EXPLORING THE ROLE OF EBP50 IN VASCULAR INFLAMMATION

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Inflammation is a necessary biological response to injury and infections, but chronic activation can lead to a host of diseases. This beneficial-turned-destructive process plays a fundamental role in the development of cardiovascular diseases, and the vascular response requires communication between multiple cell types such as vascular smooth muscle cells (VSMC) and macrophages. A PDZ-domain scaffolding protein, Ezrin-Radixin-Moesin-Binding Phosphoprotein 50 (EBP50, also known as NHERF1), is expressed at low levels in normal vessels but is upregulated following arterial wire injury and promotes neointima formation. However, the mechanisms underlying these actions are not fully understood. I hypothesized that EBP50 functions as a central mediator of macrophage activation and the response of VSMC to inflammation. EBP50 expression increased in an NF-κB-dependent manner upon LPS or TNFα treatment macrophages and VSMC. Conversely, NF-κB activation was impaired in EBP50 knockout (KO) VSMC and macrophages. Mechanistically, inflammatory stimuli induced the formation of an EBP50-PKCζ complex that promoted PKCζ membrane translocation and subsequent NF-κB signaling. Macrophage cytokine production (IL-1β, TNFα) and vascular adhesion molecule expression (ICAM-1, VCAM-1) after acute LPS or TNFα treatment were reduced in KO cells and mice compared to WT. Bridging the multicellular effects of EBP50, macrophage recruitment to lesions in the femoral artery one week after wire injury was significantly reduced in KO mice.

The effect of myeloid cell-specific EBP50 on atherosclerosis was determined in *Ldlr*$^{-/-}$ mice, a well-established model of diet-induced atherosclerosis. Mice were lethally irradiated and transplanted with bone marrow from WT or KO mice and kept on a high-cholesterol diet for 12 weeks. A complete blood count and genotyping of hematopoietic cells established the successful ablation of original bone marrow and the full reconstitution of the donor marrow. No differences in weight, blood glucose, or cholesterol levels were observed between the two groups. Oil Red O staining of sections throughout the aorta revealed a 25% reduction in lesion area in KO bone marrow recipients compared to WT.

Collectively, these new findings indicate that EBP50 potentiates macrophage activation and the response of VSMC to inflammation. Thus, EBP50 is a key regulator of vascular remodeling during both acute and chronic inflammatory states.

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ABBREVIATIONS

AGE	Advanced Glycation End Products
AngII	Angiotensin II
ApoE	Apolipoprotein E
β_2-AR	β2-Adrenergic Receptor
BMT	Bone Marrow Transplant
CHO	Chinese Hamster Ovary
CBP	CREB-Binding Protein
Cdk1	Cyclin-Dependent Kinase 1
CFTR	Cystic Fibrosis Transmembrane Regulator
CKII	Casein Kinase II
CVD	Cardiovascular Disease
EBD	Ezrin Binding Domain
EBP50	Ezrin-radixin-moesin Binding Phosphoprotein 50
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
ER	Estrogen Receptor
ERE	Estrogen Response Elements

FAK	Focal Adhesion Kinase
GAS6	Growth Arrest-Specific Protein 6
GPCR	G Protein-Coupled Receptor
GRK6A	G Protein-Coupled Receptor Kinase 6A
ICAM-1	Intercellular Adhesion Molecule 1
IFNγ	Interferon γ
IκB	Inhibitors of Kappa B
IKK	IκB Kinase
IL	Interleukin
IL-1R	Interleukin 1 Receptor
IL-1RAcP	IL-1R Accessory Protein
iNOS	Inducible Nitric Oxide Synthase
IRAK	IL-1R Associated Kinase
KD	Kinase Dead
KO	EBP50 Knockout
LDL	Low-Density Lipoprotein
LDLR	Low-Density Lipoprotein Receptor
LPS	Lipopolysaccharides
LXR	Liver X Receptor
MCP-1	Monocyte Chemotactic Protein-1
MERTK	C-MER Proto-Oncogene Tyrosine Kinase
MFGE8	Milk Fat Globulin E8
MMP	Matrix Metalloproteinase

MSK	Mitogen- and Stress-Activated Protein Kinase
MyD88	Myeloid Differentiation Primary Response Gene 88
Myr	Myristoylated
NF-κB	Nuclear Factor κB
NHERF	Na+/H+ Exchanger Regulatory Factor
NHE	Na+/H+ Exchanger
NLS	Nuclear Localization Sequence
Npt2a	Sodium-Phosphate Cotransporter Type IIa
oxLDL	Oxidized Low-Density Lipoprotein
p38 MAPK	p38 Mitogen-Activated Protein Kinase
PDGF	Platelet-Derived Growth Factor
PDGFR	Platelet-Derived Growth Factor Receptor
PDZ	Postsynaptic Density 95/Discs-Large/Zona Occludens
PI3K	Phosphatidylinositol-3-OH Kinase
PIP_2	Phosphatidylinositol 4,5-Bisphosphate
PIP_3	Phosphatidylinositol 3,4,5-Trisphosphate
PIPKIβ	Phosphatidylinositol-Phosphate Kinase β
PKA	Protein Kinase A
PKC	Protein Kinase C
PLCβ	Phospholipase Cβ
PP2A	Protein Phosphatase 2A
PTEN	Phosphatase and Tensin Homolog
PTH	Parathyroid Hormone

PTH1R	Parathyroid Hormone Receptor 1
PTHrP	Parathyroid Hormone-Related Peptide
RAGE	Receptor for Advanced Glycation End Products
RIP	Receptor Interacting Protein
RSK1	Ribosomal S6 Kinase 1
SOCS	Suppressors of Cytokine Signaling
TAD	Transactivation Domain
TGFβ	Transforming Growth Factor β
Th1	Type 1 Helper
Th2	Type 2 Helper
TIR	Toll/IL-1 Receptor
TIRF	Total Internal Reflection Fluorescence
TLR	Toll-Like Receptor
TNFα	Tumor Necrosis Factor α
TNF-R1	TNF Receptor 1
TRAF	TNF Receptor Associated Factor
VCAM-1	Vascular Cell Adhesion Molecule-1
VSMC	Vascular Smooth Muscle Cells
ZIP	Zeta-Interacting Protein

1.0 INTRODUCTION

1.1 ATHEROSCLEROSIS: AN INFLAMMATORY DISEASE

Large arteries that are more prone to atherosclerosis consist of three major layers: the intima, media, and adventitia (1). As depicted in Figure 1-1, the intima is a single layer of endothelial cells (and vascular smooth muscle cells (VSMC) in humans) that serves as a protective barrier between the blood-carrying lumen and the underlying medial VSMC. A thin layer of connective tissue and elastin bands called the internal elastic lamina separates the intima from the media, the thickest segment of an artery. Medial VSMC contract or relax and thus dictate the volume of a vessel and its blood pressure. The outermost layer of an artery, the adventitia, is divided from the media by the external elastic lamina and mainly contains fibroblasts and connective tissue. A network of capillaries within the adventitia called the vasa vasorum supplies the vessel with nutrients and can additionally serve as an alternative route of entry for leukocytes.

Dysfunction of any of these features sets off a chain of events down the path of cardiovascular disease (CVD). CVD includes, but is not limited to, myocardial infarction, heart failure, hypertension, stroke, atherosclerosis, and restenosis. Although mortality rates have declined since the 1970s, CVD is still responsible for a sizeable 32.3% of deaths every year in the United States, amounting to one death every 40 seconds (2). While much information about the mechanisms of CVD has been gathered, it is clear that there is still much more to learn.

It is increasingly evident that atherosclerosis is an inflammatory disease (3-6). Any alteration in the integrity of the endothelium triggers a coordinated response of multiple cell

types. Dysfunctional endothelium recruits monocytes and allows for their transmigration through the VSMC layer (Figure 1-1). Monocytes further mature into macrophages and engulf cholesterol, forming foam cells and eventually contributing to the development of the necrotic core. In response to the heightened inflammatory environment, VSMC proliferate and migrate to the intimal space, building a fibrous cap, while also continuing to express adhesion molecules and facilitate macrophage infiltration. An unstable lesion is inclined to rupture and release a thrombus, the ultimate complication.

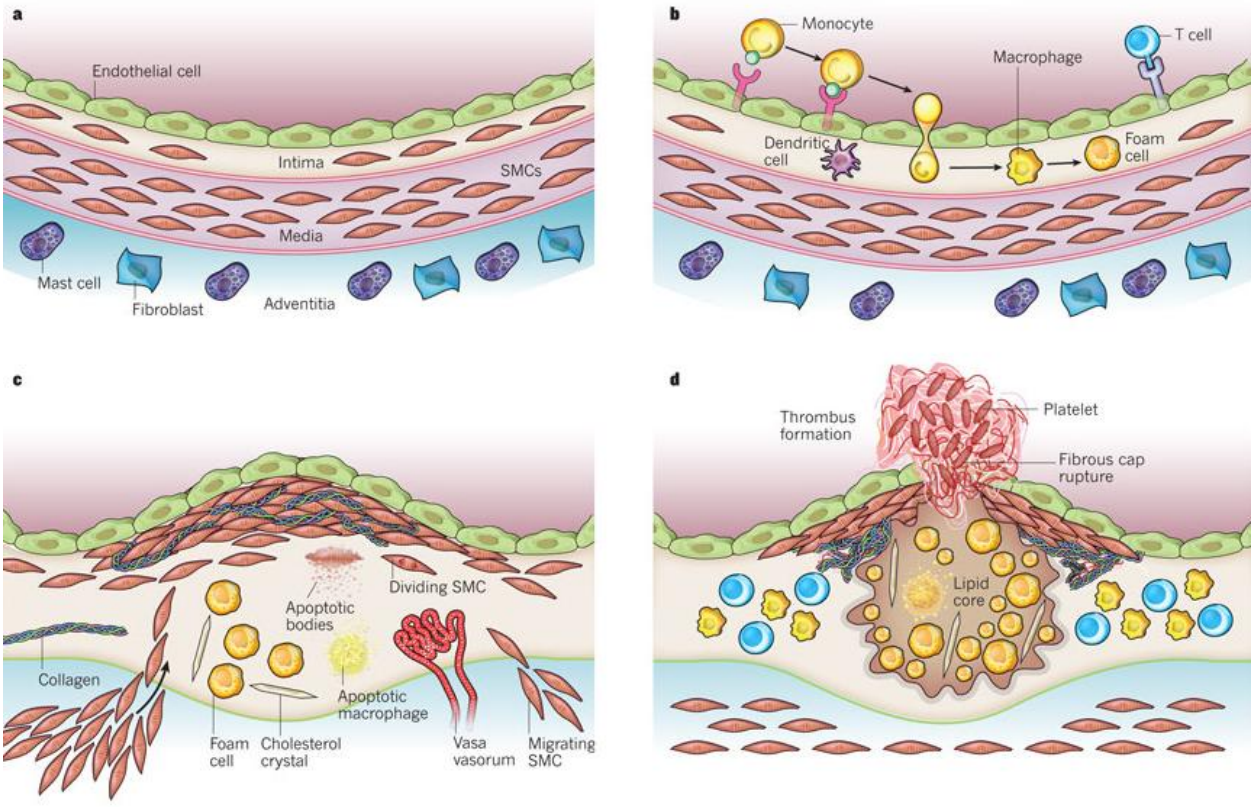

Figure 1-1: Stages of atherosclerotic lesion development.

A. The normal artery contains three layers. The innermost layer is the intima (mainly endothelium with some VSMC in humans), followed by the media (VSMC), and surrounded by the outer adventitia (fibroblasts and connective tissue). **B.** Atherosclerosis is initiated by the adhesion of circulating leukocytes to the activated endothelium and their migration into the intima. The monocytes mature into macrophages and engulf cholesterol, yielding foam cells.

C. VSMC migrate into the lesion and synthesize extracellular matrix components to constitute the fibrous cap. Lipid-laden foam cells undergo apoptosis and accumulate in what is known as the necrotic core. **D.** Thrombosis is the ultimate complication of atherosclerosis and is accelerated by a large necrotic core and thin fibrous cap.

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1.1.1 Initiation of the Fatty Streak

Lesions primarily occur in medium- and large-sized muscular and elastic arteries at branches, bifurcations, and curvatures where low or oscillatory shear stress is present (6, 8-10). This alteration in blood flow stimulates intercellular adhesion molecule 1 (ICAM-1) expression on endothelial cells (11, 12), priming the site for the adhesion of inflammatory cells. Low-density lipoprotein (LDL) can become trapped in subintimal spaces and undergo progressive oxidation, as well as other modifications (13). Exposure to high levels of cholesterol, including oxidized-LDL (oxLDL), further increases the vessel permeability with the expression of vascular cell adhesion molecule-1 (VCAM-1). Adherence of platelets to activated endothelium also enhances adhesion molecule expression as well as the release of the chemokine monocyte chemotactic protein-1 (MCP-1) in response to lipoproteins (14). MCP-1 and oxLDL attract circulating monocytes to this site where they attach to endothelial ICAM-1 and VCAM-1 via β_2-and $\alpha_4\beta_1$-integrins, respectively (15, 16). Using a host of other adhesion molecule-integrin interactions, monocytes transmigrate through the intima and eventually the media, led by VSMC-derived MCP-1 (17, 18). This contributes to the formation of the fatty streak, the earliest phase of atherosclerosis.

The fatty streak is a purely inflammatory lesion consisting only of monocyte-derived macrophages and T-cells. These lesions are common even in children and adolescents (19) and may resolve on their own or continue progressing into more mature plaques. Locally-produced macrophage colony-stimulating factor allows the monocytes to survive, replicate, and differentiate into macrophages (20, 21). Scavenger receptors on macrophages can internalize a broad range of molecules, including oxLDL, and failure to sufficiently undergo cholesterol efflux leads to the accumulation of lipid droplets in the cytoplasm (22, 23). Macrophages that are overloaded with cholesterol are referred to as foam cells. Furthermore, an overabundance of cholesterol is toxic to macrophages and triggers ER stress and apoptosis (24-26).

1.1.2 Plaque Progression and Rupture

Macrophages and T-cells accumulate at the "shoulder" region near where plaque meets normal tissue (27) and propagate a local inflammatory response inside the vessel wall. Lesional CD4+ T-cells are reactive to oxLDL (28), and ligation of the antigen receptor initiates an activation cascade culminating in either a type 1 helper (Th1) or type 2 helper (Th2) response. Th1 cells secrete pro-inflammatory cytokines such as interferon γ (IFNγ) and tumor necrosis factor α (TNFα) and are typically utilized to fight intracellular pathogens. On the other hand, Th2 cells secrete interleukin (IL) 4, IL-5, and IL-13 and are more involved in allergic inflammation (29). Atherosclerotic lesions contain Th1 cells, which can activate macrophages and augment their cytokine generation (i.e. TNFα and IL-1β), in addition to upregulating genes relevant to antigen presentation (30). Inhibition of Th1 cells with pentoxifylline, a phosphodiesterase inhibitor which reduces the Th1 polarization of T helper lymphocytes, decreases atherosclerotic severity in Apolipoprotein E (*Apoe*)$^{-/-}$ mice (31). However, switching to a strictly Th2 differentiation

induces abdominal aortic aneurysms (32), demonstrating that these processes are intricately related and not completely black and white.

Similar to T-cell polarization, macrophages are classified as M1 or M2, although this tends to be an oversimplification. “Classically activated” M1 macrophages are pro-inflammatory and depend on Th1-produced IFNγ while “alternatively activated” M2 macrophages are primarily involved in wound healing and rely on IL-4 or IL-13 secretion from Th2 cells (33). Another classification of M2 includes the IL-10-induced regulatory macrophages, which are important for resolving inflammation. After IFNγ priming, oxLDL, alone or in immune complexes, activates M1 macrophages to produce matrix metalloproteinases (MMPs), tissue factor, and cytokines including TNFα, IL-1β, and IL-6 (34-36). Macrophage activation may further occur through toll-like receptors (TLRs) and nucleotide-binding oligomerization domain-like receptors (37). In fact, C3H/H2J mice, which carry a missense mutation in signaling domains of *Tlr4*, are resistant to atherosclerosis (38). Furthermore, cytokines produced by M1 macrophages can further propagate the inflammatory response through autocrine and paracrine signaling.

Early macrophage apoptosis resulting from cholesterol overload is beneficial as it suppresses lesion cellularity, but uncontrolled cell death promotes advanced plaque necrosis. Inhibiting apoptosis in macrophages enhances atherosclerosis (39, 40). Apoptotic cells that are not cleared by phagocytes become swollen and leaky, resulting in secondary necrosis and the formation of the necrotic, or lipid, core (41, 42). Apoptotic cells can shed procoagulant membrane microparticles such as tissue factor (43). Tissue factor activity is dependent upon phosphatidylserine (44), a phospholipid that is consequently exposed during apoptosis, rendering

the necrotic core highly thrombogenic (43, 45, 46). The necrotic core is the most deleterious part of the plaque and its encapsulation and separation from the blood flow is critical.

Advanced lesions are covered by a fibrous cap formed from matrix deposition and the proliferation and migration of VSMC. This cap protects the bloodstream from the pro-thrombotic interior. Production of transforming growth factor β (TGF-β) by any cell in the atheroma stimulates VSMC and fibroblasts to produce collagen, creating a protective scar over the lesion (47-49). Additionally, cytokines and growth factors from macrophages and activated endothelium, especially platelet-derived growth factor (PDGF), promote VSMC proliferation and migration, adding to the collagenous fibrous cap (50). MMPs cleave collagen and elastin to facilitate the migration of VSMC through the elastic laminae and collagenous matrix to the intimal space (51, 52). However, the matrix-degrading abilities of MMPs also contribute to the thinning of fibrous cap, particularly in the shoulder regions (53). IFNγ from Th1 cells can inhibit VSMC proliferation and collagen production (48) and VSMC are also susceptible to apoptosis (54), further weakening plaque stability.

The thickening of the arterial wall triggers outward remodeling, and the gradual dilation of the vessel delays the onset of flow-limiting stenosis that can lead to ischemia (55, 56). MMPs may be involved in this process in order to relieve the structural constraint imposed by the matrix (57). While initially beneficial, positive remodeling may also contribute to plaque instability. Larger, outward remodeled plaques are associated with unstable clinical presentation (58). Dilation of these arteries may in fact weaken them and make them mechanically vulnerable to plaque rupture.

An unstable plaque is typically characterized by a thin fibrous cap, large necrotic core, and a substantial inflammatory cell infiltrate. When a plaque ruptures, procoagulants in the

bloodstream come into contact with tissue factor in the necrotic core and generate a thrombus. The thrombus can locally impede blood flow or dislodge and travel through the circulation as an embolus, eventually becoming lodged in another artery. This obstruction causes tissue ischemia, stroke, and myocardial infarction. Occlusive thrombi from ruptured plaques account for almost one-third of sudden coronary deaths (59). Surprisingly, plaque rupture is not always fatal (60) and the possibility exists that a thrombus can be resorbed into the plaque (61). Intraplaque hemorrhage can also occur as a result of leakage of the vasa vasorum or other plaque neovessels that oxygenate advanced plaques (62). However, both mechanisms of a non-fatal rupture contribute to plaque growth and may still lead to occlusion of the vessel.

1.1.3 Resolution of Inflammation

An inflammatory response can continue indefinitely if the offending source is not eliminated. Effective resolution of inflammatory processes requires the inhibition of inflammatory cell recruitment, clearance of apoptotic cells (efferocytosis), and promotion of inflammatory cell egress. Defects in the resolution of inflammation promote the formation of vulnerable plaques. Consequently, these functions and other anti-inflammatory signaling events are often impaired in atherosclerosis.

1.1.3.1 Anti-Inflammatory Mediators

There are a number of anti-inflammatory mediators that attempt to dial back the inflammation before it progresses to a dangerous level. These include IL-10, TGFβ, lipid mediators, and the liver X receptor (LXR) transcription factor. IL-10 targets leukocytes and induces suppressor of cytokine signaling (SOCS) 3, inhibiting pro-inflammatory nuclear factor

κB (NF-κB) signaling (63, 64). Additionally, the cytokine enhances macrophage efferocytosis (65) and its overexpression in lymphocytes decreases atherosclerotic severity in mice (66). In human cases of atherosclerosis, serum IL-10 levels are decreased and inversely related with future myocardial infarctions (67).

Lipid mediators such as 12,15-lipoxygenase and prostaglandin E_2 also have a role in lesional inflammation. Global deficiency of 12,15-lipoxygenase in *Apoe*$^{-/-}$ mice aggravates atherosclerosis while overexpression of the enzyme in macrophages is atheroprotective (68). These effects are mediated through the synthesis of lipoxin A4, protectin D1, and resolvin D4, resulting in attenuated cytokine, chemokine, and adhesion molecule expression and increased phagocytic activity (68). A product of the cyclooxygenase pathway, prostaglandin E_2 suppresses macrophage production of inflammatory cytokines while enhancing IL-10 production (69). Furthermore, *Ldlr*$^{-/-}$ mice receiving bone marrow deficient in EP4, the receptor for prostaglandin E_2, exhibit reduced early atherosclerosis (70). This is due to the suppression of pro-survival pathways and increased apoptosis of macrophages that would otherwise initiate atherosclerosis.

Activation of the LXR family of transcription factors is also atheroprotective. LXR agonists induce expression of cholesterol transporters such as ABCA1 and ABCG1 to promote cholesterol efflux in foam cells (71). Like most other anti-inflammatory mediators, these nuclear receptors repress expression of pro-atherogenic molecules (72). Finally, LXR family agonists induce a marked reduction in plaque formation in mouse models of atherosclerosis (73).

1.1.3.2 Monocyte Recruitment

There is persistent recruitment of new macrophages throughout the lifetime of the plaque due in part to the hypercholesterolemia-induced increase in the number of circulating Ly6C^{hi} monocytes (M1 precursors) (74, 75). This increase comes as a consequence of enhanced survival

and impaired conversion of Ly6C^{hi} monocytes to their Ly6C^{lo} counterparts (74). A decrease in plaque-derived MCP-1 is observed following the transplant of plaque-bearing region of the aorta from atherosclerotic *Apoe*$^{-/-}$ mice to wild-type mice, demonstrating that a decrease in the surrounding cholesterol levels could stifle macrophage recruitment (76).

1.1.3.3 Macrophage Egress

Egress of inflammatory cells is similarly impaired in the presence of hypercholesterolemia. However, plaque transfer from lipid-laden mice to normal mice not only results in lesion regression but emigration of inflammatory cells out of the vessel wall (77). Resident macrophages differentiate into migratory dendritic cells and emigrate to the lymph nodes in a CC-chemokine receptor 7-dependent manner (76, 77). This suggests that the lowering of cholesterol levels is central to proper resolution of inflammation.

1.1.3.4 Efferocytosis

Efferocytosis, the removal of apoptotic cells by phagocytes, is effective in early lesions, but as the disease progresses and more cells undergo apoptosis the plaque becomes necrotic. Indeed, efferocytosis is defective in advanced lesions (78). Not only is it advantageous to clear out dying cells, but phagocytosis of apoptotic cells inhibits pro-inflammatory cytokine production through the release of TGF-β (79). TGF-β additionally inhibits MCP-1 expression and further macrophage recruitment (80).

Foam cells undergoing apoptosis secrete “find me” factors, such as lysophosphatidylcholine, and membrane-bound “eat me” signals, including phosphatidylserine, to attract phagocytes (81). Phagocytes express a specific set of molecules that bridge the interaction with the apoptotic cells. For example, αVβ3 integrin and C-MER proto-oncogene

tyrosine kinase (MERTK) on phagocytes contact milk fat globulin E8 (MFGE8) and growth arrest-specific protein 6 (GAS6) on apoptotic cells, respectively (82, 83). Disrupting these interactions leads to an accumulation of apoptotic cells and accelerates atherosclerosis (84, 85).

There are many hypotheses as to what could be causing faulty efferocytosis in developing atheromas. One possibility is that the phagocytes are simply overwhelmed by the sheer number of cells that need to be cleared. However, individual phagocytes have a high capacity for efferocytosis and there are plenty of macrophages that are capable of the job, even with the high death rate. Foam cells are still able to engulf apoptotic cells (86) so the pool of competent phagocytes is adequate. Furthermore, phagocytes are highly resistant to cholesterol-induced death due to massive cholesterol efflux and the activation of NF-κB and Akt survival pathways after foam cell ingestion (87). A likely possibility is that the bridging molecules are somehow deficient in function or expression in advanced plaques. Cleavage of the ectodomain of MERTK in response to pro-inflammatory stimuli inhibits apoptotic cell clearance (88). In addition to the now-dysfunctional MERTK left in the membrane, the soluble portion of MERTK binds and sequesters GAS6, creating competition for intact MERTK (88). Inflammation can also downregulate MFGE8 expression similarly affecting efferocytosis (89).

Cellular age may also explain the defect in efferocytosis as the majority of deaths attributed to CVD occur after the age of 75 (2). Macrophages extracted from old mice (2 years) and humans (between 65 and 90 years) display a diminished ability to clear apoptotic cells (90, 91). Additionally, monocyte-derived dendritic cells from older subjects secrete higher levels of TNFα and IL-6 in response to inflammatory stimuli (90). Interestingly, pre-treatment of macrophages with serum from aged mice also reduces efferocytosis in comparison to treatment

with serum from young mice, suggesting the presence of an inhibitory factor or absence of an effector in aged serum (91).

1.1.4 Risk Factors

1.1.4.1 Hyperlipidemia

While a number of different factors can initiate atherosclerosis, the most obvious culprit is high cholesterol levels (92). As detailed above, modified lipids trapped in the intima can stimulate the expression of adhesion molecules, proinflammatory cytokines, chemokines, and other mediators in T-cells, macrophages, VSMC, and endothelial cells. Certain growth factors and cytokines such as PDGF, TGFβ, TNFα, and IL-1β can induce LDL receptor (LDLR) expression and thus promote LDL uptake (93-95). Therefore, as long as there is excess modified cholesterol present, a vicious cycle is maintained between lipoprotein uptake and inflammation. Antioxidants vitamin E and probucol can reduce the oxidative propensity of LDL and reduce the size and severity of atherosclerotic lesions without lowering lipid levels (96-98).

Patients with familial hypercholesterolemia, a genetic disorder characterized by high levels of cholesterol and caused primarily by mutations in the *LDLR* gene are at an increased risk of early-onset coronary heart disease without treatment (99). A popular treatment for those with familial hypercholesterolemia and others with high cholesterol is HMG-CoA reductase inhibitors (rate limiting in cholesterol synthesis), or statins. Statins are currently the main class of drugs used in the treatment of atherosclerosis. Consequently, the reduction in cholesterol synthesis by statins has prominent anti-inflammatory effects, reducing adhesion molecules, MMPs, tissue factor, and IFNγ and other cytokine production (100-102).

1.1.4.2 Obesity and Diabetes

Obesity is a worldwide epidemic affecting multiple organ systems, including the cardiovascular system, and was reclassified by the American Heart Association as a "major, modifiable risk factor" for coronary heart disease (103, 104). For each additional unit of body mass index , the risk of atherosclerosis increases by 8% (105). Like atherosclerosis, obesity is characterized by dyslipidemia, although the profile is different. While high LDL levels contribute to CVD, obesity is characterized by higher levels of very low-density lipoprotein, triacylglycerols, and total cholesterol with a decrease in high-density lipoprotein levels (106). For this reason, obese individuals require a more intensive course of statin treatment to impact plaque regression than those with a lower body mass index (107).

Adipose tissue is an active autocrine and paracrine organ that secretes "adipokines" to regulate different metabolic and vascular processes. Two prominent examples of adipokines that are dysregulated in obesity and other insulin-resistant states are leptin and adiponectin. Instead of a normal balance, leptin and most other adipokines are increased while levels of the normally anti-atherogenic adiponectin are decreased. Adiponectin can reduce VSMC proliferation and migration in addition to inhibiting MMPs following induction of IL-10 (108, 109). This suggests that adiponectin could protect against plaque rupture. Adiponectin could potentially guard against endothelial dysfunction as the adipokine can also inhibit adhesion molecule expression and foam cell formation (110, 111). Increased plasma concentrations of TNFα are correlated with reduced adiponectin levels (112) and many speculate that this is a more causative relationship. On the other hand, leptin is pro-thrombotic and stimulates VSMC proliferation and migration (113, 114).

Hyperglycemic disorders stemming from obesity such as diabetes can initiate pro-inflammatory states through non-enzymatic formation of heterogeneous advanced glycation end products (AGE). AGE modification of macromolecules in the extracellular matrix (i.e. collagen) results in crosslinking and increased arterial stiffness. In addition, this can trap oxLDL within the vessel wall (64). In contrast, binding of AGE to the receptor for AGE (RAGE) increases cytokine production, VSMC proliferation and migration, adhesion molecule expression, and reactive oxygen species in the vasculature (115). Furthermore, AGE-RAGE increases foam cell formation and inhibits cholesterol efflux, contributing to the progression of atherosclerosis (115).

1.1.4.3 Infection

While there is no direct evidence that infectious agents can induce atherosclerosis by themselves, microorganisms such as cytomegalovirus and *Chlamydia pneumonia* can accelerate lesion development in conjunction with other factors (116). *C. pneumonia* stimulates adhesion molecule and cytokine expression in endothelial cells, VSMC, and macrophages (117). These agents can release lipopolysaccharides (LPS) into the bloodstream and the reduction of atherosclerosis in a *Tlr4/Apoe* double knockout mouse model indicated a role for infections in CVD (118). LPS signals through TLR4, but oxLDL is also able to bind this receptor, complicating the results. Many plaques show signs of infection by cytomegalovirus and *C. pneumonia* (119, 120), but administration of antibiotics has no effect on prevention of CVD (121).

1.1.4.4 Other Factors

Not only is cigarette smoke a teratogen that increases risk for lung cancer, but the nicotine and other toxins inhaled can induce endothelial dysfunction and accelerate

atherogenesis. Cigarette smoke can cause an increase in soluble ICAM-1 in the plasma, a biomarker for myocardial infarction risk and sign of endothelial dysfunction (122, 123). Furthermore, cigarette smoke activates macrophages, a possible mode for the pro-atherogenic insulin resistance and decrease in adiponectin levels seen in chronic smokers (124-126).

Elevated concentrations of the vasoconstrictor angiotensin II (AngII), a major contributor to hypertension, can initiate vascular inflammation. In addition to contraction and hypertrophy, AngII stimulates VSMC to express IL-6, MCP-1, and VCAM-1 (127-129). Hypertension can also induce oxidative stress in the arterial wall (63).

1.1.5 Restenosis

Significant stenosis is usually defined as a greater than 75% reduction of cross-sectional area of the lumen (59). The current methods to circumvent arterial blockages, such as balloon angioplasties and stents, compress plaques and widen the artery but often result in counterproductive re-narrowing of the vessel (restenosis). With a simple balloon angioplasty, restenosis occurs in 30-60% of patients. The introduction of the bare-metal stents reduced the reoccurrence to 16-44%, while drug-eluting stents (i.e. sirolimus, paclitaxel) are even further improved with 0-16% (130). However, these stents are less efficacious in diabetics compared to non-diabetic patients (131, 132).

Restenosis features a hyperactive wound-healing response and, aside from foam cell formation, highlights many of the same proliferative and inflammatory processes as atherosclerosis. In a process known as denudation, the protective endothelial lining is removed, damaging the underlying vascular layer and setting into motion an array of events that lead to neointima formation. Immediately following vascular injury, a deposit of platelets forms on the

denuded surface of the vessel and release PDGF and other growth factors to the VSMC below to stimulate migration (133). Leukocytes are recruited to the platelet layer and migrate through to the medial VSMC, secreting cytokines and growth factors (6). NAD(P)H-derived reactive oxygen species also promote VSMC proliferation and migration (134). The surge of VSMC proliferation, due to the release of these stimulants, continues for two weeks post-injury in rodents (135). Migratory adventitial fibroblasts may also contribute to neointima formation (136).

Normally, VSMC proliferate at a very low rate, less than 0.1% per day (137). After stimulation with numerous growth factors and cytokines, as well as tensile stress from the balloon or stent, VSMC undergo a switch from a contractile phenotype to a synthetic phenotype. This is evidenced by cellular hypertrophy, increased proliferation and migration, and a decreased ability to contract (138). These dedifferentiated VSMC are also stimulated to synthesize matrix components, such as collagen, leading to increased extracellular matrix. In order to allow continued migration of VSMC from the media to intima, there is an increase in plasminogen activators and matrix metalloproteinases which degrade the surrounding matrix (139). Even though the VSMC stop proliferating after two weeks, the neointima continues to grow through increased cell volume and extracellular matrix deposits (137).

1.2 NUCLEAR FACTOR-KAPPA B

1.2.1 Introduction to NF-κB

NF-κB was originally described over 25 years ago as an essential DNA-binding complex for immunoglobulin kappa light chain transcription (140). This family of transcription factors consists of five subunits that may homo- or heterodimerize: RelA/p65, RelB, c-Rel, p50, and p52, where p65/p50 is the classical example. These proteins share a highly conserved 300-amino-acid Rel homology domain which is responsible for dimerization, DNA binding, and interactions with ankyrin repeats in inhibitors of κB (IκB) (141, 142). NF-κB also contains a nuclear localization sequence (NLS). The C-terminal halves of p65, RelB, and c-Rel contain a transactivation domain (TAD) for transcriptional activity, while the pre-processed forms of p50 and p52 instead have ankyrin repeats typical of the IκB family. Subunits p50 and p52 are generated by proteolytic cleavage of p105 and p100, respectively.

Active NF-κB exists as a dimer, although not all combinations of the five subunits occur. The ubiquitous example is p65/p50, while RelB only dimerizes with p50 or p52 (143). The p50/p50 homodimer lacks transactivational activity and instead binds to κB promoter regions or recruits deacetylases to inhibit κB-dependent transcription (144), although emerging evidence suggests that it may in fact positively influence certain gene transcription (145, 146). Each dimer possesses distinct properties including binding site preference, activation, subcellular localization, and tissue specificity, allowing for differential gene regulation (147, 148). In this text I will focus on the classical p65/p50 heterodimer and refer to it as NF-κB from now on.

At least 150 different stimuli activate the transcription factor, and in turn NF-κB controls transcription of at least as many genes (149). These stimuli include inflammatory mediators,

antigen receptor engagement, bacterial and viral infections, UV irradiation, and oxidative stress (148). Cell growth, proliferation, and survival is largely dependent on NF-κB for the transcription of anti-apoptotic genes (inhibitor of apoptosis proteins, Bcl family members), growth factors, and cyclins (150-153). Genetic ablation of p65 is embryonic lethal, further substantiating the importance of NF-κB in growth and development (154). NF-κB-dependent transcription of cytokines, chemokines, adhesion molecules, and acute phase proteins also regulates inflammatory processes (149). Not surprisingly, deregulation of NF-κB results in diseases such as cancer, autoimmunity, immunodeficiency, and chronic inflammatory diseases including asthma, rheumatoid arthritis, and inflammatory bowel syndrome (155, 156).

1.2.2 The NF-κB Signaling Pathway

Traditionally, inactive NF-κB is sequestered in the cytoplasm by members of the IκB family, most notably IκBα (157). However, even though IκBα masks the NLS of p65, the NLS of p50 is still accessible (158). In conjunction with the nuclear export sequence on IκBα, this results in the shuttling of the NF-κB:IκBα complex between the cytoplasm and the nucleus, although the cytoplasm is heavily favored in the steady-state localization (159, 160). Upon stimulation, upstream signals rapidly converge on the phosphorylation and induction of the multi-subunit IκB kinase (IKK) complex. Figure 1-2 illustrates the general activation pathway of NF-κB.

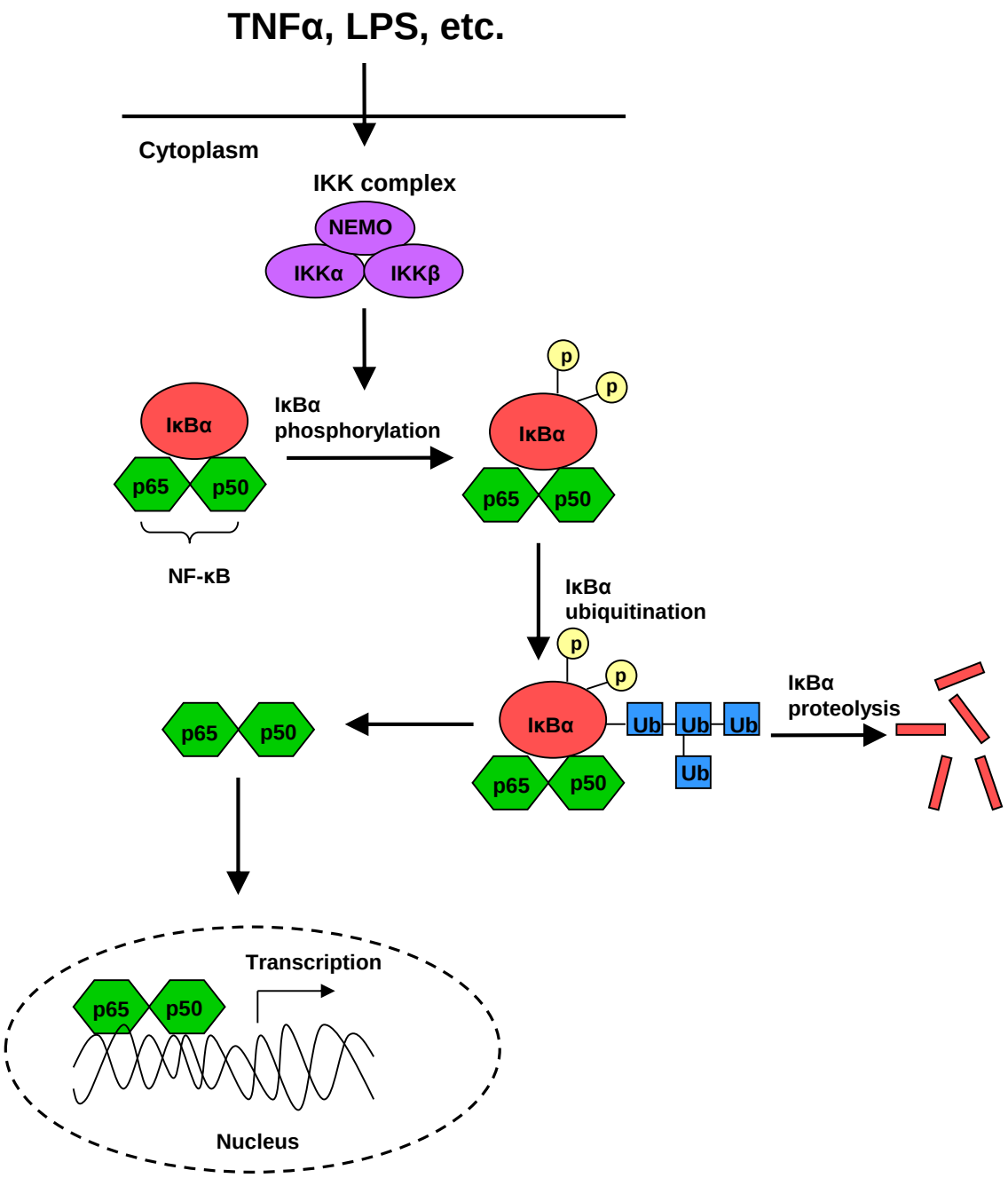

Figure 1-2: General activation of NF-κB.

NF-κB is basally sequestered in the cytoplasm by IκBα. Upon activation, upstream signals converge on IKK activation, resulting in the phosphorylation and degradation of IκBα. NF-κB is now able to translocate to the nucleus and stimulate gene transcription.

The IKK complex includes two catalytic subunits, IKKα and IKKβ, and the regulatory/scaffolding subunit IKKγ/NEMO (161). IKKα plays a minor role in this pathway and is more important for non-canonical activation of NF-κB-inducing kinase and RelB/p52 in response to B-cell activating factor, lymphotoxin-β, or CD40 ligand (162). In response to activators of the canonical pathway (i.e. TNFα, LPS, IL-1β), activation of the β subunit of the IKK complex results in the phosphorylation of S32/36 on IκBα and its subsequent proteosomal degradation, exposing the NLS of NF-κB and permitting its nuclear translocation (163, 164). Newly synthesized IκBα, one of the earliest induced NF-κB targets, can enter the nucleus and export NF-κB back to the cytosol as one method of terminating transcription (165, 166). Here I will briefly focus on the basics of the classical signaling pathways of TLR4/IL-1 receptor (IL-1R) (receptors for LPS and IL-1β, respectively) and TNF-R1 (receptor for TNFα).

Although their extracellular domains differ, IL-1R and TLR4 both contain intracellular TLR/IL-1R (TIR) domains. Upon binding IL-1β, the IL-1R accessory protein (IL-1RAcP) and myeloid differentiation primary response gene 88 (MyD88) are recruited to the TIR domain of IL-1R, followed by IL-1R associated kinase (IRAK) 1, Tollip, TNF receptor-associated factor (TRAF) 6, and IRAK-4 (167-170). Auto-phosphorylation activates IRAK-4 (171), leading to subsequent phosphorylation and activation of IRAK-1 (172). Hyperphosphorylation of IRAK-1 dissociates the kinase from Tollip and MyD88, but not TRAF6 (172). The dissociated IRAK-1/TRAF6 complex interacts with a pre-formed membrane complex consisting of TAK1/TAB1/TAB2 (173). This complex translocates to the cytoplasm but leaves IRAK-1 behind at the membrane for poly-ubiquitination and degradation (173, 174). However, the degradation of IRAK-1 is debatable and could be cell-specific (173, 175). In the cytoplasm, TRAF6 interacts with Ubc13/Uev1A, an E2 ubiquitin-conjugating enzyme complex, and

undergoes auto-poly-ubiquitination by K63-linked ubiquitin chains (176). This prompts the poly-ubiquitin binding of TAB2 and the activation of TAK1, which subsequently phosphorylates IKKβ (177).

Generally, signaling through TLR4 is similar to IL-1R with few variations (Figure 1-3). LPS first binds CD14 before transfer to MD-2 and TLR4 (178, 179). Recruitment of MyD88 to the receptor is instead dependent on TIRAP/Mal (180) and BCL10 and MALT1 are inserted in the pathway (181). In addition, there is a MyD88-independent pathway stemming from TLR4 activation. TLR4 can associate with the adaptor proteins TRIF and TRAM in early endosomes to induce delayed NF-κB and IRF-3 activation, although there is no expression of the classical inflammatory genes (182, 183).

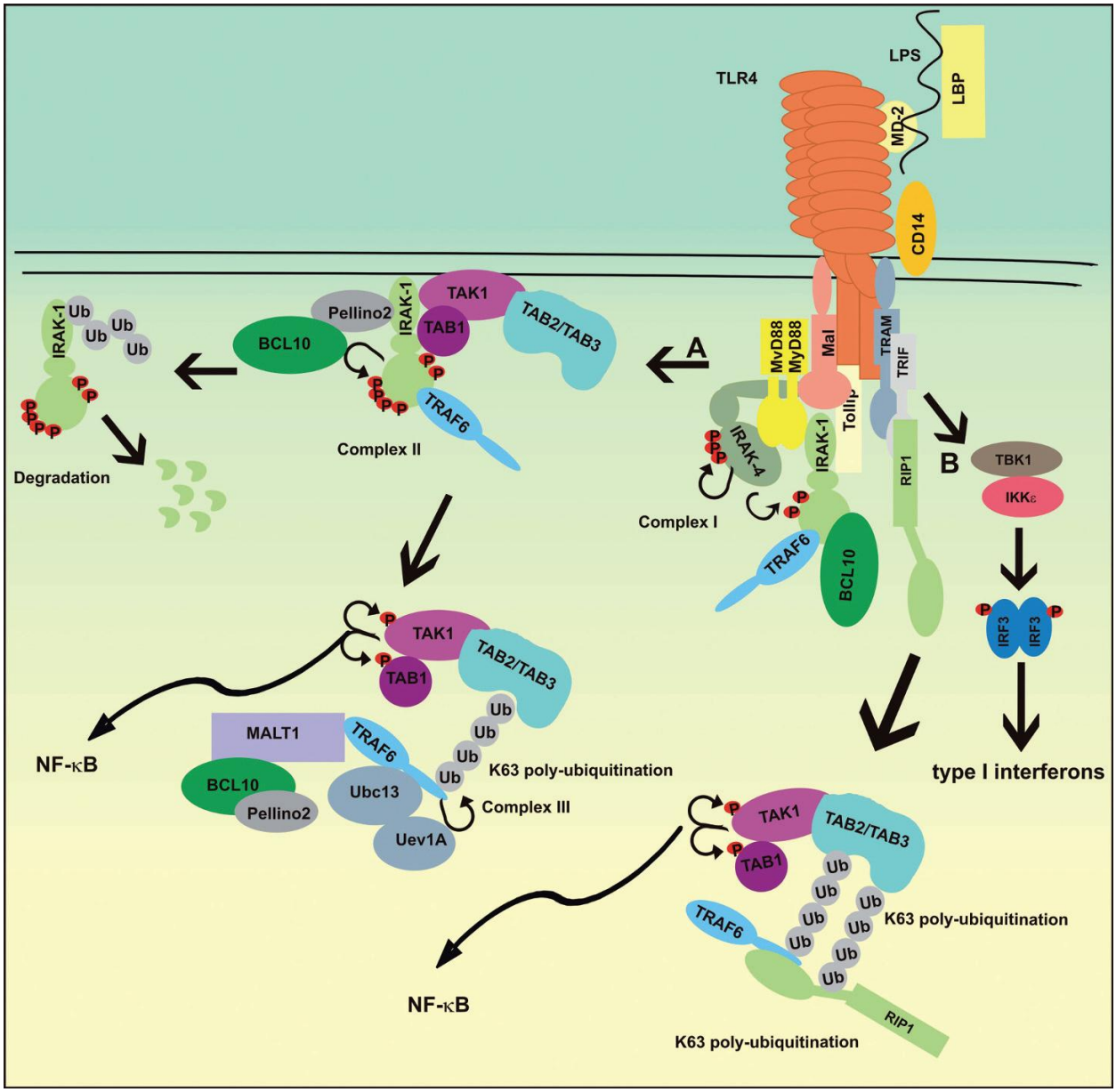

Figure 1-3: LPS signaling to NF-κB.

LPS, in complex with LPS-binding protein (LBP), binds CD14 and is transferred to MD-2 and TLR4. **A.** MyD88 is recruited to the receptor complex via Mal. The addition of Tollip, IRAK-1, IRAK-4, and TRAF6 form Complex I. Complex formation triggers IRAK-4 autophosphorylation and the phosphorylation of IRAK-1. IRAK-1 and TRAF6 leave the complex and associate with a pre-formed complex of TAK1/TAB1/TAB2/3 at the membrane (Complex II). IRAK-1 is poly-ubiquitinated and degraded. The new Complex III leaves the membrane and interacts with Ubc13/Uev1A. This results in the ubiquitination of TRAF6, TAB2 binding, and TAK1 activation, leading to IKK

phosphorylation and NF-κB activation. **B.** TLR4 also features a MyD88-independent pathway. TRAM and TRIF are recruited to endosomal TLR4. TRIF is associated with RIP1, whose poly-ubiquitination interacts with poly-ubiqutinated TRAF6 and the TAK1/TAB1 complex to induce late-phase NF-κB activation. TRIF can also initiate type 1 interferon induction through the activation of TBK1, IKKε, and IRF3.

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Like TLR4, TNF-R1 signals through distinct pathways at the membrane and after endocytosis (Fig. 1-4). TNF-R1 trimerizes in response to TNFα and interacts with TRADD through its death domains. TRADD recruits TRAF2, which undergoes K63-linked poly-ubiquitination, and receptor interacting protein (RIP), which also interacts with TRAF5 (185-187). TRAF2 similarly ubiquitinates RIP via K63-linked poly ubiquitin chains (188). The TAK1/TAB1/TAB2 complex interacts with these poly-ubiquitin chains, activates TAK1, and phosphorylates IKKβ (189, 190).

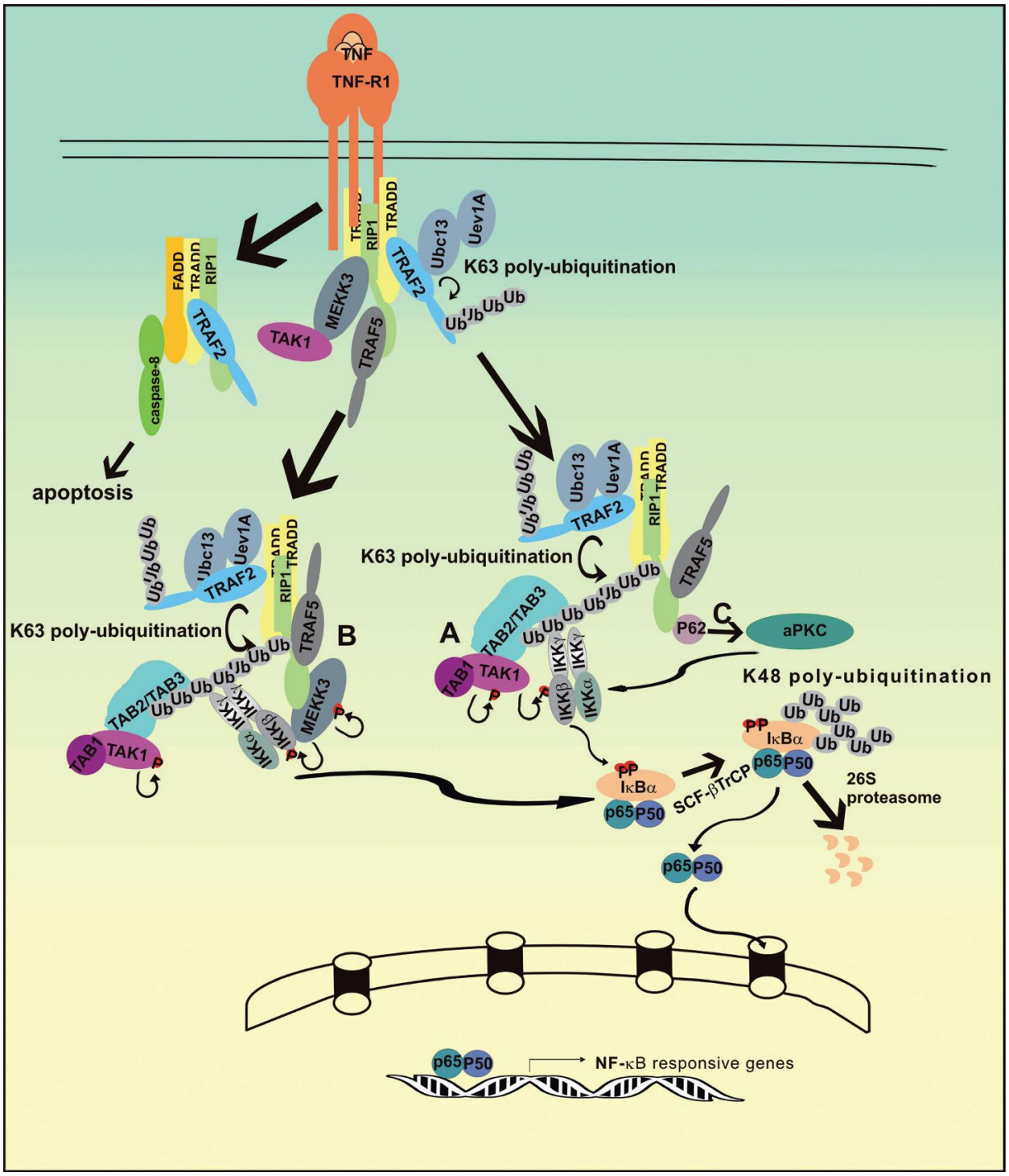

Figure 1-4: TNFα signaling to NF-κB.

TNF-R1 trimerizes in response to TNFα stimulation and recruits TRADD through its death domain. TRADD dimerizes and binds TRAF2 and RIP1, which also interacts with TRAF5. TRADD dissociates from TNF-R1 during

endocytosis and interacts with FADD, leading to caspase-8 recruitment. This initiates proapoptotic signaling. Meanwhile, TRAF2 undergoes auto-poly-ubiquitination and subsequently ubiquitinates RIP1. **A.** Poly-ubiquitinated RIP1 recruits TAB2 and IKKγ, which respectively interact with TAK1 and IKKβ, leading to NF-κB activation. **B.** RIP1 can also recruit MEKK3, which phosphorylates IKKβ. **C.** RIP1 can also interact with p62to activate atypical PKCs, leading to IKKβ phosphorylation.

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However, NF-κB activation is not always completely abrogated in TAK1-deficient cells (191, 192), suggesting the possibility of TAK1-independent routes of IKKβ activation. MEKK3 and protein kinase C (PKC) ζ are likely candidates to fill this role. After TNFα stimulation, MEKK3 can phosphorylate IKKβ independent of TAK1 (193). In contrast, MEKK3 activation in response to IL-1β involves phosphorylation of IKKα and IKKγ, not IKKβ, and subsequent phosphorylation and dissociation of IκBα from NF-κB (194). On the other hand, PKCζ is also implicated in IKK activation and will be covered in detail in a later section.

1.2.2.1 Post-Translational Modifications of p65

In order to elicit a maximal NF-κB response, the p65 subunit is additionally subjected to phosphorylation, acetylation, and ubiquitination. This extra layer of regulation influences the ability of NF-κB to recruit and interact with coactivators, bind DNA, and reassociate with IκBα.

The p65 subunit has a protein kinase A (PKA) consensus site at S276 within the Rel homology domain and coincidentally the catalytic subunit of PKA is included in the cytoplasmic NF-κB:IκBα complex (195). Degradation of IκBα activates PKAc, phosphorylating p65 at S276 and enhancing p65 binding to both DNA and the transcriptional coactivator p300/CREB-binding protein (CBP) (144, 196). Normally folded in a closed conformation, S276 phosphorylation

disrupts the inhibitory intramolecular interaction within p65 and exposes the binding sites for DNA and p300/CBP. Mitogen- and stress-activated protein kinase (MSK) 1 and MSK2 have identical substrate specificity to PKA and can also phosphorylate p65 at S276 (197). The only difference lies within subcellular localization since MSK1/2 are nuclear proteins. How the two kinases cooperate with cytosolic PKA is still unknown.

Additional p65 phosphorylation sites at S536 and S529 in the C-terminal TAD have been studied but are not as well characterized as S276. IKKα, IKKβ, ribosomal S6 kinase 1 (RSK1), and TANK-binding kinase 1 have all been implicated in phosphorylation of p65 at S536 (198-201). Phosphorylation of this residue is required for full NF-κB activity (202, 203). In contrast to other mechanisms of NF-κB induction, activation of p53 in response to DNA damage activates RSK1 and phosphorylates S536 in an IKK/IκBα-independent manner (201). This modification results in decreased affinity of p65 for IκBα, shifting the steady state localization of p65 to the nucleus and increasing its transactivational activity (201). It is not known whether phosphorylation of S536 by other kinases has similar effects. Casein kinase II (CKII) is the lone kinase implicated in the phosphorylation of S529 (204-206). Although CKII is not activated upon TNFα stimulation, it readily phosphorylates cytoplasmic p65 upon IκBα degradation and increases its transactivational potential (204, 205). However, others failed to find evidence supporting a role for S529 in NF-κB activity (202). In addition, p65 is a target for protein phosphatase 2A (PP2A) and the dephosphorylation of these serines could contribute to NF-κB inhibition (207, 208).

Inhibitory phosphorylation sites also exist within the TAD of p65. Activation of checkpoint kinase Chk1 by the ARF tumor suppressor or the chemotherapeutic drug cisplatin phosphorylates T505 and increases the association of p65 with histone deacetylases (see below)

(209, 210). This results in the repression of some anti-apoptotic genes including Bcl-xL. Phosphorylation of T435 also negatively regulates NF-κB activity, although the kinase is unknown (211). The TAD of p65 is important for binding to coactivators, so these inhibitory phosphorylations most likely regulate such associations.

Like phosphorylation, inducible acetylation of p65 provides both positive and negative levels of regulation. Acetylation of K218, -221, and -310 promote NF-κB activity while K122/123 function in an inhibitory role. K221 acetylation enhances p65 binding to DNA and, alone or together with K218 acetylation, inhibits the association of p65 with newly synthesized IκBα and retains the complex in the nucleus (212). Additionally, acetylation of K310 stimulates transactivational activity of NF-κB independent of DNA-binding and IκBα complex assembly, possibly through interactions with other coactivators (212). Furthermore, a link between p65 phosphorylation and acetylation exists in which S276 and S2536 phosphorylation leads to increased association of p65 with p300/CBP, resulting in enhanced acetylation (213). Deacetylation of these lysines by histone deacetylase 3 and sirtuin 1 results in the dissociation of p65 from κB sites and IκBα-dependent removal from the nucleus (214, 215). Conversely, K122/123 acetylation inhibits p65:DNA interactions and facilities its export from the nucleus (216).

Lastly, p65 is subject to inducible ubiquitination by the E3 ubiquitin ligases PDLIM2, SOCS1, and COMMD1. PDLIM2 binds, polyubiquitinates, and mediates targeting of p65 to PML bodies within the nucleus where it is degraded by the proteasome (217). SOCS1 is part of a complex that can couple to COMMD1 in response to stimulation (218). This association stabilizes the SOCS1-p65 interaction and also facilitates the ubiquitination of the transcription factor (218). These events result in the degradation and termination of NF-κB signaling.

1.2.2.2 Termination

While NF-κB is able to self-propagate through feed-forward and amplification loops to produce beneficial inflammation for healing and repair within the body, it is just as important that it terminates the cycle before it becomes excessive and causes damage. Negative feedback mechanisms are rapidly induced but allow for at least one round of uninhibited activation before their own induction. There are many other modes of turning off NF-κB activity in addition to newly synthesized IκBα and the post-translational modifications of p65 already described. These mechanisms target various steps of the signaling pathway and include interfering with receptor function, production of dominant-negative signaling proteins, reversal of post-translational modifications, and inhibition of nuclear p65 (219).

The IL-1 receptor and TLR4 are targeted by the nonfunctional receptor mimics ST2 and RP105, respectively. ST2 is produced in two forms, soluble secreted ST2 and transmembrane ST2. Soluble ST2 functions as a decoy receptor to IL-1 while transmembrane ST2 lacks a TIR domain and inhibits IL-1 receptor and TLR4 by competing for MyD88 binding (220). RP105, which also lacks a TIR domain and is unable to transduce signals, interacts with TLR4 and inhibits LPS binding (221).

Dominant-negative signal transducers can arise from splice variants or as a cell-specific response. Unlike the ubiquitous IRAKs, expression of IRAK-M is restricted to monocytes and macrophages (222). Rapidly induced upon TLR4 signaling, the kinase-dead IRAK-M prevents dissociation of IRAK and IRAK-4 from MyD88, effectively inhibiting NF-κB activation (223). Similarly, IRAKc is a splice variant that lacks exon 11, part of the kinase domain, and thus kinase activity. IRAKc still retains the ability to interact with IRAK2, MyD88, Tollip, and

TRAF6, but is unable to be phosphorylated by IRAK4 (224). Another example is a truncated form of MyD88, MyD88s, which fails to recruit IRAK4 (225).

An interesting protein induced after NF-κB activation is A20, an enzyme that features dual ubiquitin-editing functions. A20 is a deubiquitinase for K63-linked polyubiquitination of TRAF6, NEMO, and RIP (226-228). Other deubiquitinating enzymes important for the termination of NF-κB activation include Cezanne and Cyld (229, 230). Conversely, A20 functions as an E3 ubiquitin ligase for K48-linked degradative polyubiquitination of RIP (226). Both actions of A20 result in the suppression of NF-κB signaling upstream of the IKK complex.

Further downstream in the pathway, nuclear proteins are able to inhibit p65 binding to target promoters. The IκB family member IκBζ, induced upon stimulation, interferes with NF-κB binding to DNA without affecting nuclear translocation (231). In addition, IKKα activation phosphorylates PIAS1, enabling its association with NF-κB promoters and inhibition of a subset of genes (232).

1.2.3 Involvement of PKCζ in NF-κB Activation

PKCζ is a serine/threonine kinase classified as an atypical member of the PKC family. The structure of a PKC family member can be divided into the N-terminal regulatory region and the C-terminal kinase domain. The ten members of the kinase family can further be classified according to the topology of their regulatory domains: classical, novel, or atypical. Classical PKCs (PKCα, βI, βII, γ) contain a diacylglycerol-responsive conserved region 1 (C1) as a tandem repeat that is structurally represented as a double zinc finger, in addition to a C2 domain that senses calcium (233, 234). Novel PKCs (PKCδ, ε, η, θ) are activated by diacylglycerol but are calcium-independent while atypical PKCs (PKCζ and λ/ι) are insensitive to both, containing

a single C1 domain and lacking the C2 altogether (235). Instead, atypical PKCs have a Phox/Bem1 domain that connects them to Phox/Bem1-containing proteins such as p62 (236).

PKCζ is naturally inactive because its substrate-binding pocket in the kinase domain is auto-inhibited by the regulatory pseudosubstrate (237). Although not responsive to phorbol esters, other lipids such as phosphatidylinositols (238), phosphatidic acid (239), ceramide, and arachidonic acid (240) can activate PKCζ, presumably through release of the pseudosubstrate from the binding pocket. Indeed, overexpression or induction of phosphatidylinositol-3-OH kinase (PI3K) increases phosphatidylinositol 3,4,5-trisphosphate (PIP_3) production and activates PKCζ (238, 241). Phosphoinositide-dependent kinase 1 is activated after its pleckstrin homology domain binds PIP_3, and it is this kinase that phosphorylates the T410 priming site in the activation loop of PKCζ (242, 243). Autophosphorylation of T560 renders PKCζ catalytically competent (244).

PKCζ is a crucial regulator of the NF-κB pathway. In response to TNFα, IL-1β, and LPS, PKCζ is phosphorylated and translocated to the plasma membrane (241, 245-250). Overexpression of PKCζ activates NF-κB, even in the absence of stimuli, and a kinase-dead mutant inhibits this (245, 251, 252). A key binding partner of PKCζ is zeta-interacting protein (ZIP)/p62 (236). ZIP/p62 scaffolds PKCζ to TNF-R1 complexes including RIP and TRAF2, and additionally links the kinase to the IL-1R signaling complex with TRAF6 (247, 253). Although not yet directly examined, PKCζ may phosphorylate IRAK-1 in these complexes (254, 255). Activated PKCζ can also directly phosphorylate IKKβ but not α, serving as a TAK1 substitute (245). However, while PKCζ is necessary for NF-κB transcriptional activity in embryonic fibroblasts, it has no effect on IKK activation (246). On the other hand, PKCζ is essential for IKK activation in the lungs of these mice, demonstrating a cell-specific role for PKCζ in this step

of the pathway (246). In addition to upstream functions, PKCζ also phosphorylates S311 on the p65 subunit (256). Phosphorylation of S311 is necessary for transactivational activity and binding to p300/CBP but has no effect on DNA binding (256).

1.2.4 NF-κB in the Vasculature

NF-κB activation lies downstream of many factors implicated in vascular diseases including hypoxia, metabolic factors (homocysteine, AGE), infectious agents, T-cell activation, and cytokines (257). Modified LDL can induce NF-κB , but longer exposure suppresses its activation (19). This may contribute to the apoptosis of foam cells. Activated NF-κB is present in endothelial cells, macrophages, and VSMC within the atherosclerotic plaque, and controls the expression of many key inflammatory features of macrophage recruitment and adhesion, VSMC proliferation, and fibrous cap integrity (149, 258).

Endothelial cells and VSMC express NF-κB-dependent proteins such as MCP-1 to attract leukocytes to the injured area as well as the adhesion molecules ICAM-1, E-selectin, and VCAM-1 to facilitate the attachment and transmigration of the leukocytes along the vascular wall (18, 149). NF-κB is central to VSMC proliferation and survival, mainly by the induction of anti-apoptotic proteins (127, 129, 259). MMPs are upregulated to degrade the surrounding extracellular matrix for migrating VSMC and inhibition of NF-κB abolishes the procoagulant activity of tissue factor (20-22). Other proteins increased by NF-κB include inducible nitric oxide synthase (iNOS), PAI-1, and COX-2, which further mediate the progression of inflammation (149). Of course, many cytokines such as IL-1β and TNFα are also NF-κB-dependent and can create a positive feedback loop to further activate NF-κB (149, 257).

Many studies have established a role for NF-κB in vascular pathologies. Basal NF-κB in the rat carotid is low, but after balloon injury there is degradation of IκBα and an upregulation of NF-κB genes VCAM-1 and MCP-1 (260). Administration of an IκBα adenovirus during an angioplasty in rabbits decreased ICAM-1 and MCP-1 expression as well as macrophage recruitment and lumen narrowing (261). Similarly, transfection of NF-κB antisense oligonucleotides into a balloon-injured rat carotid artery inhibits neointima formation along with ICAM-1 and VCAM-1 expression and leukocyte infiltration (92). Additionally, activated NF-κB is present in atherosclerotic lesions (262, 263) and broad inhibition of NF-κB is correlated with an overall attenuation of atherosclerosis (128, 264, 265).

Surprisingly, NF-κB is also involved in the resolution of inflammation. Macrophage-specific deletion of IKKβ enhances atherosclerosis in *Ldlr*$^{-/-}$ mice (266). These macrophages present with a reduced early inflammatory profile, but 24 hours post-LPS treatment cytokine levels of TNFα, IL-1β, and IL-6 are similar to wild type. The concomitant decreased expression of the anti-inflammatory IL-10, another NF-κB target gene in macrophages, is unable to deactivate macrophages and inhibit inflammatory cytokine release. As described earlier, IL-10 is a critical signaling molecule for the resolution of inflammation. In addition, the p50 subunit of NF-κB can form homodimers in the nucleus to inhibit κB promoter elements (119).

Other conflicting evidence exists for a role of NF-κB in atherosclerosis. Recently, an independent group showed that ablation of IKKβ in the bone marrow actually decreases atherosclerosis in *Ldlr*$^{-/-}$ mice, even though IL-10 is similarly affected as previously described (267). Inhibition of NF-κB also results in decreased foam cell formation (267, 268). In the vessel wall, endothelium-specific inhibition of NF-κB through expression of a dominant-negative IκBα or deletion of IKKγ/NEMO protects *Apoe*$^{-/-}$ mice (269). Yet deletion of IκBα in the bone

marrow, investigated by the same group who deleted IKKβ in the macrophages above, aggravates atherosclerosis by enhancing leukocyte recruitment without affecting classical NF-κB genes (270). Clearly there is a complex and dual role for NF-κB in the control of inflammation.

In addition to coordinating NF-κB signaling in macrophages (255) and endothelial cells (271), there is evidence that PKCζ also participates in vascular function. PKCζ promotes VSMC proliferation by a variety of stimuli including norepinephrine, angiotensin II, insulin growth factor, and PDGF (272-275). In polymorphonuclear leukocytes, PKCζ mediates integrin-dependent adhesion and chemotaxis (276) and their adhesion to endothelium (275). Increased PKCζ activity is linked to athero-susceptibility in sites of disturbed flow in aortic endothelium (277). Moreover, inhibition of PKCζ by antisense oligonucleotides significantly decreases neointima formation in injured rat carotid arteries (278).

1.3 THE SCAFFOLDING PROTEIN EBP50

1.3.1 Discovery and Structure

The discovery of ezrin-radixin-moesin-binding phosphoprotein 50 (EBP50), a scaffolding protein that assembles signal transduction complexes, was the product of two independent groups focused on different areas of inquiry. The origin of the name EBP50 evolved from a search for proteins bound to ezrin. Immobilized NH_2-terminal fragments of ezrin and moesin revealed EBP50 as a binding partner (279). They also noticed that this protein migrated as multiple bands ranging from 50kDa to 53 kDa which were resolved into a single band of 50kDa

with phosphatase treatment, indicating phosphorylation (279). Concurrently, another group was pursuing a necessary regulatory cofactor of cAMP-mediated inhibition of Na^+/H^+ exchanger (NHE) activity in the renal brush border membrane (280, 281). This cofactor was a substrate for PKA phosphorylation distinct from the exchanger and coined Na+/H+ exchanger regulatory factor (NHERF) (282). These two terms for the same protein, EBP50 and NHERF1, are used interchangeably throughout the literature; in this text I will refer to the protein as EBP50.

EBP50 is comprised of two tandem postsynaptic density 95/discs-large/zona occludens (PDZ) domains (termed PDZ1 and PDZ2) followed by a C-terminal ezrin-binding domain (EBD) (Fig. 1-5). This EBD serves to anchor binding partners of EBP50 to the underlying cytoskeleton. The most abundant protein-protein interaction module, PDZ domains are typically 80-100 amino acid residues in length and composed of six β-strands and two α-helices (283). The single carboxylate binding loop within each domain is highly conserved and features a core motif of G-Φ-G-Φ, where the first glycine is permissive and Φ is a hydrophobic residue (GYGF in the case of EBP50) (284). The hydrophobic residues in this motif help form the hydrophobic ligand-binding pocket (285). As its name suggests, the carboxylate binding loop recognizes defined sequences at the extreme C-terminus of target proteins (286), although binding to internal sequences has also been reported (287-290). PDZ domains can be classified based on the motifs with which they interact: class I prefers (E/D)-S/T-X-Φ, where X is any amino acid; class II recognizes Φ-X-Φ; and class III favors D/E-X-Φ (286, 291). However, upstream residues may also be important, especially in determining specificity for PDZ1 or PDZ2 (292). The PDZ domains in EBP50 are largely considered class I (293, 294), although they can interact with other motifs (295-298). Consequently, EBP50 terminates in a consensus PDZ motif (S-N-L) and can self-associate in an intramolecular head-to-tail interaction between PDZ2 and the EBD (299).

This closed conformation hinders interactions between PDZ domains and their ligands. Binding of the EBD to ezrin unmasks the PDZ domains and restores the ability of EBP50 to assemble complexes (299).

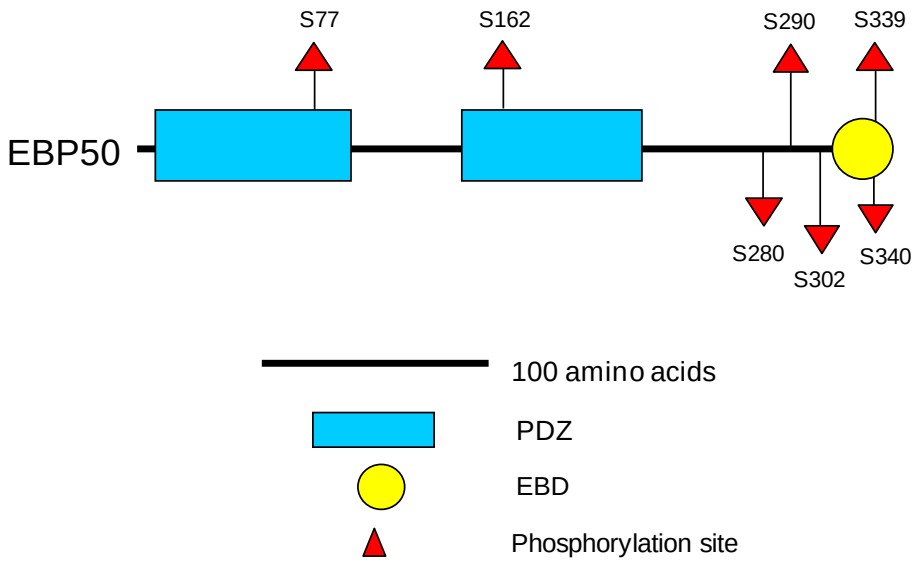

Figure 1-5: Structure of EBP50

EBP50 is 358 amino acids in length and contains two tandem PDZ domains (blue boxes) followed by a C-terminal EBD (yellow circle). As a phosphoprotein, EBP50 is regulated by phosphorylation at multiple locations (red triangles).

EBP50 is abundantly expressed in the apical membranes of polarized epithelial tissues including the kidney, small intestine, placenta, and liver (279, 300). Generally EBP50 colocalizes with ezrin, particularly in the microvilli (279), but this is not absolute (301).

Additionally, EBP50 is present in airway epithelium, ependymal cells in the brain, mammary epithelium, and to a lesser extent in other tissues (279, 300, 302, 303).

Additional related PDZ proteins have been discovered, mainly in epithelial tissues. NHERF2, shares 44% identity with EBP50 and a similar structure of two PDZ domains and a C-terminal EBD (304). However, the distribution of these two family members is mutually exclusive (301). NHERF3 and NHERF4 are distinct from the former pair with four PDZ domains and no EBD (305-307).

1.3.2 Regulation of EBP50

1.3.2.1 Transcriptional

Although other regulators have been discovered, estrogen is the main inducer of human EBP50 expression. This regulation was first discovered using a differential RNA display to identify estrogen-responsive genes in breast cancer (300). Interestingly, the human *SLC9A3R1* (EBP50) promoter lacks any palindromic full estrogen response elements (ERE) but instead contains 13 half-ERE sites consisting of TGACC or GGTCA in the first 3.5 kilobases which are sufficient for estrogen receptor (ER) binding (308). Other estrogen-responsive genes such as ovalbumin can function without any accompanying complete palindromic ERE sites (309). In addition, *SLC9A3R1* lacks a canonical CAAT or TAT box in the promoter region. These observations are consistent with studies showing a correlation between estrogen levels and EBP50 expression during the reproductive cycle (303, 310). EBP50 expression is also increased in ER+ breast cancer cells compared to ER- or normal mammary tissues (303, 311, 312). However, the mouse *Slc9a3r1* promoter is deficient in ERE sites, full or otherwise, and thus is unresponsive to estrogen (313). Other hormones such as glucocorticoids, androgens, and progestin have no effect

on EBP50 expression (300). I will describe new advances in the transcriptional regulation of EBP50 stemming from my own work in a later section.

1.3.2.2 Post-Translational

The phosphoprotein EBP50 has 31 serine and 9 threonine residues available for post-translational regulation. Depending on the residue, phosphorylation events can enhance or diminish EBP50 function. The phosphorylation sites described below are also depicted in Figure 1-5.

Although EBP50 does not contain a canonical PKA or PKC motif, there is evidence that these kinases lead to phosphorylation of EBP50. PKA does not phosphorylate EBP50 *in vitro*, but activation of the kinase using 8-bromo-cAMP induced phosphorylation of S77 (314). Therefore, additional signaling events may lie between PKA and EBP50 phosphorylation.

In contrast, activators of the PKC pathway such as parathyroid hormone (PTH), dopamine, and 1,2-dioleoylglycerol, as well as direct *in vitro* kinase assays with PKC, result in S77 phosphorylation (314-316). Located in the PDZ1 domain, this phosphorylation attenuates the interaction of EBP50 with numerous binding partners (314, 315). PKC additionally phosphorylates EBP50 at S339/340 in the EBD, weakening the EBP50-ezrin interaction and redistributing EBP50 from the membrane to the cytoplasm without affecting ezrin localization (317-319). Consequently, this generates increased PDZ interactions, possibly by disrupting the closed conformation of EBP50 and exposing the PDZ2 domain. PKC also mediates the phosphorylation of S162, although the intramolecular interactions of the EBD and PDZ2 domain in the closed conformation may initially shield this residue (318). Furthermore, a triple PKC phosphomimic (S162/339/340D) increases EBP50 turnover on microvilli (319).

Other kinases that phosphorylate EBP50 include cyclin-dependent kinase 1 (Cdk1) and G protein-coupled receptor kinase 6A (GRK6A). GRK6A is responsible for the constitutive phosphorylation of rabbit EBP50 at S289 (S290 in humans). Interestingly, GRK6A terminates in a PDZ ligand (T-R-L) and interacts with EBP50, an event that is required for S289 phosphorylation (320). This residue lies within a cluster of serines which are required for biological activity (321). EBP50 is highly phosphorylated during mitosis, more so than other stages of the cell cycle, due to the activity of Cdk1 (322). Cdk1 phosphorylates rabbit EBP50 at S279/301 (S280/302 in humans), an action that decreases microvilli formation (323).

1.3.3 Physiology

EBP50 currently has more known targets than any other PDZ protein (324). These targets include G protein-coupled receptors (GPCRs), ion transporters, receptor tyrosine kinases, and other non-receptor membrane and cytoplasmic signaling proteins. Some examples are outlined in Table 1. In this section I will describe some of the more prominent biological functions of EBP50, although there are clearly many more roles for this protein than summarized here.

Table 1-1: Common PDZ ligands for EBP50

Ligand	PDZ-Binding Motif	EBP50 PDZ Domain	Reference
GPCRs			
β2-Adrenergic Receptor	DSLL	1	(325)
Parathyroid Hormone Receptor 1	ETVM	1	(326)
κ-Opioid Receptor	NKPV	1	(296, 327)
Ion Transporters			
NHE3	STHM	2	(328)

Cystic Fibrosis Transmembrane Regulator	DSTL	1 > 2	(329, 330)
Sodium-Phosphate Cotransporter Type IIa	ATRL	1	(306)
Receptor Tyrosine Kinases			
Epidermal Growth Factor Receptor	DSFL (internal)	1	(331)
Platelet-Derived Growth Factor Receptor	DSFL	1	(332)
Other			
β-catenin	DTDL	2	(333)
Phospholipase C β1 and 2	DTPL/ESRL	1	(334)
$G_q\alpha$	YNLV	1/2	(335)

1.3.3.1 Ion Homeostasis

The EBP50 knockout mouse was originally generated by homologous recombination of exon 1 (336) and later using exons 1 to 4 (337), both cohorts reporting hypophosphatemia as a major phenotype. Mice genetically deficient in EBP50 exhibit normal blood chemistry except for a decrease in serum phosphate concentration (336). This is coupled to an increase in urinary phosphate and calcium excretion. Shenolikar *et al.* reported lower bone mineral density and an increased incidence of fractures (336), but this was not replicated in the mice generated by Morales and colleagues (337). The bone phenotype and calcium wasting was originally dismissed as secondary to the increased phosphate excretion since EBP50 is not expressed in the thick ascending limb or the distal convoluted tubule of the kidney where calcium is reabsorbed (338). However, recent studies demonstrate that EBP50 is required for osteoblast differentiation and matrix synthesis (339).

Phosphate homeostasis, on the other hand, is primarily regulated in the proximal tubule by the sodium-phosphate cotransporter type IIa (Npt2a). EBP50 tethers this ion transporter to the apical membrane, facilitating phosphate reabsorption, and the absence of EBP50 results in the

redistribution of Npt2a to cytoplasmic vesicles (336). In contrast, EBP50 is also important for the PTH-mediated inhibition of Npt2a and phosphate transport. EBP50 stabilizes the parathyroid hormone receptor 1 (PTH1R) at the plasma membrane and PTH-induced phosphorylation at S77 results in the disruption of the EBP50-Npt2a complex (314, 340, 341). Similarly, patients with *SLC9A3R1* (EBP50) mutations (L110V, R153Q, E225K) present with phosphate wasting, kidney stones, and a history of bone fractures or low bone density (342). These mutations stabilize the closed formation of EBP50, impeding the formation of a ternary complex between EBP50, Npt2a, and ezrin and thus rendering the system refractory to phosphate reabsorption (343).

True to its alternate name, EBP50 can also regulate Na^+/H^+ exchange via NHE3 binding. NHE3 is responsible for the majority of NaCl absorption in the intestine and kidney. While EBP50 does not affect the presence of NHE3 at the apical membrane, it is crucial for the cAMP-PKA-mediated phosphorylation and subsequent inhibition of NHE3 activity (281, 336, 344, 345). Furthermore, stimulation of the β_2-adrenergic receptor (β_2-AR) increases NHE3-mediated Na^+/H^+ exchange independently of G protein activation (346). This is thought to be due to the redistribution of EBP50 from NHE3 to β_2-AR, releasing the inhibitory actions of EBP50 on NHE3. Physiologically, EBP50 is downregulated in intestinal biopsies from mouse models and patients with inflammatory bowel disease, resulting in diarrhea (347).

A similar complex regulates the cystic fibrosis transmembrane regulator (CFTR) Cl^- channel, although in a positive manner. In the absence of EBP50, HCO_3^- is decreased in the duodenum (348). Additionally, stimulation of β_2-AR with isoproterenol in human airway mucosa generates cAMP, activating CFTR-dependent chloride transport (349). Together EBP50, β_2-AR, and CFTR exist in a PKA-dependent complex at the apical surface of both the small intestine and airway epithelium (348, 350, 351). EBP50 is vital for bringing CFTR in close proximity to β_2-

AR and the local concentrations of cAMP produced after stimulation (350). Additionally, about 10% of CFTR mutations in cystic fibrosis are C-terminal deletions, and the lack of interaction with EBP50 interferes with proper CFTR distribution and Cl^- transport (352).

1.3.3.2 Cancer

There is no doubt that EBP50 plays a role in a number of different cancers, but whether it functions as a tumor suppressor (acting as a molecular brake on proliferation and migration) (353-356) or an oncogene (associated with metastasis and invasiveness) (311, 357-359) is controversial. In some reports, EBP50 is overexpressed in cancers of the breast, liver, and brain (311, 333, 358, 360). Not surprisingly, there is a strong correlation between EBP50 and ER status in ER+ breast cancers (303, 311, 312) and one study even discovered an upregulation of EBP50 after serum starvation and hypoxia, suggesting a positive association with the surrounding tumor environment (311). Conversely, screening of breast cancer cell lines and primary tumors reveals that a majority are undergoing loss of heterozygosity at the *SLC9A3R1* locus (17q25.1), some also with missense mutations (356). These genotypes are associated with higher aggressiveness and poor prognosis.

In the instances where EBP50 is overexpressed in cancer tissues, there is a relocation of EBP50 from the membrane to the cytoplasm (303, 311, 312, 333, 361). Georgescu *et al.* proposed an elegant model which describes EBP50 as oncogenic when redistributed to the cytoplasm and a tumor suppressor when localized at the plasma membrane (362). Movement to the cytoplasm could disrupt protective complexes at the membrane and progress toward tumorigenic consequences. There are a number of mechanisms that support this theory. For instance, EBP50 can bridge phosphatase and tensin homolog (PTEN) to PDGFR in a ternary complex (363). PTEN is a tumor suppressor that dephosphorylates PIP_3 to phosphatidylinositol

4,5-bisphosphate (PIP_2), antagonizing PI3K activity (364). Complex assembly via EBP50 suppresses PI3K activation in response to PDGF and slows down migration (363). Another example is the regulation of β-catenin by EBP50. At the membrane, β-catenin stabilizes adherens junctions but interacts with T-cell factor/lymphoid enhancing factor in the nucleus to promote proliferation (365). EBP50 stabilizes β-catenin at the membrane and the absence or overexpression (relocation) of EBP50 generates increased β-catenin transcriptional activity (333, 354). Additionally, EBP50 mediates an association between the ERM protein merlin and the epidermal growth factor (EGF) receptor (EGFR) which inhibits the receptor's signaling (366). Dissolution of these complexes removes the checks EBP50 provides and allows for prolonged and even uncontrollable signaling and growth.

1.3.3.3 Vasculature

Significant roles for EBP50 in the vasculature have emerged in recent years and the work in our lab has been instrumental in this regard. EBP50 is normally restricted to the endothelium with little or no expression in VSMC but is robustly expressed in the proliferative VSMC of the neointima following endothelial denudation in rat femoral arteries (367). Furthermore, we reported a modest increase in EBP50 expression following parathyroid hormone-related peptide (PTHrP) treatment in VSMC, although additional stimuli are surely responsible for its compelling upregulation in injured vessels. Using a similar model, EBP50-null mice exhibit a striking decrease in neointima formation after wire injury (368).

We have identified the effect of EBP50 in two key characteristics of vascular remodeling, proliferation and migration. VSMC lacking EBP50 exhibit significantly decreased rates of these two characteristics (368, 369). Consistent with the variety of binding partners EBP50 boasts, there are at least two mechanisms behind its effects on proliferation. N-terminal fragments

(amino acids 1-36) of PTHrP, which is also increased after vascular injury, typically inhibit VSMC proliferation through a PTH1R-cAMP-dependent pathway (367, 370). However, the overexpression of EBP50 increases total and membrane expression of PTH1R and restores the receptor's ability to produce calcium signals (367). This results in an overall attenuation of the anti-proliferative effect of PTHrP (367). In addition, EBP50 negatively regulates the expression of p21 (368). This cell-cycle inhibitor is degraded by Skp2, part of the Skp1/Cullin-1/F-box E3-ligase complex (371), which also happens to interact with EBP50 (368). Active Akt increases the interaction between Skp2 and EBP50, resulting in increased expression and stability of Skp2 (368). Ultimately, EBP50 is a positive and stimulus-independent regulator of VSMC proliferation.

EBP50 also promotes the motility of VSMC (369). The non-receptor tyrosine kinase focal adhesion kinase (FAK) plays a central role in focal adhesion turnover and cell migration (372), a function that is newly dependent on EBP50. EBP50 bridges the interaction between EGFR and FAK, facilitating the phosphorylation of FAK after EGF treatment (369). Activation of FAK leads to increased focal adhesion turnover and migration, characteristics that are diminished in VSMC lacking EBP50 (369).

Concurrently, the Morel lab at the Université Catholique de Louvain in Belgium has also been looking into the functions of EBP50 in vasculature, albeit within the rat as opposed to the mouse. First, they demonstrate a noradrenaline-dependent interaction between EBP50 and moesin, leading to a decrease in the contractile response of mesenteric arteries (373). Second, Baeyens and colleagues propose that EBP50 acts as a molecular brake on migration. Curiously, knocking down EBP50 in rat VSMC results in a decrease in the number and size of focal adhesions and an overall faster migration rate, opposite of what we observe in mouse VSMC

(374). The authors conclude EBP50 inhibits the stable formation of microtubules through associations with myosin IIa, thus slowing down migration. In addition to the difference in species of VSMC utilized, these experiments were conducted in prolonged absence of serum, whereas we stimulated our cells with growth factors or maintained them in minimal serum conditions for no more than 16 hours. Therefore, the discrepancy in conditions may have led to differences in EBP50 phosphorylation and thus altered its functions. This could explain the differences in results seen between the two labs.

The role of EBP50 in the endothelium has not been as thoroughly researched with only two publications on the topic to date. In contrast to VSMC, EBP50 has no effect on PDGF-mediated proliferation or chemotaxis in porcine aortic endothelial cells (375). In a second study focused on the mitosis-specific phosphorylation of EBP50 in bovine pulmonary artery endothelial cells, overexpression of EBP50 has no effect on wound healing but a Cdk1 phosphomimic mutant (S288/301D, 280/302 in humans) slightly accelerates the process (376). Interestingly, EBP50 is strictly nuclear in these endothelial cells and only shifts to the cytoplasm when it is phosphorylated during mitosis (376). It is possible that dephosphorylation of these sites is necessary for mitotic exit, and preliminary evidence suggests this may be mediated by PP2Ac (376). Certainly further studies need to be conducted on EBP50 in the endothelium.

Although they do not always reside within the physical vessel wall, circulating leukocytes are important in the progression and resolution of vascular diseases. There is growing evidence that EBP50 helps guide important functions in macrophages and neutrophils. Phosphatidylinositol-phosphate kinase β (PIPKIβ) is required for dHL60 (a neutrophil-like cell line) cell polarity and chemotaxis, a function dependent on the C-terminal tail of the kinase (377). Consequently, EBP50 binds the C-terminus and links PIPKIβ to the cytoskeleton,

associations which are augmented in response to chemoattractants (377). EBP50 then localizes PIPKIβ to the uropod of the chemotaxing neutrophils to facilitate polarization and chemotaxis (378). EBP50 also scaffolds the interaction between CXCR2 and phospholipase C β (PLCβ) 2 in dHL60 cells (379). CXCR2, a G_i-coupled GPCR, is one of the main chemokine receptors in neutrophils mediating the chemotactic response to IL-8 (380-382). Calcium influx is additionally required for leukocyte migration and this is mediated by PLCβ2, the main PLC isoform in neutrophils (383, 384). When the CXCR2-EBP50-PLCβ2 complex is disrupted by a peptide corresponding to the PDZ motif of CXCR2, the release of calcium stores, migration, and endothelium infiltration are inhibited (379). Thus, EBP50 has multiple modes of controlling neutrophil chemotaxis.

EBP50 is furthermore important in the bactericidal function of macrophages. iNOS generates high quantities of nitric oxide for host protection and proximity to the pathogen is imperative for its efficacy (385). EBP50 has previously been shown to bind iNOS in epithelial cells, and this interaction is essential for proper targeting of iNOS to phagosomes in RAW 264.7 macrophages in response to LPS (386, 387). Remarkably, EBP50 expression is also increased after LPS treatment, a finding that may open new doors to explaining its upregulation after vascular injury (386).

1.4 RATIONALE, HYPOTHESIS, AND SPECIFIC AIMS

1.4.1 Rationale and Hypothesis

CVD is the leading cause of death in developing countries. In fact, Americans have a 50% greater risk of dying from CVD than cancer (388). Atherosclerosis, an inflammatory disease, is a common precursor to ischemia and myocardial infarction. Even with the best current treatment the risk of another major adverse cardiovascular event is 70-80% (389). The vascular response to this inflammation requires communication between multiple cell types including VSMC and macrophages. Even though a number of signaling pathways have been implicated in vascular diseases, there still exist many gaps in this knowledge. Current studies in the laboratory have identified EBP50 as a key mediator of vascular remodeling (368). However, the function of EBP50 in inflammation and atherosclerosis has never been explored and my preliminary data suggest a role as an inflammatory mediator. Collectively, my studies suggest that EBP50 is involved in the pathogenesis of atherosclerosis. **It is hypothesized that EBP50 is a central mediator of macrophage activation and the response of VSMC to inflammation.** This novel mechanism may be an important cause of atherosclerosis and its elucidation has significant translation implications for the development and targeting of pharmacological therapies.

1.4.2 Specific Aim 1: Explore the Role of NF-κB in the Regulation of EBP50 Expression

Little is known about what regulates EBP50 expression in tissues besides estrogen. One study has demonstrated an upregulation of EBP50 upon LPS treatment in macrophages (386). This suggests that EBP50 is under control of NF-κB, an LPS-inducible pathway. Initial examination

of the EBP50 promoter revealed multiple potential binding sites for NF-κB according to its consensus sequence GGGA/GNNC/TC/TCC, where N is any nucleotide (390). LPS and other inflammatory activators will be used to confirm the upregulation of EBP50 by NF-κB in VSMC and macrophages. Furthermore, pharmacological and molecular inhibitors of the NF-κB signaling pathway will be utilized to determine the importance and specificity of NF-κB on EBP50 expression.

1.4.3 Specific Aim 2: Determine the Involvement of EBP50 in Potentiating NF-κB Activity

Preliminary data indicated that EBP50 is necessary for iNOS expression following LPS treatment in VSMC. This suggests that EBP50 may be regulating the expression of iNOS and other NF-κB-inducible genes. EBP50 has already been shown to enhance NF-κB activity in stimulated bronchial epithelial cells (391). The goal of this Aim is to define the molecular mechanism by which EBP50 regulates NF-κB.

1.4.4 Specific Aim 3: Investigate the Effect of EBP50 on Macrophage Activation and Atherosclerosis in *Ldlr*$^{-/-}$ Mice

Previous data demonstrated that femoral arteries from EBP50-null mice exhibit decreased neointimal thickening after wire injury, an acute response to inflammation (368). It is therefore possible that EBP50 affects the activation of macrophages at the site of injury and thus the translation of inflammatory signals to VSMC. The goal of this Aim is to characterize the inflammatory environment *in vivo* and the activation of macrophages *in vitro.* In addition, the

contribution of EBP50 to a more chronic form of inflammation will be determined in atherogenic *Ldlr*$^{-/-}$ mice. To this end, bone marrow from C57BL/6 or EBP50$^{-/-}$ mice will be transplanted into *Ldlr*$^{-/-}$ mice. Extensive analysis of inflammation and atherosclerotic burden in these mice will be performed following 12 weeks of high-fat/high-cholesterol diet.

2.0 EBP50 AND NF-KB: A FEED-FORWARD LOOP FOR VASCULAR INFLAMMATION

The interaction between vascular cells and macrophages is critical during vascular remodeling. Here I report that the scaffolding protein, EBP50 is a central regulator of VSMC function. EBP50 is upregulated following endoluminal injury and promotes neointima formation. However, the mechanisms underlying these effects are not fully understood. Because of the fundamental role that inflammation plays in vascular diseases, I hypothesized that EBP50 mediates the response of vessels to inflammation. Indeed, EBP50 expression increased in primary macrophages and VSMC, and in the aorta of mice, upon treatment with LPS or TNFα. This increase was NF-κB-dependent. Conversely, activation of NF-κB was impaired in EBP50-null VSMC. I found that inflammatory stimuli promote the formation of an EBP50-PKCζ complex at the cell membrane that induces NF-κB signaling. Vascular inflammation after acute LPS treatment was reduced in EBP50-null cells and mice compared to WT. Furthermore, macrophage recruitment to vascular lesions was significantly reduced in EBP50 knockout mice. Thus, EBP50 and NF-κB participate in a feed-forward loop leading to an enhanced response of VSMC to inflammation.

2.1 INTRODUCTION

Inflammation is a necessary biological response to injury and infections. Yet, over-activation and chronic inflammatory status can lead to diseases such as cancer, asthma, rheumatoid arthritis, and cardiovascular disease. Over the past two decades the critical role of inflammation in the etiology of neointimal hyperplasia and atherosclerosis has emerged (3-6). Compelling evidence correlates risk factors for vascular disease (such as dyslipidemia, obesity, and hypertension) to inflammation (5, 392, 393). Moreover, a strong link between inflammation and the progression and adverse prognosis of atherosclerosis has been documented in humans (394, 395). Vascular inflammation is a major cause of the increased growth and migration of VSMC (396, 397) and for the expression of adhesion molecules (such ICAM-1 and VCAM-1) that are critical for the further recruitment of inflammatory cells to the lesion site (15). The local production of cytokines and growth factors stimulates phenotypic changes in VSMC leading to increased proliferation, motility, and matrix production ultimately resulting in plaque formation and neointimal hyperplasia.

Many of the genes implicated in vascular dysfunction are regulated by the transcription factor NF-κB (149, 257). NF-κB is rapidly induced by the cytokines IL-1β, TNFα, and infectious agents such as LPS. NF-κB is sequestered in the cytoplasm under basal conditions by IκBα (157). Activation of the IKK complex results in the phosphorylation and subsequent degradation of IκBα (163), allowing translocation of NF-κB into the nucleus. NF-κB activity is normally low in vessels but is rapidly activated during vascular injury. Degradation of IκBα and increased NF-κB-dependent gene expression was observed after balloon injury (260). Similarly, administration of an IκBα adenovirus during angioplasty decreased ICAM-1 and MCP-1 expression as well as macrophage recruitment and lumen narrowing (261).

The physical and functional interaction between vascular cells and macrophages is therefore critical during vascular remodeling and the identification of mediators of this interaction is significant for understanding the molecular mechanisms leading to vascular disease. Here we report that the PDZ-scaffolding protein EBP50 is a central mediator of this interaction.

EBP50, also known as NHERF1, is a scaffolding protein that assembles signal transduction complexes through its two PDZ domains (279, 398). This adaptor protein also contains an EBD that tethers binding partners to the cytoskeleton. EBP50 is expressed at low levels in healthy vessels, but is significantly upregulated after endoluminal injury (367). EBP50 increases VSMC proliferation and motility, and consequently neointima formation following wire injury is greatly reduced in EBP50 knockout (KO) mice (367-369). However, the molecular mediators that increase EBP50 expression in injured vessels are not known. Similarly, the effects of EBP50 on inflammation and on the response of vascular cells to inflammatory stimuli have not been established. In this study, we used a combination of *in vitro* and *in vivo* approaches to determine the mechanisms controlling EBP50 expression and the role of EBP50 on NF-κB signaling and inflammation. Our observations indicate that EBP50 and NF-κB participate in a feed-forward loop propagating vascular inflammation.

2.2 MATERIALS AND METHODS

2.2.1 Plasmids and Mutagenesis

The N-terminal Flag-tagged EBP50 mutants were constructed as previously described (369). The PKCζ-EESA mutant construct was made from wild-type PKCζ (a generous gift from Peter Parker, King's College London) by using the QuikChange site-directed mutagenesis kit from Stratagene. Mutagenic primers were designed based on rat PKCζ sequence (5'-GTCTGCTGAGGAGTCCGCGTGACTCTAGAG-3'). N-terminally CFP-tagged PKCζ was constructed by inserting WT PKCζ into a pcDNA3-CFP vector (Addgene plasmid #13030, a generous gift from Doug Golenbock). DNA sequences were confirmed by sequence analysis (GENEWIZ).

2.2.2 Experimental Animals and Surgeries

Animal surgeries were performed in 10-week old WT C57BL/6 mice and KO littermates. Mice were anesthetized using Ketamine (100 mg/kg) and Xylazine (5 mg/kg) i.m. A fixed core wire guide 0.015 inch (Cook Medical Incorporated) was inserted into the left femoral artery and passed within the artery 3 times. The right femoral artery was used as uninjured control artery. Femoral arteries were harvested 1 week post-surgery.

For the LPS studies, mice were injected with 10 mg/kg LPS i.p. for 16 h before sacrifice. The aorta and femoral arteries were harvested and analyzed as described later. All animal procedures were approved by the University of Pittsburgh Institutional Animal Care and Use Committee.

2.2.3 Cell Culture, Treatments, and Transfections

To isolate peritoneal macrophages, mice were sacrificed by CO_2 inhalation and cervical dislocation and warm RPMI media containing 10% FBS was injected into the abdominal cavity. After gentle shaking for 1-2 min, the lavage was collected and cells (3×10^6) were plated in 6-well plates and incubated at 37ºC for 2 hours (h). Cultures were vigorously washed with cold PBS to remove non-adherent cells. Macrophages were incubated overnight in 5% CO_2 at 37ºC before treatment to allow for quiescence. Primary VSMC were isolated from abdominal aortic explants and grown in DMEM containing 10% FBS in 5% CO_2 at 37ºC as previously described (369). All experiments were performed with cells between passages 5 and 15. Monolayers of clonal mouse RAW 264.7 macrophages (ATCC) were grown in DMEM containing 10% FBS.

p65-Flag (Addgene plasmid #20012, a generous gift from Dr. Stephen Smale) (399) and IκBα S32A/S34A (a generous gift from Dr. Lawrence Kane, University of Pittsburgh School of Medicine) were transfected in RAW 264.7 cells using HP XtremeGene (Roche) according to the manufacturer's instructions and used for experiments 1-2 days later. Various constructs including pcDNA3.1, p65-Flag, PKCζ, PKCζ-EESA, Flag-EBP50, Flag-EBP50-S1S2, and Flag-EBP50-ΔEBD were introduced into VSMC by electroporation using an AMAXA electroporator and the Basic Nucleofect kit for primary smooth muscle cells (Lonza) as previously described (369). For expression of different PKCζ constructs, VSMC were infected with adenoviruses encoding LacZ, WT PKCζ, myristoylated (Myr) PKCζ (containing the NH_2-terminal c-src myristoylation signal (400)), or kinase dead (KD) PKCζ (K281W), all generous gifts from Dr. Adolfo García-Ocaña (Mount Sinai Medical Center). Cells were incubated with adenovirus in serum-free media for 1 h and incubated with 10% FBS-supplemented media overnight. Experiments were performed at 48 h after infection. Chinese hamster ovary (CHO) cells were cultured in Ham's F-12 medium

supplemented with 10% FBS and transfected using X-tremeGENE HP (Roche) according to the manufacturer's instructions.

VSMC and macrophages were treated with 1 μg/ml and 100 ng/ml LPS (Sigma, catalog number L 4516, source *Escherichia coli* 0127:B8), respectively. Recombinant mouse TNFα (R&D Systems) was used at a concentration of 10 ng/ml for all cell treatments unless otherwise indicated. Cells were preincubated with inhibitors as indicated for 1 h, including 60 μM IKK Inhibitor II (Calbiochem) and 25 μM PKCζ psuedosubstrate (ζ-PS) (Calbiochem). VSMC were serum starved with 0.1% FBS in DMEM overnight prior to experiments.

2.2.4 Nuclear Fractionation

Pelleted VSMC were solubilized in cytoplasmic extract buffer (10 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM EGTA pH 8, 0.1 mM EDTA pH 8, 0.5 mM DTT,) containing a protease inhibitor cocktail and placed on ice for 5 min. Lysates were centrifuged at maximum speed at 4°C for 5 min to achieve separation of cytoplasm (supernatant) and nucleus (pellet). The nuclear pellet was briefly washed twice before resuspension in urea lysis buffer (see Immunoblot Analysis).

2.2.5 Immunoblot Analysis

Cells were solubilized in ice-cold lysis buffer (4 M urea, 62.5 mM TrisHCl, 2% SDS, 1 mM EDTA) containing a protease inhibitor cocktail. Proteins were resolved on a 10% SDS-PAGE gel and transferred to a nitrocellulose membrane. Blots were incubated with antibodies (1:500 dilution for all except p65, Flag [both 1:1000], and actin [1:2000]) to EBP50, VCAM-1, IκBα, PKCζ (Santa Cruz), phospho-IKKα/β (S176/180), IKKβ, phospho-IκBα (S32), phospho-p65

(S536) (Cell Signaling Technology), p65, iNOS (BD Biosciences), β-actin, and Flag (Sigma) with secondary mouse or rabbit antibodies (Cell Signaling Technology, 1:2000 dilution).

2.2.6 Co-Immunoprecipitation

CHO cells were transfected as described above and treated with TNFα for 2 min before lysis in RIPA buffer (Santa Cruz). Equal amounts of protein were incubated with anti-PKCζ (1:500) for 24 h at 4°C before 1 h incubation with Protein A/G beads (Santa Cruz). Samples were washed three times with cold NP-40 buffer (1 M Tris-base, 150 mM NaCl, 5 mM EDTA, 0.5% NP-40) before elution with sample buffer and subsequent immunoblotting.

2.2.7 Real-time RT-PCR

RNA was isolated from cells and tissue using the RNeasy Mini Kit (Qiagen) and cDNA was generated using Im-Prom II Reverse Transcription System (Promega). Mouse primers to EBP50, VCAM-1, ICAM-1, IL-10, and GAPDH were designed using Primer 3 software and synthesized by Invitrogen. The specific primers were as follows: EBP50, forward 5'-AGTGCAAAGTGATCCCATCC-3', reverse 5'-GAGGGCTCTGTGGAAACTTG-3'; ICAM-1, forward 5'- CAGGCTGGAGATTGATCTG-3', reverse 5'-GAGAGATGTAGAGTTGTAGTTC-3'; ICAM-1, forward 5'-GTGATGCTCAGGTATCCATC-3', reverse 5'- GTCCACTCTCGAGCTCATC-3'; IL-10, forward 5'- AGCTGCGGACTGCCTTCAGC-3', reverse 5'- ACAGCGCCTCAGCCGCATC-3'; and GAPDH, forward 5'-CTCATGACCACAGTCCATGC-3', reverse 5'-ATGTAGGCCATGAGGTCCAC-3. PCR reactions were run using the Power SYBR Green

(Applied Biosystems) reagent. The amplification program was as follows: 10 min at 95ºC, 40 cycles of 15 seconds (s) at 95ºC, 30 s at 60ºC (or 55ºC for ICAM-1), and 60 s at 72ºC. A melting curve was run for 60 s at 60ºC and 15 s at 95ºC. TaqMan primers to MCP-1, iNOS, and actin were purchased from Applied Biosystems. The amplification program was as follows: 20 s at 90ºC, 40 cycles of 1 s at 90ºC and 20 s at 60ºC.

2.2.8 Total Internal Reflection Fluorescence (TIRF) Microscopy

CHO cells expressing CFP-PKCζ with or without YFP-EBP50 were analyzed by TIRF at room temperature. After a 5 min stabilization, 10 ng/ml TNFα was added and images were collected every 20 s for 20 min. YFP-EBP50 was used to determine which cells were positive or negative for EBP50. The translocation of CFP-PKCζ to the cell membrane was calculated as a slope = ΔCFP fluorescence/time after TNFα stimulation.

2.2.9 Immunofluorescence

Femoral arteries were fixed with 4% paraformaldehyde and embedded in OCT Tissue-Tek (Sakura Finetek). Sections (10 μm) were incubated in boiled Tris-EDTA buffer (10 mM Tris-base, 8 μM EDTA, 0.05% Tween-20) for 10 min, blocked in 4% NGS, 1% BSA, 0.5% Triton X100 for 20 min at room temperature and incubated with anti-CD68 (AbD Serotec, 1:100 dilution), anti-VCAM-1 (Santa Cruz, 1:100 dilution), or anti-ICAM-1 (Santa Cruz, 1:100 dilution). As a secondary antibody, anti-rabbit Alexa546-conjugated IgG (Invitrogen, 1:250 dilution) was used before DAPI staining. Fluorescence intensities were measured with ImageJ software (National Institutes of Health).

2.2.10 Statistical Analyses

Results from each experiment were averaged and expressed as mean ± standard error (SE). Results were analyzed by one-way ANOVA with Tukey's test or Student's t-test. P-values were considered statistically significant when lower than 0.05.

2.3 RESULTS

2.3.1 Inflammatory Cytokines Increase EBP50 Expression via NF-κB

EBP50 expression increases after endoluminal arterial injury (367, 368), but the mechanisms controlling this upregulation are unknown. In the first series of experiments we tested whether inflammatory stimuli mediate EBP50 expression in VSMC and macrophages. Stimulation of primary peritoneal or RAW 264.7 macrophages and VSMC with LPS for 16 h resulted in a significant increase in EBP50 expression (Fig. 2-1A, C, D). Similarly, EBP50 mRNA expression in VSMC and RAW 264.7 increased with LPS and TNFα (Fig. 2-1B, E), indicating that the effect was not receptor specific and at the transcriptional level. Additional experiments in mice treated with LPS confirmed that inflammatory stimuli increase EBP50 transcripts in the aorta (Fig. 2-1F).

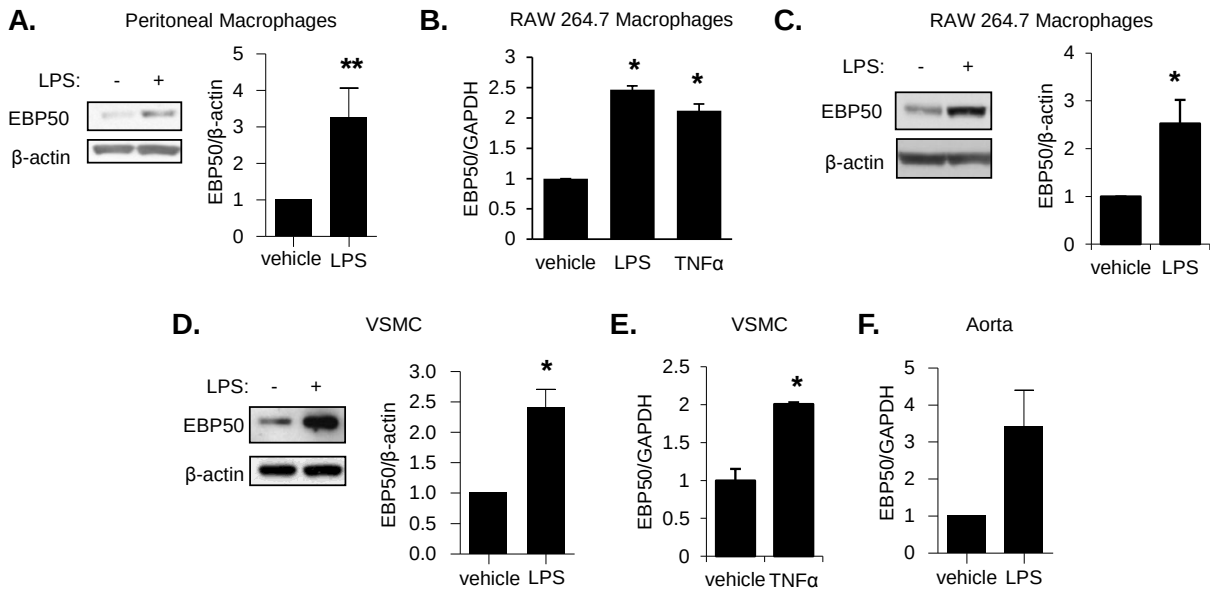

Figure 2-1: Inflammatory stimuli increase EBP50 expression.

A. Peritoneal macrophages were treated for 16 h with 100 ng/ml LPS and the protein extracts subjected to Western blotting for EBP50. **B.** RAW 264.7 cells were treated with or without 100 ng/ml LPS or 10 ng/ml TNFα for 16 h before isolating RNA. EBP50 RNA was analyzed by quantitative RT-PCR. **C.** RAW 264.7 cells were treated as in **A** and immunoblotted for EBP50. **D.** Primary VSMC were treated with 10 μg/ml LPS for 16 h. Cells were lysed and blotted for EBP50. **E.** Primary VSMC were treated with 10 ng/ml TNFα for 16 h. RNA was isolated and analyzed by quantitative RT-PCR. **F.** WT mice were injected with 10 mg/kg LPS for 16 h before sacrifice. RNA was extracted from the thoracic aorta and analyzed for EBP50 expression by RT-PCR. In all immunoblotting analyses β-actin was used as a loading control; GAPDH was the control for RT-PCR. All data represent $n \geq 3$, mean ± SE; *, $p < 0.05$; **, $p < 0.01$.

LPS and TNFα signaling converge on the activation of NF-κB, suggesting that this transcription factor mediates EBP50 expression in inflammatory conditions. Analysis of the promoter region of *Slc9a3r1* (EBP50) revealed multiple potential binding sites for NF-κB,

including two perfect matches (Table 2-1). Thus, it is possible that NF-κB binds to the *Slc9a3r1* promoter.

Table 2-1: NF-kB consensus sites are present in the *Slc9a3r1* promoter.

NF-κB consensus motif: GGGA/GNNC/TC/TCC

Promoter Region	Sequence
[-2515...-2506]	GGGGAGCCCC
[-2023…-2014]	GAGAAATCCC
[-1980…-1971]	GAGAAATCCC
[-484…-475]	GGGGAATCGC
[+136…+135]	GGGCCGTCCC
[+219…+228]	GGGGCGCCCC

We tested this hypothesis by pre-treating with the irreversible IKK Inhibitor II (wedelolactone) or transfecting with a dominant negative form of IκBα (S32A/S36A) that is unable to be phosphorylated and prevents NF-κB activation. We found that both IKK Inhibitor II and IkBα (S32A/S36A) abrogated LPS-induced EBP50 expression in peritoneal macrophages (Fig. 2-2A) and RAW 264.7 cells (Fig. 2-2B). Conversely, over-expression of the p65 subunit of NF-κB, which overwhelms the basal inhibitory machinery and mimics activation, resulted in a 2-fold increase in EBP50 expression (Fig. 2-2C). Comparable results were obtained in primary VSMC (Fig. 2-2D). These studies show that NF-κB regulates the expression of EBP50 in macrophages and VSMC under inflammatory conditions.

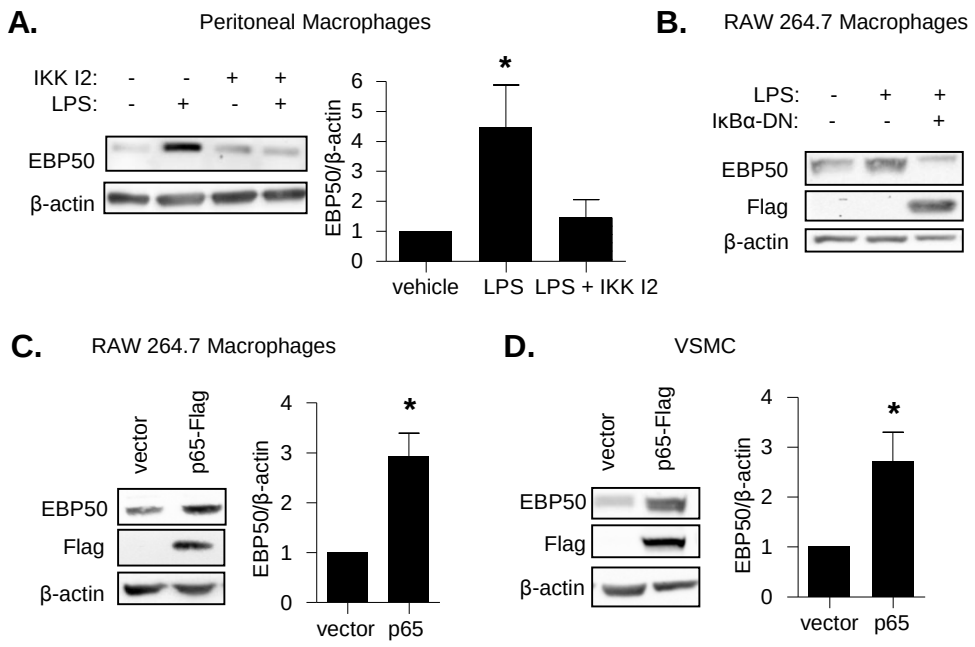

Figure 2-2: NF-κB regulates EBP50 expression.

A. Peritoneal macrophages were treated with 60 μM IKK Inhibitor II (IKK I2) for 1 h prior to overnight stimulation with LPS. EBP50 expression was measured by Western blot. **B.** RAW 264.7 cells transfected with Flag-tagged dominant negative IκBα (S32A/S36A) were treated with LPS for 16 h. Extracts were subject to Western blotting for EBP50 and Flag. **C.** RAW 264.7 were transfected with vector (pcDNA3.1) or p65-Flag before lysing and blotting for EBP50 and Flag. **D.** VSMC were electroporated with vector or p65-Flag and expression of EBP50 and Flag was determined by immunoblot. In all immunoblotting analyses β-actin was used as a loading control. All data represent $n \geq 3$, mean ± SE; *, $p < 0.05$.

2.3.2 EBP50 Promotes NF-κB Activation

During the course of the previous studies, we made an unexpected observation. As described above, LPS stimulated EBP50 expression in VSMC from WT mice. This was accompanied by a parallel increase in iNOS expression (a *bona fide* NF-κB-responsive gene) (Fig. 2-3). As

expected, EBP50 was not detected in VSMC from KO mice. Consequently, LPS did not stimulate iNOS expression in these cells, suggesting that EBP50 regulates NF-κB signaling.

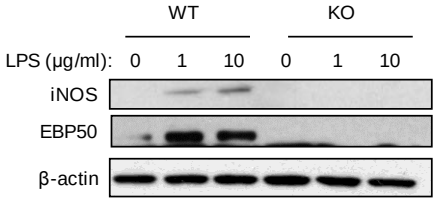

Figure 2-3: iNOS is not induced in KO VSMC.

WT and KO VSMC were treated with the indicated concentrations of LPS for 16 h before immunoblotting for iNOS and EBP50. β-actin was used as a loading control.

Indeed, LPS-induced activation of IKKβ was decreased in KO VSMC compared to WT VSMC (Fig. 2-4A). EBP50-null cells also exhibited decreased IκBα phosphorylation and degradation, and p65 phosphorylation at serine 536 (Fig. 2-4B). Importantly, LPS stimulated phosphorylation of p38 mitogen-activated protein kinase (p38 MAPK) and c-Jun in both WT and KO VSMC (Fig. 2-4C), indicating that the effect of EBP50 on NF-κB signaling is specific and not related to defects in TLR4 signaling. Additionally, nuclear translocation of the p65 subunit is decreased in KO VSMC (Fig. 2-4D).

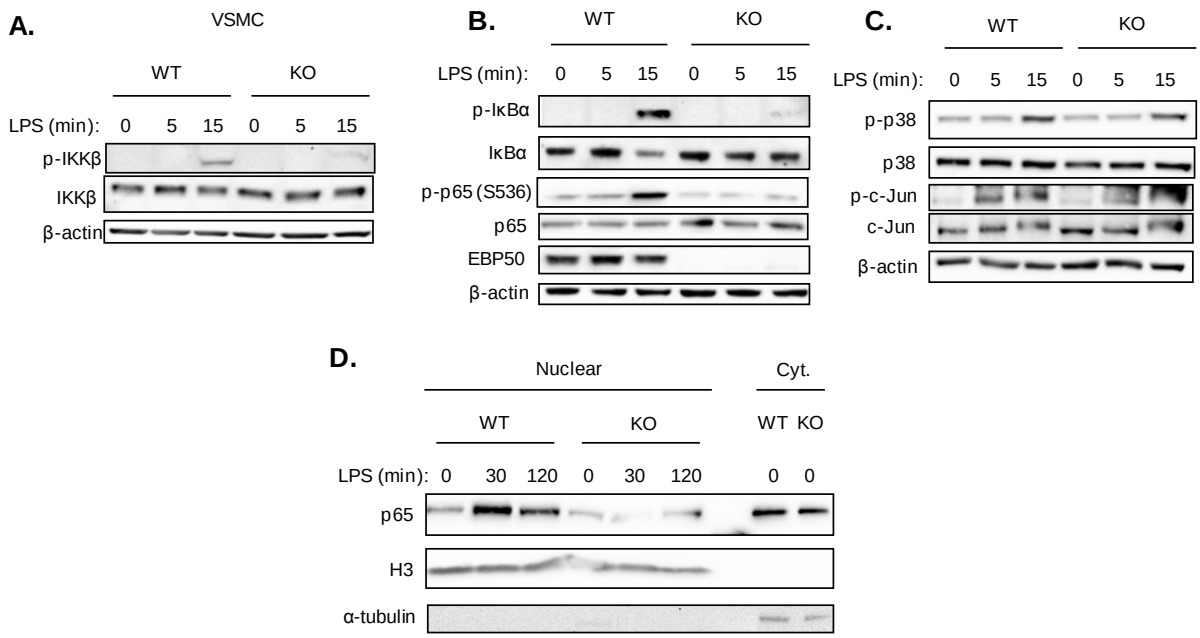

Figure 2-4: EBP50 increases NF-κB signaling.

A, B, C. WT and KO VSMC were treated with 1 μg/ml LPS for 0, 5, or 15 min. Lysates were analyzed by immunoblotting with the indicated antibodies. **D.** WT and KO VSMC were treated with 1 μg/ml LPS for 0, 30, or 120 min. Cells were fractionated into cytoplasmic and nuclear extracts and analyzed by Western blot. In all immunoblotting analyses β-actin was used as a loading control.

To further explore the role of EBP50 in NF-κB activation, we performed a rescue experiment using KO VSMC. Introduction of EBP50 into these cells restored LPS-induced IKK phosphorylation (Fig. 2-5). EBP50 contains two PDZ domains, which are important for scaffolding binding partners, and a C-terminal EBD that interacts with cytoskeletal components. To identify the domains of EBP50 that are necessary for NF-κB activation we utilized EBP50 constructs containing inactivating mutations in both PDZ domains (S1S2) or lacking the EBD domain (ΔEBD). Expression of either mutant was not sufficient to rescue IKK activation in KO VSMC (Fig. 2-5).

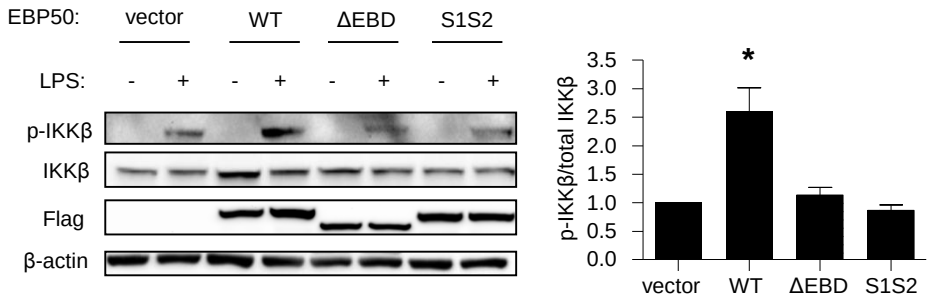

Figure 2-5: Rescue of the null phenotype with EBP50.

KO VSMC were electroporated with the indicated Flag-tagged EBP50 constructs. After 24 h, cells were treated with or without LPS for 15 min and immunoblotted. Quantification is shown in the graph at right. In all immunoblotting analyses β-actin was used as a loading control. All data represent $n \geq 3$, mean ± SE; *, $p < 0.05$.

2.3.3 EBP50 Interacts with PKCζ and Regulates NF-κB Activation

The previous experiments show that the effect of EBP50 occurs upstream of the IKK complex. Examination of potential PDZ ligands in the C-termini of common components of TLR4- and TNF-R1-mediated signaling suggested PKCζ as a potential target for EBP50. Indeed, PKCζ contains a typical type III consensus motif for PDZ binding (E-E-S-V) and participates in NF-κB activation by both LPS and TNFα (245, 246, 248). We determined the interaction between PKCζ and EBP50 in CHO cells that do not constitutively express EBP50. As shown in the co-immunoprecipitation experiment in Figure 2-6A, TNFα promoted the association between PKCζ and EBP50. This was dependent on the PDZ-binding motif in PKCζ because mutation of the C-terminal valine to alanine abrogated the interaction with EBP50 (Fig. 2-6A).

We next determined the role of the interaction between EBP50 and PKCζ on NF-κB activation. Consistent with previous reports (248, 255, 401), adenoviral expression of a kinase

dead PKCζ (KD-PKCζ) or a PKCζ pseudosubstrate inhibitor abrogated LPS-induced IKK phosphorylation in WT VSMC (Fig 2-6B, C). Conversely, adenoviral expression of a membrane-targeted myristoylated PKCζ (Myr-PKCζ) (242), but not of WT PKCζ, was sufficient to restore LPS-induced IKK phosphorylation in KO VSMC (Fig. 2-6D). These results suggested that EBP50 promotes membrane localization of PKCζ in response to inflammatory stimuli. We therefore recorded PKCζ translocation by TIRF microscopy. TNFα stimulated CFP-PKCζ translocation only in CHO cells expressing EBP50 (Fig. 2-6E). This translocation began promptly after TNFα application and continued for at least 20 min. The slope of the membrane-delimited CFP fluorescence over time after TNFα stimulation was significantly greater in EBP50-expressing cells than in naïve CHO cells (0.57±0.14 vs. 0.14±0.13 fluorescence·min^{-1} for EBP50 positive and negative cells, respectively; P = 0.031, n = 10) (Fig. 2-6F). Collectively, these experiments demonstrate that EBP50 potentiates NF-κB activity in response to inflammatory stimuli by a mechanism involving the formation of an EBP50-PKCζ complex at the cell membrane. Interestingly, the PKCζ–EESA mutant that does not interact with EBP50 significantly inhibited LPS-induced IKKβ phosphorylation (Fig. 2-6G), suggesting that this mutant functions as a partial dominant negative PKCζ for NF-κB activation.

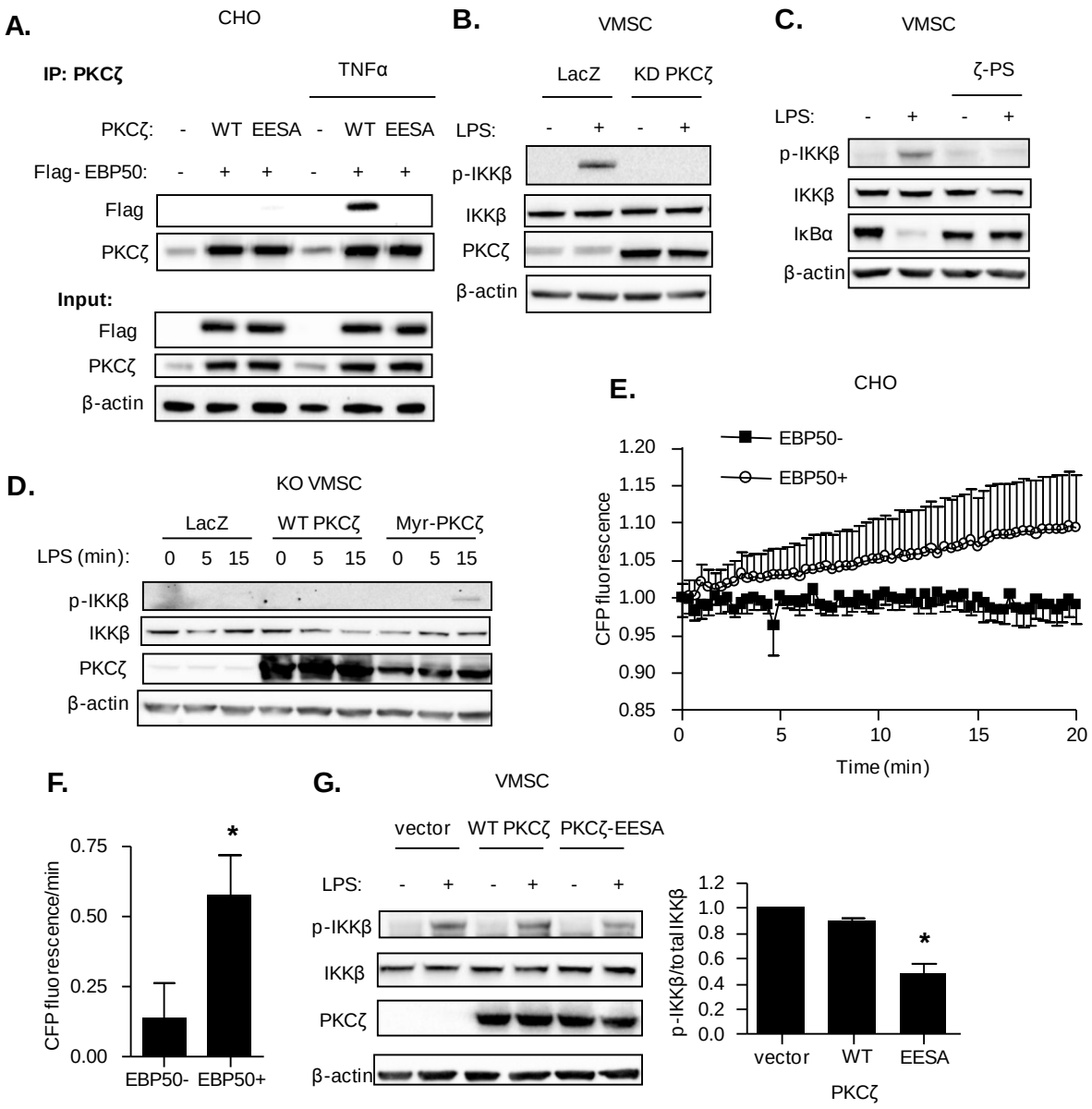

Figure 2-6: EBP50 interacts with PKCζ to promote NF-κB activation.

A. CHO cells were transfected with vector (pcDNA3.1), WT PKCζ, or PKCζ-EESA mutant in conjunction with or without Flag-EBP50 as indicated and treated with TNFα for 2 min. Cell lysates were immunoprecipitated with anti-PKCζ antibody. The immunoprecipitate (IP) and lysates (input) were immunoblotted as indicated. **B.** WT VSMC were infected with LacZ control or kinase-dead (KD) PKCζ (K281W) adenoviruses as indicated for 72 h before incubation with LPS for 15 min. Lysates were immunoblotted as shown. **C.** WT VSMC were pre-treated with 25 μM PKCζ pseudosubstrate (ζ-PS) as indicated for 1 h before 15 min LPS treatment. Lysates were immunoblotted as

shown. **D.** KO VSMC were infected with LacZ control, WT PKCζ, or myristoylated (Myr) PKCζ adenoviruses for 72 h before incubation with LPS for 0, 5, or 15 min as shown. Lysates were immunoblotted as shown. **E.** Time-resolved changes in CFP fluorescence were measured using TIRF in single CHO cells expressing CFP-PKCζ with or without YFP-EBP50. Shown are the changes in membrane-delimited CFP fluorescence over 20 min after TNFα treatment. Error bars denote SEM (n = 10). **F.** Data were quantified as the slope of the CFP fluorescence over time during the first 5 min of TNFα treatment (n = 10). **G.** WT VSMC were electroporated with vector, WT PKCζ, or PKCζ-EESA as indicated. After 24 h, cells were treated with or without LPS for 15 min, lysed and immunoblotted as indicated. In all immunoblotting analyses β-actin was used as a loading control. All data represent $n \geq 3$, mean ± SE; *, $p < 0.05$.

2.3.4 EBP50 Increases Inflammatory Responses in VSMC

The expression of adhesion molecules by vascular cells in response to inflammation is critical for the homing of macrophages to lesion sites (17, 402). We therefore tested whether EBP50 regulates the response of VSMC to inflammatory stimuli. We found that LPS-induced expression of the adhesion molecules ICAM-1 and VCAM-1, and of iNOS, were significantly reduced in KO VSMC compared to WT cells (Fig. 2-7A). In contrast, no differences in MCP-1 mRNA levels were observed. Similar observations were made upon treatment with TNFα (Fig. 2-7A), indicating that the effect of EBP50 is not receptor specific. The expression of VCAM-1 (Fig. 2-7B), determined by immunoblotting, was also reduced in KO VSMC treated with TNFα for 16 h. Thus, EBP50 promotes the response of VSMC to inflammatory stimuli.

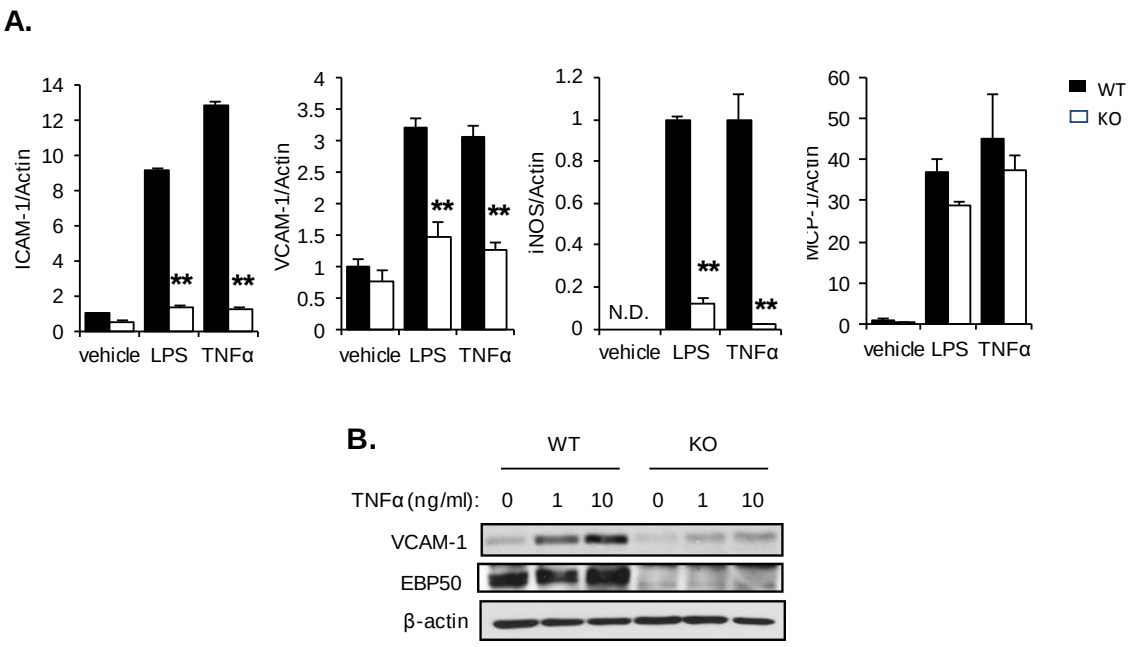

Figure 2-7: EBP50 promotes adhesion molecule expression in VSMC.

A. Primary WT and KO VSMC were treated with LPS or TNFα for 3 h. RNA was isolated and analyzed by quantitative RT-PCR with actin as the control. N.D., not detectable. **B.** WT and KO VSMC were treated with TNFα at the indicated concentrations for 16 h. Expression of VCAM-1 and EBP50 were determined by western blot and β-actin served as the loading control. All data represent $n \geq 3$, mean ± SE; **, $p < 0.01$.

2.3.5 EBP50 Increases Vascular Inflammation

To determine the role for EBP50 on vascular inflammation *in vivo* we used two distinct experimental systems: an acute LPS-induced inflammatory model and an arterial injury model. In the first setting, WT and KO mice were injected with 10 mg/kg LPS for 16 h before excision of the femoral arteries. VCAM-1 and ICAM-1 expression was low in control (PBS injected) femoral arteries (data not shown). In WT mice, LPS induced robust expression of VCAM-1 in both endothelial and VSM cells, which was reduced by ~60% in KO mice (Fig. 2-8A). LPS-

induced ICAM-1 expression occurred predominantly in the endothelium, and was also significantly reduced in KO mice (Fig. 2-8A).

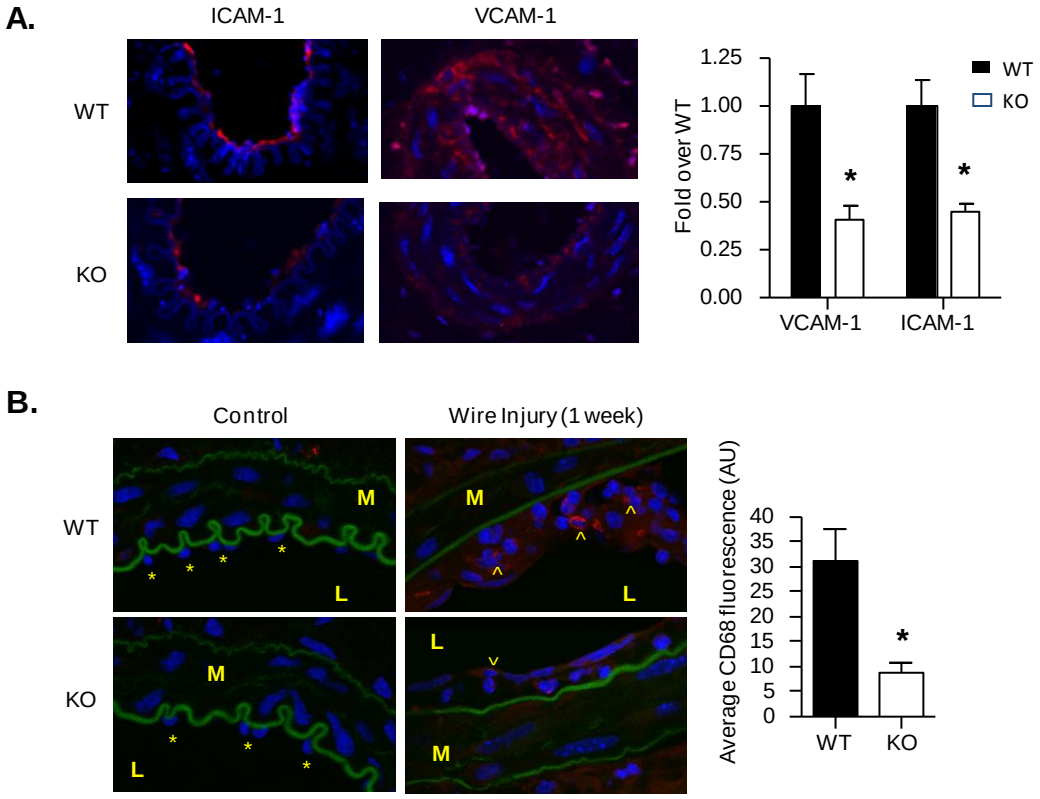

Figure 2-8: EBP50 promotes vascular inflammation *in vivo*.

A. WT and KO mice were injected with 10 mg/kg LPS for 16 h. Femoral arteries were excised and stained for VCAM-1 and ICAM-1 (in red). Nuclei were stained with DAPI (in blue). Quantitation is shown at right as the mean ± SEM; n = 4 (WT) and n = 3 (KO). **B.** Endoluminal denudation was performed in WT and KO mice. One week after surgery, injured and control femoral arteries were harvested and stained for the macrophage marker CD68 (in red). Nuclei were stained with DAPI (in blue) and autofluorescent elastin fibers are shown in green. * denote endothelial cells, ^ indicate positive CD68 staining. Quantification is shown as the mean CD68 fluorescence ± SEM;, n = 4 (WT) and n = 3 (KO). *, $p < 0.05$.

To determine if EBP50 contributes to macrophage homing to sites of vascular injury, we performed endoluminal denudation in the femoral arteries of 10 week-old WT and KO male mice. One week after injury, macrophages in the lesion site were detected by CD68 staining. We chose this marker because its expression, determined by quantitative RT-PCR, is equivalent in primary WT and KO macrophages (data not shown). As shown in Figure 2-8B, abundant macrophages populated the intimal region of the injured vessel in WT mice. In contrast, we detected significantly fewer macrophages at the lesion site in KO mice.

Collectively, these experiments show that EBP50 increases the expression of adhesion molecules under inflammatory conditions and promotes the homing of macrophages to injured vessels.

2.4 DISCUSSION

In this study we show the reciprocal relationship between EBP50 and NF-κB, whereby NF-κB increases EBP50 expression under inflammatory conditions and EBP50 propagates NF-κB signaling. Consequently, EBP50 promotes the expression of adhesion molecules and macrophage recruitment at sites of vascular injury. A schematic representation of the action of EBP50 under inflammatory conditions is shown in Fig. 2-9.

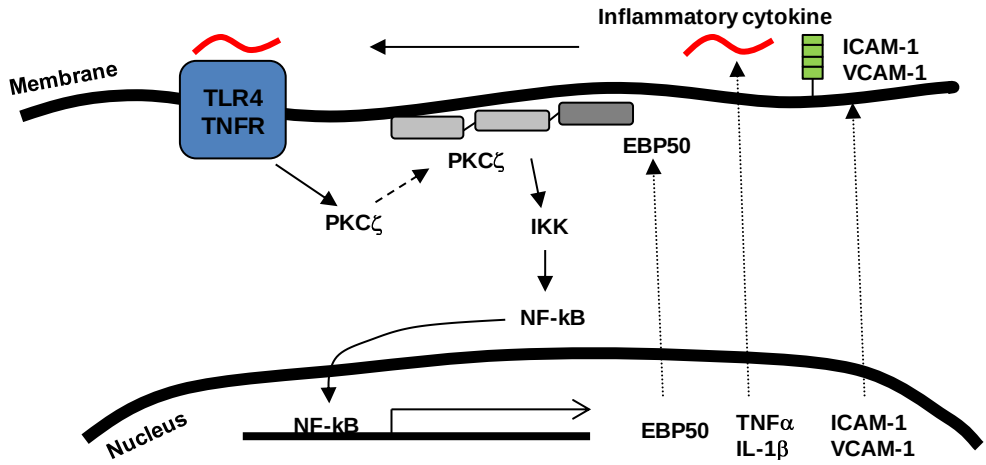

Figure 2-9: Schematic representation of the effect of EBP50 under inflammatory conditions.

In response to inflammatory stimuli, EBP50 recruits PKCζ at the cell membrane and promotes IKKβ phosphorylation and activation of NF-κB. NF-κB induces expression of EBP50, inflammatory cytokines and adhesion molecules that further promote the inflammatory response.

The upregulation of EBP50 has been reported in multiple disease states including hepatocellular carcinomas, cholangiopathies, glioblastoma, breast cancer, psoriasis, and vascular injury (311, 312, 333, 358, 368, 403, 404). However, little is known on the transcriptional regulation of EBP50 expression in normal and disease states. Estrogen is the best characterized inducer of EBP50 expression (300, 303, 308) but murine EBP50 is unresponsive to estrogen (313). We reported an increase in EBP50 expression in VSMC overexpressing PTHrP (367), yet this modest 50% increase does not account for the remarkable EBP50 upregulation upon vascular injury in rodents (367, 368). Confirming an earlier report from Davis and colleagues (386), we observed a significant increase in EBP50 expression upon treatment with either LPS or TNFα in macrophages and VSMC. Furthermore, we found that this effect was transcriptional and fully dependent on NF-κB activity. Although not yet proven, the presence of two perfect NF-κB

consensus motifs and multiple other similar sequences in the *Slc9a3r1* promoter opens up the possibility of a direct interaction between the two components. The regulation of EBP50 by NF-κB is relevant in animal models of vascular injury: NF-κB activity is normally low in uninjured arteries but IκBα degradation and upregulation of NF-κB-responsive genes (such as VCAM-1) are observed upon angioplasty (260). We recently reported that EBP50 expression is low in normal vessels and increases following endoluminal denudation in rat and mouse arteries (367, 368). Thus, both NF-κB activity and EBP50 expression increase during vascular remodeling and our studies provide a mechanistic basis for these effects.

Interestingly, we found that EBP50-null VSMC were impaired in their ability to activate NF-κB in response to inflammatory stimuli. This is consistent with a report from Estell et al. showing that NF-κB binding to DNA in bronchial epithelial cells was dependent on EBP50 (391). In our studies, the reduction in IKK phosphorylation in EBP50-null cells indicates that EBP50 exerts its effects early in the activation of NF-κB, upstream of the IKK complex. Moreover, the effect of EBP50 is not receptor-specific because it is observed with both LPS and TNFα. We reasoned that a common effector of TLR4 and TNF-R1 could be the site of action of EBP50, and focused on PKCζ because it possesses a C-terminal PDZ-binding motif and is essential for NF-κB activation. Indeed, our experiments show that inflammatory stimuli induce the formation of an EBP50-PKCζ complex at the cell membrane. The magnitude of this effect is consistent with that observed in opossum kidney cells in response to dopamine (405). PKCζ translocation to the membrane appears to be the critical step for EBP50 action, because NF-κB activity in EBP50-null cells was rescued by a membrane-targeted myristoylated form of PKCζ but not by overexpression of WT PKCζ. Consistent with this mechanism, we found that at least a functional PDZ domain and the EBD in EBP50 are required for cytokine-induced IKK

phosphorylation. Therefore, both the ability to bind PKCζ and to interact with the cytoskeleton is crucial for the effect of EBP50 on NF-κB activation.

The regulation of NF-κB signaling by EBP50 has important consequences in inflammatory states. Expression of the adhesion molecules ICAM-1 and VCAM-1 (and of iNOS) was decreased in EBP50-null VSMC and mice. Consequently, homing of macrophages to the site of arterial injury was significantly reduced in KO mice. These observations provide further insight into the critical role of EBP50 during vascular remodeling. As we previously reported, EBP50 potentiates VSMC proliferation and motility (367-369). Here we show that EBP50 regulates the response of VSMC to inflammatory stimuli and the recruitment of macrophages to injured vessels. Therefore, EBP50 promotes multiple critical events leading to aberrant vascular remodeling and genetic ablation of EBP50 confers remarkable protection from injury-induced restenosis.

In summary, we have identified EBP50 as a positive regulator of NF-κB activation. Conversely, we have also demonstrated a role for NF-κB in the upregulation of EBP50 expression. We propose that NF-κB and EBP50 participate in a positive feed-forward loop leading to enhanced responses of vascular cells to inflammation.

3.0 EBP50 ENHANCES MACROPHAGE ACTIVATION AND ATHEROSCLEROSIS

Macrophages and other leukocytes are major players in the progression of atherosclerosis and other inflammatory diseases. Here we demonstrate a role for the PDZ scaffolding protein EBP50 in macrophage activation. I have previously reported that EBP50 is a positive regulator of NF-κB activation and inflammatory responses in VSMC and wanted to extend these findings to macrophages. Additionally, I know that EBP50 can diminish vascular reactions to acute inflammation but its effect on chronic diseases such as atherosclerosis is unknown. Given these earlier findings, I hypothesized that EBP50 similarly promotes macrophage activation and atherosclerosis. Indeed, KO macrophages display diminished NF-κB signaling and subsequently decreased expression of pro-inflammatory cytokines when treated with LPS. To determine the myeloid-specific role of EBP50 in chronic inflammation, I performed transplants of WT or KO bone marrow into *Ldlr*$^{-/-}$ mice. After placing these mice on a high cholesterol diet for 12 weeks, I observed no changes in glucose or lipid levels in the plasma. However, lesion sizes throughout the aorta were significantly decreased in mice receiving KO marrow. These data demonstrate that EBP50 in myeloid cells is important for the progression of atherosclerosis. Further examination of plaque composition will yield additional insight into this mechanism.

3.1 INTRODUCTION

Atherosclerosis is considered a chronic inflammatory disease within the vessel wall, characterized by lipid-laden plaques with a fibrous coating (3-5). Elevated levels of lipids such as LDL in the blood trigger a weakness in the integrity of the endothelium. Monocytes are recruited to the site where they burrow into the intimal layer and start engulfing the lipids. These foam cells contribute to the local inflammatory environment and the formation of the necrotic core. Meanwhile, VSMC are stimulated to proliferate and migrate around the plaque to protect the lumen from the pro-thrombotic interior. VSMC additionally secrete extracellular matrix components such as collagen to aid in the construction of the fibrous cap. However, macrophages can also secrete MMPs which, while beneficial for the migration of VSMC, contribute to the thinning of the fibrous cap. It is clear that leukocytes are a major driver in the progression of this disease.

NF-κB plays a significant role in the development of atherosclerosis. Many initiating factors of this disease can activate NF-κB, including hypoxia, metabolic factors, infectious agents, T-cell activation, and cytokines (257). Activated NF-κB is present in endothelial cells, VSMC, and macrophages within the atherosclerotic plaque (262). NF-κB can induce the expression of over 150 genes, many of which are implicated in the pathogenesis of atherosclerosis including cytokines, adhesion molecules, MMPs, tissue factor, and anti-apoptotic proteins (149). Broad inhibition of NF-κB has been associated with decreased atherosclerosis (128, 264, 265) while other inhibitions of the pathway have had controversial effects. Macrophage-specific deletion of IKKβ surprisingly enhanced atherosclerosis in *Ldlr*$^{-/-}$ mice due to decreased levels of IL-10 and a defective resolution of inflammation (266). However, another group conversely found a decrease in atherosclerosis in *Ldlr*$^{-/-}$ mice with IKKβ$^{-/-}$ bone marrow

(267). Additionally, endothelial-restricted expression of a dominant-negative IκBα or deletion of IKKγ/NEMO protected *Apoe*$^{-/-}$ mice from atherosclerosis (269). Certainly NF-κB directs complex functions in the control of inflammation.

Recently, we discovered a role for EBP50 in vascular inflammation. EBP50, also known as NHERF1, is an adaptor protein that uses its tandem PDZ domains to assemble signal transduction complexes (279, 398). This scaffolding protein also contains an ezrin-binding domain at its C-terminus to tether ligands to the cytoskeleton. Normal vessels express low amounts of EBP50, which is significantly upregulated after arterial wire injury (367). EBP50 positively regulates VSMC proliferation and migration and consequently is necessary for neointima formation (367-369). In addition, inflammatory stimuli and NF-κB control EBP50 expression. Conversely, EBP50 potentiates NF-κB activation and the expression of its adhesion molecules in VSMC.

While EBP50 is important for acute stimuli such as wire injury and LPS insult, it is unknown if this scaffolding protein has an effect on chronic inflammatory diseases such as atherosclerosis. In addition, macrophage activation in an EBP50-null setting has not yet been determined. In this report, we investigate the extent of macrophage activation in KO macrophages and utilize bone marrow transplants (BMT) to explore the leukocyte-specific role of EBP50 in *Ldlr*$^{-/-}$ mice fed a diet high in cholesterol.

3.1.1 Mouse Models of Atherosclerosis

Several transgenic mouse strains have been generated that develop atherosclerotic lesions. Mice are typically put on a modified or "Western" diet enriched in fat, cholesterol, and cholate to

induce lesion formation. Inbred strains can also develop diet-induced atherosclerosis (C57BL/6 are the most sensitive) but lesions generally remain small and fail to progress beyond the fatty streak (406). *Apoe*$^{-/-}$ and *Ldlr*$^{-/-}$ mice on a C57BL/6 background are the most commonly used to model this disease. ApoE is an important mediator of lipid metabolism, facilitating high-affinity binding of cholesterol to liver LDLR for uptake and degradation (407). *Apoe*$^{-/-}$ mice are hyperlipidemic and can spontaneously develop atherosclerosis, even when fed a normal diet (408). Conversely, *Ldlr*$^{-/-}$ do not develop lesions unless fed a modified diet (409). Both models display similar degrees of diet-induced atherosclerosis, although *Apoe*$^{-/-}$ mice have higher cholesterol levels (410).

Genetic combination of these models with other transgenics is an important tool to explore the function of a gene of interest in atherosclerosis. In addition, bone marrow transplants into atherosclerotic strains are an attractive way to investigate the specific effect of genetically-modified hematopoietic cells (i.e. leukocytes, lymphocytes) on atherosclerosis. However, care must be taken as to whether the donor mice must first be back-bred onto the *Apoe*$^{-/-}$ or *Ldlr*$^{-/-}$ background. Bone marrow transplantation of *Apoe*$^{+/+}$ into *Apoe*$^{-/-}$ mice can reduce cholesterol levels and cause a regression in atherosclerosis (411, 412). ApoE is synthesized by monocytes and its expression on these cells is sufficient to rescue the ApoE-null phenotype of hypercholesterolemia. However, extrahepatic LDLR has no effect on cholesterol or lesion development (413, 414). Therefore, it is necessary to genetically cross donor mice with *Apoe*$^{-/-}$ mice, but not *Ldlr*$^{-/-}$ mice, before transplanting bone marrow into the respective atherosclerotic model. For this reason, we chose the *Ldlr*$^{-/-}$ mouse as the model for our bone marrow transplant study.

3.2 MATERIALS AND METHODS

3.2.1 Mice

Homozygous male *Ldlr*$^{-/-}$ mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and housed in sterile, filter-top cages. For analysis of atherosclerosis, animals were fed an atherogenic diet with 1.25% cholesterol and 0.5% cholate (TD.02028, Harlan Laboratories) for 12 weeks. All animal procedures were approved by the University of Pittsburgh Institutional Animal Care and Use Committee.

For the LPS studies, mice were injected with 10 mg/kg LPS i.p. for the indicated times before sacrifice. Blood was collected by cardiac puncture and the serum was used in a TNFα ELISA (R&D Systems) according to the manufacturer's instructions.

3.2.2 Bone Marrow Transplantation

One day prior to transplantation, recipient *Ldlr*$^{-/-}$ mice (10 weeks old) were subject to total body irradiation (9.5 Gy). The next day, the same mice received 7.5 x 10^6 bone marrow cells through tail vein injection. Donor bone marrow was harvested by flushing the femurs of 8-10 week old male donor WT C57BL/6 mice or KO mice with RPMI 1640 containing 2% FBS, 10 U/ml heparin, and penicillin/streptomycin. Cells were washed twice with RPMI plus 20 mM HEPES and penicillin/streptomycin before injection. Sterile drinking water contained 0.672 mg/ml Sulfatrim for two weeks post-transplantation. Mice were put on the atherogenic diet four week post-transplantation for 12 weeks.

3.2.3 Blood Analysis

Blood samples (50 μl) were obtained by tail bleeding four weeks post-transplantation to assess bone marrow reconstitution. Complete blood count was analyzed on a HEMAvet 950 (Drew Scientific). At the conclusion of the 12-week atherogenic diet, blood was drawn after an overnight fast and plasma cholesterol and triglyceride concentrations determined by enzymatic assays (BioAssay Systems). Glucose was measured using ACCU-CHEK Aviva test strips and blood glucose meter. For genotyping purposes, DNA was extracted from the blood using the QIAamp DNA blood mini kit (Qiagen). The primers used were 5'-TTCGGCCTCATTCTGGTC-3' (common), 5'-GAGAAGGGTCCAAATGGCTA-3' (WT forward), and 5'-CGCCTTCTTGACGAGTTCTT-3' (mutant forward).

3.2.4 Atherosclerotic Lesion Measurement

After 12 weeks, mice were sacrificed and perfused with 4% paraformaldehyde. Aortas were dissected and stained *en face* with Oil Red O (Sigma). Briefly, aortas were washed in 60% isopropanol for 2 min, stained with Oil Red O for 18-20 min, then washed in isopropanol followed by PBS rinses. For lesion analysis, full-length aortas were embedded in OCT Tissue-Tek (Sakura Finetek), divided into four sections per block (Fig. 3-1), and sectioned at 10 μm intervals. Sections spaced 300 μm apart were similarly stained with Oil Red O and Harris hematoxylin to calculate mean lesion area.

3.2.5 Cell Culture and Treatments

Peritoneal macrophages were isolated from the peritoneum as described in section 2.2.3. These cells were briefly cultured in RPMI medium containing FBS and penicillin/streptomycin. Monolayers of clonal mouse RAW 264.7 macrophages (ATCC) were grown in DMEM containing 10% FBS. All macrophages were treated with 100 ng/ml LPS (Sigma) as indicated.

Small interfering RNA (siRNA) for EBP50 knockdown was generated by Dhamarcon (Thermo Scientific) as follows: 5′-GAAGGAGAGCAGCCGUGAAdTdT3′ (sense) and 5′-UUCACGGCUGCUCUCCUUCdTdT-3′ (antisense). As a control siRNA, Accell Non-targeting siRNA (Dharmacon, Thermo Scientific) was used. RAW 264.7 cells were plated onto 6-well plates and transfected with siEBP50 or control siRNA using RNAimax (Invitrogen Life Technologies) in DMEM with 1% FBS in the absence of antibiotics. Cells were transfected a second time the next day and used 48 hours after the last transfection.

3.2.6 Immunoblotting

Cells were solubilized in ice-cold lysis buffer (4 M urea, 62.5 mM TrisHCl, 2% SDS, 1 mM EDTA). Proteins were resolved on a 10% SDS-PAGE gel and transferred to a nitrocellulose membrane. Blots were incubated with antibodies to EBP50 (Santa Cruz, 1:500 dilution), F4/80 (Abcam, 1:500), IL-1β (Abcam, 1:1000), and β-actin (Sigma, 1:2000) with secondary mouse or rabbit antibodies (Cell Signaling Technology, 1:2000).

3.2.7 Real-time RT-PCR

RNA was isolated from cells and tissue using the RNeasy Mini Kit (Qiagen) and cDNA was generated using Im-Prom II Reverse Transcription System (Promega). TaqMan primers to TNFα, IL-1β, IL-6, iNOS, and actin were purchased from Applied Biosystems. The amplification program was as follows: 20 s at 90ºC, 40 cycles of 1 s at 90ºC and 20 s at 60ºC.

3.2.8 Statistical Analyses

Results from each experiment were averaged and expressed as mean ± standard error (SE). Results were analyzed by Student's t-test. P-values were considered statistically significant when lower than 0.05.

3.3 RESULTS

3.3.1 Classical Macrophage Activation is Decreased in EBP50 KO Macrophages.

Macrophage activity and the secretion of cytokines are important factors for the induction of proliferation, migration, and adhesion molecule expression in VSMC during vascular remodeling (6). Many of these cytokines are regulated by NF-κB, and since we have demonstrated the importance of EBP50 in the activation of this pathway in VSMC, we next sought to determine the effect of macrophage activation. To this end, we treated peritoneal macrophages isolated

from WT and KO mice with LPS for 3 h and assessed expression of pro-inflammatory mediators by quantitative RT-PCR. We found that the induction of IL-1β, iNOS, and TNFα was significantly decreased in KO macrophages compared to WT (Fig. 3-1A). In contrast, we observed no differences for IL-6 and IL-10. We then assessed macrophage activation in WT and KO mice treated with 10 mg/kg LPS for 2 or 16 h. Serum TNFα concentrations and the expression of IL-1β in macrophages were both significantly decreased in KO compared to WT mice (Fig. 3-1B, C). These experiments show that EBP50 increases macrophage activation and the production of inflammatory cytokines both *in vitro* and *in vivo*.

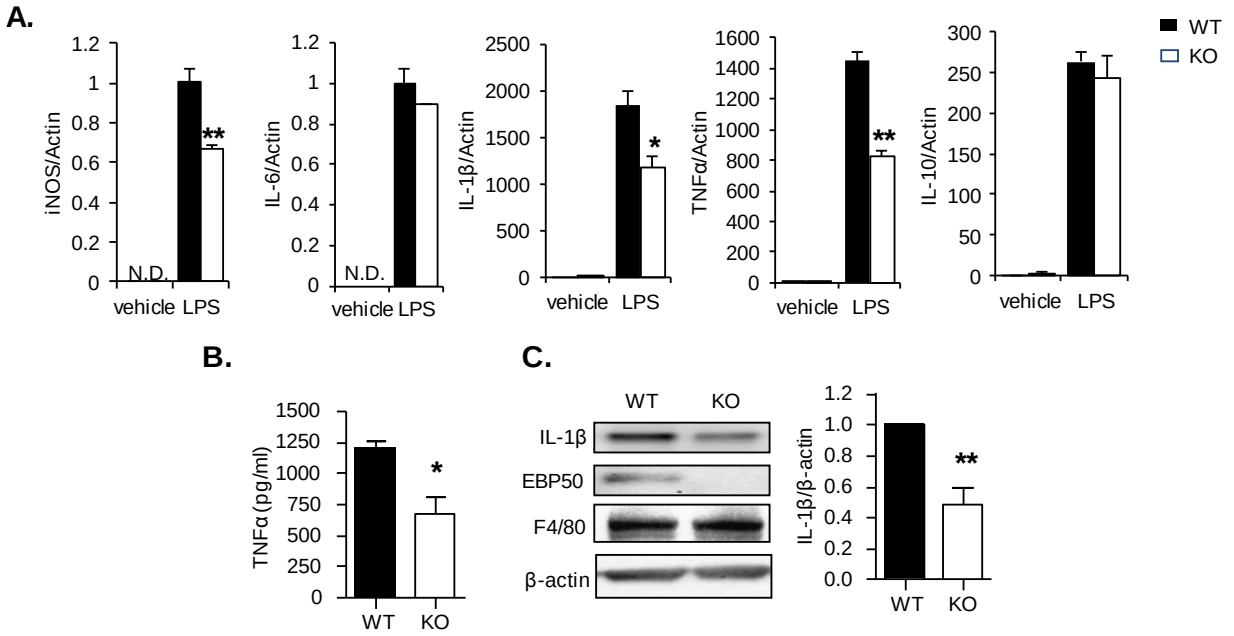

Figure 3-1: EBP50 promotes inflammatory cytokine production.

A. WT and KO peritoneal macrophages were treated with LPS for 3 h before isolating RNA. RNA was analyzed by quantitative RT-PCR. N.D., not detectable. **B.** WT and KO mice were injected with 10 mg/kg LPS (i.p.) and sacrificed after 2 h. Serum TNFα was determined by ELISA. n = 3 (WT) and 5 (KO). **C.** WT and KO mice were injected with 10 mg/kg LPS and sacrificed 16 h later. Peritoneal macrophages were harvested and purified through

adhesion to culture dishes. After 2 h, cells were collected and expression of EBP50 and IL-1β determined by western blot. Immunoblotting for F4/80, a macrophage marker, verified the equal population of macrophages in each sample. In all immunoblotting analyses β-actin was used as a loading control; Actin was the control for RT-PCR. All data represent $n \geq 3$, mean ± SE; *, $p < 0.05$; **, $p < 0.01$.

In Chapter 2, we showed that EBP50 is a positive regulator of NF-κB activity in VSMC. Since Figure 3-1 demonstrated a decrease in classical NF-κB genes in KO macrophages, we hypothesized that EBP50 was also integrated into the NF-κB pathway in these cells. Treatment of RAW 264.7 macrophages with siRNA to EBP50 resulted in >80% knockdown (Fig. 3-2A). Indeed, siEBP50 macrophages displayed a lack of IκBα degradation and decreased p65 phosphorylation (Fig.3-2B), confirming a function for EBP50 in macrophage NF-κB signaling.

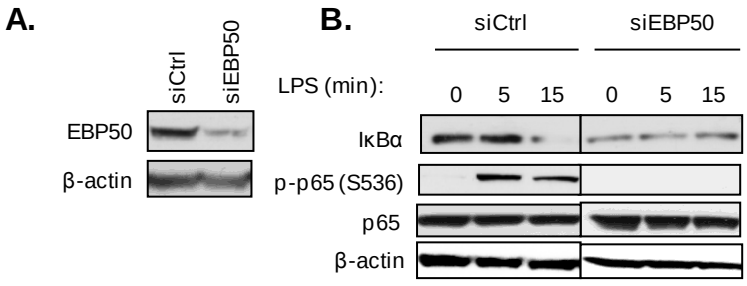

Figure 3-2: NF-κB signaling is regulated by EBP50 in macrophages.

A. RAW 264.7 cells were subjected to scrambled siRNA (siCtrl) or siEBP50 in a two-day transfection and used 24-48 hours after the second transfection. This resulted in an approximately 80% knockdown of EBP50. **B.** RAW 264.7 cells transfected with either siCtrl or siEBP50 were treated with LPS for 0, 5, or 15 min. Protein extracts were subjected to immunoblot as shown. β-actin was the loading control.

3.3.2 Deletion of EBP50 in Bone Marrow Does Not Affect Chimerism, Blood Cell Profile, or Lipid Parameters

Since EBP50-null macrophages exhibit decreased activation, we wanted to investigate the role of these macrophages in a chronic vascular inflammatory state. Bone marrow from WT C57BL/6 or KO mice was transplanted into lethally irradiated *Ldlr*$^{-/-}$ recipient mice to investigate the specific effects of EBP50 in macrophages and other myeloid cells on atherosclerosis. After four weeks recovery, blood was collected and analyzed for chimerism and successful engraftment. RT-PCR on blood cells confirmed the presence of only donor WT or KO marrow (Fig. 3-3A). A complete blood count demonstrated that white blood cell, lymphocyte, red blood cell, and platelet counts were within the normal murine ranges and not different between groups (Table 3-1).

Table 3-1: Complete Blood Counts in *Ldlr*$^{-/-}$ mice four weeks post BMT.

Mice	WT BMT	KO BMT
Red Blood Cells (M/μl)	8.6 ± 0.4	9.9 ± 0.1
White Blood Cells (K/μl)	12.8 ± 1.3	14.2 ± 2.0
Hemoglobin (g/dl)	12.1 ± 0.6	13.8 ± 0.2
Platelets (K/μl)	599.7 ± 73	592 ± 92
Neutrophils (K/μl)	2.6 ± 0.4	2.7 ± 0.4
Eosinophils (K/μl)	0.2 ± 0.08	0.09 ± 0.03
Basophils (K/μl)	0.07 ± 0.03	0.2 ± 0.01
Monocytes (K/μl)	0.4 ± 0.07	0.4 ± 0.7
Lymphocytes (K/μl)	9.5 ± 0.8	11.0 ± 1.6

Values are mean ± SE. M, million; K, thousand. N = 8 (WT), 4 (KO).

At this time, mice were put on a high-cholesterol diet for 12 weeks. Plasma glucose, total cholesterol, and triglyceride levels were similar between WT and KO recipients at the end of the diet (Table 3-2). Weight was also not different between groups.

Table 3-2: Body Weight and Plasma Glucose and Lipid Levels.

	WT BMT	KO BMT	p *(n, n)*
Weight (g)	23.4 ± 0.43	22.0 ± 0.52	0.380 *(8, 7)*
Glucose (mg/dL)	92.2 ± 6.7	95.6 ± 5.6	0.707 *(5, 5)*
Cholesterol (mg/dL)	1185.6 ± 89.6	1004.8 ± 102.5	0.21 *(7, 7)*
Triglyceride (mg/dL)	155.2 ± 26.6	91.8 ± 14.7	0.0592 *(7, 7)*

Values are mean ± SE and (n,n) denotes number of animals for (WT, KO) recipient analysis after 12 weeks of high-cholesterol diet

Additionally, peritoneal macrophages were extracted to confirm proper chimerism (Fig. 3-3B). Animals that were transplanted with KO donor marrow did not express EBP50 in the macrophages as expected.

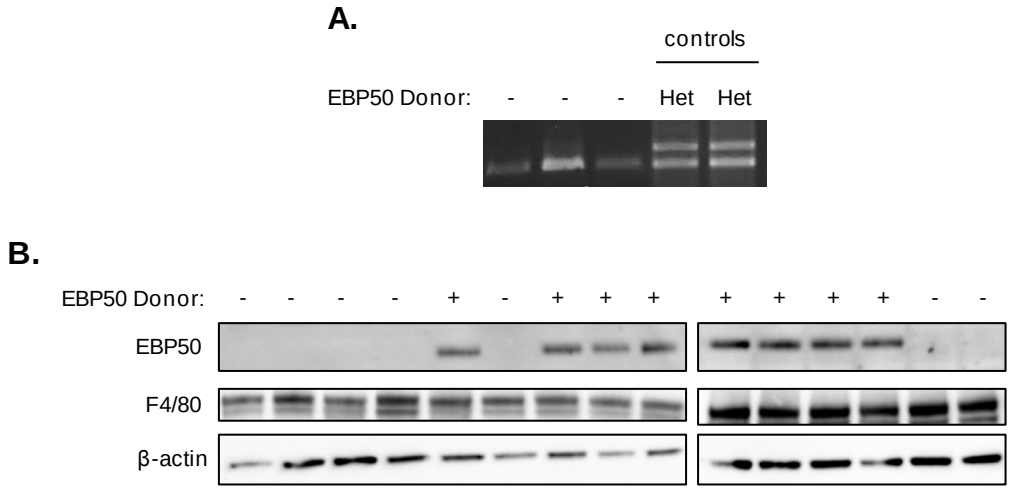

Figure 3-3: Chimerism of *Ldlr*$^{-/-}$ transplanted with indicated donor marrow.

A. Genotyping from the blood of three representative KO recipients confirms the absence of EBP50. Het controls were also run for proper band identification. The upper band signifies a WT while the lower band denotes KO. **B.** Peritoneal macrophages were extracted from *Ldlr*$^{-/-}$ mice at the conclusion of the experiment and analyzed for proper EBP50 expression with Western blot. F4/80 established equal amounts of macrophages in the lysate and β-actin was used as a loading control.

Collectively, these studies confirm the complete ablation and reconstitution of bone marrow from the donor mice. Additionally, they show that EBP50 has no effect on hematopoiesis and myeloid cell lineage specification.

3.3.3 EBP50-Null Myeloid Cells Decrease Lesion Size

After 12 weeks of a high-cholesterol diet, aortas were analyzed for atherosclerosis severity. Surprisingly, en face Oil Red O staining showed a significant increase of percent lesion area covering the aorta in KO → *Ldlr*$^{-/-}$ (KO BMT) compared to WT → *Ldlr*$^{-/-}$ (WT BMT) (Fig. 3-4A). While both groups had lesions concentrated in the aortic arch, the KO BMT cohort had an additional cluster where the diaphragm divides the thoracic from the abdominal aorta. However, examination of sectioned plaques stained with Oil Red O revealed a significant 25% decrease in KO BMT cross-sectional lesion size throughout the aorta compared to the control (Fig. 3-4B).

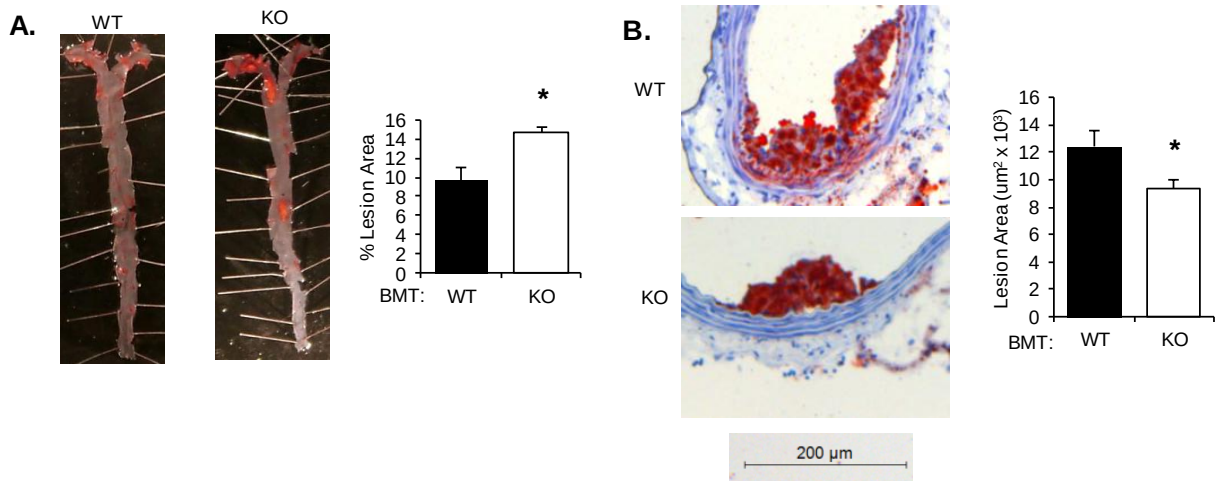

Figure 3-4: KO BMT lesions are larger in aortic surface area but smaller in cross section.

A, En face analysis of aorta after Oil Red O staining. The percent area of the aorta covered by lesions is graphed at right. N = 3 for both cohorts. B. , Cross-sectional area of lesions in the whole aorta after Oil Red O staining.

Quantification is at right. Data represent the mean ± SEM (* $P < 0.05$); n = 4 (WT BMT), 5 (KO BMT) unless otherwise noted.

3.4 DISCUSSION

In this study we demonstrate a role for EBP50 in multiple phenotypes of macrophage activation and a decrease in atherosclerosis in mice with bone marrow lacking EBP50. Additionally, EBP50 regulates NF-κB activity, confirming what we previously found in VSMC.

We show that the expressions of IL-1β and TNFα, as well as iNOS, are decreased in KO macrophages *in vitro* and *in vivo*. In contrast, there was no effect on IL-6 or IL-10. IL-10 is an anti-inflammatory cytokine that can inhibit NF-κB activation, enhance macrophage efferocytosis, and promote the conversion of M1 pro-inflammatory macrophages to a regulatory phenotype (65, 415-417). An overall decrease in pro-inflammatory cytokines and a lack of effect on IL-10 suggests that the balance between pro- and anti-inflammatory events could be altered in the absence of EBP50, leading to a more efficient resolution of inflammation.

Recent reports highlight the emerging role for EBP50 in leukocyte function. Manes et al. showed that the interaction between EBP50 and PIP5KIβ is necessary for chemoattractant-induced neutrophil polarization (378). Wu and colleagues determined that EBP50 is essential for neutrophil migration by scaffolding CXCR2 and PLCβ2 (379), and in this study we found that EBP50 is important for macrophage activation both *in vitro* and *in vivo*. It is becoming increasingly clear that EBP50 plays a central role in the activation and function of inflammatory cells.

Given that EBP50 governs the magnitude of macrophage activation, in particular the pro-inflammatory genes, we hypothesized that a mouse model with KO myeloid cells would exhibit less severe atherosclerosis. Indeed, transplantation of KO bone marrow into *Ldlr*$^{-/-}$ mice resulted in a modest but significant decrease in lesion size in the aorta following a high fat diet. Since KO macrophages only exhibit a 50% decrease in inflammatory genes, not a complete ablation, we did not expect a total rescue of atherosclerosis in these mice. The environment in these plaques is still inflammatory, albeit less so than with WT bone marrow and enough to retard the growth of these lesions by 25%. Previous studies deleting components more proximal to NF-κB such as IKKβ, which is downstream of EBP50 and can be phosphorylated by kinases other than PKCζ (184), demonstrate severely depressed expression of inflammatory genes and thus a further decrease in atherosclerosis than what we found here (267, 269). Another report utilized IKKβ$^{-/-}$ macrophages and conversely found an increase in severity due to decreased IL-10 and thus a defect in inflammation resolution (266). However, EBP50 had no effect on IL-10 expression, which may also have contributed to the diminished lesion size in the KO BMT mice.

Interestingly, KO BMT mice also exhibited increased surface area of lesions along the aorta as indicated by en face staining. This would suggest that mice receiving KO bone marrow are more sensitive to the initiation of atherosclerosis, although the protrusion of the lesion into the lumen (i.e. cross-sectional area) is not as severe as in WT BMT mice. Depending on the stability of these KO BMT lesions, having more, smaller lesions could be clinically preferable to concentrated, larger plaques. It is also important to remember that the bone marrow contains many different cells. We have demonstrated that KO macrophages have decreased cytokine secretion and others have shown EBP50 is important for polarization and migration of neutrophils (378, 379), but there is also evidence that EBP50 mediates cAMP-dependent T-cell

inhibition (418, 419). If KO T-cells are indeed more activated in the context of atherosclerosis, this could explain the increased spread of lesions seen in the KO BMT mice. In addition, the function of EBP50 in myeloid cells is still relatively unexplored and it is unknown what other consequences it could be having (foam cell formation, for example). However, we do know from the complete blood count data that EBP50 does not affect hematopoiesis, so the changes we see are not due to changes in cell number of any particular myeloid cell population.

In summary, we have demonstrated a role for EBP50 in the extent of macrophage activation. We have also confirmed previous data from VSMC on the involvement of EBP50 in the NF-κB pathway. In addition, transplantation of KO bone marrow into *Ldlr*$^{-/-}$ mice resulted in a decrease in lesion size, presumably due to the decreased inflammatory state of KO macrophages. Further assessment of the molecular and cellular composition of these plaques will yield further explanation of this phenotype.

4.0 GENERAL DISCUSSION

CVD is the leading cause of death in developing countries, and even with current therapeutics the risk of another possibly fatal event is 70-80% (389). This clearly signifies that further research is needed into more effective drugs, whether it's an addition to lipid-lowering drugs for atherosclerosis or an alternative to sirolimus- or paclitaxel-coated stents. We have been studying a scaffolding protein, EBP50, which may be a new drug target.

Our lab has been the main trailblazer on the path to determining a niche for EBP50 in the vasculature. We started with the observation that EBP50 could inhibit the anti-proliferative effects of PTHrP by switching on the G_q-dependent calcium signaling of PTH1R, essentially restoring the VSMC to their original benchmark proliferative state. EBP50 can further enhance serum-, growth factor-, and cytokine-stimulated VSMC proliferation through the stabilization of Skp2 and the subsequent degradation of p21. Additionally, EBP50 is essential for the bridging of EGFR to FAK and the initiation of cell migration. Not surprisingly, the indispensable role of EBP50 in these critical cellular responses leads to significantly decreased neointima formation in response to arterial wire injury in mice lacking this scaffolding protein.

What is surprising, however, is how a protein with such relatively low expression levels in quiescent VSMC can have such a significant influence on the injury response. One answer is that injury triggers a 4- to 5-fold increase in EBP50 expression that is only seen in the affected

VSMC. This upregulation presumably accounts for the ability of EBP50 to interact with its variety of intracellular binding partners and orchestrate these changes.

In addition to the aforementioned proliferation and migration, there are many different avenues that feed into neointima formation and other vascular diseases including inflammation, oxidative stress, lipid metabolism, and the presence of underlying pathologies. The purpose of this dissertation was to further characterize the function of EBP50 in the vasculature within an inflammatory context and to additionally extend our knowledge of this protein into the realm of atherosclerosis.

4.1 SUMMARY OF FINDINGS AND FUTURE DIRECTIONS

4.1.1 Inflammation Augments EBP50 Expression

The upregulation of EBP50 has been reported in multiple disease states including hepatocellular carcinomas, cholangiopathies, glioblastoma, breast cancer, psoriasis, and vascular injury (311, 312, 333, 358, 368, 403, 404). Estrogen is the best-described regulator of EBP50 expression (300, 303, 308) and previously our lab discovered a modest PTHrP-induced upregulation of EBP50. However, regulation by estrogen and PTHrP is not sufficient to explain the robust effect on EBP50 expression seen after wire injury. Murine EBP50 is not responsive to estrogen (313) and it is unlikely that the 50% increase induced by PTHrP solely accounts for the 4-5-fold upregulation of EBP50 (367). The purpose of this Aim was to uncover other regulators of EBP50 that could clarify this observation. While I wanted to investigate this in VSMC, I also wanted to extend the scope to macrophages.

Focusing on inflammatory mediators, LPS and TNFα were both able to increase EBP50 expression, verifying earlier results by an independent group (386). This was evident at the mRNA level and additionally translated into a 2- to 4-fold increase in protein. Furthermore, the aortas of LPS-treated mice also displayed increased EBP50 mRNA, although I did not have enough mice to reach significance. This increase was dependent upon NF-κB, a ubiquitous inflammatory transcription factor, as demonstrated by the lack of effect when simultaneously treated with an IKK inhibitor or a nondegradable IκBα construct. Simple overexpression of the p65 subunit of NF-κB was enough to mimic this effect without further stimulus. These results were manifested in both VSMC and macrophages (primary and clonal).

This considerable increase in EBP50 after treatment with inflammatory mediators takes us one step closer to explaining the upregulation we see in the neointima. This *in vivo* effect is probably the result of multiple factors including PTHrP and TNFα, and possibly others. LPS is present in very small amounts in circulation (those with bacterial infections have higher concentrations) and, depending on the status of the patient, may or may not have a physiological hand in EBP50's upregulation. Although I have not tested it, it is more likely that IL-1β takes the place of LPS in this pathway since they share very similar signaling cascades culminating in NF-κB activation. I have conducted very limited testing to determine if growth factors such as EGF and PDGF have a similar effect on EBP50 expression given that they are both able to stimulate NF-κB. So far I have not seen any effect of these stimuli on EBP50. However, these experiments are very preliminary and require further analysis to determine if other mediators with NF-κB implications are effective. If our pilot data holds and these growth factors are ineffective, it would be interesting to delineate the molecular determinants of why one stimulus is effective vs. another.

I have convincingly shown that NF-κB is necessary for EBP50 upregulation, yet I cannot conclusively say that NF-κB directly binds the promoter. There are multiple NF-κB consensus sites on the *Slc9a3r1* (EBP50) promoter, two of which are a perfect match, so the possibility does exist. However, I do not know how much "wobble" is allowed for those sites which do not 100% match the consensus sequence. The increase in EBP50 is seen relatively late in the activation of NF-κB (8-16 hours), and it is possible that this is a secondary effect of NF-κB signaling. Determining the binding site for NF-κB on the *Slc9a3r1* promoter would be one future direction. To accomplish this, the appropriate cells could be transfected with a luciferase construct under the control of the *Slc9a3r1* promoter and treated with LPS or TNFα to observe the change in transcription. Serial truncations can then be made to determine the responsible portion of the promoter. Electrophoretic mobility shift assays and chromatin immunoprecipitations could verify that this aforementioned promoter sequence binds to NF-κB.

Finally, it would be advantageous to determine the kinetics of EBP50 upregulation for the application of therapeutics in the developing neointima. We have observed that EBP50 is still increased at the peak of neointimal growth two weeks after injury, but it is unknown when this event begins. Judging by our data on cytokine influence, one would hypothesize that this happens a few days after injury when the macrophages arrive. Since we have had trouble with EBP50 immunofluorescence in the past, a more clear-cut and quantitative method would be to use pinpoint slide RNA isolation to assess EBP50 expression in the neointima versus the media.

4.1.2 EBP50 is Integrated into the NF-κB Signaling Pathway

EBP50 is a positive regulator of proliferation and migration, but I wanted to explore other possible functions for the scaffolding protein. The purpose of this Aim was to determine the

involvement of EBP50 in NF-κB activation. Indeed, EBP50 promotes NF-κB signaling. This is consistent with data from the literature showing a dependency on EBP50 for NF-κB binding to DNA in bronchial epithelial cells (391). However, my work extended these findings further up the signaling pathway. EBP50-null VSMC displayed decreased IKK, IκBα, and p65 phosphorylation, IκBα degradation, and p65 nuclear translocation. Both PDZ domains of EBP50 and the ability to bind the cytoskeleton are essential for these effects. Additionally, these VSMC exhibited decreased adhesion molecules (ICAM-1, VCAM-1). The effect of EBP50 on NF-κB activity and thus gene expression is not receptor-specific since it was observed with both LPS and TNFα. However, the lack of EBP50 does not exert a global effect on signaling as activation of p38 and c-Jun was not affected in KO VSMC. These phenomena of decreased adhesion molecules without EBP50 were also observed in an *in vivo* model of LPS-injected mice. Furthermore, I demonstrated that macrophage infiltration in our arterial injury model was decreased at the one-week time-point in KO mice.

It is interesting that EBP50 does not affect all NF-κB genes equally. Many cytokines and adhesion molecules are decreased in KO conditions, but the stimulation of MCP-1, IL-10, and IL-6 is unaffected by the absence of EBP50. While the reason for this has not been experimentally proven, it is likely that there are factors pertaining to both EBP50 and the specific promoter region which should be taken into consideration. Even though EBP50 has no effect on p38 or c-Jun pathways, the scaffolding protein could affect the activation of other transcription factors. EBP50 may positively regulate a pathway that feeds into the transcription of TNFα and IL-1β, for example, but not IL-6 or IL-10. In addition, there may be variability in NF-κB dependence among the different promoters.

Mechanistically, EBP50 interacted with PKCζ, an upstream activator of the IKK complex. This interaction is dependent upon the C-terminal PDZ motif of PKCζ (E-E-S-V), and mutation of this motif also abrogated NF-κB activation. Interestingly, overexpression of PKCζ was unable to rescue the KO phenotype, but the addition of a constitutively membrane-bound myristoylated PKCζ surprisingly restored NF-κB signaling. From this, I hypothesized that EBP50 is important for PKCζ membrane translocation. PKCζ did not accumulate at the membrane after stimulation in CHO cells, which do not express EBP50, but started to gradually increase after addition of EBP50. The magnitude of this effect is consistent with that observed in response to dopamine in OK cells (405). Together, these experiments established that EBP50 promotes NF-κB activation through the formation of an EBP50-PKCζ complex at the cell membrane.

The interaction between EBP50 and PKCζ was not only dependent on PDZ features but on the addition of a stimulus. During basal conditions, there was no evidence of an association, yet a few minutes of TNFα treatment yielded a complex containing EBP50 and PKCζ. What are the molecular determinants that permit this interaction after stimulation but not before? The C-terminal PDZ motif of PKCζ, E-E-S-V, contains a serine and phosphorylation of PDZ motifs have been shown to enhance binding (420). However, there are no reports of additional phosphorylation events within PKCζ aside from the T410 priming site and autophosphorylation at T560. Additionally, phosphorylation of the PDZ motif has more often demonstrated to inhibit binding to its respective PDZ domain (421). A more likely scenario is that EBP50 is phosphorylated in response to stimuli in a manner that enhances interactions. Phosphorylation of EBP50 has been previously shown to increase interactions, theoretically from conformational

changes (318). It is possible that EBP50 is phosphorylated in response to TNFα and other mediators, leading to its association with PKCζ.

Other future experiments pertaining to this interaction would be to 1) demonstrate that EBP50 and PKCζ do directly bind through GST pull-downs or blot overlays, and 2) determine which PDZ domain is necessary for this interaction using PDZ mutants of EBP50. Since mutation of the PKCζ PDZ motif completely abrogates its association with EBP50, the two proteins would be expected to directly bind.

In contrast to the function of PKCζ in fibroblasts yet consistent with analysis in the lung (246), I have added VSMC to the list of cell types in which PKCζ acts on IKK to activate NF-κB. I also did not check the effect of EBP50 on S311 phosphorylation on p65, a residue specifically phosphorylated by PKCζ (256). This should be explored in the future in order to make this a more complete body of work.

I chose to focus on VSMC and macrophages in this dissertation since we had previous data on EBP50 in VSMC and because macrophages also play a major role in restenosis. The endothelium is not as important in neointima formation as the majority of cells are removed. Preliminary data in the lab suggests that EBP50 has no effect on the proliferation or migration of endothelial cells, functions that could affect re-endothelialization after denudation. However, endothelium dysfunction is critical for macrophage infiltration in atherosclerosis and sepsis. I noticed that ICAM-1 and VCAM-1 expression in the endothelium *in vivo* is decreased after LPS injections in KO mice. It is possible that NF-κB signaling is also deficient in the null endothelial cells, adding to the repertoire of tissues in which EBP50 modulates inflammation.

These studies have all been performed in cells of mouse origin, and new binding partners for EBP50 are likely to emerge if human cells are investigated. While mouse TLR4 has a C-

terminus of A-T-W-T, the human homolog terminates in A-T-S-I. The difference of the final amino acid means that the human TLR4 ends in a hydrophobic residue and thus fits the consensus motif to bind EBP50. The direct interaction of these two proteins could provide a whole new level of TLR4 and NF-κB regulation in addition from PKCζ translocation. Modifying membrane TLR4 quantities could not only affect NF-κB activation but could also influence the magnitude and duration of endocytosis-mediated MyD88-independent signaling of TLR4.

It is intriguing that EBP50 and NF-κB can mutually upregulate each other's activity and/or expression, similar to the cycle in which cytokines such as IL-1β and TNFα are involved. EBP50 promotes NF-κB activation, which in turn increases EBP50 expression. This suggests that EBP50 helps keep NF-κB "turned on" for more rounds of activation in order to propagate inflammation. Since more receptors are being activated in the face of increased stimulation (as from the NF-κB-targeted TNFα or IL-1β), additional EBP50 may be needed to shuttle PKCζ to these membrane complexes. EBP50 is necessary but not sufficient for NF-κB activation, and I do not think the increased presence of EBP50 in the cell would interfere with the termination mechanisms of NF-κB. This is a scaffolding protein, not a kinase, and it does not possess constitutive activity in the absence of stimuli that could override inhibitory processes. I have completed the necessary EBP50 knockdown and null experiments, but have not performed overexpression studies. These would help clarify the purpose of EBP50 upregulation in the face of NF-κB activation.

4.1.3 EBP50 Increases Macrophage Activation and Atherosclerosis

Since I found EBP50 to govern the inflammatory response of VSMC, the first goal of this Aim was to examine the role of EBP50 in macrophage activation. EBP50 is starting to emerge as an

important modulator of leukocyte function. Through its scaffolding abilities, EBP50 is a positive regulator of neutrophil polarization (378) and migration (379) as well as the bactericidal activity of macrophages (386). I have added to that growing body of knowledge by investigating the effect of EBP50 on macrophage activation. Macrophages displayed diminished cytokine production (IL-1β, TNFα) and NF-κB activation without EBP50, and this was also reflected with *in vivo* LPS treatments. Interestingly, the absence of EBP50 did not alter IL-10 expression, suggesting that the scales could be tipped in favor of anti-inflammatory events and lead to a more efficient resolution of inflammation.

One limitation of this study is that I was not able to test the involvement of the EBP50-PKCζ complex on NF-κB activation in macrophages. I did establish a deficiency in NF-κB activation in macrophages treated with EBP50 siRNA similar to what I previously saw in KO VSMC. However, it would be interesting to see if the same mechanism I discovered in VSMC with PKCζ translates to macrophages as well. In addition, PKCζ is a main regulator of the M2 phenotype in response to IL-4 stimulation (422). EBP50 has not previously been implicated in the IL-4 pathway. I hypothesize that there would also be decreases in Jak1 and Stat6 (components of the IL-4 pathway) activation in KO cells, as well as IL-4 target genes. In addition, EBP50 and PKCζ should also experience a stimulus (IL-4)-dependent interaction, similar to what I've seen with TNFα treatment.

We have shown in previous studies and in this thesis that EBP50 is a positive regulator of acute inflammation resulting from surgical injury and LPS insult. Next, I wanted to investigate the role of EBP50 in atherosclerosis, a chronic form of inflammation. Given the interesting data I collected on EBP50 in macrophages, I wanted to focus on the contribution of

leukocyte/lymphocyte EBP50 in atherosclerosis. While there is much analysis left to do, KO BMT mice had significant reductions in lesion size.

Evaluation of the molecular and cellular composition of these plaques mainly includes macrophage, VSMC, collagen, and apoptotic content, as well as adhesion molecule expression. In addition, the ability of KO macrophages to form foam cells must be assessed. I hypothesize that the decreased M1 activation of the KO bone marrow cells is the reason for the moderate decrease in atherosclerosis. With that in mind, I would expect to see plaques with less macrophage infiltration and adhesion molecule expression. I do not anticipate changes in apoptotic areas or foam cell formation. Evaluation of the plaque composition may also shed insight into the increased spread of lesions in the KO BMT as demonstrated by the en face staining of the aorta. Other analyses include EBP50 expression in the plaque. Since we've previously shown that EBP50 expression is increased in the neointima of injured vessels (367), it would be interesting to see if this pattern continues in atherosclerosis. Both the neointima and atherosclerotic lesions are inflammatory environments so I would expect EBP50 to be similarly increased.

Since EBP50 also had an effect on the VSMC response, a double knockout (*Slc9a3r1/Ldlr*) will probably decrease atherosclerosis even further. KO macrophages secrete lower amounts of cytokines, but I would predict that the inability of KO VSMC to fully respond to a stimulus (i.e. adhesion molecule expression) would at least have an additive effect on the decreased lesion size seen here. I showed in Chapter 2 that macrophage infiltration into the neointima of KO mice is diminished, and I would expect the same result here in a double knockout background. It may be seen as disadvantageous to potentially abolish the formation of a fibrous cap due to the decreased proliferation and migration of KO VSMC, but if the plaque is

already less inflammatory due to reduced macrophage infiltration (and thus a smaller or nonexistent necrotic core) a fibrous cap would not be necessary. In fact, without a necrotic core to protect, a fibrous cap could just be an additional burden of the plaque protruding into the lumen and possibly impeding blood flow.

Similar to the bone marrow transplant methodology I conducted in this thesis, where I evaluated the contribution of KO macrophages to atherosclerosis, WT bone marrow could be transplanted into the double knockout model. This would conversely demonstrate the relative influence of KO VSMC and endothelium on the progression of atherosclerosis. Since EBP50 had a greater effect on VSMC than in macrophages, as assayed by relative decreases in gene expression, I would hypothesize that this model would display a greater decrease in lesion size than the KO BMT model I used in this dissertation. I would still expect the double knockout to have a greater rescue of atherosclerosis severity.

4.2 CONCLUDING REMARKS

The goal of this study was to further characterize the role of EBP50 in the vasculature, particularly in regards to inflammatory conditions. I have investigated the effect of EBP50 on the initiation and progression of inflammation, as well as the converse consequences of inflammation on EBP50. In doing so, I have demonstrated that:

- NF-κB is the main regulator of EBP50 expression in response to inflammatory stimuli.
- EBP50 promotes NF-κB activation in macrophages and VSMC, culminating in the expression of cytokines and adhesion molecules. In VSMC, this novel mechanism is driven by the recruitment of PKCζ to the membrane.

- EBP50-null myeloid cells in an *Ldlr*$^{-/-}$ background result in a significant decrease in atherosclerosis.

Inflammation is abundant in many other diseases including, but not limited to, cancer, asthma, rheumatoid arthritis, and sepsis. It is likely that the effect of EBP50 on inflammation and thus the findings of this thesis can extend to other disorders besides CVD.

In conclusion, this dissertation has demonstrated that EBP50 is a central mediator of macrophage activation and the response of VSMC to inflammation, culminating in atherosclerosis.

APPENDIX A

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