The Role of Natural Killer Cells in Atherosclerosis

Ahrathy Selathurai

Bachelor of Biomedical Science (Honours)

Faculty of Medicine, Nursing and Health Sciences

Monash University

&

Baker IDI Heart and Diabetes Institute

Melbourne, Victoria, Australia

A thesis submitted to Monash University in accordance with the requirements for the degree of Doctor of Philosophy

September 2011

Contents

List of Figures

Abstract

Atherosclerosis is a progressive multifactorial disease of large elastic and muscular arteries in which lesions are characterized by the deposition of cholesterol, leukocyte influx, smooth muscle cell proliferation, cell death and collagen accumulation. Inflammatory cells of the innate and adaptive immune systems contribute to lesion development and progression. NK cells have been detected in human atherosclerotic lesions in low numbers and also detected in atherosclerotic lesions of LDLR-deficient mice fed a high fat diet. However their significance for atherosclerotic lesion development is still unclear. The studies presented in this thesis unequivocally demonstrate an important role for NK cells in the development of atherosclerosis and provide novel insights as to how these cells contribute to atherosclerosis.

Chapter 3 examines the role of NK cell in the development of atherosclerosis in ApoE-deficient mice fed a high fat diet. Despite being very minor lymphocyte population in developing atherosclerotic lesions, the study has definitely demonstrated that NK cells are proatherogenic in mice. Specific depletion of NK cells by more than 90% attenuated lesion size and macrophage accumulation. Whilst not specifically examined the reduction in macrophage accumulation could be due to lack of production of NK cell derived cytokines and chemokines in the lesions.

Chapter 4 examines whether the activation of NK cells further augments development of atherosclerosis. Activation of NK cells with Poly IC exacerbates the development of atherosclerosis. Since Poly IC can also activate T cells, the effects of Poly IC were determined in T and B cells deficient $ApoE^{-/-}$ $Rag2^{-/-}$ mice. Poly IC augmented atherosclerosis in these mice which suggest a strong dependency on NK cells. To confirm the dependency of Poly IC effects on atherosclerosis on NK cells, the effects of Poly IC in NK cell depleted mice were also determined. The depletion of NK cells reduced atherosclerotic lesion development. Therefore the study indicates a dependency of Poly IC's proatherogenic effects on NK cells.

Chapter 5 examines the mechanisms by which NK cells are activated during development of atherosclerosis. NK cells can be activated by via NKT cells and/or via their activating receptor NKG2D. Activation of NKT cells has been shown to stimulate NK cell proliferation and cytotoxicity. This study demonstrates that NKT cells contribute to NK cell activation during development of atherosclerosis since the atherosclerotic lesions were similar in mice deficient in invariant NKT cells in ApoE$^{-/-}$ Jalpha18$^{-/-}$ mice and in NK cell depleted ApoE$^{-/-}$ Jalpha18$^{-/-}$ mice. As to the role of NKG2D receptors in activating NK cells during the development of atherosclerosis, the study indicate that these receptors are not involved despite the high expression of its activating ligands Rae-1δ, Rae-1ε and MULT-1.

Chapter 6 examines the mechanisms by which NK cells promote the development of atherosclerosis. Activated NK cells produce cytokines and cytotoxicity. This study examined whether NK cells participate in atherosclerosis via production of cytokines implicated in atherosclerosis, or through secretion of cytotoxic molecules. IL-2 activated NK cells from mice deficient in cytokines interferon-gamma (IFN-γ) were adoptively transferred to NK-, NKT-, T- and B- cell-deficient ApoE$^{-/-}$ Rag2$^{-/-}$ γ$^{-/-}$ mice. Interferon-gamma was found to be unnecessary for the proatherogenic actions of NK cells, as NK cells deficient in IFN-γ were capable of exacerbating atherosclerosis to the same extent as wild-type IL-2 activated NK cells. Conversely, adoptive transfer of IL-2 activated NK cells deficient in cytotoxic molecules perforin or granzyme B did not increase atherosclerosis in ApoE$^{-/-}$ Rag2$^{-/-}$ γ$^{-/-}$ mice. Therefore the atherosclerosis-promoting effects of NK cells appear to be dependent on their cytotoxic properties. Increased cytotoxicity in atherosclerotic lesions can lead to increased necrosis and inflammation via activation of inflammasomes. Lesions from mice that received perforin- or granzyme B-deficient NKT cells had smaller necrotic areas and lower expression of pro-IL-1β and caspase-1 mRNA.

In summary, the studies indicate that NK cell within the developing lesions augment atherosclerosis by secreting cytotoxic molecules perforin and granzyme B. The magnitude of effects of NK cells on lesion development is dependent not only on NK cell numbers but also on their activation status. Increased understanding of the mechanisms of NK cell actions during atherogenesis may lead to new strategies to develop immunotherapeutic treatments to attenuate atherosclerosis.

General Declaration

In accordance with Monash University Doctorate Regulation 17/Doctor of Philosophy and Master of Philosophy regulations, the following declaration is made:

This thesis, except with the Research Graduate School Committee's approval, contains no material which has been accepted for the award of any other degree or diploma in any university or other institution. To the best of my knowledge, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

Ahrathy Selathurai

Notice 1

Under the Copyright Act 1968, this thesis must be used only under the normal conditions of scholarly fair dealing. In particular no results or conclusions should be extracted from it, nor should it be copied or closely paraphrased in whole or in part without the written consent of the author. Proper written acknowledgement should be made for any assistance obtained from this thesis.

Notice 2 *(only to be included if all reasonable efforts have been made to secure copyright permissions for any significant third-party content within the thesis)*

I certify that I have made all reasonable efforts to secure copyright permissions for third-party content included in this thesis and have not knowingly added copyright content to my work without the owner's permission.

Acknowledgements

First and foremost, I would like to thank my supervisors, Professor Alex Bobik and Professor Ban-Hock Toh for their constant supervision during my PhD journey. I would like to thank them for all their support, guidance especially Alex who has always been there to guide me in my project.

I would also like to thank everyone in the Vascular Biology and Atherosclerosis Laboratory, Baker IDI who helped me with my project especially Alex Agrotis, Peter Kanellakis and Kelly To for constantly teaching techniques, answering every questions and giving me constant moral support throughout my PhD. I would also like to thank Michael, Gina, Giovanna and Christine for their help. A special thanks to Professor Peter Tipping for contributing his ideas and constant moral support. Also a special thanks to Professor Mark Smyth for contributing his knowledge on NK cells, and members from his lab for teaching me how to work with NK cells.
Thank you to Geza Paukovics for his guidance in NK cell sorting.
I am also very grateful to Baker IDI Bright Sparks Program and my sponsors Cybec foundation for their financial support during my PhD. Also would like to thank Dr Clinton Bruce and Prof Mark Febrraio from Cellular Molecular and Metabolism Laboratory, Baker IDI for giving me time off to complete my thesis during my employment in their laboratory.

Finally I would like to thank my family and friends for their constant love and support. Thank you mum, dad, Gokulan and Divya. I am extremely grateful to my husband Devan who has been there for me since day one of my PhD journey and thank you for putting up with me throughout the whole 4 years.

List of scientific presentations and awards

Scientific presentations

Selathurai, A., Agrotis, A., Toh, B.H., Bobik, A., "The role The Role of NK cells in Atherosclerosis", Oral Presentation at the 11th Australasia Autoimmunity Workshop, 2007, Melbourne, Australia

Selathurai, A., Agrotis, A., Toh, B.H., Bobik, A., "The Role of NK cells in Atherosclerosis", Poster Presentation at the Atherothrombosis IVBM Satellite Meeting: The Disease Continuum, 2008, Melbourne, Australia

Selathurai, A., Agrotis, A., Toh, B.H., Bobik, A., " Pro-Atherogenic Effects of NK cells on the Development of Atherosclerosis Require NKT cells" , Poster presentation at the 11th Meeting of the Society for Natural Immunity, 2008, Freemantle, Australia

Selathurai, A., To, K., Agrotis, A., Tipping, P., Toh, B.H., Bobik, A., "Pro-atherogenic effects of Natural Killer cells are dependent on activation by Natural Killer T cells" , Poster Presentation at the XV International Symposium on Atherosclerosis, 2009, Boston, USA.

Awards

Selathurai A. Young Investigator Award at the XV International Symposium on Atherosclerosis, 2009, Boston, Massachusetts, USA.

Preface

Parts of this thesis have been presented in my honours thesis " Role of NK cells in Atherosclerosis" Monash University, 2006, namely Chapter 3: Figure 3.3, 3.4, and 3.5.

List of Abbreviations

AII	Angiotensin II
α-GalCer	alpha-galactosylceramide
AAA	Abdominal Aortic Aneurysm
Ab	Antibody
ABCA1	ATP binding cassette transporter A1
ACS	Acute coronary syndrome
ADCC	Ab-dependent cellular cytotoxicity
AGEs	Advanced glycation products
AHA	American Heart Association
APC	Antigen presenting cell
ApoB	Apolipoprotein B
ApoE	Apolipoprotein E
β-2GPI	beta-2-glycoprotein 1
BCA	Branchiocephalic artery
BM	Bone marrow
CARD	Caspase activation and recruitment domain
CEC	Cardiac endothelial cell
CLP	Common lymphoid progenitors
CHD	Coronary heart disease
CMV	Cytomegalovirus
CIA	Collagen-induced arthritis
CRP	C-reactive protein
CVD	Cardiovascular disease

DAB	3,3'-Diaminobenzidine
DC	Dendritic cell
DsRNA	Double-stranded RNA
EAE	Autoimmune encephalomyelitis
ECM	Extracellular matrix
ECs	Endothelial cells
eNOS	endothelial nitric oxide synthase
ER	Endoplasmic reticulum
ERK-5	Enzyme extracellular signal-regulated kinase 5
Fc	Fragment, crytallizable
FCS	Fetal calf serum
FHL	Familial hemophagocytic lymphohistiocytosis
γc	Common gamma chain
G-CSF	Granulocyte colony stimulating factor
GM-CSF	Granulocyte macrophage colony stimulating factor
Grz	Granzyme
H&E	Hematoxylin and eosin
HCMV	Human cytomegalovirus
HDL	High density lipoprotein
HFD	High fat diet
HSC	Hemotopoeitic stem cell
HSP	Heat shock protein
iDCs	immature Dendritic cells
ICAM-1	Intercellular adhesion molecule-1
ICOS	Inducible costimulatory molecule

IDL	Intermediate density lipoprotein
IEL	Internal elastic lamina
IFN-γ	Interferon-gamma
Ig	Immunoglobulin
IL-1	Interleukin-1
IL-1α	Interleukin-1 alpha
IL-1β	Interleukin-1 beta
IL-1R	Interleukin-1 receptor
IL-2	Interleukin-2
IL-4	Interleukin-4
IL-5	Interleukin-5
IL-6	Interleukin-6
IL-7	Interleukin-7
IL-8	Interleukin-8
IL-10	Interleukin-10
IL-12	Interleukin-12
IL-15	Interleukin-15
IL-18	Interleukin-18
IL-21	Interleukin-21
iNKT	Invariant natural killer T
iNOS	Inducible nitric oxide synthase
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inhibitory motif
Jalpha18$^{-/-}$	J alpha 18 knockout
KIR	Killer cell immunoglobulin-like receptor

LDL	Low density lipoprotein
LDLR	Low density lipoprotein receptor
LFA-1	Leukocyte function-associated antigen 1
LIR	Leukocyte Ig-like receptors
LILR	Leukocyte Ig-like inhibitory receptors
LPS	Lipopolysaccharide
LT-α	Lymphotoxin-alpha
MCP-1	Monocyte chemotactic protein-1
M-CSF	Macrophage colony stimulating factor
MDA-5	Melanoma differentiation associated antigen 5
mDC	Myeloid dendritic cell
MDDC	Monocyte - derived dendritic cells
Mertk	Mer tyrosine kinase
MHC	Major histocompatibility complex
MI	Myocardial infarction
MIF	Macrophage migration inhibitory factor
MIP	Macrophage inflammatory protein
MMP	Matrix metalloproteinase
MPO	Myeloperoxidase
MULT-1	UL16-binding-protein-like transcript-1
MyD88	Myeloid differentiation factor 88
NCRs	Natural cytotoxicity receptors
NF-κB	Nuclear factor-κB
NK cell	Natural killer cell
NKT cell	Natural killer T cell

NO	Nitric oxide
NOD	Nonobese diabetic mice
NOS	Nitric oxide synthase
NZW	New Zealand White
OxLDL	Oxidised low density lipoprotein
PDAY	Pathobiological Determinants of Atherosclerosis in Youth
PAI-1	Plasminogen activator inhibitor-1
PAMP	Pathogen-associated molecular pattern
pDC	Plasmacytoid dendritic cell
PDGF	Platelet-derived growth factor
Pfp	Perforin
PMA	Phorbol myristate acetate
PMN	Polymorphonuclear leukocyte
Poly IC	Synthetic polyinosinic-polycytidylic acid
PRR	Pattern Recognition Receptor
RA	Rheumatoid arthritis
Rae-1	Retinoic acid early transcript-1
Rag	Recombination activating gene
RANTES	Regulated upon Activation, Normal T-cell Expressed, and Secreted
ROS	Reactive oxygen species
SAP	Sphingolipid activator protein
SCID	Severe combined immunodeficiency
SLE	Systemic lupus erythematosus
SMCs	Smooth muscle cells
SR-A	Scavenger receptor type A

T1D	Type 1 diabetes
TCR	T cell receptor
TGF-β	Transforming growth factor-beta
TGF-β.RII	Transforming growth factor-beta receptor II
Th	T helper
Th0	Naïve T cell
Th1	T helper type 1
Th2	T helper type 2
Th3	T helper type 3
Th17	T helper type 17
TICAM-1	Toll-IL-1 receptor (TIR) domain containing adaptor molecule-1
TIR	Toll-IL-1 receptor
TLR	Toll-like receptor
TNF-α	Tumour necrosis factor-alpha
Tr1	Regulatory T cell type 1
TRAIL	TNF-related apoptosis-inducing ligand
Treg cell	Regulatory T cell
TRIF	TIR-domain containing adapter inducing Interferon-β
ULBP	UL16-binding protein
VCAM-1	Vascular cell adhesion molecule-1
VLDL	Very low density lipoprotein
WHHL	Watanabe heritable hyperlipidemic
WHO	The World Health Organization
WT	Wild type

1 Introduction

1.1 Atherosclerosis

Atherosclerosis is the most common cause of heart disease and stroke. It is a chronic inflammatory disease of medium and large sized arteries characterized by excess lipid deposition [1, 2]. The disease develops slowly and can even be present in early childhood as pre atheromatous fatty streaks. More advanced disease results in plaques in the most inner layer of the arterial wall, the tunica intima, which over time grow and narrow the lumen, restricting blood flow. This restriction of blood flow to heart muscle can result in stable exercise-induced angina. Advanced plaques that are fragile are normally associated with outward vessel remodelling and can rupture, causing thrombus formation and arresting blood flow. Initially atherosclerosis was considered to be solely a lipid storage disease, the consequence of excess lipid accumulation in vessels but it is now recognized that inflammation contributes to its pathogenesis [3]. The identification of immune cells, macrophages and T cells in atherosclerotic lesions supports the involvement of immune mechanisms in atherosclerosis [4, 5].

1.1.1 Historical perspective

Atherosclerosis is a disease of ancient time. It was present in Egyptian mummies where degenerative arterial changes were identified in arteries of Egyptian mummies dating from 1500 B.C to 250 AD [6]. It was also shown that these arteries exhibited regions of lipid accumulation and medial calcification [7]. The term “atheroma” to describe a clinically significant atherosclerotic lesion, was first proposed by Albrecht von Haller in 1755. Subsequently, Joseph

Hodgson proposed that inflammation occurred in atheromatous arteries [8]. The first mechanistic insight into atherosclerosis was provided by Alexander Ignatowski who reported that rabbits fed milk and egg yolk developed atherosclerosis [8]. In 1913 Anitschkov and Chalatov demonstrated that cholesterol fed to rabbits induced atherosclerosis and proposed that excess cholesterol was a major cause of atherosclerosis.

Russell Ross proposed the "response to injury" hypothesis, to provide mechanistic insights into atherosclerosis. Specifically he proposed that endothelial injury caused by factors such as mechanical injury, modified LDL and hypertension initiated atherosclerosis [9, 10]. Subsequently, it was reported that lymphocytes and macrophages were present in atheromatous plaque [11]. Histological studies of ruptured plaques from patients with acute coronary syndrome confirmed the presence of macrophages, T cells as well as mast cells at rupture sites. The Pathobiological Determinants of Atherosclerosis in Youth (PDAY) study also identified activated T cells, macrophages, dendritic cells, and MHC class II expression in atherosclerotic lesions of juvenile American patients aged 15-34 who had no clinical symptoms, suggesting that these cells might contribute to atherosclerosis development [12]. Over the last decade, it has become apparent from clinical and preclinical studies that immune cells are important contributors to atherosclerosis. In the Framingham Heart 26 years follow up study, 35% of patients with cardiovascular disease had a total plasma cholesterol level of less than 200mg/dl, providing support that other factors in addition to cholesterol contributed to disease [13]. It is now accepted that immune responses from both innate and adaptive immune cells participate in the development of atherosclerotic lesions.

1.1.2 Epidemiology and Risk Factors

Atherosclerosis is the leading cause of death in developed countries and is most prevalent in western societies. The World Health Organization (WHO) predicts a worldwide epidemic of this disease as more developing countries adopt the western habits [14]. According to a survey by the American Heart Association (AHA) in 2010, it is estimated that 81,100,000 American adults (more than one in three) have one or more types of cardiovascular disease. These include 74,500,000 with high blood pressure, 17,600,000 with coronary heart disease (CHD) that includes those who have suffered myocardial infarction (MI) or have angina pectoris, 5,800,000 with heart failure, 6,400,000 who have suffered from strokes and 650,000 – 1,300,000 with a congenital cardiovascular disorder [15]. In Australia, reports by the National Heart Foundation of Australia in 2003, indicate that cardiovascular disease remains the largest single cause of mortality, 34% in males and 39% in females [16].

The AHA (2002) has identified a number of risk factors for CHD initiated by atherosclerosis. They include high cholesterol levels, high blood pressure, and poor lifestyle. Since atherosclerosis is an inflammatory disease, there are several factors that lead to inflammation including injury to the endothelium. Possible causes of injury to the endothelium include, elevated and modified LDL, free radicals from smoking, hypertension, diabetes mellitus, genetic abnormalities, elevated plasma homocysteine levels, and infections. In addition, predisposition to CHD increases with advancing age, gender (male), heredity, race, and genetic abnormalities.

Hypercholesterolemia plays a critical role in atherosclerosis. Low density lipoprotein (LDL) accumulating in the intima can be oxidized [17] and internalized by macrophages resulting in foam cell formation [18, 19]. In addition oxidisation of denatured lipids can initiate

inflammation by increasing the expression of adhesion molecules by the endothelium, and chemokines and pro-inflammatory cytokines by both macrophages and the endothelium.

Hypertension is an important risk factor for atherosclerosis. Angiotensin II (AII), an important hypertensive agent and vasoconstrictors can initiate inflammation by triggering the production of reactive oxygen species within the arterial wall [20]. AII can activate T cells [21] and also increase the expression of inflammatory cytokines and chemokines such as IL-6 and MCP-1 by smooth muscle cells SMCs and VCAM-1 by endothelial cells [22, 23].

Hyperglycemia causes the endothelium to increase the production of free radicals which in turn reduces nitric oxide, a chemical important in blood vessel dilation and reducing inflammation. Diabetes aggravates atherosclerosis by interrupting the function of the enzyme extracellular signal-regulated kinase 5 (ERK) [24]. ERK-5 activates endothelial nitric oxide synthase (eNOS) to produce nitric oxide and dilate blood vessels. Patients with diabetes also produce high levels of advanced glycation end products (AGEs) [25]. AGEs promote production of free radicals and inflammation and also interrupt the athero-protective role of ERK-5. Also, interruption of ERK-5 function promotes the adhesion of pro-inflammatory leukocytes and secretion of pro-inflammatory cytokines [25].

Obesity is also a risk factor for atherosclerosis and may lead to development of insulin resistance and diabetes; it can also contribute to dyslipidemia. Obesity is associated with endothelial dysfunction, partly due to reduced bioavailability of nitric oxide [26]. In addition, adipose tissue can release inflammatory cytokines such as TNF-α, IL-6, complement factor C3, AII and plasminogen activator inhibitor-1 (PAI-1), factors that promote atherosclerosis [27]. Circulating TNF-α is markedly elevated in obese patients [28]. Leptin secreted by adipocytes also plays a role in obesity-related endothelial dysfunction [29]. It stimulates in endothelial cells (ECs) the production of superoxide to increase oxidative stress in endothelial cells, CCL2 expression and endothelin-1 in these cells; it can also promote the migration and proliferation of

SMCs [30, 31]. Treatment of ApoE$^{-/-}$ mice with recombinant leptin also promotes atherosclerosis and thrombosis [32].

Chronic infections also contribute to the development of atherosclerosis [33] . Infection by *Chlamydia pneumonia* in human plaques, releases endotoxin and heat shock protein which activate vascular endothelial cells, SMCs and macrophages, enhancing inflammation during atherogenesis [34]. The presence of *C.pneumonia* in atherosclerotic lesion has been reported in a number of studies, and the presence of *C.pneumonia*-specific T lymphocytes, primarily the CD4^{+} Th1 subtype, has been demonstrated. These T lymphocytes maintain inflammatory responses due to infection and augment progression of atherosclerosis [35, 36]. *C.pneumonia* infection in ApoE$^{-/-}$ mice accelerates the development of atherosclerosis [37]. This pathogen can multiply within the cells of atherosclerotic lesions, including endothelial cells and macrophages. *In vitro* studies indicate that monocytes infected with *C.pneumonia* transmit the pathogen to endothelial cells [38], thereby upregulating the expression of adhesion molecules and secretion of pro-inflammatory cytokines. Infection of endothelial cells triggers the production of IL-8, IL-6 and PAI-1. Infected macrophages secrete pro-inflammatory cytokines including TNF-α, IL-1β, IL-6 and MCP-1, MIP-1 and IL-12 [39, 40].

Autoimmune diseases are also a major risk factor for atherosclerosis. Several autoimmune rheumatic conditions, including rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) enhance atherosclerosis and subsequently contribute to higher cardiovascular morbidity and mortality rates [41]. RA is a chronic inflammatory disease and both atherosclerosis and RA have similarities in their inflammatory pathways. Patients with RA have increased risk of cardiovascular events [41]. RA has been considered an independent risk factor for atherosclerosis [42]. Dyslipidemia is a common risk factor observed in RA which is associated with low levels of HDL and high levels of triglyceride [43]. High levels of pro-inflammatory cytokines in RA have also been associated with the development of

atherosclerosis. The chronic systemic inflammation that occurs in RA can contribute to endothelial dysfunction and oxidative stress to promote atherosclerosis [44]. TNF-α and interleukin-1 expressed in joints affected by RA, may act on other tissues and promote atherogenesis. The levels of C-reactive protein (CRP), a marker for inflammation, are also elevated in RA patients. This marker for inflammation has also been implicated in atherosclerosis [44, 45].

SLE is a complex multisystem inflammatory disease that predominantly affects young women [46]. Atherosclerosis develops early in the course of this disease. Risk factors that could contribute to atherosclerosis development are CRP, fibrinogen, IL-6, CD40/CD40L, adhesion molecules; immunological factors: αCL, anti-B2GPI, and anti-oxLDL; abnormal coagulation factors; plasminogen activator inhibitor-1, and homocycteine and the lipoprotein HDL [47].

1.1.3 Changes in Vessel Morphology Associated with Development of Atherosclerotic Lesions

The normal human arterial wall consists of three main regions, the innermost tunica intima region, the middle media region and an outer region, the adventitia which embeds the vessel in its surrounding (Figure 1.1A).

The intima is defined as the region commencing at the endothelium surface and extending to the luminal margin of the media [48]. It is composed of proteoglycan [49], occasional macrophages and SMCs. The SMCs can have a mixed phenotype, either synthetic or contractile. This layer increases in thickness with increasing age as well as in subjects who exhibit increased susceptibility to atherosclerosis.

The endothelium layer not only covers the intima but also serves as a barrier between circulating molecules and cells in blood. The endothelium synthesizes signalling molecules that can regulate both vascular tone and structure, and inhibit platelet adhesion and aggregation, leukocyte adhesion and migration, and SMCs proliferation and migration [50-52]. Functional and structural changes in ECs can contribute to the pathogenesis of atherosclerosis. Scanning electron microscopy of human carotid plaques indicate increased thickness and delamination of the connective tissue underlying the endothelium. Endothelial cells in these areas were partially separated from each other and nearly detached from the basal lamina. The endothelial cells appear to be irregular in shape, and had formed microvilli and pseudopodia. There were also areas in atherosclerotic carotid arteries where the endothelial layer was completely absent, exposing the basal lamina and covered by a fibrous reticulum with entrapped cells, mainly erythrocytes. In normal carotid arteries, the basal lamina is completely covered by a well junctioned single layer of endothelial cells [53]. Under the endothelial monolayer, the subendothelial intima of human arteries is populated by predominantly SMCs, some immune cells and extracellular matrix [54, 55]. The number and morphology of cells especially SMCs in an atherosclerotic lesion increases substantially compared to normal intima [54]. The SMCs in the intima have different morphology including being elongated, stellate, with side processes and irregularly shaped. At the site of atherosclerotic lesions, the stellate cell population increases substantially and appear to have a synthetic phenotype which is rich in rough endoplasmic reticulum. During atherogenesis, SMCs are thought to migrate from the medial layer through the fenestrations in the internal elastic lamina to the intima in response to injury, and then proliferate and produce extracellular matrix which contributes to the increase in plaque size.

A SMC subpopulation with unique characteristics such as enhanced growth capabilities, and increased proliferation and contraction ability probably contributes to pathological conditions. *In vitro* studies have suggested that the arterial media might be composed of a

phenotypically and functionally diverse subpopulation of SMCs [56]. Originally it was thought that SMC in atherosclerotic lesions were solely derived from the media, but recently it has been suggested that bone marrow progenitor cells infiltrate the intima and probably differentiate *in vivo* to form SMCs [57]. There is also evidence from human studies that after bone marrow transplant, SMCs of donor origin are increased in coronary atherosclerotic plaque [58].

The internal elastic lamina separates the media from the intima. The media is the thickest layer of normal vessels and contains 20% of SMCs of both contractile and synthetic phenotype, and 60% of collagen and elastin which provides the contractile ability for the vessel. The medial lamellar unit is both the structural and functional unit of most arteries. Medial lamellar units are oriented in concentric layers or lamellar units with SMCs and collagen in between in a fairly uniform composition and are constant in size regardless of species and vessel size [59]. The number of lamellar units are proportional to the circumferential tension in the arterial wall [59]. The media layer contains collagen to maintain the integrity of the vessel wall which includes mainly type 1 collagen [60]. Medial SMCs which are normally quiescent, can contribute to the development of atherosclerosis in response to endothelial injury, by proliferating and migrating into the subendothelial intima. In early lesions SMCs are present in various numbers, as very early lesions progress to fibrofatty streaks. Medial SMCs are also implicated in the development of Abdominal Aortic Aneurysm (AAA), which is characterized by structural deterioration of aortic wall leading to arterial dilation and subsequent ruptures also known as arterial remodelling. Patients with AAA frequently have decresed medial SMC which is associated with arterial remodelling [61]. *In vitro* and *in vivo* studies suggest that changes in media occur in response to shear stress alterations caused by arterial stenosis [62]. In order to normalize lumen diameter and shear stress, extracellular matrix in the medial layer remodels, enabling expansion of the artery and the repetition of such remodelling results in medial thinning [62].

The outermost layer of the vessel, the adventitia is separated from the media by an external elastic lamina and consists of extracellular matrix composed of connective tissues (collagen type I and type II) and fibroblasts. The human adventitial fibroblast population includes mesenchymal progenitor cells that can differentiate into adipocytes, osteoblasts, myofibroblasts and SMCs [63, 64]. This layer changes function and can contribute to pathology of atherosclerosis [65]. There is evidence for a role of lymphocytes in the adventitia in vessel disease [65-67]. In the setting of murine hyperlipidemia, lymphocytes are present in clusters within the adventitia of atherosclerotic abdominal aorta [67, 68]. Both T and B cells have been identified within the adventitia of atherosclerotic lesions [69, 70]. Complex lymphoid-like structures or nodules within the adventitia of advanced lesions are composed primarily of B cells surrounded by macrophages and T cells and it is thought that these are centres for antigen-derived B cell maturation to generate antibodies against inflammatory antigens generated by advanced lesions [67]. These lymphoid organ-like structures are also found in human plaque but not in normal blood vessels [71], and also appear to contain lymphatic vessels and drain local inflammatory cells and cytokines to the lymph nodes These structures ensure a constant delivery of active inflammatory cells and cytokines to promote chronic inflammation within the vessel [72].

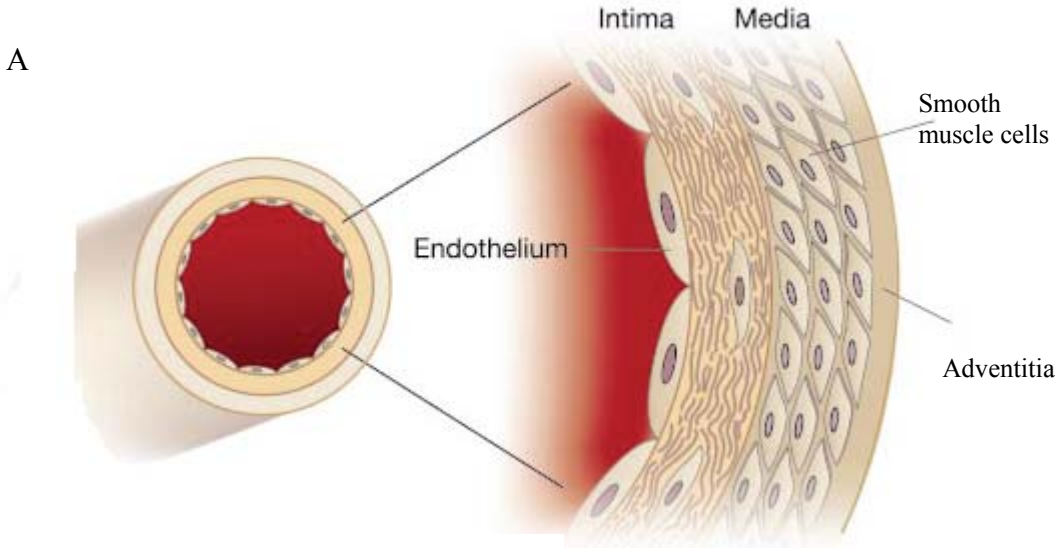


Figure 1.1: Structure of arterial wall. Schematic adapted from Libby et al. 2002 shows the intima, media and adventitia of (A) normal artery and (B) diseased artery (vulnerable plaque) with thin fibrous cap and large lipid pool [73].

1.1.4 Sites of Atherosclerosis Development

Atherosclerosis occurs at specific sites within the vasculature, mainly in large elastic and medium sized muscular arteries including the abdominal aorta and coronary arteries. It is a focal disease that localizes to reproducible sites in vessels [74]. These highly susceptible atherosclerotic sites are exposed to low shear stress, oscillatory flow and turbulent flow normally in areas that have high vessel curvatures and at branching sites. Areas of vessels exposed to laminar flow are generally highly resistant to atherosclerosis. Sites where atherosclerotic lesions develop include coronary arterial bed, major branches of arch, visceral branches of abdominal aorta, and terminal abdominal aorta and its branches (Figure 1.2).

Endothelial cells respond to changes in blood flow particularly changes to hemodynamic shear stress [75].These cells align according to the axis of laminar flow, and this alignment is disrupted in areas of interrupted flow. Endothelial cells express genes that are regulated by flow rate, including cell-surface adhesion molecules (vascular cell adhesion molecule- 1 [VCAM-1] and intracellular adhesion molecule-1 [ICAM-1]), and antioxidant enzymes (NO synthase, superoxidase dismutase). Cultured human endothelial cells upregulate VCAM-1 and ICAM-1 when exposed to oscilatory flow, effects mediated by nuclear factor-κB (NF-κB) [76, 77]. Nitric oxide (NO) is the product of conversion of L-arginine into L-citrulline by nitric oxide synthase (NOS). It plays a major role in modulating vascular tone and is regulated by laminar shear stress. Oscilatory shear stress downregulates the expression of eNOS [78]. Hypercholesterolemia downregulates the bio-availability of endothelium-derived NO which in turn increases neutrophil adherence to the endothelium [79]. A deficiency in NO also enhances SMCs proliferation and platelet aggregation and adhesion [80]. In rabbit models of hypercholesterolemia, chronic inhibition of NO synthesis accelerates the development of atherosclerosis [81]. ApoE$^{-/-}$ mice

which are deficient in endothelial nitric oxidase synthase fed a Western diet exhibit an increase in the development of atherosclerosis [82].

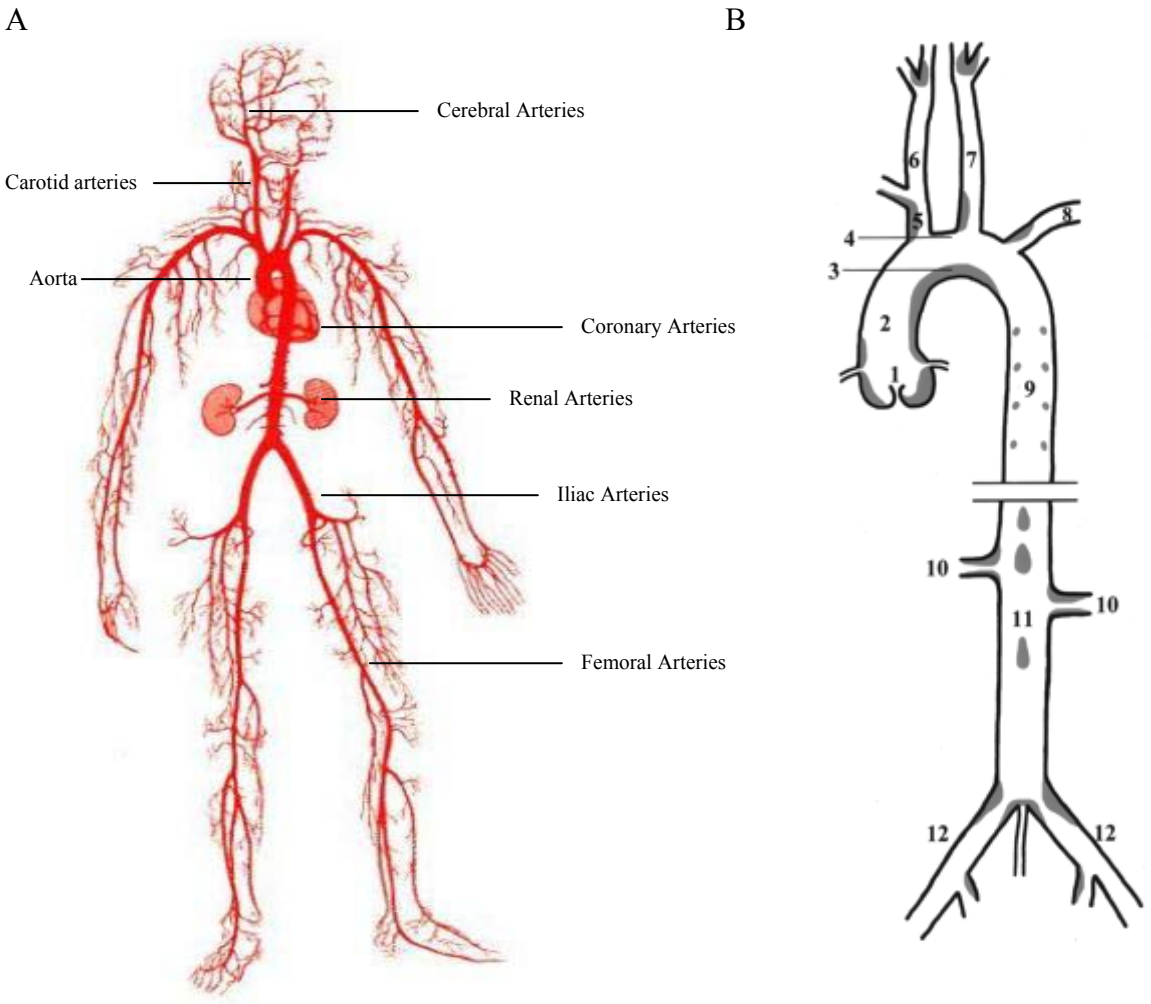


Figure 1.2: Common sites of atherosclerosis lesion formation in humans and C57BL/6 mouse strain. (A) Schematic shows the common sites of atherosclerotic formation in human vasculature [83]. (B) Schematic shows the major arterial vasculature distribution of atherosclerosis (gray shading) in the vasculatures of LDL receptor–deficient mice fed a high-fat atherogenic diet. 1, indicates aortic sinus; 2, ascending aorta; 3, lesser curvature of aortic arch; 4, greater curvature of aortic arch; 5, innominate artery; 6, right common carotid artery; 7, left common carotid artery; 8, left subclavian artery; 9, thoracic aorta; 10, renal artery; 11, abdominal aorta; and 12, iliac artery, figure adapted from VanderLaan et al. 2004 [74]

1.1.5 Atherosclerotic lesions

Atherosclerotic lesions have been classified into a number of subtypes by the American Heart Association (AHA). “Early” implies that these lesions are followed by “latter” (advanced) lesions. It also implies that they are found early in life. Neither implication is necessarily true. Early atherosclerotic lesions can be classified into types I, II and III. Type I lesions, also called intimal xanthomas, are the initial lesions in atherosclerosis. Type I lesions usually consist of small isolated groups of macrophages containing lipid droplets (Figure 1.3A). They are most apparent in regions of the intima that have an adaptive intimal thickening of the eccentric type. Most type I lesions are only visible under a microscope [84-87].

Type II lesions, commonly known as fatty streaks, are the first grossly visible early atherosclerotic lesion, frequently visible as yellow-coloured streaks, patches or spots on the intima surface [86]. They are characterized by stratified layers of macrophage-derived foam cells, lipid - containing intimal SMCs, T lymphocytes, few mast cells and small amounts of extracellular lipid droplets (Figure 1.3B) [86, 88, 89] .

Type III lesions, also called intermediate or preatheroma lesions, are a progressive form of the fatty streak. These lesions consist of increased extracellular lipid droplets and foam cells, and increased numbers of SMCs and T cells [86]. A thin fibrous cap, which is a distinct layer of connective tissue and SMCs, also starts to develop in these lesions (Figure 1.3C) [84, 90]. As the size of the intima enlarges from an influx of cells, the vessel may remodel outwards to preserve the lumen size.

1.1.5.1 Advanced and complicated atherosclerotic lesions

A Type IV lesion, also called an atheroma, is an advanced lesion according to the AHA classification [90]. It consists of a dense accumulation of extracellular lipid that occupies a well defined region of intima known as the lipid core. The lipid core develops from coalesence of small isolated pools of extracellular lipids of type III lesions [90] and first appears at sites of adaptive intimal thickening of eccentric type [87]. Between the lipid core and endothelial surface, the lesion contains macrophages, SMCs [90], T lymphocytes and mast cells (Figure 1.3D) [11].

Type V lesions are lesions in which prominent new fibrous connective tissue has formed [90]. These lesions consist of a fibrous cap overlying a large necrotic core [84]. Type V lesions may contain two or more lipid cores of different sizes (Figure 1.3E). These lesions may show fissures, haemorrhage, and thrombi. They can be further subdivided into 3 subtypes. Type Va lesions consists of fibrous tissue covering a plaque containing lipid core; if the plaque also contains a lipid core that is calcified, it is classified as a type Vb lesion. If there is significant fibrotic tissue in the lesion but little lipid, it is categorized as type Vc [90]. The later lesions are stable lesions.

Type VI lesions are the most advanced lesion types that are formed from type IV and V lesions and exhibit features such as hematoma, fissures and thrombotic deposition (Figure 1.3F) [84]. Thin fibrous atheromas are prone to thrombosis and haemorrhage [84]. Such atheromas contain either a scant population of SMCs, or none at all, due to apoptosis [91]. The major molecular mechanism contributing to plaque rupture is thought to be the loss of SMCs and excessive degradation of the extracellular scaffold of the fibrous cap via secretion of matrix-degrading metalloproteinase (MMPs) by macrophages and mast cells [92, 93]. Macrophage

derived MMPs have been shown to degrade collagen *in vitro* and *in vivo*; collagen determines the biomechanical strength of the fibrous cap of atherosclerotic lesion [94].

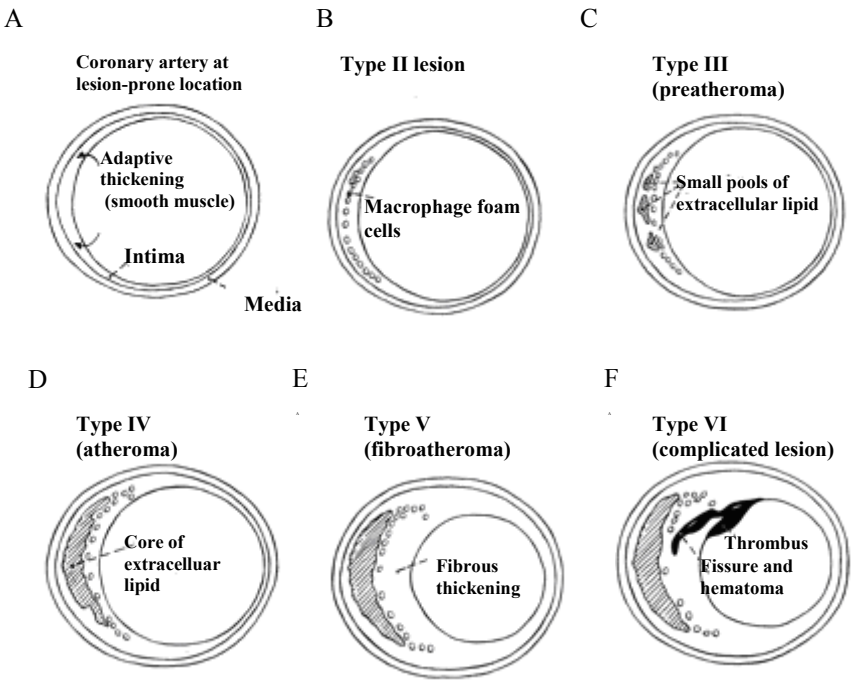


Figure 1.3: Types of atherosclerotic lesions. Schematic adapted from Stary et al. (1995) shows the cross-section and morphology of the intima ranges from (A) adaptive intimal thickening (type I), (B) type II, (C) type III, (D) type IV, (E) type V and (F) to type VI lesion in advanced atherosclerotic disease [90].

1.2 Theories on the Pathogenesis of Atherosclerosis

Several hypotheses have been proposed as to how atherosclerotic lesions develop. They include the "injury hypothesis", the "response to retention", the "inflammation hypothesis" and the "autoimmune hypothesis".

1.2.1 Response to Injury Hypothesis

The response to injury hypothesis was proposed by Russell Ross [51], who postulated that the development of atherosclerosis is the result of damage primarily to the endothelium based on observations that platelet derived serum factors stimulated the proliferation of arterial SMCs leading to development of an arterial intima [51]. The loss of endothelium exposes the underlying collagen layer and SMCs to platelet derived growth factor (PDGF) from platelets that adhere to the exposed subendothelial connective tissue. The infiltration of platelets leads to migration and proliferation of SMCs from the medial layer. The proliferation of SMCs in the intima forms new connective tissues and ultimately results in intracellular and extracellular lipid deposition. The uncontrolled migration and proliferation of SMCs was thought to eventually cause artery occlusion. Repetition and amplification of this process leads to the development of complex lesions. Ross acknowledged that the endothelium plays a more active role in the pathogenesis of atherosclerosis. It was recognized that endothelial cells are in an activated state and promote cell adhesion and invasion of monocytes, secrete oxygen free radicals; they also synthesize and secrete PDGF-like protein stimulating medial SMC migration and proliferation [95]. The mechanism of macrophage accumulation in the intima was not discussed in this hypothesis. Subsequently, the hypothesis was modified to include injury to the endothelium

caused by chronic hyperlipidemia, infections, mechanical factors and immunological injury that lead to endothelial dysfunction. Endothelial dysfunction due to endothelial injury increased the trapping of lipoprotein in branch points of the arterial tree and upregulation of adhesion molecules and chemokines on the surface of the endothelium [96, 97]. These adhesion molecules and chemokines attracted monocytes to attach to the endothelium and migrate into the intima. Monocytes then differentiate into macrophages. These macrophages take up lipoprotein to form foam cells and subsequently release inflammatory chemokines, and cytokines and growth factors to promote fatty streak and atheroma development [97].

1.2.2 Response to Retention Hypothesis

The response to retention hypothesis was proposed in 1995 by William *et al* [98]. It proposed that the extracellular trapping of cholesterol-rich atherogenic lipoprotein within the arterial intima is responsible for atherosclerosis. Atherogenic lipoproteins which enter the arterial wall bind and are retained through interactions with proteoglycans secreted by SMCs. Hypercholesteremia results in a rapid retention of lipoproteins [99] which are subsequently modified by oxidation or other enzymes. Oxidized and denatured LDL is taken up macrophages to form foam cells [100]. The retention of lipoprotein by proteoglycans was demonstrated in transgenic mice expressing human apoB100 containing low-density lipoprotein with normal proteoglycan binding or defective LDL-proteoglycan binding, where mice expressing proteoglycan-binding-defective LDL developed significantly less atherosclerosis than mice expressing wild-type control LDL due to the inability of apoB100-modified LDL to bind to proteoglycans in the vessel wall [101]. This study suggested that atherosclerosis is initiated by sub-endothelial retention of atherogenic lipoprotein.

1.2.3 Atherosclerosis: An Inflammatory Disease

In 1999 Ross proposed that atherosclerosis is an inflammatory disorder of blood vessels modifying his earlier "Response to Injury" hypothesis [102]. Damage to the endothelium was caused by agents such as modified LDL, dyslipidemia, elevated plasma homocysteine concentrations, hypertension, diabetes, and pathogens. These agents stimulated the endothelial layer to lose its normal function and increase its permeability to lipoproteins and other plasma constituents and increase the expression of adhesion molecules and growth factors which promote the adherence and migration of monocytes, macrophages and T lymphocytes. These immune cells migrate through the endothelial layer and reside at the subendothelial layer. Macrophages accumulate lipid and form foam cells, which then trigger the release of growth factors and cytokines that promote migration and proliferation of SMCs resulting in the formation of fatty streaks. Inflammation plays a fundamental role in mediating all stages of atherosclerotic lesion development from the initiation, progression to the destabilisation of atheromas. However, each stage of lesion formation can be reversible, by removing the cause of injury, or halting early inflammatory processes. There are several inflammatory mediators which predict the likelihood of developing vascular disease. These include IL-6, TNF-α, ICAM-1, and C-reactive protein (CRP) all of which have been associated with CHD [103-105]. CRP is a sensitive, unspecific marker for inflammation. In bacterial infection, CRP levels increase dramatically within 24 hours of infection [106]. Elevated CRP levels are known to be present in over 65% of patients with unstable angina [3]. CRP is capable of binding and activating complement, inducing expression of adhesion molecules, mediating LDL uptake by macrophages and inducing recruitment of monocytes and production of MCP-1[3]. There is also direct association of IL-6 and CVD, where IL-6 levels were elevated in patients with unstable angina [107].

1.2.4 Atherosclerosis: An Autoimmune Disease

Autoimmunity is an immune response to self-antigens that involves autoantibody or self-reactive T cells in pathogenesis of disease. Wick postulated that the inflammatory immunological processes characteristic of the very first stages of atherosclerosis are initiated by humoral and cellular immune reactions against heat-shock proteins 60 (HSP60) [108]. HSP60 is expressed by cells under conditions of stress such as heat and toxins. HSP60 has been identified as the autoantigen present during development of atherosclerosis. Studies have shown that immunization of normocholesterolemic rabbits with heat-killed mycobacterium and recombinant mycobacterium HSP65 leads to development of atherosclerotic lesions and this is irreversible in the presence of high levels of blood cholesterol [109, 110]. Also, cholesterol fed mice develop aggravated lesions when immunized with mHSP65 [111]. In humans, antibodies against mycobacterial HSP65 have been implicated in carotid atherosclerosis [112]. Chronic infections also play a role in the pathogenesis of atherosclerosis [113]. In a study by Bruneck, it was shown that chronic infections contribute to development of carotid atherosclerosis, where increased levels of soluble HSP60 and Abs to bacterial HSP60 are common [114]. Another autoantigen for atherosclerosis is oxidised LDL [115]. Antibodies to oxLDL have been detected in patients with atherosclerosis [116] and T cells from human atherosclerotic lesions have been shown to respond to oxLDL [117], indicating that oxLDL is an important antigen involved in immune responses in atherosclerosis. However this hypothesis, like others discussed previously, does not explain why lesions localize to specific sites of the vasculature.

1.3 Animal models of atherosclerosis

Atherosclerosis is a complex disease, which in humans develops over decades. This greatly limits approaches that can be used to study mechanisms as to how atherosclerosis develops and progresses. Thus animal models of atherosclerosis have been utilized, including rabbit, swine, non-human primates and mouse models.

Early rabbit models mostly utilized New Zealand white rabbits [8, 118]. In diet - induced atherosclerosis, rabbits are usually fed 0.5 – 2% containing cholesterol diets for 4-16 weeks depending on the severity of disease required. Lesions occur in aortic arch and ascending aorta. The Watanabe heritable hyperlipidemic (WHHL) rabbit strain is also widely used because of its hyper responsiveness to cholesterol or spontaneous hypercholesteromia [119]. The genetic defect resides in the gene for LDL receptors. This strain's cholesterol levels while on chow diet are close to these found in human familial hypercholesterolemia [118], and the lesions that develop are close to human counterparts [119]. Transgenic rabbit models have also been developed to elucidate mechanisms involved in the development of atherosclerosis. Such rabbits overexpress transgenes for human apo A, apoA-I, apoB, and lecithin: cholesterol acyltransferase (LCAT) in both NZW and WHHL strains [120]. Human apoA-I expression in both NZW and WHHL significantly reduces aortic atherosclerosis. However, expression of apoE2 in NZW rabbits increased susceptibility to atherosclerosis.

Swine are also used to study atherosclerosis, as they develop spontaneous atherosclerosis in vessels and intimal thickenings are present in coronary arteries and can be augmented by dietary interventions [121]. Early fatty streaks develop by 6 months of age and advanced lesions develop after one year. Lesion characteristics in swine are very similar to humans. They also have similar lipoprotein profiles and metabolise lipoproteins similar to humans. The use of high

fat, high cholesterol diets further elevates the total and LDL plasma cholesterol. Pigs that overexpress the human ApoE4 gene exhibit atherosclerosis-induced heart failure, implicating the ApoE4 gene as a predictor for chronic heart disease in humans [122].

Non-human primates also exhibit susceptibility to atherosclerosis similar to humans. Atherosclerosis lesions are localized to coronary arteries, abdominal aorta and iliac arteries [123]. Non-human primates used include cynomolgus, rhesus monkeys, cebus, and squirrel and pigtail monkeys. Cholesterol-laden diet in monkeys further augments the development of atherosclerosis [123]. Familial LDL receptor deficiency atherosclerosis has been reported in the rhesus monkey [124].

The most extensively studied mammal is the mouse, mostly because of the ease of genetically manipulating their genome. Thompson and Roberts were the pioneers in creating inbred strain for experimental atherosclerosis [125, 126]. The C57BL/6 mouse strain is the more susceptible strain to atherogenic diets than other strains, and lesions are present after 14 weeks on atherogenic diet [127]. Mouse strains that are more resistant to diet-induced atherosclerosis include BalB/c, C3H, A, SWR and NZB [127, 128]. C57BL/6 mice or wild type (WT) mice have normal plasma cholesterol, plasma triglyceride levels, and high density lipoprotein (HDL) is the predominant lipoprotein with only small amounts of VLDL and LDL [129]. WT mice develop small lesions when fed western diet over a long period of time. Knockout and transgenic mice on C57BL/6 background are extensively used to study atherosclerosis. Apolipoprotein E (ApoE) deficient mice and low density lipoprotein (LDL) receptor deficient mice are the most commonly studied mouse models of atherosclerosis.

1.3.1 ApoE-deficient (ApoE$^{-/-}$) mice

Apolipoprotein E- deficient (ApoE$^{-/-}$) mice are commonly used to study atherosclerosis, and were created by homologous recombination in embryonic stem cells. These mice developed severe hypercholesterolemia and atherosclerotic lesions are similar to humans [130]. The knockout model was generated by inactivating the ApoE gene [131]. The lack of a functional ApoE gene prevents these mice from producing apolipoprotein E, a glycoprotein that is essential for the transport and metabolism of lipids. Both humans and mice lacking ApoE exhibit hypercholesterolemia, near normal triglycerides, decreased HDL levels and elevated LDL and VLDL levels [132].

ApoE is a 34 kD glycoprotein synthesized in liver, brain, intestine lung and macrophages in both humans and mice [133]. The function of apoE is to facilitate binding of lipoproteins to cell surface proteins, which enhances the transfer of components such as cholesterol ester and tri-glycerols to or from cells [129]. It has a high affinity ligand for apoB and ApoE (LDL) receptor and chylomicron remnant receptor which are used facilitate uptake of ApoE-containing particles by liver [132]. ApoE also serves as an immune modulator, macrophages express apoE, the activation of T cells by macrophages induce secretion of IFN-γ, which in turn inhibit the expression of ApoE on macrophages [134]. They also downregulate TH1 immune responses [135] to maintain feedback regulation and reverse cholesterol transport.

When compared with WT mice, the ApoE$^{-/-}$ mice are healthy, have no difference in body weight or litter size, reproduce normally but exhibit hyperlipidemia with increased total plasma cholesterol, cholesterol ester and free cholesterol levels and plasma triglycerides and phospholipids. HDL levels are reduced to one third the levels seen in normal WT mice. The

major lipoproteins in the ApoE$^{-/-}$ mice are VLDL and IDL compared to WT where HDL is the predominant lipoprotein [129, 132].

ApoE$^{-/-}$ mice fed a normal mouse chow diet develop fatty streaks in the aortic wall by 3 months of age, which then progress to advanced atherosclerotic lesions by 8 months [132]. Lesions develop in aortic root and throughout the aorta. Lesions develop first in the aortic root, followed by the lesser curvature of aortic arch, branches of brachiocephalic artery, branches of superior mesenteric artery, both renal arteries, aortic bifurcation and pulmonary artery [136].

In older mice, lesions are present in the descending thoracic aorta, lower abdominal aorta, proximal coronary, common iliac, and femoral arteries. Upon feeding ApoE mice a high fat diet (HFD), lesions first appear as small yellowish-white nodules [136]. Mononuclear cell adhesion to endothelium and the presence of foam cells is also apparent.

At 15 weeks of age ApoE$^{-/-}$ mice on western diet, exhibit early fibrous plaques which contain a small necrotic core covered by a fibrous cap [136]. ApoE$^{-/-}$ mice can also develop complex atherosclerosis even when fed a chow diet [130, 132].

1.3.2 LDL Receptor-deficient (LDLR$^{-/-}$) mice

The LDLR knockout (LDLR$^{-/-}$) mouse was created by homologous recombination in embroyonic stem cells [137]. The LDL receptor is a glycoprotein receptor located at the surface of cells and binds to LDL increasing its entry into cells. The receptor has high affinity for LDL and is able to cycle cholesterol in and to the tissues, while keeping plasma LDL low; it is an important regulator of plasma cholesterol levels [138]. LDLR$^{-/-}$ mice exhibit delayed clearance of VLDL and LDL from plasma. Both female and male homozygous mice deficient in LDL receptors are viable and fertile. The total plasma cholesterol levels are two fold higher than in WT mice. They also exhibit a seven fold increase in intermediate density lipoprotein (IDL) and

LDL without significant change in HDL. Plasma triglycerides levels are normal in $LDLR^{-/-}$ mice [137]. Plasma lipid profiles resemble those in humans. Even though total plasma cholesterol is elevated in these mice compared to WT mice, 236mg/dl to 108mg/dl, aortic atherosclerotic is minimal [139]. Therefore, this knockout mouse is given a cholesterol diet to elevate the plasma cholesterol in studies of atherosclerosis [137].

$LDLR^{-/-}$ mice fed a cholesterol diet (1.25% cholesterol, 7.5% cocoa butter and 7.5% casein and 0.5% sodium cholate [127]), exhibit marked hypercholesterolemia, with total cholesterol levels 1500 mg/dl after 2 weeks on diet compared to wild type C57BL/6 mice, 160mg/dl [139]. $LDLR^{-/-}$ mice develop atherosclerosis within the aortic root similar to $ApoE^{-/-}$ [140]. These mice when fed a high fat diet develop atherosclerotic lesions more slowly compared to $ApoE^{-/-}$, however lesions reach the same level of complexity after 30 weeks [141].

1.3.3 ApoE Leiden mice

The ApoE*3 Leiden gene is one of the mutant apoE genes that is associated with familial dysbetalipoproteinemia in humans [142, 143]. It is characterized by a tandem duplication of codons 120 to 126 [144]. Transgenic mice over expressing the ApoE*3 Leiden gene exhibit increased plasma cholesterol and triglycerides when fed a chow diet. ApoE*3 Leiden mice develop hyperlipidimia which can be further enhanced by a diet rich in saturated fats, cholesterol and cholate. These mice are highly susceptible to diet-induced atherosclerosis. Lesions are predominantly lipid-laden foam cells and develop in the aortic root [145]. ApoE* Leiden mice on high fat/high cholesterol diet have increased levels of cholesterol and ApoE when compared to WT mice fed a similar diet. Atherosclerotic lesions develop in the aortic root and along the entire vasculature tree progressively with lesions similar to human pathology [143]. In these mice, DNA synthesis peaks in early lesions and apoptosis peaks in late lesions and mostly

confined to macrophage-derived foam cells. ApoE* Leiden mice are a suitable model to study the different aspects of early atherosclerosis lesion development.

1.3.4 Apo B transgenic mice

Apolipoprotein B (apoB) is involved in mammalian lipoprotein metabolism [146], as the primary apolipoprotein of LDL, β-VLDL, oxidized LDL and Lp (a) [147]. A diet rich in fats and cholesterol elevate the apoB-containing lipoprotein and this is accompanied by the development of atherosclerotic lesions. Impairment in the clearance of apoB-containing lipoprotein results in human lipoprotein disorders such as type III hyperlipoprotenemia, familial defective apolipoprotein B100 and familial hypercholesterolemia and premature atherosclerosis development [148, 149]. In both $ApoE^{-/-}$ and $LDLR^{-/-}$ mice, plasma levels of apoB-containing lipoprotein are elevated together with increased susceptibility to development of atherosclerosis [132, 139]. Transgenic mice expressing high levels of human apoB were generated using a 79.5kb genomic DNA fragment spanning the entire human apo-B gene [147]. Human apo-B transgenic mice on a C57BL/6 genetic background express high levels of human apo-B. Chow diet fed transgenic mice exhibit increased levels of LDL cholesterol in the plasma but do not develop aortic atherosclerotic lesions. However, female transgenic mice on high fat diet develop extensive atherosclerotic lesions compared to female nontransgenic mice fed a high fat diet.

These mouse models of atherosclerosis can be further modified genetically through cross-breeding with other genetically modified mice, or through the use of bone marrow transplantation, and can be used to study the actions of different cytokines, chemokines, molecules and factors that are involved in the pathogenesis of atherosclerosis (see later- Section 1.4 and 1.5).

1.4 Cytokines in Atherosclerosis

Multiple cytokines have been implicated in atherosclerosis including Th1 and Th2 cytokines, Th17 and other cytokines. They can exert either pro-atherogenic or anti-atherogenic effects and include interferon-γ, TNF-α, IL-1β, IL-2, IL-12, IL-18, IL-6, IL-4, IL-10, TGF-β as well as colony stimulating factor. Th1 cytokines are IL-1β, IL-2, IL-12, IL-18, TNF-α and IFN-γ, Th2 cytokines include IL-4, IL-5, IL-13 , IL-10 and TGF-β; Th 17 cytokines are the IL-17 family of cytokines and other miscellaneous cytokines, including CSF and TGF-β [8].

In the mid 1980s, cytokines were first identified in human plaques [150]. It was found that most cells in plaque express MHC class II antigen HLA-DR, which suggested the presence of IFN-γ. PDGF [151], TNF-α [152], IL-1[153], MCP-1 [154], and IFN-γ [155] were also detected in human plaque. A number of mouse models deficient in genes encoding the cytokines or their receptors have been generated on an $ApoE^{-/-}$ and $LDLR^{-/-}$ genetic background (Table 1.1).

1.4.1 Interferon-gamma (IFN-γ)

IFN-γ plays an important role in atherosclerosis (Refer to Table 1.1). It is a pro-inflammatory cytokine that is expressed in atherosclerotic lesions by activated macrophages, T cells, NK cells, and NKT cells. In atherosclerosis, IFN-γ has both direct and indirect effect. Administration of IFN-γ in $ApoE^{-/-}$ mice significantly increased lesion size and the number of T cells in the lesion [156]. Deficiency in endogenous IFN-γ in $ApoE^{-/-}$ mice decreased atherosclerosis and was associated with a decrease in the number and activation of T cells [157]. A deficiency in the IFN-γ receptor also reduces atherosclerotic lesion development in $ApoE^{-/-}$

mice together with a decrease in lesion cellularity and an increase in collagen [158]. The indirect effect of IFN-γ is modulated by IL-12 and IL-18. Both IL-12 and IL-18 are pro-atherogenic. The pro-atherogenic effect of IL-18 is mediated by IFN-γ; a deficiency of IFN-γ in IFNγ$^{-/-}$ApoE$^{-/-}$ mice prevents the atherosclerotic effects of recombinant IL-18 administration [159]. This pro-atherogenic effect of IL-18 can also occur in the absence of T cells, where the injection of IL-18 to SCID ApoE$^{-/-}$ mice resulted in larger lesions and increased IFN-γ expression [160]. This suggests that NK cells are the most likely source of IFN-γ under these circumstances.

1.4.2 IL-1

IL-1 is a pro-inflammatory cytokine that exists in two forms, IL-1α and IL-1β, which mediates its effects through the IL-1 receptor (IL-R). Short-term perfusion with recombinant IL-1 receptor-α (IL-1Rα) reduces fatty streak formation in ApoE$^{-/-}$ fed a cholesterol/cholate-rich diet [161]. In line, IL-1β$^{-/-}$ ApoE$^{-/-}$ exhibit reduction in atherosclerotic lesion size at 12 and 24 weeks of age compared to wild-type ApoE$^{-/-}$ mice and there was no difference in plasma lipid levels between the two groups [162]. These observations were further confirmed by another study using IL-1β$^{-/-}$ C57/BL6 mice and this study also reported that genetic deletion of IL-1α reduced lesion size [163]. Additionally this study also reported that bone marrow transplantation from IL-1α$^{-/-}$ or IL-1β$^{-/-}$ into irradiated C57/BL6 mice reduced early atherosclerotic lesion formation without altering plasma lipids [163].

1.4.3 IL-12

IL-12 plays an important role in Th1 differentiation and also in the activation of NK cells. IL-12 has been shown to be proatherogenic and appears to be important in the early phases of atherosclerotic lesion development in $ApoE^{-/-}$ mice [164]. Thirty week old $ApoE^{-/-}$ $IL\text{-}12^{-/-}$ mice exhibit an increase in lesion size while 40-week old mice had similar lesion size compared to wild-type $ApoE^{-/-}$ mice.

1.4.4 Tumor Necrosis Factor-alpha (TNF-α)

TNF-α is a pro-inflammatory cytokine that is produced primarily by monocytes and macrophages; activated NK cells also produce TNF-α, but studies examining the effects of TNF-α on atherosclerosis have been conflicting. $TNF\text{-}\alpha^{-/-}$ $ApoE^{-/-}$ mice exhibit smaller lesions compared to $ApoE^{-/-}$ mice which is associated with decreased expression of adhesion molecules ICAM-1, VCAM-1 and MCP-1 [165]. However, mice that lack TNF-α receptors [p55 (TNFR1) and p75 (TNFR2)] develop larger atherosclerotic lesions [166, 167]. Furthermore, lymphotoxin-α (LTα) a cytokine with homology to TNF-α has been shown to be proatherogenic [168].

1.4.5 IL-10

IL-10 is an anti-inflammatory cytokines produced by Th2-type T cells, CD4+Foxp3+ regulatory T cells, B cells, monocytes and macrophages. It inhibits Th1 cytokine production, antigen presentation, prevents macrophages from secreting TNF-α and overall suppresses

inflammatory responses in the vessel wall [169-171]. IL-10 has anti-atherogenic potential, where it exerts its anti-inflammatory properties on macrophages [170] and also limits inflammatory response in the vessel wall [171] (Refer to Table 1.1). IL-10 deficiency in C57BL/6 mice fed an atherogenic diet resulted in early atherosclerotic lesion formation characterized by increased infiltration of immune cells, primarily activated T cells and increased pro-inflammatory cytokines [172, 173]. Similarly, IL-10 deficiency in IL-$10^{-/-}$ $ApoE^{-/-}$ mice also promotes atherosclerotic lesion development [174]. Furthermore, bone marrow cells deficient in IL-10 transferred into $LDLR^{-/-}$ mice resulted in attenuated atherosclerosis, suggesting that the leukocyte-derived IL-10 was essential in the prevention of atherosclerotic lesion development [175].

1.4.6 Transforming growh factor-beta (TGF-β)

TGF-β is an anti-inflammatory cytokine that has an immunosuppressive and pro-fibrotic effect on atherosclerotic lesions (Refer to Table 1.1). In patients with advanced atherosclerotic lesions, serum TGF-β is severely decreased. Moreover TGF-$\beta^{+/-}$mice fed an atherogenic diet exhibit increased endothelial activation and macrophage accumulation at the aortic sinus [176, 177]. TGF-β also plays a role in SMC migration and proliferation, and probably plaque stability. Treatment of $ApoE^{-/-}$ mice with neutralizing TGF-β antibodies augments lipid lesion size and switches lesion phenotype from stable to unstable plaque by increasing infiltration of inflammatory cells and reducing collagen [178]. Bone marrow transferred from transgenic mice that express a dominant negative TGF-β receptor type II under T-cell-specific promoter, into $LDLR^{-/-}$ mice, results in an increase in differentiation of T cells. Atherosclerotic plaques from these mice exhibit increased T cell infiltration and expression of MHC class II together with

decreased SMC and collagen content [179]. Therefore, TGF-β signalling in T cells plays an important role in the modulation of atherosclerotic lesion development and progression.

1.4.7 Other cytokines

IL-2 is a pro-inflammatory cytokine produced by Th1 cells. Injection of recombinant IL-2 into $ApoE^{-/-}$ mice fed an atherogenic diet increases atherosclerotic lesion size, while anti-IL-2 antibody treatment reduces atherosclerotic lesion development, which indicates that IL-2 is pro-atherogenic [180].

IL-6 has both pro-inflammatory and anti-inflammatory properties. IL-6 has been shown to exacerbate atherosclerotic lesion development. Recombinant IL-6 treatment of C57/BL6 resulted in a 5-fold increase in atherosclerotic lesion size, whereas treatment of $ApoE^{-/-}$ mice on low or high fat diets resulted in a 2 fold increase in lesion size [181], suggesting IL-6 is a pro-atherogenic cytokine. However, older chow fed (53 weeks old) $IL\text{-}6^{-/-}$ $ApoE^{-/-}$ mice showed an increase in atherosclerotic lesion size with reduction in collagen content, release of IL-10 and recruitment of inflammatory cells [182], also these mice at one year of age had increased calcified atherosclerotic lesions [183]. However, younger (16 week old) $IL\text{-}6^{-/-}$ $ApoE^{-/-}$ mice fed a chow diet had no difference in atherosclerotic lesion size compared to $ApoE^{-/-}$ mice [183]. Similarly $IL\text{-}6^{-/-}$ $LDLR^{-/-}$ mice also did not exhibit any changes in atherosclerotic lesion size when fed either a chow, high fat or atherogenic diet [184]. Together, all reported studies have shown both a positive and negative role for IL-6 in the development of atherosclerosis as a reflection of its pro-inflammatory of anti-inflammatory activities [185].

IL-4 is a Th2 cytokine that plays a role in Th2-cell differentiation and is produced by activated T cells and mast cells and can exert both pro- and anti- inflammatory effects [186]. Studies of IL-4 in atherosclerosis have been contradicting. $IL\text{-}4^{-/-}$ $ApoE^{-/-}$ mice have been

reported to have reduced atherosclerosis compared to ApoE$^{-/-}$ mice [164], while another study has reported that IL-4 deficient C57/BL6 mice had no difference in atherosclerosis compared to wild-type C57/BL6 mice [187]. One possible effect of IL-4 that may contribute to its proatherogenic role is the induction of IFN-γ production by NK cells and NKT cells [188].

IL-18 has been reported to have a proatherogenic role. Treatment of ApoE$^{-/-}$ mice with IL-18 exacerbated atherosclerotic lesion development through the release of IFN-γ [159]. Elhage *et al*. also reported reduced atherosclerosis in IL-18$^{-/-}$ ApoE$^{-/-}$ mice compared to ApoE$^{-/-}$ mice. Since IL-18 in combination with IL-12 has been shown to activate NK cells to produce IFN-γ, this may be a possible mechanism by which IL-18 may exert their proatherogenic activity.

Table 1.1 Cytokines involved in atherosclerosis.

Cytokines	Mice/Treatment	Atherosclerotic lesion size	Lesion Composition	Reference
Th1 Cytokines				
IFN-γ	**IFN-γ$^{-/-}$ApoE$^{-/-}$** 3 months western diet	 ↓60% lipid accumulation	 ↓lesion cellularity ↑collagen	[158]
	IFNγ$^{-/-}$LDLR$^{-/-}$ 8 weeks atherogenic diet 20 weeks atherogenic	 ↓75% ↓43%	 ↓70%macrophage ↓84%SMCs ↓62%MHC 11+ cells ↓64% MHC 11 + cells	[189]
	ApoE$^{-/-}$ 30 days recombinant mouse IFN-γ injection (daily) Chow diet	 ↑ 2 fold	 ↑ T lymphocytes ↑MHC II + cells	[190]
IL-2	**ApoE$^{-/-}$** Recombinant IL-2-6 weeks atherogenic diet or Anti IL-2 antibody-6 weeks atherogenic diet	 ↑ 24% ↓ 43%	 Not reported. Not reported.	[180]
IL-12	**IL-12$^{-/-}$ApoE$^{-/-}$** 30 weeks:Chow diet 45 weeks:Chow diet	 ↓52% No change	 ↓macrophage No change.	[164]

Cytokines	Mice/Treatment	Atherosclerotic lesion size	Lesion Composition	Reference
IL-18	**ApoE$^{-/-}$** IL-18 injection 30days-chow diet **IL-18$^{-/-}$ApoE$^{-/-}$** HFD	↑2 fold ↓ 35%	↑ T cell ↑ MHC II +cells ↓ I-A^{b} ↑ SMCs	[159] [191]
IL-1	**ApoE$^{-/-}$** Recombinant IL-1Rα , minipump perfusion 4 weeks-HFD + Cholate **IL-1α$^{-/-}$C57BL6** **C57BL6:BMT, IL-1α$^{-/-}$ BM** 13 weeks-atherogenic diet **IL-1β$^{-/-}$ApoE$^{-/-}$** Chow diet (12 and 24weeks of age)	↓>50% ↓56% ↓59% ↓32-33%	Not reported. Not reported. Not reported. ↓MCP-1 and VCAM-1 mRNA expression	[161] [163] [162]

Cytokines	Mice/Treatment	Atherosclerotic lesion size	Lesion Composition	Reference
IL-6	**C57BL6** (24 weeks of age)+ recombinant IL-6 (HFD)	↑5.1 fold	Not reported	[181]
	ApoE$^{-/-}$ (9 weeks of age) + recombinant Il-6 (HFD/ LFD)	HFD:↑1.9 fold LFD:↑2.4 fold	Not reported Not reported	
	IL-6$^{-/-}$ApoE$^{-/-}$ Chow diet- 53 weeks	↑lesion	↓Macrophage ↓collagen I and V ↓MMP-9 ↓IL-10 mRNA expression	[182]
	Chow diet (16/52 weeks)	16wk:No change 52 wk:↑55%	↑314% calcification	[183]
	IL-6$^{-/-}$LDLR$^{-/-}$ Chow/HFD/ Atherogenic diet	No change.	No change.	[184]
TNF-α	**TNF-α$^{-/-}$ApoE$^{-/-}$** Chow diet-12 weeks.	↓30%	↓VCAM-1, ICAM-1 and MCP-1.	[165]
	HFD-10wk/40 wk	10wk: ↓50% 40wk: ↓60%	No change.	[192]

Cytokines	Mice/Treatment	Atherosclerotic lesion size	Lesion Composition	Reference
	TNF-$\alpha^{-/-}$C57BL6 Atherogenic diet-16 weeks	No change.	Not reported.	[168]
	LT$\alpha^{-/-}$C57BL6 Atherogenic diet-16 weeks	↓62%	Not reported	[168]
	p55$^{-/-}$C57BL6 Atherogenic-14 weeks	↑2.3 fold	↑Scavenger receptors	[166]
Th2 Cytokines				
IL-4	**LDLR$^{-/-}$ BMT; IL-4$^{-/-}$ BM** Atherogenic (high cholesterol,cholate) - 4 weeks	No change-Aortic sinus ↓69% -aortic arch ↓67%-thoracic aorta	No change.	[193]
	IL-4$^{-/-}$ApoE$^{-/-}$ Chow diet-30 and 45wk	30wk:↓27%(aortic sinus) 45wk: ↓58%(aortic arch)	No change.	[164]
	IL-4$^{-/-}$C57BL6 Atherogenic diet(high cholesterol)-15 weeks	No change	Not reported.	[187]
	C57BL6 + IL-4 Weekly IL-4 injections, HFD- 15 weeks	↓90%	Not reported.	[194]

Cytokine	Mice/Treatment	Atheroslerotic lesion size	Lesion Composition	Reference
IL-10	**IL-10$^{-/-}$C57BL6** Atherogenic diet +cholate-16 weeks	↑3 fold	↑T cells, IFN-γ ↓collagen	[172]
	LDLR$^{-/-}$ BMT of IL10 transgenic mice(controlled by IL-2 promoter) Atherogenic diet + cholate -20 weeks	↓47%	↓80%necrotic core ↓lipid accumulation	[195]
	IL-10$^{-/-}$ApoE$^{-/-}$ Chow diet	↑2.8 fold (16 wk of age females) No change (48 wk of age)	↑MMP-9 & Tissue factor activity ↑thrombosis	[196]
	LDLR$^{-/-}$: BMT; IL-10$^{-/-}$ BM High fat-cholate free- 14 weeks	↑>2 fold (thoracic aorta) ↑35% (aortic root)	↑lymphocyte & macrophage ↓collagen	[175]

Cytokines	Mice/Treatment	Atherosclerotic lesion size	Lesion composition	Reference
Miscellaneous				
TGF-β	**TGF-β$^{-/+}$**			
	Atherogenic diet-12 weeks	↑674%	↓44%SMC ↑1100% macrophage	[176]
	ApoE$^{-/-}$			
	i.p injection TGF-β1, 2 & 3 neutralizing antibody- chow diet:9 weeks	↑2fold	↑macrophage ↑57% infiltrating lymphocytes ↓collagen	[197]
	Injected TGFβRII:Fc fusion protein (2x/wk)-12 weeks. Start age: 5/17wk	5wk: NC 17wk:↓37.5%	↑T cells ↑inflammatory cells ↑lipid core size ↓collagen(17wk)	[198]
	LDLR$^{-/-}$			
	BMT: transgenic mice with dominant negative TGF-βR11 BM (atherogenic diet-high cholesterol)	↓29%	↑T cell ↑MHC class II + cells ↓SMC & collagen	[179]

1.5 Chemokines in Atherosclerosis

Chemokines are small cytokines that are involved in the recruitment of inflammatory cells into the intima. Chemokines can be divided into 4 families CC, CXC,CX3C, and XC based on the position of the first 2 cysteine residues [199]. Chemokines signal through G protein-coupled receptors to activate and aid in transmigration of cells. Endothelial cells, inflammatory cells and atherosclerotic lesions express chemokines to attract leukocytes into inflammatory sites. They can have profound effects in the development of atherosclerosis (Refer to Table 1.2).

1.5.1 MCP-1

MCP-1 is an important chemokine for the recruitment of circulating monocytes and for initiation of atherogenesis. The receptor for MCP-1 chemokine is CCR2. The lack of MCP-1 in $LDLR^{-/-}$ mice has been shown to reduce macrophage recruitment into the vessel wall and inhibit atherosclerosis via CCR2 [200]. $CCR2^{-/-}ApoE^{-/-}$ also exhibit reduction in atherosclerotic lesion development [201]. In mice overexpressing apolipoprotein B, the deletion of MCP-1 gene prevented monocyte recruitment and reduced atherosclerotic lesion size [202].

1.5.2 CCL5

CCL5, also known as RANTES, is a chemokine involved in the trafficking and homing of T and NK cells. CCL5 can also augment NK cell cytolytic activity, and in addition to responding to CCL5, activated NK cells can also produce CCL5 to attract other cell types [203].

CCL5 and its receptors CCR1 and CCR5 have been implicated in atherosclerosis. CCR1 and CCR5 are expressed on macrophages, and T lymphocytes in atherosclerotic lesions.

The blocking of CCL5/RANTES receptors with the peptide antagonist Met-RANTES has been shown to inhibit atherosclerotic lesion development and leukocyte infiltration into the vessel wall [204]. The reduction in lesion size was associated with a more stable plaque phenotype that had increased SMC and collagen content and reduced MMP-9 expression. To examine the role of CCR1 and CCR5 in atherosclerosis, several *in vivo* studies utilizing either CCR1 or CCR5 deficient mice were performed. CCR5 deficiency in ApoE $^{-/-}$ mice does not reduce early atherosclerosis, however it protects against advanced atherosclerosis [205, 206]. In contrast, the transfer of CCR5$^{-/-}$ bone marrow into LDLR$^{-/-}$ mice does not affect lesion size; however atherosclerotic lesions had a more stable phenotype with increased SMC content and less inflammation [207]. However, in the same model CCR1$^{-/-}$ bone marrow cells enhanced inflammation and atherosclerotic lesion development [208]. Similarly, deficiency in CCR5, but not CCR1, protected against neointima formation after arterial injury in mice susceptible to atherosclerosis, due to an athero-protective immune response mediated by IL-10 [209]. CCR1$^{-/-}$ mice have reduced NK cells recruitment in lymphoid tissue during pulmonary granuloma formation [210] and CCR1 has also been implicated in pro-atherogenic effects of atherosclerosis, which suggest the likely recruitment of NK cells into atherosclerotic lesion. CCR5 and CCR1 have opposite effect on atherosclerosis despite having the common ligand. This may be due to chemokines exerting antipodal effects by acting as an agonist for one receptor and an antagonist for another [211].

1.5.3 CX3CL1

CX3CL1, also known as fractalkine, is expressed in atherosclerotic lesions but not in normal vessels. The receptor for CX3CL1 is CX3CR1. NK cells, T cells and monocytes express CX3CR1 [212]. In atherosclerosis, CX3CL1 not only mediates monocyte and T cell recruitment but also SMC migration [213]. $CX3CL1^{-/-}$ $ApoE^{-/-}$ mice exhibit reduced atherosclerotic lesions and macrophage recruitment in the brachiocephalic artery but not aortic root [214, 215]. However, $LDLR^{-/-}$ mice deficient in fractalkine exhibit reduced lesion size and monocyte infiltration in both the braciocephalic artery and in the aortic root [216]. Both CCR2 and CX3CR1 are involved in the recruitment of macrophages into the lesions. The combined deficiency of CCR2 and CX3CR1 in $ApoE^{-/-}$ mice results in marked reductions in lesion size and macrophage accumulation in the artery wall; CX3CR1 and CCR2 function independently to recruit monocytes [217].

Table 1.2: Chemokines involved in atherosclerosis.

Chemokine and associated Chemokine signalling receptor	Mice/Treatment	Atherosclerotic lesion size	Lesion Composition	Reference
CX3CL1 (Fractalkine) (CX3CR1)	**CX3CR1$^{-/-}$ApoE$^{-/-}$** HFD-5,10/15wks	Aorta: 5wk: ↓43% 10wk: ↓49% 15wk: ↓36%	10wk:↓macrophage	[214]
	CX3CR1$^{-/-}$ApoE$^{-/-}$	12wk age: ↓30% (aortic root) 16wk age:No change (aortic root) 16wk age: (BCA) ↓85%	↓macrophage	[216]
	CX3CR1$^{-/-}$LDLR$^{-/-}$ (semisynthetic modified AIN76 +0.02% cholesterol)8-12 weeks.	↓35% (aortic root),↓50% (BCA) 16 wk of age	Not reported.	[215]
	CX3CR1$^{-/-}$ApoE$^{-/-}$	↓55%(aortic surface) ↓50% (aortic sinus)	↓macrophage	
CCL5 (RANTES) (CCR5)	**CCR5$^{-/-}$ApoE$^{-/-}$** Chow diet-16wks	No change	Not reported	[205]

Chemokine and associated Chemokine signalling receptor	Mice/Treatment	Atherosclerotic lesion size	Lesion Composition	Reference
CCL5 (RANTES) (CCR5)	**CCR5$^{-/-}$ApoE$^{-/-}$** Chow/HFD	26wks chow diet:No change 36wks & 65 wks chow: ↓ HFD: ↓	↓macrophage	[206]
	HFD-10 weeks	↓>50%	↓macrophage ↓CD4 mRNA ↑SMC ↑IL-10 mRNA	[208]
	CCR5$^{-/-}$ApoE$^{-/-}$ HFD-12weeks	↑(sinus & thoracoabdominal)	↑T cells ↑IFN-γ	[208]
(CCR1)	**CCR1$^{-/-}$ApoE$^{-/-}$** HFD	↑lesion size	↑T cells	[208]
	LDLR$^{-/-}$ Met-RANTES (CCL5) antagonist	↓lesion size	↓leukocyte infiltration	[204]
CXCL12 [stromal derived factor 1 (SDF-1)] (CCXR4& CCXR7)	**ApoE$^{-/-}$** CXCR4 antagonist AMD3465 treatment	↑lesion size	↑macrophages ↓SMC ↓T cell	[218]
	or ApoE$^{-/-}$; BMT mIL-8RH$^{-/-}$ homologue	↑lesion size	↑macrophages ↓SMC ↓T cell	

Chemokine and associated Chemokine signalling receptor	Mice/Treatment	Atherosclerotic lesion size	Lesion Composition	Reference
MCP-1 (CCR2)	**MCP-1$^{-/-}$LDLR$^{-/-}$** Atherogenic diet. **CCR2$^{-/-}$ApoE$^{-/-}$** HFD	↓83% ↓>50%	Not reported. Not reported.	[200] [201]

BCA: brachiocephalic artery

1.6 Immune cells and Atherosclerosis

Leukocytes in atherosclerotic arteries were reported in the early 1980s [219]. Initially it was thought that only macrophages were present in lesions, however subsequent studies have shown the presence of other leukocytes in both mouse and human atherosclerotic aortas [12].

1.6.1 Monocytes

Monocytes are short-lived cells that normally do not proliferate. They are found in the peripheral blood, bone marrow and spleen [220]. There are two distinct subsets of monocytes in the circulation of mice, (i) $Gr^{+}/Ly6C^{high}$ monocytes that migrate into tissues and differentiate into M1-type macrophages and mediate inflammation, phagocytosis and proteolysis, and (ii) $Gr^{-}/Ly6C^{low}$ monocytes that patrol in the circulation and migrate into tissue and differentiate to M2 or activated macrophages to aid wound repair and tissue remodelling [221-223]. During atherogenesis, monocytes are recruited into the intima and differentiate into macrophages, take up oxLDL and lipids and become foam cells in developing fatty streaks. The recruitment of monocytes requires activation of chemokine receptors CX3CR1, CCR2, and CCR5 [224]. $Gr^{+}/Ly6C^{high}$ monocytes migrate into atherosclerosis-prone arteries using CX3CR1, CCR2 and CCR5 [225]. $Ly6C^{high}$ monocytes increase dramatically in hypercholesterolemic ApoE-deficient mice on a high–fat diet, doubling in number every month. This hypercholesterolemia-induced monocytosis results from increased cell survival, continuous cell proliferation and attenuated $Ly6C^{high}$ conversion to $Ly6C^{low}$ cells [226]. In contrast, $Ly6C^{low}$ monocyte numbers remain constant [226]. The combined deletion of CCL2, CX3CR1 and CCR5 on $ApoE^{-/-}$ mice reduces both monocytosis and atherosclerosis dramatically [227]. Atherosclerosis in $CX3CL1^{-/-}CCR2^{-/-}$

$ApoE^{-/-}$ mice is significantly reduced compared to $CCR2^{-/-}ApoE^{-/-}$ and $CX3CL1^{-/-}ApoE^{-/-}$ mice suggesting an independent and additive contribution of the different chemokines to atherosclerosis [228]. $Gr^{-}/Ly6C^{low}$ monocytes require CCR5 for migration [225].

1.6.2 Macrophages

Monocyte-derived macrophages are the most dominant cell type in atherosclerotic lesions. They are present in fatty streaks of the intima during the very early stages of atherosclerosis and throughout all stages of atherosclerotic lesion development [1]. They produce inflammatory cytokines, metabolize lipids and facilitate vascular remodelling, and express Pattern Recognition Receptors (PRR) bridging the innate and adaptive immune response. Macrophages have 2 different phenotypes, M1 and M2, which are present in atherosclerotic lesions. Macrophages activated by IFN-γ take on an M1 phenotype to produce pro-inflammatory cytokines IL-12, IL-23, IL-6, IL-1, and TNF-α. Macrophages activated by IL-4, IL-13, IL-1 produce IL-10 and exhibit a M2 phenotype and express scavenger receptors [229].

It is known that circulating monocytes enter the intima in response to chemokines produced by vascular cells, and differentiate into macrophages. Intimal macrophages take up modified lipoproteins through their scavenger receptors (SR-AI/II, SR-BI, CD36, CD68, LOX-1) that results in the formation of lipid-rich foam cells [230]. This is a hallmark of atherosclerosis and promotes lesion expansion. Studies using SR-A or CD36-deficeint mice have shown that the deficiency in these scavenger receptors results in reduced foam cell formation and subsequent reduction in the development of atherosclerotic lesions [231-233].

Despite responding to chemokines, activated macrophages can also secrete chemokines such as monocyte chemoattractant protein-1 (MCP-1) to facilitate further recruitment of monocytes into the intima. Over-expression of MCP-1 by macrophages accelerates the

progression and development of atherosclerosis and potentiates inflammatory response by promoting more lipid oxidation [234].

Macrophage activation promotes secretion of pro-inflammatory cytokines such as TNF-α and cytotoxic substances such as peroxynitrite, a product of the reaction of NO with superoxide [235, 236]. Peroxynitrite and TNF-α released by macrophages promotes endothelial and smooth muscle cell death, reducing the number of viable smooth muscle cells required for collagen synthesis that subsequently leads to unstable atherosclerotic lesion development.

The accumulation of free cholesterol or excessive uptake of oxidized LDL can induce macrophage apoptosis [237]. Macrophage apoptosis in early lesions suppresses plaque progression and in advanced lesions macrophage apoptosis promotes necrotic core development [238]. The removal of proaptotic factor p53 in macrophages using bone marrow transplantation in apoE*3leiden transgenic mice augments atherosclerosis suggesting macrophage apoptosis suppresses development of early atherosclerotic lesion [239]. Conversely, cytokines such as TNF-α and IL-1β released by apoptotic macrophages augment inflammatory response and further increase lesion size [240]. Additionally, apoptotic macrophages can also alter the extracellular matrix of the vessel by releasing metalloproteinases (MMPs) which can cause complication during the later stages of atherosclerosis. MMPs can affect the structural integrity of the fibrous cap and lead to rupture of the vessel wall [241].

1.6.3 Dendritc cells

Dendritic cells (DCs) are members of both the innate and adaptive immune system and are potent antigen presenting cells (APCs) that take-up free- or cell-associated protein or lipid, process them, and present the fragmented molecules on MHC to T lymphocytes to initiate an

adaptive immune response. DCs are found in atherosclerotic lesions in both human and murine models [242, 243]. DCs reside in the aortic intima in areas predisposed to atherosclerotic lesion development and are absent in areas resistant to atherosclerosis [244, 245].The depletion of resident intimal DCs in $LDLR^{-/-}$ mice using the CD11c-DTR model resulted in reduced intimal lipid surface areas, foam cells, and extracellular subendothelial lipid accumulation suggesting that resident intimal DCs differentiate into the initial foam cells in emerging atherosclerotic lesions [246]. DCs are also resident in healthy adventitia, where they are localized to the sub-endothelial space and media-adventitia junction [247]. These DCs play a role in surveillance of the arterial wall and also in tolerating self-reactive T cells either by deleting them or expanding regulatory T cells [248].

Recently, DCs have been shown to regulate plasma cholesterol levels [249-251]. Increases in DC population lead to a severe decrease in plasma cholesterol in both $LDLR^{-/-}$ and $ApoE^{-/-}$ mice. In contrast, the specific depletion of conventional DCs by injection of diphtheria toxin (DT) to CD11c-DTR $ApoE^{-/-}$ mice induced elevation in plasma cholesterol suggesting that conventional DCs may contribute to correction of hyperlipidemia and hence regulate cholesterol homeostasis by favouring cholesterol lowering in hyperlipidemic environment [251].

DCs are activated in early stages of atherogenesis [252]. VCAM-1, P- and E-selectin are involved in DC recruitment into the intima [244, 253]. DC adhesion and migration is modulated by changes in endothelial function where exposure of EC to hypoxia, oxLDL, TNF-α and inhibition of endothelial NO synthase enhances their adhesive properties and transmigration into the intima [254]. Once adhered to the vessel wall, CCL2 and CCL5 chemokines play a role in recruiting DCs into the inflamed vessel wall. Fractalkine also plays a role in accumulation of

DCs in atherosclerotic lesion, asdeficiency in CX3CR1 reduced atherosclerotic lesion and DC number within the plaque [245].

Activated DCs produce IL-12 which upregulates the expression of CCR5 that leads to the recruitment of T cells in atherosclerotic plaques [255]. They also express CCL19 and CCL21 chemokines that can enhance recruitment of T lymphocytes to promote progression of atherosclerosis [256]. Within the vascular wall of human plaque, DCs are seen near T cells and NKT cells, suggesting that they might present antigen to naïve T cells and also further enhance NKT cells cytotoxic potential in atherosclerotic plaque [257]. Cell to cell contact of mature DCs and NK cells promotes IL-12 production by DCs that can trigger the effector functions of NK cells to produce IFN-γ, which might suggest the involvement of NK cells in the development of atherosclerosis [258].

1.6.4 T lymphocytes

T lymphocytes are normal residents of the adventitia of healthy arteries [259]. In atherosclerosis-prone areas, T cells are recruited into aortic intima of early and advanced atherosclerosis [260]. T cells are abundantly found in shoulder and fibrous cap regions, and constitute approximately 20% of the total cells [11].These T cells are activated $TCR\alpha\beta^{+}CD4^{+}$ and some $CD8^{+}$ [155].

1.6.4.1 $CD4^{+}$ T cells

Earlier studies have examined the proatherogenic effects of T lymphocytes, as the adoptive transfer of $CD4^{+}$ T lymphocytes into $ApoE^{-/-}$ SCID mice aggravated atherosclerosis

[261, 262]. T cells can be activated by antigens within the atherosclerotic lesions including OxLDL [263], β2-GPI and HSP65. The transfer of $CD4^+$ T cells reactive against MDA-5 or HSP65 resulted in larger atherosclerotic lesion development compared to mice transferred with naive T cells [264, 265] . However, recently the influence of different T cell subpopulation has been elucidated, and they can have opposing effects on atherosclerosis.

Recent studies have suggested that a specific subtype of $CD4^+$ T cells, $CD4^+CD28^-$ T cells may mediate plaque instability in humans and are associated with acute coronary events [266]. These cells differ from conventional $CD4^+CD28^+$ T cells in both function and phenotype. They are terminally differentiated and pro-inflammatory, producing large amounts of interferon-gamma and tumour necrosis factor-alpha [267]. They are also cytotoxic and kill endothelial cells *in vitro* [268], most likely by mechanisms dependent on perforin and granzyme B [269]. These cells also express the C-type lectin receptor NKG2D [270] and in many ways mimic the effects of NK cells. Their precise role and importance in atherosclerotic lesion rupture and erosion (endothelial denudation) associated with acute coronary events remains to be elucidated

Mature T cells, also known as $CD4^+$ T, cells can be divided into different subpopulations, including Th1, Th2 and TH17 cells. These different subtypes of T cells influence atherosclerosis differently through the production of different cytokines.

Th1 cells mainly produce IFN-γ and activate macrophages [271].The Th1 subsets of T cells are most abundant in the atherosclerosis lesion, where they recognize epitopes of OxLDL. The elevated OxLDL in arterial wall, promotes Th1 cells to stimulate atherogenesis by secreting IFN-γ, IL6 and IgG2a antibodies against modified OxLDL [272]. Patients with acute coronary syndrome (ACS) have higher levels of Th1 cells that secrete IFN-γ [273]. Also effector Th1 cells stimulate other cells like DCs, macrophages and B cells via CD40L-CD40 interactions to further promote atherogenesis [274]. Impaired Th1 differentiation by the deletion of transcription factor

T-bet leads to reduction in atherosclerosis and indicates a Th1 to Th2 shift [275]. Similarly, severe hypercholesterolemia switches the Th1 phenotype to Th2, promoting IL-4 production and reducing the severity of atherosclerosis [272].

Th2 cells produce IL-4, IL-5, IL-10 and IL-13 which are known to downregulate Th1 responses and assist B cells in antibody production and Th2 cells are usually associated with anti-inflammatory responses. However, their role in atherosclerosis is contradictory depending on the stages and sites of lesion. In mild hypercholesterolemia in atherosclerosis-resistant mouse model, Th2 cells have shown to protect against early fatty streak formation [194]. In $LDLR^{-/-}$ mice deficient in IL-4, atherosclerotic lesion development was decreased, which suggest a pro-atherogenic role for Th2 cells [193].

Th17 cells have been shown to be present in peripheral blood of patients with coronary atherosclerosis [276]. However their role in atherosclerosis is controversial. Recently, a study showed that Th17/IL-17 cells promoted the development of atherosclerotic lesions in $ApoE^{-/-}$ mice. There was an increase in the number of Th17 cells that was associated with the magnitude of the lesion development, and the expression of IL-17 in plaque was increased. Also, blockade of IL-17 with neutralizing anti-IL-17A antibody inhibited atherosclerosis [277]. However, another study showed increased levels of IL-17 provide an athero-protective effect, possibly through the reduction in endothelial VCAM-1 expression and T cell recruitment [278].

1.6.4.2 Regulatory T cells

Regulatory T (Treg) cells are known as the modulator of the immune system, where the balance of Th1 and Th2 responses are regulated by these cells [279]. These cells are a

heterogeneous population that consist of naturally occurring and acquired regulatory T cells which can inhibit functions of different leukocytes, including macrophages, $CD8^+$ T cells [280], NK cells [281], NKT cells [282] and B cells [283]. Since regulatory T cells can inhibit functions of these leukocytes, they are likely to be atheroprotective. The presence of Treg in both human and mouse atherosclerotic lesions has been established [284, 285].

There are a number of different types of regulatory T (Tr) cells that have been studied in atherosclerosis. Tr1 cells secrete high levels of IL-10 and low levels of TGF-β; Th3 cells secrete primarily TGF-β, and CD4+CD25+ T cells inhibit immune response through cell to cell contact [286]. The transfer of naturally occurring T regulatory cells ($CD4^+CD25^+$) is protective in murine atherogenesis [287]. These naturally occurring T regulatory cells suppress atherosclerosis, by reducing T cell and macrophage infiltrate into plaque, increasing lesion collagen, and switching to an anti-inflammatory cytokine profile [287]. Similarly, the adoptive transfer of cognate peptide specific T regulatory cells (Tr1) into $ApoE^{-/-}$ mice, diminishes the Th1 cytokines IFN-γ and IgG2α, and elevates IL-10 and reduces atherosclerosis in recipient $ApoE^{-/-}$ mice [286]. The expansion of Tregs by administration of anti-CD3 antibodies have been shown to reduce atherosclerosis by a mechanism dependent on TGF-β [288].

The generation and maintenance of Tregs can be regulated by a number of factors. Inducible costimulatory molecule (ICOS) has been suggested to be involved in Treg response during atherosclerosis. ICOS has been found to be important to regulate function of T regulatory cells to suppress atherosclerosis via TGF-β, as bone marrow T regulatory transfer from $ICOS^{-/-}$ mice to $LDLR^{-/-}$ mice aggravated atherosclerosis via a T-cell response [289]. The number and activity of Tregs can also be regulated by CD31, however the precise mechanism is unclear [290]. Additionally, obesity can increase the number and function of T regulatory cells in atherosclerosis. Leptin deficiency is shown to increase number and function of Treg cells that resulted in reduced atherosclerotic lesion development [291]. HSP-60 can also induce and

increase $CD4^+CD25^+Foxp3^+$ T regs. The adoptive transfer of HSP-60 activated T regs into $ApoE^{-/-}$ mice reduced atherosclerotic lesion development. This evidence suggests the possibility of antigen-specific Treg in atherosclerosis [292].

1.6.4.3 $CD8^+$ T cells

$CD8^+$ T cells have been identified in human atherosclerotic lesions [293]. $CD8^+$ T lymphocytes expressing αβT-cell receptor are uncommon in early atherosclerotic plaque however it is important in established lesion [294]. In patients with unstable angina, $CD8^+$ T cells are present in an activated state to secrete IFN-γ [273]. Intimal $CD8^+$ T cells increase with increasing severity of atherosclerotic lesions, and in advanced plaques they represent about 50% of infiltrating CD45-positive leukocytes [295]. In $ApoE^{-/-}$ mice, $CD8^+$ T cells are present in atherosclerotic lesions, although not as frequent as $CD4^+$ T cells [263]. Patients with autoimmune disease SLE have increased number $CD8^+$ T cells that account for the accelerated atherosclerosis [296]. Hypercholesterolemia leads to activation of Th1 immune responses in lymph nodes draining into atherosclerotic lesions. The activation of $CD8^+$ T cells predominate early immune response to hypercholesterolemia in $ApoE^{-/-}$ mice, and this was found to involve formation of cell autoantigen [297] .

1.6.5 NKT cells

NKT cells comprise a subpopulation of T cells that co-express T cell receptor (TCR) and the Natural Killer (NK) surface antigen CD161 in humans and NK1.1 in mice [298]. The majority of NKT cells (invariant) utilized Vα14-Jα18α-chain paired with a Vβ8 or Vβ2 β-chain.

NKT cells have been identified in both human and mice atherosclerotic lesions [299, 300]. Chemokines involved in attracting NKT cells into atherosclerotic lesion are not fully known but may involve CXCL12 [301].

NKT cells recognize the glycolipid antigen non-polymorphic MHC class I-related glycoprotein CD1d [302]. They play a pro-atherogenic role in atherosclerosis, in a CD1d-dependent manner; $CD1d^{-/-}$ $ApoE^{-/-}$ mice exhibit reduced lesions [303]. Also, $ApoE^{-/-}$ mice injected with alpha galactoscylceramide, which stimulates NKT cells via CD-1d, exhibit increased atherosclerotic lesion size associated with increases in proinflammatory cytokines such as IFN-γ, and IL-2 [303]. There are 2 major subsets of NKT cells, $CD4^+$ and $CD4^-CD8^-$ (DN) NKT cells. Adoptive transfer of these different subsets into $ApoE^{-/-}$ mice depleted of NKT cells by neonatal thymectomy demonstrated that $CD4^+$ but not DN NKT cells augment atherosclerosis lesion development. The pro-atherogenic capabilities of DN NKT cells appear to be limited by inhibitory Ly49 receptors [304]. Upon activation NKT cells can rapidly produce both Th1 and Th2 cytokines, including IFN-γ, IL-4, IL-2, IL-10, TNF-α and IL-13 [305]. IFN-γ and IL-2 production can lead to activation and increased IFN-γ secretion and cytotoxicity of NK cells and macrophages [188, 306]. It is not known whether NKT cells mediate their effects in atherosclerosis directly or via bystander lymphocyte activation, e.g. NK cell activation.

1.6.6 Mast Cells

Mast cells are found in the adventitia and also in atherosclerosis plaques [307]. Upon activation mast cells produce cytokines including TNF-α, IFN-γ, IFN-β, IL-1α, IL-1β, IL-6, IL-18, IL-10, and TGF-β, which may facilitate recruitment of lymphocytes into the vessel wall [308]. Activated mast cells also secrete proteases such as tryptase and chymase that may

promote degradation of collagen, elastin and proteoglycans. Mast cell enzymes such as chymase are capable of activating MMPs to destabilize atherosclerotic plaque [309]. Perivascular mast cell activation has been shown to promote atherosclerosis and induce plaque destabilization in $ApoE^{-/-}$ mice [310]. Inhibition of chymase abrogates atherosclerosis progression, enhancing plaque stability by increasing collagen and reducing necrotic core size [311]. Mast cell degranulation in $ApoE^{-/-}$ increases atherosclerotic lesion size, and also increases the number of macrophages and T lymphocytes [312]. Mast cell deficient mice ($Kit^{W\text{-}sh/W\text{-}sh}LDLR^{-/-}$) have increased collagen in atherosclerotic lesions and larger fibrous caps together with reduced T cells and macrophages, and smaller atherosclerotic lesions [313].

1.6.7 B cells

B cells have been detected in human atherosclerotic lesions but are mostly localized in the adjacent adventitia. In hypercholesterolemic mice, they are also present in atherosclerotic lesions [70, 314]. B cells appear to be protective against atherosclerotic lesion development by producing IgM Abs to ox-LDL [259]. Atherosclerotic lesions are increased following transfer of bone marrow from B cell-deficient mice into $LDLR^{-/-}$ probably due to reduced production of anti-oxLDL antibodies [315]. Splenectomised $ApoE^{-/-}$ mice also exhibit aggravated atherosclerosis together with a reduction in anti-OxLDL antibodies and the transfer of splenic B cells into these mice reduces atherosclerotic lesion size [316]. The protective effect of B cells appears to be mediated by IgM as $LDLR^{-/-}$ mice deficient in serum IgM exhibit exacerbated atherosclerosis [315] . However, recently it has been found that the depletion of mature B cells using anti-CD20 antibodies prevented atherosclerosis lesion development [317]. The reduction in atherosclerosis in this study was associated with reduced T cell activation and diminished T cell-derived IFN-γ secretion and enhanced IL-17 production [317]. Another study using this CD20

antibody also reported reduction in atherosclerosis and proposed that the 2 different subsets of B cells, B1 and B2 cells, have different roles in atherosclerosis. Transfer of B2 cells into lymphocyte deficient $ApoE^{-/-}Rag2^{-/-}$ common cytokine receptor γ-chain (γc)-deficient mice aggravated atherosclerosis and the transfer of B1 cells did not promote atherosclerosis [318]. Studies elucidating the significance of B cells for human atherosclerosis are yet to be performed.

1.6.8 Neutrophils

Neutrophils are professional phagocytes that sense and destroy pathogens, and have been reported in human and mouse atherosclerotic lesions. Neutrophils have also been associated with ruptured lesions which suggest a role in destabilization of the atherosclerotic lesion [319, 320]. In $LDLR^{-/-}$ mice, neutrophils associated with lesions were attached to the cap and arterial adventitia. They are not detected in early lesions, but are abundant in advanced stages and frequently localized to shoulder regions of plaques [321, 322]. $ApoE^{-/-}$ mice fed an atherogenic diet and treated with anti-polymorphonuclear leukocyte antibody (anti-PMN Ab), reducing neutrophils without affecting monocyte numbers, exhibit smaller atherosclerotic lesions at the aortic root, demonstrating that neutrophils can augment atherosclerosis development [218]. Furthermore, blocking of CXCR4 which increases neutrophils in plaques promotes inflammation, suggesting that the interaction between CXCR4 and its ligand CXCL12 plays an atheroprotective role by modulating the expansion of circulating PMN [218].

1.6.9 Basophils

Basophils have been considered to be important mediators of late-phase allergic reactions, based in increased numbers in tissues such as lung and skin after allergen challenge [323]. As they do not accumulated in atherosclerotic lesions they appear to have little if any influence on atherosclerosis. In hyperlipidemic rabbits, they mainly accumulate in the aortic adventitia, and with low numbers accumulating within both normal intima and developing fatty streaks but not in atherosclerotic lesions [324].

1.7 Natural Killer Cells

Natural Killer (NK) cells are bone marrow derived lymphocytes from $CD34^+$ hemopoietic progenitor cells. NK cells populate the lymphoid and non-lymphoid organs. They are the third major lymphocyte population of the immune system after T and B cells [325]. NK cells are effector lymphocytes of the innate immune system, which mediate effector functions via cytotoxicity, cytokine and chemokine production [325].

Originally, NK cells were described as large granular lymphocytes [326] which have the ability to spontaneously lyse susceptible tumour lines without prior sensitization [325]. They have been also described as cells that lack the T cell antigen receptors and CD3 complex [325, 327, 328]. NK cells express high levels of asialo ganglio-N-tetraosylceramide on their surface but expression is not exclusive to classical NK [325, 329].

Classical NK cells do not rearrange the gene that encodes their receptor. They possess germline encoded inhibitory and activating receptors that regulate their function [330, 331]. The development of NK cells is dependent on the microenvironment within the bone marrow [325]. NK cells provide an immediate response against parasites [332] and intracellular bacteria [333] and are critical for controlling several types of viral infections [334]. They are also potent effector cells eliminating tumors and infected cells and then providing signals that shape the adaptive immune system [330].

NK cells were accidently discovered during studies investigating the specific cytotoxic effect of lymphocytes against target cells [335-339]. In 1975, naturally occurring killer cells with cytolytic activity were described [340]. This was followed by the discovery of active and spontaneously occurring cytotoxic cells [341]. Eva Klein coined the word "natural killer" which defined this population of lymphocytes which are able to spontaneously kill tumor cells without preconditioning [340-343]. NK cells are recognized as a separate lymphocyte lineage which exerts immune response via cytotoxicity and cytokine production [325].

For NK cells to exert their effector functions, they need to be activated [344]. NK cells are most competent to exert effector function when activated by type I and type II interferons including IFN-α, IFN-β and IFN-γ [345, 346] and following interactions with accessory immune cells such as dendritic cells or NKT cells [347]. Activation of NK cells by type 1 IFN including IFN-α and IFN-β results in efficient killing of target cells, via upregulation of both CD95 ligand (FASL) and perforin [346, 348]. IFN-γ is a key inducer and coordinator of cell-mediated immune effector mechanisms such as destruction of pathogens and phagocytosis. NK cells are a major source of IFN-γ, and IL-12 stimulates NK cells to secrete IFN-γ. Activated NK cells mediate cytotoxicity by releasing various perforins especially perforin A and B which facilitate the entry of granzymes into target cells, to induce cell death [349, 350]. The NK-mediated killing of virus-

infected or tumor cells generates apoptotic or necrotic products that subsequently influence the function of monocyte - derived dendritic cells (MDDC). NK cells are capable of lysing immature DCs that do not undergo full maturation, furthermore NK cells also play an important role in ensuring the full maturation of DCs and other bystander innate cells such as mast cells or plasmacytoid DC (pDC) (see later) [351, 352].

NK cell activity is regulated by the balance of inhibitory and activating signals from cell surface receptors (see later). NK cell receptors that bind to self MHC class 1 molecules inhibit NK cell activity [353]. NK cells also recognize ligands on stressed, transformed, infected cells via their receptors [354, 355]. The phenomenon by which NK cells do not kill their own and recognize self has been termed the missing self hypothesis [353, 356, 357].

1.7.1 Human NK cells

In humans, NK cells comprise approximately 15% of all circulating lymphocytes. They are defined by their cell surface expression of CD56 and the lack of CD3 [358, 359]. NK cells are a heterogeneous blood cell population and subsets are defined by the level of expression of CD16 and CD56, an isoform of the human neural-cell adhesion molecule with unknown functions [360]. CD16 is a low affinity FcγRIII surface receptor that binds to Ab-coated targets and signals via its immunoreceptor tyrosine-based activation motif (ITAM) to direct Ab-dependent cellular cytotoxicity (ADCC) [361]. In healthy individuals, 90% of NK cells are $CD56^{dim}$, expressing high levels of Fcγ receptors III (CD16), i.e., $CD56^{dim}CD16^{+}$, and 10% are the $CD56^{bright}$ that can be further subdivided into $CD16^{dim}$ and $CD16^{-}$ fractions [362]. These distinct $CD56^{bright}$ and $CD56^{dim}$ NK cell subsets have different effector functions and recognize different receptors for NK cell activation and inhibition (discussed below) [362] .

The $CD56^{bright}$ cells express low concentrations of Killer cell immunoglobulin-like receptors (KIR) and inhibitory receptors ILT-2 as well as high levels of CD94-NKG2A inhibitory receptors, whereas the opposite is true for $CD56^{dim}$ NK cells [362-365]. $CD56^{bright}$ also express the NK activating receptor NKG2D which recognizes MHC class 1 related molecules MICA, MICB and UL-16 binding proteins [366]. $CD56^{bright}$ $CD16^{-}$ cells express high levels of the adhesion molecule L-selectin, which aids in their attachment to the endothelium [367]. They also express the chemokine receptors CCR7 and CXCR3, which interact with chemokines I-TAC and IP-10 to initiate chemotaxis [368].

$CD56^{bright}$ cells highly express the IL-2 receptor, IL-2Rαβγ. These cells rapidly proliferate in the presence of a low concentration of IL-2, both *in vivo* and *in vitro* [369-371]. This subset also expresses c-kit receptor tyrosine kinase that binds to ligands which enhance the proliferation and survival of these cells by upregulating bcl-2 [372, 373]. $CD56^{bright}$ NK cells that highly express IL-2R are capable of homing to secondary lymphoid organs through their CCR7 [368] where they interact with T cells which produce IL-2 to stimulate NK cells through their IL-2 receptor to produce IFN-γ. This promotes cytokine cross-talk between NK cells and T cells [362].

The proliferation of $CD56^{bright}$ NK cells can also be stimulated by high concentrations of IL-15 via the IL-2/15Rβγ (CD122) receptor consisting of a common beta chain for IL-2R and the IL-15R which is expressed on all NK cells [374]. $CD56^{bright}$ NK cells also express IL-1R and IL-18R monokine receptors and respond to IL-1β and IL-18 to secrete IFN-γ and other cytokines such as TNF-β (lymphotoxin) and GM-CSF [375, 376].

In contrast to $CD56^{bright}CD16^{-}$, $CD56^{dim}CD16^{+}$ NK cells exhibit high expression of KIR and low expression of inhibitory receptors CD94-NKG2A [364, 365]. They do not express

CCR7, but express chemokine receptors CXCR1 and CX3CR1 and respond to IL-8 and fractalkine [368]. They do not express L selectin but express PEN5 (NK cell restricted sulphated lactosamine epitope) which aids in their attachment to the endothelium [362, 363]. $CD56^{dim}$ NK cells also express high levels of leukocyte function-associated antigen 1 (LFA-1) [367]. These cells are $c\text{-}kit^{-}$ but express IL-2Rβγ [371]; consequently they do not proliferate even in the presence of high concentrations of IL-2 [370].

$CD56^{dim}$ are more cytotoxic than $CD56^{bright}$ cells [377]. They are also more granular in appearance and express high levels of CD16 which is required to mediate antibody dependent cellular cytotoxicity (ADCC) [377]. In contrast, $CD56^{bright}$ NK cells primarily secrete cytokines such as IFN-γ, TNF-β, IL-10, IL-13 and GM-CSF in response to monokines such as IL-1β and IL-18 [378]. It has been suggested that $CD56^{bright}$ cells are an important source of IFN-γ which could ultimately shape the adaptive immunity response [362].

1.7.2 Mouse NK cells

Unlike human NK cells, mouse NK cells do not express CD56. Mature NK cells are defined as cells that express $Mac\text{-}1^{high}$. Specifically they are $NK1.1^{+}$,TCR^{-}, $CD122^{+}$, $DX5^{high}$, $Ly49s^{+}$, $CD43^{high}$, $Mac\text{-}1^{high}$ and can be divided into $CD27^{high}$ and $CD27^{low}$ NK cells [379]. They differentiate from $CD11b^{dull}$ $CD27^{+}$ NK cells. [380]. Naïve NK cells express CD27, and their stimulation via CD27 triggers activation, cytokine production and induction of subsequent adaptive immune responses [381, 382]. CD27 is a member of the TNF receptor superfamily [382], and its interaction with CD27 ligand enhances NK proliferation, IFN-γ production and cytotoxicity [382]. CD27 is expressed both before and after IL-2 stimulation of NK cells [382].

Mac-1^{high}CD27^{low} cells highly express the inhibitory receptors Ly-49C and Ly-49I which recognize self-MHC class 1 molecules H-2^{b} in C57/BL6 mice. They also express KLRG1 the receptor for ligands E-, R-, and N-cadherins found on langerhans cells, keratinocytes and epithelial cells [383, 384]. In contrast, Mac-1^{high}CD27^{low} cells exhibit low expression of CD94/NKG2 cells. They are long-lived or senescent cells and are resistant to cytotoxic agents. This NK cell subset is predominantly found in the lung, peripheral blood, spleen and liver, but not in lymph nodes. CD27^{low} NK cells exhibit low cytotoxicity and respond poorly to NKG2D ligands. They also respond poorly to cytokines IL-12 and IL-18 and lack CXCR3 [379].

Mac^{high}CD27^{high} NK cells are predominantly found within secondary lymphoid organs suggesting an involvement in crosstalk with DCs [379, 385, 386]. Crosstalk between Mac^{high}CD27^{high} NK cells and DCs stimulates the NK cells to produce IFN-γ that promotes Th1 polarization [387, 388]. These subsets are present in the spleen, bone marrow, and liver. CD27^{high} NK cells highly express IL-7Rα (CD127) and c-kit as the different activating antigen CD69. These cells have a lower activation threshold when stimulated with IL-2, and respond more vigorously to activating ligands expressed on tumor cells, including NKG2D ligands, compared to CD27^{low} NK cells. Mac^{high}CD27^{high} NK cells also exhibit greater responsiveness to IL-12 and IL-18 produced by antigen-presenting cells (APC) stimulating IFN-γ and GM-CSF secretion. This subset is the predominant NK cell subset that resides in the lymph node which highly expresses CXCR3 and responds to CXCL10, IP-10 and CXCL11, chemokines which can be induced by IFN-γ [389]. A summary of the biology and function of mouse and human NK cell subsets is shown in Figure 1.4.

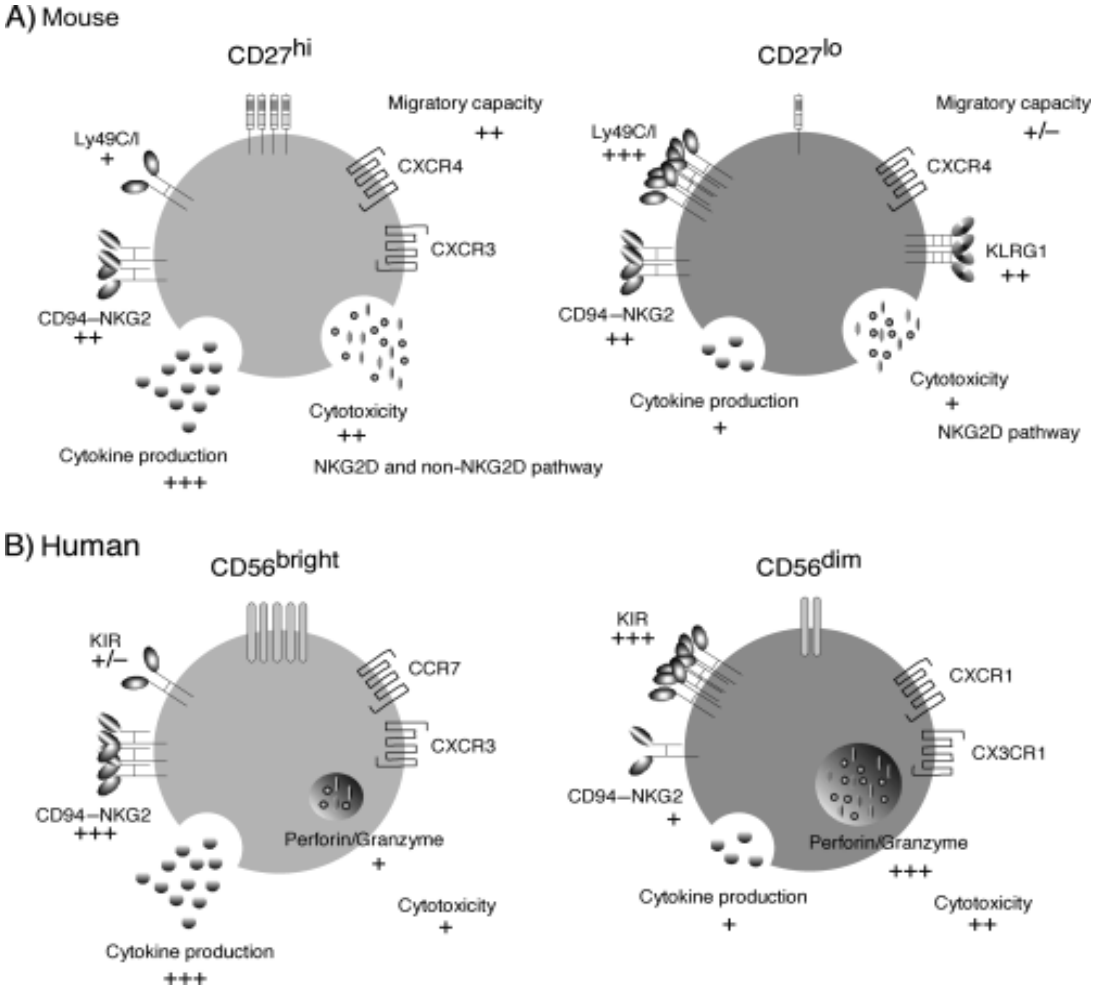


Figure1.4: Mouse CD27 NK cells and human CD56 NK cells. This figure adapted from Hayakawa *et.al* shows expression of specific markers, activating and inhibitory receptors, chemokine receptors as well as their relative ability to produce pro-inflammatory cytokines and secretes cytotoxic substances (for details refer to text) [390].

1.7.3 NK cell development and maturation

1.7.3.1 Generation of NK cell progenitors from hematopoitic stem cells (HSC)

For their development, NK cells require (1) an intact bone marrow microenvironment and (2) interactions with stromal elements, such as interactions between membrane lymphotoxin-α (LT-α)-expressing NK cell precursors and LT-α-responsive stromal cells [391-394]. It has been proposed that NK cell development occurs in 5 stages [380]. The first stage of NK cell development is the commitment of hemotopoietic stem cells (HSC) in the bone marrow (BM) to common lymphoid progenitors (CLP) that give rise to NK, T and B cells. CLP are present in bone marrow of adult mice and humans [395, 396]. The transcription factors involved in this step are the zinc-finger transcription factor Ikaros and PU.1. A deficiency in Ikaros and PU.1 results in severe defects in the development of all lymphoid cells but erythroid lineages are less affected [397]. PU.1 factor is important for early hemotopoiesis and lymphopoiesis [398]. A deficiency in PU.1 impairs NK cell differentiation but not as severely as it affects the T-cell and B-cell lineage, indicating that the transcription factor is not NK cell specific [399]. Ikaros expression is restricted to hemotopoietic cells and Ikaros$^{-/-}$ mice are deficient in mature NK and B cells and have greatly reduced numbers of T cells, again indicating that this transcription factor is not NK cell specific [400].

The second stage is the differentiation of CLP to a bipotential T/NK progenitor (T/NKP). T/NKP can give rise to T and/or NK cells [391]. CD3ε (receptors on T and NK cells) and FcεRIγ (high affinity IgE-gamma chain, a component of Fc and TCR receptors) transgenic mice exhibit selective defects in maturation which results in complete loss of both T and NK cells [401, 402]. In the third stage of NK cell development, T/NKPs differentiate to a committed NK cell progenitor (NKP). NKP cells then acquire the IL-2/15Rβ subunit (CD122) [403, 404].

Transcription factors critical for this stage of development are Ets1 and the Id2 DNA-binding protein; deficiencies in these transcription factors prevent development of NK cells but do not affect the development of T and B cells [405, 406]. NKPs are responsive to IL-15, signalling through an IL-15 receptor complex consisting of IL-15Rα, IL-2/IL-15 beta and common gamma chains; γ [407]. Mice lacking IL-15 or IL-15Rα are selectively deficient in NK cell [408]. In contrast, NK cell development is normal in mice deficient in IL-2/IL-2Rα. Deletion of IL-2Rβ or IL-2Rγ, a component of the IL-15R complex, also affects NK development [409, 410]. Mice deficient in Jak3 [411] and Stat5α/β [412] which are required for IL-15R signalling also exhibit defects on NK cell development, indicating that IL-15 is essential for normal NK cell development.

1.7.3.2 NK cell development stages from NKP to mature NK cells

The final stages of NKP development to NK cells involve the maturation of NK cell precursors into phenotypically and functionally mature NK cells. This maturation involves a series of five intermediate developmental stages. During intermediate stage I, committed NKPs express CD122 (IL-2Rβ) and subsequently at intermediate stage II, the cells acquire NK1.1 [413] and integrin αv. They also express CD94/NKG2 and NKG2D [380]. During intermediate stage III, Ly49 receptors and c-kit are acquired [391]. However, CD94/NKG2 expression is not a prerequisite for Ly49 expression as cells can express Ly49 molecules but not CD94/NKG2 [391]. During intermediate stage IV, NK cells express integrin α2 and proliferate significantly within the BM. During stage V they acquire Mac-1(αMβ1) and CD43 and proliferation markedly decreases [380]. At this stage, NK cells can be subdivided according to the level of CD27 expression [390] and by the end of this stage, NK cells acquire their full effector functions and are capable of producing IFN-γ and inducing cytotoxicity.

In mice, mature NK cells express NK1.1, CD49b, CD122, CD161, NKG2D, NKp46 and a range of Ly49 receptors, CD11b and CD43. They are capable of lysing target cells and secrete IFN-γ and TNF-α upon activation. They also produce these cytokines in response to IL-12 and IL-18 [414].

It is generally assumed that NK cell development is similar in both mice and human. However, since these developmental stages are defined by surface phenotypic markers, NK cell development stages elucidated in mice are not easily translated to humans. Some differences between human and mouse NK cell development have been noted. In humans, immature NK cells are capable of secreting cytokines and killing but in mice only mature NK cells are capable of exerting such effector functions [415]. IL-12 appears to be important for human NK cell development, but IL-12 deficiency in mice does not affect NK cell development or function [416, 417]. Human NK cell development appears dependent on IFN-γ or IL-18 but deficiencies in these cytokines in mice do not affect NK cell number [391, 418].

There is an emerging view that NK cells can complete their maturation in various organs outside the BM, and depending on the site of maturation, NK cells differ in their functions. Recent evidence suggest that NK cells can originate from human secondary lymphoid organs [419]. In lymph nodes (LN) $CD34^+$ precursors develop to functional $CD56^+$ NK cells [420]. However the relative importance of lymph nodes in NK cell development compared with BM has not been elucidated. It has also been demonstrated that a population of NK cells that express the IL-7 receptor alpha chain (CD127) arise in the thymus of C57BL/6 mice; these cells express low levels of CD11b and CD43 and resemble the immunoregulatory NK cell ($CD56^{high}CD16^-$) identified in humans [421].

1.7.4 NK cell tissue distribution

After maturation NK cells relocate to lymphoid and non-lymphoid organs [422]. Mature peripheral BM derived NK cells are most abundant in the blood, followed by the liver, spleen, bone marrow and lymph nodes [422]. In mice, the largest number of NK cells are in the spleen, with around 2-3 million cells.

There are three types of cell surface receptors involved in NK cell trafficking in mouse, CC, CXC and CX3 chemokine receptors. The chemokine receptors CCR2, CCR5, CXCR3 and CXCR1 regulate NK cell recruitment upon inflammation [422]. In inflammatory conditions, NK cells extravasate through endothelium via their chemokine receptors, migrate to inflammation sites and respond to chemokines that are expressed on inflamed tissues [423-426]. During cytomegalovirus infection in the liver, NK cells migrate to the liver and respond to CCL2 chemokines via CCR2 expressed on NK cells. However mice deficient in CCL2 exhibit a marked reduction in NK cell accumulation in the liver and a reduced ability to respond to cytomegalovirus infection [423].

The G-protein coupled sphingosine 1- phosphate receptor (S1P) has been implicated in the trafficking of NK cells in both normal and inflammatory conditions. Mice deficient in $S1P_5$ exhibit defects in the homing of NK cells to blood, spleen, lung, and inflamed liver [427]. The reduced number of NK cells in these organs suggests that other mechanisms may potentially compensate for the lack of $S1P_5$.

Whilst NK cells are not generally detectable in lymph nodes (LN), enhanced NK cell homing from blood to LN draining DC through CD62L and CXCR3-dependent extravasation has been reported [428, 429]. This recruitment of NK cells promotes antigen-specific T cell activation with NK cells in lymph nodes secreting IFN-γ for T helper cell polarization [430].

NK cells have been found in close proximity to DC and macrophages in LNs [428, 429]. This highlights a role for NK cells in bridging innate and adaptive immunity.

1.7.5 NK cell homeostasis and survival

NK cells have a half life of 7-10 days in the circulation [431] and NK cells adoptively transferred into alymphoid $Rag^{-/-}$ $\gamma c^{-/-}$ double knockout mice, proliferate and accumulate in a similar manner to T cells [431, 432]. However, when transferred into normal mice, they do not proliferate [391, 432, 433].

IL-15 is important for NK cell proliferation, development and their survival. NK cells adoptively transferred into $IL\text{-}15^{-/-}$ mice do not survive [431]. Also, blocking IL-15R signalling results in a 90% reduction of splenic NK cells [434]. The effect of IL-15 on survival of NK cells is dependent on high levels of antiapoptotic factor Bcl-2 [435]. Despite the NK cell dependence on IL-15 for survival, NK cells do not need to express IL-15Rα for their survival. However, adoptively transferred NK cells do not survive in mice deficient in $IL\text{-}15^{-/-}$ but survival is unaffected when NK cells deficient in $IL\text{-}15R\alpha^{-/-}$ are transferred into normal mice. This suggests that non NK cells expressing IL15Rα may potentially bind IL-15 and present it to NK cells facilitating their survival [436, 437].

Type I (interferon) IFN which signals through a type 1 receptor composed of IFNAR1 and IFNAR2 subunits, is also critical for the maintenance of NK cell numbers, their activation and anti-tumor responses. In both $IFNAR1^{-/-}$ and $IFNAR2^{-/-}$ mice, NK cells in spleen are significantly decreased. NK cells from these mice also express lower NK cell surface markers CD122, CD11b and Ly49 C/I and exhibit reduced cytotoxicity against sensitive tumor cells.

[438]. Type I IFN acts as a growth factor for NK cells and may regulate the expression of IL-15, since inhibiting IL-15 during interferon treatment prevents NK cell proliferation [439, 440].

1.8 Receptor-mediated NK cell responses

NK cell responses are determined by the balance of inhibitory and activating signals received from receptors on their surface [354]. The balance of signals forms the decision of whether an NK cell becomes activated or their activation is inhibited. Inhibitory receptors signal through intracellular immunoreceptor tyrosine-based inhibitory motif (ITIM) which is located in their cytoplasmic tail. After phosphorylation of the tyrosine residue, Src homology 2 domain containing phosphotases (SHP1 or 2) are recruited which inhibit NK cell activation [441]. In contrast, some activating receptors signal through immunoreceptor tyrosine-based activating motifs (ITAMs) contained in associated molecules. After phosphorylation of a tyrosine residue in the cytoplasmic tail, the Src homology 2 domain containing kinases (Syk or ZAP70) are recruited, which leads to a signalling cascade that results in degranulation and transcription of cytokine and chemokine genes. Other activating receptors such as NKG2D utilize an alternate signalling mechanism using DAP-10 or DAP-12 [441, 442].

NK cells are capable of identifying target cells via expression of MHC class I on target cells [442]. Each NK cell expresses a selection of receptors, which enables NK cells to recognize a diverse repertoire of different MHC class I molecules [443].

Since some receptor families contain both activating and inhibitory receptors, therefore each receptor will be discussed with respect to structure, function and regulation.

1.8.1 Mechanisms preventing NK cell activation

Inhibitory receptors specific for MHC class 1 molecules play a central role in 'missing self recognition'. Missing self recognition is defined as the ability of NK cells to recognize and eliminate host hemotopoetic cells that lack or have downregulated MHC class 1 molecules, which are expressed by all normal cells [356, 357]. NK cell inhibitory receptors are categorized into two different families, the Ig superfamily which include in humans the killer cell Ig-like receptor (KIR) and the leukocyte Ig-like receptors (LIR), and in mice the C-type lectin-like receptor superfamily which includes Ly49 receptors. The Heterodimeric C-type Lectin Receptor (CD94/NKG2) complex is present in mice and humans [443].

1.8.1.1 Killer cell immunoglobulin –like receptors

KIRs are encoded by 15 polymorphic genes and 2 pseudogenes on chromosome 19q134 [354]. They are expressed by subsets of NK cells (as previously described). The human KIRs evolved from the Ig superfamily and are type 1 transmembrane glycoproteins with two Ig-like domains (KIR2D) or three Ig-like domains (KIR3D) in their extracellular region. A short stalk region separates the Ig-like domain from the transmembrane segment and the cytoplasmic domain. Some receptors possesses long cytoplasmic domains that contain one or two ITIM sequences, and others have short cytoplasmic domains without ITIM [444]. KIRs with long cytoplasmic domain are usually referred to as KIR2DL and KIR3DL. KIRs with short cytoplasmic domain are referred to as KIR2DS and KIR3DS. They associate with DAP12 adaptor protein to exhibit activating signals.

KIRs recognize polymorphic epitopes on human leukocyte antigen classes HLA-A, HLA-B, HLA-C proteins [445]. The members of the KIR2DL subfamily recognize HLA-C

alleles, whereas the members of KIR3DL family recognize HLA-A and HLA-B alleles. Ligands for KIR2DS and KIR3DS molecules have not been characterized [354].

The binding of inhibitory KIR to MHC class I ligands expressed by target cells results in suppression of cytokine secretion and cytotoxicity. Recruitment of SHP-1 to tyrosine phosphorylated ITIM in KIR is the mechanism that inhibits NK cells [446, 447]. These receptors ensure NK cell tolerance to self MHC class I, allowing responses only to cells that lack MHC class I molecules. KIR prevents NK activation by inhibiting the cytoskeleton-dependent movement of activating receptors. Specifically, engagement of ITIM containing - inhibitory KIR receptors prevents the accumulation of actin at NK cell immune synapses which is essential for cytotoxic effector functions of NK cells [448].

1.8.1.2 Leukocyte Ig-like inhibitory receptors

Leukocyte Ig-like inhibitory receptors (LILR) belong to the IgG superfamily. The LILR family of 13 genes is located on chromosome 19q13.4. Two of the 13 genes, LILRB1 and LILRB2 encode inhibitory receptors that bind MHC class I molecules, however only LILRB1 is expressed by NK cells, (ranging from undetectable to ≈ 75% on peripheral NK cells). LILRB1 contains four Ig-like domains in the extracellular region and four ITIMs in its cytoplasmic domains [449]. It binds to a conserved region in the α3 domain of MHC class 1 protein, which enables the recognition of a broad spectrum of MHC class 1 proteins including HLA-A, HLA-B, HLA-C and HLA-G alleles [450].

1.8.1.3 Ly49 receptors

Murine NK cells express multiple Ly49 receptors that are type II transmembrane receptors. These receptors are expressed on subsets of mouse NK cells. The Ly49 gene is best characterized in C57Bl6 and 129/J mouse strains with 14 genes identified on chromosome 6 [451-453]. Most Ly49 genes encode inhibitory receptors (Ly49 A, C, G and I), which inhibit NK cell function upon binding of class I ligands present on target cells. These inhibitory receptors contain ITIM in their cytoplasmic domains that is phosphorylated upon stimulation, resulting in the recruitment of SHP-1 phosphotase to prevent intracellular signalling. There are also Ly49 activating receptors, Ly49D and Ly49H that do not contain ITIMs but rather associate with ITAM containing DAP-12 adapter molecule important for positive signalling by these receptors [454-456]. Ly49 receptors are functionally similar to KIRs, however the human KIRs are structurally dissimilar as they belong to the immunoglobulin receptor superfamily. Ly49A is a disulfide-linked homodimer with type II membrane orientation, which is composed of C-type lectin-like domain. It binds to H-2D at two distinct sites, one site involving the $\alpha 1$ and $\alpha 2$ domains of MHC class I and the second site that spans the $\alpha 1$, $\alpha 2$, $\alpha 3$ domains and $\beta 2$ microglobulin [457, 458].

Circulating NK cells expressing activating Ly49 receptors also coexpress Ly49 inhibitory receptors. Therefore, NK cells express Ly49D activating receptor that binds to H2-D^d and also coexpress the inhibitory receptor Ly49G2 or Ly49A at high levels which binds to the same ligand [459]. In general, the inhibitory signal overrides any activating signal generated via activating Ly49 receptors [459]. To override the inhibitory signal, NK cells require two positive signals: (1) the engagement of Ly49D activating receptors to their ligand H2-D^d and (2) stimulation by inflammatory cytokines such as IL-12 or IL-18 [460, 461]. Ligation of the Ly49D

activating receptor on NK cells stimulates the secretion of cytokines IFN-γ and TNF-α and chemokines MIP1α and MIP1β.

1.8.1.4 CD94/NKG2 Receptors

The CD94 and NKG2 family of genes are present in humans and mice. They are located on human chromosome 12p12.3-p13.2 and on mouse chromosome 6 [462]. CD94 and NKG2 C-type lectins covalently assemble to form a NK inhibitory receptor for HLA class I molecules. The receptor recognizes nonconventional MHC class Ib ligands, human HLA-E and mouse $Qa1^b$ molecules, and is expressed by most NK cells [463, 464]. CD94 associates as a disulfide-linked homodimer or as a disulphide-linked heterodimer with members of the NKG2 family including NKG2A, NKG2C or NKG2E. NKG2A has an ITIM in its cytoplasmic domain and the CD94/NKG2A complex transmits inhibiting signals [465]. In contrast, CD94/NKG2C associates with adaptor protein DAP12 to transmits activating signals [466]. Mouse CD94/NKG2C and CD94/NKG2E associates with DAP12 for stimulatory signals [467].

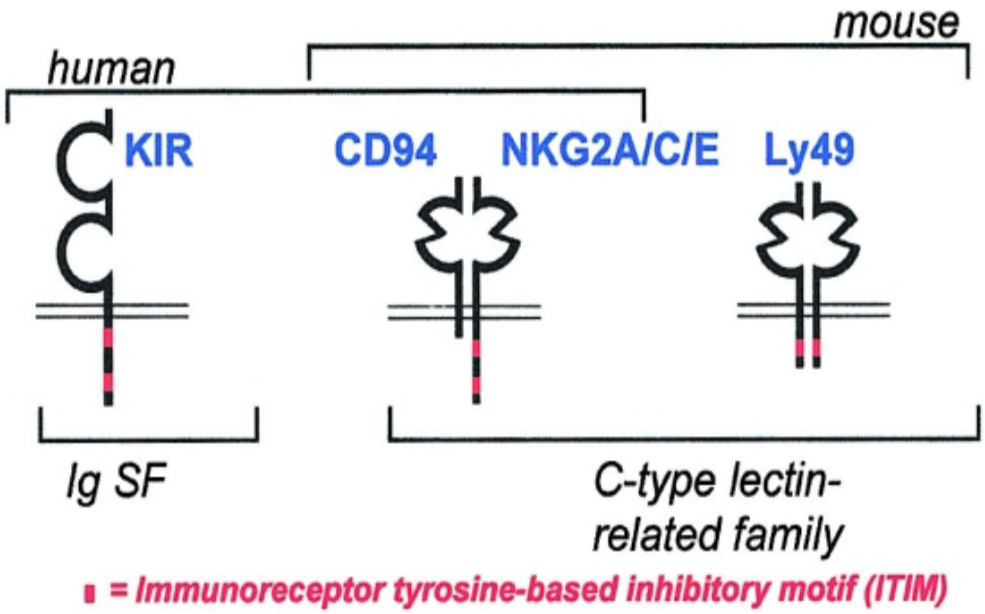


Figure 1.5: NK cell inhibitory receptors. Schematic shows NK cell inhibitory receptors. Inhibitory receptors are divided into two superfamily the IgG superfamily and C-type lectin superfamily. Human NK cells express killer Ig-like receptors and CD94/NKG2 receptors. Mouse NK cells exclusively express Ly49 receptors and also express CD94/NKG2A receptors. Inhibitory receptors have ITIM in their cytoplasmic domain [468].

1.8.2 Mechanisms that promote NK cell activation

1.8.2.1 NK cell activating receptors

NK cell activating receptors are expressed by both human and mouse NK cells and include NKG2D, 2B4 and natural cytotoxicity receptors (NCRs) NKp44, NKp46 and NKp30 [390]. Under normal circumstances NK cell activity is dominated by the signals from inhibitory receptors, which prevents NK cell effector functions. This is strongly influenced by MHC class I molecules that are expressed by all normal tissues [469] However, in tumor cells with DNA damage, virus-infected cells and stressed cells, expression of MHC-class I molecules is altered, and they express more activation ligand. Under these circumstances activating signals dominate inhibitory signals.

1.8.2.1.1 NKG2D receptors

NKG2D is the most characterized NK cell activation receptor. NKG2D receptors are expressed on the surface of virtually all NK cells [355] and are present on human and mouse NK cells [470, 471]. The NKG2D receptor is a type 11 transmembrane glycoprotein with an extracellular C-type lectin-like domain [470]. The NKG2D gene is located on human chromosome 12 and mouse chromosome 6 [471, 472]. NKG2D is a primary activation receptor for NK cell and triggers cytotoxicity. NK cells are activated by this receptor when they encounter tumor cell lines, bacterial/virus infected cells, and damaged cells that express ligands for NKG2D [366, 473, 474].

Activation of NKG2D receptors on NK cells results in a strong signal that either bypasses or overrides any inhibitory signals from major histocompatibility complex (MHC) class 1-

specific receptors, killing target cells. These receptors can provide both activating and costimulatory signals, which is achieved by selective association with DAP10 or DAP12 which contains YxxM and ITAM motifs respectively [475, 476]. NKG2D-L (long form) associates with DAP10 but not DAP12 and NKG2D-S (short form) associates with both adaptor proteins, most likely due to differences in cytoplasmic tails of the receptor isoforms [477-479]. DAP10 recruits PI3K to its YxxM motif [480] whilst DAP12 binds to Syk or ZAP70 kinases and subsequently recruits PI3K, phospholipase Cg1 and p44/42 ERK [481, 482]. Expression of these two receptor isoforms varies *in vitro* depending on culture conditions. Freshly isolated NK cells predominantly express NKG2D-L but after activation expression of NKG2D-S is increased [477]. Signalling through NKG2D-L-DAP10 in resting NK cells increases cytotoxicity [478].

Ligands for NKG2D are structurally related to MHC class I molecules. In humans, there are two members of the MHC class I-polypeptide related sequence family (MICA,MICB), and six members of the UL16-binding protein (ULBP) family that are also known as retinoic acid early transcript (RAET) proteins [366, 483]. In mice there are five retinoic acid early transcript-1 (Rae-1), three variants of H60 minor histocompatibility antigen, and murine UL16-binding-protein-like transcript-1(MULT1) [473, 484, 485]. Ligands are induced when cells are infected with a wide range of viruses [486, 487], or in solid tumours and leukaemia cells [488] and in cells containing damaged DNA [489].

Rae-1 is expressed on the surface of mouse tumor cells and on cells following viral and bacterial infection [473, 474, 490]. For example, all five retinoic acid early transcript-1 (Rae-1α to Rae-1ε) are rapidly elevated on F9 embryocarcinoma cells after treatment with retinoic acid [491]. MICA/B stress-inducible ligands are under the control of heat shock promoter and expressed on the cell surface during cellular stress, transformation, viral or bacterial infection [487, 492]. Infection with cytomegalovirus (CMV) also upregulates ligands MICA, MICB and

ULBPs on fibroblasts in humans and Rae-1 on infected peritoneal macrophages in mice [487, 490, 493]. The binding of these ligands to NKG2D receptors results in IFN-γ, TNF, lymphotoxin and GM-CSF, and chemokine CCL4 and CCL1 expression on NK cells [494-496]. It also stimulates the release of granzymes that mediate NK cell lytic functions [497].

NKG2D ligands also participate in crosstalk between NK cells and other immune cells. Lipopolysaccharides upregulate surface expression of ULBP and MICA on macrophages. This triggers NK cell mediated cytotoxicity towards these macrophages, eliminating over-stimulated macrophages, thereby regulating innate immune responses [498]. Furthermore, NK cells and dendritic cells can reciprocally activate one another by cell-cell contact upon the engagement of NKG2D receptor on NK cells to NKG2D ligands expressed on TLR activated dendritic cells [499, 500]. Thus NKG2D receptors on NK cells can not only control pathological cell number but may also regulate responses of normal cells [501].

The local cytokine microenvironment is an important regulator of NKG2D function. NKG2D expression on NK cells is upregulated by IL-15 [494]. Also, NKG2D expression can be downregulated by IL-4 or IL-12/IL-18, reducing NKG2D-ligand-dependent cytotoxic functions [502]. TGF-β, IL-12 and type 1 IFN also suppress NKG2D expression in human NK cells, as does IL-21 [503-505].

1.8.2.1.2 2B4 receptors

Receptor 2B4, also called CD244, and its ligand CD48 are members of the CD2 family of immunoglobulin-related proteins. 2B4 is expressed on all human and mouse NK cells. CD48 is

expressed on lymphocytes, viral-infected cells and tumor cells [354, 506, 507]. The activation of 2B4 with CD48 induces IFN-γ secretion and stimulates non-MHC-restricted killing by NK cells. Human NK cells express two isoforms of the receptor, 2B4-A and 2B4-B which differ in binding affinity for CD48. 2B4-A has a high affinity for CD48 and results in greater activation. This receptor mediates natural cytotoxicity against target cells expressing CD48, whereas 2B4-B is ineffective [508]. In mice, there are also 2 isoforms, a longform 2B4-L with four tyrosine motifs in the cytoplasmic domain and short form 2B4-S with one tyrosine [509]. 2B4L is an inhibitory receptor and 2B4S is an activating receptor.

2B4 receptors activate NK cells, triggering cytotoxicity and IFN-γ production. These effects are modulated via lymphocyte activation molecule-associated protein (SLAM) associated protein (SAP). However, the lack of SAP triggers inhibitory signals through 2B4 receptors [510]. The importance of SAP has been demonstrated in patients with a loss of function mutation in the X-linked SAP gene; NK cells from these patients cannot be activated via the 2B4 receptor [511]. Human 2B4 also functions as a co-receptor for other NK activating receptors such as NKp46 [512], enhancing NK cell responses under limiting-ITAM-mediated activation. NK cell activation via the 2B4 receptor results in phosphorylation of tyrosine-based motifs in the cytoplasmic tail and recruitment of SAP signalling by activation molecule associated protein and the Src-family kinase Fyn [513]. However, issues as to the mechanism by which 2B4 provides activation signals in relation with other receptors and ligand interactions, and whether 2B4 can trigger cytotoxicity independently of other receptors remains to be elucidated.

1.8.2.1.3 Natural cytotoxicity receptors

Natural cytotoxicity receptors (NCRs) include NKp46, NKp44 and NKp30. NCRs belong to the Ig superfamily and contain a charged amino acid in their transmembrane domain which associates with ITAM-bearing adaptor molecules. NKp30 and NKp46 are expressed by all NK cells, but NKp44 is only expressed by activated NK cells. NKp46 is a 46 kDa glycoprotein that contains two C2 Ig-like extracellular domains. NKp46 signals by association with the adapter molecules CD3ζ and FcεRγ which contain ITAM motifs [514]. NKp44 is a 44kDa surface glycoprotein which signals through the association and phosphorylation of the DAP12 adaptor molecule [515]. NKp44 has a IgV-like fold with a putative ligand binding site for anionic ligands [516]. NKp30 is a 30 kDa glycoprotein containing one V-type Ig-like region in its extracellular domain. It associates with CD3ζ chain to initiate activating signals [517].

Cellular ligands for these receptors are still being elucidated. Heparan sulphate proteoglycans (HSP) appear to be important in the recognition of NCR ligands by NKp46 and NKp30 [518]. Tumor cells expressing heparinase are resistant to NK cell-mediated lysis compared to normal cells that do not express heparinase [519].

Pathogen-derived structures have been shown to activate NCR. NKp46 and NKp44 receptors recognize the haemagglutinin protein of viruses, stimulating NK cell cytotoxicity [520, 521]. NKp30 binds to the pp65 protein of human cytomegalovirus (HCMV) to inhibit NK cell cytotoxicity[522]. Human NKp44 on NK cells not only recognizes ligands on virus infected cells but also recognizes bacterial pathogens such as mycobacterium [523]. NCR are also important for stimulating anti-tumor responses of NK cells; antibody-mediated inhibition of NCR inhibits NK cell-mediated lysis of tumor cells. NKp30 can cooperate with NKp46 and /or NKp44 to induce NK-mediated cytotoxicity against tumor cells [517]. It is also involved in the crosstalk between dendritic cells and NK cells (see later) [524].

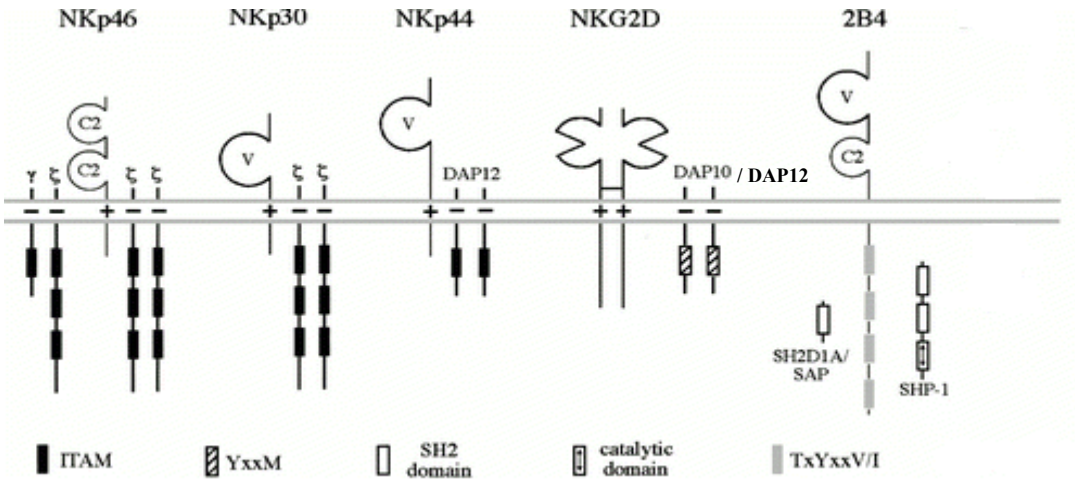


Figure 1.6: NK cell activating receptors. Schematic adapted from Moretta, et al. 2001 shows NK cell activating receptors. All NK cells express NKG2D receptors that signal via association of DAP10/DAP12 that contains YxxM and ITAM motif in their cytoplasmic domains respectively, Natural Cytotoxicity Receptors (NCR), NKp46, NKp30 and Nkp44 that signals through ITAM in their cytoplasmic domain and 2B4 receptors that signal through SAP [518].

1.8.2.2 Other receptors that activate NK cells

1.8.2.2.1 Toll-like receptor 3

NK cells express TLR3 on the cell surface and intracellularly [525-527]. TLR3 consists of an extracellular domain containing 23 leucine rich repeats (LRRs) and N- and C-terminal flanking regions, a transmembrane domain, and intracellular domain [528]. The recognition of TLR3 transmits signals via Toll-IL-1 receptor (TIR) domain containing adaptor molecule-1 (TICAM-1) also known as TRIF (TIR-domain containing adapter inducing IFN-β). Both these adaptors initiate downstream signalling pathways that lead to activation of transcription factor IF3, IRF7 and NFκb leading to induction of type 1 IFN: i.e., IFN-α and IFN-β, pro-inflammatory cytokines and molecules involved in antigen presentation [525, 529, 530].
TLR3 recognizes double-stranded RNA (dsRNA) produced during viral infections and also mammalian RNA released by necrotic cells [531, 532].

Most studies aimed at understanding TLR3 activation have used the synthetic polyinosinic-polycytidylic acid (Poly IC). Upon activation TLR3 recruits the Toll-IL-1 receptor (TIR) domain containing adaptor molecule-1 (TICAM-1) and induces IRF-3 activation followed by IFN-β promoting activation [529].

Human NK cells express TLR3 and can directly recognize poly IC [533]. Poly IC has been shown to activate TLR3 expressed in cultured human NK cells [534-536]. Upon Poly IC stimulation, TLR3 RNA levels were upregulated in NK cells, resulting in enhanced NK cell-mediated cytotoxicity, upregulation of NK cell activation marker CD69 and secretion of pro-inflammatory cytokines IL-6, IL-8 and IFN-γ. Accordingly, the activation of NK cells in response to poly IC is independent of type 1 IFNs, as the Poly IC stimulation does not induce secretion of IFN-α or IFN-β in NK cells [534].

In addition to TLR3 there is a cytosolic dsRNA receptor on NK cell, melanoma differentiation associated antigen 5 (MDA5), which recognizes Poly IC to induce IFN-α/β production. MDA5 signals via IPS1 [537], leading to activation of IRF3, IRF7, IFR1, NFκB and expression of type 1 interferon α and β [538, 539]. MDA5 consists of three functional domains, tandem-CARDs (caspase activation and recruitment domain), a DEAD box helicase-like domain and a well conserved C-terminal domain (CTD) [540]. MDA5 appears more important than TLR3 for triggering NK cell cytotoxicity, CD69 upregulation, and IFN-γ production [541]. This was evident when NK cells from $MDA5^{-/-}$ mice injected with Poly IC had significant defects in cytotoxicity, CD69 expression and IFN-γ production whereas NK cells from $TLR3^{-/-}$ mice injected with Poly IC had only had modest defects in cytotoxicity, CD69 expression and IFN-γ production. These results suggest that MDA5 may be able to compensate for the lack of TLR3, and the TLR3 contribution to NK cell activation is evident only in the absence of MDA5 [541].

NK cells can also be activated indirectly by Poly IC through $CD8\alpha^{+}$ conventional dendritic cells (DCs). Stimulation with Poly IC results in DC maturation and migration to secondary lymphoid organs [542-545]. Poly IC triggers IPS1 and TRIF signalling through TLR3 on $CD8\alpha^{+}$ DC to produce type I IFNs and IL-12p40 which subsequently activates NK cells to produce IFN-γ [546]. Poly IC stimulated dendritic cells are also important in recruiting NK cells to the draining lymph node.

Poly IC mediated NK cell activation can also occur independently of CD8α [541]. NK cells isolated from $BATF3^{-/-}$ mice which do not have $CD8\alpha^{+}$ DCs, after injection with Poly IC effectively kill RMA-S cells *in vitro*. After Poly IC injection, CD69 expression, NK cell secretion of IFN-γ, serum IFN-α and serum IL-12p40 were all upregulated in $BATFF3^{-/-}$ mice suggesting that TLR3/MDA5 on other cell types may also be important.

TLR3 is expressed in human and mouse atherosclerotic lesions. Cole *et al* observed that TLR3-deficient ApoE$^{-/-}$ mice exhibited increased early atherosclerosis compared to TLR3 expressing mice under conditions where no Poly IC was administered [547]. This observation is puzzling as the ligation of TLR3 usually promotes proinflammatory and growth promoting effects. However, additional research will be important in examining the role of Poly IC and NK cells on atherosclerosis, in relation to TLR3 and MDA5.

1.8.2.2.2 Fc receptor

Fc receptor is expressed on the surface of NK cells and interacts with a region of antibodies known as Fc (Fragment, crytallizable) region. Fc receptors bind to antibodies that are attached to infected cells or pathogens, stimulating phagocytosis or cytolysis of infected cells via antibody-mediated phagocytosis or antibody dependent cell-mediated cytotoxicity (ADCC). Fc receptors are classified on the basis of the type of antibody they recognize. Fc receptors on NK cells that recognize IgG bound to the surface of pathogen-infected cell are classified FcγRIIIα (CD16) [548]. FcγRIIIα is a type 1 transmembrane receptor containing two extracellular Ig-like domains and functions as a low affinity IgG receptor, binding IgG antibodies via their Fc region [549]. Activation of FcγRIIIα on NK cells by IgG initiates the production of cytokines such as IFN-γ or stimulates the secretion of cytotoxic mediators such as perforin and granzyme [550]. FcγRIIIα is the most potent activating receptor on NK cells and induces TNF-α and IFN-γ cytokine secretion in the absence of other exogenous signals [344]. FcγRIIIα on NK cells also mediates direct killing of virus-infected and tumor cells independent of antibody such as IgG ligation. The incubation of target cells and NK cells expressing FcγRIIIα in the presence of Fc receptor blocking antibody resulted in NK cell-mediated killing of target cells [551]. Therefore, it is possible that FcγRIIIα is also activated by other yet to be identified ligands.

1.9 Interaction between NK cells and other immune cells.

1.9.1 NK cell interaction with Dendritic cells

NK cells can be activated by DCs. Adoptive transfer of activated DCs promotes NK cell mediated antitumor responses by increasing NK cell cytolytic activity and IFN-γ production. This interaction is dependent on cytokine secretion by DCs and cell to cell contact [552]. DC derived IL-12, IL-18, IL-15 and type 1 interferon contribute to this activation [553].

NK cell - DC interactions have been observed *in vivo* at various sites of infection, for example, in Malasseezia sympodialis-induced skin lesions [554] and in lymph nodes. DCs can also stimulate NK cell proliferation. For example, $CD56^{high}CD16^{-}$ NK cells in lymph nodes proliferate in response to membrane bound IL-15 expressed by DCs [555]. NK cell activation by DCs provides the early source of IFN-γ to promote the proliferation of T cells and their polarization towards Th1 phenotype [430, 556]. Interaction between NK:DC provide a bridge between innate and adaptive immunity [381]. The direct contact interaction of NK cells with DCs also elevates IL-12 production by DCs and increases the ability of DCs to prime $CD8^{+}$ T cells [557]. The increase in IL-12 production by DCs is mediated via the NKG2D receptor on NK cells since antibody inhibiting NKG2D reduces IL-12 production [557].

Activated NK cells play a role in DC maturation. This function is exerted by direct DC stimulation or through killing those DCs that did not properly acquire a mature phenotype. Co-culture of activated human NK cells with immature monocyte-derived DCs, increased expression of DC maturation marker CD86 and DC cytokine production [558]. NK cell derived TNF-α and IFN-γ as well as cell contact are crucial for DC maturation. This maturation has been shown to be dependent on triggering of NKp30 receptors on NK cells which initiates IFN-γ and TNF-α

secretion [559]. NK cells can also lyse DCs. Higher numbers of NK cells triggers the killing of immature DCs (iDCs) but not mature DCs [558]. This killing of iDCs by NK cells is also dependent on NKp30 signalling [560]. It can be inhibited by TGF-β which strongly down-regulates NKp30 receptors on NK cells inhibiting their lytic functions [504]. Both maturation and killing of immature DCs are induced by the engagement of the NKp30 receptor by undefined ligands on DCs.

1.9.2 NK cell interactions with iNKT cell

NKT cells can activate NK cells [561, 562]. Stimulation of NKT cells with marine sponge glycolipid, alpha-galactosyleceramide (α-GalCer) activates hepatic NK cells to eliminate hepatoma cells from the liver *in vivo* [563]. Also NKT cell activation enhances anti-viral responses mediated by NK cells after cytomegalovirus infection in mice [564]. The mechanism by which NKT cells activate NK cells appears to involve IFN-γ production by NKT cells as the anti-metastatic effects of α-GalCer, which activates NKT cells, is dependent on both NK cells and IFN-γ [565]. Furthermore, Smyth et al have shown that sequential production of IFN-γ by NKT cells and NK cells is essential for the anti-metastatic affects of NK cells [566].

1.10 NK cell effector function

Activated NK cells exert their effects either via cytotoxic mechanisms, cytokine-dependent mechanisms or both mechanisms. NK cells can be highly cytotoxic. For killing, they require the direct or close contact with the target cells. NK cells release cytotoxins via exocytosis of cytoplasmic granules containing membrane-disrupting proteins such as perforin and proteases

such as granzymes to induce target cell lysis or apoptosis. NK cells also possess ligands (FASL and TRAIL, discussed below) on their surface that stimulate target cells expressing the receptor for these receptors to induce target cell apoptosis.

Granule exocytosis to release cytotoxins occurs following NK-target cell synapse formation with the granules fusing with the plasma membrane to release factors such as the pore-forming protein perforin together with proteases such as granzymes A and B [567]. The expression of perforin on NK cells is regulated by signals from NKG2D activating receptors, and cytokines IL-2, IL-15 and IL-21[568]. It facilitates the delivery of granzymes to target cells promoting apoptosis. Perforin is an important component of the NK cell killing machinery, as $Pfp^{-/-}$ mice are unable to clear intracellular infections and exhibit reduced efficiency in eliminating fibrosarcoma [569]. In humans, mutations in the perforin gene result in familial hemophagocytic lymphohistiocytosis (FHL) due to reduced NK cell cytotoxicity [570, 571].
NK cells express three granzymes, Granzyme A, B and M. These granzymes enter the cytosol of target cells to trigger apoptotic cell death in a perforin-dependent manner. Granzyme B triggers caspase-3-dependent cell death and also damages DNA by nicking ssDNA. In contrast, granzyme A mediates caspase-independent cell death of target cell by causing oligonucleosomal fragmentation [567, 572, 573].

Granzyme B is the most characterized and abundant member of the granzyme family. It is a 32 kDa serine protease. The receptor for granzyme B is mannose-6-phosphate [574]. Recently, Granzyme B has been shown to cleave extracellular matrix substances and may induce cell death by this mechanism [575]. It might also contribute to the vascular remodelling associated with atherosclerosis.

FasL is a type II membrane protein expressed by NK cells that is also packaged in the cytoplasmic granules. FasL-mediated cytotoxicity by NK cells is directed against Fas-expressing target cells [576]. It is initiated by the engagement of surface bound FasL on NK cells to Fas receptor expressed on target cell surface. This promotes the recruitment of 'death-domain' containing proteins leading to activation of the caspase cascade [577]. FasL expression on NK cells is regulated by Fc receptor stimulation (activating receptor) and by NK cell inhibitory receptors. The activation of Fc receptor (FcγRIII) upregulates the FasL expression on NK cells which upon interaction with Fas positive target cells triggers NK cell mediated target cell apoptosis [578]. FasL-mediated cytotoxicity may be downregulated in NK cells expressing CD94/NKG2A inhibitory receptor via the HLA class I ligand expressed on target cells [579]. The engagement of this inhibitory receptors inhibits the activating signal mediated by FcγRIII and inhibits the transport of cytotoxic granule contents to the cell surface of target cells [580]. NK cell derived IFN-γ upregulates Fas expression on tumor cells and this facilitates the ability of these cells to eliminate tumor cells [581].

TNF-related apoptosis-inducing ligand (TRAIL) is a type II membrane protein that is also expressed by NK cells. TRAIL is also capable of inducing apoptosis by binding with death–domain containing receptors TRAIL-R1 and TRAIL-2 thereby activating the caspase cascade. [582]. TRAIL expression is upregulated by IL-2, IL-15 and IFN-γ on NK cells [583]. TRAIL deficient mice exhibit loss of NK cell cytotoxic function [584].

Overall, NK cell cytotoxicity plays a crucial role in preventing inflammation and systemic inflammatory syndromes.

NK cells are an important source of cytokines. Upon activation, NK cells secrete IFN-γ, TNF-α, GM-CSF, IL-10 and IL-13 [378]. Cytokine production by NK cells is regulated by inflammatory mediators including the cytokines IL-2, IL-15, IL-4, IL-10 and IL-12, TLR ligands

such as dsRNA, engagement of NK cell activating receptors with ligands on target cells, and following interaction of NK cells with NKT cells, macrophages and dendritic cells. IFN-γ is the most important cytokine secreted by NK cells.

NK cell derived IFN-γ promotes macrophage activation, mediates antiviral and antibacterial immunity, promotes autophagy, enhances antigen presentation to macrophages and dendritic cells, orchestrates activation of innate immune system, coordinates lymphocyte-endothelium interaction, regulates Th1/Th2 balance, and controls cellular proliferation and apoptosis of target cells [585]. Cytokines produced by activated monocytes such as IL-12, IL-15, and IL-18 enhance the production of IFN-γ by NK cells. For example, the combined stimulation of NK cells with IL-12 and IL-18 produces very high levels of IFN-γ [586]. In contrast, IFN-γ production by NK cells can be inhibited by IL-4 [587]. Hence the cytokine repertoire of the inflammatory environment regulates the IFN-γ production by NK cells.

IFN-γ produced by NK cells provides an innate barrier to infections and also affects tumor metastasis. NK-derived IFN-γ mediates the early resistance to Myocobacterium tuberculosis [588]. Similarly, IFN-γ secreted by NK cells suppresses viral transcription during infection [589]. NK cell derived IFN-γ also inhibits the growth of various tumors [590] but the exact mechanism is still unclear [591].

1.11 NK cells and inflammatory diseases

NK cells are important in a variety of inflammatory disease including rheumatoid arthritis (RA), type 1 diabetes (T1D) and experimental autoimmune encephalomyelitis (EAE).
NK cells can influence both the development and progression of rheumatoid arthritis (RA). RA is characterized by joint inflammation leading to progressive destruction of cartilage and bones

[592]. Substantial numbers of NK cells, particularly $CD56^{bright}$ NK cells, are present in synovial fluid of affected joints [593]. NK cells appear to attenuate collagen-induced arthritis (CIA) since antibody-mediated depletion of NK cells exacerbates disease. The mechanism is unknown but may involve IFN-γ production by NK cells; IFN-γ ισ an inhibitor of Th17 cell differentiation, and Th17 cells have a pathogenic role in RA [594] . In contrast, other studies suggest that NK cell depletion prevents bone erosion and augments CIA [595].

Type 1diabetes (T1D) is characterized as an immune-mediated progressive destruction of pancreatic β cells. NK cells have been implicated in T1D and have been shown to both augment and attenuate its development. These effects appear dependent on the manner in which NK cells are activated. NK cell activation through the engagement of NKp46 receptor by unknown ligands on β cells results in destruction of β cells thereby promoting development of T1D [596]. In contrast, NK cell activation through Poly IC, an analog of dsRNA in nonobese diabetic mice (NOD) mice reduces the incidence of T1D. This protective effect is possibly mediated by promoting Th2 immune responses in response to islet autoantigen [597]. Clearly more research is required to understand the role of NK cells in diabetes.

NK cells play a regulatory role in experimental autoimmune encephalomyelitis (EAE). EAE is known to be mediated by $CD4^+$ T cells that recognizes peptides derived from encephalomyelitis proteins of the central nervous system and is characterized by a paralysis from the hindlimbs to the forelimbs [598]. *In vivo* NK cell depletion in a model of EAE induced by immunization with the myelin oligodendrocytes glycoprotein peptide, has been shown to diminish the onset of EAE by reducing T cell responses [598]. The absence of NK cells reduced the production of proinflammatory cytokines such as TNF-α and IL-6 and growth promoting

cytokines IL-2 by T cells; hence NK cells have been shown to regulate the immune responses that initiate EAE.

1.12 NK cells and cardiovascular disease

NK cells are involved in viral myocarditis, in cardiac repair and angiogenesis after myocardial infarction and atherosclerosis.

Myocarditis is an inflammatory disorder of the myocardium with necrosis of the myocytes and infiltration of inflammatory cells that decrease myocardial function and enlarge the heart. It is the consequences of viral infections including Coxsackie virus and adenovirus. In the acute phase of mycocarditis, NK cells are the predominant cell type in the myocardium [599]. NK cells infiltrate the myocardium killing virus infected myocytes in a perforin-dependent manner clearing the viral infection [600]. In this manner, NK cells provide some protection against viral-induced myocarditis by preventing virus replication in the heart tissue [601].

Myocardial infarction (MI) is associated with an intense inflammatory response and activation of the host innate immune system that enhances inflammatory cell mobilization, cardiac repair and remodelling. Studies have reported that steel factor positive (c-kit) bone marrow derived hematopoietic cells repair damaged myocardium through neovascularization and myogenesis [602, 603]. C-kit plays a major role in NK cell development and mobilization [604]. Ayach *et al* reported that c-kit signalling after MI is mediated by bone marrow derived NK cells which contributes to improved remodelling and cardiac function [602]. They also reported that the depletion of NK cells in immuno competent mice reduced their survival as a result of heart failure. More recently, a study has reported the mechanism by which NK cells promote cardiac repair. In this study, activated NK cells have been shown to bind to cardiac endothelial cells

(CEC) to induce their proliferation and promote angiogenesis via their inhibitory receptor KLGR1. Specifically, activated NK cells binds to CEC through α4β7 integrin and VCAM-1 and disrupt N-cadherin association via KLRG1, which dislocates β-catenins that removes the cell contact inhibition of proliferation that subsequently enhances CEC proliferation and angiogenesis [605].

NK cells have been detected in human atherosclerotic lesions in low numbers [606]. Also, patients with severe atherosclerosis also have higher levels of circulating NK cells [607]. They have also been identified in atherosclerotic lesions of $LDLR^{-/-}$ mice fed a high fat diet [608]. However, their significance for atherosclerotic lesion development is still unclear.

Early studies on determining the role of NK cells in atherosclerosis utilized beige mutation mice in which NK cells exhibit low cytotoxic activity [609]. Using these mice on a high fat diet (HFD), no differences in lesions were observed between control and beige mutation mice [610]. However, lesions formed in such mice are small and difficult to detect. To overcome this issue, beige mutation mice were crossed with $LDLR^{-/-}$ mice to generate beige, $LDLR^{-/-}$ mice. After feeding a HFD, lesions were greater in the beige $LDLR^{-/-}$ mice suggesting that the beige mutation was extending proatherogenic properties. This is difficult to explain with respect to immune cells as these mice have defective NK cells and cytotoxic T cells [611]. It is also possible that lysosomal trafficking is being affected on cells such as macrophages by the beige mutation [612, 613].

Other studies have utilized $LDLR^{-/-}$ mice that have received bone marrow from Ly49A transgenic mice where expression of Ly49A is under the control of the granzyme A promoter [614]. Ly49 is a cell surface receptor on NK cells. Ly49A transgenic mice exhibit an unexplained and selective reduction in NK cell numbers. Upon bone marrow transplant to

$LDLR^{-/-}$, $NK1.1^{+}$ $CD3^{-}$ cells in the spleen were not reduced and $CD3^{+}$ T lymphocytes were also unaffected as were plasma cholesterol concentrations. However, atherosclerotic lesions in these mice were reduced by 38-70% depending on the site of location of lesions. These results suggest that a reduction in NK cell activity may be responsible for the attenuated atherosclerosis although NK cell activity was not assessed in this study. Since granzyme A is also expressed on $CD8^{+}$ T cells and present in atherosclerotic lesion, however the significance for atherosclerosis has not been elucidated. However, NK cells do secrete cytokines such as IFN-γ, which are proatherogenic. A possible way of interpreting the differences between studies using beige-$LDLR^{-/-}$ and Ly49A $LDLR^{-/-}$ is that NK cells might exert proatherogenic effects via cytokine secretion rather than via cytotoxic molecules. However, this remains to be proven.

Clearly, further investigations are required to conclusively define and elucidate how NK cells affect development of atherosclerosis. Issues that still need to be addressed include (1) to what extent does selective depletion of NK cells affect development of atherosclerosis? (2) what are the mechanisms by which NK cells are activated to exert their effects in atherosclerosis? and (3) how do NK cells exert their functions in atherosclerosis? Increased understanding of these issues is required to determine whether targeting NK cells could represent a useful therapeutic approach to attenuate atherosclerosis.

2 Materials and Methods

2.1 Mice

Apolipoprotein E homozygous gene knockout ($ApoE^{-/-}$) mice on a C57BL/6J background were obtained from The Jackson Laboratories (Bar Harbor) and bred at the AMREP Animal Services (AMREP AS), Melbourne, VIC.

J alpha 18 knockout (J alpha $18^{-/-}$) mice originating from Dr Taniguchi [615] and backcrossed to the C57BL6 genetic background [616] were obtained from Dr D Goodfrey (Microbiology and Immunology, University of Melbourne, Australia). Deletion of J alpha 18 abolishes NKT cells without affecting other lymphocyte populations [615]. J $alpha18^{-/-}$ mice were crossed with $ApoE^{-/-}$ and the F2 generation, $ApoE^{+/-}$ $Jalpha18^{+/-}$ mice crossed to generate $Jalpha18^{-/-}$ $ApoE^{-/-}$ mice which were confirmed by genotyping using tail DNA, details for primer sequence described in *Ditiatkovski* ,2006 [617].

The double knockout $ApoE^{-/-}$ $Rag2^{-/-}$, and triple knockout $ApoE^{-/-}$ $Rag2^{-/-}$ $\gamma c^{-/-}$, were generated by crossing $ApoE^{-/-}$ mice with $Rag2^{-/-}$ $\gamma c^{-/-}$ and obtained from Monash Medical Centre Animal Services.

$Pfp^{-/-}$, $GrzB^{-/-}$, $IFN\gamma^{-/-}$ and C57Bl/6 mice on a C57BL/6 background were obtained from Dr Mark Smyth (Cellular Immunity Laboratory, Peter MacCallum Cancer Centre, Australia) and breed at AMREP Animal services. Genotyping of tail DNA confirmed the deleted genes (data not shown). Phenotype analysis (flow cytometry) confirmed the absence of all lymphocyte population in the $ApoE^{-/-}$ $Rag2^{-/-}$ $\gamma c^{-/-}$ mice (see Chapter 6:Results).

The mice were housed in standard conditions in a clean experiment area with food and water available *ad libitum.*

2.2 Experimental Design

At 6 weeks of age, male mice were fed with high fat diet (HFD) containing 21% butter fat and 0.15% cholesterol (SF00-19) (obtained from Specialty Feeds) *ad libitum*. All procedures performed on the mice were approved by the AMREP Animal Ethics Committee (E/0472/2006/B), (E/0588/2007/B) and (E/0804/2009/B).

Table2.1: Reagents used for in vivo and in vitro studies

Reagents	Supplier
Anti-asialo GM1	Wako Pure Chemical Industries
Normal Rabbit Serum	Vector Laboratories, CA
Polyinosinic – polycytidylic acid sodium salt, γ irradiated	Sigma
Saline 0.9% (sodium choride)	In house.
Hamster Anti-NKG2D (C7 clone)	Antibody purified in house.
Hamster IgG	Jackson ImmunoResearch Laboratories, USA.

2.3 Blood and Tissue Collections

At the completion of each study the mice were killed with an overdose of sodium pentobarbitone (1mg/10g body weight; *Abbott Laboratories*) and weighed; and blood was collected from the heart by cardiac puncture using a 26 Gauge needle. Blood was placed into EDTA (0.5M) coated

eppendorf tubes, then centrifuged at 15,000 RPM and supernatant was stored at -20º C for plasma cholesterol measurements.

Aortic roots were collected for histochemical and immunohistochemical analysis (see tissue sectioning and staining), and the aortic arch and the two carotid arteries were removed, cleaned of fat and rapidly frozen at -80 C for subsequent mRNA analysis. Liver, (perfused with 1xPBS before removal), spleen and blood were removed for subsequent FACS analysis.

2.4 Lipid analysis

Plasma cholesterol levels were measured by performing a 1:3 dilution of plasma in 0.9% saline, and using a standard commercial enzymatic assay using Beckman Coulter LX20PRO Analyzer at the Pathology Department, Monash Medical Centre, Clayton, Victoria. Reagents and calibrators were supplied by Beckman Coulter Diagnostics, New South Wales, Australia. Reactions were performed in duplicates.

2.5 Aortic sinus dissection and tissue sectioning

The aortic sinus were dissected from mice, embedded in OCT compound (*Tissue-tek, Sakura Finetek, Torrance,CA*) and frozen using cooled 2-methylbutane (*Sigma Aldrich*) and then stored at -80^{o}C. Frozen tissues were sectioned at 6μm in thickness using Microm HM220 or Leica cryostat set at -20º to -22º C. Frozen sections (6μm) were cut from the aortic sinus defined as region where the valve or valve cusps first become visible to where the left and right coronary arteries branch off [618].The aortic sinus was evaluated because this region of the aorta is particularly susceptible to the development of atherosclerosis in ApoE$^{-/-}$ mice fed a HFD. Three

successive rows of ten consecutive sections were cut and collected on SuperFrost Plus microscope slides (*Menzel-Glazer*) and air dried for at least an hour before storing at -20ºC until required for staining. For each mouse, lesion size was measured in 6 cross-sectional areas at 30μm intervals and averaged.

2.6 Tissue staining

2.6.1 Histology

2.6.1.1 Oil Red O

Oil Red O was used to determine lipid accumulation in the lesions. An Oil Red O working solution was prepared 10 minutes prior to use. Thawed frozen sections were fixed in 4% buffered formalin for 4 minutes, followed by 4 minutes in 1XPBS, and then washed in 60% isopropanol for 20-30 seconds. Sections were then stained with Oil Red O working solution for 1 hour, then differentiated in 60% isopropanol for approximately 5 seconds and washed in dH20 for 2 minutes. Sections were then counterstained with Mayer' haematoxylin for 25 minutes, washed under running tap water for 3 minutes, washed in dH20 for 3 minutes, and mounted with Aquamount and coversliped. Slides were stored in dark. (Refer to Table 2.2 for materials).

Table 2.2: Reagents for histological staining Oil Red O.

Materials	Constituents	Supplier
1XPBS		Amresco
4% buffered formalin	4% formalin in 1XPBS	BDH
Oil Red O stock solution	1% Oil Red-O stock powder 99% isopropanol	Sigma BDH
Oil Red-O working solution	30ml Oil Red-O stock solution 20ml dH20	

2.6.1.2 Haematoxylin and eosin stain

Haematoxylin and eosin (H&E) was used to stain nuclei and eosinphilic other structure of cells. This stain is used to determine necrotic core in atherosclerotic lesions. Thawed frozen sections were fixed in 100 ml of the following solution: (70ml ethanol, 10ml 37-40% formaldehyde, 5ml glacial acetic acid, and 15ml of dH2O). Sections were then dehydrated with 70% Ethanol for 30 seconds followed by two washes in dH2O for 5 minutes each. Sections were stained with Mayer' haematoxylin for 25 minutes and washed twice in tap water for 5 minutes each wash. Sections were further washed in dH2O for 5 minutes. Sections were then counterstained in Putt's Eosin solution for 40 approximately 40 seconds, washed in tap H2O twice for 5 minutes each wash, and further washed in dH2O for 5 minutes. Sections were dehydrated with two 3 minutes washes in 100% in ethanol, cleared in xylene (two 5 minutes washes) and mounted with DEPEX and coversliped.

2.6.2 Immunohistochemistry

Individual sections were outlined with a wax pen to retain solutions on the tissue specimen while staining procedures are performed. Immunohistochemistry was performed using a primary antibody followed by a secondary antibody that is conjugated to biotin. Sections were fixed in acetone for 20 minutes at -20ºC, followed by 20 minute incubation in 3% hydrogen peroxidase to block any endogenous peroxidise activity. Sections were incubated with 10% normal serum, followed by avidin blocking solution and incubated for 30 minutes, and followed by subsequent addition of primary antibody and biotin blocking solution for 1 hour, biotinylated secondary antibody for 30 minutes, then avidin biotin complex (ABC) at 1:100 dilutions for 30 minutes. One grain of imidazole and 1.0ul of H2O2 was added to 1ml of DAB solution before adding to the sections and incubating for 1-10 minutes until brown staining is observed. Sections were observed under the microscope with 6.3X magnification, and when brown staining was observed, the reaction was stopped by incubating the slides in 1xPBS, twice for 5 minutes. Sections were counter stained with Mayer's Haematoxylin for 25 minutes and then washed under running tap water for 5 minutes. Sections were dehydrated in 100% ethanol twice for 5 minutes, then dewaxed in xylene (two 5 minutes wash), and mounted with Depex and coversliped. (Refer to Table 2.3 and Table 2.4 for materials used).

Table 2.3: Reagents used in immunoperoxidase staining.

Materials	Constituent (s)	Supplier
1XPBS		Amresco
3% hydrogen peroxide (H202)	5ml 30% H202 45 ml 1XPBS	BDH
Acetone		BDH
Avidin/Biotin blocking kit	1tube Avidin blocking solution 1 tube Biotin blocking solution	Vector Laboratories
DAB (3,3' Diaminobenzidine)	Stored as 1ml aliquots at -20 C 1 DAB tablet 20ml 1XPBS	Sigma
Depex		BDH
Haematoxylin		Sigma
Imidazole		BDH
Normal Horse Serum (NHS)		Vector Laboratories
Normal Rabbit Serum (NRS)		Vector Laboratories
Vectastain ABC elite peroxidise kit		Vector Laboratories
Ethanol		Biolab
Xylene		BDH

Table 2.4: Antibodies used for immunoperoxidase staining

Staining for:	**Primary Ab**	**Dilution**	**Supplier**	**Secondary Ab**	**Dilution**	**Supplier**
Macrophages (CD68)	Rat anti-mouse CD68	1:100	Ab D Serotec	Biotin mouse anti-rat IgG	1:200	BD Pharmingen
$CD4^+$ helper T cells	Rat anti-mouse CD4	1:50	BD Pharmingen	Biotin mouse anti-rat IgG	1:200	BD Pharmingen
CD31	Rat anti-mouse CD31	1:100	BD Pharmingen	Biotin mouse anti-rat IgG	1:200	BD pharmingen
Smooth muscle cells	Rabbit anti-mouse alpha-smooth muscle actin	1:100	Abcam	Biotin goat anti-rabbit IgG	1:200	Vector Laboratories
Vascular cellular adhesion molecule-1 (VCAM-1)	Rat anti-mouse VCAM-1	1:200	BD Pharmingen	Biotin mouse anti-rat IgG	1:200	BD Pharmingen

2.7 Histology and Immunoperoxidase section analysis

All histology and immunoperoxidase sections were examined under light microscopy using 6.3X magnification and cross-sectional area quantified using image analysis software (Optimus 6.2 Video Pro-32, Bedford Park, South Australia). Region of interest for example the lesion area were traced and section stained were analysed to obtain the total area stained.

2.8 FACS Analysis of Immune Cells

Spleen collected from mice were placed in separate tubes containing 3 ml of FACS buffer and kept on ice. Spleens treated with FACS buffer were disrupted between the frosted ends of two glass slides. Cells were collected in small petri dishes and transferred to tubes whilst passing through a 100um nylon mesh, to obtain single cell suspensions. For spleen and liver cell suspensions red blood cells were lysed using 1 ml of ammonium chloride (NH_4Cl, 0.156M). Cells were counted using a Coulter Counter and 2 million cells were transferred to 96 U bottom well plate. Plates were spun at 1300 RPM for 5 minutes and the supernatant was completely removed. Each pellet was resuspended in 30 ul of an antibody cocktail (Table 2.5). Blood collected for FACS analysis was also transferred into a tube containing 4.5 ml MilliQ to lyse red blood cells. After 20 seconds, 0.5ml of 10xPBS was added to the tubes to restore osmolarity. The cell suspensions were centrifuged at 15000 rpm for 5 minutes and supernatant were removed. Then to determine the effects of different treatments on tissue lymphocyte and monocyte populations, these cell suspensions were analysed using fluorochrome-conjugate antibodies (Abs) (Refer to Table 2.5 for antibodies) on a BD FACS Calibur (*BD Bioscience*). Data analysis was performed using WEASEL 2.5 software.

Table 2.5: Antibodies used for FACS analysis. The following antibodies were used to define and quantitate NK, NKT, B, $CD4^+$ T, $CD8^+$ T cells and monocytes in peripheral blood, liver and spleen.

Staining for:	Conjugated primary antibody	Clone	Dilution	Supplier
NK cells	NK1.1 – PE or NK1.1 - PerCP	PK136	1:200	Pharmingen
NKT cells	TCR-β – APC	H57-597	1:200	Pharmingen
$CD4^+$ helper T cells	CD4 – FitC	RM-4-5	1:200	Pharmingen
$CD8^+$ cytotoxic T cells	CD8 – PerCP	53-6.7	1:200	Pharmingen
B cells	CD19 – PE or CD20 - PE	1D3	1:200	Pharmingen
Monocytes	CD11b – PerCP	M1/70	1:200	Pharmingen
Type II membrane glycoprotein	CD69- PE	L78	1:200	Pharmingen

2.9 Anti-NKG2D Receptor Antibody

2.9.1 NKG2D C7 clone Hybridoma culture

The NKG2D C7 clone hybridoma [619] was obtained from Dr Mark Smyth (Cellular Immunity Laboratory, Peter MacCallum Cancer Centre, Australia). Hybridoma obtained was cultured in 40 ml of culture medium in T75 flask (*BD Falcon*), within a few days, the hybridoma cells begin to proliferate. After 2 days, media was split into four T75 flasks and 30ml of media was added to each flask to allow cells to grow (Refer to Table 2.6 for culture medium). After 3 days (confluent culture), 30 ml of culture media were transferred from the flasks with cells into a 50 ml Falcon tube and centrifuged at 2000 RPM for 5 minutes, the supernatant was collected and stored at -20^0C in a clean bottle containing 0.02% sodium azide. To the remaining cell suspension in the T75 tissue culture flask, 30ml of fresh culture media was added for cells to grow, and culture was split every 3 days and supernatant was stored as described above.

Table 2.6: Culture media for growing hyridomas and buffers used for hybridoma purification.

Materials	Constituents
Culture media (RPMI Medium 1640) (*Gibco*)	• 10% fetal calf serum (heat inactivated at 60^0C for 30 minutes. • 1% penincilin/streptomycin (*Gibco*) • 1% Glutamax (*Gibco*)
Binding buffer	• 20mM disodium hydrogen phosphate, pH 7.0
Elution buffer	• 0.1M Glycine, pH 2.8
Neutralization buffer	• 1M Tris-HCl, pH 9.0

2.9.2 Purification of antibodies from NKG2D C7 hybridoma culture using protein G affinity column.

The protein G affinity columns (*Milipore*) were used to concentrate and enrich antigen specific antibodies and remove any non-specific proteins. Affinity column was prepared in a cold room according to the manufactures directions. The protein G column was loaded with 2cm of protein G resin beads (*Milipore*) and stored in 70% ethanol. To purify the NKG2D C7 clone, the columns containing the resin beads in 70% ethanol was washed and equilibrated with 20-30 ml binding buffer. The hybridoma supernatant that was stored at -20^0C was loaded to the column, and was passed through the column at a flow rate of approximately 1ml per minute. After passing the supernatant through, the column was washed with 20-30 ml of binding buffer, so that

the entire IgG NKG2D antibody binds to the protein G resin beads in the column. To elute the antibody, elution buffer was passed through the column and twelve 1 ml fractions of antibody were collected in 100ul of neutralization buffer in an eppendorf tube. After elution, the column was equilibrated with the binding buffer and washed with 70% ethanol for storage. The 12 fractions of antibody elution in neutralizing buffer were loaded into a UV spectrometry and the purified NKG2D antibody concentration was determined at 280nm wavelength. The absorbance reading at 280nm for all 12 purified antibody fractions was recorded. The antibody concentration was calculated using a formula (Absorbance (280nm)/1.44). The eluted antibody fractions that had a concentration of 0.7mg/ml or more were transferred to a dialyzing tube and dialysed against 1XPBS pH 7.4 for 24 hours. After dialysis, the antibody concentration was measured using UV spectrometry again and NKG2D antibody was aliquoted into 1ml volumes and stored at -20^0C.

2.10 NK depleting antibody and purification of depleting antibody

To deplete NK cells, rabbit anti-asialo GM1 was purchased from Wako Pure Chemical Industries, USA. The antibody was reconstituted in 1ml of 1XPBS (*Amresco*) and stored at 4^0C. Antibody concentration was measured using UV spectrometry at 280nm. The concentration of all anti-asialo GM1 used in the studies was in the range 2.10 to 2.20 mg/ml.

For some experiments as indicated, anti-asialo GM1 and normal rabbit serum were purified using a protein G column to remove any non-specific proteins (Refer to section 2.9.2 for purification method). The activity of purified anti-asialo GM1 was determined using FACS analysis (Refer to Chapter 4: Results).

2.11 Cytokine Analysis

Cytokine concentrations in culture medium from NK cells and saline were measured using the Bio-Plex Mouse Cytokine Assay (Bio-Rad). For the Bio-Plex Mouse Cytokine Assay, cytokine capture beads were prepared according to manufacturers instructions, added to the filter plate, and washed. Standards and cell culture supernatant samples were then added to the plate and incubated for 30 minutes. After washing, detection antibodies were added and incubated for 30 minutes, followed by another wash, and incubation with Streptavidin-PE for 10 minutes. All incubations with the BioRad kit were performed on a shaker at room temperature away from light. After the final incubation, the plate was washed and samples were resuspended in Bio-Plex Assay Buffer and analysed on a Bio-Plex Reader.

2.12 Cytotoxicity assay

2.12.1 Target cell preparation

RMA-S-Rae-1β tumor cells (target cells) were obtained from Dr Mark Smyth (Cellular Immunity Laboratory, Peter MacCallum Cancer Centre, Australia). RMA-S, a MHC class I-deficient variant of RBL-5, is one of the prototypic NK cell-sensitive target cells for in vitro and in vivo studies. The RMA-S expressing NKG2D ligand Rae-1β were grown in complete RPMI 1640 media as described in Table 2.7 for 5 days. For the cytotoxicity assay, RMA-S-Rae-1β tumor cells were harvested according to the number of cells needed for the assay. Cells were centrifuged at 200g for 5 minutes, and supernatant were aspirated. The cell pellet were washed

twice in Tris buffered saline and resuspended in 1ml per $5X10^5$ cells in Tris buffered saline. Then, 100uCi of Chromium-51 was added and incubated at standard culture conditions (37^0C, 5%CO2). After incubation, cells were centrifuged at 2500RPM for 7 minutes and supernatant containing radioactive material were discarded appropriately. Cell were then washed three times in 1ml of culture media and centrifuged at 2500RPM to remove residual radioactivity. Viable cell were counted using tryphan blue dye on a haemocytometer. Cells were resuspended to 5 X 10^4 cells per ml.

2.12.2 NK cell preparation

NK cells were prepared according to methods underlined in Section 2.13.1 to 2.13.3 (see below). NK cells were resuspended in culture media to needed concentration in a total of 100ul.

2.12.3 Cytotoxicity assay

5X 10^3 target cells were plated in a 96 well U bottom plate in duplicates. 30ug/ml of anti-NKG2D or Hamster IgG was added to each well containing target cells and incubated for 15 minutes at standard culture conditions. Then, NK cells were added to target cells in the well. Control wells were loaded with (1) for spontaneous killing (target cell + media) and (2) for maximum killing (target cell + 5% SDS to completely lyse all target cells). The 96 well plate was then centrifuged at 1200 RPM for 2 minutes to allow the target and effector cells to bind. The plate was incubated for 4 hours at standard culture conditions. After incubation, the plate was centrifuged at 1200 RPM for 5 minutes, and 100ul of supernatant was collected and loaded into the gamma counter (*Perkin Elmer*) for the amount of radioactive substance released by

target cells were counted (equal to amount of cytotoxicity). The counts per minute (CPM) of cytotoxic killing of target cell by NK cells were calculated using the formula

$$\text{Cytotoxic killing} = \frac{(\text{measured killing-spontaneous killing})}{(\text{Maximum killing-spontaneous killing})} \quad [620]$$

Table 2.7: Cytotoxicity assay buffers and culture media.

Materials	Constituents
Culture media (RPMI Medium 1640) (*Gibco*)	• 10% fetal calf serum (heat inactivated at 60^0C for 30 minutes • 5% penincilin/streptomycin (*Gibco)* • 5% Glutamax (*Gibco*)
Tris-buffered saline, pH 7.4	• Made in house
Chromium-51 (*Perkin Elmer*)	• As per manufactures description
Tryphan Blue (*Sigma*)	• As per manufactures description
5% SDS	• Made in house.

2.13 Preparation of NK cells for adoptive transfer

2.13.1 Isolation of NK cells

For adoptive transfer of NK cells into an ApoE$^{-/-}$ Rag2$^{-/-}$ γc$^{-/-}$, two spleen from C57BL/6 mice were collected and immersed in 3ml of sterile PBS$^{-/-}$. Each spleen was disrupted with the frosted ends of two glass slides into 3 ml of PBS$^{-/-}$. Cell suspension was transferred to a 15ml tube and the tube was spun at 1400 RPM for 4 minutes, and the supernatant was discarded. The cells were resuspended in 1ml of sterile red blood cell lysing buffer (*Sigma*) for 1 minutes, then the tube was filled with 10 ml of PBS$^{-/-}$ and was filtered through a 40um nylon cell strainer with additional 15 ml of PBS$^{-/-}$. The cell suspension was centrifuged for 4 minutes at 1400 RPM and the supernatant discarded (Refer to Table 2.8 for materials used for preparation of NK cells for adoptive transfer).

2.13.2 Magnetic labelling of non-NK cells and isolation of unlabeled NK cells.

The MACS mouse NK cell isolation kit (*Miltenyi Biotec*) was used to magnetically remove non-NK cells. All procedures were performed at temperatures 0 º- 4º C. The cell pellet (see above) was resuspended in 200ul of MACS staining buffer and transferred into a 15ml tube. 37.5ul of MACS Biotin-Antibody Cocktail was added and incubated at 4 ^{o}C for 30 minutes. After incubation, 150ul of MACS staining buffer and 75ul of MACS Anti-Biotin Microbeads were added and incubated for further 15 minutes in the 4 ^{o}C. The cells were then washed with by adding10ml of MACS staining buffer and centrifuged at 1400 RPM for 4 minutes and

supernatant was completely removed by aspiration and the cell pellet 2.5ml of MCAS staining buffer and filtered through a 40um nylon cell strainer.

2.13.3 Magnetic separation of NK cells

Magnetically labelled cell suspension was placed in the Automacs separator. The separation program "Depletes" was used to collect the enriched, untouched NK cell population by negative selection. The number and viability of enriched NK cells was determined using a hemocytometer.

2.13.4 Culture of enriched NK cells

To reduce the number of donor mice required for each adoptive transfer, enriched NK cells were expanded in cell culture. The enriched cells from magnetic separation were adjusted to a 7 X 10^5 cell per 1ml with cell culture media RPMI 1640. 1 ml of media containing 7 X 10^5 NK cells were plated into a 24 well flat bottom plate and 1000U of human recombinant IL-2 (rhIL-2) into each well to activate and increase the number of NK cells. NK cells were cultured in the presence of rhIL-2 for 5 days. NK cell numbers doubled after 5 days of culture with IL-2.

2.13.5 NK cells sorting

On day 5 after culture, the number and viability of NK cells were determined using haemocytometer. The cells were then labelled with 30ul of an antibody cocktail per 2 million cells containing anti-NK1.1 (NK1.1-PE and anti- TCR-β (TCR-β-APC) fluorescently labelled antibodies. The cells were incubated for 30 minutes at 4ºC in the dark. Then 500ul of MACS staining buffer was added and the cell suspension centrifuged at 1400 RPM for 4 minutes. The supernatant containing untreated antibodies was removed completely and cell pellet was resuspended in 500ul/10^7 cells of MACS staining buffer.

NK1.1 positive and TCR-β negative cells were sorted using a BD FACSAria sorter to obtain a highly pure NK cell population. These highly pure NK cells were adoptively transferred into $ApoE^{-/-}$ $Rag2^{-/-}$ $\gamma c^{-/-}$ mice.

Table 2.8: NK cell culture media and buffers for NK cell isolation

Materials	Constituents
Culture media (RPMI 1640) (*Gibco*)	• 10% fetal calf serum (heat inactivated at 60^0C for 30 minutes • 5% penincilin/streptomycin (*Gibco)* • 5% Glutamax (*Gibco*) • 50mM 2-mercaptoethanol (*Sigma*) • 5% non essential amino acid (*Gibco*) • 5% Sodium pyruvate (*Gibco*) • 5% HEPES (*Gibco*)
Human recombinant IL-2 (National Cancer Institute,USA)	• 6,000,000 U
PBS without Ca^{2+} and Mg^{2+} ($PBS^{-/-}$)	• Pre-made, media services
MACS staining buffer	• $PBS^{-/-}$ • 2mM EDTA
MACS biotin-antibody cocktail (*Miltenyi Biotec*)	• As per manufactures description.
MACS anti-biotin micro beads	• As per manufactures description.

2.14 RNA extraction

RNA was extracted from aortic arch pooled from 3-4 mice using the solution D and Dnase treatment using Qiagen clean up method (Refer to Table 2.9 and 2.10 for materials). Blood vessels were grinded into a powder under liquid nitrogen using a mortar and pestle. 500ul of solution D (4M Guanidium thiocyanate, 25mM Sodium citrate, 0.1M 2-mercoptoethanol, 0.5% Sorkosyl, dH2O) was added onto the tissue and a little liquid nitrogen was added to allow the solution D to freeze. Tissues were further disrupted and grinded into a fine powder. The powder was then transferred to a clean labelled eppendorf tube and the powder was thawed out. Combined solution of 540ul of phenol/chloroform (pH 4.7) and 45ul NaAc (2M, pH 5.2) was added to the lysate and was mixed with using pipette. The lysate was centrifuged at 14000rpm for 30 minutes at 4^{o}C. The supernatant were transferred into a clean eppendof tube containing 400ul of isopropanol and the tube was inverted slowly. The eppendorf tube was maintained at -20 ^{o}C for 5 minutes and centrifuged at 14000 RPM in 4^{o}C for 15 minutes. The supernatant was aspirated using a fine pasteur pipette and 300ul of RNA grade isoproponal was added and left at room temperature for 2 hours. After 2 hours, 300ul of RNA grade isoproponol was added and spun at 14,000 RPM at 4^{O}C for 15 minutes. The supernatant was aspirated and the pellet was washed in 400ul of 70% RNA grade ethanol and spun for 8 minutes at room temperature. The supernatant was then aspirated and the pellets were dried at 37^{o}C and resuspended in 20ul of RNase free water. RNase-free water was pipetted onto the column and tubes were spun for 1 minute at 14000 RPM. Eluted RNA can be stored at -80 C before Dnase treatment.

The Dnase treatment was performed using RNeasy mini kit from Qiagen to remove contaminating genomic DNA.

The volume of each eluted RNA in eppendorf tubes was adjusted to 100ul using RNase free H2O and 350ul of buffer RLT plus β-Mercaptoethanol (350ul RLT + 3.5ul of β-mercaptoethanol and was mixed thoroughly with a pipette. 250ul of 100% ethanol was then added to dilute the RNA and was thoroughly mixed by pipetting. The sample was applied on to the silica membrane in an Rneasy mini column placed in a 2ml collection tube and tube was centrifuged for 15 seconds at 10,000 RPM, the flow-through was and collection tube was discarded. The was then transferred to a new collection tube and 350ul of Buffer RW1 was pipetted into the Rneasy mini column and further centrifuged for 15 seconds at 10,000 RPM, and flow through was discarded. 80ul of Dnase was added directly onto the silica gel membrane of the column and incubated at room temperature for 15 minutes. After incubation, 350ul of Buffer RW1 was added into the mini column and centrifuged for 15 seconds at 10,000 RPM and flow-through was discarded. 500ul of Buffer RPE was added to wash the column and further centrifuged for 15 seconds at 10,000 RPM and flow-through was discarded. Another 500ul of Buffer RPE was added to the column and centrifuged for 2 minutes at 10,000 RPM and the column was transferred to a clean 2ml collection tube. The tube was further centrifuged to eliminate ethanol contamination at the next step. To elute, the column was transferred to a clean eppendorf tube and 30ul of RNase free H2O was pipetted directly to the silica membrane of the column, and the tube was gently centrifuged for 1 min. A further 30ul of RNase free H2O was added to the column and spun for another 1 minute. The elution was stored at -80^{o}C.

Table 2.9: Reagents used for RNA extraction.

Materials	Constituents	Supplier
Solution D	• 4M Guanidium thiocyanate, • 25mM Sodium citrate, • 0.1M 2-mercoptoethanol, • 0.5% Sarkosyl, • dH2O	In house
Phenol/choloroform (pH 4.7)	Pre made	Amresco
NaAc (2M, pH5.2)	Pre made	
RNA grade isoproponal	Pre made	
70% RNA grade ethanol	Pre made	Qiagen
RNase-free H20	Pre made	Qiagen

Table 2.10: Reagents used for Dnase treatment

Materials	Supplier
Buffer RLT	Qiagen
β-mercaptoethanol	Sigma
Buffer RW1	Qiagen
Buffer RPE	Qiagen
Buffer RDD	Qiagen
Rnase	Qiagen
Rnase free H2O	Qiagen.

2.15 RNA gel electrophoresis

RNA integrity and concentration was determined by gel electrophoresis.

A 30ml RNA (1%) gel was prepared using 21.6ml sterile H2O, 3ml 10XMOPS (RNA running buffer), and 0.3g agarose, heated and mixed until agarose fully dissolved, and allowed to cool before adding 1ul ethidium bromide and 5.4ml formaldehyde.

To prepare RNA samples for electrophoresis, 6ul buffer "F" (500ul deionized formamide, 100ul RNA running buffer, 222ul sterile H2O, 178ul formaldehyde) was added to 500ng of RNA and heated at 60º C for 15 minutes and cooled on ice for 2 minutes. 2ul of loading dye was added to prepared sample, mixed, centrifuged, and loaded onto 1% agarose gel and run at 100V in 1X MOPS for approximately 1 hour. Gel was incubated overnight in distilled water to remove ethidium bromide before observing under UV light. RNA bands were visualised under UV light and photographed using FujiFilm instant black and white film.

2.16 Real Time PCR (RT- PCR)

2.16.1 Reverse Transcription

To reverse transcribe extracted RNA, 30ng of RNA is prepared in a 10ul reaction. Reaction tubes comprise of 1ul 10X Taq Man RT Buffer, 2.2ul 25mM MgCl2, 2ul of 2.5mM deoxyNTP's, 0.5ul 50uM Random Hexamers, 0.2ul Rnase Inhibitor (20U/ul 0.25ul of Multiscribe (50U/ul), 1ug RNA and RNase-free H20 to make the total reaction mix of 10ul. Reverse Transcription was carried out on the PCR machine (Refer to Table 2.11 for thermal cycling parameters).

Table 2.11: Thermal cycling parameters for Reverse Transcription reactions

	Temp.	Time
Incubation	25º C	10 min
Reverse Transcription	48º C	30 min
Reverse Transcription activation	95º C	5 min

2.16.2 Primer designing

Primers for real-time PCR were designed using the default conditions within the *Applied Biosystems,* Primer Express program, with a minimum length specified of 18 nucleotides, and a maximum length specified of 30 nucleotides. Amplified fragment sizes ranged from 60-150 bp. Nucleotide sequences encoding the cDNA of each gene assessed were obtained from the NCBI website using either *BLAST* or *Entrez*, and the position of exons and introns were delineated by comparison to the corresponding genomic sequence. Primers for each cDNA were chosen to span the largest intron within the gene sequence, so that the possibility of amplifying trace amounts of genomic DNA was minimized; dissociation stages during the real-time run confirmed that primers for each gene amplified only cDNA. Each cDNA primer pair was also checked in BLAST to confirm non-homology to other cDNAs. The 18s internal control primers were obtained from Applied Biosystems. (Refer to Table 2.12 for primers designed)

Table 2.12: Primer sequences.

Primer	Sense Sequence (5' →3')	Anti-Sense sequence (5'→ 3')
*IL-1β	AAG AAT CTA TAC CTG TCC TGT GTA ATG AAA	TGG GTA TTG CTT GGG ATC CA
*ICE	GGC ATT AAG AAG GCC CAT ATA GAG	TGA GCC CCT GAC AGG ATG TC
*18S	CGG CTA CCA CAT CCA AGG AAG GCA	GCT GGA ATT ACC GCG GCT GCT GGC

**Primers supplied by Gene Works*.

2.16.3 Real time PCR (RT-PCR)

RT-PCR was used to detect and quantitate mRNAs encoding Interleukin-1 converting enzyme/caspase-1(ICE), and Interleukin-1beta (IL-1β) in the RNA of aortic arch. Each reaction tubes comprised 10ul of SybrGreen, 1ul sense primer, 1ul Anti Sense primer, 6ul RNase-free H20 and 2ul of cDNA which makes up 20ul reactions. Each reaction is carried out in duplicates. RT-PCR was carried out on the 7500 Fast Real Time PCR machine (*Applied Biosystems*) according to RT-PCR conditions as outlined in Table 2.13. The real time PCR data was analysed using 7500 V.2.0.4 software 9 (*Applied Biosystems*).

Table 2.13:. RT-PCR conditions

Primer	Reverse Transcription		Denaturation		Amplification						Cycles
	Temp.	Time	Temp.	Time	Denaturation		Annealing		Elongation		
					Temp.	Time	Temp.	Time	Temp.	Time	
All primers	50°C	35 min	94°C	2 min	94°C	2 sec	60°C	30 sec	70°C	1min	45

2.16.4 Comparative Ct calculations

The real time RT-PCR results are quantified by using the comparative Ct method. This method compares the Ct values of the samples of interest with a control (non-treated sample). Ct values are normalized to 18S housekeeping gene. The $2^{-[delta][delta]\ Ct}$ method is calculated where [delta][delta]Ct = [delta]Ct sample – [delta]Ct control. [delta]Ct sample is the Ct value for samples normalized to 18S housekeeping gene and [delta]Ct control is the Ct value for control also normalized to 18S housekeeping gene. Therefore the relative aortic mRNA abundance of gene of interest was calculated using $2^{-[delta][delta]\ Ct}$ (*Applied Biosystem*) [621].

2.17 Superscript one-step RT-PCR

For detection of Rae-1δ, Rae-1ε, MULT-1, and H60 expression, the Superscript one-step RT-PCR method (*Invitrogen #10928-034)* was used, essentially as described by the manufacturer. Briefly, 150 ng of RNA in a total volume of 25 ul was reverse-transcribed and

subjected to amplification with specific primers (0.2 uM each primer) using the following cycling conditions:

Table 2.14: Superscript RT-PCR conditions

Primer	***Reverse Transcription***		***Denaturation***		Amplification						***Cycles***
					Denaturation		***Annealing***		***Elongation***		
	Temp.	***Time***	***Temp.***	***Time***	***Temp.***	***Time***	***Temp.***	***Time***	***Temp.***	***Time***	
All primers	***50°C***	***30 min***	***94°C***	***2 min***	***94°C***	***15 sec***	***60°C***	***30 sec***	***70°C***	***1 min***	***35***

Following cycling, 10 ul of product was electrophoresed in 2% Agarose/1x TAE gels containing ethidium bromide to visualize the amplified products, with pX174/HaeIII DNA (*Promega*) as size markers. Gels were photographed under UV illumination.

2.18 Statistical analysis

Statistical analyses to compare two groups were performed using Student's t-test when data followed a normal distribution or Mann-Whitney U test when data did not follow a normal distribution. Normality was determined using the D'Agostino and Pearson omnibus normality test. One-way ANOVA was used to compare more than two groups, and the significance between two groups analysed by Tukey post-test. All statistical analyses were performed using the software GraphPad Prism v5.02. P-value < 0.05 was considered statistically significant.

3 NK cells promote the development of Atherosclerosis in ApoE$^{-/-}$ mice.

3.1 Introduction

Atherosclerosis is an intimal inflammatory disorder characterized by lesions that contain cholesterol, immune cells, smooth muscle cells and a necrotic core. Macrophages, dendritic cells and T cells are the major immune cells populating developing lesions but other less abundant immune cell types also contribute to pathology [622]. NK cells are present in early lesions in human [623] and also more advanced lesions [606]. They are a small population infiltrating about 0.5% of the total lymphocyte population, lying in close proximity to the luminal endothelium, under necrotic cores deep within plaques as well as in shoulder region [606]. NK cells have also been detected in atherosclerotic lesions of mice [614]. Whilst not yet proven that NK cells could be attracted to developing lesions via specific chemokines such as MCP-1 and Fraktalkine (CX3CL1) which are highly expressed in lesions [624, 625]. Developing lesions also express IL-15, a critical trophic and activating cytokine required for NK cell development, activation and proliferation [626]. NK cells have the potential to modulate atherosclerosis via NK inhibitory receptors and NK activating receptors which recognize MHC class I molecules including homologues such as MICA and MICB [627].

Despite such studies the role of NK cells in the development of atherosclerosis is still unclear. Studies using mice with a beige mutation to determine the role of NK cells in atherosclerosis have been reported. Beige mutation mice have a defect in lysosomal fusion/fission and trafficking and the deficiency causes defects in cytolytic function of NK cells

[628]. Early studies using mouse with beige mutation suggest that atherosclerosis may be unaffected by NK cells. More recently, a study examined at the role of NK cells in atherosclerosis in mice harbouring the beige mutation on a LDLR-deficient background [629]. The results from this study indicated that beige mutation mice deficient in LDLR exhibited increased atherosclerosis compared to $LDLR^{-/-}$ mice, suggesting that NK cells play an anti-atherogenic role. The mutation in the beige mutation mice involves the Lyst gene, which is a homologue to the protein implicated in autosomal recessive human Chediak Higashi syndrome that has a complex phenotype of partial albinism, immunodeficiency, bleeding diathesis and neuropthy [62]. Since the mutation in beige mice results in a complex phenotype and not just defects related to NK cell cytolytic function, therefore it is not a suitable model to determine the role of NK cells in atherosclerosis. Furthermore, since Lyst gene is involved in lysosomal trafficking and the beige mutation does no result in a total defect in lysosomal trafficking of NK cells, hence there maybe defects in lysosomal trafficking in other cell types that lead to the increase in atherosclerosis. Functional changes in lysosomal trafficking in other cells such as lesion macrophages are possible, which could increase the formation of foam cells and augment atherosclerosis.

More recent studies have utilized Ly49 transgenic mice. Whitman *et al* used the Ly49A transgenic mouse over expressing the inhibitory Ly49A receptor under the control of the granzyme A promoter [614]. Reconstitution of $LDLR^{-/-}$ mice with Ly49A transgenic bone marrow following high fat feeding resulted in a reduction in lesion size compared to control $LDLR^{-/-}$ mice given a non-transgenic bone marrow, suggesting a pro-atherogenic role for NK cells. However this model is also not totally specific for NK cells as the results maybe influenced by other cell types, particularly NKT and $CD8^+$ T cells [630]. $CD8^+$ T cells are known to express granzyme A [631] and overexpression of Ly49A attenuates their immune responses [632].

Similarly NKT cells express granzyme A [633]; Ly49A receptors on these cells attenuate their activation [634]. Thus it is clear that in these studies the importance of NK cells could be overestimated due to simultaneous expression of CD8 and NKT cells. NKT cells are also known to augment development of atherosclerosis [300, 303].

To clarify these issues in relation to the importance of NK cells in development of atherosclerosis, I investigated the role by depleting NK cells using a NK cell specific depleting antibody, anti-asialo GM1 [635]. I definitely demonstrated that NK cells, despite being a very minor immune cell type in lesions contribute to development of atherosclerotic lesions in $ApoE^{-/-}$ mice fed a high fat diet.

3.2 Methods

3.2.1 Mice and treatment

ApoE$^{-/-}$ mice (C57BL/6 background) were fed a high fat diet consisting of 21% butter fat and 0.15% cholesterol from 6 weeks of age for 8 weeks. The mice were treated by intravenous (i.v) injection with either anti-asialo GM1 antibodies (54μg/injection) or rabbit serum (NRS) (54μg/injection) every 5 days for eight weeks (Refer to Chapter 2: Materials and Methods, page 95, Table 2.1 for details of reagents used).

3.2.2 Tissue collection

At the end of the study, mice were killed by administering an overdose of pentobarbitone and body weight was determined. Blood was collected for cholesterol and triglyceride measurements, and the aortic sinus was dissected and frozen in OCT for subsequent sectioning and histological and immunohistochemical studies. Spleen and blood was also collected for FACS analysis of immune cells as described in Chapter 2: Materials and Methods, page 95. All experiments were approved by the AMREP Animal Ethics Committee.

3.2.3 Lipid analysis

At the end of study after 8 weeks of high fat diet, blood was collected by cardiac puncture and plasma lipid was measured for cholesterol, triglyceride, HDL, and LDL levels (Refer to Chapter 2: Materials and Methods; page:96).

3.2.4 Tissue Sectioning

The frozen aortic sinus were sectioned at 6 μm in thickness and collected on microscope slides for subsequent histological and immunohistochemical studies (Refer to Chapter 2: Materials and Methods; pages 96).

3.2.5 Atherosclerotic lesion size analysis

Sections of aortic sinus were stained with Oil Red O and examined using light microscopy and cross-sectional area of lipid deposition was quantified (Refer to Chapter 2: Materials and Methods; page: 97 and 102).

3.2.6 Immunohistochemistry

Antibodies against CD68, α smooth muscle actin, CD31, VCAM-1 and CD4 were used for immunohistochemical analyses of macrophages, smooth muscle cells, endothelial cells, vascular adhesion molecule-1 and $CD4^+$ T cells in frozen sections of the aortic sinus, respectively. CD68-

, α smooth muscle cell actin and VCAM-1 positively stained areas were quantified by Optimus software. I microscopically counted CD31-stained endothelial cells and CD4-stained T cells within the atherosclerotic lesions (Refer Chapter 2: Materials and Methods; pages: 99-102).

3.2.7 Flow cytometry analysis

NK cells, monocytes and lymphocyte populations in peripheral blood and spleen were analysed using flurochrome-conjugated Abs on a BD FACS calibur. For NK cells, PE-conjugated NK1.1 and allophycocyanin-conjugated TCR-β Abs were used. For non-NK lymphocytes and monocyte populations, FITC-conjugated CD4, PerCP-conjugated CD8, PE-conjugated NK1.1, allophycocyanin-conjugated TCR-β, PE -conjugated CD22 and PerCP-conjugated CD11b (Refer Chapter 2: Materials and Methods; page 102).

3.2.8 Statistical analyses

Statistical analyses were performed using Student's t-test, and analysed using the software GraphPad Prism v5.02. P-value <0.05 were considered statistically significant.

3.3 Results

3.3.1 Specificity of NK cell depletion by anti-asialo GM1 antibodies in ApoE$^{-/-}$ mice.

To determine the effectiveness of the anti-asialo GM1 antibody treatment, I examined its effects on NK cells in peripheral blood. Five days after a dose of anti-asialo GM1 antibody, NK cells in blood were reduced by more than 90% ($P < 0.05$; Figure 3.1, panel A). To determine the specificity of anti-asialo GM1 antibody, I also examined its effects on NK cells, other lymphocytes and monocyte population in spleen five days after administration of anti-asialo GM1 or NRS. NK cells in the spleen were also reduced by almost 90%, 5 days after a dose of anti-asialo GM1 ($P < 0.05$; Figure 3.2 A). Other lymphocyte and monocyte populations, namely CD4+ and CD8+ T-cells, CD22+ B-cells, NKT cells as well as CD11b^{+} monocytes were unaffected in the spleen ($P > 0.05$; Figure 3.2 A). Similarly, NK cells were still reduced by about 90% in the blood at the end of the study, after 8 weeks on HFD ($P<0.05$; Figure 3.1, panel B). At the end of the study, spleen NK cells were reduced by about 50% ($P<0.05$; Figure 3.2 B and other lymphocyte and monocyte populations were unaffected ($P>0.05$; Figure 3.2 B).

3.3.2 NK depletion attenuates development of atherosclerosis.

To determine the role of NK cells on atherosclerosis lesion development, I initially assessed lipid accumulation using Oil Red O stain at the aortic sinus. Analysis of Oil Red O-stained aortic root cross sections of atherosclerotic lesions indicated a 70% reduction in lesion size in the anti-asialo treated mice which was independent of plasma cholesterol ($P < 0.05$; Figure 3.3).

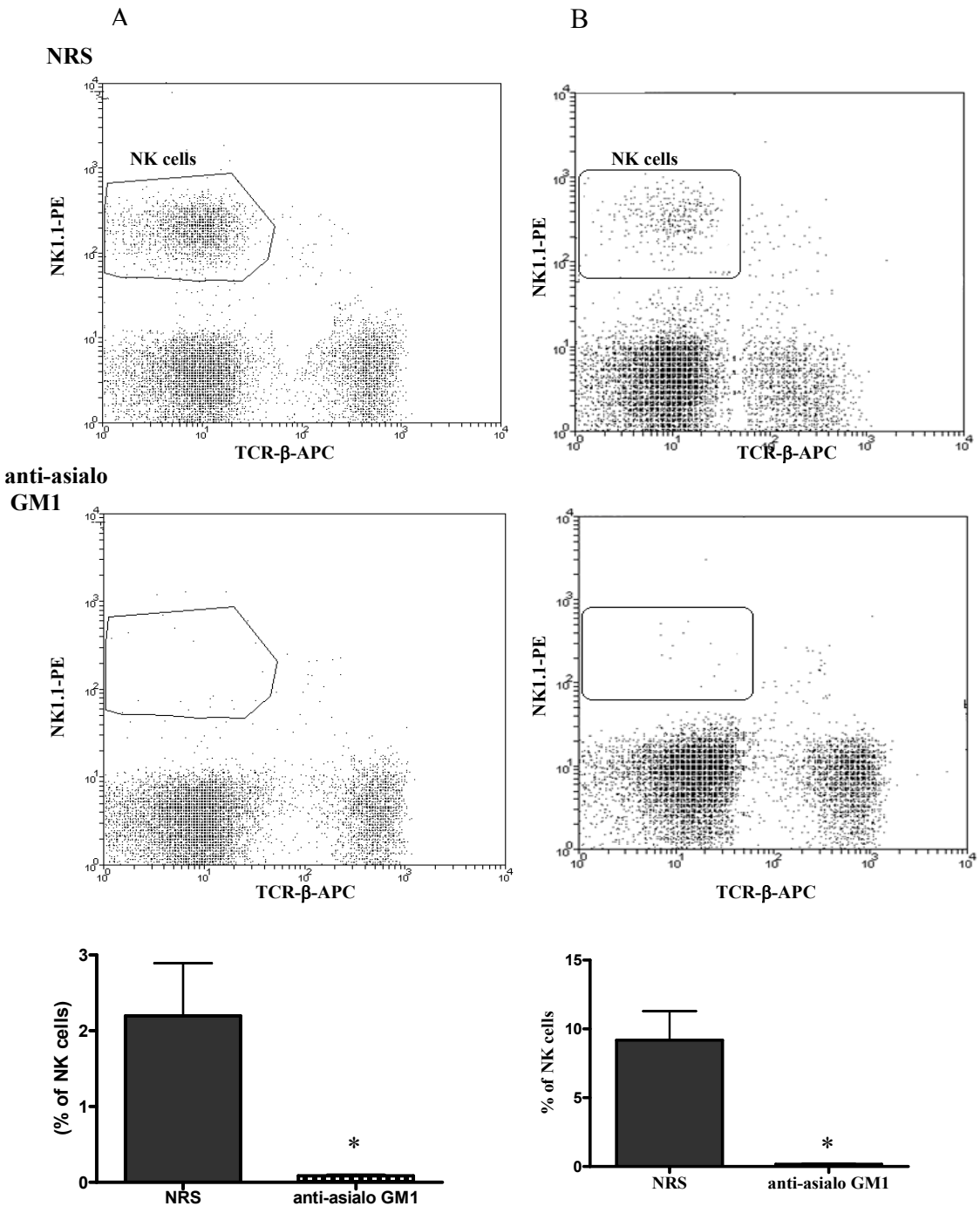


Figure 3.1:FACS analysis density plots of ApoE$^{-/-}$ mice treated with anti-asialo GM1 or NRS. Panel (A) below represent FACS analysis of peripheral blood collected 5 days after the administration NRS or anti-asialo GM1. Panel (B) below represents FACS analysis of blood at the end of the study, after 8 weeks of HFD in mice treated with NRS and anti-asialo GM1. Bar graph shows the means ± SEM from 4 mice from each group, $^{*}P < 0.05$ from NRS. Cells were stained for NK1.1 and TCR-β surface markers, NK1.1 positive and TCR-β negative population depicts NK cell population (regions on A and B marked as NK cells).

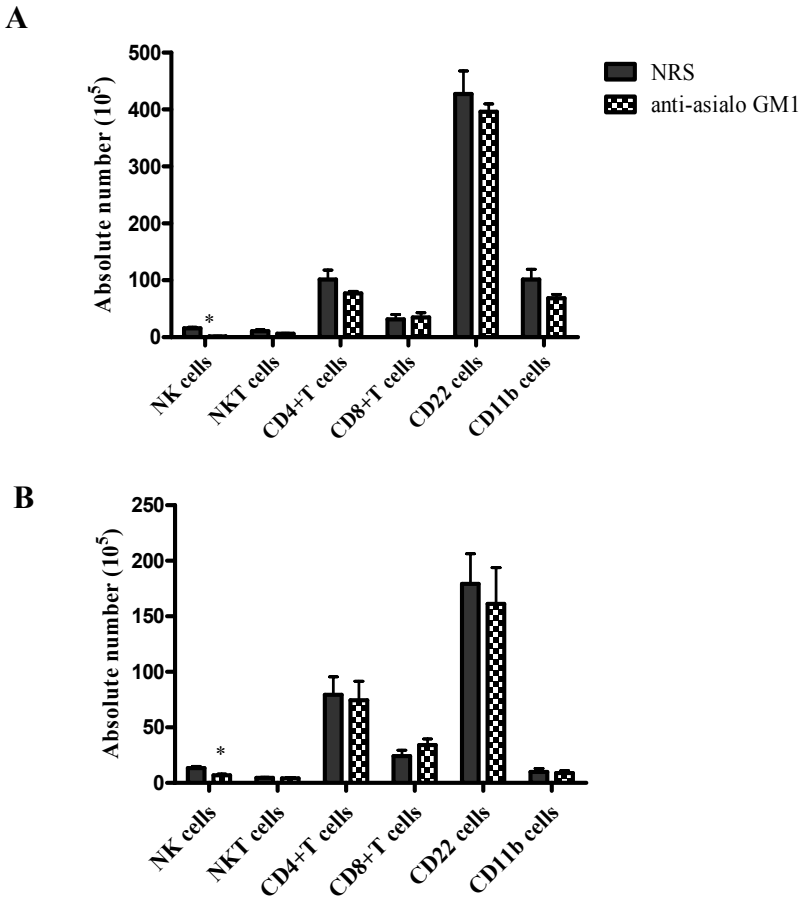
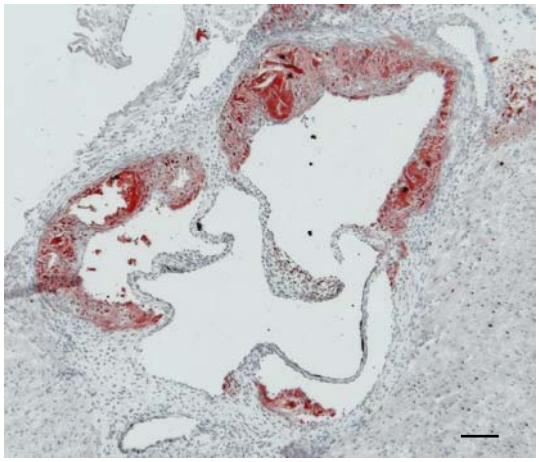
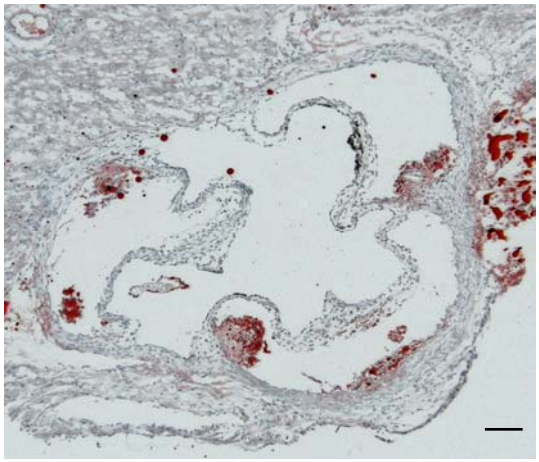


Figure 3.2: Absolute numbers of NK cells and other lymphocytes and monocyte population in the spleen of ApoE$^{-/-}$ mice treated with anti-asialo GM1 or NRS. (A) Bar graph shows the means ± SEM of total number of NK, NKT, $CD4^+$,$CD8^+$,B ($CD22^+$) cells and monocytes ($CD11b^+$) from spleen of 4 mice from each group collected 5 days after administration of NRS or anti-asialo GM1, $^*P < 0.05$ from NRS. (B) Bar graph shows the means ± SEM of total number of NK, NKT, $CD4^+$,$CD8^+$,B ($CD22^+$) cells and monocytes ($CD11b^+$) from spleen of 4 mice from each group collected at the end of the study, $^*P < 0.05$ from NRS.

A



B



C

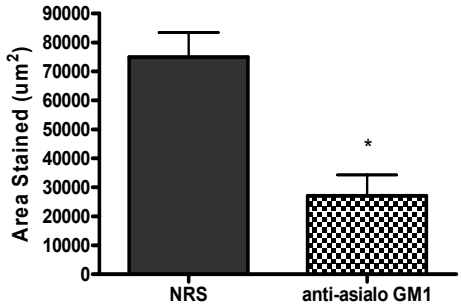


Figure 3.3: Lipid accumulation in atherosclerotic lesion in ApoE$^{-/-}$ mice treated with anti-asialo GM-1. Oil Red O stained lipid accumulation in the aortic sinus from mice treated with either (A) NRS or (B) anti-asialo GM1. (C) Bar graph represented mean ± SEM of area stained from 8 to 9 mice in a group; *P<0.05. Scale represents 100μm.

3.3.3 NK cell depletion and macrophage accumulation in atherosclerotic lesions.

Activated NK cells are known to secrete chemokines including chemokine that attract monocytes. This includes CCL3 (macrophage inflammatory protein-1α), CCL4 (macrophage inflammatory protein-1β), and CCL5 (RANTES) [203]. Also, these chemokines have been shown to attract macrophages expressing CCR5 receptors in HIV-1 infections [636]. To determine whether the depletion of NK cell affects the recruitment of macrophages to atherosclerotic lesions, I examined the macrophage accumulation at the aortic sinus. The depletion of NK cells, reduced macrophage accumulation at the aortic root by 60% in mice treated with anti-asialo GM1 antibodies compared to NRS treated mice, ($P < 0.05$; Figure 3.4).

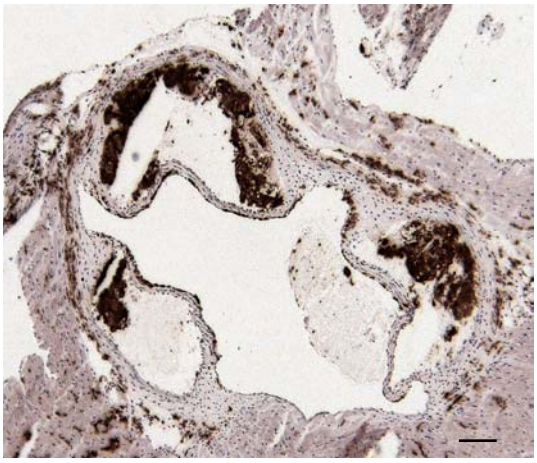
3.3.4 NK cell and smooth muscle cells and endothelial cells in atherosclerotic lesions.

NK cell derived pro-inflammatory cytokines such as IFN-γ and TNF-α are known to influence SMC proliferation and migration [637, 638]. IFN-γ has been shown to stimulate SMC proliferation [637], whilst TNF-α promotes vascular SMCs migration and mitogenesis [638]. Thus I examined whether NK cell depletion affected the accumulation of SMCs within developing atherosclerotic lesions. Alpha-smooth muscle cells were apparent in lesions within the fibrous cap of both control and the treatment groups. There was no difference in the SMC population between NRS and anti-asialo GM1 treated mice ($P > 0.05$; Figure 3.5).

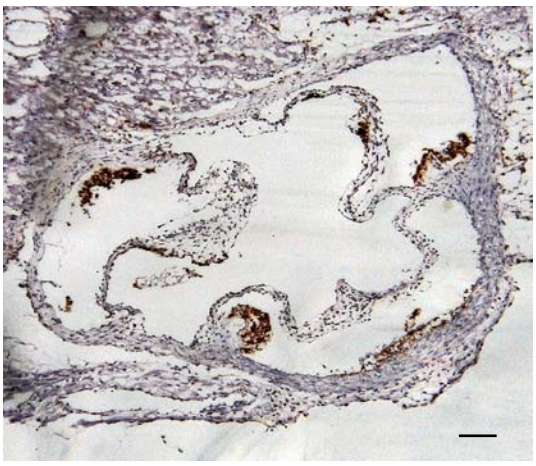
Endothelial cells (ECs) proliferation and migration enhances angiogenesis of arterial vessels to preserve cardiac functions following infarction [605]. NK cell derived IFN-γ and TNF-α can also influence the proliferation and migration of ECs. IFN-γ have been shown to inhibit ECs proliferation and migration and induces a change in endothelial cell morphology [639]. In

contrast TNF-α activates endothelial cell and enhance their proliferation and migration [640]. Therefore, I counted the intra lesional CD31, positive endothelial cells to determine the effects of NK cell depletion on luminal microvessels. Anti-asialo GM1 treatment tended to reduce CD31 positive cells in the lesions but the difference did not reach statistical significance ($P>0.05$). The number of CD31 cells per unit area of lesion was similar in both treatment groups, suggesting that the apparent reduction in CD31 positive cells reflected a smaller lesion in the anti-asialo GM1 treated group (Figure 3.6). These CD31 cells were apparent in the fibrous cap and occasionally within the lesion.

A



B



C

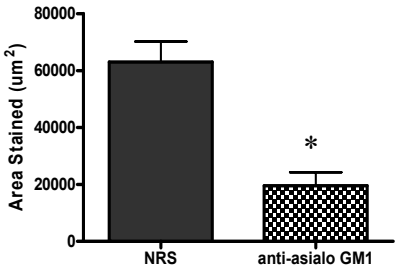


Figure 3.4: Macrophage accumulation in atherosclerotic lesions in mice treated with anti-asialo GM1. CD68 positive staining (brown stain) in the aortic sinus from mice treated with either (A) NRS or (B) anti-asialo GM1. (C) Bar graph represented mean ± SEM of area stained from 8 to 9 mice in a group; *P<0.05. Scale represents 100 μm.

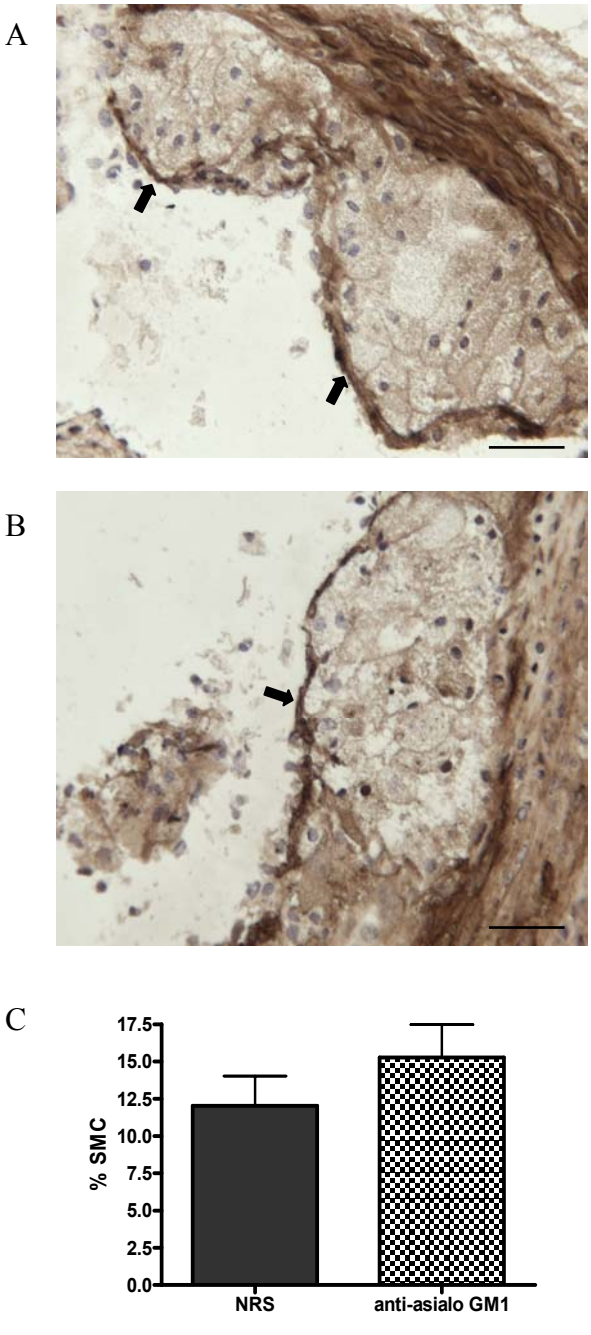
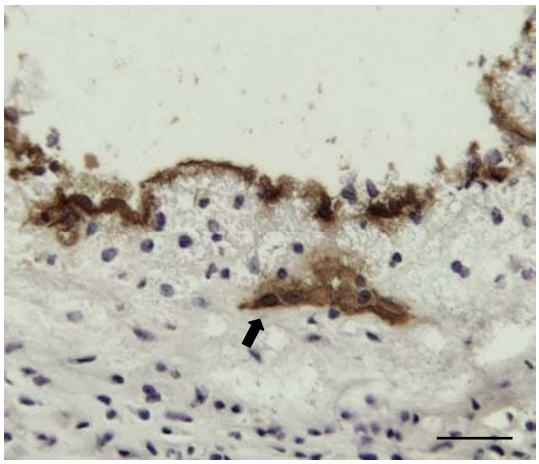
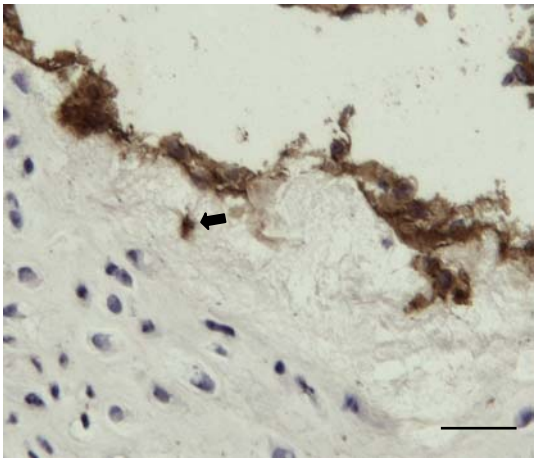


Figure 3.5: Percentage of SMC in atherosclerotic lesions in mice treated with anti-asialo GM1. Smooth muscle cell staining (dark brown stain as indicated by arrows) was observed in atherosclerotic lesions at the aortic sinus valves of both (A) NRS and (B) anti-asialo GM1 treated mice. (C) Bar graph represent mean ± SEM of percentage of SMC in atherosclerotic lesions from 8-9 mice in each group; $P>0.05$. Scale represents 40 μm.

A



B



C

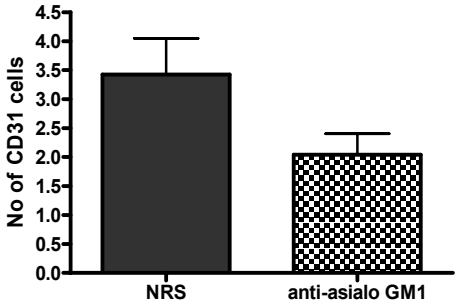


Figure 3.6: CD31 endothelial cell staining in atherosclerotic lesions of ApoE$^{-/-}$ mice treated with anti-asialo GM1. CD31 positive staining (see arrows, brown) is observed in the lesions of cross sections of aortic sinus from both (A) NRS and (B) anti-asialo treated mouse. (C) Bar graph represent mean ± SEM, number of CD31 positive cells in atherosclerotic lesions from 8-9 mice in each group; P>0.05. Scale represents 40 μm.

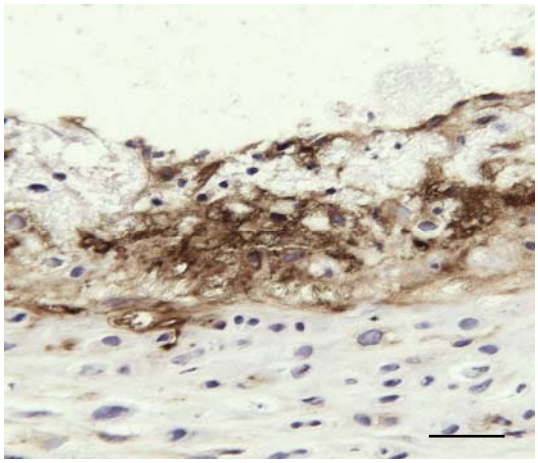
3.3.5 NK cell depletion and VCAM-1 expression.

NK cells secreted TNF-α and IFN-γ may activate endothelial cells that leads to increased in surface expression of cell adhesion molecules especially vascular cell adhesion molecule -1 (VCAM-1). VCAM-1 facilitates the recruitment of inflammatory cells to arterial wall and their transmigration [641]. VCAM-1 is expressed not only by ECs but also by macrophages, fibroblast and SMC in the arterial wall [642, 643]. Thus I examined the effects of NK depletion on VCAM-1 expression in atherosclerotic lesion.VCAM-1 within lesions was unaffected, VCAM-1 was mainly expressed in endothelial cells and occasionally within the lesions in both NRS and anti-asialo GM1 treated groups (Figure 3.7, $P > 0.05$).

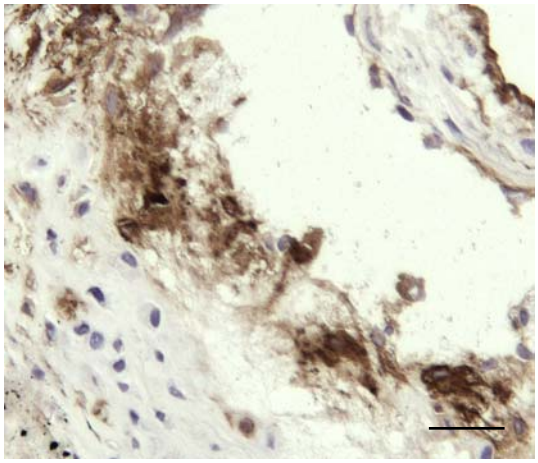
3.3.6 NK depletion and $CD4^+$ T cell recruitment.

Activated NK cells produce T cell-recruiting chemokines including IL-8, macrophage inflammatory protein-1α (MIP-1α), MCP-1 and RANTES [644]. To determine whether NK cells promote recruitment of CD4 T cells into the atherosclerotic lesions, I examine the number of $CD4^+$ T cells in the lesions. NK depletion did not affect the number of $CD4^+$ T cells recruited into the atherosclerotic lesion, the number of $CD4^+$ T cells was similar in both control and antibody treated group (Figure 3.8, $P>0.05$). T cells were located throughout the lesions in both studied group.

A



B



C

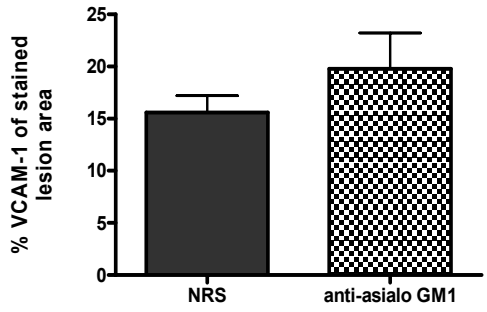
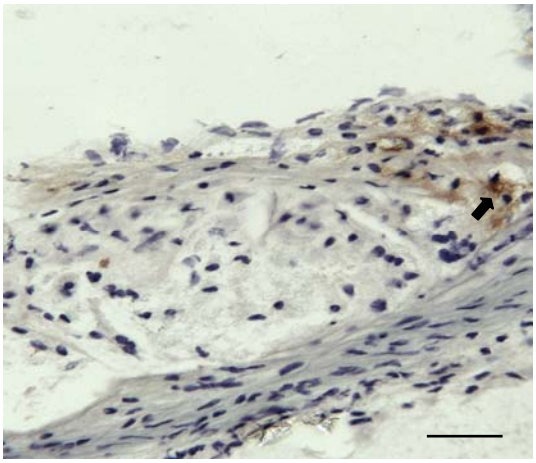
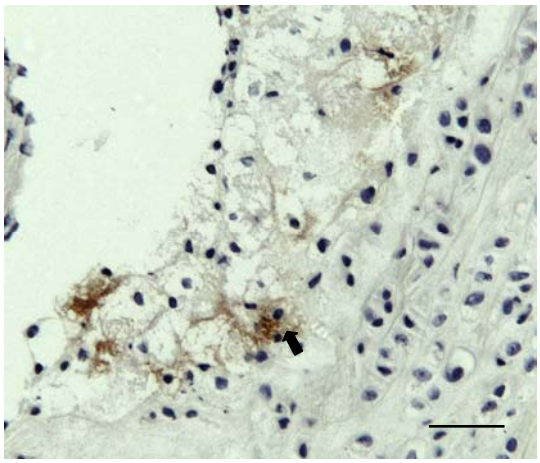


Figure 3.7: VCAM-1 expression in atherosclerotic lesions in ApoE$^{-/-}$ mice treated with anti-asialo GM1. VCAM-1 was expressed in both (A) NRS and (B) anti-asialo GM1 treated mice. (C) Bar graph represent mean ± SEM, of percentage of VCAM-1 in atherosclerotic lesions from 8-9 mice in each group; P>0.05. Scale represents 40 μm.

A



B



C

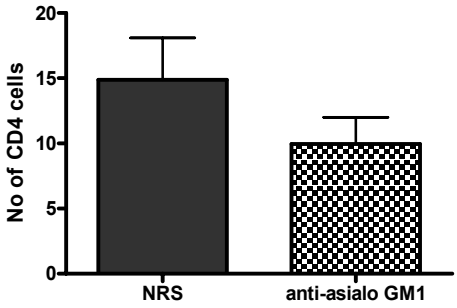


Figure 3.8: CD4+ T cell staining in atherosclerotic lesions of ApoE-/- mice treated with anti-asialo GM1. CD4 T cell positive staining (see arrows, brown) is observed in the lesions of cross sections of aortic sinus from both (A) NRS and (B) anti-asialo treated mouse. (C) Bar graph represent mean ± SEM, number of CD4 positive cells in atherosclerotic lesions from 8-9 mice in each group; $P>0.05$. Scale represents 40 μm.

3.3.7 NK cell depletion and plasma cholesterol, triglyceride, HDL and VLDL/LDL levels.

Plasma lipoprotein concentration have been shown to affect NK cell activity [645, 646]. The increase in VLDL/LDL concentration have been shown to increase NK cell cytotoxicity levels, in contrast the increase in HDL concentration decrease NK cell cytotoxicity [647]. Therefore, I examined the effects of NK cell depletion on plasma lipoprotein levels of VLDL/LDL and HDL together with total cholesterol and triglyceride levels. The NK cell depletion did not affect cholesterol, triglyceride, HDL and VLDL/LDL levels, these levels were similar in both NRS and anti-asialo GM1 treated group (Table 3.1, $P>0.05$). Despite significance differences in extent of atherosclerosis in $ApoE^{-/-}$ mice treated with anti-asialo GM1, the body weights of mice from each group were not affected.

Table 3.1: Body weight and plasma cholesterol levels of ApoE$^{-/-}$ treated with NRS or Anti-asialo GM1 fed a high fat diet for 8 weeks.

Group	*Weight (g)*	*Total Cholesterol*	*Triglyceride*	*VLDL/LDL*	*HDL*
NRS	28.3 ± 0.6 (9)	16.2 ± 1.9 (9)	2.2 ± 0.2 (9)	13.0 ± 1.5 (9)	2.1 ±0.4 (9)
Anti-asialo GM1	28.2 ± 0.6 (8)	13.7 ± 2.5 (8)	2.7 ± 0.5 (8)	11.0 ± 2.0 (8)	1.5 ± 0.4 (8)

p>0.05 compared to NRS treated mice
Total cholesterol, triglyceride, VLDL/LDL and HDL measurements in mmol/L.
Number of mice in each group is indicated in parentheses.
Data represent the mean ± SEM.

3.4 Discussion

This study has definitely demonstrated that NK cells despite being very minor lymphocyte population in developing atherosclerotic lesions profoundly influence development of atherosclerosis. Depleting NK cells by more than 90% attenuated lesion size and macrophage accumulation. Whilst not specifically examined the reduction in macrophage accumulation could be due to the lack of production of NK cell derived chemokines or cytokines in the lesions.

Treatment of ApoE$^{-/-}$ mice with anti-asialo GM1 depleting antibody every 5 days was highly effective in reducing the circulating number of NK cells by about 95% on the 5th day after the first injection. At the end of the antibody treatment for 8 weeks whilst on high fat diet, significantly reduced spleen NK cells by about 50%, determined by FACS analysis, further supporting the ability of anti-asialo GM1 in effectively depleting NK cells in fat fed ApoE$^{-/-}$ mice. FACS analysis of other lymphocyte populations namely NKT, CD4^{+} T, CD8^{+} T, and B cells were all unaffected by the antibody treatment. Since asialo GM1 antigen is also expressed by monocytes, it was possible that this population could also be affected by the antibody treatment. However the monocyte population was not affected by the antibody treatment regime. Anti-asialo GM1 appear to be highly specific for NK cells in contrast to NK1.1 as this latter antibody also depletes NKT cells which also express NK1.1 [648].

Recently treatment with anti-asialo GM1 antibodies has been also shown to deplete basophils [649]. It is unlikely that depletion of basophils contributes to the reduced atherosclerosis in ApoE$^{-/-}$ mice treated with anti-asialo GM1 antibodies as these cells do not accumulate in atherosclerotic lesions [324]. In hyperlipidemic rabbits they accumulate in the aortic adventitia and to a much smaller extent in normal intima and developing fatty streaks.

My findings that NK cell depletion attenuates atherosclerosis contrast with studies using beige mutation mice. In the first study using the beige mutation mice, focused on these mice as a model for defective platelet functions. The study demonstrated that NK cells were not involved in the development of atherosclerosis [610]. In the second study, in the fat fed beige mutation mice harbouring $LDLR^{-/-}$, NK cells were reported to be anti-atherogenic [629]. However, there were concerns regarding the use of beige mouse as a model to determine the role of NK cells in atherosclerosis. The mutation in beige mice, involving the Lyst gene resulted in complex phenotype that is beyond the defects in NK cell cytolytic activity [650, 651]. Furthermore, the beige mutation did not show a complete defect in the lysosomal trafficking; function of the Lyst protein, therefore the increase in atherosclerosis observed in the study using $LDLR^{-/-}$ mice could be due to changes in lysosomal trafficking in other cell types particularly macrophages that could result in an increase in foam cell formation hence increased in atherosclerosis [652, 653].

Rather my findings are consistent with the study using Ly49A transgenic mice. In both instances, lesions were reduced by 70%. Whilst it is possible that in the Ly49A transgenic mice, NKT cells might have also been affected, this is not reflected in lesion size. One possible explanation is that NKT cells could activate NK cells. For example, the sequential activation of NK cells by NKT cells promoted an anti-tumor response, however the inhibition of both NK and NKT cells promoted tumor growth [654]. This simultaneous inhibition of NKT and NK cells does not produce any additive effects.

Previous studies have not examined whether NK cell modulate the characteristics of developing lesions. Therefore, I investigated the effects of depleting NK cells on cell composition of the lesion, and the expression of adhesion molecules. The depletion of NK cells reduced macrophage accumulation at the aortic sinus by about 60% compared to control. The decrease in macrophages recruited into the lesion in NK cell depleted mice may be due to two

reasons, (1) NK cell maybe expressing chemokines such as CCL5 and CCL4 that attracts macrophages expressing their chemokine receptor CCR5, and (2) since activated NK cells are potent IFN-γ secretors and NK cell derived IFN-γ maybe involved in macrophage differentiation or activation in atherosclerosis. However, further investigation is required to examine the role of NK cell derived chemokines and cytokines in atherosclerosis.

I also examined the smooth muscle cell (SMC) content of the lesions. The depletion of NK cells, did not affect the SMC content of the lesions, in both instances atherosclerotic lesions were covered by a SMC rich cap. One possible reason why NK cells do not modulate SMC of ECs despite being significant produces of IFN-γ and TNF-α, is their relative small numbers in lesions. It is likely that IFN-γ and TNF-α from other immune cells in developing lesions are the major producers of these cytokines. IFN-γ is produced by T cells [655] and TNF-α is produced by activated macrophages [656].

Since the number of NK cells in atherosclerotic lesion is relatively small, the detection of NK cells using immunohistochemical studies has been difficult. Study by Whitman *et al* using the transgenic Ly49A mice, detected NK cells using asialo GM 1 antibody, and the study revealed small numbers of positively stained cells in the lesions [614]. I have also attempted to identify NK cells in atherosclerotic lesions of $ApoE^{-/-}$ mice using various antibodies such as NKp46 and also asialo GM1. However the immunohistochemistry staining resulted in non-specific binding of antibodies that prevented the detection of NK cells.

Activated NK cells have been shown to promote TNF-α–stimulated endothelial cell proliferation and enhanced angiogenesis to preserve cardiac function following infarction [605]. Furthermore, uterine NK cells have been shown to produce angiogenic growth factors including vascular endothelial growth factors (VEGF) and TGF-β1 which have been shown to facilitate

angiogenesis in arterial vessels [657, 658]. However, in my study NK depletion did not affect angiogenesis as the lesional endothelial cell expression was similar in both studied group. It is possible that NK cells might affect vascularisation of more advanced lesions [659] but this clearly require further investigations.

In summary, NK cells are pro-atherogenic and appear to regulate macrophage accumulation in lesions. It's possible that NK cell derived chemokines contribute to their pro-atherogenic effects but other mechanisms are equally possible, for example their cytolytic effects.

4 Poly IC induced NK cell activation promotes the development of atherosclerosis.

4.1 Introduction

Atherosclerosis is a chronic inflammatory disorder of the vessel wall and both innate and adaptive immune responses have been shown to influence the disease progression [3]. The innate signals within the lesion including microbial and viral products and endogenous danger signals during tissue injury promotes the development of atherosclerosis through inflammatory processes [660-662]. Activation of the innate immune system is important for inducing an inflammatory response by triggering pattern recognition receptors [537] . NK cells play a major role as effector cells of the innate immune system in anti-microbial activity. NK cells can be activated through various receptors depending on the ligands presented by target cells.

NK cells can be activated by viral dsRNA which can be mimicked by poly IC [541]. More recently endogenous mRNAs including RNA released from necrotic cells have been implicated in atherosclerosis [663]. RNA including poly IC can interact with TLR3 and MDA5 to activate NK cells. TLR3 is expressed by NK cells as well as other cells in atherosclerotic lesions and it has been shown to protect against atherosclerosis [547]. MDA-5 is also expressed by NK cells. MDA-5 is a cytosol sensor which detects poly IC that penetrates into cytosol through a yet to be defined mechanisms [537]. Both MDA-5 and TLR3 initiate downstream signalling pathway that lead to activation of a similar array of transcription factors that mediate expression of proinflammatory cytokines and molecules involved in antigen presentation [541, 664].

Given that atherosclerosis is characterized by a pro-inflammatory milieu, and associated with virus RNA infections, and degraded host RNA accumulate within atherosclerotic plaque, and activated NK cells respond to viral dsRNA and a potent producer of IFN-γ that are pro-atherogenic, I investigated the role of poly IC activated NK cells on the development of atherosclerosis.

The aim of this study was to determine whether activation by poly IC augments development of atherosclerosis and to determine whether the activation of NK cells with poly IC is the major cell type that is responsible for the increase in atherosclerosis.

4.2 Methods

4.2.1 Mice and treatment

Six week old ApoE$^{-/-}$ mice were administered (5ug/g body weight) poly IC through intra-peritoneal injections 3 times per week. Control mice were treated with sterile 0.9% saline. Mice were treated with poly IC for 8 weeks while on high fat diet (HFD).

To determine whether poly IC activation of NK cell are not dependent on T and B cells, six week old ApoE$^{-/-}$ Rag2$^{-/-}$ (C57BL/6 background) mice were administered with poly IC for 8 weeks while on HFD.

To determine whether the activation by poly IC was NK cell specific, five week old ApoE$^{-/-}$ mice were treated with 54ug of protein G column, purified anti-asialo GM1 every 5 days till the end of the study together with poly IC starting at 6 weeks of age and high fat diet feed for 8 weeks (Refer to Chapter 2: Materials and Methods, page 95 and 106 for details of reagents used).

4.2.2 Tissue collection

At the end of all experimentations mice were killed with an overdose of sodium pentobarbitone, aortic sinus was dissected and frozen in OCT for subsequent sectioning and histological and immunohistochemical analyses. All experiments are approved by the AMREP Animal Ethics Committee (Refer to Chapter 2: Materials and Methods; pages 95-96).

4.2.3 Tissue Sectioning

The frozen aortic sinus were sectioned at 6 μm in thickness and collected on microscope slides for subsequent histological and immunohistochemical studies (Refer to Chapter 2: Materials and Methods; page 96).

4.2.4 Flow cytometry analysis

To determine NK cell activation , $NK1.1^{+}$ TCR^{-} NK cells were gated using PerCP-conjugated NK1.1 and allophycocyanin-conjugated TCR-β and analysed using PE-conjugated CD69 (Refer to Chapter 2:Materials and Method page 102)

4.2.5 Atherosclerotic lesion size analysis

Sections of aortic sinus were stained with Oil Red O and examined using light microscopy and cross-sectional area of lipid deposition was quantified (Refer Chapter2: Materials and Methods; pages: 97 and 102).

4.2.6 Immunohistochemistry

Antibodies against CD68 and α smooth muscle actin were used in immunohistochemical analyses of macrophages, and smooth muscle cells in frozen sections of the aortic sinus,

respectively. CD68- and α smooth muscle cell actin positively stained areas were quantified by Optimus software. (Refer Chapter2: Materials and Methods; pages 99-102).

4.2.7 NK cell culture with Poly IC

NK cells were isolated from spleen of $ApoE^{-/-}$ mice after two i.p doses of (5μg/g) Poly IC [542] or saline after magnetic separation using Automacs. Isolated NK cells were cultured with sterile 0.9% saline for 24 hours (Refer to Chapter 2: Materials and Methods, pages 110-111 for NK cell culture method).

4.2.8 Cytokine assay

To determine the cytokines secreted by NK cells when stimulated with Poly IC, supernatant from the culture of NK cells with saline were analysed using Bio-Plex cytokine assay (*Bio Rad*). This cytokine assay detects an array of cytokines including GM-CSF, IFN-γ, IL-2, IL-12, IL-10 and TNF-α. (Refer to Chapter 2: Materials and Methods page 107).

4.2.9 Statistical analyses

Statistical analyses were performed using Student's t-test, or one way ANOVA using the software GraphPad Prism v5.02. P-value <0.05 were considered statistically significant.

4.3 Results

4.3.1 Poly IC activates NK cells in vivo

Poly IC is a well known NK cell activator to induce NK cell IFN-γ production to enhance Th1 polarization [542]. The activation status of NK and T cells was determined in $ApoE^{-/-}$ mice by the expression lymphocyte activation marker CD69 on NK and T cells. FACS analysis confirmed that the expression of CD69 on NK cells ($NK1.1^{+}$, TCR-β^{-}) and T cells (TCR-β^{+}) was upregulated in $ApoE^{-/-}$ mice 24 hours after Poly IC treatment (Figure 4.1).

4.3.2 Poly IC augments atherosclerosis

Since TLR3 activation exert a pro-inflammatory response and vascular and immune cells involved in atherosclerosis express TLR3 [665, 666], I initially examined the effects of activation of TLR3/MDA-5 by Poly IC on atherosclerosis. Lipid accumulation using Oil Red O stain at the aortic sinus was initially examined to assess the lesion size. Analysis of Oil Red O-stained aortic sinus cross sections of atherosclerotic lesions indicated a 40% increase in lesion size in the Poly IC treated mice ($^{*}P < 0.05$; Figure 4.2).

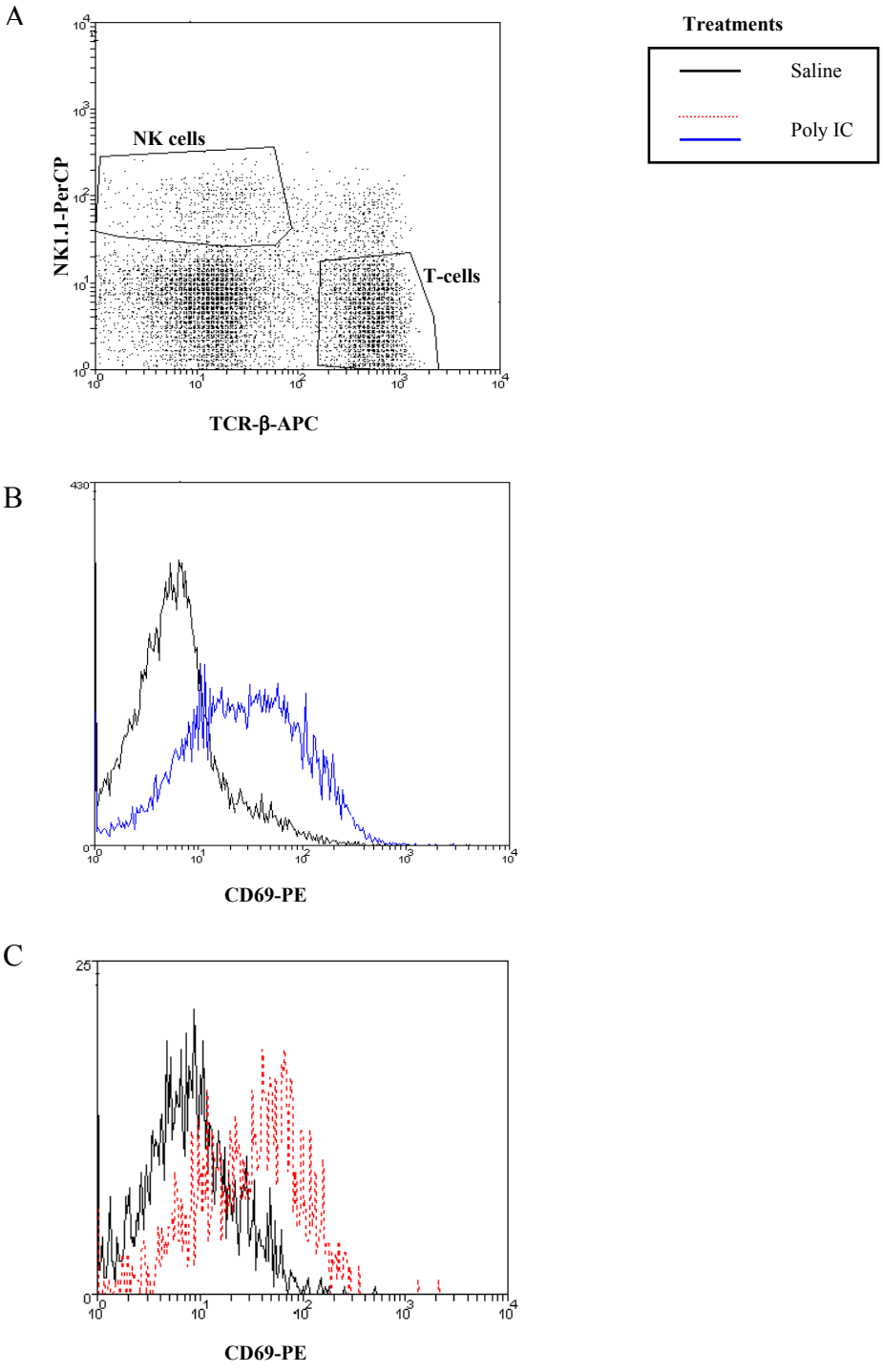


Figure 4.1: CD69 expression on spleen NK and T cells. (A) FACS dot plot represent NK cells gated as $NK1.1^{+,}$ TCR-β^{-} cells and T cell as TCR^{+} cells. Histogram represented the increased expression of CD69 positive (B) NK cells (blue line) and (C) T cells (in dotted red line) 24 hours after Poly IC treatment.

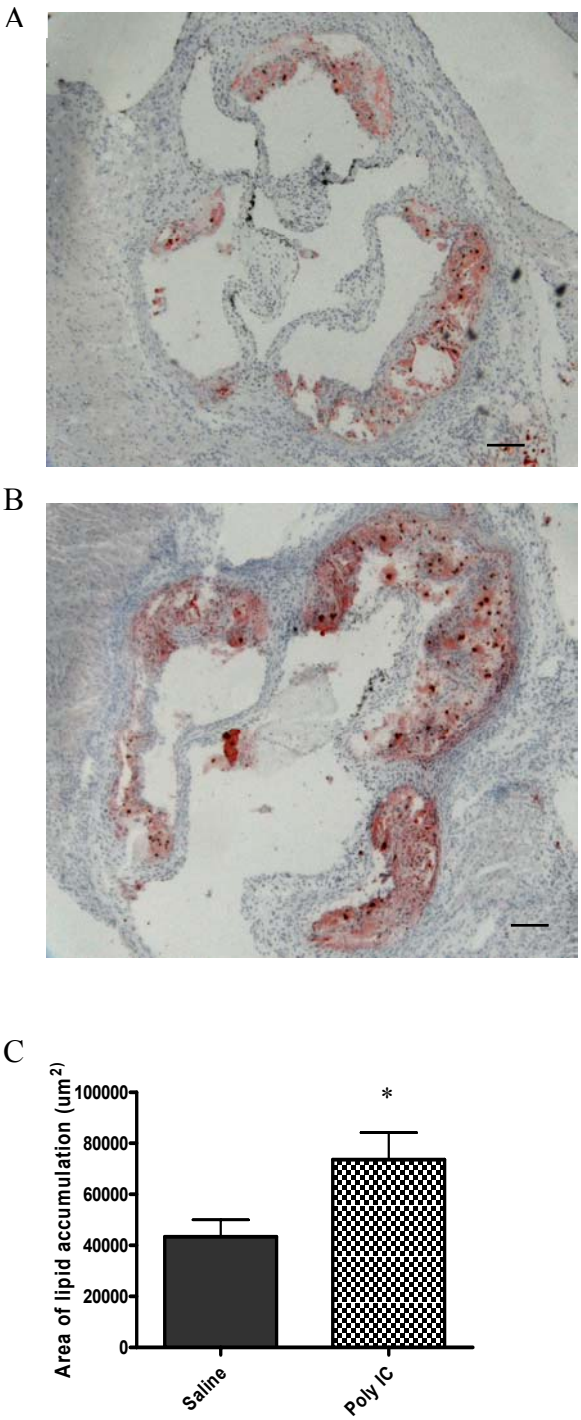


Figure 4.2: Lipid accumulation in atherosclerotic lesion in ApoE$^{-/-}$ mice treated with Poly IC. Oil Red O was used to stain lipid accumulation in the aortic root from mice treated with either (A) saline or (B) Poly IC. (C) Bar graph represented mean ± SEM of area stained from 13 to 15 mice in a group; *P<0.05. Scale represents 100 μm.

4.3.3 Poly IC and lesion cell composition

Activated NK cells have been shown to express chemokines CCL3 (macrophage inflammatory protein-1α), CCL4 (macrophage inflammatory protein-1β), and CCL5 (RANTES). These chemokines have monocyte chemotactic ability which increases the infiltration of macrophages to sites of inflammation and macrophages express the natural receptors CCR5 for these chemokines [667, 668]. Therefore I examined the effects of Poly IC activation on macrophage accumulation at the aortic sinus. Macrophage accumulation was increases by about 40% in Poly IC treated mice compared to control ($P<0.05$, Figure 4.3 A).

NK cell activation with Poly IC enhances the IFN-γ and TNF-α production and cytotoxicity of NK cells [526, 534]. As previously discussed IFN-γ and TNF-α are known to stimulate SMC migration and proliferation [637, 638]. In contrast, the increase in NK cell cytotoxicity may also promote the apoptosis of SMC which lead to unstable atherosclerotic lesion development. Thus, I examined the SMC content of the lesion, the number of alpha-smooth muscle positive cells in the lesion was unaffected by Poly IC treatment ($P>0.05$, Figure 4.3 B).

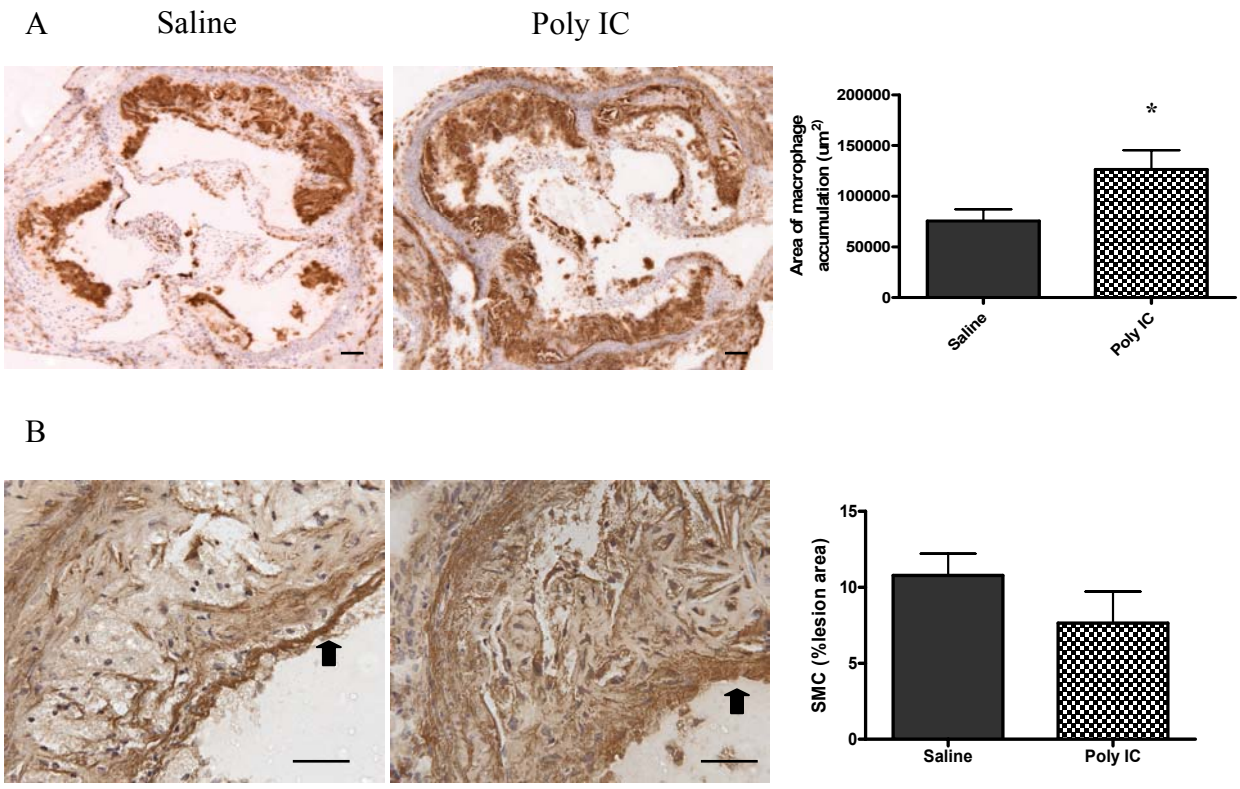


Figure 4.3: Characteristics of atherosclerotic lesions from ApoE$^{-/-}$ mice treated with Poly IC or saline. (A) Photomicrograph together with mean data demonstrating macrophage accumulation (CD-68 positive cells, brown stain) in the aortic sinus of saline and Poly IC treated mice. Scale bar represents 100 μm. (B) Photomicrograph together with mean data demonstrating smooth muscle cell content (alpha SM actin positive cells, dark brown stain shown with arrows) in aortic sinus, measured as alpha smooth muscle immunostaining (% area). Scale bar represents 250 μm. Bar graph shows means ± of at least 13 mice from each group. $^{*}P < 0.05$ from saline treated mice.

4.3.4 Affects of Poly IC on atherosclerosis are not dependent on T and B cells.

Since T cells most probably CD8 T cells were activated by Poly IC [669], and T cells have been implicated in atherosclerosis. Therefore to determine whether the effects of NK cell activation is not dependent on T cell responses, I examined atherosclerotic lesions in $ApoE^{-/-}$ $Rag2^{-/-}$ treated with Poly IC. The lesion size and lipid accumulation at the aortic sinus of $ApoE^{-/-}$ $Rag2^{-/-}$ treated with Poly IC was increased by about 50% compared to $ApoE^{-/-}$ $Rag2^{-/-}$ mice treated with saline (Figure 4.4 A, $P<0.05$).

Macrophage accumulation was also increased by about 65% in Poly IC treated $ApoE^{-/-}$ $Rag2^{-/-}$ mice (Figure 4.4 B, $P<0.05$).

4.3.5 Effects of Poly IC on lesion cell composition in $ApoE^{-/-}$ $Rag2^{-/-}$ mice

To determine if poly IC treatment affected lesion composition and inflammatory mediators in $ApoE^{-/-}$ $Rag2^{-/-}$ mice, I examined the effects of Poly IC treatment on SMC content and VCAM-1 expression. SMC content of the lesion in the absence of T cells in $ApoE^{-/-}Rag2^{-/-}$ mice was unaffected (Figure 4.5 A).

VCAM-1 has been shown to be important in atherosclerosis. VCAM-1 facilitates monocyte and T cells infiltration into the lesion [670]. The expression of VCAM-1 was unaffected by NK cell activation by Poly IC in T cell deficient mice $ApoE^{-/-}$ $Rag2^{-/-}$ (Figure 4.5 B).

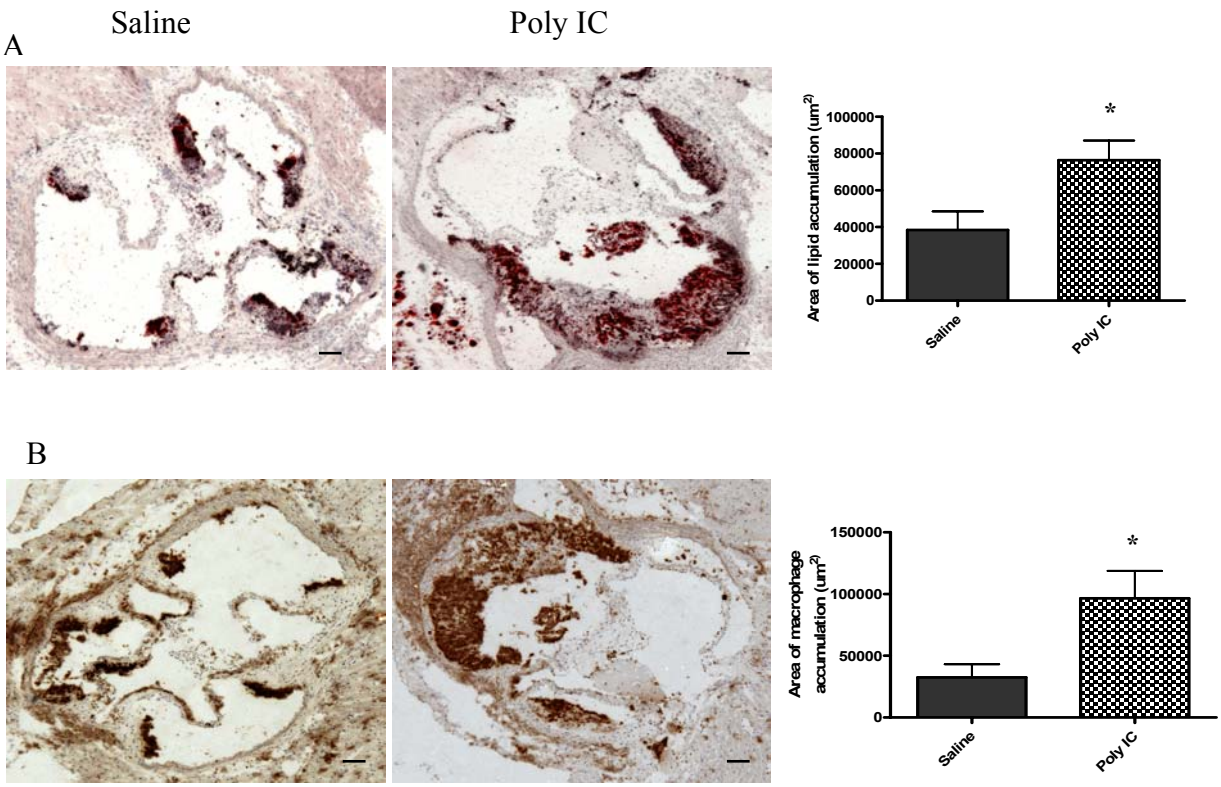
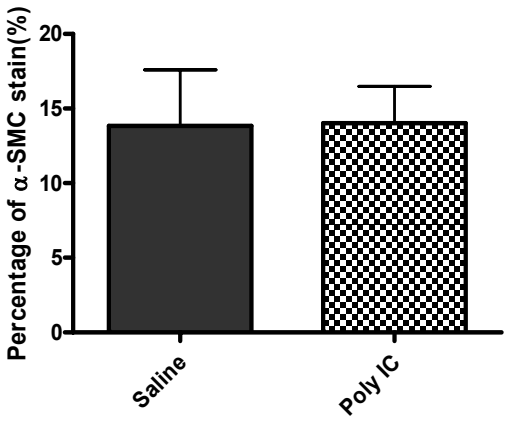


Figure 4.4: Characteristics of atherosclerotic lesions from ApoE$^{-/-}$ Rag2$^{-/-}$ mice treated with Poly IC. (A) Photomicrograph together with mean data demonstrating lipid accumulation (Oil-Red O stain, red stain) in the aortic root of Poly IC and saline treated mice. (B) Photomicrograph together with mean data demonstrating macrophage accumulation (CD-68 positive cells, brown stain) in the aortic root of Poly IC and saline treated mice. Bar graph shows SEM ± of at least 5 mice from each group. $^{*}P < 0.05$ from Poly IC treated mice. Scale bar represents 100 μm.

A



B

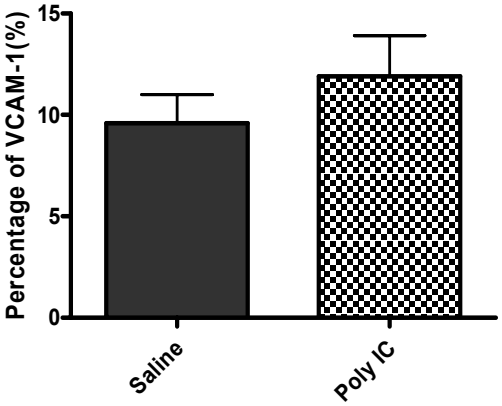


Figure 4.5: Smooth muscle cell and VCAM-1 content of ApoE$^{-/-}$ Rag2$^{-/-}$ lesions. (A) Bar graph represented mean data demonstrating smooth muscle cell content (alpha SM actin positive cells, brown stain) and (B) VCAM-1 expression in atherosclerotic lesion at the aortic root, measured as alpha smooth muscle cell and VCAM-1 positive staining (% area). Bar graph shows SEM ± of at least 5 mice from each group, $P > 0.05$.

4.3.6 NK cell depletion attenuates the effect of Poly IC on lesion size

TLR3 is expressed on macrophages and dendritic cells and these cells have been implicated in atherosclerosis. Therefore, to evaluate whether the effects of Poly IC on atherosclerosis is NK cell specific, I assessed the lesion size of $ApoE^{-/-}$ mice treated with Poly IC and anti-asialo GM1. The lesion size assessed by the accumulation of lipid in the aortic root using Oil red O stain was reduced by 40% in the Poly IC and anti-asialo GM-1 treated $ApoE^{-/-}$ mice compared to Poly IC and NRS treated mice ($^{*}P<0.05$, Figure 4.6 A). The macrophage accumulation at the aortic root was also reduced by about 40% ($^{*}P<0.05$, Figure 4.6 B). To confirm whether the decrease in lesion size is mediated by NK cells, lesion size from anti-asialo GM1 treated mice were compared with anti-asialo GM1 + Poly IC mice and only Poly IC treated mice. The reduction in lesion size in Poly IC + anti-asialo GM1 treated group was similar to mice treated with only anti-asialo GM1 treated mice compared to $ApoE^{-/-}$ mice treated with Poly IC (Figure 4.6 C).

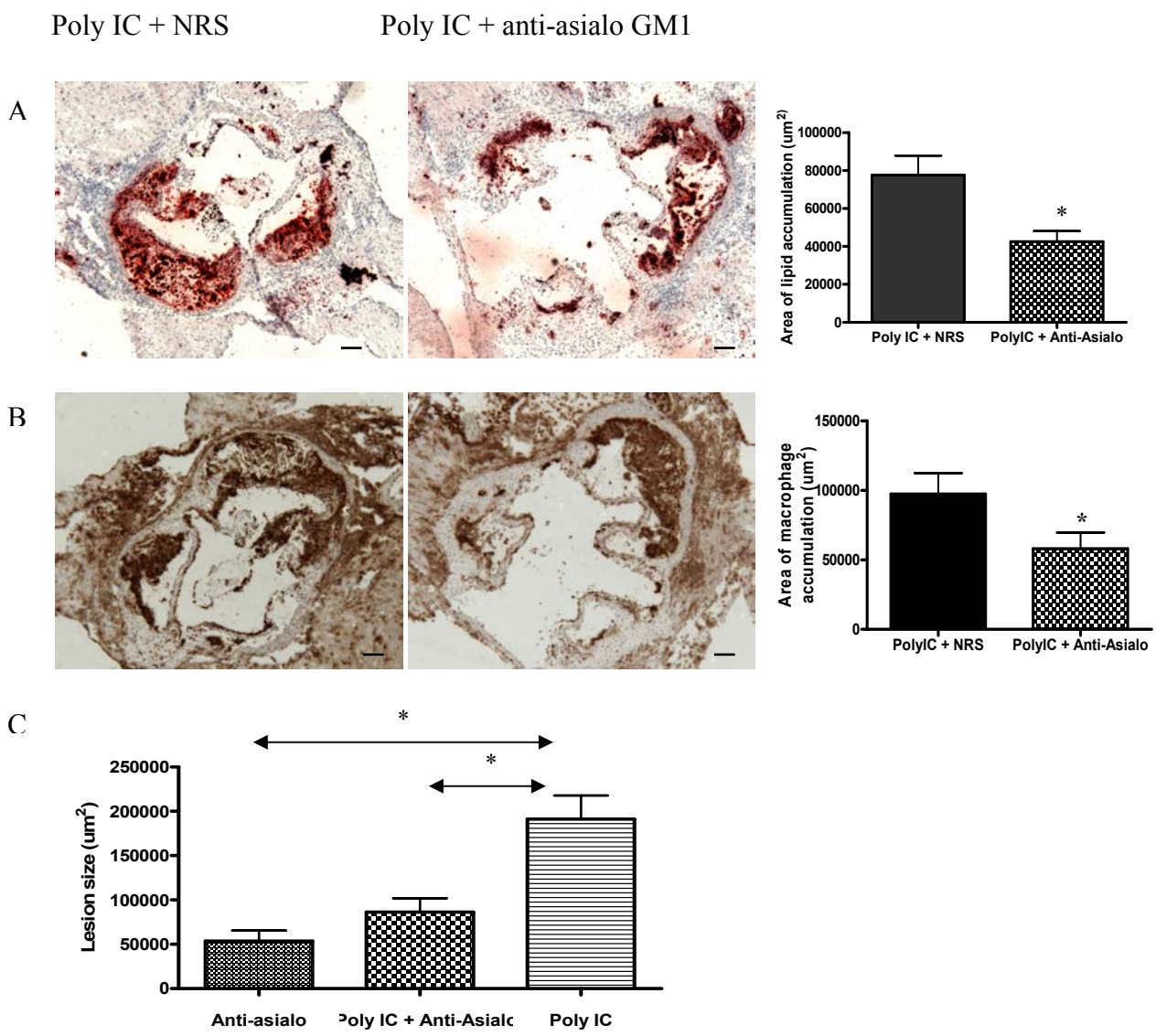


Figure 4.6: Characteristics of atherosclerotic lesions from ApoE$^{-/-}$ mice treated with Poly IC and anti-asialo GM1. (A) Photomicrograph together with mean data demonstrating lipid accumulation (Oil-Red O stain, red stain) in the aortic root of Poly IC and NRS; and Poly IC and anti-asialo GM1 treated mice. (B) Photomicrograph together with mean data demonstrating macrophage accumulation (CD-68 positive cells, brown stain) in the aortic root of Poly IC and NRS; and Poly IC and anti-asialo GM1 treated mice. (C) Bar graph represented mean data comparing lesion size of anti-asialo GM1, Poly IC and anti-asialo GM1 and Poly IC treated mice at the aortic root. Bar graph shows SEM ± of at least 13 mice from each group. $^{*}P < 0.05$ from Poly IC and NRS treated mice. Scale represents 100 μm.

4.3.7 Poly IC induced secretion of GM-CSF and IFN-γ but not TNF-α by NK cells

The activation of NK cells enhances NK cell derived cytokine including IFN-γ, TNF-α, and GM-CSF. To determine the cytokines released by NK cells in vivo when activated by Poly IC, NK cells from the spleen of mice treated with Poly IC or saline were isolated and further cultured with saline for 24 hours and supernatant from culture was used to perform a cytokine assay, assay detected a 78% increase in GM-CSF, 95% increase in IFN-γ, and no difference in TNF-α cytokines (Table 4.1).

Table 4.1: Cytokines produced by NK cells stimulated with Poly IC. Table shows the increase in NK cell derived cytokines IFN-γ, TNF-α and GM-CSF from supernatant collected following incubation of NK cells (isolated from Poly IC treated mice) with saline.

Treatment	Cytokines (pg/ml)		
	IFN-γ	**TNF-α**	**GM-CSF**
Saline	3.575	1.57	2726.225
Poly IC	75.255	1.57	12600.530

4.4 Discussion

My findings have demonstrated that synthetic analog dsRNA Poly IC augments the development of atherosclerosis and the increase in atherosclerosis lesion size was mediated by Poly IC activation of NK cells and not activation of T and B cells. These results support earlier study that concluded NK cells were pro-atherogenic where the depletion of NK cells ameliorated atherosclerosis, in turn the activation of NK cells by Poly IC exacerbated atherosclerosis.

The treatment of ApoE$^{-/-}$ mice with Poly IC augmented atherosclerosis development. Previous study has shown that Poly IC or self-RNA induces a robust inflammatory response in human coronary arteries by eliciting cytokines and chemokines production by intrinsic vascular cells and stimulating VSMCs. This inflammatory responses were potentiated by IFN-γ that upregulated dsRNA receptor expression [666]. However this study did not investigate the mechanism by which Poly IC promotes the inflammatory response, for example which immune cells that is activated by Poly IC to produce a pro-inflammatory environment and the effects of Poly IC treatment on atherosclerosis. Rather it suggests that Poly IC treatment induces a pro-inflammatory environment of the vessel which favors the development of atherosclerotic lesions.

Poly IC is a well known activator of NK cells, inducing NK cells to produce inflammatory cytokine IFN-γ, increases NK cell cytotoxicity and increases chemokines CCL3, CCL4, and CCL5 expression on NK cells [534, 542, 671, 672]. I examined the activation status of NK cells after stimulation with Poly IC in ApoE$^{-/-}$ mice, CD69 activation marker on spleen NK cells was upregulated. CD69 is a differentiation antigen expressed shortly after lymphocyte activation [673]. CD69 itself promotes NK cell mediated cytolytic activity, enhance NK cell proliferation and induce TNF-α secretion by NK cells [674, 675]. The upregulation of NK cell

activation marker indicates that NK cells in ApoE$^{-/-}$ mice are activated by Poly IC and suggest the involvement of NK cells in the increase in lesion size. However, CD69 expression on T cells was also upregulated, indicating T cell have been activated. The T cell most probably activated is CD8^{+} T cells rather than CD4^{+} T cells as Poly IC treatment have been shown to upregulated CD69 expression on CD8^{+} T cells [669]. Since poly IC is able to activate CD8^{+} T cells and CD8^{+} T cells have been implicated in atherosclerosis [295, 297], I also treated ApoE$^{-/-}$ Rag2$^{-/-}$ with Poly IC, these mice are deficient in both T and B cells but NK cells are normal to determine whether the effects of Poly IC on atherosclerosis is dependent on T cells. Poly IC treatment again increased lesion size in these mice, suggesting that Poly IC might be activating NK cell to promote atherosclerosis and T cell may be activated secondary to NK cell activation as the CD69 expression on T cells were upregulated after Poly IC treatment in ApoE$^{-/-}$ mice, NK activation results in IFN-γ secretion by NK cells that potentially could enhance Th1 cell polarization.

To confirm that the increase in lesion size is mediated by NK cell activation, I treated ApoE$^{-/-}$ mice with NK depleting antibody anti-asialo GM1 and Poly IC. NK cell depletion reduced atherosclerotic lesion in Poly IC treated mice. Additionally when compared data from ApoE$^{-/-}$ mice treated with only anti-asialo GM1 and both anti-asialo GM1 + Poly IC treated mice, lesion size of both groups were similar, this confirms that Poly IC mediated atherosclerotic lesion development is dependent on NK cell activation.

To rule out the possibilities of Poly IC activating macrophages independent of NK cells to promote atherosclerosis, I also compared data from lesion size of ApoE$^{-/-}$ mice treated with only NRS (data from Chapter 3) and ApoE$^{-/-}$ mice treated with anti-asialo GM1 + Poly IC (data not shown, as the data for lesion size of ApoE$^{-/-}$ mice treated with NRS was extracted from Chapter 3 for comparison purposes). There was a high tendency for lesion size in ApoE$^{-/-}$ mice treated with anti-asialo GM1 to be reduced compared to ApoE$^{-/-}$ mice treated with NRS, these experiments needs to be carried out concurrently to confirm these results. However, macrophage

accumulation at the aortic sinus in ApoE$^{-/-}$ mice treated with anti-asialo GM1 + Poly IC were significantly reduced compared to ApoE$^{-/-}$ mice treated with NRS + Poly IC (Figure 4.6B), indicating that Poly IC maybe specifically be activating NK cells to promote atherosclerosis and macrophages maybe activated secondary to Poly IC NK cell activation, Poly IC NK cell activation results in expression of chemokines by NK cells that potentially could attract and activate macrophages in atherosclerotic lesions.

Poly IC treatment also increased the accumulation of macrophages in the developing lesion. Poly IC has been shown to activate NK cells, and Poly IC activated NK cells highly express chemokines CCL3, CCL4 and CCL5 [672]. These chemokines on NK cells potentially form chemotaxis with macrophages that express CCR5 and assist the recruitment of macrophages into the atherosclerotic lesions. VCAM-1 facilitates the infiltration of monocytes and T cells into the inflammatory sites. The expression of VCAM-1 was unaffected in Poly IC treated double knockout mice that lack both T and B cells. This further confirms the recruitment of macrophages into atherosclerotic lesions is dependent on NK cell chemokines rather than the adhesion molecules. Further investigations are required to determine the chemokines expressed by NK cells in developing atherosclerotic lesions.

Activated NK cells are known to secrete IFN-γ, TNF-α, GM-CSF and IL-10. Cytokine assay detected an increase in these cytokines when NK cells were stimulated in vivo with Poly IC. In atherosclerosis, NK cells are most likely be secreting IFN-γ to promote the development of atherosclerosis. NK cell derived cytokines IFN-γ may be inducing T helper cell polarization and exerts a pro-inflammatory response to promote atherosclerotic lesion development; additionally IFN-γ itself may promote atherosclerotic lesion development.

TLR3 is expressed by immune cells such as NK cells, macrophages and dendritic cells. Poly IC has been shown to modulate macrophage cholesterol metabolism [676]. This study reported that Poly IC inhibits the ability of macrophage to efflux excess cholesterol through TLR3 and may regulate the development of atherosclerosis. This study also suggests a pro-atherogenic role for Poly IC in atherosclerosis. Previously, my study has revealed that macrophage recruitment into the lesion might be dependent on chemokines expressed on activated NK cells; hence Poly IC might be activating NK cells to recruit macrophages into the lesion and subsequently prevents cholesterol homeostasis that leads to atherosclerotic lesion development. Human NK cells have been reported to express TLR3, and Poly IC directly activates NK cells through TLR3 [534], however this is not the case for mouse NK cells. Mouse NK cells are activated indirectly through dendritic cells expressing TLR3 when treated with Poly IC. CD8α-DC also expresses TLR3 and Poly IC treatment has been reported to enhance CD8α-DC maturation which results in pro-inflammatory cytokine secretion such as IL-12 and IL-18 which then activates NK cells in vivo [542]. Therefore further investigation is needed to determine if Poly IC activates NK cells directly or indirectly through dendritic cells.

My study contrast with a recent study that has reported a protective role for TLR3 in the arterial wall [547]. Mice deficient in TLR3 and fed a chow diet, accelerated the onset of atherosclerosis. This study also found that Poly IC induced TLR3 expression on VSMCs, increased pro-inflammatory cytokines, anti-inflammatory cytokines and anti-apoptotic genes, increased expression of VCAM-1 and chemokines MCP-1 and CCL5, and the study stresses that the anti-inflammatory and anti-apoptotic production that mediated the TLR3 dependent protection against disease. In studies linking inflammation and atherosclerosis, increased expression of VCAM-1, chemokines and pro-inflammatory cytokines usually facilitates the leukocyte infiltration into the intima and promote the development of atherosclerosis. However this study did not report on the leukocytes infiltration in atherosclerosis, they reported that

macrophage accumulation in TLR3-deficient mice at the aortic root tended to be increased but not significant. Another explanation for the contradicting results between my study and this study is that this study did not look at the effects of Poly IC on atherosclerosis, they only looked at the effects of TLR3 perhaps Poly IC could be activating its other sensor MDA-5 to promote atherosclerosis. Poly IC activation of MDA-5 have been shown to be involved in controlling viral infection [677]. In terms of Poly IC activating NK cells, it has been reported that MDA-5 was crucial for NK cell activation, whereas TLR3 had only a minor impact on NK cell activation [541]. Therefore, whether the activation of NK cell is mediated via MDA-5 in atherosclerosis needs further investigation.

These finding further supports the pro-atherogenic role of NK cells. NK cell activation by MDA-5/TLR3 agonist promotes the development of atherosclerosis and the effects of NK cells on atherosclerosis are independent of T cells.

5 Mechanisms activating NK cells during development of atherosclerosis: Role of NKG2D receptors and NKT cells.

5.1 Introduction

Previously I have shown that NK cells contribute to the development of atherosclerosis and then activation by Poly IC further augments development of atherosclerosis. Whilst these studies clearly demonstrate a pro-atherogenic role for NK cells, the mechanisms responsible for their activation during development of atherosclerosis are not known. It is possible that either activation of NK cells via NKG2D activating receptors or via NKT cells may contribute to the pro-atherogenic effects.

Reactive oxygen species are known to be elevated during development of atherosclerosis and has been associated with DNA damage [678-680]. DNA damage has been reported in atherosclerosis [679] and can affect both macrophages and smooth muscle cells to downregulate MHC class I molecules [681]. DNA damage upregulates NKG2D ligands which in turn can activate NK cell activating receptor NKG2D [489]. NKG2D ligands upregulated include Rae-1, MICA and MICB [682, 683]. It is possible that upregulation of such ligands can contribute to NK cell activation during development of atherosclerosis.

It is also possible that NKT cells contribute to NK cell activation. Pharmacological stimulation of NKT cells with α-Galactosylceramide (α-GalCer), a glycolipid, that binds to CD1d molecules on antigen-presenting cells, have been reported to rapid downstream activation of NK cells to secrete IFN-γ, express CD69 , become more cytotoxic and proliferate. This is

partly dependent on IFN-γ production by NKT cells to activate NK cells [188]. This mechanism have also been shown to be involved in anti-tumor responses that are dependent on the production of IFN-γ by NKT cells and NK cells [684]. NKT cells have also been implicated in augmenting the development of atherosclerosis. α-GalCer activates invariant NKT cells and promotes atherosclerosis by producing pro-inflammatory cytokines IFN-γ and IL-2 [300, 303]. However, whether the mechanism of activation of NK cells by NKT cells is operative in atherosclerosis is currently unknown.

In the present study I investigated these two possibilities, specifically whether NKG2D receptor activation in $ApoE^{-/-}$ mice contributes to the development of atherosclerotic lesions by neutralizing NKG2D receptors and whether NKT cells were essential for the pro-atherogenic effects of NK cells by depleting NK cells in NKT cell deficient $ApoE^{-/-}$ $Jalpha18^{-/-}$ mice.

5.2 Methods

5.2.1 Animals and Treatment

Animals ApoE$^{-/-}$ and NKT cell deficient ApoE$^{-/-}$Jalpha18$^{-/-}$ mice aged between 6-8 weeks old were used in these studies. Both these strains were fed a high fat diet as described in Chapter 2: Materials and Methods, for 8 weeks while on treatment.

To determine the significance of NK cell activation by NKG2D receptors for atherosclerosis, ApoE$^{-/-}$ mice were treated with hamster anti-mouse NKG2D antibodies (C7 clone) or control hamster IgG (250μg i.p.) twice weekly for eight weeks [685]. The dose (250ug ip) and frequency (twice weekly) of NKG2D antibodies given to the ApoE$^{-/-}$ mice was identical to the dose administered by Smyth et al [685]. This dose of anti-NKG2D antibody enhances the sensitivity of mice to methylcholanthrene-induced fibrosarcoma and also completely suppresses NKG2D mediated rejection of RMA- S-Rae-1beta tumour cells in mice.

To determine whether NKT cells are required for the pro-atherogenic effects of NK cells, ApoE$^{-/-}$ Jalpha18$^{-/-}$ mice were treated with anti-asialo GM-1 antibodies (54ug/injection) or rabbit serum (54ug/injection) every 5 days for eight weeks whilst on a high fat diet (Refer to Chapter 2: Materials and Methods page 95). Normal rabbit serum (NRS) is a control used in this study for rabbit asialo GM1 antibody. The treatment of NRS alone does not affect the development of atherosclerosis, therefore the treatment of mice with NRS + anti-asialo GM1 would be expected to produce similar results to mice treated with asialo GM1.

5.2.2 Tissue collection

At the end of the study period, mice were administered an overdose of pentobarbitone and body weight was measured. Plasma was collected for cholesterol and triglyceride measurements, aortic sinus was dissected and frozen in OCT for subsequent sectioning and histological and immunohistochemical (Refer to Chapter 2: Materials and Methods pages 95). All experiments were approved by the AMREP Animal Ethics Committee.

5.2.3 Purification of anti-NKG2D antibodies

Monoclonal anti-NKG2D antibodies were purified from culture supernatants of C7 hybridoma cells using a protein G column [619] (Refer to Chapter 2: Materials and Methods; pages 104-106).

5.2.4 Bio-activity of anti-NKG2D neutralizing antibody

The inhibitory activity of the antibody was tested in an in vitro lysis assay using a standard 4 hour ^{51}Cr release assay, incubating ^{51}Cr labelled RMA-S-Rae1β (tumor cells) and activated spleen derived NK cells isolated from $ApoE^{-/-}$ mice (treated with IL-2 to activate NK cells) in the presence and absence of anti-NKG2D antibodies (30μg/ml and 60μg/ml) [686] . The isolation of splenic NK cells and cytotoxicity assay is described in Chapter 2: Materials and Methods; pages 107-109.

5.2.5 Plasma cholesterol analysis

Plasma samples were sent to Southern Cross Pathology Australia, (Clayton, Australia) for measurements of total plasma cholesterol, HDL-cholesterol, VLDL/LDL cholesterol and triglyceride levels using Beckman Coulter reagents as described in Chapter 2 Materials and Methods, page 96.

5.2.6 Tissue Sectioning

The frozen aortic sinus were sectioned at 6 μm in thickness and collected on microscope slides for subsequent histological and immunohistochemical studies (Refer to Chapter 2: Materials and Methods; page 96).

5.2.7 Atherosclerotic lesion size analysis

Sections of aortic sinus were stained with Oil Red O and examined using light microscopy and cross-sectional area of lipid deposition was quantified (Refer Chapter2: Materials and Methods; pages 97 and 102).

5.2.8 Histology

Sections of aortic sinus were stained with haematoxylin and eosin for analysis of necrotic core, sections were then examined using light microscopy and cross-sectional area of necrotic core was quantified (Refer Chapter2: Materials and Methods; pages 98 and 102).

5.2.9 Immunohistochemistry

Abs against CD68, and α smooth muscle actin were used in immunohistochemical analyses of macrophages and smooth muscle cells in frozen sections of the aortic sinus, respectively. CD68- and α smooth muscle cell actin positively stained areas were quantified by Optimus software (Refer Chapter 2: Materials and Methods; pages 99-102).

5.2.10 Rae-1δ, Rae-1ε, MULT-1, and H60 expression

For the detection of Rae-1δ, Rae-1ε, MULT-1, and H60 in atherosclerotic lesions, RNA was extracted from aortic arches of $ApoE^{-/-}$ mice fed a high fat diet for 8 weeks. Extracted RNA was reverse-transcribed and subjected to amplification with specific primers using the Superscript one-step RT-PCR method. The products of RT-PCR were electrophoresed in 2% Agarose gels to visualize the amplified products and gels were photographed under UV illumination. RNA extraction, primer sequences, and Superscript one-step RT-PCR methods are described in detail in Chapter 2: Materials and Methods; pages 114-117, 120.

5.2.11 Flow cytometry

NK cells in peripheral blood of ApoE$^{-/-}$ Jalpha18$^{-/-}$ was analysed using flurochrome-conjugated Abs on a BD FACS calibur following treatment with anti-asialo GM1 antibodies. For NK cells, PE-conjugated NK1.1 and allophycocyanin-conjugated TCR-β Abs were used (Refer to Chapter 2: Materials and Method page 102).

5.2.12 Statistical analyses

Statistical analyses were performed using Student's t-test, and analysed using the software GraphPad Prism v5.02. P-value <0.05 were considered statistically significant.

5.3 Results

5.3.1 NKG2D receptor activation and Atherosclerosis

5.3.1.1 mRNA encoding NKG2D ligands are expressed by cells in developing atherosclerotic lesions

Since DNA damage have been reported in atherosclerosis and NKG2D ligands are upregulated during DNA damage [489, 680], I initially investigated whether ligands that activate NKG2D receptors are expressed in atherosclerotic lesions. RT-PCR analysis of mRNA isolated from atherosclerotic lesions from $ApoE^{-/-}$ mice fed a high fat diet for 8 weeks, indicated high expression NKG2D ligands Rae-1δ, Rae-1ε and MULT-60; but not H60 (Figure 5.1).

5.3.1.2 Efficacy of anti-NKG2D antibodies

To determine the efficacy of purified anti-NKG2D antibodies, I performed a standard 4 hour Chromium-51 assay by co-culturing activated NK cells (isolated from $ApoE^{-/-}$ mice treated with IL-2) with RMA-S-Rae-1β tumor cells together with either anti-NKG2D or hamster IgG; anti-NKG2D prevented the lysis of RMA-S-Rae-1β tumor cells by NK cells (Figure 5.2). The antibody concentration used in the in vitro testing was in par to the amount used in the in vivo study in mice.

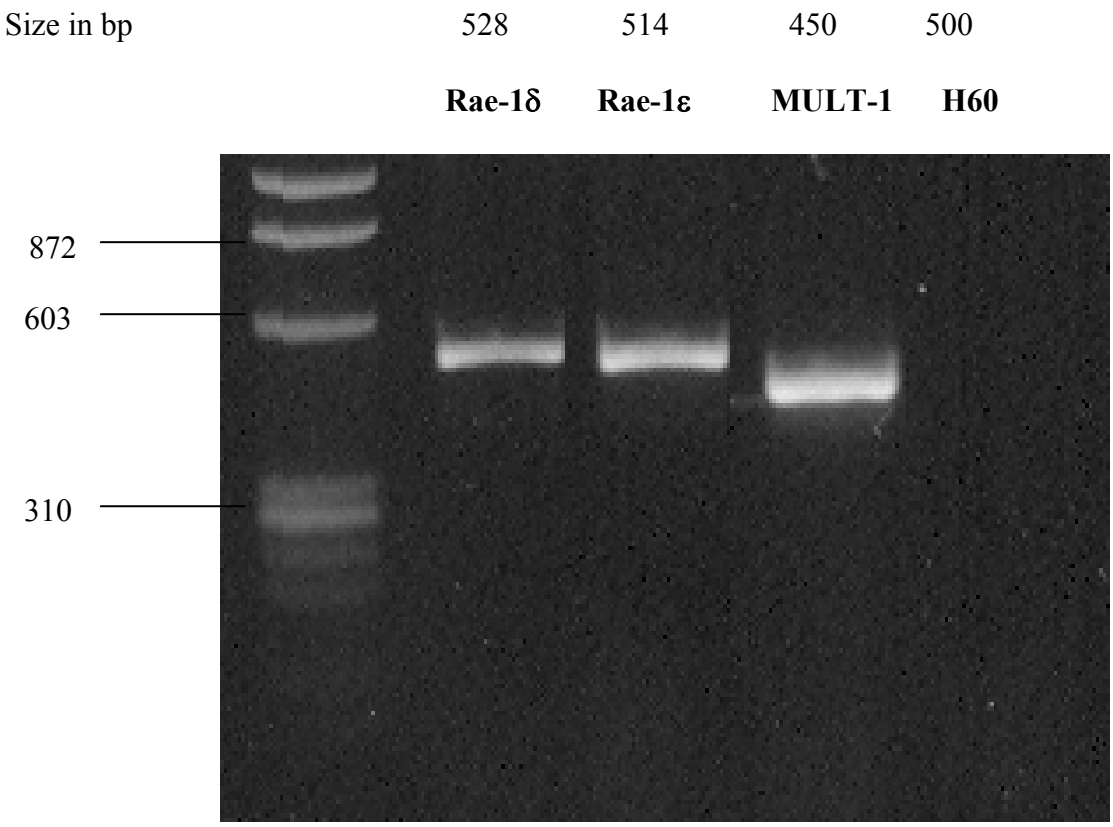


Figure 5.1: NKG2D ligands are present in atherosclerotic lesions of ApoE$^{-/-}$ mice.

RNA from aortic arches of ApoE$^{-/-}$ mice fed a high fat diet for 8 weeks was subjected to RT-PCR to detect RAE-1δ, RAE-1ε, MULT-1 and H60, ligands for NKG2D activating receptor. Expression of RAE-1δ, RAE-1ε and MULT-1 were observed but not H60.

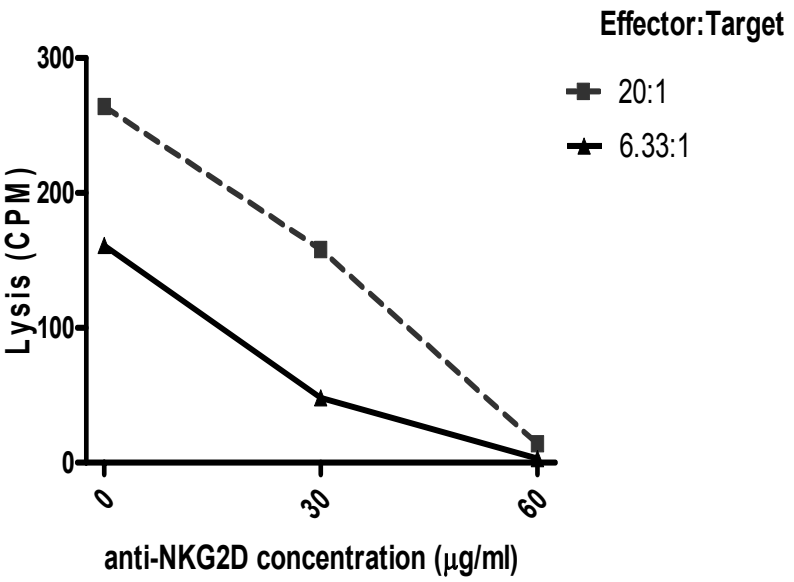


Figure 5.2: Efficacy of Anti-NKG2D neutralizing antibody. Cytotoxic activity of NK cells was tested against RMA-S-Rae-1β tumor cells in the presence of 30μg/ml and 60μg/ml of anti-NKG2D mAb or control hamster IgG by 4h ^{51}Cr release assay at several effector : target ratios (20:1 and 6.33:1 shown). Anti-NKG2D prevented the lysis of tumor cells by NK cells.

5.3.1.3 Anti-NKG2D treatment does not attenuate atherosclerosis

To determine whether the presence of ligands in lesion were functionally significant for NK cell activation during development of atherosclerosis, I treated $ApoE^{-/-}$ mice with an anti-NKG2D neutralizing antibody. Treatment with the neutralizing antibody did not affect development of atherosclerosis suggesting alternate mechanisms of activation of NK cells in atherosclerosis; the Oil Red O stained area of developed lesions in mice treated with the NKG2D neutralizing antibody was similar to controls ($P>0.05$, Figure 5.3A).

Since activated macrophages can express NKG2D ligands Rae-1, and NK cells have been reported to interact with these macrophages through its NKG2D receptors [687], I examined the effects of anti-NKG2D on the recruitment of macrophages into atherosclerotic lesions. The accumulation of CD68 positive macrophages at the aortic sinus were unaffected by the treatment and was similar in both control and antibody treated mice ($P>0.05$, Figure 5.3B).

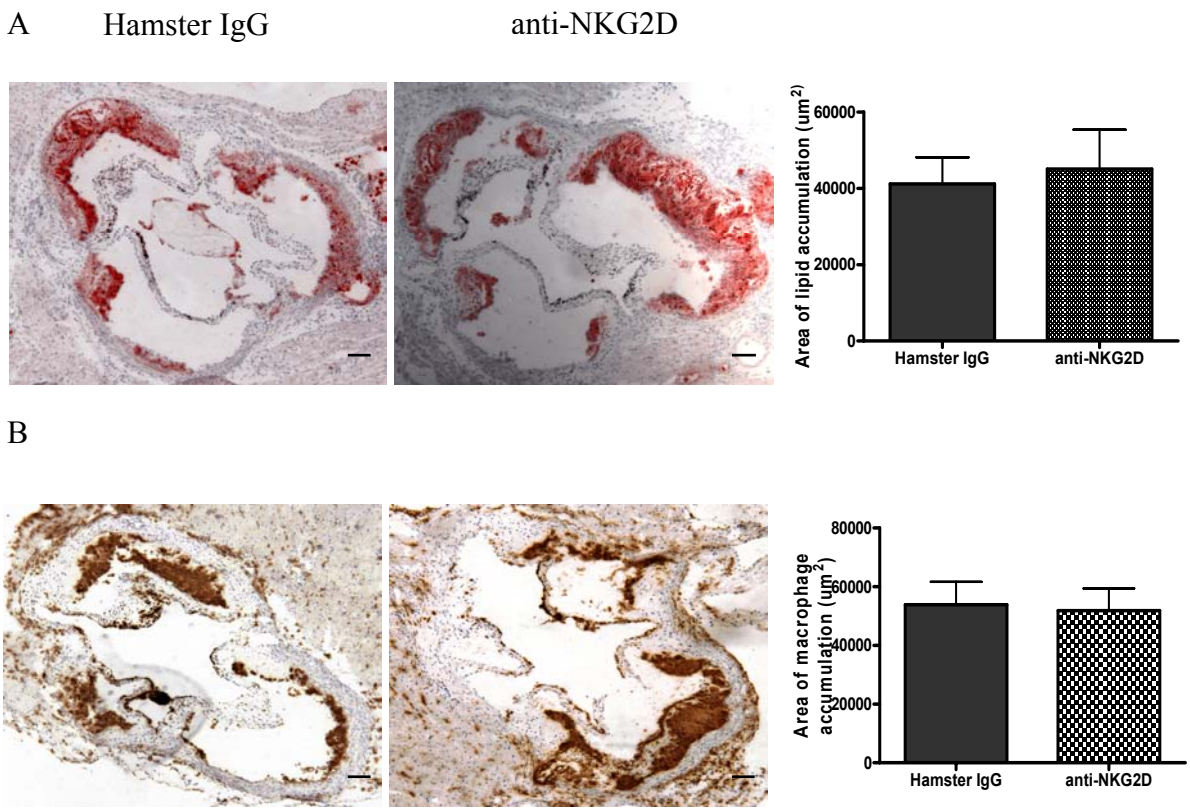


Figure 5.3: Anti-NKG2D administration does not affect development of atherosclerotic lesions in ApoE$^{-/-}$ mice. (A) Photomicrograph together with mean data demonstrating lipid accumulation (Oil-Red O stain, red stain) in the aortic root of control Hamster IgG and anti-NKG2D treated mice. (B) Photomicrograph together with mean data demonstrating macrophage accumulation (CD-68 positive cells, brown stain) in the aortic root of control Hamster IgG and anti-NKG2D treated mice. Bar graph shows SEM ± of at least 13 mice from each group. $P > 0.05$ from Hamster IgG mice. Scale represents 100 μm.

5.3.1.4 Anti-NKG2D treatment does not affect composition of atherosclerotic lesions

Since the treatment with NKG2D neutralizing antibodies did not affect the development of atherosclerotic lesion, I examined the composition of the lesions in ApoE$^{-/-}$ mice treated with anti-NKG2D. The alpha smooth muscle content of the lesion was unaffected by anti-NKG2D antibodies ($P>0.05$, Figure 5.4A).

Since NK cells lyse tumor cells via NKG2D activating receptors and the administration of anti-NKG2D neutralizing antibody should inhibit this effect, I examined for the appearance of necrotic core (accellular, result of apoptosis of cells) in the atherosclerotic lesion in these mice. The necrotic core sizes in atherosclerotic lesions were similar in both anti-NKG2D and hamster IgG treated ApoE$^{-/-}$ mice, this suggest that NK cells promote target cell apoptosis in the development of atherosclerosis, however this is not dependent on their activation by activating receptor NKG2D ($P>0.05$, Figure 5.4B).

5.3.1.5 Anti-NKG2D treatment does not affect plasma lipids or body weight

Since the increase in plasma lipoprotein increases NK cell effector functions mainly its lytic functions [647], the plasma cholesterol, triglyceride, VLDL/LDL and HDL levels were examined. At the end of 8 weeks of high fat diet, the administration of NKG2D neutralizing antibody into ApoE$^{-/-}$ mice did not affect plasma cholesterol, triglyceride, VLDL/LDL and HDL levels. In addition, the body weight of ApoE$^{-/-}$ mice was also unaffected ($P > 0.05$, Table 5.1).

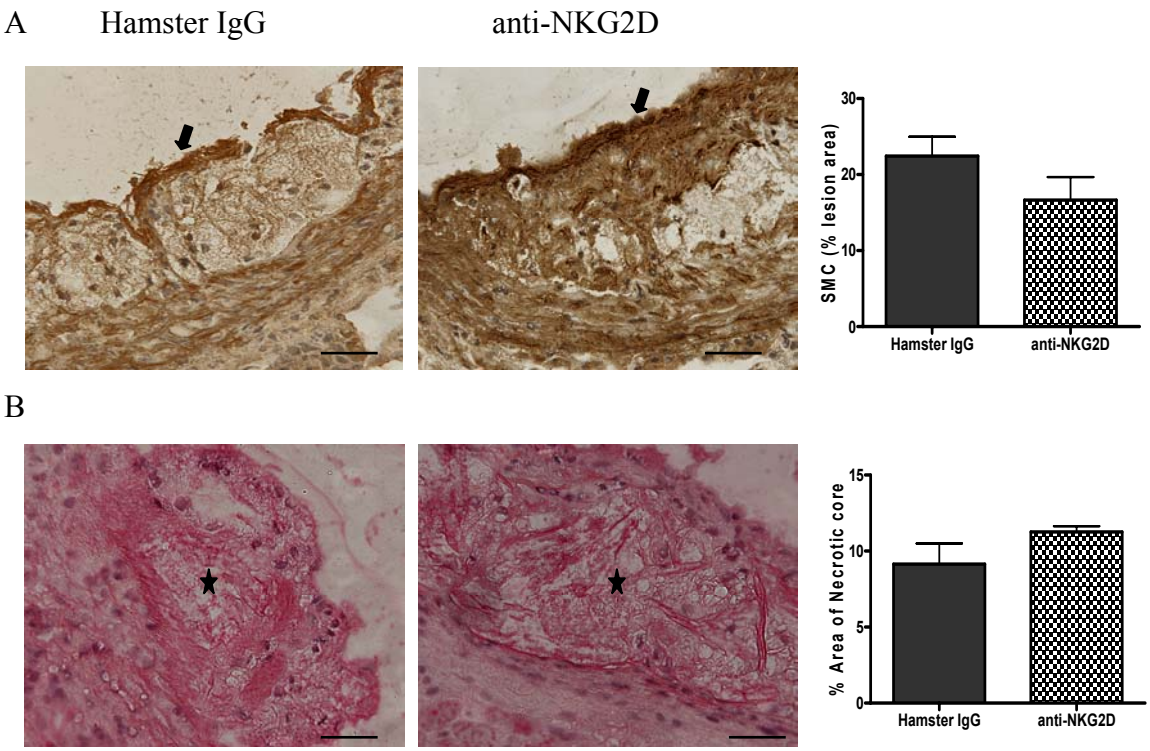


Figure 5.4: Anti-NKG2D administration does not affect atherosclerotic lesion composition in ApoE$^{-/-}$ mice. (A) Photomicrograph together with mean data demonstrating smooth muscle cell content (alpha SM actin positive cells, dark brown stain shown by arrow) in aortic sinus, measured as alpha smooth muscle immunostaining (% area). (B) Photomicrograph together with mean data demonstrating percentage of necrotic core per lesion area (stained with H&E stain, ★ marked) in atherosclerotic lesion. Bar graph represented SEM ± of at least 7 mice from each group. P >0.05 from hamster IgG treated mice. Scale represents 250 μm.

Table 5.1: Body weights and plasma cholesterol levels of ApoE$^{-/-}$ mice treated with anti-NKG2D and fed a high fat diet for 8 weeks.

Group	Weight (g)	Total Cholesterol	Triglyceride	VLDL/LDL	HDL
Hamster IgG	31.0 ± 0.8 (15)	18.7 ± 2.3 (15)	1.5 ± 0.3 (15)	15.6 ± 1.9 (15)	2.4 ± 0.2 (15)
anti-NKG2D	30.1 ± 0.6 (12)	15.4 ± 1.9 (12)	1.1 ± 0.2 (12)	12.9 ± 1.6 (12)	2.0 ± 0.3 (12)

$p > 0.05$ compared to Hamster IgG
Total cholesterol, triglyceride, VLDL/LDL and HDL measurements are in mmol/L.
Number of mice in each group is indicated in parentheses.
Data represent the mean ± SEM.

5.3.2 NK cell activation by NKT cells and Atherosclerosis

5.3.2.1 Anti-asialo GM1 antibody treatment depletes NK cells in ApoE$^{-/-}$Jalpha18$^{-/-}$ mice

Initially I determined the efficacy of anti-asialo GM1 antibody treatment of NKT cell deficient ApoE$^{-/-}$Jalpha18$^{-/-}$ mice in depleting NK cells. Mice were treated with anti-asialo GM1 or NRS and NK cell population in peripheral blood were examined 5 days after the treatment. NK cells were reduced by about 90% in the peripheral blood of ApoE$^{-/-}$Jalpha18$^{-/-}$, consistent with the data obtained from ApoE$^{-/-}$ mice see (see Chapter 3: Results) (Figure 5.5).

5.3.2.2 NK cell depletion in ApoE$^{-/-}$Jalpha18$^{-/-}$ mice does not attenuate atherosclerosis.

NK cells have also been shown to be activated by NKT cells [188, 635]. To determine whether such a mechanism might be operating in atherosclerosis we treated ApoE$^{-/-}$Jalpha18$^{-/-}$ mice with anti-asialo GM1 antibodies. Jalpha18$^{-/-}$ mice do not possess NKT cells but other lymphocyte populations-NK cells and T-lymphocyte populations are normal [615]. ApoE$^{-/-}$Jalpha18$^{-/-}$ mice when fed a high fat diet developed significant atherosclerosis although less than ApoE$^{-/-}$ mice, consistent with a significant role for invariant V alpha 14 NKT cells in the development of atherosclerosis [688]. Treatment of these mice with anti-asialo GM1, did not affect development of atherosclerosis ($P > 0.05$, Figure 5.6 A), with Oil Red O stained area at the aortic root and CD68 (macrophage) stained areas similar to that in control mice ($P > 0.05$, Figure 5.6 B). This lack of effect of anti-asialo GM1 antibodies in the ApoE$^{-/-}$Jalpha18$^{-/-}$ mice markedly contrasts with the effects of anti-asialo GM1 treatment in ApoE$^{-/-}$ mice (Figure 3.3), consistent with a role for NKT cells in activating NK cells during development of atherosclerosis.

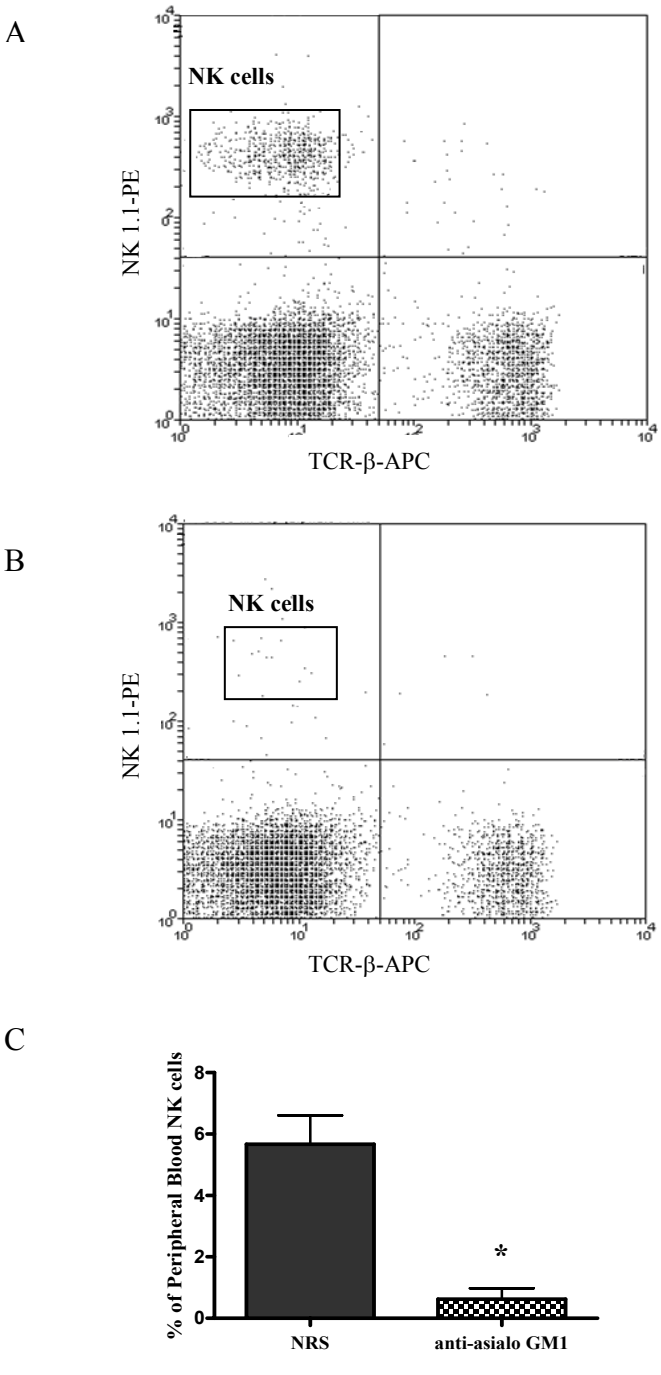


Figure 5.5: NK depletion in peripheral blood of ApoE$^{-/-}$ Jalpha18$^{-/-}$ mice. Dot plot from FACS analysis show NK1.1^{+} TCR-β^{-} (NK) cells in peripheral blood are depleted in ApoE$^{-/-}$ Jalpha18$^{-/-}$ mice 5 days after first dose of (B) anti-asialo GM1 or (A) NRS. (C) Bar graph represent the means ± SEM from 3 mice from each group, $^{*}P < 0.05$ from NRS. Cells were stained for NK1.1 and TCR-β surface markers, NK1.1 positive and TCR-β negative population depicts NK cell population (regions on A and B marked as NK cells).

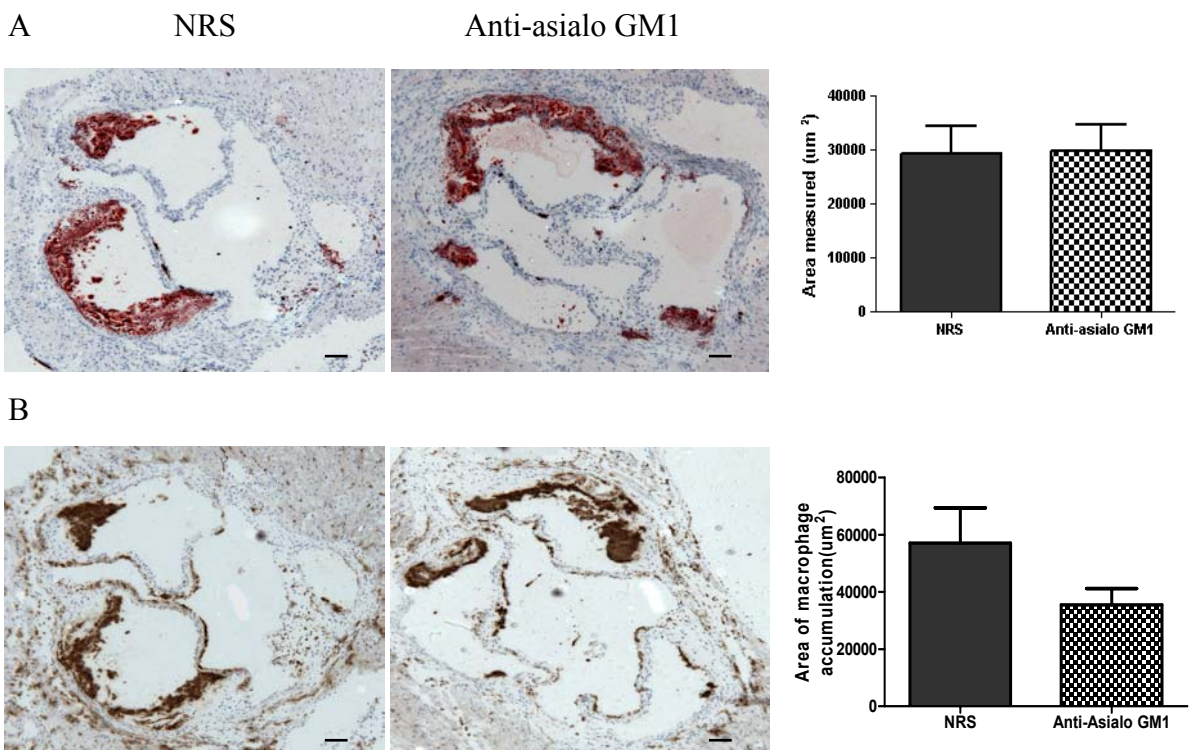


Figure 5.6: Atherosclerotic lesions in ApoE$^{-/-}$ Jalpha18$^{-/-}$ mice treated with anti-asialo GM-1. (A) Photomicrograph together with mean data demonstrating lipid accumulation (Oil-Red O stain, red stain) in the aortic root of control NRS and anti-asialo GM1 treated mice. (B) Photomicrograph together with mean data demonstrating macrophage accumulation (CD-68 positive cells, brown stain) in the aortic root of control NRS and anti-asialo GM1 treated mice. Bar graph represented mean ± SEM of area stained from 10 to 12 mice in a group; P>0.05. Scale bar represents 100 μm.

5.3.2.3 Body weight and plasma cholesterol unaffected by anti-asialo GM1 in ApoE$^{-/-}$ Jalpha18$^{-/-}$ mice.

At the end of 8 weeks of high fat diet, the administration of NK depleting antibody into ApoE$^{-/-}$ Jalpha18$^{-/-}$ mice did not affect plasma cholesterol, triglyceride, VLDL/LDL and HDL levels. In addition, the body weight of ApoE-/-Jalpha18 mice was also unaffected ($P > 0.05$, Table 5.2).

Table 5.2: Body weights and plasma cholesterol levels of ApoE$^{-/-}$Jalpha18$^{-/-}$ mice treated with anti- asialo GM1 and fed a high fat diet for 8 weeks.

Group	Weight (g)	Total Cholesterol	Triglyceride	VLDL/LDL	HDL
NRS	31.4 ± 0.8 (9)	27.4 ± 1.7 (12)	2.4 ± 0.3 (12)	22.1 ± 1.3 (12)	4.2 ± 0.3 (12)
Anti-asialo GM1	33.6 ± 0.9 (10)	27.7 ± 1.5 (11)	3.0 ± 0.3 (11)	21.0 ± 1.2 (11)	4.3 ± 0.4 (11)

$p > 0.05$ compared to NRS
Total cholesterol, triglyceride, VLDL/LDL and HDL measurements are in mmol/L.
Number of mice in each group is indicated in parentheses.
Data represent the mean ± SEM.

5.4 Discussion

In the present study, I have shown that the pro-atherogenic effects of NK cells are dependent on the presence of NKT cells and activation of NKG2D receptors on NK cells is not required for the proatherogenic effect.

NK cells can be activated by a number of mechanisms including via NKT cells and NKG2D activating receptors. NKT cell have been shown to rapidly activate NK cells to secrete IFN-γ, become more cytotoxic and proliferate [188]. This mechanism have also been shown to be involved in anti-tumor responses that are dependent on the production of IFN-γ by NKT and NK cells [566, 684]. NKT cells have also been implicated in augmenting the development of atherosclerosis. Therefore, to determine whether such a mechanism is operative in atherosclerosis I treated invariant NKT cell deficient ApoE$^{-/-}$ Jalpha18$^{-/-}$ mice with NK cell depleting antibody. In the present study ApoE$^{-/-}$ Jalpha18$^{-/-}$ mice were used to asses the importance of NKT cells for the proatherogenic effects of NK cells. Since lesions that develop in these mice are relatively small compared to ApoE$^{-/-}$ mice, about half the size, it is possible that additional mechanisms often than NKT cells might contribute to NK cell activation [689] which could not be detected using ApoE$^{-/-}$ Jalpha18$^{-/-}$ mice. Consequently, activated NKT cells using α-galactosylceramide in ApoE$^{-/-}$ mice which were depleted of NK cells by treating with anti-asialo GM1, unfortunately die prematurely and whilst purification of the anti-asialo GM1 reduced the incidence of death no relative results were obtained (data not shown).

In addition to NK cell activation by NKT cells, NK cells can be also activated by other mechanisms. NKG2D receptors can activate NK cells to produce cytokines and cytotoxicity which could contribute to the proatherogenic effect. Activating ligands for these receptors

include Retinoic acid early inducible-1(Rae-1) and murine UL-16-binding protein-like transcript 1 (MULT-1). Despite the expression of activating ligands in atherosclerotic lesions, NKG2D receptor activation was not apparent on NK cells during development of atherosclerosis, indicated by the lack of attenuation of atherosclerosis by anti-NKG2D neutralizing antibodies. The reason is to why NK cells were not activated in this manner despite the presence of activating ligands was not investigated. However, it is likely that inhibitory Ly49 receptors are involved. NKG2D receptor-mediated NK cell function is regulated by inhibitory Ly49 receptors [690]. Ly49 receptors are highly expressed on NK cells [441] and their MHC class I ligands are highly expressed on atherosclerotic lesions [304]. Thus it is possible that these inhibitory receptors prevent ligands induced NKG2D receptor activation in atherosclerotic lesions. It is also possible that NKG2D ligands are downregulated on atherosclerotic lesions. IFN-γ can down-regulate NKG2D ligand expression and impair NKG2D-mediated cytolysis of melanoma by NK cells [691]. Since IFN-γ is abundant in atherosclerotic lesion [692], it possible that these NKG2D ligands are downregulated in atherosclerotic lesions preventing NKG2D-mediated NK cell activation. Additionally it is also possible that NKG2D receptor expression on NK cells is downregulated by IL-12 [693]. IL-12 have been detected in atherosclerotic lesions and promote the development of atherosclerosis [164, 694], hence IL-12 may be involved in reducing the NKG2D receptor expression on NK cells thus preventing NKG2D mediated NK cell activity.

In summary, my findings suggest that pro-atherogenic effects of NK cells are dependent on presence of NKT cells. However, NK cells could also be activated independently of NKT cells through several other mechanisms including by dendritic cells and cytokines. The role of these mechanisms on NK cell pro-atherogenic activity needs to be elucidated.

6 Proatherogenic effects of NK cells are dependent on cytotoxicity not cytokines.

6.1 Introduction

Previously I demonstrated that NK cell pro-atherogenic activity was dependent on their activation by NKT cells. However, it is not known whether NK cells exert their pro-atherogenic effects by secreting cytokines or cytotoxic molecules. Activated NK cells produce and secrete cytokines as well as cytotoxins.

IFN-γ is a major cytokine secreted by NK cells. Whilst the role of NK cells derived IFN-γ has yet to be determined, IFN-γ is expressed in human and mouse atherosclerotic lesions. Deletion of IFN-γ receptor in $ApoE^{-/-}$ mice attenuates atherosclerosis [158]. IFN-γ stimulates expression of VCAM-1 on endothelial cells [695], MHC class II on macrophages and vascular smooth muscle cells, and lipoprotein receptors on vascular smooth muscle cells, all potentially proatherogenic effects [150, 642, 696]. It also decreases collagen synthesis by SMCs [697] and proliferation [698, 699], effects that lead to development of unstable atherosclerotic lesions . Deletion of IFN-γ receptor in $ApoE^{-/-}$ mice alters somewhat the plasma cholesterol lipoprotein profile [158]. Exogenous IFN-γ increases development of atherosclerotic lesions, simultaneously increasing $CD4^{+}$ T cells and MHC class II- presenting cells whilst slightly reducing plasma cholesterol levels [190].

NK cells could theoretically also augment atherosclerosis by killing cells. Fas ligand (FasL)-mediated cytotoxicity is exhibited in NK cells through ligation of their activating

receptors (Fas); expressing of Fas ligands accelerates atherosclerotic lesion formation [700]. Tumor necrosis factor (TNF)- related apoptosis-inducing ligand (TRAIL) is predominantly expressed by immature NK cells and its role in atherosclerosis is unclear, these cells lose expression of TRAIL upon maturation [701]. Perforin and granzyme B are also secreted by activated NK cells. Perforin and granzyme B have been detected in atherosclerotic lesions [575, 702]. It is likely that when these agents are released, they enter target cell by endocytosis, and perforin-facilitated entry of granzyme B into the nucleus of the target cells to initiate DNA fragmentation and apoptosis [703] of vascular cells in atherosclerotic lesions. However, the significance of NK cell mediated cytotoxicity through perforin and granzyme B in atherosclerosis have not been elucidated.

Apoptosis induced by NK cells mediated by cytolytic molecules such as granzyme B and perforin can lead to post apoptotic necrosis and enlarged necrotic lesion development as phagocytosis of apoptotic cells by macrophages is impaired in atherosclerosis [704]. Furthermore necrotic cells can activate the NRLP3 inflammasomes [705] leading to activation of proatherogenic cytokines IL-1 β and IL-18 [162, 191].

In the present study, I investigated the importance of NK cells derived IFN-γ, perforin and granzymeB for the development of atherosclerosis. These studies involved adoptive transfer of IL-2 stimulated NK cells from IFN-γ, perforin or granzyme B knockout mice into $ApoE^{-/-}$ $Rag2^{-/-}\gamma c^{-/-}$ that are deficient in T, B, NKT and NK cells.

6.2 Methods

6.2.1 Mice

ApoE$^{-/-}$Rag2$^{-/-}$γc$^{-/-}$ (C57BL/6 background) mice were used as recipient mice for the studies. These mice are deficient in T, B, NKT and NK cells and obtained from Monash Medical Centre, details in Chapter 2 Materials and Methods, page 94. IFN-γ$^{-/-}$, granzyme B (GrzB)$^{-/-}$, perforin (pfp)$^{-/-}$ mice (all on C57Bl/6 background) were used as donor mice for isolation of splenic NK cells. C57BL/6 mice (WT) were also used as control donor mice.

6.2.2 Isolation of NK cells and adoptive transfer

ApoE$^{-/-}$Rag2$^{-/-}$γc$^{-/-}$ mice received splenic NK cells isolated from C57BL/6, IFN-γ$^{-/-}$, GrzB$^{-/-}$, pfp$^{-/-}$ mice. NK cells were isolated by magnetic separation of non-NK cells using automacs separator. This method retains antibody labeled non-NK cells, and enriched, untouched NK cells were isolated. To obtain sufficient NK cells for adoptive transfer, isolated NK cells were expanded in cell culture using recombinant human Interleukin-2 (rhIL-2) for 5 days. The cultured NK cells were purified by BD FACSAria sorter based on staining for PE-conjugated NK1.1^{+} and APC-conjugated TCR-β^{-}. Details for the isolation of NK cells are described in Chapter 2 Materials and Methods, pages 110-113. In all cases, 3 million NK cells were adoptively transferred into 6-8 weeks old ApoE$^{-/-}$Rag2$^{-/-}$γc$^{-/-}$ mice and the mice were commenced on a high fat diet (HFD) for 8 weeks. Injections of NK cells were repeated 4 weeks later whilst mice on the HFD. Control mice were administered sterile 1X PBS.

6.2.3 Tissue collection

At the end of the study, mice were administered an overdose of pentobarbitone and body weight was measured. Plasma was collected for cholesterol and triglyceride measurements, aortic sinus was dissected and frozen in OCT for subsequent sectioning and histological and immunohistochemical analyses. Aortic arches were cleaned of fat and snap frozen in liquid nitrogen for mRNA extraction. All experiments were approved by the AMREP Animal Ethics Committee. All methods are described in detail in Chapter 2: Materials and Methods; page 95.

6.2.4 Analysis of gene expression by real-time PCR

Expression of pro-IL-1β and caspase-1 (ICE: IL-1β converting enzyme) in atherosclerotic arches was determined by real-time PCR. Primer designing, RNA extraction and real-time PCR methods are described in Chapter 2 Materials and Methods, pages 114-120.

6.2.5 Flow cytometry

Cells were stained with PE-conjugated NK1.1, and APC-conjugated anti-TCR-β antibodies to detect NK cells. Data was acquired using FACSCalibur as described in Chapter 2 Materials and Methods, page 102.

6.2.6 Tissue Sectioning

The frozen aortic sinus were sectioned at 6 μm in thickness and collected on microscope slides for subsequent histological and immunohistochemical studies (Refer to Chapter 2: Materials and Methods; pages 96).

6.2.7 Immunohistochemistry and histology

Aortic sinus were stained and analyzed for Oil red O, macrophages and VCAM-1 as described in Chapter 2 Materials and Methods, pages 97 to 102. Sections were also assessed for necrotic core size, defined as accellular area within atherosclerotic lesions, using H&E stain.

6.2.8 Plasma cholesterol analysis

Plasma samples were sent to Southern Cross Pathology Australia, (Clayton, Australia) for measurements of total plasma cholesterol, HDL-cholesterol, VLDL/LDL cholesterol and triglyceride levels using Beckman Coulter reagents as described in Chapter 2 Materials and Methods, page 96.

6.2.9 Statistical analyses

Statistical analyses were performed using Student's t-test, or one way ANOVA using the software GraphPad Prism v5.02. P-value <0.05 were considered statistically significant.

6.3 Results

6.3.1 Purity of cultured NK cells

Purity of the cells was assessed by FACS. Negative selection by automacs separator yield purity of 26% of NK cells and 5 days after culture the purity had increased to 81% (Figure 6.1A and B). Adoptive transfer of these cells indicated that this purity was insufficient for studies on atherosclerosis. FACS profiling of liver lymphocytes in $ApoE^{-/-}Rag2^{-/-}\gamma c^{-/-}$ indicated, expansion of additional lymphocyte population particularly T cells compared to PBS treated mice (Figure 6.1C and 6.1D). Thus although this approach has been used to study the anti-tumor function of adoptively transferred NK cells in vivo [591], this purity is insufficient for studies on atherosclerosis probably because of the long duration of the experiments. Further purification of the rhIL-2 cultured NK cell population by BD FACSAria sorting provided the requested purity. The sorted cells were 99.9% pure of NK cells ($NK1.1^{+}$ TCR-β^{-}) and there were no other lymphocytes expansion in liver of $ApoE^{-/-}Rag2^{-/-}\gamma c^{-/-}$ after adoptive transfer of these sorted NK cells compared to PBS treated control mice (Figure 6.2).

6.3.2 Upregulation of CD69 activation marker on cultured NK cells

Because NKT cells are required to activate NK cells during development of atherosclerosis (see Chapter 5) and they are absent in $ApoE^{-/-}Rag2^{-/-}\gamma c^{-/-}$ recipient mice, NK cells were activated whilst in cell culture by co-culture with rhIL-2. FACS analysis of the cultured IL-2 stimulated NK cells indicated high expression of CD69, an activation marker (Figure 6.3).

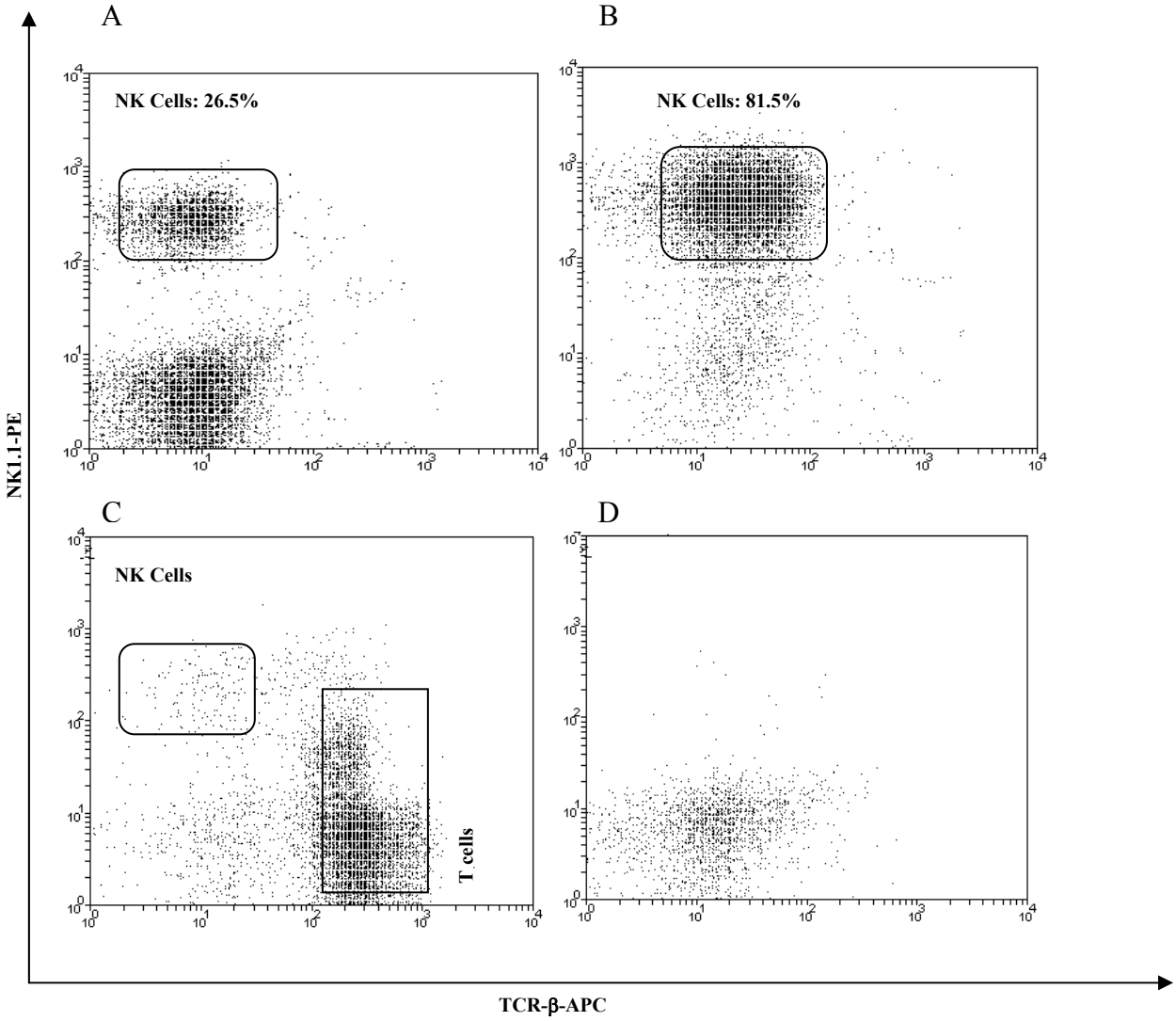


Figure 6.1: NK cell purity before and after culture, and after adoptive transfer.

(A) FACS dot plot shows NK cells ($NK1.1^{+}$-PE conjugated, TCR-β^{-}-APC-conjugated) before culture and (B) after culture with rhIL-2. (C) FACS dot plot of T and NK cells in the liver of $ApoE^{-/-}Rag2^{-/-}\gamma c^{-/-}$ recipient mice 8 weeks after adoptive transfer of NK cells of 81.5% purity showing a large increase in T cells as well as NK cells. (D) FACS dot plot of PBS treated control $ApoE^{-/-}Rag2^{-/-}\gamma c^{-/-}$ mice with no NK or T cells.

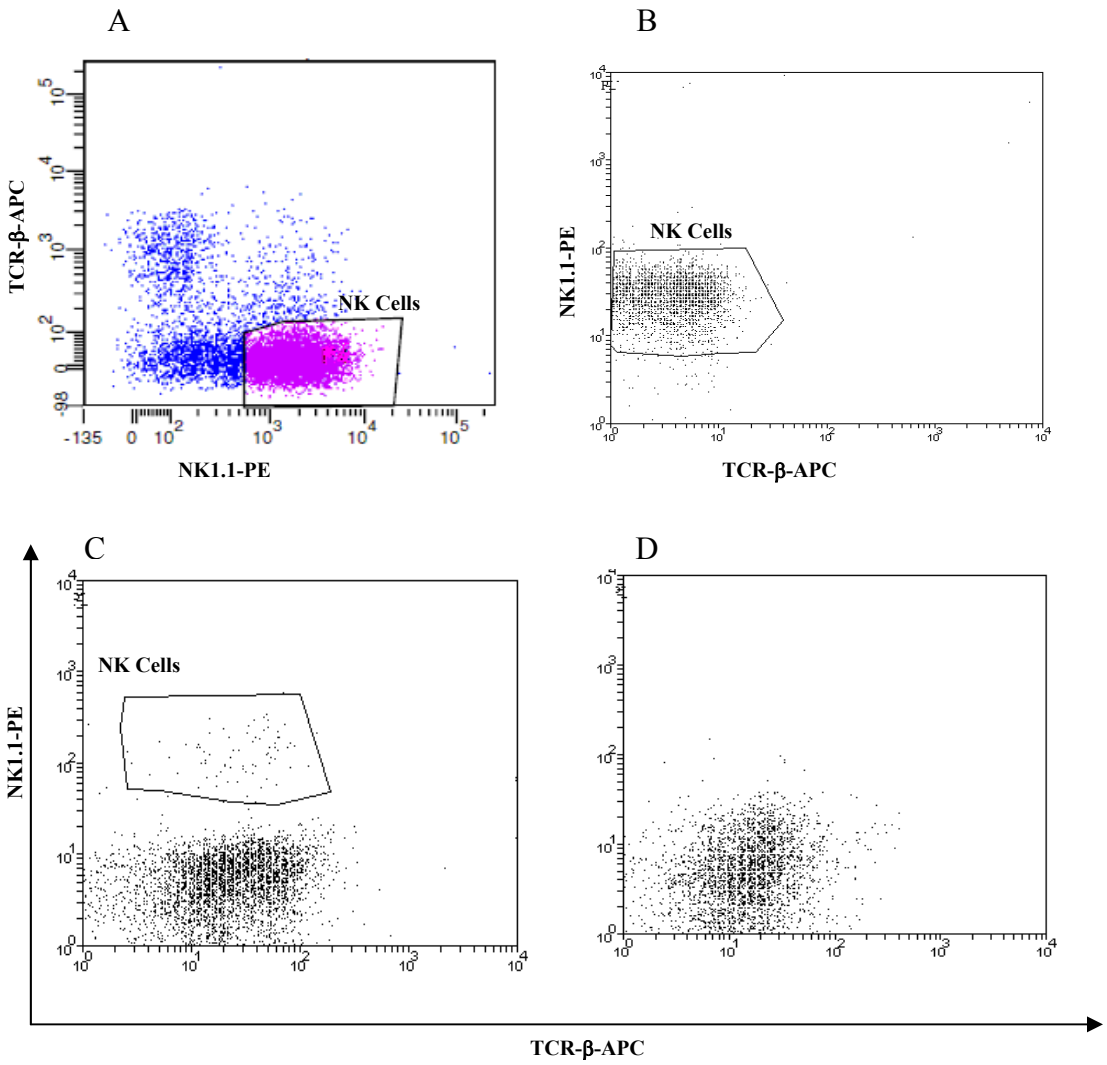


Figure 6.2: NK cell purity before and after NK cell sorting. (A) FACS dot plot shows NK cells ($NK1.1^+$ cells conjugated with PE fluorochrome conjugate) culture sample before sorting on the BD FACSAria sorting machine. (B) NK cell analysis on the BD FACS calibur after sorting, with 99.9% purity, NK cells depicted by $NK1.1^+TCR^-$ cells. (C) FACS dot plot of NK cells in the liver of $ApoE^{-/-}Rag2^{-/-}\gamma c^{-/-}$ recipient mice 8 weeks after adoptive transfer of NK cells of 99.9% purity showing an increase in NK cells only. (D) FACS dot plot of PBS treated $ApoE^{-/-}Rag2^{-/-}\gamma c^{-/-}$ mice with no NK cells

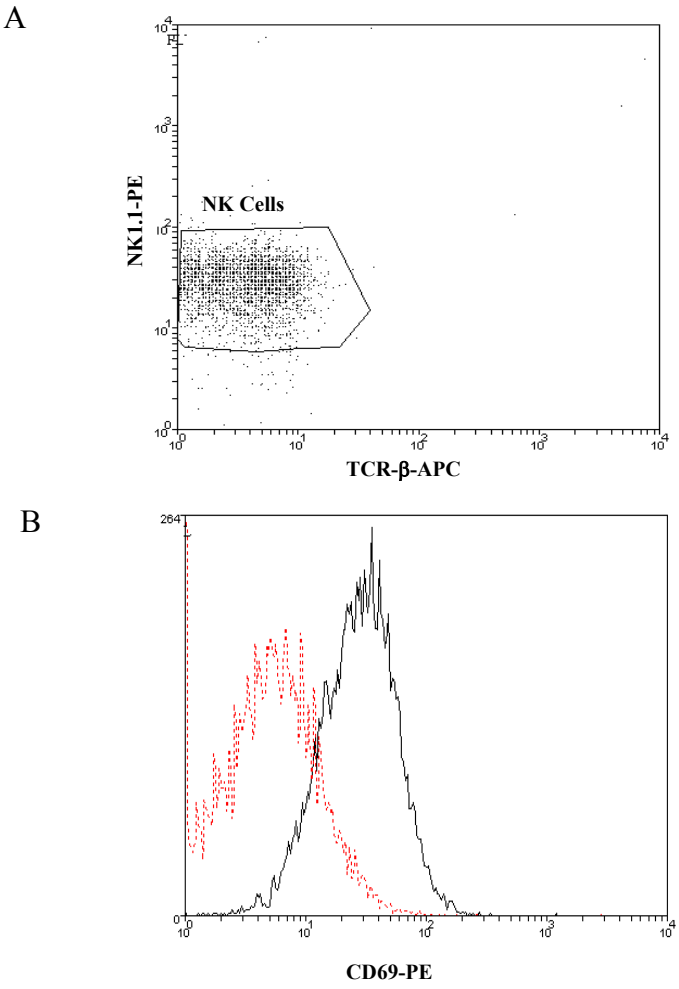


Figure 6.3: CD69 upregulation on cultured FACS purified NK cells. (A) FACS dot plot shows highly pure NK cells after culture and sort. (B) Histograms show the high expression of CD69, a marker of NK cell activation on rhIL-2 cultured and sorted NK cells (black histogram) compared to unstimulated NK cells (red, dotted histogram).

6.3.3 NK cell reconstitution in ApoE$^{-/-}$Rag2$^{-/-}$γc$^{-/-}$ mice.

To determine the fate of adoptively transferred NK cells, I analyzed the NK cell population in ApoE$^{-/-}$Rag2$^{-/-}$γc$^{-/-}$ transferred with NK cells and PBS. Four weeks after the second adoptive transfer of 3 million of sorted NK cells, there were approximately 2.6x10^5 NK cells present in the liver of ApoE$^{-/-}$Rag2$^{-/-}$γc$^{-/-}$mice (Figure 6.4). However the total number of NK cells in the liver did not reconstitute to normal levels of NK cells in ApoE$^{-/-}$ mice which is about 4.0x10^5 cells in the liver.

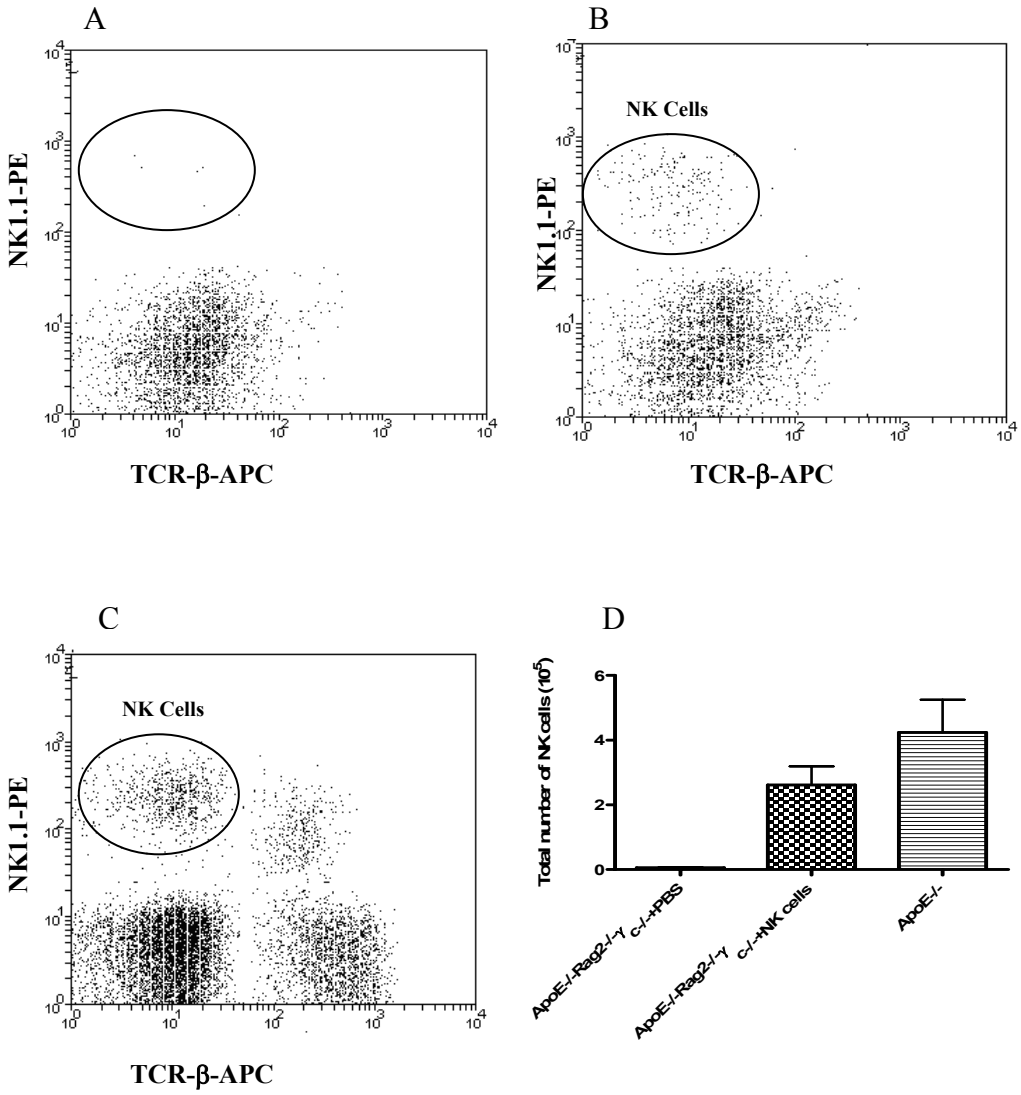


Figure 6.4: NK cells in liver of ApoE$^{-/-}$ Rag2$^{-/-}$ γc$^{-/-}$ mice four weeks after adoptive transfer. (A) FACS dot plot analysis of liver NK cells show that NK cell were absent in PBS treated ApoE$^{-/-}$ Rag2$^{-/-}$γc$^{-/-}$ (circled region), (B) approximately 2.6x10^5 NK cells were detected in the liver of recipient ApoE$^{-/-}$Rag2$^{-/-}$γc$^{-/-}$ mice compared to (C) 4.0x10^5 NK cells in normal ApoE$^{-/-}$ mice. (D) Bar graphs represented the mean ± SEM of number of NK cells in liver of 4-6 mice in a group.

6.3.4 WT NK cells and IFN-γ deficient NK cells increase atherosclerotic lesion size upon adoptive transfer to ApoE$^{-/-}$Rag2$^{-/-}$γc$^{-/-}$ mice.

After 8 weeks on a high fat diet, as expected adoptive transfer of WT NK cells augmented atherosclerosis (16230 ± 1631 μm^2 in control mice administered vehicle vs 31430 ± 3015 in mice transferred with C57BL/6 WT NK by 48% ; $p < 0.05$, Figure 6.5 A). Similarly macrophage accumulation in lesions also increased by about 52% in WT NK cells transferred group compared to control mice administered vehicle ($^*p < 0.05$, Figure 6.5 B).

Since IFN-γ is known to augment atherosclerosis [189] and activated NK cells secrete IFN-γ, I also investigated whether NK cell derived IFN-γ was required for the proatherogenic effects of NK cells. Similar to WT NK cell adoptive transfer, NK cells from IFN-γ$^{-/-}$ mice also augmented lesion size (16230 ± 1631 μm^2 in control mice administered vehicle vs 28560 ± 3524 in mice transferred with IFN-γ$^{-/-}$ NK cells; $^*p < 0.05$, Figure 6.6 A) by about 43 %. Macrophage accumulation in lesions was also tended to increase but was not significant compared to control mice ($p > 0.05$, Figure 6.6 B)

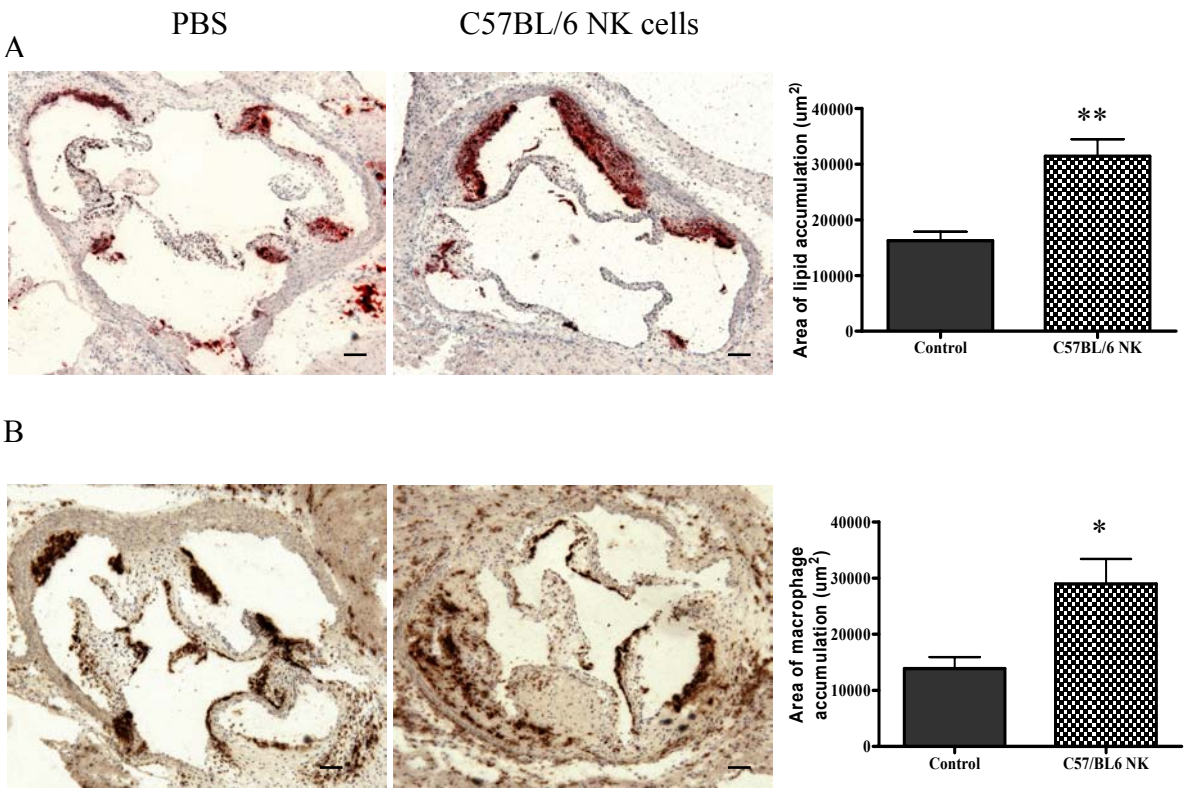


Figure 6.5: NK cells promote the development of atherosclerosis. ApoE$^{-/-}$Rag2$^{-/-}$γc$^{-/-}$ mice were injected with PBS (control, left), or C57BL/6 WT NK cells (right) and fed a high fat diet for 8 weeks. Aortic root cross-sections were stained with (A) Oil-red-O (red stain) to detect lipids, (B) anti-CD68 mAb (brown stain) to detect macrophages. Bar graphs represented the mean ± SEM of area stained from 5-6 mice in a group, $^{*}P < 0.05$; $^{**}P < 0.01$. Scale represents 100 μm.

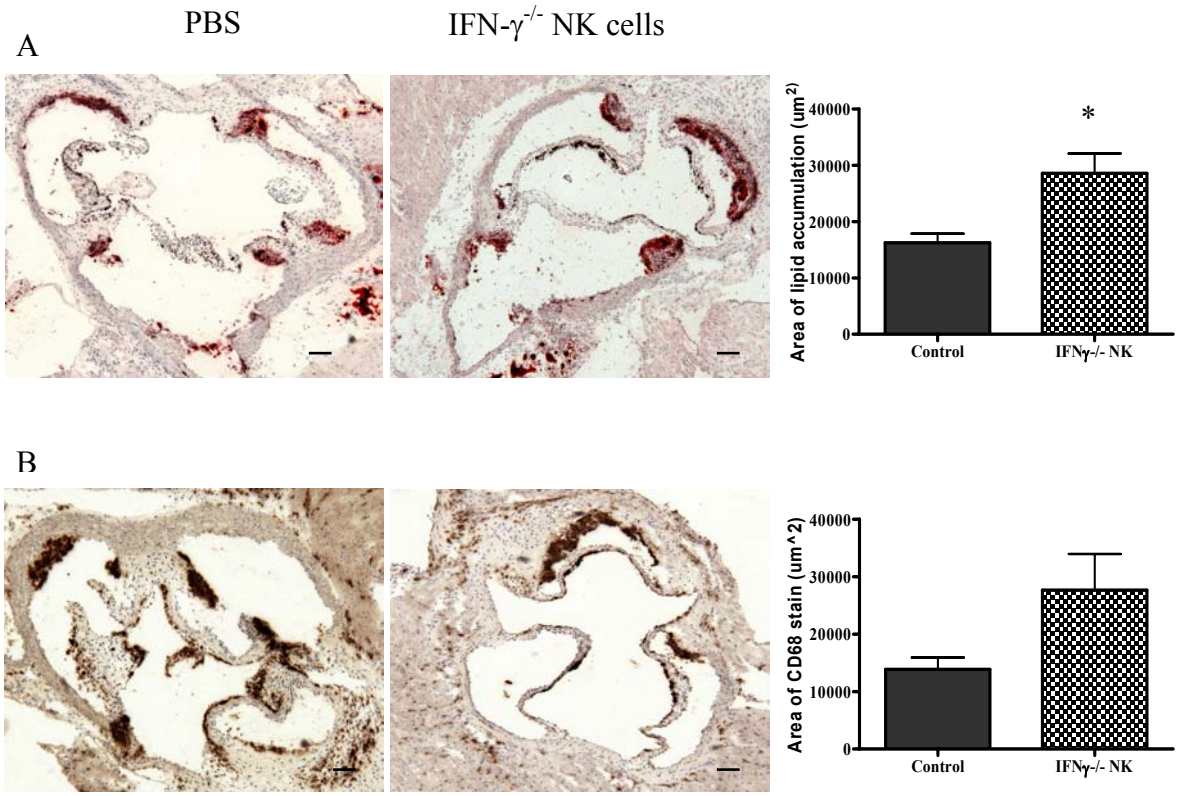


Figure 6.6: NK cell-derived IFN-γ is not required for proatherogenic activity. ApoE$^{-/-}$Rag2$^{-/-}$γc$^{-/-}$ mice were injected with PBS (control, left), or IFN-γ$^{-/-}$ NK cells (right) and fed a high fat diet for 8 weeks. Aortic root cross-sections were stained with (A) Oil-red-O (red stain) to detect lipids, (B) anti-CD68 mAb (brown stain) to detect macrophages. Bar graphs represented the mean ± SEM of area stained from 5-6 mice in a group, $^{*}P < 0.05$. Scale bar represents 100 μm.

6.3.5 Proatherogenic effects of adoptively transferred NK cells are dependent on granzyme B.

NK cells produce and secrete granzyme B, which has been implicated in cell apoptosis and inflammation [706], In atherosclerosis, granzyme B have been implicated in vascular SMC apoptosis [575]. To examine the significance of NK cell-derived granzyme B on the ability of NK cells to promote atherosclerosis, splenic NK cells were isolated from granzyme B-deficient mice, and adoptively transferred to $ApoE^{-/-}Rag2^{-/-}\gamma c^{-/-}$ mice. Granzyme B deficient NK cells did not increase lesion size in $ApoE^{-/-}Rag2^{-/-}\gamma c^{-/-}$ mice. The lesions of mice that received granzyme B-deficient NK cells had similar lipid and macrophages content to $ApoE^{-/-}Rag2^{-/-}\gamma c^{-/-}$ control mice treated with sterile PBS (Figure 6.7, $p > 0.05$).

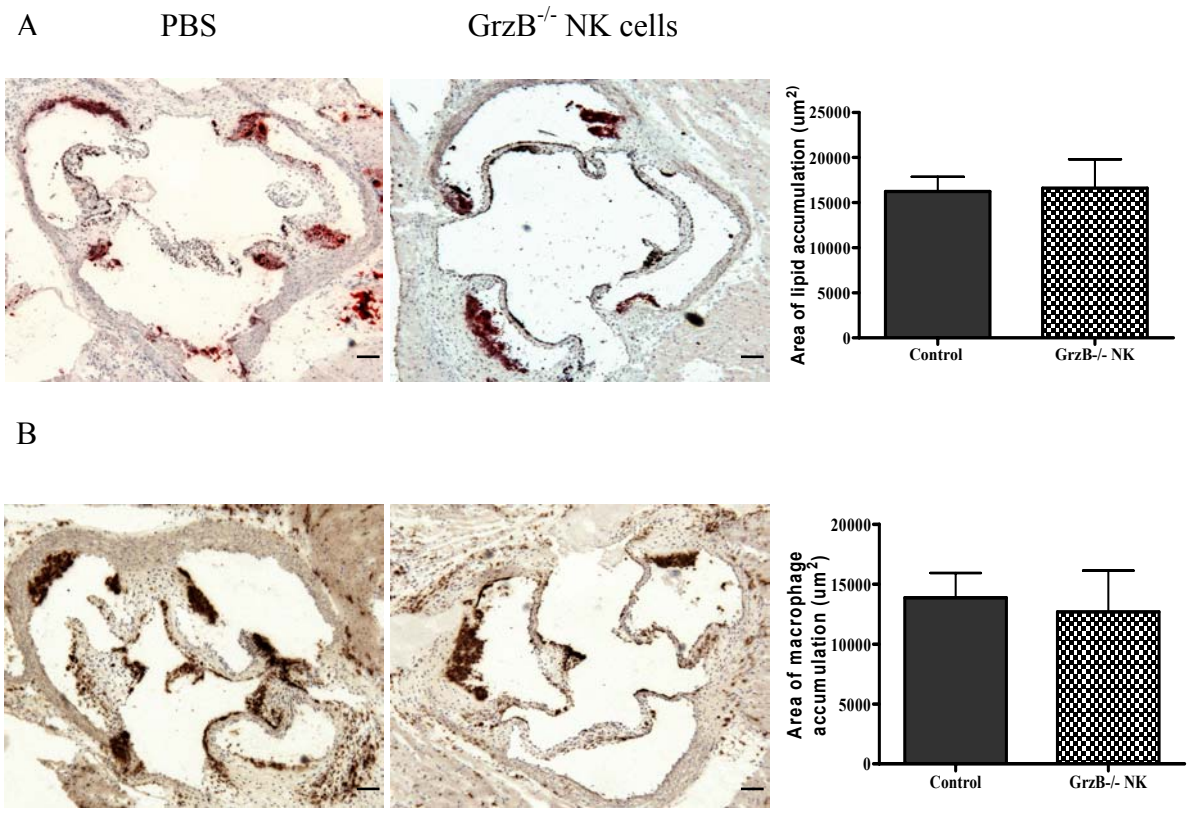


Figure 6.7: NK cell deficient in granzyme B (grzB) did not promote atherosclerosis. ApoE$^{-/-}$ Rag2$^{-/-}$γc$^{-/-}$ were injected with PBS (control, left), or grazyme B$^{-/-}$ NK cells (right) and fed a high fat diet for 8 weeks. Aortic root cross-sections were stained with (A) Oil-red-O (red stain) to detect lipids, (B) anti-CD68 mAb (brown stain) to detect macrophages. Bar graphs represented the mean ± SEM of area stained from 5-6 mice in a group, $P > 0.05$. Scale bar represents 100 μm.

6.3.6 Proatherogenic effect of adoptive transferred NK cells is dependent on perforin.

To examine the mechanism by which NK cell-derived granzyme B promotes the development of atherosclerosis, I next examined whether perforin was also required. Perforin facilitates the entry of granzyme B into the cell nucleus for induction of apoptosis [703]. Adoptive transfer of perforin-deficient NK cells to ApoE$^{-/-}$Rag2$^{-/-}$γc$^{-/-}$ and feeding a high fat diet for 8 weeks did not augment atherosclerosis. The lesion size assessed by Oil Red O stain was similar in both perforin-deficient NK cell treated mice and vehicle treated ApoE$^{-/-}$Rag2$^{-/-}$γc$^{-/-}$ mice (Figure 6.8A; $p > 0.05$). Similarly macrophage accumulation at the aortic sinus was also unaltered in mice transferred with perforin-deficient NK cells compared to control ApoE$^{-/-}$Rag2$^{-/-}$γc$^{-/-}$ mice (Figure 6.8 B; $p > 0.05$).

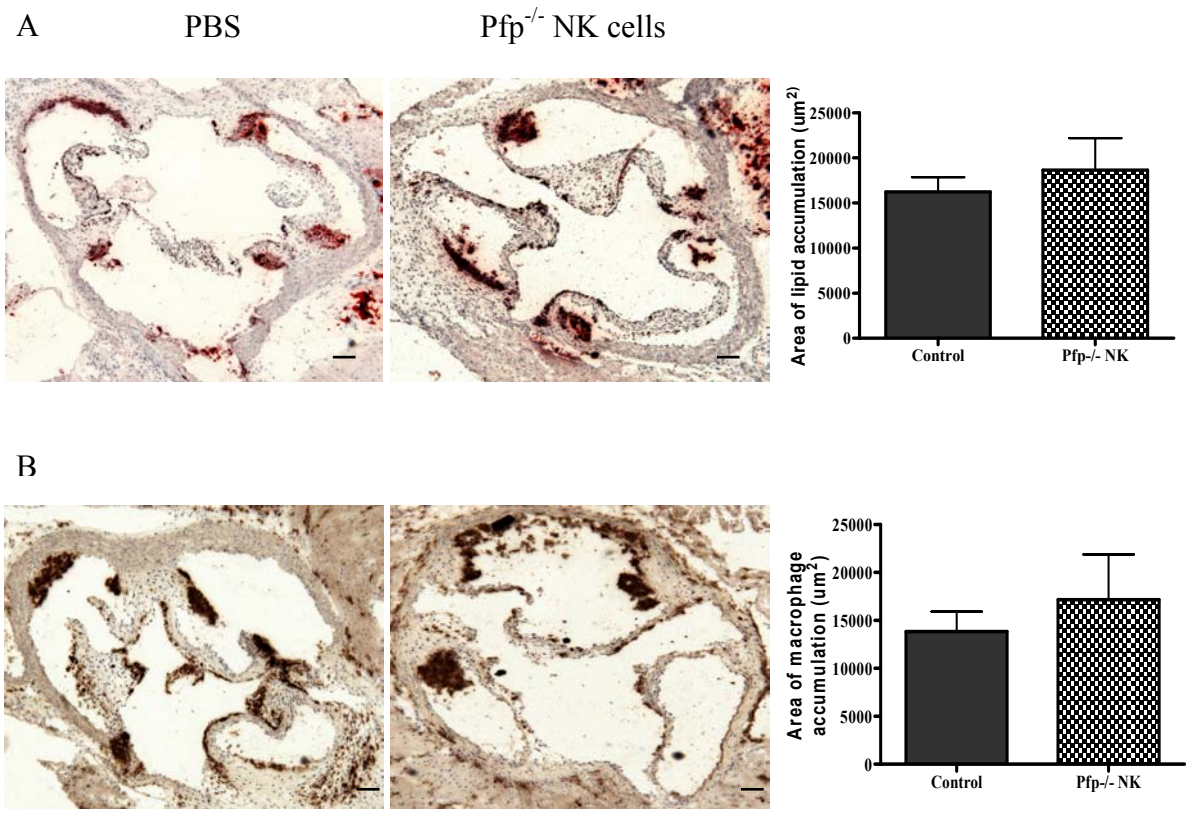


Figure 6.8: NK cell deficient in perforin (pfp) did not promote atherosclerosis. ApoE$^{-/-}$Rag2$^{-/-}$ γc$^{-/-}$ were injected with PBS (control, left), or perforin$^{-/-}$ NK cells (right) and fed a high fat diet for 8 weeks. Aortic root cross-sections were stained with (A) Oil-red-O (red stain) to detect lipids, (B) anti-CD68 mAb (brown stain) to detect macrophages. Bar graphs represented the mean ± SEM of area stained from 5-6 mice in a group, $P > 0.05$. Scale bar represents 100 μm.

6.3.7 NK cell-derived perforin and granzyme contributes to necrotic core development in atherosclerotic lesion.

NK cell-derived granzyme B and perforin are required by NK cells to mediate their proatherogenic effects, possibly through the killing of cells involved in atherosclerosis. To examine this possibility, I determined whether the size of necrotic cores associated with atherosclerotic lesions was decreased by adoptive transfer of perforin and granzyme B deficient NK cell. Necrotic core results from the loss of cells in lesion due to apoptosis and is associated with lesion instability and inflammation [707] and the defective clearance of apoptotic macrophages ,leading to secondary necrosis [238]. Adoptive transfer of perforin-deficient NK cells resulted in a decrease in necrotic core associated with aortic sinus lesions compared to mice receiving WT NK cells that express perforin. The percentage of lesion area occupied by necrotic core reduced by about 60% in mice receiving perforin-deficient NK cells compared to mice receiving WT NK cells (Figure 6.9, $P<0.05$).

Similarly adoptive transfer of granzyme B-deficient NK cells also resulted in reduced necrotic cores associated with aortic sinus lesions compared to mice receiving WT NK cells. In these mice, percentage of lesion area occupied by necrotic cores was reduced by about 70% compared to mice transferred WT NK cells (Figure 6.9, $P<0.05$).

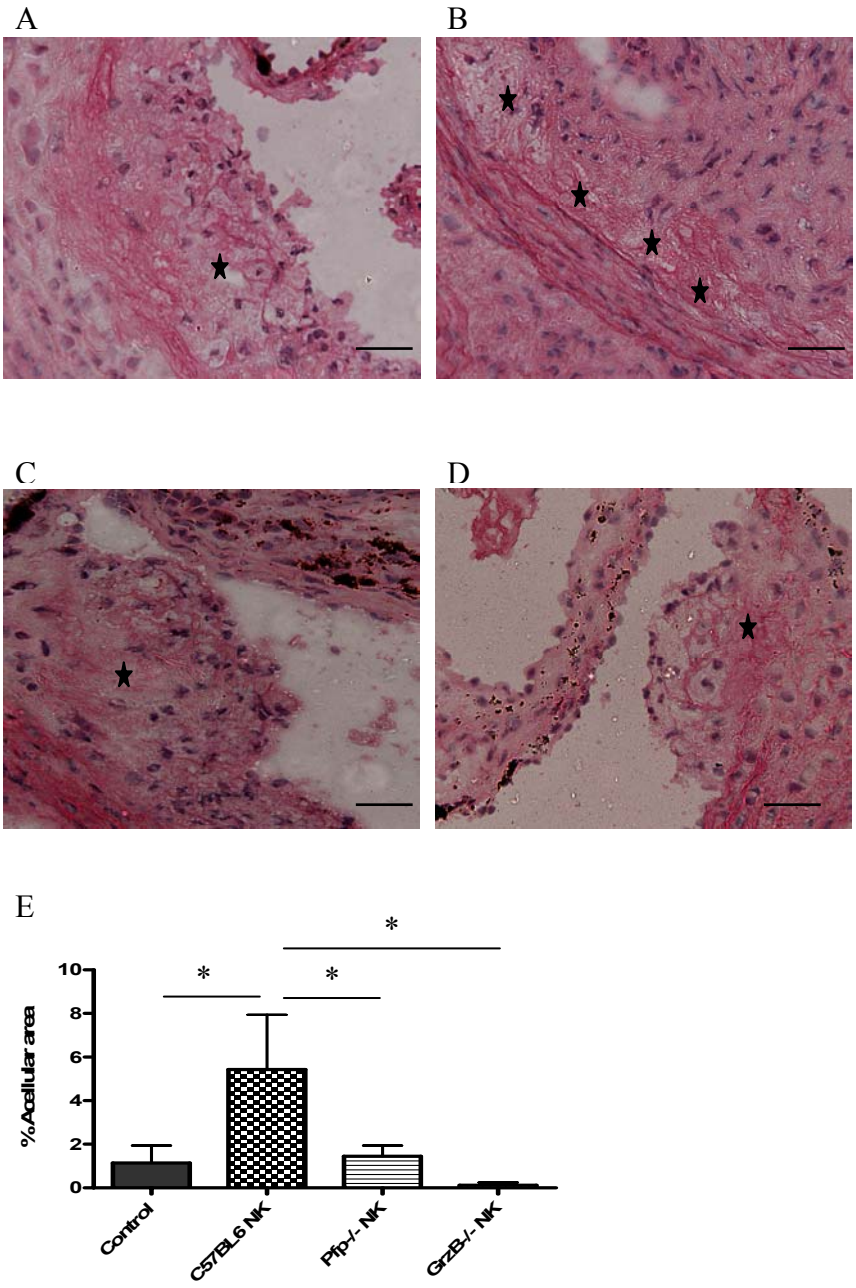


Figure 6.9: Perforin and granzyme B deficiency in NK cells reduces acellular area in lesions. ApoE$^{-/-}$Rag2$^{-/-}$γc$^{-/-}$ mice were injected with (A) PBS (Control), (B) WT NK cells (C57BL/6 NK cells), (C) pfp$^{-/-}$ NK cells or (D) grzB$^{-/-}$ NK cells and fed a high fat diet for 8 weeks. Aortic root sections were stained with haemotoxylin and counterstained with eosin. Acellular area was defined as regions lacking hemotoxylin nuclei staining (marked by ★) and expressed as percentage of lesion area. (E) Bar graph represented mean ± SEM of percentage of necrotic core in atherosclerotic lesions from 5-6 mice in each group; $^{*}P < 0.05$. Scale bar represents 250 μm.

6.3.8 NK cell-derived perforin and granzyme B contributes to inflammation in atherosclerosis

Increase in apoptosis in atherosclerosis results in accumulation of apoptotic cells and cellular debris due to impaired clearance by macrophages [708] leads to secondary necrosis [709]. The increase in necrotic cells can lead to increased inflammasomes including NLRP3 inflammasomes activation [705]. The activation of inflammasomes by necrotic cells induces caspase- 1 activation, and causes the release of proinflammatory cytokines IL-1β and IL-18 that augment inflammation and atherosclerosis [162, 191]. Therefore I examined whether the deficiency in perforin or granzyme B in NK cells in atherosclerotic lesions can potentially reduce inflammation. Real-time PCR analysis of aortic arch mRNA encoding pro-IL-1β and caspase-1 (ICE) indicated reduced mRNA levels in both these factors in lesion of mice that received perforin-deficient NK cells or grazyme B-deficient NK cells. The levels of pro-IL-1β and caspase-1 in these mice were similar to control $ApoE^{-/-}Rag2^{-/-}\gamma c^{-/-}$ (Figure 6.10, $P > 0.05$).

IL-1β increases the expression VCAM-1 on endothelial cells [710]. To determine if the increase in IL-1β in atherosclerotic lesion in mice transferred with WT NK cells affected the VCAM-1 expression, immunohistochemistry was performed on aortic sinus sections to assess the levels of VCAM-1 expression. VCAM-1 expression was unaffected by WT NK cell transfers and expressed in atherosclerotic lesions of all groups (Figure 6.11, $P>0.05$).

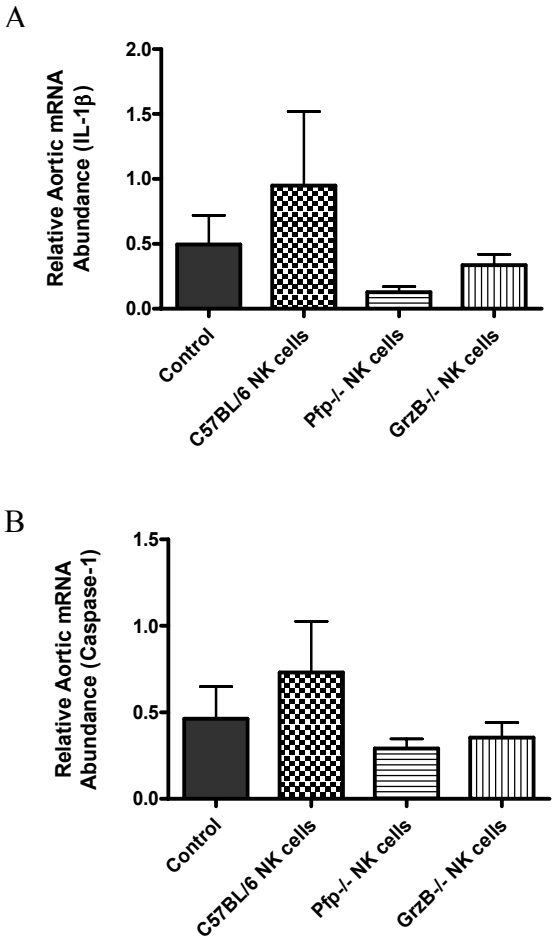


Figure 6.10: Perforin and granzyme deficiency reduces inflammation in atherosclerotic lesions. Mice injected with control (PBS), WT NK cells (C57BL/6 NK cells), $pfp^{-/-}$ NK cells or $grzB^{-/-}$ NK cells then fed a high fat diet for 8 weeks. RNA was extracted from atherosclerotic aortic arch. Lesions from mice adoptively transferred with pfp or grzB deficient NK cells showed lower expression of proinflammatory cytokine IL-1β and caspase-1.

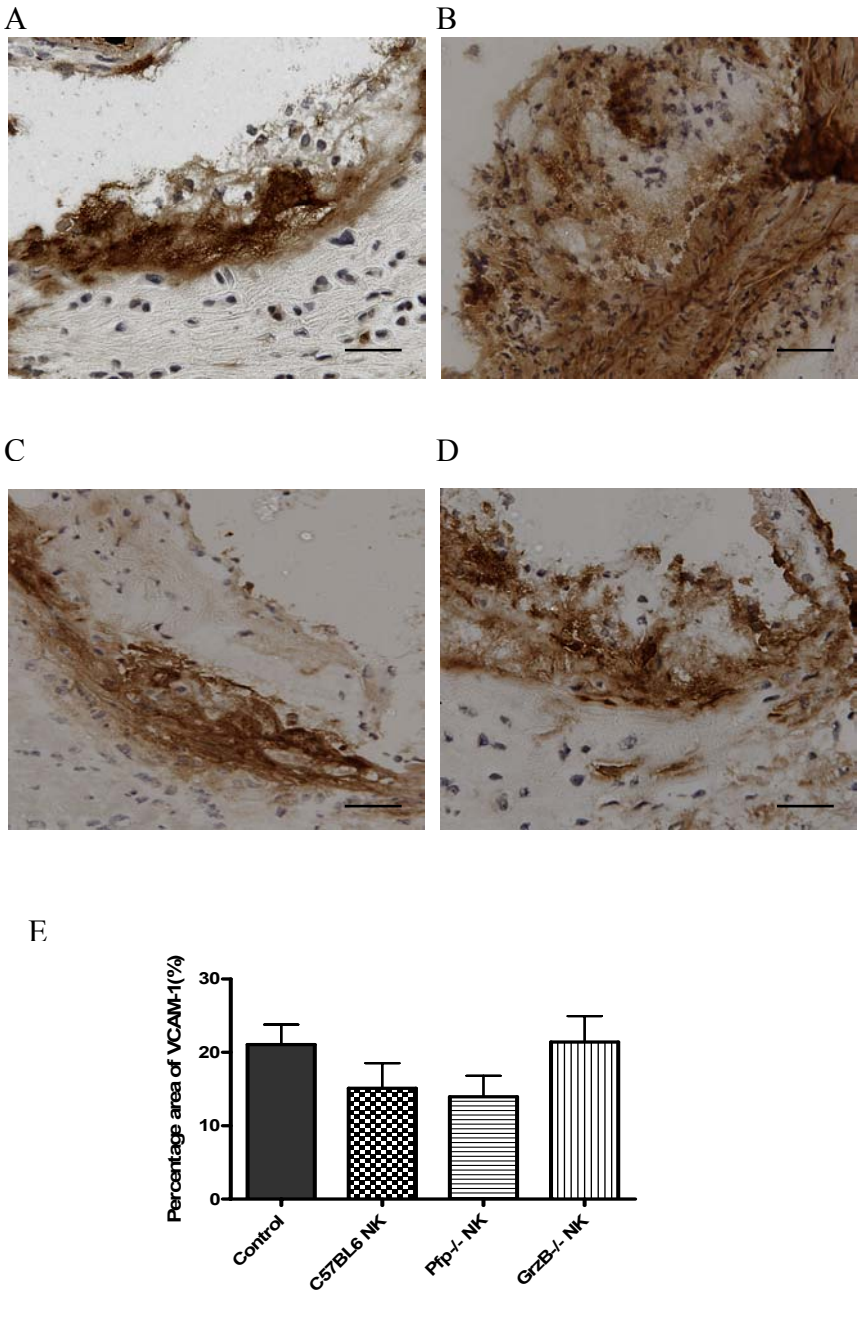


Figure 6.11: Transfer of perforin- or granzyme B-deficient NK cells did not affect VCAM-1 expression in lesions. ApoE$^{-/-}$Rag2$^{-/-}$γc$^{-/-}$ mice were injected with (A) PBS (Control), (B) WT NK cells (C57BL/6 NK cells), (C) pfp$^{-/-}$ NK cells or (D) grzB$^{-/-}$ NK cells and fed a high fat diet for 8 weeks. Aortic sinus cross-sections were stained for VCAM-1 (brown stain). (E) Bar graph represented the mean ± SEM of percentage area stained atherosclerotic lesions from 5-6 mice in each group; P>0.05. Scale bar represents 250 μm.

6.3.9 Plasma cholesterol levels and body weight of ApoE$^{-/-}$Rag2$^{-/-}$γc$^{-/-}$ with different adoptively transferred cultured NK cells.

To determine if the transfer of NK cells affects the hypercholesterolemia state of ApoE$^{-/-}$ Rag2$^{-/-}$ γc$^{-/-}$ mice, total cholesterol levels were examined. Despite the significance difference in the extent of atherosclerotic lesion size in ApoE$^{-/-}$ Rag2$^{-/-}$ γc$^{-/-}$ mice transferred with WT NK cells, IFN-γ-, granzyme B- or perforin-deficient NK cells the body weights of mice from each group were not affected. Although, the total LDL cholesterol and triglyceride were moderately higher in mice transferred with perforin-deficient mice (total triglyceride 2.9±0.2mmol/L; LDL-chol 35.2±3.0mmol/L) compared to those transferred with WT NK cells (total triglyceride 1.9±0.3mmol/L; LDL-chol 25.1±3.2mmol/L) (Table 6.1), this did not influence the severity of atherosclerosis in those mice. Atherosclerotic lesion size in mice adoptively transferred with WT NK were increased despite a significant decrease in triglyceride levels in WT NK cell transferred ApoE$^{-/-}$Rag2$^{-/-}$γc$^{-/-}$ mice compared to control mice. There was no significant difference in HDL-cholesterol levels between all groups (Table 6.1).

Table 6.1: Body weights and plasma cholesterol levels of ApoE$^{-/-}$Rag2$^{-/-}$γc$^{-/-}$ mice fed a high fat diet for 8 weeks.

Group	Weight (g)	Total Cholesterol	Triglyceride	VLDL/LDL	HDL
ApoE$^{-/-}$Rag2$^{-/-}$γc$^{-/-}$ control	33.2 ± 0.7 (5)	29.1 ± 1.4 (5)	3.8 ± 0.8 (5)	23.4 ± 0.9 (5)	4.4 ± 0.4 (5)
ApoE$^{-/-}$Rag2$^{-/-}$γc$^{-/-}$ WT NK	34.0 ± 1.2 (5)	31.3 ± 3.9 (5)	1.9 ± 0.3 (5)*	25.1 ± 3.2 (5)	5.4 ± 0.7 (5)
ApoE$^{-/-}$Rag2$^{-/-}$γc$^{-/-}$ IFN-γ$^{-/-}$ NK	35.0 ± 1.3 (5)	34.2 ± 2.6 (5)	4.6 ± 1.9 (5)	26.7 ± 2.2 (5)	5.5 ± 0.5 (5)
ApoE$^{-/-}$Rag2$^{-/-}$γc$^{-/-}$ pfp$^{-/-}$ NK	36.6 ± 0.8 (5)	39.2 ± 1.7 (5)	2.9 ± 0.2 (5)*	35.2 ± 3.0 (5)*	2.7 ± 1.6 (5)
ApoE$^{-/-}$Rag2$^{-/-}$γc$^{-/-}$ grzB$^{-/-}$ NK	33.0 ± 0.5 (5)	24.8 ± 5.1 (5)	1.7 ± 0.3 (5)	24.8 ± 5.1 (5)	3.8 ± 1.2 (5)

* $p < 0.05$ compared to ApoE$^{-/-}$Rag2$^{-/-}$γc$^{-/-}$ WT NK
Total cholesterol, triglyceride, VLDL/LDL and HDL measurements are in mmol/L.
Number of mice in each group is indicated in parentheses.
Data represent the mean ± SEM.

6.4 Discussion

The major findings of this study are that IL-2 activated NK cells promote the development of atherosclerosis via mechanisms involving the secretion of perforin and granzyme B. Adoptive transfer of perforin- or granzyme B-deficient NK cells into endogenous lymphocyte deficient mice ApoE$^{-/-}$Rag2$^{-/-}$γc$^{-/-}$ did not augment atherosclerosis compared to adoptive transfer of wild-type NK cells. Secondary necrosis mechanism seems to be involved in the proatherogenic activity of NK cells, as inflammation and lesion necrotic core (acellular regions) was reduced in mice adoptively transferred with perforin- and granzyme B deficient NK cells. Hence, NK cells are proatherogenic by exerting their cytolytic functions.

Perforin is a cytolytic protein in the cytoplasmic granules of NK cells. Upon degranulation, perforin is thought to insert itself to the surface membrane of target cells forming a pore. Although perforin are highly cytotoxic, its ability to induce target cell apoptosis is dependent on granzymes, especially granzyme B [568, 711, 712]. Granzyme B, a serine protease in the cytoplasmic granules of NK cells, cleaves target cell proteins to induce caspase-mediated and caspase-independent cell death [712]. My findings indicate that both perforin and granzyme B secretion by NK cells are important for the proatherogenic activity of NK cells. However, it is also possible that granzyme B may induce apoptosis independent of perforin by inducing apoptosis of SMCs by cleaving extracellular proteins such as fibronectin [575] but it is likely to be a minor mechanism as for both perforin and granzyme. Although the mechanism by which perforin and granzyme B augments atherosclerosis was not directly investigated, my findings indicate that the necrotic cores of atherosclerotic lesion in ApoE$^{-/-}$Rag2$^{-/-}$γc$^{-/-}$ mice adoptively transferred with perforin- and granzyme-deficient NK cells were reduced compared to mice adoptively transferred with wild-type NK cells, suggests that secondary necrosis resulting from

a defective phagocytic clearance of apoptotic debris [238], contributes to a proinflammatory response and increase in lesion size. NLP3 inflammasomes that have been previously implicated in atherosclerosis [713] is activated by necrotic cells [705]. Necrotic cells are known to activate inflammasomes resulting in increased activity of caspase-1. Caspase-1 cleaves pro-IL-1β and pro-IL-18 to their respective active forms which are pro-atherogenic [162, 191]. Necrotic cells are also known to release nucleosomes that contains substances such as HMGB1 [714] which are also proatherogenic [715]. Since it is not possible to measure active IL-1β or IL-18, I measured the pro-IL-1β and caspase-1 expression in lesions and also changes that can be mediated by IL-1β in lesions such as VCAM-1 protein expression [162]. There was an increase in pro-IL-1β and caspase-1 in $ApoE^{-/-}Rag2^{-/-}\gamma c^{-/-}$ transferred with wild-type NK cells and a decrease in mice transferred with NK cell deficient in perforin and grazyme B that suggest that the decrease in necrotic cells may potentially decrease inflammasome activation and caspase-1 activation and hence reduction in secretion of IL-1β secretion and inflammation. VCAM-1 an adhesion molecule that is essential in recruiting cells mediating the development of atherosclerosis [716]. The expression of VCAM-1 protein in lesions was not affected by adoptive transfer of wild-type, perforin-deficient and granzyme B –deficient NK cells. In this instance, the expression of VCAM-1 maybe regulated by NK cell derived IFN-γ, IFN-γ induces VCAM-1 expression on endothelial cells [717]. These results so far supports the hypothesis that secondary necrosis in atherosclerosis leads to a more severe inflammation in atherosclerotic lesions that results in augmentation of atherosclerosis. However, further investigation is required to further establish this hypothesis.

IFN-γ has been known to exert many of the effects of NK cells. NK cell derived IFN-γ plays a major role in anti-tumor responses, anti-viral and anti-bacterial responses. IFN-γ also plays a vital role in the development of atherosclerosis [158]. However, adoptive transfer of IFN-

γ-deficient NK cells into ApoE$^{-/-}$Rag2$^{-/-}$γc$^{-/-}$ mice surprisingly increased lesion size. One possible explanation for the lack of effect form NK cell-derived IFN-γ in atherosclerosis is the low numbers of NK cells present in atherosclerotic lesion [257] compared to other IFN-γ producing cells such as macrophages which are present in large numbers within the lesion.

In this study, I used a mouse model ApoE$^{-/-}$Rag2$^{-/-}$γc$^{-/-}$ which lacked endogenous lymphocytes including NK cells. Importantly, this model allowed me to focus entirely on the mechanisms that NK cells may utilize in promoting their proatherogenic activity. I explored the important molecules in the in vivo proatherogenic responses mediated by adoptively transferred NK cells such as perforin, granzyme B and IFN-γ. Therefore, the purity of NK cells obtained from these various knockout mice was crucial in elucidating the role of each of these molecule expressed on NK cells on atherosclerosis. Initially I used a well known NK cell isolation protocol that have been used to study the response of adoptively transferred NK cells on tumor inhibition [591], however the purity of NK cells obtained by this method was insufficient for the model of atherosclerosis. There were T cells contamination in liver of recipient ApoE$^{-/-}$Rag2$^{-/-}$γc$^{-/-}$ mice at the end of 8 weeks whilst on high fat diet, therefore I had to perform one additional step from the protocol described in the above source by sorting IL-2 cultured NK cells by FACS sorter to obtain a 99% pure NK cell population. The only possible explanation for the importance of purity for atherosclerosis is that other immune cell types particularly T cells can expand rapidly in lymphopenic mice [718] and account for the increase in lesion size.

Adoptively transferred NK cells are able to survive and proliferate effectively in lymphocyte deficient Rag2$^{-/-}$γc$^{-/-}$ mice [432]. Since adoptively transferred NK cells rapidly proliferate in the liver, the extent of NK cell reconstitution in the liver of ApoE$^{-/-}$Rag2$^{-/-}$γc$^{-/-}$ mice were determined after 4 weeks of transfer. Usually in a normal ApoE$^{-/-}$, NK cells comprise

around $4x10^5$ cells in the liver, however in adoptively transferred NK cell deficient $ApoE^{-/-}$ $Rag2^{-/-}\gamma c^{-/-}$ mice, there was only around $2.0x10^5$ NK cells indicating that the amount of NK cell transferred was not sufficient to reconstitute the liver, however this is enough to exacerbate atherosclerosis. Therefore, this suggest that the number of NK cells is not critical in promoting atherosclerosis, however the impact of their cytotoxic effector functions was sufficient to augment the development of atherosclerosis.

My previous findings indicate that NK cells are activated by NKT cells probably by IFN-γ to exert their proatherogenic effects and $ApoE^{-/-}Rag2^{-/-}\gamma c^{-/-}$ lack NKT cells. NK cells can be activated via number of different mechanisms. In normal conditions, there are other mediators such as IL-2 and IL-12 produced by T cells and dendritic cells that could possibly activate NK cells to exert their proatherogenic activity. Additionally, in the presence of other immune cells, activated NK cells can potentially activate bystander immune cells such as $CD8^+$ T cells, macrophages and B cells to promote an immune response [719-721] that can further exacerbate atherosclerosis. However, further studies are needed to further substantiate this hypothesis.

In summary, my findings extend the principle that NK cell is pro-atherogenic and they exert their pro-atherogenic effects through their lytic granules perforin and granzyme B, together they promote cell lysis or necrosis in atherosclerotic lesions.

7 General Discussion

The role of NK cells in development of atherosclerosis is controversial with studies suggesting no involvement or a proatherogenic involvement. My findings have unambiguously defined the role of NK cells in atherosclerosis and the mechanisms by which they promote atherosclerosis. Specifically I have shown that (1) specific depletion of NK cells ameliorates atherosclerosis whilst their activation augments atherosclerosis; (2) NK cells require NKT cells for them to exert their proatherogenic effects, presumably for activating NK cells and (3) the mechanism by which NK cells exert their proatherogenic effects is by releasing the cytotoxic molecules perforin and granzyme B rather than proinflammatory cytokines such as interferon-γ.

NK cells have been identified in both mouse and human atherosclerotic lesions [606, 614]. Despite their presence in developing atherosclerotic lesions, their role in atherosclerosis has been unclear. Studies using the beige mutation mice that exhibit defects in NK cell cytolytic function suggest that atherosclerosis may be unaffected by NK cells [610]. More recent studies examining the role of NK cells using mice harbouring the beige mutation on a LDLR-deficient background suggested that NK cells may be protective against atherosclerosis, as these mice exhibited increased atherosclerosis [608]. However, this model is not specific for NK cell as these mice exhibit a complex phenotype that is more than a deficiency in NK cell cytolytic function; cytotoxic T lymphocyte function is also impaired and during infection such cells expand in number to compensate for their reduced cytolytic activity [327]. Unfortunately NK cell numbers were not monitored in the beige mutant LDLR deficient mice. A more recent study by Whitman utilizing $LDLR^{-/-}$ chimeric mice whose bone marrow had been reconstituted with bone marrow from Ly49A transgenic mice resulted in a reduction in atherosclerosis, suggesting

a proatherogenic role for NK cells [614]. However, Ly49A receptor expression is not restricted to NK cells and NKT cells as well as CD8+ and CD4+ T cells have been reported to express Ly49A [722-724]; suppression of these cells may have contributed to the reduction in atherosclerosis as both NKT cells and CD4+ T cells have been shown to be pro-atherogenic [261, 303]. My studies indicate that NK cells augment development of atherosclerosis. I used a direct approach to determine the role of NK cells in atherosclerosis by using an antibody that specifically depletes NK cells in ApoE$^{-/-}$ mice. Other lymphocyte populations as well as monocyte cell populations were unaffected during NK cell depletion using anti-asialo GM1 antibodies confirming the selectivity of this antibody for NK cells.

As my study indicated that the depletion of NK cells ameliorates atherosclerotic lesion development, I also examined whether the activation of NK cells further augments development of atherosclerosis. Polyinosinic-polycytidylic acid (Poly I:C), a double-stranded RNA analog recognized by melanoma-differentiation associated gene 5 (MDA5) and TLR3, activates NK cells in vivo [541]. I examined whether this agent augmented development of atherosclerosis in the ApoE-/- mice fed a high fat diet. Poly IC exacerbated the development of atherosclerosis. Since Poly IC has been shown to also activate CD8+ T cells [725], I also examined its effects on atherosclerosis in ApoE$^{-/-}$ Rag2$^{-/-}$ mice. These mice are deficient in B and T cells but not NK cells [318, 726]. In these mice Poly IC also augmented development of atherosclerosis lesions suggesting a strong dependency on NK cells. To confirm the dependency of Poly IC effects on atherosclerosis on NK cells I also examined the effects of Poly IC in NK cell depleted (using NK cell depleting antibody, anti-asialo GM1) ApoE$^{-/-}$ mice. NK cell depletion in these mice reduced the development of atherosclerosis compared to ApoE$^{-/-}$ mice treated with just Poly IC. Whilst my studies indicate a dependency of Poly IC's proatherogenic effects on NK cells, it remains to be determined whether this is a consequence of directly activating NK cells or an indirect action

via activation of CD8+ dendritic cells [542]. $CD8^{+}$ dendritic cells also express MDA-5 and TLR3 and is activated by Poly IC. Also, in the present study I did not examine whether activation was the consequence of Poly IC interacting with TLR3 or MDA-5 or both receptors. However, given the results of a recent study indicating that TLR3 plays a protective role in atherosclerosis [547], it is tempting to speculate that Poly IC mediated activation may be via MDA-5. However, this remains to be proven.

An important question not previously addressed is how NK cells are activated during development of atherosclerosis. NK cells can be activated by several mechanisms, including cytokine-dependent mechanisms involving IL-2, IL-12, IL-15 or IL-18 [727], via Fc-gamma RIII [728], via NKT cells [188] and/or via their activating receptor NKG2D [729]. Activated NKT cells can stimulate NK cell proliferation and cytotoxicity [306], effects partly attributed to IFN-γ produced by NKT cells [188, 306, 566, 730]. My studies indicate that NKT cells contribute to NK cell activation during development of atherosclerosis since atherosclerotic lesions were similar in size in mice deficient in invariant NKT cells in $ApoE^{-/-}$ $Jalpha18^{-/-}$ and in NK cell depleted $ApoE^{-/-}$ $Jalpha18^{-/-}$ mice. Whether the activation of NK cells by NKT cells is dependent on IFN-γ secreted by NKT cells need further investigations. As to the role of NKG2D receptors in activating NK cells during development of atherosclerosis, my studies indicate that these receptors are not involved despite the high expression in lesions of its ligands Rae-1δ, Rae-1ε and MULT-1. It is possible that the expression of NKG2D receptors on NK cells is downregulated by inhibitory Ly49 receptors expression on NK cells, inhibiting them from exerting their proatherogenic effects [690]. Given that atherosclerotic lesions express abundant amounts of MHC class 1 which are ligands for Ly49 inhibitory receptor, far greater than expressed by normal tissue [304], this may have prevented NK activation via NKG2D receptors despite the presence of activating ligands in atherosclerotic lesions.

Theoretically, NK cells can promote atherosclerosis via a number of mechanisms, by producing pro-inflammatory cytokines or cytotoxins. Activated NK cells are high producers of IFN-γ [731], a Th1 cytokine known to augment development of atherosclerosis [158], by enhancing leukocyte recruitment, cell activation, and foam cell formation [732, 733]. Despite this importance of IFN-γ, NK cell derived IFN-γ did not contribute to development of atherosclerosis; the increase in atherosclerosis after adoptive transfer of IL-2 activated NK cells deficient in IFN-γ to ApoE$^{-/-}$ Rag2$^{-/-}$ γc$^{-/-}$ was similar to transfer of wild-type IL-2 activated NK cells. In contrast, the ability of NK cells to exert their proatherogenic response was highly dependent on their secretion of cytotoxic molecules, perforin and granzyme B. Both perforin and granzyme B are involved in NK cell target cell-mediated apoptosis. Perforin facilitates the entry of granzyme B into the nucleus of target cells to initiate DNA fragmentation and cell apoptosis [703]. Since both perforin and granzyme B are important for NK cells to promote atherosclerosis, my findings indicate that NK cell mediated apoptosis and post apoptotic necrosis is important for development of atherosclerotic lesions. This is supported by my findings that mice receiving perforin-deficient and granzyme B-deficient NK cells exhibit lesions with smaller necrotic cores. Increased frequency of cell death and impaired clearance of apoptotic cells by macrophages in lesions results in secondary cell necrosis, and development of larger necrotic cores [709]. The increase in the number of necrotic cells can potentially increase inflammation within atherosclerotic lesions, by activating inflammasomes [734]. NLRP3 inflammasomes have been implicated in atherosclerosis [713]. Their activation by necrotic cells results in the activation of caspase-1 also known as IL-1-β converting enzyme (ICE) which in turn processes pro-IL-1beta and pro-IL-18 to active (secreted) proatherogenic IL-1β and IL-18 [162, 705]. The direct effect and involvement of inflammasomes in the proatherogenic effects of NK cells was not investigated, since it is not possible to measure active IL-1β. My findings that the pro-IL-1β and caspase-1 protein expression were reduced in atherosclerotic lesions of ApoE$^{-/-}$ Rag2$^{-/-}$ γc$^{-/-}$

mice transferred with perforin– and granzyme B –deficient NK cells is consistent with the proatherogenic effects involving inflammasome activation [162]. It is possible that the reduction in necrotic cell numbers in atherosclerotic lesions of ApoE$^{-/-}$ Rag2$^{-/-}$ γc$^{-/-}$ transferred with perforin and granzyme B deficient NK cells, reduced inflammasomes activation hence reduced caspase-1 activation and active IL-1β secretion. Necrotic cells are also known to release HMGB1 [735] which is present in significant quantities in cells within atherosclerotic plaques [736] and been shown to be proatherogenic [715]. Thus it appears that NK cells indirectly mediate down stream proinflammatory and proatherogenic effects, by augmenting post apoptotic necrotic cell numbers.

In addition to investigating how NK cells promote atherosclerosis, I also examined whether NK cells modulated cell composition of atherosclerotic lesions. Activated NK cells produce cytokines and chemokines. NK cell derived pro-inflammatory cytokines IFN-γ and TNF-α are known to influence both smooth muscle cell (SMC) and endothelial cell (EC) proliferation and migration, and chemokine expressed by NK cells are known to attract other immune cells particularly macrophages and T cells [203, 637-640, 644]. Therefore I examined the effects of NK cell on lesion cell composition by examining the lesion content of SMC, EC, macrophages and CD4^{+} T cells. However, there were no changes in SMC, EC and CD4^{+} T cells content of the lesion, except for macrophage content of the lesion. Since it appears that NK cells indirectly mediate downstream proinflammatory and proatherogenic effects by augmenting post apoptotic necrotic cell numbers which may activate inflammasomes and possibly nucleosomes such as HMGB1, it is possible that these macrophages are recruited into atherosclerotic lesions via such mechanisms. Inflammasomes activation that leads to active IL-1β secretion may potentially increase MCP-1 expression by endothelium cells augmenting the recruitment of macrophages to developing lesions [162]. HMGB1 has been shown to be involved in the

migration of macrophages into atherosclerotic lesions to augment atherosclerosis [715]. Although NK cells are also capable of producing cytokines and chemokines such that chemoattract monocytes/macrophages to atherosclerotic lesions, this probably makes a very minor contribution to lesion development as the ability of NK cells to increase atherosclerosis is clearly dependent on granzyme B and perforin.

An important issue that arises from the present studies is whether pharmacological targeting of NK cells might be a useful therapeutic strategy to prevent atherosclerosis. At present one can only speculate as to how this might be achieved as there are currently no specific inhibitors that prevent NK cell activation. One possible approach may be to target specific chemokines that are responsible for the homing of NK cells to atherosclerotic lesions. Whilst I did not directly assess NK cell homing to lesions, the fact that necrotic core size in atherosclerotic lesions was dependent on NK cells and NK cell derived perforin and granzyme B indicate that NK cells exert their pro-atherogenic effects within developing atherosclerotic lesions. NK cells have been previously shown to accumulate in mouse and human atherosclerotic lesions [11, 606, 614, 623]. NK cells express chemokine receptors CCR5, CXCR3 and CX3CR1 [203, 737] for chemokines RANTES, CXCL10 and fractalkine (CX3CL1) that are expressed in atherosclerotic lesions [204, 216, 284]. Targeting one or more of these chemokines would be expected to attenuate the pro-atherogenic effects of NK cells. Similarly, targeting perforin might also be expected to attenuate their pro-atherogenic. Recently, inhibitors of perforin have been developed to prevent the cytolytic actions of NK cells and cytotoxic T lymphocytes [738]. Clearly, further experimentation is required to more critically assess these potential therapeutic strategies to prevent the proatherogenic effects of NK cells.

Another important issue is to what extent lesions that develop at the aortic sinus of mice are representative of human atherosclerotic lesions; atherosclerosis does not develop at the aortic sinus of humans. Atherosclerosis is a complex disease that affects very specific sites of the vasculature. Hemodynamic forces are largely responsible for dictating which vascular sites are susceptible to disease whilst systemic and local immune factors modulate subsequent disease development and progression. These latter factors-immune cells and cytokines are similar in human and mouse atherosclerotic lesions [8, 739], indicating that development and progression of atherosclerosis is regulated by similar cellular and inflammatory factors in both instances. The increased susceptibility of aortic sinus in mice to atherosclerosis compared with humans is most likely related to heart rate [740]. Heart rate is very rapid in mice (550 beats per minute) compared to humans (70 beats per minute) and consequently blood flow is disrupted to a much greater extent, i.e., much more turbulent than in the human aortic sinus, increasing the susceptibility of this site to atherosclerosis. Bassiouny et al have shown that heart rate is an important determinant of atherogenesis [741].

In conclusion, my studies indicate that NK cells within developing atherosclerotic lesions augment atherosclerosis by secreting the cytotoxic molecules perorin and granzyme B. The magnitude of the effects of NK cells on lesion development is dependent not only on NK cell number but also on their activation status. During development of atherosclerosis NK activation appears dependent on NKT cells whilst activating receptors on NK cells, such as NKG2D receptors have little if any role. My studies suggest that targeting NK cells may be a useful therapeutic strategy to attenuate development of atherosclerosis.

8 References

1. Glass CK, Witztum JL. Atherosclerosis: the road ahead. Cell 104(503-519, 2001.
2. Ross R. Mechanism of disease. New England Journal of Medicine 340(2): 115-126, 1999.
3. Libby P, Ridker PM, maseri A. Inflammation and atherosclerosis. Circulation 105(1135-1143, 2002.
4. Hansson GK, Jonasson L, Seifert PS, Stemme S. Immune mechanisms in atherosclerosis. Arteriosclerosis 9(567-578, 1989.
5. Libby P, Hansson GK. Involvement of the immune system in human atherogenesis: current knowledge and unanswered questions. Lab Invest 64(5-15, 1991.
6. Ruffer MA. On arterial lesions found in egyptian mummies (1580 B.C - 525 A.D). Journal of Pathol Bact 15(453-462, 1911.
7. Sandison A. Degenerative vascular disease in Egyptian mummy. Med Hist 6(77-81, 1962.
8. Tedgui A, Mallat Z. Cytokines in atherosclerosis. Physiol Rev 86(515-581, 2006.
9. Ross R, Glomset J. The pathogenesis of atherosclerosis (first of two parts). N Eng J Med 295(369-377, 1976a.
10. Ross R, Glomset J. The pathogenesis of atheroscelrosis (second of two parts). N Eng J Med 295(420-425, 1976b.
11. Jonasson L, Holm J, Skalli O, Bondjers G, Hansson G. Regional accumulations of T cells, macrophages, and smmoth muscle cells in the human atherosclerotic plaque. Arteriosclerosis 6(131-138, 1986.
12. Hansson GK, Libby P. The immune response in atherosclerosis: a double-edged sword. Nat Rev Immunol 6(508-519, 2006.
13. Castelli WP. LIpids, risk factors and ishemic heart disease. Atherosclerosis 124(S1-S9, 1996.
14. Bonow RO, Smaha LA, Smith SCJ, Mensah GA, Lenfant C. World Heart Day 2002: the international burden of cardiovascular disease: responding to the emerging global epidemic. Circulation 106(1602-1605, 2002.
15. Association AH. Heart disease and stroke statistical - 2010 update. Dallas, Texas. American Heart Association 2010 2002.
16. Vos T, Begg S. The burden of cardiovascular disease in Australia for the year 2003. National Heart Foundation of Australia 2003.
17. Steinberg D. Low density lipoprotein oxidation and its pathobiological significance. The Journal of Biological Chemistry 272(20963-20966, 1997.
18. Khoo JC, Miller E, McLouglin P, Steinberg D. Enhanced macrophage uptake of loe density lipoprotein after self-agregation. Arterioscler. Thromb. Vasc. Biol. 8(348-358, 1988.
19. Khoo JC, Miller E, Steinberg D, Witztum JL. Monoclonal antibodies against LDL further enhance macrophage uptake of LDL aggragates. Arterioscler. Thromb. Vasc. Biol. 12(1258-1266, 1992.
20. Griendling KK, Ushio-Fukai M, Lassegue;R B, Alexander W. Angiotensin II signalling in vascular smooth muscle. Hypertension 29(366-370, 1997.
21. Guzik TJ, Hoch NE, Brown KA, McCann LA, Rahman A, al. e. Role of T cell in the genesis of angiotensin II-induced hypertension and vascular dysfunction. J Exp Med 204(2449-2460, 2007.
22. Kranzhofer R, Schmidt J, Pfeiffer CAH, Hagl S, Libby P, Kubler W. Angiotensin induces inflammatory activation of human vascular smooth muscle cells. Arterioscler. Thromb. Vasc. Biol. 19(1623-1629, 1999.

23. Tummala PE, Chen X-L, Sundell CL, Laursen JB, Hammes P, Alexander RW, Harrison DG, Medford RM. Angiotensin II induces vascular adhesion molecule-1 expression in rat vasculature. Circulation 100(1223-1229, 1999.
24. Woo C-H, Shishido T, McClain C, Lim JH, Li J-D, Yang J, Yan C, Abe J-i. Extracellular signal-regulated kinase 5 SUMoylation antagonizes shear stree-induced antiinflammatory response and endothelial nitric oxide synthase expression in endothelial cells. Circulation Research 102(538-545, 2008.
25. Schmidt AM, Yan SD, Wautier J-L, Stern D. Activation of receptor for advanced glycation end products. Circulation Research 84(489-497, 1999.
26. Williams IL, Wheatcroft SB, Shah AM, Kearney MT. Obesity, atherosclerosis and the vascular endothelium: mechanisms of reduced nitric oxide bioavailability in obese humans. International Journal of Obesity 26(754-764, 2002.
27. Yudkin JS, Stehouwer CDA, Emeis JJ, Coppack SW. C-reactive protein in healthy subjects:Associations with obesity, insulin resisitance, and endothelial dysfunction. Arterioscler. Thromb. Vasc. Biol. 19(972-978, 1999.
28. Winkler G, Lakatos P, Salamon F, Nagy Z, Speer G, Kovacs M, Harmos G, Dworak O, Cseh K. Elevated serum TNF-alpha level is a link between endothelial dysfunction and insulin resisitance in normotensive obese patients. Diabetes Med 16(207-211, 1999.
29. Boulomie A, Drexler HC, Lafontan M, Busse R. Leptin, the product of Ob gene, promotes angiogenesis. Circulation Research 83(1059-1066, 1998.
30. Yang R, Barouch LA. Leptin signalling and obesity: catdiovascular consequences. Circulation Research 101(545-549, 2007.
31. Boulomie A, Marumo T, Lafontan M, Busse R. Leptin induces oxidative stress in human endothelial cells. FASEB 13(1231-1238, 1999.
32. Bodary PF, Gu S, Shen Y, Hasty AH, Buckler JM, Eitzman T. Recombinant leptin promotes atherosclerosis and thrombosis in apolipoprotein-E-deficient mice. Artrioscler Thromb. Vasc. Biol 25(e119-122, 2005.
33. Danesh J, Collins R, Peto R. Chronic infections and coronary heart disease: is there a link? Lancet 350(430-436, 1997.
34. Kol A, Bourcier T, Lichtman AH, Libby P. Chlamydial and human heat shock protein 60s activate human vascular endothelium, smmoth muscle cells,and macrophages. J Clin Invest 103(571-577, 1999.
35. Kuo CC, Campbell LA. Detection of Chlamydia pneumonia in arterial tissues. J . Infect Dis 181(S432-S436, 2000.
36. Halme S, Juvonen T, Laurila A, Juvonen J, Mosorin M, saikku P, Surcel HM. Chlamydia pneumonia reactive T lymphocytes in the walls of abdominal aortic aneeurysms. Eur J Cin Invest 29(546-552, 1999.
37. Moazed TC, Campbell LA, Rosenfeld ME, Grayston JT, Kuo CC. Chlamydia pneumonia infection accelerates the progression of atherosclerosis in apolipoprotein E-deficient mice. J Infect Dis 180(238-241, 1999.
38. Gaydos CA. Growth in vascular cells and cytokine production y Chlamydia pneumonia. J . Infect Dis 181(S473-S478, 2000.
39. Netea MG, Selzman CH, Kullberg BJ, al e. Acellular components of Chlamydia pneuonia stimulate cytokine production in human blood mononuclear cells. Eur J Imunol 30(541-549, 2000.
40. Belland RJ, Quellette SC, Gieffers J, Byrne GI. Chlamydia pneumonia and atherosclerosis. Cellular Microbiology 6(117-127, 2004.
41. del Rincon ID, Williams K, Stern MP, Freeman GL, Escalante A. High incidence of cardiovascular events in a rheumatoid arthritis cohort not explained by traditional cardiac risk factors. Arthritis & Rheumatism 44(2737-2745, 2001.

42. Pham T, Gossec L, Constantin A, Pavy S, Bruckert E, Cantagrel A, Combe B, Flipo RM, Groupille P, Le Loet X, Mariette X, Puechal X, Schaeverbeke T, Sibilia J, Tebib J, Wendling D, Dougados M. Cardiovascular risk and rheumatoid arthritis: clinical practice guidelines based on published evidence and expert opinion. Joint Bone Spine 73(379-387, 2006.
43. Sattar N, McCarey DW, Capell H, Mcinnes IB. Explaining how 'high grade' systemic inflammtion accelerates vascular risk in rheumatoid arthritis. Circulation 108(2957-2963, 2003.
44. Gonzalez-Gay MA, Gonzalez-Juanatey C, Vazquez-Rodriguez TR, al e. Endothelial dysfunction, carotid intima-media thickness, and accelerated atherosclerosis in rheumatoid arhhritis. Semin Arthritis Rheum 38(67-70, 2008.
45. Gupta A, Fomberstein B. Evaluating cardiovascular risk in rheumatoid arthritis. The Journal of Muscuoskeletal Medicine 26(301-304, 2009.
46. Sherer Y, Shoenfeld Y. Mechanisma of disease: atherosclerosis in autoimmune diseases. Nature Clinical Practise Rheumatology 2(99-106, 2006.
47. Shoenfeld Y, Gerli R, Doria A, Matsuura E, Cerinic MM, Ronda N, Jara LJ, Abu-Shakra M, Meroni PL, Sherer Y. Accelerated atherosclerosis in autoimmune rheumatic disease. Circulation 112(3337-3347, 2005.
48. Stary HC, Blankenhorn DH, Chandler AB, Glagov S, Insull JW, Richarson M, Rosenfeld ME, Schaffer SA, Schwartz CJ, Wagner WD, Wissler RW. A definition of the intima of human arteries and of its atherosclerosis-prone regions. A report from the Committee on Vascular Lesions of the Council on Arteriosclerosis, American Heart Association. Arterioscler. Thromb. Vasc. Biol. 12(120-134, 1992.
49. Richardson M, Hatton MW, Moore S. Proteoglycan distribution in the intima and media of aortas of young and aging rabbits: An ultrastructural study. Atherosclerosis 71(243-256, 1988.
50. Landmesser U, Hornig B, Drexler H. Endothelial function : A critical determinant in atherosclerosis? Circulation 109(II-27 - II-33, 2004.
51. Ross R, Glomset J. Atherosclerosis and the arterial smooth muscle cell: proliferation of smooth muscle is a key event in the genesis of the lesions of atherosclerosis. Science 180(1332, 1973.
52. Nayler WG. Atherosclerosis and endothelial damage: A brief overview. Cardiovascular Drugs and Therapy 9(S25-30, 1995.
53. Congiu T, Schembri L, Tozzi M, Guasti L, Maio RC, Cosentino M, Marino F. Scanning electron microscopy examination of endothelium morphology in human carotid plaques. Micron 41(532-536, 2010.
54. Orekhov AN, Andreeva ER, Krushinsky AV, Novikov ID, Tertov VV, Nestaiko GV, Khashimov KA, Repin VS, Smirnov VN. Intimal cells and Atherosclerosis Relationship between the number of intimal cells and major manifestations of atherosclerosis inthe human aorta. Am J Pathol 125(402-415, 1986.
55. Orekhov AN, Karpova II, Tertov VV, Rudchenko SA, Andreeva ER, Krushinsky AV, Smirnov VN. Cellular composition of aherosclerotic and uninvoled human aortic subendothelial intima. Light-microscopic study of dissociated aortic cells. Am J Pathol 115(17-24, 1984.
56. Frid MG, Aldashev AA, Dempsey EC, Stenmark KR. Smooth mucle cells isolated from discrete compartments of the mature vascular media exhibit unique phenotypes and distinct growth capabilitis. Circulation Research 81(940-952, 1997.
57. Han CI, Campbell GR, Campbell JH. Circulating bone marrow cells can contribute to neointimal formation. J Vasc Res 38(113-119, 2001.

58. Caplice NM, BUnch TJ, Stalboerger PG, Wang S, Simper D, MIller DV, Russell SJ, Litzow MR, Edwards WD. Smooth muscle cells in human coronary atherosclerosis can originate from cells administered at marrow transplantation. Proc Natl Acad Sci USA 100(4754-4759, 2003.
59. Wolinsky H, Glagov S. A lamellar unit of aortic medial structure and function in mammals. Circulation Research 20(99-111, 1967.
60. Ooshima A, Muragaki Y. Collagen metabolism in atherogenesis. Ann NY Acad Sci 598(582-584, 1990.
61. Lopez-Candales A, Holmes DR, Liao S, Scott MJ, al. e. Decreased vascular smooth muscle cell density in medial degeneration of human abdominal aortic aneurysms. American Journal of Pathology 160(993-1007, 1997.
62. Ward MR, Pasterkamp G, Yeung AC, Borst C. Arterial remodelling: mechanisms and clinical implications. Circulation 102(1186-1191, 2000.
63. Hoshino A, Chiba H, Nagai K, Ishii G, Ochiai A. Human vascular adventitial fibroblsts contain mesenchymal stem/progenitor cells. Biochemical and Biophysical Research Communications 368(305-310, 2008.
64. Hu Y, Zhang Z, Torsney E, Afzal AR, Davison F, Metzler B, Xu Q. Abundant progenitor cells in the adventitia conribute to the atherosclerosis of vein graft in ApoE-deficient mice. J Clin Invest 113(1258-1265, 2004.
65. Torsney E, Hu Y, Xu Q. Adventitial progenitor cells contribute to atherosclerosis. Trends Cardiovasc. Med. 15(64-68, 2005.
66. Shi Y, O'Brien JE, Fard A, Mannion JD, Wag D, Zalewski A. Adventitial myofibroblasts conribute to neointimal formation in injured porcine coronary arteries. Circulation 94(1655-1664, 1996.
67. Moos MPW, John N, Grabner R, Nobmann S, Gunther B, Vollandt R, Funk CD, Kaiser B, Habenicht AJR. The lamina adventitia is the major site of immune cell accumulation in standard chow-fed apolipoprotein E-deficient mice. Arterioscler. Thromb. Vasc. Biol. 25(2386-2391, 2005.
68. Galkina E, Kadl A, sanders J, Varughese D, Sarembock IJ, Ley K. Lymphocyte recruitment into the aortic wall before and during development of atherosclerosis is partially L-selectin dependent. J Exp Med 203(1273-1282, 2006.
69. Rosselaar SE, Kakkananthu PX, Daugherty A. Lymphocyte populations in atherosclerotic lesions of ApoE-/- and LDL receptor -/- mice: decreasing density with disease progression. Arterioscler. Thromb. Vasc. Biol. 16(1013-1018, 1996.
70. Zhou X, Hansson GK. Detection of B cells and pro-inflammtory cytokines in atherosclerotic plaques of hypercholesterolemia apolipoprotein E knockout mice. Scand. J. Immunol 50(25-30, 1999.
71. Houtkamp MA, de Boor OJ, van der Loos CM, van der Wal AC, Becker Anton E. Adventitial infiltrates associated with advanced atheroscelrotic plaques: structuralorganization suggests generation of local humoral immune responses. J Pathol 193(263-269, 2001.
72. Xu X, LIn H, Huixia L, Zhang M, Zhang Y. Adventitial lymphatic vessels- an important role in atherosclerosis. Medical Hypotheses 69(1238-1241, 2007.
73. Libby P. Inflammtion in atherosclerosis. Nature 420(868-874, 2002.
74. VanderLaan PA, Reardon CA, Getz GS. Site specificity of atherosclerosis. Arterioscler. Thromb. Vasc. Biol. 24(12-22, 2004.
75. Dewey CFJ, Bussolari SR, Gimbrone MAJ, Davies PF. The dynamic response of vascular endothelial cells to fluid shear stress. J Biomech Eng 103(177-185, 1981.

76. Chappell DC, Varner SE, VNerem RM, Medford RM, Alexander RW. Oscillatory shear stress stimulates adhesion molecule expression in cultured human endothelium. Circulation Research 82(532-539, 1998.
77. Mohan S, Mohan N, Sprague EA. Differential activation of NF-kB in human aortic endothelial cells conditioned to specific flow environments. Am J Physiol 273(C572-C578, 1997.
78. ZIegler T, Bouzourene K, Harrison VJ, Brunner HR, Hayoz D. Influence of occilatory and unidirectional flow environments on the expression of endothelin and nitric oxide synthase in culture endothelial cells. Arterioscler. Thromb. Vasc. Biol. 18(686-692, 1998.
79. Lefer AM, Ma XL. Decreased basal nitric oxide release in hypercholesteromia increases neutrophil adherence to rabbit coronary artery endothelium. Arterioscler. Thromb. 13(771-776, 1993.
80. Shaul PW. Regulation of endothelial nitric oxide synthase: location, location, location. Annu Rev Physiol 64(749-774, 2002.
81. Naruse K, Shimizu K, Muramatsu M, Toki Y, Miyazaki Y, Okumura K, Hashimoto H, Ito T. Long-term inhibition of NO synthesis promotes atherosclerosis in the hypercholesterolemic rabbit thoracic aorta. PGH2 does not contribute to impaired endothelium-dependent relaxation. Arterioscler. Thromb. Vasc. Biol. 14(746-752, 1994.
82. Kuhlencordt PJ, Gyurko R, Han F, Scherrer-Crosbie M, Aretz TH, Hajjar R, Picard MH, Huang PL. Accelarated atherosclerosis, aortic aneurysm formation, and ischemic heart disease in apolipoprotein E/endothelial nitric oxide synthase double-knockout mice. Circulation 104(448-454, 2001.
83. De Bakey ME, Lawrie G, Glaeser DH. Patterns of atherosclerosis and their surgical significance. Annals of Surgery 201(115-131, 1985.
84. Virmani R, Kolodgie FD, Burke AP, Farb A, Schwartz SM. Lessons from sudden coronary death: A comprehensive morphological classification scheme for atheroscelrotic lesions. Arterioscler. Thromb. Vasc. Biol. 20(1262-1275, 2000.
85. Stary HC. Macrophages, macrophae foam cells, and eccentric intimal thickening in coronary arteries of young children. Atherosclerosis 64(91-108, 1987.
86. Stary HC, Chandler AB, Glagov S, J.R G, Insull W, Rosenfeld ME, Schaffer SA, Schwartz CJ, Wagner WD, Wissler RW. A definition of initil, fatty streak, and intermediate lesions of atherosclerosis. A report from the Committee on Vascular Lesions of the Council on Arteriosclerosis, American Heart Association. Circulation 89(2462-2478, 1994.
87. Stary HC. Evolution and progression of atherosclerotic lesions in coronory arteries of children and young adults. Arteriosclerosis 9(Supp I): I-19-I-32, 1989.
88. Munro JM, van der Walt JD, Munro CS, Chalmers JA, Cox EL. An immunohistochemical analysis of human aortic fatty streaks. Hum Pathol 18(479-497, 1987.
89. Katsuda S, Boyd HC, Fligner C, Ross R, Gown AM. Human atherosclerosis, part III: immunocytochemical analysis of the cell composition of lesions of young adults. Am J Pathol 140(907-914, 1992.
90. Stary HC, Chandler AB, Dinsmore RE, Fuster V, Glagov S, Insull W, Rosenfeld ME, Schwartz CJ, Wagner WD, Wissler RW. A definition of advanced types of atherosclerotic esions and a histological classification of atherosclerosis. Arteriosclerosis, Thrombosis, and Vascular Biology 15(1512-1531, 1995.
91. Bjorkeud S, Bjorkeud B. Apoptosis is abundant in human atheroscelrotic lesions, especially in inflammtory cells (macrophages and T cells), and may contributr to the accumulation of gruel and plaque instability. Am J Pathol 149(367-380, 1996.

92. Shah PK, Falk E, Badimon JJ, al e. Human monocyte-derived macrophages induce collagen breakdown in fibrous caps of atherosclerotic plaques: potential role of matrix-degrading metalloproteinases and implications for plaque rupture. Circulation 92(1565-1569, 1995.
93. Shah PK, Galis ZS. Matrix metalloproteinase hypothesis of plaque rupture. Circulation 104(1878-1880, 2001.
94. Sukhova GK, Schonbeck U, Rabkin E, Schoen FJ, Poole R, Billinghurst RC, Libby P. Evidence for increased collagenolysis by interstitial collagenases-1 and -3 in vulnerable human atheromatous plaques. Circulation 99(2503-2509, 1999.
95. DiCorleto PE, Chisolm GM. Participation of the endothelium in the development of the atherosclerotic plaque. Prog. Lipid Res 25(365-374, 1986.
96. Mora R, Lupu F, Simionescu N. Atherosclerosis 67(143-154, 1987.
97. Ross R. The pathogenesis of atherosclerosis: a perspective for the 1990s. Nature 362(801-809, 1993.
98. Williams KJ, Tabas I. The response-to-retention hyphothesis of early atherogenesis. Arterioscler. Thromb. Vasc. Biol. 15(551-561, 1995.
99. Nievelstein PFEM, Fogelman AM, Mottino G, Frank JS. Lipid accumulation in rabbit aortic intima 2 hours after bolus infusion of low density lipoprotein. J Lab Clin Med 48(43-50, 1991.
100. Hurt-Camejo E, Camejo G, Rosengren B, Lopez F, Ahlstrom C, GFager G, Bondjers G. Effect of arteril proteoglycans and glycosaminoglycans on low density lipoprotein oxidation and its uptake by human macrophages and arterial smooth muscle cells. Arterioscler. Thromb. Vasc. Biol. 12(569-583, 1992.
101. Skalen K, Gustafsson M, Rydberg EK, HUlten LM, Wiklund O, Innerarity TL, Boren J. Subendothelial retention of atherogenic lipoproteins in early atherosclerosis. Nature 417(750-754, 2002.
102. Ross R. Atherosclerosis-an inflammatory disease. N Eng J Med 340(115-126, 1999.
103. Ridker PM, Hennekens CH, Buring JE, Rifai N. C-reactive protein and other markers of inflammtion in the prediction of cardiovascular disease in women. N Eng J Med 342(836-843, 2000.
104. Ridker PM, Rifai N, Pfeffer M, Sacks F, Lepage S, Braunwald E. Elevation of tumor necrosis factor-a and increased risk of recurrent coronary events after myocardial infarction. Circulation 101(2149-2153, 2000.
105. Ridker PM, Rifai N, Stampfer MJ, Hennekens CH. Plama concentration of interleukin-6 and the risk of future myocardial infarction among apparently healthy men. Circulation 101(1767-1772, 2000.
106. Ablij HC, Meinders AE. C-reactive protein: history and revival. Eur J Intern Med 13(412-422, 2002.
107. Biasucci LM, Vitelli A, Liuzzo G. Elevated levels of IL-6 in unstable angina. Circulation 94(506-512, 1999.
108. Wick G, Perschinka H, Millonig G. Atherosclerosis as an autoimmune disease: an update. Trends in Immunology 22(665-669, 2001.
109. Xu Q, Dietrich H, Steiner HJ, Gown AM, Schoel B, MIkuz G, Kaufmann SH, Wick G. Induction of arterisclerosis in normocholesteromic rabbits by immunization with heat shock protein 65. Arterioscler. Thromb. 12(789-799, 1992.
110. Xu Q, Kleindienst R, Schett G, Waitz W, Jindal S, Gupta RS, Dietrich H, Wick G. Regression of atherosclerotic lesions induced by immunization with heat shock protein 65-containing material in normocholesterolemia, but not hypercholesterolemic, rabbits. Atherosclerosis 123(145-155, 1996.

111. George J, Shoenfeld Y, Afek A, Gilburd B, Keren P, Shaish A, Kopolovic J, Wick G, Harats D. Enhanced fatty streak formation in C57BL/6J mice by immunization with heat shock protein-65. Arterioscler. Thromb. Vasc. Biol. 19(505-510, 1999b.
112. Xu Q, al e. Association of serum antibodies to heat-shock protein 65 with carotid atheroscelrosis. Lancet 341(255-259, 1993.
113. Voltonen VV. Role of infections in atherosclerosis. Am Heart J 138(S431-S433, 1999.
114. Kiechl S, al e. Chronic infections and the risk of carotid atherosclerosis: prospective results from a large population study. Circulation 103(1064-1070, 2001.
115. Witztum JL. The oxidation hypothesis of atherosclerosis. Lancet 344(793-795., 1994.
116. Parums DV, Brown DL, Mitchinson MJ. Serum antibodies to axideised low-density lipoprotein and ceroid periaortitis. Arch. Pathol. Lab. Med. 114(383-387, 1990.
117. Stemme S, Faber B, Holm J, Wiklund O, Witztum JL, Hansson GK. T lymphocytes from human atherosclerotic plaques recognize oxideised low density lipoprotein. Proc Natl Acad Sci USA 92(3893-3897, 1995.
118. Watanabe Y. Serial inbreeding of rabbits with heriditary hyperlipidemia (WHHL-rabbit): incidence and development of atherosclerosis and xanthoma. Atherosclerosis 36(261-268, 1980.
119. Buja LM, Goldstein JL, Browns MS. Cellular pathology of progressive atherosclerosis in WHHL rabbit. an animal model of famillial hypercholesterolemia. Arteriosclerosis 3(87, 1983.
120. Brousseau ME, Hoeg JM. Transgenic rabbits as models for atherosclerosis research. Journal of Lipid Research 40(365-375, 1999.
121. Gottlieb H, Lalich JJ. The occurence of arterisclerosis in the aorta of swine. Am J Pathol 30(851-855, 1954.
122. Jensen TW, Mazur MJ, Pettigew JE, Perez-Mendoza VG, XZachary J, Schook LB. A cloned pig model for exmining atherosclerosis induced by high fat , high cholesterol diets. Animal Biotechnology 21(179-187, 2010.
123. Bocan TMA. Animal models of atheroscelrosis and interpretation of drug intervention studies. Current Pharmaceutical Designs 4(37-52, 1998.
124. Kusumi Y, Scanu AM, McGill HC, Wissler RW. Atherosclerosis in a rhesus monkey with genetic hypercholesterolemia and elevated plasma Lp (a). Atherosclerosis 99(165-174, 1993.
125. Tompson JS. Atheromata in an inbred strain of mice. J. Atheroscler Res 10(113, 1969.
126. Roberts A, Thompson JS. Inbred mice and their hybrids as an animal model for atherosclerosis reserch. Adv Exp Med Biol 67(313, 1976.
127. Paigen B, Marrow A, Brandon C, MItchell D, Holmes PA. Variation in susceptibility to atherosclerosis among inbred strains of mice. Atherosclerosis 57(65, 1985.
128. Shi W, Wang NJ, Shih DM, Sun VZ, Wang X, Lusis AJ. Determinants of atherosclerosis susceptibility in the C3H and C57BL/6 metabolism. Circulation Research 86(1078-1084, 2000.
129. Kashyap VS, Santamaria-Fojo S, Brown DR, Parrott CL, Applebaum-Bowden D, Meyn S, Talley G, Paigen B, Maeda N, Brewer HBJ. Apolipoprotein E deficiency in mice: gene replacement and prevention of atherosclerosis using adenovirus vectors. J Clin Invest 96(1612-1620, 1995.
130. Plump AS, Smith JD, Hayek T, K A-S, Walsh A, Verstuyft JG, Rubin EM, Breslow JL. Sevare hypercholedterolemia and atherosclerosis in apolipoprotein E-deficeint mice created by homologous recombination in ES cells. Cell 71(343-353, 1992.
131. Piedrahita JA, Zhang SH, Hagaman JR, Oliver M, Maeda N. Generation of mice carrying a mutant apolipoprotein E gene inactivated by gene targeting in embryonic stem cell. Proc Natl Acad Sci USA 89(4471-4475): 1992.

132. Zhang SH, Reddick RL, Piedrahita JA, Maeda N. Spontaneous hypercholesterolemia and arterial lesions in mice lacking apolipoprotein E Science 258(468-471, 1992.
133. Tenger C, Zhou X. Apolipoprotein E modulates immune activation by acting on antigen presenting cell. Immunology 109(392-397, 2003.
134. Curtis L, Forte T, Davies P. Cord blood plasma lipoproteins inhibit mitogen-stimulated lymphocyte proliferation. Journal of Immunology 133(1379-1384, 1984.
135. Laurat E, Poirier B, Tupin E, Caliguiri G, Hansson GK, Bariety J, Nicoletti A. In vivo downregulation of T helper cell 1 immune responses reduces atherogenesis in apolipoprotein E-knockout mice. Circulation 104(197-202, 2001.
136. Nakashima Y, Plump AS, Raines EW, Breslow JL, Ross R. ApoE-deficient mice develop lesions of all phases of atherosclerosis throughout the arterial tree. Arteriosclerosis, Thrombosis, and Vascular Biology 14(133-140, 1994.
137. Ishibashi S, Brown MS, Goldstein JL, Gerard RD, Hammer RE, Herz J. Hypercholesterolemia in LDL receptor knock out mice and its reversal by adenovirus-mediated gene delivery. J Clin Invest 92(883-893, 1993.
138. Brown MS, Goldstein JL. A receptor-mediated pathway for cholesterol homeostasis. Science 232(34-47, 1986.
139. Ishibashi S, Goldstein JL, Brown MS, Herz J, Burns DK. Massive xanthomatosis and atherosclerosis in cholesterol-fed low density lipoprotein receptor-negative mice. J Clin Invest 93(1885-1893, 1994.
140. Tangirala RK, Rubin EM, Palinski W. Quantitation of atheroscelrosis in murine models: correlation between lesions in the aortic origin and in the entire aorta, and differences in the extent of lesions between sexes in LDL receptor-deficent and apolipoprotein E-deficient mice. J lipid Research 36(2320-2328, 1995.
141. Sjoland H, Eitzman DT, Gordon D, Westrick R, Nabel EG, Ginsburg D. Atherosclerosis progression in LDL-receptor deficient and apolipoprotein E-deficient mice is independent of genetic alterations in plasminogen activator inhibitor-1. Artrioscler Thromb. Vasc. Biol 20(846-852, 2000.
142. de Kniff P, van den Maagdenberg AM, Stalenhoef AF, Leuven JA, Demacker PN, Kuyt LP, Frants RR, Havekes LM. Familial dysbetalipoprotenemia associated with apolipoprotein E3-Leiden in an extended multigeneration pedigree. J Clin Invest 88(643-655, 1991.
143. Lutgens E, Daemen M, Kockx M, Doevandans P, Hofker M, Havekes L, Wellens H, Ebo D, de Muinck ED. Atherosclerosis in ApoE*3-Leiden transgenic mice: from proliferative to atheromatous stage. Circulation 99(276-283, 1999.
144. Havakes L, de Wit E, Leuven JG, Klasen E, Utermann G, Weber W, Beisiegel U. Apolipoprotein E3-Leide. A new variant of human apolipoprotein E associated with familial type 111 hyperlipoproteinemia. Hum Genet 73(157-163, 1986.
145. van Vlijmen BJM, van den Maagdenberg AMJM, Gijbels MJJ, van der Boom H, HogenEsch H, Frants RR, Hofker MH, Havekes LM. Diet-induced hyperlipoproteinemia and atherosclrosis in apolipoprotein E3-Leiden transgenic mice. J Clin Invest 93(1403-1410, 1994.
146. Young SG. Recent progress in understanding apolipoprotein B. Circulation 82(1574-1594, 1990.
147. LInton MF, Farese RV, Chiesa G, Grass DS, Chin P, Hammer RE, Hobbs HH, Young SG. Transgenic mice expressing high plasma concentrations of human apolipoprotein B100 and lipoprotein (a). J Clin Invest 92(3029-3037, 1993.
148. Innerarity TL, Mahley RW, Weisgraber KH, Bersot TP, Krauss RM, Vega GL, Grundy SM, Friedl W, Davignon J, McCarthy BJ. Familial defective apoliprotein B100: a

mutation of apolipoprotein B that causes hypercholesterolamia. J Lipid Research 31(1337-1349, 1990.
149. Purcell-Huynh DA, Farese RV, Johnson DF, Flynn LM, Pierotti V, Newland DL, LInton MF, Sanan DA, Young SG. Transgenic mice expressing high levels of human apolipoprotein B develop severe atherosclerotic lesions in response to a hig-fat diet. J Clin Invest 95(2246-2257, 1995.
150. Jonasson L, Holm J, Skalli O, Gabbiani G, Hansson GK. Expression of class II transplantation antigen on vascular smooth muscle cells in human atherosclerosis. J Clin Invest 76(125-131, 1985.
151. Ross R, Masuda J, Raines EW, Gown AM, Katsuda S, Sasahara M, Malden LT, Masuko H, Sato H. Localization of PDGF-B protein in macrophages in all phases of atherogenesis. Science 248(1009-1012, 1990.
152. Barath P, Fishlein MC, Cao J, Berenson J, Helfant RH, Forrester JS. Detection and localization of tumor necrosis factor in human atheroma. Am J Cardiol 65(297-302, 1990.
153. Schrader JW, Moyer C, Ziltener HJ, Reinisch CL. Release of the cytokine colony-stimulating factor-1,granulocyte-macrophage colony-stimulating factor, and IL-6 by cloned murine vascular smooth muscle cells. J Immunol 146(3799-3808, 1991.
154. Nelken NA, Coughlin SR, Gordon D, Wilcox JN. Monocyte-chemoattractant protein-1 in human atheromatous plaques. J Clin Invest 88(1121-1127, 1991.
155. Hansson GK, Holm J, Jonasson L. Detection of activated T lymphocytes in the human atherosclerotic plaque. Am J Pathol 135(169-175, 1989.
156. Whitman SC, Ravishankar P, Elam H, Daugherty A. Exogenous interferon-gamma enhances atherosclerosis in apolipoprotein E-/- mice. Am J Pathol 157(1819-1824, 2000.
157. Whitman SC, Ravishankar P, Daugherty A. IFN-gamma deficiency exerts gender-specific effcts on atherogenesis in apolipoprotein E-/- mice. J Interferon Cytokine Res 22(661-670, 2002.
158. Gupta S, Pablo AM, Jiang XC, Wang NJ, Tall AR, Schindler C. IFN-gamma potetiates atherosclerosis in apoEknock-out mice. J Clin Invest 99(2752-2761, 1997.
159. Whitman SC, Ravishankar P, Daugherty A. Interleukin-18 enhances atheroscelrosis in apolipoprotein E-/- mice through release of interferon-g. Circulation Research 90(E17-E22, 2002.
160. Tenger C, Sundborger A, Jawien J, Zhou X. Il-18 accelerates atheroscelrosis accompanied by elevation of IFN-gamma and CXCL16 expression independently of T cells. Arterioscler. Thromb. Vasc. Biol. 25(791-796, 2005.
161. Elhage R, Maret A, Pieraggi M-T, Thiers JC, Arnal JF, Bayard F. Differential effects of interleukin-1 receptor antagonist and tumor necrosis factor binding protein on fatty-streak formation in apolipoprotein E-deficient mice. Circulation 97(242-244, 1998.
162. Kirii H, Niwa T, Yamada Y, Wada H, Saito K, Iwakura Y, Asano M, Moriwaki H, Seishima M. Lack of interleukin-1b decreases the severity of atherosclerosis in ApoE-deficient mice. Arterioscler. Thromb. Vasc. Biol. 23(656-660, 2003.
163. Kamari Y, Wermann-Verkert R, Shaish A, Werman A, Harari A, Gonen A, al e. Differential role and tissue speicificity of interleukin-1 alpha gene expression in atherogenesis and lipid metabolism. Atherosclerosis 195(31-38, 2007.
164. Davenport P, Tipping PG. The role of interleukin-4 and interleukin-12 in the progression of atherosclerosis in apolipoprotein E-deficient mice. The American Journal of Pathology 163(1117-1125, 2003.
165. Ohta H, Wada H, Niwa T, Kirii H, Iwamoto N, Fujii H, Saito K, Sekikawa K, Seishima M. Distruption of tumor-necrosis factor-alpha gene diminishes the development of atherosclerosis in ApoE-deficient mice. Atherosclerosis 180(11-17, 2005.

166. Schreyer SA, Peschon JJ, Leboeuf RC. Accelerated atheroscelrosis in mice lacking tumor necrosis factor p55. J Biol Chem 271(26174-26178, 1996.
167. Schreyer SA, Vick CM, Leboeuf RC. Loss of lymphotoxin-alpha but not tumor necrosis factor-alpha reduces atherosclerosis. J Biol Chem 277(12364-12368, 2002.
168. Schreyer SA, Vick CM, Leboeuf RC. Loss of lymphotoxin-alpha but not tumor necrosis factor-alpha reduces atherosclerosis in mice. Arterioscler. Thromb. Vasc. Biol. 25(1256-1261, 2002.
169. Wakkach A, Cottrez F, Groux H. Can interleukin-10 be used as a true immunoregulatory cytokine? Eur Cytokine Netw 11(153-160, 2000.
170. Bogdan C, Vodovotz Y, Nathan C. Macrophage deactivation by interleukin-10. J Exp Med 174(1549-1555, 1991.
171. Tedgui A, Mallat Z. Anti-inflammtory mechanisms in the vessel wall. Circulation Research 88(877-887, 2001.
172. Mallat Z, Besnard S, Duriez M, Deleuze V, Emmanuel F, Bureau MF, Soubrier F, Esposito B, al e. Protective role of interleukin-10 in atherosclerosis. Circulation Research 85(e17-e24, 1999.
173. Pinderski Oslund LJ, Hendrick CC, Olvera T, Hagenbaugh A, Territo M, al. e. Interleukin-10 blocks atherosclerotic events in vitro and in vivo. Arterioscler. Thromb. Vasc. Biol. 19(4847-2853, 1999.
174. Caliguiri G, Rudling M, Ollivier V, Jacob MP, Michel JB, Hansson GK, Nicoletti A. Interleukin-10 deficiency increases atherosclerosis, thrombosis, and low-density lipoproteins in apolipoprotein E knockout mice. Mol Med 9(10-17, 2003.
175. Potteaux S, Esposito B, Oostrom Ov, Brun V, Ardouin P, Groux H, Tedgui A, Mallat Z. Leukocyte-derived interleukin 10 required for protection against atherosclerosis in low-density lipoprotein receptor knockout mice. Arterioscler. Thromb. Vasc. Biol. 24(1474-1478, 2004.
176. Grainger DJ, Mosedale DE, Metcalfe JC, Bottinger EP. Dietry fat and reduced levels of TGFbeta1 act synergistically to promote activation of the vascular endothelium and formation of lipid lesions. Journal of Cell Science 113(132355-132361, 2000.
177. Grainger DJ, Kemp BR, Metcalfe JC, Liu AC, Al. e. The serum concentration of active trasnforming growth factor-beta is severly depressed in advanced atherosclerosis. Nature Medicine 1(74-79, 1995.
178. Mallat Z, Gojova A, Marchiol-Fournigault C, Esposito B, Kamate C, Mervel R, Fradelizi D, Tedgui A. Inhibition of transforming groeht factor-beta signalling accelerates atheroscelrosis and induces an unstable plaque phenotype in mice. Circulation Research 89(930-934, 2001.
179. Gojova A, Brun V, Esposito B, Cottrez F, Gourdy P, Ardouin P, Tedgui A, Mallat Z, Groux H. Specific abrogation of transforming factor-b signalling in T cells alters atherosclerotic lesion size. Blood 102(4052-4058, 2003.
180. Upadya S, Mooteri S, Peckham N, Pai RG, Linda L. Atherogenic effect of interleukin-2 and antiatherogenic effect of interleukin-2 antibody in Apo-E-deficient mice. 2004 55(289-294, 2004.
181. Huber SA, Sakkinen P, Conze D, Hardin N, Tracey R. Interleukin-6 exacerbates early atherosclerosis in mice. Arterioscler. Thromb. Vasc. Biol. 19(2364-2367, 1999.
182. Schieffer B, Selle T, Hilfiker A, Hilfiker-Kleiner D, Grote K, Tietge UJF, Trautwein C, al e. Impact of interleukin-6 on plaque development and morphology in experimental atherosclerosis. Circulation 110(3493-3500, 2004.
183. Elhage R, Clamens S, Bernard S, Mallat Z, Tedgui A, Arnal JF, Maret A, Bayard F. Involvement of interleukin-6 in atherosclerosis but not in the prevention of fatty streak

formation by 17 beta-estradiol in apolipoprotein E-deficient mice. Atherosclerosis 156(315-320, 2001.
184. Song L, Schindler C. Il-6 and the acute phase response in murine atherosclerosis. Atherosclerosis 177(43-51, 2004.
185. Kishimoto T. The biology of interleukin-6. Blood 74(1-10, 1989.
186. Kleeman R, Zadelaar S, Kooistra T. Cytokines and atherosclerosis: a comprehensive review of studies in mice. Cardiovascular Research 79(360-376, 2008.
187. George J, Mulkins M, Shaish A, Casey S, Schatzman R, Sigal E, Harats D. Interleukin (IL)-4 deficiency does not influence fatty streak formation in C57BL/6 mice. Atherosclerosis 153(403-411, 2000.
188. Carnaud C, Lee D, Donnors O, Park S-H, Beavis A, Koezuka Y, Bendelac A. Cutting edge: cross-talk between cells of the innate immune system: NKT rapidly activate NK cells. J Immunol 163(4647-4650, 1999.
189. Buono C, Come CE, Stavrakis G, Maguire GF, Connelly PW, Lichtman AH. Influence of interferon-g on the extent and phenotype of diet -induced atherosclerosis in the LDLR-deficient mice. Arterioscler. Thromb. Vasc. Biol. 23(454-460, 2003.
190. Whitman SC, Ravishankar P, Elam H, Daugherty A. Exogenous interferon-g enhances atherosclerosis in apolipoprotein E-/- mice. American Journal of Pathology 157(1819-1824, 2000.
191. Elhage R, Jawien J, Rudling M, Ljunggren HG, Takeda K, Akira S, Bayard F, Hansson GK. Reduced atherosclerosis in interleukin-18 deficient apolipoprotein E-knockout mice. Cardiovascular Research 59(234-240, 2003.
192. Branen L, Hovgaard L, Nitulescu M, Bengtsson E, Nilsson J, Jovinge S. Inhibition of tumor necrosis factor-alpha reduces atherosclerosis in apolipoprotein E knockout mice. Arterioscler. Thromb. Vasc. Biol. 24(2137-2142, 2004.
193. King VL, Szivassy SJ, Daugherty A. Interleukin-4 deficiency decreases atherosclerotic lesion formation in a site-specific manner in female LDL receptor-/- mice. Arterioscler. Thromb. Vasc. Biol. 22(456-461, 2002.
194. Huber SA, Sakkinen P, David C, Newell MK, Tracey RP. T helper-cell phenotype regulates atherosclerosis in mice under conditions of mild hypercholesterolemia. Circulation 103(2610-2616, 2001.
195. Pinderski LJ, Fischbein MP, Subbanagounder G, Fishbein MC, Kubo N, Cheroutre H, Curtiss LK, Berliner JA, Boisvert WA. Overexpression of interleukin-10 activated T lymphocytes inhibits atherosclerosis in LDL receptor-deficient mice by altering lymphocyte and macrophage phenotype. Circulation Research 90(10641071, 2002.
196. Calligiuri G, Rudling M, Ollivier V, Jacob MP, Michel JB, Hansson GK, Nicoletti A. Interleukin-10 deficiency increases atherosclerosis, thrombosis and low density lipoprotein in apolipoprotein E knockout mice. Molecular Medicine 9(10-17, 2003.
197. Mallat Z, Gojova A, Marchiol-Fournigault C, Esposito B, Kamate C, Merval R, Fradelizi D, Tedgui A. Inhibition of trasforming growth factor-b signalling accelerates atherosclerosis and induces an unstable plaque phenotype in mice. Circulation Research 89(930-934, 2001.
198. Lutgens E, Gijbels M, Smook M, Heeringa P, Gotwals P, Koteliansky VE, Daeman MJAP. Transforming growth factor-b mediates balance between inflammation and fibrosis during plaque progression. Arterioscler. Thromb. Vasc. Biol. 22(975-982, 2002.
199. Zernecke A, Shagdarsuren E, Weber C. Chemokines in atherosclerosis. Arterioscler. Thromb. Vasc. Biol. 28(1897-1908, 2008.
200. Gu L, Okada Y, Clinton SK, Gerard C, Sukhova GK, Libby P, Rollins BJ. Absence of monocyte chemoattractant protein-1 reduces atherosclerosis in low density lipoprotein receptor-deficient mice. Mol Cell 2(275-281, 1998.

201. Boring L, Gosling J, al. e. Decreased lesion formation in CCR2-/- mice reveals a role for chemokines in the initiation of atherosclerosis. Nature 394(894-897, 1998.
202. Gosling J, Slaymaker S, Gu L, Tseng S, et.al. MCP-1 deficiency reduces susceptibility to atherosclerosis in mice that overexpress human apolipoprotein B. J Clin Invest 103(773-778, 1999.
203. Robertson MJ. Role of chemokines in the biology of natural killer cells. Journal of Leukocyte Biology 71(173-183, 2002.
204. Veillard NR, Kwak B, Pelli G, al. e. Antagonism of RANTES receptors reduces atheroscelrotic plaque formation in mice. Circulation Research 94(253-261, 2004.
205. Kuziel WA, Dawson TC, Quinones M, Garavito E, Chenaux G, Ahuja SS, Reddick RL, Maeda N. CCR5 deficiency is not protective in the early stages of atherogenesis in apoE knockout mice. Atherosclerosis 167(25-32, 2003.
206. Quinones MP, Martinez HG, Jimenez F, Estrada CA, Dudley M, Willmon O, Kulkarni H, Reddick RL, Fernandes G, Kuziel WA, Ahuja SK, Ahuja SS. CC chemokine receptor 5 influences late stage atherosclerosis. Atherosclerosis 195(e92-e103, 2007.
207. Potteaux S, Combadiere C, Esposito B, Lecureuil C, al. e. Role of bone marrow-derived CC-Chemokine Receptor 5 in the development of atherosclerosis of low density lipoprotein receptor knockout mice. Arterioscler. Thromb. Vasc. Biol. 26(1858-1863, 2006.
208. Braunersreuther V, Zernecke A, Arnaud C, Liehn EA, Steffens S, Shagdarsuren E, Bidzhekov K, Burger F, Pelli G, Lucknow B, Mach F, Weber C. Ccr5 nor Ccr1 deficeincy reduces development of diet-induced atherosclerosis in mice. Arterioscler. Thromb. Vasc. Biol. 27(373-379, 2007.
209. Zernecke A, Liehn EA, Gao JL, et.al. CCR5 but not CCR1 protects against neointima formation in atherosclerosis-prone mice: involvement of IL-10. Blood 107(4240-4243, 2006.
210. Shang X, Qiu B, Frait KA, Hu JS, Sonstein J, Curtis JL, Lu B, Gerard C, Chensue SW. Chemokine receptor 1 knockout abrogates natural killer cell recruitment and impairs type-1 cytokines in lymphoid tissue during pulmonary granuloma formation. Am J Pathol 157(2055-2063, 2000.
211. Braunersreuther V, Mach F, Steffens S. The specific role of chemokines in atherosclerosis. Thromb Haemost 97(714-721, 2007.
212. Kraaijeveld AO, de Jager SC, van Berkel TJ, Biessen EA, Jukema JW. Chemokines and atherosclerosis plaque progression:towards therapeutic targeting? Curr Pharm Des 13(1039-1052, 2007.
213. Ludwig A, Weber C. Transmembrane chemokines: versatile 'special agents' in vascular inflammtion. Thromb Haemost 97(694-703, 2007.
214. Lesnik P, Haskell CA, Charo IF. Decreased atherosclerosis in CX3CX1-/- mice reveals a role for fractalkine in atherogenesis. J Clin Invest 111(333-340, 2003.
215. Conmbadiere C, Potteaux S, Gao J-L, Esposito B, Casanova S, Lee EJ, Debre P, Tedgui A, Murphy PM, Mallat Z. Decreased atherosclerotic lesion formation in CX3CR1/apolipoprotein E doublr knockout mice. Circulation 107(1009-1016, 2003.
216. Teupser D, Pavlides S, Tan M, Gutierrez-Ramos JC, Kolbeck R, Breslow JL. Major reduction of atherosclerosis in fractalkine (CX3CR1)- deficient mice is at brachiocephalic artery, not the aortic root. Proc Natl Acad Sci USA 101(17795-17800, 2004.
217. Saederup N, Chan L, Lira SA, Charo IF. Fractalkine deficiency markedly reduced macrophage accumulation and atheroscelrotic lesion formation in $CCR2^{-/-}$ mice. Circulation 117(1642-1648, 2008.

218. Zernecke A, Bot I, Djalali-Talab Y, Shagdarsuren E, Bidzhekov K. Protective role of CXCR4 / CXC ligand 12 unveils the importance of neutrophils in atherosclerosis. Circulation Research 102(209-217, 2008.
219. Gerrity RG, Naito HK, Richardson M, Schwartz CJ. Dietry induced atherogenesis in swine. Morphology of the intima in prelesion stages. Am J Pathol 95(775-792, 1979.
220. Auffray C, Siewenka MH, Geissmann F. Blood monocytes: development, heterogeneity, and relationship with dendritic cells. Annu. Rev. Immunol 27(669-692, 2009.
221. Geissmann F, Auffray C, Palframan R, Wirrig C, Ciocca A, Campisi L, Narni-Mancinelli E, Lauvau G. Blood monocytes: distinct subsets, how they relate to dendritic cells, and thier possible roles in the regulation of T-cell responses. Immunol Cell Biol 86(398-408, 2008.
222. Randolph GJ, Jacubzick C, Qu C. Antigen presentation by monocytes and monocyte-derived cells. Curr. Opin. Immunol 20(52-60, 2008.
223. Woollard KJ, Geissmann F. Monocytes in atherosclerosis: subsets and functions. Nature Review Cardiology 7(77-88, 2010.
224. Geissmann F, Jung S, Litmann DR. Blood monocytes consist of two principal subsets with distinct migratory properties. Immunity 19(71-82, 2003.
225. Tacke F, Alvarez D, Kaplan TJ, Jacubzick C, Spanbroek R. Monocyte subsets differentially employ CCR2, CCR5, CX3CR1 to accumulate within atherosclerotic plaques. J Clin Invest 117(185-194, 2007.
226. Swirki FK, Libby P, Aikaw E, Alcaide P, Luscinskas FW. Ly6C^{hi} monocytes dominate hypercholesteromia-associated monocytosis and give rise to macrophages in aheromata. J Clin Invest 117(195-205, 2007.
227. Combadiere C, Potteaux S, Rodero M, Simon T, Pezard A. Combined inhibition of CCL2, CX3CR1 and CCR5 abrogates Ly6C^{hi} and Ly6C^{lo} monocytosis and almost abolishes atheroslerosis in hypercholesteromic mice. Circulation 117(1649-1657, 2008.
228. Saederup N, Chan L, Lira SA, Charo IF. Fractalkine defiency markedly reduces macrophages accumulation and atherosclerotic lesion formation in CCr2-/- mice: evidence for independent chemokine functions in athersogenesis. Circulation 117(1642-1648, 2008.
229. Mantovani A, Sica A, Locati M. Macrophage polarization comes of age. Immunity 23(344-346, 2005.
230. Greaves DR, Gordon S. Recent insights into the biology of macrophage scavenger receptors. J Lipid Research 46(11-20, 2005.
231. Suzuki H, Kurihara Y, al. e. A role for macrophage scavenger receptors in atherosclerosis and susceptibility to infection. Nature 386(292-296, 1997.
232. Babaev VR, Gleaves LA, al e. Reduced atherosclerotic lesions in mice deficient for total or macrophage-specific expression of scavenger receptor-A. Arterioscler. Thromb. Vasc. Biol. 20(2593-2599, 2000.
233. Febbraio M, Podrez EA, al. e. Targeted disruption of the class B scavenger CD36 protects against atherosclerotic lesion developmen tin mice. J Clin Invest 105(1049-1056, 2000.
234. Aiello RJ, Bourassa P-AK, Lindsey S, Weng W, Natoli E, Rollins BJ, Milos PM. Monocyte chemoattractant protein-1 accelerates atherosclerosis in apolipoprotein E-deficient mice. Arterioscler. Thromb. Vasc. Biol. 19(1518-1525, 1999.
235. Nathan CF. Secretory products of macrophages. J Clin Invest 79(319-326, 1987.
236. Dickhout JG, Hossain GS, Pozza LM, Zhou J, Lhotak S, Austin R. Peroxynitrite causes endoplasmic reticulum stress and apoptosis in human vascular endothelium. Arterioscler. Thromb. Vasc. Biol. 25(2623-2629, 2005.

237. Li Y, Gerbod-Giannone MC, Seitz H, Cui D, Thorp E, Tall AR, Matsushima GK, Tabas I. Cholesterol-induced apoptotic macrophages elicit an inflammatory response in phagocytes, which is partially attenuated by the MER receptor. . J Biol Chem 281(6707-6717, 2006.
238. Tabas I. Consequences and therapeutic implications of macrophage apoptosis in atherosclerosis: the importance of lesion stage and phagocytic efficiency. Arterioscler. Thromb. Vasc. Biol. 25(2255-2264, 2005.
239. van Vlijmen BJM, Gerritsen G, Franken AL, Boesten LSM, Kockx MM, Gijbels MJJ, Vierboom MP, al e. Macrophage p53 deficiency leads to enhanced atherosclerosis in apoE*-Leiden transgenic mice. Circulation Research 88(780-786, 2001.
240. Kim YM, Talanian RV, Li J, Billiar TR. Nitric oxide prevents IL-1 beta and IFN-gamma-inducing factor (IL-18) release from macrophages by inhibiting caspase-1 (IL-1beta-converting enzyme). J Immunol 161(4122-4128, 1998.
241. Jones CB, Sane DC, Herrington DM. Matrix metalloproteinases: A review of their structure and role in coranary syndrome. Cardiovascular Research 59(812-823, 2003.
242. Erbel C, Sato K, Meyer FB, Kopecky SL, al. e. Functional profile of activated dendritic cells in unstable atherosclerotic plaque.`. Basic Res. Cardiol 102(123-132, 2007.
243. Bobryshev YV, Taksir T, Lord RS, Freeman MW. Evidence that dendritic cells infiltrate atheroscelrotic lesions in apolipoproteinE-deficient mice. Histol Histopathol 16(801-808, 2001.
244. Jongstra-Bilen J, Haidari M, Zhu SN, Chen m, Guha D, Cybulsky MI. Low-grade chronic inflammation in regions of normal mouse arterial intima predisposed to atehrsoclerosis. J Exp Med 203(2073-2083, 2006.
245. Liu P, Yu YR, Spencer JA, Johnson AE, Vallanat CT, Fong AM, Patterson C, Patel DD. CX3CR1 deficiency impairs dendritic cell accumulation in arteril intima and reduces atherosclerotic burden. Arterioscler. Thromb. Vasc. Biol. 28(243-250, 2008.
246. Paulson KE, Zhu S-N, Chen M, Nurmohamed S, Jongstra-Bilen J, Cybulsky MI. Resident intimal dendritic cells accumulate lipid and contribute to the initiation of atherosclerosis. Circulation Research 106(383-390, 2010.
247. Ma-Krupa W, Jeon MS, Spoerl S, Tedder TF, Goronzy JJ, weyand CM. Activation of arterial wall dendritic cells and breakdown of self-tolerance in giant cell arteritis. J Exp Med 199(173-183, 2004.
248. Steinman RM, Haiwiger D, Liu K, Bonifaz L, Bonnyay D, Mahnke K, Iyoda T, Ravetch JV, Dhodapkar M, al e. Dendritic cell function in vivo during the steady state : a role in peripheral tolerance. Ann NY Acad Sci 987(15-25, 2003.
249. Gautier EL, Huby T, Witztum JL, Ouzilleau B, al. e. Macrophage apoptosis exerts divergent effcts on atherogenesis as a function of lesion stage. Circulation 119(1795-1804, 2009.
250. Landsman L, Bar-On L, Zernecke A, Kim KW, al e. CX3CR1 is required for monocyte homeostasis and atherogenesis by promoting cell survival. Blood 113(963-972, 2009.
251. Gautier EL, Huby T, Saint-Charles F, Ouzilleau B, al. e. Convential dendritic cells at the crossroads between immunity and cholesterol homeostasis in atherosclerosis. Circulation 119(2397-2375, 2009.
252. Lord RS, Bobryshev YV. Clustering of dendritic cells in athero-prone areas of aorta. Atherosclerosis 146(197-198, 1999.
253. Bobryshev YV, Lord RS, Rainer SP, Munro VF. VCAM-1 expression and network of VCAM-1 positive vascular dendritic cells in advanced atherosclerotic lesions of carotid arteries and aorta. Acta Histochem 98(185-194, 1996.
254. Weis M, Schlichting CL, Engleman EG, Cooke JP. Endothelial determinants of dendritic cell adhesion and migration. Arterioscler. Thromb. Vasc. Biol. 22(1817-1823, 2002.

255. Zhang X, Niessner A, Nakajima T, Ma-Krupa W, Kopecky SL, Frye RL, Goronzy JJ, Weyand CM. Interleukin-12 induces T cell recruitment into the atherosclerotic plaque. Circulation Research 98(524-531, 2006.
256. Erbel C, Sato K, Meyer FB, Kopecky SL, Frye RL. Functional profile of activated dendritic cells in unstable atherosclerotic plaque. Basic Res. Cardiol 102(123-132, 2007.
257. Bobryshev YV, Lord RS. Co-accumulation of dendritic cells and natural killer T cells within rpture-prone regions in human atheroscelrotic plaques. J Histochem Cytochem 53(781-785, 2005.
258. Borg C, Abdelali J, Laderach Dea. NK cell activation by dendritic cellsc (DC) require the formation of a synapses leading to Il-12 polarization in DC. Blood 104(3267-3275, 2004.
259. Galkina E, Ley K. Leukocyte influx in atherosclerosis. Curr Drug Targets 8(1239-1248, 2007.
260. Galkina E, Ley K. Vascular adhesion molecules in athersoclerosis. Artrioscler Thromb. Vasc. Biol 27(2292-2301, 2007.
261. Zhou X, Nicoletti A, Elhage R, Hansson GK. Transfer of $CD4^+$ T cells aggravates atherosclerosis in immunodeficeint apolipoprotein E knockout mice. Circulation 102(2919-2911, 2000.
262. Robertson AK, Hansson GK. T cells in atherogenesis for better or for worse? Artrioscler Thromb. Vasc. Biol 26(2421-2432, 2006.
263. Zhou X, Stemme S, Hansson GK. Evidence for a local immune response in atherosclerosis. CD4+ T cells infiltrate lesions of apoliprotein-E-deficien tmice. Am J Pathol 149(359-366, 1996.
264. George JA, Afek A, al. e. Cellular and humoral immune responses to heat shock protein 65 are both involved in promoting fatty-streak formation in LDL-receptor deficient mice. J Am Coll Cardiol 38(900-905, 2001.
265. George J, Harats D. Adoptive transfer of beta (2)-glycoprotein I-reactive lymphocytes enhances early atherosclerosis in LDL receptor-deficient mice. Circulation 102(1822-1827, 2000.
266. Liuzzo G, Biasucci LM, Trotta G, Brugaletta S. Unusual $CD4^+CD28^{null}$ T lymphocytes and recurrence of acute coronary events. J Am Coll Cardiol 50(1450-1458, 2007.
267. Weyand CM, Brands JC, Schmidt A, Fulbright JW, Goronzy JJ. Functional properties of $CD4^+CD28^-$ T cells in aging immune system. Mechanisms of Ageing and Development 102(131-147, 1998.
268. Nakajima T, Schulte S, Warrington KJ, Kopecky SL, al. e. T-cell-mediated lysis of endothelial cells in acute coronary syndromes. Circulation 105(570-575, 2002.
269. Namekawa T, Wagner UG, Goronzy JJ, Weyand CM. Functional subsets of CD4 T cells in rheumatoid synovitis. Arthritis & Rheumatism 41(2108-2116, 1998.
270. Warrington KJ, Takemura S, Goronzy JJ, Weyand CM. $CD4^+$, $CD28^-$ T cells in rheumatoid arthritis patients combine features of the innate and adaptive immune systems. Arthritis & Rheumatism 44(13-20, 2001.
271. Romagnani S. T - cell subsets (Th1 versus Th2). Ann Allergy Asthma Immunol 85(9-18, 2000.
272. Zhou X, Paulsson G, Stemme S, Hansson GK. Hypercholesteromia is associated with T helper (Th) 1/Th2 switch of the autoimmune response in atherosclerotic apo E-knockout mice. J Clin Invest 101(1717-1725, 1998.
273. Liuzzo G, Kopecky SL, al e. Pertubation of the T -cell repetoire in patients with unstable angina. Circulation 100(2135-2139, 1999.
274. Phipps RP. Atherosclerosis: the emerging role of inflammation and the CD40-CD40 ligand system. Proc Natl Acad Sci USA 97(6930-6932, 2000.

275. Buono C, Binder CJ, Stavrakis G, Witztum JL, Glimcher LH, Lichtman AH. T-bet deficiency reduces atherosclerosis and alters plaque antigen-specific imune response. Proc Natl Acad Sci USA 102(1596-1601, 2005.
276. Eid RE, Rao DA, Zhou J, Lo SL, Ranjbaran H, Gallo A, Sokol SI, Pfau S, Pober JS, Tellidas G. Interluekin-17 and interferon-g are produced comcomitantly by human coronary artery-inflitrating T cells and act synergistically on vascular smmoth muscle cells. Circulation 119(1424-1432, 2009.
277. Gao Q, Jiang Y, Ma T, Zhu F, Gao F, Zhang P, al e. A critical function of Th17 pro-inflammtory cells in the development of atherosclerotic plaque in mice. J Immunol 185(5820-5827, 2010.
278. Taleb S, Romain M, al. e. Loss os SOCS3 expression in T cells reveals a regulatory role for interleukin-17 in atherosclerosis. J Exp Med 206(2067-2077, 2009.
279. Sakaguchi S, Ono M, Setoguchi R, Yagi H, Hori S, al. e. $Foxp3^{+}CD25^{+}CD4^{+}$ natural regulatory T cells in dominant self-tolerance and autoimmune disease. Immunol Rev 212(8-27, 2006.
280. Mempel TR, Pittet MJ, al e. Regulatory T cells reversibly suppress cytotoxic T cell function independent of effector differentiation. Immunity 25(129-141, 2006.
281. Ghiringhelli F, Menard C, al e. CD4+CD25+ regulatory T cells inhibit natural killer cell functions in a trasforming growth factor-beta-dependent manner. J Exp Med 202(1075-1085, 2005.
282. Azuma T, Takahashi T, al e. Human CD4+ CD25+ regulatory T cells suppress NKT cell functions. Cancer Res 63(4516-4520, 2003.
283. Lim H, Hillsamer WP, al e. Cutting edge: direct suppression of B cells by CD4+CD25+ regulatory T cells. J Immunol 175(4180-4183, 2005.
284. Heller EA, Liu E, Tager AM, Yuan Q, al e. Chemokine CXCL10 promotes atherogenesis by modulating the local balance of effector and regulatory T cells. Circulation 113(2301-2312, 2006.
285. de Boer OJ, van der Meer JJ, Teeling P, van der Loos CM, van der Wal AC. Low numbers of FOXP3 positive regulatory T cells are present in all developmental stages of human atheroscelrotic lesion. PLoS ONE 2(e779, 2007.
286. Mallat Z, Gojova A, Brun V, Esposito B, Fournier N. Induction of a regulatory T cell type 1 responses reduces the development of atherosclerosis in apolipoprotein E-knockout mice. Circulation 108(1232-1237, 2003.
287. Ait-Oufella H, Saloman BL, Potteaux S, Robertson AK, Gourdy P. Natural regulatory T cells control the development of atherosclerosis in mice. Nat Med 12(178-180, 2006.
288. Steffens S, Burger F, al e. Short term treatment with anti-CD3 antibody reduces the developemnt and progression of aherosclerosis in mice. Circulation 114(1977-1984, 2006.
289. Gotsman I, Grabie N, Gupta R, Dacosta R, MacConmara M. Impaired regulatory T-cell response and enhanced atherosclerosis in the absence of inducible costimulatory molecule. Circulation 114(2047-2055, 2006.
290. Groyer E, Nicoletti A, Ait-Oufella H, Khallou-Laschet J, Varthaman A, al. e. Atheroprotective effect of CD31 receptor globulin through enrichment of circulating regulatory T cells. J Am Coll Cardiol 50(344-350, 2007.
291. Taleb S, Herbin O, Ait-Oufella H, Verreth W, Gourdy P. Defective leptin/leptin receptor signaling improves regulatory T cell immune response and protects mice from atherosclerosis. Artrioscler Thromb. Vasc. Biol 27(2691-2698, 2007.
292. TYang K, Li D, Luo M, Hu Y. Generation of HSP60-specific regulatory T cell and effect on atherosclerosis. Cell Immunol 243(90-95, 2006.

293. van derWal AC, Das PK, Bentz van de Berg D, Van der Loos CM, Becker AE. Atherosclerotic lesions in humans. In situ immunophenotypic analysis sugeesting an immune mediated response. Lab Invest 61(166-170, 1989.
294. Kleindienst R, Xu Q, Willeit J, Waldenberger FR, Weimann S, Wick G. Immunology of atherosclerosis. Demonstration of heat shoch protein 60 expression and T lymphocytes bearing alpha/beta or gama/delta receptor in human atherosclerotic lesions. Am J Pathol 142(1927-1937, 1993.
295. Gewaltig J, Kummer M, Koella C, Cathomas G, Biedermann BC. Requirements for CD8 T-cell mgration into the human arterial wall. Human Pathology 39(1756-1762, 2008.
296. Conen KL, Jeanneret C, Hecker B, Cathomas G, Biedermann BC. Acute occulsive large vessel leading to fatal stroke in patient with systemic lupus erythematosus: arteritis or atherosclerosis? Arthritis & Rheumatism 54(908-913, 2006.
297. Kolbus D, Ramos OH, Berg KE, Persson J, Wigren M, Bjorkbacka H, al e. $CD8^{+}$T cell activation predominate early immune responses to hypercholesterolemia in ApoE-/- mice. BMC Immunology 11(58, 2010.
298. Godfrey DI, Kronenberg M. Going both ways: immune regulation via CD-1d-dependent NKT cells. J Clin Invest 114(1379-1388, 2004.
299. Chan WL, Pejnovic N, Hamilton H, Liew TV, Popadic D, Poggi A, Khan SM. Atherosclerotic abdominal aortic aneurysm and the interaction between autologous human plaque-derived vascular smooth muscle cells, type 1 NKT, and helper T cells. Circulation Research 96(675-683, 2005.
300. Nakai Y, Iwabuchi K, Fujii S, Ishimori N, Dashtsoodol N. Natural killer T cells accelerate atherogenesis in mice. Blood 104(2051-2059, 2004.
301. Franitza S, Grabovsky V, Wald O, al e. Differential usage of VLA-4 and CXCR4 by CD3+CD56+ NKT cells and CD56+CD16+ NK cells regulates their interaction with endothalil cells. Eur J Imunol 34(1333-1341, 2004.
302. Stronge VS, Salio M, Jones EY, Cerundolo V. A closer look at CD1d molecules: new horizons in studying NKT cells. Trends Immunol 28(455-462, 2007.
303. Tupin E, Nicoletti A, Elhage R, Rudling M, Ljunggren HG. CD1d-dependent activation of NKT cells aggravates atherosclerosis. J Exp Med 199(417-422, 2004.
304. To K, Agrotis A, Besra G, Bobik A, Ban-Hock T. NKT cells subsets mediate differential proatherogenic effects in $ApoE^{-/-}$ mice. Arterioscler. Thromb. Vasc. Biol. 29(671-677, 2009.
305. Yoshimoto T, Paul WE. $CD4^{+}$, $NK1.1^{+}$ T cells promptly produce interleukin 4 in response to in vivo challenge with anti-CD3. J Exp Med 179(1285-1295, 1994.
306. Eberl G, MacDonald HR. Selective induction of NK cell proliferation and cytotoxicity by activated NKT cells. Eur J Imunol 30(985-992, 2000.
307. Lindstedt KA, Mayranpaa MI, Kovanen PT. Mast cells in vunerable atherosclerotic plaques- a view to kill. J. Cell. Mol. Med. 11(739-758, 2007.
308. Tang Y, Yang YF, Wang S, al e. Mast cell degranulator compound 48-80 promotes atheroscelrotic plaque in apolipoprotein E knockout mice with perivascular carotid collar placement. Chinese Medical Journal 122(319-325, 2009.
309. Kelley JL, Chi DS, Abou Auda W, Smith JK, Krishnaswamy G. The molecular role of mast cells in atheroscelrotic cardiovascular disease. Mol Med 6(304-308, 2000.
310. Bot I, de Jager SC, Zernecke A, Lindstedt KA, Van Berkel TJ, Weber C, al e. Perivascular mast cells promote atherogenesis and induce plaque destabilization in apolipoprotein E-deficeint mice. Circulation 115(2516-2525, 2007.
311. Bot I, Bot M, van Heiningen SA, van Santbrink PJ, Lankhuizen IM, al e. Mast cell cymase inhibition reduces atherosclerotic plaque progression and improves plaque stability in ApoE-/- mice. Cardiovascular Research 89(244-252, 2011.

312. Kaartinen M, Penttila A, Kovanen PT. Mast cells in rupture-prone areas of human coronary atheromas produce and store TNF-alpha. Circulation 94(2787-2892, 1996.
313. Sun J, Sukhova GK, Wolters PJ, Yang M, Kitamoto S. Mast cells promote atherosclerosis by releasing proinflammatory cytokines. Nat Med 13(719-724, 2007.
314. Ramshaw AL, Parums DV. Immunohistochemical characterization of inflammtory cells associated with advanced atherosclerosis. Histopathology 17(543-552, 1990.
315. Major AS, Fazio S, LInton MF. B-lymphocyte deficiency increases atherosclerosis in LDL receptor-null mice. Arterioscler. Thromb. Vasc. Biol. 22(1892-1898, 2002.
316. Caligiuri MA, Nicoletti A, Poirier B, Hansson GK. Protective immunity against atherosclerosis csrried by B cells of hypercholesterolemic mice. J Clin Invest 109(745-753, 2002.
317. Ait-Oufella H, Herbin O, Bouaziz J-D, Binder CJ, Uyttenhove C, al e. B cell de[pletion reduces the development of atherosclerosis in mice. J Exp Med 207(1579-1587, 2010.
318. Kyaw T, Tay C, Khan A, Dumouchel V, Cao A, To K, Kehry M, Dunn R, Agrotis A, Tipping P, Bobik A, Ban-Hock T. Conventional B2 B cell depletion amelirates wharas its adoptive trasfer aggaravates atherosclerosis. The Journal of Immunology 185(4410-4419, 2010.
319. Naruko T, Ueda M, Haze K, van der Wal AC, al e. Neutrophil infiltration of culprit lesions in acute coronary syndrome. Circulation 106(2894-2900, 2002.
320. Sasaki T, Kuzuya M, Nakamura K, Cheng XW, Shibata T, al e. A simple method of plaque rupture induction in apolipoprotein E-deficient mice,. Arterioscler. Thromb. Vasc. Biol. 26(1304-1309, 2006.
321. van Leeuwen M, Gijbels M, Duijvestijin A, Smook M, al e. Accumulation of myeloxidase-positive neutrophils in atheroscelrotic lesions in LDLR-/- mice. Arterioscler. Thromb. Vasc. Biol. 28(84-89, 2008.
322. Rotzius P, Thams S, Soehnlein O, al. e. Distinct infiltration of neutrophils in lesion shoulders in $ApoE^{-/-}$ mice. Am J Pathol 177(493-500, 2010.
323. Gibbs BF. Human basophils as effector and immunomodulators of allergic inflammation and innate immunity. Clin Exp Med 5(43-49, 2005.
324. Thonnard-Neumann E, Fox RR, Taylor WL. Basophilic leukocytes in the hyperlipidemic rabbit. Annals of Hematology 20(214-221, 1970.
325. Trinchieri G. Biology of natural killer cells. Adv Immunol 47(187-276, 1989.
326. Timonen T, Saksela E, Ranki A, Hayry P. Fractionation, morphological and functional characterization of effector cells responsible for human natural killer activity against cell-line targets. . Cell Immunol 48(133-148, 1979.
327. Biron CA, van den Elsen P, Tutt MM, Medveczky P, Kumar V, Terhorst C. Murine natural killer cells stimulated in vivo do not express the T cell receptor a, b, T3d, or T3e genes. J Immunol 139(1704-1710, 1987.
328. Wang B, Biron CA, She J, Higgins K, Sun-shine M-J, Lacy E, Lonberg N, Terhorst C. A block in both early T lymphocyte and natural killer cell development in transgenic mice with high-copy numbers of human CD3e gene. Proc Natl Acad Sci USA 91(9402-9406, 1994.
329. Biron CA. Activation and function of natural killer cell responses during viral infections. Curr. Opin. Immunol 9(24-34, 1997.
330. Pyzik M, Vidal SM. Nk cells stroll down the memory lane. Immunology and Cell Biology 87(251-263, 2009.
331. Murphy W, Kumar V, Bennett M. Rejection of bone marrow allografts by mice with severe combined immunodeficiency (SCID). J Exp Med 165(1212-1217, 1987.

332. Scharton TM, Scott P. Natural killer cells are a source of interferon gamma that drives differentiation of CD4+T cell subsets and induces early resistance to Leishmania major in mice. J Exp Med 178(567-577, 1993.
333. Unanue ER. Studies in listeriosis show strong symbiosis between the innate cellular system and T-cell response. Immunol Rev 158(11-25, 1997.
334. Biron CA, Byron KS, Sullivan JL. Severe herpesvirus infection in adolescent without natural killer cells. N Eng J Med 320(1731-1735, 1989.
335. Baldwin RW, Embleton MJ, Jones JS, Langman MJ. Cell-mediated and humoral immune reactions to human tumours. Int. J. Cancer 12(73-83, 1973.
336. Bubenik J, Jakoubkova J, Krakora P, Baresova M, Helbich P, Viklicky V, Malaskova V. Cellular immunity to renal carcinomas in man. Int. J. Cancer 8(503-513, 1971.
337. Hellstrom I, Hellstrom KE. Cell-mediated immune reactions to tumor antigens with particular emphasis on immunity to human neoplasms. Cancer 34((suppl 8), 1974.
338. Levental BG, Halterman RH, Rosenberg EB, Herberman RB. Immune reactivity of leukemia patients to autologous blast cells. Cancer Res 32(1820-1825, 1972.
339. O'Toole C, Stejskal V, Perlmann P, Karlsson M. Lymphoid cells mediating tumor-specific cytotoxicity to carcinoma of the urinary bladder. Separation of the effector population using surface marker. J. Exp. Med 139(457-466, 1974.
340. Kiessling R, Klein E, Wigzell H. "Natural" kiler cells in the mouse. I. Cytotoxic cells with specificity for mouse Moloney leukemia cells. Specificity and disribution according to genotype. Eur J Imunol 5(112-117, 1975.
341. Kiessling R, Klein E, Pross H, wigzell H. Natural killer cells in the mouse II.Cytotoxic cells with specificity for mouse Moloney leukemia cells. Characteristics of the killer cell. Eur J Imunol 5(117-121, 1975.
342. Herberman RB, Nunn ME, Holden HT, Larvin DH. Natural cytotoxic reactivity of mouse lymphoid cells against syngeneic and allogeneic tumors II. Characterization of effector cells. Int. J. Cancer 16(230-239, 1975a.
343. Herberman RB, Nunn ME, Lavrin DH. Natural cytotoxic reactivity of mouse lymphoid cells against syngeneic and allogeneic tumors I. Distribution of reactivity and specificity. Int. J. Cancer 16(216-229, 1975b.
344. Bryceson YT, March ME, Ljunggren HG, Long EO. Synergy among receptors on resting NK cells for the activation of natural cytotoxicity and cytokine secretion. Blood 107(159-166, 2006.
345. Biron CA, Nguyen KB, OPien GC, Cousens LP, Salazar-Mather TP. Natural killer cells in antiviral defense: function and regulation by innate cytokines. Annu. Rev. Immunol 17(189-220, 1999.
346. Jinushi M, Takehara T, Kanto T, Tatsumi T, al e. Critical role of MHC-class 1 related chain A and B expression on IFN-alpha-stimulated dendritic cells in NK cell activation: impairement in chronic hepatitis C virus infection. J Immunol 170(1249-1256, 2003.
347. Newman KC, Riley EM. Whatever turns you on: accessory-cell-dependent activation of NK cells by pathogen. Nat Rev Immunol 7(279-291, 2007.
348. Cerwenka A, Lanier L. Natural killer cells, viruses, and cancer. Nat Rev Immunol 1(41-49, 2001.
349. Lieberman J. The ABCs of granule-mediated cytotoxicity: new weapons in the arsenal. Nat Rev Immunol 3(361-370, 2003.
350. Russell JH, Ley TJ. Lymphocyte-mediated cytotoxicity. Annu Rev Immunol 20(323-370, 2002.
351. Moretta A. Natural killer cells and dendritic cells: rendeznous in abused tissues. Nat Rev Immunol 2(957-964, 2002.

352. Moretto A, Marcenaro S, Sivori S, Della Cieesa M, Vitale M, Moretta A. Early liaisons between cells of the innate immune system in inflammed peripheral tissues. Trends Immunol 26(668-675, 2005.
353. Karre K. Natural killer cell recognition of missing self Nat Immunol 9(477-480, 2008.
354. Lanier L. Nk cell recognition. Annu. Rev. Immunol 23(225-274, 2005.
355. Raulet DH. Roles of the NKG2D immunoreceptor and its ligands. Nat Rev Immunol 3(781-790, 2003.
356. Karre K, Ljunggren HG, Piontek G, Kiessling R. Selective rejection of H-2-deficient lymphoma variants suggest alternative immune defense strategy. Nature 319(675-678, 1986.
357. Ljunggren HG, Karre K. In search of the 'missing self' : MHC molecules and Nk cell recognition. Immunol Today 11(237-244, 1990.
358. Robertsons MJ, Ritz J. Biology and clinical relevance of human natural killer cells. Blood 76(2421-2438, 1990.
359. Lanier L. The relationship of CD16 (Leu-11) and Leu-19 (NKH-1) antigen expression on human peripheral blood NK cells and cytotoxic T lymphocytes. J Immunol 136(4480-4486, 1986.
360. Lanier Lea. Identity of Leu-19 (CD56) leukocyte differentiation antigen and neural cell adhesion molecule. J. Exp. Med 169(2233-2238, 1989.
361. Leibson PJ. Signal transduction during natural killer cell activation:inside the mind of a killer. Immunity 6(655-661, 1997.
362. Cooper MA, Fehniger TA, Caligiuri MA. The biology of human natural killer-cell subsets. Trends in Immunology 22(633-639, 2001.
363. Andre P. Modification of P-selectin glycoprotein ligand 1 with a natural-killer-cell-restricted sulfated lactosamine cretaes an alternate ligand for L-selectin. Proc Natl Acad Sci USA 97(3400-3405, 2000.
364. Colonna M. A common inhibitory receptor for major histocompatibility complex class I molacules on human lymphoid and myelomonocytic cells. J Exp Med 186(1809-1818, 1997.
365. Voss SD. Participation of the CD94 receptor complex in costimulation of human natural killer cells. J Immunol 160(1618-1626, 1998.
366. Bauer Sea. Activation of NK cells and T cells by NKG2D, a receptor for stress-inducible MICA. Science 285(727-729, 1999.
367. Frey M. Differential expression and function of L-selectin on $CD56^{bright}$ and $CD56^{dim}$ natural-killer-cell subsets. J Immunol 161(400-408, 1998.
368. Campbell JJ, all e. Unique subpopulations of $CD56^{+}$ NK and NK-T peripheral blood lymphocytes identified by chemokine receptor expression repertoire. J. Immunol 166(6477-6482, 2001.
369. Caligiuri MA. Selective modulation of human natural killer cells *in vivo* after prolonged infusion of low-dose recombinant interleukin-2. J Clin Invest 91(123-132, 1993.
370. Baume DM. Differential responses to interleukin-2 define functionally distinct subsets of human natural killer cells. Eur J Imunol 22(1-6, 1992.
371. Caligiuri MA, al e. Functional consequences of interleukin-2 receptor expression on resting human lymphocytes. Identification of a novel natural-killer-cell subset with high affinity receptor. J Exp Med 171(1509-1526, 1990.
372. Matos ME. Expression of a functional c-kit receptor on a subset of natural killer cells. J Exp Med 178(1079-1084, 1993.
373. Carson WE, al e. The c-kit ligand supresses apoptosis of human natural killer cells through the up-regulation of bcl-2. . Proc Natl Acad Sci USA 91(7553-7557, 1994.

374. Carson WE, al e. Interleukin(IL)-15 is a novel cytokine that activates human natural killer cells via components of the IL-2 receptor. J Exp Med 180(1395-1403, 1994.
375. Kunikata T, al e. Constitutive and induced IL-18 receptor expression by various peripheral-blood-cells- subsets as determined by anti-hIL-18R monoclonal antibody. Cell Immunol 189(135-143, 1998.
376. Cooper MA, al e. Interleukin-1b costimulates interferon-g production by human natural killer cells. Eur J Imunol 31(792-801, 2001.
377. Nagler A. Comparative studies of human FcR111-positive and -negative natural killer cells. J Immunol 143(3183-3191, 1989.
378. Cooper MA, Etal. Human natural killer cells: a unique innate immunoregulatory role for the $CD56^{bright}$ subsets. Blood 97(3146-3151, 2001.
379. Hayakawa Y, Smyth MJ. CD27 disects mature NK cells into two subsets with distinct responsiveness and migratory capacity. Journal of Immunology 176(1517-1524, 2006.
380. Kim S, Iizuka K, Kang S, Dokun A, French AR, Greco S, Yokoyama WM. In vivo developmental stages in murine natural killer cell maturation. Nat Immunol 3(523-528, 2002.
381. Kelly JMea. Induction of tumor-specific T cell memory by Nk cell-mediated tumor rejection. Nat Immunol 3(83-90, 2002.
382. Takeda K, Oshima H, Hayakawa Y, Akiba H, Atsuta M, Kobata T. CD27-mediated activation of murine NK cells. J Immunol 164(1741-1745, 2000.
383. Tang A, Amgai M, Garnger LG, Stanley JR, Udey MC. Adhesion of epidermal Langerhans cells to keratinocytes mediated by E-cadherin. Nature 361(82-85, 1993.
384. Grundemann C, al e. Cutting edge: identification of E-cadherin as a ligand for the murine likker cell lectin-like receptor G1. J Immunol 176(1311-1315, 2006.
385. Silva A, Andrews DM, Brooks AG, Smyth MJ, Hayakawa Y. Application of CD27 as a marker for distinguishing human NK cell subsets. Int. Immunol 20(625-630, 2008.
386. Vossen MTM, Matmati M, Hertoghs KML, Baars PA, Gent M-R, Leclercq G, Hamann J, Kuijpers TW, van Lier RAW. CD27 defines phenotypically and functionally different human Nk cell subsets. J Immunol 180(3739-3745, 2008.
387. Watt SV, Andrews DM, Takeda K, Smyth MJ, Hayakawa Y. IFN-g dependent recriutment of mature $CD27^{high}$ NK cells to lymph nodes primed by dendritic cells. J Immunol 181(5323-5330, 2008.
388. Martin-Fontecha A, Thomsen LL, Brett S, Gerard C, LIpp M, Lanzavecchia A, Sallusto F. Induced recruitment of NK cells to lymph nodes provides IFN-gamma for T(H)1 priming. Nat Immunol 5(1260-1265, 2004.
389. Farber JM. MIg and IP-10: CXC chemokines that target lymphocytes. J Leukocyte Biol 61(246-257, 1997.
390. Hayakawa Y, Huntington ND, Nutt SL, Smyth MJ. Functional subsets of mouse natural killer cells. Immunological Reviews 214(47-55, 2006.
391. Yokoyama WM, Kim S, French AR. The dynamic life of natural killer cells. Annu. Rev. Immunol 22(405-429, 2004.
392. Seaman WE, Blackman MA, Gindhart TD, Roubinian JR, Loeb JM, Talal N. beta-Estradiol reduces natural killer cells in mice. J Immunol 121(2193-2198, 1978.
393. Wu Q, Sun Y, Wang J, Lin X, Wang Y, at e. Signal via lymphotoxin-b R on bone marrow stromal cells is required for an early checkpoint of NK cell development. J Immunol 166(1684-1689, 2001.
394. Iizuka K, Chaplin DD, Wang Y, Wu Q, Pegg LE, al e. Requirement for membrane lyphotoxin in natural killer cell development. Proc Natl Acad Sci USA 96(6336-6340, 1999.

395. Kondo M, Weissman IL, Akashi K. Identification of clonogenic common lymphoid progenitors in mouse bone marrow. Cell 91(661-672, 1997.
396. Galy A, Travis M, Cen D, Chen B. Human T, B, natural killer, and dendritic cells arise from a common bone maroow progenitor cell subset. Immunity 3(459-473, 1995.
397. Boggs SS, Trevisan M, Patrene K, Geogopoulos K. Lack of natural killer cell precursors in fetal liver of Ikaros knockout mutant mice. Nat Immunol 16(137-145, 1998.
398. Dahl R, Simon MC. The importance of PU.1 concentration in hemapoetic lineage commitment and maturation. Blood Cell Mol Dis(229-233, 2003.
399. Colucci F, Samson SI, DeKoter RP, Lantz O, Singh H, DiSanto JP. Differential requirement fro the tracription factor PU.1 in the generation of natural killer cells versus B and T cells. Blood 97(2625-2632, 2001.
400. Miyake T, Campbell KS. Analysis and use of mutant mice exhibiting natural killer cell defects. Methods in Molecular Biology 121(323-335, 2000.
401. Wang B, Biron CA, She J, Higgins K, al e. A block in both early T lymphocyte and natural killer celldevelopment in transgenic mice with high copy numbers of the human CD3E gene. Proc Natl Acad Sci USA 91(9402-9406, 1994.
402. Flamand V, Shores EW, Tran T, Huang K, Lee E, al e. Delayed maturation of $CD4^{-}CD8^{-}$ $FcgRII/III^{+}$ T and natural killer cell precursors in FceRIg transgenic mice. J Exp Med 184(1725-1735, 1996.
403. Ikawa T, Kawamoto H, Fujimoto S, Katsura Y. Commitment of common T/Natural killer (NK) progenitors to unipotent T and NK progenitors in the murine fetal thmus revealed by a single progenitor assay. J Exp Med 190(1617-1626, 1999.
404. Rosmaraki EE, Douagi I, Roth C, Collucci F, Cumano A, Di Santo JP. Identification of committed NK cell progenitors in adult murine bone marrow. Eur J Imunol 31(1900-1909, 2001.
405. Barton K, Muthusamy N, Fischer C, Ting CN, Walnus TL, al e. The ets-1 transcription factor is required for the development of natural killer cells in mice. Immunity 9(555-563, 1998.
406. Yokota Y, Mansouri A, Mori S, Sugawara S, Adachi S, al e. Development of peripheral lymphoid organs and natural kiler cells depends on the helix-loop-helix inhibitor Id2. Nature 397(702-706, 1999.
407. Waldmann TA, Tagaya Y. The multifaceted regulation of interleukin-15 expression and the role of this cytokine in NK differentiation and host response to intracellular pathogens. Annu. Rev. Immunol 17(19-49, 1999.
408. Lodolce JP, Boone DL, Chai S, Swain RE, Dassopoulos T, al e. IL-15 receptor maintains lymphoid homeostasis by supporting lymphocyte homing and proliferation. Immunity 9(669-676, 1998.
409. Suzuki H, Duncan GS, Takimoto H, Mak Tw. Abnormal development of intestinal intraepithelial lymphocytes and peripheral natural killer cells in mice lack the IL-2 receptor b chain. J Exp Med 185(499-505, 1997.
410. Kondo M, Akashi K, Domen J, Sugamura K, Weissman IL. Bcl-2 rescues T lymphopoiesis, but not B or NK cell development in common gamma chain-deficient mice. Immunity 7(155-162, 1997.
411. Park Sy, Saijo K, Takahashi T, Osawa M, Arase H, al e. Developmental defects of lymphoid cells in Jak3 kinase-eficient mice. Immunity 3(771-782, 1995.
412. Imada K, Bloom ET, Nakajima H, Horvath-Arcidiacono JA, Udy GB, al e. Stat5b is essential for natural killer cell-mediated proliferation and cytolytic activity. J Exp Med 188(2067-2074, 1998.

413. Ryan JC, Turck J, Niemi EC, Yokoyama WM, Seamn WE. Molecular cloning of the NK1.1 antigen, a member of the NKR-P1 family of natural killer cell activation molecule. J Immunol 149(1631-1635, 1992.
414. Di Santo JP. Natural killer cell developmental pathways: a question of balance. Annu Rev Immunol 24(257-286, 2006.
415. Loza MJ, Perussia B. Final steps of natural killer cell maturation: a modelfor type 1-type 2 differentiation? J Immunol 149(1631-1635, 2001.
416. Bennett IM, Zatsepina O, Zamai L, Azoni L, Mikheeva T, Perussia B. Definition of a natural killer NKR-P1A^{+}/CD56^{-}/CD16^{-} functionally immature human NK cell subset that differentiates invitro in the presence of interleukin-12. J Exp Med 184(1845-1856, 1996.
417. Wu C, Wang X, Gadina M, O'Shea JJ, Presky DH, Magram J. IL-12 receptor beta 2 (IL-12R beta 2)-deficient mice are defective in Il-12-mediated signalling despite the presence of high affinity IL-12 binding sites. J Immunol 165(6221-6228, 2000.
418. Takeda K, Tsutsui H, Yoshimoto T, Adachi O, Yoshida N, al e. Defective NK cell activity and Th1 response in IL-18-deficient mice. Immunity 8(383-390, 1998.
419. Freud AG, Becknell B, Roychowdhury S, Mao HC, al. e. A human CD34(+) subset resides in lymph nodes and differentiates into CD56bright natural killer cells. Immunity 22(295-304, 2005.
420. Freud AG, Yokohama A, Becknell B, Lee MT, Mao HC, Ferketich AK, Caligiuri MA. Evidence for discrete stages of human natural killer cell differentiation in vivo. J Exp Med 203(1033-1043, 2006.
421. Vosshenrich CA, al e. A thymic pathway of mouse natural killer cell development characterized by expression of GATA-3 and CD127. Nat Immunol 7(1217-1224, 2006.
422. Gregoire C, Chasson L, Luci C, Tomasello E, Geissmann F, Vivier E, Walzer T. The trafficking of natural killer cells. Immunological Reviews 220(169-182, 2007.
423. Hokeness KL, Kuziel WA, Biron CA, Salazar-Mather TP. Monocyte chemoattractant protein-1 and CCr2 interactions are required for IFN-a/b-induced infalmmtory responses and antiviral defense in liver. J Immunol 174(1549-1556, 2005.
424. Thapa M, Kuziel WA, Carr D, J. Susuceptibility of CCR5-deficient mice to genital herpes simplex virus type 2 is linked to NK cell mobilization. J Virol 81(3704-3713, 2007.
425. Jiang D, Liang J, Hodge J, Lu B, al e. Regulation of pulmonary fibrosis by chemokine receptor CXCR3. J Clin Invest 114(291-299, 2004.
426. Yu YR, Fomng AM, Combadiere C, Gao J-L, Murphy PM, Patel DD. Defective antitumor response in CXCR1-deficient mice. International Journal of Cancer 121(316-322, 2007.
427. Matloubian M, Lo CG, Cinamon G, Lesneski MJ, Xu Y, al e. Lymphocyte egress from thymus and peripheral lymphoid organs is dependent on SIP receptor 1.`. Nature 427(355-360, 2004.
428. Bajenoff M, Breart B, Huang AY, Qi H, al. e. Natural killer cell behaviour in lymph nodes reveled by static and real-time imaging. J Exp Med 203(619-631, 2006.
429. Walzer T, Blery M, Chaix J, Fuseri N, Chasson L, Robbins SH, al e. Indentification, activation, and selective in vivo ablation of mouse NK cells via NKp46. Proc Natl Acad Sci USA 104(3384-3389, 2007.
430. Martin-Fontecha Aea. Induced recruitment of NK cells to the lymph nodes provides IFN-g for TH1 priming. Nature Immunol 5(1260-1265, 2004.
431. Prlic M, Blazar BR, Farrar MA, Jameson SC. In vivo survival and homeostatic proliferation of natural killer cells. J Exp Med 197(967-976, 2003.
432. Ranson T, Vosshenrich CA, Corcuff E, Richard O, Muler W, Di Santo JP. IL-15 ia an essential mediator of peripheral Nk cell homeostasis. Blood 101(4887-4893, 2003.

433. Cooper MA, Bush JE, Fehninger TA, VanDeusen JB, Waite RE, al e. In vivo evidence foe a dependence on interleukin 15 for survival of natural killer cells. Blood 100(3633-3638, 2002.
434. Ranson T, Vosshenrich CA, Corcuff E, Richard O, Muller W, Di Santo JP. IL-15 is an essential mediator of peripheral NK cell homeostasis. Blood 101(4887-4893, 2003.
435. Cooper MA, Bush JE, Fehninger TA, VanDeusen JB, Waite RE, al. e. In vivo evidence for a dependence on interleukin 15 for survival of natural killer cells. Blood 100(3633-3638, 2002.
436. Koka R, Burkett P, Chien M, Chai S, Chan F, al e. Interleukin (IL)-15Ra-deficient natural killer cells survive in normal but not IL15Ra-deficient mice. J Exp Med 197(977-984, 2003.
437. Dubois S, Mariner J, Waldmann TA, Tagaya Y. IL-15Ralpha recycles and presents IL-15 In trans to neigboring cells. Immunity 17(537-547, 2002.
438. Swann JB, Hayakawa Y, Zerafa N, Sheehan KCF, al S. Type I IFN conributes to NK cell homeostasis, activation and antitumor function. Journal of Immunology 178(7540-7549, 2007.
439. Biron CA, Sonnenfeld G, Welsh RM. Interferon induces natural killer cell blastogenesis in vivo. J Leukocyte Biol 35(31-37, 1984.
440. Nguyen KB, Salazar-Mather TP, Dalod MY, VanDeusen JB, al. e. Coordinated and distinct roles for IFN-a b, IL-12 and IL-15 regulation of NK cell responses to viral infection. J Immunol 169(4279-4287, 2002.
441. Pegram HJ, Andrews DM, Smyth MJ, Darcy PK, Kershaw MH. Activating and inhibitory receptors of natural killer cells. Immunology and Cell Biology 89(216-224, 2011.
442. Raulet DH, Vance RE. Self-tolerance of natural killer cells. Nature Immunol 6(520-531, 2006.
443. Raulet DH, Vance RE, McMahon CW. Regulation of the natural killer cell receptor repertoire. Annu. Rev. Immunol 19(291-330, 2001.
444. Moretta A, al e. Receptors for HLA class-1 molecules in human natural killer cells. Annu Rev Immunol 14(619-648, 1996.
445. Litwin V, Gumperz J, Parham P, Philipis JH, Lanier LL. An NK cell receptor involved in the recognition of polymorphic HLA-B molecules. J Exp Med 180(537-543, 1994.
446. Campbell KS, Dessing M, Lopes-Botet M, Cella M, Colonna M. Tyrosine phosphorylation of a human killer inhibitory receptor recruits protein tyrosine phosphotase. J Exp Med 184(93-100, 1996.
447. Burshtyn DN, Scharenberg AM, Wagtmann N, Rajagopalan S, Berrada K, al. e. Recruitment of tyrosine phosphotase HCP by the killer cell inhibitory receptor. Immunity 4(77-85, 1996.
448. Long EO. Negative signalling by inhibitory receptors: the NK cell paradigm. Immunological Reviews 224(70-84, 2008.
449. Colonna M, Navvarro F, Bellon T, Llano M, Gracia P, al. e. A common inhibitory receptor for major histocompatibility complex class I molecules on human lymphoid and myelomonocytic cells. J Exp Med 186(1809-1818, 1997.
450. Colonna M, Nakajima H, Cella M. Inhibitory and activating receptors involved in immune surveillance by human NK and myeloid cells. Journal of Leukocyte Biology 66(718-722, 1999.
451. Mehta IK, Wang J, Roland J, Margulies DH, Yokoyama WM. Ly49A allelic variation and MHC class 1 specificity. Immunogenetics 53(572-583, 2001.

452. Wilhelm BT, Gagnier L, Magner DL. Sequnce analysis of the ly49 cluster in C57BL/6 mice: a rapidly evolving multigene family in the immune system. Genomics 80(646-661, 2002.
453. Makrigiannis AP, Pau AT, Saleh A, Winkler-Pickett R, Ortaldo JR, Anderson SK. Class 1 MHC-binding characteristics of the 128/J Ly49 repertoire. J Immunol 166(5034-5043, 2001.
454. Smith KM, Wu J, Bakker AB, Philipis JH, Lanier LL. Cutting edge: Ly-49D and Ly-49H associate with mouse DAP12 and form activating receptors. J Immunol 161(7-10, 1998.
455. Mason LH, Gosselin P, Anderson SK, Fogler WE, Ortaldo JR, McVicar DW. Differential tyrosine phosphorylation of inhibitory versus activating Ly-49 receptor proteins and their recruitment of SHP-1 phosphatase. J Immunol 159(4187-4196, 1997.
456. Lanier LL. Nk cell receptors. Annu Rev Immunol 16(359-393, 1998.
457. Tormo J, Natarajan K, Margulies DH, Mariuzza RA. Crystal structure of a lectin-like natural killer cell receptor bound to its MHC class 1 ligand. Nature 402(623-631, 1999.
458. Wang J, Whitman MC, Natarajan K, Tormo J, Mariuzza RA, Margulies DH. Binding of the natural killer cell inhibitory receptor Ly49A to its major histocompatibility complex class 1 ligand. Crucial contacts include both H-2Dd and b2-microglobulin. J Biol Chem 277(1433-1442, 2002.
459. Ortaldo JR, Winkler-Pickett R, Wiegand G. Activating Ly-49D NK receptors: expression and function in relation to ontogeny and Ly-49 inhibitor receptors. Journal of Leukocyte Biology 68(748-756, 2000.
460. Ortaldo JR, Young HA. Expression of IFN-g upon triggering of activating Ly49D NK receptors in vitro and in vivo: costimulation with IL-12 or IL-18 overrides inhibitory receptors. J Immunol 170(1763-1769, 2003.
461. Ortaldo JR, Young HA. Mouse Ly49 NK receptors: balancing activation and inhibition. Molecular Immunology 42(445-450, 2005.
462. Carretero M, Palmieri G, LIano M, Tullio V, Santoni A, al e. Specific engagement of the CD94/NKG2-A killer inhibitory receptor by the HLA-E class 1b molecule induces SHP-1 phosphatase recruitment to tyrosine-phophorylated NKG2-A: evidence for receptor function in heterologous transfectants. Eur J Imunol 28(1280-1291, 1998.
463. Long EO. REgulation of immune responses through inhibitory receptors. Annu Rev Immunol 17(875-904, 1999.
464. Ravetch JV, Lanier LL. Immune inhibitory receptors. Science 290(84-89, 2000.
465. Houchins JP, Lanier LL, Niemi EC, Philipis JH, Ryan JC. NKG2A complexed with CD94 defines a novel inhibitory NK cell receptor. J Immunol 158(3603-3609, 1997.
466. Lanier LL, Corliss B, Wu J, Philipis JH. Association of DAP12 with activating CD94/NKG2C NK cell receptors. Immunity 8(693-701, 1998.
467. Vance RE, Jamieson AM, Raulet D. Recognition of the class Ib molecule Qa-1b by putative activating receptors CD94/NKG2C and Cd94/NKG2E on mouse natural killer cells. J Exp Med 190(1801-1812, 1999.
468. Zhang W. Immunology: Innate immune cells. 2007.
469. Hoglund P, Sundback J, Olsson-Alheim MY, Johansson M, Salsedo M, al. e. Host MHC class I gene control of NK-cell specificity in the mouse. Immunol Rev 155(11-28, 1997.
470. Houchins JP, Yabe T, Mcsherry C, Bach FH. DNA sequence analysis of NKG2, a family of related cDNA clones encoding type 11 integral membrane proteins on human natural killer cells. J Exp Med 173(1017-1020, 1991.
471. Ho EL, Heusel JW, Brown MG, Matsumoto K, Scalzo AA, Yokoyama W. Murine NKg2d and Cd94 are clustered within the natural killer complex and are expressed independently in natural killer cells. Proc Natl Acad Sci USA 95(6320-6325, 1998.

472. Vance RE, Tanamachi DM, Hanke T, Raulet DH. Cloning of a mouse homolog of CD94 extends the family of C-type lectins on murine natural killer cells. Eur J Imunol 27(3236-3241, 1997.
473. Cerwenka A, Bakker AB, McClanahan Tea. Retinoic acid early inducible genes define a ligand family for the activating NKG2D receptor in mice. Immunity 12(721-727, 2000.
474. Diefenbach A, Jamieson AM, Liu SD, Shastri N, Raulet DH. Ligands for the murine NKG2D receptor: Expression by tumor cells and activation of NK cells and macrophages. Nat Immunol 1(119-126, 2000.
475. Lanier LL, Corliss BC, Wu J, Leong C, Philipis JH. Immunoreceptor DAP12 bearing a tyrosine-based activation motif is involved in activating NK cells. Nature 391(703-707, 1998.
476. Vivier E, Nunes JA, Vely F. Natural killer cell signalling pathways. Science 306(1517-1519, 2004.
477. Diefenbach A, Tomasello E, Lucas Mea. Selective associatios with signalling proteins determine stimulatory versus costimulatory activity of NKG2D. Nat Immunol 3(1142-1149, 2002.
478. Gilfillan S, Ho EL, Cella M, Yokoyama WM, Colonna M. NKG2D recruits two distinct adapters to trigger NK cell activation and costimulation. Nat Immunol 3(1150-1155, 2002.
479. Rosen DB, Araki M, Hamerman JA, Lanier LL. A structural basis for the association of DAP12 with mouse, but not human, NKG2D. J Immunol 173(2470-2478, 2004.
480. Graham DB, Cella M, Giurisato E, Fujiikawa K, al. e. Vav1 controls DAP-10-mediated natural cytotoxicity by regulating actin and microtubule dynamics. J Immunol 177(2349-2355, 2006.
481. Long EO. Versatile signalling through NKG2D. Nature Immunol 3(1119-1120, 2002.
482. Segovis CM, Schoon RA, Dick CJ, al. e. PI3K links NKG2D signalling to a CrkL pathway involved in natural killer cell adhesion, polarity, and granule secretion. J Immunol 182(6933-6942, 2009.
483. Bacon L, Eagle RA, Meyer M, Easom N, Young NT, Trowsdale J. Two human ULBP/RAET1 molecules with transmembrane regions are ligands for NKG2D. J Immunol 173(1078-1084, 2004.
484. Carannopoulos LN, Jaieson AM, Liu SD, Shahtri N, Raulet DH. Cutting-edge:murine Ul-16-binding protein-like transcript 1: a newly described transcript encoding a high-affinity ligand for murine NKG2D. J Immunol 169(4079-4083, 2002.
485. Takada A, Yoshida S, Kajikawa Mea. Two novel NKG2D ligands of the mouse H60 family with differential expression patterns and binding affinities to NKG2D. J Immunol 180(1678-1685, 2008.
486. Vilarinho S, Ogasawara K, Nishimura S, Lanier LL, Baron JL. Bolckade of NKG2D on NKT cells preents hepatitis and the acute immuneresponse to hepatitis B virus. Proc Natl Acad Sci USA 104(18187-18192, 2007.
487. Groh V, Rhinehart R, Randolf-Habecker Jea. Costimulation od CD8alphabeta T cells by NKG2D via engagement by MIC induced virus-infected cells. Nat Immunol 2(255-260, 2001.
488. Coudert JD, Held W. The role of the NKG2D receptor for tumor immunity. Semin Cancer Biol 16(333-343, 2006.
489. Gasser S, Orsulic S, Brown EJ, Raulet DH. The DNA damage pathway regulates innate immune system ligands of the NKG2D receptor. Nature 436(1186-1190, 2005.
490. Lodoen M, Ogasawara K, Hamerman JA, Arase H, Houchins JP, Mocarski ES, Lanier LL. NKG2D-mediated natural killer cell protection against cytomegalovirus is impaired

by viral gp40 modulation of retinoic acid early inducible 1 gene molecule. J Exp Med 197(1245-1253, 2003.
491. Nomura M, Takihara Y, Shimada K. Isolation and characterization of retinoic acid-inducible cDNA clones in F9 cells: one of the early inducible clones encodes a novel protein sharing several highly homologous regions with a drosophila polyhomeotic protein. Differentiation 57(39-50, 1994.
492. Groh V, Bahram S, Bauer S, Herman A, Beuchamp M, Spies T. Cell stress-regulated human major histocompatibility complex class 1 gene expressed in gastrointestinal epithelium. Proc Natl Acad Sci USA 93(12445-12450, 1996.
493. Welte SA, al e. Selective intracellular retention of virally induced NKG2D ligands by the human cytomegalovirus UL16 glycoprotein. Eur J Imunol 33(194-203, 2003.
494. Sutherland C, al e. UL16-binding proteins, novel MHC class 1 related proteins, bind to NKG2D and activate multiple signalling pathways in primary NK cells. J Immunol 168(671-679, 2002.
495. Cosman D, al e. ULBPs, novel MHC class 1-related molecules, bind to CMV glycoprotein UL16 and stimulate cytotoxicity through the NKG2D receptor. Immunity 14(123-133, 2001.
496. Kubin M, al e. ULBP1, 2, 3: novel MHC class 1-related molecules that bind to human cytomegalovirus glycoprotein UL16, activate NK cells. Eur J Imunol 31(1428-1437, 2001.
497. Billadeau DD, Upshaw JL, Schoon RA, Dick CJ, Leibson PJ. NKG2D-DAp10 triggers human NK cell-mediated killing via Syk-independent regulatory pathway. Nat Immunol 4(557-564, 2003.
498. Nedvetzki S, Sowinski S, Eagle RA, HArris J, Vely F, al. e. Reciprocal regulation of human natural killer cells and macrophages associated with distinct immune synapses. Blood 109(3776-3785, 2007.
499. Zwirner NW, Fuertes MB, Girart MV, Domaica CI, Rossi LE. Cytokine -driven regulation of NK cell fucntions in tumour immunity: role of the MICA-NKG2D system. Cytokine Growth Factor Rev 18(159-170, 2007.
500. Ebihara T, Masuda H, Akazawa T, Shingai M, Kikuta H, Ariga T, al. e. Induction of NKG2D ligands on human dendritic cells by TLR ligand stimulation and by RNA virus infection. Int Immunol 10(1145-1155, 2007.
501. Eagle RA, Jafferji I, Barrow AD. Beyond stressed self: evidence for NKG2D ligand expression on healthy cells. Current Immunological Reviews 5(22-34, 2009.
502. Brady J, Carotta S, Thong RPL, Chan CJ, Hayakawa Y, Smyth MJ, Nutt SL. The interactions of multiple cytokines control NK cell maturation. J Immunol 185(6679-6688, 2010.
503. Burgess SJ, Mariusina AI, Pathmananthan I, Borrego F, Coligan JE. IL-21 downregulates NKG2D/DAP10 expression on human NK and CD8+T cells. J Immunol 176(1490-1497, 2006.
504. Castrioni R, Cantoni C, Della Cieesa M, Vitale M, Marcenaro E, Conte R, Biassoni R, Bottino C, Moretta L, Moretta A. Transforming growth factor beta 1 inhibits expression of NKp30 and NKG2D receptors: consequnces for the NK -mediated killing of dendritic cells. Proc Natl Acad Sci USA 100(4120-4125, 2003.
505. Munsasell A, Magri G, Pende D, Angulo A, Lopez-Botet M. Inhibition of NKG2D expression in NK cells by cytokines secreted in response to human cytomegalovirus infection. Blood 115(5170-5179, 2010.
506. Brown MH, al. e. 2B4, the natural killer and T cell immunoglobulin superfamily surface protein, is a ligandfor CD48. J Exp Med 188(2083-2090, 1998.

507. Davis SJ, van der Merwe PA. The structure and ligand interactions of Cd2: implications for T-cell function. Immunol Today 17(177-187, 1996.
508. Matthew SO, Rao KK, Kim JR, Bambard ND, Mathew PA. Functional role of human NK cell receptor 2B4 (CD244) isoforms. Eur J Imunol 39(1632-1641, 2009.
509. Stepp SE, Schatzle JD, Bennett M, Kumar V, Mathew PA. Gene structure of the murine NK cell receptor 2B4: presence of two alternatively spliced isoforms with distinct cytoplasmic domains. Eur J Imunol 29(2392-2399, 1999.
510. Tangye SG, Philipis JH, Lanier LL, Nichols KE. Cutting edge: functional requirement for SAP in 2B4-mediated activation of human natural killer cells as reveled by the X-linked lymphoproliferation syndrome. J Immunol 165(2932-2936, 2000.
511. Nakajima H, al e. Patients with X-linked lymphoproliferative disease have a defect in 2B4 receptor-mediated NK cell cytotoxicity. Eur J Imunol 30(3309-3318, 2000.
512. Sivori S, Falco M, Marcenaro E, Parolini S, Biassoni R, Bottino C, Moretta L, Moretta A. Early expression of triggering receptors and regulatory role of 2B4 in human natural killer cell precursors undergoing in vitro differentiation. Proc Natl Acad Sci USA 99(4526-4531, 2002.
513. Nakajima H, Cella M, Langen H, Friedlein A, Colonna M. Activating interactions in human NK cell recognition: the role of 2B4-CD48. Eur J Imunol 29(1676-1683, 1999.
514. Lanier LL. Natural killer cell receptor signalling. Curr Opin Immunol 15(308-314, 2003.
515. Vitale M, al. e. NKp44, a novel triggering surface molecule specifically expressed by activated natural killer cells, is involved in non-major histocompatibility complex-restricted tumor cell lysis. J Exp Med 187(2065-2072, 1998.
516. Cantoni C, al. e. The three dimensional structure of the human NK cell receptorNKp44, a triggering partner in natural cytotoxicity. Structure 11(725-734, 2003.
517. Pende D, Parolini S, Pessino A, Sivori S, al. e. Identification and molecular characterization of NKp30, a novel triggering receptor involved in natural cytotoxicity mediated by human natural killer cells. J Exp Med 190(1505-1516, 1999.
518. Moretta A, Bottino C, Vitale M, Pende D, al e. Activating receptors and co-receptors involved in human natural killer cell-mediated cytolysis. Annu Rev Immunol 19(197-223, 2001.
519. Bloushtain N, al. e. Membrane-associated heparan sulfate proteoglycans are involved in the recognition of cellular targets by NKp30 and NKp46. J Immunol 176(2392-2401, 2004.
520. Arnon TI, Lev M, Katz G, Chernobrov Y, Porgador A, MAndelboim O. Recognition of viral hemagglutinins by NKp46 but not NKp30. Eur J Imunol 31(2680-2689, 2001.
521. Mandelboim O, Leiberman N, Lev M, Paul L, Arnon TI, al. e. Reconition of hemagglutinins on virus-infected cellsby NKp46 activates lysis of human NK cells. Nature 409(1055-1060, 2001.
522. Arnon TI, Achoudt H, Levi O, Markel G, Katz G, al e. INhibition of the NKp30 activating receptor by pp65 of human cytomegalovirus. Nat Immunol 6(515-523, 2005.
523. Esin S, Batoni G, Counoupas C, Stringaro A, Brancastisano FL, al. e. Direct binding of human NK cell cytotoxicity receptor NKp44 to the surfaces of mycobacteria and other bacteria. Infection and Immunity 76(1719-1727, 2008.
524. MOretta L, Ferlazzo G, Bottino C, Vitale M, Pende D, al. e. Effector and regulatory events during natural killer-dendritic cell interactions. Immunol Rev 214(219-228, 2006.
525. Matsumoto M, Seya T. TLR3:interferon induction by double-stranded RNA including poly IC. Advanced Drug Delivery Reviews 60(805-812, 2007.
526. Wulff S, Pries R, Wollenberg B. Cytokine release of human NK cells solely triggered with Poly I:C. Cellular Immunology 263(135-137, 2010.

527. Xie L, Pries R, Kesselring R, Wulff S, Wollenberg B. Head and neck cancer triggers the internalization of TLR3 in natural killer cells. Int J Mol Med 20(493-499, 2007.
528. Bell JK, Mullen GED, Leifer CA, Mazzoni A, Davies DR, Segal DM. Leucine-rich repeats and pathogen recognition inToll-like receptors. Trends in Immunology 24(528-533, 2003.
529. Yamamoto M, Sato S, Hemmi H, Hoshino K, Kaisho T, Sanjo H, Takaeuchi O, Sugiyama M, Okabe M, Takeda K, Akira S. Role of adaptor TRIF in the MyD88-independent toll-like receptor signalling pathway. Science 301(640-643, 2003.
530. Oshiumi H, Matsumoto M, Funami K, Akazawa T, Seya T. TICAM-1, an adpator molecule that participates in Toll-like recptor 3-mediated interferon-b induction. Nat Immunol 4(161-167, 2003.
531. Alexopoulou L, Holt A, c., Medzhitov R, Flavell RA. Recognition of double-stranded RNA and activation of NF-kB by toll-like receptor 3. Nature 413(732, 2001.
532. Kariko K, Ni H, Capodici J, Lamphier M, Weissman D. mRNA is an endogenous ligand for toll-like receptor 3. Journal of Biological Chemistry 279(12542-12550, 2004.
533. Schidt KN, Leung B, Kwong M, Zarember KA, Satyal S, Navas TA, Wang F, Godowski PJ. APC-independent activation of NK cells by toll-like receptor 3 agonist double-stranded RNA. Journal of Immunology 172(138-143, 2004.
534. Schmidt KN, Leung B, Kwong M, Zarember KA, Satyal S, Navas TA, Wang F, Godowski PJ. APC-independent activation of NK cells by toll-like receptor 3 agonist double-stranded RNA. Journal of Immunology 172(138-143, 2004.
535. Lauson NM, Mian F, MacKenzie R, Ashkar AA. The direct effects of Toll-like receptor ligands on human NK cell cytokine production and cytotoxicity. . Cell Immunol 241(102-112, 2006.
536. Sivori S, Falco M, Della Cieesa M, Carlomagno S, Vitale M, Morretta L, Morreta A. CpG and double-stranded RNA trigger human NK cells by Toll-like receptors: induction of cytokine release and cytotoxicity against tumor and dendritic cells. Proc Natl Acad Sci USA 101(10116-10121, 2006.
537. Ishii KJ, Koyama S, Nakagawa A, C. C, Akira S. Host innate immune receptors and beyond:making sense of microbial infectios. Cell Host MIcrobe 3(352-363, 2008.
538. Kawai T, Akira S. Toll-like receptor and RIG-I-like receptor signalling. Ann NY Acad Sci 1143(1-20, 2008.
539. Sato Sea. Toll/IL-1 receptor domain -containing adaptor inducing IFN-b (TRIF) associates with TNF receptor -associated factor 6 and TANK-binding kinase 1, activates two distinct trancription factors, NF-kB and IFN-regulatory factor -3, in the Toll-like receptor signalling. J Immunol 171(4304-4310, 2003.
540. Takahasi K, Kumeta H, Tsukadi N, Narita R, al. e. Solution structures of cystolic RNA sensor MDA5 and LGP2 C-terminal domains. Journal of Biological Chemistry 284(17465-17474, 2009.
541. McCartney S, Vermi W, Gilfillan S, Cella M, MUrphy TL, Schreiber RD, Murphy KM, Colonna M. Distinct and complementary functions of MDA5 and TLR3 in poly(I:C)-mediated activation of mouse NK cells. J Exp Med 206(2967-2976, 2009.
542. Miyake T, Kumagai Y, Kato H, Guo Z, Matsushita K, Satoh T, Kawagoe T, Kumar H, Jang MH, Kawai T, Tani T, Takaeuchi O, Akira S. Poly I:C-induced activation of NK cells by CD8 alpha^{+} dendritic cells via IPS-1 and TRIF-dependent pathways. J Immunol 183(2522-2528, 2009.
543. Edwards AD, Diebold SS, Slack EM, Tomizawa H, Hemmi H, Kaisho T, Akira S, Reis e Sousa C. Toll-like receptor expression in murine DC subsets: lack of TLR7 expression by CD8 alpha+ DC correlates with un responsiveness to imidazoquinolines. Eur J Imunol 33(827-833, 2003.

544. Kumar H, Koyama S, Ishii KJ, Kawai T, Akira S. Cutting edge: cooperation of IPS-1 and TRIF-dependent pathways in poly IC-enhanced antibody production and cytotoxic T cell responses. J Immunol 180(683-687, 2008.
545. Lucas M, Schachterie W, Oberie K, Aichele P, Diefenbach A. Dendritic cells prime natural killer cells by trans-presenting interleukin 15. Immunity 26(503-517, 2007.
546. Mityake T, Kumagai Y, Kato H, Guo Z, Matsushita K, Satoh T, Kawagoe T, Kumar H, Jang MH, Kawai T, Tani T, Takaeuchi O, Akira S. Poly I:C-induced activation of NK cells by CD8 alpha^{+} dendritic cells via IPS-1 and TRIF-dependent pathways. J Immunol 183(2522-2528, 2009.
547. Cole J, Navin TJ, al. e. Unexpected protective role for Toll-like receptor 3 in the arterial wall. Proc Natl Acad Sci USA 108(2372-2377, 2011.
548. Trinchieri G, Valiante N. Receptors for the Fc fragment of IgG on natural killer cells. Nat Immunol 12(218-234, 1993.
549. Raghavan M, Bjorkman P. Fc receptors and their interactions with immunoglubulins. Annu Rev Cell Dev Biol 12(181-220, 1996.
550. Sun P. Structure and functions of natural killer receptors. Immunol Res 27(539-548, 2003.
551. Mandelboim O, Malik P, Davis DM, Jo CH, Boyson JE, Strominger JL. HUman CD16 as a lysis receptor mediating direct natural killer cell cytotoxicity. Proc Natl Acad Sci USA 96(5640-5644, 1999.
552. Fernandez NC, Lozier A, Flament Cea. Dendritic cells directly trigger Nk cell functions: cross-talk relevant in innate anti-tumor immune responses in vivo. Nat Med 5(405-411, 1999.
553. Iwasaki A, Medzhitov R. Toll-like receptor control of the adaptive immune responses. Nat Immunol 5(987-995, 2004.
554. Buentke E, D'Amato M, Scheynius A. *Malassezia* enhances natural killer cell-induced dendritic cell maturation. Scand. J. Immunol 59(511-516, 2004.
555. Ferlazzo G, Pack M, Thomas Dea. Distinct roles of IL-12 and IL-15 in human natural killer cell activation by dendritic cells from secondary lymphoid organs. Proc Natl Acad Sci USA 101(16606-16611, 2004.
556. Mailliard RB, Son YI, Redlinger Rea. Dendritic cells mediate Nk cell help for Th1 and CTL responses:two signal requirement for the induction of NK cell helper function. J Immunol 171(2366-2373, 2003.
557. Guan H, Moretto M, Bzik DJ, Gigley J, Khan IA. NK cells enhance dendritic cell response against parasite antigens via NKG2D. Journal of Immunology 179(590-596, 2007.
558. Piccioli D, Sbrana S, Melandri E, Valiante NM. Contact-dependent stimulation and inhibition of dendrotic cells by natural killer cells. . J Exp Med 195(335-341, 2002.
559. Vitale M, Della Cieesa M, Carlomagno Sea. Nk-dependent DC maturation is mediated by TNF-a and IFN-g released upon engagement of NKp30 triggering receptor. . Blood 106(566-571, 2005.
560. Ferlazzo G, Tsang ML, Morreta L, Melioli G, Steinman RM, Munz C. Human dendritic cells activate resting natural killer (NK) cells and are recognized via NKp30 recptor by activated NK cells. J Exp Med 195(343-351, 2002.
561. Lantz O, Bendelac A. An invariant T cell receptor a chain is used by unique subset of MHC class-1 specific CD4^{+} and CD4^{-}8^{-}T cells in mice and humans. J Exp Med 180(1097-1106, 1994.
562. Kawano T, Cui J, Koezuka Y, Toura I, Kaneko Y, Motoki K, Ueno H, Nakagawa R, Sato H, Kondo E, etal. CD1d-restricted and TCR-mediated activation of va14 NKT cells by glycosyleramides. Science 278(1626, 1997.

563. Miyagi T, Takehara T, Tatsumi T, Kanto T, Suzuki T, al. e. Cd1d-mediated stimulation of natural killer T cells selectively activates hepatic natural killer cells to eliminate experimentally disseminated hepatoma cells in murine liver. Int J Cancer 106(81-89, 2003.
564. van Dommelen SLH, Tabarias HA, Smyth MJ, Degli-Esposti MA. Activation of natural killer (NK) T cells during murine cytomegalovirus infection enhances the antiviral response mediated by NK cells. Journal of Viorology 77(1877-1884, 2003.
565. Hayakawa Y, Takeda K, Yagita H, al e. Critical contribution of IFN-gamma and NK cells, but not perforin-mediated cytotoxicity, to anti-metastatic effect of alphs-galactosylceramide. Eur J Imunol 31(1720-1727, 2001.
566. Smyth MJ, Wallace ME, Nutt SL, Yagita H, Godfrey DI, Hayakawa Y. Sequential activation of NKT cells and NK cells provides effective inntae immunotherapy of cancer. J Exp Med 201(1973-1985, 2005.
567. Smyth MJ, Cretney E, Kelly JM, Westwood JA, Street SEA, Yagita H, Takeda K, van Dommelen SLH, Degli-Esposti MA, Hayakawa Y. Activation of NK cell cytotoxicity. Molecular Immunology 42(501-510, 2005.
568. Bolitho P, Voskoboinik I, Trapani JA, Smyth MJ. Apoptosis induced by the lymphocyte effector molecule perforin. Current Opinion in Immunology 19(339-347, 2007.
569. Kagi D, Ledermann B, Burki K, Seiler P, al. e. Cytotoxicity mediated by T cells and natural killer cells is greatly impaired in perforin-deficient mice. Nature 369(31-37, 1994.
570. Stepp SE, Dufourcq-Lagelouse R, LeDeist F, Bhawan S, et.al. Perforin genedefects in familial hemophagocytic lymphohistiocytosis. Science 286(1957-1959, 1999.
571. Molleran Lee S, Villanueva J, Sumegi J, Zhang K, Kogawa K, Davis J, Filipovich AH. Cahracterisation of diverse PRF-1 mutations leading to decreased natural killer cell activity in North American families with haemophagocytic lymphohistiocytosis. J Med Genet 41(137-144, 2004.
572. Fan Z, al e. Cleaving the oxidative repair protein APe1 enhances cell death mediated by granzyme A. Nature Immunol 4(145-153, 2003.
573. Beresford PJ, Xia Z, Greenberg AH, Lieberman J. Grazyme A loading induces rapid cytolysis and a novel form of DNA damage independently of caspase activation. . Immunity 10(585-594, 1999.
574. Motyka B, Korbutt G, Pinkoski MJ, Heibein JA, Caputo A, Hobman M, al. e. Mannose 6-phosphate/insulin-like growth factor II receptor is a death receptor for granzyme B during cytotoxic T cell-induced apoptosis. Cell 103(491-500, 2000.
575. Choy JC, Hung VHY, Hunter AL, al. e. Graznzyme B induces smooth muscle cell apoptosis in the absence of perforin. Arterioscler. Thromb. Vasc. Biol. 24(2245-2250, 2004.
576. Tanaka M, Suda T, Haze K, Nakamura N, Sato K, Kimura F, al e. Fas ligand in human serum. Nat Med 2(317-322, 1996.
577. Ashkenazi A, Dixit V. Death receptors: signalling and modulation. Science 281(1305, 1998.
578. Eischen C, Schilling JD, Lynch DH, Krammer PH, Leibson PJ. Fc receptor-induced expression of Fas ligand on activated NK cells faciliates cell=mediated cytotoxicity and subsequent autocrine NK cell apoptosis. J Immunol 156(2693-2699, 1996.
579. Chua HL, Serov Y, Brahmi Z. Regulation of fasL expression in natural killer cells. Human Immunology 65(317-327, 2004.
580. Palmieri G, Tullio V, Zingoni A, Piccoli M, Frati L, Lopez-Botet M, Santoni A. CD94/NKG2-A inhibitory complex blocks CD16-triggered sykand extracellular regulated kinase activation, leading to cytotoxic function of human NK cells. J Immunol 162(7181): 1999.

581. Screpanti V, Wallin RP, Ljunggren HG, Grandien A. A central role for death-receptor-mediated apoptosis in the rejection of tumors by NK cells. J Immunol 167(2068-2073, 2001.
582. Wiley SR, Schooley K, Smolak PJ, al. e. Identification and characterization of a new member of the TNF family that induces apoptosis. Immunity 3(673-682, 1995.
583. Takeda K, Hayakawa Y, Smyth MJ, Kayagaki N, Yamaguchi N, Kakuta S, Iwakura Y, Yagita H, Okumura K. Involvement of tumor necrosis factor-related apoptosis-inducing ligand in surveillance of tumor metastasis by liver natural killer cells. Nature Medicine 7(94-100, 2001.
584. Cretney E, Takeda K, Yagita H, Glaccuma M, Peschon JJ, Smyth MJ. Increased susceptibility to tumor initiation and metastasis in TNF-related apoptosis-inducing ligand deficeint mice. J Immunol 168(1356-1361, 2002.
585. Schoenborn JR, Wilson CB. Regulation of interferon-gamma during innate and adptive immune responses. Adv Immunol 96(41-101, 2007.
586. Yu Y, Hagihara M, Ando K, Gansuvd B, Matsuzawa H, Tsuchiya T, al. e. Enhancement of human cord blood CD34+ cell-derived NKcell cytotoxicity by dendritic cells. J Immunol 166(1590-1600, 2001.
587. Hsu DH, Moore KW, Spits H. Differential effects of IL-4 and IL-10 on IL-2-induced IFN-gamma synthesis and lymphokine-activated killer activity. Int Immunol 4(556-563, 1992.
588. Feng CG, Kaviratne M, Rothfuchs AG, Cheever A, Hieny S, al e. NK cell-derived IFN-g differentially regulates innate resiistance and neutrophil response in T cell-deficient hosts infected with *Mycobacterium tuberculosis*. J Immunol 177(7086-7093, 2006.
589. Salazar-Mather TP, Hamilton TA, Biron CA. A chemokine-to-cytokine-to-chemokine cascade critical in antiviral defence. J Clin Invest 105(985-993, 2000.
590. Rabinowich H, Vitolo D, Altarac S, Herberman RB, Whiteside TL. Role of cytokines in the adoptive immunotherapy of an experimental model of human head and neck cancer by human IL-2-activated natural killer cells. J Immunol 149(340-349, 1992.
591. Pegram HJ, Haynes NM, Smyth MJ, Kershaw MH, Darcy PK. Characterizing the anti-tumor function of adoptively transferred NK cells in vivo. Cancer Immunology, Immunotherapy 59(1235-1246, 2010.
592. Brennan FM, Mcinnes IB. Evidence that cytokines play a role in rheumatoid arthritis. J Clin Invest 118(3537-3545, 2008.
593. Pridgeon C, Lennon GP, Pazmany L, Thompson RN, Christmas SE, Moots RJ. Natural killer cells in the synovial fluid of rheumatoid arthritis patients exhibit a $CD56^{bright}$, $CD94^{bright}$, $CD158^{negative}$ phenotype. Rheumatology 42(870-878, 2003.
594. Lo CKC, Lam QLK, Sun L, Wang S, KO K-H, Xu H, al. e. NAtural killer cell degeneration exacerbates experimental arthritis in mice via enhanced interleukin-17 production. Arthritis & Rheumatism 58(2700-2711, 2008.
595. Soderstrom K, Stein E, Colmenero P, Purath U, al. e. Natural killer cell trigger osteoclastogenesis and bone destruction in arthritis. Proc Natl Acad Sci USA 107(13028-13033, 2010.
596. Gur C, Porgador A, Elboim M, Gazit R, al. e. The activating receptor NKp46 is essentil for the development of type 1 diabetes. Nature Immunol 11(121-129, 2010.
597. Zhou R, Wei H, Tian Z. NK3-like NK cells are involved in protective effect of polyinosinic-polycytidylic acid on type 1 diabetes in nonpbese diabetic mice. J Immunol 178(2141-2147, 2007.
598. Winkler-Pickett R, Young HA, Cherry JM, al. e. In vivo regulation of experimental autoimmune encephalomyelitis by NK cells: alteration of primary adaptive responses. J Immunol 180(4495-4506, 2008.

599. Degushi H, Kitaura Y, Hayashi T, Kotaka M, Kawamura K. Cell-mediated immune cadiocyte injury in viral myocaditis of mice and patients. Japanese Circulation Journal 53(61-77, 1989.
600. young LHY, Joag SV, Zheng LM, Lee CP, Lee YS, Young JDE. Perforin-mediated myocardial damage in acute myocarditis. Lancet 336(1019-1021, 1990.
601. Godeny EK, Gauntt CJ. Involvement of natural killer cells in coxsackievirus B3-induced murine myocarditis. J Immunol 137(1695-1702, 1986.
602. Ayach BB, Yoshimitsu M, Dawood F, Sun M, Arab S, al. e. Stem cell factor receptor induces progenitor and natural killer cell-mediated cardiac survival and repair after myocardial infarction. Proc Natl Acad Sci USA 103(2304-2309, 2006.
603. Davani S, Deschaseaux F, Chalmers D, Tiberghien P, Kantelip JP. Can stem cells mend a broken heart? Cardiovascular Research 65(305-316, 2005.
604. Colucci F, Caligiuri MA, Di Santo JP. What does it take to make a natural killer? Nature Rev. Immunol 3(413-425, 2003.
605. Bouchentouf M, Forner KA, Cuerquis J, al. e. Induction of cardiac angiogenesis requires killer cell lectin-like receptor 1 and a4b7 integrin expression by NK cells. J Immunol 185(7014-7025, 2010.
606. Bobryshev YV, Lord RS. Identification of natural killer cells in human atherosclerotic plaque. Atherosclerosis 180(423-427, 2005.
607. Cler G, Rouz PM. Lymphocyes subsets in severe atherosclerosis before revascularization. Ann Intern Med 126(1004-1005, 1997.
608. Schiller NK, Boisvert WA, Curtis LK. Inflammtion in atherosclerosis: lesion formation in LDL receptor-deficient mice with perforin and Lyst beige mutations. Arterioscler. Thromb. Vasc. Biol. 22(1341-1346, 2002.
609. Baca ME, Mowat AM, Parrott DMV. Immunological studies of NK cell-deficient beige mice. Immunology 66(131-137, 1989.
610. Paigen B, Holmes PA, Novak EK, Swank RT. Analysis of atherosclerosis susceptibility in mice with genetic defects in platlet fucntion. Arteriosclerosis 10(648-653, 1990.
611. Saxena RK, Saxena QB, Adler WH. Defective T-cell response in beige mutation. Nature 295(240-241, 1982.
612. Wolff SM. The Chediak-Higashi syndrome: studies of host defenses. Ann Intern Med 76(293-306, 1972.
613. Vassali JD, Granelli-Piperno A, Grisceli C, Reich E. Specific protease deficiency in polymorphonuclear leukocytes of Chediak-Higashi syndrome and beige mice. J Exp Med 147(1285-1290, 1978.
614. Whitman SC, Rateri DL, Szivassy SJ, Yokoyama W, Daugherty A. Depletion of natural killer cell function decreases atherosclerosis in low-density lipoprotein receptor null mice. Arterioscler. Thromb. Vasc. Biol. 24(1049-1054, 2004.
615. Cui J, Shin T, Kawano T, Sato H, al. e. Requirement for Valpha14 NKT cells in IL-12-mediated rejection of tumors. Science 278(1623-1626, 1997.
616. Terabe M, Swann J, Ambrosino E, Sinha P, al e. NKT cell is sufficient for downregulation of tumor immunosurveillance. J Exp Med 202(1627-1633, 2005.
617. Ditiatkovski M, B.H. T, Bobik A. GM_CSF deficiency reduces macrophage PPAR-gamma expression and agravates atherosclerosis in ApoE-deficient mice. Arterioscler. Thromb. Vasc. Biol. 26(2337-2344, 2006.
618. Paigen B, Morrow A, Holmes PA, MItchell D, Williams RA. Quantitative assessment of atheroscelrotic lesions in mice. Atherosclerosis 68(231-240, 1987.
619. Ho EL, Carayannopoulos LN, Poursine-Laurent J, Kinder J, al. e. Costimulation of multiple NK cell activation receptors by NKG2D. J Immunol 169(3667-3675, 2002.

620. Schmitz M, Zhao S, al. e. Tumoricidal potential of native blood dendritic cells: direct tumor cell killing and activation of NK cell-mediated cytotoxicity. J Immunol 174(4127-4134, 2005.
621. Dussault A-A, Pouliot M. Rapid and simple comparison of messenger RNA levels using real-time PCR. Biological Procedures Online 8(1-10, 2006.
622. VandeLaan PA, Reardon CA. Thematic review series: The immune system and atherogenesis. The unusual suspects: an overview of the minor leukocyte populations in atherosclerosis. Journal of Lipid Research 46(829-838, 2005.
623. Millonig G, Malcom GT, Wick G. Early inflammatory -immunological lesions in juvenile atherosclerosis from the Pathobiological Determinants of Atherosclerosis in Youth (PDAY)-study. Atherosclerosis 160(441-448, 2002.
624. YIa-Herttuala S, Lipton BA, al. e. Expression of monocyte chemoattractant protein-1 in macrophage-rich areas of human and rabbit atherosclerotic lesions. Proc Natl Acad Sci USA 88(5252-5256, 1991.
625. Greaves DR, Hakkinen T, Lucas AD, al. e. Linked chromosome 16q13 chemokines, macrophage-derived chemokine, fractalkine, and thymus- and activation-regulated chemokine, are expressed in human atherosclerotic lesions. Arterioscler. Thromb. Vasc. Biol. 21(923-929, 2001.
626. Wuttge DM, Eriksson P, Sirsjo A, Hansson GK, Stemme S. Expression of interleukin-15 in mouse and human atherosclerotic lesions. The American Journal of Pathology 159(417-423, 2001.
627. Stephens HA. MICA and MICB genes: can the enigma of their polymorphism be resolved? Trends Immunol 22(378-385, 2001.
628. Faigel W, Raposo G, al. e. Deficient peptide loading and MHC class II endosomal sorting in a human genetic immunodeficiency disease: the Chediak-Higashi Syndrome. Journal of Cell Biology 141(1121-1134, 1998.
629. Schiller NK, Boisvert WA, Curtiss LK. Inflammtion in atherosclerosis : lesion formation in LDL receptor-deficient mice with perforin and Lyst (beige) mutations. Arterioscler. Thromb. Vasc. Biol. 22(1341-1346, 2002.
630. Linton MF, Major AS, Fazio S. Proatherogenic role of NK cell revealed. Arterioscler. Thromb. Vasc. Biol. 24(992-994, 2004.
631. Schacklett BL, Cox CA. 2004. The Journal of Immunology 173(641-648, et al.
632. Brawand P, Lemonnier FA, MacDonald HR, Cerottini JC, Held W. Trangenic expression of Ly49A on T cells impair a specific antitumor response. J Immunol 165(1871-1876, 2000.
633. Bade B, Boettcher HE, Lohrmann J, Hink-Schauer C, Bratke K, Jenne DE, Virchow JJC, Luttman W. Differential expression of the granzyme A, K,and M and perforin in human peripheral blood lymphocytes. Int Immunol 17(1419-1428, 2005.
634. Maeda N, Lohwasser S, Yamamura T, Takei F. Regulation of NKT cells by Ly49: Analysis of primary NKT cell and generation of NKT cell line. J Immunol 167(4180-4186, 2001.
635. Smyth MJ, Crowe NY, Godfrey DI. NK cells and NKT cells collabrate in host protection from methylcholanthrene-induced fibrosarcoma. Int Immunol 13(459-463, 2001.
636. Berger EA, Murphy PM, Farber JM. Chemokine receptors as HIV-1 coreceptors: roles in viral entry, tropism, and disease. Annu Rev Immunol 17(657-700, 1999.
637. Wang Y, Bai Y, al. e. Interferon-g induces human vascular smooth muscle cell proliferation and intimal expansion by phosphatidylinositol 3-kinase- dependent mammalian target of rapamycin raptor complex 1 activation. Circulation Research 101(560-569, 2007.

638. Peppel K, Zhang L, al. e. Activation of vascular smooth muscle cells by TNF and PDGF: overlapping and complementary signal transduction mechanisms. Cardiovascular Research 65(674-682, 2005.
639. Friesel R, Komoriya A, Maciag T. Inhibition of endothelial cell proliferation by gamma-interferon. The Journal of Cell Biology 104(689-696, 1987.
640. Frater-Schroder M, Risau W, Hallmann R, Gautschi P, Bohlen P. Tumor necrosis factor a, a potent inhibitor of endothelial cell growth in vitro, is angiogenic in vivo. Proc Natl Acad Sci USA 84(5277-5281, 1987.
641. Qi Y, Liang J, al. e. MCP-induced protein 1 suppresses TNF-a-induced VCAM-1 expression in human endothelial cells. FEBS Lett 584(3065-3072, 2010.
642. Li H, Cybulsky MI, Gimbrone Jr MA, Libby P. Inducible expression of vascular cell adhesion molecule-1 by vascular smooth muscle cells in vitro and within rabbit atheroma. Am J Pathol 143(1551-1559, 1993.
643. Couffinhal T, Duplaa C, Moreau C, Lamaziere JM, Bonnet J. Regulation of vascular cell adhesion molecule-1 and intracellular adhesion molecule-1 in human vascular smooth muscle cells. Circulation Research 74(225-234, 1994.
644. Roda JM, Parihar R, Magro C, Nuovo GJ, Tridandapani S, Carson III WE. Natural killer cells produce T cell-recruiting chemokines in response to antibody-coated tumor cells. Cancer Res 66(517-526, 2006.
645. De Sanctis JB, Blanca I, Bianco NE. Expression of different lipoprotein receptors in natural killer cells and their effect on natural killer proliferative and cytotoxic activity. Immunology 86(399-407, 1996.
646. De Sanctis JB, Blanca I, Bianco NE. Secretion of cytokines by natural killer cells promed with interleukin-2 and stimulated with different lipoproteins. Immunology 90(526-533, 1997.
647. De Sanctis JB, Blanca I, Bianco NE. Expression of different lipoprotein receptors in natural killer cells and their effect on natural killer proliferative and cytotoxic activity. Immunology 86(399-407, 1995.
648. Dong Z, Zhang J, Sun R, Wei H, Tian Z. Impairment of liver regeneration correlates with activated hepatic NKT cells in HBV transgenic mice. Hepatology 45(1400-1412, 2007.
649. Nishikado H, Mukai K, Kawano Y, Minegishi Y, Karasuyama H. NK cell-depleting anti-asialo GM1 antibody exhibits a lethal off-target effect on basophils in vivo. J Immunol 186(5766-5771, 2011.
650. Spritz RA. Genetic defects in Chediak-Higashi Syndrome and the *beige* mutation. Journal of Clinical Immunology 18(97-105, 1998.
651. Ward DM, Griffiths GM, Stinchcombe JC, Kaplan J. Analysis of the lysosomal storage deisease, Chediak Higashi syndrome. Traffic 1(816-822, 2001.
652. Burkhardt JK, Wiebel S, Hester S, Argon Y. The giant organelle in beige and Chediak-Higashi fibroblasts are derived from late endosomes and mature lysosomes. J Exp Med 178(1845-1856, 1993.
653. Getz GS. Do natural killer cells participate in a killer vascular disease? Arterioscler. Thromb. Vasc. Biol. 22(1251, 2002.
654. Hayakawa Y, Takeda K, Yagita H, Smyth MJ, al. e. IFN-g-mediated inhibition of tumor angiogenesis by natural killer T-cell ligand, a-glactosylceramide. Blood 100(1728-1733, 2002.
655. Schoenborn JR, Wilson CB. Regulation of interferon-gamma during innate and adaptive immune responses. Advances in Immunology 96(41-101, 2007.
656. Carswell EA, al. e. An endotoxin-induced serum factor that causes necrosis of tumors. Proc Natl Acad Sci USA 72(3666-3670, 1975.

657. Dulak J, Jozkowics A, al. e. Vascular endothelial growth factor: angiogenesis, atherogenesis or both? J Am Coll Cardiol 38(2137-2138, 2001.
658. Roberts AB, Sporn MB, al. e. Transforming growth factor type-beta: rapid induction of fibrosis and angiogenesis in vivo and stimulation of collagen formation in vitro. Proc Natl Acad Sci USA 83(4167-4171, 1986.
659. Moulton KS, Heller E, Konerding MA, Flynn E, Palinski W, Folkman J. Angiogenesis inhibitors endostatin or TNP-470 reduce intimal neovascularization and plaque growth in apolipoprotein E-deficient mice. Circulation 99(1726-1732, 1999.
660. Lundberg AM, Hansson GK. Innate immune signals in atherosclerosis. Clin Immunol 134(5-24, 2010.
661. Kol A, Libby P. The mechanisms by which infectious agents may contribute to atherosclerosis and its clinical manifestations. Trends Cardiovasc. Med. 8(191-199, 1998.
662. Gibson FC, Honh C, al. e. Innate immune recognition of invasion bacteria accelerates atherosclerosis in apolipoprotein E-deficient mice. Circulation 109(2801-2806, 2004.
663. Baiersdorfer M, Schwarz M, Seehafer K, Lehmann C, al. e. Toll-like receptor 3 mediates expression of clusterin/apolipoprotein J in vascular smooth mucle cells stimulated with RNA releases from necrotic cells. Experimental Cell Research 316(3489-3500, 2010.
664. Kawai T, Akira S. Pathogen recognition with toll-like receptors. Curr Opin Immunol 17(338-344, 2005.
665. Elfeldt K, Swedenborg J, Hansson GK, Yan ZQ. Expression of toll-like receptors in human atherosclerotic lesions: a possible pathway for plaque activation. Circulation 105(1158-1161, 2002.
666. Ahmad U, Ali R, al. e. IFN-gamma primes intact human coronary arteries and cultured coronary smooth muscle cells to double-stranded RNA- and self-RNA-induced inflammtory responses by upregulating TLR3 and melanoma differentiation-associated gene 5. J Immunol 185(1283-1294, 2010.
667. Keepers TR, Gross LK, Obrig TG. Monocyte chemoattractant protein 1, macrophage inflammatory protein 1 alpha, and RANTES recruit macrophages to the kidney in a mouse model of hemolytic-uremic syndrome. Infect Immun 75(1229-1236, 2007.
668. Zamilpa R, Kanakai R, al. e. CC chemokine receptor 5 deletion impairs macrophage activation and induces adverse remodeling following myocardial infarction. Am J Physiol Heart Circ Physiol 300(H1418-H1426, 2011.
669. Funderburg N, Lucianno AA, Jiang W, Rodriguez B, Sieg SF, Lederman MM. Toll-like receptor ligands induce human T cell activation and death, a model for HIV pathogenesis. PLoS ONE 3(e1915-e1921, 2008.
670. Ley K, Huo Y. VCAM-1 is critical in atherosclerosis. J Clin Invest 107(1209-1210, 2001.
671. Wang J, Xu J, Zhang W, Wei H, Tian Z. TLR3 ligand-induced accumulation of activated splenic natural killer cells into liver. Cell Mol Immunol 2(449-453, 2005.
672. Sawaki J, Tsutsui H, al. e. Type 1 cytokine/chemokine production by mouse NK cells following activation of their TLR/MyD88-mediated pathways. Int Immunol 19(311-320, 2007.
673. Borrego F, Pena J, Solana R. Regulation of CD69 expression on human natural killer cells: differential involvement of protein kinase C and protein tyrosine kinases. Eur J Immunol 23(1039-1043, 1993.
674. Karlhofer FM, Yokoyama W. Stimulation of murine natural killer (NK) cells by a monoclonal antibody specific for the NK1.1 antigen. IL-2-activated NK cells possess additional specific stimulation pathways. J Immunol 146(3662-3673, 1991.
675. Borrego F, al e. Nk phenotypic markers and IL-2 response in Nk cells from elderly people. Exp. Gerontol 34(253-265, 1999.

676. Castrillo A, Joseph SB, al. e. Crosstalk between LXR and toll-like receptor signalling mediates bacterial and viral antagonism of cholesterol metabolism. Mol Cell 12(805-816, 2003.
677. McCartney SA, Thackray LB, Gitlin L, Gilfillan S, Virgin HW, Colonna M. MDA-5 recognition of murine norovirus. PLoS Pathog 4(e1000108, 2008.
678. Lusis AJ. Atherosclerosis. Nature 407(233-241, 2000.
679. Martinet W, Knaapen MW, De Meyer GR, Herman AG, Kockx MM. Elevated levels of oxidative DNA damage and DNA repair enzymes in human atherosclerosis. Circulation 106(927-932, 2002.
680. Martinet W, de Meyer GR, Herman AG, Kockx M. Reactive oxygen species induce RNA damage in human atherosclerosis. Eur J Clin Invest 34(323-327, 2004.
681. Bennett MR. Reactive oxygen species and death: oxidative DNA damage in atherosclerosis. Circ Res 88(648-650, 2001.
682. Gasser S, Raulet D. The DNA damage responses arouses the immune system. Cancer Res 66(3959-3962, 2006.
683. Siemens DR, Hu N, al. e. Hypoxia increases tumor cell shedding of MHC class I chain-related molecule: role of nitric oxide. Cancer Res 68(4746-4753, 2008.
684. Smyth MJ, Crowe NY, Pellicci DG, Kyparissoudis K, Kelly JM, Takeda K, Yagita H, Godfrey DI. Sequential production of interferon-g by NK1.1^{+}T cells and natural killer cells is essential for the anti-metastatic effect of a-galactosylceramide. Blood 99(1259-1266, 2002.
685. Smyth MJ, Swann J, Cretney E, Zerafa N, Yokoyama W, Hayakawa Y. NKG2D function protects the host from tumor initiation. J Exp Med 202(583-588, 2005.
686. Smyth MJ, Swann J, Kelly JM, Cretney E, Yokoyama W, Diefenbach A, Sayers TJ, Hayakawa Y. NKG2D recognition and perforin effector function mediate effective cytokine immunotherapy of cancer. J Exp Med 200(1325-1335, 2004.
687. Hamerman JA, Ogasawara K, Lanier LL. Cutting edge: Toll-like receptor signalling in macrophages induces ligands for the NKG2D receptor. J Immunol 172(2001-2005, 2004.
688. Rogers L, Burchat S, al. e. Deficiency of invariant V alpha 14 natural killer T cells decrease atherosclerosis in LDL receptor null mice. Cardiovascular Research 78(167-174, 2008.
689. Duthie MS, Kahn SJ. NK cell activation and protection occur independently of natural killer T cells during Trypanosoma cruzi infection. Internationl Immunology 17(607-613, 2005.
690. Regunathan J, Chen Y, Wang D, Malarkannan S. NKG2D receptor-mediated NK cell function is regulated by inhibitory Ly49 receptors. Blood 105(233-240, 2005.
691. Schwinn N, Vokhminova D, al. e. INterferon-gamma down-regulates NKG2D ligand expression and impairs the NKG2D-mediated cytolysis of MHC class I-deficient melanoma by natural killer cells. Int J Cancer 124(1594-1604, 2009.
692. Young JL, Libby P, Schonbeck U. Cytokines in the pathogenesis of atherosclerosis. Thromb Haemost 88(554-567, 2002.
693. Muntasell A, Magri G, Pende D, Angulo A, Lopez-Botet M. Inhibition of NKG2D expression in NK cells by cytokines secreted in response to human cytomegalovirus infection. Blood 115(5170-5179, 2010.
694. Lee TS, Yen HC, Pan CC, Chau LY. The role of interleukin-12 in the development of atherosclerosis in ApoE-deficient mice. Arterioscler. Thromb. Vasc. Biol. 19(734-742, 1999.
695. Li H, Cybulsky MI, Gimbrone Jr MA, Libby P. An atherogenic diet rapidly induces VCAM-1, a cytokine-regulatable mononuclear leukocyte adhesion molecule, in rabbit aortic endothelium. Arterioscler. Thromb. Vasc. Biol. 13(197-204, 1993.

696. Li H, Freeman MW, Libby P. Regulation of smooth muscle cell scavenger receptor expression in vivo by atherogenic diets and in vitro by cytokines. J Clin Invest 95(122-133, 1995.
697. Libby P. Molecular bases of the acute coronary syndromes. Circulation 91(2844-2850, 1995.
698. Warner SJ, Friedman GB, Libby P. Immune interferon inhibits proliferation and induces 2'-5'-oligoadenylate synthase gene expression in human vascular smooth muscle cells. J Clin Invest 83(1174-1182, 1989.
699. Hansson GK, Hellstrand M, Rymo L, Rubbia L, Gabbiani G. Interferon gamma inhibits both proliferation and expression of differentiation-specific alpha-smooth muscle actin in arterial smooth muscle cells. J Exp Med 170(1595-1608, 1989.
700. Schneider DB, Vassalli G, al. e. Expression of Fas ligand in arteries of hypercholesterolemic rabbits accelerates atherosclerotic lesion formation. Arterioscler. Thromb. Vasc. Biol. 20(298-308, 2000.
701. Takeda K, Cretney E, al. e. TRAIL identifies immature natural killer cells in newborn mice and adult mouse liver. Blood 105(2082-2089, 2005.
702. Slevin M, Ethirajan P, al. e. Dynamin and perforin are associated with neovascularization in advanced carotid plaques. Front Biosci 13(6515-6519, 2008.
703. Trapani JA, Jans P, al. e. Peforin-dependent nuclear entry of granzyme B precedes apoptosis, and is not a consequence of nuclear membrane dysfunction. Cell Death Differ 5(488-496, 1998.
704. Schrijivers DM, De Meyer GR, Kockx MM, Herman AG, Martinet W. Phagocytosis of apoptotic cells by macrophages is impaired in atherosclerosis. Arterioscler. Thromb. Vasc. Biol. 25(1256-1261, 2005.
705. Li H, Ambade A, Re F. Cutting Edge: Necrosis activates the NLRP3 inflammasome. J Immunol 183(1528-1532, 2009.
706. Shresta S, Maclover DM, Heusel JW, Russell JH, Ley TJ. Natural killer and lymphokine-activated killercells require granzyme B for the rapid induction of apoptosis in susceptivle target cells. Proc Natl Acad Sci USA 92(5679, 1995.
707. Schaub FJ, Han DK, al. e. Fas/FADD-mediated activation of a specific program of inflammatory gene expression in vascular smooth muscle cells. Nat Med 6(790-796, 2000.
708. Kahn M, Pelengaris S, Cooper M, Smith C, Evan G, Betteridge J. Oxidised lipoproteins may promote inflammtion through the selective delay of engulfment but not binding of apoptotic cells by macrophages. Atherosclerosis 171(21-29, 2003.
709. Thorp E, Cui D, Schrijivers DM, Kuriakose G, Tabas I. Mertk receptor mutation reduces efferocytosis efficiency and promotes apoptotic cell accumulation and plaque necrosis in atherosclerotic lesion of $Apoe^{-/-}$ mice. Arterioscler. Thromb. Vasc. Biol. 28(1421-1428, 2008.
710. Marui N, Offermann MK, al. e. VAscular cell adhesion molecule-1 (VCAM-1) gene transcription and expression are regulated through an antioxidant-sensitive mechanism in human vascular endothelial cells. J Clin Invest 92(1866-1874, 1993.
711. Metkar SS, Wang B, al. e. Cytotoxic cell granule-mediated apoptosis: perforin delivers granzyme B-serglysin complexes into target cells without plasma membrane pore formation. Immunity 16(417-428, 2002.
712. Trapani JA, Smyth MJ. Functional significance of perforin/granzyme cell death pathway. Nat Rev Immunol 2(735-747, 2002.
713. Duwell P, Kono H, al. e. NLRP3 inflammasomes are required for atherogenesis and activated by cholesterol crystals. Nature 464(1357-1361, 2010.

714. Urbonaviviute V, Furnrohr BG, al. e. Induction of inflammatory and immune responses by HMGB1-nucleosome complexes: implications for the pathogenesis of SLE. J Exp Med 205(3007-3018, 2008.
715. Kanellakis P, Agrotis A, al. e. High-mobility group box protein 1 neuralization reduces development of diet-induced atherosclerosis in apolipoprotein e-deficient mice. Arterioscler. Thromb. Vasc. Biol. 31(313-319, 2011.
716. Cybulsky MI, Iiyama K, Li H, Zhu SN, Chen M, Iiyama M, Davis V, Gutierrez-Ramos JC, Connelly PW, Milstone DS. A mojor role for VCAM-1, but not ICAM-1 in early atherosclerosis. J Clin Invest 107(1255-1262, 2001.
717. Cybulsky MI, Gimbrone MA, Libby P. Inducible expression of vascular cell adhesion molecule-1 by vascular smooth muscle cells in vitro and within rabbit atheroma. Am J Pathol 143(1551-1559, 1993.
718. Singh NJ, Schwartz RH. The lymphopenic mouse in immunolgy: from patron to pariah. Immunity 25(851-855, 2006.
719. Degli-Esposti MA, Smyth MJ. Close encounters of different kinds: dendritic cells and NK cells take centre stage. Nature 5(112-124, 2005.
720. Robbins SH, Bessou G, al. e. Natural killer cells promote early CD8 T cell responses against cytomegalovirus. PLoS Pathog 3(e123, 2007.
721. Jennings P, Yuan D. NK cell enhancement of antigen presentation by B lymphocytes. J Immunol 182(2879-2887, 2009.
722. Yang TY, Sun Y, Langnas AN, Zhao Y. Prolongation of allogenic skin graft survival by injection of anti-Ly49A monoclonal antibody YE1/48. Clin Immunol 106(148-154, 2003.
723. Held W, Cado DR, D.H. Transgenic expression of the Ly49A natural killer cell receptor confers class I major histocompatibility complex (MHC)-specific inhibition and prevents bone maroow allograft rejection. J Exp Med 184(2037-2041, 1996.
724. Held W, Raulet D. LY49A transgenic mice provide evidence for a major histocompatibility complex-dependent education process in natural killer cell development. J Exp Med 185(2079-2088, 1997.
725. Salem ML, Diaz-MOntero CM, El-Nagger SA, Chen Y, Moussa O, Cole DJ. The TLR3 agonist poly (I:C) targets CD8+ T cells and augments their antigen-specific responses upon their adoptive transfer into naiv receipient mice. Vaccine 27(549-557, 2009.
726. Shinkai Y, Rathbun G, Lam KP, Oltz EM, Stewart V, al e. RAG-2-deficient mice lack mature lymphocytes owing to iability to initiate V(D)J rearrangement. Cell 68(855-867, 1992.
727. Walzer T, Dalod M, Robbins SH, Zitvogel L, Vivier E. Natural-killer cells and dendritic cells: "l' union fait la force". Blood 106(2252-2258, 2005.
728. Ting AT, Einspahr KJ, Abraham RT, Leibson PJ. Fc gamma receptor signal transduction in natural killer cells. Coupling to phospholipase C via a G protein-independent, but tyrosine kinase-dependent pathway. J Immunol 147(3122-3127, 1991.
729. Jamieson AM, Diefenbach A, Mcmaho CW, Xiaong N, Carlyle JR, Raulet DH. The role of NKG2D immunoreceptor in immune cell activation and natural killing. Immunity 17(19-29, 2002.
730. Eberl G, Brawand P, MacDonald HR. Selective bystander proliferation of memory CD4+ and CD8+ T cells upon NK T or T cell activation. J Immunol 165(4305-4311, 2000.
731. Vivier E, Tomasello E, Baratin M, Walzer T, Ugolini S. Functions of natural killer cells. Nat Immunol 9(503-510, 2008.
732. Valente AJ, Xie JF, Abramova MA, Wenzel UO, Abboud HE, Graves DT. A complex element regulates IFN-gamma-stimulated monocyte chemoattractant protein-1 gene transcription. J Immunol 161(3719-3728, 1998.

733. Panousis CG, Zuckerman SH. Interferon-gamma induces downregulation of Tangier disease gene (ATP-binding-cassette transporter 1) in macrophages-derived foam cells. Arterioscler. Thromb. Vasc. Biol. 20(1565-1571, 2000.
734. Iyer SS, Pulskens WP, al. e. Necrotic cells trigger a sterile inflammatory response through the Nlrp3 inflammasome. Proc Natl Acad Sci USA 106(20388-20393, 2009.
735. Scaffidi P, Misteli T, Bianchi ME. Release of chromatin protein HMGB1 by necrotic cells triggers inflammation. Nature 418(191-195, 2002.
736. Kalinina N, Agrotis A, Antropova Y, al. e. Increased expression of the DNA-binding cytokine HMGB1 in human atherosclerosis lesions: role of activated macrophages and cytokines. Arterioscler. Thromb. Vasc. Biol. 24(2320-2325, 2004.
737. Khan IA, Thomas SY, Moretto MM, al. e. CCR5 is essential for NK cell trafficking and host survival following Toxoplasma gondii infection. PLoS Pathogens 2(e49, 2006.
738. Lena G, Trapani JA, Sutton VR, Ciccone A, al. e. Dihydrofuro[3,4-c] pyridinones as inhibitors of the cytolytic effects of the pore-forming protein perforin. J Med Chem 51(7614-7624, 2008.
739. Little PJ, Chait A, Bobik A. Cellular and cytokine-based inflammatory processes as novel therapeutic targets for the prevention and treatment of atherosclerosis. Pharmacology & Therapeutics 131(255-268, 2011.
740. Tiemann K, Weyer D, Djoufack PC, Ghanem A, al. e. Increasing myocardial contraction and blood pressure in C57BL/6 mice during early postnatal development. Am J Heart Circ Physiol 284(H464-H474, 2003.
741. Bassiouny HS, Zarins CK, Lee DC, Skelly CL. Diurnal heart rate reactivity: A predictor of severity of experimental coronary and carotid atherosclerosis. Journal of Cardiovascular Risk 9(331-338, 2002.