ADIPOCYTE ENHANCER-BINDING PROTEIN-1 (AEBP1) IS A NOVEL PLAYER IN MACROPHAGE CHOLESTEROL HOMEOSTASIS, INFLAMMATION, AND ATHEROGENESIS

by

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DEDICATION

Foremost, I dedicate my work to almighty God for His generosity, guidance, consecration, and blessings. I also dedicate my work to Prophet Mohammed and all other prophets of God, peace be upon them, for their ever-lasting passion and care for humanity. For their continuous academic, financial, and emotional support, love, and enthusiasm, I dedicate this work to my parents, siblings, wife, and daughter "Mona".

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ABSTRACT

PPARγ1 and LXRα are nuclear receptors that play pivotal roles in macrophage cholesterol homeostasis and inflammation, key biological processes in atherogenesis. Once fully differentiated, intimal macrophages express scavenger receptors, allowing internalization of oxidized low density lipoprotein (oxLDL). PPARγ1 and LXRα become activated in response to lipid loading, and they cooperate to induce expression of the cholesterol/phospholipid ATP-binding cassette (ABC) transporter proteins and apolipoprotein E (ApoE), prominent players in macrophage cholesterol efflux. Pro-inflammatory mediators such as interleukin-1β (IL-1β), IL-6, tumor necrosis factor α (TNFα), monocyte chemoattractant protein-1 (MCP-1), cyclooxygenase-2 (COX-2), and inducible nitric oxide synthase (iNOS) promote cell recruitment to the inflamed vasculature, advancing atherogenesis.

Adipocyte enhancer-binding protein 1 (AEBP1) is an 82-kDa, ubiquitously expressed transcriptional repressor that plays key regulatory roles in various biological processes. Herein, AEBP1 is identified as a transcriptional repressor that impedes macrophage cholesterol efflux via PPARγ1 and LXRα down-regulation in a DNA binding-dependent manner. Contrary to AEBP1 deficiency (AEBP1$^{-/-}$), AEBP1 transgenic over-expression (AEBP1^{TG}) in macrophages is accompanied by decreased expression of PPARγ1 and LXRα, and their target genes ABCA1, ABCG1, apolipoprotein E, and CD36, with concomitant elevation in IL-6, TNFα, MCP-1, and iNOS levels. AEBP1-mediated repression of PPARγ1 and LXRα is physiologically relevant since AEBP1^{TG} macrophages accumulate considerable amounts of lipids compared to AEBP1 non-transgenic (AEBP1^{NT}) macrophages, making them precursors for foam cells. Indeed, AEBP1^{TG} and AEBP1$^{-/-}$ macrophages exhibit diminished and enhanced cholesterol efflux, respectively. Remarkably, AEBP1^{TG} mice, but not AEBP1^{NT} counterparts, exhibit hyperlipidemia and develop lipid-filled atherosclerotic lesions consisting of intimal macrophages in their proximal aortae, suggesting that AEBP1^{TG} mice may serve as a novel murine model of atherosclerosis.

Experimental evidence suggests that AEBP1 manifests its pro-inflammatory function by up-regulating NF-κB activity via hampering IκBα inhibitory function through DLD-mediated protein-protein interaction, rendering IκBα susceptible to increased phosphorylation and proteolytic degradation, subsequently leading to augmented NF-κB transcriptional activity. Collectively, in vitro and ex vivo experimental evidence prompts speculation that AEBP1 may potentially function as a critical pro-inflammatory, pro-atherogenic mediator with the anticipation that it may serve as a molecular target for the development of therapeutic strategies towards the treatment of atherosclerosis and inflammatory diseases.

LIST OF ABBREVIATIONS AND SYMBOLS USED

Ab	antibody
ABC	ATP-binding cassette
ACAT	acyl-CoA:cholesterol acyltransferase
ACLP	aortic carboxypeptidase-like protein
AE-1	adipocyte enhancer-1
AEBP1	adipocyte enhancer-binding protein 1
AEBP1^{TG}	AEBP1-transgenic (over-expressing)
AEBP1^{NT}	AEBP1-non-transgenic
AEBP1$^{+/+}$	AEBP1-wildtype
AEBP1$^{-/-}$	AEBP1-deficient
AEC	9-amino-3-ethylene-carbazole
AF	transactivation domain
Akt/PKB	protein kinase B
ANK	ankyrin
aP2	gene encoding adipocyte lipid-binding protein (ALBP)
Apo	apolipoprotein
AS	anti-sense
ATCC	American type culture collection
ATP	adenosine triphosphate
Bcl-3	B-cell lymphoma-3
BSA	bovine serum albumin
CC	chemokine with two adjacent cysteine (C) residues near the N-terminus
CCR2	CC chemokine receptor 2
CE	cholesteryl ester
C/EBP	CCAAT-enhancer binding protein
CHD	coronary heart disease
CHO	Chinese hamster ovary

CHX	cycloheximide
COX	cyclooxygenase
CP	carboxypeptidase
DBD	DNA binding domain
DC	dendritic cell
DD	death domain
DLD	discoidin-like domain
DMEM	Dulbecco's modified eagle's medium
DTT	dithiothreitol
E. coli	*Escherichia coli*
ECL	enhanced chemiluminescence
ECM	extracellular matrix
ECSIT	evolutionary conserved signaling intermediate in Toll pathways
EDTA	ethylenediaminetetraacetic acid
EGTA	ethyleneglycol-bis(β-aminoethylether)-tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EMSA	electrophoretic mobility gel shift assay
Erk	extracellular signal-regulated kinase
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
GFP	green fluorescent protein
GM-CSF	granulocyte-macrophage colony-stimulating factor
GST	glutathione *S*-transferase
HDL	high density lipoprotein
H&E	hemaloxylin & eosin
HEPES	4-2-hydroxyethyl-1-piperazineethanesulfonic acid
HFD	high-fat diet
HRP	horse radish peroxidase
IB	immunoblotting
ICAM-1	intracellular adhesion molecule-1

IDL	intermediate density lipoprotein
IFNγ	interferon γ
IgG	immunoglobulin G
IκB	inhibitor of NF-κB
IKK	IκB kinase
IL	interleukin
iNOS	inducible nitric oxide synthase
IP	immunoprecipitation
IP-10	γ-interferon inducible protein-10
IPTG	isopropyl-β-D-thiogalactopyranosid
IRAK	interleukin 1 receptor-associated kinase
LAL	lysosomal acid lipase
LB	Luria-Bertani
LBD	ligand binding domain
LBP	LPS binding protein
LDL	low density lipoprotein
LDLR	low density lipoprotein receptor
LPS	lipopolysaccharide
LRR	leucine-rich repeat
LUC	luciferase
LXR	liver X receptor
LXRE	LXR response element
MAPK	mitogen-activated protein kinase
MCP	monocyte chemoattractant protein
M-CSF	macrophage colony-stimulating factor
MD-2	MD-2 protein
MEK	MAPK kinase (also designated MAPKK)
MEKK	MEK kinase (also designated as MAPKKK)
MIP	macrophage inflammatory protein
MKP3	MAPK phosphatase 3
MMP	matrix metalloproteinase

MOMA-2	MOnocyte/MAcrophage-2
MyD88	myeloid differentiation factor 88
NCBI	national center for biotechnology information
nCEH	neutral cholesteryl ester hydrolase
NEMO	NF-κB essential modulator
NES	nuclear export signal
NF-κB	nuclear factor κB
NHR	nuclear hormone receptor
NIK	NF-κB inducing kinase
NLS	nuclear localization signal
NO	nitric oxide
OCT	optimum cutting temperature
OD	optical density
ONPG	o-nitrophenyl-β-galactopyranoside
OPN	osteopontin
Opti-MEM	optimal minimum essential medium
ORO	oil red O
oxLDL	oxidized low density lipoprotein
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PE	phycoerythrin
PEST	proline-glutamate/aspartate-serine-threonine
PGN	peptidoglycan
PKB/Akt	protein kinase B
PMA	phorbol-12-myristate-13-acetate
PMN	polymorphonuclear phagocyte
PMSF	phenylmethylsulfonyl fluoride
PPAR	peroxisome proliferator-activated receptor
PPRE	PPAR response element
PTEN	phosphatase and tensin homologue deleted on ch. 10

RANTES	regulated on activation normal T-cell expressed & secreted
RCT	reverse cholesterol transport
RHD	rel homology domain
RIP	receptor interacting protein
RIPA	radioimmune precipitation buffer
RPM	round per minute
RPMI	Roswell park memorial institute medium
RT	reverse transcription
RXR	retinoid X receptor
SDS	sodium dodecyl sulfate
SEM	standard error of mean
siRNA	small interfering RNA
SR-A	scavenger receptor class A
STAT	signal transducer and activator of transcription
STP	serine-, threonine-, and proline-rich region
$t_{1/2}$	half-life
TAB2	TAK1-binding protein 2
TAD	transactivation domain
TAK1	TGFβ activating kinase 1
TF	tissue factor
TBST	tris-buffered saline tween-20
TGF	tumor growth factor
TIMP	tissue inhibitor of matrix metalloproteinases
TIR	toll/interleukin-1 receptor
TLR4	toll-like receptor 4
TNFα	tumor necrosis factor α
TRADD	TNF receptor-associated death domain
TRAF	TNFR associated factor
TZD	thiazolidinedione
UCP	uncoupling protein
VCAM-1	vascular cell adhesion molecule-1

VLDL	very low density lipoprotein
VSMC	vascular smooth muscle cell
WAT	white adipose tissue
WT	wildtype

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INTRODUCTION

Mature macrophages, derived from circulating blood monocytes, have diverse and unique functions in the body. Indeed, macrophages function as a source of chemotactic molecules and cytokines, immune mediators, as well as scavenger cells. During the past two decades, many researchers have focused on identifying some of the roles that macrophages play in the initiation and progression of atherosclerosis. Atherosclerosis, which is responsible for more than 50% of the deaths in North America, Europe, and most of Asia, is a multigenic, progressive disease that starts during childhood (Breslow, 1997; Lusis, 2000). Although it is a metabolic disorder, a large body of research has identified atherosclerosis as a complex, chronic inflammatory disease (Lusis, 2000, Ross, 1999, Steffens and Mach, 2004), in which multiple cell types including macrophages, lymphocytes, mast cells, and vascular smooth muscle cells (VSMCs) are directly involved. Monocyte recruitment, rolling, attachment, penetration of the endothelial layer, and concurrent differentiation into intimal macrophages are imperative determinants in the development of atherosclerosis.

Once fully differentiated in the intima, the innermost layer of arteries, macrophages express a wide range of scavenger receptors that allow uptake and internalization of modified LDL. Lipid accumulation in macrophages leads to the activation of signaling pathways that involve activation of PPARγ1 and LXRα, nuclear receptors that function as transcription factors controlling macrophage cholesterol homeostasis (Fajas *et al.*, 1998, Tontonoz *et al.*, 1998, Venkateswaran *et al.*, 2000a, Chawla *et al.*, 2001c). Ligand-bound, activated PPARγ1 and LXRα are synergistically implicated in the transactivation of several genes whose products are critically involved in mediating cholesterol efflux from macrophages and initiating reverse cholesterol transport to the liver. Genetic defects or pharmacological inhibition of any component of

the macrophage cholesterol efflux pathway leads to an imbalance in cholesterol homeostasis. This eventually leads to massive accumulation of lipids in the cytoplasmic compartment of macrophages, which acquire a foamy appearance and develop into lipid-engorged foam cells, a hallmark of fatty streak and atherosclerotic lesion formation. Thus, macrophages have a central role in atherogenesis not only due to their ability to initiate atherosclerotic lesion formation, but also due to their ability to initiate a pro-inflammatory response in the vasculature, and thus, advancing atherogenesis (Glass and Witztum, 2001; Lusis, 2000).

Since atherosclerosis is a multigenic, complex disease, understanding the roles and expression patterns of genes with known and unknown functions is critical towards understanding the molecular mechanisms underlying atherogenesis. Furthermore, understanding the exact function(s) of key metabolic and inflammatory mediators involved in atherogenesis may allow identification of molecular targets that will allow pharmacological intervention and development of therapeutic strategies towards prevention and/or treatment of atherosclerosis.

1.1 Adipocyte Enhancer-Binding Protein 1 (AEBP1)

1.1.1 Structure and Expression of AEBP1

In human and mouse, the AEBP1 gene is located on chromosome 7 and 11, respectively. By alternative splicing, AEBP1 gene is believed to be transcribed to yield two related, but distinct, transcripts. One transcript is translated into an 82-kDa protein, AEBP1, while the other transcript is translated into a 175-kDa protein, ACLP (Aortic Carboxypeptidase-Like Protein), which has an additional 380-amino acid coding sequence at its N-terminus (Figure 1A) (Ro *et al.*, 2001). Ro's group has determined the structural organization of the AEBP1 gene, which extends over 10 kb and has 21 exons (Ro *et al.*, 2001). The AEBP1 gene is transcribed in such a way that the 9th intron is retained in the mature AEBP1 mRNA. Exon 10 in the AEBP1 gene contains an ATG start codon, which translates into methionine in the AEBP1 protein. Experimental data

indicates that ACLP and AEBP1 transcripts are generated by alternative splicing (Ro *et al.*, 2001).

Unlike ACLP, which is a 175-kDa protein targeted to the extracellular matrix (ECM) (Layne *et al.*, 2001) due to the presence a lysine- and proline-rich 11-amino acid repeating motif and a signal sequence (Layne *et al.*, 1998), AEBP1 is an intracellular protein that exists in the cytoplasm and the nucleus (Park *et al.*, 1999; A. Majdalawieh, unpublished, L. Zhang, unpublished, H. Ma, unpublished). As shown in Figure 1B, the AEBP1 protein is composed of three main domains: a discoidin-like domain (DLD) at its N-terminus, a central carboxypeptidase (CP) domain, and a structurally uncharacterized C-terminal DNA-binding domain (He *et al.*, 1995). The C-terminal domain of AEBP1 is divided into three distinct sub-domains: a lysine- and arginine-rich basic (B) region, a serine-, threonine-, and proline-rich (STP) region, and a glutamate-rich acidic (A) region towards the end (Figure 1B). Because the whole AEBP1 amino acid sequence is encoded in ACLP, both proteins seem to play similar roles in key biological processes, in which both proteins promote proliferation of preadipocytes and inhibit their differentiation into mature, fat-filled adipocytes (He *et al.*, 1995; Gagnon *et al.*, 2002). Nonetheless, AEBP1 and ACLP play very distinctive and unique roles in other biological processes as described later.

AEBP1 is ubiquitously expressed in all mouse tissues and cells tested to date, and its expression seems to be highest in white and brown adipose tissues, liver, lung, spleen, and brain (Ro *et al.*, 2001). Recently, AEBP1 was shown to be abundantly expressed in mammary gland tissue (S. Reidy and colleagues, unpublished) and macrophages (Majdalawieh *et al.*, 2006).

Figure 1. AEBP1 Gene and Protein Structure. *(A)* A schematic representation of the general organization of mouse AEBP1 gene. The mouse AEBP1 genomic DNA contains 21 exons that encode AEBP1 and ACLP transcripts. Both AEBP1 and ACLP transcripts are produced by alternative splicing that allows the former to retain intron 9 (yellow box). AEBP1 transcript is translated to yield a 748-amino acid protein (AEBP1), while ACLP transcript is translated to yield an 1128-amino acid protein (ACLP). This figure is adapted from Ro *et al.*, 2001. *(B)* AEBP1 protein possesses two well-characterized domains, a discoidin-like domain (DLD) at its N-terminus and a central carboxypeptidase (CP) domain. The C-terminus of AEBP1 contains three subdomains: a basic (B) subdomain, a serine-threonine-proline rich (STP) subdomain, and an acidic (A) subdomain.

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A
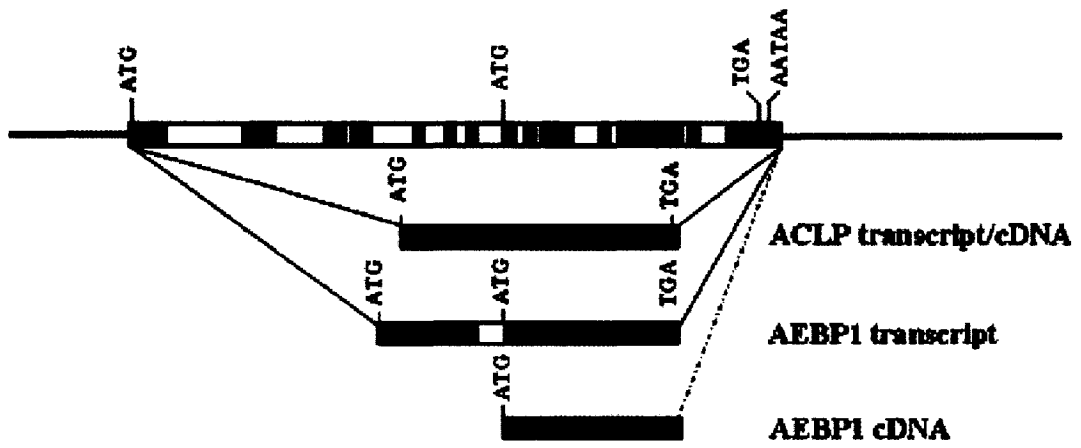
B
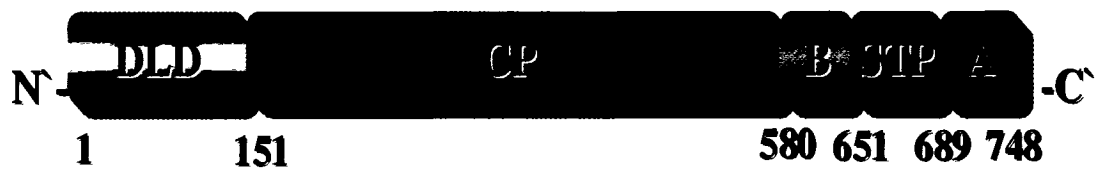
1.1.2   AEBP1 Multifunctionality in Key Biological Processes

1.1.2.1   AEBP1 is a Transcriptional Repressor Involved in Adipogenesis

Adipogenesis is a multistep process that involves growth arrest of preadipocytes and their terminal differentiation into mature adipocytes (Gregoire *et al.*, 1998). During adipogenesis, the expression pattern of several factors is positively or negatively altered (Gregoire *et al.*, 1998). One of the genes that becomes transcriptionally activated during preadipocyte differentiation into adipocytes is aP2, which encodes the adipocyte lipid-binding protein (ALBP) (Bernlohr *et al.*, 1984; Chapman *et al.*, 1984; Hunt *et al.*, 1986). The promoter region of the murine aP2 gene contains a 35-nt adipocyte enhancer-1 (AE-1) sequence (-159 CCAGGGAGAACCAAAGTTGAGAAATTTCTATTAAA -125), which functions as an enhancer (*cis*-controlling) element in the regulation of aP2 (Distel *et al.*, 1987; Herrera *et al.*, 1989; Ro and Roncari, 1991). The expression of AEBP1, which is expressed abundantly in preadipocytes, persists during the first stages of adipogenesis (He *et al.*, 1995; Kim *et al.*, 2001). However, AEBP1 levels drop dramatically as preadipocytes differentiate into mature adipocytes (fat cells), and AEBP1 expression is completely abolished in terminally differentiated, non-proliferative adipocytes (He *et al.*, 1995; Kim *et al.*, 2001, Ro *et al.*, 2001). Because of the altered expression pattern of AEBP1 during adipogenesis, AEBP1 was suspected to play a negative regulatory role in aP2 expression in preadipocytes. Indeed, *in vitro* studies have demonstrated that AEBP1 specifically binds AE-1 DNA sequence (He *et al.*, 1995), and transcriptionally represses aP2 in 3T3-L1 preadipocytes and other cell lines (He *et al.*, 1995; Kim *et al.*, 1999; Lyons *et al.*, 2005).

Transcriptional repression of aP2 by AEBP1 is physiologically significant since targeted over-expression of AEBP1 in adipose tissue leads to diet-induced obesity in mice (Zhang *et al.*, 2005). AEBP1 seems to induce massive obesity in AEBP1^{TG} mice by inducing adipocyte proliferation *in vivo*, leading to adipocyte hyperplasia in white adipose tissue (Zhang *et al.*, 2005). In contrast, AEBP1 deficiency in mice leads to 25% reduction in total body weight due to significantly reduced fat pads via enhanced apoptosis and impaired survival signal (H.S. Ro and colleagues, unpublished). Indeed,

AEBP1-over-expressing preadipocytes display augmented proliferation (Kim *et al.*, 2001; Zhang *et al.*, 2005), while AEBP1-null preadipocytes exhibit a defective proliferative potential *in vitro* (H.S. Ro and colleagues, unpublished).

1.1.2.2 AEBP1 is a Carboxypeptidase

Both zinc metallocarboxypeptidase and serine-carboxypeptidase families of proteins include exopeptidases that are capable of catalyzing the hydrolysis of peptide bonds at the C-terminus of target polypeptides (Vendrell *et al.*, 2000). Zinc metallocarboxypeptidases can be classified in different ways, but they are generally divided into digestive and regulatory subfamilies. The digestive family of carboxypeptidases is subdivided into two main groups: carboxypeptidase A-like (e.g. CPA1 and CPA2), and carboxypeptidase B-like (e.g. CPB and CPH) enzymes. The former has more preference for hydrophobic C-terminal amino acid residues, while the latter favors basic (i.e. lysine and arginine) C-terminal residues (Vendrell *et al.*, 2000). The regulatory (N/E) subfamily of carboxypeptidases include non-digestive enzymes that are capable of catalyzing hydrolysis of C-terminal amino acid residues (e.g. CPN, CPE, CPD, CPM, CPX1, and CPX2), and these enzymes function in a more specific fashion that is important for the function and biosynthesis of target peptides or proteins (Skidgel and Erdos, 1998; Reznik and Fricker, 2001).

AEBP1 shares a remarkable amino acid sequence homology with two members of the regulatory carboxypeptidase family of enzymes, CPX1 and CPX2 (Reznik and Fricker, 2001). AEBP1, CPX1, and CPX2 all contain N-terminal DLD and homologous central CP domain. Despite the fact that CPX1 and CPX2 are catalytically inactive with regard to cleaving C-terminal amino acid residues present in various carboxypeptidase substrates (Xin *et al.*, 1998; Lei *et al.*, 1999), AEBP1 was shown to function as an active carboxypeptidase capable of catalyzing hydrolysis of arginine and lysine in hippuryl-arg and hippuryl-lys synthetic CPB substrates, respectively (He *et al.*, 1995; Muise and Ro, 1999). Studies from Ro's laboratory have demonstrated that deletion of residues 429 to 460 in the CP domain, which encompasses the active site, renders AEBP1 catalytically inactive (He *et al.*, 1995). Moreover, the carboxypeptidase activity of AEBP1 has been

shown to be responsive to carboxypeptidase activators and inhibitors, and that DNA binding enhances AEBP1 hydrolytic activity (Muise and Ro, 1999), indicating that AEBP1 functions as an active carboxypeptidase.

Noteworthy, however, other studies have failed to confirm the carboxypeptidase activity of AEBP1 using different substrates and expression systems (Song and Fricker, 1997; Reznik and Fricker, 2001). Fricker and his group argue that the carboxypeptidase active site in AEBP1, as well as that of CPX1 and CPX2, lacks key residues that are essential for carboxypeptidase activity, and they suggest that these proteins may function as mediators of protein-protein interaction rather than functional carboxypeptidases (Reznik and Fricker, 2001). Recently, Dr. Ro's group has demonstrated that the putative active site of AEBP1 has distinctive characteristics compared to the active sites of other carboxypeptidases (Lyons et al., 2006). In particular, some critical residues within the active site of most functional carboxypeptidases are altered in the putative active site of the carboxypeptidase domain of AEBP1 (Lyons et al., 2006). Lyons and colleagues argue that such alterations may interfere with zinc coordination, which is required for the enzymatic activity to take place, and/or the catalytic mechanism leading to peptide cleavage (Lyons et al., 2006). Furthermore, based on protein modeling and sequence analysis of AEBP1, Lyons and colleagues speculate that AEBP1 may have specificity towards N-terminal substrate residues (Lyons et al., 2006).

1.1.2.3 AEBP1 is a Modulator of MAPK Activity

Mitogen-activated protein kinase (MAPK) pathways are a network of serine/threonine kinases and dual-specificity kinases, whose function is implicated in various key biological processes in the cell including proliferation, inflammation, and tumorigenesis (Mansour et al., 1994; Wilkinson and Miller, 2000; Platanias, 2003; Roux and Blenis, 2004). Kinases involved in MAPK pathways include JNK1/2, Erk-1/2, and other MAP kinases (MEK, MEKK, MEKKK,...., etc.). The endpoints of cascades initiated by such kinases include active transcription factors that regulate transactivation of diverse genes implicated in key biological processes such as inflammation, cell

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growth, proliferation, and immune responses (Mansour *et al.*, 1994; Wilkinson and Miller, 2000; Platanias, 2003; Roux and Blenis, 2004).

Extracellular signal-regulated kinases (Erk-1 and Erk-2) are activated by phosphorylation at tyrosine and threonine residues by the upstream dual specificity MAPK kinase (MAPKK or MEK) (Mansour *et al.*, 1994; Cobb and Goldsmith, 1995; Elion, 1998). Unlike sustained phosphorylation of Erk-1/2, transient phosphorylation is insufficient in inducing Erk-1/2 translocation into the nucleus (Alessi *et al.*, 1995). Erk-1/2 dephosphorylation by specific phosphatases leads to its inactivation. Studies have shown that MKP3 (or PYST1) is a dual-specificity cytoplasmic phosphatase that is capable of hydrolyzing the phosphate groups on phosphorylated T^{183} and T^{185} in Erk-1, and T^{202} and T^{204} in Erk-2 (Groom *et al.*, 1996; Mourey *et al.*, 1996; Muda *et al.*, 1996). Balanced regulation of MAPK activity is critical in turning several transcription factors involved in key biological processes on and off. Importantly, MAPK signaling pathways engage in a crosstalk with other signaling pathways, such as NF-κB signaling pathway (Lin and Weiss, 2001). Thus, modulation of MAPK activity may lead to modulation of transcriptional activity of many transcription factors whose function is not directly dependent on MAPK activity.

In vitro and *in vivo* experimental studies revealed that AEBP1 physically interacts with Erk-1/2 via its DLD (Kim *et al.*, 2001). This protein-protein interaction is critical for MAPK activity since it leads to protection of Erk-1/2 from dephosphorylation by its specific phosphatase (MKP3), leading to sustained activation of Erk-1/2 (Kim *et al.*, 2001). In fact, modulation of Erk-1/2 activity by AEBP1 is not associated with its transcriptional repression function. Yet, AEBP1 inhibits differentiation of preadipocytes into mature adipocytes, thus impeding adipogenesis, by means of enhancing Erk-1/2 activity in preadipocytes (Kim *et al.*, 2001). Hence, AEBP1-mediated transcriptional repression of aP2 and AEBP1-mediated sustained activation of MAPK, which impedes adipogenesis (Hu *et al.*, 1996; Font de Mora *et al.*, 1997), seem to be coordinate regulatory functions that allow AEBP1 to serve as an inhibitor of adipogenesis.

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1.1.2.4 The Role of AEBP1 in Mammary Gland Development

The mammary gland is a structurally complex, branched tubuloalveolar structure that changes in morphology during various stages of life. The mammary gland can be divided into two main compartments: (1) the parenchyma, which consists of branching ducts and alveoli, and (2) the fat-pad stroma, which surrounds the parenchyma (Sheffield, 1988; Bissell, 1998). At the onset of puberty, the mammary gland in females is signaled to grow and develop via estrogen action on mesenchymal cells (Korach *et al.*, 1996). Mammary gland development is a dynamic process, in which several morphological and cellular changes take place reversibly during regular menstrual cycles, pregnancy, lactation, and weaning (Hovey and Trott, 2004).

During mammary gland development, epithelial cell proliferation, basement membrane remodeling, and deposition of interlobular fat occur, leading to enlargement of the mammary gland (Barcellos-Hoff *et al.*, 1989; Bissell, 1998). During the last three days of pregnancy in mice (i.e. days 19-21), extensive growth and differentiation of the mammary gland takes place, and this ultimately leads to the formation of milk-filled alveolar buds, which differentiate into lobular-alveolar structures that eventually give rise to milk-secreting lobules during lactation (Richert *et al.*, 2000). During lactation, which normally takes about three weeks in mice (Richert *et al.*, 2000), milk, consisting of milk proteins and lipids, is produced and secreted by alveolar epithelial cells (Mather and Keenan, 1998). Pup weaning induces a biologically complex process called involution, in which alveolar structures collapse and the mammary gland regains its original, pre-pregnant morphology (Green and Streuli, 2004). Mammary gland involution is a process that involves epithelial cell apoptosis and tissue remodeling, in which epithelial alveolar structures are replaced by adipose tissue (Marti *et al.*, 1994; Lund *et al.*, 1996).

Upon generation of AEBP1$^{-/-}$ mice (H.S. Ro and colleagues, unpublished), it was realized that these mice are unable to nurse their pups which die within 24-48 hr, due to the lack of milk supply, if not fostered to a wildtype nursing mother (S. Reidy and colleagues, unpublished). This is an indication of defective mammary gland development during pregnancy and/or lactation. Further analysis revealed that ductal branching and alveolar development occur normally in AEBP1$^{-/-}$ pregnant females. However,

histopathological examination revealed that right after birth, AEBP1$^{-/-}$ mammary glands exhibit signs of premature involution, characterized by collapsed alveolar structures and epithelial hypotrophy (reduced cell number) and featured by increased levels of phospho-STAT3, C/EBPδ, and cleaved-caspase-3 (S. Reidy and colleagues, unpublished), markers of apoptosis (Li et al., 1996; Philp et al., 1996; Gigliotti and DeWille, 1998; Chapman et al., 1999; Green and Sreuli, 2004). Moreover, AEBP1^{TG} females, which express about 3-fold higher AEBP1 levels in their mammary glands compared to their AEBP1^{NT} female counterparts, exhibit mammary epithelial cell hyperplasia (L. Zhang and colleagues, unpublished), an early sign of mammary tumorigenesis (Cardiff and Aguilar-Cordova, 1988; Medina, 2002). Epithelial cell hyperplasia results from accelerated epithelial cell proliferation, leading to the formation of multi-layer epithelial structure surrounding the lumen (Medina, 2002). Hence, aberrant mammary gland development displayed by AEBP1$^{-/-}$ pregnant females and epithelial cell hyperplasia displayed by AEBP1^{TG} females indicate that AEBP1 plays a critical role in mammary gland development and tumorigenesis.

1.2 Atherosclerosis

Atherosclerosis is a term derived from two Greek words, "athero" for paste, and "sclerosis" for hardening. Basically, atherosclerosis is a medical condition describing hardening of the arteries, and it is the primary cause of heart disease and stroke (Lusis, 2000). Atherosclerosis is a killer disease responsible for more than 50% of deaths in the developed world (Breslow, 1997; Lusis, 2000). Atherosclerosis is associated with various medical conditions including obesity, diabetes mellitus, hypertension (Ross, 1999; Libby et al., 2002), as well as viral and bacterial infections (Libby et al., 1997; Hendrix et al., 1990; Jackson et al., 1997).

Although some researchers seem to focus on atherosclerosis as either a metabolic/lipid disorder or an inflammatory disorder, there is a general consensus among most investigators that atherosclerosis is a complex disease involving both metabolic and inflammatory dysfunctions (Steinberg, 2002).

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1.2.1 Anatomical Structure of the Arterial Wall

As for all other major types of blood vessels, major arteries have walls that are composed of three main layers (or tunics): tunica intima, tunica media, and tunica adventitia (Figure 2) (Hungerford and Little, 1999). The tunica intima is the innermost layer of the arterial wall, and it is represented by a monolayer of flattened, squamous endothelial cells that is supported by loose connective tissue that lies sub-endothelially and underlined with a basement membrane composed on elastic fibers. So, the endothelial monolayer of the intima forms a central tube lining the lumen of blood vessels, and circulating blood is in constant contact with this endothelial monolayer. The basement membrane of the tunica intima, or the internal elastic lamina, is a sheet composed of elastic fibers, and it separates the tunica intima from the tunica media. The tunica media is the thickest layer of the arterial wall, and medial smooth muscle cells are interspersed with some collagen fibers, elastic fibers, and proteoglycans. External elastic lamina is a sheet composed of elastic fibers that separate the tunica media from the tunica adventitia. The tunica adventitia is the outermost layer of the arterial wall and is composed mainly of fibroblasts, collagen fibers, and elastic fibers. The tunica adventitia is in direct contact with the connective tissue surrounding arteries. Although it is more common in veins, the vasa vasorum is a network of small arteries that nourish cells within the tunica media and tunica adventitia that can be devoid of sufficient oxygen supply due the thickness of these layers, which are not in direct contact with circulating blood in the lumen (Williams and Heistad, 1996).
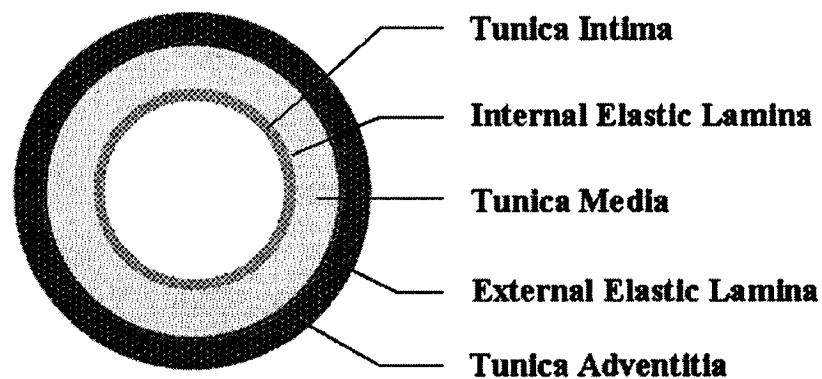
Figure 2. Anatomical Structure of the Arterial Wall.
The wall of large arteries such as the aorta is composed of
three main layers: tunica intima, tunica media, and tunica
adventitia. The tunica intima, a single layer of endothelial
cells, is the innermost layer of the arterial wall, and it sits
on a membrane called the basement membrane. The
basement membrane of the tunica intima, or the internal
elastic lamina, is a sheet composed of elastic fibers, and it
separates the tunica intima from the tunica media. The
tunica media is the medial layer of the arterial wall, and
hence its name. External elastic lamina is a sheet composed
of elastic fibers that separate the tunica media from the
tunica adventitia. The tunica adventitia is the outermost
layer of the arterial wall and is composed mainly of
fibroblasts, collagen fibers, and elastic fibers.

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1.2.2 Atherosclerosis is a Metabolic Disease

Many studies have confirmed that significant alteration in plasma cholesterol levels is a trigger for the development of atherosclerosis, and hypercholesterolemia is considered by many researchers as an initiator of atherosclerosis (Steinberg, 1989; Steinberg, 2002). In humans, a 10% reduction of plasma cholesterol levels is accompanied by about 15% reduction in coronary heart disease death cases associated with atherosclerosis (Gould et al., 1998). High cholesterol level in the blood of children with familial hypercholesterolemia is sufficient on its own to account for the development of atherosclerosis in such, otherwise healthy, young CHD patients (Steinberg, 2002). Another piece of evidence indicating that hypercholesterolemia is a sufficient cause of atherosclerosis originates from the identification of LDLR mutations in humans with extremely high plasma cholesterol levels (especially LDL cholesterol) and who develop atherosclerosis (Brown and Goldstein, 1986).

It is important to mention that atherosclerosis manifests itself as a very complex disease with many known and unknown factors involved, and that hypercholesterolemia cannot stand on its own as the only cause of atherosclerosis. This becomes very evident considering the fact that familial hypercholesterolemia siblings, who have almost identical plasma cholesterol profiles, may respond in a completely different manner with regard to development of atherosclerosis, in which one may die by myocardial infarction secondary to atherosclerosis before the age of 10 years, while the other can survive more than five decades (Goldstein et al., 1995). It is also important to mention that although high HDL levels is normally considered protective against atherosclerosis, mice that lack ApoAI expression, and thus have extremely low HDL levels, do not develop atherosclerotic lesions (Li et al., 1993b). However, mice that have very high LDL levels due to ApoE deficiency develop advanced lesions at a young age (Plump et al., 1992); indicating that low HDL levels cannot lead to atherosclerosis unless they are accompanied by high LDL levels.

Since foam cell formation is a key event in atherogenesis, inhibition of any of the pathways that are considered prerequisite for foam cell formation should result in athero-protection. As discussed above, uptake of oxLDL by intimal macrophages is the first

trigger for foam cell formation, and subsequently, lipid-filled lesion formation. Interestingly, ApoE$^{-/-}$ mice that are deficient in 12/15-lipooxygenase, which is mainly responsible for LDL oxidation *in vivo* (Kuhn *et al.*, 1994; Folcik *et al.*, 1995), have very tiny lesions compared to ApoE$^{-/-}$ mice with normal 12/15-lipooxygenase expression (Cyrus *et al.*, 1999). This indicates that oxidation of LDL is essential for the development of atherosclerosis. Nonetheless, the HDL:LDL ratio is critical in determining the fate of LDL since HDL plays atheroprotective roles not only by removing excess cholesterol from peripheral tissues, but also by reducing the oxLDL:LDL ratio. Indeed, HDL carries an esterase called paraoxonase, which is capable of hydrolyzing oxidized lipids of oxLDL and other biologically active oxidized lipid remnants (Hegele, 1999). Thus, paraoxonase protects LDL against oxidation. Interestingly, paraoxonase deficiency in ApoE$^{-/-}$ mice results in significantly enhanced LDL oxidation, and renders mice more susceptible to atherosclerosis (Shih *et al.*, 2000). Finally, it is compelling that high levels of βVLDL-chylomicron remnants and IDL, caused by ApoE or LDLR deficiency, are accompanied by the development of advanced atherosclerotic lesions in mice (Plump *et al.*, 1992; Zhang *et al.*, 1992; Ishibashi *et al.*, 1993). Clearly, genetic and environmental factors that cause an imbalance in plasma lipoprotein levels can inevitably lead to the development of atherosclerosis.

1.2.3 Atherosclerosis is an Inflammatory Disease

Until the end of the 1980s, the relationship between lipoprotein metabolism and atherosclerosis has kept researchers busy trying to dissect and pinpoint the details underlying the process of atherogenesis in the context of dyslipidemia and metabolic dysfunction. However, during the past two decades, mounting experimental evidence has been gathered suggesting an intimate relationship between inflammation and atherosclerosis (Ross, 1999; Lusis, 2000; Libby, 2002; Steinberg, 2002). The use of murine systems that are deficient in one of many immune and inflammatory genes has convinced researchers that atherosclerosis is a multifaceted disease with two main arms, metabolic and inflammatory. As Daniel Steinberg put it, hypercholesterolemia and inflammation are partners in crime (Steinberg, 2002).

Initial identification of a possible role of inflammation in atherosclerosis originated from microscopic analysis revealing prominent attachment of leukocytes to the endothelial monolayer of the intima upon challenging rabbits with an atherogenic diet (Poole and Florey, 1958). Normally, circulating blood leukocytes have no ability to attach to the endothelial monolayer due to insufficient expression of endothelial-leukocyte adhesion molecules (Libby, 2002). Monocytes, macrophages, lymphocytes, and mast cells seem to be involved in a complex interplay with endothelial cells and VSMCs leading to aggravated inflammatory response within the inflamed vasculature (Ross, 1993; Libby et al., 2002; Binder et al., 2002).

VCAM-1 and ICAM-1, which play crucial roles in leukocyte attachment to the endothelial monolayer of the intima, are among the most important mediators of inflammation in atherosclerosis since upon feeding an atherogenic diet to rabbits, their expression on endothelial cells is up-regulated in certain areas of intima that confers a suitable microenvironment for lesions to form (Cybulsky and Gimbrone, 1991; Nakashima et al., 1998). Indeed, VCAM-1 and ICAM-1 expression is selectively detectable in parts of the endothelial monolayer where lesions with intimal foam cell infiltrates are evident in ApoE$^{-/-}$ mice (Nakashima et al., 1998). To signify the role of VCAM-1 in early atherosclerosis, LDLR$^{-/-}$ mice were engineered to express a mutant, non-functional form of VCAM-1 (designated VCAM-1^{D4D}), which compensates for the lethality caused by complete abrogation of the VCAM-1 gene in mice. Compared to control LDLR$^{-/-}$ mice, mice expressing VCAM-1^{D4D} develop lesions that are 2-fold smaller, while plasma cholesterol profile is almost identical in VCAM-1^{D4D} and wildtype mice (Cybulsky et al., 2001). Similarly, ICAM-1 deficiency renders mice about 60% less susceptible to atherosclerotic lesion formation (Nageh et al., 1997; Collins et al., 2000). Moreover, ablation of P-selectin and E-selectin, which are adhesion molecules that allow leukocyte rolling on the endothelial monolayer of the intima, results in 5-fold smaller lesions in LDLR$^{-/-}$ mice (Dong et al., 1998). Studies performed in ApoE$^{-/-}$ mice also demonstrate that deficiency of P-selectin and/or E-selectin leads to smaller lesions, with deficiency of the former being more protective against atherosclerosis (Collins et al., 2000). Hence, it is obvious that adhesion molecules that confer capability of monocytes

and other leukocytes to roll and adhere to the endothelial monolayer of the intima play significant functions in the development of atherosclerosis (Blankenberg *et al.*, 2003).

MCP-1 is a CC chemokine that plays an integral role in monocyte recruitment from the blood stream into the site of lesion formation. Studies have shown the MCP-1 expression is elevated within atherosclerotic lesions (Yia-Herttuala *et al.*, 1991; Nelken *et al.*, 1991). MCP-1 is a secretory molecule that can bind specifically to its CC receptor, CC chemokine receptor 2 (CCR2), *in vitro* (Kurihara and Bravo, 1996). CCR2 deficiency in mice leads to a severe reduction in leukocyte adhesion and impaired monocyte extravasation (Boring *et al.*, 1997; Kurihara *et al.*, 1997; Kuziel *et al.*, 1997), indicating that CCR2 functions as a receptor for MCP-1 *in vivo*. The first set of studies suggesting a role of MCP-1 in atherosclerosis relied on analyzing the effect of CCR2 deficiency in ApoE$^{-/-}$ mice. Independent laboratories have shown that although CCR2 deficiency has no effect on plasma lipoprotein levels, CCR2$^{-/-}$/ApoE$^{-/-}$ mice develop significantly smaller, less advanced lesions compared to ApoE$^{-/-}$ controls (Boring *et al.*, 1998; Dawson *et al.*, 1999). Moreover, bone marrow transplantation (BMT) experiments revealed that injection of bone marrow cells from CCR2$^{-/-}$ mice into ApoE3-leiden mice, a mouse strain susceptible to diet-induced atherosclerosis, is accompanied by about 85% reduction in mean lesion area (Guo *et al.*, 2003). Such findings clearly indicate a role of MCP-1 in the progression of atherosclerosis. However, a more direct evidence of a crucial role of MCP-1 in atherosclerosis came from MCP-1 knockout models. Ablation of the MCP-1 gene not only leads to impaired monocyte recruitment (Lu *et al.*, 1998), but it also leads to inhibition of atherosclerosis in murine models of diet-induced atherosclerosis including LDLR$^{-/-}$ (Gu *et al.*, 1998) and HuBTg (Gosling *et al.*, 1999) mice. In contrast, transgenic over-expression of MCP-1 in ApoE$^{-/-}$ mice promotes progression of atherosclerosis by increasing the number of macrophages and oxidized lipoproteins in the inflamed intima, with no drastic change to plasma lipoprotein profile (Aiello *et al.*, 1999). These studies have led to the development of anti-MCP-1 gene therapies, in which blocking antibodies directed against MCP-1 successfully eliminated lesion formation in ApoE$^{-/-}$ mice (Ni *et al.*, 2001; Inoue *et al.*, 2002). Thus, experimental evidence implicating MCP-1 and its receptor, CCR2, in monocyte recruitment in atherosclerosis is very convincing.

A wide range of other pro-inflammatory mediators are implicated in the progression of atherosclerosis, including IL-1β, IL-6, IL-8, TNFα, TGFβ, M-CSF, iNOS, and COX-2 (van Reyk and Jessup, 1999). The expression of such pro-inflammatory mediators co-localizes with intimal macrophages within atherosclerotic lesions, suggesting their involvement in the inflammatory response accompanying atherosclerosis (Rosenfeld *et al.*, 1992; Kishikawa *et al.*, 1993; Tipping and Hancock, 1993; Rayment *et al.*, 1996; Apostolopoulos *et al.*, 1996; Luoma *et al.*, 1998; Sukovich *et al.*, 1998; Baker *et al.*, 1999). Knockout systems of specific inflammatory genes provided invaluable insight into the pro-atherogenic roles of such gene products. For example, iNOS$^{-/-}$/ApoE$^{-/-}$ mice, which have plasma lipoprotein levels comparable to those of control mice, develop about 40% smaller lesions (Detmers *et al.*, 2000; Kuhlencordt *et al.*, 2001). Similarly, TNFα deficiency in ApoE$^{-/-}$ mice results in 50% reduction of mean lesion area compared to control mice, with no effect on plasma lipoprotein profile (Branen *et al.*, 2004). Interestingly, transplantation of bone marrow isolated from TNFα$^{-/-}$ mice into ApoE$^{-/-}$ almost completely abolished lesion formation (Branen *et al.*, 2004). Furthermore, deficiency of IL-1β in ApoE$^{-/-}$ mice renders such mice less susceptible to lesion formation, despite a lack of effect on plasma lipoprotein levels (Kirii *et al.*, 2003). The use of pharmacological inhibitors of COX-2 has proven helpful in understanding the role of COX-2 in the development of atherosclerosis. LDLR$^{-/-}$ and ApoE$^{-/-}$ mice challenged with COX-2 inhibitors display significant regression (~ 50%) of lesions, compared to control mice challenged with a placebo (Burleigh *et al.*, 2002; Burleigh *et al.*, 2005a; Burleigh *et al.*, 2005b). Findings based on genetic deletion of IL-6 gene in murine models of atherosclerosis have been somewhat controversial, however. Two independent studies performed on IL-6$^{-/-}$/ApoE$^{-/-}$ mice revealed that IL-6 deficiency leads to significant enlargement of atherosclerotic lesions compared to control mice (Elhage *et al.*, 2001; Schieffer *et al.*, 2004). Even more controversial is that one of the two studies revealed that IL-6 deficiency does not affect plasma lipoprotein profile (Elhage *et al.*, 2001), while the other study suggested that IL-6 deficiency is accompanied by significantly elevated plasma lipoprotein levels (Schieffer *et al.*, 2004). So, the exact role of IL-6 in the progression of atherosclerosis is yet to be clarified. Nonetheless, the use of genetic deficient models and pharmacological inhibitors provides convincing evidence

suggesting a positive relationship between the role of pro-inflammatory mediators and development of atherosclerosis.

Expression and secretion of various pro-inflammatory mediators by different cell types (e.g. macrophages, T lymphocytes, endothelial cells, VSMCs, ..., etc.) within the atherosclerotic lesion promote further recruitment of leukocytes, advancing lesion development. Pro-inflammatory mediators can also advance atherosclerosis by further activating macrophages and T lymphocytes to express pro-atherogenic factors including COX-2 (Linton and Fazio, 2003), nitric oxide (NO) (Wennmalm, 1994), matrix metalloproteinases (MMP-1, MMP-2, MMP-3, MMP-9) (George 1998), IFNγ (Gupta et al., 1997), MIP-1α, MIP-1β, and RANTES (Reape et al., 1999). Due to their imperative roles in all stages of atherosclerosis, special attention has been given to pro-inflammatory mediators including IL-1β, IL-6, TNFα, P-selectin, VCAM-1, and ICAM-1 as biomarkers for predicting risks associated with atherosclerosis (Blake and Ridker, 2001). Collectively, this body of evidence points to many pro-inflammatory candidates as putative targets for therapeutic intervention using anti-inflammatory approaches aimed at preventing or treating atherosclerosis.

1.2.4 Murine Models of Atherosclerosis

During the past two decades, the development of murine models of atherosclerosis has provided insights into different mechanisms underlying atherosclerotic lesion formation in mice. Importantly, in contrast to humans, mice are naturally resistant to the development of atherosclerosis due to the fact that they have very low LDL and high HDL plasma levels (Zhang et al., 1992; Breslow, 1996). When fed low-cholesterol, low-fat diets, mice typically have cholesterol levels of less than 100 mg/dL, and the majority of that cholesterol is contained in the HDL. However, pioneering work has focused on the generation of knockout and transgenic systems as well as the use of modified diet high in cholesterol and fat to provide in vivo tools to elucidate mechanisms underlying atherogenesis. Such animal models have been proven very beneficial for identifying pro-atherogenic and anti-atherogenic roles of various specific genes. Despite the availability of other engineered murine systems, ApoE$^{-/-}$ and LDLR$^{-/-}$

mice remain to be the most well-characterized and accepted murine models of atherosclerosis, and they have been used extensively to study environmental and genetic determinants associated with atherosclerosis (Breslow, 1996).

1.2.4.1 ApoE-Deficient (ApoE$^{-/-}$) Mice

About 15 years ago, two groups generated ApoE-deficient (ApoE$^{-/-}$) mice by homologous recombination in embryonic stem cells (Piedrahita et al., 1992; Plump et al., 1992). ApoE$^{-/-}$ mice are raised on a C57/BL6 strain background, they seem to have no developmental abnormalities, and they are viable with no occurrence of premature sudden death events (Piedrahita et al., 1992; Plump et al., 1992; Zhang et al., 1992). Due to the importance of ApoE in the binding of chylomicrons remnants and VLDL remnants (i.e. IDL and LDL) to receptors, ApoE$^{-/-}$ mice suffer from hypercholesterolemia even when fed on low-fat chow diet (4.5% fat, 0.02% cholesterol). In fact, ApoE$^{-/-}$ mice have plasma cholesterol levels that are 8-fold higher than wildtype mice (~ 500 mg/dL in ApoE$^{-/-}$ mice versus 60 mg/dL in wildtype mice) (Plump et al., 1992). This difference in plasma cholesterol levels between ApoE$^{-/-}$ and wildtype mice almost doubles when the mice are challenged with Western, high-fat diet (21% fat, 0.15% cholesterol) (~ 1800 mg/dL in ApoE$^{-/-}$ mice versus ~ 130 mg/dL in wildtype mice). The majority of elevated cholesterol levels in ApoE$^{-/-}$ mice is represented by VLDL and IDL (Plump et al., 1992). Interestingly, these plasma cholesterol levels are not subject to influence by age or sex (Zhang et al., 1992). Notably, mice that are heterozygous for the ApoE gene knockout (i.e. ApoE$^{+/-}$) have plasma cholesterol levels that are comparable to wildtype mice, indicating that lack of both ApoE alleles is essential for hypercholesterolemia to develop.

Due to the severe hypercholesterolemia displayed by ApoE$^{-/-}$ mice, ApoE deficiency predisposes mice to the formation of atherosclerotic lesions in their proximal aortae, as judged by oil red O staining (Plump et al., 1992). Interestingly, ApoE$^{-/-}$ mice develop atherosclerosis regardless of whether they are fed low- or high-fat diet. However, the mean lesion area is about 3-fold larger when the mice are challenged with high-fat diet (Plump et al., 1992). Although most lesions are present in the proximal aorta, some lesions can also be detected in the coronary and pulmonary arteries. Atherosclerotic

lesions that develop in ApoE$^{-/-}$ mice are typical of those found in humans (Nakashima *et al.*, 1994; Reddick *et al.*, 1994). Although atherosclerotic lesions can be detected when ApoE$^{-/-}$ mice are about 10 weeks old, well-characterized fibrous lesions with necrotic cores do not develop until 20 weeks of age (Breslow, 1996). Interestingly, and consistent with plasma cholesterol levels, there seems to be no gender-specific differences with regard to susceptibility to atherosclerotic lesion formation in ApoE$^{-/-}$ mice (Zhang *et al.*, 1992). The fact that ApoE deficiency leads to early development of atherosclerotic lesions coupled with the fact that lesions develop even when the mice are fed on low-fat chow diet makes ApoE$^{-/-}$ mice a favorable *in vivo* murine model of atherosclerosis.

The significant role of ApoE in protection against atherosclerosis can be further demonstrated by studies utilizing bone marrow transplantation techniques to assess ApoE function in atherogenesis. Linton and colleagues have shown that transplantation of wildtype macrophages isolated from C57/BL6 mice into ApoE$^{-/-}$ mice leads to restoration of normal plasma cholesterol levels (~ 125 mg/dL) and almost complete protection against atherosclerosis (Linton *et al.*, 1995; Boisvert *et al.*, 1995). Consistently, reconstitution of C57/BL6 wildtype mice with ApoE$^{-/-}$ macrophages results in 10-fold increased susceptibility to develop atherosclerosis, but with no significant change in plasma cholesterol profile (Fazio *et al.*, 1997).

1.2.4.2 LDLR-Deficient (LDLR$^{-/-}$) Mice

LDLR$^{-/-}$ mice are considered the second most well-characterized and recognized murine model of atherosclerosis. In 1993, Ishibashi and colleagues generated LDLR$^{-/-}$ mice by homologous recombination in embryonic stem cells (Ishibashi *et al.*, 1993). Like ApoE$^{-/-}$ mice, LDLR$^{-/-}$ mice develop normally with no signs of embryonic lethality or infertility. In humans, rabbits, and monkeys, genetic defects in LDLR leads to severe hypercholesterolemia (Watanabe *et al.*, 1985; Scanu *et al.*, 1988; Hobbs *et al.*, 1992). Likewise, low-fat diet-fed LDLR$^{-/-}$ mice have 2-fold higher plasma cholesterol levels compared to wildtype mice (~ 230 mg/dL versus ~ 110 mg/dL, respectively) (Ishibashi *et al.*, 1993). LDLR heterozygotes have plasma cholesterol levels that fall in between those of LDLR$^{-/-}$ and wildtype mice. Both LDLR$^{-/-}$ males and females have comparably

elevated cholesterol levels compared to their wildtype counterparts (Ishibashi *et al.*, 1993). VLDL and IDL are the major type of lipoproteins leading to hypercholesterolemia in $LDLR^{-/-}$ mice, which have HDL levels that are equivalent to those of wildtype mice. However, challenging $LDLR^{-/-}$ mice with high-fat, high-cholesterol diet (21% fat, 1.25% cholesterol, and 0.5% cholate) leads to about 6-fold elevation of plasma cholesterol levels (~ 1500 mg/dL on high-fat, high-cholesterol diet versus ~ 230 mg/dL on low-fat, low-cholesterol diet), with a decreased HDL portion (Ishibashi *et al.*, 1994). The reason for the increased severity of ApoE deficiency compared to LDLR deficiency with regard to hypercholesterolemia in mice is that ApoE is capable of binding both LDLR and chylomicron remnant receptor, whereas LDL can only bind LDLR (Ishibashi *et al.*, 1993).

In contrast to $ApoE^{-/-}$ mice, $LDLR^{-/-}$ mice do not develop atherosclerotic lesions in their aortae when they are fed low-fat, low-cholesterol diet (Ishibashi *et al.*, 1994). However, analysis of 28-32-week old $LDLR^{-/-}$ mice challenged with high-fat, high-cholesterol diet revealed the formation of lipid-filled lesions with evidence of intimal foam cells within the aortic root (Ishibashi *et al.*, 1994). As in $ApoE^{-/-}$ mice, $LDLR^{-/-}$ mice develop typical lesions in that they have necrotic cores with foam cell infiltrate surrounded with a fibrous cap. Unlike $ApoE^{-/-}$ mice, $LDLR^{-/-}$ mice do not develop distal coronary or pulmonary lesions. Importantly, LDLR deficiency does not discriminate between males and females with regard to predisposing mice to the development of atherosclerotic lesions (Ishibashi *et al.*, 1994). Thus, $LDLR^{-/-}$ mice provide a favorable alternative to $ApoE^{-/-}$ mice as a murine model of diet-induced atherosclerosis. Interestingly, hepatic over-expression of ApoE in $LDLR^{-/-}$ mice leads to a significant inhibition of atherosclerotic lesion formation without reducing plasma cholesterol levels, indicating that ApoE can play functions other than controlling lipoprotein metabolism (Tsukamoto *et al.*, 2000).

1.2.5 Pro-atherogenic Roles of oxLDL

Besides its fundamental role in inducing cholesterol uptake by macrophages and thereby promoting foam cell formation in the aortic intima, an enormous number of

studies have shown that oxLDL play potential pro-atherogenic functions that speed up the atherogenic process (Steinberg, 2002). One of major functions attributed to oxLDL is its ability to attract circulating monocytes to the inflamed intima by means of inducing the expression of MCP-1 and M-CSF (Navab *et al.*, 1991), promoting monocyte adherence to the endothelial monolayer inducing the expression of VCAM-1 (Cybulsky and Gimbrone, 1999; Amberger *et al.*, 1997). OxLDL also induces the production of IL-1β and IL-8 (Terkeltaub *et al.*, 1994), NO (Murohara *et al.*, 1994), and MMP-2 (Rajavashisth *et al.*, 1999). Interestingly, NF-κB activity seems to be augmented in presence of oxLDL (Brand *et al.*, 1997a), which likely accounts for the oxLDL-mediated induced expression of pro-inflammatory mediators. Additionally, the apoprotein components of oxLDL can be modified and become immunogenic, and thus, they can induce a localized T lymphocyte-mediated immune reaction that can amplify the chronic inflammatory response within the inflamed intima, advancing lesion development (Stemme *et al.*, 1995; Binder *et al.*, 2002). Moreover, oxLDL has been shown to cause cytotoxicity for endothelial cells, VSMCs, and macrophages that are localized within atherosclerotic lesions (Martinet and Kockx, 2001), most likely by inducing Fas-mediated apoptosis (Sata and Walsh, 1998). Apoptotic cells within atherosclerotic lesions release enzymes and other biological factors that advance lesion development and may eventually lead to lesion rupture and atherothrombosis (Martinet and Kockx, 2001). Apparently, oxLDL contributes to lesion development by promoting foam cell formation, as well as promoting an exaggerated inflammatory response within atherosclerotic lesions.

1.3 Monocyte Recruitment and Differentiation into Intimal Macrophages

Monocyte recruitment and subsequent differentiation into intimal macrophages is a very complex process that is integral to the initiation and progression of atherosclerosis (Figure 3). In the early stages of atherosclerosis, circulating monocytes are recruited to certain areas within the luminal endothelium of major arteries. It is believed the production of MCP-1, M-CSF, GM-CSF, and other chemoattractants (Takahashi *et al.*, 2002; Steffens and Mach, 2004; Zernecke and Weber, 2005), as well as physical injury

and endothelial stress (Ross and Golmset, 1973; Ross, 1993) lead to monocyte recruitment and subsequent differentiation at those areas. Several proteins belonging to the selectin family of cellular adhesion molecules are expressed on endothelial cells (P-selectin and E-selectin) and monocytes (L-selectin), and such molecules are involved in interactions that allow monocytes to roll along the endolethlial monolayer (Springer, 1990; Price and Loscalzo, 1999). However, such interactions between selectin proteins and their receptors are not strong enough to allow monocyte adherence to the endothelium. Yet another set of proteins expressed on monocytes (β-integrins) and endothelial cells (cellular adhesion molecules such as vascular cell adhesion molecule 1, VCAM-1, and intracellular adhesion molecule 1, ICAM-1) allow firm attachment of monocytes to the endothelial monolayer of arteries (Springer, 1990; Price and Loscalzo, 1999). Once they are firmly attached, monocytes start migrating and penetrating the endothelial monolayer by means of various factors including MCP-1, M-CSF, GM-CSF, MIP-1, RANTES, TGFβ, IL-1β, IL-6, IL-12, and TNFα that are produced by monocytes, lymphocytes, and endothelial cells (Takahashi et al., 2002; Steffens and Mach, 2004; Zernecke and Weber, 2005).

As monocytes penetrate the endothelial monolayer, they start expressing cell surface molecules that are characteristic of macrophages, and the differentiation process is underway (Takahashi et al., 2002; Steffens and Mach, 2004; Zernecke and Weber, 2005; Bobryshev, 2006). However, active monocyte differentiation into macrophages intensifies once monocytes are localized to the subendothelial space of the intima. As differentiation progresses, differentiating monocytes experience enlarging cytoplasmic volume filled with vesicles, vacuoles, and lysosomes, and they acquire morphological characteristics of macrophages (Bobryshev, 2006), and the expression of several cell surface molecules (e.g. CD14) increases remarkably (Landmann et al., 1991). As well, the expression of the scavenger receptors, CD36 and SR-A, is dramatically augmented to allow uptake of modified lipoproteins (Libby, 2002). Mature macrophages participate along with other leukocytes in a local inflammatory response that not only advances atherogenesis, but also leads to complications associated with formation of atherosclerotic lesions within the intima (Libby et al., 2002).

Here, it is important to mention that oxLDL particles serve several functions that contribute to their atherogenicity (Leitinger, 2003; Jessup *et al.*, 2004). Most importantly, oxLDL particles are not only attractive candidates for internalization by intimal macrophages, but they also provoke further recruitment of circulating blood monocytes into that microenvironment by means of promoting the production of chemotactic factors (e.g. MCP-1 and MCP-3) and the expression of adhesion molecules (e.g. VCAM-1 and ICAM-1) on the surface of endothelial cells (Vainio and Ikonen, 2003). As chemotactic factors are produced, monocytes start to migrate from the blood stream towards the arterial wall, and they finally reside in the subendothelial space of the arterial intima. This allows further recruitment of monocytes into the area (Glass and Witztum, 2001).
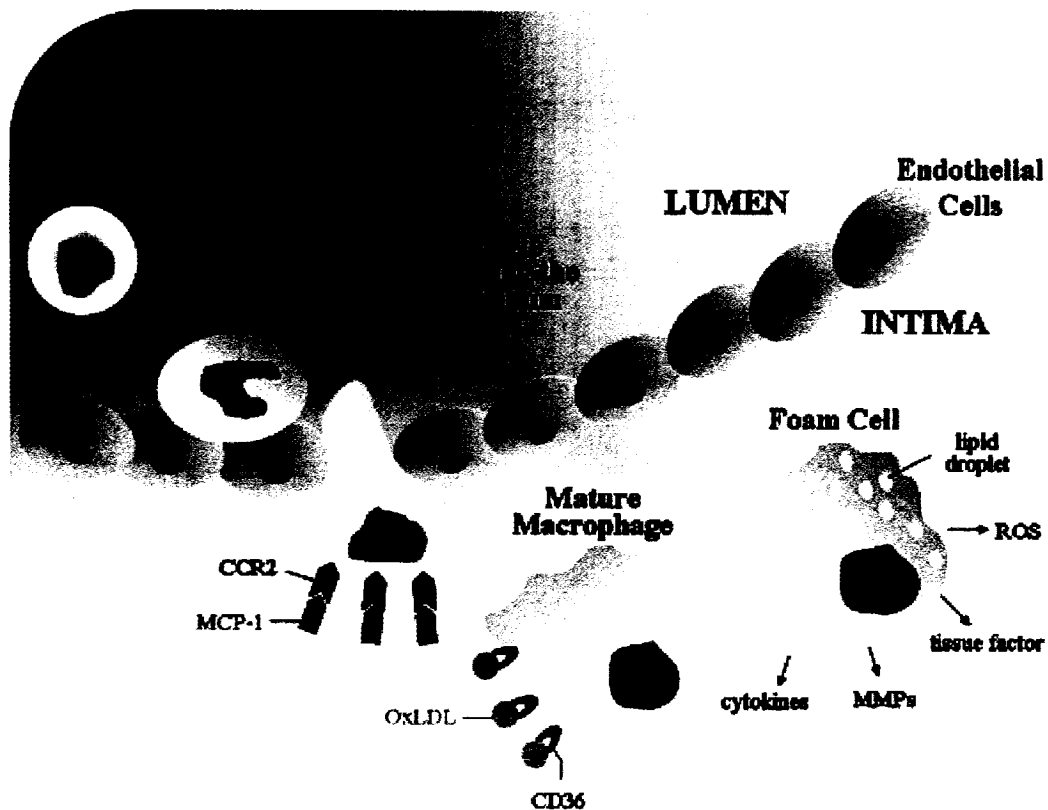
Figure 3. Monocyte Differentiation into Intimal Macrophages. Under certain circumstances, circulating monocytes are attracted to the endothelial monolayer of the arterial wall, where they start rolling by means of weak interactions between selectin molecules expressed on endothelial cells and their receptors expressed on the cell-surface of monocytes. Expression of adhesion molecules (e.g. VCAM-1 and ICAM-1) on the cell-surface of endothelial cells allows monocytes to adhere and firmly attach to the endothelial monolayer. Once fully attached, monocytes start penetrating the endothelial monolayer, and thus, active monocyte differentiation into intimal macrophages is initiated. As monocytes penetrate and differentiate, they acquire the morphological features of macrophages, and they express macrophage-specific cell-surface markers. The most important of these markers is a set of scavenger receptors that can bind modified versions of LDL and allow their internalization. Expression of scavenger receptors on intimal macrophages is a prerequisite for foam cell formation. This figure is adapted from Libby, 2002.

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1.4 Macrophage Cholesterol Homeostasis and Foam Cell Formation

1.4.1 Lipoprotein Modification and Uptake by Macrophages

The macrophage is a pivotal cell that plays major immune and non-immune functions in our bodies. Aside from their immune-related functions, macrophages facilitate clearance of cholesterol from the body via a mechanism known as reverse cholesterol transport (RCT) (Ohashi *et al.*, 2005). Indeed, macrophages are capable of taking up excess cholesterol from the periphery, process it, and transfer it to its final acceptor, high density lipoprotein (HDL) molecules, which in turn transport it to the liver for deposition (Ohashi *et al.*, 2005).

Noteworthy, however, macrophages are very inefficient in taking up LDL, the major carrier of plasma cholesterol, in its native form. This is due to the fact that macrophages express marginal levels of receptors that can recognize and associate with native LDL, and the expression of such receptors is down-regulated under environments of high levels of plasma LDL (Brown and Goldstein, 1983). Although macrophages express LDL receptor (LDLR) on their cell surface, LDL uptake by LDLR-mediated pathway does not generally lead to foam cell formation since the expression of LDLR severely diminishes as lipid levels start to rise in macrophages (Brown and Goldstein, 1997). This is a protective measure to avoid further lipid deposition, and thus, keep lipid homeostasis under control. Hence, LDL modification by oxidation, acetylation, self-aggregation, lipolysis, or proteolysis is necessary for macrophage internalization (Goldstein *et al.*, 1979). Oxidation is the most physiologically relevant means of LDL modification *in vivo* (Brown and Goldstein, 1983).

Mature macrophages express copious levels of several scavenger receptors on their cell surface, including CD36, a class B scavenger receptor (Endemann *et al.*, 1993), and SR-A, a class A scavenger receptor (Kodama *et al.*, 1988; Matsumoto *et al.*, 1990), which allow binding and internalization of oxLDL. Unlike LDLR, the expression of these scavenger receptors does not seem to become down-regulated as lipid accumulation occurs (Krieger, 1997). In fact, the expression of CD36 increases as more oxLDL is internalized (Han *et al.*, 1997; Han *et al.*, 1999). It is worth mentioning that other less

well-characterized pathways have been proposed as mechanisms that participate in cholesterol uptake in macrophages by means independent of LDLR- or scavenger receptor-mediated endocytosis (Buton *et al.*, 1999; Tabas, 2000).

1.4.2 Foam Cell Formation

Interestingly, macrophages that end up in the arterial intima face microenvironments that encourage internalization of some molecules that exist subendothelially (Skalen *et al.*, 2002; Itabe 2003). It is believed that some of the circulating lipoproteins become trapped at certain areas within major arteries where blood flow is disrupted and where endothelial cells tend to have polygonal shapes with no uniform orientation (Gimbrone, 1999). Eventually, these lipoproteins infiltrate the endothelial monolayer of the arterial wall and accumulate in the arterial intima (Oorni *et al.*, 2000; Skalen *et al.*, 2002, Itabe 2003). Once infiltrated into tiny pockets in the intima, such lipoproteins become susceptible to modification by enzymatic and non-enzymatic oxidative processes since they are no longer protected by antioxidants circulating in the blood stream (Vainio and Ikonen, 2003). Studies suggest that several cell types have the potential to participate in LDL oxidation *in vivo* including endothelial cells, smooth muscle cells, monocytes, macrophages, fibroblasts, and neutrophils (Steinberg, 1997), but other factors also play a role in such a modification (Gaut and Heinecke, 2001). Different enzymes such as NADPH oxidase, 15-lipoxygenase, and myeloperoxidase are capable of oxidating LDL *in vitro* (Steinberg, 1997), but lipooxygenases (Kuhn *et al.*, 1994; Folcik *et al.*, 1995) and myeloperoxidase (Leeuwenburgh *et al.*, 1997) seem to be responsible for *in vivo* oxidation of LDL within atherosclerotic lesions. Indeed, both the apoprotein and lipid moieties of trapped lipoproteins become oxidized, and while oxidized apoproteins contribute to receptor recognition, oxidized lipids contribute to the formation of biologically active species of lipids, namely oxysterols (Gaut and Heinecke, 2001).

Upon oxLDL uptake, macrophages become lipid-loaded, and the majority of internalized cholesterol is stored in the form of cholesteryl esters (CE), which are kept in late endosomes and lysosomes (Kruth *et al.*, 2002). It is thought that CE enters a cycle of events that involves its hydrolysis, re-esterification, and re-hydrolysis. Initially, CE is

hydrolyzed in the late lysosomes by the lysosomal acid lipase (LAL) (also known as acidic cholesteryl ester hydrolase (aCEH)), which converts CE into unesterified cholesterol. Due to its cytotoxicity, unesterified cholesterol is transported to the plasma membrane for efflux or to the endoplasmic reticulum (ER) for re-esterification by an enzyme called acyl-CoA:cholesterol acyltransferase (ACAT) (Chang *et al.*, 1997). Although unesterified cholesterol esterification followed by CE deposition in lipid droplets that protrude from the ER (van Meer, 2001) is a measure undertaken by macrophages to avoid unesterified cholesterol cytotoxicity, CE cannot be cleared from those macrophages unless they are hydrolyzed back into unesterified cholesterol and transported to the plasma membrane (Rothblat *et al.*, 1999). An enzyme called neutral cholesteryl ester hydrolase (nCEH) is a cytosolic esterase that converts CE back into unesterified cholesterol (Brown *et al.*, 1980). Now, unesterified cholesterol is transported to the plasma membrane, where it can be cleared out of macrophages and transferred onto HDL molecules for RCT to take place. Macrophage cholesterol efflux involves several factors that play critical roles in different signaling pathways that culminate in efficient cholesterol clearance from macrophages, and disruption of such pathways leads to improper cholesterol efflux and cholesterol accumulation in macrophages. So, lipid uptake by scavenger receptor-mediated pathways can potentially lead to drastic accumulation of lipid in the cytoplasmic compartment of macrophages, which eventually transform into lipid-laden cells with a foamy morphology (i.e. macrophage foam cells).

1.4.3 Role of Apolipoprotein E in Foam Cell Formation

Apolipoprotein E (ApoE) is a unique apolipoprotein in that it is secreted by cells outside the enterohepatic axis, such as macrophages and adipocytes (Mazzone, 1996; Curtiss *et al.*, 2000), unlike other apolipoproteins which are primarily produced by the liver and intestine. ApoE is a major component of chylomicron remnants, very-low density lipoproteins (VLDL), intermediate density lipoproteins (IDL), as well as in some subpopulations of HDL (Mahley, 1988). ApoE has been demonstrated to play a crucial role in macrophage cholesterol efflux, in which it has been shown to be capable of facilitating cholesterol efflux from human monocyte-derived macrophages in the absence

of cholesterol acceptors (Zhang et al., 1996). Interestingly, ApoE expression increases in macrophages upon cholesterol loading (Larkin et al., 2000), as well as in foam cells within atherosclerotic lesions (O'Brien et al., 1994), indicating a protective role of ApoE against foam cell formation. Indeed, in vitro studies have shown that ApoE enhances macrophage cholesterol efflux in presence of HDL or Apolipoprotein AI (Langer et al., 2000). Although nascent ApoE is sequestered in the lysosomal compartment in macrophages for degradation, some ApoE molecules can be liberated and secreted in the presence of HDL and participate in cholesterol clearance from macrophages (Deng et al., 1995).

In addition, ApoE has been shown to be a direct downstream target of PPARγ1 and LXRα in macrophages, and it is considered a component of the PPARγ1-LXRα signaling pathway in macrophages (Laffitte et al., 2001b). The most compelling evidence that ApoE is essential for efficient cholesterol efflux in macrophages comes from studies utilizing ApoE$^{-/-}$ macrophages, which exhibit a markedly inhibited cholesterol efflux to HDL and lipid-free ApoAI compared to wildtype macrophages (Mazzone, 1996; Langer et al., 2000).

1.5 Orphan Nuclear Hormone Receptor Superfamily

1.5.1 Peroxisome Proliferator-Activated Receptors (PPARs)

1.5.1.1 PPAR Isoforms, Expression, and Functional Specificity

PPARα, PPARβ/δ, and PPARγ are three isoforms encoded by three different genes in eukaryotic cells, and these three isoforms constitute the PPAR subfamily of the orphan nuclear hormone receptor superfamily (Berger and Moller, 2002). PPARs are traditionally known as orphan nuclear receptors due to the initial lack of knowledge about their physiological ligands, which are now known to include a wide range of biomolecules. Whereas PPARα and PPARδ can be activated by a wide range of saturated and unsaturated fatty acids (Gottlicher et al., 1992; Yu et al., 1995; Amri et al., 1995; Kliewer et al., 1997; Forman et al., 1997), PPARγ prefers polyunsaturated fatty acids as

ligands (Xu *et al.*, 1999). Fibrates, thiazolidinediones (TZDs) (e.g. rosiglitazone, pioglitazone, ciglitazone, and troglitazone), and α-substituted carboxylic acids (e.g. L-165041) are potent synthetic agonists for PPARα (Willson *et al.*, 2000), PPARγ (Lehmann *et al.*, 1995; Berger *et al.*, 1996; Willson *et al.*, 1996), and PPARβ/δ (Berger *et al.*, 1999), respectively.

PPARs are ligand-activated transcription factors that regulate the expression of a wide range of genes whose products are critically involved in lipid metabolism. PPARs are thought to be ubiquitously expressed, with differential expression patterns among the three isoforms (Berger and Moller, 2002). PPARα, the first PPAR to be identified, is expressed in many tissues and cells including the liver, kidney, skeletal muscle, heart, brown adipose tissue, monocytes, endothelial cells, and VSMCs (Berger and Moller, 2002). PPARβ/δ is expressed in a wide range of tissues and cells, but its expression seems to be highest in the brain, skin, and adipose tissue (Berger and Moller, 2002). Interestingly, the PPARγ gene is transcribed into three different mRNA molecules: PPARγ1 and PPARγ2, which are transcribed from the same promoter by differential promoter usage and subsequent alternative mRNA splicing (Zhu *et al.*, 1995), and PPARγ3, which is transcribed from an independent promoter (Fajas *et al.*, 1998). Yet, these three mRNA transcripts give rise to only two PPARγ proteins, PPARγ1 and PPARγ2, due to the fact that PPARγ3 mRNA is translated into a protein that is identical to PPARγ1 (Fajas *et al.*, 1998). PPARγ2 protein, whose expression is restricted to colon and adipose tissue (Fajas *et al.*, 1997; Fajas *et al.*, 1998), has 30 extra amino acid residues at its N-terminus compared to PPARγ1, which is ubiquitously expressed (Berger and Moller, 2002).

Upon ligand binding, PPARs become activated and they heterodimerize with RXR, which also has three isoforms designated RXRα, RXRβ, and RXRγ (Berger and Moller, 2002), all of which are activated by retinoic acid (Mangelsdorf *et al.*, 1992). PPAR-RXR obligate heterodimers bind to PPREs (direct repeats of AGGNCA separated by 1 or 2 nucleotides, DR1 and DR2 elements, respectively) within the promoter regions of their target genes, leading to gene transactivation (Berger and Moller, 2002). Such PPREs are found in the promoter regions of several genes including aP2 (Tontonoz *et al.*,

1994), phosphenolpyruvate carboxykinase (PEPCK) (Tontonoz *et al.*, 1995), lipoprotein lipase (LPL) (Schoonjans *et al.*, 1996), CD36 (Sfeir *et al.*, 1997), LXRα/β (Chawla *et al.*, 2001b), and ApoE (Galitto *et al.*, 2001).

1.5.1.2 Structure of PPARs

As nuclear receptors, PPARs possess a canonical domain structure similar to that of other members of the nuclear hormone receptor superfamily. At the N-terminus, PPARs harbor a ligand-independent transactivation (AF-1) sub-domain within the A/B domain, followed by a DNA binding domain (DBD) containing two zinc finger motifs, ligand binding domain (LBD), and a ligand-dependent transactivation (AF-2) domain towards the C-terminus (Rosen and Spiegelman, 2001; Berger and Moller, 2002). DBD and LBD are the most conserved domains among different isoforms of PPARs (Berger and Moller, 2002). LBD serves complex functions since it does not only mediate ligand binding, but it also mediates interaction with RXR as well as co-activators and co-repressors in a highly specific manner (Gearing *et al.*, 1993; Chen *et al.*, 1996).

1.5.2 Liver X Receptors (LXRs)

1.5.2.1 LXR Isoforms, Expression, and Functional Specificity

Liver X receptors (LXRs) are members of the orphan nuclear receptor superfamily that were first identified in the liver, hence their name (Apfel *et al.*, 1994; Willy *et al.*, 1995). Two isoforms have already been characterized, namely LXRα and LXRβ, the latter being ubiquitously expressed (Song *et al.*, 1994), while the expression of the former is more restricted in the kidney, spleen, adipose tissue, lung, intestine, skeletal muscle, and macrophages (Apfel *et al.*, 1994; Willy *et al.*, 1995; Peet *et al.*, 1998). It is believed that intracellular cholesterol leads to the production of LXRs specific physiological ligands, oxysterols (Repa and Mangelsdorf, 2002). 24(*S*),25-epoxycholesterol, 24(*S*)-hydroxycholesterol, and 22(*R*)-hydroxycholesterol are the most abundant and potent oxysterols capable of activating LXRs in the cell (Janowski *et al.*,

1996). Potent and specific pharmacological LXR agonists, such as T0901317 and GW3965, have been synthesized. Similar to PPARs, ligand-bound LXRs tend to form heterodimers with their obligate partner RXR, and activated LXR-RXR heterodimers are capable of binding to specific DNA binding sites known as LXRE, which consists of two direct repeats (AGGTCA) separated by four nucleotides (DR4 elements) (Willy *et al.*, 1995). LXREs have been found in the promoter regions of several genes regulated by LXRs including ABCA1 (Luo *et al.*, 2000), ABCG1 (Venkateswaran *et al.*, 2000b), PPARγ (Seo *et al.*, 2004), and ApoE (Laffitte *et al.*, 2001b).

1.5.2.2 Structure of LXRs

Structurally, LXRs are similar to other members of the nuclear hormone superfamily. LXRs contain a poorly characterized N-terminus that has AF-1 domain, followed by a central DNA binding domain (DBD), and a relatively large C-terminus containing the ligand-binding domain (LBD) and AF-2 ligand-dependent domain (Chawla *et al.*, 2001c). DBD of LXRs contains two highly conserved zinc finger motifs, characteristic of other orphan nuclear receptors, which is required for physical contact between LXR-RXR heterodimers and LXREs in the promoters of target genes. The LBD of LXRs confer ligand specificity, heterodimerization with RXRs, as well as interactions with co-activators and co-repressors (Renaud *et al.*, 1995).

1.5.3 Co-Activation and Co-Repression of PPARs and LXRs

As their names suggest, co-activators and co-repressors are transcription modulators that allow transactivation and repression of target genes, respectively, by means of associating with transcription factors that regulate expression of such genes (Rosen and Spiegelman, 2001; Edwards *et al.*, 2002). Like other nuclear hormone receptors, PPARs are involved in protein-protein interactions with co-activators and co-repressors, and such interactions are crucial for mediating physical association between PPAR-RXR heterodimers with chromatin and the basic transcription machinery (Rosen and Spiegelman, 2001). PPAR co-activators include CBP/p300 (Zhu *et al.*, 1996), steroid

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receptor co-activator (SRC)-1 (Zhu *et al.*, 1996), PPARγ co-activator (PGC)-1 (Puigserver *et al.*, 1998), and PPAR binding protein (PBP or TRAP220) (Zhu *et al.*, 1997). Nuclear receptor co-repressor (N-CoR) (Horlein *et al.*, 1995), silencing mediator of retinoic acid and thyroid hormone receptor (SMRT) (Chen *et al.*, 1996), and small heterodimer partner (SHP) (Nishizawa *et al.*, 2002) are among the well-characterized co-repressors that interact with PPAR-RXR heterodimers, inhibiting transcriptional transactivation driven by active PPAR-RXR homodimers.

Upon binding of PPAR-RXR heterodimers to PPREs of target genes, co-activators with histone acetylase activity bind to the ligand- and DNA-bound PPAR-RXR heterodimer. Such binding is thought to cause chromatin remodeling, giving access to other co-activators such as PBP, which connect the PPAR-RXR complex to the basic transcription machinery leading to gene transactivation (Berger and Moller, 2002). In contrast, co-repressors bind to ligand- and DNA-bound PPAR-RXR heterodimers and allow recruitment of histone deacetylases and/or conformational alterations that ultimately confer a condensed, inactive chromatin structure, leading to transcriptional repression (Chen and Li, 1998; Hu and Lazar, 2000; Glass and Rosenfeld, 2000; Rosenfeld and Glass, 2001).

Similar to PPARs, LXRs are involved in protein-protein interactions with co-activators and co-repressors, which upon ligand binding to LXRs, take advantage of conformational changes that allow their recruitment (Edwards *et al.*, 2002). Co-activators and co-repressors of LXRα lead to transcriptional activation and repression of LXR target genes by means of chromatin remodeling, respectively (Edwards *et al.*, 2002). Apparently, LXRs interact with co-activators (PGC-1, SRC-1, and CBP/p300) and co-repressors (N-CoR, SMRT, and SHP) that bind PPARs (Brendel *et al.*, 2002; Hu *et al.*, 2003; Unno *et al.*, 2005). Some studies have also suggested that co-repressors are constitutively bound to PPARs and LXRs, and upon ligand binding, conformational changes force simultaneous dissociation of co-repressors and recruitment of co-activators (Glass and Rosenfeld, 2000; Edwards *et al.*, 2002).

Interaction between nuclear receptors and their co-activators requires multiple LXXLL motifs located within NR boxes of co-activators (Le Douarin *et al.*, 1996; Heery *et al.*, 1997; Torchia *et al.*, 1997). Slight differences within such NR boxes are critical

determinants of NHR-coactivator specificity (Torchia *et al.*, 1997; Ding *et al.*, 1998). Likewise, co-repressors of nuclear hormone receptors contain small peptide motifs (CoRNR boxes) that mediate protein-protein interaction with nuclear hormone receptors (NHRs), and subsequently transcriptional repression of target genes (Hu and Lazar, 1999). Despite their remarkable sequence homology, CoRNR boxes within different co-repressors have unique sequences, an important determinant in NHR-corepressor specificity (Hu *et al.*, 2001; Cohen *et al.*, 2001). Of course, the exact sequence of LBD of nuclear receptors is essential in supporting or precluding NHR-coactivator and NHR-corepressor interactions (Moras and Gronemeyer, 1998).

1.5.4 PPARγ1-LXRα-ABC Signaling Pathway in Macrophages

1.5.4.1 Role of PPARγ1 & LXRα in Macrophage Cholesterol Homeostasis

PPARγ1 and LXRα are known to be potent sterol and fatty acid sensors that play fundamental roles in lipid metabolism. PPARγ/LXRα signaling pathways are involved in various key biological processes that are implicated in many conditions such as obesity, diabetes mellitus, atherosclerosis, and inflammatory diseases (Walczak and Tontonoz, 2002; Gao *et al.*, 2004). Both PPARγ1 and LXRα are expressed abundantly in macrophages, especially in lipid-laden foam cells within atherosclerotic lesions (Tontonoz *et al.*, 1998; Ricote *et al.*, 1998a; Venkateswaran *et al.*, 2000b).

Upon uptake of oxLDL by macrophages, many intermediates such as oxidized fatty acids and oxysterols are formed, providing macrophages with PPARγ1 and LXRα natural ligands (Nagy *et al.*, 1998; Janowski *et al.*, 1999; Fu *et al.*, 2001). Thus, PPARγ1 and LXRα become ligand-bound and heterodimerize with their obligate RXR molecules to become transcriptionally active. In fact, oxLDL does not only lead to PPARγ1 and LXRα activation, but it also leads to increased expression of these genes (Tontonoz *et al.*, 1998). In turn, PPARγ1 and LXRα signaling pathways are turned on, leading to the transactivation of a cascade of genes whose products are critically involved in cholesterol efflux in macrophages (Figure 4) (Chawla *et al.*, 2001b; Chinetti *et al.*, 2001).
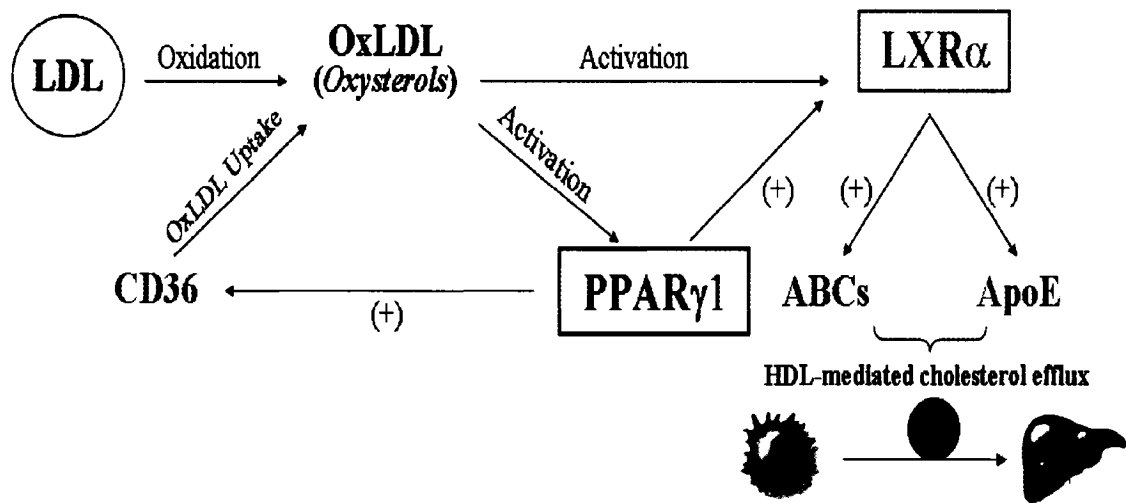
Figure 4. PPARγ1 and LXRα are Key Regulators of Macrophage Cholesterol Homeostasis. Upon uptake of oxLDL by macrophages, oxysterols are synthesized, which allows activation of PPARγ1 and LXRα. Once activated, PPARγ1 and LXRα not only induce the expression of each other, but they also induce the expression of many ABC transporters as well as that of ApoE. ABCs and ApoE play integral roles in transferring excess cholesterol to its acceptor, HDL, and this initiates the process of reverse cholesterol transport (RCT). In RCT, excess peripheral cholesterol is scavenged by tissue macrophages, which process cholesterol and transport it to the liver via HDL for excretion. This figure is adapted from Chawla et al., 2001.

1.5.4.2 ABCs are Direct Downstream Targets of PPARγ1 and LXRα

Members of the ATP-binding cassette (ABC) family of proteins are among the most well-studied downstream targets of the PPARγ1-LXRα signaling pathway initiated by lipid loading in macrophages. ABCs are transmembrane proteins with two ATP-binding domains and 12 membrane-spanning domains, and they function as efficient cholesterol transporters by forming channel-like structures in the plasma membrane (Decottignies and Goffeau, 1997). ABCs are involved in active transport of cholesterol from inside the cell onto HDL particles. In macrophages, ABCA1 and ABCG1 play major roles in HDL-mediated and ApoAI-mediated cholesterol efflux (Brooks-Wilson et al., 1999; Lawn et al., 1999). Lipid loading of macrophages increases the expression of ABCA1 (Langmann et al., 1999) and ABCG1 (Klucken et al., 2000; Venkateswaran et al., 2000b). The importance of ABCA1 function as a cholesterol efflux mediator is illustrated by Tangier disease, a genetic disorder characterized by extremely low plasma HDL levels and remarkable accumulation of cholesterol in macrophages localized in the tonsils, spleen, liver, and intestine (Serfaty-Lacroseniere et al., 1994). Numerous studies have demonstrated that familial Tangier disease mainly results from mutations in the ABCA1 gene (Rust et al., 1998; Brooks-Wilson et al., 1999; Bodzioch et al., 1999). Moreover, macrophages isolated from Tangier patients express significantly lower levels of ABCG1 (Lorkowski et al., 2001). Interestingly, pharmacological activation of PPARγ1 and/or LXRα in macrophages cannot overcome ABCA1 deficiency, which completely abolishes cholesterol efflux (Chinetti et al., 2001). Experimental evidence exists indicating that reduced levels of ABCG1 correlate with inhibited cholesterol efflux in macrophages (Wang et al., 2004). Clearly, ABCs are involved in the first step of reverse cholesterol transport (RCT), and they are also involved in the control of total plasma HDL levels, major events associated with atherogenesis. Interestingly, about 40% of Tangier patients develop symptoms of atherosclerosis at one point in their life (Serfaty-Lacroseniere et al., 1994).

Studies have shown that PPARγ1 and LXRα are involved in a positive, reciprocal relationship, in which the activation of one of them leads to the up-regulation of the other (Chawla et al., 2001b; Seo et al., 2004). However, experimental evidence suggests that

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ABCA1 and ABCG1 are not direct targets of PPARγ1 in macrophages, and that PPARγ1 activation leads to induction of ABCA1 and ABCG1 levels via LXRα up-regulation (Chawla *et al.*, 2001b). Actually, Chawla and colleagues have shown that LXRα activation results in marked induction of ABCA1 and ABCG1 in PPARγ1-deficient macrophages, indicating that PPARγ1 is dispensable for LXRα-mediated up-regulation of ABC proteins in macrophages (Chawla *et al.*, 2001b). In addition, LXRα-deficient macrophages display diminished cholesterol efflux due to lack of up-regulation of ABCA1 (Repa *et al.*, 2000), and ABCG1 (Laffitte *et al.*, 2001b), confirming that PPARγ1 is insufficient in up-regulating ABCA1 and ABCG1 in the absence of LXRα. However, studies using a PPARγ1 conditional knockout revealed that PPARγ1-deficient macrophages have reduced LXRα, ABCA1, and ABCG1 levels, and thus, diminished cholesterol efflux, and they develop into foam cells (Chawla *et al.*, 2001b; Akiyama *et al.*, 2002). These studies suggest a complex, regulatory loop implicating PPARγ1 and LXRα as key, upstream players in a signaling pathway that culminates in up-regulation of ABC proteins and cholesterol clearance from macrophages.

1.5.4.3 ApoE is a Direct Downstream Target of PPARγ1 and LXRα

An extensive body of literature demonstrates that ApoE, which is expressed abundantly in macrophages, is a prominent player in macrophage cholesterol efflux and foam cell formation (Basu *et al.*, 1982; Basu *et al.*, 1983; Dory, 1989; Mazzone and Reardon, 1994; Zhang *et al.*, 1996). ApoE-deficient macrophages display severely diminished ability to efflux cholesterol and other lipids to HDL particles or lipid-free apolipoproteins (Mazzone, 1996; Langer *et al.*, 2000), indicative of a key role of ApoE in RCT. Noteworthy, the PPARγ1-LXRα-ABC signaling pathway described above also leads to up-regulation of ApoE in macrophages, as suggested by many independent studies. It was shown that basal expression of ApoE is attenuated in PPARγ1-deficient macrophages, indicating that PPARγ1 positively regulates ApoE expression (Akiyama *et al.*, 2002). In addition, treatment of THP-1 macrophages with ciglitazone, a potent PPARγ agonist, results in a significant increase in ApoE levels (Galetto *et al.*, 2001),

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indicating that PPARγ1 activity positively correlates with ApoE expression in macrophages. However, treatment of PPARγ1-deficient macrophages with 22(*R*)-hydroxycholesterol, a natural LXRα agonist, induces ApoE expression, and thus, bypasses PPARγ1 dependency. Another line of evidence indicates that 22(*R*)-hydroxycholesterol and synthetic LXR agonist T0901317 up-regulate ApoE expression in wildtype, but not LXRα-deficient, macrophages, confirming that ApoE up-regulation in macrophages is LXRα-dependent, consistent with the fact that the ApoE promoter contains consensus LXRE (Laffitte *et al.*, 2001b). Despite the presence of PPRE within the promoter region of ApoE, solid evidence indicating a direct PPARγ1-mediated up-regulatory effect on ApoE expression is lacking. Collectively, these studies suggest that activation of PPARγ1 leads to induction of ApoE expression via LXRα up-regulation, and that ApoE is a major component of the PPARγ1-LXRα signaling paradigm. Noteworthy, defects in any component of the PPARγ1-LXRα signaling pathway in macrophages render cholesterol efflux defective, and thus, result in the transformation of macrophages into lipid-laden foam cells.

1.5.5 Anti-Inflammatory Roles of PPARγ1 and LXRα

1.5.5.1 PPARγ1 is an Anti-Inflammatory Regulator

Aside from its imperative role in controlling macrophage cholesterol homeostasis, PPARγ1 possesses potent anti-inflammatory functions in macrophages (Lee and Evans, 2002; Zelcer and Tontonoz, 2006). A variety of PPARγ synthetic agonists have been used to demonstrate that PPARγ1 exerts anti-inflammatory effects in macrophages due to their ability to induce the expression of a wide range of pro-inflammatory genes. Treatment of murine peritoneal macrophages with 15-deoxy-$\Delta^{12,14}$-prostaglandin or BRL 49653, specific PPARγ agonists, results in marked reduction in IFNγ-induced and PMA-induced expression of iNOS and MMP-9 (also known as gelatinase B), respectively (Ricote *et al.*, 1998b). Moreover, PMA-induced expression of IL-1β, IL-6, and TNFα is significantly reduced in primary human monocytes treated with two PPARγ agonists, 15-deoxy-$\Delta^{12,14}$-

prostaglandin and troglitazone (Jiang *et al.*, 1998). 15-deoxy-$\Delta^{12,14}$-prostaglandin and troglitazone also lead to inhibited TNFα promoter-driven expression in the human monocyte/macrophage cell line U937 (Jiang *et al.*, 1998). Numerous studies have shown that activation of PPARγ1 in macrophages also leads to blocked expression of IL-12 (Alleva *et al.*, 2002), iNOS (Petrova *et al.*, 1999; Bernardo *et al.*, 2000; Fahmi *et al.*, 2001) and COX-2 (Tsubouchi *et al.*, 2001). However, the effort of these studies to present PPARγ as a potent anti-inflammatory mediator is hampered by experiments performed in PPARγ-deficient macrophages. Chawla and colleagues have demonstrated that LPS treatment leads to equivalent induction of IL-6 and TNFα in wildtype and PPARγ-deficient macrophages (Chawla *et al.*, 2001a). Most importantly, PPARγ expression in macrophages or lack of it had no effect on the ability of 15-deoxy-$\Delta^{12,14}$-prostaglandin to inhibit LPS-induced expression of IL-6 and TNFα (Chawla *et al.*, 2001a). Alternatively, Hinz and colleagues demonstrated that 15-deoxy-$\Delta^{12,14}$-prostaglandin and ciglitazone resulted in reduced LPS-induced expression of IL-6, TNFα, and COX-2 in human monocytes in presence of the PPARγ antagonist, bisphenol A diglycidyl ether (BADGE) (Hinz *et al.*, 2003). Other studies have also demonstrated that PPARγ-independent anti-inflammatory effects exerted by PPARγ agonists *in vitro* and *in vivo* (Reilly *et al.*, 2000; Niino *et al.*, 2001). These findings raised questions about the exact role of PPARγ1 in macrophage inflammatory responsiveness. Later studies have shown that PPARγ agonists exert anti-inflammatory functions in macrophages via PPARγ1-dependent and PPARγ1-independent mechanisms depending on the concentration of agonists and the nature of the inflammatory signal. When PPARγ agonists are used at low receptor-specific concentrations, their anti-inflammatory function seems to be dependent on PPARγ1 expression (Welch *et al.*, 2003). At high agonist concentrations, however, PPARγ1-independent mechanism(s), possibly involving PPARβ/δ, manifests anti-inflammatory effects of such PPARγ agonists in macrophages (Welch *et al.*, 2003).

1.5.5.2 LXRα is an Anti-Inflammatory Regulator

A strong line of evidence indicates the existence of a vital crosstalk between macrophage cholesterol homeostasis and inflammation based on recent studies demonstrating a direct role of LXRα in mediating anti-inflammatory effects in macrophages. Using murine peritoneal macrophages, the LXR agonists T0901317 and GW3965 have been shown to be very effective in inhibiting LPS-induced expression of a wide range of pro-inflammatory mediators including IL-1β, IL-6, iNOS, COX-2, MCP-1, MCP-3, MIP-1β, IP-10, and MMP-9 (Joseph et al., 2003). Although lack of expression of either LXRα or LXRβ in macrophages did not interfere with LXR agonist-mediated inhibition of LPS-induced expression of these pro-inflammatory mediators, macrophages that lack both isoforms (i.e. LXRα$^{-/-}$LXRβ$^{-/-}$ macrophages) were unresponsive to the anti-inflammatory effects exerted by LXR agonists (Joseph et al., 2003). This data indicate that the loss of one LXR isoform can be compensated by the other isoform, and that LXR agonists exert their anti-inflammatory effects in macrophages in an LXR-dependent mechanism. Interestingly, in vivo experiments revealed that LXR-mediated anti-inflammatory functions are not restricted to macrophages, since LPS-induced hepatic expression of IL-6, TNFα, and iNOS, as well as LPS-induced aortic expression of IL-6, iNOS, and MMP-9 is significantly elevated in LXRα$^{-/-}$LXRβ$^{-/-}$ mice compared to wildtype mice (Joseph et al., 2003). Consistently, LPS-, IL-1β-, and TNFα-induced expression of MMP-9 is markedly inhibited by T0901317 and GW3965 in murine peritoneal macrophages (Castrillo et al., 2003). Furthermore, T0901317 and GW3965 treatment results in reduced LPS-induced expression levels of tissue factor (TF) and osteopontin (OPN), pro-inflammatory, pro-atherogenic mediators, in murine peritoneal macrophages and RAW 264.7 macrophages, respectively (Terasaka et al., 2005; Ogawa et al., 2005). In vivo, LXR agonists are also capable of reducing LPS-induced TF expression levels in the aorta, kidney, and lung (Terasaka et al., 2005).

1.5.5.3 NF-κB Role in PPARγ1- and LXRα -Mediated Anti-Inflammation

Despite the strong evidence behind the anti-inflammatory effects of PPARγ and LXR agonists, the exact mechanism underlying such effects is poorly understood. Recent findings strongly suggest that activation of PPARγ and/or LXR interferes with NF-κB, STAT, and AP-1 activity in macrophages (Chinetti *et al.*, 1998; Ricote *et al.*, 1998b; Zhou and Waxman, 1999; Joseph *et al.*, 2003; Castrillo *et al.*, 2003). Mechanisms leading to inhibited NF-κB activity by PPARγ agonists in macrophages include inhibited IKK activity, and thus, decreased IκBα phosphorylation (Straus *et al.*, 2000; Zingarelli *et al.*, 2003), covalent modifications of NF-κB subunits leading to abrogated NF-κB-DNA interaction (Chung *et al.*, 2000), and induced NF-κB nuclear export (Kelly *et al.*, 2004). Indeed, it was shown that PPARγ is capable of interfering with NF-κB activity by interacting with NF-κB subunits, rendering NF-κB transcriptionally inactive (Chung *et al.*, 2000). Furthermore, PPARγ agonists may be capable of modulating NF-κB activity by means of alkylation of cysteine residues located in the DNA-binding domains of NF-κB subunits (C^{62} in p50 and C^{38} in p65) (Straus *et al.*, 2000). It was also suggested that PPARγ agonists lead to enhanced MAPK activation, and subsequently more phosphorylation of PPARγ, which is more efficient in interacting with NF-κB when phosphorylated (Chen *et al.*, 2003). However, other studies have demonstrated that PPARγ agonists impede NF-κB signaling via PPARγ-independent mechanisms. In a study performed in RAW 264.7 macrophages, which lack endogenous PPARγ, 15-deoxy-$\Delta^{12,14}$-prostaglandin treatment resulted in significant inhibition of NF-κB activity (Straus *et al.*, 2000), suggesting that PPARγ is not required for PPARγ agonist-mediated anti-inflammatory effects in macrophages. Hence, it is not surprising that the importance of PPARγ involvement in NF-κB inhibition by PPARγ agonists is very controversial.

1.6 Sudden Cardiac Death

In humans, sudden cardiac death is an instant, unexpected death that normally happens in a very short period of time (about 1 hour) (Morady and Scheinman, 1984).

Sudden cardiac death events account for about 400,000 death cases in USA every year (Rubart and Zipes, 2005). About 80% of sudden cardiac death cases occur in patients with known coronary artery disease, including myocardial infarction and myocardial ischemia (Huikuri et al., 2001). Most sudden cardiac deaths result from cardiac arrhythmias caused by acute coronary events that can happen in individuals with no known cardiac conditions (Rubart and Zipes, 2005). Atherosclerosis is a killer disease that is considered the primary cause of sudden cardiac death (Mayers, 2003).

1.6.1 External and Internal Triggers of Atherosclerotic Plaque Rupture

It is believed that some atherosclerotic lesions are very fragile, and thus, they are prone to degradation and rupture. Plaque rupture is determined by many factors, the most important of which is the thickness of the fibrous cap surrounding the lipid-filled, cell-dense necrotic core (Mitra et al., 2004). As the fibrous cap becomes thinner, the plaque becomes more vulnerable to rupture. Plaque rupture occurs when the integrity and continuity of the fibrous cap of a plaque discontinue, leading to leakage of the contents of the necrotic lipid core (Mitra et al., 2004). Eroded fibrous cap, inflammatory infiltrate, large necrotic core, and disoriented, sparse vascular smooth muscle cells are the main characteristic of a ruptured atherosclerotic lesion (Virmani et al., 2000).

Both intrinsic factors, which have to do with the pathological properties of individual plaques, as well as extrinsic factors, which are external forces acting towards plaque rupture, are key determinants in determining plaque stability (Mitra et al., 2004). Usually, plaques have to be weakened internally before the external forces can cause rupture. Some of the intrinsic factors that determine plaque stability are the thickness of the fibrous cap, size of plaque, type of lipids within the fibrous core, cellular components within the core, and the nature of inflammatory mediators produced within the plaque. External factors that can trigger rupture of vulnerable plaques include high blood pressure, stress, increased heart rate, and hemorrhage into the plaque (Mitra et al., 2004). Here, it is important to mention that apoptosis has been linked to many cardiac diseases including atherosclerosis (Kockx et al., 1998; Mallat and Tedgui, 2001), and it has been illustrated that apoptosis of macrophages, VSMCs, and endothelial cells not only advance

atherogenesis and plaque formation but also participate in plaque vulnerability to rupture (Kockx *et al.*, 1998; Tricot *et al.*, 2000; Kolodgie *et al.*, 2001).

1.6.2 The Roles of MMPs and TIMPs in Atherosclerotic Plaque Rupture

Plaque rupture is mostly understood as an event whereby the fibrous cap surrounding an unstable lesion becomes prone to degradation (Libby, 1995). The strength and stability of a fibrous cap is determined by the amount of smooth muscle cell-produced collagen present in the fibrous cap (Lee and Libby, 1997). The collagen structure is generally very resistant to degradation by proteolysis, and thus, conferring stability and tensil strength to the majority of fibrous caps (Libby, 2002). Matrix metalloproteinases (MMPs) are zinc-dependent endopeptidases that mediate proteolysis of proteins including collagen within the extracellular matrix of the sub-endothelial basement membrane of the arterial wall and fibrous caps (Loftus *et al.*, 2002).

Although a number of MMPs (e.g. MMP-1, MMP-2, MMP-3, MMP-8, MMP-9, MMP-13, ..., etc.) are expressed in atherosclerotic lesions (Galis *et al.*, 1994; Galis *et al.*, 1995; Sukhova *et al.*, 1999; Herman *et al.*, 2001), these proteinases do not cause degradation of the fibrous cap because their activity is suppressed by a set of MMP inhibitors known as tissue inhibitors of metalloproteinases (TIMPs), which are also expressed in atherosclerotic lesions (Vine and Powell, 1991; Knox *et al.*, 1997). However, when MMPs are over-expressed in lesions, the inhibitory function of TIMPs is overcome and collagen degradation takes place (Sukhova *et al.*, 1999). Interestingly, production of pro-inflammatory mediators such as IL-1β and TNFα by cells occupying a lesion induce over-expression of many MMPs including MMP-1, MMP-2, MMP-7, MMP-9, MMP-11, MMP-12, MMP-14, and MMP-17 by VSMCs, endothelial cells, macrophages, and T lymphocytes (Saren *et al.*, 1996; Nagase, 1997; Carrell *et al.*, 2002). At later stages of atherosclerosis when inflammation intensifies, high expression of MMPs can potentially lead to collagen and fibrous cap degradation, ultimately causing plaque rupture. In fact, inflammation not only induces MMP over-expression, but it also inhibits the ability of smooth muscle cells to synthesize collagen, making the fibrous cap less stable and more prone to destruction (Libby, 2002). Of interest, higher oxLDL levels

within atherosclerotic lesions have been shown to associate with higher levels of MMPs (Rajavashisth *et al.*, 1999).

Different studies have demonstrated that pharmacological inhibitors of several MMPs impede destruction of the extracellular matrix of the fibrous cap, and they inhibit progression of atherosclerosis in various animal models (Yoon *et al.*, 1999; Prescott *et al.*, 1999). MMP inhibitors have also been shown to reduce atherosclerosis progression (Corti *et al.*, 2001) and reduce stroke risk (Plehn *et al.*, 1999). Genetic deficiency of various MMPs protects against destruction of lesions, leading to the formation of more stable lesions that are prone to rupture (Silence *et al.*, 2001; Galis *et al.*, 2002; Shi *et al.*, 2003; Kuzuya *et al.*, 2003; Luttun *et al.*, 2004; Deguchi *et al.*, 2005). In contrast, ablation of TIMP-1 or TIMP-3 leads to increased lesion destruction in ApoE$^{-/-}$ mice (Lemaitre *et al.*, 2003; Federici *et al.*, 2005), while adenovirus-mediated over-expression of TIMP-1 protects against lesion destruction and progression of atherosclerosis in ApoE$^{-/-}$ mice (Rouis *et al.*, 1999). Clearly, the interplay between MMPs and TIMPs is a critical determinant of lesion fate and progression of atherosclerosis.

1.6.3   Atherosclerotic Plaque Rupture and Atherothrombosis

Regardless of the trigger, it is well known that plaque rupture leads to thrombus formation (Mitra *et al.*, 2004), and this in turn leads to acute cardiac dysfunction including myocardial infarction (DeWood *et al.*, 1980; Falk 1983; Davies and Thomas, 1985) and thrombotic stroke (Mitra *et al.*, 2004; Viles-Gonzalez, *et al.*, 2004) due to clogging of narrow arteries within the heart or brain, respectively. Both myocardial infarction and thrombotic stroke combine to be the leading cause of death in the developed world (Ginsburg, 2005). Atherothrombosis is a term that is used to describe plaque rupture leading to the formation of thrombi that can circulate in the blood and cause life-threatening conditions including myocardial infarction and stroke (Leys, 2001; Viles-Gonzalez, *et al.*, 2004).

It is reported that thrombi can be detected in infarct-related arteries of about 90% of patients undergoing acute myocardial infarction (DeWood *et al.*, 1980). Since then, the use of thrombolytic therapy has become widespread in an attempt to degrade thrombi

before they become potentially deadly (Schellinger *et al.*, 2004). Acute myocardial infarction, which is referred to as heart attack, happens as a result of a shortage of blood, and thus oxygen, supply to certain areas of the cardiac tissue leading to death of cardiomyocytes (Alpert, 2003). Since cardiomyocytes cannot proliferate, death of cardiomyocytes due to myocardial infarction leads to the formation of fibrotic tissue at the infarct site, and this definitely weakens the cardiac function (Schwarz *et al.*, 1998).

Atherothrombosis can also lead to stroke, which is considered the primary cause of paralysis, the second leading cause of dementia, and the third leading cause of death in the developed world (Leys, 2001). As for myocardial infarction, thrombotic strokes, also known as ischemic strokes, usually happen secondary to atherosclerotic plaque rupture and thrombus formation (more than 90% of the cases) (Yuan *et al.*, 2002; Cohen *et al.*, 2002). Upon plaque rupture, thrombi are carried by the blood flow, and thus, they become potentially capable of occluding small blood vessels. Occlusion of such small vessels in the brain prevents blood/oxygen supply to that area of the brain tissue, leading to death of nerve cells. Depending on the exact site of blood flow disruption, thrombotic stroke can cause a wide range of mild and severe neurological deficits that are usually irreversible simply because nerve cells lack the potential to regenerate. Permanent disabilities associated with thrombotic strokes include general paralysis, partial paralysis, speech impairment, blindness, and sensory change, but death can also be an outcome of thrombotic stroke (Yuan *et al.*, 2002; Cohen *et al.*, 2002).

1.7 NF-κB Signaling Pathway

NF-κB is a fascinating molecule due to its ability to serve as a "cell guard" in that it senses conditions that can be destructive to cells, and it responds by inducing a vast number of defense genes (Baeuerle, 1998). What makes NF-κB a favorable candidate to undertake such a crucial task is not only the fact that it is ubiquitously expressed in almost all types of cells, but also the fact that it is a transcription factor that is sequestered in an inactive state in the cytosol but can become activated by a wide range of diverse internal and external stimuli (Figure 5) (Baeuerle, 1998). Additionally, NF-κB is a unique transcription factor in that its function is not solely dependent on its expression when

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needed. Rather, NF-κB is constitutively expressed in the cell, but it does not become active until it is called upon for action, in which it will be ready and its mission can be accomplished in a timely-regulated fashion. NF-κB activation can be achieved via the classical or alternative pathways, which are turned on by distinct stimuli and lead to different outcomes (refer to section 1.7.2.3 for more details).
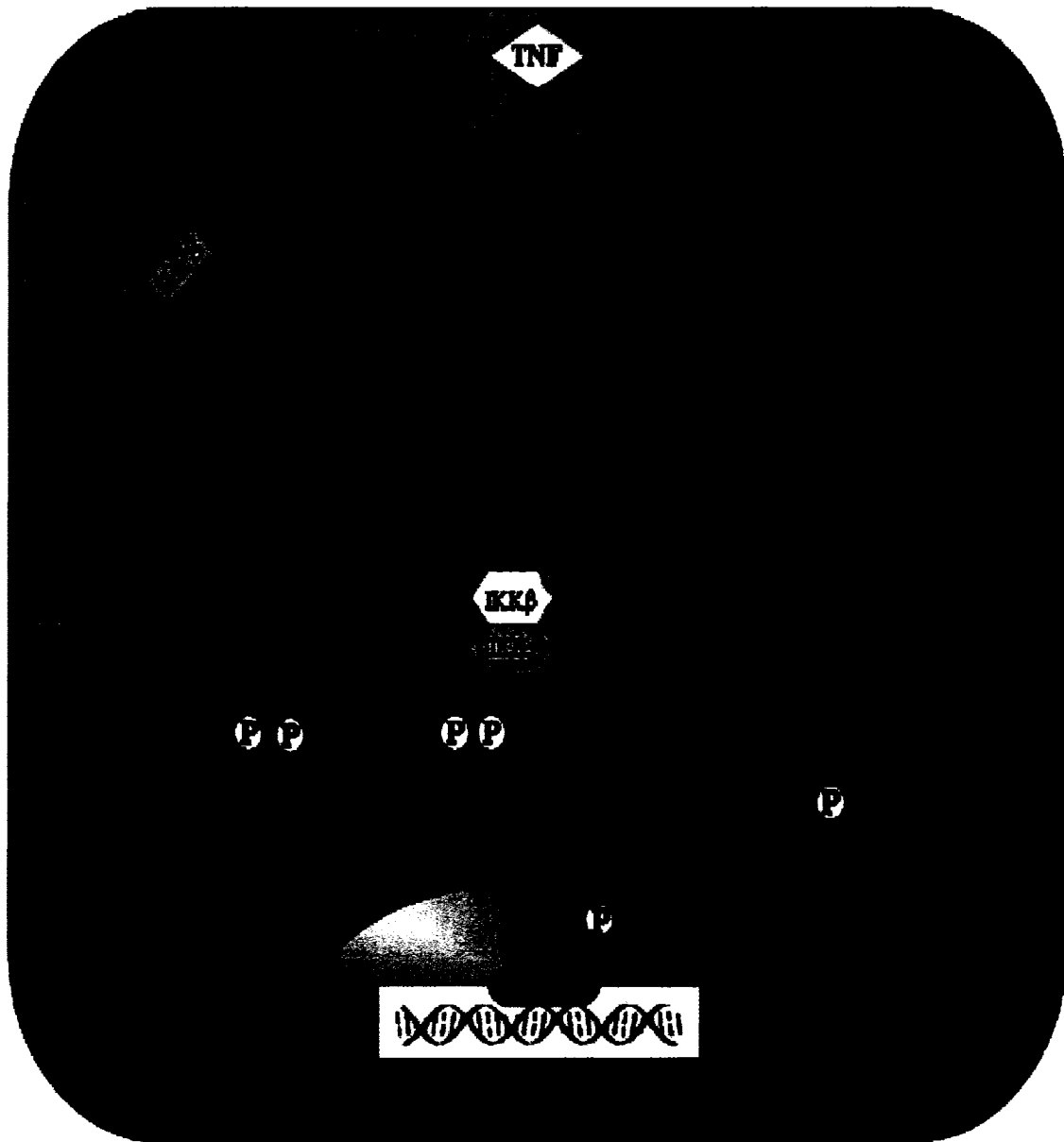
Figure 5. NF-κB Signaling Pathway. A cartoon representing the cascade of biochemical events that are initiated by various stimuli, eventually leading to NF-κB nuclear translocation and transcriptional activation. Recruitment of different adaptor molecules to different receptor complexes as well as activation of different downstream kinases is shown. This figure is adapted from www.biocarta.com.

1.7.1 NF-κB/Rel Protein Family

Nuclear factor κB (NF-κB) comprises a family of ubiquitously expressed, eukaryotic transcription factors that participate in the regulation of multiple immediate genes that are expressed at the onset of many vital biological processes such as cell growth, immunoregulation, inflammation, and apoptosis (Tak and Firestein, 2001; Yamamoto and Gaynor, 2001). Modulation of NF-κB activity can lead to many abnormal cellular processes and diseases including asthma, arthritis, atherosclerosis, obesity, and various types of cancers (Tak and Firestein, 2001; Yamamoto and Gaynor, 2001; Clevers 2004, Kumar et al., 2004, Monaco and Paleolog, 2004, Shoelson et al., 2003). NF-κB exists in cells as a heterodimer of members of the Rel family of proteins, including p50, p65 (RelA), RelB, and c-Rel, which share a high degree of structural similarity (May and Ghosh, 1997).

1.7.1.1 Structure of NF-κB/Rel Proteins

Although several homodimers and heterodimers are formed by various members of the NF-κB protein family, NF-κB is a term that is often used to describe the p50/p65 heterodimer, which was the first NF-κB dimer to be described (Kopp and Ghosh, 1995; Verma et al., 1995). Indeed, p50 and p65 are the first members of the NF-κB gene family to be cloned and characterized (Kawakami et al., 1988; Baeuerle and Baltimore, 1989; Ghosh et al., 1990; Zabel et al., 1991). As shown in Figure 6, members of the NF-κB/Rel protein family contain a highly conserved, N-terminal 300-amino acid region known as the rel homology domain (RHD), which mediates dimerization, interaction with IκB proteins, nuclear translocation due to the presence of a nuclear localization signal (NLS) within RHD, as well as binding to specific sites within the promoters of target genes (Ghosh et al., 1998). Although the majority of NF-κB dimers are capable of transactivating target genes, in vivo data demonstrated that some dimers such as p50/p50 and p52/p52 homodimers can be inactive or repressive (Kang et al., 1992; Plaksin et al., 1993; Brown et al., 1994). The fact that p50 and p52 lack a C-terminal region that is

conserved in the majority of other NF-κB proteins suggests that this region confers on NF-κB proteins a transcriptional potential, and hence, it is called the transactivation domain (TAD) (Ghosh *et al.*, 1998). Mutations of important residues within TAD render activating NF-κB dimers transcriptionally inactive (Blair *et al.*, 1994). RelB is a structurally unique member of the NF-κB protein family in that it contains a leucine zipper-like region at its N-terminus, which is required for its full transcriptional activity (Dobrzanski *et al.*, 1993).

1.7.1.2 Regulation and Activity of NF-κB/Rel Proteins

Under basal conditions, most NF-κB subunits are sequestered in the cytosol, where they are constitutively bound by members of the NF-κB inhibitor family of proteins, mainly IκBα and IκBβ (Baeuerle and Baltimore, 1989; Ghosh and Baltimore, 1990). However, diverse stimuli including inflammatory cytokines, mitogens, lipopolysaccharides, UV light, as well as bacterial and viral pathogens can transduce a signal that ultimately results in NF-κB liberation from its inhibitors, allowing NF-κB dimers to translocate to the nucleus and become transcriptionally active (May and Ghosh, 1997; Israel, 2000; Hayden and Ghosh, 2004). Except for RelB, all other NF-κB proteins contain a PKA phosphorylation site 20-30 amino acids N-terminal of the NLS within RHD, and phosphorylation of this site (S^{337} in p50 and S^{276} in p65) seems to be essential for nuclear translocation (Mosialos *et al.*, 1992; Norris and Manley, 1992). Moreover, phosphorylation of the PKA phosphorylation site is important in protecting the transcriptional and DNA-binding activities of active NF-κB dimers (Naumann and Scheidereit, 1994; Li *et al.*, 1994a; Li *et al.*, 1994b; Zhong *et al.*, 1997). Studies have shown that S^{276} in p65 is a major phosphorylation site that is subject to compartment-specific and stimulus-specific phosphorylation by PKAc in the cytoplasm (Zhong *et al.*, 1997) and the mitogen- and stress-activated protein kinase-1 (MSK1) in the nucleus (Vermeulen *et al.*, 2003). S^{276} phosphorylation is required for optimal NF-κB activity in different cell types (Zhong *et al.*, 1998; Zhong *et al.*, 2002; Vermeulen *et al.*, 2003). Other important phosphorylation sites have been demonstrated to be critical for optimal

NF-κB transactivation potential. Such sites include PKCζ-phosphorylated S^{311} (Duran *et al.*, 2003), CKII-phosphorylated S^{529} (Wang *et al.*, 2000), IKKα/β-phosphorylated S^{536} (Sakurai *et al.*, 1999; Jiang *et al.*, 2003; O'Mahony *et al.*, 2004). S^{536} in p65 has also been shown to be subject to phosphorylation by other kinases such as Akt (Madrid *et al.*, 2001; Sizemore *et al.*, 1999), ribosomal S6 kinase 1 (RSK1) (Bohuslav *et al.*, 2004), TRAF family member-associated (TANK)-binding kinase 1 (TBK1) (Fujita *et al.*, 2003; Buss *et al.*, 2004), and IKKε (Buss *et al.*, 2004) under certain circumstances.

Dimerization of NF-κB proteins is a prerequisite for NF-κB to become transcriptionally active, and it is mediated by specific motifs within RHDs of both members of NF-κB dimers (May and Ghosh, 1997). Studies have shown that the dimerization motifs are located at the C-terminus of RHD, and mutation of critical residues within such motifs interferes with dimerization (Logeat *et al.*, 1991; Ruben *et al.*, 1992; Ganchi *et al.*, 1993). Site-directed mutagenesis experiments also revealed the importance of certain residues with the dimerization motifs in determining partner specificity (Ruben *et al.*, 1992; Ganchi *et al.*, 1993; Ryseck *et al.*, 1995).

Once in the nucleus, active NF-κB dimers can bind to specific DNA-binding sites, known as κB binding sites, within the regulatory regions of their target genes, leading to gene transactivation (Kunsch *et al.*, 1992; Ghosh *et al.*, 1998). The κB site has a conserved consensus sequence of 10 nucleotides (GGGRNNYYCC where N is any base, R is a purine, and Y is a pyrimidine), and slight variations of the κB nucleotide sequence confer preference to different dimer combinations of NF-κB subunits (Kunsch *et al.*, 1992; May and Ghosh, 1997). The N-terminus of RHD is known to be essential for DNA-binding activity of NF-κB proteins (Rice *et al.*, 1992; Logeat *et al.*, 1991). Although point mutations of specific residues within this region do not interfere with dimerization, they completely abrogate the DNA-binding activity of NF-κB dimers (Logeat *et al.*, 1991; Bressler *et al.*, 1993).

1.7.2 Inhibitors of NF-κB (IκB)

Work from Baltimore's laboratory provided initial characterization of NF-κB coordinate regulation via physical interaction with its inhibitors, members of the IκB family of proteins (Baeuerle and Baltimore, 1988a; Baeuerle and Baltimore, 1988b). The observation that nuclear NF-κB exists in an IκB-unbound state indicated that IκB proteins can sequester NF-κB in an inactive state in the cytosol. Initial characterization of IκB proteins that associate with NF-κB led to the identification of 37-kDa and 43-kDa proteins, which are now known as IκBα and IκBβ, respectively (Zabel and Baeuerle, 1990). IκBα and IκBβ are the most well-characterized members of the mammalian IκB family of proteins, which contains a number of structurally-related proteins besides IκBα and IκBβ, including IκBγ1, IκBγ2, IκBδ, IκBε, IκBR, IκBL, p100, p105, and Bcl-3 (May and Ghosh, 1997; Whiteside and Israel, 1997). Recently, a new member of the IκB protein family was identified and named IκBζ (Totzke et al., 2006). Except for Bcl-3 and IκBζ, which are constitutively localized in the nucleus (Zhang et al., 1994; Totzke et al., 2006), all other IκB proteins are localized in the cytosol (Whiteside and Israel, 1997). Nuclear localization of Bcl-3 and IκBζ indicates that these proteins do not regulate NF-κB translocation into the nucleus, but rather, they seem to be involved in regulating NF-κB transcriptional and DNA-binding activities (Franzoso et al., 1992; Franzoso et al., 1993, Inoue et al., 1993; Totzke et al., 2006). Despite their structural homology, individual IκB proteins have distinctive structural features and they exhibit differential ability and preference to associate with and inhibit various combinations of NF-κB dimers in the cytosol.

1.7.2.1 Structure of IκBα and IκBβ Molecules

Structural organization of IκB proteins started to be uncovered upon molecular cloning and characterization of the IκBα gene (also known as MAD-3) in the early 1990s (Haskill et al., 1991; Davis et al., 1991). Now, it is clear that all IκB proteins known to date possess three to seven centrally-located, 30-33 amino acid repeated sequences

known as ankyrin (ANK) repeats (also known as notch-related motifs, cell cycle repeats, and cdc10/SW16 repeats) (Figure 6) (Beg and Baldwin, 1993; May and Ghosh, 1997; Whiteside and Israel, 1997). These repeats were initially identified in the SW16 protein expressed by *Saccharomyces cerevisiae* (Breeden and Nasmyth, 1987). Although the exact amino acid sequences of ANK repeats found in different IκB proteins can be distinct, ANK repeats have a consensus nucleotide sequence (XGXTPLHLAARXGHVEVVKLLLDXGADVNAXTK, where X can be any amino acid) (Lux *et al.*, 1990; Michaely and Bennett, 1992). Even within the same IκB protein, ANK repeats can be quite distinct, and this is thought to be an important determinant in the specificity and selectivity of the protein-protein interaction between IκB and NF-κB proteins (Davis and Bennett, 1990). The presence of ANK repeats in IκB proteins renders them capable of physically interacting with regions within the RHD of target NF-κB dimers (Hatada *et al.*, 1992; Inoue *et al.*, 1992b; Ganchi *et al.*, 1992). Additionally, IκBα, IκBβ, and IκBε have N-terminal signal-receiving domain (SRD) containing two highly conserved serine residues, which are known to be important phosphorylation sites involved in the regulation of IκB function (May and Ghosh, 1997; Whiteside and Israel, 1997). IκBα, IκBβ, IκBγ1, IκBγ2, IκBδ, IκBR, IκBL, p100, and p105 contain a region at the C-terminus that is rich in proline, glutamate, aspartate, serine and threonine residues, and hence, it is called the PEST domain (May and Ghosh, 1997; Whiteside and Israel, 1997). The PEST domain plays an important role in the inhibition of NF-κB DNA-binding activity (Ernst *et al.*, 1995), as well as in IκB protein stability/turnover (Verma *et al.*, 1995; Beauparlant *et al.*, 1996; McElhinny *et al.*, 1996; Lin *et al.*, 1996; Schwarz *et al.*, 1996). Although deletion of the N-terminus and/or the C-terminus does not affect IκBα ability to interact with NF-κB dimers, point mutations of certain residues within the N-terminus of IκBα render it resistant to signal-induced phosphorylation and degradation (Brockman *et al.*, 1995; Brown *et al.*, 1995; Traenckner *et al.*, 1995; Whiteside *et al.*, 1995), while deletion of the C-terminus of IκBα interferes with its ability to dissociate NF-κB from its DNA binding sites (Inoue *et al.*, 1992b; Leveillard and Verma, 1993; Ernst *et al.*, 1995). Finally, two nuclear export signal (NES) sequences have been identified in the N-terminus (Johnson *et al.*, 1999) and C-terminus of IκBα (Arenzana-

Seisdedo *et al.*, 1997). Actually, the more conserved N-terminal NES was shown to be necessary and sufficient for IκBα nuclear export (Johnson *et al.*, 1999). Efficient nuclear translocation and cytosolic re-localization (i.e. nuclear export) of IκBα is ensured by the presence of NLS and NES, respectively.

NF-κB/Rel Family
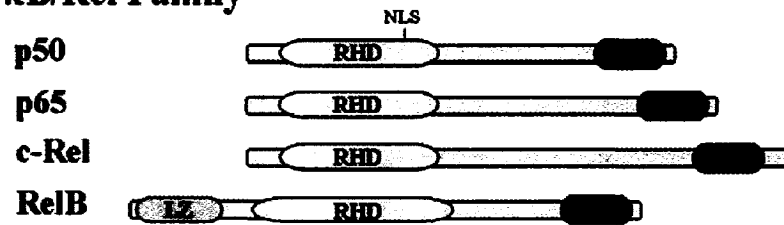
IκB Family
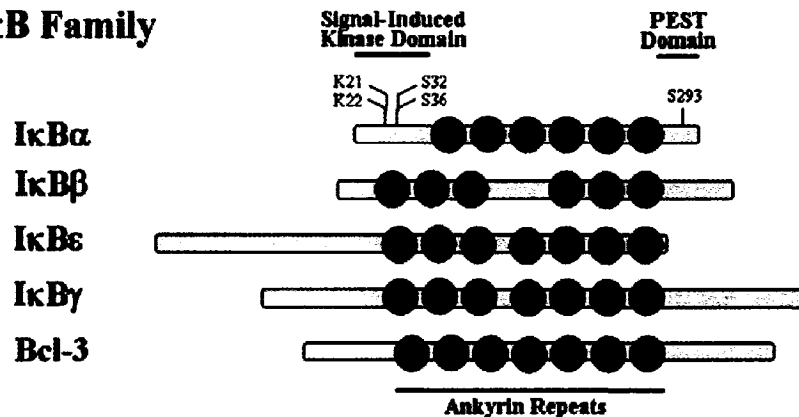
Figure 6. Structural Organization of NF-κB/Rel and IκB Proteins. *(A)* A schematic representation of some members of the Rel family of proteins. Members of this family contain a unique, highly conserved Rel homology domain (RHD) towards the N-terminus, and this domain carries a nuclear localization signal (NLS). Most members of the Rel family contain a C-terminally located transactivation domain (TAD) that is important for optimal transcriptional activity. *(B)* A schematic representation of some members of the IκB family of proteins, which are uniquely characterized by the presence of 30-33-amino acid repeats known as ankyrin (ANK) repeats. At least for IκBα and IκBβ; the most well-characterized members of the IκB family, there are two conserved serine residues at the N-terminus preceding the first ANK repeat. Phosphorylation of these two serine residues is known to be crucial for signaling IκB proteins for ubiquitination and proteolytic degradation. At the C-terminus of IκB proteins, there is a region rich with proline, glutamate, serine, and threonine residues, and hence, it is named the PEST domain. This figure is adapted from Hayden and Ghosh, 2004.

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1.7.2.2 Function of IκBα and IκBβ Molecules

Since IκBα and IκBβ are the best studied members of the IκB protein family, special emphasis will be allotted to these two molecules throughout this thesis. Members of the IκB protein family are constitutively and ubiquitously expressed proteins that localize in the cytosol, except for Bcl-3 and IκBζ which are primarily present in the nucleus (Zhang *et al.*, 1994; Totzke *et al.*, 2006). The main function of IκB proteins is to inhibit NF-κB activity when it is not required, and this happens via protein-protein interaction that takes place between IκB proteins and NF-κB dimers in the cytosol. IκBα and IκBβ interact via their ANK repeats with the RHD of NF-κB dimerized proteins in such a way that masks the positively charged regions of the NLSs within the RHDs of NF-κB dimers (Ghosh *et al.*, 1995; Muller *et al.*, 1995). As a result, NF-κB dimers are prevented from translocating to the nucleus, and thus, they are kept in an inactive, IκB-bound state in the cytosol (Beg *et al.*, 1992; Henkel *et al.*, 1992; Zabel *et al.*, 1993). Although IκB-NF-κB interaction is mediated by ANK repeats of IκB proteins, not all ANK repeats are involved in this interaction (Inoue *et al.*, 1992b; Leveillard and Verma, 1993; Dobrzanski *et al.*, 1994). In an extensive site-directed mutagenesis study performed to assess the significance of every ANK repeat within IκBα (Inoue *et al.*, 1992b), a number of interesting findings were revealed. First, the C-terminus of IκBα is required for the protein to be functional, and thus, the ANK repeats are not sufficient on their own to exert an inhibitory action towards NF-κB. Second, lack of the third ANK repeat does not impede IκBα inhibitory function, suggesting that this ANK repeat is dispensable for IκBα inhibitory function. Third, the only mutant forms of IκBα that are unable to inhibit NF-κB activity are those that were incapable of interacting with NF-κB. Another study suggests that the first ANK repeat of IκBα is mostly responsible for its inhibitory activity, and substituting the first ANK repeat in IκBβ with that of IκBα significantly enhances the former's inhibitory activity (Simeonidis *et al.*, 1999). It is evident that the ANK repeats and the C-terminal region (i.e. PEST domain) of IκBα form a tertiary structure that is capable of interacting with NF-κB proteins, and that such interaction confers NF-κB transcriptional inactivity (Inoue *et al.*, 1992b).

Remarkably, it is now known that NF-κB is itself an up-regulator of IκBα and IκBβ, in which NF-κB activation via various and distinct stimuli is usually followed by rapid induction of IκBα and IκBβ expression (Verma *et al.*, 1995; Baldwin, 1996; Suyang *et al.*, 1996) due to the presence of a κB DNA-binding site within the IκB promoter (Sun *et al.*, 1993; Cheng *et al.*, 1994; Chiao *et al.*, 1994). This negative feedback regulatory loop sets a molecular switch that ensures rapid, controlled, and transient activation of target genes by NF-κB. Induced expression of IκBα allows translocation of nascently synthesized IκBα into the nucleus, where it binds to active NF-κB dimers that are bound to κB sites within the promoters of their target genes. Interaction between nuclear IκBα and active NF-κB dimers leads to dissociation between NF-κB dimers and DNA, and it forces a conformational change in IκBα that exposes the nuclear export signal (NES), eventually leading to re-sequesterization of IκBα and NF-κB dimers in the cytosol (Zabel and Baeuerle, 1990; Arenzana-Seisdedos *et al.*, 1995; Arenzana-Seisdedos *et al.*, 1997). This highly complex, tightly regulated reciprocal regulatory process involving NF-κB and IκB proteins confers the NF-κB signaling pathway a central regulatory function in many key biological events that requires transient, short-term NF-κB activity.

1.7.2.3 Regulation of IκBα and IκBβ Molecules

There are at least two well-characterized signaling pathways leading to NF-κB activation, classical and alternative, and both rely on the catalytic activity of known IκB kinases (IKKs). The classical NF-κB signaling pathway is typically triggered by a vast number of pro-inflammatory cytokines (e.g. IL-1β and TNFα), viruses, and bacteria, and hence, it leads to a coordinate inflammatory/immune response culminating in the expression of multiple cytokines, chemokines, adhesion molecules, and pro-inflammatory proteolytic enzymes (Figure 5) (Luo *et al.*, 2005a; Viatour *et al.*, 2005). On the other hand, the alternative NF-κB signaling pathway is normally triggered by non-proinflammatory cytokines (e.g. lymphotoxin β (LTβ), B-cell activating factor (BAFF), and CD40 ligand (CD40L)) as well as some viruses (e.g. human T-cell leukemia virus

(HTLV) and Epstein-Barr virus (EBV)) (Luo *et al.*, 2005a; Viatour *et al.*, 2005). The alternative NF-κB signaling pathway is triggered to induce the expression of genes whose products play fundamental roles in the development and maintenance of secondary lymphoid organs (Luo *et al.*, 2005a). Unlike the classical pathway, the alternative pathway is NEMO-independent in that it does not require the IκB kinase (IKK) complex, which contains the scaffold protein NF-κB essential modulator (NEMO), IKKα, IKKβ, IKKγ and other adaptor proteins (Luo *et al.*, 2005a; Viatour *et al.*, 2005). Instead, the alternative pathway relies on the activity of the NF-κB inducing kinase (NIK) that transactivates IKKα-IKKα homodimers, which upon activation transduce a signal that culminates in profound NF-κB activation (Viatour *et al.*, 2005). In the next sections, special attention will be paid to the classical NF-κB signaling pathway.

1.7.2.3.1 Signal-Induced IκBα and IκBβ Phosphorylation by the IKK Complex

In order for NF-κB to become activated, it is now known that IκBα/β must become phosphorylated at specific serine residues at the N-terminus, followed by ubiquitination (not for IκBβ) and proteolytic degradation of phosphorylated IκBα and IκBβ in the cytosol. Phosphorylation and subsequent proteolytic degradation of IκBα and IκBβ liberate NF-κB dimers, which become phosphorylated, translocate to the nucleus, and bind to specific κB binding sites within the promoter/enhancer regions of their target genes, leading to their transactivation. Binding of TNFα to its receptor (TNFR1) is known to trigger NF-κB activation through the classical pathway, leading to TNF-induced cell death (Jiang *et al.*, 1999). Under basal conditions, constitutive activation of the TNF-induced cell death pathway is prevented by the blocking potential of a protein called the silencer of death domains (SODD), which binds to TNFR1 and prevents downstream signal transduction (Jiang *et al.*, 1999). Upon TNFα-TNFR1 binding, SODD dissociates from TNFR1 and this allows recruitment of adaptor molecules TNF receptor-associated death domain (TRADD), receptor interacting protein (RIP), and TNFR associated factor 2 (TRAF2), which bind to TNFR1 as a complex through TRADD.

Sequential recruitment of the NF-κB inducing kinase (NIK) and the IKK complex to the TRADD complex bound to TNFR1 is mediated by TRAF2 (Ninomiya-Tsuji *et al.*, 1999; Takeuchi and Akira, 2001). Stimulation signals triggered by LPS, IL-1β, and TNFα also leads to the recruitment and activation of MEKK1 (Nemoto *et al.*, 1998). The recruited IKK complex also contains an IκB kinase regulatory subunit called ELKS, which allows IκBα recruitment and interaction with the IKK complex at the membrane (Ducut Sigala *et al.*, 2004). Although NEMO, IKKα, IKKβ, IKKγ, and ELKS are the main components of the cytoplasmic serine-protein-kinase multi-subunit IKK complex, other proteins are identified as essential elements of the complex (Regnier *et al.*, 1997; Rothwarf *et al.*, 1998, Yamaoka *et al.*, 1998; Didonato *et al.*, 1997; Mercurio *et al.*, 1997; Woronicz *et al.*, 1997; Zandi and Karin, 1999; Mercurio *et al.*, 1999). NIK and MEKK1 are upstream up-regulators of the IKK complex, in which they phosphorylate and transactivate IKKα and IKKβ within the complex (Regnier *et al.*, 1997; Nakano *et al.*, 1998).

Membrane recruited IKK complex with catalytically active IKKα and IKKβ is responsible for phosphorylating two N-terminally-located conserved serine residues in IκBα and IκBβ (Ser^{32} and Ser^{36} in IκBα; Ser^{19} and Ser^{23} in IκBβ) (Zandi *et al.*, 1997; Wu and Ghosh, 2003). Interestingly, cell lines that lack NEMO display severe defects in NF-κB activation and they are unresponsive to a wide range of potent stimuli (Yamaoka *et al.*, 1998), indicating that catalytically active IKKα and IKKβ are insufficient in phosphorylating IκBα and IκBβ in the absence of complex formation. Although some studies have initially suggested a major role of IKKα in IκBα and IκBβ phosphorylation (DiDonato *et al.*, 1997; Mercurio *et al.*, 1997, Zandi *et al.*, 1997; Zandi *et al.*, 1998; Ling *et al.*, 1998), a study demonstrated that mutation of two serine residues within the activation loop of IKKβ, but not IKKα, renders the IKK complex catalytically inactive (Delhase *et al.*, 1999), indicating that IKKβ is the predominant kinase component of the IKK complex. This observation is supported by an experiment demonstrating that IKKα$^{-/-}$ cells display normal IKK activity towards IκBα and IκBβ upon LPS, IL-1β, and TNFα treatment (Hu *et al.*, 1999). Moreover, IKKβ$^{-/-}$ mice resemble p65$^{-/-}$ mice in that they suffer from embryonic lethality due to severe liver apoptosis (Li *et al.*, 1999). The apoptotic phenotype of IKKβ$^{-/-}$ mice combined with the observation that TNFα

deficiency eliminate embryonic lethality of p65$^{-/-}$ mice (Doi *et al.*, 1999) and that NF-κB mediates TNFα-induced apoptosis (Beg and Baltimore, 1996) strongly suggest that IKKβ catalytic activity is absolutely required for NF-κB activation. Direct experimental evidence indicates that IKKβ$^{-/-}$ embryonic stem cells and fibroblasts display defective IKK activity towards IκBα, and no NF-κB activity (Li *et al.*, 1999). These findings indicate the IKKα cannot compensate for IKKβ loss, and that IKKβ is solely responsible for phosphorylating IκBα and IκBβ *in vivo*. Interestingly, phosphorylation of S^{32}/S^{36} and S^{19}/S^{23} in IκBα and IκBβ, respectively, does not force IκBα and IκBβ dissociation from their NF-κB dimer partners in the cytosol (DiDonato *et al.*, 1995; Traenckner *et al.*, 1995), but it renders them susceptible to ubiquitination and subsequent proteolytic degradation (Beg and Baldwin, 1993; Traenckner and Baeuerle, 1995; DiDonato *et al.*, 1995; Traenckner *et al.*, 1995; Chen *et al.*, 1995; Alkalay *et al.*, 1995b; Li *et al.*, 1995).

1.7.2.3.2 Signal-Induced Degradation of IκBα and IκBβ

Proteolysis, or proteolytic degradation, is a highly-regulated cellular multi-step process that involves enzymes called proteases that are capable of hydrolyzing peptide bonds within polypeptides that are usually ubiquitinated, ultimately leading to protein degradation. Protein ubiquitination and subsequent degradation were thought to be molecular mechanisms undertaken by cells to eradicate misfolded or defective proteins (Ciechanover, 1994; Hochstrasser *et al.*, 1995). Nowadays, however, it is well recognized that protein degradation is a process that is not directed only against imperfect proteins, but also against some fully-functional proteins as a means to regulate and control various key biological processes (Ghosh *et al.*, 1998). Cyclins, proteins involved in the regulation of the cell cycle, are a prime example of functional proteins that are regulated by ubiquitination-dependent proteolytic degradation pathways (Murray, 1995; Wilkinson, 1999).

It is evident that signal-induced phosphorylation of IκBα and IκBβ must be followed by their degradation for NF-κB transactivation potential to be manifested (Henkel *et al.*, 1993; Sun *et al.*, 1994; Traenckner *et al.*, 1994; Finco *et al.*, 1994;

Miyamoto *et al.*, 1994; Lin *et al.*, 1995; Alkalay *et al.*, 1995a; DiDonato *et al.*, 1995). The most compelling evidence indicating the necessity of IκBα and IκBβ degradation for NF-κB activation came from a study showing that treatment of stimulated cells with protease inhibitors does not eliminate phosphorylation of IκBα and IκBβ, but protects NF-κB-IκBα and NF-κB-IκBβ cytosolic complexes, and thus, prevents NF-κB activation (Traenckner *et al.*, 1994; Lin *et al.*, 1995; Alkalay *et al.*, 1995a; DiDonato *et al.*, 1995; Weil *et al.*, 1997). Under stimulatory conditions, proteolytic degradation of IκBα and IκBβ occurs via an ubiquitination- and proteasome-dependent mechanism (Chen *et al.*, 1995; Alkalay *et al.*, 1995b).

The 26S proteasome is composed of a core protease, known as the 20S proteasome, and the 19S regulatory complex (RC), which is composed of at least 18 different subunits in two subcomplexes known as the lid and the base (Hartmann-Petersen and Gordon, 2004). The involvement of the 26S proteasome in signal-induced NF-κB activation was originally signified by studies demonstrating that treatment with selective inhibitors of the 26S proteasome block NF-κB activation (Palombella *et al.*, 1994; Traenckner *et al.*, 1994). Subsequent to phosphorylation of the two serine residues within the signal-induced kinase domain of IκBα, multiple 76-amino acid ubiquitin polypeptides covalently attach to the N-terminus of phosphorylated IκBα, rendering IκB proteins susceptible to 26S proteasome-dependent degradation (Chen *et al.*, 1995; Alkalay *et al.*, 1995b; Rodriguez *et al.*, 1996; Griscavage *et al.*, 1996). For IκBα, ubiquitination primarily takes place on two adjacent lysine residues (K^{21} and K^{22}) in the N-terminus of the protein, and mutation of these two lysine residues prevents IκBα ubiquitination and subsequent proteolytic degradation (Scherer *et al.*, 1995, Baldi *et al.*, 1996). Indeed, conservative substitution of K^{21} and/or K^{22} by arginine precludes not only ubiquitination, but also signal-induced degradation of IκBα, ultimately preventing NF-κB activation (Scherer *et al.*, 1995; Baldi *et al.*, 1996). During initial characterization of IκBβ regulation, it was shown that treatment of cells with protease inhibitors prevents IκBβ degradation (Weil *et al.*, 1997), suggesting that IκBβ may be under control of the ubiquitin-proteasome machinery in a phosphorylation-dependent fashion, as in IκBα. Indeed, site-directed mutagenesis of S^{19} and/or S^{23} renders IκBβ somewhat resistant to

degradation (Weil *et al.*, 1997). Strikingly, however, alanine substitution of K^9 has no effect on IκBβ degradation (Weil *et al.*, 1997), indicating that ubiquitination is not a prerequisite for IκBβ degradation. So, although phosphorylation of the two, N-terminal conserved serine residues is required for inducing IκBα and IκBβ degradation, ubiquitination of the N-terminal lysine residues is required for proteasome-dependent degradation of IκBα, but not IκBβ. Interestingly, although the PEST domain of IκBα and IκBβ is not required for S^{32}/S^{36} and S^{19}/S^{23} phosphorylation, respectively, its deletion eliminates signal-induced degradation of IκBα (Brown *et al.*, 1995; Rodriguez *et al.*, 1995; Whiteside *et al.*, 1995; Aoki *et al.*, 1996; Sun *et al.*, 1996) and IκBβ (DiDonato *et al.*, 1996; Harhaj *et al.*, 1996; McKinsey *et al.*, 1996). In sum, for signal-induced NF-κB transactivation activity to manifest, at least six main biochemical events must precede: (1) phosphorylation of IκBα and IκBβ by the IKK complex, (2) ubiquitination of phosphorylated IκBα, (3) proteasome-mediated degradation of IκBα and IκBβ, (4) phosphorylation of NF-κB dimer, (5) nuclear translocation of NF-κB dimers, and (6) NF-κB dimer-κB DNA interaction (Figure 7).
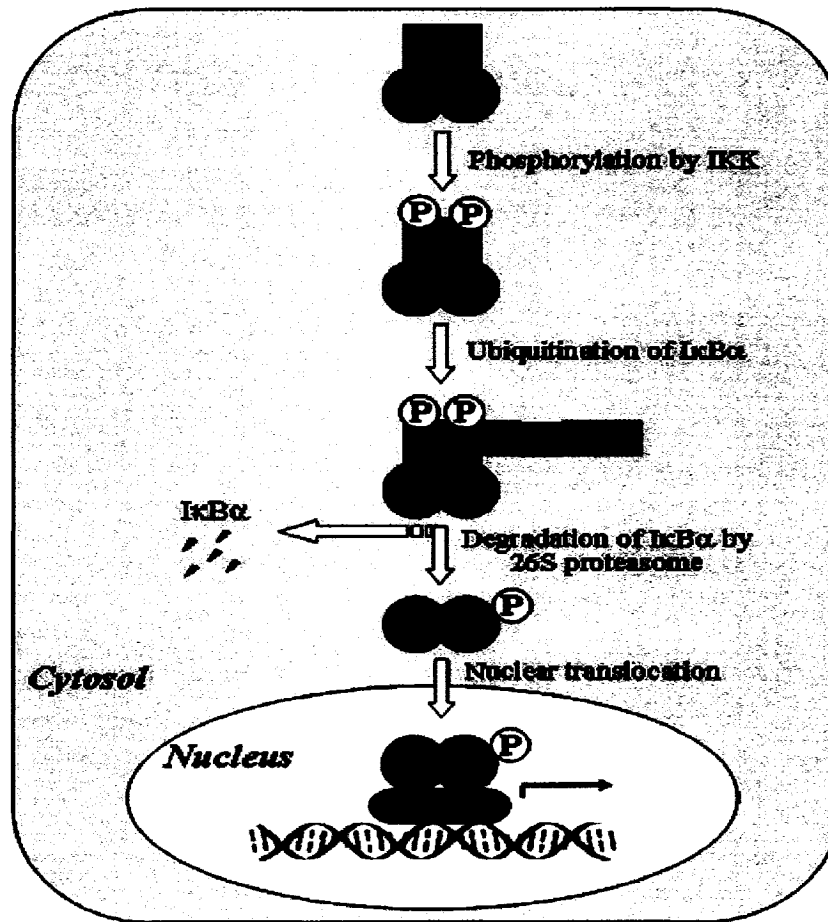
Figure 7. Regulation of IκBα and IκBβ. A cartoon representing the most critical intracellular events that lead to NF-κB transcriptional activation. Interaction between diverse ligands and their receptors eventually lead to activation of the IKK complex, which allows IκBα and IκBβ phosphorylation in the cytosol. This phosphorylation step is followed by IκBα, but not IκBβ, ubiquitination, and subsequently, IκBα and IκBβ are subjected to proteolytic degradation. Once IκBα and IκBβ molecules are degraded, NF-κB dimers are liberated, and they translocate to the nucleus subsequent to unmasking of their NLS. Once in the nucleus, NF-κB dimers bind to κB sites within the promoter/enhancer regions of their target genes, driving gene transactivation.

1.7.2.3.3 Basal Turnover/Degradation of IκBα and IκBβ

Besides signal-induced proteolytic degradation of IκBα and IκBβ, these proteins have been shown to be susceptible to degradation under basal, un-stimulatory conditions. In fact, IκBα and IκBβ have been shown to be constitutively phosphorylated in absence of stimuli (Mellits *et al.*, 1993; Naumann and Scheidereit, 1994), and specific serine/threonine residues (S^{283}, S^{289}, S^{293}, and T^{291}) within the PEST domain of IκBα have been shown to be the target of constitutive phosphorylation by casein kinase II (CKII) (Barroga *et al.*, 1995; Whiteside *et al.*, 1995; McElhinny *et al.*, 1996; Schwarz *et al.*, 1996). Phosphorylation of the PEST domain renders IκBα susceptible to degradation, indicating that the PEST domain is essential for controlling IκBα intrinsic protein stability (McElhinny *et al.*, 1996; Lin *et al.*, 1996; Schwarz *et al.*, 1996). Likewise, it was shown that the PEST domain of IκBβ is required for its degradation (Weil *et al.*, 1997). Unlike signal-induced degradation of IκBα, which required ubiquitination, basal degradation of IκBα seems to be ubiquitination-independent, in which degradation of un-ubiquitinated IκBα is evident in un-stimulated cells *in vitro* (Krappmann *et al.*, 1996). This data is supported by the observation that a mutant form of IκBα carrying lysine-to-arginine substitutions at the two ubiquitination sites (K^{21} and K^{22}) is as prone to basal degradation as the wildtype form of IκBα (Miyamoto *et al.*, 1998). In other words, ubiquitination of IκBα is a signal-induced event and is not required for basal degradation of IκBα. However, the ubiquitin-independent IκBα degradation pathway is proteasome-dependent, since proteasome inhibitors block basal, as well as signal-induced, degradation of IκBα (Krappmann *et al.*, 1996).

Until the emergence of a paper published by Phillips and Ghosh in 1997, the 26S proteasome-mediated proteolysis pathway was the only known cellular process responsible for basal and signal-induced degradation of IκBα and IκBβ. However, the use of selective proteasome inhibitors revealed the existence of a novel proteolysis pathway that leads to IκBα and IκBβ degradation in an ubiquitin-independent, proteasome-independent manner in immature B cells (Phillips and Ghosh, 1997; Shumway and Miyamoto, 2004). Indeed, such a novel pathway was subsequently shown

to be dependent on the presence of free calcium, most likely imported from outside the cell (Miyamoto *et al.*, 1998). Further examination of this pathway revealed that phosphorylation of the PEST domain of IκBα allows it to bind to the calmodulin-like domain (CaMLD) of the large subunit of the calcium-dependent thiol protease complex, calpain (Shumway *et al.*, 1999; Shumway *et al.*, 2002). Interaction between IκBα and calpain is followed by N-terminal cleavage and further proteolysis of IκBα (Shumway *et al.*, 1999; Shumway *et al.*, 2002). These studies suggest that IκBα and IκBβ can also be regulated by protease machineries other than the intrinsic, well-known 26S proteasome complex.

1.7.3   Role of NF-κB in Inflammation and Atherosclerosis

One of the major functions of NF-κB is its key involvement in inducing an effective immune/inflammatory response against viral and bacterial infections. The importance of NF-κB role in initiating a potent inflammatory response cannot be better signified than recognizing that the κB consensus sequence is found in the promoter/enhancer regions of more than 50 diverse genes whose expression is known to be crucial in driving an inflammatory response (Baeuerle and Baltimore, 1988b; May and Ghosh, 1997, Ghosh *et al.*, 1998). Inducible genes that are known to be transactivated by NF-κB include, but not limited to, IL-1β, IL-6, IL-8, TNFα, IFNγ, MCP-1, iNOS, COX-2, ICAM-1, and VCAM-1 (Wilcox *et al.*, 1989; Barath *et al.*, 1990; Brand *et al.*, 1991; Cybulsky and Gimbrone, 1991; Mackman *et al.*, 1991; Ross, 1993; Baeuerle and Henkel, 1994; Kopp and Ghosh, 1995; Baldwin, 1996; Ghosh *et al.*, 1998; Tak and Firestein, 2001; Yamamoto and Gaynor, 2001). These molecules play critical roles in key biological events involving cell recruitment, attachment, differentiation, proliferation, and activation constituting an active inflammatory response. NF-κB is also known to cooperate with other active transcription factors such as AP-1 in up-regulating the expression of some MMPs (Yokoo and Kitamura, 1996; Mengshol *et al.*, 2000), which play destructive roles in atherosclerotic lesions rendering them unstable and prone to rupture (refer to section 1.6.2 for more details).

Genetic knockout models have provided lucid evidence that NF-κB proteins are absolutely essential for the development of a normal, effective immune system, since NF-κB genetic ablation, in general, renders mice immunocompromised and prone to pathogenic infections. Specifically, p50$^{-/-}$ mice develop normally but are defective in immunoglobulin production, and thus, humoral immune responses (Sha et al., 1995). Likewise, p52$^{-/-}$ mice develop normally but their B-cell follicles and germinal centers do not develop normally, and the mice are unable to launch an adequate humoral response against T-cell-dependent antigens (Caamano et al., 1998; Franzoso et al., 1998). Although ablation of p65 causes embryonic lethality due to liver apoptosis (Beg et al., 1995), ablation of TNFα or TNFR rescues p65$^{-/-}$ from the lethal phenotype (Doi et al., 1997; Alcamo et al., 2001). However, loss of p65$^{-/-}$/TNFα$^{-/-}$ mice are highly susceptible to bacterial infections and unable to provoke an innate immune response. In addition, T and B lymphocytes of c-Rel$^{-/-}$ mice are unresponsive to various mitogenic stimuli, and the mice are unable to generate a humoral immune response (Kontgen et al., 1995). Lastly, RelB$^{-/-}$ mice are severely defective in generating adaptive immune responses (Weih et al., 1995). Thus, it is evident that NF-κB proteins are indispensable in generating effective inflammatory, innate, and adaptive immune responses against viral and bacterial pathogens.

The first experimental evidence of NF-κB role in atherosclerosis came from a study demonstrating that active NF-κB can be detected in aortae with evident atherosclerotic lesions but not in normal, non-lesional aortae (Brand et al., 1996). In fact, a strong signal of active NF-κB can be detected in endothelial cells, macrophages, and to a lesser extent, T lymphocytes within atherosclerotic lesions (Kaltschmidt et al., 1994; Brand et al., 1996). Interestingly, oxLDL is potentially capable of activating NF-κB in endothelial cells and macrophages in culture systems as well as in atherosclerotic lesions (Parhami et al., 1993; Peng et al., 1995; Rajavashisth et al., 1995; Brand et al., 1997a). In the context of atherosclerosis, NF-κB activation is believed to promote the expression of various factors that mediate various processes such as proliferation, chemotaxis, adhesion, inflammation, and thrombosis, key events in atherogenesis (Brand et al., 1997b). Furthermore, several studies have demonstrated a positive correlation between

NF-κB activity and incidence of myocardial infarction (Thourani *et al.*, 2000; Sasaki *et al.*, 2000; Sasaki *et al.*, 2001; Yoshiyama *et al.*, 2001; Izumi *et al.*, 2001; Shimizu *et al.*, 2002; Thiemermann 2004; Lu *et al.*, 2004). Due to its critical role in atherosclerosis and myocardial infarction, NF-κB is proposed to be a promising therapeutic target for reducing, if not eliminating, the risks of atherosclerosis and its complications (Jones *et al.*, 2003; Kutuk and Basaga, 2003; Monaco and Paleolog, 2004).

1.8 LPS-Induced NF-κB Activation via Toll-Like Receptors (TLRs)

1.8.1 Structure and Expression of Toll-Like Receptors

Toll is a protein that was first discovered in *Drosophila*, and it has been implicated in a signaling pathway that confers *Drosophila* with a bacterial recognition system (Belvin and Anderson, 1996). Near the end of the 1990s, Toll-Like Receptors (TLRs) were identified as mammalian homologues of the *Drosophila* Toll protein (Medzhitov *et al.*, 1997; Rock *et al.*, 1998). To date, ten and thirteen different orthologues of TLRs, designated TLR1 to TLR13, have been identified in humans and mice, respectively (Tsan and Gao, 2004; Palsson-McDermott and O'Neill, 2004), and they have been classified as members of the TLR subfamily, which along with IL-1R subfamily form the toll/interleukin-1 receptor (TIR) superfamily of proteins due to substantial sequence homology between TLRs and members of the IL-1R subfamily (Takeda *et al.*, 2003). TLRs are type I transmembrane proteins that share an extracellular leucine-rich repeat (LRR) domain and a cytoplasmic toll/interleukin-1 receptor (TIR) domain (Takeuchi and Akira, 2001; Tsan and Gao, 2004). Based on their genomic structure and amino acid sequence, TLRs are subdivided into five groups: TLR2 (contains TLR1, TLR2, TLR6, and TLR10), TLR3, TLR4, TLR5, and TLR9 (contains TLR7, TLR8, and TLR9) (Takeda *et al.*, 2003; Tsan and Gao, 2004). TLRs display distinct expression pattern, in which TLR1 is ubiquitously expressed, TLR2, TLR4, and TLR5 are abundantly expressed in monocytes, polymorphonuclear phagocytes (PMNs), and dendritic cells (DCs), whereas TLR3 is exclusively expressed in DCs (Muzio *et al.*, 2000).

1.8.2 Function of Toll-Like Receptors

Functionally, TLRs, mainly TLR2 and TLR4, have been implicated in signaling pathways triggered by various bacterial protein and lipoprotein components, indicating that TLRs are important for host innate immunity manifested by signaling pathways that are crucial for proper recognition and response to bacterial infections (Kopp and Medzhitov, 1999; Anderson, 2000; Takeuchi and Akira, 2001). Septic shock syndrome is a life-threatening condition that arises from an exaggerated, un-controlled immune reaction to various bacterial infections caused by LPS-bearing bacteria (Zhang and Ghosh, 2000). Accumulating evidence indicates that activation of the TLR signaling pathway is a main feature of septic shock events, and that members of the TLR subfamily play critical roles in mediating the LPS-induced effects leading to septic shock syndrome (Cristofaro and Opal, 2003; Miyake, 2004; Lin and Yeh, 2005).

Although initial investigations revealed that TLR2 is responsive to LPS treatment (Yang et al., 1998; Kirschning et al., 1998), subsequent in vitro and in vivo analyses revealed that TLR4 is the biologically relevant LPS receptor. The most convincing evidence that TLR4 functions as an endogenous receptor of LPS came from genetic knockout studies demonstrating that lack of TLR4 renders macrophages and B lymphocytes completely unresponsive to LPS, and that TLR4$^{-/-}$ mice are resistant to LPS-induced septic shock (Hoshino et al., 1999). Moreover, a natural point mutation within the TLR4 gene in C3H/HeJ and C57BL6/10ScCr mouse strains renders them hyporesponsive to LPS treatment (Poltorak et al., 1998; Qureshi et al., 1999). In contrast, TLR2$^{-/-}$ mice, which retain a functional TLR4 gene, undergo a LPS-induced septic shock as do their wildtype counterparts (Takeuchi et al., 1999). Finally, LPS-stimulated TLR2$^{-/-}$ and wildtype macrophages express equivalent levels of cytokines (Takeuchi et al., 1999). These findings, and others, clearly emphasize that LPS requires TLR4, but not TLR2, to transducer a signal that ultimately leads to septic shock.

Besides LPS, TLR4 has been shown to be responsive to lipoteichoic acid (LTA), a polyol phosphate polymer bearing a strong negative charge that is covalently linked to the peptidoglycan (PGN) in some Gram-positive bacteria, and loss of TLR4 renders mice unresponsive to LTA (Takeuchi et al., 1999). Many Gram-positive bacterial components

including PGN from *Staphylococcus aureus* have been demonstrated to trigger signaling pathways that are solely mediated by TLR2. Indeed, TLR2 has been shown to be responsible for mediating signaling pathways triggered by different Gram-positive bacteria such as *Staphylococcus aureus*, *Staphylococcus pneumoniae*, *Bacillus subtilis* , and *Listeria monocytogenes* (Schwandner *et al.*, 1999; Yoshimura *et al.*, 1999; Flo *et al.*, 2000). Physiologically, it is evident that TLR2 responds exclusively to components of Gram-positive bacteria, while TLR4 is responsive to components of both Gram-negative and Gram-positive bacteria.

Interestingly, TLR3 has been implicated in mediating a signaling pathway induced by double-stranded RNA (dsRNA) molecules leading to interferon production, and thus, TLR3 is believed to play critical roles in generating anti-viral innate immune responses (Schroder and Bowie, 2005). Although important regulatory roles of other TLRs in host innate immune response have been suggested, their exact function and modes of action is far from clear.

1.8.3 LPS-Induced TLR Signaling Pathways and NF-κB Activation

LPS is the primary glycolipid component of the outer membrane of Gram-negative bacteria, and it induces the expression of a wide range of inflammatory/immune factors (e.g. IL-1β, IL-6, TNFα, MCP-1, iNOS, and COX-2) through NF-κB activation (Ghosh *et al.*, 1998; May and Ghosh, 1997). It was shown that for LPS-responsiveness to manifest, a membrane-bound or soluble form of CD14 should be expressed, and that CD14, along with TLR4, functions as a co-receptor for LPS (Wright *et al.*, 1990; Ulevitch and Tobias, 1999). LPS is also known to bind specifically to a serum protein called LPS binding protein (LBP) (Tobias *et al.*, 1986), and the nascently formed LPS-LBP complex is delivered to CD14 expressed on the cell surface of target cells (Ulevitch and Tobias, 1995). CD14$^{-/-}$ mice have been shown to be unresponsive to LPS, and thus, they are resistant to LPS-induced septic shock (Haziot *et al.*, 1996). The fact that CD14 is a cell-surface glycoprotein that lacks a cytoplasmic domain suggested that other factors should be bound or in close proximity to the CD14/TLR complex. MD-2 is a secreted glycoprotein that has been shown to serve as an extracellular adaptor protein that bridges

LPS to LRR domain of TLR, initiating LPS-induced signal transduction via TLR (Figure 8) (Visintin *et al.*, 2003). In fact, a mutant derivative of MD-2 as well as genetic ablation of wildtype MD-2 lead to LPS unresponsiveness in TLR4-expressing 293 cells (Schromm *et al.*, 2001; Visintin *et al.*, 2001). Moreover, MD-2$^{-/-}$ mice are hyporesponsive to LPS treatment, and they successfully evade LPS-induced septic shock (Nagai *et al.*, 2002). Later work has identified an adaptor protein designated MyD88 (myeloid differentiation factor 88), which covalently binds to TLRs via the so-called homophilic interaction due to the fact that MyD88 has a C-terminal TIR domain that mediates interaction with the TIR domain of TLRs (Takeuchi and Akira, 2001; Tsan and Gao, 2004). MyD88 also has an N-terminal death domain (DD) that mediates MyD88 homophilic interaction with a protein serine/threonine kinase called IL-1R associated kinase 1 (IRAK1) that becomes recruited to the membrane upon receptor binding (Figure 8) (Wesche *et al.*, 1997). Upon IRAK1 recruitment and activation by IRAK4-mediated trans-phosphorylation and followed by autophosphorylation, another adaptor protein called TNFR associated factor 6 (TRAF6) is phosphorylated and recruited to the active TLR complex (Cao *et al.*, 1996).

While the C-terminal domain of TRAF6 is important for interaction with IRAK1, the N-terminal domain of TRAF6 is important for interaction with downstream signaling molecules (Takeuchi and Akira, 2001). Once TRAF6 is bound to IRAK1, TAK1-binding protein 2 (TAB2), an adaptor protein that recruits TGFβ activating kinase 1 (TAK1), and the evolutionary conserved signaling intermediate in Toll pathways (ECSIT) become recruited to the active TLR complex (Figure 8) (Ninomiya-Tsuji *et al.*, 1999; Takaesu *et al.*, 2000; Kopp *et al.*, 1999). Recruitment of TAK1 induces its autophosphorylation in its activation loop (Kishimoto *et al.*, 2000), and active TAK1 activates NIK, which in turn activates IKKβ in the NEMO/IKK complex that ultimately leads to IκBα and IκBβ degradation and NF-κB activation (Ninomiya-Tsuji *et al.*, 1999). Interaction between TRAF6 and ECSIT has also been shown to lead to the activation of MEK kinase 1 (MEKK1) that ultimately results in NF-κB activation (Figure 8) (Kopp *et al.*, 1999).

Importantly, genetic deletion of various components of the TLR complex and downstream signaling molecules that participate in a robust LPS-induced NF-κB activation signified the role of each of those proteins in LPS responsiveness. In fact,

deficiency of CD14, MD-2, MyD88, IRAK, and TRAF6 in macrophages leads to defective LPS-induced NF-κB signaling, and mice lacking these genes are resistant to LPS-induced septic shock (Haziot *et al.*, 1996; Nagai *et al.*, 2002; Kawai *et al.*, 1999; Swantek *et al.*, 2000; Lomaga *et al.*, 1999).

Figure 8. Toll-Like Receptor (TLR) Signaling Pathway.
A cartoon representing a complex series of biochemical events that are triggered by various stimuli (e.g. LPS) via Toll-like receptors (TLRs). TLR triggering eventually leads to the activation of NF-κB and MAPK signaling pathways, through which a wide range of transcription factors can be activated. TLR signaling pathways are typically triggered by infectious agents, and thus, they are critically implicated in various aspects of the innate immune response in mammals. This figure is adapted from www.biocarta.com.

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1.9 Goals of this Research

Although AEBP1 has been initially characterized as a transcriptional repressor that can potently repress the aP2 gene (He *et al.*, 1995; Kim *et al.*, 1999), no other genes have been identified as targets of transcriptional repression by AEBP1 prior to my work. Despite the fact that AEBP1 binding to the AE-1 sequence within aP2 promoter has been shown to be crucial for manifestation of AEBP1-mediated transcriptional repression of aP2 (He *et al.*, 1995), no attempts have been made to identify AE-1 homologous sequences within the regulatory regions of other possible target genes. Moreover, besides other phenotypes exhibited by AEBP1^{TG} females, an uncharacterized premature sudden death phenomenon is displayed by a substantial proportion of these mice. Given that atherosclerosis is known to be a primary cause of premature sudden death, I became very interested in investigating the potential transcriptional repression function of AEBP1 towards genes whose products are critically involved in atherosclerosis. Since PPARγ1 and LXRα are key players in macrophage cholesterol homeostasis/foam cell formation, a fundamental event in atherogenesis, I proposed that PPARγ1 and LXRα, whose promoters contain AE-1 homologous sequences, can serve as potential *in vivo* targets of AEBP1's transcriptional repression function. Thus, investigating a possible role of AEBP1 in macrophage cholesterol homeostasis and atherogenesis has been a major goal of my research.

Equally important, I was also interested in investigating a possible role of AEBP1 in macrophage inflammatory responsiveness, due to the fact that atherosclerosis is established to be a chronic inflammatory disease (Ross, 1999; Lusis, 2000; Libby, 2002; Steinberg, 2002). Furthermore, if a role of AEBP1 in modulating macrophage inflammatory responsiveness is evident, the molecular mechanism underlying such a regulatory process was to be elucidated. In sum, the ultimate goal of my research was to investigate the potential, novel molecular and physiological roles of AEBP1 in macrophage cholesterol homeostasis and macrophage inflammatory responsiveness, key biological events in atherogenesis.

Chapter 2

MATERIALS AND METHODS

2.1 Reagents and Plasmids

2.1.1 Chemicals and Isotopes

Thioglycollate broth medium, H&E stain, Mayer's hematoxylin solution, Sudan IV, and oil red O (ORO) were all purchased from Sigma (Missouri, USA). Protein A/G plus agarose beads were purchased from Santa Cruz Biotechnology (California, USA). Enhanced chemiluminescence (ECL) reagent was purchased from Amersham Biosciences (Buckinghamshire, England). DNA oligonucleotides for RT-PCR and gel shift assay were purchased from Sigma (Missouri, USA). [α-^{32}P]ATP, [γ-^{32}P]ATP, and ^{3}H-cholesterol were purchased from Amersham Biosciences (Buckinghamshire, England). Poly dI:dC was purchased from Promega Corporation (Wisconsin, USA). Lipopolysaccharide (LPS) isolated from *Escherichia coli* 055:B5, phorbol-12-myristate-13-acetate (PMA), cycloheximide (CHX), and MEK inhibitor (U0126) were all purchased from Sigma (Missouri, USA), PPARγ agonist (troglitazone) was purchased from Biomol Research Laboratories (Pennsylvania, USA), and LXRα agonist (T0901317) was purchased from Cayman Incorporation (California, USA). ApoAI was purchased from Calbiochem (California, USA), polyfect and effectene were purchased form Qiagen (Ontario, Canada), metafectene was purchased from Biontex (Munich, Germany), and lipofectamine^{2000} was purchased from Invitrogen (Ontario, Canada). Mammalian cell culture media, trypsin/EDTA, and penicillin-streptomycin antibiotic cocktail were purchased from Invitrogen (Ontario, Canada). Bovine and fetal bovine sera were purchased from Cansera (Ontario, Canada). All restriction enzymes were purchased from New England Biolabs (Massachusetts, USA).

2.1.2 Plasmids

The pJ3H-AEBP1 expression constructs were derived from the pJ3H vector (Park *et al.*, 1999; Kim *et al.*, 2001), while the pcDNA-AEBP1 expression constructs were derived from the pcDNA vector (P.J. Lyons and X. Wu, unpublished). The pCMV-β-galactosidase expression vector (pHermes-lacZ) was purchased from Stratagene (California, USA). The pGL3-PPARγ1-luciferase (Fajas *et al.*, 1997), pGL3-LXRα-luciferase (Steffensen *et al.*, 2002), pGL3-TK-PPRE-X3-luciferase (Kim *et al.*, 1998) and pGL3-TK-LXRE-X3-luciferase (Willy *et al.*, 1995), pNF-κB-TA-luciferase (Pessara and Koch, 1990), pGST-IκBα (1-54) (Zandi *et al.*, 1997), and pEGFP-N1 constructs were kindly provided by Dr. Johan Auwerx (University Louis Pasteur, Strasbourg, France), Dr. Knut Steffensen (Karolinska Institute, Huddinge, Sweden), Dr. Bruce Spiegelman (Harvard Medical School, Massachusetts, USA), Dr. David Mangelsdorf (Howard Hughes Medical Institute, Maryland, USA), Dr. Kirill Rosen (Dalhousie University, Nova Scotia, Canada), Dr. Michael Karin (University of California, San Diego, USA), and Dr. Neale Ridgway (Dalhousie University, Nova Scotia, Canada), respectively.

2.1.3 Antisera

Anti-AEBP1 polyclonal antibody, generated in rabbits against recombinant mouse AEBP1, was affinity purified from whole serum using recombinant mouse AEBP1 protein immobilized on nitrocellulose, as previously described (Olmsted, 1981). Primary antibodies directed against PPARγ1, LXRα, p65, IκBα, phospho-IκBα (phospho S^{32}/S^{36}), and IκBβ were purchased from Santa Cruz Biotechnology (California, USA). Anti-p53 polyclonal antibody from Santa Cruz Biotechnology (California, USA) is a kind gift from Dr. Patrick Lee (Dalhousie University, Nova Scotia, Canada). Anti-Erk-1/2 and anti-phospho-Erk-1/2 antibodies were purchased from Cell Signaling Technology (Massachusetts, USA). Anti-actin antibody was purchased from Sigma (Missouri, USA). Rat anti-MOMA-2 antibody and total rat IgG isotype were purchased from Serotec Incorporation (North Carolina, USA). PE-conjugated anti-mouse TLR4 and PE-conjugated mouse IgG1 isotype control antibodies were purchased from *e*Bioscience

(California, USA). FITC-conjugated anti-mouse CD11b, purified rat anti-mouse CD14, PE-conjugated anti-mouse CD40, FITC-conjugated rat IgG2b, purified rat IgG1, PE-conjugated rat IgG2a, purified mouse IgG2a, FITC-conjugated anti-rat IgG1, FITC-conjugated anti-mouse IgG2a antibodies (BD Biosciences Pharmingen, California, USA), and purified anti-mouse FcγR II/III (CD32/CD16) antibody (Cedarlane Labs, Ontario, Canada) were kindly provided by Dr. Jean Marshall (Dalhousie University, Nova Scotia, Canada). Normal IgG and HRP-conjugated secondary antibodies were purchased from Cell Signaling Technology (Massachusetts, USA).

2.2 Recombinant DNA Methods

2.2.1 Sequence Analysis

All gene sequences were obtained from the NCBI databases (http://www.ncbi.nlm.nih.gov/) as well as from the Ensembl genome database (http://www.ensembl.org/index.html). Nucleotide and amino acid alignments were generated using ClustalW (http://www.ebi.ac.uk/clustalw/) (Thompson *et al.*, 1994) and NCBI databases. Restriction endonuclease analyses were performed using Emboss UGI (http://bioinfo.pbi.nrc.ca:8090/EMBOSS/index.html) and RestrictionMapper 3.0 (http://www.restrictionmapper.org/).

2.2.2 Plasmid DNA Construction

pGL3-PPARγ1-M1-luciferase and pGL3-LXRα-M3-luciferase are deleted in the 35-bp sequence representing the putative AEBP1-binding site (i.e. AE-1 homologous sequence) within the promoter regions of PPARγ1 and LXRα in pGL3-PPARγ1-luciferase and pGL3-LXRα-luciferase, respectively.

2.2.2.1 pGL3-PPARγ1-M1-luciferase Construction

For pGL3-PPARγ1-M1-luciferase construction, pGL3-PPARγ1-luciferase (Fajas et al., 1997) (a kind gift from Dr. Auwerx) was cut with BtrI (BmgBI) (New England Biolabs), which cuts 39 bp upstream of the putative AEBP1-binding site (at 2046 bp of PPARγ1 promoter) and PacI (New England Biolabs, Massachusetts, USA), which cuts 145 bp downstream of the putative AEBP1-binding site (at 2266 bp of PPARγ1 promoter). Neither BtrI nor PacI cuts anywhere on the pGL3-basic vector, while both of them cut only once in the promoter region of PPARγ1. This digestion generates two DNA fragments (~7.5 kb and 219 bp). After resolving both fragments on 1% agarose gel, the ~7.5-kb fragment was purified using QIAquick Gel Extraction Kit (Qiagen). While BtrI yields a blunt end (5'...CACGTC...3' / 3'...GTGCAG...5'), PacI yields a 3'-overhang (5'...TTAATTAA...3' / 3'...AATTAATT...5'). So, the 3' to 5' exonuclease activity of Klenow enzyme (Invitrogen) was used to digest the 2-nt protruding 3'-overhang, yielding a DNA fragment with a blunt end. The plasmid was re-ligated using the Rapid DNA Ligation Kit (Roche Applied Science). The final product represents pGL3-PPARγ1-M1-luciferase, which lacks a 219-bp sequence containing the putative AEBP1-binding site within the PPARγ1 promoter present in pGL3-PPARγ1-luciferase. Loss of the 219-bp sequence containing the putative AEBP1-binding site within the PPARγ1 promoter was confirmed using HindIII restriction enzyme, which yields ~6.5 kb and 1010 bp fragments in pGL3-PPARγ1-luciferase, and ~6.5 kb and 791 bp fragments in pGL3-PPARγ1-M1-luciferase. This was further confirmed using BamHI restriction enzyme, which yields 2816 bp, 2353 bp, 1530 bp, and 885 bp fragments in pGL3-PPARγ1-luciferase, and 2816 bp, 2353 bp, 1530 bp, and 666 bp fragments in pGL3-PPARγ1-M1-luciferase.

2.2.2.2 pGL3-LXRα-M3-luciferase Construction

pGL3-LXRα-M3-luciferase was constructed using inverse PCR starting with pGL3-LXRα-luciferase (Steffensen et al., 2002) (a kind gift from Dr. Steffensen). In brief, two inverse primers flanking the putative AEBP1-binding site within the LXRα

promoter region were designed (upstream, 5'-CCTCTGGGAAGGCGCCAAG-3'; downstream, 5'-CACAGGCTGGGGCTTGGTG-3'). The whole plasmid excluding the putative AEBP1-binding site was amplified by *pfu* turbo DNA polymerase (Stratagene) using the MJ Research PTC-100 Thermal Cycler (Scientific Support, California, USA). The PCR cycle was as follows: 30 sec at 94 °C, followed by 30 cycles of 30 sec at 94 °C, 1 min at 61 °C, and 6 min at 72 °C, and finally 10 min at 72 °C. Subsequently, *DpnI* endonuclease (New England Biolabs) was added to the inverse PCR product in order to digest the methylated parental pGL3-LXRα-luciferase (45 min at 37 °C), followed by Klenow enzyme reaction (1 hr at 37 °C). The PCR product was then resolved on 1% agarose gel, and the ~5.5 kb linear DNA fragment was purified and subjected to ligation as described above. This yielded pGL3-LXRα-M3-luciferase, which lacks the putative AEBP1-binding site within the LXRα promoter present in pGL3-LXRα-luciferase. DHα5 *E. coli* cells were transformed with pGL3-LXRα-luciferase, which was purified using the QIAprep Spin Miniprep Kit (Qiagen), and finally sent for sequencing to confirm accuracy of plasmid construction.

All plasmids were purified using QIAprep Spin Miniprep Kit and QIAprep Maxiprep Kit (Qiagen) according to the manufacturer's recommendations. Plasmids were quantitated by UV spectrophotometry as well as by comparison with High DNA Mass Ladder (Invitrogen) on an agarose gel.

2.2.3 Transformation of Competent *E. coli* Cells

Competent DHα5 *E. coli* (Invitrogen) were thawed slowly on ice. Purified plasmid (200 ng) was added to thawed bacteria, which were kept on ice for 30 min. Bacteria were then heat shocked for 45 sec at 42 °C, placed on ice immediately, transferred to 100 µl Luria Broth (LB), and incubated at 37 °C for 1 hr. Subsequently, bacteria were streaked on LB plates supplemented with 100 µg/ml ampicillin, and grown overnight at 37 °C to allow colony formation. Bacterial stocks were also prepared in 50% glycerol solutions and stored at -80 °C.

2.3 Purification of Recombinant Proteins

2.3.1 AEBP1

Two tubes of 3 ml LB (100 μg/ml ampicillin) were inoculated with BL21 (DE3) *E. coli* bacteria previously transformed with pET-AEBP1. This inoculation was either directly from frozen glycerol stock or from a freshly transformed bacterial colony. This inoculum was grown overnight at 30 °C with shaking at 250 rpm. Subsequently, the entire 6-ml culture was transferred to 500 ml LB supplemented with 100 μg/ml ampicillin, and grown for approximately 3 hr at 37 °C with shaking at 250 rpm until the OD_{600} was between 0.5 and 0.6. Expression of AEBP1 was then induced with 1 mM IPTG (isopropyl-β-D-thiogalactopyranosid) for 3.5 hr at 37 °C with shaking at 250 rpm. The bacteria culture was centrifuged at 6,000 x g for 15 min at 4 °C. Pelleted bacteria were resuspended in 25 ml extraction/wash buffer (50 mM sodium phosphate [pH 7.0] and 300 mM NaCl) and lysed by passing through a French pressure cell twice at 14,000 psi. Immediately prior to lysis, 1 mM phenylmethylsulfonyl fluoride (PMSF) was added. Following lysis, extracts were centrifuged at 15,000 x g for 20 min at 4 °C. The supernatant was discarded and the pellet (containing insoluble inclusion bodies) was dissolved in 10 ml denaturing extraction/wash buffer (50 mM sodium phosphate [pH 7.0], 300 mM NaCl, and 6 M guanidine-HCl). This was centrifuged again at 15,000 x g for 20 min at 4 °C.

His6-tagged protein was purified using Talon metal affinity resin (Clontech). A 0.75 ml bed volume of Talon resin was equilibrated twice with 10 ml denaturing extraction/wash buffer by mixing briefly and centrifuging at 700 g for 2 min. The clarified protein sample was added to this resin and incubated for 30 min at room temperature on a rocking platform. This was then centrifuged at 700 g for 5 min, and the resin washed twice with 10 ml of denaturing extraction/wash buffer, followed by additional washing on a 5 ml gravity-flow column until the OD_{280} < 0.01. When purifying pET16b-AEBP1, second batch wash and subsequent washing on the column was done with extraction/wash buffer [pH 6.7]. Elution was performed with 3 ml imidizole elution buffer (45 mM sodium phosphate [pH 7.0], 5.4 M guanidine-HCl, 270

mM NaCl, 300 mM imidizole) and 0.5 ml fractions were collected and combined. Protein fractions were dialyzed several times for 2 hr in dialysis buffer (50 mM sodium phosphate [pH 7.5], 100 mM NaCl, 1 mM β-mercaptoethanol, and 0.2 mM $ZnCl_2$) containing 0.5, 1, 2, and 4 M urea (2 hr each), followed by dialysis overnight at 4 °C in dialysis buffer containing 10% glycerol. Finally, protein concentration was determined by Bradford Protein Assay.

2.3.2 GST-IκBα (1-54) Fusion Protein

GST-IκBα (1-54) fusion protein was expressed in DHα5 *E. coli* and purified as previously described (Kupfer and Scheinman, 2002). Briefly, a 500 ml culture of LB was inoculated with DHα5 *E. coli* transformed with pGST-IκBα (1-54) (a kind gift from Dr. Michael Karin) and grown for about 4 hr until an OD_{600} between 0.5 and 0.6 was reached. Protein expression was induced by addition of IPTG to a final concentration of 0.5 mM and growth was continued at 37 °C for 4 hr with shaking at 250 rpm. Cells were harvested by centrifugation at 6,000 x g for 15 min, and the pellet was suspended in 5 ml cold lysis buffer (20 mM Tris [pH 8.0], 10 mM $MgCl_2$, 1 μg/ml DNase I, 1 mg/ml lysozyme, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 100 μg/ml trypsin inhibitor, 1% Triton, and 5 mM EDTA). PMSF was added to a concentration of 1 mM, and the mixture was set on ice for 15 min to allow cell lysis. Subsequently, the mixture was sonicated (3x for 15 sec) on ice. Clear cell lysate was obtained by centrifugation at 15,000 x g for 20 min at 4 °C. To allow capture of GST-IκBα (1-54) fusion protein, the clear cell lysate was slowly added to 300 μl glutathione-sepharose 4B beads (Pharmacia Biotech) in an equilibrated glutathione-sepharose column (Pharmacia Biotech). The cell lysate was allowed to flow through the column at 4 °C, while GST-IκBα (1-54) fusion protein binds to the glutathione-sepharose 4B beads. The beads were then washed three times with 5 ml cold lysis buffer, and GST-IκBα (1-54) fusion protein was finally eluted three times by adding 300 μl glutathione solution (20 mM reduced glutathione, 50 mM HEPES, 30% glycerol). Protein fractions were combined and dialyzed three times (2 hr, 2 hr, overnight) in dialysis buffer (50 mM sodium phosphate [pH 7.0], 100 mM NaCl, 0.2 mM $ZnCl_2$, and 10% glycerol) at 4 °C. Purified GST-IκBα (1-54) fusion protein was

harvested and protein concentration was determined by Bradford protein assay using BSA as a standard.

2.4 Mammalian Cell Culture Methods

2.4.1 Cell Line Culture

2.4.1.1 Chinese Hamster Ovary (CHO) Cell Line

CHO cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen) supplemented with 5% fetal bovine serum (FBS) (Cansera), 1% penicillin-streptomycin antibiotic cocktail (Invitrogen), and 37 μg/ml L-proline. Every 3 days, CHO cells were washed once with warm 1X PBS, lifted in 1 ml trypsin/EDTA (Invitrogen) (5 min at 37 °C), resuspended in fresh, complete medium at a cell density of 2.0×10^5 cells/ml, and finally cultured under standard culture conditions. Where applicable, cells were treated with 1 μM troglitazone (Sigma) for 18 hr, 1 μM T0901317 (Cayman) for 18 hr, or 1 μg/ml LPS for 30 min to induce, PPARγ1, LXRα, and NF-κB activation, respectively.

2.4.1.2 J774, THP-1, and U937 Monocyte/Macrophage Cell Lines

Murine J774 (kindly provided by Dr. Timothy Lee) and human THP-1 (purchased from ATCC) and U937 (kindly provided by Dr. Robert Anderson) monocyte/macrophage cell lines were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated FBS, 1% penicillin-streptomycin, 10 mM HEPES, and 0.05 mM β-mercaptoethanol under standard culture conditions. J774, THP-1, and U937 cells were passaged every 3 days and kept at a cell density of 1.0×10^5 cells/ml. J774 cells, which are adherent cells, were lifted in 1 ml trypsin/EDTA (5 min at 37 °C) followed by scraping using a rubber policeman. Where applicable, cells were treated with 100 nM PMA for 48 hr to induce their differentiation into mature macrophages.

2.4.1.3   C6 Glioma Cell Line

Rat C6 glioma cells (kindly provided by Dr. Kenneth Renton) were cultured in DMEM supplemented with 5% FBS and 1% penicillin-streptomycin antibiotic cocktail. Every 3 days, C6 glioma cells were washed once with warm 1X PBS, lifted in 1 ml trypsin/EDTA (5 min at 37 °C), resuspended in fresh, complete medium at a cell density of 2.0 X 10^5 cells/ml, and finally cultured under standard culture conditions. Where applicable, cells were treated with 1 µg/ml LPS for 30 min to induce NF-κB activation. For protein stability studies, cells were treated with 50 µg/ml CHX for the indicated time points.

2.4.1.4   3T3-L1 Preadipocyte Cell Line

3T3-L1 (Neo-1) and anti-sense (AS/Neo-11) stably transfected preadipocytes were cultured in DMEM supplemented with 10% bovine serum (Cansera), 1% penicillin-streptomycin antibiotic cocktail, and 200 µg/ml G418 (Invitrogen). Every 3 days, cells were washed once with warm 1X PBS, lifted in 1 ml trypsin/EDTA (5 min at 37 °C), resuspended in fresh, complete medium at a cell density of 2.0 X 10^5 cells/ml, and finally cultured under standard culture conditions. For protein stability studies, cells were treated with 50 µg/ml CHX for the indicated time points.

2.4.2   Peritoneal Macrophage Isolation and Culture

Thioglycollate-elicited peritoneal macrophages were isolated as previously described (Miles *et al.*, 2000). Briefly, mice were injected intraperitoneally with 3 ml sterile 4% Brewer's thioglycollate broth solution (Sigma). Five days later, mice were sacrificed by cervical dislocation and peritoneal exudate cells were isolated by peritoneal lavage using sterile, cold RPMI-1640 medium. Peritoneal exudate cells were obtained by centrifugation for 10 min at 4 °C at 225 x g and resuspended in 5 ml of ACK lysis buffer (0.15 M NH_4Cl, 1 mM $KHCO_3$, 0.1 mM Na_2EDTA) for 2 min at 37 °C to get rid of erythrocyte contamination. Subsequently, cells were centrifuged for 10 min at 4 °C at 225

x g, and finally resuspended and cultured in RPMI-1640 medium supplemented with 10% heat-inactivated FBS, 1% penicillin-streptomycin, 10 mM HEPES, and 0.05 mM β-mercaptoethanol under standard culture conditions for 72 hr before experimentation. To induce NF-κB activation, macrophages were treated with 1 μg/ml LPS for 30 min, unless otherwise indicated. For protein half-life studies, macrophages were treated with 50 μg/ml CHX for the indicated time points.

2.4.3 Transient Transfection

CHO cells were transfected at 60-80% confluency in 60 mm^2 dishes (for Western Blot Analysis) or 12-well plates (for luciferase assay) with polyfect transfection reagent (Qiagen) according to the manufacturer's protocol (the numbers provided below correspond to transfecting CHO cells in 60 mm^2 dishes). Briefly, 6.0 X 10^5 CHO cells were seeded in a 60 mm^2 dish one day before transfection. Next day, 2.5 μg plasmid DNA was diluted in 150 μl with serum-free, antibiotic-free DMEM. 15 μl polyfect was added to diluted plasmid DNA and incubated for 10 min at room temperature to allow polyfect-DNA complex formation. Meanwhile, cells were washed once with 4 ml sterile 1X PBS, and finally incubated in 3 ml growth medium. Subsequently, 1 ml growth medium was added to the polyfect-DNA complexes, and the mixture was slowly added to cells. Finally, cells were incubated at 37 °C/5% CO$_2$ for 48 hr to allow protein expression.

C6 glioma cells were transiently transfected at 90% confluency in 60 mm^2 dishes (for Western Blot Analysis) or 12-well plates (for luciferase assay) using lipofectamine^{2000} transfection reagent (Invitrogen, Ontario, Canada) according to the manufacturer's instructions (the numbers provided below correspond to transfecting C6 glioma cells in 60 mm^2 dishes). Briefly, 1.2 X 10^6 C6 glioma cells were seeded in a 60 mm^2 dish one day before transfection. Next day, two mixtures were prepared and set for 5 min at room temperature: mixture 1 (250 μl Opti-MEM I plus 10 μl lipofectamine^{2000} reagent), and mixture 2 (250 μl Opti-MEM I plus 4 μg plasmid DNA). Subsequently, the 2 mixtures were combined and set for 20 min at room temperature to allow lipofectamine^{2000}-DNA complex formation. Meanwhile, cells were washed once with 4

ml Opti-MEM I, after which the lipofectamine^{2000}-DNA complexes were added to the cells, which were incubated for 4 hr at 37 °C/ 5% CO_2. Subsequently, the transfection mixture was aspirated, and the cells were washed once with 4 ml growth medium before incubation in 5 ml growth medium for 42 hr to allow protein expression.

2.5 Mice

2.5.1 Handling & Maintenance

Age-matched mice were kept on a 12-h light cycle in air-conditioned rooms in the Carleton Animal Care Facility at Dalhousie University. Mice were kept in filtered cages (minimum of 2 mice, maximum of 5 mice) with constant access to diet and water. Mice were fed chow (4.5% fat, 0.02% cholesterol, no cholate) (LabDiet, Indiana, USA) or high-fat diet (HFD) (45% fat, 0.05% cholesterol, no cholate) (Research Diets, New Jersey, USA) starting at 3 wk of age. Mice were monitored on a daily basis, and total body weight of mice was recorded weekly. All protocol procedures were approved by the University Committee on Laboratory Animals (UCLA), an ethics board whose functions are clearly defined by the Canadian Council on Animal Care (CCAC). Mice were sacrificed by cervical dislocation to isolate thioglycollate-elicited peritoneal macrophages for protein, RNA, and lipid analyses, flow cytometry, and staining.

2.5.2 Generation

2.5.2.1 AEBP1^{TG} Mice

Generation of AEBP1^{TG} was carried out as follows: the fat-specific AEBP1 transgene was constructed using the 5.4-kb DNA fragment containing the fat-specific promoter/enhancer (Ross et al., 1990) from the fatty acid-binding protein gene, aP2, and it was ligated to a 2.6-kb mouse AEBP1 cDNA. The aP2 promoter/enhancer directs expression in adipocytes as well as macrophages (Makowski et al., 2001). The transgene plasmid was digested with Hind III and NdeI to release the 8.7-kb aP2-AEBP1 transgene

for the injection. The construct was injected into the pronucleus of fertilized zygotes from FVB/n mice and transferred to pseudo-pregnant females. A total of 35 pups were born from four pseudo-pregnant mice with blastocysts that had been injected with the AEBP1 transgene construct. Out of 35 litters, 8 pups were shown by PCR analysis of tail DNA to have acquired the transgene. These 8 founders were mated and first sets of F_1 mice from all the mating pairs were obtained. Pups were weaned at the age of 3 weeks, and then genomic DNA was prepared from tail for PCR analysis. Mice positive for the transgene were determined by tail DNA PCR using the sense primer in the exon 18 (5'-GGACTACACCAGCGGCATGG-3') and the antisense primer from exon 21 (5'-GCGTGAGCTGTCACACGGTA-3'). This primer pair amplifies a 360-bp fragment of cDNA and ~ 900-bp genomic sequence. One transgenic littermate from each founder was sacrificed at the age of 7 wk to examine the tissue-specific expression of the AEBP1-transgene.

2.5.2.2 AEBP1$^{-/-}$ Mice

Generation of AEBP1$^{-/-}$ was carried out as follows: a replacement targeting vector was prepared in which a segment of the AEBP1 gene between the 6th and 12th introns, including the start codon of AEBP1 in exon 10, was removed and replaced with a PGK-NEO-poly(A) expression cassette (Susulic *et al.*, 2001) in the opposite orientation. The AEBP1 targeting vector was constructed by inserting a 5.1-kb *XbaI/SalI* fragment lying in the middle of intron VI, a 1.8-kb *SalI/XbaI* fragment containing a PGK-NEO-poly(A) expression cassette, and a 5.7-kb *XbaI/EcoR* I fragment beginning at the 3' end of intron XII into pGEM-11Zf vector (Promega). The targeting plasmid was linearized with *NotI* and electroporated into J1 embryonic stem cells provided by En Li and Rudolf Jaenisch (Li *et al.*, 1993a). A homologous recombination event between the targeting vector and the endogenous AEBP1 locus results in production of a modified AEBP1 locus in which a 2.1-kb fragment, including the entire exons and introns 7 to 12 along with the 3' half of intron 6, is removed. Selection with G418 was done as described (Susulic *et al.*, 2001). Drug-resistant clones were isolated and expanded followed by genomic DNA extraction for Southern blot analysis. A 1.8-kb *XhoI/BamHI* fragment lying outside of the targeting

construct was used as a 3' probe for Southern blotting analysis of DNA from ES cells and mice. A 19-kb fragment corresponding to the wild-type allele and a 10-kb fragment corresponding to the disrupted allele were detected on Southern blots. Successful targeting of AEBP1 locus was achieved in 12 of the 92 G418-resistant clones. Four independently targeted embryonic stem cell clones were used to produce male chimeras in C57BL/6J background, and germ-line transmission of the disrupted allele was obtained. The resulting heterozygous (F_1) progeny was interbred to produce F_2 offspring.

2.5.3 Genotyping

2.5.3.1 AEBP1 Genotyping

About 5 mg of mouse ear tissue (collected by ear puncher) was used to extract DNA using REDextract-N-Amp Tissue PCR Kit (Sigma) following the manufacturer's recommendations. In a total of 10 μl reaction volume, 5 μl of DNA sample was used for amplification using the following primers: GT-sense, 5'-CAATGGCTACGAGGAAATG-3'; GT-anti-sense, 5'-GTGCGTAGTAGCTGTAGACAG-3'; anti-SK, 5'-GGGGATCCACTAGTTCTAGA-3'. The MJ Research PTC-100 Thermal Cycler (Scientific Support) was used for amplification. PCR commenced at 95 °C (4 min), followed by 35 cycles at 95 °C (30 sec), 54 °C (1 min), and 72 °C (1 min), and finally 72 °C (10 min). Subsequently, 1 μl 10X loading buffer was added to PCR samples, which were run on 1% agarose gel at 70 V. The expected DNA fragments for all different genotypes are illustrated in Table 1.

Table 1. Expected PCR Products of All Possible AEBP1 Genotypes.

Genotype	Expected Fragments
AEBP1^{TG}	400 bp and 200 bp
AEBP1^{NT}	400 bp
AEBP1$^{+/+}$	400 bp
AEBP1$^{-/-}$	300 bp
AEBP1$^{+/-}$	400 bp and 300 bp

2.5.3.2 ApoE Genotyping

About 5 mg of mouse ear tissue (collected by an ear puncher) was used to extract DNA using REDextract-N-Amp Tissue PCR Kit (Sigma) following the manufacturer's recommendations. In a total of 10 μl reaction volume, 5 μl of DNA sample was used for amplification using the following primers: oIMR180, 5'-GCCTAGCCGAGGGAGAGCCG-3'; oIMR181, 5'-TGTGACTTGGGAGCTCTGCAGC-3'; oIMR182, 5'-GCCGCCCCGACTGCATCT-3'). The genotyping PCR thermocycles commenced at 95 °C (4 min), followed by 35 cycles at 95 °C (30 sec), 68 °C (1 min), and 72 °C (1 min), and finally 72 °C (10 min). The expected DNA fragments for all different genotypes are illustrated in Table 2.

Table 2. Expected PCR Products of All Possible ApoE Genotypes.

Genotype	Expected Fragments
ApoE$^{+/+}$	155 bp
ApoE$^{-/-}$	245 bp
ApoE$^{+/-}$	155 bp and 245 bp

2.6 Chemical and Immunostaining Techniques

2.6.1 Cytospin Preparation

For immunostaining purposes, cytospin samples were prepared from single cell suspension using Sheldon Cytospin III Cytocentrifuge (Global Medical Instrumentation Inc., Minnesota, USA). Briefly, 1.0×10^5 cells were suspended in 150 µl sterile growth medium containing 10% FBS, and the whole cell suspension was loaded in clear plastic cuvettes for cytocentrifugation after applying 100 µl clear medium to wash the cuvettes. Cells were cytospun on silanated microscope glass slides at 500 rpm for 5 min. Cytospin preparations were air dried or fixed with 2% formalin for 5 min for immediate use.

2.6.2 Hematoxylin & Eosin (H&E) Staining

For morphological examination of peritoneal exudate cells, 1.0×10^5 cells were cytospun on a silanated microscope glass slides at 500 rpm for 5 min. Air-dried cells were fixed in 2% formalin for 5 min, washed with H_2O for 5 min, and stained with Mayer's hematoxylin (Sigma) for 10 min. Cells were then washed with H_2O, placed in 1% acid alcohol (99 volumes 70% ethanol plus 1 volume conc. HCl) for 1 min, washed with H_2O, and stained with eosin Y solution (Sigma) for 5 min. Cells were then washed with H_2O, and finally mounted with glycerol gelatin (Sigma) for microscopic examination.

2.6.3 Oil Red O Lipid Staining

For macrophage lipid detection, 2.0×10^5 peritoneal macrophages were cultured in 8-well LabTek chamber slides (Nunc, New York, USA), and stained as follows. Macrophages were fixed in 50% isopropanol (in H_2O) for 2 min and subsequently stained for 15 min with 0.5% oil red O (ORO) (Sigma) diluted in 50% isopropanol. Sections were washed three times with H_2O and subsequently counter-stained for 1 min with Mayer's hematoxylin solution (Sigma). Finally, slides were mounted with glycerol

gelatin (Sigma) for microscopic examination. Photomicroscopy was performed on a Nikon Eclipse E600 microscope attached to a Nikon Coolpix 990 camera.

2.6.4 Sudan IV Lipid Staining

For detection of lipid-filled atherosclerotic lesions in mice aortae, refer to section 2.11.1.

2.6.5 Masson's Trichrome Staining

10 μm heart tissue cryosections were rinsed with H_2O and stained with Weigert's iron hematoxylin for 1 min to allow nuclei staining. Subsequently, sections were immersed in solution A (0.5% acid fuchsin, 0.5% xylidine ponceau, and 1% glacial acetic acid in H_2O) for 10 min. Sectioned were rinsed with H_2O and immersed in solution B (1% phosphomolybdic acid in H_2O) for 5 min. Sections were then rinsed with H_2O and immersed in solution C (2% light green SF yellowish, 2% glacial acetic acid in H_2O) for 10 min. Subsequently, sections were rinsed with H_2O and dehydrated using 70%, 95%, and 100% ethanol (2 min each). Finally, stained sections were cleared with xylene (10 min, three times), and mounted with glycerol gelatin for microscopic examination.

2.6.6 Immunocytochemistry and Immunohistochemistry

Immunostaining of cultured peritoneal macrophages and intimal macrophages within lipid-filled atherosclerotic lesions was performed as previously described (Boisvert et al., 1998) with a few modifications. 1.0×10^5 peritoneal macrophages cytospun on silanated microscope glass slides at 500 rpm for 5 min as well as aortic cryosections were air-dried for 3 hr at room temperature. Specimens were fixed in 2% formalin for 5 min, washed with 1X PBS, incubated in 2% H_2O_2 for 5 min, washed with 1X PBS, and incubated in 5% goat serum for 1 hr at room temperature. Subsequently, specimens were incubated with anti-MOMA-2 antibody (1:80) or total rat IgG isotype in a humid-chamber at 4 °C overnight. Specimens were then washed three times with 1X

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PBS, incubated in rabbit anti-rat biotinylated secondary antibody (Dako Corporation, California, USA) for 1 hr at room temperature. Subsequently, specimens were incubated in DAKO LSAB streptavidin-HRP (Dako Corporation, California, USA) for 30 min at room temperature, followed by incubation with 9-amino-3-ethylene-carbazole (AEC) (Vector Laboratories, California, USA) for 20 min at room temperature. Specimens were then counter-stained with Mayer's hematoxylin solution for 1 min, washed with H_2O, and finally mounted in glycerol gelatin (Sigma) for microscopic examination.

2.7 Protein Analysis

2.7.1 Whole Cell and Nuclear Protein Extract Preparation

Cells were washed twice with cold 1X PBS, and subsequently lysed in cold radioimmune precipitation (RIPA) buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1 mM dithiothreitol (DTT), 0.25% sodium deoxycholate, 0.1% Nonidet P-40) containing 1 mM phenylmethysulfonyl fluoride (PMSF), 50 mM $Na_4P_2O_7$, 1 mM Na_3VO_4, 1 mM NaF, 5 mM EDTA, 5 mM EGTA and complete protease inhibitor cocktail (Roche Applied Science, Quebec, Canada). Cell lysis was performed on ice for 30 min. Clear protein extracts were obtained by centrifugation at 15,000 x g for 30 min at 4 °C.

Nuclear protein extracts for Western blot analysis and EMSA were obtained using the Active Motif nuclear protein extraction kit (Active Motif, California, USA) and following the manufacturer's recommendations. Briefly, cells were washed and resuspended in 1.5 ml PBS/phosphatase inhibitor solution. Pelleted cells were obtained by centrifugation at 500 x g for 5 min at 4 °C. The pellet was resuspended in 250 μl 1X hypotonic buffer, incubated on ice for 15 min, after which 12.5 μl detergent was added. The mixture was centrifuged at 15,000 x g for 30 sec at 4 °C, and the supernatant (cytoplasmic protein fraction) was harvested. The pellet, representing nuclei, was resuspended in 50 μl complete lysis buffer, and kept on ice for 30 min. The nuclei extract was vortexed multiple times, after which it was centrifuged at 15,000 x g for 10 min at 4 °C. The supernatant (nuclear protein fraction) was harvested, and protein concentration

was determined by Bradford protein assay. Proteins were directly subjected to SDS-PAGE or stored at -80 °C for future analysis.

2.7.2 Protein Concentration Determination

Protein concentration was measured by Bradford Protein Assay (Bradford, 1976) using the Bio-Rad Protein Assay dye (Bio-Rad). In brief, 10 µl protein sample was added to 790 µl water, followed by addition of 200 µl Protein Assay Dye (BioRad). 800 µl water plus 200 µl dye served as a negative control. The mixture is set for 5 min, and absorbance was measured at A_{595} based on a protein standard curve generated based on known concentrations of bovine serum albumin (BSA) (Sigma).

2.7.3 SDS-PAGE

Clear whole cell and nuclear protein extracts as well as immunoprecipitation samples, along with pre-stained protein marker (New England Biolabs), were resolved on 8.5% polyacrylamide gels, and run at 80-120 V. Protein samples were run in 1X running buffer (25 mM Tris-Base, 192 mM glycine, and 0.1% SDS). 5X SDS sample buffer (0.5 M Tris-HCl [pH 6.8], 0.35 M SDS, 0.5 M DTT, 0.2 mM bromophenol blue, and 30% glycerol) was added to protein samples, which were boiled for 5 min prior to loading on gel.

2.7.4 Western Blot Analysis

Following SDS-PAGE, proteins were transferred onto supported nitrocellulose membranes (Bio-Rad) using Trans-Blot Semi-dry Transfer Cell (Bio-Rad) and 1X transfer buffer (48 mM Tris-HCl [pH 7.4], 39 mM glycine, 0.0375% SDS, and 20% methanol) (1 hr at 380 mA). Western blot analysis was used for specific protein detection. To that end, nitrocellulose membranes were blocked with 5% fat-free milk in 1X TBST (10 mM Tris-HCl [pH 8.0], 150 mM NaCl, 0.05% Tween-20) for 1 hr at room temperature and incubated with specific antibodies recognizing target proteins overnight

at 4 °C or 2 hr at room temperature. Table 3 provides a list of the primary antibodies used for immunoblotting, the dilution they were used at, and where they were purchased from. The membranes were then washed several times with 1X TBST for 30 min, and subsequently incubated with HRP-conjugated secondary antibody (1:3000) for 1 hr at room temperature. Thereafter, membranes were washed with 1X TBST several times for 30 min, and subsequently analyzed by ECL detection system (Amersham Biosciences, Buckinghamshire, England).

Table 3. A List of the Primary Antibodies used in Western Blot Analysis.

Antibody	Working Dilution	Company
Anti-AEBP1	1:600	Home-made
Anti-PPARγ1	1:200	Santa Cruz Biotechnology
Anti-LXRα	1:1000	Santa Cruz Biotechnology
Anti-Erk-1/2	1:1000	Cell Signaling Technology
Anti-phospho-Erk-1/2	1:1000	Cell Signaling Technology
Anti-NF-κB p65	1:1000	Santa Cruz Biotechnology
Anti-IκBα	1:1000	Santa Cruz Biotechnology
Anti-IκBβ	1:1000	Santa Cruz Biotechnology
Anti-phospho-IκBα	1:200	Santa Cruz Biotechnology
Anti-IKKα	1:1000	Santa Cruz Biotechnology
Anti-CD14	1:1000	BD Biosciences
Anti-p53	1:1000	Santa Cruz Biotechnology
Anti-GST	1:1000	Santa Cruz Biotechnology
Anti-Actin	1:2000	Sigma

2.7.5 Co-Immunoprecipitation

Whole cell protein extracts were obtained by shearing cells in cold RIPA lysis buffer as described above. Protein extracts (0.5 mg) were first incubated with protein A/G plus agarose beads (Santa Cruz Biotechnologies) for 2 hr at 4 °C. The pre-cleared protein extracts were subsequently incubated with 2 μg/ml normal IgG, anti-IκBα, or anti-IκBβ antibodies (Santa Cruz Biotechnologies) for 16 hr at 4 °C, and then with protein A/G plus agarose beads for 4 hr at 4 °C to allow precipitation of target proteins. Then, the protein A/G plus agarose beads were collected and washed twice with cold RIPA lysis buffer, once with cold RIPA lysis buffer containing 500 mM NaCl, and finally once with cold RIPA lysis buffer. Subsequently, 20 μl sample buffer was added to the immunoprecipitate samples, which were then boiled for 5 min and centrifuged at 15,000 x g for 10 min. Finally, the immunoprecipitate samples were resolved by SDS-PAGE, transferred onto nitrocellulose membranes, and subjected to immunoblotting as described above.

2.7.6 FACS Analysis (Flow Cytometry)

Peritoneal macrophages (5.0×10^5) were washed twice with immunofluorescence buffer (2% BSA, 0.02% NaN_3 in 1X PBS). Macrophages were resuspended in immunofluorescence buffer and incubated for 20 min on ice with 5 μg/ml anti-mouse FcγR II/III (CD32/CD16) antibody (Cedarlane Labs, Ontario, Canada) to block free antibody binding sites. Subsequently, macrophages were incubated for 20 min on ice with 1 μg/ml FITC-conjugated anti-mouse CD11b, purified anti-mouse CD14 antibody, PE-conjugated anti-mouse CD40, PE-conjugated anti-mouse TLR4 antibody, purified anti-mouse FcγR II/III (CD32/CD16), or isotype control antibodies (FITC-conjugated rat IgG2b, purified rat IgG1, PE-conjugated rat IgG2a, FITC-conjugated mouse IgG1, and purified mouse IgG2a, respectively). Macrophages were then washed three times with immunofluorescence buffer. For CD14 and FcγR II/III (CD32/CD16) detection, macrophages were incubated with 1 μg/ml FITC-conjugated anti-rat IgG1 and FITC-conjugated anti-mouse IgG2a antibodies, respectively, for 20 min on ice. Subsequently,

stained macrophages were washed three times with immunofluorescence buffer, and finally fixed in 1% paraformaldehyde in 1X PBS for 30 min on ice. Fixed macrophages were washed twice with immunofluorescence buffer and resuspended in 300 µl immunofluorescence buffer. For flow cytometric analysis, macrophages were acquired with a FACSCalibur (BD Biosciences, San Jose, CA, USA) and analyzed with CellQuest (BD Biosciences, California, USA). Positive staining using specific antibodies was evaluated relative to negative staining using isotype control antibodies.

2.8 RNA Analysis

2.8.1 RNA Isolation

Total RNA was isolated and purified from peritoneal macrophages using RNA STAT-60 isolation reagent (TEL-TEST, Texas, USA) following the manufacturer's recommendations. Briefly, cells were homogenized in 1 ml RNA STAT-60 reagent and placed for 5 min at room temperature to permit complete dissociation of nucleoprotein complexes. Chloroform (200 µl) was added to the homogenized sample, shaken very vigorously, kept for 3 min at room temperature, and subsequently centrifuged at 15,000 x g for 15 min at 4 °C. The top aqueous layer (containing total RNA) was harvested, 1 ml isopropanol was added to it, and the mixture was set for 15 min at room temperature. RNA was obtained by centrifugation at 15,000 x g for 10 min at 4 °C, washed once with 1 ml 75% ethanol, dried for 10 min at room temperature, and finally resuspended in 30 µl Diethylpyrocarbonate (DEPC)-H_2O (10 min at 60 °C). RNA concentration was determined by making a 1:100 dilution in DEPC-H_2O, and the Bio-Rad UV spectrophotometer was used for measurements ($A_{260}/A_{280}/A_{320}$). RNA samples were either directly used for reverse transcription, or stored at -80 °C.

2.8.2 Semi-Quantitative RT-PCR

For semi-quantitative RT-PCR analysis, 1.0 µg RNA was subjected to reverse transcription using the Omniscript reverse transcriptase kit (Qiagen) along with oligo

(dT)$_{12\text{-}18}$ primers (Invitrogen, Ontario, Canada) according to the manufacturer's directions. Upon heating RNA samples in DEPC-H$_2$O for 5 min at 65 °C, reverse transcription reaction was allowed to take place for 2 hr at 37 °C, after which 2 µl RT product (cDNAs) was used directly for amplification and the remaining sample was stored at -20 °C. Specific murine PCR primers were used to amplify PCR products corresponding to the genes of interest using Hotstar Taq DNA polymerase kit (Qiagen). The primer sets that were used to amplify the genes of interest using the MJ Research PTC-100 Thermal Cycler (Scientific Support) are listed in Table 4. All primers were purchased from Sigma. The PCR conditions and number of thermal cycles were optimized for each gene examined.

Table 4. Forward and Reverse Primer Sets used for RT-PCR Analysis.

Gene	Forward Primer (5' to 3')	Reverse Primer (5' to 3')	Amplicon Size (bp)
AEBP1	GTAGTAGCCCCAAGGAGGAC	GGCACTCCTCATTGACAGCC	712
PPARγ1	GGTTGACACAGAGATGCCATTCTGGCC	AAGCTTCCATCGGATGGTTCTTCG	421
LXRα	CAACAGTGTAACAGGCGCT	TGCAATGGGCCAAGGC	60
CD36	AGTTTTGGATCTTTGATGTGC	TTCAATAGGTTCTGAAACATC	883
ABCA1	ATTGCCAGACGGAGCCG	TGCCAAAGGGTGGCACA	102
ABCG1	AAGGCCTACTACCTGGCAAAGA	CGGATTTTGTATCTGAGGACGAA	540
ApoE	AGGATCTACGCAACCGACTC	GGCGATGCATGTCTTCCACTA	503
p65	CTCTGGGGCGGCACGTTTTAC	CATCCCACCTGTTCCCCTTGG	400
IκBα	CAGGACTGGGCCATGGAGGG	TGGCCATTGTAGTTGGTGGC	527
IκBβ	GGACACAGCCCTGCACTTGG	GTAGCCTCCAGTCTTCATCA	422
MCP-1	CAGCCAGATGCAGTTAACGC	GCCTACTCATTGGGATCATCTTG	72
iNOS	AATGGCAACATCAGGTCGGCCATCACT	GCTGTGTGTCACAGAAGTCTCGAACTC	326
MMP-9	AGGCCTCTACAGAGTCTTTG	CAGTCCAACAAGAAAGGACG	425
β-actin	CTGGCACCACACCTTCTACA	TCGTACTCCTGCTTGCTGATC	733

2.9 Lipid Analysis

2.9.1 Oil Red O Microscopic Analysis

See above under Chemical and Immunostaining Techniques (section 2.6.3).

2.9.2 ^{3}H-Cholesterol Efflux Assay

Cholesterol efflux assay was performed as previously described (Lin *et al.*, 1999) with minor modifications. Briefly, 5.0×10^5 macrophages were cultured in 5% lipoprotein-depleted serum-supplemented RPMI-1640 in presence of 0.5 μCi/ml ^{3}H-cholesterol (Amersham) for 24 hr. Macrophages were washed with 1X PBS, and incubated for 2 hr in 5% lipoprotein-depleted serum-supplemented RPMI-1640 for equilibration. Macrophages were washed with 1X PBS and incubated for 6 hr in RPMI-1640 supplemented with 10 μg/ml BSA or human ApoAI (Calbiochem). Subsequently, ^{3}H radioactivity in medium was determined, and adherent macrophages were lysed in 1 ml 0.5 N NaOH (1 hr, 37 °C). Cellular protein content and cellular ^{3}H radioactivity were determined. Percentage efflux was calculated by dividing ^{3}H radioactivity in medium by the sum of ^{3}H radioactivity in medium and cellular fractions, multiplied by 100%. Percentage ApoAI specific efflux was determined by subtracting ^{3}H radioactivity in BSA-treated samples from ^{3}H radioactivity in ApoAI-treated samples.

2.9.3 Serum Lipid Isolation and Analysis

Whole serum samples obtained from mice were analyzed for total lipids as described previously (Kuksis *et al.*, 1976). Serum lipids were analyzed on a Hewlett Packard 5890 Gas Chromatograph (GC) using a siliconized glass column packed with 3% SP-2100 on 100/20 Supelcopart. Samples were eluted using UHP helium as the carrier gas with a flow rate of 33 ml/min. Tridecanoin (Sigma) was used as the internal for these studies. Samples were detected using flame ionization detection and total serum cholesterol and triglycerides are reported as mg/dL.

2.10 Enzymatic and Non-Enzymatic Assays

2.10.1 Cytokine Enzyme-Linked Immunosorbent Assay (ELISA)

Peritoneal macrophages (2.0 X 10^5) were seeded in flat-bottom, 96-well tissue culture plates in a total medium volume of 200 µl. Adherent macrophages were left un-stimulated or stimulated with LPS (10 ng/ml), IFNγ (2 U/ml), or a combination of LPS (10 ng/ml) and IFNγ (2 U/ml) for 12 hr and 48 hr (TNFα and IL-6, respectively). Secreted cytokines were obtained by collecting the supernatants of the cultured macrophages. Cytokine concentration was determined by using the BD OptEIA™ ELISA kits (BD Pharmingen, Ontario, Canada for TNFα and IL-6, respectively) following the manufacturer's recommendations. Briefly, flat-bottom 96-well plates were pre-coated with anti-IL-6 or anti-TNFα monoclonal antibodies (1° Ab) overnight at 4 °C. The wells were then washed with washing buffer (0.05% Tween-20 in 1X PBS), and blocked with blocking buffer (10% fetal calf serum in 1X PBS) for 1 hr at room temperature. After washing, cytokine standards and macrophage supernatants were incubated in the pre-coated 96-well plates for 2 hr at room temperature. The wells were washed again, and incubated with the working detector antibody (2° Ab) for 1 hr at room temperature. After washing, TMB one-step substrate reagent was added and allowed to react for 30 min at room temperature. Subsequently, the enzymatic reaction was stopped by adding 1M H_3PO_4. Finally, light absorbance was measured at 450 nm using the Emax Precision Microplate Reader (Molecular Devices, Sunnyvale, California, USA) and the SOFTmax computer software (Molecular Devices, Sunnyvale, California, USA).

2.10.2 Luciferase Reporter Assay

Luciferase reporter activity was assessed using the luciferase assay system (Promega Corporation, Madison, WI, USA) according to the manufacturer's directions. In brief, C6 glioma cells were transiently co-transfected with luciferase reporter construct, pCMV-β-galactosidase expression vector (pHermes-lacZ), and pJ3H-AEBP1 or pcDNA-AEBP1 expression constructs in 12-well plates. Forty-eight hours later,

transfectants were washed twice in cold 1X PBS and subsequently lysed in 120 μl 1X passive lysis buffer (1X PLB) (Promega Corporation, Wisconsin, USA). Subsequently, 30 μl of protein extract was used to measure reporter activity using the BMG FLUOstar Galaxy Microplate Reader (BMG Lab technologies, North Carolina, USA). Luciferase activity was adjusted based on β-galactosidase activity to normalize for transfection efficiency.

2.10.3 β-galactosidase Assay

β-galactosidase activity was measured in order to normalize for transfection efficiency in luciferase reporter assays. To that end, 30 μl of protein extract was incubated in a combination of 3 μl Mg^{2+} buffer (10 μl 1M $MgCl_2$, 35 μl β-mercaptoethanol, 55 μl water), 66 μl ONPG (o-nitrophenyl-β-galactopyranoside) (4 mg/ml in 0.1 M $NaHPO_4/Na_2PO_4$ [pH 7.4]), and 201 μl 0.1 M $NaHPO_4/Na_2PO_4$ buffer [pH 7.4]. As a negative control, β-gal activity was measured using 30 μl of 1X PLB. β-Gal reaction was carried out at room temperature or 37 °C until color change from transparent to yellow was visible (5-30 min). The reaction was subsequently stopped by the addition of 0.5 ml 1 M Na_2CO_3, and β-gal activity was determined using a Bio-Rad spectrophotometer at A_{410}.

2.10.4 *In Vitro* Kinase Assay

Endogenously expressed IKK complex was immunoprecipitated from 0.5 mg pre-cleared protein extracts by incubation with 2 μg/ml rabbit polyclonal anti-IKKα antibody (H-744) (Santa Cruz Biotechnologies), which cross-reacts with IKKα and IKKβ, for 16 hr at 4 °C, and then with protein A/G plus agarose beads for 4 hr at 4 °C. The immunoprecipitate samples were washed twice with cold RIPA lysis buffer, once with cold RIPA lysis buffer containing 500 mM NaCl, and finally once with cold kinase reaction buffer (50 mM Tris-HCl [pH 8.0], 10 mM $MgCl_2$, 1 mM DTT, 0.1% NP-40, 1 mM Na_3VO_4, 1 mM PMSF, and 0.3 mM cold ATP). Kinase assays were performed by adding 1 μg of bacterially expressed GST-IκBα (1-54) fusion protein, as a substrate, and

5 µCi of [γ-^{32}P]ATP (Amersham Biosciences, Buckinghamshire, England) to the immunoprecipitate samples in a total of 20 µl kinase reaction buffer. The kinase reaction was carried out for 30 min at 30 °C, stopped by the adding 1 µl 2X SDS sample loading buffer, boiled for 5 min, and centrifugation for 10 min at 15,000 x g. The samples were subjected to SDS-PAGE, transferred to nitrocellulose membranes, analyzed by autoradiography, and finally subjected to immunoblotting.

2.10.5 Electrophoretic Mobility Gel Shift Assay (EMSA)

AEBP1-DNA binding activity was assessed by EMSA as previously described (Lyons *et al.*, 2005). Briefly, 500 ng recombinant AEBP1 protein and [α-^{32}P]ATP-labeled oligonucleotide sequence (probe) (25,000 CPM) are incubated in a total of 20 µl binding reaction buffer (10 mM Tris-HCl [pH 7.5], 10 mM KCl, 5 mM MgCl$_2$, 1 mM DTT, 2.5% glycerol). Specific (un-labeled probe) or non-specific (unrelated probe) competitors were used at 50X excess. The reaction was allowed to take place for 20 min at room temperature. Thereafter, 2 µl of 10X loading dye (0.4 mM bromophenol blue in binding reaction buffer) was added to the reaction samples, which were subsequently resolved on 5% 0.25X TBE polyacrylamide mini-gels. The gels were then dried and subjected to autoradiography.

For NF-κB-DNA binding, the same procedure outlined above was followed with minor modifications. In particular, 2.0 µg nuclear protein was incubated with the κB-radiolabeled probe along with 1.0 µg poly dI:dC (Promega Corporation, Madison, WI, USA). All probes were radiolabeled with [α-^{32}P]ATP (Amersham Biosciences, Buckinghamshire, England) by Klenow fill-in reaction. Nucleotide sequences of probes used in EMSA are shown in Table 5. For specific competition, an unlabeled, specific probe representing the above sequences was used. For non-specific competition, an unlabeled, unrelated sequence designated PO4 was used.

Table 5. Nucleotide Sequences of Probes used in EMSA.

Probe Designation	Nucleotide Sequence (5' to 3')
AE-1	CCAGGGAGAACCAAAGTTGAGAAATTTCTATTAAA
PPARγ1	GGTGTCAGAAACACTGCTAAGAAATTTAAGAAATT
PPARγ1-M	GGTGTCAGAAACACT**CAATTT**AAATTTAAGAAATT
LXRα	CAGGGGAGGAGGGAGGCTGGGAACACAGGCTGGGG
LXRα-M	CAGGGGAGGAGGGAG**CAATTT**AACACAGGCTGGGG
κB	AGTTGAGGGGACTTTCCCAGGC
PO4	CCCCTCACGACCCATGGACCCCCAGCAGCGGCGCATGCAG

2.11 Atherosclerotic Lesion Analysis

2.11.1 Aortic Section & Whole Aorta Preparation

For aortic cryosections, analysis of atherosclerotic lesions in the proximal aortic root was performed as previously described (Boisvert *et al.*, 1998). In brief, the heart and 1 mm of the proximal aorta were removed, washed with heparin and 1X PBS, embedded in OCT/sucrose solution (2:1), and stored at -80 °C. Ten μm-thick cryosections were obtained on superfrost/PLUS microscope glass slides (Fisher Scientific, Ontario, Canada) starting at the level of the attachment of the aortic valve cusps to the aortic ring, using a cryostat microtome (Reichert Incorporation, New York, USA).

For *en face* analysis, aorta isolation and detection of atherosclerotic lesions was performed as previously described (Palinski *et al.*, 1994). In brief, aortae were microscopically collected from the heart to the iliac bifurcation and dissected to remove any adventitial fat. Dissected aortae were fixed in 10% neutral buffered formalin, opened longitudinally, and pinned onto a flat wax surface. Subsequently, pinned aortae were immersed in Sudan IV solution containing 0.5% Sudan IV (Sigma), 35% ethanol, and 50% acetone for 30 min, after which they were washed with 80% ethanol and kept immersed in 1X PBS containing 0.02% NaN_3 for preservation. Images of the aortae *en face* were captured with a Pixelink microscopy camera (PL-A686, 6.6 megapixels) attached to a Leica MZ6 dissecting microscope.

2.11.2 Atherosclerotic Lesion Detection

For detection of lipid-filled atherosclerotic lesions in aortic cryosections, every fourth cryosection obtained from OCT-embedded aortae was stained with oil red O (Sigma) followed by Mayer's hematoxylin counter-staining as described above (section 2.6.3). For detection of intimal macrophages within lesions, cryosections obtained from OCT-embedded aortae were immunostained with anti-MOMA-2 antibody as described above (section 2.6.4). Tissue analysis and photomicroscopy were performed on a Nikon Eclipse E600 microscope attached to a Nikon Coolpix 990 camera. Stained aortic

cryosections were blindly evaluated by three examiners, and qualitative and quantitative measures were independently recorded. For detection of lipid-filled atherosclerotic lesions in aorta *en face*, Sudan IV staining was performed as described above (section 2.11.1).

2.11.3 Assessment of Mean Atherosclerotic Lesion Area

Captured images of ORO-stained aortic cryosections were analyzed using Image*J* analysis software (free download from http://rsb.info.nih.gov/ij/). For each mouse, 6-8 images were analyzed by semi-automatically measuring the cross-section luminal area covered by lesion as well as the total area of the aortic lumen. The mean lesion area was calculated by dividing the lesion-covered area by the total area of lumen, multiplied by 100. Mean lesion area is expressed as a percentage of the total aortic lumen area occupied by lesions.

2.12 Statistical Analysis

Statistical significance was determined using Student *t*-test for un-paired observations. $*P \leq 0.05$ is considered statistically significant. For each experiment, duplicate or triplicate samples were used, and the (n) value indicated in the figure legends represents the total number of samples in all corresponding experiments. The mean and SEM values were calculated based on the total number of samples (n), and data is expressed as the mean ± SEM of the indicated number of samples (n).

Chapter 3

RESULTS

3.1 AEBP1 Expression in Macrophages

3.1.1 Isolation and Purification of Peritoneal Macrophages

After harvest of peritoneal exudate depleted of erythrocytes by ACK treatment, several staining techniques were performed on cytospin samples to determine the percentage of monocyte/macrophages in the peritoneum. First, cytospin samples representing 1.0×10^5 peritoneal cells were stained with hematoxylin & eosin (H&E) to permit identification of different cell types based on distinctive morphological properties. H&E stained samples revealed that about 95% of peritoneal exudate cells are identified as monocytes/macrophages (Figure 9A). The remaining 5% of exudate cells are represented by lymphocytes, mast cells, and other leukocytes. Second, to further confirm that the vast majority of isolated peritoneal exudate cells belong to the monocyte/macrophage cell lineage, 1.0×10^5 cells were cytospun and immunostained with IgG control or antibody against MOMA-2, a specific monocyte/macrophage marker (Kraal *et al.*, 1987). Immunocytochemical analysis indicates that monocytes/macrophages represent more than 95% of peritoneal exudate cell population (Figures 9 B & C). Taking advantage of their ability to adhere to the bottom of tissue culture plates, monocytes/macrophages were further purified by culturing peritoneal exudate cells for 72 hr in standard growth medium. Lymphocytes, mast cells, and other cellular contaminants which remained in suspension, were aspirated while replacing culture medium every 24 hr.

Figure 9. Peritoneal Exudate Cells Consist Primarily of Macrophages and Monocytes. After isolation of peritoneal exudate cells from mice, red blood cells were lysed using ACK lysis buffer. Peritoneal exudate cells were then washed three times with 1X PBS, and cytospin samples of 1.0×10^5 cells were prepared as described in section 2.6.1. Peritoneal exudate cells were then stained with H&E *(A)* and immunostained with 1:80 dilution of rat IgG control *(B)* or rat anti-MOMA-2 *(C)* to allow monocyte/macrophage detection.

A   Hematoxylin & Eosin (H&E)
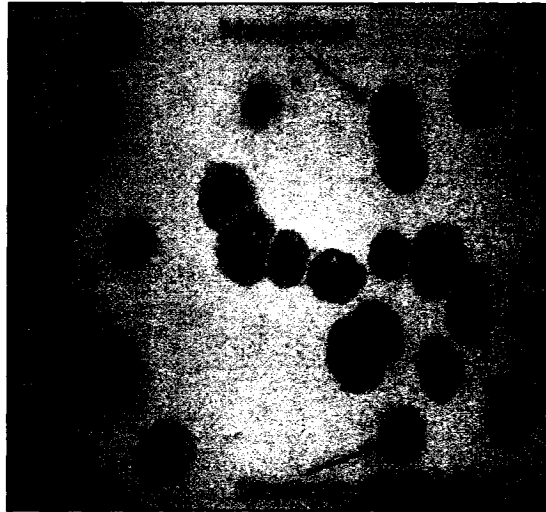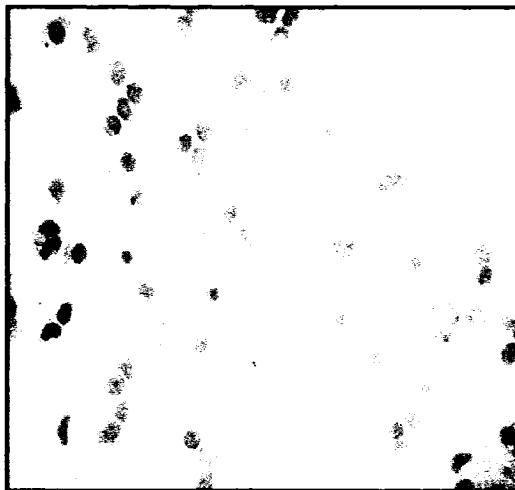
B   Normal IgG   **C   Anti-MOMA-2**
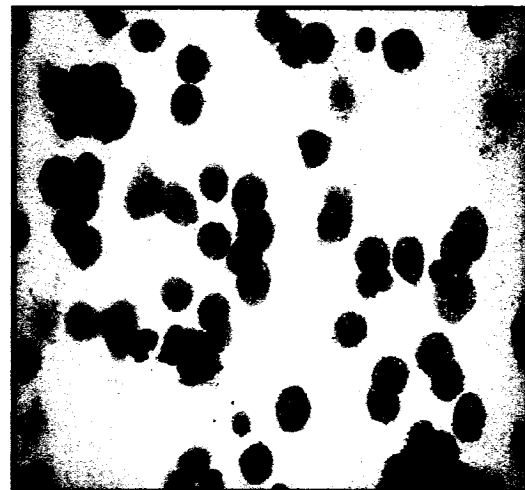
3.1.2 Macrophages Express Abundant Levels of AEBP1

AEBP1 is an 82-kDa protein that has been found to be expressed in various tissues including white adipose tissue (WAT), brown adipose tissue (BAT), liver, spleen, brain, lung, and skeletal muscle (Ro *et al.*, 2001). However, AEBP1 expression was never examined in macrophages or any other hematopoietic cell type. To assess AEBP1 expression in primary macrophages as well as in murine and human monocyte/macrophage cell lines, whole cell protein extracts were obtained from AEBP1$^{+/+}$ and AEBP1$^{-/-}$ macrophages, as well as from J774, THP-1, and U937 cells treated with DMSO (vehicle) or 100 nM PMA for 48 hr to induce their differentiation into mature macrophages. Protein extracts were subjected to SDS-PAGE and immunoblotting for AEBP1, CD14 (as a differentiation marker control), and actin (as a loading control). Western blot analysis clearly revealed that AEBP1 is abundantly expressed in peritoneal macrophages as well as in monocyte/macrophage cell lines (J774, THP-1, and U937) (Figure 10). Notably, AEBP1 expression seems to be up-regulated upon monocyte differentiation into macrophages *in vitro* (Figure 10). Indeed, a time-course experiment indicates that PMA treatment leads to increased AEBP1 expression in J774, but not in CHO cells or the mouse mammary epithelial cell line, HC11 (data not shown).
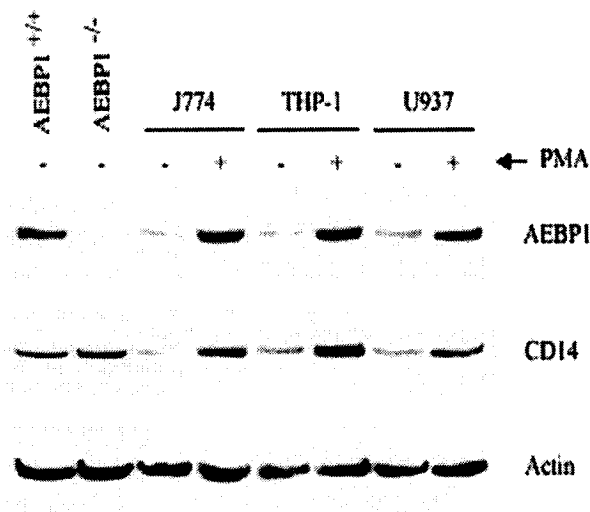
Figure 10. Primary Macrophages and Monocyte/Macrophage Cell Lines Express AEBP1. Whole cell protein extracts were obtained from macrophages isolated from 20-wk old, HFD-fed AEBP1$^{+/+}$ and AEBP1$^{-/-}$ females. Whole cell protein extracts were also obtained from DMSO-treated (-) or PMA-treated (+) J774, THP-1, and U937 cells. Protein extracts (25 µg) were resolved on 8.5% polyacrylamide gels and transferred onto nitrocellulose membranes. Subsequently, the nitrocellulose membranes were immunoblotted for AEBP1, CD14, and actin.

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3.1.3 AEBP1 Expression in AEBP1^{TG} and AEBP1$^{-/-}$ Macrophages

In order to examine the physiological significance of altered AEBP1 expression in primary macrophages, the modulated expression level of AEBP1 in primary macrophages isolated from AEBP1^{TG} mice, in which AEBP1 transgene expression is driven by the aP2 promoter, as well as from AEBP1$^{-/-}$ mice, should first be confirmed.

Increased expression of aP2 was recently documented in monocytes following stimulation with PPARγ activators (Pelton *et al.*, 1999), and oxLDL was reported to induce aP2 expression in macrophages (Fu *et al.*, 2000). These observations suggest that the regulatory elements that direct aP2 expression in adipocytes are sufficient to confer expression in macrophages isolated from AEBP1^{TG} mice. Primary macrophages from three independent transgenic lines expressing genes encoding uncoupling protein (UCP)-1, agouti, and TNFα under the control of the 5.4-kb aP2 promoter/enhancer showed over-expression of these transgenes (Makowski *et al.*, 2001). Since AEBP1 transgene expression is driven by the 5.4-kb aP2 promoter (Zhang *et al.*, 2005), AEBP1 should be over-expressed in macrophages of AEBP1^{TG} mice. Indeed, both AEBP1 mRNA and protein levels in AEBP1^{TG} macrophages are about 4-fold higher than those of AEBP1^{NT} macrophages (Figures 11 A & B). Macrophages isolated from AEBP1^{TG} females and males over-express AEBP1 to the same extent (data not shown). Expectedly, AEBP1 expression is completely abolished in AEBP1$^{-/-}$ macrophages (Figures 11 A & B).

A

B
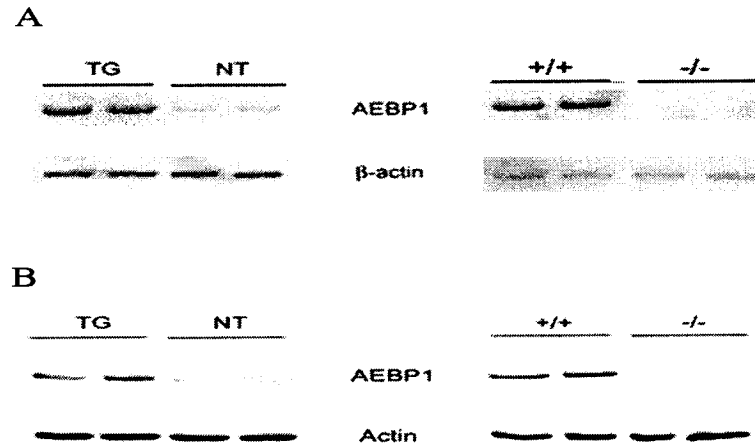
Figure 11. AEBP1^{TG} and AEBP1$^{-/-}$ Macrophages Over-Express and Lack AEBP1, Respectively. *(A)* Total RNA was obtained from macrophages isolated from 32-wk old, HFD-fed AEBP1^{TG} and AEBP1^{NT} females, as well as 20-wk old, HFD-fed AEBP1$^{+/+}$ and AEBP1$^{-/-}$ females, and reverse transcription was performed using 1.0 μg RNA. Subsequently, AEBP1 and β-actin cDNA sequences were amplified by PCR using specific primers. Two samples from each genotype are shown as representatives. *(B)* Whole cell protein extracts were isolated from macrophages obtained from 32-wk old, HFD-fed AEBP1^{TG}, AEBP1^{NT}, AEBP1$^{+/+}$, and AEBP1$^{-/-}$ females. Protein extracts (25 μg) were resolved on 8.5% polyacrylamide gels and transferred onto nitrocellulose membranes. Subsequently, the nitrocellulose membranes were immunoblotted for AEBP1 and actin. Two samples from each genotype are shown as representatives.

3.2 Evaluation of Transfection Efficiency

3.2.1 Estimation of Transfection Efficiency in CHO Cells

In order to determine transfection efficiency in CHO cells using polyfect transfection reagent, CHO cells were transfected with pEGFP-N1 that carries a sequence encoding the red-shifted variant of wildtype green fluorescent protein (GFP), designated GFPmut1, which contains the double amino acid substitution of F^{64} and S^{65} to L^{64} and T^{65}, respectively (Cormack *et al.*, 1996). This construct has been optimized for brighter fluorescence and higher expression in mammalian cells (Clontech). So, in this case, GFP is used as a transfection marker. CHO cells were co-transfected with pJ3H empty vector or pJ3H-AEBP1 construct. As a negative control, CHO cells were transfected with pJ3H empty vector alone (i.e. no pEGFP-N1 transfection). Upon transfection, CHO transfectants were cultured for 48 hr in standard growth medium. Subsequently, CHO transfectants were examined under a Zeiss Axiovert-200 inverted phase contrast microscope attached to a Zeiss AxioCam HR camera (Carl Zeiss, Göttingen, Germany), and the total number of cells and the number of GFP-positive cells were recorded. Microscopic analysis revealed that compared to 0% GFP-positive staining in CHO cells transfected with pJ3H empty vector, about 50% of CHO cells transfected with pEGFP-N1 displayed GFP-positive staining (Figure 12), indicating that polyfect achieves about 50% transfection efficiency in CHO cells under the conditions described.

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Figure 12. Transfection Efficiency in CHO Cells Using Polyfect. *(A)* CHO cells were transfected with 50 ng pEGFP-N1, and either 50 ng pJ3H empty vector (*middle panels*) or pJ3H-AEBP1 construct (*right panels*). CHO cells were transfected with 100 ng pJ3H empty vector alone (i.e. no pEGFP-N1) as a negative control (*left panels*). Cells were then cultured for 48 hr in standard growth medium, after which they were washed twice with 1X PBS and examined under a Zeiss Axiovert-200 inverted phase contrast microscope attached to a Zeiss AxioCam HR camera. Cells, in one field of view, were visualized without a fluorescence filter (*top panels*) or with a green fluorescence filter (*bottom panels*) to allow detection of GFP-positive CHO cells. *(B)* The number of GFP-positive cells was divided by the total number of cells (GFP-negative plus GFP-positive cells), and a histogram was constructed to represent the percentage of GFP-positive CHO cells. Values are expressed as a percentage that represents transfection efficiency, which is expressed as mean ± SEM (n=9).

A

No GFP	GFP + AEBP1	GFP + Empty Vector

B

116
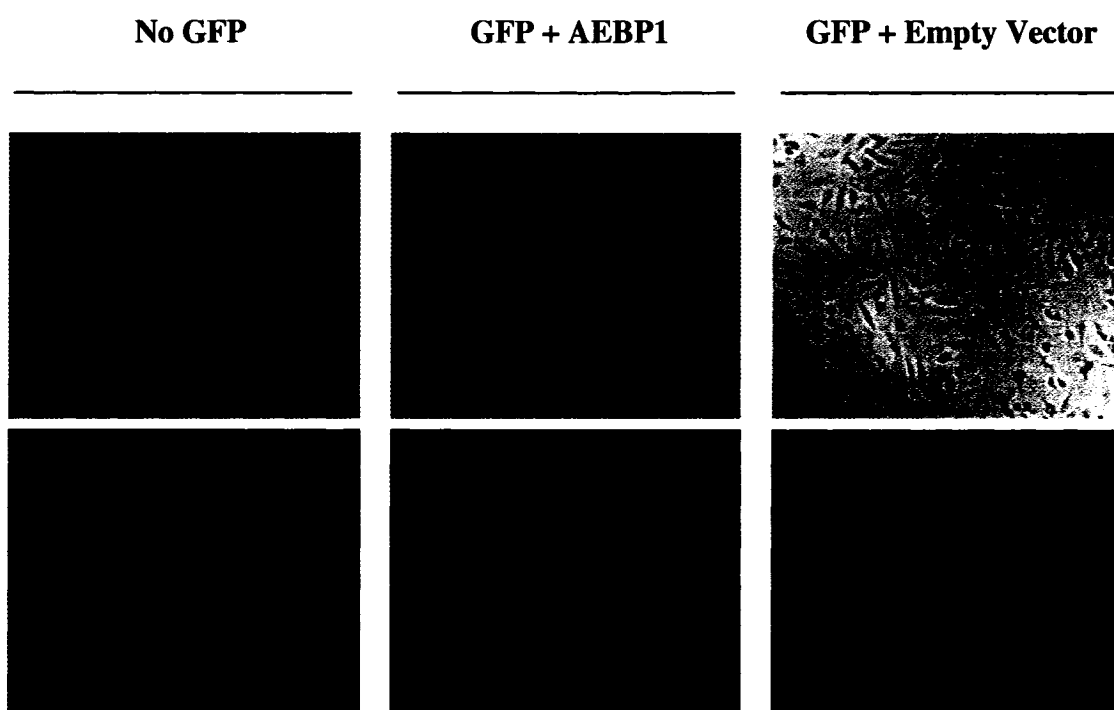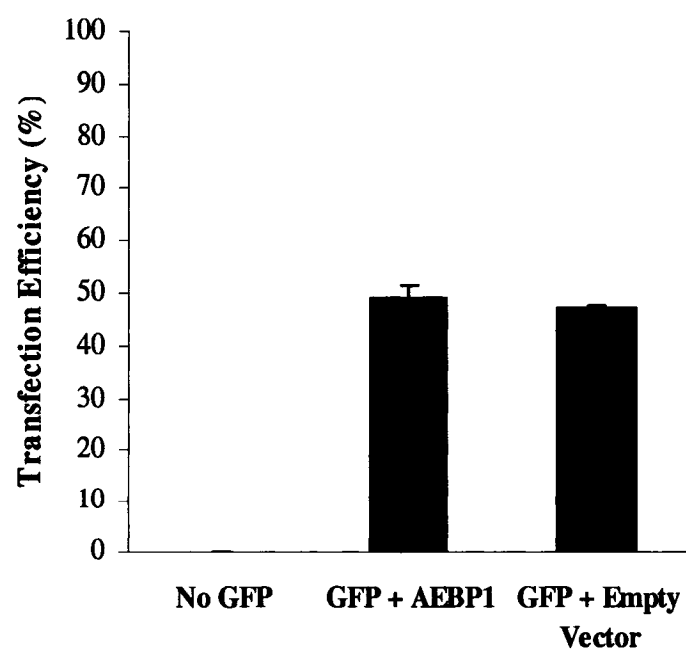
3.2.2 Assessment of Transfection Efficiency in J774, THP-1, and U937 Cells

Due to their abundance and ease of manipulation, monocyte/macrophage cell lines (e.g. J774, THP-1, and U937) were of primary interest to be used as *in vitro* systems to perform over-expression and siRNA-knockdown studies to assess the molecular function of AEBP1 in macrophages. However, the feasibility of such experimental studies hinges on successful transfection of such cell lines. Although some studies have shown that efficient transfection can be achieved in murine and human monocyte/macrophage cell lines (see below), many other studies have demonstrated that such cell lines are very resistant to transfection by conventional gene transfer methods (i.e. lipid-based transfection and electroporation) (Ohtani *et al.*, 1989; Kusumawati *et al.*, 1999; Martinet *et al.*, 2003). Hence, the ease of transfecting J774, THP-1, and U937 cells using different approaches was assessed.

3.2.2.1 Transfection using Lipid-Based Reagents

To evaluate transfection efficiency in J774, THP-1, and U937 cells by lipid-based gene transfer methods, cells were treated with 100 nM PMA for 48 hr to induce their differentiation into mature macrophages. Cells were subsequently transfected with 4, 8, and 12 µg pEGFP-N1 or empty vector using lipofectamine[2000] (Invitrogen), effectene (Qiagen), and metafectene (Biontex) transfection reagents according to the manufacturer's recommendations. PMA-untreated CHO cells, which are relatively easy to transfect using lipid-based transfection reagents, were used as a positive control. Different transfection reagent:DNA ratios were used for optimization. Subsequently, cells were cultured in fresh growth medium under standard culture conditions. Forty-eight hours post transfection, the efficiency of transfection was determined based on microscopic analysis of GFP fluorescence. Microscopic examination revealed that only 3% of J774 cells, 2% of THP-1, and 0% of U937 cells were GFP-positive (Figure 13A), with no differences among lipofectamine[2000], effectene, and metafectene (lipofectamine[2000] data is shown as a representative). These results support studies

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indicating that lipid-based transfection reagents are inefficient in causing sufficient gene transfer in J774, THP-1, and U937 monocyte/macrophage cell lines.

3.2.2.2 Transfection by Electroporation

Electroporation is a mechanical method that depends on high-voltage current that allows cell membranes to become permeable by creating transient pores, thus permitting introduction of plasmids into prokaryotic and eukaryotic cells (Melvik *et al.*, 1986; Mir *et al.*, 1988). In mammalian cells, electroporation is considered to be an efficient gene transfer technique when other conventional lipid-based transfection methods prove to be unsuccessful (Kusumawati *et al.*, 1999). It is documented that electroporation leads to efficient transfection of THP-1 (Kurosu and Takada, 2001; Hatch *et al.*, 1998; Yu *et al.*, 2002) and U937 (Withers and Hakomori, 2000, Hayes *et al.*, 2002, Zheng *et al.*, 2004) cells. It is documented that electroporation is a poor gene transfer system in J774 cells (Kusumawati *et al.*, 1999). To test transfection efficiency by electroporation in J774, THP-1, and U937 cells, cells were treated with 100 nM PMA for 48 hr to induce their differentiation into mature macrophages. Subsequently, 5-20 μg of pEGFP-N1 or empty vector was added to 0.4 cm electrode gap cuvettes containing suspended, differentiated cells, which were electroporated at different voltage settings (230 to 300 V @ 950-970 μF) using Gene Pulser II (BioRad). PMA-untreated Jurkat cells, a human leukemic T cell line, are known to be easily transfected by electroporation, and hence, they were used as a positive control. Electroporated cells were cultured in fresh growth medium for 48 hr, after which they were examined for GFP positive signal. As shown in Figure 13B, microscopic analysis indicated that even after optimization, electroporation was less than 2% efficient in transfecting J774, THP-1, and U937 cells.

3.2.2.3 Transfection by Ligand-Conjugated Reagents

Recently, a novel series of ligand-conjugated transfection reagents have been manufactured to enable transfection of relatively hard-to-transfect cells in a cell-specific manner (Polyplus Transfection, Illkirch, France). JetPEI™-macrophage is a

mannose-conjugated linear polyethylenimine transfection reagent that is claimed to achieve relatively high transfection efficiency in cells expressing mannose-specific membrane receptors, such as macrophages. To evaluate transfection efficiency in J774, THP-1, and U937 cells by lipid-based gene transfer methods, cells were treated with 100 nM PMA for 48 hr to induce their differentiation into mature macrophages. Cells were subsequently transfected with pEGFP-N1 or empty vector using JetPEI^{TM}-macrophage transfection reagent following the manufacturer's recommendations. Unfortunately, microscopic examination of transfected cells revealed that JetPEI^{TM}-macrophage did not lead to successful transfection of J774, THP-1, or U937 cells (data not shown).

Collectively, it is evident that macrophage/monocyte cell lines are very hard to transfect by conventional transfection methods, and thus, isolation and culture of AEBP1-over-expressing and AEBP1-dificient primary macrophages from AEBP1^{TG} and AEBP1$^{-/-}$ mice, respectively, is necessary to investigate possible roles of AEBP1 in macrophages.

Figure 13. Monocyte/Macrophage Cell Lines are Resistant to Transfection. *(A)* J774, THP-1, and U937 cells were treated with 100 nM PMA for 48 hr, after which they were transfected with 4, 8, and 12 μg pJ3H empty vector or pEGFP-N1 using lipofectamine^{2000} as recommended by the manufacturer. PMA-untreated CHO cells were used as a positive control. Forty-eight hours post transfection and culture in standard growth medium, cells were washed twice with 1X PBS and visualized under a Zeiss Axiovert-200 inverted phase contrast microscope attached to a Zeiss AxioCam HR camera. GFP-positive cells and total number of cells were counted, and a histogram was obtained representing percentage of cells that were successfully transfected (i.e. GFP-positive). Data show represents cells transfected with 8 μg pEGFP-N1. *(B)* J774, THP-1, and U937 cells were treated with 100 nM PMA for 48 hr, after which they were lifted by trypsin-EDTA and transfected with 5, 10, 15, and 20 μg pJ3H empty vector or pEGFP-N1 by electroporation at different voltage settings (230 to 300 V @ 950-970 μF). PMA-untreated Jurkat cells were used as a positive control. Cells were cultured for 48 hr, washed twice with 1X PBS and visualized as described in (A). A histogram is shown summarizing data representing transfection efficiency using 10 μg pEGFP-N1 at 250 V/950 μF. Transfection efficiency is expressed as mean ± SEM (n=3).

A
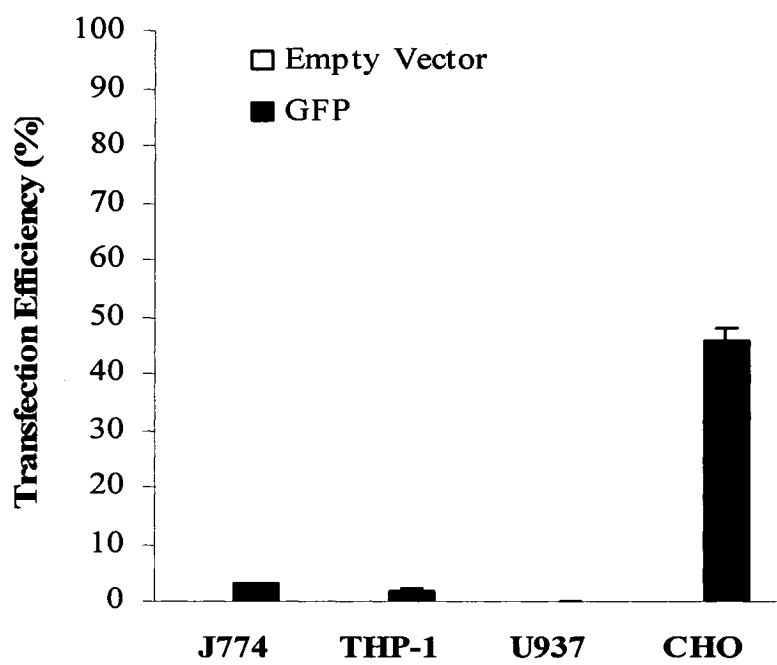
B
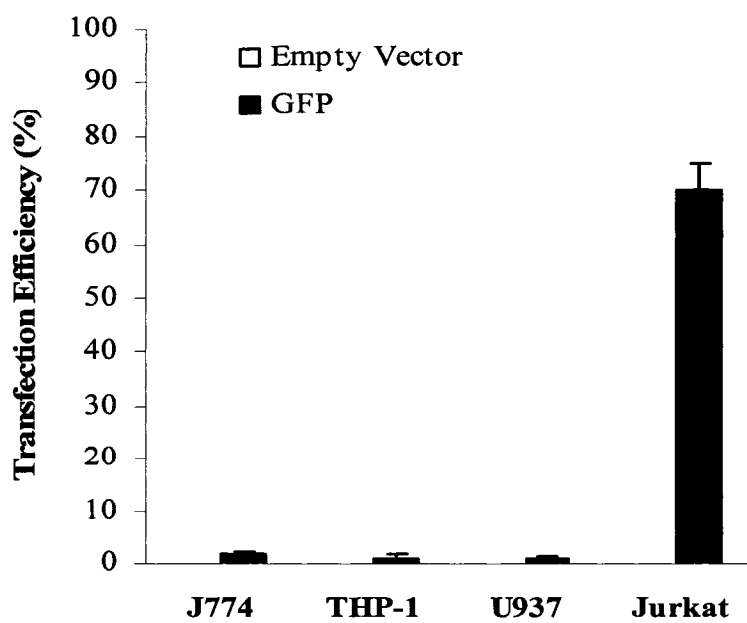
3.3 AEBP1-Mediated Transcriptional Repression of PPARγ1 and LXRα

3.3.1 PPARγ1 and LXRα Promoters Possess Putative AEBP1-Binding Sites

AEBP1 was initially identified as a transcriptional repressor that is capable of down-regulating the expression of the aP2 gene via interacting with AE-1 sites within the promoter of aP2 (He *et al.*, 1995). Careful examination of the promoter regions of PPARγ1 and LXRα revealed the presence of sequences that are homologous to the AE-1 sequence (Figure 14A). It was hypothesized that such sequences can serve as putative AEBP1-binding sites. To examine this possibility, electrophoretic mobility gel shift assay was performed using ^{32}P-labeled probes representing the AE-1 sequence as well as putative AEBP1-binding sequences within the promoter regions of PPARγ1 and LXRα. Apparently, recombinant AEBP1 protein binds as effectively and specifically to these sequences as it does to the AE-1 sequence (Figure 14B). Importantly, AEBP1-DNA complex formation was eliminated by replacing purines with pyrimidines, and vice versa, for the six most conserved nucleotides (bolded in Table 5) among the AE-1 sequence and the putative AEBP1-binding sequences within PPARγ1 and LXRα promoters (hPPARγ1-M and mLXRα-M) (Figure 14B).

Figure 14. AEBP1 Binds to AE-1-like Sequences within PPARγ1 and LXRα Promoters. (*A*) Sequence homology between the AE-1 sequence and the putative AEBP1-binding sequences within the promoter regions of mouse LXRα (mLXRα) and human PPARγ1 (hPPARγ1) genes. A line (|) represents an exact nucleotide match, whereas an asterisk (•) represents a purine:purine or a pyrimidine:pyrimidine match. The underlined sequences are deleted in the pGL3-mLXRα-luciferase and pGL3-hPPARγ1-luciferase constructs, respectively. (*B*) EMSA shows that AEBP1 specifically binds to AE-1 homologous sequences in the promoter regions of hPPARγ1 and mLXRα, but not to the mutated sequences (hPPARγ1-M and mLXRα-M) (refer to Table 5). For each probe, lane 1 represents ^{32}P-labeled probe alone, lane 2 represents probe plus purified AEBP1 protein, and lanes 3 and 4 represent probe plus purified AEBP1 protein in presence of specific and non-specific competitors, respectively. This data is representative of three independent experiments.

A

```
hPPARγ1  -650  GGTGTCAGAAACACTGCTAAGAAATTTAAGAAATT  -616
                 |   |||| ||   |•|•||||||||   •  |
AE-1           CCAGGGAGAACCAAAGTTGAGAAATTTCTATTAAA
                 | •|||||  |  •|•|•||•|||   •    |  •••
mLXRα    -103  CAGGGGAGGAGGGAGGCTGGGAACACAGGCTGGGG  -69
```

B

124
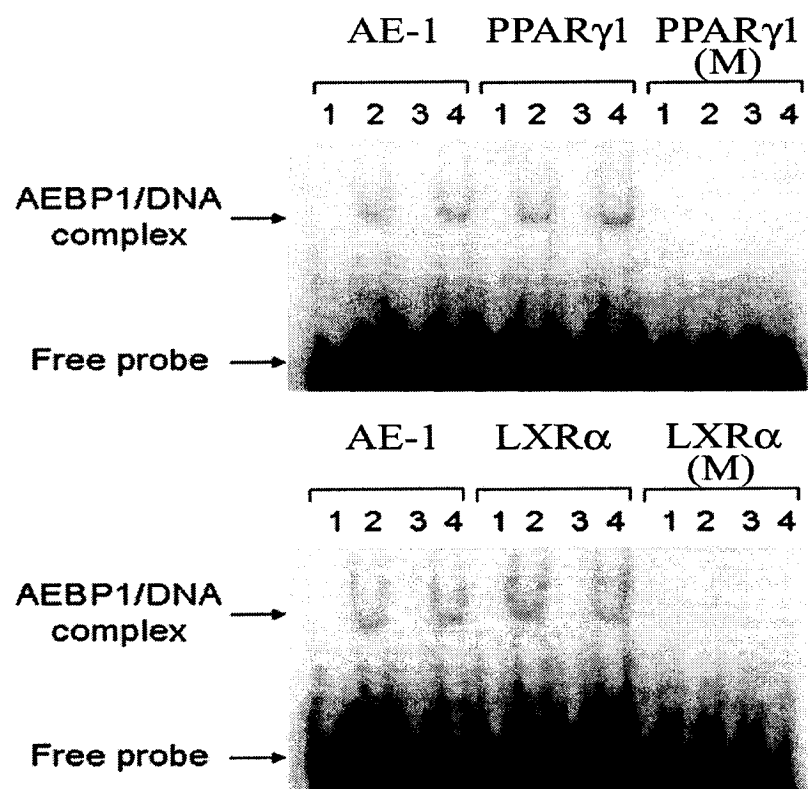
3.3.2 AEBP1 Represses PPARγ1 and LXRα Expression *in vitro*

Since AEBP1 is able to bind specifically to AE-1 homologous sequences within the promoter regions of PPARγ1 and LXRα (Figure 14), it is likely that AEBP1 may act as a transcriptional repressor of these two genes, which are critically involved in controlling macrophage cholesterol homeostasis and inflammatory responsiveness. To examine whether AEBP1 regulates PPARγ1 expression *in vitro*, luciferase reporter assays were performed in CHO cells transfected with pGL3-PPARγ1-luciferase that contains a luciferase gene whose expression is driven by the promoter of PPARγ1 (Fajas *et al.*, 1997), and co-transfected with increasing amounts of pJ3H-AEBP1 (Park *et al.*, 1999; Kim *et al.*, 2001). pJ3H empty vector was used as a negative control. As shown in Figure 15A, AEBP1 is capable of repressing PPARγ1 expression in a dose-responsive manner, despite transfection of equal DNA amount using pJ3H empty vector. Since PPARγ1 has been shown to be a direct, positive regulator of LXRα (Chawla *et al.*, 2001b), and since the proximal promoter region of LXRα gene (Alberti *et al.*, 2000) contains a putative AEBP1-binding site (Figure 14A), it is plausible that LXRα expression may be under control by AEBP1 transcriptional repression function. Expectedly, co-transfection analysis using pGL3-LXRα-luciferase containing the proximal LXRα promoter upstream of the luciferase gene (Steffensen *et al.*, 2002) and pJ3H-AEBP1 construct revealed that AEBP1 represses LXRα expression in a dose-responsive manner (Figure 15A).

To substantiate these findings, protein extracts isolated from CHO cells over-expressing AEBP1 were subjected to SDS-PAGE followed by immunoblotting to assess PPARγ1 and LXRα protein levels *in vitro*. Western blot analysis clearly demonstrates that AEBP1 over-expression in CHO cells is accompanied by significantly reduced PPARγ1 and LXRα protein levels (Figure 15B), confirming the ability of AEBP1 to transcriptionally repress PPARγ1 and LXRα *in vitro*.

Figure 15. AEBP1 Transcriptionally Represses PPARγ1 and LXRα _in vitro_. _(A)_ CHO cells were transiently transfected with 200 ng pGL3-hPPARγ1-luciferase or pGL3-mLXRα-luciferase using polyfect. Cells were co-transfected with 20 ng pHermes-lacZ, which expresses β-galactosidase, along with pJ3H empty vector or increasing amounts of pJ3H-AEBP1 (25, 50, 75, 100, and 125 ng). Total amount of plasmid DNA was equated using pJ3H empty vector. Forty-eight hours post transfection, cells were lysed and subjected to luciferase and β-galactosidase assays. Luciferase activity, normalized to β-gal activity, was set to 100% for the pJ3H empty vector (i.e. 0 ng pJ3H-AEBP1) transfection sample. Relative luciferase activity is expressed as mean ± SEM (n=8). Statistical significance was determined based on relative luciferase activity in pJ3H empty vector-transfected cells. _(B)_ CHO cells were transiently transfected with pJ3H empty vector or pJ3H-AEBP1. Forty-eight hours post transfection, whole cell protein extracts were obtained and 25 µg total protein was resolved on 8.5% gels, and nitrocellulose membranes were immunoblotted for AEBP1, PPARγ1, LXRα, and actin. Protein level was normalized based on actin expression, and normalized protein level was set to 100% in pJ3H empty vector transfection samples. A histogram illustrates densitometric analysis based on actin expression. Relative protein level is expressed as mean ± SEM (n=6). Statistical significance was determined relative to protein level in pJ3H empty vector-transfected cells.

A
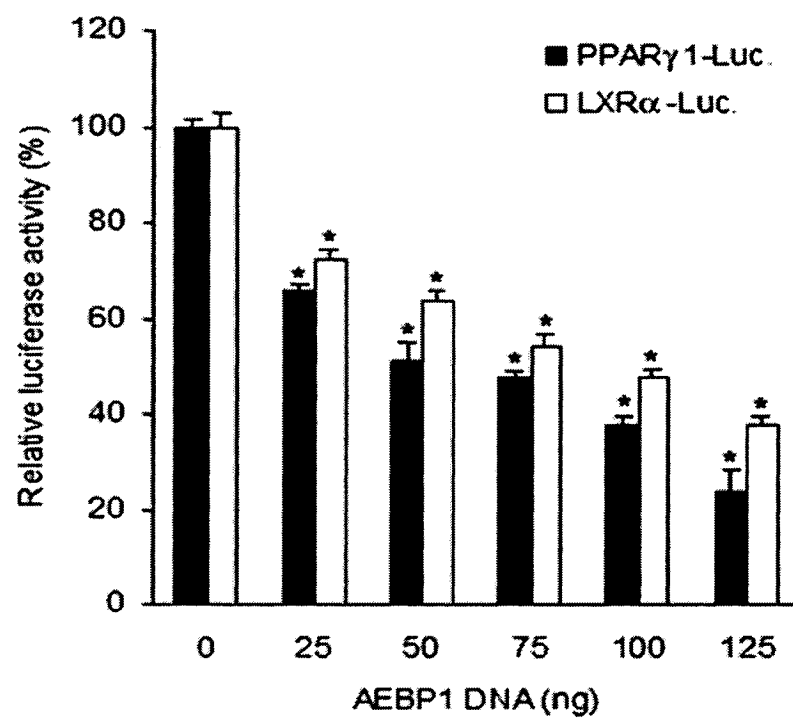
B
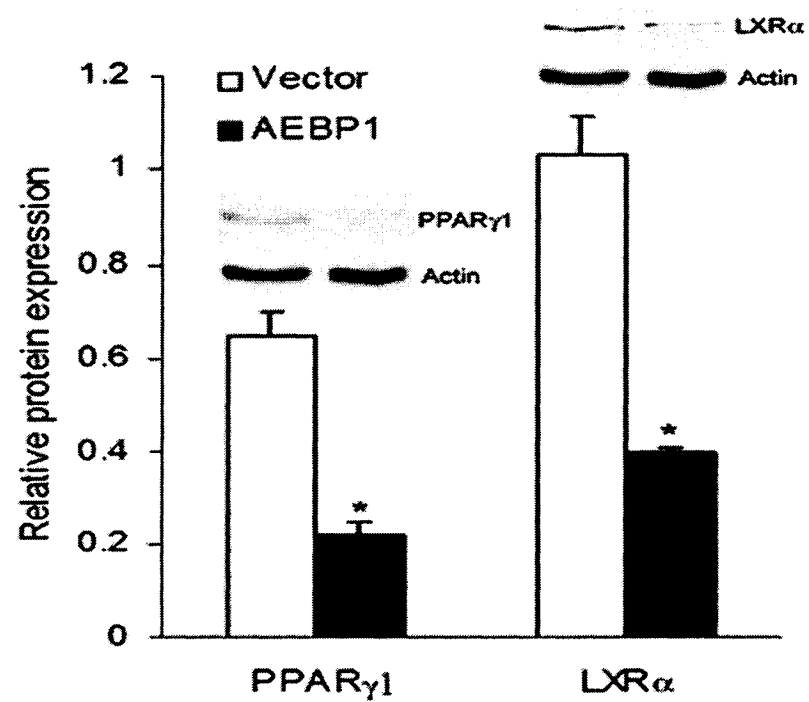
3.3.3 AEBP1 Represses PPARγ1 and LXRα Transcriptional Activity *in vitro*

Active PPARγ1 and LXRα are known to heterodimerize with RXR and subsequently bind to PPAR response elements (PPRE) and LXR response elements (LXRE), respectively, within the regulatory regions of their target genes (Willson *et al.*, 2000). Knowing that AEBP1 is capable of transcriptionally repressing the expression of PPARγ1 and LXRα *in vitro*, it is arguable that AEBP1-mediated repression of PPARγ1 and LXRα may not be sufficient to significantly suppress the transcriptional activity of these two transcription factors. To examine the ability of AEBP1 to modulate PPARγ1 and LXRα transcriptional activity, CHO cells were transfected with TK-PPRE-X3-luciferase (Kim *et al.*, 1998) or TK-LXRE-X3-luciferase (Willy *et al.*, 1995) containing three tandem repeats of consensus PPRE and LXRE, respectively, and co-transfected with pJ3H-AEBP1 or pJ3H empty vector. CHO transfectants were also co-transfected with β-gal expression vector so that β-gal activity can serve as an internal control for transfection efficiency. Luciferase reporter assays performed using extracts isolated from CHO transfectants grown in presence or absence of PPARγ1 and LXRα selective agonists (troglitazone and T0901317, respectively) demonstrate that AEBP1 over-expression is accompanied by significant reduction of PPARγ1 and LXRα transcriptional activity (Figures 16 A & B, respectively). Obviously, AEBP1-mediated suppression of PPARγ1 and LXRα transcriptional activity cannot be overcome by treating the cells with PPARγ1 and LXRα selective agonists (Figures 16 A & B, respectively), suggesting a constitutive suppression of the transcriptional activity of these two transcription factors by AEBP1. Collectively, these results suggest that AEBP1 is not only capable of repressing the expression of PPARγ1 and LXRα, but it is also capable of suppressing their transcriptional activity *in vitro*.

Figure 16. AEBP1 Suppresses PPARγ1 and LXRα Transcriptional Activity *in vitro.* CHO cells were transfected with 200 ng pGL3-TK-PPRE-X3-luciferase *(A)* or pGL3-TK-LXRE-X3-luciferase *(B)* using polyfect. Cells were co-transfected with 20 ng pHermes-lacZ along with 25 ng pJ3H empty vector or pJ3H-AEBP1. Transfected CHO cells were cultured in standard growth medium for 30 hr, after which they were treated with DMSO (vehicle), 1 μM troglitazone (TRO) *(A)*, or 1 μM T0901317 *(B)* for 18 hr. Subsequently, cells were lysed and subjected to luciferase and β-gal assays. Luciferase activity, normalized to β-gal activity, was set to 100% for the DMSO-treated, pJ3H empty vector-transfected cells. Relative luciferase activity, normalized to β-gal activity, is expressed as mean ± SEM (n=6). Statistical significance was determined based on relative luciferase activity in DMSO-, TRO-, or T0901317-treated, pJ3H empty vector-transfected cells.

A

PPRE-Luc.

B

LXRE-Luc.
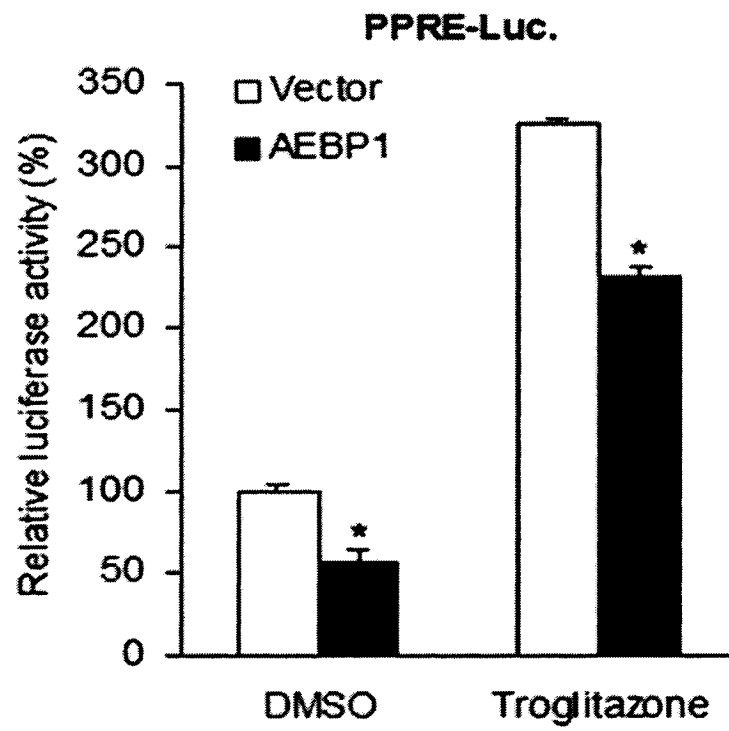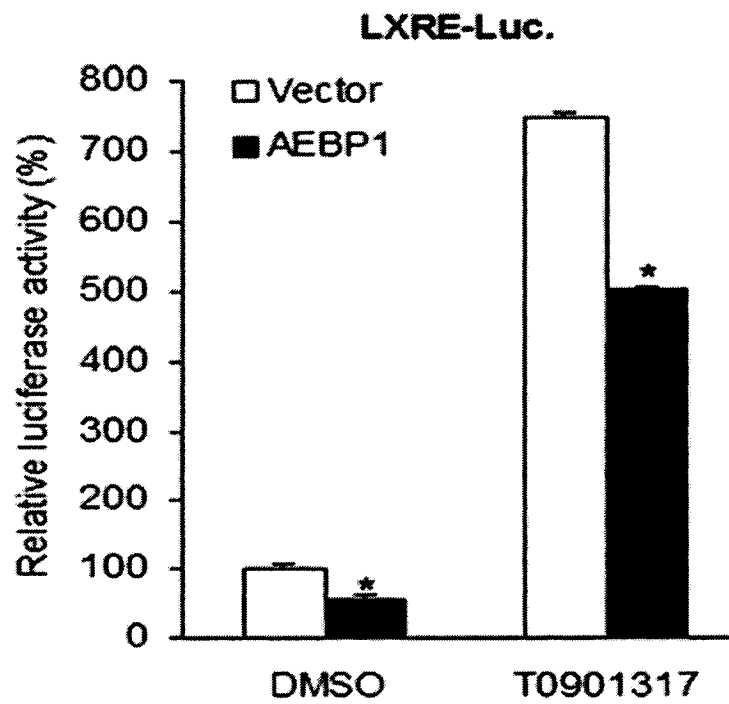
3.3.4 Suppression of PPARγ1 and LXRα Transcriptional Activity by AEBP1 is Independent of AEBP1-Mediated MAPK Sustained Activation

Since AEBP1 is known to protect MAPK against dephosphorylation via protein-protein interaction, leading to sustained MAPK activation (Kim *et al.*, 2001), and since active MAPK is believed to interfere with PPARγ1 transcriptional activity (Camp and Tafuri, 1997), it is possible that AEBP1 represses PPARγ1 and/or LXRα transcriptional activity by means of sustaining MAPK activation. To test this possibility, CHO cells were transfected with luciferase reporter constructs that carry three tandem repeats of PPRE and LXRE (pGL3-TK-PPRE-X3-luciferase and pGL3-TK-LXRE-X3-luciferase, respectively). CHO cells were co-transfected with pJ3H-AEBP1 or pJ3H empty vector, along with the β-gal expression vector as a control for transfection efficiency. CHO cells transfected with pGL3-TK-PPRE-X3-luciferase were treated with DMSO (vehicle), troglitazone (PPARγ agonist), U0126 (MEK inhibitor), or a combination of troglitazone and U0126 (Figure 17A). CHO cells transfected with pGL3-TK-LXRE-X3-luciferase were treated with or DMSO (vehicle), T0901317 (LXR agonist), U0126, or a combination of T0901317 and U0126 (Figure 17B). As clearly illustrated Figure 17, AEBP1 is capable of repressing PPARγ1 and LXRα transcriptional activity in the absence and presence of the MEK inhibitor, U0126, indicating that the AEBP1-mediated repression of PPARγ1 and LXRα transcriptional activity is independent of the status of MAPK activity.

Figure 17. Suppression of PPARγ1 and LXRα Transcriptional Activity by AEBP1 is Independent of AEBP1-Mediated MAPK Sustained Activation. CHO cells were transfected with 200 ng pGL3-TK-PPRE-X3-luciferase *(A)* or pGL3-TK-LXRE-X3-luciferase *(B)* using polyfect. Cells were co-transfected with 20 ng pHermes-lacZ along with 25 ng pJ3H empty vector or pJ3H-AEBP1. Transfected CHO cells were cultured in standard growth medium for 30 hr, after which they were treated with DMSO (vehicle), 1 μM troglitazone (TRO) (PPARγ agonist), 10 μM U0126 (MEK inhibitor), or a combination of 1 μM TRO plus 10 μM U0126 in *(A)*, or DMSO (vehicle), 1 μM T0901317 (LXR agonist), 10 μM U0126, or a combination of 1 μM T0901317 plus 10 μM U0126 in *(B)* for 18 hr. Subsequently, cells were lysed and subjected to luciferase and β-gal assays. Luciferase activity, normalized to β-gal activity, was set to 100% for the DMSO-treated, pJ3H empty vector-transfected cells. Relative luciferase activity, normalized to β-gal activity, is expressed as mean ± SEM (n=6). Statistical significance was determined based on relative luciferase activity in pJ3H empty vector-transfected cells under each condition. Statistical significance was also determined based on the difference between relative luciferase activities in pJ3H empty vector- and pJ3H-AEBP1-transfected cells cultured in presence or absence of U0126. (Δ *n.s.* indicates that difference is not statistically significant). In a separate experiment, 10 μM U0126 was shown to almost completely inhibit Erk-1/2 phosphorylation *in vitro* (data not shown).

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A **PPRE-Luc.**

□ Vector
■ AEBP1

Δ *n.s.*

Δ *n.s.*

Relative Luciferase Activity (%)

400
350
300
250
200
150
100
50
0

DMSO   TRO   U0126   TRO & U0126

B **LXRE-Luc.**

□ Vector
■ AEBP1

Δ *n.s.*

Δ *n.s.*

Relative Luciferase Activity (%)

800
700
600
500
400
300
200
100
0

DMSO   T0901317   U0126   T0901317 & U0126

3.3.5 PPARγ1 and LXRα Repression by AEBP1 is DNA Binding-Dependent

Previously, AEBP1 was shown to be capable of repressing the aP2 gene in a process that requires binding to the AE-1 sequence within the aP2 promoter (Ro and Roncari, 1991). Indeed, the C-terminal DNA-binding domain truncation mutant form of AEBP1 (AEBP1$^{\Delta Sty}$) was shown to be incapable of binding the AE-1 sequence that full-length AEBP1 is capable of binding, and thus, unable to repress aP2 (Kim *et al.*, 2001). In order to examine whether DNA-binding by AEBP1 is essential for its ability to repress PPARγ1 and LXRα *in vitro*, two experimental approaches were followed. First, the ability of full-length AEBP1 and AEBP1$^{\Delta Sty}$ forms to transcriptionally repress PPARγ1 and LXRα was assessed. To that end, CHO cells were co-transfected with pGL3-PPARγ1-luciferase or pGL3-LXRα-luciferase as well as equivalent amounts of pJ3H-AEBP1, pJ3H-AEBP1$^{\Delta DLD}$, pJ3H-AEBP1$^{\Delta Sty}$, or pJ3H empty vector. As shown in Figure 18, luciferase reporter assays reveal that in contrast to full-length AEBP1, AEBP1$^{\Delta Sty}$ form is incapable of repressing PPARγ1 or LXRα expression in CHO transfectants, while DLD deletion (i.e. AEBP1$^{\Delta DLD}$) has no effect on PPARγ1 or LXRα transcriptional repression by AEBP1. This indicates that deletion of the DNA-binding domain of AEBP1 completely eliminates the ability of AEBP1 to transcriptionally repress the expression of PPARγ1 and LXRα *in vitro*.

To further confirm these findings, the ability of AEBP1 to repress PPARγ1 and LXRα expression was assessed using luciferase reporter constructs that harbor (pGL3-PPARγ1 and pGL3-LXRα) or lack (pGL3-PPARγ1-M1 and pGL3-LXRα-M3) the putative AEBP1-binding, AE-1 homologous sites within PPARγ1 and LXRα promoters, respectively (the deleted sequences are underlined in Figure 15A). To that end, CHO cells were co-transfected with pGL3-PPARγ1, pGL3-PPARγ1-M1, pGL3-LXRα, or pGL3-LXRα-M3 luciferase reporters along with pJ3H-AEBP1 or pJ3H empty vector. Luciferase reporter assays clearly demonstrate that AEBP1 is incapable of repressing PPARγ1 or LXRα when the putative AEBP1-binding sites within the promoter regions of these two genes are completely deleted (Figures 19 A & B, respectively). Thus, deletion of the putative AEBP1-binding sites within PPARγ1 and LXRα promoters completely

eliminates transcriptional repression of these two genes by AEBP1 *in vitro*. Taken together, these findings strongly indicate that AEBP1 is capable of directly repressing PPARγ1 and LXRα expression in a DNA binding-dependent fashion.
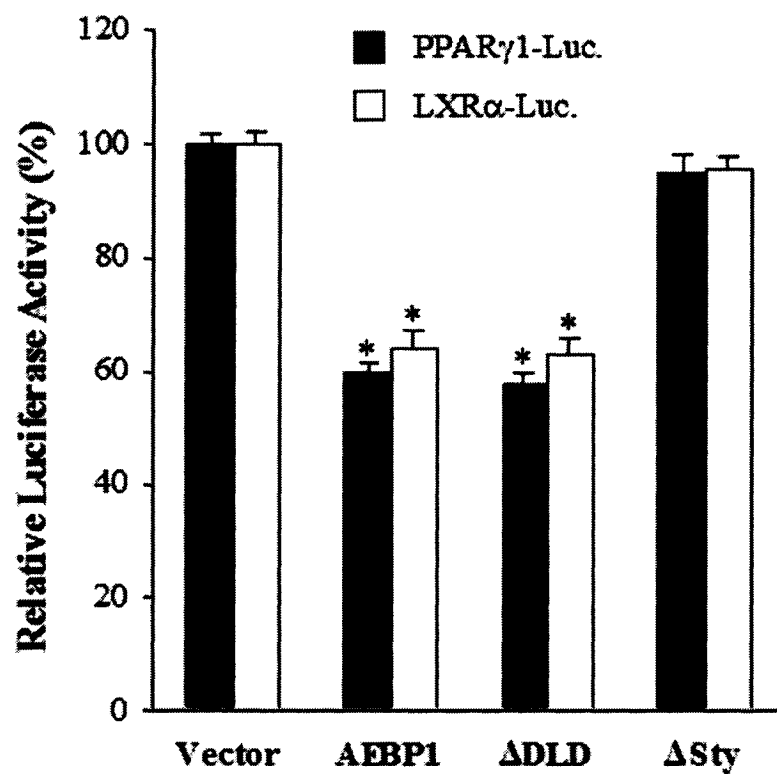
Figure 18. Deletion of DNA-Binding Domain, but not DLD, Impedes AEBP1-Mediated PPARγ1 and LXRα Transcriptional Repression. CHO cells were transiently transfected with 200 ng pGL3-hPPARγ1-luciferase or pGL3-mLXRα-luciferase using polyfect. Cells were co-transfected with 20 ng pHermes-lacZ, which expresses β-galactosidase, along with 25 ng pJ3H empty vector, pJ3H-AEBP1, pJ3H-AEBP1$^{\Delta DLD}$, or pJ3H-AEBP1$^{\Delta Sty}$. Forty-eight hours post transfection, cells were lysed and subjected to luciferase and β-gal assays. Relative luciferase activity, normalized to β-gal activity, is expressed as mean ± SEM (n=6). Statistical significance was determined based on relative luciferase activity in pJ3H empty vector-transfected cells.

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Figure 19. AEBP1-Mediated PPARγ1 and LXRα Transcriptional Repression Requires Specific Sequences within PPARγ1 and LXRα Promoters. CHO cells were transiently transfected with 200 ng pGL3-hPPARγ1-luciferase or pGL3-PPARγ1-M1-luciferase *(A)*, as well as pGL3-mLXRα-luciferase or pGL3-LXRα-M3-luciferase *(B)* using polyfect. Cells were co-transfected with 20 ng pHermes-lacZ, which expresses β-galactosidase, along with 25 ng pJ3H empty vector or pJ3H-AEBP1. Forty-eight hours post transfection, cells were lysed and subjected to luciferase and β-gal assays. Relative luciferase activity, normalized to β-gal activity, is expressed as mean ± SEM (n=6). Normalized luciferase activity represented by wildtype or mutated promoter sequences was set to 100% in pJ3H empty vector transfection samples. Statistical significance was determined based on relative luciferase activity in pJ3H empty vector-transfected cells.

A
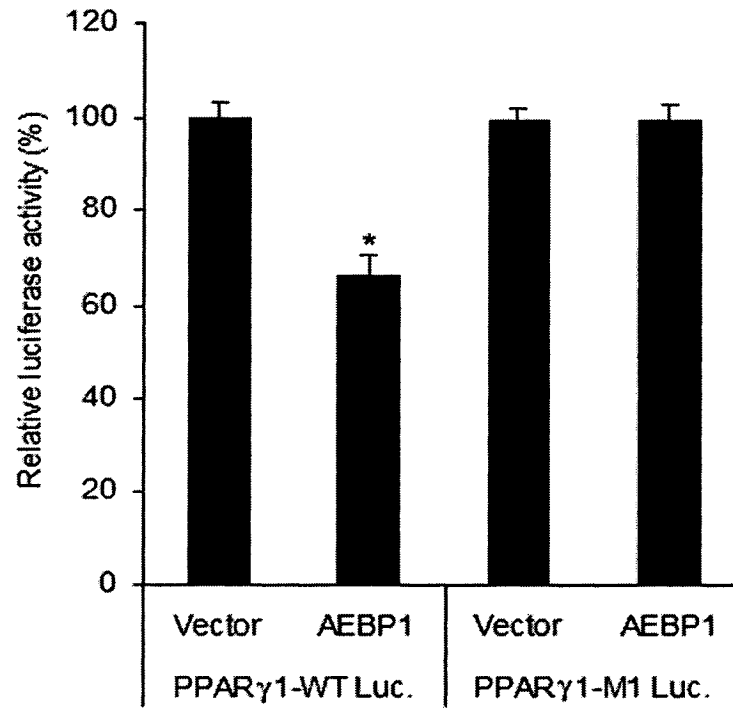
B

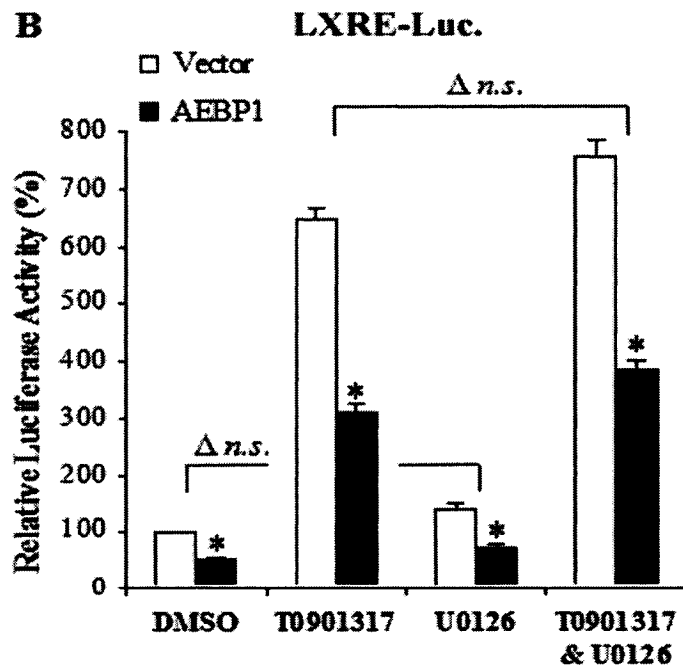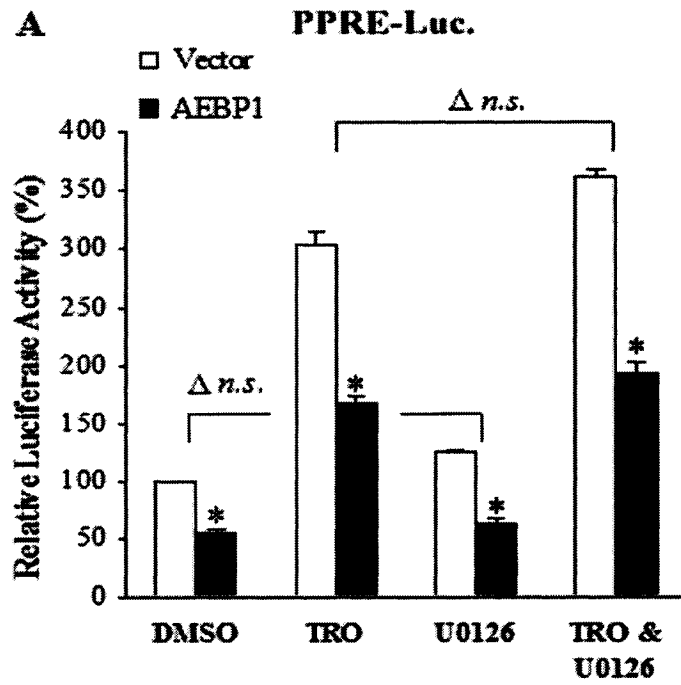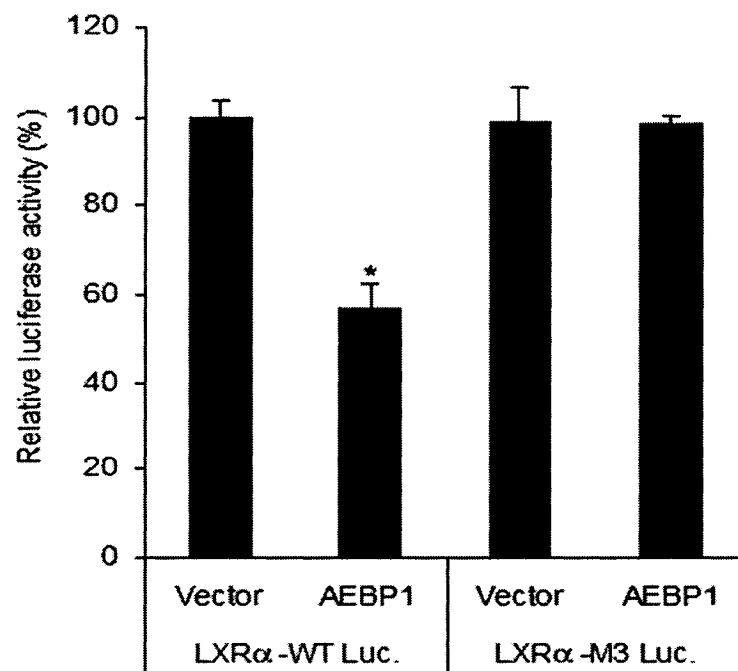### 3.3.6 AEBP1 Represses PPARγ1 and LXRα Expression in Macrophages

In vitro data strongly suggests that AEBP1 functions as a specific transcriptional repressor that is capable of inhibiting the expression and activity of PPARγ1 and LXRα in a dose-responsive, DNA binding-dependent manner (Figures 15-19). In order to examine a functional role of AEBP1-mediated transcriptional repression of PPARγ1 and LXRα in macrophages, total RNA was extracted from macrophages isolated from HFD-fed AEBP1^{TG}, AEBP1^{NT}, AEBP1$^{+/+}$, and AEBP1$^{-/-}$ mice, and was subjected to RT-PCR to assess PPARγ1 and LXRα transcript levels. As shown in Figures 20 A & B, AEBP1^{TG} macrophages express 2-3 fold lower levels of PPARγ1 and LXRα mRNA compared to AEBP1^{NT} macrophages. In contrast, AEBP1$^{-/-}$ macrophages express 3-4 fold higher levels of PPARγ1 and LXRα mRNA compared to AEBP1$^{+/+}$ macrophages (Figures 20 C & D).

To examine whether AEBP1 is capable of modulating PPARγ1 and LXRα protein levels in macrophages, total protein extracts were obtained from AEBP1^{TG}, AEBP1^{NT}, AEBP1$^{+/+}$, and AEBP1$^{-/-}$ macrophages of HFD-fed mice, and were subsequently subjected to SDS-PAGE followed by immunoblotting. Densitometric analysis of Western blots clearly revealed that AEBP1 expression negatively correlates with PPARγ1 and LXRα protein levels, based on actin normalization (Figure 21). Noteworthy, this data is obtained with macrophages isolated from young (12 wk) and old (32 wk) mice, females and males, as well as chow- and HFD-fed mice. These results strongly indicate that PPARγ1 and LXRα are downstream targets of AEBP1's transcriptional repression function not only *in vitro*, but also *in vivo*.

Figure 20. AEBP1 Transcriptionally Represses PPARγ1, LXRα, and Their Downstream Target Genes in Primary Macrophages. Total RNA samples were obtained from macrophages isolated from 32-wk old, HFD-fed AEBP1^{TG} and AEBP1^{NT} females *(A & B)*, as well as AEBP1$^{+/+}$ and AEBP1$^{-/-}$ females *(C & D)*. 1.0 μg total RNA was reverse transcribed, and the indicated transcripts were amplified by PCR using specific primers (Table 4). Densitometric analysis based on β-actin level in AEBP1^{TG} and AEBP1^{NT} macrophages *(B)*, as well as AEBP1$^{+/+}$ and AEBP1$^{-/-}$ macrophages *(D)* is shown. Normalized mRNA level is expressed as mean ± SEM (n=6). Statistical significance was determined relative to mRNA level in AEBP1^{NT} *(B)* or AEBP1$^{+/+}$ *(D)* macrophages.

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Figure 21. PPARγ1 and LXRα Protein Levels Negatively Correlate with AEBP1 Expression in Primary Macrophages. Whole cell protein extracts obtained from macrophages isolated from 32-wk old, HFD-fed AEBP1^{TG} and AEBP1^{NT} females (*A & B*), as well as AEBP1$^{+/+}$ and AEBP1$^{-/-}$ females (*C & D*) were subjected to SDS-PAGE. Nitrocellulose membranes were immunoblotted for AEBP1, PPARγ1, LXRα, and actin. Densitometric analysis based on actin level in AEBP1^{TG} and AEBP1^{NT} macrophages (*B*), as well as AEBP1$^{+/+}$ and AEBP1$^{-/-}$ macrophages (*D*) is shown. Normalized protein level is expressed as mean ± SEM (n=6). Statistical significance was determined relative to protein level in AEBP1^{NT} *(B)* or AEBP1$^{+/+}$ *(D)* macrophages.
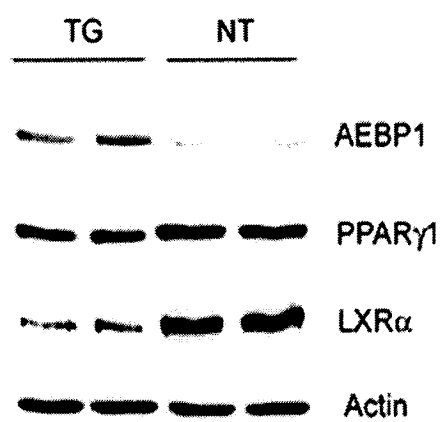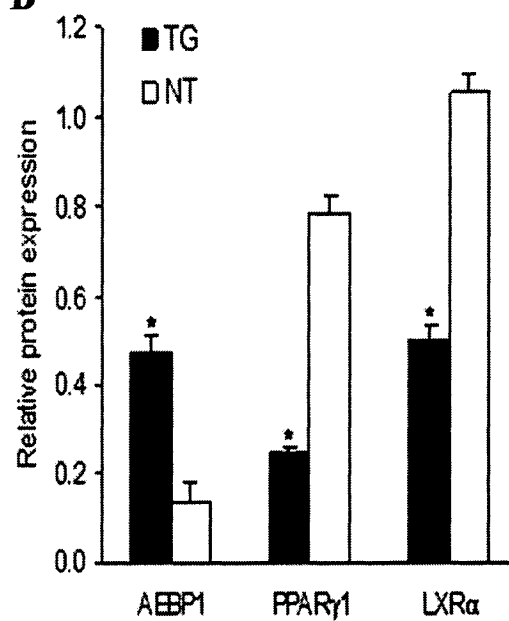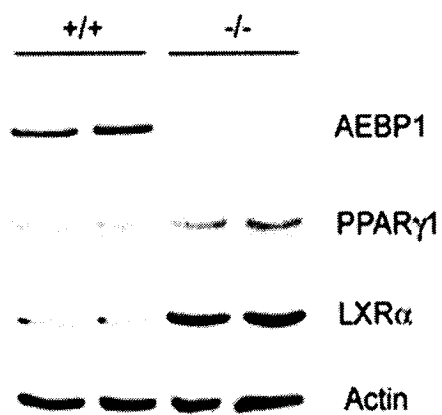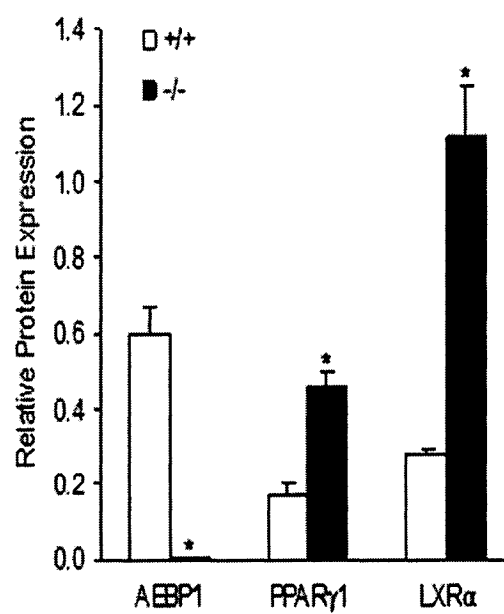
3.4 Inhibited Expression of PPARγ1/LXRα Downstream Target Genes by AEBP1

Since the expression of ABCA1 and ABCG1 was shown to be enhanced by PPARγ1 activation via transcriptional up-regulation of LXRα (Chawla *et al.*, 2001b; Costet *et al.*, 2000; Venkateswaran *et al.*, 2000b), and since ApoE has been shown to be a downstream target of PPARγ1 (Galitto *et al.*, 2001) and LXRα (Laffitte *et al.*, 2001a; Laffitte *et al.*, 2001b), the expression of these prominent players in cholesterol efflux, as well as that of CD36, was determined in macrophages that over-express or lack AEBP1. To this end, total RNA was isolated from peritoneal macrophages isolated from AEBP1^{TG}, AEBP1^{NT}, AEBP1$^{+/+}$, and AEBP1$^{-/-}$ mice, and RT-PCR was performed using specific primers recognizing AEBP1, PPARγ1, LXRα, ABCA1, ABCG1, ApoE, CD36, and β-actin transcripts (Table 4). Semi-quantitative RT-PCR analysis reveals that ABCA1, ABCG1, and ApoE mRNA levels are significantly reduced (2-3 fold) in AEBP1^{TG} macrophages compared to AEBP1^{NT} macrophages (Figures 20 A & B). In contrast, AEBP1$^{-/-}$ macrophages express significantly higher levels (2-3 fold) of ABCA1, ABCG1, and ApoE compared to AEBP1$^{+/+}$ macrophages (Figures 20 C & D). As for CD36, AEBP1 over-expression slightly, but significantly, inhibits CD36 expression (Figures 20 A & B), while AEBP1 ablation results in increased CD36 expression (Figures 20 C & D). Notably, peritoneal macrophages isolated from AEBP1^{TG} males and females display no differential pattern of AEBP1-mediated down-regulation of ABCA1, ABCG1, ApoE, and CD36 (data not shown), suggesting no gender-specific differences involved in this specific AEBP1-mediated regulation of macrophages cholesterol homeostasis mediators. Noteworthy, this data is obtained with macrophages isolated from young (12 wk) and old (32 wk) mice, as well as chow- and HFD-fed mice. Thus, these findings coupled with those indicating that AEBP1 is capable of suppressing the transcriptional activity of PPARγ1 and LXRα *in vitro* strongly suggest that AEBP1-mediated transcriptional repression of PPARγ1 and LXRα is accompanied by a significant reduction of ABCA1, ABCG1, and ApoE levels in macrophages.

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3.5 AEBP1 Promotes Foam Cell Formation

3.5.1 Qualitative Assessment of Lipid Accumulation in Macrophages

The fact that AEBP1 serves as a potent transcriptional repressor that down-regulates the expression of PPARγ1, LXRα, ABCA1, ABCG1, and ApoE, key mediators of cholesterol efflux in macrophages, prompted me to expect impeded cholesterol efflux from macrophages that over-express AEBP1, while cholesterol efflux should be more efficient in macrophages that lack AEBP1. Indeed, qualitative microscopic analysis of ORO-stained macrophages isolated from HFD-fed mice and cultured for 72 hr under normal culture conditions indicates that AEBP1^{TG} macrophages accumulate considerable amounts of lipids in their cytoplasmic compartments compared to AEBP1^{NT} counterparts (Figure 22A). This is a likely indication of disrupted cholesterol efflux that accompanies AEBP1 over-expression in macrophages. Notably, AEBP1$^{+/+}$ and AEBP1$^{-/-}$ displayed no detectable levels of lipids upon ORO staining (Figure 22A). Importantly, the ability of peritoneal macrophages isolated from AEBP1^{TG} mice to accumulate higher amounts of lipids was not gender- or age-specific (data not shown).

3.5.2 Assessment of Cholesterol Efflux Efficiency in Macrophages

For quantitative assessment of macrophage cholesterol efflux efficiency, macrophages isolated from HFD-fed AEBP1^{TG}, AEBP1^{NT}, AEBP1$^{+/+}$, and AEBP1$^{-/-}$ mice were subjected to ^{3}H-cholesterol efflux assay. Macrophages were labeled with ^{3}H-cholesterol for 24 hr, and apolipoprotein AI (ApoAI) was used to induce cholesterol efflux for 6 hr. Cholesterol efflux analysis clearly indicates that AEBP1^{TG} macrophages are significantly less efficient than their AEBP1^{NT} counterparts in terms of ApoAI-specific cholesterol efflux (0.5% efflux in AEBP1^{TG} macrophages vs. 2.5% efflux in AEBP1^{NT} macrophages) (Figure 22B). Consistently, AEBP1 ablation in macrophages is accompanied by a significant increase (3.5 fold) in cholesterol efflux efficiency (2% efflux in AEBP1$^{+/+}$ macrophages versus 7% efflux in AEBP1$^{-/-}$ macrophages) (Figure 22B). Importantly, no gender- or age-specific differences with regard to cholesterol

efflux efficiency were observed among the four different genotypes (data not shown). Hence, AEBP1^{TG} macrophages are potentially capable of presenting themselves as lipid-engorged foam cell precursors *in vivo*. Collectively, PPARγ1 and LXRα repression by AEBP1 in macrophages leads to significant reduction in the expression levels of cholesterol efflux mediators, which directly correlates with diminished macrophage cholesterol efflux and foam cell formation.

Figure 22. AEBP1 Impedes Macrophage Cholesterol Efflux and Promotes Foam Cell Formation. *(A)* Peritoneal macrophages isolated from 32-wk old, HFD-fed $AEBP1^{TG}$, $AEBP1^{NT}$, $AEBP1^{+/+}$, and $AEBP1^{-/-}$ females were cultured in standard growth medium for 72 hr. Subsequently, macrophages were stained with oil red O and counter-stained with Mayer's hematoxylin to allow lipid visualization. ORO-stained macrophages were examined under a Nikon Eclipse E600 microscope attached to a Nikon Coolpix 990 camera. (lipid = red/pink; nuclei = blue). These images are representative of at least 50 images obtained per group. *(B)* Peritoneal macrophages isolated from 32-wk old, HFD-fed $AEBP1^{TG}$, $AEBP1^{NT}$, $AEBP1^{+/+}$, and $AEBP1^{-/-}$ females were subjected to ^{3}H-cholesterol efflux assay. Data is normalized based on macrophage cholesterol efflux in absence of ApoAI (i.e. in presence of BSA alone). ApoAI specific efflux is expressed as mean ± SEM (n=12). As shown, statistical significance was determined relative to cholesterol efflux by $AEBP1^{NT}$ and $AEBP1^{+/+}$ macrophages.

A

AEBP1^{TG}

AEBP1^{NT}
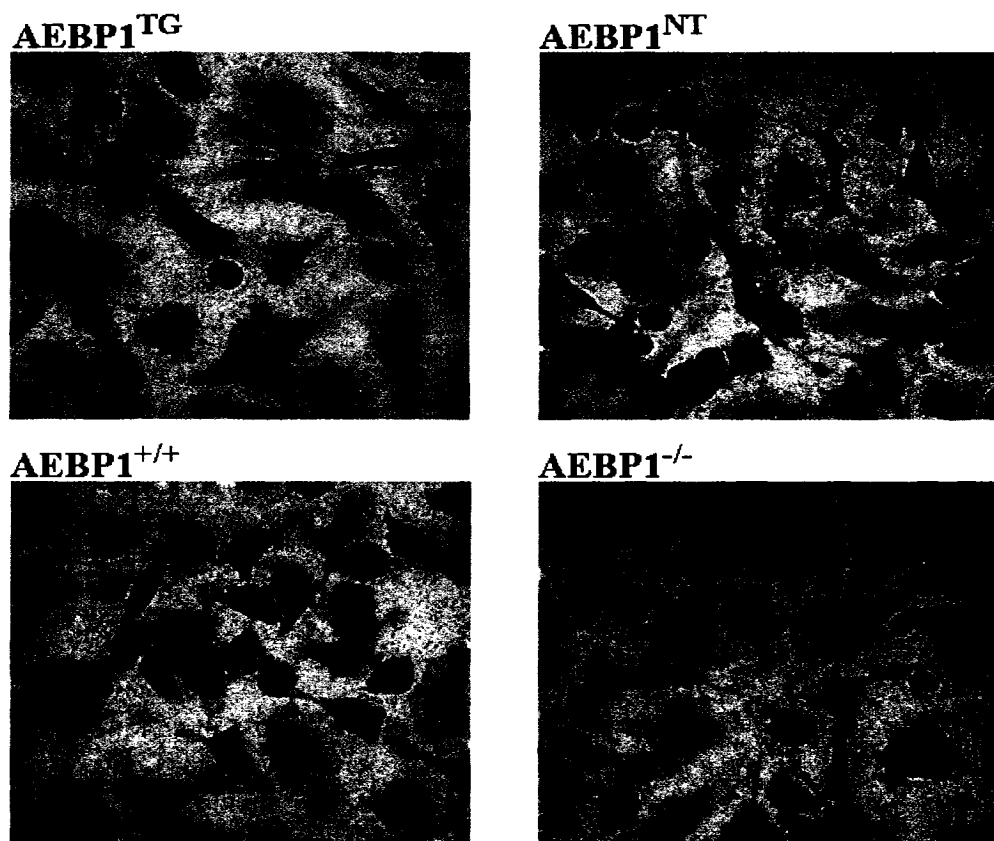
AEBP1^{+/+}

AEBP1^{-/-}

B
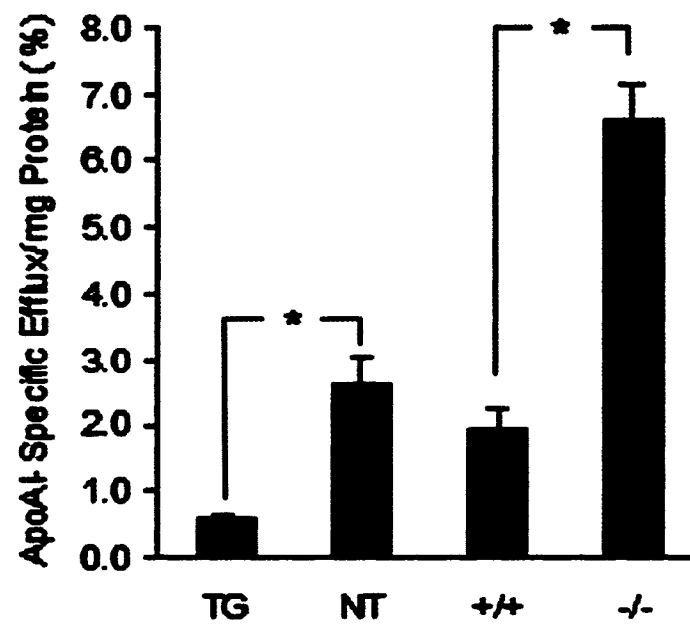
3.6 AEBP1 Enhances Macrophage Inflammatory Responsiveness

3.6.1 Assessment of IL-6 and TNFα Production by Macrophages

Due to their imperative roles in macrophage inflammatory responsiveness and atherogenesis, the expression pattern of IL-6, TNFα, MCP-1, and iNOS in macrophages that over-express or lack AEBP1 was evaluated. To assess IL-6 and TNFα production by macrophages, ELISA was performed on supernatants obtained from macrophages isolated from HFD-fed AEBP1^{TG}, AEBP1^{NT}, AEBP1$^{+/+}$, and AEBP1$^{-/-}$ mice and cultured in fresh medium or medium supplemented with LPS, IFNγ, or a combination of LPS plus IFNγ. ELISA analysis reveals that un-stimulated and stimulated AEBP1^{TG} macrophages produce significantly higher levels of IL-6 and TNFα compared to AEBP1^{NT} macrophages (Figures 23 A & B, respectively). In agreement, AEBP1$^{-/-}$ macrophages produce significantly lower IL-6 and TNFα levels compared to AEBP1$^{+/+}$ macrophages under un-stimulatory and stimulatory conditions (Figures 23 C & D, respectively). Noteworthy, comparable data was obtained with macrophages isolated from young (12 wk) and old (32 wk) mice, females and males, as well as chow- and HFD-fed mice (data not shown).

Figure 23. AEBP1 Enhances IL-6 and TNFα Production by Primary Macrophages. Peritoneal macrophages isolated from 32-wk old, HFD-fed AEBP1^{TG}, AEBP1^{NT}, AEBP1$^{+/+}$, and AEBP1$^{-/-}$ females were cultured in medium alone, or medium supplemented with 10 ng/ml LPS, 2 U/ml IFNγ, or a combination of 10 ng/ml LPS plus 2 U/ml IFNγ for 48 hr or 12 hr (IL-6 and TNFα, respectively). Subsequently, supernatants were harvested and subjected to ELISA to measure IL-6 and TNFα concentration. IL-6 concentration in supernatants obtained from AEBP1^{TG} and AEBP1^{NT} macrophages *(A)* as well as AEBP1$^{+/+}$, and AEBP1$^{-/-}$ macrophages *(C)* is shown. TNFα concentration in supernatants obtained from AEBP1^{TG} and AEBP1^{NT} macrophages *(B)* as well as AEBP1$^{+/+}$, and AEBP1$^{-/-}$ macrophages *(D)* is shown. IL-6 and TNFα concentrations in supernatants obtained from AEBP1^{NT} and AEBP1$^{+/+}$ macrophages cultured in medium alone were set to 100%. This data is representative of 4 experiments and is expressed as mean ± SEM (n=12-18). Statistical significance was determined relative to IL-6 and TNFα concentrations in supernatants obtained from AEBP1^{NT} *(A & B)* and AEBP1$^{+/+}$ *(C & D)* macrophages cultured in medium alone.

150
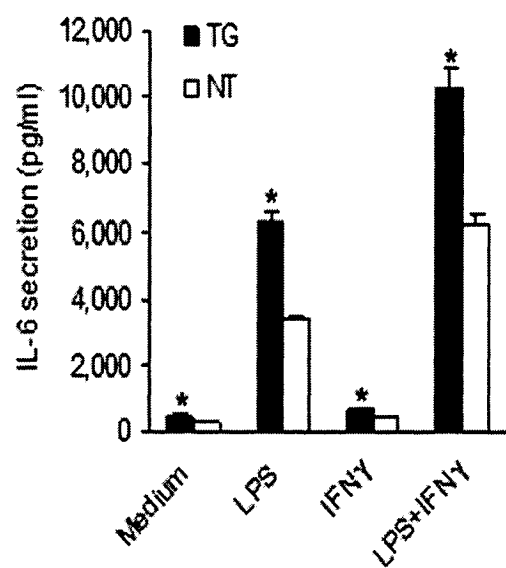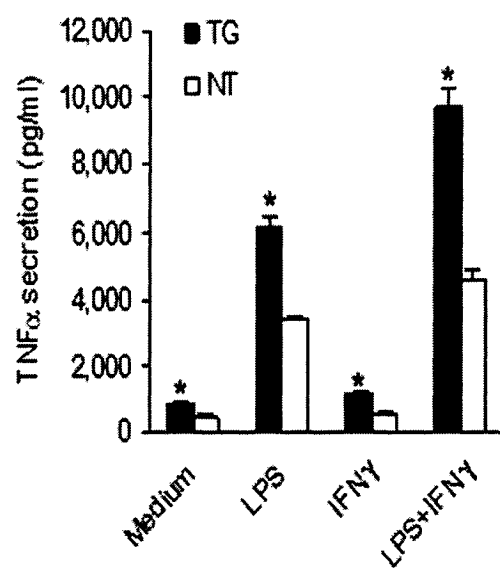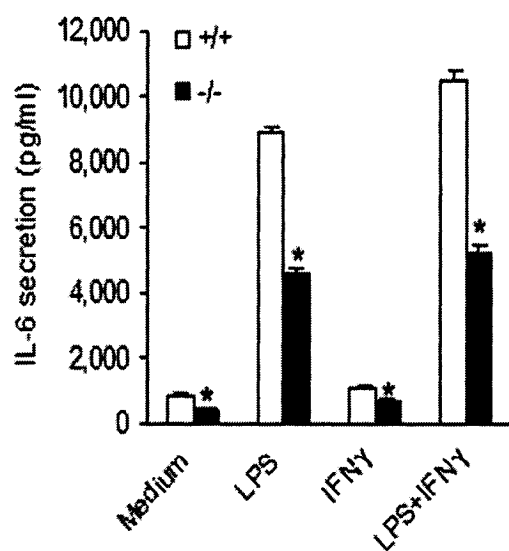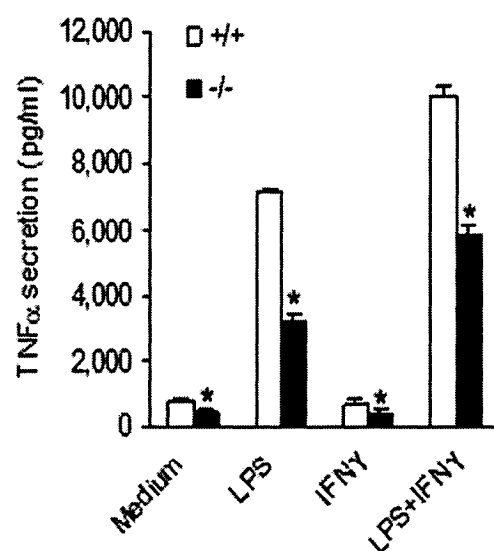
3.6.2 Assessment of MCP-1 and iNOS Expression in Macrophages

In order to assess the ability of AEBP1 to modulate MCP-1 and iNOS expression in macrophages, total RNA was isolated from macrophages isolated from HFD-fed AEBP1^{TG}, AEBP1^{NT}, AEBP1$^{+/+}$, and AEBP1$^{-/-}$ mice, and was subjected to RT-PCR analysis using specific primers (Table 4). Semi-quantitative RT-PCR analysis illustrates that AEBP1^{TG} macrophages express significantly elevated levels ($\sim$ 3 fold) of MCP-1 and iNOS compared to AEBP1^{NT} macrophages (Figures 24 A & B), while AEBP1$^{-/-}$ macrophages display significantly decreased ($\sim$ 3 fold) MCP-1 and iNOS expression compared to their AEBP1$^{+/+}$ counterparts (Figures 24 C & D). Again, it is noteworthy that modulation of macrophage inflammatory responsiveness by AEBP1 is not gender-specific (data not shown). These findings clearly suggest that AEBP1 augments the inflammatory responsiveness in macrophages under un-stimulatory and stimulatory conditions, enhancing the expression of major pro-inflammatory mediators that are known to be critically involved in various inflammatory conditions and the development of atherosclerosis. Once again, comparable data was obtained with macrophages isolated from young (12 wk) and old (32 wk) mice, females and males, as well as chow- and HFD-fed mice (data not shown).

Figure 24. AEBP1 Enhances MCP-1 and iNOS Expression in Primary Macrophages. Total RNA samples were obtained from macrophages isolated from 32-wk old, HFD-fed AEBP1^{TG} and AEBP1^{NT} females (*A & B*), as well as AEBP1$^{+/+}$ and AEBP1$^{-/-}$ females (*C & D*). 1.0 μg total RNA was reverse transcribed, and MCP-1 and iNOS transcripts were amplified by PCR using specific primers (Table 4). Densitometric analysis based on β-actin level in AEBP1^{TG} and AEBP1^{NT} macrophages (*B*), as well as AEBP1$^{+/+}$ and AEBP1$^{-/-}$ macrophages (*D*) is shown. Normalized mRNA level is expressed as mean ± SEM (n=12-18). Statistical significance was determined relative to mRNA level in AEBP1^{NT} *(B)* or AEBP1$^{+/+}$ *(D)* macrophages.

A

TG   NT

MCP-1

iNOS

β-actin

B

C

+/+   -/-

MCP-1

iNOS

β-actin

D

154
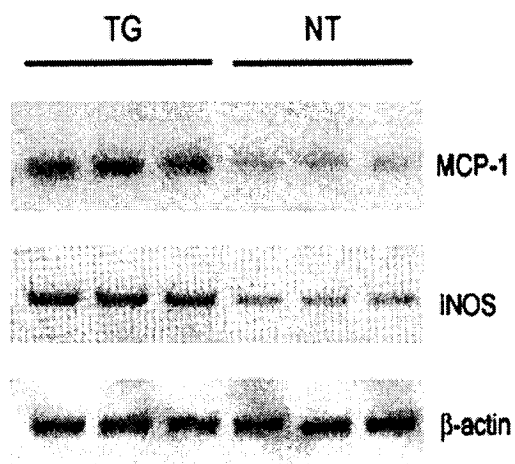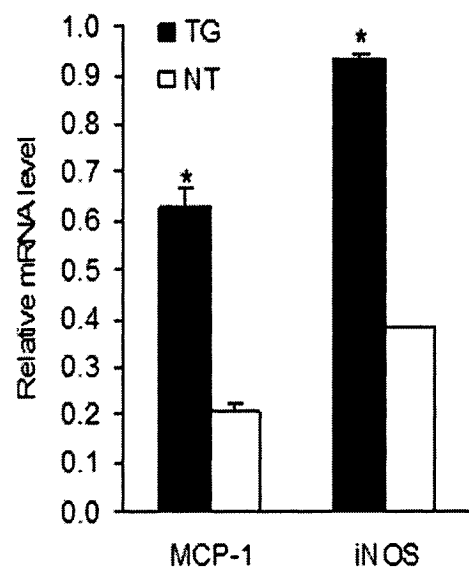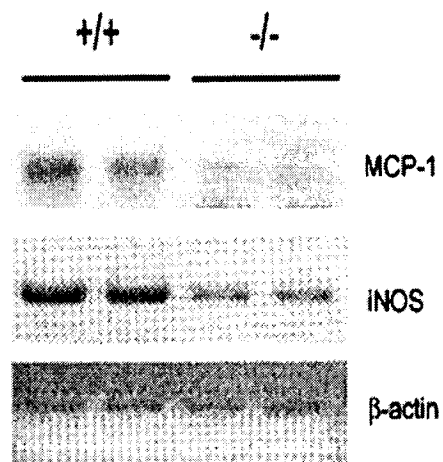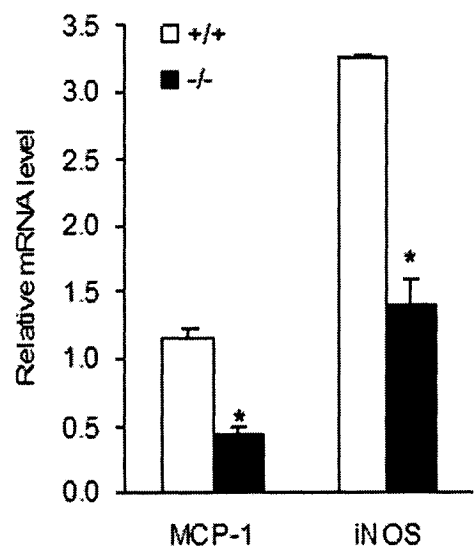
3.7 AEBP1 Promotes Hyperlipidemia and Atherosclerotic Lesion Formation

3.7.1 AEBP1 Induces Hyperlipidemia in Mice

The fact that AEBP1 is capable of causing a cholesterol homeostasis imbalance in macrophages prompted examination of the serum lipid profile in AEBP1^{TG} and AEBP1$^{-/-}$ mice. In order to assess total cholesterol and triglyceride serum levels, serum samples obtained from 32-wk old, HFD-fed AEBP1^{TG}, AEBP1^{NT}, AEBP1$^{+/+}$, and AEBP1$^{-/-}$ females and males were analyzed by gas chromatography in collaboration with Dr. Roger McLeod (Dalhousie University, Nova Scotia, Canada). Figure 25 clearly shows that AEBP1^{TG} females have significantly higher serum cholesterol and triglyceride levels compared to AEBP1^{NT} females (cholesterol, 179 ± 6.2 and 128 ± 7.3 mg/dL; triglyceride, 143 ± 12.3 and 100 ± 9.3 mg/dL, respectively). Interestingly, although AEBP1^{TG} males have higher serum cholesterol and triglyceride levels compared to AEBP1^{NT} males, this difference did not reach statistical significance (Figure 25). As for the effect of AEBP1 ablation on total cholesterol and triglyceride levels in plasma, it seems that while serum triglyceride levels are significantly lower in AEBP1$^{-/-}$ mice compared to AEBP1$^{+/+}$ mice, serum cholesterol levels are only significantly lower in AEBP1$^{-/-}$ males, not females (H.S. Ro and colleagues, unpublished). Thus, serum lipid analysis suggests that AEBP1 has the potential to modulate cholesterol and triglyceride levels in mice in a gender-specific manner.
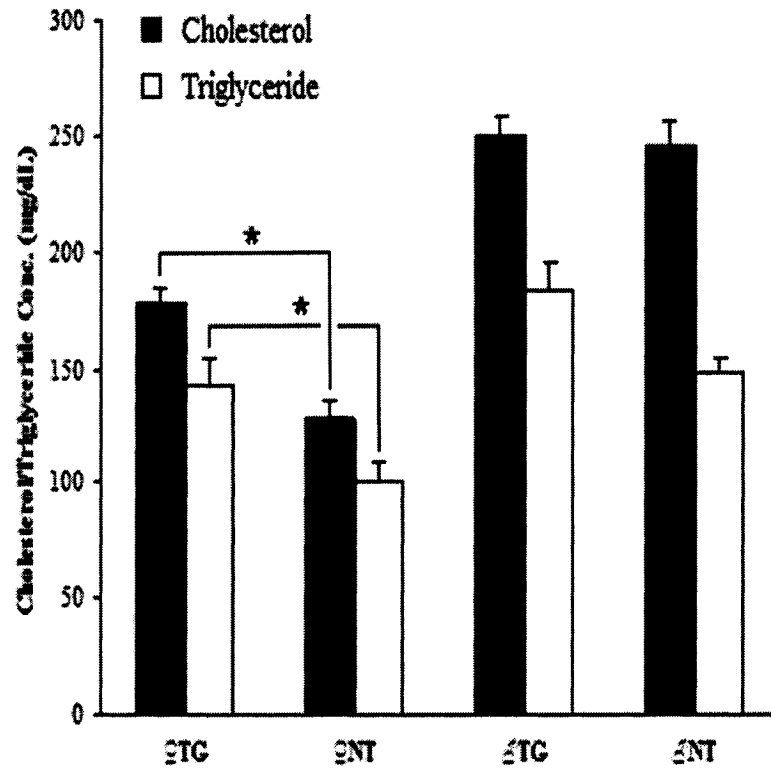
Figure 25. AEBP1 Promotes Hyperlipidemia in Mice.
Whole blood samples were obtained from 32-wk old, HFD-fed AEBP1^{TG} and AEBP1^{NT} mice to measure total cholesterol and triglyceride serum levels by gas chromatography (GC). Concentration of total cholesterol and triglycerides is expressed as mg/dL and data is demonstrated as mean ± SEM (n=3-5). Statistical significance was determined relative to cholesterol or triglyceride serum level in AEBP1^{NT} mice for each gender.

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3.7.2 AEBP1 Promotes Atherosclerotic Lesion Development in Mice

3.7.2.1 Detection and Morphological Analysis of Atherosclerotic Lesions

Thus far, AEBP1 is shown to be capable of (1) impeding macrophage cholesterol efflux and thus promoting foam cell formation, (2) increasing cholesterol and triglyceride plasma levels in a gender-specific fashion, and (3) inducing macrophage inflammatory responsiveness. Such experimental findings suggest that AEBP1 may play a potential role in atherosclerotic lesion formation in mice. To investigate this likely possibility, proximal aortic cryosections obtained from young (12 wk) and old (32 wk), chow- and HFD-fed $AEBP1^{TG}$, $AEBP1^{NT}$, $AEBP1^{+/+}$, and $AEBP1^{-/-}$ females and males were assessed for atherosclerotic lesion formation. To that end, aortic cryosections were stained with ORO and counter stained with Mayer's hematoxylin, a staining technique that allows detection of lipid-filled atherosclerotic lesions within the aortic intima. ORO-stained aortic cryosections were independently and blindly tested by three investigators, and observations were recorded accordingly. Data was gathered and tabulated in Table 6, which indicates that lipid-filled lesions were detected in the proximal aortae of 94% and 72% HFD-fed $AEBP1^{TG}$ females and males, respectively. Representative 100X and 400X images of ORO-stained aortic cryosections of 32-wk old, $AEBP1^{TG}$ and $AEBP1^{NT}$ females are shown in Figures 26 A-D. Most importantly, no lesions or histological abnormalities were observed in chow- or HFD-fed $AEBP1^{NT}$, $AEBP1^{+/+}$, or $AEBP1^{-/-}$ mice (Table 6, Figures 26 B & D). Interestingly, $AEBP1^{TG}$ mice do not develop atherosclerotic lesions when fed low-fat (4.5%)-containing chow diet.

Despite detection of lesions in young (12 wk) $AEBP1^{TG}$ mice, lesions were more advanced and easier to detect in older (32 wk) mice (Table 6). In fact, development of atherosclerosis is more prevalent in old HFD-fed $AEBP1^{TG}$ mice (females, 94%; males, 72%) compared to young HFD-fed $AEBP1^{TG}$ counterparts (females, 50%; males, 50%) (Table 6), suggesting a positive correlation between prevalence of atherogenesis and aging. Moreover, old HFD-fed $AEBP1^{TG}$ mice have significantly larger lesions compared to young HFD-fed $AEBP1^{TG}$ mice (see below).

Notably, atherosclerotic lesions detected in AEBP1^{TG} mice are distinct from those found in ApoE$^{-/-}$ and LDLR$^{-/-}$ mice. As shown in Figures 26 A & C, and in contrast to lesions found in ApoE$^{-/-}$ and LDLR$^{-/-}$ mice, AEBP1^{TG} mice develop lesions that are surrounded by a very thin, hard to characterize fibrous caps. Additionally, lesions detected in AEBP1^{TG} mice are not continuous along the endothelial monolayer. Rather, they are found at one site within the aortic lumen, while other sites are completely free of lesions (Figures 26 A & C). In comparison, lesions found in ApoE$^{-/-}$ and LDLR$^{-/-}$ mice seem to spread and form at multiple sites within the lumen. Yet, AEBP1^{TG} lesions have high lipid content, and they are cell-dense (Figures 26 A & C).

Table 6. The Effect of AEBP1 Genotype, Gender, Age, and Diet on Atherosclerotic Lesion Formation in Mice.

Gender & Genotype	Age (wk)	Diet	Total Number of Mice (n)	Lesion Prevalence (%)
♀ AEBP1^{TG}	12	Chow	4	-
♂ AEBP1^{TG}	12	Chow	4	-
♀ AEBP1^{NT}	12	Chow	3	-
♂ AEBP1^{NT}	12	Chow	3	-
♀ AEBP1^{TG}	32	Chow	18	-
♂ AEBP1^{TG}	32	Chow	9	-
♀ AEBP1^{NT}	32	Chow	8	-
♂ AEBP1^{NT}	32	Chow	8	-
♀ AEBP1^{TG}	12	HFD	8	50 (4 of 8)
♂ AEBP1^{TG}	12	HFD	8	50 (4 of 8)
♀ AEBP1^{NT}	12	HFD	9	-
♂ AEBP1^{NT}	12	HFD	8	-
♀ AEBP1^{TG}	32	HFD	35	94 (32 of 35)
♂ AEBP1^{TG}	32	HFD	32	72 (23 of 32)
♀ AEBP1^{NT}	32	HFD	32	-
♂ AEBP1^{NT}	32	HFD	29	-
♀ AEBP1$^{+/+}$	12	Chow	2	-
♂ AEBP1$^{+/+}$	12	Chow	2	-
♀ AEBP1$^{-/-}$	12	Chow	2	-
♂ AEBP1$^{-/-}$	12	Chow	2	-
♀ AEBP1$^{+/+}$	32	Chow	3	-
♂ AEBP1$^{+/+}$	32	Chow	4	-
♀ AEBP1$^{-/-}$	32	Chow	3	-
♂ AEBP1$^{-/-}$	32	Chow	3	-
♀ AEBP1$^{+/+}$	12	HFD	3	-
♂ AEBP1$^{+/+}$	12	HFD	2	-
♀ AEBP1$^{-/-}$	12	HFD	3	-
♂ AEBP1$^{-/-}$	12	HFD	3	-
♀ AEBP1$^{+/+}$	32	HFD	3	-
♂ AEBP1$^{+/+}$	32	HFD	2	-
♀ AEBP1$^{-/-}$	32	HFD	3	-
♂ AEBP1$^{-/-}$	32	HFD	3	-

Figure 26. AEBP1 Promotes Diet-Induced Atherosclerosis in Mice. To assess lipid-filled lesion formation in mice, aortic cryosections obtained from 12- and 32-wk old, chow- and HFD-fed AEBP1^{TG} and AEBP1^{NT} mice were stained with oil red O. Representative images of ORO-stained aortic cryosections from 32-wk old, HFD-fed AEBP1^{TG} *(A & C)* and AEBP1^{NT} *(B & D)* females are shown at 100X *(A & B)* and 400X *(C & D)* magnification. Cell nuclei are evident in the lipid-filled atherosclerotic lesions detected in the proximal aortae of AEBP1^{TG} mice. No such lesions are apparent in the proximal aortae of AEBP1^{NT} mice. For detection of intimal macrophages/foam cells, aortic cryosections obtained from the same mice were immunostained with anti-MOMA-2 (1:80 dilution). Representative images of anti-MOMA-2-immunostained aortic cryosections from 32-wk old, HFD-fed AEBP1^{TG} *(E & G)* and AEBP1^{NT} *(F & H)* females are shown at 100X *(E & F)* and 400X *(G & H)* magnification. 3-35 mice per group were examined (refer to Table 6 for more details).

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AEBP1^{TG}   AEBP1^{NT}
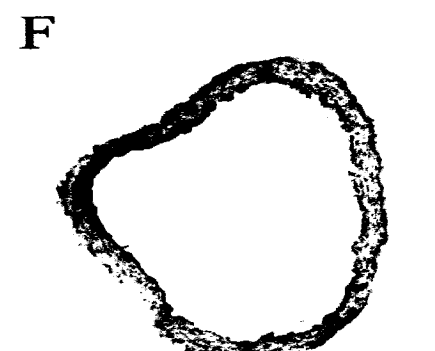
3.7.2.2 Determination of Mean Atherosclerotic Lesion Area

Besides the observed effects of gender and age on the prevalence of atherosclerotic lesion formation in AEBP1^{TG} mice (Table 6), I assessed whether gender and/or age has any significant effects on lesion size in those mice. To that end, microscopic images of ORO-stained aortic cryosections with detectable lesions found in young and old, HFD-fed AEBP1^{TG} females and males were captured, and mean lesion area was determined using ImageJ analytical software (free download from http://rsb.info.nih.gov/ij/). Computational imaging analysis revealed that there does not seem to be a significant difference in mean atherosclerotic lesion area when AEBP1^{TG} females and AEBP1^{TG} males are compared at a young age (3.26 ± 0.55% and 3.09 ± 0.38%, respectively) (Figure 27 or Table 7). Also, there is no significant difference with regard to mean atherosclerotic lesion area between young and old AEBP1^{TG} males (3.09 ± 0.38% and 3.00 ± 0.39%, respectively) (Figure 27 or Table 7). However, old AEBP1^{TG} females develop significantly larger lesions compared to their young AEBP1^{TG} female counterparts (6.76 ± 0.88% and 3.26 ± 0.55%, respectively) (Figure 27 or Table 7). Of course, the mean atherosclerotic lesion area is 0.00 ± 0.0% in AEBP1^{NT} females and males, which develop no detectable lesions (Figure 27 or Table 7). These findings indicate that in the AEBP1^{TG} murine model, although females and males develop atherosclerotic lesions that are comparable in size at a young age, such lesions tend to progress and advance more significantly in females, but not in males, as they age. Collectively, these findings suggest that compared to AEBP1^{TG} males, AEBP1^{TG} females are not only more prone to develop atherosclerotic lesions, but they also develop significantly larger lesions as they age.

Table 7. The Effects of Gender and Age on Atherosclerotic Lesion Size in AEBP1^{TG} Mice.

Gender & Genotype	Age (wk)	Diet	Total Number of Sections/Mice (n)	Mean Lesion Area (%)
♀ AEBP1^{TG}	12	HFD	14/4	3.26 ± 0.55
♂ AEBP1^{TG}	12	HFD	15/4	3.09 ± 0.38
♀ AEBP1^{NT}	12	HFD	11/4	0.00 ± 0.00
♂ AEBP1^{NT}	12	HFD	11/4	0.00 ± 0.00
♀ AEBP1^{TG}	32	HFD	28/7	6.76 ± 0.88
♂ AEBP1^{TG}	32	HFD	15/4	3.00 ± 0.39
♀ AEBP1^{NT}	32	HFD	13/4	0.00 ± 0.00
♂ AEBP1^{NT}	32	HFD	12/3	0.00 ± 0.00
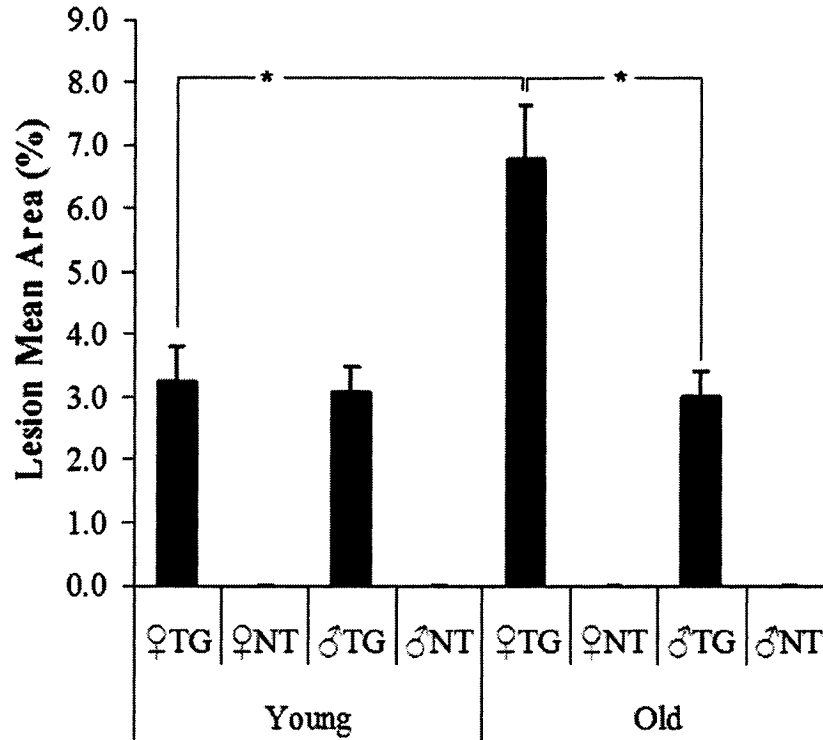
Figure 27. Assessment of Mean Atherosclerotic Lesion Area. Captured images of ORO-stained aortic cryosections from 12- and 32-wk old, HFD-fed AEBP1^{TG} and AEBP1^{NT} mice were assessed. For each mouse, 6-8 images were examined for mean atherosclerotic lesion area determination as described in section 2.11.3. Mean atherosclerotic lesion area is expressed as a percentage of the total aortic lumen area occupied by lesions. Data is expressed as mean ± SEM (n=11-28) (refer to Table 7 for more details). Statistical significance was determined relative to mean atherosclerotic lesion area between different gender and age groups.

3.7.2.3 Detection of Intimal Macrophages within Atherosclerotic Lesions

Atherosclerotic lesions detected in AEBP1^{TG} mice are characterized by the presence of cells that occupy the major size of such lipid-filled lesions (Figures 26 A & C). Since foam cells are known to accumulate in atherosclerotic lesions, immunostaining with anti-MOMA-2, a specific marker for monocytes/macrophages (Kraal *et al.*, 1987), was performed on serial aortic cryosections obtained from 12- and 32-wk old, HFD-fed AEBP1^{TG} and AEBP1^{NT} mice that develop atherosclerotic lesions to confirm the presence of foam cells in such lesions. As shown in Figures 26 E & G, MOMA-2 immunostaining revealed that positive staining co-localizes with the lipid-filled lesions detected in AEBP1^{TG} aortae (Representative 100X and 400X images of MOMA-2-immunostained aortic cryosections of 32-wk old, AEBP1^{TG} and AEBP1^{NT} females are shown). Importantly, no such positive staining was detected in MOMA-2 immunostained aortic cryosections obtained from age-matched, HFD-fed AEBP1^{NT} mice (Figures 26 F & H). Apparently, atherosclerotic lesions detected in AEBP1^{TG} mice are densely populated with foam cells.

3.8 Assessment of AEBP1 Atherogenic Effects in ApoE$^{-/-}$ Mice

Due to the critical role of AEBP1 in impeding macrophage cholesterol efflux, enhancing macrophage inflammatory responsiveness, and promoting atherosclerotic lesion formation, it is conceivable that AEBP1 deficiency in ApoE$^{-/-}$ mice (i.e. generation of AEBP1$^{-/-}$/ApoE$^{-/-}$ mice) will yield positive outcomes with regard to prevention and/or retardation of atherosclerotic lesion formation. In contrast, aP2-driven over-expression of AEBP1 in ApoE$^{-/-}$ mice is expected to advance atherogenesis, probably leading to formation of more advanced and larger lesions. Thus, AEBP1$^{-/-}$/ApoE$^{-/-}$ and AEBP1^{TG}/ApoE$^{-/-}$ hybrid mice can serve as invaluable *in vivo* tools to further elucidate the pro-atherogenic functions of AEBP1 in mice.

3.8.1 Generation of AEBP1$^{-/-}$/ApoE$^{-/-}$ Hybrid Mice

To generate AEBP1$^{-/-}$/ApoE$^{-/-}$ hybrid mice, AEBP1$^{-/-}$ females were mated with ApoE$^{-/-}$ males, a mating that generated 100% AEBP1$^{+/-}$/ApoE$^{+/-}$ F$_1$ progeny. Next, AEBP1$^{+/-}$/ApoE$^{+/-}$ mice were mated to generate double-knockout mice. In principle, AEBP1$^{+/-}$/ApoE$^{+/-}$ X AEBP1$^{+/-}$/ApoE$^{+/-}$ mating should yield one or more of the following 9 possible F$_2$ progeny genotypes:

1. AEBP1$^{+/+}$/ApoE$^{+/+}$
2. AEBP1$^{+/-}$/ApoE$^{+/+}$
3. AEBP1$^{-/-}$/ApoE$^{+/+}$
4. AEBP1$^{+/+}$/ApoE$^{-/-}$
5. AEBP1$^{+/+}$/ApoE$^{+/-}$
6. AEBP1$^{+/-}$/ApoE$^{+/-}$
7. AEBP1$^{+/-}$/ApoE$^{-/-}$
8. AEBP1$^{-/-}$/ApoE$^{+/-}$
9. AEBP1$^{-/-}$/ApoE$^{-/-}$

Next, and based on ApoE genotyping, AEBP1$^{+/-}$/ApoE$^{-/-}$ mice were identified and mated. According to Mendelian principle, AEBP1$^{+/-}$/ApoE$^{-/-}$ X AEBP1$^{+/-}$/ApoE$^{-/-}$ mating should yield one or more 3 possible F$_3$ progeny genotypes:

1. AEBP1$^{+/+}$/ApoE$^{-/-}$ (25%)

2. AEBP1$^{+/-}$/ApoE$^{-/-}$ (50%)

3. AEBP1$^{-/-}$/ApoE$^{-/-}$ (25%)

Unfortunately, out of 104 pups generated from 25 litters of several AEBP1$^{+/-}$/ApoE$^{-/-}$ X AEBP1$^{+/-}$/ApoE$^{-/-}$ crosses, none of these pups represented an AEBP1$^{-/-}$/ApoE$^{-/-}$ genotype, while 32% and 68% of those pups had AEBP1$^{+/+}$/ApoE$^{-/-}$ and AEBP1$^{+/-}$/ApoE$^{-/-}$ genotypes, respectively.

3.8.2 Generation and Characterization of AEBP1^{TG}/ApoE$^{-/-}$ Hybrid Mice

In order to generate AEBP1^{TG}/ApoE$^{-/-}$ mice, AEBP1^{TG} females that carry AEBP1 transgene on both alleles (i.e. double AEBP1^{TG} mice) were crossed with ApoE$^{-/-}$ males, which yielded 100% AEBP1^{TG}/ApoE$^{+/-}$ F$_1$ progeny carrying one AEBP1^{TG} and one AEBP1^{NT} alleles (i.e. single AEBP1^{TG} mice). Next, AEBP1^{TG}/ApoE$^{+/-}$ F$_1$ progeny mice were mated. Such mating should yield one or more of the following 9 possible F$_2$ genotypes:

1. AEBP1^{TG}/ApoE$^{+/+}$ (double AEBP1^{TG})

2. AEBP1^{TG}/ApoE$^{+/-}$ (double AEBP1^{TG})

3. AEBP1^{TG}/ApoE$^{-/-}$ (double AEBP1^{TG})

4. AEBP1^{TG}/ApoE$^{+/+}$ (single AEBP1^{TG})

5. AEBP1^{TG}/ApoE$^{+/-}$ (single AEBP1^{TG})

6. AEBP1^{TG}/ApoE$^{-/-}$ (single AEBP1^{TG})

7. AEBP1^{NT}/ApoE$^{+/+}$

8. AEBP1^{NT}/ApoE$^{+/-}$

9. AEBP1^{NT}/ApoE$^{-/-}$

Subsequently, AEBP1^{TG}/ApoE$^{-/-}$ and AEBP1^{NT}/ApoE$^{-/-}$ mice were backcrossed 6 times before F$_3$ AEBP1^{TG}/ApoE$^{-/-}$ and AEBP1^{NT}/ApoE$^{-/-}$ progeny were considered for experimentation. In total, 93 pups from 15 litters were obtained and identified to have AEBP1^{TG}/ApoE$^{-/-}$ (71%) and AEBP1^{NT}/ApoE$^{-/-}$ (29%) genotypes.

In order to assess the effect of AEBP1 over-expression on atherosclerotic lesion development in ApoE$^{-/-}$ mice, AEBP1^{TG}/ApoE$^{-/-}$ and AEBP1^{NT}/ApoE$^{-/-}$ mice were fed HFD until they were sacrificed at 32 wk of age. The entire aorta (from the aortic root until 3-5 mm after the iliac bifurcation) was isolated from mice and *en face* analysis was performed using Sudan IV to positively stain lipid-filled atherosclerotic lesions. Unfortunately, the data was strikingly disappointing since atherosclerotic lesions were very hard to detect. However, the data was much less surprising given that post-experiment genotyping revealed that the studied mice had an ApoE$^{+/-}$, not ApoE$^{-/-}$, genotype. Interestingly, however, some atherosclerotic lesions were detected in the aortae of AEBP1^{TG}/ApoE$^{+/-}$ mice, but not AEBP1^{NT}/ApoE$^{+/-}$ mice (Figure 28). This is consistent with oil red O staining of aortic cryosections of AEBP1^{TG} and AEBP1^{NT} mice showing that the former develop lipid-filled atherosclerotic lesions (Figure 26). No lesion quantification was performed due to the very small number of mice in each group (AEBP1^{TG}/ApoE$^{+/-}$ mice, n=4; AEBP1^{NT}/ApoE$^{+/-}$ mice, n=2).

Figure 28. AEBP1 Promotes Atherosclerotic Lesion Formation in ApoE$^{+/-}$ Mice. The aortae were isolated from 32-wk old, HFD-fed AEBP1^{TG}/ApoE$^{-/-}$ and AEBP1^{NT}/ApoE$^{-/-}$ mice (n=4 and n=2, respectively) and were subjected to *en face* analysis using Sudan IV stain (refer to section 2.11.1 for more details).

3.9 AEBP1^{TG} Mice Exhibit a Premature Sudden Death Phenomenon

3.9.1 Characterization of Mice that Undergo Premature Sudden Death

Upon generation of AEBP1^{TG} mice, it was noticed that a significant proportion of such mice undergo a premature sudden death at a relatively young age (Average 15 weeks). Interestingly, this premature sudden death phenomenon is gender-specific, in which only AEBP1^{TG} females, but not males, are victimized. During a 32-week trial, 38% (9 of 24) of HFD-fed and 21% (12 of 56) of chow-fed AEBP1^{TG} females had perished beginning at the age of 13 weeks, while the last casualty was observed at 30 weeks of age. Interestingly, no premature sudden death event was observed among chow- or HFD-fed AEBP1^{TG} males (n=12 and n=9, respectively), AEBP1^{NT} males (n=10 and n=8, respectively), or AEBP1^{NT} females (n=21 and n=20, respectively). Noteworthy, premature sudden death victims display no signs of lethargy, morbidity, or mortality, and they do not show any significant body weight differences compared to AEBP1^{TG} female survivors, before they perish.

3.9.2 Detection of Atherosclerotic Lesions in Sudden Death Victims

The fact that atherosclerosis is considered the primary cause of sudden cardiac death (Mayers, 2003) coupled with the observations that HFD-fed AEBP1^{TG} mice develop atherosclerotic lesions and that about 38% of HFD-fed AEBP1^{TG} females undergo a premature sudden death event, suggested that atherosclerotic lesion formation is likely associated with premature sudden death in AEBP1^{TG} females. In an attempt to identify a pathophysiological mechanism that is associated with atherosclerosis and that would satisfactorily explain the sudden death phenomenon displayed by AEBP1^{TG} females, it is important to verify lesion formation in sudden death victims. To that end, aortic cryosections obtained from AEBP1^{TG} females (n=6) that died of premature sudden death were stained with ORO to allow lesion detection. Microscopic analysis demonstrates atherosclerotic lesion formation in all the aortae of sudden death victims examined (Figure 29).
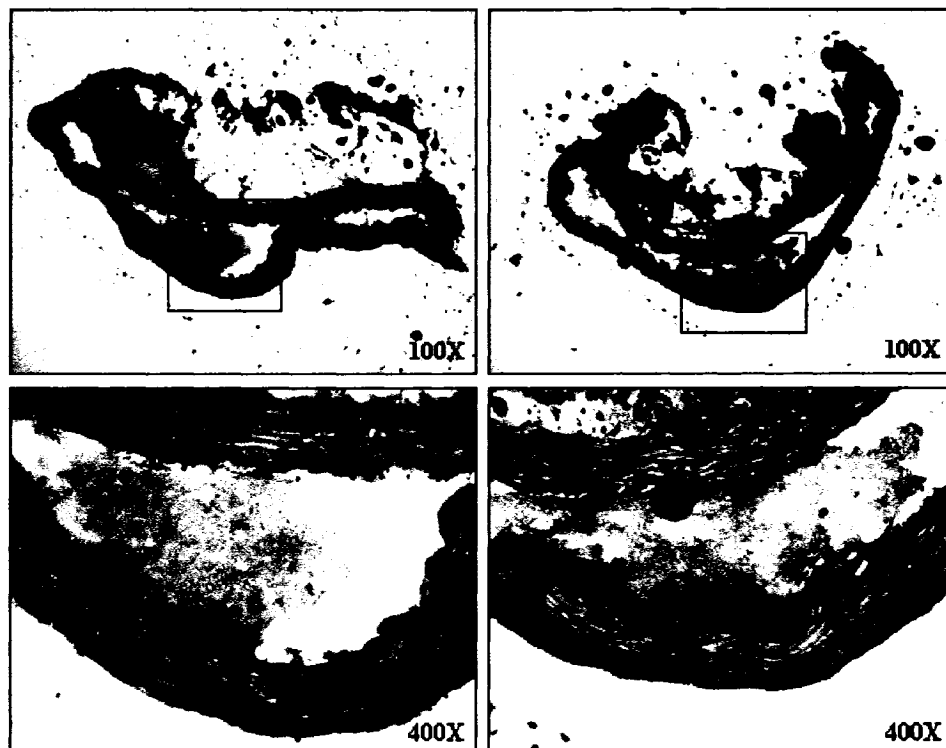
Figure 29. Sudden Death AEBP1^{TG} Victims Possess Atherosclerotic Lesions. Aortic cryosections obtained from 13-22-wk old, HFD-fed AEBP1^{TG} females that suffered from premature sudden death events were stained with ORO to allow assessment of atherosclerotic lesion development. Two representative images obtained from a 17-wk old, HFD-fed sudden death AEBP1^{TG} victim are shown at 100X and 400X magnifications.

3.9.3 Histopathological Examination of Heart Tissue

The fact that AEBP1^{TG} mice develop atherosclerotic lesions that harbor relatively thin fibrous caps makes such lesions highly vulnerable to rupture, a likely mechanism underlying premature sudden death in AEBP1^{TG} females. In an attempt to identify the pathophysiological mechanism(s) behind such a premature sudden death phenomenon, heart tissues obtained from AEBP1^{TG} sudden death victims and AEBP1^{TG} survivors were examined for any signs of myocardial infarction, a frequent event following atherosclerotic lesion rupture (Mayers, 2003). To that end, heart tissue cryosections were stained with Masson's trichrome stain, which allows detection of fibrotic regions (light blue) and dying cardiomyocytes (dark blue), characteristic abnormalities associated with myocardial infarction (Oliver *et al.*, 1997). Unfortunately, no obvious histophathological abnormalities (i.e. fibrosis or dead cardiomyocytes) were detected in heart tissues obtained from AEBP1^{TG} sudden death victims, compared to those obtained from AEBP1^{TG} female survivors (Figure 30).

Healthy AEBP1^{TG} Female   **"Sudden Death" AEBP1^{TG} Victim**
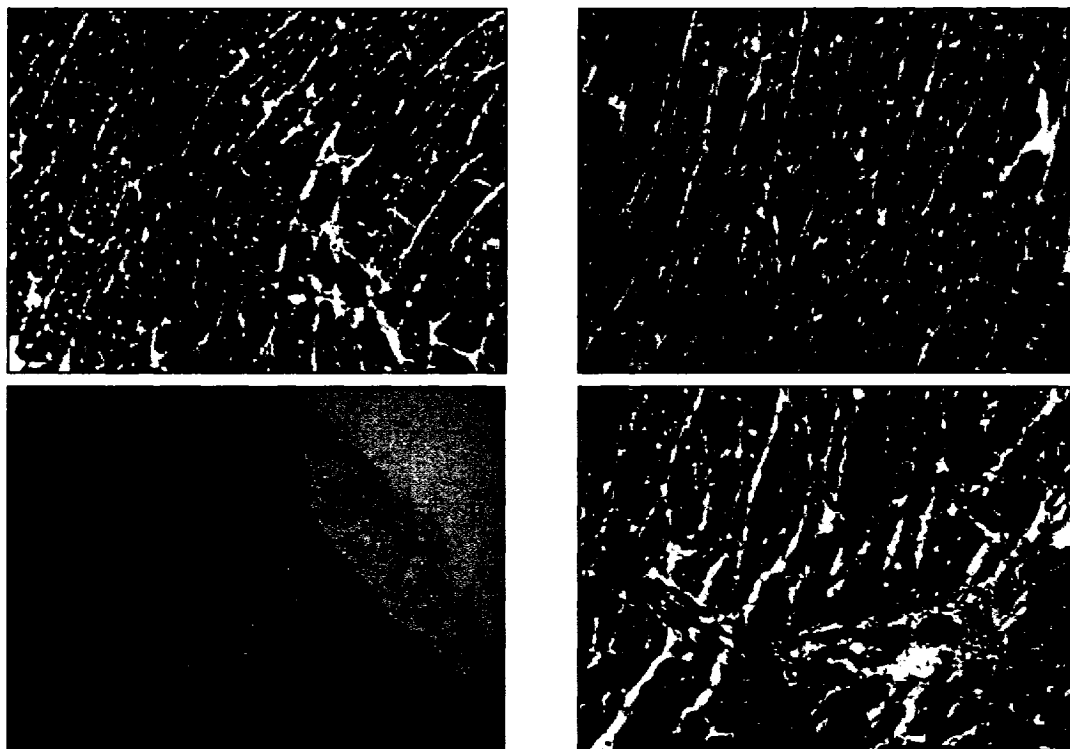
Figure 30. Examination of Heart Tissue Morphology in Sudden Death AEBP1^{TG} Victims. 10 μm-thick heart tissue cryosections obtained from 13-22-wk old, HFD-fed AEBP1^{TG} females that suffered from premature sudden death events were stained with Masson's Trichrome in an attempt to identify myocardial infarcts. Age-matching, HFD-fed healthy AEBP1^{TG} females served as a negative control. Two images representing 17-wk old, HFD-fed healthy AEBP1^{TG} and sudden death AEBP1^{TG} victim are shown.

3.10 AEBP1 Is a Positive Regulator of NF-κB Activity *In Vitro* and *In Vivo*

3.10.1 AEBP1 Up-Regulates NF-κB Activity *in vitro*

Since AEBP1 seems to play a crucial role in promoting macrophage inflammatory responsiveness by enhancing the expression of IL-6, TNFα, MCP-1, and iNOS (Figures 23 & 24), and since NF-κB activation in macrophages provokes increased expression of various inflammatory cytokines, chemokines, proteolytic enzymes, and cell surface adhesion molecules (Tak and Firestein, 2001; Yamamoto and Gaynor, 2001), a positive relationship between AEBP1 and NF-κB activity in macrophages was investigated. To test this possibility *in vitro*, C6 glioma cells, which express LPS-responsive Toll-like receptor 4 (TLR4) (Jack *et al.*, 2005) and its co-receptor CD14 (Deininger *et al.*, 2003), were made use of due to repeatedly unsuccessful attempts to transfect J774, THP-1, and U937 monocyte/macrophage cell lines (refer to section 3.2.2). C6 glioma cells were co-transfected with pNF-κB-TA-luciferase carrying four tandem repeats of the κB binding site along with increasing amounts of the pcDNA-AEBP1 expressing AEBP1 or pcDNA empty vector. C6 glioma transfectants were also co-transfected with β-gal expression vector as an internal control for transfection efficiency. C6 glioma transfectants were left un-stimulated or stimulated with 1 μg/ml LPS for 30 min before harvesting whole cell protein extracts. As shown in Figure 31, luciferase reporter assay clearly illustrates that AEBP1 significantly enhances NF-κB activity in a dose-responsive manner. Intriguingly, NF-κB activity seems to be up-regulated by AEBP1 under basal and LPS-stimulatory conditions *in vitro* (Figure 31).
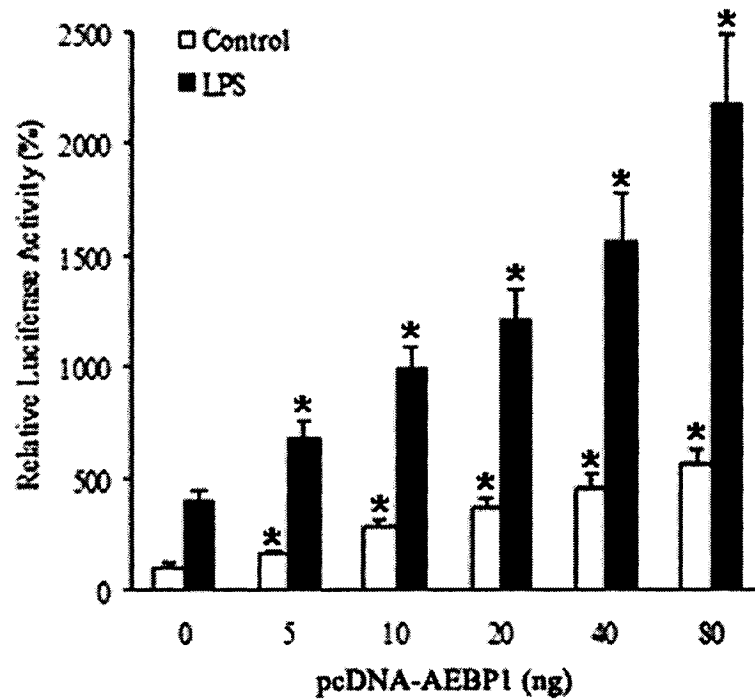
Figure 31. AEBP1 Up-Regulates NF-κB activity in a Dose-Responsive Manner *in vitro.* C6 glioma cells were transiently transfected with 200 ng pNF-κB-TA-luciferase using lipofectamine[2000]. Cells were co-transfected with 20 ng pHermes-lacZ, which expresses β-galactosidase, along with pcDNA empty vector or increasing amounts of pcDNA-AEBP1 (5, 10, 20, 40, and 80 ng). Total amount of plasmid DNA was equated using pcDNA empty vector. Forty-eight hours post transfection, cells were left un-stimulated (i.e. treated with 1X PBS) or stimulated with 1 μg/ml LPS for 30 min. Cells were subsequently lysed and subjected to luciferase and β-galactosidase assays. Luciferase activity, normalized to β-gal activity, was set to 100% for the un-stimulated, pcDNA empty vector (i.e. 0 ng pcDNA-AEBP1) transfection sample. Relative luciferase activity is expressed as mean ± SEM (n=6). Statistical significance was determined based on relative luciferase activity in un-stimulated and LPS-stimulated, pcDNA empty vector-transfected C6 glioma cells.

3.10.2 AEBP1 Up-Regulates NF-κB Activity in Macrophages

3.10.2.1 Assessment of Nuclear p65 Protein Level in Macrophages

To examine whether AEBP1 up-regulatory effects on NF-κB activity take place in macrophages, p65 protein levels in the nuclei (i.e. active p65) of un-stimulated and LPS-stimulated peritoneal macrophages isolated from AEBP1$^{+/+}$ and AEBP1$^{-/-}$ mice were evaluated by Western blot analysis. Nuclear protein extracts were subjected to SDS-PAGE followed by immunoblotting for AEBP1, p65, and actin. Densitometric analysis of Western blots clearly shows that nuclear p65 protein levels are significantly lower (3 fold and 5 fold) in AEBP1$^{-/-}$ macrophages, compared to AEBP1$^{+/+}$ macrophages, under basal and LPS-stimulatory conditions, respectively (Figure 32). Indeed, NF-κB hyperactivation by AEBP1 seems to be independent of LPS stimulation since nuclear p65 protein levels are lower in un-stimulated AEBP1$^{-/-}$ macrophages, compared to un-stimulated AEBP1$^{+/+}$ counterparts (Figure 32). In accord, un-stimulated and LPS-stimulated AEBP1^{TG} macrophages possess higher nuclear p65 protein levels compared to their AEBP1^{NT} macrophages (data not shown). Noteworthy, the ability of AEBP1 to enhance p65 nuclear localization is not gender-, age-, or diet-specific (data not shown).

Figure 32. AEBP1 Up-Regulates NF-κB Activity in Primary Macrophages. *(A)* Nuclear protein extracts (25 μg) obtained from un-stimulated (-) and LPS-stimulated (+) macrophages isolated from 20-wk old, HFD-fed AEBP1$^{+/+}$ and AEBP1$^{-/-}$ mice were resolved on 8.5% polyacrylamide gels and subjected to immunoblotting for AEBP1, p65, and actin. *(B)* Protein level was normalized based on actin expression, and normalized protein level was set to 100% in un-stimulated AEBP1$^{+/+}$ macrophages. A histogram illustrates densitometric analysis based on actin expression. Relative protein level is expressed as mean ± SEM (n=6). Statistical significance was determined relative to protein level in un-stimulated and LPS-stimulated AEBP1$^{+/+}$ macrophages.

A

AEBP1^{+/+}   AEBP1^{-/-}
1   2   1   2
−   +   −   +   −   +   −   +   ← LPS

AEBP1
p65
Actin

B

178

3.10.2.2 Assessment of NF-κB DNA-Binding Activity in Macrophages

To further confirm the ability of AEBP1 to modulate NF-κB activity in macrophages, nuclear protein extracts obtained from un-stimulated and LPS-stimulated AEBP1$^{+/+}$ and AEBP1$^{-/-}$ macrophages were subjected to electrophoretic mobility gel shift assay using ^{32}P-labeled κB DNA-binding consensus sequence to evaluate NF-κB DNA-binding activity. As shown in Figure 33, the band intensity corresponding to NF-κB-DNA complex is dramatically lower in AEBP1$^{-/-}$ macrophages, compared to AEBP1$^{+/+}$ macrophages, consistent with significantly reduced nuclear p65 protein levels in AEBP1$^{-/-}$ macrophages (Figure 32). Consistently, AEBP1 seems to up-regulate NF-κB activity in presence and absence of LPS stimulatory signal (Figure 33). Noteworthy, AEBP1 stimulatory function towards NF-κB DNA-binding activity is not gender-, age-, or diet-specific (data not shown). Together, these findings indicate that AEBP1 is able to enhance NF-κB transcriptional and DNA-binding activities *in vitro* and *in vivo* under basal and LPS-stimulatory conditions.
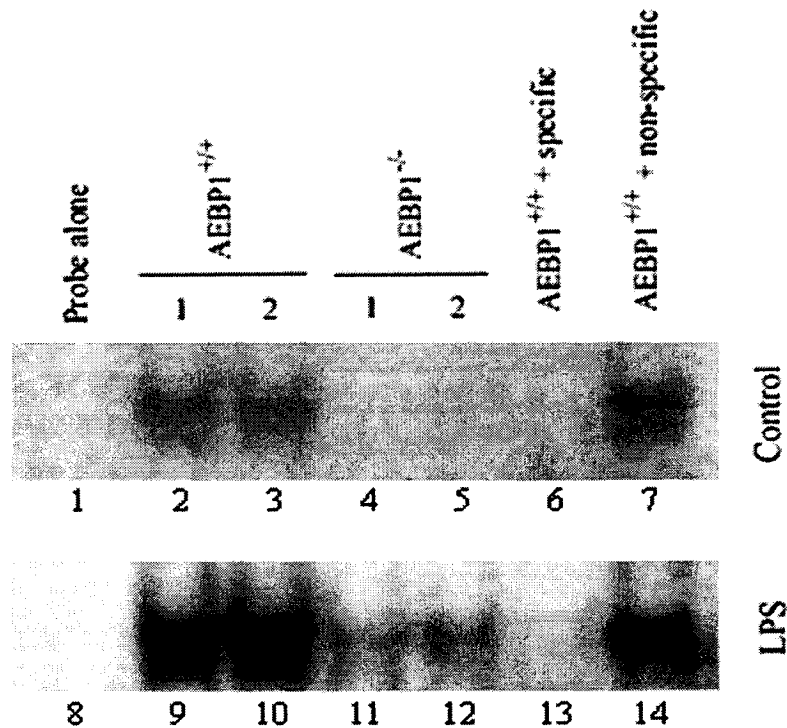
Figure 33. AEBP1 Up-Regulates NF-κB DNA-Binding Activity in Primary Macrophages. 2.0 μg nuclear protein extracts obtained from un-stimulated and LPS-stimulated macrophages isolated from 20-wk old, HFD-fed $AEBP1^{+/+}$ and $AEBP1^{-/-}$ mice were subjected to NF-κB EMSA in the presence of specific (unlabeled NF-κB probe) (*lanes 6 and 13*) or non-specific (unlabeled, unrelated probe) (*lanes 7 and 14*) competitors. ^{32}P-labeled NF-κB probe was run alone as a negative control (*lanes 1 and 8*). Two samples per genotype are shown. This data is representative of three independent experiments.

3.11 AEBP1 Does Not Transcriptionally Modulate NF-κB Activity

Since AEBP1 is known to function as a potent transcriptional repressor of several target genes (He *et al.*, 1995, Majdalawieh *et al.*, 2006), it is conceivable that AEBP1 transcriptionally represses known and/or unknown genes whose products function as negative regulators of NF-κB gene expression. Similarly, it is possible that AEBP1 directly represses the expression of IκBα and/or IκBβ genes, whose products are known to antagonize NF-κB activity. If any one of these possibilities is true, then p65 mRNA level should correlate positively with AEBP1 expression in macrophages. To examine these possibilities, total RNA was isolated from un-stimulated and LPS-stimulated macrophages isolated from 32-wk old, HFD-fed AEBP1$^{+/+}$ and AEBP1$^{-/-}$ mice, and subsequently subjected to RT-PCR in order to assess p65, IκBα, and IκBβ mRNA levels. RT-PCR and densitometric analysis based on β-actin expression clearly demonstrate that differential expression of AEBP1 has no correlation with p65 mRNA level in macrophages (Figure 34). Consistent with a role of AEBP1 in NF-κB enhanced activation, the mRNA levels of IκBα and IκBβ are higher in AEBP1$^{+/+}$ macrophages, compared to AEBP1$^{-/-}$ macrophages, under basal and LPS-stimulatory conditions (Figure 34), confirming inhibited NF-κB activity in AEBP1$^{-/-}$ macrophages. These findings suggest that AEBP1 up-regulates NF-κB activity independent of its transcriptional repression function, and that AEBP1-mediated up-regulation of NF-κB activity occurs post-transcriptionally.

Figure 34. AEBP1 Up-Regulates NF-κB Activity Independent of its Transcriptional Repression Function. *(A)* Peritoneal macrophages isolated from 20-wk old, HFD-fed AEBP1$^{+/+}$ and AEBP1$^{-/-}$ mice were left un-stimulated (-) or stimulated (+) with 1 μg/ml LPS for 1 hr. Total RNA samples were isolated from macrophages and 1.0 μg total RNA was reverse transcribed, and the indicated transcripts were amplified by PCR using specific primers (Table 4). Two samples per genotype and condition are shown. *(B)* Densitometric analysis based on β-actin level in AEBP1$^{+/+}$ macrophages is shown. Normalized mRNA level is expressed as mean ± SEM (n=6). Statistical significance was determined relative to mRNA level in un-stimulated AEBP1$^{+/+}$ macrophages.
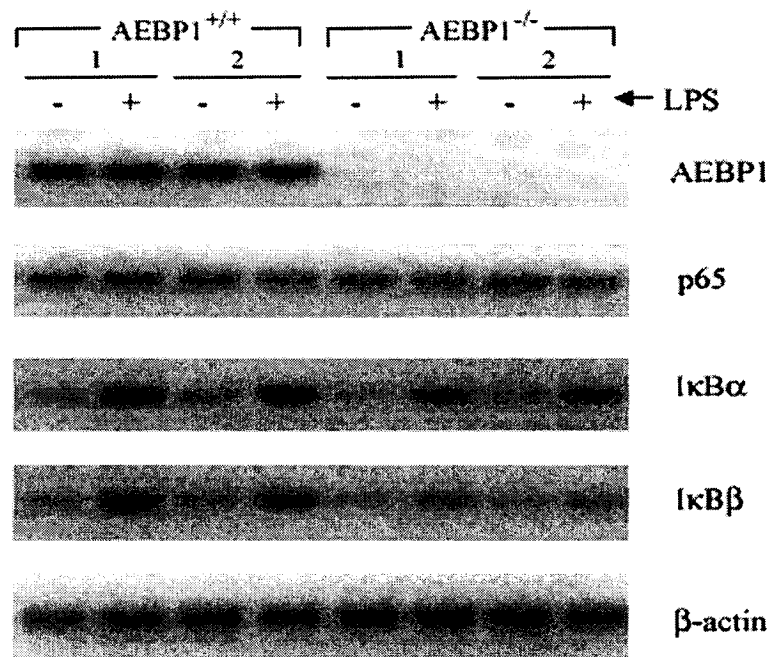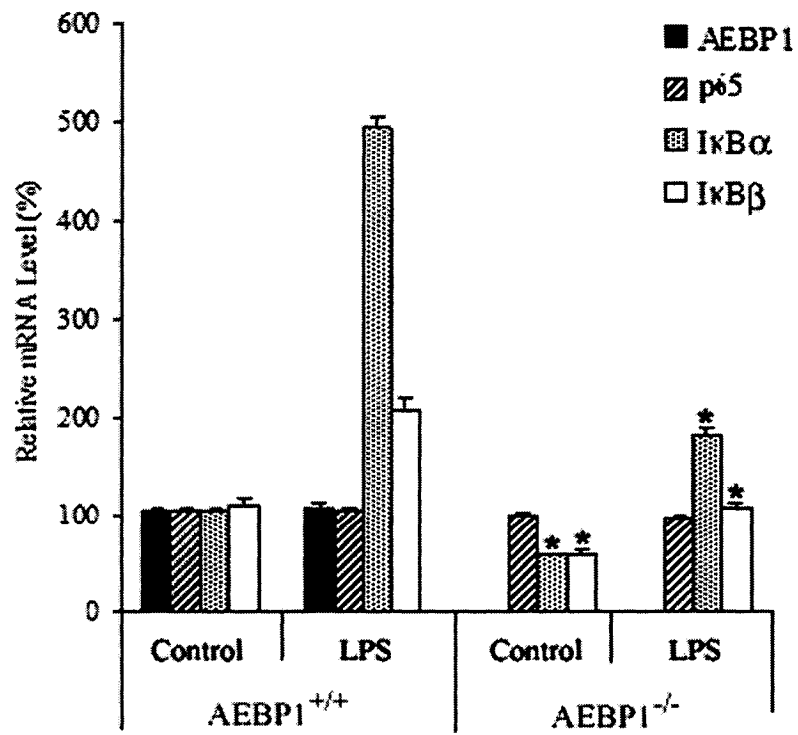
3.12 AEBP1 Is a Negative Regulator of IκBα Inhibitory Function

3.12.1 AEBP1 Reduces IκBα Protein Levels in Macrophages

In order to resolve the mechanism by which AEBP1 up-regulates NF-κB activity in macrophages, it was hypothesized that IκBα and/or IκBβ protein levels are negatively modulated by AEBP1 in macrophages. To examine this possibility, whole cell protein extracts were obtained from un-stimulated and LPS-stimulated macrophages isolated from 32-wk old, HFD-fed AEBP1$^{+/+}$ and AEBP1$^{-/-}$ mice, and were subsequently subjected to SDS-PAGE followed by immunoblotting for AEBP1, IκBα, IκBβ, and actin. In support of my hypothesis, densitometric analysis of Western blots revealed that IκBα protein levels are 3-fold higher in AEBP1$^{-/-}$ macrophages, in comparison to AEBP1$^{+/+}$ macrophages, under basal and LPS-stimulatory conditions (Figure 35). Interestingly, AEBP1$^{+/+}$ and AEBP1$^{-/-}$ macrophages display equivalent IκBβ protein levels under basal and LPS-stimulatory conditions (Figure 35), indicating that AEBP1-mediated modulation of IκBα protein levels in macrophages is very specific. Noteworthy, the ability of AEBP1 to reduce cytosolic IκBα protein level in macrophages is not gender-, age-, or diet-specific (data not shown).

3.12.2 AEBP1 Promotes IκBα Phosphorylation in Macrophages

To explain the differential IκBα protein levels in AEBP1$^{+/+}$ and AEBP1$^{-/-}$ macrophages, and since phosphorylation of IκBα on S^{32} and S^{36} is a major biochemical event regulating IκBα protein stability and function, the phosphorylation status of IκBα on those two serine residues was assessed in un-stimulated and LPS-stimulated AEBP1$^{+/+}$ and AEBP1$^{-/-}$ macrophages. Whole cell protein extracts obtained from macrophages were resolved on SDS-PAGE and immunoblotted using an anti-phospho-IκBα specific antibody. As shown in Figure 35, AEBP1 ablation in macrophages is accompanied by significantly reduced levels (~ 4 fold) of S^{32}/S^{36} phosphorylated IκBα, indicating that AEBP1 has the potential to induce IκBα phosphorylation *in vivo* under basal and LPS-

stimulatory conditions. Noteworthy, the ability of AEBP1 to enhance IκBα phosphorylation in macrophages is not gender-, age-, or diet-specific (data not shown).

Figure 35. AEBP1 Expression Negatively Correlates with IκBα Protein Levels in Primary Macrophages. *(A)* Whole cell protein extracts (25 μg) obtained from un-stimulated (-) and LPS-stimulated (+) macrophages isolated from 20-wk old, HFD-fed AEBP1$^{+/+}$ and AEBP1$^{-/-}$ mice were resolved on 8.5% polyacrylamide gels and subjected to immunoblotting for AEBP1, IκBα, P-IκBα, IκBβ, and actin. Two samples per genotype and condition are shown. *(B)* Protein level was normalized based on actin expression, and normalized protein level was set to 100% in un-stimulated AEBP1$^{+/+}$ macrophages. A histogram illustrates densitometric analysis based on actin expression. Relative protein level is expressed as mean ± SEM (n=6). Statistical significance was determined relative to protein level in un-stimulated and LPS-stimulated AEBP1$^{+/+}$ macrophages.

187
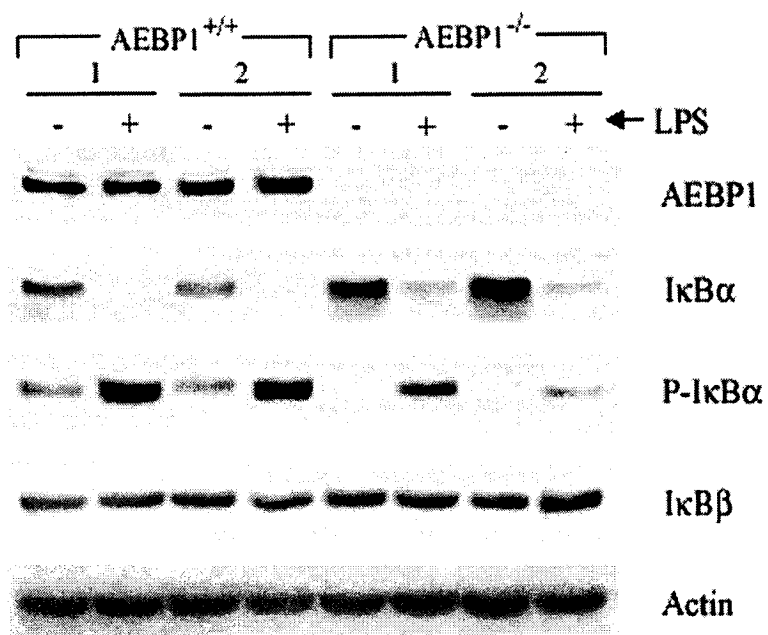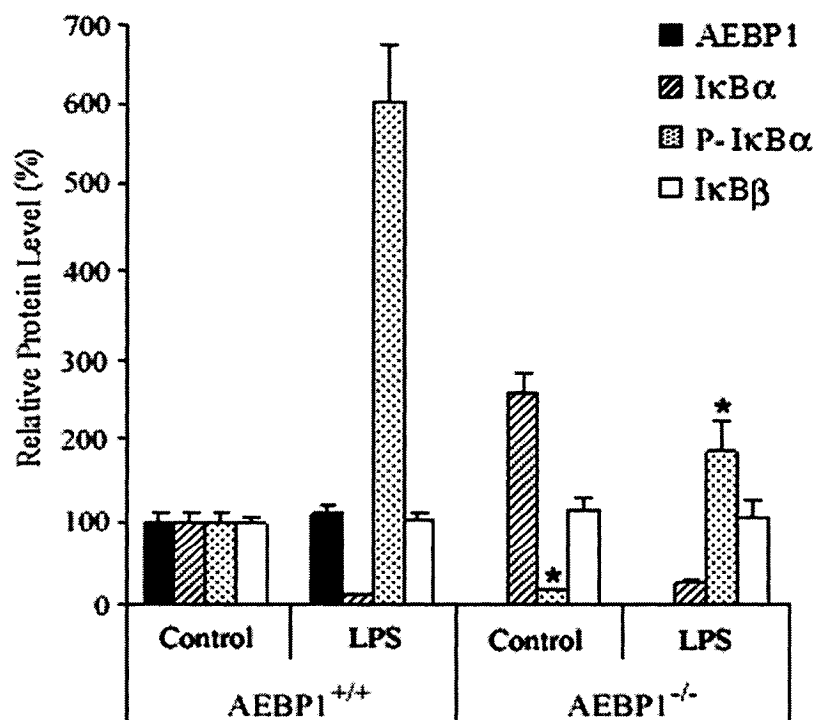
3.12.3 AEBP1 Decreases IκBα Protein Stability in Macrophages

As mentioned above, IκBα phosphorylation renders it susceptible to ubiquitination and proteolytic degradation. The observation that AEBP1$^{-/-}$ macrophages display significantly reduced levels of phosphorylated IκBα and elevated levels of total IκBα suggests that AEBP1 regulates IκBα proteolytic degradation, shortening its half-life, in macrophages. To further investigate the role of AEBP1 in modulating IκBα protein stability in macrophages, whole cell protein extracts obtained from AEBP1$^{+/+}$ and AEBP1$^{-/-}$ treated with 50 μg/ml CHX for various time points (0 to 240 min) were subjected to SDS-PAGE followed by immunoblotting. As shown in Figure 36, IκBα protein half-life in AEBP1$^{+/+}$ macrophages is estimated to be 50 min, consistent with studies suggesting that IκBα half-life is about 40 min (Miyamoto *et al.*, 1998, Shumway and Miyamoto, 2004). Interestingly, densitometric analysis revealed that IκBα protein half-life is significantly prolonged (~ 250 min) in AEBP1$^{-/-}$ macrophages (Figure 36). IκBβ protein levels in AEBP1$^{-/-}$ macrophages upon CHX treatment were comparable to those in AEBP1$^{+/+}$ macrophages (Figure 36A), confirming the differential ability of AEBP1 to modulate IκBα, but not IκBβ, protein levels in macrophages. As a control, p53 protein level was assessed in AEBP1$^{+/+}$ and AEBP1$^{-/-}$ macrophages upon CHX treatment, and p53 protein stability profile is comparable in both types of macrophages ($t_{1/2}$ = ~50 min) (Figure 36A). Actin was chosen as a loading control due to its relatively long half-life (> 48 hr). For the first time ever, this experiment demonstrates that endogenous AEBP1 protein has a half-life of about 65 min. This protein stability experiment was also performed in control 3T3-L1 preadipocytes (Neo-1) and stably transfected AEBP1 anti-sense (AS/Neo-11) preadipocytes, and very similar results were obtained (data not shown). Taken together, these findings strongly suggest that lack of AEBP1 prolongs IκBα protein stability, significantly inhibiting its turnover.

Figure 36. AEBP1 Negatively Influences IκBα Protein Stability in Primary Macrophages. *(A)* Peritoneal macrophages isolated from 20-wk old, HFD-fed AEBP1$^{+/+}$ and AEBP1$^{-/-}$ mice were treated with ethanol (vehicle) or 50 μg/ml CHX for the indicated times. Whole cell protein extracts were obtained, and 25 μg total protein extracts were resolved on 8.5% polyacrylamide gels. Nitrocellulose membranes were immunoblotted for AEBP1, IκBα, IκBβ, p53, and actin. *(B)* IκBα protein level was normalized based on actin expression, and normalized IκBα protein level was set to 100% in ethanol-treated AEBP1$^{+/+}$ and AEBP1$^{-/-}$ macrophages (i.e. 0 min samples). A line-curve illustrates densitometric analysis of IκBα protein levels shown in (A). IκBα protein levels at 0 min was taken to be 100% for every group, and IκBα protein levels at other time points are expressed relative to that at 0 min within the same group, and data is expressed as mean ± SEM (n=3). At each time point, statistical significance was determined relative to IκBα protein level in AEBP1$^{+/+}$ macrophages at each time point.

A

B

190
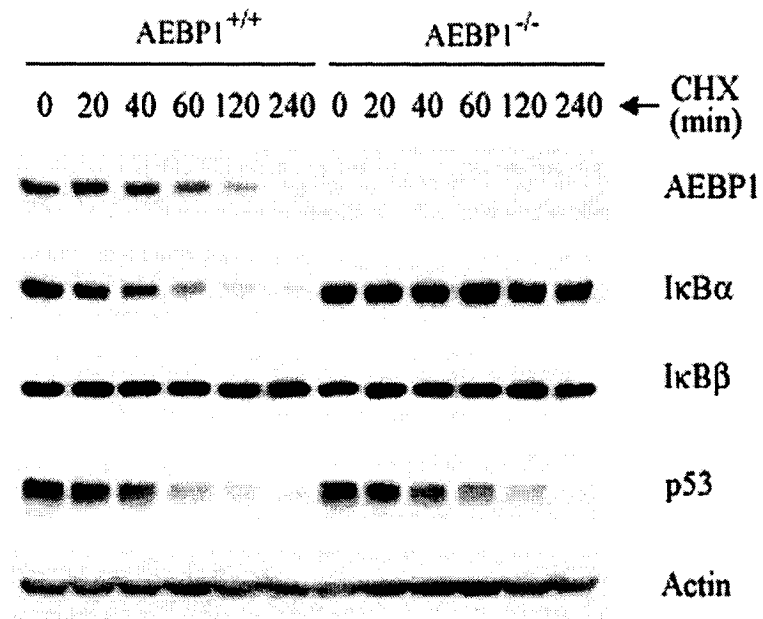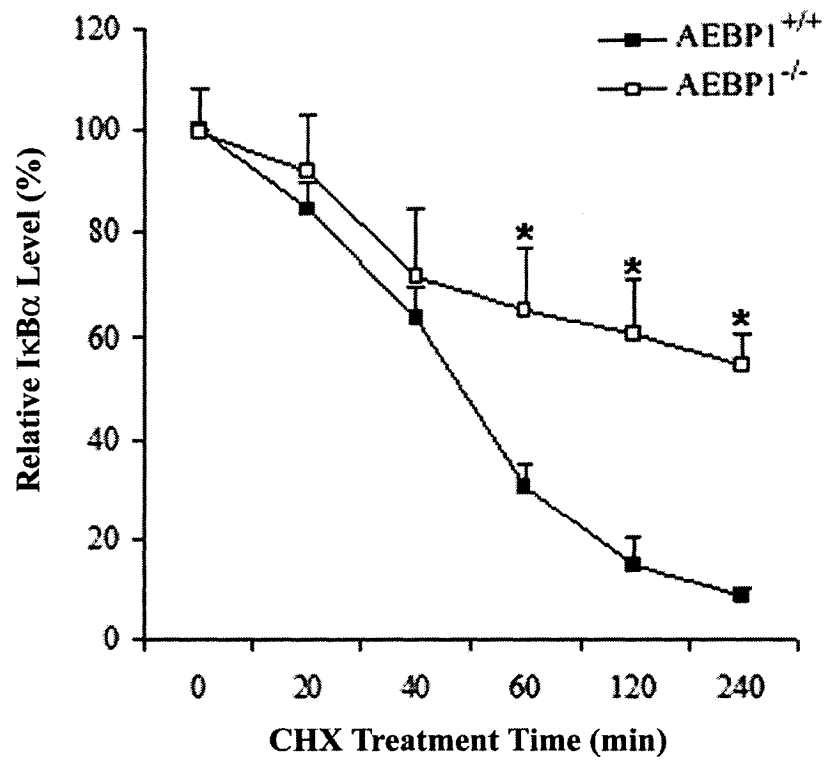
3.13 AEBP1 Does Not Influence IKKβ Catalytic Activity in Macrophages

IKKβ has been shown to be the predominant cytoplasmic kinase responsible for phosphorylating the S^{32}/S^{36} residues in IκBα *in vivo* (Li *et al.*, 1999). Since modulation of IKKβ activity is associated with modulation of IκBα phosphorylation status, and subsequently IκBα proteolytic degradation, it is possible that AEBP1 may enhance IKKβ activity in a direct or indirect manner, leading to increased phosphorylation of IκBα in macrophages. To examine this possibility, intrinsic IKKβ catalytic activity in AEBP1$^{+/+}$ and AEBP1$^{-/-}$ macrophages was assessed by *in vitro* IKK kinase assay. To this end, endogenously expressed IKK complex was immunoprecipitated from whole cell protein extracts obtained from un-stimulated and LPS-stimulated AEBP1$^{+/+}$ and AEBP1$^{-/-}$ macrophages, and IKKβ catalytic activity was assessed using a bacterially expressed GST-IκBα (1-54) fusion protein as a substrate in an *in vitro* kinase assay. Clearly, IKKβ catalytic activity is indistinguishable in AEBP1$^{+/+}$ and AEBP1$^{-/-}$ macrophages (Figures 37 A, *top panel*, & B), indicating that AEBP1 does not alter the enzymatic activity of IKKβ in macrophages. No IKKβ catalytic activity was detected in protein extracts isolated from un-stimulated macrophages, confirming LPS viability. Equivalent GST-IκBα (1-54) and IKKα immunoprecipitate levels was confirmed by blotting the membrane with anti-GST and anti-IKKα antibodies, respectively (Figure 37A, *middle panels*). Western blot analysis of whole cell lysates confirmed absence of AEBP1 in AEBP1$^{-/-}$ macrophages (Figure 37A, *bottom panel*). Taken together, these findings suggest that AEBP1 is capable of modulating IκBα protein stability, and subsequently NF-κB activity, in macrophages without altering IKKβ catalytic activity.

Figure 37. AEBP1 does not Influence IKKβ Catalytic Activity in Primary Macrophages. *(A)* Endogenously expressed IKK complex was immunoprecipitated from whole cell protein extracts obtained from un-stimulated (-) and LPS-stimulated (+) AEBP1$^{+/+}$ and AEBP1$^{-/-}$ macrophages isolated from 20-wk old, HFD-fed AEBP1$^{+/+}$ and AEBP1$^{-/-}$ mice, using anti-IKKα antibody. IKKβ catalytic activity was assessed using a bacterially expressed GST-IκBα (1-54) fusion protein as a substrate in an *in vitro* kinase assay. GST-IκBα (1-54) fusion protein alone and normal IgG immunoprecipitation samples were used as negative controls. Autoradiography (*top panel*) and immunoblotting for GST and IKKα (*middle panels*) is shown. Whole cell protein extracts were immunoblotted for AEBP1 and actin (*lower panel*) to confirm AEBP1 ablation in AEBP1$^{-/-}$ macrophages. *(B)* A histogram illustrates densitometric analysis of GST-IκBα (1-54) autoradiography shown in (A). Data is expressed as mean ± SEM (n=3). Statistical significance was determined relative to IKKβ catalytic activity in LPS-stimulated AEBP1$^{+/+}$ macrophages.

A
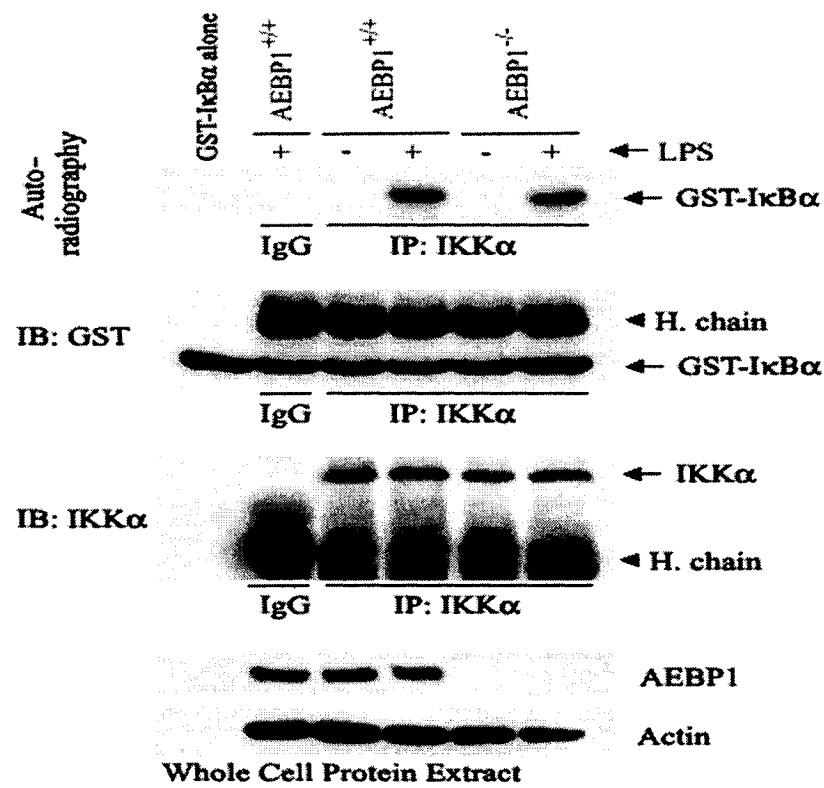
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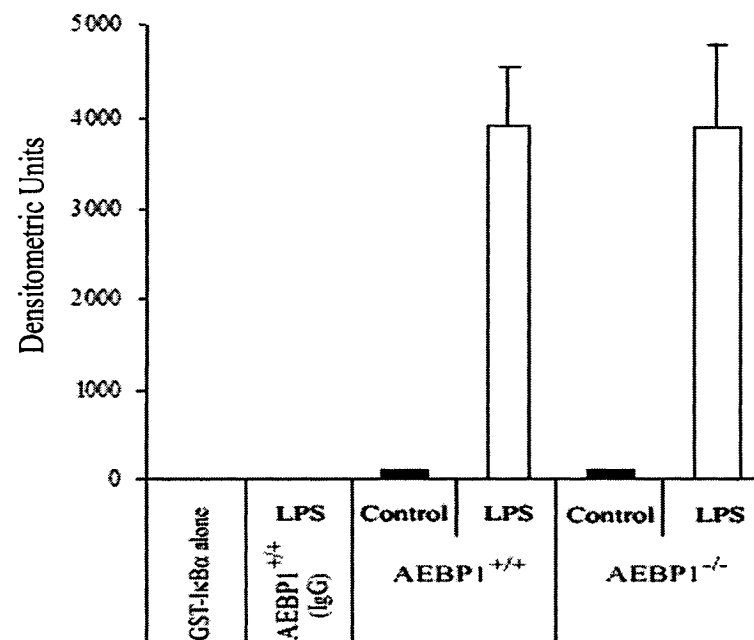
3.14 Protein-Protein Interaction between AEBP1 and IκBα

3.14.1 AEBP1 Physically Interacts with Endogenous IκBα in Macrophages

In an attempt to shed light on the molecular mechanism by which AEBP1 mediates IκBα protein destabilization, it was hypothesized the AEBP1 may be potentially capable of physically interacting with IκBα in a way that renders IκBα more prone to phosphorylation, and thus, proteolytic degradation in macrophages. In order to examine this possibility, co-immunoprecipitation studies were performed in which endogenously expressed IκBα was immunoprecipitated from whole cell protein extracts isolated from un-stimulated and LPS-stimulated AEBP1$^{+/+}$ and AEBP1$^{-/-}$ macrophages. Protein extract obtained from LPS-stimulated AEBP1$^{+/+}$ macrophages was incubated with normal IgG to serve as a negative control for IκBα co-immunoprecipitation analysis. Immune complexes and total protein extracts were subjected to SDS-PAGE followed by immunoblotting for AEBP1, IκBα, and actin. Figure 38, *top left panel*, clearly demonstrates that endogenous AEBP1 physically interacts with endogenous IκBα in macrophages. Importantly, no AEBP1 band was detectable in the normal IgG control sample or IκBα immunoprecipitate samples from AEBP1$^{-/-}$ macrophages (Figure 38, *top left panel*) despite successful IκBα immunoprecipitation (Figure 38, *middle left panel*) and equivalent AEBP1 expression in the IgG control sample (Figure 38, *bottom panel*), suggesting a specific protein-protein interaction between AEBP1 and IκBα. Notably, co-immunoprecipitation analysis demonstrates that AEBP1 physically interacts with IκBα to the same extent under basal and LPS-stimulatory conditions (Figure 38, *top left panel*).

3.14.2 AEBP1 Does Not Interact with Endogenous IκBβ in Macrophages

Due to the relatively high structural similarity between IκBα and IκBβ (May and Ghosh, 1997), it would be interesting to examine whether AEBP1 is capable of interacting with IκBβ in macrophages, as it does with IκBα. For this purpose, endogenously expressed IκBβ was immunoprecipitated from whole cell protein extracts

isolated from un-stimulated and LPS-stimulated AEBP1$^{+/+}$ and AEBP1$^{-/-}$ macrophages. Protein extract obtained from LPS-stimulated AEBP1$^{+/+}$ macrophages was incubated with normal IgG to serve as a negative control for IκBα co-immunoprecipitation analysis. Immune complexes and total protein extracts were subjected to SDS-PAGE followed by immunoblotting for AEBP1, IκBβ, and actin. Interestingly, co-immunoprecipitation studies failed to reveal any interaction between endogenously expressed AEBP1 and IκBβ in un-stimulated and LPS-stimulated macrophages (Figure 38, *top right panel*), despite successful IκBβ immunoprecipitation (Figure 38, *middle right panel*) and despite detection of AEBP1 in whole cell protein extracts (Figure 38, *bottom panel*).

Figure 38. AEBP1 Physically Interacts with Endogenous IκBα, but not IκBβ, in Primary Macrophages. Whole cell protein extracts obtained from un-stimulated (-) and LPS-stimulated (+) macrophages isolated from 20-wk old, HFD-fed AEBP1$^{+/+}$ and AEBP1$^{-/-}$ mice were subjected to co-immunoprecipitation analysis using anti-IκBα antibody (*top left panel*), anti-IκBβ antibody (*top right panel*), or normal IgG. Immunoprecipitate samples were resolved on 8.5% polyacrylamide gels, and nitrocellulose membranes were immunoblotted for AEBP1. Successful IκBα and IκBβ immunoprecipitation was confirmed by immunoblotting using anti-IκBα and anti-IκBβ antibodies (*middle panels*). Whole cell protein extracts (25 μg) were resolved on 8.5% polyacrylamide gels, and nitrocellulose membranes were immunoblotted for AEBP1 and actin to confirm AEBP1 ablation in AEBP1$^{-/-}$ macrophages (*bottom panel*). This data is representative of three independent experiments.
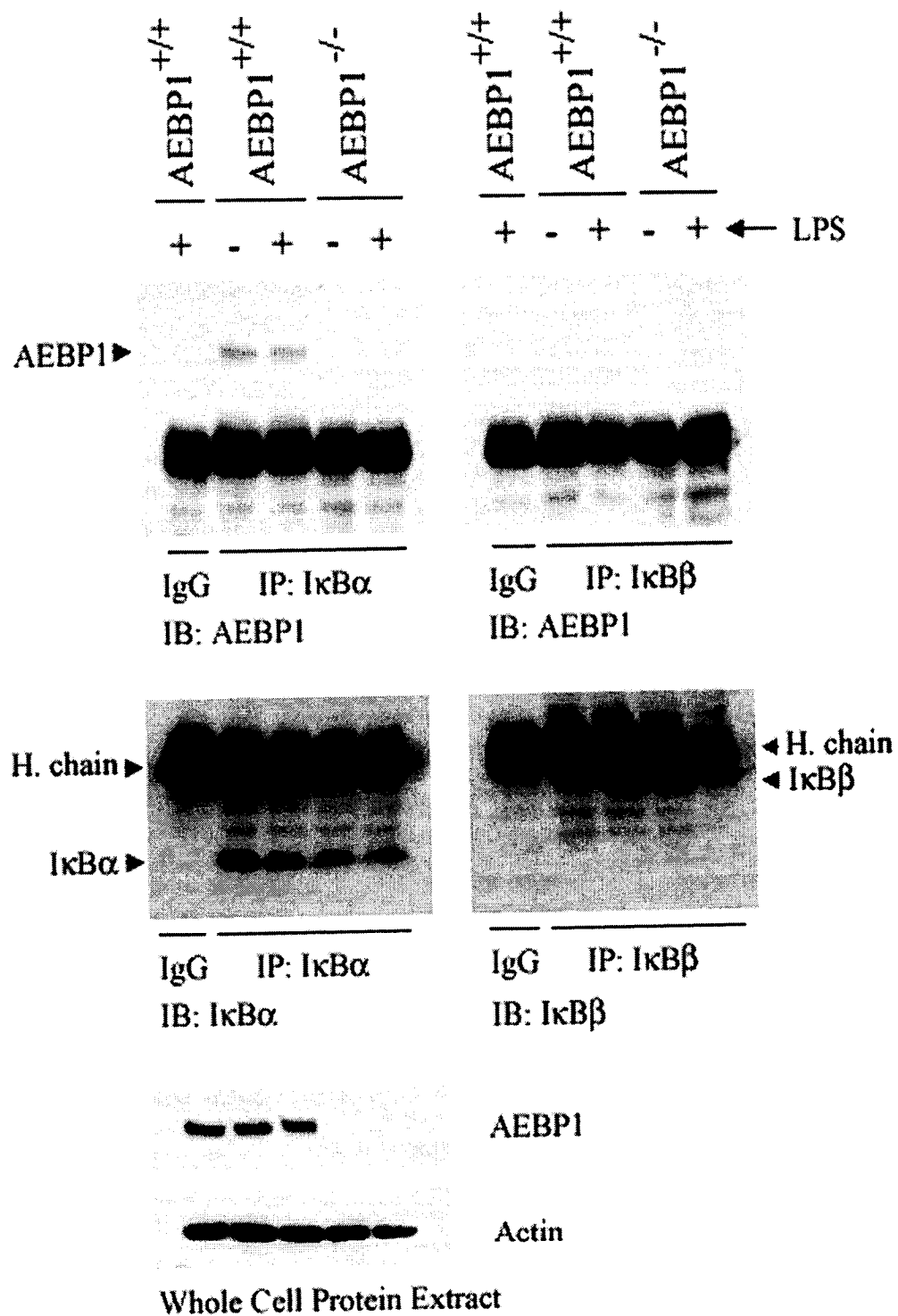
3.14.3 DLD is Required for AEBP1-IκBα Protein-Protein Interaction

In order to determine the region or domain of AEBP1 that is required for mediating protein-protein interaction with IκBα, co-immunoprecipitation studies were performed using C6 glioma transfectants expressing various mutant derivatives of AEBP1 (Figure 39A). Whole cell protein extracts were obtained from un-stimulated and LPS-stimulated C6 transfectants and endogenously expressed IκBα was immunoprecipitated. Normal IgG was incubated with protein extracts obtained from LPS-stimulated C6 glioma transfectants expressing the WT form of AEBP1. Upon immunoprecipitation, immune complexes and whole cell protein extracts were subjected to SDS-PAGE followed by immunoblotting for AEBP1, IκBα, and actin. As shown in Figure 39B, similar levels of AEBP1 derivatives were expressed in C6 glioma transfectants. Interestingly, the WT form of AEBP1 specifically co-immunoprecipitated with IκBα, in which AEBP1 band was absent in the normal IgG control sample (Figure 39C, *top panel*). It is evident that all AEBP1 derivative forms shown in Figure 39A, except ΔN and CP, are capable of binding IκBα (Figure 39C, *top panel*), despite equivalent IκBα immunoprecipitation (Figure 39C, *bottom panel*). Since ΔN and CP are the only mutant derivatives of AEBP1 that lack DLD, these findings suggest that AEBP1 physically interacts with IκBα via its DLD. Consistently, co-immunoprecipitation analysis using whole cell protein extracts obtained from un-stimulated and LPS-stimulated C6 glioma transfectants confirmed that LPS has no noticeable effect on AEBP1-IκBα protein-protein interaction *in vitro* (Figure 39C, *top panel*). Taken together, these findings suggest that AEBP1 is capable of physically interacting with IκBα, but not IκBβ, under basal and LPS-stimulatory conditions via its DLD.

Figure 39. DLD Mediates Protein-Protein Interaction between AEBP1 and IκBα. *(A)* A schematic representation illustrates AEBP1 derivatives with expected protein size. The ability of AEBP1 derivatives to physically interact with endogenous IκBα is indicated (- or +). *(B)* Whole cell protein extracts (25 μg) isolated from un-stimulated (-) and LPS-stimulated (+) C6 glioma transfectants were resolved on 8.5% polyacrylamide gels, and nitrocellulose membranes were immunoblotted with anti-AEBP1 antibody to detect exogenously expressed AEBP1 derivatives. *(C)* Endogenously expressed IκBα was immunoprecipitated from whole cell protein extracts obtained from un-stimulated (-) and LPS-stimulated (+) C6 glioma transfectants. Normal IgG was used as a negative control. Immunoprecipitation samples were resolved on 8.5% polyacrylamide gels, and nitrocellulose membranes were immunoblotted for AEBP1 (*top panel*). An asterisk represents AEBP1 derivatives that co-immunoprecipitated with endogenous IκBα. Successful IκBα immunoprecipitation was confirmed by immunoblotting the nitrocellulose membranes with anti-IκBα antibody (*bottom panel*). This data is representative of three independent experiments.

A
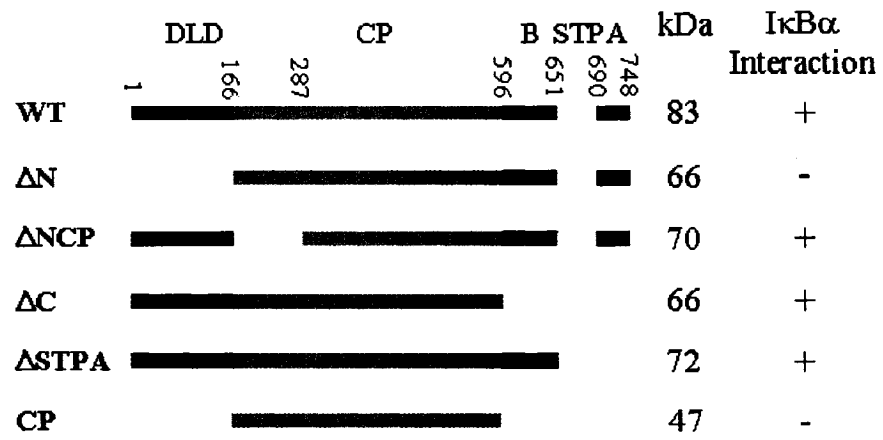
B
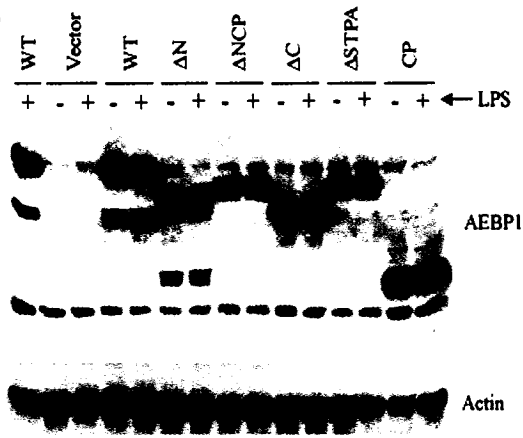
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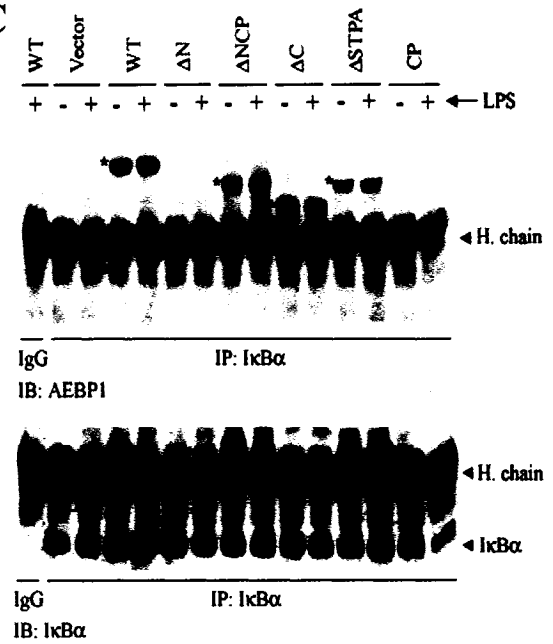
3.15 DLD is Essential for IκBα Down-Regulation and NF-κB Up-Regulation

3.15.1 DLD Deletion Eliminates AEBP1-Mediated IκBα Down-regulation

3.15.1.1 DLD Deletion Effect on IκBα Protein Level and Phosphorylation

Since AEBP1 mutant derivatives that lack DLD were unable to physically interact with IκBα, the effect of deleting DLD of AEBP1 on IκBα protein stability, as well as NF-κB transcriptional activity, was studied. Whole cell protein extracts obtained from un-stimulated and LPS-stimulated C6 glioma cells that are transiently transfected with plasmids expressing various AEBP1 derivatives were subjected to SDS-PAGE and immunoblotting to assess IκBα and IκBβ protein levels, as well as IκBα phosphorylation status. As shown in Figure 40, over-expression of WT, ΔNCP, ΔC, and ΔSTPA, but not ΔN and CP, forms of AEBP1 is accompanied by about 2-fold decreased protein levels of IκBα, but not IκBβ, under basal and LPS-stimulatory conditions, despite equal loading. Consistently, C6 glioma transfectants that express AEBP1 derivatives retaining DLD (i.e. WT, ΔNCP, ΔC, and ΔSTPA) display about 4-fold higher levels of phosphorylated IκBα, under basal and LPS-stimulatory conditions (Figure 40).

Figure 40. DLD is Required for AEBP1-Mediated Enhanced IκBα Phosphorylation. *(A)* Whole cell protein extracts (25 µg) isolated from un-stimulated (-) and LPS-stimulated (+) C6 glioma transfectants were resolved on 8.5% polyacrylamide gels, and nitrocellulose membranes were immunoblotted AEBP1, IκBα, P-IκBα, IκBβ, and actin. *(B)* Protein level was normalized based on actin expression, and normalized protein level was set to 100% in un-stimulated pcDNA empty vector-transfected C6 glioma cells. A histogram illustrates densitometric analysis based on actin expression. Relative protein level is expressed as mean ± SEM (n=3). Statistical significance was determined relative to protein level in un-stimulated and LPS-stimulated pcDNA empty vector- transfected C6 glioma cells.

A

B

LPS (1 μg/ml)

203

3.15.1.2 The Effect of DLD Deletion on IKKβ Catalytic Activity

It is arguable that exogenous over-expression of AEBP1 derivatives retaining DLD can somehow positively influence the catalytic activity of endogenously expressed IKKβ, leading to increased phosphorylation of IκBα. To rule out this possibility, IKKβ catalytic activity in C6 glioma transfectants expressing exogenous AEBP1 derivatives was assessed by *in vitro* IKK kinase assay. The IKK complex was immunoprecipitated from un-stimulated and LPS-stimulated C6 glioma transfectants and incubated with bacterially expressed GST-IκBα (1-54) fusion protein as a substrate. Autoradiography revealed that AEBP1 derivatives expressed in C6 glioma cells displayed no differences with regard to IKKβ catalytic activity, despite equal immunoprecipitation and protein loading (data not shown). Consistent with the analysis of IKKβ catalytic activity in macrophages, this data demonstrates that DLD deletion has no effect on endogenous IKKβ catalytic activity *in vitro*, indicating that DLD is directly involved in AEBP1-mediated enhanced phosphorylation and proteolytic degradation of IκBα.

3.15.1.3 The Effect of DLD Deletion on IκBα Protein Stability

In order to evaluate the physiological importance of DLD in AEBP1-mediated IκBα protein degradation, whole cell protein extracts obtained from CHX-treated C6 glioma transfectants expressing the WT, ΔN, and ΔC derivatives of AEBP1 were subjected to SDS-PAGE followed by immunoblotting to assess IκBα protein stability. Densitometric analysis of Western blots revealed that DLD deletion eliminates the ability of AEBP1 to promote IκBα proteolytic degradation (Figure 41). Importantly, the ΔC mutant form of AEBP1, which was used as a positive control, behaved similar to the WT form (Figure 41). In those cells expressing WT, ΔN, or ΔC derivatives, the protein half-life of IκBα is estimated to be 60 min, >240 min, and 60 min, respectively. In agreement with previous data, transfectants expressing various derivatives of AEBP1 displayed no significant differences with respect to IκBβ or p53 protein turnover (Figure 41A), substantiating the role of AEBP1 as a specific inducer of IκBα phosphorylation and

proteolytic degradation. These findings indicate that DLD is indispensable for AEBP1-mediated enhanced IκBα phosphorylation and proteolytic degradation.

Figure 41. DLD is Required for AEBP1-Mediated Enhanced IκBα Proteolytic Degradation. *(A)* C6 glioma transfectants expressing WT, ΔN, and ΔC forms of AEBP1 were treated with ethanol (vehicle) or 50 μg/ml CHX for the indicated times. Whole cell protein extracts were obtained, and 25 μg total protein extracts were resolved on 8.5% polyacrylamide gels. Nitrocellulose membranes were immunoblotted for AEBP1, IκBα, IκBβ, p53, and actin. *(B)* IκBα protein level was normalized based on actin expression, and normalized IκBα protein level was set to 100% in ethanol-treated samples (i.e. 0 min samples) within every transfectant group. Data is expressed as mean ± SEM (n=3). Statistical significance was determined relative to IκBα protein level in C6 glioma cells expressing WT AEBP1 at each time point.

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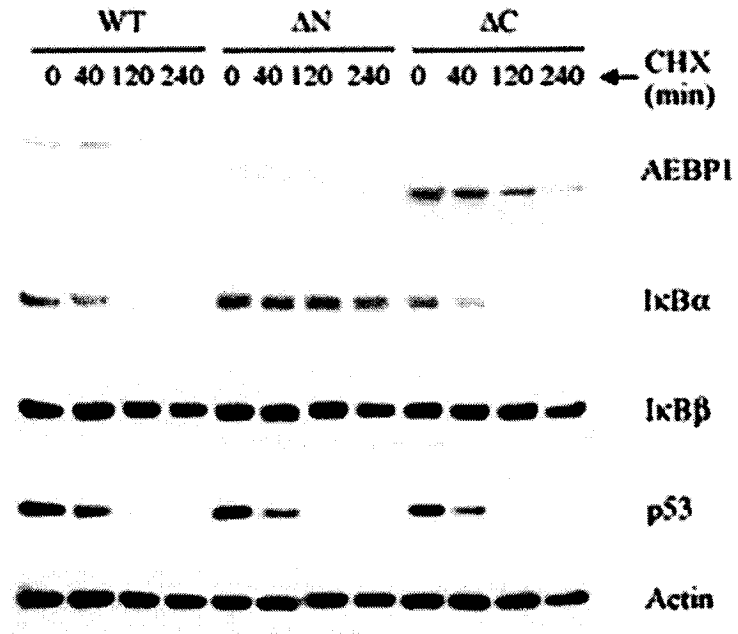
B
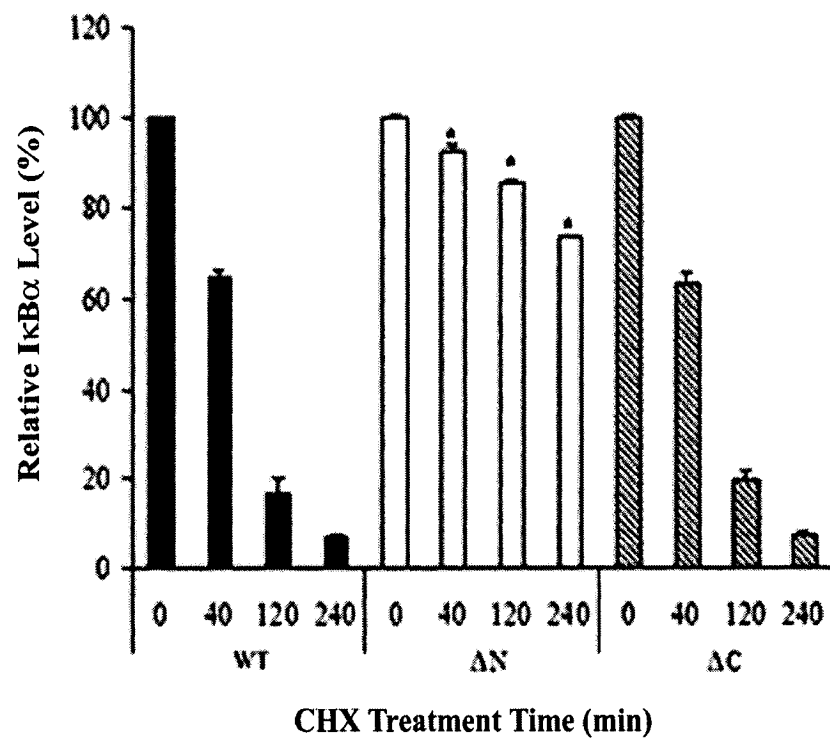
3.15.2 DLD is Required for AEBP1-Mediated NF-κB Up-regulation

3.15.2.1 The Effect of DLD Deletion on NF-κB Transcriptional Activity

Because DLD is shown to be crucial in AEBP1-mediated IκBα down-regulation *in vitro*, it is expected that DLD deletion will abolish the ability of AEBP1 to enhance NF-κB activity. To investigate this hypothesis, NF-κB transcriptional activity was evaluated in C6 glioma transfectants expressing various AEBP1 derivatives by two means. First, nuclear protein extracts obtained from un-stimulated and LPS-stimulated C6 glioma transfectants were subjected to SDS-PAGE followed by immunoblotting using anti-p65 antibody. Based on actin levels, densitometric analysis of Western blots clearly revealed that C6 glioma cells that express WT, ΔNCP, ΔC, and ΔSTPA possess higher nuclear p65 protein levels, whereas cells that express ΔN and CP have nuclear p65 protein levels that are equivalent to those in cells transfected with empty vector, under basal and LPS-stimulatory conditions (Figure 42).

Second, to further confirm the importance of DLD in AEBP1-mediated enhanced NF-κB transcriptional activity, NF-κB-luciferase reporter assay was performed using whole cell protein extracts obtained from C6 glioma transfectants expressing various AEBP1 derivatives, in which NF-κB activity was evaluated under basal and LPS-stimulatory conditions. Compared to empty vector transfection, expression of WT, ΔNCP, ΔC, or ΔSTPA forms of AEBP1 was accompanied with significant increase (5 fold) of NF-κB transcriptional activity under basal and LPS-stimulatory conditions (Figure 42C). Notably, WT, ΔNCP, ΔC, and ΔSTPA derivatives of AEBP1 exhibited no significant differential ability among themselves to enhance NF-κB transcriptional activity (Figure 42C). On the other hand, ΔN and CP derivatives had no significant effect on NF-κB transcriptional activity under the same conditions (Figure 42C), indicating a crucial role of DLD in mediating NF-κB transcriptional transactivation by AEBP1 *in vitro*.

Figure 42. DLD is Required for AEBP1-Mediated Up-Regulation of NF-κB Transcriptional Activity. *(A)* Nuclear protein extracts (25 µg) isolated from un-stimulated (-) and LPS-stimulated (+) C6 glioma transfectants expressing the indicated forms of AEBP1 were resolved on 8.5% polyacrylamide gels, and nitrocellulose membranes were immunoblotted AEBP1, p65, and actin. *(B)* p65 protein level was normalized based on actin expression, and normalized p65 protein level was set to 100% in un-stimulated, pcDNA empty vector-transfected C6 glioma cells. A histogram illustrates densitometric analysis based on actin expression. Relative p65 protein level is expressed as mean ± SEM (n=3). Statistical significance was determined relative to p65 protein level in un-stimulated and LPS-stimulated, pcDNA empty vector-transfected C6 glioma cells. *(C)* C6 glioma cells were transiently transfected with 200 ng pNF-κB-TA-luciferase using lipofectamine[2000]. Cells were co-transfected with 20 ng pHermes-lacZ, which expresses β-galactosidase, along with 10 ng pcDNA empty vector or pcDNA AEBP1-expressing constructs carrying cDNAs of the indicated AEBP1 derivatives. Forty-eight hours post transfection, cells were left un-stimulated or stimulated with 1 µg/ml LPS for 30 min. Cells were subsequently lysed and subjected to luciferase and β-galactosidase assays. Luciferase activity, normalized to β-gal activity, was set to 100% for the un-stimulated, pcDNA empty vector-transfected C6 glioma cells. Relative luciferase activity is expressed as mean ± SEM (n=8). Statistical significance was determined based on relative luciferase activity in un-stimulated and LPS-stimulated, pcDNA empty vector-transfected C6 glioma cells.

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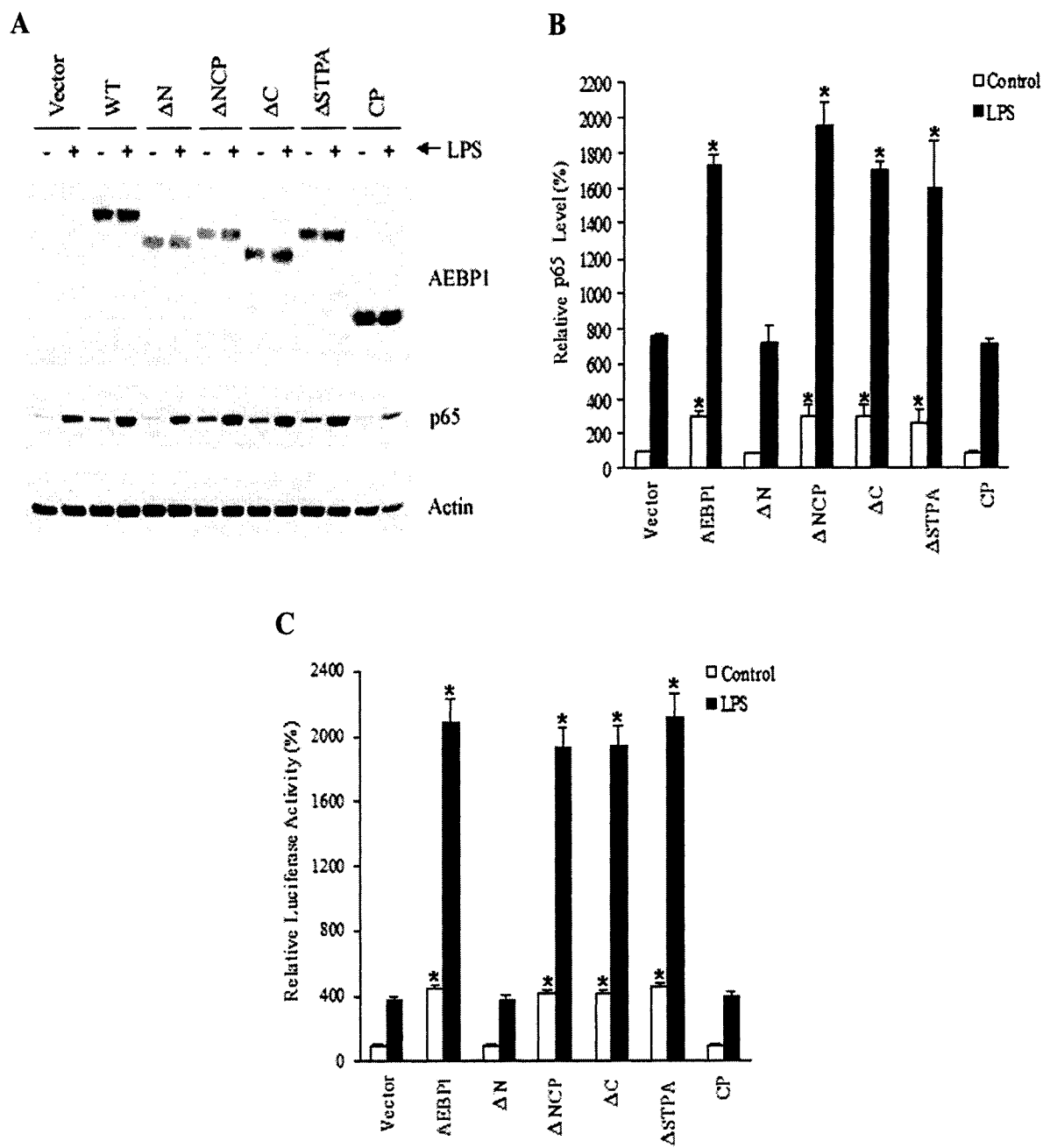
3.15.2.2 The Effect of DLD Deletion on NF-κB DNA-Binding Activity

To further confirm DLD involvement in AEBP1-mediated NF-κB up-regulation, NF-κB DNA-binding activity was evaluated in C6 glioma transfectants expressing various AEBP1 derivatives. To that end, EMSA was performed using nuclear protein extracts obtained from un-stimulated and LPS-stimulated C6 glioma transfectants. Autoradiography illustrated that AEBP1 derivatives that retain DLD are capable of enhancing NF-κB DNA-binding activity in un-stimulated and LPS-stimulated cells (Figure 43). In contrast, AEBP1 derivatives that lack DLD (i.e. ΔN and CP) were incapable of enhancing the DNA-binding activity of NF-κB (Figure 43). Collectively, these findings strongly suggest that DLD is essential for AEBP1-mediated NF-κB up-regulation, consistent with the effect of DLD deletion on the ability of AEBP1 to promote IκBα phosphorylation and proteolytic degradation.
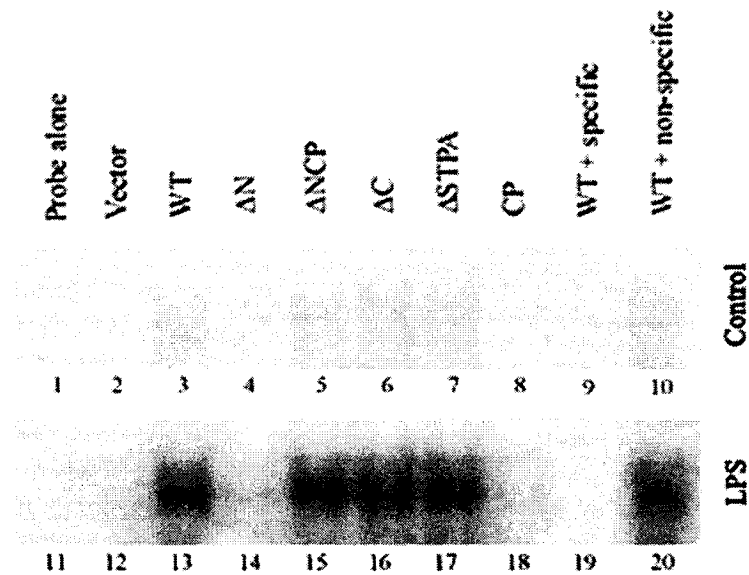
Figure 43. DLD is Required for AEBP1-Mediated Up-Regulation of NF-κB DNA-Binding Activity. 2.0 μg nuclear protein extracts obtained from un-stimulated and LPS-stimulated C6 glioma transfectants expressing the indicated forms of AEBP1 were subjected to NF-κB EMSA in presence of specific (unlabeled NF-κB probe) (*lanes 9 and 19*) or non-specific (unlabeled, unrelated probe) (*lanes 10 and 20*) competitors. ^{32}P-labeled NF-κB probe was run alone as a negative control (*lanes 1 and 11*). This data is representative of three independent experiments.

3.16 AEBP1 has no Effect on CD11b, CD14, CD40, FcγR, or TLR4 Expression

3.16.1 CD11b, CD40, and FcγR, Expression in Macrophages

During the process of monocyte differentiation into macrophages, a wide range of specific cell-surface markers (e.g. CD11b (Mac-1), CD11c, CD14, and CD40) are expressed on the plasma membrane of mature macrophages. Since sufficient expression of such markers is critical for the proper biological functions of macrophages, AEBP1^{TG}, AEBP1^{NT}, AEBP1$^{+/+}$, and AEBP1$^{-/-}$ macrophages were compared in terms of CD11b, CD40, as well as FcγR surface expression by FACS analysis. As shown in Figures 44 A & B, the expression pattern of CD11b, CD40, and FcγR is indistinguishable in macrophages isolated from AEBP1^{TG} and AEBP1^{NT} or AEBP1$^{+/+}$ and AEBP1$^{-/-}$ mice, respectively. This data suggests that AEBP1 over-expression or ablation has no effect on the expression of main cell-surface markers in macrophages.

3.16.2 CD14 and TLR4 Expression in Macrophages

TLR4 has been shown to act as the functional receptor responsive to signaling initiated by bacterial LPS (Poltorak *et al.*, 1998) following specific binding to CD14 and LPS-binding protein (LBP) (Wright *et al.*, 1990) that are expressed on various cell types including macrophages. Since NF-κB transcriptional and DNA-binding activities are assessed under basal and LPS-stimulatory condition, it is important to compare the expression of TLR4 and CD14 on the cell surface of macrophages that over-express or lack AEBP1, and thus, eliminate the possibility that the differential NF-κB activity associated with differential AEBP1 expression in macrophages is not due to altered levels of TLR4 or CD14. Hence, FACS analysis was performed on AEBP1^{TG}, AEBP1^{NT}, AEBP1$^{+/+}$, and AEBP1$^{-/-}$ un-stimulated macrophages cultured in standard medium. As shown in Figures 44 C & D, TLR4 and CD14 cell surface levels are comparable in AEBP1^{TG} and AEBP1^{NT}, as well as in AEBP1$^{+/+}$ and AEBP1$^{-/-}$ macrophages, respectively. This data indicates that AEBP1 does not influence the expression of TLR4

or CD14, suggesting indiscriminative LPS-induced signaling cascade leading to NF-κB activation in macrophages.

Figure 44. AEBP1 has no Effect on CD11b, CD14, CD40, FcγR, or TLR4 Expression in Macrophages. 5.0 X 10^5 un-treated macrophages isolated from 32-wk old, HFD-fed AEBP1^{TG} and AEBP1^{NT} mice *(A & C)*, as well as 20-wk old, HFD-fed AEBP1$^{+/+}$ and AEBP1$^{-/-}$ mice *(B & D)* were subjected to FACS analysis to assess CD11b, CD14, CD40, FcγR, and TLR4 cell-surface expression. FITC- and PE-conjugated specific and isotype control antibodies were used at 1 µg/ml working concentration. Refer to section 2.7.6 for more details. The expression profiles of each protein among different genotypes were overlaid.

A

CD11b CD40 FcγR

AEBP1TG (isotype)
AEBP1NT (isotype)
AEBP1TG (positive)
AEBP1NT (positive)

B

CD11b CD40 FcγR

AEBP1+/+ (isotype)
AEBP1-/- (isotype)
AEBP1+/+ (positive)
AEBP1-/- (positive)

C

CD14 TLR4

AEBP1TG (isotype)
AEBP1NT (isotype)
AEBP1TG (positive)
AEBP1NT (positive)

D

CD14 TLR4

AEBP1+/+ (isotype)
AEBP1-/- (isotype)
AEBP1+/+ (positive)
AEBP1-/- (positive)

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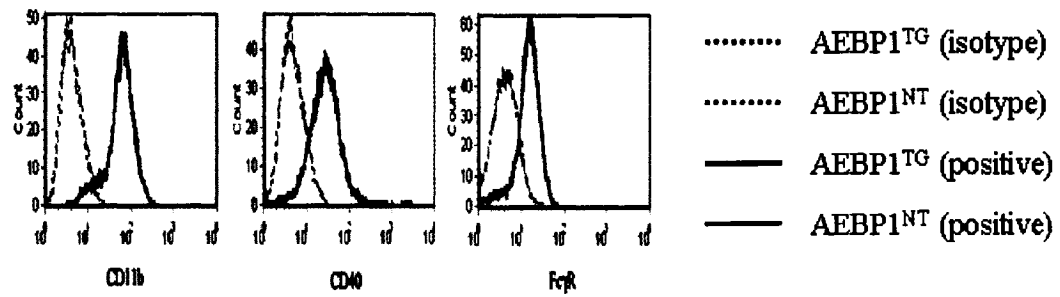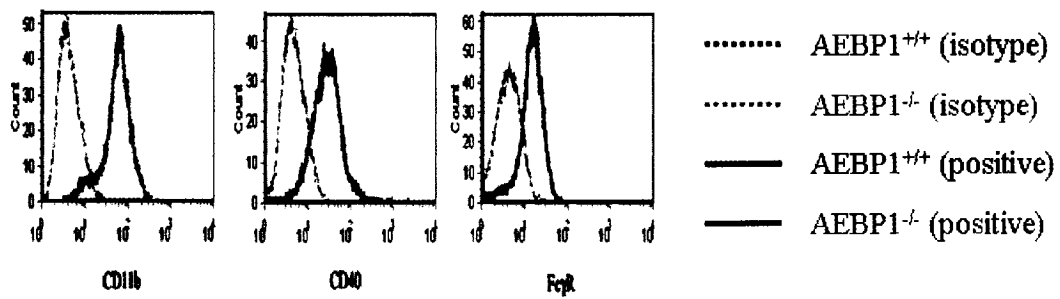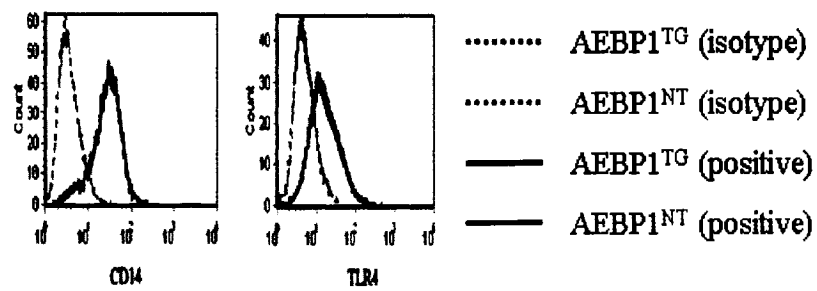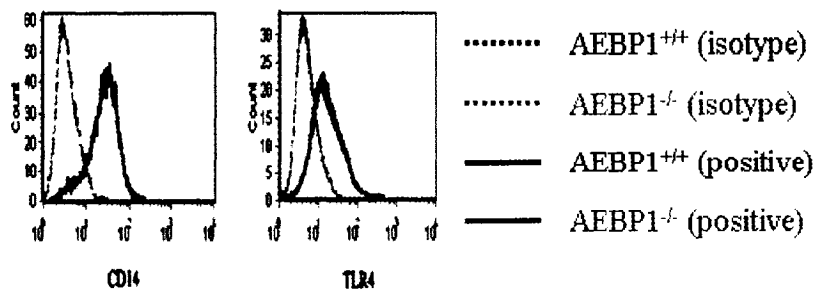
3.16.3 CD14 and TLR4 Expression in C6 Glioma Transfectants

To rule out the possibility that over-expression of AEBP1 derivatives alters the cell-surface levels of CD14 and TLR4, FACS analysis was performed using C6 glioma transfectants expressing various AEBP1 derivatives. Consistent with the observation that AEBP1 expression has no correlation with CD14 or TLR4 expression in macrophages, Figure 45 illustrates that C6 glioma transfectants expressing WT, ΔN, ΔNCP, ΔC, ΔSTPA, and CP derivatives of AEBP1 express equivalent levels of CD14 and TLR4 on their cell surface. Again, these results suggest that AEBP1 has no effect on the cell-surface expression of CD14 or TLR4.
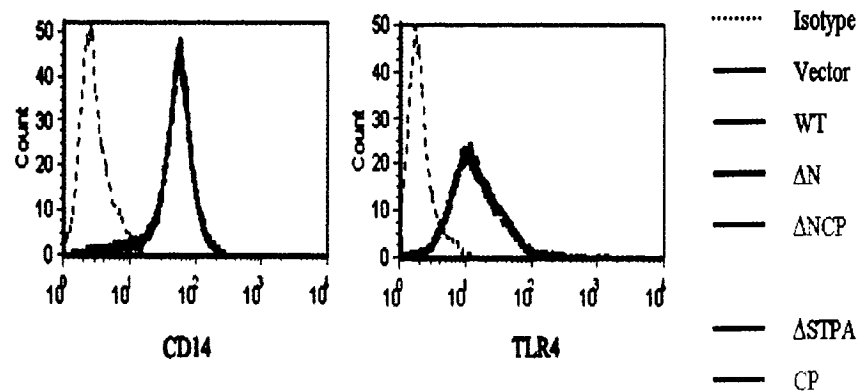
Figure 45. AEBP1 Derivatives have no Effect on CD14 or TLR4 Expression in C6 Glioma Cells. 1.2×10^6 untreated C6 glioma cells were transiently transfected with 4 µg pcDNA empty vector or pcDNA constructs carrying cDNAs of the indicated AEBP1 derivatives using lipofectamine^{2000}. Forty-eight hours post transfection, C6 glioma transfectants were washed twice with 1X PBS and lifted sing trypsin-EDTA. Cells were briefly centrifuged and subjected to FACS analysis to assess CD14 and TLR4 cell-surface expression. FITC- and PE-conjugated specific and isotype control antibodies were used at 1 µg/ml working concentration. Refer to section 2.7.6 for more details. The expression profiles of each protein among different transfectants were overlaid.

Chapter 4

DISCUSSION

4.1 The Role of AEBP1 in Macrophage Cholesterol Homeostasis

According to the model proposed by Chawla and colleagues, uptake of oxLDL leads to PPARγ1 and LXRα activation in macrophages (Chawla *et al.*, 2001). LXRα directly up-regulates ABCA1, ABCG1, and ApoE expression, promoting cholesterol clearance from macrophages. My findings strongly indicate that AEBP1 modulates macrophage cholesterol homeostasis by acting as a DNA-binding-dependent transcriptional repressor of PPARγ1 and LXRα *in vitro* and *in vivo*. Consistent with its ability to repress PPARγ1 and LXRα, AEBP1 over-expression and ablation lead to decreased and increased levels of ABCA1, ABCG1, and ApoE in macrophages, respectively (Figure 20). As expected, inhibited expression of ABCA1, ABCG1, and ApoE via PPARγ1 and LXRα transcriptional repression by AEBP1 results in inefficient cholesterol efflux from macrophages (Figure 22B). Indeed, AEBP1^{TG} macrophages accumulate considerable amounts of lipids in their cytoplasmic compartments (Figure 22A), indicating that sustained lipid accumulation is a direct indication of disrupted cholesterol efflux in macrophages that express significantly decreased levels of ABCA1, ABCG1, and ApoE due to AEBP1 over-expression. The ability of AEBP1 to interfere with macrophage cholesterol efflux predisposes AEBP1^{TG} macrophages to serve as foam cell precursors that can lead to atherosclerotic lesion formation.

These findings strengthen the model proposing that activation of PPARγ1 and LXRα is essential for up-regulated surface expression of ABC transporters and ApoE, as well as successive removal of accumulated lipids in macrophages (Chawla *et al.*, 2001; Laffitte *et al.*, 2001b). A PPAR response element (PPRE) was identified in the regulatory region of ApoE, suggesting that PPAR activation can potentially induce ApoE expression,

subsequently regulating foam cell formation and atherogenesis (Galetto *et al.*, 2001). Similarly, LXRα promotes ApoE expression in macrophages due to the presence of a conserved LXR response element (LXRE) in the regulatory region of ApoE (Laffitte *et al.*, 2001b). Thereby, negative regulation of ApoE by AEBP1 is consistent with AEBP1-mediated transcriptional repression of PPARγ1 and LXRα in macrophages (Figure 20).

PPARγ1 induction of lipid uptake via CD36 and lipid efflux via LXRα-ABCs raises the question of whether the net effect of PPARγ1 activation would be to promote or impede foam cell formation. Although PPARγ1 induces CD36 up-regulation, forcing macrophages to uptake and accumulate lipids, it concurrently induces expression of LXRα, ABCs, ApoE, and lipoprotein lipase (LPL), crucial factors favoring macrophage cholesterol efflux (Akiyama *et al.*, 2002). Meaningfully, a bone marrow transplantation experiment revealed that the PPARγ1-LXRα-ABC efflux pathway dominates *in vivo* (Chawla *et al.*, 2001). Consistently, my findings support a protective role of PPARγ1 against foam cell formation since PPARγ1 down-regulation in AEBP1^{TG} macrophages is accompanied by decreased levels of not only LXRα, ABCA1, ABCG1, and ApoE, but also CD36.

4.2 AEBP1 is a DNA Binding-Dependant Transcriptional Repressor

A large number of studies have previously suggested that AEBP1 transcriptional repression function requires DNA binding by AEBP1. In particular, AEBP1 has been shown to bind to AE-1 sequence within the promoter region of aP2 gene, and that AEBP1-AE-1 interaction is required for aP2 repression by AEBP1 (He *et al.*, 1995; A. Muise, unpublished). In fact, deletion of the terminal 205 amino acid residues in the C-terminus of AEBP1 (i.e. AEBP1$^{\Delta Sty}$) eliminates not only DNA binding, but also transcriptional repression of aP2 by AEBP1 (He *et al.*, 1995; A. Muise, unpublished). Another piece of evidence suggesting the importance of DNA-binding in the transcriptional activity of AEBP1 has been provided by a study demonstrating that mutation of T^{623} at the C-terminus of AEBP1 interferes with both DNA-binding to AE-1 and transcriptional repression of aP2 (Lyons *et al.*, 2005). Nonetheless, whether AEBP1 binding to the AE-1 sequence is essential for aP2 transcriptional repression by AEBP1 has been recently questioned (PhD

thesis of P.J. Lyons, 2005), in which it is demonstrated that AEBP1$^{\Delta Sty}$ is capable of repressing aP2 in CHO cells despite its inability to bind DNA. Interestingly, AEBP2, an AEBP1-related zinc finger transcriptional repressor, has been previously shown to be capable of binding the AE-1 sequence within the aP2 promoter, as well as aP2 repression (He *et al.*, 1999). In fact, a point mutation in the middle of the zinc finger motif of AEBP2 inhibited aP2 repression, but not binding to the AE-1 sequence. Based on these studies, the significance of DNA-binding in AEBP1-mediated repression remains elusive.

Noteworthy, however, previous studies have relied only on EMSA to evaluate DNA-binding by AEBP1, which does not necessarily provide a realistic picture of what happens at the cellular level. Interestingly, my findings provide both *in vitro* and *in vivo* experimental evidence supporting the notion that DNA-binding by AEBP1 is an essential step in its transcriptional repression function. I show that AEBP1 is capable of binding putative AE-1-like binding sites within the promoter regions of PPARγ1 and LXRα by EMSA (Figure 15B), and I also show that AEBP1, but not AEBP1$^{\Delta Sty}$, represses PPARγ1 and LXRα both *in vitro* and *in vivo* (Figure 18). Based on this data, one can infer that DNA-binding by AEBP1 is associated with its transcriptional repression function. To further assess the importance of DNA-binding at the cellular level, I relied on luciferase reporter assays using luciferase constructs carrying either wildtype PPARγ1 and LXRα promoters or mutated PPARγ1 and LXRα promoters lacking the putative AEBP1-binding sites. My findings clearly illustrate that deletion of such putative AEBP1-binding sites completely eliminates the ability of AEBP1 to transcriptionally repress PPARγ1 and LXRα (Figures 19 A & B, respectively), providing direct evidence that DNA-binding is crucial for transcriptional repression by AEBP1 *in vitro* and *in vivo*. Undoubtedly, chromatin immunoprecipitation (ChIP) assay is necessary to further confirm the significance of AEBP1-DNA interaction towards the transcriptional repression function of AEBP1 at the cellular level.

4.3 AEBP1-Mediated PPARγ1 and LXRα Inhibition: Is MAPK Involved?

Previously, MAPK was shown to be a negative regulator of PPARγ1 activity, in which active MAPK is capable of phosphorylating PPARγ1 at S^{82} *in vitro* and *in vivo*,

rendering PPARγ1 transcriptionally inactive (Camp and Tafuri, 1997). So, enhanced MAPK activation can potentially interfere with PPARγ1 functions in lipid metabolism, macrophage cholesterol homeostasis, and macrophage pro-inflammatory responsiveness. Since AEBP1 is a positive regulator of MAPK activity, it is plausible that AEBP1-mediated inhibition of PPARγ1 transcriptional activity may be attributed in part to AEBP1-mediated prolonged MAPK activity, besides the ability of AEBP1 to transcriptionally repress PPARγ1 expression *in vivo* and *in vitro*. However, this possibility seems to be invalid given that AEBP1 inhibits PPARγ1 transcriptional activity equally efficiently in presence and absence of a MAPK inhibitor, U0126, *in vitro* (Figure 17A). This is also supported by the fact that AEBP1-mediated inhibition of LXR transcriptional activity is not affected by U0126 *in vitro* (Figure 17B). Therefore, AEBP1-mediated down-regulation of PPARγ1 and LXRα transcriptional activity seems to be independent of AEBP1-mediated sustained activation of MAPK.

4.4 The Role of AEBP1 in Macrophage Inflammatory Responsiveness

A wide range of macrophage-derived pro-inflammatory mediators including cytokines, chemokines, and proteolytic enzymes are directly implicated in atherogenesis (Steffens and Mach, 2004). Interestingly, my findings clearly indicate that IL-6, TNFα, MCP-1, and iNOS expression positively correlates with AEBP1 expression in macrophages (Figures 23 and 24). It is worth mentioning that AEBP1 is not only capable of enhancing the expression of pro-inflammatory mediators upon macrophage activation by LPS or LPS plus IFNγ, but it is also capable of inducing the expression of such mediators in absence of an activation signal (Figure 23). Interestingly, this is consistent with the observation that AEBP1 up-regulates NF-κB activity in macrophages under basal and LPS-stimulatory conditions (Figures 32 and 33). This positive correlation seems to be an indispensable means by which AEBP1 exerts its pro-inflammatory, pro-atherogenic functions in mice. The ability of AEBP1 to promote macrophage inflammatory responsiveness indicates that this effect may not be localized, and that, compared to their control counterparts, AEBP1^{TG} and AEBP1$^{-/-}$ mice may display elevated and reduced levels of pro-inflammatory mediators in their plasma, respectively.

In fact, preliminary evidence suggests that plasma obtained from AEBP1$^{-/-}$ mice has markedly less circulatory IL-6 and TNFα compared to that obtained from AEBP1$^{+/+}$ mice (data not shown). Testing this possibility is critical with regard to the ability of AEBP1^{TG} and AEBP1$^{-/-}$ mice to combat bacterial and viral infections. In other words, I anticipate that AEBP1^{TG} mice are more resistant, while AEBP1$^{-/-}$ mice are more susceptible, to microbial infections based on their circulatory levels of pro-inflammatory/immune mediators. In addition, based on their differential NF-κB activity profile, and thus, pro-inflammatory/immune responsiveness, I anticipate that AEBP1^{TG} mice are more susceptible, while AEBP1$^{-/-}$ mice are more resistant, to LPS-induced septic shock.

4.5 AEBP1 Promotes Hyperlipidemia and Diet-Induced Atherosclerosis in Mice

Interestingly, AEBP1^{TG} males, which have plasma cholesterol and triglyceride levels that are statistically comparable to their AEBP1^{NT} male counterparts (Figure 25), clearly develop atherosclerotic lesions (Table 6). Here, it is important to emphasize that atherogenesis can be initiated and advanced despite normal plasma lipoprotein profile. It would be of interest to compare the serum lipid profile in AEBP1^{TG} and AEBP1^{NT} females and males that are fed chow diet in order to have a better understanding of the exact effects of serum cholesterol/triglyceride levels on atherosclerotic lesion development in AEBP1^{TG} mice. However, such data is currently lacking. Noteworthy, macrophages isolated from AEBP1^{TG} males expresses significantly higher levels of IL-6, TNFα, MCP-1, and iNOS compared to those isolated from AEBP1^{NT} mice, confirming that inflammation is a critical event in atherogenesis. In fact, there is a general consensus among researchers that hyperlipidemia is not sufficient on its own to lead to the development of advanced atherosclerotic lesions, and that inflammatory events such as monocyte recruitment, macrophage activation, cytokine and chemokine production, as well as infiltration of other immune cells (e.g. T lymphocytes, neutrophils, and mast cells) are critical for atherosclerotic lesion initiation and progression. Many studies have demonstrated that promotion or inhibition of atherogenesis is not always preceded or accompanied by changes in the plasma lipoprotein profile (Gu et al., 1998; Tsukamoto et al., 2000; Cybulsky et al., 2001; Song et al., 2001). Hence, these findings lend support to the

inflammatory aspect of atherosclerosis, which can develop despite normal plasma lipoprotein profile. Interestingly, AEBP1^{TG} mice seem to be relatively resistant to elevated cholesterol levels upon HFD challenge. While HFD-fed ApoE$^{-/-}$ and LDLR$^{-/-}$ have about 1800 mg/dL and 1500 mg/dL plasma cholesterol levels (about 6-fold higher than in wildtype mice), respectively, HFD-fed AEBP1^{TG} females have less than 2-fold elevated cholesterol levels compared to HFD-fed AEBP1^{NT} counterparts (Figure 25). So, AEBP1^{TG} mice may serve as a favorite *in vivo* model to elucidate the inflammatory events during atherogenesis in absence of severe hyperlipidemia.

Admittedly, the atherosclerotic lesions detected in AEBP1^{TG} mice are atypical and morphologically distinct from those found in ApoE$^{-/-}$ and LDLR$^{-/-}$ mice. For example, in contrast to ApoE$^{-/-}$ and LDLR$^{-/-}$ lesions, AEBP1^{TG} lesions occupy a single site of the aortic lumen (Figure 26A), and rarely two lesions can be detected within one aortic cross-section. In comparison, ApoE$^{-/-}$ and LDLR$^{-/-}$ mice develop lesions at multiple sites within the aortic lumen, and they can be found protruding into the lumen from almost every area of the endothelial monolayer. This indicates to me that aortae of AEBP1^{TG} mice are not densely occupied with lesions, and that sporadic lesions are distributed sparsely along the aortic endothelial monolayer, whereas ApoE$^{-/-}$ and LDLR$^{-/-}$ mice form lesions that almost fully occupy the entire endothelial monolayer of the aorta. Most importantly, the lipid-filled, cell-dense lesions detected in AEBP1^{TG} mice are surrounded by very thin fibrous caps that are hardly detectable. Lesions found in ApoE$^{-/-}$ and LDLR$^{-/-}$ mice contain very well-characterized, relatively thick fibrous caps (Plump *et al.*, 1992; Ishibashi *et al.*, 1994). This difference, nonetheless, is very intriguing since it urges me to speculate that the premature sudden death events seen in AEBP1^{TG} females may be at least partially due to lesion instability. In other words, I postulate that the very thin fibrous caps surrounding the lipid-filled, cell-dense lesions found in AEBP1^{TG} females become prone to degradation, leading to thrombi formation and eventually life-threatening conditions as a result of atherothrombosis. The fact that ApoE$^{-/-}$ and LDLR$^{-/-}$ mice, which develop relatively thick fibrous caps around their lesions, do not encounter premature sudden death events supports my speculation. Interestingly, some of the lesions detected in AEBP1^{TG} mice can be found semi-detached from the endothelial monolayer (data not shown), a likely indication of lesion instability and destruction.

Herein, it is important to mention that preliminary evidence suggesting increased expression of MMP-9 in macrophages isolated from AEBP1^{TG} mice (data not shown) further supports a role of plaque rupture in premature sudden death events in AEBP1^{TG} mice. However, my speculation is less convincing given that AEBP1^{TG} males, which develop lesions that are morphologically identical to those found in AEBP1^{TG} females, evade premature sudden death and are totally viable.

In addition to differences regarding the thickness of fibrous caps, lesions found in AEBP1^{TG} mice are more isolated and harder to detect. In terms of lesion content, AEBP1^{TG} lesions posses high lipid content and are cell-dense (Figures 26 A & C). Unfortunately, only the proximal aorta including the ascending aorta and arch, but excluding the aortic root leaflet regions, was sectioned and examined for lesion detection. Hence, it is impossible to comment on whether AEBP1^{TG} mice develop lesions in the aortic root, coronary and pulmonary arteries, or the distal descending aorta. I strongly believe that en face analysis of the whole aorta (from the aortic root until 3-5 mm after the iliac bifurcation) should be performed to provide better characterization of lesion localization in AEBP1^{TG} mice.

4.6 Gender-Specific Differences Regarding AEBP1's Pro-Atherogenicity

Despite the observation that atherosclerotic lesions are detected in both genders of AEBP1^{TG} mice, females develop lesions more prevalently (94% in females, 71% in males), and of a significantly larger mean lesion area (Tables 6 & 7). Here, a number of points are noteworthy. Despite a strong body of literature suggesting that males are more susceptible to the development of atherosclerosis than females (Holm, 2001; Mendelsohn, 2002; Hodgin and Maeda, 2002), my data is consistent with other studies suggesting that in HFD-fed mice, C57/BL6 females are more susceptible to atherosclerosis and develop larger lesions compared to their male counterparts (Paigen et al., 1987a; Paigen et al., 1987b; Ishii et al., 1995; Smith et al., 2001). Several explanations were provided to account for gender-specific differences with regard to athero-susceptibility (Paigen et al., 1987a; Dansky et al., 1999; Song et al., 2001). The only satisfactory explanation for the gender-specific, athero-susceptibility differences detected in AEBP1^{TG} mice is that AEBP1^{TG} females, but not

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males, have significantly elevated cholesterol and triglyceride serum levels compared to their AEBP1^{NT} counterparts (Figure 25). As mentioned earlier, the ability of AEBP1 to repress PPARγ1, LXRα, and their downstream target genes, promote foam cell formation, and enhance macrophage inflammatory responsiveness is not gender-, or age-specific. So, it is not conceivable that the gender-specific atherogenic differences seen in AEBP1^{TG} mice can be attributed to differential ability of AEBP1 to either impede macrophage cholesterol efflux or promote macrophage pro-inflammatory responsiveness in those mice.

It is worth mentioning that many studies have demonstrated that circulating estrogen (17β-estradiol) and its receptors, estrogen receptors α and β (ERα and ERβ), play protective roles against the development of atherosclerosis (Holm, 2001; Mendelsohn, 2002; Hodgin and Maeda, 2002). In fact, numerous studies have shown that 17β-estradiol treatment leads to marked reduction in mean atherosclerotic lesion area in ApoE$^{-/-}$ and LDLR$^{-/-}$ males (Tse et al., 1999; Nathan et al., 2001). Such studies suggest that females are more resistant to atherosclerosis compared to their age-matched male counterparts. However, gender-specific effects on atherosclerosis becomes confusing given that testosterone administration to C57/BL6 females leads to a significant reduction in atherosclerotic lesion formation (Paigen et al., 1987a). Interestingly, ovariectomy experiments revealed that AEBP1 expression in WAT is positively regulated by estrogen via an unknown molecular mechanism (Zhang et al., 2005). Based on such findings, one can assume that AEBP1^{TG} females would possess higher levels of AEBP1 compared to their AEBP^{TG} males in tissues and cells that are estrogen-responsive. This would also imply that AEBP1^{TG} females would be more resistant to atherosclerosis compared to AEBP1^{TG} males, due to the atheroprotective roles of estrogen. However, the positive effect of estrogen on AEBP1 expression is questionable given that WAT isolated from age-matched, chow-fed AEBP1^{TG} females and males expresses comparable AEBP1 levels (Zhang et al., 2005). So, the relationship between estrogen and AEBP1 level remains paradoxical.

Of note, experimental evidence indicates that AEBP1 expression level is comparable in macrophages isolated from age-matched AEBP1^{TG} females and males (data not shown). Interestingly, I have gathered preliminary evidence suggesting that AEBP1 represses the endogenous expression of ERα and ERβ in vivo (data not shown). Indeed,

this is an attractive finding, given that estrogen and its receptors play atheroprotective roles *in vivo* and that AEBP1^{TG} females are more susceptible to atherosclerosis compared to their AEBP1^{TG} males. So, besides the gender-specific differences between AEBP1^{TG} and AEBP1^{NT} mice regarding serum cholesterol/triglyceride profile, I speculate that AEBP1 is potentially capable of impeding the atheroprotective roles played by estrogen *in vivo*. Obviously, this is a hypothesis that needs further examination, and I anticipate that regulation of estrogen roles in atherogenesis by AEBP1 may serve as a satisfactory mechanism that further explains the gender-specific atherogenic differences displayed by AEBP1^{TG} mice.

4.7 AEBP1^{TG} Mice May Serve as a Novel Murine Model of Atherosclerosis

Although completely absent in AEBP1^{NT} mice, atherosclerotic lesions are evident in the proximal aortae of HFD-fed AEBP1^{TG} mice. Besides ApoE$^{-/-}$ (Piedrahita *et al.*, 1992; Plump *et al.*, 1992) and LDL receptor-deficient (LDLR$^{-/-}$) (Ishibashi *et al.*, 1993) mice, the most well-characterized murine models of atherosclerosis, my findings strongly suggest that AEBP1^{TG} mice may serve as a novel murine model of diet-induced atherosclerosis. Arguably, AEBP1^{TG} mice may be advantageous in many aspects. First, unlike most other models that develop atherosclerotic lesions only when fed high-cholesterol (1.25%) diet, AEBP1^{TG} mice develop lesions when fed low-cholesterol (0.05%) diet. Second, unlike some of the existing models, AEBP1^{TG} mice develop lesions when fed HFD that has no cholate (cholic acid). Atherogenic diet containing cholate was shown to induce a hepatic inflammatory response and stimulate expression of pro-atherogenic factors (Liao *et al.*, 1993; Vergnes *et al.*, 2003). Moreover, cholate-containing diet has been shown to modulate plasma ApoE levels in mice (Nishina *et al.*, 1990), suggesting that feeding mice with cholate-containing diets can interfere with data interpretation. Third, AEBP1^{TG} mice develop atherosclerotic lesions starting at a young age (12 wk), which mimics atherosclerotic lesion formation in humans (Ross, 1999; Libby *et al.*, 2002). Fourth, unlike ApoE$^{-/-}$ and LDLR$^{-/-}$ mice, AEBP1^{TG} mice display gender-specific differences regarding susceptibility to atherosclerosis and mean lesion area (Tables 6 & 7). This makes AEBP1^{TG} mice a useful model to study the effects of

gender on atherogenesis in mice. Fifth, no lesions are detected in the aortae of chow-fed mice, suggesting that AEBP1^{TG} mice resemble LDLR$^{-/-}$ mice with regard to their ability to form lesions in a diet-induced manner. This can be important for studies that do not require spontaneous formation of atherosclerotic lesions in mice. Sixth, in contrast to other murine models of atherosclerosis, AEBP1^{TG} mice exhibit a premature sudden death phenomenon, which makes them a favorable model for sudden death studies.

Importantly, AEBP1^{TG} mice were raised on FVB/N background, while ApoE$^{-/-}$ and LDLR$^{-/-}$ mice were raised on C57BL/6 background. Different strains of mice display differential athero-susceptibility (Paigen *et al.*, 1987a; Dansky *et al.*, 1999; Smith *et al.*, 2003). The mean lesion area is 7- to 9-fold and 3.5-fold higher in ApoE$^{-/-}$ mice raised on C57BL/6 background when fed chow and HFD, respectively, compared to ApoE$^{-/-}$ mice raised on FVB/N background (Dansky *et al.*, 1999). The fact that FVB/N mice are relatively athero-resistant coupled with the high athero-susceptibility exhibited by AEBP1^{TG} mice reflects the robust atherogenic potential of AEBP1. Undoubtedly, generating AEBP1^{TG} and AEBP1$^{-/-}$ mice on LDLR$^{-/-}$ genetic background will provide invaluable *in vivo* tools to further elucidate the pro-atherogenic functions of AEBP1.

4.8 AEBP1^{TG} Mice Undergo Gender-Specific Premature Sudden Death

As mentioned earlier, 21% and 38% of AEBP1^{TG} females undergo premature sudden death when fed on chow diet and HFD, respectively. Strikingly, this premature sudden death phenomenon is gender specific, since AEBP1^{TG} males are totally resistant to it. Although AEBP1^{TG} females develop atherosclerotic lesions more prevalently and of a larger mean lesion area compared to AEBP1^{TG} males, it is rather unconvincing that such differences alone can account for the dramatic gender-specific differences displayed by AEBP1^{TG} mice with regard to premature sudden death. In fact, the premature sudden death phenomenon exhibited by AEBP1^{TG} females is, if anything, very puzzling for many reasons. Firstly, although atherosclerotic lesion instability can lead to rupture followed by thrombi formation that can potentially result in myocardial infarction and/or thrombotic stroke, critical events leading to sudden death, atherosclerosis alone cannot explain this sudden death phenomenon. This is because chow-fed AEBP1^{TG} females can still be

victims to premature sudden death (21%), despite lack of detectable atherosclerotic lesions (Table 6). Secondly, if atherosclerosis on its own is the primary and only reason behind premature sudden death, then one should expect at least a small percentage of AEBP1^{TG} males, 71% of which develop atherosclerotic lesions, to undergo a premature sudden death, which is not the case. Thirdly, the fact that only 38% of HFD-fed AEBP1^{TG} females die prematurely, despite the fact that 94% of AEBP1^{TG} females develop atherosclerotic lesions, is puzzling. Fourthly, although I believe that the massive obesity displayed by HFD-fed AEBP1^{TG} females, but not AEBP1^{TG} males (Zhang *et al.*, 2005), likely contributes to premature sudden death, it is rather difficult to explain why 21% of chow-fed AEBP1^{TG} females, which have body weights comparable to their AEBP1^{NT} female counterparts, still fall victims to premature sudden death. Fifthly, despite the fact that 20% and 80% of chow- and HFD-fed AEBP1^{TG} females, respectively, display mammary epithelial cell hyperplasia that may eventually lead to breast cancer (at age > 1 yr) that can contribute to death, the fact that AEBP1^{TG} females die prematurely at a relatively young age (average 17 wk) makes it unlikely that mammary epithelial cell hyperplasia can solely be responsible for the premature sudden death phenomenon. In conclusion, I believe that AEBP1^{TG} females suffer from complications related to atherosclerosis, massive obesity, and mammary epithelial cell hyperplasia, and perhaps yet unidentified complications that may collectively predispose AEBP1^{TG} females, but not AEBP1^{TG} males, to premature sudden death.

In an attempt to identify the pathophysiological mechanism(s) behind the premature sudden death phenomenon exhibited by AEBP1^{TG} females, I attempted to investigate whether sudden death victims display any signs of myocardial infarcts in their hearts since myocardial infarction is one of the most common secondary complications associated with atherosclerosis and atherothrombosis that can lead to sudden death. Unfortunately, successful Masson's trichrome staining did not reveal any obvious signs of dead myocytes or fibrosis within cardiac tissues obtains from premature sudden death victims (Figure 30). In addition, although thrombotic strokes can potentially lead to sudden death (Mitra *et al.*, 2004; Viles-Gonzalez, *et al.*, 2004), they usually result in partial or complete paralysis. However, AEBP1^{TG} female victims show no signs of immobility or physical disability prior to death, making thrombotic stroke unlikely

mechanism underlying premature sudden death. The fact that AEBP1^{TG} females die prematurely and suddenly urges me to suspect cardiac-related events such as myocardial infarctions or acute cardiac arrhythmias, conditions that potentially cause sudden death. Needless to say, elucidation of the pathophysiological mechanism behind premature sudden death in AEBP1^{TG} females requires further investigation.

4.9 AEBP1^{TG} Females: Defects Other Than Atherosclerosis and Sudden Death

Apparently, AEBP1 transgenic over-expression seems to be associated with more complications in females than in males. In particular, AEBP1^{TG} females suffer from massive diet-induced obesity that does not affect AEBP1^{TG} males. Although the massive obesity exhibited by AEBP1^{TG} mice does not manifest until 15 wks of age, atherosclerotic lesions are evident in AEBP1^{TG} females at 12 wk of age, and some AEBP1^{TG} females fall victim to premature sudden death starting at 13 wk of age, it is possible that this massive obesity contributes to atherogenesis and premature sudden death in AEBP1^{TG} females. A positive correlation between the incidence of obesity and development of atherosclerotic lesions is basically inescapable. Moreover, the higher prevalence of atherosclerosis and the larger mean lesion area in AEBP1^{TG} females compared to their AEBP1^{TG} male counterparts may at least in part be attributed to the massive obesity exhibited exclusively by the former. Likewise, it is possible that the mammary epithelial cell hyperplasia displayed by AEBP1^{TG} females contributes to the gender-specific premature sudden death phenomenon exhibited by those mice.

In order to prove or disprove any contribution of massive obesity and/or mammary epithelial cell hyperplasia to gender-specific differences with regard to atherosclerotic lesion formation and premature sudden death, I suggest a series of bone marrow transplantation experiments. I hypothesize that diet-induced atherosclerosis and premature sudden death in AEBP1^{TG} mice is primarily caused by disruption of macrophage cholesterol efflux and induction of macrophage pro-inflammatory responsiveness by AEBP1, and that massive obesity and mammary epithelial cell hyperplasia have very minimal influence, if any. If my hypothesis is correct, one should assume normal development of atherosclerosis as well as typical occurrence of premature

sudden death in AEBP1^{NT} females transplanted with bone marrow obtained from AEBP1^{TG} females. Moreover, transplantation of AEBP1^{TG} females with bone marrow obtained from AEBP1^{NT} females would be expected to attenuate atherogenesis and premature sudden death in the former, without interfering with massive obesity and mammary epithelial cell hyperplasia. Likewise, transplantation of bone marrow obtained from AEBP1$^{-/-}$, but not AEBP1$^{+/+}$ or AEBP1^{NT}, mice into AEBP1^{TG} mice may lead to complete abrogation of atherosclerotic lesion development and premature sudden death. This set of bone marrow transplantation experiments should shed more light on the critical role of AEBP1 in the hematopoietic system towards atherogenesis and premature sudden death.

4.10 Lessons From AEBP1$^{-/-}$/ApoE$^{-/-}$ and AEBP1^{TG}/ApoE$^{-/-}$ Hybrid Mice

Due to the fact that FVB/N mice are resistant to atherosclerosis (Dansky et al., 1999), and since AEBP^{TG} mice develop atherosclerotic lesions that are atypical compared to those found in ApoE$^{-/-}$ and LDLR$^{-/-}$ mice, it was wise to attempt generating mice that lack or over-express AEBP1 on an ApoE$^{-/-}$ background. Unfortunately, however, generation of AEBP1$^{-/-}$/ApoE$^{-/-}$ has proven unsuccessful (refer to section 3.8.1). This finding suggests that dual deficiency of AEBP1 and ApoE causes embryonic lethality in mice, eliminating the likelihood of producing viable double knockout mice. This is a very interesting finding given that ApoE$^{-/-}$ mice are totally viable and reproduce normally (Piedrahita et al., 1992; Plump et al., 1992), indicating that AEBP1 plays a critical role in mouse embryonic development. This proposal is supported by the observation that the litter size of AEBP1$^{-/-}$ mice is about half that of their AEBP1$^{+/+}$ counterparts (H.S. Ro and colleagues, unpublished). In light of these findings, I strongly feel that attempts to generate AEBP1$^{-/-}$ mice on a LDLR$^{-/-}$ background should be made to further elucidate the significant role of AEBP1 in macrophage cholesterol homeostasis, pro-inflammation, and atherogenesis.

In contrast to AEBP1 deficiency in ApoE$^{-/-}$ mice, AEBP1 over-expression in ApoE$^{-/-}$ mice does not cause any noticeable developmental defects, and AEBP1^{TG}/ApoE$^{-/-}$ mice are totally viable and healthy (refer to section 3.8.2). Unfortunately, the single experiment

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performed on AEBP1^{TG}/ApoE$^{-/-}$ mice and described in section 3.8.2 failed to demonstrate whether AEBP1 over-expression in ApoE$^{-/-}$ mice advances atherosclerotic lesion formation or not due to a genotyping problem. So, repeat of this experiment is currently underway. Nonetheless, the data obtained from this experiment confirmed that AEBP1 over-expression promotes atherosclerotic lesion formation in mice, since AEBP1^{TG}/ApoE$^{+/-}$ mice clearly develop atherosclerotic lesions that are completely absent in their AEBP1^{NT}/ApoE$^{+/-}$ counterparts (Figure 28). Again, evaluation of atherosclerotic lesion formation in AEBP1^{TG}/ApoE$^{-/-}$ and AEBP1^{NT}/ApoE$^{-/-}$ mice remains the ultimate golden goal.

4.11 AEBP1 and NF-κB: A Positive Relationship

Since its initial identification by Sen and Baltimore about two decades ago (Sen and Baltimore, 1986), NF-κB has been the focus of many researchers in an attempt to understand the various molecular mechanisms involved in inflammatory diseases and cancer. Modulation of NF-κB activity can result in many abnormal cellular processes and diseases including asthma, arthritis, atherosclerosis, obesity, and various types of cancers (Tak and Firestein, 2001; Yamamoto and Gaynor, 2001; Shoelson *et al.*, 2003; Monaco and Paleolog, 2004). Throughout my project, I partially focused on examining the potential interplay between AEBP1 and NF-κB in an attempt to shed light on the molecular mechanism by which AEBP1 manifests its pro-inflammatory function in macrophages. My findings provide experimental evidence establishing a positive relationship between AEBP1 expression and NF-κB activity in macrophages. Indeed, nuclear p65 protein level is barely detectable in AEBP1$^{-/-}$ macrophages, compared to AEBP1$^{+/+}$ counterparts (Figure 32). Consistently, electrophoretic mobility gel shift assay clearly illustrates that ablation of AEBP1 expression in macrophages correlates with inhibited NF-κB DNA-binding activity (Figure 33). This positive relationship seems to be a consequence of a negative relationship between AEBP1 expression and IκBα protein stability in macrophages. Interestingly, AEBP1 is shown to promote IκBα phosphorylation followed by its proteolytic degradation, liberating the NF-κB subunits, which translocate into the nucleus and become transcriptionally active. Furthermore, I

demonstrate that such negative regulation imposed by AEBP1 on IκBα function in the cytosol is mediated by protein-protein interaction that requires DLD of AEBP1, as confirmed by co-immunoprecipitation analysis (Figure 39C). Consistent with my proposal that AEBP1-IκBα protein-protein interaction, which is mediated by DLD, provokes destabilization of IκBα shortening its half-life, ΔN and CP mutant forms of AEBP1, which are devoid of DLD, have no influence on IκBα protein stability, unlike AEBP1 derivatives retaining DLD (Figure 41). Importantly, in contrast to WT form of AEBP1, ΔN and CP mutant forms possess marginal or no up-regulatory function towards NF-κB activity (Figures 42 and 43), confirming that AEBP1-IκBα interaction is a key biological event that is crucial for AEBP1-mediated IκBα-induced degradation and subsequent NF-κB up-regulation in macrophages.

Herein, it is important to mention that abrogation of NF-κB activity has been shown to cause embryonic lethality due to liver apoptosis (Beg *et al.*, 1995; Doi *et al.*, 1997; Li *et al.*, 1999). This is very interesting given that my finding clearly indicate that NF-κB transcriptional activity is severely inhibited in AEBP1$^{-/-}$ macrophages due to prolonged IκBα protein half-life (Figure 36). If AEBP1-mediated positive regulation of NF-κB is a universal process that takes place in cells and tissues other than macrophages (e.g. liver), one would expect that AEBP1$^{-/-}$ embryos may suffer from liver apoptosis that is life-threatening. Although NF-κB activity has not been evaluated in AEBP1$^{-/-}$ hepatocytes, it is fascinating that AEBP1$^{-/-}$ mice suffer from about 50% embryonic lethality (H.S. Ro and colleagues, unpublished). Hence, it is possible that AEBP1 deficiency-mediated severely diminished NF-κB activity, which can potentially lead to liver apoptosis, may serve as a molecular mechanism underlying embryonic lethality in AEBP1$^{-/-}$ mice.

4.12   AEBP1 Physically Interacts with IκBα, but not IκBβ

Our findings suggest that AEBP1 physically interacts with IκBα via its DLD, which is known to be a protein-protein interaction mediator (Johnson *et al.*, 1993; Prag *et al.*, 2004). Indeed, DLD of AEBP1 was found to be required for protein-protein

interaction between AEBP1 and MAPK (Kim *et al.*, 2001). Similarly, this study suggests that AEBP1 is capable of physically interacting with IκBα by means of its DLD, whose deletion eliminated AEBP1-IκBα interaction (Figure 39C). It is worth mentioning that despite the structural similarities between IκBα and IκBβ, our data suggests that AEBP1 is capable of interacting with IκBα, but not IκBβ, in macrophages (Figure 38). Analysis of IκBα-IκBβ amino acid sequence alignment reveals that there are three main structural differences between IκBα and IκBβ (Figure 46). First, the first 12 amino acid residues in IκBα are absent in IκBβ. Second, there is a 41-amino acid stretch located between the third and forth ANK repeat of IκBβ that is not present in IκBα. Third, there is an 18-amino acid stretch at the C-terminus of IκBβ that is absent in IκBα. Based on sequence analysis, it is conceivable that either the presence of the first 12 amino acid residues in IκBα is required for interaction with AEBP1 or that the presence of the extra amino acid stretches in IκBβ allow the formation of a tertiary structure that does not permit protein-protein interaction with AEBP1. It is also conceivable that the extra amino acid stretches in IκBβ somehow mask the region, or domain, that is necessary for protein-protein interaction with AEBP1. Identification of the region of IκBα that mediates protein-protein interaction with AEBP1, which will shed more light on the differential ability of AEBP1 to regulate IκBα and IκBβ functions *in vivo*, is currently underway. Further investigation of the AEBP1-interacting region of IκBα will shed more light on how AEBP1 is capable of differentially regulating IκBα and IκBβ functions *in vivo*. Although modulation of IκBα expression has been previously shown to be a mechanism explaining altered NF-κB activity (Delerive *et al.*, 2000; Azuma *et al.*, 1999; Saile *et al.*, 2001), our study is the first of its kind to propose a mechanism behind modulated NF-κB activity by which IκBα protein stability is altered via protein-protein interaction.

Since about 50% of AEBP1 protein population exists in the nucleus (Park *et al.*, 1999; A. Majdalawieh, unpublished, L. Zhang, unpublished, H. Ma, unpublished), and since newly synthesized IκBα is known to translocate to the nucleus to bind DNA-bound NF-κB dimers and re-sequester them into the cytosol (Hayden and Ghosh, 2004), it is possible that AEBP1 and IκBα interact in the nucleus. This is an interesting possibility since AEBP1-IκBα interaction in the nucleus can interfere with the ability of nuclear

IκBα to bind to its target, DNA-bound NF-κB dimers, leading to sustained NF-κB-driven transactivation of target genes (e.g. pro-inflammatory genes). Thus, by virtue of its cytosolic/nuclear localization and its ability to bind IκBα, it is reasonable to propose that AEBP1 can impede IκBα inhibitory functions towards NF-κB both at the cytosolic and nuclear levels.

Figure 46. Amino Acid Sequence Comparison between IκBα and IκBβ. *(A)* Alignment of amino acid sequences of murine IκBα and IκBβ is shown. The six highly conserved ANK repeats in both proteins are highlighted in grey, yellow, green, red, pink, and blue, respectively. *(B)* A cartoon illustrating a comparison of the slightly different structural domain organizations of murine IκBα and IκBβ proteins. The signal-induced kinase domain, six ANK repeats, and PEST domain of each protein are shown.

A

```
mIkBα   MFQPAGHGQDWAMEGPRDGLKKERLVDDRHDSGLDSMKDEEYEQMVKEL-REIRLQPQEA  59
mIkBβ   ----------MAG-VACLGKTADADEWCDSGLGSLGPDAAAPGGFGLGAELGPELSWA  47
        * *     * *    .:    ****.*:    :         * *:  :  . *

mIkBα   PLAAEPWKQQLTE█████████████████████████████████QNNLQQTPLHLAV 119
mIkBβ   PLVFG----YVTE█████████████████████████████████QNDLGQTALHLAA 102
        **.   :*****: ****:**..:*   *    *    :*.:**.* **.****

mIkBα   ITNQPGIAEALLKAGCDPELRDF█████████████████████-------------- 166
mIkBβ   IIGEASTVEKLYAAGAGVLVAER██████████████████████████████DTYL 162
        * .:..  .* *  **..  :   ..:.. ** *

mIkBα   ────────██████─────────────────────████ILQATNY████████████ 198
mIkBβ   TCBQDCTPDTSHAPAAVDSQPNPENEEEPRDEDWRIQLEAENY████████████████ 222
            ***:  *:              *:* **:***  **:* .  *      :

mIkBα   ██████████████████C██████████████████████████████████████ 258
mIkBβ   ██████████████████I██████████████████████████████████████ 282
        *. *   ***.:. **  ***.****:. * ...:.****.*** . * * :*   :

mIkBα   ██████─────████████████████████████PESEDEESYDIESEFTEDELPYDDCVFGGQRLTL- 314
mIkBβ   ██████████████████████████████████DKLSPCSSSGSDSDSDNRDEGDEYDDIVVHSGRSQNR 342
        **.    :*:  *     *:     . .... *::*:  ::  *** *.  . *

mIkBα   ──────────────
mIkBβ   QFPSPASKPLPDIPNPA 359
```

B
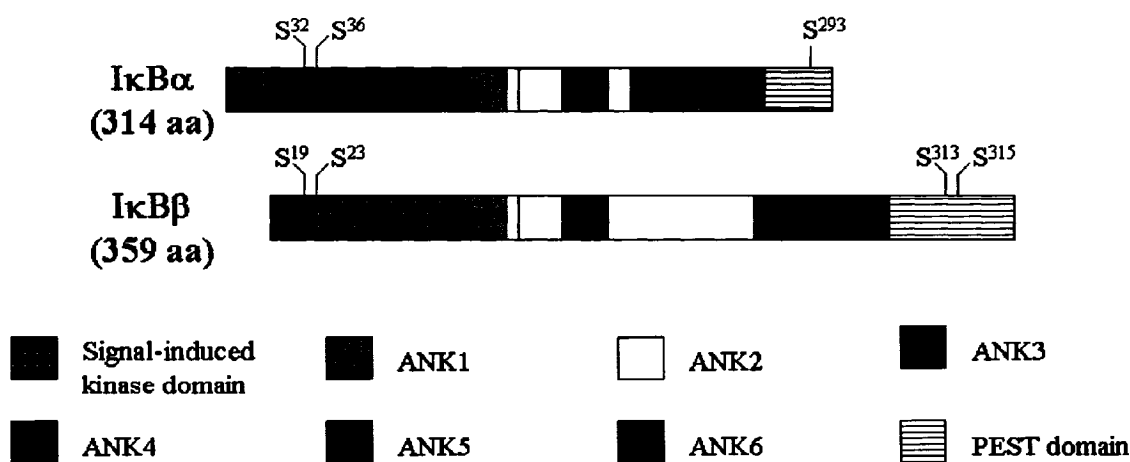
4.13 Differential Regulation of IκBα and IκBβ: A Possible Role for AEBP1?

Different IκB proteins are known to exhibit differential partner specificity with regard to NF-κB dimer interaction and inhibition (Ghosh et al., 1998). For example, both IκBα and IκBβ preferentially interact with and inhibit the activity of NF-κB dimers containing p50, p65, and c-Rel (Baeuerle and Baltimore, 1989; Thompson et al., 1995; Beg et al., 1992), IκBγ prefers p50 homodimers and p50/p65 heterodimers (Inoue et al., 1992a), IκBε prefers p65 and c-Rel homo- and heterodimers (Whiteside et al., 1997), and Bcl-3 prefers p50 and p52 homodimers (Nolan et al., 1993; Zhang et al., 1994), whereas p100 and p105 seem to bind to almost all possible dimer combinations of NF-κB proteins (Rice et al., 1992; Mercurio et al., 1993). It is also known that different NF-κB dimers display differential intrinsic preference with regard to DNA binding specificity (Kunsch et al., 1992; Fujita et al., 1992), and this DNA binding specificity confers distinct NF-κB dimers a differential transactivation potential towards a diverse set of genes (Lin et al., 1995). So, the differential specificity of IκB/NF-κB interaction combined with the differential transactivation potential of different NF-κB dimers can somewhat explain how differential regulation of distinct IκB proteins can lead to differential regulation of NF-κB dimer activity, and thus, differential expression control of discrete genes. However, due to the remarkable similarities between IκBα and IκBβ in terms of their structure and NF-κB dimer specificity, understanding the molecular mechanisms behind the differential regulatory functions undertaken by these two molecules in different cell types and under different conditions has proven to be a tremendous challenge, and as of yet, a clear explanation of such differential regulation of these two molecules is still lacking.

Despite their structural similarities, IκBα and IκBβ are known to be differentially regulated in various cell types and under several stimulatory conditions (Thompson et al., 1995, Weil et al., 1997). Several studies have demonstrated that while IκBα is subject to rapid degradation upon cell stimulation by various stimuli including LPS, IL-1β, TNFα, and PMA in most cell types, IκBβ degradation cannot be induced except by very few potent stimuli such as LPS and IL-1β in certain cell types and it tends to be a relatively

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slow process (Thompson *et al.*, 1995; Shumway and Miyamoto, 2004). However, other studies have shown that S^{19}/S^{23}-phosphorylated IκBβ is subject to degradation induced by PMA or TNFα treatment (Weil *et al.*, 1997; Pomerantz *et al.*, 2002). The slower kinetics associated with IκBβ degradation has been suggested to be probably due to the slower rate of IκBβ phosphorylation by the IKK complex, which seems to favor IκBα as a more efficient substrate (DiDonato *et al.*, 1996). So, depending on the potency of signals, IκBβ may or may not become subject to phosphorylation and subsequent degradation (Ghosh *et al.*, 1998). Here, it can be argued that AEBP1, by virtue of its differential ability to interact with IκBα, but not IκBβ, may play a determining role in making IκBα more susceptible than IκBβ to signal-induced phosphorylation and subsequent degradation.

Since the PEST domain plays a critical role in IκB protein turnover/stability (Verma *et al.*, 1995; Beauparlant *et al.*, 1996; Weil *et al.*, 1997), it is arguable that the function of this domain is differentially regulated in IκBα and IκBβ, thus leading to their differential regulation. However, studies have shown that deletion or mutations within the PEST domain confer resistance to signal-induced degradation for both IκBα (Brown *et al.*, 1995; Rodriguez *et al.*, 1995; Whiteside *et al.*, 1995; Aoki *et al.*, 1996; Sun *et al.*, 1996; Beauparlant *et al.*, 1996) and IκBβ (DiDonato *et al.*, 1996; Harhaj *et al.*, 1996; McKinsey *et al.*, 1996; Weil *et al.*, 1997). In light of these results, understanding the role of PEST domain does not seem to help in explaining the differential regulation imposed on IκBα and IκBβ. In addition, while the two N-terminal lysine residues (K^{21} and K^{22}) of IκBα are known to be ubiquitination sites that are required for signal-induced degradation of IκBα (Scherer *et al.*, 1995, Baldi *et al.*, 1996), the only N-terminal lysine residue (K^9) in IκBβ does not seem to be an exclusive ubiquitination site, and its mutation has no effect on signal-induced degradation of IκBβ (Weil *et al.*, 1997). Moreover, it was shown that IκBβ is phosphorylated on Ser^{19} and Ser^{23} in un-stimulated cells, whereas Ser^{32} and Ser^{36} phosphorylation in IκBα is only signal-induced (Weil *et al.*, 1997). Nonetheless, the exact mechanism(s) responsible for the differential regulation and function of IκBα and IκBβ remains unrevealed. My findings shed some light on a possible mechanism that can satisfactorily explain the differential regulatory functions

exhibited by these two molecules. This mechanism implicates the differential capability of AEBP1 to physically interact with IκBα, but not IκBβ.

4.14 How Does AEBP1-IκBα Interaction Lead to IκBα Proteolytic Degradation?

To date, two pathways have been suggested as molecular mechanisms responsible for IκBα proteolytic degradation. First, upon stimulation, IκBα is thought to be degraded via a classical, signal-induced proteasome-dependent pathway that involves the 26S proteasome (Alkalay et al., 1995). Second, in vitro studies using immature B cells have demonstrated that IκBα can be subject to constitutive proteasome-independent, Ca^{++}-dependent degradation under basal conditions (Miyamoto et al., 1998). It was also shown that constitutive phosphorylation of serine/threonine residues within the C-terminal PEST domain of IκBα by casein kinase II (CKII) is required for IκBα turnover (McElhinny et al., 1996; Lin et al., 1996; Schwarz et al., 1996). Also, accumulation of free IκBα in the cytosol triggers its rapid degradation through a phosphorylation, ubiquitination-independent proteasome-dependent pathway (Krappmann et al., 1996). My findings raise the question: how does AEBP1-IκBα interaction lead to IκBα phosphorylation and subsequent proteolytic degradation? Although the exact molecular mechanism underlying this regulatory process is yet to be identified, I can provide a few speculative points that may explain the role of AEBP1-IκBα interaction in promoting IκBα phosphorylation and proteolytic degradation. First, AEBP1-IκBα interaction could cause a conformational change in the latter rendering it more susceptible to Ser^{32}/Ser^{36} phosphorylation and degradation via the ubiquitination-dependent proteasome-dependent pathway. Second, IκBα-bound AEBP1 could serve as a scaffold protein that facilitates recruitment of the constitutive proteasome-independent Ca^{++}-dependent proteolytic or ubiquitination-independent proteasome-dependent machineries. Third, it is possible that AEBP1-bound IκBα is more prone to constitutive phosphorylation on serine/threonine residues within the PEST domain, inducing its proteasome-dependent proteolytic degradation. Fourth, AEBP1 may also serve as a "bridge" that brings IκBα in proximity to IKKα/β in the cytosol, forcing IκBα phosphorylation and subsequent proteolytic degradation. Finally, it

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is possible that AEBP1 somehow enhances the catalytic activity of an "unknown" kinase that can potentially phosphorylate S^{32} and S^{36} in IκBα. Conversely, it is also possible that AEBP1 may somehow interfere with an "unknown" phosphatase that can exercise its catalytic activity on S^{32}/S^{36}-phosphorylated IκBα in the cytosol. Examination of these possibilities may shed light on the exact molecular mechanism undertaken by AEBP1 to hamper IκBα inhibitory function towards NF-κB.

4.15   AEBP1 Up-Regulates NF-κB Activity Independent of PPARγ1 and LXRα

Experimental evidence suggesting that PPARγ1 and LXRα play anti-inflammatory roles is overwhelming. PPARγ ligands suppress inflammation by interferring with the NF-κB, AP-1, and STAT signaling pathways (Chinetti *et al.*, 1998; Ricote *et al.*, 1998; Chung *et al.*, 2000; Joseph *et al.*, 2003). Similarly, LXRα ligands exhibit anti-inflammatory functions in macrophages by impeding NF-κB activity (Joseph *et al.*, 2003). PPARγ1 and LXRα repression by AEBP1 serves as a mechanism that satisfactorily explains the pro-inflammatory properties exhibited by AEBP1 in macrophages. Since AEBP1 represses PPARγ1 and LXRα transcriptional activity in macrophages (Majdalawieh *et al.*, 2006), active PPARγ1 and LXRα interfere with NF-κB activity (Chinetti *et al.*, 1998; Ricote *et al.*, 1998; Chung *et al.*, 2000; Joseph *et al.*, 2003), and AEBP1 enhances NF-κB activity, it is reasonable to suggest that PPARγ1 and LXRα transcriptional repression by AEBP1 may contribute to AEBP1-mediated NF-κB up-regulation in macrophages. However, this effect may be marginal due to the following reasons. First, deletion of DLD, which does not influence the ability of AEBP1 to repress PPARγ1 or LXRα (Figure 18), completely eliminates the ability of AEBP1 to up-regulate NF-κB activity (Figures 42 and 43). In agreement, deletion of the C-terminus of AEBP1, which completely eliminates the ability of AEBP1 to repress PPARγ1 or LXRα (Figure 18) (Majdalawieh *et al.*, 2006), does not interfere with the ability of AEBP1 to up-regulate NF-κB activity (Figures 42 and 43). Second, Glass and colleagues have shown that neither treatment of RAW 264.7 macrophages with PPARγ ligand nor PPARγ1 over-expression in absence of its ligand had any anti-inflammatory effects (Ricote *et al.*,

1998). Rather, PPARγ-mediated anti-inflammatory effects are only observed when PPARγ1 is over-expressed and ligand activated (Weil *et al.*, 1997). Similarly, LXRα-mediated anti-inflammatory effects can only be observed in the presence of LXR ligands and under LPS-stimulatory conditions (Joseph *et al.*, 2003). My findings clearly demonstrate that AEBP1 enhances NF-κB activity in macrophages expressing endogenous PPARγ1 and LXRα in the absence of PPARγ or LXR ligands under both basal and LPS-stimulatory conditions. Collectively, I conclude that co-ordinate AEBP1-mediated IκBα proteolytic degradation and subsequent NF-κB up-regulation is independent of AEBP1-mediated PPARγ1 and LXRα repression in macrophages.

4.16 DLD Mediates AEBP1 Protein-Protein Interaction with Other Proteins

The N-terminus of AEBP1 contains a discoidin-like domain (DLD) that is remarkably homologous to discoidin, a lectin expressed in the slime mold *Dictyostelium discoideum* (Rosen *et al.*, 1973), and hence the name. In *Dictyostelium discoideum*, discoidin has been shown to be crucial for proper cell aggregation and migration (Springer *et al.*, 1984). DLD has been identified in several proteins including the blood coagulation cofactors V and VIII (Kane and Davie, 1986), milk-fat globule proteins (Stubbs *et al.*, 1990), discoidin domain receptor tyrosine kinase (Johnson *et al.*, 1993), and muskelin (Prag *et al.*, 2004). DLD has been suggested to mediate cell-cell adhesion (Baumgartner *et al.*, 1998), protein self-association (Prag *et al.*, 2004), and protein-protein interaction (Johnson *et al.*, 1993; Prag *et al.*, 2004). In fact, protein-protein interaction between AEBP1 and MAPK in the cytosol, which prolongs MAPK activation by protecting it from dephosphorylation by its specific phosphatase (MKP3, also known as PYST1), has been shown to be mediated by DLD of AEBP1 (Kim *et al.*, 2001). Interestingly, my findings indicate that AEBP1 is capable of physically interacting with cytosolic IκBα by means of its DLD, whose deletion eliminates AEBP1-IκBα protein-protein interaction (Figure 39C). So, these findings further support a role of DLD in protein-protein interaction in mammalian systems. Furthermore, these findings underscore the importance of DLD in mediating very critical functions undertaken by AEBP1 to control key biological processes in the cell. Intriguingly, I propose that the presence of DLD creates a molecular

competition between MAPK and IκBα in the cytosol to bind to AEBP1. This proposal is interesting given that sustained MAPK activation and IκBα proteolytic degradation followed by NF-κB activation culminate in diverse biological outcomes in different cell types. Although the molecular mechanisms that signal AEBP1 to interact predominantly with MAPK or IκBα are unknown, it is conceivable that AEBP1 can be utilized by the cell as an on/off switch to promote or inhibit MAPK and NF-κB activities via balancing AEBP1 protein-protein interaction with MAPK and IκBα.

4.17 NF-κB Modulation: An AEBP1 Roadway Towards Cancer and Apoptosis?

NF-κB has been shown to be persistently active in various types of cancer including those of liver and colon (Karin *et al.*, 2002; Lin and Karin, 2003; Greten and Karin, 2004; Gilmore, 2003; Shishodia and Aggarwal, 2004; Karin and Greten, 2005; Luo *et al.*, 2005a; Luo *et al.*, 2005b). This is consistent with studies describing NF-κB as a survival, anti-apoptotic factor that is capable of prolonging cell growth by antagonizing cell apoptosis and necrosis *in vitro* and *in vivo* (Gilmore *et al.*, 1996; Baichwal and Baeuerle, 1997). As mentioned earlier, AEBP1^{TG} females develop mammary epithelial cell hyperplasia, a state of uncontrolled, progressive proliferation of mammary epithelial cells, both under chow- (20%) and HFD-fed (80%) conditions (L. Zhang and colleagues, unpublished). In contrast, AEBP1$^{-/-}$ females display defective mammary gland development during lactation due to premature mammary gland involution driven by epithelial cell apoptosis (S. Reidy and colleagues, unpublished). Based on my findings indicating a positive relationship between AEBP1 expression and NF-κB activity in macrophages and in C6 glioma cells, I postulate that such relationship also exists in mammary epithelial cells. In other words, I suspect that mammary epithelial cell hyperplasia and apoptosis that occur in AEBP1^{TG} and AEBP1$^{-/-}$ females, respectively, is due to AEBP1-mediated modulation of NF-κB activity in those cells. In support of my hypothesis, preliminary evidence suggests that NF-κB activity is augmented and diminished in mammary gland tissues isolated from AEBP1^{TG} and AEBP1$^{-/-}$ females, respectively (L. Zhang, unpublished). This suggests that the potential of AEBP1 to up-

regulate NF-κB activity is not restricted to specific cell types, and that modulation of NF-κB activity may be a mechanism undertaken by AEBP1 to regulate distinct biological processes in various cell types. Based on these findings, I further postulate that AEBP1 is an anti-apoptotic factor that is capable of directly provoking the NF-κB survival function in cells.

4.18   AEBP1 versus ACLP

The aortic carboxypeptidase-like protein (ACLP) is a 175-kDa protein that was initially identified in human aortic smooth muscle cells, and whose expression was shown to be up-regulated during smooth muscle cell differentiation (Layne et al., 1998). Although ACLP mRNA can be detected in several tissues including the kidney, colon, aorta, brain, stomach, spleen, and thymus, ACLP protein can only be detected in the aorta (Layne et al., 1998) and adipose tissue (Layne et al., 2001). ACLP harbors a signal peptide (termed signal sequence), a lysine- and proline-rich 11-amino acid repeating motif (termed repeat sequence), a discoidin-like domain (DLD), and a poorly characterized C-terminal domain (Layne et al., 1998). Later, it was demonstrated that ACLP is an extracellular matrix (ECM)-associated secretory protein that is essential for embryonic development and dermal wound healing processes (Layne et al., 2001). Cell fractionation and immunofluorescent staining experiments revealed that ACLP is excluded from the nucleus and localized in the perinuclear space, indicative of its entry into the secretory pathway (Layne et al., 2001). Noteworthy, ACLP cannot be detected in the cytosolic compartment of mouse aortic smooth muscle cells, and is associated predominantly with the ECM (Layne et al., 2001). As mentioned repeatedly throughout my thesis, AEBP1, whose sequence is identical to that of ACLP starting at the DLD, can be detected in the nucleus and in the cytosol (Park et al., 1999, A. Majdalawieh, unpublished, L. Zhang, unpublished, H. Ma, unpublished).

Here, several points are noteworthy. First, nuclear localization of AEBP1 is consistent with its ability to bind AE-1 and AE-1 homologous nucleotide sequences, and serve as a transcriptional repressor of several target genes. Although ACLP shares the exact C-terminal DNA-binding domain of AEBP1, it is not possible for ACLP to function

as a transcriptional repressor due to its absence in the nucleus. Thus, it is rather inconceivable that ACLP can repress the expression of PPARγ1 and LXRα as does AEBP1, and hence, I doubt that ACLP can modulate macrophage cholesterol homeostasis. Second, since ACLP cannot be detected in the cytosol, it is inconceivable that ACLP can have any effect on IκBα function/NF-κB activity, despite the fact that both AEBP1 and ACLP share the DLD. Third, regardless of what has been mentioned above, it is important to emphasize that the repressed expression of PPARγ1 and LXRα, the disrupted cholesterol efflux, and the enhanced inflammatory responsiveness associated with AEBP1^{TG} macrophages can only be attributed to AEBP1, not ACLP. This is because over-expression of AEBP1 in AEBP1^{TG} macrophage is driven by a transgene carrying the cDNA of AEBP1. It is not known, hoever, whether AEBP1 over-expression has any effect on ACLP expression or cellular localization. Most importantly, immunoblotting of whole cell and nuclear protein extracts obtained from primary macrophages and J774, THP-1, and U937 monocyte/macrophage cell lines using anti-AEBP1 did not reveal expression of ACLP, despite its detection in cell extracts obtained from white and brown adipose tissue, mammary gland tissue, and heart (L. Zhang, unpublished, A. Majdalawieh, unpublished). Thus, despite the fact that they are transcribed from the same gene and share identical structural domains, AEBP1 and ACLP display different expression pattern, cellular localization, and function.

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Chapter 5

CONCLUSIONS AND REMARKS

Collectively, I provide experimental evidence indicating that AEBP1, which is expressed abundantly in macrophages, inhibits macrophage cholesterol efflux by down-regulating the expression of PPARγ1, LXRα, and their target genes, promoting foam cell formation. Indeed, I show that transcriptional repression of PPARγ1 and LXRα by AEBP1 requires AEBP1-DNA interaction. Additionally, AEBP1 is shown to be a potent pro-inflammatory factor that is capable of inducing the expression and secretion of major pro-inflammatory mediators including IL-6, TNFα, MCP-1, and iNOS, key players in atherogenesis, by macrophages. The ability of AEBP1 to impede macrophage cholesterol efflux and enhance macrophage inflammatory responsiveness allows AEBP1 to manifest itself as a potent pro-atherogenic factor. Figure 47 depicts a model implicating AEBP1 as a novel regulator of macrophage cholesterol homeostasis and macrophage inflammatory responsiveness, and thus, as a novel pro-atherogenic mediator. The pro-atherogenic properties exhibited by AEBP1 seem to be a byproduct of a vital interplay of its ability to antagonize PPARγ1 and LXRα cholesterol efflux functions in macrophages and its ability to promote pro-inflammation via enhanced NF-κB transcriptional activity. Moreover, my findings add to the accepted prospect implicating environmental (i.e. diet) and genetic determinants in the development of atherosclerosis. I postulate that AEBP1^{TG} mice can potentially serve as a novel murine model of diet-induced atherosclerosis with the anticipation that this invaluable *in vivo* tool will help identify potential atherosclerosis modifiers, presenting AEBP1 as a likely molecular target for developing novel therapeutic strategies for atherosclerosis.
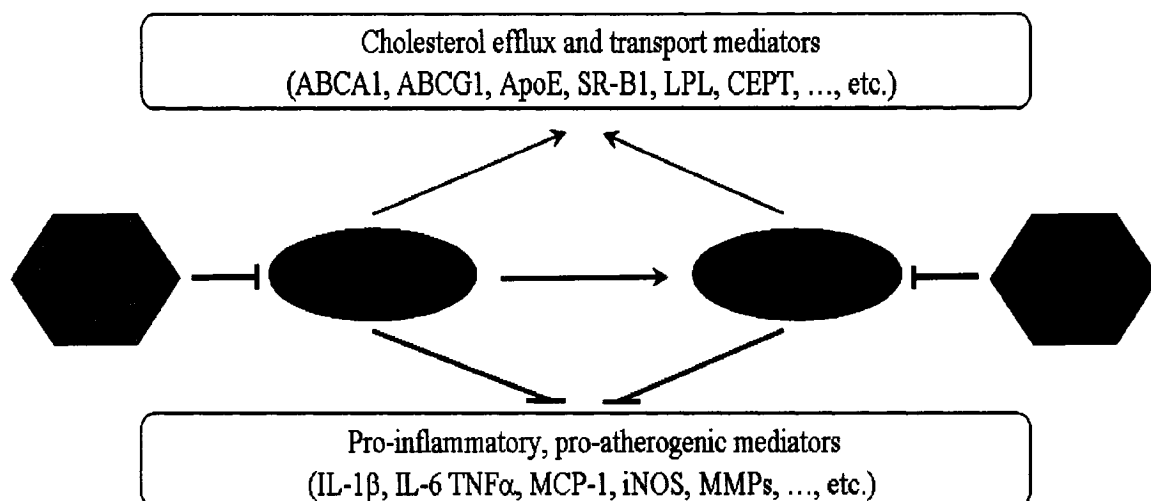
Figure 47. A Model Implicating AEBP1 as a Potentially Critical Player in Macrophage Cholesterol Homeostasis and Atherogenesis. In macrophages, PPARγ1 and LXRα cooperate to induce the expression of major cholesterol efflux mediators that are critically involved in transferring excess cholesterol to its acceptor (i.e. HDL) in plasma. PPARγ1 and LXRα also play imperative anti-inflammatory functions by antagonizing the expression of key inflammatory mediators in macrophages. AEBP1 is proposed to impede macrophage cholesterol efflux, induce foam cell formation, and provoke pro-inflammation. Thus, AEBP1 is anticipated to function as a likely pro-atherogenic factor, promoting both metabolic and inflammatory processes involved in atherogenesis.

I also provide experimental evidence indicating that AEBP1 exercises its pro-inflammatory function in macrophages by hampering IκBα function, thus enhancing NF-κB transcriptional activity leading to augmented expression of pro-inflammatory mediators. Figure 48 depicts a model implicating AEBP1 as an IκBα physical interacting partner that enhances macrophage inflammatory responsiveness by promoting IκBα phosphorylation and proteolytic degradation, subsequently leading to NF-κB activation. Experimental evidence indicates that AEBP1 physically interacts with endogenous IκBα via its DLD, and this protein-protein interaction somehow renders IκBα prone to phosphorylation and proteolytic degradation. This AEBP1-mediated IκBα down-regulatory process is physiologically significant since it is accompanied by enhanced NF-κB activity leading to augmented macrophage inflammatory responsiveness. Since NF-κB is involved in biological processes implicated in many diseases and medical conditions, I anticipate that AEBP1 may potentially serve as a molecular candidate towards the development of therapeutic strategies for the treatment of inflammatory diseases, obesity, and various types of cancer.
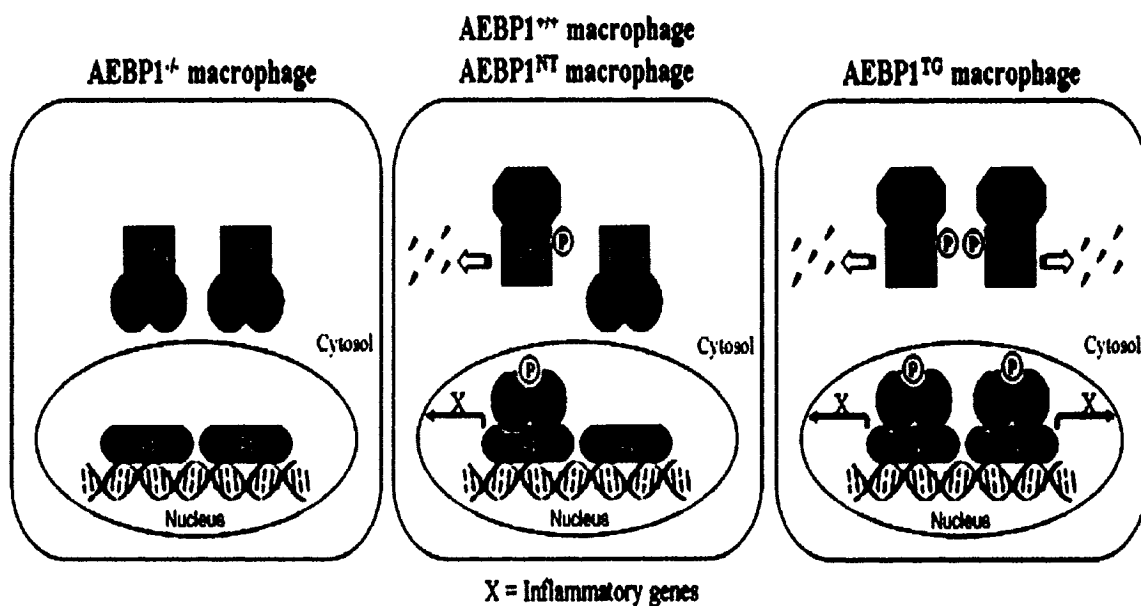
Figure 48. A Model Implicating AEBP1 as a Positive Regulator of NF-κB Activity in Macrophages. In this model, AEBP1 is presented as a cytosolic factor capable of promoting IκBα phosphorylation and proteolytic degradation, leading to enhanced NF-κB activation in macrophages. Experimental evidence suggests that AEBP1 promotes IκBα phosphorylation and proteolytic degradation via protein-protein interaction that is DLD-dependent. Hence, AEBP1 over-expression and deficiency in macrophages is postulated to ultimately result in enhanced and suppressed NF-κB transcriptional and DNA-binding activities, respectively. This is a plausible molecular mechanism underlying the pro-inflammatory function exerted by AEBP1 in macrophages.

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