Inactivation of IL-15 gene expression does not reduce atherosclerosis in a mouse model of carotid artery narrowing

Inactivation of IL-15 gene expression does not reduce atherosclerosis in a mouse model of carotid artery narrowing

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Abstract

IL-15 is a pleiotropic cytokine which influences a variety of immune and inflammatory responses. Our lab has previously demonstrated that IL-15 and IL-15Rα promote atherosclerosis. However, the mechanisms by which IL-15 affect atherosclerosis development were not fully defined. In this study, we reported that overexpression of the IL-15 gene resulted in an increase of granzyme B level in the atherosclerotic plaque of ApoE deficient mice. Furthermore, we observed that leukocytes-specific genetic deletion of IL-15Rα reduced the granzyme B level within atherosclerotic lesions from LDLr deficient mice. Collectively, our data shows one of the mechanistic pathways by which IL-15 promotes atherosclerosis development.

Moreover, we tested the role of IL-15 in carotid artery disease. It has been reported previously that immunization of low density lipoprotein receptor deficient mice against IL-15, by inoculating them with bacteria harboring an IL-15 expression plasmid, led to reduced development of diet induced atherosclerosis in carotid arteries whose diameter was restricted to induce a hemodynamic stress. Others, however, reported that injection of wild type mice with an antibody against IL-15 triggered increased neointima formation in carotid arteries that were partially ligated. In our study, we found no differences in the amount of collar/diet induced atherosclerosis in control apoE KO mice and in IL-15/apoE dKO mice. Therefore,

inactivation of IL-15 gene expression does not appear to affect the rapid onset of atherosclerosis in carotid arteries of ApoE KO mice induced by a combination of high fat diet and hemodynamic stress.

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Finally I would like to dedicate this work to my parents, Abdullah and Afra: I will remain forever unable to repay your loving kindness and compassion that began before I was born and continues unabated.

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ADCC	Antibody-dependent cell-mediated cytotoxicity
APC	Allophycocyanin
ApoB	Apolipoprotein B
ApoE	Apolipoprotein E
CAD	Coronary artery disease
CD	Cluster of differentiation
DAPI	4',6-diamidino-2-phenylindole
ECM	Extracellular matrix
FasL	Fas ligand
FITC	Fluorescein isothiocyanate
FPLC	Fast protein liquid chromatography
HDL	High Density Lipoprotein
ICAM-I	Intracellular adhesion molecule-I
IFN-γ	Interferon gamma
IL	Interleukin
IL-15	Interleukin 15
IL-15Rα	Interleukin 15-receptor alpha
IL-15Rβγ	Apolipoprotein E
JAK	Janus Kinase
JNK	c-Jun NH2-Terminal Kinase
KO	Knockout
LDL	Low Density Lipoprotein

LDLr	Low Density Lipoprotein receptor
M-CSF	Macrophage colony-stimulating factor
MHC-I	Major histocompatibility class I
MHC-II	Major histocompatibility class II
mRNA	Messenger ribonucleic acid
NF-kB	Nuclear factor kappa-light-chain-enhancer of
NK cells	Natural Killer cells
NKT cells	Natural Killer T cells
NOS	Nitric oxide synthase
ORO	Oil Red O
PBS	phosphate buffer saline
PD-1	Programmed cell death protein 1
PE	Phycoerythrin
Rag	recombination-activating genes
RBC	Red Blood Cells
SAA	Serum amyloid A
SMCs	Smooth muscle cells
SRA	Scavenger receptor A
STAT	signal transducer and activator of transcription
TCR	T cell receptor
TNF-α	Tumor necrosis factor-alpha
TRAIL	*TNF-related apoptosis-inducing ligand*
VCAM-I	Vascular cell adhesion molecule-I

1. General Introduction and Objectives:

1.1 Overview of atherosclerosis:

1.1.1 Epidemiology and definition of atherosclerosis:

Epidemiological studies revealed that cardiovascular diseases are the most common cause of death in developed countries. Current statistics state that 17.3 million deaths are due to cardiovascular diseases and this number is expected to increase to more than 23.6 million by 2030[1]. In addition to the global burden of the disease, the Canadian economic cost of cardiovascular diseases is more than $20.9 billion every year and such massive expenses will have an effect on the Canadian economy as the prevalence rates continue to rise[2].Therefore, there is an urgent need to better understand molecular mechanisms of cardiovascular diseases to provide us with more effective prevention and treatment of the most common cause of death in the modern era.

Atherosclerosis is a fundamental aspect of the etiology of cardiovascular diseases such as coronary artery disease (CAD) and cerebrovascular disease and it is defined as a formation of atherosclerotic lesions in the intima of large- and medium- sized arteries[3]. These lesions consist of necrotic cores, calcified region, accumulated modified lipids, smooth muscle cells, leukocytes, and foam cells[4]. For many years, it was thought that the development of atherosclerosis was a result of accumulation of lipid in the arterial wall[5]. This view was supported by

considerable evidence that shows a correlation between serum cholesterol level and atherosclerosis development[6]. Although high level of low-density lipoprotein (LDL) remains the most important risk factor for atherosclerosis, immune and inflammatory mechanisms of atherosclerosis have received considerable attention in the past few decades[7].Today, atherosclerosis is viewed as a more complex disease which involves vascular, metabolic, and immune systems with local and systemic manifestation.

1.1.2 Pathophysiology of atherosclerosis:

It is well-known that high concentrations of cholesterol in the blood promote atherosclerosis. Since lipids such as cholesterol and triglycerides are insoluble in the blood, they are transported by low density lipoprotein (LDL) particles. These particles carry esterified cholesterol and triglycerides internally and shielded from the blood by a phospholipid monolayer with free cholesterol and apolipoprotein B[8]. Atherosclerosis is initiated when apolipoprotein B-containing LDL particles infiltrate into the arterial intima, the innermost layer of the artery, where they are retained in the intima as a result of interactions between apolipoprotein-B 100 and the extracellular matrix[4]. As result of this retention, LDL particles are trapped in the intima where they become exposed to oxidative modification[9]. These oxidized LDL particles as well as the turbulent blood flow at arterial branch sites cause local activation of endothelial cells which is considered as an early marker of

atherosclerosis[10]. Activated endothelial cells express a number of adhesion molecules such as ICAM and VCAM which facilitate the recruitment of monocytes and lymphocytes to the sub-endothelial space where the atherosclerotic plaque develops. Activated endothelial cells also secrete chemokines such as macrophage colony stimulating factor (M-CSF) which differentiate recruited monocytes into macrophages. M-CSF also influences macrophage functions such as expression of scavenger receptors[10]. Two scavenger receptors, primarily SRA and CD36, appear to be responsible for endocytosis of oxidized LDL particles leading to accumulation of cholesterol particles as cytosolic droplets in macrophages which are eventually transformed into foam cells, hallmark cells of the atherosclerotic lesion[11]. Moreover, scavenger receptor-mediated endocytosis of oxidized LDL results in degradation of oxLDL components, and presentation oxLDL-derived antigens to T cells.

T cells are recruited to the site of the atherosclerotic lesion by mechanisms similar to monocyte recruitment involving adhesion molecules and chemokines, and are present in all stages of atherosclerotic plaques. Activated T cells, along with macrophages, produce a wide range of cytokines with pro- atherogenic effects which highly contribute to the development of atherosclerosis[12]. Cytokines of Th1 pathway, such as TNF-alpha and IL-1beta, exacerbate the immune response, inhibit collagen synthesis and increase accumulation of apoptotic cells and

extracellular degradation thereby promoting thinning of the fibrous cap and plaque rupture[13]. All these observations indicate that the local inflammatory response is one of the major mechanisms related to atherosclerosis development and there are a variety of inflammatory and immune cells are implicated in the atherogenic process. Therefore, understanding the inflammatory process associated with atherosclerosis development may lead to discovering potential targets for therapeutic intervention.

1.1.3 Model of atherosclerosis in animal studies:

During the last few decades, several animal species and atherogenic stimuli have enhanced our understanding of atherosclerosis development. Mice models are primarily used in medical research fields because of its well-known genetics, the availability of a number of inbred genetic backgrounds, ease of breeding, and low cost of maintenance. Wild type mouse strains are normally resistant to the development of atherosclerosis. This is due to the normal high level of circulating high density lipoprotein (HDL) and low level of circulating low-density lipoprotein (LDL) in mice[14]. Therefore, deletion of genes involved in lipoprotein transport render mice susceptible to atherosclerosis. ApoE is a primary ligand for several lipoprotein receptors and plays a major role in the clearance of lipid from the circulation[15]. ApoE knockout mice develop spontaneous atherosclerosis at the aortic sinus as early as 6weeks under high fat diet conditions and 12 weeks under

normal chow diet. However, the lipoprotein profile in apoE deficient mice differs from the lipoprotein profile in human atherosclerotic patients. In apoE deficient mice, VLDL is the major cholesterol carrying molecule, but in humans it is LDL[14].

LDLr plays a major role in the clearance of plasma LDL cholesterol. Unlike apoE knockout mice, LDLr knockout mice require high fat diet to develop atherosclerosis. One advantage of this model is that the lipoprotein profile is similar to those in human atherosclerotic patients[16]. Although these models have been commonly used, another mouse model which develops site-controlled lesion formation in response to arterial injury has gained considerable advantages. For instance, rapid atherosclerosis seen in this model allows researchers to test many potential therapeutic reagents, particularly those with limited duration of effectiveness.

Perivascular collar placement has been used as a tool for localized atherosclerosis induction in animals. This model, originally established in rabbits and recently adapted to mice, develops rapid atherosclerosis in response to carotid arterial injury [17]. Carotid arteries are resistant to atherosclerosis under normal conditions and this is due to the sustained laminar flow and high shear stress [18]. Alteration in laminar flow and low shear stress as a result of collar placement around carotid arteries in conjunction with cholesterol feeding induces rapid site-control neointima formation [19]. Whereas neointima formation is homogenous and

cellular in LDLr deficient mice, it is heterogeneous and acellular in apoE deficient mice. In both strains, collar-induced atherosclerotic plaque has significant extracellular matrix deposition and it is associated with intimal accumulation of both lipid-rich foam cells and smooth muscle cells. Furthermore, the presence of the collar significantly increases the expression of adhesion molecules, ICAM and VCAM, and decreases eNOS at the proximal site[19].

Although the above mentioned models of atherosclerosis have some limitations, these models have been widely used as means to dissect the mechanisms of atherogenesis. Mouse models provide a view of atherosclerosis varying from fatty streaks to mild, moderate and severe complex plaques. Importantly, mouse models of atherosclerosis present the opportunity to explore a variety of risk factors and modulators of atherogenesis.

1.2 Role of NK, NKT and CD8 T cells in atherosclerosis:

1.2.1 Development, activation and effector functions of cytotoxic T cells:

Cytotoxic T cells are a subset of T cells and play an important role in the adaptive immune system by eliminating viral infected and cancerous cells. T cell development takes place within the thymus, a specialized organ where T cell progenitors mature and differentiate. Interaction between T cell receptor (TCR) on the surface of T cells and MHC-peptide complex displayed by thymic cortical epithelial cells plays an important role in defining the fate of T cells. At this point,

the developing T cells express both CD4 and CD8 as well as TCR receptors. Cells that have a receptor able to recognize MHC-I molecule receive both survival and maturation signal. These cells eventually stop CD4 expression, maintain CD8 expression and are positively selected as CD8 T cells. Cells that have a receptor able to recognize MHC-II molecule receive also a survival signal and different maturation signal. These cells eventually stop CD8 expression, maintain CD4 expression and are positively selected as CD4 T cells. The surviving T cells are now mature naïve CD4 and CD8 T cells and they migrate to the circulation and secondary lymphoid system[20].

CD8 T cells require antigen specific stimulation and cytokine signalling to become activated effector cells. Generally, antigen-presenting cells such as macrophages and dendritic cells present antigen-MHC-I complex to TCR on the surface of CD8 T cells but it is also possible that exogenous antigen can be presented to CD8 T cells via a process known as cross presentation. Once they are activated, CD8 T cells proliferate for 4-5 days and differentiate into effector CD8 T cells[21]. Activated CD8 cytotoxic T cells induce apoptosis in infected cells by the perforin/granzyme B pathway in which perforin creates a pore through which perforin-granzyme complexes enter the infected cell and granzymes are released and activate cytosolic enzyme pathways leading to cell death[21]. The death receptor pathway is another mechanism by which effector CD8 T cells induce apoptosis in

infected cells. In this pathway, Fas ligand on CD8 T cells triggers the apoptotic signal in infected cells through interacting with death receptors. Moreover, activated CD8 T cells secrete proinflammatory cytokines such as TNF-α and IFN-γ which can recruit more leukocytes to the site of infection and eventually exacerbate the inflammatory responses[21,22].

1.2.2 Role of cytotoxic T cells in atherosclerosis:

A growing body of evidence has revealed that cytotoxic T cells are involved in pathogenesis of several immune diseases. In atherosclerosis, cytotoxic T cells are the major lymphocyte population and they account for 29% of leukocytes in the early atherosclerotic plaque increasing to 50% in advanced plaques in humans[23]. The atherogenic roles of CD8 T cells and their molecular mechanisms have been studied during the past few years. It has been shown that activation of CD8 T cells by injection of an agonist to the TNF-like receptor CD137 increases the recruitment of CD8 T cells to the atherosclerotic lesion resulting in increased formation of atherosclerotic plaque in apoE deficient mice[24]. In line with this, genetic disruption of programed death-ligand 1/2, which are ligands of PD-1 immunoreceptor, lead to increased atherosclerosis development in aortic sinus and aorta of LDLr deficient mice, with massive infiltration of CD4 and CD8 T cells in the lesion[25]. Recently, CD8 T cells were found to promote atherosclerosis through their cytotoxic mediators, notably granzyme B and perforin, and through their

inflammatory cytokine TNF-α[26]. Although these observations indicate that CD8 T cells promote atherosclerotic plaque development, one paper has reported that reconstitution of Rag deficient mice with CD8 T cells decreases neointima formation possibly by targeting endothelial and smooth muscle cells[27]. One limitation of this study is that it was not done on atherosclerosis-prone mice. Therefore, these findings demonstrate that CD8 T cells are prominent contributors of atherosclerotic plaque in the aortic sinus and more studies are needed to examine the effects of CD8 T cells in atherosclerosis in different arterial sites. Modulating numbers or activities of these cells may provide novel opportunities for preventing atherosclerosis.

1.2.3Activation and effector functions of Natural Killer cells:

Natural killer cells are a unique subset of lymphocyte and are important effector cells in the innate immune system. NK cells represent 5-15% of the total circulating lymphocyte populations and develop from precursor cells in the bone marrow and eventually emigrate to the lymphoid and non-lymphoid tissues, including BM, lymph nodes (LN), spleen, peripheral blood, lung and liver[28]. They are called natural killer cells because they do not require prior sensitization to demonstrate cytotoxic activities against tumor and virally-infected cells[29]. Several lines of evidence have shown that NK cells are completely dependent on IL-15 for their development, survival and activation[3031]. It has been shown that dendritic

cells present IL-15 through its private receptor IL-15Rα to its cognate receptor IL-15Rβγ on NK cells[32]. NK cells express a variety of cell surface receptors that play key function in sustaining NK cell activity. NK cell activation is regulated by a balance between the inhibitory receptors and activating receptors[33]. Under normal conditions, the inhibitory receptors which bind to MHC-I molecules found on almost all cell types, deliver a negative signal to NK cells and prevent NK activation. Under stress conditions, cells down-regulate the MHC-1 molecule expression on their surface, which leads to activation of NK cells as they no longer receive the inhibitory signalling in a process called missing-self recognition[33]. In addition, NK cells are stimulated through up-regulating stress-induced ligands by stressed cells and thereby outweighing the inhibitory signalling delivered by MHC-1 molecules; this is known as stress-induced self recognition[33]. In both conditions, activated NK cells can use several methods to exert their cytotoxic effects. These include cytotoxic granules-mediated cell apoptosis, death-receptor ligation and antibody-dependant cell cytotoxicity (ADCC)[34]. Several studies have shown that perforin, FasL and TRAIL are important effectors in NK cell cytotoxicity against both tumor and pathogen-infected tissues[34].

1.2.4 Natural Killer cells in atherosclerosis:

While NK cells may provide beneficial roles in surveillance against cancer and infections, there is emerging evidence that this cell lineage may have a

detrimental role in some inflammatory and autoimmune disorders. In atherosclerosis, NK cells were found present in atherosclerotic plaque in human and mice[35]. In an animal model where the inhibitory MHC-1 receptor Ly49A was overexpressed causing a defective in NK cell cytotoxicity, it has been reported that atherosclerosis is significantly decreased in both the ascending aorta and the aortic arch in HFD-fed LDLr KO mice[36]. Although previous findings suggests that NK cells promote atherosclerosis through their activating receptor Ly49A, it was not clear whether other T cells, particularly NKT and CD8 T cells, also contribute to these effects since they also express the activating receptor Ly49A. A more recent study examining the effects of NK cells on atherosclerosis has provided us with new evidence showing proatherogenic roles of NK cells. In this study, injecting ApoE deficient mice with anti-Asilao-GM1 antibodies, resulting in depleting NK cells without targeting other cells, leads to significant reduction in the aortic sinus atherosclerosis[37]. Furthermore, adoptive transfer of NK cells into $Rag^{-/-}$ $ApoE^{-/-}$ mice which lack all lymphocyte populations increases atherosclerotic lesion development. This increase in atherosclerosis is associated with increased accumulation of macrophage, MCP-1 expression and necrotic core size. Interestingly, these adoptively transferred NK cells failed to increase atherosclerosis when they were deficient in perforin and granzyme B suggesting that atherogenic activities of NK cells depend on perforin and granzyme B[37]. These

attributes show that the NK cell is a potential target of therapy in preventing or treating atherosclerosis and its complications.

1.2.5Activation and effector functions of Natural Killer T cells:

NKT cells are a distinct subset of T lymphocyte which represents 5% of peripheral blood lymphocyte. However, NKT cells are present in a high numbers in liver accounting for 20-35% of total lymphocyte in mouse and 10-15% in human[38]. NKT cells carry both conventional T cell surface markers, TCR and CD4, as well as natural killer cell surface markers, NK1.1 and Ly49[39]. Unlike T cells which recognize antigens presented by the MHC system, NKT cells recognize a wide array of glycolipid and cholesterol antigens presented by the CD1d molecule on the antigen-presenting cells[40]. Upon activation, NKT cells secrete large amounts of Th1-associated cytokines, such as INF-γ and TNF-α, and Th2-associated cytokines such as IL-4 and IL-10 which reflect the complexity of NKT cell function and highlight the importance of studying the roles of these cells in different conditions[39].

1.2.5 Natural Killer T cells in atherosclerosis:

Although NKT cells play a protective role in autoimmune disease and metastatic cancers, NKT cells have been implicated in many inflammatory diseases and promote the development of Th1-immune responses. In fact, there are several lines of evidence supporting the link between NKT cells and the development of

atherosclerotic plaque. First, NKT cells are present in the atherosclerotic plaque of human and mice[41]. Moreover, genetic deletion of the CD1 molecule, which causes a loss of NKT cells, resulted in a reduction in atherosclerosis progression in apoE deficient mice[42]. It has been shown that administration of α-GalCer, which activates NKT cells, increases atherosclerosis in apoE mice[43]. To further examine the roles of NKT cells in atherosclerosis, adoptive transfer NKT cells into $Rag^{-/-}$ $LDLr^{-/-}$ double knockout augmented atherosclerosis development and that effect disappeared when these cells were deficient in perforin and granzyme B[44].

Collectively, these data suggest that NKT cells promote atherosclerosis in a perforin and granzyme-dependant manner. Although most studies reported a proatherogenic role for NKT cells, one paper has found a protective role of NKT cells in atherosclerosis[45]. In this study, activation of NKT cells with α-GalCer protects LDLr but not apoE deficient mice from collar-induced atherosclerosis in carotid artery. However, this finding may be a result of using a different model or that NKT cells may have different effect in different arterial sites. In fact, NKT cells are repeatedly reported to have atherogenic effects in the aortic sinus but not the carotid artery and these effects are dependent on perforin and granzyme B. Thus, modulating NKT cell numbers or activities may be useful in attenuating atherosclerosis in a site specific manner.

1.2.6 Intracellular and extracellular activities of granzyme B:

The capacity of cytotoxic lymphocytes to eliminate infected or transformed cells is a central principle of immune system. The major types of cytotoxic lymphocytes are NK, NKT and CD8 T cells. Despite the differences in how these cells are activated and how they recognize their targets, the mechanism by which they induce apoptosis in their target cells are similar. The granzyme B/perforin cytotoxic pathway has been considered as a primary mechanism used by these cells. Granzymes belong to serine protease family and are mostly found in the granules of cytotoxic T cells, NK cells and NKT cells. There are 5 granzymes in humans and 11 in mice. Granzyme B is one of most abundant granzymes and has been studied extensively during the last decade. In addition to cytotoxic lymphocytes, Granzyme B can be expressed in a number of cell types under proinflammatory conditions. These cells include macrophages dendritic cells, neutrophils, smooth muscle cells and mast cells. It has been shown that the killing efficiency in effector cells is significantly reduced when these cells lack granzyme B, reflecting the importance of this protease in eliminating tumorigenic or infected cells[46].

Along with perforin, granzyme B is the major constituent of cytotoxic granules within the effector cells and their lytic activities are blocked by the acidic pH of granules so that they do not lyse the host cells. Once effector cells recognize

target cells, these granules move to the plasma membrane adjacent to the target cell. Next, these granules are fused with the plasma membrane and then secreted to the space between the two cells, known as the immunological synapse. Upon degranulation, perforin binds to the target cell's membrane and oligomerizes in a $Ca2^{+}$ dependant manner to form a pore in the plasma membrane. This allows for passive diffusion of granzymes inside the cells. Although perforin deficiency impaired lymphocyte-mediated cytotoxicity, others have reported other mechanisms facilitating the endocytosis of granzymes independent of perforin. These mechanisms include mannose 6 phosphate receptors, heat shock protein-70 and serglycin-granzymes complexes. This might suggest that perforin has a role beyond facilitating granzyme cell entry[47].

Once it is released into the cytoplasm, granzyme B induces target cell death through multiple pathways that eventually lead to apoptosis. The major mechanism is through the direction of the BH3-family protein Bid to the mitochondrial membrane which results in interaction with proapoptotic protein Bax and/or Bak. This eventually leads to the release of cytochrome c into the cytosol and apoptosome formation. Furthermore, Granzyme B is also reported to directly initiate the cleavage of caspases such as caspase-3, -7 and -8 resulting in caspase cascade activation and manifestation of apoptosis phenotype[48].

As I mentioned above, granzyme B has been shown to be expressed in a variety of cell types including immune cells such as macrophages and B cells and non-immune cells such as keratinocytes and smooth muscle cells. As some of these cells either do not express perforin or do not form immunological synapse, granzyme B is secreted extracellularly. Previously, the extracellular activity of granzyme B was underestimated for scientific and therapeutic interests. However, recent discoveries that report the existence of extracellular granzyme B in body fluids and its association with pathological conditions have turned the attention to the role of granzyme B independent of perforin and cytotoxicity. Indeed, granzyme B is present in plasma, synovial fluids and cerebrospinal fluids and its level is elevated in chronic inflammatory diseases such as atherosclerosis and rheumatoid arthritis. In addition to intracellular apoptosis, extracellular granzyme B can degrade a variety of extracellular matrix substrates such as betaglycan, vitronectin and laminin which lead to remodelling of the extracellular matrix and collagen disorganization[48].

In short, the granzyme/perforin cytotoxic pathway is the major mechanism by which cytotoxic immune cells kill their targets. Recent work has shown granzyme B can be secreted by both immune and non-immune cells and the functions of this protease include intracellular apoptosis as well as matrix degradation.

1.2.6 Granzyme B in atherosclerosis:

Granzyme B is present in atherosclerotic plaque and its expression level is correlated with the disease severity[49]. Within the atherosclerotic lesion, there are several potential sources of granzyme B including cytotoxic lymphocytes, macrophages and smooth muscle cells. Recently, granzyme $B^{-/-}$ $apoE^{-/-}$ mice reportedly developed less plaque area in the descending aorta compared to the control apoE-/- mice. Although deficiency of granzyme B did not affect the plaque development in the aortic sinus, atherosclerotic plaque from granzyme $B^{-/-}$ $apoE^{-/-}$ mice had greater collagen and decorin contents compared to the control $apoE^{-/-}$ group[49]. One mechanism by which granzyme B contributes to atherosclerosis progression is through induction of macrophage and smooth muscle cell apoptosis as well as degradation of extracellular matrix. In line with this, granzyme B is found to localize to TUNEL-positive foam cells[50]. Moreover, others have shown that granzyme B induces apoptosis in SMCs even in the absence of perforin trough disruption the SMC-ECM interactions and consequently induction of anoikis[51]. These attributes suggest that granzyme B not only promotes atherosclerosis development but also contributes to plaque instability. Indeed, the highest level of granzyme B in plasma is detected in patients with unstable plaques[52]. Furthermore, it was found that the production of granzyme B by mononuclear cells in the blood isolated from patients with unstable angina pectoris is significantly higher than

those from patients with stable angina pectoris[53]. These findings show that granzyme B is involved in the pathogenesis of atherosclerosis through both intracellular and extracellular activities resulting in formation of necrotic core and plaque rupture.

1.3 Role of Interleukin 15 (IL-15) in atherosclerosis:

1.3.1 Molecular characteristics and signalling of Interleukin 15:

Interleukin 15 (IL-15) is a cytokine belonging to the 4 α-helix bundle family of cytokines. It is encoded by the IL15 gene, which is located on chromosome 4 in humans and chromosome 8 in mice. Human IL-15 shares 73% sequence identity with murine IL-15[54]. Two mRNA isoforms of IL- 15 have been reported in humans and mice. Although both isoforms encode an identical mature IL-15 protein, they differ in their signal sequence, translation efficiency and cellular trafficking. One isoform, which has a long signal peptide of 48 amino acids (IL-15 LSP), is directed to the endoplasmic reticulum and Golgi apparatus. It exists in two forms: secreted or membrane-bound, particularly in antigen presenting cells. The other isoform, which has a short signal peptide of 21 amino acids (IL-15 SSP), is not secreted and found only in the nucleus and cytoplasm[55].

IL-15 gene is expressed in a variety of cell types including, monocyte-derived macrophages, dendritic cells, skeletal muscle cells, fibroblast, endothelial cells and smooth muscle cells[54]. In contrast to the widespread nature of IL-15

mRNA expression, it has been difficult to demonstrate IL-15 protein in the supernatants of many cells expressing message for this cytokine. This discrepancy is because IL-15 is post-transcriptionally regulated[56].

Biologically, IL-15 signals are delivered through its heterotrimeric receptor including a γ subunit shared with IL-2, IL-4, IL-7, IL-9 and IL-21, and a β subunit shared with IL-2[54]. The third component is a private subunit IL-15Rα which binds to IL-15 alone with high affinity. IL-15Rα is widely expressed in a variety of cell types independently of the IL-15Rβγ chain complex[57]. It has been reported that dendritic cells lacking IL-15Rα cannot secrete IL-15, suggesting a critical role of IL-15Rα subunit in the IL-15 production from the cells[32]. In line with this, a recent study has found that IL-15 in plasma from lymphodepleted melanoma patients and mice was associated with IL-15Rα[58]. Moreover, it has been shown that once IL-15Rα binds to IL-15, the complex may be retained on or disassociate from the cell surface in the form of soluble IL-15/IL-15Ra complex[32].

The main mechanism of IL-15 signalling is trans-presentation where the membrane-bound complex of IL-15/IL-15Rα is presented by macrophages or dendritic cells to the NK, NKT or CD8+ T cells, expressing the heterodimeric receptor IL-2/IL-15Rβγ. This signalling activates the phosphorylation of the receptor-associated JAK1 and subsequently phosphorylates STAT3 via the β-chain and JAK3/STAT5 via the γ-chain. Once phosphorylated, STAT3 and STAT5

proteins form a heterodimer which translocates to the nucleus where it binds to the promoters of various IL-15-responsive genes[59].

In contrast to lymphocytes, a recent study has shown that IL-15 delivers its biological roles in myeloid cells through its private receptor IL-15Rα independent of IL-2/IL-15Rβγ and this ligation results in JNK and NF-κB activation[60].

1.3.2 Physiological roles of IL-15:

The IL-15 gene is expressed in a variety of cell types including, monocyte-derived macrophages, dendritic cells, skeletal muscle cells, fibroblast, endothelial cells and smooth muscle cells. This broad tissue distribution of IL-15 mRNA explains the several non-redundant functions of IL-15 in innate immunity and inflammation. Studies investigating the bioactivity of IL-15 have shown that this cytokine plays important roles in the development and survival of different immune cells including, NK, NKT and CD8 T cells.IL-15 is a critical survival factor for NK NKT and CD8 T cells primarily through up-regulating the anti-apoptotic Bcl-2 proteins such as Mcl-1 and down-regulating the proapoptotic proteins such as Bim and Noxa[30,61]. Therefore, gene-targeting studies have shown that NK NKT and CD8 T cells are reduced in IL-15 deficient mice and overexpression of IL-15 increases NK, NKT and CD8 T cells numbers[62]. Moreover, IL-15 activates NK, NKT and CD8 T cells and the outcome of this activation is the increased production of granzyme B, perforin, IFN-γ and TNF-α[63].

IL-15 is able to directly activate monocyte-derived macrophages to produce proinflammtory cytokines. It has been shown that RANTES production was significantly elevated in IL-15-stimulated bone marrow-derived cells from $Rag^{-/-}\gamma c^{-/-}$ mice[60]. Ongoing research in our laboratory has demonstrated that IL-15 in vitro accelerated the acetylated LDL-mediated foam cell formation, increased the total monocyte count, including the proinflammtory monocyte subset, and triggered macrophage to produce proinflammtory mediators including MCP-1, IL-6, iNOS and RANTES in vitro[64].

Altogether, these findings show that IL-15 has inflammatory effects on various cells types. Because of its inflammatory properties, IL-15 is thought to be atherogenic and promote atherosclerotic lesion formation and progression.

1.3.3 Effects of IL-15 expression in atherosclerosis at the aortic sinus:

IL-15 expression is substantially increased under various autoimmune diseases and conditions with aberrant immune responses. Unlike pathogen infections, the increased IL-15 expression is associated with pathogenesis of these diseases. It has been demonstrated that IL-15 mRNA is present in atherosclerotic plaque in humans and atherosclerosis susceptible mice, suggesting that there is a link between IL-15 expression and the atherosclerosis progression[65]. Indeed, our lab has proposed that genetic deletion of IL-15 reduces the development of spontaneous atherosclerosis in the aortic sinus of ApoE mice fed normal chow

diet[64]. This reduction is accompanied by decreased accumulation of $CD8^+$ cells, CD11b staining and MCP-1 staining within the atherosclerotic vessel. Consistent with that, overexpression of IL-15 promotes atherosclerotic plaque formation in the aortic sinus of ApoE mice, increases $CD8^+$ cells abundance within the atherosclerotic vessel[64].

Furthermore, our lab has studied the effects of IL-15Rα in atherosclerosis by transplanting IL-15Rα KO bone marrow cells into lethally irritated LDLr deficient mice to generate atherogenic-prone mice lackingIL-15Rα in bone marrow-derived leukocytes. IL-15Rα inactivation in leukocytes significantly attenuated aortic sinus atherosclerosis without affecting IL-6 and MCP-1 levels within atherosclerotic plaque[64]. These findings clearly show that IL-15 and its private receptor IL-15Rα contribute to atherosclerosis development in the aortic sinus.

1.3.4 The effects of IL-15 expression in atherosclerosis at the carotid artery:

The perivascular carotid collar model of atherosclerosis is a method of arterial manipulation in which site-controlled neointima formation is induced by placing a non-constrictive collar around the carotid artery. This collar approach increases the turbulence in the blood flow at the proximal area of the collar leading to triggering endothelial cell activation and initiating atherosclerosis development[19]. The effects of IL-15 on the carotid model of atherosclerosis have not been well studied. In carotid arteries that were partially ligated, injection wild

type mice with an antibody against IL-15 triggered increased neointima formation, suggesting that IL-15 has a protective role in this model[66]. This finding is consistent with the in vitro data that shows IL-15 inhibited smooth muscle cell proliferation by down-regulating the chemokine franklin (FKN) and its receptor CX3CR118[66]. One limitation of this paper was that it was not evaluated on atherosclerosis prone mice. On the other hand, others have shown that immunization of low density lipoprotein receptor deficient mice against IL-15, by inoculating them with bacteria harboring an IL-15 expression plasmid, led to reduced development of diet induced atherosclerosis in carotid arteries whose diameter was restricted to induce a hemodynamic stress[67]. However, this study has some complications in regards to the vaccination strategy since macrophage numbers are significantly reduced in multiple organs. This appeared to be due to the cytotoxic T cell response which targets these cells that overexpress bacterially delivered IL-15. Despite these observations, the role of IL-15 in carotid artery disease is not clear.

1.4 Objectives and rationale:

Atherosclerosis is a major health disorder worldwide and is associated with the development of numerous cardiovascular diseases. Recently, atherosclerosis has been described as a complex disease characterized by chronic infiltration and migration of inflammatory cells to the arterial injured site. Atherosclerotic lesions

are composed of immune cells, proinflammatory cytokines and chemokines, lipids and cell debris. Therefore, determining how immune modulators such as proinflammatory cytokines, like IL-15, influence atherosclerotic plaque development is crucial in exploring the role of immune response in the development of atherosclerosis.

Granzyme B has been proposed as a critical factor by which NK cells, NKT cells and CD8 T cells promote atherosclerosis. IL-15 is known as a survival factor of different immune cells including, NK, NKT and CD8 T cells. Given that IL-15 is repeatedly reported to increase the expression of granzyme B in cytotoxic lymphocytes, we have hypothesized that **manipulation of the gene expression of IL-15 or its private receptor IL-15Rα cause significant difference in the level of granzyme B within the atherosclerotic plaque**. The overall objective of this thesis was to investigate the mechanisms by which IL-15 promotes atherosclerosis with the following aims:

1. To determine the effects of overexpressing IL-15 gene on the level of granzyme B within the atherosclerotic plaque.
2. To define the effects of genetic deletion of IL-15Rα in leukocytes on granzyme B level in the atherosclerotic plaque.
3. To characterize the effects of inactivation of IL-15 gene in the carotid injury model of atherosclerosis.

2. Materials and methods:

2.1 Mice:

All mice were on a C57BL/6 inbred background. ApoE$^{-/-}$, LDLR$^{-/-}$ and C57BL6/J mice were obtained from Jackson Laboratories. IL-15^{Tg}, IL-15$^{-/-}$ and IL-15Rα$^{-/-}$ mice were obtained from A. Ashkar (McMaster University)[68]. IL-15^{Tg}ApoE$^{-/-}$ and IL-15$^{-/-}$ApoE$^{-/-}$ mice were generated by Dr O.Dadoo by crossing IL-15^{Tg} or IL-15$^{-/-}$ with apoE$^{-/-}$ mice and were maintained by M. MacDonald in the Trigatti lab. Mice were housed in the Thrombosis and Atherosclerosis Research Institute (McMaster University) with free access to food and automatic watering. All research involving animals was approved by the Animal Research Ethics Board of McMaster University and was in accordance with guidelines set out by the Canadian Council on Animal Care.

2.2 Collar Placement on the Right Common Carotid Artery:

The surgery was based on a previously described method[19]. Surgeries were performed by Peng Liao with my assistance. Collars were prepared from silicone tube (Helix Medical Inc.) for carotid collar placement (length, 2mm; internal diameter, 0.3 mm). They were sterilized with 70% ethanol and then the ethanol was washed out by sterile PBS 24 hours ahead of the surgery time. Eight week old mice were anaesthetized by Ketamine (125mg/kg) + Xylazine (12.5mg/kg) + Atropine (0.25mg/kg) mixture via intraperitoneal injection. The neck and the chest

area were shaved and then sterilized with 10% Povidine followed by 70% ethanol. Afterwards, the right carotid artery was dissected from the surrounding connective tissues. The collar was placed carefully around the right common carotid artery then tied with three circumferential silk ties at their axial edges. Subsequently, the carotid arteries were then returned to their original position and the entry wound was sutured, and the animals were started on a Western-type diet for two weeks until they were harvested. Prior to harvesting, mice were fasted for 6 hours and then anesthetized by isoflurane gas. After the right atria had been nicked, hearts were perfused in situ through the left ventricle with 10 U/ml heparinized saline followed by 10% formalin. Hearts, carotid arteries, spleen, livers and aortas were then excised, fixed overnight in 10% formalin, cryoprotected in 30 % sucrose for 24 hours and embedded in Cryomatrix (Thermo Scientific, Ottawa, Ontario, Canada). Formalin fixation was omitted for arteries and spleen that were to be stained for CD8 T cells; these were immediately snap-frozen in liquid nitrogen after having been embedded in Cryomatrix compound, whereas the remaining arteries were left in 10% formalin overnight before freezing. Transverse 10µm cryosections were prepared in a proximal direction from the carotid bifurcation and mounted in order on a parallel series of slides. The specimens were stored at -80°C until further use.

2.3 Atherosclerosis analysis:

Frozen sections were obtained by cutting the carotid artery in cross section from 1400μm distance from the collar. Sections were 10μm thick. 14 sections, spaced 100um apart, were stained for each carotid artery with Oil Red O (sigma Aldrich, St Louis MO, USA) and counterstained with haematoxylin (sigma Aldrich, St Louis MO, USA) as described previously[69]. Cross sections of atherosclerotic plaques were quantified within 1400μm distance from the collar and measured by quantitative morphometry using Zeiss Axiovert 200M microscope and Zeiss Axiovision software, and then analyzed. Plaque volume was determined by multiplying the plaque area by the length of the assessment site (1400μm) of the carotid artery.

2.4 Plasma Lipoprotein Assay:

Plasma was collected at sacrifice, and pools of plasma samples from each group were separated by fast protein liquid chromatography (FPLC) as described previously[70]. Total cholesterol (Cholesterol Infinity, Thermo) content was measured in FPLC fractions using enzymatic assay kits.

2.5 Immunohistochemistry and immunofluorescence:

Immunofluorescence staining for granzyme B was performed using rabbit anti-mouse granzyme B antibody (dilution 1:100, Abcam, Cambridge MA, USA) and Alexa 569-anti rabbit secondary antibody (dilution 1:200, ThermoFisher,

Burlington, Canada). Immunofluorescence staining for smooth muscle cells was performed using rabbit anti-mouse α-SMC actin antibody (dilution 1:50, Abcam, Cambridge MA, USA) and Alexa 488-anti rabbit secondary antibody (dilution 1:200,ThermoFisher, Burlington, Canada). Immunofluorescence staining for macrophages was performed using rat anti-mouse CD107b "Mac-3" antibody (dilution 1:50, BD Pharmingen, Mississauga, Canada) and Alexa 594-anti rat secondary antibody (dilution 1:200, ThermoFisher, Burlington, Canada). All sections were counterstained for nuclear DNA with 2-(4-amidinophenyl)-6-indolecarbamidine (DAPI) (Sigma-Aldrich, St Louis MO, USA). Immunofluorescence images were taken using a Zeiss Axiovert 200 M inverted microscope and the average area of staining or the intensity of staining was quantified using Image J software and normalized to the atherosclerotic plaque area.

Immunohistochemical staining was performed by the McMaster Immunology Research Centre Core Histology Facility using rat anti-CD8 (BD Pharmingen, Mississauga, Canada) antibody. Endogenous peroxidase was blocked with 0.3% hydrogen peroxide. Sections were blocked with Rodent Block M (Biocare Medical, Concord, CA, USA). The primary antibody was detected with a Rat on Mouse kit (Biocare Medical, Concord, CA, USA) and visualized with AEC chromogen. Sections were counterstained with hematoxylin (Sigma-Aldrich, St

Louis MO, USA). Immunohistochemistry sections were quantified by dividing the number of CD8 T cells by the atherosclerotic plaque area (for carotid sections) and spleen section area (for spleen sections) and then presented as "number of cells/mm^2".

2.6 Flow cytometry analysis:

Heparinized blood samples were collected via cardiac puncture. Next, blood samples were treated with RBC lysis buffer (R&D Systems) which lyses erythrocytes under gentle hypotonic conditions while preserving the leukocytes. Following several washing steps, leukocytes were stained with PE labeled NK1.1, FITC labeled CD3, FITC labeled CD11b, APC labeled Ly6c antibodies (BD Pharmingen) and subjected to flow cytometry. FlowJo software (Tree Star, Inc.) was used to analyze flow cytometry data.

2.7 Statistical analysis:

All the data are averages ± SEM. Data was subjected to pairwise comparisons by the Student's T-test or Mann-Whitney Rank Sum test as indicated. Two-way ANOVA was used for multiple comparisons. Differences were considered significant if $P<0.05$.

3. Results:

3.1 Overexpression of IL-15 increases granzyme B staining in atherosclerotic vessels of the aortic sinus in ApoE KO mice:

Granzyme B is a key effector molecule in lymphocyte-mediated cytotoxicity[48]. Moreover, previous studies found IL-15 increases the production of granzyme B in NK cells and CD8 T cells[71–73]. Research conducted in our lab revealed that overexpression of IL-15 in ApoE KO mice increased spontaneous atherosclerosis and the abundance of CD8 T cells within the atherosclerotic plaque at the aortic sinus of ApoE KO mice[64] (Appendix A). Because it has been suggested that cytotoxic $CD8^+$ T cells contribute to atherosclerotic plaque formation through perforin and granzyme B dependent cytotoxicity[26], we evaluated the abundance of granzyme B in atherosclerotic plaques from IL-15tg ApoE KO and il-15wt ApoE KO mice. We used sections of aortic sinus collected by Omid Dadoo, a former PhD student in our lab. Immunostaining revealed increased levels of granzyme B in sections of atherosclerotic aortic sinus from il-15tg ApoE KO compared to IL-15wt ApoE KO mice (Figure 1C). This data suggests that overexpression of IL-15 increases granzyme B levels in plaques of $ApoE^{-/-}$ mice and raises the possibility that this may contribute to the increased atherosclerosis detected.

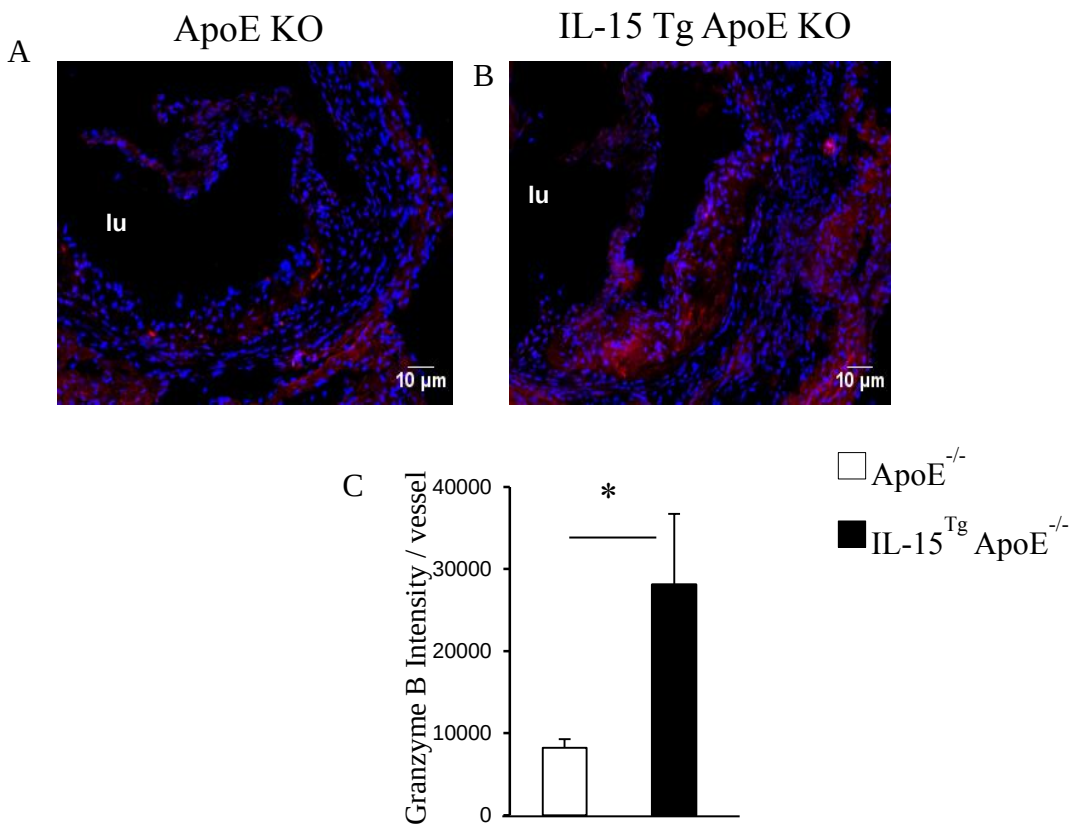


Figure 1: Overexpression of IL-15 increases granzyme B level in atherosclerotic plaques from ApoE KO mice.

Representative immunofluorescence images for granzyme B staining (red) and DAPI counterstaining (blue) in atherosclerotic plaques from A) $ApoE^{-/-}$ and B) $IL\text{-}15^{tg}ApoE^{-/-}$ mice and C) quantification of the average intensity of granzyme B staining per vessel for N=6 $ApoE^{-/-}$ mice and N=5 $IL\text{-}15^{tg}ApoE^{-/-}$ mice. Error bars represent SEM. Statistical significance was determined by the Mann-Whitney Rank Sum Test (* indicates P=0.03; ** indicates P<0.004).

3.2 Inactivation of IL-15Rα gene expression in leukocytes decreases granzyme B staining in atherosclerotic plaque from LDLr KO mice:

IL-15Rα is a private receptor that has a high affinity to IL-15 and plays an important role in augmenting IL-15-mediated biological responses. Recently, it was shown that biological activity of IL-15 results in 50-fold increase when it is complexed with IL-15Rα[74]. Furthermore, IL-15/IL-15Rα complex enhanced the effector functions and expression of granzyme B in NK and CD8 T cells[75]. Research conducted in our lab revealed that deficiency of IL-15Rα gene expression in leukocytes attenuates atherosclerosis in LDLr KO mice (Appendix B)[64]. This reduction is accompanied by a reduction in $CD8^{+}$T cells and NK cells in circulation, consistent with findings of others[76]. Given that these cells promote atherosclerosis through perforin and granzyme B-dependant cytotoxicity[26,37], we analyzed the effects of leukocytes-specific genetic deletion of IL-15Rα in the abundance of granzyme B within the plaque. Immunostaining of sections from aortic sinus collected by Dadoo revealed that western diet fed LDLR KO mice lacking IL-15Rα in leukocytes exhibited reduced levels of granzyme B staining in the atherosclerotic plaque compared to the control group (Figure 2C).

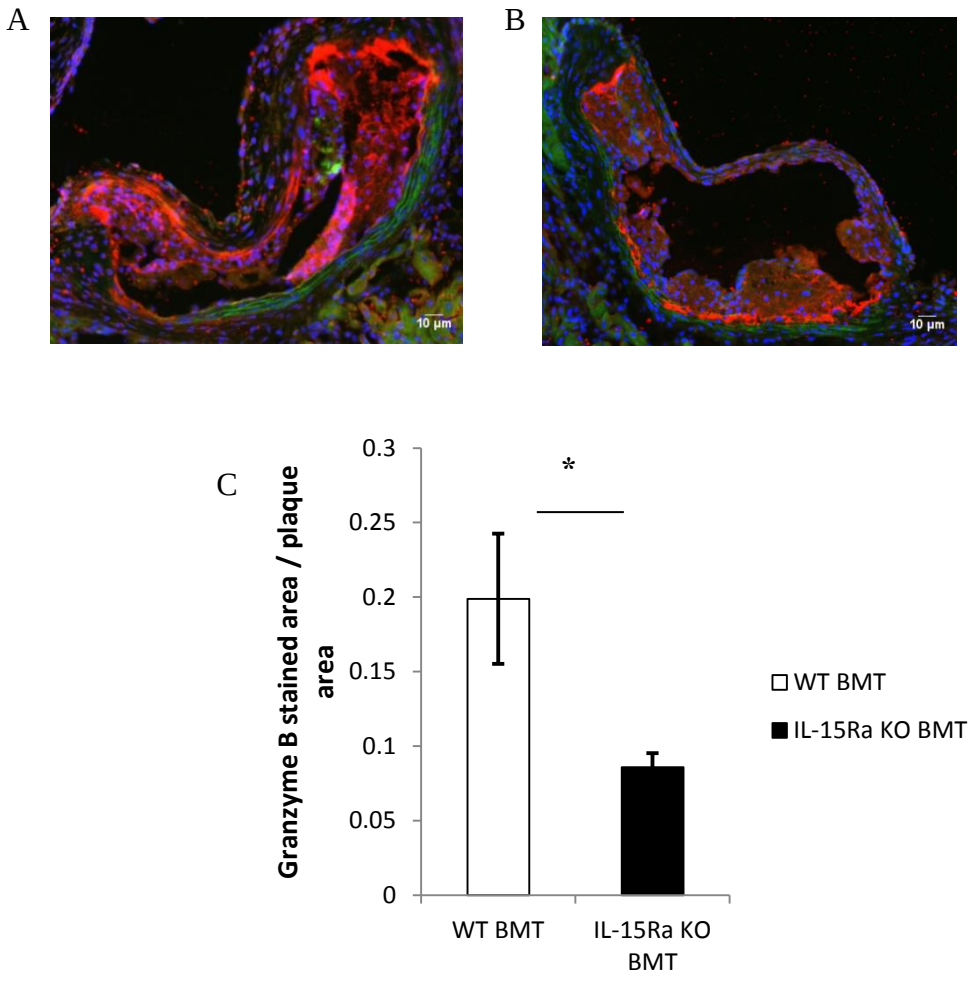


Figure 2: Leukocyte-specific inactivation of IL-15Ra reduces granzyme B level in atherosclerotic plaques from LDLr KO mice:

Representative images of atherosclerotic plaque from A) WT BMT mice and B) IL-15Rα KO BMT mice stained for granzyme B. C) Average stained area of granzyme B normalized to plaque area was determined for N=6 mice (IL=15Ra BMT) and N= 4 mice (WT BMT). Statistical significance was analyzed by Student's T-test. (p value<0.05)

3.3 Inactivation of IL-15 gene expression did not affect lipoprotein cholesterol profiles in high fat diet fed ApoE KO mice:

To test the effects of inactivating IL-15 on carotid artery atherosclerosis, we used a model system similar to that used by others who reported that immunization against IL-15 protected against carotid artery atherosclerosis[67]. We generated $ApoE^{-/-}$mice which lack IL-15 (IL-$15^{-/-}$ $ApoE^{-/-}$) and placed a collar into the midsection of the right common carotid artery. After the surgery, both groups (IL-$15^{-/-}$$ApoE^{-/-}$mice & $ApoE^{-/-}$mice) were challenged with Western Diet for two weeks. We first tested if there were differences in plasma lipoprotein cholesterol levels in the western diet fed mice that were either IL -$15^{-/-}$$ApoE^{-/-}$ or $ApoE^{-/-}$ mice. Plasma samples from IL-$15^{-/-}$$ApoE^{-/-}$ mice and $ApoE^{-/-}$ were collect at harvest. Three pools of plasma samples (3 mice per pool) from each group were separated by gel filtration chromatography and cholesterol levels were quantified corresponding to different types of lipoproteins. We found no difference between both groups (IL-15 $^{-/-}$ ApoE $^{-/-}$ mice & ApoE $^{-/-}$mice) (Figure 3).

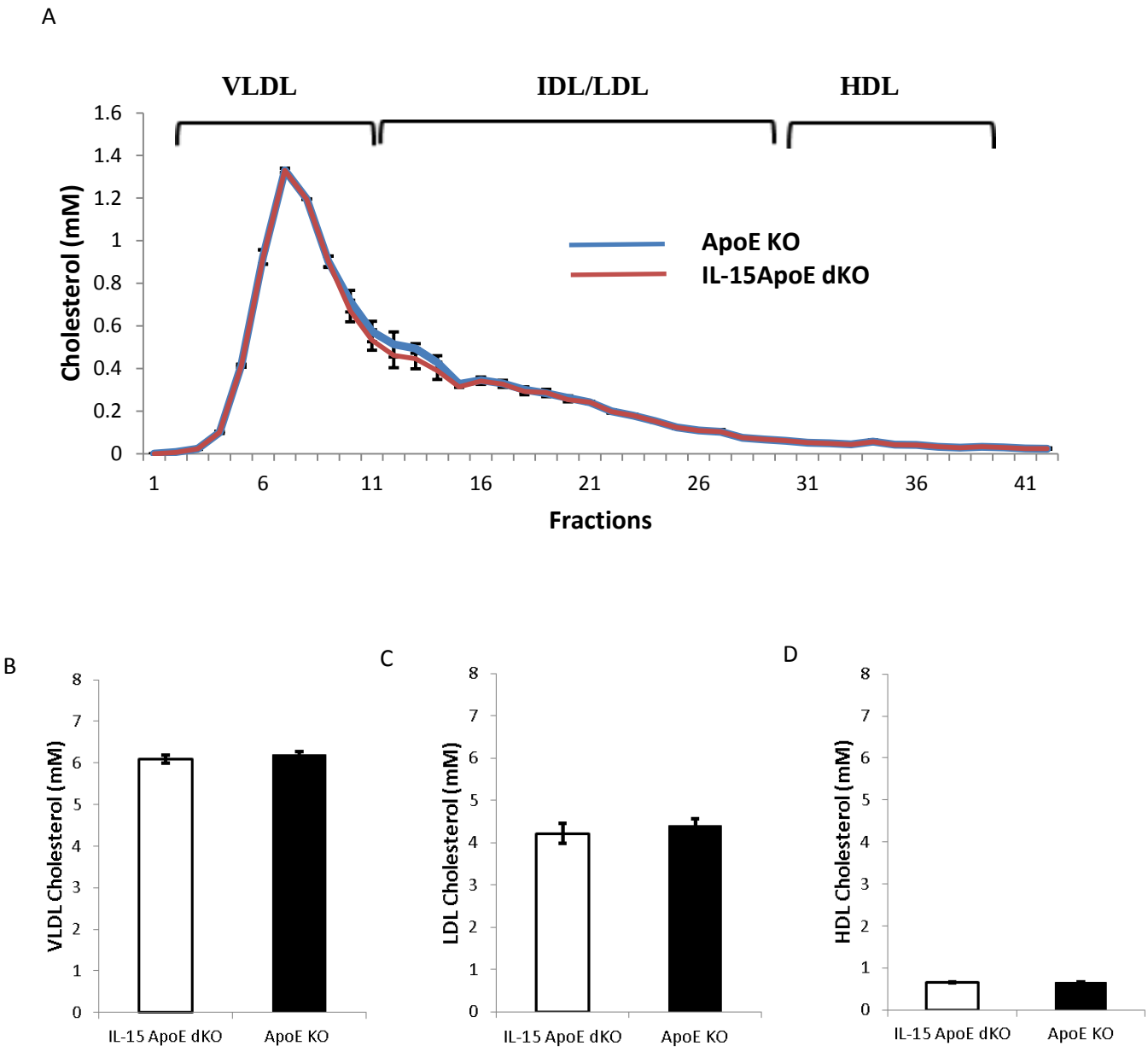


Figure 3: Lipoprotein profile of ApoE KO mice and IL-15 ApoE dKO:

A) Representative plasma lipoprotein profiles and average (± SEM) amounts of cholesterol associated with VLDL-, LDL- and HDL-sized fractions. Three pools of plasma samples (3 mice per pool) from each group were separated by FPLC and cholesterol levels were quantified corresponding to different types of lipoproteins. Averages of cholesterol level were calculated for B) VLDL, C) LDL, and D) HDL fractions (1 to 11, 12 to 27, and 28 to 41, respectively). Statistical significance was analyzed by two-way ANOVA ($p>0.05$).

3.4 Flowcytometry Analysis of NK cells, NKT cells and monocytes:

IL-15 has been shown to affect the development of NK, NKT, CD8+ T cells and monocytes[77]. Therefore, we first confirmed that manipulating IL-15 levels in ApoE$^{-/-}$ mice affected levels of these cells. Consistent with the findings of others, absence of IL-15 is accompanied with virtual elimination of NK cells (Figure 4C). We found also that there was also a trend towards lower NKT cells but the difference did not reach statistical significance, likely due to the low numbers of NKT cells detected in blood in ApoE$^{-/-}$mice (Figure 4D). Knockout of IL-15 reduced the abundance of circulating monocytes in apoE$^{-/-}$ mice (Figure 5C), and no differences were noted in the proportions of monocytes that were Ly6Chi among ApoE$^{-/-}$ mice and IL-15 ApoE$^{-/-}$ mice (Figure 5D).

3.5 CD8 T cells numbers in spleen from ApoE$^{-/-}$ and IL-15$^{-/-}$ ApoE$^{-/-}$ mice:

IL-15 is capable of stimulating the production of CD8 T cells. Therefore, we wanted to determine if the absence of IL-15 had altered CD8 T cell numbers in the spleen. CD8a immunohistochemistry on IL-15$^{-/-}$ApoE$^{-/-}$ and the control ApoE$^{-/-}$ spleen sections revealed that there was a trend towards an increase in the number of CD8 T cells in ApoE$^{-/-}$ spleen in comparison to IL-15$^{-/-}$ ApoE$^{-/-}$ spleen (Figure 6C). More samples are needed to reach the statistical significance.

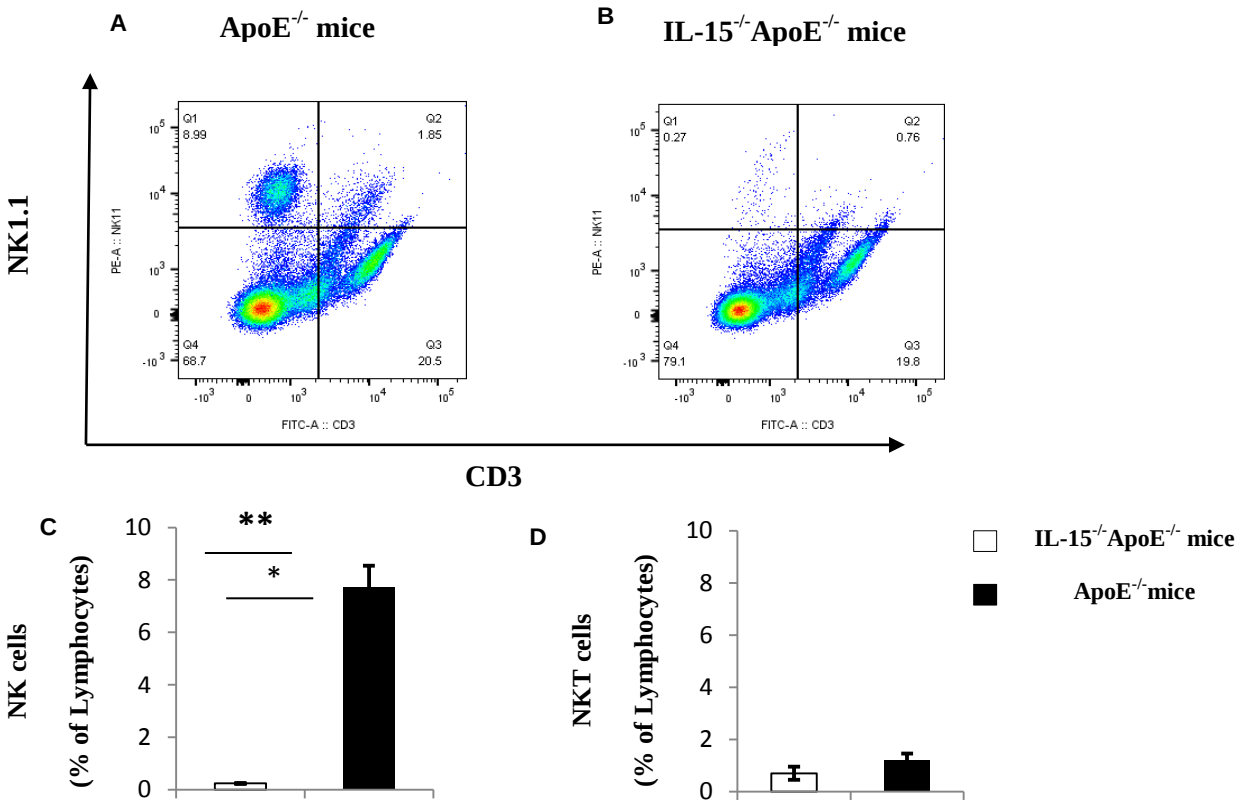


Figure 4: Circulating blood lymphocytes in IL-15$^{-/-}$ApoE$^{-/-}$ mice and ApoE$^{-/-}$ mice.

Flow cytometry analysis of blood lymphocyte stained with NK1.1 and CD3 from A) ApoE$^{-/-}$ mice and B) IL-15$^{-/-}$ApoE$^{-/-}$ mice. C) NK cells (NK1.1^{+} CD3^{-}); D) NKT cells (NK1.1^{+}CD3^{+}). Data are averages from N= 5, male 10 week old mice; error bars represent SEM. * indicates $P<0.05$ as determined by Student's T test.

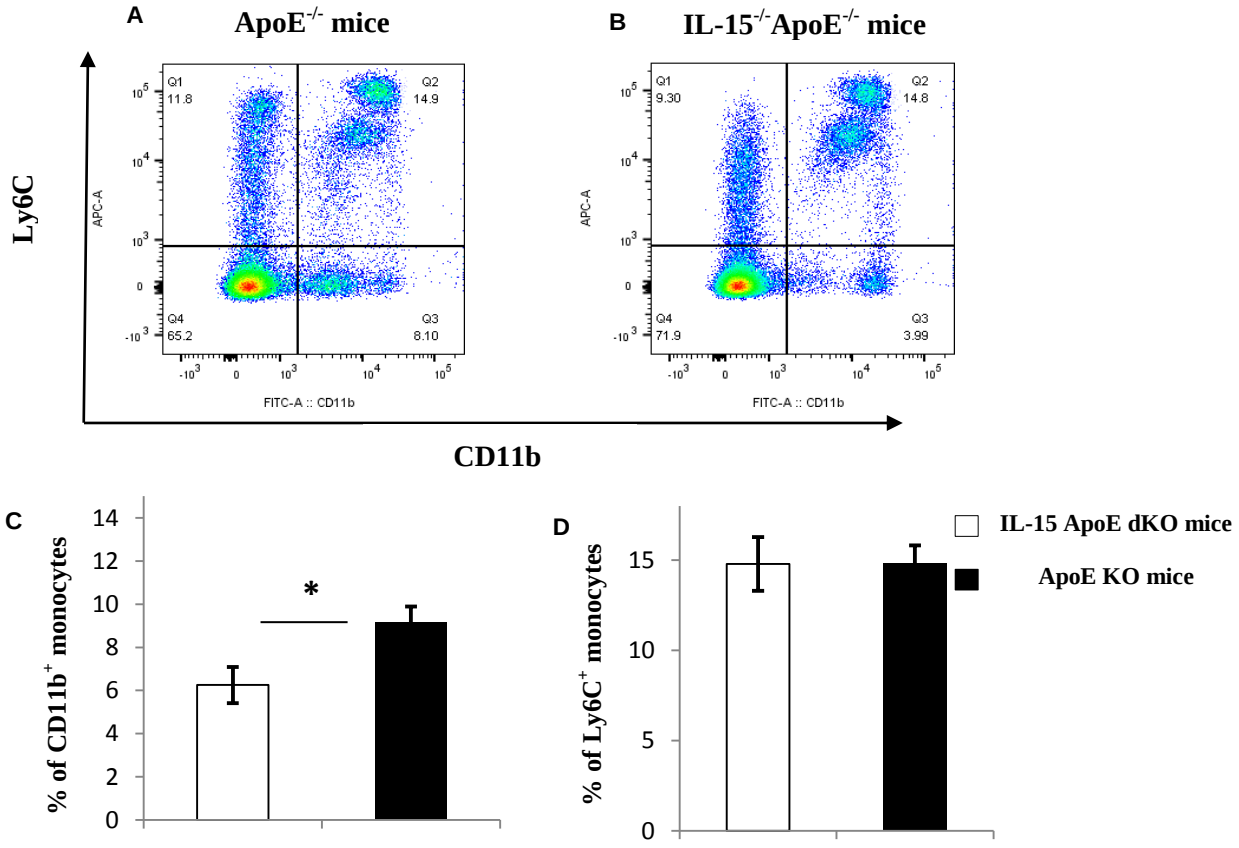


Figure 5: Circulating blood monocytes in IL-15$^{-/-}$ApoE$^{-/-}$ mice and ApoE$^{-/-}$ mice.

Representative flow cytometry scatter plots of CD11b and Ly6C staining of leukocytes from A) ApoE$^{-/-}$ mice and B) IL-15$^{-/-}$ApoE$^{-/-}$ mice. C) monocytes (Ly6C^{+}CD11b^{+}); D) Ly6C^{+} monocytes (Ly6C^{+}CD11b^{+}). Data are averages from N= 5, male 10 week old mice; error bars represent SEM. * indicates P<0.05 as determined by Student's T test.

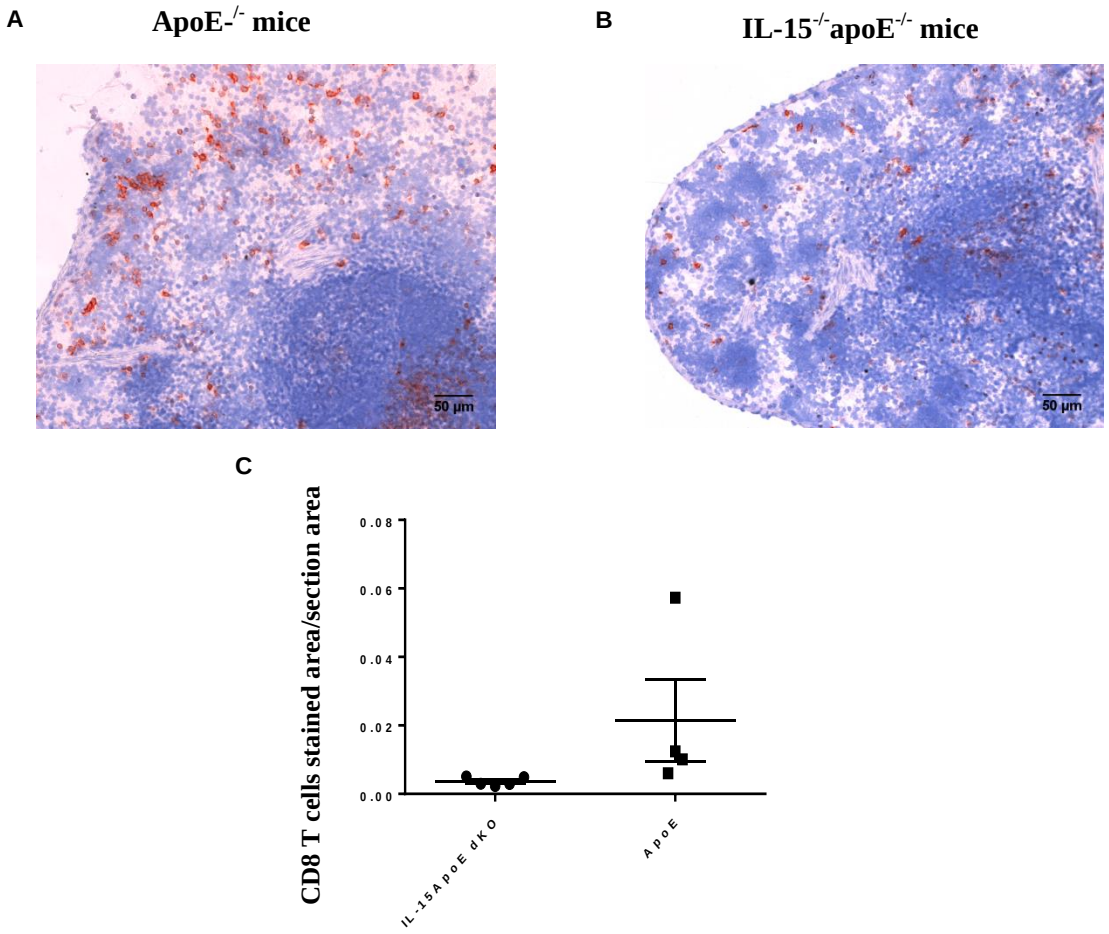
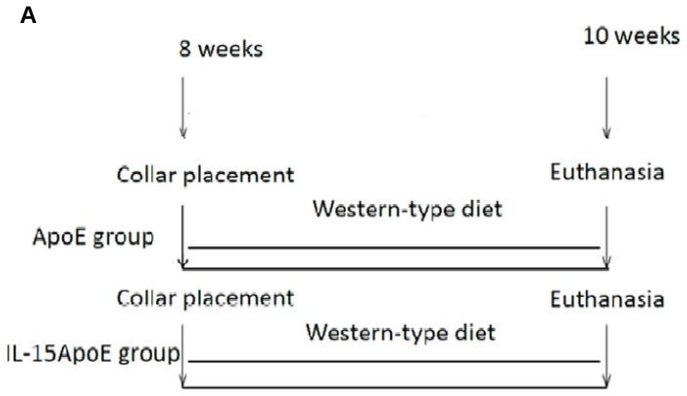


Figure 6: Splenic CD8 T cells in IL-15$^{-/-}$ApoE$^{-/-}$ mice and ApoE$^{-/-}$ mice.

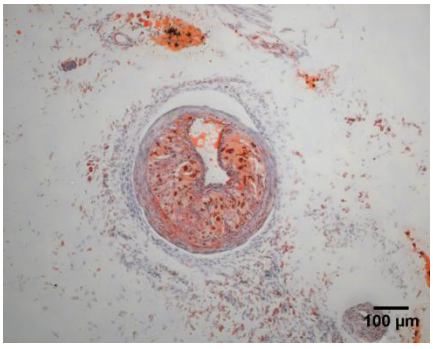
Representative images of splenic frozen section from 10 week old A) apoE$^{-/-}$ mice and B) IL-15$^{-/-}$ apoE$^{-/-}$ mice, were analyzed by Immunohistochemical staining for CD8 T cells. Data are averages from N= 4 and 5, error bars represent SEM. Statistical significance was analyzed by Student's T-test (p value=0.23). Staining was performed by Mary Jo Smith from the McMaster University Immunology Research Centre.

3.6 Inactivation of IL-15 gene expression did not attenuate collar-induced atherosclerosis in apoE$^{-/-}$mice:

In order to examine the effects of genetic deletion of IL-15 in the carotid model of atherosclerosis, we measured the collar-induced atherosclerotic plaque volume in the collared right common carotid arteries from IL-15$^{-/-}$ApoE$^{-/-}$ mice and ApoE$^{-/-}$ mice fed Western diet for two weeks (Figure 7A). We observed a formation of complex atherosclerotic plaques in the collared right common carotid arteries compared to the non-collared left common carotid artery which showed no plaque formation (Figure 7 B&C). Furthermore, collared arteries in both groups (IL-15 $^{-/-}$ ApoE $^{-/-}$ & ApoE $^{-/-}$) developed a plaque volume which reaches to 1350 µm distance from the collar (Figure 8G). More importantly, we found that there is no significant difference in plaque volume between the two groups, suggesting that the absence of IL-15 did not affect the atherosclerotic plaque formation in the collared carotid arteries (Figure 9C).



B Collared right common carotid artery



C Non-collared left common carotid artery

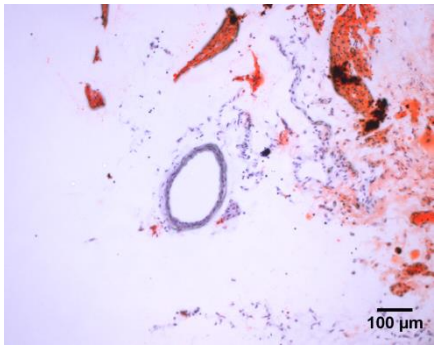


Figure 7: Experimental design for the collar-induced carotid artery atherosclerosis.

A) Time schedule of the experiment examining the impact of genetic deletion of IL-15 on the collar-induced acceleration of carotid atherosclerotic plaque development. Representative cross sectional images of B) collared right common carotid artery and, C) non collared left common carotid artery.

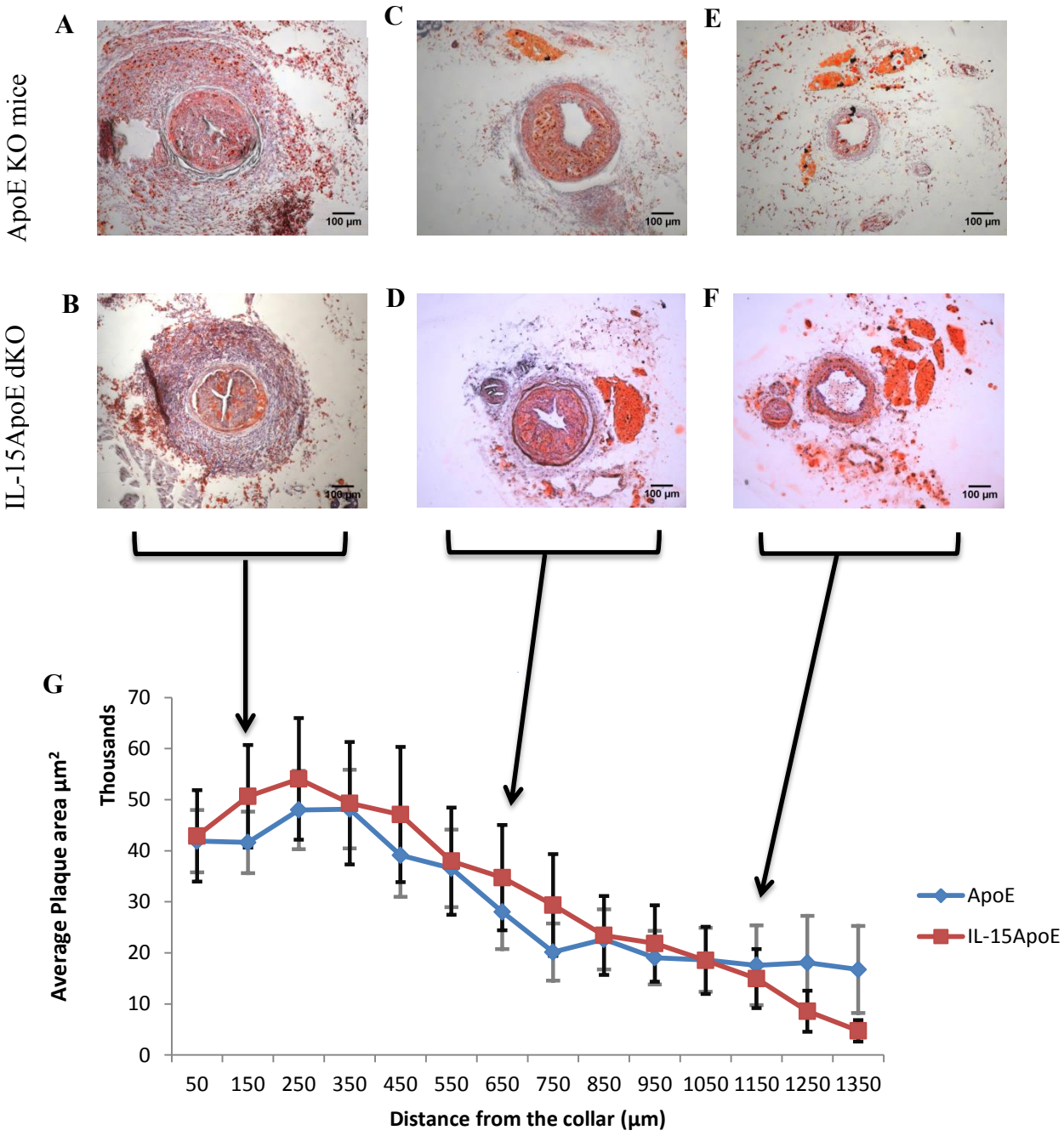


Figure 8: Collar-induced atherosclerosis on the carotid artery in ApoE$^{-/-}$ mice and IL-15$^{-/-}$ ApoE$^{-/-}$ mice.

A) & B) are representative lesions 150µm away from the collar. C) & D) are representative lesions 650µm away from the collar. E) & F) are representative lesions 1150µm away from the collar. G) Collar-induced atherosclerotic plaque area, measured at every 100 µm distance from collar, in ApoE -/- mice (n=14) and IL-15 -/- ApoE -/- mice (n=12)

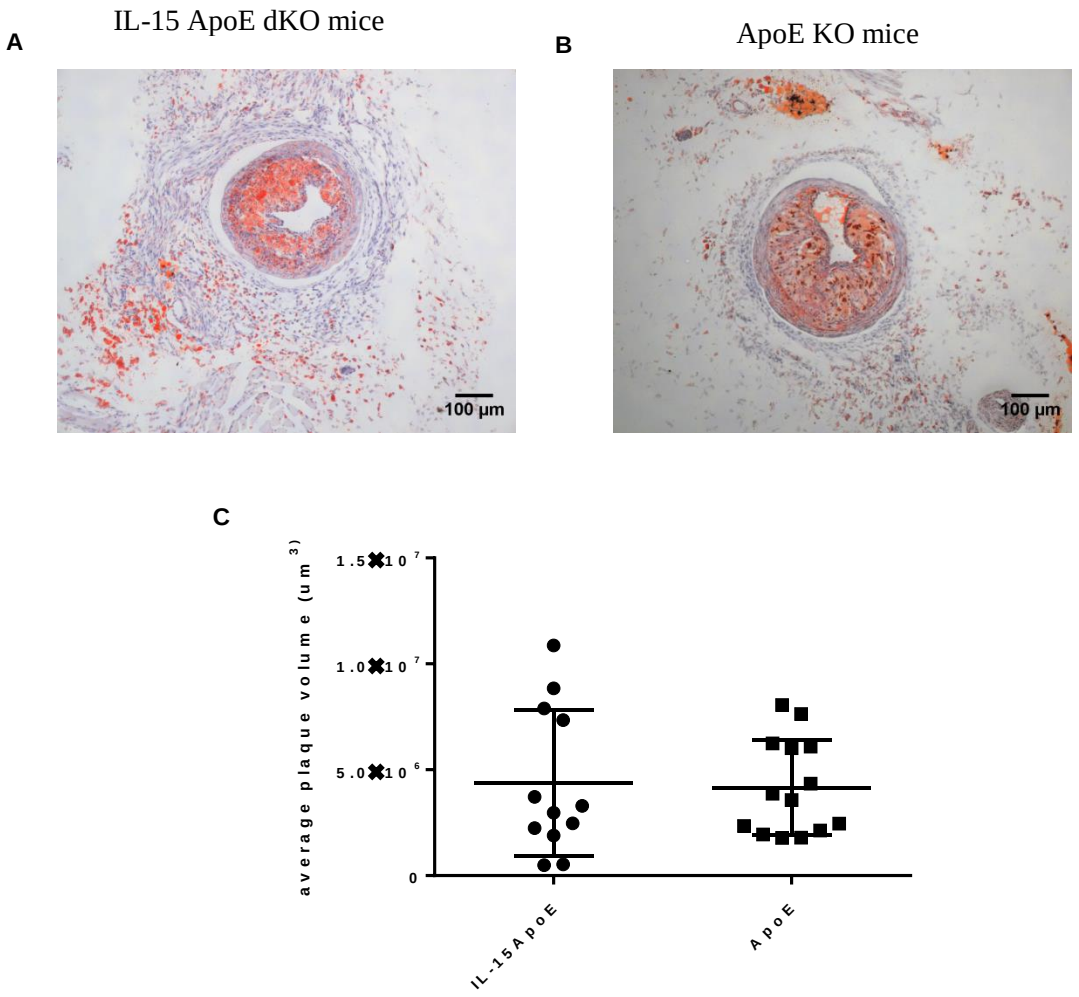


Figure 9: Atherosclerotic plaque volume at the collared Carotid Artery:

Representative images of lesions 650 um away from the collar of A) IL-15 $^{-/-}$ ApoE $^{-/-}$ mice and B) ApoE $^{-/-}$ mice of collar-induce atherosclerosis. C) Atherosclerotic plaque volume in the carotid artery, Plaque volume is expressed in μm^3. Statistical significance is determined by Student's T-test (p value = 0.85, n= 12, 14)

3.7 Plaque composition of collar-induced atherosclerosis in ApoE$^{-/-}$mice and IL-15$^{-/-}$ApoE$^{-/-}$mice:

Although the absence of IL-15 did not reduce the plaque volume in ApoE$^{-/-}$ mice, it might change the plaque composition. Previous studies have shown that IL-15 affects SMC proliferation and macrophage content in collar-induced atherosclerotic plaques[66,67]. Therefore, we sought to analyze the plaque composition of both strains. SMCs have been reported to accumulate in the atherosclerotic plaque and promote plaque formation[4]. Experimental studies in animals showed that IL-15 also inhibits the proliferation SMC in the arterial duct, and blockade of IL-15 increased neointimal formation in a model for cuff induced plaque formation[66,78]. To determine the effects of IL-15 genetic deletion in SMC proliferation, we evaluated the abundance of SMC in the collar-induced atherosclerotic plaque by using immunofluorescent antibody against α-smooth muscle actin. Consistent with others[79], the intensity of α-SMC actin staining was stronger in the peripheral area of the neointima compared to the inner core of the atherosclerotic plaque. Importantly, Sections of collared carotid arteries from ApoE deficient mice and IL-15 ApoE deficient mice exhibit a similar level of immunostaining for α-SMC actin (Figure 10C). This data suggest that genetic deletion of IL-15 did not have an effect on SMC content of collar-induced atherosclerotic plaque.

Macrophages are largely abundant within the atherosclerotic plaques and accelerate the progression of atherosclerosis by promoting the formation of foam cells and secretion of cytokines and chemotactic factors[4]. Others reported that immunization against IL-15 reduced collar induced plaque development was accompanied by reduced macrophage content in plaques suggesting that IL-15 affects macrophage accumulation in atherosclerotic plaques[67]. In this study, we wanted to determine whether the absence of IL-15 affect the macrophage content within collar-induced atherosclerosis. In the ApoE deficient mice, the macrophages stained with macrophage-specific marker Mac-3 were detected throughout the atherosclerotic plaque. Absence of IL-15 did not change the immunostaining level of Mac-3 (Figure 10F). This data suggests that in collar-induced atherosclerosis IL-15 does not have an effect in the macrophage content.

CD8 T cells accumulate in all stages of atherosclerosis development in the aortic sinus in mouse models[4]. IL-15 is a known survival factor of CD8 T cells[77]. Our lab has reported that overexpression of IL-15 increases while elimination of IL-15 decreases the infiltration of CD8 T cells into the atherosclerotic lesion of the aortic sinus[64]. In the present study, we wanted to examine the effects of IL-15 deficiency in the abundance of CD8 T cells in the collar-induced atherosclerosis. Immunohistochemical analysis using antibody against CD8α shows that only three of six $ApoE^{-/-}$ mice exhibited a small number of CD8 T cells with an average 28

cell/mm^2 of the plaque area (Figure 10L). Compared with the atherosclerotic plaque within aortic sinus where the average number of CD8 T cells in ApoE$^{-/-}$ mice was approximately 200 cell/mm^2 of the plaque area, there is approximately 10 times reduction of CD8 T cell number in the carotid injury model. Therefore, this finding suggests that CD8 T cells play less of a contribution if any to collar-induced carotid atherosclerotic plaques in this model than to development of spontaneous plaques in the aortic sinus. Consistent with our finding, others have found that there were no difference in neointima formation between Tap1 deficient mice, which is associated with almost a complete loss of CD8 T cells, and wild type mice suggesting that CD8 T cells did not affect the development of collar-induced atherosclerosis[80]. None of the carotid artery atherosclerotic plaque sections from the IL-15/apoE dKO mice analyzed showed any evidence of CD8+ cell staining. Because of the low abundance of CD8$^+$T cells in carotid plaques in the apoE KO mice in this model, it is hard to draw a firm conclusion in regard to the effects of IL-15 deficiency in CD8 T cell content within the collar-induced atherosclerosis.

Next, we analyze the effects of IL-15 deficiency on granzyme B level within the atherosclerotic plaque in the carotid injury model. We observed no difference in the level of granzyme B staining in the collar-induced atherosclerosis from ApoE deficient mice and IL-15 ApoE deficient mice (Figure 10I). As the

Immunohistochemical analysis reveals that the abundance of CD8T cells were low in the collar-induced atherosclerotic plaque, one or more other cell type may be the source of granzyme B in these plaques. Others have reported that both SMC's and macrophages can express granzyme B suggesting that either or both of these cells may be the source of granzyme B in the in the collar-induced atherosclerotic plaques carotid artery model.

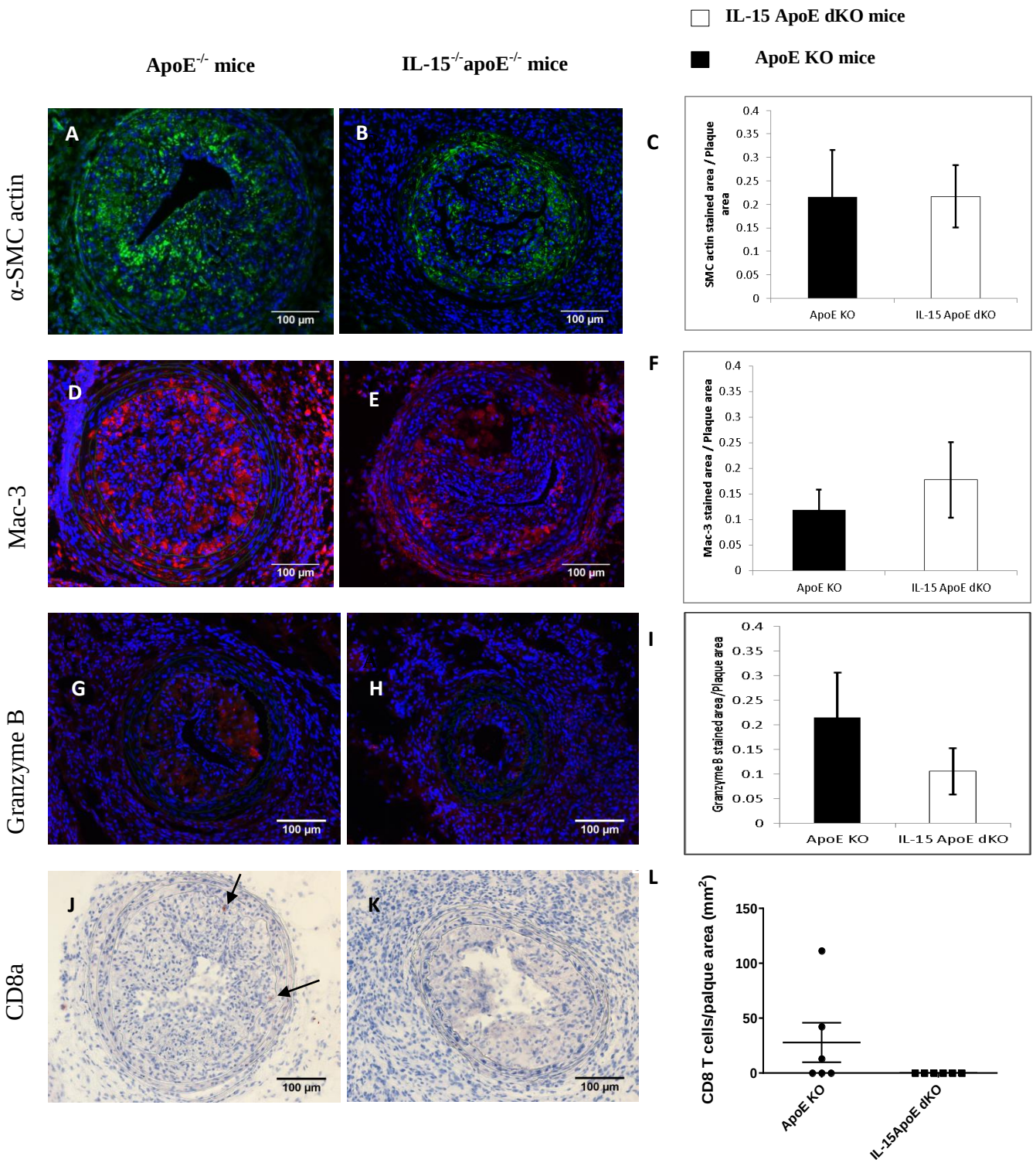

ApoE-/- mice
IL-15-/-apoE-/- mice
IL-15 ApoE dKO mice
ApoE KO mice
α-SMC actin
Mac-3
Granzyme B
CD8a
A
B
C
D
E
F
G
H
I
J
K
L
100 µm
SMC actin stained area / Plaque area
Mac-3 stained area / Plaque area
Granzyme B stained area /Plaque area
CD8 T cells/palque area (mm2)
ApoE KO
IL-15 ApoE dKO
IL-15ApoE dKO

Figure 10: Plaque phenotypic characteristics in $ApoE^{-/-}$ and $IL\text{-}15^{-/-}ApoE^{-/-}$ mice.

Collar-induced atherosclerotic plaque in carotid artery from ApoE-/- and IL-15-/-ApoE-/- mice stained for (A&B) SMCs, (D&E) Mac-3 (G&H) granzyme B (J&K) CD8a. C) SMCs expression (green) in plaque was quantified and normalized to the total plaque area. F) Macrophage expression (red) in plaques was quantified and normalized to the total plaque area. I) granzyme B expression (red) in plaques was quantified and normalized to the total plaque area. Nuclei were stained by DAPI (blue) L) Average numbers of CD8 T cells was normalized to the total plaque area. Error bars represent SEM. * indicates $P<0.05$ as determined by Student's T test. All images are adjusted equivalently using bright/contrast function of ImageJ. CD8 T cell staining was performed by Mary Jo Smith from the McMaster University Immunology Research Centre.

4. Discussion:

IL-15 performs key functions that may contribute to atherosclerosis plaque progression, and these include supporting the development of NK, NKT, CD8 T cells, increasing total monocyte numbers, accelerating macrophage-derived foam cell formation and directly activating macrophage mediated proinflammtory cytokines[64,77]. These effects contributed to the finding that manipulation of IL-15 gene expression affects atherosclerosis development in the aortic sinus of ApoE KO mice,

One of the main products secreted by cytotoxic lymphocytes is the serine protease granzyme B that initiates apoptotic cell death in target cells[48]. IL-15 was found to increase the intracellular level of granzyme B and lytic capacity in CD8 T cells[75]. Recent studies have shown that granzyme B contributes to atherosclerosis progression in experimental mice[49]. In this thesis, I examined the effects of IL-15 and its private receptor IL-15Rα in granzyme B level within the atherosclerotic lesion. First, we found that overexpression of IL-15 gene resulted in an increase of granzyme B level in the atherosclerotic plaque of ApoE deficient mice. Furthermore, we observed that leukocytes-specific genetic deletion of IL-15Rα reduced the granzyme B level within atherosclerotic lesions from LDLr deficient mice. Our data together show one of the mechanistic pathways by which IL-15 promote atherosclerosis development.

The role of granzyme B within atherosclerotic lesions has been studied recently. Granzyme B is capable of inducing macrophage and smooth muscle cell apoptosis, and degrading multiple extracellular matrix proteins including fibronectin, vitronectin and laminin[47]. In this thesis, our data also suggest a scenario in which IL-15 and its private receptor IL-15Rα promote the production of cytotoxic granzyme B molecules, by NK, NKT and CD8+ T cells which increases the apoptotic cell death and extracellular matrix degradation, leading to weakening of the fibrous cap of atherosclerotic plaques.

While the proatherogenic effects of IL-15 on the aortic root appear clear-cut[64], the effects of IL-15 on the carotid model of atherosclerosis have not been well studied. In the present study, we examined the effects of genetic deletion IL-15 in $ApoE^{-/-}$mice using a collar-induced carotid artery atherosclerosis model. Our results showed that inactivation of IL-15 gene expression did not reduce collar-induced atherosclerosis in the carotid artery. These results contradict a study by Van and coworkers where they observed that vaccination against IL-15 reduced collar-induced atherosclerosis in LDLr deficient mice[67]. The reasons for the different outcomes remain to be clarified but may involve differences in the model used. Immunization against IL-15 by inoculating mice with bacteria harboring an IL-15 expression plasmid leads to the killing of cells overexpressing IL-15. However, this vaccination strategy may result in induction of a cytotoxic CD8 T

cell response against cells overexpressing IL-15, including macrophages[67]. Another possibility could be that IL-15 has opposing effects which maintained the plaque volume in both groups. While IL-15 increases macrophage and CD8 T cell abundance within the plaque, others have reported that IL-15 inhibits neointima formation and smooth muscle proliferation in the arterial injury model[66]. As a result, we verified whether genetic disruption of IL-15 changed the collar-induced atherosclerotic plaque composition. We found that the macrophage content, SMC content and granzyme B staining were not affected by the absence of IL-15 in this model. Interestingly, we found that the number of CD8 T cells is much lower in atherosclerotic plaques in carotid arteries induced by the restrictive collar and high fat diet feeding, than what we and others have previously found in atherosclerotic plaques developing in the aortic sinus. In apoE deficient mice, we found that the average number of CD8 T cells within the collar-induced atherosclerosis is approximately 28cell/mm^2, which is 10 times lower than what our lab has and other reported at the aortic sinus where the average number of CD8 T cells is 200 cell/mm^2 of the lesion area[26,64]. Consistent with our findings, others have reported that inactivation of CD8+ T cells does not reduce the development of atherosclerosis in carotid arteries in response to carotid artery injury[80].

This data suggest that collar-induced atherosclerosis in the carotid artery is not sensitive to the inactivation of IL-15 gene expression, and it is not consistent

with the effects of IL-15 in the age-dependant atherosclerosis on the aortic sinus. These differential effects suggest that the atherogenic effects of IL-15 are specific to CD8 T cells and when CD8 T cells does not contribute to the collar-induced atherosclerosis, inactivation of IL-15 could not reduce atherosclerosis development in this model. Another possibility for differential effects of IL-15 may be the differences in the local hemodynamic profile or the variances in the rate of lesion growth and development between the carotid artery and the aortic sinus lead to this discrepancy. Differences in IL-15 gene expression may also exist between the carotid artery and the aortic sinus which reflect the atherogenic effects of IL-15 at these different vascular sites. These findings are not unprecedented. In a study which analyzed the effects of the liver x receptor agonist in atherosclerosis progression, it is reported that this agonist showed a great athero-protective effect in the innominate artery but not in the aortic sinus[81]. Our data suggest that more detailed studies of different arterial regions are needed to understand the effects of IL-15 in atherosclerosis development.

In conclusion, we showed that manipulation IL-15 and IL-15Rα gene expression changed granzyme B level in the atherosclerotic lesions of murine aortic sinus. On the other hand, our research demonstrated that inactivation of IL-15 gene expression did not reduce atherosclerosis development in the injured carotid arteries of ApoE KO mice.

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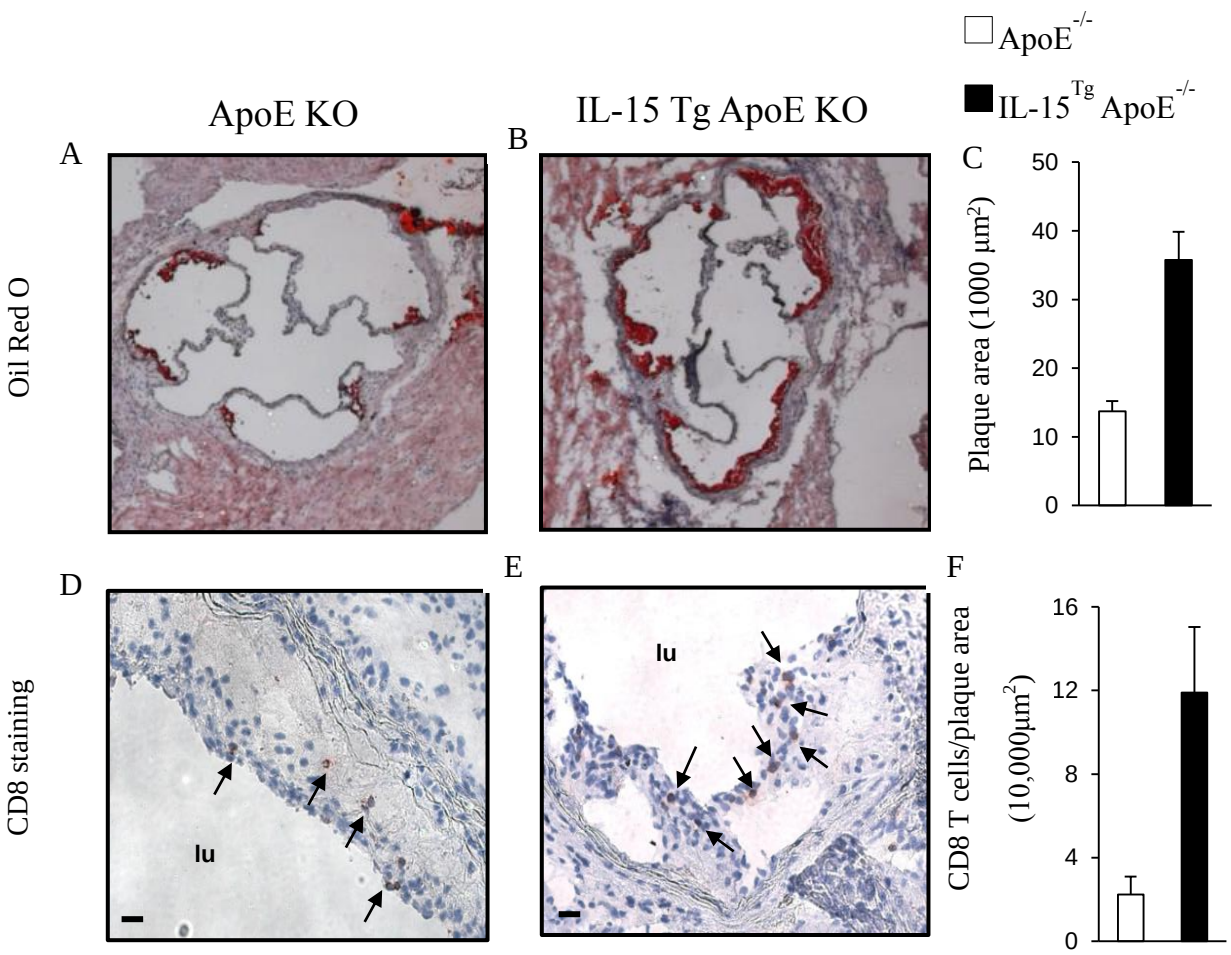
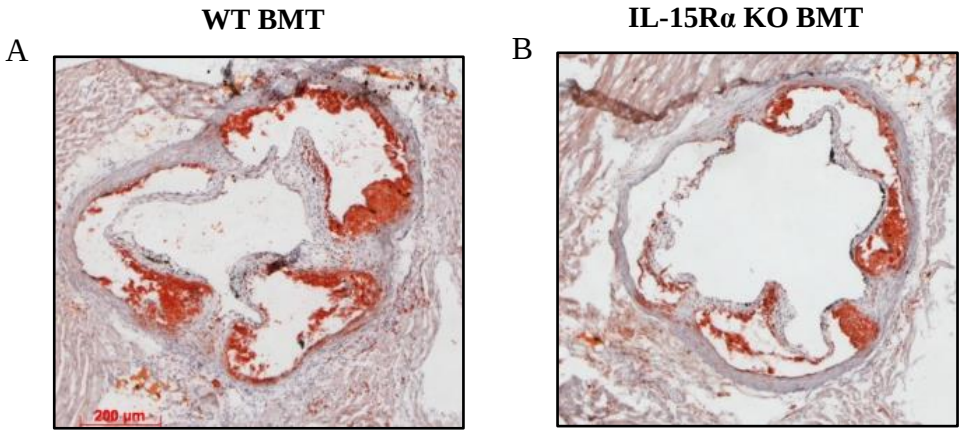
6. Appendix:

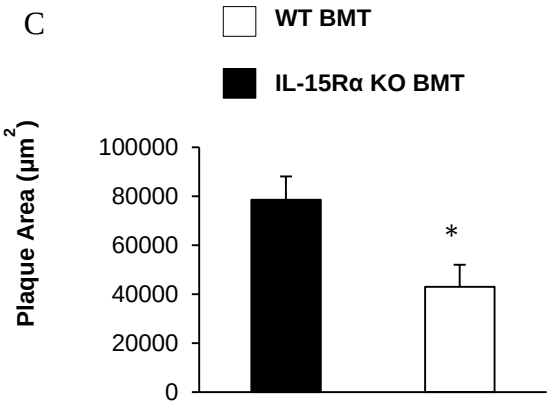
All data in Appendix are collected by Dr. Omid Dadoo



Appendix A: Increased atherosclerosis and CD8+ cells in vessel walls of il-15^{Tg}ApoE$^{KO/KO}$ mice.

Representative images of oil red O/haematoxylin stained plaques from A) ApoE$^{-/-}$and B) an IL-15^{tg}ApoE$^{-/-}$mice. C) Average plaque sizes quantified for N= 9 mice per group. Representative images of Immunohistochemical staining for CD8 in atherosclerotic plaques from D) ApoE$^{-/-}$and E) IL-15^{tg}ApoE$^{-/-}$ mice. F) Average numbers of CD8^{+} cells detected in atherosclerotic plaques were determined for N=4 mice per group. Error bars represent SEM. Statistical significance was determined by the Mann-Whitney Rank Sum Test (* indicates P=0.03; ** indicates P<0.004).





Appendix B: Leukocyte specific KO of IL-15Rα attenuates diet induced atherosclerosis in LDLR KO mice.

Representative oil red O stained sections from male LDLR KO mice reconstituted with bone marrow from A) wild type or B) IL-15Rα KO donors were fed the Western atherogenic diet for 9 weeks and (C) quantification of atherosclerotic plaque sizes in the aortic sinuses.