Induction of ABCA1 expression is correlated with increased CREB phosphorylation and altered cytokine secretion

by Maryam Zaid

A thesis submitted to the Faculty of Graduate and Postdoctoral Studies

in partial fulfillment of the requirements

for the degree of

Master of Science in Biochemistry

Submitted in December 2010

Department of Biology, Microbiology, and Immunology

Faculty of Medicine

University of Ottawa

Ottawa Ontario, Canada

ABSTRACT

ABCA1 is believed to affect macrophage inflammatory responses, but the mechanism by which ABCA1 may impact cytokine secretion in macrophages has yet to be fully defined. We observed that the induction of ABCA1 expression in three different cell lines, namely BHK, RAW 264.7 macrophages, and primary bone marrow derived macrophages (BMDMs), results in a significant increase in phosphorylated CREB, a known protein kinase A (PKA) substrate. In RAW macrophages, induction of ABCA1 expression by the LXR-agonist T0901317 is correlated with a decrease in LPS-stimulated secretion of proinflammatory cytokines IL-6 and TNF-α. Additionally, the secretion of anti-inflammatory cytokine IL-10 was increased upon ABCA1 induction. A similar trend was observed in BMDMS: ABCA1-expressing BMDMs released less TNF-α and more IL-10 compared to ABCA1-knockout BMDMs. We speculated that the inflammation modulating effects of ABCA1 in macrophages could be a result of PKA activation. Indeed, we found that the LXR-induced ABCA1 phenotype can be mimicked by cAMP in macrophages. 8-bromo-cAMP, a PKA activator, dose-dependently suppressed inflammatory cytokine secretion while promoting IL-10 release in the absence of ABCA1 expression. Finally, we found that the T0901317-induced ABCA1 expression is correlated with higher expression levels of MKP-1, a downstream target of PKA known to suppress inflammatory responses. Together, our results suggest that ABCA1 expression may activate PKA and CREB and that such activation may contribute to the inflammatory modulating effects of ABCA1.

Keywords: ABCA1, atherosclerosis, CREB, LXR-agonist, MKP-1, PKA, inflammation

Acknowledgements

I would like to sincerely thank my supervisor, Dr. Xiaohui Zha, for her guidance, patience, and encouragement. This work would not be possible without her help.

I am indebted to Dr. Fumin Dong and Loretta Ma for their help, advice, and suggestions for troubleshooting.

I would also like to thank the members of my Thesis Advisory Committee: Dr. Ross Milne and Dr. Paul MacPherson for their guidance.

Lastly, I would like to thank my family for their continued support and optimism.

Table of Contents

List of Figures

Title: **Page:**

List of Abbreviations

M	molar
mM	millmolar
μM	micromolar
nM	nanomolar
ABCA1	ATP-binding cassette transporter A1
AC	adenylyl cyclase
AKAP	A-kinase anchoring protein
apoA-I	apolipoprotein A1
apoE	apolipoprotein E
ATP	adenosine-5'-triphosphate
BHK	baby hamster kidney
BMDMs	bone marrow-derived macrophages
BSA	bovine serum albumin
CaMK	calmodulin kinase
cAMP	3'-5'-cyclic adenosine monophosphate
CPM	counts per minute
CRE	cAMP response element
CREB	cAMP response element-binding protein
CsA	cyclosporin A
DMEM	Dulbecco's Modified Eagle Medium
ELISA	enzyme-linked immunosorbent assay
Epac	exchange protein activated by cAMP
ER	endoplasmic reticulum
ERK	extracellular signal-regulated kinase
FBS	fetal bovine serum
FK506	tacrolimus or fujimycin
ggPP	geranylgeranyl pyrophosphate
GST	glutathione S-transferase
GM-CSF	granulocyte macrophage colony-stimulating factor
HDL	high-density lipoprotein
HSP70	heat-shock protein 70
ICAM-1	inter-cellular adhesion molecule 1
IKK	IκB kinase
IL-6	interleukin-6
IL-10	interleukin-10
IL-1β	interleukin-1 β
IRF3	interferon regulatory factor 3
LDL	low-density lipoprotein
LPS	lipopolysaccharide
LXR	liver-X receptor
MAPK	mitogen-activated protein kinase
M-CSF	macrophage colony-stimulating factor

MKP	MAPK phosphatase
MKP-1	MAPK phosphatase 1
NBD	nucleotide binding domain
NFAT	nuclear factor of activated of T cell
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
oxLDL	oxidized LDL
PAMP	pathogen-associated molecular pattern
PBS	phosphate buffered saline
PKA	protein kinase A
PPAR	peroxisome proliferator-activated receptor
PVDF	polyvinylidene fluoride
PXR	pregnane-X receptor
ROS	reactive oxygen species
RCT	reverse cholesterol transport
RXR	retinoic-X receptor
SD	standard deviation
SDS	sodium dodecyl sulfate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
SEM	standard error of the mean
TLR	Toll-like receptor
TLR4	Toll-like receptor 4
TNF-α	tumor necrosis factor –alpha
VCAM-1	vascular cell-adhesion molecule 1
VLA-4	very late antigen 4
wt	wild type

Chapter I: Introduction

Cardiovascular diseases are defined as diseases affecting the heart and blood vessels throughout the body and the brain (Maton, 1993). Cardiovascular disease is the leading cause of deaths throughout the world (World Health Organization, 2008) and atherosclerosis is a major underlying factor (Murray and Lopez, 1997). Atherosclerosis is a condition in which the accumulation of lipids in arteries results in the thickening of the arterial wall, leading to heart attack or stroke (Hansson and Libby, 2006). Several risk factors that contribute to the development of atherosclerosis have been identified. These risk factors include hyperlipidemia, insulin resistance, hyperglycemia, hypertension, cigarette smoking and insufficient physical activity (Worthley et al., 2001). Genetics is also believed to be a major risk factor of the development of atherosclerosis, yet the causative genes in the majority of atherosclerosis cases are unclear (Seo et al., 2006) as they likely augment the impact of environmental risk factors such as smoking and diet (Seo and Goldschmidt-Clermont, 2008). Preventative measures against the development of atherosclerosis currently are aimed at controlling these risk factors. A variety of therapeutics, that lower blood cholesterol levels, have been implemented in the treatment of atherosclerosis and other cardiovascular diseases. These include statins, fibrates, bile acid sequestrants, cholesterol absorption inhibitors, and nicotinic acid agents (Howard, 2010; Nissen et al., 2006; Toth, 2010). Lifestyle changes, such as diet and weight loss, are the most beneficial approaches for long term prevention. Another approach for the treatment of atherosclerosis is by preventing the formation of blood clots that normally lead to myocardial infarction and stroke (Loubele et al., 2010) through the use of anti-coagulants.

Although there are a variety of drugs and treatment strategies for atherosclerosis and cardiovascular disease, these diseases have been and will continue to be the leading causes of morbidity and mortality worldwide (Omoigui, 2007). Thus, further insight and study into the signaling pathways of atherosclerosis is necessary in order to discover more effective treatments of cardiovascular disease.

1.1 - Atherosclerosis

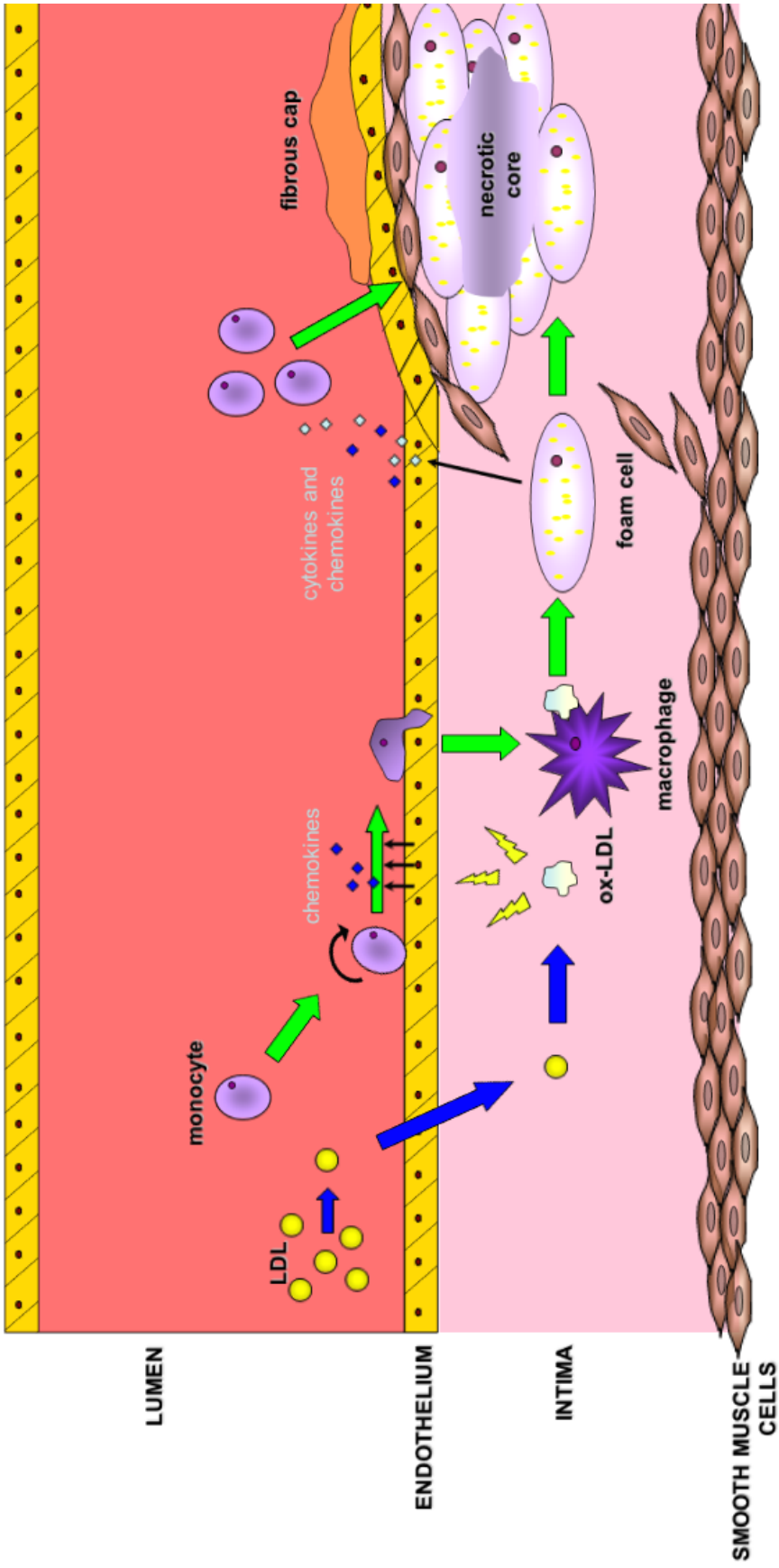
Accumulation of cholesterol in the cardiovascular system is one of the direct causes of atherosclerosis. Atherosclerosis is characterized by the presence of low-grade chronic inflammation that leads to plaque formation within the arterial wall (Li and Sun, 2007). These plaques are composed of a build-up of lipids, leukocytes, endothelial cells, and smooth muscle cells as well as calcified regions and necrotic cores (Woollard and Geissmann, 2010). Although several studies have shown that many factors can lead to atherosclerotic plaque formation, such as altered blood rheology, increased homocysteine levels (Woollard and Geissmann, 2010) or infection of vascular smooth muscle cells with herpes virus (Fabricant and Fabricant, 1999; Hsu et al., 1995), a higher level of circulating plasma cholesterol is the generally accepted cause of plaque formation associated with cardiovascular disease (Steinberg, 2005). There is a strong correlation between high levels of circulating low-density lipoprotein (LDL), a carrier of cholesterol, and risk of atherosclerosis (Toutouzas et al., 2010). Thus, the widely accepted and simplified paradigm of plaque formation begins with high levels of circulating LDL that damage the arterial wall (**Figure 1**). This triggers the infiltration of LDL into the intima, the innermost lining of the artery. Once in the intima, LDL is modified to oxidized LDL (oxLDL) by enzymes and oxygen radicals (Hansson and Libby, 2006; Kleemann et al., 2008). Modified LDL then stimulates endothelial cells to secrete chemokines (Ishibashi et al., 1994; Piedrahita et al., 1992) and express leukocyte adhesion molecules, such as vascular cell-adhesion molecule 1 (VCAM-1) and inter-cellular adhesion molecule 1 (ICAM-1) (Hansson and Libby, 2006). As a result, circulating leukocytes migrate towards the injured endothelium following chemokine gradients, roll along the endothelial cell surface, and adhere to the endothelial cell surface through the binding of ligands onto endothelial adhesion molecules. For example, very late antigen 4

(VLA-4), found on both T cells and monocytes, binds the endothelial VCAM-1. The adherence then activates monocytes so that they may extravasate between endothelial cells and enter the intima (Gumina et al., 1997).

Once inside the intima, monocytes differentiate into macrophages in response to macrophage colony-stimulating factor (M-CSF) and engulf the oxidized LDL. Under high LDL conditions, macrophages become overloaded with oxLDL, leading to an accumulation of oxysterols, and also cholesterol, and fatty acids as lipid droplets in the macrophage cytoplasm (Tabas, 2010). This results in formation of foam cells (Park et al., 2009). These foam cells, however, do not remain inert in the intima (Angeli et al., 2004) and release pro-inflammatory cytokines and chemokines. This leads to the recruitment of additional macrophages, T cells, and dendritic cells to the arterial site of injury (Linton and Fazio, 2003). The accumulation of foam cells, T cells and other inflammatory cells, along with endothelial cells and smooth muscle cells, then forms lipid-rich plaques that lead to the narrowing of the artery (Woollard and Geissmann, 2010). A fibrous cap, made of fibrin, red blood cells (RBCs), platelets, and endothelial cells, then overlies the lipid core of the plaque (Libby and Aikawa, 2002; Virmani et al., 2002). This fibrous cap is often not stable, as local macrophages secrete matrix-degrading proteases that cause thinning of the fibrous cap. This renders it vulnerable to rupture. Thrombosis, blood clot formation in the blood vessel, and consequently, myocardial infarction and stroke are the common consequences of plaque rupture (Kleemann et al., 2008; Tabas, 2010; Woollard and Geissmann, 2010).

The most prominent feature of atherosclerotic plaques is foam cells. These cells augment inflammation by releasing pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α), interleukin 6 (IL-6), interferon-β (IFN-β), macrophage inflammatory protein 1α (MIP-1α), and granulocyte-macrophage colony-stimulating factor (GM-CSF)

Figure 1. A simplified schematic of plaque formation in an atherosclerotic artery. High levels of circulating LDL lead to the damage of the arterial wall and entry of LDL into the intima. LDL becomes oxidized by enzymes and oxygen radicals found in the intima. Endothelial cells then secrete chemokines and express leukocyte adhesion markers. Circulating monocytes roll along the endothelial cell surface and adhere to the endothelial cell surface through binding of their ligands to endothelial adhesion markers. Monocytes change shape and extravasate between endothelial cells to enter the intima. Once inside the intima, monocytes differentiate into macrophages and engulf the oxidized LDL. Continued uptake of oxidized LDL causes macrophages to become lipid-laden foam cells. Foam cells remain in the intima and release pro-inflammatory cytokines and chemokines to recruit additional macrophages, T cells, and dendritic cells to the arterial site of injury. A fibrous cap, red blood cells (RBCs), platelets, endothelial cells, and intimal smooth muscle cells overly the lipid pool and provide plaque stability. Local macrophages secrete matrix-degrading proteases that cause thinning of the fibrous cap, thus increasing plaque vulnerability to rupture. Once ruptured, the plaque debris can cause thrombosis which may lead to myocardial infarction and stroke.


LUMEN
ENDOTHELIUM
INTIMA
SMOOTH MUSCLE CELLS
LDL
monocyte
chemokines
cytokines and chemokines
fibrous cap
ox-LDL
macrophage
foam cell
necrotic core

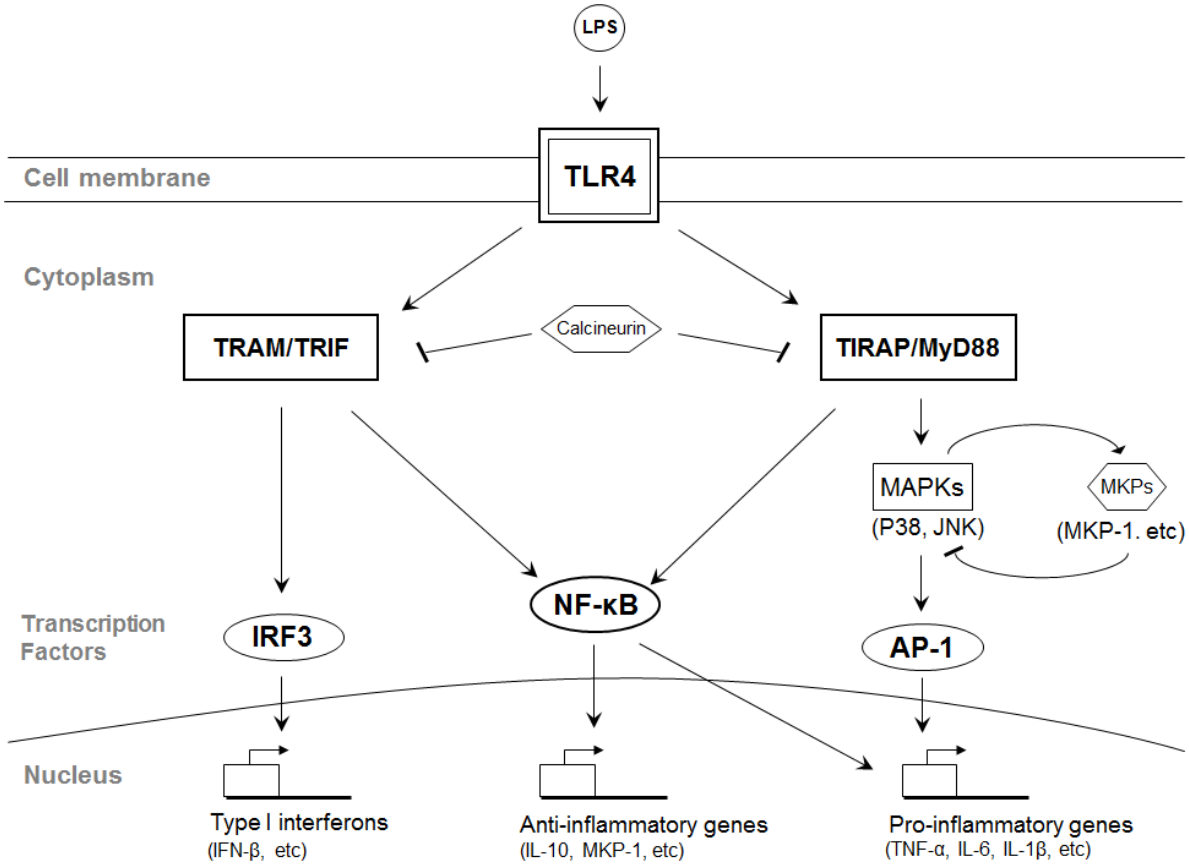
(Kleemann et al., 2008; Rocha and Libby, 2009; Woollard and Geissmann, 2010). Release of these cytokines further recruits monocytes that take up lipid particles in the intima, resulting in more foam cell accumulation. In addition, lipid-overloaded foam cells are susceptible to apoptosis (Tabas, 2010) which further exacerbates atherosclerotic plaques through the release of antigenic and thrombogenic debris (Rocha and Libby, 2009). Thus, macrophage-derived foam cells are one of the primary factors that initiate and fuel the progression of atherosclerosis.

1.2 - TLR4 Pathway in atherosclerosis

Atherosclerosis is now widely recognized not only as a lipid disorder in the cardiovascular system, but also as a low-grade chronic inflammatory disease (Hansson and Libby, 2006). The low-grade inflammation in atherosclerotic arteries has been linked to the toll-like receptor 4 (TLR4) signal transduction pathway. Indeed, TLR4, a member of the toll-like receptor (TLR) family, is expressed by macrophages in both human and murine atherosclerotic plaques (Xu et al., 2001). Significantly, a greater than three-fold increase in TLR4 expression in human atherosclerotic lesions was reported in comparison to TLR4 expression levels in normal arteries (Edfeldt et al., 2002). Immunohistochemical staining of normal and atherosclerotic arteries have revealed that TLR4 levels are considerably augmented in atherosclerotic vessels and are most prominently observed in endothelial cells, macrophages, and vascular smooth muscle cells (VSMC) (Edfeldt et al., 2002). Furthermore, global knockout of TLR4 in hypercholesterolemic apoE-/- mice, a mouse model that is prone to atherosclerosis, results in decreased atherosclerotic lesion size, macrophage infiltration and lipid accumulation (Michelsen et al., 2004).

The TLR4 signaling pathway is activated by pathogen-associated molecular patterns (PAMPs), such as lipopolysaccharide (LPS), fusion protein from respiratory syncytial virus and the envelope protein from mouse mammary tumor (Kurt-Jones et al., 2000; Poltorak et al., 1998; Rassa et al., 2002). PAMPs activate TLR4 signal transduction by interacting with CD-14, a glycosylphosphatidylinositol-anchored protein, to form a complex with MD-2 and TLR4 (Lu et al., 2008). This TLR4 complex further recruits adaptor proteins TIRAP/MyD88 or TRAM/TRIF to activate either a MyD88-dependent or a MyD88-independent pathway, respectively (**Figure 2**) (Fitzgerald et al., 2004). Activation of these pathways induces the expression of inflammatory and Type I interferon genes through activation of MAPKs and

Figure 2. LPS stimulation of the TLR4 pathway in macrophages. LPS activates TLR4 to induce both MyD88-dependent and TRIF-dependent pathways of inflammation. Activation of the MyD88-dependent pathway leads to the activation of MAPKs and activation of the transcription factors NF-κB and AP-1. This results in activation of pro-inflammatory cytokine genes as well as downstream activation of anti-inflammatory genes. Stimulation of the TRIF-dependent pathway leads to NF-κB activation, and also the activation of IRF3 transcription factor to induce the expression of Type I interferons. MKPs provide regulatory control to the inflammatory response by inhibiting MAPK function, thus preventing an excessive inflammatory response.


LPS
Cell membrane
TLR4
Cytoplasm
TRAM/TRIF
Calcineurin
TIRAP/MyD88
MAPKs
(P38, JNK)
MKPs
(MKP-1. etc)
Transcription Factors
IRF3
NF-κB
AP-1
Nucleus
Type I interferons
(IFN-β, etc)
Anti-inflammatory genes
(IL-10, MKP-1, etc)
Pro-inflammatory genes
(TNF-α, IL-6, IL-1β, etc)

transcription factors, such as interferon regulatory factor 3 (IRF3) and nuclear factor κB (NF-κB) (Lu et al., 2008).

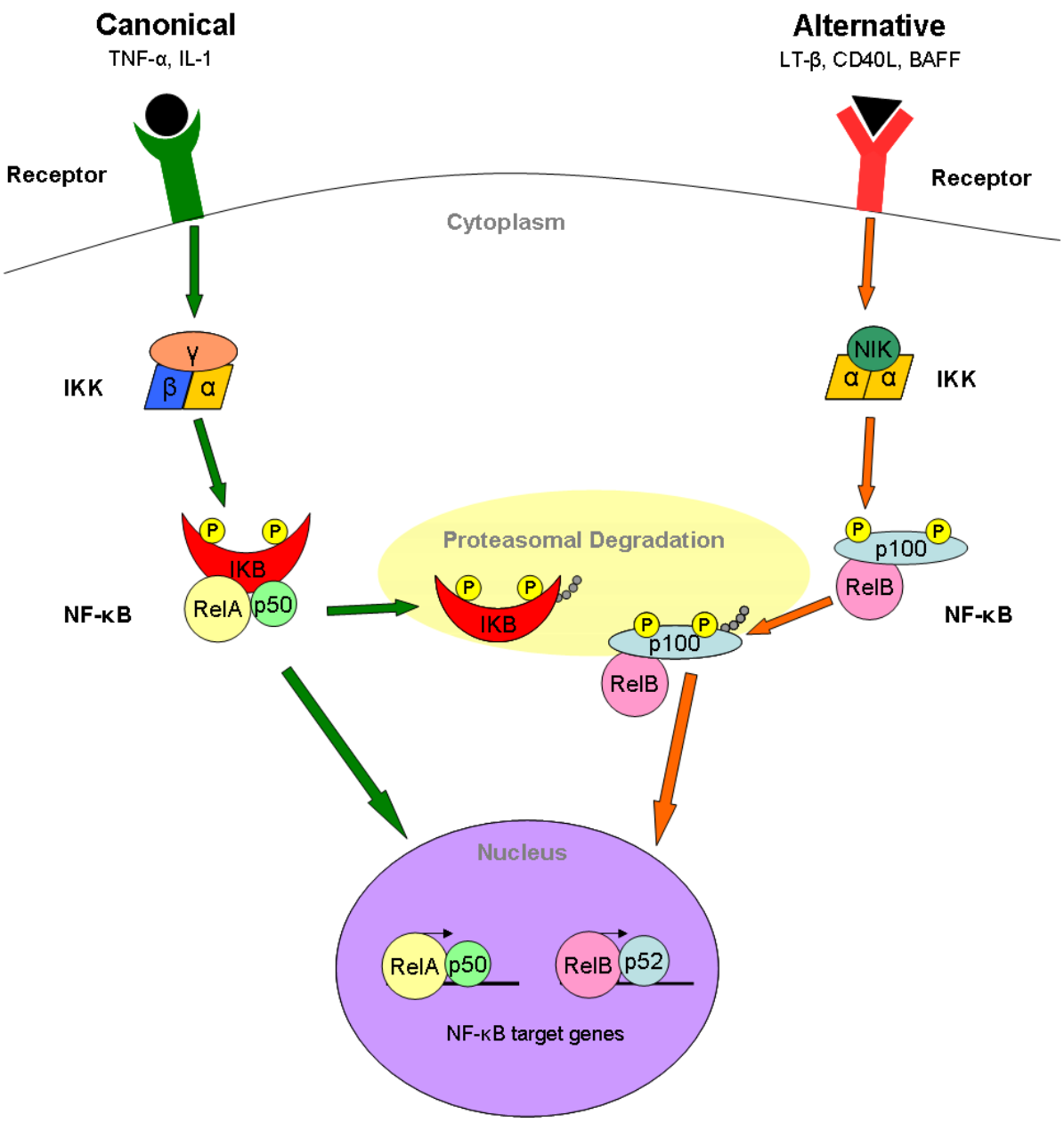
In certain disease conditions, such as cancers, obesity or chronic inflammatory disorders, endogenous ligands may activate TLRs to advance inflammatory pathways in the absence of PAMPs or infection (Karin et al., 2006). For example, chronic inflammation has been implicated in the development of prostate cancer (Nelson et al., 2004) and both murine and human prostate cancer cell lines have been found to secrete increased amounts of Hsp70 (Wang et al., 2004a). Interestingly, Hsp70 has been shown to trigger inflammatory signaling pathways in RAW 264.7 macrophages and 293T fibroblast through the activation of TLR4 and subsequent recruitment of the MyD88 adaptor protein (Vabulas et al., 2002). In obesity and atherosclerosis, elevated free fatty acid levels in the plasma have been implicated in TLR4 signal activation. Several studies have found that saturated fatty acids stimulate TLR4-dependent signaling (Lee et al., 2001; Lee et al., 2003; Schaeffler et al., 2009; Shi et al., 2006). However, one of these studies has found that LPS, but not saturated fatty acids stearic acid and oleic acid, binds directly to a soluble complex of extracellular TLR-4 fused with full length MD-2, suggesting that there is no direct binding of saturated fatty acids to TLR4 (Schaeffler et al., 2009). Also, an extensive study by Samani and Erridge has proposed that saturated fatty acids, such as lauric, myristic and stearic acids, do not directly stimulate TLR4 signaling in TLR4/MD2-transfected HEK293 cells, RAW macrophages, endothelial cells, aortic smooth muscle cells, 3T3-L1 adipocytes, or peripheral blood mononuclear cells (Erridge and Samani, 2009). Instead, Samani and Erridge proposed that the previous findings of other groups were a result of LPS and lipopeptide contamination of the commercially bought reagents fatty acid-free bovine serum albumin (BSA). As this was a recent study,

more indepth studies are needed to further determine whether fatty acids can act as endogenous ligands of TLR4. Identification of endogenous ligands that activate TLR4 is important since TLR activation will contribute to atherosclerotic plaque formation. This will then allow for new preventative measures against plaque formation. Overall, TLRs are beneficial in that they enable the immune system to sense danger, even when it originates from the body, and initiate an inflammation response through signaling pathways such as nuclear factor-κB (NF-κB) activation. It is the over-activation of TLRs, including TLR4, that drives pathological conditions.

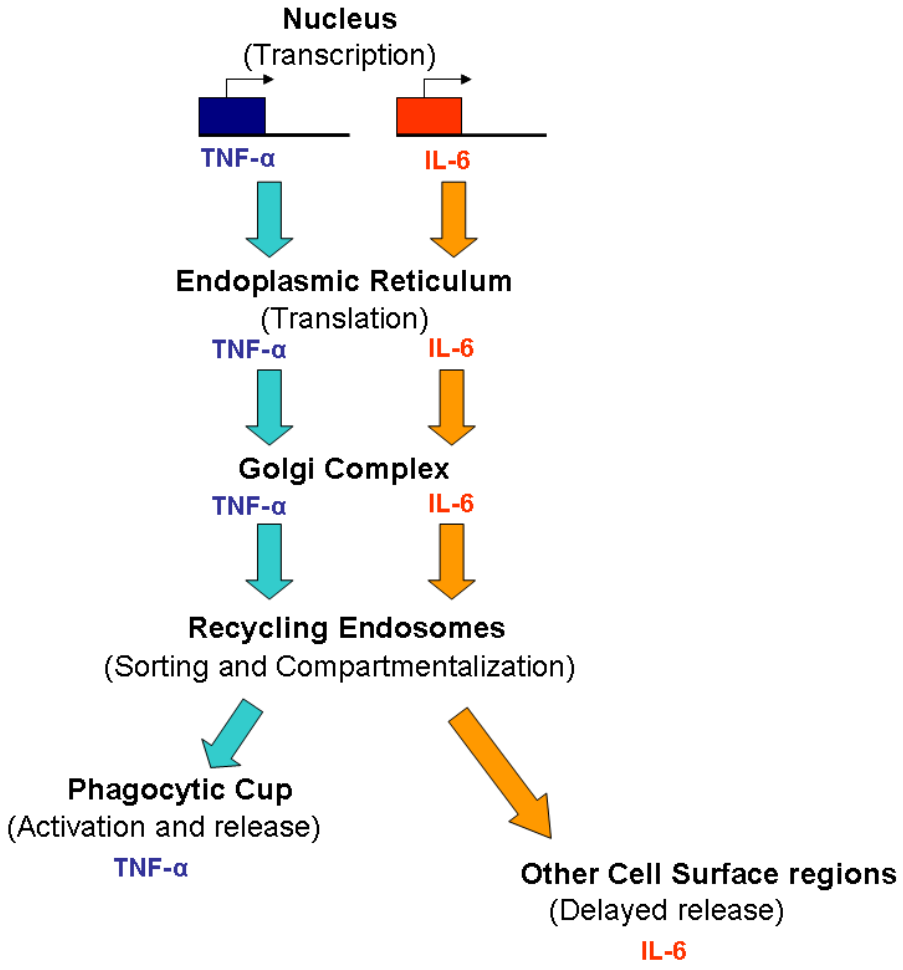
NF-κB transcription factors play a major role in the regulation of inflammation (Liang et al., 2004). The NF-κB family consists of five subunits, RelA, RelB, c-Rel, p50 and p52, which can form a variety of homodimers and heterodimers for the regulation of different genes. In the resting state of cells, inhibitory proteins IκBα, IκBβ, IκBε, p105, and p100 sequester NF-κB in the cytoplasm (Liu et al., 2009). Activation of the inflammatory pathway in cells by various stimuli leads to the phosphorylation by IκB kinase (IKK) and subsequent degradation of the inhibitory proteins. This releases NF-κB and allows it to translocate to the nucleus and activate transcription (Hayden and Ghosh, 2004). There are two major signaling pathways of NF-κB activation: canonical and alternative (**Figure 3 A**) (Beinke and Ley, 2004). The canonical pathway is stimulated by PAMPs and proinflammatory cytokines such as IL-1 and TNF-α, leading to the activation of RelA complexes that regulate cell survival and proinflammatory gene expression (Karin and Ben-Neriah, 2000). The alternative pathway is triggered by TNF-family cytokines, such as lymphotoxin β, B cell activating factor (BAFF), and CD40 ligand, but not TNF-α (Matsushima et al., 2001). The main consequence of alternative stimulation is the activation of RelB/p52 complex (Bonizzi et al., 2004). It is believed that the alternative pathway

Figure 3. Pathway of cytokine expression and secretion in macrophages. A) The canonical and alternative NF-κB signalling pathways (*modified from Lawrence 2009*). Signal initiation begins at the receptors with binding of their respective ligands. This process leads to the assembly and activation of IKK complexes. These complexes phosphorylate IκB and p100, the inhibitors of NF-κB. Once phosphorylated, IκB is degraded through the proteosomal degradation pathway. p100 is partially degraded to form the p52 subunit of NF-κB. Activation of RelA/p50 and RelB/p52 complexes allows for their translocation to the nucleus and activation of target genes. Activation of the canonical pathway regulates expression of proinflammatory and cell survival genes. The alternative pathway regulates genes involved in lymph-organogenesis and B-cell activation. **B)** Stimulation of macrophages with LPS leads to the activation of proinflammatory TNF-α and IL-6 genes via NF-κB activation. Both cytokines are transported by certain carriers to the Golgi after translation in the Endoplasmic Reticulum. Different SNARE complexes then assist in the transport of TNF-α and IL-6 to recycling endosomes where they are sorted and compartmentalized. TNF-α is transported in a polarized manner with parts of the recycling endosomes to the phagocytic cup. TNF-α-specific enzymes cleave and activate TNF-α for the containment of microbes or dying cells. IL-6 is transported by proteins and complexes to other cell surface regions for release into the extracellular space.

A


Canonical
TNF-α, IL-1
Alternative
LT-β, CD40L, BAFF
Receptor
Receptor
Cytoplasm
IKK
γ
β
α
NIK
α
α
IKK
P
P
IKB
RelA
p50
NF-κB
Proteasomal Degradation
P
P
IKB
P
P
p100
RelB
P
P
p100
RelB
NF-κB
Nucleus
RelA
p50
RelB
p52
NF-κB target genes

B


Nucleus
(Transcription)
TNF-α
IL-6
Endoplasmic Reticulum
(Translation)
TNF-α
IL-6
Golgi Complex
TNF-α
IL-6
Recycling Endosomes
(Sorting and Compartmentalization)
Phagocytic Cup
(Activation and release)
TNF-α
Other Cell Surface regions
(Delayed release)
IL-6

activates NF-κB target genes that are distinct from the canonical pathway (Bakkar and Guttridge, 2010). Together, these two pathways enable NF-κB to activate various genes involved in cell differentiation and growth, inflammation and apoptosis (Liu et al., 2009). These genes include growth and differentiation genes VEGF-C, M-CSF, GM-CSF, CD-69, platelet activating factor receptor, inflammatory cytokines TNF-α, IL-6, IL-1β, and the cell cycle regulator p53 (Wu and Lozano, 1994).

TLR4 signal transduction in macrophages can also be indirectly activated by cyclosporin A (CsA) and tacrolimus (FK506). These drugs have been previously used as T cell immunosuppressants through their inhibition of calcineurin and, consequently, of nuclear factor of activated T-cell (NFAT) (Rao et al., 1997). In T cells, after T cell receptor ligation, calcineurin transduces the signal from Ca^{2+} flux by dephosphorylating and activating NFAT transcriptional factors, leading to the expression of pro-inflammatory cytokines Il-2, IL-4 and TNF-α (Conboy et al., 1999) as well as other genes that encode cell signaling proteins, cell surface receptors and effector proteins (Rao et al., 1997). Contrary to its function in T cells, calcineurin negatively regulates TLR4 responses in macrophages by inhibiting the receptor-proximal signaling events such as TLR4 interaction with adaptor proteins MyD88 and TRIF (Jennings et al., 2009; Kang et al., 2007). Thus, inhibition of calcineurin in macrophages releases TLR4, allowing it to interact with downstream adaptors. This, in turn, triggers NF-κB activation. Similar to LPS, (see **Figure 2**) FK506 treatment in macrophages also results in MAPK activation, NF-κB activation, and increased TNF-α secretion (Kang et al., 2007). Thus, the inhibition of calcineurin by CsA and FK506 results in an immunosuppressive effect in T cells, whereas in macrophages it leads to a more inflammatory profile through NF-κB activation (Conboy et al., 1999).

Relevant to this thesis, the expression and secretion of IFN-β, IL-6, and TNF-α , pro-inflammatory cytokines triggered by TLR4 activation, have been identified in atherosclerotic plaques (Kleemann et al., 2008). These cytokines recruit additional leukocytes to the intimal site of injury, further exacerbating the progression of atherosclerotic plaques (Linton and Fazio, 2003). In this context, lipid-laden foam cells are particularly hazardous in that, when stimulated, they secrete more pro-inflammatory cytokines compared to non-loaded macrophages (Groeneweg et al., 2006). Foam cells, therefore, are not only the major component of atherosclerotic lesions, but also significantly contribute to the chronic inflammation of atherosclerosis. Targeting foam cells and their inflammatory functions may provide an effective means to suppress the prevalence of plaques and atherosclerosis (Tabas, 2010). In foam cells, the plasma membrane protein Adenosine triphosphate (ATP)-binding cassette transporter A1 (ABCA1) has an established role in relieving lipid load as well as in reducing the secretion of pro-inflammatory cytokines (van Eck et al., 2002; Van Eck et al., 2006; Yin et al., 2010; Zhu et al., 2008).

1.3 - ABCA1 structure and function

ABCA1 belongs to an evolutionarily preserved ATP-binding cassette transporter (ABC) super-family, which primarily functions to transport various substrates across biological membranes (Zarubica et al., 2007). This is generally achieved through the hydrolysis of ATP to provide energy for active transport (Nagao et al., 2010). The ABC family of proteins is divided into 7 groups based on gene sequence similarity. The ABC-A group contains the full-sized ABC transporter proteins, such as ABCA1 (Stefkova et al., 2004). The topological arrangement of ABCA1 includes two transmembrane spanning domains, each consisting of six transmembrane segments and a nucleotide binding domain (NBD) (Fitzgerald et al., 2002b). These NBDs allow the hydrolysis of ATP to provide energy necessary for substrate transport (Roosbeek et al., 2004). The ABCA transporters have a highly conserved topological arrangement (Broccardo et al., 2001; Bungert et al., 2001) except for their large extracellular loop regions. The ABCA family extracellular loop areas experience sequence hypervariability that may contribute to functional specificity of individual members (Zarubica et al., 2007).

ABCA1 plays a vital role in reverse cholesterol transport (RCT) (Zarubica et al., 2007; Zhu et al., 2008). In this process ABCA1 mediates the efflux of cholesterol and phospholipids from peripheral tissue to lipid-poor apoproteins such as apolipoprotein A1 (apoA-I) or apolipoprotein E (apoE) (Oram et al., 2000). The transport of cholesterol and phospholipids to apoA-I produces high density lipoprotein (HDL) particles (Schmitz and Grandl, 2009). The formation of HDL particles by ABCA1 not only eliminates excess intracellular cholesterol from peripheral tissues, but also facilitates cholesterol transport to the liver and steroidogenic tissue for excretion, bile acid synthesis, or steroid hormone synthesis (Bogan and Hennebold, 2010; Ohashi et al., 2005; Zarubica et al., 2007).

Mutations in the human ABCA1 gene lead to Tangier disease (Bodzioch et al., 1999; Rust et al., 1999). This disorder is characterized by nearly complete absence of plasma HDL (Nofer and Remaley, 2005), the result of impaired ability to efflux cholesterol and phospholipids to lipid-free apoA-I (Francis et al., 1995; Rogler et al., 1995; Walter et al., 1994). Additionally, Tangier patients have increased atherosclerosis (Nofer and Remaley, 2005). Genetic inactivation of ABCA1 in two atherosclerosis mouse models (apoE$^{-/-}$ and Ldlr$^{-/-}$) also produces extremely low HDL levels and severe accumulation of foam cells (Aiello et al., 2002). Interestingly, liver- and intestine-specific ABCA1 knockout mice had 80% and 20% decrease in plasma HDL levels, respectively, compared to wild type littermates (Brunham et al., 2006; Timmins et al., 2005). Macrophage-specific ABCA1 knockout does not significantly affect circulating levels of plasma HDL (Haghpassand et al., 2001). Therefore, ABCA1 in the liver and intestine is responsible for maintaining plasma HDL levels, at least in rodents.

1.4 - ABCA1 expression and regulation

There are four major transcription factors that modulate ABCA1 expression, namely liver-X receptors (LXRα and LXRβ), retinoic-X receptor (RXR), peroxisome proliferator-activated receptor (PPARα and γ), and pregnane-X receptor (PXR) (Costet et al., 2000; Schmitz and Langmann, 2005; Tall et al., 2000). Activation by oxysterols, retinoids, or their agonists causes the formation of LXR/RXR heterodimers. The LXR/RXR heterodimer recognizes and binds to the DR4 DNA response elements (Fitzgerald et al., 2002a) found in the promoter region of ABCA1, as well as other genes involved in lipid homeostasis (Li and Glass, 2004; Zarubica et al., 2007). This leads to increased ABCA1 gene transcription and increased ABCA1-dependent cholesterol efflux to apoA-I (Venkateswaran et al., 2000).

PPARα and PPARγ also increase the expression of ABCA1, albeit indirectly, through the enhanced transcription of LXRα (Chawla et al., 2001; Chinetti et al., 2001). Activation of PXRs and geranylgeranyl pyrophosphate (ggPP) leads to the down-regulation of ABCA1 expression via the DR4 element (Schmitz and Langmann, 2005).

Presence of a cyclic-AMP response element (CRE) in the promoter region of murine ABCA1 provides further regulation of ABCA1 expression (Denis et al., 2003; Oram et al., 2000). Cyclic adenosine 3',5'-monophosphate, or cyclic-AMP (cAMP), upregulates murine ABCA1 expression through the activation of the cAMP response element binding protein (CREB) and its consequent interaction with CRE. Treatment with cAMP has no effect on gene expression of human ABCA1 due to its lack of CRE sites in the promoter region (Denis et al., 2003; Le Goff et al., 2006) suggesting alternative regulatory pathways between species.

ABCA1 is highly expressed in macrophages. In addition, ABCA1 is present in many different cell types and tissues, such as liver, small intestine, lungs, and placenta (Aiello et al., 2003; Christiansen-Weber et al., 2000; Langmann et al., 1999). Although macrophage ABCA1 does not directly contribute to circulating levels of HDL, it has a well-established anti-atherogenic role. Mice lacking macrophage ABCA1 have accelerated atherosclerosis and foam cell accumulation (Aiello et al., 2002; van Eck et al., 2002) and, conversely, mice overexpressing macrophage ABCA1 have significantly decreased prevalence of atherosclerosis (Van Eck et al., 2006). Interestingly, genetic disruption of macrophage ABCA1 leads not only to foam cell formation (Li and Sun, 2007; Zhu et al., 2008), but also to an exaggerated inflammatory response (Zhu et al., 2008).

1.5 - ABCA1 and inflammation

In addition to mediating cholesterol efflux, ABCA1 also regulates inflammation. A study by Zhu and colleagues has shown that ABCA1 knockout (ABCA1-/-) mouse macrophages have increased expression and secretion of pro-inflammatory cytokines TNF-α, IL-6, and IL-12p40 upon LPS challenge (Zhu et al., 2008). Although the molecular mechanism by which macrophage ABCA1 dampens inflammation is not fully elucidated, it is most likely linked to ABCA1's primary function in regulating lipid homeostasis. This same group has revealed that the normalization of free cholesterol content in both wt and ABCA1-/- murine macrophages results in simlar inflammatory response to LPS. Also, they have found that increased levels of cellular free cholesterol in ABCA1 -/- mouse macrophages leads to an enhanced pro-inflammatory response. These results suggest a strong connection between the two functions of ABCA1 and that inflammation in macrophages may be linked to lipid homeostasis. In support of such notion, another ABC transporter activated by LXR, ABCG1, has been also shown to have a suppressive effect on inflammation in macrophages (Yvan-Charvet et al., 2008). ABCG1 mediates the efflux of cholesterol, phospholipids, and sphingomyelin to mature HDL particles, as well as LDL and phospholipid vesicles (Kobayashi et al., 2006; Wang et al., 2004b), thus promoting cholesterol removal from macrophages. Despite the capability of ABCG1 to deplete intracellular cholesterol and suppress the inflammatory response (Yvan-Charvet et al., 2008), its effect on atherosclerosis remains unclear. ABCG1 knockout in mouse macrophages leads to no change (Yvan-Charvet et al., 2008), a decrease (Baldan et al., 2006; Ranalletta et al., 2006), or an increase (Out et al., 2006) in atherosclerotic lesion development, suggesting that there are other complex factors that affect the function of ABCG1 in atherosclerosis. Also,

there are no known human diseases or defects caused by mutations in the ABCG1 gene. It is thus likely that ABCA1 is more physiologically relevant to atherosclerosis.

1.6 - Cytokine secretion from macrophages

Macrophages secrete cytokines to directly destroy microbes or to stimulate and recruit other immune cells during inflammation (Stow et al., 2009). As discussed above, the secretion of cytokines is triggered by PAMPs or endogenous ligands binding to pattern recognition receptors, such as TLRs (Lawrence, 2009). Secreted cytokines can also affect cellular function in an autocrine or paracrine manner leading to inhibition or stimulation of the inflammatory response (Lazar-Molnar et al., 2000).

One of the most extensively studied stimulants of inflammation and cytokine secretion is lipopolysaccharide (LPS) (Lu et al., 2008). LPS, found in the outer membrane of Gram-negative bacteria, consists of three components: lipid A, a core oligosaccharide, and an O side chain (Miller et al., 2005; Raetz and Whitfield, 2002). LPS stimulation of macrophages leads to the activation and transcription of proinflammatory cytokine genes TNF-α and IL-6 and the anti-inflammatory cytokine gene IL-10 (Lu et al., 2008). Although the pathways and machinery involved in cytokine secretion are not fully understood, several studies suggest that cytokines are transported alongside other cellular trafficking processes (Murray et al., 2005; Stow et al., 2009). As proposed by Stow et al (see **Figure 3 B)**, TNF-α and IL-6 are secreted from macrophages via the recycling endosome. After protein translation in the endoplasmic reticulum (ER), TNF-α and IL-6 are transported to the Golgi complex. SNARE proteins and complexes facilitate the transport of these cytokines to the recycling endosome through their essential function in the fusion of vesicles to their target

membranes (Cao et al., 2006). Interestingly, the transport of TNF-α from the recycling endosome to the cell surface is achieved alongside phagocytosis. Because the endosome provides extra membrane to form the phagocytic cup that engulfs microbes and damaged cells, it brings along TNF-α to the formation site of the phagocytic cup. This allows for the quick release and activation of TNF-α upon infection. The combined secretion of TNF-α and engulfment of the microbe allows for containment and clearance of infection in a prompt manner. Although IL-6 accumulates with TNF-α in the Golgi and both cytokines colocalize in the recycling endosome (Stow et al., 2009), IL-6 is not transported alongside TNF-α to the cell surface of the phagocytic cup. Instead, protein carriers transport IL-6 to other cell surface areas for secretion. Thus, it is believed that protein sorting exists in the recycling endosome as there is selective and temporal cytokine transportation to different cellular regions (Manderson et al., 2007). In LPS-stimulated macrophages, TNF-α is first secreted as an early response cytokine, followed by IL-6 (Bopst et al., 1998). Anti-inflammatory cytokines IL-10, IL-4, and TGF-β are secreted at later time intervals. Although the secretory pathways for anti-inflammatory cytokines are not fully understood, their delayed release is believed to be a way of moderating the inflammatory response by ensuring the containment of inflammation (Elenkov and Chrousos, 2002).

1.7 - Cyclic-AMP, protein kinase A and cAMP response element binding protein

Upon stimulation by PAMPs, cytokine secretion from macrophages is further regulated by a complex interplay of many factors. Among these, cAMP is thought to be an important modulator as it has been shown to have suppressive effects on macrophage inflammation (Wall et al., 2009).

cAMP is a secondary messenger that regulates diverse biological processes such as embryogenesis, respiration, heart contractility, lipolysis, learning and memory (Sadana and Dessauer, 2009). cAMP is generated by transmembrane and soluble adenylyl cyclases (ACs) (Pavan et al., 2009; Sadana and Dessauer, 2009). Transmembrane ACs are activated via ligand-binding to G-protein coupled receptors, such as the receptor for prostaglandin E2 (PGE_2), and soluble ACs are activated directly by cellular metabolites, such as ATP (Pavan et al., 2009). cAMP then binds to its target proteins, such as protein kinase A (PKA), to initiate a signal cascade. This cascade is terminated by the breakdown of cAMP to 5'-AMP through phosphodiesterases (Murata et al., 2009).

The newly identified guanine exchange protein activated by cAMP (Epac) allows for an alternate pathway of cAMP signaling to regulate cell functions independent of PKA activation. Epac1 and Epac2 are two genes that express this exchange protein (de Rooij et al., 1998). Epac1 is ubiquitously expressed, with higher levels of expression in skeletal muscle, kidney, thyroid, ovary, and certain areas of the brain, whereas Epac2 is mainly expressed in the brain and adrenal glands (de Rooij et al., 1998; Kawasaki et al., 1998). In cerebellar neurons, Epac was shown to regulate neuronal excitability through the activation of p38MAPK and subsequent modulation of calcium ion-dependent K+ channels (Ster et al., 2007). Little is known about the function of Epac in macrophages. Only quite recently has

there been identification of Epac in macrophages. Macrophage Epac-1 was found to mediate the inhibition of phagocytosis of antibody-coated targets and, along with PKA, Epac-1 can mediate the inhibition of bacterial killing by the generation of reactive oxygen species (ROS) (Aronoff et al., 2005).

Protein kinase A (PKA) is a cAMP-dependent protein kinase that consists of two regulatory and two catalytic subunits in its inactive state. Binding of cAMP to each regulatory subunit of the PKA holoenzyme results in a conformational change and the dissociation of the two catalytic subunits (Tasken and Aandahl, 2004). These catalytic subunits then go on to phosphorylate serine and threonine residues on target substrates (Gonzalez and Montminy, 1989). PKA is involved in many different cell signalling processes, such as proliferation, differentiation, and apoptosis (Naviglio et al., 2009). As PKA affects a wide variety of pathways, spatial and temporal regulation of PKA activity is crucial in mediating the specificity of pathway activation. This tight regulation of PKA activity is in part achieved through A-kinase-anchoring proteins (AKAPs). Through anchorage, AKAPs target PKA to its substrates and optimally place PKA in areas of activation nearby local cAMP pools (Pidoux and Tasken, 2010).

One of the main targets of PKA is the nuclear transcription factor CREB (Gonzalez and Montminy, 1989). CREB is known to regulate genes involved in immune regulation, cell proliferation, differentiation, as well as cell survival (Mayr and Montminy, 2001). This transcription factor is activated by the phosphorylation of its Ser133 residue by PKA. CREB can also be phosphorylated by other kinases. These include mitogen-activated protein kinases (MAPKs), such as extracellular signal-regulated kinase (ERK) and p38MAPK, and calmodulin kinases (CaMK) I, II, and IV (Sheng et al., 1991; Wu et al., 2001; Xing et al., 1996), as well as MSK-1 (Wiggin et al., 2002). Once CREB is phosphorylated, CREB

binding protein (CBP) binds CREB and together they activate the transcription of target genes by binding to the c-AMP response element in the gene promoter region. The transcriptional coactivator CBP activates gene transcription via its histone acetyl transferase activity and also induces CREB acetylation and transcriptional activity (Lu et al., 2003). Target genes of CREB that are associated with inflammation include IL-10, cycloxygenase 2 (COX-2), TNF-α (Mayr and Montminy, 2001) and MAPK phosphatase 1 (MKP-1) (Casals-Casas et al., 2009).

1.8 - MAPKs, MKP-1 and inflammation

MAPKs are also key players in TLR4 signal transduction. These kinases provide further augmentation of the inflammatory response by the phosphorylation and consequent activation of several transcription factors, such as Elk-1, c-Jun, c-Fos, ATF-1, ATF-2, and SRF, that regulate the expression of pro-inflammatory genes (Guha and Mackman, 2001).

MAPKs are a family of highly conserved serine/threonine protein kinases that modulate diverse cellular processes including inflammation, gene regulation, cell survival, proliferation and differentiation (Johnson and Lapadat, 2002; Thalhamer et al., 2008). In mammals, there are three main classes of MAPKs: ERKs, JNK, and p38. Activation of MAPKs requires phosphorylation by the dual-specific MAPK kinases (MKKs) on threonine and tyrosine residues. In turn, MKK stimulation requires serine/threonine phosphorylation by MAPK kinase kinases (MKKKs). Thus, the MAPK signal cascade, conserved from yeast to mammal (Zhang and Dong, 2005), leads to phosphorylation of nuclear and cytoplasmic target proteins to affect key cellular processes (Thalhamer et al., 2008).

Stimulation of macrophages with LPS leads to simultaneous activation of all three MAPKs (Geppert et al., 1994), resulting in the expression of pro-inflammatory genes (Denkers et al., 2004). Inhibition of these MAPKs decreases inflammatory cytokine production. For example, inhibition of p38 and ERK abolishes TNF-α production in LPS-treated mice (Dumitru et al., 2000; Kontoyiannis et al., 1999) and genetic ablation of JNK leads to defective induction of IL-6 and IL-12 in LPS-treated murine fibroblasts (Chu et al., 1999). Although activation of MAPKs is essential for inflammatory response, tight regulation of such activation is also necessary. This ensures a proper immune reaction against pathogens without excessive damage to the cell or tissue (Avni et al., 2010). Activation of negative feedback mechanisms to limit MAPK pathway activation is thus crucial. MAPK phosphatases (MKPs) are part of this negative feedback mechanism (Feng et al., 1999). At present, there are eleven MKP members that have been identified. Knockout of MKP1 or MKP5 leads to increased proinflammatory cytokine production and, in the case of MKP-1, a susceptibility to endotoxic shock (Zhao et al., 2006). Among the 11 MKPs, MKP-1 is transcriptionally regulated by CREB via two cAMP response elements in its promoter region (Sommer et al., 2000). MKP-1 is a dual-specific protein phosphatase that is known to inhibit MAPK activation through the dephosphorylation of tyrosine and threonine residues on the MAPK activation loop (Casals-Casas et al., 2009).

MKP-1 is most commonly known to inactivate ERK1/2, leading to the downregulation of inflammation (Casals-Casas et al., 2009). However, MKP-1's enzymatic activity is not limited to ERK as it has been found to dephosphorylate JNK and p38 MAPK as well (Sommer et al., 2000), depending on the cell and tissue type (Farooq and Zhou, 2004). Studies have shown that MKP-1 deficient macrophages have increased TNF-α, IL-6, and NO production upon LPS stimulation (Liu et al., 2007; Wang et al., 2007). Similarly, in

MKP-1 knock-out mice, there is increased production of TNF-α, IL-6, MIP-1α, GM-CSF and NO compared to wt mice in response to bacterial infection (Hammer et al., 2006; Salojin et al., 2006; Wang et al., 2007; Zhao et al., 2006). The increase in pro-inflammatory cytokine production in MKP-1 mice is accompanied by susceptibility to endotoxic shock observed through increased organ failure and mortality (Zhao et al., 2006).

In searching downstream targets of ABCA1 that promote anti-inflammatory functions, we noted the two CRE sites in the promoter region of the MKP-1 gene. This suggests that MKP-1, transcriptionally regulated by CREB activation, potentially through PKA, may be one of the pathways ABCA1 exerts its inflammation regulatory function. Interestingly, previous studies, including ours, have identified PKA as a critical regulator of ABCA1 function. Our lab has previously found that inhibition of PKA completely abolishes cholesterol efflux to apoA-I. As MKP-1 expression functions to suppress inflammation, we speculated that MKP-1 may be an important factor in linking ABCA1 to the mechanism of inflammation resolution.

1.9 - Specific Aims

Our aim was to study the potential mechanism by which macrophage ABCA1 regulates inflammation and cytokine secretion. We first aimed to characterize CREB phosphorylation in the context of ABCA1 expression. Next we aimed to establish in our cell model system that factors that phosphorylate CREB also modulate the inflammatory response. Our third objective was to identify the effect of the induction of ABCA1 on downstream targets of CREB, such as the MAPK phosphatase MKP-1.

1.10 - Hypothesis:

ABCA1 expression activates CREB, potentially through PKA, which in turn regulates cytokine secretion in macrophages.

1.11 - Significance:

Although the expression of macrophage ABCA1 has been shown to have anti-inflammatory effects, the mechanism linking ABCA1 to inflammation modulation has yet to be fully established. This study aims to identify players involved in the mechanism linking ABCA1 to inflammation modulation. Work ongoing in our laboratory has indicated involvement of PKA in ABCA1 function. As PKA activity leads to suppression of the inflammatory response, the anti-inflammatory effects of ABCA1 may be a consequence of this signalling pathway. PKA is activated by cAMP and thus we aimed to determine whether ABCA1's involvement in cytokine regulation is through cAMP signalling to activate PKA and/or through phosphorylation of the transcription factor CREB.

Chapter II: Materials and Methods

2.1 - Chemicals and Antibodies

Dulbecco's Modified Eagle Medium (DMEM), OPTI-MEM, fetal bovine serum (FBS), and penicillin/streptomycin (10,000units/mL penicillin and 10,000ug/mL streptomycin) were purchased from *Gibco® Invitrogen* (Burlington, ON). 8-Br-cAMP , T0901317, bovine serum albumin (BSA), lipopolysaccharide (LPS), and cyclosporin A (CsA) were obtained from Sigma-Aldrich (Oakville, ON). Tacrolimus (FK506) was supplied by A.G. Scientific Inc. (Sand Diego, CA). Myristoylated protein kinase inhibitor (PKI) (14-22) amide was purchased from Enzo Life Sciences (Farmingdale, NY). [^{3}H]cholesterol was supplied by Perkinelmer (Woodbridge, ON). Scintillation liquid ScintiSafe Gel Cocktail was purchased from Fisher (Whitby, ON). PhosSTOP phosphatase inhibitor cocktail and FuGene HD Transfection Reagent were purchased from Roche (Laval, QC). Bio-Rad Protein Assay dye reagent concentrate was obtained from Bio-Rad Laboratories (Montreal, QC). PageRuler^{TM} Plus Prestained Protein Ladder was purchased from Fermentas Life Sciences (Burlington, ON). SuperSignal® West Pico Chemiluminescent Substrate was purchased from ThermoScientific (Mississauga, ON). Immunoblot film was supplied by Interscience (Markham, ON). Enzyme-linked immunosorbent assay (ELISA) for murine IL-10, IL-6, and TNF-α were purchased from R & D Systems Inc. (Minneapolis, MN). Mouse ABCA1-shRNA in pLKO.1 lentiviral vectors were obtained from Open Biosystems (Huntsville, AL).

Mouse anti-ABCA1 was purchased from Millipore (Toronto, ON). Rabbit anti-phospho-CREB (Ser133), mouse anti-CREB, and rabbit anti-phospho-PKA substrate (RRXS/T)(100G7E) were purchased from Cell Signaling (Pickering, ON). Mouse anti-MKP-

1 (V-15) was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Mouse anti-hsp70 was purchased from BD Transduction Laboratories (San Jose, CA). Peroxidase-conjugated sheep anti-mouse and donkey anti-rabbit IgGs were purchased from Jackson Immunoresearch Laboratories (West Grove, PA).

2.2 - Methods

Cell Culture – all cell lines were cultured in DMEM (DMEM high glucose powder, 44mM $NaHCO_{3,}$, pH 7.2) and supplemented with 10% FBS and 1% penicillin/streptomycin (100 units/mL penicillin and 100µg/mL streptomycin) at 37^{o}C with 5% CO_2. RAW 264.7 mouse macrophages were purchased from *ATCC*. These macrophages endogenously express ABCA1 upon induction with a cAMP analog, 8-Bromo-cAMP, or with an LXR-agonist, T0901317. Baby Hamster Kidney (BHK) cells that are stably transfected with a mifepristone-inducible plasmid encoding the *abca1* gene (ABCA1) or a noncoding sequence (MOCK) were obtained from Drs. Vaughan and Oram (University of Washington). Wildtype (wt) and ABCA1 knockout (ABCA1-/-) primary mouse bone marrow-derived macrophages (BMDMs) were provided by Dr. Marcel (University of Ottawa Heart Institute). Femurs of wt and ABCA1-/- C57 mice were flushed, and cells were incubated with DMEM containing 10% FBS and 15% L929 conditioned medium for 7 days to obtain BMDMs.

Induction of ABCA1 expression – RAW cells were seeded in either 35mm dishes ($3x10^5$cells/dish) or 60mm dishes ($2.5x10^6$ cells/well). After adherence to plate (~4h), cells were washed with 37^{o}C 1X PBS (137mM NaCl, 2.7mM KCl, 4.3mM Na_2HPO_4, 1.47mM KH_2PO_4, pH adjusted to 7.4) incubated with either 50µM 8-Br-cAMP or 10µM T0901317 for 18-20h. For BHK cells, both MOCK and ABCA1 cells were seeded in 35mm dishes

($2.2x10^5$cells/dish) and induced with 10nM mifepristone for 18-20h. Wt and ABCA1 -/- primary BMDMs in 6-well plates with 35mm diameter (~3 $x10^5$cells/well) were induced with either 50μM 8-Br-cAMP or 10μM T0901317 for 18-20h. During induction, cells were maintained with either DMEM containing 1mg/mL bovine serum albumin (BSA) or DMEM containing 10% FBS.

Cholesterol Efflux – Cells were seeded in 12-well plates (RAW: $2x10^5$ cells/well and BHK: $1.5x10^5$ cells/well) and labeled with 1μCi/ml [^{3}H]cholesterol for 24h. Afterwards, RAW cells were either induced with 10μM T0901317 or maintained in media alone for 18-20h. Both MOCK and ABCA1 BHK cells were induced with 10nM mifepristone for 18-20h. For efflux period, cells were treated for 4h with DMEM + 1mg/mL BSA containing 5 μg/mL apoA-I and 0-200μM 8-Br-cAMP. After efflux, the medium was collected and the cells were lysed with 1M NaOH. Radioactivity levels in the medium and cell lysates was quantified in counts per minute (CPM) using liquid scintillation counting (Beckman LS 6500). A count time of 5 minutes per sample was maintained. Cholesterol efflux was calculated as a percentage of total cholesterol counts: $CPM_{medium}/(CPM_{medium} + CPM_{cell})$.

Cell lysis – cells were placed on ice, media was aspirated, and cells were washed twice with cold 1X PBS. Cells were lysed with 1X SDS buffer (50mM Tris-Cl pH 6.8, 100mM dithiothreitol, 2% SDS, 10% glycerol, and 1 tablet PhosSTOP per 10mL buffer). A volume of 100μL lysis buffer was used in each well of a 6-well plate and 150uL for each 6cm diameter plate. Cells were immediately scraped off plates and transferred to microcentrifuge tubes on ice. Lysates were sonicated for 10-15 seconds (to shear DNA) and then heated to

72^{o}C for 5 min. Lysates were centrifuged for 2 min at 9000g and protein levels were measured as described below.

Protein Assay – 200uL of Bradford protein assay dye reagent was added to an 800µL solution containing water and 2-5µL of sample. Mixture was placed in 10mm disposable cuvets (*Fisherbrand*) and absorbance was measured at 595nm using the Ultrospec^{TM} 3100 pro UV/visible spectrophotometer. Protein levels were calculated from a standard curve of 0 – 40µg/µL BSA.

SDS Polyacrylamide gel electrophoresis (SDS-PAGE) and Immunoblotting – 4X SDS loading buffer (200mM Tris HCl pH 6.8, 400mM dithiothreitol, 8% SDS, 40% glycerol, 0.4% bromophenol blue) was added to 30–45µg of cell lysates. Samples were loaded onto 10% acrylamide resolving gels (10% acrylamide mix (9.67% acrylamide, 0.33% bis), 375mM Tris (pH 8.8), 1% SDS, 1% ammonium persulfate, 0.04% TEMED) and subjected to SDS-PAGE at 180V for 90 min. Separated proteins were electro-transferred to PVDF membranes at 70V for 150min. PVDF membranes were blocked with TBS-T (50mM Tris HCl, (pH adjusted to 7.4) 150 mM NaCl, 0.1% Tween-20) containing 2.5% BSA for 30min. Membranes were incubated with primary antibody (1:1000 or 1:2000) with gentle agitation overnight at 4^{o}C. Membranes were than washed with TBS-T three times for 5min each. Membrane was incubated and gently agitated for 2h in secondary antibody (1:2000) diluted in TBS-T with 5% BSA. Once again, membranes were washed three times for 5min each with TBS-T. PVDF membranes were incubated for 1 minute with 1-2mL chemiluminescent substrate, excess substrate was drained, and membranes were covered in plastic wrap. In a

dark room, membranes were exposed to x-ray film for appropriate exposure time and then processed using Raytech Medical Film Processor SRX-101A.

Immunoblot stripping – Stripping buffer (2% SDS, 100mM β-mercapto-ethanol, 50mM Tris pH 6.8) was heated to 50^{o}C in water bath. Membrane was incubated with buffer at 50^{o}C for 30min with gentle shaking. Blot was rinsed three times with 1X TBS-T.

Enzyme-linked Immunosorbent Assay (ELISA) – RAW and BMDM cells were first induced for ABCA1 expression with 50μM 8-Br-cAMP or 10μM T0901317 for 18-20h. Cells were treated with either 100ng/mL LPS, 25μM FK506, or 10μM CsA for 4h. Media was collected in microcentrifuge tubes and cell debris was pelleted at 12,000g for 5min. Cells were lysed as described above. Media and cell lysates were stored in microcentrifuge tubes at -20^{o}C until ready to assay. IL-10, TNF-α, or IL-6 levels in the media were determined following protocol in R&D Systems Inc. Absorbance at 450nm and 540nm wavelengths in the 96-well plates was measured using FLUOstar Galaxy plate reader. Protein levels in cell lysates were determined to normalize cytokine levels in the media.

ABCA1 shRNA transient transfections – 2μg of the five pLKO.1 vectors (0.4μg each) containing different ABCA1-shRNAs or a pUB-GFP control vector (containing ubiquitin promoter region only) were incubated with 6μL of FuGene HD transfection reagent and 100μL of OPTI-MEM for 15min. RAW mouse macrophages were seeded at a confluency of 4x10^{5} cells/well into 6-well plates containing DMEM + FBS (no antibiotics). Transfection mixture was added immediately after transferring cells to the plates. Cells were placed at

37°C with 5% CO_2 for 18h. Cells were gently washed with 1X PBS and media was replaced with DMEM+FBS. Cells were lysed between 24h and 48h after transfection.

8-Br-cAMP dose experiment in RAW cells – RAW macrophages were seeded into 6-well plates at a confluency of $3x10^5$ cells/well and left to grow overnight at 37°C with 5% CO_2. After 18h, cells were washed and incubated for 4h with different concentrations of 8-Br-cAMP (0-300μM) in DMEM containing 1mg/mL BSA. After incubation, cells were washed gently with 1X PBS before lysis.

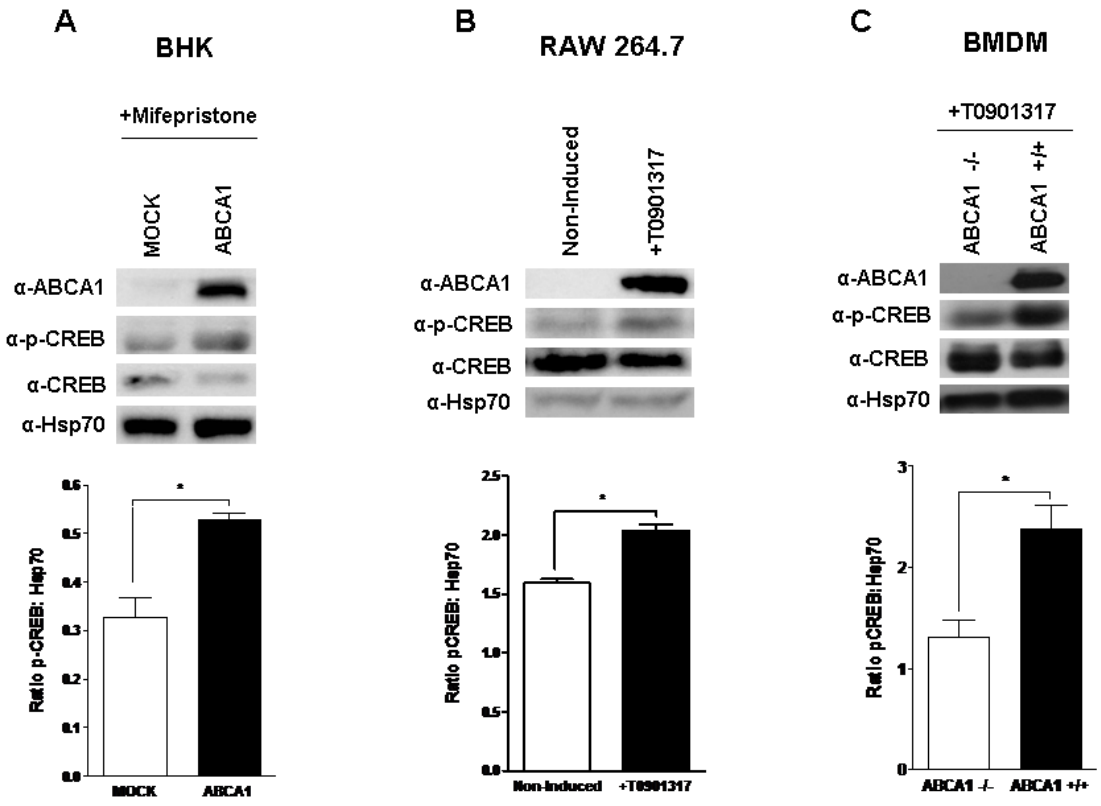
Statistical Analyses – Data for Western blot analyses and ELISA experiments are presented as the mean +/- the standard error of the mean (SEM) or standard deviation (SD) as indicated. For quantification of immunoblots, relative unit values were measured using ImageJ software. Cholesterol efflux data was presented as the mean +/- SD. Statistical comparisons and significance was determined using unpaired t-tests or analysis of variance (ANOVA), followed by post-hoc tests using GraphPad InStat v3.05. In all cases, $p<0.05$ was considered significant.

Chapter III: Results

3.1 - Characterization of CREB activation in the context of ABCA1 induction

We first set out to determine whether the induction of ABCA1 expression has an effect on CREB activation. CREB activation was analyzed by the level of its phosphorylation, relative to the total CREB levels, in three cell models. First, BHK-ABCA1 and -Mock cells were incubated with 5 nM mifepristone overnight (~18h). Under such condition, only BHK-ABCA1 cells express ABCA1 as BHK-Mock cells do not contain the ABCA1 insert. As shown in **Figure 4 A**, with ABCA1 expression, there is a significant increase in CREB phosphorylation in BHK-ABCA1 cells compared to MOCK cells ($p<0.05$). Secondly, RAW 264.7 mouse macrophages were treated with the LXR-agonist T0901317 overnight to induce ABCA1 expression We observed an increase in CREB phosphorylation in T0901317-treated RAW macrophages compared to non-treated RAW macrophages ($p<0.05$) (**Figure 4 B**). Lastly, to further confirm the causal relationship between ABCA1 expression and CREB phosphorylation, we treated wt and $ABCA1^{-/-}$ BMDMs with T0901317 overnight. As expected, T0901317 induced ABCA1 expression in wt BMDMs but not in $ABCA1^{-/-}$ BMDMs. We again observed significantly higher levels of CREB phosphorylation in BMDMs expressing ABCA1 than in $ABCA1^{-/-}$ BMDMs **(Figure 4 C)** ($p<0.05$). Suprisingly, the total CREB level signal in all the blots appear to be decreased with increased CREB phosphorylation. Nevertheless, the above observations support the notion that ABCA1 expression activates CREB as evidenced by elevated CREB phosphorylation.

Figure 4. **ABCA1 induction and CREB phosphorylation.** Immunoblots of phospho-CREB (p-CREB) levels in ABCA1-expressing and non-expressing cells. **A)** Baby hamster kidney (BHK) cells with no ABCA1 expression (MOCK) and with ABCA1 expression (ABCA1) were induced with 5nM mifepristone for 18h (n=3). **B)** RAW 264.7 macrophages non-induced and induced (+T0901317) for ABCA1 expression. ABCA1 expression was induced with 10uM T0901317 for 18h (n=3). **C)** Bone marrow-derived macrophages (BMDM) that are wildtype (ABCA1+/+) and ABCA1 knockout (ABCA1-/-) were induced with 10uM T0901317 overnight (~18h) for ABCA1 expression (n=4). ABCA1, phospho-CREB (p-CREB), and total CREB levels were probed. Hsp70 was used as a loading control. 30µg of protein was loaded per well. Graphs below immunoblots show quantification of phospho-CREB to Hsp70 levels from immunoblots. Cell lysates are from separately cultured plates of BHK, RAW, or BMDM. Data represent the means ±SEM. Statistical significance was assessed by a t-test, *= $p<0.05$.


A
BHK
+Mifepristone
MOCK
ABCA1
α-ABCA1
α-p-CREB
α-CREB
α-Hsp70
Ratio p-CREB: Hsp70
MOCK
ABCA1
B
RAW 264.7
Non-Induced
+T0901317
α-ABCA1
α-p-CREB
α-CREB
α-Hsp70
Ratio pCREB: Hsp70
Non-Induced
+T0901317
C
BMDM
+T0901317
ABCA1 -/-
ABCA1 +/+
α-ABCA1
α-p-CREB
α-CREB
α-Hsp70
Ratio pCREB:Hsp70
ABCA1 -/-
ABCA1 +/+

Furthermore, we transiently knocked down ABCA1 expression in RAW macrophages. Transient transfection of RAW macrophages with abca1-shRNA resulted in a significant knockdown of ABCA1 (~74%) (**Figure 5 A**). Concomitantly, there is approximately a 65% decrease in apoA-I dependent cholesterol efflux compared to control transfected RAW macrophages ($p<0.001$) (**Figure 5 B**), consistent with effective ABCA1 knockdown. We then analyzed CREB activity in these knockdown cells. In agreement with our results described above, we observed a decrease in phosphorylated CREB levels in ABCA1 knockdown RAW macrophages (**Figure 5 A**).

To explore the upstream kinases that may be responsible for the phosphorylation of CREB in the context of ABCA1 expression, we asked whether steady state activity of PKA, a major kinase for CREB activation, is higher in ABCA1-expressing cells. As the inhibition of PKA abolishes ABCA1 function, such as in cholesterol efflux, PKA may be a key player in increased CREB phosphorylation with the induction of ABCA1 expression. We, therefore, analyzed steady state PKA activity in RAW macrophages. This was achieved by using a phospho-PKA substrate antibody that recognizes phosphorylated PKA consensus sequences (RRXS/T). Higher levels of phospho-PKA substrate thus reflect higher overall PKA activity. We indeed observed an elevated level of PKA-phosphorylated protiens when treated with T0901317 (**Figure 6 A**). This finding was confirmed in BMDMs by another member in our laboratory: higher levels of PKA-phosphorylated proteins in wt BMDMs, relative to ABCA1-/- BMDMs (**Figure 6 B**). Both cell types were incubated with T0901317 overnight, which induced ABCA1 expression only in wt BMDM as expected.

Figure 5. ABCA1 knockdown in RAW 264.7 macrophages. RAW mouse macrophages were transiently transfected with vectors containing either ABCA1 shRNA or a control pUB-GFP vector. **A)** An immunoblot of transiently transfected cells induced with 50uM 8-Br-cAMP for ABCA1 expression. Within 72h post-transfection, there was approximately 74% knockdown of ABCA1 protein with the abca1 shRNA vector. 30µg of protein was loaded per well (n=1). **B)** Cholesterol efflux of transiently transfected RAW macrophages (n=3 per condition) with or without 5 µg/mL apoA-I for 2h. Both shRNA and control-transfected macrophages were induced with 50µM 8-Br-cAMP 18 hours prior to efflux. Data represent the means ±S.D. (assayed in triplicate). Statistical significance was assessed by ANOVA followed by post-hoc Tukey-Kramer multiple comparison test, *** = $p<0.001$.

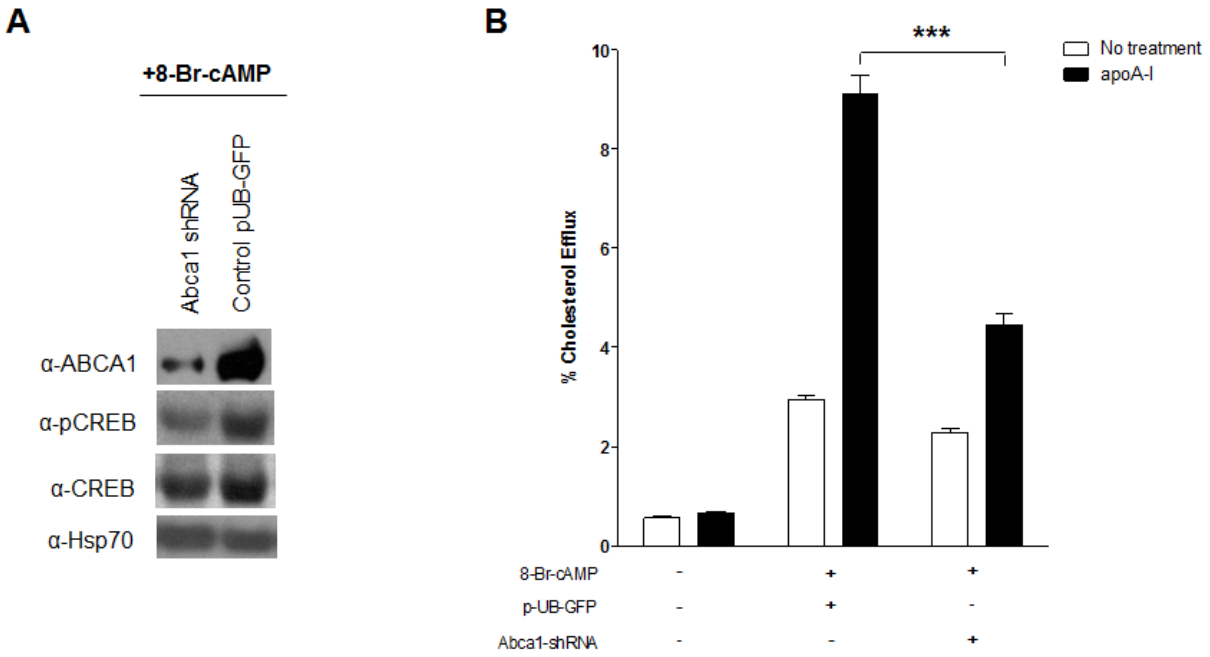
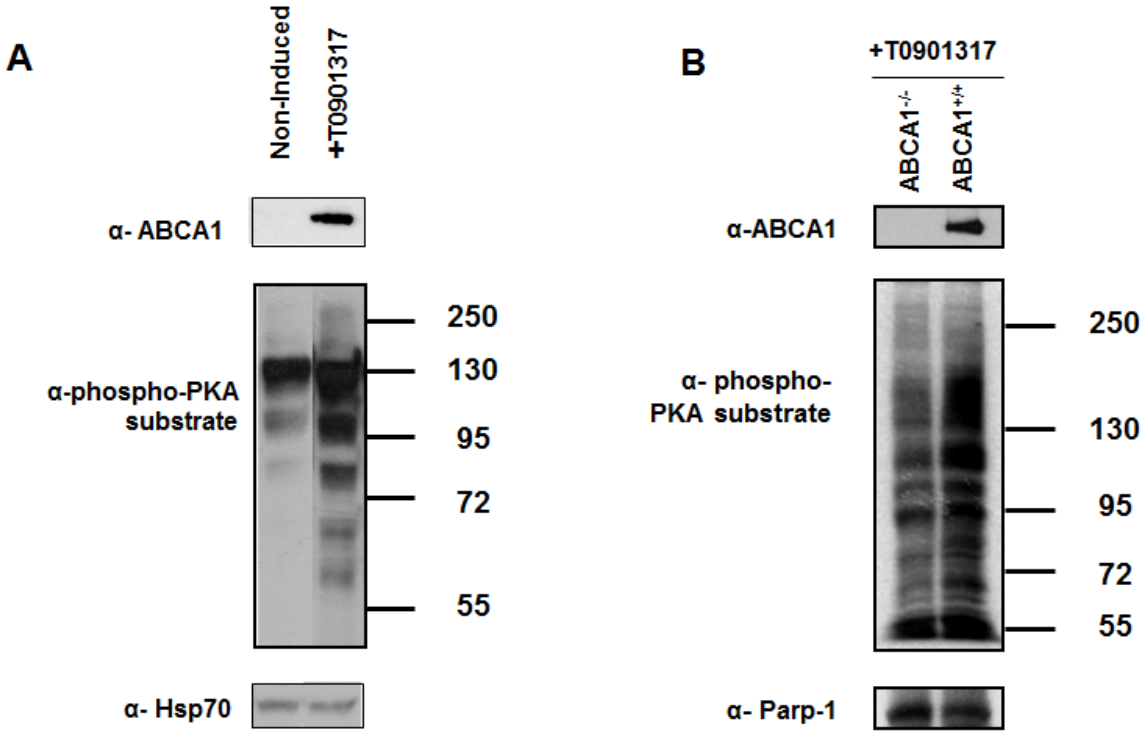

A
+8-Br-cAMP
Abca1 shRNA
Control pUB-GFP
α-ABCA1
α-pCREB
α-CREB
α-Hsp70
B
***
No treatment
apoA-I
% Cholesterol Efflux
10
8
6
4
2
0
8-Br-cAMP - + +
p-UB-GFP - + -
Abca1-shRNA - - +

Figure 6. Phospho-PKA substrate levels in RAW cells. A) An immunoblot of phospho-PKA substrate levels in RAW 264.7 macrophages not induced for ABCA1 expression (Non-Induced) and induced with 10uM T0901317 (+T0901317) for ABCA1 expression. ABCA1 expression was induced 18 hours prior to cell lysis. Hsp70 was used as a loading control. 30µg of protein was loaded per well on a 10% polyacrylamide gel (n=1). **B**) An immunoblot of phospho-PKA substrate levels in abca1 knock-out (ABCA1-/-) and wt (ABCA1+/+) BMDMs.Both cell phenotypes were induced with 10uM T0901317 for 18h. Parp-1 was used as a loading control. 35ug protein was loaded per well on a 6% polyacrylamide gel (n=1). Molecular weight markers on the right are in kilodaltons (55-250kDa). Results from *Dr. Fumin Dong*.

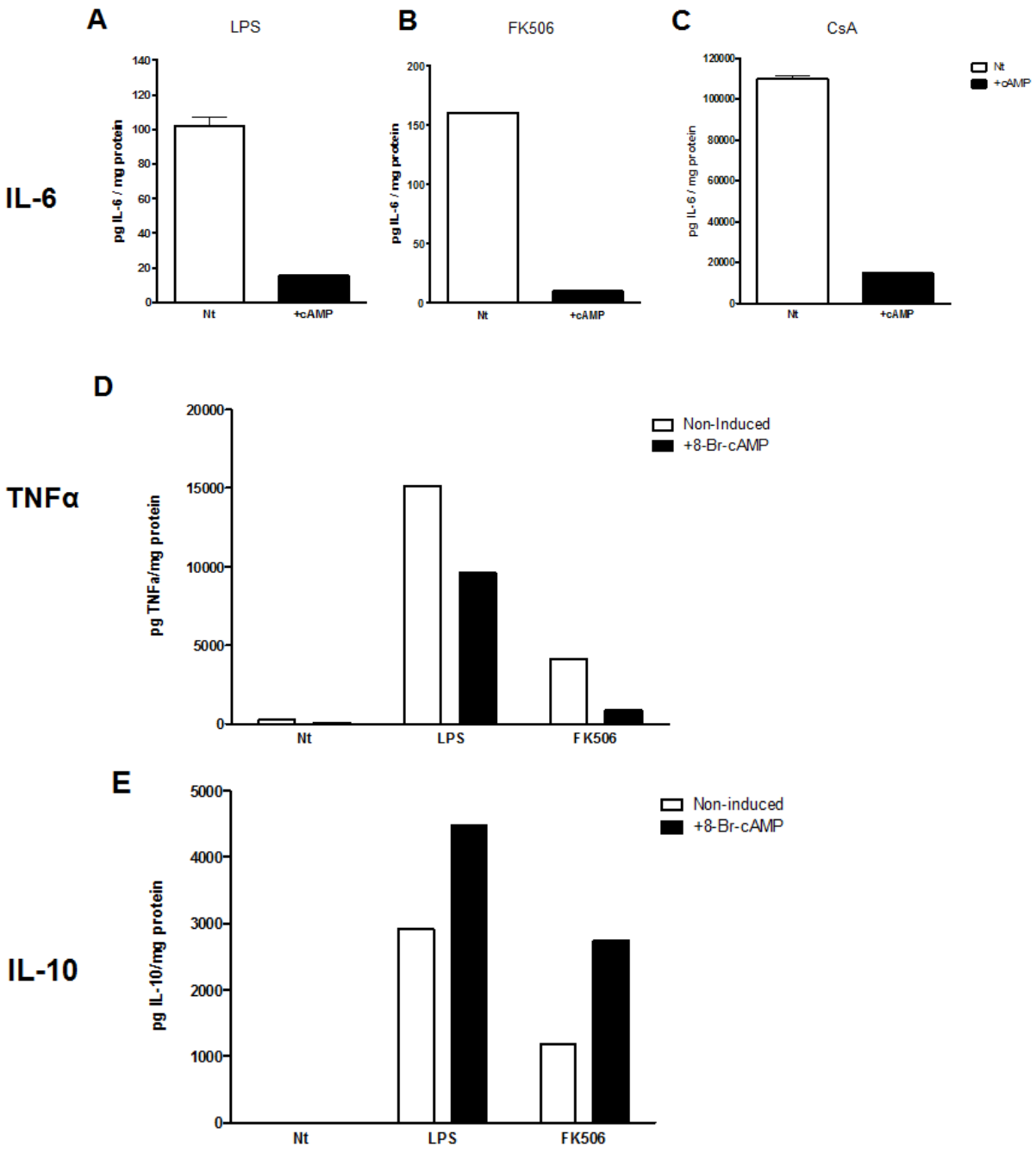

A
Non-Induced
+T0901317
α- ABCA1
α-phospho-PKA substrate
250
130
95
72
55
α- Hsp70
B
+T0901317
ABCA1-/-
ABCA1+/+
α-ABCA1
α- phospho-PKA substrate
250
130
95
72
55
α- Parp-1

3.2 - The effect of cAMP and LXR-agonist on the inflammatory response

We next studied the effect of inducing ABCA1 expression on cytokine secretion using an enzyme-linked immunosorbent assay (ELISA). In **Figure 7 A**, upon LPS stimulation, RAW macrophages releases large amounts of proinflammatory cytokine IL-6. However, in RAW macrophages treated with 50μM 8-Br-cAMP for 18 h, where ABCA1 expression is also strongly induced (**Figure 6 A**), LPS treatment resulted in decreased IL-6 secretion. Stimulation of the TLR4 pathway in RAW macrophages with other compounds, such as FK506 or CsA, also results in IL-6 secretion and the induction of ABCA1 with 8-Br-cAMP similarly reduced IL-6 release (**Figure 7 B & C**). An identical pattern in response to LPS was also observed in the secretion of another pro-inflammatory cytokine, TNF-α (**Figure 7 D**). To ensure that the effect of cAMP treatment on cytokine secretion is not an overall suppression of TLR4 signaling, we also analyzed IL-10, an anti-inflammatory cytokine. IL-10 release is also induced by TLR4 ligands but predominantly plays a modulatory function in inflammation (Medzhitov and Horng, 2009). We observed a trend of increased IL-10 secretion upon LPS challenge in RAW macrophages that have been incubated with 8-Br-cAMP (**Figure 7 E**).

Although cAMP can strongly induce ABCA1 expression in murine macropahges, it can also impact cytokine secretion independent of ABCA1 (Wall et al., 2009). To address the role of ABCA1 independent of cAMP effect, we used an LXR agonist to induce ABCA1 expression. As shown in **Figure 8 A**, ABCA1 expression was effectively induced by an LXR agonist, T0901317, in RAW macrophages. We once again observed less TNF-α secretion (**Figure 8 B**) and more IL-10 release (**Figure 8 C**) from T0901317-treated cells relative to untreated control. Lastly, to further delineate the role of ABCA1, we treated wt and ABCA1-/- BMDMs with T0901317 and analyzed LPS-stimulated TNF-α release. ABCA1 expression

Figure 7. Cytokine Secretion from cAMP-induced RAW cells. Secretion of cytokines from non-induced (Nt) or 8-Br-cAMP-induced RAW macrophages (+cAMP). 50 μM 8-Br-cAMP was used to induce ABCA1 expression 18h prior to TLR4 pathway-stimulation. Cells were treated with 100ng/mL LPS, 25uM FK506, or 10uM cyclosporin A (CsA) for 4h to activate TLR4 signaling. IL-6 levels from RAW cells treated with **A)** 100ng/mL LPS, **B)** 25uM FK506 or **C)** 10uM CsA were detected with enzyme-linked immunosorbent assay (ELISA). Data represent the means ±S.D. (assayed in duplicate) from one experiment (n=1). ELISA of **D)** TNF-α and **E)** IL-10 secreted from RAW cells treated with 100ng/mL LPS or 25uM FK506 for 4h. TNF-α and IL-10 ELISAs were performed once with only one sample per condition (n=1).


IL-6
A
LPS
pg IL-6 / mg protein
140
120
100
80
60
40
20
0
Nt
+cAMP
B
FK506
pg IL-6 / mg protein
200
150
100
50
0
Nt
+cAMP
C
CsA
pg IL-6 / mg protein
120000
100000
80000
60000
40000
20000
0
Nt
+cAMP
Nt
+cAMP
TNFα
D
pg TNFa/mg protein
20000
15000
10000
5000
0
Nt
LPS
FK506
Non-Induced
+8-Br-cAMP
IL-10
E
pg IL-10/mg protein
5000
4000
3000
2000
1000
0
Nt
LPS
FK506
Non-induced
+8-Br-cAMP

is efficiently induced in wt BMDM by T0901317 (**Figure 6 B**). Once again, we observed less TNF-α secretion from ABCA1-expressing wt BMDMs, compared to ABCA1-/- BMDMs, in response to LPS (**Figure 8 D**). We also noticed that, in the absence of T0901317 (non-treated or NT), wt BMDMs also released less TNF-α than ABCA1-/- BMDM.

Another member in our laboratory has performed a more comprehensive study on cytokines secreted from mouse BMDMs. It was observed with the knock-out of ABCA1 in mouse BMDMs there was an enhancement of pro-inflammatory cytokine IL-6, IL-12p40, TNF-α, and GCSF secretion upon LPS challenge (**Figure 9**). Similar with the observations in RAW macrophages, there appeared to be a decrease in the secretion of anti-inflammatory cytokine IL-10 from ABCA1-/- BMDMs.

Figure 8. Cytokine secretion from T0901317-induced RAW cells and BMDMs. A) Immunoblot of ABCA1 levels in RAW 264.7 macrophages non-induced (Nt), induced with 50μM 8-Br-cAMP (cAMP) or 10 μM T0901317 (T09) for 18h. 30μg of protein was loaded per well. **B-D)** ELISA of cytokines secreted from RAW 264.7 macrophages that are non-treated or treated with 10μM T0901317 for induction of ABCA1 expression. Cells were induced for ABCA1 expression with 10uM T0901317 18 hours prior to LPS-stimulation. **B)** Secretion of inflammatory TNFα from RAW cells treated with 100ng/mL LPS for 4h (n=2). Data represent the means ±SEM. **C)** Secretion of anti-inflammatory IL-10 from RAW cells treated with 100ng/mL LPS for 4h (n=2). TNFα and IL-10 levels were measured as fold difference to normalize values from two independent experiments carried out on separate days. Data represent the means ±SEM. **D)** TNF-α levels secreted from BMDMs that were either non-treated (NT) or treated with 10μM T0901317 (T09) (duplicates) 18h prior to the 4h incubation with 100ng/mL LPS. T0901317-treated (T09) BMDM data represent the means ±S.D (assayed in duplicates). Data is from one experiment (n=1).

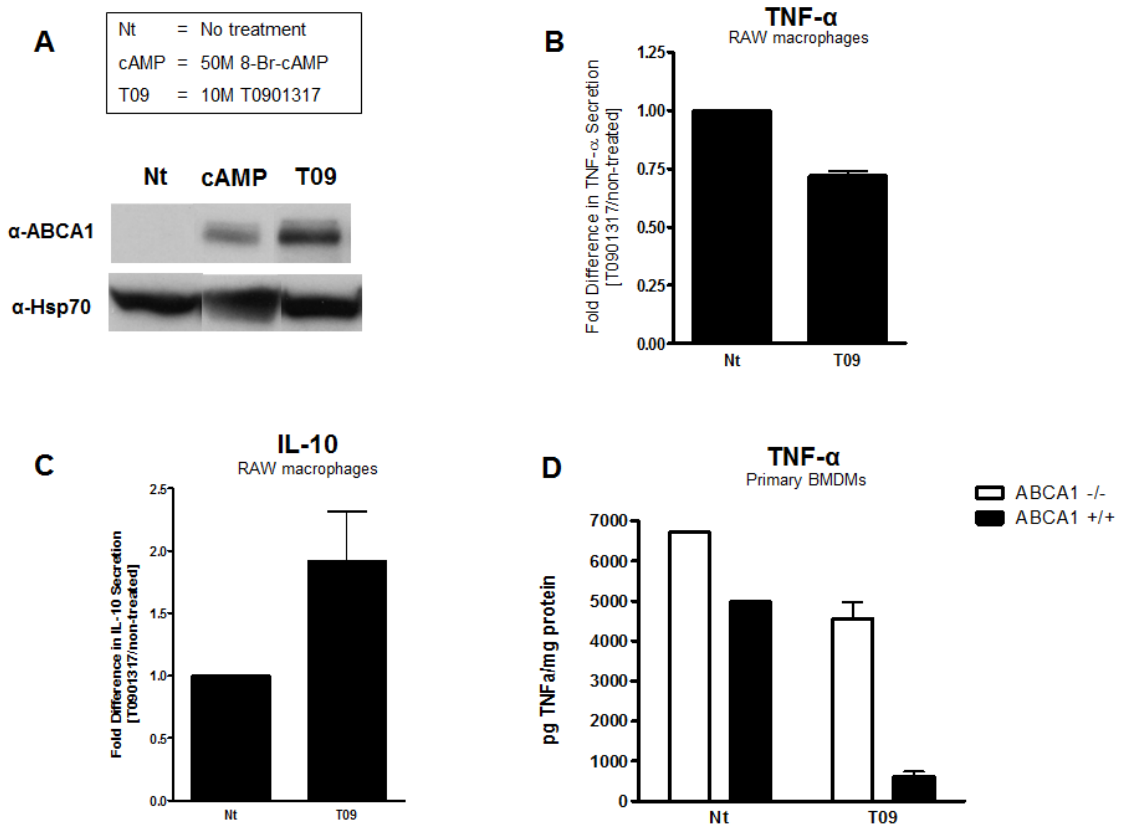
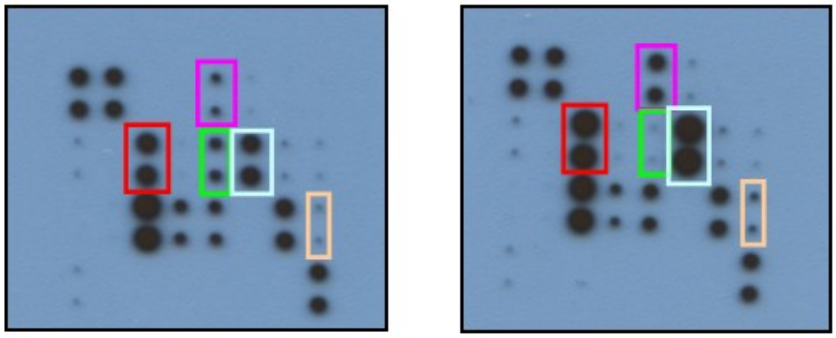

A
Nt = No treatment
cAMP = 50M 8-Br-cAMP
T09 = 10M T0901317
Nt
cAMP
T09
α-ABCA1
α-Hsp70
B
TNF-α
RAW macrophages
Fold Difference in TNF-α Secretion [T0901317/non-treated]
1.25
1.00
0.75
0.50
0.25
0.00
Nt
T09
C
IL-10
RAW macrophages
Fold Difference in IL-10 Secretion [T0901317/non-treated]
2.5
2.0
1.5
1.0
0.5
0.0
Nt
T09
D
TNF-α
Primary BMDMs
ABCA1 -/-
ABCA1 +/+
pg TNFa/mg protein
7000
6000
5000
4000
3000
2000
1000
0
Nt
T09

Figure 9. Cytokine Secretion from wt and ABCA1 -/- BMDMs. A) A map of the cytokine array (Ray Bio ® Cytokine Antibody Array 1) in **B**) Cytokine Array of bone marrow-derived macrophages with ABCA1 expression (ABCA1+/+) and ABCA1 knockout (ABCA1-/-). Macrophages were induced with ac-LDL for ABCA1 expression and then treated with 100ng/mL LPS. Cytokines IL-6 and IL-12p40/p70 are upregulated in ABCA1-/- macrophages, whereas IL-10 and GCSF are downregulated. **C**) Quantification of the cytokine array, showing cytokines that are significantly affected by the loss of ABCA1. Data represent the means ±SEM. (n=2). Results obtained from *Loretta Ma*.

A

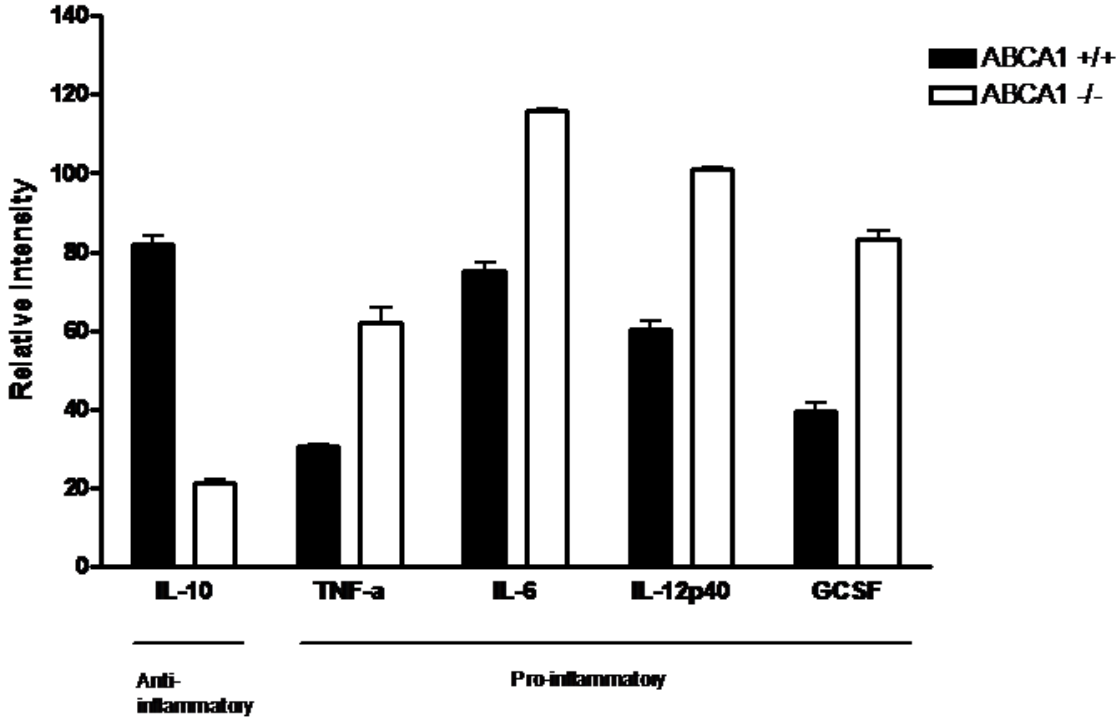
	A	B	C	D	E	F	G	H
1	Pos	Pos	Neg	Neg	GCSF	GM-CSF	IL-2	IL-3
2	Pos	Pos	Neg	Neg	GCSF	GM-CSF	IL-2	IL-3
3	IL-4	IL-5	IL-6	IL-9	IL-10	IL-12 p40p70	IL-12p70	IL-13
4	IL-4	IL-5	IL-6	IL-9	IL-10	IL-12 p40p70	IL-12p70	IL-13
5	IL-17	IFN-γ	MCP-1	MCP-5	RANTES	SCF	sTNFRI	TNF-α
6	IL-17	IFN-γ	MCP-1	MCP-5	RANTES	SCF	sTNFRI	TNF-α
7	Thrombopoietin	VEGF	Blank	Blank	Blank	Blank	Blank	Pos
8	Thrombopoietin	VEGF	Blank	Blank	Blank	Blank	Blank	Pos

B



ABCA1$^{+/+}$ ABCA1$^{-/-}$

C

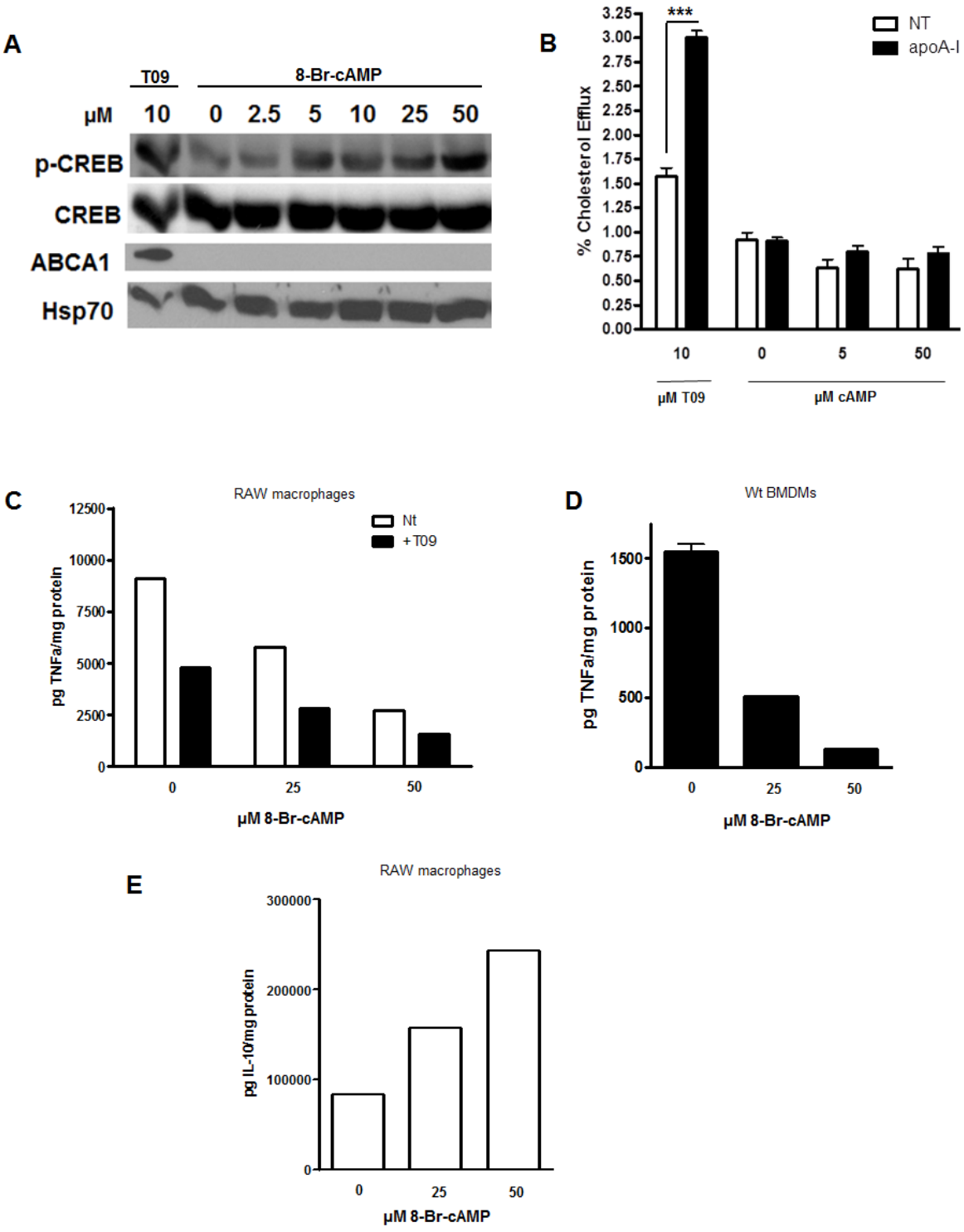


3.3 - The effect of PKA activation on the inflammatory response

We have observed so far that induction of ABCA1 expression results in (1) higher CREB phosphorylation, potentially due to PKA activation, (**Figures 4 A & C, Figure 5 A and Figure 6 B**), and (2) modulated inflammatory responses (**Figure 8 D and Figure 9**). It is plausible that increased CREB activity may cause a more anti-inflammatory profile in ABCA1-expressing cells. We thus considered increasing CREB phosphorylation with cAMP, in the absence of ABCA1 expression, to phosphorylation levels similar to those observed in ABCA1-expressing cells. From this, we could then compare the effects of CREB activation to ABCA1 expression on cytokine secretion. For this, we used 8-Br-cAMP to acutely activate PKA in RAW macrophages. Cells were incubated with increasing concentrations of 8-Br-cAMP for 4 h. As expected, 8-Br-cAMP increased phospho-CREB levels in a dose dependent manner (**Figure 10 A**). Although cAMP can also induce murine ABCA1 expression, we could not detect ABCA1 expression at low doses of cAMP (≤ 50μM) within 4h. This was further verified using a cholesterol efflux assay. ABCA1 activity, as assessed by an increase in apoA-I-dependent cholesterol efflux, was not evident at cAMP concentrations of ≤ 50μM (**Figure 10 B**). Thus, we limited our experimental condition to a cAMP range of 25 – 50μM to study the ABCA1-independent effects of cAMP on cytokine secretion.

RAW macrophages were then treated with various concentrations of cAMP and stimulated with LPS. We indeed observed a dose-dependent suppression of TNF-α secretion by cAMP (**Figure 10 C**), regardless of LXR-activated ABCA1 expression. Interestingly, treating macrophages that do not express ABCA1 with 25-50μM 8-Br-cAMP (**Figure 10 A:** lanes 25 and 50) produced levels of CREB phosphorylation similar to those expressing

Figure 10. **cAMP dose effect on CREB phosphorylation and cytokine secretion. A)** Immunoblot of RAW 264.7 mouse macrophages incubated with increasing concentrations 8-Br-cAMP for 4h. ABCA1 expression was induced in some cells with 10uM LXR agonist T0901317 (T09). **B)** Cholesterol efflux of RAW macrophages treated with increasing concentrations of 8-Br-cAMP (0-50uM). Cells were treated with or without 5 μg/mL apoA-I for 4h. ABCA1 expression was induced in some wells with 10uM T0901317 for 18h prior to efflux. Data represent the means ±S.D. (assayed in triplicate), representative of four independent experiments. Statistical significance was assessed by a one-way ANOVA followed by a post-hoc Tukey-Kramer multiple comparison test, *** = $p<0.001$. **C)** 8-Br-cAMP effect on TNFα secretion into media from RAW macrophages non-induced (Nt) and induced for ABCA1 expression (+T09). Cells were treated with 100ng/mL LPS for 4h (n=1 per condition). ABCA1 expression was induced with 10uM T0901317 overnight (~18h). **D)** Wt mouse BMDMs treated with increasing concentrations of cAMP (25uM and 50uM), and the effect on TNFα secretion with 100ng/mL LPS. Data represent the means ±S.D. (n=1, assayed in duplicate). **E)** The effect of cAMP on levels of IL-10 secretion into media from non-induced RAW macrophages (n=1).

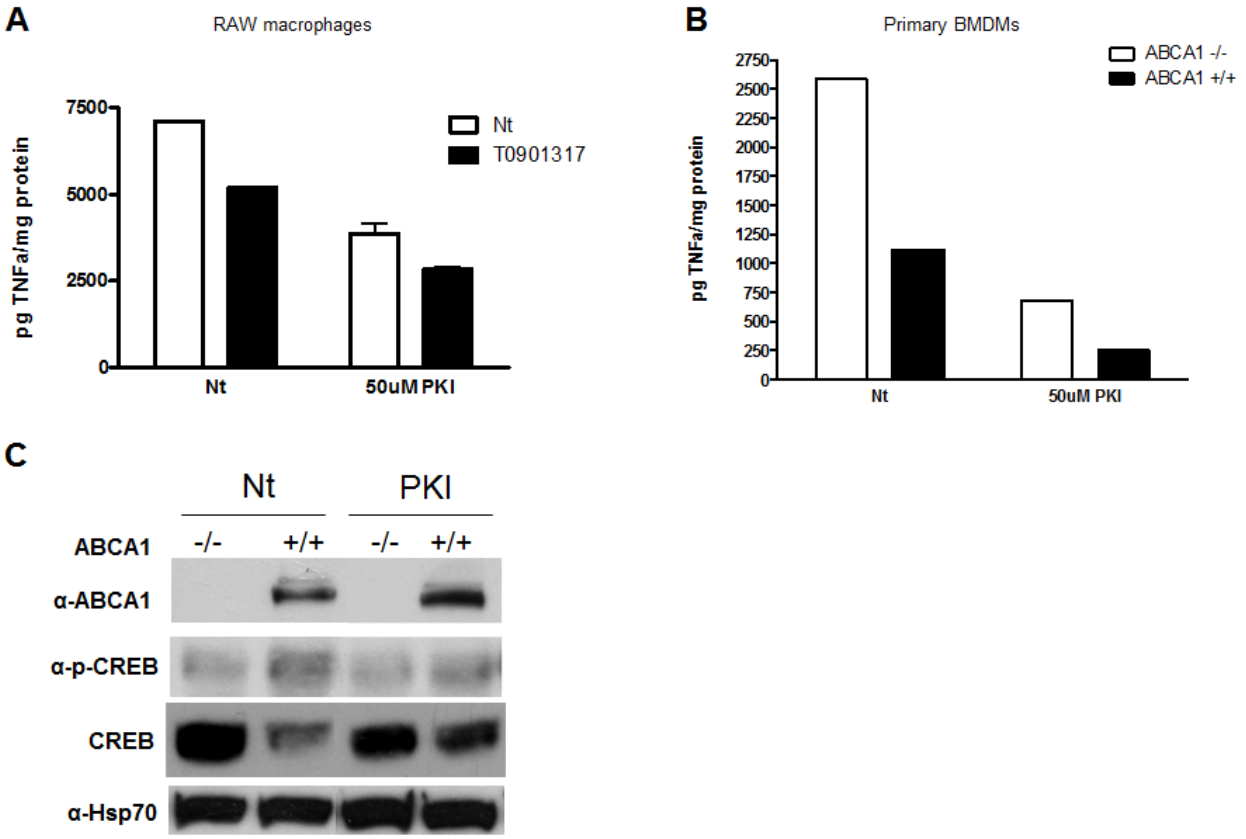

A
T09
8-Br-cAMP
μM
10
0
2.5
5
10
25
50
p-CREB
CREB
ABCA1
Hsp70
B
***
NT
apoA-I
% Cholesterol Efflux
3.25
3.00
2.75
2.50
2.25
2.00
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
10
0
5
50
μM T09
μM cAMP
C
RAW macrophages
Nt
+T09
pg TNFa/mg protein
12500
10000
7500
5000
2500
0
0
25
50
μM 8-Br-cAMP
D
Wt BMDMs
pg TNFa/mg protein
1500
1000
500
0
0
25
50
μM 8-Br-cAMP
E
RAW macrophages
pg IL-10/mg protein
300000
200000
100000
0
0
25
50
μM 8-Br-cAMP

ABCA1 (**Figure 10 A**: lane T). These RAW macrophages also released similar levels of TNF-α cytokine as T0901317-treated RAW macrophages (**Figure 10 C**).

Similarly, treatment with 8-Br-cAMP leads to a dose-dependent decrease in TNF-α secretion in wt BMDMs (**Figure 10 D**). We found that IL-10 release is increased with the incremental addition of 8-Br-cAMP in RAW macrophages (**Figure 10 E**). This is consistent with our earlier observation (**Figure 9 C**) that decreased TNF-α secretion is accompanied by rise in IL-10 release.

As cAMP can activate CREB through PKA, we next looked at the effect of inhibiting PKA on cytokine secretion. Since PKA activation by cAMP in RAW macrophages lead to a decrease in TNF-α, we anticipate that inhibition of PKA will led to an increase in TNF-α secretion. However, treatment with 50μM PKI lead to the suppression of pro-inflammatory TNF-α secretion in RAW macrophages (**Figure 11 A**) and BMDMs (**Figure 11 B**), regardless of ABCA1 expression. These observations suggest that PKA may have much broader functions, particularly in TLR4 signaling. PKI may blunt the initial events proximal to LPS and TLR4 ligation, leading an overall supression. Interestingly, we found that 4 hour treatment of PKI seemed to decrease phosphorylated CREB levels in ABCA1-expressing cells, although the data is too preliminary to draw any firm conclusions (**Figure 11 C**).

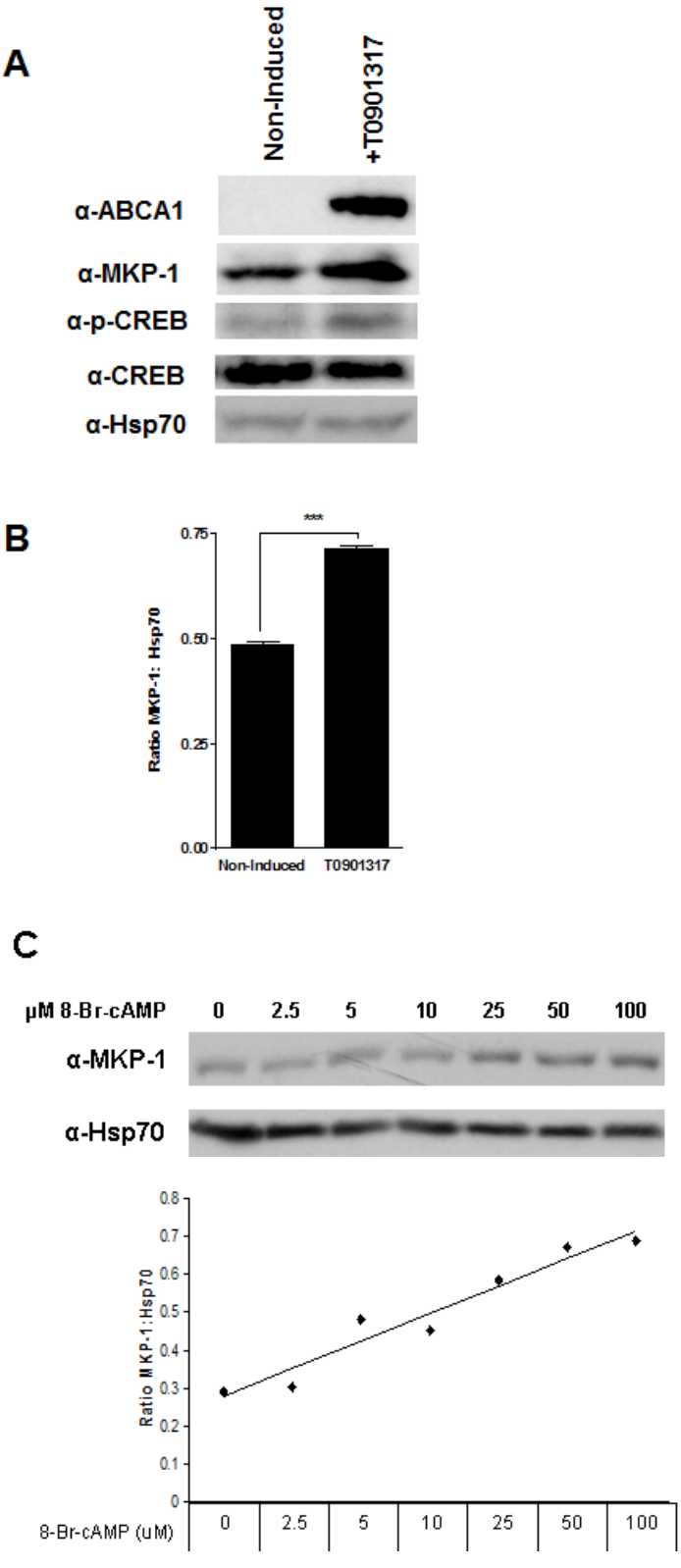
Figure 11 . The effect of PKI on cytokine secretion and CREB phosphorylation. **A)** TNF-α levels secreted from non-induced (Nt) and T0901317-induced RAW macrophages treated with 100ng/mL LPS and 50μM protein kinase A inhibitor (PKI). Cells were pre-treated with PKI for 30min prior to addition of LPS. PKI-treated data represent the means ±S.D. (n=1, assayed in duplicate). **B)** TNF-α levels secreted from wt (ABCA1 +/+) and abca1-knockout (ABCA1 -/-) BMDMs induced with 10μM T0901317 for 18h prior to 100ng/mL LPS and 50 μM PKI treatment. Cells were pre-treated with PKI for 30min prior to addition of LPS (n=1 per condition). **C)** Immunoblot of BMDM cell lysates induced with 10uM T0901317 that were either not-treated (Nt) or 25μM PKI-treated. 30μg of protein was loaded per well and hsp70 was used as a loading control.


A
RAW macrophages
pg TNFa/mg protein
7500
5000
2500
0
Nt
T0901317
Nt
50uM PKI
B
Primary BMDMs
ABCA1 -/-
ABCA1 +/+
pg TNFa/mg protein
2750
2500
2250
2000
1750
1500
1250
1000
750
500
250
0
Nt
50uM PKI
C
Nt
PKI
ABCA1
-/-
+/+
-/-
+/+
α-ABCA1
α-p-CREB
CREB
α-Hsp70

3.4 - The effect of LXR agonist on downstream targets of CREB

To provide insight into the mechanism linking ABCA1 to immune regulation, we next looked for proteins that are involved in the immune modulation and are regulated by CREB. Interestingly, among 11 MKPs, only MKP-1 is transcriptionally regulated by the CREB pathway (Sommer et al., 2000). We therefore wondered whether increased phospho-CREB levels in ABCA1 expressing cells would lead to higher levels of MKP-1, which could in part contribute to inflammation modulation by ABCA1. Indeed, we observed a significant increase in MKP-1 levels ($p<0.0001$) in RAW macrophages when induced with LXR agonist T0901317 (**Figure 12 A & B**). Similarly, with the incremental addition of 8-Br-cAMP, we detected a trend of increased MKP-1 levels (**Figure 12 C**).

Figure 12. MKP-1 levels increase with ABCA1 induction and cAMP treatment. A) Immunoblot of RAW 264.7 macrophage lysates. ABCA1 was induced with 10uM T0901317 overnight (~18h). Same immunoblot was probed for MKP-1 and p-CREB, stripped and re-probed for total CREB levels. Hsp70 was used as a loading control. **B)** Quantification of MKP-1 to hsp70 levels in the immunoblots (n=3). Data represent the means ±SEM *** = $p<0.0001$. **C)** An immunoblot of RAW macrophages treated with increasing concentrations of cAMP (0-100μM). 30ug protein was loaded per well. Graph below quantifies the ratio of MKP-1 to Hsp70 in the immunoblot from a single experiment and line of best fit indicates the general trend with increasing concentration of 8-Br-cAMP (n=1)**.**


A
Non-Induced
+T0901317
α-ABCA1
α-MKP-1
α-p-CREB
α-CREB
α-Hsp70
B
***
Ratio MKP-1: Hsp70
0.75
0.50
0.25
0.00
Non-Induced
T0901317
C
μM 8-Br-cAMP
0
2.5
5
10
25
50
100
α-MKP-1
α-Hsp70
Ratio MKP-1:Hsp70
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
8-Br-cAMP (uM)
0
2.5
5
10
25
50
100

Chapter IV: Discussion

4.1 - Increased CREB phosphorylation and PKA activation upon ABCA1 expression

CREB phosphorylation can be an indicator of PKA activity, as this transcription factor is readily phosphorylated by PKA catalytic subunits (Gonzalez and Montminy, 1989). We have shown in BHK and primary BMDMs that ABCA1 expression is accompanied by a significant increase in CREB phosphorylation (**Figure 4 A & C**). To further support this trend, the knockdown of ABCA1 with transient transfections of abca1-shRNA vectors was carried out. The results revealed a decrease in phospho-CREB levels with the knockdown of ABCA1 (**Figure 5 A**). Importantly, we found that the levels of PKA-phosphorylated proteins appear to be elevated in both ABCA1-expressing wt BMDMs and RAW macrophages, compared to ABCA1-/- BMDMs and non-induced RAW (**Figure 6 B**). This implies that elevated phospho-CREB levels could be the consequence of higher PKA activity in ABCA1-expressing cells. Certainly, the phosphorylation of CREB can be carried out by other kinases, such as MAPKs and CaMKs. However, since PKA is a major player in CREB phosphorylation (Ichiki, 2006) and since there is apparent higher steady state PKA activity in ABCA1-expressing cells, it is plausible that the increase in phospho-CREB levels is a result of an increase in PKA activation. Thus, our results presented here present a novel link between ABCA1 and CREB, potentially through PKA.

For the analysis of phosphorylated CREB levels, we also probed for CREB protein levels in parallel to determine whether the increase in CREB phosphorylation was due to an overall increase in total protein levels of CREB. Total CREB levels appear to be lower in BHK cells and BMDMs when phosphorylated CREB levels are high (i.e. whenABCA1 is expressed). This is likely due to the experimental procedure we used and antibody

specificity. According to manufacturer instructions, we had to first probe for phospho-CREB levels and then for total CREB levels. Because these two antibodies recognize a shared region on CREB, probing with one antibody will interfere with the probing of the other, even with immunoblot stripping. Neverthless, this technical limitation should not impact our data interpretation: total CREB will be under-estimated only when phospho-CREB is high. It may, on the contrary, actually improve detection sensitivity, since it amplifies the changes in the ratio of phospho-CREB/total CREB.

4.2 - LXR activation, cAMP treatment and ABCA1 expression correlate with an anti-inflammatory profile

A link between ABCA1 and inflammation has been previously demonstrated (Yvan-Charvet et al., 2008; Zhu et al., 2008), yet the pathways that form this link are not fully characterized. Thus, to study the pathways governing ABCA1 and inflammation we attempted to establish the link between factors that induce ABCA1 expression and also modulate inflammation.

We first show that factors that increase ABCA1 expression also lead to a more inflammation modulation phenotype, namely an increase in anti-inflammatory cytokine secretion and a decrease in pro-inflammatory cytokine release. As we have shown, treatment of RAW macrophages with cAMP or T0901317 increase ABCA1 protein levels (**Figure 6 A**). cAMP, through CREB, specifically activates the CRE site on the promoter region of murine ABCA1 to activate its expression (Zarubica et al., 2007). The LXR agonist T0901317 acts in a similar fashion to oxysterols and other natural agonists to bind LXR. This activates LXR and induces the formation of LXR/RXR heterodimers. LXR/RXR heterodimers then bind to DR4 DNA response elements to initiate ABCA1 expression

(Fitzgerald et al., 2002a). Both of these treatments lead to not only increased ABCA1 expression, but also to a more anti-inflammatory profile in response to LPS, as we have seen with increased secretion of anti-inflammatory IL-10 and decreased secretion of pro-inflammatory cytokines IL-6 and TNF-α (**Figure 7** and **Figure 8 B & C**). These observations suggest that factors that increase the expression of ABCA1 are associated with a more anti-inflammatory profile, upon LPS challenge, in RAW macrophages.

In addition to inducing ABCA1 expression, both cAMP and T0901317 under our experimental conditions may have effects on cytokine secretion, independent of ABCA1. Indeed, one study has found that LPS-stimulated expression and secretion of cytokines in RAW macrophages is significantly affected by cAMP via acute PGE_2 or 8-Br-cAMP treatment (Wall et al., 2009). Acute treatment with cAMP does not significantly induce ABCA1. Interestingly, this study has established that the anti-inflammatory effects of cAMP are specific to PKA activation and not the alternate EPAC signaling pathway as only the PKA-selective cAMP analog, 6Bz-cAMP, resulted in similar anti-inflammatory effects as 8-br-cAMP on cytokine secretion.

LXRs were first identified as regulators of genes involved in lipid metabolism. However, these nuclear receptors are now widely known to also regulate genes involved in the inflammatory response (Calkin and Tontonoz, 2010). The activation of LXR by LXR agonists also provides anti-inflammatory effects through the effective inhibition of pro-inflammatory IL-6, COX-2, IκBα and IL-1β expression (Joseph et al., 2003). Consistent with this notion, we observed that the LXR agonist T0901317 caused a small but significant suppression on TNF-α secretion in LPS-challenged ABCA1-/- BMDMs (**Figure 8 D**). Nevertheless, when identical T0901317 treatment was applied to ABCA1+/+ BMDMs, where ABCA1 is strongly expressed, there is a much more profound suppression of TNF-α

release. This clearly suggests a role for ABCA1 in cytokine secretion, independent of a relatively small modulating effect of T0901317.

Furthermore, another experiment from our laboratory has confirmed what has been observed in many other studies, that ABCA1 expression is correlated with an anti-inflammatory effect. This experiment, involving the screening of many LPS-stimulated cytokines, confirmed that the loss of ABCA1 leads to a great decrease in the secretion of anti-inflammatory cytokine IL-10 and an increase in secretion of pro-inflammatory cytokines IL-6, IL-12p40, TNF-α, and GCSF (**Figure 9**).

Taken together, our findings suggest that cAMP treatment, LXR activation, and ABCA1 expression all result in a more anti-inflammatory profile. Thus, there is a possibility of a common mechanism shared by ABCA1 expression, LXR activation and PKA activation on cytokine expression and secretion from murine macrophages.

4.3 - cAMP treatment, LXR activation, and ABCA1 expression correlate with increased CREB phosphorylation

With LXR treatment in RAW macrophages or induction of ABCA1 expression in BMDMs and BHK cells, we have consistently seen an increase in phospho-CREB levels (**Figure 4**), and also an increase in levels of PKA-phosphorylated proteins (**Figure 6**). These results suggest that ABCA1 expression and the factors that induce ABCA1 expression may result in increased CREB activation, potentially through PKA activation. As these factors have all shown a trend of inflammation modulation (**Figure 7 and Figure 8 B–D**), PKA/CREB may be the downstream effectors of ABCA1 that regulate cytokine secretion.

To further study the link between CREB activation and cytokine secretion, we first attempted to mimic the CREB phosphorylation found in ABCA1-expressing cells by using acute treatments of cAMP. We found that, in RAW 264.7 macrophages, acute 8-Br-cAMP treatment with concentrations of 25-50μM produced similar level of CREB phosphorylation as in ABCA1-expressing cells induced with 10μM T0901317 (**Figure 10 A**). With acute 8-Br-cAMP treatment, ABCA1 expression was not noticeable at concentrations of < 50μM, which was also confirmed by ABCA1 function assay, i.e. cholesterol efflux to apoA-I (**Figure 10 B**). Most interestingly, 8-Br-cAMP at concentrations of 25-50μM have similar effects on TNF-α suppression as T0901317-induced ABCA1-expressing cells (**Figure 10 C**). These results suggest that the levels of CREB phosphorylation seen in ABCA1-expressing cells are sufficient in modulating cytokine secretion.

4.4 - ABCA1-independent effects of cAMP and T0901317

Although we have seen significant increases in CREB activation with T0901317 and cAMP, we must contemplate the many effects these compounds have on the cell, independent of ABCA1. Firstly, cAMP is a major secondary messenger involved in many other signaling processes (Sadana and Dessauer, 2009). Thus, we would certainly expect many other ABCA1-independent effects on signaling pathways of the cell. This secondary messenger quickly and directly activates PKA, which in turn activates CREB (Tasken and Aandahl, 2004). This signaling process is much faster than the combined transcription and translation of ABCA1, so we expect major changes in the signaling pathways of the cell even before ABCA1 is upregulated. Thus we certainly cannot induce ABCA1 expression in RAW macrophages with cAMP to study the impact of ABCA1 on cytokine secretion.

cAMP has been shown to have ABCA1-independent effects on cytokine secretion (Wall et al., 2009). Both IL-6 and IL-10 have cAMP response elements in their promoter regions, and thus their expression is strongly affected by cAMP activation of CREB (Ichiki, 2006). Although the mechanism of how cAMP enhances the expression of IL-10 in the presence of LPS is not fully understood, it is clear that cAMP alone cannot enhance the secretion of IL-10 (**Figure 7 E** + data not shown). This leads us to speculate that other co-factors and enhancer proteins are necessary for the complete expression of these cytokines and that they are recruited with the stimulation of the inflammatory pathway, for example. by LPS. In LPS-stimulated peripheral human macrophages (lacking a CRE site in the ABCA1 gene) there is increased IL-10 promoter activation, expression, and secretion with increased intracellular cAMP levels (Platzer et al., 1995; Platzer et al., 1999). IL-10 downregulates the synthesis of pro-inflammatory cytokines TNF-α, IL-1, IL-6, IL-8, and GM-CSF (Howard et al., 1992). This indicates that independent of ABCA1, cAMP upregulates IL-10 expression

and this may be one of the factors that lead to the general anti-inflammatory effect observed in cAMP-treated RAW macrophages. Changes in TNF-α expression may also play a role in the cAMP-induced anti-inflammatory effect. Similar to IL-10, TNF-α also has a CRE site and its expression is affected by CREB (Kuprash et al., 1999), albeit through a more complex and not fully understood mechanism (Avni et al., 2010). In addition, increases in intracellular cAMP levels have been shown to result in decreased TNF-α expression in the human acute monocytic leukemia cell line THP-1 (Newell et al., 1994). In RAW macrophages, Wall et al have found that the effects of cAMP on LPS-induced cytokine secretion were dependent on PKA activation and its cellular localization to phosphorylate and prevent the degradation of p105, an IκB protein and an inhibitor of NF-κB activation (Wall et al., 2009). This group suggests that PKA phosphorylates p105 at an adjacent site to the IKK phosphorylation site, thereby inhibiting its degradation, and thus results in the suppression of NF-κB activation by LPS. Through the suppression of NF-κB activation and the activation of CREB, PKA inhibits pro-inflammatory cytokine expression and enhances anti-inflammatory IL-10 expression (see **Figure 2**). Thus, cAMP itself most likely has anti-inflammatory effects on downstream TLR4 signaling that are independent of ABCA1 expression, but dependent on PKA activation.

While treatment of macrophages with T0901317 or any other LXR-agonist may have less non-specific effects on cytokine secretion in comparison to treatment with cAMP, its effect is still not limited to the induction of ABCA1 expression. LXR agonists activate other genes involved in lipid metabolism, such as sterol-regulatory element binding protein (Repa et al., 2000), fatty acid synthase (Peet et al., 1998), cholesterol ester transfer protein (Luo and Tall, 2000), and other ABC sterol transporters (Joseph et al., 2003). All the genes activated by LXR agonists may have effects on CREB phosphorylation. Also, it has been shown that

LXR agonists not only increase expression of genes involved in lipid metabolism, but also increase the expression of genes involved in the inflammatory pathway. Pro-inflammatory IL-6, MIP-1β, G-CSF, and IL-1β genes have decreased expression in LPS-activated macrophages that have been treated with an LXR agonist (Joseph et al., 2003). Thus, similar to cAMP, using LXR-agonist T0901317 to induce ABCA1 expression has its own drawbacks when studying cytokine secretion.

4.5 - ABCA1 expression directly influences phosphorylated CREB levels and cytokine secretion

Thus, in order to characterize the impact of ABCA1 expression on PKA/CREB activation and on cytokine secretion, we transiently transfected RAW macrophages with abca1-shRNA or control vector and treated both sets of macrophages with 8-Br-cAMP. This lead to a 70% knockdown of ABCA1. Significantly, knockdown of ABCA1 significantly reduced steady state phosphorylated CREB levels: (**Figure 5 A**), implicating direct CREB activation by ABCA1.

An alternate method used to overcome the non-specific effects of cAMP and T0901317 is to use wt and ABCA1 deficient BMDMs. Unfortunately, we currently only have limited access to these primary cells. Nevertheless, in the primary BMDM tested, we observed a similar trend as in RAW macrophages that have underwent ABCA1 knockdown: wt BMDMs have elevated levels of phosphorylated CREB compared to ABCA1-/- BMDMs, whether treated with 8-Br-cAMP (**Figure 4 C**) or T0901317 (data not shown).

To address the effects of T0901317 on macrophage cytokine secretion, we compared LPS-stimulated cytokine secretion from ABCA1-/- BMDMs with or without overnight T0901317 incubation. In the absence of ABCA1 expression, i.e. in ABCA1-/- BMDMs,

there was a slight decrease in TNF-α secretion with T0901317 treatment, suggesting that T0901317 has general, albeit minor, anti-inflammatory effects (**Figure 8 D**). However, when we applied similar treatment to wt BMDMs, the decrease due to T0901317 is much more profound (**Figure 8 D**). Since the only difference between T0901317 treated ABCA1-/- and wt BMDMs is, presumably, ABCA1 expression, we concluded that ABCA1 is a major contributor to inflammation modulation function seen by T0901317 treatment in macrophages.

4.6 - PKA activation by 8-Br-cAMP and inhibition by PKI

In RAW macrophages, we were able to mimic a T0901317-activated phenotype simply through the addition of cAMP to activate PKA. This was evident through the increase in CREB phosphorylation of macrophages by the incremental addition of 8-Br-cAMP. Accordingly, we see a similar cytokine secretion pattern as observed in T0901317-treated macrophages (**Figure 10 A & C**).

To further confirm that the effect of ABCA1 expression in promoting a more anti-inflammatory profile is through PKA activation, we attempted to reverse the effect through inhibition of PKA activity. Unfortunately, we observed a general inhibition of TNF-α secretion in both RAW macrophages and primary BMDMs, regardless of ABCA1 expression (**Figure 11 B & C**). Although the result of PKA inhibition does not provide support to the anti-inflammatory effect of PKA activation, we must consider that treatment with PKI globally inhibits PKA activity in macrophages and, thus, has many other consequences. PKI inhibits the activation of all pathways that require PKA signaling, which could include initial LPS/TLR4 interaction events. Thus, the general decrease in pro-inflammatory TNF-α secretion, regardless of ABCA1 expression, may be a result of inhibition of all PKA

signaling pathways, including initial TLR4 signaling and not specifically downstream CREB pathways. Also, PKI inhibits PKA activity by binding to the free catalytic subunit (Dalton and Dewey, 2006). As a result, PKI can only inhibit PKA when PKA is actively phosphorylating its target proteins. Thus, under steady state conditions, basal PKA activation may already phosphorylate its target proteins prior to PKI treatment and these target proteins could still be fully functional for some time in the presence of PKI. Indeed, we observed limited effect of PKI on the steady state levels of phosphorylated CREB in either BMDMs (**Figure 11 D**) or RAW macrophages (data not shown). One solution to overcome this lack of effect would be to lengthen the PKI incubation time. However, longer periods of PKI incubation may drastically alter other major cell signaling processes that may consequently have an effect on CREB phosphorylation and cytokine secretion. Also, As PKA is involved in many different cellular signaling pathways (Sadana and Dessauer, 2009), overall inhibition of PKA with PKI most likely has other consequences that cloak ABCA1-specific effects on cytokine secretion and CREB phosphorylation. Thus, overall PKI treatment in macrophages may not be the ideal approach to inhibit ABCA1-dependent PKA activation as PKI affects many other cell signaling pathways.

The lack of PKI effect on CREB phosphorylation could also be indicative of other kinases involved in phosphorylating CREB, such as ERK, CaMKs, and p38MAPK (Sheng et al., 1991; Wu et al., 2001; Xing et al., 1996). Also, cAMP can activate other proteins, such as Epac-1, although Epac-1 is not involved in cAMP induced inflammation modulation shown by Wall and colleagues (Wall et al., 2009). Another study has also shown that, in alveolar macrophages, TNFα production is not affected by Epac1 agonists but is suppressed with PKA agonists (Aronoff et al., 2005). Similar to our predicament, both of these studies could

not reverse the effects of PGE_2 with pre-incubations of standard PKA inhibitors: PKI or H-89 (Aronoff et al., 2005; Wall et al., 2009).

4.7 - MKP-1 levels increase as a result of LXR stimulation and CREB activation

Having established an association between CREB activation, potentially through ABCA1 expression, and suppression of inflammatory cytokine secretion, we next looked for proteins that modulate inflammatory pathway but themselves are regulated by CREB. This would provide insight into the potential mechanism linking LXR induction of ABCA1 to immune regulation. One such protein, MKP-1, is involved in the modulation of the TLR4 pathway and is transcriptionally regulated by phospho-CREB through binding to the CRE sites in the gene promoter region (Sommer et al., 2000). We indeed observed that, with the incremental addition of cAMP, there is a trend of increased MKP-1 levels (**Figure 12 C**). Importantly, when RAW macrophages were treated T0901317 overnight, which among other things upregulates ABCA1 expression, there is a significant increase in MKP-1 levels (**Figure 12 A & B**). Altogether, these results suggest that the anti-inflammatory effects of LXR activation may be in part through CREB activation of the MAPK-phosphatase MKP-1.

Increased CREB phosphorylation can be indicative of increased PKA activity. However, we also need to consider several other important factors, as PKA is not the sole activator of CREB. Other kinases that have been shown to activate CREB include MAPKs (Wu et al., 2001), CaMKs (Sheng et al., 1991), and MSK-1 (Wiggin et al., 2002). Secondly, CREB phosphorylation is not a sure sign of its activation. Phosphorylated CREB requires the formation of a complex with CBP in order to bind the promoter CRE site of target genes. Thus, CREB phosphorylation alone, is not sufficient to activate target genes containing CRE

sites since recruitment of CBP and other cofactors also play a strong role in CREB target gene activation (Zhang et al., 2005).

4.8 - Possible shared mechanism between ABCA1 expression, LXR activation, and PKA activation on inflammatory signaling

The study by Wall et al. has demonstrated that the anti-inflammatory effects of cAMP occur through a PKA-specific signaling pathway and not through Epac signaling (Wall et al., 2009). They observed a decrease in secretion of pro-inflammatory TNF-α and MIP-1α and an increase in secretion of anti-inflammatory IL-10 and GCSF with the PKA-selective cAMP analog 6Bz-cAMP. The Epac-selective cAMP analog, 8pCPT-2'OMe-cAMP, had no effect on LPS-stimulated cytokine secretion, suggesting a PKA-specific signaling pathway necessary for the anti-inflammatory effects of cAMP. As we have observed, both LXR activation in RAW cells and ABCA1 expression in BMDMs led to increased PKA activation and anti-inflammatory effects. This raises the possibility that LXR activation and ABCA1 expression may utilize a PKA-specific mechanism to regulate the inflammatory response, similar to that of inducing PKA activation with cAMP or PGE_2.

4.9 - Experimental limitations

In this thesis, we have presented evidence that supports a novel module, namely PKA/CREB, as essential regulator of inflammatory responses in ABCA1-expressing cells. Admittedly, we were limited by the availability of experimental tools to firmly establish the link between ABCA1 expression, PKA/CREB activation and cytokine secretion. To properly compare the effects of ABCA1 expression to cAMP treatment and LXR activation in RAW macrophages, one ideal solution would be to obtain macrophages with various degrees of

ABCA1 knock-down. I had, in fact, spent several months, without much success, attempting to generate stable RAW macrophage cell lines of ABCA1 knock-down. For the effective knockdown ABCA1, we had used a set of vectors containing five different shRNAs that coincide with different regions of the ABCA1 mRNA. The failed attempts at generating stable cell lines may have been as a result of a combination of the large size of ABCA1 (250kD), the low transfection efficiency of RAW macrophages, and the difficulty in identifying clones that express all five shRNAs, a likely requirement for effective ABCA1 knockdown. In the future, we may be able to increase the knockdown efficiency, if we can find a more efficient technique to transfect RAW macrophages, such as viral-mediated transfection or electroporation.

Another option to avoid the use of drugs for ABCA1 expression is to use a cell model that over-expresses ABCA1. This can be achieved through the stable transfection of constitutively active ABCA1 and dominant negative ABCA1 vectors into the RAW macrophage cell line. These cells will provide a more accurate system of analyzing cytokine secretion and PKA activity with respect to differences in ABCA1 expression. Complete deletion of ABCA1, such as that in the BMDM cell line, may also be a good system for analyzing the effect of ABCA1 on inflammation and PKA activation, but it has its drawbacks. The major disadvantage of using gene knockout models is absence of certain phenotypes or functional redundancies (Leon, 2005) For example, in ABCA1-/- primary macrophages, there is a possibility of compensatory action of the other ABC transporter, ABCG1, specifically when analyzing inflammatory cytokine release (Out et al., 2008; Yvan-Charvet et al., 2008).

We also need to taken into account several technical limitations in order to understand some trends and results we have observed. First, the decreased total CREB levels

detected in cells treated with T0901317 or cells expressing ABCA1 is likely a technical issue. Because we first probed our immunoblots for serine 133 phosphorylated CREB, there was interference with the detection of total CREB using the monoclonal CREB antibody. This antibody is produced through the immunization of mice with GST-CREB full length fusion protein, and thus its binding is affected by the interference of the phospho-CREB antibody. Stripping of the immunoblot membranes prior to total CREB detection either had little effect on α- phospho-CREB binding or was too harsh on the immunoblot, resulting in protein band stripping (data not shown). Avoidance of this problem in the future may entail use of a different CREB monoclonal antibody, generated by immunizing mice with a synthetic peptide containing only certain regions of CREB instead of the full-length protein. Injection of mice with a synthetic protein containing residues of the carboxy-terminus of CREB may provide a suitable total CREB antibody. Although it is difficult to determine if overall CREB expression has changed with the antibodies used, we can still confirm changes in phosphorylated CREB by using the total CREB antibody. The total CREB antibody binds more readily to immunoblot regions that had less binding of the phospho-CREB antibody and, thus, less phospho-CREB levels.

Secondly, the control vector used for the ABCA1 shRNA transient transfection was pUB-GFP, a vector containing an ubiquitin promoter alone. In hindsight, a more appropriate control would be a vector containing a scrambled form of shRNA. An additional wildtype-untransfected control is necessary if the experiment were to be repeated. This would ensure that no other genes or cellular processes are affected by transfection with shRNA.

4.10 - Future directions

To further confirm the results we have uncovered with respect to ABCA1 and inflammation, several future experimental approaches can be taken. Firstly, we need to obtain a more appropriate *in vitro* model for analyzing the effects of ABCA1 expression on cytokine secretion and PKA activation. We propose stable transfections of dominant negative along with wt ABCA1 in a murine macrophage line, such as RAW 264.7 macrophages. With this *in vitro* model we can properly evaluate the secretion of the cytokines TNF-α, IL-6, and IL-10 in relation to ABCA1 expression. Other studies have shown that ABCA1 induction alters the production as well as the expression of cytokines TNF-α, IL-6, and IL-10 (Zhu et al., 2008). To confirm this, we must also look at the mRNA levels of each of these cytokines in relation to their secretion level in the presence and absence of active ABCA1.

Perhaps most importantly, we need to understand how ABCA1 expression correlates with PKA activation. One approach to do so would be to analyze the potential molecular interactions between PKA and ABCA1. In the preliminary experiments from others in the laboratory, PKA can be co-immunoprecipitated with ABCA1. This implies that ABCA1 may influence the cellular distribution of PKA and thereby alter PKA activation. If we can modify the interaction between PKA and ABCA1 this will allow us to more specifically identify the mechanism in which PKA affects cytokine secretion. As PKA localization is known to affect the specificity of its downstream signaling (Carnegie et al., 2009), we can attempt to alter the ABCA1-PKA interaction through manipulating various A-kinase anchoring proteins (AKAPs). If we can establish the role of AKAPs in this process, it will lead to a better understanding towards the effect of ABCA1 on cytokine secretion.

We can also more thoroughly assess the activity of CREB via CBP-binding (immunoprecipitation), phosphorylation levels of CBP, and the acetylation status of CREB. Gene targets of CREB activation also need further study. We suggest measuring promoter activity of MKP-1 in relation to ABCA1 activity using a luciferase reporter assay.

Using the same *in vitro* model, we also propose to knockdown and over-express MKP-1 either through stable or transient transfections. Expression and secretion of cytokines of interest will be measured. This will allow us to determine if ABCA1, indeed, acts via MKP-1 to alter the expression and production of inflammatory cytokines.

All of the above proposed future experiments will allow for a better understanding of the mechanism linking ABCA1 to the regulation of cytokine secretion.

4.11 - Summary

We report that, with ABCA1 expression in BHK and primary bone-marrow derived mouse macrophages, there is a significant increase in phospho-CREB levels. ABCA1 expression also leads to an elevated level of PKA phosphorylated proteins. This suggests potential PKA activation by ABCA1. In addition, expression of ABCA1 results in a more anti-inflammatory profile: upon LPS stimulation, ABCA1-expressing cells release less pro-inflammatory cytokines IL-6 and TNF-α and more anti-inflammatory cytokine IL-10. A similar pattern was observed in RAW macrophages treated with a LXR agonist, an experimental condition that also strongly induces ABCA1 expression. To establish that increased phospho-CREB level or PKA activity can influence LPS-stimulated cytokine secretion in our experimental model, we treated RAW macrophages with cAMP in the absence of ABCA1 expression. We were able to show that 25-50μM 8-Br-cAMP can increase CREB phosphorylation to the level seen in LXR-activated cells. Such 8-Br-cAMP treatment was sufficient to suppress TNF-α secretion. Taken together, our observations suggest that CREB phosphorylation may be the central mechanism by which LXR agonist and perhaps ABCA1 expression regulate inflammation. To explore potential downstream targets of phospho-CREB or PKA that may contribute to inflammation modulation, we focused on MKP-1, a phosphatase known to dampen inflammation. We observed an increase in MKP-1 levels in LXR agonist-treated RAW macrophages. This suggests that MKP-1 may be a contributor to CREB-mediated inflammation modulation. As LXR agonist also strongly induces ABCA1 expression, our study raises a possibility that ABCA1 may regulate inflammation through CREB and PKA.

Further study of the mechanism linking ABCA1 to cytokine secretion may help to develope future therapeutics for the treatment of atherosclerosis, as well as other chronic inflammatory diseases.

Contributions of Collaborators

I would like to sincerely thank Dr. Fumin Dong and Loretta Ma for their contributions to this thesis. Dr. Fumin Dong has provided an immunoblot of phospho-PKA substrate levels in BMDMs (Figure 6 B). TNF-α cytokine secretion from primary BMDMs (Figure 8 D) was measured in a joint effort between myself and Ms. Loretta Ma. Results from primary BMDM cytokine secretion (Figure 9) were provided by Ms. Loretta Ma.

References

Aiello, R.J., Brees, D., Bourassa, P.A., Royer, L., Lindsey, S., Coskran, T., Haghpassand, M., and Francone, O.L. (2002). Increased atherosclerosis in hyperlipidemic mice with inactivation of ABCA1 in macrophages. Arterioscler. Thromb. Vasc. Biol. *22,* 630-637.

Aiello, R.J., Brees, D., and Francone, O.L. (2003). ABCA1-deficient mice: insights into the role of monocyte lipid efflux in HDL formation and inflammation. Arterioscler. Thromb. Vasc. Biol. *23,* 972-980.

Angeli, V., Llodra, J., Rong, J.X., Satoh, K., Ishii, S., Shimizu, T., Fisher, E.A., and Randolph, G.J. (2004). Dyslipidemia associated with atherosclerotic disease systemically alters dendritic cell mobilization. Immunity *21,* 561-574.

Aronoff, D.M., Canetti, C., Serezani, C.H., Luo, M., and Peters-Golden, M. (2005). Cutting edge: macrophage inhibition by cyclic AMP (cAMP): differential roles of protein kinase A and exchange protein directly activated by cAMP-1. J. Immunol. *174,* 595-599.

Avni, D., Ernst, O., Philosoph, A., and Zor, T. (2010). Role of CREB in modulation of TNFalpha and IL-10 expression in LPS-stimulated RAW264.7 macrophages. Mol. Immunol. *47,* 1396-1403.

Bakkar, N., and Guttridge, D.C. (2010). NF-kappaB signaling: a tale of two pathways in skeletal myogenesis. Physiol. Rev. *90,* 495-511.

Baldan, A., Pei, L., Lee, R., Tarr, P., Tangirala, R.K., Weinstein, M.M., Frank, J., Li, A.C., Tontonoz, P., and Edwards, P.A. (2006). Impaired development of atherosclerosis in hyperlipidemic Ldlr-/- and ApoE-/- mice transplanted with Abcg1-/- bone marrow. Arterioscler. Thromb. Vasc. Biol. *26,* 2301-2307.

Beinke, S., and Ley, S.C. (2004). Functions of NF-kappaB1 and NF-kappaB2 in immune cell biology. Biochem. J. *382,* 393-409.

Bodzioch, M., Orso, E., Klucken, J., Langmann, T., Bottcher, A., Diederich, W., Drobnik, W., Barlage, S., Buchler, C., Porsch-Ozcurumez, M.*, et al.* (1999). The gene encoding ATP-binding cassette transporter 1 is mutated in Tangier disease. Nat. Genet. *22,* 347-351.

Bogan, R.L., and Hennebold, J.D. (2010). The reverse cholesterol transport system as a potential mediator of luteolysis in the primate corpus luteum. Reproduction *139,* 163-176.

Bonizzi, G., Bebien, M., Otero, D.C., Johnson-Vroom, K.E., Cao, Y., Vu, D., Jegga, A.G., Aronow, B.J., Ghosh, G., Rickert, R.C., and Karin, M. (2004). Activation of IKKalpha target genes depends on recognition of specific kappaB binding sites by RelB:p52 dimers. EMBO J. *23,* 4202-4210.

Bopst, M., Haas, C., Car, B., and Eugster, H.P. (1998). The combined inactivation of tumor necrosis factor and interleukin-6 prevents induction of the major acute phase proteins by endotoxin. Eur. J. Immunol. *28,* 4130-4137.

Broccardo, C., Osorio, J., Luciani, M.F., Schriml, L.M., Prades, C., Shulenin, S., Arnould, I., Naudin, L., Lafargue, C., Rosier, M.*, et al.* (2001). Comparative analysis of the promoter structure and genomic organization of the human and mouse ABCA7 gene encoding a novel ABCA transporter. Cytogenet. Cell Genet. *92,* 264-270.

Brunham, L.R., Kruit, J.K., Iqbal, J., Fievet, C., Timmins, J.M., Pape, T.D., Coburn, B.A., Bissada, N., Staels, B., Groen, A.K.*, et al.* (2006). Intestinal ABCA1 directly contributes to HDL biogenesis in vivo. J. Clin. Invest. *116,* 1052-1062.

Bungert, S., Molday, L.L., and Molday, R.S. (2001). Membrane topology of the ATP binding cassette transporter ABCR and its relationship to ABC1 and related ABCA transporters: identification of N-linked glycosylation sites. J. Biol. Chem. *276,* 23539-23546.

Calkin, A.C., and Tontonoz, P. (2010). Liver x receptor signaling pathways and atherosclerosis. Arterioscler. Thromb. Vasc. Biol. *30,* 1513-1518.

Cao, S., Zhang, X., Edwards, J.P., and Mosser, D.M. (2006). NF-kappaB1 (p50) homodimers differentially regulate pro- and anti-inflammatory cytokines in macrophages. J. Biol. Chem. *281,* 26041-26050.

Carnegie, G.K., Means, C.K., and Scott, J.D. (2009). A-kinase anchoring proteins: from protein complexes to physiology and disease. IUBMB Life *61,* 394-406.

Casals-Casas, C., Alvarez, E., Serra, M., de la Torre, C., Farrera, C., Sanchez-Tillo, E., Caelles, C., Lloberas, J., and Celada, A. (2009). CREB and AP-1 activation regulates MKP-1 induction by LPS or M-CSF and their kinetics correlate with macrophage activation versus proliferation. Eur. J. Immunol. *39,* 1902-1913.

Chawla, A., Boisvert, W.A., Lee, C.H., Laffitte, B.A., Barak, Y., Joseph, S.B., Liao, D., Nagy, L., Edwards, P.A., Curtiss, L.K., Evans, R.M., and Tontonoz, P. (2001). A PPAR gamma-LXR-ABCA1 pathway in macrophages is involved in cholesterol efflux and atherogenesis. Mol. Cell *7,* 161-171.

Chinetti, G., Lestavel, S., Bocher, V., Remaley, A.T., Neve, B., Torra, I.P., Teissier, E., Minnich, A., Jaye, M., Duverger, N.*, et al.* (2001). PPAR-alpha and PPAR-gamma activators induce cholesterol removal from human macrophage foam cells through stimulation of the ABCA1 pathway. Nat. Med. *7,* 53-58.

Christiansen-Weber, T.A., Voland, J.R., Wu, Y., Ngo, K., Roland, B.L., Nguyen, S., Peterson, P.A., and Fung-Leung, W.P. (2000). Functional loss of ABCA1 in mice causes severe placental malformation, aberrant lipid distribution, and kidney glomerulonephritis as well as high-density lipoprotein cholesterol deficiency. Am. J. Pathol. *157,* 1017-1029.

Chu, W.M., Ostertag, D., Li, Z.W., Chang, L., Chen, Y., Hu, Y., Williams, B., Perrault, J., and Karin, M. (1999). JNK2 and IKKbeta are required for activating the innate response to viral infection. Immunity *11,* 721-731.

Conboy, I.M., Manoli, D., Mhaiskar, V., and Jones, P.P. (1999). Calcineurin and vacuolar-type H+-ATPase modulate macrophage effector functions. Proc. Natl. Acad. Sci. U. S. A. *96,* 6324-6329.

Costet, P., Luo, Y., Wang, N., and Tall, A.R. (2000). Sterol-dependent transactivation of the ABC1 promoter by the liver X receptor/retinoid X receptor. J. Biol. Chem. *275,* 28240-28245.

Dalton, G.D., and Dewey, W.L. (2006). Protein kinase inhibitor peptide (PKI): a family of endogenous neuropeptides that modulate neuronal cAMP-dependent protein kinase function. Neuropeptides *40,* 23-34.

de Rooij, J., Zwartkruis, F.J., Verheijen, M.H., Cool, R.H., Nijman, S.M., Wittinghofer, A., and Bos, J.L. (1998). Epac is a Rap1 guanine-nucleotide-exchange factor directly activated by cyclic AMP. Nature *396,* 474-477.

Denis, M., Bissonnette, R., Haidar, B., Krimbou, L., Bouvier, M., and Genest, J. (2003). Expression, regulation, and activity of ABCA1 in human cell lines. Mol. Genet. Metab. *78,* 265-274.

Denkers, E.Y., Butcher, B.A., Del Rio, L., and Kim, L. (2004). Manipulation of mitogen-activated protein kinase/nuclear factor-kappaB-signaling cascades during intracellular Toxoplasma gondii infection. Immunol. Rev. *201,* 191-205.

Dumitru, C.D., Ceci, J.D., Tsatsanis, C., Kontoyiannis, D., Stamatakis, K., Lin, J.H., Patriotis, C., Jenkins, N.A., Copeland, N.G., Kollias, G., and Tsichlis, P.N. (2000). TNF-alpha induction by LPS is regulated posttranscriptionally via a Tpl2/ERK-dependent pathway. Cell *103,* 1071-1083.

Edfeldt, K., Swedenborg, J., Hansson, G.K., and Yan, Z.Q. (2002). Expression of toll-like receptors in human atherosclerotic lesions: a possible pathway for plaque activation. Circulation *105,* 1158-1161.

Elenkov, I.J., and Chrousos, G.P. (2002). Stress hormones, proinflammatory and antiinflammatory cytokines, and autoimmunity. Ann. N. Y. Acad. Sci. *966,* 290-303.

Erridge, C., and Samani, N.J. (2009). Saturated fatty acids do not directly stimulate Toll-like receptor signaling. Arterioscler. Thromb. Vasc. Biol. *29,* 1944-1949.

Fabricant, C.G., and Fabricant, J. (1999). Atherosclerosis induced by infection with Marek's disease herpesvirus in chickens. Am. Heart J. *138,* S465-8.

Farooq, A., and Zhou, M.M. (2004). Structure and regulation of MAPK phosphatases. Cell. Signal. *16,* 769-779.

Feng, G.J., Goodridge, H.S., Harnett, M.M., Wei, X.Q., Nikolaev, A.V., Higson, A.P., and Liew, F.Y. (1999). Extracellular signal-related kinase (ERK) and p38 mitogen-activated protein (MAP) kinases differentially regulate the lipopolysaccharide-mediated induction of inducible nitric oxide synthase and IL-12 in macrophages: Leishmania phosphoglycans subvert macrophage IL-12 production by targeting ERK MAP kinase. J. Immunol. *163,* 6403-6412.

Fitzgerald, K.A., Rowe, D.C., and Golenbock, D.T. (2004). Endotoxin recognition and signal transduction by the TLR4/MD2-complex. Microbes Infect. *6,* 1361-1367.

Fitzgerald, M.L., Moore, K.J., and Freeman, M.W. (2002a). Nuclear hormone receptors and cholesterol trafficking: the orphans find a new home. J. Mol. Med. *80,* 271-281.

Fitzgerald, M.L., Morris, A.L., Rhee, J.S., Andersson, L.P., Mendez, A.J., and Freeman, M.W. (2002b). Naturally occurring mutations in the largest extracellular loops of ABCA1 can disrupt its direct interaction with apolipoprotein A-I. J. Biol. Chem. *277,* 33178-33187.

Francis, G.A., Knopp, R.H., and Oram, J.F. (1995). Defective removal of cellular cholesterol and phospholipids by apolipoprotein A-I in Tangier Disease. J. Clin. Invest. *96,* 78-87.

Geppert, T.D., Whitehurst, C.E., Thompson, P., and Beutler, B. (1994). Lipopolysaccharide signals activation of tumor necrosis factor biosynthesis through the ras/raf-1/MEK/MAPK pathway. Mol. Med. *1,* 93-103.

Gonzalez, G.A., and Montminy, M.R. (1989). Cyclic AMP stimulates somatostatin gene transcription by phosphorylation of CREB at serine 133. Cell *59,* 675-680.

Groeneweg, M., Kanters, E., Vergouwe, M.N., Duerink, H., Kraal, G., Hofker, M.H., and de Winther, M.P. (2006). Lipopolysaccharide-induced gene expression in murine macrophages is enhanced by prior exposure to oxLDL. J. Lipid Res. *47,* 2259-2267.

Guha, M., and Mackman, N. (2001). LPS induction of gene expression in human monocytes. Cell. Signal. *13,* 85-94.

Gumina, R.J., Newman, P.J., Kenny, D., Warltier, D.C., and Gross, G.J. (1997). The leukocyte cell adhesion cascade and its role in myocardial ischemia-reperfusion injury. Basic Res. Cardiol. *92,* 201-213.

Haghpassand, M., Bourassa, P.A., Francone, O.L., and Aiello, R.J. (2001). Monocyte/macrophage expression of ABCA1 has minimal contribution to plasma HDL levels. J. Clin. Invest. *108,* 1315-1320.

Hammer, M., Mages, J., Dietrich, H., Servatius, A., Howells, N., Cato, A.C., and Lang, R. (2006). Dual specificity phosphatase 1 (DUSP1) regulates a subset of LPS-induced genes and protects mice from lethal endotoxin shock. J. Exp. Med. *203,* 15-20.

Hansson, G.K., and Libby, P. (2006). The immune response in atherosclerosis: a double-edged sword. Nat. Rev. Immunol. *6,* 508-519.

Hayden, M.S., and Ghosh, S. (2004). Signaling to NF-kappaB. Genes Dev. *18,* 2195-2224.

Howard, M., O'Garra, A., Ishida, H., de Waal Malefyt, R., and de Vries, J. (1992). Biological properties of interleukin 10. J. Clin. Immunol. *12,* 239-247.

Howard, W.J. (2010). The role of ezetimibe in the prevention of cardiovascular disease: Where do we stand after ARBITER 6-HALTS. Nutr. Metab. Cardiovasc. Dis.

Hsu, H.Y., Nicholson, A.C., Pomerantz, K.B., Kaner, R.J., and Hajjar, D.P. (1995). Altered cholesterol trafficking in herpesvirus-infected arterial cells. Evidence for viral protein kinase-mediated cholesterol accumulation. J. Biol. Chem. *270,* 19630-19637.

Ichiki, T. (2006). Role of cAMP response element binding protein in cardiovascular remodeling: good, bad, or both? Arterioscler. Thromb. Vasc. Biol. *26,* 449-455.

Ishibashi, S., Goldstein, J.L., Brown, M.S., Herz, J., and Burns, D.K. (1994). Massive xanthomatosis and atherosclerosis in cholesterol-fed low density lipoprotein receptor-negative mice. J. Clin. Invest. *93,* 1885-1893.

Jennings, C., Kusler, B., and Jones, P.P. (2009). Calcineurin inactivation leads to decreased responsiveness to LPS in macrophages and dendritic cells and protects against LPS-induced toxicity in vivo. Innate Immun. *15,* 109-120.

Johnson, G.L., and Lapadat, R. (2002). Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases. Science *298,* 1911-1912.

Joseph, S.B., Castrillo, A., Laffitte, B.A., Mangelsdorf, D.J., and Tontonoz, P. (2003). Reciprocal regulation of inflammation and lipid metabolism by liver X receptors. Nat. Med. *9,* 213-219.

Kang, Y.J., Kusler, B., Otsuka, M., Hughes, M., Suzuki, N., Suzuki, S., Yeh, W.C., Akira, S., Han, J., and Jones, P.P. (2007). Calcineurin negatively regulates TLR-mediated activation pathways. J. Immunol. *179,* 4598-4607.

Karin, M., and Ben-Neriah, Y. (2000). Phosphorylation meets ubiquitination: the control of NF-[kappa]B activity. Annu. Rev. Immunol. *18,* 621-663.

Karin, M., Lawrence, T., and Nizet, V. (2006). Innate immunity gone awry: linking microbial infections to chronic inflammation and cancer. Cell *124,* 823-835.

Kawasaki, H., Springett, G.M., Mochizuki, N., Toki, S., Nakaya, M., Matsuda, M., Housman, D.E., and Graybiel, A.M. (1998). A family of cAMP-binding proteins that directly activate Rap1. Science *282,* 2275-2279.

Kleemann, R., Zadelaar, S., and Kooistra, T. (2008). Cytokines and atherosclerosis: a comprehensive review of studies in mice. Cardiovasc. Res. *79,* 360-376.

Kobayashi, A., Takanezawa, Y., Hirata, T., Shimizu, Y., Misasa, K., Kioka, N., Arai, H., Ueda, K., and Matsuo, M. (2006). Efflux of sphingomyelin, cholesterol, and phosphatidylcholine by ABCG1. J. Lipid Res. *47,* 1791-1802.

Kontoyiannis, D., Pasparakis, M., Pizarro, T.T., Cominelli, F., and Kollias, G. (1999). Impaired on/off regulation of TNF biosynthesis in mice lacking TNF AU-rich elements: implications for joint and gut-associated immunopathologies. Immunity *10,* 387-398.

Kuprash, D.V., Udalova, I.A., Turetskaya, R.L., Kwiatkowski, D., Rice, N.R., and Nedospasov, S.A. (1999). Similarities and differences between human and murine TNF promoters in their response to lipopolysaccharide. J. Immunol. *162,* 4045-4052.

Kurt-Jones, E.A., Popova, L., Kwinn, L., Haynes, L.M., Jones, L.P., Tripp, R.A., Walsh, E.E., Freeman, M.W., Golenbock, D.T., Anderson, L.J., and Finberg, R.W. (2000). Pattern recognition receptors TLR4 and CD14 mediate response to respiratory syncytial virus. Nat. Immunol. *1,* 398-401.

Langmann, T., Klucken, J., Reil, M., Liebisch, G., Luciani, M.F., Chimini, G., Kaminski, W.E., and Schmitz, G. (1999). Molecular cloning of the human ATP-binding cassette transporter 1 (hABC1): evidence for sterol-dependent regulation in macrophages. Biochem. Biophys. Res. Commun. *257,* 29-33.

Lawrence, T. (2009). The nuclear factor NF-kappaB pathway in inflammation. Cold Spring Harb Perspect. Biol. *1,* a001651.

Lazar-Molnar, E., Hegyesi, H., Toth, S., and Falus, A. (2000). Autocrine and paracrine regulation by cytokines and growth factors in melanoma. Cytokine *12,* 547-554.

Le Goff, W., Zheng, P., Brubaker, G., and Smith, J.D. (2006). Identification of the cAMP-responsive enhancer of the murine ABCA1 gene: requirement for CREB1 and STAT3/4 elements. Arterioscler. Thromb. Vasc. Biol. *26,* 527-533.

Lee, J.Y., Sohn, K.H., Rhee, S.H., and Hwang, D. (2001). Saturated fatty acids, but not unsaturated fatty acids, induce the expression of cyclooxygenase-2 mediated through Toll-like receptor 4. J. Biol. Chem. *276,* 16683-16689.

Lee, J.Y., Ye, J., Gao, Z., Youn, H.S., Lee, W.H., Zhao, L., Sizemore, N., and Hwang, D.H. (2003). Reciprocal modulation of Toll-like receptor-4 signaling pathways involving MyD88 and phosphatidylinositol 3-kinase/AKT by saturated and polyunsaturated fatty acids. J. Biol. Chem. *278,* 37041-37051.

Leon, L.R. (2005). The use of gene knockout mice in thermoregulation studies. J. Therm. Biol. *30,* 273-288.

Li, A.C., and Glass, C.K. (2004). PPAR- and LXR-dependent pathways controlling lipid metabolism and the development of atherosclerosis. J. Lipid Res. *45,* 2161-2173.

Li, H., and Sun, B. (2007). Toll-like receptor 4 in atherosclerosis. J. Cell. Mol. Med. *11,* 88-95.

Liang, Y., Zhou, Y., and Shen, P. (2004). NF-kappaB and its regulation on the immune system. Cell. Mol. Immunol. *1,* 343-350.

Libby, P., and Aikawa, M. (2002). Stabilization of atherosclerotic plaques: new mechanisms and clinical targets. Nat. Med. *8,* 1257-1262.

Linton, M.F., and Fazio, S. (2003). Macrophages, inflammation, and atherosclerosis. Int. J. Obes. Relat. Metab. Disord. *27 Suppl 3,* S35-40.

Liu, X., Wang, B., Ma, X., and Guo, Y. (2009). NF-kappaB activation through the alternative pathway correlates with chemoresistance and poor survival in extranodal NK/T-cell lymphoma, nasal type. Jpn. J. Clin. Oncol. *39,* 418-424.

Liu, Y., Shepherd, E.G., and Nelin, L.D. (2007). MAPK phosphatases--regulating the immune response. Nat. Rev. Immunol. *7,* 202-212.

Loubele, S.T., ten Cate, H., and Spronk, H.M. (2010). Anticoagulant therapy in critical organ ischaemia/reperfusion injury. Thromb. Haemost. *104,* 136-142.

Lu, Q., Hutchins, A.E., Doyle, C.M., Lundblad, J.R., and Kwok, R.P. (2003). Acetylation of cAMP-responsive element-binding protein (CREB) by CREB-binding protein enhances CREB-dependent transcription. J. Biol. Chem. *278,* 15727-15734.

Lu, Y.C., Yeh, W.C., and Ohashi, P.S. (2008). LPS/TLR4 signal transduction pathway. Cytokine *42,* 145-151.

Luo, Y., and Tall, A.R. (2000). Sterol upregulation of human CETP expression in vitro and in transgenic mice by an LXR element. J. Clin. Invest. *105,* 513-520.

Manderson, A.P., Kay, J.G., Hammond, L.A., Brown, D.L., and Stow, J.L. (2007). Subcompartments of the macrophage recycling endosome direct the differential secretion of IL-6 and TNFalpha. J. Cell Biol. *178,* 57-69.

Maton, A. (1993). *Human Biology and Health* (Englewood Cliffs, New Jersey: Prentice Hall).

Matsushima, A., Kaisho, T., Rennert, P.D., Nakano, H., Kurosawa, K., Uchida, D., Takeda, K., Akira, S., and Matsumoto, M. (2001). Essential role of nuclear factor (NF)-kappaB-inducing kinase and inhibitor of kappaB (IkappaB) kinase alpha in NF-kappaB activation through lymphotoxin beta receptor, but not through tumor necrosis factor receptor I. J. Exp. Med. *193,* 631-636.

Mayr, B., and Montminy, M. (2001). Transcriptional regulation by the phosphorylation-dependent factor CREB. Nat. Rev. Mol. Cell Biol. *2,* 599-609.

Medzhitov, R., and Horng, T. (2009). Transcriptional control of the inflammatory response. Nat. Rev. Immunol. *9,* 692-703.

Michelsen, K.S., Wong, M.H., Shah, P.K., Zhang, W., Yano, J., Doherty, T.M., Akira, S., Rajavashisth, T.B., and Arditi, M. (2004). Lack of Toll-like receptor 4 or myeloid differentiation factor 88 reduces atherosclerosis and alters plaque phenotype in mice deficient in apolipoprotein E. Proc. Natl. Acad. Sci. U. S. A. *101,* 10679-10684.

Miller, S.I., Ernst, R.K., and Bader, M.W. (2005). LPS, TLR4 and infectious disease diversity. Nat. Rev. Microbiol. *3,* 36-46.

Murata, T., Shimizu, K., Hiramoto, K., and Tagawa, T. (2009). Phosphodiesterase 3 (PDE3): structure, localization and function. Cardiovasc. Hematol. Agents Med. Chem. *7,* 206-211.

Murray, C.J., and Lopez, A.D. (1997). Global mortality, disability, and the contribution of risk factors: Global Burden of Disease Study. Lancet *349,* 1436-1442.

Murray, R.Z., Kay, J.G., Sangermani, D.G., and Stow, J.L. (2005). A role for the phagosome in cytokine secretion. Science *310,* 1492-1495.

Nagao, K., Kimura, Y., Mastuo, M., and Ueda, K. (2010). Lipid outward translocation by ABC proteins. FEBS Lett. *584,* 2717-2723.

Naviglio, S., Caraglia, M., Abbruzzese, A., Chiosi, E., Di Gesto, D., Marra, M., Romano, M., Sorrentino, A., Sorvillo, L., Spina, A., and Illiano, G. (2009). Protein kinase A as a biological target in cancer therapy. Expert Opin. Ther. Targets *13,* 83-92.

Nelson, W.G., De Marzo, A.M., DeWeese, T.L., and Isaacs, W.B. (2004). The role of inflammation in the pathogenesis of prostate cancer. J. Urol. *172,* S6-11; discussion S11-2.

Newell, C.L., Deisseroth, A.B., and Lopez-Berestein, G. (1994). Interaction of nuclear proteins with an AP-1/CRE-like promoter sequence in the human TNF-alpha gene. J. Leukoc. Biol. *56,* 27-35.

Nissen, S.E., Nicholls, S.J., Sipahi, I., Libby, P., Raichlen, J.S., Ballantyne, C.M., Davignon, J., Erbel, R., Fruchart, J.C., Tardif, J.C*., et al.* (2006). Effect of very high-intensity statin therapy on regression of coronary atherosclerosis: the ASTEROID trial. JAMA *295,* 1556-1565.

Nofer, J.R., and Remaley, A.T. (2005). Tangier disease: still more questions than answers. Cell Mol. Life Sci. *62,* 2150-2160.

Ohashi, R., Mu, H., Wang, X., Yao, Q., and Chen, C. (2005). Reverse cholesterol transport and cholesterol efflux in atherosclerosis. QJM *98,* 845-856.

Omoigui, S. (2007). The Interleukin-6 inflammation pathway from cholesterol to aging--role of statins, bisphosphonates and plant polyphenols in aging and age-related diseases. Immun. Ageing *4,* 1.

Oram, J.F., Lawn, R.M., Garvin, M.R., and Wade, D.P. (2000). ABCA1 is the cAMP-inducible apolipoprotein receptor that mediates cholesterol secretion from macrophages. J. Biol. Chem. *275,* 34508-34511.

Out, R., Hoekstra, M., Habets, K., Meurs, I., de Waard, V., Hildebrand, R.B., Wang, Y., Chimini, G., Kuiper, J., Van Berkel, T.J., and Van Eck, M. (2008). Combined deletion of macrophage ABCA1 and ABCG1 leads to massive lipid accumulation in tissue macrophages and distinct atherosclerosis at relatively low plasma cholesterol levels. Arterioscler. Thromb. Vasc. Biol. *28,* 258-264.

Out, R., Hoekstra, M., Hildebrand, R.B., Kruit, J.K., Meurs, I., Li, Z., Kuipers, F., Van Berkel, T.J., and Van Eck, M. (2006). Macrophage ABCG1 deletion disrupts lipid homeostasis in alveolar macrophages and moderately influences atherosclerotic lesion development in LDL receptor-deficient mice. Arterioscler. Thromb. Vasc. Biol. *26,* 2295-2300.

Park, Y.M., Febbraio, M., and Silverstein, R.L. (2009). CD36 modulates migration of mouse and human macrophages in response to oxidized LDL and may contribute to macrophage trapping in the arterial intima. J. Clin. Invest. *119,* 136-145.

Pavan, B., Biondi, C., and Dalpiaz, A. (2009). Adenylyl cyclases as innovative therapeutic goals. Drug Discov. Today *14,* 982-991.

Peet, D.J., Turley, S.D., Ma, W., Janowski, B.A., Lobaccaro, J.M., Hammer, R.E., and Mangelsdorf, D.J. (1998). Cholesterol and bile acid metabolism are impaired in mice lacking the nuclear oxysterol receptor LXR alpha. Cell *93,* 693-704.

Pidoux, G., and Tasken, K. (2010). Specificity and spatial dynamics of protein kinase A signaling organized by A-kinase-anchoring proteins. J. Mol. Endocrinol. *44,* 271-284.

Piedrahita, J.A., Zhang, S.H., Hagaman, J.R., Oliver, P.M., and Maeda, N. (1992). Generation of mice carrying a mutant apolipoprotein E gene inactivated by gene targeting in embryonic stem cells. Proc. Natl. Acad. Sci. U. S. A. *89,* 4471-4475.

Platzer, C., Fritsch, E., Elsner, T., Lehmann, M.H., Volk, H.D., and Prosch, S. (1999). Cyclic adenosine monophosphate-responsive elements are involved in the transcriptional activation of the human IL-10 gene in monocytic cells. Eur. J. Immunol. *29,* 3098-3104.

Platzer, C., Meisel, C., Vogt, K., Platzer, M., and Volk, H.D. (1995). Up-regulation of monocytic IL-10 by tumor necrosis factor-alpha and cAMP elevating drugs. Int. Immunol. *7,* 517-523.

Poltorak, A., He, X., Smirnova, I., Liu, M.Y., Van Huffel, C., Du, X., Birdwell, D., Alejos, E., Silva, M., Galanos, C.*, et al.* (1998). Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. Science *282,* 2085-2088.

Raetz, C.R., and Whitfield, C. (2002). Lipopolysaccharide endotoxins. Annu. Rev. Biochem. *71,* 635-700.

Ranalletta, M., Wang, N., Han, S., Yvan-Charvet, L., Welch, C., and Tall, A.R. (2006). Decreased atherosclerosis in low-density lipoprotein receptor knockout mice transplanted with Abcg1-/- bone marrow. Arterioscler. Thromb. Vasc. Biol. *26,* 2308-2315.

Rao, A., Luo, C., and Hogan, P.G. (1997). Transcription factors of the NFAT family: regulation and function. Annu. Rev. Immunol. *15,* 707-747.

Rassa, J.C., Meyers, J.L., Zhang, Y., Kudaravalli, R., and Ross, S.R. (2002). Murine retroviruses activate B cells via interaction with toll-like receptor 4. Proc. Natl. Acad. Sci. U. S. A. *99,* 2281-2286.

Repa, J.J., Liang, G., Ou, J., Bashmakov, Y., Lobaccaro, J.M., Shimomura, I., Shan, B., Brown, M.S., Goldstein, J.L., and Mangelsdorf, D.J. (2000). Regulation of mouse sterol regulatory element-binding protein-1c gene (SREBP-1c) by oxysterol receptors, LXRalpha and LXRbeta. Genes Dev. *14,* 2819-2830.

Rocha, V.Z., and Libby, P. (2009). Obesity, inflammation, and atherosclerosis. Nat. Rev. Cardiol. *6,* 399-409.

Rogler, G., Trumbach, B., Klima, B., Lackner, K.J., and Schmitz, G. (1995). HDL-mediated efflux of intracellular cholesterol is impaired in fibroblasts from Tangier disease patients. Arterioscler. Thromb. Vasc. Biol. *15,* 683-690.

Roosbeek, S., Caster, H., Liu, Q.Z., Berne, P.F., Duverger, N., Christiaens, B., Vandekerckhove, J., Peelman, F., Labeur, C., and Rosseneu, M. (2004). Expression and activity of the nucleotide-binding domains of the human ABCA1 transporter. Protein Expr. Purif. *35,* 102-110.

Rust, S., Rosier, M., Funke, H., Real, J., Amoura, Z., Piette, J.C., Deleuze, J.F., Brewer, H.B., Duverger, N., Denefle, P., and Assmann, G. (1999). Tangier disease is caused by mutations in the gene encoding ATP-binding cassette transporter 1. Nat. Genet. *22,* 352-355.

Sadana, R., and Dessauer, C.W. (2009). Physiological roles for G protein-regulated adenylyl cyclase isoforms: insights from knockout and overexpression studies. Neurosignals *17,* 5-22.

Salojin, K.V., Owusu, I.B., Millerchip, K.A., Potter, M., Platt, K.A., and Oravecz, T. (2006). Essential role of MAPK phosphatase-1 in the negative control of innate immune responses. J. Immunol. *176,* 1899-1907.

Schaeffler, A., Gross, P., Buettner, R., Bollheimer, C., Buechler, C., Neumeier, M., Kopp, A., Schoelmerich, J., and Falk, W. (2009). Fatty acid-induced induction of Toll-like receptor-4/nuclear factor-kappaB pathway in adipocytes links nutritional signalling with innate immunity. Immunology *126,* 233-245.

Schmitz, G., and Grandl, M. (2009). The molecular mechanisms of HDL and associated vesicular trafficking mechanisms to mediate cellular lipid homeostasis. Arterioscler. Thromb. Vasc. Biol. *29,* 1718-1722.

Schmitz, G., and Langmann, T. (2005). Transcriptional regulatory networks in lipid metabolism control ABCA1 expression. Biochim. Biophys. Acta *1735,* 1-19.

Seo, D., Ginsburg, G.S., and Goldschmidt-Clermont, P.J. (2006). Gene expression analysis of cardiovascular diseases: novel insights into biology and clinical applications. J. Am. Coll. Cardiol. *48,* 227-235.

Seo, D., and Goldschmidt-Clermont, P.J. (2008). Cardiovascular genetic medicine: the genetics of coronary heart disease. J. Cardiovasc. Transl. Res. *1,* 166-170.

Sheng, M., Thompson, M.A., and Greenberg, M.E. (1991). CREB: a Ca(2+)-regulated transcription factor phosphorylated by calmodulin-dependent kinases. Science *252,* 1427-1430.

Shi, H., Kokoeva, M.V., Inouye, K., Tzameli, I., Yin, H., and Flier, J.S. (2006). TLR4 links innate immunity and fatty acid-induced insulin resistance. J. Clin. Invest. *116,* 3015-3025.

Sommer, A., Burkhardt, H., Keyse, S.M., and Luscher, B. (2000). Synergistic activation of the mkp-1 gene by protein kinase A signaling and USF, but not c-Myc. FEBS Lett. *474,* 146-150.

Stefkova, J., Poledne, R., and Hubacek, J.A. (2004). ATP-binding cassette (ABC) transporters in human metabolism and diseases. Physiol. Res. *53,* 235-243.

Steinberg, D. (2005). Thematic review series: the pathogenesis of atherosclerosis: an interpretive history of the cholesterol controversy, part III: mechanistically defining the role of hyperlipidemia. J. Lipid Res. *46,* 2037-2051.

Ster, J., De Bock, F., Guerineau, N.C., Janossy, A., Barrere-Lemaire, S., Bos, J.L., Bockaert, J., and Fagni, L. (2007). Exchange protein activated by cAMP (Epac) mediates cAMP activation of p38 MAPK and modulation of Ca2+-dependent K+ channels in cerebellar neurons. Proc. Natl. Acad. Sci. U. S. A. *104,* 2519-2524.

Stow, J.L., Ching Low, P., Offenhauser, C., and Sangermani, D. (2009). Cytokine secretion in macrophages and other cells: pathways and mediators. Immunobiology *214,* 601-612.

Tabas, I. (2010). Macrophage death and defective inflammation resolution in atherosclerosis. Nat. Rev. Immunol. *10,* 36-46.

Tall, A.R., Costet, P., and Luo, Y. (2000). 'Orphans' meet cholesterol. Nat. Med. *6,* 1104-1105.

Tasken, K., and Aandahl, E.M. (2004). Localized effects of cAMP mediated by distinct routes of protein kinase A. Physiol. Rev. *84,* 137-167.

Thalhamer, T., McGrath, M.A., and Harnett, M.M. (2008). MAPKs and their relevance to arthritis and inflammation. Rheumatology (Oxford) *47,* 409-414.

Timmins, J.M., Lee, J.Y., Boudyguina, E., Kluckman, K.D., Brunham, L.R., Mulya, A., Gebre, A.K., Coutinho, J.M., Colvin, P.L., Smith, T.L.*, et al.* (2005). Targeted inactivation of hepatic Abca1 causes profound hypoalphalipoproteinemia and kidney hypercatabolism of apoA-I. J. Clin. Invest. *115,* 1333-1342.

Toth, P.P. (2010). Drug treatment of hyperlipidaemia: a guide to the rational use of lipid-lowering drugs. Drugs *70,* 1363-1379.

Toutouzas, K., Drakopoulou, M., Skoumas, I., and Stefanadis, C. (2010). Advancing therapy for hypercholesterolemia. Expert Opin. Pharmacother. *11,* 1659-1672.

Vabulas, R.M., Ahmad-Nejad, P., Ghose, S., Kirschning, C.J., Issels, R.D., and Wagner, H. (2002). HSP70 as endogenous stimulus of the Toll/interleukin-1 receptor signal pathway. J. Biol. Chem. *277,* 15107-15112.

van Eck, M., Bos, I.S., Kaminski, W.E., Orso, E., Rothe, G., Twisk, J., Bottcher, A., Van Amersfoort, E.S., Christiansen-Weber, T.A., Fung-Leung, W.P., Van Berkel, T.J., and Schmitz, G. (2002). Leukocyte ABCA1 controls susceptibility to atherosclerosis and macrophage recruitment into tissues. Proc. Natl. Acad. Sci. U. S. A. *99,* 6298-6303.

Van Eck, M., Singaraja, R.R., Ye, D., Hildebrand, R.B., James, E.R., Hayden, M.R., and Van Berkel, T.J. (2006). Macrophage ATP-binding cassette transporter A1 overexpression inhibits atherosclerotic lesion progression in low-density lipoprotein receptor knockout mice. Arterioscler. Thromb. Vasc. Biol. *26,* 929-934.

Venkateswaran, A., Laffitte, B.A., Joseph, S.B., Mak, P.A., Wilpitz, D.C., Edwards, P.A., and Tontonoz, P. (2000). Control of cellular cholesterol efflux by the nuclear oxysterol receptor LXR alpha. Proc. Natl. Acad. Sci. U. S. A. *97,* 12097-12102.

Virmani, R., Burke, A.P., Kolodgie, F.D., and Farb, A. (2002). Vulnerable plaque: the pathology of unstable coronary lesions. J. Interv. Cardiol. *15,* 439-446.

Wall, E.A., Zavzavadjian, J.R., Chang, M.S., Randhawa, B., Zhu, X., Hsueh, R.C., Liu, J., Driver, A., Bao, X.R., Sternweis, P.C., Simon, M.I., and Fraser, I.D. (2009). Suppression of LPS-induced TNF-alpha production in macrophages by cAMP is mediated by PKA-AKAP95-p105. Sci. Signal. *2,* ra28.

Walter, M., Gerdes, U., Seedorf, U., and Assmann, G. (1994). The high density lipoprotein- and apolipoprotein A-I-induced mobilization of cellular cholesterol is impaired in fibroblasts from Tangier disease subjects. Biochem. Biophys. Res. Commun. *205,* 850-856.

Wang, M.H., Grossmann, M.E., and Young, C.Y. (2004a). Forced expression of heat-shock protein 70 increases the secretion of Hsp70 and provides protection against tumour growth. Br. J. Cancer *90,* 926-931.

Wang, N., Lan, D., Chen, W., Matsuura, F., and Tall, A.R. (2004b). ATP-binding cassette transporters G1 and G4 mediate cellular cholesterol efflux to high-density lipoproteins. Proc. Natl. Acad. Sci. U. S. A. *101,* 9774-9779.

Wang, X., Meng, X., Kuhlman, J.R., Nelin, L.D., Nicol, K.K., English, B.K., and Liu, Y. (2007). Knockout of Mkp-1 enhances the host inflammatory responses to gram-positive bacteria. J. Immunol. *178,* 5312-5320.

Wiggin, G.R., Soloaga, A., Foster, J.M., Murray-Tait, V., Cohen, P., and Arthur, J.S. (2002). MSK1 and MSK2 are required for the mitogen- and stress-induced phosphorylation of CREB and ATF1 in fibroblasts. Mol. Cell. Biol. *22,* 2871-2881.

Woollard, K.J., and Geissmann, F. (2010). Monocytes in atherosclerosis: subsets and functions. Nat. Rev. Cardiol. *7,* 77-86.

World Health Organization. (2008). Leading causes of death. In The global burden of disease: 2004 Update, (Switzerland: WHO Press) pp. 11.

Worthley, S.G., Osende, J.I., Helft, G., Badimon, J.J., and Fuster, V. (2001). Coronary artery disease: pathogenesis and acute coronary syndromes. Mt. Sinai J. Med. *68,* 167-181.

Wu, G.Y., Deisseroth, K., and Tsien, R.W. (2001). Activity-dependent CREB phosphorylation: convergence of a fast, sensitive calmodulin kinase pathway and a slow, less sensitive mitogen-activated protein kinase pathway. Proc. Natl. Acad. Sci. U. S. A. *98,* 2808-2813.

Wu, H., and Lozano, G. (1994). NF-kappa B activation of p53. A potential mechanism for suppressing cell growth in response to stress. J. Biol. Chem. *269,* 20067-20074.

Xing, J., Ginty, D.D., and Greenberg, M.E. (1996). Coupling of the RAS-MAPK pathway to gene activation by RSK2, a growth factor-regulated CREB kinase. Science *273,* 959-963.

Xu, X.H., Shah, P.K., Faure, E., Equils, O., Thomas, L., Fishbein, M.C., Luthringer, D., Xu, X.P., Rajavashisth, T.B., Yano, J., Kaul, S., and Arditi, M. (2001). Toll-like receptor-4 is expressed by macrophages in murine and human lipid-rich atherosclerotic plaques and upregulated by oxidized LDL. Circulation *104,* 3103-3108.

Yin, K., Liao, D., and Tang, C. (2010). ABCA1: A Possible Link Between Inflammation And Reverse Cholesterol Transport. Mol. Med.

Yvan-Charvet, L., Welch, C., Pagler, T.A., Ranalletta, M., Lamkanfi, M., Han, S., Ishibashi, M., Li, R., Wang, N., and Tall, A.R. (2008). Increased inflammatory gene expression in ABC transporter-deficient macrophages: free cholesterol accumulation, increased signaling via toll-like receptors, and neutrophil infiltration of atherosclerotic lesions. Circulation *118,* 1837-1847.

Zarubica, A., Trompier, D., and Chimini, G. (2007). ABCA1, from pathology to membrane function. Pflugers Arch. *453,* 569-579.

Zhang, X., Odom, D.T., Koo, S.H., Conkright, M.D., Canettieri, G., Best, J., Chen, H., Jenner, R., Herbolsheimer, E., Jacobsen, E.*, et al.* (2005). Genome-wide analysis of cAMP-response element binding protein occupancy, phosphorylation, and target gene activation in human tissues. Proc. Natl. Acad. Sci. U. S. A. *102,* 4459-4464.

Zhang, Y.L., and Dong, C. (2005). MAP kinases in immune responses. Cell. Mol. Immunol. *2,* 20-27.

Zhao, Q., Wang, X., Nelin, L.D., Yao, Y., Matta, R., Manson, M.E., Baliga, R.S., Meng, X., Smith, C.V., Bauer, J.A., Chang, C.H., and Liu, Y. (2006). MAP kinase phosphatase 1 controls innate immune responses and suppresses endotoxic shock. J. Exp. Med. *203,* 131-140.

Zhu, X., Lee, J.Y., Timmins, J.M., Brown, J.M., Boudyguina, E., Mulya, A., Gebre, A.K., Willingham, M.C., Hiltbold, E.M., Mishra, N., Maeda, N., and Parks, J.S. (2008). Increased cellular free cholesterol in macrophage-specific Abca1 knock-out mice enhances pro-inflammatory response of macrophages. J. Biol. Chem. *283,* 22930-22941.

Curriculum Vitae

Maryam Zaid
Faculty of Medicine
Department of Biochemistry, Microbiology, and Immunology
Ottawa Regional Cancer Centre

Education

2008 – 2011 **University of Ottawa – Biochemistry, *MSc. Candidate***
Supervisor: Dr. Xiaohui Zha
Thesis title: "*Induction of ABCA1 expression is correlated with increased CREB phosphorylation and altered cytokine secretion"*
Ottawa Hospital Research Institute
Ottawa, ON

2004 – 2008 **University of Ottawa – Biopharmaceutical Sciences**, ***BSc. Honours***
Supervisor: Dr. Douglas Johnson
Thesis title: "*Improving Soybean Peroxidase Enzyme by in vitro Mutagenesis*"
Center for Advanced Research in Environmental Genomics (CAREG)
Ottawa, ON

Scientific Presentations

Zaid M, Zha, X. "Activation of the Transcription Factor CREB through ABCA1 Expression and its impact on cytokine secretion." Ottawa Hospital Research Institute – Work In Progress. Seminar Presentation. April 2010, Ottawa, ON.

Zaid M, Zha, X. "Activation of the Transcription Factor CREB through ABCA1 Expression and its impact on cytokine secretion." No. 65 in Abstract of Biochemistry, Microbiology, and Immunology Symposium Research Seminars. Seminar Presentation. February 2010, Ottawa, ON.

Zaid M, Zha, X. "Activation of the Transcription Factor CREB through ABCA1 Expression and its impact on cytokine secretion." Poster Presentation. No.32 in 9th Annual OHRI Research Day. November 2009, Ottawa, ON.

Zaid M, Johnson, D. "Improving Soybean Peroxidase Enzyme by in vitro Mutagenesis." University of Ottawa: Honours Biology Poster Symposium. Poster Presentation. April 2008, Ottawa, ON