EFFECT OF MURINE NOROVIRUS-3 (MNV-3) INFECTION ON ATHEROSCLEROSIS DEVELOPMENT IN MACROPHAGE CELL LINES AND C57BL/6 MICE

MUHAMMAD HAYATUDDEEN RUMAH

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by

MUHAMMAD HAYATUDDEEN RUMAH

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It is with humility that I dedicate this thesis to my late parents, Alhaji Muhammad Abubakar Rumah and Asmau Muhammad Rumah, whose endless love, encouragement, support, and prayers have guided me through every stage of my life. May the Almighty Allah, the Sustainer of the universe, forgive their shortcomings and grant them Jannatul Firdaus.

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ABCA-1	ATP-binding cassette transporter-1
APC	Antigen-presenting cells
ApoB	Apolipoprotein B
$ApoE^{-/-}$	Apolipoprotein E deficient mice
BMDC	Bone marrow dendritic cells
C57BL/6	C57 black 6 mice
CD11b	Cluster of differentiation 11b
CD36	Cluster of Differentiation 36
CD88	Cluster of Differentiation 88
cDNA	complementary DNA
CO2	Carbon dioxide
CPE	Cytopathic effect
CVD	Cardiovascular disease
DCs	Dendritic cells
DMEM	Dulbecco's Modified Eagle Medium
ECs	Endothelial cells
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence-Activated Cell Sorting
FBS	Fetal Bovine Serum
FITC	Fluorescein isothiocyanate
Foxp3	Foxhead transcription factor
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase

H&E	Haematoxylin and Eosin
HCL	Hydrochloride acid
HDL	High-density lipoproteins
HFD	High-fat diet
HIV	Human immunodeficiency virus
HRP	Horseradish peroxidase
HuNV	Human norovirus
IL	Interleukin
$Ldlr^{-/-}$	Low-density lipoprotein receptor mice
M-CSF	Macrophages colony-stimulating factor
MFI	Median fluorescence intensity
MHC	Major histocompatibility complex
MNV	Murine norovirus
MOI	Multiplicity of infection
ND	Normal diet
NFW	Nuclease free water
NO	Nitric oxide
ORO	Oil red O
oxLDL	Oxidized Low-density lipoprotein
P. gingivalis	Porphyromonas gingivalis
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
Pen Strep	Penicillin streptomycin
PMA	Phorbol 12-myristate-13-acetate
qPCR	Quantitative real Time-Polymerase chain reaction

RNA	Ribonucleic acid
RORyt	Retinoid-related orphan receptor gamma
RPMI-1640	Roswell Park Memorial Institute-1640
SMCs	Smooth muscle cells
STAT	Signal transducer activator of transcription
T-bet	T-box expressed in T cells
TC	Total cholesterol
TCID50	50% tissue culture infective dose
TCR	T-cell receptor
TGF	Transforming growth factor
Th	T-helper cells
TLR	Toll-like receptor
TMB	Tetramethylbenzidine
TNF	Tumor nuclear factor
Treg	Regulatory T cells
UV	Ultraviolet

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KESAN JANGKITAN NOROVIRUS MENCIT-3 (MNV-3) TERHADAP PERKEMBANGAN ATEROSKLEROSIS DALAM TURUNAN SEL MAKROFAJ DAN MENCIT C57BL/6

ABSTRAK

Aterosklerosis, penyakit keradangan kronik, dicirikan oleh pengumpulan lipid di dinding arteri dan dikawal oleh tindak balas imun semula jadi dan adaptif. Peranan penting dalam permulaan dan perkembangan penyakit ini dimainkan oleh makrofaj dan pelbagai subset sel T. Kesan jangkitan murine norovirus-3 (MNV-3) terhadap perkembangan aterosklerosis telah disiasat dalam kajian ini menggunakan model *in vitro* dan *in vivo*. Bagi model *in vitro*, aterosklerosis diaruh oleh lipoprotein berketumpatan rendah-teroksida (oxLDL), manakala bagi model *in vivo*, diet kaya kolesterol digunakan untuk mengaruh aterosklerosis dalam mencit C57BL/6. Dalam eksperimen *in vitro*, pengurangan aterosklerosis diperhatikan dalam sel RAW 264.7-pra-jangkit MNV-3 disebabkan oleh penurunan tahap kolesterol total (TC), rembesan sitokin pro-radang (TNF-α, IL-6, IL-1β), dan tahap ekspresi protein permukaan makrofaj (CD88, CD36, CD11b). Sebaliknya, MNV-3 didapati mempromosikan aterosklerosis dalam sel RAW 264.7-pasca-jangkit (dirawat awal dengan oxLDL) dengan meningkatkan tahap TC, meningkatkan rembesan TNF-α, IL-6, IL-1β, dan mempromosikan tahap ekspresi CD88, CD36, CD11b. Akhirnya, tiada perubahan ketara dalam aterosklerosis diperhatikan dalam sel RAW 264.7-jangkit dan rawat bersama, kerana tahap TC, rembesan TNF-α, IL-6, IL-1β, dan tahap ekspresi CD88, CD36, CD11b tidak terjejas dengan ketara. Bagi makrofaj yang berasal dari THP-1, MNV-3 mempromosikan aterosklerosis dengan meningkatkan rembesan TNF-α, IL-6, IL-1β dan mempromosikan tahap ekspresi CD88, CD36, CD11b. Eksperimen *in*

vitro lanjut mendedahkan bahawa MNV-3 dalam sel RAW 264.7 yang dirawat awal dengan oxLDL meningkatkan pembezaan sel T CD4+ naif menjadi subset Th1, Th2, dan Th17 dengan meningkatkan T-bet, GATA-3, dan RORγt, sambil tidak menjejaskan Foxp3 (Treg). Ia juga meningkatkan ekspresi CD88, CD36, dan CD11b serta meningkatkan TNF-α, IL-6, IL-1β, sambil mengurangkan rembesan TGF-β. Dalam kajian *in vivo*, didapati bahawa MNV-3 mengubah profil lipid dalam serum tikus C57BL/6 yang diberi diet tinggi lemak (HFD) dengan meningkatkan lipoprotein berketumpatan rendah (LDL) dan kolesterol total (TC), dan menurunkan trigliserida (TG) dan lipoprotein berketumpatan tinggi (HDL) semasa aterosklerosis. MNV-3 juga didapati meningkatkan tahap mRNA CD88, CD36, dan CD11b dalam tikus yang diberi HFD. Selain itu, jangkitan MNV-3 mengubah keseimbangan sel Th17/Treg dalam splenosit tikus yang diberi HFD, yang menunjukkan peningkatan ketara dalam saiz plak aterosklerotik. Secara keseluruhan, kajian ini telah mendedahkan kesan pengawalseliaan MNV-3 dalam perkembangan aterosklerosis dengan mempengaruhi penanda makrofaj dan sel T CD4+. Walau bagaimanapun, penyiasatan lanjut mengenai mekanistik virus dan implikasi yang lebih luas adalah diperlukan untuk menguruskan aterosklerosis. Selain itu, penghapusan MNV-3 dalam fasiliti penyelidikan haiwan adalah penting untuk mengelakkan perubahan yang tidak diingini dalam hasil penyelidikan yang berkaitan dengan penyakit keradangan manusia menggunakan model mencit.

EFFECT OF MURINE NOROVIRUS-3 (MNV-3) INFECTION ON ATHEROSCLEROSIS DEVELOPMENT IN MACROPHAGE CELL LINES AND C57BL/6 MICE

ABSTRACT

Atherosclerosis, a chronic inflammatory disease, is characterized by lipid build-up in the arterial wall and regulated by innate and adaptive immune responses. Significant roles in the disease's onset and progression are played by macrophages and various T-cell subsets. The effect of murine noroviruses-3 (MNV-3) infection on atherosclerosis development was investigated in this study using *in vitro* and *in vivo* models. In the *in vitro* model, atherosclerosis was induced by oxLDL, whereas in the *in vivo* model, a cholesterol-rich diet was used to induce atherosclerosis in C57BL/6 mice. In the *in vitro* experiment, a reduction in atherosclerosis was observed in pre-infected RAW 264.7 cells due to decreased TCE , pro-inflammatory cytokine secretion (TNF-α, IL-6, IL-1β), and macrophage surface protein expression (CD88, CD36, CD11b) levels. Conversely, MNV-3 infection promoted atherosclerosis in post-infected RAW 264.7 cells by elevating TCE levels, boosting TNF-α, IL-6, IL-1β secretions, and promoting CD88, CD36, CD11b expression levels. Lastly, no significant alteration in atherosclerosis was observed in co-infected RAW 264.7 cells, as TCE levels, TNF-α, IL-6, IL-1β secretions, and CD88, CD36, CD11b expression levels were not significantly affected. In THP-1 derived macrophages, MNV-3 promoted atherosclerosis by boosting TNF-α, IL-6, IL-1β secretions and promoting CD88, CD36, CD11b expression levels. Further *in vitro* experiment revealed that MNV-3 in oxLDL-pre-treated RAW 264.7 cells enhanced the differentiation of naïve CD4+ T cells into Th1, Th2, and Th17 subsets by upregulating T-bet, GATA-3, and

RORγt, while not affecting Foxp3 (Treg). It also increased CD88, CD36, and CD11b expression and boosted TNF-α, IL-6, IL-1β, while reducing TGF-β secretions. In the *in vivo* study, MNV-3 was found to alter lipid profiles in the serum of C57BL/6 mice fed with a high-fat diet (HFD) by increasing low-density lipoprotein (LDL) and total cholesterol (TC), and lowering triglycerides (TG) and high-density lipoprotein (HDL) during atherosclerosis. MNV-3 also was found to increase the mRNA levels of CD88, CD36, and CD11b in HFD-fed mice. Furthermore, MNV-3 infection altered the Th17/Treg cell imbalance in the splenocytes of HFD-fed mice, suggestive of leading to a significant increase in atherosclerotic plaque size. Overall, this study has unveiled the regulatory effect of MNV-3 in atherosclerosis development by impacting macrophage and $CD4^+$ T cell markers. However, further investigations into the virus's mechanistic impact and its broader implications are warranted for managing atherosclerosis. Additionally, the elimination of MNV-3 in animal research settings is essential to prevent unintended alterations in research outcomes related to murine models of human inflammatory diseases.

CHAPTER 1

GENERAL INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

Atherosclerosis stands as a non-communicable, chronic inflammatory vascular disease capable of impacting various organs such as the heart, kidneys, legs, pelvis, arm, and brain. Arterial disease presents in various forms depending on the arteries impacted, including Coronary Artery Disease (CAD), Peripheral Artery Disease (PAD), Carotid Artery Disease, Renal Artery Stenosis, Vertebral Artery Disease, or Mesenteric Artery Ischemia. In the context of atherosclerosis progression, there exists a correlation with infection, attributed to the inflammatory reactions in response to pathogen infiltration (Shah, 2019). The progression of atherosclerosis may be influenced by bacterial and viral agents, employing both direct and indirect mechanisms (Campbell and Rosenfeld, 2015).

Norovirus is the leading cause of gastroenteritis globally, with prolonged infections in immunocompromised individuals (Haessler and Granowitz, 2013). Murine norovirus (MNV) serves as a key research model due to challenges in cultivating human norovirus (Ettayebi et al., 2016). MNV targets macrophages and dendritic cells and alters cytokine secretion in infected macrophages, which are key in atherosclerosis development (Hsu et al., 2015). Studies indicate MNV variable impact on atherosclerosis plaque development. Understanding how immune cells like macrophages, dendritic cells, and lymphocytes influence atherosclerosis and MNV's role in foam cell formation is crucial to advancing knowledge of atherosclerosis pathophysiology.

1.2 Cardiovascular disease

Cardiovascular disease (CVD) is the leading global cause of death, accounting for about 35% of all deaths, with 17.6 million fatalities in 2016 and projections rising to 23.6 million by 2030 (Benjamin et al., 2019). In Malaysia, heart disease led to 18.4% of deaths in 2022, followed by pneumonia, cerebrovascular diseases, COVID-19, and road accidents. Atherosclerosis, driven by chronic inflammation and lipid accumulation, leads to arterial narrowing, obstructing blood flow, and increasing CVD risk (Frostegård, 2013; Gistera and Hansson, 2017). In the vascular environment, cellular interactions and changed lipids lead to chronic inflammation as a result of the delicate interplay between innate and adaptive immune responses (Cekici et al., 2014). This condition, influenced by both innate and adaptive immune responses, plays a crucial role in CVD's progression and thrombotic complications (Hansson and Hermansson, 2011; Miteva et al., 2018).

1.3 Atherosclerosis

Atherosclerosis is a major cause of death in Western societies, often remaining asymptomatic until plaque rupture or artery blockage occurs (Hansson and Libby, 2006). The disease involves immune cells, lipoproteins, and vascular cells interacting to promote chronic inflammation, ultimately leading to cardiovascular disease (Bentzon et al., 2014; Garrido-Urbani et al., 2014). Atherosclerotic plaques evolve through stages from fatty streaks to complex lesions, featuring a mix of inflammatory cells, calcified regions, and necrotic cores, which narrow the arterial lumen, causing ischemia and stenosis (Gaudio et al., 2006; Hansson and Hermansson, 2011).

1.3.1 Arterial structure

Normal arteries have three layers namely, tunica intima, media, and adventitia. The monolayer of endothelial cells envelops the tunica intima, establishing direct contact with the bloodstream, partitioned by a basement membrane. It serves as a barrier between circulating molecules and cells in the blood and regulates vascular homeostasis. Synthesis of signaling molecules by endothelial cells acts as a deterrent to platelet aggregation and adhesion, inhibits SMCs proliferation, and hampers leukocyte adhesion and migration. Alterations in endothelial cell structure and function are implicated in atherosclerosis development (Milutinović et al., 2020). In the human arterial intima, macrophages, endothelial cells, and SMCs are the principal cellular components. Additionally, there are isolated macrophages and sparse mast cells present in the intima (Susser and Rayner, 2022). The sub-endothelial layer within the intima is composed of two distinct layers. The inner layer, predominantly made up of a proteoglycan matrix, contains non-fibrous connective tissue, a limited amount of elastic fibers, and SMCs with both synthetic and contractile phenotypes Situated beneath the proteoglycan layer is the musculoelastic layer, a thicker stratum containing a substantial number of contractile phenotype SMCs and elastic fibers (Libby et al., 2011).

Upon exposure to growth regulatory molecules like growth factors, the proliferation of SMCs within the developing intima escalates through mitosis. Initially, the prevailing belief was that SMCs present in atherosclerotic lesions originated solely from the media. However, recent discoveries propose that bone marrow progenitor cells infiltrate the intima, potentially undergoing *in vivo* differentiation to give rise to SMCs (Han et al., 2009). SMCs in pro-atherogenic environments have the potential to differentiate into macrophage-like cells, demonstrating the ability to ingest lipids and

convert them into foam cells (Xiang et al., 2022). Collagen serves a pivotal function in upholding the integrity of endothelial cells by tethering them to the subendothelial matrix. The principal collagen types prevalent in the arterial wall are the interstitial collagens, specifically types I and III. Within the subendothelial region of the intima, type III collagen is specifically situated and may potentially be synthesized by endothelial cells. Increased concentrations of type I collagen might signal heightened metabolic activity of an expanded SMCs population within the intima, as evidenced by studies indicating that cultured SMCs can generate both type I and III collagen (Stary et al., 1992).

Often classified as part of the media, the internal elastic lamina delineates the boundary between the intima and media layers. At sites of vascular transitions, the internal elastic lamina may be lacking, resulting in the intima and media appearing as a cohesive unit. The tunica media, situated in the middle, represents the thickest layer of normal blood vessels. Comprising 20% SMCs of both contractile and synthetic types, and 60% collagen and elastin, it furnishes the vessel with contractile capabilities. Through proliferation and migration towards the subendothelial intima, medial SMCs may participate in atherosclerosis development. Serving as the outer layer, the tunica adventitia is distinguished by its sparse collagen fibrils, nerve endings, and vasa vasorum, which provide its own nutrient arterial supply. It is separated from the media by the external elastic lamina. Sparse fibroblasts and mast cells represent the principal cell types within the adventitia (Stary et al., 1992). This layer can modify its function and impact the advancement of atherosclerosis. Within human plaque adventitia, a lymphoid organ-like structure is present, primarily comprising B and T cells (Milutinović et al., 2020).

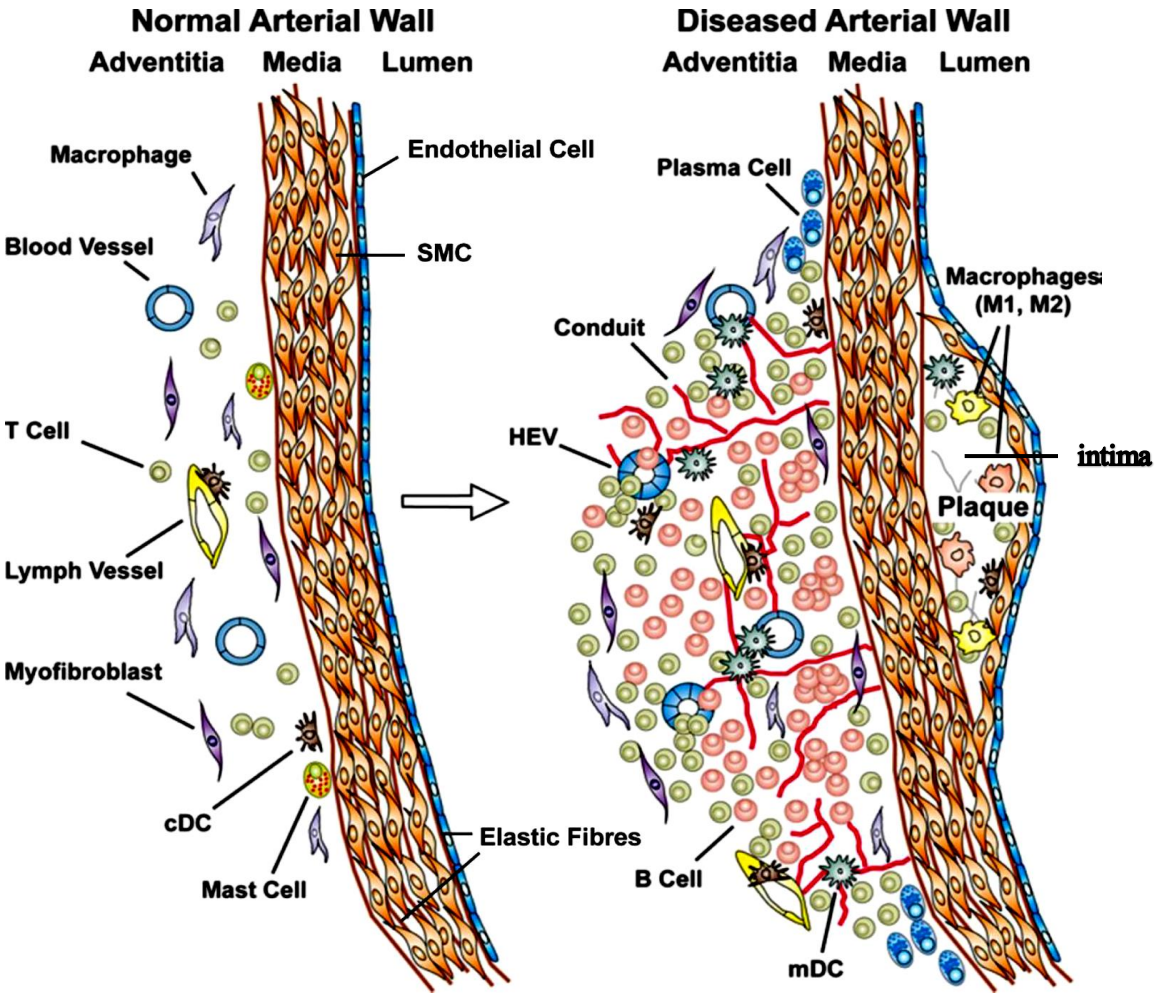

Figure 1.1: The diagrammatical representation of a healthy and diseased artery. Both healthy and diseased arteries consist of three layers: the tunica intima, tunica media, and tunica adventitia. The typical intima is characterized by a single layer of endothelial cells. The tunica media consists of numerous layers of SMCs. Below the monolayer of endothelial cells, a small number of T cells and regular dendritic cells are visible, whereas the adventitia contains mast cells, macrophages, T cells, and myofibroblasts (Mohanta et al., 2014).

1.3.2 Pathogenesis of Atherosclerosis

Atherosclerosis is a complex, multifactorial disease characterized by the accumulation of lipids, inflammatory cells, and fibrous elements in the large and medium-sized arteries, leading to plaque formation and vascular obstruction. The pathogenesis of atherosclerosis begins with chronic endothelial injury, which can be triggered by factors such as hypertension, smoking, diabetes, and elevated levels of LDL cholesterol. Endothelial cells, which line the inner surface of blood vessels, play a crucial role in maintaining vascular homeostasis. When damaged, these cells undergo functional alterations that disrupt their normal balance, increasing arterial wall permeability and allowing lipoproteins like LDL and VLDL to infiltrate the intima. Once inside, these lipoproteins undergo oxidative modification to form oxLDL, which induces the expression of adhesion molecules such as VCAM-1 and ICAM-1, facilitating the recruitment and adherence of leukocytes, primarily monocytes, to the endothelial surface (Singh et al., 2002; Linton et al., 2019).

Monocytes that adhere to the endothelial surface migrate into the subendothelial space, where they differentiate into macrophages. These macrophages engulf oxLDL through scavenger receptors, transforming into foam cells loaded with lipids, which are a hallmark of early atherosclerotic lesions known as fatty streaks. As foam cells accumulate, they release pro-inflammatory cytokines and chemokines that amplify the inflammatory response within the plaque by recruiting additional immune cells, including T cells. The inflammation within the intima perpetuates a cycle of monocyte recruitment, foam cell formation, and secretion of matrix metalloproteinases (MMPs) that degrade the extracellular matrix, contributing to the remodeling of the plaque structure (Rafieian-Kopaei et al., 2014; Lee-Rueckert et al., 2022).

As atherosclerosis progresses, SMCs from the arterial media migrate into the intima, proliferate, and synthesize extracellular matrix components such as collagen, elastin, and proteoglycans. These components form a fibrous cap over the lipid-rich necrotic core, stabilizing the plaque and preventing thrombogenic material from contacting circulating blood. However, SMCs can also undergo phenotypic switching, acquiring properties similar to macrophages, including the ability to engulf lipids and form foam cells, further contributing to plaque growth and instability. In advanced stages, plaques become more complex, containing necrotic cores, calcified regions, microvessels, and cholesterol crystals, which promote further growth and instability. Ultimately, the most dangerous stage occurs when plaques rupture or erode, exposing thrombogenic contents to the bloodstream and leading to the formation of an intraluminal thrombus that can obstruct blood flow, resulting in ischemic events such as myocardial infarctions and strokes (Loftus, 2011; Meng et al., 2016; Mehu et al., 2022).

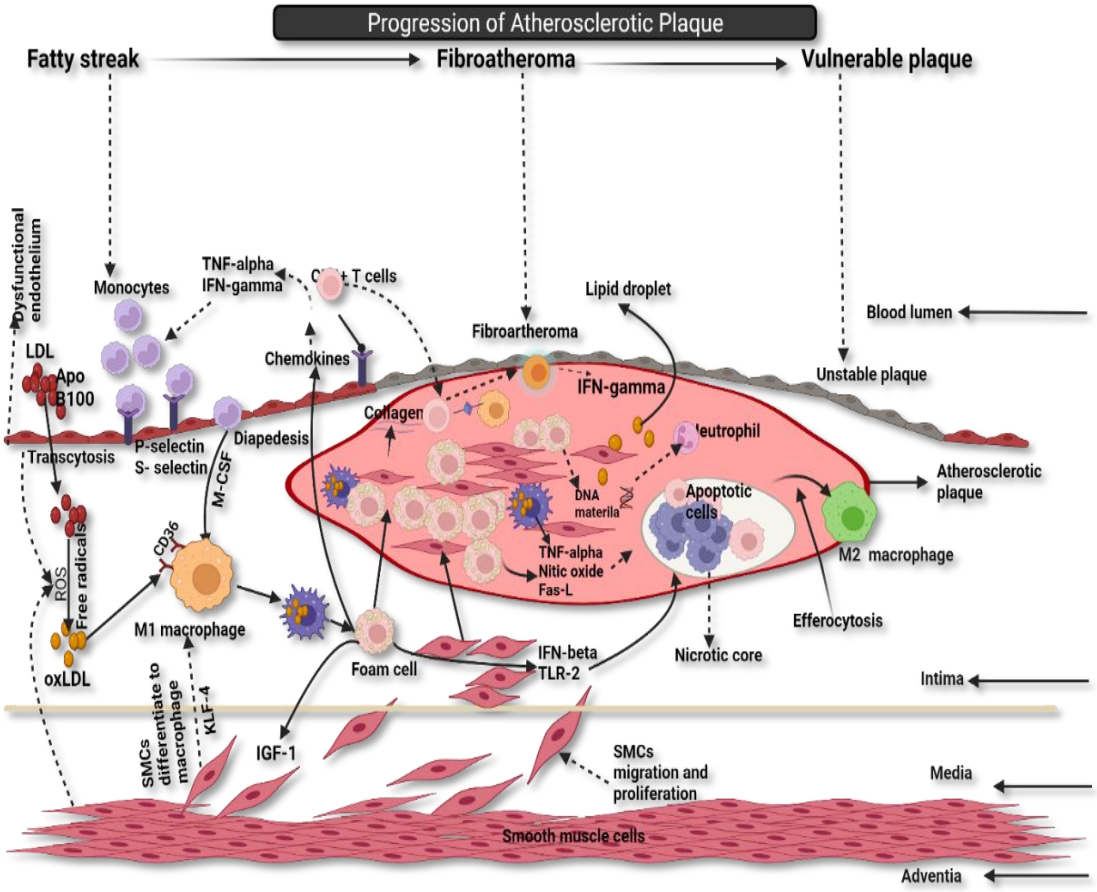

Figure 1.2: The engagement of diverse immune cells in the evolution of atherosclerotic plaque from fatty streak to unstable plaque via fibroatheroma. The entry of LDL into the intima via the subendothelial cell space leads to oxidation facilitated by reactive oxygen species (ROS) produced by impaired endothelial cells and SMCs. The recruitment of monocytes into the intima is facilitated by adhesion molecules like P and S selectin, leading to their differentiation into macrophages with the aid of M-CSF. CD36 enables macrophages to take up oxLDL, transforming them into foam cells, consequently fostering a pro-inflammatory environment by secreting chemokines that attract additional monocytes. Foam cells release IGF-1, which stimulates SMCs proliferation and migration into the intima. Subsequently, upon foam cell death, lipids and DNA attract neutrophils, while inflammatory cytokines from macrophages and neutrophils worsen inflammation within atherosclerotic plaques. Through interactions with T cells, macrophages promote the release of IFN-γ, escalating inflammation and activating endothelial cells, consequently attracting more monocytes to the site of inflammation. (Created using Bio render).

1.3.3 Risk factors of atherosclerosis

Multiple classical risk factors for atherosclerosis characterized as modifiable, unmodifiable, environmental, and lifestyle have been identified over the years (Figure 1.3). These factors encompass hypercholesterolemia, hypertension, smoking, diabetes, and a genetic predisposition or family history of cardiac disease (Lee et al., 2017). Hypercholesterolemia is a unique risk factor for atherosclerosis since it is important to foam cell production (Maganto-Garcia et al., 2012). There is strong evidence relating serum cholesterol levels to cardiovascular disease (Jung et al., 2022). LDL, along with total cholesterol, is important in risk assessment in CAD (Jung et al., 2022). Numerous studies have connected clinical atherosclerotic disease to LDL cholesterol levels (Pirillo et al., 2013; Chistiakov et al., 2016; Centa et al., 2019;). However, HDL cholesterol, which oppositely transports cholesterol, reduces cardiovascular disease risk (Mahdy et al., 2012). The ratio of HDL to total or LDL cholesterol also indicates cardiovascular risk (Millán et al., 2009).

Hypertension is a prominent risk factor for coronary and cerebrovascular events in men and women of any age (Hajar, 2017). Conversely, the onset of elevated blood pressure is associated with a diverse range of environmental, physiological, and genetic elements, as reported by Taylor et al. in 2009. The risk of atherosclerosis increases when hypertension is coupled with additional factors like obesity, abnormal lipid profiles, and insulin resistance (Klop et al. 2013). Over the past few decades, smoking has been linked to coronary artery disease (CDC, 2010). Smoking increases non-smokers' risk of ischemic heart disease or myocardial infarction (Gallucci et al., 2020). Tobacco smoking may increase atherogenesis through endothelial damage, platelet adherence and aggregation, oxidative stress, fibrinogen levels, and arterial spasm (Bernhard and Messner, 2014).

Both type 1 and type 2 diabetes significantly increase susceptibility to atherosclerosis and its complications compared to non-diabetic individuals (Poznyak et al., 2020). The exact mechanisms remain unclear, but elevated levels of lipids, insulin, and glucose may impact platelet and endothelial function. Diabetes is also associated with increased oxidative stress and reduced antioxidants, key factors in atherosclerosis (Matough et al., 2012). Advances in understanding genetic mutations, such as familial hypercholesterolemia and apolipoprotein B deficiencies, have improved insights into atherosclerotic plaque formation and cardiovascular disease risk (Sheweita et al., 2014). The role of genetic factors is further emphasized by the link between a family history of early coronary disease and endothelial dysfunction, even in the absence of other risk factors (Kessler and Schunkert, 2021). Histopathological studies indicate that atherosclerosis results from vascular injury and inflammation, with infections potentially exacerbating the condition by promoting cytokine release, adhesion molecule expression, and lipid accumulation (Ambrose and Martinez, 2002; Lopez-Candales et al., 2017).

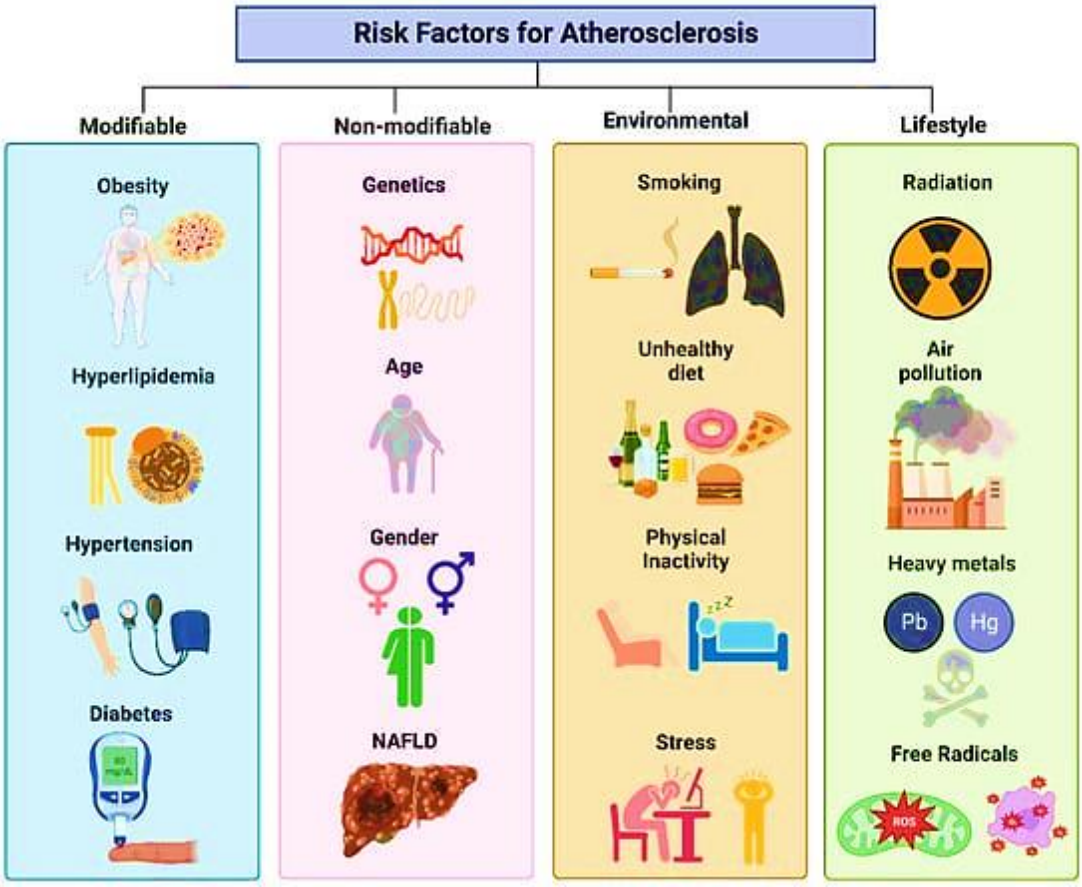

Figure 1.3: Association of risk factors linked with atherosclerosis. Factors that increase the likelihood of atherosclerosis encompass modifiable factors such as hypertension, and hyperlipidemia, non-modifiable factors like age and gender, environmental factors like air pollution and radiation, and lifestyle elements like smoking and unhealthy diet (Imtiaz et al., 2023).

1.3.4 Animal Models of Atherosclerosis

More than a hundred years ago, Ignatowski discovered that rabbits given lipid-rich animal proteins had thicker arteries, marking the first animal study of atherosclerotic plaques (Ignatowski et al., 1908). Since then, researchers have utilized a variety of animal species, including mice, pigs, rabbits, and monkeys, to study atherosclerosis (Kapourchali et al., 2014). The C57BL/6 strain of mice is the most commonly used model due to its vulnerability to an atherogenic diet (Schreyer et al., 1998). Nevertheless, C57BL/6 mice consistently exhibit notably low plasma cholesterol levels, particularly HDL, even under high-fat dietary conditions. Rats subjected to prolonged consumption of an atherogenic diet develop only minor lesions. The $Apoe^{-/-}$ and $Ldlr^{-/-}$ mice represent the prevailing transgenic mouse models extensively employed in atherosclerosis research.

1.3.4(a) ApoE deficient mice

Apolipoprotein E (ApoE), a 34 kDa glycoprotein, is synthesized in various tissues including the liver, brain, intestine, lung, and macrophages, and is essential for lipoprotein metabolism except for LDL (Tenger and Zhou, 2003). ApoE facilitates the hepatic clearance of chylomicrons and very low-density lipoprotein (VLDL) remnants by serving as a ligand for cell surface lipoprotein receptors (Alagarsamy et al., 2022). The $Apoe^{-/-}$ exhibit elevated plasma cholesterol levels, primarily in VLDL and chylomicron fractions, and develop hyperlipidemia even on a standard chow diet (Busnelli et al., 2023). These mice show lipid streaks and foam cell formation within 6-8 weeks, progressing to complex plaques with necrotic cores and fibrous caps by 20 weeks (Getz and Reardon, 2016). The progression of atherosclerosis is further exacerbated by a Western diet, with female mice being more susceptible than males

(Wang et al., 2022). However, the use of Apoe$^{-/-}$ mice is limited due to ApoE's diverse roles beyond lipoprotein metabolism, including functions in macrophage biology, immune response, and adipose tissue. This complexity complicates efforts to restore cholesterol efflux and normalize plasma cholesterol levels through Apoe$^{-/-}$ bone marrow transplantation (Daugherty et al., 2017).

1.3.4(b) Mice lacking LDL receptors (Ldlr$^{-/-}$)

The LDL receptor, a 160 kDa membrane protein, is crucial for regulating LDL cholesterol levels by mediating the endocytosis of LDL particles enriched with cholesterol (Go and Mani, 2012). It facilitates the uptake of lipoproteins containing ApoB and ApoE. Unlike Apoe$^{-/-}$ mice, which exhibit severe hyperlipidemia and advanced atherosclerosis, Ldlr$^{-/-}$ mice generally show only modest increases in plasma cholesterol and develop minimal atherosclerosis on a standard diet. To accelerate atherosclerosis in Ldl$^{r-/-}$ mice, a Western diet is employed, which increases intermediate-density lipoprotein (IDL) and larger LDL particles, while HDL and triglyceride levels remain unchanged (Ishibashi et al., 1993). The progression of plaques in Ldlr$^{-/-}$ mice mirrors that in Apoe$^{-/-}$ mice, starting in the proximal aorta and extending distally. Western diet consumption leads to larger and more advanced lesions with a collagen-rich fibrous cap and a necrotic core (Hartvigsen et al., 2007).

In Ldlr$^{-/-}$ mice, plasma cholesterol is predominantly carried by LDL particles, resembling human lipid profiles. The lack of LDL receptors primarily affects lipid levels without significantly altering inflammatory responses, which distinguishes it from ApoE deficiency (Getz and Reardon, 2012). Additionally, Ldlr-/- mice model human familial hypercholesterolemia effectively, reflecting the effects of a non-functional LDL receptor (Emini Veseli et al., 2017).

1.3.4(c) High-Fat Diet Induce (HFD) Atherosclerosis

Atherosclerosis, a primary factor in cardiovascular disease, involves the buildup of lipids, cholesterol, and fibrous components within arterial walls. One common experimental approach to study atherosclerosis is inducing it in mice using HFDs. These diets are rich in cholesterol and saturated fats, simulate the hyperlipidemia and vascular inflammation characteristic of human atherosclerosis. HFDs are designed to induce hypercholesterolemia, a critical risk factor for atherosclerosis. Unlike humans, wild-type mice are resistant to atherosclerosis due to their high plasma levels of HDL and low plasma levels of LDL cholesterol. Therefore, specific mouse strains, such as $ApoE^{-/-}$ and $LDLR^{-/-}$ mice with C57BL/6 background, are frequently used in combination with HFD to induce lipid metabolism abnormalities that mimic human atherosclerotic conditions (Getz and Reardon, 2012). In atherosclerosis research, HFDs are generally divided into two categories: Western-type diets and cholate-containing diets.

- **Western Diets**: These diets resemble the dietary patterns associated with atherosclerosis in humans, characterized by high fat (21% weight), high cholesterol (0.2% weight), and low fiber content. The fats are primarily saturated and unsaturated fatty acids, which contribute to hyperlipidemia and lipid deposition in arterial walls (Xu et al., 2015). $ApoE^{-/-}$ and $LDLR^{-/-}$ mice fed Western diets for 8 to 16 weeks typically exhibit significant atherosclerotic lesions in the aorta, particularly in the aortic root (Feig et al., 2014).
- **Cholate Diets**: These diets include cholate, a bile acid, along with 1.25% cholesterol and 15% fat. Cholate enhances cholesterol absorption in the intestines and hinders cholesterol removal from peripheral tissues, leading to

more severe atherosclerosis. However, cholate-containing diets are frequently avoided due to their association with liver toxicity and inflammation, complicating the interpretation of atherosclerosis-specific outcomes (Getz and Reardon, 2018).

1.3.4(d) Effects of HFDs on Atherosclerosis Development

The consumption of HFD leads to numerous physiological changes that play a role in the progression of atherosclerosis. HFDs lead to elevated plasma cholesterol levels, particularly increasing LDL cholesterol and VLDL cholesterol, which accumulate in the arterial intima. OxLDL promotes endothelial dysfunction and triggers a pro-inflammatory response, initiating plaque formation. Over time, macrophages infiltrate the vessel wall, engulf lipids to become foam cells, and contribute to plaque expansion (Hansson and Libby, 2006). Mice fed HFDs exhibit elevated levels of pro-inflammatory cytokines, including TNF-α, IL-6, and IL-1β, which drive the recruitment of immune cells such as monocytes and T cells to the developing plaques (Fatkhullina et al., 2016). Chronic exposure to high-fat diets drives the development of atherosclerosis and contributes to metabolic disruptions such as insulin resistance and obesity. These conditions, in turn, are linked to persistent low-grade inflammation and intensified vascular damage in mouse models (Paik et al., 2011).

1.3.5 Immunocytes in Atherosclerosis

Atherosclerosis features a diverse array of immune cell populations, including T cells, B cells, natural killer cells, macrophages, monocytes, dendritic cells, neutrophils, and mast cells (Xiong et al., 2023). Immune cells within atherosclerotic

plaques exhibit considerable heterogeneity in terms of tissue affinities, genetic variations, functions, immune kinetics, transcriptional regulation, metabolic changes, and intercellular signaling (Winkels et al., 2018). Macrophages and T cells play crucial roles, with macrophages being central to the disease's onset and progression through the secretions of TNF-α, IL-6, IL-1β, cholesterol uptake, and apoptosis initiation (Lawrence and Natoli, 2011). Additionally, macrophages and dendritic cells present antigens to T cells, promoting the production of various inflammatory cytokines (Mehu et al., 2022). Adaptive immune cells such as T and B cells are important for the progression of atherosclerosis but they are not necessary in the initiation of the disease.

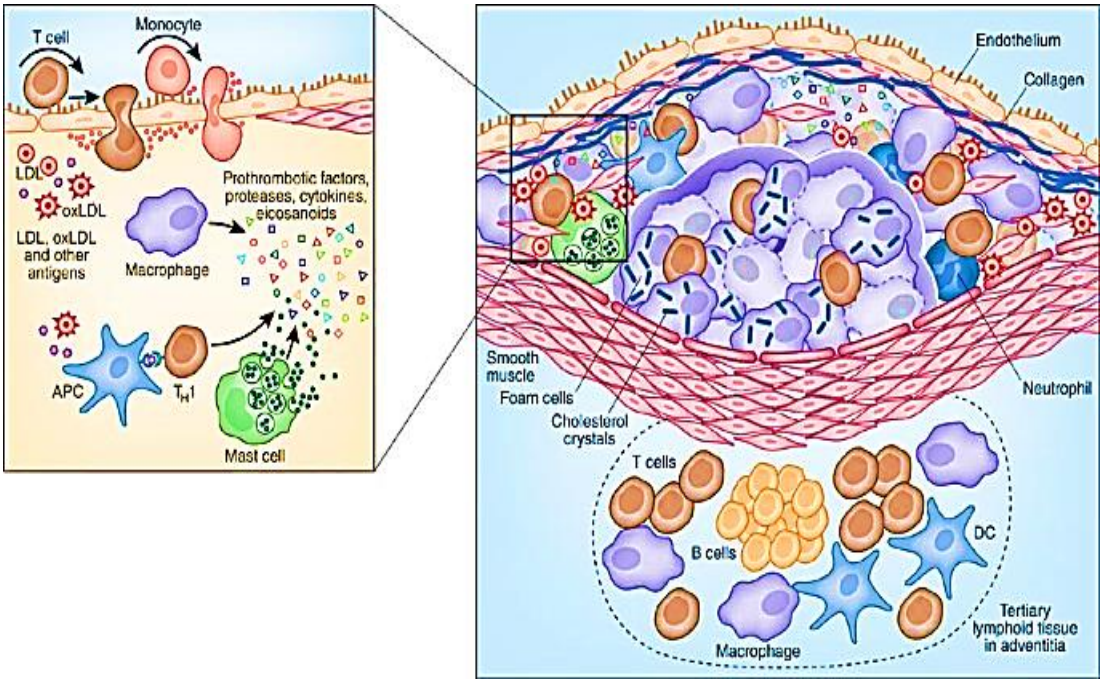

Figure 1.4: Immune cells present within atherosclerotic plaques. The core of the intimal atheroma, containing lipids, cholesterol crystals, and active and apoptotic cells, is ensheathed by a fibrous cap predominantly constituted of SMCs and collagen. This composition houses various immune cells, such as macrophages, T cells, SMCs, mast cells, and DCs. The accumulation of oxLDL is noted in the subendothelial space of the intima (Hansson and Hermansson, 2011).

1.3.5(a) Macrophage

Macrophages play a critical role throughout the progression of atherosclerosis, from plaque formation to the development of vulnerable plaques. They include tissue-resident and monocyte-derived macrophages, each influencing the disease's advancement or regression differently (Theofilis et al., 2023). Macrophages that fail to efficiently clear dead cells contribute to necrotic core formation and plaque instability. A recent publication by Neels et al. (2023) found that macrophages transform into foam cells due to excessive uptake of oxLDL, which impairs their efferocytosis ability and leads to apoptosis.

Macrophages can originate from yolk sac progenitors during fetal development or from bone marrow-derived monocytes postnatally (Nourshargh and Alon, 2014).

Different macrophage subtypes, arising from both embryonic and adult sources, are maintained by local proliferation and recruitment of circulating monocytes (Epelman et al., 2014). Tissue-resident macrophages, predominantly in cardiac tissue, originate from yolk sac progenitors (Epelman et al., 2014), while bone marrow-derived monocytes differentiate into macrophages in response to M-CSF (Moore and Tabas, 2011). Ly6Chi monocytes are the main macrophage population in inflammatory conditions (Epelman et al., 2014). Macrophages are generally classified into M1 (proinflammatory) and M2 (anti-inflammatory) types, producing cytokines such as TNF-α, IL-1β, IL-6, and TGF-β, respectively (Arora et al., 2018).

Macrophage surface proteins play a crucial role in atherogenesis, impacting various stages of atherosclerosis (Bobryshev et al., 2016; Farahi et al., 2021). Scavenger receptors like CD36 enable macrophages to recognize and ingest modified lipoproteins such as oxLDL, leading to foam cell formation, a key feature of early atherosclerosis. The inflammatory process within the arterial wall is influenced by chemotactic signals mediated by CD88, which promotes immune cell recruitment and disease progression (Kiss and Binde, 2022). CD11b facilitates macrophage adhesion to endothelial cells, aiding their migration into the sub-endothelial space and contributing to plaque formation. Additionally, macrophage surface proteins regulate cytokine and chemokine activity, essential for cell communication and inflammation in atherosclerosis (Ramji and Davies, 2015). Targeting macrophage polarization offers a promising strategy for controlling atherosclerosis, as different macrophage phenotypes can influence foam cell formation and inflammation (Farahi et al., 2021; Blagov et al., 2023).

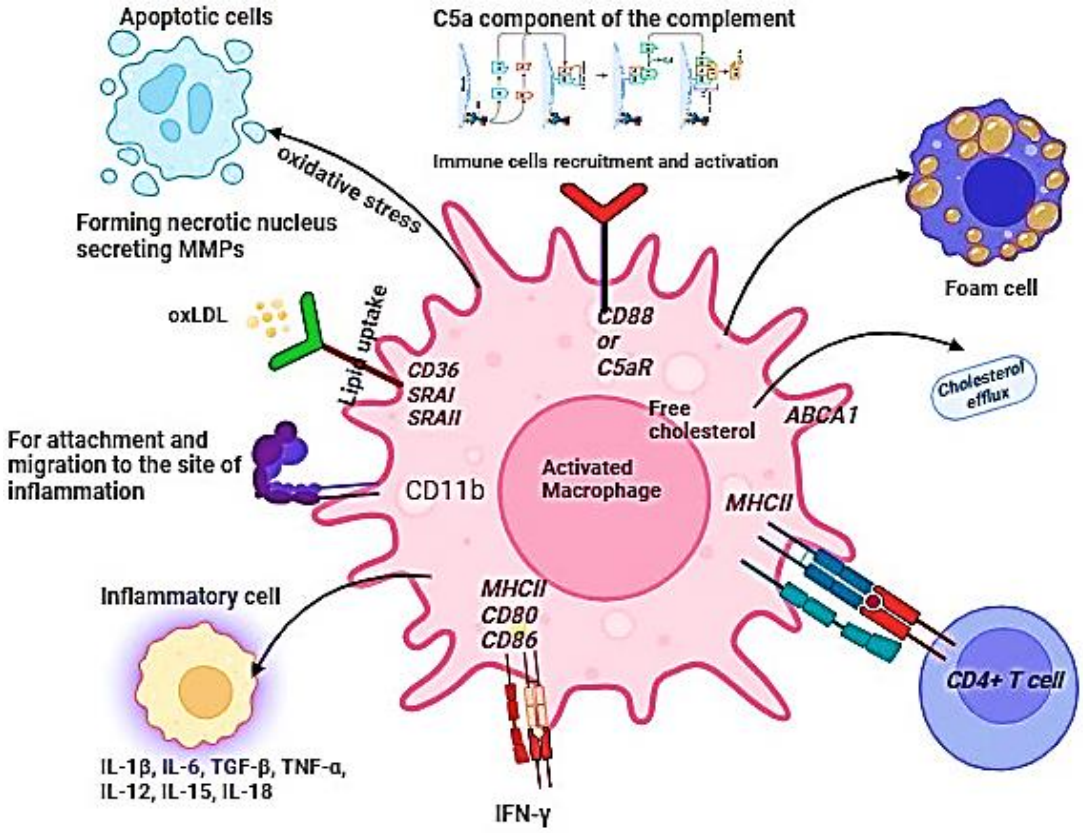

Figure 1.5: The functions of macrophages in atherosclerosis. Macrophages play a crucial role in various aspects of atherosclerosis development, including lipid metabolism, inflammatory responses, the formation of necrotic cores, and plaque instability. Macrophages directly form foam, apoptotic, and inflamed cells, while indirectly aiding CD4+ T cell activation in atherosclerosis (Fang et al., 2022).

1.3.5(b) Dendritic cells (DCs)

An expanding body of literature indicates that specialized innate immune cells, known as DCs, serve as pivotal coordinators of the immune response, crucial for both the development and maintenance of proatherogenic conditions (Koltsova and Ley, 2011) and regulatory responses within the arterial wall (Subramanian and Tabas, 2014). Dendritic cells, often denoted as DCs, have a pivotal role in instigating the adaptive immune response to atherosclerosis. Due to their similar phenotypic and functional traits, distinguishing between the functions DCs and macrophages in atherosclerotic lesions poses a challenge (Koltsova and Ley, 2011). In addition, DCs

are involved in the uptake of oxLDL via scavenger receptors, leading to foam cell formation and thus contributing to the progression of atherosclerotic lesions (Subramanian and Tabas, 2014). After absorbing oxLDL, DCs undergo maturation, move to draining lymph nodes, and present antigens to T cells (Schaftenaar et al., 2016). The presentation of antigens by DCs initiates the activation of T cells and their transformation into various subsets including Th1, Th2, Treg, and Th17 (Figure 1.6).

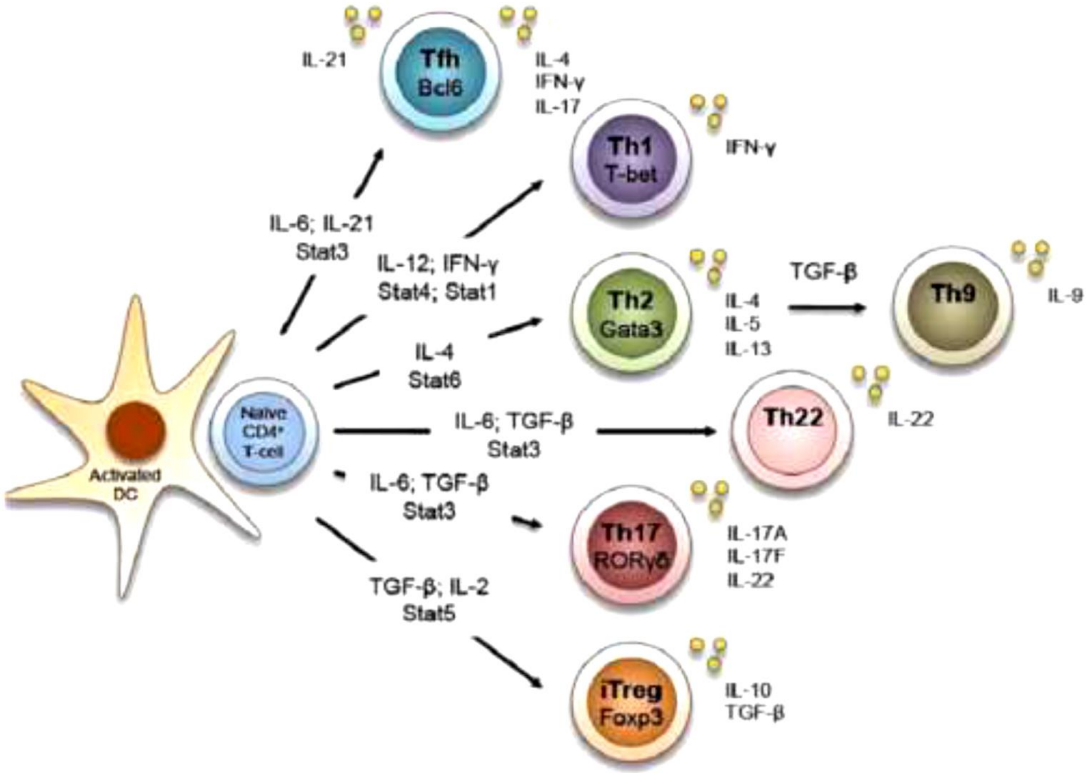

Figure 1.6: Activated DCs drive the CD4+ T cells differentiation into various T cell subsets. The activation of naive CD4+ T cells results in their differentiation into Th1, Th2, Th17, and Treg cells, which is governed by cytokine expression and transcription factors (Adapted from Paats et al., 2012).

1.3.5(c) T cells

T cells are crucial in atherosclerosis, with $CD4^+$T cells such as Th1 contributing to disease progression while Treg cells exert anti-atherogenic effects (Saigusa et al., 2020). The functions of Th2 and Th17 in atherosclerosis are less understood (Getz and Reardon, 2018). $CD8^+$T cells are involved in inducing macrophage death and forming necrotic cores within plaques (Schäfer and Zernecke, 2020). Although the exact antigenic targets driving T-cell responses during atherosclerosis are not fully known, T cells, originating from the thymus and maturing in lymph nodes and the spleen, are key to both promoting and inhibiting atherosclerosis. Research using various advanced techniques has stressed the significant role of T cells in influencing atherosclerosis,

with their activation and differentiation into subsets like Th1, Th2, Treg, and Th17 being driven by antigen presentation from DCs or macrophages (Figure 1.7).

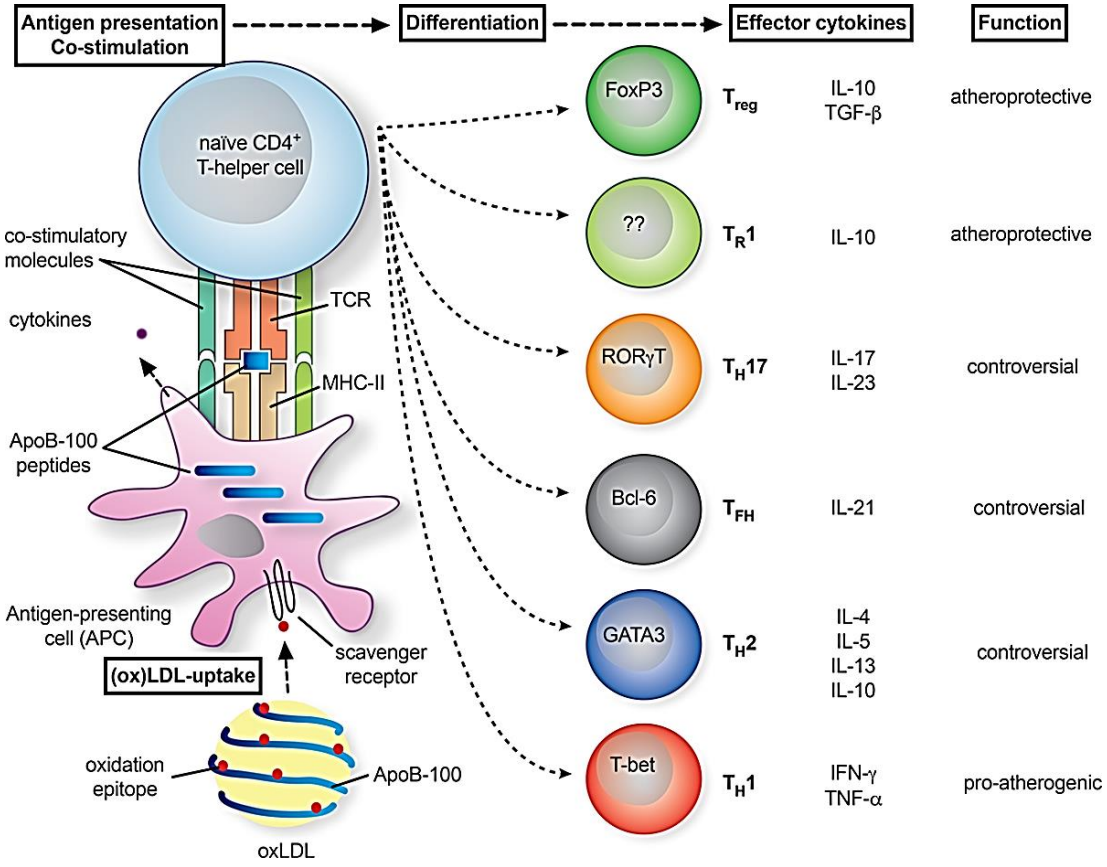

Figure 1.7: Dendritic cells stimulate naïve $CD4^+$ T-cell differentiation during atherosclerosis development. Antigen-presenting cells provide antigenic peptides to naive $CD4^+$ T cells, which thereafter become effector T cells in the plaque. OxLDL cholesterol-bound APCs process and appear on MHC class II. T-cell receptors recognize this complex. Costimulatory ligands and T-cell receptors guide the process. APC costimulatory signals and cytokines induce T cell transcription factors that enable subset differentiation. These T cell subsets express atheroprotective or proatherogenic cytokines. The role of some T cell subsets in atherosclerosis is debated. Bcl-6, Foxp3, IL, oxLDL, RORγt, and TGF are acronyms for B-cell lymphoma 6, forkhead box P3, interleukin, oxLDL, and transforming growth factor, respectively (Wolf and Ley, 2019).

1.3.5(c)(i) Th1

Atherosclerosis is a chronic inflammatory condition affecting the arterial wall, with T cells, particularly Th1 cells, playing a central role in its progression (Saigusa et al., 2020). Th1 cells, a subset of CD4+ T helper cells, secrete cytokines such as IFN-γ, which are crucial in atherosclerosis pathogenesis (Ley, 2020). IFN-γ activates macrophages and DCs, enhancing antigen presentation and further promoting Th1 polarization. This activation results in the secretion of inflammatory and cytotoxic molecules, including TNF-α and IL-1, which impact ECs and SMCs. These cytokines trigger the release of IL-6, leading to the production of acute-phase reactants like fibrinogen and C-reactive protein (Tanaka et al., 2014).

The differentiation of naive $CD4^+$ T cells into Th1 is driven by antigen exposure from APCs and involves transcription factors such as STAT4 and T-bet. Evidence of the pro-atherogenic role of Th1 is provided by studies showing reduced atherosclerosis in $Lldr^{-/-}$ mice with T-bet deficiency (Buono et al., 2005). Th1 cell-secret cytokines like IFN-γ and TNF-α contribute to inflammation and atherosclerosis development (Kany et al., 2019). Research by Buono et al. (2003) demonstrated that mice deficient in IFN-γ or its receptor had reduced lesion loads, whereas IFN administration increased lesion size. Similarly, atherosclerosis was diminished in IL-12 and IL-18-deficient animals, while their infusion enhanced lesion formation (Davenport and Tipping, 2003).

1.3.5(c)(ii) Th2

Th2 plays a significant role in regulating immune responses related to allergies, parasitic infections, and some autoimmune conditions (Paul and Zhu, 2010). They produce cytokines such as IL-4, IL-5, IL-10, and IL-13, which help suppress Th1