CELL SURFACE PHENOTYPE OF LIPOPOLYSACCHARIDES-STIMULATED PERIPHERAL BLOOD MONONUCLEAR CELLS-DERIVED MICROPARTICLES

by

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

ADCC	Antibody-dependent cell-mediated cytotoxicity
APC	Allophycocyanin
ApoB	Apolipoprotein B
BSA	Bovine serum albumin
CCR	C-C chemokine receptor
CMP	Common myeloid progenitor
CSF	Colony stimulating factor
CX3CR	CX3C chemokine receptor
CXCR	CXC chemokine receptor
DC	Dendritic cells
EDTA	Ethylene diamine tetra-acetic acid
FACS	Fluorescence activated cell sorting
FCS	Flow cytometry software
FITC	Fluorescein isothiocyanate
FSC	Forward scatter
GMP	Granulocytes macrophage progenitor
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
ICAM	Intercellular adhesion molecule
IgG	Immunoglobulin G
IKKb	IkB kinase beta
IL	Interleukin
KDR	Kinase insert domain
LPS	Lipopolysaccharides

MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
mMP	Monocytic microparticles
MP	Microparticles
MPO	Myeloperoxidase
NFkB2	Nuclear factor kappa B
NK	Natural killer cells
NO	Nitric oxide
PAMP	Pathogen associated molecular pattern
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PE	Phycoerythrin
PerCP	Peridinin chlorophyll A protein
PS	Phosphatidylserine
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RPMI	Rosewell Park Memorial Institute
SAPK	Stress-activated protein kinase
SEM	Standard error of mean
SSC	Side scatter
TLR	Toll-like receptor
TNFR	Tumor necrosis factor receptor
TNF-α	Tumor necrosis factor alpha
VCAM	Vascular cell adhesion molecule
VEGF	Vascular endothelial growth factor

ABSTRAK

Mikropartikal monosit (mMP) boleh ditakrifkan sebagai populasi vesikel kecil yang berukuran 0.1 hingga 1.0 μm berasal daripada monosit. Peningkatan paras edaran mikropartikal (MP) yang ketara telah dilihat dalam pelbagai jenis penyakit, dan peningkatan ini berkait rapat dengan tahap penyakit. Kajian terdahulu mencadangkan bahawa MP mempamerkan 'phosphatidyserine' (PS) pada permukaannya dan molekul ini menyumbang kepada inflamasi. Pengeluaran MP dapat ditingkatkan oleh kecederaan sel, pengaktifan sel, atau apoptosis dan ia boleh dirangsang oleh lipopolisakarida (LPS). MP mempamerkan antigen permukaan yang menyerupai sel induk. Walau bagaimanapun, kajian terhadap mMP adalah terhad. Sehingga kini, ciri-ciri permukaan sel mMP masih belum jelas. Kajian ini bertujuan untuk mengkaji ciri-ciri antigen permukaan mMP termasuk 'CD14', 'CD16', dan PS. Kajian ini juga bertujuan untuk membandingkan pengeluaran mMP oleh sel mononuklear darah periferi (PBMC) yang dirangsang dengan LPS dan tanpa rangsangan selama 18 dan 24 jam. Pengeluaran mMP telah dinilai menggunakan 'anti-human CD14', 'anti-human CD16', dan 'Annexin V'. Manakala, sel PBMC yang dirangsang dengan LPS telah dinilai menggunakan 'anti-human CD14', 'anti-human CD16', dan 'anti-human CD11b'. Kesemua analisis telah dilakukan menggunakan teknik aliran sitometri. Data menunjukkan bahawa 'CD14' dan 'Annexin-V' dapat dikesan pada mMP. Sebaliknya, 'CD16' hanya dikesan pada kadar yang sangat rendah. Selain itu, pengeluaran mMP adalah berkadar terus dengan tempoh rangsangan. PBMC yang dirangsang mengeluarkan lebih banyak mMP berbanding PBMC yang tidak dirangsang apabila dikultur selama 18 dan 24 jam. Viabiliti sel PBMC yang dirangsang tidak berubah. Tambahan pula, perubahan purata keamatan pendafluor 'ΔMFI' bagi semua penanda yang digunakan untuk PBMC yang tidak

dirangsang adalah lebih tinggi berbanding PBMC yang dirangsang. Hasil kajian kami menunjukkan bahawa 'Annexin-V' dan CD14 adalah penanda permukaan sel yang berpotensi untuk pengesanan mMP. Kajian ini juga menunjukkan LPS berupaya untuk mengurangkan ekspresi CD14, CD16, dan CD11b pada PBMC yang dirangsang.

ABSTRACT

Monocytic microparticles (mMP) can be defined as a heterogeneous population of small vesicles with approximate size 0.1 to 1 μm derived from peripheral blood monocytes. Notable elevated levels of circulating microparticles (MP) have been observed in various clinical states, and are significantly associated with disease severity. Previous studies suggest that MP express phosphatidylserine (PS) which contribute in inflammation process. Their release is enhanced by cell injury, cell activation, or apoptosis and can be triggered by lipopolysaccharides (LPS) stimulation. MP display phenotypic and cytosolic compositions that resemble their parental cells. However, study on mMP is limited. To date, the characterization of cell surface mMP is unclear. In this study, we intended to characterize mMP by measuring cell surface expression of mMP including CD14, CD16 and PS as well as comparing the level of mMP secretion between stimulated and unstimulated peripheral blood mononuclear cells (PBMC). PBMC were cultured in the presence or absence of LPS for 18 and 24 hours. Monocytic MP secretion was assessed in the supernatants using anti-human CD14, anti-human CD16 and Annexin-V. Meanwhile, stimulated PBMC was assessed in culture pellet using anti-human CD14, anti-human CD16, and anti-human CD11b. All analyses were performed using flow cytometry. Our experimental data showed that CD14 and Annexin-V marker were clearly detected on mMP. In contrast, CD16 marker was undetectable. We observed that mMP production was proportional to stimulation period. LPS-stimulated PBMC secreted higher level of mMP compared to unstimulated PBMC when cultured for 18 and 24 hours. Cell viability of stimulated PBMC remains unchanged. In addition, changes in mean fluorescense intensity 'ΔMFI' of all markers in unstimulated PBMC was higher than stimulated PBMC. These finding suggest that

Annexin-V in combination with CD14 are the potential cell surface markers for mMP detection, while we confirmed that LPS down-regulate the expression of CD14, CD16 and CD11b on stimulated PBMC.

CHAPTER 1

INTRODUCTION

1.1 Background of study and problem statement

This study was conducted to investigate the phenotypic characteristic of monocyte-derived microparticles (mMP) in circulating human blood. Human blood monocytes were collected from peripheral blood mononuclear cells (PBMC) which consist of monocytes, macrophage, natural killer cells (NK) and dendritic cells (DC) (Končarević *et al.,* 2014). The isolation of PBMC is facilitated by Ficoll-Hypaque density gradient centrifugation, and they were collected at the interphase of Ficoll layer. Cell activation and apoptosis greatly induce the production and up regulation of mMP. In addition, they also can be induced by a component of Gram-negative bacterial membrane known as lipopolysaccharide (LPS) (Bernimoulin *et al.,* 2009). Previous studies have shown that stimulation of cells as well as microparticles (MP) promotes the expression of procoagulant and tumor factors. Moreover, MP and parental cells are thought to have similar surface antigens, which can be identified by flow cytometry. Significant elevation of MP can be clearly observed in various clinical conditions such as in inflammation, diabetes mellitus, sickle-cell disease, and pre-eclampsia (Lynch & Ludlam, 2007). Many researchers have suggested MP as useful biomarkers for disease activity and it can be used as ultimate diagnostic tools in identification and confirmation of various diseases. Although studies on surface components of extracellular MP are widely conducted, there are some discrepancies regarding antigen expression on MP. Moreover, research on mMP has not been widely established and the expression of marker on mMP remains controversial.

1.2 Hypothesis

We hypothesized that mMP-derived LPS-stimulated PBMC display various cell surface phenotypes.

1.3 Objectives

1.3.1 General objevtives

To determine cell surface phenotype of LPS-stimulated monocytes microparticle derived-PBMC.

1.3.2 Specific objectives

1. To determine cell viability of LPS-stimulated PBMC.
2. To assess cell surface phenotype of mMP-derived LPS-stimulated PBMC
3. To determine cell surface phenotype of LPS-stimulated PBMC

1.4 Significance of the study

Monocytic MP is an extracellular vesicles derived from monocytes in response to stress and other stimuli. Monocytic MP has been suggested to play important roles in inflammation due to the expression of phospholipids, which act as procoagulants (Nomura *et al.,* 2008). The study on mMP is limited in the literatures. Therefore, this study was conducted to characterise surface marker expression on mMP derived from PBMC.

This study provides preliminary data of mMP particularly on cell surface phenotype of mMP derived from PBMC. To date, the expression of antigens on mMP is still unclear. Once the antigens expressed on mMP can be identified, these markers can be used as potential biomarkers in diagnostic method to detect various diseases. In addition, this basic data can be used to design treatments for inflammation-related diseases. Additionally, cell surface expression of LPS-stimulated PBMC was also assessed in this study.

CHAPTER 2

LITERATURE REVIEW

2.1. Human monocytes

Peripheral blood mononuclear cells (PBMC) are the population of white blood cells, which have a single round of nucleus. PBMC have emerged as the most blood cells studied in many medical fields include immunology, haematology, infectious diseases, and oncology (Chan *et al.,* 2013). In immunology research area, PBMC have been widely studied in defining and monitoring immunological functions in both normal and disease state. For instance, PBMC primarily comprised of monocytes, lymphocytes, natural killer (NK) cells, and dendritic (DC) cells, which possess crucial roles in body defence system monitoring immune-relevant event and respond competently during inflammation (Haudek-Prinz *et al.,* 2012). Isolation of PBMC is facilitated by simple means of Ficoll-Hypaque density gradient centrifugation without the need for advanced machines or complex procedures. Basically, blood cells are separated into different layers according to their density, in which lower and higher density cells are arranged on the top and bottom layers respectively. With specific gravity of 1.077, Ficoll-hypaque solution permits a successful distribution of blood cells population as platelets and PBMC appeared on the top of Ficoll-Hypaque layer, while red blood cells and granulocytes are collected at the bottom of Ficoll-Hypaque layer (Fuss *et al.,* 2001) (Figure 2.1). PBMC are normally used as a predominant source of monocytic cells, which is primarily for human immune system-related study.

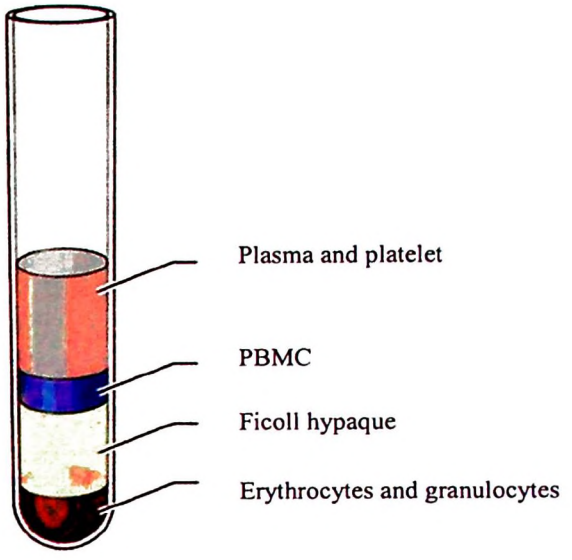

Figure 2.1: Separation of blood components on a Ficoll-Hypaque gradient. Blood components are separated based on their density. PBMC are collected at interphase of Ficoll layer. (Adapted from Fuss *et al.*, 2001)

Monocytes are the largest blood cells of leukocytes and they can be distinguished easily due to their morphology of nucleus that are characterised by kidney or horseshoe-shape (Robbins & Swirski, 2010). Monocytes have been identified as blood cells originated from the bone marrow, which circulate in the peripheral blood typically about 10% of human PBMC. Monocytes migrate from the hematopoietic stem cells in the bone marrow (Yona & Jung, 2010) from common myeloid progenitor (CMP), which later this precursor differentiate into the granulocyte macrophage progenitor (GMP) (Iwasaki & Akashi, 2007). Monocytes circulate in the blood, subsequently mature and migrate to infection sites following stimulation. The life span of circulating human monocytes is approximately three days before undergoing programmed-cell death. Human monocytes are best categorized based on their cell surface marker expression (Ziegler-Heitbrock *et al.*, 2010).

2.1.1 Monocyte subsets and function

The classification of monocytes subset are well-established and as far, it has been reported that monocyte subsets are equipped with particular phenotypic and functional traits (Auffray *et al.*, 2009). Previous studies suggest that human monocytes consist of three subsets, based on the expression of CD14 and CD16 antigens. CD14 is a lipopolysaccharide receptor (Skrzeczyńska-Moncznik *et al.*, 2008), which serves a significant role for bacterial lipopolysaccharide (LPS) recognition. Meanwhile, CD16 is an Fcγ III receptor (Shantsila *et al.*, 2011) specifically for triggering phagocytosis activity. The three monocyte subsets are classical $CD14^{++}CD16^{-}$ monocytes, intermediate $CD14^{++}CD16^{+}$ monocytes, and non-classical $CD14^{+}CD16^{++}$ monocytes (Table 2.1). The classical monocytes expressed high level of CD14 compared to CD16, while the intermediate monocytes expressed high level of both antigens. In contrast, non-classical monocytes expressed high level of CD16 compared to CD14.

Table 2.1: Classification of monocyte subsets.

Human monocytes	
Classification	**Cell surface phenotype**
Classical	$CD14^{++}CD16^{-}$
Intermediate	$CD14^{++}CD16^{+}$
Non-classical	$CD14^{+}CD16^{++}$

The classical $CD14^{++}CD16^{-}$ monocytes are the most abundant circulating subsets approximately 85% to 90% of total monocytes in the circulation. In addition, it is being cited as 'classical' as its phenotypes is likely the same with the original designation of monocytes. From the experiment conducted by Shantsila *et al.* (2011), they found that high intensity of expression of IL6 receptor, CD64, CCR2 and CD163 on classical $CD14^{++}CD16^{-}$ monocytes. In contrast, VCAM receptor and CD204 have low expression on classical $CD14^{++}CD16^{-}$ monocytes. In particular, the classical $CD14^{++}CD16^{-}$ monocytes are the main player in response to inflammation, as it is able to migrate promptly to inflamed tissue via extravasation (Reynolds & Haniffa, 2015). Moreover, with the capability to perform phagocytosis, classical monocytes also greatly cooperate with the stimulation of LPS by releasing chemokines. The classical $CD14^{++}CD16^{-}$ monocytes show significant increase in production of IL-10 and low levels of TNF-α, as well as elevated peroxidise activity as a reaction to LPS stimulation. (Geissmann *et al.*, 2003). Besides that, it also exhibit high myeloperoxidase (MPO) activity, elevated antibody-dependant cell-mediated cytotoxicity (ADCC), suppress antigen-activated lymphocytes and produce colony-stimulating factor (CSF) (Robbins & Swirski, 2010).

Meanwhile, $CD14^{+}CD16^{++}$ monocytes are characterized by their small size, less dense and known as the non-classical monocytes. Usually, it is less frequent and estimated about less than 15% of total circulating monocytes. Compared to other subsets, non-classical $CD14^{+}CD16^{++}$ monocytes have high expression of VCAM1 receptor and CD204, while decreased levels of IL-6 receptor, CD64, CCR2 and CD163. This characteristic may implies that non-classical $CD14^{+}CD16^{++}$ is a monocytes represent developmentally independent subset rather than different stage of monocyte maturation (Shantsila *et al.*, 2011). However, others have reported that classical $CD14^{++}CD16^{-}$

monocytes and non-classical $CD14^{+}CD16^{++}$ monocytes have similar features such as morphology, cytochemistry and surface marker. Non-classical $CD14^{+}CD16^{++}$ monocytes have lower phagocytic activity, diminished capacity of producing reactive oxygen species (ROS) and express notable high level of CX3CR1 receptor (Geissmann *et al.,* 2003). Previous studies have affirmed that the non-classical $CD14^{+}CD16^{++}$ monocytes were not actively involves in inflammatory response, which was marked by low level of IKKb, an enzyme that engage in promoting the cellular response to inflammation. The non-classical $CD14^{+}CD16^{++}$ monocytes is directly involves in patrolling activity in blood vessel and involve in antigen presenting process as they expressed significantly high level of CD80 and CD86 (Mukherjee *et al.,* 2015). Unlike the classical monocytes which respond to LPS, non-classical $CD14^{+}CD16^{++}$ monocytes are responsive to viral stimulation by producing IFN-α (Cros *et al.,* 2010). Non-classical $CD14^{+}CD16^{++}$ monocytes actively engage with innate local surveillance of tissue in accordance to cell death (Yoshimoto *et al.,* 2007). The data from previous study that non-classical $CD14^{+}CD16^{++}$ monocytes serves as vital agent that produce high level of TNF-α, however only in response to LPS (Skrzeczyńska-Moncznik *et al.,* 2008).

The intermediate $CD14^{++}CD16^{+}$ monocytes possess an intermediate phenotype between the classical $CD14^{++}CD16^{-}$ and non-classical $CD14^{+}CD16^{++}$ monocytes. These less frequent monocytes respond to cytokine treatment and inflammatory activity (Moniuszko *et al.,* 2009). The intermediate $CD14^{++}CD16^{+}$ monocytes show greater expression of ICAM receptor, Tie2, CXCR4, CD163, VEGF receptor 1, KDR, ferittin, ApoB and CD115 compared to other subsets (Shantsila *et al.,* 2011). In addition, the intermediate monocytes up-regulate CD16, VCAM1 receptor, and CD204. Intermediate $CD14^{++}CD16^{+}$ monocytes was cited to have a unique functional role in inflammatory.

These subset reveals lower level of IKKb compared to classical $CD14^{++}CD16^{-}$ monocytes and similar to non-classical $CD14^{+}CD16^{++}$ monocytes, which indicates different role from proinflammatory classical $CD14^{++}CD16^{-}$ monocytes. Intermediate $CD14^{++}CD16^{+}$ monocytes express higher TLRs 2, 4 and 5 compared to other monocytes subset, indicating a primarily proinflammatory function (Mukherjee *et al.*, 2015). According to Ginhoux & Jung (2014), intermediate $CD14^{++}CD16^{+}$ monocytes express CD80, CD85, and HL-DR receptors, which are specifically associated to antigen presentation and activation of T cell. This subset is also thought to have capacity in expressing CCR2 and CCR5, which eventually respond to macrophage inflammatory protein-1α. Moreover, intermediate $CD14^{++}CD16^{+}$ monocytes shows significant low level of peroxidise activity, higher capability in IL-1β and TNF-α production, and major producer of IL-10 in order to react to LPS (Cros *et al.*, 2010).

Usually, these three monocyte subsets appear to develop from classical by intermediate to nonclassical, which subsequently increase the level of circulating intermediate monocytes followed by nonclassical monocytes (Ziegler-Heitbrock *et al.*, 2010). Moreover, previous literatures suggest that up regulation of CD16 is possibly performed by $CD16^{-}$ monocytes and the $CD16^{+}$ subset is a mature form compared to $CD16^{-}$.

2.1.2 Toll-Like Receptors

Toll-like receptors (TLRs) are a class of proteins that are expressed by different types of cell such as monocytes, granulocytes, B lymphocytes, epithelial cells and endothelial cells. They are family of cell receptors that recognize non-self antigen or pathogen associated molecular patterns (PAMPs). For example, they are able to recognize cell wall and RNA of bacteria (Takeuchi & Akira, 2010). TLRs are vital parts of the cells as they essentially participate in innate immune response activity. Eventually, this activity results in proinflammatory cytokine productions, expanded antigen presentation, production of antibody, proliferation, and differentiation of the inflammatory cells (Tadema *et al.,* 2011). To date, there are ten human TLRs that have been discovered and successfully identified, which are TLR1-10. These TLRs are categorized as type I integral membrane receptors with two distinct portions including extracellular leucine rich regions and intracellular portion (Figure 2.2).

Many researchers suggest that cells usually expressed TLR1, TLR2, TLR4, and TLR6 both on the surface and intracellularly, in order to permits recruitment event by phagolysosomes. Meanwhile, TLR3, TLR7 TLR8, and TLR9 are specialized for recognition of bacterial and viral nucleic acid, as they were confined mainly on intracellular portion of the cell (Parker *et al.,* 2005). The expression of TLR3, TLR6, TLR7, and TLR10 were described to be lower in monocytes population. However, previous literature suggests that TLR2 and TL4 are primarily expressed on monocytes. Visintin *et al.* (2001) have studied human TLRs that present on monocytes and they found that monocytes express a significant number of TLRs, however the expression was decline abruptly with corresponding to formation of immature dendritic cell.

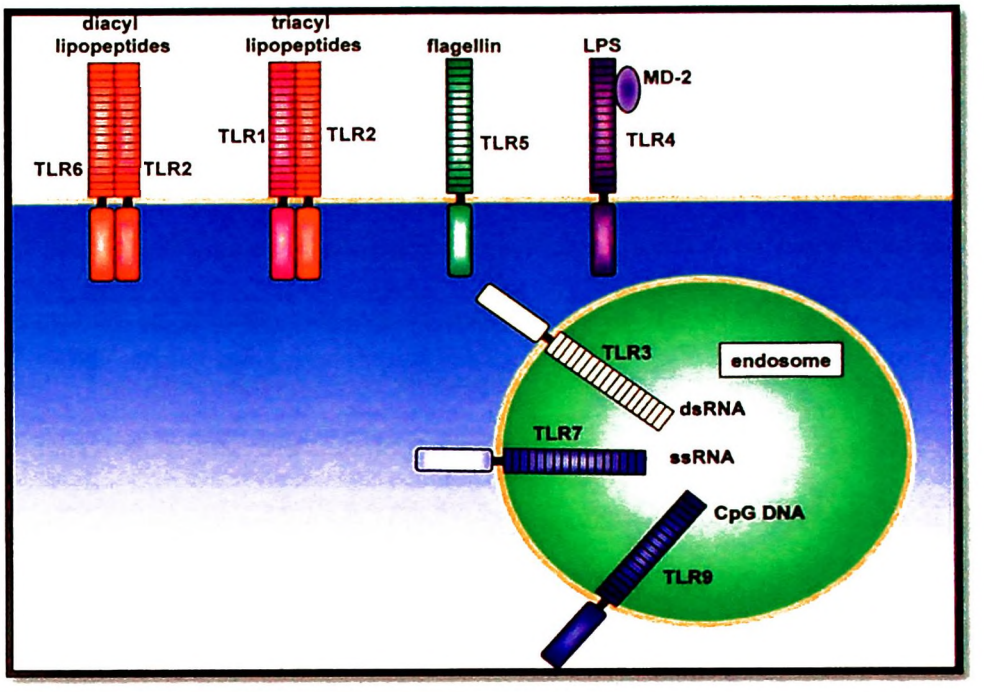

Figure 2.2: TLRs and their ligands. TLR1, TLR2, TLR4, TL5, TLR6 are expressed extracellularly, while TLR3, TLR7, and TLR8 are expressed intracellularly. (Adapted from Takeda & Akira, 2005)

TLR4 is one of the predominant receptor expressed on monocytes which binds to LPS. The recognition of LPS by TLR 4 occur together with myeloid differentiation factor 2 (MD2) on monocytes surface (Takeuchi & Akira, 2010). The complex crystal structure, which comprises of TLR4, MD2 and LPS revealed the two comples of TLR4-MD2-LPS interact symmetrically to form a TLR4 homodimer (Park *et al.,* 2009). Gram-negative LPS is considered as a highly potent antigen, which act as an immune-activator. Takeda & Akira (2005) have shown that small amount of LPS is able to confer activation of TLR4. Additionally, TLR4 has been shown to participate in identification of endogenous ligand, however only in a notable high concentration.

2.2 The role of monocytes in inflammation

Monocytes are actively engage with inflammatory response, including innate and adaptive immune system. The main function of monocytes include phagocytosis, cytokines production and antigen presentation. Phagocytosis is an engulfment activity of the particles or foreign substances that conducted by specialized cells, which subsequently being internalized into a vacuole (Hespanhol & Mantovani, 2002). The basis of phagocytosis is to kill invading pathogens and dispose debris, waste, and dead cells. In addition, monocytes usually eliminate and remove ruptured red blood cells and debris at the inflamed or damage tissue (Dale *et al.*, 2008).

Other role of monocytes during inflammation includes cytokine production. Monocytes produce cytokines during natural and acquired immune response. Once TLRs were ligated, monocytes and macrophages will produce and release some types of cytokines (Ziegler-Heitbrock, 2007). During inflammation, IL-1α is released predominantly by phagocytes include monocytes and macrophages, as well as nonimmune cells such as fibroblasts and endothelial cells. IL-1β have the ability to trigger own expression, which have been proved by human IL-1β administration prior to murin IL-1β induction (Shaftel *et al.*, 2007). Meanwhile, TNF-α is another cytokines that are produced by monocytes in response to inflammation and it displays important role in inflammatory response and being a key role in pain models. TNF-α are involved in different signalling pathway via TNFR1 and TNFR2 with the purpose of regulation apoptotic pathway, initiation of inflammation and activation of stress-activated protein kinase (SAPKs) (Zhang & An, 2007).

2.3 Monocytic Microparticles

Extracellular vesicles (EV) are tiny particles produced by cells, which serve as important mediators of physiological process in normal and pathological cells (György *et al.*, 2011). EV are categorized into three subpopulations include exosome, microparticles (MP), and apoptotic bodies (AP). The classification of these three subpopulations are based on size, cellular composition, and biogenesis.

The size of MP which also described as microvesicles (MV) derived from cellular compartments is approximately 0.1 μm- 1.0 μm (Crompot *et al.*, 2015). Usually, MP contain cellular component such as proteins, including cytoplasmic and membrane protein as well as nucleic acid (Choi *et al.*, 2013) such as mRNA, microRNA, non-coding RNA, and DNA. MP are able to deliver these components to other cells via different mechanisms (Mause & Weber, 2010).

Monocytic microparticles (mMP) can be defined as cell membrane-derived fragments that shed from monocytes origin cells in response to activation or apoptosis (Figure 2.3). Previous literatures reported that they are involve actively in coagulation and inflammation (Meziani *et al.*, 2010). Monocytic MP was shown to survive longer than their parental cells presumably due to their size, which prevent from optimal exposure to senescence signal. Moreover, they are varying in terms of cellular origin as they can be shed from various cell types. Monocytic MP may share the same phenotype with their parental cell (Distlera *et al.*, 2006) (Figure 2.4), therefore their origin can be determine by assessment of antigen expressed on their surface.

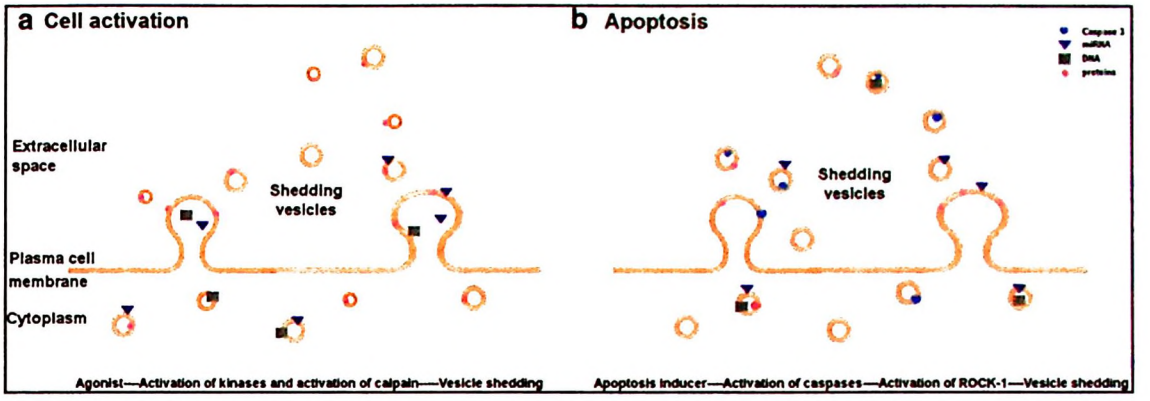

Figure 2.3: Formation of microparticles. Microparticles formed by cell activation or cell apoptosis. Vesicles shed from plasma cell membrane of parental cells via exocytosis and released into extracellular environment. (Adapted from Barteneva *et al.*, 2013)

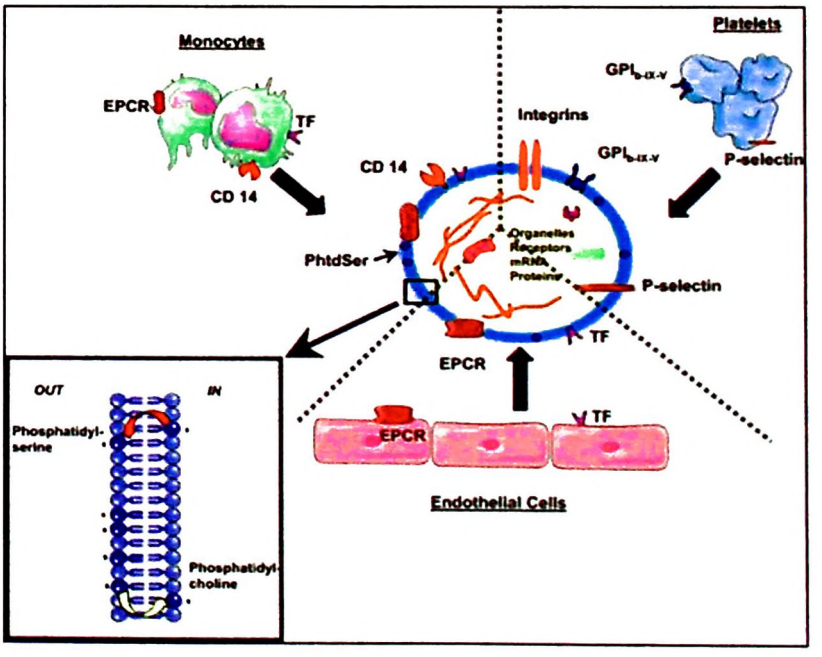

Figure 2.4: Surface structure of mMP. Monocytic MP expresses the same antigen as their parental cells such as CD14, and externalized phosphatidylserine (PS). (Adapted from Meziani et. al., 2010)

In addition, mMP can be found in body fluids (Freyssinet, 2003) and they can be distinguished from other vesicle that are derived from cell membrane such as exosomes and apoptotic bodies. Monocytic MP are measured by size, density and irregular shape, which help to distinguish them from exosome and apoptotic bodies (Freyssinet, 2003). Usually, exosomes have smaller size, while apoptotic bodies display a larger size compared to mMP. The expression of phosphatidylserine (PS), a signal for phagocytosis on the mMP allows them to be recognized as 'microparticle', while exosome and apoptotic bodies do not express phosphatidylserine (Thery *et al.*, 2002).

Monocytic MP may play a number of roles in normal and disease setting. Typically, mMP are able to trigger effect on downstream targets as they deliver parental antigens and lipids (Hugel *et al.*, 2005). Previous studies have shown that mMP contain various bioactive effector including cytoadhessions, bioactive phospholipids, cytoplasmic components and various antigen characteristic, in which predominantly originated from parent cells (Freyssinet, 2003). In addition, hundreds of proteins were discovered from the proteome of mMP and this suggests that mMP have specific roles in clinical disease state. With the presence of defined proteins, mMP can be used as ultimate biomarkers specifically for various disease processes (Smalley *et al.*, 2007). Moreover, level of mMP is observed to increase drastically in the disease state compared to normal health state. The presence of low level of mMP in normal individual could be due to normal cell death events, which are typically involved in normal homeostasis regulation. Monocytic MP may promote biological response involve in vascular homeostasis as they are competent to change the vascular function (Morel *et al.*, 2006). Circulating mMP are able to promote vascular dysfunction, lower nitric oxide (NO) formation and elevate reactive oxygen species (ROS) level, eventually promote oxidative stress

(Mortaza *et al.*, 2009). Moreover, significant increase of mMP concentration are described in most of the thrombotic disease, angiogenesis, diabetes mellitus, and immune-related disease. However, the association of mMP with disease whether it directly confers to a disease state or only acts as indicator, which reflect disease condition remains controversial.

The mechanism of mMP formation was clearly cited in most of the recent studies. Formation of mMP occurs by continuously being released from the cell surface. However, cell activation or apoptosis can actively trigger and up regulate the formation of microparticles (Lynch & Ludlam, 2007). Cell activation or apoptosis leads to markedly increase in concentration of cytosolic calcium, followed by cytoskeletal alteration. It has been thought that leaflet that localized at inner layer of phospholipid contain negatively charge aminophospholipid including PS. Plasma membrane lipid bilayer stability is changed resulting from cytoskeletal alteration, and consequently exposed the phospholipid on the surface of cell membrane (Nomura *et al.*, 2008) via flip-flopping action. This eventually leads to mMP with PS-exposing being released from the cells.

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

3.1.1 Medium

3.1.1.1 Preparation of AB sera

Heat-inactivated frozen human AB serum (Sigma-Aldrich, UK) was thawed and stored in 10 ml aliquots at -20°C until used.

3.1.1.2 Preparation of complete AB Medium

Complete AB medium was prepared by mixing Rosewell Park Memorial Institute (RPMI) -1640 (Hyclone) with 10% human AB serum, 100 U/ml penicillin, 100 µg/ml streptomycin, 2mM L-glutamine, 1 mM of sodium pyruvate, 0.1 mM non-essential amino acids, 1M HEPES buffer, and 50 µM 2-mercaptoethanol. The media was mixed thoroughly and stored at 4°C.

3.1.2 Preparation of buffers and reagents

3.1.2.1 Phosphate-buffered saline (PBS), (1X)

PBS tablet (Amresco, US) was dissolved in 100 ml of distilled water. Buffer was measured at pH 7.4 and autoclaved at 121°C for 15 minutes.

3.1.2.2 Fluorescence Activated Cell Sorting (FACS) Buffer

FACS buffer was prepared by adding 5% Bovine Serum Albumin (BSA) and 0.5% sodium azide into 1X PBS. The solution was filtered using 0.22 µm filter and stored at 4°C.

3.1.2.3 Binding buffer (1X)

Binding buffer (10X) containing 0.2 µm sterile filtered 0.1M Hepes (pH 7.4), 1.4M NaCl, and 25 mM CaCl2 solution was brought from BD Bioscience. 10X concentration were diluted to 1:10 with distilled water and stored at 4°C.

3.1.2.4 Lipopolysaccharides (LPS) preparation

Lyophilized LPS powder from *Escherichia coli* 026:B6 (Sigma-Aldrich, UK) was dissolved in distilled water to the final working concentration of 1µg/ml, 10 µg/ml and 50µg/ml and stored at -80°C.

3.1.3 List of antibodies

Type of antibodies	Descriptions
FITC AnnexinV (BD Bioscience)	Catalog No.: 556420 Vol. per Test: 5 µl
PE Mouse Anti-Human CD14 (BD Bioscience)	Catalog No.: 557742 Vol. per Test: 5 µl Clone: M5E2
PE Mouse Anti-Human IgG (BD Bioscience)	Catalog No.: 555787 Vol. per Test: 5 µl Clone: G18-145
PerCP/Cy5.5 anti-human CD11b (BioLegend®)	Catalog No.: 301327 Vol. per Test: 5 µl Clone: ICRF44
APC-Cy™ 7 Anti-Human CD16 (BD Bioscience)	Catalog No.: 561726 Vol. Per Test: 5 µl Clone: 3G8

3.1.4 List of reagents

Reagents	Description
0.4% Trypan blue	Catalog No.: 93595 Vol. per Test: 10 µl

3.2 Methods

3.2.1 Autoclaving

All heat-resistant apparatus, equipments, and materials were autoclaved at 121°C for 15 minutes.

3.2.2 Centrifugation

Unless otherwise indicated, centrifugation of blood sample was performed in a Universal 320 centrifuge (Hettichzentrifugation) at 544 x *g* for 20 minutes and 10 minutes at 22°C for the purpose of PBMC isolation and washing step respectively. Centrifugation of cultured PBMC was performed in a Universal 320 centrifuge (Hettichzentrifugation) at 500 x *g* for 5 minutes at 22°C to pellet cells. Centrifugation of both culture pellets and supernatants were performed prior to flow cytometry analysis. Two step centrifugation were subjected on culture supernatants for flow cytometry analysis. First step centrifugation of culture supernatants was performed in a Universal 320 centrifuge (Hettichzentrifugation) at 1200 x *g* for 5 minutes at 22°C and supernatants was collected. Second step centrifugation or ultracentrifugation of supernatants was performed in Mikro 22R centrifuge (Hettichzentrifugation) at 20000 x *g* for 60 minutes at 4°C to pellet microparticles. Meanwhile, centrifugation of both cultured cell in pellet and labelled cells were performed in a Universal 320 centrifuge (Hettichzentrifugation) at 500 x *g* for 5 minutes at 22°C and 4°C respectively.

3.2.3 Assessment of cell viability

Cell viability was assessed under light microscope (Leica) using haemocytometer (Invitrogen). Cells were diluted with Trypan blue (Sigma-Aldrich, UK) with the dilution factor 1:2. Ten microlitres of the dilution was transferred to a haemacytometer couting chamber and live cells were counted by exclusion of Trypan blue stained dead cells. Cell concentration was calculated using the following formula:

Concentration of viable cell/ ml = average number of viable cells x dilution factor x 10^4

3.2.4 Cell morphology

The morphology of cells during culture were assessed under light microscope (Leica) from low (10x) to high magnification (40x).

3.2.5 Peripheral Blood Mononuclear Cells (PBMC) preparation

Blood was collected from healthy donors with appropriate informed consent as approved by the Research Ethics Committee (Human) USM (USM/JEPeM/14120522). The blood was collected into EDTA anticoagulant tubes via standard venipuncture in 10 ml volume. Blood was transferred into a 50 ml tube and diluted 1:2 with sterile 1X PBS (Amresco, US). Blood was gently mixed and underlaid with 10 ml Ficoll-Paque PLUS solution (GE Healthcare, UK) to the bottom of the blood mixture, followed by centrifugation at 22°C, 544 x *g* (without brakes) for 20 minutes. PBMC was carefully

collected from a buffy coat by using transfer pipette into a 50 ml Falcon tube. PBS was added into the tube containing PBMC up to 50 ml. Cell count was performed and the cell was centrifuged at 22°C, for 10 minutes at 544 x *g*. Supernatant was discarded and the pellet was resuspended in 2 ml complete AB medium.

3.2.6 Peripheral blood mononuclear cells (PBMC) culture

PBMC was isolated from healthy donor blood and was cultured in complete AB medium. All washing steps were performed using 1X phosphate-buffered saline (PBS) (Amresco, US) unless otherwise stated. Cells were allowed to grow in a 37°C, 55 CO_2/air atmosphere incubator.

3.2.7 Monocytic microparticles generation

PBMC were cultured in a 96-well plate (TPP, German) (2 x 10^5 cells/well) in complete AB medium. Cells were stimulated with LPS from *Escherichia coli* 026:B6 (Sigma-Aldrich, UK), with various concentrations (100 ng/ml, 1 µg/ml, 5 µg/ml) (Wen *et al.*, 2014, & Wang *et al.*, 2011) in triplicates. The cells were resuspended in complete AB media and 20 µl were added to the final concentration of 100 ng/ml, 1 µg/ml, and 5µg/ml accordingly into each respective wells. The cells were allowed to grow in an incubator at 37°C in 5% CO_2 atmosphere for 18 hours. Then, cell viability of each wells were assessed by using Trypan blue (Sigma-Aldrich, UK). The stimulated cells were transferred into microcentrifuge tubes, and centrifuged at 500 x *g*, room temperature for 5 minutes. Both supernatants and pellets were collected and kept at -20°C or directly stained with appropriate antibodies.

3.2.8 General staining for flow cytometry

Following culture, cells were centrifuged at room temperature for 5 minutes, at 544 x *g* in 1 ml FACS buffer. Both pellets and supernatants were collected in separate tubes. For cell surface staining, cell pellet (2 x 10^5 cells/well) were washed once in FACS buffer and subsequently incubated with human phycoerythrin (PE)-conjugated anti-human CD14 (BD Bioscience), allophycocyanin (APC-Cy™ 7)-conjugated anti-human CD16 (Bioscience), and peridinin chlorophyll A protein (PerCP/Cy5.5)-conjugated anti-human CD11b (BioLegend®) for 20 minutes in dark at room temperature. Labelled cells were centrifuged at 500 x *g* for 5 minutes at 4°C. Then, FACS buffer was added up to 400µl.

3.2.9 Staining for monocytic microparticles

Supernatants were centrifuged twice at 1200 x *g*, for 5 minutes, at room temperature and ultracentrifugation at 20,000 x *g*, for an hour, at 4°C. The pellets were collected and 50 µl of supernatants were transferred into FACS tubes. For cell surface staining, 100 ml supernatant were resuspended in Binding buffer, and subsequently incubated with Annexin V- FITC (BD Bioscience), phycoerythrin (PE)-conjugated anti-human CD14 (BD Bioscience), and allophycocyanin (APC-Cy™ 7)-conjugated anti-human CD16 (BD Bioscience) for 20 minutes in the dark at 4°C. Then, Binding Buffer was then added up to 400µl before being analysed by flow cytometry.

3.2.10 Data acquisition

Cell acquisition was performed using FACS Canto II flow cytometer (BD Bioscience) for both mMP and cell antigen surface expression. A specific setting with higher threshold of flow cytometry was established to analyse mMP, while normal cell setting of flow cytometry was used to analyse cells.

3.2.11 Data analysis

Data analysis was performed using FCS Express V software (De Novo software). Appropriate flow cytometric colour compensation was performed to minimize the spectral overlap. The expression of cell surface markers on microparticles were measured based on double positive staining for the expression of Annexin-V and appropriate cell surface markers. While, expression of markers on cells were measured based on the mean fluorescence intensity (MFI).

$$\Delta \text{ MFI sample} = \text{MFI sample} - \text{MFI control}$$