CELLULAR RESPONSE IN THE DEVELOPMENT OF ATHEROSCLEROSIS BY *GYNURA PROCUMBENS*

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CELLULAR RESPONSE IN THE DEVELOPMENT OF ATHEROSCLEROSIS BY *GYNURA PROCUMBENS*

by

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LIST OF SYMBOLS

μ	Micro
<	Less than
α	Alpha
β	Beta
γ	Gamma
TM	Trademark
®	Registered

LIST OF ABBREVIATIONS

ABCA-1	ATP-binding cassette transporter-1
APC	Antigen presenting cells
ApoB	Apolipoprotein B
ApoE	Apolipoprotein E
BMDC	Bone marrow dendritic cells
bp	base pair
CCR	C-C chemokine receptor
CD	Cluster of differentiation
cDNA	complementary DNA
COX	Cyclooxygenase
CRP	C-reactive protein
CTLs	Cytotoxic T lymphocytes
CVD	Cardiovascular disease
DCs	Dendritic cells
DLL	Delta like ligands
EAE	Experimental autoimmune encephalitis
ECs	Endothelial cells
ECM	Extracellular matrix
ELISA	Enzyme linked immunosorbent assay
ESI	Electrospray ionization
FACS	Fluorescence activated cell sorting
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
Foxp3	Foxhead/winged helix transcription factor
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GC-MS	Gas Chromatography- Mass Spectrometry
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HDL	High density lipoproteins
HSP65	Heat shock protein 65
ICAM-1	Intracellular adhesion molecule 1
IFN	Interferon

iNOS	Inducible nitric oxide synthase
IL	Interleukin
iTreg	inducible Treg cells
LC-MS	Liquid Chromatography-Mass Spectrometry
LDL	Low-density lipoprotein
LOX-1	Lectin-like oxidized LDL receptor-1
MCP-1	Monocyte chemotactic protein 1
M-CSF	Macrophages colony stimulating factor
MDA	Malondialdehyde
MHC	Major histocompatibility complex
MAE	Microwave-assisted extraction
MMP	Matrix metalloproteinase
ORO	Oil red O
NC	Normal control
NF-kB	Nuclear factor kappa B
NK	Natural killer
NO	Nitric oxide
NPD1	Neuroprotectin D1
oxLDL	Oxidized LDL
PAI-1	Plasminogen activator inhibitor 1
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PDGF	Platelet derived growth factor
PRRs	Pattern recognition receptors
qPCR	quantitativeReal Time-Polymerase chain reaction
RNA	Ribonucleic acid
RORγt	Retinoid related orphan receptor gamma
ROS	Reactive oxygen species
RPMI-1640	Roswell Park Memorial Institute-1640
SFE	Supercritical fluid extraction
SMCs	Smooth muscle cells
STAT	Signal transducer activator of transcription
T-bet	T-box expressed in T cells
TC	Total cholesterol

TCR	T-cell receptor
TGF	Transforming growth factor
Th	T-helper
TLR	Toll like receptor
TMB	Tetramethylbenzidine
TNF	Tumor nuclear factor
Treg	T regulatory cells
UAE	Ultrasound-assisted extraction
VCAM 1	Vascular adhesion molecule 1
VEGF	Vascular endothelial growth factor
VLDL	Very low-density lipoprotein
WHO	World Health Organization

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GERAK BALAS SELULAR DALAM PERKEMBANGAN ATEROSKLEROSIS OLEH *GYNURA PROCUMBENS*

ABSTRAK

Aterosklerosis adalah factor penting penyakit kardiovaskular disebabkan oleh keradangan saluran darah kronik, yang diransang oleh lipoprotein berketumpatan rendah teroksidasi (oxLDL) dan leukosit. *Gynura procumbens* atau nama tempatan sambung nyawa mempunyai kesan pelindung kardio. Kajian ini dijalankan untuk mengkaji unsur kimia ekstrak etanol *G. procumbens* dan fraksi, kesannya terhadap pembentukan sel buih yang berasal dari makrofaj dan pembezaan sel T $CD4^+$. Pertama, LC-MS mengesan komponen bioaktif dari kumpulan asid lemak, flavonoid, sesquiterpenoids dan produk pemecahan klorofil manakala GC-MS menunjukkan bahawa ekstrak etanol *G. procumbens* dan fraksinya mengandungi pelbagai sebatian mudah berubah. Kedua, ekstrak etanol *G. procumbens* dan pecahannya mengurangkan pengumpulan titisan lipid dan jumlah kolesterol dalam makrofaj yang dirawat dengan oxLDL bersama dengan pengurangan rembesan TNF-α dan IL-1β yang signifikan dalam supernatan mereka. Sebagai tambahan, ekstrak etanol *G. procumbens* dan fraksinnya mengurangkan pengekspresan gen LOX-1 dan meningkatkan gen ABCA-1 secara signifikan dalam makrofaj yang dirawat dengan oxLDL. Akhir sekali, ekstrak etanol *G. procumbens* dan fraksinya meningkatkan ekspresi CD11c, MHC kelas II dan CD80 dalam BMDC yang dirawat dengan oxLDL. Ekstrak etanol *G. procumbens* dan fraksinya merencat pengekspresan gen T-bet, GATA-3 dan RORγt tetapi meningkatkan pengekspresan gen Foxp3 dalam sel T $CD4^+$ yang dibezakan. Selanjutnya, ekstrak etanol *G. procumbens* dan fraksinya juga meningkatkan gen DLL-3 tetapi merencat pengekspresan gen Jagged-1 dalam BMDC yang diaktifkan.

Kesimpulannya, kesan anti-aterogenik ekstrak etanol *G. procumbens* dan fraksinya dengan menghalang komponen sel yang terlibat dalam pembentukan aterosklerosis akan memberi gambaran yang jelas untuk pembangunan terapi bersasar yang baru untuk rawatan aterosklerosis.

CELLULAR RESPONSE IN THE DEVELOPMENT OF ATHEROSCLEROSIS BY *GYNURA PROCUMBENS*

ABSTRACT

Atherosclerosis is the main fundamental root of cardiovascular disease (CVD) caused by a chronic inflammatory of blood vessels, namely atherosclerosis which induced by oxidised low-density lipoprotein (oxLDL) and leukocytes. *Gynura procumbens* or locally called as sambung nyawa has cardio-protective effect. The current study was undertaken to elucidate the chemical constituents of *G. procumbens* ethanol extract and its fractions, their effects on macrophage derived foam cells formation and $CD4^+$ T cell differentiation. Firstly, LC-MS analysis detected bioactive constituents from group of fatty acid, flavonoid, sesquiterpenoids and products of chlorophyll breakdown whereby GC-MS showed that *G. procumbens* ethanol extract and its fractions contained varied volatile compounds such as hexadecane, phytol and stigmasterol. *G. procumbens* ethanol extract and its fractions exhibited potent cell viability as all the concentrations induced proliferation of RAW264.7 macrophages as the percentages of cell viability were above 100% compared to untreated cells. Secondly, *G. procumbens* ethanol extract and its fractions reduced lipid droplet accumulation and total cholesterol in oxLDL-treated macrophages together with significant reduction of TNF-α and IL-1β secretions in their supernatant. In addition, *G. procumbens* ethanol extract and its fractions significantly reduced LOX-1 gene expression and increased ABCA-1 gene in oxLDL-treated macrophages. Finally, *G. procumbens* ethanol extract and its fractions up-regulated the expression of CD11c, MHC class II and CD80 in oxLDL-loaded BMDC. *G. procumbens* ethanol extract and its fractions suppressed T-bet, GATA-3 and RORγt gene expression but increased the

expression of Foxp3 gene in differentiated $CD4^+$ T cells. Furthermore, *G. procumbens* ethanol extract and its fractions also increased DLL-3 gene but suppressed Jagged-1 gene expression in activated BMDC. In conclusion, *G. procumbens* ethanol extract and its fractions possess anti-atherogenic effect via inhibiting cellular components involve in atherogenesis, thus give clear insight for the development of novel therapeutic target for atherosclerosis treatment.

CHAPTER 1

INTRODUCTION

1.1 Cardiovascular disease and atherosclerosis: An overview

Cardiovascular disease (CVD) is the leading cause of mortality worldwide which estimated 17.6 million deaths per year in 2016 and expected to reach 23.6 million by 2030 (Benjamin *et al.*, 2019). CVD have been a main root of death and illness in Malaysia since early 1970s. CVD were four principal causes of death in Malaysia comprise heart disease (5.6%), diabetes mellitus (3.3%), stroke (1.7%), and hypertension (1.6%) (Mohammad *et al.*, 2018). The pathological condition of CVD is atherosclerosis, a slowly progressing chronic inflammation disorder associated with lipid accumulation in the large and medium-sized arteries (Gistera & Hansson, 2017). This lipid build-up causes hardening and narrowing of the artery lumen which obstructs the blood flow that limits the oxygen supply to various organs and tissues leading to further complications (Hansson & Hermansson, 2011). There are numerous risk factors responsible for the development and complications of atherosclerosis including hyperlipidaemia and hypertension which known as the primary risk factors (Ramji & Davies, 2015). Previously, it was believed that atherosclerosis was merely passive accumulation of cholesterol in the artery wall (Hansson & Hermansson, 2011). However, recent studies have displayed atherosclerosis as a chronic inflammatory disease regulated by both innate and adaptive immune responses that mediate the initiation, progression, and ultimate thrombotic complications of the disease (Miteva *et al.*, 2018). Both the innate and adaptive immune responses form a complex interaction in vascular environment, modified lipids and cellular interactions which caused chronic inflammation (Garrido-Urbani *et al.*, 2014).

1.2 Atherosclerosis

Atherosclerosis is an inflammatory disease consists of intense immunological activity that leads to CVD which drastically threatens human health globally (Hansson & Hermansson, 2011). According to World Health Organization (WHO) classification, atherosclerosis disease progression involves three different phases of development known as fatty streak, atheroma, fibrous plaque, and complex lesions (Gaudio *et al*., 2006). The atherosclerotic plaque is structurally complex compared to fatty streak and protected by a fibrous cap of variable thickness. The shoulder' regions of the fibrous cap infiltrated by activated T cells, macrophages and mast cells that secreted various pro-inflammatory mediators and enzymes. The atherosclerotic plaque comprises of necrotic cores, calcified regions, oxidised lipoprotein (oxLDL), inflamed smooth muscle cells (SMCs), endothelial cells (ECs), leukocytes, and foam cells. The plaque eventually leads to stenosis (narrowing of the lumen) which results in ischemia in the surrounding tissue (Hansson and Hermansson, 2011; Poledne & Kralova Lesna, 2018).

1.3 Diversity of immune cells in atherosclerosis

Atherosclerosis is initiated by the activation of endothelium by oxLDL which expressed adhesion molecules, integrin and chemokines that facilitate the recruitment of monocytes, macrophages, T cells, dendritic cells (DCs), other inflammatory cells like B cells, mast cells to migrate into the intima (Ilhan & Kalkanli, 2015; Taleb, 2016). Under the influence of macrophage-colony stimulating factor (M-CSF), monocytes tend to differentiate into macrophages inside the intima (Bilen *et al.*, 2006). The uptake of oxLDL by macrophages is mediated by scavenger receptors such as Lectin-like oxLDL receptor-1 (LOX-1), and also the efflux is mediated by ATP-binding cassette (ABC)

transporters, particularly ABCA-1 (Westerterp *et al.*, 2014; Schaftenaar *et al.*, 2016). The excessive accumulation of oxLDL led to the formation of foam cells.

DCs also play a huge role in initiating the adaptive immune reaction towards atherosclerosis. DCs have similar phenotype and functional properties with macrophages and it's difficult to differentiate between the role of DCs and macrophages in atherosclerotic lesion (Geissmann *et al.*, 2010). DCs also ingest oxLDL via scavenger receptors, form foam cells hence contributing to atherosclerotic lesion development (Bobryshev, 2010; Subramanian & Tabas, 2014). DCs involve in maturation, migration and antigen presentation to T cells in draining lymph nodes subsequent to uptake of oxLDL (Schaftenaar *et al.*, 2016). The antigen presentation to T cells by DCs leads to T cells activation and differentiation into various T cell subsets including T helper 1 (Th1), Th2, Regulatory T (Treg) cells and Th17 cells.

Th1 cells are the predominant T cell subsets in atherosclerotic lesion (Zhu & Paul, 2010). Th1 cells produce various pro-inflammatory cytokines such as TNF-α, IFN-γ, IL-2, and IL-12 and express the transcription factor T-bet and called pro-atherogenic as they enhance oxLDL uptake, reducing collagen production SMCs, and increase leukocyte recruitment (Tse *et al.*, 2013). Th2 cells are accountable for secretion of various pro-inflammatory cytokines including IL-4, IL-5, IL-10 and IL-13 as well as potent in activating B cells to produce antibodies (Taleb, 2016). The naïve $CD4^+$ T cells differentiate into Th2 with Notch receptors 1 and 2 on the cells enhancing the expression of GATA-3 (Auderset *et al.*, 2012). The role Th2 in atherosclerosis continues to be unclear as firstly Th2 was projected as atheroprotective by inhibiting Th1 response (Taleb, 2016). Treg cells are classified into two types, natural and induced Treg counting on their origin (Liu *et al,*, 2011). Natural Tregs (nTreg), categorised by the

expression of CD4, CD25 and also the transcriptional factor, Foxp3. During a vigorous immune response, induced Treg (iTreg) are produced within the periphery and the naïve $CD4^+CD25^-$ cells in the periphery characterised by the phenotype $CD4^+CD25^+Foxp3^+$ in the presence of TGF-β and IL-10 (Workman *et al.*, 2009). Both nTreg and iTreg play significant role in reducing atherosclerosis by inhibiting lesion formation and progression (Taleb, 2016). Another T helper subset is Th17 cells, which do not belong to the Th1 and Th2 family. (Damsker *et al.*, 2010). Th17 generate interleukins, like IL-17A, IL-17F, IL-21 and IL-22 and expressed transcription factor, RORγt (Taleb *et al.*, 2015). Different cytokines are suggested to stimulate Th17 differentiation, including IL-23, IL-6 and TGF-β (Burkett *et al.*, 2015).

1.4 Medicinal plants

Plants have been the foundation of traditional medicine, which has existed for thousands of years to treat various human diseases and also to provide new therapies for manhood (Rahman *et al.*, 2013). According to WHO valuation, to date medicinal plants have existed as the significant natural substitutions to synthetic drugs since roughly 80% of the world inhabitants' hinge on plants as their prime health care (Rahman *et al.*, 2013).

Secondary metabolites of medical plants are accountable for ailment prevention and promoting healthiness via different efficient underlying mechanisms. Numerous studies involving *in vitro* and *in vivo* studies as well as clinical based studies have been performed for detection and isolation of the chemical constituents to establish their biological effectiveness. The secondary metabolites of medicinal plants possess various vital functions including antioxidant, antimicrobial, antifungal, regulation of detoxification enzymes, immune system stimulation, platelet aggregation reduction,

hormone metabolism modulation, antihyperlipidemic, antihypertension and anticarcinogenic (Saxena *et al.*, 2013; Al-snafi, 2015). These chemical constituents could act individually or synergistically for better therapeutics effects; for instance, phenolic compounds serve as antioxidant agent while alkaloids aid in mood improvement which provide a sense of well-being (Rasoanaivo *et al.*, 2011). Additionally, traditional and allopathic medicines are arises side by side in a complimentary way (Al-snafi, 2015).

Plant-based active compounds such as phenols, flavonoids, and antioxidants serve as therapies on atherosclerosis prompting factors, hence prevents the disease and associated harmful complications (Gul *et al.*, 2016). The active compounds of medicinal plants play a crucial role in treating atherosclerosis and preventing its progression by lowering cholesterol level, averting increase in free radicals and lessening vascular plaque plus resistance (Sedigh et al., 2017). Moreover, plant-derived compounds alone or in combination with hypocholesterolaemia medications, can be potential effective therapeutic remedies for patients with hyperlipidaemia complications (Sedigh *et al.*, 2017).

1.4.1 *Gynura Procumbens*

Gynura procumbens (Lorr.) Merr. (*G. procumbens*) a fast-growing herbaceous plant with fleshy stem, belongs to the family of Astereceae and found throughout South-East Asia including Indonesia, Malaysia and Thailand (Tan *et al.*, 2016). *G. procumbens* locally known as sambung nyawa, which means "prolongation of life" (Rohin *et al.*, 2018). This plant has been widely used as traditional medicine to treat various diseases such as cancer, kidney disease, migraines, hypertension and diabetes, eruptive fever,

migraines, constipation, diabetes mellitus, and cancer (Afandi *et al.*, 2014). Studies have shown that *G. procumbens* extracts comprises numerous pharmacological activities such as anti-hyperglycaemic (Hassan *et al.*, 2010), anti-inflammatory (Dwijayanti & Rifa'I, 2015), anti-hypertensive effects (Poh *et al.*, 2013), antioxidant (Akowuah *et al.*, 2012), blood hypertension reduction capabilities (Kaur *et al.*, 2012) and anti-proliferative actions (Kim *et al.*, 2011; Nisa *et al.*, 2012; Shwter *et al.*, 2014).

These pharmacological activities attributed to the bioactive compounds such as flavonoids, saponins, tannins, terpenoids, steroil glycosides, rutin and kaempferol (Zahra *et al.*, 2011; Akowuah et al., 2012; Kaewseejan *et al.*, 2012). Our studies similarly found that *G. procumbens* ethanol extract and its fractions composed of bioactive constituents from variety of groups including fatty acids, flavonoids, sesquiterpenoids and product of chlorophyll breakdown (Manogaran *et al.*, 2019).

1.5 Problem statements and hypothesis

Several studies have demonstrated that *G. procumbens* extract has anti-hypertensive effect. Hypertension play an important role in atherogenesis by enhancing the development of vulnerable plaques which in turn lead to thrombosis and vessel occlusion. Occasionally hypertensive patients experience inadequate control of blood pressure which leads to rise of the monotherapy dose or need to use drug combinations that increases the risk of side effects. Therefore plant-derived compounds alone or in combination with hypertensive properties, can be potential effective therapeutic remedy for patients with no or less side effects. Hence, anti-hypertensive effect of *G. procumbens* may reduce atherogenesis by controlling hypertension. However, the exact mechanism how *G. procumbens* regulate atherosclerosis development need to be investigated. Since no study have been carried out on the direct effect of *G. procumbens*

on atherosclerosis, we hypothesise that *G. procumbens* ethanol extract and fractions may have anti-atherogenic effect by inhibiting certain cellular components which accumulate in the atherosclerotic plaques. The flow chart of the study is illustrated in Figure 1.1.

1.6 Objectives of the study

The aim of this study is to elucidate the regulation of cellular response involved atherosclerosis development by *G. procumbens*. The specific objectives of the study are listed below.

1. To determine the bioactive compounds in *G. procumbens* ethanol extract and its fractions by using LC-MS and GC-MS analysis.
2. To investigate the effect of *G. procumbens* ethanol extract and its fractions on the macrophage derived foam cell formation.
3. To determine the effect of *G. procumbens* ethanol extract and its fractions on the bone marrow dendritic cells (BMDC) in the atherosclerotic lesion.
4. To determine the effect *G. procumbens* ethanol extract and its fractions on the differentiation of $CD4^+$ T cells into Th1, Th2, Th17 and Treg cells in the atherosclerotic lesion.

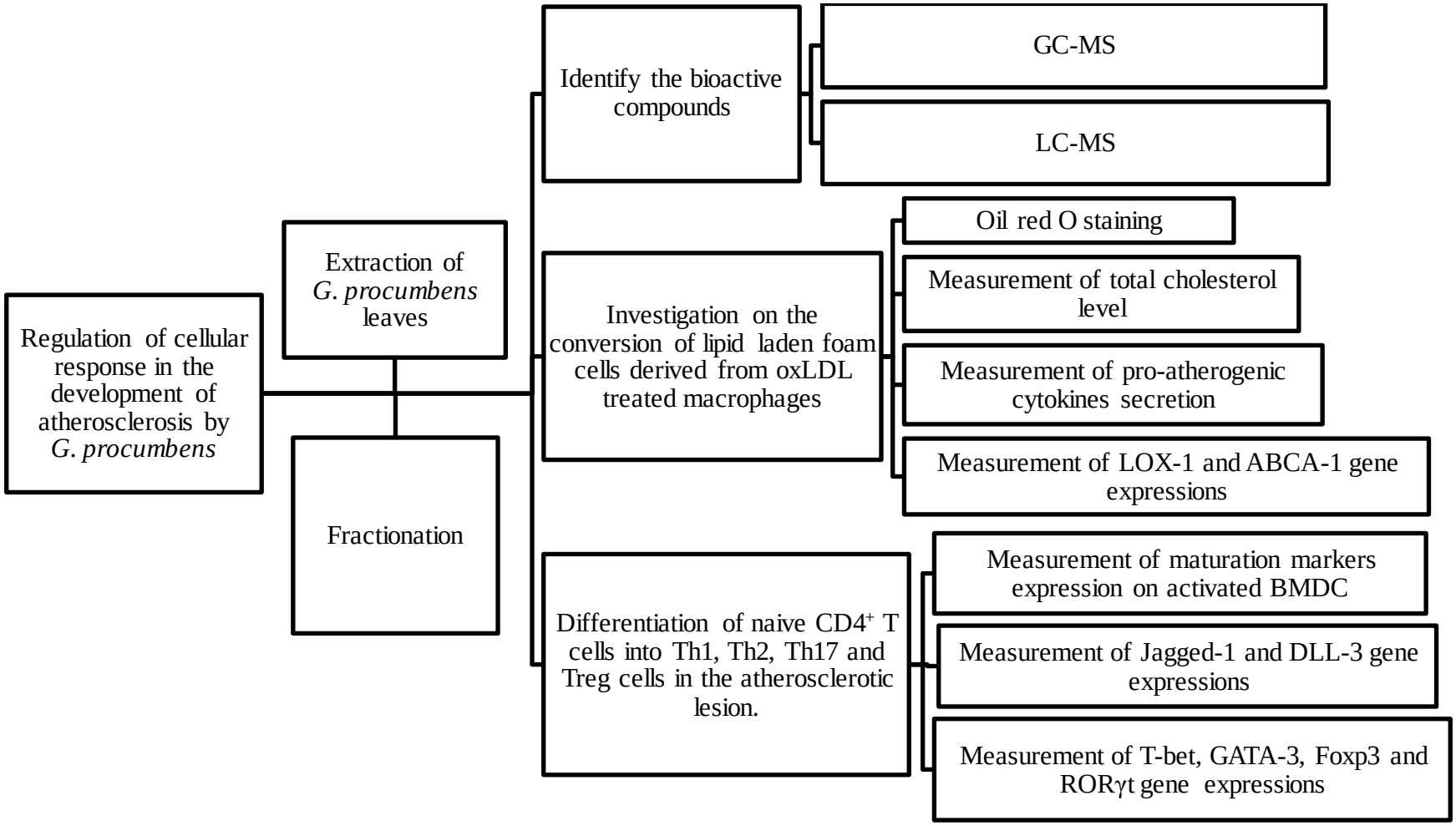

Figure 1.1: Flow chart of the study

CHAPTER 2

LITERATURE REVIEW

2.1 Atherosclerosis

The disruption of homeostasis in the cardiovascular system elicit the development of multifaceted diseases such as atherosclerosis which lead to various clinical complications including myocardial infarction and stroke (Sack *et al.*, 2017). Atherosclerosis is the major cause of death worldwide due to increase potential risk factors such as obesity and diabetes (World Health Organization, 2014). The development of atherosclerosis begins in the early of teen-ages and progresses over 50 years (Insull, 2009).

Atherosclerosis is a chronic inflammatory disease of arteries which occurs at susceptible sites in the main conduit arteries due to lipid retaining, oxidation and alteration that eventually leads to cell death, fibrosis, thrombosis or stenosis (Hansson & Hermansson, 2011). The accumulation of lipids known as lesion begin in the inner layer of artery, tunica intima and gradually affect the whole arterial wall, including the middle layer, tunica media and the outer layer, tunica adventitia (Chistiakov *et al.*, 2017). The atherosclerotic lesion matures progressively and gain new features known as atherosclerotic plaque which also consist of numerous immune cells such as macrophages, dendritic cells (DCs) and lymphocytes. Over the time, the atherosclerotic plaque develops into a more complex form that made up of apoptotic and necrotic cells, cell debris and cholesterol crystals that ultimately hardens and narrow the lumen of the artery which causes ischemia to the surrounding tissue (Hansson & Hermansson, 2011). Finally, thrombosis occur due to rupture of fibrous cap of the plaque which leads to release of thrombogenic materials in the core and form a thrombus that obstructs the

blood flow as well as restrict oxygen supply to various vital organs such as heart, brain, legs and other organs which cause further severe complications (Otsuka *et al.*, 2014).

2.1.1 Risk factors of atherosclerosis

The complications of cardiovascular diseases (CVD) that arise from atherosclerosis is increasing due to the persistence exposed to risk factors known as conventional and novel risk factors (Witztum & Lichtman, 2014). The conventional risk factors including high level of low-density lipoprotein (LDL), low level of high-density lipoprotein (HDL), hypertension, smoking, diabetes mellitus, obesity, sedentary lifestyle and age (Owen *et al.*, 2011; Weber & Noels, 2011). Meanwhile, the novel risk factors that are responsible for the development of atherosclerosis have been summarised in Table 2.1.

Table 2.1: Novel risk factors involved in atherosclerosis

Novel risk factors	Example
Haemostasis/Thrombosis markers	Fibrinogen Fibrinogen involves in lesion development and thrombosis as it induces cellular proliferation, contracts of damaged cellular walls, stimulate platelet aggregation, and regulate cell adhesion (Asgary *et al.*, 2013).
Platelet-related factors	Platelet reactivity Platelet size, function and accumulation highly induces the risk of developing atherosclerotic cardiovascular complications (Mangiacapra *et al.*, 2010).
Lipid-related factors	Lipoprotein(a) High levels of apolipoprotein B (ApoB) leads to atherosclerotic plaques and thrombogenic properties which indicate the risk of heart disease precisely compared with LDL or total cholesterol levels (Hansson & Hermansson, 2011).

	Small dense LDL Small dense LDL exhibits higher level of atherogenicity due to its capacity to infiltrate into the subintimal space, greater susceptibility to oxidation and increased binding to intimal proteoglycans (Meiliana & Wijaya, 2012).
Inflammatory markers	C-reactive protein (CRP) CRP is the key factor in advancing vascular wall impairment as it activates platelets to bind with endothelial cells thus induces monocytes and lymphocytes migration into the endothelial walls. CRP also stimulates the proliferation vascular SMC which leads to accretion of these cells in the intima (Teupser *et al.*, 2011). Adhesion molecules; Intracellular adhesion molecule-1 (ICAM-1) ICAM-1 facilitates the adhesion and migration of monocytes to the vessel wall which increases the activation of endothelial cell and inflammation (Moore & Tabas, 2011).
Infectious agents	*Chlamydia pneumoniae* (*C. pneumoniae*) *C. pneumoniae* replicates and sustains in macrophages and endothelial cells which results in the initiation of inflammatory progression and acutely aggravating the response. It exhibits direct effect on atheroma through plaque inflammation, thus contributing to plaque disruption (Rafieian-Kopaei *et al.*, 2014).
Other factors	Homocysteine Reduced homocysteine metabolism causes oxidative stress and induces pro-inflammatory vascular condition which leads to development of atherosclerosis (McCully, 2016).

2.1.2 Autoantigens in atherosclerosis

There are wide range of atherosclerosis-related antigens involved in the atherogenesis such as oxidised low-density lipoproteins (oxLDL), heat shock proteins (HSP) and foreign antigens which included viruses and bacteria. OxLDL is the utmost studied autoantigens in the atherosclerosis progression (Shi, 2010).

2.1.2(a) oxidized Low-Density Lipoprotein (oxLDL)

The risk factors of atherosclerosis highly influenced by enhanced oxidative stress condition that oxidises native LDL-cholesterol (Mitra *et al.*, 2011). Oxidative stress is known as the excess production of reactive oxygen species (ROS) that damages cellular lipids, cell structures, proteins and nucleic acids that leads to various complications such as atherosclerosis which characterized as inflammatory oxidative conditions (Kattoor *et al.*, 2017). LDL is a lipoprotein with a 22 nm diameter and 1.019 to 1.063 grams per mL density. The core of LDL is hydrophobic which made up of mainly of cholesteryl ester with a small number of triglycerides. On the other hand, the surface of LDL is comprising of phospholipids (phosphatidylcholine and sphingomyelin free cholesterol) and a single molecule of a large protein, apolipoprotein B100 (apoB-100) (Milioti *et al.*, 2008) (Figure 2.1).

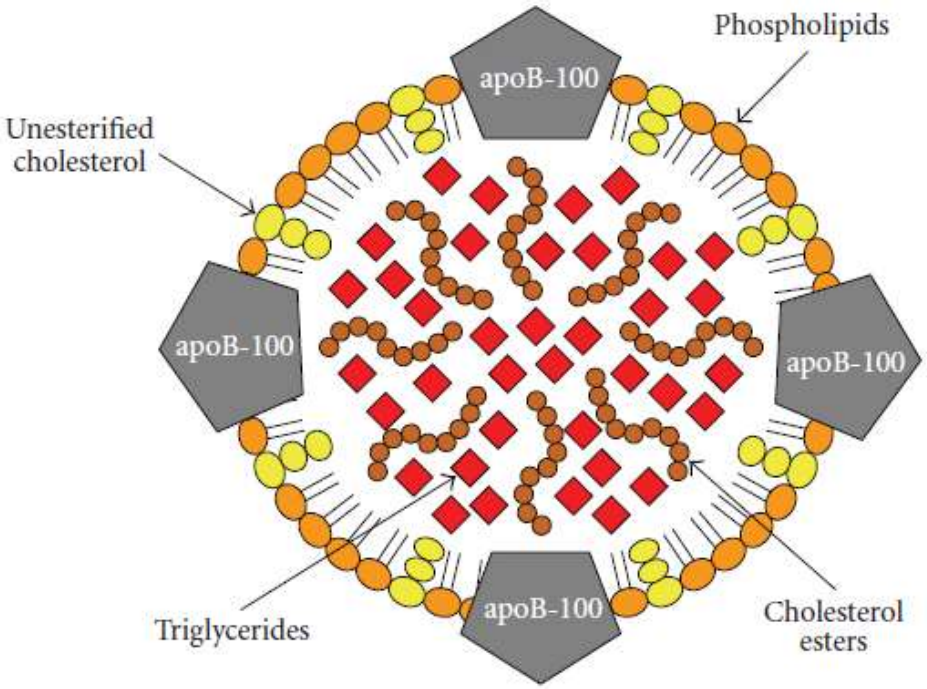

Figure 2.1: Low-density lipoprotein (LDL). LDL is approximately 21–24nm in size and the foremost transporter of unesterified cholesterol, cholesterol esters, and triglycerides in the blood. It made up of phospholipids, unesterified cholesterol and apolipoprotein B-100 (apoB-100) as outer layer and the core mainly composed of cholesterol esters and triglycerides (Mitra *et al.*, 2011).

Uncoupling of endothelial nitric oxide synthase (eNOS) is a vital source of ROS as it is attributed to constraint the availability of its cofactor tetrahydrobiopterin (BH4) and oxidation of BH4 to BH2 that produces nitric oxide, which in turn can oxidize native unmodified LDL to oxLDL (Gielis *et al.*, 2011). Scavenger receptors (SRs) are the cell surface receptors expressed on the surfaces of macrophages and other vascular cells that recognize and internalize oxLDL rather than native LDL (Gao & Liu, 2017). There are several SRs such as cluster differentiating 36 (CD36), SR-BI, cluster differentiating 68 (CD68), scavenger receptor for phosphatidylserine and oxidized lipoprotein (SRPSOX) and lectin-like oxidized LDL receptor-1 (LOX-1) (Trpkovic *et al.*, 2015). The high levels of intracellular cholesterol will not affect or down regulate the SRs, thus the uptake of oxLDL by macrophages via SRs elicit intracellular

cholesterol, induces the conversion of macrophages into foam cells and promotes the progression of atherosclerotic lesions (Moore & Tabas, 2011). On the other hand, the oxLDL deposition in the subendothelial space also activates atherogenesis and plaque formation through endothelial cell activation and dysfunction, smooth muscle cells (SMCs) migration and proliferation by the retained oxLDL particles, together with induction of inflammatory cytokines secretion and expression of adhesion molecules that promote endothelial cells (ECs) dysfunction and leukocyte extravasation (Hansson & Hermansson, 2011). T cells also tend to interact with oxLDL peptide bind with MHC complex presented on the surface of antigen-presenting cells, become activated, and release proinflammatory cytokines (Ismail, 2013).

2.1.3 Pathogenesis of atherosclerosis

Atherosclerosis is a chronic inflammatory disease of arteries that occurs in the subendothelial space known as tunica intima due to accumulation of oxLDL that initiate endothelial dysfunction which facilitate the infiltration of various immune cells including monocytes, macrophages, SMCs and lymphocytes that secrete various pro-atherogenic cytokines (Tabas *et al.*, 2015). The structure of normal arterial wall comprises of three different layers namely tunica intima, tunica media and tunica adventitia (Figure 2.2a). Tunica intima is the innermost layer of artery made up by one-layer ECs that faces the lumen which is in direct contact with blood and composed of connective tissue and occasional macrophages or SMCs. Intima is highly responsible in sustaining the vascular homeostasis and its smoothness and elasticity such that it does not block the blood flow (Zhang, 2019). The internal elastic laminae is located between media and intima while the external elastic laminae is situated among adventitia and media (Kilany *et al.*, 2020).

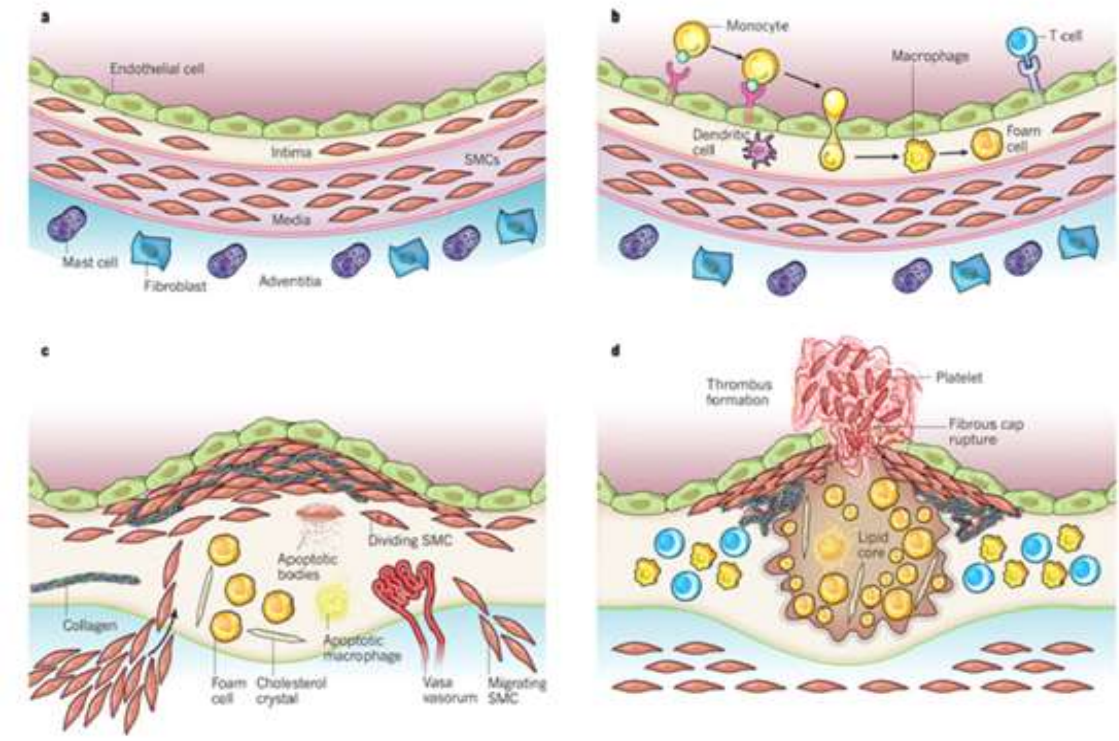

Figure 2.2: Stages of atherosclerotic plaque development. a) Normal artery consists of three different layers known as tunica intima the inner layer, tunica media the middle layer and tunica adventitia the outer layer. b) The primary stages of atherosclerosis are the adhesion of leukocytes to the activated ECs which recruited the leukocytes into the intima, differentiation of monocytes into macrophages by M-CSF, and their excessive lipid uptake that leads to foam cells development. c) Lesion progression includes the migration and proliferation of SMCs from the media to the intima that increases the synthesis of ECM. Plaque macrophages and SMCs die in advancing lesions through apoptosis and the accumulation of extracellular lipid in the central region of the plaque known as necrotic core. d) Thrombosis, the final complication of atherosclerosis due to a physical disruption of the plaque's fibrous cap which causes blood coagulation components to burst out and contact tissue factors in the plaque's interior which triggers the thrombus that outspreads into the vessel lumen and block blood flow (Libby, 2011).

The middle layer of artery is known as tunica media which made up of SMCs that accountable for maintaining the contractility of the vessel and aid in storing kinetic energy required for the transmission of pulsatile flow. External elastic lamina that bound on the outside of media separates the media from the adventitia. Tunica adventitia is the outmost layer of the artery that consists of connective tissues, mast cells, fibroblasts, SMCs, immune cells including T cells, monocytes and macrophages. Adventitia

composed of matrix comprising collagen and proteoglycans and contained vasa vasorum that supply oxygen to various cellular components of the wall (Zhang, 2019).

2.1.3(a) Fatty streak development

LDL is a lipoprotein that responsible for transportation of cholesterol in blood. The initial sign of atherosclerosis development is the fatty streak formation which trigger by the entry of LDL into intima from the bloodstream which begins in childhood (Figure 2.2b) (Cheraghi *et al.*, 2019). The binding of LDL apoB-100 to proteoglycans of the extracellular matrix (ECM) by ionic interactions is the key aspect in early atherogenesis as it causes subendothelial retention in which LDL particles trapped in the intima and subjected to oxidative alterations triggered by enzymes including myeloperoxidase and lipoxygenases together with ROS such as phenoxyl radical intermediates which leads to innate inflammatory responses on atherosclerosis (Hansson & Hermansson, 2011).

Inflammation initiates as the modified LDL known as oxLDL induces ECs to express adhesion molecules such as intracellular adhesion molecule-1 (ICAM-1), vascular adhesion molecule (VCAM-1) and P- and E-selectin molecules that sited at the susceptible site to lesion formation with turbulent blood flow (Gistera & Hansson, 2017). At the same time, proinflammatory cytokines secretion such as tumor necrosis factor alpha (TNF-α), Interleukin 1 beta (IL-1β), and Interleukin 6 (IL-6) activates by oxLDL increases adhesion molecules expression on ECs to form a malicious cycle. The expression of adhesion molecules activates the recruitment of leukocytes including monocytes, neutrophils, lymphocytes and mast cells into the arterial wall (Libby *et al.*, 2011). This acts in synergy with chemotactic factor such as monocyte chemotactic

protein-1 (MCP-1) which facilitate the migration of monocytes, DCs and T cells into intima through ECs (Pirillo *et al.*, 2013).

Infiltrating monocytes in the intima differentiate into macrophages under influence of macrophage-colony stimulating factor (M-CSF) produced by activated ECs (Gleissner, 2012). This leads to up-regulation of scavenger receptors, subtypes of pattern recognition receptors (PRRs) and toll like receptors (TLRs) that enable macrophages to capture oxLDL which eventually turns macrophages into the foam cells and activates inflammation via series of cellular signalling pathway (Hansson & Hermansson, 2011; Gistera & Hansson, 2017). Activated inflammation responses triggers other immune cells such as DCs and $CD4^+$ T cells which results in activation of adaptive immunity responses (Niessner & Weyand, 2010).

2.1.3(b) Formation of the fibrous cap (early fibro-atheroma or complex lesions)

Early fibroatheroma starts at the age of 20s and continues throughout lifetime (Figure 2.2c) (Insull, 2009). The formation of fibrous cap is symptomless process which can develop as a complex atheroma or revert to a simpler plaque (Mughal *et al.*, 2011). The accumulation of lipid results in "activation" or "phenotypic switching" of SMCs where the quiescent, completely contractile SMCs down-regulate smooth muscle α-actin (Acta2) and smooth muscle myosin heavy chain (Myh11) genes and secrete proinflammatory cytokines such as IL-1β and TNF-α by adjacent SMCs causes the migration and proliferation of SMCs into the intima or sub-endothelial space (Alexander & Owens, 2012; Rafieian-Kopaei *et al.*, 2014). This leads to secretion of numerous ECM proteins by SMCs such as collagen, fibrin and proteoglycan forming a fibrous cap (Mughal *et al.*, 2011). A necrotic core made up of increased extracellular lipid accumulation, activated inflammatory cells such as natural killer (NK) cells,

lymphocytes, and DCs along with release of apoptotic factors bulges in the central part of intima that comprises 30% to 50% of the arterial wall volume and decrease the blood flow (Martinet *et al.*, 2011). The various elevated immune cells deteriorate the fibrous cap as production of meta proteinase by macrophages lysis the ECM while TNF-α secretion by T cells prevents the collagen synthesis of SMCs (Rafieian-Kopaei *et al*., 2014). At this phase the fibrous cap may remain intact that help to stabilises the plaque or continue to grow and becomes more vulnerable to rupture (Martinet *et al.*, 2011).

2.1.3(c) Advanced atheroma and atherosclerotic plaque rupture

The advanced atheroma usually occurs in the ages of 55 to 65 years where lastly the plaque may rupture and causes severe effects such as myocardial infarction and stroke (Figure 2.2d) (Insull, 2009). The fibrous cap is prone to rupture as it becomes thin and weakened at a few sites due to continuous proteolytic enzyme activity which dissolves the fibrous cap (Finn *et al.*, 2010). This physical disruption leads to exposure of clotting factors to pro-coagulants expressed in the lesions and produces a thrombus that extends into the arterial lumen due to the disruption of micro-vessels within the plaques that makes the vessel fragile and weak. Generation of thrombin stimulates SMCs migration and proliferation by triggering platelets to produce platelet derived growth factor (PDGF) and transforming growth factor (TGF-β) which rises the silent micro-vascular haemorrhage in the atherosclerotic intima. Immune cells such as macrophages exist in the plaque secrete angiogenic mediators such as acidic and basic fibroblast growth factor and vascular endothelial growth factor (VEGF) which also contributes to rupture of the plaque (Greenberg & Jin, 2013). Proinflammatory cytokines such as interferon gamma (IFN-γ) decreases collagen production by SMCs through apoptosis and stimulating overexpression of matrix metalloproteinase (MMP),

as well augment plaque vulnerability. Production of collagen and new fibrous tissue eventually will restore the plaque disruption but successive development of the atherogenesis may rapture the plaque again (Bennett *et al.*, 2016).

2.1.4 Diversity of immune cells involved in atherosclerosis

Atherosclerosis is a complex progressive disease characterized by the formation of atherosclerotic plaques which made up of necrotic cores, calcified regions, accumulated modified lipids and various inflammatory cells such as SMCs, ECs, leukocytes, and foam cells (Park & Lee, 2019). Both innate and adaptive immune responses involve in the pathogenesis of the disease. Alterations of oxLDL is a vital process that initiates endothelial dysfunction and activation of immune cells in atherosclerosis development (Park & Lee, 2019). The deposition and accumulation of oxLDL causes ECs dysfunction which activates the adhesion molecules for recruiting monocytes that later transform into macrophages then foam cells. Other immune cells such as T helper cell type 1 (Th1) contributes to the plaque progression by secreting IFN-γ that aggravate the inflammatory responses (Feil *et al.*, 2014; Gisters & Hansson, 2017). The balance between progression and resolution of the plaque inflammation is differentially affected by the heterogeneity of immune cells (Tabas & Lichtman, 2017). Correspondingly, immune cells possess various functions in metabolic stimulation of atherosclerosis development. Therefore, intense study needs to be performed to understand the interdependence of immune cell fate and metabolism since they are interconnected at cellular, molecular, organism and organ level (Figure 2.3) (Park & Lee, 2019).

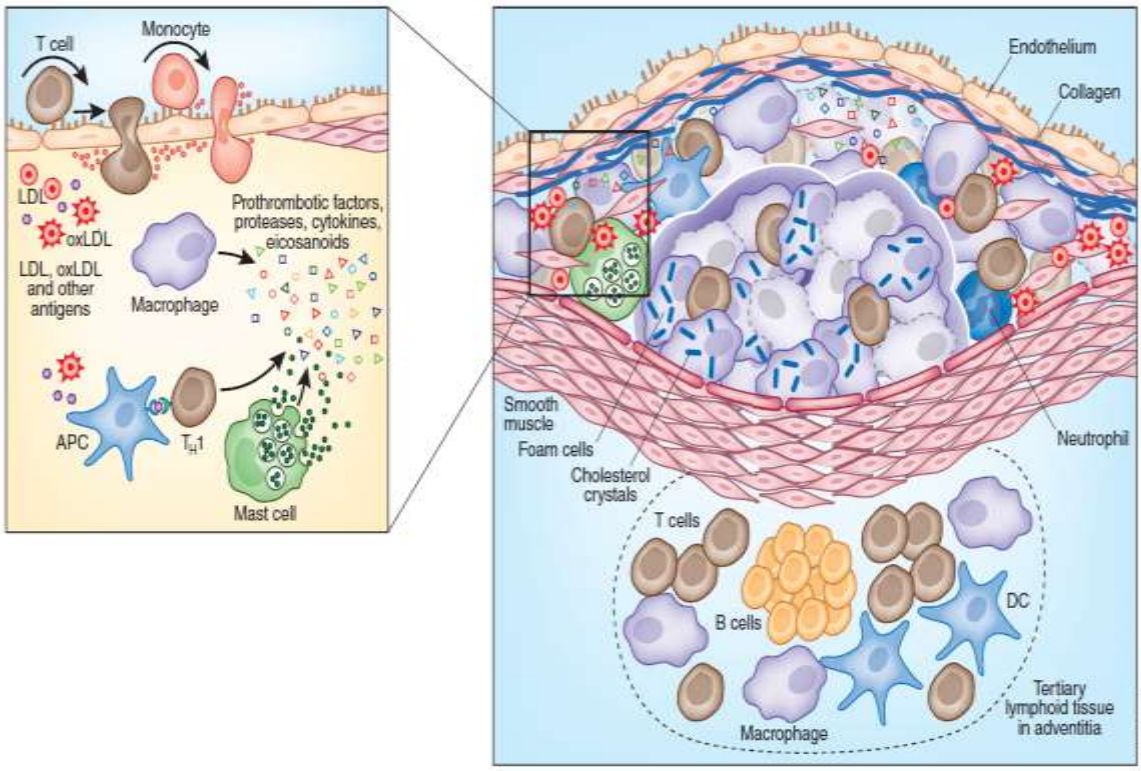

Figure 2.3: Immune cells in atherosclerotic plaque. Core of the atheroma in intima which compose of lipids, cholesterol crystals, active and apoptotic cells with a fibrous cap of SMCs and collagen. Atheroma comprises of numerous types of immune cells such as macrophages, T cells, SMCs, mast cells and DCs. OxLDL deposits in the subendothelial space of intima (Hansson & Hermansson, 2011).

Monocyte-derived macrophages are the key component in all stages of the atherosclerosis development since macrophages being the most abundant immune cells in atherosclerotic plaque (Tabas *et al.*, 2015; Cochain *et al.*, 2018). Monocytes transformed into macrophages under the influence of M-CSF upon entry from circulation into intima of arterial wall. M-CSF induces higher expression of SRs that increases the cytokines and growth factors production of macrophages which aid for survival and co-mitogenic stimulus. Both human and experimental atherosclerotic plaques exhibit overexpression of M-CSF (Gleissner, 2012). The aggregation of lipid in the arterial intima leads to increase of the SRs expression such as CD36, SR-A1 and SR-A2, SR-BI, TLRs, subtypes of PRRs and LOX-1 which bind oxLDL such that

cholesteryl esters in cytoplasmic droplets (Kzhyshkowska *et al.*, 2012) (Figure 2.4). CD36 and SR-A receptors have the maximum affinity for oxLDL which accountable for up to 90% of uptake by macrophages. These lipid-laden macrophages identified as foam cells initiate the formation of atherosclerotic lesion which stimulate cellular signalling cascades that trigger inflammation that connect the innate and adaptive immune response during atherosclerosis. Stimulation by oxLDL also results in secretion of various pro-inflammatory and growth factors by macrophages that activate both $CD4^+$ T cells and $CD8^+$ T cells which involves in lesion progression and complications (Ilhan & Kalkanli, 2015).

Macrophages are known as plastic cells as they present in several phenotypes within the plaque and possess contradictory roles throughout the inflammation. These macrophage phenotype changes according to the local microenvironment within the plaque (Park & Lee, 2019). Several factors influence the polarization of macrophage phenotype switching such as growth factors, lipids and cytokines (Seneviratne *et al.*, 2012). These macrophages phenotype known as M1 and M2 type macrophages. M1 macrophages are more prone to inflammatory responses which involves in the plaque vulnerability induced by the actions of IFN-γ and lipopolysaccharide, while M2 macrophages are less inflammatory and responsible for the plaque stability by the activation of IL-4 or IL-13 (Gistera & Hansson, 2017). According to histological analysis, M1 macrophages shows lipid augmentation while M2 macrophages possess a reduced amount of lipids and located further away from the lipid core (Chinetti-Gbaguidi*et al.*, 2011). Consequently, the disproportion of M1 and M2 macrophages ratio results in the plaque instability (Park & Lee, 2019). Macrophage induce by oxidized phospholipids (Mox) is a novel subset that characterized by plenty of nuclear factor erythroid 2-related factor 2 (NRF2)-mediated redox-regulatory genes together

with decreased chemotactic and phagocytic capacities. Advanced atherosclerotic plaque contains 30% of Mox macrophages (Kadl *et al.*, 2010).

Macrophages also capture oxLDL via several receptors including LOX-1 which stimulate foam cell formation (Figure 2.4). LOX-1 is a type II integral membrane glycoprotein comprising of a short N-terminal cytoplasmic domain, a transmembrane domain, a neck region, which controls receptor oligomerization, and an extracellular C-type lectin-like extracellular domain, involved in ligand binding (Pirillo *et al.*, 2013). LOX-1 serve as the primary receptor of oxLDL uptake in ECs (Sawamura *et al.*, 2012). The stimulated LOX-1 by oxLDL causes endothelial activation and dysfunction through reduced endothelium-dependent relaxation and augmented monocyte adhesion to ECs along with senescence and apoptosis of ECs (Xu *et al.*, 2013). LOX-1 initiates redox sensitive nuclear factor-kappa B (NF-κB) signalling pathway, a primary regulator for enhanced expression of numerous adhesion molecules which leads to adhesion of monocytes to ECs (Chen *et al.*, 2011). Several factors such as oxLDL, proinflammatory cytokines, high-glucose levels and lipoprotein lipase, upregulates LOX-1 expression in macrophages (Xu *et al.*, 2012). This suggests that LOX-1 plays a vital role in oxLDL uptake by macrophages in inflamed microenvironments which comprises of plentiful proinflammatory cytokines (Xu *et al.*, 2012). Histology analysis have shown the participation of LOX-1 in the weakening unstable atherosclerotic plaques. Study on Watanabe heritable hyperlipidaemic (WHHL) rabbit showed that advanced plaque possesses LOX-1 with thin fibrous cap and macrophage-rich lipid core. MMP expression, decrease in collagen content and apoptosis of SMC are the factors contributing LOX-1 modulation in plaque instability (Xu *et al.*, 2013).

Macrophages remove the excessive lipid by transporting out cholesterol that resides within the cell and through foam cells efflux via ATP-binding cassette

transporter A family member 1 (ABCA-1), ATP-binding cassette sub-family G member 1 (ABCG-1) and scavenger receptor class B type 1 (SR-B1) (Figure 2.4) (Yvan-Charvet *et al.*, 2010). ABCA-1 responsible for promoting intracellular cholesterol and phospholipids to apolipoprotein A1 (apoA-I), a component of high-density lipoproteins (HDL) (Moore *et al.*, 2013). ApoA-I is originally produced and secreted in liver which promptly deals with liver ABCA-1 but then some apo-I travels to the periphery and interact with ABCA-1 on cholesterol loaded cells which is mainly macrophages. The ABCA-1 bound apoA-I quickly obtains free cholesterol and phospholipids, becoming partially lapidated, and the matured HDL distributes cholesteryl esters to liver after bind to SR-B1 to excrete as bile. SR-B1 is a receptor of HDL which responsible for the transferences of cholesteryl esters into hepatocytes (Yvan-Charvet *et al.*, 2010).

ABCA-1 reverse the lipids from inner to outer membrane through the ATPase-dependent process by forming a channel in the membrane (Oram, 2003; Tang *et al.*, 2017). ABCA-1 protect the cells by integrating excessive free cholesterol to the endoplasmic reticulum that may interrupt the peptide biosynthetic machinery. OxLDL and cell debris immersed by macrophages serve as the primary source of cholesterol that undergo the reverse cholesterol transport pathway thru peripheral ABCA-1. Meanwhile, dietary and lipoproteins transported through chylomicron and LDL receptors to liver being the key source of liver ABCA-1 secreted cholesterol. These lipids processed by the liver involves in effective biliary secretion in the form HDL particles (Yvan-Charvet *et al.*, 2010). Upregulation of ABCA-1 expression hinders foam cell formation in arterial macrophages that leads to increase in liver ABCA-1 activity that raise the HDL level, thus augment the various atheroprotective roles of this lipoprotein subclass (Koldamova *et al.*,2014; Wang & Tontonoz, 2018).

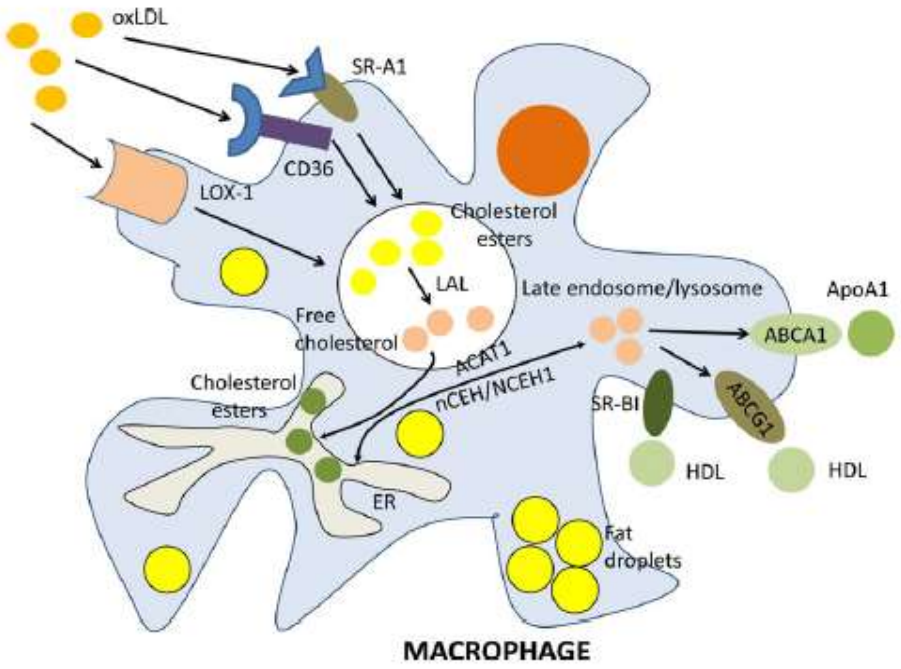

Figure 2.4: Cholesterol metabolism in macrophages. Lipid homoeostasis interruption in macrophages causes cholesterol build-up and development of foam cells. Macrophages take up oxLDL through LOX-1. Cholesterol esters release free cholesterol from macrophages by ABCA-1 and SR-BI. HDL and apoA-1 are the main acceptors of free cholesterol of SR-B1 and ABCA-1 respectively (Chistiakov *et al*., 2016).

2.1.4(a) Dendritic cells

Steinman and Cohn discovered that dendritic cells (DCs), an antigen-presenting cells (APCs) that is capable to integrate between the innate and adaptive immune responses by capturing, processing and presenting peptides to T cells and responsible for primary and secondary immune responses (Cohn & Steinman, 1973; Chistiakov *et al*., 2014) (Figure 2.5). DCs involves in innate immune system by secreting protecting cytokines upon receiving the danger indications while in adaptive immune system, DCs identify and respond to hazardous by provoking the progress of primary immune responses suitable for the nature of threat. DCs are capable of activating T cells including naive, memory and effector T cells through the effective antigen-presenting capacity along with accountable for natural killer T (NKT) cells stimulation. DCs also

play a role in maintenance of tolerance towards antigens (Merad *et al.*, 2013). DCs interacts with T cells in response to the peptide present on major histocompatibility complex (MHC) class II and class I molecules complex on DCs surfaces throughout the progress of adaptive immune responses. Costimulatory molecules such as CD80 and CD86 are essential during DCs and T cells contacts for T cell stimulation and differentiation into effector cells. T cells can undergo apoptosis or anergy state during DCs and T cells interaction due to absence of costimulatory molecules signals (Sanchez *et al.*, 2012; Merad *et al.*, 2013). DCs produce a wide range of cytokines such as IL-12, IL-23 and IL-10. These cytokines help DCs regulation in differentiation of naive T cells into Th1, Th2, Th17 cells or T regulatory (Treg) cells (Figure 2.5) (Merad *et al.*, 2013).

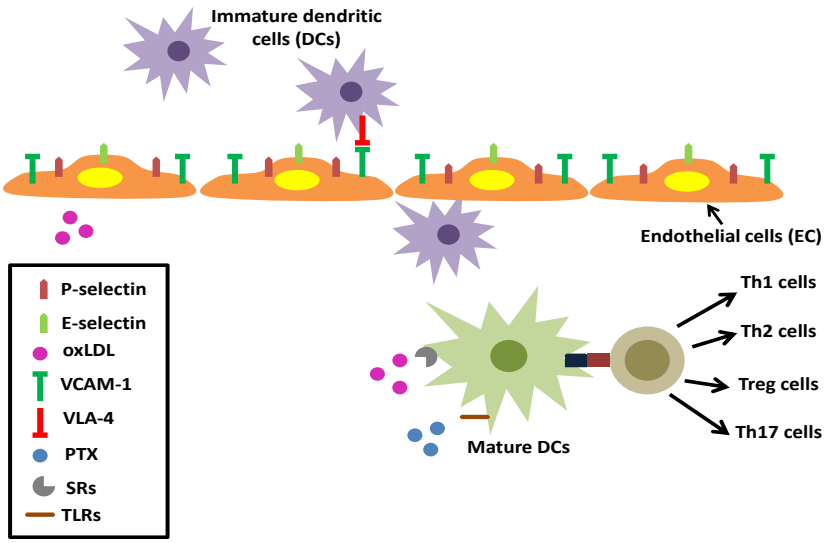

Figure 2.5: Recruitment of DCs into atherosclerotic plaques and differentiation of T-cell subsets. The activation of ECs by oxLDL triggers adhesion molecules that enable the migration of DCs into atherosclerotic plaques. oxLDL uptake by DCs causes maturation of DCs that present peptides to naive T cells which leads to their differentiation into Th1, Th2, Treg and Th17 cells. (Merad *et al.*, 2013).

DCs originated from $CD34^+$ progenitor in the bone marrow and the precursors leave bone marrow to circulate into the bloodstream which resides in various peripheral tissues to trigger T cells activation. The period of the precursors circulates in bloodstream differ as it dependent on targeted anatomical localizations. The DCs