MECHANISMS OF ATHEROSCLEROSIS DEVELOPMENT

CELLULAR AND MOLECULAR MECHANISMS BY WHICH INTERLEUKIN-15 CONTRIBUTES TO ATHEROSCLEROSIS

BY:

OMID DADOO, B.Sc.

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AUTHOR: Omid Dadoo, B.Sc.

SUPERVISOR: Bernardo L. Trigatti, Ph.D.

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ABSTRACT (GENERAL PUBLIC)

Cardiovascular diseases (CVD) are leading causes of death in Canada and among different types of CVD's, atherosclerosis is the most dominant type. During the progression of this disease, immune cells and cholesterol carrying lipoproteins trespass the wall of arteries where, immune cells such as macrophages engulf cholesterol resulting in narrowing of lumens of arteries. Studies show that elements of our immune system together with other factors such as the levels of lipids in circulation, determine the severity of atherosclerosis development and consequently the occurrence of heart attack and stroke. Our findings demonstrate that Interleukin-15, a protein which regulates many aspects of immune system, plays an important role in atherosclerosis development. Using a variety of biological approaches, we have shown less atherosclerotic plaques in the absence of Interleukin-15 in mouse models, suggesting that this protein can be potentially targeted therapeutically in patients who have high levels of Interleukin-15.

ABSTRACT

Interleukin-15 is a well known pro-inflammatory cytokine which regulates many activities of immune cells including macrophages, CD8 T, natural killer, and natural killer T cells. All these cell types have been shown to contribute to atherogenesis. Here we report that Interleukin-15 plays a key role in the progression of atherosclerosis in both aortic sinus and coronary arteries. Mice lacking Interleukine-15 had lower levels of atherosclerosis while mice overexpressing Interleukin-15 had increased plaque size. We also showed that this effect on atherosclerosis is partially independent of natural killer, natural killer T, and CD8 T cells. Immunodepletion of NK1.1+ cells led to less atherosclerosis in IL-15+/- ApoE-/- compared with IL-15+/+ ApoE-/- mice. Furthermore the absence of IL-15 in IL15-/- ApoE-/- mice resulted in lower expression of MCP-1 and less presence of CD11b+ cells in plaques compared with ApoE-/- control mice. In addition, in vitro treatment of macrophages with Interleukin-15 increased the expression of inflammatory cytokines, while a soluble Interleukin-15 receptor α suppressed the pro-inflammatory effects of Interleukin-15. These findings are consistent with others' findings that Interleukine-15 affects macrophages through Interleukin-15 receptor α. To further examine this in vivo, we transplanted bone marrow from wild-type or Interleukin-15 receptor α knockout mice into LDLR-/- recipients. Mice lacking Interleukin-

15 receptor α in their bone marrow derived cells developed smaller plaques. Also, we confirmed that Interleukin-15's pro-inflammatory effect in macrophages isolated from Interleukin-15 receptor α knockout mice was partially suppressed, in comparison with macrophages isolated from wild-type mice.

It has been shown that the absence of HDL receptor (SR-BI) and ApoE in SR-BI/ApoE dKO mice leads to severe coronary artery atherosclerosis and consequently heart attack. We demonstrated that a lack of Interleukin-15 in IL-15/SR-BI/ApoE tKO mice, improved the survival of these mice compared with SR-BI/ApoE dKO control animals and also reduced the levels of atherosclerosis both in aortic sinus and coronary arteries.

In conclusion, these findings indicate that Interleukin-15 promotes atherosclerosis through multiple cellular and molecular mechanisms including monocyte/macrophage activation and survival/maturation of natural killer and CD8 T cells.

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LIST OF ABBREVIATIONS

ABC	ATP binding cassette
ACAT-1	acyl-coenzyme A:cholesterol acyltransferase 1
AcLDL	acetyl-LDL
ANOVA	analysis of variance
Apo	apolipoprotein
BAK	BCL2-antagonist/killer
BAX	Bcl-2-associated X protein
Bcl-2	B cell lymphoma 2
BM	bone marrow
γc	common γ chain receptor
CAA	coronary artery atherosclerosis
CD36	cluster of differentiation 36
CE	cholesteryl ester
CHOP	CEBP-homologous protein
CM	chylomicron

CVD	cardiovascular disease
DC	dendritic cell
EC	endothelia cell
ER	endoplasmic reticulum
FATP4	fatty acid transport protein 4
FC	free cholesterol
GFP	green fluorescent protein
HDL	high density lipoprotein
HMGCR	3-hydroxy-3-methylglutaryl coenzyme A reductase
ICAM-1	intracellular cell adhesion molecule 1
IFNγ	interferon gamma
IL	interleukin
iNOS	inducible nitric oxide synthase
JAK	janus kinase
LDL	low density lipoprotein

LDLR	low density lipoprotein receptor
LFA-1	lymphocyte function-associated antigen 1
LOX-1	lectin-like oxidized LDL receptor 1
LPL	lipoprotein lipase
LRP-1	LDL-related protein-1
LSP	long signal peptide
LXR	liver X receptor
Ly6C	lymphocyte antigen 6 complex
MCP-1	monocyte chemotactic protein-1
MCSF	macrophage colony stimulating factor
MHC-I	major histocompatibility complex I
MI	myocardial infarction
MTTP	microsomal triglyceride transfer protein
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cell
NK	natural killer

NKT	natural killer T
NPC1L1	Niemann-Pick C1-like 1
OxLDL	oxidised low density lipoprotein
PCSK-9	proprotein convertase subtilisin/kexin type 9
PI3K	phosphatidylinositol-3-kinase
P-selectin	platelet selectin
RAG-2	recombination activating gene 2
rIL-15	recombinant interleukin-15
RIP	receptor-interacting protein
SAA	serum amyloid A
SMC	smooth muscle cell
SR-A	scavenger receptor class A
SR-BI	scavenger receptor class B type I
SSP	short signal peptide
STAT	signal transducer and activator of transcription

TAK-1	transforming growth factor β activated kinase-1
TCR	T cell receptor
TG	triglyceride
TGFβ	transforming growth factor beta
TLR	toll-like receptor
TNF-α	tumour-necrosis factor α
TRADD	TNF receptor-1 associated death domain protein
TRAF2	tumour-necrosis factor receptor-associated factor 2
UPR	unfolded protein response
USP31	ubiquitin specific protease 31
VCAM-1	vascular cell-adhesion molecule 1
VLA-4	very late antigen 4
VLDL	very low density lipoprotein
vWF	von Willebrand Factor

CHAPTER 1: Introduction

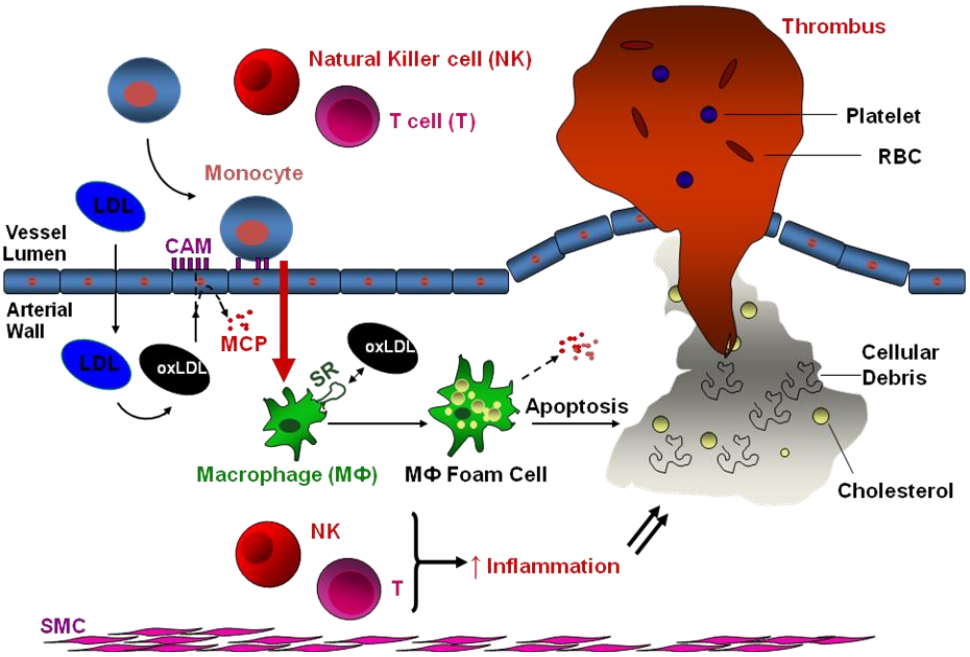
1.1 Atherosclerosis

Cardiovascular diseases are major causes of death among developed nations.[1] In human populations, one of the main causes of cardiovascular disease is atherosclerosis which is developed over decades. Atherosclerosis is an inflammatory disorder which is regulated by changes in immune responses and lipid metabolism.[1] As a result of endothelial cell (EC) damage which can be caused by a wide range of stimuli, low density lipoprotein (LDL) particles penetrate into the arterial intima, where they become oxidized.[2] These modified lipoproteins induce ECs to increase expression of adhesion molecules which in turn facilitate the recruitment of immune cells such as monocytes and lymphocytes into the intima in response to chemotactic proteins such as monocyte chemotactic protein-1 (MCP-1) and RANTES.[3] Leukocytes under the influence of inflammatory regulators including cytokines and chemokines become activated. For example, monocytes in response to macrophage colony stimulating factor (MCSF) differentiate into macrophages.[4] Furthermore, macrophages through scavenger receptors such as Scavenger Receptor A (SR-A) and cluster of differentiation 36 (CD36) take up modified lipoproteins including OxLDL resulting in the formation foam cells.[1] Eventually these macrophage foam cells undergo apoptosis through mechanisms that will be discussed below and accumulation of cellular debris and cholesterol

leads to the development of a lipid rich necrotic core.[2] In advanced plaques smooth muscle cells (SMC) migrate from adventitia to intima and secrete collagens which form a thick fibrous cap, preventing the plaque from rupture.[2] As atherosclerotic plaques advance, the arterial lumen narrows which in small arteries could result in blockade of blood flow. In addition, activated immune cells in atherosclerotic plaques can produce cytokines such as IFNγ [2] which induces secretion of proteases which in turn can cleave collagens and weaken the fibrous cap.[1, 2] Thin fibrous caps are susceptible to rupture in which case the interaction between the material in plaques with thrombotic factors in blood leads to blood coagulation.[1] These clots can block blood flow in coronary arteries or arteries feeding the brain and cause heart attack or stroke respectively which could lead to death (Figure 1.1).

Figure 1.1 Overview of atherosclerosis development

LDL lipoproteins can diffuse from circulation into the intimal layer of arteries, where LDL particles are modified to form oxidized LDLs (OxLDL). These modified particles induce endothelial cells to overexpress cell adhesion molecules such as ICAM-1 and VCAM-1. These cell adhesion molecules facilitate blood cells recruitment into the intimal layer. Monocytes differentiate into macrophages while migrating into the sub-endothelial layer. Through scavenger receptors, macrophages engulf modified lipoproteins and become lipid rich foam cells. Macrophage foam cells eventually undergo apoptosis and the accumulation of cholesterol and cellular debris contributes to the formation of lipid rich necrotic cores and advanced atherosclerotic plaques. These plaques are protected by collagen rich fibrous caps which when rupture as a result of blood turbulence, it triggers thrombosis and finally leads to heart attack and stroke.


Thrombus
Natural Killer cell (NK)
T cell (T)
Platelet
RBC
Monocyte
LDL
Vessel Lumen
CAM
Arterial Wall
MCP
LDL
oxLDL
SR
oxLDL
Cellular Debris
Apoptosis
Macrophage (MΦ)
MΦ Foam Cell
Cholesterol
NK
T
↑ Inflammation
SMC

1.2 Lipoprotein Metabolism

Lipoproteins are particles which facilitate the trafficking of cholesterol and triglyceride (TG) throughout body. These particles are categorized based on their size and density, but also the apoproteins associated with them. Lipoproteins have a hydrophobic core containing cholesterol ester (CE) and TG, and a hydrophilic coat formed from free cholesterol (FC), phospholipids, and proteins (apoproteins). Chylomicron (CM) and very low density lipoproteins (VLDL) are the main TG carrying lipoproteins, while LDL and high density lipoproteins (HDL) are the main cholesterol carrying lipoproteins. Lipoprotein metabolism is regulated by multiple factors including the intestinal absorption of lipids, lipoprotein secretion by the liver, and clearance of lipoproteins by peripheral tissues and the liver (Figure 1.2).

At the absorption level, intestinal cells (enterocytes) absorb cholesterol via the Niemann-Pick C1-like 1 (NPC1L1) transporter and secret some into the intestine by ATP binding cassette G5/G8 (ABCG5/G8). Hydrolyzed dietary fats enter intestinal cells through transporters such as fatty acid transporter protein 4 (FATP4).[5] Then triglyceride (TG) which consists of a glycerol group and three fatty acid chains is packaged with cholesterol and apoB48 into CMs, mediated by microsomal TG transfer protein (MTTP). CMs are secreted into the lymphatic system and then circulate in blood. In circulation, TG is hydrolyzed through the interaction between CM

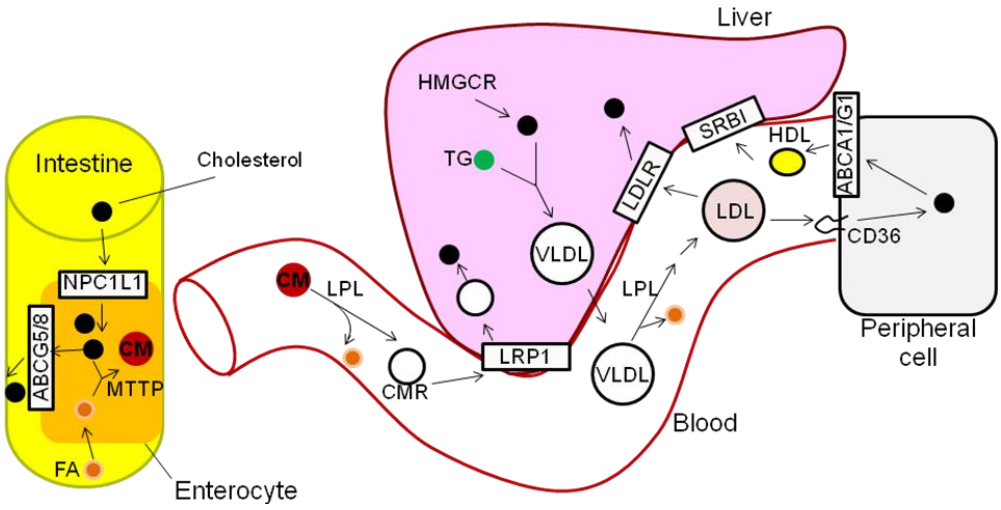
and lipoprotein lipase (LPL) and the CM remnants are taken up by hepatocytes in the liver via LDLR and LDL-related protein-1 (LRP1). [6 7] TG is degraded to FA and glycerol and cholesterol ester (CE) to free cholesterol (FC) and FA. FA, glycerol and FC are then used to re-synthesize TG and CE and are re-packaged into nascent VLDL for secretion into blood.[6] Only ~20% of cholesterol in blood originates from the diet and the other 80% is synthesized by the liver 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCR) enzyme.[8] VLDL particles are associated with ApoB100 and ApoE. Once in circulation, VLDL will interact with LPL in peripheral tissues and loose TG and eventually forms LDL particle.

Cholesterol rich LDL is endocytosed by peripheral cells or the liver cells by the LDLR. In the liver, proprotein convertase subtilisin/kexin type 9 (PCSK9) can bind to LDLR, leading to its trafficking to the lysosome and degradation. As a result, PCSK9 can block LDL cholesterol clearance from circulation.[8] As modified forms of LDL such as oxidized LDL can be taken up by macrophages and contribute to foam cell formation, these particles are known as pro-atherogenic lipoproteins. On the other hand, HDL can remove cholesterol from peripheral tissues including macrophages and participate in the reverse cholesterol transport to the liver and hence it is known as protective against atherosclerosis. HDL consists of several

apoproteins such as ApoAI which is the major apoprotein of HDL and is produced and secreted by the liver. After secretion, ApoAI acts as an acceptor of cholesterol from peripheral tissues such as macrophages through ATP-binding cassette transporter (ABC) A1 /G1 to form cholesterol rich HDL particles. Furthermore, in the liver, these particles transfer their cholesterol to hepatocytes, a process that is mediated by scavenger receptor class B type I (SR-BI) on the cell membrane of hepatocytes.[8]

Figure 1.2 Overview of lipoprotein metabolism

Dietary fatty acids and sterols enter enterocytes via FA transporters and NPC1L1 transporter respectively. Some sterols are released into the intestinal lumen via ABCG5/8. In enterocytes MTTP packages cholesterol and TG together with ApoB48 into chylomicrons which are hydrolyzed in blood and the CM remnants will be taken up. Liver is the main source of cholesterol synthesis facilitated by HMGCR. In hepatocytes, cholesterol, TG, and ApoB100 are packaged into VLDL particles. In circulation VLDL is hydrolyzed and form cholesterol rich LDL lipoproteins. LDL particles are taken up by hepatocytes via LDLR, or by peripheral cells such as macrophages via scavenger receptors including CD36 and SRA. Excess cholesterol in peripheral cells is transported into HDL lipoproteins via ABCA1/G1. Furthermore, HDL, via ApoA1/SR-BI interactions, mediates cholesterol transport into hepatocytes.


Liver
HMGCR
TG
SRBI
HDL
ABCA1/G1
LDLR
LDL
CD36
VLDL
Intestine
Cholesterol
NPC1L1
ABCG5/8
CM
MTTP
FA
Enterocyte
CM
LPL
CMR
LRP1
VLDL
LPL
Blood
Peripheral cell

1.3 The Immune System and Atherosclerosis

Immune cells are involved in different stages of atherosclerosis development. Table 1 summarizes key cellular players in atherogenesis. For example monocytes play central roles in all stages of plaque formation from initiation to development of advanced plaques. Platelets have been shown to facilitate monocyte recruitment to ECs [9]. Other immune cells such as lymphocytes have been shown to contribute to atherogenesis through multiple molecular and cellular pathways. Lymphocytes are involved in generation of antibodies against modified lipoprotein and in programming of other immune cells' response to modified lipoproteins. Dendritic cells (DCs) are antigen presenting cells (APC) that are involved in many stages of atherogenesis and are capable of producing inflammatory cytokines such as IL-6 and TNFα.[10] They also participate in the presentation of lipid derived antigens to lymphocytes and hence induce T cell activation.[10] In addition, immune cell sub-populations can be found in different regions in a plaque. For instance, while macrophages are found throughout atherosclerotic plaques, T cells are abundant in the shoulder region of the plaque.[11]

1.3.1 Recruitment of Immune Cells in the Site of Plaque Development

ECs in arteries express cell adhesion molecules (CAM) such as vascular cell-adhesion molecule 1 (VCAM-1), intercellular cell-adhesion molecule 1 (ICAM-1), P-selectin, and E-selectin in response to accumulated

Table 1. Immune cells are involved in different stages of atherosclerosis.

M1 and M2 macrophages refer to pro-inflammatory and anti-inflammatory phenotypes. DC; dendritic cell, NK: natural killer, NKT: natural killer T.

Cell type	Common marker(s)	Major mediators	Role in atherosclerosis	REF
Inflammatory monocyte	CX3CR1-CCR2+Ly6C+	CCR2	Ability to be recruited in plaques and differentiate into APCs	[2, 12, 13]
Resident monocyte	CX3CR1+CCR2-Ly6C-	CX3CR1, CCR5	Ability to differentiate into DCs with potential atheroregression capacity	[2, 12-14]
M1 macrophage	Cd11b+CD11c-, CD68+, F4/80+	IL-1β, IL-6, TNFα, iNOS	Phagocytic activities, promote inflammation, lipid uptake	[1-3, 15]
M2 macrophage	Cd11b+CD11c-, CD68+, F4/80+	IL-10, Arg-1	Resolve inflammation	[1-3, 15]
DC	CD11b+ CD11c+	CCL19, CCR7	Lipoprotein derived antigen presentation to CD4 Th1 cells	[3, 16, 17]
Neutrophil	Ly6G+	ROS, proteases	Proteolytic activity, destabilize plaques	[18, 19]
CD4 T cell	CD3+CD4+	IFNγ	Become activated by APCs via MHC-II, promotes inflammation, lysis of SMCs	[19-21]
CD8 T cell	CD3+CD8+	IFNγ	Cytotoxic activity, induce SMC apoptosis	[1, 2, 20, 21]
NK cell	NK1.1+CD3-, DX5+	IFNγ, granzyme B	Induce apoptosis through perforin and granzyme B secretion	[22-24]
NKT cell	NK1.1+CD3+	IFNγ	Become activated through lipid presentation by APCs via CD1d	[24-26]
SMC	SMA+	Collagens	Produce collagens	[27-29]

cholesterol in sub-endothelial space, shear stress, and disturbed blood flow. [30-34] Monocytes and lymphocytes can bind to CAM expressing ECs through the interaction between CAMs and leukocytes' cell membrane proteins such as very late antigen 4 (VLA-4) which interacts with VCAM-1 [35] and lymphocyte function-associated antigen 1 (LFA-1) which binds to ICAM-1.[36] These interactions mediate the leukocyte rolling on the surface of ECs. Finally leukocytes respond to local chemokines such as monocyte chemotactic protein 1 (MCP-1) [37, 38] and CCL5 T cell attractants (also known as RANTES) [39] by migrating into the intimal space. The absence of MCP-1 (also known as CCL2) or its receptor (CCR2) has been shown to attenuate atherosclerosis by limiting the entry of monocytes and T cells in the site of atherosclerotic plaque development.[37, 38]

In addition, a large body of evidence suggests a role for platelets in triggering monocyte arrest on inflamed endothelium.[33] Platelets are thought to decorate monocytes through interaction of platelet selectin (P-selectin) and monocyte P-selectin glycoprotein ligand 1. Platelets bind to von Willebrand Factor (vWF) which is a cell adhesion protein and expressed by ECs and hence facilitate monocyte recruitment to inflamed endothelium.[9, 40]

1.3.2 Mononuclear Phagocytes

Monocytes are mononuclear phagocytes and also progenitors of other phagocytes including macrophages. Monocytic cells are the most numerous among immune cells in atherosclerotic plaques.[1] Monocytes are released from bone marrow and circulate for several days. [41] They are recruited into the sites of inflammation, secrete a variety of inflammatory cytokines and differentiate into macrophages and DCs.[41] There are two distinct subsets of monocytes based on their migratory properties.[42] Lymphocyte antigen 6 complex expressing (Ly6C^{high}) monocytes are pro-inflammatory and are likely to be recruited in the sites of inflammation such as atherosclerotic plaques.[43] Ly6C^{high} monocytes have been shown to express more functional P-selectin glycoprotein ligand 1 than Ly6C^{low} monocytes.[34] On the other hand, Ly6C^{low} monocytes are thought to patrol vasculature and have reparative roles and are less likely to be recruited in plaques.[33] Ly6C^{high} monocyte infiltration into the site of plaque development is highly regulated by chemokines interactions including CCL2-CCR2 and CCL5-CCR5.[12] Disruption of these chemokine-dependent pathways has been shown to attenuate atherosclerosis in mouse models.[12, 44]

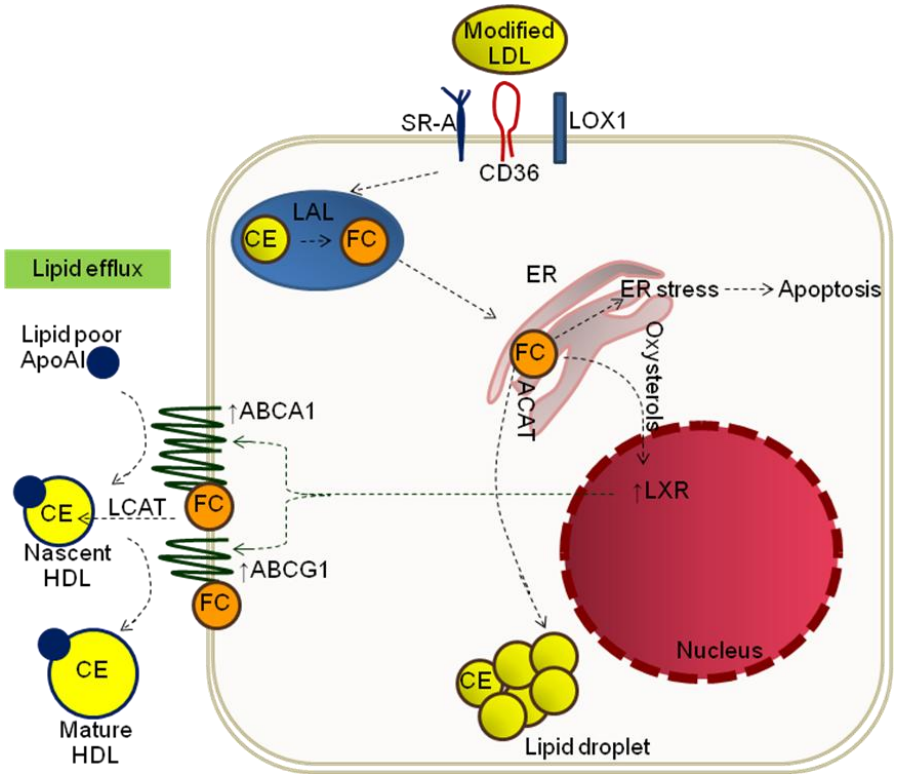
Macrophages play a central role in atherosclerosis and are the most prominent cellular contributors of plaque development.[13] Mice that are deficient in macrophage-colony stimulating factor (M-CSF) and as a result

unable to differentiate monocytes into macrophages, are resistant to developing atherosclerosis.[45] Macrophages are professional phagocytes that can sense and internalize lipids through different approaches such as scavenger receptor-mediated pathways.[1] Lipid uptake by members of scavenger receptors (SR) family including SR-A, CD36, and lectin-like oxidized LDL receptor 1 (LOX-1) leads to formation of macrophage foam cells (Figure 1.3).[15] CE derived from lipoproteins are hydrolyzed to free cholesterols which are then transported to endoplasmic reticulum (ER), where they become re-esterified by acetyl-coenzyme A:cholesterol acetyltransferase 1 (ACAT-1) and are stored in cytosolic lipid droplets.[46] Accumulation of cholesterol in foam cells is a key event during atherogenesis.[47] Dysfunctional ACAT-1 can increase the ratio of FC to phospholipids in the ER membrane, leading to ER stress.[48] This will cause the induction of the unfolded protein response (UPR) that increases the expression of the pro-apoptotic protein CEBP-homologous protein (CHOP).[49] CHOP activation is shown to induce apoptosis via release of Ca^{2+} from ER.[50] Macrophage foam cells respond to accumulation of cholesterol and other lipids by promoting several mechanisms to efflux lipids including ATP-binding cassette subfamily A member 1 (ABCA-1) and ABCG-1.[15] Elevated levels of cholesterol derivatives such as oxysterols and desmosterol are sensed by liver X receptors (LXRs), which are transcription factors, that upregulate ABCA1 and ABCG1

expression.[51]ABCA1 and ABCG1 are cell membrane proteins which transfer free cholesterol to apoprotein A1 (ApoAI) carrying HDL.[15] HDL particles then transport the excess cholesterol to the liver where scavenger receptor class B type I (SR-BI) takes up cholesterol from HDL.[52] There is a large body of evidence indicating that not only the number, but also the inflammatory phenotype of macrophages influences the atherosclerotic plaque fate. Macrophages that are activated by Toll-like receptor ligands (TLR) and interferon-γ (IFNγ) are considered pro-inflammatory M1 type. These cells are more likely differentiated from $Ly6C^{high}$ monocytes and secrete pro-atherogenic cytokines such as IL-6, IL-1β, and TNFα.[53] Studies in mice lacking TNFα[54] or IL-1β[55] showed reduced atherosclerosis which was accompanied by a reduced expression of VCAM-1 and MCP-1 and consequently less monocyte recruitment into atherosclerotic plaques. Treatment of mice with IL-6 resulted in ~ 5 fold increase in atherosclerosis, although the mechanisms through which IL-6 promotes atherosclerosis is unclear.[56] On the other hand $Ly6C^{low}$ monocytes are likely to differentiate into M2 anti-atherogenic macrophages and produce anti-inflammatory cytokines such as IL-10 and TGFβ.[57] Several reports have shown that IL-10 deficiency leads to increased atherosclerosis potentially due to enhanced T cell infiltration into plaques.[58, 59] Finally TGFβ has been shown to protect against atherosclerosis by stabilizing plaques through collagen production.[60]

Figure 1.3 Pathways involved in macrophage foam cell formation

Lipoproteins can be taken up by scavenger receptors including SR-A, LOX1, and CD36. CEs are hydrolyzed in lysosomes by lipoprotein acid lipase (LAL) which leads to the formation of FCs. Furthermore, FCs traffics to other cellular compartments such as ER. In ER, FC is converted to CE via ACAT activity and accumulation of CE in lipid droplets contribute to the formation of foam cells. In the absence of ACAT activity, excess FC can cause ER stress which can lead to apoptosis. High levels of cellular cholesterol can also trigger formation of oxysterol, an activator of LXR, which in turn induces the expression of ABCA1/G1. These two recent proteins facilitate the efflux of cholesterol into ApoA1 to form HDL particles. As a result, the amount of cellular cholesterol is determined by lipid uptake and also lipid efflux.


Modified LDL
SR-A
LOX1
CD36
LAL
CE
FC
Lipid efflux
ER
ER stress
Apoptosis
Lipid poor ApoAI
FC
ACAT
Oxysterols
↑ABCA1
CE
LCAT
FC
↑LXR
Nascent HDL
↑ABCG1
FC
Nucleus
CE
Mature HDL
CE
Lipid droplet

1.3.3 Lymphocytes

Many lymphocytic cells such as T cells, natural killer (NK), and natural killer T (NKT) cells have been detected in human and mouse plaques[61]. T cell responses in atherosclerosis are likely driven by antigen presentation of LDL-associated antigens such as peptides derived from apoprotein B 100 (ApoB-100) that is the major apoprotein associated with LDL particles.[62, 63] CD8 T cells have been shown to promote atherosclerosis mainly by perforin and granzyme B mediated apoptosis of macrophages, smooth muscle cells, and endothelial cells which, in turn results in secretion of TNFα, MCP-1, IL-1β, and IFNγ.[64] Both perforin and granzyme B have been shown to promote atherosclerosis in the descending aorta, although no effect was observed in the aortic sinus atherosclerosis.[65]

NK cells have been shown to play a role in atherosclerosis.[23] NK cells are the major sources of IFNγ which is known to contribute to atherosclerosis through multiple mechanisms including induction of MCP-1 expression in ECs.[25] In addition, NK cells induce apoptosis in their target cells through perforin and granzyme B cytotoxic activities.[66] Recently, it was reported that NK cells require perforin and granzyme B activities in order to promote atheroslcerosis.[67]

Natural killer T NKT cells have also been detected in human atherosclerotic plaques [68] and shown to contribute to atherosclerosis in

murine models.[26] NKT cells are a subset of T lymphocytes that through their T cell receptor (TCR) can recognize lipid antigens that are bound to CD1d molecules on the surface of antigen presenting cells.[69] After stimulation, NKT cells can rapidly secret a broad range of cytokines including IFNγ, MCP-1, TNFα, and IL-6 and hence can promote atherosclerosis.[26]

In conclusion, many immune cells and inflammatory cytokines are involved in atherosclerosis development. In the past decade, many research groups have investigated the roles of different cytokines in atherogenesis, in the hope to identify therapeutic targets that can be used for the treatment of atherosclerosis without major negative physiological side effects. One of the cytokines which has been shown to be strongly pro-inflammatory is Interleukin-15.

1.3.4 Interleukin-15

Mature Interleukin (IL)-15 is a 14 KDa cytokine with 114 amino acids (aa). There are two IL-15 precursor isoforms, one with a 48 aa long signal peptide (LSP: long signal peptide) in the N-terminal and the other with 21 aa long SP (SSP: short signal peptide).[70, 71] Signal peptides determine the intracellular localization and also secretion of the corresponding mature protein. LSP-IL15 has been detected in ER and Golgi apparatus (secretory pathways) and hence is secreted, whereas SSP-IL15 appears to be

restricted to the cytoplasm and nucleus.[72-74] IL-15 mRNA is expressed by multiple tissues and cell types including skeletal muscle, kidney, lung, heart, monocytes, DCs, and macrophages.[75] Studies have revealed the presence of binding sites within the human and mouse IL-15 promoter regions for the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB).[76] IL-15 has been shown to be essential for the survival and/or maturation of NK, NKT, and CD8 T cells through pathways which will be discussed below.[77] IL-15 has three receptors; IL-15Rα, IL-2/15Rβ (Rβ), and the common γ chain (γ_c).[77] IL-15 has high affinity for IL-15Rα ($K_a > 10^{11}$ M^{-1}) while it has intermediate affinity ($K_a \sim 10^9$ M^{-1}) for IL-2/15R$\beta\gamma_c$ complex in the absence of IL-15Rα.[77, 78] IL-15Rα is a type-1 transmembrane protein with a signal peptide (32 amino acids), an extracellular domain (173 amino acids), a transmembrane domain (21 amino acids), and a cytoplasmic tail (37 amino acids).[79] There are 8 different isoforms of IL-15Rα including forms that were found on membrane and forms which were shed and found as soluble in circulation.[79-81] Those forms of IL-15Rα that lack exon 2 are unable to bind IL-15, suggesting that exon 2 encodes a binding domain for IL-15.[79-81] Only isoforms containing exon 2 have been found in the nuclear membrane where it is proposed that IL-15Rα and SSP-IL15 forms the IL-15/ IL-15Rα complex.[79-81] Recently it was shown that all circulating IL-15

was associated with soluble IL-15Rα, indicating the high binding affinity between the receptor and ligand.[82]

1.3.4.1 IL-15 and Immunity

It has been shown that IL-15 and its IL-15Rα are expressed in macrophages and DCs and as they interact with each other with high affinity, they form IL-15/ IL-15Rα complex which after production are transported to the cell membrane. On the cell membrane, IL-15Rα can trans-present IL-15 to target cells such as NK, NKT and CD8 T cells. These target cells express IL-2/15Rβγ_c. IL-15 can bind to IL-12Rβ and also γ_c with lower affinity compared with IL-15Rα. The common γ chain receptor is common among several cytokines including IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21 which all are involved in regulating lymphocytes development and functions.[83] Through these receptors, IL-15 induces the JAK (Janus kinase) and STAT (signal transducer and activator of transcription) molecular signaling pathway leading to the survival and maturation of these target cells.[77, 84] The cytosolic domains of IL-2/15Rβ and γ_c in target cells are associated with JAK 1 and 3 respectively. Upon induction by IL-15, JAK 1 and 3 are phosphorylated and in turn phosphorylate STAT 3 and 5 respectively.[84] Phosphorylated STAT 3 and 5 form a heterodimer which translocates into the nucleus where it promotes the expression of anti-apoptotic B cell lymphoma (Bcl) -2 gene. The Bcl-2 protein inhibits the pro-apoptotic proteins, BAX and BAK from

undergoing a conformational change under stress conditions. The conformational change leads to activation of BAX and BAK causing the disruption of the mitochondrial outer membrane, leading to release of cytochrome c and consequently the formation of the apoptosome and activation of cell apoptosis.[85] IL-15 has also been shown to induce other pathways including the induction of the phosphatidylinositol-3-kinase-AKT (PI3K/AKT) pathway.[84] The adaptor protein, Shc, which is associated with IL2/15Rβ, activates PI3K which in turn induces activation of Akt leading to phosphorylation of FOXO3a. Activation of FOXO3a suppresses upregulation of the pro-apoptotic protein, Bim.[84, 86]

However, Chenoweth M. and colleagues have also shown that IL-15 can activate myeloid cells through an NF-κB dependent pathway, and independently of the JAK/STAT pathway.[87] In this report, it was demonstrated that IL-15 induces RANTES production in myeloid cells in a manner that was suppressed by soluble IL15Rα, but not affected by the inactivation of IL2/15Rβ or γ_c.[87] NF-κB is a transcription factor of many genes including inflammatory cytokines. In un-stimulated cells, NF-κB is located in the cytosol bound to inhibitor of NF-κB (IκB).[88] Upon stimulation by inducers, IκB kinase (IKK) phosphorylates IκB , tagging it for ubiquitination and proteasomal degradation.[88] Consequently, the released NF-κB complex translocates into nucleus where it induces the expression

of a wide range of genes including those encoding inflammatory cytokines.[88] In addition NF-κB has been shown to promote several inflammatory diseases such as atherosclerosis.[89, 90]

1.3.4.2 IL-15 and Metabolic Disease

IL-15 is a potent inflammatory cytokine and its role in several autoimmune and inflammatory diseases has been documented.[77] In Rheumatoid arthritis (RA), a chronic inflammatory condition of the joints, IL-15 is proposed to assist T cell infiltration and induction of TNFα expression by macrophages.[91] IL-15 has been linked to obesity before.[92] In obesity, adipose tissue becomes an inflammatory environment as a result of leukocyte infiltration and secretion of pro-inflammatory cytokines.[92, 93] Obesity is known to contribute to atherosclerosis development through inflammatory pathways such as IL-1β and IL-18 activation.[94-96] It has been demonstrated that obese patients have lower circulating IL-15 levels than lean subjects.[97] Also, IL-15 KO mice exhibit greater body fat than WT mice while IL-15 administration reduced fat mass without any effect on food intake.[97] Furthermore, IL-15 has been shown to inhibit preadipocyte differentiation and lipid deposition in mouse adipocytes in vitro.[97] This work suggests that IL-15 can directly modulate pre-adipocytes and mature adipocytes, however more work is needed to characterize the molecular pathway through which IL-15 regulates adipocytes behaviour.

1.3.4.3 IL-15 and atherosclerosis:

Many cytokines have been shown to play roles in atherosclerosis. Table 2 summarizes several key pro- and anti-inflammatory cytokines' roles in atherosclerosis development. IL-15 has been shown to be present in both human and mouse atherosclerotic plaques[98 99]. Also, it has been reported that individuals with cardiovascular diseases have higher levels of serum IL-15 compared to healthy individuals [100]. Furthermore, vaccination of LDLR KO mice with attenuated S. typhimurium bacteria against IL-15 reduces shear stress-induced atherosclerosis in carotid arteries. However, this could be due to the elimination of IL-15 expressing cells including macrophages. [101]. As mentioned before, macrophages are major cellular contributors of atherogenesis and hence more experiments are required to clarify whether the reduction of atherosclerosis was due to the elimination of macrophages or the absence of IL-15.

1.4 Mouse Models of Atherosclerosis in Aortic Sinus and Coronary Arteries

Wild-type mice are resistant to developing atherosclerosis even on high fat and high cholesterol diets, likely because HDL is the major plasma lipoprotein in wild type mice. Elimination of essential genes (apolipoprotein E, LDL receptor) involved in the clearance of VLDL and LDL can result in the development of advanced plaques in the aortic sinus and the descending aorta. ApoE KO and Ldlr KO mice are particularly fruitful

Table 2- Cytokines and atherosclerosis. HF: high fat, chol: cholesterol, ↑: increased, ↔: no change, ↓: decreased, ND; not determined.

Cytokine	Mouse model	Diet	Cholesterol	Lesion size	REF
CCL2	CCL2-/-LDLR-/-	HF, 21% fat/ 0.15% chol	25%↑	83%↓	[38]
CCL5	ApoE-/-,CCL5-/- BMT	HF, 21% fat/ 0.15% chol	ND	40%↓	[102]
IL-1β	IL-1β-/-ApoE-/-	Chow, 4.5% fat/ 0.02% chol	↔	33%↓	[55]
IL-2	ApoE-/-, anti IL-2 antibody	HF, 21% fat/ 0.15% chol	ND	45%↓	[103]
IL-4	IL-4-/-ApoE-/-	Chow, 4.5% fat/ 0.02% chol	↔	27%↓	[104]
IL-5	LDLR-/-,IL-5-/-BMT	HF, 15.8% fat/1.25% chol	ND	50%↑	[105]
IL-6	IL-6-/-ApoE-/- IL-6-/-ApoE-/- ApoE-/-, rmIL-6	Chow, 4.5% fat/ 0.02% chol Chow, 4.5% fat/ 0.02% chol HF, 20% fat/1.5% chol/ 0.5% cholate	26%↑ 60%↑ ↔	↔ 40%↑ 90%↑	[106] [107] [56]
IL-10	IL-10-/-ApoE-/-	Chow, 4.5% fat/ 0.02% chol	↔	200%↑	[108]
IL-12	IL-12-/-ApoE-/-	Chow, 4.5% fat/ 0.02% chol	↔	52%↓	[104]
IL-15	LDLR-/-, IL-15 Vac	HF, 21% fat/ 0.15% chol	↔	75%↓	[101]
IL-18	IL-18-/-ApoE-/-	Chow, 4.5% fat/ 0.02% chol	50%↑	35%↓	[109]
IFNγ	IFNγ-/-ApoE-/- IFNγR-/-ApoE-/-	Chow, 4.5% fat/ 0.02% chol HF, 21% fat/ 0.15% chol	↔ ↑	42%↓ 60%↓	[24] [25]
MCSF	Op/op/ApoE-/-	Chow, 4.5% fat/ 0.02% chol	190%↑	90↓	[45]
TNFα	TNFα-/-ApoE-/-	HF, 21% fat/ 0.15% chol	↔	50%↓	[110]

mouse models to study molecular and cellular pathways of atherosclerosis. In 1992, the first murine model of atherosclerosis was developed by genetic inactivation of the apolipoprotein E (ApoE) gene. ApoE, a 34 KD protein, is found in CMs and VLDLs and is a ligand for the receptors involved in the clearance of these lipoproteins. ApoE KO mice have higher total cholesterol and also increased VLDL and LDL cholesterol, while they have substantially reduced HDL cholesterol. ApoE KO mice were generated by insertion of a neomycin resistance cassette in place of exon 3 and intron 3 of the gene. No ApoE was detected in plasma of ApoE KO mice.[111]

LDLR KO mice on normal chow diet have more than 2 fold increased total cholesterol compared with wild-type mice due to the inefficient LDL clearance as a result of LDL receptor deficiency, but they do not develop substantial atherosclerosis. When LDLR KO mice are fed high fat and/or high cholesterol diets, however, the TC is increased by more than 10 fold compared with LDLR KO mice fed a normal chow diet and these mice develop substantial atherosclerosis.[112] LDLR KO mice were generated in 1993 by insertion of a neomycin resistance cassette which deleted exon 4 resulting in a non-functional truncated version of the LDLR protein lacking a membrane spanning segment.[112]

ApoE and LDL receptor KO mice develop spontaneous or diet induced atherosclerosis in the aorta or primary branches of the aorta. Atherosclerosis development is most often studied in the aortic sinus, the descending aorta and in the brachiocephalic arteries of these mice. However they do not develop substantial of atherosclerosis in their coronary arteries, limiting their usefulness for studies of atherosclerosis accompanied by its downstream effects, such as myocardial infarction (MI). On the other hand, inactivation of the gene encoding SR-BI in either apoE KO or LDL receptor KO mice results in the spontaneous or diet induced (respectively) development of coronary artery atherosclerosis (CAA), MI and early death of these mice. SR-BI/apoE dKO mice develop spontaneous occlusive CAA by 5 weeks of age and do not survive beyond 9 weeks of age.[113] SR-BI/LDLR dKO mice develop diet induced occlusive CAA, MI and death within 3.5 weeks, depending on the diet[114]. Early death of these mice is thought to be the outcome of severe occlusive coronary arteries together with thrombosis formation leading to heart attack[113].

Several groups have used this mouse model to study the effects of different genes on CAA. Others have investigated the role of hepatic lipase deficiency by generating triple knockout mice that were deficient in not only SR-BI and ApoE, but also in hepatic lipase. Hepatic lipase

deficiency led to longer survival and lower coronary artery atherosclerosis in this study. [115] The same group has also used this animal model to understand the effect of lymphocyte deficiency in CAA by generating recombination activating gene 2 (RAG2), SR-BI, ApoE triple KO mice and did not detect any significant difference in the survival of the mice lacking RAG2.[116] In other studies, the effects of pharmacological candidates including Ezetimibe, an inhibitor of the intestinal absorption of cholesterol, and probucol, a lipid-lowering drug, on CAA were investigated using the srbi/apoE dKO mouse models and in both cases an improvement in the survival of mice which had received pharmacological treatment was seen.[117-119]

1.5 Specific Hypotheses

IL-15 has a wide range of effects on different cell types through multiple pathways and receptors. While IL-15 through IL2/15Rβ and γ_c participate in survival of NK and CD8 T cells which are essential for clearance of tumour cells, it can induce inflammatory responses in myeloid cells through IL-15Rα.[87] ***As NK, NKT, CD8 T cells and myeloid cells such as macrophages have previously been demonstrated to play critical roles in different stages of atherosclerosis development, and because IL-15 impacts all these cell types, we hypothesized that IL-15 promotes atherosclerosis.*** To test this hypothesis we used multiple in vitro and in vivo approaches including mouse models of atherosclerosis. ***In***

addition, because IL15Rα in bone marrow derived cells has a critical role in the survival/maturation of NK, NKT, and CD8 T cells, and also in myeloid cell activation of inflammation, we further proposed that IL15Rα in bone marrow derived cells is required for atherogenic effects of IL-15. Finally, due to the importance of coronary artery atherosclerosis and MI in human cardiovascular disease, we set out to test the role of IL-15 in occlusive coronary artery disease. ***We hypothesized that deletion of IL-15 in heart attack mice (discussed below) reduces coronary artery atherosclerosis and improves the survival of these mice.***

1.6 Experimental Design

1.6.1 Aim 1: To test the effect of IL-15 in atherosclerosis development in IL-15 overexpressing and knockout ApoE deficient mice.

To test our first hypothesis, we generated 4 strains of mice with different IL-15 expression levels that were deficient in apoprotein E; *il15*$^{tg/wt}$*apoE*$^{ko/ko}$, *il15*$^{wt/wt}$ *apoE* $^{ko/ko}$, *il15*$^{wt/ko}$ *apoE* $^{ko/ko}$, *il15* $^{ko/ko}$ *apoE* $^{ko/ko}$. To generate *il15*$^{tg/wt}$*apoE*$^{ko/ko}$ (referred as IL15Tg ApoE KO) mice we crossed IL15Tg mice that overexpress IL-15 to apoE KO mice. We acquired IL15Tg mice in which the global overexpression of IL-15 was achieved by the MHC-I promoter.[120] As MHC-I protein is expressed globally in mice, IL-15 is expressed by all cell types throughout body and

hence the levels of IL-15 are higher in IL15Tg mice than wild-type animals[120]. In addition, to generate *il15*$^{wt/ko}$ *apoE* $^{ko/ko}$ (IL15+/- ApoE KO) and *il15* $^{ko/ko}$ *apoE* $^{ko/ko}$ (il15/ApoE dKO), we crossed IL15KO to ApoE KO mice. The IL-15 KO mice were generated by replacing exons 3-5 with a PGK-neo cassette.[121]

1.6.2 Aim 2: To evaluate the role of IL15Rα in bone marrow derived cells in the development of atherosclerosis.

To test the hypothesis that IL-15 Ra in leukocytes was involved in atherosclerosis development, we used LDLR KO mice as the recipient of BM from WT or IL15Rα KO donors. LDLR KO mice do not develop atherosclerosis spontaneously.[122] This provides the opportunity to turn on and off progression of atherosclerosis if required.[122] In order to study BM derived cells, transplantation of BM from donors is required, followed by several weeks of recovery. In order to avoid atherosclerosis development during the recovery period which can complicate the experiments, researchers prefer to use LDLR KO mice instead of ApoE KO animals. Also, the BM derived cells in recipient mice will express ApoE. Performing BMT from a wild-type mouse to ApoE KO mice leads to secretion of ApoE in circulation of recipient and hence will lower lipids and consequently have large impact on atherosclerosis. This is not a concern for LDLR KO mouse model as LDLR in BM derived cells does not affect atherosclerosis.[122] The IL15Rα KO mice were generated by replacing

exons 2 and 3 by a neomycin resistance cassette[123]. Exons 2 and 3 encode the extracellular ligand binding domain of the receptor.[124] Furthermore, peritoneal macrophages were isolated from wild-type and IL-15Ralpha KO mice to determine the importance of the receptor in driving the effects of IL-15 on macrophage activation and foam cell formation.

1.6.3 Aim 3: To test the effect of IL-15 deficiency on CAA and MI in mice lacking SR-BI and ApoE.

Finally to test our third hypothesis, we generated mice that lacked IL-15, SR-BI, and ApoE (tKO) and compared them with srbi/ApoE dKO control group. Atherosclerosis, markers of inflammation, lipids levels, and the composition of plaques were analyzed in these mice.

CHAPTER 2: Interleukin-15 promotes atherosclerosis in apoE-/- mice through effects on multiple inflammatory cells

Foreword

The following study investigates the role of Interleukin-15, a pro-inflammatory cytokine in the development of atherosclerosis in apolipoprotein E deficient mouse models. Here we report that overexpression of Interleukin -15 increases atherosclerosis and deletion of this cytokine leads to a reduction in the disease development. We demonstrated that Interleukin-15 promotes atherosclerosis through multiple cellular and molecular pathways which involve macrophages and lymphocytes.

The following individuals contributed to this work: Omid Dadoo, Mark Fuller, Magdalena Ciecierska, David Y Wang, Melissa E MacDonald, Suleiman A Igdoura, Carl D Richards, Ali A Ashkar, Bernardo L Trigatti.

B.L.T and O.D. conceived and designed the experiments. O.D., M.F., D.W., M.M., and M.C. performed the experiments. O.D. and B.L.T. analysed the data wrote the manuscript. A.A., C.R, and S.I. contributed intellectually.

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2.1 Abstract:

Interleukin (IL)-15 is essential for the survival and maturation of natural killer (NK) and $CD8^+$ T cells and directly activates macrophages. In the present study, we examined the effects of overexpressing or inactivating IL-15 on atherosclerosis in *apoE*$^{-/-}$ mice. IL-15 knockout reduced whereas overexpression increased atherosclerosis despite opposite effects on plasma lipoprotein cholesterol levels. IL-15 deficiency reduced while overexpression increased circulating pools of monocytes, NK and $CD8^+$ T cells in *apoE*$^{-/-}$ mice. $CD8^+$ T cells were detected in atherosclerotic plaques from *apoE*$^{-/-}$ mice, were increased in plaques from *il-15*^{Tg}*apoE*$^{-/-}$ mice and were virtually absent from plaques from *il15*$^{-/-}$ *apoE*$^{-/-}$ mice. Elimination of only one il15 allele markedly reduced atherosclerosis, without altering circulating NK, CD8+T or monocyte cell numbers. Similar results were obtained in mice depleted of NK1.1+ cells which include NK, NKT and CD8+T cells, demonstrating that IL-15 has pro-atherogenic effects other than its ability to regulate the amounts or activities of these circulating inflammatory cells. Treatment of macrophages with recombinant IL-15 *in vitro* induced inflammatory cytokines and acetyl-LDL induced foam cell formation. Deletion of one or both IL-15 alleles reduced levels of MCP-1 immuno-reactivity in atherosclerotic plaques. IL-15 plays a significant role in promoting atherosclerosis through pathways affecting multiple inflammatory cells including the direct activation of macrophages.

2.2 Introduction:

Atherosclerosis is an inflammatory disorder which is largely triggered by dysregulated lipoprotein metabolism and inflammatory responses to modified lipoproteins [1]. Leukocyte recruitment to the sites of atherosclerotic plaque formation and the consequent pro-inflammatory activities of such cell types contribute to the development of advanced plaques in the arterial walls [11]. Monocytes recruited to the vessel wall migrate into the arterial intima, differentiate into phagocytic macrophages [45], take up modified lipoproteins such as oxidized LDL and are converted into lipid engorged foam cells[125] which are typical hallmark cellular constituents of atherosclerotic plaques. Lymphocytes such as natural killer (NK), NK T and $CD8^+$ T cells, major sources of the pro-atherogenic cytokine IFNγ [126,24], have also been shown to contribute to atherosclerotic plaque development[7-10]. Inflammatory cytokines and chemokines are major regulators of immune cells and have been demonstrated to play critical roles at different stages of atherogenesis [127, 128]. While some function as chemoattractant proteins (e.g. MCP-1 and RANTES [38, 129]) to induce immune cell migration into the sites of inflammation, others (e.g. IFNγ, IL-1β and TNFα [130]) activate macrophages to produce additional inflammatory factors or to form foam cells by taking up modified LDL particles. In addition to activation of macrophages, cytokines have been

shown to program T cell responses to produce factors which eventually contribute to plaque advancement and instability [1].

Interleukin-15 (IL-15) is a 14 kDa cytokine secreted by a variety of cell types including macrophages and endothelial cells that plays a key role in both the survival and maturation of NK, NKT and $CD8^+$ T cells [22, 77, 131] and also has pro-inflammatory properties that affect macrophage activation [87]. IL-15 interacts with IL-15 receptor α (Rα), IL-2/IL-15 receptor β (Rβ) and common chain receptor $R\gamma_c$ [132]. IL-15 together with its Rα is produced by macrophages and dendritic cells and is trans-presented to NK, NKT, and $CD8^+$ T cells[133]. There, interaction of trans-presented IL-15 with the $R\beta/R\gamma_c$ complex induces JAK/STAT signaling pathways leading to production of anti-apoptotic factors; thus IL-15 is an essential survival factor for NK, NKT and $CD8^+$ T cells[134]. On the other hand, IL-15 has also been shown to directly activate macrophages to induce expression of pro-inflammatory and pro-atherogenic genes [reference 22-Vaccine paper] including RANTES, which appears to involve IL-15 Rα and activation of NF-κB[87].

IL-15 has been detected in both human and mouse atherosclerotic plaques[98 99]. Patients with cardiovascular diseases have been reported to have higher levels of serum IL-15 compared to healthy individuals [100]. IL-15 and IL-15 Rα have been detected in smooth muscle cells in humans

and mice and antibody mediated blockade of IL-15 was reported to enhance neo-intima formation in mice in response to carotid arterial injury mediated by a perivascular cuff[135], suggesting that IL-15 may suppresses vascular injury associated with atherosclerosis. On the other hand, vaccination of LDL receptor deficient (*ldlr*$^{-/-}$) mice against IL-15 reduced atherosclerosis in carotid arteries in response high fat diet combined with placement of a perivascular cuff[135]. In that study, vaccination against IL-15 was achieved by infection with attenuated S. typhimurium bacteria that harbored a plasmid conferring expression of IL-15[101]. The reduction in atherosclerosis, however, appeared to involve immune mediated elimination of IL-15 expressing cells, including macrophages, the major cellular constituent of atherosclerotic plaques [101]. Therefore the role of IL-15, itself, on atherosclerosis development remains unclear.

In this study we directly tested the role of IL-15 on atherosclerosis by determining the effects of overexpressing IL-15 or of deleting one or both copies of the *il15* gene in *apoE*$^{-/-}$ mice. Overexpression of IL-15 increased atherosclerosis despite reduced plasma cholesterol levels, while complete inactivation of IL-15 reduced atherosclerosis in *apoE*$^{-/-}$ mice despite significantly increased plasma cholesterol levels. IL-15 overexpression increased while its deletion eliminated NK cells and reduced circulating $CD8^+$ T cells and monocytes. On the other hand, elimination of only one

copy of IL-15 did not affect circulating NK cells, $CD8^+$T cells or monocytes, or plasma lipoprotein cholesterol levels, but resulted in a marked reduction in atherosclerosis development. This was observed even in mice treated with an anti-NK1.1 antibody to immuno-deplete $NK1.1^+$ cells including NK and $CD8^+$T cells, demonstrating that the effects of IL-15 were at least in part mediated by cells other than NK or CD8T cells. IL-15 treatment of macrophages increased iNOS expression and activity, RANTES secretion and MCP-1, IL-6, IL-1β and TNF-α expression, and increased foam cell formation induced by acetyl-LDL. Overexpression of IL-15 increased and reduction of 1 or 2 copies reduced MCP-1 immunoreactivity in atherosclerotic plaques consistent with direct effects on macrophage activation in atherosclerotic plaques. These findings demonstrated that IL-15 promotes atherosclerosis through effects on multiple inflammatory cells including NK/NKT, $CD8^+$ T cells as well as direct activation of macrophages in atherosclerotic plaques.

2.3 Materials and Methods:

2.3.1 Mice and diets: All experiments involving mice were approved by the McMaster University Animal Research Ethics Board and were in accordance with the Canadian Council on Animal Care Guidelines, which conform to the NIH guidelines (Guide for the care and use of laboratory animals). All mice were on a C57BL6/J background. *il15*^{tg} mice were kindly provided by M. Caligiuri (Ohio State University, School of Medicine, Columbus, OH). *il15* $^{-/-}$ mice were obtained from Immunex (currently Amgen). *apoE* $^{-/-}$ mice were obtained from Jackson Laboratories. *il15* $^{-/-}$ *apoE* $^{-/-}$, *il15* $^{+/-}$*apoE* $^{-/-}$, *il15* $^{+/+}$*apoE* $^{-/-}$, *and il15* ^{tg}*apoE* $^{-/-}$ mice were generated by mating *il15* $^{-/-}$ *or il15* ^{tg} with *apoE* $^{-/-}$ mice and intercrossing their offspring through two generations. Mice were housed in the Central Animal Facility at McMaster University and with free access to food and water unless otherwise noted. For atherosclerosis induction in female C57BL/6 and *il-15*^{tg} mice, animals were fed a diet containing 15 % fat, 1.25 % cholesterol and 0.5 % sodium cholate (Teklad Research Diets, TD88051) beginning at 13 weeks of age, for a total of 15 weeks. All other mice were fed a normal chow diet (Teklad 18 % protein diet, Harlan Research Labs, USA).

2.3.2 Antibody-mediated depletion of NK1.1+ cells in vivo. 8 week old mice were injected with 200 µg of NK1.1 depleting antibody (PK136 mouse immunoglobulin G2a hybridoma HB191; ATCC, Manassas, VA,

USA) for two consecutive days, followed by 100 µg of the antibody every three days for a total duration of 7 weeks.

2.3.3 Atherosclerosis analysis: Mice were fasted for 12 hours. Mice were anesthetized with isoflurane gas and heparinized blood was collected by cardiac puncture. Thoracotomy was then performed and issues were perfused in situ with saline followed by formalin prior to removal of the heart and aorta. Aortas fixed in formalin prior to staining with Sudan IV for en face analysis of plaque coverage as described previously [136]. Images were acquired, and Sudan IV–positive area and total area of aorta were measured. Hearts were embedded in Cryomatrix (Thermo Scientific, Ottawa, ON) prior to collection of frozen sections from the aortic sinus of hearts, staining with oil red O and hematoxylin, and analysis as described previously [113, 137].

2.3.4 Lipid and lipoprotein analysis: Plasma was prepared from heparinized blood and assayed for total cholesterol (Cholesterol Infintiy, Thermo Scientific, Ottawa, ON) and triglyceride (Wako Diagnostics, Richmond, VA). Plasma fractions were collected using FPLC and total cholesterol associated with each fraction was measured using chemical assay.

2.3.5 Immunohistochemistry and immunofluorescence:

Immunostaining was performed using rat anti-CD8 (BD Pharmingen, Mississauga, Canada), rabbit anti-MCP-1 (Abcam, Cambridge, MA) and biotinylated anti-CD11b (BD Pharmingen, Mississauga, Canada) antibodies. Antigens were visualized using goat anti-rat IgG secondary antibody, goat anti-rabbit conjugated to AlexaFluor 594, and Strapavidin conjugated to AlexaFlour 468 (Molecular Probes, Burlington, ON, Canada). Sections were counterstained for nuclei with DAPI (Invitrogen/Molecular Probes, Burlington, Ontario) for immunofluorescence.

2.3.6 Cell culture. Murine Raw264.7 cells (ATCC, TIB-71, Manassas, VA, USA) were incubated in DMEM supplemented with 10% fetal bovine serum (FBS), for 24 hr in the presence or absence of mouse rIL-15 (100 ng/ml) (Peprotech, 210-15, Rocky Hill, NJ, USA).

2.3.7 Foam cell formation. C57BL/6 mice were euthanized by asphyxiation with CO_2. Macrophages were flushed from the peritoneal cavity with 10 ml of PBS and cultured in RPMI medium supplemented with 3% newborn calf lipoprotein-deficient serum [138]. After incubation for 24 hours with or without 250 ng/mL rIL15, 100µg/mL acetylated LDL

(Biomedical Technologies, Inc,Ward Hill, MA, USA) was added for 8 hours before staining with oil Red O and DAPI.

2.3.8 Flow cytometry: Blood cells were stained with PE labeled NK1.1, FITC labeled CD3, APC-H7 labeled CD8, FITC labeled CD11b, FITC or APC labeled Ly6c antibodies (BD Pharmingen, Mississauga, Canada) and subjected to flow cytometry using BD LSR II flow cytometer.

2.3.9 Plasma lipoprotein, chemical assays, and ELISA analysis. Plasma was collected at sacrifice, and plasma lipoprotein fractions were separated as described previously [139]. Total cholesterol (Cholesterol Infintiy, Thermo Scientific, Ottawa, ON, Canada) and triglyceride (Wako Diagnostics, Richmond, VA, USA) content was measured using enzymatic assay kits. IL-6 and RANTES (R&D Systems, Minneapolis, MN, USA) and SAA (Life Technologies, Carlsbad, CA, USA)were measured by ELISA kits. iNOS activity was measured as the amount of nitrite in the culture medium using Griess reagent (Sigma-Aldrich, St Louis MO, USA).

2.3.10 RNA purification and analysis by RT-PCR. Total RNA was extracted from cells using TRIzol (Life Technologies, Carlsbad, CA, USA). RNA was reverse transcribed using the Ambion RETROscript kit (Life Technologies, Carlsbad, CA, USA). Quantitative real time PCR analysis of individual cDNA was performed using TaqMan Gene Expression

Assays (Applied Biosystems Japan Ltd., Tokyo, Japan; NOS-2: Mm00440502_m1 , IL-1β: Mm00434228_m1 , TNFα: Mm00443258_m1, MCP-1: Mm00441242_m1, IL-6: Mm00446190_m1).

2.3.11 Statistical analysis: All the data are averages ± SEM unless otherwise indicated. Data was subjected to one way ANOVA or pairwise comparisons by the Student's T-test or Mann-Whitney Rank Sum test or one way ANOVA with Holm-Sidak post-hoc test as indicated. Differences were considered significant if $P<0.05$.

2.4 Results:

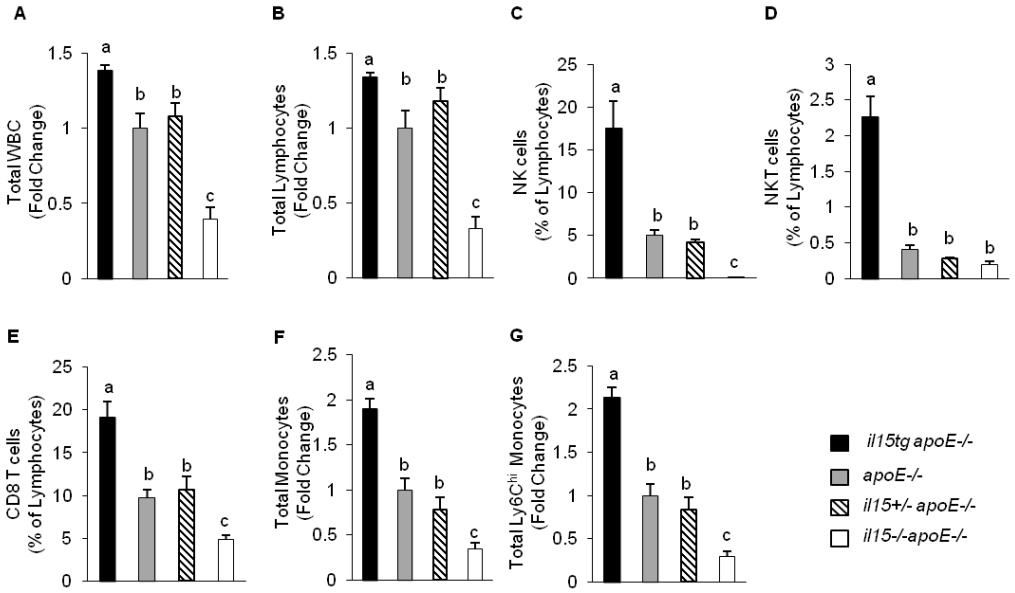
2.4.1 Effects of manipulating IL-15 expression levels on circulating NK, NKT and CD8+ T cells in apoE-/- mice.

IL-15 has been shown to affect the development of NK, NKT, and $CD8^+$ T cells [77]. We crossed *il15*$^{tg/wt}$ (hereafter referred to as *il15*^{tg}) or deficient (*il15*$^{-/-}$) with *apoE*$^{-/-}$ mice, to generate *apoE*$^{-/-}$ mice with different levels of IL-15 expression: *il15*^{tg} *apoE*$^{-/-}$, which overexpress IL-15 and, *il15*$^{+/+}$ *apoE*$^{-/-}$, *il15*$^{+/-}$*apoE*$^{-/-}$ and *il15*$^{-/-}$ *apoE*$^{-/-}$ mice, which have two, one or no functional copies of the *il15* gene. We chose to analyze *il15*^{tg}*/apoE*$^{-/-}$ mice at 15 weeks of age because older *il15*^{tg} *apoE*$^{-/-}$ mice exhibited severe ulcerative skin lesions as previously reported for *il15*^{tg} mice; furthermore, older *il15*^{tg} mice develop acute lymphoblastic leukemia and die within 1 year of age.[120, 140] We analyzed *il-15*$^{+/-}$*apoE*$^{-/-}$ and *il-15*$^{-/-}$*apoE*$^{-/-}$ mice at 25 weeks of age to allow for an examination of the effects of reduced IL-15 levels on more mature atherosclerotic plaques (see below). First, we confirmed that genetic manipulation of IL-15 levels affected levels of NK, NKT, and $CD8^+$ T cells in *apoE*$^{-/-}$ mice. Overexpression of IL-15 increased and knockout of IL-15 reduced the total numbers of white blood cells and lymphocytes (Figure 2.1 A, B) when compared with age and gender matched *il15*$^{+/+}$*apoE*$^{-/-}$ mice, consistent with findings of others [120]. IL-15 overexpression increased NK, NKT and $CD8^+$ T cells, while knockout of IL-15 virtually eliminated NK cells and reduced $CD8^+$ T cells (Figure 2.1 C-E) in

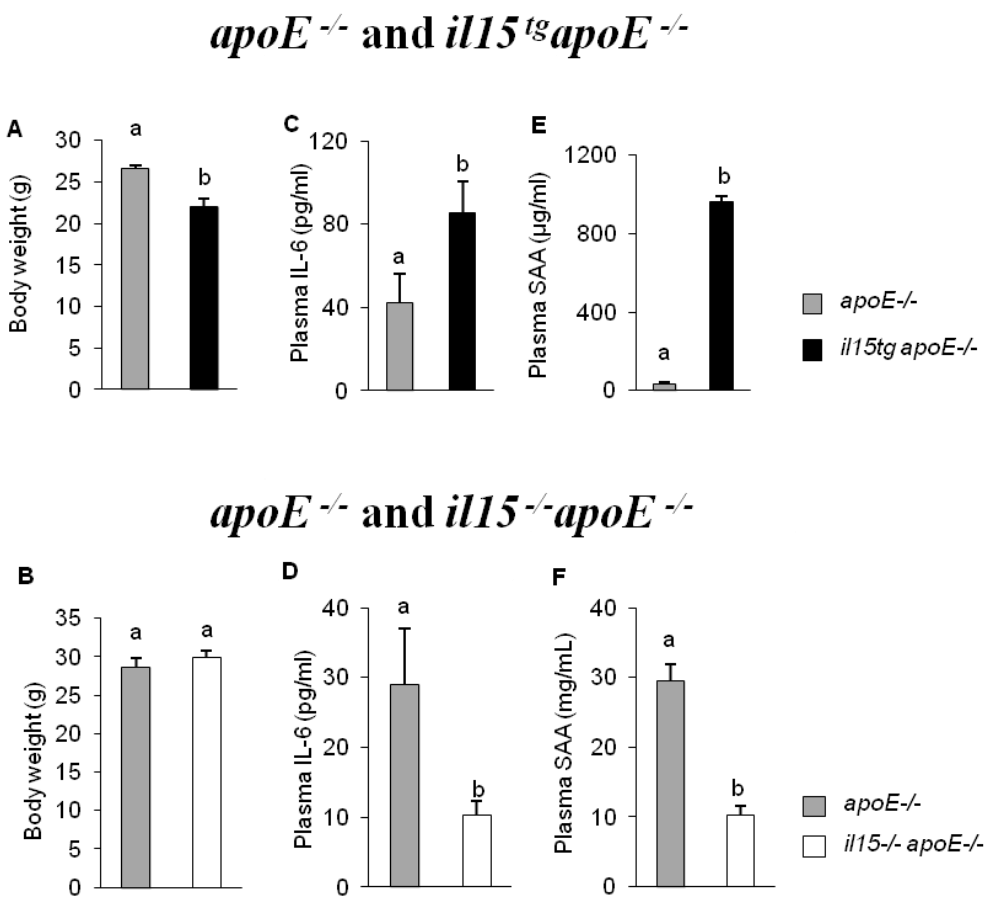
il15$^{+/+}$*apoE*$^{-/-}$ mice as previously reported for otherwise wild type mice [133]. Circulating NKT cells have previously been reported to be low in *apoE*$^{-/-}$ mice.[141] Consistent with this, circulating NKT cells in *il15*$^{+/+}$*apoE*$^{-/-}$ mice were at the lower limit of detection and we did not see further reductions in *il15*$^{+/-}$*apoE*$^{-/-}$ or *il15*$^{-/-}$*apoE*$^{-/-}$ mice (Figure 2.1D). Overexpression of IL-15 increased and knockout of IL-15 substantially reduced levels of circulating monocytes (Figure 2.1 F), without affecting the proportions of monocytes with different cell surface Ly6C levels. Therefore the total numbers of circulating LyC6^{hi} monocytes were correspondingly increased by overexpression and reduced by complete inactivation of IL-15 (Figure 2.1 G). On the other hand, elimination of only one copy of the *il15* gene did not reduce total white blood cell or lymphocyte numbers, or the proportions of monocytes, NK or CD8^{+} T cells in circulation (Figure 2.1A-G).

Figure 2.1 Blood cells levels in apoE-/- mice with different levels of IL-15 expression.

(A, B, and F) Total white blood cell, lymphocyte and monocyte populations were determined using a Hemavet Multi-Species Hematology System (Drew Scientific) (N=6 mice per group). (C) NK, (D) NKT, and (E) CD8+ T cells were analyzed by flow cytometry using CD3 (FITC) and NK1.1 (PE), or CD8 (APC-H7) antibodies. Data represent the averages of 5 mice per group. Ly6Chi monocytes were detected by flow cytometry for Ly6C and CD11b. (G) The total number of Ly6Chi monocytes was calculated by multiplying the proportion of Ly6C hi monocytes by the total number of monocytes. N=6 for all the groups. Error bars indicate SEM. Different lower case letters indicate statistically significant differences ($P<0.05$) as determined by one way ANOVA followed by the Holm-Sidak post-hoc test.


A
Total WBC (Fold Change)
B
Total Lymphocytes (Fold Change)
C
NK cells (% of Lymphocytes)
D
NKT cells (% of Lymphocytes)
E
CD8 T cells (% of Lymphocytes)
F
Total Monocytes (Fold Change)
G
Total Ly6C^{hi} Monocytes (Fold Change)
il15tg apoE-/-
apoE-/-
il15+/- apoE-/-
il15-/-apoE-/-

Supplementary Figure 2.1. Body weights and markers of inflammation. (A, C, and E) Body weights , IL6 and SAA measurements in Male il15 tgapoE -/- and apoE -/- mice. (A) Body weights (P=0.001 by Mann-Whitney Rank Sum Test). (C) Plasma IL-6 (N=6 for both groups) and (E) SAA (N=7 for both groups) were measured by ELISA (P<0.03) determined by Student's T-test and Mann-Whitney Rank Sum Test. Within each panel, different lower case letters indicate statistically significant differences (P<0.05). (B) Body weights were not statistically significant, N=10 for each group. (D) Plasma IL-6 (N=7 for both groups) and (E) SAA (N=13 for both groups) were measured by ELISA. Data in (D) passed analyses for normality and equal variance, and statistical significance was analyzed by Student's T test (P=0.04). Data in C was analyzed using Mann-Whitney Rank Sum Test (P<0.001).


apoE -/- and il15 tg apoE -/-
A
Body weight (g)
30
25
20
15
10
5
0
a
b
C
Plasma IL-6 (pg/ml)
120
80
40
0
a
b
E
Plasma SAA (µg/ml)
1200
800
400
0
a
b
apoE-/-
il15tg apoE-/-
apoE -/- and il15 -/- apoE -/-
B
Body weight (g)
35
30
25
20
15
10
5
0
a
a
D
Plasma IL-6 (pg/ml)
40
30
20
10
0
a
b
F
Plasma SAA (mg/mL)
40
30
20
10
0
a
b
apoE-/-
il15-/- apoE-/-

2.4.2 Effects of IL-15 expression levels on plasma lipids. At 15 weeks of age, male *il15*^{tg} *apoE*$^{-/-}$ mice exhibited a small but statistically significant reduction in body weight compared to control male *apoE*$^{-/-}$ mice (Supplementary Figure 2.1A). In contrast, we detected no differences in body weights in 25 week old *il-15*$^{-/-}$*apoE*$^{-/-}$ or *il-15*$^{+/+}$*apoE*$^{-/-}$ mice (supplementary Figure 2.1B). *il15*^{tg} *apoE*$^{-/-}$ mice exhibited reduced plasma total cholesterol and reduced cholesterol associated with VLDL sized lipoproteins, compared to age and gender matched *apoE*$^{-/-}$ mice (Figure 2.2A-D). Plasma total cholesterol was increased in *il15*$^{-/-}$ *apoE*$^{-/-}$ mice compared to *il15*$^{+/+}$*apoE*$^{-/-}$ controls in both male (Figure 2.2E) and female mice (Supplementary Figure 2.2). The increased plasma total cholesterol in the *il15*$^{-/-}$ *apoE*$^{-/-}$ mice compared to age matched control *il-15*$^{+/+}$*apoE*$^{-/-}$ mice was accounted for mainly by increased cholesterol associated with VLDL sized lipoproteins (Figure 2G, H; Supplementary Figure 2.2). In contrast, plasma cholesterol was not altered in *il-15*$^{+/-}$*apoE*$^{-/-}$ mice compared to *il-15*$^{+/+}$*apoE*$^{-/-}$ controls (Supplementary Figure 2.2). No differences were detected in plasma triglycerides (Figure 2.2B and F; Supplementary Figure 2.2B). Overexpression of IL-15 also resulted in an approximately two-fold increase in plasma IL-6 and an almost 30-fold increase in plasma serum amyloid A (SAA), while *il15*$^{-/-}$ *apoE*$^{-/-}$ mice exhibited statistically significantly (~67%) reduced plasma levels of both IL-6 and SAA compared to age and gender matched control *apoE*$^{-/-}$ mice

(Supplementary Figure 2.1C-F). These results suggest that IL-15 plays important roles in the regulation of lipoprotein cholesterol and systemic inflammation in *apoE*$^{-/-}$ mice.

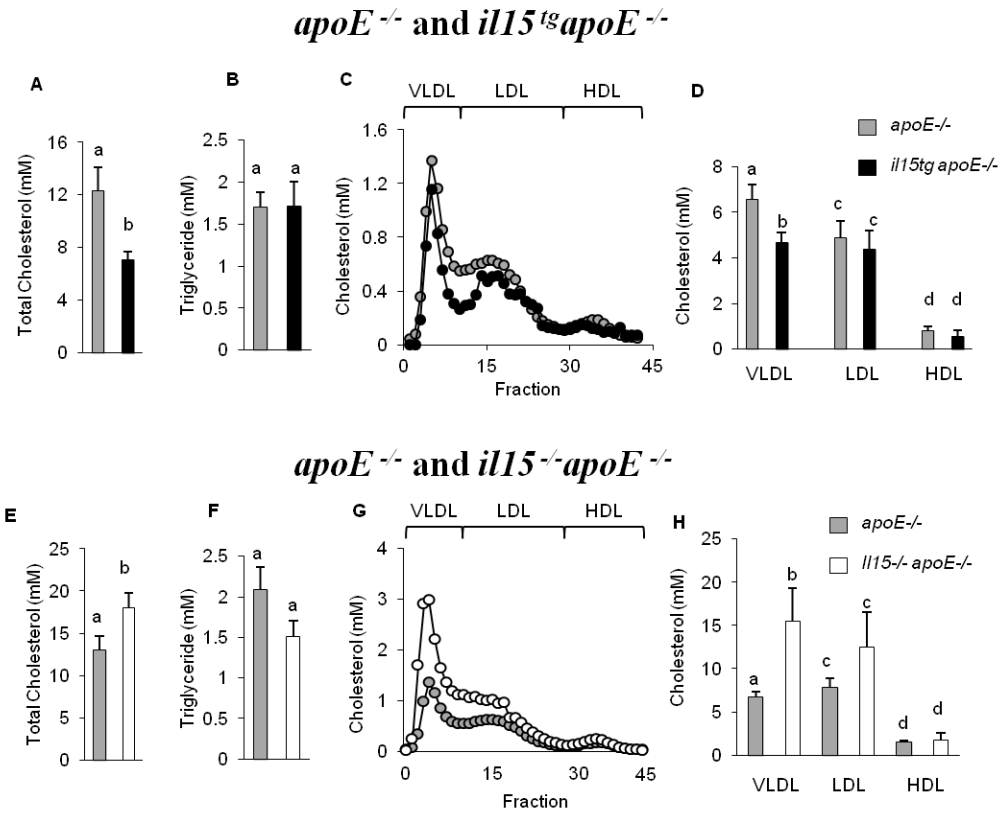
2.4.3 Overexpression of IL-15 promotes while IL-15 deficiency reduces atherosclerosis in apoE-/- mice. To begin to test the effects of IL-15 on atherosclerosis, we first analyzed aortic sinus atherosclerosis in male *il15*^{tg}*apoE*$^{-/-}$ or control *apoE*$^{-/-}$ mice aged 15 weeks. Control male *apoE*$^{-/-}$ mice exhibited small lipid rich lesions (mainly fatty streaks) in their aortic sinus. *il15*^{tg} *apoE*$^{-/-}$ mice exhibited on average, approximately 2.7-fold larger plaque cross sectional areas than the control *apoE*$^{-/-}$ mice (Figure 2.3A-C). We saw similar results when we examined diet induced fatty streak formation in the aortic sinus of *il15*^{tg} and control wild type C57BL6/J mice that had been fed a high fat/high cholesterol/cholate-containing atherogenic diet for 15 weeks (Supplementary Figure 2.3). These data demonstrated that overexpression of IL-15 could enhance atherosclerosis in mice.

To test if normal levels of IL-15 expression contributed to the formation of atherosclerotic plaques, we tested the effects of knocking out IL-15 on atherosclerosis in *apoE*$^{-/-}$ mice. At 25 weeks of age, male *apoE*$^{-/-}$ mice developed extensive atherosclerotic plaques in their aortic sinuses (Figure 2.3D). In contrast, 25 week old male *il15*$^{-/-}$ *apoE*$^{-/-}$ mice developed

approximately 75 % smaller atherosclerotic plaques (Figure 2.3E, F). Elimination of IL-15 had similar effects in female mice, reducing atherosclerotic plaque size in the aortic sinus by approximately 75 % (see Figure 2.5A). The percentage of the inner surface of the aorta covered by atherosclerotic plaques was also reduced (approximately 50 %) in both female and male *il-15*$^{-/-}$*apoE*$^{-/-}$ mice compared to control *il-15*$^{+/+}$*apoE*$^{-/-}$ *mice* (Figure 2.3G).

Figure 2.2. Effects of altered IL-15 expression on plasma lipids.

(A-D) Male il15 tgapoE -/- and apoE -/- littermates at 15 weeks of age and fed a normal chow diet, were analyzed. (A) Total cholesterol (Mann-Whitney Rank Sum Test; p=0.02) and (B) triglycerides were measured in plasma collected from fasted mice. (N=9 for il15 tgapoE -/- and N=11 for apoE -/- mice). Representative plasma lipoprotein profiles (C) and average (± SEM) amounts of cholesterol associated with fractions containing VLDL-, LDL- and HDL-sized lipoproteins (D). Data in D passed analyses for normality and equal variance, and statistical significance was analyzed by Student's T test (P =0.039). N=4 for il15 tgapoE -/- and N=6 for apoE -/- groups. (E-H) Male apoE -/- and il15 -/-apoE -/- at 25 weeks of age on normal chow diet were analyzed. (E) Plasma total cholesterol and (F) triglycerides for N=8 mice per each group (data passed analyses for normality and equal variance, and statistical significance was analyzed by Student's T test). Representative plasma lipoprotein profiles (G) and average (± SEM) amounts of cholesterol associated with fractions containing VLDL-, LDL- and HDL-sized lipoproteins (H) for N=4 mice per group. Data in H passed analyses for normality and equal variance, and statistical significance was analyzed by Student's T test. Different lower case letters indicate statistically significant differences.


apoE -/- and il15 tg apoE -/-
A
B
C
D
Total Cholesterol (mM)
Triglyceride (mM)
Cholesterol (mM)
VLDL
LDL
HDL
Fraction
apoE-/-
il15tg apoE-/-
apoE -/- and il15 -/- apoE -/-
E
F
G
H
Total Cholesterol (mM)
Triglyceride (mM)
Cholesterol (mM)
VLDL
LDL
HDL
Fraction
apoE-/-
Il15-/- apoE-/-

Supplementary Figure 2.2. Lipids measurement in female 25 week old mice.

(A) Plasma total cholesterol and (B) triglycerides were measured in female apoE-/- , il15+/- apoE-/- and il15-/-apoE-/- for N=8 mice per each group (data passed analyses for normality and equal variance, and statistical significance was analyzed by Student's T test). Representative plasma lipoprotein profiles (C) and average (± SEM) amounts of cholesterol associated with fractions containing VLDL-, LDL- and HDL-sized lipoproteins (D) for N=4 mice per group in apoE-/- and il15-/-apoE-/- . Data in D passed analyses for normality and equal variance, and statistical significance was analyzed by Student's T test. Different lower case letters indicate statistically significant differences.

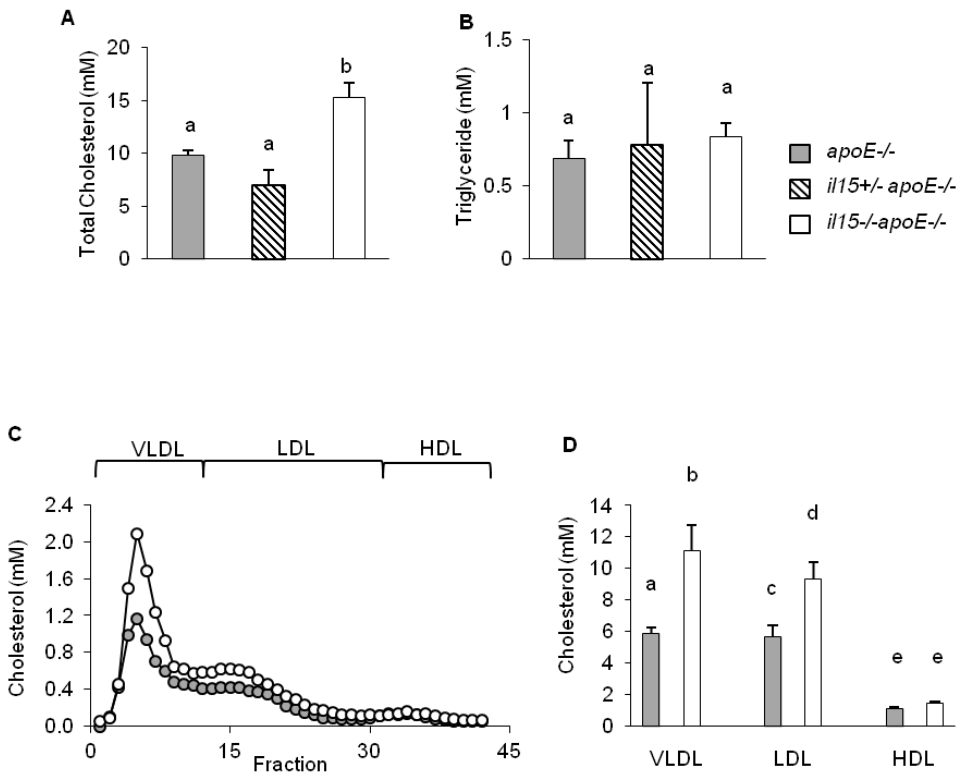
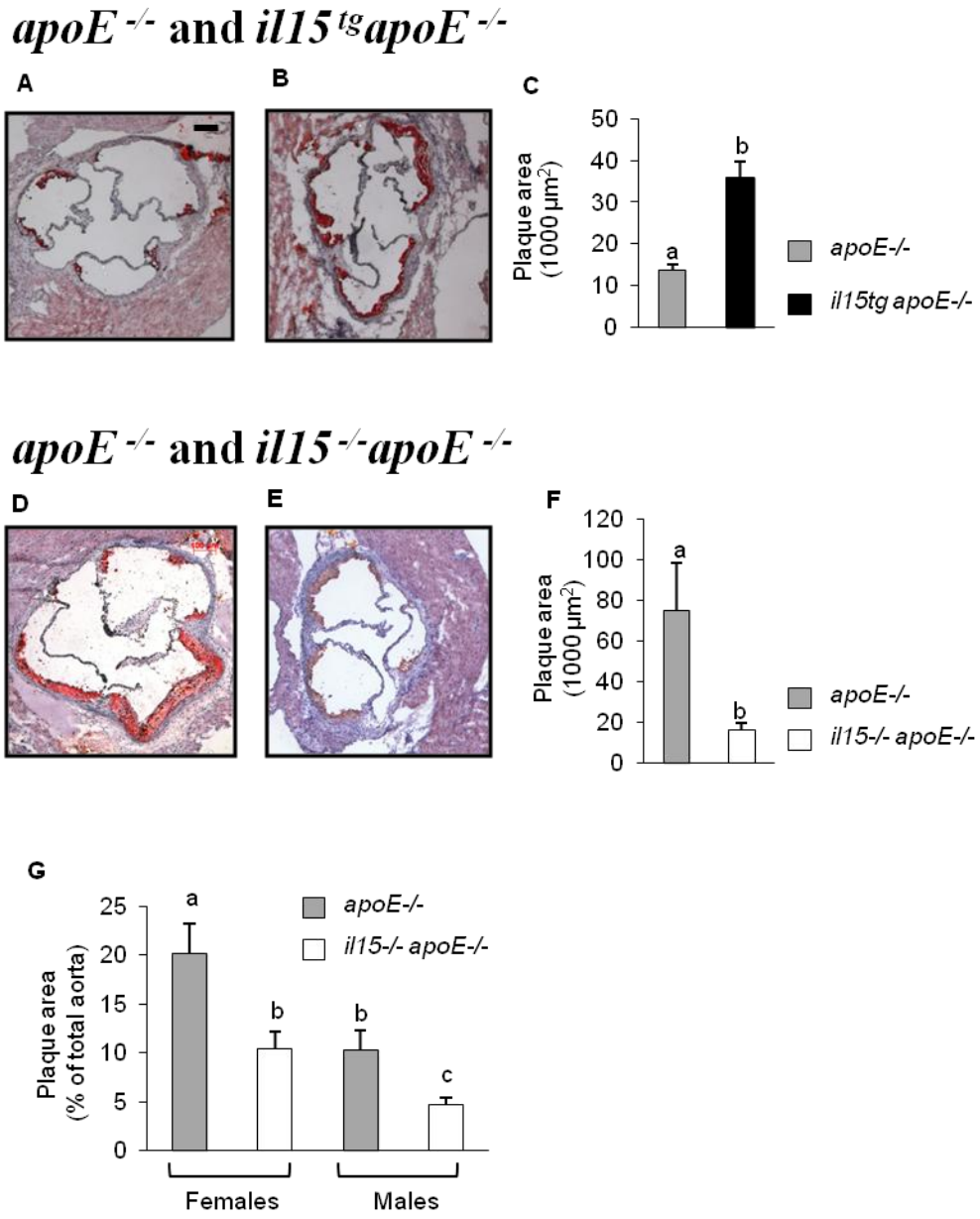
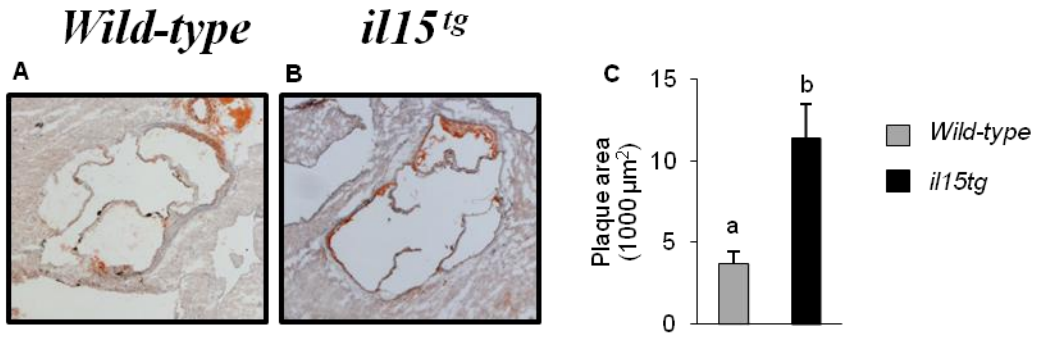

A
Total Cholesterol (mM)
20
15
10
5
0
a
a
b
B
Triglyceride (mM)
1.5
1
0.5
0
a
a
a
apoE-/-
il15+/- apoE-/-
il15-/-apoE-/-
C
VLDL
LDL
HDL
Cholesterol (mM)
2.4
2.0
1.6
1.2
0.8
0.4
0.0
0
15
30
45
Fraction
D
Cholesterol (mM)
14
12
10
8
6
4
2
0
a
b
c
d
e
e
VLDL
LDL
HDL

Figure 2.3. Effects of altered IL-15 levels on atherosclerosis in apoE-/- mice. A-C) Atherosclerotic plaques in the aortic roots were identified oil red O/ hematoxylin staining. Representative images for apoE -/- (A) and il15 tgapoE -/- (B) mice are shown, and average plaque sizes were quantified (C) (N= 9 mice per group). Data are averages ± SEM. P=0.003 by the Mann Whitney rank sum test. (D-F) Male 25 week old mice fed normal chow were analyzed. Representative oil red O stained images of atherosclerotic plaques in the aortic roots of (D) apoE -/- and (E) il15 -/- apoE -/- mice. F) Atherosclerotic plaque sizes were quantified for N=10 mice per group. Data are averages ± SEM. Different lower case letters indicate statistically significant differences (P<0.001, determined by the Mann Whitney rank sum test). (G) Atherosclerosis on the surface of descending aorta were stained using Sudan IV in both 25 week old males and females. The area of Sudan IV staining normalized to the total area of the aorta was taken as a measure of the atherosclerotic plaque area. (F and I) Average± SEM plaque areas for N=8 per group . Lower case letters indicate statistically significant differences. P<0.05 by the Mann-Whitney Rank Sum Test.


apoE -/- and il15 tg apoE -/-
A
B
C
Plaque area (1000 µm2)
50
40
30
20
10
0
a
b
apoE-/-
il15tg apoE-/-
apoE -/- and il15 -/- apoE -/-
D
E
F
Plaque area (1000 µm2)
120
100
80
60
40
20
0
a
b
apoE-/-
il15-/- apoE-/-
G
Plaque area (% of total aorta)
25
20
15
10
5
0
a
b
b
c
apoE-/-
il15-/- apoE-/-
Females
Males

Supplementary Figure 2.3. Overexpression of IL-15 promotes diet induced fatty streak formation.

12 week old female A) C57BL6 or B) *il15*^{tg} mice were fed a high fat (15.8%), high cholesterol (1.25%), sodium cholate (0.5%) containing diet for 15 weeks. Mice were harvested at 27 weeks of age. C) Average± SEM atherosclerotic plaque sizes for N=10 C57BL6 and N=6 *il15*^{tg} mice (P=0.006 using Mann-Whitney Rank Sum Test).


Wild-type
il15tg
A
B
C
15
10
5
0
Plaque area (1000 µm²)
a
b
Wild-type
il15tg

2.4.4 Abundance of CD8+ cells in atherosclerotic plaques is affected by IL-15. CD8 T cells accumulate in atherosclerotic plaques and promote plaque formation[64]. Immunostaining revealed abundant $CD8^+$ cells in atherosclerotic plaques from apoE KO mice (Figure 2.4A, D). Overexpression of IL-15 increased (Figure 2.4B,C) while complete inactivation of IL-15 dramatically reduced (Figure 2.4E, F) the numbers of $CD8^+$ cells in atherosclerotic plaques compared to age and gender matched control *apoE*$^{-/-}$ mice. These findings are consistent with the expansion of CD8 T cell populations in IL-15 overexpressing mice and reduced levels of CD8 T cells in mice lacking IL-15 (Figure 2.1E).

2.4.5 IL-15 has NK, NKT and CD8+ T cell independent effects on atherosclerosis. As observed for male mice, elimination of both copies of IL-15 in 25 week old female *apoE*$^{-/-}$ mice resulted in a significant 75 % reduction in atherosclerotic plaque size (Figure 2.5A). To examine if a partial reduction of IL-15 affected atherosclerosis, we compared atherosclerotic plaque sizes in *il15*$^{+/-}$*apoE*$^{-/-}$ and age and gender matched *il15*$^{+/+}$*apoE*$^{-/-}$ mice (Figure 2.5A). Mice lacking only one copy of the IL-15 gene exhibited a significant, 35 % reduction in the size of atherosclerotic plaques in the aortic sinus compared to mice with normal IL-15 expression (Figure 2.5A). However elimination of one copy of IL-15 did not significantly alter numbers of circulating NK or $CD8^+$T cells or monocytes

(Figure 2.1C, E,F), suggesting that the effects of IL-15 on atherosclerosis extend beyond regulation of the amounts of these pro-atherogenic cell types in circulation. To confirm this we sought to test whether elimination of one copy of the IL-15 gene had the same atheroprotective effect in the absence of circulating NK, NKT and $CD8^+$T cells. We did this by immuno-depleting $NK1.1^+$ cells from *il15*$^{+/+}$*apoE*$^{-/-}$ and *il15*$^{+/-}$ *apoE*$^{-/-}$ mice using the anti-NK1.1 mAb, since NK1.1 is found on NK cells, most NKT cells and activated CD8T cells [142]. We began treatments in 8 week old mice because we found, in a separate experiment, that female *apoE*$^{-/-}$ mice at that age exhibited little atherosclerosis in their aortic sinus (data not shown). Treatments continued for 7 weeks, until the mice were 15 weeks of age. Therefore under these conditions, atherosclerosis development occurred in the absence of $NK1.1^+$ cells, as confirmed by flow cytometric analysis (Supplementary Figure 2.4A, B). Comparison of atherosclerosis in the NK1.1 immunodepleted *il15*$^{+/+}$*apoE*$^{-/-}$ and *il15*$^{+/-}$ *apoE*$^{-/-}$ mice revealed that even in the absence of $NK1.1^+$ cells, elimination of one copy of the IL-15 gene significantly reduced atherosclerosis by 40 % (Figure 2.5 B). Immuno-depletion of $NK1.1^+$ cells did not affect plasma lipoprotein cholesterol profiles or plasma levels of SAA (Supplementary figure 2.4C, D) but it did drastically reduce immunodetectable $CD8^+$ cells in atherosclerotic plaques when compared to reference saline-treated *apoE*$^{-/-}$ mice (Supplementary Figure 2.4E). The reduced plaque sizes observed in

apoE$^{-/-}$ mice treated with the anti-NK1.1 antibody, compared to reference saline treated *apoE*$^{-/-}$ mice are consistent with previous reports that NK/NKT cell function and CD8^{+}T cells contribute to atherosclerotic plaque development[23, 143,64] . The observation that atherosclerosis was reduced in *il15*$^{+/-}$*apoE*$^{-/-}$ mice compared to control *il15*$^{+/+}$*apoE*$^{-/-}$ mice when both were immunodepleted of NK1.1+ cells for the duration of atherosclerosis development demonstrates that IL-15 has pro-atherogenic effects that are independent of NK1.1 expressing NK, NKT and CD8^{+} T cells.

Figure 2.4. IL-15 expression affects CD8+ cell presence in atherosclerotic plaques. (A-C) Atherosclerotic plaques from 15 week old male *apoE* $^{-/-}$ (A) and *il15*^{tg}*apoE* $^{-/-}$ (B) mice were stained for CD8^{+} cells. C) Average numbers of CD8+ cells normalized to plaque area was determined for N=4 mice per group. Different lower case letters in panel C designate statistically significant differences (P=0.029) determined by theMann-Whitney Rank Sum Test. Representative images of CD8^{+} cell staining in atherosclerotic plaques of *apoE* $^{-/-}$ (A) and *il15* $^{-/-}$*apoE* $^{-/-}$ (B) mice. (C) Quantification of CD8+ cell staining in atherosclerotic plaques. N=4 for both groups. Data in C passed analyses for normality and equal variance, and statistical significance was analyzed by Student's T test (P=0.01).

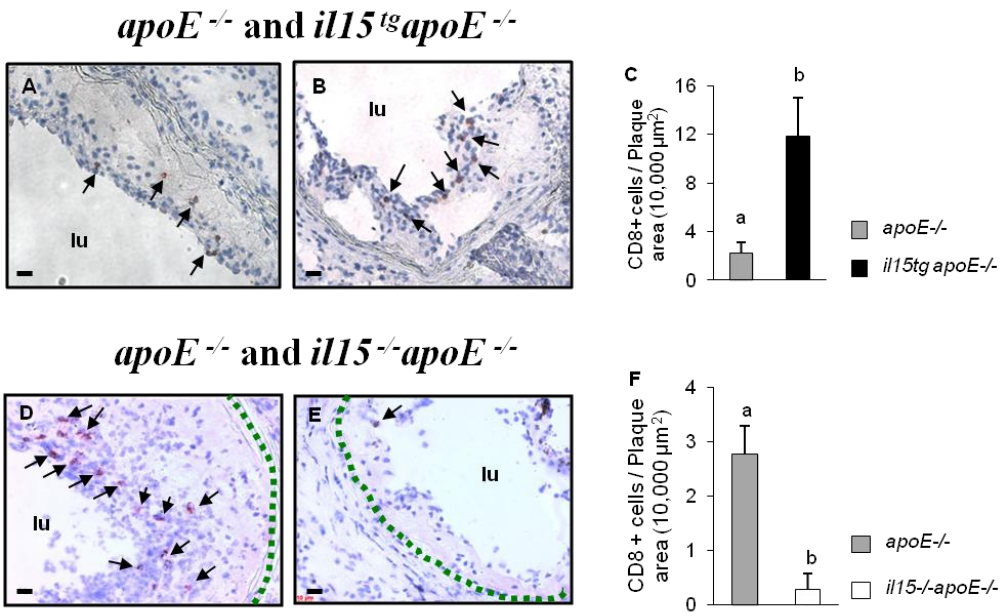
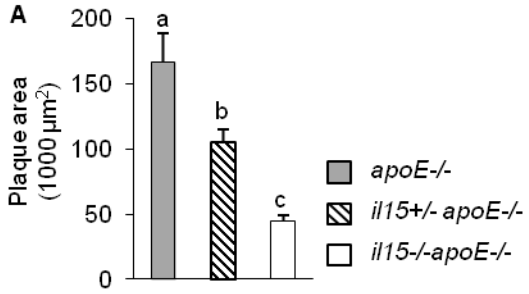
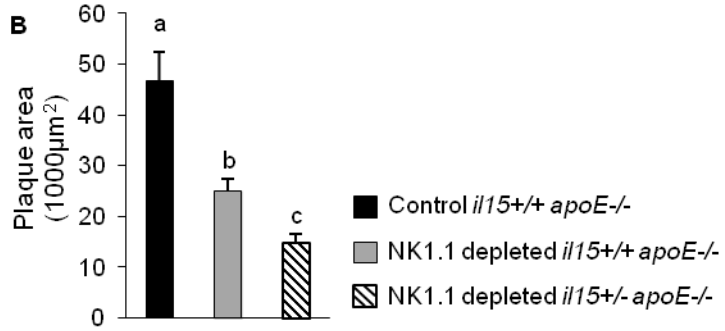
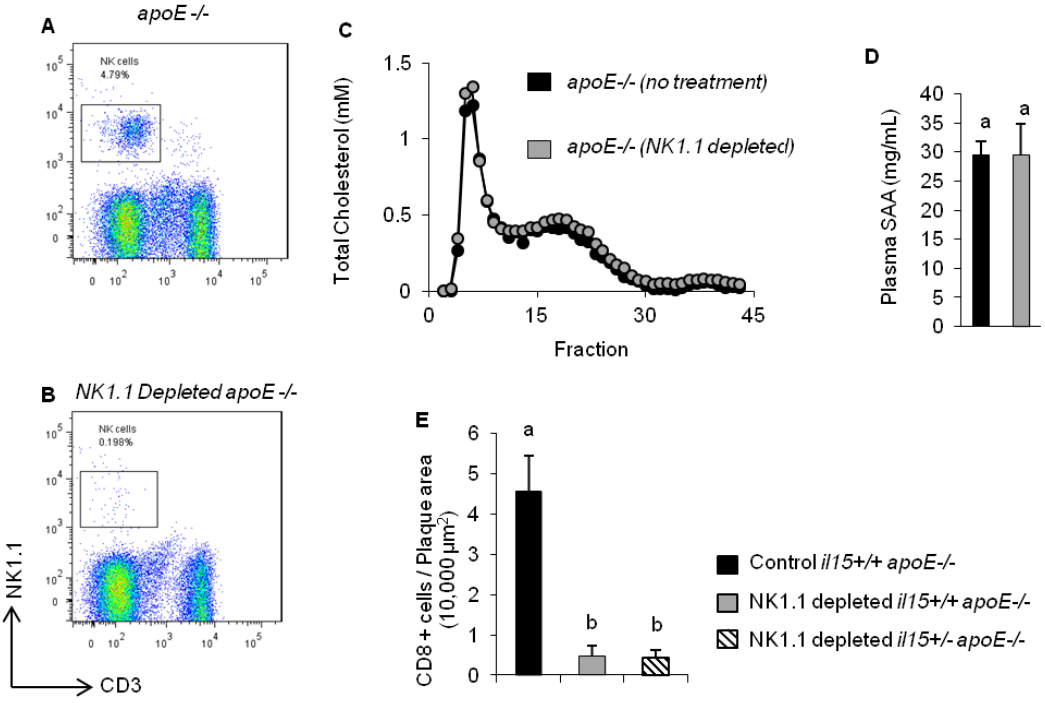

apoE -/- and il15 tg apoE -/-
A
lu
B
lu
C
CD8+ cells / Plaque area (10,000 µm2)
16
12
8
4
0
a
b
apoE-/-
il15tg apoE-/-
apoE -/- and il15 -/- apoE -/-
D
lu
E
lu
F
CD8 + cells / Plaque area (10,000 µm2)
4
3
2
1
0
a
b
apoE-/-
il15-/-apoE-/-

Figure 2.5. Effects of immunodepletion of NK1.1+ cells on atherosclerosis. (A) Atherosclerosis in female 25 week old mice fed normal chow were analyzed in *apoE* $^{-/-}$, *il15* $^{+/-}$*apoE* $^{-/-}$ and *il15* $^{-/-}$*apoE* $^{-/-}$ mice. Atherosclerotic plaque sizes were quantified for N=10 mice per group. Data are averages ± SEM. Different lower case letters indicate statistically significant differences (P<0.001). (B) A*poE* $^{-/-}$ and *il15* $^{+/-}$*apoE* $^{-/-}$ mice were injected without or with NK1.1 monoclonal antibody starting at 8 weeks of age for 7 weeks. Atherosclerotic plaque areas for N=7 mice per group. P=0.01 between *apoE* $^{-/-}$ versus NK1.1 depleted *apoE* $^{-/-}$. P<0.05 between NK1.1 depleted *apoE* $^{-/-}$ versus NK1.1 depleted *il15*$^{+/-}$*apoE* $^{-/-}$ mice. All statistical significances were assessed using Mann-Whitney Rank Sum Test.


A
Plaque area (1000 µm²)
200
150
100
50
0
a
b
c
apoE-/-
il15+/- apoE-/-
il15-/-apoE-/-


B
Plaque area (1000µm²)
60
50
40
30
20
10
0
a
b
c
Control il15+/+ apoE-/-
NK1.1 depleted il15+/+ apoE-/-
NK1.1 depleted il15+/- apoE-/-

Supplementary Figure 2.4. Effects of immunodepletion of NK1.1+ cells in apoE-/- mice. (D and E) Flow cytometry for NK1.1 and CD3 of blood cells from (D) control *apoE*$^{-/-}$ and (E) NK1.1 immunodepleted *apoE*$^{-/-}$ mice (representative profiles shown; boxed area indicates NK1.1^{+} CD3^{-}, NK cells). (C) Plasma samples were collected from *apoE* $^{-/-}$ with no treatment and *apoE* $^{-/-}$ treated with NK1.1 depleting antibody. Plasma samples from three mice per group were pooled and lipoprotein fractions were separated by FPLC and analyzed for cholesterol content. Representative profiles are shown. (D) SAA levels in plasma were measured by ELISA for N=7 mice per group. SAA levels were not statistically significantly different (Mann Whitney Rank Sum Test). (E) CD8^{+} cells in plaques were detected by immunohistochemistry and quantified (N=4 mice per group). P=0.02 between *apoE* $^{-/-}$ (no treatment) versus the other two groups (NK1.1 depleted mice). Different lower case letters indicate statistically significant differences (P<0.05). All statistical significances were assessed using Mann-Whitney Rank Sum Test.


A
apoE -/-
NK cells
4.79%
B
NK1.1 Depleted apoE -/-
NK cells
0.198%
NK1.1
CD3
C
Total Cholesterol (mM)
apoE-/- (no treatment)
apoE-/- (NK1.1 depleted)
Fraction
D
Plasma SAA (mg/mL)
a
a
E
CD8+ cells / Plaque area (10,000 µm²)
a
b
b
Control il15+/+ apoE-/-
NK1.1 depleted il15+/+ apoE-/-
NK1.1 depleted il15+/- apoE-/-

2.4.6 IL-15 stimulates macrophage activation and foam cell formation. It has previously been reported that IL-15 can directly activate myeloid cells including macrophages to produce the chemokine RANTES[87], suggesting a role for IL-15 in macrophage activation. Consistent with this, we observed that murine RAW264.7 macrophage-like cells treated with recombinant murine (rm) IL-15 secreted increased levels of RANTES into culture medium (Figure 6A). In addition, rmIL-15-treated cells exhibited increased iNOS activity, and IL-6 and MCP-1 gene expression compared to control untreated cells (Figure 2.6B-D). As has been previously reported for IL-15 stimulated RANTES production, these effects were all attenuated by co-incubation with the soluble form of recombinant IL-15 receptor α, suggesting that they involve a pathway similar to that previously described for IL-15 induced RANTES production by macrophages. Resident mouse peritoneal macrophages isolated from wild type mice and treated with rmIL-15 in culture also showed increased levels of expression of iNOS, IL-6, MCP-1 as well as IL-1β and TNFα in an IL-15 concentration dependent manner (Figure 2.6E-I). When resident peritoneal macrophages isolated from wild type mice were pre-treated with rmIL-15 for 24 hrs and then challenged with acetyl-LDL for a further 8 hrs, we observed a 2-fold increase in neutral lipid accumulation, as measured by oil red O staining, compared to control cells incubated without added IL-15 (Figure 6J-N). This indicated that in addition to stimulating

macrophage activation, IL-15 also stimulates macrophage foam cell formation in vitro.

2.4.7 Reduction of IL-15 lowers MCP-1 levels in atherosclerotic plaques. We therefore sought to determine whether altered levels of IL-15 affected local inflammation in atherosclerotic plaques in vivo. We used immunofluorescence to determine the levels of MCP-1 in atherosclerotic plaques from *apoE*$^{-/-}$ mice with reduced levels of IL-15. *Il15*$^{-/-}$*apoE*$^{-/-}$ mice, completely lacking IL-15, exhibited an approximate 60 % reduction in the levels of immunodectable MCP-1 in atherosclerotic plaques (when normalized to plaque size) compared to those in *apoE*$^{-/-}$ mice (Figure 2.7A-C). Consistent with the reduction in MCP-1 levels in atherosclerotic plaques and with the reduced levels of circulating monocytes (Figure 2.1F), atherosclerotic plaques from *il15*$^{-/-}$*apoE*$^{-/-}$ mice also exhibited substantially reduced staining for CD11b, a marker of monocytic cells, (Figure 2.7 D-F) when compared to those from *apoE*$^{-/-}$ mice. Therefore, the complete absence of IL-15 is associated with reduced abundance of monocytic cells in atherosclerotic plaques, consistent with the reduced levels of MCP-1 within plaques and reduced numbers of monocytes in circulation. In a separate experiment, we also compared the levels of MCP-1 within atherosclerotic plaques in *il15*$^{+/-}$*apoE*$^{-/-}$ and *il15*$^{+/+}$*apoE*$^{-/-}$ mice. Mice lacking only one copy of the *il15* gene exhibited a statistically

significant 30 % reduction in the level of immunodetectable MCP-1 within atherosclerotic plaques (normalized to plaque size) when compared to *apoE*$^{-/-}$ mice with two intact copies of the *il15* gene (Figure 2.7 G-I). CD11b immunostaining in atherosclerotic plaques, however, was not statistically significantly different between *il15*$^{+/-}$*apoE*$^{-/-}$ and *il15*$^{+/+}$*apoE*$^{-/-}$ mice (Figure 2.7 J-L), consistent with the observation that deletion of only one copy of the il-15 gene in *il15*$^{+/-}$*apoE*$^{-/-}$ mice did not result in reduced numbers of circulating monocytes (Figure 2.1F). Together these results suggest that reduction of IL-15 levels leads to reduced local inflammation within atherosclerotic plaques, independent of reductions in the numbers of monocytic cells within atherosclerotic plaques, and are consistent with our findings that IL-15 induces cytokine production and increases foam cell formation in macrophages in vitro.

Figure 2.6. The impact of rIL-15 on macrophage activation and foam cell formation. (A-D) RAW 264.7 cells were incubated with or without rIL-15 (100 ng/ml) in the presence or absence of sIL-15Rα (200 ng/ml). After 24 hours, (A) RANTES and (B) nitrite levels were measured in the supernatant, and gene expression of (C) IL-6 and (D) MCP-1 were analyzed. (E-I) Resident peritoneal macrophages cells were incubated without or with different concentrations of rIL-15 (25, 100, and 500 ng/ml). After 4 hours, gene expression of (E) TNFα, (F) IL-1β, (G) iNOS, (H) IL-6, and (I) MCP-1 were analyzed. (J-N) Resident peritoneal macrophages collected from C57BL6 mice were cultured in the absence or presence of rIL-15 (250ng/ml) for 24 hours followed by 100 µg/ml AcLDL for 8 hours. Cells were fixed and stained with oil red O (red) and DAPI (blue). (J-M) Representative fluorescence images of untreated cells (J), or cells treated with IL-15 alone (K), only AcLDL (L), or both IL-15 and AcLDL (M). (N) Quantification of lipid accumulation was conducted by normalizing the oil red O staining intensity by the number of DAPI stained nuclei in several images per well for three wells per condition. Different lower case letters in indicate statistically significant differences ($P < 0.001$ determined by one way ANOVA followed by the Holm-Sidak post-hoc test.

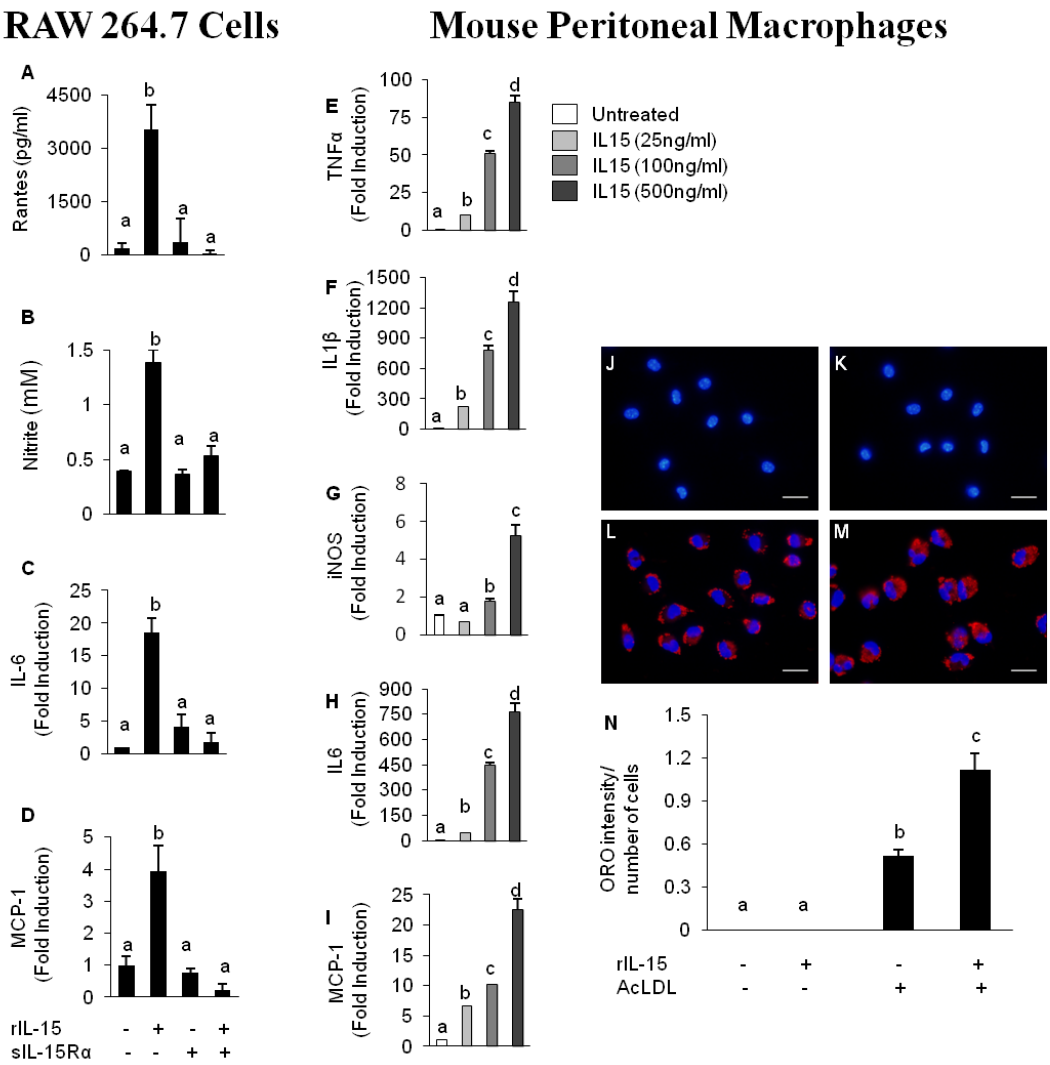
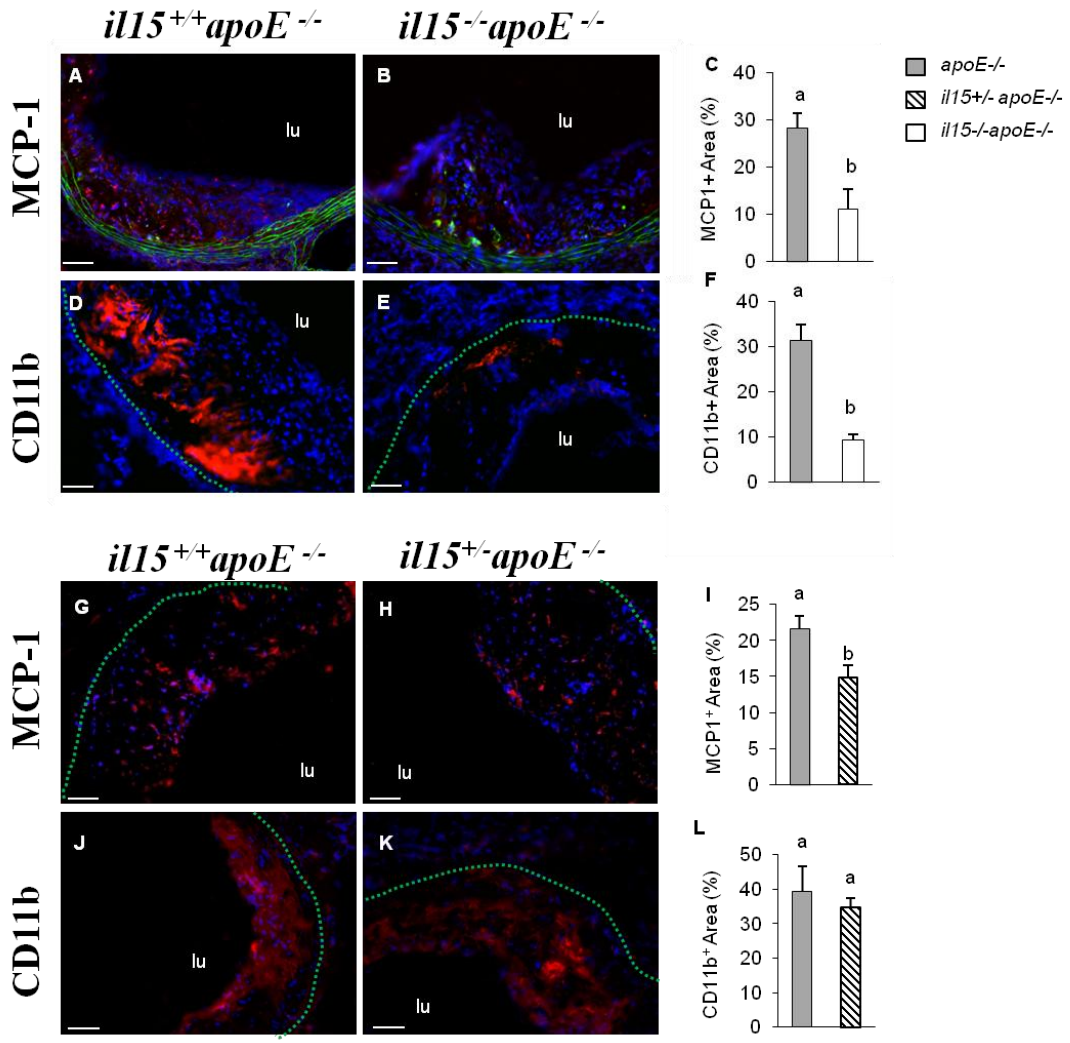

RAW 264.7 Cells
Mouse Peritoneal Macrophages
A
Rantes (pg/ml)
B
Nitrite (mM)
C
IL-6 (Fold Induction)
D
MCP-1 (Fold Induction)
rIL-15
sIL-15Rα
E
TNFα (Fold Induction)
F
IL1β (Fold Induction)
G
iNOS (Fold Induction)
H
IL6 (Fold Induction)
I
MCP-1 (Fold Induction)
Untreated
IL15 (25ng/ml)
IL15 (100ng/ml)
IL15 (500ng/ml)
J
K
L
M
N
ORO intensity/ number of cells
rIL-15
AcLDL

Figure 2.7. Effects of altered IL-15 expression on MCP-1 and CD11b^{+} cells in atherosclerotic plaques. (A-C) MCP-1 expression (red) in plaques from 25 week old female *apoE* $^{-/-}$ (A) and *il15* $^{-/-}$*apoE* $^{-/-}$ (B) mice was quantified (C) and normalized to the total plaque area. Nuclei were stained by DAPI (blue). (D-F) CD11b+ area (red) in plaques from *apoE* $^{-/-}$ (D) and *il15* $^{-/-}$*apoE* $^{-/-}$ (E) mice was quantified (F) and normalized to the total plaque area. Nuclei were stained by DAPI (blue). Data in C and F represent averages ± SEM of N=7 samples per group. In a separate experiment MCP-1 (G-I) and CD11b+ (J-L) were detected in 25 week old female *apoE* $^{-/-}$ (G and J) and *il15*$^{+/-}$*apoE* $^{-/-}$ (H and K) mice. Data in I and L represent averages ± SEM of N=6 samples per group. Data was subjected to the Mann-Whitney Rank Sum test. Different lower case letters in each panel indicate statistically significant differences ($P<0.02$).


il15+/+apoE-/-
il15-/-apoE-/-
MCP-1
CD11b
A
B
C
D
E
F
lu
apoE-/-
il15+/- apoE-/-
il15-/-apoE-/-
MCP1+ Area (%)
CD11b+ Area (%)
a
b
il15+/+apoE-/-
il15+/-apoE-/-
G
H
I
J
K
L
MCP1+ Area (%)
CD11b+ Area (%)

2.5 Discussion:

Atherosclerosis is an inflammatory disorder caused largely by dysregulation of lipid metabolism and immune responses. In recent years, published evidence suggests that regulating the inflammatory responses may be effective in delaying atherosclerosis development [2]. IL-15 is a known immune-mediator that is present in atherosclerotic plaques[98-100], and hence could play a role in atherosclerosis development. It has been reported that antibody mediated blockade of IL-15 enhanced neo-intima formation in carotid arteries of C57BL6 mice in response to injury mediated by perivascular placement of a non-occlusive collar around the carotid artery[135]. In that study, IL-15 treatment of murine aortic smooth muscle cells in culture reduced serum induced proliferation and expression of CX3CR1 and CX3CL1, whereas antibody mediated blockade of IL-15 in injured carotid arteries increased CX3CR1 and CX3CL1[135] . That study, therefore, suggested that IL-15 normally attenuates smooth muscle mediated neo-intima formation in response to arterial injury and therefore may protect against atherosclerosis. On the other hand infection of *ldlr* $^{-/-}$ mice with S. typhimurium bacteria harbouring a mammalian expression plasmid containing a recombinant IL-15 cDNA, protected them against diet induced carotid artery atherosclerosis in response to perivascular cuff placement [101]. However this could have been secondary to the reported immune mediated elimination of IL-15

expressing cells, including macrophages, rather than reflecting effects of reduced IL-15[101]. Thus, the direct role of IL-15 in development of lipid rich atheromatous plaques remained ulclear.

To address the direct role of IL-15 in atherosclerosis development, we tested the effects of overexpression or elimination of one or both copies of the *il15* gene in the apoE deficient mouse model of spontaneous atherosclerosis. Here we provide mechanistic evidence that IL-15 is upstream of many inflammatory responses that impact atherosclerosis development and promotes atherosclerosis through multiple pathways. IL-15 has recently been shown to directly activate macrophage production of the chemokine RANTES via a pathway involving IL-15 Rα and NF-κB signaling [87]. Others have demonstrated that treatment of mouse RAW 264.7 macrophage like cells with IL-15 induced TNFα, IL-1β, CXCL1, CCL2 (MCP-1) and CCR2)[101] . Likewise, we show that in addition to stimulating increased RANTES, MCP-1, TNFα and IL-1β, IL-15 treatment of murine RAW 264.7 cells and peritoneal macrophages increased IL-6 expression and iNOS expression and activity, and that these were suppressed by soluble recombinant IL-15Rα (Figure 2.6), suggesting that they may involve a pathway similar to that described for IL-15 induced RANTES production[87]. Consistent with these effects of IL-15 on macrophage activation, IL-15 pre-treatment of resting peritoneal macrophages isolated from wild type mice resulted in increased acetyl-

LDL mediated foam cell formation (Figure 2.6J-N). Increased macrophage activation and foam cell formation induced by IL-15 suggest that it may have pro-atherogenic effects. We demonstrated that overexpression of IL-15 increases while elimination of IL-15 reduces atherosclerosis development in apoE deficient mice (Figures 2.3 and 2.5). These effects on atherosclerosis occur despite opposing effects on levels of cholesterol associated with atherogenic VLDL- and LDL-sized lipoproteins (Figure 2 and Supplementary Figure 2.2). The hypercholesterolemic effects of eliminating IL-15 were not replicated by immunodepletion of $NK1.1^+$ cells (Supplementary Figure 2.4), suggesting that they were not mediated by the loss of NK/NKT cells or activated $CD8^+$ T cells. Eliminating only one copy of the IL-15 gene did not affect steady state plasma cholesterol levels (Supplementary Figure 2.2A). Similarly, vaccination of LDL receptor deficient mice against IL-15, which was reported to reduce diet induced carotid artery injury mediated atherosclerosis, did not affect plasma cholesterol levels[101], suggesting the possibility that vaccination may not have resulted in complete elimination of IL-15. The mechanism by which eliminating IL-15 increased and overexpressing IL-15 reduced lipoprotein cholesterol remains unclear. However the hypercholesterolemic effects of eliminating IL-15 are reminiscent of other reports of increased levels of plasma and/or lipoprotein associated cholesterol/triglyceride in *mcp1* $^{-/-}$ [38],

il6$^{-/-}$ [107], *mcsf* $^{-/-}$[45], and *ifnγr*$^{-/-}$ [25] mice , consistent with a general role for cytokine signaling in lipid metabolism.

It has previously been shown that overexpression of IL-15 reduces while elimination of IL-15 increases adipose tissue mass and body weight in mice that express wild-type levels of apoE [97]. In our experiments, IL-15 overexpression was associated with only a slight reduction and elimination of IL-15 with no change in body weights of *apoE* $^{-/-}$ mice (Supplementary Figure 2.1A, B). This is consistent with the reported resistance of *apoE* $^{-/-}$ mice to obesity[144, 145] and afforded us the ability to examine the effects of IL-15 on atherosclerosis independent of any effects on obesity.

IL-15 is essential for the survival of NK and NKT cells and promotes the survival of $CD8^+$ T cells[22, 77]. Consistent with this, overexpression of IL-15 increased numbers of circulating NK, NKT and $CD8^+$ T cells while complete knockout of IL-15 virtually eliminated circulating NK cells and reduced $CD8^+$ T cells (Figure 2.1). NK and NKT cell function has previously been shown to promote atherosclerosis development in mice[23, 143]. We immuno-depleted NK and NKT cells from *apoE* $^{-/-}$ mice starting at 8 weeks of age (when we found little detectable atherosclerosis in their aortic sinus — data not shown) and continuing to 15 weeks of age when sizable plaques are expected to develop in *apoE* $^{-/-}$ mice (see Figure 2.4B, control). Thus atherosclerosis development occurred under conditions of virtually no circulating $NK1.1^+$ cells (Supplementary Figure 2.4B). We

found that NK1.1 mAb treatment resulted in a 33 % reduction of atherosclerotic plaque size when compared to reference, untreated *apoE*$^{-/-}$ mice (Figure 2.4B), consistent with previous reports that NK and NKT cell function promoted atherosclerosis[23, 143]. Furthermore, plaques from NK1.1 mAb treated mice were virtually devoid of $CD8^+$ T cells (Supplementary Figure 2.4E), as was observed for plaques from *il15* $^{-/-}$ *apoE* $^{-/-}$ mice (Figure 2.4D-F). However complete elimination of IL-15, which led to similar reductions in NK and $CD8^+$T cells, resulted in a much greater, 75 % reduction in atherosclerosis (Figure 2.3 D-F and Figure 2.5A), suggesting that IL-15 exerts NK/NKT and $CD8^+$ T cell independent effects on atherosclerosis. Consistent with this, elimination of 1 copy of the IL-15 gene in heterozygous mice, reduced atherosclerosis by 35-40 % both in untreated mice (in which NK or $CD8^+$T cell numbers in circulation were not affected) and in NK1.1+ cell immuno-depleted mice, demonstrating that even when detectable circulating NK or NKT cells, or CD8+ cells within plaques, were absent, reducing IL-15 levels still significantly reduced atherosclerosis.

Complete knockout of IL-15 resulted in reduced levels of circulating monocytes, including total numbers (but not proportions) of $Ly6C^{hi}$ monocytes which have previously been reported to be preferentially recruited to sites of inflammation including atherosclerotic plaques [3, 146]. Indeed we observed reduced immunostaining for the monocyte marker

CD11b in plaques from *il15* $^{-/-}$ *apoE* $^{-/-}$ mice compared to age matched *apoE* $^{-/-}$ controls. In contrast, elimination of only one copy of IL-15 in *il15* $^{+/-}$ *apoE* $^{-/-}$ mice reduced atherosclerosis by 35-40 % compared to control *apoE* $^{-/-}$ despite having no effect on circulating monocyte levels. Therefore IL-15 can influence atherosclerosis via pathways other than regulation of the numbers of pro-inflammatory monocytes, NK or NKT cells in circulation or $CD8^+$ cells in plaques. Instead, we found that atherosclerotic plaques from *il-15*$^{+/-}$*apoE*$^{-/-}$ or *il-15*$^{-/-}$*apoE*$^{-/-}$ mice had reduced levels of MCP-1 immunostaining compared to control plaques from *apoE*$^{-/-}$ mice supporting a role for IL-15 in regulation of local inflammation in atherosclerotic plaques.

In conclusion, we have demonstrated that elimination of IL-15 reduces atherosclerosis through effects on multiple inflammatory pathways, including regulation of the survival of NK and NKT cells, the recruitment of $CD8^+$ T cells to plaques, effects on circulating monocytes and the activation of macrophages, foam cell formation and plaque inflammation. All of these beneficial effects of reducing IL-15 on atherosclerosis occur despite increased levels of VLDL and LDL cholesterol. The mechanisms by which reducing IL-15 increases VLDL and LDL cholesterol are not currently known, and this observation may present a limitation on potential usefulness of IL-15 as a target for prevention of atherosclerosis. However, IL-15 appears to exert effects via various receptors and future studies,

aimed at dissecting the contribution of IL-15 receptors to the atherogenic versus anti-cholesterolemic effects of IL-15, may allow for strategies to selectively modulate IL-15's pro-atherogenic effects without negatively affecting cholesterol metabolism.

CHAPTER 3: Inactivation of IL-15Rα in bone marrow derived cells attenuates diet induced atherosclerosis in LDL receptor deficient mice

Foreword

The following study investigates the effects of Interleukin-15 receptor α in bone marrow derived cells on atherogenesis in LDLR deficient mice. Inactivation of IL-15Rα in bone marrow derived cells results in reduced levels of atherosclerosis, accompanied by an elevation of cholesterol associated with VLDL sized lipoproteins. We have also shown that the effect of IL-15 on macrophage foam cell formation is dependent on the presence of IL-15Rα in macrophages.

The authors of the following manuscript are; Omid Dadoo, Mark Fuller, David Y Wang, Melissa E MacDonald, Suleiman A Igdoura, Vidhya Nair, Carl D Richards, Ali A Ashkar, Bernardo L Trigatti.

B.L.T and O.D. designed the experiments. O.D., M.F., D.W., and M.M. performed the experiments. O.D. and B.L.T. interpreted the data wrote the manuscript. V.N., A.A., C.R, and S.I. contributed intellectually.

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scholarships from Heart and Stroke Foundation of Ontario and Canadian Institutes of Health Research.

3.1 Abstract

Interleukin-15 (IL-15) has been shown to promote atherosclerosis via several bone lymphocytic and monocytic cell populations. We set out to test the role of leukocytes IL-15 receptor α (IL-15Rα) in atherosclerosis in ldlr deficient recipient mice. We first demonstrated that IL-15Rα was immunodectable and co-localized with CD68, a marker of macrophages, in human atherosclerotic plaques. Macrophages from IL-15Rα KO mice exhibited attenuated IL-15 induction of IL-6, IL-1β and TNF-α compared to macrophages from wild type mice. IL-15 treatment of macrophages from wild type but not IL-15Rα KO mice led to increased acetylated low density lipoprotein-induced foam cell formation in culture. This led us to hypothesize that inactivation of IL-15Rα in leukocytes might attenuate atherosclerosis. To test this we used bone marrow transplantation to generate LDL receptor KO mice lacking IL-15Rα in leukocytes and compared diet induced atherosclerosis development to control LDL receptor KO mice transplanted with bone marrow from wild type controls. Inactivation of IL-15Rα in leukocytes was accompanied by reduced numbers of circulating NK and CD8 T cells, but an increase in cholesterol associated with VLDL and LDL sized lipoproteins in high fat diet fed mice. Atherosclerotic plaque sizes were reduced by ~40% in mice lacking IL-15Rα in leukocytes, although we detected no differences in inflammatory markers within plaques. These findings confirm that IL-15Rα is expressed

in macrophages in atherosclerotic plaques and mediates their responses to IL-15. Inactivation of IL-15Rα in leukocytes attenuates the development of diet induced atherosclerosis in LDL receptor deficient mice.

3.2 Introduction

Atherosclerosis is an inflammatory disease that is driven by dysregulation of lipoprotein metabolism and immune responses.[1] The infiltration of leukocytes such as monocytes and lymphocytes in response to activation of the endothelium and their secretion of a wide range of cytokines including IL-6, TNFα, and IL-1β , lead to the development of a pro-inflammatory niche in the wall of the aorta.[2] Monocytes under the influence of such factors differentiate into activated macrophages which in turn take up modified lipoproteins resulting in the formation of foam cells and drive the expansion of the atherosclerotic plaque.[1, 2] These lipid rich cells eventually undergo apoptosis, which, in the context of defective efferocytosis, leads to the accumulation of cellular debris and cholesterol and the development of a lipid-rich necrotic core within the atherosclerotic plaque.[33] Other cells types such as lymphocytes can secret cytokines which cause further development of advanced plaques.[1] Natural killer (NK) cells and CD8 cytotoxic T cells have been suggested to promote atherosclerosis by the secretion of IFNγ and the induction of apoptosis in target cells via perforin and granzyme B dependent mechanisms. Furthermore, NK T cells have been shown to play a role in atherogenesis as a result of stimulation by CD1d dependent lipid antigen presentation which in turn produces pro-inflammatory cytokines including MCP-1 and IFNγ. [26, 143]

IL-15 is an inflammatory cytokine which is essential for the survival and maturation of NK, NKT, and CD8 T cells [147] [133] IL-15 can also directly activate macrophages to produce inflammatory cytokines.[87] [132] IL15 has three receptors; IL-15 receptor (R) α, which is highly specific for IL-15, IL-2/15 Rβ which is shared between IL-2 and IL-15, and the common gamma chain cytokine receptor (γ_c), which participates in signalling in response to a variety of different cytokines.[77] The heterodimeric complex between IL-2/15Rβ and γ_c can bind with relatively low affinity and signal in response to IL-15; however its affinity for and signaling in response to IL-15 is greatly enhanced by the binding of IL-15 to either soluble or membrane associated IL-15Rα[77, 132]. The trans-presentation of IL-15 bound to IL-15Rα on the surface of other cells such as macrophages appears to be critical for IL-2/15Rβ/γ_c signaling leading to the activation of survival pathways in NK, NKT and CD8 T cells[133, 147]. In addition to trans-presentation of IL-15 to other cell types, macrophages appear to be able to directly respond to IL-15, themselves[87]. For example, IL-15 treatment of macrophages leads to the induction of RANTES expression and secretion via a pathway that is suppressed by soluble IL-15Rα and not affected by deletion of either IL-2/15Rβ or γ_c, suggesting that it does not require trans-presentation of IL-15 to signaling receptors on macrophages.[87] Instead, evidence points to the involvement of IL-15Rα on macrophages in mediating IL-15 dependent induction of RANTES via a pathway involving

NF-κB activation.[87]IL-15 has previously been detected in both mouse and human atherosclerotic plaques.[98] We recently found that IL15 contributes to the development of atherosclerosis and this appears to involve its roles in the activation macrophages as well as in the survival of NK cells and CD8 T lymphocytes. We demonstrated that overexpression of IL-15 increases atherosclerotic plaque development while elimination of IL-15 reduces atherosclerosis. These effects occur despite increased levels of plasma cholesterol in mice lacking IL-15 and reductions in plasma cholesterol in mice overexpressing IL-15. IL-15Rα has also recently been detected in human atherosclerotic plaques[98,99], but its distribution in plaque resident macrophages has not been described. Given the important role of IL-15Rα in mediating both direct signaling in response to IL-15 and in mediating its trans-presentation to support IL-2/15Rβ/γc dependent IL-15 signaling, we hypothesized that IL-15Rα may mediate IL-15's effects on atherosclerosis.

In this study, we demonstrate that IL-15Rα is present in macrophages in human atherosclerotic plaques, and that macrophages from mice in which IL-15Rα expression has been inactivated exhibit impaired IL-15 mediated activation of IL-6, IL-1β and TNF-α expression, as well as IL-15 enhancement of acetylated LDL-driven foam cell formation. To test the role of IL-15Rα in experimental atherosclerosis in mice, we used bone

marrow transplantation to generate ldl receptor deficient mice that specifically lacked IL-15Rα in bone marrow derived cells, which includes macrophage derived foam cells in atherosclerotic plaques. Consistent with previous reports that IL15Rα in macrophages and dendritic cells are required for NK and CD8 T cell homeostasis[131], IL-15Rα deficiency in bone marrow derived cells resulted in depletion of NK cells and reduction of CD8 T cells in circulation. Surprisingly, we found that LDLR KO mice lacking IL-15Rα in leukocytes exhibited increased rates of production of VLDL and steady state levels of cholesterol associated with VLDL and LDL sized lipoproteins. Despite these increases in lipoprotein cholesterol, IL-15Rα deficiency in bone marrow derived cells reduced diet induced atherosclerosis development. These findings demonstrate that IL-15Rα in bone marrow derived cells promotes the development of atherosclerosis and suggest that this may involve multiple cellular pathways, including direct IL-15 mediated activation of macrophage foam cell formation and through modulation of NK and CD8 T cell survival.

3.3. Materials and Methods:

3.3.1 Mice, bone marrow transplantation and diets: All experiments involving mice were approved by the McMaster University Animal Research Ethics Board and are in accordance with guidelines set out by the Canadian Council on Animal Care which conform to the NIH guidelines (Guide for the care and use of laboratory animals). All mice were obtained from the Jackson Laboratories and housed in the Animal Facility at the Thrombosis and Atherosclerosis Research Institute (McMaster University) with free access to food and water. Mice were fed a normal chow diet (Teklad 18 % protein diet, Harlan Research Labs, USA). Recipient mice were irradiated (split dose of 7.33 and 3.67 Gy separated by 3 hrs) using a Gammacell 3000 small animal irradiator [148] . Mice were immediately injected i.v. with 3 million bone marrow cells prepared from the femurs and tibias of donor WT or IL-15Rα KO mice. Mice were maintained on normal chow as they were allowed to recover for 4 weeks. 4 weeks after BMT, atherosclerosis was induced by switching the mice to the Western Type diet (21% butter fat and 0.15% cholesterol; Dyets Inc. Bethlehem, PA). Mice were euthanized and analyzed after 9 weeks of Western diet feeding.

3.3.2 Atherosclerosis analysis: Mice were fasted for 12 hours prior to euthanasia. Mice were anesthetized with isoflurane gas and euthanized by thoracotomy while still under anesthesia . Heparinized blood was collected for preparation of plasma. Hearts were frozen in Shandon

cryomatrix and 10 µm cryosections were collected. Sections from the aortic sinus of hearts were stained with oil red O and hematoxylin as described previously[113, 137]. Necrotic core sizes were quantified after hematoxylin and eosin staining.

3.3.3 Immunofluorescence: Analysis of human samples was approved by the Hamilton Integrated Research Ethics Board. Human thoracic aorta aneurysm and coronary artery samples were collected by autopsy. Paraffin sections of human atherosclerotic lesions were deparaffinised and treated with antigen unmasking citrate-based solution (Vector Laboratories, Burlington, ON) prior to immunostaining using goat anti-human IL15Rα (R&D Systems, Minneapolis, MN) and mouse anti-human CD68 (Novus Biologicals Inc, Littleton, CO). Rabbit anti-goat IgG conjugated to AlexaFluor 594 and rabbit anti-mouse IgG conjugated to AlexaFluor 488 (Molecular Probes, Burlington, ON) were used as secondary antibodies. Cryosections of mouse atherosclerotic plaques were immunostained using rat anti-mouse CD68 (AbD Serotec, Burlington, ON), rabbit anti-IL-6 (Abcam, Cambridge, MA), rabbit anti-MCP-1 (Abcam, Cambridge, MA), rabbit anti-Arg1 (Santa Cruz, Dallas, TX). Goat anti-rat IgG conjugated to AlexaFluor 488 and goat anti-rabbit IgG conjugated to AlexaFluor 594 (Molecular Probes, Burlington, ON) were used as secondary antibodies. All samples were counter stained with DAPI (Invitrogen/Molecular Probes, Burlington, ON)

3.3.4 Cell culture and foam cell formation. Resident peritoneal macrophages were isolated from C57BL/6 and *il15ra-/-* mice and cultured in RPMI medium supplemented with 3% newborn calf lipoprotein-deficient serum[138] . Macrophages were incubated for 4 hours with or without 250 ng/mL rIL15 (Peprotech, Rocky Hill, NJ). For gene expression analysis, total RNA was extracted after incubation with rIL-15. For foam cell formation, 100µg/mL acetylated LDL (Biomedical Technologies, Inc,Ward Hill, MA, USA) was added for 8 hours after 24 hours incubation with rIL-15 and cells were then fixed in methanol and stained with oil Red O and DAPI.

3.3.5 RNA purification and analysis by RT-PCR*:* Total RNA was extracted using the Rneasy Mini kit (Qiagen, Toronto, ON) and was used as a template for cDNA synthesis using the Qiagen Quantitect Reverse Transcription kit. Quantitative real time PCR analysis of individual cDNA was performed using TaqMan Gene Expression Assays (Applied Biosystems Japan Ltd., Tokyo, Japan; IL-1β: Mm00434228_m1 , TNFα: Mm00443258_m1, IL-6: Mm00446190_m1) using an Applied Biosystems 7900HT Fast Real-Time PCR System.

3.3.6 Flow cytometry: Blood cells were stained for lymphocyte populations with PE labeled NK1.1, FITC labeled CD3, APC-H7 labeled CD8, and stained for monocytes with PE labeled CD115, FITC labeled CD11b, APC labeled Ly6C antibodies (BD Pharmingen, Mississauga,

Canada) and subjected to flow cytometry using BD LSR II flow cytometer at McMaster University Medical Sciences Facility.

3.3.7 Plasma lipoprotein, chemical assays, and ELISA analysis. Plasma lipoprotein fractions were separated by fast protein liquid chromatography (FPLC) [139]. Total cholesterol (Cholesterol Infintiy, Thermo Scientific, Ottawa, ON) and triglyceride (Wako Diagnostics, Richmond, VA) content were measured using enzymatic assay kits. IL-6 and TNFα (Biolegend, San Diego, CA) were measured by ELISA kits.

3.3.8 Triglyceride secretion: Mice were fasted for 12 hours and then injected (i.v.) with 500mg/kg of Tyloxapol (Sigma-Aldrich, St Louis MO) in PBS. Blood samples were collected just before and 3 hours after injection with Tyloxapol. Plasma was prepared and triglyceride concentration was analyzed as described above.

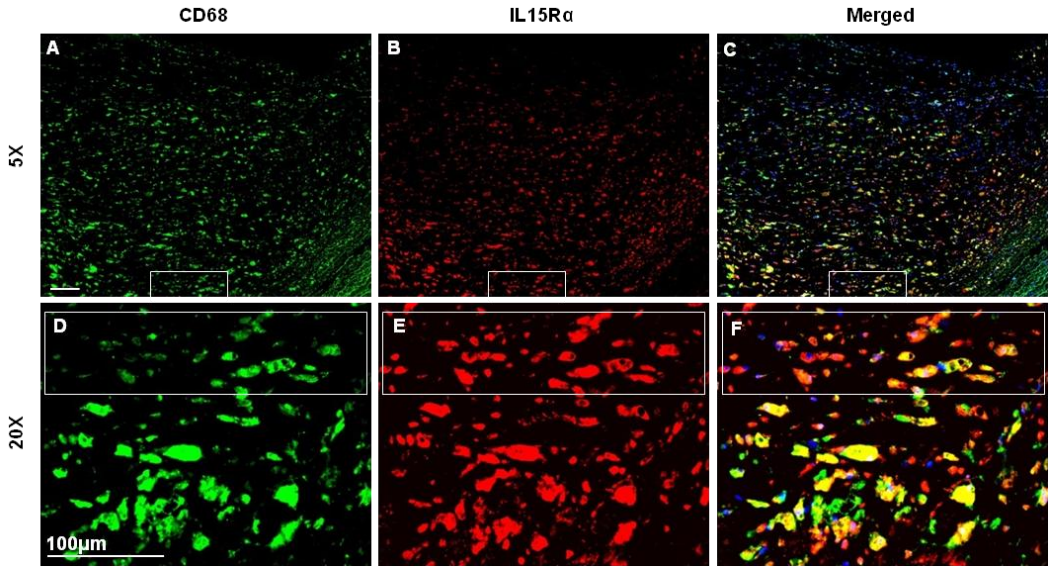
3.3.9 Statistical analysis: All the data are averages ± SEM. Data was subjected to one way ANOVA or pairwise comparisons by the Student's T-test or Mann-Whitney Rank Sum test.

3.4 Results

3.4.1 Co-localization of IL15Rα and CD68 cells in human atherosclerotic plaques.

Co-immunofluorescence staining of human atherosclerotic plaque sections (Figure 3.1) revealed that the majority of cells staining strongly for the macrophage marker, CD68, also stained for IL-15Rα. This suggests that macrophages express IL-15Rα within human atherosclerotic plaques. We also noticed that some IL-15Rα positive cells demonstrated only weak or little staining for CD68, consistent with previous reports that IL-15Rα is also expressed in vascular smooth muscle cells within human atherosclerotic plaques.[149]

Figure 3.1. Expression of IL15Rα and CD68 in human atherosclerotic plaques. (A-C) Lower magnification (5X) and (D-F) higher magnification (20X) of human plaques stained for (A and D) CD68 (green), (B and E) IL15Rα (red), and merged images together with DAPI (C and F). Sections were deparaffinised and treated with antigen unmasking citrate-based solution before staining. White boxes in A-C are the lower magnification of images in D-F.


CD68
IL15Rα
Merged
5X
20X
A
B
C
D
E
F
100µm

3.4.2 IL-15 induced activation of cytokine expression and enhancement of foam cell formation are attenuated in macrophages lacking IL-15Rα. Our observation that IL-15Rα is expressed in macrophages in human atherosclerotic plaques led us to examine its role in IL-15 mediated activation of macrophage expression of pro-inflammatory cytokines and IL-15 stimulated enhancement of macrophage foam cell formation. We cultured resting peritoneal macrophages (essentially all of the cells stained positively for CD68; data not shown) from either wild type or IL-15Rα deficient mice in the absence or the presence of 250ng/ml recombinant mouse (rm) IL-15 for 24 hrs. Figure 3.2A demonstrates that stimulation of resting peritoneal macrophages from wild type mice with rmIL-15 led to a 650-fold induction in the levels of IL-6, a 1350-fold induction in IL-1β and a ~120 fold induction in TNFα detected by RT-PCR. In contrast, IL-15 mediated induction of IL-6, IL-1β and TNF-α was reduced by more than 3-, 5- and 12-times in resting peritoneal macrophages from IL-15Rα KO mice. It is noteworthy that although the levels of IL-15 mediated induction of IL-6, IL-1β and TNFα were reduced in the IL-15Rα KO cells, they were still induced by IL-15 treatment, albeit to lesser extents. The reason for this is unclear but it may suggest either that IL-15Rα is not completely inactivated in the KO cells, or that rmIL-15 may be able to stimulate alternative IL-15Rα

independent pathways leading to increased cytokine expression in macrophages. Further research is required to resolve this issue.

Macrophage conversion to foam cells is an important process that is thought to be one of the factors that drive the formation of atherosclerotic plaques[2, 33]. To examine the effects of IL-15 and IL-15Rα on macrophage foam cell formation, we prepared resting peritoneal macrophages from wild type and IL-15Rα gene targeted mice and cultured them for 24 hrs in the presence or absence of 250ng/ml IL-15. We then added 100 µg/ml acetyl-LDL for an additional 8 hrs to drive foam cell formation. The cells were then fixed and stained with oil red O, for neutral lipid, a hallmark of foam cell formation. IL-15 treatment of wild type peritoneal macrophages increased both the numbers of macrophages accumulating neutral lipids as well as the extent of the neutral lipid accumulation within cells indicating that treatment with IL-15 enhanced AcLDL driven foam cell formation (Figure 3. 2D,E). In the absence of IL-15 treatment, macrophages from IL-15Rα deficient mice accumulated similar amounts of neutral lipid as macrophages from wild type mice (Figure 3.2, panels D and F). In contrast to wild type macrophages, IL-15 treatment did not stimulate enhanced neutral lipid accumulation in macrophages from IL-15Rα deficient mice (Figure 3.2 panels F and G; quantified in H). Therefore the ability of IL-15 to enhance AcLDL driven foam cell formation in wild type macrophages

was completely lost in macrophages from IL-15Rα deficient mice. This suggested that the ability of IL-15 to enhance macrophage foam cell formation was entirely dependent on IL-15Rα.

Figure 3.2. The effect of IL15Rα on macrophage activation and foam cell formation. (A-C) Peritoneal macrophages from wild-type and il15ra KO mice were incubated with or without rIL-15 (250 ng/ml). After 4 hours, (A) IL6, (B) IL-1β, and (C) TNFα gene expression levels were analyzed. Resident peritoneal macrophages collected from wild-type and il15ra KO mice were cultured in the absence or presence of rIL-15 (250ng/ml) for 24 hours followed by 100 µg/ml AcLDL for 8 hours. Cells were fixed and stained with oil red O (red) and DAPI (blue). (D-G) Representative images of untreated WT cells (D), or WT cells treated with IL-15 (E), il15ra KO untreated cells (F), or il15ra KO treated cells (G). (H) Quantification of foam cell formation was performed by normalizing the oil red O staining intensity by the number of cells. * or Different lower case letters in indicate statistically significant differences ($P < 0.001$ determined by two way ANOVA followed by the Holm-Sidak post-hoc test.

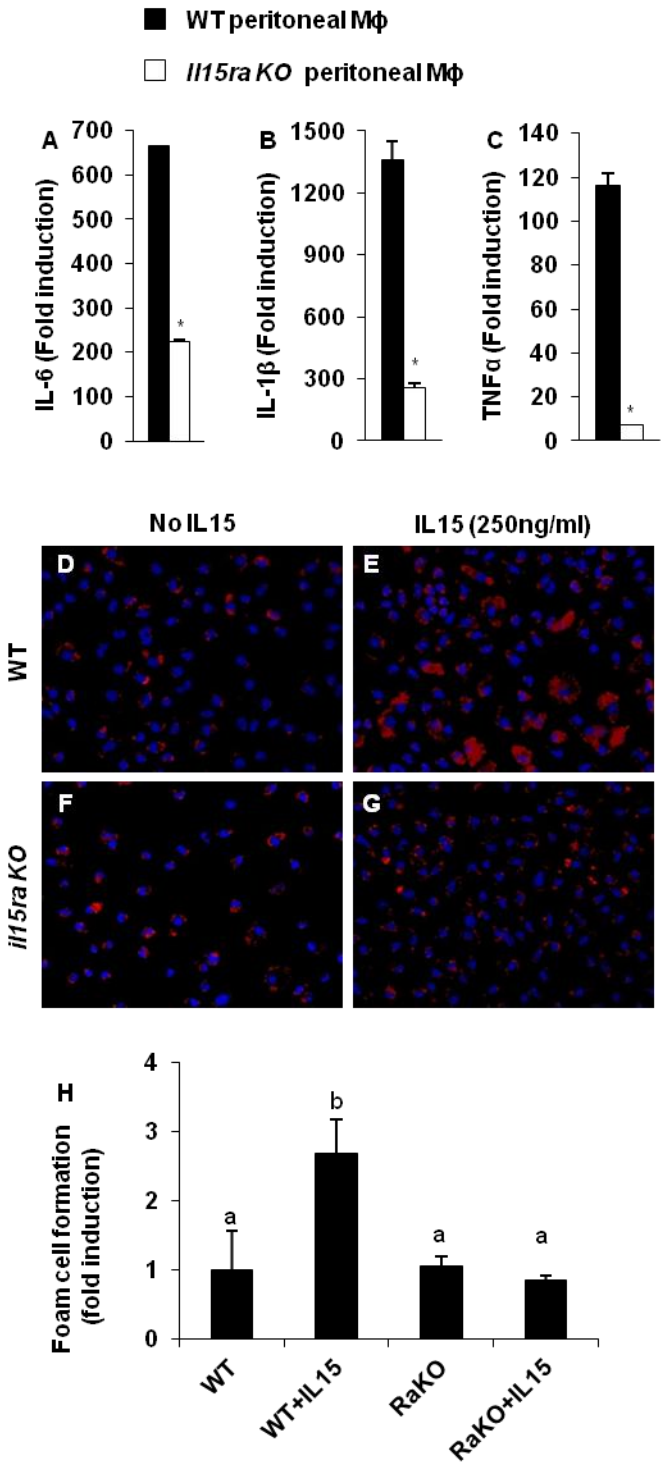
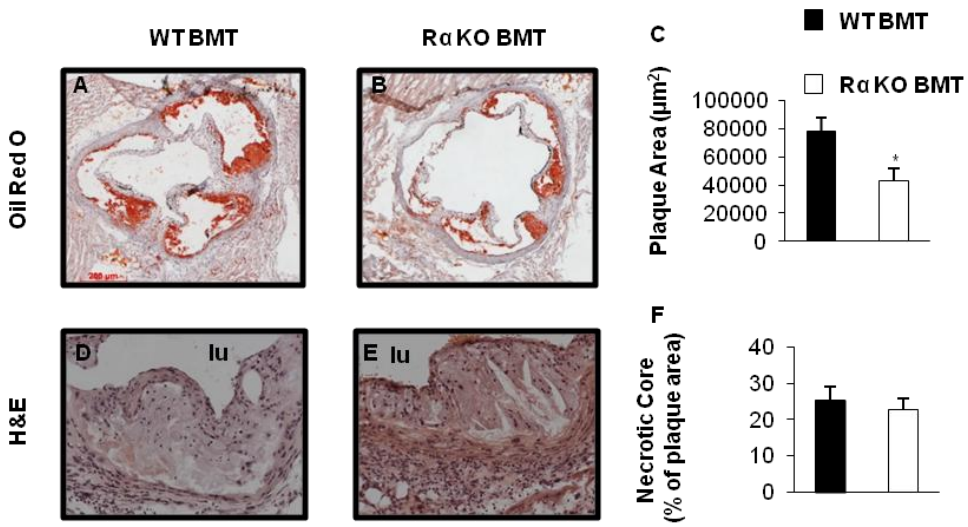

WT peritoneal Mϕ
Il15ra KO peritoneal Mϕ
A
IL-6 (Fold induction)
700
600
500
400
300
200
100
0
B
IL-1β (Fold induction)
1500
1200
900
600
300
0
C
TNFα (Fold induction)
140
120
100
80
60
40
20
0
No IL15
IL15 (250ng/ml)
WT
il15ra KO
D
E
F
G
H
Foam cell formation (fold induction)
4
3
2
1
0
a
b
a
a
WT
WT+IL15
RaKO
RaKO+IL15

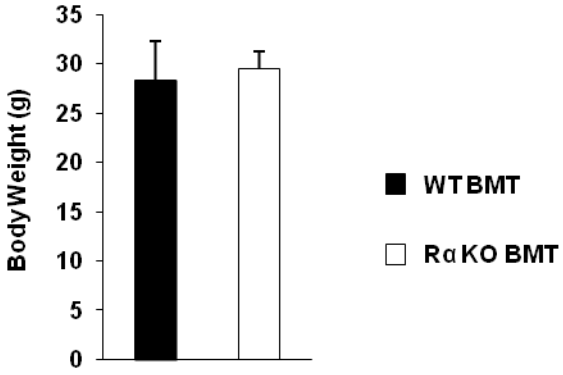
Figure 3.3. Effects of IL15Rα in BM derived cells on atherosclerosis in LDLR-/- mice. A-C) Atherosclerosis in the aortic roots were measured using oil red O staining. Representative images for *ldlr* $^{-/-}$ mice transplanted with WT bone marrow (A) and *ldlr* $^{-/-}$ mice transplanted with IL15Rα deficient bone marrow (B) are shown, and average plaque sizes were quantified (C) (N= 10 mice per group). Data are averages ± SEM. P=0.02 by the Mann Whitney rank sum test. (D-F) Atherosclerotic plaques were stained with hematoxylin and eosin for identifying necrotic cores in *ldlr* $^{-/-}$ mice transplanted with WT bone marrow (D) or IL15Ra deficient bone marrow (E). (F) Necrotic core sizes were normalized to the total plaque area.


WT BMT
RαKO BMT
Oil Red O
H&E
A
B
C
D
E
F
lu
lu
WT BMT
RαKO BMT
Plaque Area (μm²)
100000
80000
60000
40000
20000
0
*
Necrotic Core (% of plaque area)
40
30
20
10
0

3.4.3 IL15Rα deficiency in BM derived cells reduces atherosclerosis in ldlr-/- mice. To test the involvement of leukocyte IL-15Rα in atherosclerosis, we generated ldlr-deficient mice in which IL-15Rα was selectively inactivated in bone marrow derived cells, by transplanting bone marrow from IL-15Rα -/- donors into lethally irradiated ldlr KO recipients. As controls we transplanted bone marrow from IL-15Rα$^{+/+}$ donors into ldlr KO recipients. After recovery from BMT for 4 weeks, the mice were fed a high fat, high cholesterol atherogenic diet for 9 weeks, prior to analysis. We saw no differences in the body weights of the mice after the atherogenic diet feeding period (Supplementary Figure 3.1). Lipid rich atherosclerotic plaques in the aortic sinus were stained with oil red O and the cross-sectional areas of atherosclerotic plaques were measured. Representative oil red O stained sections are shown in Figure 3.3A and B. Atherosclerotic plaques in ldlr deficient mice that had been transplanted with bone marrow from IL-15Rα deficient mice were approximately 40 % smaller than plaques from ldlr deficient mice that had been transplanted with bone marrow from wild type donors. Despite the smaller sizes of the atherosclerotic plaques, the relative size of the necrotic cores was not affected by the absence of IL-15Rα expression in bone marrow derived cells. We also found that the relative extent of macrophage staining within the atherosclerotic plaques was not different in mice transplanted with either wild type or IL-15Rα deficient bone marrow. Furthermore, we

detected no differences in the abundance of IL-6 or MCP-1 or in the abundance of arginase-1 a marker of M2 macrophage polarization (Figure 3.4 D-L). Similarly, we saw no differences in the levels of IL-6 or TNF-α in plasma (Supplementary Figure 3.2). These data suggested that inactivation of IL-15Rα in bone marrow derived cells reduced atherosclerosis development but did not alter local inflammation or necrotic core formation within atherosclerotic plaques, consistent with the observation that IL-15 was still able to at least partially induce cytokine production in cultured macrophages from IL-15Rα deficient mice, albeit to a lesser extent than in wild type macrophages, but that IL-15 mediated enhancement of macrophage foam cell formation was prevented in the absence of IL-15Rα (Figure 3.2).

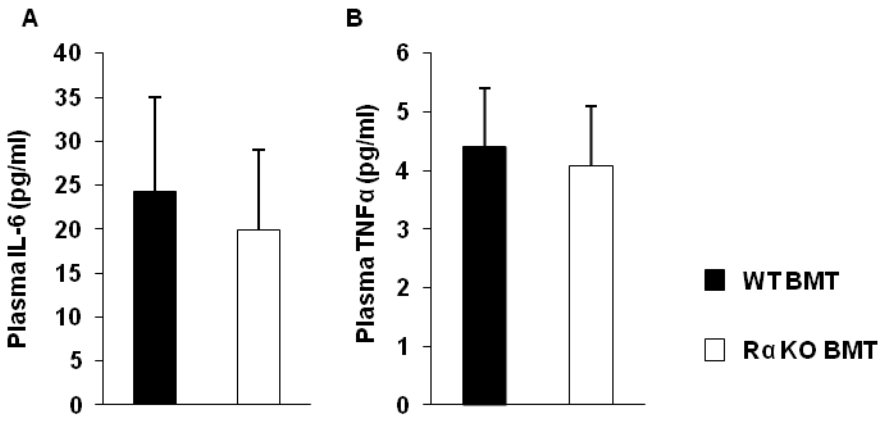
Supplementary Figure 3.1. Body weights in transplanted mice. Body weights of mice transplanted with WT and IL15Ra deficient BM derived cells were quantified at the point of harvest. N=10 per each group. Data was subjected to Mann-Whitney Rank Sum Test.


35
30
25
20
15
10
5
0
Body Weight (g)
WT BMT
RαKO BMT

3.4.4 Effect of bone marrow specific IL15Rα deficiency on circulating leukocytes. Atherosclerosis development in the artery wall is driven by the recruitment of different types of leukocytes, a process that is, in part, dependent on the abundance of circulating leukocyte populations. IL-15Rα deficient mice did not display differences in total white blood cell, lymphocyte or monocyte counts as determined using a Hemavet blood cell analysis system (Supplementary Figure 3.1). Consistent with this, bone marrow specific IL15Rα deficiency had no effects on either the numbers of total or Ly6C^{hi} monocytes in circulation as determined by flow cytometry (Figure 3.5). On the other hand, the abundance of NK cells and CD8^{+}T cells in circulation were substantially reduced when IL-15Rα was inactivated in bone marrow derived cells. We also noticed 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. Therefore, as expected from studies of bone marrow specific deficiency of IL-15Rα in otherwise wild type mice, inactivation of IL-15Rα in bone marrow derived cells of LDL receptor deficient mice leads to reductions in the numbers of circulating NK and CD8T cells consistent with the role of IL-15Rα in leukocytes in IL-15 mediated survival of these cells. Therefore, in addition to the effects of inactivation of IL-15Rα on IL-15 stimulated macrophage foam cell formation, the reductions in NK and CD8T cells may contribute

to the reduced atherosclerosis observed when IL-15Rα is inactivated in BM derived cells of LDLR KO mice.

Supplementary Figure 3.2. Markers of inflammation in plasma samples. Plasma IL-6 (A) and TNFα (B) were measured by ELISA. Data was subjected to Mann-Whitney Rank Sum Test. N=10 per each group.


A
B
Plasma IL-6 (pg/ml)
Plasma TNFα (pg/ml)
40
35
30
25
20
15
10
5
0
6
5
4
3
2
1
0
WT BMT
RαKO BMT

Supplementary Figure 3.3. Blood cells levels in WT and il15ra KO mice. (A, B, and C) Total white blood cell, lymphocyte and monocyte populations were determined using a Hemavet Multi-Species Hematology System. Data was subjected to Mann-Whitney Rank Sum Test.

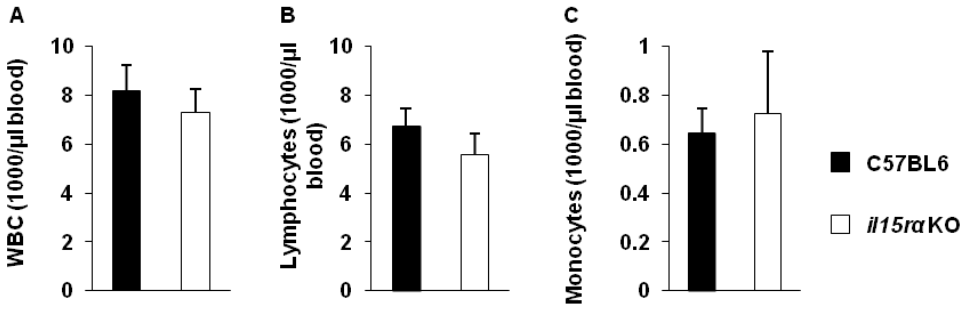
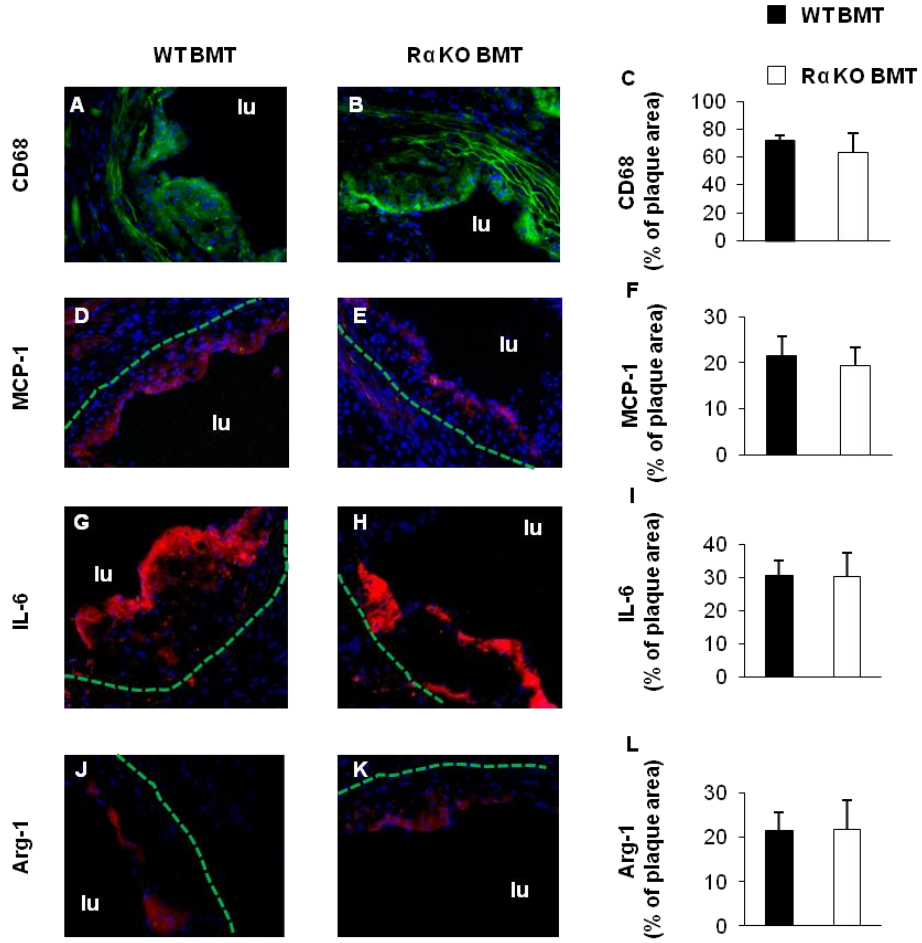

A
WBC (1000/µl blood)
10
8
6
4
2
0
B
Lymphocytes (1000/µl blood)
10
8
6
4
2
0
C
Monocytes (1000/µl blood)
1
0.8
0.6
0.4
0.2
0
C57BL6
il15rαKO

Figure 3.4. Effects of IL-15Rα expression in BM derived cells on CD68+ cells, MCP-1, IL-6, amd Arg-1 expression in atherosclerotic plaques. (A-C) CD68+ area (green), (D-F) MCP-1 (red), (G-I) IL-6 (red), and (J-L) Arg-1 (red) in plaques from *ldlr* $^{-/-}$ mice transplanted with WT bone marrow (A, D, G, J) and *ldlr* $^{-/-}$ mice transplanted with IL15Rα deficient bone marrow (B, E, H, K) was quantified (C, F, I, L) and normalized to the total plaque area. Nuclei were stained by DAPI (blue). Data represent averages ± SEM of N=7 samples per group. Data was subjected to the Mann-Whitney Rank Sum test.


WT BMT
RαKO BMT
CD68
MCP-1
IL-6
Arg-1
A
B
C
D
E
F
G
H
I
J
K
L
lu
WT BMT
RαKO BMT
CD68 (% of plaque area)
MCP-1 (% of plaque area)
IL-6 (% of plaque area)
Arg-1 (% of plaque area)
0
10
20
30
40
60
80
100

3.4.5 Deletion of IL15Rα in BM derived cells affects lipoprotein cholesterol metabolism. Diet induced atherosclerosis in LDL receptor knockout mice is strongly influenced by alterations in lipoprotein cholesterol metabolism. Therefore to control for such effects, we measured total cholesterol and triglyceride levels in plasma from fasted mice after 9 weeks of Western type diet feeding. Surprisingly, we observed increased plasma total cholesterol levels (Figure 3.6A). Fractionation of plasma lipoproteins by size exclusion FPLC revealed that total cholesterol associated with fractions containing VLDL sized lipoproteins were statistically significantly increased. Likewise, cholesterol in fractions containing LDL sized lipoproteins showed a trend towards increased levels but this did not reach statistical significance (Figure 3.6 B, C). On the other hand, there were no statistically significant differences in triglyceride concentrations, although there was a trend towards elevated plasma and VLDL triglycerides that did not reach statistical significance (Figure 3.6 D-F). To examine the likely cause of the increased steady state lipoprotein cholesterol, we used a well established assay for hepatic production of triglyceride-rich VLDL. Western type diet fed LDL receptor KO mice transplanted with either WT or IL-15Rα deficient bone marrow were first fasted and then were injected with Tyloxapol, an inhibitor of lipoprotein lipase activity. Plasma was collected at time 0 (time of injection with Tyloxapol) and 3 hrs later and the accumulation of triglyceride was

measured. We observed substantially increased accumulation of triglyceride in plasma of LDL receptor KO mice transplanted with IL-15Rα deficient bone marrow than those transplanted with wild type bone marrow 3 hrs after Tyloxapol injection. This suggests that inactivation of IL-15Rα in bone marrow derived cells increases hepatic production of VLDL, thereby increasing steady state levels of plasma LDL, the product of VLDL remodelling.

Together, these findings demonstrate that inactivation IL-15Rα in bone marrow derived cells reduces atherosclerosis despite increasing hepatic VLDL production and steady state LDL levels in western type diet fed LDL receptor KO mice. The reduced atherosclerosis may be the consequence of reduced IL-15 stimulated foam cell formation as well as reduced circulating levels of NK and CD8T cells.

Figure 3.5. Blood cells levels in *Idlr*$^{-/-}$ mice transplanted with WT and IL-15Rα deficient BM cells. (A) NK, (B) NKT, (C) CD8^{+} T cells , (D) monocytes, and (E) Ly6C+ monocytes were analyzed by flow cytometry using CD3 (FITC), NK1.1 (PE), CD8 (APC-H7), CD11b (FITC), CD115 (PE), and Ly6C (APC) antibodies. Data represent the averages of 4 mice per group. * indicate statistically significant differences (P<0.01) determined by student t test. Normality and equal variance passed.

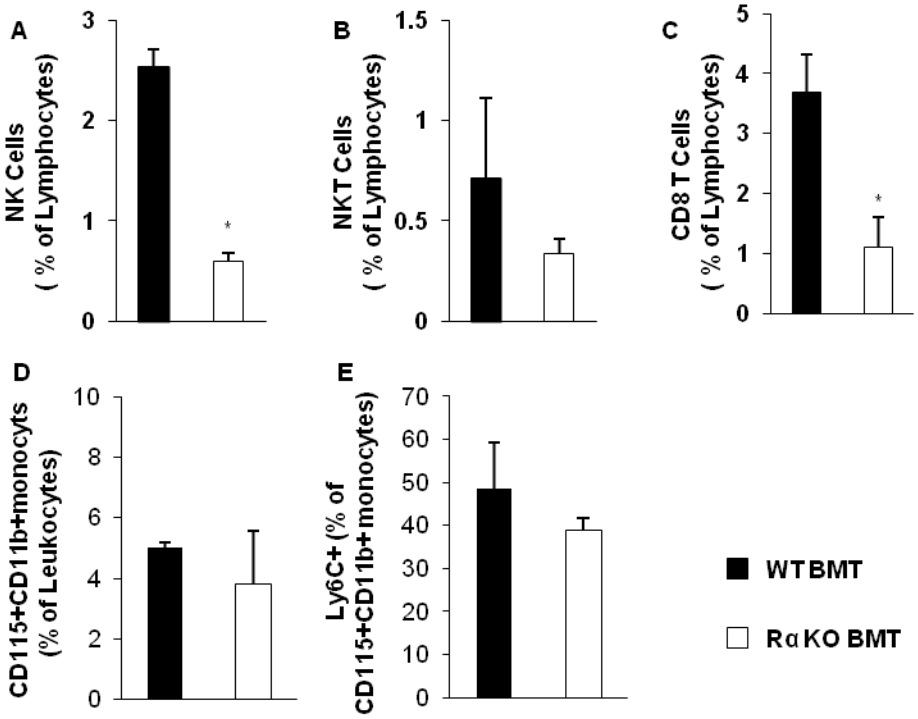
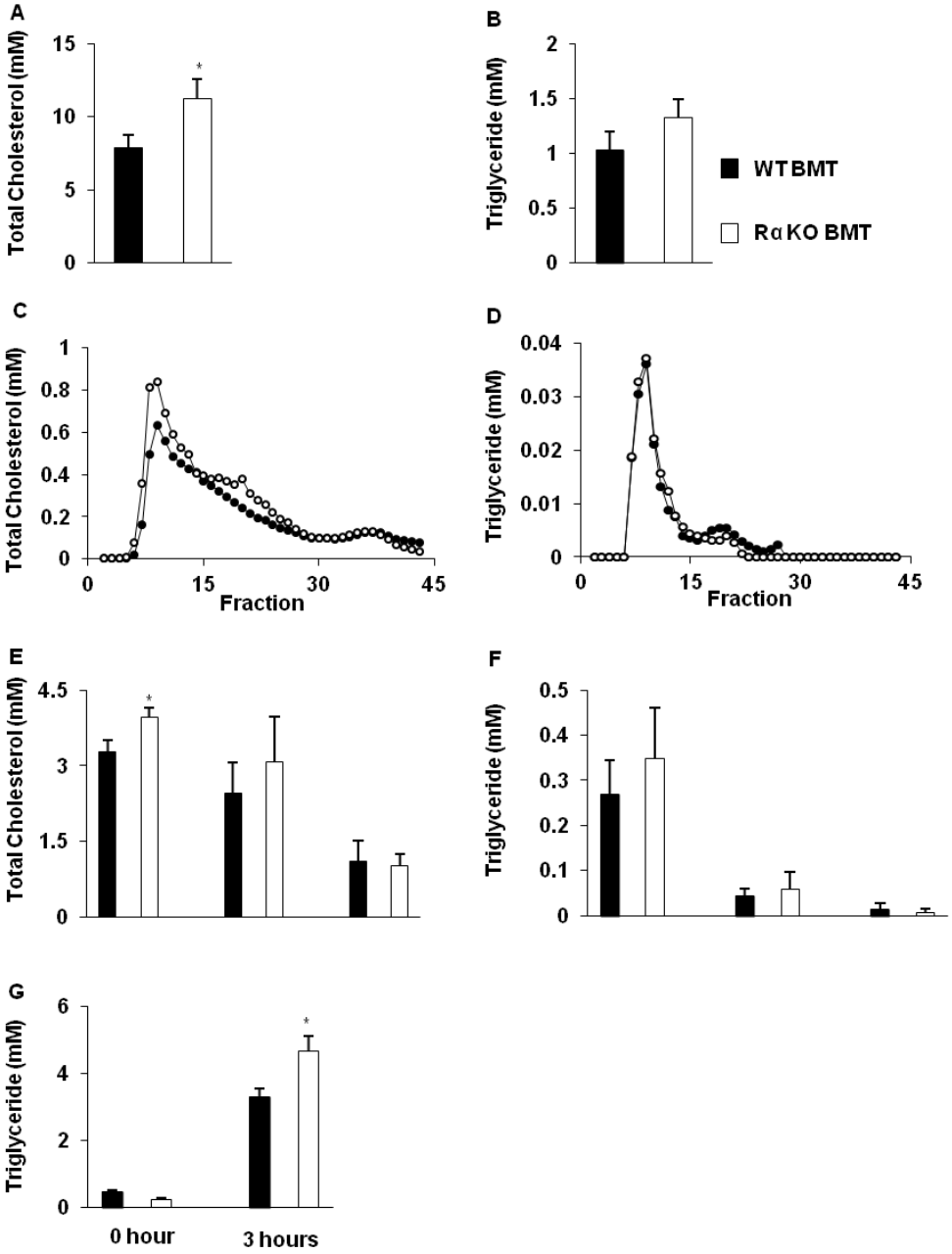
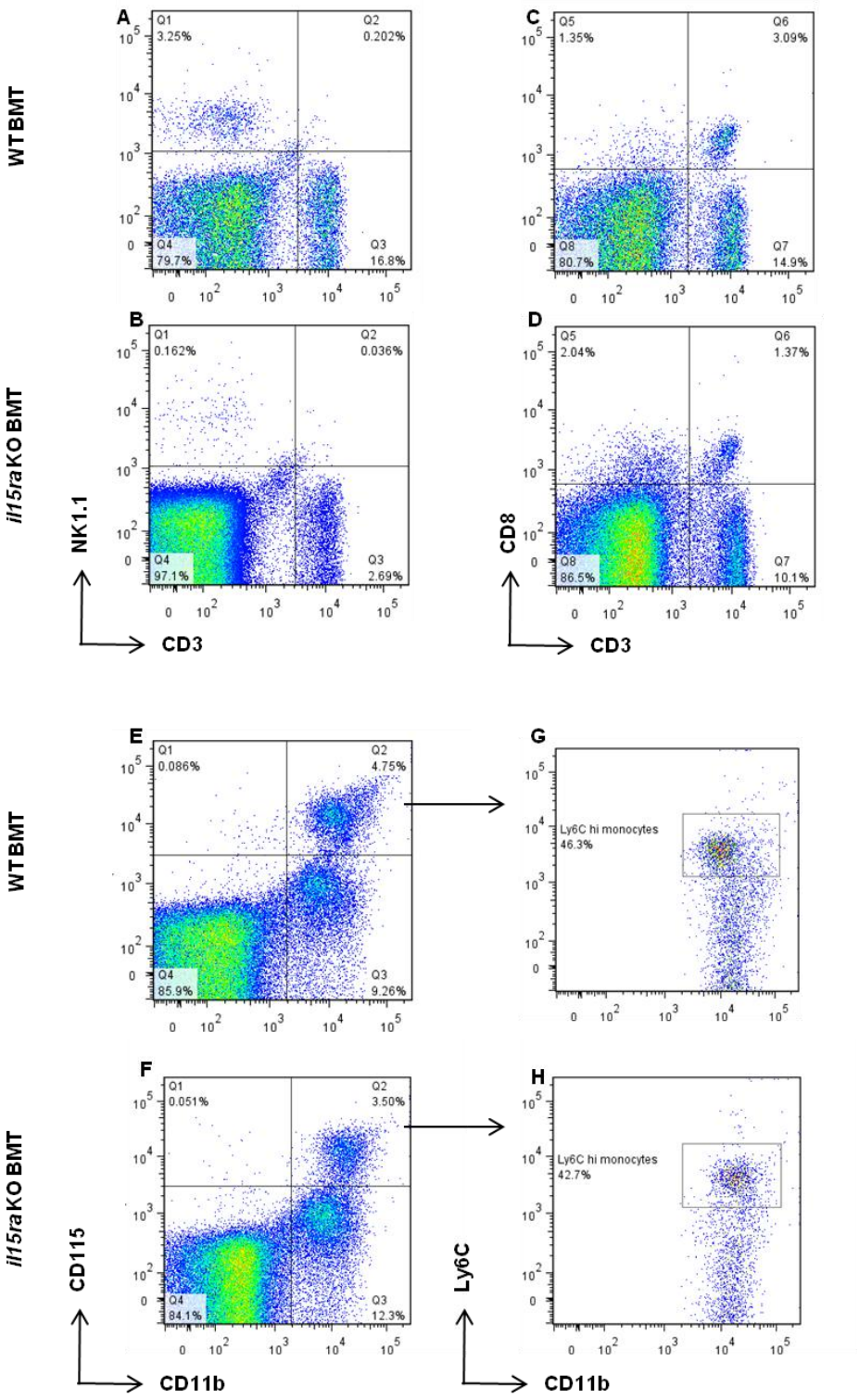

A
NK Cells
(% of Lymphocytes)
3
2
1
0
*
B
NKT Cells
(% of Lymphocytes)
1.5
1
0.5
0
C
CD8 T Cells
(% of Lymphocytes)
5
4
3
2
1
0
*
D
CD115+CD11b+monocyts
(% of Leukocytes)
10
8
6
4
2
0
E
Ly6C+ (% of
CD115+CD11b+monocytes)
70
60
50
40
30
20
10
0
WT BMT
RαKO BMT

Figure 3.6. Effects of IL-15Rα expression in BM derived cells on plasma lipids. (A) Total cholesterol (Mann-Whitney Rank Sum Test; p=0.03) and (B) triglycerides were measured in plasma collected from fasted mice. (N=10 per group). Representative plasma lipoprotein profiles (C and D) and average (± SEM) amounts of cholesterol (C) and triglyceride (D) associated with fractions containing VLDL-, LDL- and HDL-sized lipoproteins (E and F). Data in E and F passed analyses for normality and equal variance, and statistical significance was analyzed by Student's T test (P =0.03 for VLDL cholesterol). N=3 pools of three plasma samples per group. (G) Transplanted mice were fed a high fat diet for 9 weeks. Then they were fasted for 12 hours and injected (i.v.) with 500mg/kg of Tyloxapol in PBS. Triglyceride levels were measured before injection and 3 hours after injection with Tyloxapol. N=4 mice per group. Data in G passed analyses for normality and equal variance, and statistical significance was analyzed by Student's T test (P =0.03).


A
Total Cholesterol (mM)
15
10
5
0
*
B
Triglyceride (mM)
2
1.5
1
0.5
0
WT BMT
RαKO BMT
C
Total Cholesterol (mM)
1
0.8
0.6
0.4
0.2
0
0
15
30
45
Fraction
D
Triglyceride (mM)
0.04
0.03
0.02
0.01
0
0
15
30
45
Fraction
E
Total Cholesterol (mM)
4.5
3
1.5
0
*
F
Triglyceride (mM)
0.5
0.4
0.3
0.2
0.1
0
G
Triglyceride (mM)
6
4
2
0
*
0 hour
3 hours

Supplementary Figure 3.4. Flow cytometry representatives from blood samples of mice transplanted with WT and il15ra KO mice. (A and B) NK, NKT, (C and D) $CD8^+$ T cells, (E and F) CD115+CD11B+ monocytes, and (G and H) Ly6C+ monocytes were analyzed by flow cytometry. Blood cells were collected at the point of harvest from mice transplanted with WT bone marrow (A, C, E, G) and il15ra KO bone marrow (B, D, F, H).


WT BMT
Il15ra KO BMT
A
Q1 3.25%
Q2 0.202%
Q4 79.7%
Q3 16.8%
B
Q1 0.162%
Q2 0.036%
Q4 97.1%
Q3 2.69%
NK1.1
CD3
C
Q5 1.35%
Q6 3.09%
Q8 80.7%
Q7 14.9%
D
Q5 2.04%
Q6 1.37%
Q8 86.5%
Q7 10.1%
CD8
CD3
E
Q1 0.086%
Q2 4.75%
Q4 85.9%
Q3 9.26%
F
Q1 0.051%
Q2 3.50%
Q4 84.1%
Q3 12.3%
CD115
CD11b
G
Ly6C hi monocytes 46.3%
H
Ly6C hi monocytes 42.7%
Ly6C
CD11b

3.5 Discussion

IL-15 is an inflammatory cytokine which is involved in activation/maturation of several key immune cells that play important roles in atherosclerosis. IL-15Rα participates in IL-15 signaling in different ways: it is required for trans-presentation of IL-15 to the heterodimeric IL-2/15Rβ-γ_c complex in NK, NKT and CD8T cells which activates anti-apoptotic signaling required for the survival of these cells. Consistent with this, we found that NK cells were absent and CD8T cells were reduced in circulation when LDLR KO mice were reconstituted with BM from IL-15Rα KO donors. IL-15Rα can also directly mediate IL-15 dependent activation of cytokine production in macrophages[87] and, as we have shown herein, enhances acetyl-LDL driven macrophage foam cell formation. Both IL-15 and IL-15Rα have been reported to be present in atherosclerotic plaques. IL-15Rα has previously been reported to be present in regions of human atherosclerotic vessels that also showed abundant staining for smooth muscle alpha actin, leading to the conclusion that it was present in smooth muscle cells in atherosclerotic vessels[99]. The localization of IL-15Rα in macrophages in atherosclerotic plaques was not tested however. Here we used co-immunofluorescence microscopy to demonstrate that in atherosclerotic plaques from humans, IL-15Rα staining is abundant in cells that also stain strongly for CD68, a marker for macrophages. In research to be published elsewhere, we found that overexpression of IL-15 increases while

inactivation of IL-15 expression reduces atherosclerosis in apoE KO mice in a dose-dependent manner. Similarly, others have reported that vaccination against IL-15 reduced high fat diet induced atherosclerosis in LDLR KO mice.[101]

Macrophage derived foam cells are among the most abundant cell types in lipid rich atherosclerotic plaques and are thought to drive plaque development [33]. Similarly, NK, NKT and CD8T cells have all been reported to be present in atherosclerotic plaques and have been shown to contribute to plaque development [23, 26, 64, 67, 143]. Inactivation of NK or NKT cells or the immunodepletion of NK, NKT or CD8T cells has been shown to reduce atherosclerosis development [4, 5, 20-22]. Given these findings, the importance of IL-15Rα for IL-15 signaling leading to survival of NK, NKT and CD8T cells, as well as macrophage activation and foam cell formation, and the observation that IL-15Rα is present in macrophages in atherosclerotic plaques in humans, we reasoned that targeting IL-15Rα may be effective at reducing atherosclerosis development. We demonstrated this by generating LDLR KO mice in which IL-15Rα was deleted in leukocytes by performing BMT using IL-15Rα KO donors. When compared with LDLR KO mice that were reconstituted with BM from control wild type donors, we found that diet induced atherosclerosis was

indeed significantly reduced in the LDLR KO mice lacking IL-15Rα in leukocytes.

It is noteworthy that while we observed reduced IL-15 dependent induction of IL-6, TNFα and IL-1β in macrophages from IL-15Rα KO compared to wild type mice in culture, we did not observe altered levels of inflammatory cell activation within atherosclerotic plaques (indicated by no changes in IL-6, MCP-1 and Arg-1 staining). This differs from our previous observation that inactivation of IL-15 in apoE KO mice reduced inflammatory activation in atherosclerotic plaques (Dadoo, Richards, Ashkar and Trigatti, manuscript under review). Analysis of cytokine induction in macrophages in culture suggests that IL-15 mediated induction of IL-6 and other cytokines (IL-1β and TNFα) is not completely abrogated in the IL-15Rα KO macrophages. The reasons for this are unclear. Perhaps, however, the residual activity is sufficient to maintain inflammatory activation within atherosclerotic plaques in LDLR KO mice reconstituted with BM from IL-15Rα KO donors.

A surprising finding was that impairing the activity of IL-15Rα in leukocytes of LDLR KO mice resulted in increased hepatic VLDL secretion (as measured by plasma accumulation of triglyceride after inhibition of LPL in fasted mice) and increased steady state VLDL cholesterol levels. Consistent with this, in data to be reported elsewhere, we found that

overexpression of IL-15 reduced, while inactivation of IL-15 increased steady state VLDL cholesterol levels (chapter 2). This does not appear to be the result of alterations in NK, NKT or CD8T cells because immunodepletion of these cells using an NK1.1 antibody was not accompanied by changes in steady state lipoprotein cholesterol levels (chapter 2). The mechanisms linking impaired IL-15Rα in leukocytes and hepatic VLDL production are currently unclear. However these findings are consistent with previous reports that impairing different cytokine signaling pathways either by knockout of the cytokine or its receptor, including IL-6, IFN-γ, MCS-F, and MCP-1 have all been shown to result in increased steady state cholesterol and/or triglyceride levels.[38, 45 25, 107] Despite the increased steady state levels of VLDL, considered to be an atherogenic lipoprotein, imparing IL-15Rα in leukocytes (this study) or inactivation of IL-15 expression (chapter 2), substantially reduced atherosclerosis, suggesting that the atheroprotective effects of impaired inflammatory and immune functions mediated by IL-15-IL15Rα outweigh the atherogenic effects of increasing VLDL levels.

In conclusion, we demonstrate that IL-15Rα is expressed in macrophages in human atherosclerotic plaques, and that it is required for IL-15's ability to enhance macrophage foam cell formation driven by uptake of modified LDL. Furthermore, impairing IL-15Rα in leukocytes in LDLR KO mice

results in reduced levels of circulating NK and CD8T cells but increased steady state VLDL cholesterol. In balance, however, the anti-atherogenic effects of impairing IL-15Rα outweigh the potentially pro-atherogenic effects of increased cholesterol levels resulting in a net reduction in development of diet induced atherosclerosis These findings suggest that IL-15Rα might be a potential therapeutic target to lower atherosclerosis.

CHAPTER 4: Interleukin 15 Deficiency Reduces Aortic Sinus and Coronary Artery Atherosclerosis in SR-BI/ApoE Double Knockout Mice.

Foreword

The following study investigates the effect of IL-15 deficiency on atherosclerosis in aortic sinus and coronary arteries, and also myocardial infarction in SR-BI/ApoE dKO mouse models. Here we report that elimination of IL-15 improves the survival of SR-BI/ApoE dKO mice compared with the control group. This study confirms the previous findings that IL-15 plays an important role in the development of atherosclerosis and hence validates this cytokine as a therapeutic target for the treatment of atherosclerosis.

The authors of this report are; Omid Dadoo, Mark Fuller, Melissa E MacDonald, Carl D Richards, Ali A Ashkar, Bernardo L Trigatti. OD and BLT designed the project. OD, MF, and MM conducted the experiments. AA and CR contributed intellectually.

This research was funded by Heart and Stroke Foundation of Canada (G13-0002833) to BLT. OD was a recipient of McMaster University International Excellence Award. MF was a recipient of scholarships from Heart and Stroke Foundation of Ontario and Canadian Institutes of Health Research.

4.1 Abstract:

Interleukin (IL)-15 is a survival factor of natural killer (NK) and $CD8^+$ T cells and had been previously shown to play an important role in the development of aortic sinus atherosclerosis. In the present study, we examined the effect of inactivating IL-15 on coronary artery atherosclerosis and survival of IL-15/SR-BI/ApoE triple KO (referred as tKO) mice compared with SR-BI/ApoE double KO (referred as dKO) control group. IL-15 deficiency improved the survival of tKO mice compared with dKO animals. While 50% of dKO mice did not survived beyond 50 days, half of tKO mice survived for longer than 60 days. In a separate experiment, dKO and tKO mice were harvested at 50 days of age. Mice lacking IL-15 showed reduced atherosclerosis in both aortic sinus and coronary arteries. IL-15 elimination reduced NK and $CD8^+$ T cells in tKO mice compared to dKO control group. No significant change was observed in the levels of plasma IL-6 and TNF-α. Total cholesterol and VLDL-sized cholesterol levels were lower in the absence of IL-15 in tKO animals. The levels of CD68+ (macrophage) or fibrotic areas were not significantly different. In conclusion, IL-15 plays an important role in the development of coronary artery atherosclerosis and also the survival of SR-BI/ApoE dKO mice through potentially lowering cholesterol and also NK and CD8 T cell levels.

4.2 INTRODUCTION

Atherosclerosis, an inflammatory process of lipid rich plaques development in vascular walls, is one of the major contributors of heart diseases and can lead to myocardial infarction (MI) and death.[2] During atherogenesis, inflammatory cells infiltrate into the site of atherosclerosis development where they become activated and produce a wide range of inflammatory regulators.[1] For example, monocytes migrate into the sub-endothelial space and differentiate into macrophages.[2] These macrophages become activated by expressing scavenger receptors including SR-A and CD36 and take up modified lipoproteins which results in macrophage foam cell formation.[2] These cells eventually undergo apoptosis and accumulation of cholesterol and cellular debris leads to the formation of lipid-rich necrotic cores.[150] The rupture of advanced plaques causes thrombosis formation which in turn blocks the blood flow in coronary arteries.[1] Disruption of the blood flow in these arteries leads to myocardial infarction (MI) and death.

The roles of many inflammatory cytokines in the development of aortic sinus atherosclerosis have been studied in the past 20 years using mouse models. However, wild-type mice are resistant to developing coronary artery atherosclerosis (CAA). Mice lacking both ApoE and SR-BI, on the other hand, can develop extensive CAA and MI[113] similar to SR-BI/LDLR dKO[114], and some other mouse models. [151] [152] SR-BI/ApoEdKO mice die

from cardiovascular complications after only 5-8 weeks of age. [113] It has also been shown that these dKO mice develop occlusive coronary atherosclerosis and multiple MIs, which are key features of human cardiovascular disease. SR-BI is a receptor of HDL and has been demonstrated to have several atheroprotective functions.[52, 114, 136, 139] In the liver, SR-BI facilitates the transfer of cholesterol from HDL particles into hepatocytes and hence it contributes to the reverse cholesterol transport (RCT) from peripheral tissues to the liver.[153] SR-BI deficiency leads to the formation of enlarged HDL particles as a result of non-functional RCT.[139] Functional HDLs not only contribute to RCT and hence is protective against atherosclerosis, but also protect against inflammation in macrophages in vitro. HDL has been shown to suppress inflammatory pathways in endothelial cells[154] and macrophages[155] through SR-BI. In conclusion, through several effects on lipoprotein metabolism and inflammatory responses, SR-BI deficiency can promote severe levels of atherosclerosis not only in the root of the aorta, but also in coronary arteries.

IL-15 is an inflammatory cytokine which is essential for the survival and maturation of NK[22], NKT[156] and CD8 T cell lymphocytes[157]. These cells have been detected in atherosclerotic plaques and have been shown to play a role in the disease development.[23, 26, 64, 67, 143] Despite the role lymphocytes play in atherosclerosis, it has been shown that mice deficient

in B and T cells did not survive longer than control mice. In this study recombination activating gene 2 (RAG2)/SR-BI/ApoE tKO mice were compared with SR-BI/ApoE dKO animals. RAG2 is essential for the development of T and B lymphocytes but not NK cells. Hence, it is not clear whether NK cells are required for rapid development of coronary artery atherosclerosis.

On the other hand, it is known that IL-15 can directly activate macrophages to produce inflammatory mediators such as RANTES[87], IL-1β, TNFα, and IL-6.[158] We previously reported that IL-15 can promote aortic sinus atherosclerosis through multiple cellular and molecular pathways (chapter 2). To test the effect of IL-15 deficiency in CAA, we generated il15/srbi/apoE tKO mice and compared their survival, lipid measurements, atherosclerosis levels, and blood cell components to srbi/apoE dKO mice. Here we report that elimination of IL-15 from dKO mice not only reduces aortic sinus atherosclerosis, but also reduces the CAA levels and improves the survival of tKO mice compared to control srbi/apoE dKOgroup.

Consistent with the published reports, IL-15 deficiency led to NK cell depletion and lower CD8 T cell populations. We measured plasma lipids and we found that IL-15 deficiency in tKO mice lowers the cholesterol level associated with VLDL and LDL sized fractions. While female and male dKO mice lived for around 46 days in average, female and male tKO mice

lived for 55 and 52 days, respectively. The improved survival was accompanied by less atherosclerosis in the aortic sinus, although the macrophage content of the plaques were the same. Consistent with the aortic sinus atherosclerosis, CAA level was lower in tKO mice. Only 18% of coronary arteries in tKO mice were completely occluded whereas 33% of the CAs in dKO mice were 100% occluded. Even though there was less atherosclerosis in both CAs and in aortic sinus, no difference was observed in the level of fibrosis in the myocardium as a result of IL-15 deficiency. Together these findings confirm a critical role for IL15 in the development of atherosclerosis not only in the aortic sinus but also in CAs.

4.3 Materials and Methods:

4.3.1 Mice and diets: All animal experiments were approved by the McMaster University Animal Research Ethics Board conform to the Canadian Council on Animal Care Guidelines. IL15/ApoE dKO mice on C57BL6 background (described in chapter 2) were crossed to SR-BI+/-ApoE-/- mice on 129/C57BL6 background [118] to generate IL15+/-SR-BI+/-ApoE-/- mice. These were crossed together to generate IL15-/-SR-BI+/-ApoE-/- and IL15+/+SR-BI+/-ApoE-/- . IL15-/-SR-BI+/-ApoE-/- mice were intercrossed to generate IL15/SR-BI/ApoE triple knockout mice. IL15+/+SR-BI+/-ApoE-/- mice were intercrossed to generate the control SR-BI/ApoE dKO mice. Animals were housed in the TaARI animal facilities at McMaster University with free access to normal chow diet (Teklad 18% protein diet, Harlan Laboratories, Mississauga, ON) and water. Survival analysis was done in both female and male mice. Briefly, mice were monitored for development of hunched posture, abnormal hunched gait, ruffled fur, panting, and reduced activity. Mice displaying all of those features were considered to be at endpoint and were euthanized humanely. All other analyses were conducted in male animals.

4.3.2 Atherosclerosis analysis: After 12 hours of fasting, male mice were anesthetized with isoflurane gas and heparinized blood was collected by cardiac puncture. All the mice were euthanized while still under anesthesia. Mice were perfused with PBS containing 10 U of

heparin/ml followed by formalin. Hearts were frozen in Shandon Cyromatrix (Thermo Fisher Scientific, Ottawa, ON, Canada) and stored at −80 °C. Aortic sinus and coronary artery atherosclerosis were measured as described before.[114] Coronary arteries were categorized into 5 groups: No atherosclerosis, fatty streaks, atherosclerotic plaques occluding less than 50% , more than 50% , or 100% of the lumen of the artery.

4.3.3 Immunofluorescence and fibrosis analysis: Frozen sections were immunostained using rat anti-mouse CD68 primary antibody (AbD Serotec, Burlington, ON), goat anti-rat IgG conjugated to AlexaFluor 594 secondary antibody (Molecular Probes, Burlington, ON) and DAPI (Invitrogen/Molecular Probes, Burlington, ON) as described before (capter 3) Sections were fixed with methanol prior to blocking with 10% goat serum in PBS. Fibrosis was measured following the Masson's Trichrome staining protocol provided by the manufacturer (Thermo Fisher Scientific, Ottawa,ON) and as described before[118], which stains healthy myocardium red and collagen fibers blue.

4.3.4 Flow cytometry: Blood samples were collected and red blood cells were lyzed using mouse blood lysis buffer (R&D Systems, Burlington, ON). Cells were stained for NK, NKT (PE labeled NK1.1 and FITC labeled CD3 antibodies), and CD8 T cells (APC-H7 labeled CD8 and FITC labeled CD3 antibodies). NK cells are CD3-NK1.1+ and NKT cells are defined as CD3+NK1.1+ lymphocytes. CD8 T cells are CD3+CD8a+ lymphocytes. All

antibodies were purchased from BD Pharmingen, Mississauga, Canada. Samples were subjected to flow cytometry using BD LSR II flow cytometer at McMaster University Medical Sciences Facility.

4.3.5 Plasma lipids and ELISA analysis. Plasma lipoproteins were separated as described previously, using FPLC [139]. Plasma total cholesterol, cholesterol associated with different lipoprotein sizes (Cholesterol Infintiy, Thermo Scientific, Ottawa, ON) and triglyceride (Wako Diagnostics, Richmond, VA) contents were measured using enzymatic assay kits. IL-6 and TNFα levels in plasma (BioLegend, Burlington, ON) were measured by ELISA kits.

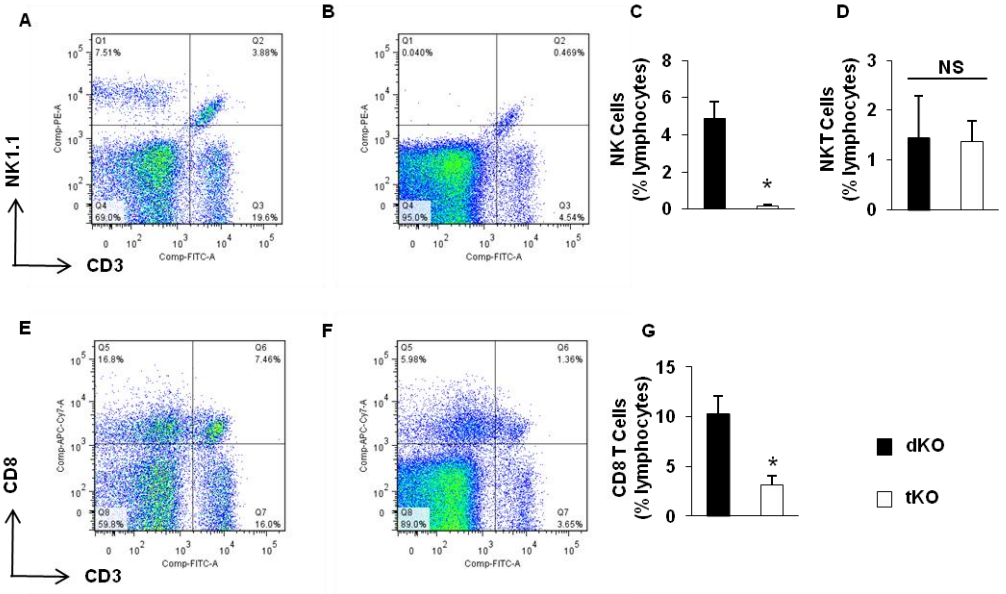
4.3.6 Statistical analysis: All the data are shown as averages ± SEM and subjected to one way ANOVA or pairwise comparisons by the Student's T-test or Mann-Whitney Rank Sum test or one way ANOVA with Holm-Sidak post-hoc test as indicated.

4.4 RESULTS

4.4.1 A lack of IL-15 depletes NK cell and reduces CD8 T cell populations.

Lymphocytes play roles in different stages of atherosclerosis development. On the other hand, IL-15 has been shown to be an essential survival/maturation factor for NK, NKT and CD8 T lymphocytes. Hence, we assessed these lymphocyte populations using flow cytometry. Consistent with other reports, IL-15 deficiency results in depletion of NK cells and also a reduction in the CD8 T cell populations in tKO mice compared with dKO control group (Figure 4.1).

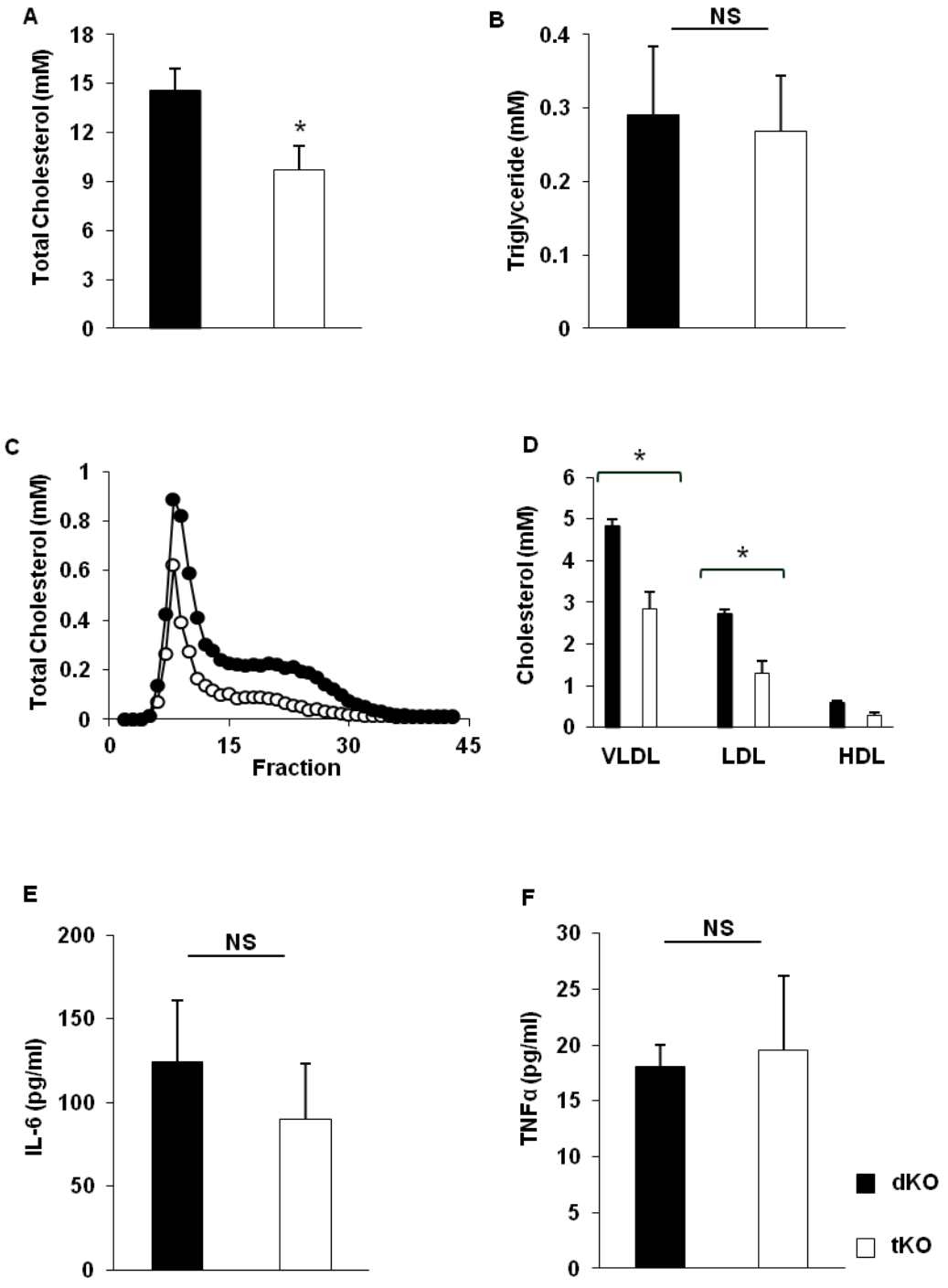
Figure 4.1. IL-15 expression affects blood cells in SR-BI/ApoE dKO mice. (A-D) The percentage of lymphocytes that were NK (NK1.1+CD3-) and NKT (NK1.1+CD3+) cells were quantified (C and D) using NK1.1 and CD3 antibodies. N=4 per each group. A and B are representative flow cytometery of dKO and tKO mice respectively. (E-G) $CD8^+$ T cells (CD8a+CD3+) were assessed by CD8a and CD3 antibodies. N=4 per each group. E and F are representative of dKO and tKO flow cytometery respectively. * indicates statistically significant differences (P=0.02) determined by the Mann-Whitney Rank Sum test.


A
B
C
D
NK1.1
CD3
Q1 7.51%
Q2 3.88%
Q4 69.0%
Q3 19.6%
Q1 0.040%
Q2 0.469%
Q4 95.0%
Q3 4.54%
NK Cells (% lymphocytes)
NKT Cells (% lymphocytes)
NS
E
F
G
CD8
CD3
Q5 16.8%
Q6 7.46%
Q8 59.8%
Q7 16.0%
Q5 5.98%
Q6 1.36%
Q8 89.0%
Q7 3.65%
CD8 T Cells (% lymphocytes)
dKO
tKO
Comp-PE-A
Comp-FITC-A
Comp-APC-Cy7-A

4.4.2 IL-15 deficiency reduces cholesterol levels and results in no difference in the levels of inflammatory cytokines in plasma. It has been reported [113] that CAA in the dKO mice is accompanied by a severe hypercholesterolemia in these mice with an increase in the cholesterol levels associated with VLDL sized lipoproteins. We had previously demonstrated that IL-15 deficiency in apoE KO mice leads to increased cholesterol levels associated with VLDL size lipoproteins. However we found that a lack of IL-15 in il15/srbi/apoE tKO mice results in lower levels of VLDL and LDL size cholesterol compared to srbi/apoE dKO mice (Figure 4.5 A-D).

Furthermore, we had reported that IL-15 deficiency in il15/apoE dKO mice reduces plasma IL-6 suggesting a role for IL-15 to regulate systemic inflammation in apoE KO mice. As a result we set out to measure the levels of plasma IL-6 and TNFα in these two groups and found no differences in the levels of these inflammatory cytokines (Figure 4.5 E and F).

Figure 4.2. Effects of IL-15 expression on plasma lipids and cytokines. (A) Total cholesterol and (B) triglyceride were measured in plasma. (N=10 per group). Data was subjected to the Mann-Whitney Rank Sum Test (P=0.03 for total cholesterol). Representative plasma lipoprotein profile and average (± SEM) amounts of cholesterol associated with VLDL-, LDL- and HDL-sized lipoproteins are shown in C and D. Data was analyzed by Mann-Whitney Rank Sum Test (P =0.02 for VLDL and 0.03 for LDL cholesterol). N=3 per group. (E and F) Plasma IL-6 (E) and TNFα (F) were measured using ELISA kits. Data was subjected to the Mann-Whitney Rank Sum Test. N=10 per group.


A
Total Cholesterol (mM)
18
15
12
9
6
3
0
*
B
NS
Triglyceride (mM)
0.4
0.3
0.2
0.1
0
C
Total Cholesterol (mM)
1
0.8
0.6
0.4
0.2
0
0
15
30
45
Fraction
D
Cholesterol (mM)
6
5
4
3
2
1
0
*
*
VLDL
LDL
HDL
E
NS
IL-6 (pg/ml)
200
150
100
50
0
F
NS
TNFα (pg/ml)
30
25
20
15
10
5
0
dKO
tKO

4.4.3 Deletion of IL-15 reduces aortic sinus atherosclerosis in dKO mice. In order to assess atherosclerosis, we chose to sacrifice the mice at 50 days of age by which point, ~50% of dKO mice die. Triple knockout mice exhibited reduced atherosclerosis in the aortic sinus compared to dKO mice matched for age and gender (Figure 4.3 A-C). Furthermore, we evaluated the macrophage content of the plaques and saw no difference in the CD68+ cell content of atherosclerotic lesions between dKO and tKO groups (Figure 4.3 D-F).

4.4.4 Elimination of IL-15 lowers the ratio of completely occluded coronary arteries in tKO mice. dKO mice develop extensive coronary artery atherosclerosis in only 5-9 weeks on a normal chow diet. At 50 days of age, 48% of coronary arteries in dKO mice were completely free of plaques while 33% of coronary arteries (CA's) are fully occluded by plaques. Elimination of IL-15 reduced the percent of fully occluded CA's to 18% (Figure 4.4) and raised the percent of coronary arteries with no atherosclerosis to 66%.

Figure 4.3. IL-15 deficiency attenuates atherosclerosis in the root of aorta in SR-BI/ApoE dKO mice. (A-C) Atherosclerosis in the aortic sinus in 50 day old male dKO (A) and tKO (B) mice was quantified (C). Atherosclerotic plaques were stained with oil red O and hematoxylin to detect lipid rich areas. N=10 per group. (D-E) CD68+ area (red) in the aortic sinus atherosclerotic plaques from dKO (D) and tKO (E) was quantified (F) and normalized to the total plaque area. Nuclei were stained by DAPI (blue). N=7 per group. Data are averages ± SEM and were subjected to Mann Whitney rank sum test (*P<0.05).

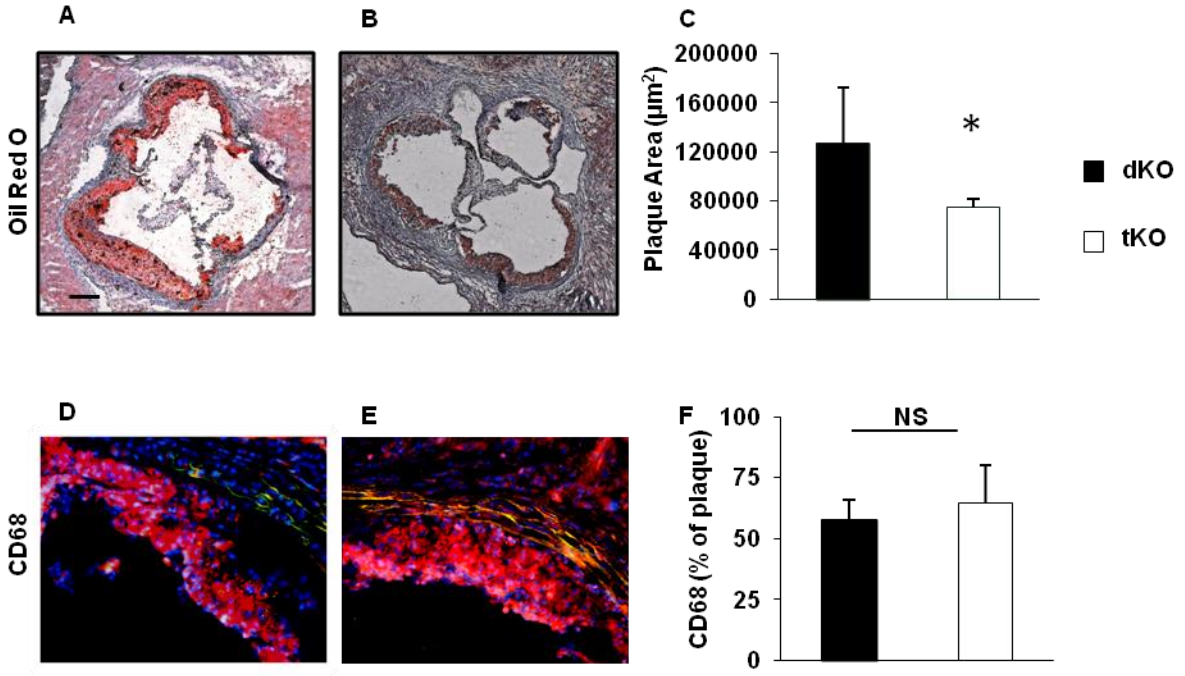
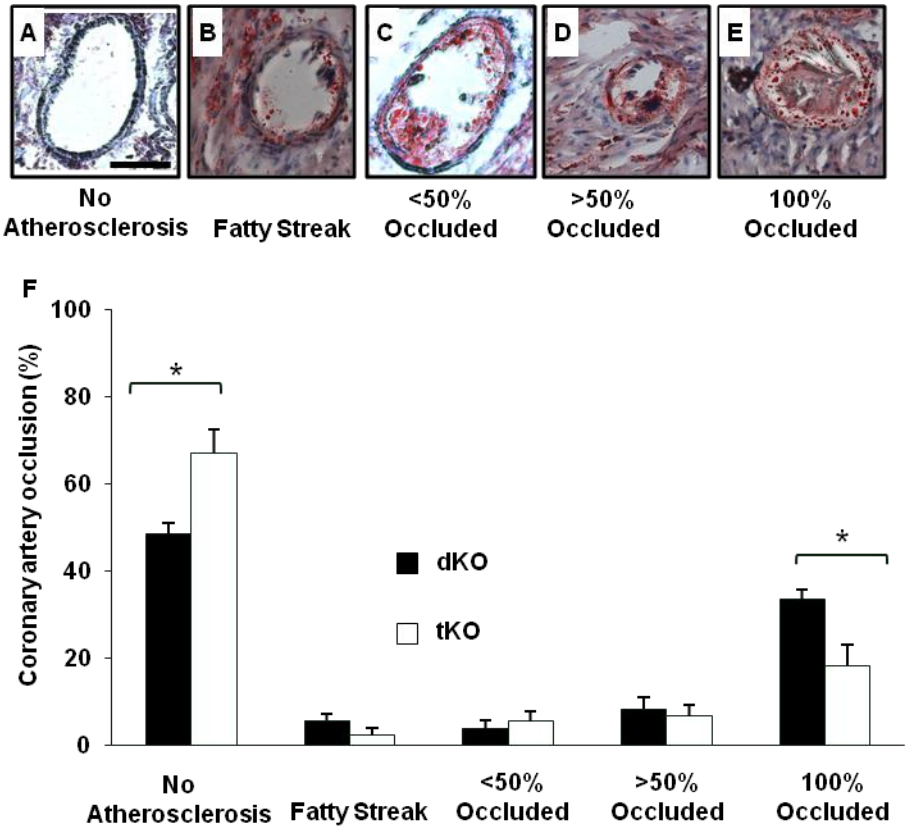
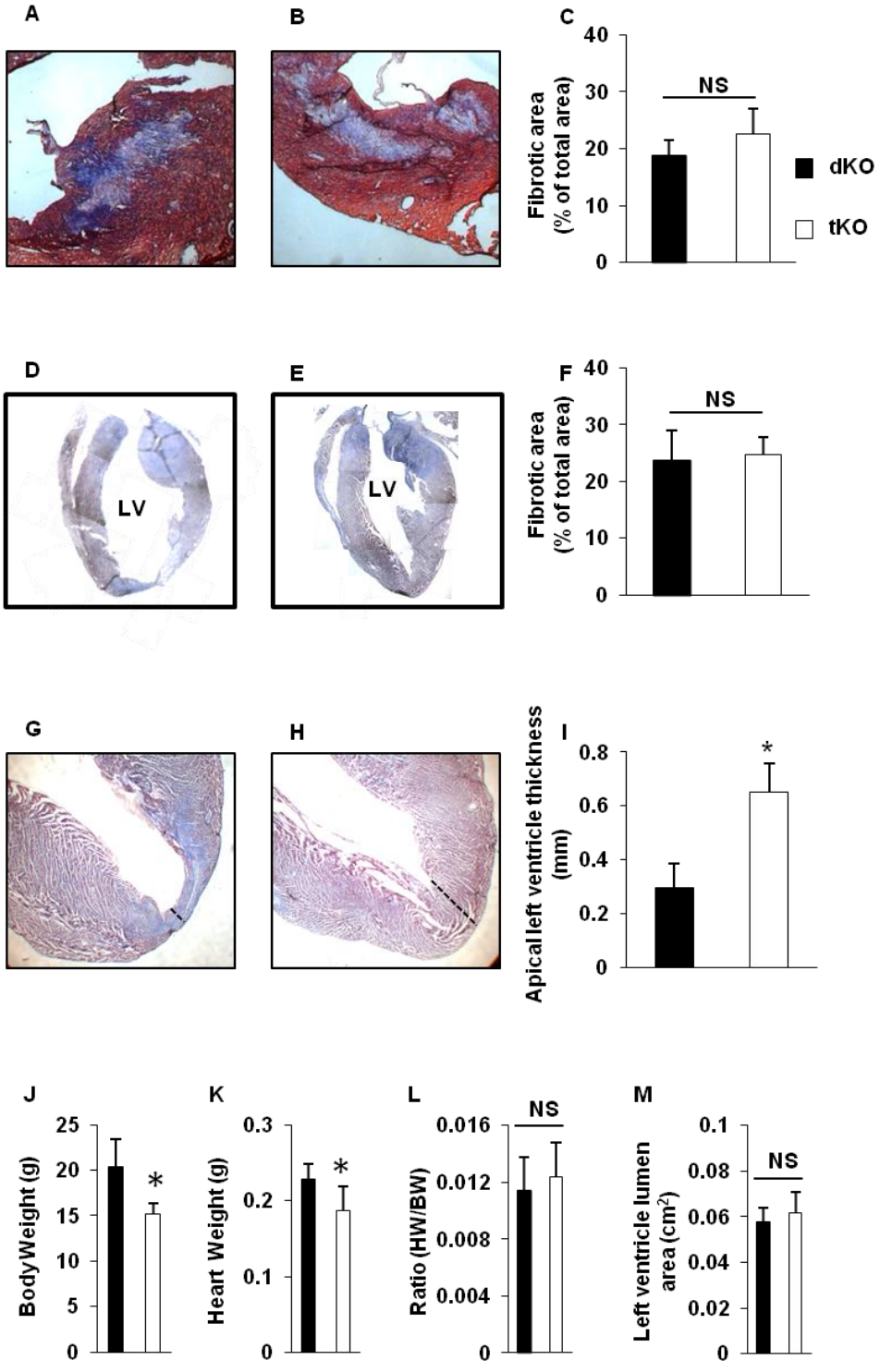

A
B
C
Oil Red O
Plaque Area (μm²)
200000
160000
120000
80000
40000
0
*
dKO
tKO
D
E
F
CD68
CD68 (% of plaque)
100
75
50
25
0
NS

Figure 4.4. IL-15 deficiency reduces atherosclerosis in coronary arteries in SR-BI/ApoE dKO mice. Cross sections of hearts were stained with oil red O and Hematoxylin. Coronary arteries in three sections, at a distance of 100μm from each other, with different levels of atherosclerosis including CA's with no atherosclerosis (A), fatty streak (B), less than 50% occlusion (C), more than 50% occlusion (D), and 100% occlusion (E) were quantified and reported (F) as averages ± SEM for each category. N=10 per group. Data was analyzed by Student T Test ($P<0.05$).


A
B
C
D
E
No Atherosclerosis
Fatty Streak
<50% Occluded
>50% Occluded
100% Occluded
F
Coronary artery occlusion (%)
100
80
60
40
20
0
*
*
dKO
tKO
No Atherosclerosis
Fatty Streak
<50% Occluded
>50% Occluded
100% Occluded

4.4.5 IL-15 deletion does not reduce cardiac fibrosis. dKOs' hearts are enlarged relative to apoE KO mice and exhibit pale patches indicative of extensive MI.[113] Staining heart sections with trichrome which stains healthy myocardium red and fibrotic tissue blue revealed that in dKO mice, approximately 20-25 % of the cross sectional and also longitudinal area of hearts are fibrotic. However a lack of IL-15 did not change the percent of fibrotic area in tKO mice (Figure 4.5). On the other hand quantification of the left ventricular wall's thickness (Figure 4.5 G-I) showed a two fold increase in tKO mice compared to dKO controls. In addition, heart enlargement is one of the outcomes of MI during which, cardiac cells die from lack of nutrition and as a result, in order to compensate for the cardiac failure, more muscle cells will be built up and consequently heart becomes enlarged. In our study, the improved survival in tKO mice is accompanied by a significant reduction in the body and heart weights compared to dKO mice, even though the ratio of heart to body weight is not different (Figure 4.6). Finally, we did not observe a significant difference in the area of left ventricular lumen between dKO and tKO groups.

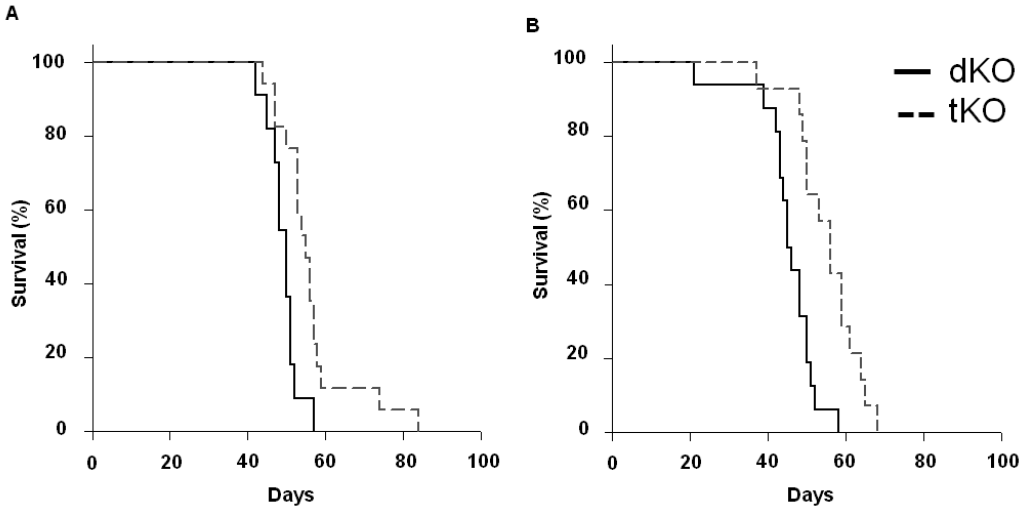
Figure 4.5. Effects of IL-15 deletion in myocardial fibrosis in SR-BI/ApoE dKO mice. (A-C) Cross sectional (A-C) and longitudinal (D-F) area of fibrotic tissues were quantified using trichrome staining which stains healthy myocardium red and fibrotic tissues blue. (A) and (D) represent cross sections and longitudinal sections of dKO hearts, respectively. (B) and (E) represent cross sections and longitudinal sections of hearts from tKO mice. N=5 per group. Data are averages ± SEM and were subjected to Mann Whitney rank sum test. (G-I) Cardiac wall thickness at the apex of the left ventricle was measured in longitudinal sections of hearts form both dKO (G) and tKO (H) mice (N=5 per group) ($P<0.05$). Body weights (B) and heart weights (C) were measured at 50 days of age. Heart weights were normalized to body weights for each group (D). N=10 per group. The area of left ventricle was measured in longitudinal sections of hearts (M). Data were subjected to the Mann Whitney rank sum test (*$P<0.05$).


A
B
C
Fibrotic area (% of total area)
NS
dKO
tKO
D
E
LV
LV
F
Fibrotic area (% of total area)
NS
G
H
I
Apical left ventricle thickness (mm)
*
J
Body Weight (g)
*
K
Heart Weight (g)
*
L
Ratio (HW/BW)
NS
M
Left ventricle lumen area (cm²)
NS

4.4.6 TKO mice show improved survival over dKO control mice. dKO mice fed a normal chow diet die prematurely, accompanied by extensive cardiovascular disease. All the dKO mice died between 5 to 9 weeks of age and ~50% of the dKO mice died before 50 days of age. In average dKO female and male mice survived for ~46 days while female and male tKO mice survived for 55 and 52 days in average, respectively (Figure 4.6 A and B).

Figure 4.6. Effects of IL-15 elimination on the survival of dKO mice.

Male (A) and female (B) IL-15/SR-BI/ApoE tKO mice (N=17 for males and 14 for females) exhibit increased survival compared with male dKO control animals (N=11 for males and 16 for females) using Kaplan-Meier survival curve ($P<0.01$ for both genders).


A
B
100
80
60
40
20
0
Survival (%)
0
20
40
60
80
100
Days
dKO
tKO

4.5 DISCUSSION

Here we report that the level of atherosclerosis in aortic sinus and coronary artery is reduced in tKO mice lacking IL-15 compared with dKO control mice. These findings are consistent with our previous report that a lack of IL-15 in il15/apoE dKO mice results in a significant reduction of atherosclerosis in the root of the aorta of apoE KO mice. Coronary artery atherosclerosis is the main cause of heart attack in humans and hence, characterization of CAA is more clinically relevant. These effects on atherosclerosis were accompanied by an improvement in the survival of tKO mice compared with control group. However, more work is required to determine whether improved survival of tKO mice compared to their dKO controls, is due to reduced coronary artery atherosclerosis. In addition, analysis of platelets in CAA plaques will help us to test the involvement of IL-15 in thrombosis formation in dKO mice.

In heart tissues, fibrosis is the formation of connective tissues in the organ in a reparative response to injuries caused by cardiomyocytes death as the result of myocardial infarction. We did not observe any difference in the cardiac fibrosis between the two groups. Furthermore, insufficient oxygen delivery to heart muscles (e.g. due to coronary artery disease) causes thinning of the cardiac wall including the apex of left ventricular wall which is defined as Ischemic cardiomyopathy.[159] SR-BI deficiency in atherogenic mice has been shown to result in ischemic cardiomyopathy.[160]

Here in this report, we demonstrated a significant increase in the thickness apex of the left ventricle wall in tKO mice compared to dKO controls, suggesting that IL-15 deficiency may potentially improve heart function of dKO mice. More studies are needed to understand how IL-15 may affect functional and morphological changes in hearts of dKO mice. In mouse cardiomyocytes, Il-15 has been shown to protect against hypoxia driven apoptosis[161], suggesting a role for IL-15 in heart function, independent of CAA.

While the levels of aortic sinus atherosclerosis were lower in tKO mice compared with dKO animals, the CD68+ content of plaques was unchanged at around 60% in both dKO and tKO groups. Also the level of systemic inflammation measured by IL-6 and TNFα in plasma showed no significant difference between the two groups, indicating that the presence of IL-15 is not critical to regulate systemic inflammation in srbi/aoE dKO mouse model.

Cholesterol measurements in dKO and tKO mice revealed a reduction in the cholesterol levels associated with VLDL and LDL lipoproteins. This is the opposite of our findings in il15/apoE dKO mice compared with apoE KO controls. Further experiments are needed in order to explain these different findings. The main difference in the two models is the absence of SR-BI which is a receptor for HDL. In the absence of SR-BI, HDL particles have been shown to become enlarged, potentially due to dysfunctional

reverse cholesterol transport. [119] Atherogenic mice lacking SR-BI have been demonstrated to have higher ratio of surface lipid (free cholesterol and phospholipids) to core lipids (cholesterol ester and TG) which results in the formation of abnormal vesicular VLDL-sized lipoproteins.[119] These abnormal lipoproteins in the VLDL-size range are shown to contain ApoAI and lack ApoB, indicating that these enlarged particles could be classified as HDLs.[137] The mechanisms through which IL-15 affects lipoprotein metabolism remain to be determined. Lipoprotein analysis in these mice suggests that IL-15 deficiency may reduce atherosclerosis partially through changes in lipids levels.

Finally, we confirmed that a lack of IL-15 depletes NK cells and reduces CD8 T cell populations in tKO mice compared to dKO controls. As NK and CD8 T cells have been shown to play a role in atherosclerosis development, the effects of IL-15 deficiency on these lymphocyte populations can partially explain the lower levels of atherosclerosis in tKO mice compared with control mice. NK and CD8 T cells are major producers of IFNγ[77], a cytokine which has been demonstrated to promote atherosclerosis[25]. Also, NK cell have been illustrated to play a role in atherosclerosis through granzyme B and perforin functions[67]. Others have shown that B and T lymphocytes are not essential for the rapid development of coronary artery disease[116] by studying SR-BI/ApoE/ RAG2 triple knockout mice. They showed that CA atherosclerosis was

not different in tKO mice compared with control dKO mice. RAG2 is essential for the development of B and T cells, while NK cells are not believed to require RAG2. As a result, RAG2/SR-BI/ApoE tKO mice are deficient in B and T lymphocytes, but not in NK cells.

Altogether these findings confirm a key role for IL-15 in the development of atherosclerosis and also its potential role in promoting CAA and that its blockade could reduce CAA in mice and perhaps in human populations.

CHAPTER 5: Discussion

5.1 Summary of findings

Cardiovascular diseases are among the leading causes of death worldwide. Atherosclerosis, a chronic inflammatory disorder in the vascular wall, is one of the most dominant drivers of cardiovascular complications. Atherosclerosis is greatly regulated by inflammatory factors including a wide range of cytokines. IL-15 is an inflammatory cytokine and its role in atherosclerosis is the focus of this thesis. We demonstrated that elimination of IL-15 reduces the aortic sinus and more importantly coronary artery atherosclerosis, potentially through suppressing atherogenic activities of NK, NKT, CD8 T cells, and monocytes/ macrophages. In addition we showed that IL-15Rα in bone marrow derived cells is required for many of these effects. Moreover, we found that altering IL-15 or IL-15Rα expression affects lipoprotein metabolism. This section will discuss these findings in more detail and provide suggestions for future experiments which can shed light on cellular and molecular mechanisms through which IL-15 affects atherosclerosis.

5.2 IL-15, NK cells, CD8T lymphocytes, and atherosclerosis

IL-15 plays a critical role in the development and functions of NK, NKT, and CD 8 T lymphocytes. These cell types are involved in the development of atherosclerosis through mechanisms that include IFNγ and perforin/granzyme B. As has been reported for other strains of mice,

overexpression of IL-15 in apoE deficient mice in our studies, expands circulating NK, NKT and CD8 T cells while deletion of both copies of the IL-15 gene in either apoE deficient or SR-BI/apoE double KO mice, or bone marrow specific inactivation of IL-15 Rα in LDL receptor deficient mice results in depletion of circulating NK and reduced circulating CD8 T cell populations. Interestingly, deletion of only one copy of IL-15 in apoE deficient mice did not affect any of these circulating lymphocytic populations. In our studies, the largest effects on atherosclerosis were seen in the context of genetic manipulations that resulted in the greatest changes in the abundance of these lymphocytic cells in circulation.

NK cell deficiency reduces atherosclerosis, potentially through suppression of IFNγ-dependent activation of macrophages and ECs[25]. NK cells can induce apoptosis via perforin and granzyme B cytotoxic activities[66]. Apoptosis of smooth muscle cells, endothelial cells and macrophages has been shown to promote atherosclerosis in advanced atherosclerotic plaques.[48] NKT cells can promote atherosclerosis after activation via presentation of lipid derived antigens by antigen presenting cells (APCs) through a CD1d dependent pathway.[26, 143, 162] Following activation, NKT cells produce inflammatory cytokines including IFNγ, MCP-1, TNFα, and IL-6[26], all of which have been shown to promote atherosclerosis development. CD8 T cells have been shown to play a role

in atherosclerosis through both IFNγ secretion as well as perforin/granzyme B mediated apoptosis of macrophages, SMCs, and ECs.

To confirm the role of IL-15 in atherosclerosis development through lymphocytic populations, we depleted NK1.1+ cells by a monoclonal antibody (mAb). It has been shown that NK, NKT, and activated CD8 T lymphocytes[142] express NK1.1. In our studies, depletion of NK1.1+ cells in apoE KO mice reduced atherosclerosis compared with control untreated apoE KO mice. Depletion of NK1.1+ cells was accompanied by the absence of CD8+ cells in atherosclerotic plaques. It is possible that CD8 T cells need to be primed to be recruited by NK/NKT cells. It has been suggested that CD8 T cells require stimulation by NK cells for some of their functions such as production of IFNγ to lyse infected cells with *Mycobacterium tuberculosis*.[163] Finally immune-staining of the atherosclerotic plaques revealed dramatically reduced CD8 T cells in il15/apoEdKO mice compared to apoE KO control mice. This could be due to the direct or indirect effects of IL-15 deficiency.

Three lines of evidence, however, suggest that the effects of eliminating IL-15 expression on atherosclerosis are not solely mediated by the loss of circulating NK cells and reduced CD8T cells. First, female IL15+/-ApoE-/- mice had lower atherosclerosis than corresponding ApoE-/- control mice,

despite there being no differences in NK, NKT, and CD8 T cell populations. Secondly, immunodepletion with an antibody against NK1.1, a marker of NK, NKT and activated CD8T cells, beginning before the start of atherosclerosis development in apoE deficient mice and continuing throughout atherosclerosis development for 7 weeks, resulted in less of a reduction of atherosclerosis development than did the elimination of IL-15 expression, even though NK cells in circulation and CD8T cells within atherosclerotic plaques were similarly reduced. Thirdly, inactivation of one IL-15 gene in the context of NK1.1+ cell depletion (throughout atherosclerosis development), itself resulted in a reduction in atherosclerosis. This provides strong evidence that at least some of the pro-atherogenic effects of IL-15 are independent of NK cells in circulation or CD8 T cells within atherosclerotic plaques. Therefore, we decided to study the effect of IL-15 on monocytic cells.

5.3 Monocytes are involved in IL-15 atherogenic effects

Monocytes are major cellular contributors of atherosclerosis development. The number of monocytes in circulation has been shown to be directly correlated with the levels of atherosclerosis and coronary heart disease.[164] Also, the degree to which monocytes are recruited into plaques is another factor that determines the size and complexity of atherosclerotic lesions. Monocyte recruitment is affected by several factors which are discussed in the introduction. These factors include platelets, CAMs expression by

ECs, chemotactic proteins expression in the site of plaque development, and finally the monocyte number and phenotype (Ly6C high vs. Ly6C low).

In our studies, no significant difference was observed in the Ly6C high monocytes, when either IL-15 was overexpressed, deleted globally, or IL15Rα was deleted in BM derived cells. However, mice overexpressing IL-15 had higher numbers of circulating leukocytes and monocytes consistent with the findings of others.[140] Also, mice completely lacking IL15 had lower numbers of monocytes, although deletion of only one copy of IL-15 did not affect monocytes in circulation. Furthermore, a lack of IL-15 in il15/apoEdkO mice reduced MCP-1 expression in plaques compared with ApoE KO control mice by approximately 60% and deletion of one copy of IL-15 in il15+/-apoE-/- mice reduced MCP-1 expression by ~30%.

In order to determine if IL-15 can promote monocyte recruitment, I suggest to monitor monocyte migration in vitro in response to IL-15 compared with no treatment or positive control, MCP-1, a well known chemoattractant protein for monocyte migration. Furthermore, to show whether IL-15 overexpressing monocytes are more likely to be recruited in plaques compared with WT or IL-15 KO monocytes, the following experiment could be done. The same number of CD11+CD115+ monocytes (extracted by FACS Cell Sorter) from RFP+IL15Tg and GFP+WT mice can be adoptively transferred to the same IL15/ApoE dKO group and after 24 to

72 hours aortic sinuses can be sectioned to quantify GFP and RFP positive cells in plaques. Similarly, monocytes isolated from RFP+IL15rα KO and GFP+WT animals can be adoptively transferred into IL15/apoE dKO mice to understand the role of IL15Rα in monocyte recruitment in vivo. 24 to 72 hour time point is chosen based on our findings[148] that show monocytes are recruited into the plaques rapidly even after one day.

Alternatively monocyte recruitment into the developing atherosclerotic plaques can be monitored using fluorescent latex beads. These beads after being injected intravenously have been shown to be taken up by circulating monocytes.[12] Quantifying the number of monocytes which are positive for these latex beads can help us to determine if overexpression and deletion of IL-15 affects monocytes recruitment in vivo.

5.4 IL-15 promotes macrophage foam cell formation

After recruitment to the site of plaque development, monocytes differentiate into macrophages and DCs. Macrophages are professional phagocytes and can take up modified lipoproteins (see introduction for more details) and eventually become lipid rich foam cells. The extent of lipid content of macrophages in the atherosclerotic plaque is determined by multiple factors such as lipid (particularly cholesterol) uptake and efflux. While cholesterol uptake via lipoproteins is regulated by scavenger receptors including SR-A and CD36, cholesterol efflux is controlled by

ABCA1 and ABCG1. Macrophages deficient in both SR-A and CD36 have been shown to have little or no lipid droplet accumulation after incubation with modified LDL, suggesting that these two receptors are major mediators of foam cell formation in macrophages.[165] Several types of LDL modification have been demonstrated to allow cholesterol uptake in vitro including acetylation and oxidation. Approximately 80% of AcLDL uptake is mediated by SRA, whereas oxLDL uptake is mediated by several scavenger receptors including SRA and CD36.[166, 167]After internalization of cholesterol ester (CE) rich lipoproteins by macrophage receptors, CE is hydrolyzed in lysosomes by lysosomal acid lipase (LAL) to free cholesterol (FC).[168] FC can then be transported to the plasma membrane for cholesterol efflux or to the ER, where ACAT1 esterifies FC to form CE and the accumulation of CE forms lipid droplets of the foam cell.[169] FC can also be converted to oxysterols (OS), which in the nucleus can act as ligands for the liver X receptor (LXR) transcription factor, which in turn upregulates the expression of ABCA1 and ABCG1.[169] These two trans-membrane proteins facilitate cholesterol mobilization from lipid droplets and efflux from the plasma membrane to ApoA-I and mature HDL respectively.[168, 170, 171] In conclusion, lipid droplet accumulation in macrophages is a dynamic phenomenon which is regulated by cholesterol influx (mainly through SRA and CD36) and cholesterol efflux which is

influenced by ABCA1, ABCG1, ACAT1, LXR, ApoA-1, and mature HDL activities.

In our experiments, we set out to test the role of IL-15 in foam cell formation in vitro. We used AcLDL as an inducer of foam cell formation which is used by researchers frequently. Although oxLDL is the major modified LDL that is found in human plaques and as a result is more clinically relevant, it has been shown to induce inflammatory responses[172] and consequently, using oxLDL may alter the effects of IL-15 in foam cell formation. We showed that incubation of macrophages with IL15 in vitro followed by addition of AcLDL increases foam cell formation by 2 fold compared to incubation of macrophages with only AcLDL and no IL-15. Interestingly this effect of Il-15 on the induction of accumulation of lipid droplets was completely blocked in macrophages isolated from il15ra-/- mice, indicating the essential role of IL-15Rα in driving the effect of IL-15 in this phenomenon. However, more work is required to understand the molecular pathways through which IL-15 induces foam cell formation. Additionally, it is necessary to test the effect of IL-15 in foam cell formation in vivo.

In order to determine the mechanisms through which IL-15 acts as an inducer of foam cell formation, I propose to incubate macrophages with rIL-15 and analyze the gene expression and/or protein activity of the

genes involved in cholesterol influx and efflux such as SRA, CD36, ABCA1, ABCG1, ACAT, and LXR. Previously, ACAT1 was shown to be up-regulated in macrophages by other inflammatory cytokines such as IFNγ[173] and TNFα[174]. Also, others have shown that pro-atherogenic cytokines such as IFNγ[175] and IL-1β[176] inhibit the expression of macrophage ABCA1, while anti-atherogenic cytokines including IL-10[176] up-regulate ABCA1 expression in macrophages.[177] All the mentioned pro-inflammatory cytokines (IFNγ[178], TNFα[174], IL1β[130]) have been demonstrated to promote macrophage foam cell formation.[179] This experiment will shed light on the effects of IL-15 on the major proteins involved in cholesterol influx and efflux. Using peritoneal macrophages isolated from mice lacking IL2/15Rβ, or γ_c, we will be able to determine the role of different IL-15 receptor subunits in macrophage foam cell formation. To determine how IL-15 may affect lipid efflux, macrophages can be loaded by ^{3}H cholesterol followed by addition of HDL to lipoprotein deficient serum in the presence or absence of IL-15. These experiments will help us to understand the molecular pathways of macrophage foam cell formation in vitro. To link our findings to physiological conditions, the key findings can be tested in vivo. Others have shown that peritoneal macrophages isolated from 10 week old apoE-/- mice, fed a Western Diet for 6 weeks (starting at 4 weeks of age) had substantial lipid droplets.[180] il15/apoEdKO, apoE KO, and il15tg/apoE KO mice could be fed with a

Western Type diet for 6 weeks starting at 4 weeks of age, followed by isolation of peritoneal macrophages for oil red O staining and quantification of lipid droplet accumulation. However, it is critical to be aware of the fact that dKO mice have higher levels of atherogenic lipoproteins and Il15tg/apoE KO mice have lower lipoprotein cholesterol compared with apoE KO control mice. These different levels of cholesterol associated with VLDL/LDL particles may affect foam cell formation in vivo. Alternatively, peritoneal macrophages from GFP^{+}WT and RFP^{+}IL15ra-/- mice can be isolated and equal number of macrophages from each donor group can be injected to ApoE KO mice fed for 6 weeks with Western Type diet. Three days after, peritoneal macrophages will be isolated and lipid droplets in GFP+ and RFP+ cells will be quantified. The three day time point is chosen based on published findings that confirm macrophages can become foam cells after only three days in vivo.[180]

5.5 IL15 activates macrophage inflammatory responses directly

Others have shown that IL-15 induces the expression of TNFα, IL1β, and IL6 by macrophages in vitro.[158] IL-15 has also been shown to increase the production of RANTES by myeloid cells in vitro via NFκB pathway activation. In the same study a soluble IL15Rα suppressed IL-15's effect in myeloid cells, while IL2/15Rβ blockade or γ_c deficiency could not inhibit IL-15 dependent induction of RANTES. [87] These data together suggest that IL-15 may activate macrophages and myeloid cells to express more pro-

inflammatory cytokines that have been shown to be pro-atherogenic, potentially through IL-15Rα (independent of IL2/15Rβ and γ_c) and via induction of the NFκB pathway. We confirmed that addition of IL-15 to RAW 264.7 murine macrophage like cells in culture increases the expression of RANTES, IL-6, MCP-1, and iNOS activity. Consistent with others' findings[87], we observed that IL-15's effect in macrophage activation was completely suppressed by addition of soluble IL-15Rα. Furthermore, we used peritoneal macrophages from WT and il15rα-/- mice to investigate the outcome of IL15Rα deficiency on treatment with rIL-15. While WT and il15rα-/- derived peritoneal macrophages responded to rIL-15 by expressing more TNFα, ILβ, and IL6 compared to untreated cells measured by RT-PCR, il15ra-/- macrophages' responses were ~3 to 10 fold lower than those of WT macrophages. It is unclear however, why a lack of IL15Rα in macrophages does not inhibit IL-15's effect completely. This may suggest that alternative receptor(s) may be involved in the induction of pro-inflammatory cytokines in macrophages by IL-15. Also, it is important to note that IL-15Rα−/− mice were generated by deleting exons 2 and 3 of the IL-15Rα gene which encode the ligand binding and extracellular domains of IL-15Rα. Hence, these mice have a truncated version of IL-15Rα gene and may not be completely deficient in IL-15Rα.[124] Together, these findings indicate an important role for IL15 in

macrophage activation. It also suggests that the above effect is driven by IL15Rα, at least partially.

In order to determine whether another receptor is involved in the induction of macrophages by IL-15, it is suggested that all three known receptors (IL-15Rα, IL-2/IL-15Rβ, and γ_c) can be knocked down, for example by using siRNA. Then macrophages will be treated with IL-15 to analyze the expressions of inflammatory cytokines such as IL-6, IL-1β, and TNF-α. This experiment will only demonstrate whether there is a fourth receptor involved in IL-15 dependent activation of macrophages.

Furthermore, we tested the effects of altering expression of IL15 and its receptor α in systemic and local inflammation in vivo. We measured plasma IL6 and serum amyloid A (SAA) in il15tg/apoE KO, apoE KO, and il15/apoEdKO mice. While overexpression of IL-15 increased both markers of inflammation, deletion of IL-15 reduced the levels of these proteins in plasma. Additionally, we observed a reduction of MCP-1 expression in atherosclerotic plaques from dKO mice compared with apoE KO control mice. Quantification of plasma IL6 and TNFα in ldlr-/- mice that had received BM from WT or il15ra-/- donors showed no significant difference. Atherosclerotic plaques of these two groups of mice were immunostained for MCP-1, IL6 and Arg1 and no differences were found. These results indicate that while global overexpression or deletion of IL-15

in apoE KO mice have an impact on systemic and local inflammation within atherosclerotic plaques, IL15Rα deficiency in BM derived cells in ldlr-/- recipient mice does not lead to a significant difference in inflammatory cytokines in vivo. This could be due to differences in mouse models as our group has found that ldlr-/- mice on a Western Type diet has a low basal levels of serum IL6 and TNFα. In addition, liver, adipose tissues, and skeletal muscles can produce inflammatory cytokines. As a result, global modification of IL-15 gene expression may have greater impact on inflammatory cytokines production compared to deletion of IL15Rα only in BM derived cells.

In conclusion, it is suggested that IL-15 can induce NFκB dependent pro-inflammatory molecular pathway through IL-15Rα in macrophages[87]. However, it is not clear through what series of events IL15Rα can activate inflammation in macrophages. It has been reported that upon stimulation with IL-15, tumour-necrosis factor receptor-associated factor 2 (TRAF2) is recruited to the cytoplasmic domain of IL15Rα in fibroblasts.[181] This report proposed that IL-15Rα competes with TNFα receptor to recruit TRAF-2. It is likely that TNF receptor-1 associated death domain protein (TRADD) recruits TRAF-2 and receptor-interacting protein (RIP) to the cytoplasmic domain of IL15Rα. TRAF-2 has ubiquitin ligase function and after activation, it catalyzes polyubiquitination of itself. This leads to the release

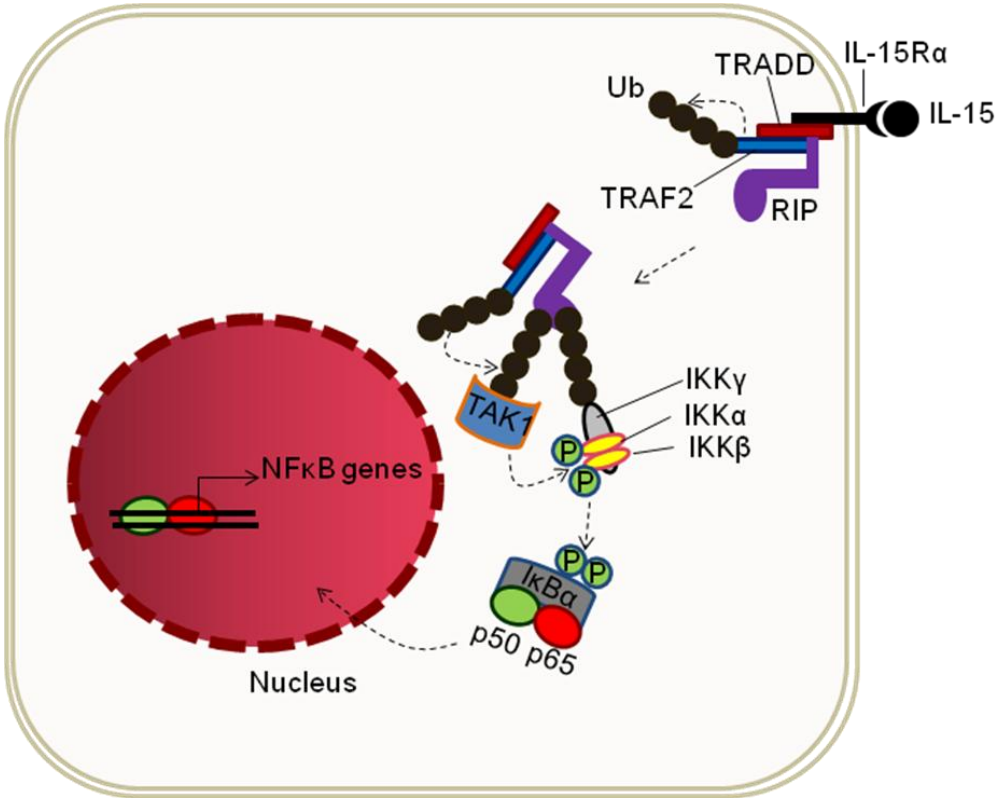
of TRADD-TRAF2-RIP complex into the cytosol and TRAF2-mediated polyubiquitination of RIP.[182, 183] Polyubiquitinated RIP binds to the "transforming growth factor β activated kinase-1" (TAK-1) complex[184, 185] and IκB kinase γ (IKKγ)[186, 187]. This brings TAK-1 and IKK complexes in close proximity which leads to phosphorylation of IKKαβ by TAK-1.[182] Furthermore, phosphorylated IKKαβ complex phosphorylates IκBα in the NFκB complex (IκBα/p50/p65), which leads to the release of p50/p65 dimmers.[182] This results in the expression of pro-inflammatory genes.[182]

Here I suggest a series of experiments that could potentially help us to understand the molecular pathways involved in IL-15/IL15Rα dependent activation of macrophages as hypothesized in Figure 5.1. To test whether RIP, TRADD, TRAF2, TAK1, IKKs are essential for IL-15's effect on macrophage activation, we can inhibit the expression of these proteins (using siRNA) separately and evaluate the effect of IL-15 on macrophage inflammation in vitro. To determine if ubiquitination of TRAF2 and RIP are necessary for IL-15 to induce NFκB activation, I propose to overexpress or knockdown de-ubiquitinating enzymes (DUBs) including USP31 and A20. Ubiquitin specific protease 31 (USP31) has been shown to de-ubiquitinateTRAF-2 and consequently inhibit NFκB activation.[188] A20 overexpression has been shown to de-ubiquitinate RIP and suppresses TNFα-mediated NFκB activation.[189, 190] Furthermore, phosphorylation of

IKKα and IKKβ can be monitored in macrophages after stimulation with IL-15 to confirm the involvement of IKK complex in IL-15 mediated activation of macrophages. Finally, WT and il15rα-/- macrophages upon stimulation with IL-15, can be stained with antibodies against p65 and p50 to find out if they are localized in nucleus or cytosol. This will help us to understand whether IL-15Rα is directly involved in NF-κB activation in macrophages or not. In addition, key findings from in vitro experiments can be tested in vivo. Co-staining of macrophages (CD68 or F4/80) and phosphorylated IKKα (or IKKβ) in atherosclerotic plaques can potentially link the findings in vitro and in vivo. Moreover, NF-κB inhibition has been shown to reduce macrophage foam cell formation.[191] Hence, similar experiments as suggested above can be conducted to determine the molecular pathways through which IL-15 promotes macrophage foam cell formation.

Figure 5.1 Proposed mechanisms through which IL-15 may activate macrophages

IL-15/IL-15Rα interaction leads to the recruitment of TRADD, and consequently TRAF2 and RIP. TRAF2 can catalyse its own polyubiquitination, which leads to its release into the cytosol. Furthermore, TRAF2 facilitates RIP polyubiquitination. Activated RIP is recognized by both TAK-1 and IKKαβγ complex. TAK-1 can then phosphorylate IKKαβ, resulting in phosphorylation of IκBα and release of p50/65 complex. In nucleus, p50/65 activates the expression of several genes including IL-1β, IL-6, and TNF-α.


IL-15Rα
TRADD
Ub
IL-15
TRAF2
RIP
IKKγ
IKKα
IKKβ
TAK1
P
P
NFκB genes
P
P
IκBα
p50 p65
Nucleus

5.6 Interleukin-15 and lipoprotein metabolism

We found that a lack of IL-15 in apoE-/- mice increased total cholesterol (TC) and VLDL cholesterol, while overexpression of IL-15 reduced TC and cholesterol levels associated with VLDL particles compared with apoE-/- control animals. Consistent with this, knocking out IL15Rα from BM derived cells in ldlr-/- recipients led to increased level of TC and VLDL cholesterol. However, IL-15 deficiency in il15/srbi/apoEtKO mice resulted in a reduction of TC and cholesterol associated with VLDL-size lipoproteins compared with srbi/apoEdKO control group. No significant differences were observed in TG levels. Finally, depletion of NK1.1+ cells (NK, NKT, and activated CD8 T cells) did not change cholesterol levels associated with different lipoprotein particles.

In separate experiments we assessed TG secretion in ldlr-/- mice transplanted with WT or il15ra-/- BM. After 9 weeks of high fat diet, fasted mice were injected with Tyloxapol which indirectly indicates the VLDL production level by the liver. Tyloxapol has been shown to block LPL activity and hence inhibits TG hydrolysis. Therefore accumulation of TG levels in plasma after Tyloxapol treatment of fasted mice are taken as a measure of VLDL produced by the liver.[192] We observed higher TG secreted by ldlr-/- mice that received il15ra-/- BM compared to ldlr-/- animals that received wild-type BM. These results together suggest a role for IL-15 in lipoprotein metabolism. However the conflicting findings in

srbi/apoEdKO mice compared to either apoE KO or ldlr KO mice indicates that depending on the mouse model, the effect of IL-15 on lipids measurements may be different.

It has been shown by others that multiple inflammatory cytokines and several immune cell types affect cholesterol homeostasis. For example, DC depletion in both ApoE KO and LDLR KO mice has been reported to increase plasma cholesterol levels and also elevation in DC populations decrease plasma TC in mice.[193] It has been suggested that DCs may contribute to lipoprotein uptake and clearance. Similarly, a lack of MCSF in ApoE KO mice led to reduced number of monocytes and macrophages and was accompanied by a 3 fold increase in cholesterol levels.[4] We have shown that IL-15 can affect the number of circulating monocytes and also promote lipoprotein uptake in macrophages, a process that is facilitated by IL15Rα in macrophages. Lipoprotein uptake by monocytic cells such as DCs and macrophages in an IL-15 dependent manner can partially explain the increased cholesterol level in the absence of IL-15 and reduced TC in overexpressing ApoE KO mice. This hypothesis remains to be tested in the future. Alternatively, IL-15Rα in BM derived cells plays a role in regulation of hepatic lipoprotein synthesis as shown in the third chapter.

Hepatic synthesis of cholesterol is the major source of TC (~80%). Hence it is important to determine whether IL-15 can affect HMGCR expression/

activity. HMGCR is an enzyme that mediates one of the early steps in the synthesis of cholesterol. Liver produces and secretes VLDL containing TG, cholesterol, and apoB48. Assessment of the liver MTTP and apoB48 expression is required to understand whether IL-15 can affect VLDL production by the liver. LPL interacts with VLDL and hydrolyzes TG and the remnant lipoprotein particle forms IDL and consequently LDL. Measuring the LPL activity/ expression in mice with different expression of IL-15 will help us to understand whether IL-15 can affect lipoprotein levels via LPL activity or not. In addition, quantifying the liver LDLR and the levels of PCSK9 expression in the context of IL-15 expression will determine if IL-15 contributes to plasma LDL clearance through hepatic pathway or not.

Our findings in the LDLR KO BMT model and ApoE KO mice seem to be consistent with the current knowledge about the potential role of immune cells and inflammatory cytokines in lipoprotein metabolism. However, the reason for the reduced level of TC and VLDL cholesterol in tKO mice compared to srbi/apoE dKO control group is unknown and requires further experiments. It has been shown that mice lacking SR-BI and apoE have abnormal vesicular and discoidal lipoprotein particles in the VLDL-size range that were rich in free cholesterol. Formation of these abnormal particles is perhaps due to the high ratio of lipoprotein surface to core as

the result of increased FC but not CE levels.[119] It is proposed that these FC rich lipoproteins contribute to the rapid formation of occlusive coronary atherosclerosis and MI in these mice.[119] Moreover, it has been suggested that VLDL sized lipoprotein fractions from srbi/aspoE dKO mice compose of particles that contain ApoAI and lack ApoB[137]. As a result, the absence of SR-BI and ApoE leads to different and more complex conditions compared to other mouse models. Hence, IL-15 may have different roles in lipoprotein metabolism depending on which mouse model is used to study.

Characterization of lipid components such as FC, CE, phospholipids, ApoB and ApoAI levels both in the plasma and VLDL-size fractions can shed light on the differences in the nature of lipoproteins in dKO and tKO mice. Correction of the lipoprotein profile and lipid measurements in tKO and dKO mice to the level of apoE KO by restoring the hepatic expression of SR-BI via adenovirus transduction[153], will help us to determine whether the opposite effects of IL-15 in SR-BI/ApoE dKO background is driven by the lack of SR-BI and consequently formation of the abnormal lipoproteins or not. It has been shown that in SR-BI KO mice, expression of wild-type levels of hepatic SR-BI, restored normal HDL size distribution and plasma FC/TC ratio.[153]

5.7 Significance:

Interleukin-15 has been reported to be present in human atherosclerotic plaques and at higher levels in serum from patients with cardiovascular diseases. We have tested the role of IL-15 in atherosclerosis by analyzing the effects of either overexpression or deletion of IL-15 on atherogenesis in the ApoE KO mice. We demonstrated that overexpressing IL-15 increases, while reducing IL-15 expression reduces atherosclerosis. Our data implicates multiple inflammatory pathways including IL-15 mediated macrophage activation and foam cell formation in its effects on atherosclerosis development. Furthermore, we showed that many of IL-15's atherogenic effects are through IL-15Rα on BM derived cells. Finally we illustrated that a lack of IL-15 in SRBI/ApoE dKO mice improved their survival and also lowered the severity of both aortic sinus and coronary artery atherosclerosis. These genetic data provide proof of principle that IL-15 may be an attractive target for therapeutic intervention.

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