that whole monocytes, $CD14^+$ monocytes, and $CD16^+$ monocytes expressed both CD14 and CD16 on their surface at different intensities and mMP inherit their origin cell identity. Distinct cell types may give rise to mMP with different phenotypic profiles. Additionally, Annexin-V in combination with CD14 or CD16 are potential cell surface markers for mMP detection. Moreover, the coagulation potential of mMP was assessed in this study. Flow cytometry analysis demonstrated that PS and TF were expressed on whole monocytes, $CD14^+$ monocytes, and $CD16^+$ monocytes. This suggests their attribution in the coagulation process. Likewise, PT analysis showed that mMP derived from stimulated monocytes shortened the plasma clotting time. Based on our findings, mMP display TF-dependent procoagulant property. In addition, the potential role of mMP in endothelial cell activation was further assessed. Monocytic MP derived from both unstimulated and stimulated monocytes enhanced the expression of CD31, ICAM-1, and VCAM-1 by endothelial cells, as well as induced the vesiculation of eMP in a culture condition. These results provide further evidence on the important role of mMP as biological messengers in cell signalling during physiological and pathological states.

5.1 Study limitation and future study

This study has some limitations. Thus, further studies are recommended as follows:

1. The concentration of monocytes as well as $CD14^+$ monocytes and $CD16^+$ monocytes was limited since circulating monocytes population constitutes only 2% to 10% of human blood. Therefore, the use of apheresis technique is highly recommended for leucocyte isolation in order to increase monocyte concentrations.

2. The morphology and size of mMP derived from monocyte subsets has not been identified. Therefore, characterisation of mMP by using scanning electron microscope (SEM), and nanoparticle tracking analysis (NTA) is recommended.

3. This study demonstrated that the presence of TF and PS on the surface of mMP exhibited procoagulant property. However, the exact role of either TF or PS in coagulation by mMP remains unclear. Therefore, further studies to assess thrombin generation, fibrin generation and inhibitory test are recommended to identify the possible mechanism in mMP-dependent coagulation.

4. This study showed that mMP up regulated the expression of CD31 and adhesion molecules by endothelial cell in a culture condition. Previous study has reported the potential role of mMP in enhancing cytokines secretion by monocytes which resulted in endothelial cell activation. Therefore, it is recommended to assess the role of cytokines released by monocytes in the presence of mMP such as IL-1β and IL-6 in promoting endothelial cell activation.

5. Information regarding cytoplasmic protein, lipids and genetic profile of mMP remains unclear. Therefore, it is recommended to determine protein, lipids and miRNA profile in mMP.

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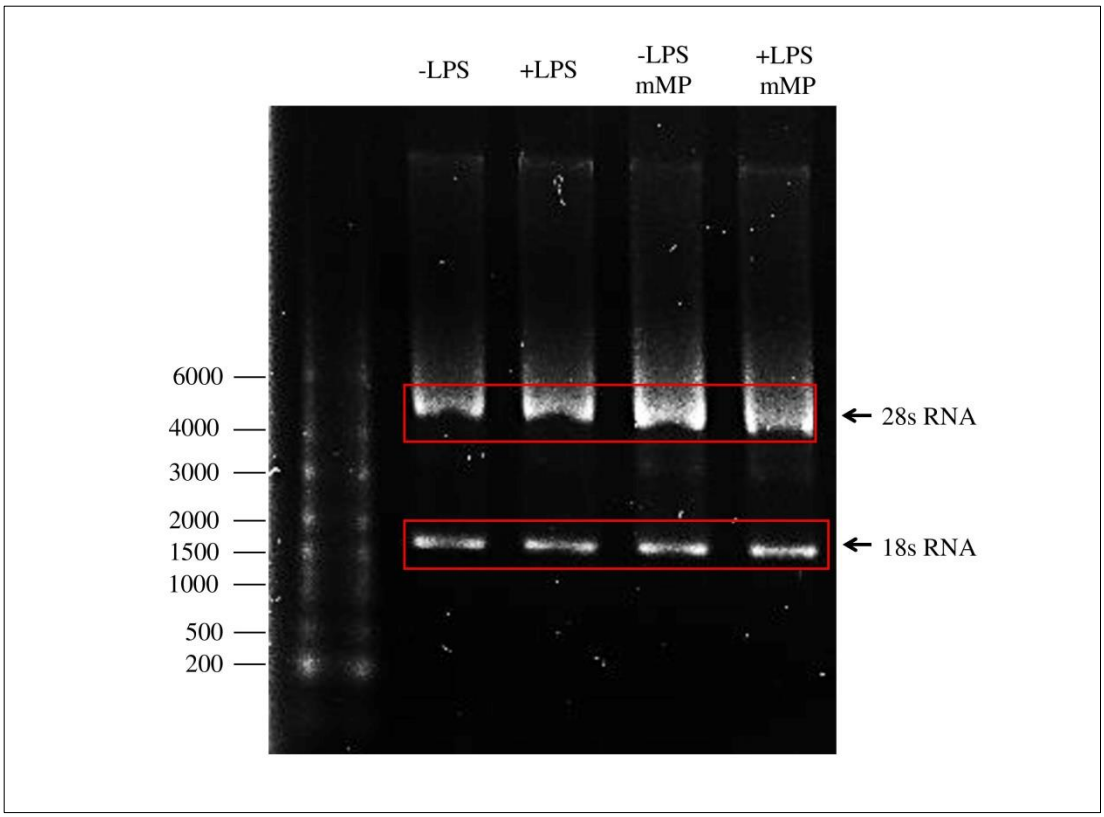
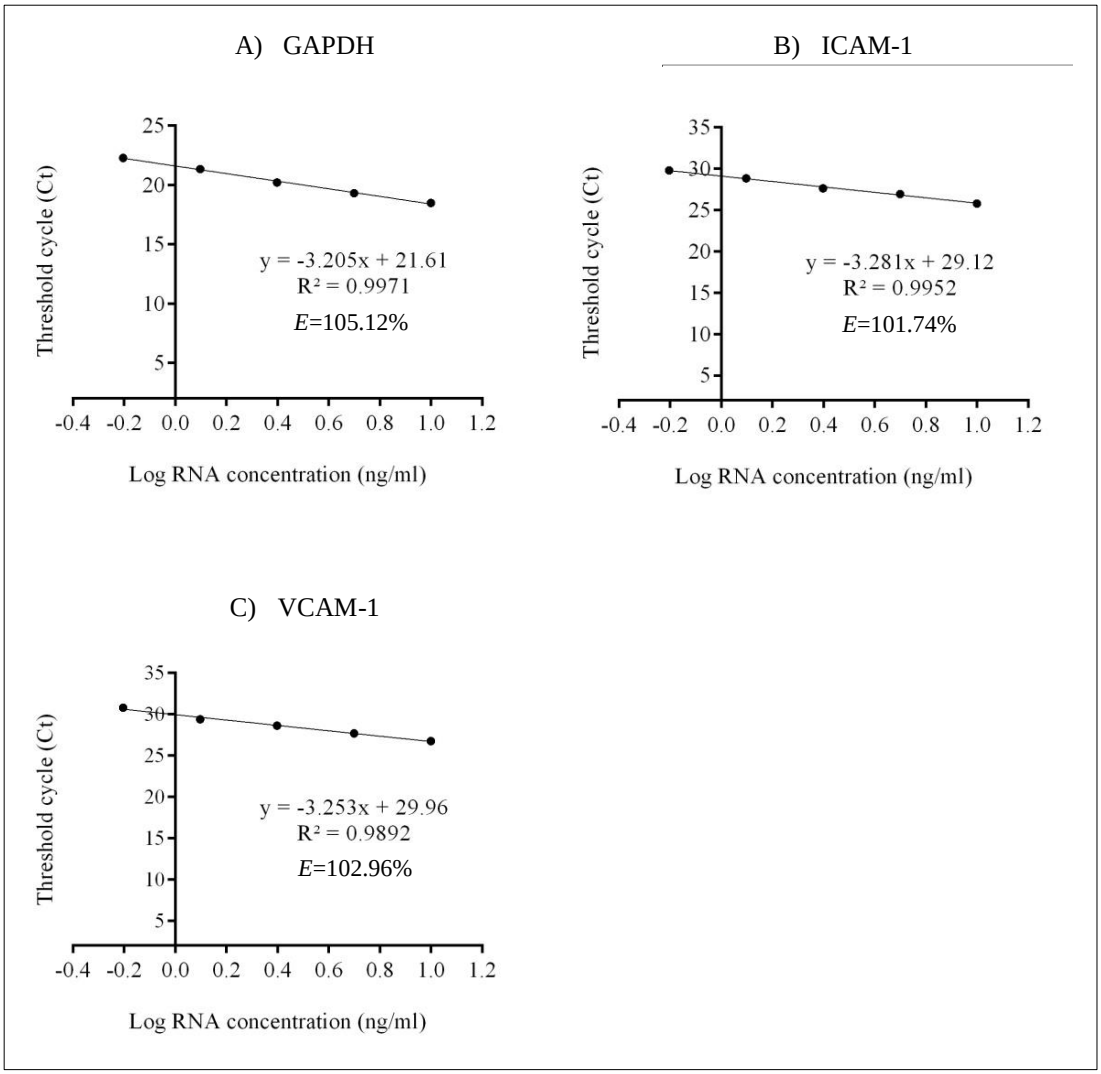
APPENDICES

Appendix A: RNA integrity test



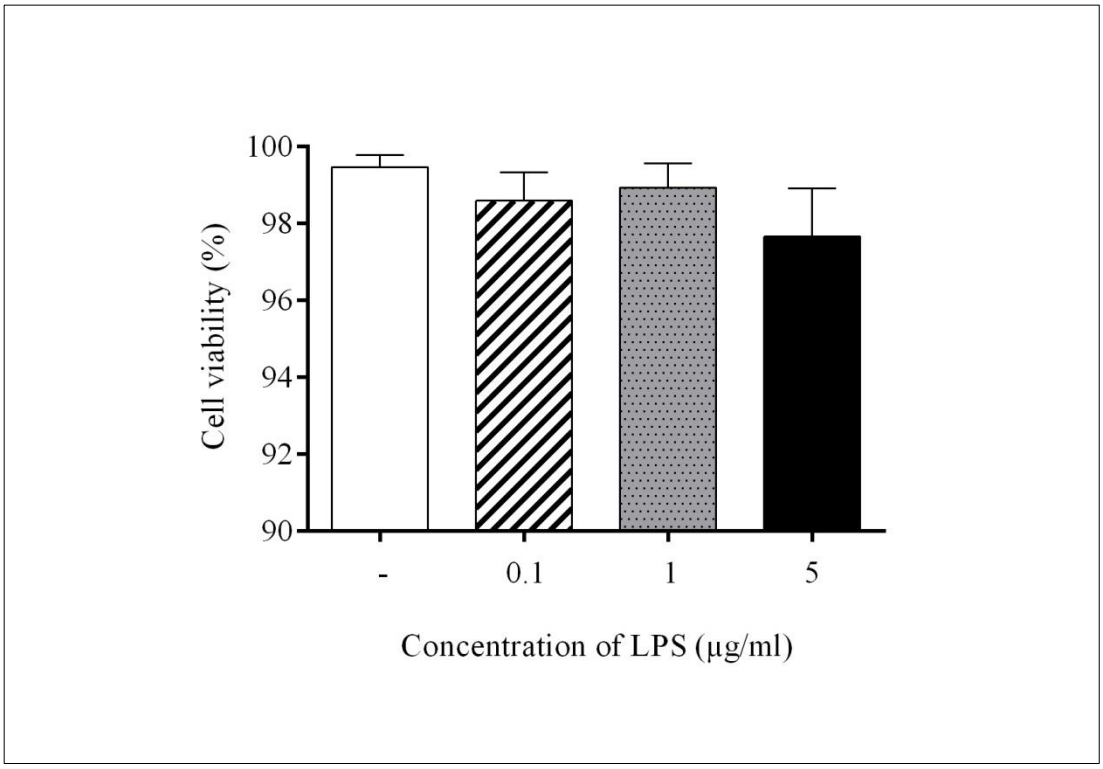
Appendix A: RNA integrity test. Gel electrophoresis was conducted for 50 minutes at 70 V. Top band shows 28s RNA and lower band shows 18s RNA indicating high integrity of total RNA used.

Appendix B: Standard curve for GAPDH, ICAM-1 and VCAM-1



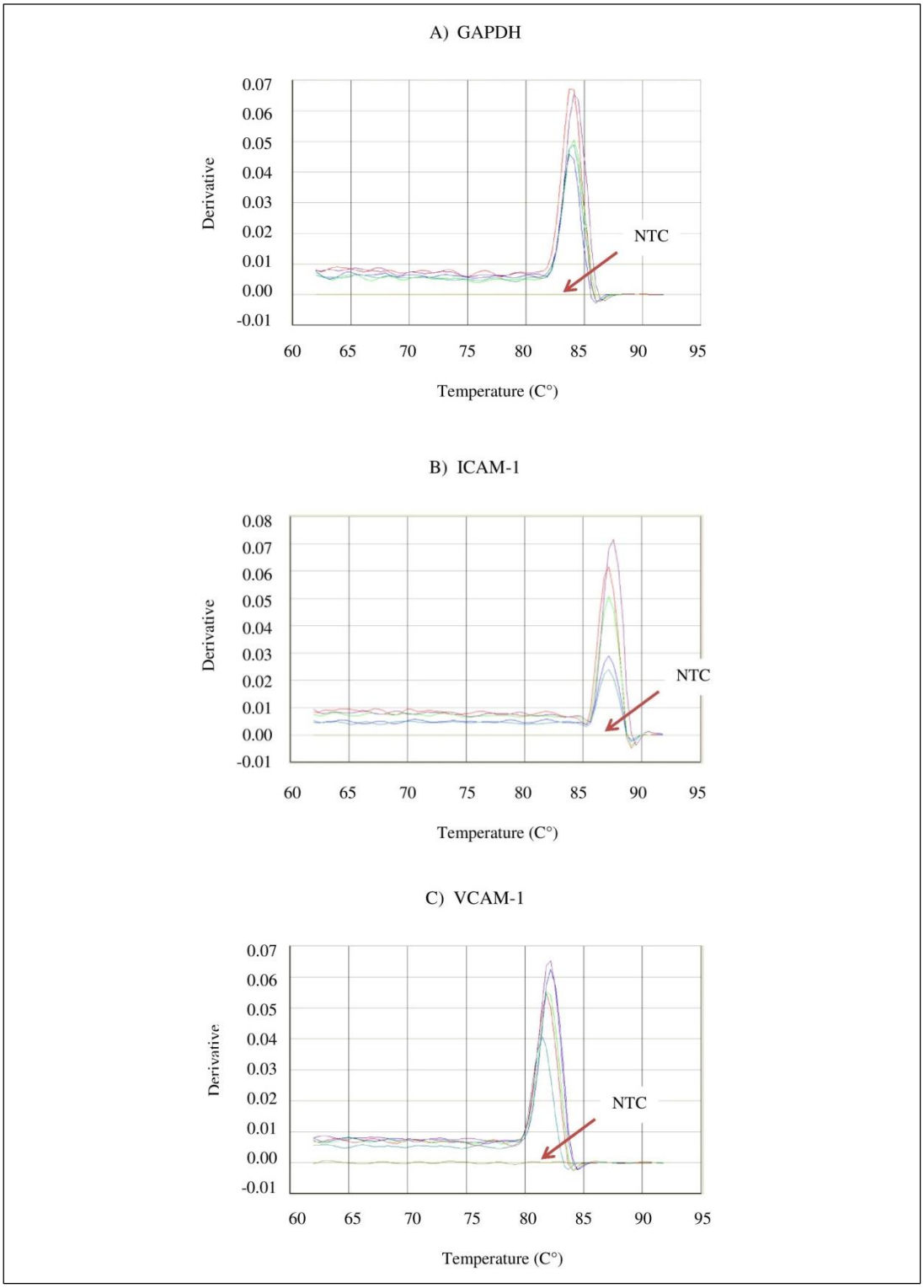
Appendix B: Efficiency of real-time PCR analysis. Standard curves of each targeted genes; A) GAPDH, B) ICAM-1 and C) VCAM-1 are generated using 2-fold dilution. Linearity of slopes (r^2) are used for determination of amplification efficiency.

Appendix C: Optimization of LPS concentration



Appendix C: Cell viability of PBMC. Peripheral blood mononuclear cells (PBMC) were incubated with 100 ng/mL, 1 µg/mL and 5 µg/mL LPS. Following 18 hours culture, cell viability was measured by Trypan blue assay. Data are representative of three independent experiments. Error bar represent mean ± standard error of the mean (SEM) (n= 3).

Appendix D: Dissociation curves for GAPDH, ICAM-1 and VCAM-1



Appendix D: Dissociation curves of GAPDH, ICAM-1 and VCAM-1. Dissociation curves of A) GAPDH, B) ICAM-1 and C) VCAM-1 show a single peak which indicate the primer used are specifically targeted on the interest genes. Non-template control (NTC) is used as a negative control.

Appendix E: Human ethical approval

Jawatankuasa Etika Penyelidikan Manusia USM (JEPeM)
Human Research Ethics Committee USM (HREC)

Universiti Sains Malaysia
Kampus Kesihatan,
16150 Kubang Kerian,
Kelantan. Malaysia.
T: 609 - 767 3000 *samb.* 2354/2362
F: 609 - 767 2351
E: jepem@usm.my
www.jepem.kk.usm.my

19th October 2015

Dr. Maryam Azlan
School of Health Sciences
Universiti Sains Malaysia
16150 Kubang Kerian, Kelantan.

JEPeM Code : USM/JEPeM/15040128
Protocol Title : The Role of Monocytic Microparticles (mMP) in Endothelial Cell Function.

Dear Dr.,

We wish to inform you that your study protocol has been reviewed and is hereby granted approval for implementation by the Jawatankuasa Etika Penyelidikan Manusia Universiti Sains Malaysia (JEPeM-USM). Your study has been assigned study protocol code **USM/JEPeM/15040128**, which should be used for all communication to the JEPeM-USM related to this study. This ethical clearance is valid from **November 2015** until **October 2016.**

The following documents have been approved for use in the study.

1. Research Proposal

In addition to the abovementioned documents, the following technical document was included in the review on which this approval was based:

1. Patient Information Sheet and Consent Form (English version)
2. Patient Information Sheet and Consent Form (Malay version)
3. Data Collection Sheet

Attached document is the list of members of JEPeM-USM present during the full board meeting reviewing your protocol.

While the study is in progress, we request you to submit to us the following documents:

1. Application for renewal of ethical approval 60 days before the expiration date of this approval through submission of **JEPeM-USM FORM 3(B) 2014: Continuing Review Application Form.** Subsequently this need to be done yearly as long as the research goes on.
2. Any changes in the protocol, especially those that may adversely affect the safety of the participants during the conduct of the trial including changes in personnel, must be submitted or reported using **JEPeM-USM FORM 3(A) 2014: Study Protocol Amendment Submission Form.**
3. Revisions in the informed consent form using the **JEPeM-USM FORM 3(A) 2014: Study Protocol Amendment Submission Form.**
4. Reports of adverse events including from other study sites (national, international) using the **JEPeM-USM FORM 3(G) 2014: Adverse Events Report.**
5. Notice of early termination of the study and reasons for such using **JEPeM-USM FORM 3(E) 2014.**
6. Any event which may have ethical significance.
7. Any information which is needed by the JEPeM-USM to do ongoing review.
8. Notice of time of completion of the study using **JEPeM-USM FORM 3(C) 2014: Final Report Form.**

Please note that forms may be downloaded from the JEPeM-USM website: **www.jepem.kk.usm.my**

Jawatankuasa Etika Penyelidikan (Manusia), JEPeM-USM is in compliance with the Declaration of Helsinki, International Conference on Harmonization (ICH) Guidelines, Good Clinical Practice (GCP) Standards, Council for International Organizations of Medical Sciences (CIOMS) Guidelines, World Health Organization (WHO) Standards and Operational Guidance for Ethics Review of Health-Related Research and Surveying and Evaluating Ethical Review Practices, EC/IRB Standard Operating Procedures (SOPs), and Local Regulations and Standards in Ethical Review.

Thank you.

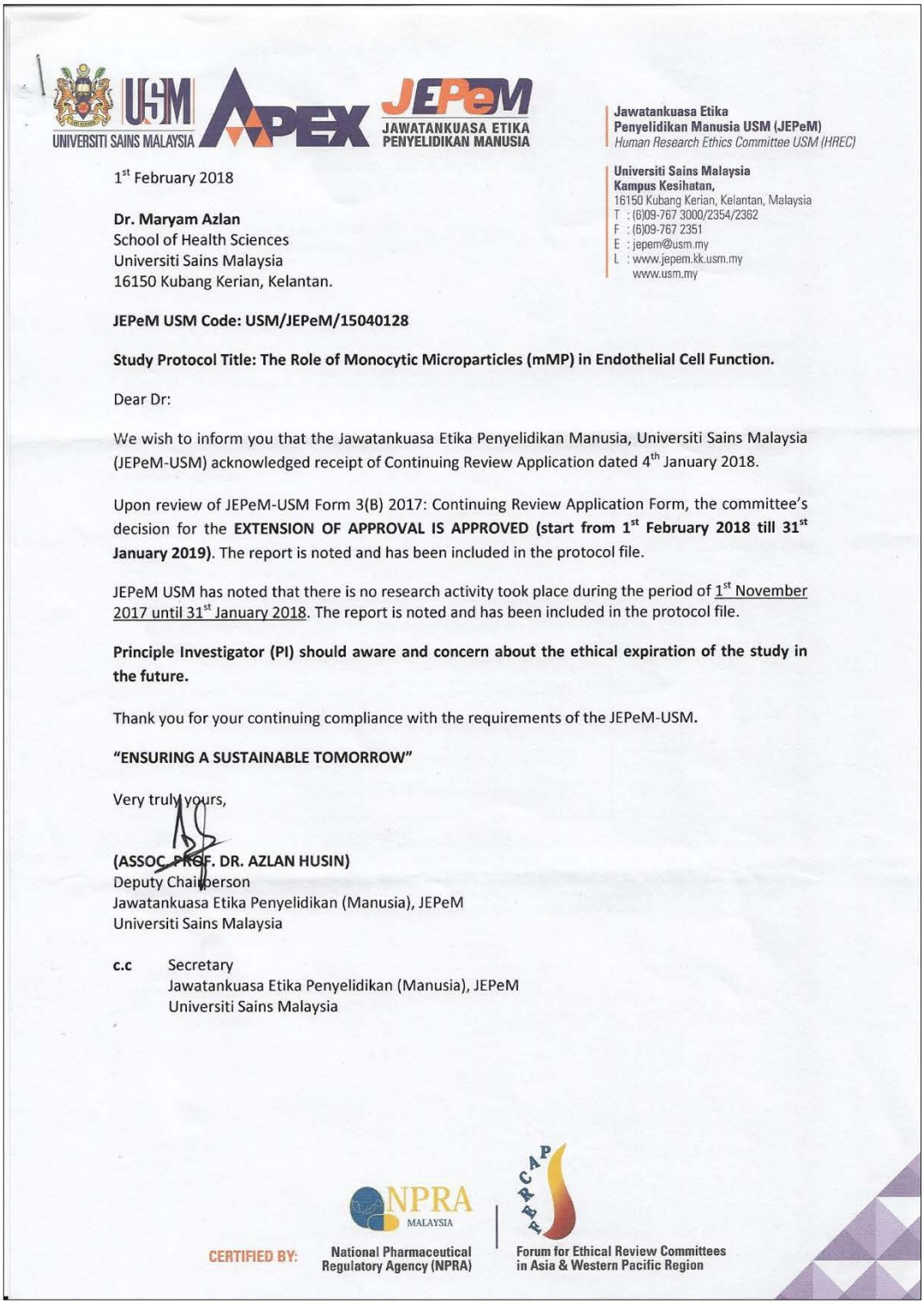
"ENSURING A SUSTAINABLE TOMORROW"

Very truly yours,

PROF. DR. HANS AMIN VAN ROSTENBERGHE
Chairperson
Jawatankuasa Etika Penyelidikan (Manusia) JEPeM
Universiti Sains Malaysia

Appendix E: Human ethical approval. Study protocol has been approved by Human Research Ethics Committee USM (HREC).

Appendix F: Human ethical approval (continuation)



UNIVERSITI SAINS MALAYSIA

JAWATANKUASA ETIKA PENYELIDIKAN MANUSIA

Jawatankuasa Etika Penyelidikan Manusia USM (JEPeM)
Human Research Ethics Committee USM (HREC)

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1st February 2018

Dr. Maryam Azlan
School of Health Sciences
Universiti Sains Malaysia
16150 Kubang Kerian, Kelantan.

JEPeM USM Code: USM/JEPeM/15040128

Study Protocol Title: The Role of Monocytic Microparticles (mMP) in Endothelial Cell Function.

Dear Dr:

We wish to inform you that the Jawatankuasa Etika Penyelidikan Manusia, Universiti Sains Malaysia (JEPeM-USM) acknowledged receipt of Continuing Review Application dated 4th January 2018.

Upon review of JEPeM-USM Form 3(B) 2017: Continuing Review Application Form, the committee's decision for the **EXTENSION OF APPROVAL IS APPROVED (start from 1st February 2018 till 31st January 2019)**. The report is noted and has been included in the protocol file.

JEPeM USM has noted that there is no research activity took place during the period of 1st November 2017 until 31st January 2018. The report is noted and has been included in the protocol file.

Principle Investigator (PI) should aware and concern about the ethical expiration of the study in the future.

Thank you for your continuing compliance with the requirements of the JEPeM-USM.

"ENSURING A SUSTAINABLE TOMORROW"

Very truly yours,

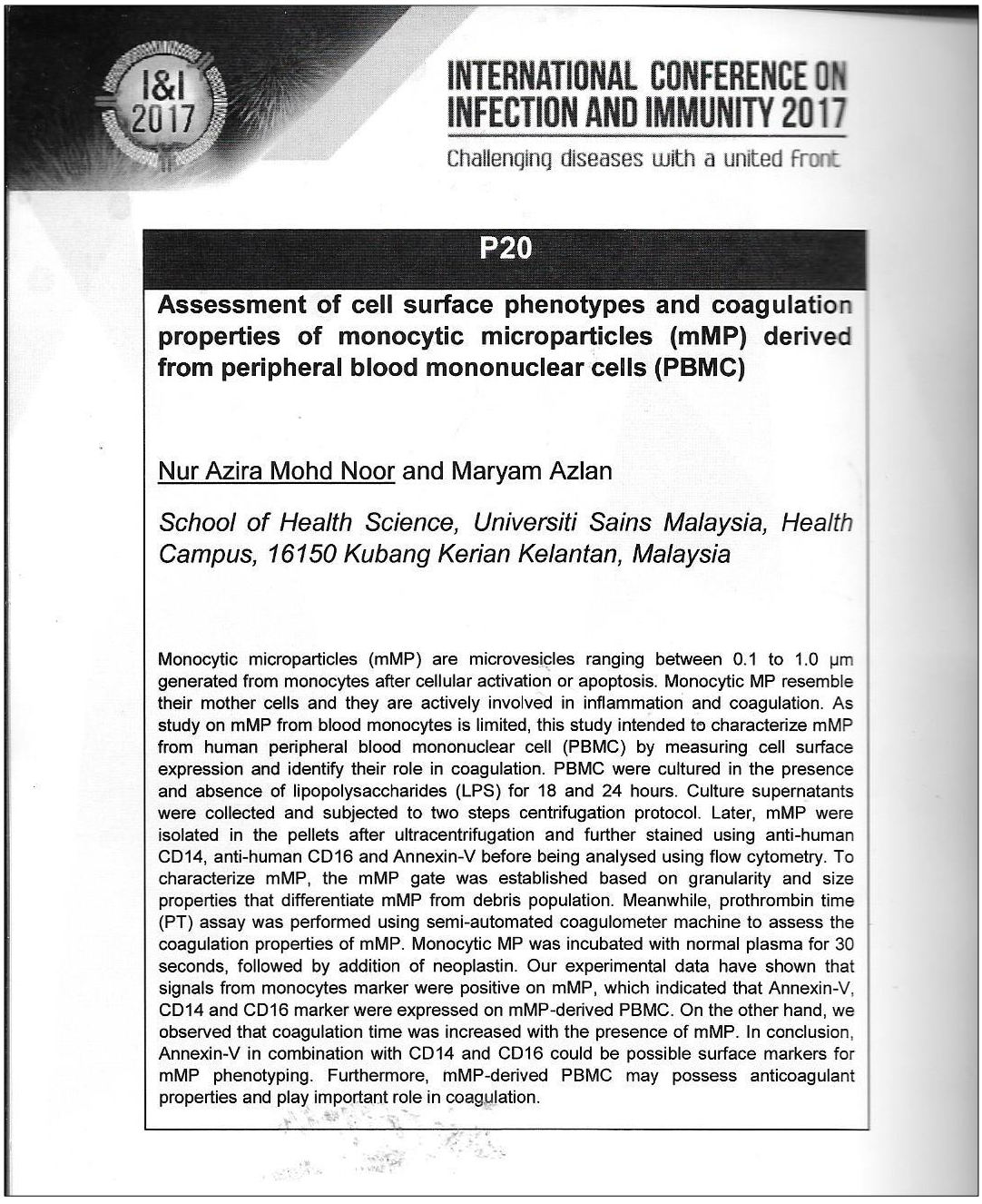
(ASSOC. PROF. DR. AZLAN HUSIN)
Deputy Chairperson
Jawatankuasa Etika Penyelidikan (Manusia), JEPeM
Universiti Sains Malaysia

c.c Secretary
Jawatankuasa Etika Penyelidikan (Manusia), JEPeM
Universiti Sains Malaysia

CERTIFIED BY: National Pharmaceutical Regulatory Agency (NPRA) | Forum for Ethical Review Committees in Asia & Western Pacific Region

Appendix F: Approval for continuation of human ethics. Continuation of study protocol has been approved by Human Research Ethics Committee USM (HREC).

Appendix G: Conference presentation



I&I 2017

INTERNATIONAL CONFERENCE ON INFECTION AND IMMUNITY 2017

Challenging diseases with a united front

P20

Assessment of cell surface phenotypes and coagulation properties of monocytic microparticles (mMP) derived from peripheral blood mononuclear cells (PBMC)

Nur Azira Mohd Noor and Maryam Azlan

School of Health Science, Universiti Sains Malaysia, Health Campus, 16150 Kubang Kerian Kelantan, Malaysia

Monocytic microparticles (mMP) are microvesicles ranging between 0.1 to 1.0 μm generated from monocytes after cellular activation or apoptosis. Monocytic MP resemble their mother cells and they are actively involved in inflammation and coagulation. As study on mMP from blood monocytes is limited, this study intended to characterize mMP from human peripheral blood mononuclear cell (PBMC) by measuring cell surface expression and identify their role in coagulation. PBMC were cultured in the presence and absence of lipopolysaccharides (LPS) for 18 and 24 hours. Culture supernatants were collected and subjected to two steps centrifugation protocol. Later, mMP were isolated in the pellets after ultracentrifugation and further stained using anti-human CD14, anti-human CD16 and Annexin-V before being analysed using flow cytometry. To characterize mMP, the mMP gate was established based on granularity and size properties that differentiate mMP from debris population. Meanwhile, prothrombin time (PT) assay was performed using semi-automated coagulometer machine to assess the coagulation properties of mMP. Monocytic MP was incubated with normal plasma for 30 seconds, followed by addition of neoplastin. Our experimental data have shown that signals from monocytes marker were positive on mMP, which indicated that Annexin-V, CD14 and CD16 marker were expressed on mMP-derived PBMC. On the other hand, we observed that coagulation time was increased with the presence of mMP. In conclusion, Annexin-V in combination with CD14 and CD16 could be possible surface markers for mMP phenotyping. Furthermore, mMP-derived PBMC may possess anticoagulant properties and play important role in coagulation.

Appendix G: Abstract for poster presentation. The title of presentation was assessment of cell surface phenotypes and coagulation properties of monocytic microparticles (mMP) derived from peripheral blood mononuclear cells (PBMC). The International Conference on Infection and Immunity 2017 was held on 5th and 6th April 2017 at Renaissance Hotel, Kota Bharu, Kelantan.

Appendix H: Certificate of award

Appendix H: Certificate of award. The International Conference on Infection and Immunity 2017 was held on 5th and 6th April 2017 at Renaissance Hotel, Kota Bharu, Kelantan.