ADIPOSE TISSUE INFLAMMATION IS ASSOCIATED WITH IMMUNE DYSFUNCTION DURING INFLUENZA VIRUS INFECTION

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ABSTRACT

KATHRYN A. COLE: Adipose Tissue Inflammation Is Associated With Immune Dysfunction During Influenza Virus Infection
(Under the direction of Melinda A. Beck, Ph.D.)

Adipose tissue in the obese state expresses increased concentrations of inflammatory mediators and has an increased number of macrophages and T lymphocytes. This raises questions about the role that adipose tissue plays during an infectious disease. This dissertation describes research in diet-induced obese mice that 1) provides a timeline of changes in body composition, hormonal and metabolic alterations, and the onset of inflammation in the liver and three adipose tissue depots; and 2) demonstrates that during an infectious disease, distant adipose tissue depots undergo changes in their inflammatory state and number of leukocytes. A third, conceptually distinct part of this dissertation is the study of a mechanism linking preadipocyte proliferation with differentiation.

The differentiation of preadipocytes contributes to increased adipose tissue mass through increased capacity to store triacylglycerol. Differentiation-induced 3T3-L1 preadipocytes undergo several rounds of mitotic clonal expansion while concurrently initiating a cascade of transcription factor expression that culminates in the adipocyte phenotype. We demonstrated that G1 of the cell cycle and the initiation of differentiation are functionally linked by the interaction of hypophosphorylated Rb and C/EBPβ.

In order to establish a timeline of the development of inflammation in diet-induced obesity, weanling C57BL/6 mice were fed either a low-fat or a high-fat diet for 16 weeks.

Our data show that the inflammatory state in adipose tissue is depot-specific, and occurs prior to the development of liver steatosis, and increased fasting serum leptin and insulin concentrations.

Previous work in our laboratory demonstrated that compared to lean mice, diet-induced obese mice have higher morbidity and mortality after influenza virus infection. We demonstrate that during influenza A/PR/8/34 virus infection, relative to lean mice, adipose tissues in obese mice have a greater pro-inflammatory cytokine and chemokine gene expression, with a decrease in the number of macrophages and T lymphocytes in the gonadal adipose tissue depot.

Taken together, our studies demonstrate that obesity can have a profound influence on the immune response to an infectious disease and that adipose tissue itself may be a major component of the dysregulated immune response during influenza infection.

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LIST OF ABBREVIATIONS

ACTH	adrenocorticotropic hormone
ALT	alanine aminotransferase
APC	antigen presenting cell
APP	acute-phase protein
ARA	arachidonic acid
AST	aspartate aminotransferase
AT	adipose tissue
BSA	bovine serum albumin
BMI	body mass index
CD	cluster of differentiation
Cdk	cyclin-dependent kinase
C/EBP	CCAAT/enhancer binding protein
Con A	concanavalin A
CVD	cardiovascular disease
D	dexamethasone
DC	dendritic cell
DHA	docosahexanoic acid
DIO	diet-induced obesity
EC	endothelial cells
EMSA	electrophoretic mobility shift assay
EPA	eicosapentanoic acid

F	fibroblasts
Fc	fragment crystallizable
G0	growth arrest
G1	Gap 1 (phase of cell cycle)
G2	Gap 2 (phase of cell cycle)
G3PDH	glyceraldehyde-3-phosphate dehydrogenase
GC	glucocorticoids
HA	hemagglutinin
HAU	hemagglutinating unit
HF	high fat
Influenza PR8	influenza A/Puerto Rico/8/34
I	insulin
ICAM-1	intercellular adhesion molecule-1
Ig	immunoglobin
IL	interleukin
KC	Kupffer cells
LF	low fat
M	mitosis
MCP-1	monocyte chemotactic protein-1
MDI	methyl-3-isobutylxanthine, dexamethasone and insulin
MHC	major histocompatability complex
MIP-1α	macrophage inflammatory protein-1alpha
MIX	1-methyl-3-isobutylxanthine

NA	neuraminidase
NAFLD	nonalcoholic fatty liver disease
NASH	nonalcoholic steatohepatitis
NF-κB	nuclear factor kappa B
NK	natural killer cell
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PC	parenchymal cells
PD	pocket domain
PPAR	peroxisome proliferator-activated receptor
PUFA	polyunsaturated fatty acids
qRT-PCR	quantitative real time polymerase chain reaction
R	restriction point (of cell cycle)
Rb	Retinoblastoma protein
ROS	reactive oxygen species
S	Synthesis (phase of cell cycle)
STAT	signal transducers and activators of transcription
TAG	triacylglycerol
TCR	T-cell receptor
Th	T-helper cell
TLR	toll-like receptor
TNFα	tumor necrosis factor alpha
VCAM-1	vascular cell adhesion molecule-1

CHAPTER I

Introduction

A. Public health impact of obesity

Obesity is a complex disease that involves environmental, genetic, physiologic, metabolic and psychological components. Worldwide, obesity affects 300 million adults. In the U.S., approximately 127 million adults are overweight, 60 million are obese, and 9 million are severely obese [1]. Body mass index (BMI), expressed as weight/height^2 (BMI; kg/m^2), is commonly used to determine overweight (BMI equal to 25.0-29.9), obesity (BMI greater than or equal to 30.0), and severe obesity (BMI greater than 40), because for most adults, it correlates with their amount of body fat.

Obese individuals are at increased risk for many diseases and health conditions, including the following: hypertension, osteoarthritis, dyslipidemia, type 2 diabetes, coronary heart disease, stroke, gallbladder disease, sleep apnea and respiratory problems, and endometrial, breast and colon cancers [1]. Obesity's strong association with hypertension is due to central and peripheral abnormalities that increase arterial pressure, such as activation of the sympathetic nervous system and the renin-angiotensin–aldosterone system. Obesity is also associated with endothelial dysfunction and changes in renal function that may contribute to hypertension [2]. Type 2 diabetes is associated with obesity, and stems from insulin resistance coupled with the inability to produce enough insulin to overcome this resistant state. Several adipocyte-derived factors have been shown to contribute to systemic insulin resistance, including resistin, TNFα, IL-6, and MCP-1. Also, adipocyte-derived free fatty acids contribute to insulin resistance in liver and muscle [3]. Obesity and its comorbidities increase the risk of coronary heart disease. When the arterial endothelium is exposed to high concentrations of non-esterified fatty acids or inflammatory cytokines derived from excess adipose tissue, vasoconstrictor hormones (in hypertension), and/or the products of

glycoxidation (in insulin resistance), these cells increase the expression of adhesion molecules [4]. Leukocyte adhesion and transmigration in the arterial intima initiate an inflammatory response, which is followed by lesion formation and atherosclerotic plaque formation [4]. Blood flow occlusion may result in a myocardial infarction or a stroke. The relationship of obesity to gallbladder disease remains to be elucidated, but may be due to obesity's association with insulin resistance. Insulin resistance causes a decrease in gallbladder contractility that may result in gallstone formation [5]. Obstructive sleep apnea is characterized by episodes of upper airway obstruction with a subsequent fall in blood oxygen saturation. Sleep apnea develops due to increased mechanical loads on the soft tissues of the upper airway and defects in compensatory neuromuscular responses [6]. Cancer risk is increased in obesity. In obese women, increased exposure to endogenous steroids may increase brease cancer risk. However, other factors may also be important. For example, increased serum insulin, insulin-like growth factor-I, and leptin may facilitate angiogenesis necessary for tumor growth [7]. Similarly, the increased risk of colon cancer in obesity may be the result of insulin resistance. Increased circulating levels of insulin, triglycerides, and non-esterified fatty acids are a proliferative stimulus for colonic epithelial cells and also expose them to reactive oxygen intermediates, which may promote colon cancer [8].

The adverse health consequences of obesity have an immense economic impact. The World Bank estimated that in the late 1990s, obesity cost the U.S. 12% of its national health care budget, or $117 billion ($61 billion direct and $56 billion indirect health care costs). Moreover, the prevalence of overweight and obesity among children, adolescents and adults is rapidly increasing. Therefore, the future economic and health impacts of obesity are likely to be both substantial and long-lasting.

B. *In vitro* adipogenesis in the study of obesity

Obesity is the accumulation of excess adipose tissue (AT) mass. In obesity, adipocyte hyperplasia occurs through the recruitment and proliferation of preadipose cells in the vascular stroma of the adipose tissue [9-12]. This hyperplasia is mimicked *in vitro* by inducing fibroblastic preadipocytes to undergo mitotic clonal expansion, which culminates in the expression of genes required for specialization in triacylglycerol (TAG) storage and the mature adipocyte phenotype.

1. Overview of *in vitro* adipogenesis

Adipocyte development can be reproduced in cell culture. Much of what is known about adipocyte development has been elucidated from established cell lines that mimic adipocyte proliferation and differentiation *in vivo*. The 3T3-L1 preadipocyte cell line provides a well-characterized model for the study of adipocyte-specific terminal differentiation. 3T3-L1 preadipoctyes acquire the ability to differentiate after they reach confluence and become growth arrested in G0 of the cell cycle. The addition of 1-methyl-3-isobutylxanthine (MIX), dexamethasone (D), and high doses of insulin (I) or physiological doses of Insulin-like Growth Factor-1 to the serum-containing medium (MDI protocol) concurrently activates two different processes: the synchronous reentry of the preadipoctyes into the cell cycle and the initiation of differentiation. Therefore, one preadipocyte, which does not store large amounts of TAG, gives rise to 2-4 adipocytes that specialize in TAG storage [13].

2. Adipogenic transcription factors

The major transcriptional regulators that govern adipogenesis are peroxisome proliferator activated receptor gamma (PPARγ) and the CCAAT-enhancer binding protein (C/EBP) family members [14-17]. Several loss- and gain-of-function studies have shown that PPARγ is both

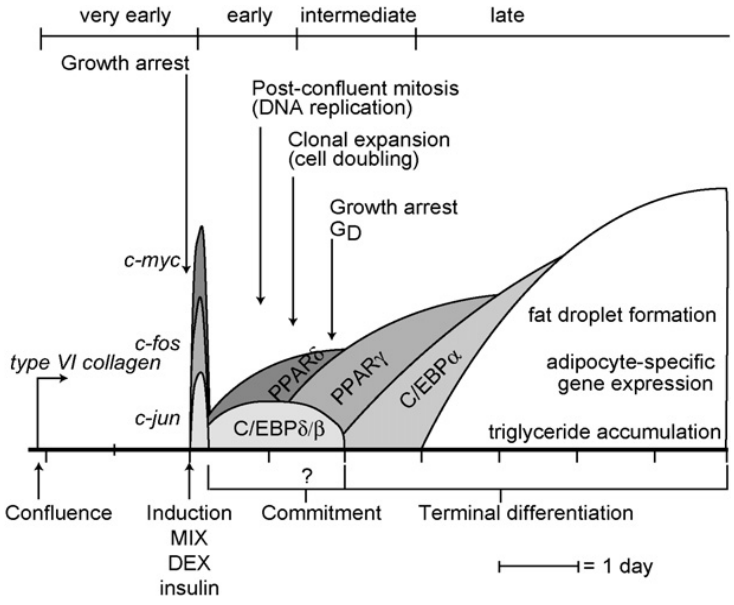
necessary and sufficient for *in vitro* adipogenesis [14, 18], as well as for *in vivo* adipogenesis [19, 20]. Although PPAR-/- mice are not viable, adipose tissue-specific PPARγ-/- mice have much less adipose tissue than wild type mice [21]. The deletional strategies used to attain adipose tissue-specific PPARγ-/- mice provide important information about the essential functions of PPARγ at different developmental stages. When PPARγ is deleted before adipose development, the mice are not viable. In contrast, when the deletional strategy is linked to aP2 gene expression, which does not peak until the adipocyte is mature, PPARγ is deleted at a later developmental stage [21]. The resulting adipose tissue-specific PPARγ-/- mice are viable, but have much less white adipose tissue. Similarly, C/EBPα-/- mice are not viable [22], and adipose tissue-specific C/EBPα-/- mice have much less fat than wild-type mice [23]. *In vitro*, C/EBPα is sufficient for the differentiation of 3T3-L1 preadipoctyes, as inducible expression of C/EBPα in 3T3-L1 preadipocytes resulted in their differentiation without requiring the addition of the MDI induction cocktail [24].

Transcription factors in the C/EBP family function in a cascade-like manner to activate adipogenesis [25, 26]. C/EBPβ and C/EBPδ function synergistically to initiate the cascade [27]. *In vitro,* C/EBPβ/δ-deficient mouse embryonic fibroblasts fail to synthesize C/EBPα and PPARγ, and cannot differentiate into mature adipocytes with the aid of MDI [28]. However, ectopic expression of PPARγ2 and activation with its ligands induces their differentiation into adipocytes [29]. The resulting C/EBPβ/δ-/- adipocytes have less GLUT4 and IRS-2 expression, and reduced insulin-responsive glucose uptake. *In vivo,* C/EBPβ·C/EBPδ double knockout mice have low viability, with survivors having 70% less AT than wild-type mice [28]. Therefore, while steps in the transcriptional cascade can be bypassed *in vitro*, C/EBPβ and C/EBPδ are important for *in vivo* adipocyte differentiation.

While C/EBPβ and C/EBPδ are synthesized and translocated to the nucleus within four hours after the induction of differentiation, they do not bind to DNA until eight hours later

[30]. The mechanism for this delay is unknown. C/EBPβ induces its own synthesis and cooperates with C/EBPδ to activate the synthesis of peroxisome proliferators-activated receptor gamma (PPARγ) and C/EBPα, which are critical adipogenic transcription factors [31]. C/EBPα is a differentiation-specific transcription factor with antiproliferative activity [32].C/EBPα is expressed between day 2 and day 5, and further induces the expression of PPARγ [33]. C/EBPα and PPARγ function synergistically to increase the expression of adipocytes-specific genes. By the fourth day after the induction of differentiation, the cells exhibit biochemical and morphological characteristics of mature adipocytes [34]**.** PPARγ transactivation of its target genes is required for adipocyte differentiation [35].

Figure 1.1: Progression of 3T3-L1 preadipocyte differentiation. Major events of preadipocyte differentiation with periods of gene expression and stages of differentiation presented chronologically. C/EBP, CCAAT/enhancer binding protein; PPAR, peroxisome proliferator activated receptor; MIX, methylisobutylxanthine; DEX, dexamethasone.



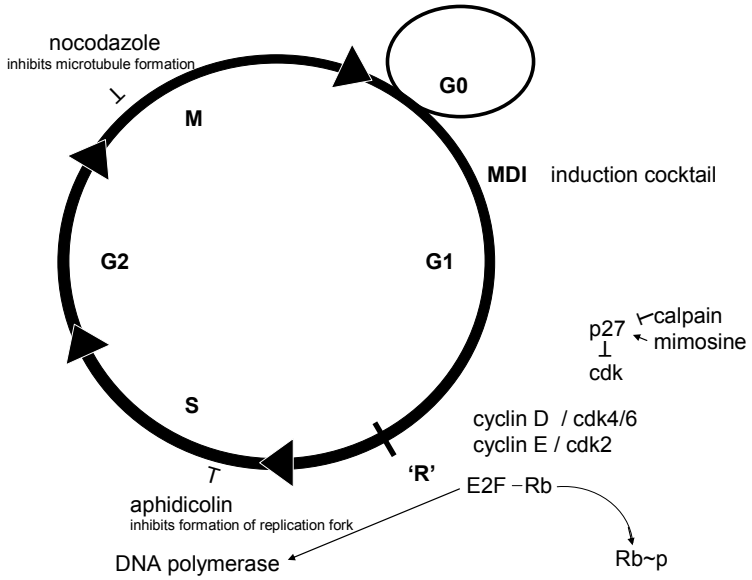
Modified from Ntambi JM and Kim Y-C, 2000, *J Nutr* **130**(12): 3124S, [36].

3. The cell cycle

One round of the cell cycle produces 2 daughter cells. The cell cycle consists of four phases. Gap1 (G1) is the interval between mitosis and DNA replication that is characterized by cell growth. Late in G1 there is a transition, called the restriction point (R), when the cells are committed to complete the cell cycle even if mitogenic stimulation is withdrawn. Replication of DNA occurs during the synthesis (S) phase, which is followed by another gap phase (G2), during which growth and preparation for cell division occurs. Mitosis occurs in the M phase. Multiple regulatory mechanisms control the onset and completion of the cell-cycle. The standard MDI protocol induces two-day postconfluent 3T3-L1 preadipocytes to undergo one to two rounds of mitotic clonal expansion.

The progression of the cell cycle is controlled by cyclins and cyclin-dependent protein kinases (cdks). Cdks lack kinase activity unless they are complexed to a cyclin molecule. The regulated synthesis and degradation of the cyclins leads to the cyclical ability of the cdks to phosphorylate target proteins, and thereby change their activation state. Cyclin/cdk complexes phosphorylate the Retinoblastoma protein (Rb).

Figure 1.2: Schematic diagram of the cell cycle. The major events of the cell cycle upon differentiation induction with MDI. Areas labeled within the circles represent stages of the cell cycle: G0, cell cycle arrest; G1, gap 1; S, synthesis; G2, gap 2; M, mitosis. Cdk, cyclin-dependent kinase; MDI, 1-methyl-3-isobutylxanthine and dexamethasone and insulin; Rb, retinoblastoma protein.



4. The Retinoblastoma protein

The Retinoblastoma protein (Rb) is a nuclear phosphoprotein that restricts the progression of the cell cycle, and in some tissues, promotes differentiation [37-39]. Progression of the cell cycle from the G1 to the S phase is regulated by the sequential phosphorylation of Rb by cyclin-dependent kinases. In G1, the hypophosphorylated form of Rb binds and sequesters members of the E2F family of transcription factors that are required for the expression of genes necessary for DNA synthesis [40-42]. Phosphorylation of Rb late

in G1 causes these transcription factors to be released, permitting cell cycle progression. In addition to interacting with transcription factors, Rb can interact directly with elements of the transcriptional machinery [43]. Almost all of these proteins interact with the hypophosphorylated form of Rb and not with the hyperphosphorylated form of Rb. Thus, Rb's regulatory function in cell cycle progression plays a key role in cell proliferation. In addition to its role in proliferation, Rb is critical in differentiation. Rb binds to transcription factors involved in differentiation [39, 44, 45], including members of the CCAAT/enhancer binding protein (C/EBP) family of transcription factors [37, 38, 46]. A direct interaction between Rb and the C/EBPβ isoform was detected in U937 cells induced to differentiate into monocytes and macrophages [37, 46]. Rb appears to increase or decrease the DNA-binding activity of C/EBPβ depending on the promoter being investigated. For example, Rb transfection increased the C/EBPβ-activation of an SP-D promoter-reporter [38]. In contrast Rb transfection decreased C/EBPβ-activation of the C/EBPα promoter-reporter gene [47].

C. Obesity, infection, and immune dysfunction

1. Obesity and infection

a. Obesity and infection in humans

Obesity increases susceptibility to infections. Data on the incidence and outcome of specific infections in obese people are limited. The available data suggest that obese people are more likely than people of normal weight to develop postoperative infections and nosocomial infections, as well to develop community-acquired infections [48].

Among surgical patients, obesity increases the risk of nosocomial infections such as wound infections, and bacteraemia [49]. For example, obese women undergoing elective hysterectomy for fibromas have an increased risk of surgical site infection, particularly if no

antimicrobial prophylaxis has been administered [50]. Morbid obesity is an independent risk factor for surgical site infection after spinal surgery [51, 52]. Obesity increases the incidence of midiastinitis as well as of deep and superficial sternal infection after surgery for coronary artery bypass graft [53-55].

In the hospital, obese patients are more likely to develop infections. For example, obese women are nearly two times more likely to develop postpartum urinary tract infections [56]. In a matched cohort study of 170 mechanically ventilated obese patients with 170 normal weight patients, the obese patients had two-fold higher mortality. This higher mortality was explained by higher incidence of infectious complications including sepsis, ventilator-associated pneumonia, and central venous catheter-related infections [56]. In a matched cohort of 15 obese burn patients with 15 normal weight burn patients, the obese patients had higher incidence of bacteremia and sepsis [57].

Obesity increases the risk of community-acquired infections. In a large population study of 26429 men aged 44-79 years from the Health Professionals Follow-up Study and 78062 women aged 27-44 years from the Nurses Health Study II, overweight and obesity were associated with increased risk of community-acquired pneumonia [58]. In a Polish cross-sectional field study of 1129 9-year old school children, overweight and obese children had twice the risk of acute respiratory infection than normal weight children [59]. Therefore, in humans, obesity increases the risk of infection.

b. Diet-induced obesity and infection in animals

Reports of the incidence of community-acquired infection among diet-induced obese animals are lacking. However, there is evidence that mice with diet-induced obesity have impaired resistance to experimentally-induced bacterial infection, parasitic infection, and

viral infection. Relative to their lean counterparts, diet-induced obese mice that were experimentally infected with *Klebsiella pneumoniae* or *Salmonella typhimurium* had increased morbidity [60]. Additionally, diet-induced obese mice have greater liver pathology due to *Schistosomiasis mansoni* relative to lean mice [61]. Diet-induced obese mice infected with influenza A/PR/8/34 had higher lung pathology and 6-fold higher mortality relative to lean mice [62].

c. Genetic obesity in humans, *ob/ob and db/db* animals and infection

Obese humans with leptin and leptin-receptor gene mutations are at increased risk for infections, including urinary tract infections [63] and upper respiratory tract infections [64]. However, data on the increased risk of infection from specific pathogens is unknown.

Genetically obese *ob/ob* mice are highly susceptible to bacterial infections. *Ob/ob* mice intranasally infected with *Mycobacterium tuberculosis* [65], *Streptococcus pneumoniae* [66], or *Klebsiella pneumonae* [67, 68] have increased bacterial load and decreased survival post-infection relative to mice with normal serum leptin levels. Similarly, *ob/ob* and *db/db* mice intravenously infected with *Listeria monocytogenes* have increased bacterial growth in their livers and increased mortality relative to heterozygous littermates [69].

2. Obesity and immune dysfunction

a. Obesity and immune dysfunction in humans

Obesity is associated with immune dysfunction [62, 70, 71]. Relative to lean individuals, obese individuals have higher risk of infections [49], require longer hospitalizations and longer periods of antibiotic treatment [72]. The cellular and molecular mechanisms responsible for the immune dysfunction are largely unknown. Some mechanisms of the immune dysfunction are impaired cell-mediated immune responses [73], lower

lymphoproliferative response to mitogen stimulation [74], and decreased antibody response to vaccines, such as hepatitis and tetanus vaccines [75-80]. However, not all studies support the hypothesis that obesity negatively affects the immune response [70, 81]. Many of the reasons that may explain the conflicting findings can be broadly categorized as subject heterogeneity, such as the degree of adiposity, age, gender, and co-morbidities. Another explanation for discrepant conclusions is that different parameters of the immune system were studied.

b. Obesity and immune dysfunction in animals

Results from studies in rodents support the results from human studies demonstrating impairment of the innate and adaptive immune responses in diet-induced obesity (DIO). The following are impaired or reduced in DIO mice and rats relative to their lean counterparts: macrophage phagocytic activity [82]; contact hypersensitivity [83]; natural killer cell cytotoxicity [62, 84]; lymphoproliferative response to mitogens [84], with altered splenocyte cytokine production [85].

Although DIO mice most closely mimic human obesity, genetic mouse models of obesity such as *ob/ob* and *db/db* mice are widely studied. The *ob/ob* mice lack functional leptin, while *db/db* mice lack a functional leptin receptor. Because leptin receptors are found in all cells of the innate and adaptive immune response [86, 87], leptin can affect many aspects of the immune system [88]. The following are impaired or reduced in *ob/ob* mice relative to wild type mice: macrophage phagocytotic activity [89-91]; T lymphocyte number and thymus and spleen weights [92-94]; Dendritic cell antigen presentation [95].

3. Obesity and inflammation

Obesity is associated with a state of chronic, low-grade inflammation. The classic symptoms of inflammation (redness, warmth, swelling, and pain) are absent in obesity. Instead, other features of inflammation are present. For example, compared to normal weight individuals, obese individuals have higher serum concentrations of inflammatory mediators, such as C-reactive protein [96], serum amyloid A [97], MCP-1 and IL-8 [98], TNFα and IL-6 [99-101]. In addition, obesity increases the numbers and activation state of macrophages in adipose tissue [102].

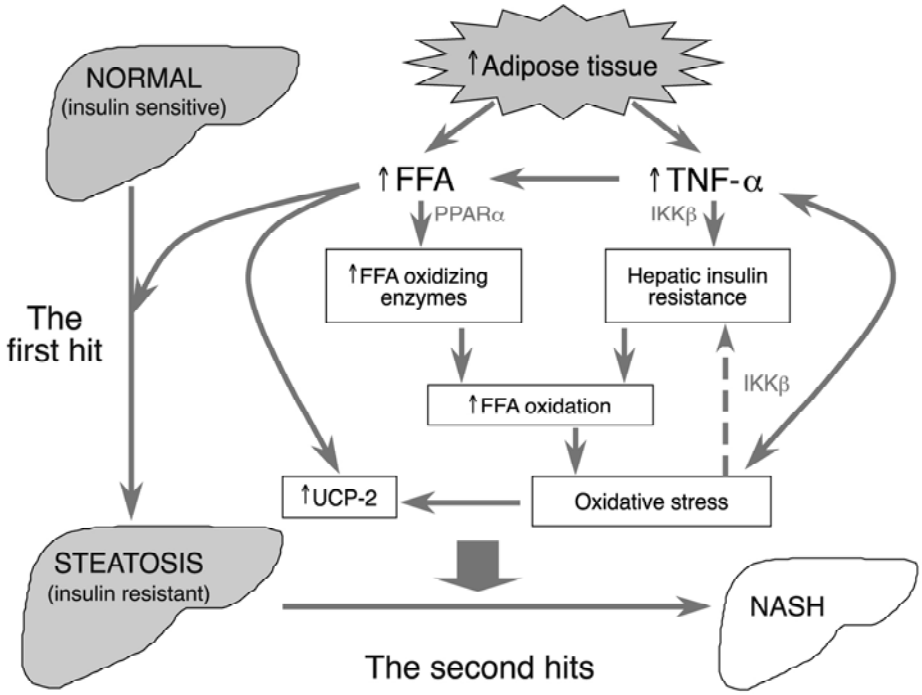
a. Obesity and inflammation in the liver

Obesity is associated with nonalcoholic fatty liver disease (NAFLD), which may or may not include inflammatory changes. Although the prevalence of NAFLD in the normal weight adult population is 10-15% [103, 104], in obese adults the prevalence of NAFLD increases to 74%; in morbidly obese adults, the prevalence of NAFLD increases to 90% [105, 106]. NAFLD comprises a spectrum of disorders ranging from simple fatty liver (steatosis), to nonalcoholic steatohepatitis (NASH) [104, 107]. In the liver, inflammation is typically defined by histopathological parameters. However, a liver biopsy is required for diagnostic purposes. Because biopsies are invasive and have the potential for adverse consequences such as infection and internal bleeding, alternative screening techniques have been investigated. Ultrasound, computerized tomography, and magnetic resonance imaging can detect NAFLD, but they do not distinguish between simple liver steatosis and more advanced forms of liver disease, such as NASH [108]. Elevated levels of serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) are indicators of hepatocyte damage but are not well correlated with the changes in the degree of liver steatosis or fibrosis

[109, 110]. Indeed, Garcia-Monzon et al. reported that 54% of adults with severe NASH had normal AST and ALT levels [111]. Central adiposity, serum TAG, insulin, C-peptide, and leptin concentrations have also been used to estimate the presence of NAFLD [112].

Hepatocyte TAG accumulation in excess of 5% of the total mass is considered liver steatosis. TAG accumulation occurs when there is increased free fatty acid flux to the liver, such as during excess food consumption. The 'two-hit' theory of NAFLD by James and Day [113] proposes that liver TAG accumulation (the first hit) makes the liver more susceptible to damage from a second hit [114]. The second hit may result from increased free fatty acid (FFA) delivery to the liver and/or increased TNFα concentrations [114]. In obesity, much of the FFAs come from adipose tissue, which will be discussed in a later section. The liver becomes increasingly insulin resistant due to FFA activation of hepatic PKC-δ [115], which inhibits IRS-1/2 tyrosine phosphorylation. In addition, the FFAs activate IKK-β. IKK-β phosphorylation of IκB-α causes IκB-α to dissociate from NF-κB. NF-κB is a transcriptional activator for inflammatory cytokines such as TNFα, IL-1β, IL-6, and MCP-1 [115]. The increase in TNFα expression not only exacerbates insulin resistance by impairing the insulin signaling pathway, TNFα impairs the flow of electrons in the electron transport chain, resulting in the generation of reactive oxygen species (ROS) [114]. In the liver, the increased ROS (the second hit) in the presence of steatosis (the first hit) results in lipid peroxidation, further ROS production, TNFα expression, insulin resistance and subsequent NASH [114].

Figure 1.3: The role of TNFα and free fatty acids in the pathogenesis of NASH. Visceral obesity increases serum FFA and TNFα concentrations. FFA release in adipocytes is exacerbated by TNFα-induced insulin resistance mediated by IKKβ activation. FFA, free fatty acids; IKKβ, inhibitor kappa kinase beta; NASH, non-alcoholic steatohepatitis; PPARα, peroxisome proliferator-activated receptor α; UCP-2, uncoupling protein-2.



From Day CP, 2002, *Best Practice & Research Clinical Gastroenterology*,**16**(5):668, [114].

i. Cellular infiltrate

Park et al. reported that among individuals with NAFLD, those whose disease had progressed to NASH had more CD68+ Kupffer cells than those who had liver steatosis [110]. Obese individuals with NAFLD have increased liver infiltration of CD8+ T lymphocytes and CD11c+ macrophages [111]. Although the authors of these studies refer to the CD68+ cells as Kupffer cells, and CD11c+ cells as macrophages, respectively, this distinction is not entirely clear. CD68 is a scavenger receptor that is expressed not only on Kupffer cells, but

also on monocytes/macrophages, neutrophils, and dendritic cells (DC) [116]. Similarly, CD11c is expressed not only on macrophages, but is also expressed on monocytes, neutrophils, natural killer (NK) cells, and DC [116]. Therefore, in NAFLD the precise identification of the infiltrating cells is unknown, but the infiltrate consists predominantly of cells associated with inflammation and the innate immune system.

ii. Inflammatory mediators

The cellular infiltrate associated with NAFLD is a potential source of inflammatory mediators, as are the resident Kupffer cells and hepatocytes themselves. Inflammatory cytokines and chemokines have recently been studied in association with NAFLD. In humans, NAFLD is associated with elevated serum levels of IL-6 and MCP-1 [117]. Crespo et al. found that obese patients with NASH compared to those without it have increased liver expression of TNFα [118]. A liver biopsy to measure cytokine and chemokine mRNA poses some health risks. Therefore, liver gene expression has been measured mainly in people at risk of liver disease which makes the study's findings less generalizable to the population at large. Mice have been used to study liver steatosis and cytokine expression. C57BL/6 mice fed a high fat (HF) diet developed liver steatosis after 10 weeks [119]. Cytokines expressed by the liver may be detected in the serum. Although serum TNFα, and IL-6 levels were not increased, serum MCP-1 levels were increased in DIO mice [120, 121]. In contrast to the serum cytokines, liver mRNAs encoding IL-6, IL-1β and TNF-α were increased several fold in DIO mice relative to chow-fed controls [122].

b. Obesity and inflammation in the adipose tissue

In the obese state, visceral adipose tissue (AT) has increased numbers of macrophages and T lymphocytes and increased concentrations of inflammatory mediators.

i. Regional differences in adipose tissue inflammation

Adipose tissues have important regional differences. In addition to metabolic differences [123], AT depots differ in the degree of cellular infiltration during obesity. In DIO and *db/db* mice [124, 125], and severely obese humans [126], visceral AT contatins a higher number of macrophages than subcutaneous AT. Differences in local production of chemokines may account for this discrepancy. For example, the expression of IL-8 and MCP-1 is greater in visceral AT than subcutaneous AT [127, 128]. The depot-specific difference in protein expression extends beyond chemokines to include hormones and cytokines. In humans, relative to visceral AT, subcutaneous AT expresses more adiponectin mRNA [129] and leptin mRNA [130], and releases more leptin protein [131]. In contrast, the expression of IL-6 occurs predominantly in the visceral depots [132]. While these regional differences may be of physiological and clinical importance, there has not been a systematic study of depot-specific changes in macrophage infiltration or cytokine gene expression during the onset of obesity.

ii. Cellular infiltrate

Recently, inflammatory lesions have been identified in the AT of obese animals and humans [133, 134]. Macrophages are the predominant infiltrate [124, 135]; however, a small proportion of T cells [136] and neutrophils may also be present [133]. In obese children, but not in lean children, microgranulomas were found in the AT. Some microgranulomas contained macrophages with necrotic adipocytes surrounded by fibrosis [133], indicative of chronic inflammation in the AT during early stages of obesity. Subsequent weight loss results in decreased macrophage infiltration of the AT [137]. In DIO mice, macrophages have been found to aggregate around non-viable adipocytes, apparently scavenging adipocyte fragments

and residual lipid droplets [134]. However, it is not known to what degree the infiltrating cells ameliorate or exacerbate the inflammation in the AT.

iii. Inflammatory mediators

In human obesity, serum concentrations of the inflammatory cytokines and chemokines IL-6, TNFα, and MCP-1 are elevated [99-101]. These cytokines and chemokines are expressed in AT [138] and may originate from either adipocytes [139] or from cells in the stromal vascular fraction, such as macrophages [124]. Significant amounts of plasma IL-6 originate from AT, especially from the visceral AT depots [140, 141]. In contrast, while TNFα gene expression in AT increases in obesity, it likely acts in an autocrine fashion, because it does not contribute significantly to circulating concentrations of TNFα [139] [142]. It is hypothesized that the increase in cytokine expression results from the increased FFA concentrations. The FFAs activate IKK-β. IKK-β phosphorylation of IκB-α causes IκB-α to dissociate from NF-κB. NF-κB is a transcriptional activator for inflammatory cytokines such as IL-6, TNFα, IL-1β, and MCP-1 [115].

D. Influenza A virus

Viruses are infectious agents that are obligate intracellular parasites. They infect and use host cellular factors for their replication.

1. Public health impact of influenza virus infection

Influenza virus infection is a major cause of morbidity and mortality worldwide. Symptoms of influenza infection include headache, cough, sore throat, nasal congestion, sneezing, body aches, and fever. During a pandemic, 20-40% of the world's population may be infected [143]. In an average year, influenza virus infects approximately 10-20% of the U.S. population. Annually in the U.S., influenza viruses infect about 50 million people, are

responsible for approximately 36,000 deaths and 114,000 hospitalizations [143]. In the late 1990's it was estimated that the direct medical cost of influenza infection was $1-3 billion; indirect costs ranged from $10-15 billion [144]. Given the health care costs and high infection rates, influenza is a significant socioeconomic burden [144].

2. Overview of influenza A virus

Influenza viruses belong to the Orthomyxoviridae family of viruses, which have a negative-sense, single-stranded, segmented RNA genome [145]. There are 5 different genera in the family due to antigenic differences between their nucleocapsid and matrix proteins. Influenza A viruses are further divided based on the antigenic differences of their hemagglutinin (HA) and neuraminidase (NA) glycoproteins.

The influenza A virus has a lipid envelope that is derived from the plasma membrane of the host cell. This envelope contains the HA and NA proteins that form projections on the surface of the virus. Inside the envelope the ribonucleoprotein (RNP) complex consists of 8 RNA segments in association with nucleoproteins, and the RNA-polymerase proteins.

3. Mechanism of influenza A virus infection

To start the process of infection, the influenza virus attaches via HA proteins to sialic acid residues of glycoproteins on the host cell surface. The HA of human influenza viruses preferentially bind to sialic acid attached to galactose by an α2,6 linkage. This limits host range because avian influenza viruses predominantly bind to an α2,3 linkage. However, this specificity is not absolute because humans and birds contain both linkages [145]. Also, viruses can adapt to a host. In addition, influenza viruses can adapt to infect mice, which are not naturally infected with the virus, by the natural selection of mutations in the receptor-binding site of the viral HA.

After attachment, the viruses enter cells by receptor-mediated endocytosis. In the low pH of the endosome, the HA undergoes a conformational change which enables it to fuse with the endosome membrane and open a pore that releases the viral RNP complex into the cell. Subsequently, the cell's nuclear import machinery traffics the viral RNA and nucleoproteins to the nucleus for viral RNA synthesis. Influenza virus mRNA synthesis is dependent on cellular RNA polymerase II activity. This is because it requires a 5′ capped primer, which it cleaves from host mRNA to initiate viral mRNA synthesis [145]. RNA viruses must encode an RNA polymerase since cells do not normally replicate RNA. In the case of all RNA viruses, virus-encoded proteins are required to form a replicase complex to replicate the viral RNA since cells do not possess RNA to RNA copying enzymes. In the case of the minus-strand RNA viruses, these RNA synthesizing enzymes also synthesize mRNA and are packaged in the virion, because their genomes cannot function as messengers. Viral replication occurs in 2 steps. First, a full-length positive-sense copy of the vRNA is transcribed (cRNA). Next, it is used as a template to produce more vRNA.

Influenza viruses assemble and form buds at the plasma membrane on the apical surface of the cell. This is important in the pathogenesis of influenza because the infection is generally restricted to the cells lining the respiratory tract. After the membranes fuse at the base of the bud, the enveloped virus particle is released. Influenza viruses must be cleaved from the cell membrane because their HA binds to the host cell's sialic acid receptors. The viral NA enzyme removes the sialic acid, releasing the virus and allowing the virus to spread. Influenza viruses do not cause persistent or latent infections. They survive in the population by being transmitted from one host to another, typically in aerosolized droplets during coughing or sneezing.

Figure 1.4: Illustration of the influenza virus replication cycle.

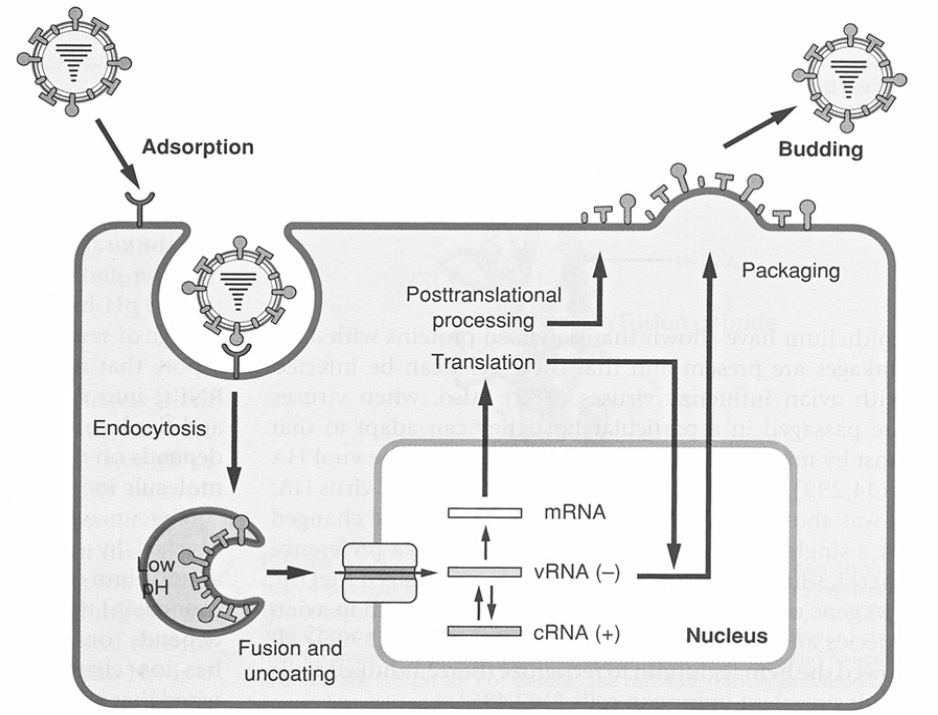


Illustration from Knipe DM, and Howley PM, *Fields Virology 5th ed.*, Chapter 47 [145].

E. Immune response to influenza A virus infection

The influenza virus targets airway epithelial cells; however, it can also infect monocytes and alveolar macrophages [146]. Although the influenza virus infection is typically limited to the respiratory mucosa, it not only causes respiratory symptoms such as nasal discharge, sore throat and cough, but also causes systemic symptoms such as chills, myalgia, anorexia, headache, sleepiness and fever (reviewed in [147]). These symptoms are caused by inflammatory mediators that are released at the site of infection and initiate a cascade of events necessary for the elimination of the virus and tissue repair. For example, fever is generally beneficial to host defense because most pathogens grow better at lower temperatures and adaptive immune responses are moe intense at elevated temperatures [148].

The initial release of inflammatory mediators by infected lung epithelial cells and alveolar macrophages results in the extravasation of neutrophils, macrophages and then T cells from the blood into the infected lung tissue [149]. Therefore, the immune response to influenza virus infection includes both innate and adaptive immune processes, which function interdependently.

1. Innate immune response to influenza A virus infection

The influenza A virus primarily targets epithelial cells of the respiratory tract. These cells produce large numbers of virion, which can then infect the resident alveolar macrophages [146]. Virus infection activates members of the toll-like receptor (TLR) family, which subsequently activates the transcription factors involved in chemokine and cytokine gene expression. These include nuclear factor kappa B (NF-κB), interferon regulatory factors, and signal transducers and activators of transcription (STATs) [150]. Cytokines and chemokines are reviewed later in the introduction. The increased expression of inflammatory chemokines and cytokines causes a rapid infiltration of neutrophils [151], which is followed by infiltration of other inflammatory cells such as macrophages and natural killer (NK) cells. Because viruses are intracellular pathogens, cell-mediated mechanisms are responsible for their elimination. As will be discussed in the section on adaptive immunity, cytotoxic T (Tc) lymphocytes are mainly responsible for the clearance of influenza virus infections; however, there is a 4-6 day lag before there are sufficient numbers of antigen-specific Tc cells to successfully eliminate the infected cells [116]. During this delay, neutrophils, macrophages, and NK cells help to control viral spread. Neutrophils and macrophages phagocytose influenza virus-infected cells [152, 153], while NK cells lyse virally-infected cells.

2. Adaptive immune response to influenza A virus infection

During the adaptive immune response, naïve antigen-specific B and T cells are activated to undergo clonal expansion and development of memory cells that can prevent reinfection with an antigenically similar pathogen [116]. An adaptive immune response is necessary to recover from influenza infection, because CD4-/- and CD8-/- animals die from influenza infection [154].

a. Humoral immune response

Humoral immunity refers to antibody-mediated protection. B-cells, with help from T cells, produce antibodies. In influenza A virus infection, B-cell activation is mediated by T-cells and activating cytokines. Typically, virions are phagocytosed by antigen presenting cells (APC) such as DC or macrophages. The APC then processes the protein portions of the virion into peptide fragments and re-expresses these peptides on the surface of the APC in association with MHC-II molecules [155]. The complex of the antigen/MHC-II molecule can be recognized by the T-cell receptor (TCR) on the surface of the T-helper (Th) cells. Then, the CD4+ coreceptor binds to the MHC-II molecule. This induces the expression of the CD40 ligand (CD40L) on the Th cell. B-cell binding to the CD40L induces their expression of cytokine receptors. The activated Th cells secrete cytokines such as IL-4 that induce the B-cells to proliferate and terminally differentiate into antibody forming plasma cells [155]. Because antigen-specific B-cells are rare, it takes several days for sufficient proliferation of B cells to occur.

While humoral immunity does little to contain the primary influenza virus infection, it is important for resistance to subsequent influenza infections by virions that are antigenically similar. During a second exposure, the infection can be blocked by antibodies that are

secreted into the mucous. Antibodies directed against peptides from viral HA and NA proteins are especially important. Antibodies to epitopes of the virus' HA protein prevent the virus from binding to the sialic acid residues on the host cell's receptors, whereas the NA antibodies prevent the virus from being released by the infected cell [145]. Antibodies that bind to viruses engage Fc receptors on phagocytes, facilitating phagosomal destruction [116]. Similarly, antibodies that bind to viral peptides displayed on the surfaces of infected host cells can engage the Fc receptors of NK cells, neutrophils and macrophages, inducing antibody-dependent cell-mediated cytotoxicity [116].

b. Cell-mediated immune response

Cytotoxic T (Tc) lymphocyes are the main effector cells for cell-mediated immune responses. Tc lymphocytes are also designated CD8+ T-cells, because the CD8 molecule is part of the coreceptor that recognizes the MHC-I proteins (in contrast to CD4+ T-cells that recognize MHC-II).

During influenza infection, viruses are taken up by APCs via receptor-mediated endocytosis or phagocytosis. The APCs, especially dendritic cells, migrate from the site of infection to efferent lymph nodes where they present viral peptides for recognition by antigen-specific T cells. The intracellular nature of viral replication means that viral antigens are processed by the host cell's endogenous antigen-processing pathway and viral peptides are displayed on MHC-I molecules [116]. Tc lymphocytes kill virus-infected cells by secreting perforin or by inducing Fas-mediated apoptosis which activates caspases [116]. Tc lymphocytes rapidly kill an inflected host cell, which prevents the virus from spreading.

3. Cytokines and chemokines function in innate and adaptive immunity

Cytokines are necessary for both the initiation and control of innate and adaptive immune responses. Cytokines act as intercellular messengers among cells, especially among leukocytes, yet because of their small size, low effective concentration, and variable half-life, cytokine activities are difficult to evaluate *in vivo*. Most studies have shown that cytokine signaling is predominantly autocrine and paracrine; however, cytokine signaling may also induce a systemic response. Chemokines are cytokines that function as chemoattractants for leukocytes. By binding to their receptors on cell surfaces, cytokines trigger intracellular signaling pathways that induce gene expression which results in activation, proliferation, survival, apoptosis, migration, and differentiation [116]. Furthermore, many cytokines have functional redundancy because receptors from different families can share component intercellular chains [156].

Because the cytokine network is so complex, it is useful to group cytokines into categories based on their immune function [157].

Table 1.1: Cytokines and chemokines that participate in inflammation and systemic responses to infection.

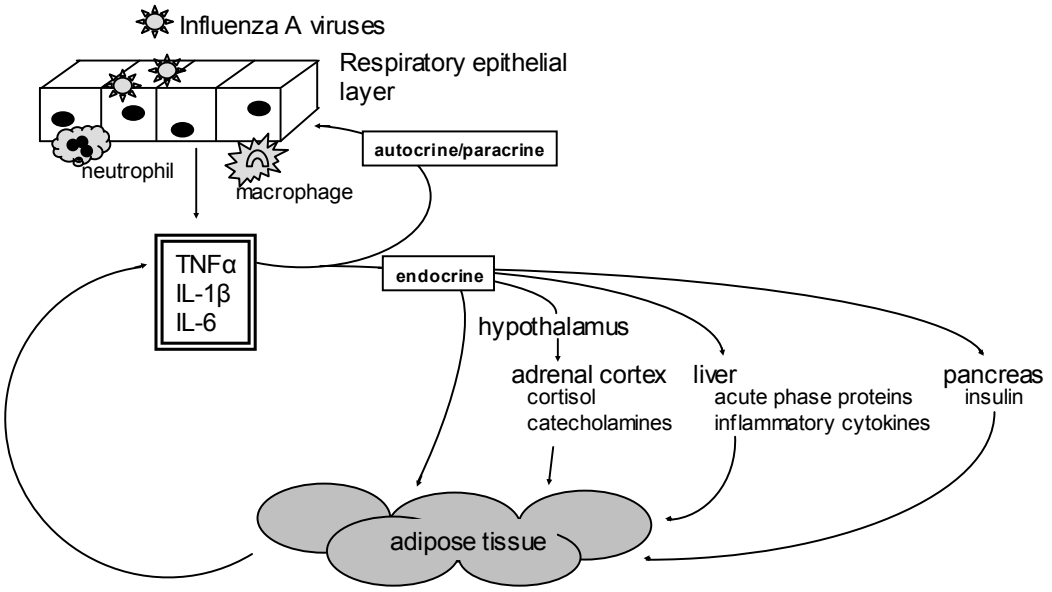
Cytokines participating in inflammation and systemic responses to infection	**Response**
TNFα Inflammation Extracellular pathogen Intracellular pathogen	↑ APP by hepatocytes ↑ macrophage production of IL-1, IL-6, TNFα ↑ neutrophil production of IL-1, IL-6, IL-8 ↑ neutrophil, macrophage microbicidal killing ↑ activation, adhesion, and extravasation of neutrophils, macrophages, Tc lymphocytes ↑ MHC-II on APC ↑ MHC-I on target cells ↑ B cell proliferation, antibody production ↑ T cell production of IFN
IL-1β Inflammation	↑ APP by hepatocytes ↑ adhesion molecules on endothelial cells & leukocytes ↑ IL-6, TNFα, MIP-1α production
IL-6 Inflammation Extracellular pathogen	↑ APP by hepatocytes ↑ neutrophil microbicidal functions ↑ B cell terminal differentiation
Chemokines favoring inflammation	**Response**
MCP-1/CCL2 (CCR2, CCR4 receptors)	Chemoattractant for monocytes, activated T cells, Th2, basophils, NK cells. Not neutrophils
MIP-1α/CCL3 (CCR1, CCR4, CCR5)	Chemoattractant for monocytes, macrophages, Tc, Th1, DC, NK cells, basophils

Modified from Mak TW, and Saunders ME, 2006, *The Immune Response,* Elsevier Academic Press, p511-513 [116].

4. Influenza virus infection may alter adipose tissue inflammation.

As previously discussed, the influenza virus infection is typically localized to the respiratory mucosa. Cytokines produced at the site of infection are responsible for initiation of the local inflammatory response, as well as some systemic effects [158]. TNFα, IL-1β, and IL-6, acting alone or in combination, have been demonstrated to affect various tissues and organs indirectly and directly. For example, there is evidence that cytokines cross the blood brain barrier to act on the hypothalamus [159], although they may also communicate with the CNS via their actions on the vagus nerve [160]. The hypothalamic-pituitary-adrenal (HPA) axis plays a critical role in the immune response [161] by regulating cortisol and catecholamines. Therefore, acting through the hypothalamus, cytokines indirectly affect the release of cortisol and catecholamines by the adrenal gland. In addition, cytokines can directly affect the acute phase response by the liver [162], and insulin secretion by the pancreas [163]. Changes in the concentrations of hormones, neuropeptides, cytokines, and other proteins can alter AT gene expression. Figure 1.5 illustrates how an influenza virus infection may alter AT gene expression.

Figure 1.5: Influenza A virus infection induces systemic changes that may alter adipose tissue gene expression. Influenza virus infection of respiratory mucosal cells induces expression of inflammatory cytokines which have local and systemic effects. Cytokines can act directly or indirectly on various tissues and organs to alter hormone, neuropeptide, and protein concentrations, including their own, which may alter AT expression of cytokines.



In obesity, inflammatory cytokines originating from the adipose tissue may contribute to an adverse outcome during infection. Increased serum concentrations of IL-6, as seen in obese humans, have been associated with a higher mortality from pneumonia [164]. Increased mRNA transcripts for TNFα and IL-1β, which have been detected in AT of obese animals [165-167], may also alter the outcome of infection. Increased serum concentrations of TNFα exacerbate the inflammatory infiltrate in the lungs during influenza A viral pneumonia [168]. In contrast, IL-1β appears to be important in clearing the virus [169].

5. Effect of dietary lipids on the immune response

The effect of diet on the immune response has gained much attention. Recently, there has been interest in how specific lipids may modulate the immune response. One link between fatty acids, inflammation and immunity are eicosanoids. Eicosanoids, a collective term for C_{20} polyunsaturated fatty acids (PUFA), mediate inflammation and immune responses [170].

The most prevalent dietary precursor for eicosanoids is linoleic acid [170]. Through a series of elongation and desaturation steps, linoleic acid is converted to arachidonic acid (ARA; 20:4*n*-6). However, linolenic acid and other *n*-3 fatty acids (found in oily fish, soybean and canola oils) are also substrates for eicosanoid synthesis. After elongation and desaturation, linolenic acid is converted to eicosapentanoic acid (EPA; 20:5*n*-3). ARA and EPA are incorporated into the sn-2 position of phospholipids in the plasma membrane. Phospholipase A2 activation releases the ARA or EPA from the phospholipid. The ARA and EPA are substrates for both cyclooxygenase and lipoxygenase enzymes. When EPA is used as the substrate instead of ARA, class-3 , rather than class-2 prostaglandins, thromboxanes and leukotrienes are synthesized. These class-3 molecules derived from EPA have a slightly different structure, and are less potent inflammatory mediators than those formed from ARA [170]. Thus, diets high in *n*-3 fatty acids, including EPA and DHA may decrease inflammation.

Figure 1.6: Synthesis of long-chain *n*-6 and *n*-3 PUFAs from their precursors.

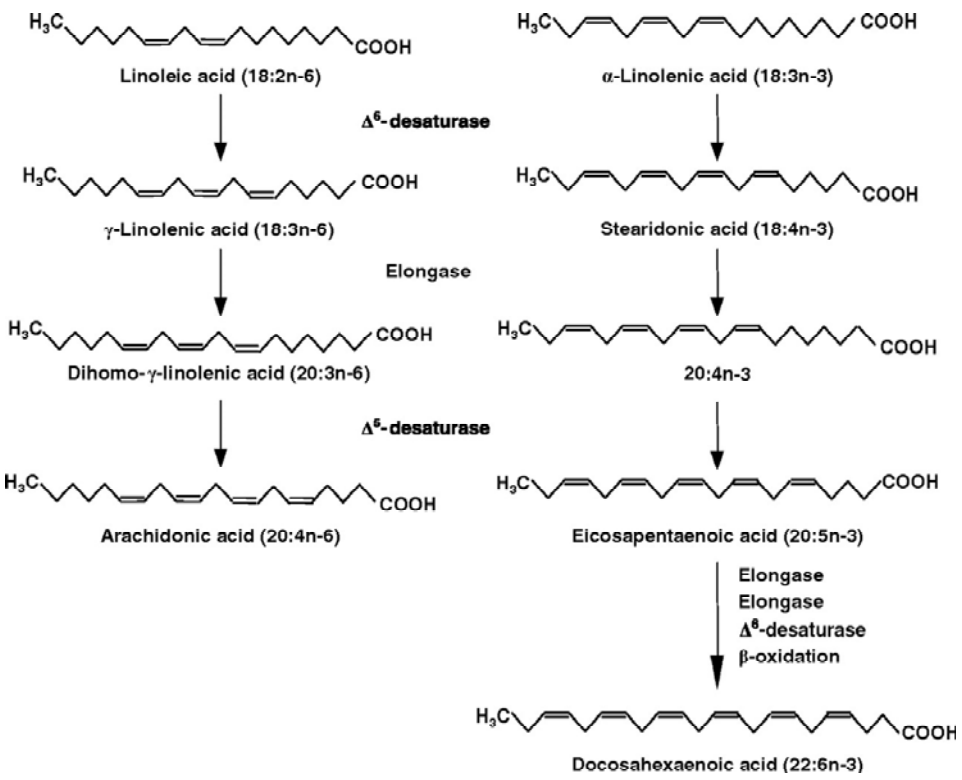


Figure from Wanten GJ, et al., 2007, Am J Clin Nutr **85:**1171-1184 [171].

PUFAs are the most studied type of dietary lipid in immune function. Because obese individuals have increased serum concentrations of TNFα and IL-6 [172], two studies in obese men investigated the effects of supplementation with fish oil, which is rich in EPA and docosahexanoic acid (DHA; 22:6*n*-3), on serum TNFα and IL-6 concentrations. Jellema et al. provided 1g EPA and 1g DHA, while Chan et al. provided 3g EPA and 4g DHA [173, 174]. Compliance was ascertained compliance by counting the remaining capsules at the end of the study. Fish oil supplementation increased plasma EPA and DHA concentrations but did not affect the plasma TNFα or IL-6 concentrations in either study. This suggests that long chain *n*-3 PUFA do not reduce the low-grade inflammatory state in obesity [170].

There have been many studies on the effects of long-chain n-3 PUFA supplementation on markers of immune function. A review by Sijben et al. concluded that overall, in healthy adults, *ex vivo* T-cell proliferation and cytokine production induced by Con A was not reduced, and monocyte production of TNFα, IL-1β and IL-6 in response to LPS was not reduced [170]. Therefore, although there are numerous studies on the effect of long-chain *n*-3 PUFA, it appears that factors such as genetic variation, health status or disease, type of immune stimulation, and age, confound the effect of long-chain *n*-3 PUFA on the immune system. Studies conducted in animals that investigated the influence of dietary *n*-3 fatty acids on host survival and/or pathogen clearance are inconclusive, some showing that *n*-3 PUFAs improve host defense and others showing impairment [175].

While studies on dietary long chain *n*-3 PUFAs have not demonstrated consistent effects on markers of inflammation or immune function, studies of dietary saturated fatty acids (SFAs) have been even less informative. Consumption of diets high in SFA has been associated with increased risk of cardiovascular disease. Therefore it has been postulated that SFA may alter the inflammatory responses. However, there is little experimental research to support the epidemiological evidence. For example, in a double-blind cross-over study design in hypercholesterolemic adults, 30% of calories were provided from butter, stick margarine, or soybean oil [176]. Relative to the other diets, the butter diet had twice the amount of SFA, the stick margarine diet had four-fold more trans-fatty acids, and the soybean oil diet had four-fold more PUFA. Among the groups, there were no differences in conA-stimulated PGE_2 or IL-2 production by PBMCs, delayed type hypersensitivity response, or in lymphocyte proliferative response [176]. Hence, consumption of diets with

varying degrees and types of fat saturation did not adversely affect these indices of cellular immunity.

Waitzberg et al. reviewed the effect of lipids administered parenterally [177]. While several studies reported that certain lipid emulsions altered monocyte/macrophage and neutrophil phagocytosis, other studies of the same lipid emulsions reported no effect. In mice fed either a LF diet or HF diet with 20% hydrogenated coconut oil (w/w), there was no difference in peritoneal macrophage superoxide, hydrogen peroxide, or nitric oxide production, PGE2, TXB2, or LTB4, TNFα, IL-1β or IL-6 production [178]. In lymphocytes, there was no difference in proliferation or in the production of IL-2, IL-4, IL-10, IFNγ [179].

The high-fat diets consumed by mice in our studies reflected diets could reasonably be consumed by humans. Described as percent kilocalories protein:carbohydrate:fat, the high-fat mouse diet was 20:35:45. The typical American diet is 16:50:34 [180]. Therefore, the increased fat content, considering kilocalories, trans fatty acids and saturated fat, and *n*-3:*n*-6 PUFAs, is roughly equivalent to what a typical American could consume by consuming one "super-size" fast food meal per day.

F. Questions raised by the literature

It has long been recognized that malnutrition is associated with immune impairment. However, only recently have the effects of obesity on the immune response received attention. Evidence indicates that obesity results in a low-grade, chronic inflammatory state. This raises the question about the role that adipose tissue itself plays during infection. Studies suggest that adipose tissue depots differ with respect to their inflammatory state, yet this information is incomplete due to the lack of research on the retroperitoneal depot. Furthermore, many studies on inflammation in adipose tissue were conducted after the onset

of insulin resistance, thereby confounding whether the inflammation is due to obesity or the insulin resistance. Chapter III provides new data on the development of inflammation in a time-dependent and tissue-specific manner.

Although adipose tissue in obese humans and mice produces pro-inflammatory mediators and accumulates macrophages, there are no studies demonstrating how this inflammation may affect the response to an infectious disease. Previous work in our laboratory demonstrated that obese mice have a worse outcome than lean mice after an influenza infection. In Chapter IV, the effect of influenza infection on adipose tissue and liver inflammation are reported.

Taken together, our studies demonstrate that obesity can have a profound influence on the immune response to an infectious disease and that the adipose tissue itself may be a major component of the dysregulated immune response during influenza infection.

Chapter II

Rb regulates C/EBPβ-DNA-binding activity during 3T3-L1 adipogenesis

Kathryn A. Cole, Anne W. Harmon, Joyce B. Harp, and Yashomati M. Patel.

Am J Physiol Cell Physiol, 2004, Vol. 286(2):C349-54

A. Abstract

Two pathways are initiated upon 3T3-L1 preadipocyte differentiation: the reentry of cells into the cell cycle and the initiation of a cascade of transcriptional events that "prime" the cell for differentiation. The "priming" event involves the synthesis of members of the CCAAT/enhancer binding protein (C/EBP) family of transcription factors. However, the relationship between these two pathways is unknown. Here we report that in the 3T3-L1 preadipocytes induced to differentiate, cell cycle progression and the initiation of differentiation are linked by a cell cycle-dependent Rb-C/EBPβ interaction. Cell cycle arrest in G1 by L-mimosine inhibited differentiation-induced C/EBPβ-DNA-binding activity and Rb phosphorylation. However, cell cycle arrest after the G1/S transition by aphidicolin or nocodazole did not prevent C/EBPβ-DNA-binding activity or Rb phosphorylation. Furthermore, hypophosphorylated Rb and C/EBPβ coimmunoprecipitated, whereas phosphorylated Rb and C/EBPβ did not. Electrophoretic mobility shift assays demonstrated that recombinant hypophosphorylated Rb decreased C/EBPβ-DNA-binding activity and that Rb overexpression inhibited C/EBPβ-induced transcriptional activation of a C/EBPβ-promoter-luciferase reporter gene. We conclude that C/EBPβ-DNA-binding activity is regulated by its interaction with hypophosphorylated Rb, thereby linking the progression of the cell cycle to the initiation of differentiation during 3T3-L1 adipogenesis.

B. Introduction

The obesity epidemic has heightened the demand for effective prevention and treatment methods. An insight into the molecular mechanisms underlying adipogenesis may lead to the development of effective strategies for reducing the prevalence of obesity. Much of what is known about adipocyte development has been elucidated from established cell lines that mimic adipocyte proliferation and differentiation in vivo [181]. The 3T3-L1 preadipocyte cell line provides a well-characterized model for the study of adipocyte-specific terminal differentiation [27, 182-185]. On reaching confluence, 3T3-L1 preadipocytes growth arrest at the G0/G1 cell cycle boundary. The addition of 1-methyl-3-isobutylxanthine (M), dexamethasone (D), and a pharmacological dose of insulin (I) to serum-containing medium (MDI protocol) concurrently activates two different processes: 1) the synchronous reentry of preadipocytes into the cell cycle, and 2) the initiation of differentiation. Whereas the role of the differentiation pathway is well characterized, the role of the cell cycle during 3T3-L1 preadipocyte differentiation is poorly understood.

Progression of the cell cycle from G1 to S phase is regulated by the phosphorylation status of the retinoblastoma protein Rb. In G1, hypophosphorylated Rb binds and sequesters transcription factors that are required for the expression of genes necessary for DNA synthesis [41, 42]. Rb phosphorylation at the G1/S boundary results in its dissociation from these transcription factors and triggers cell cycle progression. Rb not only binds transcription factors involved in cell cycle progression, it also interacts with transcription factors involved in differentiation [39, 44, 45]. Rb has been shown to bind to members of the CCAAT/enhancer binding protein (C/EBP) family of transcription factors [37, 38, 46].

Transcription factors in the C/EBP family function in a cascade-like manner to activate adipogenesis [25, 26]. The MDI protocol induces the synthesis of C/EBPβ and C/EBPδ, which prime the differentiation pathway [26]. C/EBPβ and C/EBPδ activate the synthesis of two critical adipogenic transcription factors, peroxisome proliferator-activated receptor-γ and C/EBPα, which are required for the expression of adipocyte genes. Whereas C/EBPβ and C/EBPδ are synthesized and translocated to the nucleus within 4 h after the induction of differentiation, they do not bind to DNA for another 8 h [30]. This delay in DNA-binding activity is not well characterized.

Previous studies [186] have suggested that C/EBPβ-DNAbinding activity may be linked to cell cycle progression during differentiation. Preadipocytes induced to differentiate in the presence of a G1 cell cycle inhibitor, expressed C/EBPβ but had decreased C/EBPβ-DNA-binding activity and decreased C/EBPβ-mediated transactivation of a reporter gene [186]. In this report, we show that C/EBPβ acquires the ability to bind to DNA in a cell cycle-dependent manner (after the G1/S transition). Importantly, we demonstrate that although hypophosphorylated Rb associates with C/EBPβ, phosphorylated Rb does not. We also show that Rb inhibits C/EBPβ-DNA binding activity in vitro and overexpression of Rb prevents C/EBPβ-mediated transactivation of a C/EBPα-promoter-reporter gene. These studies suggest that Rb is a critical regulator of C/EBPβ-DNA binding activity during the early stages of 3T3-L1 adipogenesis.

C. Materials and methods

Materials. L-Mimosine, aphidicolin, and nocodazole were obtained from Cal-Biochem (San Diego, CA). Aprotinin, leupeptin, antipain, benzamidine,chymostatin, and pepstatin-A were purchased from Sigma-Aldrich (St. Louis, MO). C/EBPβ mouse monoclonal antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The Rb monoclonal antibody was purchased from PharMingen (San Diego, CA). The C/EBPβ rabbit polyclonal antibody was generously provided by Dr. M. Daniel Lane (Johns Hopkins University). Recombinant full-length Rb and a truncated Rb protein, p56Rb (amino acids 379–928), were purchased from QED Bioscience (San Diego, CA). The recombinant truncated Rb p25Rb (amino acids 773–928) was purchased from Upstate (Charlottesville, VA).

Cell culture. 3T3-L1 preadipocytes were cultured in Dulbecco's Modified Eagle medium (DMEM) containing 10% calf serum (CS) until confluence and then maintained for 48 h (day 0). Preadipocytes were then maintained in CS (time 0) or were induced to differentiate with 0.5 mM 3-isobutyl-1-methylxanthine, 1 μM dexamethasone, and
1 μg/ml insulin in DMEM containing 10% fetal bovine serum for 48 h (MDI protocol). Where indicated, cell cycle inhibitors (5 μM aphidicolin, 500 μM mimosine, or 10 μM nocodazole) were added at the time of induction.

Immunoblot analysis. 3T3-L1 preadipocyte whole cell lysates were prepared as previously described [187]. Protein concentrations were determined and protein (50 μg) was subjected to SDS-PAGE and transferred to Immobilon-P membranes (Millipore). The membranes were incubated with antisera as indicated, followed by incubation with a peroxidase-conjugated secondary antibody. Proteins were visualized by enhanced chemiluminescence (Amersham Pharmacia Biotech).

Nuclear extract preparation. Nuclear extracts were prepared from 3T3-L1 preadipocytes as described previously [188, 189]. Cells were washed with PBS and scraped in a hypotonic lysis buffer containing 20 mM Tris, pH 7.5, 10 mM NaCl, 3 mM MgCl2, 1 mM dithiothreitol (DTT), and 2 μl/ml of protease inhibitor cocktails PIC I and PIC II. PIC I contains (in mg/ml) 1 leupeptin, 1 antipain, and 10 benzamidine in 1 aprotinin in distilled water. PIC II contains 1 mg/ml chymostatin and 1 mg/ml pepstatin-A in dimethyl sulfoxide. Nonidet P-40 (NP-40) was added to a final concentration of 1%. The samples were Dounce homogenized and centrifuged at 2,200 x g for 5 min at 4°C. The crude nuclear pellet was resuspended in hypotonic lysis buffer and centrifuged at 2,200 x g for 5 min at 4°C. The pellet was resuspended in a nuclei storage buffer containing 40% glycerol, 1 M Tris pH 8.0, 3 mM MgCl2, 1 mM DTT, and 2 μl/ml PIC I and PIC II and was centrifuged at 6,500 x g for 10 seconds at 4°C. The pellet was resuspended in NUN buffer (0.3 M NaCl, 1 M urea, 1% NP-40, 25 mM HEPES, pH 7.9, 1 mM DTT, and 2 μl/ml PIC I and PIC II), incubated on ice for 30 min, and centrifuged at 14,000 x g for 10 min at 4°C. Supernatants were collected and glycerol was added to a final concentration of 10%.

EMSA. A [32P]-labeled double-stranded oligonucleotide (20 bp), corresponding to the C/EBP site in the C/EBPα promoter was used for EMSA. The labeled probe and 10 μg of nuclear protein were incubated on ice for 15 min and then subjected to nondenaturing PAGE. Protein-DNA complexes were visualized by autoradiography. For gel supershift experiments, 2.5 μg of the indicated antibody were added to the nuclear protein 15 min before the addition of the labeled probe. All recombinant Rb proteins were resuspended in a solution containing 20 mM sodium phosphate, 200 mM NaCl, 1 mM EDTA, and 10% glycerol, pH 7.5. In competition experiments, 1 μg of either p56Rb or p25Rb was added to 12 μg of nuclear

protein 15 min before the addition of labeled probe. In dose-dependent competition experiments, 0.05–0.5 μg of purified, full-length recombinant Rb was added to the nuclear protein. Protein concentration was equalized with BSA, and the samples contained equal amounts of Rb diluent.

Immunoprecipitation. 3T3-L1 preadipocytes were washed twice in ice-cold PBS containing 1 mM orthovanadate, scraped in ice-cold gentle lysis buffer (25 mM Tris-HCl, pH 7.5, 1% NP-40, 10% glycerol, 50 mM NaF, 10 mM NaH2PO4, 137 mM NaCl, 2 mM Na3VO4, 1 mM PMSF, and 10 μg/ml aprotinin), sonicated, and then centrifuged at 6,000 x g at 4°C for 20 min. Supernatants were removed, and protein concentrations were determined. Antibodies (10 μg/ml) to either C/EBPβ or Rb were added to protein lysates (800 μg) and allowed to incubate at 4°C for 1 h. Agarose beads (protein A/G PLUS-agarose beads) were mixed with the immunoprecipitates for 1 h at 4°C. Immunoprecipitates were recovered by centrifugation at 2,500 x g and washed three times with ice-cold lysis buffer. Immunoprecipitated proteins were dissolved in 2X Laemmli buffer, heated at 95°C for 5 min, subjected to SDS-PAGE, and transferred to Immobilon-P membranes (Millipore). Membranes were incubated with the indicated primary antibodies and visualized by enhanced chemiluminescence.

Transfection. 3T3-L1 preadipocytes were transiently cotransfected

on day 0 by calcium phosphate co-precipitation with a C/EBPα promoter-luciferase construct alone [30] or with either a cytomegalovirus (CMV)-C/EBPβ expression vector (generously provided by Dr.M. Daniel Lane, Johns Hopkins University) and/or a CMV-Rb or a Rb mutant vector with a deletion corresponding to amino acid 389–580 (Rbmut) (generously provided by Dr. Jonathan M. Horowitz, North Carolina State University)[190]. Cells were

then maintained in DMEM containing 10% CS for 24 h. Differentiation was induced as described previously. Cell lysates were prepared 24 h after induction and assayed for luciferase activity, which was normalized to cells transfected with the C/EBPα-promoter-luciferase construct alone and treated with MDI.

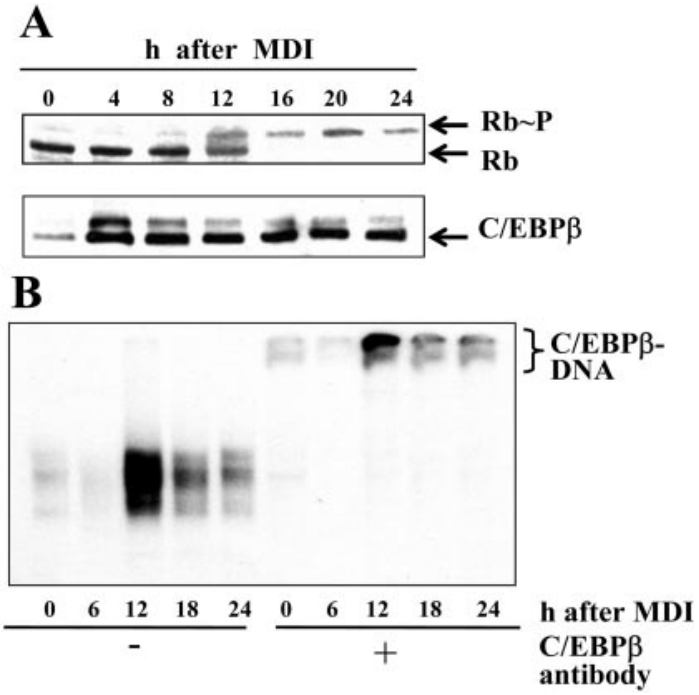
D. Results

C/EBPβ-DNA-binding activity correlates with Rb phosphorylation. Because both C/EBPβ-DNA-binding activity and cell cycle progression are induced early during 3T3-L1 adipogenesis, we planned to determine the relationship between these two events. Day 0 (2-day postconfluent preadipocytes) were maintained in CS (time 0) or were induced to differentiate, using the MDI protocol. Whole cell lysates were collected every 4 h (for 24 h) and subjected to immunoblot analysis with the use of an Rb antibody (Fig. 2.1A, top) or a C/EBPβ antibody (Fig. 2.1A, bottom). Although Rb is constitutively expressed, its activity is regulated by cell cycle-dependent phosphorylation. Rb is hypophosphorylated at the 0, 4, and 8 h time points (Fig. 2.1A, top). Two bands are detected 12 h after MDI treatment as the cells cross the G1/S boundary: the hypophosphorylated form of Rb and a slower-migrating hyperphosphorylated form of Rb. The hyperphosphorylated form of Rb predominates at the subsequent time points. Next, we characterized C/EBPβ expression during this time period (Fig. 2.1A, bottom). C/EBPβ mRNA can be alternately translated to produce both 35- and 18-kDa proteins, termed Liver Activating Protein (LAP) and Liver-Inactivating Protein, respectively. C/EBPβ (LAP) is expressed at maximal levels within 4 h after MDI treatment (Fig. 2.1A, bottom).

To determine the temporal pattern of C/EBPβ-DNA-binding activity in relation to the G1/S transition of the cell cycle, day 0 preadipocytes were maintained in CS (time 0) or were treated with the MDI protocol. Nuclear extracts were prepared at 6-h intervals (for 24 h) and subjected to EMSA with the use of an oligonucleotide corresponding to the C/EBP binding site of the C/EBPα promoter. C/EBPβ-DNA-binding activity increased dramatically 12 h after the induction of differentiation (Fig. 2.1B). Nuclear extracts incubated with a C/EBPβ-

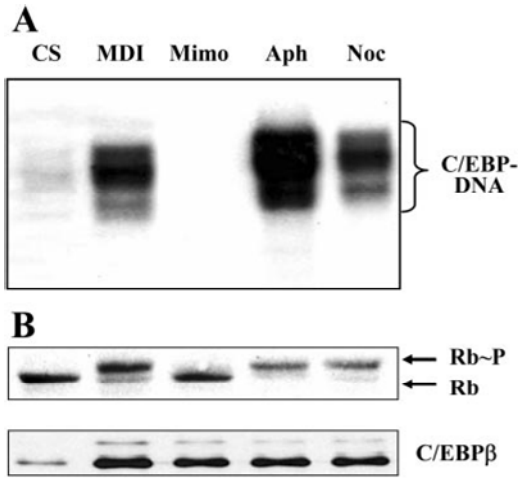
specific antibody supershifted the protein-DNA complex, thereby confirming that C/EBPβ was the predominant C/EBP isoform binding to the oligonucleotide at these time points (Fig. 2.1B). Because both Rb phosphorylation and C/EBPβ-DNA-binding activity occur about 12 h after induction of differentiation, we wanted to determine whether these two events link the cell cycle to the priming of differentiation during adipogenesis.

Figure 2.1: Rb phosphorylation (Rb~P) coincides with CCAAT/enhancer binding protein beta (C/EBPβ)-DNA-binding activity. Day 0 (2-day postconfluent) 3T3-L1 preadipocytes were induced to differentiate using the standard 1-methyl-3-isobutylxanthine, dexamethasone, and insulin (MDI) protocol (see EXPERIMENTAL PROCEDURES). A: whole cell lysates were prepared at the times indicated and subjected to SDS-PAGE and Western blot analysis with the use of an antibody directed against Rb (top) or C/EBPβ (bottom). B: ^{32}P-labeled oligonucleotide probe containing the C/EBP binding site of the C/EBPα promoter was incubated with 10 μg of nuclear protein, prepared at the times indicated, and subjected to EMSA. Supershift analysis was performed with the use of a C/EBPβ-specific antibody. The data are representative of three independent experiments.



Cell cycle inhibition affects C/EBPβ-DNA-binding activity. Because C/EBPβ is maximally expressed 4 h after the induction of differentiation but does not bind to DNA for another 8 h, we wanted to determine whether the delay in C/EBPβ-DNA-binding activity is related to cell cycle progression. Day 0 preadipocytes were maintained in 10% CS or were induced to differentiate with the MDI protocol in the absence or presence of various cell cycle inhibitors. Twenty-four hours after induction, nuclear extracts were prepared and 10 μg of nuclear protein were subjected to EMSA, as described under EXPERIMENTAL PROCEDURES (Fig. 2.2A). Whole cell lysates were also prepared 24 h after treatment and subjected to immunoblot analysis using either an Rb or C/EBPβ-specific antibody (Fig. 2.2B, top and bottom, respectively). The cell cycle inhibitor L-mimosine (a late G1 cell cycle inhibitor) inhibited C/EBPβ-DNA binding activity without affecting C/EBPβ expression (Fig. 2.2, A and B). As expected, L-mimosine treatment prevented the phosphorylation of Rb (Fig. 2.2B). In contrast, preadipocytes induced to differentiate in the presence of either aphidicolin (an agent that prevents DNA synthesis without preventing Rb phosphorylation) or nocodazole (an agent that arrests the cell cycle at the G2/M transition) had similar levels of C/EBPβ expression and C/EBPβ-DNA-binding activity as preadipocytes treated with MDI alone (Fig. 2.2, A and B). Rb was hyperphosphorylated in preadipocytes treated with either aphidicolin or nocodazole because both agents arrest the cell cycle after the G1/S transition (Fig. 2.2B). Cell cycle arrest by the various inhibitors was confirmed by fluorescence-activated cell sorting analysis (data not shown).

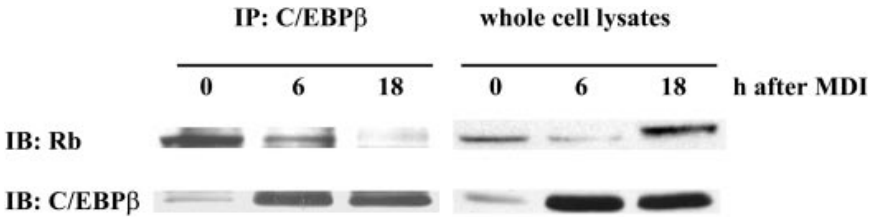
Figure 2.2: C/EBPβ-DNA-binding acitivity requires G_1/S transition. Day 0 3T3-L1 preadipocytes were maintained in 10% calf serum (CS) or were induced to differentiate by the standard MDI protocol (MDI) in the absence or presence of L-mimosine (Mimo), aphidicolin (Aph), or nocodazole (Noc). A: nuclear extracts were prepared 24 h after treatment. Ten micrograms of nuclear protein were subjected to EMSA, as described in EXPERIMENTAL PROCEDURES. B: cell lysates were prepared 24 h after the various treatments and subjected to SDS-PAGE and Western blot analysis using Rb antisera (top) or C/EBPβ antisera (bottom). The results are representative of three independent experiments.



Rb and C/EBPβ interact in a cell cycle-dependent manner. Before its hyperphosphorylation at the G1/S transition, Rb binds and sequesters transcription factors required for DNA synthesis. Rb has also been shown to interact with members of the C/EBP family, including C/EBPβ [37, 38, 46] . Because the DNA-binding activity of C/EBPβ correlates with the hyperphosphorylation of Rb (Figs. 2.1 and 2.2), we wanted to determine whether C/EBPβ and Rb interact in a cell cycle-dependent manner. Preadipocytes are in G1 6 h after MDI induction and have progressed beyond the G1/S transition point by 18 h [191]. Day 0 3T3-L1 preadipocytes were either maintained in CS (time 0) or induced to differentiate by the MDI protocol for 6 or 18 h. Coimmunoprecipitation assays were conducted with the use of a C/EBPβ-specific antibody and immunoblotted by using either an Rb or a C/EBPβ antibody (Fig. 2.3). Immunoprecipitation reactions yielded only

hypophosphorylated Rb (compare the migration pattern in the whole cell lysates with that in the immunoprecipitates). Immunoprecipitated C/EBPβ associates with Rb strongly at 0 and 6 h, when Rb is hypophosphorylated, but only weakly at 18 h (Fig. 2.3), when most of the Rb is hyperphosphorylated. These studies suggest that the Rb-C/EBPβ interaction depends on the phosphorylation state of Rb. The pattern of C/EBPβ expression in whole cell lysates and immunoprecipitates was similar (Fig. 2.3).

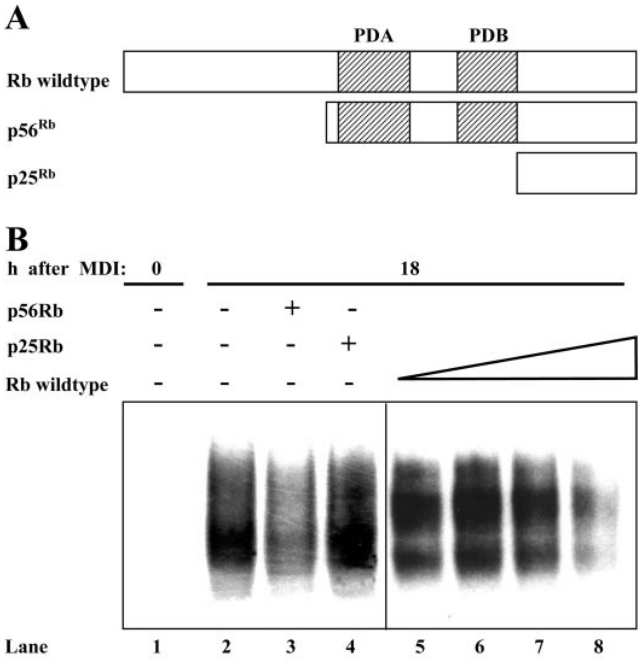
Figure 2.3: Rb-C/EBPβ interaction is regulated by phosphorylation of Rb. Day 0 3T3-L1 preadipocytes were induced to differentiate by the standard MDI protocol. Lysates were collected at 0, 6, and 18 h after the induction of differentiation, subjected to coimmunoprecipitation (IP) using a C/EBPβ-specific antibody, and immunoblotted (IB) using either an Rb antibody (top) or a C/EBPβ antibody (bottom). Whole cell lysates from the time points indicated were included as controls. The figure is representative of three independent experiments.



Rb decreases C/EBPβ-DNA-binding activity. Because Rb and C/EBPβ interact at time points when Rb is hypophosphorylated and C/EBPβ-DNA-binding activity is low, we wanted to determine whether unphosphorylated Rb alters C/EBPβ-DNAbinding activity. Two-day postconfluent 3T3-L1 preadipocytes were maintained in 10% CS (0 h) or were treated with MDI for 18 h; nuclear extracts were prepared as described in EXPERIMENTAL PROCEDURES. C/EBPβ-DNA-binding activity is maximal at 12 h and continues to be high 18 h after induction (Fig.2.4B). Nuclear extracts from 0 and 18 h were subjected to EMSA. Recombinant, full-length unphosphorylated Rb protein (0, 0.05, 0.1, 0.25, and 0.5 μg) was

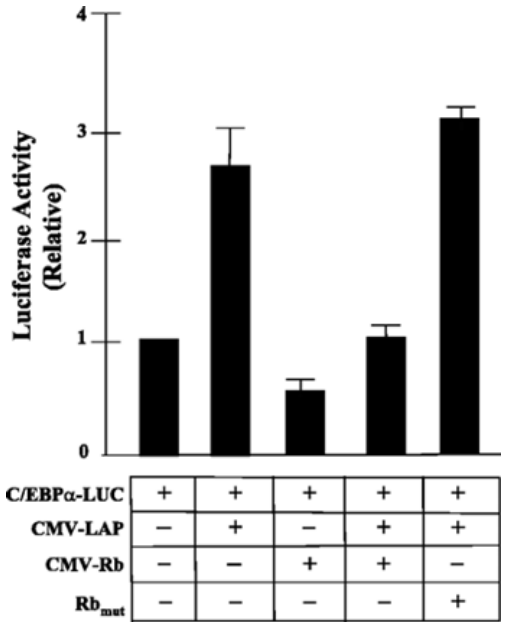
incubated with 18 h nuclear extracts and subjected to EMSA (Fig. 2.4B, lanes 2 and 5–8). Full-length unphosphorylated Rb decreased C/EBPβ-DNA binding activity. Because previous studies have demonstrated that C/EBPβ can bind to the two pocket domains (PDA and PDB) of Rb (Fig. 2.4A) (3), we examined the effect of truncated Rb proteins (p56Rb or p25Rb) on C/EBPβ-DNA-binding activity in 18 h nuclear extracts (Fig. 2.4B, lanes 3 and 4). The addition of p56Rb decreased C/EBPβ-DNA-binding activity, whereas p25Rb did not (Fig. 2.4B). These findings suggest that unphosphorylated Rb can inhibit the DNA-binding activity of C/EBPβ even after the G1/S transition.

Figure 2.4: Rb decreases C/EBPβ-DNA binding activity. A: schematic representation of the full-length Rb protein and the Rb truncated proteins $p56^{Rb}$ and $p25^{Rb}$. Pocket domains A and B (PDA and PDB) are binding sites for C/EBPβ. B: day 0 3T3-L1 preadipocytes were either untreated (0 h) or were induced to differentiate by the standard MDI protocol for 18 h. Nuclear extracts were prepared, and 12 μg of nuclear protein were subjected to EMSA (lanes 1 and 2). One microgram of either $p56^{Rb}$ or $p25^{Rb}$ protein was incubated with 18 h nuclear extracts (lanes 3 and 4). Recombinant full-length Rb protein (0.05, 0.1, 0.25, or 0.5 μg) was incubated with 18 h nuclear extracts and subjected to EMSA (lanes 5-8). The results are representative of three independent experiments.



Overexpression of Rb inhibits C/EBPβ-mediated transcription. Because association with Rb inhibits the DNA-bindingactivity of C/EBPβ in vitro, we wanted to determine whether the overexpression of Rb would inhibit C/EBPβ-mediated transcriptional activation. The C/EBPα-promoter contains a C/EBP binding site that mediates transactivation by members of the C/EBP family. To determine whether Rb can alter C/EBPβ-mediated transcriptional activation, 3T3-L1 preadipocytes were mock transfected or were transfected with the C/EBPα-promoter-luciferase reporter transgene alone or with a CMV-Rb, a Rbmut (deletion of the PDA domain) (27), and/or a CMV-LAP expression vector by calcium-phosphate coprecipitation. Transfected preadipocytes were then induced to differentiate with MDI for 24 h. Luciferase activity was assayed 24 h after MDI induction (results are presented as means ±SD, n=4). Cells cotransfected with a C/EBPα promoter-luciferase reporter gene and a CMV-LAP expression vector resulted in approximately a threefold increase in luciferase activity compared with cells transfected with the C/EBPα-luciferase reporter gene alone (Fig. 2.5). Cotransfection of the C/EBPα-luciferase reporter gene and a CMV-Rb expression vector resulted in a slight inhibition of luciferase activity compared with the level in cells transfected with a C/EBPα-luciferase reporter gene alone. Overexpression of Rb inhibited the C/EBPβ-mediated transcriptional activation of the C/EBPα promoter to a level similar to cells transfected with the C/EBPα-luciferase reporter gene alone (Fig. 2.5). Transfection of an Rb expression vector (Rbmut) lacking the PDA domain (a C/EBP-binding site) did not inhibit C/EBPβ-mediated transcriptional activation of the C/EBPα promoter. Taken together, these findings suggest that Rb regulates the transcriptional activity of C/EBPβ by directly interacting with it and preventing DNA binding.

Figure 2.5: Overexpression of Rb reduces C/EBPβ-mediated transcription from the C/EBPα promoter. Day 0 3T3-L1 preadipocytes were transiently cotransfected with a C/EBPα-promoter-luciferase (LUC) reporter alone or with either a C/EBPβ and/or an Rb expression vector or an Rb expression vector lacking the PDA domain of Rb (Rb_{mut}). After 24 h, cells were induced to differentiate by the standard MDI protocol. Luciferase activity was assayed 24 h after induction. Luciferase activity from transfected cells was normalized to the levels present in cells transfected with the C/EBPα-luciferase expression vector alone, which was set to one. CMV, cytomegalovirus; LAP, liver-activating protein. Data represent four independent experiments (means ± SD, $n = 4$).



E. Discussion

Induction of 3T3-L1 adipogenesis initiates two processes: reentry into the cell cycle (termed mitotic clonal expansion) and the priming of differentiation. Whereas the role of the priming of differentiation pathway is well characterized, the role of mitotic clonal expansion is poorly understood. Moreover, the requirement of mitotic clonal expansion remains controversial [13, 34]. Our studies were aimed at determining the relationship between mitotic clonal expansion and the priming of differentiation during the early stages of adipogenesis. Our findings suggest that these processes are linked by a cell cycle-dependent interaction between Rb and C/EBPβ before the G1/S transition. These findings aid in our understanding the temporal delay between C/EBPβ expression and C/EBPβ-DNA-binding activity during 3T3-L1 preadipocyte differentiation and also further elucidate the relationship between proliferation and differentiation.

A critical component of 3T3-L1 adipogenesis is the temporal expression and activation of C/EBPβ and C/EBPδ. Although C/EBPβ is at maximal levels 4 h after induction, it does not bind to DNA for another 8 h (Fig. 1) [30]. Several mechanisms have been proposed in the regulation of C/EBPβ activity, including conformational change, interaction with dimerization partners, and phosphorylation [26, 192-194]. In this report, we demonstrate that C/EBPβ-DNA-binding activity is also regulated by the cell cycle (Figs. 1 and 2). Our previous studies have shown that C/EBPβ-DNA-binding activity is inhibited in the presence of the calpain inhibitor ALLN, which arrests cells in G1 [186]. To determine whether calpain activity or cell cycle progression mediates C/EBPβ-DNA-binding activity, we employed another known G1 cell cycle inhibitor. Inhibition of the cell cycle in G1 by L-mimosine also blocked C/EBPβ-DNA binding activity even after 24 h (Fig. 2). In contrast, inhibition of the

cell cycle after the G1/S transition by either aphidicolin (cell cycle arrest in S phase) or nocodazole (arrest at G2/M) did not prevent C/EBPβ-DNA-binding activity. Because all of these studies were conducted 24 h after MDI induction, our findings suggests that C/EBPβ-DNA-binding activity is cell cycle-dependent, not time dependent, during the early stages of adipogenesis.

Linking C/EBPβ-DNA-binding activity to the cell cycle further suggests a connection between the initiation of differentiation and cell cycle progression. Our studies demonstrate a cell cycle-dependent interaction between C/EBPβ and Rb (Fig. 3). Rb is a critical regulator of cell cycle progression at the G1/S transition. Previous studies have shown that Rb and C/EBP family members are able to interact in in vitro experiments [37]. While these studies demonstrate an interaction between Rb and C/EBP isoforms, that interaction was not investigated in the context of cell cycle progression. Our study is the first to examine the interaction between Rb and C/EBPβ as a function of the cell cycle. We propose a mechanism for cell cycle-mediated C/EBPβ-DNA-binding activity during 3T3-L1 adipogenesis. Specifically, our studies show that hypophosphorylated Rb (6 h after induction) binds to C/EBPβ, whereas hyperphosphorylated Rb (18 h) does not (Fig. 3). During time points when Rb and C/EBPβ interact, the DNA binding activity of C/EBPβ is minimal (Figs. 1 and 3). Conversely, maximal C/EBPβ-DNA-binding activity occurs when Rb and C/EBPβ no longer associate (Figs. 1 and 3). Previous studies (4) have shown that C/EBPβ is able to bind to Rb by interacting with the pocket domains PDA and PDB. Our studies demonstrate that recombinant Rb proteins that contain the pocket domains (either full-length or p56Rb) reduce C/EBPβ-DNA-binding activity in 18 h nuclear extracts, whereas p25Rb, which lacks both C/EBPβ-DNA-binding domains (PDA and PDB), does not affect C/EBPβ-DNA-binding

activity (Fig. 4). Because hypophosphorylated Rb interacts with many different proteins with varying affinities [39], relatively high concentrations of recombinant Rb proteins were used to alter C/EBPβ-DNA-binding activity in competition assays. The addition of full-length recombinant hypophosphorylated Rb reduced C/EBPβ-DNA-binding activity when the concentration of exogenous Rb was about 4% of the total protein in the assay (Fig. 4). Furthermore, we show that overexpression of Rb inhibited the C/EBPβ-induced transactivation of the C/EBPα-promoter-luciferase reporter (Fig. 5). Collectively, our findings suggest that the hypophosphorylated Rb-C/EBPβ interaction couples cell cycle progression with the initiation of differentiation.

In conclusion, this study demonstrates that hypophosphorylated Rb is a negative regulator of C/EBPβ-DNA binding activity during 3T3-L1 preadipocyte differentiation. These studies add to the growing body of evidence that demonstrate a requirement for mitotic clonal expansion during 3T3-L1 adipogenesis. Our findings also provide evidence that clonal expansion participates in the temporal regulation of transcriptional effects required for differentiation (C/EBPβ-DNA-binding activity). These studies provide a link between mitotic clonal expansion and initiation of differentiation. Although this study proposes a mechanism for the delay in C/EBPβ-DNA binding activity, further studies are required to determine the purpose of this delay.

Chapter III

Adipose tissue inflammation is depot-dependent in diet-induced obese mice

Kathryn A. Cole, Alexia G. Smith, Patricia A. Sheridan, Nicole M.J. Schwerbrock, Qing Shi and Melinda A. Beck

A. Abstract

Objective: To investigate the sequence of events mediating inflammatory changes in subcutaneous and visceral adipose tissues in obesity.

Research Methods and Procedures: Male C57BL/6 weanlings were fed a high fat (HF) or low fat (LF) diet for 16 weeks. At two-week intervals, weight, body composition, liver triacylglycerol content, and fasting serum concentrations of glucose, insulin, corticosterone, leptin, and cytokines were measured. Liver and adipose tissue (AT) from inguinal, epididymal, and retroperitoneal depots were studied for cytokine and chemokine gene expression. ATs were studied for adipocyte size and macrophage infiltration.

Results: Compared to LF diet, the HF diet resulted in a higher body weight after 2 weeks and more body fat after 6 weeks. At week 8 in HF mice, epididymal adipocyte size peaked. Also, TNFα, IL-1β, IL-6 mRNA transcripts in the retroperitoneal AT increased, and MCP-1 and MIP-1α mRNAs in all AT depots increased. Inguinal MCP-1 and MIP-1α expression was transient, but epididymal and retroperitoneal expression persisted. At week 10, the HF mice had liver steatosis, higher serum leptin concentrations and the initial formation of macrophage aggregates in epididymal AT; aggregates were never significant in inguinal AT. At week 14, in HF mice had higher fasting serum insulin concentrations relative to the LF mice.

Discussion: Before significant hormonal or metabolic changes associated with obesity occur, epididymal adipocyte hypertrophy induced by a HF diet coincides with increased MCP-1 and MIP-1α mRNA in this depot, and precedes macrophage aggregation. In contrast, inguinal adipose tissue MCP-1 and MIP-1α gene expression is transient and is not followed by macrophage aggregation despite the presence of similar size adipocytes.

B. Introduction

Obesity, in both humans and animals, induces a state of low-grade chronic inflammation [133, 138, 195-198]. Attention has focused on the liver and adipose tissue as sources of the inflammatory mediators which exacerbate the health consequences of obesity [99, 199-201]. Within the adipose tissue, macrophages are reported to be the predominant source of most inflammatory cytokines and chemokines [124, 135, 202]. The macrophage infiltrate in the adipose tissue of lean C57BL/6 mice is rare and dispersed, while in overtly obese mice, the macrophages are plentiful [124], and aggregate around non-viable adipocytes [134]. Several studies have shown that in adipose tissue, adipocytes and macrophage express mRNA for TNFα, IL-1β and IL-6 [139, 140, 142] [124, 135, 203, 204], which are the prototypic inflammatory cytokines studied in association with obesity. The autocrine/paracrine effects of these cytokines include impairing local insulin signaling and facilitating the recruitment and activation of macrophages in the adipose tissue. Additionally, the chemokines MCP-1 and MIP-1α are elevated in the adipose tissue of obese animals and humans [128, 135, 202, 205, 206]. These chemokines may increase monocyte and macrophage trafficking to the tissue.

While there are clear differences between visceral adipose tissue and subcutaneous adipose tissue with regard to their metabolic profiles and health risks [207-209], no studies have examined their differences in the development of inflammation. Therefore, this study was designed to determine if there are inflammatory differences between visceral and subcutaneous adipose tissue in lean and diet-induced obese mice. In addition, rather than examining mice at one time point, we studied mice at numerous time points during induction of obesity to better determine the sequence of events that leads to the inflammatory state.

Every 2 weeks for 16 weeks, we measured adipocyte size, liver triacylglycerol (TAG) content, liver and adipose tissue mRNA for inflammatory cytokines and chemokines and the presence of adipose tissue macrophages.

C. Materials and methods

Animals and diets. Four-week old male C57BL/6 mice purchased from Jackson Labs (Bar Harbor, ME) were housed 4 per cage and were fed Purina standard laboratory chow for 4 days. At week 0 of the study, mice were randomized to receive either a high fat (HF) or low fat (LF) diet. Diets were purchased from Research Diets (New Brunswick, NJ). The HF diet (D01060502) contained 20% kcal from protein, 35% carbohydrate (corn starch), and 45% fat (soybean oil and lard). The LF diet (D01060501) contained 20% kcal from protein, 70% carbohydrate (corn starch), and 10% fat (soybean oil and lard). All experiments in this study used four to eight mice per group on each of the days examined. Animals were maintained under the strict animal care guidelines outlined by the Animal Welfare Act and the NIH "Guide for the Care and Use of Laboratory Animals". The animal facility is fully accredited by the AAALAC and all mouse procedures were approved by the UNC at Chapel Hill Institutional Animal Care and Use Committee.

Body composition. At baseline (week 0) and at 2 week intervals for 16 weeks, cages were randomly selected and the mice were weighed and their body composition was analyzed by PIXImus® densitometer (GE Lunar Corporation; Waukesha, WI), to measure whole body (subcranial) lean and fat tissue.

Fasting serum glucose, insulin, corticosterone, leptin, and cytokine concentrations. Following a 4-hour fast (0400-0800) mice were killed by cervical dislocation and blood was collected from a cardiac puncture. Serum was separated by centrifugation (2000 x g; 15 min) and stored at –20°C. Serum glucose was measured with Wako Glucose C2 kit. (Wako Diagnostics, Richmond, VA). Serum insulin was measured by ELISA (LINCO Research, St. Charles, MO). Serum corticosterone was measured by RIA (MP Biomedical, Solon, OH).

Serum leptin was measured by ELISA (R&D Systems, Minneapolis, MN). Serum cytokines were measured by 10-plex Antibody Bead Kit (Biosource, Camarillo, CA).

Measurement of liver triacylglycerol content. Approximately 100 mg liver tissue was homogenized in tissue lysis buffer. Lipids were extracted with 2:1 (v/v) chloroform: methanol and final extracts were dissolved in 3:1:1 (v/v/v) tert-butanol, methanol, Triton X-100. Triacylglycerol concentrations were measured using and enzymatic colorimetric assay. Values are expressed as milligrams of TAG per gram of liver tissue. The analysis of iver triacylglycerol was limited to 14 weeks of dietary intervention.

Quantitation of liver and adipose tissue gene expression. Liver and white adipose tissue (AT) from the inguinal, epididymal and retroperitoneal depots were flash frozen in liquid nitrogen and stored at −80°C. Total RNA from adipose tissue and liver was isolated using the RNeasy Lipid Tissue-, and RNeasy Mini kits (Qiagen, Valencia, CA), respectively. Reverse transcription was carried out using Superscript II First Strand Synthesis kit (Invitrogen, Carlsbad, CA) using oligo (dT) primers. Relative expression of mRNA transcripts for murine TNFα, IL-1β, IL-6, MCP-1, MIP-1α and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were determined using quantitative RT-PCR, with fluorescent reporters detected by a Bio-Rad iCycler PCR machine. Primers and probes were designed using Primer Express 1.5 (Applied Biosystems, Foster City, CA). The relative mRNA transcript expressions for GAPDH were determined for all samples and were used to normalize gene expression. All data are expressed as fold induction of mice fed the HF diet relative to mice fed the LF diet.

Immunohistochemistry. Paraformaldehyde-fixed, paraffin-embedded inguinal and epididymal adipose tissues were cut in 5μm sections. Sections were deparaffinized,

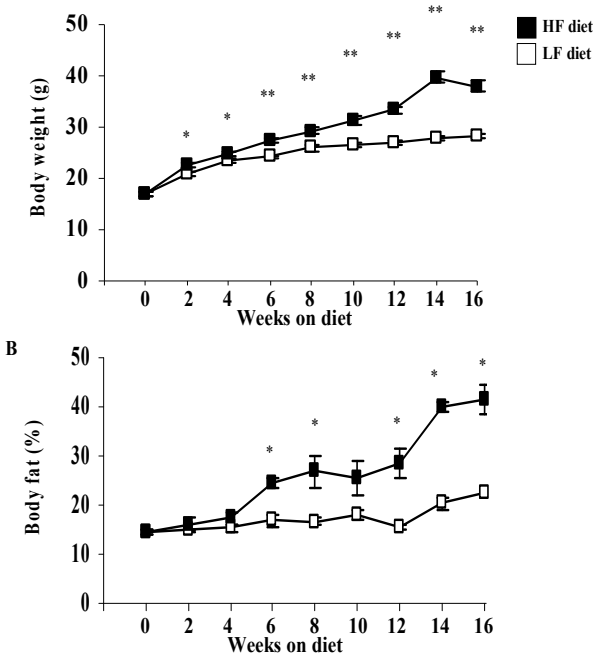
rehydrated, and endogenous peroxidases were quenched with 3% H_2O_2 for 20 min. Antigen retrieval was performed using Retrievagen A (BD Pharmingen, San Jose, CA). Endogenous biotin was blocked with diluted egg white for 20 min, rinsed with dH2O, and remaining avidin was quenched with 5% milk. Non-specific binding was blocked with 10% goat serum with 1% BSA in TBS. On each slide, the negative control was incubated in diluent, and the other tissue section was incubated with F4/80 antibody (Abcam, Cambridge, MA) overnight at 4°C. After washing in TBS, biotinylated goat anti-rat secondary antibody (BD Pharmingen) was applied for 30 min, followed by streptavidin-HRP, DAB substrate solution, and then counterstained with hematoxylin. Average adipocyte cross sectional area was determined from an average of 500 adipocytes in ≥5 fields at 20x magnification using ImageJ software. Macrophage formations of 'ring' aggregates were counted on the entire cross section under 40x magnification, A 'ring' aggregate was defined as DAB-positive stained macrophages encircling ≥ 75% of an adipocyte's circumference.

Statistical analysis. All data were analyzed using JMP 6© Statistical Software (SAS Institute Inc. Cary, NC). Data were analyzed by 2-way ANOVA. When the *F*-test was significant, data were analyzed using Wilcoxon rank scores. Differences were considered significant at *$p \leq 0.05$; ** $p \leq 0.001$. Values are expressed as means ± SE of 4-8 mice/group.

D. Results

Mice fed a high fat diet become obese. As expected, the HF diet induced obesity during the 16 week study. After only 2 weeks on the HF diet, the mice weighed significantly more than the LF group, and the difference in weight between the two groups increased over time (Fig. 3.1A). At week 12, the HF group weighed 20% more than the LF group. By week 16, the HF group had gained nearly twice as much weight as the LF group. Body composition determination by PIXImus™ revealed that while the groups had similar lean body mass (data not shown), the HF group had a higher percent body fat at week 6 (Fig. 3.1B). By week 8, the HF group had twice the body fat mass of the LF group. Relative to the LF group, the HF group gained 3.3 times more fat mass during the 16 week study. Thus, as previously shown [85, 135, 210], feeding a HF diet to C57BL/6 mice induces obesity.

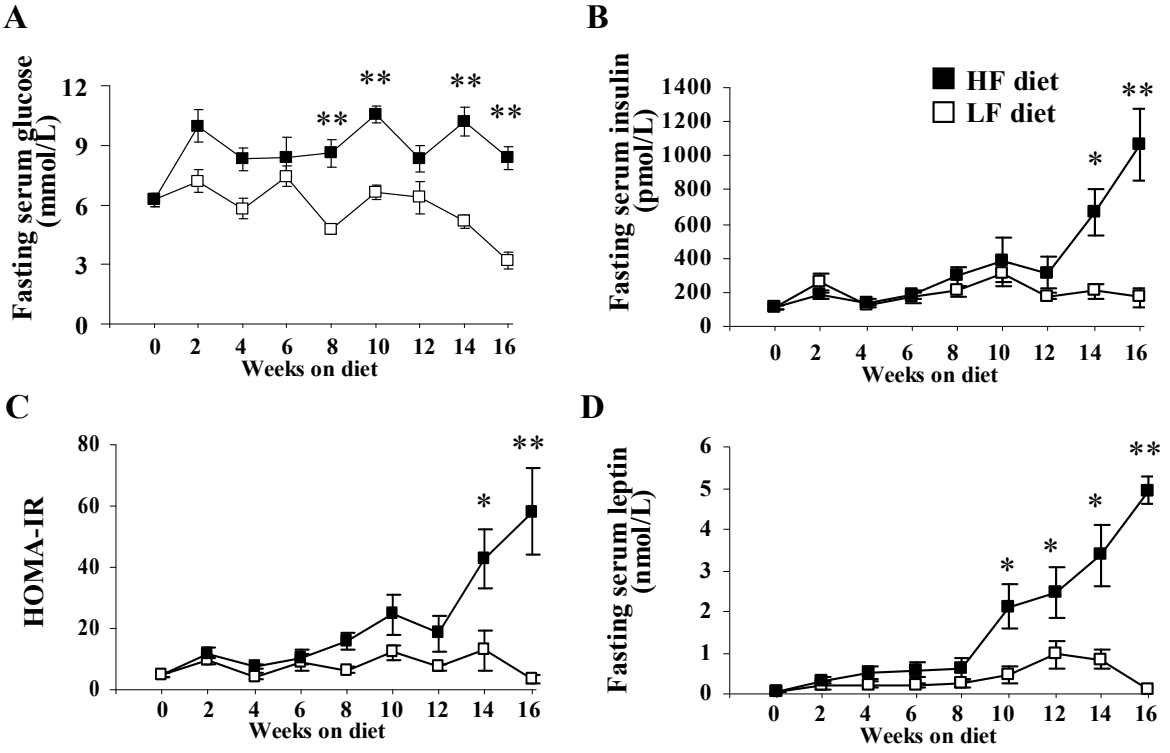
Figure 3.1: Increase in body weight and body fat during 16 weeks of HF feeding. (A) Body weight (grams) of male mice receiving either a HF or LF diet. Values are expressed as mean ± SEM, *n* = 12-16. (B) Percent body fat as determined by PIXImus™. Values are expressed as mean ± SEM, *n* = 6-8. *p≤0.05; **p≤0.001 of HF relative to LF group, Student's *t* test.



Elevated fasting serum glucose and insulin concentrations in mice fed a HF diet. Obesity and inflammation are associated with glucose intolerance and insulin resistance; therefore, we measured fasting serum glucose and insulin concentrations in the HF and LF groups. As shown in Fig. 3.2A, the mean fasting glucose concentrations in the HF group exceeded 8.3 mmol/L (~ 150 mg/dl) at each time point. This is above the fasting glucose concentrations found in the LF mice, as has been previously described [211, 212]. Because corticosterone is known to increase serum glucose and counteract the effects of insulin, we measured serum corticosterone concentrations. There were no differences in fasting serum corticosterone levels between the groups (data not shown). Fasting serum insulin concentrations were similar between the groups through week 12 (Fig. 3.2B). However, at weeks 14 and 16, the respective fasting serum insulin in the HF group was 4-, and 6-fold higher than the LF group. Furthermore, the homeostatic model assessment for insulin resistance (HOMA-IR) was higher in the HF group relative to the LF group at weeks 14 and 16 (Fig. 3.2C).

Increased fasting serum leptin concentrations in mice fed a HF diet. It has been well established that the serum concentration of leptin is increased in obesity. Leptin not only functions in energy homeostasis, it is also important in the inflammatory response. By week 10 of the study, mice in the HF group had elevated fasting serum leptin concentrations (Fig. 3.2D).

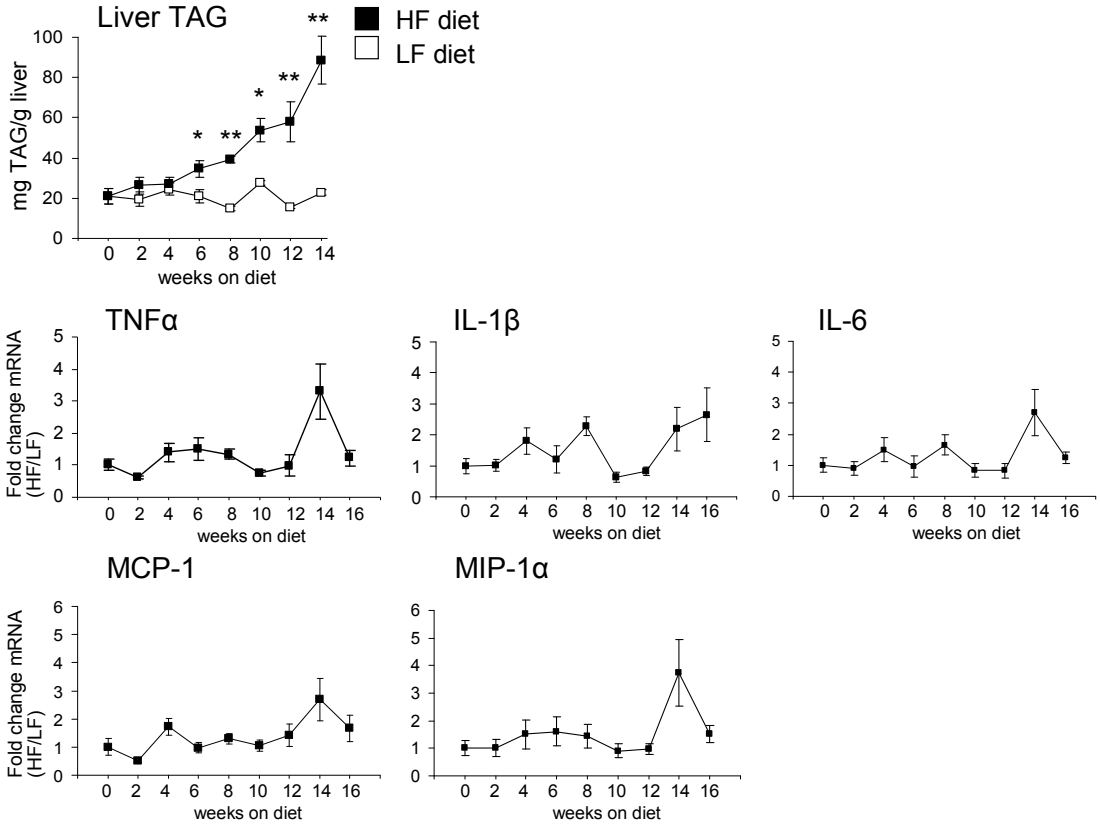
Figure 3.2: Changes in metabolic and hormonal parameters during the 16 weeks of HF feeding. (A) Fasting serum glucose (mmol/L); (B) fasting serum insulin (pmol/L), (C) homeostatic modeling assessment of insulin resistance (HOMA-IR); (D) fasting serum leptin (nmol/L) in mice receiving a HF or LF diet. Mice were fasted for 4 hours (0400-0800) prior to blood draw. Values are expressed as means ± SEM, n = 6-8. *p≤0.05; **p≤0.001 of HF relative to LF group, Student's t test.



Increased liver triacylglycerol is not associated with increased liver cytokine or chemokine mRNA. Obesity and several metabolic disturbances involving inflammation are associated with hepatic steatosis. Therefore, we measured the hepatic triacylglycerol (TAG) content in mice receiving either the LF or HF diet. When compared to the LF group at week 6, the HF group had significantly more liver TAG (Fig. 3.3A). At week 10, over 5% of the liver weight in the HF group was TAG, indicating steatosis. By week 14, relative to the LF group, the HF group had nearly 4 times more liver TAG. To determine whether or not inflammatory cytokines and chemokines were being expressed in the livers of mice on the

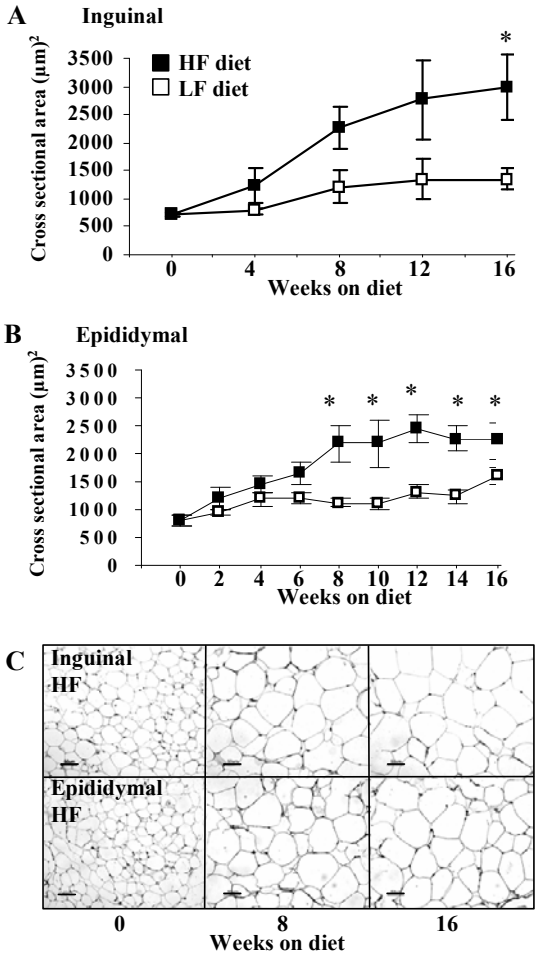
HF diet, we measured TNFα, IL-1β, IL-6, MCP-1 and MIP-1α gene expression by qRT-PCR (Fig. 3.3). Statistical analysis by 2-way ANOVA revealed that there was not a main effect of diet on cytokine and chemokine transcript gene expression. Therefore, changes in cytokine and chemokine transcript expression in the liver are likely due to an effect of age rather an effect of obesity. To determine if serum cytokine concentration was increased in the HF group, we analyzed the serum of both groups for proinflammatory cytokines. Serum TNFα, IL-1β and IL-6 were below the detection limit for the assay (data not shown).

Figure 3.3: Hepatic triacylglycerol content and inflammatory cytokine and chemokine gene expression increases during HF feeding. Mean hepatic triacylglycerol content. Data are expressed as milligrams of triacylglycerol per gram of liver tissue ± SEM. *p≤0.05; **p≤0.001 of HF relative to LF group by Student's *t* test. Relative gene expression for liver cytokines and chemokines: TNFα, IL-1β, IL-6, MCP-1, and MIP-1α. Target gene expression was normalized to GAPDH gene expression. Values represent the mean of the HF group expressed as fold change relative to the mean of the LF group ± SEM, *n* = 6-8. Two-way ANOVA indicated that there was not a main effect of diet.



Differences in the onset of increased adipocyte size are depot-dependent in mice fed the HF diet. Hepatic steatosis, and increased fasting serum insulin and leptin concentrations are correlated with obesity and increased visceral adipocyte size [213-215]. Although visceral adipose tissue is often studied in obesity, much less is known about subcutaneous adipose depots. Therefore, during the study we measured the mean cross-sectional area of adipocytes in inguinal (subcutaneous) and epididymal (visceral) adipose tissue. Throughout the study, the inguinal adipose depot from mice on the HF diet displayed more variability in adipocyte size. At week 16, the inguinal adipocytes of mice fed the HF diet were significantly larger than adipocytes from mice fed the LF diet (Fig. 3.4A). In contrast, the epididymal adipocytes of the HF group were significantly larger than the LF group at week 8, when they reached a plateau in size (Fig. 3.4B).

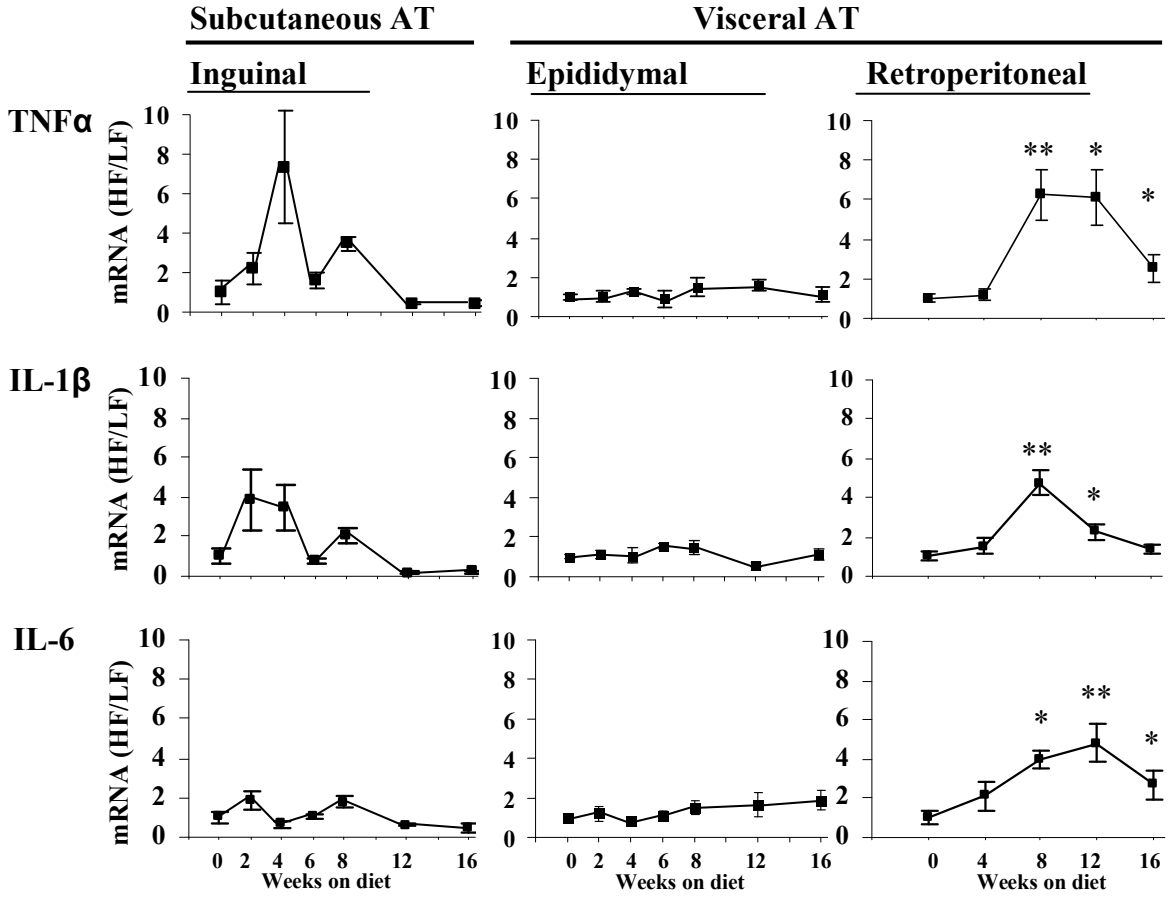
Figure 3.4: Mean adipocyte cross sectional area of mice receiving either a HF diet or LF diet. (A) Inguinal and (B) epididymal adipocyte cross sectional area (μm^2) in mice receiving a HF diet or LF diet. Values represent the mean ± SEM, n = 4-6. *$p \leq 0.05$ of HF group relative to the LF group, Kruskal-Wallis test. (C) Representative histological sections of inguinal (top panel) and epididymal (bottom panel) adipose tissue from mice after 0, 8 and 16 weeks of HF feeding. Scale bar is 50 μm; magnification X20.



Inflammatory cytokine mRNA levels vary by adipose tissue depot in mice fed a HF diet. Increased adipocyte size is positively correlated with increased expression of several inflammatory mediators [214]. However, because adipose tissue displays depot-specific metabolic characteristics, we measured cytokine gene expression in both subcutaneous (SQ) and visceral (V) depots: inguinal (SQ); epididymal (V), and retroperitoneal (V). By week 8,

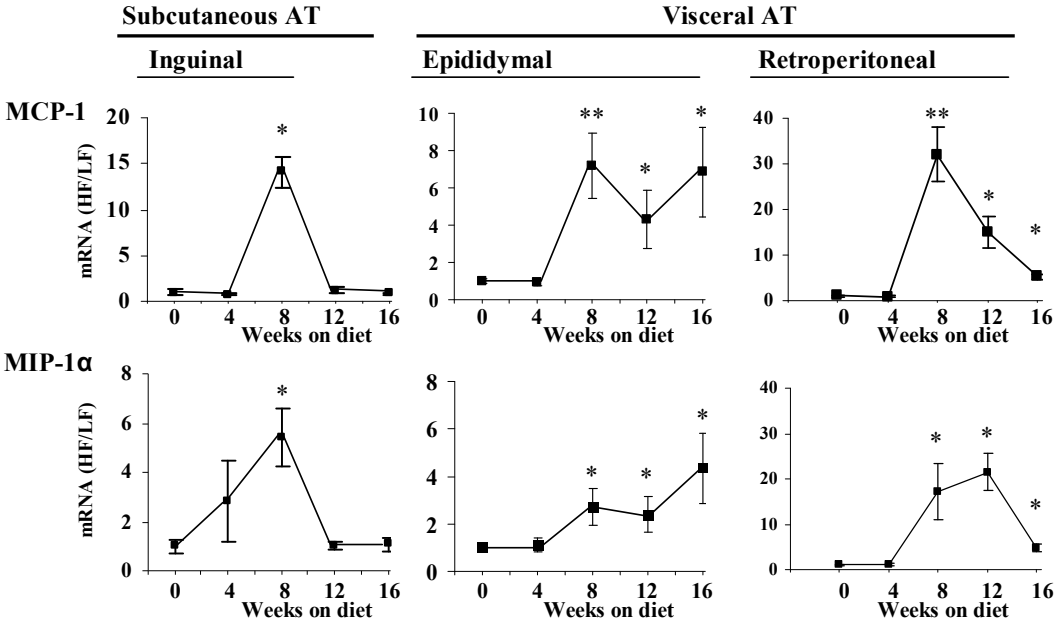
the amount of mRNA transcripts for TNFα, IL-1β, and IL-6 were significantly higher in the retroperitoneal adipose tissue of the HF fed group relative to the LF fed group (Fig. 3.5). In contrast, in the inguinal and epididymal adipose depots, none of the cytokine mRNA concentrations were elevated.

Figure 3.5: Relative abundance of cytokine mRNA in subcutaneous and visceral adipose tissue of mice receiving a HF diet. TNFα gene expression (top row) in inguinal (left), epididymal (center), and retroperitoneal adipose tissue (right column). IL-1β gene expression (center row) in inguinal (left), epididymal (center), and retroperitoneal adipose tissue (right column). IL-6 gene expression (bottom row) in inguinal (left), epididymal (center), and retroperitoneal adipose tissue (right column). Values represent the mean of the HF group expressed as fold change relative to the mean of the LF group ± SEM, *n* = 6-8. *p≤0.05; **p≤0.001 of HF relative to LF group, Kruskal-Wallis test.



Inflammatory chemokine mRNA levels vary by adipose tissue depot in HF fed mice. In addition to adipose tissue of obese mice and humans producing inflammatory cytokines, inflammatory chemokines, such as monocyte chemotactic protein-1 (MCP-1) and macrophage inflammatory protein-1α (MIP-1α) are also elevated [128, 135, 202, 205, 206]. We found that, as for inflammatory cytokine expression, chemokine expression in the AT was also increased in mice fed the HF diet. However, although proinflammatory changes were limited to the retroperitoneal depot, the increase in chemokine mRNA transcripts for MIP-1α and MCP-1 occurred in all 3 depots. As shown in Figure 3.6, the pattern of gene induction differed between the subcutaneous and visceral adipose tissue. In the inguinal adipose tissue, the increase in chemokine gene expression was transient. In the visceral adipose tissue of the mice fed the HF diet, MCP-1 and MIP-1α gene expression was elevated at week 8 and persisted through week 16.

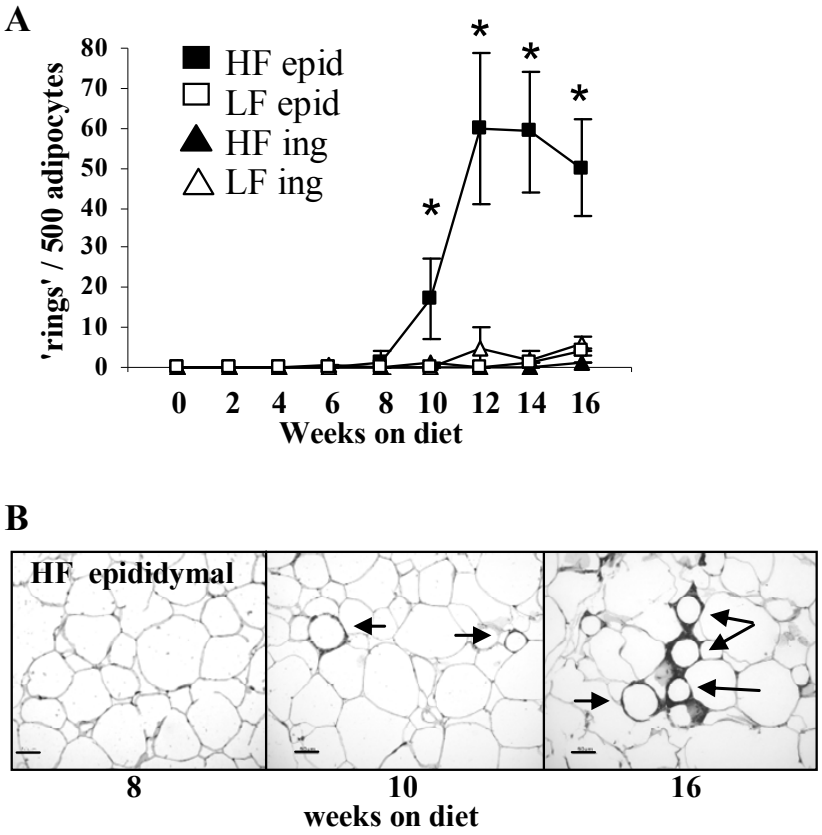
Figure 3.6: Relative abundance of chemokine gene expression in subcutaneous and visceral adipose tissue of mice receiving a HF diet. MCP-1 gene expression (top row) in inguinal (left), epididymal (center), and retroperitoneal adipose tissue (right column). MIP-1α gene expression (bottom row) in inguinal (left), epididymal (center), and retroperitoneal adipose tissue (right column). Each data point represents the mean of the HF group expressed as fold change relative to the mean of the LF group, ± SE of 6-8 mice. *p≤0.05; **p≤0.001 of HF relative to LF group, Kruskal-Wallis test.



Macrophage infiltration occurs after chemokine gene expression in epididymal AT. MCP-1 and MIP-1α are chemoattractants for macrophages. Although several studies have demonstrated macrophage infiltrate in the visceral AT of obese animals and humans [124, 135] no studies have examined macrophage infiltration in different AT depots over time. In the epididymal AT, significant macrophage accumulation began at week 10 in mice fed the HF diet (Fig. 3.7A). Importantly, the macrophage infiltration follows the increase in chemokine gene expression (week 8). In contrast, in the inguinal AT, were there was no

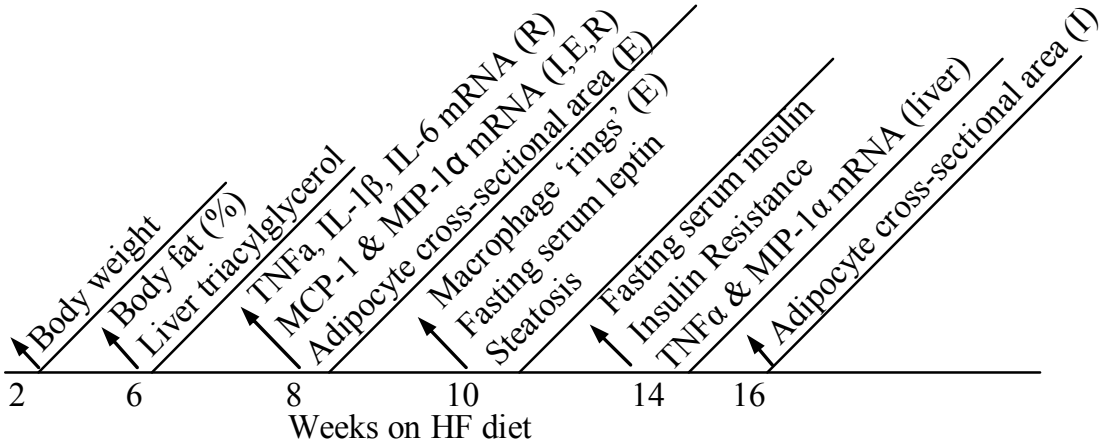
sustained upregulation of chemokine expression, no significant macrophage accumulation occurred, even after 16 weeks on the HF diet.

Figure 3.7: Macrophage formations of 'ring' aggregates in adipose tissue of mice fed a HF or LF diet for 16 weeks. (A) Macrophage 'ring' aggregates per 500 adipocytes in inguinal (ing) and epididymal (epid) adipose tissue of HF and LF groups. A 'ring' was defined as DAB-positive stained macrophages encircling ≥ 75% of an adipocyte's circumference. Mean ± SEM. n = 4-6. *p<0.05 of HF relative to LF group, Kruskal-Wallis test. (B) Immunohistochemical staining of epididymal adipose tissue F4/80$^+$ macrophage 'ring' aggregates (arrows) after 8, 10 and 16 weeks of HF feeding. Scale bar is 50 μm; magnification x 20.



Timing of hormonal and metabolic changes. Figure 3.8 summarizes the onset of hormonal and metabolic changes and inflammation in various adipose tissue depots during 16 weeks of HF feeding.

Figure 3.8: Timeline of changes in metabolic, hormonal and liver characteristics, and the onset of inflammation in adipose tissue of mice receiving a HF diet relative to mice receiving the LF diet. Abbreviations: R = retroperitoneal adipose tissue; I = inguinal adipose tissue; E = epididymal adipose tissue.



E. Discussion

Adipose tissue's contribution to a state of chronic low-grade inflammation during obesity has recently received much attention [133, 138, 195, 196, 198]. The findings that increased numbers of macrophages were in the visceral adipose tissue of obese humans and animals contributed to the idea that the coincident increase in inflammatory mediators was derived from the infiltrating macrophages. Because AT depots are known to have disparate metabolic profiles [207, 208], we reasoned that the individual depots may also have differences in macrophage accumulation and inflammatory profiles.

To study inflammation in AT, we utilized the well-characterized C57BL/6 model of diet-induced obesity. C57Bl/6 mice fed a HF diet have previously been shown to consume more calories than mice on a LF diet, and to become obese [216]. Mice fed either a HF or LF diet were studied at 2-week intervals for 16 weeks. Metabolic and hormonal changes induced by the HF diet were determined by measuring fasting serum glucose, insulin and leptin concentration, and liver TAG content. As previously demonstrated [211, 212], elevated fasting serum glucose concentration occurred soon after starting the high fat diet (within 2 weeks) without a consequent increase in fasting serum insulin concentration. Toye et al. showed that C57BL/6 mice with normal insulin sensitivity have impaired glucose-stimulated insulin secretion [211]. In our study, fasting serum insulin concentrations and HOMA-IR values were not elevated in the HF group until week 14, which is consistent with other reports [212, 217]. Although we assessed insulin resistance using HOMA-IR, and not using the hyperinsulinemic-euglycemic clamp or the frequently sampled intravenous glucose tolerance test that are widely considered to be more accurate, HOMA-IR has been validated as a surrogate marker of insulin resistance in several studies and compares reasonably well

with clamp data [218-221]. Also, as expected [217], fasting serum leptin concentrations in the HF mice were greatly increased by week 10.

Elevated liver triacylglycerol (TAG) concentratons have been reported in diet-induced obese mice [120]; therefore, we measured liver TAG content over time. In hepatic steatosis, TAG comprises 5% or more of the liver weight. While the HF group had higher hepatic TAG content by week 6, the onset of steatosis occurred at week 10. Therefore, steatosis, which coincided with elevated fasting serum leptin concentrations, occurred prior to the increase of fasting serum insulin concentrations. Because hepatic steatosis may induce the expression of inflammatory cytokines [122] and chemokines, we measured hepatic cytokine and chemokine gene expression. Relative to mice consuming the LF diet, mice consuming the HF diet did not have increased TNFα, IL-1β, IL-6, MCP-1, or MIP-1α mRNA transcripts. Cai et al. reported that in diet-induced obese mice, liver TNFα and IL-1β mRNA increased 3-fold, and IL-6 increased 6-fold increase in hepatic IL-1β and IL-6 mRNA, respectively during their study of diet-induced obese mice [122]. The differences between the results of our studies may be due to differences in experimental design, such as the choice of control diet (e.g. the chow diet used for the LF mice in the Cai et al. study normally contains 4% fat vs. 10 % fat in our purified LF diet).

Although increased serum concentrations of inflammatory cytokines have been reported in humans and genetic animal models of obesity [222-226], in our diet-induced obese mice, serum cytokine concentrations were below the detection limit for the assay (assay sensitivity for TNFα ≥ 5 pg/ml; IL-1β ≥ 10 pg/ml; IL-6 ≥ 10 pg/ml). Lack of serum cytokines in diet-induced obese mice was also reported by Weisberg et al. (12), with the exception of very low concentrations of IL-6 (8 pg/ml).

Previous studies have demonstrated increased mRNA transcripts for inflammatory chemokines in the visceral adipose tissue of mice fed a HF diet [120, 205, 227]. Two studies have examined MCP-1 and MIP-1α during the development of inflammation in diet-induced obese mice. Xu et al. reported that C57BL/6 mice fed a HF diet for 16 weeks have increased mRNA levels for MIP-1α and MCP-1 in their epididymal adipose tissue [135]. However, inguinal and retroperitoneal adipose tissues were not examined. Chen et al. designed a study to avoid age-related effects of the diet [227]. They demonstrated increased MCP-1 mRNA levels in both inguinal and epididymal AT in 4 month old mice after 1 week of HF feeding. The Chen et al. study differed from our study in the age of the mice when the diet was introduced (12-15 week old mice vs. 4 week old mice), as well as the percent fat in the LF and HF diets (5% vs 10% LF and 59.4% vs 45% HF). In our study, we examined 3 AT depots (inguinal, epididymal and retroperitoneal) for MCP-1 and MIP-1α the gene expression. In contrast to Chen et al., we found no expression of these chemokines until week 8 on the HF diet, at which time all 3 depots had increased expression of mRNA for both MCP-1 and MIP-1α. Interestingly, the subcutaneous AT expressed increased mRNA for these chemokines only at week 8, whereas the two visceral AT depots had elevated mRNA levels at weeks 8 through 16. The highest induction occurred in the retroperitoneal AT. By week 16, although mRNA levels for MCP-1 and MIP-1α were decreased in the retroperitoneal compared to week 8 values, the level of mRNA expression in the retroperitoneal was similar to the level in the epididymal AT. Thus, although both epididymal and retroperitoneal AT are visceral depots, their pattern of chemokine gene expression differs, suggesting that not all visceral depots are alike.

In addition to chemokines, increased mRNA levels for cytokines has been demonstrated in the visceral adipose tissue from obese rodents and humans [139, 228, 229]. Until quite recently, the various intraabdominal adipose tissue depots have been collectively referred to as visceral adipose tissue, therefore, distinctions among mesenteric, perigonadal (epididymal or parametrial) and retroperitoneal depots were not made. Recently, the perigonadal depot has received more attention than the retroperitoneal adipose tissue. Indeed, very little is known about cytokine gene expression in the retroperitoneal depot. In obese rats, Morin et al. reported that the retroperitoneal adipose tissue expresses more TNFα mRNA than the epididymal depot [230]. We are the first to report increased gene expression of the inflammatory cytokines TNFα, IL-1β and IL-6 in the retroperitoneal adipose tissue of diet-induced obese mice. In contrast to the increased gene expression of inflammatory cytokines in the retroperitoneal depot, the epididymal depot did not express any of these cytokines. Furthermore, the subcutaneous adipose tissue did not upregulate the expression of the inflammatory cytokines.

What induces the AT to express proinflammatory cytokines and chemokines in mice fed the HF diet? One proposal is adipocyte size. It has been demonstrated that the expression of inflammatory chemokines is greater in larger adipocytes compared to smaller adipocytes [231]. In our study, the inflammatory gene expression in the epididymal adipose tissue coincided with a plateau in the size of the adipocytes in the HF group. Proposed mechanisms of the association between adipocyte hypertrophy and inflammation include increased shear stress [232], reactive oxygen species [233, 234], impaired insulin signaling [235-237], and hypoxia-induced cytokine gene expression [238]. However, our study suggests that the size of the adipocyte is not the primary driving force for the induction of pro-inflammatory

mediators. We measured adipocyte size in two different adipose depots, representing subcutaneous (inguinal) and visceral (epididymal) depots. Each of these depots had unique pro-inflammatory characteristics, independent of adipocyte size. Thus, we found that although the subcutaneous inguinal and the visceral epididymal adipocytes of the HF mice increased equivalently in size over time, the subcutaneous adipose tissue did not sustain elevated levels of mRNA for MCP-1 and MIP-1α. Studies are underway to determine whether or not retroperitoneal adipocyte size is correlated with inflammatory cytokine and chemokine gene expression. At the writing of this manuscript, our data do not support the hypothesis that adipocyte size caused an upregulation of these genes.

Given the increased gene expression of macrophage chemoattractants in mice fed the HF diet, we looked for the presence of macrophage infiltration in subcutaneous and visceral adipose tissue. We found that macrophages began to aggregate in the epididymal adipose tissue at week 10, reaching its highest level at week 12. The macrophage infiltrate occurred after the increase of MIP-1α and MCP-1 mRNA, suggesting that instead of macrophages being responsible for the initial inflammatory signals, the adipocytes produced the pro-inflammatory cytokines and chemokines prior to macrophage arrival. Related to the lack of sustained chemokine production by the subcutaneous adipose tissue in the HF group, we found no significant increase in macrophage aggregation in this depot. This dramatically contrasts with the visceral fat, which had an increase in macrophage aggregation that occurred after the increase in chemokine gene expression. It has been suggested that macrophages may be present in the AT tissue due to hypertrophy-induced adipocyte death [134]. Thus, as the adipocyte reaches a maximal size, it dies by necrosis and the macrophages surrounding the dead adipocyte are there to remove the dead/dying adipocytes. Indeed, 2

weeks after the onset of epididymal adipocyte hypertrophy and induction of inflammatory chemokines, macrophage aggregates were detected. However, the subcutaneous adipocytes also reached a maximal size, but macrophage infiltration was not found in this depot. Few studies have investigated macrophage accumulation in the subcutaneous adipose tissue. Weisberg et al. reported that, relative to female lean mice, male diet-induced obese mice had a higher percentage of F4/80+ macrophages in the subcutaneous AT at 12 weeks on a high fat diet [124]. Although the Weisberg et al. study did find an increase in the macrophage cell population in the subcutaneous AT, it was less than half that found in visceral tissue. In addition, unlike our study, they did not count the presence of macrophage 'ring' aggregates, but rather counted the number of nuclei in cells that stained positive for F480. Thus, there are clear differences in the macrophage trafficking pattern between visceral and subcutaneous adipose tissue.

As for the cytokine/chemokine mRNA expression, the differences between subcutaneous and visceral AT gene expression are likely independent of size alone. Metabolically, it is known that visceral and subcutaneous AT are not equivalent. Studies have indicated that gene expression differs between fat depots in both rodents and humans [128, 208]. Genes involved in inflammation predominate in the visceral adipose tissue depots [239], while genes promoting lipid accumulation predominate in the subcutaneous depots [240]. The importance of this is underscored by the depot's location, as excess visceral adipose tissue is associated with many of the adverse consequences of obesity, such as coronary artery disease, hypertension and type 2 diabetes. Gesta et al. demonstrated that adipocytes isolated from different depots retain their unique gene expression, even in culture [208]. Therefore, it is likely that the subcutaneous and visceral depots have different regulatory mechanisms for

cytokine gene expression that are independent of their microenvironment. In addition, even within the visceral depots, there are differences based on location of the AT (as seen by chemokine/cytokine mRNA differences between two visceral depots, epididymal and retroperitoneal).

This study reports for the first time, metabolic changes, liver triacylglycerol accumulation, and adipose tissue inflammation in 3 different depots over a 16 week period of high fat feeding. A specific time line can be built from our study that demonstrates a distinct pattern of metabolic changes and development of inflammation. We found that serum glucose levels increased as early as week 2 on the HF diet and liver triacylglycerol, which was significantly higher by week 6, resulted in liver steatosis by week 10. Of particular importance, we found significant differences between subcutaneous and visceral adipose tissue. Although both depots contained adipocytes of similar size, only the visceral adipose tissue demonstrated sustained chemokine and cytokine mRNA levels beginning at week 8 on the HF diet. In addition, macrophage infiltration occurred after chemokine expression was upregulated, and only in visceral tissue. At this time, it is unclear why the subcutaneous tissue is not as susceptible to inflammation as visceral adipose tissue. Further studies are needed to elucidate the mechanisms underlying the disparate inflammatory response between subcutaneous and visceral adipose tissue in obesity.

Chapter IV

Adipose tissue cytokine response to influenza virus infection in diet-induced obese mice

Kathryn A. Cole, Alexia G. Smith, Patricia A. Sheridan, Nicole M.J. Schwerbrock, and Melinda A. Beck

A. Abstract

Obesity in mice is associated with an impaired immune response to influenza virus infection. Influenza viruses induce an inflammatory response in the infected respiratory mucosa. Because obesity itself results in an inflammatory state, we hypothesized that inflammation in the liver and adipose tissues may contribute to the immune dysfunction during influenza infection. C57BL/6 weanling mice were fed a LF or HF diet. Body composition, serum cytokines, liver TAG, and liver and adipose tissue cytokine and chemokine mRNA transcripts were measured. After 22 weeks on the respective diets, the mice were intranasally infected with influenza A/PR8/34. Influenza infection induced liver TNFα mRNA expression in both lean and obese mice, but only in obese mice were IL-1β, IL-6, and MCP-1 induced. Adipose tissue (AT) responded to influenza infection in a depot-specific manner. Inguinal AT MIP-1α mRNA transcripts increased in both lean and obese mice, but IL-1β and IL-6 only increased in obese mice. Gonadal AT IL-1β and MIP-1α mRNA transcripts increased in both groups, while IL-6 and MCP-1 transcripts were already higher in obese mice prior to infection. Gonadal and retroperitoneal AT TNFα mRNA transcripts decreased in lean but not obese mice during infection. Relative to lean mice, obese mice had higher retroperitoneal AT IL-1β, IL-6 and MIP-1α mRNA transcripts 3 d post-infection. During the influenza infection, in obese mice, but not in lean mice, the number of macrophages and T cells in the gonadal AT decreased. This study provides evidence suggesting that in obesity, the liver and adipose tissue depots may contribute to immune dysfunction during influenza virus infection.

B. Introduction

Obesity is associated with many adverse health consequences, including immune dysfunction. Diet-induced obese mice infected with influenza virus have higher mortality and increased inflammatory cellular infiltrate in their lungs than their lean counterparts [62]. Adipose tissue (AT) of obese animals and humans is associated with a chronic inflammatory state that is characterized by increased expression of inflammatory cytokines, chemokines, and increased numbers of macrophages and T lymphocytes. Because of the pro-inflammatory state of the AT in obese mice, we hypothesized that the AT itself may be contributing to the exaggerated inflammatory response following influenza infection. In addition, the liver may contribute to the inflammatory state in obesity as increased free fatty acid flux to hepatocytes activates IKK-β, resulting in subsequent NFκB-mediated gene transcription of inflammatory cytokines such as TNFα, IL-1β, IL-6 and MCP-1 [115].

Feeding a high fat diet induces obesity and liver steatosis. Although there are several reports about inflammatory gene expression in adipose tissue, there have been fewer reports about inflammatory gene expression in livers with steatosis [122, 241]. Inflammation in the liver is primarily defined histologically rather than by the presence of biochemical mediators of inflammation. Because leukocyte activation and migration are mediated by inflammatory cytokines and chemokines, changes in hepatic inflammatory cytokine and chemokine expression due to obesity may be important determinants in the outcome of an influenza virus infection.

Diet-induced obese mice have increased levels of inflammatory cytokines and chemokines in their AT. However, the inflammatory profiles among the AT depots differ. Compared to the subcutaneous AT, the visceral depots express quantitatively more

inflammatory cytokines, chemokines, and also contain more cytotoxic T lymphocytes and macrophages. Of the visceral depots, the gonadal AT has been most widely studied, owing to its large size and accessible location. In contrast, the retroperitoneal depot has received comparatively little attention. Because the individual AT depots display different inflammatory and transcriptional profiles, AT distribution, not only between subcutaneous and visceral, but also between the visceral gonadal and retroperitoneal depots may have important health consequences. Although the pro-inflammatory state of AT has been studied by others, there are no reports of the response of AT to infection.

Here, we demonstrate that in obese mice, the AT itself may contribute to the immune dysfunction during influenza virus infection through increased inflammatory cytokine and chemokine gene expression, and alterations in macrophage and T cell chemotaxis. Our study raises the question of whether the increased macrophage and T-cell populations of the AT contribute to the lung pathology of the influenza-infected obese mice.

C. Materials and methods

Animals. Four-week old C57BL/6 mice were purchased from Jackson Labs (Bar Harbor, ME) and had ad libitum access to food and water. Mice were housed 4 per cage at the University of North Carolina Animal Facility, which is fully accredited by the American Association for Accreditation of Laboratory Animal Care. Mice were maintained under protocols approved by the UNC at Chapel Hill Institutional Animal Care and Use Committee. Each experiment used five to eight mice per group on each of the days examined.

Diets: Weanling mice were fed Purina standard laboratory chow for 4 days. They were then randomized to receive either a low-fat (LF) diet or a high-fat (HF) diet for the remainder of the study (#D12328 or D12331, Research Diets, New Brunswick, NJ).

Body composition. At 2-week intervals for 16 weeks, and prior to infection at 22 weeks, mice were weighed and their body composition was analyzed by PIXImus™ densitometer (GE Lunar Corporation; Waukesha, WI).

Measurement of liver triacylglycerol content. Approximately 100 mg liver tissue was homogenized in tissue lysis buffer. Lipids were extracted with 2:1 (v/v) chloroform: methanol, and final extracts were dissolved in 3:1:1 (v/v/v) tert-butanol, methanol, Triton X-100. Triacylglycerol (TAG) concentrations were measured using and enzymatic colorimetric assay. Values are expressed as milligrams of TAG per gram of liver tissue. The liver TAG portion of experiment was limited to 14 weeks of dietary intervention.

Virus and infection. The mouse-adapted strain of Influenza A/Puerto Rico/8/34 (A/PR/8) was obtained from the American Type Culture Collection (Rockville, MD), and was propagated in the allantoic fluid of fertilized hen eggs. Infectious allantoic fluid was

collected, clarified by centrifugation, and the viral titer was determined by hemagglutination assay (HAU) [242]. After 22 weeks on the diets, mice were anesthetized with an intramuscular injection of a ketamine (0.6 mg/kg)/xylazine (0.35 mg/kg) solution and infected intranasally with 2 HAU of A/PR8 virus in 0.02 mL PBS. Previous studies in our laboratory established that this dose of virus elicits an immune response while causing little mortality in infected control mice.

Measurement of serum cytokine concentration. At 2-week intervals for 16 weeks, and then prior to infection at 22 weeks (0 d), and 3, and 6 d post-infection (p.i.), mice were killed by cervical dislocation and blood was collected from a cardiac puncture. Serum was separated by centrifugation (2000 x g; 15 min) and stored at –20°C. Serum cytokines were measured by 10-plex Antibody Bead Kit (Biosource, Camarillo, CA).

Quantitation of liver and adipose tissue cytokine and chemokine mRNA transcripts. Liver and adipose tissues from the inguinal, gonadal, and retroperitoneal depots were removed at 2-week intervals for 16 weeks, and then prior to infection at 22 weeks (0 d), 3, and 6 d p.i. In the inguinal depot, the lymph node was carefully removed and discarded. Total RNA from adipose tissue and liver was isolated using the RNeasy Lipid Tissue, and RNeasy Mini kits (Qiagen, Valencia, CA), respectively. Reverse transcription was carried out using Superscript II First Strand Synthesis kit (Invitrogen, Carlsbad, CA) using oligo (dT) primers. Concentrations of mRNA levels for murine TNFα, IL-1β, IL-6, MCP-1, MIP-1α, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were determined using quantitative RT-PCR (qRT-PCR) with fluorescent reporters detected by a Bio-Rad iCycler PCR machine. Probes and intron-exon junction spanning primers were designed using Primer Express 1.5 (Applied Biosystems, Foster City, CA). The mRNA levels for GAPDH were determined for

all samples, and the Ct values were analyzed by 2-way ANOVA. Because Ct values did not differ by day or diet, GAPDH was used to normalize gene expression.

Immunohistochemistry. Paraformaldehyde-fixed, paraffin-embedded adipose tissues were cut in serial 5μm sections, with 2 sections per slide. Sections were deparaffinized, rehydrated, and endogenous peroxidases were quenched with 3% H_2O_2 for 20 min. Antigen retrieval was performed using Retrievagen A (BD Pharmingen, San Jose, CA). Endogenous biotin was blocked with diluted egg white for 20 min, rinsed with dH2O, and remaining avidin was quenched with 5% milk. Non-specific binding was blocked with 10% goat serum with 1% BSA in TBS. On each slide, the first section was the negative control and was incubated in diluent without the primary antibody. For macrophage identification, the second section was incubated with F4/80 antibody (Abcam, Cambridge, MA) overnight at 4°C. For T-cell identification, the second tissue section was incubated with CD3 antibody overnight at 4°C. After washing in TBS, biotinylated goat anti-rat secondary antibody (BD Pharmingen) was applied for 30 min, followed by streptavidin-HRP, DAB substrate solution, and then counterstained with hematoxylin. DAB-positive cells were counted under 40 x magnification. Adipocyte mean cross sectional area was calculated from 5 sections at 20 x magnification by Image J software (1.37v.) downloaded from http://rsb.info.nih.gov/ij/.

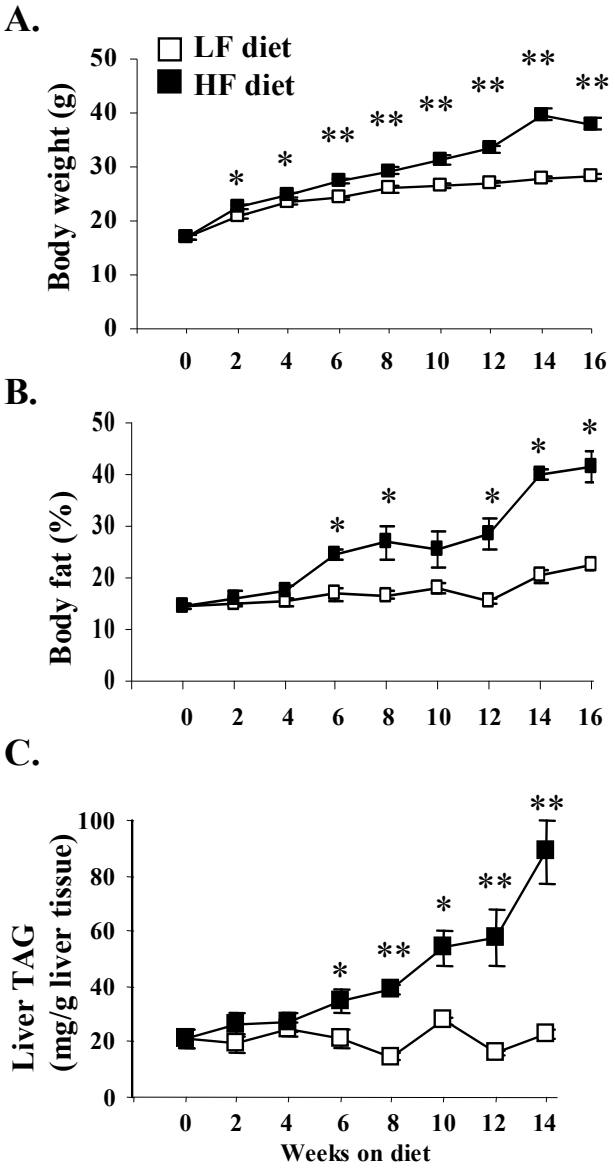
Statistical analysis. Statistical analyses were performed using JMP 6© Statistical Software (SAS Institute Inc, Cary, NC). Normally distributed data were analyzed by 2-way ANOVA with diet and day as main effects. Post-hoc, Student's *t* test was used for comparison between the dietary groups and Tukey-Kramer HSD was used for comparisons among the days. Nonparametric data were analyzed using the Kruskal Wallis test. Differences were considered significant at $P \leq 0.05$.

D. Results

High fat feeding induces obesity and liver steatosis. As reported previously [85, 135, 210], feeding a high fat diet to mice leads to rapid weight gain (Fig. 4.1A). After two weeks, mice on the HF diet weighed significantly more than mice on the LF diet, and the difference between the two groups increased over time. At week 12, the HF group weighed 20% more than the LF group and by week 16 the HF group had gained twice as much weight as the LF group. PIXImus™ analysis revealed, as expected, that the weight gain was due to an increase in body fat (Fig. 4.1B). By week 8, the mice on the HF diet had gained twice the body fat relative to the mice on the LF diet. By week 16, the mice on the HF diet had 3.3 times the fat mass compared to the mice on the LF diet.

Liver steatosis is associated with obesity and is also associated with metabolic disturbances involving inflammation. Liver triacylglycerol (TAG) content increased over time in the mice consuming the HF diet (Fig. 4.1C). By week 10, mice on the HF diet had over 5% of their liver weight as TAG, indicating steatosis. By week 14, the mice fed the HF diet had 4 times more liver TAG than the mice on the LF diet.

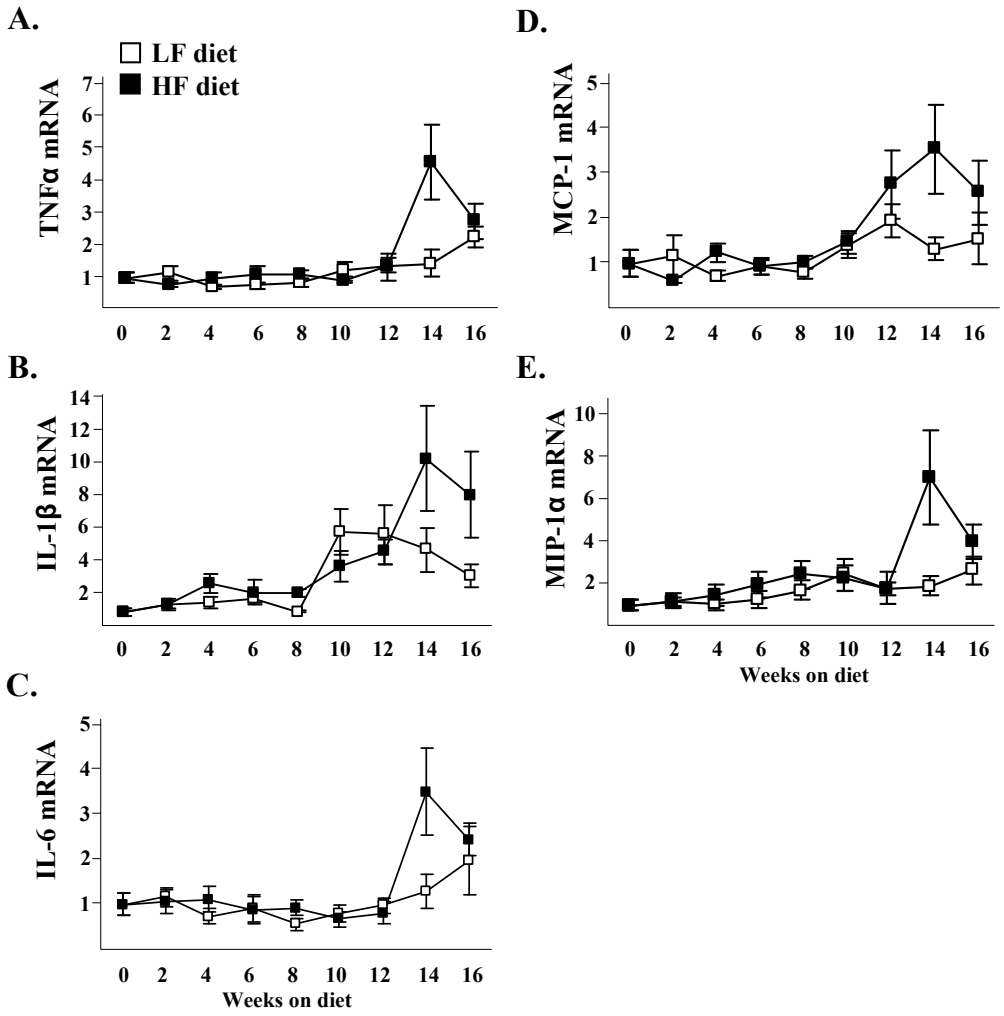
Figure 4.1: Progressive increase in body weight, body fat, and liver triacylglycerol (TAG) during HF feeding. (A) Body weight (grams) of mice receiving either a LF diet or HF diet, *n* = 12-16. (B) Percent body fat as determined by PIXImus™, *n* = 6-8. (C) Hepatic TAG content expressed as mg of TAG per gram of liver tissue, *n* = 6-8. Values represent mean ± SEM. *p≤0.05; **p≤0.001 of HF relative to LF group by Student's *t* test.



Because of the development of liver steatosis, we measured gene expression for proinflammatory cytokines and chemokines to determine if the TAG accumulation was

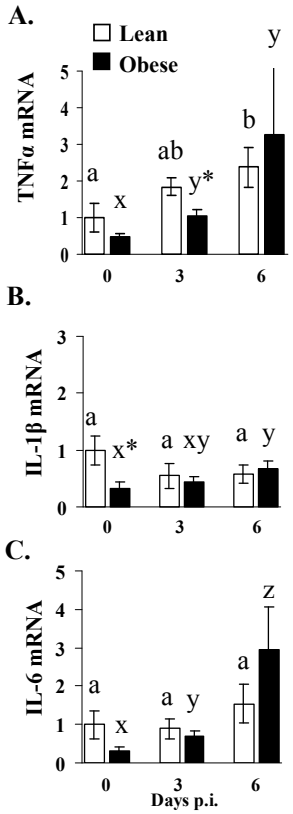
associated with liver inflammation. Using qRT-PCR, we measured liver transcript levels for TNFα, IL-1B, IL-6, MCP-1 and MIP-1α. As shown in Figure 4.2, induction of inflammatory cytokines and chemokines was not related to diet, but rather increased in both groups over time, likely reflecting an age, not diet, effect.

Figure 4.2: Liver inflammatory cytokine and chemokine gene expression during HF feeding. Liver gene expression of (A) TNFα, (B) IL-1β, (C) IL-6, (D) MCP-1, and (E) MIP-1α in mice receiving either a LF or HF diet. mRNA expression was determined by quantitative RT-PCR using total RNA extracted from the liver. Values are normalized to GAPDH, and are expressed relative to the mean value at week 0, which was arbitrarily set to 1. Each data point represents the mean ± SEM, n = 6-8. Two-way ANOVA indicated that there was not a main effect of diet.



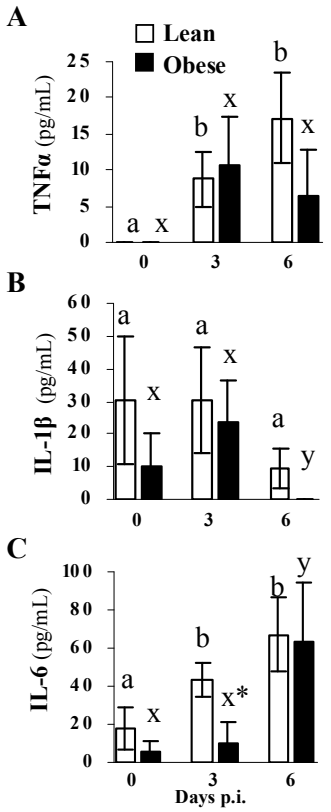
Increased inflammatory cytokine gene expression in livers of obese mice infected with influenza virus. Although obesity itself did not increase inflammatory mRNA cytokine production in the liver of the obese mice, infection with influenza virus had a profound effect. As shown in Figure 4.3, in the obese mice 6 days after influenza infection, liver mRNA transcripts for TNF-α and IL-6 increased 6-fold, and mRNA transcripts for IL-1β doubled. Thus, obesity contributed to an increased inflammatory response in the livers of influenza-infected mice.

Figure 4.3: Liver inflammatory cytokine gene expression during influenza A virus infection. Liver gene expression of (A) TNFα, (B) IL-1β, and (C) IL-6 were determined by quantitative RT-PCR using total RNA extracted from the liver. Values are normalized to GAPDH, and are expressed relative to the mean value of lean, uninfected mice (0 d p.i.), which was arbitrarily set to 1. *Different from lean at that time; means for a group without a common letter differ by Kruskal-Wallis test, $P \leq 0.05$. n = 6-8.



Increase in proinflammatory cytokines in the serum of influenza infected lean and obese mice. In order to determine if obesity influences serum cytokine concentrations during an influenza infection, we measured cytokine concentrations 0, 3, and 6 d post-infection (p.i.). Interestingly, in lean mice but not in obese mice, serum TNFα increased (Fig. 4.4A). Serum IL-6 concentration increased in both lean and obese mice during infection; however, the increase was delayed in obese mice (Fig. 4.4C). In contrast, IL-1β concentration decreased in obese mice at 6 d p.i. (Fig. 4.4B). Therefore, obesity altered serum cytokine concentrations during influenza virus infection.

Figure 4.4: Serum cytokine concentrations during influenza A virus infection. Serum concentrations of (A) TNFα, (B) IL-1β, and (C) IL-6 during influenza virus infection in lean and obese mice. Values are the mean (pg/mL) ± SEM. *Different from lean at that time; means for a group without a common letter differ by Kruskal-Wallis test, $P \leq 0.05$. n = 6-8.



Infection with influenza virus alters the adipose tissue mRNA proinflammatory patterns in both lean and obese animals. In our previous report [62], we demonstrated that obese mice infected with influenza virus had higher mortality and an altered immune response in the lungs compared with infected lean mice. Because the adipose tissue (AT) itself contributes to an inflammatory state in obesity, we examined AT depots to determine if infection alters the inflammatory pattern in obese animals. We measured TNFα, IL-1β, IL-6, MCP-1 and MIP-1α transcript levels in inguinal, gonadal, and retroperitoneal AT of lean and obese mice 0, 3, and 6 d post-infection with influenza virus.

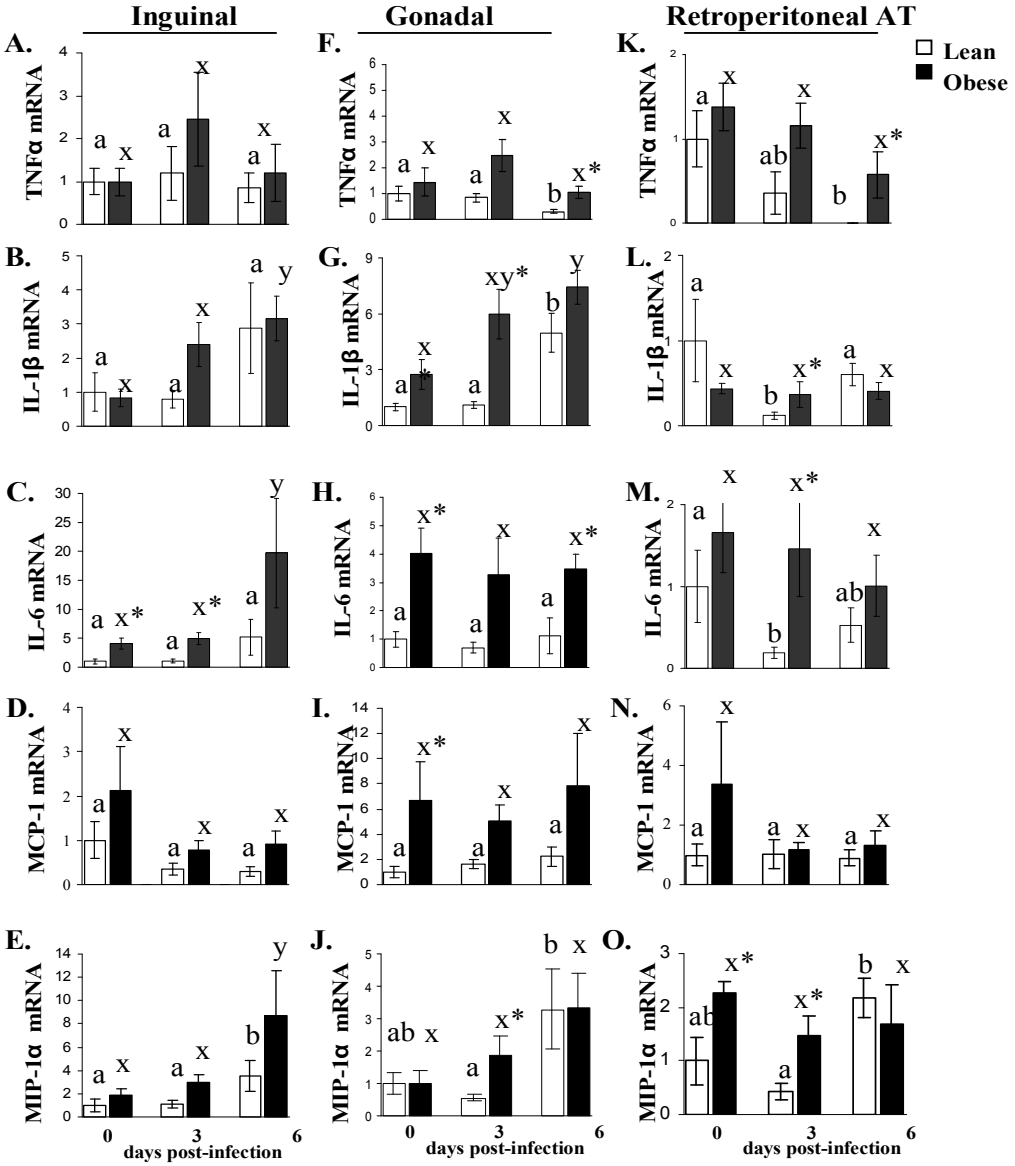
Inguinal AT: During influenza infection, inguinal AT MIP-1α gene expression increased in both lean and obese mice (Fig. 4.5E). TNFα and MCP-1 gene expression did not change due to influenza infection (Fig. 4.5A and D, respectively). However, in obese mice, but not in lean mice, mRNA transcripts for IL-1β and IL-6 also increased (Fig. 4.5B and C, respectively). Thus, during influenza infection, obesity increased the inflammatory state in the inguinal AT.

Gonadal AT: Gonadal IL-6 and MCP-1 gene expression were higher in obese mice than lean mice (Fig. 4.5H and I, respectively). TNFα decreased in the lean mice during influenza infection, such that at 6 d p.i., TNFα transcripts in the lean mice were lower than in obese mice (Fig. 4.5F). IL-1β and MIP-1α mRNA transcripts increased in both groups during infection (Fig. 4.5G and J, respectively). Therefore, the overall inflammatory milieu in gonadal AT was greater in obese mice than in lean mice during influenza infection.

Retroperitoneal AT: Retroperitoneal TNFα mRNA decreased in the lean mice, but not in the obese mice during infection (Fig. 4.5K). At 3 d p.i., IL-1β, IL-6, and MIP-1α were higher in obese mice than in lean mice (Figs. 4.5L, M, and O). Hence, during influenza

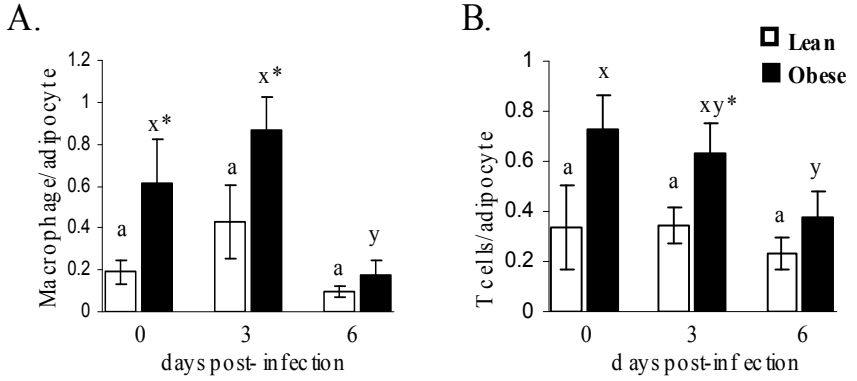
infection, the retroperitoneal AT of obese mice had a greater proinflammatory state compared to the same AT depot lean mice.

Figure 4.5: Adipose tissue inflammatory gene expression during influenza A virus infection. During influenza virus infection, inguinal, gonadal and retroperitoneal ATs were analyzed for TNFα, IL-1β, IL-6, MCP-1, and MIP-1α gene expression. Gene expression was determined by quantitative RT-PCR using total RNA extracted from the AT. Values are normalized to GAPDH, and are expressed relative to the mean value of lean, uninfected mice (0 d p.i.), which was arbitrarily set to 1. *Different from lean at that time; means for a group without a common letter differ by Kruskal-Wallis test, $P \leq 0.05$, $n = 5$-6.



Macrophage and T-cell- populations in gonadal AT decrease in influenza-infected obese mice. During influenza infection, cells in the respiratory tract express inflammatory chemokines and cytokines that attract and activate immune cells in order to fight the infecton. Therefore, the differences in the expression of these inflammatory mediators in the AT of lean and obese mice may alter the trafficking of immune cells. In order to determine whether or not influenza affects the number of macrophages and T lymphocytes in AT of lean and obese mice, we calculated their populations in the gonadal AT depot during influenza infection. In obese mice infected with influenza, the number of macrophages and T cells in the adipose tissue declined (Figs. 4.6A and B, respectively). Therefore, influenza infection decreases the number of immune cells in the AT of obese mice.

Figure 4.6: Macrophage and T lymphocyte populations in gonadal AT during influenza infection. Gonadal ATs were analyzed for (A) $F4/80^{+}$ macrophages and (B) $CD3^{+}$ T lymphocytes by immunohistochemistry at 0, 3, and 6 d post influenza infection. Values expressed are the mean number of target cells per adipocyte in the entire cross-sectional area of the tissue sample, counted at magnification X 40. *Different from lean at that time; means for a group without a common letter differ by Kruskal-Wallis test, $P \leq 0.05$. $n = 5$.



E. Discussion

Previously, we demonstrated that relative to lean mice, diet-induced obese mice had a worse outcome when infected with influenza virus [62]. In addition, the immune response in the lungs of the obese mice was altered. Lately, a great deal of attention has been focused on the "chronic inflammation" that occurs in the AT of obese humans and animals [102, 135, 196, 243-246]. In diet-induced obesity, the AT has been shown to have elevated expression of inflammatory genes as well as an influx of macrophages and T cells [120, 121, 124, 135, 136, 167, 243, 247], which does not occur to the same extent in the AT of lean animals. Because the pathology induced by influenza virus infection is primarily due to an inflammatory response in the lungs, we reasoned that the AT in obese mice may have an exaggerated inflammatory response following influenza virus infection. In addition, because TAG accumulates in the liver during high fat feeding, we sought to determine whether a fatty liver is associated with increased hepatic inflammatory cytokine and chemokine gene expression that may also contribute to immune dysregulation during an influenza virus infection.

Prior to infection, we first established the metabolic disruptions in mice on the HF diet. As reported by others, feeding a high fat diet induces weight gain which results in obesity [85, 135, 210]. In agreement with the results of Hong et al., the onset of liver steatosis occurred after 10 weeks on the HF diet [119]. However, the increase in TAG accumulation in the liver was not associated with increased inflammatory gene expression. Cai et al. reported a 3- to 6-fold increase of liver TNFα, IL-1β, and IL6 mRNA levels in diet-induced obese mice relative to lean mice [122]. The discrepancy between the results of our study with the Cai et al. study may be due to differences in the choice of the control diet. Our study controls

consumed a purified LF diet that contains 10% fat. In contrast, the study controls used by Cai et al. consumed a chow diet which normally contains 4% fat. Another possible study design difference is the age of the animals, as Cai et al. did not specify this. Because age affects gene expression in the liver [248], if older mice were used by Cai et al., they may have elevated inflammatory transcript levels. During our study, liver MCP-1 mRNA levels did not increase. However, Ito et al. reported increased liver MCP-1 mRNA levels in mice that consumed a HF diet for 50 weeks [241]. Differences in duration between the studies (16 and 22 weeks vs. 50 weeks) and therefore, age and extent of obesity, may account for the discrepant results. Few studies have investigated liver MIP-1α levels in obesity. In humans, liver MIP-1α mRNA levels were increased in individuals with liver steatosis, compared to those with normal liver TAG levels [249]. To our knowledge, in mice with liver steatosis or diet-induced obesity, liver MIP-1α mRNA levels have not previously been reported. In summary, although the livers of mice fed the HF diet had 4 times the TAG content relative to mice fed the LF diet, this degree of steatosis did not increase inflammatory gene expression in the liver.

During influenza A virus infection, the livers of obese mice had inflammatory changes. TNFα mRNA levels increased in the livers of both lean and obese influenza-infected mice. However, in obese mice, but not in lean mice, liver transcripts of IL-1β and IL-6 increased during influenza infection. Compared to lean mice that had been infected with influenza virus for 6 d, the livers in obese mice had higher transcript levels of MCP-1. MCP-1 is important in clearing influenza virus infection [250, 251]. MCP-1 acts as a chemoattractant for leukocytes, including activated T cells and NK cells. During influenza infection, the obese mice had fewer NK cells in the lungs [62], raising the question of whether increased liver

MCP-1 in the obese mice may have directed leukocyte trafficking away from the site of infection in the lungs. There is evidence that leukocytes traffic through the liver during influenza infection. Polakos et al. demonstrated that antigen-specific CD8+ T cells migrate through the liver during influenza infection [252]. In addition to the role of the inflammatory chemokine MCP-1, the upregulated inflammatory cytokine gene expression in the livers of the infected obese mice may have altered the immune response. The increase in inflammatory cytokine transcripts in the liver may have resulted in the subsequent expression of molecules involved in adhesion and extravasation. Therefore, the increased inflammatory profile in the livers of obese mice may have contributed to a dysregulated immune response to the infection in the lungs.

During an influenza virus infection, cytokines expressed by the infected respiratory mucosa can enter the circulation to activate a systemic response. While moderate serum levels of inflammatory cytokines are beneficial in controlling the viral infection, excess levels have been associated with increased mortality. For example, high IL-6 levels are associated with increased mortality from pneumonia of all causes [164], and very high levels of TNFα may contribute to mortality from influenza virus infection [168]. Although cytokine excess may contribute to increased pathology and mortality, the lack of an appropriate cytokine response may be equally detrimental. For example, compared to wild type mice, mice deficient in IL-1 receptor and IL-1β have higher mortality from influenza virus [169, 253, 254]. In our study, obese mice differed from lean mice in that their serum TNFα levels failed to increase, IL-1β declined and the increase in IL-6 was delayed. For TNFα, and IL-1β, this is in contrast to the gene expression in the liver and lungs [62]. TNFα mRNA levels increased in the livers and lungs of obese mice during influenza infection without a

coincident increase in serum TNFα levels. Similarly, IL-1β mRNA levels increased in the livers of infected obese mice without a coincident increase in serum IL-1β levels.

Serum cytokine levels are determined not only by their rate of synthesis, but also by receptor uptake. The discrepancy between increased gene expression in the lungs and livers without a concordant increase in serum levels may be due to increased uptake at the site of cytokine synthesis. There is evidence to suggest that increased cytokine receptor expression in the liver may occur in obesity and infection. For example, in obesity-associated steatosis, liver IL-1RI mRNA is upregulated [255], while in obese humans whose steatosis has progressed to nonalcoholic steatohepatitis, both IL-1RI and TNFRI (p55) are upregulated [118, 255]. In our influenza study, the increase in serum IL-6 levels paralleled the increase in liver and lung mRNA. IL-6 has been shown to increase liver expression of IL-1RI, and TNFRI [256, 257]. Future studies are necessary to determine whether hepatic cytokine receptor expression is changed in obesity, and to identify what impact that change has in conjunction with alterations in cytokine expression on immune function.

Several studies have shown that obese humans and genetic animal models of obesity have elevated levels of inflammatory mediators in their serum [222-226]. However, during 16 weeks of HF feeding, serum TNFα, IL-1β and IL-6 levels were below the detection limits for our assays (assay sensitivity for TNFα $\geq$ 5 pg/mL; IL-1β $\geq$ 10 pg/mL; IL-6 $\geq$ 10 pg/mL). Serum TNFα levels remained below the detection limits after 22 weeks of HF feeding, while serum IL-1β and IL-6 levels were within detection limits but were similar between the lean and obese mice. Hong et al. compared the serum TNFα levels between ob/ob and C57BL/6 diet-induced obese mice [119]. Both *ob/ob* and DIO mice that consumed a HF diet between 10 to 20 weeks of age had mean serum TNFa levels of 3.5 pg/mL. Therefore, although the

DIO mice in our study consumed the HF diet 6 and 12 weeks longer, serum TNFα did not reach the lower detection limit for our assay of 5 pg/mL. Pitombo et al. measured serum TNFα, IL-6 and IL-1β in Swiss mice fed either a standard chow or a HF diet [225]. Compared to mice consuming the chow diet, Swiss mice on the HF diet had higher serum TNFα, IL-1β, and IL-6 concentrations. Serum cytokine levels in the Swiss mice fed either the chow or HF diet were an order of magnitude greater than serum cytokine levels reported in C57BL/6 mice, therefore the discrepancy in results between the studies may be due to the strain of mice being studied. To our knowledge, serum IL-6 levels in DIO C57BL/6 mice have not been reported; IL-6 levels in chow fed C57BL/6 mice are approximately 6 pg/mL[122]. Taken together, these results indicate that unlike obese humans, some strains of diet-induced obese mice, and genetically obese mice, diet-induced obese C57BL/6 mice do not have elevated inflammatory serum cytokine levels.

The chronic, low-grade inflammation associated with obesity is characterized by increased expression of inflammatory mediators by the AT. In this report we provide evidence to suggest that in obesity, the AT itself contributes to immune dysfunction during an infection through increased inflammatory cytokine and chemokine gene expression. While we did not expect the influenza infection to increase inflammation in the inguinal AT because of this depot's distance from the lungs and comparatively low metabolic activity and cytokine/chemokine expression, not only did influenza increase MIP-1α gene expression, but it increased IL-1β and IL-6 levels specifically in the obese mice. Thus, although excess subcutaneous AT carries less risk for chronic diseases than visceral AT, it may contribute to alterations in immune function during an infection. As previously mentioned, relative to the inguinal AT, the visceral AT of obese mice had a higher inflammatory profile before

infection. Influenza infection exacerbated this inflammatory profile, especially in the obese mice. There has been considerable interest in how much adipose tissue contributes to circulating cytokine levels. As with the lungs and livers of these obese mice, the increased cytokine expression by the AT probably serves an autocrine function, since serum levels do not parallel the increase in gene expression in the AT. In the obese state, adipose tissue not only increases its expression of inflammatory mediators, but there is some evidence that it also increases its expresion of receptors [258]. More research is necessary to elucidate receptor expression in adipose tissue. A mechanism which could account for the discrepancy between TNFα gene expression in AT and serum levels is that the membrane bound form of TNFα is increased in obesity [258]. Although our study does not determine whether the increased inflammation in the AT of obese mice contributes to their poorer outcome during influenza infection, it demonstrates that influenza virus infection increases the inflammatory milieu in the adipose tissue. This increase in inflammation is especially evident in obese mice.

Adipose tissues of obese mice have increased populations of macrophages and T cells [124, 135, 136]. MCP-1 and MIP-1α attract monocytes and macrophages as well as T lymphocytes [116]. During influenza infection, MCP-1 mRNA levels remained constant while MIP-1α increased. Interestingly, the number monocytes/macrophages and T lymphocytes in the AT did not change in the lean mice, but in the obese mice the number of these cells decreased. Smith et al. showed that the obese influenza-infected mice had increased cellular infiltrate [62]. Although we did not follow macrophage and T cell trafficking in the mice, our study raises the question of whether an exodus of macrophages

and T-cells from the adipose tissue of obese mice contributed to their lung pathology during influenza infection.

This study raises many important questions about the roles being played by the liver and adipose tissues during infection in obese individuals. To our knowledge, there are no reports about the incidence or severity of influenza A virus infections in obesity. Given the prevalence of obesity and the health threat posed by influenza infection, further research is necessary to understand the mechanisms involved in the immune dysfunction associated with obesity.

Chapter V

Summary and Concluding Remarks

A. Introduction

This dissertation is composed of two conceptually distinct parts. The common link between these two parts is the study of adipocytes and adipose tissue. In the first part of my graduate research, because the control of cellular proliferation and differentiation is tightly regulated, we sought to determine whether these processes were functionally connected. The second part of my graduate research focused on the inflammatory state of adipose tissue in obesity. Because adipose tissue expresses inflammatory mediators that may alter the immune response, we measured inflammatory cytokine and chemokine gene expression in the absence and presence of influenza A virus infection. This chapter summarizes the results of these two parts of my research and discusses the implications of the data.

B. Summary and implications of mechanisms linking preadipocyte proliferation and differentiation

Adipose tissue plays a critical role in energy storage and metabolism. In adipose tissue, preadipocytes have the ability to proliferate, but lack the ability of differentiated adipocytes to store large amounts of TAG and participate in insulin-stimulated glucose uptake. Much of what we know about cell cycle regulation and adipocyte differentiation has been elucidated from clonal cell lines, such as the 3T3-L1 preadipocyte cell line. When contact-inhibited 3T3-L1 preadipocytes are induced to differentiate, they reenter the cell cycle and undergo one to two rounds of proliferation, called mitotic clonal expansion. During the same time, they initiate a cascade of transcription factor expression that culminates in the expression of genes necessary for terminal differentiation. C/EBPβ plays a key role as an immediate/early transcription factor in this cascade. We show that although C/EBPβ is expressed and located in the nucleus within 4 h after induction of differentiation, its DNA binding activity is inhibited by its interaction with hypophosphorylated Rb. After Rb is phosphorylated at the

G1/S checkpoint, Rb and C/EBPβ dissociate. Then, C/EBPβ is able to bind to the C/EBP response element on the C/EBPα promoter and activate gene transcription. Therefore, during 3T3-L1 adipogenesis, the initiation of differentiation is functionally linked to proliferation by the interaction between the C/EBPβ and Rb proteins.

Rb is a critical regulator of the cell cycle, as cells lacking a functional Rb protein frequently fail to control their cellular proliferation and are therefore associated with tumorigenesis. C/EBPβ is critical in the differentiation of a number of cell types, including adipocytes, hepatocytes, and the mammary gland. Because fully differentiated cells lack the ability to proliferate, C/EBPβ has attracted attention as a potential target for constraining tissue growth, as in obesity and cancer. In obesity, however, because adipogenesis is a normal adaptation to excess calorie intake, attempts to modify obesity by limiting adipogenesis are likely to result in ectopic TAG accumulation and cause adverse health consequences. In cancer, adenoviral C/EBPβ vectors are being investigated for their ability to reactivate the differentiation program in order to stem tumorigenesis. Hence, our *in vitro* studies in 3T3-L1 preadipocytes contribute toward a better understanding of mechanisms that may link mitotic clonal expansion and differentiation in other cell types.

C. Summary and implications of adipose tissue inflammation in obesity in the absence and presence of influenza A virus infection

Establishing a timeline of events among changes in body composition, hormonal and metabolic parameters, liver and adipose tissue inflammation during the accumulation of adipose tissue is necessary in order to understand their relationships. Our studies are the first to establish a timeline of inflammatory changes that occur during the onset of obesity. Studies published previously have reported increased levels of inflammatory mediators during obesity. Therefore, critics may argue that our findings are not novel. However, careful

review of the literature reveals many gaps. For example, studies demonstrating increased levels of inflammatory mediators have frequently been conducted in genetic rodent models of obesity. Although evidence supporting a genetic component to the development of obesity is overwhelming, the prevalence of these genetic defects is rare in humans compared to the prevalence of obesity. Hence, the diet-induced obese mouse model that we used in our studies most closely resembles human obesity. Another attribute of our study is that we established changes in metabolic and inflammatory parameters over time. Many other studies which reported increased inflammation in obesity have been conducted well after obesity has been established. These studies fail to elucidate if the increase in inflammatory mediators occurs gradually over the course of adipose tissue accumulation, i.e. whether inflammation occurs when the animals are merely overweight, or if the inflammatory state occurs only after the animals cross the threshold to obesity. Inflammation in adipose tissue has received a considerable amount of attention. Studies have demonstrated that in obesity, the adipose tissue is characterized by increased gene expression for inflammatory mediators and increased macrophage and T lymphocyte infiltration. Relative to the subcutaneous adipose tissue, the visceral adipose tissue has higher inflammatory gene expression and one of these visceral depots, the gonadal adipose depot, has higher macrophage cell infiltration. Among visceral adipose tissue depots, the gonadal adipose tissues (epididymal in males; parametrial in females) have been most widely studied. In contrast, the retroperitoneal depot has received very little attention. Indeed, inflammation in the murine retroperitoneal depot has not been reported. In the retroperitoneal adipose tissue, we are currently investigating macrophage and T lymphocyte cell infiltration. Our research demonstrates that in diet-induced obesity, compared to the inguinal or gonadal depots, the retroperitoneal adipose tissue has the greatest

increase in inflammatory cytokine and chemokine gene expression in male, but not in female mice. Although the retroperitoneal adipose depot is small compared to the gonadal adipose depot, it may hold important information about gender-specific health risks associated with the accumulation of visceral adipose tissue in the upper abdomen.

Previous studies in our lab demonstrated that obese mice infected with influenza A virus have an impaired immune response and higher mortality relative to their lean counterparts. Although others have reported that certain components of the immune system are impaired in obesity, we are the first to demonstrate that adipose tissue itself may contribute to the impairment. Obese mice have a heightened inflammatory response in adipose tissue depots that are distant from the influenza virus infection. Inflammatory cytokines and chemokines facilitate and direct leukocyte trafficking. Therefore, increased expression of these mediators in the adipose tissue may disrupt trafficking to the lungs. In addition, we demonstrate that in obese mice infected with influenza A virus, macrophage and T cell infiltration in the adipose tissue decreases at the same time cellular infiltrate increases in the lungs.

D. Public health impact of influenza A virus infection in the obese population

The threat of influenza epidemics and pandemics is heightened by increased global travel and commerce [259]. The spread of influenza infection causes tremendous economic and social loss worldwide due to its toll on the morbidity and mortality of the population. Therefore, it is imperative that that we study influenza infection in at-risk populations so that we better understand and respond to disease threats. Obese individuals may be at risk for increased morbidity and mortality than normal-weight individuals. This study demonstrates that in obesity, adipose tissue inflammation is associated with immune dysfunction during

influenza A virus infection, and emphasizes the need for future research that will enable appropriate allocation of preventive measures.

Influenza virus infection may result in two concurrent processes that exacerbate morbidity and mortality in obese individuals. One process may be an increase in the virulence of the virus, while the second process is the dysfunctional immune response of the obese host. Given that in the United States alone, 60 million people are obese [1] and 50 million people are infected with influenza virus each year [143], the potential public health impact of influenza virus infection in the obese population is tremendous.

Although there are no direct data linking obesity with increased morbidity and mortality from influenza A virus infection, there are data that support this hypothesis. Analysis of data from NHANES I, II, and III indicates that a BMI greater than or equal to 35 is associated with a two-fold increase in the risk of noncancer, non-CVD mortality, which includes, among other causes, acute respiratory disease and infection [260]. Although the noncancer, non-CVD deaths accounted for 39% of the total deaths in 2004, they are often not considered to be weight related [260]. Given that obese mice infected with influenza A virus have increased mortality relative to lean mice [62], the association between human obesity and increased mortality in this category should be elucidated further.

In the population, vaccination is an effective strategy for preventing infection when the influenza vaccine matches the circulating influenza strain [148]. For example, pneumonia and influenza mortality decreased by 26% in the portion of the population that was targeted by a large-scale, cost-free influenza vaccination campaign in Brazil, while those not targeted by the vaccination campaign did not have a significant decrease in mortality [261]. However, vaccination might not be as effective in obese individuals as people of normal weight,

because obese individuals have reduced antibody titers to several vaccines [75, 76, 79]. The effectiveness of influenza vaccines in obese individuals relative to lean individuals is not known. Therefore, future research is needed to determine whether influenza vaccination confers adequate protection to obese individuals.

Low vaccination rates [261] and obesity [262] disproportionately affect the poor. In 2005, 20 states tightened Medicaid eligibility criteria, and 21 states reduced the program benefits and/or increased cost-sharing, which has resulted in reduced vaccination rates [263]. To compound the problem, between 2000 and 2005, the prevalence of severe poverty, defined as less than 50% of the federal poverty threshold, increased by nearly 23% [264]. As family budgets are strained by the rising cost of health care and insurance, the ability to purchase healthy foods is impaired. The apparent paradox of obesity and poverty is because low-cost foods provide adequate kilocalories to meet or exceed energy requirements, but lack the dietary quality needed to promote optimal health and prevent chronic disease. For example, low-cost diets contain more high-carbohydrate, high-fat foods, and less fruits and vegetables, and consequently are lower in antioxidants such as vitamin C and beta carotene than the average adult diet [262, 265]. Therefore, the current decline in US household income is likely to intensify demands on the health care system in both the short and long term, through the decline in vaccination rates and the cumulative effect of poor diet and obesity, especially in children [264].

Our lab has demonstrated that viruses may become more virulent in an oxidatively stressed host [75, 76, 79, 266-270]. As previously mentioned, low-cost diets favor the consumption of high kilocalorie foods which are lower in antioxidants than the average adult diet [262, 265]. Most programs aimed at alleviating food insecurity and hunger have

neglected the increasing problem of obesity. Therefore, these programs should be redirected to enable low-income families to access a more healthful diet that includes foods high in antioxidants, such as fruits and vegetables. Oxidative stress is increased in the obese state [271]. While nutrient deficiencies of antioxidants such as vitamin E and selenium increase the virulence of several viruses [266-270], it is not known if oxidative stress resulting from obesity increases the virulence of influenza A virus. Therefore, additional research is required to determine if obesity alters viral genomes.

The World Health Organization and the G-8 summit concur that governments will need to work together to develop collaborative strategies in confronting shared problems, including the spread of infectious diseases across international borders [259]. At present, there is a major mismatch between global expenditure on health research and the nature and extent of the global mortality: only 5% of the world's health research budget is being spent on conditions responsible for 93% of the global mortality [272]. Given the obesity pandemic and the high morbidity and mortality not only from annual influenza epidemics, but also the potential for a pandemic, research and public health programs designed to mitigate the effects of influenza virus infection in the obese population deserve immediate attention.

E. Concluding remarks and future directions

In conclusion, data obtained from experiments in diet-induced mice indicate that inflammation in adipose tissue occurs prior to macrophage infiltration, and prior to the significant increases of fasting serum leptin and insulin concentration that are associated with obesity. Furthermore, inflammation in adipose tissue is gender and depot-specific, with the retroperitoneal adipose tissue in male mice demonstrating higher levels of inflammatory gene expression than gonadal or inguinal adipose tissues, and more than any of the adipose tissue

depots in obese female mice. Because the retroperitoneal adipose depot is very small in the lean mice that are used as the control group, future study designs will need to include more mice in this group, because both of the retroperitoneal depots are required to obtain a sufficient amount of cytokine mRNA for gene expression analysis by qRT-PCR. Furthermore, because of the low numbers of infiltrating cells, pooling of the samples will be necessary to measure cellular infiltrate by fluorescence-activated cell sorting.

During influenza A virus infection, our data suggest that the adipose tissue itself may be contributing to the impaired immune response in the obese animals. While we may speculate that increased inflammatory mediators in the adipose tissue caused a disruption in leukocyte trafficking, additional studies are necessary to confirm or refute this hypothesis. The F4/80 protein is expressed in macrophages, but not in adipocytes. Transgenic mice expressing GFP-tagged F4/80 protein could be developed. Adipose tissue depots from lean and diet-induced obese transgenic mice could be transplanted into wild-type mice. After allowing sufficient time for post operative recovery, the wild-type mice could then be infected with influenza A virus. Lung sections viewed by fluorescence microscopy would show whether or not macrophages from the adipose tissue traffic to the lungs during influenza virus infection, and whether the trafficking differs between lean and obese mice. Our data demonstrate that during influenza virus infection, inflammation in adipose tissue increases, especially in obese mice. This is significant because the adipose depots were distant from the site of infection (the lungs). It is possible that an infection located close to the adipose tissue depots would cause an even greater inflammatory response. For example, the rotavirus infects the epithelial cells of the small intestine [273]. Symptoms of fever, vomiting, and diarrhea have been attributed to inflammatory cytokines such as TNFα and IL-6 that are produced by infected

cells and leukocytes responding to the infection [274]. However, because adipose tissue in obesity has increased inflammatory cytokine gene expression and has increased numbers of macrophages and T lymphocytes, it is possible that excess adipose tissue may exacerbate the symptoms of a rotavirus infection. At the present time, there are no studies that examine whether or not there is a relationship between obesity and the severity of a rotavirus infection. The first step would be to review the hospital records of case-control studies that have investigated rotavirus infection, especially those studies that also measured serum cytokine concentration [274], and determine whether severity of illness and/or serum cytokine concentration was correlated with BMI. Subsequently, animal studies could be designed to elucidate the role that inflammation in adipose tissue plays during the infection.

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