The Role of Scavenger Receptor-A in Heat Shock Protein 27-mediated Atheroprotection: Mechanistic Insights into a Novel Anti-Atherogenic Therapy

Joshua Edward Raizman

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Department of Biochemistry, Microbiology, and Immunology
Faculty of Medicine
University of Ottawa

ABSTRACT

Heat shock protein (HSP)27 is traditionally described as an intracellular chaperone and signaling molecule, but growing evidence suggests it is released from immune cells where it plays an anti-inflammatory role during atherogenesis. Previously, the O'Brien lab found that overexpression of HSP27 led to augmented HSP27 serum levels in female apolipoprotein E knockout ($ApoE^{-/-}$) mice, attenuated atherogenesis, and inhibited macrophage foam cell formation via physical binding with scavenger receptor (SR)-A. However, the precise mechanism of atheroprotection remained elusive. This thesis sought to ascertain the mechanism(s) by which HSP27 prevents foam cell formation, and determine if SR-A, a key receptor involved in the uptake of lipid into macrophages, plays an important role in HSP27-mediated atheroprotection. Pre-treatment of human macrophages with recombinant HSP27 (rHSP27) inhibited acytelated low density lipoprotein (acLDL) binding and uptake independent from receptor competition effect. Reduction in uptake was associated with attenuation of expression of SR-A mRNA, total protein, and cell surface expression. To explore the signaling mechanism by which HSP27 modulated SR-A expression it was hypothesized that nuclear factor-kappa B (NF-κB), a major regulator of many atherosclerosis gene programs, is altered by extracellular HSP27. Indeed, rHSP27 markedly activated NF-κB signaling in macrophages. Using an inhibitor of NF-κB signaling there was an attenuation of rHSP27-induced inhibition of SR-A gene and protein expression, as well as lipid uptake, suggesting that SR-A expression is regulated by NF-κB activation. Lastly, to investigate if SR-A is required for HSP27-mediated atheroprotection *in vivo*, $ApoE^{-/-}$ and $ApoE^{-/-}SR\text{-}A^{-/-}$ mice fed a high fat diet were treated with rHSP25, the mouse orthologue of HSP27, or PBS for 3 weeks. While rHSP25 therapy equally reduced serum cholesterol

levels in the mouse cohorts, aortic atherogenesis, assessed using *en face* and sinus cross-sectional analyses, was attenuated in $ApoE^{-/-}$ mice but not $ApoE^{-/-}SR\text{-}A^{-/-}$ mice. In conclusion, rHSP27 inhibits foam cell formation by downregulating SR-A expression. This effect may be associated with NF-κB activation. Reductions in atherosclerotic burden by rHSP27 require SR-A, and are independent of changes in serum cholesterol levels, highlighting the importance of macrophage lipid uptake in atherogenesis. Results presented in this thesis demonstrate that SR-A is a major target for HSP27 atheroprotection in the vessel wall, and provide an impetus for further studies that investigate the potential therapeutic value of HSP27.

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In the modern scientific era major discoveries and generation of novel ideas are no longer concocted by the brilliance of a single investigator. Rather, creation of great science requires a cocktail of interdisciplinary ingredients. This team-work approach has pushed the solo scientist to the brink of extinction. The work created during the course of my PhD that culminated in this thesis is truly no exception and reflects the dedication, support, and efforts by my supervisor, thesis advisory committee members, and countless colleagues, amazing friends, and loving family. Like the pages that collate to create this thesis, so too are the individual people that comprise my team of supporters that made my PhD possible.

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LIST OF ABBREVIATIONS

ABC	adenosine triphosphate-binding cassette receptor
ACAT	acetyl-coenzyme A acetyl transferase
ACEH	acid cholesterol ester hydrolase
acLDL	acetylated low density lipoprotein
AGE	advanced glycation end product
ANOVA	analysis of variance
ApoB	apolipoprotein B
ApoE	apolipoprotein E
Arg	arginase
ATP	adenosine triphosphate
B.I.D	*bis in die* (twice daily)
BMDM	bone marrow derived macrophages
bp	base pairs
BSA	bovine serum albumin
CAD	coronary artery disease
CCL	chemokine (cc-motif) ligand
CD	cluster of differentiating factor
CE	cholesterol esterase
CHO	chinese hamster ovary cells
CMV	cytomegalovirus
EDTA	ethylenediaminetetraacetic acid
ER	endoplasmic reticulum

ERE	estrogen response element
ERK	extracellular signal-regulated kinase
ERβ	estrogen receptor beta
FBS	fetal bovine serum
GWAS	genome wide association study
HDL	high density lipoprotein
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
hMDM	human monocyte derived macrophages
HSE	heat shock element
HSF	heat shock factor
HSP27	heat shock protein-27
ICAM	intercellular adhesion molecule
IKK	Iκβ kinase
IFNγ	interferon gamma
IL	interleukin
iNOS	inducible nitric oxide synthase
JNF	jun N-terminal kinase
kDa	kilodalton
LAL	lysosomal acid lypase
LDL	low density lipoprotein
LDLr	low density lipoprotein receptor
LPS	lipopolysaccharide
LXR	liver x receptor

M1	classically activated macrophage state
M2	alternatively activate macrophage state
MAPK	mitogen-activated protein kinase
MAPKAP	mitogen-activated protein kinase activated protein
MARCO	macrophage receptor with collagenous structure
MCP	monocyte chemoattractant protein
M-CSF	macrophage colony stimulating factor
MI	myocardial infarction
MMP	matrix metalloproteinase
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NCEH	neutral cholesterol ester hydrolase
NF-κβ	nuclear factor kappa-light-chain-enhancer of activated B cells
Ni^{+}NTA	nickel-nitrilotriacetic acid
NO	nitric oxide
NPC	neimanm pick type C protein
PAK	p21 activated kinase
PBMC	peripheral blood mononuclear cells
PBS	phosophate buffer saline
PCR	polymerize chain reaction
PDGF	platelet derived growth factor
PE	phycoerythrin
PKC	protein kinase C
PMA	phorbol-12-myristate-13-acetate

PMB	polymixin B
PPAR	peroxisome proliferators-activator receptor
PVDF	polyvinylidene fluoride
qPCR	quantitative real time polymerize chain reaction
ROS	reactive oxygen species
S.c	subcutaneous
SDS-PAGE	sodium dodecyl sulfate polyacrylamide
SEAP	secreted embryonic alkaline phosphatase
SEM	standard error of the mean
SR-A	scavenger receptor-A
SRCC	scavenger receptor with C-type lectin type I/II
SREBP	sterol regulatory element-binding proteins
TGF-β	transforming growth factor beta
TIMPs	tissue inhibitor of matrix metalloproteinase
TNF-α	tumor necrosis factor alpha
UPR	unfolded protein response
VCAM	vascular cell adhesion molecule
VLDL	very low density lipoprotein

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1.0 INTRODUCTION

This chapter will provide background information relevant to the topics addressed in this thesis. The first section will begin with an introduction to atherosclerosis, or "hardening of the arteries", giving a general overview about its etiology, socio-economic impact and risk factors, as well as primary and secondary treatment strategies. The second section will describe in detail the mechanisms of atherosclerosis pathophysiology addressing different stages of lesion development and highlighting important concepts and hypotheses currently accepted in the literature. Given that the macrophage is the major cell type studied in this thesis, the next section will specifically address the current understanding of the role of macrophage activation and its relation to inflammation in the vessel wall. The subsequent section logically transitions into an overview describing mechanisms of lipid uptake to provide a link between the dynamic phenotypic changes of macrophages during atherogenesis and the formation of foam cells during this process. Although scavenger-receptor A (SR-A) is a regulator of foam cell formation and a focus in this study, controversies outlined in the literatures are discussed in this section as well as some concluding remarks about the current understanding of SR-A as a therapeutic target for atherosclerosis. The subsequent section is designed to introduce the reader to heat shock protein 27 (HSP27), the central theme and research pursuit of the O'Brien laboratory. A brief introduction to highlight major discoveries in the field of molecular chaperone function will be followed by a discourse describing both the intracellular and extracellular function of HSP27 and its importance in atherogenesis. Finally, this chapter will conclude with a background describing seminal HSP27 discoveries in the O'Brien lab related to macrophage biology and protection against atherosclerosis. Readers will be provided with a current state

of knowledge at the initiation of experimental work to illustrate gaps in the understanding of HSP27 atheroprotection, and objectives to support appropriate hypothesis generation.

1.1 An introduction to atherosclerosis

Atherosclerosis is a common disease process that is characterized by an inflammatory response in the vessel wall. Its initiation is thought to occur primarily due to localized damage to the endothelium followed by the accumulation of cholesterol deposits in the subendothelial space, which lies between the endothelium and intimal elastic membrane of the vessel. Over time, these initial steps in lesion formation may progress and lead to the formation of an advanced plaque that consists of lipid, fibrous material, calcification and infiltration of a number of cell types such as smooth muscle cells (SMC), macrophages, lymphocytes, and T-cells. Plaque growth occurs as accumulation of low density lipoproteins (LDL) and infiltration of blood-borne leukocytes counterbalances anti-atherogenic processes such as removal of cholesterol via high density lipoprotein (HDL), and egression of macrophages from the arterial wall. Although clinically relevant manifestation of atherosclerosis typically presents in the fifth or sixth decade of life, there are certainly exceptions, with some patients showing premature evidence of the disease – typically in the context of a strong family history of vascular disease. Atherosclerosis may be evident in autopsy specimens of young adults, with plaques or fatty streaks often observed well before clinical events occur (1). Despite the presence of the disease, it is currently held that clinical manifestations of atherosclerosis in the heart or cerebral circulation may be absent until plaque rupture occurs - an event characterized by the sudden rupture into the side-wall of an artery, typically at the site of modest narrowing of the vessel lumen - resulting in vessel occlusion due to thrombus formation.

1.1.1 Risk factors, causes, and clinical outcomes

Atherosclerosis was once considered to be a disorder of cholesterol handling in the liver. It was originally hypothesized that cholesterol lowering drugs would be sufficient to prevent plaque build up in the arterial wall (2). Yet, the inability of cholesterol lowering drugs to completely abolish this disease provided evidence that atherosclerosis is a multi-faceted condition with an underlying pathology beyond inappropriate cholesterol storage. It is now accepted that atherosclerosis involves complex interactions between the systemic immune system and local inflammatory mediators. This immune-inflammatory axis is thought to interact with environmental and genetic risk factors to instigate, regulate, and propagate lesions in the vessel wall (2).

Hypercholesterolemia is one of the primary and strongest risk factors for the development of atherosclerosis. Based on results of the Framingham study and other clinical trials assessing the risk to benefit ratio of cholesterol lowering drugs, it was found that for every 10% reduction in serum cholesterol levels, coronary artery disease (CAD) mortality was reduced by 15% and total mortality by 11% (3). Whether these encouraging initial results remain valid in the current age of intense lipid lowering with “statin” drugs and other complementary medical treatments is unclear, as certainly we continue to see examples of refractory atherosclerotic disease states despite remarkably low cholesterol levels. Increased ratio of LDL (“bad” cholesterol) to HDL (“good” cholesterol) is also known as a risk factor. Whereas tobacco smoke, sedentary life style, hypertension, and diabetes are considered modifiable risk factors (4), non-modifiable factors that play a role are sex, age (i.e., males), family history, and genetic abnormalities like familial hypercholesteremia. Other risk factors thought to be associated with atherosclerosis are obesity, psychosocial stress (e.g. cortical

levels), diet low in fresh fruits and vegetables, and sedentary lifestyle (4).Currently, there is increasing interest in determining the role of genetics in cardiovascular risk, with some studies arguing that genetics alone accounts for more than half of the disease etiology (5). Recent advances in gene profiling technology have made it possible to efficiently perform genome-wide association studies (GWAS) to identify specific regions of genes or loci associated with disease (5). For example, a locus located at chromosome 9p21 was found to have a significant association with CAD, independent of common risk factors (6,7). Interestingly, ancient Egyptian mummies were found to have evidence of atherosclerosis, suggesting that atherosclerosis is not necessarily a disease exclusively linked to risk factors typically associated with modern society (8).

Many cardiovascular disorders are caused by atherosclerosis including peripheral vascular disease (e.g., aortic aneurysms, cerebrovascular disease) and CAD. Plaque development during atherogenesis is categorized into three developmental stages (discussed in detail below): fatty streak formation, intermediate lesions, and advanced lesions. As the plaque matures over time, it can be further categorized into two broad subclasses: stable or unstable. Plaque type is clinically relevant because in the stable form it tends to be asymptomatic due to build up of supporting extracellular matrix and cellular components such as smooth muscle cells, in turn, helping to resist the plaque's vulnerability to rupture. However, unstable or vulnerable plaques which are characterized by accumulation of foam cells as well as cellular debris and dying cells, develop an outer layer or fibrous cap that separates its contents from the arterial lumen. Unstable plaques tend to be weak, rendering them prone to rupture leading to intraluminal thrombi that can themselves block arterial blood supply. In addition to plaque rupture, physical narrowing of the arterial lumen due to

stenosis of the expanding plaque can restrict the blood supply that provides vital oxygen and nutrients to the myocardium. Chronic ischemia to the mycoardium manifests as angina pain.

1.1.2 Socioeconomic impact on Canadian society

Although rates of heart disease have generally declined over the last four decades due to improvements in diagnostics, treatment, and management, cardiovascular disease is still the leading causes of mortality and morbidity in Canada. According to data from Statistics Canada (found at http://www.heartandstroke.com/), in 2008, 29% of all deaths in Canada were attributed to cardiovascular and its related diseases with 54, 20, and 23% as a result of ischemic heart disease, heart attack, and stroke, respectively (9). As a reflection of its prevalence, it was reported by the Public Health Agency of Canada in 2007 that 1.3 million Canadians (4.8% of the population) were living with the disease (10). Cardiovascular disease continues to impart a great burden on the Canadian economy. A report published by the Conference Board of Canada estimated that cardiovascular disease costs Canadian tax payers more than $20.9 billion annually based on hospitalization services, lost wages, and decreased productivity (11). Moreover, hospital admission rates due to complications arising from atherosclerosis are steadily rising, accounting for 19.9% of total hospitalizations annually – the highest of all diseases in Canada (10). As the life expectancy in Canada continues to rise in the future, finding novel preventative strategies in addition to acute treatment strategies is an important consideration from both a public health and economic standpoint.

1.1.3 Treatment strategies

Strategies to manage atherosclerosis are based on primary and secondary prevention as well as acute care. The Bogalusa Heart study revealed that the severity and number of atherosclerotic-related complications, as assessed by the size of lesions present in post-mortem arterial specimens, positively correlated with the number of risk factors present in children and young adults (12-14). Since this landmark study, primary prevention strategies have shown beneficial effects in reducing the risk of developing clinically relevant atherosclerotic events later in life by focusing on limiting modifiable risk factors, e.g. increasing physical exercise, reducing LDL cholesterol and triglycerides through diet, and smoking cessation.

Secondary treatment strategies, on the other hand, are focused on reducing the risk of atherosclerotic events or myocardial infarctions (MI) when the disease is already established. HMG-coA reductase inhibitors or "statins" are the most commonly prescribed pharmacological therapy for the prevention of atherosclerosis-related events. The principal vascular protective effects of statins occur via lowering serum cholesterol levels by inhibition of cholesterol synthesis in the liver. Although not fully understood, statins are also known to have a variety of off-target, pleiotropic properties such as antioxidant and anti-inflammatory effects, induction of endothelial nitric oxide, recruitment of endothelial progenitor cells, and plaque stabilization (15). The Scandinavian Simvastatin Survival Study, the first large clinical study to determine the efficacy of statins in prevention of atherosclerotic events, found a relative 30% reduction in death in a group of patients on simvastatin with established CAD (16). Other large randomized clinical studies such as the West of Scotland Coronary Prevention Study (WESTCOPS) trial (17), the Myocardial

Ischemia Reduction with Acute Cholesterol Lowering (MIRACL) trial (18), and the Pravastatin or Atorvastatin Evaluation and Infection Therapy (PROVE-IT) trial (19), have demonstrated favorable outcomes for the use of statins in reducing serum cholesterol levels and lessening the risk of heart attacks or death in patients with hypercholesteremia or CAD.

Despite the beneficial outcomes of these strategies, atherosclerosis continues to be the most common cause of death in the Western world. Strategies to manage hypercholesterolemia using statin treatment were initially anticipated to erase the disease by the end of the 20th century, yet the rising incidence of atherosclerotic risk factors like obesity and diabetes coupled to increasing rates of life expectancy will likely continue to increase the prevalence of cardiovascular disease well into the 21st century. In addition, small cohorts of patients are intolerant to statin therapy rending their use ineffective in the treatment of the disease. Thus, development of better and more targeted therapies, either as complementary to existing interventions, or as stand-alone therapeutics, is certainly warranted.

Currently, much effort to prevent atherogenesis or slow its progression is united by research to elucidate novel molecular pathways. The macrophage has emerged as an essential component of the atherogenesis process – from early lesion development to advanced plaque formation (reviewed below). Understanding macrophage biology relating to regulation of inflammation and cholesterol handling in the artery wall may provide important insight into development of these therapeutic targets (20).

1.2 Pathophysiology of Atherosclerosis

Atherosclerosis pathophysiology is multi-factorial involving physiological systems and processes that converge on various cell types. The development of animal models to study complex human diseases has elucidated important advances in knowledge about its

molecular and cellular pathways that can only be reproduced in a living system. Apolipoprotein E (ApoE) and LDL receptor (LDLr) transgenic mice are the most widely studied models in the atherosclerosis field. As ApoE and LDLr are involved in cholesterol transport, metabolism, and storage, their absence has been shown to initiate development of atherosclerotic lesions after a relatively short interval in rodents (early lesions at ~3-4 weeks and advanced disease by ~3 months), making murine models very efficient and cost effective. Importantly, vascular lesions in these mouse models share similarities to those observed in humans (21), and therefore are suited to study not only underlying biochemical and cellular interactions, but also provide fertile grounds for testing the development of novel therapeutic strategies. This section explores the underlying molecular and cellular pathways that participate in the progression of lesion development.

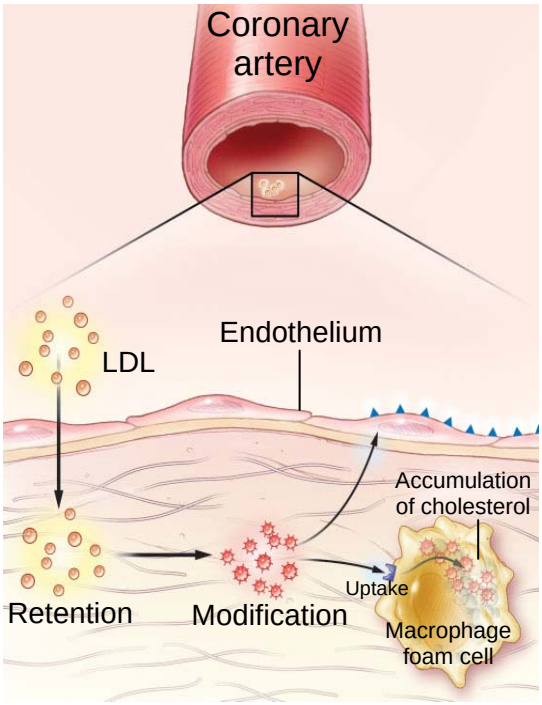
1.2.1 Early stages of lesion development: initiation of fatty streaks

The healthy arterial endothelium normally acts as a selective barrier that protects the vessel wall from potentially damaging factors present in the circulation. Elevated sheer stress, inflammation, and serum cholesterol are known to cause endothelial dysfunction. Endothelial dysfunction appears to preferentially occur in susceptible areas of the arterial tree subjected to high hemodynamic load. It is thought that patterns of turbulent blood flow create shear stress on the vessel wall resulting in activation of endothelial cells, in turn, leading to perturbations in gene expression, including the upregulation of surface adhesion molecules (22). Loss of endothelial integrity allows contents of the plasma such as LDL to gain access to the underlying subendothelial space of the vessel wall. The positively charged domain of apolipoprotein B (ApoB), the major protein constituent of LDL, is thought to promote molecular bonds with negatively charged moieties of the extracellular matrix

leading to LDL retention in the arterial intima (23). The *oxidative modification hypothesis* proposed by Steinberg and colleagues suggests that these trapped lipoprotein complexes become prone to chemical or physical modification through oxidation, glycoxylation, or aggregation rendering them pro-inflammatory and readily taken up by resident macrophages (24,25). In addition, a number of enzymes located within the vessel wall have been shown to alter LDL including secretory phospholipase A2 (26) and sphingomyelinase (27). Accumulation of modified LDL in the subendothelial space further damages the endothelium leading to initiation of a chemotactic gradient that attracts circulating monocytes and T-cells to the site of injury – the hallmark of the *response-to-retention theory* **(Figure 1)**.

Activation and recruitment of macrophages into the vessel wall involve multiple coordinated steps. Monocyte rolling and adherence are initially mediated by P-selectins expressed on the surface of activated endothelial cells (28-31). Next, capture and adhesion of monocytes to the endothelium occurs primarily through the interactions of αβ integrin subunits - expressed on the surface of activated monocytes - with fibronectin and other extracellular components (29,32,33). Integrins such as vascular-cell-adhesion molecule 1 (VCAM) and intercellular adhesion molecule-1 (ICAM-1) also interact with adhesion receptors expressed on endothelial cells (34-39). Once firmly adhered, monocytes

A)


Coronary artery
LDL
Endothelium
Accumulation of cholesterol
Uptake
Retention
Modification
Macrophage foam cell

B)

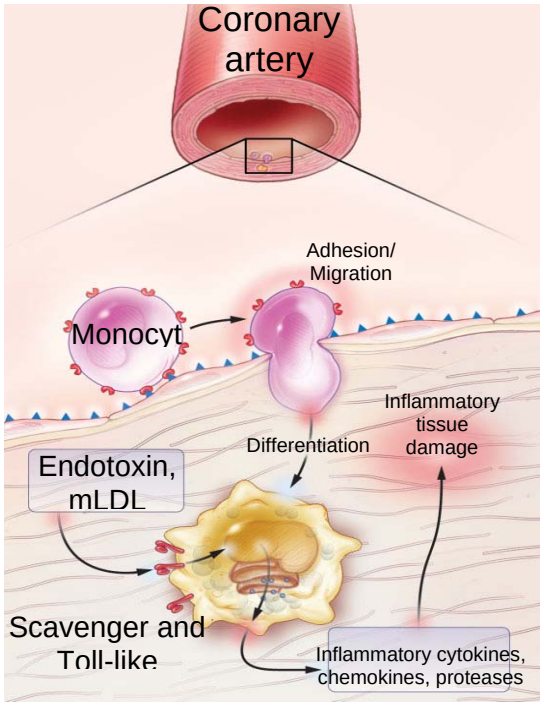

Coronary artery
Adhesion/ Migration
Monocyt
Inflammatory tissue damage
Differentiation
Endotoxin, mLDL
Scavenger and Toll-like
Inflammatory cytokines, chemokines, proteases

Figure 1: Infiltration of lipids into the vessel wall and activation of macrophages during early lesion development

(A) Lesion genesis occurs with the formation of a fatty streak. Low density lipoprotein (LDL) translocates into the subendothelial space of the arterial wall, particularly at sites of high hemodynamic strain. LDL becomes retained in the extracellular matrix through its association with proteoglycans leading to its modification by enzymes and oxygen radicals present in the extracellular milieu. This leads to formation of oxidized (ox)LDL. OxLDL and together with secreted pro-inflammatory factors secreted from resident macrophages, induces activation of endothelial cells to express adhesion molecules such as vascular cell-adhesion molecule 1 (VCAM1). Expression of pattern-recognition receptors increases, such as scavenger receptors (SR) and toll-like receptor (TLR). SRs mediate uptake of oxLDL particles, leading to intracellular cholesterol build up and formation of foam cells. Foam cells accumulate in the subendothelalial space where they contribute to the net pro-inflammatory response during atherogenesis.

(B) Monocytes and T cells are recruited to the damaged endothelium, bind to VCAM1 expressing endothelial cells and transmigrate into the vessel wall in response to locally produced chemokines. Monocytes then differentiate into macrophages by responding to local chemokine such as macrophage colony-stimulating factor (M-CSF). Pro-inflammatory factors in the subendothelial space activate scavenger receptors and toll-like receptors on these cells. While SRs mediate oxLDL uptake, TLRs bind to lipopolysaccharide (LPS), heat shock protein 60 (HSP60), oxLDL and other ligands inducing secretion of many pro-inflammatory cytokines, chemokines, oxygen and nitrogen radicals, other inflammatory molecules, which ultimately leads to inflammation and tissue damage.

Adapted from Hansson *et al*. (2) with minor modifications.

transmigrate across the endothelium into the subendothelial space in response to a local gradient of chemokines (33) **(Figure 1).**

During the initial stages of lesion formation the generation of a chemoattractant gradient plays an essential role in affecting the recruitment of monocytes to the vessel wall. Cytokines such as platelet derived growth factor (PDGF), tumor necrosis factor alpha (TNF-α), interleukin-1 (IL-1), interferon gamma (IFNγ) (40), monocyte chemoattractant protein (MCP)-1 (41-43) and chemokine (C-C motif) ligand (CCL)-5 (44) are all expressed in the plaque and known to be involved in chemotaxis of monocytes to the subendothelial space. The chemokine receptor, CX3CR1, plays a major role in lesion development by promoting monocyte arrest as demonstrated in $ApoE^{-/-}$ $Cx3CR1^{-/-}$ mice, which are protected against atherosclerosis via reduced accumulation of monocyte-derived macrophages in the lesion (45,46). In addition, various transgenic animal models deficient in MCP-1 (43,47-49) or macrophage colony stimulating factor (M-CSF) (50) exhibit atheroprotection that is associated with reduced macrophage content and foam cell formation.

Once in the subendothelial space, monocytes upregulate pattern-recognition receptors such as scavenger and toll like receptors as well as other markers consistent with mature macrophages (51,52). Macrophages act to phagocytose modified lipids that become trapped in the subendothelial space - an effort that is initially adaptive to maintain proper homeostasis to initiate a healing process in the vessel wall. Over time, the gross accumulation of trapped lipids overwhelms the ability of the macrophage to remove lipid from the inflammatory milieu – rendering macrophage conversion into foam cells. Named for the appearance of defined cytosolic lipid droplets in their cytoplasm, foam cells are lipid-laden macrophages that accumulate in the fatty streak. They are thought to generate an

inflammatory response by secreting various cytokines, reactive oxygen species (ROS), and proteases. An important concomitant process in the maintenance of vessel wall homeostasis is the clearance of dying macrophages by other macrophages or efferocytosis. Unfortunately, as part of the disease process, efferocytosis may also become defective; hence, an increase in macrophage number tips the local pro-inflammatory/anti-inflammatory balance in favor of a pro-inflammatory milieu (53,54). At this stage, the fatty streak is visible in the arterial wall.

1.2.2 Development of intermediate and advanced lesions

The architecture of the lesion undergoes structural remodeling as the normal vessel wall extracellular scaffold is replaced by a lipid core containing free cholesterol, cholesterol crystals, and calcium. As the plaque evolves, macrophages are joined by other cells such as T cells, dendritic cells, neutrophils, mast cells, and to a lesser extent smooth muscle cells. The formation of an atheroma, which is now a visible structure, is characteristic of the intermediate stage of atherogenesis. Hypersecretory smooth muscle cells migrate from the vessel media into the intima, proliferate and secrete extracellular matrix components (e.g. collagen, fibronectin, and elastin) in response to local growth factors such as PDGF (55,56) and TGF-β (57).

Foam cell formation initially promotes macrophage survival. However, over time unregulated uptake of lipids and accumulation of free cholesterol in the cell is thought to promote endoplasmic reticulum stress leading to activation of the unfolded protein response (UPR), which initiates macrophage apoptosis in the plaque (reviewed in (58)). Free cholesterol, calcium, extracellular matrix components, and cellular debris form a complex necrotic core surrounded by a fibrous cap containing smooth muscle cells and extracellular

matrix (59). Given that the contents of the necrotic core are potentially thrombolytic and prone to rupture, the fibrous cap functions as a barrier between the neointima and blood constituents (59). As the expanding neointima protrudes into the lumen, the vessel wall initially compensates by undergoing expansive (or positive) remodeling to increase its surface area, and in turn, preserve the lumen area (60). Continued synthesis of extracellular matrix components occurs as a result of increased activity of matrix metalloproteinase (MMPs) that overwhelms tissue inhibitors of MMPs enzymes (TIMPs) that would otherwise inhibit MMP activity. This leads to remodeling of the microenvironment and degradation of the fibrous cap (61-63). The destabilized plaque is now vulnerable and prone to rupture. Thrombosis occurs if the plaque ruptures, occluding the vessel lumen, which causes obstruction of blood flow, and ultimately myocardial ischemia (64).

The vast majority of acute coronary syndromes are caused by plaque rupture (64). Identification of molecular targets involved in plaque stability is therefore clinically relevant and an area of much importance for development of novel therapies to help prevent rupture (59,65) or stimulate regression via removal of inflammatory foam cells (66-68)

1.3 Role of the macrophages in atherosclerosis

Macrophages are members of the mononuclear phagocytic system and function as homeostatic surveillance cells of the innate immune system (69). They are distributed across different tissues of the body displaying heterogeneity and specific functional phenotypes based on their anatomical location (69). Some of these subpopulations include osteoclasts in the bone, alveolar macrophages in the lungs, histiocytes in the interstitial connective tissues, Kupffer cells in the liver, and foam cells in the atherosclerotic lesion. Macrophages are derived from hematopoietic stem cells that originate in the bone marrow. In the circulation

they exist as monocytes, functioning mainly as a reservoir to replenish the pool of tissue resident macrophages (69).

In response to a number of signals such as inflammatory mediators during early fatty streak formation, monocytes hone to the site of injury, migrate into tissues, and differentiate into macrophages where they become phagocytic cells that are defined by their ability to internalize cellular debris, apoptotic cells, and pathogens (66,70). As such, macrophages exist as the "poster" cell of the inflammatory response in the atherosclerotic plaque, acting as a rapid line of defense to clear potentially damaging factors in the vessel wall.

Macrophages are also involved in antigen presentation, demonstrating their pleiotropic actions as an important connection point between the innate and adaptive immune system. Under steady state conditions a number of negative feedback systems regulate macrophage activation. They display inherent anti-inflammatory phenotypes thereby preventing unfavorable inflammatory reactions from taking place (71-73). As a result, macrophages also play an important role in suppressing the immune system. These fine tuned checkpoints ultimately occur to prevent self antigens from being recognized as non-self, thereby mitigating inappropriate destruction and remodeling of tissues.

Macrophages comprise the major leukocyte population during early lesion genesis. It is not surprising then that atherosclerosis is characterized as a chronic inflammatory disease arising primarily from the persistent infiltration of monocytes into the subendothelial space. Unregulated engulfment of modified lipoproteins ultimately gives rise to foam cells and the subsequent inflammatory response that ensues thereafter (74,75). This section will focus on macrophage activation in the context of the developing lesion with particular emphasis on regulation of foam cell formation.

1.3.1 Activation states and phenotypes

According to the review by Johnson and Newby (76), macrophages in the plaque can fall into a number of categories that broadly describes their roles in (a) the innate or adaptive immune system, (b) tissue destruction or repair, (c) immigration or emigration, (d) cholesterol accumulation or release, and (e) proinflammatory or anti-inflammatory. Within these dichotomous categories, macrophages have been shown to exhibit a plethora of phenotypic plasticity in response to acute changes in the particular local microenvironment they reside (77-84). Over the last decade there has been a growing appreciation for the temporal distribution of macrophage subpopulations at different stages of plaque development. In the mid 2000's Siamon Gordon summarized two different macrophage activation states or phenotypes based on expression of polarized markers - referred to as classical (M1) and alternatively (M2) activated cells (reviewed in (83,84)). As a result, a vast number of *in vitro* studies have been conducted to determine the relevance of macrophage polarization under different atherogenic conditions (recently reviewed in (33,76,85).

Classically activated or M1 macrophages are known to be activated by bacterial stimuli (e.g. lipopolysaccharide, LPS), as well as cytokines such as IFNγ, TNF-α, and GM-CSF. They are also known to express arginase II (ArgII) and inducible nitric oxide synthase (iNOS) (86). M1 activation leads to production of a host of inflammatory effector molecules and cytokines that contribute to tissue degeneration and pro-inflammatory responses, including reactive oxygen and nitrogen species, IL1β, TNF-α, IL-6, and IL-12 (87). Human monocytes treated with GM-CSF typically differentiate into unpolarized (M0) macrophages

(87). However, in the presence of LPS and IFN-γ their differentiation states become skewed into an M1 phenotype with elevated expression of TNF-α and IL-12 (87).

The alternate macrophage activation state, or M2, is induced by IL-3, IL-13, IL-10, corticosteroids such as glucocorticoid, members of the transforming growth factor (TGF)-β family, and IL-12 (87). They express high levels of ArgI, IL-10, IL-1, as well as scavenger, mannose, and galactose receptors. M2 cells generally function to promote tissue remodeling and angiogenesis. Recent data demonstrate that peroxisome proliferator-activator receptor (PPAR)-γ is a novel marker for M2 macrophages in both *in vitro* (88,89) and human tissue specimens (88). In one study, Bouhlel *et al.*, demonstrated that expression of PPAR-γ in human endartectomy specimens colocalized with cluster of differentiation (CD)68^{+} expressing macrophages (88). PPAR-γ is a transcription factor known to regulate genes involved in cholesterol efflux via upregulation of liver X receptor-α (LXRα) and ATP-binding cassette (ABC)-G1 (90). PPAR-γ activation was also found to reduce foam cell formation *in vitro* (91) though repression of SR-A expression (90,92), further providing evidence for the anti-inflammatory status of M2 macrophages.

In the healthy vessel the majority of resident macrophages are considered positive for Mac-1 ($Mac1^{+}$), CD11b, CD68 (or macrosialin – a class D scavenger receptor), and the macrophage marker F4/80 (28,93,94). These macrophages serve an immuno-surveillance function by maintaining vessel wall homeostasis. However, during the inflammatory response, circulating levels of M-CSF signals for monocyte recruitment from bone-marrow precursor cells, and their differentiation into cells of a monocyte-macrophage lineage (95). Accordingly, plaque macrophages are found to be positive for the macrophage marker $CD14^{+}$ (96). Although the role of macrophage heterogeneity in the early versus advanced

lesion remains unclear, it is hypothesized that the classic pro-inflammatory M1 macrophage is the predominant population in the established plaque (97). However, M2 markers have also been identified in early plaques as well (85,98,99), making interpretations of macrophage phenotype confusing. Interestingly, aortas from $LDLr^{-/-}$ mice fed a high fat diet for 30 weeks were analyzed for a number of macrophage markers and revealed that 39% were $CD86^{+}$ or of the M1 phenotype, and 21% expressed the mannose receptor CD206, specifying the M2 phenotype (99). Moreover, it has been shown that infiltration by $iNOS^{-}$ $IL1^{-}ArgI^{+}$ (M2) macrophages during early lesion development in $ApoE^{-/-}$ mice are gradually replaced by $iNOS^{+}IL1^{+}ArgII^{+}$ (M1) macrophages in the chronic lesion (100). M2 activation may exert atheroprotective actions in the early stages of atherosclerosis helping to dampen the pro-inflammatory response through clearing of atherogenic debris and dying cells (101). This effect may help counterbalance factors that favour lesion development, a process that may act similarly to an acute wound healing response on the surface of the skin, for instance. As the plaque develops, it is thought that a switch to the pro-inflammatory M1 phenotype may coincide with changes to the cytokine profile in the vessel wall, producing a net effect that signals a gradual shift to a more pro-inflammatory profile (101).

The balance of environmental signals during atherogenesis and its influence on macrophage gene programs and phenotype are complex. In addition, the interplay between macrophage activation states and foam cell formation is ambiguous and not fully appreciated. Indeed, it can be hypothesized that macrophage infiltration in the early stages of atherosclerosis functions to restore balance to the injured endothelium through secretion of anti-inflammatory mediators, uptake of pro-inflammatory lipids, and efferocytosis/egression of macrophages from the vessel wall. However, a positive feedback cycle develops probably

originating with oxidation of trapped LDL in the subendothelial space, which signals a gradual change in the microenvironment of the lesion including alterations in the cytokine profiles of the plaque, and gene expression patterns in macrophages. Ultimately, these steps lead to shifts in the anti/pro inflammatory balance that fall in favor of foam cell formation and the chronic inflammatory milieu giving rise to predominantly M1 macrophages in the advanced lesion. Do foam cells display heterogeneity with respect to macrophage markers and the stage of lesion formation? Can they be altered to change their inflammatory profiles in favour of M2 over M1 activation states? Certainly, future work will seek to identify the factors that drive foam cell phenotype into that of an apoptotic state in the necrotic core of the advanced plaque in an attempt to find targets to prevent plaque progression.

1.3.2 Macrophage foam cell formation

Cholesterol is an essential molecule involved in cell viability, plasma membrane integrity, and metabolism of hormones. The body derives its sources of cholesterol through dietary intake and intracellular synthesis in the ER. In the blood, cholesterol is transported by LDL and is taken up into the cell by its receptor, LDLr. The cell has a number of tightly controlled feedback mechanisms that balance metabolism and transport of intracellular cholesterol involving circuits that route cholesterol for storage, and export outside of the cell for excretion (102). Once inside the cell cholesterol and cholesterol esters of LDL are trafficked within the endocytic pathway involving movement from endosome vesicles to the lysosome for degradation, and then to the endoplasmic reticulum for further sorting (102). Under normal physiological conditions, acid cholesterol ester hydrolases (ACEH) converts cholesterol esters to free cholesterol within the lysosome (103). Free cholesterol is then routed through a number of different pathways depending on the homeostatic balance of

cholesterol sensed by the cell. In response to increasing cholesterol levels, free cholesterol is delivered to the endoplasmic reticulum where it is re-esterified by acyl CoA:cholesterol acyltransferase (ACAT) and sequestered into lipid droplets (104). Neutral cholesterol ester hydrolase (NCEH) located in lipid droplets cleave cholesterol esters back into free cholesterol where it is used by the cell for biochemical processes such as membrane assembly or sterol biosynthesis, as well as shuttled to extracellular cholesterol acceptors such as HDL (103,104). Free cholesterol is required to facilitate cholesterol efflux outside of the cell as a mechanism to control intracellular cholesterol levels; however, at high levels it is cytotoxic and thought to mediate cell death pathways (105) characteristic of macrophage morphology in the necrotic core of advanced plaques (106).

In response to high levels of intracellular cholesterol stores LDLr is down regulated - a negative feedback cycle important to maintains homeostatic cholesterol levels by preventing its over-accumulation (107). Alterations in the chemical structure of LDL by enzymatic or chemical modification in the subendothelial space of atherosclerotic arteries renders LDL unrecognizable by its cognate receptor, LDLr; instead modified LDL is taken up by alternative mechanisms, particularly via scavenger receptors (108). Given that scavenger receptors are not suppressed when cellular cholesterol content rises, the continuous accumulation of lipids results in untoward storage of intracellular cholesterol esters (108) **(Figure 2)**. Hence, it is now a widely accepted paradigm that endocytosis of

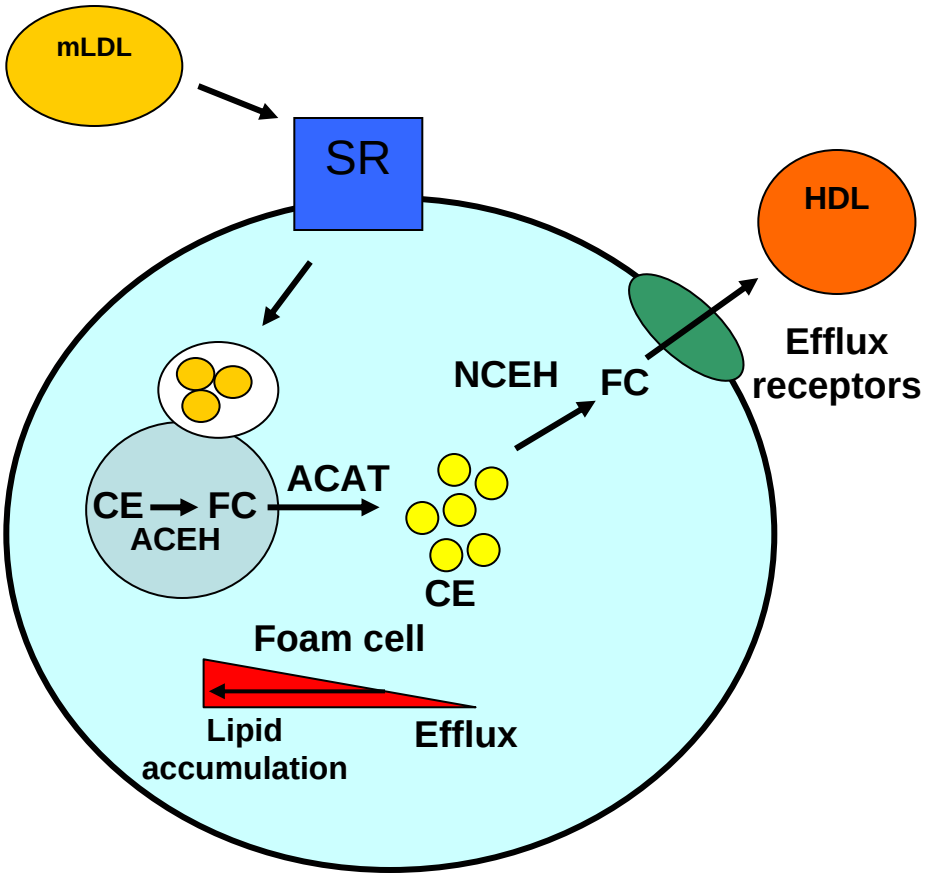


Figure 2. Cholesterol trafficking in macrophages and formation of foam cells.

Scavenger receptors (SR) on the surface of macrophages internalize modified LDL (mLDL) into vesicles that subsequently fuses with acid lysosomes. In lysosomes, cholesterol esters (CE) are cleaved into free cholesterol (FC) by acid cholesterol ester hydrolase (ACEH). FE is then transported out of the lysosome and re-esterified by ACAT in the cytosol to form cholesterol esters, which get sequestered and stored in lipid droplets. At steady state cholesterol metabolism, cholesterol esters can be cleaved to FC by neutral cholesterol ester hydrolase (NCEH). FC can then be transported to cholesterol acceptors (HDL) in the extracellular space by efflux transporters such as ABCA1. Imbalance in cholesterol accumulation to cholesterol efflux shifts the pathway in favor of excessive cholesterol accumulation leading to foam cell formation.

Adapted from Daugherty A *et al.* (109) with modifications. © Wolters Kluwer Health; Reprinted with permission from Wolters Kluwer Health.

modified lipoproteins – mediated, in part, by scavenger receptors – regulates *bona fide* foam cell formation, helping to drive the inflammatory response during atherogenesis.

Once modified lipoproteins are endocytosed by scavenger receptors, cholesterol and cholesterol esters are delivered to lysosomes in a similar process to the endocytosis pathway of native LDL. Lysosomal acid lipase (LAL), a subclass of ACEH, located in lysosomes hydrolyze cholesterol esters into its constitutive parts: free cholesterol and fatty acids (110). Excess free cholesterol is then trafficked to the ER (103) resulting in suppression of the sterol-regulatory element binding (SREB) pathway leading to inhibition of endogenous synthesis of cholesterol, and subsequent downregulation of the LDLr gene (111). ACAT re-esterifies free cholesterol to cholesterol fatty acid esters in the ER (112) as a detoxification mechanism to limit the level of toxic free cholesterol (113). When metabolism and export systems become saturated, free cholesterol is then sequestered into peripheral components of the cell - namely in membrane bound lipid inclusion droplets that facilitate its storage, as well as lysosomes that continually attempt to promote its degradation (112). It is thought that lipid storage droplets form a continual extension of the ER, and it is this accumulation of cholesterol esters which gives the foam cell its morphological "foamy" appearance (114) **(Figure 2)**.

As expected, lipid loading alters macrophage biology and this contributes to changes in macrophage function. For example, a recent study used a proteomic approach to elucidate differentially expressed proteins in peritoneal macrophages isolated from $LDLr^{-/-}$ mice fed a high fat diet versus a normal chow diet (115). The authors found that lipid loaded macrophages form networks involved in cytoskeletal regulation, vesicle-mediated transport, and lipid binding. Other reports have shown that lipid-laden macrophages demonstrate high

levels of DNA damage (116) that coincide with activation of ER-dependent stress pathways (58). These stress pathways likely contribute to apoptosis (116,117) and necrotic death (118) observed in the advanced states of lesion development.

The endosomal membrane spanning proteins Neimann-Pick Type C (NPC)1 and NPC2 are implicated in delivering esterified cholesterol from late endosomes into lipid vesicles for storage (104). Genetic mutations in NPC1 and NPC2 result in Neimann Pick disease, a lipid storage disorder characterized by disproportionate levels of free cholesterol accumulation in lysosmes, which reduces the accessibility of cholesterol to bind the surface receptors, ABCG1 or ABCA1, for efflux out of the cell (119,120). Cholesterol efflux is an important process involved in reducing lipid accumulation in macrophages, and therefore an important factor in plaque regression. It involves trafficking of free cholesterol from lysosomes to the plasma membrane for removal via ABCG1 or ABCA1 to HDL or ApoA1, respectively (121,122) **(Figure 2)**. Mutations in the ABCA1 gene cause Tangier disease, which is characterized by defects in reverse cholesterol transport. Low circulating levels of serum HDL coupled to lipid poor ApoA levels leads to massive accumulation of foam cells in different regions of the body (123). Moreover, deletions of ABCG1 and ABCA1 in mice models of atherosclerosis are associated with increased lesion formation and enhanced foam cell formation (121). Taken together, these concepts highlight the central role of cholesterol efflux receptors in balancing removal of cholesterol, which in turn, helps protect the cell against elevated intracellular free cholesterol.

It is clear that macrophages are major mediators of atherosclerosis, playing important roles in all stages of lesion development. During fatty streak formation, the inflammatory response that occurs as a result of trapped lipoproteins in the subendothelial space leads to

macrophage infiltration and serves as an adaptive mechanism to remove these pro-inflammatory factors. Yet, the macrophage recognizes these lipoproteins as "non-self" thereby activating "scavenging" pathways via upregulation of scavenger receptors to facilitate lipoprotein removal (113). Thus, it has been suggested that lipid uptake pathways are independent from cholesterol metabolism systems that work to maintain proper cholesterol biogenesis for normal cell function (113). Indeed, internalization of cholesterol in the developing lesion initially elicits an adaptive response that involves cycling of cholesterol esters, its hydrolysis into free cholesterol, and upregulation of cholesterol efflux pathways. Under conditions of unregulated cholesterol uptake, these cytoprotective mechanisms ultimately fail. Cholesterol esters become trapped in lysosomal compartments as the macrophage becomes saturated with newly forming lipid droplets in the face of defective cholesterol esterification and efflux of free cholesterol. In addition, defective rates of efferocytosis and macrophage emigration from the plaque (28) lead to the overall net persistence of foam cells within the vessel wall, thereby contributing to the continual growth of the plaque.

Besides modified LDL, other factors also contribute to foam cell formation and the atherogenesis process. As reviewed by Seigel-Axel *et al.* platelets may contribute to a proatherogenic phenotype by binding and internalization of both modified as well as native LDL in the circulation (124). Moreover, emerging evidence suggests that scavenger receptors such as SR-A, CD36, and LOX-1 are expressed on the surface of platelets which may provide an explanation linking platelets to their lipid accumulation pathways (124). OxLDL loaded platelets that leak into the injured endothelium of the vessel wall (125) are phagocytosed by macrophages of the developing lesion - a process proposed as an

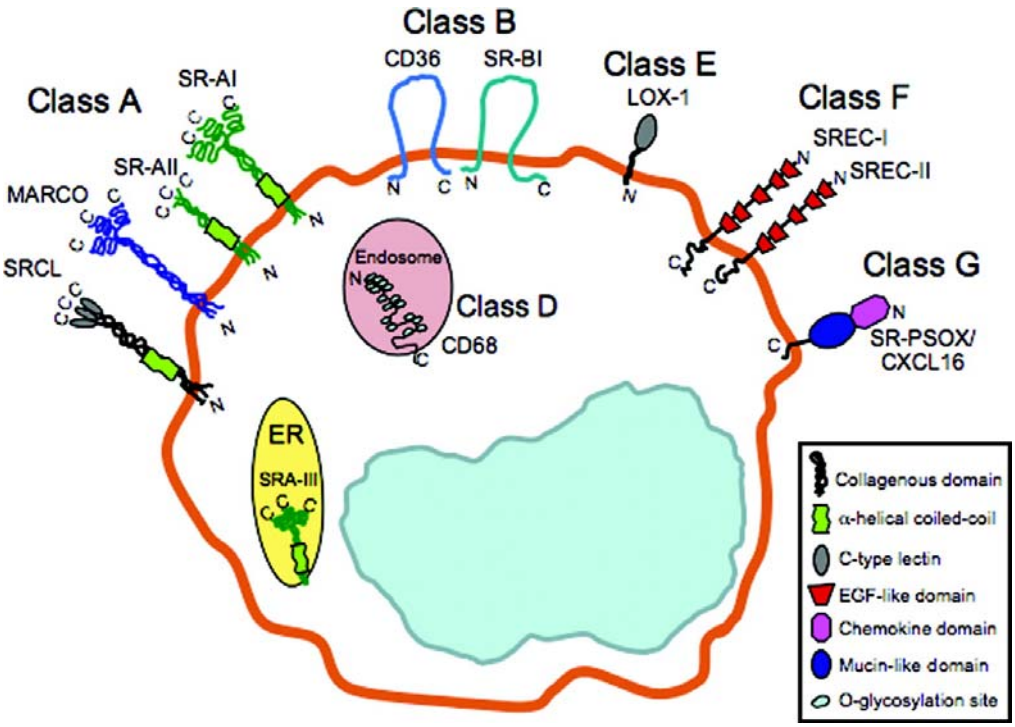
alternative mechanism for foam cell formation (126-128). Phagocytosis of platelets is also found to be mediated by scavenger receptors on the surface of intimal macrophages, and in this way may facilitate the transfer of modified LDL into macrophages further exacerbating foam cell formation (129-131). Lastly, beta-amyloid deposits in the plaque (132,133), and the presence of advanced glycation end products (AGE) common in hyperglycemia conditions (134) may also contribute to accelerated foam cell formation.

Hence, finding strategies to mitigate lipid uptake and cholesterol deposition in the early stages of foam cell formation is an important objective for the development of anti-atherogenic therapies (113). As scavenger receptors are likely candidates for developing interventions to prevent foam cell formation, the next section will focus on these key receptors.

1.4 Mechanisms of lipid uptake: Scavenger receptor A

Lipid-loading pathways that culminate in the morphogenesis of macrophage foam cells are a salient feature in the development and evolution in the pathogenesis of atherosclerotic lesions. The identification of oxidized forms of LDL in mouse and human atherosclerotic lesions has strengthened the *oxidative modification hypothesis* (135). When these lipids are purified from the vessel wall, they are found to contain oxidation epitopes that are preferentially recognized and tightly bound to pattern recognition receptors, namely scavenger receptors (136-139). Over the last 30 years eight subclasses of scavenger receptors, A-H, have been identified for their roles in binding and internalizing modified forms of LDL (135) **(Figure 3A)**. This section will focus on scavenger receptor-A (SR-A) of the class A family of scavenger receptors.

A)


Class A
SR-AI
SR-AII
MARCO
SRCL
Class B
CD36
SR-BI
Class E
LOX-1
Class F
SREC-I
SREC-II
Endosome
Class D
CD68
Class G
SR-PSOX/
CXCL16
ER
SRA-III
Collagenous domain
α-helical coiled-coil
C-type lectin
EGF-like domain
Chemokine domain
Mucin-like domain
O-glycosylation site

B)

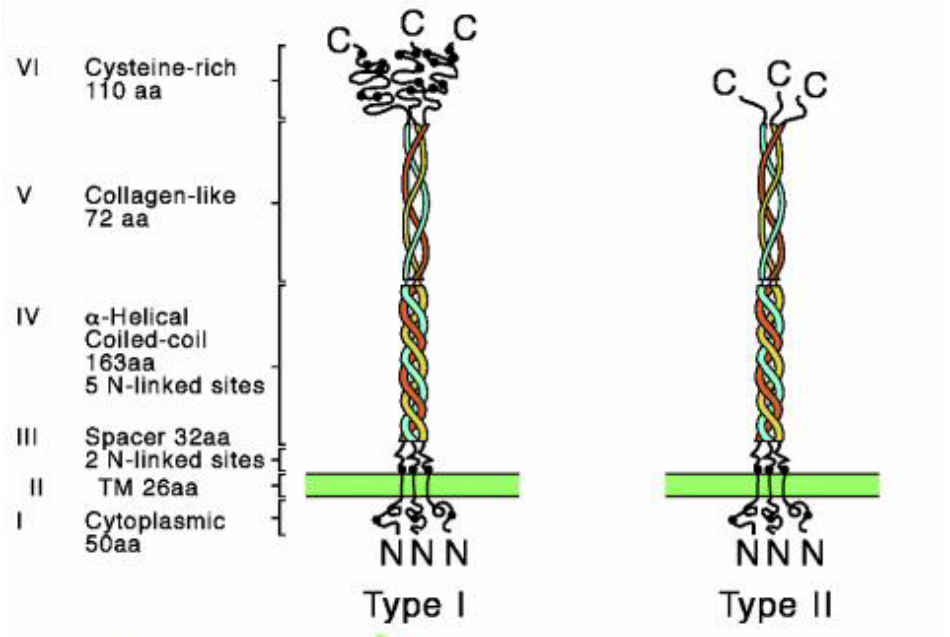

VI Cysteine-rich 110 aa
V Collagen-like 72 aa
IV α-Helical Coiled-coil 163aa 5 N-linked sites
III Spacer 32aa 2 N-linked sites
II TM 26aa
I Cytoplasmic 50aa
NNN
Type I
NNN
Type II

Figure 3. Structural features of scavenger receptors involved in macrophage lipid uptake during atherosclerosis

(A) Schematic diagram illustrating different classes of scavenger receptors (class H is not shown). Modified forms of LDL are recognized by these receptors expressed on the surface of macrophages in the developing lesion. SR-A is a member of the class A family and is the most widely studied for its role in foam cell formation. Adapted from Moore and Freeman (140).

(B) Domain organization of SR-A type I and II. See text for details. Adapted from Platt and Gordon (141).

1.4.1 Scavenger receptor-A structure and functional properties

SR-A was the first identified member of the scavenger receptor family. SR-A is a type II, single membrane-spanning, trimeric transmembrane glycoprotein that was purified in 1988 as a novel acetylated (ac)LDL binding site (142), and cloned from bovine macrophages in 1990 (143). The SR-A gene is located on chromosome 8p22, contains 11 exons (144), and produces three forms that are products of alternative mRNA splicing: type I, II, and III (145). Although SR-A type I and II are typically co-expressed, functional differences have yet to be ascribed between them. A third subtype, SR-A-III, does not reach the cell surface, but rather is found sequestered in the ER and shown to have a dominant negative effect on SR-A expression in transfected cells (146).

SR-A1 is the best characterized member of the scavenger receptor A family containing 6 distinct structural domains: N-terminus cytoplasmic (I), membrane (II), spacer (III), α-helical coil-coil (IV), collagen-like (V), and C-terminus cysteine rich (VI) domains. Type II and III express a truncated C-terminus (147) with a cysteine domain approximately half the length of SR-AI (146) **(Figure 3B)**. Based on structural homology in their cysteine rich domains, SR-A is further sub-classified with other members of the class A family.

These include the macrophage receptor with collagenous structure (MARCO), which is expressed in macrophages but has roles mostly in pathogen host defense (reviewed in (148)); as well as a newly described member, SCARA5, which was found expressed on endothelial cells but does not bind modified LDL (149). Interestingly, another member of the class A scavenger receptor, scavenger receptor with C-type lectin type I/II (SRCL) has high homology to SR-A and MARCO but does not contain a cysteine rich domain nor does it recognize modified lipids (148). Although the cysteine-rich structure is highly conserved within class A members, its function remains ambiguous because its presence or absence does not appear to be directly associated with mediating binding of modified lipids (148).

The collagen-like domain is another hallmark structural feature found in all the members of the class A scavenger receptors **(Figure 3B)**. It contains positively charged arginine and lysine clusters thought to mediate electrostatic interactions with negatively charged ligands (150). This domain has also been implicated in mediating cell adhesion to collagen substrate (151).

A third structural feature of class A receptors is the α-helical coiled-coil domain. It is thought to contribute to structural flexibility acting like a "hinge" enabling SR-A to fold onto itself upon ligand binding (152) **(Figure 3B)**. In addition, this domain participates in receptor processing in the lysosome proceeding internalization (153,154). Histidine residues are thought to regulate the lysosomal pH to facilitate ligand dissociation from the receptor. Lastly, the α-helical coiled-coil domain may also be involved in ligand recognition and adhesion. A monoclonal antibody directed against this region (the 2F8 clone) was shown to alter the structure of the receptor, particularly at the collagenous domain interface by blocking acLDL uptake and adhesion to collagen coated surfaces (151,155).

1.4.2 Expression and regulation of SR-A

SR-A1/II are expressed not only by macrophages but also by aortic endothelial cell and liver sinusoidal endothelial cells, albeit to a lesser level (156,157). Although human monocytes isolated from peripheral blood mononuclear cells express minimal levels of SR-A, mRNA levels appear to rise rapidly during differentiation into macrophages in culture (158,159). SR-A isoforms are highly expressed in foam (144,156,160,161) and smooth muscle cells (162) in human atherosclerotic lesions. In addition, both modified lipid uptake and expression of SR-A were higher in human monocyte-derived-macrophages isolated from healthy elderly individuals free of CAD than their younger counterparts (163), implying that SR-A expression may have a protective role in the aging process. Furthermore, SR-A gene levels in peripheral blood mononuclear cells were found to rise in patients experiencing acute coronary events compared to healthy controls further reflecting the role of SR-A in pathological states (164).

Many factors and cytokines regulate SR-A expression. *In vitro*, MCP (165), M-CSF (166), oxLDL as well as acLDL (167,168) have all been documented to positively regulate SR-A expression in macrophages. Phorbol esters (i.e. phorbol myristate acetate - PMA) are well known to differentiate the monocytic cell line, THP-1 cells, into macrophages, which is associated with increased functional expression of SR-A (169,170). Mechanical strain upregulates SR-A expression - a mechanism suggested to explain accelerated atherosclerosis in the context of hypertension (171). In contrast, TNF-α (172,173), TGF-β (174), GM-CSF (175), INFγ (176,177), and activation of PPAR transcription factors (178) downregulate SR-A expression. LPS was shown to reduce SR-A expression in human THP-1 macrophages (172,179), but increases expression in mouse peritoneal macrophages (179).

In 1992, Monty Krieger coined the term "molecular flypaper" (180) to describe SR-A for its *promiscuous* ability to bind a broad range of overlapping but sometimes distinct negatively charged (polyanionic) ligands involved many physiological and pathological processes (181). These include lipoproteins or proteins such as acLDL, oxLDL, malondialdehyde-modified LDL/albumin, AGE-modified LDL/albumin (182,183), ApoA, and ApoE (184); polyanions such as polyinosine and polyguanosine (185); polysaccharides such as dextran sulfate, fucoidan, polyinosinic acid, carrageenan, and beta-amyloid fibrils (186); bacterial products such as LPS and lipoteichoic acid (186); and phospholipids (e.g. phosphatidylserine) (187). Although it remains unclear why SR-A binds such a broad range of ligands, Bowdish and Gordon have suggested it may reflect both SR-A's ancient roles in cell adhesion, and phagocytic functions concomitant with more recent evolutionary roles in host-defense, self vs. non-self recognition, and homeostatic clearance of lipoproteins (188). From an evolutionary perspective many domains of SR-A are highly conserved among vertebrate lineages; as well, parts of its gene may also be evolutionary conserved in less complex organisms (188).

The physiological relevance of SR-A was brought into focus by the pioneering work of Brown and Goldstein in the late 1970's who discovered that unregulated receptor-mediated uptake and degradation of acLDL, but not native LDL, triggers massive accumulation of intracellular lipids (189). Scavenger receptors were initially denoted as "acLDL binding sites" because of their role in "foraging" modified forms of LDL unrecognized by the classical LDL receptor. SR-A plays an important role in atherogenesis because its gene is upregulated by macrophages in response to both binding of its modified lipid at the cell surface and increasing accumulation of intracellular lipid stores. In a

clathrin-dependent process (190) SR-A internalizes acLDL through a mechanism that involves its N-terminal cytoplasmic tail (143,190,191). Once internalized the clathrin-LDL complex is trafficked from early endosomes to lysosomes for processing (107,192-194). In the acidic environment of the lysosome, the ligand dissociates from SR-A allowing SR-A to be recycled back to the plasma membrane through the trans-Golgi network (195). As free cholesterol is toxic, it is either shuttled out of the cell or esterified for deposition into lipid droplets for storage. However, when efflux pathways become overwhelmed, SR-A expression is not shut down promoting progressive accumulation of lipid droplets. A positive feedback cascade is established involving continual recycling of SR-A to the cell surface in the face of continued modified lipid interaction at the cell surface, thereby perpetuating intracellular lipid stores, and ultimately leading to foam cell formation **(Figure 4)**.

The precise mechanism(s) by which scavenger receptors mediate phagocytosis of its ligand is not entirely resolved. It has been hypothesized that intracellular signaling pathways are activated either directly through the cytoplasmic tail, or occur indirectly through adaptor proteins recruited to this region. The cytoplasmic tail has a unique internalization motif called VXFD. In mutant cells missing this SR-A motif, surface SR-A expression and internalization of ligands are abolished (196). It has been shown that acLDL binding to SR-A changes the confirmation state of the receptor complex, leading to phosphorylation at any one of the three conserved serine or one conserved threonine residues located on the C-terminus monomers (197,198). This initiates a classical pertussis toxin-sensitive signaling cascade (i.e. G protein-coupled receptor cascade) as well as activation of membrane protein kinase C (PKC), and involvement of the inhibitory G-coupled receptor domain (Gi/o)

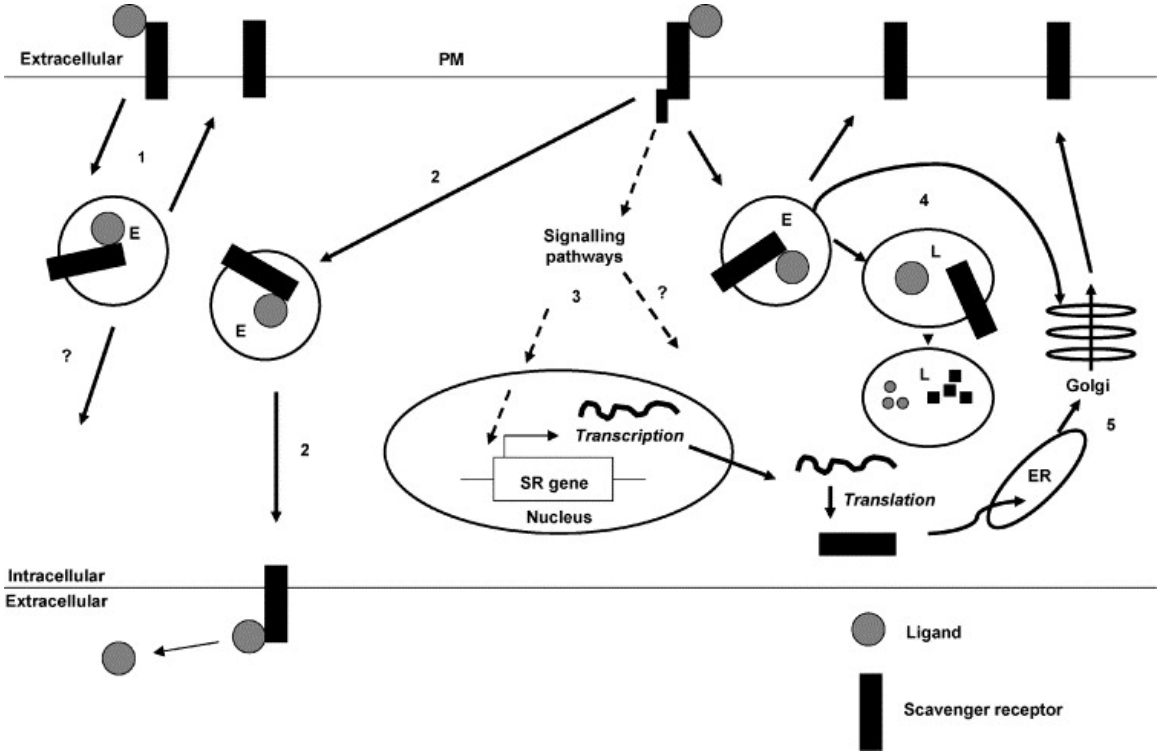


Figure 4. SR-A mediated uptake of cholesterol, its intracellular trafficking, and signaling leading to foam cell formation

SR-A function in the macrophage is tightly coordinated by trafficking and intracellular signaling pathways. Constitutive cycling of SRA between the plasma membrane (PM) and endosomes (E) may be altered by receptor binding of modified lipid (grey circle). This triggers one or a combination of positive feedback responses, ultimately leading to accumulation of cholesterol esters inside the cell: (1) endocytosis and recycling of SR-A back to the cell surface; (2) receptor relocation from one region of the plasma membrane to another via transcytosis; (3) intracellular signaling pathways that lead to increased SR-A gene expression; (4) degradation of both ligand and receptor in lysosomes (L) leading to recycling of the SR-A back to the cell surface; and (5) transcriptional regulation of *de novo* receptors. Please note that this figure is overly simplified, especially the intracellular signaling pathways, and meant only to provide an global appreciation for the pathways that regulate SR-A.

Adapted from Murphy *et al.* (199).

(200,201). Treatment of mouse macrophages with pertussis toxin, a specific inhibitor of G protein-coupled receptor signaling, was found to prevent acLDL uptake without affecting SR-A expression receptors implying the role of G-coupled protein signaling cascades in a feedback process that positively regulates acLDL internalization (202,203). Furthermore, Jun N-terminal kinase (JNK)-2 mediated phosphorylation of SR-A appears to be required for foam cell formation – an effect associated with protection against atherosclerosis in $ApoE^{-/-}$ mice in which JNK2 is specifically deleted in macrophages (204). In another study, SR-A ligands, fucoidan and oxLDL, were found to stimulate secretion of the pro-inflammatory cytokines IL-1 and TNF-α by regulating signal transduction cascades involving protein kinase-mediated mitogen-activated protein kinase (MAPK) and PKC (205). This signaling pathway involves upstream regulation by p21-activated kinase (PAK), the extracellular signal-regulated kinase (ERK), JNK, and p38 (205). Activation of SR-A by fucoidan was also found to induce NO production in macrophages in a p38 and NF-κB-dependent mechanism (206), implying the involvement of SR-A in controlling vessel wall vasodilation.

Induction of the SR-A gene, MSR1, occurs as a result of binding of the transcription factors PU.1/Spi-1, to highly conserved ETS-domains in the promoter regions of the gene (207). Transcriptional control of SR-A is also mediated by promoter regions identified as activator protein (AP)-1 binding sites (207). Both ETS-domain and ATP-1 binding sites are well known to be involved in cell differentiation, growth, and survival - linking activation of the MSR1 to the process of macrophage differentiation. Using a transgene reporter system, regulatory elements in the SR-A promoter were found to direct macrophage-specific expression of the human growth hormone receptor in mice as well in bone-marrow derived

macrophages in response to M-CSF (208). Transgene expression as well as foam cell formation was abolished in atherosclerotic mice when the PU.1 promoter region was mutated, consistent with the importance of SR-A regulatory genes in differentiation of macrophages and foam cell formation (208). A number of studies have demonstrated an influence of extracellular cytokines in controlling the activity of the MSR gene. M-CSF is known to upregulate SR-A in macrophages via GTPase (Ras)-dependent signal transduction pathways involving PKC activity (209,210). The Ras pathway is known to activate transcription through AP-1/ETS motifs in promoters of target genes (211,212); this provides evidence for a link between cytokines and transcriptional control of SR-A expression.

1.4.3 Cellular Functions of SR-A

The contribution of SR-A to lipid uptake in atherosclerosis was initially evaluated by Suzuki *et al.* using SR-A null macrophages. SR-A was found to be responsible for binding and internalization of 80% of acLDL, and 50% of oxLDL (213). Development of SR-A and CD36 (a class B member of the scavenger receptor family also implicated in foam cell formation and atherogenesis) null mice further established that 90% of modified lipid uptake occurred via the combined effects of SR-A and CD36, validating the importance of scavenger receptor mediated endocytosis of lipids as a process central for foam cell formation (214).

In addition to its role in lipid uptake, SR-A has been shown to have pleiotropic functions including cellular adhesion, as well as recognition and clearance of bacteria (215) and their constituents (i.e. lipid A component of LPS) (216,217). SR-A dependent macrophage adhesion requires G protein-coupled receptors signaling and activation of PI3-kinase (218,219), as well as actin cytoskeleton regulating Rho-GTPases molecules, Rac and

Cdc42 (220). SR-A is also involved in both clearance of apoptotic cells and in controlling apoptosis (148). Accumulation of intracellular free cholesterol induces a cellular stress response involving the ER (unfolded protein response) that is dependent on SR-A-mediated signaling mechanisms (221). The absence of both SR-A and CD36 in ApoE-/- mice fed a Western diet for 12 weeks is associated with reduced plaque macrophage apoptosis and necrotic core formation (222). Moreover, the demonstration that SR-A null mice are more susceptible to endotoxic shock in an infection model using *bacillus Calmette Guerin* led to the hypothesis that SR-A plays an important role in clearance of bacteria (217). Lastly, the ability of SR-A expressing dendritic cells to capture antigens for presentation to T-cells further provides important insight into the involvement of SR-A in mediating adaptive immune functions in the vessel wall (223).

1.4.4 Role of SR-A in atherosclerosis

To gain better insight into the physiological relevance of SR-A *in vivo,* it is helpful to examine the various atherosclerosis-prone transgenic animal studies reported over the last 15 years. **Table 1** provides an up-to-date summary on the major results of these animal studies. Although loss of function and gain of function studies have confirmed the impact of SR-A in atherosclerosis, inconsistencies and surprising results have raised new questions surrounding the expected functions of SR-A.

Suzuki *et al*. were the first to show that SR-A deficiency is atheroprotective by crossing SR-A null mice ($SR\text{-}A^{-/-}$) onto an $ApoE^{-/-}$ background after feeding with a normal chow diet (213). Targeted disruption of MSR1 reduced lesion size by greater than 55%. Peritoneal macrophages derived from these mice showed decreased modified lipid uptake *in vitro* compared to wild type controls. OxLDL uptake was also reduced by 15% in

Table 1. Role of SR-A in murine models on atherosclerosis

Murine model	Background strain	Number of backcross generations	Sex	Diet type and length	Effect on lesion size	*Ex vivo* effect on modified lipid uptake/accumulation	Ref.
Msr-/-xApoE-/-	C57BL/6	N/A	N/A	High fat – 11wks	↓58%	↓ in peritoneal macs	(213)
Msr-/-xLDLr-/-	C57BL/6-129/ICR chimera	N/A	F	High fat – 4 & 12wks	↓20%	↓ in peritoneal macs.	(224)
Msr-/-xApoE3 Leiden tg	C57BL/6	11	M/F	High fat – 10wks	N.S.	Not studied	(225)
Msr-/-x LDLr-/-	C57BL/6	6	M/F	Butter fat – 30wk	↓ >81%	Not studied	(226)
Msr-/-xApoE-/-	C46BL/6	7	M/F	Western – 8 wk	↑40% in males	↓ in peritoneal macs.	(227)
Msr-/-x CD36-/- x ApoE-/-	C57BL/6	7	M/F	Western – 12 wk	*N.S	N.S	(222)
Msr-/-xApoE-/-	C57BL/6	7	M/F	High fat – 12wk	↓32% in female	Not studied	(228)
BMT SRAo/e→ApoE-/-	C57BL/6	3	N/A	Normal chow	N.S	↑ in peritononeal macs.	(229)
BMT SRAo/e→LDLr-/-	C57BL/6	4	N/A	Western 12wk post transplant	N.S	↑ in peritoneal macs.	(230)
BMT SRAo/e→LDLr-/-	C57BL/6	10	N/A	High fat diet 8 wk post transplant	↓70%	Not studied	(231)

N/A = not available
N.S. = no differences (not significant) between SR-A$^{-/-}$ null mice and littermate controls
BMT = bone marrow transplant
Ref = reference
*Although CD36 and SRA deficiency did not impact the size of the lesion, content was affected by reducing expression of inflammatory genes as well as macrophage apoptosis and plague necrosis

macrophages that lacked SR-A. To further clarify the role of SR-A, Sakaguchi *et al.* demonstrated reduced lesion size in SR-A$^{-/-}$ mice on a LDLr background after 4 or 12 weeks of a high-fat diet compared to LDLr single null mice (224). In addition, Babaev *et al.* showed a >80% decrease in lesion size in SR-A-/- mice backcrossed to LDLr$^{-/-}$ C57/BL6 mice fed a butter-fat diet for 30 weeks (232). Despite these initial reports that agreed with the established concepts for the role of SR-A in foam cell formation and its expected pro-atherogenic contribution, disparate results obtained in subsequent studies sparked much debate.

Unlike the Suzuki *et al* study, SR-A null mice in on a hyperlipidemic ApoE3 Leiden background in the study by de Winther *et al.* showed a trend toward development of *larger* lesions in the double knockout mice (225). Moreover, Moore *et al.* showed a 40% increase, but slight decrease, in aortic sinus lesion area in male and female SR-A null mice on an ApoE background fed a Western diet for 8 weeks, respectively; however, these results were not statistically significant (227). Interestingly, mean cholesterol levels in the male ApoE$^{-/-}$ SR-A$^{-/-}$ mice were 40% higher compared to ApoE$^{-/-}$ mice, which paralleled the percent differences observed for lesion area. The increases in lesion formation in the absence of SR-A in the male mice were accompanied by accumulation of foam cell formation *in vivo* as observed by electron microscopy, suggesting that SR-A in this study is in fact protective against atherosclerosis and argued against the classical paradigm that attributes a casual role of SR-A in foam cell formation. The accumulation of foam cells in the absence of SR-A further implies that other scavenger receptors may compensate, or that alternative lipid uptake mechanisms may occur. Indeed, a recent study by Makinen *et al.* demonstrated that short hairpin RNA silencing of SR-A in mouse macrophages led to reciprocal upregulation

of CD36 expression (and vice versa) (233), providing a possible explanation why foam cell formation may continue to occur even in the absence of one or the other scavenger receptors. Interestingly, Moore *et al.* found that foam cell formation occurred independent from SR-A when SR-A null macrophages were incubated with aggregated LDL rather than with chemically modified LDL (acetylation or oxidation), providing more evidence that other non-receptor mediated uptake mechanisms may be playing a role (227). Kruth and colleagues have demonstrated a non-receptor mediated pathway via macropinocytosis of LDL that may explain accumulation of lipids and formation of foam cells independent from scavenger receptor function (234-236). Moroever, sex differences have been shown to impact the role of SR-A in atherosclerosis. Kuchibhotla et al. demonstrated a gender specific role of SR-A by showing that female but not male SR-A null mice backcrossed onto $ApoE^{-/-}$ C57/BL6 exhibited a 32% reduction in lesion area fed a high fat diet for 12 weeks (228), offering yet another interpretation into the disparity of SR-A in the literature.

In another study to further explore the impact of both SR-A and CD36 in development of foam cells and lesion formation in an advanced model of atherosclerosis, Manning-Tobin *et al.* crossed SR-A and CD36 null mice onto an ApoE background and fed the mice a Western diet for 12 weeks (222). No changes were reported in aortic *en face* and aortic sinus lesion as well as foam cell formation in the vessel wall of neither female or male $ApoE^{-/-}Cd36^{-/-}SR\text{-}A^{-/-}$ compared to $ApoE^{-/-}$ littermates. Given that independent contribution of both SR-A and CD36 to foam cell formation is clearly documented in many earlier studies, the lack of protection effect afforded by combined deletion of both scavenger receptors was surprising and further ads to the confusion underlying the central understanding of scavenger receptor function in atherosclerosis. Although the deletion of

SR-A and CD36 in tandem did not alter atherosclerosis, a careful analysis of previous studies brings forth a number of technical and methodology caveats that may help shed light into the different results reported in each study. Whereas the Moore (227) and Manning-Tobin (222) study cited above use SR-A mice backcrossed into the C57BL/6 strain 7 times, earlier studies by Babaev, for example, report backcrossing onto this background strain 6 times (226) **(Table 1)**. Strain background is well known to influence the outcome of murine atherosclerosis models. For example, a 6% lower C57BL/6 strain genome as a result of crossing a target gene 6 fewer generations into $LDLr^{-/-}$ mice caused 40% less atherosclerosis than the mice backcrossed more generations (237). Thus, the reports by Moore and Tobin-Manning that backcross the SR-A null mice 7 generations are more genetically homogeneous than previous studies. Furthermore, the early studies by Suzuki (213) and (224) reported significant reductions in lesion size in SR-A null mice on a chimeric C57BL/6-129/ICR background. While the commonly used C57/BL6 strain used in recent SR-A atherosclerosis models is considered more susceptible to plaque formation, the ICR/129 strain used in earlier studies is considered atherosclerosis-resistant (213,238). In addition, SR-A expression and uptake of lipids is shown to be higher in macrophages isolated from mice strains resistant to hypercholestermia compared to strains more susceptible to the disease (239).

The length and type of diet as well as the atherosclerosis background model is certainly a factor known to impact the size and composition of atherosclerotic lesions (21,240). Van *et al.* reported a 12 week study of atherosclerosis in $ApoE^{-/-}$ mice transplanted with marrow cells overexpressing (o/e) SR-A and fed a normal chow diet supplemented with 5.7% fat (229). On the other hand Herigjgers *et al.* (241) reported another 12 week study but

in LDRr$^{-/-}$ transplanted with SR-Ao/e cells and fed a Western diet. Despite the use of different atherosclerotic transgenic models these studies also failed to detect any impact of SR-A on atherosclerosis. In other studies, bone marrow from SR-A overexpressing mice transplanted into irradiated LDLR$^{-/-}$ mice, and fed a high fat diet for 8 weeks showed reduced lesion formation by over 70% (231). The earlier study by Babaev *et al.* also demonstrated significant reduction in lesion size in SR-A$^{-/-}$ LDLr$^{-/-}$ fed a butter fat diet for 30 weeks (226). Certainly, differences in ApoE and LDL null mice may account for such differences in results. LDL derived from the serum of ApoE$^{-/-}$, for example, is more potent in promoting binding to SR-A and CD36 than that of LDL from LDR null mice (242), demonstrating that the "atherogenicity" between these two transgenic models may provide possible explanations for the divergent impact of SR-A in the above studies. Lastly, in the study by Moore *et al.* macrophages isolated from SR-A null mice clearly exhibited impairment in their ability to take up modified lipids *in vitro*, however, this effect does not closely parallel inhibition of foam cell formation in the vessel wall (227). I*n vitro* preparations of modified LDL are likely to display study-to-study variability in not only the extent of modification, but also contextual differences in the type of modification, which may account for the discordance of results observed between lipid uptake observed *in vitro* and foam cell formation reported *in vivo*. While acLDL is a known ligand for SR-A, the degree to which these *in vitro* preparations resemble modified lipids in the vessel wall of humans or animal models is unknown. It is well known that enzymatic modification of LDL by secretory phospholipase A2 can also induce foam cell formation in the plaque, similar to that observed with biochemical modification of LDL using acetylation or oxidation techniques (243). This provides evidence that other forms of modified lipids may render

foam cells *in vivo* involving alternative lipid uptake mechanisms independent from SR-A or CD36, and therefore may account for the progression of atherosclerosis previously observed despite the absence of SR-A.

A number of genetic studies have implicated the involvement of SR-A in human atherosclerosis but its contribution to lipid uptake pathways continues to remain unclear. SR-A is considered a polymorphic gene that contains many regions of genetic variability including single-nucleotide polymorphisms and frame-shift mutations (188). Studies performed on a founder family within a French-Canadian community revealed that a locus containing the SR-A gene may be associated with CAD (244). In another Canadian pedigree, overexpression of SR-A was observed in planar xanthomas tumors - a disease characterized by formation of papules in localized regions of the body containing foam cell deposits (245). Interestingly, no member of this family died due to CAD despite many patients displaying high plasma cholesterol profiles, further raising questions about the causative role of SR-A in lesion development.

Even in the face of apparent inconsistencies in atherosclerotic models as well as unexpected outcomes of SR-A in human diseases, it is evident that SR-A plays a pivotal role in influencing macrophage biology. Thus, development of drugs that could modulate scavenger receptor function and/or expression could potentially provide a strong basis for novel anti-atherosclerotic therapies, which could ultimately assist in further navigating the impact of SR-A *in vivo*.

1.4.5 SR-A as a therapeutic target

Given the role of SR-A in mediating lipid uptake in macrophages, SR-A is considered a potential therapeutic target for atherosclerosis (246). **Table II** summarizes a number of studies that test natural or synthetic compounds as exogenous therapies to block foam cell formation. Using different macrophage cell types, these studies demonstrate that antagonizing scavenger receptor function or expression leads to reduction in foam cell formation, and the subsequent inflammatory response characteristic of atherogenesis.

Table 2. Compounds that block foam cell formation by inhibiting SR-A function and/or expression

Compound name	Compound source/type	Type of macrophage studied	Effect on SRA mRNA/protein	Effect on lipid uptake/accumulation	Uptake assay method	Ref.
Interleukin-4	Cytokine	Mouse peritoneal and J774	↓ protein	↓	I-125-acLDL	(247)
Interleukin-33	Cytokine	THP-1 and human MDM	↓ mRNA/protein	↓	DiI-acLDL	(248)
Over-expressed GRP-78	Endoplasmic reticulum chaperone	Mouse peritoneal and THP1	N.S	↓	DiI-acLDL	(249)
Peptide H11	Screened from a phage-displayed peptide library	THP1	↓ protein	↓	DiI-acLDL	(250)
Human protein S	Anti-coagulation cofactor	THP1, U937, human MDM	↓ mRNA/protein	↓	Alexa Fluor 488-acLDL	(251)
Heregulin-β1	Growth factor	Human MDM	↓ mRNA/protein	↓	I-125-acLDL	(252)
LY379196	PKCβ inhibitor	Human MDM and THP1	↓ mRNA/protein	↓	DiI-acLDL	(253)
Nobiletin	Citrus flavonoid	J774	N.S	↓	H3-oleate	(254)
DTF	Metabolite of nobiletin	Human MDM and THP1	↓ mRNA/surface protein	↓	DiI-ac/oxLDL	(255)
Lycopene	Plant arotenoid	Human MDM and THP1	↓ mRNA in THP1s	↓ CE synthesis	H3-oleate	(256)
1.35—$1,25(OH)_2D3$	Vitamin D	Human MDM from diabetic patients	↓ mRNA/protein	↓	DiI-acLDL	(257)
Adiponectin	Adipocyte-plasma hormone	Human MDM and THP1	↓ mRNA/protein	↓	DiI-acLDL	(258)
Phenolic acids	Blueberries	Mouse perit. cacs from blueberry fed mice $^{-/-}$	↓mRNA/protein	↓ foam cells *ex vivo*	Oil-red-O staining	(259)
Lovastatin	Statin	THP-1	↓mRNA/protein			(260)
17β-estradiol	Estrogen	THP-1	Not studied	↓	I-125-acLDL	(261)
HSP27	Heat shock protein	U937	Not studied	↓	DiI-acLDL	(262)

Gene therapy has become a novel approach to modulate SR-A expression or activity (see review(246)). For example, an adenoviral vector expressed in macrophages to direct synthesis and secretion of the extracellular domain of SR-A yields a soluble SR-A molecule that may act as a "decoy" (263). This compound was found to block uptake of modified lipids and inhibit foam cell formation *in vitro* (263). Similarly, injection of a recombinant adenovirus vector encoding a similar soluble SR-A decoy into $LDLr^{-/-}$ mice results in a modest reduction in lesion burden (264,265). As such, blocking macrophage lipid accumulation through antagonism of SR-A may impede foam cell formation, offering a new strategy for prevention of morbidity and mortality associated with atherosclerosis.

1.5 NF-κB and atherosclerosis

The nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) is a key transcription factor that controls a wide variety of cellular functions including inflammation, immune regulation, survival, apoptosis, and proliferation (266).NF-κB is a key transcription factor that mediates important macrophage functions including survival, apoptosis, proliferation and inflammation in atherosclerotic plaques (267,268). The classical NF-κB pathway includes the inactive heterodimer, p50/p65, that is present in the cytoplasm and under basal conditions binds to IκB proteins that inhibit NF-κB. In response to inflammatory stimuli, a cascade of phosphorylation events increases the activity of the IκB kinase (IKK) complex that acts to phosphorylate IκBα, thereby leading to its degradation by the proteasome, and consequently, dissociation from the NF-κB dimers. Release of IκBα from p50/p65 promotes translocation of these subunits to the nucleus where they interact with specific promoter regions to modulate transcription of target genes (269). The alternative pathway **(see Figure 32)** is activated by members of the TNF-α family such as

lymphotoxin-β (LyT-β) and involves IKKα phosphorylation of p100 leading to nuclear translocation of the p52 transcription factor (270).

NF-κB has been associated with pro-inflammatory responses in atherosclerosis (271,272); however, emerging *in vivo* data argues that NF-κB also regulates anti-inflammatory processes in the atherosclerotic plaque (273). For example, in a study of atherosclerosis prone $LDLr^{-/-}$ mice with a macrophage-restricted deletion of IKK2 it was observed that the mice developed more severe atherosclerosis and had markedly decreased levels of the anti-inflammatory cytokine, IL-10 (274). In addition, macrophages deficient in the NF-κB p50 subunit have a prolonged production of the inflammatory cytokine, TNF-α in response to LPS and while atherosclerosis prone $LDLr^{-/-}$ mice with a hematopoeitic deficiency for the p50 subunit have smaller lesions (41%) in the aortic root compared to $LDLr^{-/-}$ control mice, these lesions are characterized by an increase in accumulation of inflammatory leukocytes (275). In humans, there is evidence that a genetic deletion in the promoter of the *NFKB1* gene results in decreased levels of the p50 subunit and is associated with a higher risk for coronary heart disease (276-278). Hence, these studies suggest that there are protective roles for NF-κB signaling in atherosclerosis and highlight the importance of macrophages in balancing pro- and anti-inflammatory signals in the developing lesion. Therefore, understanding the environmental conditions that promote the anti-inflammatory properties of NF-κB signaling in macrophages is crucial in the development of novel anti-atherosclerosis therapeutics. Interestingly, Lawrence *et al.* demonstrated that IKKα negatively regulates macrophage activation and inflammation by increasing degradation of p65, as well as via blocking p65 binding to promoter regions of pro-inflammatory genes (279). In addition, inactivation of IKKα in mice enhances

inflammation and bacterial clearance (279). These findings have led to the hypothesis that IKKα balances cross-talk between the alternative and canonical NF-κB pathway. This cross-talk works to limit the classical pro-inflammatory IKKβ– activation response, while regulating adaptive immunity through the canonical pathway (280). NF-κB regulates transcription of a number of inflammatory genes that have varying functions in the cell including membrane proteins, receptors, ligands, kinases, inhibitors, cytokines, adhesion molecules, apoptotic and anti-apoptotic factors, and transcription factors. Examples of genes regulated that play important roles in atherogenesis include the cytokines IL-1β (pro-inflammatory) (281), IL-10 (anti-inflammatory) (282,283), and GM-CSF (284) .

1.6 Heat Shock Protein-27

1.6.1. General introduction about heat shock proteins

In 1962, a co-worker in the Ritossa laboratory accidentally raised the water bath temperature in which larva salivary gland preps from Drosophila were incubating. Upon sub-lethal application of heat, the chromosomes within the cells of these salivary glands displayed a “puffing” pattern. The altered chromosomal pattern as a result of heat activation was interpreted as changes to the expression of particular proteins within the cells of the gland (285). This cellular process was termed the “heat shock response” and the proteins responsible for this effect were called “heat shock proteins”. HSP70 was later discovered as the protein upregulated during Ritossa’s serendipitous discovery, thus heralding the birth of the HSP field.

Over a decade later in 1978, Ron Laskey coined the term “molecular chaperone” to describe the function of nucleoplasmin, a nuclear protein, which was shown to aid in the

formation of the nucleosome, in part, by preventing untoward aggregation between histone complexes and DNA (286). By 1987 molecular chaperones were found to facilitate post-translational folding of proteins by binding with polypeptides as soon as they exit the ribosome complex thereby assisting with their tertiary structure and preventing newly synthesized proteins from aggregation (287).

Heat shock proteins are evolutionarily conserved proteins with high sequence homology across different species. Mammals and bacteria are known, for example, to share up to 50% sequence identity (288). Heat shock proteins are ubiquitously and constitutively expressed in all tissues of the body making up approximately 10% of total cellular protein content under normal conditions, and 15% upon activation of their genes (289).

HSP proteins have been previously classified by size, structure and function (290). The most commonly used classification method in the literature divides HSPs according to seven families based on molecular weights: HSP10, small HSPs (15-30kDa), HSP40, HSP60, HSP70, HSP90, and HSP110. HSPs are also named according to gene symbols with families often sharing similar members as those classified under the molecular weight system. For instance, the small HSP family, also called HSPB, possess 10 members including HSPB1 (or HSP27) and HSPB5 ($\alpha\beta$ crysallin). These members share structural properties due to the presence of the α-crystallin domain core in the protein sequence and are known to be upregulated under pathological conditions (291). Recently, an updated nomenclature system has been proposed to account for the growing diversity of family members within different organisms (reviewed in (292)). Briefly, these include the HSP70 superfamily (HSPA and HSPH families), the DNAJ (HSP40) family, the HSPB (small heat

shock proteins) family, the HSP90/HSPC family, and the human chaperonin families (HSBD/E and CCT).

Heat shock protein function is ascribed to maintaining cellular homeostasis as a chaperone molecule where they assist in assembly, folding, and translocation of target proteins (289). The cytosol is extremely concentrated due to high levels of protein turnover at any given time; therefore, molecular chaperones also function to prevent inappropriate aggregation of newly synthesized unfolded proteins as they exit the ribosome complex (287). They play important roles in cellular surveillance by stabilizing the three dimensional structure of proteins when cells are subject to a variety of stress conditions such as temperature changes (e.g. hyperthermia), nutritional deficiency, ultraviolet radiation exposure, oxidative stress or hypoxia, reperfusion following ischemic injury, and exposure to cytokines (289). Although functional redundancy exists between most members of heat shock protein families, each HSP is known to perform a specific task in the cell. HSP60 for instance, aids in refolding nonnative proteins in an adenosine triphosphate (ATP)-dependent manner, while HSP70 assists in refolding of aggregated proteins (293). Heat shock protein transcription is tightly regulated by heat shock factor (HSF1), a transcription factor that interacts with heat shock elements promoter regions of HSP genes (294).

In the decades that followed their discovery in the 1960's, molecular chaperones were believed to reside purely as intracellular proteins with entirely intracellular functions. However, emerging evidence now beckons the notion that they should in fact be viewed as "multiphase proteins" found both in the extracellular milieu as circulating blood factors, and bound to the cell surface. In 1989, Hightower and Guidon were the first to report that members of the HSP70 family were released from cultured embryonic cells (295); this study

supported previously ignored findings by Tytell showing that molecular chaperones were transported in the extracellular milieu from glial cells to neurons (296). Molecular chaperones are now widely studied as key players in immunological responses where they are secreted from antigen presenting cells and participate in both paracrine and autocrine cell signaling processes.

HSPs play a number of important roles relating to maintaining homeostasis of the vessel wall. The vessel wall and subendothelial space are continually exposed to a number of stress factors such as hemodynamic strain, as well as damaging factors within the blood itself (i.e. pathogens, carcinogens, and oxLDL). Therefore, vascular cells are known to produce high levels of HSPs in an attempt to protect against the potentially damaging effects of pro-inflammatory mediators (297). Accumulating evidence suggests that HSP27 is one of these HSPs that participate not only as an intracellular protective factor but also as an extracellular molecule. HSP27 is upregulated under stress conditions and capable of transducing signaling responses in a number of vascular cells including macrophages. The next series of subsections will focus on HSP27.

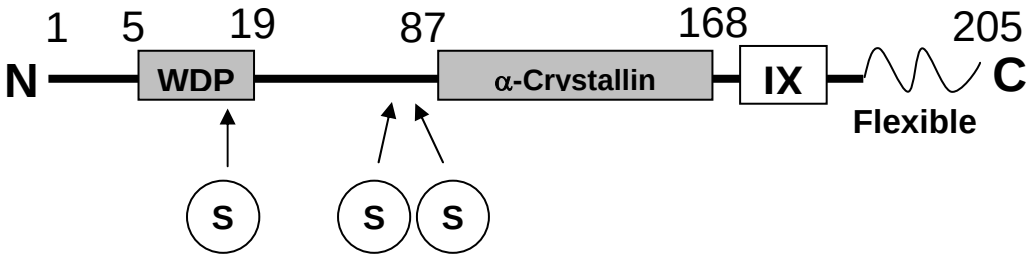
1.6.2 HSP27: Structure and Function

Human HSP27 (and its mouse homolog, HSP25) is an ATP-independent molecular chaperone and a member of the small HSP (sHSP) family. There are 10 known sHSP genes, HSPB1-10, spanning nine chromosomes that produce protein products with different molecular weights, some displaying alternative splicing giving rise to different isoforms with varying homology (reviewed in (298). The HSP27 gene, HSPB1, encodes a sequence of 205 amino acids and contains a number of heat shock binding elements (HSE) (298). Binding of HSFs to HSEs are known to play key roles in cell survival (299,300), division

(301), differentiation (301), and ischemic stress (302). The promoter region of HSP27 also contains an estrogen response element (ERE) that regulates HSP27 transcription in response to estrogen-induced stimulation and binding of estrogen receptors to this promoter region (303,303)

The HSP27 structure is divided into domain sequences (**Figure 5A**). The α-crystallin domain in the C-terminus is highly conserved among species and comprises approximately 40% of the HSP27 amino acid sequence (304). The α-crystallin domain contains β-sheets and is involved in providing a flexible mechanical hinge essential for dimerization and chaperone function (304). The IXI domain is also highly conserved; it is located in the C-terminus and believed to participate in self-assembly of the protein by formation of hydrophobic intermolecular interactions (305). At the flanking region of the C-terminus, although not fully understood, the flexible domain is believed to play a role in binding with target proteins in addition to involvement in oligomerization and solubility of the protein (306). The N-terminus contains phosphorylation sites as well a tandem sequence of amino acid residues containing tryptophan, aspartic acid, praline, and phenylalanine, known as the WDPF motif – these domains are important for oligomerization, as well as chaperone activities (304,306). The phosphorylation sites are also involved in intracellular cell signaling pathways (discussed below).

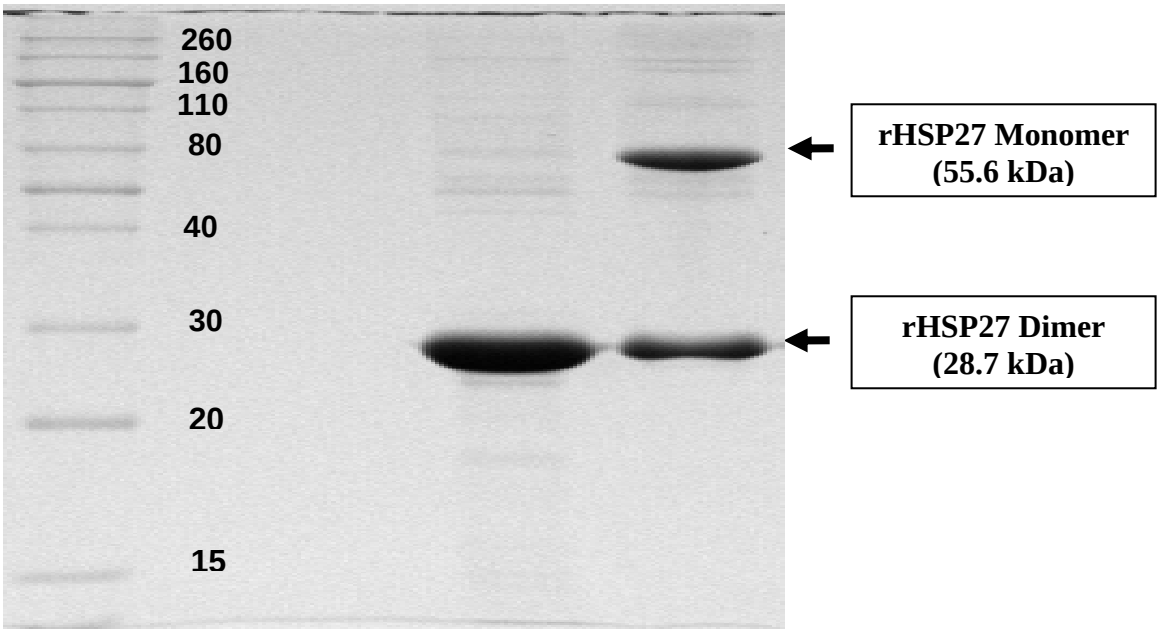
A)


1
5
19
87
168
205
N
WDP
α-Crystallin
IX
C
Flexible
S
S
S

B)


HSP27 WT
1
15
78
82
87
167
179
182
205
N
IXI
C
C1 -HSP27(93-205)
93
167
179
182
205
IXI
C

C)


260
160
110
80
40
30
20
15
rHSP27 Monomer
(55.6 kDa)
rHSP27 Dimer
(28.7 kDa)

D)

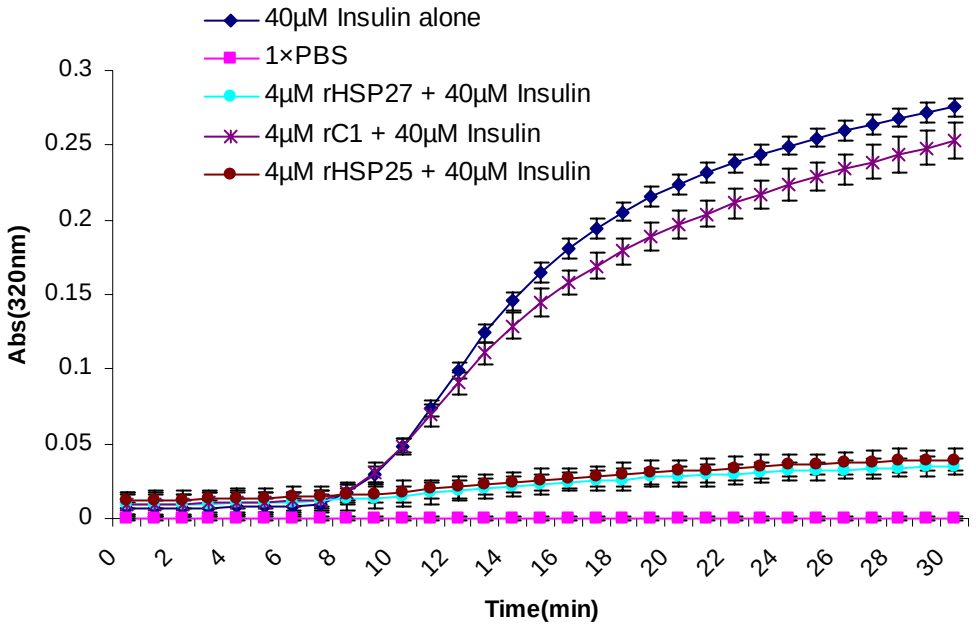


Figure 5. Structural, biochemical and functional features of rHSP27 generated in the O'Brien lab

(A) HSP27 consists of 205 amino acids and has multiple domains including the highly conserved α-crystallin domain, the IXI site, the C-terminus flanking flexible domain, three serine phosphorylation sites in the N-terminus (S15, S78, S82) and the partially conserved WPDF domain (see text for further discussion on structure/function).
(B) Structural schematic of full length and truncated rHSP27 domain sequences generated in the O'Brien lab. rC1 lacks 60% of the full length HSP27 amino acid sequence. Black box: α-crystallin domain; White circles: serine phosphorylation sites
(C) rHSP27 was run on an SDS-PAGE gel under reducing and non-reducing conditions to illustrate its monomeric (reducing conditions) and dimer confirmation (native).
(D) For chaperone activity assays, insulin was incubated with DTT in the presence or absence of rHSP27, rHSP25, or rC1. Aggregation of insulin was determined by measuring absorbance using spectrometry as a function of time (courtesy of Mr. Thomas Hu of the O'Brien Lab).

HSP27 is subject to several post-translational modifications involving classical phosphorylation cascades, many of which are associated with survival pathways following stress conditions (298). These signaling cascades occur extremely rapidly following exposure to stress even before transcriptional activation of HSP27 transcripts can occur - reflecting the role of HSP27 as an early stress responder (298). HSP27 phosphorylation sites are located on serine residues 15, 78, and 82 in the N-terminus of the amino acid sequence (306) and are directly catalyzed by the MAP kinase activated protein kinase (MAPKAP)-2/3 (307-309). The mouse homologue, HSP25, on the other hand contains phosphorylation sites on serine 15 and 86 (306). PKC (310) activates HSP27 phosphorylation in response to phorbol esters (311). Both MAPKAP and PKC are regulated upstream by phosphorylation of p38 MAP kinase (312), implicating many signaling effectors such as growth factors, differentiating cytokines, and oxidative stress in modulating HSP27 phosphorylation states.

HSP27 oligomer size and chaperone activity are tightly controlled by phosphorylation activation states. Unphosphorylated and phosphorylated HSP27 are in constant steady-state, balancing chaperone activity with nascent cellular functions. Under normal conditions phosphorylation tends to decrease the size oligomers promoting actin stabilization (313), whereas conditions of stress tends to induces dephosphorylation and favors assembly of large oligomeric structures involved in chaperone activity, in turn, promoting resistance against cellular damage by helping to restore protein refolding (313). While the large unphosphorylated oligomers have greater potential to provide a sink for misfolded polypeptides serving a cytoprotective function through their chaperone activity (314), phosphorylated dimers function to stabilize the cytoskeleton at the level of F-actin turnover (314) as well as interaction with apoptosis factors (315). Large oligomers also

target denatured proteins to the proteasome (316). Unlike other chaperone molecules, HSP27 functions without the requirement of ATP. Therefore, HSP27 oligomers act as a reservoir to triage misfolded polypeptides until they can become processed by ATP-dependent chaperones (317) or degraded by the proteosome (316).

1.6.3 Intracellular cellular functions

HSP27 is involved in a number of intracellular cellular processes. Phosphorylated HSP27 has been shown to bind to estrogen receptor (ER)-β and influence its cellular localization in response to estrogen signaling (318). As circulating estrogen is known to be protective against atherosclerosis, HSP27 may be an important regulator of estrogen function in the vessel wall (319). Moreover, activation of p38 MAPK, an upstream activator of HSP27, (320) regulates the induction of IL-10 secretion from human monocytes (321). HSP27 blocks TNF-α signaling (322), and suppresses NF-κB activation (323), implicating HSP27 as an anti-inflammatory stimulus. In addition, phosphorylated HSP27 negatively regulates smooth muscle cell function (324) by modulating actin filament dynamics and focal adhesions (306). As implied above, HSP27 regulates remodeling of cytoskeletal elements such as F-actin (325), microtubules (326) and intermediate filaments (327), further supporting its involvement in cellular migration and adhesion pathways (328,329).

HSP27 is cytoprotective under a variety of conditions. As an anti-oxidant, HSP27 protects cells exposed to oxidative challenges via decreasing levels of ROS and NO production (314,330,331). Another mechanism of HSP27 cytoprotection is its ability to inhibit apoptosis by blocking upstream signals that trigger cytochrome c release from mitochondria (332,333), in turn, preventing activation of downstream caspase 3 activity (334,335). Furthermore, HSP27 expression is noted in regions localized to the fibrous cap of

advanced atherosclerotic plaque (336). Taken together with its pro-survival actions, these findings may provide an explanation for HSP27's role in prevention of plaque instability and rupture (337).

Reducing HSP27 expression promotes apoptosis (333,338-340). Since high levels of HSP27 are found in many cancer cells (341,342), inhibiting HSP27 expression is currently a potential target in development of strategies to interfere with cancer progression (313,343). The cytoprotective functions of HSP27 has been further explored in a variety of other pathological conditions such as stroke (344-346), seizures (347), renal disease (348), gastric ulcers (349), myocardial infarction (350,351), Alzheimer's disease (346), and Huntington's disease (352,353). Lastly, the discovery that inherited neuropathologies like Charcot-Marie-Tooth disease are associated with HSP27 mutations (354) supports HSP27 as an important intracellular cytoprotective factor.

1.6.4 Role of HSP27 in atherosclerosis

There is emerging evidence that molecular chaperones have "moon-lighting" functions acting both as traditional intracellular chaperones but also functioning outside the cell as immunoregulatory pluripotent modulators (288). A number of heat shock proteins such as HSP70 (355,356), HSP90, and calnexin are secreted from cells via vesicle-like exosomes (357) in response to stress stimuli. This provides evidence that intracellular chaperone molecules have the ability to exit the cell. In addition, members of the HSP70 superfamily bind the cytoplasmic domain of SR-A in macrophages (357,358). Exogenous HSP27 prevents differentiation of monocytes into dendritic cells by modulating the inflammatory response to TLR4 at the cell surface (359) strengthening the notion that HSPs mediate adaptive and innate immune responses in the context of atherosclerosis.

The O'Brien laboratory, in conjunction with two other laboratories, simultaneous provided evidence to suggest that HSP27 may be a biomarker of atherosclerotic disease. Martin-Ventura et al. found that patients suffering from carotid atherosclerotic disease had lower serum HSP27 level compared to healthy controls (360). The O'Brien lab (336) and the Rose lab (361) reported diminished expression of HSP27 in atherosclerotic plaques compared to vessels free of disease further validating the association between augmented HSP27 levels and protection against atherosclerosis. Despite using a small sample size, Park *et al.* reported elevated HSP27 levels in patients with acute coronary syndrome, and diminished levels of HSP27 in plaque compared to adjacent disease free regions (362). Lastly, a proteomic study demonstrated that HSP27 was elevated in stable compared to unstable plaques further implicating HSP27 in events relating to plaque vulnerability (363).

It is clear that there is a link between HSP27 levels in the serum and expression in the vessel wall with atherosclerosis in humans. Yet, it remained ambiguous whether HSP27 levels are secondary to atherosclerosis as an artifact or stress response during the disease process, or if HSP27 plays a causal role in directly regulating its pathology. The next section provides the chronology of milestone HSP27-related discoveries in the O'Brien lab, and sets up a contextual framework for the work presented in this thesis.

1.7 Background on HSP27 in the O'Brien Lab

It is well known that premenopausal women are protected from CAD compared to age-matched men; however, rates of CAD rise in women after menopause and surpass that of men later in life (364). The differences in CAD between men and women were originally hypothesized by the O'Brien lab to occur through the loss of ovarian estrogen levels in postmenopausal women. Estrogens mediate its biological effects through activation of

estrogen receptors (ER) that bind to promoter regions of target genes. While ERα is largely studied as a regulator of reproductive tissue function as well as in pathological conditions such as uterine and breast cancers, ERβ is found differentially expressed in non-reproductive tissue such as in the heart. For example, Christian *et al.* showed that ERβ but not ERα was abundantly expressed in coronary arteries of post-menopausal women and correlated with advanced atherosclerosis suggesting that estrogen mediates its cardioprotective actions through regulation of ERβ in the vessel wall (365). Hence, the O'Brien lab postulated that novel proteins may associate with ERβ to regulate its protective functions. In 2005, the O'Brien lab discovered HSP27 as an ER-β associated protein that functions as a co-repressor of estrogen signaling *in vitro* (336). Interestingly, this study further showed that HSP27 expression decreases, and is proportional to the increased stage of CAD in humans – an effect that was independent from ERβ in the vessel wall. As previously indicated above, simultaneous findings by De Souza *et al.* and Martin-Ventura *et al.* confirmed that HSP27 levels were diminished in regions of the vessel wall that contain plaque versus those regions which are disease free (360,361). In 2008, to further explore the role of HSP27 in atherosclerosis, the O'Brien lab crossed $ApoE^{-/-}$ mice with HSP27 overexpressing mice (262). When HSP27 overexpressing mice were fed a high fat diet they exhibited higher HSP27 serum levels (>10 fold increase) and atheroprotection compared to littermate controls, but only in the females. In fact, correlation of HSP27 serum levels with lesion size revealed decreased HSP27 serum levels associated with increased lesion size, suggesting that augmented levels of HSP27 is required to confer its protective effects. In addition, *in vitro* data showed that estrogens promoted the release of HSP27 from macrophages into the serum.

Next, two papers were published in the O'Brien lab demonstrating the role of estrogen in HSP27 mediated atheroprotection effects. Using an ovariectomy model, removal of estrogens from HSP27 overexpressing mice blunted the reductions in lesion formation compared to mice with intact ovaries (366). However, infusion of estrogen therapy to the ovariectomized transgenic mice rescued the protective effect previously observed in the HSP27 overexpressing animals, demonstrating that HSP27 serum levels are directly associated with atherosclerosis. Moreover, treatment of $ApoE^{-/-}$ mice with 8βVE2, an ERβ specific agonist, reduced lesion size and induced secretion of HSP27 from macrophages *in vitro* (367).

To explore the cells that were instrumental in producing HSP27 mediated atheroprotection (e.g., vascular smooth muscle cells vs. cells of hematopoietic origin) transplantation of HSP27 overexpressing bone marrow cells into $ApoE^{-/-}$ recipients was performed. $ApoE^{-/-}$ recipients receiving HSP27 overexpressing cells showed dramatic reductions in lesion burden demonstrating that immune cells are central for HSP27 responses *in vivo* (368). This study supported the 2008 report from the O'Brien lab showing that extracellular HSP27 modulates macrophage biology by competing with SR-A for acLDL thereby inhibiting foam cell formation and favoring a sequelae of downstream inflammatory responses involving secretion of the anti-inflammatory IL-10, and blocking release of the pro-inflammatory IL-1β (262).

In a chronic model of atherosclerosis where HSP27 overexpressing $ApoE^{-/-}$ mice were fed a high fat diet for 12 weeks, the protective response of HSP27 was not limited to female mice (as observed in the previous acute model). Atheroprotection was achieved in both male and female mice in the aforementioned chronic experiment and was accompanied

by elevated HSP27 serum levels as well as reduced lipid, macrophage content and apoptosis in the plaque (369). However, it remained unclear how HSP27 was regulating cholesterol handing in the lesion and how it interacted with SR-A at the surface of macrophages to attenuate foam cell formation.

To explore the utility of extracellular HSP27 as an anti-atherogenic therapeutic, the O'Brien lab recently generated purified rHSP27 using bacterial expression systems. Initial studies revealed that treatment of female and male ApoE$^{-/-}$ mice fed a high fat diet concomitant with subcutaneous injections of rHSP27 for 3 weeks lead to a reduction in lesions compared to control groups (370). This was accompanied by reductions in total serum cholesterol levels, as well as decreases in macrophage content and apoptosis in the lesion. Finally, the number of apoptotic cells and macrophage content in the plaque were reduced by 80% and 45% respectively with rHSP27 treatment. Preliminary pharmokinetics revealed that injection with rHSP27 was sufficient to raise serum HSP27 levels 100-fold higher than levels observed in the HSP27o/e model, e.g. 200 ng/ml at the half life of rHSP27 injection (90 min) compared with 2 ng/ml in the HSP27o/e mice, which may provide an explanation for the ability of HSP27 to overcome the aforementioned sex-dependent effects (communication with Dr. Charles Cuerrier). As will be described, the O'Brien lab has also produced rHSP25 (murine homologue) plus a truncated form C-terminus form (C1) that appears to be a biologically inactive control.

2.0 RATIONALE AND STATEMENT OF HYPOTHESIS / OBJECTIVES

2.1 Rationale

The focus of this thesis is HSP27, a protein that the O'Brien lab initially discovered to associate with ERβ and subsequently as an anti-atherogenesis (or "atheroprotective") factor (336). Moreover, while heat shock proteins are generally thought to function inside the cell as molecular chaperones, it was the release of HSP27 into the extracellular space / serum that was found to be critical for the modulation of atherogenesis (262). Three important observations arose from these initial studies: 1) Extracellular HSP27 interacts with SR-A in macrophages where it favorably modulates downstream anti-inflammatory processes, 2) Atheroprotection in HSP27 o/e ApoE$^{-/-}$ mice is associated with reduced macrophage and cholesterol accumulation in the lesion, and 3) rHSP27 administered systemically during the early stages of atherogenesis (3 weeks of a high fat diet or HFD) in ApoE$^{-/-}$ mice led to therapeutic benefits (370). There is convincing evidence that HSP27 appears to modulate macrophage biology by regulating cholesterol uptake in the vessel wall. Nonetheless, gaps remain in the understanding of how HSP27 confers atheroprotection. Specifically, how HSP27 interacts with SR-A in macrophages to control foam cell formation and accumulation of macrophages in the lesion is yet to be determined. SR-A is a crucial player in initiating foam cell formation and the inflammatory response central for lesion development. Given the role of SR-A in atherogenesis, this thesis asks two important questions: 1) How does HSP27 interact with SR-A to regulate foam cell accumulation?; and 2) Is SR-A is an essential target for HSP27 atheroprotection? Therefore, in order to advance the exploration for the utility of HSP27 as a new, endogenous therapeutic for vascular

disease, the central purpose of this thesis is to elucidate the mechanism(s) by which HSP27 is atheroprotective - focusing specifically on its effects on SR-A.

2.2 Statement of hypothesis / objectives

2.2.1 Hypothesis

HSP27-mediated atheroprotection requires the presence of SR-A.

More specifically, three sub- hypotheses include:

A) Extracellular HSP27 inhibits foam cell formation by blocking acLDL uptake in macrophages via modulation of SR-A expression in macrophages.

B) The effect of HSP27 on SR-A expression is NF-κB-dependent.

C) SR-A is important for HSP27 atheroprotection *in vivo*.

2.2.2 Global Objectives

The global objectives of this thesis are to elucidate HSP27's effect on SR-A to inhibit foam cell formation, and whether this interplay is important for HSP27 atheroprotection *in vivo*.

Four specific objectives were pursued:

A) Explore whether extracellular HSP27 modulates acLDL uptake and binding in human and mouse macrophages.

B) Determine if HSP27 modulates SR-A expression at the gene and protein level.

C) Determine whether HSP27 mediates signaling events downstream of the cell surface, and whether interaction with NF-κB is involved in these biological responses.

D) Determine if SR-A is required to mediate HSP27 anti-atherogenic responses *in vivo* by injecting rHSP27 into $ApoE^{-/-}$ $SR\text{-}A^{-/-}$ mice.

3.0 METHODS AND MATERIALS

3.1 O'Brien Lab recombinant proteins

3.1.1 Production and synthesis

N-terminal His-tagged full length HSP27 or HSP25 DNA was constructed into a pET-21a vector. The plasmids were then transformed into *Escherichia coli* (E.coli) expression strain (Rosetta cell set, Calbiochem - Mississauga, Canada). For the HSP27 truncated mutant (rC1), amino acid 1-92 of the full length HSP27 N-terminus was cloned as previously described. Recombinant proteins were purified with Ni-NTA resin (Qiagen, Duesseldorf, Germany) and refolded by dialysis. Endotoxin was determined using the Limulus Amebocyte Lysate (LAL) test as per manufacturer's instructions (Lonza, Basel, Switzerland). Removal of endotoxin was performed using Detoxi-Gel Endotoxin Removing Gel as per manufacturer's instructions (Thermo Scientific, Ottawa, Canada). After purification, endotoxin levels were determined to be less than 5 endotoxin units (EU) per mg consistent with acceptable levels for cell culture (371,372), and studies using murine models (373). Purity of the final recombinant proteins were determined to be > 95% by sodium dodecyl sulfate polyacrylamide (SDS-PAGE). After production and purification of each lot of recombinant proteins were run on a 10% an SDS-PAGE gel either in the absence or presence of DTT (to bread disulfide bonds) to confirm proper folding and oligerimization.

3.1.2 Chaperone Activity Assay

To measure the functional chaperone activity of recombinant proteins DTT-induced aggregation of insulin was performed in the absence or presence of rHSP27, rHSP25, rC1 and PBS at pH 7.4. Insulin (40 μM. Novo Nordisk – Denmark) was incubated with 4μM rHSP27, rHSP25 or rC1 in a PBS solution containing 20mM DTT (Sigma, St. Louis, MO)

and incubated for 10min at 43°C. Aggregation was monitored by measuring the absorbance at 320nm in a Bioteck Synergy Mx (Winooski, VT) spectrophotometer for 1 min. intervals up to 30min (374).

3.2 Cell culture systems

3.2.1 Human acute monocytic leukemia cell line (THP-1)

The human THP-1 monocytic leukemia cell line was obtained from American Type Culture Collection (ATTC, Manassas, VA) and grown in complete RPMI 1640 media (Hyclone, Ottawa, Canada) containing 10U/ml penicillin/streptomycin (Gibco, Burlington, On), 5mM sodium pyruvate and 10% fetal bovine serum (FBS) (Gibco) at 37°C and 5% CO_2 according to manufacturer's instructions. Subcultures were split every three or four days and maintained at 0.2-1x10^6 cells/ml as per manufactures instructions. Cell counts were routinely performed with a Beckman Coulter counter (Mississauga, Ontario) system using trypan blue dye exclusion for determination of viability. For all experiments, except where otherwise indicated, undifferentiated cells, between passage 3 and 16, were seeded at 1x10^6 cells/well in 24-well plates, and stimulated with 50 ng/ml phorbol 12-myristate 13-acetate (PMA, Sigma - St. Louis, MO) to induce differentiation into a macrophage-like phenotype as previously described (169,375). Cells were considered differentiated into mature macrophages after exposure to PMA for 2 days and used as the primary *in vitro* model to reflect the function and regulation of macrophages in the vessel wall (376).

3.2.2 Human monocyte derived macrophages (hMDM)

Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood of healthy individuals by Histopaque (Sigma, St. Louis, MO) gradient centrifugation. PBMCs

were seeded at 10 x 10^6 cells in 24-well plates in complete RPMI media (Gibco, Burlington, On). Media was changed 2 hours after initial seeding, then once every 48 hours thereafter to remove non-adherent contaminating cell populations such as platelets, and lymphocytes. After 7 days, spindle shaped adherent cells were considered differentiated macrophages as described previously (377).

3.2.3 Mouse bone-marrow derived macrophages (BMDM)

Bone marrow cells were isolated from at least 8 week old female or male $ApoE^{-/-}$ or $SR\text{-}A^{-/-}$ $ApoE^{-/-}$ mice. Bone marrow from the tibia and femur of excised hind limbs were flushed out, and seeded at 1 x 10^6 cells/well in 24 well plates. Cells were grown and maintained in DMEM (Gibco, Burlington, On) supplemented with 20% L929 (ATCC, Manassas, VA) conditioned media (containing secreted M-CSF), 10% FBS, and 10U/ml penicillin/streptomycin. M-CSF is known to play a role in differentiation of macrophage populations and used in previous studies as a macrophage differentiation factor for cells of bone marrow origin (378,379). Incubation for 10 days rendered the cells into differentiated macrophages with previously described characteristics including spindle morphology, Mac-2 staining, and acLDL uptake (379).

3.2.4 THP-1 Blue Cells – NF-□B reporter cell line

THP-1 Blue cells were purchased from Invitrogen (San Diego, CA). They are a human monocytic cell line stably transfected with an NF-κB inducible secreted embryonic alkaline phosphatase (SEAP) gene. Stimulation of the NF-κB pathway activates the SEAP reporter gene, which leads to translation of SEAP protein and subsequent secretion into the media. It has previously been reported (ref) that assayed SEAP levels directly reflect levels

of NF-κB activity. THP-1 Blue cells were maintained at 0.2-1 x 10^6 cells/ml, and subcultured in complete RPMI-1640 media supplemented with Zeocin (100ug/ml, InvivoGen - San Diego, California) –to select for positive reporter clones. For NF-κB activity experiments, cells were seeded at 1 x 10^5 cells in 96 well plates in complete RPMI media in the absence of Zeocin. Cells were differentiated with PMA (50ng/ml) for 24 hours followed by addition of fresh media without PMA. Following experimental treatment, conditioned media from each replicate group was measured for the presence of SEAP using Quanti-blue medium as per manufacturer's instructions (Invitrogen, San Diego, CA).

3.2.5 Chinese hamster ovary (CHO) cells stably transfected with mouse SR-A

CHO-SR-A and mock (CHO-K1) cells were obtained from Dr. Dawn Bowdish (McMaster University, Hamilton, Ontario, Canada). CHO cells which otherwise do not express SR-A, were stably transfected with a mouse SR-A1 plasmid under the control of a *Cytomegalovirus* (CMV) promoter to induce constitutively active expression of the target gene. Cells were used between passages 2-5. They were maintained and subcultured in DMEM supplemented with 5% FBS and 10U/ml penicillin/streptomycin. For uptake and immunoblotting assays, cells were seeded at 1 x 10^6 cells well in 24 well dishes.

3.3 Treatment strategy for recombinant proteins and other blockers or inhibitors

Cells were treated with molar equivalent concentrations of recombinant proteins for consistency between the different proteins (rC1 and rHSP27/25 have different amino acid sequence lengths) and ease of interpreting the data in the text. Since the sequence of rC1 is 60% less than rHSP27 and rHSP25, the molar equivalent of the full length proteins were determined based on the number of amino acids present in the sequence and the amount of

protein used in the experiment. For example, 9.6 μM is equivalent to 250 μg/ml of rHSP27 (and rHSP25) and 150 μg/ml of rC1 For experiments involving THP-1 cells, recombinant proteins were added to the media either at the same time as addition of PMA (then incubated for 2 days to induce differentiation), or 24 hours after the 2 day PMA differentiation process. For uptake assays involving receptor competition experiments, recombinant proteins were added either 1 hr prior to addition of acLDL or simultaneously. In SR-A gene experiments involving qPCR, cells were treated for either 6 or 24 hours after PMA differentiation as indicated. Moreover, in some experiments 10 ng/ml polymixin B (PMB, Calbiochem), was added to the media at the same time as the recombinant proteins or LPS (Sigma and Calbiochem) to neutralize endotoxin contamination (380,381). For experiments involving THP-1 Blue, hMDM, or BMDM cells were treated with recombinant proteins or LPS for 24 hours after the respective differentiation processes. Cells were pre-treated with fucoidan (10 and 50μg/ml - Sigma), and antibodies against SR-A for 1 hour before addition of DiI-acLDL. Anti-hSR-A1 (R&D Systems, Minneapolis, MN) was used for THP-1 cells and the 2F8 clone used for CHO-SR-A1 cells (AbD Serotec, Raleigh, NC). In some experiments, 250 μg/ml unlabeled acLDL (Biomedical Technologies, Stoughton, MA) was added to cells at the same time as DiI-acLDL. In experiments testing the involvement of the NF-κB pathway, cells were pre-treated with the NF-κB inhibitor, BAY11-7082 (Calbiochem) at the accepted concentration (10 μM) (382) for 1 hour prior to addition of rHSP27 or LPS.

3.4 AcLDL uptake assays

Uptake of acLDL was confirmed by multiple techniques including flow cytometry, confocal microscopy, fluorescent plate reader method, and oil red O staining. Except for oil red O, these methods involved the use of a fluorescent probe, 1,1'-dioctadecyl-3,3,3'3'-tetramethylindocarbocyanine perchlorate (DiI) conjugated to acLDL (Molecular Probes, Invitrogen) (383,384). Unless otherwise specified, cells were incubated with DiI-acLDL at 5μg/ml for 3 hours prior to being assayed for the level of incorporated fluorescence using the techniques as described below.

3.4.1 Flow cytometry analysis

For flow cytometric analysis following incubation with DiI-acLDL, cells were washed twice with ice cold PBS to rinse away excess dye and cell debris. Cells were then incubated with detachment buffer (5mM EDTA in PBS pH 7.4 and RPMI 1640 containing 10% FBS) for 15 min at 37°C, then lifted by gently flushing of the well with a pipetter. Cells were filtered by passing the cell suspension solution through a 70μM strainer (BD Biosciences, Franklin Lakes, NJ) before being spun at 400g for 5mins at 4°C. The pellet was resuspended in wash buffer (0.13% EDTA and 1% FBS in PBS) to prevent cell clumping, spun again, and then resuspended in 300ul of PBS containing 1% BSA (Sigma). At least 100 000 cells were analyzed in a BD FACSAria (San Jose, California) using system software (BD FACS Diva version 6.1.2). Baseline fluorescence values and gating were based on cells not incubated with labeled lipoproteins. Representative histograms are displayed next to each graph and show the amount of fluorescence as a function of cell population. Percent positive Dil-acLDL cells were interpreted as the amount of labeled lipoproteins incorporated

into the cell. For some datasets, the percent positive DiI-acLDL cells in each experimental group were adjusted and plotted as a percent of the no treatment control.

3.4.2 Confocal microcopy and live cell imaging

Confocal microscopy imaging of live cells was performed to simultaneously visualize incorporation of acLDL into the cell and measure the amount of dye being taken up. THP-1 cells were seeded at 6 x 10^5 cells/well into 24 well plates on glass coverslips. Following treatment and incubation with DiI-acLDL, cells were washed twice with PBS then incubated with 25 μg/ml Hoechst (Sigma) to label nuclei. Coverslips were mounted onto live cell imaging chamber slides in media supplemented with 10mM Hepes buffer to maintain pH during processing of images. Slides were then placed into a heated chamber fixed onto the microscope stage. Using identical parameters for each control or experimental group, fluorescent images in the DiI channel (excitation: 551nm; emission; 565 nm) and Hoechst channel (excitation 353nm; emission 435 nm) were captured in triplicates at 1000x magnification using oil immersion. To quantify the level of fluorescence, integration of fluorescence intensity was determined by software analysis in each DiI image and normalized to the number nuclei counted in the 1000x field. The ratio of DiI fluorescence/cell number was further plotted as a percent of the no treatment control.

3.4.3 Fluorescent plate reader method

THP-1 cells were seeded at 1x10^5 cells/well into 96 well plates. DiI-acLDL was added to the cells for 3 hours following pre-treatment with recombinant proteins. Cells were washed at least twice with PBS to remove excess dye. Complete media was then added to the cells before fluorescent signal was measured in a SynergyMX (BioTech) fluorescent

plate reader at an excitation of 520nm and emission of 580nm. Cells were then incubated with Hoechst (25μg/ml) for 15 min at 37°C then washed three times with PBS. Hoechst fluorescence was measured at an excitation of 348 nm and emission of 455 nm. AcLDL uptake was analyzed by calculating a ratio of the DiI to Hoechst fluorescence.

3.4.4 Oil Red O foam cell assay

BMDM isolated from ApoE$^{-/-}$ and ApoE$^{-/-}$SR-A$^{-/-}$ were seeded onto glass coverslips and differentiated as previously described above. To confirm the impact of SR-A in foam cell formation, macrophages were incubated with 50 μg/ml acLDL (Biomedical Technologies) for 24hr then evaluated for foam cell formation as previously described (385). Briefly, cells were washed twice with PBS then fixed in 4% paraformadehyde (PFA, Sigma) in PBS for 10 min. Cells were then rinsed in 60% isopropanol for 15 seconds to facilitate staining of neutral lipids followed by staining with 6:4 filtered oil Red O (Sigma) working solution to water at 37°C for 10 min (stock solution prepared using 0.5% w:v Oil red O in 100% isopropanol pre-warmed to 60°C). Cells were rinsed with 60% isopropanol for 15 sec then rinsed 3X with PBS for 3 min. Cells were then counterstained with hematoxylin, washed with PBS to remove excess stain then mounted on glass coverslips using 0.5% glycerol solution. Images were captured using a light microscope with oil immersion at 1000X magnification. Positive-stained (red) cells were considered macrophages-derived foam cells.

3.5 AcLDL binding assays

Macrophages were seeded at 1x10^5 cells/well in quadruplicate in a 96-well plate. Binding assays involved incubation of cell with DiI-acLDL at 4°C for 2hrs (rather than 37°C for uptake assays to prevents endocytosis of ligand/receptor complex into the cell (386).

Cells were then washed extensively to remove unbound dye. Fluorescent signal was measured using the SynergyMx plate reader at 520nm excitation and 580nm emission specta.

3.6 Quantitative Real Time Polymerase Chain Reaction (q-PCR)

Total RNA was extracted from THP-1 cells with TRIZOL reagent (Invitrogen) according to manufacturer's instructions. RNA quality (integrity, purity, and degradation) were assessed by size chromatography on RNA 6000 Agilent Nano chips using the Agilent 2100 Bioanalyzer instrument and software (Mississauga, ON). Samples with relative index numbers (RIN) above 7 were considered acceptable for further processing as per manufacturer's instructions and published guidelines (387). RNA concentration was determined using a Nanodrop Spectrophotometer ND-100 (Fisher Scientific, Ottawa, Canada) before purification by RNAeasy Mini Kit as per manufacturer's instructions (Qiagen, Duesseldorf, Germany). Total RNA was converted to cDNA with a Transcriptor First Strand cDNA synthesis kit (Roche Scientific, Laval, Quebec) in a final volume of 20 μl using 1 μg RNA and anchored-oligo(dT)18 primers according to manufacturer's instructions. qPCR was performed in a Roche 480 LightCycler. All qPCR experiments were performed in triplicate using the LightCycler 480 SYBR Green 1 Master kit (Roche). Each reaction (20ul) contained 5μl template cDNA, 0.5μM of each primers, 10 μl Master Mix (containing buffer, dNTPs, SYBR Green, and Tag polymerase), and 3 μl PCR-grade water. The following primers were used for SR-A: sense - TGGAATAGTGGCAGCTCAACTCC; antisense – CATTTCCTCTTCGCTGTC ATTTCCTT, and for β-actin: sense - GCCATCCTGCGCTGACTGACTACC; antisense - GGCGACGTAGCACAGCTTCTCC. The amplification program consisted of 1 cycle at 95°C for 5 minutes, followed by 45 cycles with a melting phase at 95°C for 10 seconds, an annealing phase at 60°C for 5 seconds, and

an elongation phase at 72°C for 8 seconds. A melting curve analysis was performed after amplification to verify the accuracy of the primer product. For verification of the correct amplification, a selection of PCR products was visualized on an ethidium bromide-stained 1% agarose gel. A calibration curve was generated for SR-A and β-actin primers to determine their efficiencies using the following purified amplicon serial dilutions: $1x10^4$, $1x10^5$, $1x10^6$, and $1x10^7$. Crossing point values were determined using the Roche LightCycler 1.5 software. Relative expression ratios between experimental and control groups using target and reference gene efficiencies and crossing points (Cps), were calculated using the Pfaffl method as previously described (388).

3.7 Western Blotting

Immunoblotting was used to semi-quantitatively measure the level of SR-A protein expression in total cellular lysate. Media was aspirated from THP-1 and CHO cells followed by washing twice with ice cold PBS. Cells were lysed with RIPA buffer (5M NaCL, 1M Tris, 10% SDS, Triton X-100, 0.25g Na-deoxycholate, and 1 tablet of complete protease inhibitors (Roche, Scientific), and incubated on ice for 30 min to allow protein digestion to occur. Insoluble cell components were pelleted by spinning lysate at 13.3 x 10^3 RPM at 4°C for 20min. The supernatant containing soluble proteins were removed and quantified using a BCA protein assay kit (Thermo Scientific, Ottawa, Canada). Cell lysates were then prepared in 5X non reducing sample buffer (0.25M Tris-Cl pH 6.8, 10% SDS, 50% glycerol, and bromophenol blue). 25 μM of protein was resolved by 8% SDS-PAGE for anti-hSR-A blots and 6% gels for anti-CD204 (2F8 clone). Proteins were transferred to polyvinylidene difluoride (PVDF) membranes for 7 min at 20 volts using the semi-dry iBlot apparatus (Invitrogen, San Diego, CA). PVDF membranes were blocked with 5% skim milk in tris

buffered saline supplemented with 0.5% Triton X (TBS-T) for 1hr, probed with mouse anti-human SR-A1 (1:500, R&D), or at anti-mouse CD204 (1:700, Serotec), or mouse anti-β-actin (1:100 000, Abcam - Cambridge, MA) antibodies. Blots were then incubated with goat anti-mouse IgG conjugated to horseradish peroxidase (HRP) secondary antibody (1:5000 for hSR-A1 blots; 1:10000 for β–actin blots, Santa Cruz - Santa Cruz, California), or goat anti-IgG-HRP (1:5000, Millipore - Billerica, MA) for CD204 blots. Finally, blots were incubated for 5 min with a chemiluminescence substrate, ECL plus (GE Health Care - Baie d'Urfe, Quebec). Bands were detected using an Alpha Innotech (Santa Clara, California) imaging station and densitometry performed using AlphaEaseFc software. For each experiment, quantification of target protein band density was standardized to β-actin as a control.

3.8 SR-A surface expression

3.8.1 Flow cytometry

To quantify surface expression of SR-A using flow cytometry, cells were lifted with detachment buffer, filtered, spun, and washed once in washing buffer as previously indicated above for acLDL uptake assays. Cells were resuspended in 2% BSA in PBS, and then incubated with 0.5μg of anti-human SR-A1 or IgG2B isotype control conjugated to phycoerythrin (PE, antibodies obtained from R&D). Unbound antibody was removed by washing the cells twice in PBS. At least 100 000 events were analyzed in a BD FACSAria using the PE channel. Gating was set according to basal fluorescence in unlabeled cells. The level of surface SR-A was calculated by plotting the percent positive PE labeled cells as a percentage of the no treatment control.

3.8.2 Confocal microscopy

Confocal microscopy was also used to visualize the SR-A surface expression. Live cell imaging was performed as previously described.

3.9 Cell viability and apoptosis assays

3.9.1 MTT assays

To rule out that differences between experimental and controls are the result of reduced cell number due to cytotoxicity effects, cell viability was assessed with a (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells were incubated with MTT solution (5mg/ml, Sigma) for ~4hr. Media containing MTT was aspirated followed by solublization of blue crystals with 2-propanol and 0.2M HCL (7.3:1 mixture). Absorbance, which is proportional to the number of surviving cells, was read at 570 nM in a SynergMx spectrophotometer.

3.9.2 Annexin-V staining

To rule out the possibility that recombinant protein treatment is activating apoptosis, cells were labeled with PE-Annexin V and the cell viability marker 7-AAD as per manufacturer's instructions (PE Annexin V Apoptosis Detection Kit I, BD Scientific -). Cells were processed for flow cytometry analysis as indicated above. At least 100 000 events were run and analyzed using appropriate gating and colour compensation in cells that received no antibody and dual stains, respectively.

3.10 Mouse models

3.10.1 Genotyping

All animals used in this study conform to the University of Ottawa Animal Care and Veterinary Services (ACVS) standard operating procedures with respect to handling, husbandry, and ethics guidelines. $ApoE^{-/-}SR\text{-}A^{-/-}$ (double knockout, KO) mice were received from Dr. Kathryn Moore (New York University, New York) backbred at least 10 generations into the C57/BL6 strain. Littermates were generated from crossing heterozygous $SR\text{-}A^{+/-}ApoE^{-/-}$ mice to each other to generate $SR\text{-}A^{+/+}ApoE^{-/-}$ followed by inbreeding these mice to produce $ApoE^{-/-}$ mice. Genomic DNA from ear and/or tail clippings were extracted using QIAGEN DNeasy Blood and Tissue kit according to manufacturer's instructions and genotyping for MSR and ApoE PCR were performed as previously described (227). Briefly, genotyping of ApoE and SR-A knockout mice involve the use of three primers that span either the coding region or interrupting insert of the respective model. For genotyping of ApoE mice the following primers were used: primer #1: GCCTAGCCGAGG GAGAGCCG; primer #2: TGTGACTTGGGAGCTCTGCAGC; primer #3: GCCGCCCCGACTGCATCT. Primer #1 and #2 amplifies 155 base pairs (bp) of the wild type allele while primer #1 and #3 amplifies 245 bp of the targeted allele. For genotyping of SR-A mice the following primers were used: primer #1: AGAATTTCAGCATGGCA ACTG; primer #2:CCATCTGCACGAGACTAGTGAGAC; and primer #3: ACGGACTCTGA CATGCAGTG. Primer #1 and #2 will amplify a 400 bp band from the targeted allele while primer #1 and Primer #3 will amplify a 200 bp band from the wild type allele. DNA samples were loaded onto agarose gels supplemented with 25μg/ml ethidium bromide (Sigma).

3.10.2 HSP25 injection strategy

Mice were fed a normal chow diet (Harlan, Indianapolis, IN) until 7 or 8 weeks of age. Concomitant with the start of twice daily (B.I.D) subcutaneous injections (S.C) of 50μg rHSP25 or PBS (both 50μl), mice were placed on a high-fat diet *ad libum* consisting of 1.25% cholesterol and 15.8% fat (Harlan). S.C injections commenced for three weeks with a minimum of 8 hours between the morning and afternoon doses. Injections were performed at the nape region of the neck using 1cc insulin syringes (Terumo, Somerset, NJ). The dose of rHSP27 and 3 week timeline was chosen based on previous optimization experiments from the O'Brien using different over 3 weeks. In addition, given the large amount of recombinant protein required to carry out these experiments, the 3 weeks duration was chosen due to the limited quantity available at this time. Three weeks of a high fat diet induced early lesion formation and the extent of lesion formation in our model was significant and measurable at the time of sacrifice as compared to other studies using similar models (389). Body weight was measured on the first and last day of injection.

3.11 Serum cholesterol measurements

After completion of the final injection, mice were euthanized and blood samples were collected in serum separator tubes (BD Scientific) via puncture of the left ventricle for analysis of total serum cholesterol using an enzymatic assay kit as per manufacturer's instructions (Wako Pure Chemical Industries, Ltd, Osaka, Japan). Serum samples were run using high performance liquid chromatography (HPLC) to obtain cholesterol profiles of lipid fractions (262).

3.12 Preparation of the en face aorta and evaluation of atherosclerosis

The aorta was microdissected from the ascending aorta to the thoracic segments and the adventitial tissue was removed before it was pinned to a black wax surface using micro-needles and imaged using an Olympus BX50 microscope (Center Valley, Pennsylvania). Thereafter, the aorta was opened longitudinally with the primary incision following the lesser curvature of the arch. To obtain a flat preparation for imaging, a second incision was made along the greater curvature of the arch down to the level of the left subclavian artery. Lipid-rich intraluminal lesions were stained with oil red O (0.5% in propylene glycol) and imaged as previously described (262). The *en face* atherosclerotic lesions of aorta were analyzed by three blinded independent observers using Image-Pro software (Media Cybernetics, Silver Spring, MD) to calculate the total and atherosclerotic lesion areas. As described previously the extent of atherosclerosis was expressed as the percentage of surface area of the entire aorta covered by lesions (262,390).

3.13 Preparation of aortic sinus and evaluation of atherosclerosis

The top half of the heart containing the aortic root was frozen in Tissue-Tek O.C.T. media. Serial 10-μm sections of the aortic sinus with valves were cut, beginning at the level where the aortic valve first appears, and stained with hematoxylin and eosin (H&E), Masson's trichrome and oil red O. Additional tissue sections were used for immunohistochemical / immunofluorescence labeling. For the quantification of atherosclerotic lesion areas all micrographs were captured with a bright field / fluorescence microscope (Olympus BX60, magnification at 40-1000) and analyzed by two observers using Image-Pro software. Lesion area data for each mouse is presented as the mean lesion area of six sections.

3.14 Quantification of macrophage content in lesions

Immunolabeling of tissue sections for macrophage content was performed using the immunofluorescence method as described previously (391). Briefly, the *en face* aortia or serial 10-μm sections of aortic sinus were pre-incubated with 10% normal horse serum in PBS for 5 minutes followed by an rat anti-mouse macrophage primary antibody (Mac-2; Accurate Chemical and Scientific Corp., Westbury, New York, USA) diluted in PBS 1:500 at 4°C overnight. After repetitive rinsing with PBS, the sections were incubated with a FITC-conjugated anti-rat IgG second antibody (1:100; Vector Laboratories - Burlington, Ontario) for 20 minutes at room temperature (RT) before addition of the nuclear fluorochrome Hoechst 33258 for 5 minutes at RT (0.1 μg/ml; Sigma). The immunopositive areas in cross sections were quantified using Image-Pro software according to previously described techniques (262,390).

3.15 Immunohistochemical expression of SR-A in the vessel wall

Immunolabeling of tissue sections for SR-A content was performed using the avidin-biotin-alkaline phosphatase method (Vector Laboratories, as described previously (390). Briefly, serial 10-μm sections of aortic sinus were pre-incubated with 10% normal horse serum in PBS for 5 minutes followed by an goat anti-mouse SR-A primary antibody (A-20, Santa Cruz) diluted in PBS 1:50 at 4°C overnight. After repetitive rinsing with PBS, the sections were incubated with a biotinylated rabbit anti-goat secondary antibody (1:100, Vector Laboratories) for 10 minutes at room temperature. The endogenous peroxidase was quenched with 3% H_2O_2 for 10 minutes. Antibody reactivity was detected using an ABC kit (Vector Laboratories, and visualized with diaminobenzidine (DAB) / hydrogen peroxidase

as chromogenic substrate, resulting in a brown-colored precipitate at the antigen site. As a negative control, tissue sections from each mouse were subjected to the same immunohistochemical protocols but in the absence of the primary antibody. Sections were counterstained with hematoxylin to identify the nucleus, cleared, and mounted.

3.16 Statistics

All data sets of at least three independent experiments are expressed as mean ± standard error of the mean (SEM), unless otherwise indicated. Graphs and statistics were analyzed using Sigma Stat 3.5 software (San Jose, California). Comparisons across two groups were analyzed by a t-test, while comparisons between multiple groups were performed using One Way Analysis of Variance (ANOVA) followed by the post-hoc test, Student Newman–Keuls. Statistical significance was considered at $p<0.05$.

4.0 RESULTS

4.1 Recombinant proteins

To test the hypotheses generated in this thesis, the experiments described herein use three recombinant proteins that were synthesized in the O'Brien Lab: rHSP27, rHSP25, and rC1, a truncated HSP27 variant which lacks amino acid 1-92 of the full length N-terminus **(Figure 5B)**. These proteins are >95% pure, with the LAL endotoxin test consistently measuring less than 5 EU/mg protein rendering them sufficient for the treatment of cultured cells (392). Following dialysis of purified proteins to aid with protein refolding and removal of superfluous agents and ions that accumulated after isolation and purification, biochemical and chaperone properties were assessed to ensure proper folding and function.

4.1.1 Folding properties

Purified rHSP27 was loaded onto an SDS-PAGE gel either in the absence or presence of DTT to break disulfide bridges of the tertiary and quaternary protein structure **(Figure 5C)**. While a single band was detected at the predicted molecular weight of 27 kDa in the sample with DTT, two bands were also detected at ~27 and 54 kDa in the sample without DTT, suggesting that HSP27 can form multimers.

4.1.2 Chaperone Activity

Given heat shock proteins are traditionally thought of as intracellular chaperones it was also important to test their fidelity using in vitro protein re-folding chaperone assays. Aggregated insulin not properly re-assembled after addition of DTT to disrupt disulfide bridges, showed higher absorbance units than insulin in the presence of rHSP27 or rHSP25 (Figure 5D). Despite being synthesized in nearly the identical manner as the full length

protein, addition of rC1 to insulin had virtually no effect on the aggregation absorbance levels, which paralleled those of insulin alone.

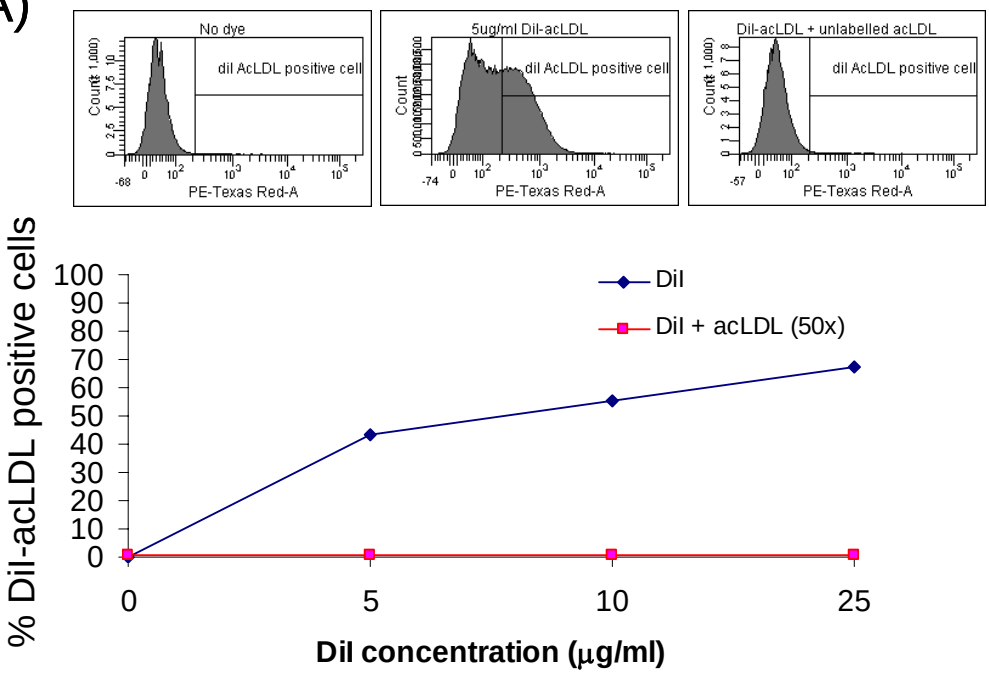
4.2 Optimization of acLDL uptake and binding macrophages

4.2.1 Dose and time-dependent analysis of acLDL uptake

Since this thesis focuses on SR-A related to foam cell formation it was important to first determine the kinetics of acLDL uptake in the macrophage model used herein. The ability of macrophages to internalize DiI-acLDL as a function of time and concentration was tested in THP-1 macrophages to determine optimal conditions for subsequent experiments. THP-1 monocytes were allowed to differentiate for 2 days with 50 ng/ml PMA.During this time, cells displayed morphological and biological characteristics of differentiated macrophages such as adherence to the culture dish, cell spreading (including formation of lamallipodia), and expression of macrophage markers such as SR-A (see below), and CD68 (not shown). Differentiated macrophages were incubated with increasing concentrations of DiI-acLDL (5, 10, and 25 μg/ml) for 3 and 24 hours. At each time point (3 and 24 hours) the increasing doses of DiI-acLDL coincided with a proportional elevation in the amount of cells taking up dye **(Figure 6A and Figure 6B)**.

As well, concomitant addition of unlabelled acLDL at 50-fold excess of each DiI-acLDL concentration diminished uptake of DiI-acLDL via competitive inhibition. The amount of dye incorporated was further enhanced with a longer incubation time reflecting the positive feedback characteristics of this ligand / receptor interaction. For example, incubation of cells with 5 μg/ml acLDL for 3 hours resulted in approximately 45% and 70% of cells taking up the dye at 3 and 24 hours, respectively. Uptake was saturable, as the curve plateaued with doses beyond 5 μg/ml for both 3 and 24 hours demonstrating the receptor

A)


No dye
dil AcLDL positive cell
5ug/ml DiI-acLDL
dil AcLDL positive cell
DiI-acLDL + unlabelled acLDL
dil AcLDL positive cell
Count
PE-Texas Red-A
% DiI-acLDL positive cells
DiI
DiI + acLDL (50x)
DiI concentration (µg/ml)

B)

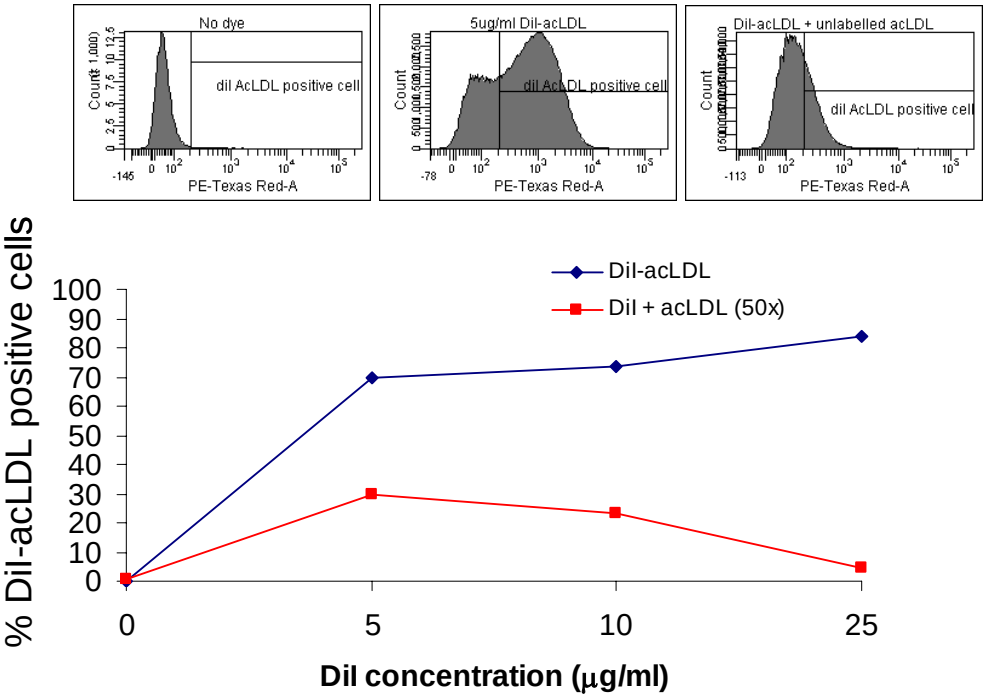

No dye
dil AcLDL positive cell
5ug/ml DiI-acLDL
dil AcLDL positive cell
DiI-acLDL + unlabelled acLDL
dil AcLDL positive cell
Count
PE-Texas Red-A
% DiI-acLDL positive cells
DiI-acLDL
DiI + acLDL (50x)
DiI concentration (µg/ml)

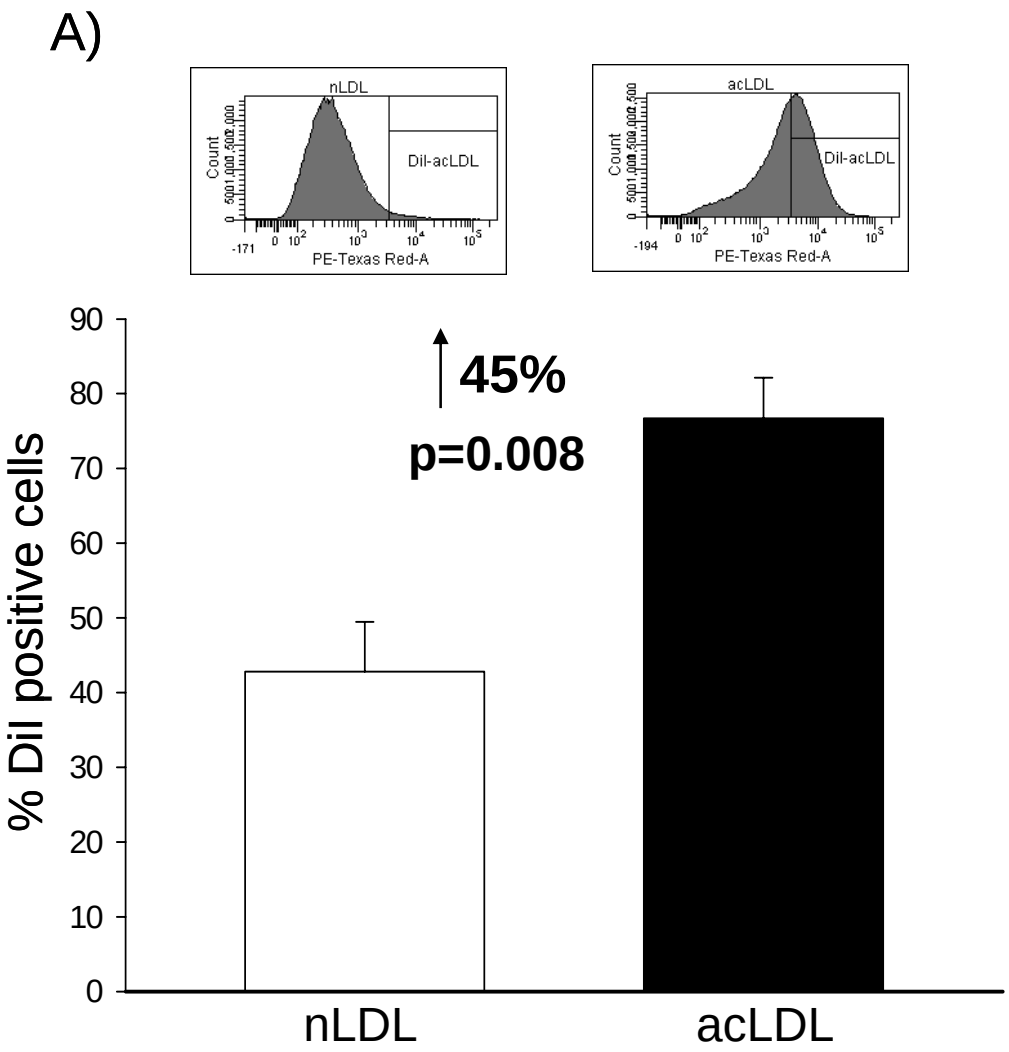
Figure 6. Dose and time-dependent effects of DiI-acLDL on acLDL uptake in macrophages

THP-1 macrophages were differentiated with PMA (50 ng/ml) for 2 days followed by incubation with increasing doses of DiI-acLDL, and/or 50-fold excess unlabelled acLDL for **(A)** 3 and **(B)** 24 hours. The amount of DiI-acLDL positive cells were determined using flow cytometry analysis. Representative flow histograms are depicted at the top of each graph and show the fluorescent signal against cell number.

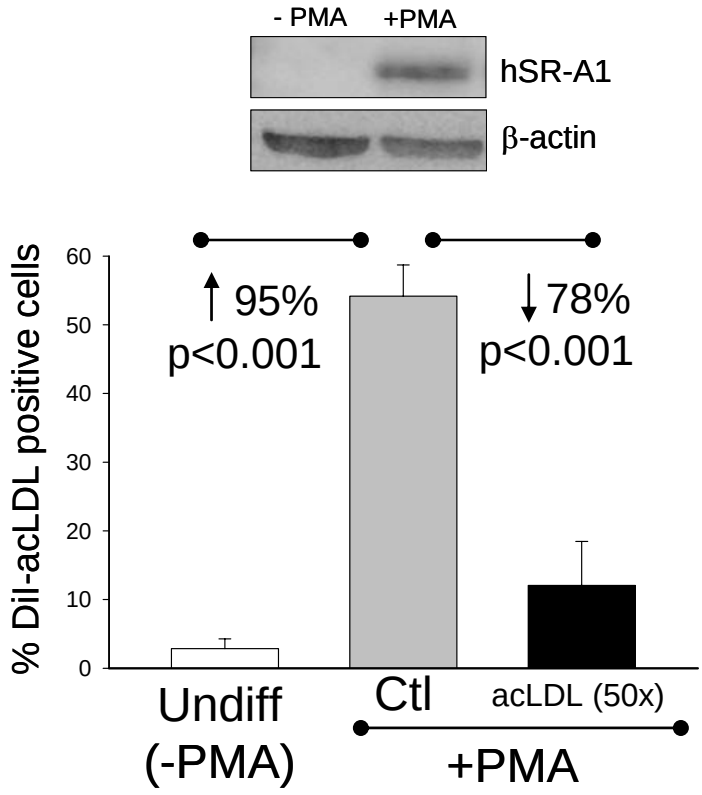
dependence of SR-A for acLDL. Thus, 5 μg/ml DiI-acLDL was determined to be the optimal dose and was used for subsequent uptake assays.

4.2.2 Specificity of acLDL uptake and binding via SR-A function

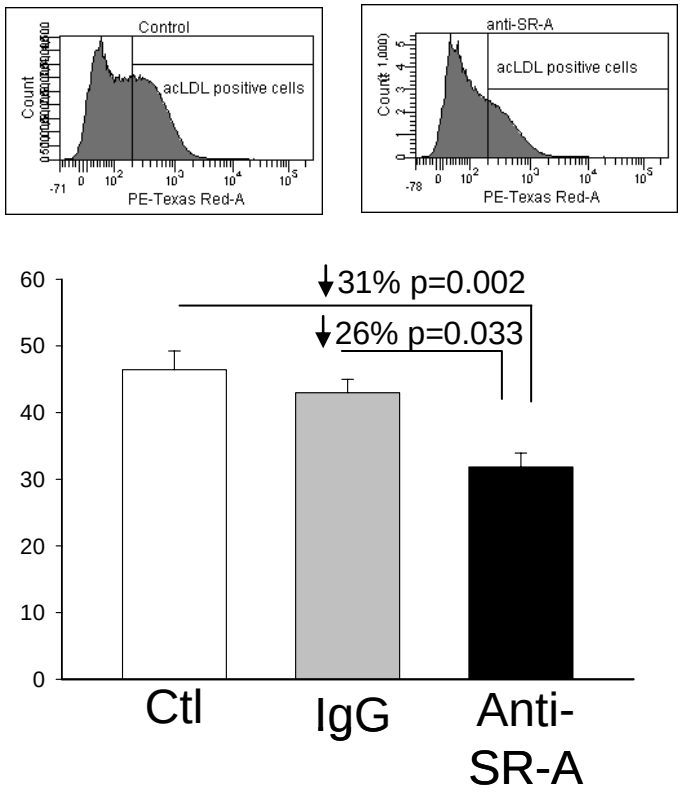
A number of approaches were used to describe the functional characteristics of acLDL uptake in THP-1 macrophages, and to demonstrate its specificity for SR-A. First, 45% ($p=0.008$) more cells incorporated the dye when incubated with acLDL compared to nLDL **(Figure 7A).** Second, acLDL uptake could be blocked by 78% ($p<0.001$) with excess (50-fold) amounts of unlabeled acLDL reflecting a receptor competition effect and supporting acLDL specificity **(Figure 7B)**. Third, undifferentiated cells not incubated with PMA are SR-A deficient as demonstrated by immunoblotting, and showed no uptake relative to PMA differentiated cells that express SR-A **(Figure 7B)**. Fourth, a neutralizing antibody against hSR-A1 blocked uptake by 31% ($p=0.002$) versus control **(Figure 7C**), while the IgG negative control did not affect uptake. Finally, fucoidan, a known SR-A competitive ligand (393), inhibited uptake in a dose-dependent manner (e.g., 41 and 63% reductions at concentrations of 10 and 50 μg/ml respectively; $p<0.001$ for both, **Figure 7D)**.


A)
nLDL
Count
DiI-acLDL
PE-Texas Red-A
acLDL
Count
DiI-acLDL
PE-Texas Red-A
45%
p=0.008
% DiI positive cells
90
80
70
60
50
40
30
20
10
0
nLDL
acLDL

B)


- PMA
+PMA
hSR-A1
β-actin
% DiI-acLDL positive cells
60
50
40
30
20
10
0
↑ 95%
p<0.001
↓ 78%
p<0.001
Undiff
(-PMA)
Ctl
acLDL (50x)
+PMA

C)


Control
acLDL positive cells
Count
PE-Texas Red-A
anti-SR-A
acLDL positive cells
Count (1,000)
PE-Texas Red-A
↓31% p=0.002
↓26% p=0.033
60
50
40
30
20
10
0
Ctl
IgG
Anti-
SR-A

D)

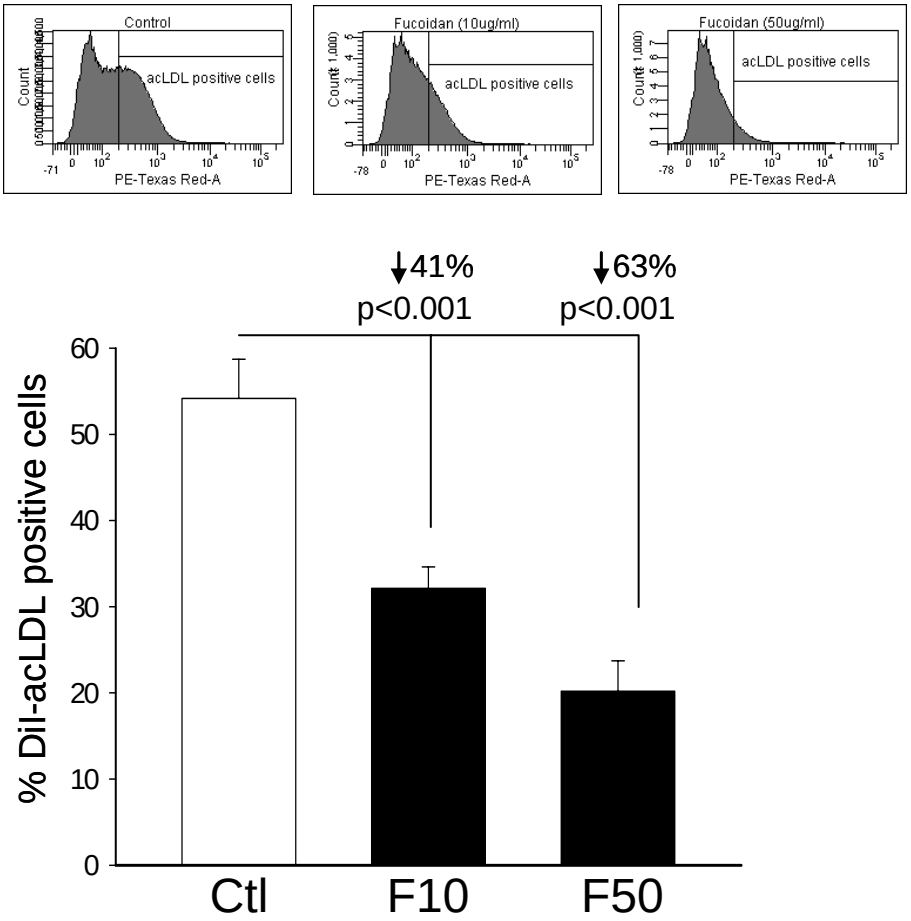


Figure 7. Role of PMA and SR-A ligands or blockers in acLDL uptake

(A) THP-1 differentiated macrophages were incubated with either nLDL or acLDL for 3 hours. Percent DiI-acLDL positive cells was measured using flow cyotmetry showing that acLDL is taken up more readily than nLDL (n=4). **(B)** Immunoblot using an antibody against hSRA-1 and β-actin, and uptake assays showing the role of PMA in upregulating SR-A expression and stimulating acLDL uptake compared to undifferentiated cells not incubated with PMA (n=6). Incubation of differentiated THP-1 cells with anti-SR-A (5 μg/ml) or mouse IgG (**C**, n=3-9) and fuciodan (F) at indicated concentrations (μg/ml) (**D**, n=6) demonstrating the role of SR-A in acLDL uptake under baseline conditions. Representative flow histograms are depicted above each uptake graphs.

Consistent with these results, addition of unlabelled acLDL, fucoidan and the SR-A neutralizing antibody also blocked acLDL binding at 4°C **(Figure 8)** further demonstrating functional acLDL binding sites on the cell surface.

Uptake of acLDL was also tested in BMDM isolated from ApoE$^{-/-}$ and ApoE$^{-/-}$ SR-A$^{-/-}$ mice using Oil red O staining and flow cytometry. ApoE$^{-/-}$SR-A$^{-/-}$ cells showed less accumulation of Oil red O compared to ApoE$^{-/-}$ cells **(Figure 9A)**. This was confirmed with flow cytometry that revealed a 37% reduction (p=0.04) of acLDL uptake in ApoE$^{-/-}$SR-A$^{-/-}$ cells relative to ApoE$^{-/-}$ cells **(Figure 9B)**. To further demonstrate the role of SR-A in these cells, additional experiments revealed that acLDL could be blocked in the presence of unlabelled acLDL and fucoidan (data not shown).

4.3 Effect of recombinant proteins on acLDL uptake or binding in macrophages

The first objective of this study was to explore whether extracellular HSP27 could modulates macrophage biology, and test the hypothesis that rHSP27 could alter uptake and binding of acLDL – a key step during foam cell formation.

THP-1 macrophages were pre-treated with increasing molar equivalent doses of recombinant proteins (1.9, 3.8, and 9.6 μM) before addition of DiI-acLDL at 1, 3, and 24 hours. Pre-treatment of cells with rHSP27 reduced acLDL uptake in a dose-dependent manner at each incubation time point compared to no treatment control using flow cytometry **(Figure 10)**. Although 9.6 μM showed the maximal effect on uptake, the potency of rHSP27 in blocking acLDL uptake was diminished at each incremental time point. rHSP27 treatment reduced uptake by 58% (p=0.008, **Figure 10A**), 34% (p=0.002, **Figure 10B**), and 31% (p=0.008, **Figure 10C**) at 1, 3, and 24 hours, respectively compared to the

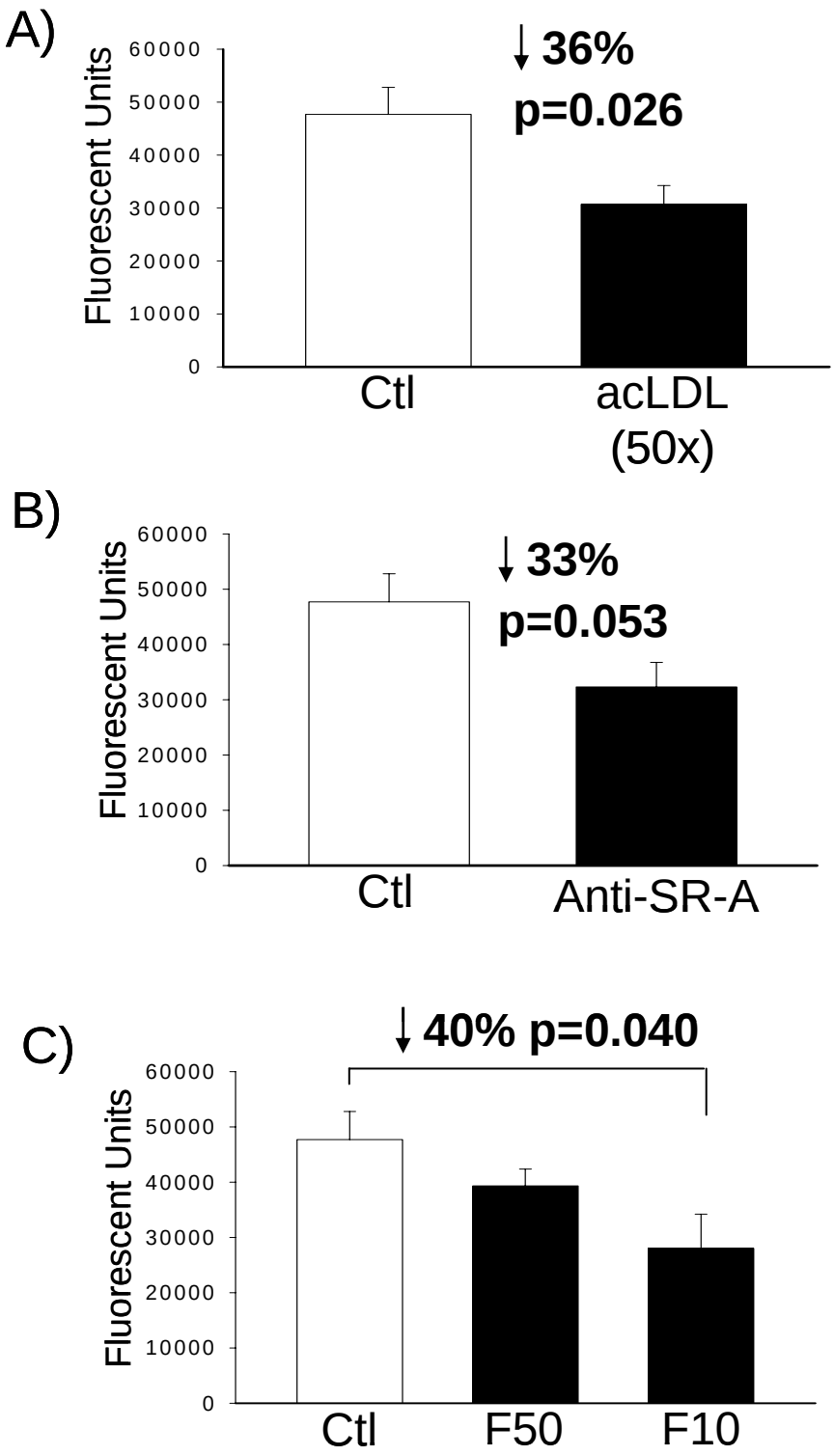


Figure 8. Role of SR-A ligands or blockers in binding of acLDL

The functional properties of acLDL binding to the cell surface of THP-1 macrophages were assessed using SR-A ligands and blockers. Cells were incubated with DiI-acLDL at 4°C for 2 hours following 1 hr pre-treatment with 50× unlabeled acLDL **(A)**; anti-SR-A (5 μg/ml) **B)**; and indicated concentrations (μg/ml) of fucoidan (F) **(C)**. F=fucoidan. Fluorescence intensity was measured (arbitrary units) using a fluorescent plate reader. n=5.

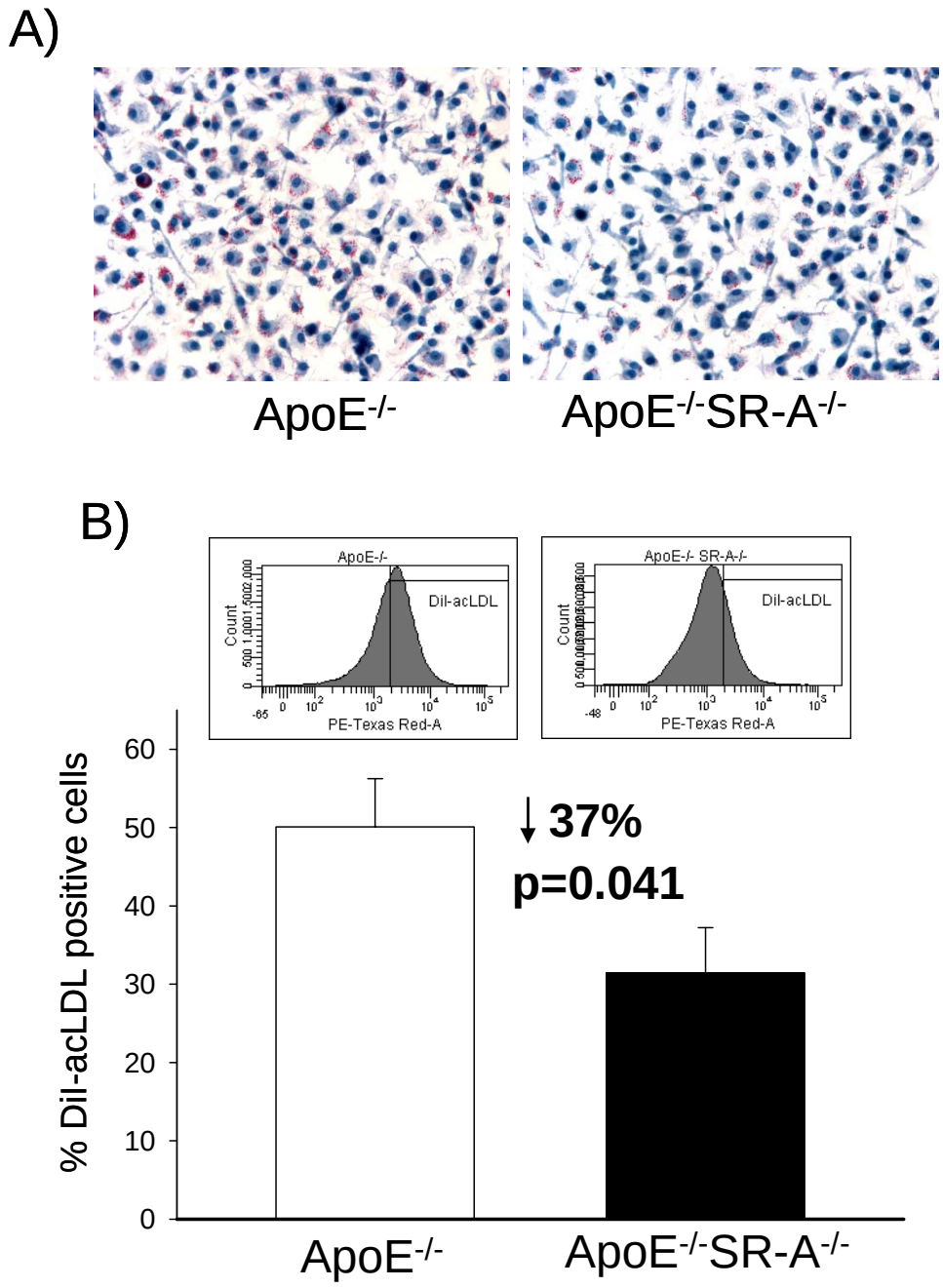


Figure 9. Deletion of SR-A affects acLDL uptake

Bone marrow derived macrophages (BMDM) isolated from ApoE$^{-/-}$ SR-A$^{-/-}$ mice exhibit less acLDL uptake than ApoE$^{-/-}$ cells when stained with Oil red O (red) **(A)**, or incubation with DiI-acLDL (3 hr) using flow cytometry (**B**, n=6). Representative flow histograms are illustrated for flow cytometry analysis.

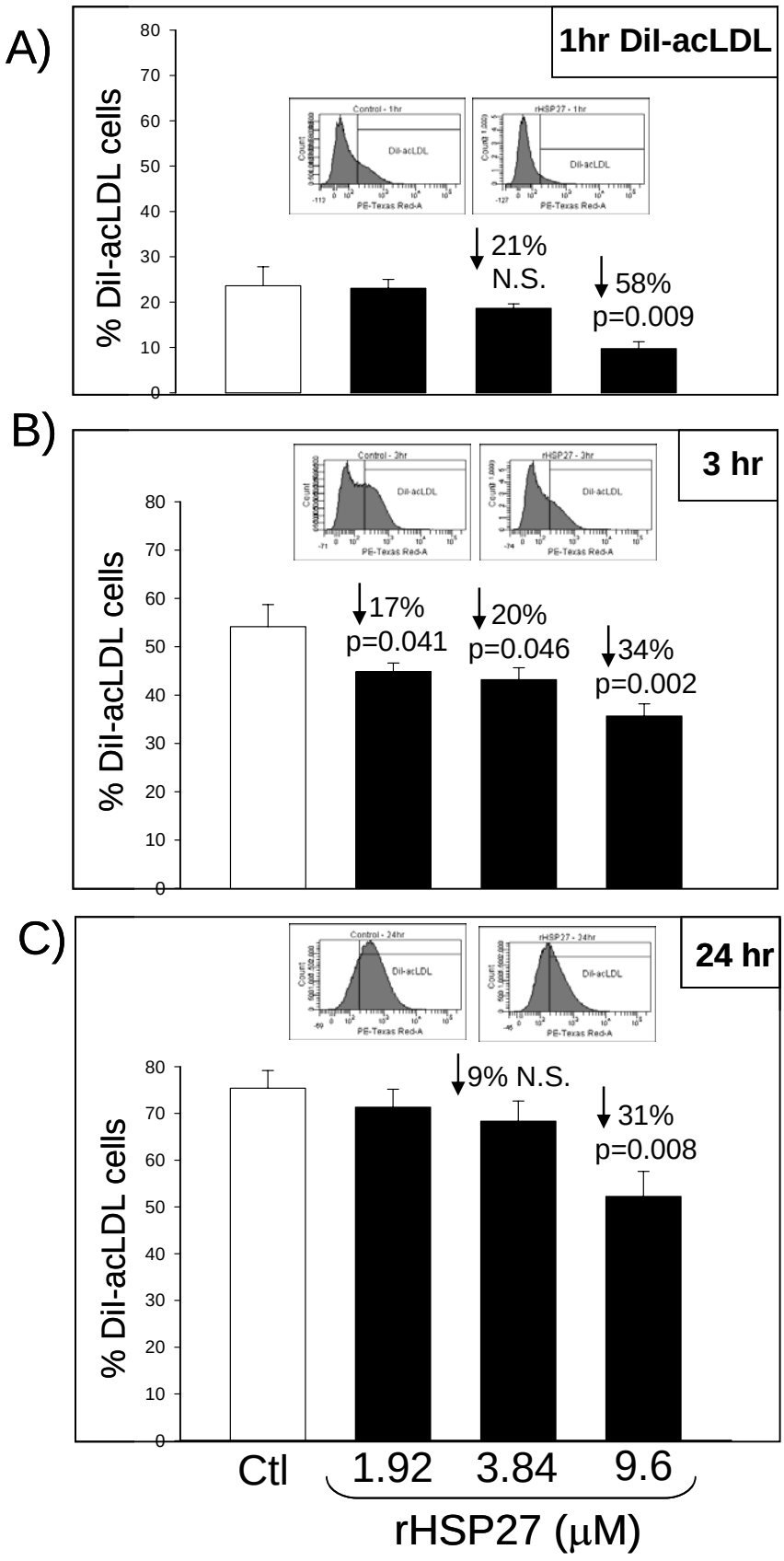

A)
1hr DiI-acLDL
% DiI-acLDL cells
Control - 1hr
rHSP27 - 1hr
DiI-acLDL
PE-Texas Red-A
21%
N.S.
58%
p=0.009
B)
3 hr
Control - 3hr
rHSP27 - 3hr
17%
p=0.041
20%
p=0.046
34%
p=0.002
C)
24 hr
Control - 24hr
rHSP27 - 24hr
9% N.S.
31%
p=0.008
Ctl
1.92
3.84
9.6
rHSP27 (μM)

Figure 10. Dose-dependent effect of HSP27 pretreatment on acLDL uptake

THP-1 macrophages were pre-treated with or without increasing doses of rHSP27 for 24 hours followed by incubation with DiI-acLDL for 1 (**A**, n=4), 3 (**B**, n=6), and 24 (**C**, n=6) hours. The percent positive DiI-acLDL cells were determined using flow cytometry. Representative histograms illustrated above each graph depicting the no treatment control and the highest dose of rHSP27 used in this experiment (9.6 μM equivalents or 250 μg/ml).

no treatment control. A similar dose-dependent profile was observed when cells were pre-treated with rHSP25, with 9.6μM maximally inhibiting uptake by 64% ($p<0.001$, **Figure 11**).

Pre-treatment with the truncated mutant, rC1, failed to influence acLDL uptake at either 1, 3, or 24 hours despite using equimolar concentrations **(Figure 12)**. Similar effects of rHSP27 (and rHSP25) were demonstrated in hMDM isolated from healthy donors. There were 39% ($p=0.017$) and 37% ($p<0.033$) reductions in acLDL uptake when hMDMs were treated with rHSP27 versus control and rC1, respectively **(Figure 13)**. To test the baseline characteristics of acLDL in these cells, DiI-acLDL was incubated with unlabelled acLDL and fucoidan resulting in inhibition of acLDL uptake (data not shown).

Next, to test if HSP27 could alter acLDL binding to the cell surface, THP-1 macrophages pre-treated with recombinant proteins were incubated with DiI-acLDL at 4°C to prevent endocytosis of ligand/receptor complex into the cell. Fluorescent signal was measured using a fluorescent plate reader. rHSP27 dose-dependently reduced binding of acLDL, with 9.6 μM demonstrating the most potent affect ($p=0.008$, **Figure 14A**). Similarly, treatment with rHSP25 also reduced acLDL binding by 21% ($p=0.026$) compared to control **(Figure 14B).** No affect on binding occurred with increasing does of rC1 **(Figure 14C)**.

To simulate a receptor competition phenomenon in acLDL uptake assays, recombinant proteins were added to the media of THP-1 cells either 1 hour before or

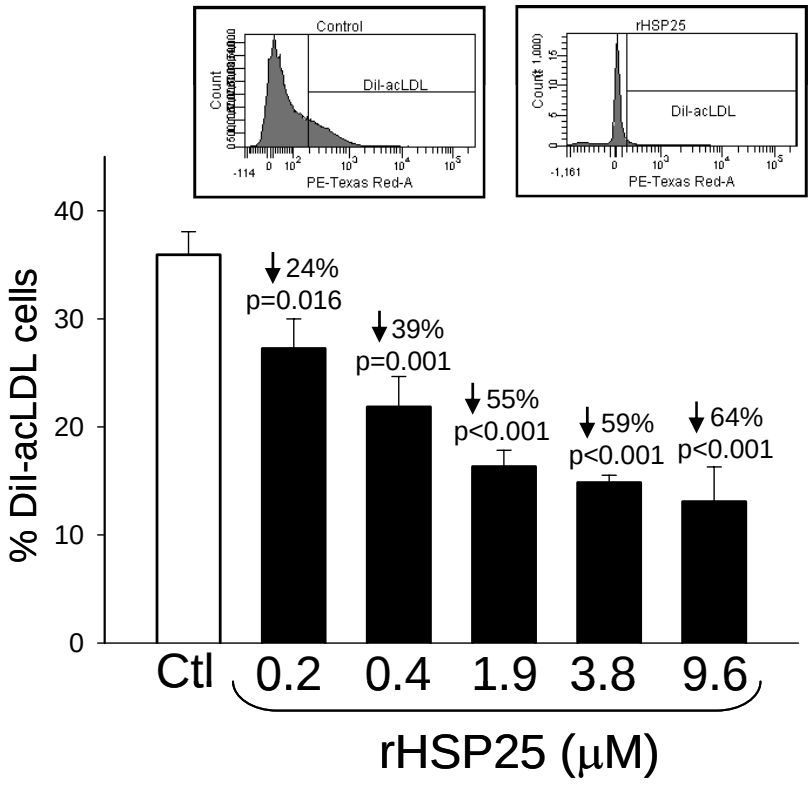


Figure 11. Dose-dependent effect of HSP25 pretreatment on acLDL uptake

THP-1 macrophages were pre-treated in the presence or absence of increasing doses of rHSP25 for 24 hours following incubation with DiI-acLDL for 3hrs. Percent positive DiI-acLDL cells were determined using flow cytometry. Representative histograms depict the no treatment control and the highest dose of rHSP25 used in this experiment (9.6 μM equivalents or 250 μg/ml). n=5.

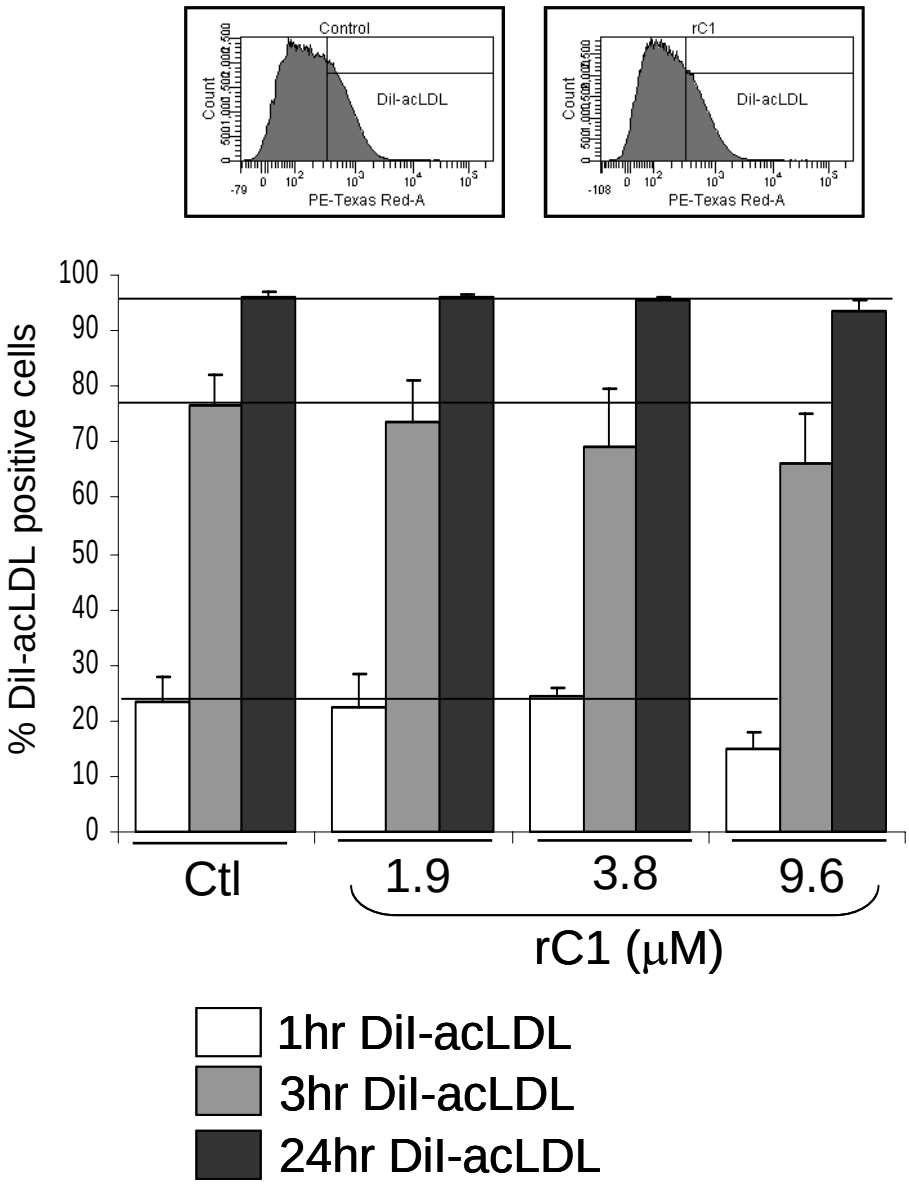


Figure 12. Dose-dependent effect of C1 pre-treatment on acLDL uptake

THP-1 macrophages were pre-treated in the presence or absence of increasing doses of rC1 for 24 hours following incubation with DiI-acLDL for 1 (white bars), 3 (grey bars), and 24 (black bars) hours. Percent positive DiI-acLDL cells were determined using flow cytometry. Representative histograms depict the no treatment control and the highest dose of rC1 (9.6 μM equivalents or 150 μg/ml) used at the 3hr time point.

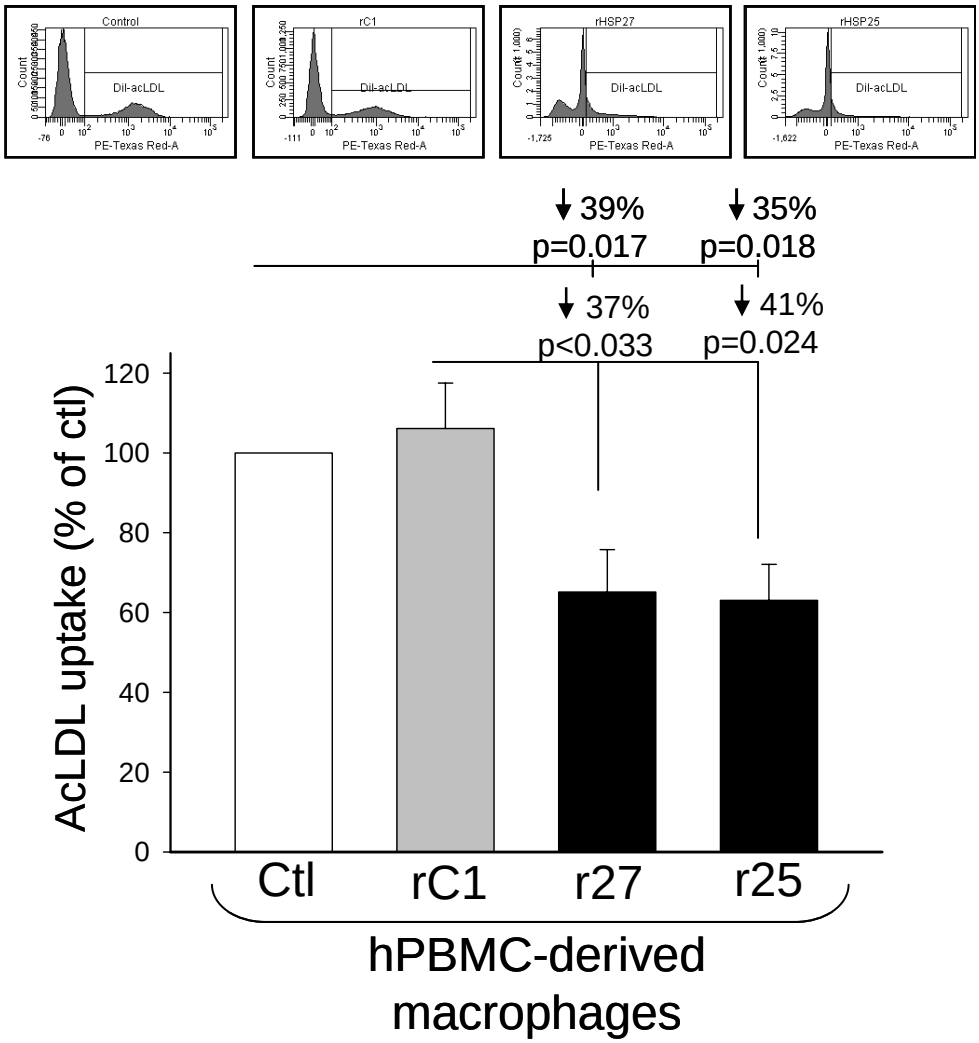
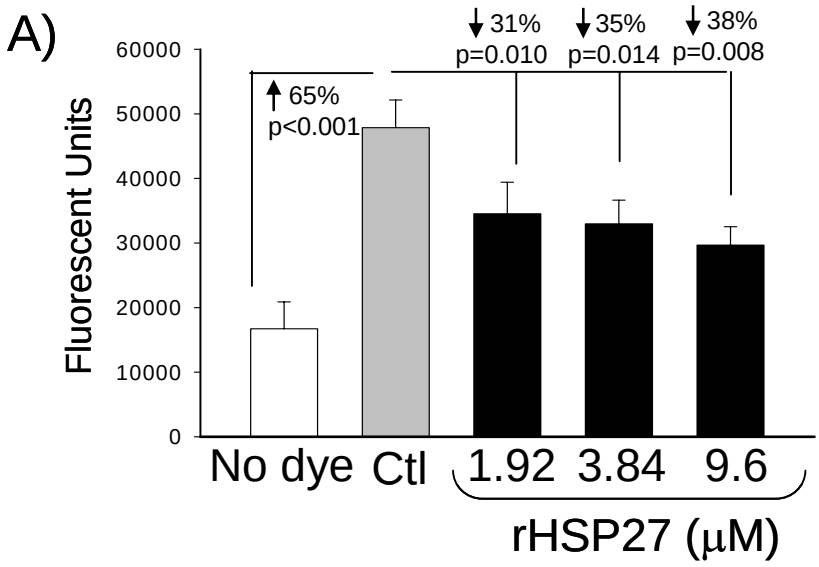
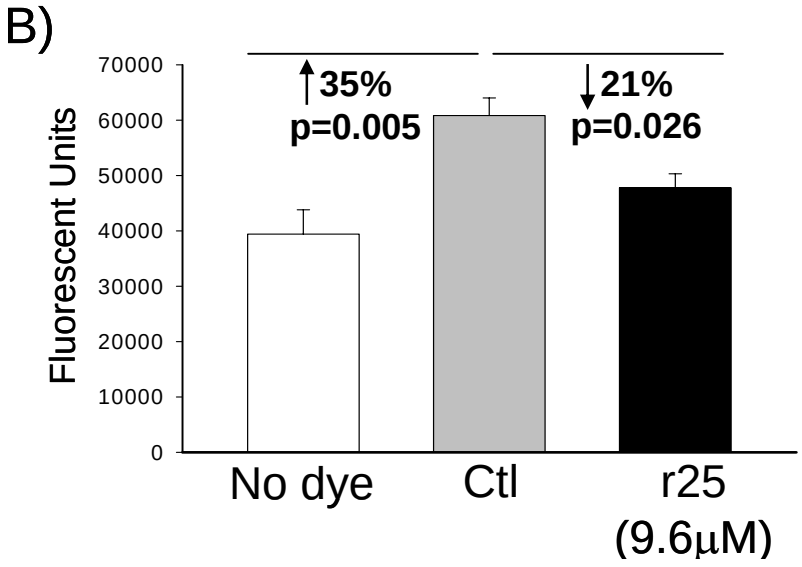


Figure 13. Effect of HSP27 on acLDL uptake in primary human macrophages

Peripheral blood mononuclear cells (PBMC) isolated from healthy individuals were differentiated into macrophages for 7 days, and then treated with or without 9.6μM rC1, rHSP27, or rHSP25 for 24 hours before addition of DiI-acLDL for 3 hours. Cells were subjected to flow cytometry and the percent DiI-acLDL cells in each treatment group were plotted as a percent of the no treated control (Ctl). Representative flow cytometry histograms are shown. n=4.


A)
↓31% p=0.010
↓35% p=0.014
↓38% p=0.008
↑65% p<0.001
Fluorescent Units
60000
50000
40000
30000
20000
10000
0
No dye
Ctl
1.92
3.84
9.6
rHSP27 (μM)


B)
↑35% p=0.005
↓21% p=0.026
Fluorescent Units
70000
60000
50000
40000
30000
20000
10000
0
No dye
Ctl
r25 (9.6μM)

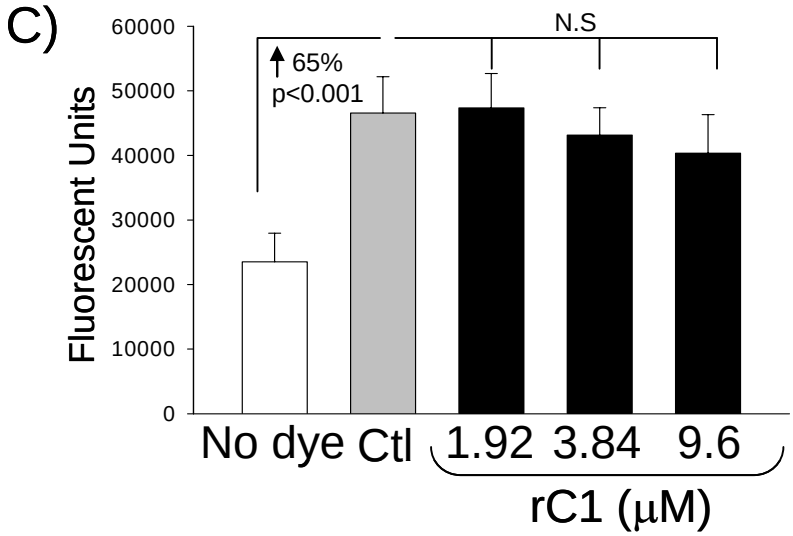

C)
N.S
↑65% p<0.001
Fluorescent Units
60000
50000
40000
30000
20000
10000
0
No dye
Ctl
1.92
3.84
9.6
rC1 (μM)

Figure 14. Effect of HSP27 on acLDL binding in THP-1 cells

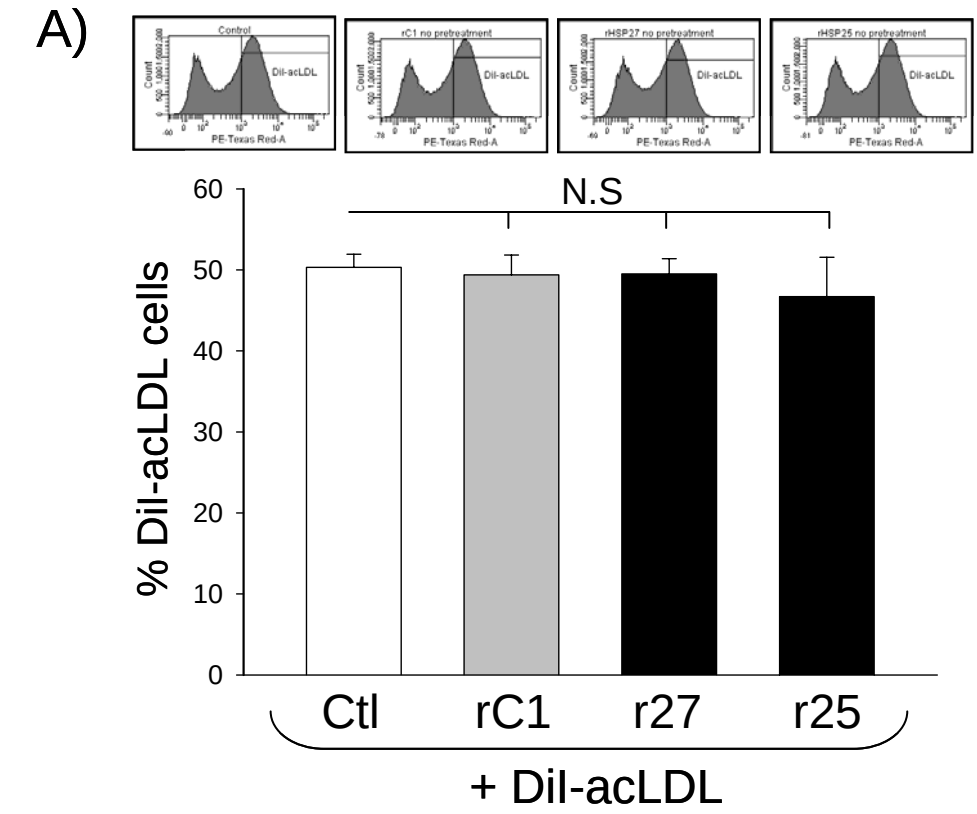
THP-1 macrophages were incubated with DiI-acLDL at 4°C for 2 hours before 24 hour pre-treatment with or without rHSP27 (**A**, n=6), rHSP25 (**B**, n=4), and rC1 (**C**, n=5) at indicated doses. Fluorescence intensity in each group was measured using a fluorescent plate reader. Autoflourescence of the cells alone are illustrated as the no dye control. n=6.

simultaneously with acLDL. In contrast to the inhibition effect by rHSP27 *pre-treatment* in THP-1 macrophages as outlined above, addition of rHSP27 *simultaneously* with acLDL did not influence acLDL uptake **(Figure 15A)** or binding **(Figure 15B)**. Taken together, these results suggest that rHSP27 modulates acLDL uptake and binding in macrophages and that treatment timing is crucial for HSP27's biological action.

4.4 Effect of HSP27 on acLDL uptake in SR-A null macrophages

Given the role of SR-A in the biological effects of HSP27 in macrophages, the next logical step was to test the specific requirement of SR-A on acLDL uptake in SR-A null cells. BMDC were isolated from $ApoE^{-/-}$ and $ApoE^{-/-}SR\text{-}A^{-/-}$ mice, differentiated for 10 days and treated with rHSP27 in acLDL uptake experiments. Flow cytometry analysis revealed that treatment of $ApoE^{-/-}$ cells with rHSP27 reduced acLDL uptake by 38% ($p<0.001$) compared to controls consistent with HSP27's effect in other macrophage cell types **(Figure 16A)**. As expected, rC1 had no effect on uptake. In the $ApoE^{-/-}$ $SR\text{-}A^{-/-}$ cells rHSP27 and rHSP25 showed a trend toward reduced uptake by 22% and 9%, respectively compared to non-treatment control ($p=0.059$) **(Figure 16B)**. A similar trend was also observed when the treatment groups were compared to rC1, which had no effect on uptake in these cells versus the non-treatment control. Two way ANOVA analyses revealed no statistical differences

between the HSP27 treated ApoE$^{-/-}$ and ApoE$^{-/-}$SR-A$^{-/-}$ cells.



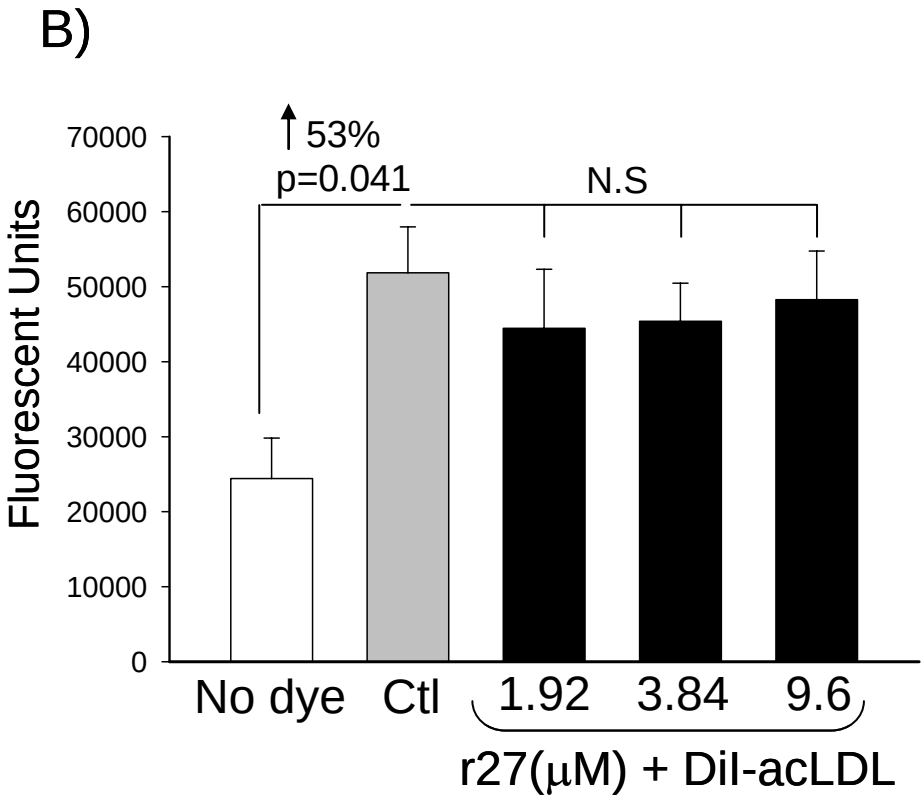
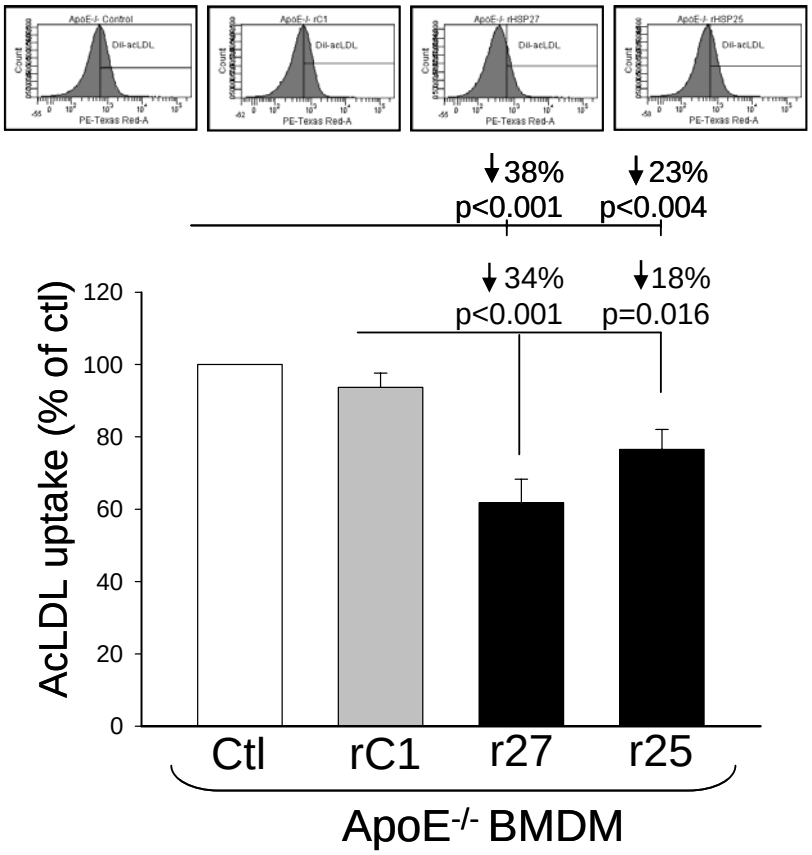


Figure 15. Receptor competition effect of HSP27 on acLDL uptake and binding

rHSP27, rHSP25, and rC1 were incubated simultaneously with DiI-acLDL for 3 hours at 37°C and for flow cytometry uptake assays (**A**, N=3-5), and DiI-acLDL at 4°C for 2 hours for binding assays using a fluorescent plate reader (**B**, N=5). Representative flow cytometry histograms are illustrated in panel A. Autoflourescence is shown as the no dye control

A)


ApoE-/- Control
ApoE-/- rC1
ApoE-/- rHSP27
ApoE-/- rHSP25
DiI-acLDL
Count
PE-Texas Red-A
↓38%
p<0.001
↓23%
p<0.004
↓34%
p<0.001
↓18%
p=0.016
AcLDL uptake (% of ctl)
120
100
80
60
40
20
0
Ctl
rC1
r27
r25
ApoE-/- BMDM

B)

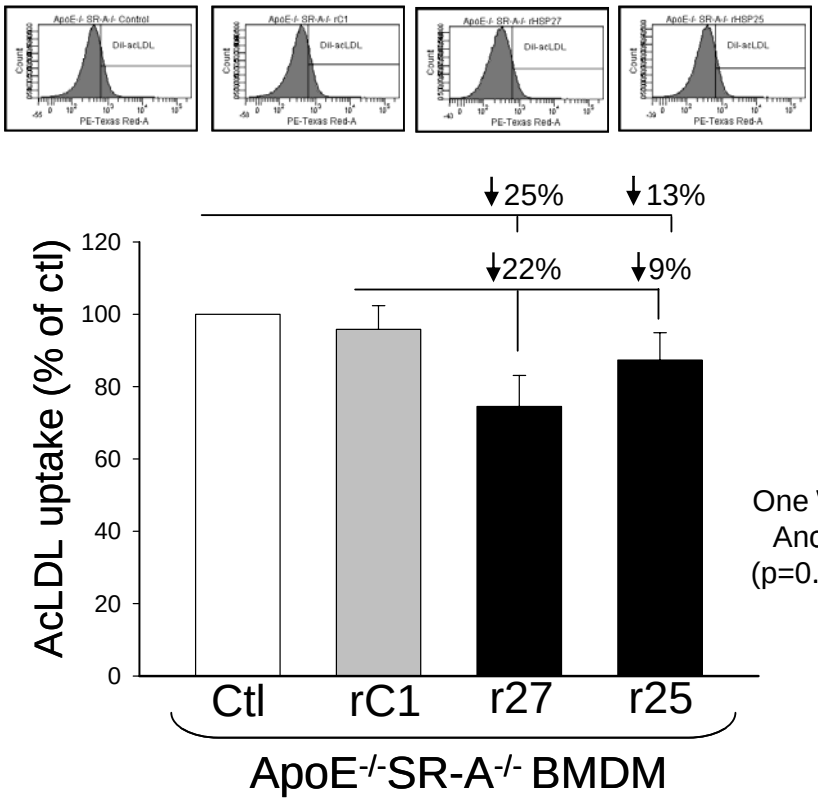

ApoE-/- SR-A-/- Control
ApoE-/- SR-A-/- rC1
ApoE-/- SR-A-/- rHSP27
ApoE-/- SR-A-/- rHSP25
DiI-acLDL
Count
PE-Texas Red-A
↓25%
↓13%
↓22%
↓9%
AcLDL uptake (% of ctl)
120
100
80
60
40
20
0
Ctl
rC1
r27
r25
ApoE-/-SR-A-/- BMDM
One Way
Anova
(p=0.059)

Figure 16. Effect of HSP27 on acLDL uptake in SR-A null macrophages

BMDM isolated from ApoE$^{-/-}$ (**A**, n=6) and ApoE$^{-/-}$SR-A$^{-/-}$ (**B**, n=8) mice were treated with indicated recombinant proteins for 24 hours before being incubated with DiI-acLDL for 3 hours. The percent positive DiI-acLDL cells in macrophages isolated from each genotype were analyzed by flow cytometry and plotted as a percent of the no treatment control (Ctl). Representative flow histograms are illustrated.

4.5 Alternative methods to quantify acLDL uptake

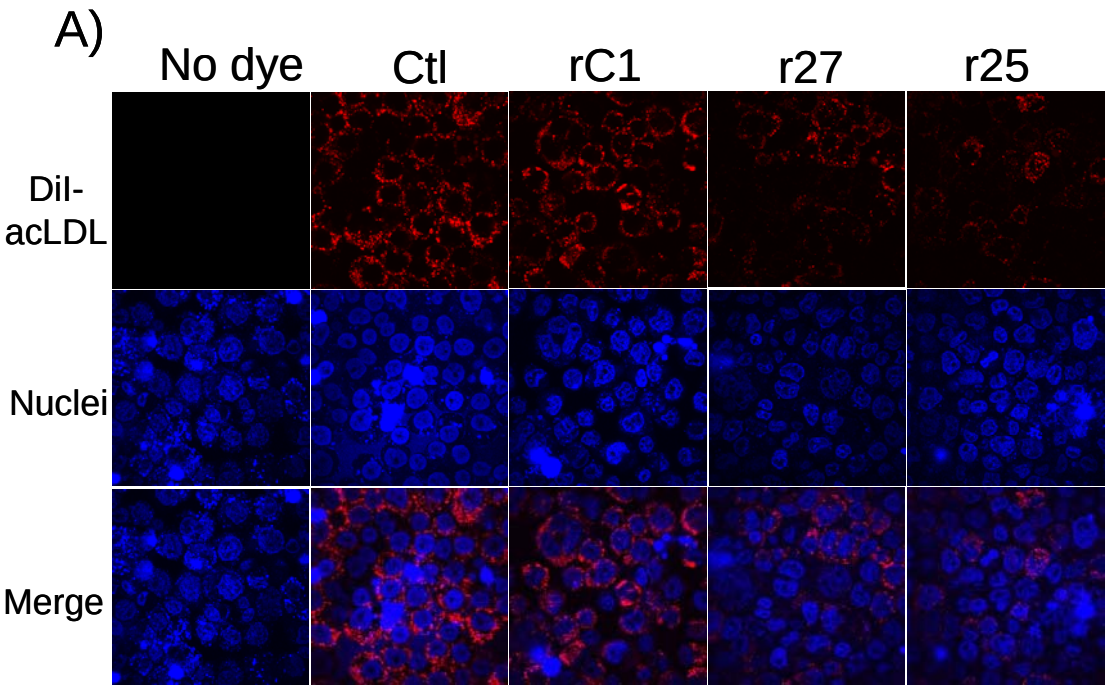
The inhibitory effects of rHSP27 on acLDL uptake were further demonstrated using alternative methodologies: confocal microscopy and recordings from a fluorescent plate reader. Confocal microscopy images illustrated diminished acLDL accumulation in THP-1 cells treated with rHSP27/25 versus controls **(Figure 17A)**. Quantification of fluorescent intensity in these images revealed a 36% reduction ($p<0.001$) in DiI-acLDL signal when normalized to the number of nuclei in each field **(Figure 17B)**. The use of a fluorescent plate reader also demonstrated reductions ($p<0.002$) in acLDL uptake by rHSP27 as normalized to Hoechst fluorescence **(Figure 17C).**

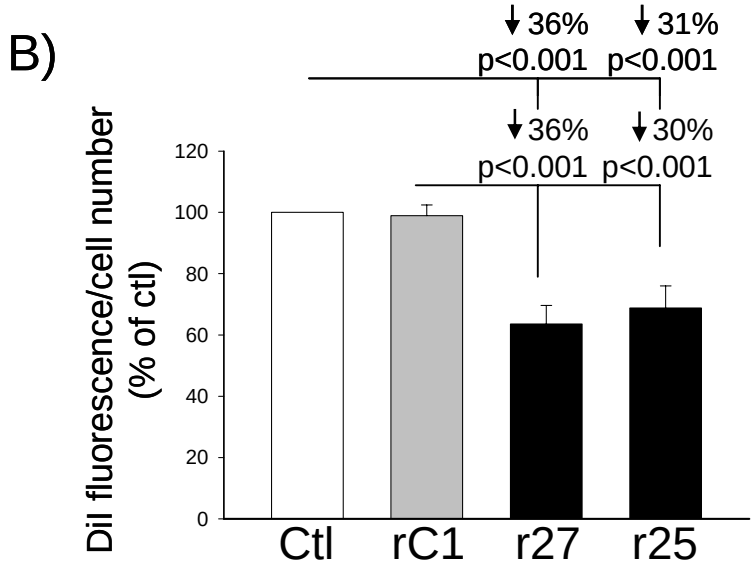
4.6 Effect of rHSP27 on SR-A expression in THP-1 macrophages

To investigate the hypothesis that the reduction in acLDL uptake and binding is the result of altered SR-A expression, mRNA and protein were measured in THP-1 cells.

4.6.1 Gene expression

qPCR was performed to measure RNA transcript levels of human SR-A in the presence or absence of rHSP27 (9.6 μM) treatment for 6 and 24 hours in THP-1 macrophages. β-actin was used as a reference gene. Treatment of cells with rHSP27 for 6 hours reduced SR-A mRNA levels by 46% ($p=0.009$) compared to no treatment controls


A)
No dye
Ctl
rC1
r27
r25
DiI-acLDL
Nuclei
Merge


B)
36%
p<0.001
31%
p<0.001
36%
p<0.001
30%
p<0.001
DiI fluorescence/cell number (% of ctl)
120
100
80
60
40
20
0
Ctl
rC1
r27
r25

C)

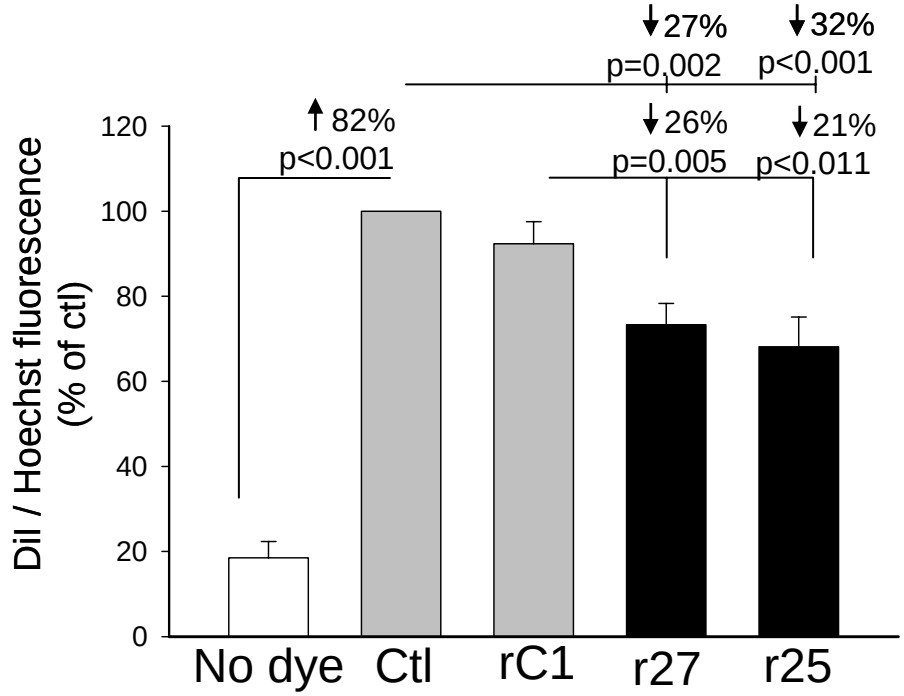


Figure 17. Effect of HSP27 on acLDL uptake in THP-1 cells using alternative methods

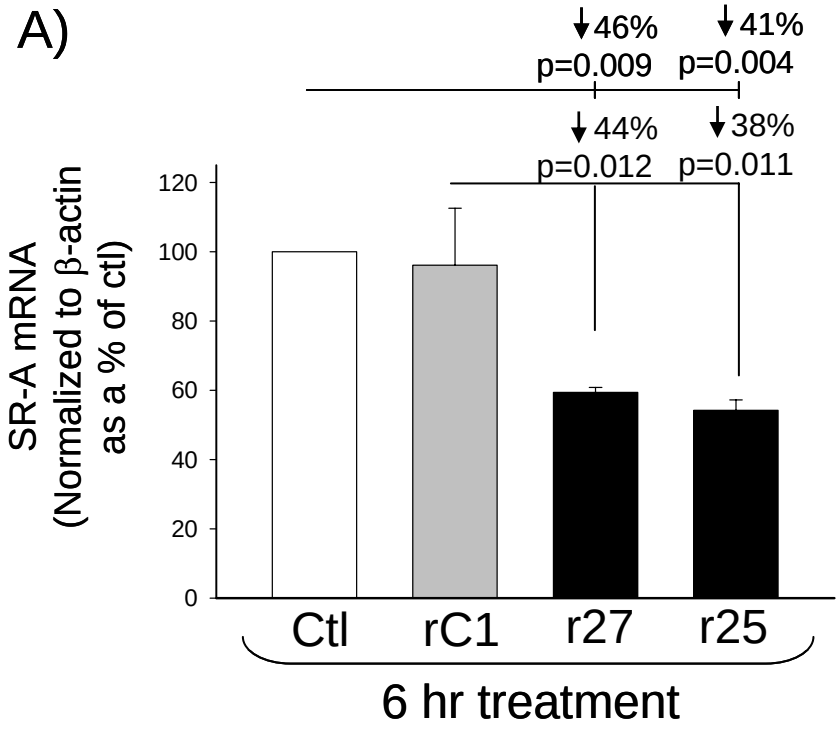
THP-1 macrophages were pretreated with rC1, rHSP27, and rHSP25 for 24 hours followed by incubation with DiI-acLDL for 3 hours. (A) Representative live cell images (1000x magnification) using confocal microscopy illustrate reduced accumulation of DiI-acLDL (red) into the cell. Nuclei are stained with Hoechst (blue). (B) DiI signal in each treatment or control groups were quantified using confocal fluorescent integration software as a ratio of the number of cells in each field. The data was then plotted as a percent of the non-treatment control (Ctl); n=5-6. (C) DiI fluorescence was measured in cells using a fluorescent plate reader and normalized to Hoechst fluorescence. Data was expressed as a percent of the no treatment control (Ctl). n=5.

(Figure 18A). A similar inhibitory effect was observed for rHSP25. However, when cells were treated with rHSP27 for 24 hours there was a 25% increase ($p=0.025$) in SR-A transcript levels; no change was observed in cells treated with rHSP25 **(Figure 18B)**. rC1 treatment did not have an effect on SR-A mRNA at both time points.

4.6.2 Protein expression

To test if the reductions in SR-A mRNA are reflective of SR-A protein levels, total lysate was collected from THP-1 cells treated in the presence or absence of rHSP27 or rHSP25 for 24 hours. Immunoblotting revealed 63% ($p<0.001$), and 55% ($p=0.002$) reductions in SR-A1 protein versus no treatment and rC1, respectively **(Figure 19A)**. In addition, rHSP25 dose-dependently attenuated the abundance of SR-A protein, with 9.6 μM showing the maximum response ($p=0.002$, **Figure 19B**).

Next, flow cytometry was performed to test if reductions in total cellular SR-A levels were associated with altered SR-A levels at the cell surface. There was a 36% reduction ($p=0.009$) in surface SR-A levels in HSP27 treated cells compared to controls **(Figure 20A)**, an effect further validated using confocal microscopy showing reduced SR-A staining at the cell surface **(Figure 20B)**. While no affect on SR-A levels were observed for rC1 compared to no treatment controls, a similar inhibitory affect on SR-A surface expression was observed for rHSP25. No SR-A expression was observed at the cell surface of undifferentiated THP-1 cells. In addition, the IgG negative control failed to show a fluorescent signal in confocal images and flow cytometry analysis demonstrating specificity of the antibody used in this system.


A)
↓46%
p=0.009
↓41%
p=0.004
↓44%
p=0.012
↓38%
p=0.011
SR-A mRNA
(Normalized to β-actin
as a % of ctl)
120
100
80
60
40
20
0
Ctl
rC1
r27
r25
6 hr treatment

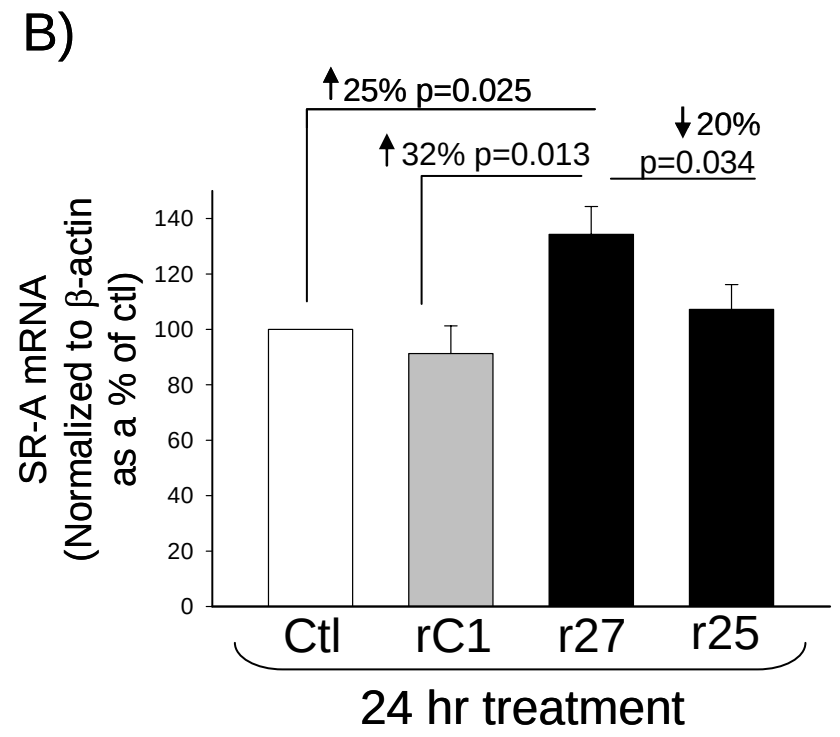

B)
↑25% p=0.025
↑32% p=0.013
↓20%
p=0.034
SR-A mRNA
(Normalized to β-actin
as a % of ctl)
140
120
100
80
60
40
20
0
Ctl
rC1
r27
r25
24 hr treatment

Figure 18. Effect of HSP27 on SR-A gene expression in THP-1 macrophages

THP-1 macrophages were treated with 9.6μM rC1, rHSP27, or rHSP25 for 6 (**A**, n=3-5), and 24 (**B**, n=4-6) hrs. qPCR analysis was performed using primers complimentary to hSR-A and β-actin. Cp ratios were calculated from SR-A and β-actin fluorescent signals, and then plotted as a percent of the no treatment controls (Ctl).

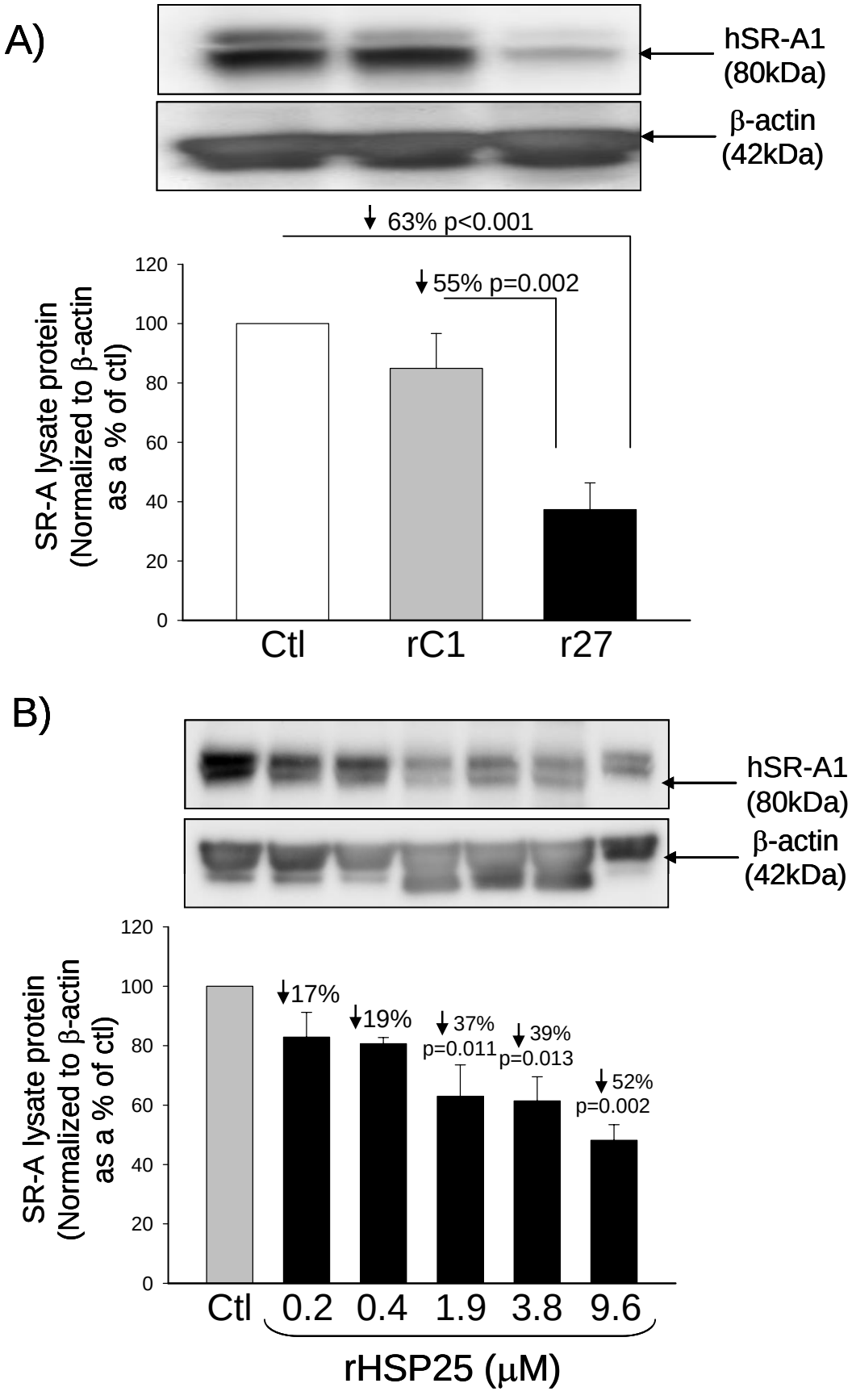
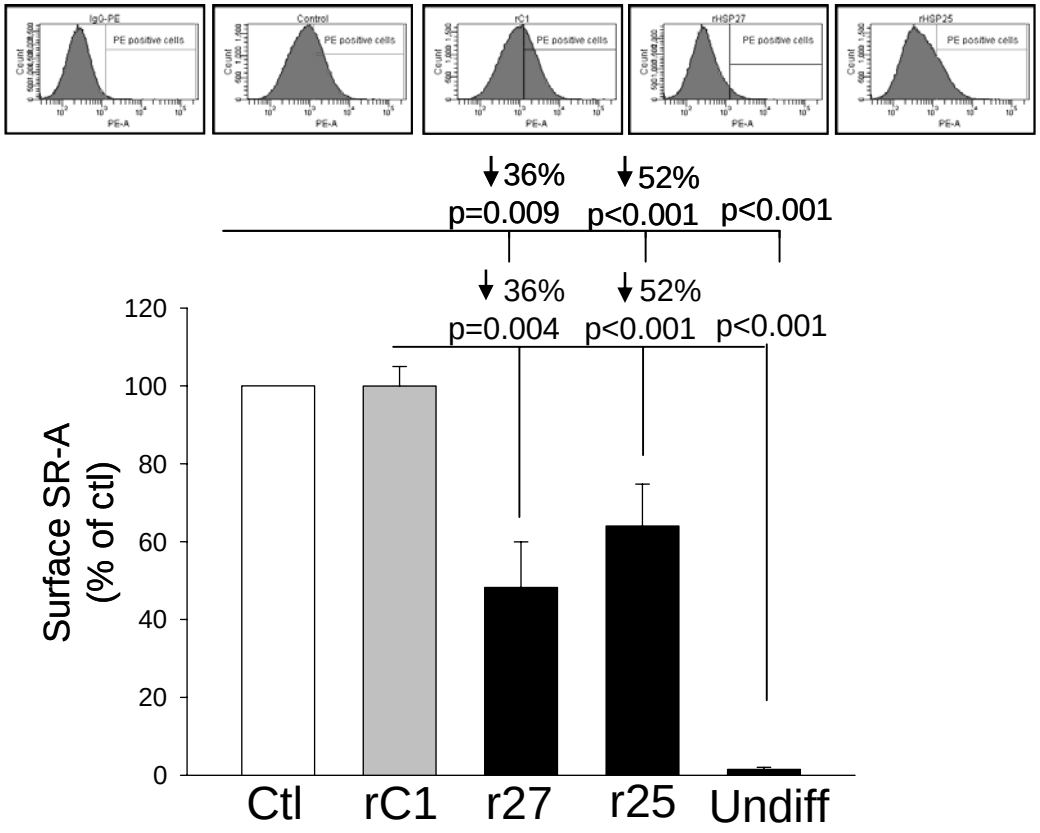

A)
hSR-A1
(80kDa)
β-actin
(42kDa)
63% p<0.001
55% p=0.002
SR-A lysate protein
(Normalized to β-actin
as a % of ctl)
120
100
80
60
40
20
0
Ctl
rC1
r27
B)
hSR-A1
(80kDa)
β-actin
(42kDa)
SR-A lysate protein
(Normalized to β-actin
as a % of ctl)
120
100
80
60
40
20
0
17%
19%
37%
p=0.011
39%
p=0.013
52%
p=0.002
Ctl
0.2
0.4
1.9
3.8
9.6
rHSP25 (μM)

Figure 19. Effect or HSP27 on SR-A protein expression in THP-1 macrophages

Total cell lysate from THP-1 macrophages treated with rC1 and rHSP27 (**A**, n=5), or rHSP25 (**B**, n=3) for 24 hours were subjected to Western Blotting analysis using antibodies against hSR-A and β-actin. Amount of SR-A expression was determined using densitometry ratios between SR-A and β-actin in each control or treatment group. Ratios were then plotted as a percentage of the no treatment control (Ctl). Representative immunoblots are illustrated above each graph.

A)


IgG-PE
Control
rC1
rHSP27
rHSP25
PE positive cells
PE-A
Count
36%
52%
p=0.009
p<0.001
p<0.001
36%
52%
p=0.004
p<0.001
p<0.001
Surface SR-A
(% of ctl)
120
100
80
60
40
20
0
Ctl
rC1
r27
r25
Undiff

B)

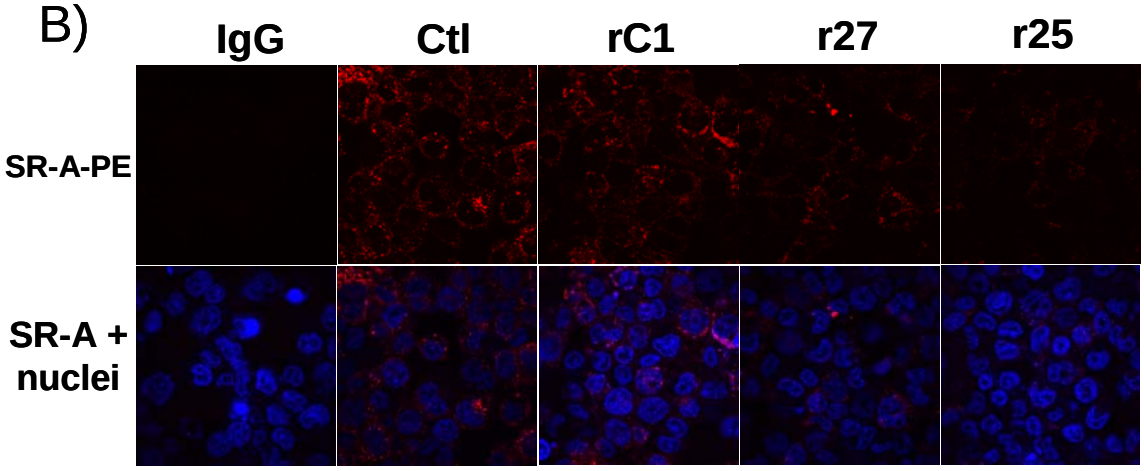

IgG
Ctl
rC1
r27
r25
SR-A-PE
SR-A +
nuclei

Figure 20. Effect of HSP27 on SR-A surface expression

(A) THP-1 macrophages treated with specified recombinant proteins were lifted, and then incubated with an SR-A1 antibody conjugated to phycoerythryn (PE). Flow cytometry analysis was performed to determine the percent positive PE cells, and then plotted as a percent of the no treatment controls (Ctl). Representative histograms are exhibited. n=3-10. **(B)** Representative confocal microscopy images illustrating SR-A-PE (red) and nuclei (blue) of live THP-1 cells treated with recombinant proteins.

Taken together, these results provide evidence that HSP27 affects SR-A mRNA levels, the outcome of which leads to reductions in total SR-A expression at the cell surface.

4.7 Chinese Hamster Ovary Cells engineered to express SR-A

To test whether HSP27 could alter SR-A expression and acLDL uptake in a cell system where SR-A promoter activity is constitutively active, CHO cells – which otherwise do not express SR-A – were stably transfected with a mouse SR-A plasmid under control of a CMV promoter. The rationale was that if HSP27 could alter SR-A expression in a cell system that lacks regulation of SR-A gene expression, it would provide clues about whether SR-A is regulated at the post-translational level. Indeed, immunoblotting and flow cytometry analysis revealed no differences between HSP27 treatments and controls for both acLDL uptake **(Figure 21A)**, and SR-A protein expression **(Figure 21B)**.

To validate the functional role of SR-A in these cells, uptake assays were assessed using unlabelled acLDL, a neutralizing antibody against SR-A, and fucoidan. These conditions were effective in blocking acLDL uptake **(Figure 22)** demonstrating the functional utility of SR-A overexpression in this cell line. Mock transfected CHO cells (CHO-K1) showed low levels of acLDL uptake compared to transfected cells further confirming the fidelity of this cell model and additionally validating specificity of acLDL for SR-A.

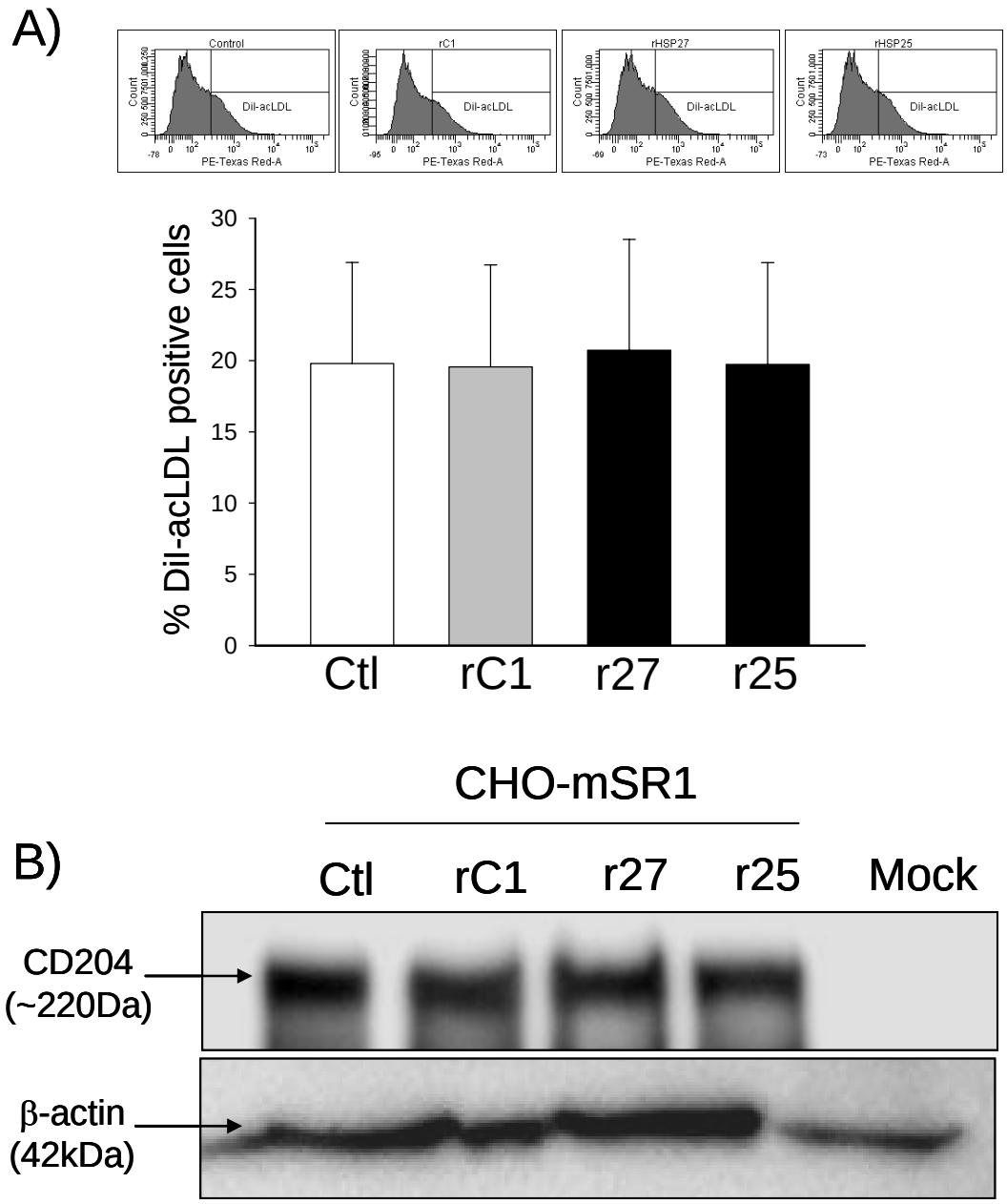


Figure 21. Effect of HSP27 on acLDL uptake and SR-A expression in CHO-SR-A cells

CHO cells stably transfected with mSRA-1 on a CMV promoter were treated with indicated recombinant proteins for 24 hours. **(A)** Cells were incubated with DiI-acLDL for 3 hours followed by flow cytometry analysis of percent positive DiI-acLDL cells Representative histograms are depicted. n=3. **(B)** Total cell lysate was subjected to immunoblotting for mSR-A and β-actin. Representative immunoblots are shown.

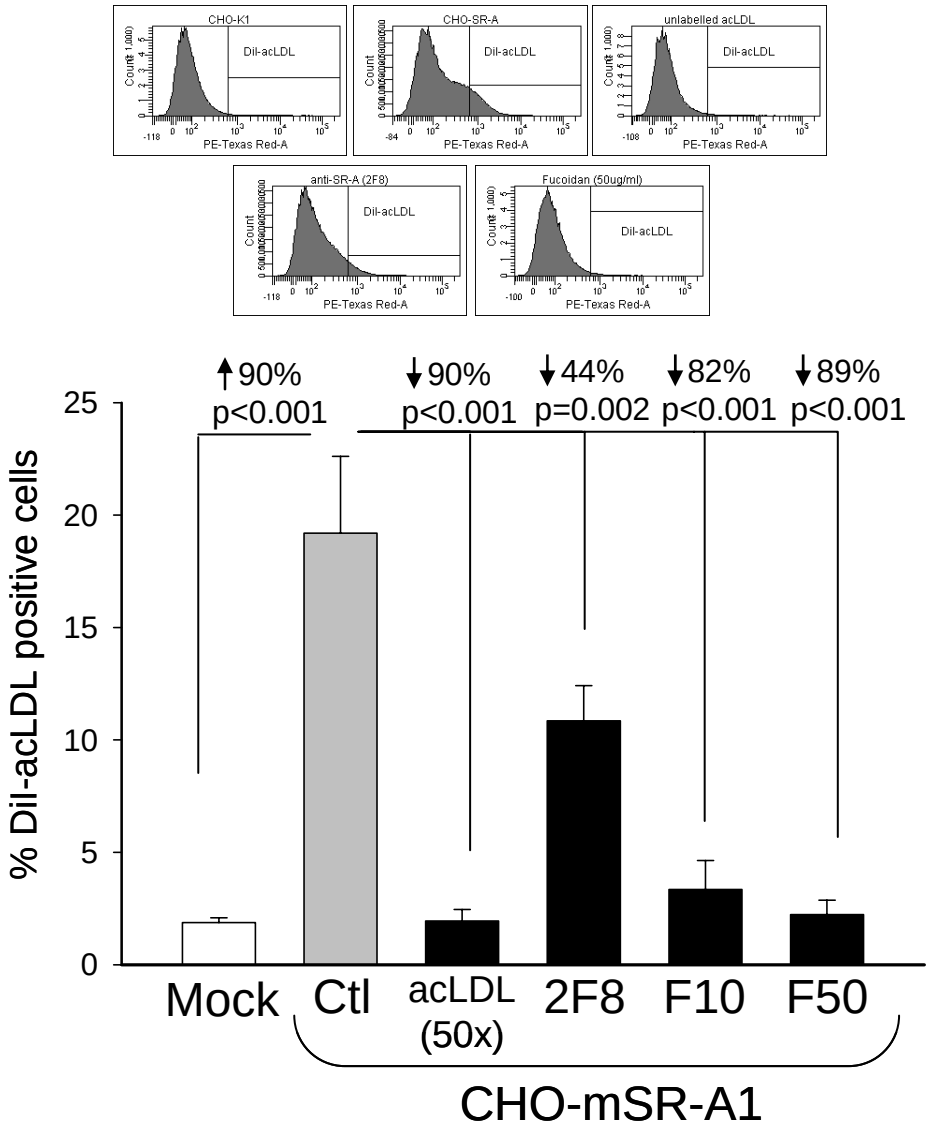


Figure 22. Effect of SR-A ligands and blockers on acLDL uptake in CHO-SR-A cells

The percentage of DiI-acLDL positive cells were quantified using flow cytometry after CHO-SRA1 cells were treated with indicated SR-A ligands and blockers. Mock transfected cells (CHO-K1) served as a negative control. Representative histograms are illustrated. n=4.

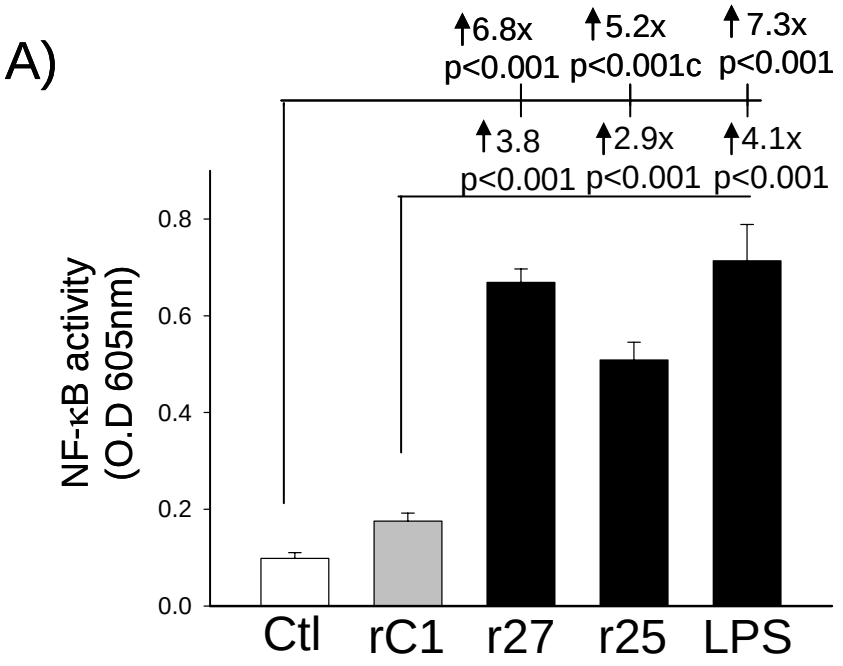
4.8 The role of NF-κb in HSP27 mediated responses in macrophages

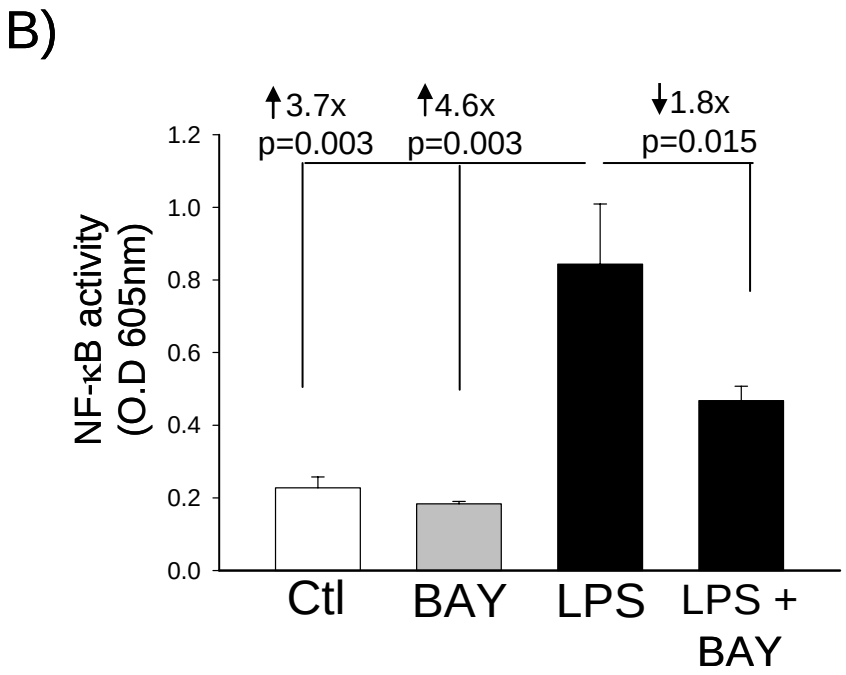
Previously, the O'Brien lab demonstrated that HSP27 favorably modulates macrophage inflammatory mediators such as IL1β and IL10 (262) – cytokines known to be regulated by NF-κB signaling. NF-κB is a key transcription factor that controls a battery of atherosclerotic gene programs, primarily via differential regulation of cytokine expression patterns that, in turn, regulate macrophage phenotype and activation in the vessel wall during lesion formation. To determine the molecular mechanism by which HSP27 alters SR-A expression, it was hypothesized that HSP27 mediated responses (e.g., acLDL uptake and SR-A expression) are dependent on intact NF-κB signaling mechanisms.

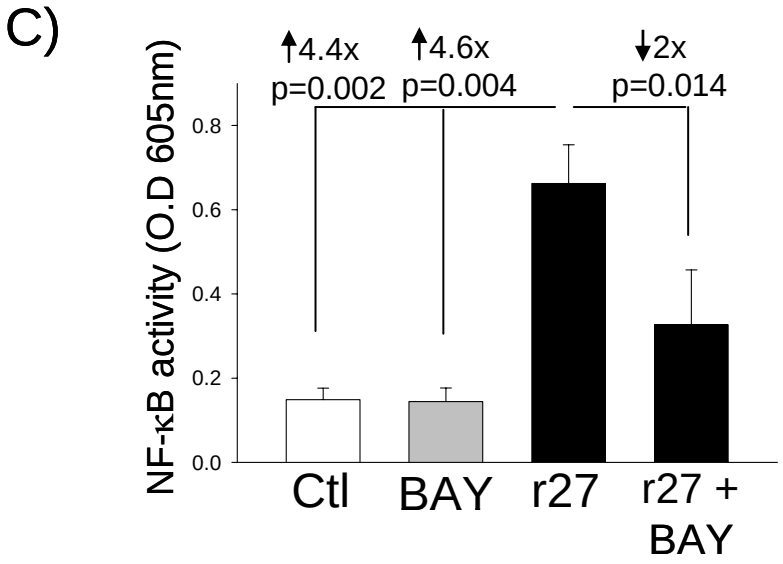
4.8.1 Effect of rHSP27 on NF-κB activation

THP-1 Blue cells stably transfected with an NF-κB promoter upstream of a secreted embryonic alkaline phosphatase (SEAP) gene provided a useful tool to study NF-κB activity, as expression and secretion of SEAP into the media is proportional to NF-κB activity. LPS is a potent activator of NF-κB. As a positive control, THP-1 Blue Cells were treated with LPS. This led to a 7.3 fold ($p<0.001$) increase in absorbance (i.e. NF-κB, **Figure 23A)** compared to no treatment control. Next, treatment of cells with rHSP27 and rHSP25 induced 6.8 and 5.2 fold respective increases in NF-κB activity versus control ($p<0.001$ for both; **Figure 23A)**. rC1 did not significantly increase NF-κB activation above control levels further demonstrating that rC1 lacks biological activity **(Figure 23A)** and is a reasonable protein null control.

To illustrate the specificity of NF-κB activation in the THP-1 Blue cell reporter assay, an NF-κB inhibitor (BAY11-7082) was tested in this system. BAY11-7082 blocks


A)
↑6.8x
p<0.001
↑5.2x
p<0.001c
↑7.3x
p<0.001
↑3.8
p<0.001
↑2.9x
p<0.001
↑4.1x
p<0.001
NF-κB activity
(O.D 605nm)
0.0
0.2
0.4
0.6
0.8
Ctl
rC1
r27
r25
LPS


B)
↑3.7x
p=0.003
↑4.6x
p=0.003
↓1.8x
p=0.015
NF-κB activity
(O.D 605nm)
0.0
0.2
0.4
0.6
0.8
1.0
1.2
Ctl
BAY
LPS
LPS +
BAY



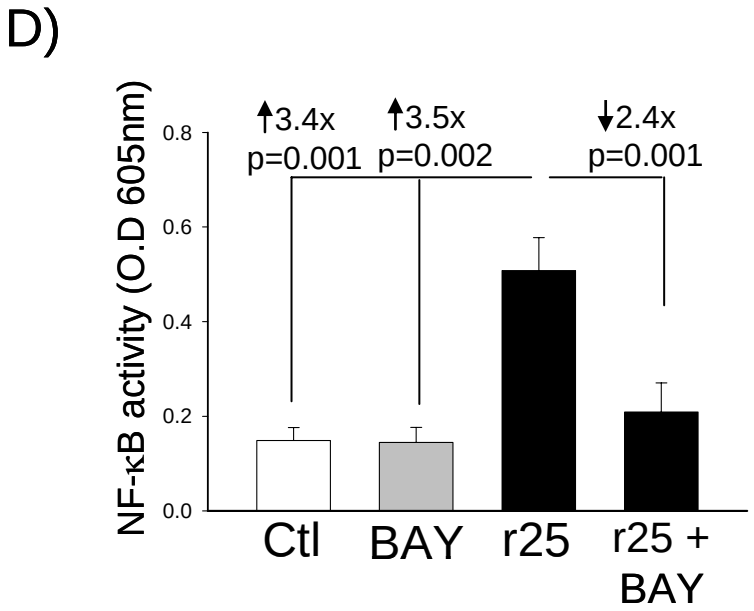


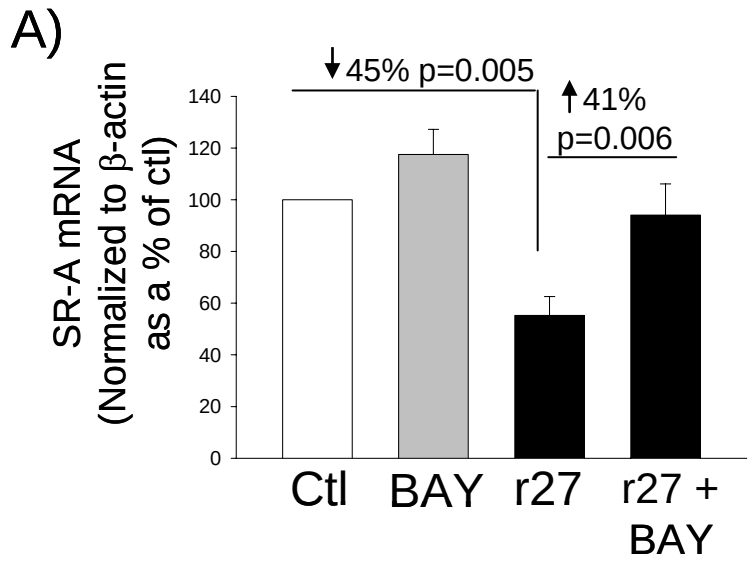
Figure 23. Effect of HSP27 on NF-κB activity in THP-1 Blue Cells

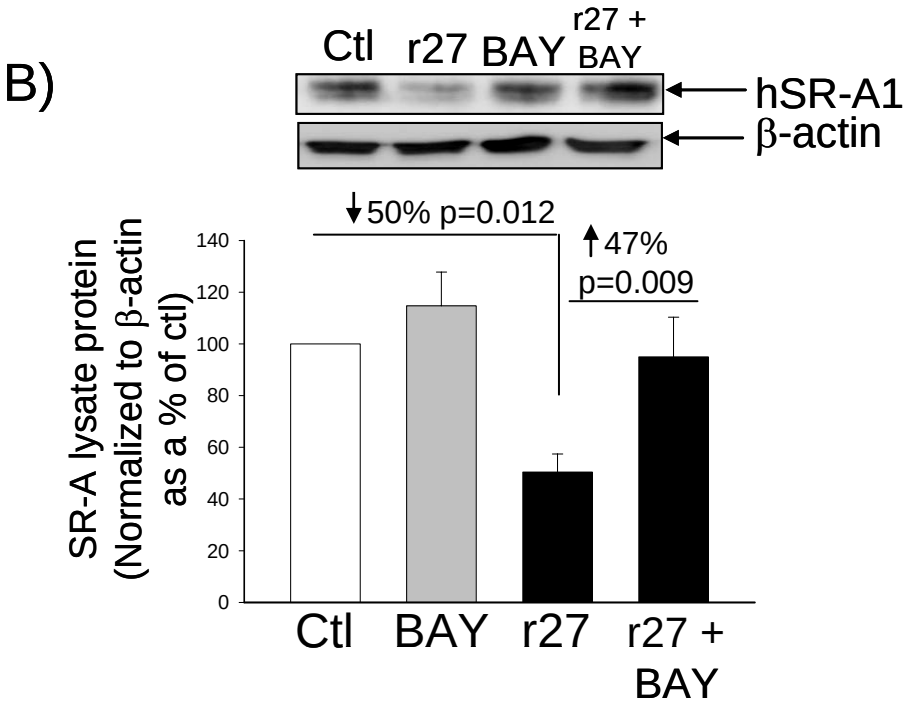
An NF-κB reporter assay was performed using THP-1 macrophages stably transfected with an NF-κB reporter. **(A)** PMA-differentiated cells were treated in the presence or absence of LPS (1 μg/ml), rHSP27 (9.6 μM), rHSP25 (9.6 μM), or rC1 (9.6 μM) for 24 hours (n≥5). An NF-κB antagonist, BAY11-7082 (10 μM), was added alone or 1 hour before treatment with LPS (1 μg/ml) (**B**, n=3); rHSP27 (**C**, n=4); or rHSP25 (**D**, n=4). The absorbance of secreted embryonic alkaline phosphatase (SEAP) was measured in conditioned media of each treatment and control group using a spectrometer.

translocation of the NF-κB-p65 subunit into the nucleus inhibiting NF-κB activity. LPS induction of NF-κB was partially blocked by 1.8 fold (p=0.015) in the presence of BAY11-7082 **(Figure 23B)**. No effect was observed when the BAY compound was added to the cells alone. THP-1 Blue cells were then treated with rHSP27 (and rHSP25) in the presence of BAY11-7082. There was a partial inhibition of NF-κB activity in cells treated with rHSP27 (2.0 fold; p=0.014, **Figure 23C**), and rHSP25 (2.4 fold; p=0.001, **Figure 23D**) in the presence of BAY compared with cells that received only the respective recombinant proteins without the BAY compound.

4.8.2 Effect of NF-κB inhibition on HSP27-mediated responses

Given that HSP27 specifically increased NF-κB activity in macrophages, the next goal was to explore the dependence of this pathway on SR-A expression. It was hypothesized that inhibition of NF-κB would reverse HSP27-mediated responses on SR-A expression, in turn, affecting acLDL uptake. Indeed, the presence of BAY11-7082 restored the inhibitory effect of rHSP27 on SR-A mRNA by 41% (p=0.006, **Figure 24A**), and SR-A protein by 47% (p=0.009, **Figure 24B**) compared to cells treated with rHSP27 alone – effects that showed SR-A mRNA and protein returning to baseline levels in the presence of BAY. The rescue effect on SR-A expression by BAY11-7082 in HSP27 treated cells was further associated with attenuation of HSP27 inhibition of acLDL uptake (36%; p=0.004, **Figure 24C**). These results demonstrate not only the dependence of NF-κB in regulating SR-A expression, but also illustrate that altering SR-A protein levels reflects the acLDL uptake in these cells. Thus, intact NF-κβ signaling is required for HSP27 regulation of SR-A expression, especially at the level of transcription.


A)
SR-A mRNA
(Normalized to β-actin
as a % of ctl)
45% p=0.005
41%
p=0.006
140
120
100
80
60
40
20
0
Ctl
BAY
r27
r27 +
BAY


B)
Ctl
r27
BAY
r27 +
BAY
hSR-A1
β-actin
SR-A lysate protein
(Normalized to β-actin
as a % of ctl)
50% p=0.012
47%
p=0.009
140
120
100
80
60
40
20
0
Ctl
BAY
r27
r27 +
BAY

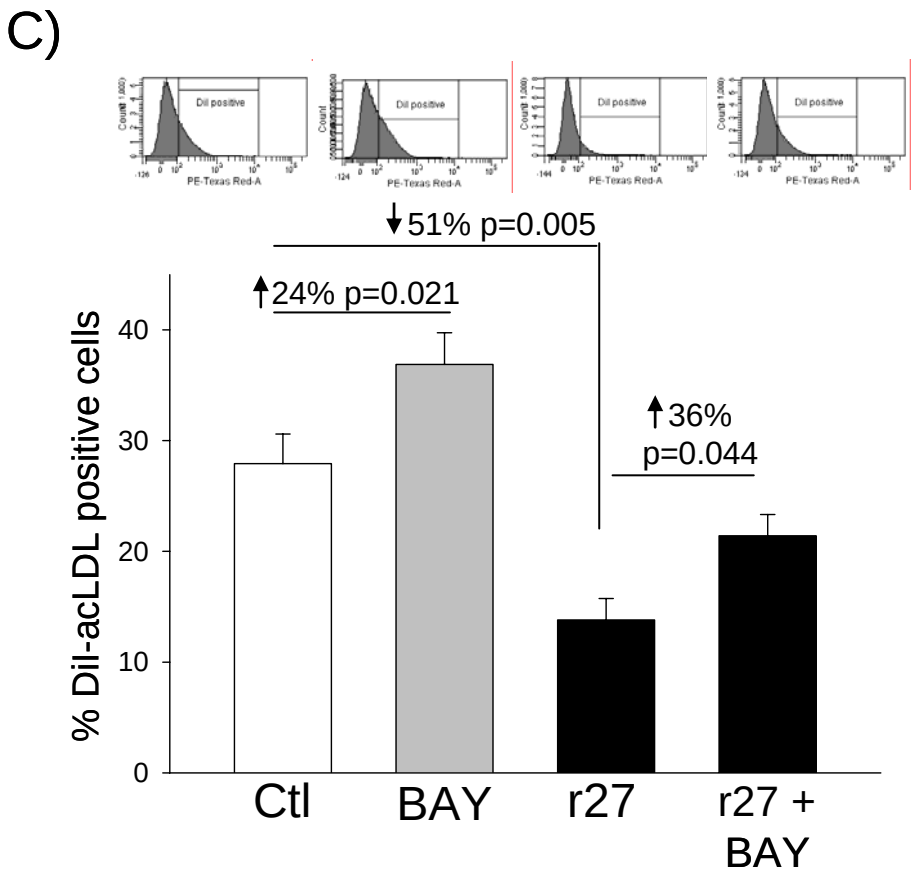


Figure 24. Effect of NF-κB inhibition on SR-A expression and acLDL uptake

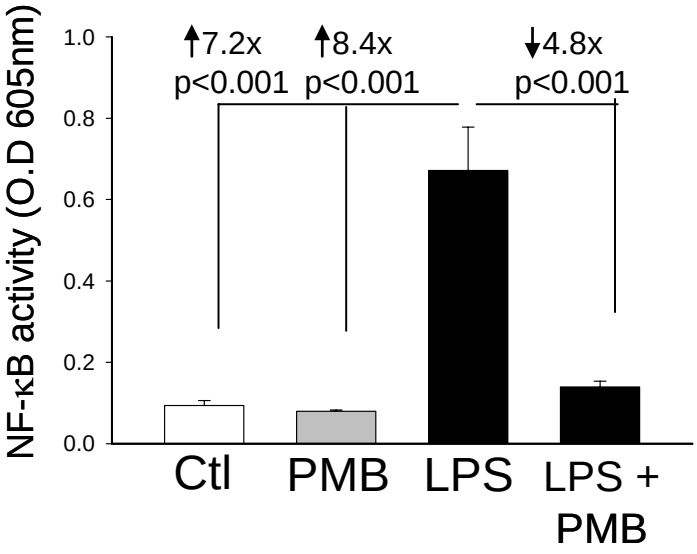
THP-1 macrophages were incubated with BAY11-7082 (10 μM) alone or 1 hour before treatment with rHSP27 for 24 hours. **(A)** qPCR analysis using primers complimentary to hSR-A1 and β-actin. Ratio of SR-A and β-actin Cps were plotted as a percent of the no treatment control (Ctl; n=5). **(B)** Total cell lysate was subject to Western Blotting for hSR-A1 and β-actin loading control. Quantification of SR-A protein was exhibited as the ratio of densometric units between SR-A and β-actin bands, and then expressed as a percentage of the no treatment controls (Ctl; n=5). **(C)** For uptake assays, cell were incubated with DiI-acLDL for 3 hours, then subjected to flow cytometry analysis. Data is expressed as the percentage number of DiI-acLDL positive cells. Representative flow histograms are illustrated (n=4).

4.9 Controlling for endotoxin and cytotoxic effects of rHSP27

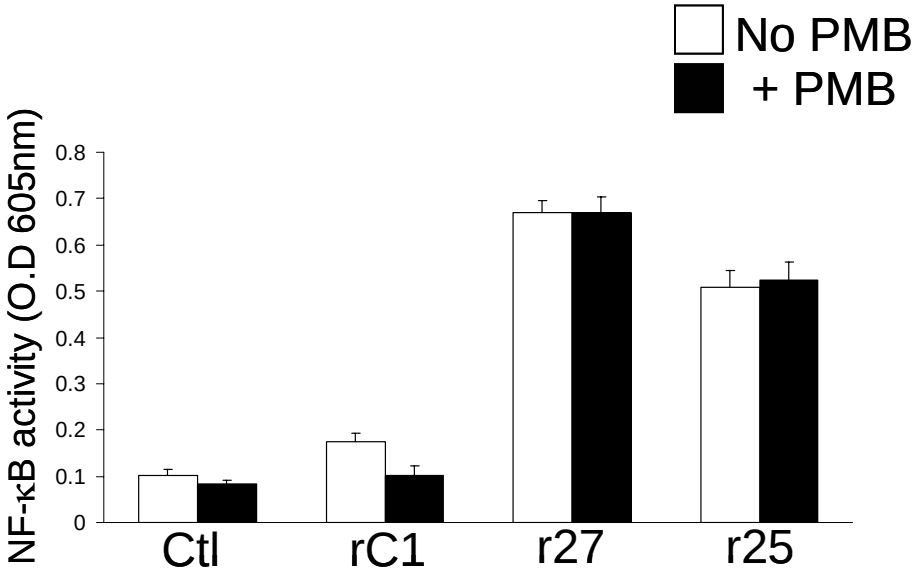
4.9.1 Effect of polymixin B (PMB) on HSP27-mediated responses

Despite best attempts to remove 99% of residual endotoxin associated with synthesis and purification of the HSP recombinant proteins generated from bacteria, it was necessary to perform a number of control experiments to ensure that rHSP27 effects on macrophages were not secondary to residual endotoxins that are present after synthesis. PMB is an antibiotic known to bind and neutralize LPS. As proof-of-principle to demonstrate the efficacy of PMB in neutralizing endotoxin mediated activation of NF-κB, THP-1 Blue cells were incubated with LPS in the presence of PMB. As expected, the 8.4 fold ($p<0.001$) increase in NF-κβ activity by LPS was completely blocked with PMB ($p<0.001$) - returning NF-κB to baseline levels (**Figure 25A**). There was no affect on NF-κB activity when PMB was added to the cells alone. Moroever, addition of PMB did not alter the effects of rHSP27 or rHSP25 on activation of NF-κB signaling compared to no treatment controls and rC1 **(Figure 25B)**. Similarly, when PMB was added to normal THP-1 cells treated with rHSP27 or rHSP25 in acLDL uptake **(Figure 25C)** or SR-A immunobloting experiments **(Figure 25D)**, no additional effect of PMB was observed on these endpoints, suggesting that endotoxin related effects do not play a role in HSP27 mediated responses.

A)


NF-κB activity (O.D 605nm)
1.0
0.8
0.6
0.4
0.2
0.0
↑7.2x
p<0.001
↑8.4x
p<0.001
↓4.8x
p<0.001
Ctl
PMB
LPS
LPS + PMB

B)


No PMB
+ PMB
NF-κB activity (O.D 605nm)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Ctl
rC1
r27
r25

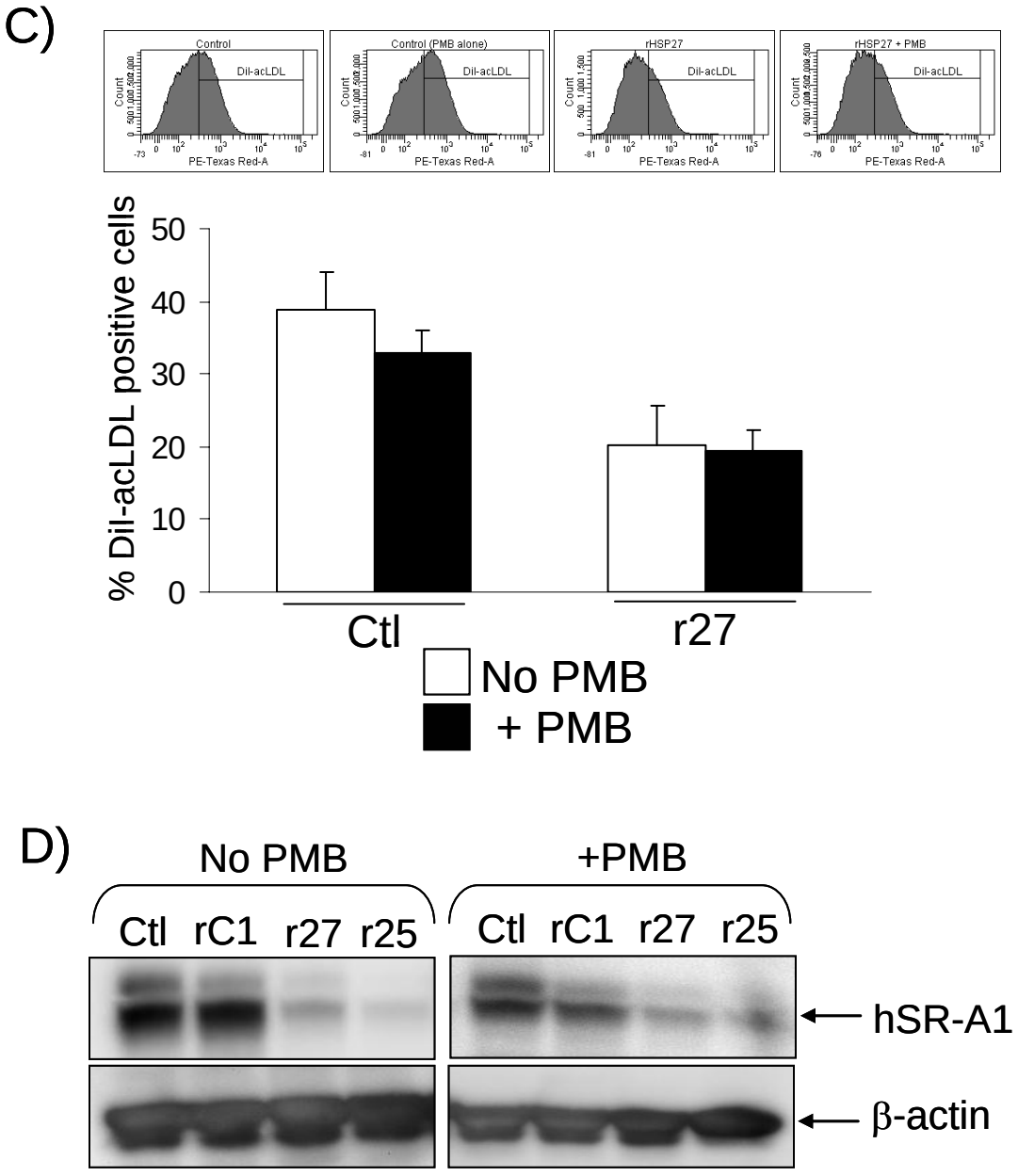


Figure 25. Effect of polymixin B (PMB) on HSP27 mediated biological responses

THP1 Blue cells were incubated with LPS (1μg/ml) (**A**, n=6) or indicated recombinant proteins (**B**, n=5-6) for 24 hours in the presence or absence of PMB (10 μM). NF-κB activity was determined by assaying the conditioned media for SEAP using a spectrometer. Normal THP-1 macrophages were incubated with indicated recombinant proteins for 24 in the presence or absence of PMB before being subject to flow cytometry for acLDL uptake assays (**C**, n=5-9) and immunoblotting using an antibody against hSR-A1 and β-actin as a loading control **(D)**. Representative flow histograms and immunoblots are illustrated.

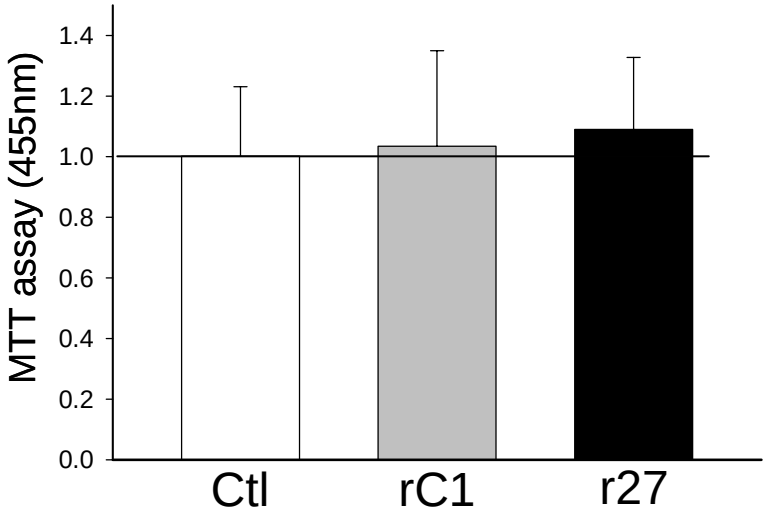
4.9.2 Cell viability and apoptosis

To rule out the possibility that recombinant protein treatments in the above experiments affect cell viability as well as apoptosis, MTT assays and Annexin-V (an early apoptosis marker) staining were performed using flow cytometry in THP-1 cells. MTT assays revealed no difference in cell viability between cells receiving rHSP27, rHSP25, rC1 and a no treatment control **(Figure 26A and 26B)**. In addition, using flow cytometry, no increase in early apoptosis was observed above baseline controls when cells were treated with the recombinant proteins **(Figure 26C)**.

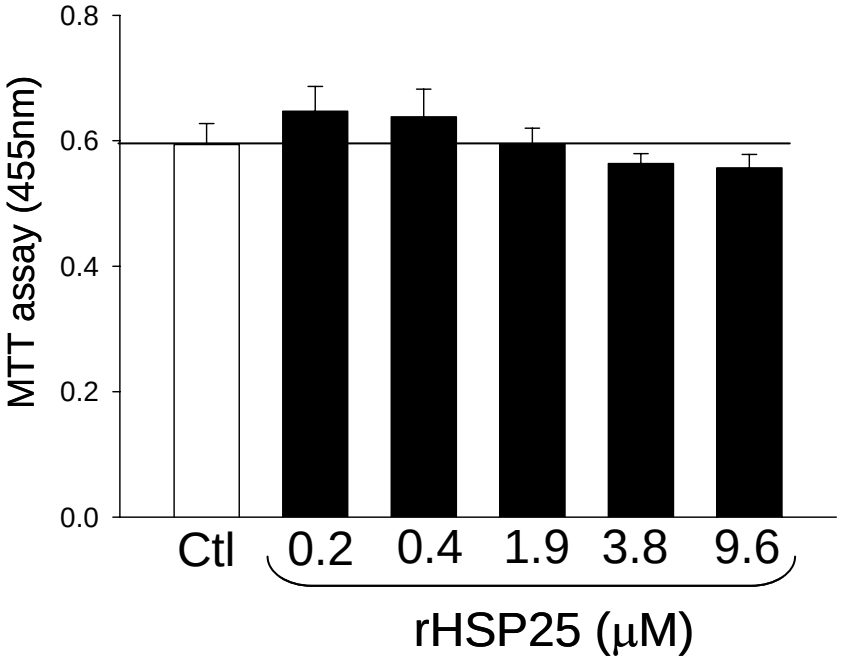
4.10 Effects of rHSP25 injection on atherosclerosis

Previous results from the O'Brien lab demonstrated that b.i.d s.c injection of 50 μg rHSP27 into $ApoE^{-/-}$ mice fed a high fat diet for 3 weeks was effective in reducing lesion burden and maintaining elevated levels of serum HSP27, thereby suggesting that extracellular HSP27 may represent a novel anti-atherogenic therapeutic (370). However, the precise role of SR-A in HSP27 atheroprotection *in vivo* remained elusive. Accordingly, it was hypothesized that (a) HSP25 (the mouse homologue of HSP27) administered as an exogenous therapy would be as efficacious as rHSP27 in reducing lesion size, and (b) HSP25-mediated protection would be dependent on the presence of SR-A by using $ApoE^{-/-}$ $SR\text{-}A^{-/-}$ mice, and therefore provide an *in vivo* mechanistic explanation for the atheroprotective actions of HSP27. Genotyping was performed in the $ApoE^{-/-}SR\text{-}A^{-/-}$ (double knout out, DKO) and $ApoE^{-/-}$ mice to determine the fidelity of the gene deletions, and confirm the presense of the DNA disruption. DNA was isolated from tail or ear clippings while cDNA was synthesized from liver RNA. Primer #2 binds to a region in the

A)


MTT assay (455nm)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Ctl
rC1
r27

B)


MTT assay (455nm)
0.8
0.6
0.4
0.2
0.0
Ctl
0.2
0.4
1.9
3.8
9.6
rHSP25 (μM)

C)

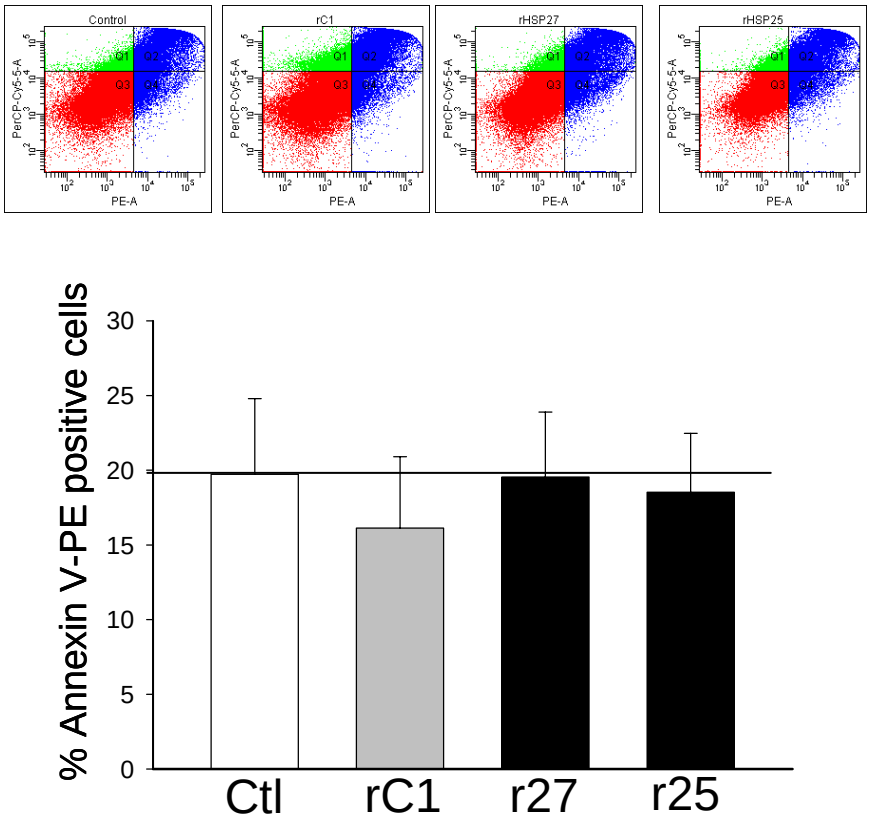


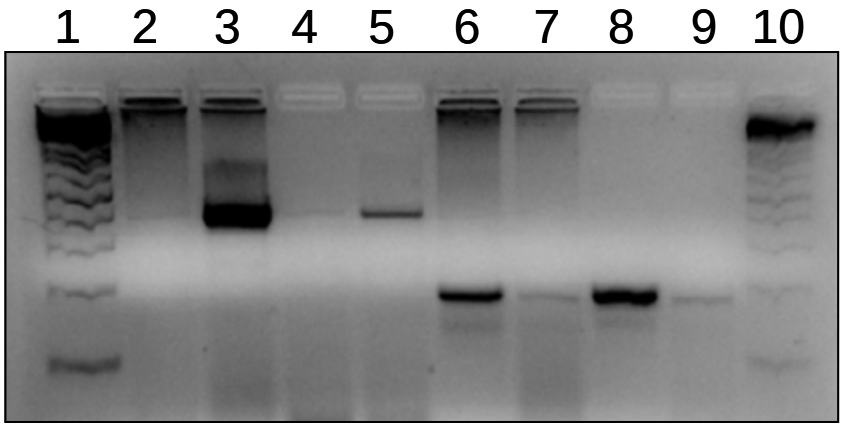
Figure 26. Cell viability and annexin-V staining assays

THP-1 macrophages treated with indicated recombinant proteins for 24 hours were assayed for cell viability with MTT tests using a spectrometer (**A & B,** n=4), and Annexin-V staining using flow cytometry (**C**, n=5). Representative flow cytometry density plots are illustrated of cells stained with Annexin-PE against the viability marker 4-AA7.

exogenously added insert of the SR-A gene, while primers #1 and #3 bind regions flanking upstream and downstream of the SR-A gene, respectively. PCR reactions using primers #1 and #2 revealed the presence of bands at 400kbp in both DNA and cDNA samples from the DKO mice (**Figure 27A, lanes 3 & 5**, respectively). As expected, no bands were observed in the ApoE$^{-/-}$ mice using this primer combination **(Figure 27A, lanes 2 & 4)**, suggesting the presence of insert in the ApoE$^{-/-}$SR-A$^{-/-}$. Bands at ~200kbp were present in PCR reactions using primers #1 and #3 in DNA and cDNA samples isolated from ApoE$^{-/-}$, but not in samples from ApoE$^{-/-}$SR-A$^{-/-}$ mice **(Figure 27, lanes 6 & 8, and 7 & 9).** The heavier sized bands seen in the DNA from ApoE$^{-/-}$SR-A$^{-/-}$ compared to ApoE$^{-/-}$ mice reflect the presence of a large product in the SR-A null sequence as demonstrated with sequencing results **(Figure 27B)**.

Next, female and male ApoE$^{-/-}$ and ApoE$^{-/-}$ SR-A$^{-/-}$ mice were fed a high fat diet (HFD) for 3 weeks while receiving rHSP25 (50 μg in 50 μl) or PBS (50 μl) subcutaneously twice daily (B.I.D). There were no differences in body weights between rHSP25 and PBS groups in both genotypes after the 3 week time-course (data not shown). Analysis of *en face* aortas of female ApoE$^{-/-}$ mice treated with rHSP25 revealed a 39% ($p<0.001$) reduction in lesion size compared to PBS control **(Figure 28A)**, confirming previous atheroprotective effects for HSP27 (370). A similar trend was observed for male mice, with a 38% decrease in *en face* lesions in ApoE$^{-/-}$ mice compared to PBS control **(**$p=0.06$; **Figure 28B)**. However, when both female **(Figure 28A)** and male **(Figure 28B)** ApoE$^{-/-}$ SR-A$^{-/-}$ mice were treated with rHSP25 there was no difference in lesion size compared to PBS ($p=0.791$ for females and $p=0.627$) for males, suggesting that rHSP25-mediated atheroprotection is abolished in the absence of SR-A.

A)



B)

>Exon #1 in KO mouse

CAGCTCAGCTTTTGAATTGGGAAATGAAGAACTGCTTAGTTTGTTCA
CTTAACACAAGTGACACATCTCAAGGTCCTATGGAAAAAGAAAATA
CCAGTAAAGTGGAAATGAGATTTACAATTATCATGGAACACATGAA
GGACATGGAGGAGAGAATCGAAAGCATTTCAAACTCAAAAGCCGA
CCTTATAGACACGGAACGCTTCCAGAATTCAGCATGGCAACTGACC
AAAGACTTAATGATATTCTTCTGCAGTTAAATTCCTTGATTTCGTCAG
TCCAGGAACATGGGAATTCCTGCAGCCCGGGGGATCCACTAGTTCT
AGTCGAGGAATTCTACCGGGTAGGGGAGGCGCTTTTCCCAAGGCAG
TCTGGAGCATGCGCTTTAGCAGCCCCGCTGGGCACTTGGCGCTACA
CAAGTGGCCTCTGGCCTCGCACACATTCCACATCCACCGGTAGGCG
CCAACCGGCTCCGTTCTTTTGGTGGCCCCTTCGCGCCACCTTCTACT
CCTCCCCTAGTCAGGAAGTTCCCCCCCGCCCCGCAGCTCGCGTCGT
GCAGGACGTGACAAATGGAAGTAGCAC**GTCTCACTAGTCTCGTGCA**
GATGGA ----->
ACTGGATGCAATCTCCAAGTCCTTGCAGAGTCTGAATATGACACTG
CTTGATGTTCAACTCCATACAGAAACACTGAATGTCAGAGTCCGTG
AATCTACAGCAAAGCAACAGGAGG

Figure 27. Genotyping of SR-A-/- mice

(A) Agarose gel showing bands that correspond to PCR reactions of SR-A amplicon products from genomic DNA and cDNA of $ApoE^{-/-}$ (WT) and $ApoE^{-/-}$ $SR\text{-}A^{-/-}$ (KO) mice. Lane 1 and 10 are DNA ladders. Lanes 2 and 3 are genomic DNA products from WT and KO mice, respectively, amplified using primers #1 & #2. Lanes 4 and 5 are cDNA products from WT and KO mice, respectively, amplified using primers #1 and #2. Lanes 6 and 7 are genomic DNA products from WT and KO mice, respectively, amplified using primers #1 & #3. Lanes 8 and 9 are cDNA products from WT and KO mice, respectively, amplified using primers #1 and #3.

(B) mRNA sequence showing exon #1 of the KO mouse MSR gene. Illustrated are the locations of the canonical gene (turquoise) and insert (black) as well as the locations of the primers in the sequence – primer #1 is in red, primer #2 spans the insert and is in orange, and primer #3 is in dark yellow.

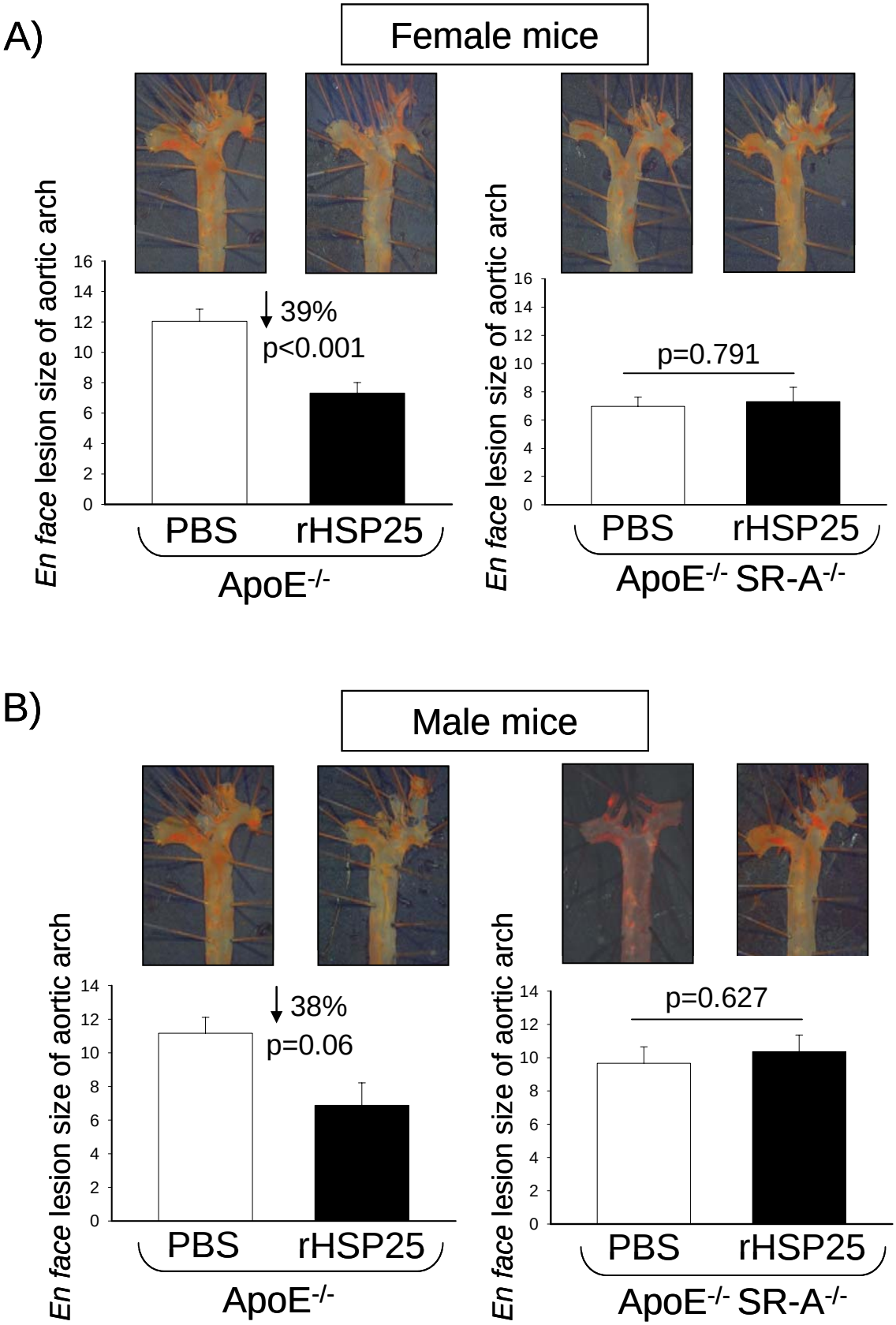
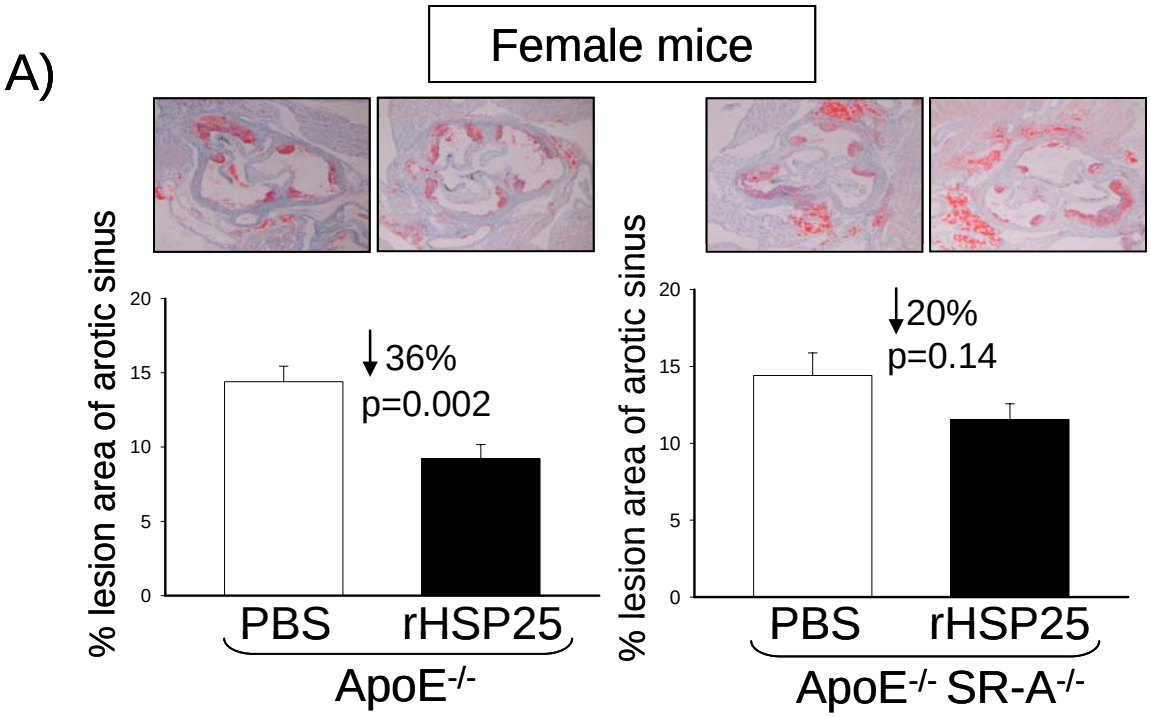
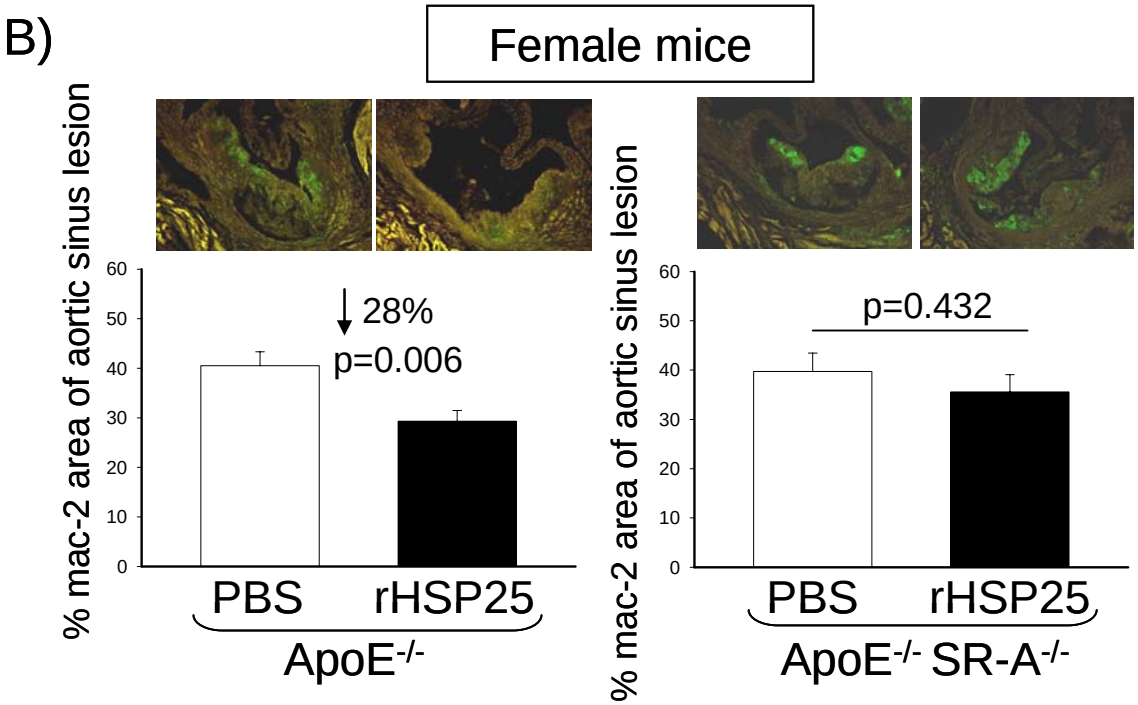

A)
Female mice
En face lesion size of aortic arch
16
14
12
10
8
6
4
2
0
39%
p<0.001
PBS
rHSP25
ApoE-/-
p=0.791
PBS
rHSP25
ApoE-/- SR-A-/-
B)
Male mice
En face lesion size of aortic arch
14
12
10
8
6
4
2
0
38%
p=0.06
PBS
rHSP25
ApoE-/-
p=0.627
PBS
rHSP25
ApoE-/- SR-A-/-

Figure 28. The impact of SR-A expression in HSP25-mediated atheroprotection on aortic en face lesions in ApoE$^{-/-}$SR-A$^{-/-}$ male and female mice

rHSP25 (50 μg in 50 μl) or PBS (50 μl) was administered subcutaneously B.I.D for 3 weeks to female (**A**, n=8-9) and male (**B**, n=4-8) ApoE$^{-/-}$ and ApoE$^{-/-}$SR-A$^{-/-}$ mice. Area of Oil red O staining in the *en face* preparation were quantified as a percentage of the aortic area. Representative aortic *en face* images are exhibited.

It should be noted that the female PBS control ApoE$^{-/-}$ SR-A$^{-/-}$ mice displayed a ~48% reduction in lesion size compared to PBS control ApoE$^{-/-}$ mice, suggesting an effect of SR-A on lesion size in the *en face* lesions. Interestingly, in the males, SR-A did not have an effect on *en face* lesions at baseline. Furthermore, rHSP25 treated female ApoE$^{-/-}$ mice led to 36% (p=0.002) and 28% (p=0.006) reductions in aortic sinus lesion area (**Figure 29A)** and macrophage content (as assessed by mac-2 staining) **(Figure 29B)**. Although there was a trend toward reduction of aortic sinus lesions and macrophage content by 25% and 10%, respectively in the rHSP25 treated ApoE$^{-/-}$ SR-A$^{-/-}$ mice compared to their respective PBS littermates, these effects were not statistically significant (p=0.14 and p=0.432, respectively) **(Figure 29C)**. In addition, two way ANOVA revealed no difference between the rHSP27 treated ApoE$^{-/-}$ and ApoE$^{-/-}$SR-A$^{-/-}$ mice groups. Unlike the *en face* lesion in the female mice, there was no effect of SR-A in lesions (as well as macrophage content) quantified in the aortic sinus when PBS control ApoE$^{-/-}$ mice were compared to PBS control ApoE$^{-/-}$ SR-A$^{-/-}$ mice. Taken together, although the effect of rHSP27 was lost in the absence of SR-A in both the *en face* and aortic sinus of both female and male mice, there appeared to be sex- and site-dependent affect of SR-A on atherosclerosis (e.g., at baseline the absence of SR-A reduced *en face* lesions size in female but not male mice yet had no effect in the aortic sinus in the females).


A)
Female mice
% lesion area of arotic sinus
20
15
10
5
0
36%
p=0.002
PBS
rHSP25
ApoE-/-
20%
p=0.14
PBS
rHSP25
ApoE-/- SR-A-/-


B)
Female mice
% mac-2 area of aortic sinus lesion
60
50
40
30
20
10
0
28%
p=0.006
PBS
rHSP25
ApoE-/-
p=0.432
PBS
rHSP25
ApoE-/- SR-A-/-

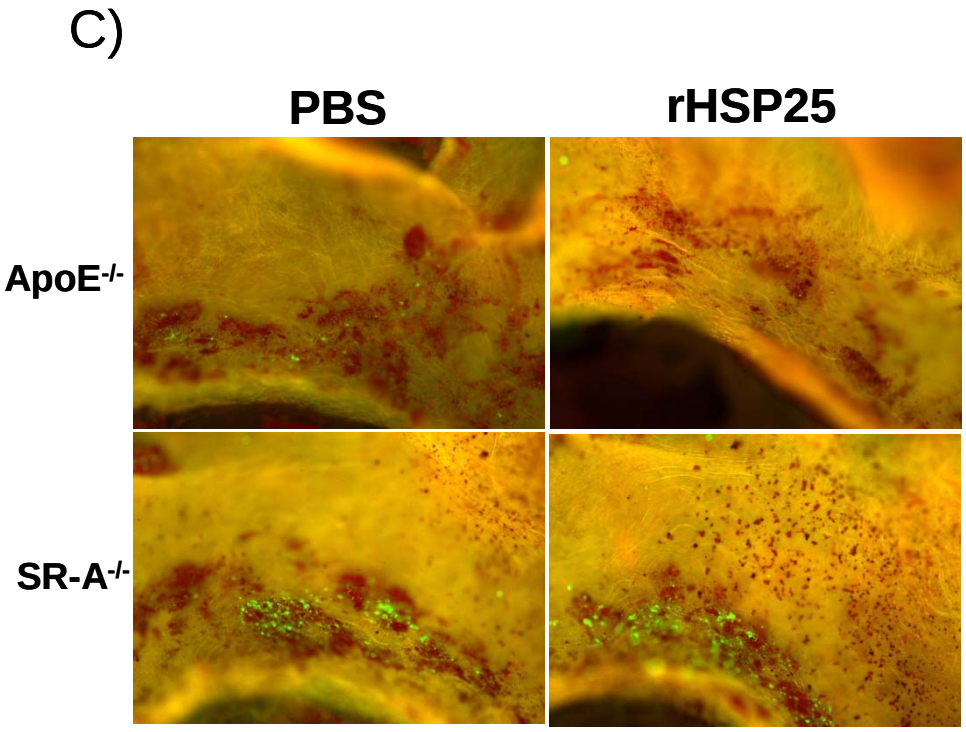


Figure 29. The impact of SR-A expression in HSP25-mediated atheroprotection on lesion size and macrophage content in female ApoE$^{-/-}$ SR-A$^{-/-}$ mice

rHSP25 (50 μg in 50 μl) or PBS (50 μl) was administered subcutaneously B.I.D for 3 weeks to female ApoE$^{-/-}$ and ApoE$^{-/-}$SR-A$^{-/-}$ mice. **(A)** Area of Oil red O staining was quantified as a percent of the aortic sinus. Representative aortic sinus images shown. n=9 for all groups. Macrophage content was quantified as the area of mac-2 staining (green) of the aortic sinus lesion **(B)**, and also illustrated in the *en face* aortic arch lesions **(C).** Representative mac-2 staining images are shown. n=9 for all groups.

The anti-atherogenic effect of rHSP25 treatment was associated with reduced SR-A expression in the intima of ApoE$^{-/-}$ mice as observed qualitatively by immunohistochemistry **(Figure 30A-C)**. Absence of SR-A immunolabeling in the ApoE$^{-/-}$ SR-A$^{-/-}$ tissue illustrated the specificity of the antibody against SR-A *in vivo* **(Figure 30D).**

Interestingly, HSP25 treatment resulted in a decrease in total serum cholesterol levels in female ApoE$^{-/-}$ mice by 32% (p=0.002, **Figure 31A**), as well as ApoE$^{-/-}$ SR-A$^{-/-}$ mice by 25% (p=0.026, **Figure 31B**) compared to PBS controls of their respective littermates, validating previous findings with rHSP27 (370). Serum lipid profiles revealed a reduction in the VLDL and LDL fraction in the ApoE$^{-/-}$ mice treated with rHSP25, an effect less pronounced in the ApoE$^{-/-}$ SR-A$^{-/-}$ mice. In contrast, rHSP25 treatment did not alter cholesterol levels in male mice **(Figure 31C)** despite the observed atheroprotective effect in the vessel wall.

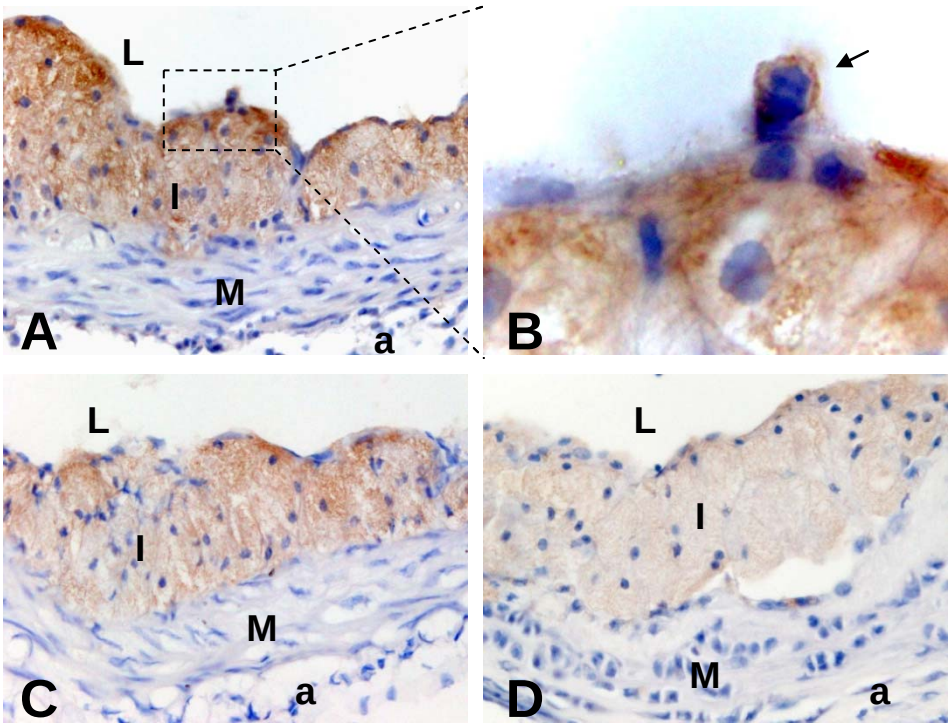
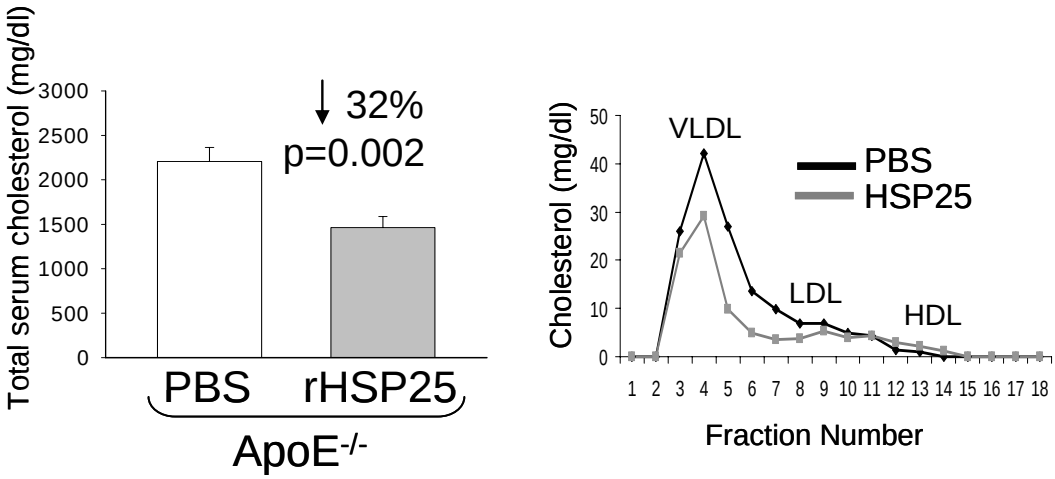


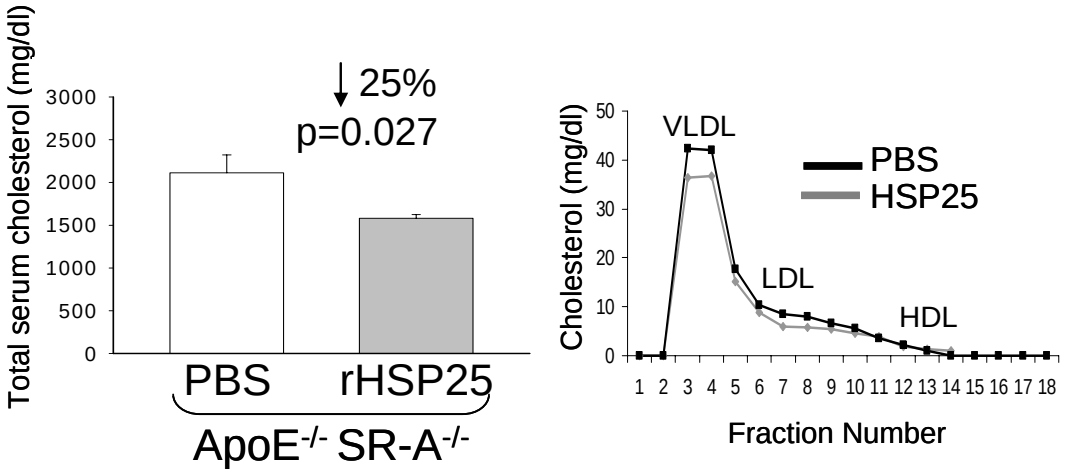
Figure 30. Effect of rHSP25 treatment on SR-A expression *in vivo*

Immunohistochemistry of mouse SR-A (brown staining) in aortic sinus sections of ApoE$^{-/-}$ mice treated with PBS control **(A & B)** or rHSP25 **(C)**. SR-A staining in ApoE$^{-/-}$ SR-A$^{-/-}$ mice was performed as a negative control to demonstrate antibody specificity **(D)**. Tissues were counterstained with haematoxylin and eosin. L=lumen side, I=intima, M=media, A=adventitia. Magnification = 400x for A, C, and D; 1000x for B.

A)


Total serum cholesterol (mg/dl)
3000
2500
2000
1500
1000
500
0
32%
p=0.002
PBS
rHSP25
ApoE-/-
Cholesterol (mg/dl)
50
40
30
20
10
0
VLDL
LDL
HDL
PBS
HSP25
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
Fraction Number

B)


Total serum cholesterol (mg/dl)
3000
2500
2000
1500
1000
500
0
25%
p=0.027
PBS
rHSP25
ApoE-/- SR-A-/-
Cholesterol (mg/dl)
50
40
30
20
10
0
VLDL
LDL
HDL
PBS
HSP25
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
Fraction Number

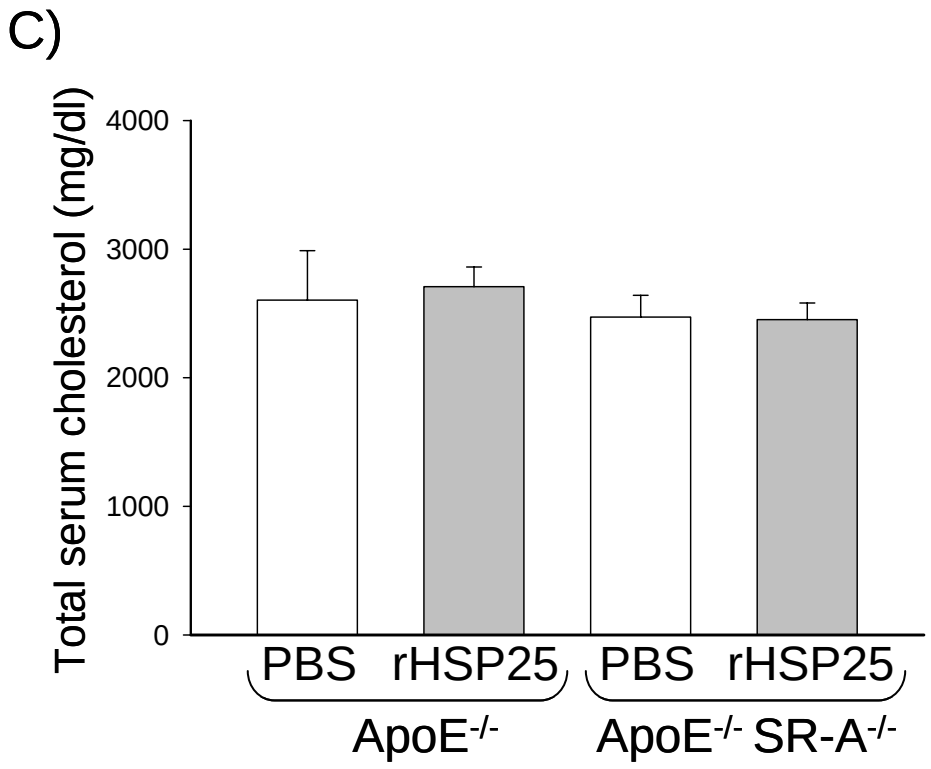


Figure 31. Total serum cholesterol levels and profiles in rHSP25 treated ApoE$^{-/-}$ and ApoE$^{-/-}$ SR-A$^{-/-}$ mice.

Cholesterol levels were assayed from total and FPLC fractions of serum isolated from female ApoE$^{-/-}$ (**A**, n=8) and ApoE$^{-/-}$ SR-A$^{-/-}$ (**B**, n=9) mice injected with rHSP25 or PBS. Total serum cholesterol levels were also measured in male mice (**C**, n=3-8). HSP25 treatment led to reductions in total cholesterol levels in both ApoE$^{-/-}$ and ApoE$^{-/-}$ SR-A$^{-/-}$ in female but not male mice.

5.0 DISCUSSION

The data presented in this thesis is an extension of previous work in the O'Brien laboratory demonstrating that augmented serum HSP27 levels attenuate lesion burden by modulating macrophage function in the vessel wall (262,370). However, this thesis expands upon the current understanding on how HSP27 acts to protect against atherosclerosis, and provides new data underlying how HSP27-mediated atheroprotection is achieved. Specifically, this thesis sought to determine how SR-A is associated with HSP27 mediated inhibition of foam cell formation, an effect previously identified as a consequence of the physical interaction between HSP27 and SR-A in macrophages *in vitro* (262). Secondly, this thesis asks whether SR-A is important for HSP27 therapeutic atheroprotection *in vivo*. Three findings are made: 1) Extracellular HSP27 blocks uptake of modified lipids into macrophages by reducing SR-A expression, possibly through gene regulation, leading to reduction of receptors at the cell surface, 2) HSP27 effects on SR-A expression may be associated with the NF-κB pathway, and 3) HSP27-mediated atheroprotection requires intact SR-A expression because in the absence of SR-A the therapeutic effect of HSP27 is abolished. The loss of protection in the absence of SR-A occurred despite achieving reductions in total serum cholesterol levels in all of the HSP25 treated genotypes. These findings provide evidence that the therapeutic actions of HSP27 are pleitropic and include blocking foam cell formation via modulation of SR-A expression in the vessel wall but also by reducing total serum cholesterol. As such, these observations support previous findings from the O'Brien lab and agree with the working hypothesis presented in this thesis showing that SR-A may be a target for HSP27 protection. Please note that HSP27 and HSP25 are used interchangeably in the Discussion chapter.

rHSP27 modululates SR-A expression

Previous studies was the O'Brien lab demonstrate that HSP27 is actively secreted from macrophages via endosomal vesicles (262). Once in the extracellular space it was postulated that HSP27 interacts with SR-A to modulate inflammatory foam cell formation by competing with SR-A for acLDL uptake. Specifically, the O'Brien lab demonstrated that binding of HSP27 to macrophages was completely abolished in the absence of SR-A, suggesting that SR-A is a principle target for HSP27 (262). This thesis expands upon these observations showing that rHSP27 may not necessarily physically interact with SR-A on the surface of macrophages, *per se*, as previously described, but rather downregulate SR-A expression at the cell surface as a means to block foam cell formation.

There is a growing body of literature to suggest that traditionally described intracellular chaperone molecules are released from cells of the immune system into the extracellular space where they act as danger signals via physical interactions with pattern recognition receptors (394-396). Indeed, extracellular HSP27, used in the form of a recombinant protein throughout this study, affects macrophage biology by blocking uptake of acLDL, as well as protection against atherosclerosis *in vivo*. This supports the argument in favor of exogenous HSP27 as an important player in the extracellular milieu of the lesion, acting to inhibit foam cell formation during the developing lesion, and therefore dampening the inflammatory response characteristic of atherosclerosis.

A number of studies have demonstrated that other HSPs physically interact with pattern-recognition receptors on the surface of antigen-presenting cells. For example, the endoplasmic reticulum heat shock protein, gp96, was found to bind with SR-A on the surface of macrophages and become internalized by this receptor (397). Extracellular HSP70

binds with members of the scavenger receptor family (358,398,399) as well as TLR2/4 (400). The results presented in this thesis illustrate that rHSP27 may not physically bind with SR-A on the surface of macrophages or compete with acLDL for uptake into the cell as was originally described (262). Rather, it was found herein that HSP27 blocks lipid uptake by acting to downregulate SR-A expression, possibly at the gene level. When rHSP27 is added simultaneously to the culture media with acLDL, binding and uptake of acLDL is not blocked, suggesting an alternate mechanism as opposed to competing with SR-A for acLDL uptake. Instead, a pre-treatment strategy was required in order for HSP27 to be effective in inhibiting acLDL binding and uptake.

The length of time in which acLDL was added to the culture media also influences the efficacy of HSP27 to inhibit uptake. For example, at the maximal dose of 9.6 μM, rHSP27 inhibited uptake by 54%, 34%, and 31% at 1, 3, and 24 hours of acLDL challenge following pre-treatment of HSP27, respectively. The loss of inhibition with increased time of acLDL incubation most likely reflects 1) a saturation effect of DiI-acLDL into the cell, and/or 2) an alternative receptor or mechanisms of acLDL uptake. It is clear that acLDL is a ligand for SR-A, as demonstrated in differentiated THP-1 macrophages and CHO-SR-A cells using unlabelled acLDL for receptor competition assays, a neutralizing antibody against SR-A, and fucoidan. Also, SR-A null bone-marrow-derived macrophages are more resistant to foam cell formation compared to wild type cells as demonstrated using oil-red-O staining as well as flow cytometry (37% reduction in acLDL uptake compared to single-knockout $ApoE^{-/-}$ cells). However, 63% of acLDL remains in the cells in the absence of SR-A, which is significant compared to other studies demonstrating that SR-A accounts for more than 70% of acLDL being taken up into macrophages (213,214). Since a significant

amount of acLDL appears to be taken up by the cells even in the absence of SR-A, these experiments do not fully support the hypothesis that the diminished uptake of acLDL in THP-1 cells in response to HSP27 is specific for the proportion that is SR-A mediated uptake. Although the effect of HSP27 on acLDL uptake is diminished in the SR-A$^{-/-}$ macrophages and is not statistically lower than control, uptake is still decreased by ~25%. Indeed, other scavenger receptors such as CD36, and LOX-1 are known to bind and take up acLDL. Future experiments using CD36$^{-/-}$ or LDLr$^{-/-}$ would help clarify whether HSP27 affects other lipoprotein ligands, such as LDL or oxLDL and provide important controls for not only the specificity of the effect for SR-A but also the effect of acLDL as a SR-A specific ligand. Certainly, a limitation in these experiments is the use of commercially purchased acLDL. The degree of modification is known to affect receptor specificity and its uptake in macrophages (168). Since, acLDL can become oxidized during its shelf life, one explanation for the high levels of residual acLDL in the ApoE$^{-/-}$SR-A$^{-/-}$ cells is that that preparation become oxidized over time and thus rendering it a ligand for CD36.

Nevertheless, given that inhibition of acLDL binding and uptake by HSP27 is time-dependent, and that acLDL is a well established ligand for SR-A, it was hypothesized that HSP27 mediated downregulation of SR-A would lead to loss of receptors at the cell surface and might explain the inhibitory effect of HSP27 on acLDL binding/uptake. Indeed, the observation that HSP27 reduces total cellular SR-A, an effect associated with reduction of receptors at the cell surface, confirms this hypothesis and is further supportive of the anti-foam cell role of HSP27. Furthermore, HSP27 reduced SR-A expression at the gene level – an effect that was only observed at 6 hours. At 24 hours, on the other hand, HSP27 returned mRNA levels beyond baseline levels. The SR-A gene is tightly controlled in macrophages

(207,208). THP-1 cells used in this experiment are differentiated with PMA, which is a potent PKC activator, and positive regulator of SR-A expression (401). Therefore, it is likely that a feedback regulation effect occurs whereby early changes in SR-A message levels may stimulate a proportional increase in message levels at later times. The temporal changes in SR-A levels by HSP27 treatment may illustrate the importance of the macrophage in maintaining SR-A transcriptional equilibrium. Translation of a gene message into functional protein is a highly regulated process. Many factors such as timing, the signaling pathways involved, transcription factor binding, and mRNA stability/half-life all influence delays between coding of a transcript message and production of its subsequent protein on the ribosome complex. Changes in SR-A gene expression by HSP27 at 6 hours does not appear to reflect the protein stoichiometry at 24 hours. It is likely that the increase in SR-A message at 24 hours compensates for decreased levels observed at early time points. How this biphasic response is translated to a sustained decrease in SR-A *in vitro* or *in vivo*, that ultimately alters atherogenesis, is unclear. Certainly, in order to maintain elevated HSP25 serum levels and in turn atheroprotection, a twice daily injection regimen was required and this could ultimately translate into reducing SR-A mRNA and sustained reduction in protein over time. Although immunohistochemistry staining of lesions provided evidence that SR-A expression may be diminished in the rHSP25 treated mice, this qualitative data is only suggestive. Longer time-course studies for mRNA and protein in THP-1 cells is required to fully appreciate the long term effect of HSP27 treatment on SR-A expression. Furthermore, given that macrophage content in the plaque were reduced in the rHSP25 treated mice it is unclear if SR-A expression is actually lower on a per cell basis or the result of decreased

numbers of macrophages in the lesion. Measurements of SR-A mRNA expression in the aorta and normalization to Mac-2 staining would help answer these questions.

To further confirm whether the influence of HSP27 on SR-A expression occur at the transcriptional level, CHO cells were engineered to express SR-A on a plasmid with a CMV promoter. Treatment of these cells with rHSP27 failed to reveal changes in either SR-A protein or acLDL uptake compared to control. The CMV promoter constitutively drives SR-A expression, which prevents SR-A gene activity from being regulated. As such, these results provide some evidence against post-translational modification as an explanation for HSP27's effect on SR-A expression. However, it should be noted that there is no evidence in this thesis that HSP27 or HSP25 effects the binding and/or uptake of acLDL in the same manner as they do with THP-1 cells. Since CHO cells do not normally express SR-A, they likely lack other receptors and signaling moieties important for HSP27 signaling responses compared to THP-1 macrophages. Therefore, caution must be taken in the interpretation of these results linking HSP27 to regulation of SR-A expression specifically at the gene level rather than regulation via intracellular trafficking and signaling pathways that involve lysosomal or ubiquitin-mediated degradation (199). In preliminary experiments performed (not shown in the thesis) using known inhibitors of the proteosomal and lysosomal degradation pathways, MG123 (402) and E64/pepstatin (403), respectively HSP27 mediated inhibition of acLDL and SR-A protein expression were restored, suggesting that SR-A may be regulated by a proteosomal pathway. Interestingly, MG132 has also been found to inhibit NF-κB activation (404), agreeing with the hypothesis that HSP27 regulates SR-A through an NF-κB pathway.

Previous observations from the O'Brien lab demonstrate that HSP27 binds to SR-A on the surface of macrophages, and in this way competes with acLDL uptake. On the other hand, results presented in this thesis provide evidence that HSP27 may not physically interact with SR-A but rather downregulate its expression. A number of factors may provide an explanation for the differences in results between this study and the earlier O'Brien reports. In Rayner *et al.*, HSP27-transfected macrophages were stimulated to secrete HSP27 tagged with EGPF (262). Conditioned media containing HSP27-EGFP was then applied to naïve macrophages and using immunoprecipitation techniques found to interact with SR-A. The HSP27 that is secreted from macrophages likely has different conformation states than the rHSP27 protein produced using *in vitro* methodologies. Processing of *de novo* proteins in macrophages is under control of physiologically relevant machinery compared to rHSP27 being synthesized artificially in bacterial expression systems. Thus, differences in the confirmation or oligomeric states of eukaryotic HSP27 versus bacterially generated rHSP27, may have contributed to the differences in their binding capacities to surface receptors. While the results presented in this thesis suggest that HSP27 does not compete with acLDL for uptake via SR-A, it does not exclude the possibility that HSP27 still binds to SR-A in a manner that is distinct from the acLDL binding site and activates intracellular signaling pathways, consistent with previous findings by Rayner *et al.*. Oligerimization states play important roles in the biological functions of intracellular HSP27 (405,406). Although biochemical analysis of O'Brien rHSP27 reveals that it exists as a dimer when added to the SDS-PAGE under non-reducing conditions, it is currently unknown exactly what confirmation or folding states are required for its physical binding or association with its cognate receptor (or SR-A) in the extracellular space. Furthermore, the variability in protein

characteristics such as oligomerization states or folding properties among different batches of rHSP27 are important limitations that need to be acknowledged in this study. Certainly, discovery of novel HSP27 interacting factors may provide an alternative target to stimulate HSP27's release into the circulation as previously demonstrated by estrogens (366), or ERβ isoform selective agonists (367). However, these targets may themselves possess off-target side-effects. Therefore, the most efficacious strategy to augment HSP27 serum levels, either by direct injection of rHSP27 protein or stimulation of its release from cells, is an area that requires further research.

Extracellular HSP27 and NF-κB activity

Macrophages express a variety of NF-κB-dependent genes that promote a balance of both anti-inflammatory and pro-inflammatory responses during atherogenesis (407,408). The O'Brien lab previously demonstrated that extracellular HSP27 favorably modulates macrophage inflammatory responses by increasing IL-10 secretion into the culture media while decreasing secretion of IL-1β (262) – cytokines known to be controlled by NF-κB. In addition, HSP27 was found to modulate a number of macrophage functions such as adhesion , migration (262) and apoptosis in the vessel wall (369,370). Therefore, it was hypothesized that extracellular HSP27 alters the inflammatory profile of macrophges by specifically modulating NF-κB activity (409). Using differentiated THP-1 macrophages stably transfected with an NF-κB-inducible secreted alkaline phosphatase gene, it was demonstrated that rHSP27 and rHSP25 activated NF-κB activity – results that are in agreement with previous observations from the O'Brien lab (409). Induction of the canonical (i.e. classical) NF-κB pathway by LPS or HSP27 could be blocked in the presence

of BAY-7082 - an NF-κB inhibitor that precludes phosphorylation of IκBα, in turn, preventing its degradation and subsequent translocation of its binding proteins, p50/p65 transcription factors, into the nucleus **(Figure 32)**. This demonstrates specificity of NF-κB induction by rHSP27 and provides "proof-of-principle" for the antagonistic actions of the BAY compound on NF-κB activation.

Given that HSP27 appears to signal through NF-κB in macrophages, it was still unclear what the functional importance of this pathway entailed. Therefore, it was hypothesized that HSP27 regulates SR-A expression and acLDL uptake through NF-κB activation. THP-1 macrophages were treated with rHSP27 in the presence of the NF-κB inhibitor before being assayed for SR-A mRNA, protein, and acLDL levels. Indeed, blocking NF-κB activation restored SR-A gene and protein levels to baseline compared to non treated control levels. These results were confirmed functionally in uptake assays as *rescuing* SR-A expression by NF-κB inhibition was associated with a return of acLDL uptake levels to baseline. These experiments provide evidence that extracellular HSP27 may may activate NF-κB leading to downregulation of SR-A gene expression and inhibition of lipid uptake.

Although it is unknown whether extracellular HSP27 relays its signal via a surface receptor or is translocated directly or indirectly across the plasma membrane where it may activate signaling partners upstream of NF-κB, a number of studies have demonstrated an interaction between HSPs and NF-κB activity. Parcellier *et al*. demonstrated that overexpression of HSP27 in human U937 macrophages stimulates NF-κB activity, in turn, promoting cell survival in response to the apoptosis inducer etoposide (404). In another study, Lui *et al.* transfected THP-1 cells with an HSP27 construct and found that HSP27

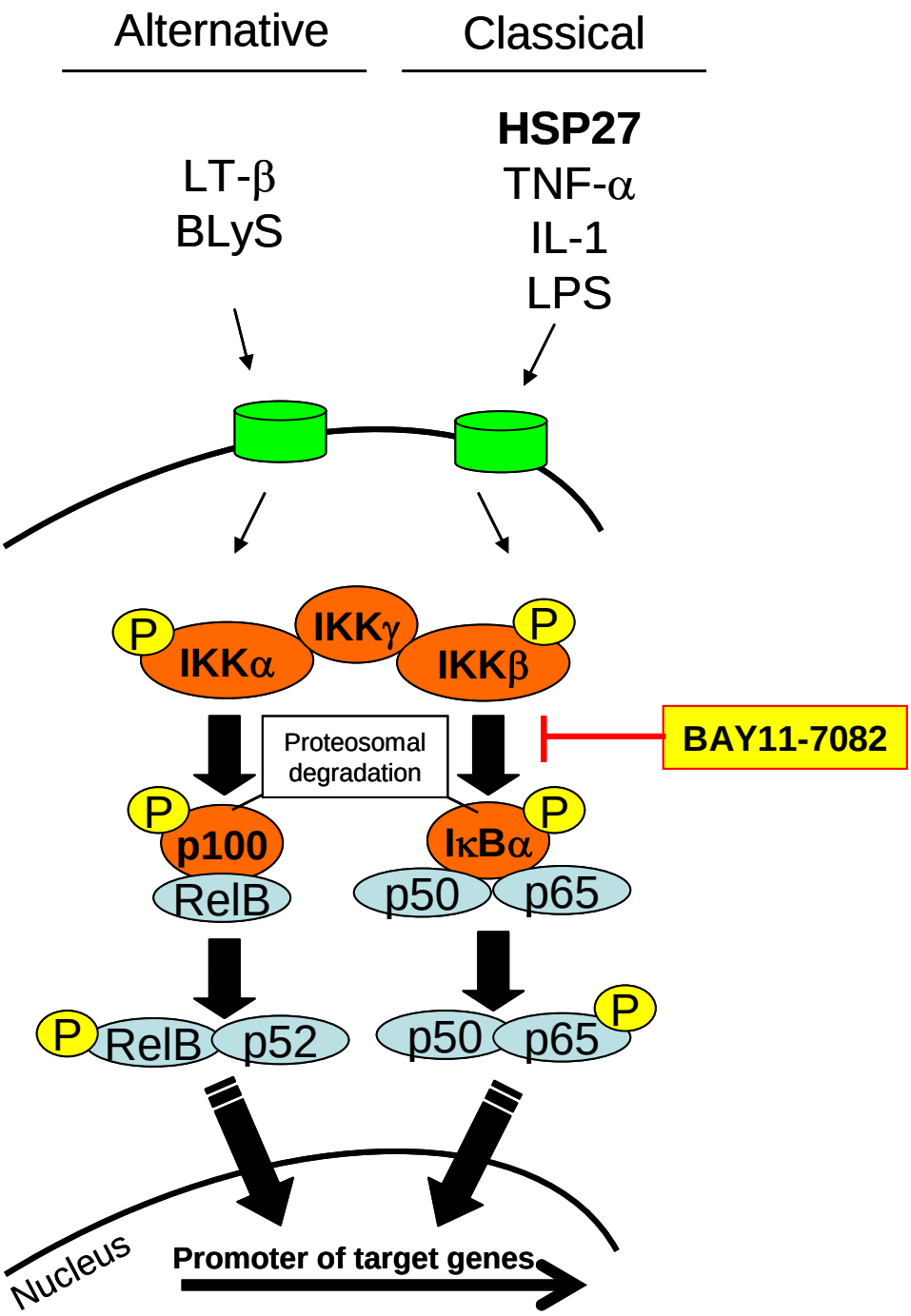


Figure 32. Canonical and non-canonical NF-κB pathways

The canonical or classical NF-kb pathway is activated by TNF-α, IL-1, or LPS. Phosphorylation of the IKKβ catalytic subunit targets IκB for ubiquination and its degradation in the proteosome. Dissociation of p50 and p65 from IKKβ leads to their tanslocation into the nucleus and activation of target genes involved in inflammation and cell survival. The BAY compound was used in this study to inhibit the classical NF-κB

pathway by blocking IKKβ phosphorylation of IκB thereby preventing its degradation and subsequent translocation of the p50/p65 transcription factors into the nucleus. The non-canonical or alternative pathway is triggered by members of the TNF-α family (e.g. LT-B and BLys) and is dependent on IKKα phosphorylation of p100 leading to release of the RelB subunit. The alternative pathway is known to play a role in innate immunity as well as regulation of inflammation by the canonical pathway. Although This thesis demonstrates that HSP27 likely activates the classical pathway.
Figure adapted from Karin and Lin (270) with modifications. © Nature Publishing Group; reprinted with permission from Nature Publishing Group.

enhanced LPS stimulation of iNOS and COX-2 via increased NF-κB activity, but not MAPK activation (410). Although the latter two studies agree with the results presented in this thesis in human THP-1 cells, HSP27 has been shown to have variable effects on NF-κB activity in human and rodent cell lines. Conversely, studies have shown that HSP27 is a negative regulator of NF-κB activity in rat skeletal muscle (411), aortic smooth muscle cells (412), as well as in the rat heart (412). Comparisons between the above studies and the results generated in this thesis are difficult to make, considering most studies focus on intracellular HSP27, which likely affects intracellular signaling pathways differently then signals originating from outside the cells. Asea *et al*., however, illustrated that exogenously added rHSP70 was found to bind TLR2 and TLR4 on the surface of monocytes leading to stimulation of NF-κB activity and activation of downstream cytokines (400), which is in agreement with the role of extracellular heat shock proteins such as HSP27 in modulating macrophage biology.

SR-A is also known to be differentially regulated in human and murine cells. For example, Fitzgerald *et al.* demonstrated that LPS reduced SR-A expression in human THP-1 macrophages, but increased SR-A expression in mouse macrophages (179). Since LPS is a well described NF-κB activator, this suggests that NF-κB and SR-A may a) not be linked and b) differentially regulated in these different cell systems. However, HSP27 was

previously shown in the O'Brien lab to stimulate NF-κB activity in a RAW mouse macrophage cell line stably transfected with an NF-κB reporter as well as stimulate translocation of p65 into the nucleus using immunolabelling experiments in both human THP-1 and peritoneal macrophages (personal communication with Tara Seibert in the O'Brien lab), suggesting comparable responses between species. Despite these observations an alternative interpretation could be the following: if HSP27 affects SR-A similarly in mouse and human cells, but has differential effect on NF-κB this may suggest that HSP27 acts through some other pathway to regulate SR-A expression. It should be noted that NF-κB antagonist, BAY11-7082, used in this study is not totally specific to NF-κB. It has been shown to affect other kinase regulated pathways such as p38 MAPK, ERK, and JNK (413,414). Since these signaling kinases have also been shown to be major regulators of SR-A expression and function in different macrophage cell types (173,204,205,219,220,401), it can be argued that HSP27 may also be regulating SR-A expression and foam cell formation through alternative mechanisms. Other NF-κB or MAPK pharmcocological inhibitors or siRNA technology to compliment the BAY studies in this thesis would certainly be needed to confirm the specificity of HSP27 activation of NF-κB vs. other signaling factors such as MAPK and how these are involved in modulation of SR-A expression.

Activated NF-κB was found to be present in atherosclerotic lesions compared to vascular tissue that is devoid of lesion formation (415). Moreover, inhibition of its activation has been shown to reduce foam cell formation, implicating NF-κB as an important player in mediating lesion development (416). Many studies attribute NF-κB activation as pro-inflammatory with consequent pro-atherogenic actions. Yet, a burgeoning body of literature has revealed new anti-inflammatory roles of NF-κB (417) that agree with the general

framework of this thesis - implicating NF-κB as an anti-inflammatory target for HSP27. For example, Lawrence *et al*. demonstrated that NF-κB plays a role in preserving anti-inflammatory gene programs during leukocyte apoptosis (417). In the context of atherosclerosis, Kanters *et al*. demonstrated that recipient $LDLr^{-/-}$ mice transplanted with NF-κB deficient bone marrow cells have more extensive lesions compared to controls – an effect that was associated with reduction in IL-10 secretion *in vivo* (381). In a follow up study, Kanters *et al.* showed that silencing of the p50 subunit of NF-κB attenuated lipid uptake into bone-marrow-derived macrophages via downregulation of SR-A expression (418).

There is evidence to support the working hypothesis that HSP27 acts as an anti-inflammatory stimulus. For example, incubation of rHSP27 with human monocytes was previously shown by De *et al*. to activate IL-10 secretion (321). IL-10 is a strong anti-inflammatory target gene for NF-κB. Furthermore, IL-10 is expressed in human advanced plaques (282) underscoring its atheroprotective properties in a number of animal models (283,419). Recently, incubation of THP-1 macrophages with IL-10 was found to prevent oxLDL uptake, an effect explained partially by downregulation of SR-A expression (420). Given that treatment of macrophages with rHSP27 induced IL-10 secretion (262), these findings further support HSP27 as an important regulator of inflammation and lipid uptake.

In the context of macrophage activation states, a number of studies demonstrate that deletion of IKKβ decreased alternative (M2) macrophage markers relative to classical (M1) activation states in a variety of macrophage cell types (421,422). As M2 macrophages have anti-inflammatory features, these studies implicate NF-κB activation as a contributing factor in HSP27-mediated atheroprotection. The role of NF-κB during foam cell formation is

difficult to reconcile for a number of reasons. First, NF-κB can simultaneously control both pro and anti-inflammatory atherosclerotic gene programs, affecting lipid uptake and handling in macrophages. Second, foam cell formation may have beneficial and harmful actions during different stages of lesion development. In early stages of fatty streak formation foam cell formation may be the consequence of macrophages acting to clear pro-inflammatory lipids. However, foam cells also contribute to plaque progression and necrotic core formation of the advanced plaque. Therefore, the role of NF-κB on lipid uptake pathways and SR-A expression may ultimately depend on the context of both macrophage function and the stage of lesion development.

During foam cell formation, regulatory elements in the promoter regions of the SR-A gene are known to be activated by AP-1 and ets-domain transcription factors (207,208). Although it is unclear whether NF-κB transcriptions factors (i.e., p65/p50) directly influences SR-A gene regulation in these promoter regions, previous results from the O'Brien lab demonstrate translocation of the p65 into the nucleus in response to rHSP27 (368). In addition, LPS (179,423) and TNF-α (172), are known to downregulate SR-A expression in THP-1 macrophages. Both LPS and TNF-α are potent activators of NF-κB signaling, supporting the idea that p65 may bind to promoter elements in the SR-A gene, silencing its message, and leading to downregulation of SR-A expression. Moreover, Bassuk *et al*. demonstrated that NF-κB interacts with Ets domains to regulate T-cell activation (424), providing indirect evidence for NF-κB dependent regulation of SR-A gene expression.

Although this thesis highlights a novel intracellular signaling pathway for exogenous HSP27 atheroprotection, several interesting questions arise from the current work. Namely, it remains unclear precisely how HSP27 activates NF-κB signaling, if a cognate receptor is

required for this effect, what the identity of this receptor is, and finally whether HSP27 internalization is required. Certainly, identification of a receptor is the focus of ongoing studies, which may potentially yield a novel therapeutic target.

Limitations in this thesis are acknowledged that are implicit in utilizing recombinant proteins. Recombinant proteins generated in bacterial expression systems are known to contain contaminating endotoxin. Despite best attempts to remove the majority of this contamination using polymixin B coated columns, residual endotoxin levels may still remain, which may contribute to NF-κB activation or other untoward effects independent from the true effect of the recombinant protein under study (392). Furthermore, endotoxin has been shown to reduce SR-A expression in THP-1 macrophages (423), and previously found to be a confounding factor in studying the role of rHSP70 (371) and rHSP60 (372) in induction of inflammatory responses in macrophages. Polymixin B (PMB) is known to neutralize LPS and prevent its binding to surface receptors such as TLR2/4 (380) as demonstrated by its ability to completely suppress LPS induction of NF-κB in THP-1 Blue cells. When PMB was added to the culture media in the presence of rHSP27 or rHSP25 no confounding effect was observed on NF-κB, SR-A protein, and lipid uptake. As well, the truncated rC1 served as a protein null control given that it showed no activity in chaperone or subsequent assays used in this study. At equimolar concentrations any endotoxin-related effects would have also been observed with rC1 since it was generated in the same manner as full length rHSP27. Therefore, these results demonstrate that the effect of rHSP27 on macrophage biology (i.e. NF-κB activation, SR-A expression, lipid uptake) reflects the properties of the protein itself rather than residual endotoxin contamination that may conceivably be present in the recombinant protein preparation.

Absence of SR-A leads to loss of atheroprotection in both males and females

It is well established that expression of macrophage SR-A during lesion development is associated with foam cell formation (161) and that SR-A represents an important therapeutic target for the prevention of atherogenesis. In the absence of SR-A, HSP25 protection is completely lost in the *en face* lesions of females and males. While the data is not statistically significant, there appears to be a trend to a HSP25-induced reduction in atherosclerosis in aortic root lesions of female ApoE$^{-/-}$SR-A $^{-/-}$ mice treated with rHSP25 compared to controls. In addition, at baseline, SR-A appears to alter lesion development in females, but only when lesions are measured *en face*. On the other hand, both the female aortic sinus and male *en face* lesions were unaffected by the absence of SR-A. The HSP27 inhibition effect on acLDL uptake in bone-marrow derived macrophages isolated from ApoE$^{-/-}$ mice is partially lost in the ApoE$^{-/-}$ SR-A$^{-/-}$ cells but comparisons between HSP27 treated the single and double knockout cells revealed no statistical difference. The uptake results of the uptake experiments in the SR-A$^{-/-}$ suggest that SR-A may play a incomplete role in HSP27-mediated inhibition of foam cell formation.

If SR-A is in fact a key target for HSP25 during foam cell formation one would expect that in its absence, rHSP25 would no longer be efficacious in reducing lesion formation. Certainly, when the gene for SR-A is not present, NF-κB dependent HSP27 signaling is also lost during foam cell formation, presumably because NF-κB activation specifically targets the SR-A promoter or related proteins involved in the transcription of this gene. Therefore, in the absence of SR-A, modified lipids may be taken up by alternative mechanisms driving foam cell formation and rendering rHSP25 no longer atheroprotective in ApoE$^{-/-}$SR-A$^{-/-}$ mice. However, HSP25 may have some effects in the SR-A$^{-/-}$ mice given

that there was a modest protective effect (p=0.14) when lesions were measured in the aortic sinus. This suggests that SR-A may not be the only target for HSP25 and lends support for another mechanism to explain its atheroprotection. GM-CSF, a hematopoietic growth factor linked to salutary effects on lesion and serum cholesterol levels in rabbits (425,426), was previously shown by the O'Brien lab to be upregulated (and secreted) from macrophages in response to extracellular HSP27 (409). At the time this thesis was written, studies in ApoE$^{-/-}$ GM-CSF$^{-/-}$ mice were in progress to determine whether this cytokine plays a role in HSP27 atheroprotection.

Other classes of scavenger receptors like CD36, LOX-1, CD68 and SR-PSOX are expressed in macrophages. A wealth of animal studies describing their role in atherosclerosis has provided evidence linking them to lipid uptake pathways (135,140,427). Recently, receptor-independent forms of lipid endocytosis have emerged as an alternate mechanism thought to incite foam cell formation. Macropinocytosis of LDL has been described by Kruth and colleagues as a non-saturable fluid phase endocytosis pathway involving well characterized signaling cascades such as Rho GTPase and PI3-kinase. Accumulation of intracellular cholesterol occurs in lipid droplets much in the same way as described for receptor-mediated endocytosis – a process that is independent from downregulation of LDL receptors (234,428). In a recent review, Kruth *et al*. provides evidence that fluid-phase macropinocytosis occurs in the atherosclerotic plaque and may be an important target in limiting cholesterol accumulation into the vessel well (236). In the absence of SR-A, HSP27 mediated protection is completely abolished in both female and male mice. Although not tested in this thesis, it is conceivable that other receptors or sources of lipid uptake other than SR-A are not affected by HSP27 in the ApoE$^{-/-}$ SR-A$^{-/-}$ mice. This

would allow pro-atherogenic processes to proceed unperturbed leading to foam cell formation and increased lesion burden as demonstrated in the absence of SR-A.

While HSP27-mediated atheroprotection is lost in both male and female ApoE$^{-/-}$ SR-A$^{-/-}$ mice, it is important to note that in female mice the absence of SR-A alone is associated with a reduction in *en face* lesion size, but not in the aortic sinus. Furthermore, the effect of SR-A deletion on atherosclerosis was not observed in the aorta of male mice. In spite of these conclusions, this implies that SR-A may not necessarily play a role in the development of lesions in these mice. However, injection of HSP25, which possibly acts through diminished expression of SR-A, conferred protection in the single knockout ApoE$^{-/-}$ mice. Based on these results one could argue against HSP25-mediated effect on SR-A expression affecting atherosclerosis. A first explanation for this anomalous result might include methodological limitations. Since 3 weeks of a high fat diet is considered early stage lesion formation, it is possible that the extent of atherosclerosis development in the ApoE$^{-/-}$ mice (389) would not be great enough to observe differences in lesions in the aortic sinus. Lesion formation along different regions of the aortic tree has previously been shown to develop at varying rates (429). Therefore, a longer time-course is required to test if observable changes in lesion development occur as a result of SR-A deletion, especially in the aortic sinus.

Other studies have documented site-specific effects of various knockout models and therapeutic interventions. For example, Goel *et al*. reported increased lesion size in the aortic sinus but reduced lesions in the lesser curvature of the aorta in Pecam-1$^{-/-}$LDLr$^{-/-}$ mice (430). Other reports using therapeutic intervention such as procubol (431) and the estrogen

receptor β agonist, 8BVE2 (367), have all documented site-specific anti-atherogenic effects in ApoE-/- mice.

The role of SR-A in lesion development is complex and begs the question: are there sex-specific (or even ovarian hormone specific) as well as site-specific factors that confound the analysis of the atherogenesis in SR-A null mice? Although it is not entirely clear how sex hormones or vessel regions affect SR-A during atherogenesis and whether they are even related, they are not surprising given that a number of studies have reported divergent functions of SR-A in atherosclerosis, many of which show sex as well as region (e.g. aortic versus sinus lesion) specific differences (see **Table I**). For example, Moore *et al.* found a 40% increase in aortic sinus lesion size in male $ApoE^{-/-}SR\text{-}A^{-/-}$ mice fed a high fat diet for 8 weeks compared to $ApoE^{-/-}$ females (227). Kuchibhotla *et al.*, on the other hand, reported a 32% decrease in sinus lesion size in female mice on a high fat diet for 12 weeks compared to male counterparts (228). Differential patterns of SR-A as well as CD36 expression within different geographical vessel regions have also been noted within the plaque (432), providing evidence that lipid uptake mechanisms may be unequal in the aortic arch versus the sinus regions. Indeed, SR-A expression is known to be co-localized in macrophages of human atherosclerotic lesions compared to diffuse non-atherosclerotic intimal thickening or in the adjacent healthy arterial tissue (156). However, the intensity of SR-A immunoreactivity is known to peak in the fatty streak because studies have shown decreased expression of SR-A in advanced lesions, suggesting that SR-A may have differential roles and expression profiles in every stage of lesion development (156). During early lesions formation SR-A functions to phagocytose modified lipids in the vessel wall contributing to fatty streak formation, yet in the advance plaque where cell death contributes to the necrotic

core, SR-A is known to play an important role in not only initiating these cell death pathways (221) but also in the clearance of dying cells (433). SR-A may have both anti- or pro-atherogenic roles depending on the stage of lesion formation. Early clearance of modified lipids from the vessel wall acts to dampen the inflammatory response, but when lipid uptake and cholesterol metabolisms pathways become overwhelmed, foam cell formation ensues leading to macrophage apoptosis and the formation of the necrotic core (140). Given that SR-A is implicated in other important cellular process beyond lipid uptake, such as innate immunity, cell migration/adhesion, and clearance of apoptotic cells (140), its absence in atherosclerotic animal models may influence the normal maintenance of the vessel wall – these pleiotropic effects ultimately impact the effect of atherosclerotic lesion formation and provides an explanation for intra- and inter-study discrepancies.

While estrogen has been shown to decrease foam cell formation *in vitro* by modulating SR-A message levels (261), the sex- and region-dependent differences observed in the ApoE$^{-/-}$ SR-A$^{-/-}$ can not be easily explained. The model of atherosclerosis presented in this thesis reflects an early stage of atherogenesis (i.e., due to 3 weeks of a high fat diet). Therefore, the function of SR-A during lesion development likely represents a model of early lipid uptake and foam cell formation during the initiation of lesion development. Since SR-A may play different roles in macrophage function in advanced stages of disease, future work is warranted to test whether SR-A is required for the beneficial effects of HSP27 treatment during formation of the necrotic core in chronic atherosclerosis experiments (>12 weeks on a high fat diet), or even after lesions are already established. Although SR-A expression in this thesis was shown to be diminished in the intima of HSP25 treated ApoE$^{-/-}$ mice using immnohistochemistry (qualitative) techniques, it also remains unclear if the

attenuation of SR-A expression specifically in macrophages directly leads to reduction in lesion formation. Analysis of lesions stained with CD68 or Mac-2 would help clarify whether HSP27 directly affects SR-A expression relative to macrophage content in the lesion.

Lastly, it should be recognized that the dose and delivery route of rHSP25 in this thesis was selected based on initial preliminary mouse trials in the O'Brien lab (370). Follow up studies are warranted to optimize dosage, timing, and delivery methods (i.e. oral vs. intravenous vs. subcutaneous). Furthermore, rC1 was only used in the *in vitro* studies of this thesis and hence future injection studies are required to confirm whether it has no affect on atherosclerosis compared to rHSP27/25 and validate its utility as a protein null control *in vivo*.

Augmented HSP27 levels is required for atheroprotection in males and females

Lesion burden in arteries of ApoE$^{-/-}$ HSP27o/e mice fed a HFD are strongly associated with reduction in HSP27 serum levels, but only in females (262,366). These observations led to the hypothesis, and confirmed in this study, that raising extracellular HSP27 (or the mouse ortholoug, HSP25) levels via therapeutic administration of recombinant protein would be sufficient to confer a similar protection profile seen previously in HSP27o/e female mice fed a HFD – effects shown to be dependent on estrogen (366). This study, however, provides evidence that that the sex-dependent actions of HSP27-atheroprotection are overcome, since injection of HSP25 into male ApoE$^{-/-}$ mice trends toward similar reductions in *en face* lesions ($p=0.06$) observed in their female counterparts. Given the small sample size analyzed for the male mice, increasing the power of each group would likely achieve the desired statistical significance in this experiment.

The current study makes the important observation that realizing sufficient levels of HSP27 in the serum, through exogenous administration of recombinant protein, can reduce atherosclerotic burden. Furthermore, the observation that low levels of serum HSP27 are associated with progression of atherosclerosis in patients (360), supports the hypothesis that augmentation of HSP27 levels is required to realize its atheroprotective potential. Pharmacokinetic analysis of HSP27 concentration in the serum after subcutaneous injections revealed that at its half life HSP27 levels are 100 fold higher (200 ng/ml) compared to levels observed in the HSP27o/e mice (2 ng/ml) (personal communication with Dr. Charles Cuerrier). At such high levels it is likely that HSP27 no longer requires the presence of estrogen to carry out its biological actions – thus making modulation of HSP27 serum levels a potential therapeutic target in both males and females. *In vitro*, 250 μg/ml (9.6 μM) HSP27/25 was used as the optimal dose to demonstrate the biological effects observed herein. At a concentration of 9.6 μM, for example, HSP27 would theoretically represent one of the top ten proteins found in the serum, which is probably not very likely given the potential toxic side-effect of using these high amounts. However, the use of high concentrations of HSP27 *in vitro* to confer its biological actions may reflect the requirement of high localized levels of HSP27 required at the site of the developing lesion. Cytokines acting in an autocrine or paracrine fashion are certainly concentrated at their localized sites of actions. Although more work must be carried out to determine optimal doses and administration routes *in vivo*, elevated levels of HSP27 present in targeted regions of the vessel wall may provide an explanation for both its protective effects in males as well as its use at high concentrations *in vitro*. Lastly, another limitation of this study is the inability to properly measure circulating levels HSP25 (or HSP27) after injections because commercial

ELISAs have proven unreliable and ineffective. Therefore, refinement or development of better detection methods is warranted.

Serum cholesterol levels and lesion burden

The mechanism by which HSP25 lowers serum cholesterol levels remains unidentified and is an ongoing research pursuit in the O'Brien lab. Interestingly, the cholesterol lowering effect of rHSP25 is also observed in ApoE$^{-/-}$ SR-A$^{-/-}$ mice despite loss of atheroprotection. Cholesterol profiles in both groups of mice indicate that HSP27 reduces both VLDL and LDL cholesterol levels. High VLDL/LDL is a major predictor of cardiovascular disease outcomes and reduction in their levels are associated with improved outcome of cardiovascular events (3).

Like statins, HSP25 may have pleiotropic functions that modulate both serum cholesterol levels as well as cellular factors in the vessel wall important. Although statins are known for their cholesterol lowering properties via mechanisms that target a key enzyme involved in the synthesis of cholesterol moieties, there are additional (pleiotropic) functions of statins that may contribute to reductions in foam cell formation (434). For example, THP-1 cells incubated with a lovastatin show reduced SR-A mRNA expression (260). In *ex vivo* experiments Fuhrman *et al.* demonstrated that monocytes isolated from hypercholesterolemic patients taking statins had both reduced oxLDL uptake, scavenger receptor expression, and ROS production (435). *In vivo*, several studies demonstrate that statins can directly modulate vessel wall biology by modulating lipid accumulation and foam cell formation in rabbit (436) and human (437) atherosclerotic lesions

It is important to note that in the absence of SR-A, HSP25 is no longer atheroprotective despite being associated with a reduction in serum cholesterol levels. Therefore, it can be reasoned that the pro-atherogenic factors controlling foam cell formation in the early stages of atherosclerosis offset or counterbalance the anti-atherogenic effects of reductions in serum levels that one might expect would be sufficient to attenuate lesion development in the $ApoE^{-/-}$ $SR\text{-}A^{-/-}$ mice. Stated differently, these data lead us to postulate that the anti-atherogenesis effects of HSP27 may be more related to inhibition of foam cell formation via reductions in SR-A expression than reductions in serum cholesterol levels.

While the concept that reductions of serum cholesterol levels may not be the dominant means by which lesion formation can be prevented is somewhat iconoclastic, there are other examples that help support the tenet that serum cholesterol levels are not necessarily predictive of atherogenesis. Ezetimibe is a cholesterol lowering drug that binds NPC1-like protein in endothelial cells of the intestine and blocks cholesterol absorption into the circulation. Results from the ENHANCE trial demonstrated that despite reductions in serum cholesterol levels in hypercholesterolemic patients treated with both ezetimibe and statins, there was no added benefit of ezetimibe in preventing increases in arterial intima-media thickness compared to patients taking statins alone (438). Although the ENHANCE trial was not a clinical-outcome trial that assessed cardiovascular events, it provided evidence that reducing cholesterol levels may not be directly associated with attenuation of atherosclerotic plaque burden. Moreover, the ARBITER 6–HALTS trial enrolled patients already taking statins to test whether the additive effect of ezetimibe was more beneficial than niacin in reducing plaque burden, raising HDL, and preventing cardiovascular events

(439) Paradoxically, patients supplemented with ezetimibe showed an increase in plaque size concomitant with elevated incidence of cardiovascular events compared to those supplemented with niacin alone. Despite reductions in LDL cholesterol with enzetimibe treatment, this trial further illustrated that traditional serum cholesterol risk factors for atherosclerosis may not be entirely predictive of the extent of lesion development. Certainly, fatty streak formation consisting largely of foam cells has been observed in young children and adults who display normal serum cholesterol levels (440) further supporting the argument against serum cholesterol levels as strong predictors of disease outcome.

Other studies in mice also demonstrate that interventions that are targeted specifically to the artery wall can influence atherosclerosis without affecting plasma cholesterol levels. Zhang *et al*. demonstrated that high-fat diet-fed chimeric Neimann-Pick C (NPC)$1^{-/-}$ mice reconstituted with $Ldlr^{-/-}Npc1^{-/-}$ macrophages displayed worse lesions despite presenting with lower serum cholesterol levels compared with mice reconstituted with wild-type macrophages (441). The authors demonstrate that the discordance between the low serum lipoprotein levels and the presence of aortic atherosclerosis was due to the intrinsic alterations in macrophage sterol metabolism in the chimeric $Npc1^{-/-}$ mice and they proposed that this effect in the vessel wall played a greater role in atherosclerotic lesion formation than did serum lipoprotein levels.

Given the observed magnitude of serum cholesterol reduction (25-32%) remains of uncertain physiological significance, only conservative conclusions should be drawn regarding its responsibility for atheroprotection. Instead, the observed atheroprotective effects may stem from a synergistic combination of both serum cholesterol reduction and SR-A downregulation in the vessel wall, thereby inhibiting foam cell formation. Finally, it is

clear that HSP27 has other pleiotropic effects that are recognized, for example, in its downstream effects on gene transcription via NF-κB activation, which will require further study as previously described (409).

6.0 SUMMARY AND CONCLUSIONS

This thesis was designed to examine the mechanism of HSP27-mediated atheroprotection, focusing specifically on how HSP27 is associated with SR-A in macrophages to inhibit foam cell formation. Previous results from the O'Brien lab and other groups have demonstrated that HSP27 is actively released from macrophages where it acts as a signaling molecule in the extracellular space to regulate anti-inflammatory responses. Given that foam cell accumulation in the lesion drives, in part, the pro-inflammatory response characteristic of atherosclerosis, inhibition of foam cell formation is considered a therapeutic target. A number of factors thought to influence lipid uptake into macrophages *in vitro* were considered herein, such as the ability of HSP27 to either prevent lipid accumulation into macrophages by directly competing with SR-A, or alternatively by limiting the amount of receptors at the cell surface. The results presented in this thesis demonstrate for the first time that augmented HSP27 levels (using rHSP27) inhibits lipid uptake into macrophages by downregulating SR-A expression. This effect may occurs at the gene level further implicating extracellular HSP27 in signaling events downstream from the cell surface. The signaling events by which HSP27 regulates SR-A expression may involve NF-κB activation but further work is required to conclude whether NF-κB definitively plays a role in modulating SR-A. Lastly, the importance of SR-A in atherosclerosis was explored by injecting $ApoE^{-/-}$ $SR\text{-}A^{-/-}$ mice fed a high fat diet with HSP25, the mouse homologue of HSP27. Despite sex- and region-specific effects of SR-A in atherosclerosis, it was revealed that 1) the atheroprotection effect of HSP25 are lost in the absence of SR-A in both female and male mice, and 2) loss of protection occurred in the presence of equal reductions in total serum cholesterol levels in both female $ApoE^{-/-}$ and $ApoE^{-/-}$ $SR\text{-}A^{-/-}$ mice groups. Hence, the

studies in this thesis describe a possible mechanism by which HSP27 inhibits foam cell formation through downregulation of SR-A expression - an effect that may be targeted to the vessel wall **(Figure 33).** Given the discordance of results on the impact of SR-A among male and females along different regions of the arotic tree, these *in vivo* studies highlighted the complex role of SR-A in mediating HSP27 atheroprotection. Though HSP27 likely has other pleiotropic targets that might contribute to the attenuation of atherosclerosis, this thesis provides evidence that SR-A may be one of these targets.

Hence, this work confirms the utility of HSP27 as a novel extracellular anti-atherogenic therapy and provides important insight into development of HSP27 as a potential therapeutic moiety that can be tested in future animal and clinical trials. Furthermore, given that rC1 was shown to lack biological activity in all the *in vitro* bioassays presented in this thesis, additional studies will take aim at identifying the specific sequence or part of the HSP27 molecule that is required to attenuate SR-A expression. The challenge of future work (as described in the following sections) will be to find the most efficacious methods to direct HSP27 protective actions in the vessel wall to prevent foam cell formation.

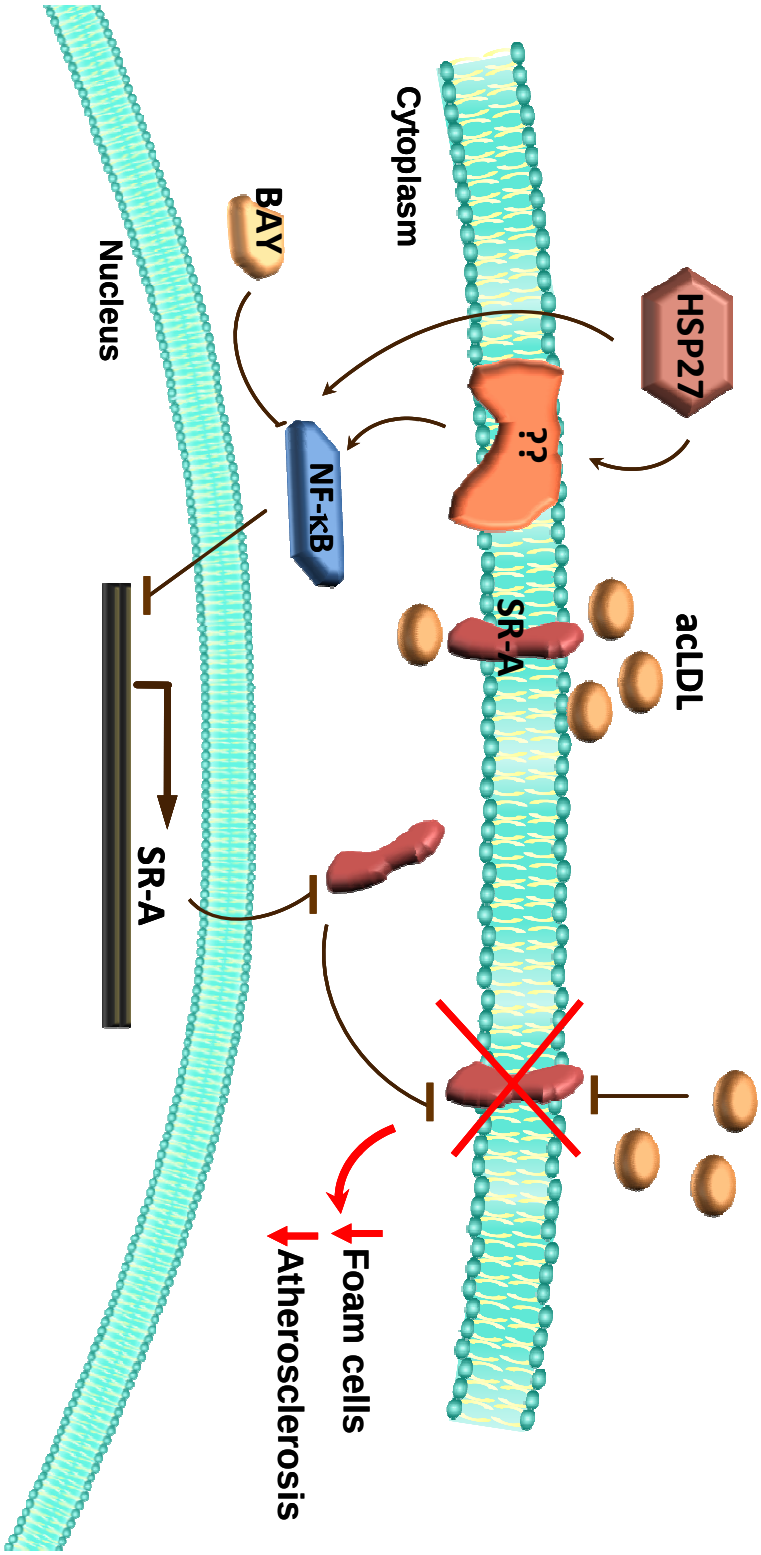

Cytoplasm
Nucleus
BAY
HSP27
??
NF-κB
SR-A
acLDL
SR-A
Foam cells
Atherosclerosis

Figure 33. Extracellular HSP27 inhibits foam cell formation by downregulating SR-A expression

This thesis highlights a novel pathway by which extracellular HSP27 leads to inhibition of foam cell formation and protection against atherosclerosis. Activation of NF-κB either through an unknown cognate receptor or direct translocation across the plasma membrane (PM) was found to downregulate SR-A gene expression. This leads to reduced levels of SR-A at the cell surface, in turn, preventing accumulation of modified lipid into the macrophage.

7.0 FUTURE DIRECTIONS AND RECOMMENDATIONS

This chapter will expand on some unanswered questions that stem from the results presented in this thesis. Three recommendations will be made to carry out future experiments.

This study has investigated the signaling properties of extracellular HSP27 and its relationship between NF-κB activation and SR-A expression. It would be of foremost interest to further elucidate how HSP27 is activating NF-κB to modulate SR-A expression, particularly focusing on whether these effects are mediated through a cognate surface receptor or whether HSP27 is translocated across the plasma membrane. Using fluorescently labeling techniques, previous studies have shown that HSP70 binds to the surface of macrophages as well as to CHO cells transfected with a number of plasmids expressing receptor candidates involved in signaling such as TLR2/4, CD14, and CD40 (442). HSP60, HSP70, and HSP90 have also been shown to interact with scavenger receptors at the cell surface to facilitate their internalization into the cell. In addition, previous studies have illustrated that extracellular HSP70 activated NF-κB activity by interacting with TL2/4, and CD14 on the surface human monocytes (400). These receptor screening experiments may provide clues that HSP27 likely binds to signaling receptors expressed in macrophages. A similar approach is recommended herein, where HSP27 (or rC1 protein null controls) is conjugated to a fluorescent probe either via labeling the native protein with a fluorescent probe (e.g. Alexa 488) after purification, or generated in bacterial expression systems using an HSP27 vector containing the green fluorescent protein (GFP) sequence. Macrophages can then be screened for HSP27 binding or internalization by incubating the cells with the fluorescent protein at 4°C or 37°C, respectively. Protein binding or internalization can then

be assessed either visually using confocal microscopy or quantified using a fluorescent plate reader. Although these experiments do not assay for individual receptors for HSP27 binding, they provide general insight into whether HSP27 is interacting with a surface receptor, being incorporated inside the cell, or both. Indeed, at the time this thesis was written pull down assays were being performed involving interactions between rHSP27 and preparations of THP-1 cell membranes. Elusion of HSP27 in columns using Ni^{+}NT beads and subsequent analysis of putative proteins using mass spectrometry will potentially reveal novel HSP27 interacting partners. Uncovering a receptor for rHSP27 will provide further targets to improve HSP27 anti-atherogenic actions in the vessel wall and expand the current knowledge about extracellular HSPs as important regulators of the inflammatory response.

Moreover, the results of this thesis provide evidence that NF-κB regulates SR-A expression at the gene level. Although p65 was previously shown in the O'Brien lab to translocate into the nucleus when macrophages were treated with rHSP27 (409), it remains unclear in this dataset whether NF-κB transcription factors directly interacts with SR-A promoter regions to modulate expression of SR-A message. As such, it would be of reasonable interest to specifically determine if HSP27 alters SR-A transcriptional activity. One approach would be to transfect THP-1 cells with a plasmid encoding the SR-A promoter linked to a luciferase reporter gene as previously described (443,444). Incubation of cells with HSP27 in the presence or absence of the NF-κB inhibitor, BAY11-7082, would confirm whether HSP27 suppresses SR-A gene expression and whether this effect is directly dependent on NF-κB activity. Another approach would be to measure SR-A mRNA, protein, as well as acLDL uptake in NF-$\kappa B^{-/-}$ macrophages isolated from NF-κB knockout mice (381,418,444). Given that SR-A expression and acLDL uptake are modulated by NF-κB as

assessed by pharmacological inhibition strategies, a similar biological effect would be expected in the absence of the NF-κB gene, further validating the importance of NF-κB activity in HSP27 responses.

Lastly, the development and design of small molecule drugs that target foam cell formation and macrophage mediated pro-inflammatory processes in the vessel wall is an attractive research pursuit (444,445). It is clear that the N-terminus truncated HSP27 variant, rC1, lacks biological activity demonstrating not only its utility as a protein null control, but also that the N-terminus contains a sequence important for HSP27's biological activity. This matches with previous findings that exemplify the N-terminus (e.g. WDTF domain and phosphorylation sits) essential for cell protection, maintenance of the oligomeric structure, and chaperone activity of the protein (446-448). Moreover, overexpression of an N-terminus truncated variant in neurons, but not the C-terminus, protected against ischemia *in vitro* (344), further validating the N-terminus as an important requirement in mediating HSP27's intracellular activity. Further development of recombinant truncated HSP27 mutants would provide insight into which specific region of the N-terminus is essential for activation of NF-κB and downregulation of SR-A expression. Injection of $ApoE^{-/-}$ and $ApoE^{-/-}$ $SR\text{-}A^{-/-}$ with these truncated variants would provide information about its sequence specific atheroprotection actions focusing explicitly on inhibiting SR-A expression in the lesion. This holds significance in development of small peptide drugs for exploiting specific anti-atherogenic targets while minimizing off target side effects.

In an attempt to develop HSP27 as a therapeutic to inhibit foam cell formation *in vivo* it would be of clinical interest to ask whether statins could provide an additive benefit in HSP27 treated mice. Given that statins modulate SR-A (434) and HSP27 expression (449)

in vitro it is currently unclear in the literature how SR-A is involved in these statin-dependent effects *in vivo.* Analysis of lesion burden in SR-A null mice given statins in the presence of rHSP27 compared to mice treated with statins or rHSP27 alone would help further substantiate if SR-A expression is a major target for HSP27 therapy. This experiment would provide insight into the mechanism of rHSP27 atheroprotection, describing important information in the context of a clinically relevant drug therapy model.

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9.0 CONTRIBUTIONS OF COLLABORATORS

1. The recombinant proteins used throughout this study was produced and purified by Mr. Thomas Hu in the O'Brien lab (University of Ottawa Heart Institute). Mr. Hu also performed the SDS-PAGE analysis and chaperone activity assays presented in Section 4.1.

2. The CHO cells stably transfected with SR-A as presented in Section 4.7 were received from Dr. Dawn Bowdish (McMaster University, Hamilton, Ontario).

3. Although a different data set, similar results presented in Section 4.8 using the NF-κB reporter cell line comprised a major part of Ms. Samira Salari's Master's thesis presented to the University of Ottawa, Department of Biochemistry (Ottawa, Canada).

4. Breeding and genotyping of the mice models presented in Section 4.10 were performed by Mrs. Xaoling Zhao in the O'Brien lab (University of Ottawa Heart Institute).

5. The $ApoE^{-/-}$ $SR\text{-}A^{-/-}$ mice presented in Section 4.10 were received from Dr. Kathryn Moore (New York University, Langone Medical Center, New York City, U.S.A).

6. The aortic tissue cross-sections presented in Section 4.10 were cut, stained, and immunolabelled by Dr. Yong-Xian Chen in the O'Brien lab (University of Ottawa Heart Institute).

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11.0 CURRICULUM VITAE

JOSHUA E. RAIZMAN

DATE OF BIRTH September 13, 1981

EDUCATION

- Clinical Chemistry Fellowship — July 1, 2012
  University of Toronto
  Toronto, Ontario

- PhD in Biochemistry — Sept., 2007-April. 2012
  Supervisor: Dr. Edward O'Brien
  University of Ottawa
  Ottawa, Ontario

- MSc in Physiology — Sept., 2003-April 2006
  Supervisor: Dr. Ian Dixon
  University of Manitoba
  Winnipeg, Manitoba

- BSc in Biochemistry — Sept., 1999-April 2003
  (4-year major)
  University of Winnipeg
  Winnipeg, Manitoba

RESEARCH EXPERIENCE

- Lab Technician III — Dec. 2006- June 2007
  Department of Pharmacology and Therapeutics,
  University of Manitoba, Winnipeg, Canada

- Summer Student — April 2003-Aug 2003
  Institute of Cardiovascular Science
  St. Boniface Research Centre
  University of Manitoba, Winnipeg, Canada

- Summer Student — May 2002-August 2002
  Department of Biological Chemistry
  Weizmann Institute of Sciences
  Rehovot, Israel

PUBLICATIONS

A) Peer-reviewed papers

1 **Raizman JE,** Chen Y-C, Hibbert B, Hu T, Salari S, Zhao X, Ma, Seibert T, Currier C, Simard T, Caravaggio J, Rayner K, Bowdish D, Moore K., O'Brien ER. Extracellular Heat Shock Protein-27 Mediated Atheroprotection: Down-regulation of Scavenger Receptor-A Expression via NF-κB Signaling. Submitted.

2. Seibert T, Chen Y-X, Hibbert B, Simard T, Cuerrier CM, **Raizman JE**, Rayner K, Hu T, Zhao X, Wells DJ, de BelleRoche J, O'Brien ER. Decreased Serum Heat Shock Protein 27 Levels are associated with Human CAD: Use of Recombinant HSP27 to Attenuate Experimental Atherogenesis and Stabilize Established Plaques. Submitted.

3. Simard T, Hibbert B, **Raizman JE**, Ramirez D, Pourdjabbar A, O'Brien ER. Pathogenesis of in-stent restenosis – current understanding and future novel targets. Submitted.

4. Freed DH, Chilton L, Li Y, Dangerfield, A, **Raizman JE**, Rattan S, Visen N, Hryshko L, and Dixon IM. Role of Myosin Light Chain Kinase in Cardiotrophin-1 Induced Cardiac Myofibroblast Cell Migration. Am J Physiol Heart Circ Physiol. 2011 Aug;301(2):H514-22

5. Fediuk DJ, Wang T, Raizman JE, Parkinson FE, Gu X. Tissue Deposition of the Insect Repellent DEET and the Sunscreen Oxybenzone From Repeated Topical Skin Applications in Rats. Int J Toxicol. 2010 Dec;29(6):594-603.

6. Santiago JJ, Dangerfield AL, Rattan SG, Bathe KL, Cunnington RH, Raizman JE, Bedosky KM, Freed DH, Kardami E, Dixon IM. Cardiac fibroblast to myofibroblast differentiation in vivo and in vitro: expression of focal adhesion components in neonatal and adult rat ventricular myofibroblasts. Dev Dyn. 2010 Jun;239(6):1573-84.

7. Raizman JE, Komljenovic J, Chang R, Deng C, Elimban VV, Freed DH, and Dixon IMC. The participation of the Na+-Ca2+ exchanger (NCX1) in primary cardiac myofibroblast migration, contraction, and proliferation. J Cell Physiol. 2007 Nov;213(2):540-51

8. Drobic V, Cunnington RH, Bedosky K, Raizman JE, Elimban VV, Rattan SG, and Dixon IMC. Differential and combined effects of Cardiotrophin-1 and TGF-β1 on cardiac myofibroblast proliferation and contraction. Am J Physiol Heart Circ Physiol. 2007 Aug;293(2):H1053-64.

9. Dixon IMC and Raizman JE. Cardiac cells of the living dead: Concurrent upregulation of both anti- and pro-apoptotic proteins in cells from post-MI hearts fuels the argument for the concept of stasis in programmed cell death. Ann Thorac Surg 2005 79: 1337

B) Meeting abstracts

1. **Raizman JE**, Hu T, Chen Y-C, Salari S, Seibert T, Ma X, Hibbert B, Simard T, Zhao X, Rayner K, Moore K, O'Brien ER.Heat Shock Protein 27 Mediated Atheroprotetion Requires Scavenger Receptor-A: Mechanistic Insight Into a Novel Therapeutic. Circulation. 2011; 124: A15504. Oral presentation. American Heart Association Scientific Sessions 2011 in Orlando Florida.

2. **Raizman J**, Seibert T, Salari S, Chen YX, McNulty M, Hu T, Zhao X, Sun J, Rayner K, O'Brien E. Extracellular HSP27 and atheroprotection: NF-kB signaling mechanisms. Presented at the International Congress on Stress Response in Medicine and Biology in Quebec City August 21-25, 2011

3. **Raizman JE,** Tieqiang Hu, Salari S, Gill J, Rayner K, McNulty M, Chen YX, and O'Brien ER. Identification of a Novel Scavenger Receptor-A Dependent Mechanism for Heat Shock Protein 27- mediated atheroprotection. Presented at the Arteriosclerosis, Thrombosis, and Vascular Biology Scientific Sessions Meeting in Chicago April 28-30, 2011.

4. **Raizman JE**, Hui T, Salari S, Seibert S, O'Brien ER. Extracellular HSP27 blocks uptake and binding of modified lipids by modulating expression of SRA: A novel anti-atherogenesis therapeutic opportunity? October2010, Volume 26, Supplement SD. Presented at Cardiovascular Congress, Montréal, Canada, October 22-26, 2011

5. Ma X., Hibbert B., Dhaliwal B., Rayner K., Chen Y-X, Sun J., Zhao X, Seibert S, Raizman JE, O'Brien ER. Glycogen Synthase Kinase 3 Beta Inhibitor Coated Stents Inhibit Neointimal Formation and Enhance Re-endothelialization Through Improved Endothelial Progenitor Cell Recruitment and Function .Circulation. 2008;118:S508

6. Freed DH, Dangerfield AL, Raizman JE, Rattan SG, Visen N, Hryshko LV, Giles WR and Dixon IMC. Role of calcium in cardiotrophin-1 induced cardiac myofibroblast cell migration. ISHR-American Section Meeting, Toronto, Canada, June 13, 2006. J Mol Cell Cardiol 40:871, 2006

7. Qiuzhi Chang, Xijun Deng, **Josh E. Raizman**, Sunil G. Rattan, Ian M.C. Dixon. Myofibroblasts as agent of cardiac wound healing post MI: The effect of Na Ca exchanger inhibition and L-type calcium channel blockade in primary cardiac myofibroblast contractile responses. Canada-Wide Science Fair, Chicoutimi, Quebec, May 2006

8. **Raizman JE**, Komljenovic J, Elimban VV, Freed DH, Rattan SG and Dixon IMC. Myofibroblasts as agents of cardiac wound healing: Role of NCX1.1 and NSCCs in myofibroblast migration and contraction. 3rd Annual CIHR Young Investigator Forum, Winnipeg, Canada, April 27-30, 2006 Exp Clin Cardiol 11:56, 2006

9. **Raizman JE**, Komljenovic J, Elimban VV, Freed DH, Rattan SG and Dixon IMC. NCX1.1 and NSCCs participate in PDGF-BB mediated cardiac myofibroblast migration and contraction. Submitted as a late-breaking abstract to Experimental Biology 2006 in San Francisco, California, USA, April 1-5, 2006

10. Freed DH, Dangerfield AL, Jones SC, Sutton JA, **Raizman JE**, Pascoe EA and Dixon IMC. Effect of angiotensin antagonism on myocardial infarct scar cardiotrophin-1 expression and myofibroblast proliferation *in vivo*. Canadian Cardiovascular Society, Montreal, Canada, October 27 – 30, 2005. Can J Cardiol, 2005

11. **Raizman JE**, Freed DH, Hryshko LV and Dixon IMC. Regulation of PDGF-induced motility of cardiac myofibroblasts is dependent on plasmalemmal NSCC and NCX function. Annual Canadian Student Health Research Forum, University of Manitoba, Faculty of Medicine, June 7, 2005

12. **Raizman JE**, Hryshko LV, Freed DH and Dixon IMC. Regulation of cytokine-induced motility of cardiac myofibroblasts is dependent on plasmalemmal NCX and NSCC function. 2nd Annual CIHR Young Investigator Forum, Winnipeg, Canada, April 29-May 1, 2005. Exp Clin Cardiol 10:53, 2005

13. Freed DH, Dangerfield AL, Sutton JA, Jones S, **Raizman JE**, Dixon IMC. Cardiotrophin-1 expression and myofibroblast involvement in post myocardial infarct wound healing. Poster presented at the Annual Canadian Student Health Research Forum, University of Manitoba, Faculty of Medicine, June 2004

AWARDS & SCHOLARSHIPS

- Ministry of Health Fellowship for Training in Clinical Chemistry — July 2012

- University of Ottawa Excellence Scholarship Tuition award — May 2008 – present

- CIHR Frederick Banting and Charles Best Canada Graduate Scholarship Doctoral Award Application rated top 2% in Canada — May 2008 – May 2011

- Department of Biochemistry Seminar Day University of Ottawa 2nd Prize for PhD Oral Presentation — Feb. 2011

- Department of Biochemistry Poster Day University of Ottawa Honourary mention — May 2008

- St. Boniface General Hospital Research Foundation — April 2004-2006
  MSc Scholarship

- Canadian Student Health Research Forum — June 2005
  University of Manitoba
  Honourary mention

- Max and Jean Ruder Perpetual Scholarship — May 2003
  Jewish Federation of Manitoba

- Chemistry Club Award — Oct. 2002
  Department of Chemistry
  University of Winnipeg

TEACHING EXPERIENCE

- Teaching Assistant — Jan 2012-present
  BCH2333: Introduction to Biochemistry
  University of Ottawa,
  Department of Biochemistry

- Teaching and marking assistant — Sept 2010-Dec 2011
  BCH3356: Molecular Biology Laboratory
  University of Ottawa
  Department of Biochemistry

- Teaching Assistant — Jan 2010-April 2010
  BCH3346: Biochemistry Laboratory II
  University of Ottawa,
  Department of Biochemistry

VOLUNTEER EXPERIENCE

- Executive Board Member — Sept 2011-present
  Public Relations VP
  The Ottawa Hospital Toastmaster Club

- Executive Board Member — Sept 2011-present
  Communications VP
  jnet Ottawa Networking Initiative

- Heart Institute Rep — Sept 2010-present
  Member at large
  Biochemistry Microbiology Immunology

Graduate Student Council.

- President — Sept 2007-present
  Jewish Graduate Student Association
  University of Ottawa

- Lets Talk Science (LTS) volunteer — Sept 2007-present

- LTS Aboriginal Mentorship Program Rep — Sept 2010-May 2011
  Mentoring grade 11&12 students
  Cornwall High School

- Science Fair Judge — April-May 2010
  Ottawa Regional Science Fair
  Canada Wide Science Fair

- Research Supervisor — Oct. 2005-April 2006
  Sanofi Aventis Biotalent Challenge
  Institute of Cardiovascular Sciences
  St. Boniface Research Centre

- Science Fair Judge — May 203 2005 & 2006
  Manitoba School Science Symposium

- Journal Club Organizer — Sept 2004-April 2005
  St. Boniface Research Centre

- Visiting Scientist Seminar Lunches Series — Sept 2003-March 2005
  St. Boniface Research Centre

PROFESSIONAL MEMERSHIPS

- Canadian Society of Clinical Biochemists
- American Association for Clinical Chemistry
- North American Vascular Biology Organization
- American Heart Association

OTHER TRAINING

- Competent Communicator and Leadership — May 2009-present
  Ottawa Hospital Toastmasters Club

- NAVBO Vasculata Workshop on Vascular Biology — August 2008
  University of Washingto Seattle, Washington

- Cardiovascular Genetics course — June 2007
  Heart and Stroke Foundation of Ontario
  Program Grant Course
  University of Ottawa Heart Institute