The role of cIAP2 in early and late atherosclerosis lesion development

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ABSTRACT

Cellular Inhibitor of Apoptosis 2 (cIAP2) belongs to the IAP family, a group of endogenous proteins that inhibit apoptosis. However, the physiological role of cIAP2 remains poorly defined. Knock-out (KO) and wild type (WT) mice were used to examine the effect of cIAP2 protein on the progression of atherosclerosis in apoE $^{-/-}$ mice. Following the high-fat diet period of 4 and 12 wks, tissues were harvested and analysis focused on the aortic root, the aortic arch, the descending aorta, and the blood. *Ex vivo* results show a significant decrease in aortic arch lesion area in KO vs. WT in both study groups. Results also show a decrease in aortic root lesion size in KO vs. WT in both study groups. These results support that cIAP2 is an important survival factor for lesion-associated macrophages, since loss of cIAP2 expression in this mouse model reduced atherosclerotic lesion development.

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List of abbreviations

ACAT:	Acyl CoA Cholesterol acyl transferase
AIM	Apoptosis inhibitor of macrophage
Apaf-1	Apoptotic protease activating factor 1
ApoE	Apolipoprotein E
ATP	Adenosine triphosphate
BIR	Baculovirus IAP Repeat
BMT	Bone Marrow Transplant
CAD	Coronary Artery Disease
CARD	Caspase activation and recruitment domains
cIAP1	Cellular inhibitor of apoptosis protein 1
cIAP2	Cellular inhibitor of apoptosis protein 2
CT	Computed Tomography
CVD	Cardiovascular Disease
DAB	Diaminobenzidinetetrahydrochlorid
DIABLO	Direct IAP binding protein with low pI
E1	Ubiquitin-activating enzyme
E2	Ubiquitin-conjugating enzyme
E3	Ubiquitin ligase
ER	Endoplasmic reticulum
F1	Filial 1
F2	Filial 2
FADD	Fas-associated via death domain

FC	Free cholesterol
F^{18}-FDG	Flouro-deoxy-glucose
FLIP	FLICE-inhibitory protein
FPLC	Fast Protein Liquid Chromatography
HDL	High Density Lipoprotein
IAP	Inhibitor of Apoptosis Protein
IBM	IAP Binding Motif
IDL	Intermediate density lipoprotein
KO	Knock out
LDL	Low Density Lipoprotein
LPS	Lipopolysaccharides
MCP-1	Monocytes chemotactic protein-1
MPTP	Mitochondrion permeability transition pores
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cell
OCT	Optimal cutting temperature
oxLDL	Oxidized LDL
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PET	Positron Imaging Tomography
RAS	Renin-Angiotensin-System
RING	Really Interesting New Gene
Rip1	Receptor-interacting protein 1
Smac	Second mitochondrial activator of caspases

SR	Scavenger Receptor
SR-A	Scavenger Receptor-A
TNF-α	Tumor Necrosis Factor - α
TNFR1	Tumor necrosis factor receptor 1
TNFR2	Tumor necrosis factor receptor 2
TRADD	TNFR-associated death domain
TRAF	TNF receptor associated factor
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
VLDL	Very Low Density Lipoprotein
WT	Wild Type
XIAP	X-linked Inhibitor of Apoptosis

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I would like to thank the late Dr. Whitman for all his support, encouragement and direction that he has provided me with this project. His interest and expertise has made my experience as part of his lab extremely rewarding. I would also like to extend many thanks: to my advisory committee Dr. Martin Holcik and Dr. Erik Suuronen for their help in directing my project; to Dr. Ross Milne for his continuous support and advise; and to Dr. Rob Beanlands for providing me the opportunity to have a positive experience, applying high caliber of research, and working with a fantastic team of people. Finally, I would like to extend my gratitude to the many people who put their time and effort into this project in a variety of ways, including: the outstanding laboratory technicians Jessica Gage, Mirela Hasu, Mohamed Thabet, Sarah Burchat and Stephanie Thorn.

In the end, I would like to dedicate this thesis to my Mom and Dad, sisters, and husband for their unconditional support and care.

CHAPTER 1: Introduction

Atherosclerosis is the underlying cause of most cardiovascular diseases, including coronary artery disease (CAD), ischemic gangrene, abdominal aortic aneurysms, and many cases of heart failure and stroke. Atherosclerosis is now considered an inflammatory disease involving inflammatory, smooth muscle and endothelial cells and their signaling pathways. Macrophages play a critical role in the disease progression where their regulation is highly impacted by the rate at which they are cleared from the intima (reviewed by Tabas I., 2005). Apoptosis of macrophages, whether at an early or late stage during the disease, plays an important role in the pathogenesis of atherosclerosis disease. Apoptosis is a highly regulated process involving multiple signaling pathways. Under different circumstances, both anti- and pro-apoptotic mechanisms contribute to the development and progression of atherosclerosis, yet the molecular events leading to these processes are not clearly understood. Cellular Inhibitor of Apoptosis 2 (cIAP2) belongs to the inhibitors of apoptosis (IAP) family, a group of endogenous proteins that inhibit apoptosis. As such, cIAP2 can protect cells (such as macrophages) from apoptosis by inhibiting the activity of certain caspases. Interestingly, preliminary unpublished studies from Dr. Whitman's laboratory outline a role for cIAP2 in atherosclerotic disease progression in the low density lipoprotein receptor (*LDLr* $^{-/-}$) mouse models (can be found in the index on pg. 89). The present study examines, for the first time, the novel role of cIAP2 in atherosclerotic lesion development at an early and late stage in the Apolipoprotein E (*apoE* $^{-/-}$) mouse model.

CHAPTER 2: Background

2.1 Atherosclerotic Cardiovascular Disease

According to the World Health Organization, Atherosclerotic cardiovascular disease (CVD) remains one of the leading causes of death in the Western world. Of the many complications that accompany atherosclerotic CVD, myocardial infarctions (MIs), peripheral artery disease and strokes remain to be the most precarious (reviewed in Lucas AD. and Greaves DR., 2001). The mechanism of atherosclerosis progression remains to be heavily researched, as it involves many molecular and cellular processes. The disease is mainly characterized by the build up of lipid-rich plaques in the walls of large arteries (reviewed in Lusis J., 2000). These plaques are referred to as "atheromas" and contain a soft, yellow core of lipids covered by a white fibrous "cap" (reviewed in Ross R., 1999). As the atheromas grow in size, they can obstruct blood flow to the target organ and the underlying vessel wall can weaken causing the plaque or atheroma to rupture. These events lead to thrombosis of the vessel resulting in strokes or MIs (reviewed in Lusis J. 2000). Of the many processes involved in the progression of the disease, macrophage recruitment to the site of injury plays the most pivotal role as first described by in vitro studies of isolated macrophages demonstrating their role in internalizing, storing and secreting large amounts of lipoprotein cholesterol (reviewed in Lusis J, 2000; Fowler S. et al., 1979). The focus of this project is to examine the roles played by regulatory mechanisms of macrophages in the progression of atherosclerotic disease.

2.1.1 Working model of atherosclerosis

Extensive studies have shown that during atherogenesis, the vessels undergo a series of changes involving blood-derived inflammatory cells (such as monocytes and macrophages) (reviewed in Lusis J., 2000). Furthermore, early cell culture studies have shown that disease initiation and progression is largely impacted by the accumulation of oxidized LDLs in the intima (Reviewed in Lusis J. 2000; Goldstein JL. et al., 1979). Together, these events contribute to monocyte recruitment and foam cell formation.

Generally, the endothelium functions as a barrier between the blood and tissues where it is also able to control the remodeling of the vasculature, inflammation as well as thrombus formation (reviewed in Gimbrone MA., 1999, Mantovani A, et al., 1992). However, it is now clear that endothelial cells' morphology and even modification of gene expression is affected in areas where there is high shear stress (Reviewed in Gimbrone MA., 1999; Rasnick N, et al., 1995). Endothelial cells in regions where the artery branches show signs of increased permeability to molecules, such as circulating LDLs, which enter the subendothelial space and contribute to the process of atherosclerosis (*DePaoblo N, et al., 1992*). Once in the subendothelial space, "trapped" LDLs are exposed to matrix proteoglycans and undergo modifications such as oxidation, lipolysis, proteolysis and aggregation (Goldstein JL. et al., 1979). Injury to the endothelium also leads to the over-expression of certain molecules that are responsible for the adherence, migration and accumulation of monocytes and T cells into the endothelial (reviewed in Ross R., 1999; reviewed in Lusis AJ, 2000). Such molecules include platelet-endothelial-cell adhesion molecules and chemo-attractant molecules (such as MCP-1 or monocyte chemoattractant molecule -1 as well as modified LDLs) (Reviewed in Ross R., 1999; Yu X, et al., 1992; Kita T, et al., 1990; reviewed in Lusis AJ, 2000). The

arterial wall eventually thickens and compensates by gradually dilating whereas the radius of the lumen maintains the same size and dimension – this is also known as the 'remodeling' phenomena as described by Stastny and colleagues (Stastny J. *et al.,* 1986). Upon activation, the inflammatory cells induce additional damage that leads to focal necrosis. This is thought to be mediated via cytokines, chemokines, hydrolytic enzymes and growth factors that are released (reviewed in Lusis J., 2000; reviewed in Ross R., 1979). An outline of the events leading to the initiation of atherosclerosis is represented in **Figure 1.**

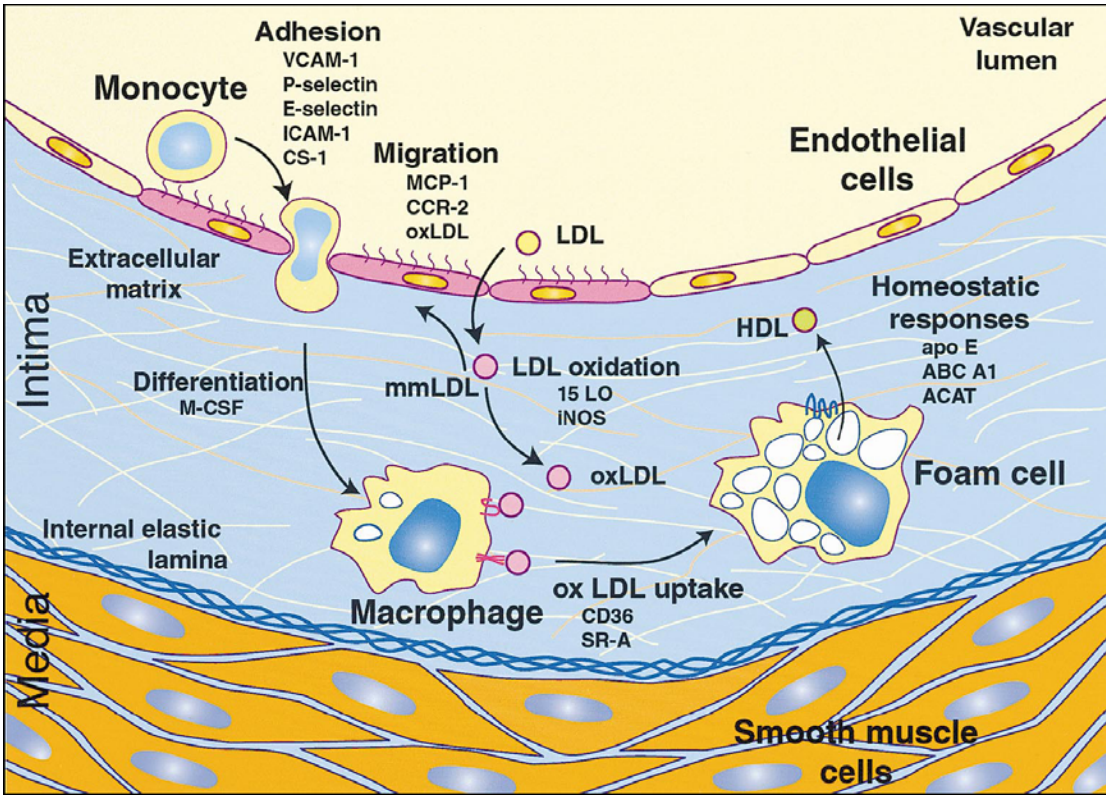

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Figure 1. **Initiating Events in the Development of a Fatty Streak Lesion**

"LDL is subject to oxidative modifications in the subendothelial space, progressing from minimally modified LDL (mmLDL), to extensively oxidized LDL (oxLDL). Monocytes attach to endothelial cells that have been induced to express cell adhesion molecules by mmLDL and inflammatory cytokines. Adherent monocytes migrate into the subendothelial space and differentiate into macrophages. Uptake of oxLDL via scavenger receptors leads to foam cell formation. OxLDL cholesterol taken up by scavenger receptors is subject to esterification and storage in lipid droplets, is converted to more soluble forms, or is exported to extracellular HDL (high density lipoprotein) acceptors via cholesterol transporters, such as *ABC-A1*." (Christopher K. Glass and Joseph L. Witztum., 2001, pg. 506)

There are six stages of atherosclerosis progression as reviewed by Whitman SC (Whitman SC, 2004). Clinical manifestations of atherosclerosis are often only visible very late in the disease process when lesions reach stage VI (Whitman SC., 2004). The earliest lesions (type I lesions) contain lipid deposits microscopically and chemically detectable in the intimal space (Wissler RW., 1992). Type I lesions eventually lead to Type II lesions or fatty streaks which mainly consist of microscopically evident macrophage foam cells (Reviewed in Stary HC. *et al.,* 1994; Nakashima Y. et al., 1994). More advanced Type II lesions with a larger number of macrophages (+7 layers thick) are termed Type III lesions (*ibid*). Macrophage recruitment and lipid deposition eventually lead to the enlargement and restructuring of the lesions. This continues until the lesions develop a core of lipids and necrotic tissue that become covered by a fibrous cap (Whitman SC, 2004). The first lesions to be considered advanced are stage IV lesions with a widespread, although well defined, area in the tunica intima filled with extracellular lipids (Whitman SC, 2004). Stage V is reached when there is fibrous tissue accumulation on the lumen side of the lipid core resulting in "cap" formation (Whitman SC., 2004). When stages IV and V contain thrombotic deposits or hematoma, they are considered Stage VI. Stage IV and V have been shown to form in the lumen of *apoE*$^{-/-}$ mice fed an atherogenic diet for a prolonged period (14 to 20 weeks), however, stage VI lesions are uncommon (Whitman SC, 2004). An outline of the events leading to lesion progression is represented in **Figure 2.**

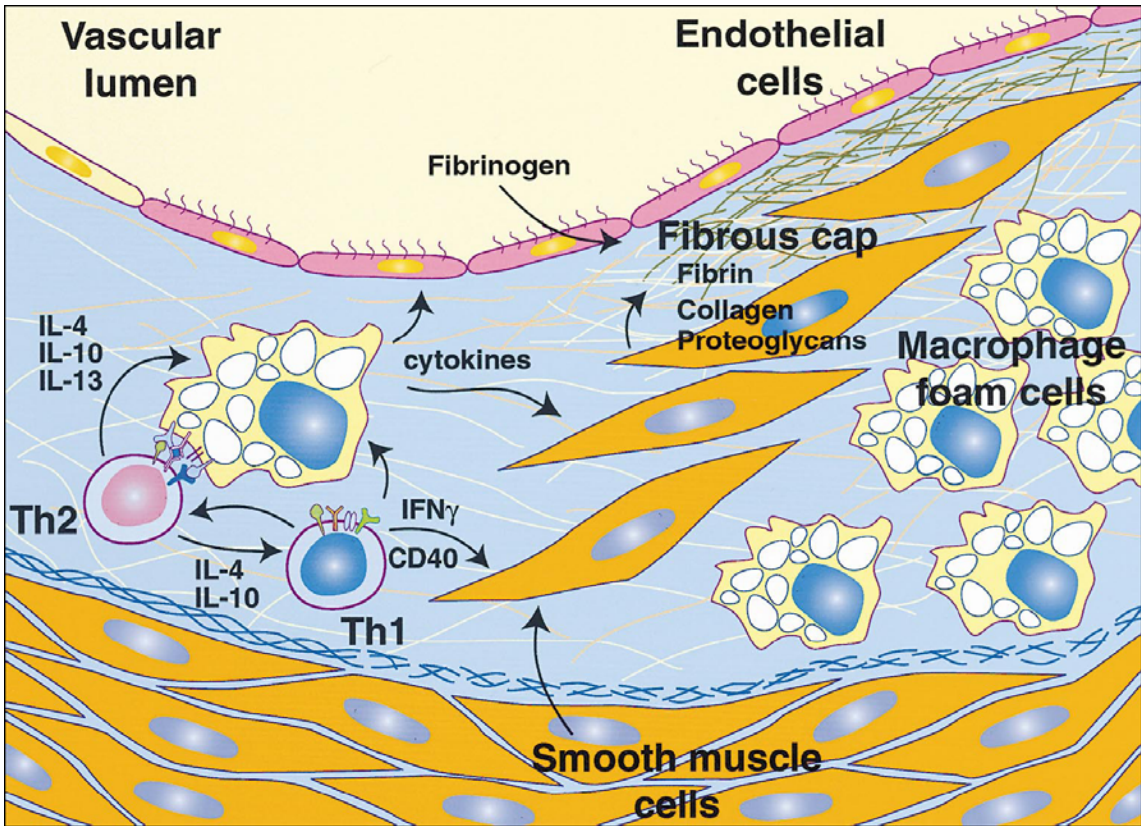

Figure from Cell, Vol. 104, 503–516, February 23, 2001.
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Figure 2. Atherosclerotic lesion progression.

"Interactions between macrophage foam cells, Th1 and Th2 cells establish a chronic inflammatory process. Cytokines secreted by lymphocytes and macrophages exert both pro- and antiatherogenic effects on each of the cellular elements of the vessel wall. Smooth muscle cells migrate from the medial portion of the arterial wall, proliferate and secrete extracellular matrix proteins that form a fibrous plaque." (Christopher K. Glass and Joseph L. Witztum., 2001, pg. 507)

MIs and strokes are usually a result of plaque rupture as opposed to the progressive narrowing of the lumen (reviewed in Libby P. *et al.,* 1997). Thrombosis and platelet adherence are products of a coagulation cascade that starts with the exposure of lipids from the ruptured plaque to blood components and tissue factor (reviewed in Libby P. *et al.,* 1997). The shoulder region of the plaque has a relatively high concentration of lipid-laden macrophages compromising a large necrotic core, and once exposed to high shear stress is more likely to rupture (Davies MJ. *et al.,* 1993). The dynamic nature and complexity of the mechanisms leading to atherosclerosis make it difficult to pin point the cause and effect relationship especially when trying to examine the role of a specific molecule whether it is a protein or a gene of interest.

In order to better understand how atherosclerotic disease is initiated, the mechanisms leading to foam cell formation need to be fully examined. Much research in the field has led to a somewhat complex picture of these events as described in the next section.

2.1.2 Foam cell formation

As previously mentioned, Bell and colleagues first identified the route by which lipoproteins travel from the plasma to the sub-endothelium which is believed to be through mechanisms of enhanced focal endothelial or via transient inter-endothelial cell gaps transcytosis (Reviewed in Weinbaum S and Chien S., 1993; Bell FP. *et al*., 1974). Once the lipoproteins are in the sub-endothelial space, they can either return to the circulation or bind to proteoglycans and become "trapped" in the sub-endothelial matrix (Williams KJ. and Tabas I., 1995). Native LDLs can be taken up by macrophages via their classical LDL receptor (LDLr-mediated endocytosis) (Reviewed in Vainio S. and Ikonen E., 2003;

Goldstein JL. *et al.*,1983). Transcription of the *LDLr* gene is regulated by the amount of cholesterol in the cell where LDLr expression decreases as cholesterol becomes more abundant (*ibid*). Since the uptake of cholesterol via the LDLr is down regulated by increasing amounts of cholesterol, this route does not lead to excessive lipid accumulation within the cell. Therefore, macrophage uptake of native LDL does not contribute largely to the formation of foam cells during the early stages of atherosclerosis.

Endothelial cells, SMCs, as well as macrophages are all able to convert LDL into oxidized LDL (oxLDL) (Tiwari RL. *et al.,* 2008). oxLDL's uptake is regulated by mechanisms different than those of native LDL uptake (Reviewed in Vainio S. and Ikonen E., 2003; Goldstein JL. *et al.*,1983). Modified LDLs are taken up by scavenger receptors (SR) which are a different class of receptors specific to macrophages (*ibid*). As reviewed by Vainio and Ikonen, scavenger receptor A (SR-A) expressed on the macrophages surface identify and enable the internalization of oxLDL (reviewed in Vainio S. and Ikonen E., 2003). In this cholesterol uptake pathway, oxLDL stimulates its own uptake by up-regulating the expression of surface receptors. This uptake of oxLDL by macrophages generates a lipid-laden foam cell. High oxLDL concentrations are one factor contributing to macrophage apoptosis in the plaque (Lutgens E. *et al.,* 1999).

The section below discusses the different factors affecting macrophage apoptosis in atherosclerotic lesions and the impact this has on lesion stability.

2.2 Atherosclerosis and Apoptosis

As described by Lutgens and colleagues, as the plaque becomes more advanced, there is a high abundance of apoptosing macrophages in the lesion triggered by several upstream

signaling pathways (Lutgens E. *et al.,* 1999). The most studied of these are ones involving the cell surface death receptors as well as those generated through disruption of the mitochondria or endoplasmic reticulum (reviewed in Tabas I., 2005). The lipid-laden macrophages found in the atherosclerotic plaques are influenced by multiple factors that can lead to their apoptosis (*ibid.*). Based on *in vitro* studies that mimic the molecular and cellular conditions of atherosclerotic lesions, pro-apoptotic factors have been identified. Some of these pro-apoptotic factors are tumor necrosis factor-alpha (TNF-α), Fas ligand, growth factor withdrawal, hypoxia/adenosine triphosphate (ATP) depletion, and intracellular accumulation of un-esterified cholesterol (Stoneman VE and Bennet MR., 2004). It has been suggested that early and late atherosclerotic lesions are influenced, at least in part, by different death inducers (*ibid.*).

As indicated by Savill *et al.,* the final outcome of macrophage apoptosis is mostly dependent on how well the phagocytes can clear the apoptotic bodies whether it is at an early or late stage (Savill J. and Fadok V., 2000). Normally, apoptotic cells are identified and internalized by phagocytes in a quick and efficient manner. This interaction results in inhibition of inflammatory signaling and anti-inflammatory activation (Savill J. and Fadok V., 2000). The sections below briefly describe the apoptotic events associated with early and late atherosclerotic lesions.

Apoptosis in early atherosclerotic lesions

Current thinking, as noted by Schrijvers *et al.,* suggest that macrophage apoptotic bodies are cleared efficiently and that this clearance is beneficial (Schrijvers DM. *et al.,* 2005). *In vitro* studies show that macrophages that are cholesteryl ester-loaded identify and engulf apoptotic macrophages more effectively (*ibid.*). The beneficial effect of early lesion macrophage apoptosis on atherosclerosis appears to be largely due to the ability of phagocytes to efficiently clear dead macrophages. This results in reduced macrophage content in the plaque and hence a reduced plaque size. On the other hand, effective macrophage phagocytosis could also promote plaque progression by signaling to circulating macrophages and recruiting them to the site of injury thereby increasing macrophage content in the plaque. This likely explains why increasing early lesion macrophage apoptosis has been considered to have both anti- and pro-atherogenic consequences (Schrijvers DM. *et al.,* 2005). Overall, however, it is generally accepted that early macrophage apoptosis has a beneficial effect on plaque progression and size.

Apoptosis in advanced and late atherosclerotic lesions

An extensive review by Ira Tabas discusses the role of macrophage apoptosis late atherosclerotic lesion development (Tabas I., 2005). One of the possible mechanisms of apoptosis in late atherosclerosis involves defects in Acyl CoA: cholesterol acyl transferase (ACAT)-mediated cholesterol esterification and cholesterol efflux leading to the buildup of free cholesterol (FC) in the plaque causing it to rupture which may ultimately result in strokes and MIs (reviewed in Tabas I., 2005). Studies using cell cultured macrophages show that the

recruitment, proliferation and survival of macrophages can be altered when the cells undergoing apoptosis are phagocytosed. In this sense, phagocytosis of apoptosing macrophages causes high modification and alteration to their normal function (reviewed in Tabas I., 2005). For example, direct tissue damage can result from intracellular proteases as well as VEGF released from secondarily necrotic apoptotic cells causing cell growth, coagulation and thrombosis (Reviewed in Tabas I., 2005; Golpon HA. *et al.*, 2004). The endothelial repair process is altered as a result of defective phagocytes, leading to the recruitment of monocytes to the site of injury and decreased survival of any remaining phagocytic cells (*ibid*).

A study by Schrijvers and colleagues examined human carotid atherosclerotic lesions and found that they have a high amount of apoptotic cells that were not engulphed by phagocytes (Schrijvers DM. *et al.*, 2005). Tonsillar tissues, on the otherhand, with normally high apoptotic cell percentage had most of their apoptotic cells engluphed and contained within phagocytes (*ibid*). This supports the fact that apoptotic macrophages are more numerous in advanced vs. early lesions due to defective phagocytosis of the apoptotic bodies. In his review, Ira Tabas discusses that "promotion of late lesion progression results in a number of consequences that lead to the accumulation of non-cleared apoptotic cells and post-apoptotic necrotic cells: (a) tissue factors can be secreted from non-cleared apoptotic cells; (b) the emigration of macrophages from atherosclerotic lesions may be inhibited by necrotic core formation; (c) macrophages may secrete matrix-degrading proteases when induced by inflammatory factors in the plaques necrotic core (d) finally, failure of phagocytes to internalize apoptotic cells may make them more susceptible to apoptosis leading to a

reduced number of phagocytes in lesions which amplifies the events mentioned above" (reviewed in Tabas I., 2005. pg. 9).

There are several pro- and anti- apoptotic components that can be targeted for studying whether macrophage apoptosis is more beneficial in early or advanced atherosclerosis. Clever pro-apoptotic strategies, such as the ones discussed later in section 2.3.3, reveal some of the potential targets. This study examines the role of an anti-apoptotic or inhibitor of apoptosis protein in the development of the atherosclerotic plaque.

2.2.1 Mechanism of apoptosis

Mechanisms of cell death can be activated by various stimuli with the aid of a central group of proteolytic enzymes known as caspases (cystein aspartate-specific proteases) (Reviewed in Creagh EM and Martin SJ., 2001; Alnemri *et al.*, 1996). All caspases are found within the cell as inactive precursor enzymes known as zymogens. Studies have revealed that caspases are initially synthesized as pro-enzymes consisting of a large internal domain with a large catalytic active site, a small C-terminal domain, and an NH2-terminus pro-domain or the so-called 'death domain' (DD) *(ibid).* The transmission of apoptotic signals is the responsibility of the large N-terminal pro-domains also called "the death domain superfamily" (reviewed in Creagh EM and Martin SJ., 2001). The DD is further made up of two subdomains; the DED or "death effector domain" and the "caspase-recruitment domain" (CARD) who's 3D structure was first described by Huang and colleagues (Reviewed in Creagh EM and Martin SJ., 2001; Huang *et al.,* 1996). These two subdomains constitute the downstream portion of the signaling pathway and their main task is to recruit caspases to the plasma membrane before they become activated (Fesik SW., 2000; Huang *et al.,* 1996).

Activated caspases then lead to apoptosis or controlled cell death. Caspases involved in apoptosis activation are documented as caspases -3, -4, -5, -6, -7, -8, -9, and -10 (reviewed in Creagh EM and Martin SJ., 2001).

There are two sub-groups of caspases; the effector caspases and the initiator caspases. Caspases that activate apoptosis signaling are known as the upstream or initiator caspases. Caspases responsible for the destruction of the cell are the downstream effector caspases (reviewed in Creagh EM and Martin SJ., 2001). In order to understand apoptosis, one should first understand caspase activation and regulation mechanisms which is reviewed in detail in Movassagh M and Foo R and briefly described in the section below (reviewed in Movassagh M. and Foo R., 2008)

2.2.2 Intrinsic and Extrinsic Apoptotic Pathways.

Intense research over the past 30 years has revealed details of two highly regulated central apoptosis pathways; (1) the intrinsic pathway, regulated by mitochondria and endoplasmic reticulum and (2) the extrinsic pathway, regulated by cell surface death receptors (reviewed in Movassagh M. and Foo R., 2008).

The extrinsic pathway involves death ligands such as the Fas-L or the CD95L, tumor-necrosis factors alpha (TNF-α), TNF-related apoptosis inducing ligand, and TNF ligand superfamily member 10 or TNFSF10 (reviewed in Movassagh M. and Foo R., 2008). These death receptors interact or bind to their respective receptors, being; Fas receptor or CD95, TNF-receptor 1 (TNFR1), death receptor 4 (DR4) or TRAILR1 and DR5 or TRAILR2 (*ibid.)*. In case of the Fas receptor pathway, the binding of the ligand to the receptor results in the re-organization of the receptor complex and stimulating the recruitment of adaptor proteins such as FADD or Fas associated death domain (*ibid.*). This in turn recruits procaspase-8 and

procaspase-10 into another complex called death inducing signaling complex or DISC which results in the activation of the caspases (Kischkel FC, *et al.,* 1995).

The activation and processing of the downstream caspases can depend on multiple factors. In some cells, processed caspase-8 is enough to directly activate other caspases and execute apoptosis (reviewed in Chowdhury I. *et al.,* 2006). In other cells, however, it requires further amplification whereby caspase 8 mediates the cleavage and binding to the Bcl-2 pro-apoptotic family members (*ibid.*). This then leads to the release of pro-apoptotic mitochondrial factors and thus tying in with the intrinsic apoptotic pathway.

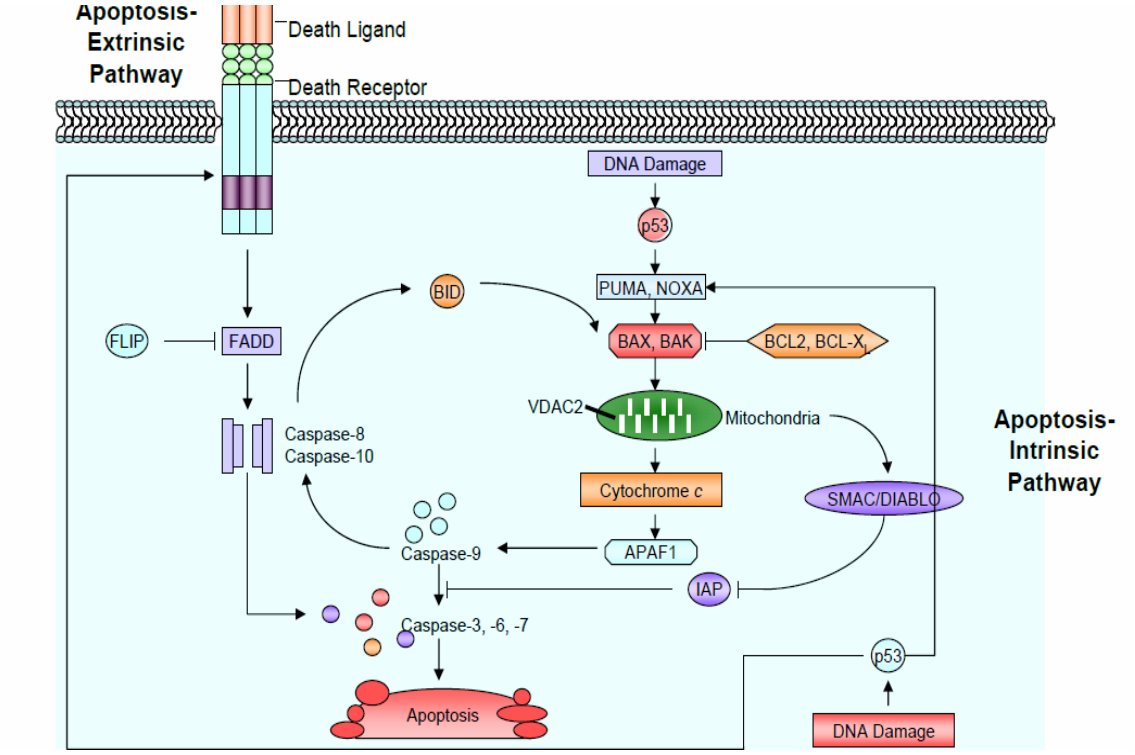
While extrinsic apoptosis is more involved in transmitting death signals from the plasma membrane, a wide variety of extracellular and intracellular signals are transduced via the intrinsic pathway (reviewed in Chowdhury I. *et al.,* 2006). Apoptotic stimuli include loss of survival, toxins, DNA damage, radiation, hypoxia/reperfusion, and oxidative stress which result in the activation of the apoptotic cascade through downstream signaling leading to activation of the death machinery on the mitochondria and ER (*ibid.*). Briefly, once the death signaling cascade reaches the mitochondria, it result in what is known as mitochondrial outer membrane permeabilization (MOMP) which is a crucial event during apoptosis (Goldstein JC, *et al*., 2000). Following those events, cytochrome c (a very important apoptogen) is released from the inter-membrane mitochondrial space (*ibid.*). Once released into the cytosol, cytochrome c binds to adapter molecule, Apaf-1. The nucleotide binding site on Apaf-1 is exposed and allows for cytochrome c to binds to it which induces a conformation change that oligomerizes Apaf-1 into an apoptosome (Li P, *et al.,* 1997). This apoptosome activates

caspase-9 which then activates many downstream effector caspases leading to the execution of apoptosis (*ibid.*). The different apoptotic pathways are outlined in **Figure 3.**

The current study examines the role of an inhibitor of apoptosis protein (cIAP2), and thus understanding the different apoptotic pathways helps clarify further the possible pathways this protein might be involved in.

2.2.3 Regulation of Apoptosis

Different mammalian cells are controlled by different transcriptional and post-transcriptional regulators of pro-caspase genes (Shi Y., 2004). Caspases are tightly regulated by many molecules whose function ranges from blocking caspase activation at the DISC to the inhibition of caspase enzymatic activity (*ibid.*). The present study examines the inhibitory effects of one specific IAP, cIAP2, and its role in early and late atherosclerosis development.



Nature Reviews; Cancer; Vol. 2, June 2002.|
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Figure 3. Overview of the extrinsic and intrinsic apoptotic pathways.

"The intrinsic apoptotic pathway is triggered from within the cell by developmental cues or severe cell stress, such as DNA damage. The extrinsic pathway is activated when a pro-apoptotic ligand, such as tumour necrosis factor alpha (TNF-α), endogenous Fas ligand binds to pro-apoptotic receptors, such as TNF-R1 or Fas receptor. Destruction of the cell is ultimately carried out by initiator caspases (8 and 9), which in turn bring about activation of executioner caspases 3, 6 and 7. Caspase 3 is the most active effecter caspase in both the intrinsic and extrinsic pathways, where it is processed and activated by caspase-9 and caspase-8, respectively. As is apparent above and in figure 2, it is a regulated process where there are many factors affecting apoptosis. " (Ashkenazi A. and Dixit VM., 2002)

2.3 Inhibitors of Apoptosis

As mentioned earlier, apoptosis must be tightly regulated in order to prevent any unnecessary cell death. Any unregulated apoptotic mechanisms usually lead to uncontrollable cell death, which results in the pathogenesis seen in many diseases and injury models (reviewed in Lotocki G. *et al.,* 2002). A group of proteins known as IAPs, first recognized in insect cells infected by a baculovirus, have been key in the regulation of the caspase cascade and in the apoptotic signaling pathway (Salvesen GS., 2002; Cheng EH. e*t al.,* 1996; Duckett CS. *et al.,* 1996). The IAP proteins share at least 1 cystein-rich domain of around seventy amino acids known as the baculoviral IAP repeat or BIR (*ibid).* Despite all IAPs possessing a BIR domain, some of them do not necessarily act as inhibitors of apoptosis. Eight human IAPs have been discovered to date, and include; BIRC1/NAIP, BIRC2/IAP1/HIAP2, BIRC3/IAP2/HIAP1, BIRC5/survivin, BIRC4/XIAP/hILP, BIRC6/Apollon, BIRC7/ML-IAP and BIRC8/ILP2 (reviewed in Deveraux and Reed, 1999). **Figure 4** illustrates the structure of the mammalian IAPs.

The generation of IAP-deleted mice has revealed the importance of this family of proteins in cell survival, propagation and some differentiation pathways. NAIP, cIAP2 and XIAP are shown to be involved in the survival of neurons, cardiomyocytes and macrophages in stress conditions, respectively (Potts MB. *et al.,* 2005; Conte D. *et al.,* 2006*;* Olayioye MA. *et al.,* 2005).

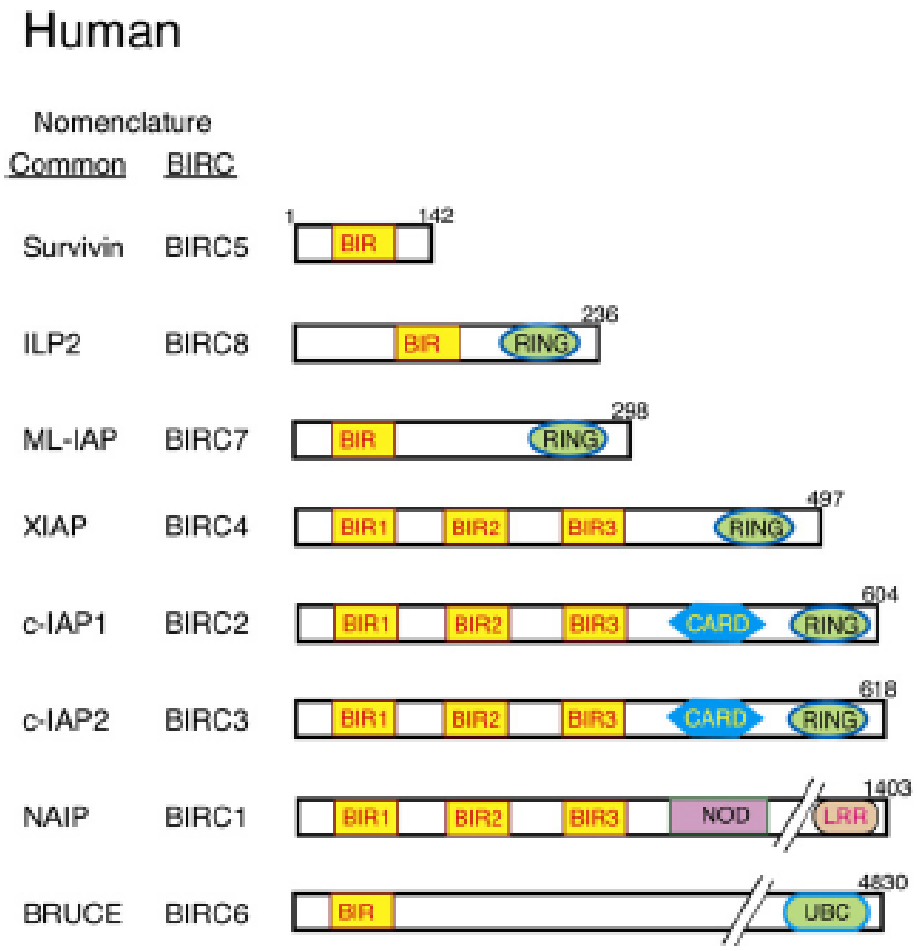

Figure from Mol Cell. 2008 Apr 25; 30(2):123-35.
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Figure 4. Illustration of the IAP family structure.

"The number of residues in each IAP as well as the functional motifs that they contain is shown. The BIRC nomenclature equivalents are provided. Abbreviations: CARD, caspase-associated recruitment domain; UBC, ubiquitin-conjugation; NOD, nucleotide-binding oligomerization domain; and LRR, leucine-rich repeats." (Srinivasula SM, Ashwell JD., 2008, pg. 124).

A recent review published by Srinivasula SM. *et al.,* discusses in great details the structural and functional features of IAPs (reviewed in Srinivasula and Ashwell J, 2008). The authors discuss that most IAPs have at least 1-3 BIR repeats, as well as an additional functional domain such as RING-regions and/or caspase-associated recruitment domains (CARD) closer to the C-terminal of the IAP molecule (Srinivasula SM and Ashwell JD, 2008). IAPs that contain multiple BIR regions usually have highly conserved properties where the BIR2 and BIR3 are involved in binding with proteins containing IBMs (IAP-binding motifs), whereas the BIR1 usually interacts with several other signaling intermediates (*ibid.*). Ubiquitylation is a modification process that is post-translational and is carried out by three ubiquitin enzymes, E1, E2 and E3 (reviewed in Wilkinson AD., 1987). Target proteins that are labeled with ubiquitin can either be poly- or mono-ubiquitylated (*ibid*.). RING domains present in most IAPs are involved in ubiquitylation and hence express E3 or ubiquitin ligase activity which, along with E1 or ubiquitin activating enzyme and E2 or ubiquitin conjugating enzyme, works on catalyzing the transfer of ubiquitin molecules to end proteins (Lorick KL. *et al.,* 1999). Polyubiquitylation labels the targeted protein for shipping to the proteosomal degradation complex via signaling to the protein-transport machinery (*ibid*.). Mono-ubiquitylation can have a less dramatic effect on a cell's fate, possibly impacting the cell through the lysosomal complex where it affects cellular sub-location, function or degradation (*ibid*.). IAPs have also been shown to participate in TNF-α and Transforming Growth Factor-β (TGFβ) signaling pathways discussed later (Geisbrecht ER. and Montell DJ., 2004).

The present study focuses on the role of one member of the IAP family, namely cIAP2, in atherosclerosis development. The section below delineates the mechanism of action of cIAP2 and its role in apoptosis inhibition and cell proliferation.

2.3.1 Cellular inhibitor of apoptosis 2 (cIAP2)

Among mammalian IAPs, XIAP, cIAP1 and cIAP2 are the most studied and have been found to be associated with modulating the TNF superfamily members along with other death receptor complexes (reviewed in Silke J and Brink R., 2010). Studies aiming to reveal components of the TNFR signaling complex first discovered cIAP1/2 to be recruited by the TNFR associated factor-2 (TRAF2) (Rothe M. *et al.,* 1995; Uren AG., *et al.,* 1996). cIAP1 and cIAP2 share many structural similarities including three BIR domains, a CARD domain, and a RING domain near the C-terminal (Mace PD. *et al.,* 2010). The BIR1 domain of cIAP1 and 2 has been shown to function in many signaling pathways via oligomerization of several binding partners (Mace PD. *et al.,* 2010). The only domain that binds to TRAF2 and is responsible for the recruitment of cIAP1 and 2 to the TNFR complex is the BIR1 domain (Samuel T. *et al.,* 2006).

Chung and colleagues demonstrated that mammalian TRAF proteins recruit other proteins to the TNFR and have been shown to regulate many processes such as immune function and regulated cell death (Chung JY. *et al.,* 2002). TNF signaling is regulated by cIAP1 and cIAP2‘s E3 ubiquitin ligase function, and the recruitment of cIAP2 to the TNFR1 by TRAF2 is necessary for the activation of NF-kB by the TNFR1 (Vince JE *et al.,* 2009). Studies have demonstrated that despite their wide expression, cIAP1 and 2 have a normally low abundance in tissues (Mace PD. *et al.,* 2010). *In vitro* studies have shown that cIAP2

expression is regulated by the E3 ubiquitin ligase activity of cIAP1, which results in its degradation and low abundance in cells (Wu CJ. *et al.,* 2005). However, when studying the effect of cIAP1 knock-out on cells, the cells did not exhibit an elevation in cIAP2 mRNA suggesting that the physiological levels of cIAP1 regulate cIAP2 post-transcriptionally via ubiquitylation and subsequent degradation (*ibid.*). cIAP1 and 2 have been involved in many signal transduction pathways some of which will be discussed in the section below.

A study by Mahoney and colleagues carefully examined the role cIAP1 and 2 play in the regulation of NF-κβ via TNF-α (Mahoney DJ. *et al.,* 2008). The group used a genetic knock-down method using siRNA techniques and demonstrated that the loss of cIAP2 diminishes NF-κβ signaling via TNF-α (*ibid*). The study also demonstrated the important role that cIAP2 plays in Rip1 poly-ubiquitylation and helps activates NF-κβ once stimulated by TNF- α (*ibid.*). TNF-α is a cytokine that is involved in many processes such as immune system function and responses and responses to injury (Wajant H. *et al.,* 2003).

Conte and colleagues examined the role the cIAP2 plays in macrophage survival (Conte D. *et al.,* 2006). Among other things, NF-κβ regulates the degree that a cytokine responds to an inflammatory factor such as lipopolysachcharides or LPS (Conte D. *et al.,* 2006). When a macrophage is activated by an inflammatory factor such as LPS, it induces the production of TNF- α which in turns helps the macrophage function in this "aggressive" environment by increasing its resistance to apoptosis (Kumar A. *et al.,* 1996). However, the study was able to demonstrate that once macrophages have been depleted of cIAP2, their resistance to cell death reduces (Conte D. *et al.,* 2006). This lead to the assumption that cIAP2 helps active macrophages survival when under stressful pro-apoptotic stimuli (Conte D. *et al.,* 2006). What is most striking about this finding is that it suggests

that cIAP1 and cIAP2, despite their high similarity in structure and function, may be working independently of each other and do not substitute for one another.

Although IAPs have been scrutinized in cancer research, investigators' attention to their role in atherosclerosis has been limited. A study by Blanc-Brude and colleague examined the role that survivin (an IAP family member) plays in the formation of atherosclerotic plaques (Blanc-Brude OP. *et al.,* 2007). Western blot analysis of excised human plaque revealed an increase in XIAP as well as cIAP2 protein expression levels when compared to regular healthy aorta (Blanc-Brude OP. *et al.,* 2007). A representative diagram of the proposed mechanism of action of cIAP1/2 in cells is shown in **Figure 5**.

IAPs (including cIAP2) are themselves regulated by at least three different mechanisms; transcriptional and post-transcriptional control, stability regulation, and IAP activity control by regulatory molecules. Understanding the regulation of IAPs (and ultimately cIAP2) helps in the development of potential pharmaceutical agents that will target specific IAPs and mimic the action of their regulators/inhibitors.

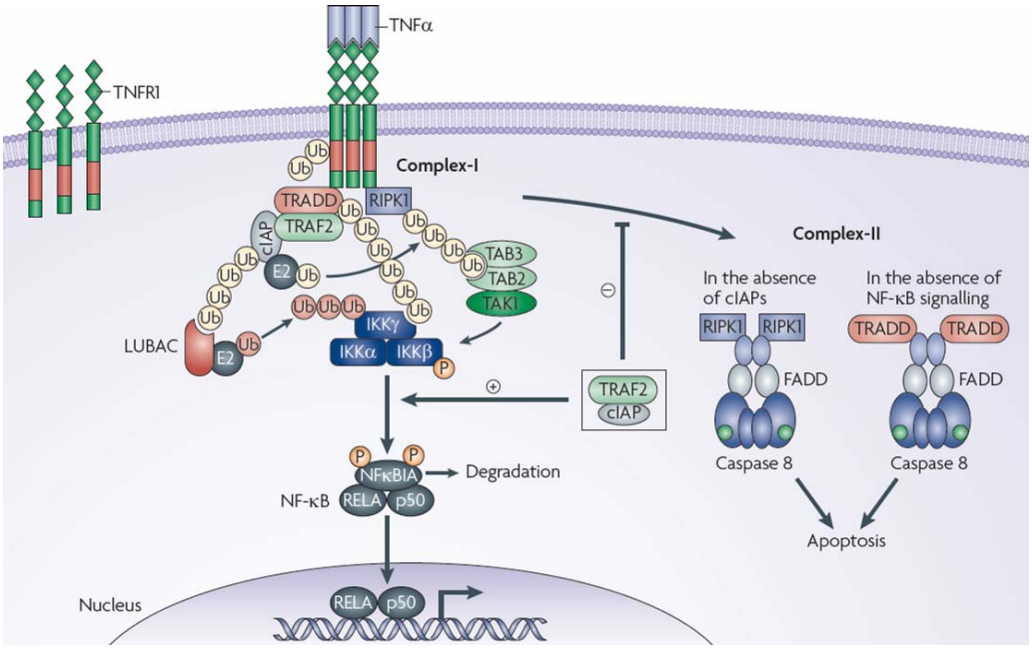

Figure from Nat Rev Cancer. 2010 Aug; 10(8):561-74.
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Figure 5. Mechanism of action of cIAP1 and 2 in cells.

"Activation of TNFR1 stimulates the formation of complex-I that consists of TNFR1, TRADD, RIPK1, TRAF2 and cIAP1/2. cIAPs ubiquitylate several components of this complex, which causes ubiquitin (Ub)-dependent recruitment of linear ubiquitin chain assembly complex (LUBAC), TGFβ-activated kinase (TAK1)–TAB2–TAB3 and IKKγ–IKKα–IKKβ through their respective Ub-binding domains (UBDs). Once recruited, LUBAC conjugates M1-linked Ub chains (shown in pink) on to IKK, and probably other components of complex-I. In addition to stimulating TNFα-mediated activation of nuclear factor-κB (NF-κB), cIAPs also suppress the formation of the death-inducing complex-II, which is the activation platform for caspase 8 that induces death by the extrinsic pathway. In the absence of cIAPs, this complex is formed by RIPK1, but in the absence of NF-κB activation it is formed by TRADD. FADD, Fas-associated via death domain; NF-κBIA, NF-kB inhibitor-α." (Gyrd-Hansen M. and Meier P., 2010, pg 567)

2.3.2 Regulation of inhibitors of apoptosis

IAP interacting molecule smac/DIABLO (direct IAP binding protein with low pI), is a nucleus-encoded protein composed of 239-residues; however, in non-apoptotic cells it is localized to the inter-membrane space of the mitochondria (Du C. *et al.,* 2000; Verhagen AM. *et al.,* 2000). During the apoptotic process, a cell releases smac/DIABLO proteins into the cytosolic space which allows for the displacement of the IAPS from the IBM of caspases and inhibiting the apoptotic process (Du C. *et al.,* 2000). As a result, smac/DIABLO enhance apoptosis and as such are considered negative regulator of IAPs (Du C. *et al.,* 2000). Today's advanced studies have revealed the mechanism by which smac/DIABLO regulate XIAP. Given that the BIRs and other structures are highly conserved across most IAPs, one can assume a similar biochemical pathway within the group. However, little is known about the triggers of ubiquitylation, how it is achieved and whether or not caspase activation is involved.

It has been found that treatment with smac-mimetic compounds encourages the auto-ubiquitylation and further degradation of IAP proteins (Varfolomeev E. *et al.,* 2007; Vince JE. *et al.,* 2007). The loss of cIAPs by either treatment with smac-mimetic compounds or by gene knock-out studies lead to apoptotic death of cells when stimulated with TNF (Bertrand MJ. *et al.,* 2008; Varfolomeev E. *et al.* 2007). This re-iterates the importance of cIAPs in the TNFR-complex, suggesting that apoptosis can be induced by perhaps upsetting the recruitment of cIAPs by the TNF receptor complex.

Despite having understood most of the complex mechanisms leading to atherosclerosis lesion development and the pathways involved in the process, the majority of the studies are

conducted *in vitro*. To better understand how foam cells lead to the pathogenesis and progression of the disease, animal disease models mimicking human disease models must be closely examined. For example, in atherosclerosis research, genetic alteration in mouse lipoproteins results in an atherosclerotic disease model that closely resembles the human hyperlipidemic/hypercholesterolemic patients and enables the study of the disease in mice with more confidence. The section below discusses the atherosclerotic mouse models briefly.

2.4 Mouse models for atherosclerosis

Atherosclerosis in humans is usually evident at the advanced or complex stages but often not detected until after the patient has suffered an adverse atherosclerotic related event. As such, currently, the early events leading to the development of atherosclerotic lesions cannot be studied effectively in humans. Experimental models of the disease have enhanced the understanding of the patho-physiological process leading to vascular obstruction in both spontaneous and accelerated atherosclerosis and thrombosis (reviewed in Fuster V. *et al.*, 1991).

Mice have been invaluable in giving insight into many human disease patho-physiological processes and for studying the genetic contributions to diseases. The ability to perform genetic manipulations using transgenic and gene-targeting technologies is one of the many advantages for using the mouse model. Other advantages of mouse models are that they are easy to breed, can be generated within 3 weeks from mating, inbred strains are widely available. Despite the use of many animal models for studying atherosclerosis, the mouse model has become a powerful tool for investigating biology and molecular pathways involved in the earliest stage lesions.

By nature, mice are very resistant to developing atherosclerosis except for the C57BL/6 mouse strain known to be spontaneously atherogenic. When this strain is put on a an atherogenic diet composed of 1.25% cholesterol , 15% fat, , and 9.5% cholic acid (more than the regular human cholesterol diet intake by 10-20 times), the C57BL/6 mice build up atherogenic plaques in the intima of the ascending aorta (Paigen B., 1990). However, in order to make mice even more susceptible to atherosclerosis, their lipid metabolism must be genetically altered. This study uses the apoE KO model which is discussed in more details below.

2.4.1 Apolipoprotein E and the apoE null mouse model

Being the primary ligand for the LDLr, the main function of apoE is to mediate LDLr-mediated lipoprotein removal from the circulation. ApoE is implicated in three lipoprotein metabolism pathways: fat transport from the diet, endogenous transport of fat, and reverse cholesterol transport (Rall SC Jr. *et al.,* 1989). Despite being recognized by two different receptors, apoE remains to be the most potent physiological ligand for the LDLr. ApoE, as a ligand, mediates the clearance of apoE-containing lipoprotein particles (chylomicrons, VLDL). If apoE is absent or has binding defects, atherosclerosis is likely to develop.

There are many reviews delineating the importance of the apoE KO mouse model as the most extreme of the viable phenotypes observed in lipoprotein transport (Zhong S., 1994; Rubin EM., 1994). The apoE-null mice become severely hyper-lipidemic because their capability to clear plasma lipoproteins from the blood is damaged (Rubin EM., 1994). When put on a regular chow diet, mice exhibit plasma cholesterol levels of around 500mg/dl (5.64 mmol/L), as opposed to control WT mice with cholesterol plasma levels of 75mg/dl (0.84 mmol/L) (Plump AS. *et al.,* 1992; Zhang SH. *et al.,* 1992).

A study by Hakamata and colleagues showed that the cholesteryl ester level of cells increased along with the concentration of VLDL/IDL when the peritoneal macrophages obtained from apoE null mice were incubated with the lipoprotein portion including VLDL and IDL (Hakamata H. *et al.,* 1998). Because the mass of the cellular cholesteryl ester also increased in the mice lacking apoE molecules (apoE KO), it was concluded that the VLDL and IDL resulted in a significant cholesteryl ester accumulation in macrophages through an apoE-independent pathway (Hakamata H. *et al.,* 1998). This pathway may somewhat explain the mechanisms by which foam cell are formed in the arterial wall and the way atherosclerotic plaques progress in apoE KO mice model (Hakamata H. *et al.,* 1998).

There are important differences between the ApoE-deficient mice and apoE defficient humans. For example, plasma cholesterol levels in apoE KO mice fed a low fat diet are slightly higher than those in humans who eat high fat diets. Plump and Breslow suggested that this could mean; a) small amounts of residual apoE are found in humans deficient of ApoE or b) compared to humans, mouse apoE is responsible for mediating the clearance of lipoprotein (reviewed in Plump and Breslow, 1995). Myant suggested that these observations could be explained by mice exhibiting a higher amount or percentage of apoB48 in their circulation (Myant N, 2990). Human apoB48,however, plays the role of a structural protein in chylomicrons as well as chylomicron remnants and is exclusively synthesized by the small intestines (*Chen S-H et al., 1987*). While in mice, however, apoB48 acts as a chylomicron structural protein and is also synthesized in the liver. ApoE is required for receptor mediated uptake of particles that contain ApoB48 as their primary structural protein. Myant discusses that since mouse plasma contains a higher percentage of apoB48 it requires a greater need for apoE-mediated receptor clearance (Myant N. 1990). This could explain why mice deficient in

ApoE (or ApoE KO mice) show such high levels or hypercholesterolemia when compared to humans deficient in apoE. Despite this fact, when studying atherosclerosis in mice, lesion development is very seminal to that observed in humans (reviewed in Whitman SC., 2004).

Mice and human HDL and LDL levels differ where by nature, mice exhibit elevated HDL levels and low LDL levels, in contrast to humans who have high LDL and low HDL levels (reviewed in Jawien J et al., 2004). This also means that most of the plasma cholesterol present is carried via VLDL rather than LDL as in humans. Mice also lack the cholesteryl ester transfer protein CETP, an enzyme responsible for the transfer of cholesterol esters from HDL to VLDL and LDL which could pose a problem in the transfer of esterified cholesterol derived from tissue to the liver (Eisenberg S. et al., 1984). Despite these abnormalities in lipoprotein profiles, the characteristics of the atherosclerotic lesions formed in the apoE KO mice show some of the closest similarities to humans than any available animal model (reviewed in Daughrety A., 2002). An elegant study by Nakashima and colleagues carried out a thorough sequential analysis of atherosclerosis in the apoE- KO mouse model and demonstrated that lesion formation events taking place are similar to those in humans (Nakashima Y. et al., 1994). Whether or not lipoprotein metabolism differences contribute to advanced stage VI lesions and spontaneous rupture events in mice is, to the knowledge of this investigator, still under debate.

Despite sharing a significant number of genes with mice, many processes in the human body are different. Therefore, atherosclerotic disease models are not expected to be exactly the same between humans and mice. Considerable differences in atherosclerotic lesions have been and continue to be studied (Allayee H *et al.,* 2003). This study examines

atherosclerosis lesion development in a mouse model keeping in mind that the mechanisms involved mimic the ones in the human disease model.

2.4.2 Comparison between atherosclerosis in humans and mice

As noted in section 2.2.1, plaque development is a multifaceted and active process. Based on morphological criteria, the atherosclerotic lesion will pass through predicted stages of development as the disease proceeds. These stages have been thoroughly reviewed by Stewart Whitman (Whitman SC., 2004). The morphological features of the apoE null mouse and humans are very comparable (Stary HC., 1990; Stary HC., 1992). Mouse atherosclerotic lesions, similar to those found in humans, are made up of an irregular buildup of lipoproteins and a "cellular gathering" consisting mainly of macrophages and T-cells (Zhou X. and Hansson GK., 1999).

Whether or not advanced lesions (stage IV and V) develop in mice, the cellular and molecular progression of early atherosclerosis in mice mirrors the development of atherosclerosis in humans. This is very helpful not only for understanding the mechanism but also potentially for treating the disease with specific pharmaceutical agents. In order to decide if a certain pharmaceutical agent or genetic loss of function affects atherosclerosis, there has to be a predefined method for measuring lesion size. The section below briefly describes a well developed method for quantifying atherosclerotic lesions that is used in this study.

2.4.3 Quantification of atherosclerosis in mice

The size of an atherosclerotic lesion should not be the only measure for quantifying the extent of atherosclerosis. Given the complex disease pathogenesis, investigation can be challenging (Whitman SC., 2004). A number of detailed reviews of the literature on methods of quantifying atherosclerosis are well compiled in a review by Daugherty *et al.* (reviewed in Daugherty *et al.,* 2009). The three vascular regions that are most commonly analyzed in mice are (1) the aortic root and ascending aorta cut in cross-section (2) the aortic tree on both sides of the arch, thoracic and abdominal regions of the aorta opened longitudinally to expose the area of definable lesion covering the luminal *en face* surface and (3) serial sectioning of 1 mm of the proximal brachio-cephalic trunk (innominate artery) which is the first major branch off the aorta at the region the aortic arch (Whitman SC., 2004). An extensive step-by-step description of atherosclerosis quantification in all three vascular regions is provided by Daughtery and Whitman (Daugherty A. and Whitman SC., 2003).

CHAPTER 3: Rationale and Objective

3.1 Rationale

Since IAPs play an important role in the control of apoptosis, they may play a critical role in the development of atherosclerosis. Previous work from Dr. Whitman's laboratory (details found in index, page 89) showed that cIAP2 (in hematopoietic cells) plays a significant role in decreasing atherosclerotic lesion development in the *LDLr*$^{-/-}$ mice put on an 8wk HFD. The current project proposes to further examine the role of cIAP2 in early and late atherosclerosis in an *apoE*$^{-/-}$ mouse model. Compared to *LDLr*$^{-/-}$ mice, the development of more complex, advance-stage lesions has been more extensively characterized in the *apoE*$^{-/-}$

strain (Ishibashi S *et al.,* 1994; Rosenfeld ME. *et al.,* 2000; Calara F. *et al.,* 2001). The *apoE*$^{-/-}$ mouse model is also considered a closer representation of human atherosclerosis than the *LDLr*$^{-/-}$ model (Stary HC., 1992). Hypothetically, cIAP2 deletion would lead to termination of its role as a suppressor of apoptosis and thus apoptosis could persist given the appropriate apoptotic stimuli. This would theoretically mean that macrophages can contribute to a lesser extent in the initiation of atherosclerotic plaques since the stimuli for apoptosis would trigger macrophage cell death and hence reduce foam cell formation. **Figure 6** demonstrates the working hypothesis.

Total body KO of the cIAP2 gene will give insight into the role of cIAP2 in the development of atherosclerosis and enable better understanding of the mechanisms underlying the disease as well as provide a basis for future investigations into therapy.

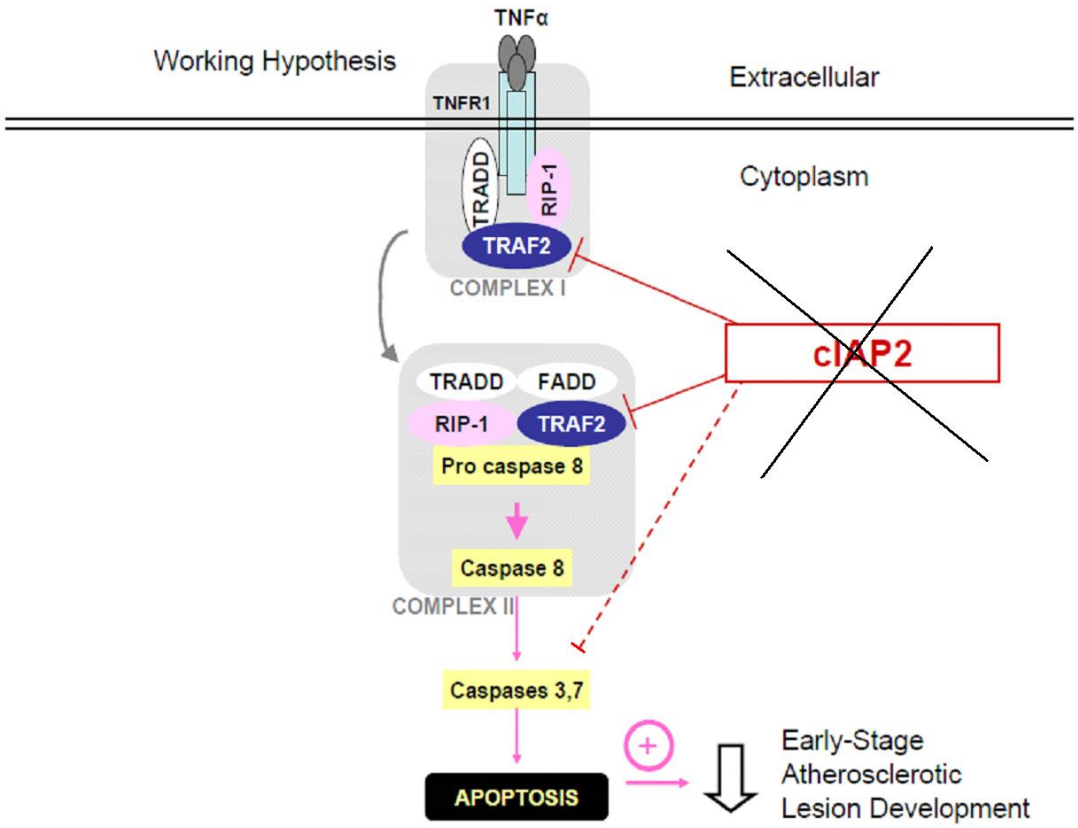


Figure adapted from Dubrez-Daloz L *et al.*, 2008 with Dr. Whitman

Figure 6. Working hypothesis.

cIAP2 protein is believed to bind to the TRAF2 complex that mediates receptor-induced apoptosis. Deletion of cIAP2 gene alleviates the inhibitory effect of cIAP2 protein on the TRAF2 complexes (as represented by solid red line) and thus indirectly inhibiting the effector caspases -3 and -7 (as represented with dotted red line). This enables the progression of the apoptotic pathway. If this were to occur in a macrophage cell, receptor-mediated apoptosis would occur and the lipid-laden foam cell would apoptose early as opposed to becoming a foam cell and later necrose. cIAP2 deletion is hypothesized to reduce atherosclerotic lesion size.

3.2 Objective

Objective # 1. To determine the role of *cIAP2* in atherosclerosis lesion development.

Hypothesis # 1. *cIAP2* plays a role in atherosclerosis lesion development.

Objective # 2. To determine whether *cIAP2* deletion reduces atherosclerotic lesion size (both early and late) in an animal model of atherosclerosis.

Hypothesis # 3. ***cIAP2***$^{-/-}$ **x** ***apoE***$^{-/-}$ mice have reduced atherosclerotic lesion areas (both early (4weeks) and late (12 weeks)) compared to wild type ***cIAP2***$^{+/+}$ **x** ***apoE***$^{-/-}$ litter mates.

Objective #3. To determine whether *cIAP2* deletion leads to reduced macrophage number and hence atherosclerotic lesion size.

Hypothesis # 2. ***cIAP2***$^{-/-}$ **x** ***apoE***$^{-/-}$ mice have reduced macrophage number (and hence atherosclerotic lesion size) compared to control wild type ***cIAP2***$^{+/+}$ **x** ***apoE***$^{-/-}$ litter mates.

3.3 General hypothesis

cIAP2$^{-/-}$ **x** ***apoE*** $^{-/-}$ **mice develop smaller atherosclerotic lesions (both in early and advanced late stage) compared to the wild type control** ***cIAP2***$^{+/+}$ **x** ***apoE***$^{-/-}$ **mice.**

CHAPTER 4: Methods

4.1 Animals:

All animal studies were performed in accordance with the policies and guidelines of the University of Ottawa Animal Care Committee. This study was designed to compare lesion development in sibling male *apoE*$^{-/-}$ mice that were *cIAP2*$^{+/+}$ versus *cIAP2* $^{-/-}$ after being put on a high-fat high-cholesterol diet. Male *cIAP2*$^{-/-}$ mice were obtained from Dr. Robert G. Korneluk at the Apoptosis Research Center at CHEO and bred with female atherosclerotic susceptible *apoE*$^{-/-}$ mice originally obtained from the Jackson Laboratories, (ME, USA). Mouse colonies were maintained in the Animal Care Facility at the University of Ottawa Heart Institute. Both strains of mice were backcrossed onto the C57BL/6 background for more than 10 generations. After the original cross, filial 1 (F1) heterozygotes were mated with *apo* $^{-/-}$ mice to obtain a filial 2 (F2) generation that served as the breeding colony for this experiment. The F3 progeny from crossing *cIAP2* $^{-/-}$ x *apoE*$^{-/-}$ were genotyped and *cIAP*$^{+/+}$ x *apoE*$^{-/-}$ and *cIAP2*$^{-/-}$ x *apoE*$^{-/-}$ male littermates were used for experiments. The *cIAP2*$^{-/-}$ mice lost the expression of cIAP2 in all tissues (total body knock-out) and are viable, fertile, reach adulthood and have not been found to develop any type of "spontaneous" diseases. The mice showed no overt phenotypes and appeared healthy up to 52 wks of age. PCR (polymerase chain reaction) was carried out on DNA from mouse ear notch samples to screen for the *apoE* and *cIAP2* genes. Results of a typical PCR carried out during the study for genotyping cIAP2 in mice can be found in the appendix.

Mouse Groups:

WT: *cIAP2* $^{+/+}$ x *apoE* $^{-/-}$

KO: *cIAP2*$^{-/-}$ x *apoE* $^{-/-}$

4.1.1 Diet

All mouse groups were fed a diet enriched in cholesterol and fat for either 4- or 12-weeks. At the end of each dietary period, the mice were sacrificed with an overdose of Somnotol followed by tissue collection. The diet is a commercially prepared mouse food supplemented with 21% (wt/wt) butterfat, 0.15% (wt/wt) cholesterol, 19.5% (wt/wt) casein and no sodium cholate (#112286, Dytes Inc., Bethlehem, PA). Approval from the University of Ottawa Animal Care Committee has been obtained.

4.1.2 Study outline

At the age of 8 weeks, the mice were put on a high-fat high-cholesterol Western diet for a period of:

a. Four weeks, to study the effects of cIAP2 KO on early lesion development.

b. Twelve weeks, to study the effects of cIAP2 KO on the development of advanced late lesions.

Each study compromised of 30 mice in total; 15 WT and 15 KO. Refer to **Figure 7** for detailed experimental set up.

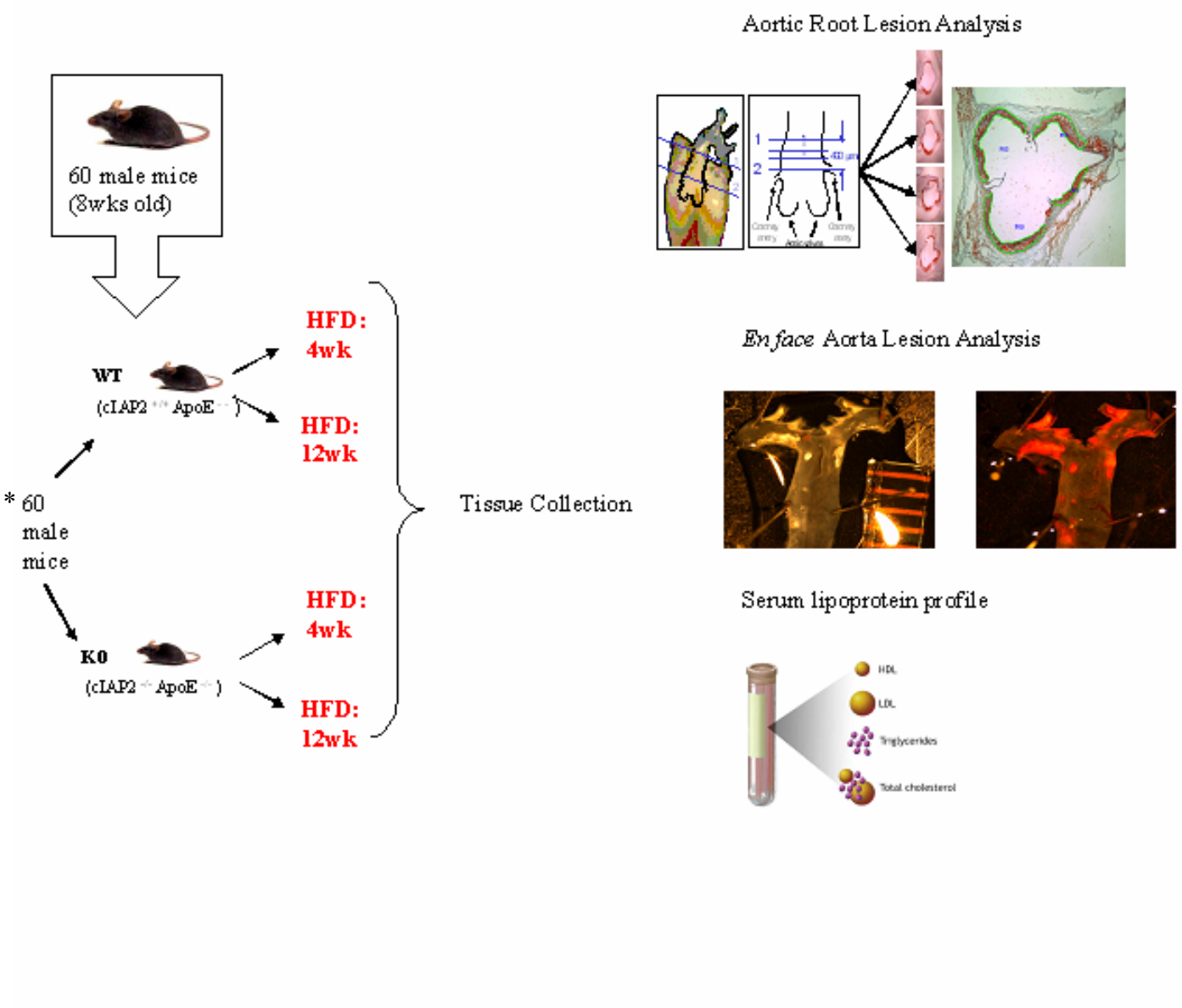


Figure 7. Experimental set up for *in vivo* mouse study.

At the age of 8wks, *cIAP2* $^{+/+}$ *ApoE* $^{-/-}$ (WT) and *cIAP2* $^{-/-}$ *ApoE* $^{-/-}$ (KO) mice were put on a HFD for 4 and 12wks. Tissue was collected at the end of the study. Hearts were mounted in OCT and snap frozen in liquid nitrogen. Aorta was cut and cleaned from adventitial fat and pinned for *en face* analysis. Blood was collected and serum lipoprotein and total cholesterol was analyzed.
(* each HFD group in the study contains 15mice, adding up to a total of 60mice)

4.2 Tissue collection

Following the dietary period, all mice were perfused with 1X phosphate buffered saline (PBS) by means of a puncture to the left ventricle with the perfusate being drained from the severed right atrium. The hearts were separated from the aorta at the base, placed in a plastic base mold, embedded in optimum cutting temperature (OCT) medium, and snap frozen on a metal plate cooled in liquid nitrogen. Samples were wrapped in Parafilm and stored at -20 °C. Liver and spleen were collected and weights were recorded as a method to screen for metabolic abnormalities that might skew results. Spleen was also dissected in half (i.e. one half was snap frozen in liquid nitrogen and the other half was embedded in OCT and snap frozen similar to the hearts) to preserve RNA in case it is required for later experiments.

4.3 *en face* aorta lesion analysis

At euthanasia, the entire mouse aorta was dissected from the proximal ascending aorta to the bifurcation of the iliac artery under a dissecting microscope. Adventitial fat was removed and the aorta was opened longitudinally, pinned flat onto dissecting wax and photographed at a fixed magnification (alongside a measuring scale to ensure that 3 mm is consistently inferior to the heart when quantified). The aorta was then stained with Sudan IV or ORO to better visualise lesions. Photographs were digitized and the total aortic areas and lesion areas were subsequently calculated using Image Pro Software. Final results are reported as a % of the total aortic arch and descending aorta that contained lesions. To reduce bias during data analysis, the data was analyzed by a second observer that was blinded to the study.

4.4 Tissue cryosectioning and aortic root lesion analysis

The extent of atherosclerotic lesion development was also determined through analysis of the ascending aorta. To determine atherosclerotic size within the ascending aorta, the mean lesion area was derived from 4 serial Sudan IV stained sections that were cut 10μm thick, and collected 100μm apart over a 1 mm segment of the aortic root. The mean lesion area was taken as the lesion size for each mouse and analysis of the lesions began at the region where the aortic sinus becomes the ascending aorta (Whitman SC. *et al.,* 2000; Whitman *et al.,* 2002b; Whitman SC. *et al.,* 2002a; Whitman *et al.,* 2004). Sudan IV is a fat-soluble diazo dye used for lipid staining, as well as for staining frozen paraffin sections of triglycerides and lipoproteins. Using the Sudan IV stained sections as a guide, the lesion area defined as intimal tissue within the internal elastic lamina was determined from images created by using the digital CoolSNAP *cf* camera (Roper Scientific Inc., Duluth, GA) using Image-Pro software (Media Cybernetic, Silver Springs, MD). Aortic root analysis was performed on all animals in this study. See **Figure 8** for detailed methods of aortic root tissue collection.

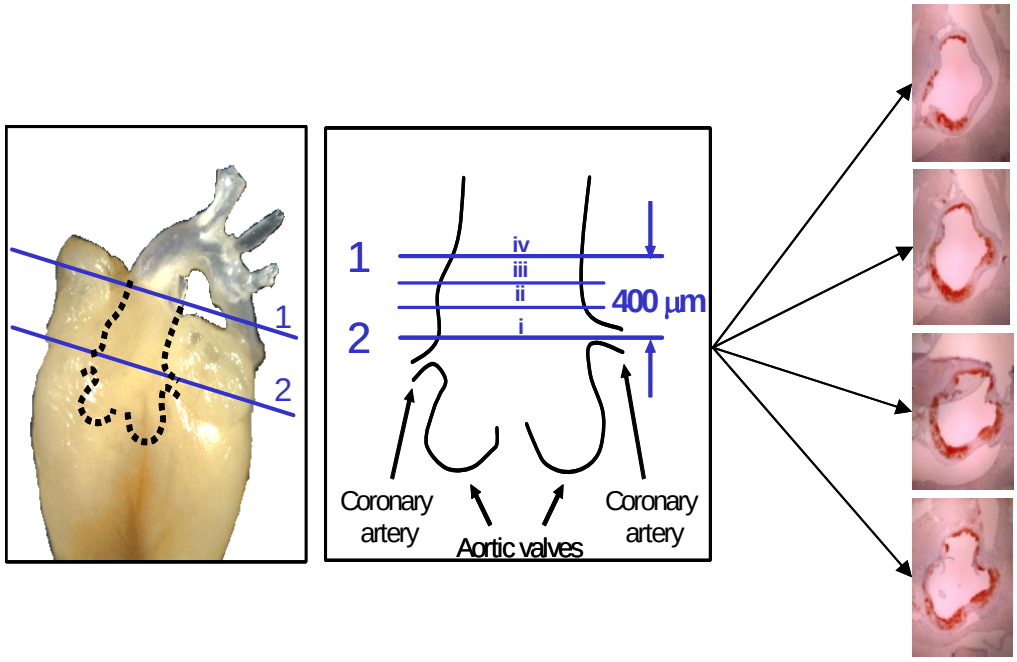


Figure 8. Methodology of atherosclerosis lesion analysis

Above is a diagram of the mouse heart showing the serial sections of the ascending aorta collected for the measurement of atherosclerotic lesion size. To determine atherosclerotic size within the ascending aorta, the mean lesion area was derived from 4 serial Sudan IV stained sections that were cut 10um thick collected 100um apart over a 1mm segment of the aortic root.

4.5 Aortic root macrophage immunostaining

The extent of macrophage infiltration in atherosclerotic lesions was determined through the analysis of CD68 positive staining of frozen cryosections. The initial steps for tissue collection are outlined in section 4.4 above. The CD68 staining protocol was adopted from a procedure performed in Dr. Ross Milne's laboratory. Briefly, frozen sections were dried with cold acetone for 5 minutes followed by several washes with 1X PBS. Samples were then treated with 0.3% hydrogen peroxide in methanol for 15 minutes to block endogenous peroxidase. After washing with 1X PBS, samples were blocked with 1% skimmed milk for 30mins. Samples were then washed and incubated for 1 hour with anti-CD68 primary antibody (Serotec, MCA1957) diluted 1:100 in 1% skimmed milk. After washing with 1X PBS, samples were incubated for 30min with goat anti-rat HRP secondary antibody diluted 1:50 in 1% skimmed milk. The samples were washed and exposed for approximately 2mins to di-aminobenzidinetetrahydrochloride (DAB) peroxidase Substrate Kit (Vector, SK4100).

4.6 Plasma cholesterol and lipoprotein profiles

At the end of the dietary period, terminal blood samples were collected from each mouse by puncture to the right ventricle. Plasma was collected and subsequently total cholesterol levels were determined with enzymatic assay kits. A commercially available calorimetric assay (Wako Bioproducts, Richmonds, VA) was used to determine serum total cholesterol concentrations present in terminal blood samples. Lipoprotein cholesterol distributions were evaluated in individual serum samples (60μL) by Fast Protein Liquid

Chromatography (FPLC) separation. Serum samples from 5 mice per group were evaluated and the means for each group was plotted and compared.

4.7 Statistical Analysis

Data analysis was performed using SigmaPlot 9.0 software (SPSS Inc, Chicago, IL). For each parameter, the mean and standard deviation of the mean were calculated. Statistical analysis between groups was evaluated by repeated measures ANOVA analysis. Post-hoc unpaired student t-testing was performed when differences were detected. Power calculations from pervious similar studies indicate that the outlined experiments would use a sufficient number of animals to attain a power of 0.8 for 20% change in lesion and provide statistically reliable results if no effects of atherosclerosis are observed. Values of $p < 0.05$ were considered statistically significant.

CHAPTER 5: Results

5.1 cIAP2 KO mice have reduced early atherosclerotic lesions in ascending aorta and descending aorta compared to WT mice on a 4wk HFD

To assess the difference in atherosclerotic lesion size between the WT and KO groups, *en face* lesion analysis was carried out following a 4 week high-fat dietary period. The number of aortic samples per group available for analysis was less than the number of mice studied as a result of the loss of some aortic samples during collection due to technical issues during cutting, cleaning or pinning.

As represented in **Figure 9**, the KO group (n=11, 0.58% ± 0.37) showed a significant decrease in lesion area when compared to the WT group (n=14, 1.51% ± 0.79). This decrease in lesion size translates into a 60.7% difference between the KO group and WT group, (P = 0.001). Representative images from this study group are shown in **Figure 9.1.**

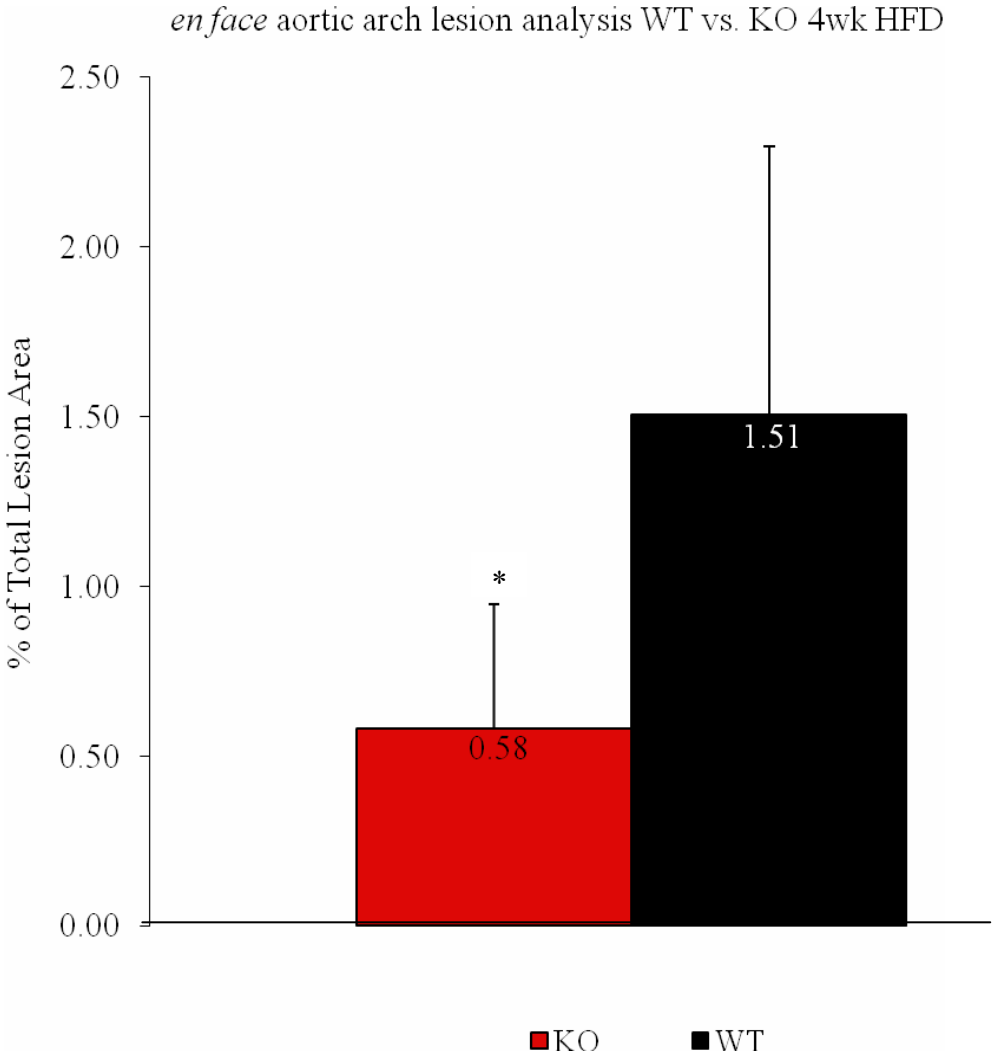
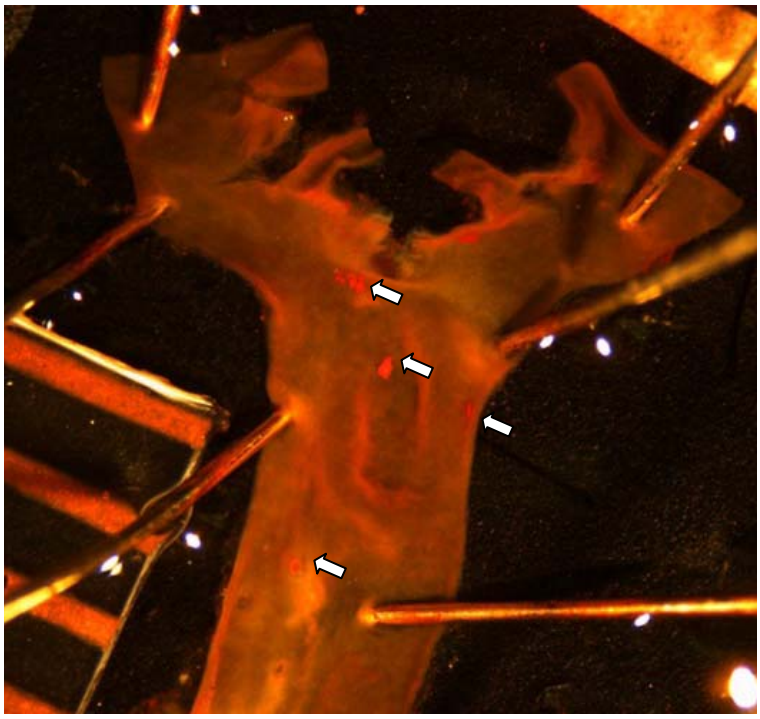


Figure 9. *en face* lesion analysis of *cIAP2* $^{-/-}$*x apoE* $^{-/-}$ (KO) vs. *cIAP2* $^{+/+}$*x apoE* $^{-/-}$ (WT) mice on a 4wk HFD.

Lesion area was quantified as percent lesion area of total aorta. As represented by the graph, the KO group showed a significant reduction in lesion area n= 11, 0.58% ± 0.37) vs. WT group. (n= 14, 1.51% ± 0.79) (* P = 0.001 using analysis of variance and *post hoc* unpaired student t-test).

A) 4wk KO



B) 4wk WT

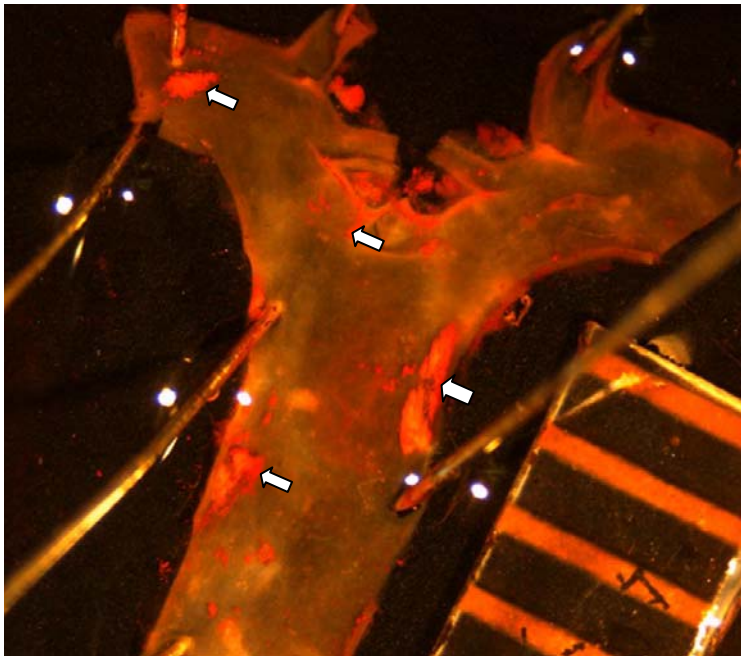


Fig 9.1. **Representative images from the mice on a 4wk HFD.**

Panel **A** shows an *en face* aorta of a KO (*cIAP2*$^{-/-}$ *x ApoE* $^{-/-}$). Panel **B** shows *en face* aorta of a WT (*cIAP2*$^{+/+}$ *x ApoE* $^{-/-}$). Lesion area is indicated with a white arrow.

5.2 cIAP2 KO mice have reduced early atherosclerotic lesions in aortic root compared to WT mice on a 4wk HFD

To assess the role of cIAP2 in atherosclerotic lesion development in a different vascular area (other than ascending and descending aorta), aortic root lesion analysis was carried out. Lesion area was calculated and results were represented in mm^2 ± SDEV. As represented in **Figure 10**, KO mice showed a significant reduction in lesion area (n= 11, 0.0327 ± 0.0145 mm^2) vs. WT mice (n=14, 0.0492 ± 0.0238 mm^2), (P = 0.047). Measurements for each level of the aortic root from KO and WT groups are represented in the **Table 1**, starting at the coronary ostia (level 0). The difference is also significant when examining two of the four levels ($p<0.05$). Differences in lesion area in levels 0 and 300 did not reach significance with p= 0.18 for level 0 and p= 0.100 for level 300. Representative images from the aortic root analysis for this study group are shown in **Figure 10.1.**

Table 1. Average of aortic root atherosclerotic lesion areas in mm^2 for KO and WT groups on 4wk HFD.

4wk KO mice

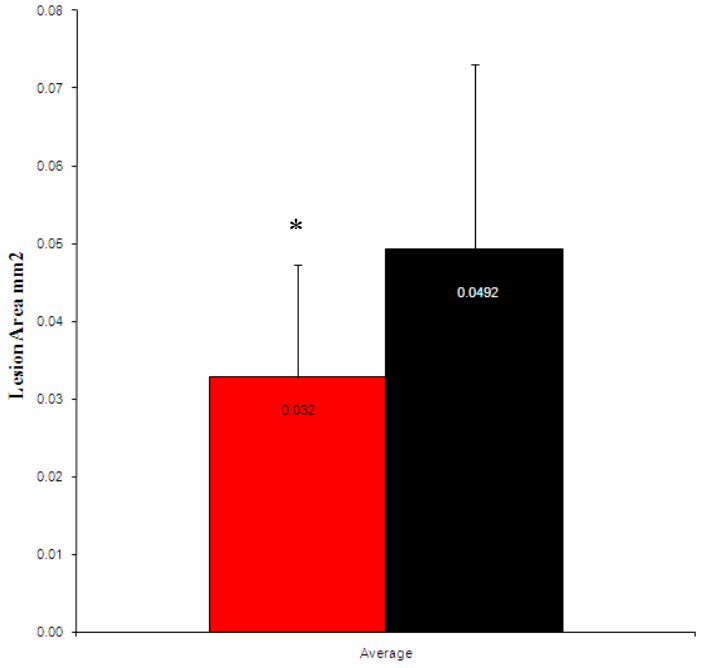
	0	**100**	**200**	**300**	**Average**
Average	0.0768	0.0359*	0.0108*	0.0074	0.0327*
STD	0.0330	0.0202	0.0049	0.0071	0.0145
STDERR	0.0099	0.0060	0.0014	0.0021	0.0043

4wk WT mice

	0	**100**	**200**	**300**	**Average**
Average	0.0919	0.0536	0.0309	0.0138	0.0492
STD	0.0500	0.0300	0.0200	0.0100	0.0238
STDERR	0.0140	0.0080	0.0050	0.0030	0.0064

* $p < 0.05$ vs. WT

A) Average of lesion area



B) Levels from coronary ostia

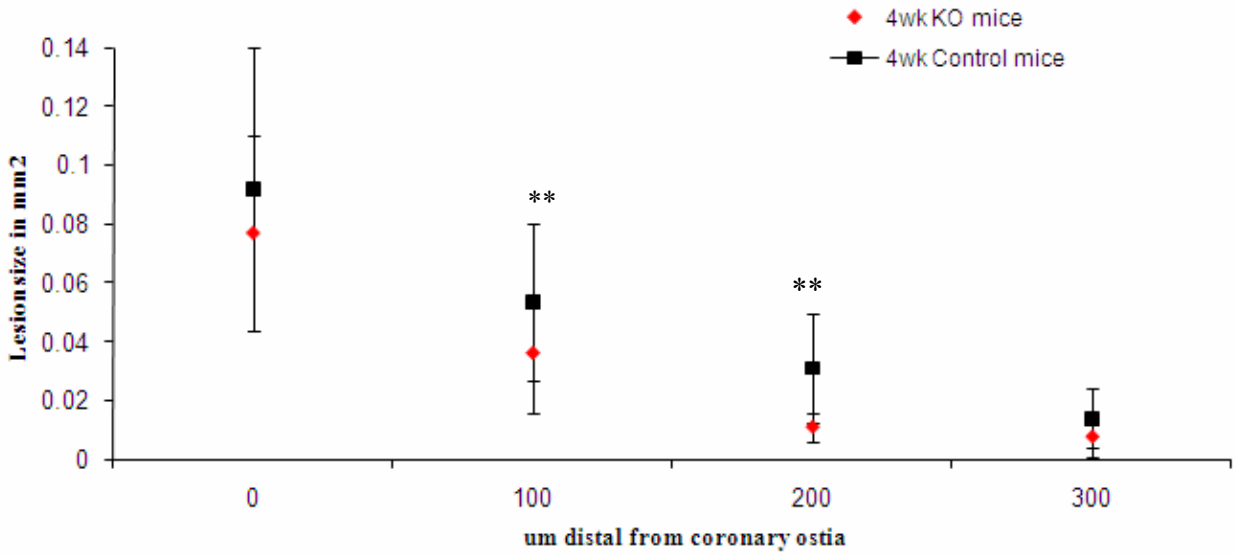
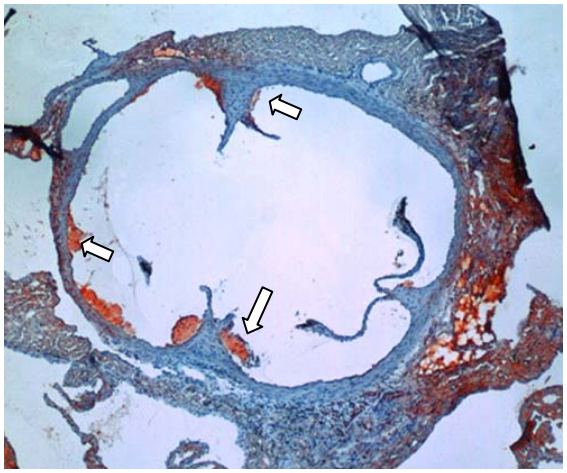


Figure 10. Aortic root lesion analysis of *cIAP2* $^{-/-}$*x apoE* $^{-/-}$ (KO) vs. *cIAP2* $^{+/+}$*x apoE* $^{-/-}$ (WT) mice on a 4wk HFD.

As represented by the graph In (A), the KO group showed a significant reduction in average lesion area (n=11, 0.0327 ± 0.0145 mm^2) vs. WT mice (n=14, 0.049 ± 0.0238 mm^2) (*P = 0.047) Further, in (B) the difference is also significant when examining two of the four levels of the aortic root starting at the coronary ostia (** P < 0.05).

A) 4wk KO



B) 4wk WT

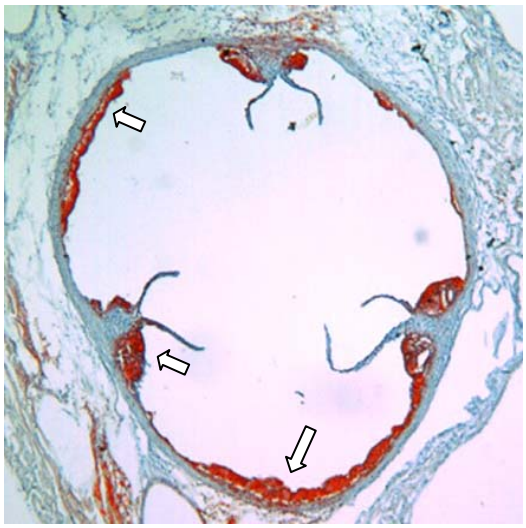


Figure 10.1. Representative images from the mice on a 4wk HFD.

Panel A shows *en face* aortic root of a KO (*cIAP2*$^{-/-}$ *x ApoE* $^{-/-}$). Panel B shows *en face* aortic root of a WT (*cIAP2*$^{+/+}$ *x ApoE* $^{-/-}$). Lesion areas are indicated with white arrows.

5.3 cIAP2 KO mice have reduced late atherosclerotic lesions in ascending aorta and descending aorta compared to WT mice on 12 wk HFD.

To asses the difference in atherosclerotic lesion size between the WT and KO groups, *en face* lesion analysis was carried out after a 12 weeks HFD period. Two additional KO animals were studied because of the loss of some aortic samples from the WT group during collection (technical issues during cutting, cleaning or pinning). As represented in **Figure 11**, the KO group showed a significant reduction in lesion area (n=17, 9.34 % ± 2.85) vs. WT group (n=15, 17.65% ± 6.24) with p = 0.002. This reduction in lesion size translates into a 53% difference between the knock-out group and wild type group, (P = 0.002). **Figure 11.1** shows representative images from the study groups.

En face aortic arch lesion analysis WT vs. KO 12wk HFD

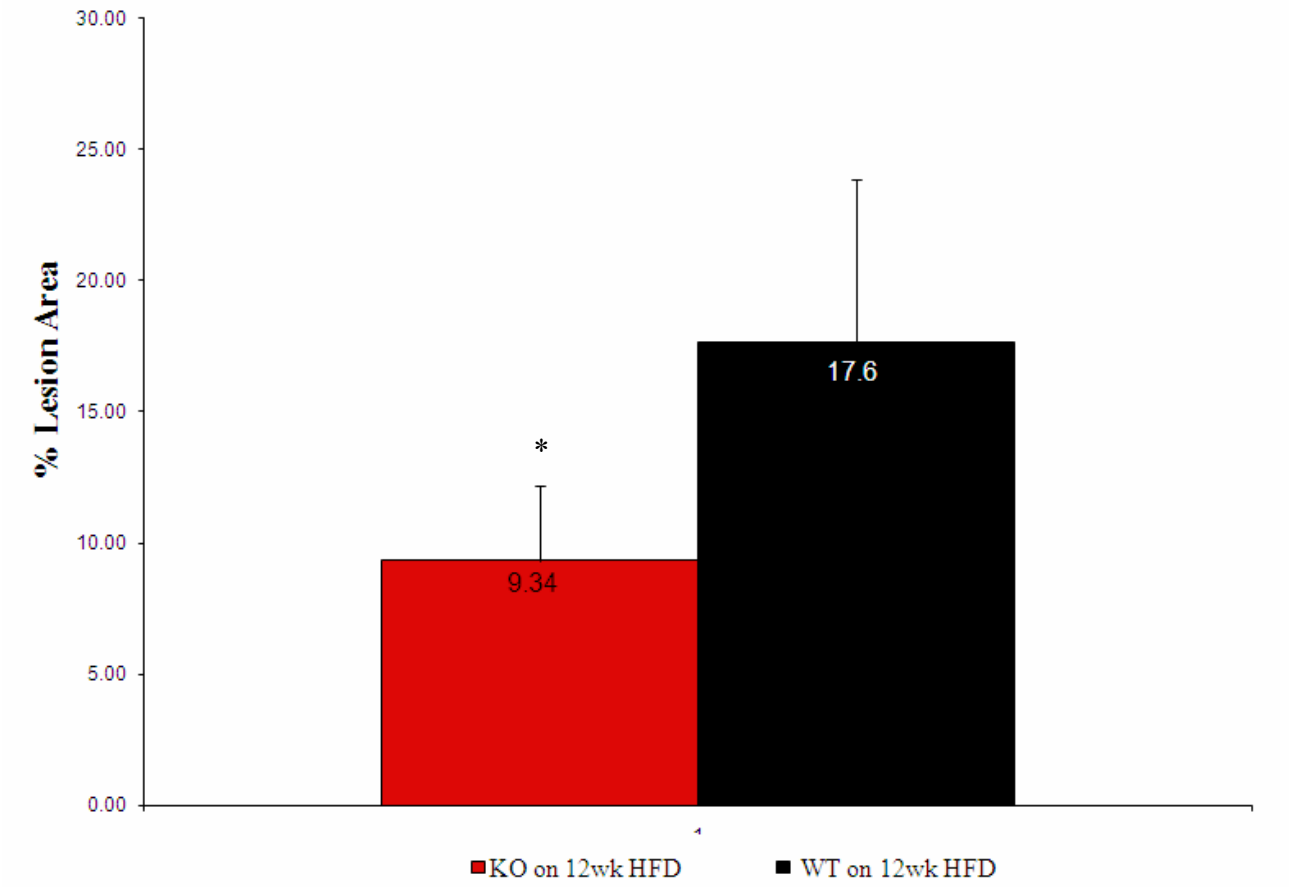
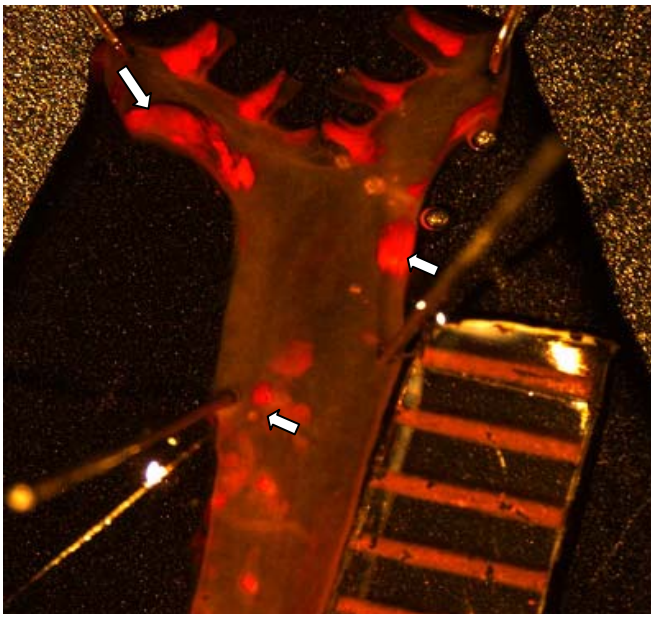


Figure 11. *En face* **lesion analysis of *cIAP2* $^{-/-}$*x apoE* $^{-/-}$ (KO) vs. *cIAP2* $^{+/+}$*x apoE* $^{-/-}$ (WT) mice on a 12wk HFD.**

Lesion area was quantified as percent lesion area of total aorta. As represented by the graph, the KO group showed a significant reduction in lesion area (9.34 % ± 2.85) vs. WT group (17.65% ± 6.24). (* P = 0.002).

A) 12wk KO



B) 12wk WT

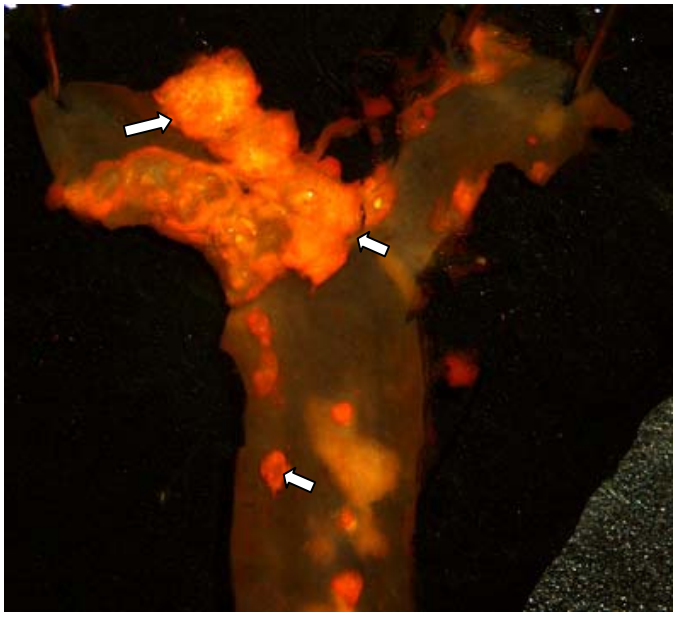


Figure 11.1 Representative images from the mice on a 12wk HFD.

Panel **A** shows an *en face* aorta of a KO (*cIAP2*$^{-/-}$ *x apoE* $^{-/-}$). Panel **B** shows *en face* aorta of a WT (*cIAP2*$^{+/+}$ *x apoE* $^{-/-}$). Lesion areas are indicated with white arrows.

5.4 cIAP2 KO mice have reduced late atherosclerotic lesions in aortic root compared to WT mice on 12 wk HFD

Atherosclerotic lesion development was assed in the aortic root of mice in order to examine a different vascular area at 12 weeks. As represented in **Figure 12**, KO mice showed a statistically significant reduction in average lesion area (n= 11, 0.3450 ± 0.1338 mm^2) vs. WT mice (n=14, 0.4482 ± 0.1567 mm^2) (p = 0.006). Measurements for each level from KO and WT groups are represented in the **Table 2** below. The mean values for KO mice were lower than the WT, reaching statistical significance at level 200 and a trend at level 300 (*p= 0.03; p= 0.085,* respectively*).* Differences in lesion area in levels 0 and 100 did not reach significance with p= 0.172 for level 0 and p= 0.179 for level 100. **Figure 12.1** shows representative images from each group.

Table 2. Average of aortic root atherosclerotic lesion areas in mm^2 for KO and WT groups on 12wk HFD**.**

12wk KO mice

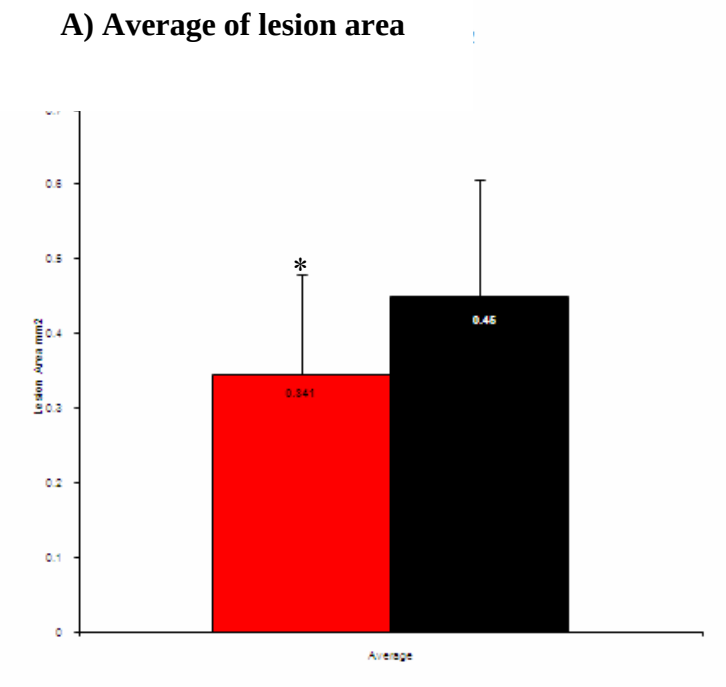
	0	**100**	**200**	**300**	**Average**
Average	0.4607	0.4025	0.2964*	0.2212**	0.3450*
STD	0.1198	0.1090	0.0938	0.1057	0.1338
STDERR	0.0378	0.0344	0.0296	0.0334	0.0403

12wk WT mice

	0	**100**	**200**	**300**	**Average**
Average	0.5520	0.4925	0.4237	0.3246	0.4482
STD	0.1501	0.1722	0.1722	0.1566	0.1567
STDERR	0.0433	0.0497	0.0497	0.0451	0.0420

* $p < 0.05$ vs. WT; ** p =0.085 vs. WT

A) Average of lesion area



B) Levels from coronary ostia

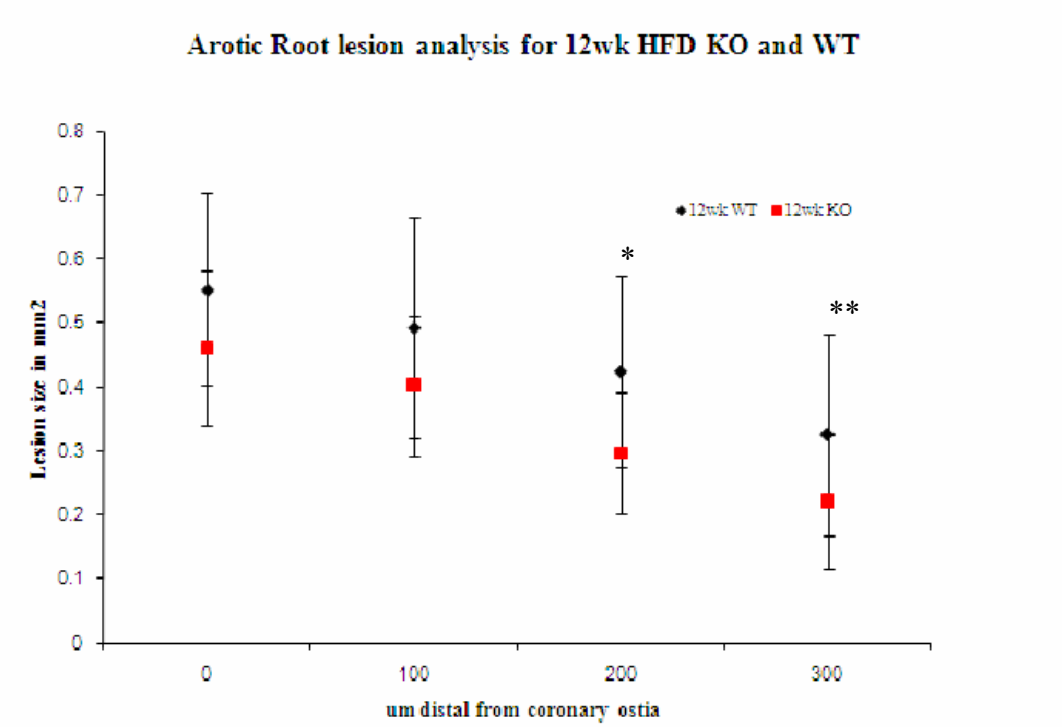
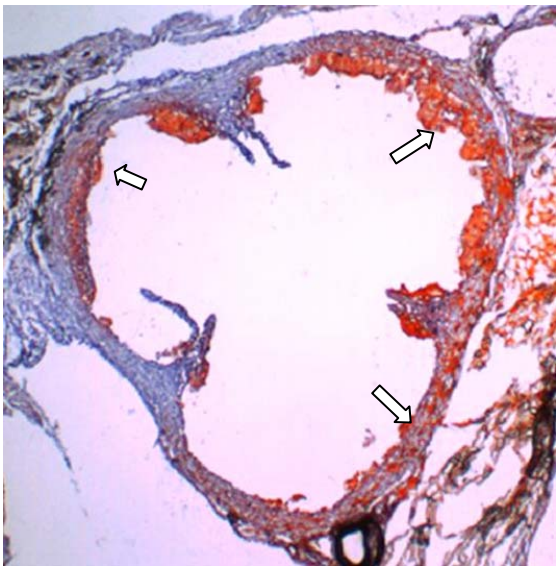


Figure 12. Aortic root lesion analysis of *cIAP2* $^{-/-}$*x apoE* $^{-/-}$ (KO) vs. *cIAP2* $^{+/+}$*x apoE* $^{-/-}$ (WT) mice on a 12wk HFD.

The graph in (**A**) the KO mice showed a significant reduction in lesion area (n= 11, 0.345 ± 0.1338 mm^2) vs. WT mice (n=14, 0.4482 ± 0.1567 mm^2). * P = 0.006 using an unpaired student t-test. In (**B**), the KO group showed a significant reduction in lesion area only at the later levels (200) (0.2964 ± 0.0938 mm^2) vs. WT mice (0.4237 ± 0.1722 mm^2) with p = 0.038. Aortic root at level 300 trends for significance where ***; p = 0.085*

A) 12wk KO



B) 12wk WT

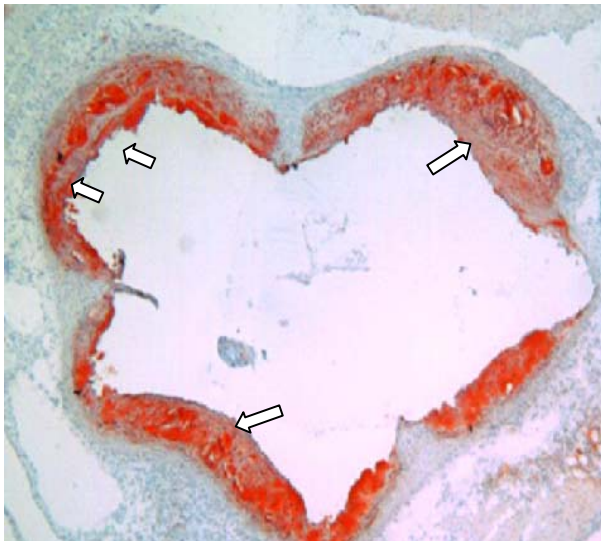


Figure 12.1 Representative images from the mice on a 12wk HFD.

Panel **A** shows aortic root of a KO mouse (*cIAP2*$^{-/-}$ *x apoE* $^{-/-}$). Panel **B** shows aortic root from a WT mouse (*cIAP2*$^{+/+}$ *x apoE* $^{-/-}$). Lesion area is indicated with white arrows.

5.5. CD68 Macrophage staining for WT and KO mice on a 4wk HFD.

Aortic root sections were stained for macrophage using an antibody specific for CD68 (a surface receptor expressed on active macrophages). Detailed staining methods are found in section 4.5. The sections used for this staining method are at a 10μm distance from the sections previously used for the Sudan IV staining. Lesion analysis was similar to that of aortic root lesion analysis explained in section 4.4. This method enables the analysis and quantification in the CD68 mouse groups to be easily compared with analysis in previous sections stained with Sudan IV from the same mouse group (i.e. 4wk KO Sudan IV vs. 4wk KO CD68 and similarly for the 4wk WT group). As represented in **Figure 13 (A),** the KO group showed a trend for reduction in average lesion area compared to the WT (KO mice, n = 6 (0.0306 ± 0.0080 mm^2) vs. WT mice, n = 5 (0.0485 ± 0.0210 mm^2) where p = 0.068). **Figure 13** (**B**) shows the difference in lesion size at the different levels from the coronary ostia. Differences in the CD68 staining area (at each level from coronary ostia) for KO and WT did not reach statistical significance and are represented in **Table 3** below. The results show relatively high variability due to the smaller sample size. **Figure 13.1** shows representative figures from aortic root lesion stained for macrophages.

Table 3. CD68 stained lesions in the different levels of the aortic root (in mm^2) of the KO and WT mice.

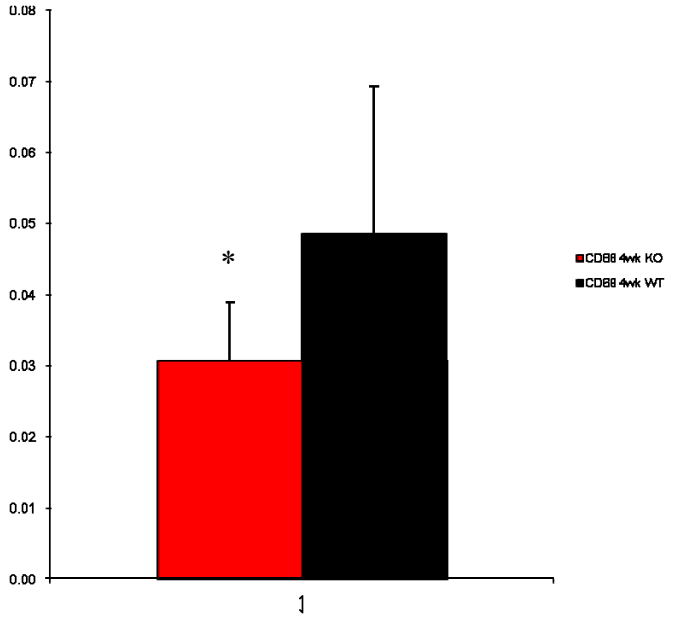
12wk KO mice

	0	100	200	300	Average
Average	0.0863	0.0358	0.0145	0.0058	0.0306
STD	0.0290	0.0260	0.0080	0.0040	0.0080
STDERR	0.0120	0.0110	0.0030	0.0020	0.0040

12wk WT mice

	0	100	200	300	Average
Average	0.0976	0.0546	0.0287	0.0131	0.0485
STD	0.0580	0.0260	0.0200	0.0090	0.0210
STDERR	0.0260	0.0011	0.0090	0.0040	0.0090

A) Average of lesion area



B) Levels from coronary ostia

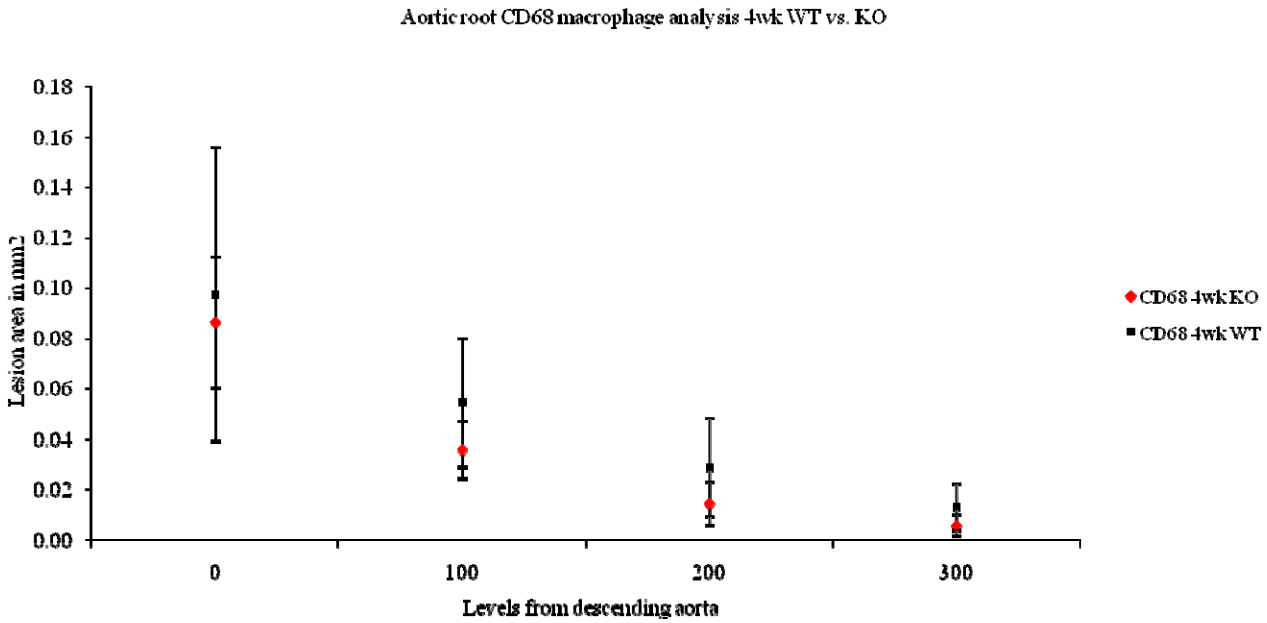


Figure 13. Aortic root lesion analysis of CD68 macrophage staining in WT and KO groups of 4wk HFD.

The graph represents the average of the lesion areas in (**A**) where the KO group showed a reduction (trending towards significance) in lesion area compared to the WT group. KO mice, n=6 (0.0306 ± 0.008 mm^2) vs. WT mice, n = 5 (0.0485 ± 0.021 mm^2). Panel (**B**) shows the difference in mm^2 at different levels from the coronary ostia.

5.6 Aortic root lesions analysis of Sudan IV and CD68 in mice fed a 4wk HFD in WT and KO groups.

Lesion analysis for CD68 and Sudan IV staining from both 4wk KO and WT groups were overlapped in order to examine the relation between lesion macrophage infiltration and lipid staining. As represented in **Figure 14**, CD68 macrophage staining and Sudan IV from the 4wk KO group overlap with no significant difference between the two approaches. A similar trend is seen in the CD68 macrophage staining and the Sudan IV staining for the 4wk WT group. The KO mice had a CD68 macrophage average staining area of 0.0356 ± 0.0142 mm^2 and Sudan IV stained average area of 0.0327 ± 0.0141 mm^2. The WT mice had a CD68 macrophage average staining area of 0.0485 ± 0.0216 mm^2 and Sudan IV stained average area of 0.0501 ± 0.0246 mm^2. The average area as well as specific lesion area at each level in aortic root for 4wk CD68 stained sections are represented in **Table 3** above. **Figure 14.1** shows representative images from this analysis. In order to calculate the correlation between the CD68 and Sudan IV staining for the 4wk HFD KO and WT groups, a correlation coeffient was calculated. **Figure 15** shows the correlation between CD68 and Sudan IV staining for 4wk KO and WT mice with data from both methods available. There is a strong correlation between the two parameters ($r^2 = 0.9912$).

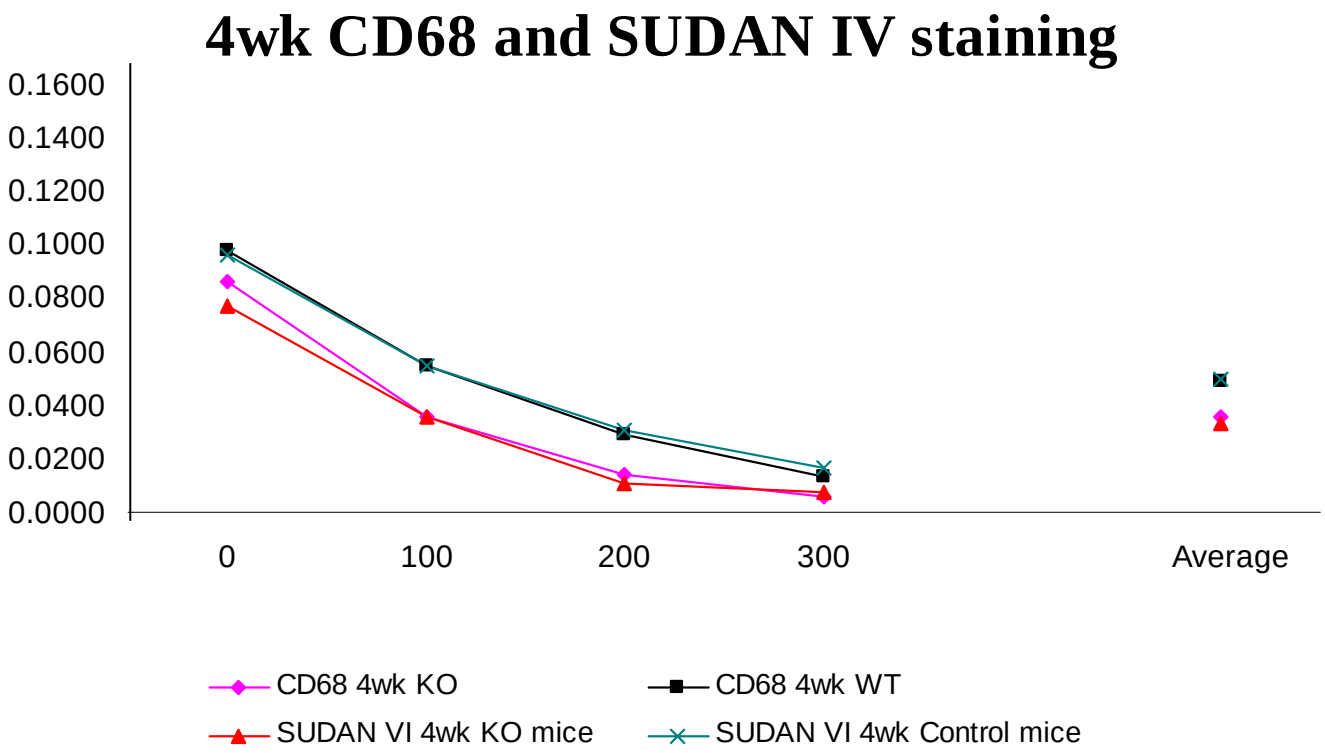


Figure 14. CD68 macrophage and Sudan IV staining for 4wk HFD WT and KO groups.

The graph represented the WT and KO mouse groups on a 4wk HFD for both Sudan IV and CD68 macrophage staining. The KO mice had a CD68 macrophage staining area average of 0.0356 ± 0.0142 mm^2 and Sudan IV stained average area of 0.0327 ± 0.0141 mm^2. The WT mice had a CD68 macrophage staining area average of 0.0485 ± 0.0216 mm^2 and Sudan IV stained average area of 0.0501 ± 0.0246 mm^2.

A) 4wk KO **B) 4wk WT**

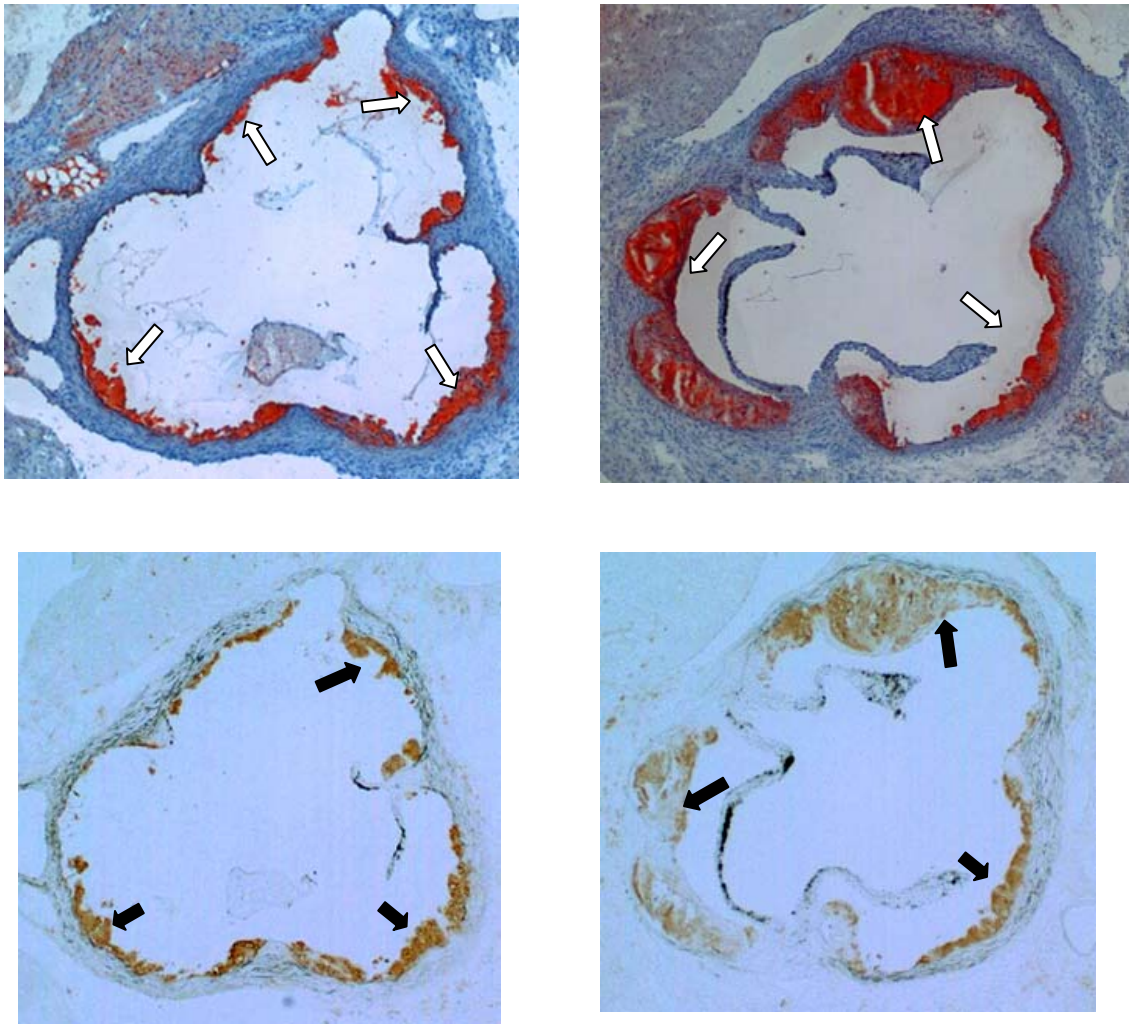


Figure 14.1 Representative figures from aortic root lesion analysis.

Panel **A** shows images from 4wk KO group and panel **B** shows images from 4wk WT group. The top panels show the same aortic root stained for Sudan IV and the bottom panels show aortic root from the same mouse stained for CD68 macrophages. Lesion areas are indicated with white arrows and macrophage filled areas are indicated with black arrows.

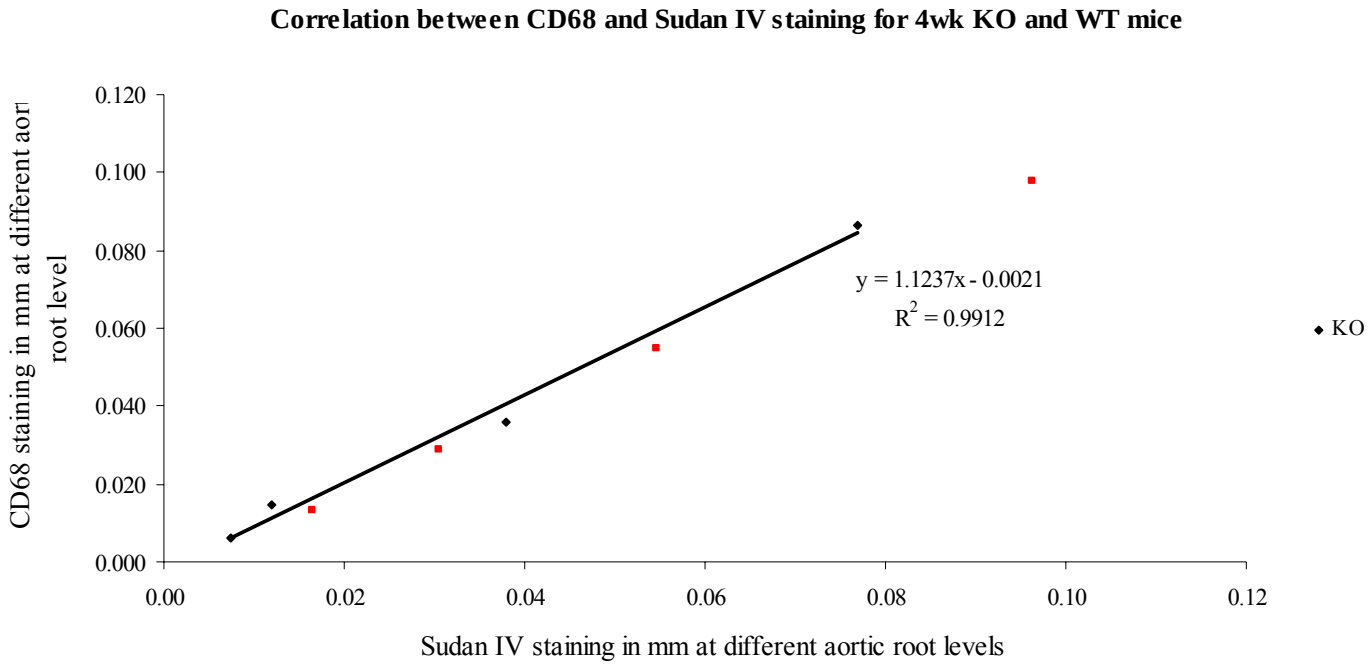


Figure 15. Correlation between CD68 staining and Sudan IV staining for 4wk HFD KO and WT mice.

Above graph represents the correlation between the CD68 staining measurements and Sudan IV staining measurements for the 4wk HFD KO and WT mice. CD68 lesion areas (mm^2) were plotted on the y-axis vs. Sudan IV stained areas (mm^2) plotted on the x-axis. A correlation coeffient was calculated for both KO WT data points with $r^2 = 0.9912$.

5.7 Plasma lipoprotein and cholesterol levels

Compared to apoE $^{-/-}$ mice deficient in cIAP2 gene, the wild type group did not show any significant differences in the serum total cholesterol levels at the end of the study shown in **Figure 16**. Analysis by size exclusion chromatography (FPLC) also confirmed that there were no differences in lipoprotein-cholesterol profiles between *cIAP2* $^{-/-}$ *x apoE* $^{-/-}$ and *cIAP2* $^{+/+}$ *x apoE* $^{-/-}$ mice represented in **Figure 17.**

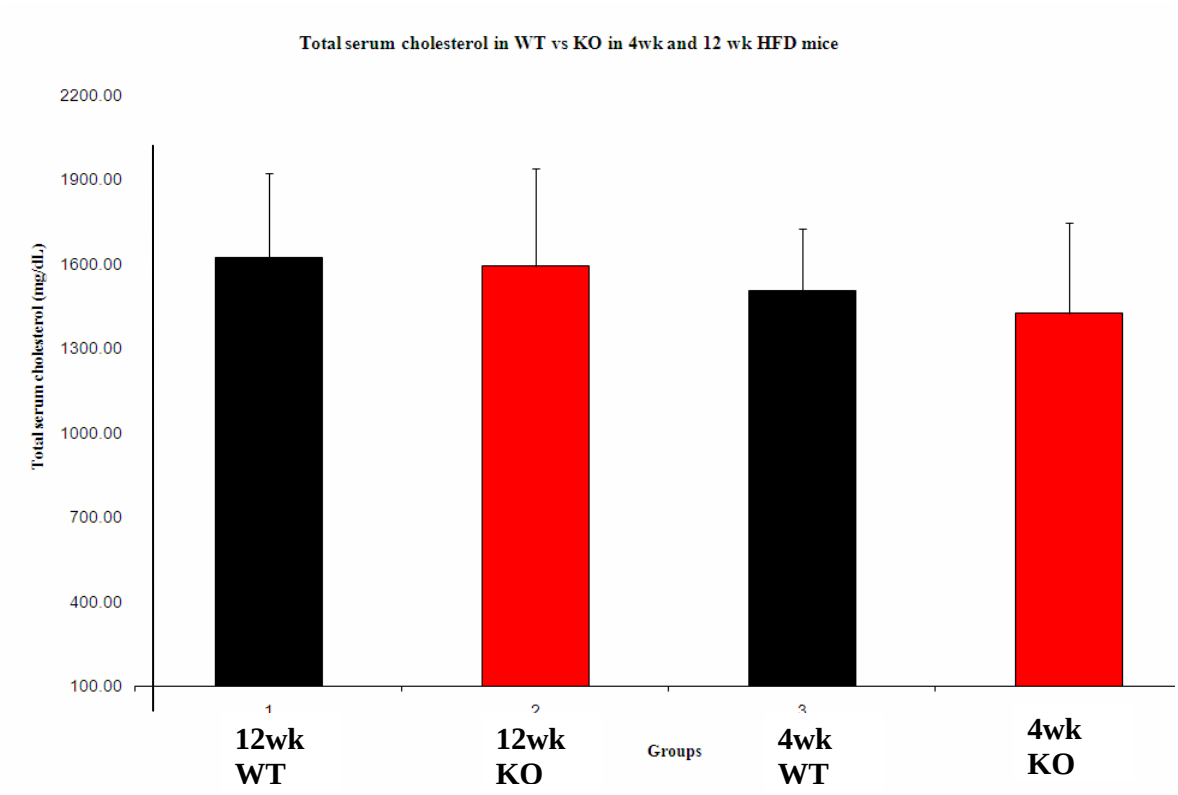


Figure 16. Serum total cholesterol analysis.

Total blood serum cholesterol levels between all four mouse groups (4wk KO, 4wk KO, 12wk KO and 12wk WT) showed no significant difference.

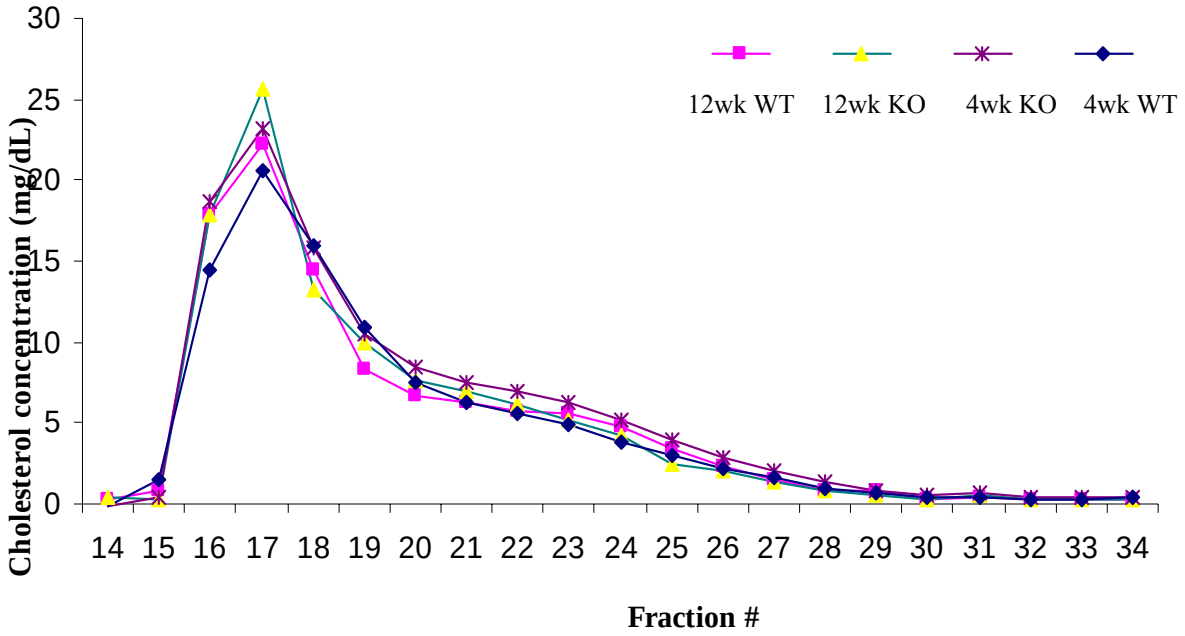


Figure 17. Lipoprotein cholesterol profiling in all 4 mouse groups.

Above graph shows the average difference in cholesterol concentrations within blood serum fractions. No significant difference can be seen for all four different mouse group's lipoprotein profiling.

CHAPTER 6: Discussion

The present study shows, for the first time, that loss of cIAP2 reduces atherosclerotic lesions at early and advanced stages of the disease in an *apoE*$^{-/-}$ mouse model. Using a cIAP2 KO mouse model, we were able to elucidate the effect of cIAP2 deletion on the progression of the disease at two different stages. We also show that not only are the atherosclerotic lesions smaller in size in the KO model, but also contain a smaller number of macrophages when compared to WT mice. This data suggests an important role for cIAP2 in the pathogenesis of the disease and unveils potential opportunities for drug therapies.

6.1 The role of apoptosis

Accumulating *in vivo* data published to date show that decreased early lesion macrophage apoptosis in mice increases lesion area (Schrijvers DM. *et al.,* 2005). It is assumed that inflammation is promoted when secondary necrosis by macrophages is not quickly and efficiently cleared at the sight of injury. Previous studies have demonstrated that the apoptosis promotion in more advanced and late atherosclerotic plaques would promote further lesion progression which could generate clinical events. However, fundamental evidence for these processes has been controversial.

The present study examines the novel role of an anti-apoptotic protein (cIAP2) in both early and late stage atherosclerosis by using a KO model lacking the cIAP2 protein. As represented in **Figures 9, 9.1, 10** and **10.1**, the KO model reduces lesion size in different vascular regions (aortic root, aortic arch and ascending aorta) in mice on a 4wk HFD. More prominently, this trend is sustained in the later more advanced stages as the mice are fed a HFD for 12 wks as is represented in **Figures 11, 11.1, 12** and **12.1**.

6.2 The role of early macrophage apoptosis in lesion development

Several genetic manipulations in experimental mouse models result in an increase or decrease in early lesion macrophage apoptosis, and the joint outcome of these studies suggest an opposite relationship between early lesion macrophage apoptosis and the size of early lesion area. A BMT study by Van Vlijman *et al.* reconstituted an apoE-3 atherosclerotic mouse model with bone marrow from mice lacking the pro-apoptotic protein p53 (Van Vlijmen BJ. *et al.,* 2001). The "receiving" mice that were transplanted with *p53*$^{-/-}$ bone marrow displayed decreased macrophage cell death when compared to the WT bone marrow recipient group. This decrease in macrophage apoptosis came with a 2.3-fold increase in early atherosclerotic lesion area possibly explaining that reduction in early lesion macrophage apoptosis supports early atherosclerotic lesions growth. However, a similar study using p53-BMT into *LDLr*$^{-/-}$ mouse models discovered that a reduction in macrophage apoptosis correlated with an increase in early atherosclerotic lesion development (Merched AJ. *et al.,* 2003). In this study, the decrease in the apoptosis of macrophage was a result of an increase in macrophage cell propagation which most likely explains why the finding is controversial (Merched AJ. *et al.,* 2003). More evidence pertaining to the importance of lesion macrophage apoptosis in early atherosclerosis was gathered from a study using *LDLr*$^{-/-}$ mice transplanted with bone marrow lacking the *Bax* gene - a pro-apoptotic protein (Liu JJ. *et al.,* 2005). As suspected, early lesion macrophage apoptosis was reduced in the mice receiving *Bax*$^{-/-}$ bone marrow vs. WT bone marrow (*ibid.)*. This data indicates that macrophage apoptosis normally takes place in early atherosclerosis possibly through one or more pathways that could involve *p53* and *Bax* proteins (*ibid.)*. Work by Arai *et al.* examined the role of an inhibitory factor

AIM, which is an apoptosis inhibitor expressed on macrophages in older tissue (Arai S. *et al.*, 2005). In this study, early atherosclerotic lesions were significantly reduced in *AIM*$^{-/-}$ x *LDLr*$^{-/-}$ KO mice when compared to the *AIM*$^{+/+}$ x *LDLr*$^{-/-}$ controls. In conclusion, the above studies demonstrate that in mouse early lesions, foam cells apoptosis seems to have a limiting effect on lesion cellularity and progression. In the present study, however, cIAP2$^{-/-}$ x apoE$^{-/-}$ double KO mice show reduced lesion cellularity at early stages when compared to their wild type mice as represented in **Figures 9** and **10**. As delineated in **Figure 11**, the lesions in the KO mice also show reduced macrophage content when compared to the WT group.

cIAP2 is believed to inhibit apoptosis by binding to the TNF receptor complex (reviewed in Wu H. *et al.*, 2007). By deleting the *cIAP2* gene, the effect of the cIAP2 protein is alleviated in all cell lineage. TRAF2 freed from cIAP2 diminishes the recruitment of the kinase Rip1 to TNFR1 complex. In the process, the canonical NF-κB pathway is deactivated, halting production of TNFα by a large amount of active macrophage cells. In a neutrophil, were the non-canonical pathway is predominant, cIAP2 deletion leads to stabilization of NIK proteins and increased concentrations allowing the ubiquitylation and activation of the NF-kB pathway (reviewed in Meike Broemer and Pascal Meier, 2009). Unlike the Fas Ligand, TNFα does not always induce apoptosis; however, it leads to cell activation and survival by means of activation of NF-κB and MAP kinases. Studies in the past have demonstrated that a secondary TRADD-RIP1-FADD-caspase- 8 complex that (devoid of TNFR1) is also able to promote TNFα-induced cell death (Micheau O. and Tschopp J., 2003). Along these lines, apoptosis induced by smac-mimetic compounds requires Rip1 molecules even in the presence of extra-cellular TNFα for smac-mimetic resistant cells alone which react to TNFα co-stimulation (Petersen SL. et al., 2007).

In a macrophage undergoing stress from lipid-infiltration, receptor mediated apoptosis is induced and drives macrophages toward self destruction. With the absence of the inhibitory effect of cIAP2, macrophage cells are destroyed and, in effect, the apoptotic bodies are rapidly and safely removed by phagocytes. The fact that we observe reduced macrophage content in the KO model suggests that their apoptotic clearance is efficient as represented in **Figures 9** and **10** by having a reduced plaque size. These findings mirror the work from Arai *et al.* examining the role of inhibitory factor AIM and show that its removal reduces atherosclerotic plaque size (Arai S. *et al.,* 2005). The effect of increased early lesion macrophage apoptosis resulted in reduced fatty streak formation as was measured by *en face* analysis in the KO model compared to the WT.

The present study also examined the extent of macrophage content in early atherosclerotic plaque. **Figure 13** represents aortic root macrophage content in KO and WT mice on a 4wk HFD. The total lesion area filled with macrophages is higher in WT vs. KO mice. This supports the notion that cIAP2 deletion increases macrophage apoptosis and results in effective phagocytic clearance of the apoptotic bodies leading to reduced plaque cellularity and size. This is reflected in **Figure 14** and **14.1**, which summarizes the results of both Sudan IV and CD68 macrophage staining. The graph represents the relation between macrophage content and plaque size in the early lesions. **Figure 15** further emphasizes the strong correlation between the CD68 stained area and Sudan IV. In the KO group, the lesion sizes at different levels from the coronary ostia overlap from both analysis. A similar observation is made with the WT mice, where both Sudan IV and CD68 macrophage staining curves overlap and both measurements are well correlated. There is a decrease in lesion size as well as macrophage infiltration size in all the groups. Analysis was not carried out for the

12wk HFD mice due to technical difficulties leading to a small sample size (discussed in section 6.8 below). However, there is sufficient evidence from the 4wk CD68 staining data to demonstrate a trend that is consistent with the significant Sudan IV findings. That it did not reach statistical significance likely reflects the sample size tested with CD68. Even so, the trend in the CD68 staining data lends support for the notion regarding the role that cIAP2 plays a role in early lesion development and progression. Moreover, it is important to outline the effects of cIAP2 on early lesions since the outcomes of this stage predict its later effect on advanced late stages. For effective drug applications, a pharmaceutical agent that targets early lesion progression may help prevent later clinical manifestations of the disease

6.3 The role of macrophage apoptosis in advanced lesions

By feeding mice a HFD for 12wks, we were able to examine the effect of cIAP2 deletion on advanced atherosclerotic lesions. As expected, the extent of atherosclerotic plaque in the group of mice fed a 12wk HFD was greater than the group fed a HFD for 4 weeks as indicated by the *en face* analysis (4wk: **Fig. 9**; 12wk: **Fig 11**) and the aortic root analysis (4wk: **Fig. 10**; 12wk: **Fig 12**).

Despite differences in lesion stages (early for 4wk and advanced for 12wk), the same trend was observed regarding the size of the atherosclerotic plaque. **Figures 11** and **11.1** shows that *cIAP2*$^{-/-}$ x *apoE*$^{-/-}$ mice on 12wk HFD had significantly reduced lesion area when compared to the WT controls through *en face* analysis. The reduction in atherosclerotic lesions was also seen in aortic root lesion analysis, represented in **Figures 12** and **12.1**. As the mice are put on a prolonged HFD, the atherosclerotic plaque increases in size. However, the *cIAP2*$^{-/-}$ x *apoE*$^{-/-}$ mice still maintains a reduced lesion size when compared to the WT control

group. This implies that finding the right target for reducing atherosclerosis progression at an early stage could help reduce advanced lesions from developing. Ultimately, this may be expected to reduce the chance of plaque rupture and thrombus formation that leads to strokes and myocardial infarctions.

In a study by Schrijvers *et al*. on human atherosclerotic lesions derived from the carotids, it was shown that there was a considerably large number of apoptotic cells that were not cleared by phagocytes (Schrijvers DM. *et al.,* 2005). Moreover, a few studies have observed that in advanced lesions, the area adjacent to the necrotic core was filled with apoptotic macrophages (Hegyi L. *et al.,* 1996; Akishima Y. *et al.,* 2005). The necrotic core is mainly composed of dead macrophages loaded with inflammatory cytokines which follows the notion that that faulty phagocytosis leads to necrosis of post apoptotic macrophage resulting in a severe inflammatory condition. Chronic inflammatory signals and pathways have been carefully studies in the past, and a similar relationship has been established for atherosclerosis at least between faulty macrophage apoptotic clearance and high inflammation (Vandivier RW. *et al.,* 2002). Many clinical manifestations resulting from plaque occlusions are believed to be a result of bad clearance of the apoptotic macrophage bodies by phagocytes, at least in an advanced atherosclerotic plaque setting (Vandivier RW. *et al.,* 2002). Although late inhibition of cIAP2 may in fact be detrimental, this notion was not the focus of our investigation.

This study suggests a role for cIAP2 in early disease progression. However, more in-depth examination of the role that cIAP2 plays in the advanced lesions is necessary to understand its implication on disease manifestations. In other words, how cIAP2 inhibition might change lesion cellularity, fibrous cap formation, plaque content and ultimately its

vulnerability. As argued in several reviews, macrophage apoptosis at later more advanced stages of the disease could be detrimental. Therefore, administering smac-mimetics at a later time point may potentially have a negative impact on disease pathogenesis. However, this requires further study.

6.4 cIAP2 and lipoprotein profile.

Many risk factors have been identified to be linked with the initiation and progression of atherosclerosis. While it was encouraging that no differences in plasma cholesterol or triglycerides were observed between any of the groups in this study, it was also essential to examine total lipid profile levels in the mice. It is possible that even though there is no difference in total lipids, lipid profiles may be altered between groups and manifesting itself, for instance, as a shift towards pro-atherogenic particles such as VLDL. To confirm that the KO model was not advantaged by reductions in cholesterol and lipoprotein levels, we separated plasma lipoproteins by size using FPLC methods and determined that there were no differences in the lipoprotein distribution between any groups. This ensures that deletion of cIAP2 does not modulate cholesterol levels in the *apoE*$^{-/-}$ model. Therefore, any observed differences in atherosclerosis in the experimental mouse model can be attributed solely to the participatory role of cIAP2 in lesion development.

6.5 cIAP2 and lesion formation

It was observed, while performing the lesion analysis on all the study groups, that cIAP2 does in fact play a role in the stages of the disease at different time points. However, it must be re-iterated that atherosclerosis is a dynamic inflammatory disease and that there are

multiple factors affecting the lesion stage at one point in time. When studying early atherosclerotic lesions, the group lacking cIAP2 (KO) appeared to have fewer fatty streaks than the control groups. Furthermore, even at a later stage of the disease, the KO group had fewer advanced lesions (stage IV and V) when compared to the WT group. This observation ties in with the finding that the KO have reduced atherosclerotic lesion area and fewer macrophage numbers when compared to the control group.

A possible explanation could be made with regards to the role cIAP2 plays in TNF-α activation. Loss of cIAPs due to smac-mimetic treatment can help in TNFα- induced apoptosis as a study by Boesten L. and colleagues examined the role TNF-α plays in advanced atherosclerotic lesion progression using a mouse model devoid of apoE-3 (Boesten L. *et al.,* 2005). The study shows that TNF-α promotes advanced lesion formation, and that the degree of necrosis was increased. Since TNF-α was shown to increase the relation between necrosis and apoptosis, it was concluded that TNF-α may make the lesion more unstable. Moreover, *in vitro* studies suggest a role for TNF-α in the instability of the plaque where it induces both the macrophages and SMC to synthesize matrix proteases which end up degrading the fibrous "cap" (Galis ZS. *et al.,* 1994). The decrease in necrosis of the lesion once TNF-α was deleted, corresponds with more apoptosis in the lesion. TNF-α has been shown to act, at least in part, via the activation of the canonical NF-κβ pathway (Ashkenazi A. and Dixit VM. 1998). cIAP2 is suggested to bind to the TNF-receptor complex I, and by doing so contributes in the ubiquitylation of several members of the complex leading to activation of the canonical NF- κβ pathway producing more TNF- α (Eugene Varfolomeev, et al., 2008). Deletion of cIAP2 should, hypothetically, inhibit its effect on NK-κβ activation and therefore reduce TNF- α production. cIAP2 is also involved in the negative regulation of

the non-canonical NF- κβ pathway in neutrophils where it ubiquitylates TRAF2, increases NIK concentration and inhibiting the activation of the pathway (Vallabhapurpu S. et al., 2008; Zarnegar B. et al., 2008). In the case of early atherosclerotic lesions, deletion of cIAP2 is could be beneficial because it activates the non-canonical NF- κβ pathways in neutrophils thereby inducing early lesion apoptosis through TNF- α production. This leads to more macrophage apoptosis, enhanced apoptotic body clearance and reduced plaque size and cellularity. Reduced lesion size and cellularity is still observed in the late stage atherosclerotic lesions, but to a lesser degree. KO lesions are less necrotic than the WT lesions; however, the difference is not significant. This could possibly mean that cIAP2 deletion is more beneficial at early stages of the disease than at later stages, where macrophage apoptosis is reduced and TNF- α secretion increases the rate of necrosis.

6.6 Proposed mechanism of action of cIAP2

It was thought that cIAP2 inhibits apoptosis by directly binding to caspases *in vitro*, however, this idea has been abolished as it has later been shown that cIAP2 is involved in various transduction pathways including NF-kB activation in response to TNFα (Rothe M. *et al.,* 1995; Samuel T. *et al.,* 2006).

When a macrophage cell (in an atherogenic milieu) ingests lipids and becomes a foam cell, different environmental cues and stimuli induce receptor-mediated apoptosis. In a normal environment (e.g. in WT control mice), cIAP2 binds to the TNF receptor complex and, through its RING domain, leads to the poly- or trans-ubiquitylating of the Rip1 protein, targeting it for either degradation or other cellular localization. This series of actions destruct the TNF receptor complex and essentially inhibits its downstream effect on the effecter

caspases leading to apoptosis. However, in the cIAP2 KO model, where cIAP2 protein is absent, this inhibitory effect of cIAP2 is eliminated and the receptor induced apoptotic stimuli can ultimately lead to macrophage apoptosis. Indeed, studies have demonstrated that by acting as E3 ubiquitin ligases for Rip1, cIAP1/2 proteins are able to positively regulate the activation of canonical NF-κB through TNF-α, as well as negatively regulate signaling of non-canonical NF-κB through the ubiquitination and activation of the NF-κB-inducing kinases (Blankenship JW. *et al.,* 2009). The proteosomal degradation as well as rapid auto-ubiquitylations of cIAPs could be induced through the cIAP's E3 ubiquitin ligase activity. As mentioned above, since cIAPs are also capable of regulating the non-canonical NF-κB pathway via NIK ubiquitylation, the loss of the cIAP proteins means that the non-canonical pathway can be freely activated through NIK stabilization (Blankenship JW. *et al.,* 2009). As a result of the activation of NF-κB, NF-κB target genes (such as TNF-α) are induced. In the case where cIAP proteins are absent, TNF- α is able to activate caspase-8 by binding to TNFR1 and therefore activating the apoptotic cascade (Blankenship JW. *et al.,* 2009).

6.7 The role of Smac-mimetic drugs.

The ability of the IAPs' over-expression to protect cells against the pro-apoptotic stimuli ranging from chemotherapies to DNA damage due to UV light, has been well demonstrated by several studies and shows that IAPs can act as a sign for unhealthy or poor progression of many malignant and solid tumors (Salvesen GS. *et al.* 2002; reviewed in Liston P. *et al.* 2003; reviewed in Nachmias B. *et al.* 2004).On the other hand, the suppression and/or inhibition of the levels of IAP protein can make cancer cells more sensitive to diverse pro-apoptotic therapeutics (Kasof GM. *et al.,* 2001). As mentioned

earlier, all IAPs share at least one BIR domain and in order to have successful inhibition of a caspase-dependent apoptosis, there must be antagonism of the IAP-mediated pathway. This can be achieved by the cytosolic releases of endogenous Smac/DIABLO in response to stimuli that are pro-apoptotic (Du C. *et al.,* 2000; Verhagen AM. *et al.,* 2000). Many studies have scrutinized the specific electrostatic interaction between specific BIR domains of the IAP proteins and the Smac/DIABLO proteins (Wu G. *et al.,* 2000; Franklin MC. *et al.,* 2003). In summary, Smac/DIABLO and caspases compete for the same BIRII domain on IAPs and by doing so, Smac/DIABLO blocks caspase-interaction. *In vivo* and *in vitro* studies have been able to demonstrate the capability of Smac-derived peptides to sensitize several tumor cell lines to apoptosis induced by many different pro-apoptotic molecules. **Figure 18** illustrates the proposed pathways for the action of Smac-mimetic on cIAP2. One may hypothesize that, in addition to these potential cancer applications, Smac-mimetic drugs could be administered in order to reduce early atherosclerotic lesion development through IAP inhibition via promotion of apoptosis in plaque macrophages.

6.8 Methodological and technical considerations

As mentioned earlier, the sample sizes in some experiments did not add up to the 15 mice per group as had been initially proposed due to technical difficulties during sample collection and processing. Moreover, aortic root section collection can be challenging. The inside temperature of the cryosectioning machine is very critical and in some occasions samples were not collected properly and/or did not adhere to the slides adequately. Furthermore, there are many washing steps during staining (both for CD68 and Sudan IV)

and this occasionally results in the sections separating from the slide and dissolving into the solution.

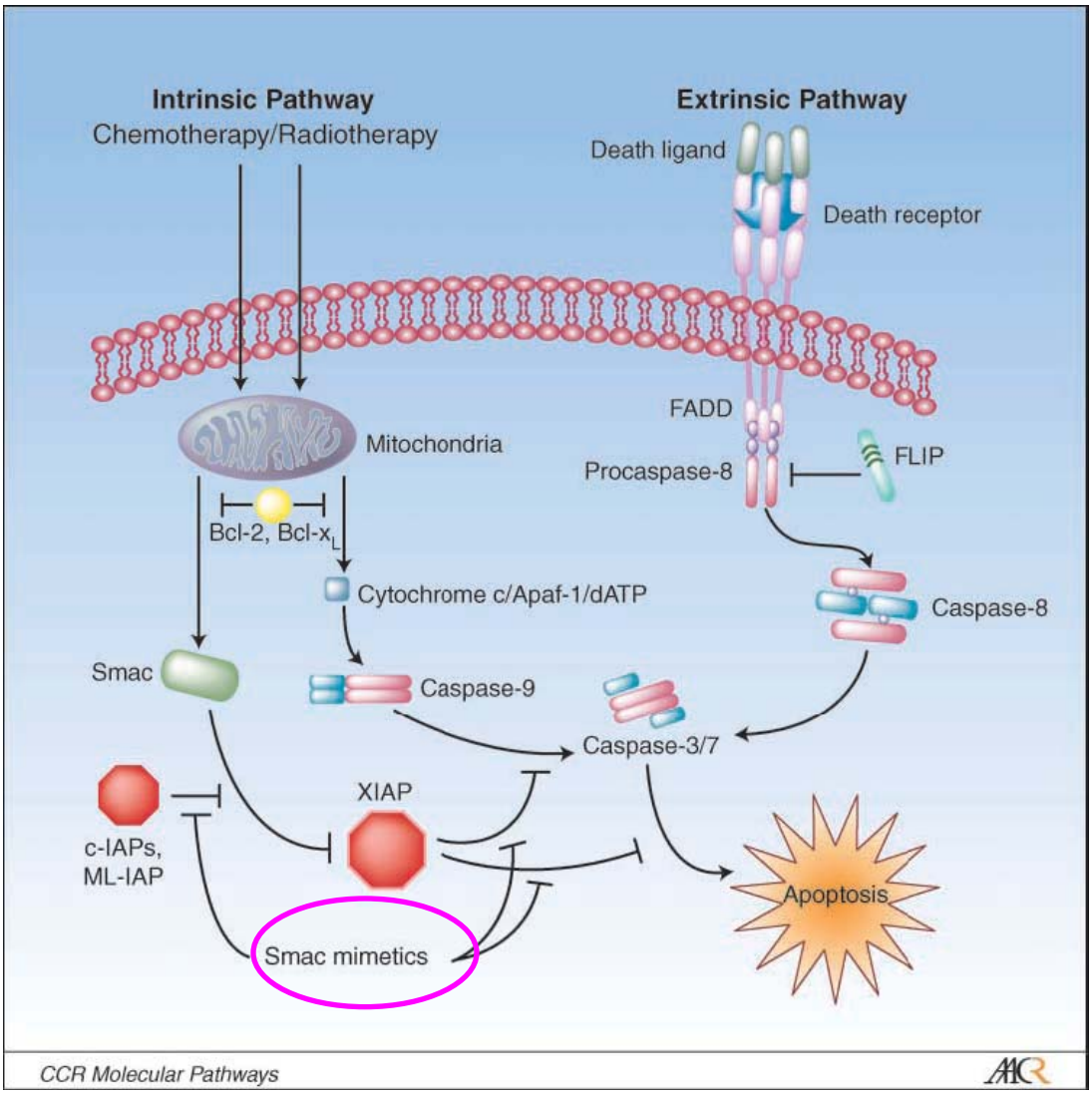


Figure adapted from Domagoj Vucic and Wayne J. Fairbrother (2007).
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Figure 18 – Cell Death Induced by Smac Mimetic.

This diagram illustrates the mechanism of action of the Smac-mimetic drugs (highlighted within the pink oval) and their hypothesized role in inhibiting the action of IAPs.

Some of the mice on a 12wk HFD underwent cardiac perfusion with 4% PFA in order to fix the animal for further analysis (for imaging purposes discussed in the "future directions" section). This fixation method was performed before deciding to stain for macrophages using CD68 antibody. Cardiac fixation of the whole mouse "fixes" the tissues and interferes with staining techniques where the antibody can no longer bind the desired target. Therefore, a significant amount of 12wk macrophage aortic root analysis could not be performed. However, the group of mice on 4wk HFD was not all fixed with 4% PFA cardiac perfusion and so could be stained and quantified.

Variability within the group, especially in an animal model, is inevitable. Despite all the mice being from the same gene pool, each mouse in this study ingests a different amount of food, metabolizes it differently, have different energy levels and expenditures. This translated into different disease rate progression and/or differences in lesion sizes. These differences were observed during analysis where some mice displayed lesion sizes very differently from the other mice in the study group.

6.9 Study Limitations

The current study used *cIAP2*$^{-/-}$ x *apoE*$^{-/-}$ male mice to study the effect of cIAP2 on atherosclerotic lesion development. Only male mice were used because several studies have reported a wide variation in lesion size in female mice attributed mainly to fluctuating estrogen levels believe to play a protective role in atherosclerosis (Nakagami F. *et al.,* 2010; reviewed in Thomas CM and Smart EJ., 2007). However, it would be helpful to examine how female mice would react to cIAP2 deletion.

In order to fully understand the mechanism of action of cIAP2, *in vitro* data using peritoneal macrophages derived from the different study groups could be performed. Caspase activity assays (in different macrophage cells (4wk/12wk KO and WT)) would help understand to what extend cIAP2 affects the effector caspases-3 and/or -7.

Measuring apoptosis in the plaque could certainly have been a relevant component of the analysis considering that we were examining the role of an inhibitor of apoptosis protein and its effect on lesion progression. Accordingly, we have started staining using fluorescent TUNEL assays on aortic root sections. The aim was to asses both CD68 staining along with TUNEL staining using dual fluorescent staining methods. This could enable us to quantify the apoptotic macrophages in the lesions. The selected aortic root sections were 10μm distant from the sections previously stained for lipids using Sudan IV. However, this fluorescent TUNEL (as well as CD68 fluorescent staining) assay was problematic as the fluorescent stains decayed very quickly and restricted us from quantifying the data properly. Furthermore, the rate of apoptosis in the study sections did not seem to be well-reflected by this staining method. However, using these methods of apoptosis detection in the early atherosclerotic lesions does not give us a clear representation of how much of the apoptotic process is taking place. Clarke and colleagues argue that the cleaved capspase-3 antigen could be detected for a short period of time which the cell is actually undergoing apoptosis, and so the frequency of the actual apoptotic rate could be hugely under-estimated (Clarke CH. and Bennet MR. CH., 2008). Likewise, having TUNEL-positive bodies after the apoptotic process has finished may largely exaggerate the frequency (as may have been the case in the 12wk HFD mouse group). Notably, these techniques look into measuring the frequency of apoptosis and not the rate, since rate cannot be measured in this setting (Clarke CH. and Bennet MR.

CH., 2008). No one to date has figured out what is the duration of the apoptotic process *in vivo*, and the majority of studies have examined less than two time points during apoptosis and thus can't calculate these rates (Clarke CH. and Bennet MR. CH., 2008). To add to the complexity of things we also don't know, at least *in vivo,* how long it takes for apoptotic bodies to clear. There are previous studies that claim to have identified "delayed clearance" but, again, they have only looked at one time point. And because there are multiple planes of view, it is almost impossible to try to decipher whether an apoptotic body is located inside or outside a cell. However, TUNEL staining can sometimes yield false-positive results as some of the forms of apoptosis follow a caspase-independent route. Other techniques are available to examine apoptotic cells *in vivo* and make use of staining methods using labeled annexin V which binds to externalized phosphatidylserine along with finding discrete morphological features of apoptotic cells, such as the condensation of nuclei.

CHAPTER 7: Conclusion and future directions

In summary, we show for the first time, using an atherosclerotic *apoE*$^{-/-}$ mouse model, that cIAP2 deletion reduces atherosclerotic lesion development at an early and later disease stage. We also show trends for reduction of macrophage infiltration in the early disease stage in the KO mouse model (*cIAP2* $^{-/-}$ x *apoE* $^{-/-}$) compared to WT mice consistent with a role for cIAP2 in macrophage longevity. This study presents new evidence for the role of cIAP2 protein in atherosclerosis progression and outlines its possible mechanism of action.

Using a total body KO model for cIAP2 as well as a WT model, aided us in identifying the role which cIAP2 plays in the progression of atherosclerotic disease. This was done using standard atherosclerosis quantification techniques such as *en face* and aortic root

lesion analysis techniques. Measuring total serum cholesterol and lipoprotein profiling was employed to eliminate this risk factor as a cause for differences in disease progression. Analysis indicates that cIAP2 deletion was linked with reduced lesion size in both early and late animal models of the disease when compared to the wild type littermates. cIAP2 is hypothesized to inhibit macrophage apoptosis in the atherosclerotic plaque. This was supported by quantifying the extent of active macrophage infiltration in the atherosclerotic plaque by using CD68 antibody specific to active macrophages. Analysis using mice on a 4wk HFD indicated reduced macrophage infiltration in the KO model, which goes hand in hand with reduced atherosclerotic lesions. In conclusion, cIAP2 deletion helps protect mice from developing atherosclerotic lesions. The data suggest that this occurs by reducing the extent of macrophage infiltration in the plaque.

Future directions

An issue in the literature in this field (as well as in the current project) is determination of cause and effect rather than relationship. Because apoptosis is a multifactor process, a study examining and manipulating a single cell's apoptotic pathway or mechanism can safely assume a direct consequence of that cell type. It becomes very complicated to try to interpret results from a study examining, for example, changes in the composition of the plaque or apoptosis using different drugs and/or genetic manipulations. These modifications or stimuli affect so many different cell lines and have multiple effects on many processes other than apoptosis.

Future studies will need to explore the early apoptosis concept by using *cIAP2*$^{-/-}$ BMT experiments to detect the specific role of cIAP2 in macrophage of *apoE*$^{-/-}$ mice. This

experimental approach will ensure that only hematopoietic cells deficient in cIAP2 protein are responsible for the decrease in atherosclerosis. This will pin-point the reduction in atherosclerosis to macrophage cells alone as opposed to vascular smooth muscle cells or other cell types found in the atherosclerotic vessel milieu that may be affected by a total body KO of cIAP2. This BMT concept has been proposed to test in *apoE*$^{-/-}$ mice fed an atherosclerosis-promoting diet for a period known to induce early lesions in control mice (Whitman SC., 2004). The study should further explore the role of cIAP2 in both sexes, male and female.

If the hypotheses are supported, such findings would then indicate that cIAP2 may be a specific therapy target. Smac-mimetic drugs, specific cIAP2 antagonists, could delay or limit atherosclerosis at early stages. On the other hand, smac-mimetic may be detrimental to late complex atherosclerotic plaque. Future *in vitro* studies will also need to explore the specific interaction of cIAP2 protein with caspases in peritoneal derived macrophages. Despite evidence in this study, more experiments should be conducted in cIAP1 KO models to understand if it also plays a role in atherosclerosis. This is important since cIAP1 and 2 are very homologous and may share many pathways and mechanisms of action.

Admiration for the complicated cellular and molecular mechanisms of atherosclerosis and vascular inflammation makes way for intelligent approaches to disease characterizations through imaging techniques. The general objective for approaches using molecular imaging is to accelerate and better diagnostics, offer insights to improved understanding of the disease, and more importantly, guide and monitor therapeutic effects. As a future direction, the possibility to apply imaging to quantify changes over time in atherosclerotic lesion size will be explored. F-18-fluorodeoxyglugose (F^{18}-FDG) can be used as a glucose analogue that is taken up by active macrophages usually found in the atherosclerotic plaque (Rudd JH. *et al.,*

2008). Using this marker of monocyte metabolism and plaque inflammation, we aim to image these mice using positron imaging tomography (PET) as well as computed tomography (CT) in order to monitor disease progression and therapy affects in humans. Our laboratory shows promising preliminary results from mice on HFD showing FDG uptake in the atherosclerotic plaques.

Ultimately, the progression and/or regressions of the disease could be followed via such plaque imaging techniques. These imaging techniques will, for example, target different molecules that are characteristic of different disease stages and thereby act a as biomarker to monitor the effectiveness of the treatment. Various methods, including ultrasonography, CT, and MRI have been employed for this purpose. However, most of these structural imaging techniques have major limitations. SPECT and PET/CT combined functional and structural whole-body imaging modality hold the most potential for this purpose.

The data from the current project set the stage for future interventions to prevent inhibition of apoptosis in early atherosclerosis. They also set the platform for developing means to serially evaluate disease progression and response to therapy *in vivo*. In the future, these approaches and treatments may be translatable to humans. The data from this proposal help to identify a potential target for therapy that may reduce atherosclerotic burden in patients and thereby reduce long term adverse clinical outcomes

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Preliminary studies

Dr. Stewart Whitman's laboratory carried out preliminary experiments that acted as the foundations of this project. Studies used the technique of bone-marrow transplantation (BMT) as a way to examine whether hematopoietic-specific deficiency of two proteins, cIAP2 and caspase-3, would modulate lesion development in LDL receptor null (*LDLr* $^{-/-}$) mice. In these studies, *LDLr* $^{-/-}$ mice (C57BL/6) underwent lethal irradiation followed by the injection of marrow cells isolated from the femurs and tibias of wild type control mice (C57BL/6 strain) or experimental mice (also C57BL/6 strain) that were either *cIAP2*$^{-/-}$ or *caspase-3* $^{-/-}$. Following sufficient time to enable full marrow repopulation, the chimeric *LDLr* $^{-/-}$ mice were put on an atherogenic diet for 8 wks. When the dietary period ends, the study mice we culled and then underwent cholesterol analysis as well as atherosclerotic lesion analysis.

cIAP2 BMT study – hematopoietic-specific deficiency of cIAP2 lead to a major reduction in the size lesions of plaque in the ascending aorta of chimeric *LDLr* $^{-/-}$ mice compared to control wilt-type mice with no significant change in serum total cholesterol concentrations as a result of hematopoietic-specific deficiency of cIAP2. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining was utilized as a method to detect apoptosis in frozen sections. More apoptotic cells (+ve TUNEL staining) were detected in mice that were regenerated with cIAP2 $^{-/-}$ bone marrow.

Caspase-3 study – hematopoietic-specific deficiency of caspase-3 lead to a significant reduction in the size of the atherosclerotic size in the ascending aorta of chimeric *LDLr* $^{-/-}$

mice compared to mice that have hematopoietic cells expressing competent caspase-3. No significant change in serum total cholesterol was found as a result of this hematopoietic-specific deficiency of caspase-3.

The fact that cIAP2 deficiency has a significant effect on lesion development, despite the fact that there would have been a functional cIAP1 protein present in *cIAP2*$^{-/-}$ cells, indicates that a unique function can be attributed to cIAP2 during the promotion of atherosclerosis and therefore justifies the focus of this research project. The fact that casp-3 deficiency lead to decreased atherosclerosis suggests that cIAP2 exerts its effect in a casp-3-independent manner.

PCR of cIAP2 in study mice.

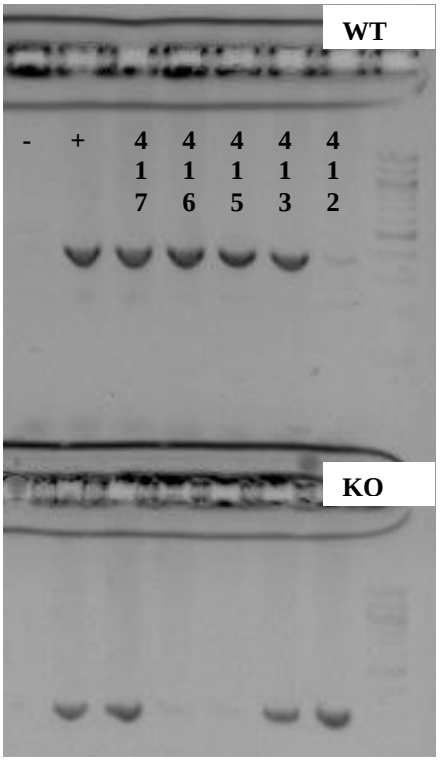

Fig 19. Representative PCR of cIAP2 in four different study mice; 413, 415, 416, 417. The cIAP2 KO mice was created via insertion of a lackZ gene. A band in the KO PCR indicates that the mouse allele is deficient in cIAP2. In order for a mouse to be a total KO (eg., 412), it would have one band in the KO and no band in the WT PCR. 415 and 416 are, on the other hand, WT mice.

References

Akishima Y, Akasaka Y, Ishikawa Y, Lijun Z, Kiguchi H, Ito K, Itabe H, Ishii T. (2005) Role of macrophage and smooth muscle cell apoptosis in association with oxidized low-density lipoprotein in the atherosclerotic development. Mod Pathol. Mar;18(3):365-7.

Arai S, Shelton JM, Chen M, Bradley MN, Castrillo A, Bookout AL, Mak PA, Edwards PA, Mangelsdorf DJ, Tontonoz P, Miyazaki T. (2005). A role for the apoptosis inhibitory factor AIM/Spalpha/Api6 in atherosclerosis development. Cell Metab. 2005 Mar;1(3):201-13.

Ashkenazi A, Dixit VM.(1998) Death receptors: signaling and modulation. Science. Aug 28;281(5381):1305-8.

Ashkenazi A.(2002) Targeting death and decoy receptors of the tumour-necrosis factor superfamily. Nat Rev Cancer. Jun;2(6):420-30.

Allayee H, Ghazalpour A, Lusis AJ. (2003) Using mice to dissect genetic factors in atherosclerosis. Arterioscler Thromb Vasc Biol 2003;23:1501-9.

Andrew S. Plump and Jan L. Breslow. (1995). Apolipoprotein E and the apolipoprotein E-deficient mouse. Annu. Rev. Nutr.. 15; 495-518.

Bertrand MJ, Milutinovic S, Dickson KM, Ho WC, Boudreault A, Durkin J, Gillard JW, Jaquith JB, Morris SJ, Barker PA. (2008) cIAP1 and cIAP2 facilitate cancer cell survival by functioning as E3 ligases that promote RIP1 ubiquitination. Mol Cell. 2008 Jun 20;30(6):689-700.

Bell FP, Gallus AS, Schwartz CJ (1974). Focal and regional patterns of uptake and the transmural distribution of I-fibrinogen in the pig aorta in vivo. Exp Mol Puthol 20, 281.

Blanc-Brude OP, Teissier E, Castier Y, Lesèche G, Bijnens AP, Daemen M, Staels B, Mallat Z, Tedgui A. (2007). IAP survivin regulates atherosclerotic macrophage survival. Arterioscler Thromb Vasc Biol. 2007 Apr;27(4):901-7.

Blankenship JW, Varfolomeev E, Goncharov T, Fedorova AV, Kirkpatrick DS, Izrael-Tomasevic A, Phu L, Arnott D, Aghajan M, Zobel K, Bazan JF, Fairbrother WJ, Deshayes K, Vucic D. (2009) Ubiquitin binding modulates IAP antagonist-stimulated proteasomal degradation of c-IAP1 and c-IAP2(1). Biochem J. Jan 1;417(1):149-60.

Boesten LS, Zadelaar AS, van Nieuwkoop A, Gijbels MJ, de Winther MP, Havekes LM, van Vlijmen BJ. (2005) Tumor necrosis factor-alpha promotes atherosclerotic lesion progression in APOE*3-Leiden transgenic mice. Cardiovasc Res. 2005 Apr 1;66(1):179-85.

Goldstein JL, Brown MS. (1979) The LDL receptor locus and the genetics of familial hypercholesterolemia. Annu Rev Genet. 1979;13:259-89.

Cheng EH, Levine B, Boise LH, Thompson CB, Hardwick JM. Nature. (1996). Bax-independent inhibition of apoptosis by Bcl-XL. 1996 Feb 8;379(6565):554-6.

Chen S-H, Habib G, Yang C-Y, Gu ZW, Lee BR, et al. (1987). Apolipoprotein B-48 is the product of a messanger RNA with an organ-specific in-frame stop codon. Science 238: 363-66.

Christopher K. Glass and Joseph L. Witztum. (2001). Atherosclerosis: the road ahead. Cell, Vol. 104, 503–516, February 23.

Chowdhury, I., Tharakan, B., Bhat, G.K., 2006. Current concepts in apoptosis: the physiological suicide program revisited. Cell Mol. Biol. Lett. 11, 506–525.

Chung JY, Park YC, Ye H, Wu H. (2002) All TRAFs are not created equal: common and distinct molecular mechanisms of TRAF-mediated signal transduction. J Cell Sci. 2002 Feb 15;115(Pt 4):679-88.

Clarke MC, Bennett MR. (2009). Cause or consequence: what does macrophage apoptosis do in atherosclerosis? Arterioscler Thromb Vasc Biol. 2009 Feb;29(2):153-5.

Conte D, Holcik M, Lefebvre CA, Lacasse E, Picketts DJ, Wright KE, Korneluk RG. (2006) Inhibitor of apoptosis protein cIAP2 is essential for lipopolysaccharide-induced macrophage survival. Mol Cell Biol. 2006 Jan;26(2):699-708.

Creagh EM, Martin SJ. (2001) Caspases: cellular demolition experts. Biochem Soc Trans. Nov;29(Pt 6):696-702.

Daugherty A, Lu H, Howatt DA, Rateri DL. (2009). Modes of defining atherosclerosis in mouse models: relative merits and evolving standards. Methods Mol Biol. 2009;573:1-15.

Daugherty A, Whitman SC. (2003). Quantification of atherosclerosis in mice. Methods Mol Biol. 2003;209:293-309.

Davies MJ, Gordon JL, Gearing AJ, Pigott R, Woolf N, Katz D, Kyriakopoulos A. (1993) The expression of the adhesion molecules ICAM-1, VCAM-1, PECAM, and E-selectin in human atherosclerosis. J Pathol. Nov;171(3):223-9

DePaola N, Gimbrone MA, Jr, Davies PF, Dewey CF Jr. (1992).Vascular endothelium responds to fluid shear stress gradients. Arterioscler Thromb Vasc Biol;12:1254-1257.

Deveraux QL, Reed JC. (1999) IAP family proteins--suppressors of apoptosis. Genes Dev. Feb 1;13(3):239-52.

Domagoj Vucic and Wayne J. Fairbrother (2007). The Inhibitor of Apoptosis Proteins as Therapeutic targets in cancer. Clin Cancer Res 2007;1 5995 3(20) October 15.

Du C., Fang M., Li Y., Li L, Wang X. (2000) Smac, a mitochondrial protein that promotes cytochrome c-dependent caspase activation by eliminating IAP inhibition. Cell. Jul 7;102(1):33-42.

Dubrez-Daloz L, Dupoux A, Cartier J. (2008). IAPs: more than just inhibitors of apoptosis proteins. Cell Cycle. 2008 Apr 15;7(8):1036-46.

Duckett CS, Nava VE, Gedrich RW, Clem RJ, Van Dongen JL, Gilfillan MC, Shiels H, Hardwick JM, Thompson CB. (1996) A conserved family of cellular genes related to the baculovirus iap gene and encoding apoptosis inhibitors. EMBO J. Jun 3;15(11):2685-94.

Eugene Varfolomeev, Tatiana Goncharov, Anna V. Fedorova, Jasmin N. Dynek, Kerry Zobel, Kurt Deshayes, Wayne J. Fairbrother, and Domagoj Vucic. (2008). c-IAP1 and c-IAP2 Are Critical Mediators of Tumor Necrosis Factor □□ (TNF□□)-induced NF-□□B Activation J Biol Chem. 2008 Sep 5;283(36):24295-9.

Fesik, S.W., 2000. Insights into programmed cell death through structural biology. Cell 103, 273–282.

Fowler S, Shio H, Haley NJ. (1979). Characterization of lipid-laden aortic cells from cholesterol-fed rabbits. IV. Investigation of macrophage-like properties of aortic cell populations.Lab Invest. Oct;41(4):372-8.

Franklin MC, Kadkhodayan S, Ackerly H, Alexandru D, Distefano MD, Elliott LO, Flygare JA, Mausisa G, Okawa DC, Ong D, Vucic D, Deshayes K, Fairbrother WJ. (2003) Structure and function analysis of peptide antagonists of melanoma inhibitor of apoptosis (ML-IAP). *Biochemistry.* Jul 15;42(27):8223-31.

Fuster V, Badimon L, Badimon JJ, Ip JH, Chesebro JH. (1991) The porcine model for the understanding of thrombogenesis and atherogenesis. *Mayo Clin Proc.* Aug;66(8):818-31.

Galis ZS, Sukhova GK, Lark MW, Libby P. (1994) Increased expression of matrix metalloproteinases and matrix degrading activity in vulnerable regions of human atherosclerotic plaques. J Clin Invest. Dec;94(6):2493-503.

Geisbrecht ER, Montell DJ. (2000) A role for Drosophila IAP1-mediated caspase inhibition in Rac-dependent cell migration. Cell 2004; 118:111-25. Getz GS.(2000) When is atherosclerosis not atherosclerosis? Arterioscler Thromb Vasc Biol. Jun;20(6):1694.

Gimbrone MA Jr. (1999). Endothelial dysfunction, hemodynamic forces, and atherosclerosis. Thromb Haemost. 1999 Aug;82(2):722-6.

Goldstein JC, Waterhouse NJ, Juin P, Evan GI, Green DR (2000). The coordinate release of cytochrome c during apoptosis is rapid, complete and kinetically invariant. Nat Cell Biol 2(3):156–162.

Goldstein JL, Ho YK, Basu SK, Brown MS. (1979). Binding sites on macrophages that mediate uptake and degradation of acetylated low density lipoprotein, producing massive cholesterol deposition. Proc. Natl Acad. Sci. USA;76:333–337.

Golpon HA, Fadok VA, Taraseviciene-Stewart L, Scerbavicius R, Sauer C, Welte T, Henson PM, Voelkel NF. (2004). Life after corpse engulfment: phagocytosis of apoptotic cells leads to VEGF secretion and cell growth. FASEB J;18:1716–1718.

Gyrd-Hansen M, Meier P. (2010). IAPs: from caspase inhibitors to modulators of NF-kappaB, inflammation and cancer. Nat Rev Cancer. 2010 Aug;10(8):561-74.

Hakamata H, Sakaguchi H, Zhang C, Sakashita N, Suzuki H, Miyazaki A, *et al*. (1998) The very low- and intermediate-density lipoprotein fraction isolated from apolipoprotein E-knockout mice transforms macrophages to foam cells through an apolipoprotein E independent pathway. Biochemistry 1998; 37: 13720-7.

Hegyi L, Skepper JN, Cary NR, Mitchinson MJ. (1996) Foam cell apoptosis and the development of the lipid core of human atherosclerosis. J Pathol. Dec;180(4):423-9.

Huang, M. Eberstadt, E.T. Olejniczak, R.P. Meadows and S.W. Fesik, (1996). NMR structure and mutagenesis of the Fas (APO-1/CD95) death domain. Nature, 384, pp. 638–641.

J. Jawien, P. Nastalek, R. Korbut (2004). Mouse Models of Experimental Atherosclerosis Journal Of Physiology and Pharmacology, 55, 3, 503–517.

Kasof GM, Gomes BC. (2001) Livin, a novel inhibitor of apoptosis protein family member. J Biol Chem. 2001 Feb 2;276(5):3238-46.

Kita T, Ishii K, Yokode M, Kume N, Nagano Y, Arai H, Kawai C (1990). The role of oxidized low density lipoprotein in the pathogenesis of atherosclerosis..Eur Heart J. 1990 Aug;11 Suppl E:122-7.

Kischkel FC, Hellbardt S, Behrmann I et al (1995) Cytotoxicity- dependent APO-1 (Fas/CD95)-associated proteins form a death- inducing signaling complex (DISC) with the receptor. Embo J 14(22):5579–5588.

Kumar A, Thota V, Dee L, Olson J, Uretz E, Parrillo JE. (1996) Tumor necrosis factor alpha and interleukin 1beta are responsible for in vitro myocardial cell depression induced by human septic shock serum. J Exp Med.

Mar 1; 183(3):949-58.

Li P. Nijhawan D, Budihardjo I et al (1997) Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initi- ates an apoptotic protease cascade. Cell 91(4):479–489

Libby P, Sukhova G, Lee RT, Liao JK. (1997) Molecular biology of atherosclerosis. *Int J Cardiol.* 1997 Dec 31;62 Suppl 2:S23-9.

Liston P, Fong WG, Korneluk RG. (2003). The inhibitors of apoptosis: there is more to life than Bcl2. Oncogene. 2003 Nov 24;22(53):8568-80.

Liu JJ, Huang RW, Lin DJ, Peng J, Wu XY, Lin Q, Pan XL, Song YQ, Zhang MH, Hou M, Chen F. (2005) Expression of survivin and bax/bcl-2 in peroxisome proliferator activated receptor-gamma ligands induces apoptosis on human myeloid leukemia cells in vitro. Ann Oncol. Mar;16(3):455-9.

Lotocki G, Keane RW. (2002). Inhibitors of apoptosis proteins in injury and disease. UBMB Life. 2002 Nov;54(5):231-40.

Lorick KL, Jensen JP, Fang S, Ong AM, Hatakeyama S, Weissman AM. (1999) RING fingers mediate ubiquitin-conjugating enzyme (E2)-dependent ubiquitination. Proc Natl Acad Sci U S A. Sep 28;96(20):11364-9.

Lucas AD, Greaves DR. (2001) Atherosclerosis: role of chemokines and macrophages. *Expert Rev Mol Med.* Nov 5;3(25):1-18.

Lusis AJ.(2000) Atherosclerosis. *Nature*. 2000 Sep 14;407(6801):233-41. Review.

Lutgens E, Daemen M, Kockx M, Doevendans P, Hofker M, Havekes L, Wellens H, de Muinck ED. (1999) Atherosclerosis in APOE*3-Leiden transgenic mice: from proliferative to atheromatous stage. Circulation. Jan 19;99(2):276-83.

Mace PD, Smits C, Vaux DL, Silke J, Day CL. (2010). Asymmetric recruitment of cIAPs by TRAF2. Asymmetric recruitment of cIAPs by TRAF2.

Mahoney DJ, Cheung HH, Mrad RL, Plenchette S, Simard C, Enwere E, Arora V, Mak TW, Lacasse EC, Waring J, Korneluk RG. (2008). Both cIAP1 and cIAP2 regulate TNFalpha-mediated NF-kappaB activation. Proc Natl Acad Sci U S A. 2008 Aug 19;105(33):11778-83.

Mantovani A, Bussolino AF, Dejana E. (1992). Cytokine regulation of endothelial cell function. FASEB J;6:2591-2599.

Myant N. (1990). Cholesterol Metabolism, LDL, and the LDL Receptor. Sandiego: Academic

Merched AJ, Williams E, Chan L. (2003) Macrophage-specific p53 expression plays a crucial role in atherosclerosis development and plaque remodeling. Arterioscler Thromb Vasc Biol. 2003 Sep 1;23(9):1608-14.

Micheau O, Tschopp J. (2003) Induction of TNF receptor I-mediated apoptosis via two sequential signaling complexes. Cell. 2003 Jul 25;114(2):181-90.

Movassagh M, Foo RS. (2008). Simplified apoptotic cascades. Heart Fail Rev. 2008 Jun;13(2):111-9.

Nakagami F, Nakagami H, Osako MK, Iwabayashi M, Taniyama Y, Doi T, Shimizu H, Shimamura M, Rakugi H, Morishita R. (2010) Estrogen attenuates vascular remodeling in Lp(a) transgenic mice. Atherosclerosis. Jul;211(1):41-7.

Nakashima Y, Plump AS, Raines EW, Breslow JL, Ross R. (1994). ApoE - deficient mice develop lesions of all phases of atherosclerosis throughout the arterial tree. Arterioscler Thromb; 14: 133-140.

Nachmias B, Ashhab Y, Ben-Yehuda D. The inhibitor of apoptosis protein family (IAPs): an emerging therapeutic target in cancer. Semin Cancer Biol. 2004 Aug;14(4):231-43.

Olayioye MA, Kaufmann H, Pakusch M, Vaux DL, Lindeman GJ, Visvader JE. XIAPdeficiency leads to delayed lobuloalveolar development in the mammary gland. Cell Death Differ 2005; 12:87-90.

Paigen B, Ishida BY, Verstuyft J, Winters RB, Albee D. (1990) Atherosclerosis susceptibility differences among progenitors of recombinant inbred strains of mice. Arteriosclerosis. Mar-Apr;10(2):316-23.

Petersen SL, Wang L, Yalcin-Chin A, Li L, Peyton M, Minna J, Harran P, Wang X. (2007) Autocrine TNFalpha signaling renders human cancer cells susceptible to Smac-mimetic-induced apoptosis. Cancer Cell. 2007 Nov;12(5):445-56.

Plump AS, Breslow JL. (1995) Apolipoprotein E and the apolipoprotein E-deficient mouse. Annu Rev Nutr. 15:495-518.

Potts MB, Vaughn AE, McDonough H, Patterson C, Deshmukh M. Reduced Apaf-1 levels in cardiomyocytes engage strict regulation of apoptosis by endogenous XIAP. J Cell Biol 2005; 171:925-30.

Rall SC Jr, Newhouse YM, Clarke HR, Weisgraber KH, McCarthy BJ, Mahley RW, Bersot TP. (1989) Type III hyperlipoproteinemia associated with apolipoprotein E phenotype E3/3. Structure and genetics of an apolipoprotein E3 variant. J Clin Invest. 1989 Apr;83(4):1095-101.

Rothe M, Pan MG, Henzel WJ, Ayres TM, Goeddel DV. (1995) The TNFR2-TRAF signaling complex contains two novel proteins related to baculoviral inhibitor of apoptosis proteins. Cell. Dec 29;83(7):1243-52.

Ross R. (1999) Atherosclerosis - an inflammatory disease. N Engl J Med. 1999 Jan 14;340(2):115-26.

Rubin EM. (1994) Studies of lipoprotein (a) and high density lipoproteins in transgenic mice. Atherosclerosis. Oct;110 Suppl:S77-81.

Rudd JH, Myers KS, Bansilal S, Machac J, Pinto CA, Tong C, Rafique A, Hargeaves R, Farkouh M, Fuster V, Fayad ZA.(2008) Atherosclerosis inflammation imaging with 18F-FDG PET: carotid, iliac, and femoral uptake reproducibility, quantification methods, and recommendations. J Nucl Med. 2008 Jun;49(6):871-8.

Samuel T, Welsh K, Lober T, Togo SH, Zapata JM, Reed JC. (2006) Distinct BIR domains of cIAP1 mediate binding to and ubiquitination of tumor necrosis factor receptor-associated factor 2 and second mitochondrial activator of caspases. J Biol Chem. Jan 13;281(2):1080-90.

Salvesen GS, Duckett CS. (2002) IAP proteins: blocking the road to death's door. Nat Rev Mol Cell Biol. Jun;3(6):401-10.

Savill J, Fadok V. (2000) Corpse clearance defines the meaning of cell death. Nature. Oct 12;407(6805):784-8. Review

Silke J, Brink R. (2010) Regulation of TNFRSF and innate immune signalling complexes by TRAFs and cIAPs. Cell Death Differ. 2010 Jan;17(1):35-45.

Singhal A. (2009) The early origins of atherosclerosis. *Adv Exp Med Biol.* 2009;646:51-8.

Srinivasula SM, Ashwell JD. (2008) IAPs: what's in a name? Mol Cell. Apr 25;30(2):123-35.

Schrijvers DM, De Meyer GR, Kockx MM, Herman AG, Martinet W. (2005) Phagocytosis of apoptotic cells by macrophages is impaired in atherosclerosis. Arterioscler Thromb Vasc Biol. Jun;25(6):1256-61

Shi Y. (2004). Caspase activation: revisiting the induced proximity model. Cell. 2004 Jun 25;117(7):855-8.

Stary HC, Chandler AB, Glagov S, Guyton JR, Insull W Jr, Rosenfeld ME, Schaffer SA, Schwartz CJ, Wagner WD, Wissler RW. (1994) A definition of initial, fatty streak, and intermediate lesions of atherosclerosis. A report from the Committee on Vascular Lesions of the Council on Arteriosclerosis, American Heart Association. Circulation. 1994 May;89(5):2462-78.

Stary HC. (1990) The sequence of cell and matrix changes in atherosclerotic lesions of coronary arteries in the first forty years of life. Eur Heart J. Aug;11 Suppl E:3-19.

Stary HC. (1992). Composition and classification of human atherosclerotic lesions. Virchows Arch A Pathol Anat Histopathol 1992;421:277-90.

Stastny J, Fosslien E, Robertson AL Jr. (1986) Human aortic intima protein composition during initial stages of atherogenesis. Atherosclerosis. May;60(2):131-9. Erratum in: Atherosclerosis 1986 Dec;62(3):277.

Stoneman VE, Bennett MR. (2004). The Role of apoptosis in atherosclerosis and its therapeutic implications. Clin Sci (Lond). Oct;107(4):343-54.

Tabas I. (2005) Consequences and therapeutic implications of macrophage apoptosis in atherosclerosis: the importance of lesion stage and phagocytic efficiency. Arterioscler Thromb Vasc Biol. Nov;25(11):2255-64.

Tiwari RL, Singh V, Barthwal MK. (2008) Macrophages: an elusive yet emerging therapeutic target of atherosclerosis. *Med Res Rev*. Jul;28(4):483-544.

Thomas CM, Smart EJ. (2007) Gender as a regulator of atherosclerosis in murine models. Curr Drug Targets. Nov;8(11):1172-80.

Uren AG, Pakusch M, Hawkins CJ, Puls KL, Vaux DL. (1996) Cloning and expression of apoptosis inhibitory protein homologs that function to inhibit apoptosis and/or bind tumor necrosis factor receptor-associated factors. Proc Natl Acad Sci U S A. 1996 May 14;93(10):4974-8.

Vainio S, Ikonen E. (2003) Macrophage cholesterol transport: a critical player in foam cell formation. Ann Med. 35(3):146-55.

Vallabhapurapu, S. et al. (2008) Nonredundant and complementary functions of TRAF2 and TRAF3 in a ubiquitination cascade that activates NIK-dependent alternative NF-kB signaling. Nat. Immunol. 9, 1364–1370.

Vandivier RW, Fadok VA, Ogden CA, Hoffmann PR, Brain JD, Accurso FJ, Fisher JH, Greene KE, Henson PM. (2002). Impaired clearance of apoptotic cells from cystic fibrosis airways. Chest. 2002 Mar;121(3 Suppl):89S.

van Vlijmen BJ, Gerritsen G, Franken AL, Boesten LS, Kockx MM, Gijbels MJ, Vierboom MP, van Eck M, van De Water B, van Berkel TJ, Havekes LM. (2001) Macrophage p53 deficiency leads to enhanced atherosclerosis in APOE*3-Leiden transgenic mice. Circ Res. Apr 27;88(8):780-6.f

Varfolomeev E, Wayson SM, Dixit VM, Fairbrother WJ, Vucic D.(2006). The inhibitor of apoptosis protein fusion c-IAP2.MALT1 stimulates NF-kappaB activation independently of TRAF1 AND TRAF2. J Biol Chem. 2006 Sep 29;281(39):29022-9.

Verhagen AM, Ekert PG, Pakusch M, Silke J, Connolly LM, Reid GE, Moritz RL, Simpson RJ, Vaux DL. (2000) Identification of DIABLO, a mammalian protein that promotes apoptosis by binding to and antagonizing IAP proteins. Cell. 2000 Jul 7;102(1):43-53.

Vince JE, Pantaki D, Feltham R, Mace PD, Cordier SM, Schmukle AC, Davidson AJ, Callus BA, Wong WW, Gentle IE, Carter H, Lee EF, Walczak H, Day CL, Vaux DL, Silke J. (2009) TRAF2 must bind to cellular inhibitors of apoptosis for tumor necrosis factor (tnf) to efficiently activate nf-{kappa}b and to prevent tnf-induced apoptosis. J Biol Chem. 2009 Dec 18;284(51):35906-15.

Weinbaum S, Chien S. (1993) Lipid transport aspects of atherogenesis. J Biomech Eng. Nov;115(4B):602-10.

Whitman SC (2004). A practical approach to using mice in atherosclerosis research. *Clin Biochem Rev* 25: 81-93.

Williams KJ, Tabas I. (1995) The response-to-retention hypothesis of early atherogenesis. *Arterioscler Thromb Vasc Biol*. 1995 May;15(5):551-61.

Wilkinson KD. (1987) Protein ubiquitination: a regulatory post-translational modification. Anticancer Drug Des. 1987 Oct;2(2):211-29.

Wissler RW. (1992). Theories and new horizons in the pathogenesis of atherosclerosis and the mechanisms of clinical effects. Arch Pathol Lab Med. 1992 Dec; 116 (12):1281-91.

Wu CJ, Conze DB, Li X, Ying SX, Hanover JA, Ashwell JD. (2005) TNF-alpha induced c-IAP1/TRAF2 complex translocation to a Ubc6-containing compartment and TRAF2 ubiquitination. EMBO J. May 18;24(10):1886-98.

Wu H, Tschopp J, Lin SC. (2007) Smac mimetics and TNFalpha: a dangerous liaison? Cell. 2007 Nov 16;131(4):655-8.

Wu G, Chai J, Suber TL, Wu JW, Du C, Wang X, Shi Y. (2000) Structural basis of IAP recognition by Smac/DIABLO. *Nature*. Dec 21-28;408(6815):1008-12.

Yu X, Dluz S, Graves DT, Zhang L, Antoniades HN, Hollander W, Prusty S, Valente AJ, Schwartz CJ, Sonenshein GE. (1992). Elevated expression of monocyte chemoattractant protein 1 by vascular smooth muscle cells in hypercholesterolemic primates. Proc Natl Acad Sci U S A. Aug 1;89(15):6953-7

Zarnegar, B. et al. (2008) Control of canonical NF-kB activation through the NIK-IKK complex pathway. Proc. Natl. Acad. Sci. U. S. A. 105, 3503–3508.

Zhong S, Goldberg IJ, Bruce C, Rubin E, Breslow JL, Tall A. (1994) Human ApoA-II inhibits the hydrolysis of HDL triglyceride and the decrease of HDL size induced by hypertriglyceridemia and cholesteryl ester transfer protein in transgenic mice. J Clin Invest. Dec;94(6):2457-67.

Zhang SH, Reddick RL, Piedrahita JA, Maeda N. (1992) Spontaneous hypercholesterolemia and arterial lesions in mice lacking apolipoprotein E. Science. Oct 16;258(5081):468-71.

Zhou X, Hansson GK. (1999) Detection of B cells and proinflammatory cytokines in atherosclerotic plaques of hypercholesterolaemic apolipoprotein E knockout mice. Scand J Immunol. Jul;50(1):25.