REGULATION OF MACROPHAGE FUNCTION
RELATED TO ATHEROSCLEROSIS DEVELOPMENT

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Abstract

Atherosclerosis has been identified as a chronic inflammatory disease resulting from interactions between immune cells and their microenvironment in the walls of susceptible arteries. Macrophages, critical players in innate immunity, participate in major events throughout all stages of atherosclerosis progression. Work in this dissertation is specifically interested in regulation of macrophage functions in the context of atherosclerosis. First, using bone marrow transplantation, we investigated how loss of eukaryotic elongation factor 2 kinase (eEF2K) activity affected atherosclerosis progression. Compared to mice transplanted with wild-type bone marrow, mice transplanted with bone marrow from eEF2K deficient mice had reduced atherosclerosis development after being fed with high-fat diet for 16 weeks. Impaired tumor necrosis factor-α release and subsequent adhesion molecule expression in the eEF2K inactive group may account for the reduction of atherosclerotic plaques. This finding suggested that the eEF2K inhibitor may be clinically useful to treat atherosclerosis. Second, we examined how oxLDL was involved in macrophage plasticity. We found that oxLDL polarized macrophages towards a novel subtype named MoL, which was characterized by high expression levels of heme oxygenase-1 and macrophage inhibitory cytokine-1. MoL cells are polarized, in part, through the activation of the PI3K/Akt pathway. MoL cells specifically up-regulates vascular cell adhesion molecule-1. This finding provided a more comprehensive view of macrophage plasticity in the context of atherosclerosis. Finally, we investigated signal transduction of ceramide 1-phosphate induced vascular endothelial growth factor release in macrophages. Using pharmaceutical inhibitors, we ruled out contributions of p38 MAPK and PKC-ζ signalling, and showed that the PI3K/Akt and MEK/ERK pathways were responsible for this process. Although we have no solid evidence to conclude on the mechanisms by which signals transduced from the
outside of the cell to the inside, a rational hypothesis that this transduction requires ceramide 1-phosphate to be either embedded into, or actively transported through, the plasma membrane has been proposed. In summary, work in this dissertation contributes to our understanding of how macrophages functions are regulated by interacting with factors in the microenvironment. Results from this dissertation provide potential new avenues for discovery of novel therapeutic approaches to treat atherosclerosis.
Preface

Chapter 1: All figures in Chapter 1 were reprinted with permission. Please refer to legend for details.

Chapter 3: A version of this material had been published as Zhang, P. et al. Impairing eukaryotic elongation factor 2 kinase activity decreases atherosclerotic plaque formation. Can J Cardiol 30, 1684–1688 (2014). I performed all of the experiments, data analysis, and manuscript writing. Contributions from additional authors are: Dr. Maziar Riazy and Dr. Shu-Huei Tsai provided thoughtful comments on the work. Dr. Maziar Riazy helped design part of bone marrow transplantation and atherosclerosis analyses. Matthew Gold and Dr. Kelly McNagny helped with bone marrow transplantation and observation of mice post-transplantation. They also provided the venue and equipment to perform micro dissection on mice post-transplant. Dr. Christopher Proud provided eef2k kinase dead (knock in) KI mice and valuable discussions. Dr. Vincent Duronio supervised my work and helped write the manuscript for the published paper.

Chapter 4: A version of this material has been submitted to Journal of Clinical & Cellular Immunology. I performed all of the experiments, data analysis, and manuscript writing. Contributions from additional authors are: Dr. Laura Sly provided training on macrophage plasticity and valuable discussions. Dr. Vincent Duronio supervised my work and helped write the manuscript submitted.

Chapter 5: A version of this material will be assembled into a manuscript with additional data from Dr. Antonio Gómez-Muñoz for manuscript submission. I performed and analyzed all the
data presented in Chapter 5. Dr. Vincent Duronio supervised my work. Dr. Antonio Gómez-Muñoz provided a valuable collaboration, which will likely result in a publication.

Experiments were performed in compliance with regulations at the University of British Columbia. Animal experiments were performed in compliance with institutional requirements at the University of British Columbia and in accordance with guidelines set forth by the Canadian Council on Animal Care. Animals were obtained and handled according to University of British Columbia ethics protocols A11-0141 (eEF2K in atherosclerosis), A10-0087 (mouse breeding), A10-0071 (eEF2K back cross), and A14-0301 (mouse breeding).
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<th>Description</th>
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<tbody>
<tr>
<td>AA</td>
<td>Arachidonic Acid</td>
</tr>
<tr>
<td>ACAT</td>
<td>Acyl-CoA:Cholesterol Ester Transferase</td>
</tr>
<tr>
<td>Ac-LDL</td>
<td>Acetylated LDL</td>
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<td>ApoB</td>
<td>Apolipoprotein B</td>
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<tr>
<td>ApoE</td>
<td>Apolipoprotein E</td>
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<td>Arg I</td>
<td>Arginase I</td>
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<td>BHT</td>
<td>Butylated Hydroxytoluene</td>
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<td>BSA</td>
<td>Bovine Serum Albumin</td>
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<tr>
<td>C1P</td>
<td>Ceramide 1-Phosphate</td>
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<tr>
<td>CCL2</td>
<td>Chemokine (C-C motif) Ligand 2</td>
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<td>CCL5</td>
<td>Chemokine (C-C motif) Ligand 5</td>
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<td>CCR2</td>
<td>C-C Chemokine Receptor Type 2</td>
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<tr>
<td>CCR5</td>
<td>C-C Chemokine Receptor Type 5</td>
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<tr>
<td>CD</td>
<td>Cluster of Differentiation</td>
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<tr>
<td>CerK</td>
<td>Ceramide Kinase</td>
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<tr>
<td>CHD</td>
<td>Coronary Heart Disease</td>
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<tr>
<td>CHOP</td>
<td>C/EBP Homology Protein</td>
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<td>CM</td>
<td>Chylomicron</td>
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<td>COX2</td>
<td>Cyclooxygenase-2</td>
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<td>cPLA2</td>
<td>Cytosolic Phospholipase A2</td>
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<td>CPTP</td>
<td>Ceramide 1-Phosphate Transfer Protein</td>
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<td>CVD</td>
<td>Cardiovascular Diseases</td>
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<td>CX3CL1</td>
<td>Chemokine (C-X3-C motif) Ligand</td>
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<td>CX3CR1</td>
<td>CX3C Chemokine Receptor 1</td>
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<td>DCs</td>
<td>Dendritic Cells</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle’s Medium</td>
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<tr>
<td>DPBS</td>
<td>Dulbecco’s Phosphate Buffered Saline</td>
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<tr>
<td>E3L</td>
<td>ApoE*3-Leiden</td>
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<tr>
<td>EBM</td>
<td>Endothelial Basal Medium</td>
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<tr>
<td>ECM</td>
<td>Extracellular Matrix</td>
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<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic Acid</td>
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<tr>
<td>eNOS</td>
<td>Endothelial Nitric Oxide Synthase</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic Reticulum</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic Reticulum</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellularly Regulated Kinases</td>
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<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
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<tr>
<td>FGF</td>
<td>Fibroblast Growth Factor</td>
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<tr>
<td>Fizz1</td>
<td>Resistin-Like- α 1</td>
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<tr>
<td>GAG</td>
<td>Glycosaminoglycan</td>
</tr>
<tr>
<td>GMCSF</td>
<td>Granulocyte/Macrophage Colony-Stimulating Factor</td>
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<tr>
<td>GPCR</td>
<td>G Protein-Coupled Receptor</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Hematoxylin And Eosin</td>
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<tr>
<td>HDL</td>
<td>High-Density Lipoprotein</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-Hydroxyethyl)-1-Piperazineethanesulfonic Acid</td>
</tr>
<tr>
<td>HFD</td>
<td>High-Fat Diet</td>
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Hmox-1  Heme Oxygenase 1
HuVEC  Human Umbilical Vein Endothelial Cells
IC  Immune Complexes
ICAM-1  Intercellular Adhesion Molecule-1
IFN-γ  Interferon gamma
IL-3  Interleukin 3
IL-4  Interleukin-4
IMDM  Iscove’s Modified Dulbecco’s Medium
KI  Kinase Inactive
KLF4  Krüppel-Like Factor 4
LCM  L929-cell conditioned media
LDL  Low-density Lipoprotein
LDLR  LDL Receptor
LFA-1  Lymphocyte Function-Associated Antigen-1
LPL  Lipoprotein Lipase
LPS  Lipopolysaccharide
LRP  Low Density Lipoprotein Receptor-Related Protein
LXR  Liver X Receptor
LY  LY294002
MCP-1  Monocyte Chemotactic Protein-1
MCs  Mast Cells
MCSF  Macrophage Colony-Stimulating Factor
MDPs  Macrophage/Dendritic cell progenitors
MEK  Mitogen-Activated Protein Kinase Kinase
MerTK  Mer receptor Tyrosine Kinase
MHC-II  Major Histocompatibility Complex-II
MM-LDL  Minimally Modified Low-Density Lipoprotein
MMP  Matrix Metalloproteinase
MPO  Myeloperoxidase
MTS  3-(4,5-Dimethylthiazol-2-yl)-5-(3-Carboxymethoxyphenyl)-2-(4- Sulfophenyl)-
2H-Tetrazolium, Inner Salt
NAD(P)H  Nicotinamide Adenine Dinucleotide (Phosphate)
nCEH  Neutral Cholesterol Ester Hydrolase
NK Cells  Natural Killer Cells
NKT Cells  Nature Killer T Cells
NO  Nitric Oxide
NPC  Niemann-Pick type C
NR4A1  Nuclear Receptor Subfamily 4, Group A, Member 1
oxLDL  Oxidize Low Density Lipoprotein
OxPAPC  Oxidized 1-Palmitoyl-2-Arachidonoyl-sn-3-Glycero-Phosphorylcholine
PAMP  Pathogen-Associated Molecular Patterns
PD  PD098059
PDGF  Platelet-Derived Growth Factor
PF4  Platelet Factor 4
PI3K  Phosphatidylinositol 3-Kinase
PMS  Phenazine Methosulfate
<table>
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<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>PPAR γ</td>
<td>Peroxisome Proliferator Activated Receptor-γ</td>
</tr>
<tr>
<td>PRR</td>
<td>Pattern Recognition Receptor</td>
</tr>
<tr>
<td>PTX</td>
<td>Pertussis Toxin</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
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<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute</td>
</tr>
<tr>
<td>SB</td>
<td>SB239063</td>
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<tr>
<td>SEL</td>
<td>Suppressor/Enhancer of Lin-12</td>
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<td>Sphingomyelinase</td>
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<td>SMC</td>
<td>Smooth Muscle Cells</td>
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<td>sPLA2</td>
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<td>SPT</td>
<td>Serine Palmitoyltransferase</td>
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<td>SR</td>
<td>Scavenger Receptor</td>
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<td>SREBP</td>
<td>Sterol regulatory element-binding proteins</td>
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<td>sSMase</td>
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<td>TIMP</td>
<td>Tissue Inhibitors of Metalloproteinase</td>
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<td>TLR</td>
<td>Toll-Like Receptor</td>
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<td>Treg Cells</td>
<td>Regulatory T Cells</td>
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<td>Full Name</td>
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<tr>
<td>UPR</td>
<td>Unfolded Protein Response</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>Vascular Cell Adhesion Molecule-1</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>VLA-4</td>
<td>Very Late Antigen</td>
</tr>
<tr>
<td>VLDL</td>
<td>Very Low-Density Lipoprotein</td>
</tr>
<tr>
<td>WHHL</td>
<td>Watanabe hereditary hypercholesterolemic</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
<tr>
<td>WM</td>
<td>Wortmannin</td>
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<td>WT</td>
<td>Wild Type</td>
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<tr>
<td>XO</td>
<td>Xanthine Oxidase</td>
</tr>
<tr>
<td>YM1</td>
<td>Chitinase 3-Like 3</td>
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</table>
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Dedicated to my little princess Evelyn Yachen Zhang and my little prince Sloyn Wenqian Zhang.

You melt my heart and make me complete. My life could not be happier.

No matter what, along your road, I will always be there for you.

Love you!
Chapter 1: Introduction

1.1 Atherosclerosis

1.1.1 Definition and epidemiology

Atherosclerosis is a chronic inflammatory disease that develops in the intimal area of large and medium-sized arteries. During the development of atherosclerosis, lipids, cells, and extracellular matrix deposit in the arterial walls, leading to the expansion of the intima. The intima lies adjacent to the outer smooth muscle layer and protrudes into the blood vessel lumen. Although the partial blockade of the artery itself often has minimal clinical manifestations, the rupture of the protruding plaques will likely result in thrombus formation and subsequent ischemia in the distal regions supplied by that artery, which is the underlying cause of complications like coronary heart disease (CHD, also known as heart attack) and stroke.\(^1\)

Atherosclerosis and its main consequence, cardiovascular diseases (CVD), is the leading cause of mortality worldwide. In 2008, more than 17 million people died from CVD, out of which CHD, one manifestation of atherosclerotic diseases, accounts for 7.3 million.\(^2\) In North America, implementation of smoking bans and increased attention to the control of risk factors have led to a change in lifestyle, which has resulted in a decline of cardiovascular deaths. However, the death rate and the burden caused by CVD still remain high. In Canada, although barely surpassed by cancer, CVD was still responsible for 28.7% (68,342) of deaths (238,418) in 2009.\(^3\) Similarly, in the United States, 31.9% (787,650) of deaths (2,468,435) were attributed to CVD, which means that on average, one American died from CVD every 40 seconds. In 2010 alone, the total cost of CVD in the United States was estimated to be $315.4 billion.\(^4\)
Multiple risk factors of atherosclerosis have been identified. According to a WHO report, there are three categories of risk factors: behavioral risk factors, which include the use of tobacco, inadequate physical activity, high-salt and high-fat diet, and alcohol abuse; metabolic risk factors, which include hypertension, diabetes, hyperlipidemia, and obesity; and “other” risk factors, which include advancing age, gender, genetic deposition, psychological disorders, and even poverty and lack of education. The underlying causal cellular and molecular mechanisms, namely the pathogenesis of atherosclerosis, have been investigated for more than a century, but some of the key steps are still unclear.

1.1.2 Pathogenesis of atherosclerosis

1.1.2.1 The lipid theory

Atherosclerosis was believed to be part of the inevitable aging process and nothing could be done to prevent it from happening. However, in 1913, Anitschkow and his student, Chalotow published their work showing that feeding rabbits with purified cholesterol lead to the development of vascular lesions, which closely resemble human atherosclerosis. Although not well accepted at that time, Anitschkow’s work shed some light on the pathogenesis of atherosclerosis and gave birth to the famous lipid theory (a hypothesis at that time and for decades after) suggesting that serum lipids, primarily cholesterol delivered by apolipoprotein B (apoB) containing lipoproteins, causally contribute to atherogenesis.

The lipid theory was not widely appreciated until three noted epidemiological studies were unveiled. The Seven Countries study conducted by Keys’ group examined people’s diet, cholesterol levels in the blood, and the death rates of CHD in seven countries. They found that
the fatal CHD rate is proportional to the serum cholesterol level, which is proportional to the saturated fat in the diet\textsuperscript{7,8}. It is also worth mentioning that some studies criticized that the authors picked data from the Seven Countries Study. Original data included 22 countries, and the analysis of all data revealed less clear statistics of correlation between dietary fat intake and CHD rate\textsuperscript{9}. To further clarify that the differences in cholesterol levels and death rates are not due to genetic differences in the populations, the Japanese migration studies were carried out. Early generations of Japanese immigrants settled in the US, compared to native Japanese non-immigrants, showed a significant increase in blood cholesterol level and its correlated death rate of CHD\textsuperscript{10}. The Framingham Heart study, initiated in 1948 and still ongoing to this day, investigated common factors and characteristics that contribute to CVD using a cohort of residents (and their offspring) in Framingham, Massachusetts. The results also supported a strong association between cholesterol levels in the blood and the death due to CHD\textsuperscript{11,12}. These studies had proven that serum lipids, mostly cholesterol, at least contribute to atherogenesis. However whether it plays a causal role remained controversial, until the discovery and wide use of statins to lower cholesterol levels in 1980s\textsuperscript{13}.

In pre-statins years, researchers had discovered drugs that can lower blood cholesterol levels. For instance, cholestyramine, a bile acid sequestrant, used in the 1984 NIH Coronary Primary Prevention Trial lowered total cholesterol in the blood by only 10\%, which barely showed a statistically significant reduction in heart attack rate\textsuperscript{14,15}. By contrast, simvastatin, used in the 4S study (Scandinavian Simvastatin Survival Study), drastically reduced total cholesterol level by 25\%, and CHD deaths by an astonishing 42\%\textsuperscript{16}. A recent meta-analysis of 90056 participants in 14 statin trials showed that, over a period of 5 years, every mmol/L reduction in low-density
lipoprotein (LDL) cholesterol correlated with about a 20% reduction in the incidence of major vascular events (including major coronary events, coronary revascularization and stroke). These unarguable facts eventually turned the lipid theory from a controversial hypothesis into a true theory.

1.1.2.2 The response-to-injury hypothesis

The lipid theory defined cholesterol as a causal risk factor in atherogenesis. However, it failed to explain all the features of atherogenesis. For example, why is the aortic root prone to developing atherosclerosis? Recently, it has been confirmed that atherosclerotic plaques primarily develop in large and medium-sized arteries, with preferred areas being the inner walls of arterial curvatures and outer walls of bifurcations. This phenomenon cannot be explained by the effect of any aforementioned risk factors since all of their effects are systematic. In 1976, Ross and his colleague, Glomset, proposed the response-to-injury hypothesis, which states that a direct (albeit unknown) injury to the endothelium results in endothelial desquamation and triggers atherosclerosis. They suggested that exposed underlying connective tissue at sites of endothelial desquamation permits the platelets to adhere and aggregate. Aggregated platelets release platelet-derived growth factor (PDGF) and other factors and lead to migration and proliferation of smooth muscle cells (SMC).

Over decades, the response-to-injury hypothesis has been reinvented. Today, we believe that endothelial dysfunction instead of endothelial desquamation triggers the consequent atherosclerosis. Altered shear stress, especially low or oscillatory shear stress, has been identified as the main causal factor of endothelial dysfunction. Endothelial structures such as
cilia and integrins act as mechanical sensors and transduce the changes in blood flow into the underlying endothelial cells\textsuperscript{23}. Under low or oscillatory shear stress conditions, endothelial cells first undergo a phenotype change from an ellipsoidal morphology to a polygonal morphology\textsuperscript{24}, which increases the permeability of macromolecules such as LDL. Meanwhile, the endothelial gene expression profile is shifted in favor of atherogenesis\textsuperscript{25}. Low or oscillatory shear stress induces the expression of monocyte chemotactic protein-1 (MCP-1)\textsuperscript{26}, nuclear factor-κB (NF-κB)\textsuperscript{27} and its downstream factors including vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1)\textsuperscript{28}. These alterations promote monocyte recruitment and subsequent inflammatory processes. In addition, low or oscillatory shear stress causes sustained activation of sterol regulatory element-binding proteins (SREBPs)\textsuperscript{29}, elevated levels of nicotinamide adenine dinucleotide (phosphate) oxidase (NAD(P)H oxidase) and xanthine oxidases (XO) at endothelial cell membranes, as well as decoupling of endothelial nitric oxide synthase (eNOS)\textsuperscript{30}. These synergistic events facilitate lipid deposits and modification in the arterial walls. Taken together, endothelial dysfunction caused by ‘response-to-injury’ has been proposed to be the initial step of atherosclerotic plaque development. And this hypothesis successfully explained the site-specific predilection of atherogenesis.

1.1.2.3 The response-to-retention hypothesis

In 1995, William and Tabas proposed that the subendothelial retention of atherogenic lipoproteins, among other early atherosclerotic events, is the central pathogenic process in atherosclerosis\textsuperscript{31}. Other events, including endothelial dysfunction, are either not required or not sufficient to provoke atherosclerotic lesion formation. According to this hypothesis, the retention of apoB containing lipoproteins sets the scene for lipoprotein modification and consequent
inflammatory response that ultimately leads to atherosclerosis\textsuperscript{32}. ApoB containing lipoproteins bind to glycosaminoglycan (GAG) chains of proteoglycans in the matrix of the intima through electrostatic forces\textsuperscript{33}. The highly positively charged site B (residue 3359-3369) is believed to be the main proteoglycan-binding site in apoB100\textsuperscript{34}. However, this site does not exist in apoB48, a truncated isoform of apoB that can be found in chylomicrons, which shows a similar proteoglycan-binding affinity. In fact apoB48 binds to proteoglycans via a different site, site B-Ib (residues 84 to 94), whereas in apoB100, site B-Ib is blocked by the C-terminus\textsuperscript{35}. A study of mice having proteoglycan-binding-defective LDL showed significantly less atherosclerosis\textsuperscript{36}. This was the first \textit{in vivo} evidence supporting the ‘response-to-retention’ hypothesis.

Modification of lipoproteins may contribute (and in some cases even accelerate) the retention of lipoproteins. LDLs modified by secretory phospholipase A\textsubscript{2} (sPLA\textsubscript{2}) expose another proteoglycan-binding site, site A (residue 3148-3158), which has higher affinity for proteoglycan\textsuperscript{37}. Other contributing elements include secretory sphingomyelinase (sSMase) and lipoprotein lipase (LPL)\textsuperscript{38}. No matter what the mechanisms are, the retention of lipoproteins results in prolonged exposure of lipoproteins to susceptible modifications and triggers the successive development of atherosclerosis. This hypothesis has provided a strong mechanistic link between serum lipids and atherosclerosis, which is essential but lacking in the lipid theory (Section 1.1.2.1).

\textbf{1.1.2.4 The response-to-oxidation hypothesis}

The ‘response-to-retention’ hypothesis cannot explain the apparent paradox that in patients expressing absolutely no functional LDL receptor, macrophages are still able to uptake
cholesterol and become lipid-laden foam cells, which are the basis of the first visible atherosclerotic lesion, the fatty streak\textsuperscript{39}. This observation suggested that native LDL has to undergo modifications in order to provoke atherosclerosis. Incubation with endothelial cells alters LDL into a form that can be rapidly engulfed by macrophages\textsuperscript{40}. Later studies verified this discovery and identified the modified LDL to be oxidized LDL (oxLDL)\textsuperscript{41} (Discussed in detail in Section 1.4.2). Steinberg and Witztum summarized the evidences linking oxLDL to atherosclerosis and proposed the ‘response-to-oxidation’ hypothesis\textsuperscript{42}.

There is a huge body of evidence supporting the oxidative modification hypothesis. First of all, oxLDL can be detected in both plasma and atherosclerotic lesions in humans\textsuperscript{43}. Mice as well as humans who have atherosclerosis show a higher level of oxLDL autoantibodies\textsuperscript{44-46}. Besides, oxLDL bears a great number of pro-atherogenic properties. It contributes to endothelial dysfunction by inducing apoptosis\textsuperscript{47}, compromising nitric oxide (NO) regulation\textsuperscript{48} and upregulating pro-inflammatory factors such as MCP-1\textsuperscript{49}, macrophage colony-stimulating factor (MCSF)\textsuperscript{50} and adhesion molecules\textsuperscript{51,52}. In addition to its effects on endothelial cells, oxLDL itself can promote monocyte recruitment and inhibit macrophage migration, which together facilitate macrophage accumulation and set the stage for foam cell formation\textsuperscript{53}. Accumulated macrophages can rapidly uptake oxLDL and become foam cells\textsuperscript{41}. OxLDL can also stimulate macrophages to release a variety of pro-inflammatory cytokines, as well as reactive oxygen species (ROS)\textsuperscript{54,55}. Moreover, other studies have also shown that oxLDL also contributes to proliferation, migration and collagen expression in smooth muscle cells\textsuperscript{56,57}. In advanced lesions, oxLDL stimulates tissue factor expression\textsuperscript{58} and matrix metalloproteinase (MMP) release\textsuperscript{59,60}, which together destabilize the plaque and promote thrombus formation.
1.1.3 Progression of atherosclerosis

1.1.3.1 Atherosclerosis and inflammation

No matter what mechanisms trigger the initiation of atherosclerosis, it will eventually result in the start of a chronic inflammatory response in arterial walls\(^2\). The immune system defends the body by quickly reacting to harmful pathogens. Often times the pathogens are foreign infecting microbes, and the immune system limits their entry and growth. In the case of atherosclerosis, there is no definitive infectious agent. Instead, a process called sterile inflammation occurs. The triggers of sterile inflammation remain unknown, however oxLDL has been thought to be immunogenic and may be responsible for the initial steps of inflammation\(^61,62\).

Both the innate and adaptive immune systems are involved in the progression of atherosclerosis (recently reviewed in \(^62-65\)). The innate immune system is the first line of defense in the body, which recognizes and responds to pathogens in a fast and non-specific manner\(^66\). Monocytes/macrophages, as a cellular constitutive part of the innate immune system, play a central role in the progression of atherosclerosis (discussed in detail in section 1.3). Other innate immune cells such as mast cells (MCs), neutrophils, natural killer (NK) cells, as well as dendritic cells (DCs), also exist in atherosclerotic plaques\(^67\). MCs, which are traditionally linked to allergy and asthma, were found to contribute to atherosclerosis based on both \textit{in vitro} and \textit{in vivo} studies\(^68,69\). Neutrophils, the main granulocytes present in atherosclerotic plaques, promote the progression by releasing ROS and granule proteins such as MMP-2, MMP-9 as well as myeloperoxidase (MPO)\(^70\). Although NK cells has been confirmed to exist in atherosclerotic lesions in both humans and mice, their role in disease development remains unclear. A very
recent study has shown that NK cells accelerate atherosclerosis progression by cytotoxic-dependent mechanisms. DCs serve as a link between the innate and adaptive immune systems. DCs can uptake modified lipid and contribute to foam cell formation, meanwhile their potent antigen presenting activity regulates T cell homeostasis. Platelets were known to participate in thrombus formation upon the rupture of atherosclerotic plaques. Those particles also contain abundant pro-inflammatory mediators and contribute to the inflammatory response in atherogenesis. In addition to the cellular constituents of the innate immune system, the complement system also participates in atherosclerosis, although its role may be considered controversial.

The adaptive immune system is composed of highly specialized cell types. Although its reaction is slow and needs to be initiated by innate immune system responses, it creates an immunological memory leading to an enhanced response towards the same pathogen in the future, which is the basis of vaccination. The presence of T cells in atherosclerotic lesions was first reported in 1985. In general, T helper-1 (Th1) cells are proatherogenic through Th1 cytokines such as IFN-γ, IL-12 and IL-18, whereas T helper-2 (Th2) cells are atheroprotective through production of IL-5 and IL-33. However, IL-4, as a typical Th2 cytokine, shows contrasting effects on atherosclerosis. Regulatory T (Treg) cells, both natural and induced by IL-10 or TGF-β, protect against atherosclerosis. Some studies have shown that Th17 cells are involved in promoting atherogenesis through the expression of IL-17, whereas others have shown an atheroprotective property of Th17 cells. B cells generate IgM and IgG antibodies against oxLDL. Those antibodies present in early stage of atherosclerosis and bear a protective role. Inconsistent with these observations, studies depleting B cells in mice resulted in reduced
atherosclerosis development\textsuperscript{86,87}. Natural killer T (NKT) cells express both NK and T cell markers, thus they may serve as an intermediate of the innate and adaptive immune systems. NKT\textsuperscript{-/-} mice developed less atherosclerotic lesions, which indicates an atherogenic role of NKT cells\textsuperscript{88,89}.

\subsection*{1.1.3.2 Different stages of atherosclerosis}
Atherosclerosis can be considered a chronic inflammatory process that often progresses over decades in humans. Although the clinical manifestation varies, the development of atherosclerotic lesions can be classified into six stages named Stary classification type I-VI (Figure 1.1)\textsuperscript{90,91}. Type I lesions, also known as initial lesions, are characterized by the thickening of intima area and infiltration of monocytes. Infiltrated monocytes differentiate into macrophages, which uptake lipids that are deposited into lipid droplets in the cell and these cells become lipid-laden foam cells\textsuperscript{90}. More foam cells accumulate in the intima and make the lesion visible as “fatty streaks”, which is the marker of type II lesions. Also in type II, other immune cells like T cells are recruited to the site of lesions\textsuperscript{90}. Type III lesions are the intermediate lesions between early and advanced stages. Extracellular lipid droplet deposits occur at this stage\textsuperscript{90}. Accumulated extracellular lipid droplets will gradually form a distinct “lipid core” called atheroma, which are characteristic of type IV lesions\textsuperscript{91}. As the lesion progresses into type V, a fibrous cap is formed between the atheroma and the endothelial cells\textsuperscript{91}. Type VI lesions are surface defective and often times associate with thrombosis\textsuperscript{91}. A recent update on the Stary classification proposed two other stages: type VII, in which calcification predominates and type VIII, in which fibrous tissue changes predominate\textsuperscript{92}. Any regression or change of lipid in lesion types IV-VI may lead to type VII-VIII lesions\textsuperscript{92}. 
Figure 1.1 Different stages of atherosclerosis progression.

A. Early lesion of atherosclerosis is marked by the infiltration of monocytes and formation of foam cells. Monocytes are recruited to the site and transmigrate into intima area. Those monocytes then interact with microenvironment in the blood vessel wall and differentiate into macrophages. Macrophages then engulf modified (especially oxidized) LDL and become lipid laden-foam cells. B. Intermediate/advanced lesion is characterized by the fibrous cap underlying the endothelium as well as the lipid cored formed in the lesion. Lipid-laden foam cells accumulate in this stage. Smooth muscle cells and other immune cells such as T-cells are recruited and migrate to the intima. Smooth muscle cells secrete extracellular matrix and form the fibrous cap, which in general protects the lesion from rupture. Accumulated foam cells undergo apoptosis and secondary necrosis, which eventually generate a lipid core in the lesion. C. Complicated lesion is identified by plaque rupture and thrombosis. Fibrous cap becomes thinner and eventually ruptured. Thrombogenic factors from the intima get in to blood vessel, which leads to coagulation and formation of thrombus. Blood vessels may be blocked by the thrombus and results in all the complications. Reprinted with permission from Glass, C. K. & Witztum, J. L. Atherosclerosis. the road ahead. Cell 104, 503–516 (2001).
Type I through III lesions are always clinically silent. However beyond this point, there is no exact correlation between lesion types and clinical manifestations. Even type IV lesions may significantly narrow the lumen area and result in fatal clinical events, while type VI lesions may remain silent. In general, the complications and fatal events, such as myocardial infarction and stroke, are associated with type VI lesions, and often include thrombosis.

1.1.3.3 Early lesions (type I, II): foam cell formation

Foam cells are the very first visible events in atherosclerosis development. A “fatty streak” that is composed of foam cells characterizes the early lesions. Macrophages that are recruited to the site of the lesion engulf infiltrated lipoproteins, deposit the lipids in the cell body and transform into lipid-laden foam cells. Under normal conditions, the metabolism of lipids, primarily cholesterol, in macrophages is tightly regulated by the intracellular sterol content. Low levels of intracellular sterols activate SREBPs leading to upregulation of LDL receptor (LDLR) and fatty acid synthesis. In contrast, the presence of sterols inhibits lipid synthesis and uptake. This negative feedback loop explains why the retention of native LDL is not sufficient for foam cell formation.

As stated in Section 1.1.2.4, native LDL undergoes some modifications, which bypasses the negative feedback loop and facilitates foam cell formation. Acetylated LDL (Ac-LDL) can be rapidly engulfed by macrophages through a LDLR independent pathway, and this pathway has been named the scavenger receptor pathway. Although acetylated LDL does not exist in vivo, researchers predicted that LDL undergoes a similar modification that results in the uptake through the scavenger receptor pathway. Incubation of LDL with endothelial cells generates a
type of modified LDL that promotes foam cell formation. This modification was identified as peroxidation of the unsaturated fatty acid component of LDL\textsuperscript{41}. Other forms of lipoproteins such as aggregated lipoprotein\textsuperscript{97} and lipoprotein immune complexes\textsuperscript{98} also contribute to foam cell formation. Recently, Kruth et al. found that native LDL can bypass SREBPs feedback by micropinocytosis\textsuperscript{99,100}.

In addition to macrophages, smooth muscle cells also express scavenger receptors and deposit cholesterol in the cell body\textsuperscript{101,102}. SMC derived foam cells are barely detected in mouse lesions, however are abundant in human lesions\textsuperscript{103}. Like macrophage-derived foam cells, SMC foam cells promote atherosclerosis development and contribute to the subsequent lipid core formation\textsuperscript{104}.

### 1.1.3.4 Intermediate/advanced lesions (type III, IV, V): lipid core and fibrous cap formation

Foam cells accumulate in the atherosclerotic lesions and eventually lead to the formation of a lipid core. Both macrophage-derived foam cells and SMC-derived foam cells participate in this event\textsuperscript{105,106}. Apoptosis, which shows a generally atheroprotective role (through efficient efferocytosis) in early lesions, detrimentally contributes to lipid core formation\textsuperscript{107}. Impaired clearance of apoptotic cells, together with other factors such as oxidative stress, depletion of cellular ATP, and elevated intracellular calcium concentrations, result in an irreversible necrotic cell death, which significantly enlarges the lipid core\textsuperscript{108}.
At this stage, the lipid core is covered by a fibrous cap, which is rich in collagens and elastin\textsuperscript{109}. SMCs are considered the main source of collagens that contribute to fibrous cap formation\textsuperscript{110}. The cap lies between the endothelium and the necrotic lipid core, and stabilizes the atherosclerotic plaque\textsuperscript{111}. The components and thickness of the fibrous cap often predicts the vulnerability of the plaque.

### 1.1.3.5 Complicated lesions (type VI): plaque rupture and thrombosis

Although type IV-V lesions may develop such that they are large enough to block the vessel lumen and result in clinical manifestations, in most cases, it is the rupture of the complicated lesions and subsequent thrombosis that lead to fatal events\textsuperscript{92}. The rupture exposes intima constituents to the blood components, thereby initiating the coagulation cascade. Thrombi formed in this process often fully block downstream arteries and, depending upon the location, may induce complications like myocardial infarction and stroke\textsuperscript{1}. Timely administration of antiplatelet or anticoagulant therapy is the key to benefit patients who have undergone this stage. Interestingly, the hemostatic system can cross-talk with atherosclerotic lesions and may be able to modulate plaque composition\textsuperscript{112}.

The rupture normally happens in the shoulder area of the plaque and is strongly associated with destruction of the fibrous cap\textsuperscript{110}. SMCs as well as macrophages release MMPs, which degrade the extracellular matrix and destabilize the plaque\textsuperscript{109,113}. Tissue inhibitor of metalloproteinases (TIMPs) neutralize MMPs effects and can prevent plaque rupture\textsuperscript{114}. 

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1.2 Animal models of atherosclerosis

1.2.1 Small animal models, large animal models, and human

Researchers have been using animal models to study atherosclerosis for more than a hundred years\(^6\). A large variety of animals have been used widely, including mice, rats, rabbits, dogs, and monkeys\(^{115}\). Among them, mice are the most frequently used models in atherosclerosis due to a number of advantages, such as low cost of breeding and maintenance, the ease of genetic manipulations, short periods of disease development, and limited ethical concerns\(^{116}\). The use of mouse models has significantly enriched our understanding of atherogenesis. However, due to the distant genetic background, the knowledge translation from mouse models to human should be taken with caution\(^{117}\).

Large animal models, although bearing many of disadvantages as opposed to the advantages of mouse models, are appreciated as being closer mimics of human disease. In addition to the use in non-invasive diagnosis studies and preclinical toxicity tests, large animal models are extremely useful in studying plaque rupture, which is the most fatal complication of atherosclerosis and occurs only rarely in mouse models\(^{118}\). It should be noted that although the human resemblance of large animal models is widely valued, no models perfectly mimic human pathogenesis of atherosclerosis. Any information gained through animal models studies should be interpreted with great caution.

1.2.2 Small animal models

Mouse models are the most popular animal models used in atherosclerosis studies. However, interestingly, mice normally do not develop severe atherosclerotic plaques. Feeding with high
cholesterol diet for a prolonged period of time only induces early lesion (fatty streak) formation. In 1990s, Brown and Goldstein derived LDLR deficient \((Ldlr^{-/-})\) mice, in which the regulation of serum cholesterol is compromised due to a lack of LDLRs in hepatocytes\(^{119}\). These mice develop hypercholesterolemia and atherosclerosis when fed with high cholesterol diet\(^{120}\). Around the same time, the Breslow laboratory derived apolipoprotein-E (ApoE) deficient \((ApoE^{-/-})\) mice, and these mice develop atherosclerotic disease even with normal chow\(^{121}\). By far, these two models are still the most widely used mouse models in atherosclerosis research. Other models such as apoE*3-Leiden (E3L)\(^{122}\) and transgenic apoB\(^{123}\) were developed later, but are not used as frequently. Genetic background also affects the susceptibility of fatty streak formation. Among commonly used strains, C57Bl/6 mice are the most susceptible to atherosclerosis\(^{124}\). Most of the currently used models use the C57Bl/6 mice.

A common use of these mouse models is to crossbreed with another transgenic strain that has null mutation or overexpression of a certain gene. Additionally \(ldlr^{-/-}\) mice can be used in bone marrow transplantations. Reconstitutions of bone marrows from genetically modified mice into irradiated \(ldlr^{-/-}\) mice allow people to specifically study the functions of hematopoietic cells in the context of atherosclerosis\(^{125}\). \(ApoE^{-/-}\) mice cannot be used as bone marrow recipients unless the donor mice are \(ApoE^{-/-}\), because the bone marrow cells from \(ApoE^{+/+}\) mice can contribute to reconstitution of apoE in the circulation.

1.2.3 **Large animal models**

Large animal models of atherosclerosis that have been used include rabbits, pigs and non-human primates. Rats and dogs are occasionally used in some studies, but they are not good models
because, like mice, they do not develop severe atherosclerosis even with hypercholesterolemia\textsuperscript{126,127}.

Rabbits are very sensitive to a high cholesterol diet. Upon high cholesterol diet treatment, rabbits develop atherosclerotic lesions rapidly\textsuperscript{128}. However, unlike in human atherosclerosis where the major lipoproteins is LDL, remnant lipoproteins predominate in these rabbits\textsuperscript{128}. Moreover, foam cells observed in these rabbits are mostly macrophage-derived, whereas in human lesions both macrophage-derived and SMC-derived foam cells exists\textsuperscript{128}. Besides cholesterol-fed rabbits, a closer mimic of human atherosclerosis is the Watanabe hereditary hypercholesterolemic (WHHL) rabbit, a familial hypercholesterolemia strain\textsuperscript{129}. The WHHL rabbit has a defect in the LDLR, which is also observed in human familial hypercholesterolemia. In contrast to cholesterol-fed rabbits, the WHHL rabbits use LDL as the predominant lipoproteins. And like in human lesions, SMC-derived foam cells present in the lesions of the WHHL rabbits\textsuperscript{130}.

Pigs share a lot of similarities with humans in the context of atherosclerosis. First, pigs spontaneously develop atherosclerosis during aging\textsuperscript{131}. This process can be accelerated by high cholesterol intake\textsuperscript{132}. Secondly, the anatomy of the heart, especially the coronary system is greatly comparable\textsuperscript{133}. As in humans, coronary arteries are also sites with higher susceptibility to atherosclerosis in pigs. Furthermore, pigs have similar lipoprotein profiles to humans\textsuperscript{134}. Having those similarities makes pig a much better model than small animals. Additionally, the size of pig organs allows them to be used in non-invasive measurement studies. However, it is very difficult to make any genetic modifications in pigs, which largely limits its use in research.
Non-human primates have been used in atherosclerosis research as well. They are the closest species to humans, however the costs and ethical issues make the use of those animals much more rare for most scientific studies\textsuperscript{116}.

1.3 Macrophages in atherosclerosis

1.3.1 Monocyte recruitment and heterogeneity

Circulating monocytes are originally differentiated from hematopoietic stem cells in the bone marrow\textsuperscript{135}. Those cells are indefinitely renewable and are precursor cells for the whole hematopoietic system. Macrophage/Dendritic cell progenitors (MDPs) are the immediate precursors of monocytes. Differentiated monocytes are released from bone marrow into the circulation and further differentiated into macrophages and DCs in tissues upon stimulation\textsuperscript{135}. In atherosclerosis, the number of circulating monocytes is greatly increased, partly due to the release of interleukin 3 (IL-3) and granulocyte/macrophage colony-stimulating factor (GMCSF) stimulated by hypercholesterolemia\textsuperscript{136}. By contrast, increasing cholesterol efflux suppresses the proliferation of monocyte precursors, thus reducing the number of circulating monocytes\textsuperscript{136}.

Monocytes are recruited to the site of atherosclerosis by its overlying activated endothelium. The classical leukocyte adhesion cascade involves the following steps: capture, rolling, adhesion and transmigration\textsuperscript{137}. P-selectin has been reported to play a critical role in monocyte rolling in atherosclerosis\textsuperscript{138}. E-selectin is also involved in leukocyte rolling in atherosclerosis but the nature of the leukocyte is not clear\textsuperscript{139}. A combined deficiency of P- and E-selectin reduces 80\% of early atherosclerotic lesions and 40\% of advanced lesions\textsuperscript{140}. The firm adhesion is mainly achieved by the binding pairs of VCAM-1/very late antigen-4 (VLA-4) and ICAM-1/lymphocyte
function-associated antigen-1 (LFA-1)\textsuperscript{139}. Endothelial cells, macrophages and SMCs can release chemokines that trigger the transmigration after the firm adhesion of monocytes to the endothelium. MCP-1 (also known as chemokine (C-C motif) ligand 2 (CCL2)), chemokine (C-X3-C motif) ligand (CX3CL1) and chemokine (C-C motif) ligand 5 (CCL5), which bind to C-C chemokine receptor type 2 (CCR2 or cluster of differentiation 192 (CD192)), CX3C chemokine receptor 1 (CX3CR1) and C-C chemokine receptor type 5 (CCR5 or CD195), respectively, are among the most important ones\textsuperscript{141}. Inhibition of these three chemokine/receptor pairs together results in a 90% reduction of atherosclerosis in ApoE\textsuperscript{-/-} mice\textsuperscript{142}.

In mice, monocytes can be further categorized into Ly6C\textsuperscript{high} and Ly6C\textsuperscript{low}\textsuperscript{143}. Ly6C\textsuperscript{high} monocytes live a short life in the circulation, but their numbers increase in response to inflammation and hypercholesterolemia\textsuperscript{141,144}. They are the major monocyte subset that gets recruited to the inflammatory sites, thus they are considered to be pro-inflammatory. By contrast, Ly6C\textsuperscript{low} monocytes patrol in the circulation and differentiate into resident macrophages and DCs in the tissues. These monocytes are important in maintaining homeostasis. Ly6C\textsuperscript{high} monocytes express high levels of CCR2, whereas Ly6C\textsuperscript{low} monocytes express high levels of CX3CR1\textsuperscript{143,145}. They also have a different expression profile of selectins and antigens\textsuperscript{4}. In humans, CD14\textsuperscript{high}CD16\textsuperscript{-} and CD14\textsuperscript{+}CD16\textsuperscript{+} monocytes resemble mouse Ly6C\textsuperscript{high} and Ly6C\textsuperscript{low} monocytes, respectively\textsuperscript{143}. CD14\textsuperscript{high}CD16\textsuperscript{-} monocytes express CCR2, L-selectin and CD64, whereas CD14\textsuperscript{+}CD16\textsuperscript{+} monocytes express CX3CR1, CD32 as well as major histocompatibility complex-II (MHC-II). Recently, CD14\textsuperscript{dim}CD16\textsuperscript{+}, a minor subset of CD16\textsuperscript{+} monocytes, has been reported\textsuperscript{146}. These cells are part of the patrolling monocyte population and participate in the innate surveillance of local tissues. The current classification system of monocytes is based on surface marker expression
profile. It is still under debate whether monocyte subsets are inter-convertible among different phenotypes.

1.3.2 Macrophage heterogeneity and plasticity

Macrophages, differentiated from monocytes, play a central role in all stages of atherosclerosis (discussed in detail below). Macrophages have been categorized into different subtypes based on expression profiles. In 1992, people reported the first discovery of alternative activation of murine macrophages by IL-4\textsuperscript{147}. Those IL-4 skewed macrophages express high levels of mannose receptors. Since then, the M1/M2 paradigm has been widely used to describe macrophages phenotypes\textsuperscript{148}. In mouse, the classically activated macrophages (M1) are characterized by a pro-inflammatory transcriptome including the upregulation of TNF-\(\alpha\), IL-1, IL-6, IL-12, inducible nitric oxide synthase (iNOS). Alternatively activated macrophages (M2), on the other hand, express high levels of mannose receptors, arginase-I (Arg I), chitinase 3-like 3 (YM1), resistin-like-\(\alpha\) (Fizz1). It has been suggested that M1 macrophages originate from Ly6C\textsuperscript{high} monocytes, whereas M2 macrophages are generated from Ly6C\textsuperscript{low} monocytes. Histological analysis of human plaque has shown that M2 macrophages dominate early stages of atherosclerosis, however along with Ly6C\textsuperscript{high} monocyte infiltration, the population of M1 macrophages increases\textsuperscript{149}. Studies on lineage of monocyte-macrophage development are needed to fully address this question.

The classification is defined slightly different in human as follows\textsuperscript{150,151}. MCSF differentiates monocytes into naïve macrophages (M0). Lipopolysaccharides (LPS) plus interferon-\(\gamma\) (IFN-\(\gamma\)) polarizes naïve macrophages into M1 macrophages, which are considered proinflammatory. By
contrast, naïve macrophages can be polarized into anti-inflammatory phenotypes, the M2 macrophages. M2 cells can be sub-divided into different subtypes. IL-4 or IL-13 leads to the activation of M2a; Immune complexes (IC) and IL-1β or LPS leads to M2b; and IL-10 or tumor growth factor β (TGF-β) or glucocorticoids leads to M2c. More recently, based on different homeostatic activities, macrophages are subcategorized into three new groups: classically activated macrophages in host defense, wound-healing macrophages and regulatory macrophages. The authors proposed that like the primary colors, these three basic subtypes can blend into various other ‘shades’ of activation (Figure 1.2)\textsuperscript{152}. If mapping the conventional M1/M2 paradigm to this new grouping system, M1 macrophages are classically activated macrophages, M2a macrophages are close to wound-healing macrophages, and M2b and M2c macrophages are like regulatory macrophages (Figure 1.3).
Figure 1.2 Color wheel of macrophage activation.

Figure 1.3 Cytokines produced by immune cells skew macrophages towards distinct subtypes.

Even with the new groupings, this macrophage classification system cannot fulfill all circumstances and is still under active investigation. CXCL4, also known as platelet factor 4 (PF4), without the presence of M-CSF, differentiates monocytes into a distinct subtype of macrophages, M4 macrophages\textsuperscript{153}. M4 macrophages express low levels of hemoglobin receptor, also known as CD163, and play a general atherogenic role\textsuperscript{154}. Exposure to oxidized 1-palmitoyl-2-arachidonoyl-sn-3-glycero-phosphorylcholine (OxPAPC) polarizes mouse macrophages into a novel subtype named Mox\textsuperscript{155}. Mox macrophages have elevated levels of atheroprotective heme oxygenase-1 (Hmox-1), however their exact role in atherosclerosis has not been revealed.

Intraplaque hemorrhage also leads to a unique macrophage phenotype called HA-mac at first and then renamed to Mhem\textsuperscript{156}. In contrast to M4 macrophages, Mhem macrophages express high levels of CD163 and are often referred as CD163\textsuperscript{+} macrophages. Mhem macrophages show many atheroprotective features including upregulation of Hmox-1 (like Mox), resistance to foam cell formation and increased release of IL-10, an anti-inflammatory factor.

\textit{In vivo} existence of macrophage heterogeneity has been widely reported\textsuperscript{154,155,157}. The M1/M2 paradigm has been used to interpret many observations. Nuclear receptor subfamily 4, group A, member 1 (NR4A1) deletion in \textit{ApoE}\textsuperscript{−/−} mice worsens atherosclerosis by enhancing M1 macrophage polarization\textsuperscript{158}. Bone marrow specific deficiency of NR4A1 has shown controversial results\textsuperscript{159,160}. Similarly, deletion of Krüppel-like factor 4 (KLF4) favors M1 polarization and accelerates atherosclerosis\textsuperscript{161}. IL-13, on the other hand, boosts M2 polarization and suppresses atherosclerosis progression\textsuperscript{162}. In general, shifting the M1/M2 balance towards the anti-inflammatory phenotype protects the body from atherosclerosis.
However, we need to keep in mind that \textit{in vitro} defined subtypes cannot be directly translated to a corresponding scenario in animals. Macrophage phenotypes \textit{in vivo} are likely to be different from those well-characterized \textit{in vitro} subtypes, partially due to the fact that macrophage subtypes can be skewed depending upon surrounding environmental changes. The microenvironments in atherosclerotic plaques are complex and often contain diverse, even opposing, driving forces. Pure extreme forms of macrophage phenotypes may barely exist in plaques, and instead, a full spectrum of macrophage phenotypes between aforementioned subtypes is expected in atherosclerotic plaques.

1.3.3 Macrophages in foam cell formation

Foam cells are the main component of the first visible lesion (fatty streak) in atherosclerosis progression. Although intima dendritic cells and smooth muscle cells are reported to contribute to foam cell formation, macrophages play a central role in this process. As briefly introduced in section 1.1.3.3, macrophages uptake modified lipoproteins and become lipid-laden cells. Scavenger receptors, a subgroup of pattern recognition receptors (PRRs), bind to modified lipoproteins and mediate the internalization\textsuperscript{163}. Among those receptors, scavenger receptor A (SR-A) and CD36 are extensively studied. Double knock-out of SR-A and CD36 inhibits 75\%-90\% modified lipoprotein uptake\textsuperscript{164}. Despite their remarkable effects on foam cell formation, their roles in atherosclerosis progression remain debatable. SR-A deficiency leads to a reduction of atherosclerosis in some studies\textsuperscript{165-167}, but results in more complex lesions in another\textsuperscript{168}. CD36 deficiency seems to be more consistent in reducing atherosclerosis\textsuperscript{169,170}. However, in a more recent study, neither SR-A nor CD36 deficiency protects from atherosclerosis\textsuperscript{171}. These
inconsistent results indicate that scavenger receptors have functions beyond facilitating lipid uptake (discussed in 1.4.2.2).

Various proteases and lipases, such as lipoprotein lipase, sphingomyelinase and phospholipase A2 family members, can modify lipoproteins\textsuperscript{172,173}. These enzymatically modified lipoproteins aggregate in the intima and can be then ingested via scavenger receptor-independent pathways, namely fluid-phase uptake or pinocytosis\textsuperscript{99}. Recently, it has been discovered that unbound native LDL can also be taken up through pinocytosis\textsuperscript{174}. Pinocytosis in macrophages is upregulated by pro-inflammatory factors, including oxLDL. OxLDL binds to TLR4, which in turn activates Syk pathways\textsuperscript{175}. Scavenger receptor dependent and independent pathways work in a synergetic way that promotes the cholesterol-loading process in macrophages.

Once taken up, the cholesteryl esters of lipoproteins are hydrolyzed to free fatty acid and free cholesterol in lysosomes (Figure 1.4)\textsuperscript{176}. The free cholesterol is then transported to endoplasmic reticulum through not fully understood mechanisms, which may involve Niemann-Pick type C1 (NPC1) and NPC2\textsuperscript{177,178}. Free cholesterol in ER suppresses SREBPs, which downregulates LDLR-mediated native LDL uptake\textsuperscript{94}. However the aforementioned SR-dependent pathways and pinocytosis are not affected by the SREBPs. Excessive free cholesterol in the ER is cytotoxic and may induce cell death\textsuperscript{179}. The cells avoid this by either re-esterifying free cholesterol into cholesteryl esters or exporting it out of the cell. The re-esterification is mediated by an ER enzyme acyl-CoA:cholesterol ester transferase (ACAT)\textsuperscript{180}. The resulting cholesteryl esters are then stored in intracellular lipid droplets, which are often referred to as the ‘foam’ of foam cells. This process can be reversed by the activation of neutral cholesterol ester hydrolase (nCEH),
which hydrolyzes cholesteryl ester to free cholesterol for efflux out the cells\textsuperscript{181}. Exporting free cholesterol out of the cells, or cholesterol efflux, is considered as the most critical process in atherosclerotic plaque regression\textsuperscript{182}. The first step of cholesterol efflux is trafficking of free cholesterol from ER to the cell membrane, which involves the trans-Golgi network\textsuperscript{183}. At the membrane, the free cholesterol was then actively exported via ABCA1 and ABCG1 to lipid-poor ApoA1 and HDL, respectively\textsuperscript{182,184}. Deficiency of both ABCA1 and ABCG1 accelerates foam cell formation and worsens atherosclerosis in mice\textsuperscript{182}. In macrophages, liver X receptors (LXRs) regulate the expression levels of ABCA1 and ABCG1, and can serve as potential therapeutic targets for atherosclerosis\textsuperscript{185}. Passive diffusion with or without the facilitation of SR-BI also contributes to the efflux of free cholesterol\textsuperscript{186}. In addition, as the recipients of cholesterol, ApoA1 and HDL provide potential therapeutic targets for atherosclerosis as well. Variants of ApoA1 or HDL with higher cholesterol clearance efficiency may be used to promote atherosclerotic plaque regression.
Figure 1.4 Intracellular cholesterol transport.

Once taken up, the cholesteryl esters of lipoproteins are hydrolyzed to free fatty acid and free cholesterol in lysosomes. The free cholesterol is transported to endoplasmic reticulum. The cholesterol then was either re-esterified into cholesteryl esters mediated by ACAT or exporting it out of the cell. The first step of cholesterol efflux is trafficking of free cholesterol from ER to the cell membrane, which involves trans-Golgi network. At the membrane, the free cholesterol was then actively exported via ABCA1 and ABCG1 to lipid-poor ApoA1 and HDL, respectively. Reprinted with permission from Maxfield, F. R. & Tabas, I. Role of cholesterol and lipid organization in disease. Nature 438, 612–621 (2005).
Since the discovery of macrophage heterogeneity, people have been debating whether macrophage-derived foam cells fit into the M1/M2 paradigm or they are a unique subtype of macrophages. Based on protein expression profiles, M2 macrophages express more SRs than M1 macrophages, which indicates M2 cells have much higher phagocytic ability, thus it is likely that M2 macrophages contribute more to foam cell formation\textsuperscript{148,151}. OxLDL, the most important foam cell-inducing factor, has been reported to have conflicting effects on macrophage polarization. OxLDL can bind to TLR4 triggering M1 polarization as well as increase M2 macrophage marker Arg I via the activation of peroxisome proliferator activated receptor-γ (PPARγ)\textsuperscript{187}. In addition, oxidized phospholipid, a component of oxLDL polarizes macrophages towards a Mox subtype\textsuperscript{155}. These observations are too preliminary to draw any firm conclusions. More systematic and comprehensive studies are thus needed.

1.3.4 Macrophages in inflammation

Macrophages, as one of the most important effectors in the innate immune system, participate actively in the chronic inflammatory process during atherosclerosis progression (Figure 1.5). Toll-like receptors (TLRs) highly expressed by macrophages are a family of pattern recognition receptors (PRRs) that recognize pathogen-associated molecular patterns (PAMPs) in microbes and initiate inflammation. In normal conditions, TLR-mediated responses are beneficial in keeping homeostasis, however in atherosclerosis, TLR responses could be atherogenic\textsuperscript{188,189}. In the context of atherosclerosis, TLR2 and TLR4 have been extensively studied. Deletion of TLR2 in both \textit{ldlr}\textsuperscript{-/-} mice and \textit{apoE}\textsuperscript{-/-} mice shows a protective effect against atherosclerosis\textsuperscript{190,191}. \textit{ApoE}\textsuperscript{-/-} mice lacking TLR4 also confirm that attenuation of TLR-mediated inflammation prevents atherosclerosis progression\textsuperscript{192}. Heterodimers of TLR4 and TLR6 assemble as a result of CD36
signals triggered by oxLDL, and activate NF-κB and its downstream proinflammatory factors\textsuperscript{193}. Other TLRs may also participate in atherosclerosis\textsuperscript{194}. For example, TLR3 and TLR9 bind to murine cytomegalovirus, which has been reported to cause early atherosclerotic lesions\textsuperscript{195}. TLR7 and its ligand Coxsackie B virus also promote lipid accumulation\textsuperscript{196}, but solid proof from animal studies is needed in order to confirm their roles in atherosclerosis.
Figure 1.5 Signaling pathways in macrophages that are involved in inflammation.

Various TLRs are activated and trigger downstream pro-inflammatory cascades. NALP3-inflammasome pathways are also activated and contribute to the pro-inflammatory effects of macrophages. Reprinted with permission from Moore, K. J. & Tabas, I. Macrophages in the pathogenesis of atherosclerosis. Cell 145, 341–355 (2011).
Studies on common TLR signaling adaptor proteins also support the involvement of TLR signaling in atherosclerosis progression. Myeloid differentiation factor 88 (MyD88)-deficient mice develop less atherosclerotic plaques on ApoE\(^{-/-}\) models\(^{192,197}\). Similar results are seen in mice lacking TIR-domain-containing adapter-inducing interferon-β (TRIF)\(^{198}\), TNF receptor-associated factor 6 (TRAF6)\(^{199}\) and IL-1 receptor-associated kinase 4 (IRAK4)\(^{200}\).

Besides the traditional TLR signaling pathways, recently, macrophage inflammasome signaling has gained a lot of attention. Both human and mouse macrophages can take up cholesterol crystals, which triggers lysosomal destabilization and the release of proteases and ROS into the cytosol, and in turn induces NOD-, LRR- and pyrin domain-containing 3 (NLRP3) inflammasome\(^{201}\). The inflammasome will then process and release IL-1β and IL-18, both of which are atherogenic. In addition to the uptake of cholesterol crystals, lipid-loaded macrophages can produce intracellular cholesterol crystals via de novo pathways\(^{202}\). This process requires CD36 and triggers NLRP3 inflammasome as well. Reconstitution of macrophages from NLRP3-null mice to ldlr\(^{-/-}\) mice results in reduced plaque formation\(^{201}\). However ubiquitous deletion of NLRP3 in ApoE\(^{-/-}\) mice does not show any difference in the development of atherosclerosis\(^{203}\). The real reason behind this discrepancy is unclear, but it is a good example showing the importance of cell specificity in atherosclerosis studies. To study the role of macrophages, the bone marrow transplantation models may be better than ubiquitous knockout models. An even more appropriate model would be a myeloid-specific knockout mouse. These issues will be discussed below in the context of studies carried out in my projects (Chapter 3).
1.3.5 Macrophage apoptosis and efferocytosis

Macrophages and other cells may undergo programmed cell death, or apoptosis. Apoptotic cells are then taken up by nearby macrophages and other phagocytes, which are called efferocytes, and this process is called efferocytosis. This is a typical process that happens in the progress of inflammation. Efferocytosis is considered as a key step promoting the resolution of inflammation\textsuperscript{204}. Efferocytosis benefits the anti-inflammatory process in at least two ways\textsuperscript{204}: (1) It clears apoptotic cells and cell fragments before any toxic substance leaks out. (2) It induces the expression of anti-inflammatory factors, such as IL-10 and TGF-β. During this process, the survival of efferocytes themselves is promoted, which guarantees they do not become apoptotic cells and need the attention of other efferocytes.

Macrophage apoptosis and efferocytosis happen throughout the chronic progress of atherosclerosis\textsuperscript{205}. Macrophage apoptosis can be induced by various factors including the lack of sufficient growth factors, the activation of death receptor signaling pathways, as well as oxidative stress\textsuperscript{206}. Apoptosis alone is not sufficient to result in any detrimental consequence, but the balance between apoptosis and efferocytosis regulates atherosclerosis progression\textsuperscript{207}. The widely accepted hypothesis is that in early lesions, macrophage apoptosis and efficient efferocytosis protect against atherosclerosis (by resolving inflammation), whereas in advanced lesions, enhanced apoptosis and defective efferocytosis contribute detrimentally to the disease by promoting secondary necrosis (Figure 1.6)\textsuperscript{107}. In other words, the enhancement of apoptosis and deficiency of efferocytosis is the turning point of atherosclerosis development.
Figure 1.6 Macrophage apoptosis and efferocytosis.

A. In early lesions, sufficient efferocytosis resolves inflammation and decrease plaque progression. B. In advanced lesions, efferocytosis is impaired and macrophage apoptosis will lead to secondary necrosis, which in turn promotes the formation of necrotic lipid core. Reprinted with permission from Tabas, I. Macrophage death and defective inflammation resolution in atherosclerosis. Nat. Rev. Immunol. 10, 36–46 (2010).
The enhanced apoptosis in advance stage is often associated with prolonged ER stress\textsuperscript{208}. In atherosclerosis, ER stress is usually caused by excessive free cholesterol and subsequent activation of the unfolded protein response (UPR)\textsuperscript{209}. Prolonged expression of C/EBP homology protein (CHOP), a marker of UPR activation, leads to the activation of apoptotic cascades in macrophages\textsuperscript{210}. Deficiency of CHOP in both \textit{ApoE}\textsuperscript{-/-} and \textit{Ldlr}\textsuperscript{-/-} mice shows a reduced apoptosis and secondary necrosis\textsuperscript{211}. In humans, expression levels of CHOP and the apoptosis rate correlate throughout the different stages of coronary atherosclerotic lesions\textsuperscript{212}. Some \textit{in vivo} studies have shown that ER stress \textit{in vivo} is subtle and is not likely to cause apoptosis by itself, but it sets the stage for the “second hit” by triggering SR and/or TLR signaling pathways\textsuperscript{213}.

Defective efferocytosis, on the other hand, is another key event switching the inflammation from reversible to irreversible. Comparing advanced plaques and tonsils in humans reveals that, in tonsils, phagocytes accompany most apoptotic cells, while in advanced plaques, there are many free apoptotic cells\textsuperscript{214}. This observation provides direct evidence showing efferocytosis is indeed impaired in advanced atherosclerosis. The mechanism of defective efferocytosis remains unknown, however many studies have provided some possible explanations. First, oxidative stress suppresses efferocytosis by impairing cholesterol efflux in phagocytes\textsuperscript{215}. Oxidized phosphatidylserine, an apoptotic marker and a ligand for efferocytes on apoptotic cell surfaces, is hydrolyzed by phospholipase in advanced lesions, which in turn damages the recognition of apoptotic cells\textsuperscript{216}. Efferocytosis receptor Mer receptor tyrosine kinase (MerTK) can be cleaved in the extracellular domain by MMPs that are abundant in advanced lesions. The resulting soluble MerTK inhibits apoptotic cell clearance\textsuperscript{217}. Lastly, exposure of macrophages to saturated fatty
acids leads to reduced phagocytosis, which is partly due to structural changes at the plasma membrane\textsuperscript{218}.

Macrophage plasticity may regulate the balance of apoptosis and efferocytosis. Based on expression profile (especially surface receptor expression levels), efferocytosis is a function of M2 macrophages (section 1.3.2). However, surprisingly, M2 macrophages are more sensitive to oxidative stress induced lipotoxicity\textsuperscript{219}. It is also plausible that in late stages of atherosclerosis the microenvironment drives macrophages towards M1 or other subtypes, and as a result, efferocytosis is impaired due to lack of M2 phenotype. Again, a careful investigation on the distribution of M1/M2/other subtypes throughout different stages of atherosclerotic lesions will be informative and helpful.

### 1.3.6 Macrophages in advanced atherosclerosis

In advanced atherosclerosis, in addition to necrotic core formation, macrophages also contribute to the regulation of plaque stability. The advanced lesions are covered by fibrous cap and the thickness of the cap correlates with the stability of the plaque. Macrophages secrete MMPs, which can degrade various types of extracellular matrix (ECM)\textsuperscript{220}. Many studies have shown that deletion of MMPs strengthens plaque stability in mouse models\textsuperscript{221}. Similar observations are made in tissue inhibitor of metalloproteinase (TIMP) transgenic mice, which would also have reduced MMP activity\textsuperscript{222}. MMP release is highly related with macrophage phenotypes. M1 driving factors such as LPS upregulate MMPs release via NF-κB dependent and independent signaling pathways\textsuperscript{220}. On the other hand, M2 factors like IL-4 and IL-10 inhibits MMPs through
PPAR-γ and LXR pathways. In addition to MMPs, macrophage-derived serine proteases and cysteine proteases also contribute to the degradation of elastin and collagen.

Macrophages also indirectly regulate plaque stability by regulating smooth muscle cells, which are the main source of fibrous cap collagen. In advanced lesions, macrophages trigger apoptosis of SMCs via activation of Fas apoptotic pathways and/or secretion of apoptotic factors such as TNF-α and nitric oxide (NO). On the other hand, defective efferocytosis significantly reduces the production of TGF-β, a potent inducer of collagen synthesis in SMCs. Apparently, macrophage heterogeneity plays a role here as well. TNF-α is a classical M1 marker whereas TGF-β is a M2 marker. As mentioned before, mouse models are not perfect for studying plaque stability since their plaques do not rupture. The creation of new mouse models that undergo plaque rupture would be very helpful in advanced atherosclerosis studies.

1.4 Oxidized low-density lipoprotein and ceramide 1-phosphate in atherosclerosis

1.4.1 Lipids and lipoproteins

Lipids, in general, are a group of hydrophobic or amphiphilic molecules that occur naturally. These molecules participate in various physiological and pathological processes, such as forming membranes, storing energy as well as signaling. However unlike genes and proteins, which have been more extensively studied in genomic and proteomic projects, these molecules and their biological functions have been under-emphasized until recently. With more extensive use of mass spectrometric techniques, a comprehensive classification system for lipids has been reported. Based on the core structure of their carbon backbones, lipids have been categorized into 8 groups: fatty acyls, glycerolipids, glycerophospholipids, spingolipids, sterol lipids, prenol
lipids, saccharolipids, and polyketides\textsuperscript{229,230}. Due to their hydrophobic property, lipids in free form cannot be found or transported in aqueous phases. In plasma, lipids are associated with apolipoproteins to form water-soluble complexes, namely lipoproteins. Based on hydrated density, lipoproteins can be classified into 4 categories: chylomicron (CM, d<0.94 g/mL), very low-density lipoprotein (VLDL, 0.94<d<1.006 g/mL), low-density lipoprotein (LDL, 1.020<d<1.063 g/mL), and high-density lipoprotein (HDL, 1.063<d<1.21 g/mL)\textsuperscript{231}. Based on this classification, preparative ultracentrifugation can be used for separation of lipoproteins\textsuperscript{232}. Another widely used method of classification is based on lipoprotein mobility on agarose gel electrophoresis. Mature HDL shows α migration, VLDL and nascent HDL show pre-β migration, and LDL shows β migration\textsuperscript{227,233}. Plasma lipoproteins not only facilitate transport of lipids in the circulation, but also have various functions in signaling. Among numerous types of lipids and lipoproteins, our laboratory has focused our interests on LDL (especially oxLDL) and sphingolipids, both of which play critical roles in the progression of atherosclerosis, as discussed below.

1.4.2 Oxidized low-density lipoprotein in atherosclerosis

1.4.2.1 Definition of oxidized low-density lipoprotein

Oxidized low-density lipoprotein, as suggested by its name, refers to a product of circulating LDL that undergoes oxidative modification. As we know, LDL is a complex particle containing various types of lipids and apolipoprotein. Both lipid and protein components could be potentially oxidized during the modification\textsuperscript{42}. In general, oxidized LDL can be sub-divided into minimally modified LDL (MM-LDL) and extensively modified LDL. MM-LDL contains lipid peroxides or their degradation products, but no protein modifications. This type of oxLDL is still
recognized by LDL-R, but not scavenger receptors\textsuperscript{234}. Although the difference between MM-LDL and native LDL is minimal, it is sufficient to develop atherogenic properties\textsuperscript{49,234,235}. More often, when referring to oxidized LDL (as in this dissertation), it means extensively oxidized LDL. This type of oxLDL bears drastic changes on both lipid and protein components, and is no longer recognized by the LDL-R. Extensively oxidized LDL is involved in many events during atherosclerosis progression, which will be discussed later in this section. Oxidants like myeloperoxidase (MPO) or peroxynitrite generate oxidized LDL that has more protein oxidation and less lipid oxidation, but is considered as extensively oxidized LDL\textsuperscript{236}. In some cases, lipid peroxides such as malondialdehyde (MDA) generated from platelets or elsewhere in the body can bind to circulating LDL. The associated LDL shares at least partial biological properties with oxLDL and is often considered as oxidized LDL as well\textsuperscript{237}. So a better definition of oxidized LDL will be: circulating LDL-derived particles that contain peroxides or their degradation products.

\textbf{1.4.2.2 Potential routes of LDL oxidation}

When cultured \textit{in vitro} with endothelial cells, macrophages and smooth muscle cells, LDL undergoes oxidization rapidly\textsuperscript{13,238}. This process may involve both enzymatic and non-enzymatic reactions. The exact route for LDL oxidation \textit{in vivo} remains unclear, but many candidates are proposed: 1. \textit{Transition metals such as iron and copper}. High concentrations of free metal ions can oxidize LDL \textit{in vitro}\textsuperscript{239}. Chelators of transition metals can block the cell-mediated LDL oxidation mentioned above\textsuperscript{42,238}. A direct correlation between iron levels and cholesterol accumulation in human artery samples has been reported as well\textsuperscript{240}. 2. \textit{Lipoxygenase}.

Lipoxygenase is one of the intracellular oxidation enzymes that directly oxidize polyunsaturated
fatty acids\textsuperscript{241}. In human atherosclerotic lesions, co-localization of lipoxygenase and oxidized LDL has been observed\textsuperscript{242,243}. The co-localization and subsequent oxidation were possibly mediated by low-density lipoprotein receptor-related protein (LRP)\textsuperscript{244-246}. Deficiency of lipoxygenase in mice compromises atherosclerosis progression\textsuperscript{247-249}. Meanwhile, overexpression of lipoxygenase accelerates atherosclerosis\textsuperscript{250}. Although lipoxygenase is considered to be atherogenic in general, some studies have shown a protective role of lipoxygenase, which makes its role in atherosclerosis a matter of discussion\textsuperscript{251}.

3. **Myeloperoxidase (MPO).** MPO catalyzes the formation of hypochlorous acid (HOCl) from hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}) and chloride. Typically HOCl plays a role in host defense against pathogens, however it is also a highly reactive oxidant. MPO has been found co-localized with foam cells in human atherosclerotic lesions\textsuperscript{252}. Moreover, 3-chlorotyrosine, a marker of MPO/HOCl-mediated oxidation, is increased in LDL isolated from human atherosclerotic plaques\textsuperscript{253}. Epidemiological studies have identified MPO as an independent risk factor for cardiovascular disease\textsuperscript{254}. Similar with lipoxygenase, animal studies on MPO have been controversial. Overexpression of human MPO in mouse promotes atherosclerosis\textsuperscript{255}. However, MPO-deficient mice also show an increased atherosclerosis\textsuperscript{256}.

4. **NO and ROS.** In the presence of superoxide, NO reacts with it to form peroxynitrite (ONOO\textsuperscript{−})\textsuperscript{257,258}. There is evidence showing involvement of NADPH oxidase\textsuperscript{259,260}, as well as XO\textsuperscript{30}. Uncoupled mitochondrial respiratory chain\textsuperscript{261}, and uncoupled endothelial nitric oxide synthase (eNOS)\textsuperscript{262} can also contribute to superoxide formation, and in turn promote oxidation of LDL.
1.4.2.3 Functions of oxLDL in atherosclerosis progression

The response-to-oxidation theory claims that oxidation of LDL plays a crucial (if not required) role in atherosclerosis initiation. It is well established that oxLDL is engulfed by macrophages via scavenger receptors to form lipid-laden foam cells (section 1.1.2.4 and section 1.3.3). However, rather than being passively taken up by macrophages, oxLDL has many other functions beyond that. First of all, oxLDL components (such as lysophosphatidylcholine) can act as chemotactic factors for monocytes. Monocytes are then bound to oxLDL-stimulated endothelial cells via ICAM-1 and VCAM-1. OxLDL also stimulates macrophage proliferation, as well as inhibits macrophage apoptosis, via multiple signaling pathways (Figure 1.7). Specifically, oxLDL prevents macrophage apoptosis following MCSF withdrawal by inhibiting acid sphingomyelinase, which in turn prevents ceramide generation, as well as by activating the PI3K/Akt pathway. Blockage of eEF2K signaling pathways also impairs oxLDL-mediated macrophage survival. All of these effects of oxLDL may promote the accumulation of macrophages in the intima and set the stage for foam cell formation. Besides foam cell formation, oxLDL contributes to atherosclerosis progression by regulating SMC migration and proliferation via the stimulation of PDGF and fibroblast growth factor (FGF) respectively. Contradictory effects of oxLDL have been revealed in fibrous cap regulation. OxLDL stimulates collagen production, which protects plaque from rupture. However, on the other hand, oxLDL leads to the release of MMPs and apoptosis of SMCs, both of which contribute to fibrous cap degradation.
Figure 1.7 OxLDL mediated macrophage survival.

Receptors mediating these functions remain unclear. Scavenger receptors like CD36 and LOX-1 may be involved and responsible for not only lipid uptake, but also signal transduction\textsuperscript{276-278}. However as pointed out by our laboratory, oxLDL-mediated macrophage survival is not altered in the absence of most of the major pattern recognition receptors\textsuperscript{279}.

1.4.2.4 Human findings and clinical trials of oxLDL in atherosclerosis

The progression of oxidation produces immunogenic epitopes in LDL particles, which leads to the generation of autoantibodies against oxLDL. These antibodies are detectable in human atherosclerotic lesions and can be used as a biomarker of LDL oxidation\textsuperscript{280,281}. Cohort experiments investigating the association between LDL oxidation and cardiovascular events have revealed highly conflicting results. 14 out of 22 cohort studies have shown a positive association between oxLDL measurement and cardiovascular events, whereas the other 8 studies have shown no association (summarized by Maiolino\textsuperscript{282}). Clinical trials using antioxidants cannot agree on any conclusion as well; many have shown a beneficial effect of the therapy whereas others have not\textsuperscript{282}. This discrepancy can be partly explained by different parameters used in these trials, such as the intervention start time, the length of follow up period, even the control group selection\textsuperscript{282}. One more factor to mention is that most of clinical trials used Vitamin E as the antioxidant. However, the efficacy of vitamin E to suppress LDL oxidation in humans has not been confirmed. More potent or selective antioxidants may be important to test in future studies.
1.4.3  Ceramide 1-phosphate in atherosclerosis

1.4.3.1  Ceramide and ceramide 1-phosphate metabolism

Ceramides are a group of lipids consisting of a sphingosine backbone that is N-acylated with fatty acids. They play a central role in the metabolism of sphingolipids, which are building blocks of eukaryotic cell membranes. The de novo synthesis of ceramides starts with the condensation of serine and palmitoyl CoA to form 3-ketosphinganine in the endoplasmic reticulum (ER). This reaction is catalyzed by serine palmitoyltransferase (SPT). 3-ketosphinganine is then reduced to form sphinganine, which is N-acylated to form dihydroceramides, which in turn are converted to ceramides by the action of desaturases (Figure 1.8). In cells, ceramide can be generated through hydrolysis of sphingomyelin (SM) and glucoceramides via sphingomyelinase (SMase) and glucoceramidase respectively. Additionally a so-called salvage pathway also contributes to ceramide generation. Complex sphingolipids can be degraded in the lysosomes and form sphingosine, which can then leave the lysosome and be converted back to ceramide in the cytosol.
Ceramide can be synthesized through *De novo* synthesis pathway as well as salvage synthesis pathway. In mammalian cells, the only known synthesis pathway for ceramide 1-phosphate is phosphorylation of ceramide. However SMase D mediated pathway and acylation of sphingosine 1-phosphate may also be possible. Reprinted with permission from Gomez-Muñoz, A. et al. New insights on the role of ceramide 1-phosphate in inflammation. Biochim. Biophys. Acta 1831, 1060–1066 (2013).
Ceramide 1-phosphate (C1P) is primarily generated by phosphorylation of ceramide catalyzed by ceramide kinase (CerK)\textsuperscript{283}. Although this is the only known mechanism in mammalian cells, the observation of significant levels of C1P in CerK-null macrophages indicates the existence of other mechanisms\textsuperscript{284}. Two possible pathways may involve SMase D mediated cleavage of SM and a putative acyl transferase mediated N-acylation of sphingosine 1-phosphate, both of which are observed in lower animals but have not been reported in mammals thus far.

In the body, C1P acts as a signaling molecule involved in a diverse range of physiological and pathological processes, including inflammation. It is well established that C1P promotes growth and survival in various cell types such as macrophages, myoblasts and fibroblasts\textsuperscript{285-288}. It has also been reported that C1P serves as an apoptosis blocker\textsuperscript{289-291}. C1P antagonizes most ceramide effects in the body, thus the balance between C1P and ceramide is the key in maintaining homeostasis\textsuperscript{292}. Recently, C1P/ceramide pathways have been connected with many diseases including cancer, cardiovascular disease as well as Alzheimer’s disease\textsuperscript{293-296}.

\textbf{1.4.3.2 Ceramide 1-phosphate in inflammation}

The direct evidence of C1P’s involvement in atherosclerosis has not been revealed. However, it is well known that C1P participates in inflammation\textsuperscript{297}. C1P stimulates arachidonic acid (AA) release, the rate-limiting step of eicosanoids synthesis, via the direct activation of cytosolic phospholipase A2 (cPLA\textsubscript{2})\textsuperscript{298}. This process is regulated by a specific C1P transfer protein (CPTP), the depletion of which results in the retention of C1P in Golgi complex that in turn promotes inflammation by accelerating AA release\textsuperscript{299}. C1P-regulated AA release can also coordinate with S1P-induced cyclooxygenase-2 (COX-2) activity to synthesize prostaglandins\textsuperscript{300}. 
Besides the regulation of AA release, C1P contributes to inflammation in many other ways. C1P potently induces macrophage migration via a G protein-coupled receptor (GPCR) that can be inactivated by pertusis toxin (PTX). Additionally, C1P also stimulates phagocytosis in neutrophils and induces degranulation in mast cells.

C1P’s role in inflammation becomes complicated when one considers the increasing evidence suggesting that C1P can have anti-inflammatory properties. First, C1P counteracts the pro-inflammatory effects of ceramide by inhibiting the accumulation of Acid SMase-derived ceramides. Second, C1P negatively regulates TNF-α production by inhibiting TNF-α converting enzyme (TACE). Moreover, C1P applied exogenously suppresses LPS-induced NF-κB transcription and subsequent production of IL-6, IL-8 and IL-1β. PCERA-1, a C1P analog, has been shown to suppress TNF-α expression and induce IL-10 production via cAMP-PKA pathways. Recently, the same group reported that PCERA-1 also inhibits cPLA2 and consequent prostaglandin formation. These studies shed light on potential therapy using C1P analogs to prevent inflammatory diseases, including atherosclerosis.

1.5 Eukaryotic elongation factor-2 kinase

Eukaryotic elongation factor 2 (eEF2) kinase was first discovered as a Ca<sup>2+</sup>/calmodulin dependent protein kinase III (CaMKIII) which was purified together with its ~100kDa substrate from a variety of mammalian tissues. Not long after its discovery, the substrate was identified as eEF2, and the kinase was then named eukaryotic elongation factor 2 kinase (eEF2K). After thirty years of research, eEF2 is still the only known substrate for eEF2K in the body. eEF2 plays a crucial role in protein elongation by facilitating the translocation of
peptidyl-tRNA from the A-site to P-site in ribosomes\textsuperscript{315}. Inhibitory phosphorylation of eEF2 by eEF2K impairs protein synthesis by reducing the affinity of eEF2 for the ribosome complex\textsuperscript{316,317}. This regulatory mechanism has been found throughout eukaryotic organisms from yeast to mammals\textsuperscript{318-320}. While it has been reported that Thr53, Thr56 and Thr58 on eEF2 can be phosphorylated by eEF2K \textit{in vitro}, Thr56 and Thr58 are the main functional sites\textsuperscript{321,322}. Phosphorylation of these two sites is an ordered process, in which phosphorylation of Thr56 is the first event, which is required for subsequent Thr58 phosphorylation\textsuperscript{322}. However, mono-phosphorylation of Thr56 is also sufficient for suppressing the rate of protein synthesis\textsuperscript{322}. More recently, a novel phosphorylation site at Ser595 was discovered. Phosphorylation of Ser595 promotes, and is required for, efficient phosphorylation of Thr56\textsuperscript{323}. In reversing this process, protein phosphatase 2A (PP2A) is the main phosphatase responsible for the dephosphorylation of eEF2 on Thr56\textsuperscript{322,324}.

\subsection*{1.5.1 Structure of eEF2K}

The majority of eukaryotic protein kinases share 12 conserved subdomains within their catalytic domain\textsuperscript{325}. However, cloning and sequencing of eEF2K revealed a novel family of protein kinases whose catalytic domain does not have significant homology to conventional eukaryotic kinases\textsuperscript{326,327}. The members of this family, including eEF2K, MHCKA\textsuperscript{328}, ChaK1 and ChaK2\textsuperscript{329}, phosphorylate amino acid residues located in \(\alpha\)-helices, and thus they were named ‘\(\alpha\)-kinases’\textsuperscript{330}. Phosphorylation on threonine residues in \(\alpha\)-helices (such as Thr56 in eEF2) can destabilize the \(\alpha\)-helices and lead to a conformational change of the substrate protein structure, which in turn affects the function of the substrate\textsuperscript{331}.
The general structural features of eEF2K are depicted in Figure 1.9. As in other α-kinases, the catalytic domain is located near the N-terminus\textsuperscript{332,333}. Immediately N-terminal of the catalytic domain is the Ca\textsuperscript{2+}/calmodulin binding domain\textsuperscript{332}. The function of the very end of the N-terminus is still unknown. Four predicted α-helices lie in the C-terminal region, which resemble Suppressor/Enhancer of Lin-12 (SEL)-1 like domains\textsuperscript{334}. The extreme C-terminus is a highly conserved area, which is important in phosphorylation of eEF2 but is not directly involved in phosphorylation. Thus this area is predicted to be responsible for eEF2 interaction\textsuperscript{332,334}.
Figure 1.9 Structure and up-stream signaling pathways of eEF2K.

Catalytic domain lies near the N-terminal with a calcium/calmodulin-binding domain further towards N-terminal. The extreme of C-terminal is an eEF2 binding domain with a SEL1-like domain lies nearby. Pathways leading to activation and inhibition of eEF2K have been discussed in detail in the text. In brief, Calcium/Calmodulin, AMPK and PKA activate eEF2K by phosphorylating multiple Serine and Threonine sites. By contrast, various regulatory factors, such as mTOR, Erk1/2 and p38 MAPK, lead to negative phosphorylation events on eEF2K.
1.5.2 Regulation of eEF2K

eEF2K, as a key factor in regulation of the protein elongation process, is tightly regulated by multiple signaling pathways (summarized in Figure 1.9). First of all, as a Ca\(^{2+}\)/calmodulin-dependent protein kinase, elevation of intracellular Ca\(^{2+}\) concentration activates eEF2K following Ca\(^{2+}\)/calmodulin binding. Upon Ca\(^{2+}\) stimulation, eEF2K must undergo extensive auto-phosphorylation before it becomes active\(^{335,336}\). Multiple serine and threonine sites, such as Thr348, Thr353, Ser445, Ser474 and Ser500, are rapidly auto-phosphorylated\(^{337,338}\). The phosphorylation of these residues requires the putative SEL-1 like domains of eEF2K\(^{334}\). The auto-phosphorylation allows eEF2K to retain Ca\(^{2+}\)-independent kinase activity, since the kinase remains active even after the intracellular Ca\(^{2+}\) concentration returns to basal levels\(^{339}\). cAMP-dependent protein kinase (PKA) phosphorylates eEF2K at Ser500 which is also able to induce Ca\(^{2+}\)-independent kinase activity\(^{339,340}\). This regulatory pathway has been confirmed in various cell lines\(^{341-343}\). In cardiomyocytes, AMP-activated protein kinase (AMPK) was shown to activate eEF2K by phosphorylating Ser398\(^{344,345}\). This is considered to be a protective mechanism during hypoxia-induced ER stress and hypertrophy\(^{346,347}\). In addition to regulation via eEF2K phosphorylation, it has been shown that AMPK can also directly phosphorylate eEF2 and thereby suppress protein synthesis\(^{348,349}\), but this has not been universally observed.

In many physiological processes, where protein synthesis rate is upregulated, eEF2K must undergo inhibitory phosphorylation. As a consequence, dephosphorylation of eEF2 activates its GTP-dependent translocase activity and promotes translational elongation. In 1996, Redpath and colleagues found that insulin inhibits eEF2K activity via a rapamycin-sensitive pathway\(^{350}\). As the central factor in rapamycin-sensitive pathways, mammalian target of rapamycin (mTOR)
plays a key role in negative regulation of eEF2K. Activation of downstream kinase p70S6K leads to the inhibitory phosphorylation of eEF2K at Ser366\(^{351}\). Through an as yet unidentified pathway, mTOR can also induce phosphorylation of Ser78, which impairs the binding of calmodulin to eEF2K\(^{352}\). The CDK1-cyclin B complex phosphorylates eEF2K at Ser359, which may also be regulated by mTOR\(^{353}\). In addition to mTOR pathways, p70S6K can be activated by PDK1\(^{351}\). PDK1 and erk1/2 activate p90\(^{RSK}\), which phosphorylates the same Ser366 site as p70\(^{S6K}\)\(^{351}\). Under stress conditions, SAPK2/p38\(\alpha\) and SAPK4/p38\(\delta\) phosphorylate eEF2K on Ser359 and Ser396, which suppress its activity\(^{354,355}\). Coincidentally, phosphorylation of Ser377 via MAPKKAP-K2/K3 was also observed. However the functional role of this phosphorylation site remains unknown\(^{355}\). In addition to regulating eEF2K activity, phosphorylation can also affect eEF2K protein stability. A point mutation of the AMPK phosphorylation site Ser398 to an alanine lengthens the half life of eEF2K from 6-8 hours to more than 24 hours\(^{356}\).

In addition to post-translational modifications, eEF2K is regulated at other levels. Heat shock protein 90 (HSP90), a well-characterized chaperone, can be co-purified with eEF2K\(^{357,358}\). Disruption of the eEF2K/HSP90 interaction decreases the total cellular level of eEF2K\(^{359}\). This process is mediated by the ubiquitin-proteasome pathway\(^{360}\). A recent paper shows that the proteasomal degradation of eEF2K is further regulated by PKA and SCF\(\beta^{TRCP}\) ubiquitin E3 ligase\(^{361}\). This discovery matches the early reports which showed that under the stimulation of nerve growth factor (NGF), PKA negatively regulates eEF2K expression level\(^{362,363}\). Last but not least, eEF2K is regulated by environmental pH. Compared to neutral pH (7.2-7.4), a slightly acidic pH (6.6-6.8) increases eEF2K activity by several fold\(^{364}\).
1.5.3 **Physiological and pathological roles of eEF2K**

It may not be surprising that a kinase such as eEF2K, whose activity plays a key role in modulating protein translation, is involved in the regulation of multiple physiological and pathological events. Here we summarize eEF2K’s role in a number of disease processes that have been reported to date.

1.5.3.1 **eEF2K in cancer**

The first report of eEF2K’s involvement in cancer dates back to 1993 in a report showing the detection of eEF2 phosphorylation in proliferating malignant glial cells\(^{365}\). Subsequently, the same group showed that phosphorylation of eEF2 is detectable in normal proliferating cells, but not in non-proliferating cells\(^{366}\). The phosphorylation of eEF2 is regulated by eEF2K, which has been found to be constitutively activated in multiple cancer cells\(^ {367,368}\). During nutrient depletion, activated eEF2K phosphorylates eEF2 and slows down protein synthesis in order to save energy and keep cells alive\(^ {369}\). This is one mechanism used by hibernating animals to overcome harsh environments\(^ {370}\). A recent study has shown in greater detail how cancer cells have likely adopted this mechanism to overcome the nutrient deprivation during tumorigenesis\(^ {371}\).

1.5.3.2 **eEF2K in neuronal development and disease**

eEF2K also plays a role in many neuronal processes. The expression of eEF2K mRNA in nervous system starts at as early as E13, and is transiently up-regulated in the cerebral cortex and hippocampal pyramidal cell layer during late embryonic and early postnatal stages\(^ {372}\). Specifically, a study on nerve growth cones showed that eEF2K regulated the local
phosphorylation level of eEF2 and controlled neurite outgrowth\(^{373}\), indicating eEF2K may be involved in early neuronal development.

Synaptic plasticity, especially long-term plasticity, requires *de novo* protein synthesis. Thus, eEF2K-mediated translational regulation is proposed to be one of the mechanisms regulating neuronal plasticity. During chemical long-term potentiation (LTP), 1 hour after induction, eEF2K-mediated phosphorylation of eEF2 inhibits local protein synthesis and contributes to persistent forms of LTP\(^{374}\). On the other hand, group I metabotropic glutamate receptors induced long-term depression (mGluR-LTD), which was also found to depend on eEF2K, which binds to the mGluR. After induction of LTD, eEF2K dissociates when mGluR is activated, allowing it to phosphorylate eEF2. The subsequent slow-down of protein synthesis increases Arc/Arg3.1 translation, which is essential for mGluR-LTD\(^{375}\). eEF2K also regulates dendritic spine stability and synaptic structure by modulating activity-dependent dendritic BDNF synthesis, coupling neuronal activity to spine plasticity\(^{376}\). A study using Aplysia showed that eEF2K was involved in 5-hydroxytryptamine (5-HT)-induced long-term facilitation (LTF)\(^{377}\), which also reinforces the evolutionary conservation of eEF2K function in neuronal activity.

1.5.3.3 **eEF2K in cardiovascular disease.**

eEF2K’s role in cardiovascular disease is still not fully understood. In 2009, our laboratory provided the first potential link between eEF2K and atherosclerosis\(^{269}\). Oxidized low density lipoprotein (oxLDL), a key pathogenic factor in atherosclerosis, activates eEF2K by triggering intracellular calcium oscillation in macrophages\(^{269}\). Activated eEF2K promotes macrophage survival, which in turn may facilitate foam cell formation, a critical cellular marker in
atherosclerotic pathogenesis\textsuperscript{269}. Lethal irradiated LDL-R\textsuperscript{−/−} mice were reconstituted by bone marrow transplantation from either eEF2K kinase inactive (eEF2K-KI) or wild-type (WT) mice, and then a high fat diet was given for 16 weeks, which allowed the development of atherosclerosis. Compared to the WT group, chimeric mice with a reconstituted hematopoietic compartment having suppressed eEF2K activity showed a significant reduction of atherosclerotic plaque formation\textsuperscript{378}. These studies are described in detail in Chapter 3 below. Although the detailed mechanism by which eEF2K mediates this effect will require further investigation, these results immediately predict a potential use for eEF2K inhibitors in the treatment of atherosclerosis.

In other studies, eEF2K has also been shown to be up-regulated in endothelial cells in a hypertension disease model\textsuperscript{379}, supporting additional roles for eEF2K in cardiovascular disease. eEF2K was found to contribute to hypertension development by regulating vascular inflammation. Targeted silencing of eEF2K by interfering RNA inhibited the induction of VCAM-1 and E-selectin as well as monocyte adhesion\textsuperscript{379}.

\subsection*{1.5.4 Pharmaceutical approaches targeting eEF2K}

Other than known calmodulin antagonists, rottlerin was the first reported inhibitor for eEF2K, efficiently suppressing eEF2K mediated eEF2 phosphorylation with an IC\textsubscript{50} of 5.3μM\textsuperscript{380}. However, it is not an ideal inhibitor due to its lack of specificity. Rottlerin inhibits PKC-δ with an IC\textsubscript{50} of 3-6μM, thus having similar potency compared to its effects on eEF2K\textsuperscript{381}. Besides its non-selective inhibition, rottlerin participates in many other cellular processes, such as activation
of multiple Ca$^{2+}$ sensitive K$^+$ channels and uncoupling of mitochondria, which in turn activates AMPK and affects reactive oxygen species (ROS) production\textsuperscript{382}.  

With an IC$_{50}$ of 60nM \textit{in vitro}, NH125 was thought to be the most potent eEF2K inhibitor\textsuperscript{383}. Soon after its discovery, NH125 was reported to have anti-tumor efficacy against a broad spectrum of human cancer cell lines\textsuperscript{383,384}. Since then, NH125 has been used in numerous research studies\textsuperscript{379,385,386}. Recently, Chen et al. claimed that NH125 had different effects on cancer cells compared to siRNA interference and the eEF2K inhibitor A-484954\textsuperscript{387}. Surprisingly, NH125 increased eEF2 phosphorylation in various cell lines\textsuperscript{387}, which is the opposite effect expected of an eEF2K inhibitor. This finding was further supported by an independent group, which showed that NH125 is a weak inhibitor of eEF2K in a standard \textit{in vitro} kinase assay (IC$_{50}$=18μM)\textsuperscript{388}. The latter study also showed that instead of inhibiting eEF2K activity, NH125 induced phosphorylation of eEF2\textsuperscript{388}. The real effect of NH125 on eEF2K signaling pathway therefore remains unclear. A revised interpretation of previous work using NH125 as an eEF2K inhibitor is clearly necessary.

TS-2 and TS-4, which are 1,3-selenazine derivatives, have been reported to inhibit eEF2K (IC$_{50}$=0.36μM for TS-2 and IC$_{50}$=0.31μM for TS-4), with activity that is more efficient and more specific than rottlerin\textsuperscript{389}. However, for unknown reasons, these inhibitors are not widely available. The same group later discovered that TX-1918 is a potent eEF2K inhibitor with an IC$_{50}$ of 0.44μM during a screening of protein tyrosine kinase inhibitors\textsuperscript{390}. TX-1918 is commercially available but has had limited use, but is one of the inhibitors used in the research study from our laboratory\textsuperscript{269}. Recently, thieno[2,3-b]pyridine analogues have been optimized to
target eEF2K$^{391}$. The most potent candidate showed an IC$_{50}$ of 170nM against eEF2K$^{391}$. However, it has not been tested in any other reports published to date. A small molecule, A-484954, has been proven to inhibit eEF2K in a more selective way with an IC$_{50}$ of 0.28μM$^{387}$. Like TX-1918, A-484954 is available but has been used in only one research study$^{379}$.

### 1.6 Objectives

Work in this dissertation was focused on regulation of macrophage functions by oxLDL and lipid signals such as C1P. Previous work in our laboratory showed that an oxLDL-mediated eEF2K signaling pathway is involved in atherosclerosis, possibly through regulating macrophage apoptosis$^{269}$. However, whether this mechanism applies in animal models was unknown. The objectives of Chapter 3 were to determine how the eEF2K signaling pathway affects the progression of atherosclerosis in vivo. To address this, bone marrow from eEF2K kinase inactive (KI) mice were transplanted to irradiated LDL-R$^{-/-}$ mice. Diet-induced atherosclerosis plaques were then analyzed to determine disease progression and possible pathogenic mechanisms.

Recently, diverse phenotypes of macrophages have been reported to play roles in different physiological and pathological events. Although there are only limited studies of macrophage plasticity in the context of atherosclerosis, oxidized phospholipid has been shown to affect macrophage phenotype$^{155}$. As a more physiologically relevant particle, whether oxLDL contributes to macrophage polarization and how this fits in with atherosclerosis progression became interesting questions. Studies in Chapter 4 were designed to address these issues. The phenotype of oxLDL-polarized macrophages was characterized based on expression profiles.
Possible signaling pathways involved in the polarization, as well as potential functions of these macrophages are also discussed.

Ceramide 1-phosphate, as a lipid messenger, participates in inflammatory diseases in different ways. In our laboratory, we found C1P contributes to atherosclerosis partly through the induction of VEGF release by macrophages. However the underlying signaling pathways remains undefined. The goal of Chapter 5 was to identify signaling pathways involved in C1P-induced VEGF release. Possible receptors were also investigated. To accomplish this goal, pharmaceutical inhibitors were used in these studies.
Chapter 2: Materials and methods

2.1 Materials

Dulbecco’s modified Eagle’s medium (DMEM), Roswell Park Memorial Institute (RPMI)
medium, Iscove’s modified Dulbecco’s Medium (IMDM), fetal bovine serum (FBS), Dulbecco’s
phosphate buffered saline (DPBS), Hanks balanced salt solution, L-glutamine, sodium pyruvate,
penicillin and streptomycin were purchased from GE Healthcare Life Sciences Hyclone
Laboratories (South Logan, UT). Ethylenediaminetetraacetic acid (EDTA), butylated
hydroxytoluene (BHT), LPS, IFN-γ, phenazine methosulfate (PMS), monothioglycerol (MTG),
Tween 20, Triton X-100, sodium bromide, sodium molybdate, sodium orthovanadate,
paraformaldehyde, sucrose, Sudan IV, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
(HEPES) and 10% formaldehyde were obtained from Sigma Aldrich (St. Louis, MO). 3-(4,5-
dimethylthiazol-2-yl)-5- (3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt
(MTS) was from Promega (Madison, WI). Recombinant mouse interleukin-4 (IL-4) and
recombinant mouse macrophage colony-stimulating factor (MCSF) were purchased from
Stemcell Technologies (Vancouver, BC). Lonza (Basel, Switzerland) supplied Endothelial Basal
Medium (EBM) and EGM-2 Bullet Kit. TS-4 was a kind gift from Dr. Y. Uehara (National
Institute of Infectious Diseases, Tokyo, Japan). LY294002 and all other inhibitors used in this
study were purchased from EMD Millipore (Etobicoke, ON). Anti-phospho Akt antibody and
Anti-phospho Erk1/2 antibody were obtained from Stressgen (Enzo Life Sciences, Farmingdale,
NY). Anti-phospho eEF2 antibody was purchased from Cell Signaling Technology (Danvers,
MA).
2.2 Animals

Wild-type C57BL/6 mice (strain code: 027) were purchased from Charles River Laboratories (Sherbrooke, Canada). *eef2k*-KI mice expressing a catalytically inactivate eEF2K kinase gene were originally generated in Dr. Christopher Proud’s lab at University of British Columbia, and were obtained as a gift when Dr. Proud left UBC. These mice were then crossed with WT C57BL/6 for 6 generations to express kinase-inactive eEF2K in the C57BL/6 background. *ldlr*−/− C57BL/6 mice (strain code: B6.129S7-Ldlr<sup>tm1Her</sup>/J, stock number: 002207) were purchased from Jackson Laboratory (Bar Harbor, MA).

2.3 Cell culture

L929 cells (kindly provided by Dr. J.W. Schrader, Biomedical Research Centre, UBC) were seeded in Corning 75cm<sup>2</sup> rectangular canted neck cell culture flask with vent cap (Corning, Tewksbury, MA) at a density of 1.5 x 10<sup>4</sup> cells per cm<sup>2</sup> and cultured in media (DMEM, 10% FBS, 2 mmol/L L-glutamine, 1 mmol/L sodium pyruvate, 50 U/mL penicillin, 50 μg/mL streptomycin) at 37°C with 5% CO<sub>2</sub>. After two weeks, the media was removed and centrifuged at 800 x g for 10 minutes. Then the supernatant was filter sterilized through a 0.22 micron filter. This L929-cell conditioned media (LCM) was then used as a crude source of MCSF containing approximately 10,000 U/mL of activity<sup>392</sup>.

Bone marrow cells were isolated from femurs of 6-8 week old female wild-type or genetically modified mice as previously described<sup>268</sup>. Cells used in Chapter 3 were plated in 10-cm diameter tissue culture plates (Corning, Tewbury, MA) and cultured in DMEM media containing L-glutamine, sodium pyruvate, 10% FBS, and 10% LCM. Non-adherent cells (monocytes) were
then collected, and differentiated into macrophages by a further 5-day incubation in the same media. Cells were washed to remove non-adherent cells and lifted using a rubber cell scraper (Sarstedt, Montreal, QC, Canada). Cells used in Chapter 4 were plated in Corning 75cm² rectangular canted neck cell culture flask with vent cap (Corning, Tewksbury, MA) and cultured in IMDM medium containing 10 ng/mL recombinant MCSF, 150 μM MTG and 10% FBS at 37°C in a 95% humidified atmosphere containing 5% CO₂. Medium was changed at Day 4 and Day 8. Differentiated macrophages were always used for experiments at Day 11. For M1 or M2 or MoL differentiation, 10 ng/mL LPS and 10 U/mL IFN-γ, or 10 ng/mL IL-4, or 25 μg/mL oxLDL were added at Day 8. Cells were lifted using Cell Dissociation Solution (non-enzymatic) from Sigma-Aldrich (St. Louis, MO).

RAW 264.7 cells were purchased from American Type Culture Collection (ATCC, Manassas, VA) and maintained in DMEM supplemented with 10% FBS, 1 mM L-glutamine and sodium pyruvate. Cells were harvested using a rubber cell scraper (Sarstedt, Montreal, QC, Canada).

Human umbilical vein endothelial cells (HuVEC), kindly provided by Dr. Alex Scott (University of British Columbia), were cultured in EBM supplemented with EGM-2 BulletKit at 37°C in a 95% humidified atmosphere containing 5% CO₂. Cells were harvested using a rubber cell scraper (Sarstedt, Montreal, QC, Canada).

2.4 Bone marrow transplantation

The ldlr⁻/⁻ recipients were lethally irradiated using 14 minutes of exposure to 850-rad gamma irradiation (two 7-minute sessions). Donor bone marrow cells were isolated from 6-8 week WT
or *eef2k*-KI mice as previously described. The cells were suspended, counted and diluted to 1 x 10⁷ cells per mL in Hanks balanced salt solution. 200 µL of diluted cell suspension was then injected into the tail veins of the irradiated recipients. Chimeric animals recovered for 4 weeks before starting feeding of the high fat diet (HFD, #D12108C, 20% fat with 1.25% cholesterol, Research Diets Inc., NJ). Animals were routinely monitored, and no major differences in weight or behavior were noted between the groups.

### 2.5 Aorta isolation and cross-sections preparation

Chimeric mice fed with HFD for 8 or 16 weeks were anaesthetized using intraperitoneal injection of 200 µL/10g body weight of 2.5% avertin in phosphate-buffered saline (PBS). Then 1 mL of blood was collected from the heart for serum lipid profiling. After that, the mice were perfused with 10mL of 4% paraformaldehyde, 7.5% sucrose, and 0.5 mM EDTA in PBS and flushed with 10mL of 0.5 mM EDTA in PBS. Hearts were harvested and stored in 10% formaldehyde. Upper portions of the hearts were sent to Wax-It Histology Service Inc. (Vancouver, BC, Canada) for OCT-based cryo-sectioning and hematoxylin and eosin and Oil-Red-O staining. Aortas were harvested by cutting open the mice longitudinally under the dissection microscope using mini-Vanna scissors and forceps. Lipids deposited on the outside were removed carefully without touching the aorta wall. Whole aortas (including the major branches near the aortic root) were then stored in 10% formaldehyde for en face staining.

### 2.6 En face staining

Aortas were cut from iliac arteries (3 mm past the bifurcation) anteriorly till aortic arch, where the cut was angled to the inside curve of the arch. A second cut started from the right carotid
towards the common carotid until it passed the arch area, so that at this point, the aortas can be flattened and resemble a Y shape. Then aortas are pinned onto a standard black wax dissection pan. The pan was filled with PBS to prevent the vessels dehydrating. The aortas were then stained in the following procedures: PBS was drained and replaced by 70% ethanol for 5 minutes. The 70% ethanol was drained and replaced by Sudan IV solution for 15 minutes. The Sudan IV was then drained and the vessels were destained using 80% ethanol for 3 minutes. The stained vessels were rinsed under running water to remove all ethanol. The pan was then filled with PBS before images of aortas were captured. The extent of atherosclerosis is determined by calculating the ratios of lipid-rich area to the total aortic area using ImageJ software.

2.7 H&E staining and Oil-Red-O staining

Cross-sections were obtained from Wax-It Histology Service Inc. (Vancouver, BC, Canada). H&E staining and Oil-Red-O staining were also performed by Wax-It. Images were obtained under microscopes, and were analyzed using ImageJ software. The ratios of lipid-rich area to the total lesion area were calculated to determine the extent of atherosclerosis.

2.8 Serum lipid profiling

Blood samples for serum analysis were obtained from the heart before the mice were perfused. Concentrations of LDL, HDL and total cholesterol were measured using HDL and LDL/VLDL Cholesterol Assay Kit (Abcam Cat. No. ab65390). Concentrations of triglyceride were measured using a Triglyceride Quantification Kit (Abcam Cat. No. ab65336).
2.9 TUNEL assay

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) was performed using TdT In Situ Apoptosis Detection Kit –DAB (R&D Systems, Minneapolis, MN) according to the manufacturer’s instructions. Images were obtained under microscope, and were analyzed using ImageJ software. Quantification of relative TUNEL positive area over plaque area in aortic root, which was performed by a blinded observer, was used to evaluate apoptosis in the lesions.

2.10 Cell viability assay

BMDMs were seeded in 96-well plates (Corning, Tewsbury, MA) at $5 \times 10^4$ cells per cm$^2$. Cells were incubated overnight to allow adherence. Wells were then washed twice with PBS and cells were incubated in fresh media with or without MCSF. Treatments of different factors as indicated were added to the media without the presence of MCSF. After 24 hours, a mixture of MTS and PMS solution was then added to each well to a final concentration of 333 μg/mL MTS and 25 ng/mL PMS. After 2 hours incubation at 37°C, the absorbance at 490 nm was recorded using microplate reader. Each condition was performed for at least three independent experiments.

2.11 Lipoprotein isolation and oxidation

Native LDL (d = 1.019 to 1.063 g/mL) was isolated by sequential ultracentrifugation of EDTA-anticoagulated fasting plasma obtained from healthy normolipidemic volunteers as described previously$^{267}$. Briefly, plasma was separated by centrifugation at 800 x g for 20 minutes at 4°C. The density of plasma was adjusted to 1.020 g/mL using sodium bromide (d = 1.5 g/mL), and centrifuged at 200,000 x g for 20 hours at 4°C with no brake during deceleration. The bottom
layer (d > 1.020 g/mL) was collected and re-adjusted to a density of 1.065 g/mL. The solution again was centrifuged at 200,000 x g for 20 hours at 4 °C with no brake. Then the top layer (d < 1.065 g/mL) was collected and dialyzed against DPBS containing 10 μM EDTA for 48 hours (DPBS was changed after 24 hours). After filtration through a 0.45 micron filter, final protein concentration of LDL was determined using BCA protein assay.

Copper oxidation was performed by incubating LDL (200 μg/mL) with 5 μmol/L copper sulfate in DPBS containing 0.90 mM calcium chloride and 0.49 mM magnesium chloride for 24 hours at 37°C. This reaction was terminated by adding 40 μM BHT and 300 μM EDTA. The oxidized LDL was then washed with DPBS with 10 μM EDTA and concentrated to approximately 1.5 mg/mL using Amicon Ultra-15 Centrifugal Filter Units (30K NMWL) from EMD Millipore (Etobicoke, ON). After filtration through a 0.45 micron filter, protein concentration of oxLDL was determined using BCA protein assay.

2.12 Lipoprotein characterization

Native and oxidized LDL particles were separated on the TITAN gel for relative electrophoretic mobility assessment. Bovine serum albumin (BSA) was added to lipoproteins as carrier protein to ensure reproducible migration distances. The electrophoresis was then performed using Ciba-Corning (East Walpole, MA) electrophoresis apparatus according to manufacturer’s instructions. Lipoprotein bands were visualized by Fat Red staining. Relative electrophoretic mobility (R_f) value of each lipoprotein was calculated as the ratio of distance traveled during electrophoresis to the distance traveled by native LDL. All oxLDL used in this study was extensively modified with an R_f value greater than 3.
Endotoxin contamination of lipoprotein samples was evaluated by running an LAL Chromogenic Endpoint Assay (Hycult Biotech, Plymouth Meeting, PA) according to manufacturer’s instruction. The endotoxin levels of all native LDL and oxLDL used in this study were below the detection limit (0.04 EU/mL).

2.13 Quantitative real-time PCR

RNA was extracted from cell pellets using GeneJET RNA Purification Kit (Thermo Scientific, Waltham, MA). Total RNA concentration and purity were tested by measuring absorbance at 260 and 280 nm with a NanoDrop 2000 UV-Vis Spectrophotometer (Thermo Scientific, Waltham, WA). cDNA was synthesized by using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Life Technologies, Burlington, ON). qPCR was then performed using SYBR Green qPCR MasterMix with either an StepOnePlus Real-Time PCR Systems (Applied Biosystems, Life Technologies, Burlington, ON) or an Applied Biosystems 7300 Real-Time PCR Systems (Applied Biosystems, Life Technologies, Burlington, ON). Primers used in this study were synthesized by Invitrogen (Table 2.1) (Invitrogen, Life Technologies, Burlington, ON). All qPCR experiments were performed in triplicate.
Table 2.1 Primers used in this dissertation.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer (5’ to 3’)</th>
<th>Reverse Primer (5’ to 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse GAPDH</td>
<td>TCACCACCATGGAGAAGGC</td>
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<td>Mouse ManR</td>
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2.14 ELISA

Cells were treated as indicated and conditioned medium was collected and centrifuged at 13,600 x g for 10 minutes. For TNF-α ELISA, the concentrations of TNF-α were measured using Mouse TNF ELISA Kit (BD Biosciences, Mississauga, ON). For VEGF ELISA, the concentrations of VEGF were determined using Quantikine human and mouse VEGF ELISA Kit (R&D Systems, Minneapolis, MN). The results were normalized to total cell protein content.

2.15 Immunohistochemistry/Immunofluorescence

Formalin-fixed frozen aortic cross-sections obtained by Wax-It Histology Service Inc. (Vancouver, BC, Canada) were used for immunohistochemistry/immunofluorescence study. Various antigen retrieval methods were used before incubating with primary antibodies (Table 2.2). Biotinylated secondary antibodies and avidin-biotin-HRP were used for immunohistochemistry, whereas fluorescein-conjugated secondary antibodies were used for immunofluorescence. Developed sections were counterstained with DAPI. Images were acquired and analyzed with ImageJ software.
Table 2.2 Histology staining conditions used.

<table>
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<tr>
<th>Fixation</th>
<th>Antigen Retrieval Methods</th>
<th>Heat Retrieval Buffers</th>
<th>Antibodies</th>
<th>Incubation Times</th>
<th>Detection</th>
<th>Tissue</th>
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</thead>
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<tr>
<td>No Fixation</td>
<td>No antigen retrieval</td>
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<td>F4/80 (Abcam)</td>
<td>1 hour</td>
<td>Immunohistochemistry</td>
<td>Formalin-fixed frozen</td>
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<td>Acetone</td>
<td>Steamer</td>
<td>EDTA</td>
<td>(1:100 and 1:10)</td>
<td>Overnight</td>
<td>(DAB)</td>
<td>mouse aortic root</td>
</tr>
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<td>Tris-EDTA</td>
<td>F4/80 (BRC)</td>
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<td>Immunofluorescence</td>
<td>Formalin-fixed frozen</td>
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<td>Ethanol/Acetic Acid (2:1)</td>
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<td>MOMA-2</td>
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<tr>
<td></td>
<td></td>
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<td>LDL-R</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>CD45</td>
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<td></td>
<td></td>
<td></td>
<td>Mic-1</td>
<td></td>
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</table>
2.16 Immunoblotting

Cells were washed with DPBS twice before lysis in ice-cold solubilization buffer containing 20 mM HEPES pH 7.4, 1% Triton X-100, 150 mM NaCl and freshly added 2 mM sodium molybdate and 2 mM sodium orthovanadate, in addition to protease inhibitor cocktail (Roche, Basel, Switzerland). Thereafter, lysates were centrifuged at 14,000 rpm for 10 minutes. Protein content of the supernatant was determined by BCA assay. Then sample buffer was added to the lysates and heated to 90°C for 10 minutes. 30-50 μg of protein from each sample was loaded onto a SDS-PAGE gel. Spectra Multicolor Broad Range Protein Ladder (Life Technologies, Burlington, ON) was used for calibration. Proteins were then transferred to nitrocellulose membrane and then blocked in tris-buffered saline (TBS) containing either 5% skim milk or 5% BSA with 0.1% Tween 20. The membrane was then incubated overnight with the relevant primary antibody diluted in the blocking buffer. After three washes with TBS containing 0.1% Tween 20, membranes were incubated with IRDye 800CW or IRDye 680RD secondary antibody (LI-COR Biosciences – Biotechnology, Lincoln, NE) for 1 hour at room temperature. After three washes with TBS containing 0.1% Tween 20, membranes were visualized using Odyssey CLx Infrared Imaging System (LI-COR Biosciences – Biotechnology, Lincoln, NE).

2.17 Statistical analyses

Unless stated otherwise, all results are expressed as means and standard deviation of pooled data from 3 experiments. QPCR results are expressed as means and range (upper and lower limits). Comparison of two groups was done using a two-tailed Student’s t-test. Comparison of three or more groups was done using one-way ANOVA test followed by Tukey’s post hoc analysis. The level of significance of difference is indicated in the graphs as * p<0.05.
Chapter 3: Impairing hematopoietic eukaryotic elongation factor 2 kinase activity decreases atherosclerosis plaque formation

3.1 Introduction

As discussed in Section 1.5, eEF2K regulates protein synthesis via phosphorylation of its only known substrate, eukaryotic elongation factor 2 (eEF2) on Thr 56. This impairs the binding of eEF2 to the ribosome and eEF2 dissociates from the ribosome complex, which compromises the translocation of peptidyl-transfer RNAs from the A site to the P site. Numerous signaling pathways, including mTORC1, MAPK and AMPK, regulate phosphorylation of eEF2K at multiple activating or inhibitory sites, indicating its central role in regulating protein synthesis. eEF2K expression is upregulated in many cancers, and indeed eEF2K promotes tumor survival by inhibiting protein synthesis during nutrient deprivation. eEF2K is also involved in neuronal development and synaptic plasticity. Deficiency of eEF2K activity induces fast-acting behavioural antidepressant effects, suggesting a potential use of eEF2K inhibitors as antidepressants.

Atherosclerosis is recognized as a chronic inflammatory disease with a complex etiology. Endothelial dysfunction, lipid retention and an oxidative microenvironment are all thought to contribute to macrophage activation, survival and/or proliferation and the subsequent development of early atherosclerotic lesions. A key mediator in the pathogenesis of this disease, oxidized low-density lipoprotein has been shown to block apoptosis of macrophages through various signaling pathways, most prominently via PI3K/AKT.
Recently, our studies showed that a Ca\textsuperscript{2+}-dependent eEF2K signaling pathway was activated by oxLDL\textsuperscript{269}. Impairment of eEF2K activity suppressed the pro-survival effect of oxLDL and resulted in macrophage apoptosis, thus implicating eEF2K as a protein kinase that could influence the impact of macrophages on atherosclerosis. We used a targeted knock-in mouse strain in which a mutation (D273A) has been introduced into the kinase domain of eEF2K, decreasing its activity by >99%\textsuperscript{394}. We have now investigated whether eEF2K deficiency affects the development of atherosclerosis \textit{in vivo} in LDL receptor-deficient mice. Bone marrow from either wild type or \textit{eef2k}-kinase inactive (KI) mice were transplanted into irradiated \textit{ldlr}\textsuperscript{−/−} mice. These hematopoietic chimeras were then fed a ‘western style’, i.e. high fat diet (HFD), for 8 or 16 weeks allowing them to develop atherosclerosis. The extent of atherosclerosis in these mice was subsequently analyzed by en face staining of the aorta, as well as H&E and Oil-Red-O staining of aortic root cross-sections. Our findings reveal a significant reduction in atherosclerosis in mice transplanted with bone marrow reconstituted from \textit{eef2k}-KI mice, and thus reveal eEF2K as a potential target for therapy.

3.2 Results

3.2.1 Impairing eEF2K activity significantly reduced atherosclerosis plaque formation

With the availability of \textit{eef2k}-KI mice, we were able to test eEF2K’s role \textit{in vivo}. Successful inactivation of eEF2K activity in KI mice was critical to all subsequent work in this chapter. To verify this, we extracted bone marrow from Wild type (WT) and KI mice and differentiated it into macrophages. Mature macrophages were then deprived of MCSF and then stimulated with oxLDL. Assay for phosphorylation of eEF2 showed increases in response to oxLDL treatment in cells from WT mice, but not in cells derived from KI mice (Figure 3.1).
Figure 3.1 eEF2K activity assay.

Macrophages are extracted from WT or KI mice and cultured for 7 days before treatment. MCSF was deprived in all groups except “non-deprived” for 4 hours. 25 ug/mL oxLDL was added to all groups except “non-deprived”. Cells were collected after time indicated. Phosphorylation of eEF2 was examined by western blot probed by p-eEF2 antibody. (* p<0.05 two-way ANOVA test followed by Student’s t-test)
WT or KI bone marrow was transplanted into irradiated \( ldlr^{-/-} \) mice. These hematopoietic chimeras were then fed a high-fat diet (HFD) for 8 or 16 weeks, allowing them to develop atherosclerosis. Hearts and aortas were harvested from chimeric mice. En face staining of aortas in situ with Sudan IV was used to measure the lipid-containing surface area, which can be used as a measure of the extent of atherosclerotic plaques. In mice that received bone marrow from KI mice, a striking reduction in lipid staining was observed after 16 weeks of receiving the HFD. Plaque areas were reduced from 13.0% (WT) to 7.7% (KI), a reduction of just > 40% (Figure 3.2).
Figure 3.2 En face staining of plaque area after 16 weeks of high fat diet feeding.

Chimeric ldlr<sup>−/−</sup> mice were generated via lethal irradiation and transplantation with bone marrow from BL/6 (wild type [WT]) or eef2k-KI (knock-in [KI]) mice. Chimeric aortas were harvested after 16 weeks of high-fat diet feeding. (A) Representative images of Sudan IV-stained aorta show the difference in plaque area between WT and KI chimeric groups. (B) Quantification of relative plaque area (plaque area divided by total aorta area), which was performed by a blinded observer, shows that reduced activity of eukaryotic elongation factor 2 kinase in the hematopoietic compartment reduces plaque area from 13.0% (WT) to 7.7% (KI). (WT: n = 12; KI: n = 10; * p<0.05, Student’s t-test)
Hematoxylin and eosin (H&E) and Oil-Red-O staining were used to determine the extent of lipid deposits in aortic root cross-sections from harvested hearts. The ratio of lipid-rich area to total lesion area estimates the extent of atherosclerosis development. Again, in 16-week group, there was a significant decrease in plaque area in mice that received a transplant of bone marrow from KI mice, 37.7%, compared with 45.2% in mice with WT bone marrow (Figure 3.3).
Figure 3.3 Plaque areas in the aortic root after 16 weeks of high-fat diet.

Chimeric animal hearts were harvested after 16 weeks of high-fat diet and compared with wild type (WT).

Representative images of hematoxylin and eosin stained (A) and Oil-Red-O stained (B) aortic root show the difference of plaque area between WT and eef2k-KI (KI) groups. (C) Quantification of relative plaque area (plaque area over total aortic sectional area) in the aortic root, which was performed by a blinded observer, shows that reduced activity of eukaryotic elongation factor 2 kinase in the hematopoietic cells reduces plaque area from 45.2% (WT) to 37.7% (KI). (WT: n = 6; KI: n = 6; * p<0.05, Student’s t-test)
We saw a small decrease in atherosclerosis in the KI group after 8-week HFD feeding, however it was not statistically significant in both en face staining and Oil-Red-O staining analysis (Figure 3.4). This may be attributed to the low degree of atherosclerosis observed at this very early time point.
Figure 3.4 En face staining of plaque area following 8-week HFD.

As in Figure 3.2, hearts from ldlr<sup>−/−</sup> chimeric mice were harvested after 8-week high fat diet feeding. Representative images of Sudan IV stained aorta (A) and the corresponding graphical representation of the results (B) show no significant difference between WT and KI group. (WT: n=12; KI: n=12, p>0.05, Student’s t-test).
3.2.2 Serum lipid profile was not changed between WT and KI groups

To eliminate the possibility that eEF2K affects lipid metabolism and the two groups of mice may have had different lipid profiles following their HFD feeding, we measured lipid components in the serum collected from the same mice used for en face staining and Oil-Red-O staining. All measures, including cholesterol (LDL and HDL) and triglyceride levels were not significantly different in the two groups of mice, confirming that no metabolic or lipid uptake changes on eEF2K inactivation (Figure 3.5).
Figure 3.5 Serum lipid profile following 16-week HFD.

Blood samples were harvested from $ldlr^{-/-}$ chimeric mice after 16-weeks on a high fat diet. Low-density lipoprotein (LDL), high-density lipoprotein (HDL), total cholesterol (A) and triglyceride (B) in the serum were measured using colorimetric assays. ($WT: n = 12; KI: n = 10; p>0.05$, Student’s t-test)
3.2.3 **Macrophage viability showed no significant difference between WT and KI groups**

Our laboratory showed that eliminating eEF2K activity suppresses oxLDL-mediated macrophage survival\(^{269}\). Impaired macrophage viability may decrease the accumulation of macrophages, thus decrease foam cell formation, which in turn suppresses atherosclerotic plaque formation. To test whether macrophage viability is affected in KI mice, we evaluated the extent of macrophage apoptosis by performing a TUNEL assay using cross-sectional samples from the same mice used for en face staining and Oil-Red-O staining. Although a small decrease in viability was seen in KI groups, it was not statistically significant (Figure 3.6). In each group we analyzed only 6 samples, which may not be large enough to show the difference. However it has to be noted that, in our previous work, the *eef2k*-KI mice used was on CD-1 background. However, among all strains, C57BL/6 mice are the most susceptible to atherosclerosis development\(^{124}\). In order to transplant bone marrows to *lدلr*\(^{-/-}\) C57BL/6 mice, we backcrossed *eef2k*-KI mice with wild type C57BL/6 mice for 6 generations. To eliminate the possibility that genetic background affects macrophage viability in response to oxLDL, we re-performed the *in vitro* viability assay using WT and KI on C57BL/6 background. Surprisingly, we found no difference between WT and KI macrophages in response to oxLDL treatment (Figure 3.7). Therefore, our previous hypothesis that eEF2K signaling pathway is involved in atherosclerosis via regulation of oxLDL-promoted macrophage survival may not be valid. Thus, at this point we cannot define the mechanism by which differences in eEF2K activity caused the difference between WT and KI mice in atherosclerosis development.
Figure 3.6 16-week TUNEL staining on aortic root sections.

Bone marrow from wild-type (WT) or eef2k-KI (KI) mice were reconstituted in ldlr⁻/⁻ mice. Hearts were harvested after 16-week high fat diet feeding. Representative images (A) of 2.5X and 10X magnifications show a positive TUNEL staining. Quantification of relative TUNEL positive area over plaque areas in aortic root (B), which was performed by a blinded observer, shows no significant difference between WT and KI groups. (n=6, p>0.05, Student’s t-test).
Figure 3.7 eEF2 kinase activity does not contribute to the pro-survival effect of oxLDL.

BMDM from wild type mice or eef2k-KI mice were cultured for 24 hours in the presence of 10% M-CSF conditioned media, native LDL (nLDL) or different concentrations of oxLDL as indicated. Viability was measured by the bioreduction of MTS and normalized to values for cells incubated with MCSF. (n=3, p>0.05, two-way ANOVA test)
3.2.4 eEF2K deficiency resulted in the downregulation of adhesion molecules in endothelial cells partly via the regulation of TNF-α

As a source of cytokines, macrophages have paracrine effects on neighbouring cells, such as endothelial cells, and thus we tested if such activity might be altered by a deficiency in eEF2K activity. Conditioned medium (CM) from macrophage cultures were tested for their effects on surface marker expression on endothelial cells. To mimic potential effects of inflammatory vs. healer (M1 vs. M2) macrophages, we compared macrophage CM from M0, M1, and M2 cells, each isolated from WT or KI mice. CM from M1 cells showed much greater activity than the others in inducing expression of E-selectin, VCAM-1, and ICAM-1. CM from similarly induced cells but lacking eEF2K activity showed a marked reduction in activity (Figure 3.8). P-selectin mRNA levels were not changed (Figure 3.8).
Figure 3.8 Lack of eukaryotic elongation factor 2 kinase activity compromises M1 cytokine-induced adhesion molecule expression in HUVECs.

Macrophages were cultured in macrophage colony stimulating factor-containing media for 11 days. Cells were counted and seeded at the same concentration. 10 ng/mL LPS and 10 U/mL IFN-γ or 10 ng/mL IL-4 were added to polarize macrophages toward either the M1 or M2 subtype, respectively. Conditioned media (CM) was collected after 24 hours. VCAM-1 (A), ICAM-1 (B), E-selectin (SELE; C), and P-selectin (SELP; D) gene expression were determined in HUVEC cells incubated with the CM. CM from cells with inactivated eukaryotic elongation factor 2 kinase (KI groups) resulted in a reduced level of induction by the M1-conditioned media for VCAM-1, ICAM-1, and SELE, but not SELP. No significant changes were observed in the effects of CM from M0 or M2 cells. Results shown are from a single experiment using triplicate samples, same trend was observed in 3 separate experiments (with slightly different design).
TNF-α is a key factor secreted by macrophages that regulates endothelial cell adhesion molecule expression. TNF-α concentration in macrophage CM was higher in M1 conditioned media, as expected, and barely detected in M2 or M0 CM. Comparison of CM from WT or KI cells showed that the TNF-α produced in the absence of eEF2K activity was reduced, matching the pattern of CM-induced adhesion molecule expression levels in endothelial cells (Figure 3.9).
Figure 3.9 Lack of eEF2K activity suppresses TNF-α release.

Macrophages were cultured in macrophage colony stimulating factor-containing media for 11 days. Cells were counted and seeded at the same concentration. 10 ng/mL LPS and 10 U/mL IFN-γ or 10 ng/mL IL-4 were added to polarize macrophages toward either the M1 or M2 subtype, respectively. Conditioned media (CM) was collected after 24 hours. Concentrations of TNF-α were detected using ELISA. Only M1 cultures produced significant levels of TNF-α, and M1 cells from eef2k-KI (KI) mice produced less TNF-α compared with the wild type (WT) cells. Results shown are from a single experiment using triplicate samples.
3.3 Discussion

The development of atherosclerotic plaques occurs over an extended time period owing to a complex series of events. The initial processes at the endothelial lining that cause activation of macrophages and their recruitment into the vessel walls may take years (in humans) to begin causing any evidence of plaque formation. Ultimately, plaque buildup causes partial or complete blockage of arteries, as well as possible plaque rupture that can lead to blockage of smaller vessels. Macrophages are key players in that they respond to signals at the endothelial lining, as well as differentiate into foam cells as a result of accumulation of cholesterol and its byproducts from uptake of altered forms of LDL. We have shown previously that survival of macrophages in the presence of oxLDL involves eEF2K, a regulator of protein synthesis\textsuperscript{269}. Based upon the premise that survival of macrophages contributes to their persistence in the intima, we postulated that blocking eEF2K activity may suppress development of atherosclerosis. We have now tested this hypothesis in the \textit{ldlr}^{-/-} mouse model. To study the possible involvement of eEF2K in the hematopoietic compartment, we generated hematopoietic chimeras using bone marrow cells from mice expressing kinase-defective eEF2K to engraft \textit{ldlr}^{-/-} recipients that had been lethally irradiated to destroy their endogenous hematopoietic compartment. Following recovery, these chimeras were fed a high fat diet to induce atherosclerosis and those mice with the mutant eEF2K had a greatly reduced level of atherosclerosis, despite displaying the same circulating lipid profile as the control animals.

The ability of eEF2K to suppress protein synthesis via phosphorylation of eEF2, is well characterized and is important in cellular responses to stress. Nutrient deprivation leading to activation of adenosine monophosphate-activated kinases, can contribute to eEF2K activation
and the reduced protein synthesis serves as an important energy-saving mechanism. Before our study, no link had been reported between eEF2K activity and the development of atherosclerosis, other than the initial in vitro study alluded to previously in which our laboratory reported a role for eEF2K in macrophage survival in response to oxLDL. However, it is significant that at least our initial analysis was not able to confirm that eEF2K regulates atherosclerosis through regulating macrophage viability.

We have also shown that when eEF2K activity is impaired, the secretory profile of macrophages is changed, which might affect macrophage function in regulating the microenvironment and neighbouring cells. We have assessed this function by measuring the expression of adhesion molecules, key factors that facilitate rolling and tethering of leukocytes, in HUVEC cells incubated with macrophage CM. Elevated levels of VCAM-1, ICAM-1, and E-selectin were induced by TNF-α produced by M1 macrophages, and these proteins are known to result in enhanced progression of atherosclerosis. It should be noted that the lack of change in P-selectin message level might not be surprising because its regulation occurs mostly at the level of surface expression of preformed protein. M1 cells derived from KI mice had considerably less activity in their CM, corresponding with a decrease in TNF-α. We have also tried to confirm these findings in vivo by doing immunohistochemistry or immunofluorescence on aortic root cross-sections. However, in order to stain lipid components, all sections were fixed in formaldehyde and embedded in OCT for cryo-sectioning. This procedure blocks the epitope of antigens in a way that makes them much more difficult to be retrieved. Combinations of multiple antigen retrieval methods were tried, and none of which worked (Table 2.2). Unfortunately, a lack of resources that would have allowed us to repeat the experiments have limited my ability to
obtain additional samples to test. In future, paraffin-embedded sections will need to be acquired so that we can analyze the changes in numbers or characteristics of macrophages that are present in plaques. Using these sections, we will also want to determine if suppression of endothelial cell surface proteins can be observed in the absence of eEF2K \textit{in vivo}. Of course, it should also be noted that to date I have only been able to show the increase in gene expression of these endothelial markers, and while it is expected that a corresponding increase in protein would be observed\textsuperscript{397}, it will be important to verify this result.

Finally, it will be important to begin testing eEF2K inhibitors \textit{in vivo} for their effectiveness in blocking atherosclerosis development. Although our studies show a partial block of atherosclerosis when eEF2K activity is suppressed, eEF2K inhibitors might become even more valuable when used together with other atherosclerosis drug therapies. Recent studies have implicated eEF2K activity in other conditions that also highlight its potential as an important drug target, including in cancer\textsuperscript{371}. In the future, it will be important to test newer specific eEF2K inhibitors in multiple animal models, to evaluate their potential as therapies for multiple disease targets.
Chapter 4: Oxidized low-density lipoprotein polarizes macrophages into a novel phenotype, with a possible role in atherosclerosis

4.1 Introduction

Macrophages have been subcategorized into different subtypes based on expression profiles. M1/M2 classification, which mirrors the Th1/Th2 paradigm in T cells, have been widely characterized and used in many studies. As summarized in Section 1.3.2, in mice, the classically activated macrophages (M1) are characterized by a pro-inflammatory proteome including the upregulation of TNF-α, IL-1, IL-6, IL-12 and inducible nitric oxide synthase (iNOS). In contrast, alternatively activated macrophages (M2) express high levels of mannose receptors, arginase-I (Arg I), chitinase 3-like 3 (YM1) and resistin-like-α (Fizz1). The classification is defined slightly differently in humans as follows. MCSF differentiates monocytes into naïve macrophages (M0). LPS plus IFN-γ polarizes naïve macrophages into M1 macrophages, which are considered as proinflammatory. By contrast, naïve macrophages can be polarized into an anti-inflammatory phenotype, the M2 macrophages. M2 cells can be sub-divided into different subtypes. IL-4 or IL-13 leads to the production of M2a; Immune complexes (IC) and IL-1β or LPS leads to M2b; and IL-10 or tumor growth factor β (TGF-β) or glucocorticoids leads to M2c. More recently, based on different homeostatic activities, macrophages are subcategorized into three new groups: classically activated macrophages in host defense, wound-healing macrophages and regulatory macrophages. The authors proposed that like the primary colors, these three basic subtypes can blend into various other ‘shades’ of activation (Figure 1.2).
This macrophage classification system cannot fulfill all circumstances and is still under active investigation. CXCL4, also known as platelet factor 4 (PF4), without the presence of MCSF, differentiates monocytes into a distinct subtype of macrophages, M4 macrophages\textsuperscript{153}. M4 macrophages express low levels of hemoglobin receptor, also known as CD163, and play a general atherogenic role\textsuperscript{154}. Intraplaque hemorrhage also leads to a unique macrophage phenotype called HA-mac at first and then renamed to Mhem\textsuperscript{156}. In contrast to M4 macrophages, Mhem macrophages express high levels of CD163 and are often referred as CD163\textsuperscript{+} macrophages.

Most studies on macrophage plasticity discuss how those subtypes are involved in inflammation, which indirectly affects their role in atherosclerosis\textsuperscript{399}. Observation of different macrophage subtypes in atherosclerotic plaques has been reported\textsuperscript{149}. However, their exact roles in the context of atherosclerosis are still unknown. Oxidized LDL, the most important foam cell-inducing factor, has been reported to have conflicting effects on macrophage polarization. OxLDL can bind to TLR4 triggering M1 polarization as well as increase M2 macrophage marker Arg I via the activation of peroxisome proliferator activated receptor-\(\gamma\) (PPAR\(\gamma\))\textsuperscript{187}. In addition, oxidized phospholipid, a component of oxLDL polarizes macrophages towards a Mox subtype\textsuperscript{155}. In this study, we focused on the characterization of oxLDL-polarized macrophages and their potential role in atherosclerosis.
4.2 Results

4.2.1 Oxidized LDL affected macrophage phenotype but did not induce either M1 or M2 polarization

First of all, we tested whether oxidized LDL has the ability to affect macrophage phenotype. To approach this, we followed the M1/M2 classification and tested whether oxLDL mediated M1 or M2 polarization. Naïve macrophages (M0) were unstimulated and left in normal medium (control), or incubated in the presence of 10 ng/mL LPS plus 10 U/mL IFN-γ (M1 inducers), 10 ng/mL IL-4 (M2 inducer), 50 μg/mL native LDL or 50μg/mL oxLDL. OxLDL-treated macrophages did not express either M1 markers (like iNOS, Figure 4.1A) or M2 markers (like ManR, Figure 4.1B). Multiple M1 markers and M2 markers were tested, and markers shown in the figures (including Figure 4.1, 4.2 and 4.3) are just typical representatives. This implies that oxLDL does not induce M1 or M2 polarization.
Figure 4.1 Oxidized LDL cannot induce M1/M2 marker expression in bone marrow derived macrophages.

BMDMs were cultured in MCSF+ RPMI for 11 days. Naïve macrophages were then stimulated with 10 ng/mL LPS and 10 U/mL IFN-γ (M1), 10 ng/mL IL-4 (M2), 50 μg/mL native LDL, 50 μg/mL oxLDL or medium only (M0). RNA was isolated after 3 hours of stimulation and reverse-transcribed. Quantitative PCR was performed and expressions of the M1 marker, Inducible Nitric Oxide Synthase (A) and the M2 marker, Mannose Receptor (B) were tested. (n=3, * p<0.05 one way ANOVA test followed by Tukey’s post hoc analysis)
The balance between M1 and M2 macrophages has been proposed as a key in inflammatory disease pathogenesis. Whether oxLDL affects M1/M2 balance was unknown and was tested by skewing macrophages to either mature M1 or M2 macrophages, followed by incubation in the presence of oxLDL to determine its effect on key markers of these two cell types (Figure 4.2 and 4.3). In M1 macrophages, oxLDL significantly reduced both M1 and M2 markers (Figure 4.2) and the same effects applied to M2 macrophages (Figure 4.3). This interesting finding showed that oxLDL indeed has the ability to affect macrophage phenotype, but it was still unclear whether oxLDL simply draws macrophages back to M0 or oxLDL polarizes macrophages towards another macrophage subtype.
Figure 4.2 OxLDL reduces expression of M1 and M2 markers in M1 cells.

BMDMs were cultured in MCSF+ RPMI for 11 days. 10 ng/mL LPS and 10 U/mL IFN-γ were added at Day 8 to skew BMDMs to M1 subtype. At day 11, the M1 cells were incubated in the same media (Control) or 25 μg/mL oxLDL was added for 8 hours. Both the M1 marker, Inducible Nitric Oxide Synthase (A) and the M2 marker, Mannose Receptor (B) were examined. (n=3, * p<0.05 Student’s t-test)
Figure 4.3 OxLDL reduces expression of M1 and M2 markers in M2 cells.

BMDMs were cultured in MCSF+ RPMI for 11 days. 10 ng/mL IL-4 were added at Day 8 to skew BMDMs to M2 subtype. At day 11, the M2 cells were incubated in the same media (Control) or 25 μg/mL oxLDL was added for 8 hours. Both the M1 marker, Inducible Nitric Oxide Synthase (A) and the M2 marker, Mannose Receptor (B) were examined. (n=3, * p<0.05 Student’s t-test)
4.2.2 Oxidized LDL polarized macrophages towards a unique MoL subtype

At the time that these experiments were actively being pursued in our laboratory, a report appeared in the literature showing that exposure to oxidized 1-palmitoyl-2-arachidonoyl-sn-3-glycero-phosphorylcholine (OxPAPC) polarizes mouse macrophages into a novel subtype named Mox; a complete gene array was performed on these cells and a key marker reported in that study was the expression of elevated levels of heme oxygenase-1 (Hmox-1)\(^{155}\). OxLDL particles contain phosphorylcholine, which makes it plausible that oxLDL may polarize macrophages towards something similar to Mox. OxLDL-treated M0 cells indeed expressed elevated levels of Hmox-1 (Figure 4.4A). In addition, oxLDL-polarized macrophages also express increased levels of macrophage inhibitory cytokine 1 (Mic-1) (Figure 4.4B). These increases are specific to oxLDL-induced polarization and cannot be observed in M1 or M2 polarization, nor in response to incubation with normal LDL (Figure 4.4 A&B). To figure out whether oxLDL-induced macrophages are equivalent to the reported Mox cells, 9 genes were selected from the Mox marker gene microarray data, and their level of gene expression was determined in response to oxLDL treatment (Table 4.1). Out of the nine genes tested, five showed a similar trend as had been reported in Mox cells. However the other 4 genes showed a different expression pattern. Thus oxLDL polarized macrophages towards a subtype similar to, but not exactly the same as Mox, and we have referred to these cells as MoL.
Figure 4.4 Oxidized LDL induced gene expression in bone marrow derived macrophages.

BMDMs were cultured in MCSF + RPMI for 11 days. Naïve macrophages were then stimulated with 10 ng/mL LPS and 10 U/mL IFN-γ (M1), 10 ng/mL IL-4 (M2), 50 μg/mL native LDL, 50 μg/mL oxLDL or medium only (M0). RNA was isolated after 3 hours of stimulation and reverse-transcribed. Quantitative PCR was performed and expressions of Heme Oxygenase-1 (A) or Macrophage Inhibitory Cytokine-1 (B) were tested. (n=3, * p<0.05 one-way ANOVA test followed by Tukey’s post hoc analysis)
Table 4.1 Genes selected shown to be elevated from Mox microarray data and verified by qPCR.

<table>
<thead>
<tr>
<th>Genes</th>
<th>Fold change observed in Mox microarray</th>
<th>Fold change in oxLDL treatment</th>
<th>Significant increase in expression observed with oxLDL treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nr4a2</td>
<td>6.7313</td>
<td>0.637</td>
<td>No</td>
</tr>
<tr>
<td>Gclc</td>
<td>3.0466</td>
<td>1.318</td>
<td>Yes</td>
</tr>
<tr>
<td>Mic-1</td>
<td>2.8885</td>
<td>6.997</td>
<td>Yes</td>
</tr>
<tr>
<td>Osm</td>
<td>2.5047</td>
<td>0.761</td>
<td>No</td>
</tr>
<tr>
<td>Gclm</td>
<td>2.3606</td>
<td>1.333</td>
<td>Yes</td>
</tr>
<tr>
<td>Txnrd1</td>
<td>2.1888</td>
<td>1.194</td>
<td>Yes</td>
</tr>
<tr>
<td>Vegfa</td>
<td>2.1762</td>
<td>1.003</td>
<td>No</td>
</tr>
<tr>
<td>Pim1</td>
<td>2.138</td>
<td>0.921</td>
<td>No</td>
</tr>
<tr>
<td>Hmox1</td>
<td>2.1143</td>
<td>2.008</td>
<td>Yes</td>
</tr>
</tbody>
</table>
4.2.3 PI3K signaling pathway was involved in MoL polarization

As a newly described macrophage subtype, the mechanism of MoL polarization is, of course, largely unknown. To test potential signaling pathways involved in MoL polarization, we added pharmaceutical inhibitors to cell culture before oxLDL simulation, so that we could investigate whether shutdown of certain signaling pathways impairs oxLDL-mediated polarization. Using Mic-1 and Hmox-1 as MoL markers, we found that PI3K is involved in MoL polarization (Figure 4.5). Other pathways activated by oxLDL, such as Erk1/2, p38, and eEF2K signaling pathways were not responsible for this process (Figure 4.6).
Figure 4.5 LY294002 blocked oxLDL-induced gene expression in macrophages.

Bone marrow derived macrophages were cultured in MCSF+ RPMI media for 11 days before use. BMDMs then stimulated by either nothing or 50 μg/mL oxidized LDL for 3 hours before harvested for quantitative PCR analysis. In conditions with inhibitors, the inhibitor was added 45 minutes before stimulation. mRNA expression of Heme Oxygenase-1 (A) or Macrophage Inhibitory Cytokine-1 (B) were tested. (n=3, * p<0.05 one-way ANOVA test followed by Tukey's post hoc analysis)
Figure 4.6 MEK/ERK, p38MAPK, eEF2K signaling pathways were not involved in oxLDL-induced gene expression in macrophages.

Bone marrow derived macrophages were cultured in MCSF+ RPMI media for 11 days before use. BMDMs then stimulated by either nothing or 50 μg/mL oxidized LDL for 3 hours before harvested for quantitative PCR analysis. In conditions with inhibitors, the inhibitor was added 45 minutes before stimulation. PD: PD 98059, MEK/ERK inhibitor. SB: SB 239063, p38 MAPK inhibitor. TX: TX1918, eEF2K inhibitor. mRNA expression of Macrophage Inhibitory Cytokine-1 (A) or Heme Oxygenase-1 (B) were tested. (n=3)
4.2.4 MoL secretes elevate VCAM-1 expression on endothelial cells.

To examine MoL’s potential role in atherosclerosis, we performed functional assays using MoL-conditioned media, similar to those described in Chapter 3. Compared to mature M1- or M2-conditioned media, MoL-conditioned media promoted VCAM-1 expression on HuVEC cells (Figure 4.7 A). ICAM-1 and P-Selectin were also slightly elevated, but E-Selectin remained at basal levels (Figure 4.7 B-D). This data implicates a pro-inflammatory function for these cells, via production of an extracellular factor, and thus would be consistent with a pro-atherogenic role for MoL cells.
Bone marrow derived macrophages were cultured in MCSF+ RPMI media for 11 Days before use. Conditioned media from BMDMs were harvested after 24-hour treatment of different polarizing factors, including 10 ng/mL LPS and 10 U/mL IFN-γ (M1), 10 ng/mL IL-4 (M2), 50 μg/mL native LDL and 50 μg/mL oxidized LDL. No cell control groups were set in parallel. After 5-hour treatment of conditioned media, HuVECs were harvested for RNA extraction followed by quantitative PCR analysis. Expression levels of VCAM-1 (A), ICAM-1(B), SELP(C), and SELE(D) were evaluated. (n=3, * p<0.05 one-way ANOVA test followed by Tukey’s post hoc analysis)

Figure 4.7 Relative quantification of adhesion molecules in HuVECs treated by MoL-conditioned media.
4.3 Discussion

It is well established that oxLDL and macrophages are important participants in atherosclerosis\textsuperscript{1,187,206}. This study provides a new insight on how these two players interact with each other. The discovery of MoL not only adds a new subtype to the macrophage heterogeneity map, but also provides a new angle to approach atherosclerosis pathogenesis. It partially explains the fact that in atherosclerotic lesions, M1 and M2 macrophages only account for 40% and 20% of macrophage population respectively\textsuperscript{155}, particularly since we showed that the presence of oxLDL can actually cause suppression of key M1 and M2 markers.

Although OxPAPC-induced Mox cells are similar to MoL, the concentration (50 μg/mL) used to polarize Mox was too high to be physiologically relevant. Even though the exact concentrations of oxLDL in atherosclerotic lesions are not known, the circulating oxLDL concentration is about 1-1.5 μg/mL\textsuperscript{400,401}. Considering the fact that in atherosclerotic lesions, oxLDL can be accumulated and packed in a relatively small area, the concentration of oxLDL in the microenvironment interacting with macrophages could be much higher, which easily falls into the concentration range (25-50 μg/mL) used in this study. It is claimed that Mox cells account for about 35% of macrophages population in atherosclerotic plaques, however Hmox-1, a shared marker between Mox and MoL, was the only one used in that study\textsuperscript{155}. Thus it is very plausible that the Mox population observed \textit{in vivo} may be in fact be the MoL population that is derived due to the presence of oxLDL, as has been described here. Transcriptome studies of MoL using high-throughput techniques will be needed to develop distinctive MoL markers. Those markers can then be used to probe MoL \textit{in vivo}, as well as to investigate potential functions of MoL.
Using conditioned media, we found MoL promotes adhesion molecule expression in endothelial cells, possibly through paracrine effects. However, factors in the conditioned media that may contribute to this effect will need to be identified. Besides activating endothelial cells, MoL may contribute to atherosclerosis in other ways. It has been well known that macrophages take up oxLDL via scavenger receptors and become lipid-laden foam cells. Whether this foam cell formation process is separate from MoL polarization is a very interesting question. Polarizing BMDMs from scavenger receptor-null mice towards MoL and testing MoL marker expression may be one possible way to address this question. Since MoL cells have a distinctive expression profile, other paracrine effects of macrophages, such as smooth muscle cell migration and metalloproteinase release should also be investigated. Answering these questions will provide a greater understanding of MoL’s potential role in atherosclerosis.

To consider possible MoL regulation as a therapeutic target for atherosclerosis, the mechanism of MoL polarization must be comprehensively investigated. Here we showed that PI3K, but neither Erk1/2, p38, nor eEF2K was involved in MoL polarization. It has to be noted that pharmaceutical inhibitors have no perfect specificity. PI3K inhibitor, LY 294002, used in this study also targets mammalian target of rapamycin, DNA-dependent protein kinase as well as casein kinase 2 (also discussed in Section 5.3). Those pathways need to be ruled out, in order to claim PI3K’s involvement. It is generally expected that more specific methods need to be incorporated for this purpose, such as siRNA knockdown. However, we know that siRNA incorporation into primary macrophages cannot be accomplished (previous work in our laboratory as well as personal communication with our collaborator Dr. Anton Munoz-Gomez).
One more thing to point out is that in Figure 4.7, I showed a specific increase of VCAM-1 in endothelial cells treated by oxLDL-stimulated macrophage conditioned media. However in Figure 3.8, I showed a specific increase of VCAM-1 in endothelial cells treated by M1-conditioned media. The discrepancy between these two results is due to the difference in the selection of control group. In Figure 4.7, all results from qPCR are divided by according “no-cell controls” (endothelial cells treated by media containing the driving factors, like LPS/IFN-γ, but without exposing to macrophages). However, in Figure 3.8, I was simply comparing WT-conditioned media with KI-conditioned media. There was no “no-cell control” for Figure 3.8.

To summarize, data in this chapter have supported the characterization of MoL as a novel macrophage subtype derived by oxLDL-mediated polarization. MoL macrophages bear properties expected of pro-atherogenic macrophages and data suggest that they are polarized at least partly via a PI3K-dependent pathway. These results provide new insights on macrophage plasticity as well as atherosclerosis pathogenesis, and shed some lights on potential new therapeutic approaches that may be considered in treatment of atherosclerosis.
Chapter 5: Signaling pathways involved in ceramide 1-phosphate induced VEGF release in macrophages

5.1 Introduction

As summarized in Section 1.4.3.2, ceramide 1-phosphate (C1P), which has a dual role in the process of inflammation, has not been linked to atherosclerosis directly. However, it is very interesting that previous studies have shown that many effects of oxLDL, a key factor in atherosclerosis, can be mimicked by C1P. For instance, both oxLDL and C1P promote macrophage survival and block macrophage apoptosis. They also utilize similar cell signaling pathways. In our laboratory, we found that oxLDL and C1P both increase vascular endothelial growth factor (VEGF) release by macrophages but possibly through different mechanisms (Dr. Maziar Riazy, unpublished data).

VEGF’s role in promoting angiogenesis and neovascularization has been well established. This cytokine maintains endothelium integrity, thus may have an anti-atherogenic role. Some clinical trials have suggested a protective role for VEGF in the management of coronary artery disease. However neovascularization within the vessel wall can promote the growth of atherosclerotic plaques (especially the larger plaques) since it provides oxygen to the area that is more distant from the arterial lumen and thus is not receiving sufficient oxygen by diffusion. Reconstruction of 3D structure of atherosclerotic aorta in apoB100/apoE double knockout mice showed a correlation between the extent of intimal angiogenesis and lesion progression. Neovascularization frequency in large diameter plaques is 9 times higher than that in small plaques. Moreover, inhibitors suppressing VEGF-induced intimal neovascularization reduce
atherosclerosis in animal models\textsuperscript{405}. Besides its function in angiogenesis, VEGF can stimulate the release of MCP-1 in SMC and endothelial cells, which in turn promotes macrophage migration to the lesion sites, thus promoting atherosclerosis\textsuperscript{406,407}.

Elevated levels of VEGF have been reported in atherosclerotic plaques\textsuperscript{408-411}. As one of the inducers of VEGF, it is important to investigate signaling pathways involved in C1P-induced VEGF release in macrophages. Most C1P effects mentioned in Section 1.4.3.2 take place within the cell. Therefore there must be an intracellular receptor for C1P. In addition, C1P serves as an extracellular ligand and binds to a G\textsubscript{i}-coupled receptor\textsuperscript{301}. In this study, we also investigated receptors responsible for C1P-induced VEGF release.

5.2 Results

5.2.1 PI3K/Akt signaling pathway was involved in C1P-induced VEGF release

It has been reported that C1P activates the PI3K/Akt signaling pathway in macrophages, which in turn increases their release of MCP-1\textsuperscript{412}. Whether this pathway accounts for the VEGF release is unknown. However, oxLDL triggers VEGF release in macrophage via PI3K/Akt\textsuperscript{413}. With the fact that many oxLDL effects are mimicked by C1P, it was reasonable to test PI3K’s involvement in C1P-induced VEGF release in macrophages. LY 294002 (LY) and wortmannin (WM) were used to block PI3K signaling pathways in these experiments\textsuperscript{414,415}. We found that both LY and WM effectively blocked the phosphorylation of Akt in our experimental settings (Figure 5.1). ELISA was used to detect VEGF released in conditioned media of macrophages. LY significantly reduced the amount of VEGF induced by C1P without affecting the basal levels of VEGF release in macrophages (Figure 5.2A). Similarly, 500 nM of WM decreased VEGF
levels to basal levels despite the presence of C1P (Figure 5.2B). Thus, PI3K/Akt signaling pathway is involved in C1P-induced VEGF release.
Figure 5.1 Both LY 294002 (LY) and wortmannin (WM) suppresses PI3K/Akt signaling pathway in RAW 264.7 cells. RAW cells were treated with 50 μM C1P for indicated period of time. Where indicated, inhibitors were applied 45 minutes before C1P treatments. Cells were then harvested for immunoblotting using anti-phospho-PKB. Kinetics of PI3K activation showed that C1P induced PI3K/Akt signaling pathway. This activity peaked at 15 minutes and was successfully suppressed by either 15 μM LY or 500 nM WM. Anti-vinculin blot was used as a total protein loading control. (n=3, gel images showed here are composites from two separate gels)
Figure 5.2 Ceramide 1-phosphate (C1P) induces VEGF release through PI3K/Akt signaling pathway.

RAW 264.7 cells were incubated with (CP) or without (NT) 50 μM C1P for 24 hours. In conditions with inhibitors, the inhibitor was applied 45 minutes before C1P treatment. Conditioned medium were then harvested, and used to measure VEGF concentration by ELISA. Both LY294002 (A) and wortmannin (B) were able to inhibit VEGF release upon C1P treatment. (n=3, * and ** p<0.05 one-way ANOVA test followed by Tukey’s post hoc analysis)
5.2.2 MEK/ERK signaling pathway was involved in C1P-induced VEGF release

Similar to the PI3K/Akt signaling pathway, both C1P and oxLDL activate the MEK/ERK signaling pathway in macrophages\textsuperscript{412,416}. To test whether this signaling pathway participates in C1P-induced VEGF release, PD 98059 (PD) was applied to cells to inhibit MEK and the subsequent activation of ERK\textsuperscript{417}. The efficacy of PD was verified by immunoblot of phospho-Erk1/2 (Figure 5.3). ELISA of conditioned media from PD-treated cells showed a significant decrease in VEGF concentration only in C1P-stimulated groups, which indicates that MEK/ERK signaling pathway also plays a role in C1P-induced VEGF release (Figure 5.4).
Figure 5.3 PD 98095 (PD) suppresses MEK/ERK signaling pathway in RAW 264.7 cells.

RAW cells were treated with 50 μM C1P for indicated period of time. Where indicated, PD was applied 45 minutes before C1P treatments. Cells where then harvested for immunoblot against phospho-Erk1/2. (n=3, gel images showed here are composites from two separate gels)
Figure 5.4 Ceramide 1-phosphate (C1P) induces VEGF release through MEK/ERK signaling pathway.

RAW 264.7 cells were incubated with (C1P) or without (NT) 50 μM C1P for 24 hours. In conditions with inhibitors, the inhibitor was applied 45 minutes before C1P treatment. Conditioned medium were then harvested, and used to measure VEGF concentration by ELISA. PD98059 was able to inhibit VEGF release upon C1P treatment. (n=3, * and ** p<0.05 one-way ANOVA test followed by Tukey’s post hoc analysis)
5.2.3 Neither p38 MAPK signaling pathway nor PKC-ζ signaling was required for C1P-induced VEGF release

Like PI3K/Akt, the p38 MAPK signaling pathway was shown to play a role in C1P-induced MCP-1 release\textsuperscript{412}. When activated by oxLDL, the p38 MAPK signaling pathway is reportedly involved in regulation of CD36, which in turn regulates foam cell formation\textsuperscript{418}. In this study, we used SB 239063 (SB) to inhibit p38 MAPK in macrophages\textsuperscript{419}. Blockade of p38 MAPK activity decreased the basal levels of VEGF release from macrophages, in a similar manner to that seen in cells treated with C1P (Figure 5.5). Therefore, the p38 MAPK signaling pathway is not a specific effector of C1P treatment.
Figure 5.5 p38 MAPK was not involved in C1P induced VEGF release.

RAW 264.7 cells were incubated with (C1P) or without (NT) 50 μM C1P for 24 hours. In conditions with inhibitors, the inhibitor was applied 45 minutes before C1P treatment. Conditioned medium were then harvested, and used to measure VEGF concentration by ELISA. SB 239063 suppressed basal levels of VEGF release. (n=3, one-way ANOVA test followed by Tukey’s post hoc analysis)
Previous work in our laboratory found that PKC-ζ is involved in oxLDL induced VEGF release, and a pseudosubstrate of PKC-ζ significantly blocked the effect\textsuperscript{413}. The same pseudosubstrate was applied in this study to investigate whether PKC-ζ is a key regulator of C1P-induced VEGF release. Interestingly, as opposed to p38 MAPK inactivation, blocking PKC-ζ activity showed a tendency to elevate levels of VEGF release in basal groups and had no effect on C1P-induced cells (Figure 5.6). Therefore PKC-ζ was not involved in C1P-induced VEGF release.
Figure 5.6 PKC-ζ was not involved in C1P induced VEGF release.

RAW 264.7 cells were incubated with (C1P) or without (NT) 50 μM C1P for 24 hours. In conditions with inhibitors, the inhibitor was applied 45 minutes before C1P treatment. Conditioned media were then harvested, and used to measure VEGF concentration by ELISA. (n=3, one-way ANOVA test followed by Tukey’s post hoc analysis)
5.2.4 The putative Gi-coupled receptor for C1P was not responsible for C1P-induced VEGF release

Unlike cytosolic C1P, extracellular C1P triggers a Gi-coupled receptor from the outside, which then transduces the signals into the cells\(^{301}\). This receptor was confirmed to participate in C1P-induced MCP-1 release\(^ {412}\). Here, we tested whether this receptor transduces signal for VEGF release. As in the MCP-1 study\(^ {412}\), pertussis toxin (PTX) was used to specifically block the Gi-coupled receptor. However, inactivation of the Gi-coupled receptor showed no effect on C1P-induced VEGF release (Figure 5.7). Interestingly, in control groups where no C1P was applied, PTX itself elevated VEGF levels (Figure 5.7), similar to what was observed with PKC-ζ inhibition. OxLDL-treated groups were used as a negative control here, since oxLDL does not signal through Gi-coupled receptors. These observations showed that C1P induces VEGF release via mechanisms other than the PTX-sensitive G protein-coupled receptors.
Figure 5.7 G<sub>i</sub>-coupled receptor was not responsible for signal transduction in C1P-induced VEGF release.

RAW 264.7 cells were incubated with medium alone (NT), 50 μM C1P (C1P) or 25 μg/mL oxLDL (oxLDL) for 24 hours. In conditions with inhibitors, the inhibitor was applied 45 minutes before C1P treatment. Conditioned media were then harvested, and used to measure VEGF concentration by ELISA. Inhibition of PTX-sensitive G protein-coupled receptor increased VEGF release in NT group, but had no effects on both C1P- and oxLDL-induced VEGF release. (n=3, one-way ANOVA test followed by Tukey’s post hoc analysis)
5.2.5 Long chain ceramide 1-phosphate had more potent effects triggering VEGF release.

No matter which mechanism C1P uses to transduce the signal from the outside to the inside, the signal has to pass through the cell membrane, which presents a hydrophobic barrier between the outside and inside of the cell. The kinetics of this process may vary drastically depending upon the length of the carbon-backbone of C1P, since short chain C1Ps are generally more hydrophilic whereas longer chain C1Ps are more hydrophobic. Therefore, we postulated that different backbone lengths of C1P act differently in triggering VEGF release. We compared C2, C8 and C16 C1P with the mixed length C1P used for previous experiments. This mixed length C1P was extracted from bovine brain and is mainly comprised of C18 and C24 C1Ps (Note: it also contains C16, C20, C22 C1Ps, but to a lesser extent). We found that at the same concentrations (molarity), the longer the carbon backbone lengths, the stronger the effects that were observed, and in turn more VEGF was released (Figure 5.8).
Figure 5.8 Long chain ceramide 1-phosphate has more potent effects on inducing VEGF release.

RAW 264.7 cells were cultured with medium alone (Ctrl), mixed-backbone C1P (Sigma-C1P), C2-C1P, C8-C1P, C16-C1P, and 25 μg/mL oxLDL (oxLDL) for 24 hours. All ceramide 1-phosphate used in this experiment were at 50 μM concentration. Conditioned medium were then harvested and used to measure VEGF concentration by ELISA. All data presented are average of two independent repeats of the same experiment.
5.2.6 **Low temperature blocked the transduction of ceramide 1-phosphate-triggered signal.**

The kinetics of C1P signaling transduction depends on not only the length of carbon backbone, but also the rigidity of cell membrane. While incubated at low temperature (e.g. at 15 degree Celsius), cell membranes will become more rigid, which may prevent C1P from being embedded into or transported through the membrane. We determined whether C1P (with different backbone lengths) triggered PI3K activity at 15°C and 37°C in parallel, and found that at normal culture temperature, C1Ps with longer backbone chain trigger higher phosphorylation signals of Akt, which is consistent with VEGF levels measured by ELISA (Figure 5.9). However, at low temperature, most of the Akt phosphorylation was blocked, indicating that C1P cannot induce downstream signaling without being embedded into or transported through the membrane (Figure 5.9). OxLDL-induced Akt phosphorylation was not affected by temperature, which served as a positive control. Unfortunately, this experiment could only be carried out once and this places a major limitation on the conclusions that can be drawn.
RAW 264.7 cells were treated with media alone (Ctrl), mixed-backbone C1P (Sigma-C1P), C2-C1P, C8-C1P, C16-C1P and 25 μg/mL oxLDL (oxLDL) for 15 minutes at 15°C and 37°C in parallel. All ceramide 1-phosphate used in this experiments were at 50 μM concentration. Cells were harvested for western blot probing phospho-PKB. At 37°C, the pattern of PKB phosphorylation matches the pattern of VEGF concentration in the medium (Figure 5.8). However at 15°C, most of C1P-induced PKB phosphorylation was blocked, whereas oxLDL-induced phosphorylation was not affected. This result indicates that C1P-induced PI3K activity requires certain membrane fluidity that allows C1P to be either embedded into or transported through the membrane. Results are from a single experiment.
5.3 Discussion

Not until recently has C1P been considered as an important lipid messenger. It is clear however that C1P participates in a number of critical physiological processes in the cell, such as proliferation and survival. However the underlying mechanisms of C1P’s involvement in these processes are still largely unknown. The knowledge of these mechanisms may provide novel therapeutic approaches to treat diseases like cancer, cardiovascular disease as well as Alzheimer’s disease, where C1P has been found to play a role.

In the context of atherosclerosis, like many other factors, C1P (as well as VEGF) potentially has a dual role, both atherogenic and atheroprotective. The balance between the two highly depends on the interaction of C1P with its microenvironment such as nearby cells. This study, for the first time, reports that C1P can stimulate VEGF release in macrophages. Investigations on the underlying receptor and signaling pathways help understand the full spectrum of how C1P is involved in this process, which will likely provide potential targets for treating atherosclerosis as well as other angiogenesis-related diseases.

In this study, we used a number of pharmaceutical inhibitors to block certain signaling pathways, however it has to be noted that pharmaceutical inhibitors often cannot specifically inhibit single pathways and may shutdown off-target pathways. For instance, LY used in this study has been reported to block other kinases such as CK2, GSK3α and GSK3β. Although in many studies (including this one) LY294002 and wortmannin are used as inhibitors to block PI3K activities, differential effects of LY and WM have also been reported. The discrepancy between LY and WM is partially due to their non-specificity, however the different mechanisms of their
actions may also play a role. LY act as a competitive antagonist for the ATP binding site of PI3K, where as WM covalently modifies PI3K, which is an irreversible effect. To overcome side effects of pharmaceutical tools, target-specific gene silencing methods can be utilized. Moreover cells from genetically modified animals can be used. Such studies in future would help in validating the conclusions that can be made from results presented here.

Signaling pathways like PI3K have a broad range of downstream effects, which makes them difficult to use as therapeutic targets. However, specific isoforms of these kinases can be targeted very precisely. Many PI3K isoform-specific inhibitors are approved or in the process of being approved for clinical use. The next step in this study will be to test which isoform of PI3K is responsible for C1P-induced VEGF release, and in these studies, siRNA or isoform-specific inhibitors can be applied.

At the end of this study, we provided some leads trying to figure out how C1P signal is transduced from the outside to the inside of the cell. Although the results are too preliminary to draw any firm conclusions based on the data we have, a rational hypothesis can be made. Having ruled out a PTX-sensitive G protein-coupled receptor, there are three other possible mechanisms by which C1P may be acting. a) C1P passively diffuses or gets transported into the cells. b) C1P binds to other receptors on the cell surface. c) C1P is embedded into the membrane and flipped over to the inside, where it binds to downstream effectors.

If C1P passively diffuses into the cells, it will be easier for short chain C1Ps to pass the membrane and trigger the signal, because the long chain C1Ps are more hydrophobic and are
likely trapped in the membrane. However our data showed the opposite trend, since longer chain C1Ps have greater induction of VEGF release, so that passive diffusion into the cell is likely ruled out (Figure 5.8).

If C1P binds to other receptors on the surface, lowering the temperature should have limited effects on the signal transduction, as was shown with the oxLDL-induced signal in Figure 5.9. However C1P signals were blocked at 15°C, which indicates that the rigidity of the membrane plays a critical role here. Thus, it is less likely that this signal is mediated via a protein receptor at the cell surface. However, it is also worth mentioning that in some cases, membrane rigidity does affect signal transduction via receptors. Given the limited data obtained in this study, the conclusions may be considered premature at this point. However, it can be suggested that in order to transduce the signal and activate VEGF release, C1P is either embedded into the membrane or is actively transported into the cells. Further experiments will be needed to prove (or disprove) this hypothesis. Non-polar small fluorescent tags like Anthrylvinyl have been used to probe ceramide, which can then be evaluated in real time. Similar techniques can be applied to C1P in future studies.
Chapter 6: Summary

Atherosclerosis has been identified as a chronic inflammatory disease\textsuperscript{1,206}. The development of atherosclerotic plaques is a result of interactions between immune cells and their microenvironment. Macrophages, as critical players in innate immunity, are among the first immune cells recruited to atherosclerotic sites. Upon their arrival, macrophages participate in all major events throughout all stages of atherosclerosis progression, including foam cell formation, recruitment and activation of adaptive immune cells (as well as smooth muscle cells), formation of necrotic lipid core, as well as destabilization and rupture of the plaque. Our laboratory has had a continuous interests in macrophages’ involvement in atherosclerosis, especially based upon their interaction with oxidized LDL (and other lipid messengers). We found that oxLDL promotes macrophage survival in the absence of MCSF, and this prolonged survival breaks the balance between apoptosis and efferocytosis in the early stages of atherosclerosis, which sets the stage for foam cell formation. There are at least three possible mechanisms utilized by oxLDL to promote macrophage survival. A) oxLDL activates the PI3K/Akt pathway in macrophages\textsuperscript{267}. B) oxLDL blocks ceramide generation, which in turn maintains Akt activation and Bcl-xL levels in macrophages\textsuperscript{268}. C) oxLDL activates eEF2K signaling pathway in macrophages\textsuperscript{269}.

This first project of this dissertation was a follow-up of a previous graduate student’s work, showing that oxLDL induced eEF2K activation in bone marrow-derived macrophages. In the previous work, he found that oxLDL increases eEF2K activity in macrophages and this activation was required for the pro-survival effect of oxLDL\textsuperscript{269}. Thus blocking eEF2K activity impairs oxLDL-induced macrophage survival, and potentially reduces foam cell formation and decreases atherosclerosis progression. However, all of those experiments were conducted \textit{in}}
vitro. My work was able to utilize a mouse model system with impaired eEF2K activity to verify whether blockade of eEF2K activity indeed decreases atherosclerosis development in the animal. With the availability of a catalytically inactive eEF2K (KI) mouse model, I collected bone marrow cells from both wild-type and KI mice and transplanted them into \( ldlr^{-/-} \) mice, which served as an atherosclerosis model. This required prior irradiation of the recipient mice to kill all of their endogenous bone marrow cells. Compared to ubiquitous gene knock out, bone marrow transplantation limited the changes of eEF2K activity only to bone marrow cells, and thus the hematopoietic compartment of the animals, which allowed me to investigate eEF2K function in a more specific way. After feeding with high-fat diet for 16 weeks, recipients of KI bone marrow cells showed a significant reduction in atherosclerotic plaque size. Plaque areas in whole aorta reduced from 13% to 7.7%, a reduction of over 40%. Plaque sizes in aortic root cross-sections were also reduced from 45.2% to 37.7%. To further investigate the potential mechanisms involved, I performed multiple functional assays including TUNEL assay, in-vitro viability assay, lipid profile assay, quantification of adhesion molecules, as well as ELISA for cytokines. While still at an early stage of characterization, and with many avenues of investigation still required for a complete follow-up, at this point there are a number of conclusions that can be made. I have been able to show that apoptosis of macrophages was not affected in vivo or in vitro. On the other hand, loss of eEF2K activity affected cytokine release in macrophages, which in turn decreased adhesion molecules expression in endothelial cells. Based on our results thus far, The in vivo verification of adhesion molecule expression was unsuccessful due to the inability to perform IHC/IF in the samples we have obtained to date (discussed in Section 3.3). This can be easily fixed in a subsequent set of tissues from these types of experiments by preparing both cryo-sections as well as paraffin-embedded cross-sections.
Regarding future directions, many experiments can be proposed to follow up this work. First of all, eEF2K conditional knock out (eef2k\textsuperscript{flo}x) mice have now been generated by Dr. Christopher Proud at University of Southampton, and these have recently been crossed with atherosclerosis model ldlr\textsuperscript{-/-} mice. Our future plan would be to cross these mice with mice having Cre recombinase under the control of a myeloid-specific promoter. The resulting mice would delete the eef2k gene in myeloid lineage cells that express Cre. Using these mice, we would first want to confirm the hypothesis that eEF2K activity in macrophages (or other myeloid cells) affects atherosclerosis. This would be an improvement over having to do transplants of bone marrow cells, which results in mice with defective eEF2K activity in all hematopoietic cells. Both cryo-sections and paraffin-embedded cross-sections will have to be prepared for proper IHC/IF experiments that can examine multiple markers for macrophage and endothelial cells. If needed, lymphoid-specific Cre mice can be crossed with eef2k\textsuperscript{flo}x/ldlr\textsuperscript{-/-} mice, so that we can rule out a role for eEF2K loss from lymphoid cells, which could not be ruled out from the bone marrow transplant studies. However, this may only be required if we unexpectedly were unable to confirm our hypothesis in the myeloid-specific knockouts. Secondly, a comprehensive study regarding cytokines affected by eEF2K loss will need to be conducted. In one initial effort to achieve this goal, I have been working with one of our collaborators, Dr. Horacio Bach, trying to identify a full spectrum of cytokines affected by the lack of eEF2K activity in macrophages using mass spectrometry. Furthermore, based on our findings, eEF2K inhibitors are proposed as a therapeutic drug to treat atherosclerosis. A collaborative project has been initiated with the Centre for Drug Research and Development at the University of British Columbia, aiming to select novel and effective eEF2K inhibitors with a potential use in clinical therapy. In summary,
the work in Chapter 3 of this dissertation is the first set of evidence showing eEF2K activity affects the development of atherosclerosis in animal models, which supports the potential use of eEF2K inhibitors as a potential treatment for atherosclerosis.

While the pro-survival function of oxLDL in macrophages is one of the major areas of investigation in our research group, our laboratory is also interested in other aspects of oxLDL function in macrophages. For instance, we have been trying to identify receptors that are responsible for the transmission of oxLDL signals. I have also devoted my efforts investigating other potential ways in which oxLDL may affect macrophages and their involvement in atherosclerosis. Recently, evidence has accumulated, based on mRNA expression profiles, that macrophages can be categorized into multiple subtypes. These subtypes can interchange under certain circumstances in processes that are defined as macrophage plasticity. I was particularly interested in how oxLDL may be involved in altering macrophage plasticity, which is the work detailed in Chapter 4. First of all, I tested whether oxLDL can affect macrophage plasticity based on the most widely used means of differentiating macrophages to the subtypes referred to as M1 or M2. Although I found that oxLDL cannot polarize macrophages towards either M1 or M2, it can skew macrophages away from both M1 or M2 subtypes, decreasing the level of expression of the key markers in both instances. With further experiments, I found that oxLDL, when incubated with naïve (undifferentiated) macrophages, polarizes the cells towards a novel subtype that we have named MoL, with Hmox-1 and Mic-1 as two of its key markers. This subtype is similar, but not identical, to macrophages that have been incubated with oxidized phospholipids, which reportedly leads to differentiation to the Mox subtype. Half of the reported Mox markers that we tested were not MoL markers. While much
more characterization will be necessary to fully describe the MoL subtype, initial studies investigated the signaling pathways involved in MoL polarization. Using pharmaceutical inhibitors, I showed that blockade of PI3K using LY blocked MoL polarization. In trying one functional assay, I was able to show that, compared to M1 and M2 subtypes, conditioned medium from MoL cells had activity that specifically increased adhesion molecules on endothelial cells. While at this stage we have not been able to connect the findings in MoL cells to those of macrophages lacking eEF2K activity, it will certainly be interesting to know the activity in MoL cells is dependent upon eEF2K activity. To date, I have not found any role for eEF2K in MoL cells, but a more definitive study would be to utilize cells that are completely lacking eEF2K.

Another limitation of our analysis of MoL cells was that due to limited funding and resources we had on this project, all measurements were limited to gene expression analysis at the mRNA level. Verifications of protein levels for each of the markers would be critical to validate the importance of the markers and will hopefully be performed in near future. Although preliminary at this stage, this work has made the exciting discovery of a novel macrophage subtype that is highly related to atherosclerosis development. This may partially explained the fact that in atherosclerotic lesions, M1 and M2 macrophages only account for 40% and 20% of macrophage population, respectively\textsuperscript{155} and thus the MoL (or Mox) subtypes may be an important contributor. A more comprehensive characterization of the macrophage subtypes in atherosclerotic lesions is the next phase of development of this part of the project.
An important step to further characterize MoL macrophages is to perform a high-throughput characterization of the MoL expression profile. Several techniques can be applied here. For instance, RNA-Seq can be used to reveal a snapshot of the transcriptome, which will allow us to get a full spectrum of the genes expressed in different macrophages subtypes. To interpret the results, any genes of interest can be sub-categorized into three groups: cytosolic, secretory and surface markers. Cytosolic gene expression would be verified by immunoblotting. Potentially interesting genes in this group would be kinases or transcription factors, which can be further verified to see if they are critical in MoL polarization, which could be tested by determining whether genetic silencing of critical kinases or transcription factors will result in MoL-null mice. The next step in such analyses would be crossing MoL-null mice with ldlr<sup>-/-</sup> mice to allow us to investigate loss of MoL activity in the context of atherosclerosis development. Other factors, such as secretory markers, can be verified by ELISA. Genes in this group may be related in MoL functions (through autocrine effects or paracrine effects). Any gene that has a known effect in atherosclerosis development will also lead us to experiments that can test possible MoL functions. Last but not least, surface markers can be verified by immunoblot and antibodies can be used to probe MoL cells by flow cytometry or using IHC/IF. Additionally, receptors would potentially be identified among the surface markers. Identification of potential receptors transducing oxLDL signals would be an ideal outcome from these experiments. All experiments that I proposed above have been written in a grant proposal that was recently submitted.

Besides oxLDL, our laboratory has also been working on other lipid messengers that are involved in regulation of macrophages. Collaborating with Dr. Antonio Gómez-Muñoz (University of the Basque Country, Bilbao, Spain), we have shown that similar to responses to
oxLDL, ceramide 1-phosphate can promote macrophage survival through PI3K/Akt pathways. We have also found that both oxLDL and ceramide 1-phosphate elevated VEGF levels in macrophages. In Chapter 5, I followed up this work and investigated which signaling pathways were involved in C1P-induced VEGF release. First of all, I used a broad selection of pharmaceutical inhibitors and found that PI3K/Akt pathway and MEK/ERK pathway are involved in C1P-induced VEGF release, whereas p38 MAPK and PKC-ζ are not involved. Then I made efforts to reveal how exogenous C1P signal is transduced into the cells. While it is too early to draw any firm conclusions based on the data obtained to date, it is reasonable to hypothesize that to transduce its signal, C1P is embedded into the membrane or is actively transported into the cells.

As discussed in Section 5.3, the use of pharmaceutical inhibitors has many caveats. Our collaborator, Dr. Antonio Gómez-Muñoz, is working on specific inhibition of PI3K/Akt and MEK/ERK pathways by siRNA. Such an approach can be used to solidify the underlying signaling pathways regulating C1P-induced VEGF release. Regarding the hypothesis of the role of C1P internalization and/or insertion into the plasma membrane as a component of its signal transduction mechanism, further experiments would be needed to prove or disprove it. Non-polar small fluorescent tags like Anthrylvinyl have been used to probe ceramide, which can be evaluated in real time. Similar techniques can be applied to C1P. Unveiling the mechanism of C1P signal transduction can provide novel clinical targets to regulate VEGF expression, and in turn regulate atherosclerosis development.
In summary, work in this dissertation approached macrophages functions in the context of atherosclerosis from three different angles, and contributed to how macrophages functions are regulated by interacting with factors in the microenvironment. Results from this dissertation provide potential new avenues for discovery of novel therapeutic approaches to treat atherosclerosis.
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