Activation of NF-κB drives the enhanced survival of adipose tissue macrophages in an obesogenic environment

By

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ABSTRACT

Objective: Obesity has become a major worldwide health issue over the past few years and often leads to insulin resistance (IR) and type 2 diabetes (T2D). Macrophage accumulation in adipose tissue (AT) during obesity contributes to inflammation and IR. In the decade since macrophages were shown to accumulate in AT, the majority of studies have focused on *recruitment-dependent* mechanisms for their accrual. However, recent evidence suggests that *recruitment-independent* mechanisms, including *increased proliferation* and *decreased egress*, may also regulate pro-inflammatory AT macrophage (ATM) numbers. Interestingly the regulation of longevity in ATM accrual in obesity had not been explored. The work in my dissertation shows that *increased ATM survival* during obesity is a *recruitment-independent* mechanism that contributes to ATM accumulation. **Results:** My studies demonstrated that cleaved caspase 3 activation is significantly reduced in the ATMs of diet-induced and genetically obese mice. This data suggests that activation of apoptotic pathways is significantly reduced in ATMs from diet-induced and genetically obese mice. Concurrently, pro-survival Bcl-2 family member protein levels and localization to the mitochondria was elevated in ATMs from obese mice. Conversely, the activities of pro-apoptotic proteins Bax and Bak were decreased in ATMs from obese compared to lean mice. Interestingly, this increased pro-survival signaling in obese ATMs was associated with elevated activation of the p65 subunit of the transcription factor, NF-κB. Furthermore, NF-κB was more nuclear localized in

ATMs of obese mice, resulting in increased expression of NF-κB pro-survival target genes, XIAP and cIAP. Finally, an obesogenic milieu increased ATM viability only when NF-κB signaling pathways were functional. **Conclusions:** Our data demonstrate that obesity promotes survival of inflammatory ATMs, possibly through an NF-κB-regulated mechanism.

DEDICATION

This thesis is dedicated to

My parents, Angela L. Hill-Stewart and Eugene Stewart,

My grandmother, Ruby L. Williams, and

My husband, Jephthah K. McAlester. Thank you all for supporting me through this journey. Words cannot express how blessed and grateful I am to have such wonderful people in my life.

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"Wait on the Lord; be strong and take heart and wait for the Lord."- PsaIms 27:14. If there is one lesson that I have struggled with, and now have been taught, throughout this journey that would be the lesson of patience. Letting go and letting God has help me learn that life doesn't have to be rushed and when the time comes things will go as they should. This is a message that I will take heed to throughout the rest of my life. I would like to thank the members of the Schrader Lane Church of Christ for all of your love, support, and being a church home away from home. I feel very blessed for God to have placed me among such great people that help and encourage me to grow in my faith.

Lastly, I would like to thank all of my family, friends and loved ones. All of you have been my biggest fans. I do not know how far I would have come without all of your love and support. To my mother, Angela, I am so blessed to have you as my mother. I wanted you to know that all of the work you put in to provide, take care of, and make opportunities for me was not in vain. It brings me such joy to see how proud you are of me and my accomplishments. I achieved this

accomplishment not only for myself, but for you as well. To my stepfather, Eugene, thank you for being such a great support to me and treating as your own. To my grandmother, Ruby Lee, thank you for being such a solid foundation in our family. Your faith in God, love, and support of your family has been the glue that holds us all together. Lastly, I would like to thank my amazing husband, Jephthah. Words cannot express how much you mean to me. We have known each other since the age of 5 and still, to this day, I know that you were put on this earth for me and I for you. Thank you for always being there for me. I love you so much and look forward to our journey together.

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LIST OF ABBREVIATIONS

AT: adipose tissue

ATM: adipose tissue macrophage

Bak: Bcl2-Antagonist/Killer 1

Bcl2: B-cell CLL/Lymphoma 2

Bcl-xl: B-cell lymphoma-extra large

Birc: Baculoviral IAP Repeat Containing

BMS: BMS-345541

CLS: crown-like structure

ER: endoplasmic reticulum

FFA: free fatty acid

GTT: glucose tolerance test

HFD: high fat diet

IFN-γ: interferon-gamma

IκBα: inhibitor of Kappa Light Chain Gene enhancer in B cells

IKK-β: inhibitor of κB kinase

IL: interleukin

KO: knockout

LFD: low fat diet

LPS: lipopolysaccharide

MCP: monocyte-chemoattractant protein

MetaC: Metabolic activation cocktail

MGL1: macrophage galactose-type C-type lectin 1

NGL: NF-κB-GFP-Luciferase mouse

NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells

PI3K: phosphatidylinositol 3-kinase

PIP_3: phosphatidylinositol (3,4,5)-trisphosphate

PKB: protein kinase

SFA: saturated fatty acids

SVF: stromal vascular fraction

T2D: type 2 diabetes

TG: triglycerides

TNF-α: tumor necrosis factor-α

TUNEL: Terminal deoxynucleotidyl transferase dUTP nick end labeling

UFA: unsaturated fatty acid

WT: wild-type

XIAP: X-linked Inhibitor of Apoptosis, E3 Ubiquitin Protein

CHAPTER I

INTRODUCTION

Portions of this Introduction have been published in a review article titled *A Decade of Progress in Adipose Tissue Macrophage Biology* written by Hill, Bolus and Hasty (1).

Our laboratory studies Immunometabolism, where we focus on macrophage function and their contribution to pathology in obesity and type 2 diabetes (T2D). A hallmark of obese adipose tissue (AT) is increased numbers of pro-inflammatory adipose tissue macrophages (ATMs). These macrophages largely contribute to the inflammatory state of obese AT and therefore the development of insulin resistance (IR). Our field has been increasingly interested in understanding the mechanism involved in regulating this increased accrual of pro-inflammatory ATMs in obese AT. The most well studied mechanism thought to regulate this process is *recruitment*. However, many studies demonstrate that deficiencies in pathways involved in recruitment do not fully attenuate macrophage accumulation in AT. In light of this, the focus has now turned to studying *recruitment-independent* mechanisms, where recent studies have elucidated the roles of *increased proliferation* and *decreased egress* as mechanisms that contribute to ATM accumulation in AT. My dissertation work shows that *increased ATM survival* serves as an additional *recruitment-independent* mechanism that controls ATM accrual in AT. In order to bring this

new finding into perspective, I first will describe the obesity epidemic, its health and economic impact, and why it still remains a major focus in biomedical research. Secondly, I will define how the body responds to nutrient intake and how the insulin signaling pathway regulates the uptake of dietary glucose and lipids under normal conditions. Next, I will detail the history of the discoveries made in the metabolism field in regards to obesity-induced IR and T2D that set the foundation for the work performed in this dissertation. I then will detail how the inflammatory state of obesity interrupts the ability of the insulin signaling pathway to properly control glucose uptake leading to the development of IR and T2D. As AT is one of the main organs affected in obesity, its role in regulating energy balance and its inflammatory nature during obesity will be detailed. Furthermore, I will describe how ATMs have been defined as the inflammatory source of AT and describe the distinctions between ATMs found in the lean compared to the obese state. Finally, I will describe the proposed research in this dissertation investigating the role of an ATM inflammatory mediator, NF-κB, as a recruitment-independent mechanism that promotes pro-inflammatory ATM survival and accrual in obese AT.

The Obesity Epidemic

Obesity has become a major worldwide health issue over the past two decades and is defined as having a body mass index of greater than or equal to 30 kg/m^2. Center for Disease Control (CDC) data shows that more the one-third of U.S. adults are obese. Even more alarming, childhood obesity is on the rise affecting

approximately 12.7 billion children and adolescents worldwide. The prevalence of obesity seems to affect some groups more than others, with low-income groups having increased prevalence due to the inaccessibility of nutritious foods. As the rate of obesity rises, the rate of the development of many obesity-related metabolic disorders also increases. Cancer, sleep apnea, cardiovascular disease, and premature mortality are all associated with obesity (2). Of particular interest to my lab, the prevalence of obesity-induced IR and T2D is also on the rise. The American Diabetes Association estimates that the economic cost of obesity-related complications with T2D to be $245 billion annually (www.diabetes.org).

Nutrient Disposition, Insulin Signaling, Insulin Resistance and Type 2 Diabetes

Obesity predisposes individuals to the development of IR and T2D. As of 2014, the Center for Disease Control suggests that 86 million adults are living with pre-diabetes and 15-30% of those individuals will develop T2D within five years. Over the years, the scientific community has made great advancements in defining the mechanisms that are involved in the development of IR and T2D but still have no reliable method of decreasing its prevalence. Continuing to research and define the mechanisms involved in the development of IR and T2D is necessary to prevent its continued progression.

Nutrient Disposition and Insulin Signaling

After ingestion of a meal, the body activates signaling pathways to uptake dietary glucose and lipids. Glucose and lipids are transported through the blood throughout the body. In response to glucose, β cells in the pancreas release insulin to signal the uptake of glucose and lipids to be stored or used as an energy source in the liver, muscle and AT (3). In these tissues, insulin signaling leads to the autophosphorylation of the tyrosine kinase insulin receptor, promoting the phosphorylation of insulin receptor substrate (IRS) family proteins. IRS1 phosphorylation leads to the activation of phosphatidylinositol 3-kinase (PI3K) at the plasma membrane. PI3K catalyzes the formation of a lipid second messenger phosphatidylinositol (3,4,5)-trisphosphate (PIP_3). Ultimately, PIP_3 and protein kinase-1 (PDK-1) phosphorylate and activate the serine-threonine kinase, AKT/protein kinase B, leading to glycogen synthesis and glucose uptake in the liver and muscle (Figure 1.1) (3, 4). Insulin signaling in the liver results in decreased glucose production and increased lipogenesis. In the muscle, glucose is taken up by the glucose transporters, GLUT1 and GLUT4, and stored as glycogen (3). Insulin action on the AT leads to inhibition of lipolysis and storage of lipids in the form of triglycerides. In the fasting state, lower blood glucose levels result in glycogen breakdown in the liver and muscle. Furthermore, lipolyzed lipids are released from AT in the form of free fatty acids to be used for fatty acid oxidation by the muscle and other tissues.

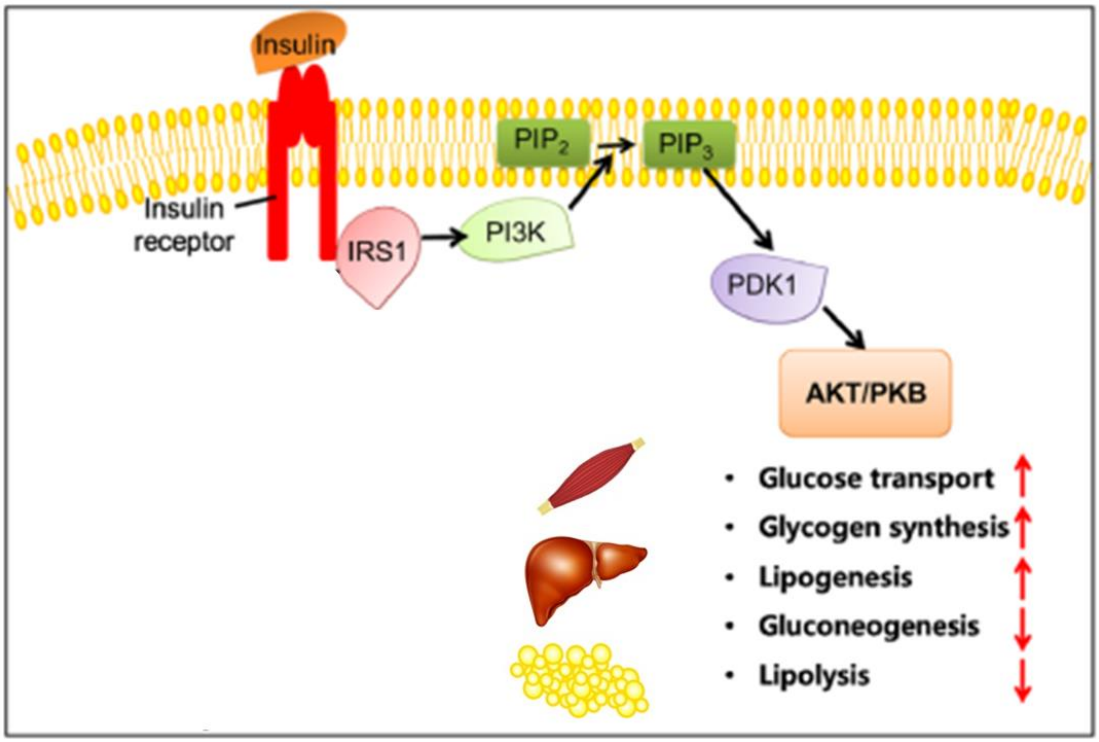

Figure 1.1. The insulin signaling pathway. Adapted from Jung and Choi. Int. J. Mol. Sci. 2014: 6184-6223. Insulin binds to the insulin receptor resulting in its autophosphorylation. A series of phosphorylation steps ultimately leads to the activation of AKT/PKB. As a result, glucose is taken up in the liver and muscle for storage as glycogen. In addition, lipolysis is inhibited in the AT.

IR and T2D

In the obese setting, insulin signaling pathways can become resistant to insulin. The IR state is manifested by decreased insulin-stimulated glucose uptake by the muscle and dysregulated hepatic glucose output, resulting in elevated levels of glucose in the blood (5). To compensate for the decreased response to insulin, the body maintains normoglycemia by increasing insulin production by the pancreas (6). This state of hyperinsulinemia allows for insulin signaling to occur and blood glucose levels to be normalized.

In the obese state, it has been well established that inflammation promotes IR [reviewed in (7)]. Obesity is often described as a state of low-grade chronic inflammation, where inflammatory chemokines such as TNFα activate the inflammatory mediator, c-Jun N-terminal kinase (JNK), resulting in inhibition of insulin receptor substrate (IRS) and insulin action (8). Additionally, I kappa B kinase (IKK) activation by TNFα leads to downstream signaling through NF-κB, leading to production of inflammatory cytokines that perpetuate the inflammatory state (9). Activation of these inflammatory pathways leads to dysregulated lipolysis in AT resulting in ectopic lipid storage in the muscle, liver, and pancreas. As a result, these tissues lose their ability to function properly leading to increased hepatic glucose production by the liver and decreased glucose uptake by the muscle. Elevated levels of glucose in the blood signal to β cells to secrete more insulin in order to promote glucose uptake. However, the increased demand for the production of insulin causes increased cellular stress and this, coupled with exposure to excessive lipids from AT, can result in β cell dysfunction and death (10). With the lack of a mechanism to alleviate elevated blood glucose levels, β cells lose their ability to compensate and individuals progress towards developing T2D (10). Furthermore, the sustained elevation of glucose levels in T2D leads to pathologies such diabetic neuropathy, diabetic retinopathy, and mortality (2).

AT in Regulating Energy Balance

AT is a metabolically active endocrine organ that plays an essential role in energy balance. During energy excess, AT stores lipids in adipocytes in the form of triglycerides. Conversely, during nutrient shortage, free fatty acids (FFAs) lipolyzed from AT serve as an energy source for muscle and liver (11). In addition to its lipid buffering capacity, AT also secretes adipokines such as leptin and adiponectin (11). Leptin has been demonstrated to decrease food intake and energy expenditure. Its action on the brain serves as a signal for long-term energy stores when energy in the body is sufficient. Another adipokine, adiponectin, enhances insulin sensitivity, increases fatty acid oxidation, and reduces hepatic glucose output among many other things (3, 11). This adipokine is specifically secreted from adipocytes and is inversely correlated with the degree of adiposity. In addition to these adipokines, AT is known to secrete inflammatory cytokines, such as TNFα, IL-6, MCP-1 and IL-1β, during obese conditions. It has been demonstrated that AT-derived TNFα, IL-6, MCP-1, and IL-1β all contribute to the insulin resistant state of obesity (12-15).

Historical Perspective on Adipose Tissue Inflammation

An association between the immune system and metabolism had been appreciated clinically for many decades; however, the impact of immune cell-secreted inflammatory cytokines on adipocyte function was not studied in detail until the mid-1980's. These initial studies showed that endotoxin-treated macrophages secrete products that can promote lipolysis in adipocytes (16) and

that a macrophage-secreted factor, cachectin, has the metabolic effect of inducing cachexia (17). Simultaneously, TNF-α was being studied for its cytotoxic, anti-tumorigenic, and inflammatory properties. It was soon discovered that cachectin and TNF-α are the same protein, which has since been referred to as TNF-α(18, 19). This marks the beginning of our understanding of the effects of inflammatory factors secreted by macrophages on metabolic processes. The mechanism underlying this new concept lies at the intersection of inflammatory and insulin signaling pathways [reviewed in (20, 21). In the mid-1990's, it was discovered that inflammatory mediators, including TNF-α, IL-6, iNOS, and CCL2, are elevated in obese compared to lean AT (13, 22-24). Furthermore, it was discovered that genetic deficiency of TNF-α (25, 26), iNOS (23), and JNK1 (8) improve systemic insulin sensitivity in obese models. These studies gave insight into the possible role of AT inflammation in metabolic homeostasis.

Macrophages are the Inflammatory Source in Adipose Tissue

Despite the growing body of evidence linking inflammation and metabolism, the cellular sources of inflammatory mediators in AT were unknown. Localization of macrophages to AT had been mentioned by several groups (27-30); however, the functional contribution of AT macrophages (ATMs) to obesity-related metabolic diseases remained unappreciated. In 2003, two seminal manuscripts were published by Weisberg *et al*. (31) and Xu *et al*. (32). These groups used microarray analysis to establish differences in gene expression between AT from lean and obese mice. They found many differences in genes related to

macrophages including surface markers and secreted products. Separation of AT into its two primary components, the stromal vascular fraction (SVF) and adipocytes, showed that canonical macrophage inflammatory genes are most highly expressed in the SVF of obese AT. Flow cytometry and immunohistochemical analyses confirmed the increased presence of macrophages in AT of obese mice. Importantly, this increase in ATMs occurs independently of the etiology of obesity: monogenetic forms of obesity and diet-induced obesity both result in increased ATMs (31, 32). Notably, this dramatic accumulation of macrophages was not found in liver, muscle, lung, or spleen (32). In addition, human subjects displayed a similar elevation in macrophages in obese compared to lean individuals (31). Thus, these two groups unequivocally demonstrated that macrophage number and inflammatory potential increase in AT in obesity. Furthermore, it should be noted that nearly every type of immune cell is present in AT, with their phenotypes and proportions changing in obesity (Figure 1.2) [reviewed in (33-36)].

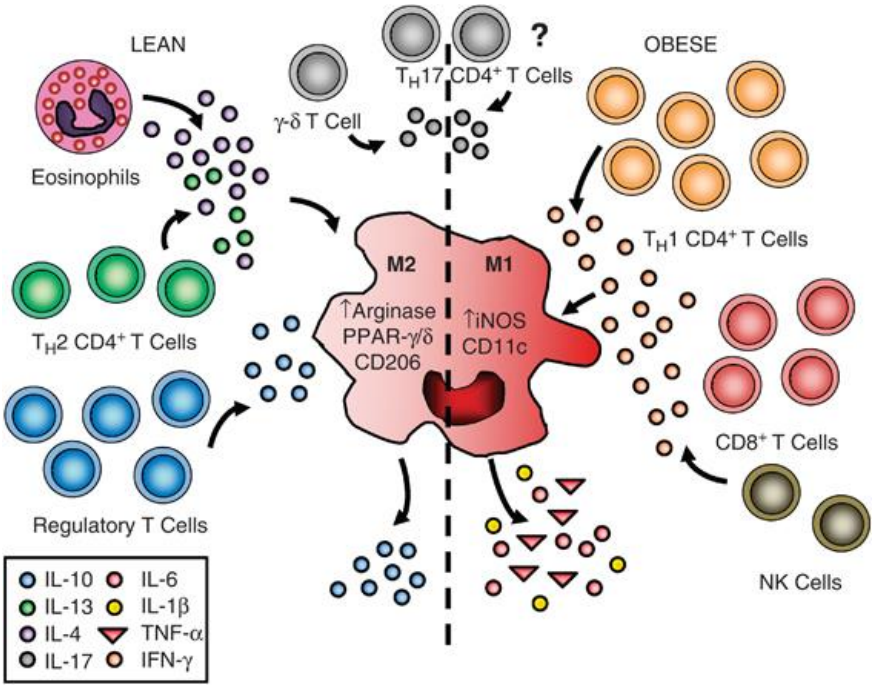

Figure 1.2. Immune cell types in AT. From Winer and Winer. Immunology and Cell Biology. 2012: 755-762. Macrophages are the most abundant immune cell in AT. However, T-cells, NK cells, and eosinophils are also present in AT. Furthermore, many of these cell types play a role in macrophage polarization state in AT. Additionally, the levels of the immune cells types change from the lean to the obese state, with many of these immune cell levels decreasing during obesity.

Relevance of ATMs to Metabolic Disease

As Dixit points out in his commentary (37), 1 g of AT can contain up to 5 million stromal vascular cells, greater than 50% of which are leukocytes. Thus, even in lean individuals, AT cannot be excluded as a major contributor to systemic immune regulation – including immunometabolism. The newly discovered increase in pro-inflammatory macrophages has significant implications for IR and metabolic disease associated with obesity (Figure 1.3). In fact, Xu *et al*. showed that the increased AT inflammatory response in obesity preceded rises in plasma

insulin, an indication of insulin resistance (32). Macrophages can impact AT function 1) by inducing adipocyte insulin resistance leading to dysregulation of basal lipolysis and ectopic lipid storage, 2) by inducing adipocyte chemokine and cytokine production, or 3) by impacting AT expansion capacity during obesity Thus, continued exploration of ATM function in lean and obese conditions enables a better understanding of how AT impacts systemic insulin action and glucose metabolism. Obesity-related accumulation of ATMs in humans is less robust than in mice, but it has been clearly demonstrated by multiple groups (38-41). The majority of human ATMs accumulate in omental rather than subcutaneous depots (40-42). Importantly, omental ATMs have been shown to correlate positively with fasting glucose and insulin levels, suggesting a link between AT inflammation and metabolic disease (40, 43). In addition, it has been demonstrated that weight loss decreases macrophage content in omental AT, improving glucose homeostasis (42). Thus, the preponderance of current literature supports a role for ATMs in metabolic homeostasis in rodents and humans.

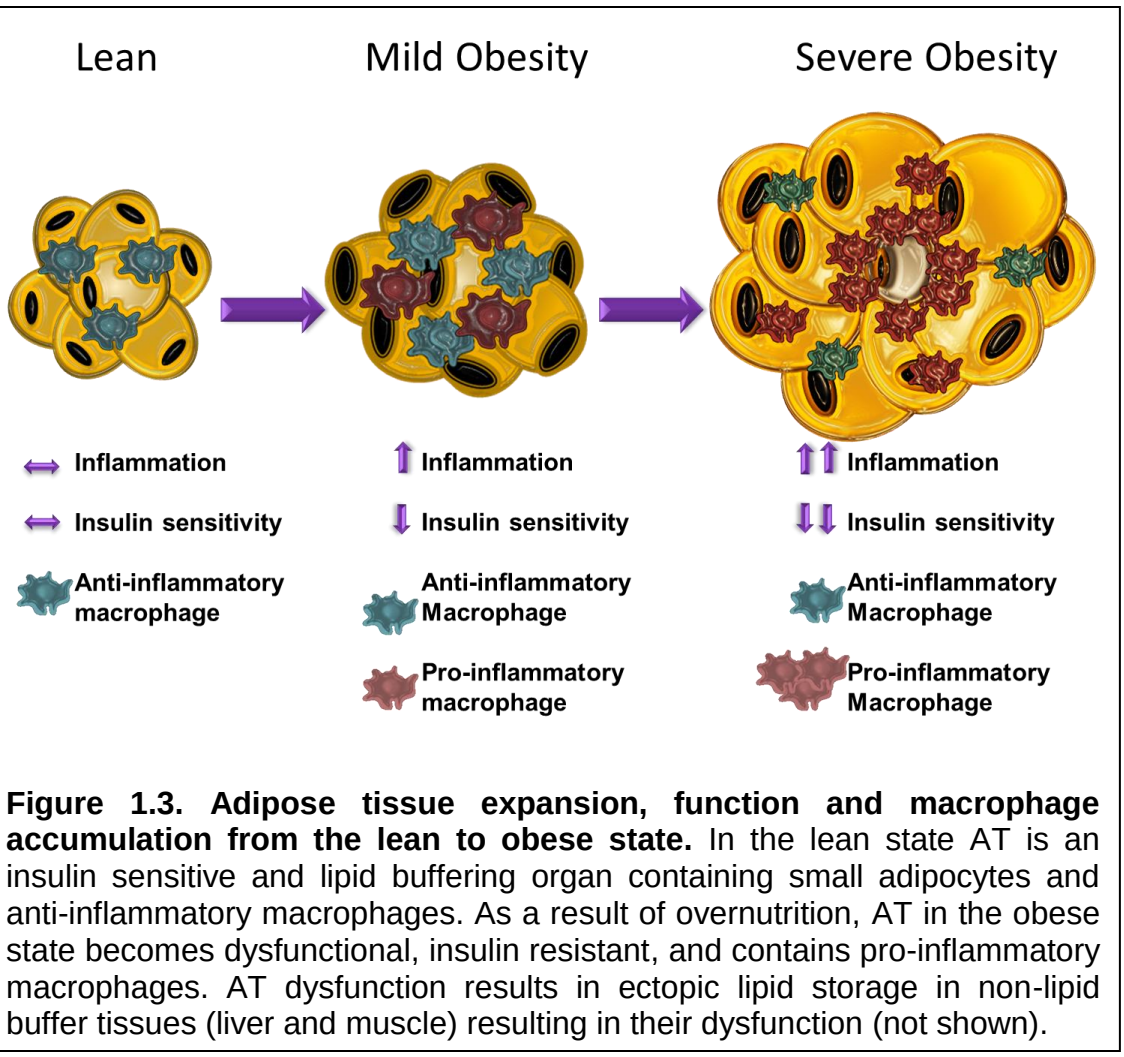

Figure 1.3. Adipose tissue expansion, function and macrophage accumulation from the lean to obese state. In the lean state AT is an insulin sensitive and lipid buffering organ containing small adipocytes and anti-inflammatory macrophages. As a result of overnutrition, AT in the obese state becomes dysfunctional, insulin resistant, and contains pro-inflammatory macrophages. AT dysfunction results in ectopic lipid storage in non-lipid buffer tissues (liver and muscle) resulting in their dysfunction (not shown).

AT Macrophage Heterogeneity

It was noted in publications by Weisberg and Xu (31, 32) that lean AT also contains macrophages, albeit at lower levels than obese AT. Thus, researchers became interested in the phenotypic differences between ATMs in lean and obese AT. Although macrophage phenotypes span a continuum and no single system of nomenclature can provide all of the required definitions, investigators have gravitated to identifying ATMs as either M1-like or M2-like. Regardless of the nomenclature used, pioneering investigators in the AT field have pursued the

notion that resident M2-like ATMs have a role in AT homeostasis, while recruited M1-like macrophages contribute to inflammation and insulin resistance.

M1 and M2 Categorization

M1, or “classically activated,” macrophages are produced upon exposure to T_H1 cytokines or inflammatory mediators such as IFNγ and LPS. Thereafter, they generate reactive oxygen species and release inflammatory cytokines such as TNF-α or IL-6. M2, or “alternatively activated,” macrophages are produced upon exposure to T_H2 cytokines such as IL-4 and IL-13 and express factors including IL-10 and arginase (Figure 1.4) (44, 45). Macrophage fuel utilization varies by polarization as M1 macrophages primarily utilize glucose, whereas M2 macrophages utilize fatty acids (46, 47).

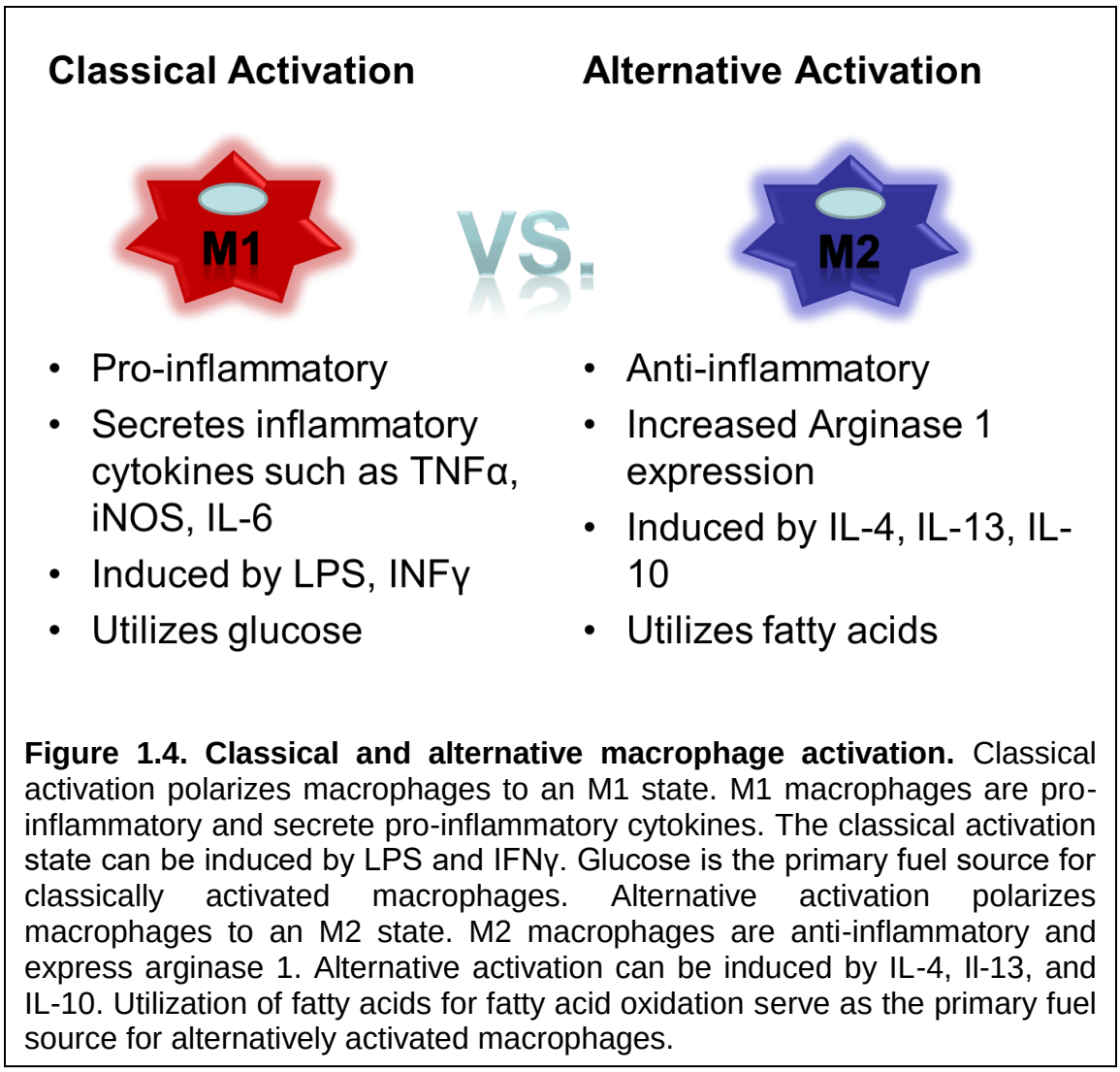

Figure 1.4. Classical and alternative macrophage activation. Classical activation polarizes macrophages to an M1 state. M1 macrophages are pro-inflammatory and secrete pro-inflammatory cytokines. The classical activation state can be induced by LPS and IFNγ. Glucose is the primary fuel source for classically activated macrophages. Alternative activation polarizes macrophages to an M2 state. M2 macrophages are anti-inflammatory and express arginase 1. Alternative activation can be induced by IL-4, Il-13, and IL-10. Utilization of fatty acids for fatty acid oxidation serve as the primary fuel source for alternatively activated macrophages.

Throughout the body, M1 macrophages are involved in inflammatory processes (such as combating infectious agents), while M2 macrophages play a role in immunosuppressive activities (such as tissue repair). Polarization of macrophages in AT is thought to confer similar properties. For example resident M2 macrophages likely contribute to AT homeostasis, while M1 macrophages in obese AT likely promote inflammation leading to IR. These properties are discussed in detail below.

M1 versus M2 ATM Localization and Plasticity

Localization of macrophages within AT differs in lean and obese mice, demonstrating yet another difference in ATM subpopulations. While the resident macrophages in lean AT are interstitially spaced, Cinti *et al.* (48) demonstrated that a preponderance of all macrophages in obese AT are localized in clusters referred to as “crown-like structures (CLSs)” – a term that is now commonly used in the Immunometabolism field. Lumeng and colleagues made the novel observation that ATMs within CLSs express M1 markers such as CCR2 and TLR4, while interstitially spaced ATMs express M2 markers such as Mgl1 and IL-10 (49). Using PKH26 labeling studies, they showed that recruited macrophages primarily localize to CLSs.

Although obesity induces a dramatic increase in M1-like ATMs, M2 macrophages increase in absolute number as well, even if their proportion compared to M1 ATMs decreases (50). Not all of the macrophages within the CLSs are M1 macrophages, indicating that some M2 ATMs are retained in obesity (49, 51). In addition, CD11c+ ATMs in obese mice express varying levels of Mgl1, indicating a broad range of phenotypes and an increase in M2 as well as M1 ATMs (50).

Standardization of Macrophage Categorization

It is becoming clearer that a caveat to the current M1 and M2 macrophage categorization is its inability to encompass the larger spectrum of macrophage phenotypes. In light of this, there has been a push to categorize macrophages

based on source of origin (bone marrow versus embryonic-derived), stimuli they are exposed to, and activation state. Many studies now demonstrate that macrophage phenotype is largely regulated by the tissue milieu it is present in (52). Recent studies have demonstrated that ATMs from obese mice display a mechanistically distinct cell surface marker profile and pro-inflammatory phenotype that differs from the traditional markers that categorize a macrophage as M1 or M2. Kratz and colleagues termed this activation state as “metabolic activation”, where ATMs express pro-inflammatory, *Tnfα* and *Il-1β*, and lipid metabolism genes, *Abca1* and *Plin2*, which are not present in traditional classically activated macrophages (53). For simplification of terminology, the remainder of this dissertation will refer to lean ATM polarization as “anti-inflammatory” and obese ATM polarization as “pro-inflammatory”.

Mediators of Anti-inflammatory Polarization

Anti-inflammatory macrophages are thought to promote AT homeostasis and to protect against IR. Through their efforts to elucidate the origin of anti-inflammatory macrophages in AT, investigators have defined multiple AT-specific mediators of anti-inflammatory polarization, including transcription factors, adipokines, fatty acids, and other immune cells. For the purpose of my dissertation, I will focus only on fatty acid induced anti-inflammatory polarization.

Unsaturated Fatty Acids

Macrophages in AT are exposed to various types of fatty acids released from adipocytes in both basal and demand lipolysis. The degree of fatty acid saturation greatly impacts macrophage polarization: saturated fatty acids (SFAs) induce a pro-inflammatory phenotype while unsaturated fatty acids (UFAs) induce an anti-inflammatory phenotype. For example, the Hasty lab has shown that macrophages treated with the UFA oleic acid express increased levels of the anti-inflammatory markers Clec10a and Cd163 (54). UFAs can also reverse the effects of SFA-induced pro-inflammatory polarization of macrophages. L'homme et al. demonstrated that treatment of human monocytes/macrophages with UFAs prevented SFAs activation of the NLRP3 inflammasome (55). Furthermore, Chan and colleagues have also demonstrated that treatment of BMDMs with palmitoleate prevented palmitate-induced inflammatory polarization (56).

Mediators of Pro-inflammatory Polarization

Reports from both Weisberg *et al.* (31) and Xu *et al.* (32) describe the inflammatory nature of macrophages in obese AT. Based upon the knowledge that inflammatory cytokines can induce IR in multiple cell types – including adipocytes – subsequent studies focused on blocking inflammatory pathways to ameliorate AT IR. Extracellular signals that can induce inflammatory signaling pathways include lipids and other molecules that activate pattern recognition receptors in ATMs. With regards to intracellular signaling pathways, NLRP3

inflammasome and NF-κB pathways have both been areas of relevance to ATMs, specifically M1 polarization.

Lipid-Mediated Activation of ATMs

ATMs are exposed to excess lipids via at least three different routes: delivery from dietary chylomicrons, basal and demand lipolysis from adipocytes, and adipocyte cell death. Subsequent to dietary fat ingestion, chylomicrons and chylomicron remnants are routed to AT where lipoprotein lipase facilitates release of fatty acids for uptake and storage in adipocytes. Fatty acids from very low density lipoproteins (VLDL) can also be delivered to AT for storage. The Hasty laboratory reasoned that in obesity, hyperlipidemia could result in increased exposure of ATMs to lipolyzed fatty acids, thereby promoting inflammation in a paradigm similar to what is known for arterial macrophages in atherosclerotic lesions. In support of this, they showed that exposure of macrophages to VLDL (57) and SFAs (58) induces secretion of pro-inflammatory cytokines, typical of a pro-inflammatory phenotype. Of relevance, fatty acid composition in the AT and plasma is partially dependent on dietary intake(59). Although there is conflicting evidence of fatty acid composition in AT, studies suggests that intraadominal AT is enriched in SFAs. If so, increased dietary intake of SFAs could increase the exposure of ATMs to SFAs, thus promoting a pro-inflammatory phenotype.

Adipocyte death may also contribute to lipid-related changes in ATM phenotype. Phagocytic ingestion of dead cells results in lipid droplet

accumulation in ATMs (48). In fact, macrophages that surround dead adipocytes display a morphology similar to that of foam cells in atherosclerotic plaques (48, 60-62), although they most likely contain TG rather than cholesterol (60). Thus, lipid debris from dead adipocytes could also be lipotoxic to the ATMs promoting inflammation and pro-inflammatory polarization. As noted above, Kratz and colleagues demonstrated that ATMs present in AT are “metabolically activated” presumably from the uptake of the lipid debris from dead adipocytes and the lipolyzed SFAs (53).

Inflammasome-Mediated Activation of ATMs

ATMs can activate many pathways that promote secretion of pro-inflammatory cytokines in response to pathogens through pattern recognition receptors such as the Toll-like receptors. This can also be achieved through recognition of danger-associated molecular patterns (DAMPs). DAMP signaling results in the activation of the Nlrp3 inflammasome, which involves the formation of a multiprotein scaffold complex, including Nlrp3 and caspase-1. Formation of this complex is required for caspase 1 to obtain full activation allowing for cleavage and release of IL-1β and IL-18 [reviewed in (63)]. Stienstra and colleagues have shown that global deficiency of caspase 1 or Nlrp3 results in improved insulin sensitivity. They concluded this to be due to effects on adipocytes rather than ATMs (64). In contrast, Dixit and colleagues demonstrated that Nlrp3 co-localized to lipid-engorged ATMs (65). In their study, expression of IL-1β and Nlrp3 in visceral AT was positively correlated with body weight and adiposity (65), and

conversely, chronic caloric restriction reversed these effects and resulted in improved insulin sensitivity in mice and in human subjects. Elimination of Nlrp3 resulted in decreased caspase-1 cleavage, along with reduced IL-18 and IFN-γ expression concomitant with improved insulin sensitivity (65). Importantly, the $Nlrp3^{-/-}$ mice had increased anti-inflammatory gene expression along with decreased pro-inflammatory gene expression in AT, possibly accounting for the improved insulin sensitivity detected. The Nlrp3 inflammasome has been shown to recognize DAMPs such as ATP, urate, asbestos, β amyloid and SFAs. With relevance to ATMs, Dixit and colleagues demonstrated that the Nlrp3 inflammasome also recognizes ceramides (65). This new role of the inflammasome in lipid-laden macrophages uncovered a mechanism by which toxic lipid species (SFAs and ceramides) may act as danger signals to ATMs and promote an inflammatory phenotype.

NF-κB-Mediated Activation of ATMs

Many of the above mentioned macrophage polarization mediators intersect with the NF-κB signaling pathway, a key mediator of macrophage polarization. NF-κB is a multi-subunit transcription factor composed of Rel subunits such as RelA (p65), RelB, c-Rel, p50, and p52, which form various homo- and hetero-dimers that bind DNA to induce transcription of a plethora of genes. The p65/p50 heterodimer is the most common form of NF-κB and regulates transcription of inflammatory genes in many cell types, including macrophages. NF-κB activation is regulated by its activator I kappa kinases (IKKs) and its inhibitor, I kappa B

alpha (IκBα). Common obesity-related stimuli such as TNFα, LPS, and other inflammatory cytokines can induce activation of the NF-κB pathway to promote even further inflammation. In this regard, researchers have been interested in ATM NF-κB activation.

Dampening of the NF-κB pathway has been known for some time to improve systemic insulin sensitivity (66); however, AT-specific effects of NF-κB were not discovered until more recently. Using NF-κB reporter mice, Chiang *et al.* demonstrated HFD-fed mice displayed a 2-fold increase in luminescence in the AT depots compared to chow-fed controls (67). Furthermore, they demonstrated that ATMs from obese mice have increased IKK and NF-κB activity compared to ATMs from their lean counterparts. Upon closer examination by confocal microscopy, it was shown that luciferase illumination and nuclear localization of the NF-κB p65 subunit was only enriched in ATMs in HFD-fed mice.

Manipulating upstream IKK activators of NF-κB has been a major focus in understanding the role of NF-κB-induced activation of inflammatory pathways in ATMs. For example, mice lacking IKK-β in myeloid cells retain insulin sensitivity; however, whether this protection is due to reduced ATM inflammation was not determined (9). Another IKK, IKKε, was shown to be significantly upregulated in pro-inflammatory ATMs of HFD-fed mice compared to controls. Furthermore, IKKε deficiency attenuated inflammation and insulin resistance in HFD-fed mice (67).

One modulator of NF-κB in ATMs could be SFAs. As noted above, exposure to SFAs and PUFAs resulting in inflammatory or anti-inflammatory

polarization of ATMs has been extensively studied. Dysregulated lipolysis in obesity exposes ATMs to excessive amounts of SFAs. Although the mechanism remains to be determined, many studies suggest that SFAs activate NF-κB pathways in macrophages promoting an inflammatory phenotype. Reports from Suganami and colleagues have shown that treatment of macrophages with the SFA palmitate significantly induces NF-κB activation (68). This activation was followed by a significant increase in expression of NF-κB regulated inflammatory molecules TNF-α and CCL2. Thus, NF-κB activation is a likely player in driving the pro-inflammatory phenotype in ATMs. Chapter III of this dissertation will explore the contribution of exacerbated NF-κB activation in ATMs to ATM accumulation and disease progression during obesity.

Mechanisms for Macrophage Accrual in AT

As detailed above, the AT milieu plays a significant role in regulating the polarization state of ATMs. Lean AT supports an anti-inflammatory phenotype; whereas, obese AT supports a pro-inflammatory phenotype. Interestingly, the lean and obese AT environment also have additional distinctions between them with regards to the macrophages present in each environment. In particular, the obese AT milieu displays a significant increase in overall macrophage content as a result of a large influx of pro-inflammatory macrophages present in the tissue. Due to the detrimental effects pro-inflammatory macrophages have on AT function and their promotion of disease progression in obesity, much of the

Immunometabolism field has focused on understanding the mechanisms that regulate macrophage accrual in AT.

The number of cells that accumulate in any given tissue can be theoretically attributed to fluxes in at least 4 different mechanisms: recruitment, egress, proliferation, or death (Figure 1.5). Although the overwhelming number of studies in AT have focused on the recruitment side of the equation, there is evidence in the literature for all four of these mechanisms contributing to macrophage accumulation in AT. The discussion of this topic has been divided into two categories: recruitment-dependent and recruitment-independent mechanisms of macrophages accrual in AT.

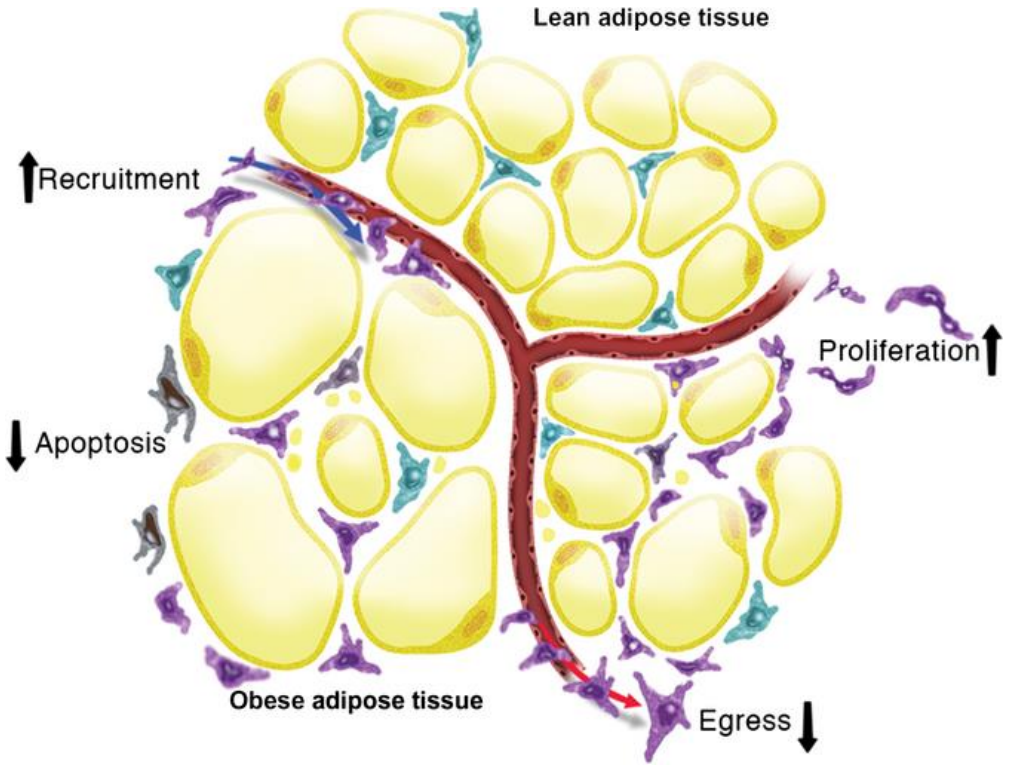

Figure 1.5. Mechanisms of macrophage accrual in AT. From Hill *et al.* Immunological Reviews. 2014: 132-152. Many publications have focused on recruitment-dependent mechanisms that can account for adipose tissue macrophage accrual. In addition, there is recent evidence for recruitment-independent mechanisms such as proliferation, egress, and apoptosis to also contribute to total adipose tissue macrophage numbers.

Recruitment-Dependent Pathways of Macrophage Accrual in AT

After the discovery of increased macrophages in obese AT, Weisberg *et al.* performed traceable bone marrow transplants in newly obese mice to determine the origin of ATMs with obesity (31). These studies demonstrated that the majority of the macrophages in the obese AT are bone marrow derived, and thus, recruited (Figure 1.6). Since this landmark publication, many studies have aimed to identify the major factors responsible for monocyte recruitment, underlying

ATM accumulation during AT hypertrophy. These factors include adipocyte death, chemokines, adipokines, and lipids**.**

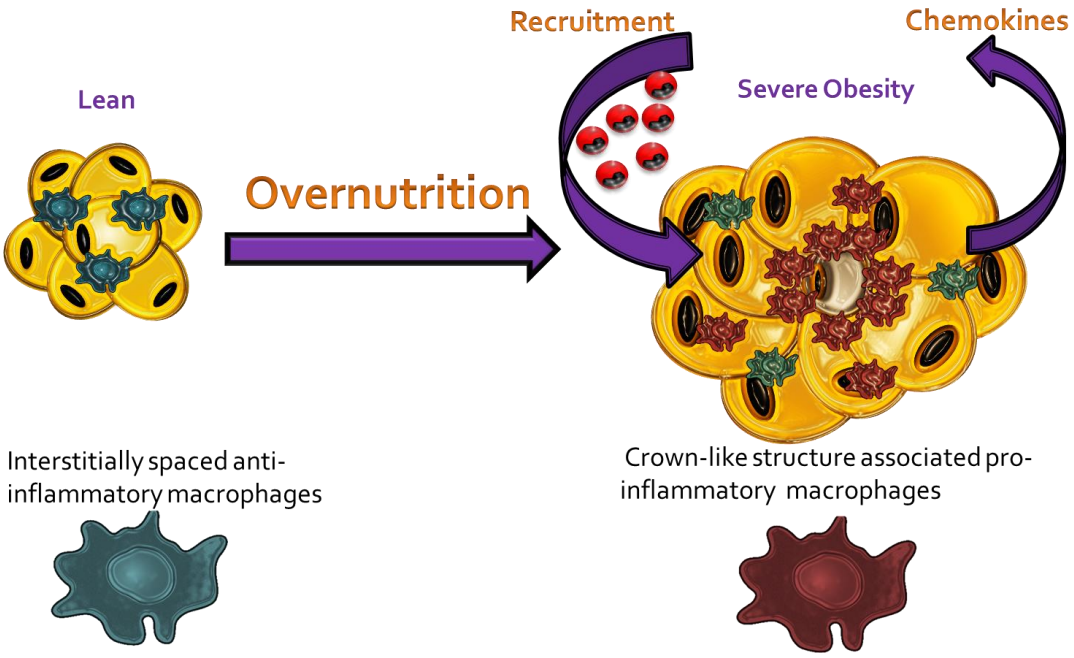

Figure 1.6. Recruitment-dependent mechanisms of macrophage accrual in AT. Inflammatory macrophage accrual in obese AT is thought to be a result of the recruitment of inflammatory monocytes to obese AT by chemotactic signals secreted from the AT. Defined as a chronic inflammatory state, obese AT recruitment cycle continues presumably resulting in inflammatory macrophages accumulation.

Adipocyte Death

Hypoxia can occur in AT when adipocytes expand in excess of microvasculature growth, or when adipocyte size exceeds the diffusion of nutrients, leading to adipocyte cell death [reviewed in (69)]. Similar to macrophage functions in other tissues, it has been hypothesized that ATMs are recruited to phagocytose cellular

debris following adipocyte apoptosis. This has been visualized by light microscopy in the CLSs of obese AT where multinucleated ATMs form as a result of engulfing residual adipocyte lipid droplets (48). In an obese mouse model of massive adipocyte apoptosis, macrophage recruitment to dead adipocytes, as well as number and CLSs dramatically increase compared to control mice (70). Like in normal models of obesity, this influx of macrophages was largely of the pro-inflammatory phenotype and further promoted inflammation in the obese state.

Chemokines / Chemokine Receptors

In humans and mice, expression of many different chemokines and chemokine receptors is elevated in obese compared to lean AT (31, 32, 71). The MCP1 (CCL2)/CCR2 chemokine/chemokine receptor axis is one of the most potent for monocyte recruitment in inflammatory settings. Further support for a potential role of CCL2/CCR2 in ATM recruitment stems from the fact that AT gene expression of CCR2 and its ligands (CCL2, CCL7, and CCL8) is increased 2-7 fold in obese compared to lean mice (72). Thus, several groups have assessed CCL2 and CCR2 deficient mice to determine whether ATM recruitment is reduced. Kanda *et al.* showed increased levels of CCL2 both in AT and plasma of obese mice corresponding with increased AT macrophage content (14), and identified adipocytes as one source of CCL2. Transgenic AT-specific overexpression of CCL2 increases AT macrophage infiltration, IR, fasting blood glucose, serum free fatty acid (FFA), and hepatic steatosis, even in the lean

state. From the other end of the spectrum, $CCL2^{-/-}$ mice in their studies had reduced HFD-induced ATM accumulation, associated with decreased IR, serum FFA, and hepatic steatosis.

In stark contrast to this, studies by Inouye *et al.* (73) and Kirk *et al.* (74) saw no reduction in ATM accumulation in $CCL2^{-/-}$ mice challenged with short-term or long-term HFD. In both of these studies, the $CCL2^{-/-}$ mice gained more weight and had slightly worsened IR compared to controls (73, 74). Thus, although the published literature is mixed, there is more support for an absence of effect of CCL2 on macrophage recruitment to AT.

Because CCR2 is a receptor for several chemokines in addition to CCL2, and CCR2 deficiency results in a near absence of circulating $Ly6C^{hi}$ inflammatory monocyte precursors (75), it is plausible that CCR2 deficiency could have a greater impact than CCL2 deficiency on macrophage recruitment to AT. Weisberg *et al*. compared weight-matched $CCR2^{-/-}$ and wild type mice and found that $CCR2^{-/-}$ mice fed HFD for 24 weeks display reduced ATMs concomitant with lower fasting blood glucose and insulin levels as well as higher plasma adiponectin (72). This finding was reproduced by Sullivan *et al.* in mice fed HFD for 20 weeks (76) and by Lumeng *et al.* who detected reduced recruitment of ATMs to CLSs in $CCR2^{-/-}$ mice (49). The Hasty laboratory performed a time course study of HFD-feeding in $CCR2^{-/-}$ mice, and were only able to detect a reduction in ATMs after 20 weeks (77). Thus, the age of mice and time on HFD may be important to detecting effects of CCR2 deficiency.

Many other chemokines and their receptors have also been studied with regards to their role in macrophage recruitment to AT. Similar to the findings with CCL2 and CCR2, the results have been mixed. For example, CCR5 is the receptor for CCL3 (macrophage inflammatory 1α), CCL4 (macrophage inflammatory 1β), and CCL5 (RANTES). The Hasty laboratory has shown no effect of CCL3 or CCR5 deficiency on macrophage recruitment to AT (78, 79). However, Kitade *et al*. reported that CCR5 deficient mice have reduced ATM numbers and inflammatory gene expression (80), resulting in improved insulin sensitivity.

Lipids

As previously discussed, it is known that various fatty acids can alter the inflammatory potential of ATMs. Furthermore, ATMs in expanding AT form multinucleated syncytia filled with large lipid droplets (48) and increased expression of genes associated with lipid metabolism (49). In fact, lipolysis (pharmacologically-induced or through short-term fasting) is associated with increased macrophage recruitment to AT (62). The levels of FFAs in circulation are also positively associated with increased AT chemokine expression and lipid uptake by ATMs. Inversely, reduced lipolysis (through genetic or dietary means) leads to reduced accumulation of macrophages in AT. Furthermore, blocking lipolysis prevents macrophage influx into AT. These findings suggest that as ATMs accumulate lipids they take on a foam-like state so that they can function to buffer local increases in lipid concentrations.

The studies discussed above demonstrate that adipocyte cell death, the absence of a single chemokine or chemokine-like molecule, and lipids can substantially impact macrophage accumulation in AT. Furthermore, a large portion of these studies focus on chemokine-dependent mechanisms of recruitment of macrophages to AT. The inconsistencies from laboratory to laboratory in the case of chemokine-mediated recruitment of macrophages to AT suggests that chemokines have redundant roles and can compensate for one another. Ultimately, it appears that no single chemokine or factor is single-handedly responsible for the recruitment of circulating monocytes to AT. Although there is a large body of evidence suggesting a role of recruitment as the key mechanism for macrophage accrual, inhibition of recruitment does not attenuate the accumulation of macrophages in AT. This suggests that recruitment alone does not control this process. Therefore, recruitment-independent mechanisms may be the missing link in understanding how macrophages accumulate in obese AT.

Recruitment-Independent Pathways of Macrophage Accrual in AT

While recruitment of circulating macrophages to AT was the focus of many early experiments, several of these published studies unexpectedly revealed the likelihood of recruitment-independent mechanisms for ATM accrual. For example, in CCR2$^{-/-}$ and MGL1$^{-/-}$ mice, there are significantly lower levels of circulating Ly6C^{hi} monocytes (75, 81). If circulating Ly6C^{hi} monocytes are a primary driver of macrophage accrual in AT, it would be expected that the mice

would have a near absence of macrophage recruitment in obesity. Interestingly, HFD-fed MGL1$^{-/-}$ mice have only a 30% reduction CD11b^{+} macrophages and no significant differences in AT expression of F4/80 compared to controls (81). Furthermore, in CCR2$^{-/-}$ mice, a difference in number of ATMs is only detected after prolonged periods of HFD feeding (72, 77). If circulating Ly6C^{hi} monocytes are the cells recruited to AT in obesity, CCR2$^{-/-}$ mice would be expected to have dramatic reductions in ATMs even early after HFD-feeding. Thus, recruitment-independent mechanisms for macrophage accrual in AT have been the topic of recent publications. These studies demonstrate a role for proliferation, egress and apoptosis in driving ATM accumulation in obese AT (Figure 1.7).

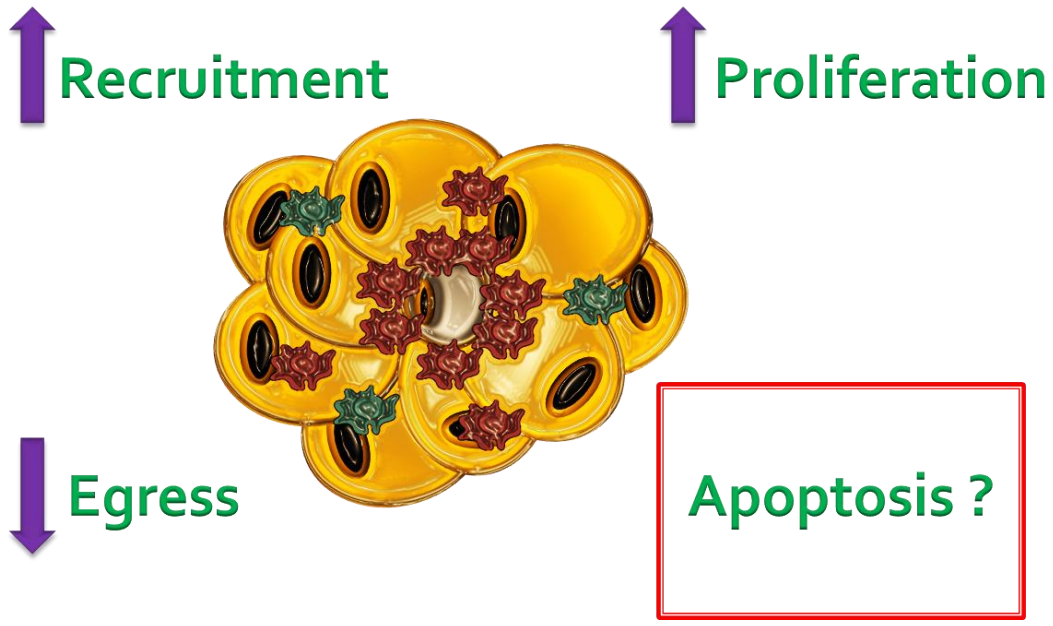

Figure 1.7. Mechanisms of Macrophage Accumulation in AT. Recruitment of macrophages plays a significant role in increased ATM content during obesity. In recent years the significant roles recruitment-independent mechanisms (proliferation, egress) have now been identified as major contributors to increase ATM number in obese AT. In Chapter III of this dissertation I will focus on the role of decreased ATM apoptosis in macrophage accumulation in obese AT.

Proliferation

Proliferation has only very recently been shown to contribute to increased ATM content in obese AT. Two groups published the novel finding that macrophages in obese AT proliferate at higher rates than those in lean AT (82, 83). Using Ki67 and EdU staining coupled with immunofluorescence and flow cytometric assays, Amano and colleagues demonstrated that 10-17% of ATMs are proliferating in obese ob/ob or diet-induced obese mice (82). This process is not impacted by the presence or absence of circulating monocytes and does not occur in other organs such as liver, spleen, or blood. CCL2/MCP-1 was determined to be a

likely AT-specific macrophage proliferation cue. It was also suggested that this increase in proliferation contributed to AT inflammation. A concurrent report by Hasse *et al*. similarly demonstrated that macrophages in AT proliferate at higher rates in obese compared to lean mice (83). In both studies, the proliferation was shown to occur mostly in the CLS-localized ATMs. However, Hasse *et al*. uniquely observed that the proliferating ATMs expressed markers of anti-inflammatory rather than pro-inflammatory polarization. With only two major studies addressing ATM proliferation, many questions remain regarding the contribution of proliferation to ATM numbers and will certainly be the topic of future investigation in the field.

Egress

Moore and colleagues have given the first insight into the role of retention in the accumulation of macrophage in obese AT (84). Their studies focus on the neuronal molecule Netrin-1 and its target receptor Unc5b. Activation of Unc5b by Netrin-1 results in chemorepulsive signaling that decreases cell migration out of tissues. Interestingly, they reported an increased expression of Netrin-1 and Unc5b in the AT of mice fed HFD compared to chow-fed controls. This expression was localized to CLSs in the AT. Interestingly, this localized expression was also seen in AT from obese humans. Importantly, they showed that hematopoietic Netrin-1 deficiency facilitates the ability of macrophages to emigrate from the AT. The model outlined by the Moore group suggests that Netrin-1 promotes defective ATM migration and accumulation in AT by blocking

chemokine induced migration. Overall, this study brings an innovative idea of involvement of egress signaling in ATM accumulation.

Apoptosis

Apoptosis is a common mechanism for cell turnover and the maintenance of homeostatic cell number in many tissues. The possibility of ATM apoptosis being important for maintenance of AT homeostasis is suggested by two lines of evidence. First, clodronate liposome-mediated depletion of ATMs from obese mice reduces AT inflammation and improves insulin sensitivity (85, 86). These data indicate that loss of inflammatory macrophages can improve AT function. In support of the relevance of this observation to humans, Kern and colleagues have shown that treatment of humans with the insulin sensitizing drug pioglitazone reduces the number of macrophages in AT via apoptotic mechanisms (87). Determining how the modulation of apoptosis regulates ATM number is the basis of the work performed in this dissertation.

Signaling Pathways Studied in this Dissertation

The balance between death and survival can be regulated by many pathways. Detailed below are the signaling pathways that are involved in regulating the intrinsic pathway of cell death and survival, which is the focus of the studies in my dissertation.

Apoptosis Signaling Pathway: Apoptosis (programmed cell death) is known to be a vital process involved in homeostatic maintenance of cell number in many tissues. Alterations in apoptotic signaling pathways have been linked to the development of cancer, autoimmune, neurodegenerative and many other diseases (88). Signaling through two pathways, the extrinsic and intrinsic pathways, largely controls activation of apoptosis. These pathways induce apoptosis in two distinct but overlapping mechanisms (Figure 1.8). The extrinsic pathway involves signaling through death receptor, FasL or TnfR, resulting in caspase 8 activation. Intrinsic activation of apoptosis is largely induced by cellular stress and mitochondrial outer membrane permeabilization (MOMP). Both signaling pathways converge on the activation of the executor caspase, caspase 3. Caspase 3 cleavage leads to a series of biochemical reactions in the cell such as protein cleavage, DNA damage and changes in cellular morphology that allows it to be phagocytized and removed from the tissue site (89). The regulation of apoptosis in ATMs in the lean and obese state will be explored in Chapter III.

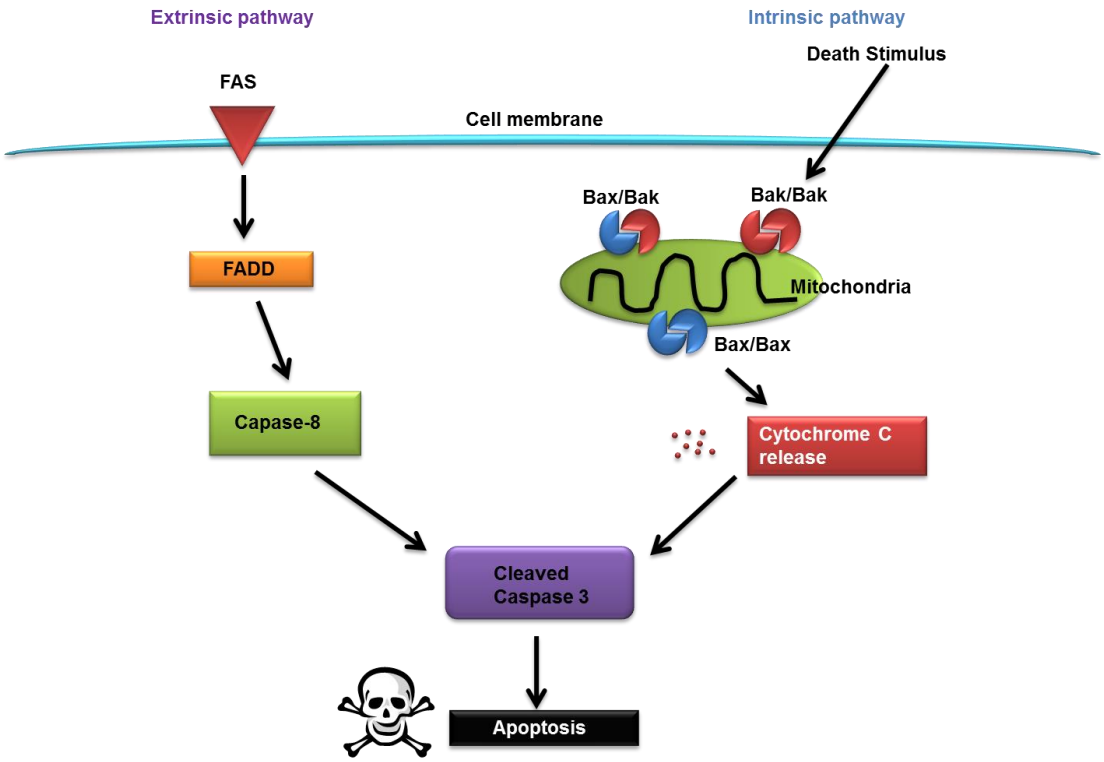

Figure 1.8. Apoptosis signaling pathway. Activation of apoptosis occurs primarily through two signaling pathways, extrinsic and intrinsic, which ultimately converge at the activation of cleaved caspase 3. The extrinsic pathway signaling is activated through of the death receptor (FasL) whereas intrinsic signaling is activated as a result of cellular stress. The intrinsic pathway is the major focus of the work presented in this dissertation.

Bcl-2 Family Cell Survival/Apoptosis Regulatory Pathway: The Bcl-2 gene was first discovered in B-cell follicular lymphomas and subsequently linked to increased survival of cancer cells [reviewed in (90)]. Since its discovery, a series of proteins sharing similar BH3 domain homology have been identified. Bcl-2 family members have been grouped into two major classes: pro-apoptotic (Bax, Bak) and pro-survival (Bcl-2 and Bcl-xl). These proteins play a significant role in the balance of cell survival and apoptosis through the disruption or maintenance of the mitochondrial outer membrane (Figure 1.9). Pro-apoptotic Bax and Bak promote cellular apoptosis by oligomerizing at the outer membrane of the mitochondria, inducing pore formation and the release of cytochrome c. Cytochrome c release in to the cytosol initiates the formation of the apoptosome, ultimately leading to the activation of cleaved caspase 3 and apoptosis. The pro-survival Bcl-2 and Bcl-xl proteins antagonize MOMP by preventing Bax and Bak oligomerization. Although the mechanism in not completely clear, Bcl-2 and Bcl-xl are thought to bind to the BH3-only domains of Bax and Bak sequestering the proteins away from each other [reviewed in (91)]. The balance of cell death and cell survival through MOMP can be largely associated with the relative ratio of proteins levels and mitochondrial localization of Bax and Bcl-2. The pro-survival Bcl-2 proteins have been generally associated with increasing cell survival under pathological conditions [reviewed in (92, 93)]. Studies performed in this dissertation will explore the differential modulation of pro-survival and pro-apoptotic proteins in ATMs of lean and obese mice.

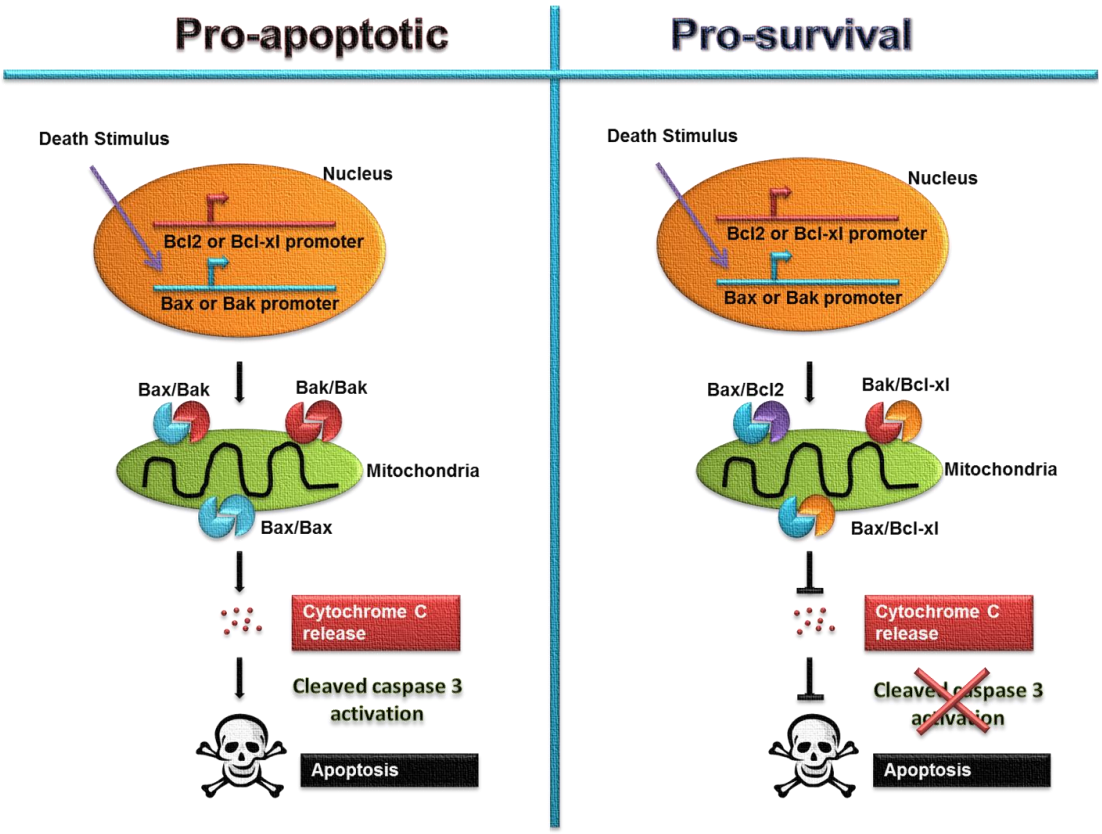

Figure 1.9. The Bcl-2 family signaling pathway. The Bcl-2 family members regulate cellular apoptosis and survival through the disrupting or maintaining the integrity of the mitochondrial outer membrane. Pro-apoptotic proteins, Bax and Bak, induce pore-formation in the outer membrane of the mitochondria. This results in cytochrome c release, cleaved caspase 3 activation and apoptosis. Bcl-2 and Bcl-xl oppose the activities of Bax and Bak, promoting cell survival.

Inhibitors of Apoptosis Regulatory Pathway: Inhibitors of Apoptosis (IAPs) belong to a family of proteins that are involved in the inhibition of apoptosis (Figure 1.10). They provide protection from a variety of apoptotic stimuli. Although first discovered in baculoviruses, various homologs of this protein family have also been discovered in mammalian cells [reviewed in (94)]. The ability of IAPs to suppress apoptosis is primarily through direct inhibition of pro-caspase and caspase. Their activities are thought to be redundant and have been demonstrated to inhibit activated caspase 3 and 7 activity [reviewed in (94)]. However, XIAP is thought to be the most powerful regulator of the IAP family due to its ability to directly inhibit caspase activity (95). In addition, IAPs demonstrate the ability to suppress apoptosis through non-caspase inhibitory mechanisms involving their transcriptional activator NF-κB. Interestingly, XIAP has been demonstrated to directly promote NF-κB signaling by promoting the degradation of the NF-κB inhibitor, IκBα, allowing for NF-κB nuclear translocation (96). Furthermore, IAPs play a significant role in inhibiting cell death through the modulation of cell cycle progression and cell division. With importance to the studies performed in this dissertation, studies have demonstrated that IAPs play a significant role in increasing macrophage survival under pathological conditions (97-99).

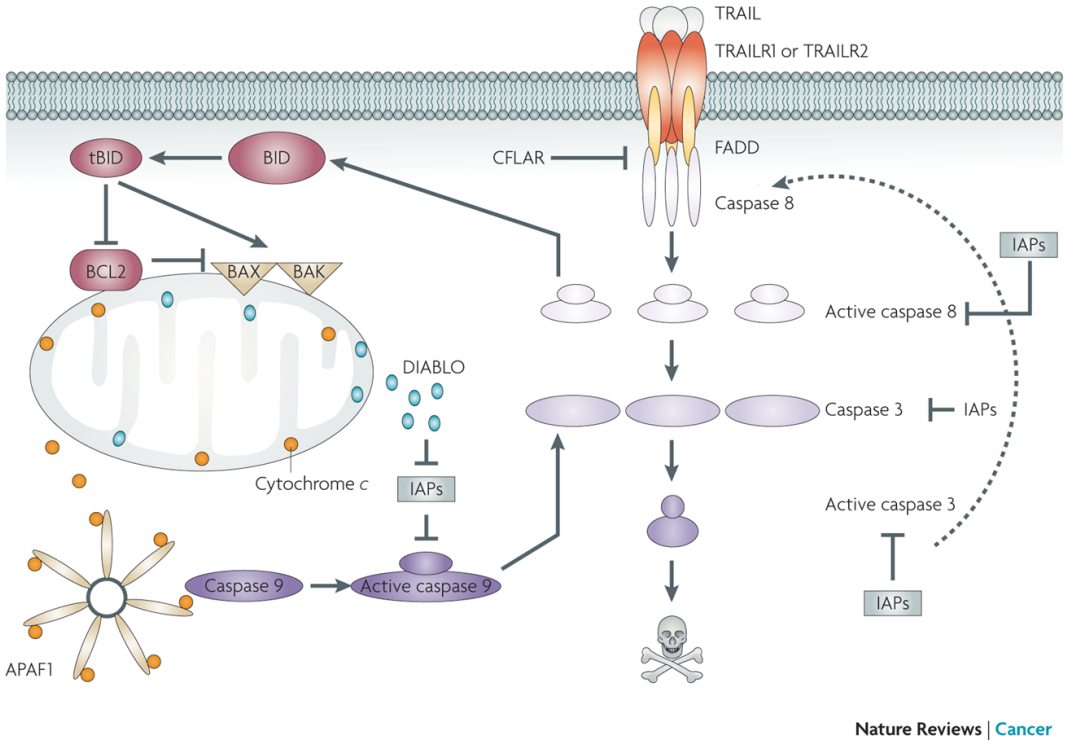

Figure 1.10. The Inhibitors of Apoptosis (IAPs) family signaling pathway. From Johnstone *et al.* Nature Reviews Cancer. 2008: 782-798. The IAP family of proteins regulate apoptosis through the inhibition of caspases. The most prominent IAP, XIAP, has been known to directly inhibit the activity of active cleaved-caspase 3. Additionally, other IAPs have also been shown to inhibit caspase7, 8 and 9. Furthermore, IAPs also been shown to promote the activation of NF-κB.

NF-κB Signaling Pathway in Cell Survival: Nuclear factor kappa-light-chain-enhancer of B cells (NF-κB) was discovered almost 30 years ago and has been demonstrated to be involved in many pathological pathways (100, 101). The NF-κB complex consists of several proteins (RelA (p65), RelB, c-rel, NF-κB1 and NF-κB2) that form various homo and hetero-dimers involved in gene transcription (102). NF-κB is largely known to induce the transcription of many inflammatory genes; however, its regulatory control spans various genes, including pro-survival factors (Figure 1.11). Activation of NF-κB is regulated through the canonical and alternative pathways. Exposure of cells to cytokines, antigens and TLR ligands activate the canonical signaling pathway. Canonical signaling leads to the activation of IκB kinases (IKKs) that phosphorylate the NF-κB inhibitor, IκBα, resulting in its ubiquitination and its degradation by the proteasome. After IκBα degradation, NF-κB (p65) translocates to the nucleus to induce the transcription of genes related to innate immunity and cell survival [reviewed in (103)]. The alternative signaling pathway activated by the TNF ligand and receptor family, results in activation of NF-κB inducing kinase (NIK) and IKKα. IKKα activity results in the phosphorylation and proteolytic processing of NF-κB1 and NF-κB2 leading to the induction of genes related to the adaptive immune system [reviewed in (103)]. NF-κB activity has been demonstrated to play a major role in the inflammatory nature of macrophages in AT (67); however, its mediation of signaling pathways beyond inflammatory pathways in ATMs has yet to be elucidated. Chapter III of this dissertation will focus on the role of its pro-survival signaling arm in ATM survival during obesity.

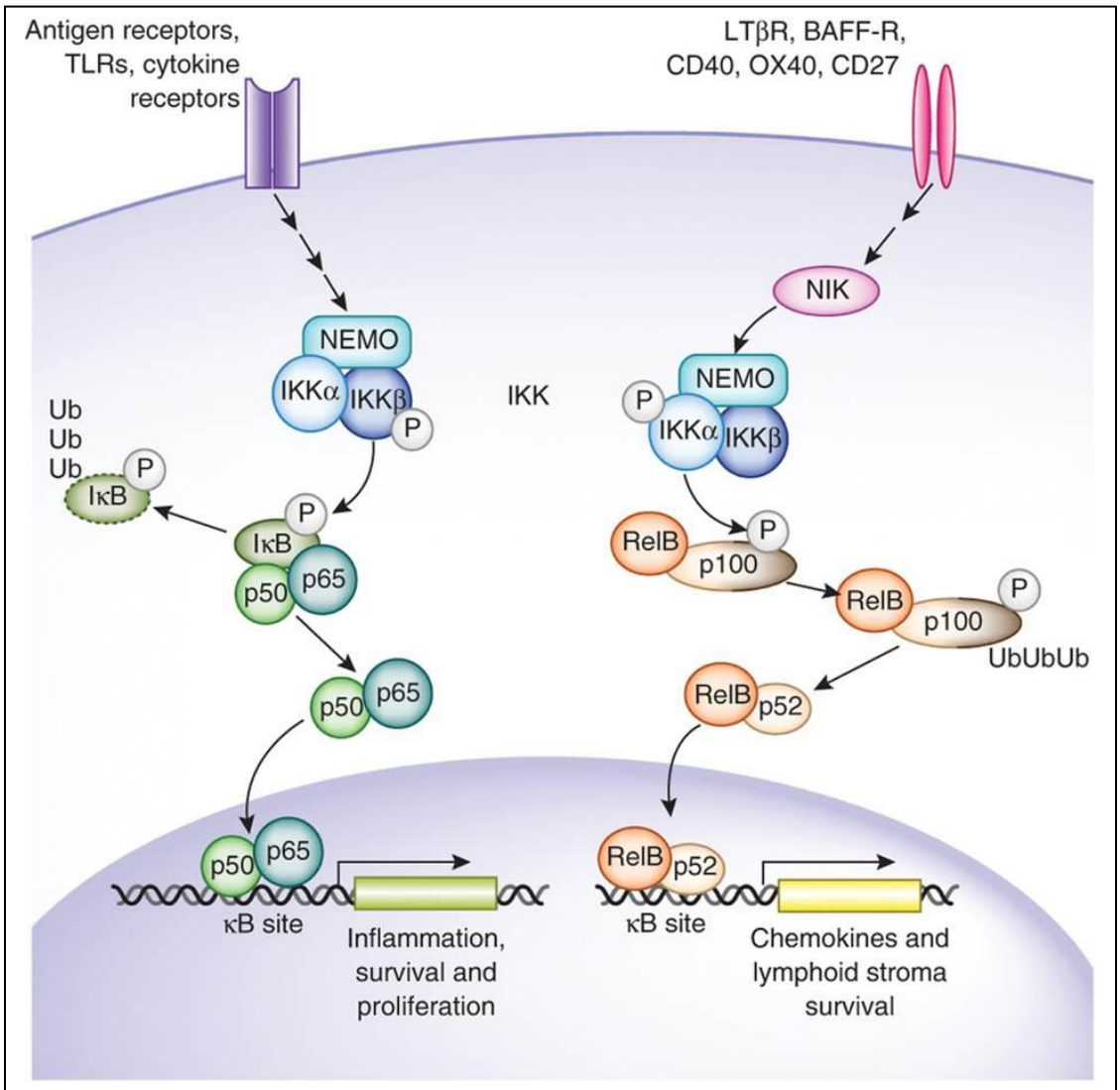

Figure 1.11. NF-κB signaling pathway. From Gerondakis *et al*. Nature Immunology. 2013: 15-25. NF-κB is a transcription factor involved in the regulation of many genes. It is comprised of various subunits (RelA (p65), RelB, c-rel, NF-κB1 and NF-κB2) that form homo and hetero-dimers. It is activated through two pathways, classical and alternative, that induce a series of events that lead to NF-κB nuclear translocation and gene transcription. The classical pathway, involving p65, results in the expression of genes involved in inflammation, survival, and proliferation. The classical activation will be focused on in Chapter III of this dissertation.

Significance

It is apparent that the increase in pro-inflammatory macrophage content in obese AT plays a major role in the progression of AT inflammation and dysfunction. Although recruitment-dependent mechanisms of ATM accrual in AT have been most well studied, it is becoming clear that they may not be the sole mechanism involved in this process. In the past couple of years, Immunometabolism investigators have expanded their thinking and hypotheses to embrace the idea that recruitment-independent mechanisms may also play a role in macrophage accrual in AT during obesity. In light of the recent studies elucidating the roles of proliferation and egress as contributing factors, ATM apoptosis or survival are likely to be contributing factors to increased pro-inflammatory ATM content in obesity. In Chapter III, I detail my work showing that ATM apoptosis is repressed in obesity through NF-κB-dependent mechanisms allowing for increased ATM survival and contributing to ATM accrual in during obesity. Overall, the studies performed in my dissertation have expanded the understanding of mechanisms that regulate ATM number under normal and metabolic conditions. These findings could promote the development of novel therapies that target multiple signaling pathways to reduce ATM content and AT inflammation, thus decreasing the metabolic pathology of obesity.

CHAPTER II

MATERIALS AND METHODS

Animal Usage and Phenotyping

All animal procedures were performed with prior approval from the Institutional Animal Care and Usage Committee of Vanderbilt University. Male C57Bl/6 mice were purchased from Jackson Laboratories. At 8-weeks of age, mice were placed on diets containing either 10% (low fat diet, LFD; Research Diets #D12450B) or 60% (high fat diet, HFD; Research Diets #D12492) of kcal from fat. The diets are protein and micronutrient-matched, providing equivalent quantities of vitamins and minerals. Ob/ob mice (stock number 000632) and lean littermate controls were purchased from Jackson Laboratories at 7 weeks of age and maintained on standard chow diet (LabDiet 5001) until 9-10 weeks of age. NF-κB-GFP-Luciferase (NGL) mice ubiquitously express an enhanced GFP (EGFP)/luciferase gene that is controlled by an enhanced promoter containing two NF-κB binding sites (104). All mice were given free access to food and water. When indicated, total fat and lean mass were quantified by nuclear magnetic resonance using a Bruker Minispec instrument (Woodlands, TX) in the Vanderbilt Mouse Metabolic Phenotyping Center. Mice were fasted for 5 h before tail vein collection of blood for the determination of glucose levels using a LifeScan One Touch Ultra glucometer (Johnson & Johnson, Northridge, CA).

Tissue Cell Isolations

Isolation of AT Stromal Vascular Fraction (SVF)

Mice were euthanized and perfused through the left ventricle with 20 mL of PBS. Epididymal AT was removed and 0.25-0.5 g of tissue was minced in 3 mL PBS with 0.5% FBS (FBS/PBS). Subsequently, 3 mL of 2 mg/mL collagenase II (Sigma-Aldrich, St. Louis, MO) was added to achieve a final concentration of 1 mg/mL. Tissue was incubated at 37 °C for 20-30 min while shaking at 200 RPM. The cell suspension was then filtered through a 100 µM cell strainer. Cells were spun at 500 x g for 10 min to separate adipocytes from the SVF. The SVF was re-suspended in 3 mL ACK buffer to lyse red blood cells. Cells were washed 2X with PBS, then lysed for Western blot or real-time RT-PCR analysis, or counted using a Cellometer Auto T4 and plated to select for macrophages based upon their strong adhesive properties (see Section 2.2.3 below).

Isolation of Hepatocyte and F4/80-enriched Fractions from the Liver

Mice were euthanized and perfused as described above. The liver was removed and minced in 3 mL RPMI with 5% FBS. Next, 3 mL of 2 mg/mL collagenase II was added and tissue was incubated at 37 °C for 30 min while shaking at 200 RPM. The cell suspension was filtered through a 100 µM cell strainer and spun at 300 RPM for 3 min. The hepatocyte fraction (pellet) was collected for Western blot analysis, while the supernatant (non-parenchymal fraction) was spun at 1500 RPM for 10 min. Cells were then re-suspended in a 33% normo-osmotic Percol solution containing 10 U/mL heparin and spun at 500 x g for 15 min.

Subsequently, cells were washed and incubated with Fc block for 10 min and then stained with anti-mouse F4/80-APC (eBioscience, San Diego, CA) at a concentration of 5 x 10^6 cells/mL. Cells were incubated with anti-APC magnetic beads for 15 min at 4 °C, washed, re-suspended in FACS buffer, and sorted using a Miltenyi AutoMACs magnetic cell sorter. The F4/80-enriched fraction was collected for Western blot analysis.

ATM Selection by Adherence

Isolated SVF cells (Chapter III) were plated in 5% DMEM for 2 h in tissue culture dishes with well sizes specific to the subsequent application purpose. The plate was then washed 2X with PBS, leaving any adherent ATMs attached and eliminating all other cells. Attached cells were verified as macrophages based upon positive immunostaining for F4/80 (86.88% ± 0.77% from LFD mice and 87.74% ± 0.72% from HFD mice, quantified from 10 images/group). ATMs were used for the following assays: 1) fixed for immunofluorescence staining (Figures 3.9-3.10 and 3.12), 2) Real-time RT-PCR (Figures 3.10 and 3.12), or 3) metabolic cocktail studies (Figure 3.12).

Western Blot Analysis

SVF cells and the F4/80-enriched fraction isolated from the liver were collected in lysis buffer containing 20 mM Tris-HCL (pH 8.0), 150 nM NaCl, 1 mM EDTA, 1 mM EGTA, 0.1 % Nonidet P-40, 2.5 mM sodium pyrophosphate, 1 mM sodium orthovanadate, and 0.5 mM PMSF. A modified Lowry protocol was used to

quantify protein concentration. Whole AT, hepatocytes, and spleen were sonicated in 500-700 µL of 2% SDS containing 2.5 mM sodium pyrophosphate and 0.5 mM PMSF. Protein was quantified using a bicinchoninic acid (BCA) assay (Thermo Scientific, Waltham, MA). Subsequently, 10-15 µg of protein was electrophoresed through 4-12% Bis-Tris gels (Invitrogen, Grand Island, NY), transferred to a nitrocellulose membrane, and immunoblotted with the following antibodies: cleaved caspase-3, Bax, Bak, Bcl-2, Bcl-xl and phospho-p65. All antibodies were obtained from Cell Signaling Technology (Boston, MA). Blots were developed using either Western Lightning enhanced chemiluminescence substrate and film (Perkin Elmer, Waltham, MA) followed by band intensity quantification using ImageJ64 software, or were imaged using Odyssey Blocking Buffer and the Li-Cor Odyssey Infrared Imaging System (Li-Cor, Lincoln, NE) followed by band intensity quantification using Image Studio Lite Version 3.1 software. A list of the antibodies, company, product number and concentrations are detailed in **Table 1** below**.**

Gene Expression by real-time RT-PCR

SVF cells were collected in TRIzol reagent (Invitrogen, Carlsbad, CA). Total RNA was isolated using a phenol-chloroform extraction, according to the manufacturer's instructions. An iScript cDNA synthesis kit (BioRad, Hercules, CA) was used for reverse transcriptase reactions. Real-time RT-PCR analysis was performed using an iQ5 multicolor real-time PCR detection system (Bio-Rad). Primer-probe sets (Assays-on-Demand) were purchased from Applied

Biosystems (Foster City, CA). All genes were analyzed using the Pfaffl method (105) and normalized to *Rplp0*. The expression of the following genes was assessed: *Emr1* (Mm00802530_m1), *Rplp0* (Mm00725448_s1), *Bax* (Mm00432051_m1), *Bak1* (Mm00432045_m1), *Bcl2* (Mm00477631_m1), *Bcl2l1* (Mm00437783_m1), *Tnf* (Mm00443258_m1), *Xiap* (Mm01311594_mH) and *Birc3* (Mm01168413_m1), *Abca1* (Mm00442646_m1), and *Plin2* (Mm00475794_m1).

Immunofluorescence Microscopy and Analysis

Confocal Staining of Whole AT for TUNEL^{+} Macrophages

PBS perfused epididymal AT was harvested and immediately fixed in 1% paraformaldehyde for 1 h. Tissue was blocked in 5% goat serum in PBS for 1 h and stained with a rat anti-mouse F4/80 antibody (Abcam, Cambridge, MA) overnight at 4 °C. After washing with PBS, tissue was incubated with an Alexa 488-conjugated anti-rat secondary antibody (Cell Signaling Technology) for 1 h at RT. TUNEL staining was performed using the In Situ Cell Death Detection Kit (Roche-Applied Science, Indianapolis, IN), according to manufacturer's instructions. Tissue was then counter-stained with DAPI (0.2 mg/mL) and imaged at 40X magnification using an Olympus FV-1000 Inverted Confocal Microscope. In order to avoid endogenous tissue autofluorescence, tissues were first imaged under the DAPI filter. There was no apparent pattern to which areas of AT displayed autofluorescence. CLSs were determined by eye as a small adipocyte surrounded by macrophages as reported by other groups (106-108). All other macrophages were considered interstitially spaced macrophages. Areas with no

autofluorescence were then selected for imaging. At least 3 images were captured from 4-7 mice per group.

Automated and Confocal Imaging for Nuclear and Mitochondrial Co-localization

The Image Xpress Automated Micro XL Microscope with Meta Xpress analysis software in the High-Throughput Screening Core at Vanderbilt University was used for these studies. SVF was collected and ATMs were adherence-selected in a 96-well plate, as described above. Adherent ATMs were then fixed with 4% PFA for 1 h. ATMs were stained with antibodies against F4/80 and Bax, Bcl-2, or p65 (Cell Signaling Technology) in order to determine co-localization with nuclear (DAPI) and mitochondrial (Cox IV, Abcam, Cambridge, MA) markers. Images were acquired from 4 areas per well at 40X magnification on the Image Xpress Automated Micro XL Microscope. An analysis software module was developed to allow for quantification of the overlap of the fluorescence signal of a specific protein with a defined organelle compartment of interest (nucleus or mitochondria). Analysis parameters were set to identify macrophages ($F4/80^{+}$) with intact nuclei (DAPI positive, diameter of 2-8 μm) and mitochondria (Cox IV, diameter of 1-3 μm). Co-localization data was collected from 10,000- 30,000 ATMs per mouse from 6-7 mice per group. For statistical purposes the average co-localization from all the macrophages of an individual mouse were counted as a single biological replicate. To obtain higher quality images for the purpose of visualization and confirmation of these computed changes, the representative

images displayed in Figures 3.9 and 3.10 were performed using an Olympus FV-1000 Inverted Confocal Microscope at 100X or 60X magnification with a 1.5 or 4.5 zoom. All images were taken at the same magnification, voltage, and gain level required for proper imaging in each channel. To perform these studies, ATMs were plated in 8 well chamber slides for 2 h to allow for selection by macrophage adherence. ATMs were then fixed for 1 h with 4% PFA, and stained for DAPI, p65, Bax and Bcl-2 as described. Mitochondria were stained using MitoTracker Deep Red FM (Life Technologies, Grand Island, NY) at 100 nM for 25 min. A list of the antibodies, company, product number and concentration are detailed in **Table 1** below**.**

Ex vivo Studies in Isolated ATMs

NF-κB-regulated Luciferase Reporter Assay

ATMs were collected from NGL mice by SVF isolation and macrophage selection by adherence, as described above. ATMs were washed once with PBS followed by the addition of 20 µL of luciferase lysis buffer (Promega, Madison, WI). Luciferase substrate was added to the sample and luminescence was immediately read on a Monolight 3010 (BD PharMingen, San Diego, CA).

Metabolic Activation of ATMs

ATMs were treated with a metabolic cocktail (MetaC) containing 30 mM glucose, 10 nM insulin and 0.4 mM palmitic acid as previously described (53). Palmitic acid was dissolved in ethanol and added to DMEM containing 5% FBS, 30 mM

glucose and 10 nM insulin. ATMs were treated with the MetaC in the presence or absence of 10 μM BMS-345541 (Sigma-Aldrich) to inhibit NF-κB. ATMs in the BMS treatment groups were pretreated with 20 μM BMS-345541 for 1 h prior to time-course studies. ATMs were exposed to MetaC for a time-course of 0-8 h.

Cell-Titer Blue Assay

ATMs were adherence-selected and plated in 96-well plates. Metabolic activation cocktail studies were performed as described in Section 2.6.2. Cell-Titer Blue reagent (Promega) was added at a volume of 20 μL to wells containing 100 μL of media 2 h prior to end of each time-point. Fluorescence was measured at $560_{Ex}/521_{Em}$ using the GloMax Discover System (Promega). Background fluorescence was measured in wells containing media and Cell-Titer Blue only (*i.e*. without cells) and was subtracted from each experimental measurement.

Statistical Analysis

GraphPad Prism 5.0 software was used for all statistical analyses. Data was analyzed using a two-tailed unpaired *t*-test to determine differences between two groups or a one-way ANOVA when more than two treatment groups were compared. Outliers were excluded from the data for each individual parameter using the Grubbs outlier test (109). A p value of <0.05 was considered significant.

Table 1

Western blot antibodies			
Antibody	**Company**	**Product #**	**Concentration**
Bax	Cell signaling	2772s	1:100
Bak	Cell signaling	12105s	1:100
Bcl-2	Cell signaling	3498s	1:100
Bcl-xl	Cell signaling	2764s	1:100
Cleaved Caspase 3	Cell signaling	9664s	1:50
Phosp-p65	Cell signaling	3033s	1:100
Immunofluorescence antibodies			
Antibody	**Company**	**Product #**	**Concentration**
Bax	Abcam	Ab5174	1:100
Bcl-2	Abcam	Ab692	1:100
CoxIV	Abcam	Ab16056	1:100
Dapi	BD Bioscience	564907	1:2000
F4/80	Abcam	Ab6640	1:100
MitoTracker	Life Technologies	M22426	100 nM
p65	Cell signaling	8242	1:200

CHAPTER III

ACTIVATION OF NF-κB DRIVES THE ENHANCED SURVIVAL OF ADIPOSE TISSUE MACROPHAGES IN AN OBESOGENIC ENVIRONMENT

INTRODUCTION

Portions of this Introduction have been published in my manuscript titled *Activation of NF-κB drives the enhanced survival of adipose tissue macrophages in an obesogenic environment* written by Hill, Anderson-Baucum, Webb, Kennedy, Yull and Hasty, Molecular Metabolism, 2015.

In 2003, two seminal papers demonstrated that macrophages accumulate in adipose tissue (AT) during obesity (31, 32). AT macrophage (ATM) number positively correlates with adiposity, systemic inflammation, and insulin resistance (IR), suggesting that these immune cells play an essential role in the pathogenesis of obesity. Recent findings also demonstrate a role for other immune cell subsets, including T cells (110-113), B cells (114), eosinophils (115), and neutrophils (116) in the control of AT inflammation. However, in mice, macrophages are the most prevalent immune cell type in AT and are a major source of inflammatory cytokines and chemokines secreted from AT during obesity (31, 32). This heightened immune response changes the types and amounts of lipids and adipokines released from AT, which can then negatively impact other tissues and promote metabolic disease (117). In fact, increased AT inflammation is now considered one of the primary drivers of IR associated with

obesity (reviewed in (21)). Thus, the immune system is now at the forefront of metabolic research, and extensive efforts have focused on determining mechanisms by which macrophages accumulate in obese AT.

Obesity increases expression of numerous chemokines and chemokine receptors in AT (118). Furthermore, labeling studies have shown that obesity results in recruitment of monocytes from the bone marrow into AT (31, 49). Therefore, to date, the majority of published studies have sought to determine whether reducing the chemoattractant potential of AT can inhibit ATM accumulation during obesity. However, in many instances, obese mice genetically lacking certain chemokines or chemokine receptors exhibit no change in ATM number and no improvement in metabolic abnormalities (73, 74, 119-121). Additionally, even in studies showing that deficiency or antagonism of chemokines decreases ATM number during obesity, macrophage accumulation during high fat diet (HFD) feeding is never completely abolished (14, 49, 72, 122-124). Furthermore, several models with deficiencies in chemoattractant molecules demonstrate a pronounced decrease in circulating inflammatory monocytes without a corresponding large reduction in ATM number (72, 81). Together these findings suggest that recruitment-independent mechanisms may also contribute to the accumulation of pro-inflammatory macrophages in obese AT. Indeed, recently published studies have highlighted that alterations in macrophage proliferation (82, 83) and egress (84) contribute to the increased number of ATMs in obesity. In addition to macrophage recruitment, proliferation,

and egress, modification of cell survival/death pathways are another mechanism by which tissue cell number could be modulated.

Regulation of cell survival through the proper control of programmed cell death (apoptosis) is essential for homeostatic maintenance of cell number in many tissues [reviewed in (125)]. For example, accelerated apoptosis is observed in neurodegenerative disorders, while impaired apoptosis can contribute to tumorigenesis, autoimmunity, and inflammatory disorders [reviewed in (89)]. Interestingly, it is not known whether macrophage apoptosis/survival is modulated in AT during obesity.

The control of cell survival is intricately balanced by the activation of pro-apoptotic and pro-survival signaling pathways. Apoptosis is initiated by either intrinsic or extrinsic pathways, both of which proximally activate the caspase cascade [reviewed in (89)]. To oppose apoptosis, cells can activate pro-survival pathways. Much of the balance between death and survival in a cell is controlled via transcriptional and post-transcriptional regulation of vital factors that maintain mitochondrial outer membrane integrity (90). The transcription factor, NF-κB, is a key regulator of pro-survival factors such as the Bcl-2 family and inhibitors of apoptosis proteins (IAPs). These proteins are important in preventing caspase-induced cell death, thus allowing for increased survival in many cell types. NF-κB-induced progression of multiple diseases through promotion of cell survival has been well documented (102, 126). Of relevance, a previous study has demonstrated increased nuclear translocation of the p65 subunit of NF-κB in ATMs of obese compared to lean mice (67).

Our findings presented below demonstrate that activation of NF-κB in ATMs during obesity may promote cell survival. Therefore, NF-κB-dependent modulation of the balance between cell survival and death may be an additional mechanism – along with recruitment, proliferation, and retention – that promotes macrophage accumulation in AT during obesity.

RESULTS

Diet-induced Obesity Decreases ATM Apoptosis

To determine the impact of obesity on ATM apoptosis and survival, mice were fed 10% LFD or 60% HFD for 9 weeks (Figure 3.1A). As expected, mice fed HFD became obese, gained lean and fat mass, and were hyperglycemic compared to LFD-fed controls (Figure 3.1B-E). Additionally, expression of *Emr1* (the gene for F4/80) in the stromal vascular fraction (SVF) of AT was significantly increased by obesity ($p<0.05$, Figure 3.1F), confirming that 9 weeks of HFD feeding is sufficient to promote the accumulation of macrophages in AT.

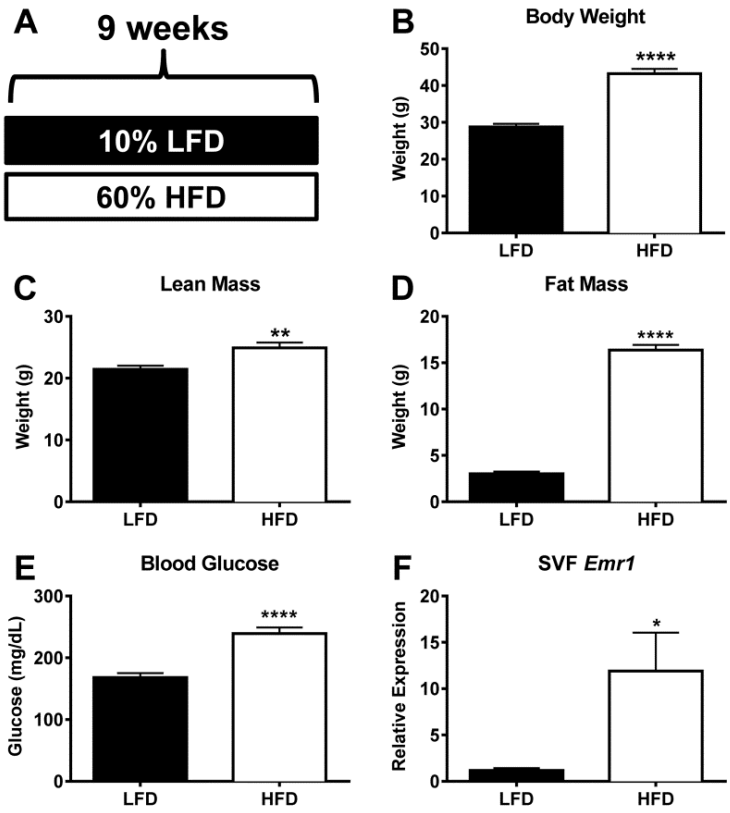

Figure 3.1: Metabolic phenotype of lean and obese mice. A) Study design: male C57Bl/6 mice were placed on a 10% low fat diet (LFD) or 60% high fat diet (HFD) for 9 weeks. B-E) Metabolic parameters were assessed at sacrifice: B) body weight, C) lean mass, D) fat mass, and E) fasting blood glucose concentrations. F) Real-time RT-PCR quantification of *Emr1* (F4/80) gene expression in the SVF of the AT normalized to *Rplpo*. Data are presented as mean ± SEM, B) n = 12/ group, C-E) n = 7-12/group, F) n = 7/group. * p<0.05, ** p<0.01, **** p<0.0001 between groups.

Recent studies suggest that obesity increases apoptosis in whole AT, likely due to adipocyte cell death resulting from local hypoxia and/or decreased vasculature (106, 108, 127, 128). Consistent with these findings, HFD feeding increased expression of the pro-apoptotic proteins Bax (p<0.001) and Bak (p<0.01) in AT, although it did not affect caspase-3 cleavage (Figure 3.2). These data support the concept that obesity increases apoptosis in whole AT. However,

the metabolic regulation of apoptosis in ATMs during obesity has not been explored.

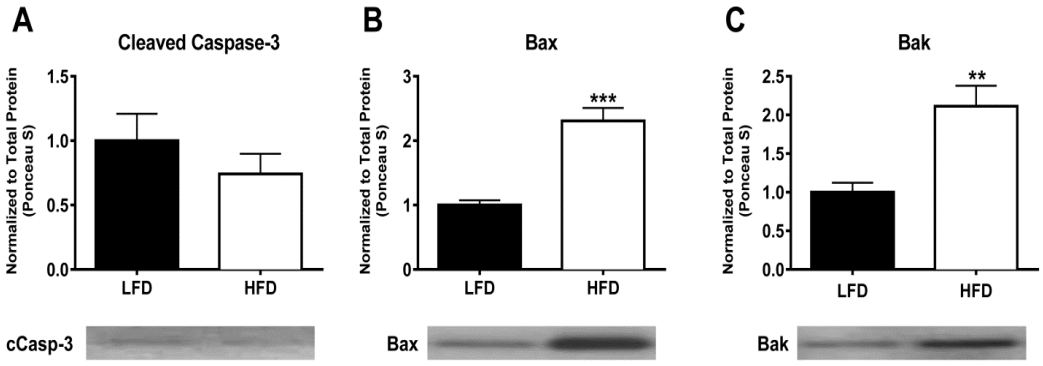

Figure 3.2: Obesity increases apoptotic markers in whole AT. Male C57Bl/6 mice were placed on a LFD or HFD for 9 weeks. A-C) Whole AT was sonicated in 2% SDS and apoptotic markers were analyzed using Western blot: A) cleaved caspase-3, B) Bax, and C) Bak. Levels of specific proteins were normalized to total protein level, as measured by Ponceau S staining. Data are presented as mean ± SEM, n = 5/group. ** p <0.01, *** p<0.001 between groups.

To determine if obesity alters apoptotic signaling in AT immune cells, protein was isolated from the SVF of AT and assessed for apoptotic markers by Western blot analysis. Remarkably, HFD-fed mice demonstrated a 50% decrease in SVF cleaved caspase-3 protein levels compared to LFD-fed controls, suggesting decreased AT immune cell apoptosis during obesity ($p<0.001$, Figure 3.3A). Although the SVF is a macrophage-enriched cell preparation, other leukocytes and pre-adipocytes are also contained within this fraction. To determine if obesity decreases apoptosis specifically in macrophages, AT was stained for F4/80, DAPI, and the apoptosis marker TUNEL. In both LFD- and

HFD-fed mice, around 10-20% of cells with TUNEL$^+$ nuclei were F4/80 negative, and about 80-90% of the apoptotic cells were macrophages, indicating that macrophages are the major cell type undergoing apoptosis in the AT (data not shown). Interestingly, quantification of confocal images demonstrated that ~17% of the macrophages in lean AT were TUNEL$^+$ (apoptotic), while only ~4% of macrophages in obese AT were TUNEL$^+$ ($p<0.0001$, Figure 3.3B-C). This decrease in apoptotic ATMs was also detected when quantified as number per high power field ($p<0.01$; Figure 3.3D). As expected because almost all ATMs in lean AT are interstitially spaced, the apoptotic ATMs in lean mice were also interstitially spaced (Figure 3.3E). Even in obese AT, about 50% of the apoptotic ATMs were localized to interstitial spaces.

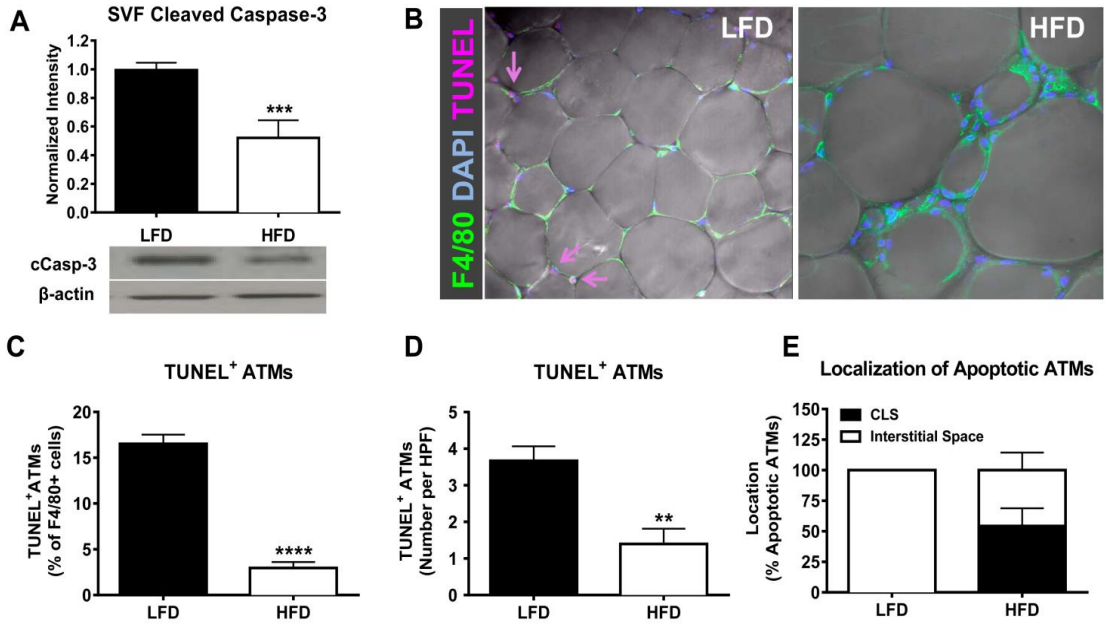

Figure 3.3: HFD feeding decreases apoptosis of ATMs. Male C57Bl/6 mice were placed on LFD or HFD for 9 weeks. A) SVF was collected and cleaved caspase-3 was analyzed using Western blot. B) AT explants were collected and analyzed by confocal staining for the macrophage marker F4/80 (green), nuclear stain DAPI (blue), and apoptosis marker TUNEL (pink). Magnification: 40X. C-D) Quantification of TUNEL positive ATMs by percent of F4/80 positive cells (C) or by number per high-power field (D). E) Quantification of localization of apoptotic ATMs. Data are presented as mean ± SEM, A) n = 11-14/group, C-D) n = 4-8/group for confocal imaging.
** p <0.01, *** p <0.001, **** p <0.0001 between groups.

To determine if the degree of obesity altered ATM apoptosis, mice fed HFD for an extended time period of 16 weeks were analyzed. These mice displayed an even further 75% decrease in cleaved caspase-3 levels in the SVF ($p<0.0001$), and a decrease in TUNEL positive ATMs ($p<0.05$) compared to mice on LFD for 16 weeks (Figure 3.4A-B). Interestingly, when the data from mice fed LFD or HFD for either 9 or 16 weeks were combined (Figure 3.4C), a clear negative correlation between body weight and the level of cleaved caspase-3 in SVF was found ($r^2 = 0.48$, $p<0.0001$). Furthermore, even when only HFD-fed

mice (9 and 16 weeks HFD) were included in the analysis (Figure 3.4D), SVF cleaved caspase-3 levels remained negatively correlated with body weight (r^2 = 0.24, $p<0.05$), suggesting that obesity drives the decrease in immune cell apoptosis.

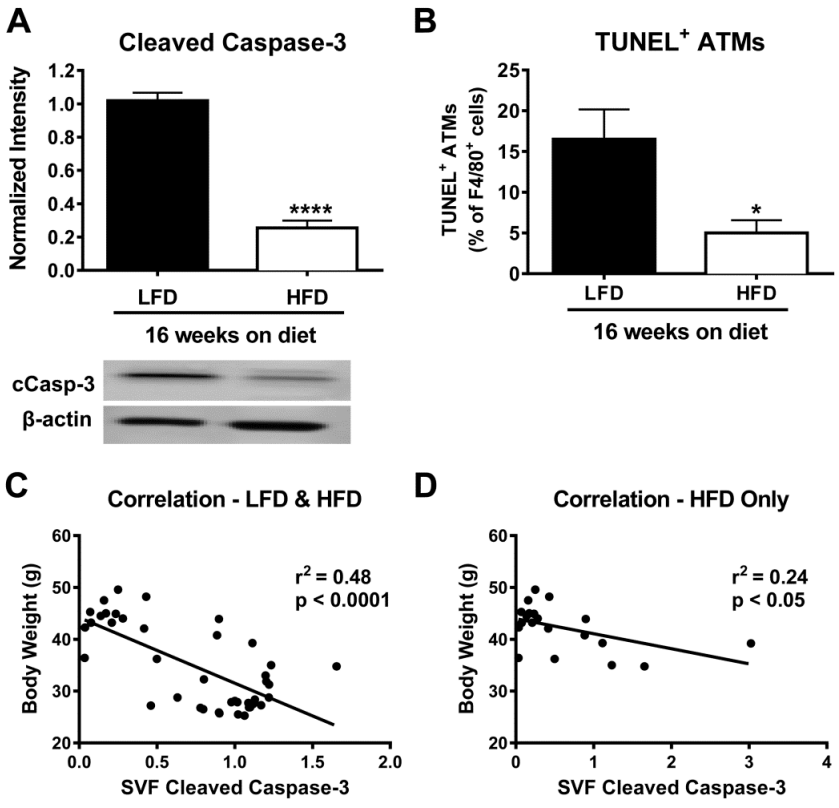

Figure 3.4: Apoptosis of ATMs is negatively correlated with body weight. Male C57Bl/6 mice were placed on a LFD or HFD for 16 weeks. A) SVF was collected and cleaved caspase-3 was analyzed using Western blot. B) Quantification of TUNEL positive ATMs by percent of F4/80 positive cells. C) Correlation of SVF cleaved caspase-3 with body weight of mice fed LFD and HFD for either 9 or 16 weeks. D) Correlation of SVF cleaved caspase-3 with body weight for HFD fed mice only (9 and 16 weeks). A-B) Data are presented as mean ± SEM, n = 5-6/group.
* p <0.05, **** p <0.0001 between groups.

Genetic Obesity Decreases ATM Apoptosis

To determine if the decreased macrophage apoptosis observed in obese AT was the result of dietary intervention or due to overt obesity, a mouse model of genetic obesity was analyzed. Leptin-deficient ob/ob mice and lean littermate controls were maintained on a chow diet until 9-10 weeks of age, at which point they were of similar weight to the mice fed HFD for 9 weeks. As expected, ob/ob mice were obese compared to lean littermate control mice ($p<0.0001$; Figure 3.5A), were hyperglycemic ($p<0.001$; Figure 3.5B) and the increase in body weight in the ob/ob mice was due to elevated fat mass, rather than lean mass ($p<0.0001$; Figure 3.5C-D).

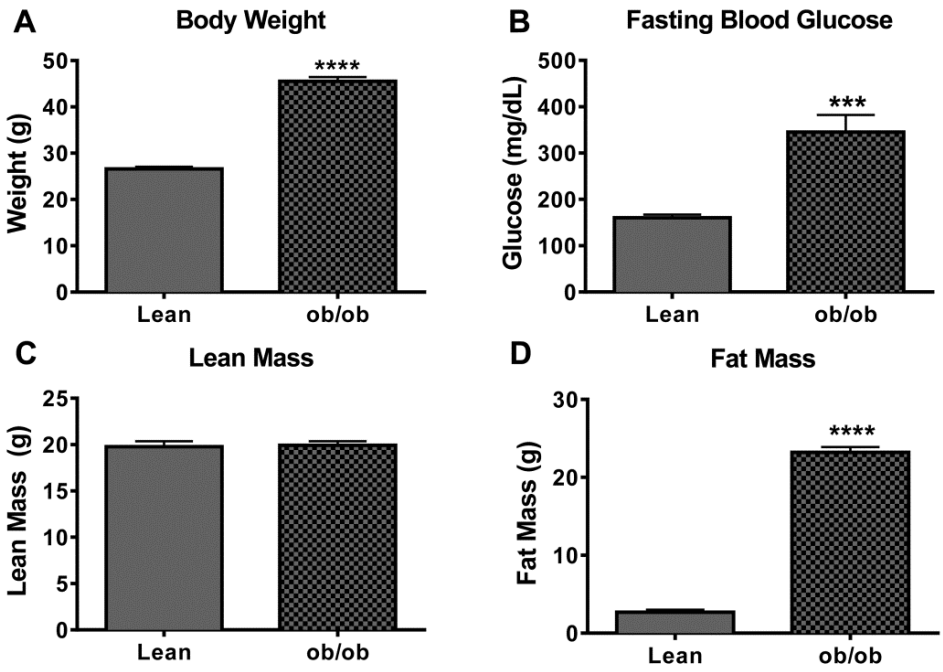

Figure 3.5: Metabolic phenotype of ob/ob mice and lean littermate controls. Metabolic parameters of ob/ob mice and lean littermate controls were assessed at sacrifice: A) body weight, B) fasting blood glucose, C) total lean mass, and D) total fat mass. Data are presented as mean ± SEM, A-B) n = 8/group, C-D) n = 5/group. *** $p<0.001$, **** $p<0.0001$ between groups.

Caspase-3 cleavage was significantly decreased in the SVF of ob/ob mice compared to lean controls ($p<0.01$, Figure 3.6A). AT was evaluated by confocal microscopy to determine if genetic obesity decreased apoptosis specifically in macrophages. Quantification of confocal images demonstrated that macrophage apoptosis is significantly decreased in the AT of ob/ob mice compared to lean controls ($p<0.01$ and $p<0.05$, Figure 3.6B-D). Additionally, 50% of apoptotic macrophages were localized to interstitial spaces, rather than crown-like structures (Figure 3.6E). Thus, both diet-induced and genetic obesity result in decreased ATM apoptosis.

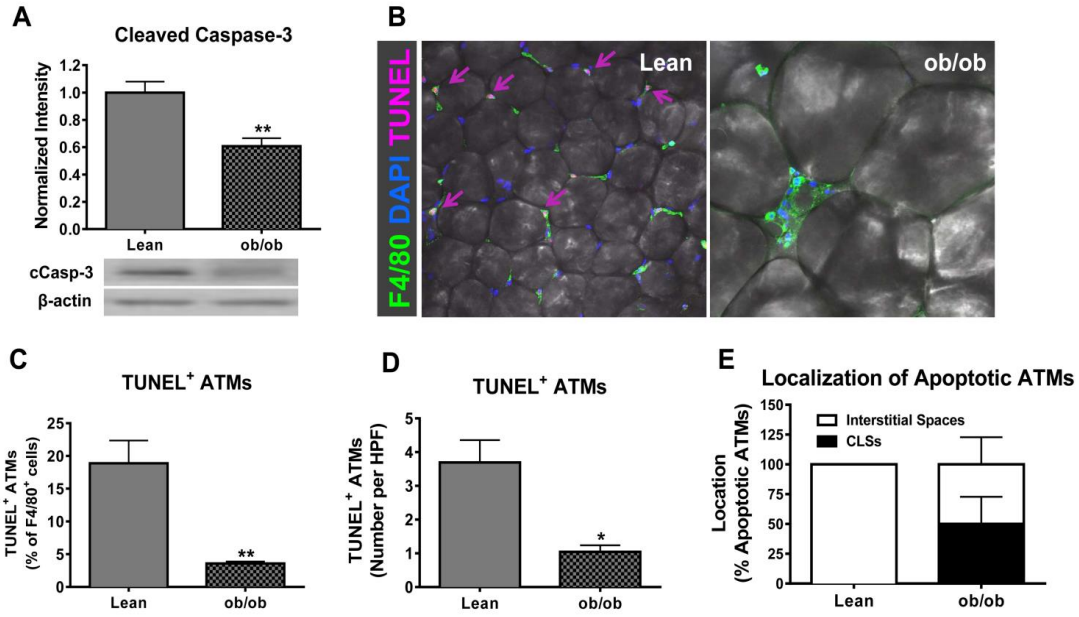

Figure 3.6: Genetic model of obesity decreases apoptosis of ATMs. Male C57Bl/6 lean or ob/ob mice were maintained on chow diet until 9-10 weeks of age. A) SVF was collected and cleaved caspase-3 was analyzed using Western blot. B) AT explants were collected and analyzed by confocal staining for the macrophage marker F4/80 (green), nuclear stain DAPI (blue), and apoptosis marker TUNEL (pink). Magnification: 40X. C-D) Quantification of TUNEL positive ATMs by percent of F4/80 positive cells (C) or by number per high-power field (D). E) Quantification of localization of apoptotic ATMs. Data are presented as mean ± SEM, A) n = 5/group, C-E) n = 4-6/group.
* p <0.05, ** p <0.01 between groups.

The Obesity-related Decreases in Macrophage Apoptosis is AT-specific

To determine if obesity modulates apoptosis in a similar manner in other metabolically-relevant tissues, protein was isolated from hepatocytes, an F4/80-enriched non-hepatocyte fraction of the liver and whole spleen of mice fed LFD and HFD for 9 weeks. Markers of apoptosis, including protein levels of cleaved caspase-3, Bax, and Bak, were not modified by HFD feeding in either the hepatocyte fraction (Figure 3.7A-C) or the F4/80-enriched fraction of the liver

(Figure 3.7D-F). Additionally, obesity did not impact markers of apoptosis in the spleen, an immune cell-enriched organ (Figure 3.7G-I). Therefore, while obesity decreased macrophage apoptosis in AT, this signaling pathway was not modulated in macrophages of the liver or whole spleen, indicating that this regulation is specific to AT.

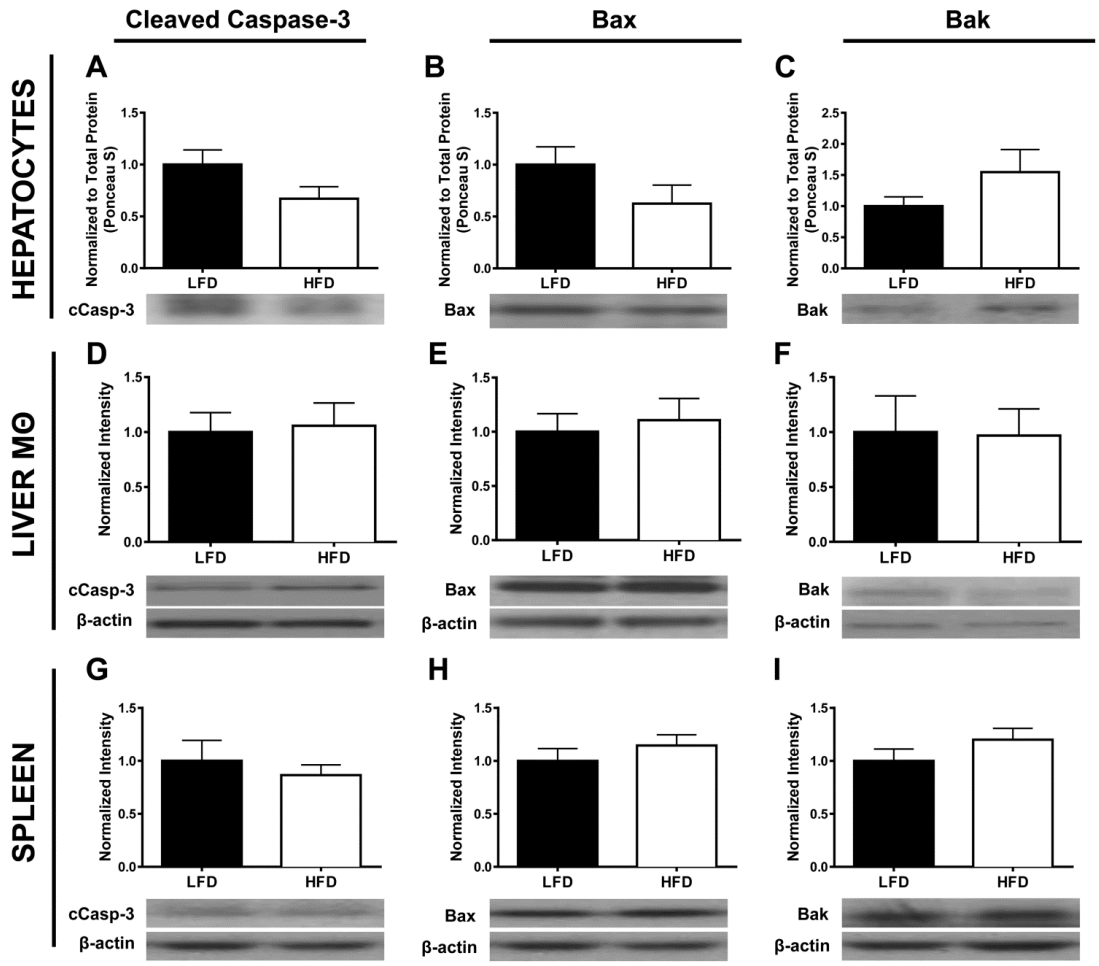

Figure 3.7: Obesity does not modulate apoptotic markers in hepatocytes, an F4/80-enriched fraction of the liver, or in the spleen. Male C57Bl/6 mice were placed on LFD or HFD for 9 weeks. A-C) The hepatocyte fraction of the liver was sonicated in 2% SDS and apoptotic markers were analyzed using Western blot with data normalized to total protein as measured by Ponceau S staining: A) cleaved caspase-3, B) Bax, and C) Bak. D-F) The non-parenchymal fraction of the liver was enriched for $F4/80^{+}$ macrophages using magnetic beads. Western blot analysis was performed for markers of apoptosis: D) cleaved caspase-3, E) Bax, and F) Bak. G-I) Whole spleen was sonicated in 2% SDS and Western blot analysis was performed: G) cleaved caspase-3, H) Bax, and I) Bak. Data are presented as mean ± SEM, A-C) n = 6-9/group, D-E) n = 7-8/group, F) n = 4/group, G-I), n = 6/group.

Decreased ATM Apoptosis Correlates with Increased Protein Levels of Total and Mitochondrial-localized Pro-survival Bcl-2 Protein

A common intrinsic mechanism to regulate apoptosis is maintenance of the integrity of the mitochondrial outer membrane. Within the Bcl-2 family, Bax and Bak are pro-apoptotic, promote mitochondrial outer membrane permeablization (MOMP), and activate the caspase cascade. Conversely, Bcl-2 and Bcl-xl are pro-survival and inhibit the pore-forming activities of Bax and Bak. To determine if obesity increases immune cell survival through the modulation of Bcl-2 family members, RNA and protein were isolated from the SVF of mice placed on LFD or HFD for 9 weeks. SVF from obese mice displayed increased gene expression of *Bax* ($p<0.01$, Figure 3.8A), with no change in the gene expression of other Bcl-2 family members (*Bak1, Bcl2, Bcl2l1* (gene for Bcl-xl), Figures 3.8B-D). At the protein level, there was a significant increase in Bax ($p<0.0001$, Figure 3.8E) in SVF of obese mice, while Bak protein expression was significantly decreased ($p<0.0001$, Figure 3.8F). Interestingly, levels of the pro-survival protein, Bcl-2, were 2.5-fold elevated in SVF of obese compared to lean mice ($p<0.001$, Figure 3.8G), while no change was seen in Bcl-xl (Figure 3.8H). These data demonstrate that obesity modifies the protein expression of both pro-apoptotic and pro-survival members of the Bcl-2 family in AT immune cells.

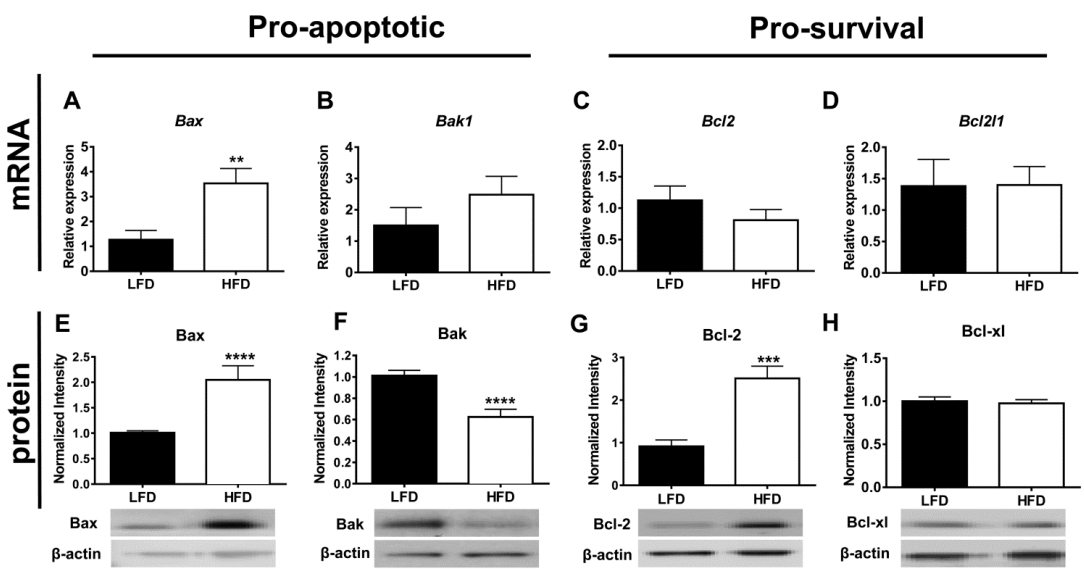

Figure 3.8: Pro-apoptotic/survival Bcl-2 family members are differentially regulated in the SVF of AT of obese mice. Male C57Bl/6 mice were placed on LFD or HFD for 9 weeks. A-D) SVF was collected and Bcl-2 family pro-apoptotic/survival gene expression was analyzed using real-time RT-PCR: A) *Bax*, B) *Bak1*, C) *Bcl2*, and D) *Bcl2l1*. E-H) SVF was collected and Bcl-2 family pro-apoptotic/survival protein levels were analyzed using Western blot: E) Bax, F) Bak, G) Bcl-2, and H) Bcl-xl. mRNA levels were normalized to housekeeping gene *Rplpo* and levels of specific proteins were normalized to β-actin. Data are presented as mean ± SEM, A-D) n= 7/group, E-F) n = 17-19/group, G-H), n = 4-8/group.
** p <0.01, *** p <0.001, **** p <0.0001 between groups.

The cellular level of Bcl-2 family members is not the sole determinant of cell survival versus apoptosis. Instead, their localization to the outer membrane of the mitochondria is essential (reviewed in (90, 129, 130)). To determine if the subcellular localization of the major Bcl-2 family members, Bax (pro-apoptotic) and Bcl-2 (pro-survival), was altered specifically in ATMs during obesity, adherence selected macrophages from the SVF of LFD and HFD mice were assessed. To quantitatively analyze the mitochondrial localization of Bax and Bcl-2, we used automated high-throughput fluorescent microscopy and analysis

software described in Chapter II. We first used the Image Xpress imaging technique to quantitatively determine the amounts of Bax and Bcl-2 protein localized to the mitochondria in ATMs of lean and obese mice. Although total protein levels of Bax were increased in the SVF during obesity (Figure 3.8E), there was no difference in the localization of Bax to the mitochondria (based upon co-localization with Cox IV) ATMs of LFD versus HFD fed mice (Figure 3.9A). Interestingly, obesity increased the localization of the pro-survival protein, Bcl-2, to the mitochondria of ATMs ($p < 0.05$, Figure 3.9B). To confirm the changes quantified using Image Xpress software, we also used confocal microscopy to visualize the differences in Bax and Bcl-2 mitochondrial localization (based upon co-localization with MitoTracker Deep Red). In support of our Image Xpress quantification data, the confocal images show that there was no apparent difference in Bax protein co-localization with mitochondria in ATMs from lean and obese mice by immunofluorescence staining (Figure 3.9C). Furthermore, Bcl-2 protein was highly co-localized to the mitochondria in ATMs of obese compared to lean mice (Figure 3.9D). Together, the data from Figures 3.8 and 3.9 suggest that the increased protein and mitochondrial localized levels of Bcl-2 may allow for increased ATM survival observed during obesity.

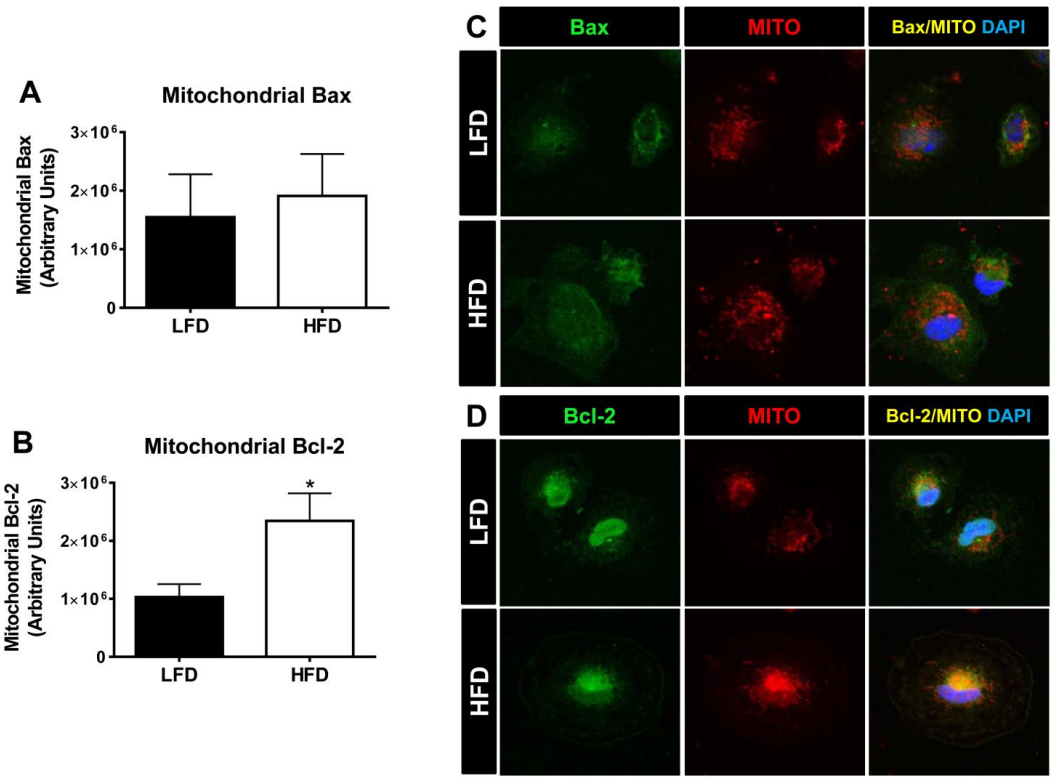

Figure 3.9: Mitochondrial localization of the pro-survival protein Bcl-2 is increased in ATMs of obese mice. Male C57Bl/6 mice were placed on a LFD or HFD diet for 9 weeks. ATMs were obtained using a 2 h macrophage selection by adhesion assay and stained for quantification of Bax and Bcl-2 mitochondrial localized protein levels by Image Xpress Automated HTS Fluorescence Microscopy or visualization by confocal microscopy. A) Quantification of the co-localization of Bax to the mitochondria of ATMs. B) Quantification of the co-localization of Bcl-2 to the mitochondria of ATMs. Magnification for quantifications: 40X. C) Representative images of Bax (green) mitochondrial (red, MitoTracker (Mito)) localization by confocal microscopy. D) Representative images of Bcl-2 (green) mitochondrial (red, MitoTracker (Mito)) localization by confocal microscopy. Magnification for representative images: 60X with a 4.5 zoom. Data are presented as mean ± SEM, n = 6-7/group.

NF-κB Activity and its Pro-survival Gene Targets are Activated in ATMs of Obese Mice

A key mediator of inflammatory gene expression in macrophages is the transcription factor NF-κB. Previous studies have demonstrated greater

localization of the p65 subunit of NF-κB to the nucleus in ATMs during obesity (67). In addition to its control of inflammatory gene expression, NF-κB also promotes cell survival through the transcription of pro-survival Bcl-2 family members (131). Therefore, we hypothesized that increased NF-κB activity during obesity may promote ATM survival. To confirm NF-κB activation in ATMs from obese mice, the protein level of the phosphorylated (active) form of the p65 subunit (P-p65) was assessed in the SVF of mice fed LFD or HFD for 9 weeks. P-p65 was significantly increased in the SVF of HFD mice ($p<0.05$, Figure 3.10A). Furthermore, nuclear localization of p65 was increased in adherence-selected ATMs from obese compared to lean mice (Figure 3.10B) and as quantified by Image Xpress Automated HTS Fluorescent Microscopy ($p<0.001$; HFD: $1.3 \times 10^6 \pm 1.1 \times 10^5$ RLU, and LFD: $0.82 \times 10^6 \pm 0.44 \times 10^5$ RLU, N=7). Next, we determined the transcriptional activity of NF-κB in adherence-selected ATMs through the use of NF-κB promoter-driven GFP-Luciferase reporter mice (NGL) described previously (104). Luciferase activity was significantly increased in ATMs of obese mice, indicating elevated NF-κB transcriptional activity ($p<0.05$, Figure 3.10C). To determine if increased NF-κB transcriptional activity resulted in elevated expression of NF-κB target genes, expression of the inflammatory cytokine, *Tnf*, and the pro-survival inhibitors of apoptosis, *Xiap* and *Birc3* (gene name for cIAP), was analyzed specifically in adherence-selected ATMs. As expected, *Tnf* gene expression was significantly increased in ATMs of HFD-fed mice ($p<0.05$, Figure 3.10D). Interestingly, there was a trend towards an increase in *Xiap* expression ($p=0.07$) and a significant increase in *Birc3* expression

(p<0.05) in ATMs from obese mice (Figure 3.10D). These data demonstrate that increased NF-κB transcriptional activity in ATMs promotes the expression of pro-survival genes. Therefore, it is likely that NF-κB contributes to the increased ATM survival observed during obesity.

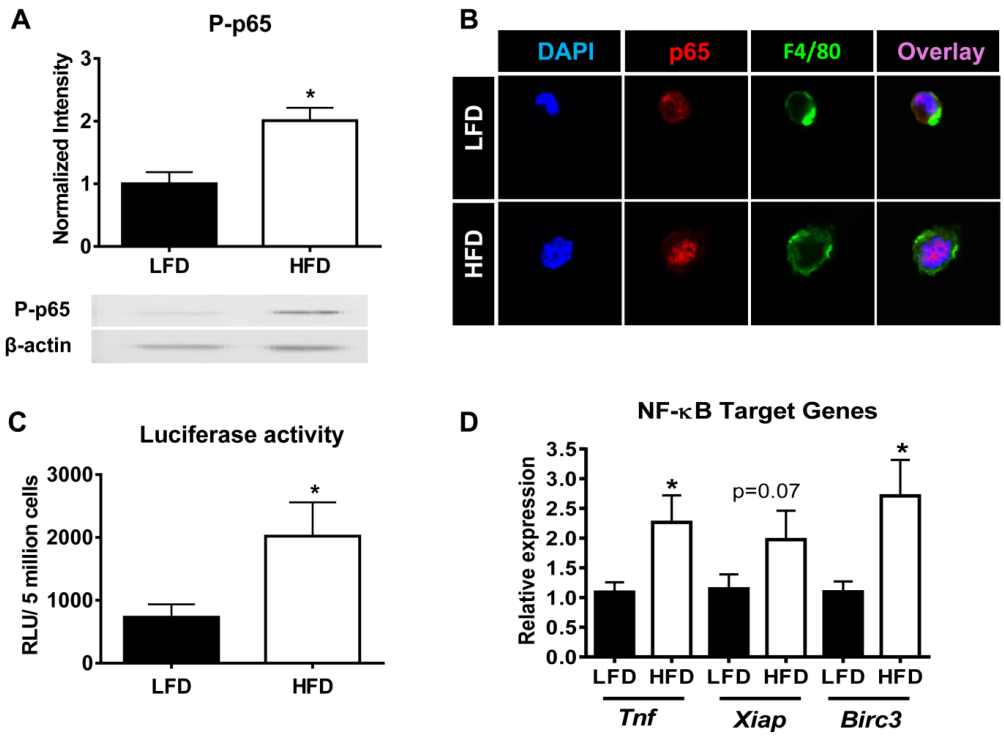

Figure 3.10: NF-κB activity and its pro-survival target genes are increased in ATMs of obese mice. Male C57Bl/6 mice were placed on a LFD or a HFD for 9 weeks. A) SVF was collected and phosphorylated p65 (P-p65) was analyzed using Western blot. B) Nuclear localization of the p65 subunit of NF-κB. ATMs were obtained using a 2 h macrophage selection by adhesion assay and stained for nuclear stain DAPI (blue), p65 (red), F4/80 (green). Magnification: 100X with a 4.5 zoom. C) Transcriptional activity of NF-κB in ATMs using NF-κB-GFP-Luciferase mice. D) Real-time RT-PCR analysis of NF-κB-driven pro-inflammatory and pro-survival target genes in ATMs (*Tnf, Xiap, Birc3)*. Data are presented as mean ± SEM, A) n = 4/group, C) n = 9-10/group, and D) n = 7-8/group.
* p <0.05 between groups.

Metabolic Activation-induced Survival of ATMs is Blunted by Inhibition of NF-κB

In previous studies, it has been shown that exposing bone marrow-derived macrophages (BMDMs) to high levels of glucose, insulin and palmitate ("metabolic activation") induces a gene expression profile/phenotype similar to ATMs of obese mice (53). I sought to determine if exposure of ATMs to this metabolic activation cocktail (MetaC; 30 mM glucose, 10 nM insulin and 0.4 mM palmitic acid) would result in increased NF-κB activation, augmented expression of its pro-survival target genes, and increased cell viability. I felt it was important to perform these studies specifically in ATMs, as recent data from the Immunological Genome Project emphasize the fact that macrophages derived from different tissue/cellular sources have vastly different transcriptomes (132). Therefore, in order to obtain a sufficient number of ATMs for these studies, mice were fed HFD for 3 weeks prior to the isolation of adherence-selected ATMs. This short-term HFD feeding did not significantly increase NF-κB activity, as measured by NGL luciferase activity (Figure 3.11A). Subsequently, the adherence-selected ATMs were exposed to control or MetaC conditions for 30 min and p65 nuclear localization was visualized using confocal microscopy. In support of my ex vivo results, exposure of ATMs to the obesogenic milieu (MetaC) increased nuclear localization of the p65 subunit of NF-κB (Figure 3.12A). Furthermore, 2 h of metabolic activation of ATMs recapitulated the reported (53) gene expression profile of ATMs in vivo (*Tnf*; $p<0.05$, *Abca1*; $p=0.09$, *Plin2*; $p=0.06$). Of note, metabolic activation in ATMs also significantly

increased the levels of NF-κB pro-survival target genes *Bcl2* ($p<0.05$) and *Xiap* ($p<0.05$), while modestly increasing *Birc3* (Figure 3.12B). These findings demonstrate that metabolic activation of ATMs increases NF-κB transcriptional activity and pro-survival gene expression, similar to what we observed *in vivo* during obesity. Furthermore, these studies were attempted using adipocyte conditioned media; however, HFD adipocyte conditioned media did not increase NF-κB activity compared to control. This is further detailed in Appendix A. Interestingly, exposure of ATMs to MetaC alone significantly increased cell viability at 6 and 8 h post-treatment ($p<0.01$ and $p<0.05$, respectively; Figure 3.12C), supporting my earlier data demonstrating that the *in vivo* obese milieu promotes ATM survival. I next used this model system to determine the role of NF-κB activation in this increased ATM survival under obesogenic conditions by treating ATMs with MetaC in the presence or absence of the highly selective NF-κB inhibitor, BMS-345541(133). Importantly, MetaC increased and BMS inhibited NGL luciferase activity in metabolically activated ATMs from chow-fed mice, indicating that BMS does, in fact, decrease NF-κB activity (Figure 3.11B). In support of my hypothesis, inhibition of NF-κB in ATMs, reduced the pro-survival effect of MetaC, a finding that trended at 4 and 8 h of treatment and was significant ($p<0.05$) at 6 h of treatment (Figure 3.12C). Taken together, these data suggest that NF-κB activation in ATMs in the obese state increases their ability to survive.

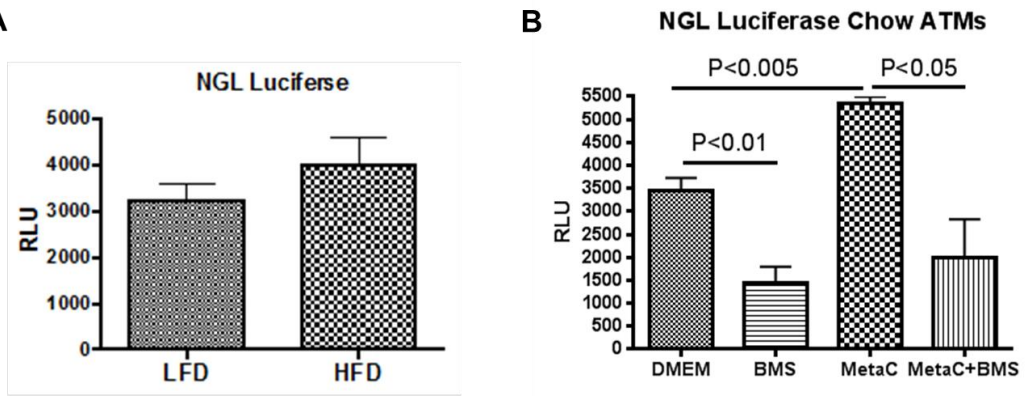

Figure 3.11: ATMs from chow diet-fed NGL mice respond to MetaC and BMS. A) NGL Luciferase Activity in ATMs of mice fed LFD or HFD for 4 weeks. Four weeks of HFD feeding doesn't significantly increase NF-κB activity in ATMs from NGL mice. N=ATMs from 4 mice/per group. B) ATMs were collected from chow-fed NGL mice and treated with BMS, MetaC, or MetaC+BMS as described for Figure 3.12. After 2 hours, cells were lysed and Luciferase activity measure. n = ATMs from 3-4 mice/group.

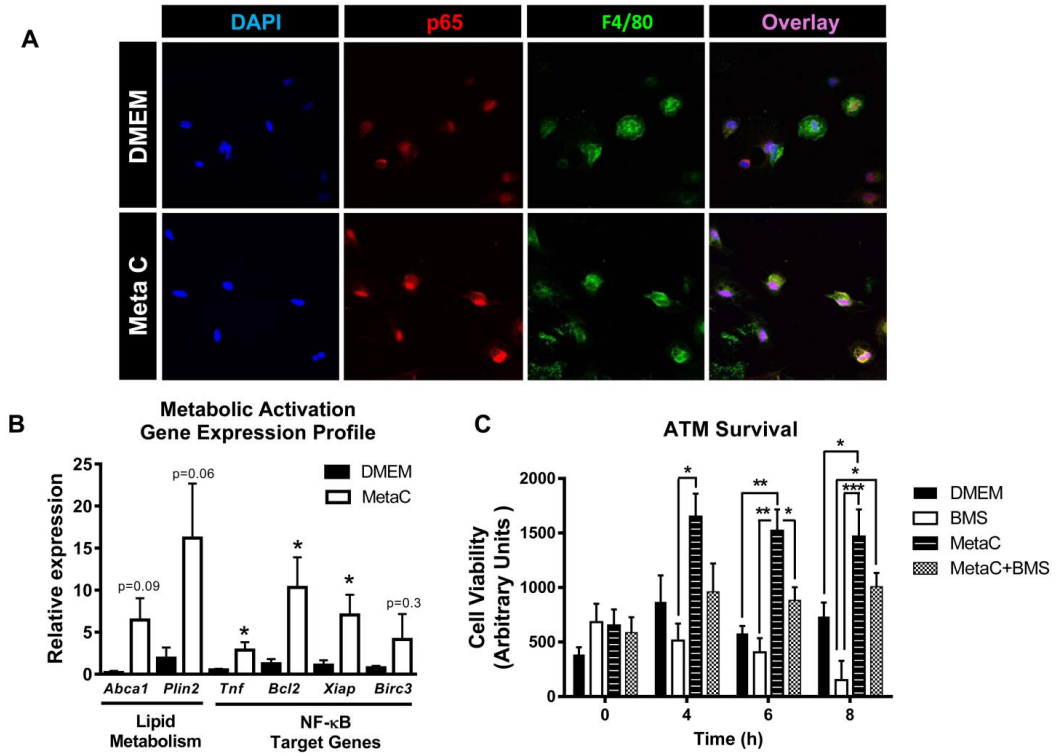

Figure 3.12: Inhibition of NF-κB activity decreases ATM survival in an obesogenic setting. Male C57Bl/6 mice were placed on a HFD for 3 weeks to obtain sufficient numbers of ATMs for ex vivo studies. A) Nuclear translocation of NF-κB. Adhesion-selected ATMs were treated with the metabolic cocktail (MetaC, 30 mM glucose, 10 nM insulin, 0.4 mM of palmitic acid) for 30 minutes and subsequently stained with DAPI (blue), p65 (red), F4/80 (green). Magnification: 60X with a 1.5 zoom. B) Gene expression. ATMs were treated with the MetaC for 2 h and RNA isolated for real-time RT-PCR analysis of expression of lipid metabolism (*Abca1* and *Plin2*) as well as NF-κB-driven pro-inflammatory (*Tnf*) and pro-survival (*Bcl2*, *Xiap*, *Birc3*) genes. C) Cell viability. ATMs were treated with DMEM (control), MetaC, BMS-34551 (BMS), or MetaC + BMS for 0-8 h. Cell viability was detected using the Cell-Titer Blue assay as described in the Chapter II. Data are presented as mean ± SEM, n = 4-5/group.
* $p<0.05$, ** $p<0.01$, *** $p<0.001$ between groups.

Discussion

During obesity, pro-inflammatory macrophages accumulate in metabolic tissues, including AT (31, 32), and contribute to obesity-associated IR both locally and systemically (21). Since this novel discovery, much effort has been focused on determining mechanisms by which this accumulation occurs. The overwhelming majority of these studies have sought to identify recruitment-dependent mechanisms for the increase in ATM number during obesity.

Much work has focused around the central hypothesis that obesity increases circulating inflammatory Ly6C^{hi} monocytes that are then recruited to AT via chemoattractants. Thus, recruitment-dependent mechanisms for increased ATMs should be contingent upon increased circulating inflammatory monocytes and chemotaxis of these cells to AT during HFD feeding. Despite the logical nature of this hypothesis, recent findings have called into question whether chemoattractant-mediated monocyte recruitment is the sole mechanism regulating ATM number during obesity. First, single gene deletion of multiple chemokines or chemokine receptors such as *Ccl3* (119), *Ccr5* (120), and *Cx3cr1* (121), does not modulate ATM number during HFD feeding. Even in studies demonstrating that a chemokine or its receptor plays a role in promoting macrophage accumulation in obese AT, ATM number is not normalized to levels observed in lean AT (72, 77, 89, 123, 124) . Second, in *Ccr2-/-* mice, there is a near absence of circulating Ly6C^{hi} cells (75), yet there is either no difference in ATM numbers or these differences are noted only after long periods of HFD feeding (49, 72, 77). Third, MGL1 has been identified as a critical factor

regulating the survival and migration of Ly6C^{hi} monocytes, as animals deficient in MGL1 do not mobilize Ly6C^{hi} monocytes from the bone marrow to the blood in response to HFD feeding (81). However, despite the near absence of circulating pro-inflammatory monocyte populations, deletion of *Mgl1* does not normalize ATM number to levels observed in lean AT (81). This dissociation between the chemoattractant potential of AT, circulating blood monocyte number, and ATM content suggests that an increase in the recruitment of inflammatory Ly6C^{hi} monocytes is not the only mechanism regulating ATM accrual during obesity. Indeed, taken together, these published reports suggest that recruitment-independent mechanisms for macrophage accrual in obese AT should be considered.

Potential recruitment-independent mechanisms that could also play a role in the regulation of ATM number during obesity include: increased proliferation of macrophages within AT, decreased egress of macrophages from AT, or increased ATM survival. Recent studies now show that increased proliferation and decreased egress of ATMs can, in fact, contribute to ATM accumulation during obesity (82-84). I now show that macrophage longevity is an additional metabolically regulated process that, when dysregulated during obesity, promotes macrophage survival and accumulation in AT, thus contributing to the diminished function of the tissue.

In agreement with my findings, recent studies show that macrophage apoptosis occurs infrequently in obese AT. For example, sophisticated imaging studies in AT explants demonstrate very few apoptotic macrophages in obese AT

(134). Furthermore, these studies showed that macrophages within the CLS of obese AT were stable, showing no shrinkage or cell death, over the 7-day imaging time-course, suggesting that ATMs in obese AT are long-lived (134). Beyond this, there have also been hints in the literature that decreasing macrophage survival in AT of obese mice and humans reduces the metabolic abnormalities associated with obesity. Feng, *et al*. showed that activation of ATM apoptosis via treatment with liposomal clodronate decreased AT inflammation and improved systemic glucose tolerance and insulin sensitivity in a mouse model of obesity (85). Additionally, Kern and colleagues reported that pioglitazone, an insulin sensitizing TZD, increased macrophage apoptosis in human AT, possibly contributing to the reduced ATM number observed after TZD treatment (135, 136). These findings demonstrate that pharmacological activation of macrophage apoptosis in obese AT reduces ATM content and improves metabolic function. If ATM apoptosis can be manipulated to improve AT function, it is logical that macrophage survival may also be regulated in a physiologically relevant manner to control macrophage content of AT.

Macrophage Apoptosis in Healthy Tissues

My studies showed that ~17% of ATMs were TUNEL$^+$ in the lean AT. This is quite surprising, given that one might expect efferocytic processes to quickly clear the apoptotic cells. However, this finding is in agreement with published literature. In their work using AT-specific p65 knockout mice (discussed in more detail below), Gao, *et al.* also showed a fair amount of TUNEL staining in wild

type lean AT (137). Furthermore, studies by Cai, *et al*. demonstrated significant macrophage apoptosis (22% of macrophages were TUNEL positive) and turnover (35% turnover rate in 48 h) in interstitial macrophages from the lung tissue of healthy rhesus macaques (138). In agreement with these findings, tissue macrophages in murine lung were found to have substantial turnover during a 21-day study, and these macrophages were replaced through a self-renewal process (139). Together, these studies support the idea that significant macrophage apoptosis/turnover occurs in multiple tissues of healthy animals. It should also be noted that about 10-20% of the $TUNEL^{+}$ were not macrophages. These cells could be adipocytes, as has been reported (127); however they could also include other immune cells such as neutrophils, T cells, B cells, or eosinophils. Further studies are needed to determine whether apoptosis of other leukocytes takes place in AT and whether this is of relevance to AT homeostasis.

In other metabolic settings, control of macrophage death/survival is known to be important for disease progression. For example, in atherosclerotic lesions, macrophage apoptosis and clearance by other efferocytic macrophages protects from early lesion formation (140, 141). Conversely, decreased macrophage apoptosis or impaired efferocytosis in advance lesions contributes to plaque instability (142). Of potential relevance to ATMs, Tabas and colleagues have shown that prior engagement of Toll Like Receptor 4, *i.e.* activation of an acute inflammatory pathway, protects macrophages from subsequent apoptosis in settings of sustained ER stress (143). This pathway is suggested to prolong cell survival to allow for continued production of inflammatory cytokines and

antimicrobial proteins in order to remove the infectious insult. Because chronic activation of ATMs during obesity can activate similar inflammatory pathways, these cells may be "tricked" into survival, with the ultimate result being detrimental rather than protective. In fact, both my *in vivo* and *ex vivo* studies support this notion that activation of macrophages increases their survival. These data suggest that modulation of macrophage survival is beneficial in the setting of microbial infection, but that activation of these same pathways may be a significant contributor to the pathological processes occurring during obesity by promoting both the survival and the inflammatory nature of ATMs.

NF-κB Signaling and ATM Survival

Inflammatory activation of macrophages is largely regulated by the transcription factor, NF-κB. Studies by Chiang *et al.* demonstrated increased nuclear translocation of the p65 subunit of NF-κB ATMs from obese compared to lean mice (112), suggesting that ATM inflammation may be driven by NF-κB-dependent mechanisms. Although NF-κB is often only appreciated for its pro-inflammatory role, this transcription factor also acts as a potent pro-survival factor. My data demonstrate that the pro-survival axis of NF-κB is initiated in ATMs during obesity, as indicated by increased p65 nuclear localization, elevated NF-κB–driven luciferase activation, increased Bcl-2 protein levels and mitochondrial localization, and elevated expression of IAP genes. These data demonstrate that obesity-driven NF-κB activity not only promotes an inflammatory phenotype in ATMs in obese AT, but also increases the expression

of pro-survival genes/proteins. Further, my *ex vivo* studies suggest that NF-κB activity is necessary for increase ATM survival under obesogenic conditions.

The use of the inhibitor BMS-34551 to inhibit NF-κB specifically in ATMs is a novel model that has given great insight in to the contribution of this pathway to ATM survival. BMS-345541 has been previously demonstrated to be highly effective at inhibiting NF-κB transcriptional activity via its specificity to the NF-κB activator, IKK (133). Importantly, the compound was tested against a panel of 15 other kinases, including c-Jun, STAT3, and MAPK, and failed to inhibit the activity of these inflammatory factors (133). Furthermore, it has been demonstrated that treatment of NGL bone marrow derived macrophages with BMS-345541 significantly decreased LPS induced NF-κB transcriptional activity (104, 133, 144). MetaC-mediated upregulation of NF-κB was also inhibited by BMS in my ATMs (Figure 3.12B). Although this specificity has been shown in other cell types, I cannot rule out that there could be off target effects on pathways other than NF-κB that control ATM apoptosis. Of note, this could alter the interpretation of my pharmacological data. In light of this, future studies, such as siRNA manipulation of NF-κB in ATMs, should be performed to better identify the importance of this transcription factor in regulating ATM survival.

As this manuscript was in preparation, Gao, *et al.* reported their findings regarding inflammation in an AT-specific p65 knockout model (driven by the ap2 promoter, *i.e.* deletion in adipocytes and likely macrophages) (137). Surprisingly, absence of p65 resulted in different effects in lean versus obese mice. They demonstrated that absence of the p65 subunit of NF-κB reduced inflammation

and ATM content in lean mice – presumably due to the absence of the inflammatory and survival arm of NF-κB signaling in the lean setting. In contrast, absence of p65 in obese mice led to adipocyte apoptosis. In addition, ATM numbers and overall AT inflammation were increased during obesity – most likely as a consequence of the adipocyte death. Although ATM content was increased, the authors also noted that ATM apoptosis was elevated in the p65-null obese mice, a finding they attributed to the absence of p65-mediated pro-survival signaling in the ATMs (137). These results suggest that adipocyte apoptosis and macrophage apoptosis may have different outcomes in regards to increasing or decreasing macrophage number in AT. The increased ATM content found in this model may be solely due to an immense and overwhelming amount of adipocyte cell death, which likely induced macrophage recruitment to AT. In this case, deletion of p65 in both the adipocytes and macrophages makes it complicated to determine the contribution of each process to the regulation of ATM number. However, these data further support my finding that NF-κB controls ATM apoptosis/survival.

Many of my studies focused of the role of NF-κB-induced ATM survival *in vitro*. I first set out to determine the role of NF-κB in ATM survival *in vivo*; however, I had difficulty in finding a usable model system. These studies are detailed in Appendices B and C. In light of this, determining the *in vivo* contribution of NF-κB to ATM survival and number in obese AT still remains to be elucidated. As discussed above, understanding ATM origin may also be

beneficial in understanding their role in restoring AT homeostasis and how, under metabolic conditions, this may be detrimental.

Conclusion

In light of the new knowledge obtained from the studies performed in this dissertation and the recent findings demonstrating the role of recruitment-independent mechanisms in AT accrual, many questions still remain. No one recruitment-dependent or independent mechanism has been unequivocally identified as the sole regulator of macrophage accumulation in AT. In fact, all of these mechanisms may be working jointly to regulate ATM number. It will be interesting to understand the relative contributions of each mechanism in promoting pro-longed macrophage longevity. Even more importantly, should all be targeted or are other undiscovered mechanisms the culprit?

The data presented in Chapter III of this dissertation demonstrate that the obese AT micro-environment metabolically activates ATMs in a way that may promote their survival. Furthermore, NF-κB appears to be at the center of controlling this life and death balance. These findings, combined with recent literature demonstrating that increased proliferation and reduced egress of macrophages promote increased ATM content in obese AT, indicate that recruitment-independent mechanisms indeed also modulate ATM number during obesity. A further understanding of the relative contributions of recruitment, proliferation, egress, and survival to the control of ATM number and inflammatory

status could pave the way for the development of novel therapeutics for the treatment of metabolic disorders.

CHAPTER IV

DISSCUSSION AND FUTURE DIRECTIONS

The mechanisms that control inflammatory macrophage accumulation in AT have long been a question of interest. Although recruitment is the most largely studied mechanism regarding this process, it is now being understood that recruitment-independent mechanisms (proliferation and egress) are also contributing factors. The data presented in my dissertation now show that regulation of life/death signals in ATMs also contributes to their accrual in AT (Figure 4.1).

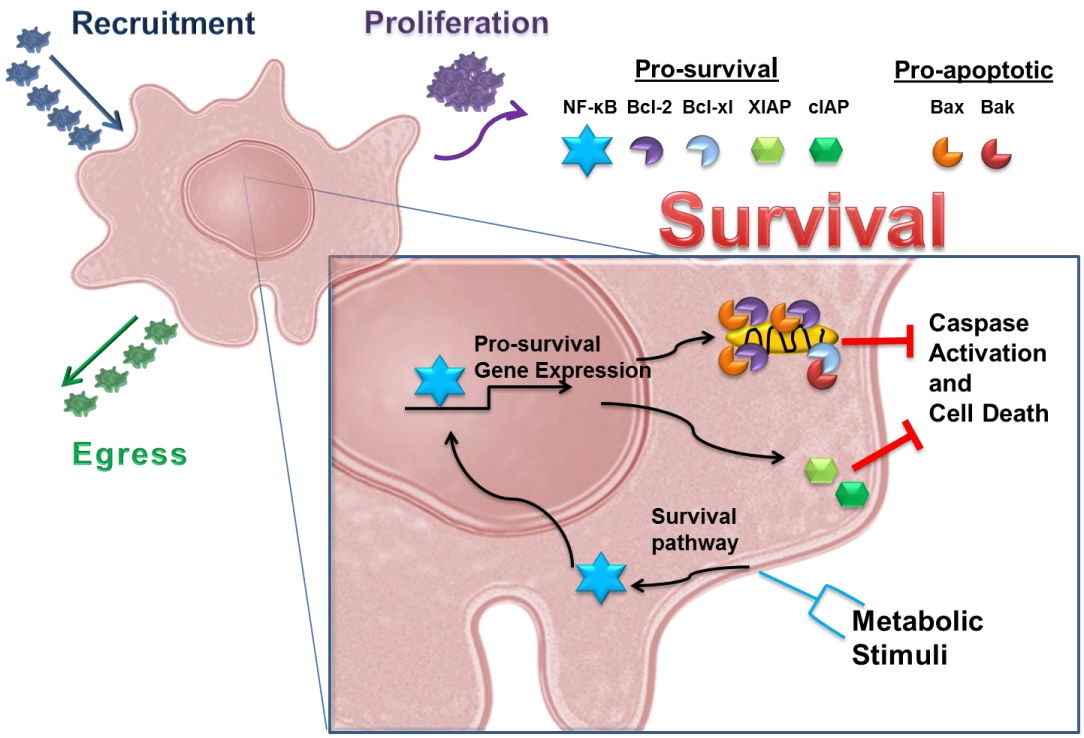

Figure 4.1. Survival as an additional mechanism of macrophage accrual in AT.

The original hypothesis leading into my studies was that the obese state would promote a more pro-apoptotic (rather than pro-survival) phenotype in ATMs due to the exposure of increased levels of SFAs in obese AT. In support of this idea, work I was involved in when I began my time in the Hasty laboratory had demonstrated that SFA lipid loading of macrophages *in vitro* induced apoptosis, suggesting that SFAs in the obese milieu would be toxic to ATMs (58). Surprisingly, during the completion of my dissertation, I found the opposite to be true in the *in vivo* setting. My work presented in this dissertation now demonstrates the novel finding that the obese environment actually promotes increased survival in ATMs and their accumulation in obese AT. Furthermore, I demonstrate that NF-κB controls macrophage survival through increasing the expression of pro-survival Bcl-2 and IAPs family proteins. These findings are distinctive because macrophage longevity in AT had not previously been studied. Furthermore, this is the first time, in the context of obesity, that the NF-κB survival arm has been described as a factor regulating ATM function in AT. These new findings bring great insight into how the obese environment can perpetuate the pathology of obesity by increasing inflammatory ATM survival and promoting their accrual in AT. This insight opens up a plethora of questions, in regards to ATM longevity in AT. The following questions will be discussed in this chapter: 1) Why do the results of the *in vivo* studies performed in this dissertation differ from previous *in vitro* SFA-accumulation studies in regards ATM survival? 2) Do anti-inflammatory or pro-inflammatory macrophages preferentially undergo apoptosis? 3) Is the NF-κB pro-survival arm activated in macrophages in other

lipid-rich metabolic settings? 4) Is this pro-survival activation intentional or a consequence of inflammatory activation? 5) Are other pro-survival pathways involved? 6) Is macrophage turnover a normal aspect of tissue homeostasis and does it apply to AT? 7) What controls macrophage turnover?

Why do the results of the *in vivo* studies performed in this dissertation differ from previous *in vitro* SFA-accumulation studies in regards ATM survival?

Previous studies performed in the Hasty laboratory demonstrated that lipid loading of macrophages with SFAs induced apoptosis. In these studies, peritoneal macrophages were treated with a long fatty acyl CoA synthetase inhibitor, Triacsin C, to inhibit their ability to metabolize lipids. As a result, SFAs were unable to be stored in the form of triglycerides (TG) and caused increased levels of intracellular free fatty acids (FFAs). This lipotoxic state resulted in endoplasmic reticulum (ER) stress-mediated apoptosis (58). In obese AT, dysregulated lipolysis exposes macrophages to increased levels of FFAs, including SFAs (145). In light of these findings, it was hypothesized that the lipotoxic environment found in obese AT would induce their apoptosis *in vivo*. Surprisingly, my studies demonstrated that the *in vivo* obese setting increased ATM survival. The inconsistencies in the results found between these studies could be explained by the modulation of lipid metabolism in macrophages in both settings. Under normal physiological conditions, lipid metabolism would not be disabled in macrophages. As a result, FFAs can be properly stored as TG and

presumably prevent lipotoxic-induced ER stress-mediated apoptosis. In fact, Kratz and colleagues demonstrated that ATMs of obese AT display increased expression of lipid metabolism genes, *Abca1* and *Plin2* (53). Furthermore, my *in vitro* studies demonstrate that exposure of ATMs to a SFA-rich metabolic cocktail increased their survival as seen in the *in vivo* setting. In the previous *in vitro* SFA studies, the inhibition of lipid metabolism in macrophages did not fully recapitulate the lipid handling abilities of macrophages in the *in vivo* setting. In light of this, the interpretation of these results, in regards to macrophage apoptosis *in vivo*, were not exact. Although these studies differ in their outcomes, they both provide insight into how macrophage lipid metabolism can control their survival. In fact, altering lipid metabolism genes and pro-survival pathways in ATMs could serve as therapeutic targets to increase their apoptosis and reduce their numbers in obese AT.

Do anti-inflammatory or pro-inflammatory macrophages preferentially undergo apoptosis?

The data presented in Chapter III demonstrate that macrophages in lean AT undergo apoptosis more frequently than macrophages in obese AT. As detailed in the Introduction, interstitially spaced macrophages, found in lean and obese AT, tend to be M2-like (anti-inflammatory) versus M1-like (pro-inflammatory) CLS-localized ATMs present in obese AT. Furthermore, the data presented in Figure 3.1E shows that the majority of apoptotic ATMs in the lean or obese state are interstitially spaced with few localized to CLSs. Taken together, these

findings would suggest that interstitially spaced anti-inflammatory macrophages are also more pro-apoptotic. However, it is not known whether anti-inflammatory or pro-inflammatory macrophages in AT preferentially undergo apoptosis and how this relates to maintaining or restoring AT homeostasis. My data in figure 3.1 would suggest that interstitially spaced ATMs, presumably of an anti-inflammatory phenotype, are more prone to apoptosis unlike CLS pro-inflammatory macrophages. This dichotomy could be explained by differential regulation of the NF-κB pro-survival signaling pathway in both phenotypes. The data presented in Figures 3.10 and 3.11 clearly demonstrate that ATM turnover in obesity is subdued, due to the activation of the NF-κB pro-survival arm. This pro-survival phenotype is not apparent in ATMs found in the lean state. Taken together, this would suggest that the lower level of NF-κB activity present in anti-inflammatory ATMs found in lean AT makes them more prone to apoptosis. Additionally, interstitially spaced ATMs in obese AT also seem to be infrequently undergoing apoptosis compared to ATMs in the lean state. Of note, macrophages are thought to be plastic in nature whereby the polarization state spans a continuum and can be controlled by the tissue microenvironment(146). In obese AT, interstitially spaced anti-inflammatory ATMs could be progressing towards a more pro-inflammatory phenotype due to the inflammatory environment of obese AT. As a result, NF-κB pro-survival signaling can occur, resulting in decreased susceptibility to apoptosis. If so, this would suggest that even a progressive increase in NF-κB activity in anti-inflammatory ATMs in obese AT could promote their survival. In efforts to understand whether anti- or pro-

inflammatory macrophages preferentially undergo apoptosis, it would be interesting to determine to what extent the NF-κB pro-survival arm has to be activated to promote longevity. Future *in vitro* studies could modulate the activation of NF-κB, using various concentrations of stimuli, in ATMs under apoptotic conditions to determine the threshold of NF-κB pro-survival activation required to prevent apoptosis. Furthermore, anti- and pro-inflammatory ATM NF-κB activity can be compared to the data from these studies to determine if one phenotype is more susceptible to apoptosis.

Is the NF-κB pro-survival arm activated in macrophages in other lipid-rich metabolic settings?

Interestingly, NF-κB induction in the context of inflammatory diseases is often associated with increased cell survival [reviewed in (102, 147, 148)]. In the obese setting, the glucose and lipid–rich microenvironment of AT seems to play a critical role in activating NF-κB survival pathways in ATMs. Although my data suggest that the changes in macrophage apoptosis/survival were specific to AT and were not seen in liver or spleen, there are other obesity-related metabolic diseases that display similar characteristics. It has been suggested that lipids secreted from dysfunctional AT promote bone loss in obesity (149). SFA enhanced survival of osteoclasts (bone macrophages) is implicated in contributing to bone loss in obesity due to the ability of osteoclast to resorb bone cells, induce inflammation, and prevent new bone formation. Oh and colleagues demonstrated that treatment of BMDM-derived osteoclasts with palmitic acid

significantly induced their survival via an NF-κB, MyD88, Mip-1α dependent mechanism (149). Furthermore, it has been shown that inhibition of NF-κB in osteoclast induced apoptosis (150). In other metabolic settings, such as atherosclerosis, exposure of macrophages to elevated levels of glucose and oxidized low-density lipoprotein (oxLDL) is responsible for foam cell formation in atherosclerotic lesions. These lipid-laden cells play a central role in plaque formation, progression, and instability. Interestingly, their increased survival in advanced lesions is suggested to play a role in the growth and destabilization of advanced atherosclerotic plaques (151). For example, *in vitro* studies in BMDMs treated with oxLDL demonstrated significant increases in pro-survival proteins Bcl-2 and Bcl-xl (152). This increase in survival factors was not seen in BMDMs treated with native LDL. Furthermore, the extent to which the levels of the pro-survival Bcl-2 proteins were elevated mirrored levels induced by a known macrophage pro-survival protein activator, CSF-1. Protein levels of pro-survival IAPs, XIAP and cIAP, were also elevated in BMDMs treated with ox-LDL. Of note, the ability of oxLDL to induce a pro-survival phenotype was largely dependent on the macrophages utilization of glucose (152). The PI3K pathway is accredited for this increase survival; however, studies demonstrate the PI3K hyperactivates NF-κB signaling in macrophages (150).It is not clear why certain lipid species activate NF-κB. It would be interesting to determine the mechanisms by which certain lipid species result in activation of pro-survival pathways in macrophages. Is their structure recognized as an antigen? Do they cause rigidity of the cellular membrane? Are there specific lipids relevant to pro-survival

activation of ATMs compared to other macrophages? Taken together, these studies further suggest the integral role of NF-κB in macrophage survival in the progression of metabolic diseases.

Is this pro-survival activation intentional or a consequence of inflammatory activation?

It is not clear whether the association between NF-κB-induced inflammation and survival in ATMs is direct or indirect. This brings to question whether 1) a macrophage "knowingly" activates the survival arm of NF-κB under inflammatory conditions or 2) if it's a "side effect" of classical NF-κB activation. In the case of scenario 1, this activation may provide protection against lipotoxicity present in the local AT environment. Furthermore, this idea suggests a level of control that the macrophage has in regards to its survival. Conversely, scenario 2 implies that increased survival is merely a consequence of inflammatory activation and is unable to be dampened even when it promotes pathology. If macrophages "knowingly" activate the survival arm of NF-κB, it would be interesting to understand the signaling pathways that control NF-κB-induced survival of ATMs in inflammatory settings and use them as therapeutic targets for decreasing ATM survival and content in obese AT. Inhibition of the NF-κB inflammatory signaling pathway could allow for its pro-survival arm to be more easily studied. This would allow for both the pro-inflammatory and pro-survival signaling arms of NF-κB to be evaluated and the ability to determine whether

there is cross talk between both pathways or if they act independently of each other.

Are other pro-survival pathways involved?

My studies demonstrate that increased markers of ATM survival in the obesogenic environment may be NF-κB-dependent. However, ATM survival may also be regulated by additional mechanisms. Kratz *et al.* demonstrates that ATMs in obese AT display a "metabolic activation" phenotype (53). An aspect of this phenotype is the induction of sequestome-1 (p62). These authors suggested that uptake of palmitic acid induces not only NF-κB activation, but also results in impaired autophagy. Interestingly, several studies suggest a role for both autophagic degradation and NF-κB signaling pathways in regulating cell survival (153). Interestingly, NF-κB activates pro-survival regulators, Bcl-2 and Bcl-xl, which inhibit key players in autophagy, including Beclin 1. The inhibition of autophagosome formation can lead to the accumulation of p62. Together, the above findings suggest that decreased autophagy in ATMs during obesity may be an additional mechanism contributing to increased cell survival. However, much of the literature suggests that the role of autophagy in inhibition or activation of cell survival is context dependent. Genetic manipulation of p62 *in vivo* or *in vitro*, under obese metabolic conditions, could help determine whether p62 is involved in ATM survival in obesity. Furthermore, this could help determine if p62 activates or antagonizes NF-κB-induced ATM survival in the obese setting.

An additional mechanism that may be involved in ATM survival is signaling through the nuclear factor E2-realted factor-2 (Nrf2) pathway. Nrf2 is a transcription factor that is induced under oxidative stress conditions where it plays a role in inducing the transcription of antioxidant genes to counter the dangerous effects of reactive oxygen species. Like NF-κB, Nrf2 promotes survival by inducing the transcription of pro-survival proteins Bcl-2 and Bcl-xl (154, 155). In regards to improving the AT inflammatory state, global deficiency of Nrf2 protects against diet-induced obesity (156). Furthermore, studies demonstrate that myeloid specific deletion of Nrf2 decreased the number of CLSs in HFD fed mice; however this deficiency did not protect from HFD-induced AT inflammation and IR (107). Future studies are needed to better elucidate the role of Nrf2 in ATM number and AT inflammation during obesity as well as whether Nrf2 and NF-kB pathways intersect in ATMs. Inhibition of these pathways, through genetic or pharmacologic manipulation, in ATMs under obesogenic conditions could help provide some insight into if these pathways play a role ATM survival.

Is macrophage turnover a normal aspect of tissue homeostasis and does it apply to AT?

Interestingly, studies suggest that macrophage turnover is a normal process that occurs to maintain and restore tissue homeostasis in other tissues. This brings into light the importance of macrophage self-renewal in the maintenance of homeostasis (139, 157). Embryonic-derived resident macrophages are present

through the development of different tissues and replenish macrophage content in tissues over a period of time to maintain tissue homeostasis. Conversely, monocyte-derived macrophages are recruited from the bone marrow, particularly during times of infection or inflammation. Under inflammatory conditions or pathological stress, recruited macrophages replace resident macrophages in different tissues – a process likely relevant in AT. In AT, anti-inflammatory macrophages are thought to be resident macrophages, whereas pro-inflammatory macrophages are recruited. If macrophage self-renewal is applicable to AT, it is possible that resident and recruited macrophages are derived from different precursors and respond differently to metabolic inflammation. If so, the inflammatory status of obese AT could result in the inability of resident ATMs to self-renew during the early stages of obesity-resulting in their replacement by recruited monocyte-derived ATMs. Although replacement of resident ATMs with monocyte derived cells is likely a compensatory mechanism to restore AT homeostasis, the increased NF-κB activity present in these ATMs could possibly prevent them from undergoing apoptosis unlike their embryo-derived counterparts. From a physiological standpoint, these ATMs maybe attempting to restore AT homeostasis; however, the obese milieu may indirectly drive their perpetuation of AT dysfunction through promoting their survival and inflammatory state by activating NF-κB. It would be interesting to use lineage tracing studies to determine whether resident and recruited macrophages are derived from different precursors. If so, their

susceptibility to apoptosis could be determined using *in vitro* and *ex vivo* MetaC studies.

What controls macrophage turnover?

The data presented in this dissertation details how ATM apoptosis is inhibited; however, the mechanisms that control ATM turnover in a normal setting still remain unknown. The interaction of ATMs in the lean state with other immune cells may provide some insight. In lean AT, eosinophils are present in greater numbers than in the obese state. Eosinophils have been demonstrated to control macrophage phenotype by secreting anti-inflammatory phenotype polarizing agents, IL-4 and IL-13(115, 158). Furthermore, eosinophils have been demonstrated to release pre-resolvins to aid in the attenuation of peritonitis (159). Interestingly, these molecules increased the expression of adiponectin, which has been shown to promote an anti-inflammatory phenotype (160). Like eosinophils, studies demonstrate that resolvins are increased in the lean compared to obese state and are important in restoring AT homeostasis during weight loss (161). One would speculate that if anti-inflammatory macrophages preferentially undergo apoptosis, the interaction between eosinophils and ATMs could be regulating their phenotype as well as turnover. In fact, preliminary studies from the Hasty laboratory demonstrate, in a model of eosinophilia, that the increased presence of eosinophils is strongly correlated with reduced numbers of ATMs. In opposition, the almost complete absence of eosinophils in obese AT may perpetuate the inflammatory activation state and survival of

ATMs. Others have hypothesized that eosinophils are major regulators of tissue homeostasis in both health and disease state (162). It is likely that regulation of ATM apoptosis by eosinophils may be a part of normal maintenance of tissue homeostasis.

In conclusion, the studies performed during the completion of this dissertation have provided insight into the regulation of ATM survival and accrual in during obesity. These findings suggest that activation of NF-κB in ATMs is responsible for their pro-survival phenotype. Furthermore, this body of work has increased the understanding of mechanisms and consequences of immune cell accumulation in AT.

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Appendix A

NF-κB Inhibition in ATMs Cultured in Adipocyte Conditioned Media

Introduction. NF-κB has been shown to promote survival in many cell types in the presence of a death stimulus *in vivo* and *in vitro* (163, 164). Obese AT is thought to be a toxic environment that exposes macrophages to secreted molecules that could induce apoptosis in ATMs and other stromal vascular cells (SVCs). The goal of these studies was to determine whether inhibition of NF-κB can promote apoptosis of ATMs *ex vivo*.

Experimental design: The macrophage selection by adhesion assay was performed on the SVF of obese mice fed HFD for 9 weeks to obtain ATMs for *ex vivo* studies. AT from 9 week HFD obese mice were cultured for 24 h to obtain adipocyte conditioned media. The conditioned media from these adipocytes was used to culture ATMs collected from an additional obese mouse for 2 hours in the presence or absence of 10μM of the NF-κB inhibitor, BMS-345541 (BMS). Adipocyte conditioned media (AD media) was also obtained from 9 LFD mice for control purposes. ATMs in DMEM alone or LFD conditioned media should not promote a pro-inflammatory phenotype or activation of NF-κB. Western blot analysis for phosphorylated p65 (P-p65) and cleaved caspase 3 was utilized to assess NF-κB activation and apoptosis in ATMs cultured in DMEM, LFD AD media, and HFD AD media alone or in the presence of BMS. ATMs cultured in HFD AD media alone should have increased NF-κB activation compared to

DMEM and LFD AD media conditions. Addition of BMS should induce apoptosis in all groups but have a greater effect on ATMs in the HFD AD media condition.

Results. To determine the role of NF-κB activation in ATM survival under obesogenic conditions, I treated ATMs with AD media in the presence of absence of the NF-κB inhibitor BMS. HFD AD media alone did not significantly increase P-p65 activation in ATMs compared to DMEM or LFD AD media treated ATMs (Figure A1.1A). Furthermore, addition of BMS decreased p65 activation in all treatment groups (Figure A1.1A). However, cleaved caspase 3 activation was not increase in ATMs in HFD AD media conditions compared to DMEM or LFD AD media treated groups (Figure A1.1 B).

Conclusions. My hypothesis suggested that elevated levels of NF-κB activation in ATMs protects them from the toxic environment of obese AT. In these *ex vivo* studies, treatment of ATMs with HFD AD media did not recapitulate the increased protein levels of Pp65 in ATMs in HFD compared to the LFD mice as seen in Figure 3.10 of Chapter III. As a result, I concluded that the HFD AD media would not activate NF-κB in ATMs to level that would allow for the role of NF-κB in ATM survival in the obese setting to be adequately assessed. An alternative method of NF-κB activation was used to circumvent this issue. This is described in greater detail below.

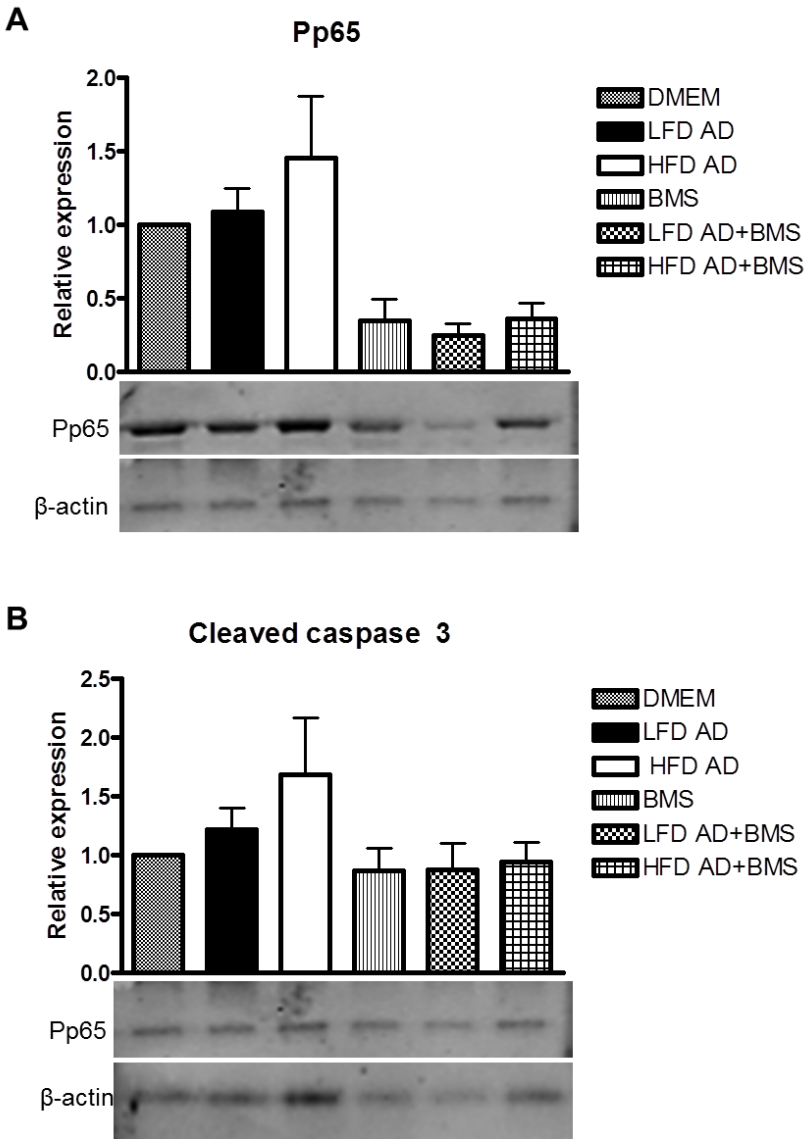

Figure A1.1: Adipocyte conditioned media and BMS treatments in ATMs. ATMs from male C57BL/6 mice where treated with DMEM or AD media from LFD or HFD fed mice in the presence or absence of BMS for 8 h. Protein was collected for Western blot analysis. Treatment groups are as follows: DMEM, LFD AD media (LFD AD), HFD AD media (HFD AD) with or without BMS. A) Protein levels of phosphorylated p65. B) Protein levels of cleaved caspase 3. Data are presented as mean ± SEM, n = 4/group.

Alternative Experimental Design. To circumvent the issues with the above experiment, an alternative obesogenic treatment cocktail was used to induce

increased activation of p65 under HFD obesogenic conditions (Figure A1.2A). Previous studies by Kratz et al. demonstrated that metabolic activation of BMDMs induced a genetic profile that resembled that of ATMs found in the obese state (53). I used this model system to determine the role of NF-κB in ATM survival *ex vivo*. Before beginning my studies, I wanted to determine if metabolic activation of ATMs from 3 week HFD mice would induce NF-κB activation in ATMs from male NGL mice were treated with DMEM or MetaC for 0-24 h. The metabolic activation cocktail (MetaC) in the Kratz et al. studies contained 30mM glucose, 10nM insulin, and 0.4 mM palmitic acid. I also used a MetaC cocktail containing oleic acid to determine if the saturation state of the fatty acid played a role in NF-κB activation. As detailed in Chapter I, SFAs activate the NF-κB signaling pathway and are highly increased in obesity, whereas UFAs are not. I would expect that the UFA, oleic acid, would not induce NF-κB activation like the SFA, palmitic acid.

Results. MetaC containing palmitic acid induced a significant increase in NF-κB-induced luciferase activity at 1 h ($p<0.01$) and 4 h ($p<0.05$) after treatment compared to DMEM or MetaC containing oleic acid (Figure A1.2A). This difference was not apparent after 6-24 h of treatment.

Conclusions. The above finding were important because it demonstrated 1) that this model system would be a useful tool to study the role NF-κB in ATM survival *ex vivo* and 2) recapitulated the idea that SFAs significantly induced the NF-κB

signaling pathway compared to UFAs, further confirming that components of the obesogenic environment promoted NF-κB activation. This metabolic activation cocktail was used to perform the experiments in Figure 3.12 of Chapter III.

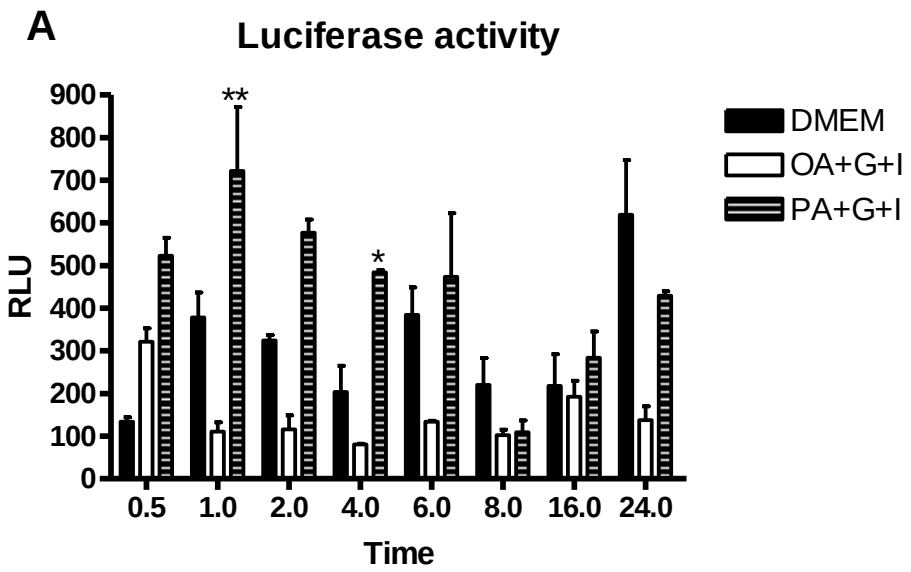

Figure A1.2: Obesogenic metabolic cocktail (MetaC) increases NF-κB-induced luciferase activity in ATMs. ATMs from male NGL mice were treated with DMEM, MetaC containing oleic acid (OA+G+I: 0.4 mM oleic acid, 30mM glucose, 10nM insulin) or MetaC containing palmitic acid (PA+G+I: 0.4 mM palmitic acid, 30mM glucose, 10nM insulin) for 0-24 h. After treatment, a luciferase assay was performed to determine luciferase activity in ATMs at each timepoint in the study. Data are presented as mean ± SEM, n = 2-3/group.
* $p<0.05$ and ** $p<0.01$ between groups.

Appendix B

Macrophage-specific, Inducible NF-κB Inhibition Model

Introduction. My initial Thesis Proposal contained a description of *in vivo* experiments to determine the role of NF-κB in ATM apoptosis in obese mice. Drs. Timothy Blackwell and Fiona Yull from Vanderbilt University generated inducible transgenic mouse models that allow for macrophage-specific inhibition of NF-κB. This model is referred to as NF-κBi. Previous studies have demonstrated that treatment of these transgenic mice with doxycycline (dox) is sufficient to induce inhibition of NF-κB in macrophages *in vivo (165)*. The goal of these studies was to use this model to inhibit NF-κB in ATMs and to determine the impact of this on their ability to survive in the obesogenic environment.

Experimental design. The *cfms* promoter was used to target expression of the reverse tetracycline transactivator specifically in macrophages to drive the expression of a tetracycline operon that controls the dominant negative form of the NF-κB inhibitor, IκBα (Figure B1.1). C57BL/6 male inducible NF-κBi transgenic mice expressing a dominant negative IκBα (DN-IκBα) were used for these experiments. NF-κBi transgenic mice were placed on HFD for 8 weeks then continued on diet and given dox for 1 or 4 weeks as previously described (165). Dox was administered in the drinking water at a dose of 2 mg/ml. Littermate controls that do not express the transgene (control) and NF-κBi mice

without dox treatment (vehicle) under the same conditions were used as controls. Prior to the initiation of the proposed studies, WT mice were placed on dox to rule out any effects of this drug on ATM apoptosis. Introduction of dox should induce activation DN-IκBα specifically in macrophages, thus resulting in NF-κB inhibition. Confirmation of DN-IκBα was determined via mRNA expression of the SVF collected from treated mice. The primary endpoint measurements for this experiment are listed below in (Table B1).

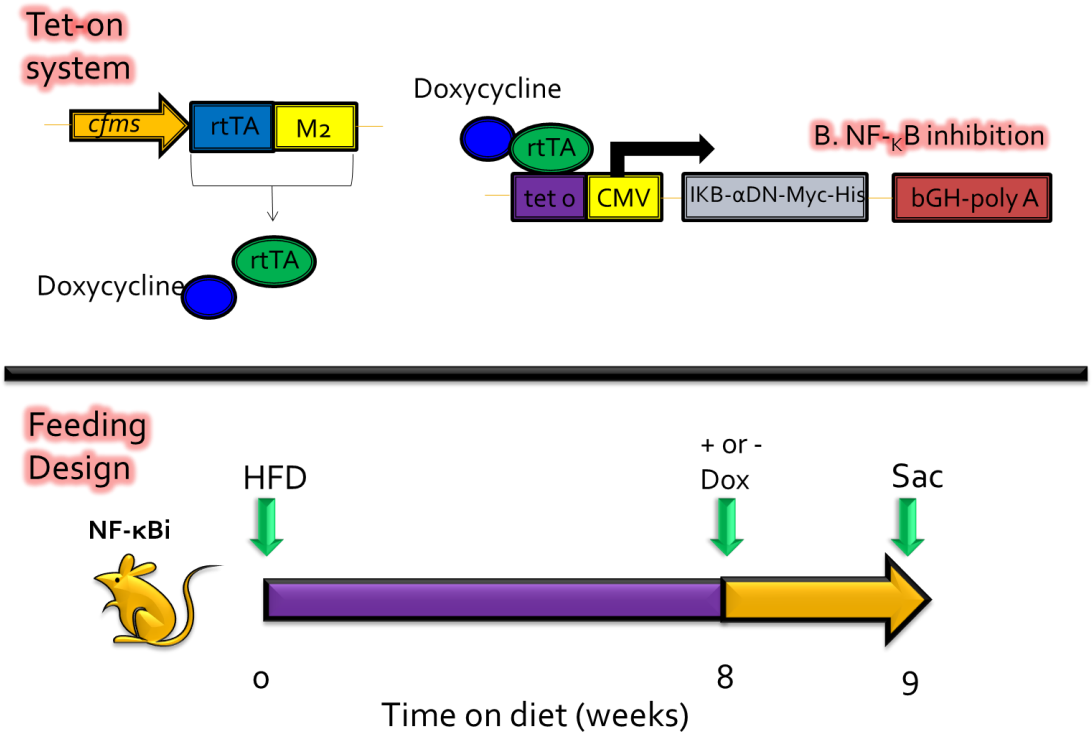

Figure B1.1. Tet-on system and feeding study design.

Table B1	Measure	Method
Activation of NF-κB in ATMs	p65 translocation into the nucleus of ATMs	Immunofluorescence for NF-κB, F4/80
NF-κB targeted inflammatory genes	Expression of TNF-α, Bcl-2 family genes, IAPs, iNOS	Real-time PCR
ATM Apoptosis	Apoptosis	TUNEL staining and Cleaved caspase 3
Changes in pro/ anti-survival protein expression ATMs	NF-κB, Bcl-2 family proteins, caspase 3	F4/80 sort of ATMs, Western blot, Immunofluorescence for NF-κB, F4/80 and Cleaved caspase 3 in AT explants
AT insulin sensitivity	AKT phosphorylation in AT explants	Insulin injections, Western blot
Subcellular localization of Bcl2 and BAX	Localization of Bcl2 and Bax to the mitochondria	Western blot for Bcl2 and Bax in the mitochondrial fraction

Results. After 1 week of dox treatment, mRNA expression of the DN-IκBα transgene was seen in the NF-κBi compared to the WT mice (Figure B1.2A). However, DN-IκBα protein expression was not able to be verified, suggesting that the protein was not being expressed (Figure B1.3A). Additionally, phosphorylated p65 (Pp65) protein expression was not decreased in dox treated NF-κBi mice (Figure B1.3A). In light of these findings, these data suggested that inhibition of NF-κB in ATMs did not occur. Furthermore, there was no difference in TUNEL staining between groups (Figure B1.4A). When both groups were treated with dox only for 4 weeks, there was increased expression of mRNA levels of DN-IκBα in NF-κBi mice (Figure B1.5A-B). However, expression of the DN-IκBα protein was not seen after 4 weeks of dox treatment (Figure B1.6A-B). Additionally, TUNEL staining was not detected in ATMs from 4 week dox treated NF-κBi mice (Figure B1.7A-B).

Conclusions: In light of the above results, I concluded that the *cfms* promoter was not strong enough to drive protein expression of the DN-IκBα transgene in

NF-κBi mice even after extended dox treatments. Therefore, NF-κB activity would not be inhibited and its role in ATM apoptosis *in vivo* cannot be tested. Furthermore, the *cfms* promoter is not expressed in all macrophages. This could result in the promoter not being expressed in ATMs and would further complicate the study. Due to the inability to verify the protein expression of the DN-IκBα transgene, I was unable to move forward with any of the proposed experiments for this model.

Figure B1.2. mRNA gene expression levels of DN-IκBα in 1 week vehicle or dox treated control and NF-κBi mice. Mice were treated with or without dox for 1 week. SVF was collected for mRNA expression analysis. GAPDH was used as a loading control. n= 5-6/group

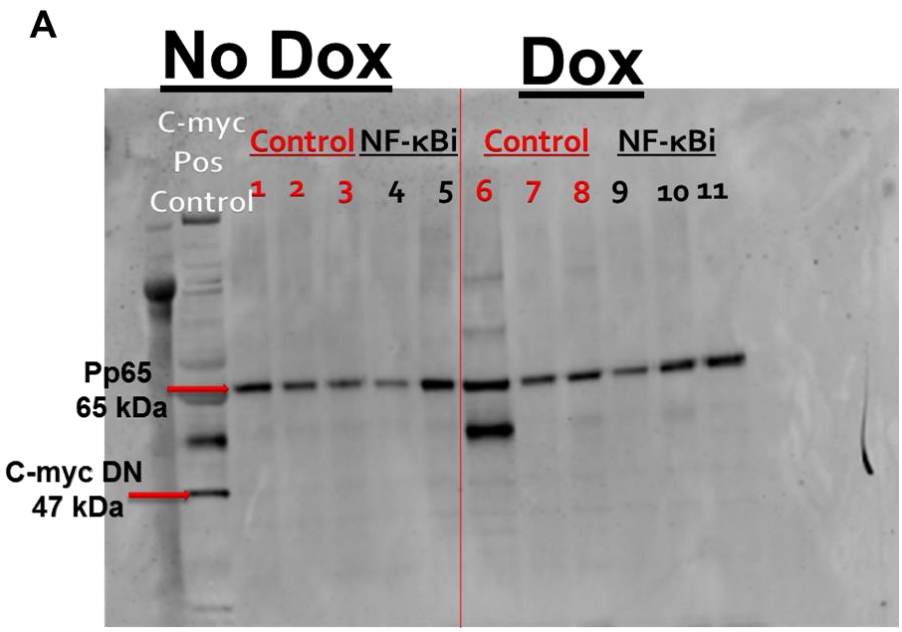

Figure B1.3. Protein expression levels of Pp65 and DN-IκBα in 1 week vehicle or dox treated control and NF-κBi mice. Mice were treated with or without dox for 1 week. Protein was collected from the SVF for Western blot analysis. Protein levels of Pp65 and the DN-IκBα transgene was analyzed. n= 5-6/group

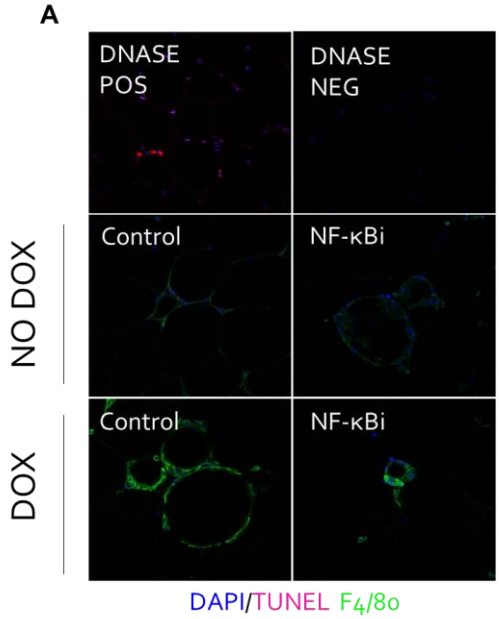

Figure B1.4. TUNEL staining in AT of 1 week vehicle and dox treated control and NF-κBi mice. Mice were treated with or without dox for 1 week. AT was collected for immunofluorescence staining of Dapi (blue), F4/80 (green) and apoptosis marker, TUNEL (pink). n= 5-6/group.

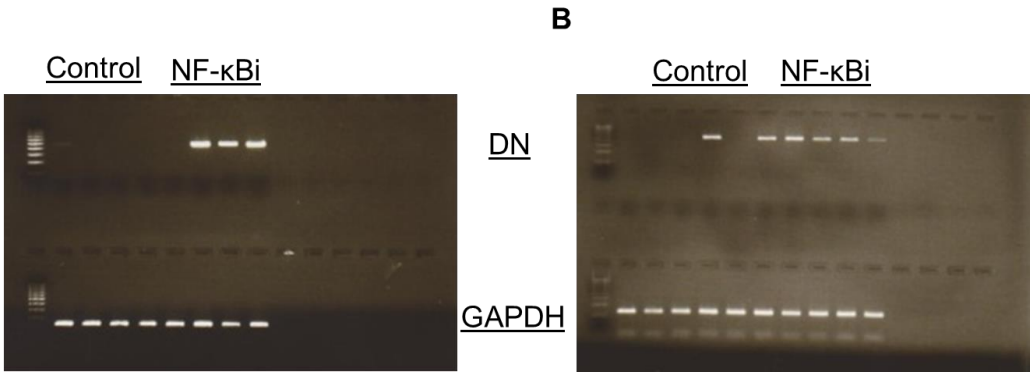

Figure B1.5. mRNA gene expression level of DN-IκBα in 4 week dox treated NF-κBi mice. Mice were treated with dox for 4 weeks. SVF was collected for mRNA expression analysis. GAPDH was used as a loading control. n= 4/group

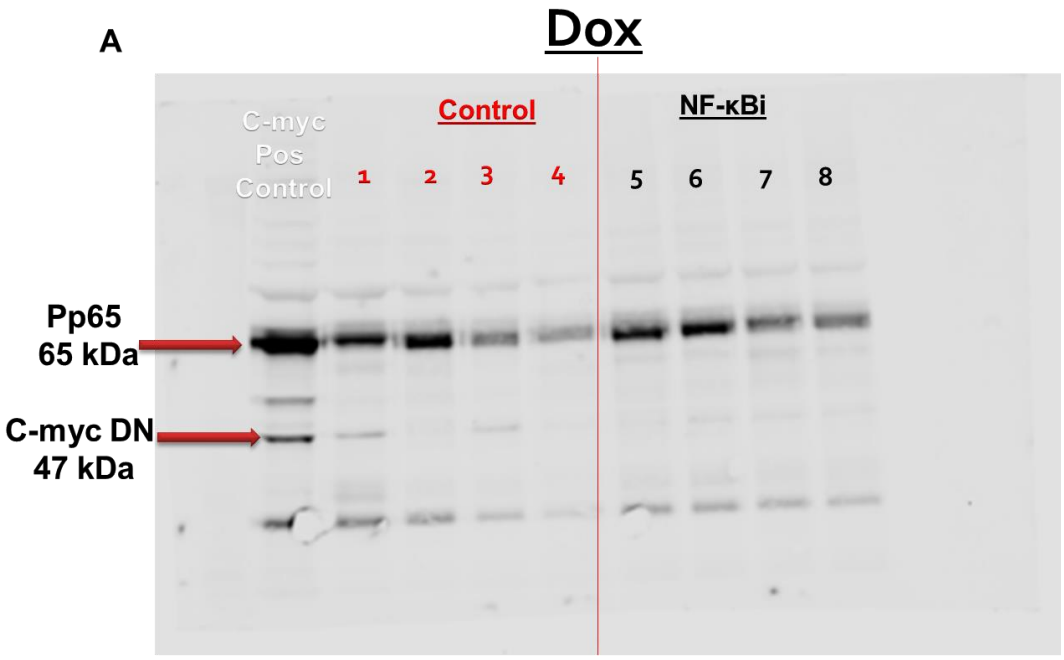

Figure B1.6. Protein expression levels of Pp65 and DN-IκBα in 4 week dox treated control and NF-κBi mice. Mice were treated with dox for 4 weeks. Protein was collected from the SVF for Western blot analysis. Protein levels of Pp65 and the DN-IκBα transgene was analyzed. n = 4/group.

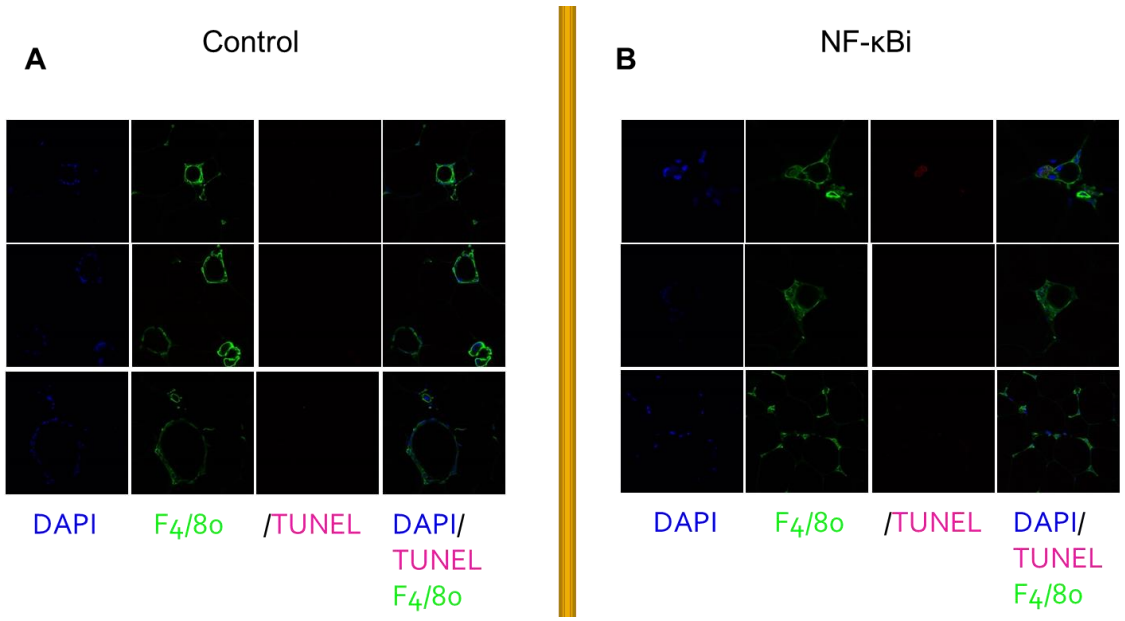

Figure B1.7. TUNEL staining in AT of 4 week dox treated control and NF-κBi mice. Mice were treated with dox for 4 weeks. AT was collected for immunofluorescence staining of Dapi (blue), F480 (green), and apoptosis marker, TUNEL (pink), in ATMs. n= 3/group.

Appendix C

Pharmacological Inhibition of NF-κB In Vivo

Introduction. As an alternative to the above studies, I performed *in vivo* pharmacological inhibition of NF-κB in ATM studies using BMS-34551 (BMS). Previous studies have demonstrated treatment of an LPS NGL model with 75ug/g significantly decreased LPS induced luciferase activity (104). The goal of my studies was to use pharmacological inhibition of NF-κB *in vivo* to determine the role of NF-κB in ATM survival.

Experimental Design. All studies were performed after obtaining IACUC approval. Eight week old male NGL mice fed LFD and HFD for 9 weeks were used for these studies. The selective IKK inhibitor, BMS-345541 (BMS), was administered intravenously at doses of 0 µg/g- 100 µg/g once a day for a period of up to 24 h. Previous studies demonstrate that administration of 100 µg/g per day for up to 6 weeks did not display any toxicological effects (166). Vehicle (3% Tween 80 and sterile water) was administered as a control. BMS was obtained from Sigma-Aldrich. The compound was formulated as a 7.5 mg/ml solution in 3% Tween 80 and sterile water. One hour prior to sacrifice a selective caspase inhibitor probe, FLIVO, would be administered intravenously. Mice would be injected with 100mcl of a 1x concentration of FLIVO (ImmunoChemistry Technologies). Prior to these studies, a BMS treatment timecourse study was performed on 9 week LFD and HFD-fed NGL mice to determine the proper time

ATM apoptosis should be assessed after treatment (Figure C1.1). This study is further detailed below. A significant decrease in body luminescence intensity (BLI) would suggest that NF-κB transcriptional activity is decreased and therefore ATM apoptosis may occur. NGL mice were treated with BMS as described above and then injected with the luciferase substrate, luciferin, at 1mg/mouse. BLI was measured using the Xenogen IVIS 200 imaging system in the Vanderbilt imaging core at 1, 6, 8 and 24 h after BMS treatment. Experimental groups were as follows: LFD/Vehicle, HFD/Vehicle and HFD/BMS.

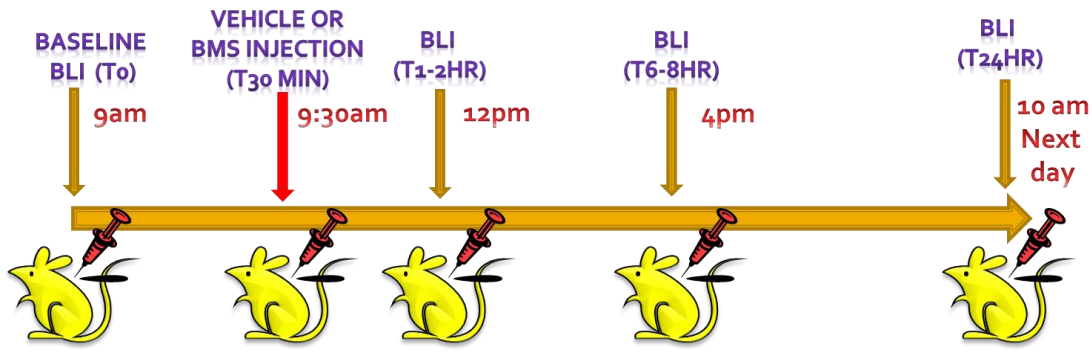

Figure C1.1.Timecourse experimental design for BLI imaging of BMS treated NGL mice. Vehicle and BMS treated NGL mice were injected with the luciferase substrate, luciferin, at 1mg/mouse and BLI was measured immediately at each designated timepoint. Images were taken using the Xenogen IVIS 200 bioluminescent and fluorescent imaging system at Vanderbilt Imaging Institute.

Results. To determine the role of NF-κB in ATM survival *in vivo*, 9 week LFD or HFD-fed NGL mice were treated with vehicle or the NF-κB inhibitor, BMS for 0-24 h (Figure C1.1). At treatment T0, all mice displayed luciferase activity, as seen by BLI, demonstrating that all mice can be used for NF-κB-induce luciferase activity measurements (Figure C1.2A). Unexpectedly, HFD fed mice did not display increased NF-κB-induce BLI compared to lean mice at T0. Control and BMS treated mice displayed decreased BLI at T1, T6 and T24 h compared to T0 (Figure C1.2A-D). However, BMS treatment of HFD mice did not decrease luciferase activity compared to the LFD and HFD vehicle treated groups (Figure C1.2C-D). Furthermore, luciferase activity returned to baseline levels 24 h after treatment (Figure C1.2E).

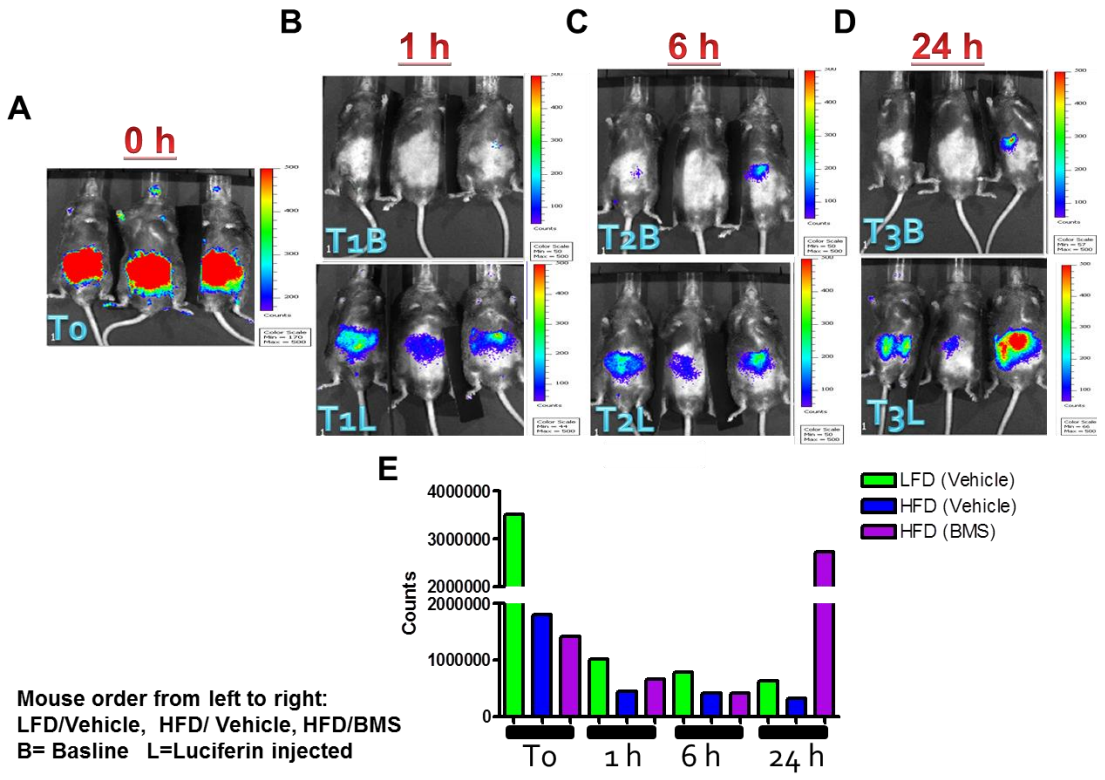

Figure C1.2. Live Imaging of vehicle and BMS treated LFD and HFD NGL mice. NGL mice were treated with BMS and imaged for luciferase activity at 1, 6 and 24 h after injection. A) Time zero (T0) whole BLI before vehicle or BMS administration. B) Baseline measurement before luciferin injection for 1 h timepoint (T1) (top panel). BLI 1 h after BMS treatment (bottom panel). C) Baseline measurement before luciferin injection for 6 h timepoint (T6) (top panel). BLI 6 h after BMS treatment (bottom panel). D) Baseline measurement before luciferin injection 24 h timepoint (T24) (top panel). BLI 24 h after BMS treatment (bottom panel).E) Graph of BLI counts for each experimental group at each time point. n = 1 per group

Conclusions. These observations suggested that there was no difference in the levels of luciferase activity between control and BMS treatment groups at T0 or throughout the timecourse study. This may be due to the high baseline levels of NF-κB-induced luciferase activity in various tissues with high proliferation such as the intestines. If so, imaging through the abdomen of the mouse would make it

difficult to determine luciferase activity in AT. To circumvent this issue, the AT would have to be imaged alone. This may it difficult to determine if ATM apoptosis was due to NF-κB inhibition or technical matters. Due to the inability to determine if this technique would be useful and cost effective, I chose not to move forward with these studies.