IDENTIFICATION OF A CD36 SCAVENGER RECEPTOR BINDING PEPTIDE USING A 12-MER PEPTIDE M13 PHAGE DISPLAY LIBRARY

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by

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I dedicate this thesis to my beloved mother, Puan Khoriah and supportive father, Encik Ayob for always give me cares, loves, concerns, supports, prayers and motivations on whatever I'm doing in my life. Heartfell thanks to all my siblings for always being there for me and encouraged me all these while.

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LIST OF ABBREVATIONS

AFL	Antigen Fragment Libraries
AGE	Advance Glycation End
AHA	American Heart Association
AMPK	Activate Protein Kinase
BSA	Bovine Serum Albumin
CAD	Coronary Artery Disease
CCR	Chemokine Receptor
CD36	Cluster of Differentiation 36
CHD	Coronary Heart Disease
CHO-CD36	Chinese Hamster Ovary-CD36
CLESH	CD36 LIMP II Emp Structural Homology
CPP	Cell Penetrating Peptide
CRP	C-Reactive Protein
CTB	Subunit of Cholera Toxin
CVD	Cardiovascular Disease
DC	Dendritic Cell
dsDNA	Double Stranded DNA
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
DPP-4	Dipeptidyl Peptidase 4
EC	Endothelial Cell
ECM	Extracellular Matrix
EDTA	Ehtylenediaminetetraacetic Acid

ELISA	Enzyme-linked Immnosorbent Assay
FAT	Fatty Acid Translocase
FABPc	Cytoplasmic Fatty Acid Binding Protein
Ff	Filamentous phage
FITC	Fluorescein Isothiocyanate
FBS	Fetal Bovine Serum
GHRPs	Growth Hormone Releasing Peptides
GLP-1	Glucagon-like Peptide 1
HDL	High Density Lipoprotein
HOC	Higly Immunogenic Outer Capsid
HPM	Histidine-Proline-Methionine
HRGP	Histidine-rice Glycoprotein
HRP	Horseradish Peroxidase
HSP	Heat Shock Protein
ICAM-1	Intracellular Adhesion Molecule-1
IFN	Interferon
IDL	Intermediate-Density Lipoprotein
IL	Interleukin
IPTG	Isopropyl β-D-1-Thiogalactopyranoside
JNK	Jun N-Terminal Kinase
LOX-1	Lectin-type Oxidised LDL receptor-1
LPS	Lipopolysaccharide
MCP-1	Monocyte Chemo attractant Protein-1
MAPK	Mitogen-Activated Protein Kinase
MMP	Matrix Metalloproteinase

MXT	Methotrexate
OD	Optical Density
oxLDL	Oxidised Low Density Lipoprotein
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
PE	Phycoerythrin
PFU	Plaque Forming Unit
PPARγ	Peroxisome Proliferator-Activated Receptor gamma
RPMI	Roswell Park Memorial Institute
SMC	Smooth Muscle Cell
TMB	3,3'5,5'-Tetramethylbenzidine
TNF	Tumor necrosis Factor
TLR	Toll-like receptor
TSP-1	Thrombospondin-1

IDENTIFIKASI PEPTIDA MELEKAT RESEPTOR PENGAUT CD36 MENGGUNAKAN PAPARAN FAJ M13 PEPTIDA 12-MER

ABSTRAK

Reseptor pengaut CD36 tergolong dalam keluarga reseptor pengaut kelas B dan terlibat secara langsung dalam pengambilan lipoprotein teroksida. Pengumpulan lipid menyumbang kepada pembentukan sel busa, yang merupakan pencetus kepada plak aterosklerosis. Kajian ini bertujuan untuk mengenal pasti peptida faj CD36 kerana ia berpotensi untuk dibangunkan sebagai peptida perencat dan digunakan sebagai kaedah molekul untuk merawat masalah radang aterosklerosis. Dalam kajian ini, Ph.D-12™ Phage Display Peptide Library yang telah disahkan melalui pemilihan faj streptavidin, kemudiannya digunakan dalam pemilihan terhadap CD36. Ujian kebolehan mengikat CD36 dijalankan dengan menginkubasi klon faj yang telah dipilih dengan dua jenis sel yang mengekspres CD36, sel CHO-CD36 dan U937 dan satu jenis sel tanpa pengekspresan CD36, sel HeLa, diikuti dengan pelabelan dengan antibodi anti-faj M13-PE dan isotaip IgG2b-PE. Semua sel tersebut dianalisis menggunakan mesin *flow cytometer* BD FACS Calibur™ dan data yang diperoleh dianalisis menggunakan perisian FLOWJO 7.6.5. Ujian spesifikasi ikatan dijalankan menggunakan ELISA berasaskan sel. Klon faj diinkubasi dengan sel dan dilabel dengan antibodi anti-faj M13-HRP. TMB digunakan dalam pengesanan faj dan isyarat dibaca menggunakan pembaca mikroplat. Struktur sekunder faj CD36 dan interaksi peptida terhadap reseptor CD36 masing-masing diramalkan menggunakan perisian I-TASSER dan CABS-dock. Menerusi kajian ini, peptida faj dengan motif streptavidin, HPM, telah dipilih melalui pemilihan streptavidin, seterusnya mengesahkan kit yang telah digunakan. Pemilihan peptida CD36 menghasilkan klon faj tanpa sebarang motif

yang sama dengan motif peptida yang pernah diterbitkan apabila dianalisis menggunakan pangkalan data atas talian Target Unrelated Peptide Scanner (SAROTUP) dan Mimotope Database and Beyond (MimoDB). Analisis *flow cytometry* bagi ujian kebolehan mengikat menunjukkan 4 klon: klon B10, B14, B16 dan B7 mempunyai kebolehan mengikat yang lebih tinggi terhadap sel CHO-CD36 berbanding klon kawalan. Manakala, 3 klon: klon A15, B17 dan B22 mempunyai kebolehan mengikat yang lebih tinggi terhadap sel CHO-CD36 dan U937 berbanding klon kawalan. Ujian spesifikasi ikatan menggunakan ELISA menunjukkan klon B22 lebih spesifik terhadap sel CHO-CD36 berbanding klon kawalan. Analisis interaksi menunjukkan klon B22 berinteraksi dengan reseptor CD36 melalui 3 bahagian interaksi putatif yang utama pada reseptor. Kesimpulannya, peptida faj CD36 yang spesifik berjaya dikenal pasti dengan menggunakan teknik pemilihan paparan faj. Peptida faj CD36, klon B22 mempunyai keupayaan mengikat dengan spesifikasi yang tinggi terhadap sel CHO-CD36 berbanding klon kawalan. Lebih menarik lagi, B22 mengikat reseptor CD36 di satu tapak yang tidak pernah dilaporkan sebelum ini. Interaksi antara B22 dan CD36 yang novel ini menjadi satu pengetahuan asas untuk kajian di masa hadapan dalam menetukan rawatan yang berpotensi untuk radang atherosclerosis.

IDENTIFICATION OF A CD36 SCAVENGER RECEPTOR BINDING PEPTIDE USING A 12-MER PEPTIDE M13 PHAGE DISPLAY LIBRARY

ABSTRACT

CD36 is a class B scavenger receptor which is involved significantly in the uptake of oxidised low-density lipoprotein (oxLDL). Accumulation of lipid contributes to foam cells formation, which is the precursor to atherosclerosis plaque. The aim of this study was to identify specific peptides which interact and bind to CD36 as it has the potential to be developed into a peptide inhibitor and as a molecular intervention in treating athero-inflammatory disorders. In this study, 12-mer Ph.D-12^{TM} Phage Display Peptide Library, which was validated in streptavidin panning, was used in selection against CD36. CD36-binding assay was performed by incubating the selected phages with two CD36-expressing cell lines, CHO-CD36 and U937 and a CD36-non expressing cell line, HeLa, followed by anti-M13 phage-PE and mouse IgG2b-PE isotype antibodies staining. All stained cells were acquired on BD FACS Calibur^{TM} flow cytometer and analysed using FlowJo 7.6.5 software. Binding specificity was assayed using cell-based ELISA. The phage clones were incubated with the cells and stained with anti-M13-HRP. TMB was used in phage detection and the signal was read using a microplate reader. Secondary structure of CD36 phage and binding interaction of the peptide to CD36 receptor were predicted using I-TASSER and CABS-dock software, respectively. Phage bearing specific streptavidin motif, HPM, was selected from streptavidin panning and validated the library used in the selection. Fourth round of CD36 panning yielded unique phage clones that had no common motif with any published peptide when analysed using online database Target

Unrelated Peptide Scanner (SAROTUP) and Mimotope Database and Beyond (MimoDB). Flow cytometric analysis of phage binding assay showed four clones: clones B10, B14, B16 and B7 had higher binding ability toward CHO-CD36 cells compared to the control clone. In addition, three other clones: clone A15, B17 and B22 had greater binding ability towards CHO-CD36 and U937 cells compared to the control clone. ELISA binding specificity test showed that clone B22 had higher specificity towards CHO-CD36 cells compared to the control clone. Docking analysis showed B22 interacted with CD36 receptor through three putative major contacts on the receptor binding sites. In conclusion, specific CD36 peptide phage was successfully identified by using phage display selection technique. The CD36 phage, B22 had higher binding ability and specificity towards CHO-CD36 cells compared to the control clone. More interestingly, B22 binds to CD36 receptor at a site which had not been reported before. This novel interaction between B22 and CD36 serves as a basis for future experiments in identifying a potential intervention and treatment for athero-inflammatory disorders.

CHAPTER 1

1 INTRODUCTION AND LITERATURE REVIEW

1.1 Research overview

CD36 is a multi-ligand scavenger receptor which is expressed in various types of cells such as endothelial, adipocytes, epithelial, platelets, monocytes and macrophages. The receptor is responsible for several physiological processes including phagocytosis of apoptotic cells and microbial pathogens. It also plays a major role in pathological processes including angiogenesis, inflammation and atherosclerosis. CD36 binds to several ligands including *Plasmodium falciparum* malaria infected erythrocytes, long chain fatty acids and oxidised low density lipoprotein (oxLDL).

CD36 is able to bind oxLDL and causes the internalisation and uptake of oxLDL into macrophages. This reaction activates the transcription factor peroxisome proliferator-activated receptor gamma (PPARγ) which facilitates further uptake of oxLDL and enlarges pools of oxLDL. The interaction between CD36 and oxLDL induces secretion of pro-inflammatory cytokines and causes more immune cell infiltration in the arterial intima. Severe inflammation induces arterial narrowing or atherosclerosis, which result in cardiovascular disease (CVD) such as coronary heart disease. Therefore, manipulation of CD36 receptor is crucial in atherosclerosis intervention.

Identification of specific CD36 molecules using phage display selection offers new approach in treating atherosclerosis. Specific CD36 peptide can be developed into a peptide inhibitor that blocks the binding sites of oxLDL on the CD36 receptor. This in turn will prevent the oxLDL internalisation into macrophages, inhibit foam cells formation and reduce the risks of atherosclerosis.

1.2 What is scavenger receptor CD36?

CD36 receptor was first isolated and characterised from the platelet membrane (Tandon et al., 1989b) and was originally known as platelet integral membrane glycoprotein IV (Tandon et al., 1989a; Pepino et al., 2014), an adhesion receptor for thrombospondin-1 (TSP-1) (Dawson et al., 1997; Silverstein and Febbraio, 2009). Recently, CD36 was classified as scavenger receptor-B2 (SR-B2) (Prabhudas et al., 2014). CD36 is expressed in various cell types including macrophages and monocytes, adipocytes, platelet, epithelial cells in the kidney, cardiac myocytes (Febbraio et al., 2001), microvasculature endothelial (Swerlick et al., 1992) and smooth muscle cells (Matsumoto et al., 2000).

CD36 is involved in many physiological processes including clearance of apoptotic cells (Ferracini et al., 2013), phagocytosis of microbial pathogens (Baranova et al., 2008) and viral infection (Cooper et al., 2016), uptake of long chain fatty acids (Koonen et al., 2005) as well as regulation of angiogenesis (Osz et al., 2014). CD36 is also involved in several pathological process such as inflammation (Erdman et al., 2009), pathogenesis of malaria (Gowda et al., 2013), pathogenesis of metabolic disorder such as diabetes and obesity (Kennedy and Kashyap, 2011), atherosclerosis (Park, 2014) and thrombosis (Nergiz-Unal et al., 2011).

CD36 is a multi-ligand receptor which binds to several ligands including oxLDL (Endemann et al., 1993), long chain fatty acids (Nicholson et al., 1995), anionic phospholipids (Ryeom et al., 1996), apoptotic cells (Ren et al., 1995), Advanced Glycation End (AGE) product (Ohgami et al., 2001) and growth hormone releasing peptide (GHRPs), Hexarelin (Demers et al., 2004). It also serves as an adhesion receptor for collagen (Leung et al., 1992) and *plasmodium falciparum*

malaria infected erythrocytes (Febbraio et al., 1999) and shed photoreceptor outer segments (Savill et al., 1992).

1.2.1 Molecular structure of CD36

Human CD36 gene is located on chromosome 7 at q11.2 locus (Fernandez-Ruiz et al., 1993). There are 15 exons in CD36 gene, however, exons 1, 2 and 15 are non-coding. N-terminal and C-terminal domains are encoded by exons 3 and 14 (Armesilla and Vega, 1994). As shown in Figure 1.1, 5'-untranslated regions (5'-UTR) consists of exons 1a, 1b, 1c, 1e, 1f, 2 and part of exons 3 while the remaining segment of exon 14 and exon 15 make up the 3'-untranslated region (3-UTR) (Armesilla and Vega, 1994; Rac et al., 2007).

CD36 receptor contains one short intracellular domain at both N- and C-terminal, two trans-membrane domains and one large extracellular domain (Rac et al., 2007). The extracellular region which is rich in N-linked glycosylation sites and bears a hydrophobic domains (184-204) could potentially interact with the plasma membrane, a proline rich region (242-333) (Greenwalt et al., 1992; Lauzier et al., 2011).

Several binding site specific for CD36 ligands are located at the extracellular domain. Each of these ligands bind to CD36 receptor at specific domains, as shown in Figure 1.2. Domain 28-93 and 120-155 are responsible for binding to oxLDL (Puente Navazo et al., 1996; Pearce et al., 1998). Domain 155-183 is responsible for growth GHRPs, hexarelin (Navazo et al., 1996) and AGE (Ohgami et al., 2001). Domain 93-120 is responsible for TSP-1 and -2 (Frieda et al., 1995; Yamashita et al., 2007).

Domains 155-183 and 120-155 which are referred as CLESH (CD36 LIMP-II Emp sequence homology) are implicated in binding and endocytosis of apoptotic neutrophils (Navazo et al., 1996). This endocytosis happens in the present of integrin

complex αvβ3 and the CD36 ligand, TSP (Savill et al., 1992; Yamashita et al., 2007). In addition to that, two domains, domain 146-164 or 145-171 mediate the binding to *P. falciparum* erythrocytes membrane protein-1, which is specifically expressed by *P falciparum* infected erythrocytes (Baruch et al., 1999).

CD36 contains 472 amino acids. The expected molecular mass is 53 kDa, however because of post translational protein modification, the apparent molecular mass is 78-88 kDa (Tang et al., 1994; Martin et al., 2007). Acylation occurs at residues 1-6 and 461-472 of the intracellular domain and residues 7-28 and 439-460 of the trans-membrane domain. There are seven glycosylation sites (Hoosdally et al., 2009), a phosphorylation site (Lauzier et al., 2011) and disulphide bridges are present on extracellular domain (Rasmussen et al., 1998). There are also ubiquitination sites at residue Lys 469 and Lys 472 on the C-terminal (Smith et al., 2008).

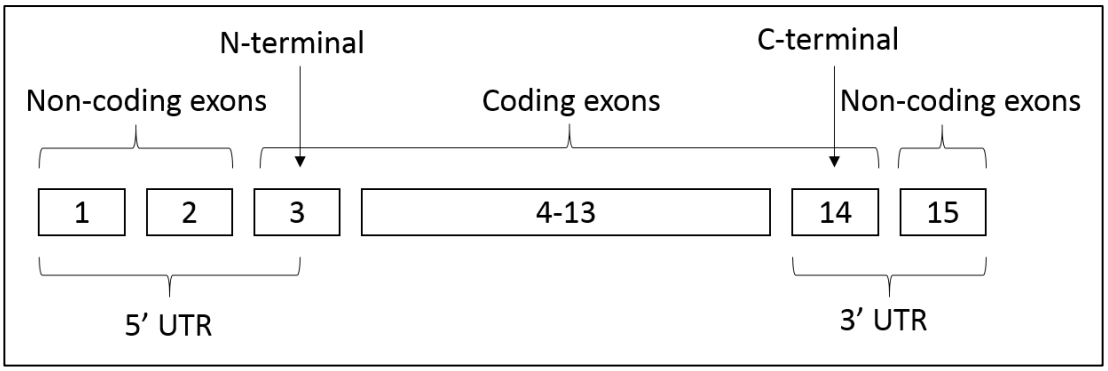

Figure 1.1: Genomic distribution of CD36. It consist of 15 exons. Exons 1, 2 and 15 are non-coding while exons 3 to 14 are coding regions. Exon 3 and exon 14 encodes for N- and C-terminal, respectively. 5'-untranslated region consists of exons 1, 2 and part of exon 3 while 3'-untranslated region consists of exons 14 and 15. This figure was adapted from Rac et al., 2007.

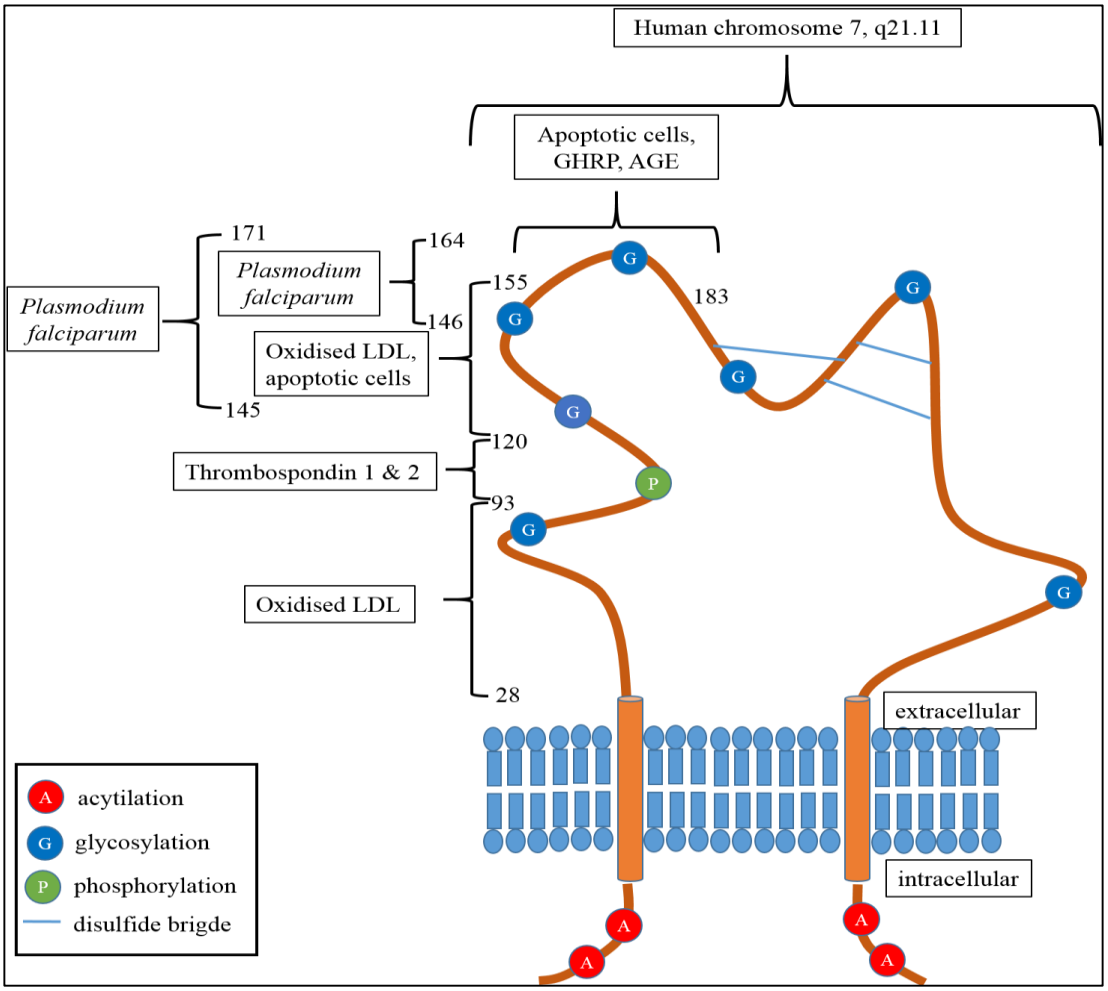

Figure 1.2: Ligands for CD36 receptor. CD36 receptor domain bind to several ligands such as oxLDL and thrombospondin. Several protein post-translational modification sites including glycosylation also present on CD36 receptor domain. This figure was adapted from Lauzier et al., 2011 and Yamashita et al., 2007.

1.2.2 Function of CD36

1.2.2(a) CD36 is involved in phagocytosis of pathogens and apoptotic cells

CD36 scavenger receptor function was first identified by Savil et al when he investigated the recognition of apoptotic cells by mononuclear phagocytes (Savill et al., 1991) which involved several receptor systems including CD36 in cooperation with $\alpha_v\beta_3$ integrin on the phagocyte surface (Savill et al., 1992).

CD36 uptakes and phagocytoses various bacterium including *Escherichia coli, Klebsiella pneumoniae, Salmonella typhiurium*, *S. aureus* and *Enterococcus faecalis*. CD36 is responsible in a TLR2/4 independent manner when induced by Gram-negative bacteria and lipopolysaccharide (LPS) via a Jun N-terminal kinases (JNK)-mediated signalling pathway (Baranova et al., 2008).

1.2.2(b) CD36 as a fatty acid translocase (FAT)

Long chain fatty acid is a nutritional compound that serves various roles in the body. The main function of fatty acid is for cellular energy production, formation of phospholipids membrane and acts in selected transduction events such as during protein phosphorylation (Glatz and Luiken, 2015).

Studies on fatty acid uptake into isolated adipocytes revealed the FAT to be the rat homologue of CD36. CD36 facilitates the fatty acid uptake in adipocytes (Abumrad et al., 1993; Pohl et al., 2005), heart and skeletal muscles and intestine (Drover et al., 2008). CD36 that is overexpressed in skeletal muscles causes a decrease in circulating fatty acids and triacylglycerols, enhances fatty acid oxidation and decreases fat deposition (Ibrahimi et al., 1999; Koonen et al., 2005). Studies done in CD36 knock-out mice showed reduction in fatty acid uptake rates in heart (-50 to -80%), skeletal muscle (-40 to -75%) and adipose tissue (-60 to -70%) (Coburn et al., 2000).

At the extracellular site, CD36 promotes the partitioning of the fatty acids into the outer leaflet of the lipid bilayer. Then, fatty acids translocate from outer to the inner leaflet of the membrane without assistance from the membrane proteins (Pohl et al., 2005; Hamilton, 2007).

At the inner sites of the membrane, fatty acids bind to the cytoplasmic fatty acid binding protein (FABPc). CD36 provides a docking site for FABPc or for enzymes that act on fatty acids such as acyl-CoA synthetase. CD36 organises the fatty acids within specific membrane domains to make it readily available for transport. At the extracellular sites, CD36 interacts with FABPc to facilitate cellular fatty acid uptake. A study showed transfection of CD36 in a rat heart muscle cell line without FABPc does not increase the fatty acid uptake (Van Nieuwenhoven et al., 1998), suggesting that CD36 only functions in the presence of FABPc.

1.2.2(c) CD36 is involved in regulation of angiogenesis

Tumor suppressor genes from hamster cells that have the ability to control the inhibition of angiogenesis were found to be similar to TSP-1. This raises the possibility that TSP-1 acts as a target molecule in restrainings tumor growth (Good et al., 1990). Further studies showed that CD36 in microvascular endothelial cells mediates the inhibition of angiogenesis in response to TSP-1. This demonstrated that endothelial CD36 may be essential for the inhibition of angiogenesis by TSP-1 (Dawson et al., 1997). CD36 also mediates anti-angiogenic activity through TSP-2 by activating a specific signalling cascade that causes pro-angiogenic response (Silverstein and Febbraio, 2007).

Histidine-rich glycoprotein (HRGP) binds with high affinity to TSP-1. HRGP co-localises with TSP-1 in the stroma of human breast cancer and masks the anti-angiogenic epitope of TSP-1. Thus, it can modulate the anti-angiogenic effect of TSP-

1 (Simantov et al., 2001). HRGP contains a CD36 homology domain and blocks the anti-angiogenic activities of TSP, thus promoting angiogenesis (Silverstein and Febbraio, 2007).

The interactions between TSP-1 and CD36 exert an anti-angiogenic response. CD36 in endothelial cells (ECs) associates with non-receptor protein tyrosine kinase fyn, lyn and yes. Interaction of TSP-1 towards TSP-1 results to the recruitment of fyn to the CD36 membrane complex. This results in the activation of kinase and subsequent p38 mitogen-activated protein kinase (MAPK). Other than that, induction of EC apoptosis via caspase-3-like effector is also linked to the antiangiogenic activity of TSP-1 (Jimenez et al., 2000).

1.2.2(d) CD36 is involved in inflammation

Since CD36 is highly expressed in several types of immune cells such as macrophages which are responsible for internalising a variety of particles, it also plays an important role in inflammatory responses to many of these ligands. The inflammatory responses results in the association of several type of diseases such as atherosclerosis, malaria, obesity, lung injury, brain injury, insulin resistance as well as stroke (Febbraio et al., 2001). CD36 interacts with several type of receptors such as TLR to promote inflammatory responses as well as phagocytosis. This leads to the production of several pro-inflammatory cytokines by macrophages (Lee et al., 2012).

CD36 was first shown to interact with TLR2 and TLR6 heterodimers. The cooperation of CD36 with these TLR promote tumor necrosis factor (TNF) production and enhance immune responses. TLR2 is required for recognition of numerous bacteria components, fungi and protozoa (Hoebe et al., 2005). CD36, through TLR4 and TLR6 mediates inflammatory response to oxLDL and amyloid-β fibrils. Macrophages which

have a deficiency of CD36 together with TLR4 and TLR6, are unable to promote inflammation against oxLDL and amyloid-β fibrils respectively (Stewart et al., 2010).

CD36 mediates phagocytosis and internalisation of *plasmodium falciparum* malaria-parasitised erythrocytes (PEs), which activates the TLRs. However, there are no secretion of cytokines from the primary macrophages in responses to TLR2. This indicate that CD36 cooperate with other TRLs to promote cytokine responses. Therefore, it can be concluded that the internalisation of PE by CD36 is not dependent on the TLR signalling and are non-inflammatory. However, internalisation of PE by CD36 can be enhanced by interactions with other TLRs to promote cytokine production (Erdman et al., 2009). The non-inflammatory response of PE internalisation by CD36 is more likely to resemble the uptake of apoptotic cells rather than bacteria. Thus, reducing the host inflammatory response can decelerate the PE clearance.

1.3 Atherosclerosis

Atherosclerosis is a worldwide disease where the fatty acid substances known as plaque builds up and accumulates inside the arteries and causes the arteries to become obstructed. Over time, the plaque which is made up of fat, cholesterol, calcium and other substances found in the blood, hardens and narrows the arteries. The blockage can restrict the blood flow and limit the flow of oxygen-rich blood to organs such as the heart, brain and other parts of the body. These events lead to serious cardiovascular diseases (CVD) such as coronary artery disease (CAD), coronary heart disease (CHD), peripheral arterial disease and cerebrovascular disease.

1.3.1 Epidemiology of Atherosclerosis

According to World Health Organisation (WHO), CVD remains the number one, non-communicable disease cause of death globally followed by cancer,

respiratory diseases and diabetes in year 2017. It was estimated that CVD caused 17.7 million people to die in 2015 which represented 31% of all death globally. From this number, 7.4 million deaths were due to CHD and 6.7 million were due to stroke (World Health Organisation 2017).

WHO also reported that, CAD caused the highest death rate in Ukraine and Russian Federation which was 718 and 654 deaths per 100 000 population respectively while the lowest death rate was in South Korea and Japan with 36.5 and 47.0 deaths per 100 000 respectively. Locally, based on the report of WHO, CAD caused 22, 158 deaths in 2010 and the number increased to 24, 900 deaths in 2012 or about one fifth of all death. 498 deaths per 100 000 population were reported in 2015. This made CAD the most common cause of death as it contributed to 20.1% of all deaths (World Health Organisation 2017).

National Cardiovascular Disease database (NCVD) reported that Malaysians obtained ACS at a young age mean between 55.9 to 59.1 years compared to developed countries where the mean age was between 63.4 to 68 years (Lu and Nordin, 2013). In 2006 to 2010, it was found that among 13,591 ACS patients, 24.2% were women and had more risk factors and higher mortality (Lu et al., 2014).

1.3.2 Risk factor of atherosclerosis

Risk factors of atherosclerosis are divided into two types which is life styles and health conditions. Having a lifestyle which included smoking (Yang et al., 2017), unhealthy diet (Fernstrom et al., 2017) and alcohol consumption (Patel et al., 2017) could result in atherosclerosis. Several conditions in the blood such as high cholesterol (Varbo and Nordestgaard, 2016), low density lipoprotein (LDL) (Lin et al., 2016), low level of high density lipoprotein (HDL) (Ahmed et al., 2016), insulin resistance

(Yamazoe et al., 2016), hyperglycemia (high blood sugar) (Tanaka et al., 2014), high blood pressure and obesity could also lead to atherosclerosis.

There are also other risk factors such as sleep apnea, old age (Johnson et al., 2017), hypertension (Korsager Larsen and Matchkov, 2016), and family history of heart disease (Khera and Kathiresan, 2017). In addition, there are several emerging risk factors that can cause atherosclerosis related diseases such as C-reactive protein (CRP), fibrinogen, and homocysteine (Hackam and Anand, 2003; Gupta et al., 2013).

In Malaysia, WHO reported that 11.6% of adults have raised blood glucose, 28.8% have raised blood pressure, 10.4% are obese and 43% of adult males are smokers. Data from National Health and Morbidity Survey (NHMS) stated that 32.7% were found to be hypertensive, 15.2% had diabetes and 35.1% had hypercholesterolemia among 18,231 adults at aged 18 years diagnosed in 2011 (World Health Organisation 2017).

1.3.2(a) Life style

Smoking has been reported to increase the incidence of CAD and myocardial infarction in male and female (Centers for Disease and Prevention, 2002). Cigarette smoke is associated with the reduction of nitric oxide (NO) availability which is primarily responsible for the vasodilatory function of the endothelium (Ota et al., 1997; Ambrose and Barua, 2004).

Cigarette smoke also increases the level of several inflammatory markers including CRP, interleukin-6 (IL-6) and Tκα (Bermudez et al., 2002). It can also affect the lipid profile by elevating the serum cholesterol, triglyceride and LDL levels, and decreasing the HDL levels (Craig et al., 1989; Ambrose and Barua, 2004). Smoking increases the oxidation of LDL which is actively taken up by macrophages to form foam-cells (Heitzer et al., 1996; Ambrose and Barua, 2004).

The dietary components of a person are important to the development, prevention and treatment of cardiovascular diseases. Alternate Healthy Eating Index (AHEI) scores were associated with significant reductions of cardiovascular disease risk in men and women (McCullough et al., 2002). An unhealthy diet such as higher intake of fat, red meat and carbohydrate and less intake of fruits and vegetables would extend the coronary artery lesions in CAD patients.

Practising a healthy life style such as not smoking, not consuming alcohol and consuming a balanced diet would prevent the majority of coronary diseases in women (Stampfer et al., 2000). Several physical activities such as walking, running or swimming would prevent the development of CAD and would reduce symptoms associated to the establishment of cardiovascular disease (Thompson, 2003).

1.3.2(b) Cholesterol

HDL, LDL, and several types of apolipoproteins such as apo A-1 and apo B are the dependent risk factors for atherosclerosis. Low levels of HDL and elevated levels of lipoprotein triglyceride contributed to the elevated cardiovascular risk (Chapman et al., 2011). Atherogenic lipoprotein are divided into three categories which are very low-density lipoprotein, LDL and intermediate-density lipoprotein (IDL). It is also sub-classified into phenotype A, large LDL particles, phenotype B, small LDL particles and intermediated phenotype (Austin et al., 1990; Carmena et al., 2004). Oxidised LDL and small, dense LDL are more atherogenic compared to buoyant LDL particles.

LDL particle size, which is smaller than 25.6 nm showed significant increase around 2.2 fold of ischemic heart disease compare to LDL particles which are larger than 25.6 nm in men (Lamarche et al., 2001). Apo B-100 is the major component of atherogenic lipoprotein and is used for estimating coronary risk. Since apo B is a

stronger risk predictor compared to LDL, apo B is more important marker in the diagnostic and treatment of patient with low levels of LDL (Walldius et al., 2001).

1.3.2(c) Hyperglycemia

Hyperglycemia, condition where the blood glucose level is high, is the hallmark sign of diabetes type 1 and type 2. A normal person blood glucose level between 70-100 mg/dL. Clinical studies showed that hyperglycemia promotes cardiovascular events in type 2 diabetes (Brown et al., 2010; Mazzone, 2010). Once the glucose level increases, the body will produce insulin to trigger a response to inhibit the body from using fat for energy production.

This results in the glucose from the bloodstream being used to make energy. However, insulin resistance can occur where the body fails to respond to the insulin which will result in higher levels of glucose in the blood. Clinical evidence have showed that insulin resistance promote CAD in the absence of hyperglycemia (DeFronzo, 2010) which suggests that insulin resistance is independent risk factor for atherosclerosis.

1.3.2(d) Inflammation

Inflammation is the central aspect of atherosclerosis and it triggers the vascular events by mediating plaque instability. During inflammation, CRP is synthesised in the liver by stimulation of IL-6 and other pro-inflammatory cytokines (Libby, 2002). CRP is responsible for the prediction of events or mortality rate in patients with ischemic stroke (van Exel et al., 2002), acute coronary syndrome (Heeschen et al., 2000) and peripheral vascular disease (Ridker et al., 2001).

CRP expression is elevated in plaque and promotes the uptake of low-density lipoprotein by macrophages. It also up-regulates the intracellular adhesion molecule

by endothelial, thus promoting the recruitment of monocytes to plaque sites (Zwaka et al., 2001).

1.3.3 Pathogenesis of atherosclerosis

In atherosclerosis, the accumulation of oxLDL and apoliprotein B in the endothelial cellular layer leads to the recruitment of immune cells such as monocytes-derived macrophages (Williams and Tabas, 1995; Moore and Tabas, 2011). Recruitment of oxLDL through CD36 triggers the signalling cascades for inflammatory responses (Park, 2014). Accumulation of T-cells and monocytes-derived macrophages forms foam cells loaded with lipids known as early fatty streak lesion.

Lesion which consists of foam cell and necrotic core replaces the fibrous cap on the fibro atheromatous plaques (Yla-Herttuala et al., 2011). This leads to plaque rupture and formation of thrombus. Atherosclerosis is characterised by several events including activation and dysfunction of endothelial, inflammation of vascular, accumulation of modified lipid and formation of inflammatory cells in plaques form within vascular wall.

1.3.3(a) Activation and dysfunction of endothelial

Injured endothelial caused by exposure to metabolic or chemical stress including cigarettes smoke and diabetes mellitus lead to endothelial dysfunction. Plaque formations found at specific sites of endothelial results in endoplasmic reticulum stress which occurs with decreased athero-protective nitric oxide (NO) and increased superoxide production. Nitric acids produced by the endothelial nitric oxide synthase (NOS) are potentially atheroprotective and has vasolidator functions (Glass and Witztum, 2001). However, rapid interaction between NO and superoxide produces perovynitrite (ONOO-) that lead to the damage of proteins and lipids. Although peroxidase is needed as a signalling molecule in vascular, excess production is highly

pathological and linked to atherosclerosis This increase the endothelial permeability and lipid accumulation in sub-endothelial cells (Sima et al., 2009).

The endothelial cells become activated in response to the oxLDL. LDL which is made up of phospholipids, is composed of fatty acids bound to a glycerol backbone with a polar head group, and is susceptible to free-radical enzymatic oxidation including reactive oxygen and nitrogen species. Enzymes present in vessel walls such as myeloperoxidase, lipoxygenase and other related enzymes generate reactive oxygen and nitrogen species (Hansson, 2001). One of the oxLDL receptor in endothelial dysfunction is lectin-type oxidised LDL receptor-1 (LOX-1). LOX-1 induces endothelial dysfunction by several mechanisms and induces pro-inflammatory cytokines (Xu et al., 2013). LOX-1 is highly expressed in endothelial dysfunction during early and advanced stages of atherosclerotic plaques. It is also expressed in smooth muscle cells and macrophages, suggesting that LOX-1 plays a role in endothelial activation and foam cell formation (Hansson, 2005).

1.3.3(b) Leukocytes recruitment

Endothelial activation causes induction of several cell surface adhesion molecules which mediates the attraction and attachment of leukocytes (monocytes and T cell). One of the adhesion molecules, monocyte chemo attractant protein-1 (MCP-1) is a chemotactic protein for monocytes. The production of MCP-1 requires the activation of MAPK by oxLDL (Li and Mehta, 2000). MCP-1 potently attracts both monocytes and T-cell and plays vital role in recruitment of these cells.

Activation of endothelial in response to oxLDL also up-regulates other chemokines including IL-8, which plays a role in monocytes-macrophages trafficking, chemokine (C-X-C) ligands 2 and 3 (Mattaliano et al., 2009). OxLDL also significantly increases E-selectin, P-selectin, vascular cell adhesion molecule-1

(VCAM-1) as well as intracellular adhesion molecules-1 (ICAM-1) expression which contributes to the recruitment of leukocytes to the atherosclerotic lesions (Hansson, 2005). OxLDL also activates nuclear factor kappa B (NF-κB) which modulates expression of pro-inflammatory genes including cytokines, adhesion molecules and chemokines (Zhu et al., 2005).

1.3.3(c) Macrophages CD36 mediates foam cell formation

Foam cell formation is the critical step of atherosclerosis. Within the intima, blood-circulating monocytes transmigrates into the arterial intima. At this site, monocytes differentiate into macrophages due to the presence of differentiation factors such as macrophage colony-stimulating factors (Sordet et al., 2002). At the early stage of atherosclerotic lesions, the majority of monocytes become cells with macrophages and/or dendritic cell-like structure features. Macrophages internalises the atherogenic lipoprotein, oxLDL via scavenger receptor CD36 (Park, 2014) and scavenger receptor-A (SR-A) (Ricci et al., 2004). Oxidation of LDL promotes the specific ligands for nuclear hormone receptor PPAR-γ. This will up-regulate the expression of CD36 and further facilitate the uptake of oxLDL.

Binding of macrophages to oxLDL via CD36 induces several transcriptional changes including up-regulation of CD36 expression. Following oxLDL uptake by macrophages via CD36, transcription factor PPARγ is transactivated via a p38 MAPK-dependent pathway (Zhao et al., 2002). It also heterodimerises with retinoid X receptor (RXR). Interaction of RXR to PPAR response element in the CD36 promoter induces CD36 expression (Tontonoz et al., 1998). CD36 up-regulation mediated by oxLDL involves the initial activation of protein kinase C (PKC) (Feng et al., 2000) and protein kinase B (PKB) (Munteanu et al., 2006) dependent pathway with subsequent PPARγ activation.

Over-expression of PKB in macrophages is stimulated by CD36 promoter and PPARγ element-driven reporter gene. CD36 up-regulation by oxLDL is necessary for the differentiation of macrophages into foam cells (Rahaman et al., 2006). PPARγ also up-regulates CD36 expression in vascular smooth muscle cell (SMC) which is responsible for differentiation of SMC into foam cells (Lim et al., 2006). OxLDL stimulation cause the secretion of inflammatory cytokines including TNFα/β, IL-1β, IL-6 and interferon beta and gamma (IFNβ/γ) (Janabi et al., 2000). In addition, apoptosis occurs in macrophages and foam cells induced by oxLDL via a mechanism that is dependent on CD36 (Wintergerst et al., 2000).

OxLDL activates eosinophils which up-regulates pro-inflammatory cytokines and down-regulates anti-inflammatory cytokines such as IL-4. Activation of eosinophils and CRP promotes pro-inflammatory M1 macrophages polarisation and down-regulation of M2 macrophages phenotype (Devaraj and Jialal, 2011). Activation of macrophages will secrete myeloperoxidase which oxidises more LDL, thus enlarging the pool of oxLDL (Carr et al., 2000). The interaction between CD36 and oxLDL induces secretion of several cytokines that causes infiltration of immune cell in the arterial intima (Jiang et al., 2012). This leads to foam cells formation and arterial inflammation which induces narrowing of arterial as shown in Figure 1.3.

CD36 signalling cascade also activates NF-κβ, induces IL-1β and TNF-α (Yamashita et al., 2007). Internalisation of oxLDL via CD36 is mediated by a member of the small GTPase family, IRGM1 which is highly expressed by macrophages (Xia et al., 2013). Other receptors such as TLR 2 also enhances the CD36-mediated uptake of oxLDL (Erdman et al., 2009).

Macrophages CD36 interact with oxLDL induce the phosphorylation of lyn and subsequently activate JNK 1 and 2. The uptake of oxLDL is mediated by JNK

activation (Rahaman et al., 2006) and the interaction also activates other MAPK such as p38 and ERK ½ (Zhao et al., 2002). CD36 signalling cascade is mediated by Vav, guanine nucleotide exchange factor. OxLDL induces the activation of macrophages by Vav. Inhibition of Vav-interacting protein, dynamin blocks the uptake of oxLDL (Rahaman et al., 2011).

Several animal studies have shown that genetic deletion of CD36, which blocks the CD36 function in the signalling pathway, is able to reduce the atherosclerosis lesion formation. This can be seen in a study where in CD36-null mice, the uptake of oxLDL by macrophages are disrupted or defective (Febbraio et al., 2000). CD36-null mice including mice with *ApoE* null or *LDL receptor* null genotype showed less atherosclerotic lesion formation compared to *ApoE* null or *LDL receptor* null mice on high-fat diet (Guy et al., 2007; Kuchibhotla et al., 2008).

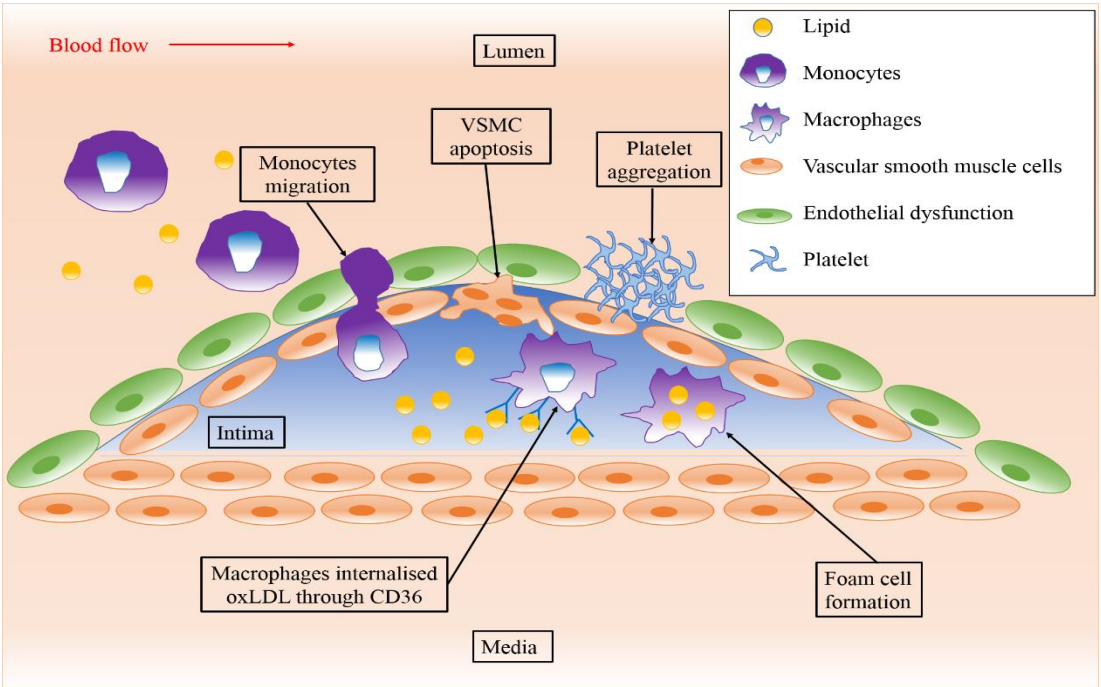

Figure 1.3: Pathogenesis of atherosclerosis. Monocytes transmigrate from lumen into intima and causes endothelial cell dysfunction. Then, monocytes differentiate into macrophages and internalise oxLDL through CD36 receptor causing formation of foam cells. Vascular smooth muscle cells undergo apoptosis due to collagen degradation. This promotes plaque rupture and platelet aggregation. This figure was adapted from Park, 2014.

1.3.3(d) Plaque stabilisation and atherosclerotic lesion

In atherosclerosis, inflammation does not resolve spontaneously like acute inflammation which is resolved by the emigration of immune cells. Atherosclerotic inflammation causes macrophages to be trapped in the lesions. There are clear mechanism on how oxLDL is responsible for inhibition of macrophages emigration *in-vitro* and *in-vivo* (Park et al., 2009; Park et al., 2012). Macrophage cell polarity is one of the reasons of macrophage trapping. Macrophages loss its polarity due to activation of Vav/Rac pathway that is induced by oxLDL. This leads to the subsequent activation of non-muscle myosin II (Park et al., 2012).

Although the macrophages are trapped inside the lesion, they do not remain static due to the behaviour of different cell types including endothelial cells, SMC, macrophages as well as platelets. Plaques beneath the fibrous cap of extracellular matrix and vascular smooth muscle cells do not change the external arterial diameter, and this causes narrowing of the lumen. Plaque remain stable as long as the endothelium and fibrous cap are intact and become unstable once the cellular composition and structure rapidly change due to the presence of large lipid core and thin fibrous cap (Moore and Tabas, 2011).

Large lipid core is formed from due to macrophages being trapped inside the lesion. Fibrous cap becomes thin due to several cellular factors. Decrease of collagen synthesised by SMC contributes to the collagen degradation, and promotes plaque rupture. Macrophages triggers apoptosis by activating Fas apoptosis pathway, secretion of pro-apoptotic TNF-α and nitric oxide (Boyle et al., 2003).

Macrophages secrete matrix metalloproteinases (MMPs) enzymes which degrade various type of extracellular matrix (ECM) proteins, thus contributing to the thin fibrous cap (Schneider et al., 2008). There are also several macrophages-derived

serine protease such as cysteine protease that is responsible for degradation of elastin and collagen (Liu et al., 2004).

Plaque rupture leads to thrombus formation. Blood comes into contact with highly thrombogenic core and causes platelet aggregation. CD36 activates platelets to induce expression of P-selectin and activation of integrin $\alpha_{2b}\beta_3$ (Podrez et al., 2007). Platelet becomes hyperactive and activate signalling pathway that mediates oxLDL/CD36-induced platelet activation (Zimman et al., 2014). Rapid expansion of plaque and subsequent thrombus formation leads to acute coronary syndrome and myocardial infarction.

1.3.4 Current treatments of atherosclerosis

Since there are a lot of risk factors that contributes to atherosclerosis, treatment of atherosclerosis includes several strategies such as lipid lowering drugs, anti-inflammatory drugs, peptide vaccines and peptide inhibitor. Each strategy has its own advantages and disadvantages.

1.3.4(a) Lipid lowering drug

Recently, lipid lowering drugs such as statin successfully showed anti-inflammatory property when delivered to atherosclerotic plaque using nanoparticle carrier, reconstituted high density lipoprotein (rHDL) (Duivenvoorden et al., 2014). Statin was able to provide primary and secondary prevention of atherosclerosis. However, several side effects of statin were identified including contribution to the coronary artery calcification and mitochondrial toxin accumulation that impaired muscle function in heart and blood vessels (Okuyama et al., 2015).

1.3.4(b) Anti-inflammatory drug

Several emerging therapeutic approaches were identified including Darapladib and methotrexate (MXT), and are both in clinical trial phase three. Darapladib is able

to significantly reduce major coronary artery events in stable coronary heart disease but not in acute coronary heart disease (O'Donoghue et al., 2014). MXT reduces intima media thickness (IMT) in patients with rheumatoid arthritis (RA) compared to the RA patient who did not receive MXT.

1.3.4(c) Peptide vaccine

Recently, several vaccination strategies in the form of peptide were established including apo B-100 peptide vaccine, heat shock protein (HSP) peptide vaccine and dendritic cells vaccine.

Immunisation of atherosclerotic mice with major histocompatibility complex (MHC) class II restricted to 16 mer peptide derived from apo B-100 was able to significantly reduce atherosclerotic plaque and was able to induce CD4+ T regulatory cell, chemokine receptor 5 (CCR5) and secrete significant amounts of IL-10, suggesting the ability of this peptide to give atheroprotective effect against atherosclerosis (Kimura et al., 2017).

Peptide derived from bacterial HSP 60 was used to immunise apo E deficient mice orally. HSP60 peptide was able to decrease lesion size, increase splenic T regulatory cell number and increase secretion of IL-10 mRNA levels in the aorta. This proved that HSP60 could be a potential candidate for vaccine development against atherosclerosis (Grundtman et al., 2015).

Since dendritic cells (DC) is an antigen presenting cell, DCs were manipulated to provoke tolerance responses. Dendritic cells vaccine was achieved by loading the DC with specific antigen such as apo B-100 antigen, and transferred onto naive recipients. DC that had been pulsed with apo B-100 protein antigen were intravenously injected into low density lipoprotein deficient mice in combination with immunosuppresive cytokine, IL-10. DC-pulsed apo B-100 peptide was able to

significantly reduce 70% of atherosclerotic lesion in aorta. It also inhibited the production of interferon-γ and decreased CD4+ T cell infiltration. Therefore, DC pulse apo B-100 peptide is a potential condidate to reduce autoimmune response against low density lipoprotein and in treatment of atheroslecrosis (Hermansson et al., 2011).

1.3.4(d) Peptide inhibitor

Several peptide inhibitors for atherosclerosis were identified which inhibited specific proteins or receptors such as glucagon-like peptide (GLP-1), MMP, dipeptidyl peptidas-4 (DPP-4), apo B-100 and apo AI.

GLP-1 such as exendin-4 and liraglutide significantly suppressed the oxLDL-induced foam cell formation in human monocytes-derived macrophages while only liraglutide significantly retarded the atherosclerotic lesion and suppressed foam cell formation in macrophages of apolipoprotein E-deficient mice. (Tashiro et al., 2014). Liraglutide also inhibitfd the induction of vascular smooth muscle proliferation by activation of activated protein kinase (AMPK) signalling as well as inducing cell cycle arrest, thus delay the progression of atherosclerosis (Jojima et al., 2017). However, Thazhath showed a slightly different result as he reported that exendin-4 increased arterial blood pressure and heart rate of diabetes type 2 patients (Thazhath et al., 2017).

MMP-9 and MMP-3 were expressed in atherosclerosis plaque of human patients undergoing peripheral revascularisation (Orbe et al., 2003). Less atherosclerotic burden was seen in double knockout mice which had deficiency in apolipoprotein and MMP-9 but not in MMP-12 knockout mice. This suggests that MMP-9 plays a major role in plaque growth (Luttun et al., 2004). Phosphinic peptide (RXP470.1) derived from murine MMP-12 inhbitor significantly reduced atherosclerotic plaque cross-sectional area by 50% in apolipoprotein E knockout mice.

This finding suggests that MMP-12 peptide inhibitor is able to delay athreosclerosis development (Johnson et al., 2011).

DPP-4 inhibitor, Anagliptin is able to suppress the DPP-4 activity and increase the GLP-1 and GIP levels upon anagliptin treatment. Anagliptin also decreased the lesion formation in coronary arteries of Japanese white rabbits and reduced the expression of proinflammatory cytokines, tumor necrosis factor-alpha and interleukin-6 in carotid arteries (Hirano et al., 2016).

Several studies showed that Apo B-100 peptide antigen inhibits atherosclerosis. Fusion protein of B subunit of cholera toxin (CTB) with apoB-100 peptide (p210) were used to immunise apoE deficient mice via nasal administration. After 12 weeks, aortic lesion size was reduced, regulatory T cells was induced to suppress effector T cells and number of interleukin 10 (IL-10) of $CD4^+$ T cells was increased (Klingenberg et al., 2010). ApoB-100 derived peptide without adjuvant subcutaneuosly infused to the same animal model reduced plaque formation associated with vascular inflammation reduction and increased collagen. It also regulated cell response associated with the reduction of both T helper cell-1 and T helper cell-2 (Herbin et al., 2012).

1.3.5 Commercially available CD36 peptide

Apart from the peptides used for treatment of atherosclerosis mentioned above, there are several commercialised CD36 peptides available. Synthetic CD36 blocking peptide such as CD36 blocking peptide from Abcam company, (domain 150-250 aa and 350-450 aa) (Abcam 2017) and from Cayman company (98-114aa) (Cayman Chemical 2017) , blocks the non-specific interaction of non-target antigen to the antibody epitope for molecular application such as western blot analysis,

immunohistochemistry, ELISA, flow cytometry and other antibody based applications.

Other CD36 peptides such as CD36 peptide P (93-110) and (139-155) were used as stimulator or inhibitor in CD36-TSP interactions. These peptides function in a two-step process of ligand receptor interaction. CD36 sequences of 139-155 represents a part of OMK5, monoclonal antibody-CD36 antibody epitopes. Application of P139-155 enhances the binding of CD36 to TSP while P93-100 blocks binding of CD36 to TSP. However, in the presence of P139-155, P39-100 is able to bind to CD36 with higher affinity (Leung et al., 1992).

The identification of specific peptides against any molecule can be done by using phage display selection technique. By using this techniques, specific CD36 peptide can be screened and identified.

1.4 Phage display

Phage display technology describes a selection technique by which a foreign polypeptide is fused to the capsid protein on lysogenic filamentous bacteriophages (Bazan et al., 2012a; Molek et al., 2011). Phage display is an extremely powerful tool for selection of peptides or proteins such as novel enzyme substrates and inhibitors (Skerra, 2007) and polypeptides with novel function and selection of new antibodies (Gronwall and Stahl, 2009).

Phage display technology provides a physical linkage between phenotype (the displayed peptide) and genotype (the encoding DNA). During screening process, binding clones are separated from non-binding clones by affinity purification. Bio-panning is the selection process by which the display library is incubated with an immobilised target, followed by an extensive washing to remove unspecific phages. Binders are eluted using acid, high salt or specific target ligand and enriched by