Obesity Alters the Immune Response to Influenza Virus Infection-
A Mechanism for Immune Modulation

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ABSTRACT

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Obesity Affects the Immune Response to Influenza Virus Infection - A Mechanism for Immune Modulation
(Under the direction of Melinda A. Beck)

The incidence of obesity worldwide has reached epidemic proportions. This has had a significant economic and social impact due to the increase in co-morbidities associated with obesity (1). Obesity leads to changes in immune parameters under basal conditions (2), suggesting that obesity may result in impaired immune responses during an infection. Influenza virus infection is a leading cause of morbidity and mortality worldwide, and proper immune regulation is necessary for clearance of virus with minimal damage to the host. Given that obesity leads to impairment in immune function, we hypothesized that obesity would result in an impaired immune response following infection with influenza virus.

Using a mouse model of diet-induced obesity, we found that influenza infected mice had minimal and delayed activation of the innate immune response, as well as a mortality rate that was eight times greater than normal weight mice. The lack of response was partially due to a reduction in mononuclear cell infiltration into the lungs during the early stages of infection. Obese mice also had impaired dendritic cell function, which is a critical link between the innate and cell-mediated immune responses, suggesting that cell-mediated responses during influenza infection were also affected by obesity. Indeed, obese mice had

lower induction of cytokines that promote anti-viral T cell responses, impaired proliferation and cytokine production by T cells, and delayed infiltration of T cells to the infected lung.

What was the cause of the immune dysfunction? Once possibility was the high circulating leptin levels found in obese mice. Leptin is a hormone that plays a role in both innate and cell-mediated immunity through phosphorylation of Signal transducer and activator of transcription 3 (STAT3) subsequent to binding to its receptor. Despite high circulating leptin, we found that obese mice had reduced STAT3 phosphorylation in immune cells during infection, indicating leptin signaling was impaired and may have mediated some of the immune changes that occurred in obese mice. When we analyzed the impact of direct leptin on immune cell function, we found that direct leptin signaling was not required for normal function. This was shown by correction of immune cell function when a functional neuro-specific leptin receptor was expressed in leptin receptor-deficient, *db/db* mice. Therefore, the impairment in immune responses that occur in obese mice is, in part, likely due to a lack of centrally mediated effects of leptin.

Overall, this work implicates obesity as a risk factor for impaired immune responses during influenza virus infection and, furthermore, suggests that responses to other infections may be affected by obesity. Thus, the rapidly growing obese population in this country and other may at substantial risk for increased morbidity and mortality during the next influenza pandemic.

Dedication

First and foremost I want to thank my advisor, Melinda Beck, for being the person she is. Melinda allows her students the freedom to imagine possibilities, while patiently instilling the values that are necessary for succeeding in the reality of science. Secondly, I want to thank Patricia Sheridan, a great friend on whose shoulders I have stood throughout my time in graduate school. Her influence on me as a person and scientist is more than I would have ever expected, and for which I am eternally grateful. Finally I want to thank my mom, my sister, and my husband for their unwaivering love and support.

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AA	arachidonic acid
AgRP	agouti-related protein
AM	alveolar macrophage
AMPK	AMP-activated protein kinase
APC	antigen presenting cell
α-MSH	α-melanocyte-stimulating hormone
BALF	bronchoaveolar lavage fluid
cDC	conventional dendritic cell
d	day
DC	dendritic cell
DIO	diet induced obesity
ELISA	Enzyme-Linked ImmunoSorbent Assay
ELISPOT	Enzyme-linked Immunosorbent Spot
EPI	epinephrine
FACS	fluorescence-activated cell sorting
FITC	fluorescein
FFA	free fatty acid
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
HAU	hemagglutinating units
H&E	hemotoxylin and eosin
HF	high fat

IFN	interferon
IL	interleukin
IRF	interferon regulatory factor
IRS	insulin receptor substrate
JAK	janus kinase
JNK	c-Jun N-terminal kinase
LF	low-fat
LN	lymph node
MAPK	mitogen-activated protein kinase
MCP	monocyte chemotactic protein
MDCK	Madine Darby canine kidney
MHC	major histocompatibility complex
MIP	macrophage inflammatory protein
NE	norepinephrine
NK	natural killer
NPY	neuropeptide Y
Ob-Rb	leptin receptor
OVA	ovalbumin
pDC	plasmacytoid dendritic cell
PE	phycoerythrin
PHA	phytahemagglutinin
p.i.	post infection
PI3K	phosphatidylinositol 3-kinase

PolyI:C	Polyinosinic:polycytidylic acid
POMC	pro-opiomelanocortin
qRT-PCR	quantitative reverse transcriptase polymerase chain reaction
RANTES	Regulated upon Activation, Normal T-cell Expressed, and Secreted
ROS	reactive oxygen species
SNS	sympathetic nervous system
SOCS3	suppressor of cytokine signaling-3
STAT	signal transducer and activator of transcription
$TCID_{50}$	50% tissue culture infective dose
Th1	T helper cell 1
Th2	T helper cell 2
TLR	Toll-like receptor
TNFα	tumor necrosis factor-α
WT	wild type

CHAPTER I
BACKGROUND AND SIGNIFICANCE

A. Specific Aims

The incidence of obesity worldwide has reached epidemic proportions. This has had a significant economic and social impact due to the increase in co-morbidities associated with obesity (1, 20). Obesity leads to changes in immune parameters under basal conditions (2), and therefore suggests that obesity may result in impaired immune responses during an infection.

Influenza virus infection is a leading cause of morbidity and mortality, and proper immune regulation is necessary for clearance of virus with minimal damage to the host. Given the impairment in immune function observed in obesity, **we hypothesized that obesity would result in dysregulation of the immune responses during influenza virus infection**. The present studies outline a series of experiments that addressed the role of obesity on modulating innate and cell-mediated immune responses during influenza virus infection.

Specific Aims

1. Determine if innate immune responses are impaired in influenza infected obese mice.
2. Determine if the cell mediated immune responses are impaired in obese mice infected with influenza virus.
3. Determine the role of leptin in immune changes in obese mice during influenza infection.

B. Obesity: A Public Health Perspective

Obesity rates, worldwide, are at an all time high and continue to escalate. In the United States greater than 60% of the population is overweight or obese. Because obesity is a major risk factor for metabolic syndrome, Type 2 Diabetes, and cardiovascular disease, prevalence of these diseases has increased as well. In addition to its adverse effects on metabolic diseases, obesity also results in impaired immune function (23-25), although little is known about the impact of obesity on infectious disease. Obesity is a significant contributor to health care costs due to the increase in co-morbidities associated with it. Raebel et al reported that the cost of healthcare for an obese individual was 250 dollars more per year than non-obese individuals (28). More importantly, the pervasiveness of obesity has extended to the young and it is so dramatic that a potential decline in lifespan has been proposed for the next generation (29).

C. Animal Models of Obesity

Obesity in humans is primarily the result of an imbalance in caloric intake and caloric expenditure (30, 31). In mice, obesity can develop in the same way through feeding of a high fat diet. C57BL/6 mice are one of the most commonly used strains for diet induced obesity. When fed a high fat diet these mice develop significant increases in adipose tissue mass, circulating glucose, insulin, and leptin (32, 33). The effects of the high fat diet in C57BL/6 mice are analogous to humans, making it an excellent model for studies related to obesity.

Many other mouse models of obesity exist that do not rely on dietary manipulation. These include single-gene loss-of-function, both natural and artificially derived, transgenic models, and polygenic models (34). Two of the most commonly used genetic mouse models

of obesity are the *ob/ob* and *db/db* models, both of which have single-gene loss-of-function leading to a premature stop codon in the leptin gene and abnormal splicing of the long form of leptin receptor, respectively. The lack of leptin signaling in these mice leads to extreme hyperphagia, hyperglycemia, and hyperleptinemia, as well as hypercorticosteronemia and infertility (35, 36), effects which are not normally seen in obese humans (37).

D. Obesity as a Modulator of Immune Function

Obesity is implicated in immunosuppression in both humans and mice (23, 25, 38). In humans, obesity has been shown to be a risk factor for infections and poor wound healing after a variety of surgical procedures (23, 25), as well as for increasing the risk of infection and sepsis in burn patients (24). Additionally, obesity results in changes in the circulating T cell population (2) and diminished bacterial killing capacity of polymorphonuclear cells (39).

Numerous studies done in genetically obese mice indicate a global impairment in immune function in these mice (40-45). For example, they have marked thymic atrophy, and fewer splenic mononuclear cells and T cells (41, 43). Additionally, genetically obese mice have decreased resistance to bacterial and viral infections (38, 43), as well as reduced cell mediated cytotoxicity (45, 46). While the use of these mice allows for research in a model with extreme adiposity, the complete lack of leptin signaling leads to phenotypic changes that can affect immune responses and are considerably more pronounced that what occurs in diet-induced obesity in humans and mice (17, 47).

Studies of immune function in diet-induced obese (DIO) mice have focused primarily on mononuclear cell function *ex vivo* (48-51) with little work done in regard to immune responses to infectious disease (52). However, the current research does indicate that diet induced obesity also impairs immune function. For example, diet-induced obese mice have impaired antigen presentation and stimulation of T cell proliferation (53), while rats fed a cafeteria diet to induce obesity, display helper T cell lymphopenia and decreased natural killer (NK) cell function (48, 49). Additionally, DIO mice have decreased hepatic NKT cells and alterations in mitogen stimulated splenocyte proliferation and cytokine production (50, 51). So, there is clearly a dysregulation of immune function in DIO mice.

It is currently unknown why obesity leads to changes in immune function, but many pathways that are altered during obesity have important roles in immune responses. These include: nuclear factor kappa B (NF-κB), Phosphoinositide-3 kinase (PI3K), glucocorticoids, catecholamines, and adipokines (See Table 1.1). Adipokine modulation of immune function, particularly leptin, is the best characterized link between obesity and immune function where it appears to be essential for appropriate function (8).

Table 1.1. Factors that may link obesity and immune function

Factor	Metabolic Effect	Immune Effect	During obesity	References
Adiponectin	+ AMPK + GLUT4 - SREBP1	Anti-inflammatory - NF-κB + PPARα - Scavenger R on macrophage	Reduced	(3-5)
Leptin	+ Lypolysis - Food intake + PI3K	Inflammatory +NFκB +JAK/STAT + Th1	Increased (but downregulation of signal)	(6-8)
Glucose (High)	+ Insulin +ROS +PKC	Inflammatory +NFκB +JNK, p38	Increased	(9, 10)
FFA	- Insulin-stimulated glucose uptake + PKCθ	+ IKK + JNK	Increased/no change (no change in DIO mice)	(11-13)
Insulin	+ Glucose uptake - Food intake - Lypolysis	- Inflammation	Increased (but downregulation of signal)	(14)
Macrophage in WAT	- PI3K	+ Local NFκB +inflammation	Increased	(15) (16)
Catecholamine NE EPI	+Lypolysis - Adipocyte proliferation + Thermogenesis	+/- Th2/Th1 - NK cell activity	Increased NE Decreased EPI	(17-19)
Glucocorticoids	+ Adipogenesis + Gluconeogenesis + Food intake	-NK cell activity - Inflammation	Increased / no change	(17, 21) (22)
Neuropeptides NPY POMC	- Lipolysis + Food intake	+ Ag presentation - T cell response	Increased/no change NPY Decreased POMC	(26, 27)

1. Adipokine release from adipose tissue

Adipose tissue is now recognized as a major endocrine and secretory organ (54), secreting molecules that function in a variety of physiological pathways, such as regulation of food intake (55), angiogenesis (56), and immune responses (57). The molecules that mediate these effects are collectively known as adipokines, which are are secreted by adipose tissue and act on cells in an autocrine and paracrine manner. For example the adipokine leptin is produced mainly by adipocytes and acts in the hypothalamus to decrease food intake, but also signals adipocytes to induce lipolysis (58). Leptin and adiponectin are the most abundantly produced adipokines, but resistin and visfatin are also important adipokines (3). Other adipokines include molecules that are classically associated with the immune response, such as IL-6, TNF-α, and C-reactive protein (59).

2. Leptin

Leptin is the 16 kDa product of the *ob* gene that is produced primarily by adipocytes and is member of the family of long helical cytokines (60). Leptin increases proportionately to adipose mass, and therefore circulates at high levels in obesity (61). Through central and peripheral actions leptin regulates adiposity, fertility, glucose tolerance, and immune function (62-64).

Leptin acts in several regions of the hypothalamus, but leptin signaling is most readily detectable in the arcuate nucleus in two distinct neuronal populations. One population synthesizes neuropeptide Y (NPY) and agouti-related peptide (AgRP) and the other synthesizes pro-opiomelanocortin (POMC). NPY and AgRP are orexigenic neuropeptides

that suppress Ob-Rb mediated growth and reproduction. Leptin inhibits their expression while at the same time activating the anorexigenic neuropeptide POMC and its downstream effector α melanocyte-stimulating hormone (αMSH) (65, 66).

Leptin also mediates it effects via direct action on peripheral cells. In adipose tissue, for example, adenovirus induced overexpression of leptin leads to fat depletion in both intact and denervated fat pads, whereas no change in mass occurs in fat pads from *fa/fa* rats which lack leptin receptors (67). Similarly, leptin also appears to exert direct action on immune cells. For instance, Tian et al. demonstrated that leptin directly enhances NK cell cytolytic activity. When *ex vivo* cytotoxicity assays were performed in the presence of IL-15, an activator of NK cells, NK cells from both WT and *db/db* mice display normal killing capacity. However, the addition of leptin augmented the cytotoxic function of WT NK cells, whereas in NK cells from *db/db* mice it had no effect (68).

2.1 Leptin Signaling

The leptin receptor protein is a member of the class I cytokine receptor family (69). Many splice variants of leptin receptor exist and include Ob-Ra, Ob-Rb, Ob-Rc, Ob-Rd, Ob-Re, and Ob-Rf (70), which are classified as short, long, and secreted. All five isoforms share a common ligand binding domain, but differ in the length of their intracellular domains. The secreted form of Ob-R (E) has been shown to bind circulating leptin, while the function of the short forms (A, C, D, and F) are thought to aid in leptin transport across the blood brain barrier (71). Only the B isoform has signaling capabilities as its intracellular domain contains the various motifs required for the interaction of signaling molecules (70, 72).

Upon binding to its receptor, leptin stimulates three main signaling cascades that begin with the phosphorylation of tyrosine residues on the receptor's intracellular domain by a noncovalently bound Janus kinase (JAK) (73). Specifically, JAK2 leads to the activation of signal transducers and activators of transcription (STAT) signaling pathway, the PI3K pathway, and the mitogen-activated protein kinase (MAPK) pathway.

Activation of the PI3K pathway occurs through insulin-receptor substrate (IRS)-2 and mediates some of leptin's anorectic effects. This is evidenced by an increase in feeding and a decrease metabolic rate in IRS-2 knock out mice despite of high leptin levels (74). Moreover, inhibition of PI3K activity in the hypothalamus attenuates leptin-mediated hyperpolarization of NPY/AgRP neurons (75). PI3K signaling is also required for leptin mediated sympathetic nervous system activation (76).

The effects of leptin activation on the MAPK signaling pathway are less well defined, but it appears that the immunomodulatory effects of leptin are mediated through constituents of this pathway. Leptin signaling through the Ras/Raf/MAPK cascade leads to the activation of genes involved in cell proliferation and differentiation (65). Additionally, leptin also increases p38 phosphorylation in mononuclear cells, suggesting that its ability to promote T cell survival (77) is mediated through this pathway (78). Activation of Ob-Rb also stimulates JNK (c-Jun N-terminal kinase), a stress-activated protein kinase that is activated by cytokines. Interestingly, activation of p38 and JNK have been shown to enhance IL-1β-mediated vascular epithelial cell adhesion molecule (VCAM)-1 in human tracheal smooth

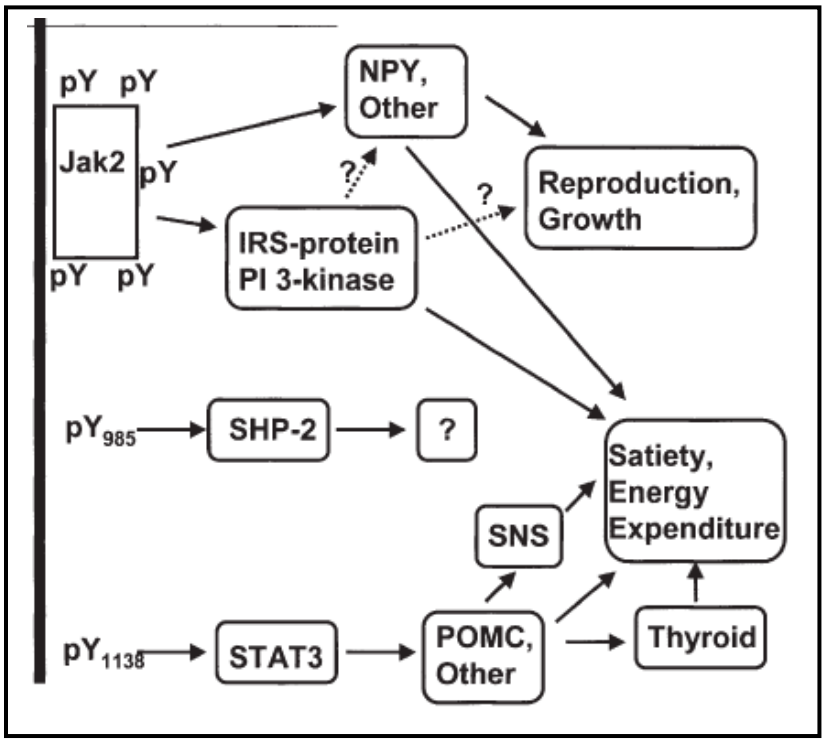
muscle cells (79), an effect that is important for leukocyte extravasion across the vascular endothelium (80). Additionally, leptin has been shown to enhance TNFα production in Kupffer cells through this pathway (81). Therefore, leptin augmentation of immune responses may be mediated through this pathway.

The phosphorylated Ob-Rb also recruits signal transducers and activators of transcription (STAT) to the receptor complex. STAT3, 5, and 6 mediate the transcriptional effects of leptin upon entry into the nucleus (82). STAT3, in particular, has been shown to mediate a number of leptin's effects. This was shown in mice that had a mutation in the tyrosine residue required for STAT3 recruitment to Ob-Rb (*s/s* mice). In comparison to *db/db* mice, *s/s* mice were; more fertile, normal length, and less hyperglycemic. Additionally, these mice had relatively normal hypothalamic NPY levels, but had low POMC expression and were extremely obese (83). This indicates that STAT3 mediates leptin's effects on the melanocortin system and adipose accumulation, and points towards another pathway for affecting NPY, reproduction, and glucose homeostasis. Additionally STAT3 mediates leptin's effects of growth and function of cells through activation of genes such as activator protein-1, c-fos, and egr-1 (84, 85).

STAT3 functions in a number of other pathways, including IL-6, IL-10 and IL-27 signaling (86). The overall effect of these pathways appears to be anti-inflammatory, since mice that have a targeted STAT3 deletion in macrophage and granulocytes develop enterocolitis and a hypersensitivity to LPS shock (87). Given that leptin generally promotes inflammation, it is unclear what role the STAT3 pathway has on leptin modulation of immune responses.

Leptin signaling is negatively regulated by feedback inhibitors and autoregulation. In accordance with other activators of JAK/STAT signaling, STAT3 induces suppressor of cytokine signaling proteins, (SOCS), and specifically SOCS3. SOCS3 is recruited to the phosphorylated tyrosines on the receptor and prevents JAK2 phosphorylation (88) leading to attenuation of Ob-Rb signaling. An increase in SOCS3 leads to downregulation of all Ob-Rb signaling pathways (88, 89). Additional regulation occurs at the receptor level where leptin signaling causes a downregulation of Ob-Rb expression (90).

Figure 1.1 Leptin signaling through Ob-Rb (71)



2.2 Leptin Resistance

With the discovery of leptin, many thought the cure for obesity had been found. However, the limited effect exogenous leptin had on obese humans, and the fact that they already had high circulating leptin, indicated that signaling by leptin may be diminished (71) Evidence for both central and peripheral leptin resistance is mounting. For instance, attenuated leptin signaling with a concomitant increase in SOCS3 levels occurred in the hypothalamus during leptin administration to *ob/ob* mice (91). In diet-induced obese rats, chronically high leptin resulted in downregulated expression of leptin receptor in liver and kidney. In the kidney, the number of leptin binding sites was inversely correlated with the concentration of serum leptin (92). In another rat model of diet-induced obesity, a decrease in leptin receptor expression was seen under basal conditions and leptin binding to its receptor was reduced upon bolus injection of leptin. Furthermore, post receptor signaling was completely abolished in the livers of obese rats following leptin injection compared to lean controls (93). This has been more recently shown in T cells from obese mice. Diet-induced obese mice were injected with leptin and STAT3 DNA binding was examined in T cells. This study found that while there was a 5 fold increase in STAT3 binding in lean mice, there was no change from baseline in obese mice (77). In this study, it was hypothesized that an upregulation of SOCS3 led to the reduction in STAT3, because there was no difference in Ob-Rb on the cell surface.

The mechanism of leptin resistance is predominantly thought to be an increase in SOCS3 protein (71) due to continual stimulation of Ob-Rb in obesity. However, other studies indicate that decreased central and peripheral Ob-Rb mRNA expression (90, 94) in the

setting of chronically high leptin, as well as a reduction in leptin binding sites (92) may be to blame. Therefore in an environment where leptin concentration is continually elevated, leptin signaling may be impaired.

2.3 Leptin and Immune Function

Though originally described as a satiety hormone, leptin's ability to modulate immune function was discovered when *db/db* mice were found to have thymic atrophy (55). It is now clear that leptin has direct and indirect effects on both innate and cell-mediated immune response. In fact, the drop in leptin is thought to be, in part, responsible for the immunosuppression that occurs during starvation, as leptin administration abrogated the immune defects in mice following a 48h fast (6). Immunomodulatory effects of leptin include; NK cell development and cytotoxicity (95), upregulation of pro-inflammatory cytokines, and T cell proliferation and activation (6, 8, 96, 97). Leptin can induce cytokine production by dendritic cells (98) and macrophages, as well as promoting NK cell cytotoxicity and type I helper T cell responses (8).

2.3.1 Innate Immune Responses

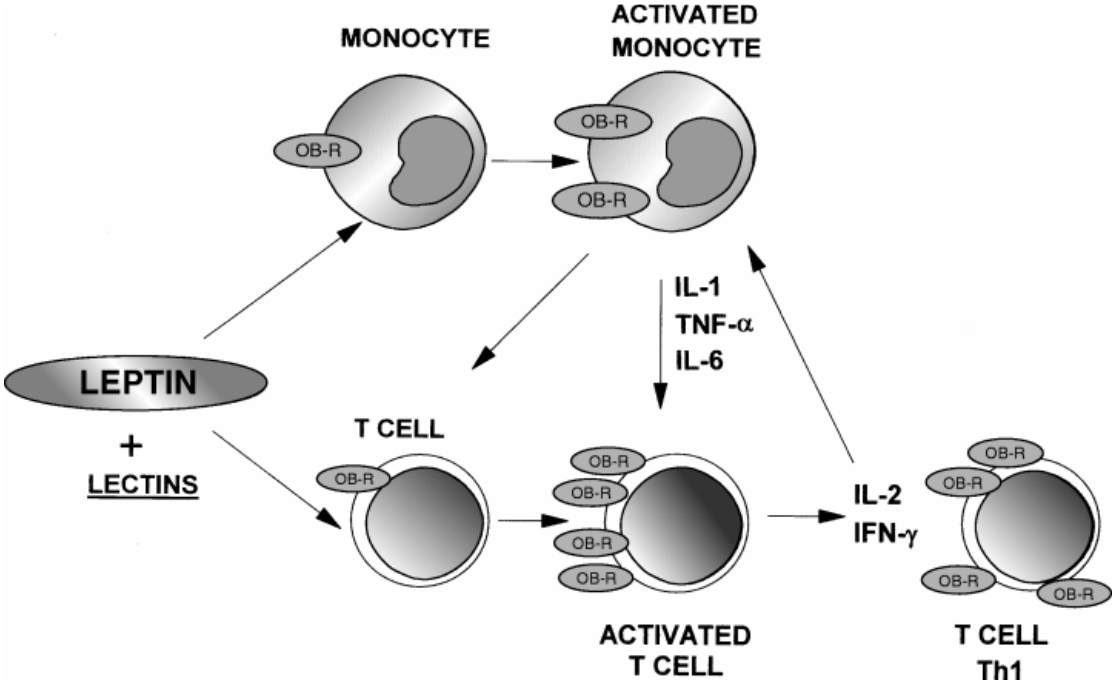
Leptin's effects on innate immune responses are generally thought to increase an inflammatory response, seeing that leptin increases during an infection and augments the secretion of the pro-inflammatory cytokines IL-1β, TNFα and IL-6 (99). Leptin's role in inflammation is also demonstrated in models of inflammatory diseases. In experimentally induced colitis, mice that lack leptin (40) are protected from the intestinal inflammation and have less pro-inflammatory cytokine secretion. However, administration of leptin increases cytokine production in these mice to that of controls (100). In addition to its role in

inflammation, leptin also promotes the secretion of cytokines from dendritic cells. Analysis of dendritic cells stimulated with lipopolysaccharide (101) and leptin, demonstrated synergistic effects on cytokine production than cells cultured with LPS alone (98). Similarly, leptin increases phagocytosis and cytokine secretion from monocytes and macrophage (97). Moreover, leptin promotes NK cell development and cytotoxicity as demonstrated by impaired NK cell function in *db/db* mice, which lack the leptin receptor (102).

2.3.2 Cell-Mediated Immune Responses

Analogous to innate responses, cell mediated immune responses are also affected by leptin. In CD4 T cells, leptin promotes IFNγ production, thus polarizing a Th1 response (103, 104). Evidence of this has been shown in children who lack leptin. These children have significantly impaired T cell proliferative responses and IFNγ production following mitogen stimulation. This is corrected, however, once leptin supplementation is initiated (103). Leptin also induces T cell proliferation and survival, IL-2 secretion, and adhesion molecule expression (6, 77, 97). Administration of leptin to *ob/ob*, as well as mice with starvation-induced leptin deficiency, reverses immunosuppression (6).

Figure 1.2. Leptin's action on cells of the immune system



E. Influenza A: A Public Health Perspective

Influenza infection affects millions every year. In the United States it is responsible for over 36,000 deaths and 200,000 hospitalizations, making it the sixth leading cause of death (105). Worldwide it is estimated 5-40% of the population is infected with influenza annually (106). Individuals with chronic medical conditions such as diabetes or heart disease are at increased risk of influenza-associated morbidity and mortality (107).

F. Influenza A: Life Cycle

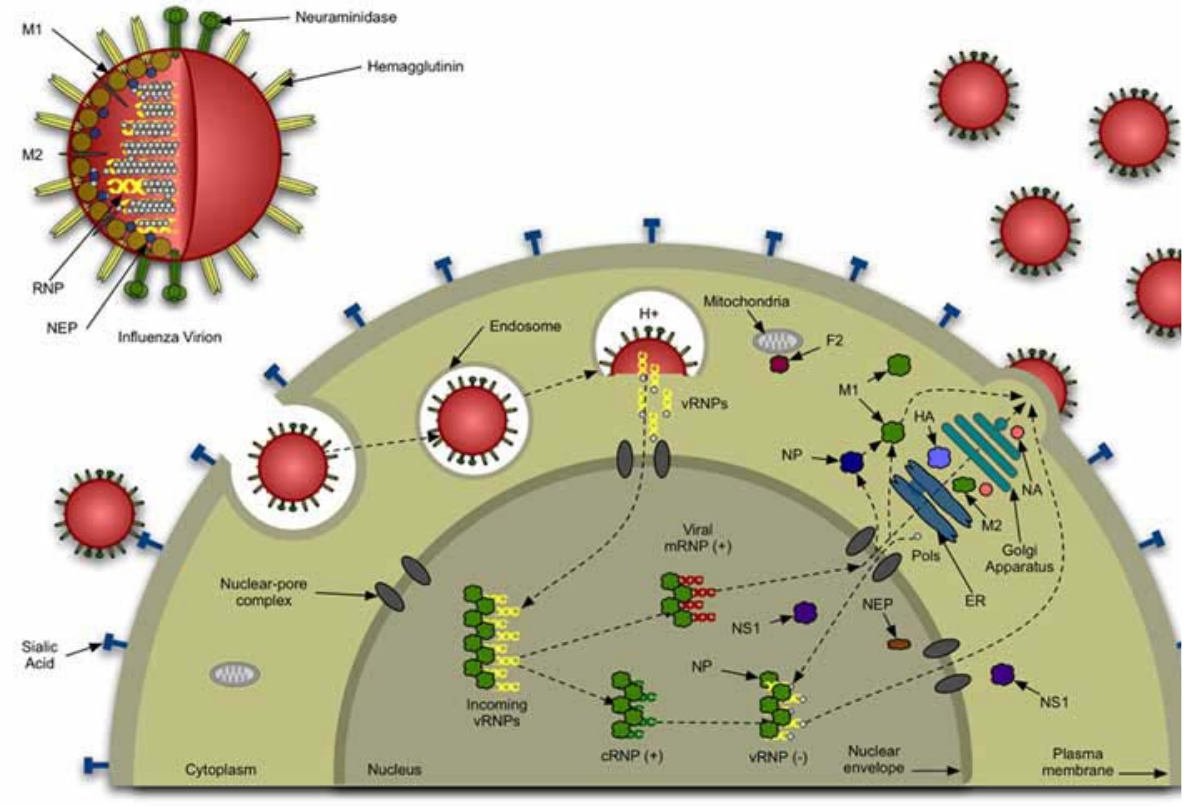
Influenza virus, a member of the Orthomyxovirus family, is a single-stranded, negative sensed, segmented RNA virus that infects epithelial cells that line the respiratory tract (108). Influenza is classified by its surface hemagglutinin (HA) and neuraminidase (25) glycoproteins. Infection begins when the viral hemagglutinin (HA) molecule binds to sialic acid-containing receptors on the surface of the host cell, and the viral envelope fuses with the host cell membrane. Infectivity requires cleavage of HA into disulfide linked products, which in the case of Influenza A/PR8, is performed by a ubiquitous set of proteases (109). The virus is internalized by clathrin-coated pits and is transported by endosomes where HA-mediated fusion of the viral envelope with the endosomal membrane occurs. This fusion is succeeded by entry of viral ribonucleoproteins into the nucleus (110).

In the nucleus, host cellular pre-mRNA is cleaved by an influenza-encoded cap dependent endonuclease. This allows for the generation of capped primers followed by initiation of viral mRNA synthesis (111). Viral replication begins after the synthesis of

mRNA and requires the formation of viral proteins. Viral progeny are produced from complementary RNA that was generated from the parental RNA (112).

Newly generated vRNAs are exported from the nucleus. This is mediated by the interaction of viral nucleoprotein (NP) and matrix protein (M1) (113). The encapsidated vRNAs assemble at the plasma membrane where they are packaged into virions. The interaction M1 with HA and neuraminidase (25) facilitate concentration of viral components and exclusion of host proteins. Virions egress the cell by budding through the plasma membrane resulting in viral envelope that is derived from the host cell membrane (114).

Figure 1.3. Life Cycle of Influenza Virus (115)



G. Immune Response to Influenza Infection

Host response to and recovery from influenza virus infection requires an intact innate and acquired immune system. A coordinated pattern of cytokine secretion (116) is responsible for inhibiting viral replication (117), stimulating an inflammatory response (118), and recruiting immune cells to the site of infection (119).

1. Innate Immune Response

The innate immune response is an immediate, relatively non-specific, response to pathogen. The purpose of the innate immune system during influenza is to control the spread of virus and concomitantly activate a virus specific response. This is done primarily through the actions of cytokines and chemokines secreted from virally infected cells

1.1 Respiratory Epithelial Cells

Infection of lung epithelial cells with influenza leads to the production of cytokines and chemokines that initiate immune responses. Infection of human primary lung bronchiolar and alveolar epithelial cell cultures with influenza indicate that these cells produce interleukin (IL)-β, IL-6, and regulated on activation, normal T cell expressed and secreted (RANTES) in responses to influenza (120). These cells are also a major source of interferon (IFN)-α/β (121). Infected epithelial cells die by necrosis which triggers cytokine production and activation of immune responses.

1.2 Macrophage

Recruitment of monocytes/macrophages into the lung is a hallmark of the innate immune response. These cells are fully susceptible to infection, however they die by apoptosis before the virus completes one replicative cycle (122). Infected monocytes and macrophages contribute to the host response by the transcription and production of pro-inflammatory cytokines, such as tumor necrosis factor (TNF)-α, IL-1β, IL-6 and IFN-α/β, and the chemokines RANTES, monocyte chemotactic protein (MCP)-1 and macrophage inflammatory protein (MIP)-1α (123, 124). The production of these cytokines induces the activation and migration of additional blood monocytes, as well as T and B cells to the site of infection. In the lung and LN, macrophage present antigen to T cells, this leads to the killing of virus-infected cells and initiation antigen specific T cell responses, respectively.

1.3 Dendritic Cells (DC)

Dendritic cells (DC) are principal antigen presenting cells and are the initiators and modulators of the immune response (125). Like macrophage, DCs can be directly infected with influenza, however, they are also very good at viral uptake through macropinocytosis or phagocytosis. Endocytosed virus is processed it into peptide fragments which are then presented to T cells on the major histocompatibility complexes. Presentation of antigen by DCs is far superior than that of macrophage due to their high expression of MHC-peptide complexes and co-stimulatory molecules. Antigen presentation to T cells occurs following migration of the dendritic cell to the draining lymph node through which T cells are circulating (125). The cytokines produced by DCs during antigen presentation play an important role in the activation and polarization of T cell responses. For example, during

influenza infection, DCs produce IL-12 which acts as a potent stimulator of Th1 responses and also functions directly on $CD8^+$ T cells to augment cytolytic activity and IFNγ production (125, 126). Through their production of IFNα/β and IL-12 dendritic cells also play a critical role in NK cell expansion and activation (127).

1.4 Natural Killer (NK) Cells

Natural Killer (NK) cells are cytotoxic lymphocytes that kill virally infected cells. The ability of NK cells to recognize virally infected cells relies on a system of recognition by their killer-activating receptors and killer-inhibitory receptors. The killer-activating receptors recognize molecules present on the surface of all host cells whereas the killer-inhibitory receptors recognize MHC class I molecules. All nucleated cells have MHC Class I and thus engage the inhibitory receptor on the NK cell to prevent NK cell activation. Therefore, the down regulation of MHC Class I molecules by influenza virus, which can help it evade some immune responses, attenuates the signal from the killer-inhibitory receptor and leads to activation of NK cell killing (128).

NK cell activity is augmented by IFN α/β and IL-12, the latter also induces IFNγ production by the NK cell, which activates antiviral gene expression and increases antigen presentation by macrophage (127).

2. Cell-Mediated Immune Responses

The cell-mediated, or adaptive, immune response, is the classification given to cells that participate in the arm of the immune response that is specific for the invading pathogen.

Due to the fact the cell-mediated immune response must be generated *de novo* and relies on "information" from the innate immune response, peak activation of this response occurs days after initial antigen insult. During influenza infection, the cell-mediated response is characterized predominantly by T cells, which are recruited to the infected lung and eradicate virally infected cells.

2.1 Major Histocompatibility Complex (MHC) Molecules

For a specific immune response to be generated, antigen must first be presented to naïve T cells via major histocompatibility complex (MHC) molecules. These molecules are expressed on the surface of cells and function to display both self and non-self antigens. Two main classes of MHC exist, Class I and Class II. Class I is present on all nucleated cells and presents peptides from cytosolic antigens (128) to T cells with the CD8 receptor. Class II, however, is exclusively on antigen presenting cells. This MHC molecule presents antigenic peptides generated from lysosomal degradation to T cells with a CD4 receptor.

2.2 T cells

T cells mature in the thymus where they become either CD4 or CD8 positive. Single positive, naïve, T cells egress from the thymus and circulate through the body. Circulating T cells migrate through peripheral lymphoid tissues, such as the lymph node, where they sample MHC-peptide complexes on antigen presenting cells for antigen recognition. This sampling increases the probability of the T cell to encounter antigen, which is crucial given that one naive T cell in 10^4-10^6 is likely to be specific for a particular antigen (129). When the T cell receptor (TCR) recognizes antigen an activation signal is transmitted by the CD3

complex of molecules on the T cell. This leads to rapid clonal expansion to produce the large numbers of progeny that will differentiate into armed effector T cells. Activation also induces synthesis of IL-2, which is produced by the activated T cell and drives clonal expansion and differentiation. Therefore one T cell can give rise to thousands of progeny that all have the same receptor for antigen (129).

There are two classes of T cells that are distinguished by the expression of the cell-surface proteins CD4 and CD8. These two types of T cell differ in the class of MHC molecule they recognize; CD4 binds to MHC class II and CD8 binds to MHC class I.

2.2.1 $CD4^+$ T cells

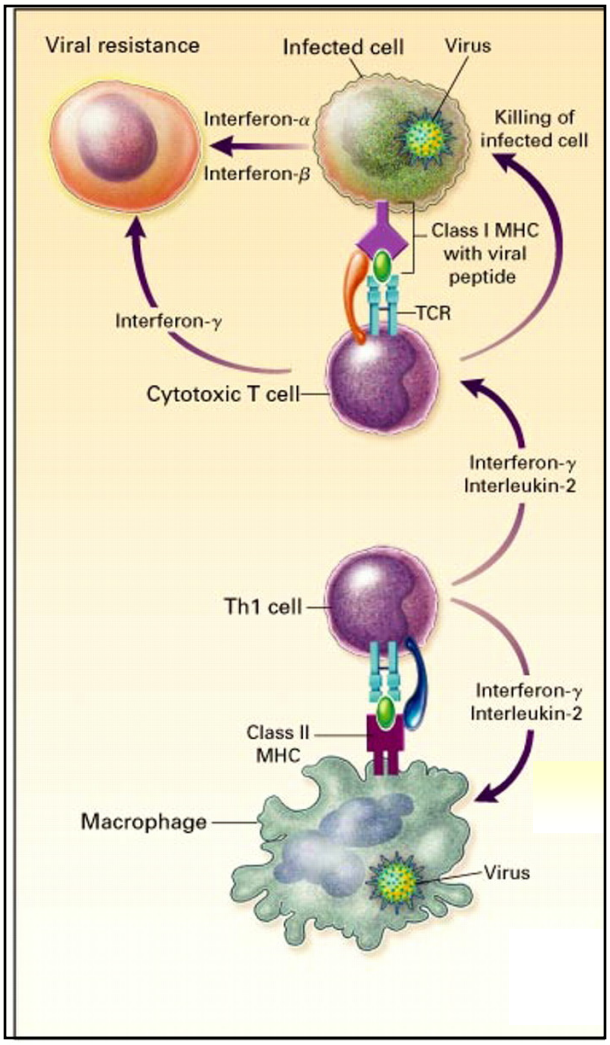
Naive $CD4^+$ T cells can differentiate into helper T cells with either a Th1 or Th2 phenotype. To which helper subtype the $CD4^+$ T cell differentiates depends on the pathogen and by the cytokines that are secreting during presentation on MHC Class II. During influenza infection, IL-12 secretion by the DC will polarize the $CD4^+$ T cell toward a Th1 phenotype. This results in IL-2 and IFNγ production by the $CD4^+$ cells which activate $CD8^+$ T cells. Conversely, induction of a Th2 response leads to the secretion of IL-4, IL-5, and IL-10, which downregulate Th1 responses and promotes B cell activation and antibody production. Although $CD4^+$ T cells help promote $CD8^+$ activation during influenza infection, it is not required as mice that lack MHC Class II molecules, elicit a normal $CD8^+$ effector T cell response (130).

2.2.2 $CD8^+$ T cells

Unlike naïve $CD4^+$ T cells, naive $CD8^+$ T cells are programmed to become cytotoxic cells. Proliferating $CD8^+$ T cells gain effector function upon MHC Class I-peptide binding in concert with co-activating molecules. During influenza infection, $CD8^+$ T cells traffic from the lymph node into the tissue parenchyma, where effector function is critical for appropriate clearance of virus. During an active infection, $CD8^+$ T cells compose almost 70% of the lung lavage population, of which, almost 90% are specific for four viral epitopes (131), (132).

Upon encounter with antigen, $CD8^+$ T cells produce IFNγ which will inhibit viral replication. Additional elimination of virus infected cells by $CD8^+$ cells occurs through direct lysis (133) by two different pathways: perforin/granzyme B or FasL. In the perforin/granzyme B pathway, $CD8^+$ T cells insert perforins into the membranes of infected cells which is followed by the release granzymes. This activates caspases and results in apoptosis. Alternatively, caspase activity can be activated by Fas/FasL interaction.

Figure 1.4. Activation of Th1 response by virus infected macrophage (128)



3. Virus Induced Cytokine Signaling

Infected cells limit viral replication via receptor and cytokine signaling pathways. Viral RNA is recognized through various receptors found on cell surfaces and in endosomal compartments. Binding of viral components initiates signaling cascades that result in cytokine production by infected cells. Cytokines signal primarily through receptors that require the Janus kinase/ Signal transducer and activator of transcription (JAK/STAT) to activate components of the immune response (**Figure 1.5**).

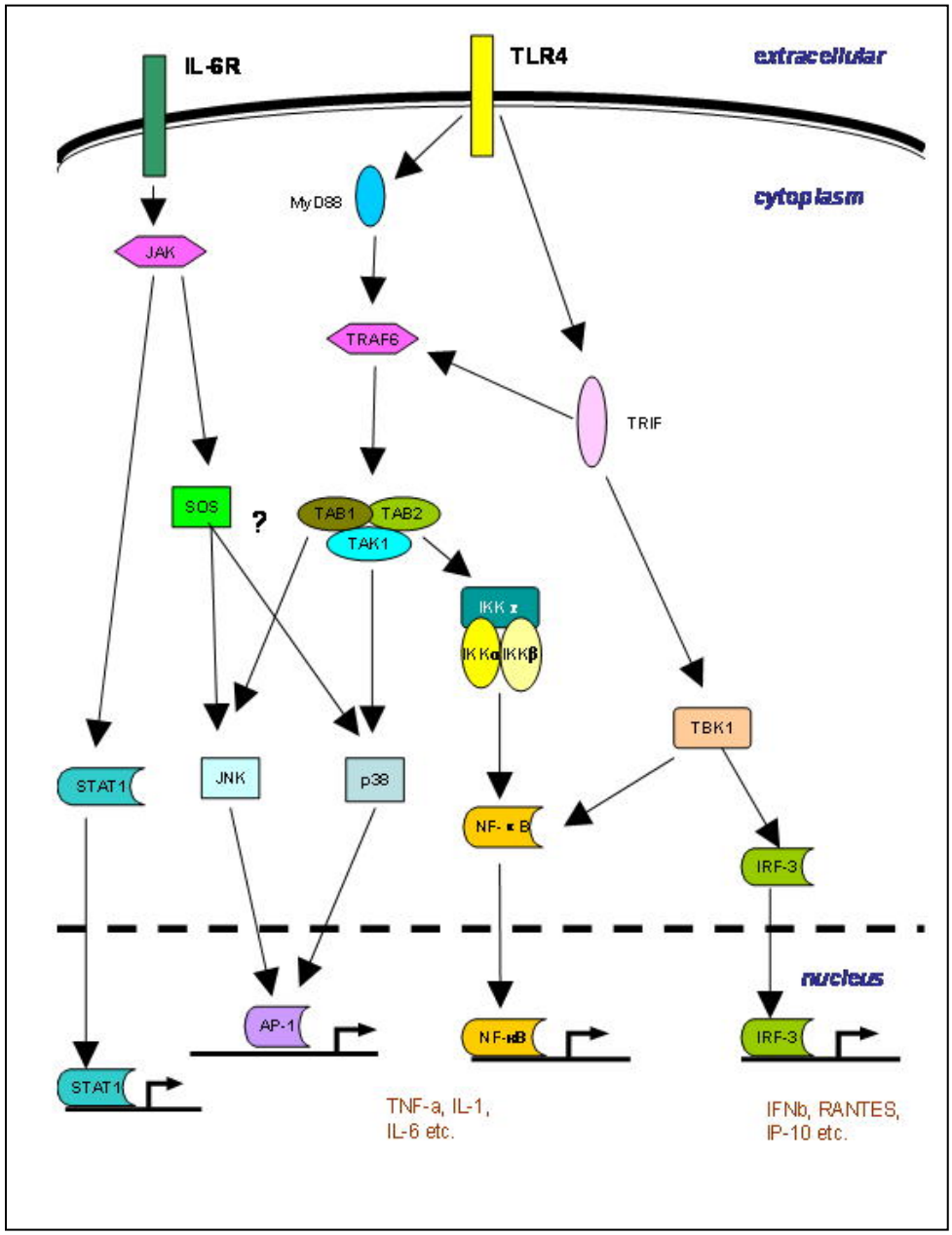
3.1 Viral Recognition

Influenza viral RNA is recognized through various receptors including; Toll-like receptors (TLR 3, 7, and 9), retinoic acid inducible gene I (Rig), and Rig-like receptors (134). Binding of viral RNA to these receptors activates separate, but often overlapping, signaling pathways which result in the transcriptional activation of Type I IFNs (IFNα/β) via interferon regulatory factor (IRF) DNA binding (135). TLR's also activate NF-κB and MAPK signaling pathways which promotes the transcription of cytokines and chemokines (136). Additionally, viral nucleic acids activate protein kinases and RNase L which non-specifically block translation and degrade RNA, respectively (137, 138) and lead to apoptosis (139).

3.2 Type I Interferons

Type I IFN can be produced in all nucleated cells in response to viral infection and all cells can respond to them (140). These cytokines act in a positive feedback loop through IFNA receptors (IFNAR) activation of STATs 1 and 2 to increase antiviral protein

Figure 1.5. Overview of cytokine/receptor signaling pathways (141)



expression, increase viral recognition receptors, as well as increase their own expression (135). Their importance in immune activation is illustrated by the ability to promote differentiation of monocytes to DCs (142), activate NK cells (127), upregulate MHC Class I and II expression (143), enhance priming of Th1 cells (144), and induce NF-kB and MAPK activation (145, 146). Their role in IFNγ production by NK cells is mediated through STAT4 activation (127). The necessity of Type I IFN during influenza infection is unclear, as some studies have reported higher viral titers and increased mortality in mice lacking the IFNβ gene (147), while others have shown little effect on viral replication or $CD8^+$ T cells responses in mice lack IFNAR (148). Type I IFNs are necessary for NK cell cytotoxicity, however (127) and general disruption of STAT1 results in extreme susceptibility to infection (149). (See Table 1.3 for list of cytokines/chemokine induced during influenza virus infection).

3.3 Type II Interferon

IFNγ, Type II IFN, can also promote NF-κB and MAPK activation through synergy with TLRs in macrophage (150). Unlike Type I IFNs, which are induced by viral nucleic acids, IFNγ is induced by T cell receptor crosslinking, as well as by APC derived cytokines TNFα and IL-12, and by autocrine action (151, 152). It mediates numerous antiviral effects by STAT 1 induction of IRFs which act as secondary transcription factors and activate genes involved in the following: upregulation class II MHC molecules; regulation of leukocyte-endothelium interaction; polarization of Th1 and suppression of Th2 responses; induction of IL-12 and TNFα; and numerous others (153). CD4 T cell secretion of IFNγ is a defining

characteristic of the Th1 response and IFNγ is critical for antigen-specific CD8 T cell homeostasis during influenza infection (154).

3.4 IL-12

IL-12 is produced by DC, macrophage, and neutrophils (155, 156) and signals by recruiting STAT3 and 4 to phosphorylated JAK2 on the IL-12R. This mediates IL-12 effector functions which includes induction of IFNγ by NK cells (157). Importantly, IL-12 acts in a non-redundant way to promote Th1 polarization (158) and is itself induced by IFNγ (159). Mice that lack STAT4 have minimal IFNγ production, reduced NK cytotoxicity, and less proliferation of activated lymphocytes (158).

3.5 IL-6

IL-6 is cytokine that is induced by TLR signaling in macrophage. It signals through a gp130 receptor and transduces its effects through JAK/STAT signaling. Macrophage production of IL-6 induce antiviral responses through STAT1 activation of IFN-induced genes (160), but also inhibits LPS-induced TNFα production through STAT3 (161), thereby acting to both induce and repress immune responses. A role for IL-6 in T cell homeostasis was shown in T cell specific STAT3 knock out mice. These mice had reduced T cell proliferation due to increased apoptosis which was prevented by IL-6 administration in normal cells (162).

More recently, it has been proposed that IL-6 contributes to chronic inflammation in obese human as serum IL-6 levels correlate with body fat (163). These findings are not consistent however (164). Currently it is unclear how low levels of IL-6 affect metabolic or

immune function since diet-induced obese rodents do not have increased circulating cytokines therefore making it difficult to study experimentally.

3.6 IL-10

IL-10 signals through the IL-10 receptor with downstream effects mediated by Jak1 and STAT3. IL-10 is considered "anti-inflammatory", hence it suppresses the expression of inflammatory genes (162, 165). Overexpression of IL-10 in antigen presenting cells has been shown to inhibit clearance of antigen and therefore expression must be tightly regulated (166). Conversely, a lack of IL-10 during bacterial infection lead to overactivation of CD4 T cells, IL-12, IFNγ, and TNFα (86).

Table 1.2 Cytokines and chemokines expressed during influenza virus infection [Adapted from (133)]

Chemokine/cytokine	Function	Produced by
IFNα/β	Inhibit viral replication Promote NK cell proliferation and cytoxicity Activate antigen-specific T cells	Dendritic cells Respiratory epithelium
IFNγ	Inhibits viral replication Stimulates CTL mediated killing Increases MHC I expression Activates macrophage and neutrophils Promotes T-cell proliferation	T cells NK cells
TNFα	Direct antiviral effects Neutrophil chemoattractant Stimulates macrophage phagocytosis and production of IL-1 Increases vascular permeability	T cells Monocytes/macrophage Dendritic cells Neutrophils
IL-1	Increases expression of adhesion factors on endothelium Increases vascular permeability Stimulates IL-6 production	Monocytes/macrophage Dendritic cells
IL-6	Pro-inflammatory cytokine Activates T cells	Respiratory epithelium T cells Monocytes/macrophage Dendritic cells
IL-12	Promotes differentiation of naïve T cells to Th1 Enhances cytotoxic activity of NK cells and T cells Stimulates IFNγ production by dendritic cells and NK cells	Macrophage Dendritic cells
MIP-1α/β	Monocytes/ T cell chemoattractant Activates neutrophils	Monocytes/macrophage Neutrophils T cells Dendritic cells
RANTES	Monocyte/T cell/Dendritic cell chemoattractant Activates T cells	T cells Respiratory epithelium

3.7 Signaling deactivation

Phosphatases have been shown to play a role in JAK/STAT deactivation. For example PTP SHP2, a ubiquitously expressed phosphatase, is recruited to the phosphorylated tyrosines on the gp130 receptor where it inhibits receptor signaling (167). SOCS proteins are another class of molecules that attenuate cytokine signaling. These are recognized as classic feedback inhibitors, acting on the JAK/STAT pathway from which they were induced. SOCS 1 and 3 bind to the activation loop of JAKs and inhibit phosphorylation of the receptor, STAT, and JAK (168), thereby attenuating activation signals from the receptor. A list of SOCS proteins and there target are presented in **Table 1.3**. Additional regulation occurs via cytokines that act as negative regulators (169).

Table 1.3. SOCS proteins and their target molecules (170)

Name	Inhibits signaling by:	Associates with:
CIS	IL-2; IL-3; prolactin; Epo; IGF-1; GH	Epo receptor; IL-3 receptor ; GH receptor; IL-2 receptor; PCK
SOCS1	IL-2; IL-3; IL-4; IL-6; GH; prolactin; Epo; LIF ; IFN-; IFN-; OSM; TSLP; Tpo; IGF-1	JAK1;JAK2; JAK3; Grb2; Vav; FGF receptor; PYK2; GH receptor; Kit receptor; Flt3 receptor; IGF-1 receptor
SOCS2	GH; IL-6; LIF; IGF-1; prolactin	IGF-1 receptor; prolactin receptor; GH receptor
SOCS3	IL-2; IL-3; IL-4; IL-6; GH ; prolactin; Epo; LIF; IFN-; IFN-; CNTF; leptin; OSM; IGF-1; insulin	Lck; FGF receptor; PYK2; GH receptor; Epo receptor; leptin receptor; gp130; IGF-1 receptor
SOCS4	Not known	Not known
SOCS5	IL-6	Not known
SOCS6	Not known	Not known
SOCS7	Not known	Ash; Nck; PLC

Abbreviations: PKC = protein kinase C; OSM = oncostatin M; Tpo = thrombopoietin; CNTF = ciliary neurotrophic factor; TSLP = thymic stromal lymphopoietin

CHAPTER II

INCREASED MORTALITY AND ALTERED IMMUNE FUNCTION IN DIET INDUCED OBESE MICE INFECTED WITH INFLUENZA VIRUS

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ABSTRACT

Obesity is associated with an impaired immune response, an increased susceptibility to bacterial infection, and a chronic increase in pro-inflammatory cytokines such as IL-6 and TNFα. However, few studies have examined the effect of obesity on the immune response to viral infections. Because infection with influenza is a leading cause of morbidity and mortality worldwide, we investigated the effect of obesity on early immune responses to influenza virus exposure. Diet-induced obese and lean control C57BL/6 mice were infected with influenza A/PR8/34 and lung pathology and immune responses were examined at d 0 (uninfected), 3, and 6 post infection. Following infection, diet induced obese mice had a significantly higher mortality rate than the lean controls and elevated lung pathology. Anti-viral and pro-inflammatory cytokine mRNA production in the lungs of the infected mice was markedly different between obese and lean mice. IFNα and β were only minimally expressed in the infected lungs of obese mice and there was a notable delay in expression of the pro-inflammatory cytokines IL-6 and TNFα. Additionally, the obese mice had a substantial reduction in NK cell cytotoxicity. These data indicate that obesity inhibits the ability of the

immune system to appropriately respond to influenza infection and suggests obesity may lead to increased morbidity and mortality from viral infections.

B. INTRODUCTION

Obesity has been associated with immune dysfunction (24, 171, 172). In humans, responses of peripheral lymphocytes to mitogen stimulation are reduced (39). Some studies have reported higher T cell counts (173), whereas others report no change (174) or lower T cell counts (175), while still others demonstrate changes in the T cell population frequency (176). In addition, obese individuals have a low level of circulating TNF-α and IL-6, suggesting a state of chronic inflammation. Studies using animal models of obesity, both genetic and diet induced, also report immune dysfunction (43, 49, 63). *In vitro* response to mitogens is diminished in obese animals (177) and secretion of IL-4 and IFN-γ is altered (51). Because obesity is associated with immune dysfunction, it is plausible that obese mice infected with a viral pathogen would be unable to mount an effective immune response. To test this hypothesis, we infected lean and diet induced obese mice with influenza virus, a serious human pathogen. Studies examining the effect of obesity on infection are minimal and to our knowledge, this is the first report on the effect of obesity on an influenza virus infection.

Influenza infection is a major cause of morbidity and mortality worldwide. Once infected, an appropriate coordination of both innate and adaptive immune responses is necessary for elimination and recovery from the virus. Groups at high risk for increased influenza virus mortality include the elderly, the very young, and individuals with chronic

pulmonary and/or cardiovascular conditions (178). Interestingly, obesity is also associated with chronic pulmonary and cardiovascular diseases (179, 180). Upon infection with influenza virus, dendritic cells, macrophages, and lung epithelial cells upregulate the expression of cytokines and chemokines. These molecules play essential roles in the early inhibition of viral replication (181), the stimulation of an inflammatory response (118) and recruitment of immune cells to the site of infection (119). Additionally, cytokines activate natural killer (NK) cells which are among the first cell types to become mobilized during an influenza infection. NK cells assist in eliminating infected cells and help limit viral spread until a specific cell-mediated response can be assembled (182). The expression of cytokines and chemokines during an influenza virus infection occurs in a coordinated and specific cascade. Antiviral and pro-inflammatory cytokines are induced first, followed by IL-6 expression, and finally chemokines, such as monocyte chemotactic protein (MCP)-1 and macrophage inflammatory protein (MIP)-1α are expressed (183). Changes in the expression of any of these molecules can alter subsequent immune responses (184).

In this study, we asked whether obesity could affect the early immune response to influenza virus infection, and therefore alter viral pathogenesis. We found that diet-induced obese mice had a marked increase in mortality compared to lean mice. Furthermore, significant alterations in immune function, including alterations in cytokine and chemokine profiles and reduced NK cell function, were found in obese mice. These data indicate that obesity is a potent mediator of immune responses during influenza infection and, therefore, may increase susceptibility to the adverse effects associated with the virus.

C. MATERIALS AND METHODS

Animals

Weanling C57BL/6J mice were obtained from Jackson Laboratories (Bar Harbor, MA). All mice were housed in the University of North Carolina Animal Facility, which is fully accredited by the American Association for Accreditation of Laboratory Animal Care. Animals were maintained under protocols approved by the Institutional Animal Use and Care Committee. Mice were randomized to receive either a low fat/no sucrose diet (LF) or a high fat/high sucrose (HF) diet for 22 wk. Mice were housed four per cage with free access to food and water, with the exception of an 8 h food fast prior to blood draws for glucose and insulin measurements as well as NK cell cytotoxicity.

Diets

The diets, which have been previously described (185), were obtained from Research Diets, Inc. (New Brunswick, NJ). The LF diet (D12328) consisted of 16.4% protein, 73.1% carbohydrate (83% starch, 17% maltodextrin), 10.5% fat (38% soybean, 62% coconut oil). The HF diet (D12331) consisted of 16.4% protein, 25.5% carbohydrate (51% sucrose, 49% maltodextrin), 58% fat (93% coconut oil, 7% soybean oil).

Virus and Infection

The mouse adapted strain of Influenza A/Puerto Rico/8/34 (A/PR8) (American Type Culture Collection, Manassas, VA) was propagated in the allantoic fluid of fertilized chicken eggs and the viral titer was determined by hemagglutination assay (186). After 22 wk on the diets, mice were anesthetized with an intramuscular injection of a ketamine

(0.6mg/kg)/xylazine (0.35mg/kg) solution and infected intranasally with 0.05 mL of 2 hemagglutinating units (HAU) of A/PR8 virus diluted in PBS. Preliminary studies from our lab determined that this dose of virus is sufficient to effectively elicit an immune response while causing little mortality in infected control mice.

Measurement of blood glucose, serum insulin and leptin

To determine if blood glucose and insulin levels were affected by infection, mice were fasted for 8 h and blood samples were collected prior to infection (after 22 wk on the diet) and at d 3 and 7 p.i. Blood glucose levels were measured with a Freestyle blood glucose monitor (Abbott Laboratories, Abbott Park, IL). Serum insulin was measured by ELISA (LINCO Research, St. Charles, MO). Serum leptin was measured by ELISA (R & D Systems, Minneapolis, MN) in unfasted mice prior to infection and at d 3 and 6 p.i.

Histopathology of lungs

Lungs were removed at d 0, 3, and 6 p.i. and perfused with 4% paraformaldehyde, paraffin embedded, cut in 6μm sections and stained with hemotoxylin and eosin (H&E). Pathology was graded semi-quantitatively as previously described (187).

Quantitation of viral titers

Lung viral titers were determined by a modified Tissue Culture Infections Dose 50 ($TCID_{50}$) using hemagglutination as an endpoint, as previously described (188). Briefly, half of the right lobe of the lung was removed, weighed, and ground in 150 uL minimal essential media. Samples were spun and the supernatant was serially diluted starting at 1:5 in MEM

containing 20ug/ml trypsin. Each diluted supernatant (100μL) was added, in duplicate, to 80% confluent MDCK cells and incubated at 37°C for 72 h. A 0.5% suspension of human O RBC (50μL) was added to each well and incubated at room temperature for 2 h. Viral titer was expressed as the reciprocal of the highest dilution at which the RBC agglutinated. This value was then normalized to total protein in the sample. Total protein was determined by bicinchoninic acid assay, as previously described (189).

Quantitation of lung mRNA cytokine levels

Half of the right lobe of the lung was removed at d 0 (uninfected), 3, and 6 p.i. Total RNA was isolated using the TRIzol method (Invitrogen, Carlsbad, CA). Reverse transcription was carried out using Superscript II First Strand Synthesis kit (Invitrogen) using oligo (dT) primers. Levels of mRNA levels for murine IFNα, IFNβ, IFNγ, IL-1β, IL-6, IL-10, IL-12, TNFα, MCP-1, MIP-1α, regulated upon activation, normal T-cell expressed, and secreted (RANTES), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were determined using qRT-PCR. Fluorescent reporters were detected using Bio-Rad (Hercules, CA) iCycler PCR machine and primers and probes were designed using Primer Express 1.5 (Applied Biosystems, Foster City, CA). The levels of mRNA for GAPDH were determined for all samples and were used to normalize gene expression. All data are expressed as fold induction over lean, uninfected controls. There were no statistical differences between lean and obese uninfected controls.

Enumeration of NK cell populations in the lung and spleen

Lungs were removed at d 3 p.i. and incubated in a collagenase solution (1500 units/set of lungs) for one hour. Both spleen and lung tissue was processed into single cell suspensions by mechanical agitation of a Stomacher (Seward, UK) and strained through a 40μm nylon filter. Cells were stained with fluorescein (FITC)-labeled anti-DX5 and Phycoerythrin (PE)-labeled anti-CD3 (BD Pharmingen, San Diego, CA) and analyzed by FACSCaliber. The lymphocyte population was gated and NK cells were identified as $CD3^-$ $DX5^+$ within that gate.

Determination of natural killer cell cytotoxicity

To determine if obesity had an effect on the ability of NK cells to lyse a target, isolated lung and spleen cells were analyzed in a standard NK cell cytotoxicity assay (190), using ^{51}Cr-labeled YAC-1 tumor cells (ATCC) as targets. Briefly, serially dilutions of 0.1 mL lung or spleen effector cells, starting at 1×10^7 cells/mL, were plated with 1×10^4 cells ^{51}Cr labeled YAC-1 target cells in a 96-well V-bottom microplate. The resulting effector to target (E:T) ratios were 100:1, 50:1, and 25:1. All samples were prepared in triplicate. To determine maximum lysis, 0.1mL of 10% sodiumdodecylsulfate was added to labeled YAC-1 cells. To determine spontaneous release 0.1 mL of the culture media was added to radioactive YAC-1 cells. Release of ^{51}Cr content was analyzed using a gamma counter (Cobra II, Hewlett Packard) and percent specific lysis was calculated by the following equation:

$$\% \text{ specific lysis} = \frac{\text{cpm (sample)} - \text{cpm (spontaneous release)}}{\text{cpm (maximum release)} - \text{cpm (spontaneous release)}} \times 100$$

Statistical Analysis

Statistical analyses were performed using JMP Statistical Software (SAS Insititute, Cary, NC). Normally distributed data was analyzed by two-way ANOVA with diet and day post infection as main effects. Where appropriate, Student's t-test was used for post hoc analysis $\alpha = 0.05$. Non-parametric data was analyzed using Kruskal Wallis test, $P \leq 0.05$.

D. RESULTS

Elevated insulin levels in obese mice during influenza infection

Because diabetes is a risk factor for complications of an influenza infection (191), we wanted to determine if mice fed the HF diet had elevated glucose and insulin levels both before and during infection. As shown in Figure 2.1A insulin levels at baseline were similar between lean and obese mice, but increased during infection in the obese mice. Glucose levels were significantly elevated at baseline and at d 3 p.i. in the obese mice, but decreased by d 7 p.i to levels similar to lean mice (Figure 2.1B). Conversely, glucose levels in lean mice were unaffected by influenza infection. The fact that insulin levels are significantly elevated in obese mice during infection suggests that the obese mice developed transient insulin resistance during influenza infection.

Obesity reduces serum leptin levels during influenza infection.

Previous studies have shown that serum leptin increases in response to infection in normal weight mice (6, 99, 192), and given the fact that obesity results in chronically elevated leptin levels, we wanted to determine what effect influenza infection had on serum leptin levels and if this was altered by obesity. As expected, baseline leptin values were higher in obese mice compared to lean mice (Figure 2.1C). Interestingly, leptin responses

during infection differed between the two groups. In lean mice, circulating leptin levels tended to increase transiently following infection, with the concentration at d 6 p.i. being similar to the uninfected levels, but significantly less than at d3 p.i. On the other hand, obese mice had a transient decrease in serum leptin during infection, with levels at d3 p.i. being significantly lower than at d 0 or 6 p.i. (Figure 2.1C).

Obesity results in high mortality during influenza infection.

As shown in Figure 2.2A, obese mice had a mortality rate that was 8 fold greater than lean mice (5.5% in lean versus 42% in obese). The obese mice died at d 8 p.i.

Viral titers and lung pathology are similar between lean and obese mice.

The high mortality rate in the obese mice indicated that an influenza virus infection is significantly altered by obesity. To determine if the increased mortality was associated with an increased viral load in the obese mice, we measured lung viral titers at d 3 and 6 p.i. As shown in Figure 2.2B, viral titers were similar between lean and obese mice at d 3 p.i., although by d 6 p.i. the lung virus titer was decreased in the obese mice compare to lean. However, viral titers were still high in both groups.

Lung inflammation is a significant contributor to increased mortality in influenza-infected mice. To determine if obese mice had significantly elevated lung pathology compared to lean mice, we examined lung tissue following infection. Figure 2.2C shows that although lung pathology increased in both groups after influenza infection, the obese mice tended to have increased lung pathology compared to lean mice ($P \leq 0.1$). These data

indicate that although an excessive amount of influenza virus in the lungs was not the cause of the high mortality in the obese mice, augmented lung pathology may have contributed to the higher mortality rate.

Obese mice have reduced expression of antiviral cytokines.

The expression of IFNα and IFNβ is induced by double stranded RNA (193) early during infection and these cytokines function to control viral replication. Additionally, they act as potent activators of NK cell cytoxicity (127). As shown in Figure 2.3, lean mice had a robust increase in mRNA expression of IFNα and IDNβ at d 3 p.i. unlike obese mice whose antiviral cytokine expression remained low throughout the infection. It is important to point out that lung viral titers are equivalent between lean and obese mice at d 3 p.i., suggesting that an unequal amount of virus did not cause the difference in IFNα/β expression.

Reduced NK cytotoxicity in influenza infected obese mice

Because IFNα and β were greatly reduced in obese mice, we reasoned that NK cell cytoxicity may be impaired in these mice. To determine if NK cell activity was affected by obesity, we measured lung and splenic NK cell cytotoxicity both prior to and three days after infection. NK cell cytoxicity prior to infection was low in both lean and obese mice, with cytotoxicty in the spleen being less than 10%, and in the lungs, cytotoxicity was below the detection limit of the assay (data not shown). However, during infection, obese mice had significant reductions in NK cell mediated killing compared to lean mice. As shown in Figure 2.4A, lung NK cell cytotoxicity was reduced by more than 50% in obese mice at d 3 p.i. This reduction may be due, in part, to the decreased proportion of NK cells in the lungs

of obese mice. As shown in Figure 4B, the proportion of NK cells in the lungs of obese mice was 22% whereas NK cells made up 30% of the lung lymphocyte population in lean mice. Analogous to the lungs, NK cell killing in the spleens of obese mice was also dramatically reduced (Figure 2.4C). However, unlike the lung, this was not due to a difference in NK cell frequency, because the percentage of splenic $DX5^{+}CD3^{-}$ cells were equivalent between lean and obese mice (Figure 2.4D).

Since NK cell cytotoxicity can be affected by other cytokines, we also measured the mRNA expression of IL-12 and IL-18 which are known to enhance NK cell cytotoxicity (194, 195). Although IL-12 mRNA levels were not different between lean and obese mice, IL-18 mRNA levels were significantly lower in obese mice post infection (Figure 2.4E and 2.4F), suggesting a role for IL-18 in the decreased cytotoxicity of NK cells in obese mice.

Lung mRNA expression of pro-inflammatory cytokines and the anti-inflammatory cytokine IL-10.

In addition to antiviral cytokine responses and NK cells, inflammatory responses are a key part of the innate immune response to influenza infection. Paradoxically, they can also contribute to lung pathology due to increased inflammatory infiltrate. We examined gene expression of the pro-inflammatory cytokines IL-6, TNFα, and IL-1β in the lungs of obese and lean mice. As shown in Figure 2.5, lung mRNA expression at d 3 p.i. for all three cytokines was significantly lower in obese mice. Interestingly, the expression of TNFα and IL-6 was down regulated by d 6 p.i. in lean mice, whereas obesity led to enhanced expression at this time point. These data suggest that during an influenza infection, obesity causes a delay in the pro-inflammatory cytokine response.

To determine if the differences in pro-inflammatory cytokine expression at d 3 p.i. could be attributed to upregulation of IL-10, a cytokine that counteracts the pro-inflammatory effect, we measured its expression in the lungs of obese and lean mice. Similar to the pro-inflammatory cytokines, IL-10 mRNA levels were significantly lower in the obese mice at d 3 p.i. compared to the lean mice (Figure 2.5). Unlike IL-6, however, there was no significant increase in the expression of IL-10 at d 6 p.i., indicating that the lungs of obese mice were in a pro-inflammatory state 6 d after the initial infection.

Decreased lung MCP-1 and RANTES mRNA expression in influenza infected obese mice.

The induction of chemokines is an important component of infection with influenza virus because they function as attractants for immune cells at the site of infection (184). As shown in Figure 2.6, lung mRNA expression of MCP-1 and RANTES was significantly decreased in obese mice 3 d post influenza infection. However, there was no difference in the expression of MIP-1α between lean and obese mice, indicating that obesity may cause selective recruitment of immune cells to the site of infection.

D. DISCUSSION

Although there are reports of genetically obese mice (*ob/ob* or *db/db*) having impaired immune function (38, 63), there are few studies that have looked at the effect of diet-induced obesity on immune function. Diet-induced obesity is a more physiologically relevant model of human obesity, as only a small number of individuals are obese due to mutations in the leptin gene (196). Furthermore, there are no reported studies on the effect of

diet induced obesity on infection with influenza virus. With over 30% of the population in the U.S. obese, and influenza virus infecting 5-20% of the entire U.S. population each year, it is important to understand how obesity may impact the host's ability to respond to infection with influenza virus.

In our study, diet-induced obese mice had an 8-fold higher mortality rate post-infection. Along with the increase in mortality was an altered immune response, including diminished NK cell cytotoxicity and delayed pro-inflammatory cytokine expression. To our knowledge, this is the first report of obesity interfering with normal host responses to influenza infection.

It is well established that leptin levels in diet-induced obese mice are higher compared to mice of normal weight (61). This chronic elevation, however, appears to cause a state of leptin resistance (77, 197), which may be disadvantageous given that numerous studies have demonstrated a significant role for leptin in the immune response (6, 63, 198). For example, when wild type mice were infected with *Klebsiella pnuemoniae*, leptin levels increased in the blood and lung homogenate and these mice had a normal survival time following infection. Conversely, when mice lacking leptin were infected, there was no rise in leptin levels following infection and these mice had reduced macrophage function as well as a considerably high mortality rate (63). Therefore, we measured circulating leptin levels in influenza infected lean and obese mice. We found that leptin levels in lean mice transiently increased during influenza infection. Surprisingly, in obese mice serum leptin decreased significantly during infection. The cause for this reduction is unknown, but given that the

infection was more detrimental to obese mice than lean, the obese group may have stopped eating. Limited food intake will result in lower serum leptin levels (6, 199). This decrease in food intake may also have occurred in the lean mice given the high levels of IL-1β and TNFα. However, in addition to their anorectic effects, these cytokines increase leptin production (200, 201), therefore leptin levels in the lean mice may have been appropriately balanced.

Given leptin's influence on immune function, the reduction in leptin may have impaired the innate immune responses in the obese mice. However, it should be noted that although the obese mice had a drop in leptin at d 3 p.i., the concentration of serum leptin at this time was similar to lean mice. Therefore, if obese mice were still sensitive to leptin, this level should be sufficient to generate a response. Usually during an infection, leptin's effects on the innate immune system are generally thought to increase an inflammatory response. Leptin acts on macrophages and DCs and augments their production of the pro-inflammatory cytokines IL-1β, TNFα and IL-6 (99). In our study, we found that obesity resulted in a notable delay in lung pro-inflammatory cytokine expression; obese mice did not significantly upregulate these cytokines until d 6 p.i., whereas the lean mice had the expected peak expression at d 3 p.i. Given the lack of an early pro-inflammatory response in obese mice, it is plausible that leukocytes from the obese mice no longer respond to leptin. Evidence for this comes from a recent report by Papathanassoglou et. al. who demonstrated that signal transducer and activator of transcription 3 (STAT3) nuclear translocation is attenuated in T cells from obese mice following leptin stimulation. A leptin concentration of 10ng/mL caused a five fold change in STAT3 DNA binding in T cells from lean mice, whereas no

change was observed from cells taken from the obese mice (77). This study clearly shows obesity can cause T cells to become insensitive to leptin and suggests that other cells of the immune system would be similarly affected.

An alternative explanation for the reduced early cytokine expression in obese mice is a reduction in number and/or maturation of macrophages in the lungs during infection. Work by Krishnan et al. demonstrated that obese humans have similar numbers of circulating monocytes, but the number of monocytes that matured into macrophages was almost three times less in these individuals (171). Moreover, the ability of a mature macrophage to elicit an anti-microbial and cytotoxic response may be inhibited, as Cousin et al. showed an impairment in these functions in thioglycollate elicited macrophages from *ob/ob* mice (202). Because macrophages are a major contributor to pro-inflammatory cytokine production, fewer macrophages in the lungs, as well as a decrease in their functional capacity, could explain the reduction in cytokine levels. It is of note, however, that infiltration of monocytes may also be reduced in the obese mice as they expressed significantly lower levels of MCP-1 at d 3 p.i.

Despite the early lack of induction of inflammatory genes in obese mice, expression of IL-6 and TNFα did increase by d6 p.i. to levels comparable to what lean mice expressed at d 3 p.i. This late response may be due to increased numbers of infected lung cells and infiltration of cytokine producing T cells into the lung. Importantly, the late rise in inflammatory gene expression was not balanced by a concomitant rise in IL-10 expression, indicating that the lungs of obese mice were in a heightened pro-inflammatory state. Indeed,

lung pathology tended to be more severe in obese mice, and it is likely that the upsurge in cellular infiltrate was due to this high level of inflammatory gene expression. Furthermore, because T cells contribute significantly to lung pathology and peak T cell responses do not occur until 8-10 d after the initial influenza infection (203), it is possible that lung pathology continued to escalate after d 6 p.i. and resulted in the death of the obese mice.

In addition to dysregulation of pro-inflammatory mediators, obesity resulted in the impairment of other important components of the early immune response. The induction of the antiviral cytokine response, induced primarily by viral factors, was severely blunted in obese mice despite equivalent lung viral titers. The lack of IFNα/β expression in obese mice indicates that obesity interferes with induction of mRNA expression of these cytokines. This may be due to impaired janus kinases (JAK)/STAT signaling caused by an elevation in suppressor of cytokine signaling (SOCS) proteins in obese mice. SOCS proteins are activated by cytokines signaling through JAK/STAT pathways and function as negative regulators of the pathways. However in obese animals, these suppressor proteins are persistently elevated (204, 205). Specifically, SOCS1 and 3 are amplified in obese animals and these proteins inhibit signaling by type I interferons (206, 207). Since IFNα/β upregulate their own expression (181), it is possible the lack of induction of these cytokines is due to impediment of the signal.

Because IFNα/β are required for NK cell proliferation and cytotoxicity (127), a deficiency in NK activity may have resulted as a consequence of reduced expression of these cytokines. NK cells are among the first cells to respond to viral infection and together with

type I interferons (INFα/β), provide an innate defense against infection (208). In response to intranasal infection with influenza A virus, C57BL/6 NK activity peaks within 3-4 d in the spleen, lung, and draining lymph nodes (209-211). In the current study, obese mice had over a 50% reduction in lung and splenic NK cell activity at d 3 p.i. The reduction of NK activity in the obese mice is in agreement with previous studies showing that NK cell function is decreased in rats fed a high fat diet (212, 213). This reduction in activity may be caused by a decrease in cellular arachidonic acid (AA), as splenic lymphocytes from rats fed diets high in saturated fat, show reduced levels of AA (212). AA plays an important role in the cytolytic activity of NK cells, likely through the production of leukotriene B4 (214, 215). Thus, the reduced cytotoxicity of NK cells in obese mice may be a result of a change in splenocyte fatty acid composition, leading to an impaired immune response.

Additionally, since obese mice had a lower proportion of NK cells in their lungs, the reduction in lung cytoxicity may also be the result of impaired NK cell recruitment to the site of infection. Previous studies have shown that MCP-1 and RANTES are important in NK cell recruitment (216), therefore the reduced expression of these cytokines in the lungs of obese mice may be responsible for the decreased proportion of NK cells.

The importance of the current findings is underscored by the fact that millions worldwide are affected by influenza infection every year and the universal prevalence of obesity has reached epidemic proportions. In this study, we found that obesity led to dysregulated innate immune responses to influenza infection and increased mortality. Because the innate immune response also activates and polarizes the appropriate cell

mediated response, these data indicate that overall immune function may be affected by obesity. Furthermore, these data suggest that in addition to influenza infection, obesity may increase susceptibility to other viral infections by way of immune system dysregulation.

Figure 2.1. Blood glucose and serum insulin and leptin levels in influenza infected obese and lean mice. Following an 8h fast (2400 to 0800), serum insulin **(A)** and blood glucose **(B)** was measured at 0 (uninfected), 3, and 7 day p.i. **(C)** Serum leptin was measured in fed mice at days 0 (uninfected), 3, and 6 p.i. Values are expressed as mean ± SEM (n=6-8). *, p< 0.05 vs. lean, #p< 0.05.

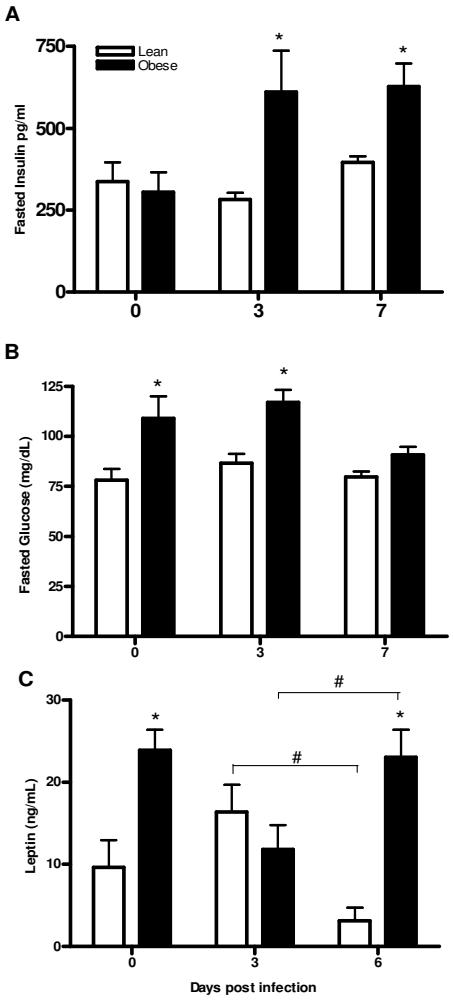


Figure 2.2. Effect of influenza virus infection on mortality, viral titers, and lung pathology. **(A)** Percent survival was calculated based on the mice remaining in the experiment at day 8 p.i., as mice were sacrificed at day 0, 3, and 6 p.i. for the experiment. **(B)** To measure viral titers, lungs were removed from mice at day 0 (uninfected), 3, and 6 post-infection and homogenized. Supernatants were serially diluted and added to MDCK cells and incubated for 72 h. Human O red blood cells were added to each well and viral titer was expressed as the reciprocal of the lowest dilution at which the RBCs agglutinated. This value was then normalized to total protein in the sample and is expressed as the mean normalized titer ± SEM (n = 6-8). **(C)** Lung pathology was scored following blinded examination of hemotoxylin-eosin stained sections of paraffin embedded lungs at day 0, 3, and 6 p.i. Pathology was scored semi-quantitatively on a scale ranging from 0-4. Values are shown as mean ± SEM (n = 6-8). *, $p<0.05$ vs. lean, #, $p<0.05$ vs. baseline.

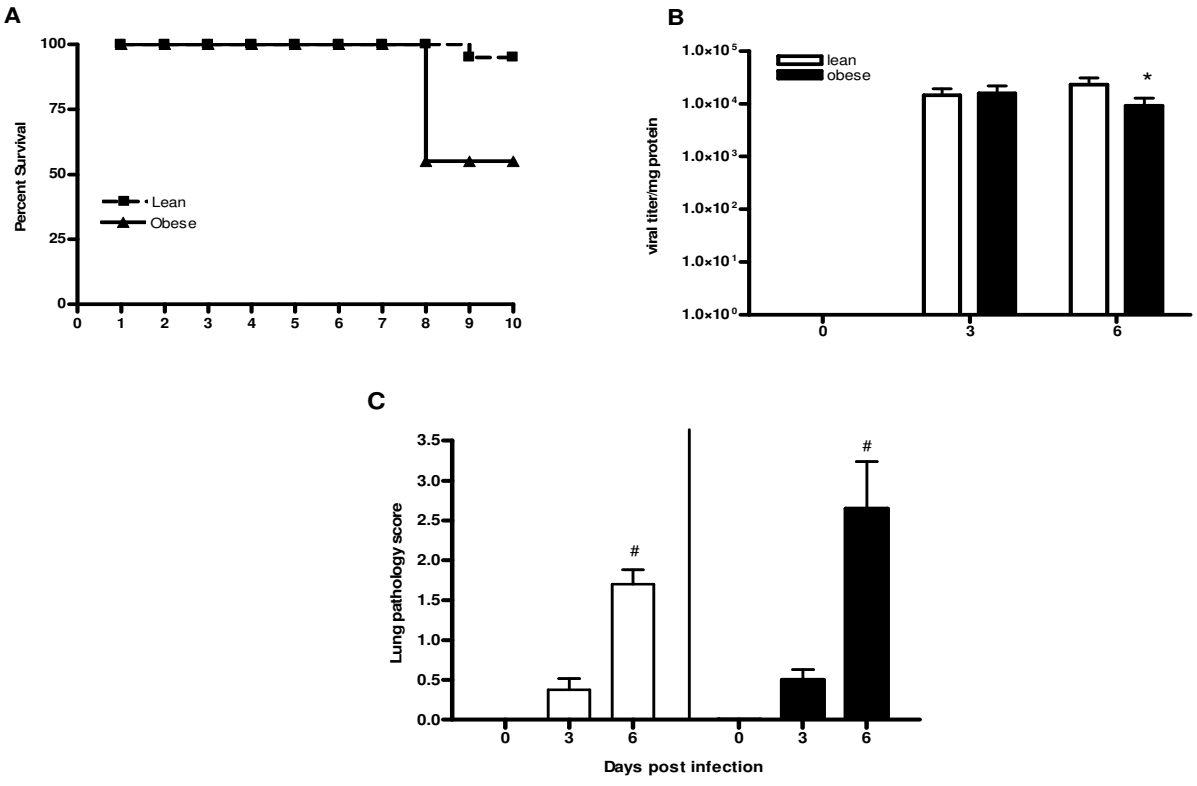


Figure 2.3. Lung mRNA expression of antiviral cytokines. The mRNA expression of antiviral cytokines IFNα and IFNβ was quantified by real-time PCR using total RNA extracted from lungs of 6-8 mice per group for days 0, 3, and 6 p.i. Values are normalized to GAPDH and are expressed as mean fold increase ± SEM over lean, uninfected controls. *, $p<0.05$ vs lean mice

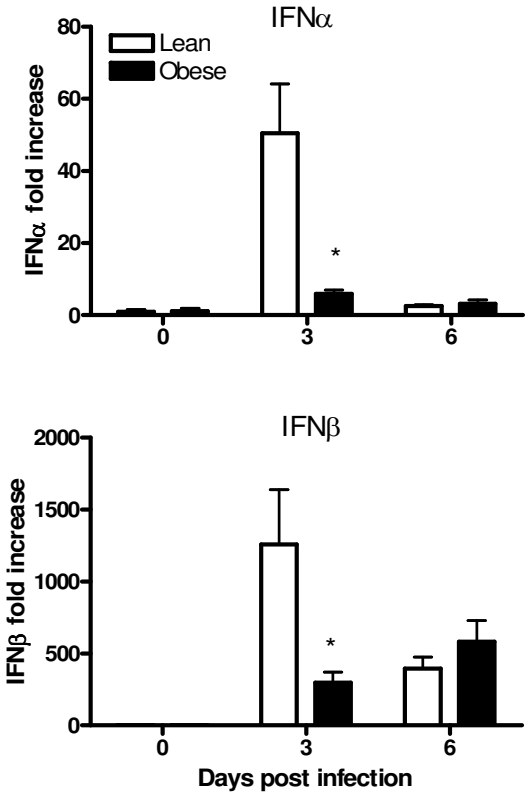


Figure 2.4. NK cell cytotoxicity and lung mRNA expression. **(A and C)** At day 3 p.i. isolated spleen and lung cells were analyzed in a standard NK cell cytotoxicity assay, using ^{51}Cr-labeled YAC-1 tumor cells as targets. The resulting effector to target (E:T) ratios were 100:1, 50:1, and 25:1. Data are shown as mean ± SEM (n=6-8). *, P≤ 0.01 vs. lean mice, **, P≤0.00001 vs lean mice. **(B and D)** To enumerate NK cells and determine their frequency in the lymphocyte population, cells were stained with FITC-labeled anti-DX5 and PE-labeled anti-CD3 and analyzed by flow cytometry. The lymphocyte population was gated and NK cells were identified as $CD3^{-}DX5^{+}$. Data are shown as mean ± SEM (n=6-8). **(E and F)** IL-12 (p40) and IL-18 mRNA expression was determined by real time PCR from total lung RNA. Lungs were removed from 6-8 mice at day 0, 3, and 6 p.i. mRNA was normalized to GAPDH and values are expressed as mean fold increase over uninfected, lean controls ± SEM. #P<0.05 vs lean mice.

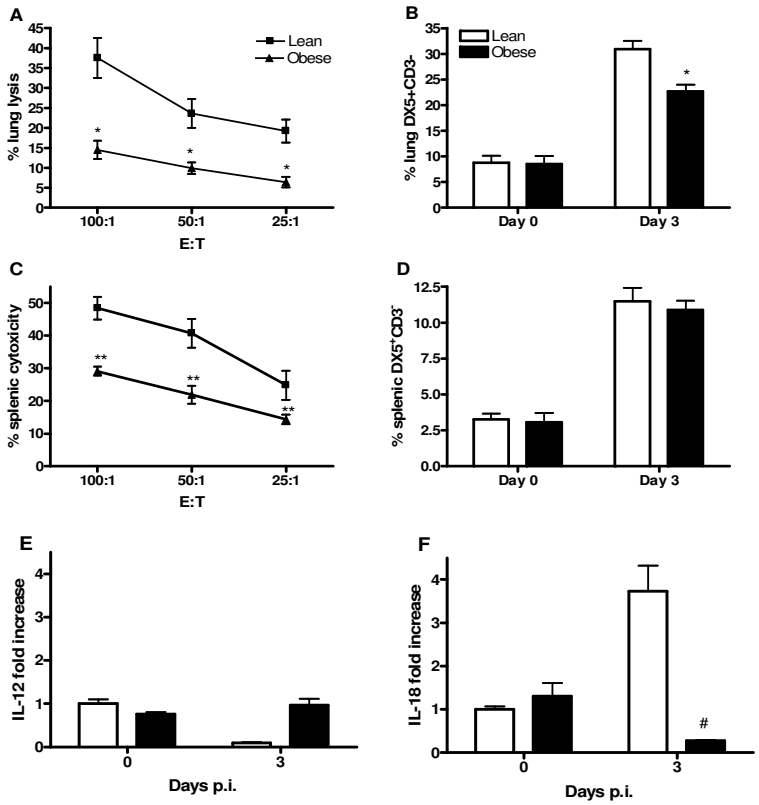


Figure 2.5. Pro-inflammatory cytokine expression in influenza infected lungs from lean and obese mice. Lung mRNA expression of cytokines IL-6, TNFα, IL-1β, and IL-10 were quantified by real-time PCR using total RNA extracted from lungs at days 0, 3, and 6 p.i. Values are normalized to GAPDH and are expressed as mean fold increase ± SEM over lean, uninfected controls (n=6-8). *, $p<0.05$ vs lean mice.

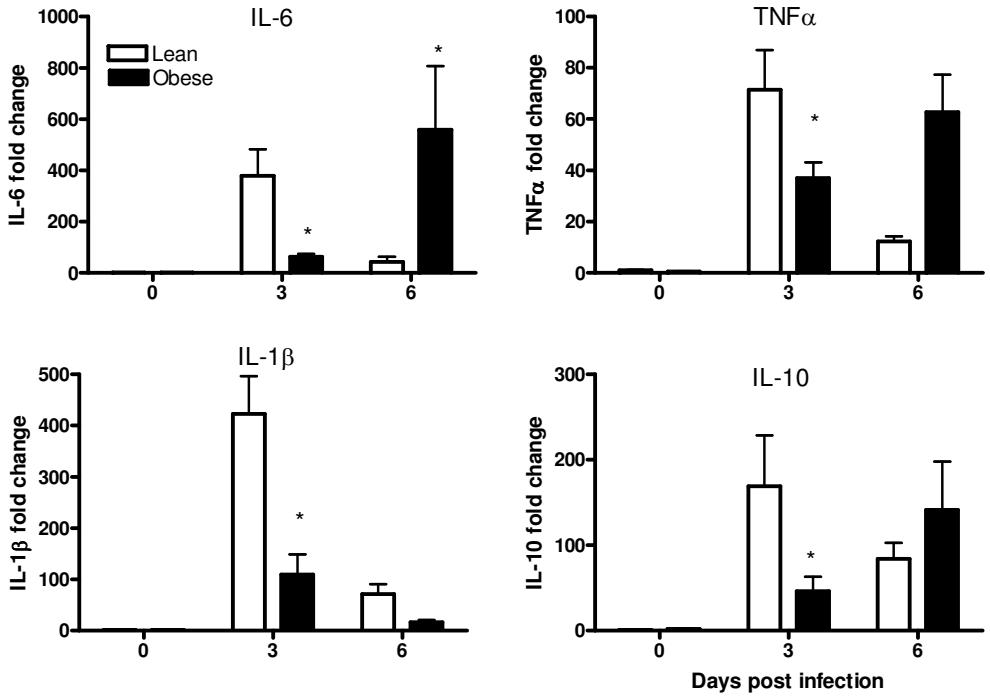
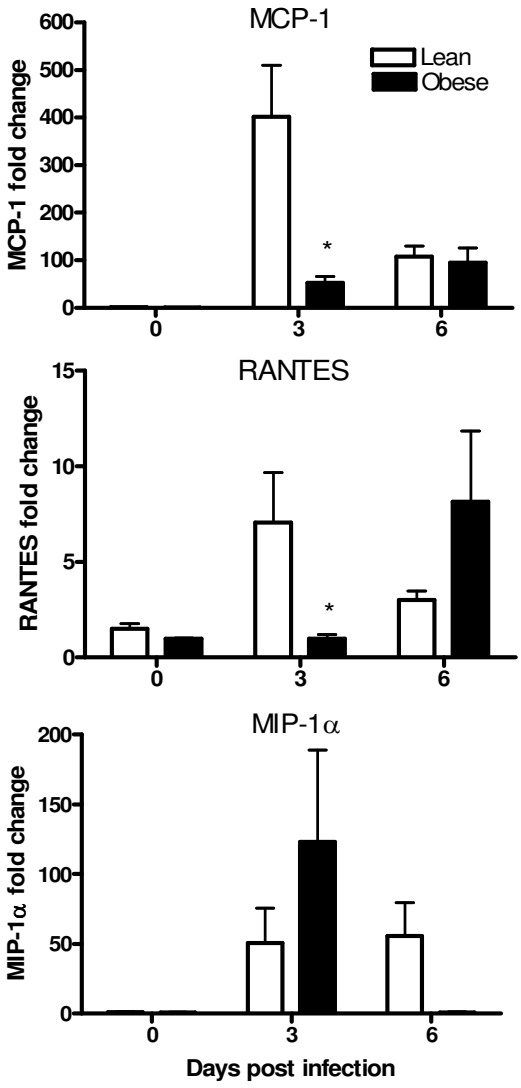


Figure 2.6. Lung mRNA expression of chemokines in influenza infected lean and obese C57BL/6 mice. The mRNA expressions of chemokines MCP-1, RANTES, and MIP-1α were quantified by real-time PCR using total RNA extracted from lungs of 6-8 mice per group for days 0, 3, and 6 p.i. Values are normalized to GAPDH and are expressed as mean fold increase ± SEM over lean, uninfected controls. *, $p<0.05$ vs lean mice.



CHAPTER III

SELECTIVE IMPAIRMENT IN DENDRITIC CELL FUNCTION AND ALTERED ANTIGEN-SPECIFIC $CD8^+$ T CELL RESPONSES IN DIET-INDUCED OBESE MICE

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A. ABSTRACT

There is a clear link between obesity and metabolic disorders, however little is known about obesity's effect on immune function, particularly during an infection. We have previously reported that diet-induced obese mice are more susceptible to morbidity and mortality during influenza infection than lean mice. Obese mice displayed aberrant innate immune responses characterized by minimal induction of IFNα/β, delayed expression of pro-inflammatory cytokines and chemokines, and impaired natural cell cytotoxicity. To explore the cause of this, we analyzed the cell populations in the lungs of lean and diet-induced obese mice. We found delayed mononuclear cell entry with a noted decrease in dendritic cells (DC) throughout the infection. Given the critical role of the DC in activating the cell-mediated immune response, we analyzed the functional capacity of DC from obese mice. We found that while obesity does not interfere with antigen uptake and migration, it does impair DC antigen presentation. This is likely due to an altered cytokine milieu, as IL-2, IL-12, and IL-6

were differentially regulated in the obese mice. Overall, this did not impact the total number of virus-specific $CD8^+$ T cells that were elicited, but did affect the number and frequency of $CD3^+$ and $CD8^+$ T cells in the lung. Thus, obesity interferes with cellular responses during influenza infection, leading to alterations in the T cell population that ultimately may be detrimental to the host.

B. INTRODUCTION

Obesity is clearly associated with metabolic disturbances, such as insulin resistance and non-alcoholic fatty liver disease (217, 218). What is less well studied is the impact of obesity on immune function. In humans, obesity has been shown to be a risk factor for infections, poor wound healing (23, 25), and decreased response to vaccination (219, 220). Additionally, obese individual have changes in the circulating T cell population, displaying an increased frequency of $CD4^+$, but reduced frequency of $CD8^+$ T cells (176) and diminished bacterial killing capacity of polymorphonuclear cells (39). Studies in genetically obese mice demonstrate decreased resistance to infections (38, 221) as well as reduced cell mediated cytotoxicity (46, 202). Diet-induced obese mice (DIO) have shown impaired antigen presentation (53), as well as altered mitogen-stimulated proliferation of splenocytes (51), and diminished NK cell function (49).

During infection with influenza virus, dendritic cells (DC) and macrophages are the initiators and modulators of the immune response (125). DC, in particular, are efficient at endocytosing viral antigen and processing it into peptide fragments which are then presented on major histocompatibility complexes (MHC). Presentation of antigen by the DC to T cells

occurs following migration of the DC to the draining lymph node (LN) through which T cells are circulating (125). This function is performed primarily by conventional DC (cDC), whereas plasmacytoid DC (pDC) have limited antigen presenting capacity, but secrete high levels of IFNα in the inflamed tissue (222, 223). Both DC subtypes reside in peripheral tissues in an immature state and mature after exposure to inflammatory stimuli, but high expression of B220 on pDC allows them to be differentiated from cDC.

In our previous study (224) we demonstrated that diet-induced obese mice infected with influenza virus had increased morbidity and mortality. The immune response in the obese mice was characterized by minimal expression of Type I inteferons (IFN) and delayed expression of pro-inflammatory cytokines and chemokines. These results suggested that obese mice have improper recruitment of mononuclear cells to the influenza-infected lungs and therefore activation of ensuing responses may be impaired.

In this paper we demonstrate that recruitment of mononuclear cells to the lung during influenza infection is delayed in DIO mice, and the number of DC remains significantly lower throughout infection. Despite this, DC were capable of taking up antigen and migrating to the LN, but their ability to present antigen to T cells is impaired. As a result, the total number of CD8 T cells is reduced in obese mice, but they are capable of eliciting a quantitatively normal virus-specific CD8 T cell response.

C. MATERIALS AND METHODS

Animals

Weanling C57BL/6J mice were obtained from Jackson Laboratories. All mice were housed in the University of North Carolina Animal Facility, which is fully accredited by the American Association for Accreditation of Laboratory Animal Care. Animals were maintained under protocols approved by the Institutional Animal Use and Care Committee. Mice were randomized to receive either a low fat/no sucrose diet or a high fat/high sucrose diet for 22 wk. Mice were housed four per cage with free access to food and water.

Diets

The diets, which have been previously described (185), were obtained from Research Diets, Inc.

Virus and Infection

The mouse adapted strain of Influenza A/Puerto Rico/8/34 (A/PR8) (American Type Culture Collection) was propagated in the allantoic fluid of fertilized chicken eggs and the viral titer was determined by standard plaque assay on MDCK cells. After 22 wk on the diets, mice were anesthetized with an intramuscular injection of a ketamine (0.6mg/kg)/xylazine (0.35mg/kg) solution and infected intranasally with 0.05 mL of 50 plaque forming units (pfu) of A/PR8 virus diluted in PBS.

Lung cell isolation and staining for flow cytometry

Lungs were removed, incubated in collagenase solution, then processed into single cell suspensions by mechanical agitation of a Stomacher and strained through a 40μm nylon filter. Cells were subjected to RBC lysis using ACK lysis buffer for 5 min at room

temperature, washed, and counted. At least 5 x 10^5 cells were stained with fluorescein (FITC)- anti-CD11b, allophycocyanin (APC)-anti-CD11c, phycoerythrin (PE) Cy7- anti-B220 (eBiosciences) and peridinin-chlorophyll (PerCp)-anti-CD8 and analyzed by FACSCaliber. Intracellular staining was performed on total lung cells from influenza infected mice that were incubated for 4-6 h with Brefaldin A (BD Biosciences). Cells were then Fc blocked with anti-FcγII/III, surface stained, and permeabilized for IL-6 staining. PE-goat-anti-mouse-IgG (Sigma) was used as a staining control, and non-specific staining was subtracted from the values presented. Antibodies were obtained from BD Biosciences unless otherwise noted. Gates were set for alveolar macrophage (AM), DC, and monocytes based on previous reports (225, 226). cDC and pDC were identified from the gated DC population as $CD11c^+B220^-$ and $CD11c^+B220^+$, respectively.

FITC uptake and DC migration to lymph node

Anesthetized mice were given 250 μg FITC-ovalbumin (OVA) (Molecular Probes) intranasally in 50 μl PBS. Control mice were given unconjugated ovalbumin which served as gating control for FITC-OVA+ cells. Mediastinal lymph nodes (LN) were harvested 24 h later and processed into single cell suspensions as described above. Prior to filtering and RBC lysis, cells were resuspended in calcium and magnesium free HBSS containing 10mM EDTA and incubated at room temperature with agitation for 5 min. 1 x 10^5 cells were stained in FACS-EDTA (227) buffer using the antibodies already described and analyzed by FACSCalibur. DC were identified by CD11c expression.

Quantitation of LN mRNA cytokine levels

Total RNA was isolated from the LN at various d p.i. using the TRIzol method (Invitrogen). Reverse transcription was carried out using Superscript II First Strand Synthesis kit (Invitrogen) using oligo (dT) primers. Levels of mRNA levels for murine IL-2, IL-12 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were determined using qRT-PCR. Fluorescent reporters were detected using Bio-Rad iCycler PCR machine and primers and probes were designed using Primer Express 1.5 (Applied Biosystems). The levels of mRNA for GAPDH were determined for all samples and were used to normalize gene expression. All data are expressed as fold induction over lean, controls at d 3 p.i.

Antigen presentation assay

Spleens from uninfected mice were removed and processed into single cell suspensions by mechanical agitation as described above and pooled (n = 4-5 mice/group). To isolate as many DC as possible, cells suspensions were treated with EDTA in a similar manner that was described in the FITC-OVA experiment. Cells were then counted and 1 x 10^8 cells were used to isolate DC using the DC enrichment kit from Dynal. The purity of the CD11c+ population was assessed by flow cytometry and found to be 76% pure. About 20% of the population was composed of CD11c-B220+ cells, likely B cells (228), which have been shown to be poor stimulators of T cells when antigen is not continuously present in the stimulation culture (229).

T cells were isolated from spleens of lean mice that had been infected with influenza 7 d prior. Single cell suspensions were incubated on plastic for 1 h and nonadherent cells

were collected. This population was shown to be greater than 92% pure by FACS analysis of CD3+ cells.

DC were incubated at an MOI of 2 with 56^{o}C heat-inactivated influenza A/PR8 for 2 h followed by extensive washing to remove any excess virus. Serial dilutions of 0.1 mL DC, starting at 1 x 10^6 cells/mL, were plated with 1 x 10^5 T cells in a 96-well round-bottom microplate. The resulting DC to T cell ratios were 1:1, 1:2, and 1:4. All samples were prepared in triplicate and incubated for 2 h at 37^{o}C, followed by the addition of Golgi Plug (BD Biosciences) and incubation for an additional 4 h. Prior to surface staining, Fc receptors were blocked with anti-FcγII/III. Cells were then stained with FITC-anti-CD3, APC-anti-CD5, PerCp-anti-CD8 (BD Biosciences), followed by fixation and permeabilization for subsequent intracellular staining with PE-anti-IFNγ. PE-goat-anti-mouse-IgG (Sigma) was used as a staining control.

Tetramer staining for antigen-specific T cells analysis

Lungs were removed and processed as described above into single cell suspensions. 1 x 10^6 cells were used for staining. Cells were Fc blocked, then stained with the D^bNP_{366}-PE (ASNENMETM) tetramer, FITC-anti-CD3, APC-anti-CD4, PerCp-anti-CD8 for 45 min at 4^{o}C and analyzed by flow cytometry. Uninfected mice were used as a staining control and the percent nonspecific binding was subtracted from values obtained from infected mice.

ELISPOT (Enzyme-Linked Immunosorbent Spot)

Lungs were lavaged three times with PBS 7 d after infection to obtain bronchoaveolar lavage fluid (BALF). RBC were lysed, cells were counted, and Lyt-2+ $CD8^+$ T cells were isolated by positive selection using magnetic beads (Dynal Biotech), according to the manufacturers directions. Ninety-six–well filtration plates were coated with capture antimouse IFN-γ mAb, washed, and blocked according to the manufacturer's protocol (BD Biosciences). Mitomycin C treated splenocytes, infected with Influenza A/PR8, were used as antigen presenting cells and 1×10^5 cells were added to wells in duplicate. Isolated lung CD8+ cells were added to each well at a concentration of 1×10^4. Plates were incubated for 12 hours at 37°C in 5% CO_2. Splenocytes alone served as negative controls. After washing with PBS/0.1% Tween 20, a biotinylated IFN-γ mAb was added, followed by streptavidin-alkaline phosphatase. The developed spots were then counted manually.

Statistics

Statistical analyses were performed using JMP Statistical Software (SAS Insititute). All results are expressed as mean ± SEM. Statistical significance was calculated using Tukey-Kramer HSD or Wilcoxon signed ranks test, $\alpha = 0.05$ and $P < 0.05$, respectively.

D. RESULTS

Reduced cellularity in influenza infected lungs of obese mice.

Previous work from our lab indicated that innate immune responses were dyregulated during influenza infection in diet-induced obese mice. We observed minimal Type I IFN expression as well as a delay in IL-6 production in obese mice. To determine the reason for

this impairment, we infected lean and obese mice and analyzed the cellularity of the lung at d 0, 3, and 6 p.i. Lung populations were identified by their CD11b and CD11c expression, as previously described (225, 230) (Figure 3.1). As expected the total number of cells in the lungs of lean mice increased during infection due to mononuclear cell infiltrate (Table 3.1). However, the progressive infiltration of mononuclear cells was not observed in obese mice until d 6 p.i. While the number of monocytes and DC double by day 3 p.i. and continue to increase through d 6 in lean mice, there is little change in these cell populations in obese mice until d 6 p.i., yet the number of DC remains significantly lower. Additionally, the population of cells not expressing CD11c or CD11b, which likely includes naïve T cells (231) and epithelial cells (232)was similar between then groups at d 0 and 3, but was significantly lower in obese mice at d 6 p.i. These data indicate that the temporal infiltration of monocytes and lymphocytes during influenza infection is affected by obesity, and therefore may result in impairment of succeeding responses.

Enhanced IL-6 production by mononuclear cells of obese mice.

Given the difference in lung cellularity, we determined if the delay in IL-6 production previously observed was due to the reduction in cell number or to an inability of the mononuclear cells to produce IL-6. As shown in Figure 3.2A, the total number of IL-6^+ cells was significantly reduced at day 3 p.i. in obese mice. Interestingly, the reason for this reduction appears to be solely due to the decrease in cell number. When the leukocyte populations were analyzed for IL-6 production, AM and monocytes from lungs of obese mice were found to have a higher proportion of IL-6^+ cells (Figure 3.2B). Furthermore, the amount IL-6 produced by the AM and monocytes from obese mice was also greater (AM: lean MFI =

50.9 ± 1.7 vs obese MFI = 58.9± 1.2, monocyte: lean MFI = 40 ± 0.9 vs obese MFI = 44.2 ± 0.8, $P < 0.05$) By day 6, cells from both lean and obese mice produce high levels of IL-6 (AM: lean MFI = 106 ± 24.8 vs obese MFI = 68 ± 6.7, monocyte: lean MFI = 196 ± 45 vs obese MFI = 182 ± 45) and the total number of IL-6^+ cells was equivalent (Figure 2A). These data indicate that while mononuclear cells are slower to enter the lungs of obese mice following infection, resident cells may be hyper-activated early after infection in obese mice.

Obese mice have less lung migrating DC during infection

DC are a critical link between the innate and cell-mediated immune responses. There are several distinct subsets of DC including cDC and plasmacytoid pDC. Belz et. al. have demonstrated there are other major subsets within the cDC population which include the $CD8^-B220^-CD11c^+$ cells (DN). These cells appear to play a major role in antigen uptake in the lungs and subsequent migration to the LN. Within the LN, migrating DN cells can present antigen to T cells, as well as transfer antigen to LN resident DCs ($CD8^+B220^-CD11c^+$) for additional presentation (223). Since the total number of DCs remained significantly lower in obese mice throughout infection, we hypothesized that the number of DN DCs would also be lower, thus indicating that obese mice may have reduced antigen trafficking to the LN. Therefore we determined the number of DN cells in the lungs of lean and obese mice prior to and during infection. Prior to infection we found no difference in the number of resident DN DCs between lean and obese mice (Figure 3.3A). By day 3 p.i. however, the total number of DN DCs was less in obese mice and this was not simply due to a reduction in total DC cell number as the percent of DN cells in the DC population was also significantly lower in obese mice at d 3 p.i. (Figure 3.3B). This suggests that infiltration of

new DN DCs does not occur in obese mice early during infection which could lead to reduced antigen accumulation in the LN. In contrast to the DN DC population, the percent of pDC ($CD11c^+$ $B220^+$) during infection was greater in obese mice compared to lean mice (Figure 3.3B). Despite the amplified increase in the percent pDCs, obese mice had significantly less total pDCs in their lungs at day 3 p.i. than lean mice, though the total number remained low in both groups (Figure 3.3C).

DC accumulate in the LNs of obese mice following OVA instillation

To determine if DC transport antigen normally in obese mice we instilled FITC-OVA intranasally and measured accumulation of $FITC^+$ in $CD11c^+$ cells in LN after 24h. Surprisingly, we found that the total number of $CD11c^+$ cells was higher in LN from obese mice (Figure 3.4A). With the increase in the total number of DCs we expected to see more antigen in the LNs of obese mice. However, we found that obese mice had an equivalent number of $FITC^+$ DCs as lean mice (Figure 3.4A). Accordingly, the percent of the DC population that contained $FITC^+$ was less in obese mice (Figure 3.4B).

Since the proportion of lung DN DC decreased following infection in obese mice (Figure 3.2B), it was possible that more DN cells had phagocytosed antigen and migrated to the LN soon after infection. We determined if this was the case after FITC-OVA instillation. When we analyzed the population of DC in the LN we found more DN DC in obese mice (Figure 3.4C), though the proportion that made up the DC population was similar between the groups (data not shown). In addition, the number of DN DC that contained FITC was greater in obese mice. In both groups a significant proportion of the DN DC were $FITC^-$

(Figure 3.4D). These data indicate that DC in obese mice are fully capable of migrating to the LN following antigen uptake.

Impaired antigen presentation by DC from obese mice

The capacity of the DC to stimulate the appropriate antigen-specific T cell responses relies, not only on its ability to migrate to the LN following antigen uptake, but to present antigen in conjunction with the production of polarizing cytokines such as IL-12 (233). We observed decreased IL-12 expression in LN from obese during influenza infection (Figure 3.5A). The initial onset of T cell proliferation during influenza A/PR8 infection begins around d 3 p.i. (234) and thus the reduction of IL-12 expression at d 3 p.i. in the LN suggested that DC stimulation of CD8+ T cells in obese mice was impaired, despite efficient migration to the LN.

To address whether DC from obese mice were able to stimulate T cells , we negatively selected $CD11c^+$ cells from spleens of uninfected lean and obese mice and stimulated T cells derived from spleens of influenza-primed lean mice. Infection of DC with heat-inactivated influenza has been shown to effectively induce DC maturation and subsequent stimulation of T cell proliferation, since the virus retains fusogenic capacity but is nonreplicative (235). IFNγ production by $CD8^+$ T cells was used to determine the effectiveness of the DC to present antigen. As shown in Figure 3.5B, influenza pulsed DC from lean mice effectively present antigen to $CD8^+$ T cells and elicit an IFNγ response. However, there is a 30% reduction in the proportion of $CD8^+$ T cells that produce IFNγ from obese mice when DC are present at a 1:1 or 1:2 ratio to T cells. Representative flow

cytometry gates for the 1:2 DC:T cell ratio are shown in Figure 3.5C. While we cannot rule out an inability of the DC to phagocytose the virus as a reason for this difference, the FITC-OVA data suggests that antigen uptake by DCs occurs normally and therefore is likely not a factor in the reduced ability of the DC to stimulate $CD8^+$ T cell IFNγ production.

Obese mice have altered antigen specific CD8 T cells responses during infection

The reduced effectiveness of DC from obese mice to stimulate CD8 T cells, suggested that DC MHC Class I presentation was impaired and also suggested that obese mice may have altered T cell responses. To address this issue, we analyzed T cells in the lung at day 7 p.i. to determine the magnitude of the antigen specific response in influenza infected obese mice. Additionally we evaluated MHC Class I expression on the DC during infection. Virus-specific $CD8^+$ T cells were identified by D^bNP_{366}-PE tetramer binding, a peptide derived from the viral nucleoprotein which has been shown to be an epitope recognized by a high percentage of CD8+ T cells (236). Though we found no difference in DC MHC Class I expression between lean and obese mice (data not shown), we did find differences in their antigen specific $CD8^+$ T cell response. Surprisingly, we found the frequency of NP_{366}-specific $CD8^+$ T cells was significantly higher in obese mice compared to lean (Figure 3.6A). In agreement with previous studies (237), about 14% of $CD8^+$ T cells from lean mice were NP_{366}-specific, whereas in obese mice, 26% of $CD8^+$ T cells were influenza-specific. This increase was also seen when the proportion of antigen-specific $CD8^+$ T cells from BALF was determined by IFNγ ELISPOT (Figure 3.6B). In this assay,

splenocytes were pulsed with influenza and therefore present a variety of viral peptides. We found a 40% increase in the frequency of influenza-specific $CD8^+$ T cells in obese mice. The similarity of the response between the two assays indicates that obese mice have a general increase in the percentage of antigen-specific $CD8^+$ T cells. Additionally, the ELISPOT indicates that antiviral responses are functional in the effector CD8+ T cells.

Despite a higher proportion of antigen-specific $CD8^+$ T cells, obese mice did not have more of these cells in their lungs. When the cell population in the lung was enumerated at d 7 p.i., we found that obese mice had significantly less $CD8^+$ T cells than lean mice and the total T cell population was also less in this group (Figure 3.6C). A similar trend was seen in the BALF, though the differences were not significant (Figure 3.6D). Given the fewer number of $CD8^+$ T cells in obese mice, the total number of $CD8^+$ T cells specific for antigen is comparable between the groups (3.6E and F).

The decrease in the number of $CD8^+$ T cells may be related to a decrease in IL-2 production since IL-2 supports $CD8^+$ T cell proliferation (238). Indeed we found that at day 3 and 7 p.i., expression of IL-2 in the LN of obese mice was significantly diminished (Figure 3.6G).

E. DISCUSSION

In this work, we demonstrate that obesity results in impairment in DC recruitment and function during influenza infection. Our previous studies showed that influenza infection in obese mice resulted in delayed pro-inflammatory responses and inhibited Type I IFN and

chemokine expression (224). This suggested that infiltration of cells into the lungs during infection may be altered in obese mice. Indeed, we saw a delay in the recruitment of monocytes and DC in the lungs of obese mice, with a sustained decrease in DC numbers throughout infection.

Two major groups of DC exist, pDC and cDC and these can be further subdivided based on the expression of cell surface makers (239). pDC express B220 and are major producers of IFNα/β (240). We found that the percent of pDC in the DC population of obese mice increases during infection and makes up a significantly larger percent of the population than in lean mice, but there are still less total pDC in the lungs of obese mice. Therefore a reduction in the number of pDC during infection may explain the diminished expression of IFNα we previously observed in influenza infected obese mice (224). Given the low number of pDC, however, it is also likely that the low level of IFNα/β expression during influenza infection in obese mice is due to impairment in production by respiratory epithelial cells (121).

In contrast, the proportion of the cDC DN subset decreased during infection in obese mice. cDC have high expression of CD11c and are very efficient at stimulating T cell responses following migration to the LN. Studies from Carbone et al, have demonstrated that cDC with the DN phenotype bring antigen from the lungs to the LN where it can be transferred to LN-resident DC or be presented by the DN DC themselves (223, 241, 242). The decrease in the number of DN cells in the lungs of obese mice could have been due to

these cells already migrating out to the LN with no new DC coming in, whereas lean mice maintain a homeostatic level in the lung.

Indeed, we did observe a lack of recruitment of cDC to the lungs of obese mice. This may be due to the reduction in CCL2 (MCP-1) and CCL5 (RANTES) expression we have previously reported at d 3p.i. infection in obese mice (224). Immature DC respond to these ligands through interactions with receptors on the cell surface which promotes chemotaxis of the immature cell to the inflamed tissue (243). The lack of chemokine induction may also explain the reduced recruitment of monocytes to the lung. Additionally, given that obese mice have less TNFα expression at day 3 p.i. (224) differentiation of DC from monocyte precursors could also be impaired (160) (244).

It is unclear what effect the lack of increase in lung DC by d 3 p.i. has on the trafficking of antigen to the LN. Legge et al, showed that accelerated migration to the LN during influenza infection only occurs within the first 24-48 h p.i. (234, 245). However, new DC continue to enter the LN, but whether these cells are lung derived and play a role in T cell responses is unknown (245, 246). When we assessed the migratory function of lung-derived DC, we found that obesity resulted in comparable antigen trafficking to the LN 24 h after intranasal FITC-OVA instillation indicating that accelerated migration following initial antigenic insult is intact in obese mice. These results are similar to another study that used *ob/ob* mice and demonstrated normal migratory capacity of Langherans cells out of the epidermis in response to TNFα induced migration (247). Although migration of DC from the lung was similar between lean and obese mice, obese mice had an augmented number of

FITC$^-$ DC in their lymph nodes. This suggests that obese mice may have an increased number of DC in their LN at baseline. Indeed, studies from Verwaerde et al. and Macia et al. indicate that this increase is due to greater baseline cellularity. Verwaerde et al. demonstrated that the number of splenic DC was greater in diet-induced obese mice prior to ovalbumin immunization, however this difference was abolished 5 d following antigenic stimulation as both groups had increased the number of DC to a comparable level (53). Similarly, Macia et al, found higher numbers of Langerhans cells in the epidermis of *ob/ob* mice compared to lean mice (247). Whether these resident DC affect the type of response that ensues remains to be determined.

The inability of DC from obese mice to properly stimulate T cells may be due to altered cytokine production. This was shown in *ob/ob* mice where the altered cytokine milieu resulted in less potent stimulation of allogeneic T cells by bone marrow derived DC, despite similar expression of co-stimulatory molecules. (247). In our study, the dampened expression of IL-12 in obese mice may mediate the reduction in T cell responses given that IL-12 acts directly on CD8+ T cells to augment cytolytic activity and IFNγ production (125, 126). Likewise, the overproduction of IL-6 in the AM and monocyte population of obese mice could impair DC presentation. Exaggerated IL-6 production can inhibit monocyte differentiation into DCs and prevent their maturation (248, 249). Previous reports indicate that IL-6 can switch DC progenitor commitment away from a DC toward a phenotype that can phagocytose but cannot present antigen (249). Additionally, IL-6 blocks MHC Class II, CD80, and CD86 expression on DC, but does not affect MHC Class I (248). Therefore the impairment in antigen presentation, without a reduction in MHC Class I expression we find

in DC from obese mice, could be due to the fact their macrophage are producing more IL-6 than lean mice.

In spite of a reduced ability of the DC from obese mice to stimulate $CD8^+$ T cells from lean mice, influenza-specific $CD8^+$ T cell responses *in vivo* remained relatively intact in obese mice. Due to the essentiality of an effective immune response, the immune system has evolved to dynamically eradicate infection when components of the response are deficient (131, 154, 237, 250). In this study we found that while influenza infected obese mice had less $CD8^+$ T cells infiltrating the lung, the frequency that were virus-specific was higher which resulted in a total number of antigen-specific cells that was comparable to lean mice. The CD8 response elicited by obese mice suggests that more of the CD8 precursor pool is being utilized to clear virus (130). Although this is effective for clearing a primary influenza infection, it may result in a compromised memory response (130, 237).

The decrease in total CD8 T cells in obese mice could be due to reduced trafficking to the lungs or to reduced proliferation. IL-2 from $CD4^+$ T cells plays a significant role in $CD8^+$ T cell proliferation (238) and its expression inversely correlated with SOCS3 (suppressor of cytokine signaing protein 3) expression (251). SOCS proteins are a family of cytokine-inducible negative feedback inhibtors that target cytokine receptors and cytoplasmic signaling adaptor molecules (252). In diet-induced obesity, SOCS3 upregulation has been suggested as a mediator of the impaired STAT3 DNA binding that occurs in T cells from these mice (77). Therefore it is possible that the reduction in $CD8^+$ T cell number we observed in obese mice was due SOCS3 inhibited proliferation via prevention of IL-2

expression. Another possibility is that if antigen is not efficiently presented then it would not be surprising to have less T cell activation and proliferation (234).

In conclusion, we demonstrated that diet-induced obesity results in selective impairment of DC functions. Our data indicate that during influenza infection, obesity leads to delayed recruitment of mononuclear cells to the infected lung. DC, in particular, are noticeably low throughout infection. While migration of antigen-loaded DC to the LN is normal in obese mice, the ability of the DC to present antigen to CD8 T cells is impaired. This may be due to lack of co-stimulation by the DC. The outcome of these alterations was an elevated frequency of virus-specific $CD8^+$ T cell response in obese mice. Further studies are needed to elucidate the exact mechanism behind these changes in obese mice and determine if these alterations result in defective memory responses.

Figure 3.1. Gating strategy to identify cell populations in lungs. CD11b and CD11c expression was used to identify alveolar macrophage (R1), dendritic cells (R2), monocytes/interstitial macrophage (R3).

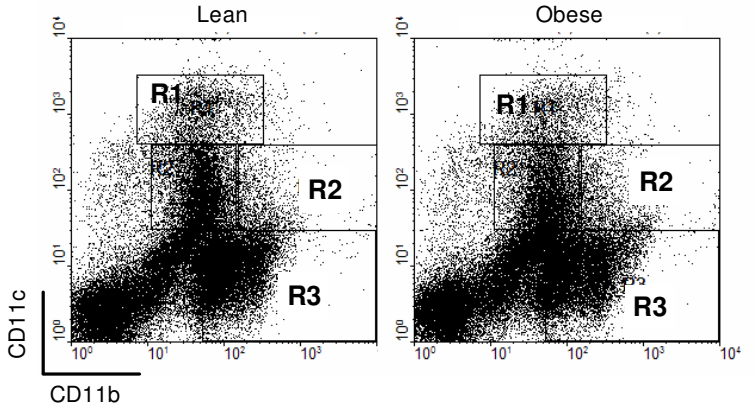


Table 3.1. Total number of cells in the lung during influenza infection.

Cell populations from total lung were identified by CD11b and CD11c expression at various time points p.i. Data are expressed as mean (SEM) of 3-6 animals per group per time point. *Indicates significantly different from lean mice, $P \leq 0.05$.

Cell Type	Phenotype	Day 0 Cell # x 10^5 (SEM x 10^5)	Day 3 Cell # x 10^5 (SEM x 10^5)	Day 6 Cell # x 10^5 (SEM x 10^5)
Total cells	Lean	101 (13.0)	118 (11.9)	294 (35.0)
	Obese	74.5 (11.6)	*67.2 (8.6)	188 (32.8)
Alveolar Mac	Lean	6.4 (0.69)	5.9 (0.49)	13.2 (2.3)
	Obese	*3.1 (0.96)	*4.4 (0.31)	7.9 (1.6)
Monocyte/Interstitial Macrophage	Lean	18.8 (3.1)	44.7 (5.2)	80.8 (14.9)
	Obese	17.8 (2.4)	*18.9 (3.0)	59.4 (14.1)
Dendritic Cell	Lean	15.6 (2.2)	25.8 (1.7)	90.0 (12.3)
	Obese	16.0 (2.4)	*11.6 (1.2)	*55.6 (9.3)
CD11b-/CD11c-	Lean	62.0 (7.2)	41.0 (4.0)	101 (13.6)
	Obese	54.1 (9.7	31.4 (3.6)	*59.1 (10.2)

Figure 3.2. IL-6^+ cells in lung during infection. Single cell suspensions from influenza infected lungs were surface stained with anti-CD11b and anti-CD11c following a 4-6 h incubation with Brefaldin A. (A). Total number of IL-6^+ cells in lung. (B) The percent IL-6 production at day 3 p.i. was determined for AM and monocyte populations using gating strategy described earlier (see Figure 3.1). Data are expressed as mean SEM ± of 5-6 animals per group per time point. *Indicates significantly different from lean mice, $P \leq 0.05$.

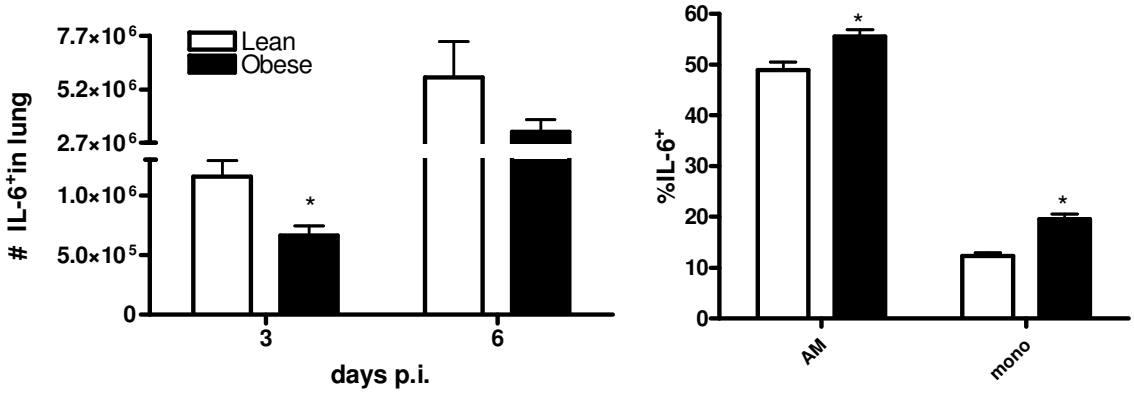


Figure 3.3. Population of DN and pDC in total DC population during influenza virus infection. In the lung DC were identified by flow CD11c CD11b expression (A) DN cells were identified as CD8-B220- at d 0 and 3 p.i. (B) Gating strategy to identify the proportion of DN DC and pDC. Values listed represent the mean percentage in the DC population. The percent pDC includes all B220+ cells in the DC gate. (C) Based on the percent of pDC found in (B), the total number of pDCs in the lung was calculated. Data are expressed as mean (SEM) of 3-6 animals per group per timepoint. *Indicates significantly different from lean mice, $P < 0.05$.

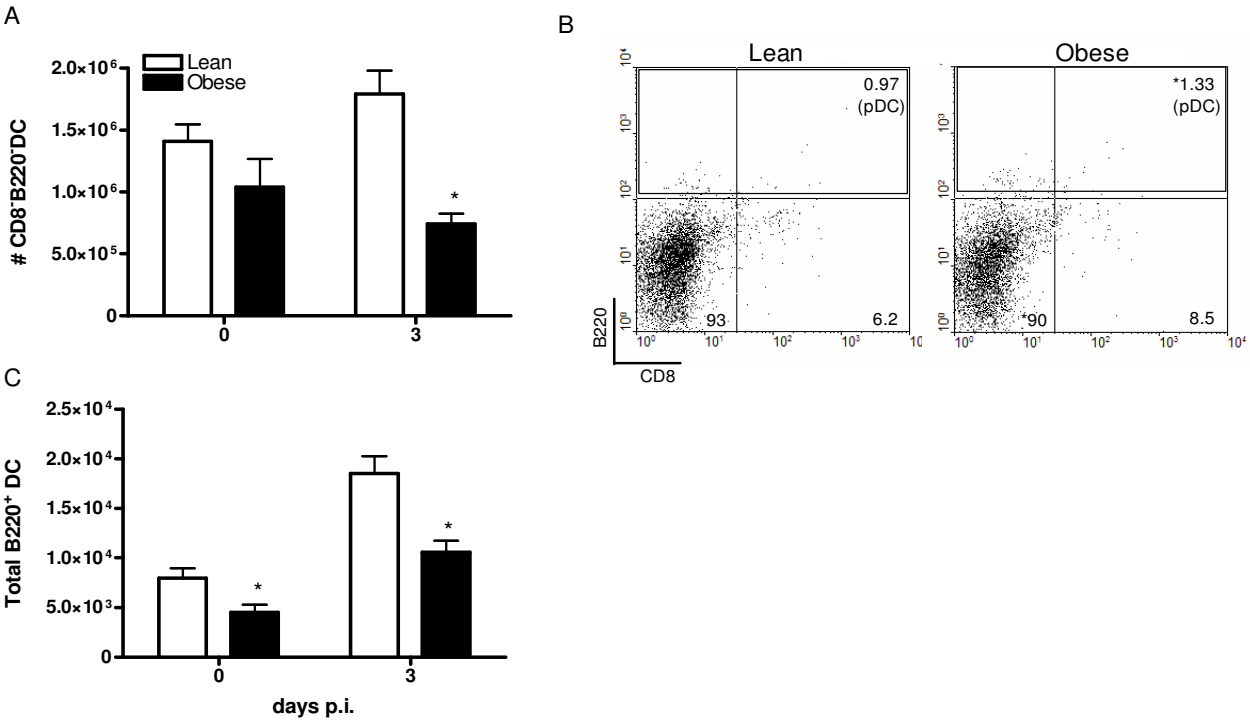


Figure 3.4. Migration of lung DC to LN. DC in the LN were identified by their expression of CD11c. (A) The total number of FITC+ DC was identified 24 h after intranasal FITC-OVA instillation. (B) Numbers indicate percent of FITC+ cells in the DC population. (C) DN DCs were identified by the lack of CD8 and B220 from CD11c+ cell population. The total number of FITC+ DN DCs was determined by analyzing the percent of DN DCs that were FITC+ in the LN 24 h after FITC-OVA treatment (D). Data in bar graphs are expressed as mean ± SEM of 3 experiments with 3-5 animals per group per experiment. *Indicates significantly different from lean mice, $P \leq 0.05$

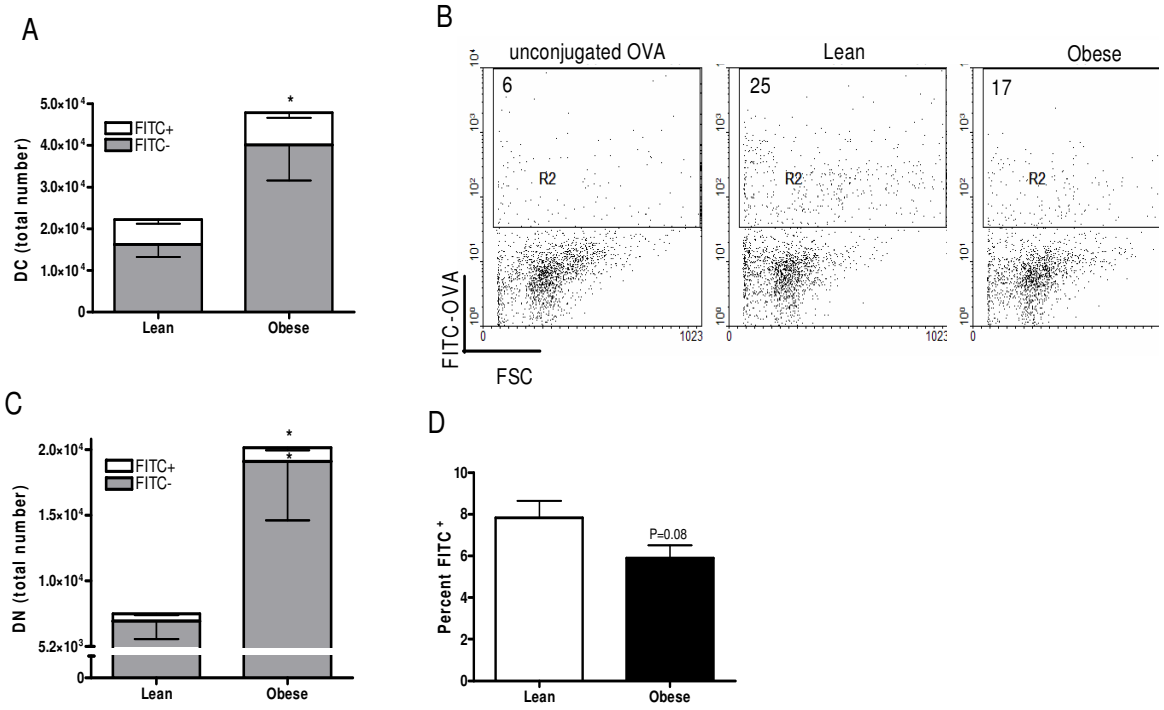


Figure 3.5. Stimulation of T cells with viral antigen presented by DC. (A) LN mRNA expression of IL-12 was quantified by real-time PCR using total RNA extracted from lungs of 6-8 mice per group. Values are normalized to GAPDH and are expressed as mean fold increase ± SEM over lean controls at d 3 p.i. (B) An antigen presentation assay was performed using T cells from influenza infected chow-fed mice at d 7 p.i. mixed with varying numbers of influenza-loaded DCs from uninfected lean and obese mice. The percent of CD8+ cells in the CD3+ lymphocyte population that produced IFNγ after 6 hours of stimulation was determined by ICS. Data are representative of two separate experiments (n= 4-5 mice/group). (C) Gating strategy for determining the percent of CD8+ T cells with IFNγ. Data are expressed as mean ± SEM, *, significantly different from lean mice, $P \leq 0.05$

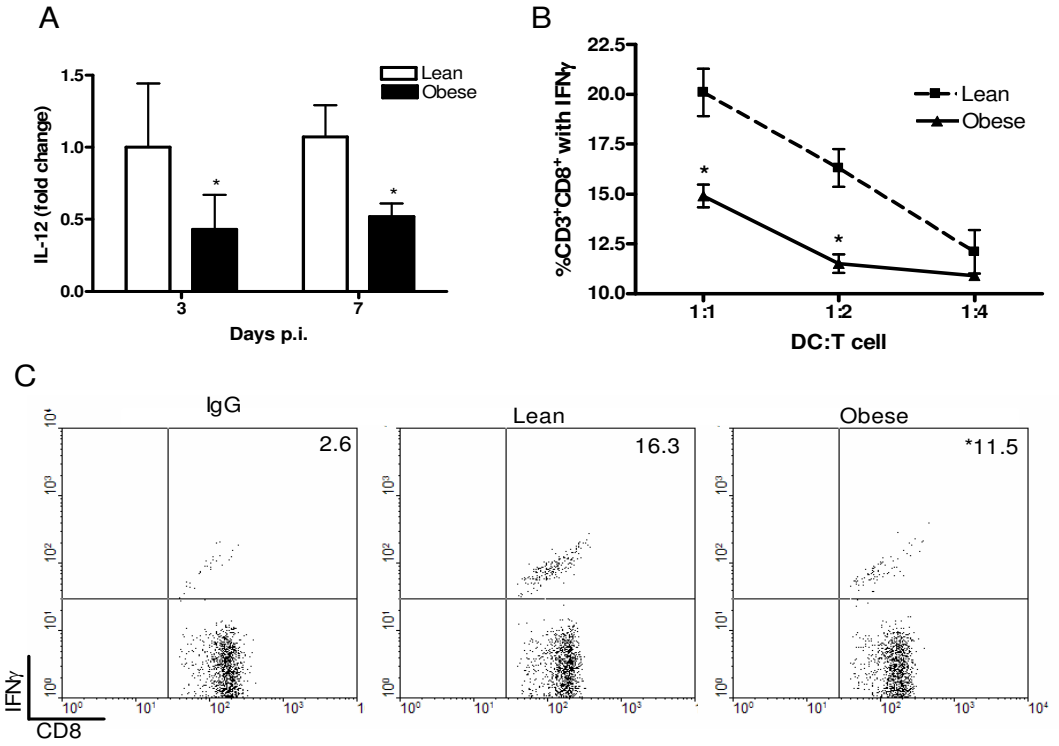
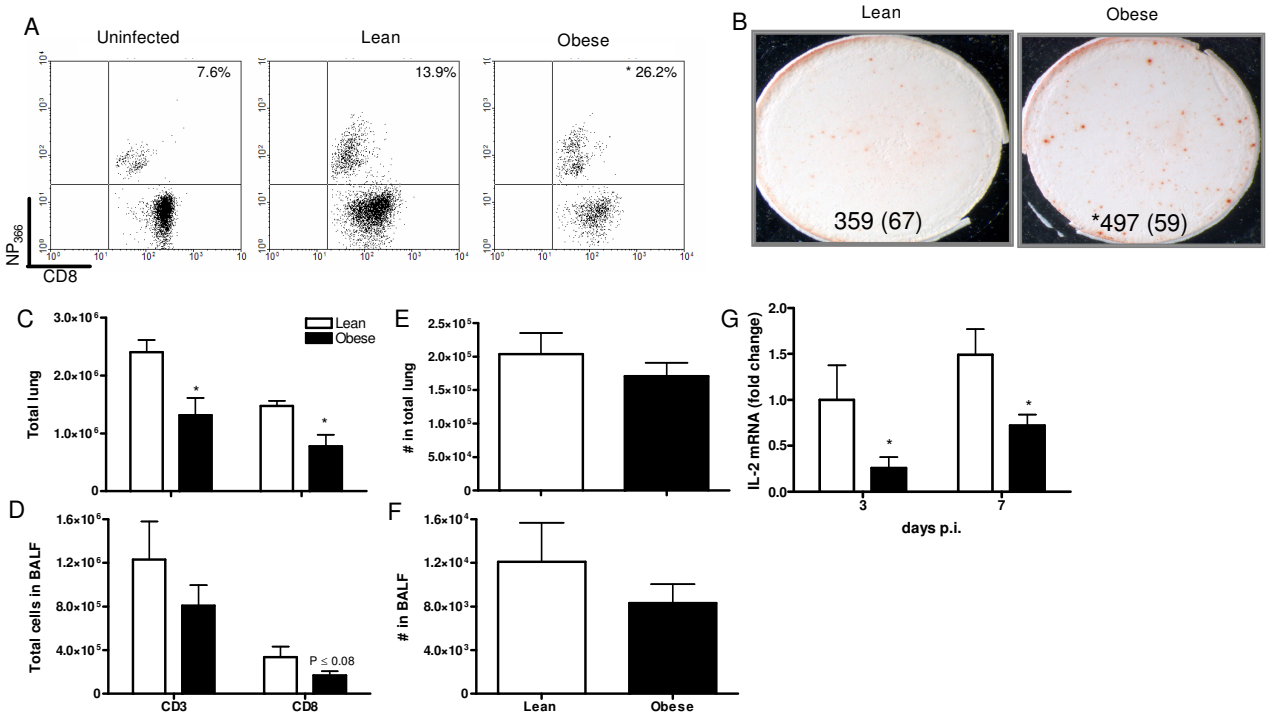


Figure 3.6. Increased frequency of antigen specific CD8+ T cells in obese mice. (A) The percent of NP_{366}^{+} in the CD8+ cell population in the total lung was determined by flow cytometry at d 7 p.i. The values presented for lean and obese account for the non-speicfic staining shown in the first panel (B) An IFNγ ELISPOT was performed with 10^4 CD8+ T cells obtained from the BALF by negative isolation. Influenza pulsed splenocytes were used as APCs, values are presented as mean (SEM). Flow cytometry was used to enumerate total CD3 and CD8 in the lung, (C) and BALF (D). The total number of antigen specific CD8+ T cells in the lung (E) and BALF (F) was calculated by multiplying the percent of antigen specific CD8+ cells determined by flow cytometry and ELISPOT, respectively, by the total number of CD8+ T cells in the CD3 population. Data are expressed as mean ± SEM, *, significantly different from lean mice, $P \leq 0.05$



CHAPTER IV
CENTRAL LEPTIN RECEPTOR SIGNALING RESCUES DEFECT IN NATURAL KILLER CELL CYTOXICITY IN *db/db* MICE DURING INFLUENZA INFECTION.

A. ABSTRACT

The incidence and prevalence of obesity worldwide are increasing at an alarming rate. Obesity increases the risk of metabolic disease and has been shown to increase susceptibility to infection. Adipokines, such as leptin, have been identified as a direct link between adipose tissue and immune responses. We have previously observed dysregulated innate and cell-mediated immune responses in obese mice during influenza infection, including reduced T cell numbers and impaired NK cell cytotoxicity. To investigate the mechanism that underlies these phenomena, we analyzed splenocyte signaling and function in obese mice. We observed a lack of STAT3 activation in obese mice during infection as well as reduced PHA-induced splenocyte proliferation. Since leptin signals through STAT3 and effects both T cell proliferation and NK cell cytotoxicity, we determined a role for leptin signaling in the aberrant immune responses. Using a transgenic mouse model, we show that central leptin signaling is sufficient to rescue NK cell function in the *db/db* mouse that lacks functional leptin receptor. Thus our data suggest that impaired central leptin signaling may mediate some of the changes in immune function that occur in obese mice infected with influenza virus

B. INTRODUCTION

The incidence and prevalence of obesity worldwide are increasing at an alarming rate. Obesity increases the risk of metabolic disease and has been shown to increase susceptibility to infection (23-25). While it has been clear for some time that a lack of adipose tissue, such as in starvation, leads to immune dysfunction, high obesity rates have generated interest in modulation of immune function by excess adipose tissue (6). In recent years a link between adipose tissue and the immune system has been identified, though the exact mechanisms by which they communicate are still largely unknown (7, 253-255).

Various molecules secreted from adipose tissue, collectively known as adipokines, have been identified as potential modulators of immune function (3, 96). Leptin and adiponectin are two adipokines that are abundantly produced by adipocytes. Adiponectin activates peroxisome proliferators activated receptor-α (PPARα) and AMPK which increase glucose uptake and fatty acid oxidation (4). Adiponectin also has anti-inflammatory properties and the low levels that occur in obesity and insulin resistance are associated with increased levels of C-reactive protein and IL-6 (256, 257). Conversely, leptin circulates in proportion to fat mass and acts centrally and peripherally to regulate food intake, hematopoiesis, reproduction, glucose homeostasis, and immune responses (65). Thus mice that lack leptin, or its receptor, have dysregulation of these functions.

Leptin's effects are mediated through its interaction with receptors found on almost all cell types including neuronal cells (36), NK cells (258), dendritic cells (98), macrophage (97), and T cells (6). Upon binding to its receptor, leptin stimulates a signaling cascade that

begins with the phosphorylation of tyrosine (pY) residues on the receptor's intracellular domain by a noncovalently bound Janus kinase (JAK) (259) (66). The phosphorylated receptor recruits a tyrosine phosphorylated-signal transducers and activators of transcription (STAT pY) to the receptor complex. STAT3, 5, and 6 mediate the transcriptional effects of leptin upon entry into the nucleus (82). Negative regulation of leptin signaling occurs through the recruitment of suppressor of cytokine signaling 3 (SOCS3), which is recruited to the pY on the receptor and prevents JAK2 phosphorylation (88). Recent evidence suggests that obesity leads to upregulation of SOCS3 and causes attenuation of STAT3 DNA binding in T cells (77) and liver (93). Therefore, if leptin signaling in immune cells is inhibited, immune responses may be dysregulated.

The effect of leptin on immune function is generally thought to be pro-inflammatory, therefore, it is not surprising that leptin increases during infection (6, 63). Leptin's role in inflammation was demonstrated in models of inflammatory diseases. In experimentally induced colitis, mice that lack leptin are protected from the intestinal inflammation and have less pro-inflammatory cytokine secretion. However, administration of leptin increases cytokine production in these mice to that of controls (100). Similarly, humans that are congenitally deficient in leptin, have reduced numbers of circulating $CD4^+$ T cells and impaired T cell proliferation and cytokine release, all of which were reversed by leptin administration (260). Moreover, leptin promotes NK cell development and cytotoxicity as demonstrated by impaired NK cell function in *db/db* mice, which lack the leptin receptor (102). Whether the correction in these responses is due to direct action of leptin on immune cells or indirectly through neuronal signals is unknown.

Our lab has previously shown diminished and delayed inflammatory responses, impaired NK cell cytotoxicity, as well as reduced T cell proliferation in obese mice infected with influenza. In this paper we assess the role of leptin in these changes.

C. MATERIALS AND METHODS

Animals

Male weanling C57BL/6J mice were obtained from Jackson Laboratories (Bar Harbor, MA). All mice were housed in the University of North Carolina Animal Facility, which is fully accredited by the American Association for Accreditation of Laboratory Animal Care. Animals were maintained under protocols approved by the Institutional Animal Use and Care Committee. Mice were randomized to receive either a low fat/no sucrose diet (LF) or a high fat/high sucrose (HF/HS) diet for 20 weeks. Mice were housed four per cage with free access to food and water.

Diets

The diets were obtained from Research Diets, Inc. (New Brunswick, NJ). The LF diet (D12328) consisted of 16.4% protein, 73.1% carbohydrate (83% starch, 17% maltodextrin), 10.5% fat (38% soybean, 62% coconut oil). The HF/HS diet (D12331) consisted of 16.4% protein, 25.5% carbohydrate (51% sucrose, 49% maltodextrin), 58% fat (93% coconut oil, 7% soybean oil).

Virus and Infection

Mouse adapted Influenza A/Puerto Rico/8/34 (A/PR8) (American Type Culture Collection) was propagated in the allantoic fluid of fertilized chicken eggs and the viral titer was determined by hemagglutination assay (48). After 20 weeks on the diets, mice were anesthetized with an intramuscular injection of a ketamine (0.022mg)/xylazine (0.0156mg) solution and infected intranasally with 0.05 mL of 50 plaque forming units (pfu) of influenza A/PR8 virus diluted in PBS.

Cell proliferation

Splenocytes were isolated from lean and obese mice (n=3/group) and pooled. 2.5 x 10^6 cells were plated in each well of a 96 well plate. Cells were stimulated for 48 h with 2.5 μg/mL phytahemagglutinin (PHA) (Sigma). During the last 4 hours of stimulation, cells were pulsed with 2 μCi [^{3}H] thymidine and proliferation was assessed by thymidine incorporation, measured by liquid scintillation counting.

Western blot

Protein extracts were prepared from splenocytes at d 0 and 7 p.i. using Pessin's Lysis Buffer. Protein extracts were separated by 10% SDS-PAGE, electroblotted to Immobilon-P membranes (Millipore, Burlington, MA) and probed with anti-STAT3 pY and anti-β-actin (Abcam, Cambridge, MA). The antigen-antibody complexes on immunoblots were treated with horseradish peroxidase–conjugated secondary antibodies (Santa Cruz Biotechnology) and visualized using autoradiography or the Gene Gnome imaging system (Syngene, Frederick, MD).

Serum glucose, corticosterone, and adipokines

Blood was collected from mice, allowed to clot at room temperature for 20 min, centrifuged at 2500 rpm for 20 min, and serum was collected and stored at -20 until analysis. Full-length adiponectin and leptin were analyzed by ELISA according to the manufacturer's instructions (R & D Systems). Blood glucose levels were measured with a Freestyle blood glucose monitor (Abbott Laboratories). Corticosterone was measured by RIA (MP Biomedical) from blood collected at 0800 by retrooribital bleed on anesthetized mice prior to infection. Following infection, mice were sacrificed and blood was collected by cardiac puncture.

Transgenic mice (Nse-Rb db/db)

Transgenic expression of the long form of leptin receptor (Ob-Rb) under the control of the neuron-specific enolase (Nse) promoter was shown to partially correct the abberent phenotype of db/db mice (36). Expression of the functional receptor was limited to the hypothalamus, the cortex, and the cerebellum, with low levels seen in the adrenals, white adipose tissue, and the testis. The mice in the published report were backcrossed three times to db/+ C57BL/6J background which were then mated to generate Nse-Rb *db/db* mice. In our study the Nse-Rb *db/db* were the FVB strain. Like C57BL/6 *db/db* mice, FVB *db/db* mice develop severe obesity, hyperglycemia, hyperinsulinemia, and hyperleptinemia.

Tables 4.1 and 4.2 (36) document the phenotype of the heterozygous transgenic mice. In addition to partial normalization of body weight, glucose, insulin, and leptin, these mice have near normal thermogenesis and reproduction, though female mice were not able to

produce a litter. In male Nse-Rb *db/db* mice POMC, AgRP, and NPY are relatively normal, while MCH was not affected.

Analysis of genomic DNA

Transgenic mice were identified using PCR analysis of DNA isolated from tail snips. Tissue was digested in overnight at 65^{o}C and DNA was isolated using the phenol chloroform method. Amplification of the trangene was performed using the following primers: Nse forward 5'-CCACCGGCTGAGTCTGCAGT-3' and rGHint reverse 5'-TTGGCGCGCCGAGAGTCTAGAAAGAGAGACA-3'. If the transgene was present a 242 bp product was produced, whereas there was no product if the trangene was absent (36). The db/db genotype was determined by PCR amplification followed by RsaI restriction digest. Primers to the leptin receptor were as follows: db forward 5'-AGAACGGACACTCTTTGAAGTCTC-3' and reverse 5'-CATTCAAACCATAGTTTAGGTTTGTGT -3'. 50µl of amplified product was digested 1U of RsaI restriction enzyme at 37^{o}C for 3 h. Digests were run on a 3% agarose gel made with 1% low melting agarose. If a 108 and 27 bp product was present the genotype was considered db/db and if a 135 bp fragment was present the genotype was wild type.

Enumeration of NK cell populations in the lung and spleen

At d 3 p.i. spleens were removed, processed into single cell suspensions by mechanical agitation of a Stomacher (Seward, UK), and strained through a 40µm nylon filter. Cells were stained with fluorescein (FITC)-labeled anti-DX5 and Phycoerythrin (PE)-labeled

anti-CD3 (BD Pharmingen, San Diego, CA) and analyzed by FACSCaliber. The lymphocyte population was gated and NK cells were identified as $CD3^{-}DX5^{+}$ within that gate.

Determination of natural killer cell cytotoxicity

Spleen cells were analyzed in a standard NK cell cytotoxicity assay (190), using ^{51}Cr-labeled YAC-1 tumor cells (ATCC) as targets. Briefly, serial dilutions of 0.1 mL lung or spleen effector cells, starting at 1 x 10^7 cells/mL, were plated with 1 x 10^4 cells ^{51}Cr labeled YAC-1 target cells in a 96-well V-bottom microplate. The resulting effector to target (E:T) ratios were 100:1, 50:1, and 25:1. All samples were prepared in triplicate. To determine maximum lysis, 0.1mL of 10% sodiumdodecylsulfate was added to labeled YAC-1 cells. To determine spontaneous release 0.1 mL of the culture media was added to radioactive YAC-1 cells. Release of ^{51}Cr content was analyzed using a gamma counter (Cobra II, Hewlett Packard) and percent specific lysis was calculated by the following equation:

$$\% \text{ specific lysis} = \frac{\text{cpm (sample)} - \text{cpm (spontaneous release)}}{\text{cpm (maximum release)} - \text{cpm (spontaneous release)}} \times 100$$

Statistical Analysis

Statistical analyses were performed using JMP Statistical Software (SAS Insititute). All results are expressed as mean ± SEM. Statistical significance was calculated using Tukey-Kramer HSD or Wilcoxon signed ranks test, $\alpha = 0.05$ and $P < 0.05$, respectively.

D. RESULTS

Adiponectin and leptin are unchanged during infection in obese mice

As, expected a high fat diet induced elevated serum leptin levels (Figure 4.1A). We found that after 1 week on a high fat diet, mice fed the HF diet have elevated leptin levels**,** which continue to increase as the time on the diet progresses. By day 0 post infection (week 20 of the diet), obese mice have serum leptin levels that are 7 times higher than lean mice. Following influenza infection, lean mice have a transient increase in leptin, whereas serum leptin in obese mice remains elevated and does not change, possibly because leptin levels are already maximized (Figure 4.1B). These data indicate leptin levels in obese mice are unresponsive to infection, and suggest that leptin's role in the stimulation of immune responses may be altered in obese mice.

Adiponectin was significantly reduced at baseline in obese mice, in agreement with previous studies (261). During infection, adiponectin decreased in lean mice, but remained low and unchanged in obese mice (Figure 4.1C).

Corticosterone levels were significantly elevated in obese mice prior to infection (Figure 4.1D). Interestingly, during infection corticosterone levels increased in lean mice, whereas obese mice have a temporal decrease in circulating levels.

Less STAT3 phosphorylation in splenocytes of obese mice during infection.

The effects of leptin are mediated through STAT3 DNA binding (65). For STAT3 to dimerize and enter the nucleus it must be phosphorylated by JAK2. Given that leptin was elevated prior to infection in obese mice, we wanted to determine if STAT3 was

differentially activated prior to and during influenza infection. Analysis of splenocytes prior to infection revealed that both lean and obese mice had low constitutive STAT3 phosphorylation (Figure 4.2). During infection though, STAT3 phosphorylation increased in lean mice, whereas levels remained unchanged in obese mice. Given that the spleen is a secondary lymphoid organ, high numbers of T cells, macrophage, NK cells, and dendritic cells can be found there during infection. Therefore these data suggests that responding immune cells from obese mice are resistant to leptin signaling.

Reduced proliferation and IFNγ production in splenocytes from obese mice.

Leptin signaling through STAT3 promotes cell proliferation and the production of IFNγ by CD4 T cells (87, 162, 262). The lack of STAT3 activation during infection in obese mice suggested these responses may be impaired. We stimulated proliferation in splenocytes from uninfected lean and obese mice with PHA, a lectin that mimics antigen-receptor signaling and induces IL-2 and IL-2R in T cells (263). As shown in Figure 3A, we found that splenocytes from obese mice had reduced basal proliferation. PHA induced a strong proliferative response in both groups, but proliferation remained almost 40% lower in obese mice (Figure 4.3A). Interestingly, the proliferative response was augmented in obese mice, as indicated by a stimulation index (SI) of 9.7 versus 6.5 in lean (Figure 4.3B).

IFNγ is produced by CD4 T cells in response to antigen presented within a specific cytokine milieu. Signaling by leptin has been shown to potentiate IFNγ production by these cells. We found that IFNγ production by PHA-stimulated splenocytes from obese mice was reduced 7-fold compared to lean mice (Figure 4.3C). These data indicate that T cells in obese

mice are capable of proliferating upon activation, but the Th1 polarizing effects of leptin may be absent.

Direct action of leptin is not necessary for normal NK cell function

The lack of STAT3 activation and minimal IFNγ production in splenocytes of obese mice, suggested that inhibition of leptin signaling may mediate some of the immune dysregulation that occurs during influenza infection in obese mice. We have previously shown that obese mice have a 50% reduction in lung and splenic NK cell cytotoxicity (see Chapter 2). Leptin is a potent activator of NK cell cytotoxicity, mediating its effects, at least in human NK cells lines, through STAT3 activation of IL-2 and perforin (258). To explore the possibility that direct inhibition of leptin signaling in NK cells resulted in their poor activation in obese mice, we used a transgenic mouse model that expresses function leptin receptor exclusively in the brain. Thus, it provides an excellent model for evaluating the effect of leptin on periphery cells without the complicating metabolic abnormalities.

As shown in Table 4.3, prior to infection, the body weight of mice expressing the transgene was significantly lower than *db/db*, but still higher than Nse-Rb *db/db* (WT) controls. At d 3 p.i. *db/db* mice were the only group that did not lose weight with infection. This lends support to the idea that leptin is involved in inflammatory-mediated anorexia (6, 201). Additionally, non-fasted blood glucose levels were significantly higher in *db/db* mice compared to both Nse-Rb *db/db* and WT mice (Table 4.3). During infection though, all groups had elevated blood glucose levels which is a normal physiologic occurrence during stress (264).

Splenocyte cellularity was similar between the groups, but the frequency of NK cells was 10% less in *db/db* mice and Nse-Rb *db/db* than WT (Table 4.3). Previous reports have shown that while total cellularity is similar in WT and *db/db* mice, the percent of NK cells in the spleen and blood are lower (68). The affect of the transgene on NK cell cytotoxicity was striking, however. As shown in Figure 4.4A, the presence of the Ob-Rb transgene in the brain fully corrected the NK cell impairment. At a 100:1 ratio of E:T cells, both the Nse-Rb *db/db* and WT mice had approximately 60% splenic NK cell cytoxicity at d 3 p.i., whereas *db/db* had half that. These data indicate that for full NK cell function, leptin receptor signaling is not necessary.

E. DISCUSSION

In this paper we have documented a potential role for obesity-induced inhibition of STAT3 signaling in dysregulated immune responses during influenza infection. We found that splenocytes from obese mice have no change in STAT3 signaling during influenza infection. This was related to a negligible change in circulating leptin levels, whereas lean mice displayed elevated STAT3 pY during infection with concomitant increases in leptin. Chronically high circulating leptin has been shown to downregulate its own signaling pathway, both by an increase in SOCS3 and downregulation of Ob-Rb (88, 91, 93). Therefore this lack of induction during infection in obese mice is likely due to foregoing inhibition of the STAT3 signaling caused by high leptin. The comparable levels of STAT3pY at baseline in lean and obese mice indicate this is the case, since obese mice have higher circulating leptin, but no elevation in STAT3 pY.

It was somewhat surprising that adiponectin decreased during infection in lean mice, given that it increases anti-inflammatory molecules like IL-10. However, it also induces apoptosis and suppression of scavenger receptors on macrophage, so lower levels during infection may be beneficial (265).

A lack of STAT3 can have numerous effects on immune responses, given it mediates the transcriptional effects of leptin, IL-6, IL-10, and IL-27 (266, 267). In T cells a lack of STAT3 leads to impairment of IL-6-induced and CD3/IL-2-mediated proliferation by preventing apoptosis and inducing IL-2R, respectively (267). The reduced proliferation we found in PHA stimulated splenocytes from obese mice may be due to lack of IL-6 signaling as the induction of T cell proliferation in response to PHA has been shown to require IL-6 produced by monocytes (268). Additionally, STAT3 is a part of the IL-27 signaling pathway which initiates Th1 responses in naïve CD4 cells, but suppresses inflammatory responses in activated CD4 T cells (266). Therefore, in splenocyte of obese mice that do not upregulate STAT3 signaling, the lack of IFNγ production could be due to hampered IL-27 induced Th1 polarization. It should be noted, however, that we have previously reported impaired IL-12 expression in LN from obese mice. IL-12 is a potent inducer of Th1 responses and is primarily produced by macrophage and DC. Thus, it is possible that the lack of IFNγ production in obese mice, is due to impaired IL-12 production by mononuclear cells.

In this study, we also tested the hypothesis that inhibiting direct leptin signaling on cells of the immune response leads to impaired function. We have previously observed significant impairment in NK cell cytoxicity in obese mice infected with influenza. Given the

fact that leptin induces and augments NK cell cytoxicity (258), we wanted to know if the effects we see during obesity could be explained by a lack of leptin signaling in NK cells. To do this, we used a mouse model in which expression of a functional leptin receptor is restricted to the specific regions of the brain, and therefore leptin signaling in NK cells is absent. This model is preferred over the *db/db* model in which a functional Ob-Rb is completely absent. In addition to the severe metabolic changes that occur in *db/db* mice, these mice also have impaired hematopoiesis, thymic atrophy (269), reduced B and T cells, and less colony forming potential in lymphoid and myeloid lineage stem cells (270). This indicates that these mice are predisposed to immune dysfunction, thus elucidating the direct role of leptin on immune cells during infection would be difficult in these mice.

Nse-Rb expression in *db/db* mice normalizes metabolic and neuropeptide defects relative to *db/db* mice (36). These mice still weigh more than WT mice due to greater adiposity, but they are normoglycemic and have near-normal levels of POMC and AgRP/NPY. Though various neuronal factors affect obesity and hyperglycemia (36), normalization of these neuropeptides suggests that these are the mediators of some of leptin's peripheral effects.

NK cell activation, accumulation, and cytoxicity involve various cytokines and signaling pathways. Their killing ability relies on IFNα/β signaling through STAT1, which also induces IL-15 a cytokine needed for their accumulation and survival (127). In studies of NK cell cytoxicity in *db/db* mice, the addition of IL-15 or PolyI:C (a ligand for TLR3) to NK cells induced their killing ability to levels similar to NK cells from WT mice but the

augmented effect of leptin addition on NK killing did not occur in the *db/db* mice (68). These experiments suggest leptin acts directly on its receptor to augment NK cell cytotoxicity, as any indirect upregulation of TLR3 or IL-15 by leptin would be expected to lead to further enhancement of cytotoxicity in *db/db* mice. Therefore it is surprising to find that Nse-Rb *db/db* mice have NK cell cytotoxicity that is similar to WT mice despite the lack of Ob-Rb signaling.

To this point, recent research suggests that the milieu of the *db/db* animal may play more of a role in immune function than direct leptin receptor signaling. When bone marrow cells from *db/db* mice were transplanted into WT mice, thymic repopulation and antigen stimulated proliferation was similar to control transplants. Moreover, when bone marrow cells from the WT mice were transplanted into *db/db* hosts, thymic atrophy and impaired repopulation of the LN occurred (269). Similarly, Unger et al, demonstrated that transplantation of adipose tissue depots from WT rats into *fa/fa* rats, followed by adenovirus-induced overexpression of leptin, induced no fat depleting effects, whereas WT to WT transfers did (271). They proposed a lack of leptin-stimulated catecholamine production or a blockade of leptin binding as mechanisms for this effect. Indeed, treatment with β-adrenergic antagonists have been shown to mimic the thymic atrophy in *db/db* mice, and administration of norepinephrine (NE) to *ob/ob* mice reverses the loss of natural killer T cells (272). Therefore, the rescue of NK cell cytoxicity that occurs in Nse-Rb *db/db* mice, may be due to normalization of central nervous system signals.

This idea in obesity is exemplified by the fact that obesity is associated with higher circulating levels of NE (17, 273). This indicates that obesity upregulates the sympathetic nervous system (SNS) which can impair immune cell function (274). Evidence for this in NK cells was shown by Gan et al, (2002). This group demonstrated that pre-incubation of lymphocytes with NE reduced NK cell killing ability, indicating that NE inhibits the programming of the NK cell for cytotoxicity.

Alernatively, the impaired NK cell function in obese (224) and *db/db* mice may be due to high circulating corticosterone levels. We observed elevated corticosterone prior to infection in obese mice, a phenotype that is also characteristic of *db/db* mice (275). Glucocorticoids, in addition to opioids, play a significant role in the suppression of NK cell recruitment and cytotoxicty and low leptin is associated with increases in corticosterone (8, 276). Whether blocking glucocorticoid action would rescue the defect in NK cell cytotoxicity in *db/db* or obese mice, independent of central leptin signaling, is unknown, but would help elucidate the mechanism by which central leptin signaling mediates its effects.

Overall, this paper demonstrates that obesity interferes with normal JAK/STAT signaling pathways in splenocyte during influenza infection, and inhibits their proliferative capacity and Th1 cytokine production. Though all of these altered responses can be attributed to reduced leptin signaling by the splenocytes themselves, we did not find evidence that direct leptin signaling is necessary for normal NK cell responses. In fact, the influence of leptin on immune function, at least in terms of the NK cell, can be attributed to its central

effects. Importantly, these results provide evidence that central leptin resistance mediates some of the obesity-induced immune dysfunction.

Table 4.1. Phenotype of Nse-Rb *db/db* transgenic mice (36)

TABLE 1
Indices of adiposity in male and female mice indicating effects of LEPR deficiency and the NSE-Rb transgene

Genotype (*n*)	Body weight (g)	BMI	Fat pad mass		Leptin (ng/ml)	Leptin/fat mass
			g	% Body weight		
Females						
db^{3J}/+ (11)	31.1 ± 1.5	0.36 ± 0.01	4.7 ± 0.8	14.5 ± 1.8	34 ± 10	5.7 ± 1.1
db^{3J}/db^{3J} (13)	73.1 ± 2.6*	0.74 ± 0.02*	22.9 ± 1.3*	31.1 ± 0.9*	248 ± 10*	11.2 ± 0.7*
NSE-Rb db^{3J}/db^{3J} (5)	44.0 ± 1.4*†	0.47 ± 0.01*†	12.0 ± 1.0*†	27.0 ± 1.5*	90 ± 11*†	6.3 ± 1.7†
Males						
db^{3J}/+ (12)	40.2 ± 1.4	0.41 ± 0.01	5.5 ± 0.3	13.6 ± 0.6	54 ± 6	9.6 ± 0.8
db^{3J}/db^{3J} (8)	74.1 ± 1.4*	0.70 ± 0.01*	18.8 ± 0.91*	25.3 ± 1.1*	222 ± 11*	12.1 ± 1.0*
NSE-Rb db^{3J}/db^{3J} (7)	48.4 ± 1.9*†	0.47 ± 0.02*†	6.9 ± 0.6†	14.2 ± 0.8†	77 ± 9†	11 ± 0.6

Data are means ± SE. Mice were 24–27 weeks old. *$P < 0.05$ vs. lean mice (db^{3J}/+) of the same sex; †$P < 0.05$ vs. obese mice (db^{3J}/db^{3J}) of the same sex.

Table 4.2. Serum glucose, insulin, and leptin in Nse-Rb *db/db* mice (36)

TABLE 2
Response to a fast in two circulating hormones, leptin and insulin

	$db^{3J}/+$, +/+	db^{3J}/db^{3J}	NSE-Rb db^{3J}/db^{3J}
Males			
n	4	5	4
Body weight (g)	33.9 ± 1.5	57 ± 1.6*	40.7 ± 1.7*†
Leptin			
Fed (ng/ml)	21.4 ± 3.5	111.0 ± 15.9*	44.1 ± 8.9*†
24-h Fasted (ng/ml)	10.8 ± 3.3‡	135.0 ± 19.9	43.6 ± 8.6
Glucose homeostasis			
Glucose			
Fed (mg/dl)	142 ± 3	378 ± 38*	131 ± 8
24-h Fasted (mg/dl)	93 ± 4‡	97 ± 9‡	94 ± 6‡
Insulin			
Fed (ng/ml)	2.9 ± 0.9	15.6 ± 3.8*	5.2 ± 1.8†
24-h Fasted (ng/ml)	0.2 ± 0.1‡	3.4 ± 0.4	1.1 ± 0.1
Females			
n	3	3	3
Body weight (g)	25.6 ± 0.2	62.2 ± 0.7*	36.5 ± 0.8*†
Leptin			
Fed (ng/ml)	17.2 ± 2.0	169.0 ± 10.2*	52.4 ± 3.6*†
24-h Fasted (ng/ml)	13.1 ± 2.6‡	218.0 ± 30.2*	52.4 ± 3.9*†
Glucose homeostasis			
Glucose			
Fed (mg/dl)	118 ± 5	314 ± 16	109 ± 5
24-h Fasted (mg/dl)	56 ± 4‡	83 ± 16‡	58 ± 5‡
Insulin			
Fed (ng/ml)	0.6 ± 0.1	19.1 ± 0.3*	2.3 ± 0.4*†
24-h Fasted (ng/ml)	0.3 ± 0.1	10.1 ± 3.9	2.3 ± 1.2

Data are means ± SE. Mice were 15–16 weeks old. *$P < 0.05$ vs. lean ($db^{3J}/+$, +/+) mice; †$P < 0.05$ vs. db^{3J}/db^{3J} mice; ‡$P < 0.05$ vs. fed mice of same genotype.

Figure 4.1. Leptin levels are chronically elevated in obese mice and do not change with infection. Serum leptin (A) was measured every other week during high fat feeding and during infection; d 0 corresponds to week 20 of the diet. Serum adiponectin (B) and corticosterone (C) were measured during infection by ELISA and RIA, respectively. Values are expressed as mean ± SEM (n=6-8). *, $P < 0.05$ vs. lean, groups not sharing a letter are significantly different, $P < 0.05$.

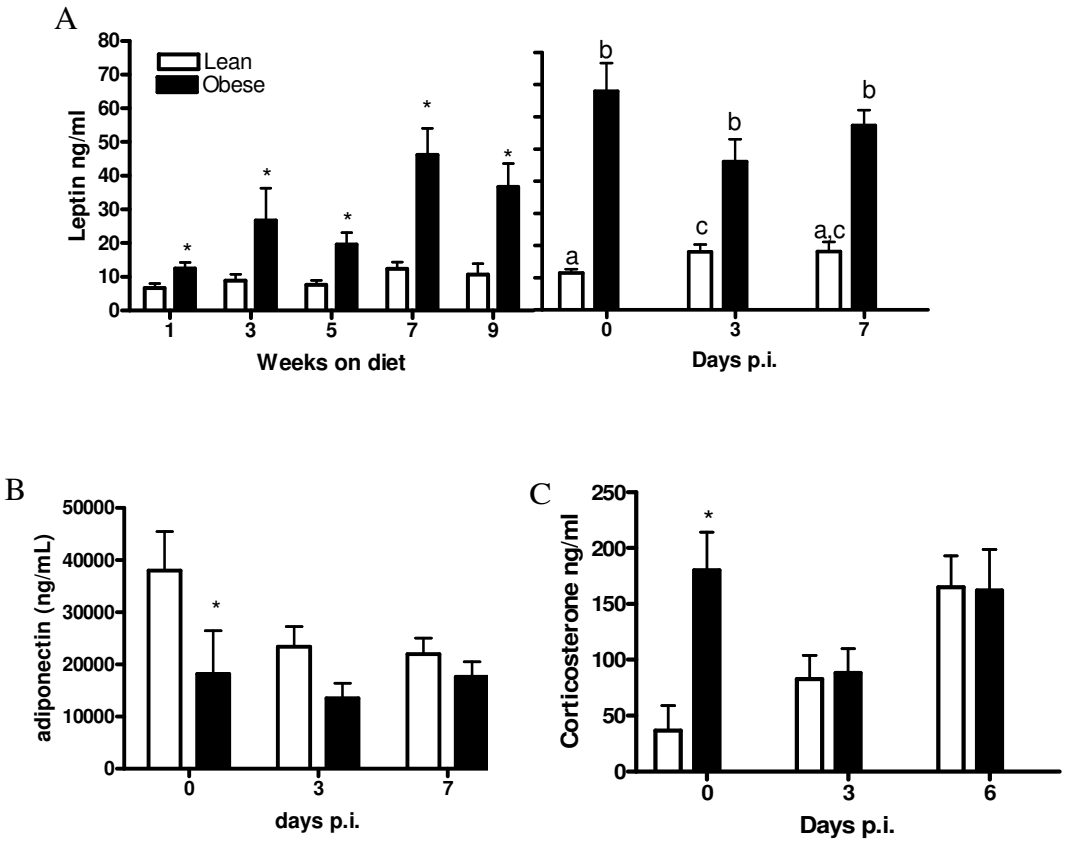
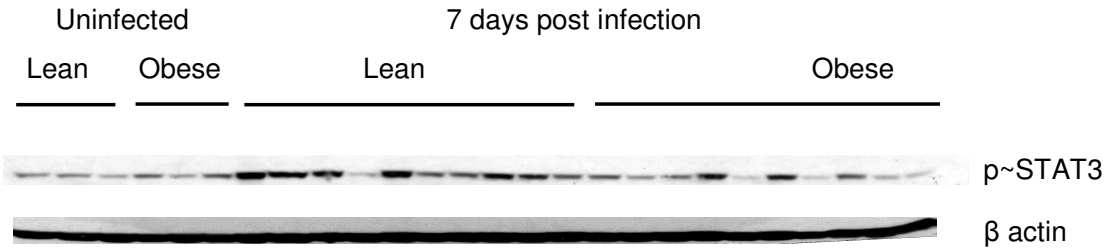


Figure 4.2. STAT3 activation is attenuated in obese mice during influenza infection. Protein extracts were prepared from splenocytes at d 0 and 7 p.i. and probed with anti-STAT3 pY and β-actin. Values on graphs represent the density of the band and data are expressed as mean ± SEM (n = 10/group).



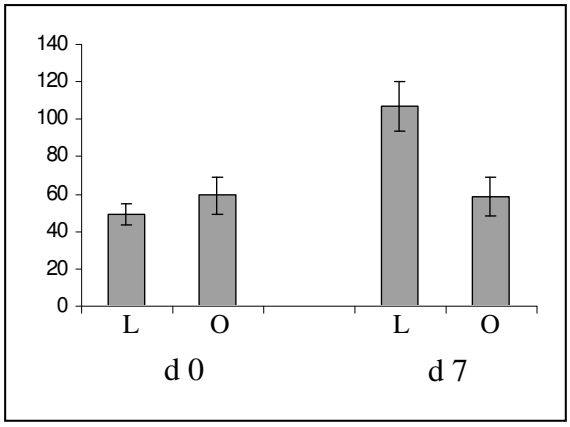


Figure 4.3. Obese mice have reduced proliferation and IFNγ production in response to PHA. Splenocytes were isolated from lean and obese mice (n=3/group) and pooled. Cells were stimulated for 48 h with 2.5 μg/mL PHA and pulsed with 2 μCi [^{3}H] thymidine during the final 4 h. Values *, $P < 0.05$ vs. lean.

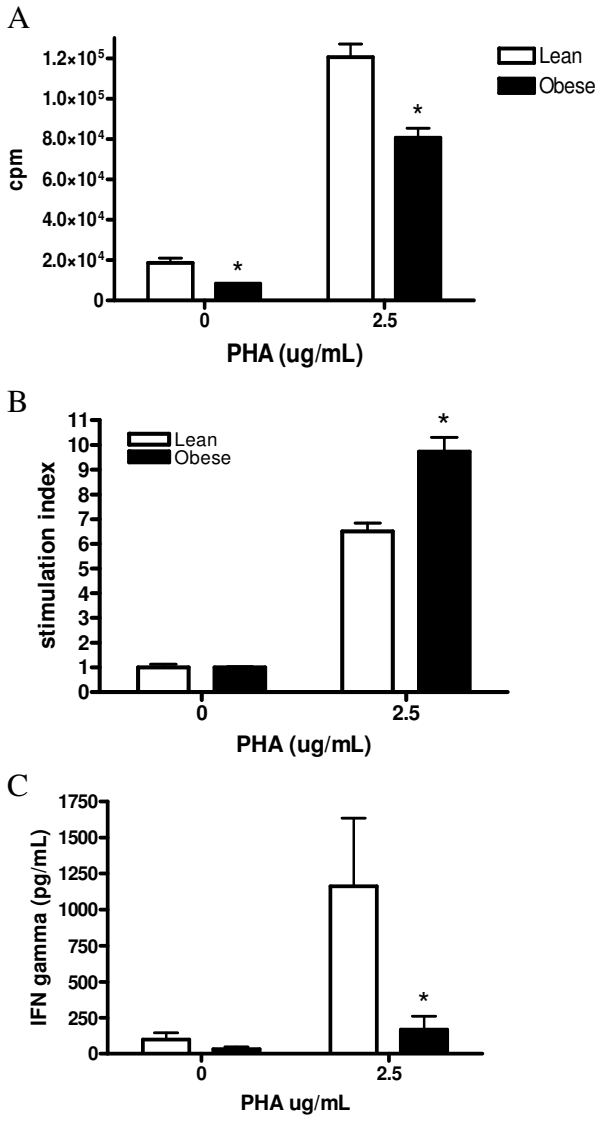


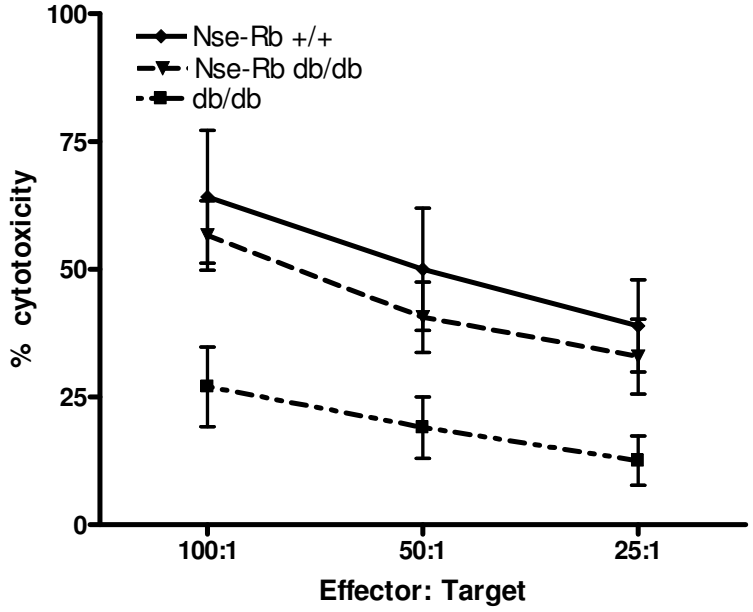
Table 4.3. Effects of influenza infection on *db/db*, Nse-Rb *db/db*, and Nse-Rb +/+ mice. Blood was collected from fed mice to determine glucose levels. Percent of NK cells in the splenocyte population were determine by flow cytometry. Values are expressed as mean (SEM) (n=3/genotype).

	Body Wt (g) (SEM)		**Blood glucose** (mg/dL)		**% NK cell** (SEM)
Genotype	d 0	d3	d0	d3	d3
db/db	67 (2.5)	68 (3.2)	245 (31.5)	410 (92.7)	30.6 (3.6)
Nse-Rb *db/db*	49 (2.8)[a,b]	47 (2.3)[a,b]	135 (10.3)[b]	250 (32)	31.3 (3.7)
Nse-Rb +/+ (WT)	28 (0.67)[b]	25 (1.2)[b]	135 (7.9)[b]	200 (30.6)	40.8 (0.96)

[a] $P < 0.05$ from WT

[b] $P < 0.05$ from *db/db*

Figure 4.4. Central leptin signaling corrects impaired NK cytotoxicity in *db/db* mice infected with influenza virus. At day 3 p.i. isolated spleen cells were analyzed in a standard NK cell cytotoxicity assay, using ^{51}Cr-labeled YAC-1 tumor cells as targets. The resulting effector to target (E:T) ratios were 100:1, 50:1, and 25:1. Data are shown as mean ± SEM (n=3).



CHAPTER V
SYNTHESIS

A. OVERVIEW OF RESEARCH FINDINGS

The incidence of obesity worldwide has reached epidemic proportions. This has had a significant economic and social impact due to the increase in co-morbidities associated with obesity. Obesity leads to changes in immune parameters under basal conditions which, suggests that obesity may result in impaired immune responses during an infection.

Influenza virus infection is a leading cause of morbidity and mortality, and proper immune regulation is necessary for clearance of virus with minimal damage to the host. Given the impairment in immune function observed in obesity, it is plausible that obesity leads to dysregulated immune responses during influenza infection, resulting in deleterious effects on the host. In this work, we characterized both the innate and cell-mediated immune responses to influenza infection in obese mice. Furthermore, we examined the role of leptin signaling on immune cell function. This section summarizes our research and provides a synthesis our findings.

B. Increased Mortality and Altered Immune Function in Diet Induced Obese Mice Infected with Influenza Virus

The initial foray into the study of obesity and the immune response to influenza infection, was prompted by previous work that demonstrated obese individuals were less responsive to Hepatitis B vaccine (220). Additional work from our laboratory indicated that the antibody response to influenza vaccine in obese humans was similarly diminished. This suggested obesity leads to impaired immune responses to influenza virus antigen.

To test this, we used the diet-induced obesity mouse model since it closely models human obesity in terms of metabolic, hormonal, and adipose tissue changes (217). Following infection with influenza virus, 42% of the obese mice died, despite the fact they were infected with a dose of virus that does not lead to excessive mortality, as only 5% of the lean mice died. Since infection with influenza stimulates immune cell infiltration into the lung, which can result in mortality, this high mortality suggested that immune responses were aberrant in obese mice.

We began by analyzing constituents of the innate immune response, since these initiate the inflammatory response and establish the cell-mediated response. We found that obese mice had minimal induction in antiviral cytokine responses, as well as a delay in pro-inflammatory cytokine expression. Furthermore, their NK cell cytotoxicity was reduced. In this study we also found that the induction of chemokines was delayed in obese mice which indicated that recruitment of mononuclear cells to the infected lung may not be occurring.

Despite the impaired response, obese mice were able to control their viral titer, indicating that an excessive amount of virus was not the cause of death. It has been previously demonstrated, by our lab and others, that viral titers do not directly correlate with the outcome of influenza infection in terms of viral clearance, lung pathology, and death (187, 277). This suggests the immune responses determine the outcome of an influenza infection. Although, we did not observe a significant difference in lung pathology between the dietary groups, the6 obese mice tended to have greater pathology at d 6 p.i. Whether the lung pathology continued to escalate in these mice is unknown, however, the peak in pro-inflammatory cytokine induction at d 6 p.i. suggests that it does.

Both obesity and infection are associated with an increase in circulating leptin. We found that obese mice have chronically high circulating levels of leptin which did not increase further with infection. Leptin activates cells of the immune response and its pro-inflammatory role during infection is thought to be mediated by its central and peripheral actions (8, 77, 94). The lack of increase in leptin during infection suggested that immune cells from obese mice may not be fully activated.

These data demonstrated, for the first time, that obesity impaired immune responses that are central to the innate immune response to influenza virus infection. They also indicated that impaired leptin signaling may mediate some of these alterations. Furthermore, because of the critical role of the innate immune response in activating the cell mediated response, these data suggested that obesity also caused dysregulation of cell mediated immune responses during influenza infection.

C. Selective impairment in dendritic cell function and altered antigen-specific $CD8^+$ T cell responses in diet-induced obese mice infected with influenza virus

In the previous study we found that obesity led to diminished and delayed cytokine production during influenza infection. To determine if this was the result of impaired cellular recruitment or defective cell function, we analyzed the lung cell population during influenza infection. We found that obese mice have delayed recruitment of mononuclear cells into their lungs. This impaired recruitment may be due to a lack of induction of chemotactic stimuli from the infected macrophage and epithelial cells. Indeed, we saw significantly lower expression of chemokines in the lungs of obese mice at d 3 p.i. Therefore, it appears that obese mice have slower infiltration of immune cells to the infected lung, which may explain, in part, their delay in pro-inflammatory cytokine expression.

To determine if the altered cytokine levels in obese mice was also due to an impairment in the cells capacity to produce cytokines, we analyzed the ability of lung cells to produce IL-6 during influenza infection. We found that despite a lower number of mononuclear cells at d 3 p.i. in obese mice, these cells were capable of producing IL-6 and, in fact, produced more IL-6 than mononuclear cells from lean mice.

The overproduction of IL-6 by the monocyte population may be due to minimal STAT3 activation (Chapter 4). STAT3 mediates IL-10 suppression of IL-6. So although IL-6 also signals through STAT3, the role of this signaling molecule is predominantly anti-inflammatory (86). This indicates that a defect in the activation of this pathway could result

in “hyperproduction” of inflammatory cytokines, such as IL-6. A defect in STAT3 signaling could also explain the heightened induction of pro-inflammatory cytokines that occurs at d 6 p.i. in obese mice (Chapter 2). So although mononuclear cells from obese mice are slower to enter the lung during influenza infection, once they are there they may be in an augmented pro-inflammatory state.

Analysis of lung cellularity also revealed that obese mice had a continual reduction in the number of DC that entered the lung during infection. Since DC play a critical role in establishing the cell-mediated immune response we explored their functional capacity and the subsequent T cell response. As shown in Chapter 3, we found that DC from obese mice had limited ability to stimulate CD8+ T cells. However, their capacity to take antigen up and migrate to the LN was normal. Therefore obesity affects specific components of DC function. The possibility arises, though, that DC were capable of presenting antigen, but were not able to appropriately stimulate T cells due to impaired IL-12 production. Evidence for this comes from the fact that we found similar MHC Class I expression between lean and obese mice, but IL-12 was significantly reduced in obese mice. However, the inability to induce T cell responses could be due to both a defect in antigen presentation as well impaired IL-12 expression. The overproduction of IL-6 by monocytes/macrophages suggests this may be what is occurring. IL-6 impairs both DC differentiation and maturation resulting in a lower number of DC, as well as DC that are ineffective at stimulating T cells. Therefore it is possible that defective STAT3 signaling leads to exaggerated production of IL-6 in macrophage, which then interferes with normal DC maturation and function.

Since the major role of the DC is to stimulate T cell responses, and we found that DCs from obese mice were limited in their capacity to do this *ex vivo*, we analyzed the population of T cells in the lungs of obese mice during influenza infection. Using two different methods we found that obese mice had a higher frequency, but similar number of virus-specific CD8+ T cells and less total CD3+ and CD3+CD8+ T cells. Additionally, upon antigen encounter they were able to produce IFNγ. These data indicate that: 1. T cell proliferation is reduced or apoptosis is increased in obese mice 2. CD8+ precursor T cells are fully utilized and thus depleted 3. T cell antiviral function is preserved.

The inability of T cells to proliferate could be due to alterations in the cytokine milieu during influenza infection in obese mice, and/or to a lack of STAT3 signaling. As described in Chapter 4, we found that STAT3 signaling in splenocytes at d 7 p.i. was impaired. STAT3 mediates IL-6 induced T cell entry into the G_1 phase of the cell cycle resulting in proliferation and IL-2 production (278). Thus, the diminished IL-2 expression we observed in the LN during at times of peak T cell proliferation indicates that T cell responses are impaired and this may be due to altered IL-6 signaling. This is further suggested by the fact that splenocyte proliferation was lower in obese mice following PHA-stimulation (Chapter 4). PHA-stimulated T cell proliferation has been shown to be IL-6 dependent (268). Since IL-6 mediates it effects via STAT3, these data therefore suggest, that the lower number of T cells in the lungs of obese mice is due to reduced STAT3 mediated cell proliferation. Notably, leptin also signals through STAT3 to increase T cell proliferation. This suggests that limited STAT3 signaling may also blunt any proliferative signal induced by leptin. There is, however, there is an alternative explanation for the lower T cells numbers that occur in

obese mice. It may be that T cells in obese mice are more prone to apoptosis. Evidence for this, comes from the fact that both leptin and IL-6 suppress apoptosis through STAT3 activation. Thus, overall it appears that the reduced number of T cells in obese mice is likely due to impaired STAT3 signaling.

Additionally, the higher frequency of antigen specific cells within the stunted T cell population in obese mice, suggested that the pool of precursor CD8+ cells were fully utilized to eradicate virus. During a normal CD8+ T cell response, there is a proportion of the antigen specific population that does not gain effector function, i.e. the precursor CD8+ T cell. These cells appear to be important in establishing the memory response to influenza. However, when the number of T cells is limiting, the proportion of these cells that are used as effectors increases (130). So while the higher frequency of antigen-specific effector CD8+ T cells was probably beneficial to the obese mice, in that it allowed them to clear antigen, it may have deleterious effects on subsequent immune responses.

Finally, the ELISPOT data revealed that T cells from obese mice were capable of eliciting antiviral responses. This was indicated by the production of IFNγ by T cells following antigenic stimulation. This is in contrast to the limited production of IFNγ from PHA-stimulated splenocytes from obese mice (Chapter 4). This difference may be due to the fact that the ELISPOT is performed with antigen presenting cells that are derived from lean mice. Therefore, if a lack of DC derived cytokines mediates the impairment in IFNγ production in obese mice, any defect in IFNγ production by T cells will not observed in the ELISPOT assay.

These data demonstrated that cell-mediated responses in obese mice were affected by obesity. Many of the alterations, however, may be caused by impaired innate immune responses. From our data, it is reasonable to hypothesize that impaired STAT3 signaling plays a significant role in the innate and cell-mediated immune dysfunction we observed in obese mice.

D. Central leptin receptor signaling rescues defect in natural killer cell cytoxicity in *db/db* mice during influenza infection.

At this point in our investigation, we did not know if STAT3 activation was altered. What we did know was that pro-inflammatory cytokine expression was delayed, NK cell cytotoxicity was impaired, and the number of T cells was less in obese mice. All of these changes were consistent with the idea that obesity leads to impaired leptin signaling in immune cells, since leptin has been shown to augment pro-inflammatory cytokine production, enhance NK cell mediated killing, and stimulate T cell proliferation and Th1 polarization. Additionally, there was evidence that leptin-induced STAT3 signaling was inhibited in T cells from DIO mice (77). Therefore, we hypothesized that obesity caused a reduction in leptin signaling in immune cells, thereby preventing full activation of normal immune responses.

To determine if leptin receptor signaling in immune cells was necessary for complete activation of immune responses, we analyzed NK cell cytoxicity. This marker of immune function was chosen because we had previously observed a 50% reduction in NK cell cytotoxicity in obese mice (Chapter 2) and because leptin receptor has been shown to be

necessary for leptin-induced augmentation of NK cell cytotoxicity. We used a transgenic mouse model in which expression of a functional leptin receptor is restricted to the brain, and therefore leptin signaling in NK cells is absent. When we analyzed the ability of NK cells from these mice to kill target cells, we found that NK cell killing in the transgenic mice was restored to the level of WT mice. However, the *db/db* mice had significantly impaired NK cell cytotoxicity. These data indicate that direct leptin signaling on NK cells is not required for complete activation of NK cell cytotoxicity.

Evidence for direct leptin-mediated augmentation of pro-inflammatory effects comes from *in vitro* and *ex vivo* studies where exogenous leptin is added to cells and responses are measured. Because immune cells have receptors for leptin, it is not surprising that leptin transduces a signal and activates target responses in these models. Our data suggest, though, that this is not necessarily how leptin's effects are mediated *in vivo*. To this point, both leptin and obesity can alter neuromodulators, such as catecholamines, glucocorticoids, and neuropeptides, all of which have significant effects on immune responses (279). Therefore, *in vivo,* leptin's effects on immune function may be mediated by these factors.

It is important to note, however, that we only tested NK cell function. Macrophage, DC, and T cells all possess leptin receptors and have been shown to be responsive to leptin. Furthermore, our data indicate a defect in STAT3 signaling during infection in obese mice, therefore any cytokine whose function is mediated by STAT3, namely IL-6, IL-10, leptin, and IL-27, may play a role in the dysregulated immune responses we observe in obese mice.

` Finally, the fact that neurospecific leptin signaling rescued NK cell function has important implications. There is increasing evidence for the idea that obesity leads to defective leptin signaling in the brain (280). Thus, if central leptin signaling is critical for maintaining normal immune function, it is quite possible that the immune defects that occur in obese humans are, in large part, due to impaired leptin signaling in the brain.

E. FUTURE STUDIES

1. Catecholamine/neuropeptide mediated changes in immune function

Obese mice and humans have an upregulated sympathetic nervous system which can modulate immune function. Given the fact that leptin alters neurohormone and neuropeptide levels, the effects we see on immune function in obese mice may be mediated through these molecules. The studies done in Chapter 4 clearly demonstrate that leptin is involved in neuroendocrine regulation of immune function. Therefore, determining the mediators of central leptin signaling would provide a mechanism by which obesity leads to immune dysregulation.

2. Central versus peripheral mediation of leptin's effects on other immune responses

Despite our findings that central leptin receptor signaling was adequate to restore NK cell cytoxicity, there is a reasonable amount of data in support direct leptin signaling on other immune cells. Since we saw defects in monocyte/macrophage, dendritic cells and T cells, all of whose functions can be modulated by leptin, it would be important to test these other immune effectors to determine if leptin is necessary for their function. The fact the these cells

signal through pathways similar to NK cells, determining how leptin signaling affects their function, may lead to a better understanding about how leptin's effects are mediated.

3. Memory response to influenza infection

Both B and T cells are an important component of the memory response. This response leads to sustained immunity to infectious pathogens and is the basis for vaccination. Memory, however, is derived from the initial immune response to infection. Thus, the primary response is a major determinant of the memory response. The immune defects that occur in obese mice during their primary response to influenza infection, strongly suggest that activation of memory responses will be impaired. Thus it will be important to determine if obese mice are able to induce effective memory responses, as it will indicate a whether effective responses to vaccines are affected by obesity.

4. Different dietary fat sources

Dietary fat, in particular polyunsaturated fatty acids, modulate immune responses. Whether this modulation also occurs with different sources of saturated fat is unclear. Therefore studies that manipulate of the fatty acid source can be used to study the effects of fatty acids on immune function, as well as tease apart the effect of obesity versus fatty acids on immune function.

F. Public Health Significance

The present studies provide evidence of significant dysregulation of host immune responses to influenza infection due to obesity. This has considerable impact given the

unparalleled increase in obesity rates worldwide, and the annual widespread incidence of influenza infection. Obesity is associated with a number of co-morbidities, some of which are also risk factors for influenza infection-related morbidity and mortality. These include: Type 2 Diabetes; Cardiovascular disease; and Chronic Pulmonary Obstructive Disorder (281). However, our work points to the fact that obesity in the absence of these co-morbidities, also increases the risk of morbidity and mortality during influenza infection.

Interestingly, the influenza vaccine is recommended for individuals with chronic diseases, such as those mentioned above. Our data illustrate that the effectiveness of the influenza vaccine by be minimized by obesity due to its dysregulation of primary immune responses. Therefore, regardless of vaccination status, obesity may result in increased susceptibility to the adverse effects of influenza virus infection. This is especially important, given the threat of a 'flu' pandemic.

Outside the scope of influenza specifically, the studies presented here, point towards a mechanism by which obesity generally modulates immune responses. Therefore, research targeting the mechanism (282) by which occurs will be useful in preventing obesity-induced immune dysregulation.

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