THE INFLUENCE OF DIET-INDUCED OBESITY ON THE GENERATION, FUNCTION AND MAINTENANCE OF INFLUENZA-SPECIFIC MEMORY CD8+ T CELLS

Erik Albert Karlsson

A dissertation submitted to the faculty of the University of North Carolina at Chapel Hill in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Nutrition, Gillings School of Global Public Health

Chapel Hill

2010

Approved by:
Advisor: Melinda Beck, Ph.D.
Reader: Jean Handy, Ph.D.
Reader: Ilona Jaspers, Ph.D.
Reader: Liza Makowski, Ph.D.
Reader: David Peden, M.D.
Reader: Daniel Pomp, Ph.D.
Reader: Patricia Sheridan, Ph. D

ABSTRACT

Erik Albert Karlsson

THE INFLUENCE OF DIET-INDUCED OBESITY ON THE GENERATION, FUNCTION AND MAINTENANCE OF INFLUENZA-SPECIFIC MEMORY CD8+ T CELLS
(Under the direction of Melinda A. Beck, Ph.D.)

In both humans and animal models, obesity leads to a dysregulated immune response; however, the effect of obesity on response to viral infection is largely unknown. Yearly outbreaks of influenza virus are a major cause of morbidity and mortality and obesity may increase this risk. Indeed, when challenged with influenza virus, obese mice exhibit a decreased immune response to influenza infection leading to increased morbidity and mortality. Memory $CD8^+$ T cells generated during primary influenza infection target internal proteins common among influenza viruses, making them effective against encounters with heterologous strains. Since previous studies in our laboratory have shown that diet-induced obese mice have a significantly altered primary immune response to influenza infection, we hypothesized that obese mice would have an impaired memory $CD8^+$ T cell response to secondary influenza infection. In male, diet-induced obese C57Bl/6 mice, a secondary H1N1 influenza challenge following a primary H3N2 infection led to a 25% mortality rate (with no loss of lean controls), 25% increase in lung pathology, failure to regain weight and 10 to 100 fold higher lung viral titers. Furthermore, mRNA expression for interferon γ (IFN-γ) was >60% less in lungs of obese mice along with one third the number influenza-specific $CD8^+$ T cells producing IFN-γ post secondary infection versus lean controls. Memory $CD8^+$ T cells from obese mice had a >50% reduction in IFN-γ production when stimulated with influenza-pulsed dendritic cells from lean mice. In addition, maintenance of influenza-specific memory T

cells was impaired in obese mice with a 10% reduction in this population 84 days post primary infection. Thus, the function and maintenance of influenza-specific memory T cells is significantly reduced and ineffective in lungs of obese mice. The reality of a worldwide obesity epidemic combined with yearly influenza outbreaks and the current threat of an H1N1 pandemic makes it imperative to understand how influenza virus infection behaves differently in an obese host. Moreover, impairment of memory responses has significant implications for vaccine efficacy in an obese population.

To my advisor, Dr. Melinda Beck: Thank you for all of the support, encouragement and help. I am especially grateful for your guidance along this winding research path

To Dr. Patricia Sheridan: Thank you for the support, sanity and insightful discussion without which much of this work would not have happened

To my parents: Thank you for the unconditional love and support and for encouraging to always keep doors open

TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

LIST OF ABBREVIATIONS

αMSH	α Melanocyte-Stimulating Hormone
Ab	Antibody
ACIP	Advisory Committee on Immunization Practices
AICD	Activation-Induced Cell Death
APC	Antigen Presenting Cell
ARC	Arcuate
AgRP	Agouti-Related Peptide
BMI	Body Mass Index
BRFSS	Behavioral Risk Factor Surveillance System
CD	Cluster of Differentiation
CDC	Centers for Disease Control and Prevention
CRP	C-Reactive Protein
CTL or T_c	Cytotoxic T Cell
CVD	Cardiovascular Disease
CXCR3	Chemokine Receptor 3
d	Day
db	Diabetes gene
DC	Dendritic Cell
DIO	Diet-Induced Obesity
EID_{50}	50% Egg Infectious Dose
Eomes	Eomesodermin
FACS	Fluorescence-activated Cell Sorting
FDA	Food and Drug Administration

FITC	Fluorescein
G3PDH	Glyceraldehyde-3-phosphate Dehydrogenase
HA	Hemagglutinin
HF	High Fat
IFN	Interferon
IL	Interleukin
i.p.	Intraperitoneally
JAK	Janus Kinase
LF	Low Fat
LFA	Lymphocyte Function-Associated Antigen
LN	Lymph Node
MAPK	Mitogen-Activated Protein Kinase
MC4R	Melanocortin-4 Receptor
MCP	Monocyte Chemoattractant Protein
MHC	Major Histocompatibility Complex
MIP	Macrophage Inflammatory Protein
MPEC	Memory Precursor Effector Cells
MΦ	Macrophage
mTOR	Mammalian Target of Rapamycin
NA	Neuraminidase
NEP	Nuclear Export Protein
NHANES	National Health and Nutrition Examination Survey
NK	Natural Killer Cell
NP	Nucleoprotein

NPY	Neuropeptide Y
ObR	Leptin Receptor
PE	Phycoerythrin
p.i.	Post Infection
PI3K	Phosphoinositide 3-kinase
POMC	Pro-Opiomelanocortin
qRT-PCR	Quantitative Reverse Transcriptase Polymerase Chain Reaction
RNP	Ribonucleoprotein
SLEC	Short-Lived Effector Cells
SOCS	Suppressor of Cytokine Signaling
STAT	Signal Transducer and Activator of Transcription
T_{CM}	Central Memory T Cells
T_{EM}	Effector Memory T Cell
T_H	Helper T Cell
T_{REG}	Regulatory T Cells (formerly suppressor T cells)
T2D	Type 2 Diabetes
$TCID_{50}$	50% Tissue Culture Infectious Dose
TCR	T Cell Receptor
TFNα	Tumor Necrosis Factor Alpha
Tyr	Tyrosine
WAT	White Adipose Tissue
WHO	World Health Organization
WT	Wild Type

CHAPTER I

BACKGROUND AND SIGNFICANCE

A. Specific Aims

In both humans and animal models, obesity leads to a dysregulated immune response. Deleterious changes in the immune function of obese subjects have been described such as increased susceptibility to infection, decreased ability to produce beneficial immune cytokines and lowered numbers of immune specific cells and subsequent cellular responses. Because of these associated problems with the immune system, one would expect a large body of data on the consequences of viral challenge during the obese state; however, the effect of obesity on response to viral infection is largely unknown. To understand how obesity could affect the immune response to viral infection, influenza virus infection was used as a model. Yearly outbreaks of influenza virus are a major cause of morbidity and mortality and obesity may increase this risk. Indeed, when challenged with influenza virus, obese mice exhibit a decreased immune response to influenza infection leading to increased morbidity and mortality, yet the exact mechanisms that cause this altered response have yet to be elucidated.

Effective clearance of the influenza virus is dependent on both innate and adaptive immune mechanisms. Diet-induced obese mice challenged with influenza virus exhibit increased lung pathology, decreased antiviral cytokine response, delayed expression of beneficial inflammatory cytokines and chemokines as well as increased influenza-specific $CD8^+$ T cells. Primary infection with influenza virus leads to the generation and maintenance

of a pool of protective memory $CD8^+$ T cells which can more rapidly respond to a secondary encounter. The initial primary response to infection as well as lung microenvironmental conditions can have a major impact on memory T cell generation and functionality. Since previous studies in our laboratory have shown that diet-induced obese mice have a significantly altered primary immune response to influenza infection, **we hypothesized that obese mice would have an impaired memory $CD8^+$ T cell response to secondary influenza infection.**

Specific Aims

1. To determine if diet-induced obesity in mice alters the $CD8^+$ T cell memory response (recall response) to a second infection with influenza virus
2. To determine if diet-induced obesity in mice affects the maintenance of influenza antigen-specific memory $CD8^+$ T cell subpopulations
3. To determine if diet-induced obesity in mice affects the generation of influenza-specific $CD8^+$ memory T cells during a primary influenza infection

B. Obesity: Definition and Public Health Perspective

Obesity has become a worldwide epidemic. In 2005, the World Health Organization (WHO) projected that approximately 1.6 billion adults were overweight (Body Mass Index, BMI ≥25), and at least 400 million of these adults were clinically obese (BMI ≥30). Furthermore, the WHO predicts that by the year 2015, approximately 2.3 billion adults will be overweight with greater than 700 million of these adults being obese (1). In the United States (US) alone, the National Health and Nutrition Examination Survey (NHANES) predicted that over 66% of the population was overweight or obese (2). Recent projections based on NHANES predict that if the current trends continue, more than half (51.1%) of US adults are likely to be obese and 86.3% are likely to be overweight or obese by 2030 (3). Analysis of obesity trends by Hill et al. in 2003, suggested that the obesity epidemic has arisen from gradual yearly weight gain in the population produced from a slight consistent degree of positive energy balance. Their findings indicate that the average adult in the United States has gained an average of 1 to 2 lb/y for the past 2 to 3 decades (2,4).

Obesity has traditionally been considered a problem affecting adults in high-income countries; however, obesity rates are also rising rapidly in low- to middle-income countries, especially in urban settings. Childhood obesity is also on the rise. Globally in 2005, there were more than 20 million overweight children under the age of 5 (1). In addition to a large portion of the population being overweight, it appears that incidence of clinically severe obesity (BMI ≥40) is growing at an exponential rate. Based on data from the Behavioral Risk Factor Surveillance System (BRFSS), between 1986 and 2000, prevalence of obesity (BMI ≥30) doubled from 1 in 10 US adults to 1 in 5. However, when focused in on clinically severe obesity, prevalence of BMI ≥40 quadrupled from about 1 in 200 US adults to 1 in 50 and prevalence of BMI ≥50 increased from 1 in 2000 to 1 in 400, a five fold increase. Therefore,

TABLE 1.1: BMI RANGES

	BMI RANGE
STARVATION	<15
UNDERWEIGHT	<18
NORMAL	18-24.9
OVERWEIGHT	25-29.9
OBESE	30-39.9
MORBID OBESITY	>40

obesity is not only increasing in the US, prevalence of super obese individuals is also increasing at an alarming rate (5). Recently, a study by Flegal et al. (2010) have reported that the prevalence of obesity in the US may be leveling off according to the most recent sets of NHANES data. However, according to the 2007-2008 NHANES data, 68.0% of the US population still has a BMI ≥ 25 meaning that 2 out of every 3 people are overweight or obese (6). Therefore, even if the prevalence of obesity is leveling in the US a significant portion of the population is still at risk for the comorbidities associated with obesity. In addition, rates of obesity are increasing worldwide, not just in the American population.

Although humans, as a species, are relatively well adapted to periods of lean food intake, they are poorly adapted to overnutrition. Human biology, which has evolved in times of frequent famine, is now for all intents and purposes maladaptive in our calorie-rich, sedentary environment (7,8). The obese state can lead to serious health consequences and subsequently, increases in health care requirements and economic burden. Caused by a change in energy balance of increased caloric intake versus expenditure (9), obesity has been linked to numerous health problems and chronic diseases (10-12). These comorbidities associated with obesity have been attributed with hormonal and metabolic changes related to increased adipose tissue mass (13-15). Obesity is an independent risk factor for cardiovascular disease (CVD), type 2 diabetes (T2D), hypertension, arthritis, sleep apnea and some cancers. Obese individuals are at increased risk for high blood pressure and unfavorable blood lipid profiles which can lead to CVD. Obesity can also be directly related to CVD apart from blood pressure and lipid levels (16,17). Individuals with a BMI ≥30 have a 60- to 80-fold increased risk of developing T2D; however, this huge increase in risk may be due to an intrinsic relationship between obesity and T2D termed “diabesity” (18). Indeed, in the Diabetes Prevention Program, weight loss of 5 to 6% along with increased physical activity in persons with a BMI of 34 kg/m^2 resulted in a 58% reduction in the

incidence of diabetes (19). In regards to cancer, Calle et. al. (2003) and others have reported that increased body weight is associated with increased death rates for all cancers combined and for cancers at multiple specific sites (20). Obesity also has been found to have adverse effects on the respiratory system including alteration in respiratory mechanics and pulmonary function tests. Most commonly these complaints are evident with shortness of breath during periods of exertion (17,21). Aside from all of the comorbidities, obesity can be directly related to a reduction of lifespan and a premature risk of death. High BMI levels are risk factors for all cause mortality. In addition, increased BMI was directly related to increased mortality risk in both the Nurses' Health Study and the US Health Professionals Follow-Up Study (22-26). Based on all of the relative risks, Oster et al. (1999) calculated that a weight loss of 10% in obese individuals could increase life expectancy by 27 months (27,28).

In addition to the health problems, chronic diseases and reduction of lifespan, obese individuals also face other challenges in the health care system. Many physician offices and hospitals are not equipped to take care of severely obese individuals. Obese patients may not fit in standard medical equipment such as imaging scanners, hospital beds or even wheelchairs. Additionally, the lack of the ability to weigh individuals over 300 lbs in ambulatory settings could affect the quality of care given to obese patients (5). It is also important to note that the increasing prevalence of obesity can translate directly into increased medical care and disability costs. Obese adults incur annual medical expenditures which are approximately 36% higher than normal weight individuals (29,30). This increase can be further divided into BMI classes, resulting in a BMI ≥40 having double (~100% greater) the healthcare costs of a normal weight individual (31). In 1998, approximately 5.7% ($51.6 billion) of the total health care expenditures in the United States could be attributable to direct medical costs associated with obesity and its cormorbidities. Additionally, obesity has indirect costs attributable to loss of work caused by morbidity and

mortality and can be estimated at $47.6 billion annually but may be even more impactful than direct costs at the personal and societal levels (32). In 1999, Colditz reported that the costs of obesity were estimated to be 7% of the total health care costs in the US and between 1% to 5% in Europe (33,34). In 2002, the annual medical spending attributable to overweight and obesity was estimated at 9.1% of total health care costs (30). Although the increased costs of obesity are generally attributed to its associated comorbidities such as T2D and CVD, impaired immunity in obese individuals may also contribute to this increased cost.

C. Animal Models of Obesity

The use of animal models has been and will be the cornerstone of our understanding of obesity and its effects on physiology including the immune system. Since many studies, especially those involving infectious pathogens and experimental analysis of vital organs, cannot be conducted in human populations, mouse models of obesity have been generated to study the physiological and biochemical effects of obesity. The use of animal models to study phenomenon that underlie obesity or result as a consequence of obesity has provided an enormous amount of information that impacts our understanding of the obese state. While these models provide a good starting point for understanding the deleterious effects of obesity, it must be recognized that animal models may not be directly comparable to human populations and studies still need to be conducted in obese individuals (35).

C.1 Genetic Models of Rodent Obesity

Energy homeostasis is a very complex, long-term process comprised of multiple interacting homeostatic and behavioral pathways, including glucose and lipid homeostasis,

the hypothalamic-pituitary-adrenal axis and satiety as well as other macronutrient pathways that all act in concert to maintain constant levels of energy stores (36) A small number of energy balance genes are known to be essential for normal body-weight regulation, with loss of function mutations in a single gene leading to obesity in laboratory animal models and in small, rare populations of human subjects (37). Discovery of these models can be attributed to astute colony managers noticing abnormal obesity phenotypes and then selectively to bring forth these traits in homozygotes (38). Historically, five such mutations (*agouti, fat, tubby, obese* and *diabetes*) were identified and a number of transgenic and knockout models of obesity and other body weight regulatory conditions have been created (39-42). It is now clear that any disruption in the development, differentiation or metabolism of the adipocyte can cause obesity or obesity resistance (36,43,44). Obesity itself is a highly complex trait and polygenic mouse models of obesity such as recombinant inbred lines, long-term selection lines and heterogeneous stocks are beginning to be studied intensely using newer and more powerful gene discovery tools (45).

C.2 Diet-induced Models of Rodent Obesity

Loss-of-function mutations, whether they be single genes or polygenic, reveal their products to be essential for normal body weight regulation in mammals. While these models are essential for basic scientific discovery, the occurrence of these mutations in human populations is extremely rare and does not help to elucidate the cause and/or effects of obesity in the majority of humans (46,47). Generally, it is assumed that most obesity is a result of small changes in a number of genes subjected to an obesigenic environment (diet, physical activity, built environment, socio-economic status, etc). Thus, since current lifestyles have reduced physical activity in conjunction with easy access to palatable and energy dense diets, it can be hypothesized that diet can be the major contributor to the

majority of human obesity. Therefore, it would be beneficial to incorporate diet-induced obesity (DIO) into the design of studies in laboratory rodents and, in fact, DIO models are beginning to be employed more and more in the laboratory setting. It must be noted, however, that the dietary manipulations performed need to be carefully considered to determine the most appropriate diet to model common human obesity.

There is wide variation in the types of diets used to induce obesity. Generally, DIO is achieved using the incorporation of increased dietary fats into the diet. However, the amount of fat can vary between 20% to 60% of energy. Adding further complexity, the fat macronutrient itself may be derived from either animal or plant products such as lard or coconut oil. DIO models include rodent, canine and other species and, within those species, a number of different strains (48,49). Several mouse strains have been described in the literature, including those by Surwit et al. (1988), West et al. (1992), and Reifsnyder et al. (2000), in which susceptibility to obesity has been found to be strain dependent. For example, some strains of mice (eg: C57BL/6) are more susceptible to DIO while other strains (eg: SWR/J) appear to be resistant (50-52). Moreover, these susceptibility and resistance dynamics can be affected by the dietary components (53). Even when specific strain/diet combinations are used, duration of dietary exposure, such as 4 months versus 6 months of treatment, could provide variability in level of obesity and its effect on outcome. Overall, the objective of DIO models is to mimic the palatability-driven over-consumption that exemplifies the majority of human obesity. Because of the extreme variability in all aspects of DIO induction, it is important that studies that use DIO models are only compared to experiments utilizing similar, if not exactly equivalent, dietary study designs (49).

C.3 Non-traditional Models of Obesity

In addition to more traditional rodent models of obesity, there are several studies which look at other obese systems such as seasonal models, companion animals, exotic models and non-human primates (38). Many small mammals exhibit annual cycles of reproduction that are accompanied by variations in body mass and adiposity. Hamsters, voles, lemmings and squirrels have all been studied for seasonal changes in adiposity mainly by linking them to photoperiod-triggered changes in leptin signaling (54,55). Hamsters have also been shown to be highly susceptible to obesigenic dietary treatment (56). Companion animals such as dogs and cats have also been noted to become obese when fed higher levels of dietary fat (57). Interestingly, the magnitude of this effect may be strain dependent which indicates an underlying genetic component in some strains (58).

Other studies involve more exotic models, usually involving wild species which undergo natural patterns of variability in their fat mass such as seals and bats (59,60). Several models of spontaneous obesity have also been noted in nonhuman primates, most notably macaque and rhesus monkeys (58,61). Additionally, controlled dietary studies in primate models have observed the association of high-fat diets with body composition (58).

D. Obesity as a Modulator of Immune Function

Obesity is well established as a risk factor for increased morbidity and mortality; however, its effects on susceptibility to infection are just beginning to be understood. Conventionally, obesity can be considered an overaccumulation of white adipose tissue (WAT). Although adipocytes occupy the bulk of the volume of WAT, adipose tissue also includes many more cells types. Nonadipocytic cells in WAT consist of a diverse population of preadipocytes, macrophages, endothelial cells, fibroblasts and leukocytes (62). WAT is

now considered an important part of body regulation and can act as an endocrine organ, secreting numerous factors, which can affect several metabolic pathways and even the function of the immune system.

D.1 Adipokines (Table 1.2)

In the past two decades, research has pushed the concept of WAT as an endocrine organ in its own right versus just serving as a storage depot for fats. Indeed, WAT has been found to produce close to 100 cytokines and other molecules including leptin, adiponectin, resistin, visfatin, apelin, vaspin, hepcidine, TNF-α, omentin and monocyte chemoattractant protein 1 (MCP-1). These "adipokines" participate in a wide variety of physiological or pysiopathological processes including food intake, insulin sensitivity and inflammation. In addition, many of the adipokines have been found to play an intricate role in various aspects of the innate and adaptive immune response (63-66). Obesity was the original condition which motivated the study of WAT. In the obese state, secretion of these adipokines is altered in correlation to the increased adipose tissue mass (14,67-69). For example, secretion of leptin, interleukin (IL)-6 and tumor necrosis factor alpha (TNFα) are all increased in obese individuals while adiponectin secretion is decreased. Adding to this

TABLE 1.2: ADIPOKINES IN OBESITY AND THEIR EFFECT ON THE IMMUNE SYSTEM

FACTOR	METABOLIC EFFECT	IMMUNE EFFECT	DURING OBESITY	REFS
ADIPONECTIN	↓ GLUCONEOGENESIS ↑ GLUCOSE UPTAKE B-OXIDATION INSULIN SENSITIVITY WEIGHT LOSS ENERGY METABOLISM	ANTI-INFLAMMATORY ↓ T CELL RESPONSES ↓ B CELL LYMPHOPOESIS	REDUCED	(62,66,70,71)
LEPTIN	↑LIPOLYSIS ↓ FOOD INTAKE	INFLAMMATORY ↑ T CELL PROLIFERATION ↑ LYMPHOPOESIS ↑ THYMOCYTE SURVIVAL ↑ TH1 RESPONSE	INCREASED (SIGNAL REDUCED)	(72-77)
VISFATIN/PBEF	↑INSULIN SENSITIVITY	INFLAMMATORY	INCREASED	(14,62,63)
RESISTIN	DIABETOGENIC	INFLAMMATORY	INCREASED	(78)
CHEMERIN	↑ LIPOLYSIS ADIPOCYTE DIFFERENTIATION	CHEMOATTRACTANT	INCREASED	(62,79)
APELIN	↑ INSULIN SENSITIVITY ↓INSULIN SECRETION		INCREASED	(80)
OMENTIN		ANTI-INFLAMMATORY	REDUCED	
VASPIN	↑ INSULIN SENSITIVITY		INCREASED	(81)
ADIPSIN	↓ TAG PRODUCTION	COMPLEMENT ACTIVIATION	INCREASED	
HEPCIDINE	IRON HOMEOSTASIS	↓ IRON RELEASE FROM MACROPHAGES	INCREASED	(82)
GLUCOSE (HIGH)	↑ INSULIN	INFLAMMATORY	INCREASED	(83,84)
INSULIN	↑ GLUCOSE UPTAKE ↓ FOOD INTAKE ↓ LYPOLYSIS	INFLAMMATION	INCREASED (SIGNAL REDUCED)	(85)
IL-6	↓ INSULIN SENSITIVITY ↑ LIPOLYSIS	INFLAMMATORY	INCREASED	(86)
TNFα	↓ INSULIN SENSITIVITY ↑ LIPOLYSIS	INFLAMMATORY	INCREASED	(87)
MCP-1		CHEMOATTRACTANT	INCREASED	(62,88)

complexity is the heterogeneity of the secretory profiles of each adipose tissue. Different fat depots in the body play distinct roles and secrete different sets of cytokines with visceral adipose tissue considered to be the most detrimental in the obesigenic state. For example, visceral adipose tissue has been found to release more resistin, TNFα and IL-6 per gram of tissue than does abdominal subcutaneous adipose tissue (62,69,89,90).

The heterogeneous composition and secretory profile of WAT, especially visceral WAT, is considered an important mediator of metabolism and inflammation (62). A low-grade state of inflammation has been associated with obesity. Although not completely demonstrated, the current working hypothesis is that adipokines, cytokines and other factors produced and released by WAT in obese states such as IL-6, TNFα and C-reactive protein (CRP) are responsible for this chronic inflammation (14,91). The presence of this low-grade, systemic chronic inflammation has been linked to the increased risk of development of CVD and T2D in obesity, being especially associated with visceral adiposity. As stated above, WAT is composed of a number of different cell types apart from adipocytes, including immune cells such as macrophages. The number of macrophages present in WAT is directly correlated to adiposity and adipocyte size in both obese animals and human subjects (92-94). As adipose size increases with obesity, adipocyte size increases accompanied by an influx of macrophages and other immune cells. The circulating levels of TNFα and IL-6 seen in obese subjects are considered to come from these macrophages since macrophages are considered to be the major source of TNFα from WAT and 50% of WAT-derived IL-6 (93,95).

Based on WATs function as an endocrine organ and its ability to influence inflammatory processes within the body, it is not surprising that local, obesity-driven changes in adipokine secretion have a systemic impact on several branches of the immune system. One adipokine that has been shown to have particular immunomodulatory

properties is leptin. Leptin and leptin-associated signaling pathways may provide a direct link between obesity and immune function.

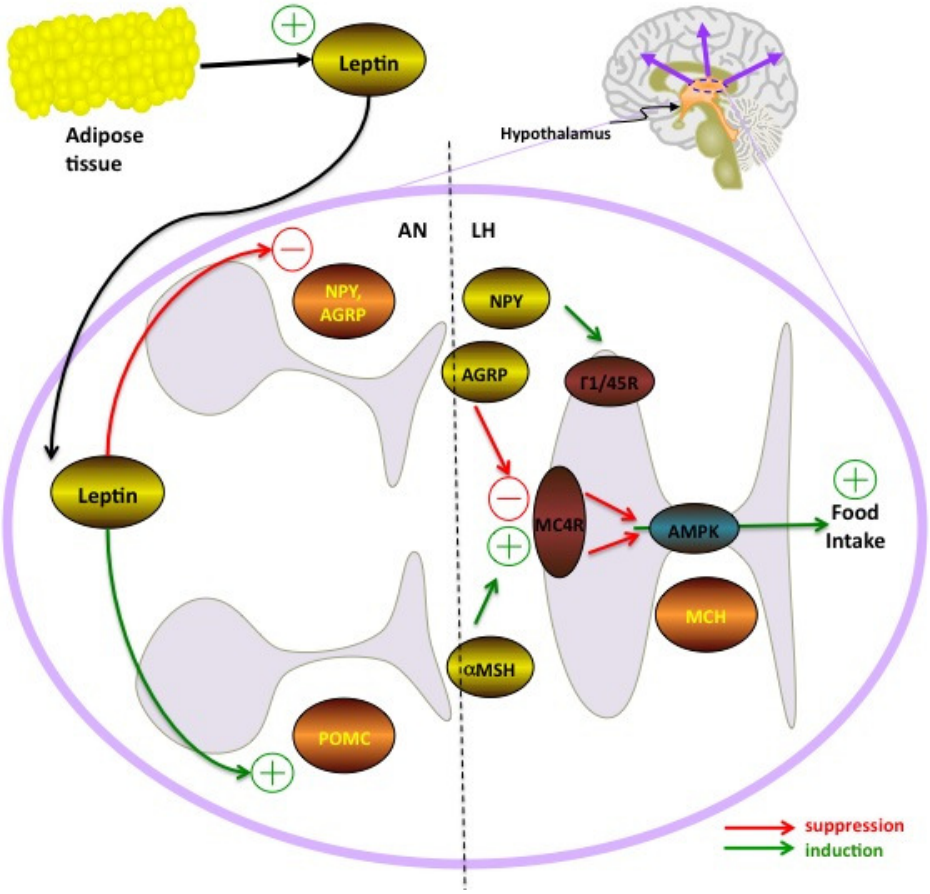
D.2 Leptin

In the late 1950's a genetic defect that caused severe obesity due to overeating and decreased energy expenditure was identified in a population of mice (96). The gene was named *obese* (*ob*) and obese mice carrying the mutation were identified as *ob/*ob (97). The discovery of leptin, which is produced by adipocytes, in 1994 by Friedman and colleagues as the molecular defect for the *ob/ob* phenotype (98) prompted the paradigm shift that WAT was truly an endocrine organ in itself. Leptin is a 16 kDa anorexic peptide that functions primarily in the hypothalamus to decrease food intake and increase energy expenditure (98,99). Initially, the effects of leptin were thought to be only centrally mediated; however, the functional pleiotropy that leptin shares with other members of the long-chain helical cytokine family have shown it to be capable of stimulating a variety of biological responses in a wide spectrum of cell types. Leptin levels can be proportional to insulin levels and inversely proportional to glucocorticoid concentrations (77,100,101). Leptin synthesis is regulated by food intake and eating-related hormones, but it also depends on energy status, sex hormones and a wide range of inflammatory mediators (102,103). In accordance, leptin synthesis is increased following acute infection or sepsis.

D.2.1. Leptin Control of Food Intake (Figure 1.1)

The early studies of leptin focused on its anorexigenic action. Leptin levels act as a general signal of energy reserves and to modulate food intake and, therefore, leptin concentrations increase proportionately to adipose mass (and BMI) that results in high

FIGURE 1.1: LEPTIN CONTROL OF FOOD INTAKE



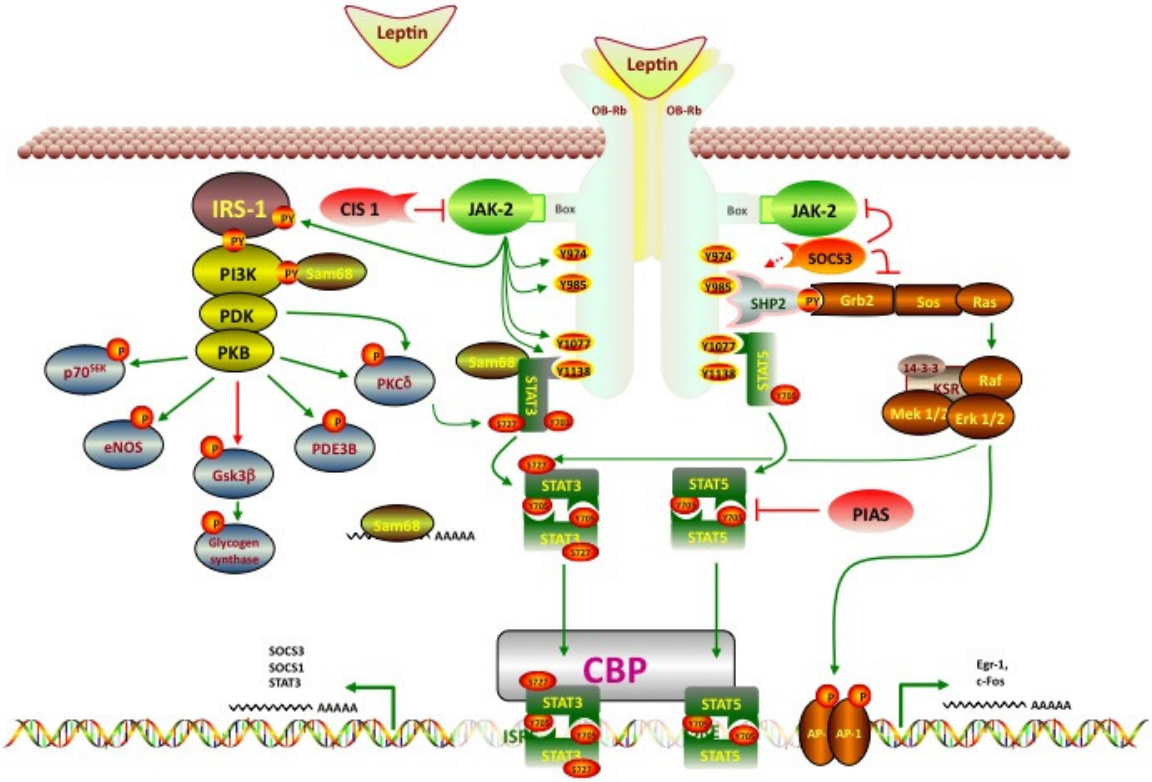
circulating levels in obese individuals (104-108). Defects in the genes encoding leptin and its receptors give rise to severe obesity and diabetes. Leptin acts to control food intake by acting on an intricate neuronal circuit involving hypothalamic and brainstem nuclei where it integrates a variety of different orexigenic and anorexigenic signals (109).

Leptin receptors are found in the highest levels in the neurons of several nuclei of the hypothalamus, especially in the arcuate (ARC) nuclei (110,111). In the ARC, leptin stimulates the production of pro-opiomelanocortin (POMC) that is then processed to produce α-melanocyte-stimulating hormone (αMSH) in neurons, which signals anorexia by activating the melanocortin-4 receptor (MC4R). In contrast, another population of ARC neurons synthesizes neuropeptide Y (NPY), an orexigenic hormone, and agouti-related peptide (AgRP) which is an antagonist of αMSH/MC4R signaling. Leptin acts via its receptor to inhibit these NPY/AgRP neurons and to suppress the expression of these neuropeptides. Therefore, leptin stimulation in the ARC results in the production of anorectic neuropeptides and suppresses levels of orexigenic peptides. Conversely, decreased leptin activity (e.g.: during starvation) stimulates appetite by suppressing synthesis of anorectic neuropeptides (POMC) and increasing orexigenic peptides (NPY and AgRP) (112-116) (Figure 1.1).

D.2.2. Leptin Receptors and Signaling (Figure 1.2)

Leptin exerts its biological actions by binding to its receptor. The leptin receptor (Ob-R) was identified shortly after the discovery of leptin itself (117). Ob-R was found to be a product of the diabetes (*db*) gene and *db/db* mice were shown to be resistant to leptin (118). The Ob-R belongs to the class I cytokine receptor family and alternative splicing give rise to six receptor isoforms: Ob-Ra, Ob-Rb, Ob-Rc,Ob-Rd, Ob-Re and Ob-Rf. The long form, Ob-Rb, is found in almost all tissues and appears to be the only form capable of transducing

FIGURE 1.2: LEPTIN RECEPTOR SIGNALING



the leptin signal while Ob-Re binds to circulating leptin and the short forms (a, c, d and f) are thought to aid in leptin transport across the blood-brain barrier (113,118-120).

Similar to other class I cytokine receptors, the main route by which leptin mediates its effects through receptors is the Janus kinase (JAK)-signal transducer and activator of transcription (STAT) pathway (119). Following leptin binding to the Ob-R, three main signaling cascades begin with the phosphorylation of tyrosine (Tyr) residues on the intracellular domain of Ob-R by noncovalently bound JAK2 (121). Tyr^{985} phosphorylation results in the activation of the Ras/Raf/ERK signaling cascade, whereas Tyr^{1077} and Tyr^{1138} activate STAT proteins. JAK2 activity leads to the activation of the STAT signaling pathway, the (PI3K) pathway and the mitogen-activated protein kinase (MAPK) pathway.

Activated JAKs transphosphorylate each other, as well as other tyrosine residues (Tyr^{985}, Tyr^{1138} and Tyr^{1077}) of the Ob-R providing “docking sites” for downstream molecules such as STATs. At the end, Ob-R signaling by leptin results in STAT3 binding and the activation of STAT1, STAT5 and STAT6 which mediate the transcriptional effects of leptin upon entry into the nucleus. STAT3 has been shown to be particularly important for mediating the effect of leptin (74,122). Recruited STATs then become tyrosine-phosphorylated by JAKs and dissociate from the receptor forming homo- or heterodimers. STAT dimers then translocate to the nucleus and act as transcription factors by binding specific response elements in the promoter regions of their target genes. (See Figure 1.2) (120,123,124).

The JAK/STAT pathway of cytokine signaling is under negative-feedback control of suppressors of cytokine signaling (SOCS) proteins. Members of the SOCS family are induced by a variety of cytokines and act as negative regulators of signaling by binding to phosphorylated Jak proteins or by indirect interaction with tyrosine phosphorylated receptors (106,125). Both SOCS1 and SOCS3 can inhibit Ob-R signaling; however, they each use different mechanisms. SOCS1 directly interacts with the kinase domain of JAK2 while

TABLE 1.3: CYTOKINE INDUCTION AND ACTION OF SOCS1 AND SOCS3 (ADAPTED FROM (126))

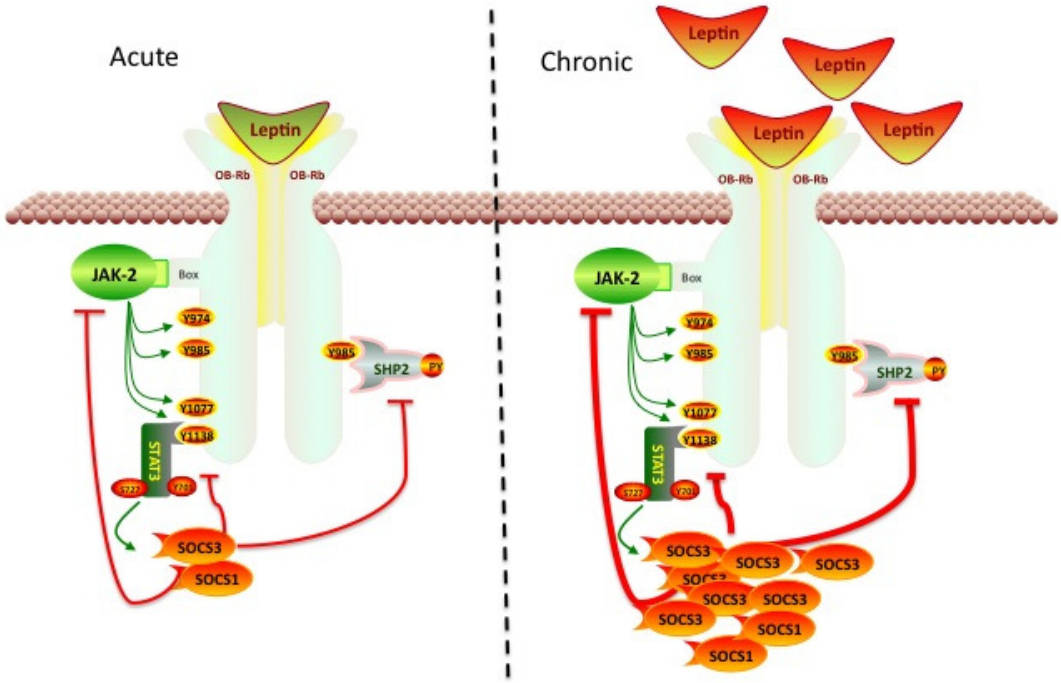
SOCS PROTEIN	**SIGNALING THAT INDUCES EXPRESSION**	**SIGNALING THAT IS SENSITIVE TO INHIBITION**	**REFERENCES**
SOCS1	IL-2, IL-4, IL-6, IL-10, INSULIN, IFN-α/β, IFN-γ, TNFα	IL-2, IL-4, IL-6, IL-7, INSULIN, LEPTIN	(127-134)
SOCS3	IL-1, IL-2, IL-6, IL-10, INSULIN, LEPTIN, TNFα	IL-2, IL-4, IL-6, INSULIN, LEPTIN, IFN-α/β, IFN-γ	(127,132-136)

SOCS3 expression inhibits the tyrosine phsophorylation of Ob-R, thus providing an important feedback mechanism for receptor signaling (136,137). SOCS proteins are induced upon cytokine stimulation and attenuate signaling by various cytokine receptors allowing possible cross-regulation among several cytokine systems. For example, SOCS3 is induced by IL-2, IL-6 and leptin and can inhibit leptin, IL-2, IL-4, IL-6, IFN-α/β and IFN-γ signaling (126,127,132,133,135)(See Table 1.3).

D.2.3. Leptin Resistance

Following the discovery of leptin, it was initially hoped that exogenous administration of leptin might induce satiety and weight loss in humans (107,138). However, leptin therapy was found not to be an effective treatment for "normal" morbid obesity that is not due to rare congenital deficiency of leptin or leptin receptors (46,139,140). In the obese state, leptin concentrations are already high as a consequence of increased fat mass. The persistence of obesity and no significant response to this increased fat mass with a reduction in food intake in spite of increased leptin levels suggest that chronically elevated leptin levels can induce a state of leptin resistance (113). The identity of the crucial mediator(s) of leptin resistance still remains unclear; however, it is hypothesized that leptin resistance may occur due to a leptin-induced increase in SOCS3, which blocks the intracellular transmission of the leptin signal. Indeed, hypothalamic expression of SOCS3 is elevated in several rodent models of obesity (136,137,141). How does this occur? *In vitro* data shows that chronic, high-level Ob-R activation results in its own feedback inhibition, probably via SOCS3, effectively limiting leptin signaling during chronic exposure (Figure 1.3). It is also possible that other signals may increase SOCS3 or other mediators of leptin signaling *in vivo* including inflammatory mediators such as IL-6, TNFα, fatty acids and other lipids, and other activators of counter regulatory signals. These too have been found to be

FIGURE 1.3: SOCS SUPPRESSION OF LEPTIN SIGNALING AND LEPTIN RESISTANCE



elevated in rodent models and in human obesity. The data for central leptin resistance in the ARC is quite strong and suggests that the ARC is the most important site in the development of leptin resistance. The data for peripheral leptin resistance, such as in cells of the immune system is less clear (104,105,113,142-145).

D.2.4. Leptin and the Immune System

In addition to adipocytes, many other cell types are now known to secrete and/or respond to leptin (146). Leptin's role in regulating immunity has been fueled by early observations of thymic atrophy in *db/db* mice (147). In addition, *ob/ob* mice are immunodeficient with increased susceptibility to bacterial and viral infections (148-150). The general consensus seems to be that leptin exerts a proinflammatory role, while at the same time protecting against infections (14). In experimental animal models, inflammatory stimuli acutely induce leptin mRNA and increase serum leptin levels (100). Leptin seems to exert its effects on immune cells through the JAK/STAT pathway. In peripheral blood mononuclear cells, leptin increases JAK2/3 and STAT3 phosphorylation, which promote proliferation and activation of T lymphocytes upon mitogen-stimulation (72)

Leptin protects T lymphocytes from apoptosis and regulates T cell proliferation and activation. Leptin induces a shift toward a Th1 cytokine-production profile (151,152), which is necessary for recovery from a number of viral infections, including influenza. In mouse models, leptin is reported to stimulate the proliferation of T cell *in vitro*, to promote T_H1 responses and to protect T cells from apoptosis. *Ob/ob* mice display an increased thymocyte apoptosis and reduced thymic cellularity compared to wildtype controls and peripheral administration of leptin reverses these defects (101,153-155).

In addition to its effects on T cells, leptin has been shown to affect the innate immune response. Leptin has been shown to influence monocyte activation, phagocytosis and

cytokine production. Macrophages from leptin-deficient mice have decreased phagocytic function and altered cytokine expression. Additionally, leptin can enhance oxidative species production in stimulated polymorphonuclear leukocytes and can inhibit the migration of neutrophils in response to classical chemoattractants (156-158). Overall, it appears that leptin increases the activity of phagocytes and may thereby contribute to host defense (153).

Most of the data available on the effects of leptin on immunity stems from experiments utilizing mice. However, the effect of leptin on the human immune system is just now being explored. The presence of leptin receptors has been documented in human circulating $CD4^+$ and $CD8^+$ T lymphocytes. The few cases of leptin deficiency described in humans have demonstrated reduced numbers of circulating $CD4^+$ T cells, impaired T cell proliferation and cytokine release, all of which are reversed by recombinant human leptin administration (159,160). It is unclear at this time whether leptin resistance, which is more common than leptin deficiency in adult obesity, is also associated with these same changes in T cells and cytokines, or whether leptin plays a role in immune alteration of obesity at all.

Similar to leptin deficiency, severe malnutrition has been associated with thymic atrophy, reduced T-cell function and increased susceptibility to infection. These changes are in correlation with the sharp reduction in leptin levels observed at extremely low BMIs (161-163). Taken together, experimental data indicate that chronic leptin deficiency differentially affects innate versus adaptive immune responses. Innate responses are altered by inadequate control of the inflammatory response while adaptive responses are severely attenuated. If peripheral leptin resistance causes a state similar to that of leptin deficiency in immune cells, this resistance could account for the immunodeficiences observed in obese individuals.

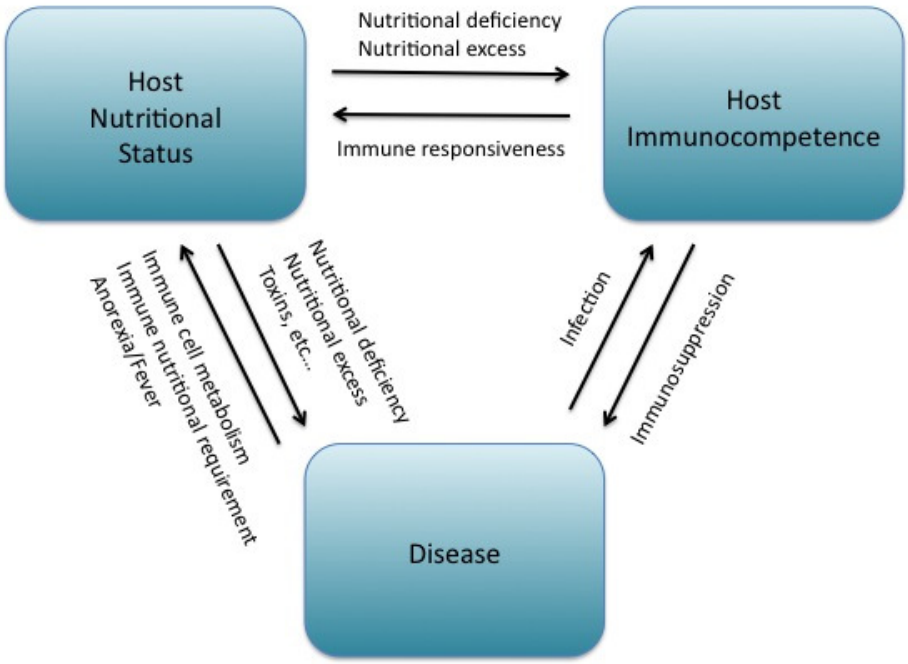
D.3 Obesity and Immunity

Nutrition and immune function are intimately linked. Therefore, immunocompetence is dependent on nutritional status and can be easily dysregulated in states of imbalanced nutrition such as obesity (Figure 1.4). Lymphoid tissues have an extremely rapid turnover and appear to be particularly sensitive to nutrient imbalances, especially those which affect metabolic pathways and functions necessary for adequate immune defense (164). It is currently unknown exactly why obesity leads to changes in the immune system; however, many pathways such as nuclear factor kappa B (NF-κB) and Phosphoinositide-3 kinase (PI3K) are altered in the obese subject which have an important role in the immune response (164,165).

D.3.1. Obesity and Immunity in Humans

Epidemiological data support the idea that obesity can affect immune function in humans. In the hospital setting, obese patients are more likely to develop secondary infections and complications such as sepsis, pneumonia, bacteraemia and wound and catheter-related infections. Patients with increased BMI and adiposity also present a higher incidence of surgical site infections, which have been associated with increased risk of other wound complications, increased length of stay and increased risk of death (166-171). Obesity negatively affects pulmonary function, and hospitalized obese patients have been shown to be at increased risk for pulmonary aspiration and community-related respiratory tract infections (21,172). In the Health Professionals Follow-up Study and the Nurses Health Study II, increased BMI and weight gain (versus weight maintenance) were directly associated with increased risk for community-acquired pneumonia in women (173,174). Increased susceptibility to acute respiratory tract infection has also been shown to be associated with BMI in overweight children (175). In addition to increased susceptibility to

FIGURE 1.4: NUTRITION AND IMMUNE FUNCTION RELATIONSHIPS (ADAPTED FROM (164))



respiratory tract infection, population-based studies have defined a significant association between obesity and asthma. In one of the more recent studies, Beuther and Sutherland (2007) report that the odds of developing asthma nearly double in obese (BMI ≥30) compared with normal-weight individuals. In addition, it was estimated that approximately 250,000 new cases/year of asthma be attributable to excess weight in the United States alone (176-178).

More recent studies have found clinically important changes in the immune system of obese humans (179). Studies of cellular function in obese humans follow the trend of diet-induced obese animal models and are mainly focused on *ex vivo* cellular functionality. Numerous studies exhibit a lowered capacity of lymphocytes from obese individuals to respond to mitogen stimulation (180). Neiman et. al. (1999) showed obesity was related to elevated leukocyte and lymphocyte subsets with lowered T and B-cell proliferation in response to mitogen stimulation (181,182). These alterations in T cell subsets have been suggested to be linked to increases in TNFα and expression of other cytokines (183-186). Even less is known about the impact of obesity on the response to vaccination. A small set of studies has shown that obesity can impact seroconversion and that obese adults and children have reduced antibody titers in response to Hepatits B and tetanus vaccines (187-189).

Several studies have assessed immune functionality in obese individuals following weight loss or dietary restriction. The majority of these studies show increased immune responsiveness and improvement. For example, a study by Tanaka et al. (2001) showed increased T cell responsiveness to mitogen following a weight reduction program (190). It must be noted that these improvements have not been assessed in the long term after subjects have achieved and maintained “healthy” weight. Further research in this area would greatly increase the number of conclusions that could be drawn on the impact of obesity and the positive effect of weight loss on immunity.

D.3.2. Obesity and Immunity in Animal Models

Numerous studies using genetically obese rodents (mainly as a result of leptin or leptin receptor deficiency) demonstrate a global impairment in immune function in these animals. Genetically obese animals exhibit marked thymic atrophy as well as diminished T and B cell populations (147,191). Genetically obese mice express decreased levels of IL-2 versus lean animals, which may explain the lowered capacity of T cells to proliferate in these animals (192). In addition to reduced IL-2, decreased glucose uptake by lymphocytes in these animals may also inhibit proliferation (193). Apart from the reduced proliferation seen in genetically obese models, TNFα production has also been found to be increased in genetically obese animals which may influence lymphoid tissue development and induce apoptosis in lymphoid cells (180,194). Increased serum free fatty acids may also inhibit T lymphocyte signaling (192). As stated above, genetically obese animals provide an excellent model for observing the effects of extreme obesity; however, the complete lack of leptin signaling in these models lead to changes in immune system development and function which are considerably more pronounced than in diet-induced obese rodents and in obese human subjects.

In diet-induced obese animals, similar, though less pronounced, impairment of the immune system has been found (180). The majority of these studies are similar to those in obese humans where immune cell function was assessed *ex vivo*. Studies in diet-induced obese animal models have shown obese mice had lower levels of mitogen-induced IL-2, although IFN-γ and IL-4 production was increased (195). Takahashi et al. (2003) found that diet-induced obese mice had increased levels of adipocyte derived mRNA for MCP-1 (monocyte chemoattractant protein-1) as well as higher protein levels of MCP-1 in the plasma. $CD11b^+$ macrophage/monocyte population also was increased in the obese mice

(88). Additionally, studies with mice fed a high fat diet show impairment of DC function and altered T cell responsiveness (196).

Genetically obese animals exhibit decreased resistance to bacterial and viral infections; however, there are few studies which observe the effects of infection in diet-induced obese models (197,198). Previous studies in our lab have demonstrated that mice with diet-induced obesity have a dysregulated primary immune response to influenza infection. Following dietary treatment, influenza-infected DIO mice had 7 times greater mortality compared to lean controls. Degree of lung infiltration and pathology was seen to be increased at day 6 post infection. DIO mice had significantly decreased expression of IFNα/β and increased and delayed expression of pro-inflammatory cytokines and chemokines (199). In addition, dendritic cells from obese mice failed to efficiently present influenza antigen to T cells (200).

Although the evidence from both animal models of obesity and obese humans demonstrates immune impairment, surprisingly, very little is known about immune responses to viral infections in obese subjects. One virus, which constitutes a very real public health threat, is the influenza virus. An impaired response to influenza infection in obese individuals, especially in an increasingly obese population, would have grave consequences.

E. Influenza and Public Health

Influenza is a respiratory illness caused by the influenza virus. Spread through airborne transmission, these viruses are highly contagious and are responsible for a great deal of morbidity and mortality in the world (201). In any given year, 5-15% of the world population is infected with influenza virus resulting in 3-5 million cases of severe illness and 500,000 deaths from influenza and influenza-related complications (202). Young children,

the elderly and people with chronic diseases are particularly susceptible to influenza-related complications including viral or bacterial pneumonia, dehydration, and death. (203). Very recently, the CDC has suggested that obese individuals are at a greater risk of morbidity and mortality from pandemic novel influenza H1N1 strain. Speculation on the cause ranges from excess adipose tissue constricting lung volume to chronic inflammation influencing the immune response (204-206). The estimated annual economic impact of influenza in the United States alone is $12-14 billion (207).

In temperate climates, such as in the United States, flu outbreaks typically occur during the winter months from late November/December through March, but the onset, peak, duration and severity of the flu season can vary considerably from year-to-year. Typical outbreaks move though a community in 12 or more weeks; however, recent evidence has shown that there is no such thing as a typical year. For example, influenza A/Fujian/411/02 (H3N2) began to produce severe illness in children in October of 2003 (208). Occasionally, and at relatively regular intervals, severe worldwide pandemic outbreaks occur which are less constrained by season. Historically, these pandemics occur at 10-40 year intervals and originate mainly in Asia (209). Three confirmed pandemics have occurred in the past century. First, the Spanish influenza pandemic in 1918-1919 claimed an estimated 50 million lives. In addition to the 1918 pandemic, the Asian (1957) and Hong Kong (1968-1969) pandemics resulted in 1-2 million deaths (210).

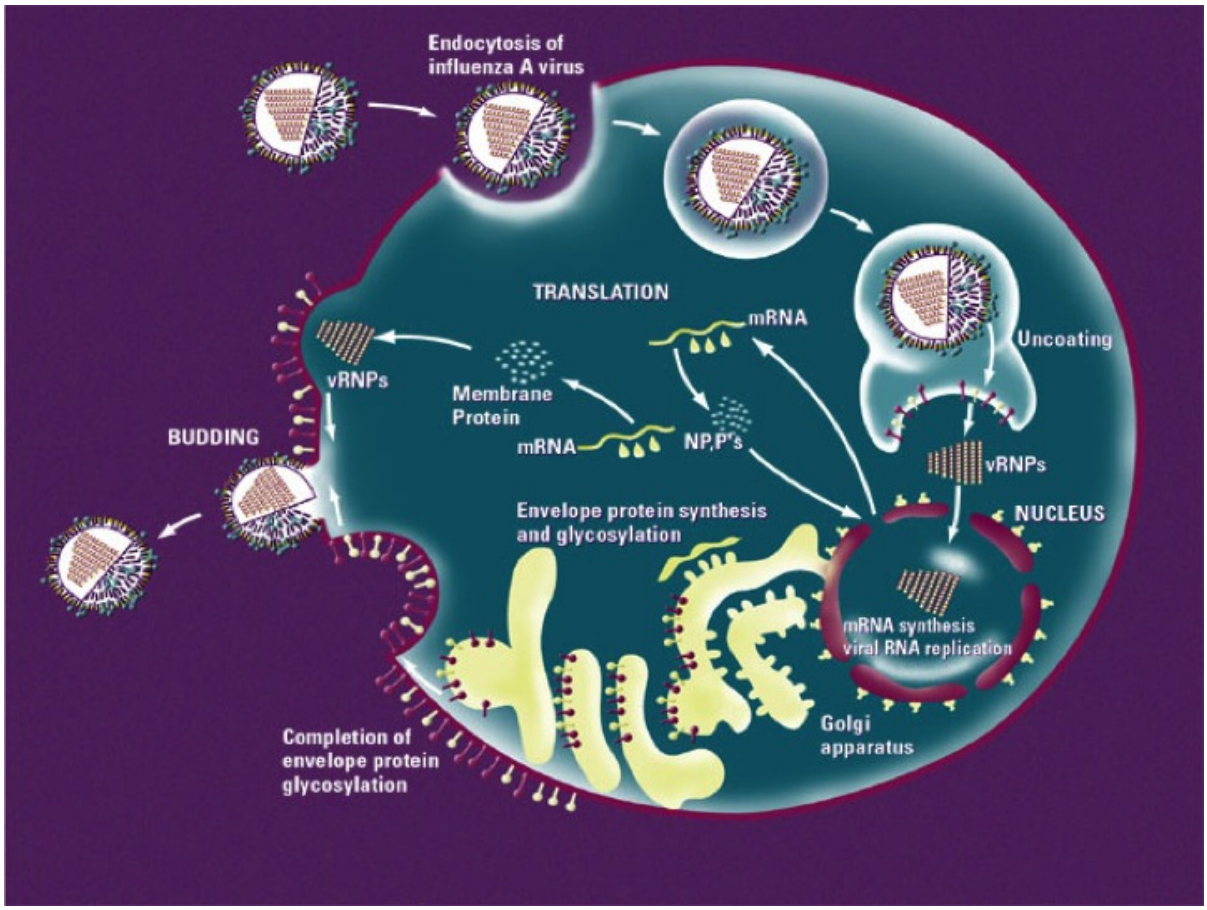
During April 2009, a novel H1N1 virus was detected in two independently-collected, unrelated cases of influenza-like virus in California which was then traced back to a number of cases of severe respiratory illness in Mexico beginning in the town of La Gloria, Veracruz in mid-February 2009 (211,212). The virus was found to be genetically and antigenically unrelated to currently circulating human seasonal influenza strains and genetically related to viruses circulating in swine. This fact, in addition to the outbreak being localized in a Mexican pig farming region, raised immediate suspicion that the virus had gone through a

pandemic shift (212,213). Indeed, genetic analysis of the virus revealed that it is a triple reassortment of strains from human, avian and swine sources (214). By the end of April, international spread and clusters of human-to-human transmission in multiple countries prompted the WHO to declare an imminent pandemic (211). On June 11, 2009 the WHO issued a pandemic alert level of six which indicated the H1/N1/09 strain had reached all the criteria for a pandemic outbreak (215,216). The H1N1/09 pandemic strain has been found to have increased severity of illness versus the seasonal H3N2 virus (217,218); however, to date the virus seems to have not reached its pandemic projections. There is great concern that the H1N1/09 pandemic strain will go through antigenic drift over the coming influenza seasons and change its pathological potential, which could theoretically result in numbers of deaths similar to previous pandemic years. (219).

F. Influenza Biology and Replication (Figure 1.5)

Influenza viruses are members of the *Orthomyxoviridae* family and are characterized by segmented, negative-strand RNA genomes. The most common of these viruses are the A, B and C genera with only influenza A and B able to infect humans. Influenza A viruses are further classified by subtype based upon their surface glycoproteins: hemagglutinin (HA) and neuraminidase (NA). Structurally, the influenza A virus is studded with these HA and NA glycoproteins (at a ratio of approximately four to one) projecting from a host cell-derived lipid membrane. A smaller number of matrix ion channel proteins (M2) span the lipid envelope. The viral envelope overlays an internal protein matrix (M1) which encloses the virion core. Inside the virion core reside the nuclear export protein (NEP) and the ribonucleoprotein (RNP) complex consisting of eight negative-sense, single-stranded viral RNA (vRNA) segments coated with nucleoprotein (NP) and the heterotrimeric RNA-dependent RNA polymerase (220,221).

Figure 1.5: Lifecycle and replication of the influenza virus



Following infection, HA spikes on the surface of influenza viruses recognize and attach to sialic acid-containing receptors on host cells of the respiratory epithelium.

Following attachment, the virus is endocytosed via clatherin-coated pits. Inside the endosome, the viral envelope fuses with the endosomal membrane releasing viral RNPs from the viral matrix into the cellular cytoplasm. Nuclear localization signals from viral proteins direct host cellular proteins to import the RNPs and other viral proteins into the host cell nucleus (222). Inside the nucleus, the viral RNA-dependent RNA polymerase uses the negative-sense vRNA to synthesize two positive-sense RNAs: messenger RNA templates for viral protein synthesis and complimentary RNA intermediates for subsequent transcription of additional negative-sense vRNA (223). Newly generated envelope proteins HA, NA and M2 as well as newly synthesized vRNA exported from the nucleus assemble into new virions at the apical plasma membrane of the infected, polarized epithelial cells. Virions egress the cells through budding resulting in a new viral envelope derived from the host cell membrane (221,224,225).

G. Immune Response to Influenza Infection (Table 1.4, Figure 1.6)

Infection with influenza virus leads to a coordinated immune response beginning with innate immune defenses responsible for limiting viral infectivity and replication. These innate defenses then signal to adaptive immune systems which are responsible for clearing the rest of the infecting virus and protecting the body again reinfection.

G.1. Innate Immune Response

The innate (non-specific) immune response is the first line of defense. Found in most living organisms, this system detects invading pathogens and promotes a coordinated pattern of cytokine secretion which acts to inhibit viral replication and induce

TABLE 1.4 CYTOKINES AND CHEMOKINES EXPRESSED DURING INFLUENZA VIRUS INFECTION [ADAPTED FROM (226)]

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 ANTIVIAL 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-2	GROWTH, DIFFERENTIATION AND SURVIVAL OF ANTIGEN-SELECTED T CELLS	T 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
MCP-1	RECRUITS MONOCYTES/MACROPHAGE, DENDRITIC CELLS AND T CELLS	RESPIRATORY EPITHELIUM? MACROPHAGE ??
MIP-1α/β	RECRUITS MONOCYTES/MACROPHAGE, T CELLS ACTIVATES NEUTROPHILS	MONOCYTES/MACROPHAGE DENDRITIC CELLS T CELLS NEUTROPHILS
RANTES	RECRUITS T CELLS, EOSINOPHILS AND BASOPHILS, MONOCYTES/MACROPHAGES, DENDRITIC CELLS ACTIVATES T CELLS	RESPIRATORY EPITHELIUM T CELLS

proinflammatory factors which aid in reducing viral spread. Influenza viruses preferentially infect the epithelial cells and macrophages of the respiratory tract (221). Influenza-infected epithelial cells and macrophages respond to infection by producing antiviral, chemotactic, proinflammatory and other immunoreguatory cytokines (221,227,228). Type I Interferons (IFNα and IFNβ), interleukins 1 (IL-1) and 6 (IL-6) and tumor necrosis factor alpha (TNFα) are all part of these signals. IFNα and IFNβ are major antiviral cytokines and can mediate direct antiviral activity of cells. In addition, these cytokines also have antiproliferative and immunomodulatory function. Proinflammatory cytokines IL-1β, IL-6 and TNFα are readily produced by infected macrophages but do not contribute directly to antiviral immunity (229). IL-1β and TNFα enhance the inflammatory response and further activation of antigen presentation. IFNα/β increases the recruitment of monocytes/macrophages and T cells to the site of infection, enhances antigen presentation on macrophages and dendritic cells and acts to promote the T_H1 response (221,229-232).

Innate immune mechanisms exist to restrict the replication of influenza viruses within the host early during infection allowing for the development of pathogen-specific adaptive responses which are needed for viral clearance. Many of the key players in innate immunity act to signal and promote the adaptive response. Dendritic cells (DC) are principal antigen presenting cells and are the initiators and modulators of the adaptive immune response (233). DCs can be directly infected with influenza; however, they uptake virus for presentation to T cells on the major histocompatibility complexes. Cytokines produced by DCs during antigen presentation play an important role in the activation and polarization of T cell responses. During influenza infection, DCs produce IL-12 which stimulates Th1 responses and functions directly on $CD8^+$ T cells to augment viral clearance and IFNγ production (233,234). DCs also produce IFN-α/β. The production of IFN-α/β by DCs, plus their production of IL-12 greatly impacts natural killer (NK) cell expansion and activation

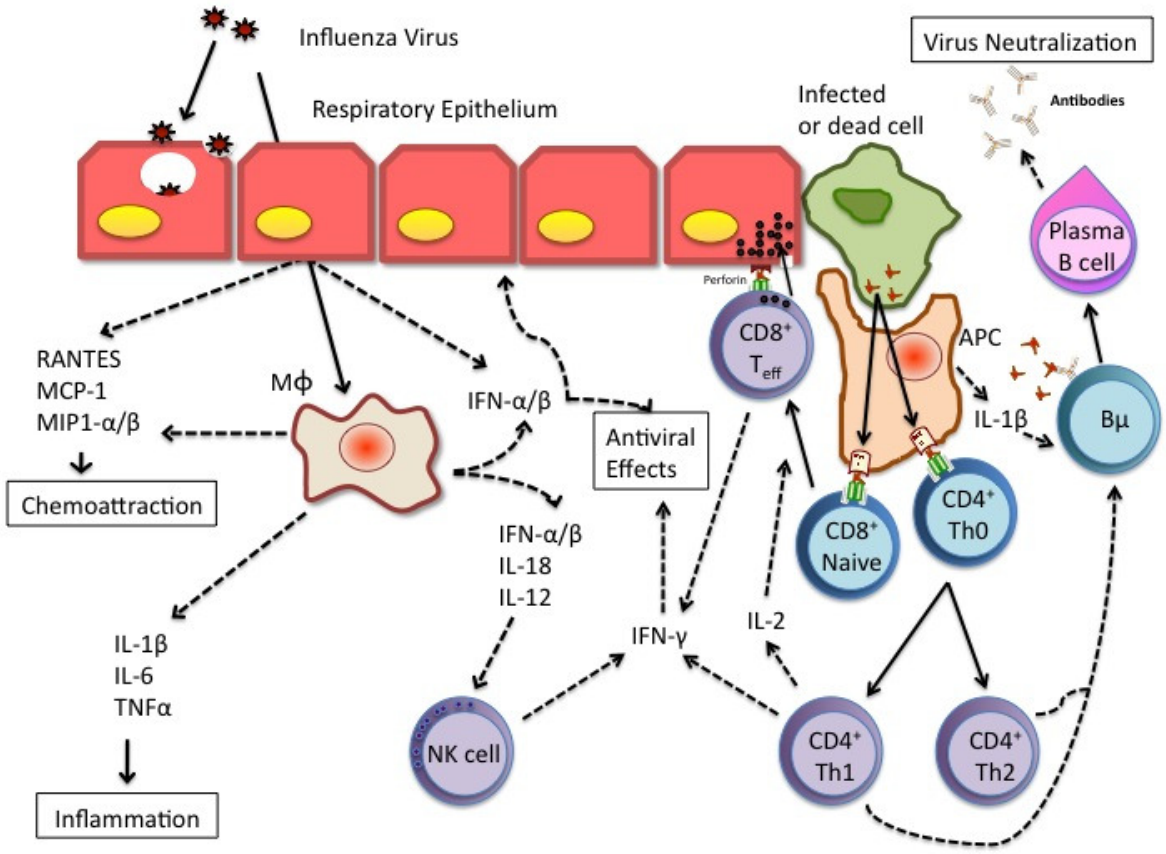
(235). NK cells are cytotoxic lymphocytes that kill virally infected cells. The ability of NK cells to recognize virally infected cells relies on a system of recognition by their receptors on NK cell surfaces. Killer-inhibitory receptors recognize MHC class I molecules expressed by all nucleated cells. Infection with influenza results in the down regulation of MHC Class I molecules leading to activation of NK cell killing (236). Therefore, dysregulation of the innate response can lead to greater viral spread and decreased promotion of viral clearance by the cells of the adaptive immune response (237).

G.2. Adaptive Immune Response

Composed of highly specialized, systemic cells, the adaptive immune system acts to eliminate pathogens from the body. Development of an effective adaptive response is highly dependent on signals received during the innate response (See Figure 1.6). Secretion of cytokines by innate cells early during infection promotes the development of $CD8^+$ cytotoxic T cells and $CD4^+$ helper T cells. During the initial stages of infection, APC such as DC help to “select” cells that exhibit the appropriate pathogen-specific receptors. Upon viral exposure, dendritic cells of the innate immune response in the lungs mature, take up antigen or are directly infected by the virus, and selectively migrate to draining lymph node (LN; cervical and mediastinal) where they display antigen peptides to naïve T cells. Interaction between APCs and its matching T cell receptor (TCR) promotes the proliferation and development of a population of antigen-specific T cells that enable the adaptive response (238). Additionally, optimal T-cell activation requires co-stimulation of the T cell receptor CD28 contact with CD80/CD86 on the APC (239).

Naive $CD4^+$ T cells can differentiate into helper T cells (T_h) with either a Th1 or Th2 phenotype, regulatory T (T_{reg}) cells or T_h17 cells. $CD4^+$ T cell differentiation depends on the

FIGURE 1.6: IMMUNE RESPONSE TO INFLUENZA INFECTION



type of pathogen and by the cytokines that are secreted during presentation on MHC Class II. During influenza infection, IL-12 secretion from DC polarize the $CD4^+$ T cell toward a Th1 phenotype, resulting in IL-2 and IFN-γ production which then activate $CD8^+$ T cells. 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 (240).

Resolution of a viral infection often relies on the induction of a $CD8^+$ T cell response (241). $CD8^+$ T cells actively identify and eliminate virally infected cells. During an influenza virus infection, T cell responses peak between day 7-10 post infection (p.i.) resulting in viral clearance by day 15 (242-245). Upon contact with an infected cell, $CD8^+$ T cells act to inhibit viral replication and to destroy virally infected cells. These functions are achieved through the secretion of IFNγ and perforin/granzyme B as well as Fas/FasL pathway (246). $CD8^+$ CTLs recognize MHC class I antigenic-peptide complexes on virus-infected epithelial cells and kill cells using perforin and granzymes (247-249). In the perforin/granzyme B pathway, $CD8^+$ T cells insert perforins into the membranes of infected cells which is followed by the release of granzymes. This activates caspases and results in apoptosis. Alternatively, caspase activity can be activated by Fas/FasL interaction. IFN-γ, which is produced by cytokine stimulation or by cell-cell interactions, is not directly involved in the lysis of infected cells; however, IFN-γ secretion enhances the development of cell-mediated immunity, activates macrophages, and increases antigen presentation (250-252).

Apart from the T cell response, the B-cell mediated response to influenza infection has long been thought to play a less important role; however, studies have shown that B-cell deficient mice have significantly reduced survival when challenged with A/PR/8 but not when challenged with influenza X-31. These studies indicate that B cells make a significant contribution to recovery during a substantial challenge with influenza (253). Following viral

infection, influenza-specific naïve B cells recognize viral particles or are presented antigen by helper T cells. The activated B cells then proliferate and differentiate into resting memory cells and antibody-secreting plasma cells (237). Antibodies directed towards the viral proteins then function to help eliminate viral replication and spread. Ergo, a coordinated cross-talk between the innate and adaptive immune responses is important for a robust response and protection against infection with influenza virus.

H. Protection against Influenza Infection

H.1. Vaccination

Traditionally, protection against influenza has been achieved using inactivated influenza virus vaccines, which were first employed over 60 years ago and are still the principal mode of immunization, although live attenuated vaccines are beginning to be used worldwide (254,255). Each year, an advisory committee of the Food and Drug Administration and WHO uses laboratory studies of flu strains and epidemiological data of flu activity to determine three viruses (two different subtypes of influenza A viruses and one influenza B virus) to target in generating the flu vaccine for the following fall and winter. Usually one or two virus strains that the vaccine targets are changed each year. In the US, the Advisory Committee on Immunization Practices for the prevention and control of influenza have identified groups that should be targeted for annual vaccination including persons at risk for complications of influenza as well as those likely to transmit influenza to other high-risk persons. All persons wishing to avoid influenza are encouraged to receive the vaccine (256)

Studies of influenza vaccine efficacy and effectiveness have used a variety of outcomes or case definitions that can dramatically affect the numbers reported (257). In the

best case scenario, criteria for the effectiveness of the vaccine is based on the induction of an adequate level of virus-neutralizing (hemagglutination-inhibiting) antibodies in the serum. Antibodies against influenza virus are primarily directed against the envelope glycoproteins of the virus, the hemagglutinin and the neuraminidase. It has been well-established that the major target for neutralization of the virus is the hemagglutinin, and circulating levels of antibody against the hemagglutinin can prevent infection with influenza (258). However, the overall efficacy rate of vaccination, particularly in the high-risk elderly populations, is approximately 60%. One of the reasons suggested for the suboptimal efficacy is age-related changes in the cell-mediated component of the immune response. Antibody production by B cells requires a $CD4^+$ T cell response, and thus a suboptimal $CD4^+$ T cell population will lead to lowered antibody production (237).

The effectiveness of the flu vaccine in protecting individuals against illness or serious complications of flu depends primarily on the immunocompetence of the person receiving the vaccine, previous exposure to influenza and flu vaccine, and the similarity between the virus strains in the vaccine and those infecting the population (259). When the match between vaccine and circulating strains is close in a given flu season, flu vaccine prevents illness in approximately 70%-90% of healthy persons younger than age 65 years. Among community-dwelling elderly persons, flu vaccine is 30%-70% effective in preventing hospitalization for pneumonia and influenza. Among elderly nursing home residents, flu vaccine can be 50%-60% effective in preventing hospitalization or pneumonia and 80% effective in preventing death from influenza (260-262)

H.2. Antigenic Drift and Shift

Hosts which survive a primary infection with influenza virus are generally immune to reinfection with the same virus. Despite this protection, influenza infections continue to infect individuals yearly. This lack of immune protection from repeated infection stems from the fact that influenza structural proteins have robust structural plasticity and can tolerate a number of amino acid substitutions. The genomes of influenza viruses consist of eight separate segments of single-stranded, negative-sense RNA. During viral replication inside of the cell, the RNA-dependent RNA polymerase is highly error-prone resulting in a large variation in viral RNA. Since antibodies to HA neutralize viral infectivity, influenza virus strains evolve frequent, single amino acid changes in the HA molecule. These relatively minor changes, termed *antigenic drift*, accumulate and eventually result in a strain that is no longer effectively neutralized by host antibodies and can produce a productive infection (263).

In addition to the small mutations resulting from drift, the segmented genome of the influenza virus enables occasional gene reassortment during a dual infection with two different influenza viruses. In this situation, the resultant influenza virus strain acquires the HA segment, and possibly the NA segment, from a completely different influenza virus subtype. This *antigenic shift* can happen in cells infected with different human and animal influenza viruses and may result in a completely novel set of surface glycoproteins which the human populations has not previously encountered. A pandemic occurs when the human population is susceptible, but immunologically naïve, to this new virus. Indeed, it has been confirmed that both the Asian (H2N2) and Hong Kong (H3N2) pandemics were reassortants containing genes derived from previously circulating human influenza subtypes and genes that appeared to be derived from avian subtypes (263,264). Interestingly, gene sequences

of the 1918 pandemic strain from formalin-fixed lung autopsy samples and from unfixed tissue from an Alaskan pandemic victim indicate that the 1918 virus may have arisen solely from adaptive mutations and not by reassortment (265,266).

H.3. Humoral versus Cell-mediated Immune Memory

A vaccine which promotes a robust memory B cell antibody-based response to the surface proteins of one strain of influenza is likely to be ineffective for a strain encountered the next season expressing different surface proteins (267). However, there are several internal viral proteins that are highly conserved among influenza viruses, and therefore do not experience the drift and shift problems seen with the external proteins. Although these proteins do not generate an effective antibody response because they are not exposed on the surface of the virus and, therefore, not accessible to B cells, influenza-specific T cells can recognize these proteins. Memory T cells generated during a primary influenza infection can target these internal proteins common to influenza strains, making them effective against encounters with heterologous virus stains. The ability to generate functional memory T cells, either during primary infection or by vaccination, has proven to be protective against potentially lethal influenza strains exhibiting completely different surface antigens (268). Indeed, exploiting this know capability of memory $CD8^+$ T cells to recognize a broad range of heterosubtypic viruses is currently a hot focus of vaccine development (269,270)

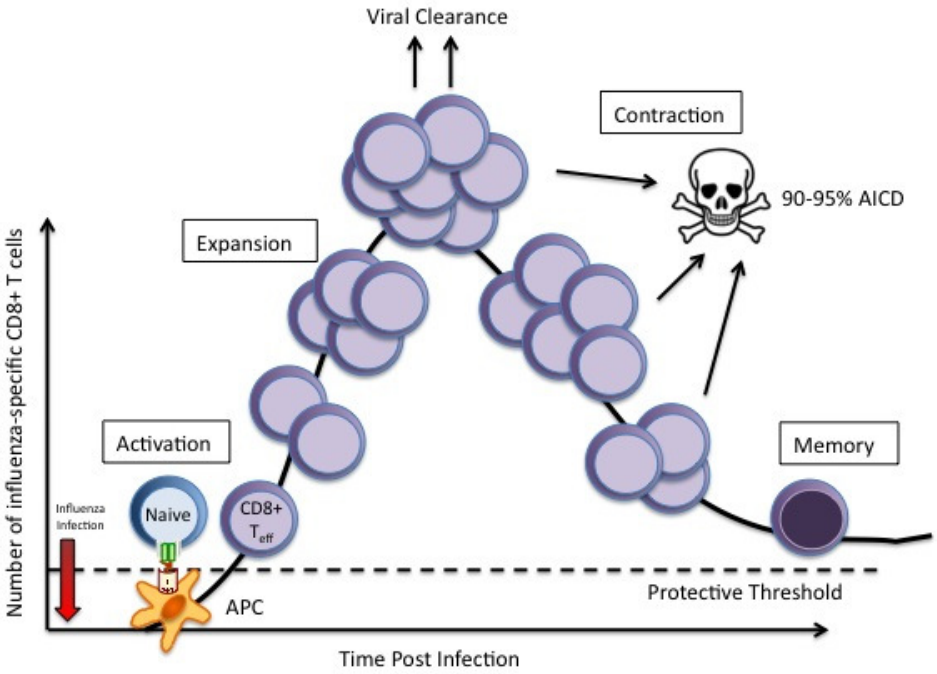
H.3.1. Memory $CD8^+$ T cell Generation

$CD8^+$ T cell responses have evolved to specifically eliminate pathogens and to protect against reinfection (271,272). The generation of long term immunity is dependent on the formation a pool of long-lived memory T cells. During infection with influenza A/PR/8 in

a rodent model, lack of $CD8^+$ cells results in decreased viral clearance and eventual morbidity (273). Initial recognition by a naïve $CD8^+$ T cell of a foreign peptide/major histocompatibility complex (MHC) class I on an antigen presenting cell (APC) results in rapid clonal expansion and gain of effector function. The extent of the influenza-specific $CD8^+$ effector T cell response can be related to viral load which is, presumably, reflective of antigen load on DCs (274). As in the primary response, upon contact with an infected cell, influenza-specific memory $CD8^+$ T cells act to inhibit viral replication and to destroy virally infected cells. These functions are achieved through the secretion of IFNγ and perforin/granzyme B as well as Fas/FasL pathway (246). Following infection, activation and expansion of naive influenza-specific $CD8^+$ T cells occur in the draining LN (242,244) These cells then migrate and localize to the lungs and infected airway epithelium where they exert their effector functions, producing antiviral cytokines and lysing target cells presenting viral determinants for which they bear a specific T-cell receptor (242,244,247)

Following viral clearance, the activated effector T cells are no longer needed and activated short-lived effector cells (SLEC) responding to the primary infection are eliminated from the antigen-specific population (237,275). Contraction of activated $CD8^+$ T cells results in a pool of memory cells representing ~5 to 10% of effector $CD8^+$ T cells found at the peak of expansion during primary infection (276-278). Cessation of the antigenic stimulus prompts the activated effector cells to undergo activation-induced cell death (AICD). This apoptosis can be induced by a number of mechanisms including interaction of the Fas molecule on the T cell surface with the Fas ligand, a member of the TNF family of cytokines (237). Downregulation of the protective Bcl-2 gene during activation of effector $CD8^+$ T cells leaves the cells vulnerable to Fas/caspase contraction, mainly mediated through the proapoptotic molecule Bim. Activation of Bim leads to proapoptotic factors being released from the inner mitochondrial membrane into the cytosol, such as cytochrome c which contributes to the formation of the apoptosome and the subsequent activation of the caspase cascade (237). T

FIGURE 1.7: GENERATION OF MEMORY T CELLS DURING PRIMARY INFECTION



cells lacking Bim undergo normal expansion but are more resistant to contraction (279). Effector $CD8^+$ T cells with increased Bim and reduced Bcl-2 expression, generated through a knockout of the Id2 transcription factor, have increased contraction following the effector phase (280). The extent of contraction can be modulated by the extent of infection duration and resulting inflammatory signals. Short-term pathogen infection decreases the size of the resultant memory pool while insufficient duration or quality of TCR signal or other signals can lead to increased contraction and a reduction of the memory cell pool (276,278,281).

H.3.1.1. Delineation of Memory versus Primary Effector T cells (Figure 1.8)

Recent studies of the generation of long-term immunity and efficacious vaccination against viral agents have begun to focus on the generation of large numbers of long-lived, antigen-specific $CD8^+$ memory T cells. From these studies, it has become apparent that generation of function $CD8^+$ memory T cells requires a “balancing act” between memory cell potential and terminal differentiation into full effector T cells (282). Antigen density on the dendritic cell and time of contact with the APC, costimulatory factors and signals and levels of inflammatory cytokines could play a role in programming the development of memory potential (283). The lack of an early definitive marker that can distinguish an effector cell from a memory cell hampers a clear definition of when *bona fide* memory cells are generated during a $CD8^+$ T-cell response; however, studies have shown altered T cell responses can lead to changes in memory cell populations and function. Timing, location and amount of exposure to antigen during $CD8^+$ T cell development appear to be very important.

Memory $CD8^+$ T cells are preferentially established in draining lymph nodes at early (days 1-3) and late (day 28) but not acute (days 7-10) time-points after influenza infection. The least efficient population for transfer of memory is the highly activated day 8-10 population (284). While it is clear that early and late events also shape $CD8^+$ T-cell memory,

FIGURE 1.8: MODELS OF MEMORY T CELL DIFFERENTIATION

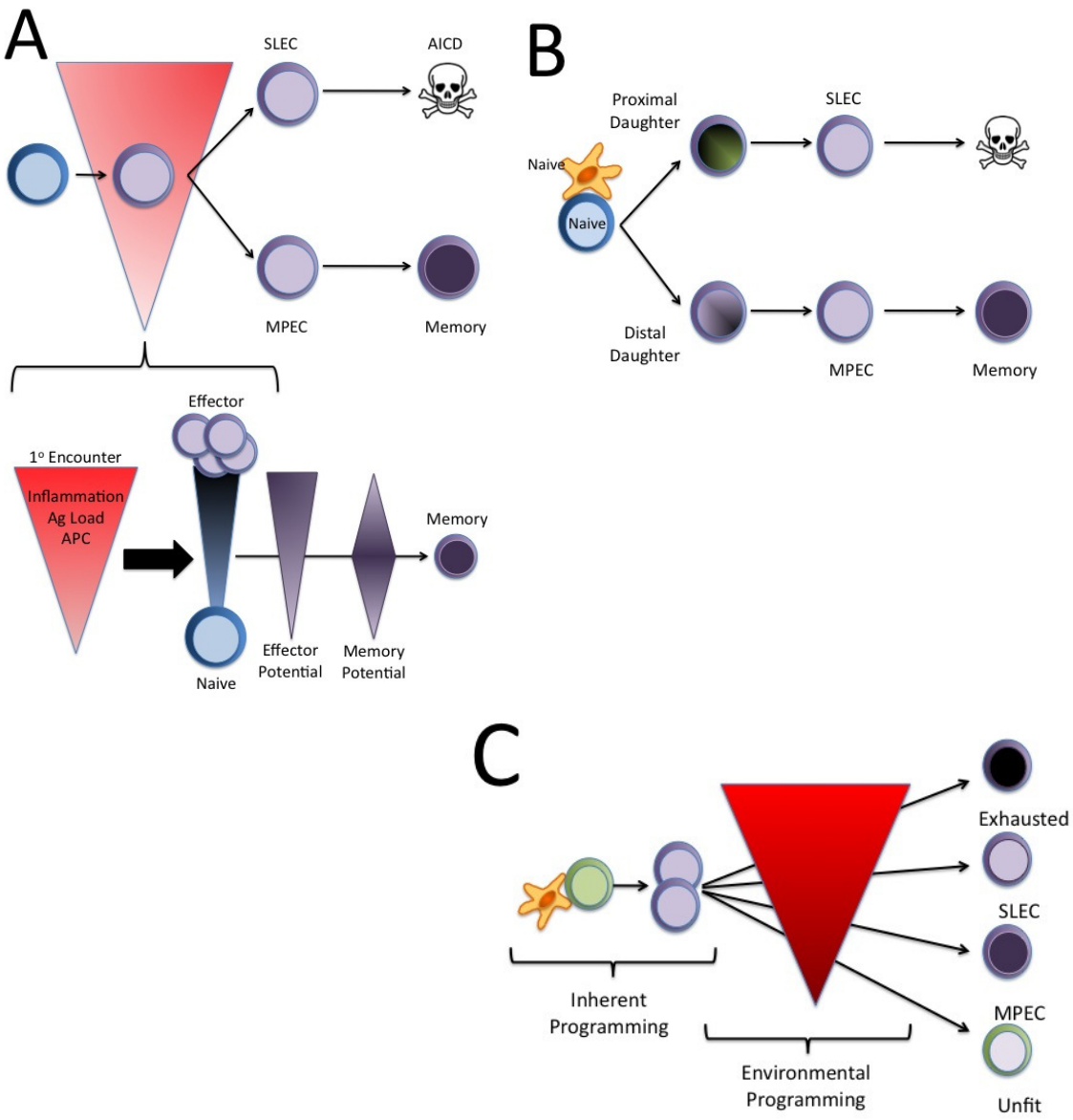


TABLE 1.4: PHENOTYPIC CHARACTERISTICS OF SLEC AND MPEC (ADAPTED FROM (285-287))

	SLEC	MPEC
T-BET	HI	LO
EOMES	LO	HI
BLIMP-1	HI	LO (TCM) /INT (TEM)
BCL-2	LO	HI
IL-7R	LO	HI
KLRG1	HI	LO
CD62L	LO	HI (TCM) /LO (TEM)
CCR7	LO	HI (TCM) /LO (TEM)
GRANZYME B	+	-
IFN-γ	+	-

studies show that a brief contact with antigen might be all it takes to set up a developmental program that will ultimately generate T cells with memory characteristics. Initial dose, but not the duration of acute infection, determines the magnitude of $CD8^+$ T-cell expansion. Also, expansion is highly dependent on the numbers of naïve T cell precursors recruited to the response (276). Programming of the CTL and memory responses have been shown to be accomplished during the first 2-3 days of infection, indicating the quality and context of the antigenic signal received is very important for determining $CD8^+$ T cell fate (288).

Compared with primary effector T cells, memory $CD8^+$ T cells exhibit less terminally differentiated phenotypes because they are multipotent, go through self-renewal and have a high proliferative potential and increased longevity (289). The ability to differentiate between effector $CD8^+$ T cells that are destined to survive and become long lived memory $CD8^+$ T cells (memory precursor effector cells; MPEC) from those which will undergo activation-induced cell death (AICD) post clearance (short-lived effector cells; SLEC) has greatly improved using a combination of functional criteria and surface marker expression. For instance, IL-7R and KLRG1 are inversely expressed on MPEC and SLEC with MPEC being $KLRG1^{low}IL\text{-}7R^{high}$ while SLEC are $KLRG1^{high}IL\text{-}7R^{low}$ (290). However, it has been difficult to track the developmental lineage between MPEC and SLEC because the memory T cell population appears to continuously evolve over time post infection (290-293). Also, it must be noted that MPECs do not “bypass” the effector cell stage and therefore “memory” and “effector” fates does not mean that the “memory” fated T cells do not acquire effector functions during the primary infection. Several developmental models exist to explain how $CD8^+$ T cells gain or maintain memory cell potential while others do not.

The two most currently debated models are the “linear differentiation model” and the “bifurcative differentiation model” (Figure 1.8). In the linear differentiation model (Figure 1.8A), stimulation of naïve T cells with antigen in the presence of environmental factors such as costimulation and inflammation leads to a population of effector T cells. Effector T cells

can then become either MPEC or SLEC depending on the extent of environmental cues (239,282,294). These environmental cues can be considered to have a “Goldilocks effect” (Figure 1.8A) where increased levels of inflammation, antigen presentation, costimulation, etc. can drive the differentiation towards an increased effector population and thereby decreasing memory potential (275,294-296)

In the bifurcative differentiation model (Figure 1.8B), differentiation between SLEC and MPEC occurs based on initial antigen recognition and that one T cell can give rise to two daughter cells with different differentiation fates. Following T cell contact with an APC, the distal daughter (the daughter arising from the side of antigen contact) gives rise to MPEC and the proximal daughter (the daughter cell arising from the side opposite of antigen contact) gives rise to SLEC (294) The current hypothesis suggests that both the linear and bifurcative differentiation models can be combined (Figure 1.8C) resulting in a model where memory T cell versus effector T cell fate then becomes a very fine tightrope between SLEC and MPEC generation. Initial antigen contact stimulates inherent programming of effector T cells. The remainder of the expansion phase is then controlled by the duration and intensity of other signals including inflammation and costimulation which may control the expression of key transcription factors such as T-bet and Blimp-1 (275,282,297).

H.3.1.2. Effect of Antigen Presentation and Costimulation on Memory T cell Generation

Infection results in the upregulation of innate anti-viral responses which act to defend against a pathogen. Subsequently, cross talk between cells of both arms of the immune system promotes the activation of an adaptive response. APCs, such as dendritic cells (DCs) and macrophages (MΦ), act as mediators in this cross talk. Initiation of the CTL response has been shown to be dependent on MHC Class I presentation of peptide

displayed by bone-marrow derived APCs such as DCs and macrophages (298). Both DCs and MΦ are capable of stimulating naïve T cells *in vitro*; however, *in vivo* studies have strongly suggested that DCs are essential for the priming of naïve precursors while MΦ may not play as prominent of a role (299,300). The presence of DCs, their interaction with T cells and even their level of activation have all been found to affect T cell priming and the subsequent primary and secondary T cell responses (301,302). Vaccination with peptide coated dendritic cells results in accelerated generation of the $CD8^+$ memory response (303).

Costimulation has also been found to be important for the generation and functionality of memory T cells. While costimulation has beneficial effects on proliferation and cytokine production in naïve T cells, increased costimulation (or lack of costimulation) can have a negative effect on the generation and subsequent functionality of effector/memory $CD8^+$ T cells (304). The activation of naïve T cells requires receiving two signals from professional APC. The first signal is provided by the TCR following recognition of a viral antigen presented by the MHC. The second signal is provided by CD28 on the naïve T cell binding to its ligands, CD80 and CD86, on the APC. CD28 promotes the assembly of the T cell signaling complex and amplifies the signal generated by the TCR (305). Additionally, CD28 also increases cell cycle activity via enhanced production of IL-2 and promotes survival by upregulating antiapoptotic molecules (306-308). Lack of CD28 costimulation leads to a greatly reduced primary response and subsequent deficiency in memory cell generation (309-311). In addition to CD28, several TNF receptor family molecules including CD27 and CD40 have also been considered to be important costimulatory receptors (312,313). CD27 is expressed on lymphoid cells and its expression is upregulated upon priming. The ligand of CD27, CD70, is exclusively expressed on activated T cells and mature DCs (314,315). Thus, CD27-CD70 interaction is important for both T cell-DC interaction in the lymphoid organs as well as T cell-T cell communication in peripheral sites of infection. Lack of CD27 in mice during an influenza infection results in

decreased generation of antigen-specific $CD8^+$ T cells in the lungs and, therefore, decreased memory populations over time (316,317). CD40 is expressed constitutively on all APCs while its ligand (CD40L; CD154) is expressed mainly on activated $CD4^+$ T cells(318). Binding of CD40 to CD40L results in APC activation and can be considered a form of $CD4^+$ "help" to promote and induce antiviral $CD8^+$ T cell responses as well as production of memory cell populations (319-321). Expression of both CD40 and CD40L have been shown to affect memory cell generation, most notably with decreased numbers of memory cells generated in $CD40L^{-/-}$ mice and absence of CD40 signaling results in impaired $CD8^+$ T cell memory irrelevant of the presence of $CD4^+$ T cell help (322,323).

H.3.1.3. Effect of Inflammation on Memory T cell Generation

Recent evidence also points out that inflammatory signals received during the primary infection can also determine the size and function of the memory cell pool. Inflammation can act as a "third signal" in the determination of the balance of effector and memory differentiation. Increased inflammation is associated with increased primary effectors but decreased memory precursors and differentiation into long-term memory. Reduced inflammation leads to reduced T cell contraction and greater numbers of $CD8^+$ memory T cells (281). Increased expression of the proinflammatory cytokine IL-12 results in decreased memory generation. Mice lacking IL-12 (*p35*$^{-/-}$) have greater numbers of memory cells, reduced contraction and increased protection from secondary infection, despite a weaker primary $CD8^+$ response (295,324). Induction of inflammation without altering the duration of DC antigen display prevented the rapid generation of $CD8^+$ memory cells in wildtype mice, but not in mice lacking IFN-γ receptor (303). Additionally, IFN-γ knockout mice have increased levels of influenza-specific $CD8^+$ T cells in the spleen and reduced levels in bronchioalveolar lavage fluid following infection (325). The adverse effect of inflammation on

T cell development may help to ensure proper viral clearance and only allow antigen-specific memory cell generation after resolution of infection (326).

Several transcription factors have been shown to coordinate and regulate the balance between MPEC and SLEC (275,282,290,297,327) (Table 1.4). The transcription factor T-bet (*Tbx21*) is the master regulator of type I effector differentiation whose expression is considerably enhanced and sustained in the presence of IL-12 (282,290,328). Recent evidence suggests that inflammation-induced T-bet can control effector and memory fate decisions in $CD8^+$ T cells because increased differential expression of T-bet leads to differentiation towards SLEC and away from MPEC. In an experimental model of inflammation, T-bet was expressed in $CD8^+$ T cells according to the amount of inflammation creating a gradient of T-bet in which high T-bet expression induced SLECs and low expression promoted MPECs (282,290,295,324). Another transcription factor, eomesodermin (*Eomes*) is proposed to promote memory formation (327). Moreover, IL-12 induces T-bet but inhibits eomesodermin expression to favor effector versus memory generation (329). Interestingly, another transcription factor, Blimp-1, is also thought to be involved in MPEC versus SLEC differentiation. Increased expression of Blimp-1 is found to enhance the formation of SLEC and Blimp-1 deficiency promotes the formation of MPEC (330-332).

H.3.1.4. The Effect of Cellular Metabolism and Nutrient Sensing on Memory T cell Generation

Adding to the complexity of MPEC versus SLEC differentiation, $CD8^+$ T cell metabolic switching has now also been associated with effector versus memory generation (333). The energy-sensitive kinase mammalian target of rapamycin (mTOR) has the ability to sense cellular metabolic state, extracellular nutrient availability, presence of growth

factors/cytokines, and control key cellular processes that govern cell fate such as apoptosis/autophagy, proliferation, and cell growth (334-338). Very recently, mTOR activity has also been associated with generation of memory $CD8^+$ T cells. Araki et al. (2009) have shown that modulation of mTOR function by rapamycin treatment affected memory generation during both the expansion and contraction phases of the T-cell response. During the expansion phase it increased the number of memory precursors and during the contraction phase it accelerated differentiation into memory T cells (339). Taking this idea one step further, Rao et al. (2010) have shown that mTOR can act as a "rheostat" in programming of naive $CD8^+$ T cells for MPEC and/or SLEC fate by regulating expression of T-bet and Eomes. Inhibition of mTOR activity by rapamycin blocked T-bet expression and promoted memory-precursor generation (340)

H.3.2. Phenotypic Characteristics of Memory $CD8^+$ T cells

Following influenza infection, long-lived influenza-specific $CD8^+$ memory cells can be found in the lung airways, spleen, lymphatic system, liver and circulation (341). In addition to anatomical location, subsets of memory $CD8^+$ T cells can be distinguished by phenotype (surface marker expression) and function. In the classical differentiation, central memory T cells (T_{CM}) are found in secondary lymphoid organs and express CD62L and CCR7. Effector memory T cells (T_{EM}) lack the expression of CD62L and CCR7 and have a wider distribution in the peripheral organs and tissues (341-343). Memory $CD8^+$ T cells can also be classified based upon "activation phenotype", independent of effector-/central-memory phenotype. Antigen specific $CD8^+$ T cells are separated based on expression of markers which distinguish quiescent and semi-activated memory T cell subsets and can be used to define three distinct subpopulations ($CD27^{hi}/CD43^{hi}$, $CD27^{hi}/CD43^{lo}$, $CD27^{lo}/CD43^{lo}$) (292). Also outside of effector and memory phenotype, extent of expression of CD127, 4-1BB and OX40

directly correlate to the extent of memory cell generation and their capacity for secondary expansion (316,317).

H.3.3. Memory $CD8^+$ T cells and Protection Against Influenza Infection

It is quite clear that influenza-specific memory $CD8^+$ T cells are protective against infection with heterologous strains. The protective effect of memory T cell populations against influenza infection has been shown in a number of models including against the highly pathogenic H5N1 viruses (344). Prime-challenge with an H9N2 isolate was protective against A/Hong Kong/156/97 (H5N1) (345) and a double-priming with both X-31 and PR8 was protective against one of the most lethal H5N1 strains (268). However, there are a number of factors that are vital for an effective memory T cell response including anatomical location of infection and memory cell populations, quantity and quality of memory T cells present as well as timing of the memory $CD8^+$ T cell response.

H.3.3.1. Memory T cells in the Respiratory Tract

For a memory T cell to be effective in participation against reinfection, it must be present at the site of secondary infection. Memory $CD8^+$ T cells persist in both the airways and parenchyma of the lungs following the resolution of a respiratory virus infection (346-348). Airway memory cells are localized primarily in the upper respiratory tract and can be easily obtained by bronchoaveolar lavage (349). These memory cells are exclusively of T_{EM} phenotype and have also been shown to express activation markers IL-7Rα, CXCR3, CD44, CD43, CD25 and CD69 (350-352). Specific to lung airway environment, $CD8^+$ memory cells in the airway downregulate the T cell migration factor LFA-1 (CD11a) which is thought to trap them in the lung and prevent them from reentering circulation (353-355). In contrast to

the cells of the lung airways, memory cells found in the lung parenchyma (BALT/NALT associated) are composed of a mixture of T_{CM} and T_{EM} phenotypes and have a high expression of LFA-1 (356,357). Memory cells found in the secondary lymphoid organs (LN, spleen) are also of mixed T_{EM}/T_{CM} phenotype; however, phenotypic composition gradually stabilizes on a more T_{CM} population over time post infection (358,359).

Influenza-specific memory $CD8^+$ T cells will preferentially migrate to the lung tissue. Indeed, memory $CD8^+$ T cells will preferentially migrate towards the site of greatest antigen specificity. Tissue-specific homing of memory cells allows for increased efficiency of adaptive response by providing increased numbers of antigen-specific T cells at the anatomical site most likely for reinfection (360). Migration of T_{CM} from lymphoid to nonlymphoid tissue results in the conversion to a T_{EM} phenotype (361). Additionally, the lung airway environment itself has been shown to directly influence surface markers and maintenance on $CD8^+$ memory cells in the absence of antigenic exposure. Memory cells isolated from the spleen and transferred intratracheally into the lungs downregulate LFA-1, CD27, CD127 and Ly6C and increase CD69 while the same cells given intraperitoneally do not go through this change (353,362). Gene expression profiles of $CD8^+$ memory cells in lung airway and spleen generated during influenza infection reveal a “focusing” of genes to favor effector function in the airway environment (363).

H.3.3.2. Memory T cell Function during Secondary Influenza Challenge

The fundamental characteristic in describing a T-cell response is its magnitude. This magnitude is generally defined as both number of responding cells and their ability to clear the viral infection. Antigen-specific memory $CD8^+$ T cells inherently have greatly increased activity and effector function (Thomas et al, 2006). Following a secondary challenge with influenza virus, non-proliferating memory $CD8^+T_{EM}$ in the lung airways are able to respond to

initial viral loads by generating inflammatory signals (such as IFN-γ) to induce the antiviral response (364,365).

Quantity of available memory T cells is also important. The protective value of $CD8^+$ T_{EM} memory cells is strongly correlated to the ability to exert effector function at the site of infection (366). Reduction in the relative number of memory T cells in the lung airways has been correlated to a decline in the overall recall response to viral challenge regardless of stable numbers of splenic T_{CM} (346,367). In addition, during a secondary response, recruitment of T_{EM} to the lungs is antigen-independent and based solely on inflammatory signals; however, the absolute number of cells which can be recruited is dependent on the number of antigen-specific memory T cells located in the periphery (365,368).

H.3.3.3. Timing of the Memory T cell Response to Secondary Influenza Challenge

It is suggested that T_{EM} are more effective at controlling infections at the very early stages, especially outside of secondary lymphoid organs. However, if the infectious challenge is greater than the capacity of T_{EM} to control, then new cells need to be recruited from the T_{CM} population in the peripheral lymphoid organs (275).

Secondary influenza-specific CTL responses arise approximately 2 days faster than the primary response (268). Following a secondary challenge with influenza virus, non-proliferating memory $CD8^+T_{EM}$ in the lung airways are able to respond to initial viral loads (364,365). These cells are not able to clear virus completely since they are rapidly eliminated by apoptosis during the resultant inflammatory response, but can significantly reduce viral loads at early time points. Circulating T_{EM} are then recruited to the lung airways by inflammatory signals and start trafficking to the lung and increase in number by day 2 post infection. Since neither airway resident nor circulating T_{EM} can proliferate and provide a sustained response, resolution of viral infection is dependent on the recruitment of

proliferating T_{EM} generated from T_{CM} in the lymphoid organs. Proliferating effector cells generated from T_{CM} then dominate the later response to clear the infection. These cells have been restimulated with antigen and proliferate in the spleen, draining LN and lung parenchyma before entering the circulation and being recruited to the lung airway around day 4 post infection and peaking around day 7 (369).

There is some evidence that DC interaction is important for activating this phase of $CD8^+$ memory response; however, requirements for costimulation are considerably lower that of naïve precursors (302,370). Following infection, numbers of effector T cells decline, resulting in memory cell generation and the replacement of the original resident $CD8^+$ memory cells by circulating memory cells which continue to enter the airway after the resolution of inflammation (371). It is apparent that the temporal nature of the recall response is highly dependent on both inflammatory signals and relative numbers of available memory cells. Early response to virus is dependent on airway resident T_{EM} to initiate antiviral response as well as T_{EM} recruited from circulation by the resultant inflammatory signals.

H.3.4. Memory T cell Survival and Maintenance

Requirements for T cell survival and proliferation differ depending on the maturation and activation state of the T cell itself. Following clearance or containment of a pathogen, 90-95% of antigen-specific cells die, probably via “neglect” because of loss of contact with the common cytokine-receptor γ-chain (γ_c) family of cytokines and/or TCR signals (372-374). In order to provide protection, it is important to maintain a population of antigen-specific memory T cells capable of responding following exposure to the same or similar pathogens. Memory $CD8^+$ T cells have been found to survive long term in both mouse (>2 years) and human (>50 years) populations (375,376). Originally, it was thought that low levels of

antigen were responsible for the maintenance of a viable memory cell pool. It is now apparent that antigen is not needed for the persistence of memory T cells and that memory T cells continue to divide at a slow rate in the absence of antigen (283,373,375). Two members of the γ_c cytokine are particularly important for antigen-specific $CD8^+$ memory T cells. IL-7 and its respective receptor, IL-7Rα (CD127) are related to memory T cell survival while IL-15 and its receptors govern the intermittent homeostatic proliferation necessary for maintaining a population of memory T cells (377,378).

H.3.4.1. IL-7/CD127 (IL-7Rα)

IL-7 signaling appears to be important for the long-term survival of antigen-specific memory T cells (379). IL-7 binds to the receptor consisting of the high-affinity IL-7R alpha chain (IL-7Rα) and the γ_c chain (373,374,380). Expression of IL-7Rα has been directly linked to the memory precursor effector population and maintenance of IL-7Rα expression during infection leads to greater propensity to join the memory T cell pool. In terms of survival, abrogation of IL-7Rα expression results in decreased memory T cell persistence (381,382). The requirement of IL-7Rα signaling for the survival of antigen specific memory T cells is tied to the regulation of downstream antiapoptotic signaling molecules. Binding of IL-7 to its receptor results in the activation of the JAK-STAT pathway. Notably the activation of JAK1, JAK3 and STAT5 is followed by the upregulation of the antiapoptotic molecule Bcl-2. Bcl-2 leads to enhanced cell survival by inhibiting the pro-apoptotic protein Bcl-2-interacting mediator of cell death (BIM). Expression of IL-7Rα is dynamically regulated by cytokines and TCR signaling. Prosurvival cytokines such as IL-2, IL-6, IL-7 and IL-15 can suppress the expression of IL-7Rα while expression of cytokines with anti-proliferative or proapoptotic properties, such as IFN-α/β and TNFα may upregulate IL-7Rα on T cells to enhance their

survival (378,383). Additionally, differential expression of IL-7Rα is regulated on the genetic level with various transcriptional factors and epigenetic mechanisms regulating *IL7Ra* gene expression. (384,385).

H.3.4.2. IL-15/CD122 (IL-2/15Rβ)

IL-15 is an essential γ_c cytokine that induces the regulation of slow, intermittent basal homeostatic proliferation of antigen-specific $CD8^+$ memory T cells (379). Viral challenge of $IL15^{-/-}$ and IL-15R$\alpha^{-/-}$ mice show that IL-15 is dispensable for the generation of antigen-specific $CD8^+$ memory T cells; however, the memory T cell pool decreased over time (386,387). In IL-$15^{-/-}$ mice, bystander proliferation fails to occur. Wildtype memory $CD8^+$ T cells transferred into these mice fail to proliferate and disappear rapidly (388). Overexpression of IL-15 increased the numbers of memory $CD8^+$ cells (389,390). These results indicate that IL-15 is not necessary for the generation of memory $CD8^+$ T cells but it is required for the homeostatic proliferation to maintain populations of memory cells over a lifetime. The IL-15 receptor (IL-15R) consists of high-affinity IL-15Rα, IL-2/15Rβ and γ_c subchains. These form a heterotrimeric receptor complex. Since IL-15 and IL-2 share signaling receptor subunits, IL-15 signaling is similar to that induced by IL-2. IL-15 binding to the complex IL-2/I5Rβ (CD122) and γ_c induces activation of Jak1/4-STAT5, PI3K/Akt and Ras/Raf/MAPK pathways in lymphocytes. These pathways induce cell proliferation and augment the effector functions of lymphocytes. It must be noted that IL-15, unlike Il-2, mediates these diverse signaling pathways dependently on cell type and on activation status of the responding cells (Budagian, Bulanova, Paus, 2006). Memory $CD8^+$ T cells express high levels of CD122. In fact, antigen-experienced memory T cells upregulate CD122, whereas surface expression of CD122 is undetectable on naïve $CD8^+$ T cells (391,392).

I. Obesity and Cell-mediated Immune Memory

Reduction in the ability to generate and maintain functional influenza-specific memory T cells in a large portion of the obese population could greatly magnify the numbers of complications and deaths during a pandemic year.

I.1. Obesity and Memory $CD8^+$ T cell Generation and Function

Diet-induced obesity in mice has been shown to alter dendritic cell steady state number and function (196,393). Additionally, antigen presentation by dendritic cells is impaired in obese animals (200). Since exposure to antigen is so important for memory cell generation, decreased DC function or numbers could lead to altered $CD8^+$ T cell priming. Differential expression of CD62L has been observed in obese versus lean patients. Morbidly obese subjects downregulate CD62L expression on neutrophils which may affect the ability of these cells to activate and migrate to sites of potential infection (394). Depressed expression of CD62L may affect both initial response, resulting in altered memory generation or it could directly affect memory populations, decreasing the numbers of central memory phenotype cells available for recruitment. It is important to note that the early phases in the memory response are able to reduce the levels of viral amplification until the proliferative phase of the response generates a sustained supply of new effector cells (352). Reduction of either the function or number of either T_{EM} or T_{CM} in the obesigenic environment could lead to the inability to mount an improved secondary response.

Obesity has also been associated with a low-grade inflammatory state which has been implicated in the development of several obesity-associated disease states such as type 2 diabetes mellitus and atherosclerosis (395) . Current research of adipose tissue as an endocrine organ has also added to this theory, demonstrating its ability to secrete

inflammatory mediators such as TNF-α and IL-6 (396,397). Increased obesity and/or adiposity in humans has been associated with increased serum levels of CRP, TNF-α, IL-6 and IL-18 and these cytokines have been shown to decrease after weight loss and moderate exercise (398-404). Inflammation is also altered during infection in the obese host (199). The possibility of a chronic inflammatory state as well as greater expression of inflammatory mediators during infection could tip the balance of memory cell generation, pushing development towards a greater number of primary effector CTL and diminished memory cell precursors.

Additionally, since the lung maintains tight control over T cell function in the airways, the inflammatory state associated with obesity may alter the ability to respond to a secondary infection. Any disturbance in this balance of regulation could alter the memory response of influenza-specific $CD8^+$ T cells during secondary challenge.

I.3. Obesity and Memory $CD8^+$ T cell Maintenance

As discussed previously, IL-7 and IL-15 have clearly been found to promote T-cell immunity. Aberrant production of these cytokines could cause harm or dysregulate the maintenance/survival of memory $CD8^+$ T cells. Little is known about the association of IL-15 and IL-7 and obesity. In terms of adipose tissue, IL-15 is produced by the skeletal muscle and has been found to greatly affect lipid metabolism. It is hypothesized that IL-15 is released from the skeletal muscle with the aim of controlling fat deposition (405,406). IL-7 may be secreted by adipose tissue and, therefore, upregulated in obesity (407). It is important to note that NK cells are highly dependent on IL-15 for survival, which may be of note since NK function was decreased in our diet-induced obese mice (408). Any alterations in these maintenance cytokines could cause a dysregulated recall response in obese animals.

J. Conclusions

Generation, function and maintenance of a protective population of antigen-specific memory T cells are highly dependent on a concert of a number of complex interactions occurring during a primary immune response. Altering the balance of this primary response, such as we see in the obese state, could greatly impact the subsequent protective capacity of the resultant antigen-specific memory T cell pool. Both obesity and influenza pose significant public health threats. Therefore, this work aims to determine the impact of obesity on the generation, function and maintenance of influenza-specific memory T cells in an obese host.

CHAPTER II

DIET-INDUCED OBESITY IMPAIRS THE T CELL MEMORY RESPONSE TO INFLUENZA VIRUS INFECTION

Authors: Erik A. Karlsson, Patricia A. Sheridan, Melinda A. Beck

Published in: Journal of Immunology. 2010;

A. ABSTRACT

The CDC has suggested that obesity may be an independent risk factor for increased severity of illness from the H1N1 pandemic strain. Memory T cells generated during primary influenza infection target internal proteins common among influenza viruses, making them effective against encounters with heterologous strains. In male, diet-induced obese C57Bl/6 mice, a secondary H1N1 influenza challenge following a primary H3N2 infection led to a 25% mortality rate (with no loss of lean controls), 25% increase in lung pathology, failure to regain weight and 10 to 100 fold higher lung viral titers. Furthermore, mRNA expression for interferon γ (IFN-γ) was >60% less in lungs of obese mice along with one third the number influenza-specific $CD8^+$ T cells producing IFN-γ post secondary infection versus lean controls. Memory $CD8^+$ T cells from obese mice had a >50% reduction in IFN-γ production when stimulated with influenza-pulsed dendritic cells from lean mice. Thus, the function of influenza-specific memory T cells is significantly reduced and ineffective in lungs of obese mice. The reality of a worldwide obesity epidemic combined with yearly influenza outbreaks and the current pandemic makes it imperative to understand how influenza virus infection behaves differently in an obese host. Moreover, impairment of memory responses has significant implications for vaccine efficacy in an obese population.

B. INTRODUCTION

Obesity has become a worldwide epidemic. The World Health Organization (WHO) predicts that by the year 2015, 2.3 billion adults will be overweight (BMI ≥ 25) with 700 million being classified as clinically obese (BMI ≥ 30) (1). Obesity has been linked to numerous health problems and chronic diseases, including type 2 diabetes, hypertension, dyslipidemia, certain cancers, and cardiovascular diseases (10). These co-morbidities have been associated with hormonal and metabolic changes related to an increase in adipose tissue mass (14). Obesity has been well established as a risk factor for increased morbidity and mortality; however, its effects on susceptibility to infection are just beginning to be understood. In the hospital setting, obese patients are more likely to develop secondary infections and complications such as sepsis, pneumonia, bacteremia and wound and catheter-related infections. Patients with increased BMI and adiposity also present a higher incidence of surgical site infections, which have been associated with increased risk of other wound complications, increased length of stay and increased risk of death (166-168,170,171). Obesity also negatively affects pulmonary function and BMI has been correlated to increased susceptibility to community-related respiratory tract infections (21,172,174,175).

Influenza is a seasonal respiratory illness caused by the influenza virus. Spread through airborne transmission, these viruses are highly contagious and are responsible for a great deal of morbidity and mortality in the world (201). In any given year, 5-15% of the world population is infected with influenza virus resulting in 3-5 million cases of severe illness and 500,000 deaths from influenza and influenza-related complications (202). Young children, the elderly and people with chronic diseases are particularly susceptible to influenza-related complications and mortality (203). Influenza viruses undergo continual mutation (drift) in surface antigens and occasional gene reassortment (shift) resulting in

heterologous strains with serologically distinct surface proteins. Therefore, a vaccine which promotes a robust memory B cell antibody-based response to the surface proteins of one strain of influenza could be ineffective for a strain encountered the next season expressing different surface proteins (267). By comparison, memory T cells generated during primary influenza infection can target internal proteins common and less variable among influenza strains, making them more effective against encounters with heterologous virus stains (268).

Very recently, the CDC has suggested that obese individuals are at a greater risk of morbidity and mortality from pandemic novel influenza H1N1 strain. Speculation on the cause ranges from excess adipose tissue constricting lung volume to chronic inflammation influencing the immune response (205). We hypothesize this increased severity may be due to obesity decreasing memory cell-mediated defenses against heterosubtypic influenza infection. Previously, we demonstrated that influenza infected diet-induced obese (DIO) mice developed increased lung pathology, decreased expression of IFNα/β and delayed expression of pro-inflammatory cytokines and chemokines (199). In addition, dendritic cells (DC) from obese mice failed to efficiently present influenza antigen to T cells (200). Because development and subsequent functionality of T cell mediated immune memory depends on the primary response (283), we hypothesized that obese mice would have an impaired memory T cell response to a secondary influenza infection.

C. MATERIALS AND METHODS

Animals.

Weanling, male C57BL/6J mice were obtained from Jackson Laboratories (Bar Harbor, ME, USA). All mice were housed at the University of North Carolina Animal Facility, which is fully accredited by the American Association for Accreditation of Laboratory Animal Care. Animals were housed 4/cage under pathogen-free/viral Ab-free conditions and 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 20 wk. The diets, previously described by Surwit et al.(50,409), were obtained from Research Diets (New Brunswick, NJ, USA). The low-fat diet (D12328) consisted of 16.4% protein, 73.1% carbohydrate (83% starch, 17% maltodextrin), 10.5% fat (38% soybean, 62% coconut oil). The high-fat diet (D12331) consisted of 16.4% protein, 25.5% carbohydrate (51% sucrose, 49% maltodextrin), 58% fat (93% coconut oil, 7% soybean oil). Previous studies in our lab (199) and others(410,411) have confirmed that these diets result in significant diet-induced obesity (increased body weight, increased body fat mass) in these mice. This induction of obesity had also been shown to be reversed in these animals by switching from high to low-fat diet (411).

Influenza viruses and infection.

Mouse-adapted influenza virus strain X-31 (H3N2) (a generous gift from David Woodland, Trudeau Institute, Saranac Lake, NY, USA) and A/Puerto Rico/8/34 (PR8, American Type Culture Collection, Manassas, VA, USA) were grown in the allantoic fluid of embryonated hen's eggs. Created by Edward Kilbourne (1969), influenza X-31 is a mouse-adapted recombinant influenza virus consisting of the external hemagluttanin (HA) and neuramidase (NA) proteins of A/Aichi/2/68 (H3N2) and the internal proteins of A/Puerto

Rico/8/34 (H1N1) (412). The X-31 strain is sublethal and efficient at producing memory T cells which are able to fight a secondary A/PR/8 infection (413). For primary infection, lean and obese mice were anesthetized i.p. with ketamine/xylazine and subsequently inoculated intranasally with 300 EID_{50} live X-31 virus in 0.03 mL sterile PBS. Thirty one days post infection (p.i.), no X-31 virus was detected by qRT-PCR or $TCID_{50}$ in mouse lungs (n=8) in either lean or obese mice. Mice were then challenged at 31 days post X-31 infection intranasally with a secondary infection of 5000 $TCID_{50}$ (100x LD_{50} in lean mice during a primary infection) live PR8 virus in 0.05 mL sterile PBS.

Lung histopathology:

As previously described (414), lungs were removed at d 10 p.i. and perfused with Optimal Cutting Temperature Compound (Tissue-Tek, Torrance, CA, USA) and frozen on dry ice. Frozen sections (6μm) were stained with hematoxylin and eosin. The extent of lung pathology was graded in a semiquantitative manner according to the relative degree (from lung to lung) of mononuclear cell infiltrate. Briefly, The scoring is on a scale from 0 to 4: 0-no inflammation, 1-mild influx of inflammatory cells, 2-increased inflammation with approximately 25-50% of the total lung involved, 3-severe inflammation involving 50-75% of the lung and 4-almost all lung tissue contains inflammatory infiltrates.

Quantitation of viral titers in lungs:

Lung viral titers were determined by a modified tissue culture infections dose 50 ($TCID_{50}$) using hemagglutination as an endpoint, as previously described (199). Briefly, half of the right lobe of the lung was removed, weighed, and ground in 0.2 mL minimal essential medium (MEM). Samples were centrifuged at 9000 x *g* for 20 min and the supernatant was serially diluted starting at 1:10 in MEM containing 20 mg/L trypsin. Each diluted supernatant (0.1 mL) was added, in duplicate, to 80% confluent Madin-Darby canine kidney cells and

incubated at 37°C for 96 h. A 0.5% suspension of human O RBC (0.05 mL) was added to each well and incubated at room temperature for 2 h. $TCID_{50}$ was determined by the method of Reed and Muench (415). Values were normalized to weight of the original tissue used.

Quantitation of lung mRNA cytokine levels:

Lung samples were collected on d 0 (uninfected), 1, 2, 3, 7 and 10 p.i. and total RNA was isolated using the TRIzol method. Reverse transcription was carried out with Superscript II First Strand Synthesis kit (Invitrogen, Carlsbad, CA, USA) using oligo (dT) primers. Following previously described methods (199), mRNA levels for murine interferon IFNα, IFNß, IFN-γ, IL-6, and TNFα, and G3PDH were determined using quantitative real time polymerase chain reaction (qRT-PCR).

Isolation of cells from the lungs and spleen:

As previously described (200), lungs from lean and obese mice were removed, digested in HBSS (with calcium and magnesium) supplemented with 160 U/mL Collagenase type 1 (Worthington, Lakewood, NJ, USA). Spleen cells were isolated in unsupplemented HBSS. Samples were processed into single-cell suspensions by mechanical agitation of a Stomacher (Seward, West Sussex, UK) and strained through a 40-µm nylon filter. Cells were subjected to red blood cell lysis using ACK lysis buffer for 5 min at room temperature, washed, counted then subjected to analysis by flow cytometry.

Flow Cytometry:

At least 1 x 10^6 cells were stained with fluorescein isothiocyanate (FITC)-anti-CD44, Pacific Blue-anti-CD62L (both from eBioscience, San Diego, CA, USA) and peridinin-chlorophyll-protein complex (PerCP)-anti-CD8α (BD Biosciences, San Jose, CA, USA). $CD8^+$ T cells specific for the major epitope of the PR8 nucleoprotein were identified using a

phycoerythrin (PE)-labeled $D^bNP_{366-374}$ tetramer. Non-specific tetramer staining was analyzed using an irrelevant tetramer towards herpes simplex virus. Intracellular staining was performed on total lung cells from influenza-infected mice that were incubated for 4 hr with GolgiPlug (BD Biosciences, San Jose, CA, USA). Cells were then Fc-blocked with anti-CD16/CD32, surface stained, and permeabilized with Perm/Wash (BD Biosciences, San Jose, CA, USA) for staining with allophycocyanin (APC)-anti-IFN-γ. An irrelevant APC-immunoglobulin G (IgG) was used as a staining control. For dendritic cells, samples were stained with FITC-anti-CD11b and eFluor 450-anti-CD11c (eBioscience, San Diego, CA, USA). Samples were analyzed on a Cyan ADP flow cytometer (Beckman Coulter, Fullerton, CA, USA) and data was analyzed using FlowJo software (TreeStar, San Jose, CA, USA). Gates were set for DC based on previous reports (200,416,417). Briefly, gates were set based on CD11b and CD11c expression and DC were determined by intermediate levels of CD11b and CD11c.

Antigen presentation by dendritic cells:

Using previously published methods(200), lungs from lean and obese mice infected 31 days previously with influenza X-31 were removed and processed into single cell suspensions as described above. Lung isolates were then pooled into groups of 3 mice based on diet. To isolate as many dendritic cells as possible, cells were resuspended in Ca^{2+} and Mg^{2+} free HBSS containing 10 mM EDTA and incubated at room temperature with agitation for 5 min. Cells were counted using a hemocytometer and 5×10^7 cells were used to isolate DC from each pool using a DC enrichment kit (Invitrogen Dynal AS, Oslo, Norway). T cells were isolated from a separate group of lean and obese mice previously infected with influenza X-31. Pooled suspensions as described above were isolated using a T cell negative isolation kit (Invitrogen Dynal AS, Oslo, Norway). DC were incubated with heat-inactivated influenza A/PR/8 at an MOI of 2 for 2 hours followed by extensive washing

to remove excess virus. DC were plated with T cells in a 96-well plate at a DC to T cell ratio of 1:2. Samples were then incubated for 2 h at 37°C followed by the addition of Golgi Plug (BD Biosciences) and incubated for an additional 4 h. Cells were stained with anti-CD16/CD32, FITC-anti-CD3 and PerCP-Cy5.5-anti-CD8 (eBioscience, San Diego, CA, USA) followed by fixation and permeabilization for subsequent intracellular staining with APC-anti-IFNγ. Cells were run on an Accuri C6 flow cytometer (Accuri, Ann Arbor, MI, USA) and samples were analyzed using FlowJo software (TreeStar, San Jose, CA, USA).

Serum leptin was measured using a commercially available ELISA (R&D Systems, Minneapolis, MN, USA).

Statistical Analysis.

Statistical analyses were performed using JMP Statistical Software (SAS Institute, Cary, NC, USA). Nonparametric data were analyzed using Kruskal Wallis test (α=0.05). Normally distributed data were analyzed by 2-way ANOVA with diet and day post infection as main effects. Student's *t* test was used for post-hoc comparison between the dietary groups and Tukey's HSD was used for post-hoc comparisons among the days p.i. Differences were considered significant at $p < 0.05$.

D. RESULTS

DIO mice have increased morbidity and mortality when challenged with a secondary influenza infection.

To test the effect of obesity on influenza-specific cell mediated immunity, lean (35.7 ± 0.4g) and obese (52.2 ± 0.4g) mice were primed with the recombinant influenza X31 strain (H3N2), followed by a dose of influenza A/PR/8/34 (PR8; H1N1) strain 4 weeks later which is normally lethal in naïve mice. Primary and memory T cell response experiments can be conducted with mouse-adapted X-31 and PR8 because they differ in surface protein expression, thus minimizing antibody mediated clearance of secondary infection (341). By day 7 post PR8 challenge, 25% of the obese mice had died with no loss of lean controls (Fig. 1A).Consistent with infection, both groups lost weight following PR8 challenge, although the infection was more severe in the obese mice as indicated by the lack of weight regain (Fig. 1B). This increased severity of infection was confirmed by the increased lung pathology (Fig. 2A and 2B) and viral titers (Fig. 2C) in obese mice.

DIO alters antiviral cytokine expression in the lungs following secondary challenge.

Respiratory epithelial cells are a primary target of influenza virus and play an important role in the pathogenesis of influenza infection. Infection of respiratory epithelial cells with influenza virus results in activation of retinoic acid-inducible gene-I and Toll-like receptor 3 which then signal to begin production of IFN-α and IFN-β (418,419). These key cytokines function to control viral replication as well as induce both the innate response and the subsequent generation of the adaptive immune response (420-422). Concordant with our observations of primary influenza infection in obese mice (199), lean mice had a robust increase in IFNα/β mRNA expression at day 2 post secondary challenge; however, obese

mice had no increase in IFNα and the increase in IFNβ expression was significantly reduced versus lean controls (Fig. 3).

Obesity reduces the ability of the mice to produce a proinflammatory response post secondary infection.

Obesity leads to a low-grade inflammatory state (423) resulting in increased serum levels of TNF-α and IL-6 which are reversed by weight loss and moderate exercise (398,424-426). Although obesity leads to an inflammatory state in the serum, we also show that the lungs of obese mice were in a state of elevated inflammation even before secondary challenge (Fig. 4A). It is possible that because of this increased inflammatory state of the lungs, obese mice challenged with a secondary influenza infection failed to upregulate lung mRNA expression of inflammatory cytokines Interleukin (IL)-6 (Fig. 4B) and tumor necrosis factor α (TNFα) (Fig. 4C) resulting in a significantly blunted response relative to lean controls.

IFN-γ expression is significantly reduced in the lungs of DIO mice following secondary challenge.

Following a secondary challenge with influenza virus, non-proliferating memory $CD8^+$ T cells in lung airways are able to respond to viral replication by generating inflammatory signals to induce the antiviral response(364,365). These cells cannot clear virus completely, but can significantly reduce viral loads through IFN-γ production at early time points. DIO mice had significantly reduced expression of INF-γ mRNA in the lungs at d 1, 3 and 7 post secondary challenge (Fig. 5A). Although the overall percentage of memory T cells did not differ between mice (Fig. 5B), percentage of influenza-specific effector memory T cells ($CD8^+/D^bNP_{366}{}^+/CD44^+/CD62L^-$) producing IFN-γ was reduced in DIO mice at day 5 (Fig.

5C). In addition, the numbers of influenza-specific effector memory T cells producing IFN-γ at d 5 post secondary challenge was significantly reduced with less than half the total numbers responding in obese mice versus lean (Fig. 5D). Mean fluorescence intensity of IFN-γ production was significantly reduced in obese mice, further indicating a functional difference (Fig. 5E).

Diminished capacity of lung-resident memory T cells from obese mice to respond to influenza-specific antigen presentation.

Despite reduced percentage (Fig 6A) and number (Fig. 6B) of DC in the lungs of obese mice, obese DC were able to present antigen effectively to lean memory T cells *ex vivo* (Fig 6C). In contrast, obese memory T cells, when stimulated with lean DC, were less able to respond to antigen presentation with a >50% reduction in IFN-γ producing cells (Fig. 6C).

Obesity leads to an altered serum leptin profile post secondary infection.

Leptin has been identified as an important immunomodulatory molecule for both the innate and adaptive response. Indeed, leptin is produced at high levels during inflammation and is involved in a number of immune pathways and cell types (165). As previously demonstrated (427,428), serum leptin levels are significantly elevated in diet-induced obese mice. In lean mice, leptin levels are significantly elevated post infection, however this response did not occur in the obese mice (Fig 7).

D. DISCUSSION

To our knowledge, this is the first time that diet-induced obesity has been shown to affect the memory response to a viral infection. It is apparent that diet-induced obesity results in an ineffective memory response to influenza infection impacting both memory T cell function and reducing the DC population in the lung. The protective effect of memory T cell populations against influenza infection has been shown in a number of models including against the highly pathogenic H5N1 viruses (344). Prime-challenge with a H9N2 isolate was protective against A/Hong Kong/156/97 (345) and a double-priming with both X-31 and PR8 was protective against one of the most lethal H5N1 strains (268). Indeed, in our study, protection against a heterologous strain of influenza was observed in the lean mice with 100% survival against a lethal dose of PR8 following X-31 priming; however, obesity reduced this protective capacity. Early innate antiviral cytokines IFNα and IFNβ were significantly delayed and reduced in the lungs of obese mice which may have contributed to the higher viral titers and increased infiltrate found in lungs of obese mice. IFNα/β expression has also been associated with immune memory generation and function and with a direct signal from IFNα/β required for memory T cells to survive the contraction phase of the primary response (429,430).

During the course of an influenza virus infection, heterogeneous pools of persistent memory $CD8^+$ T cells are established. Influenza-specific $CD8^+$ T cells have been observed in both humans and animal models months to years post influenza infection (346-348). A significant number of memory cells can be found in secondary lymphoid organs such as the draining lymph nodes and spleen; however, as many as half of the influenza-specific $CD8^+$ memory T cells can be found in peripheral (nonlymphoid) organs (341,346,431). When functioning properly, populations of memory T cells serve not to prevent viral infection but to reduce the level of viral amplification until a sustained supply of new effector cells can be

generated (352). Reduction of either the function or number of these cells could lead to the inability to mount an efficient secondary response. Based on the increased morbidity and mortality seen in obese mice, along with the reduced effector function of obese influenza-specific memory T cells versus the lean controls, it is clear that DIO mice have a significantly reduced capacity to respond to a novel influenza virus strain.

The exact mechanism by which T cell memory develops remains controversial; however, the main consensus appears to be an adequate/balanced primary response. Development of a functionally protective $CD8^+$ memory response depends on the integration of multiple signals both by responding T cells and by other immune mediators such as antigen presenting cells (APC). Antigen density and time of contact with APC, levels of inflammatory cytokines and overall primary effector response all play a role in programming the development of effective memory T cell responses (283). The reduced protective capacity of the memory response in obese mice could be caused by defects in a single or combination of factors during the primary response.

APC depend on inflammatory signals for potent stimulation of T cell responses; however, it has become clear that T cells also receive signals from inflammatory cytokines. In the obese host, inflammation is altered during infection. We have shown inflammatory signals are delayed but increased in obese mice during primary influenza infection (199). The amount of inflammatory signals received appears to balance effector and memory generation in primary $CD8^+$ T cells. Higher levels of inflammatory cytokines indicate the need for a stronger initial response, which may lead to lowered memory generation (295). During primary influenza infection, we observed an increased antigen-specific T cell response (200), potentially resulting in decreased memory T cell generation. This chronic inflammatory state, as well as greater expression of inflammatory mediators during the primary infection, could tip the balance of memory cell generation to a “too much, too late” scenario. This scenario could push development towards greater generation of primary

influenza-specific effector T cells resulting in diminished numbers or function in memory cell precursors.

$CD8^+$ T cell responses have evolved to specifically eliminate pathogens and to protect against reinfection (271,272). During infection with PR8, lack of $CD8^+$ cells results in decreased viral clearance and eventual morbidity (273,432). Antigen-specific memory T cells are protective since they are present in higher numbers than naïve precursors and respond rapidly upon reencounter with pathogen (433). Secondary influenza-specific T cell responses arise approximately 2 days faster than the primary response and have greatly increased activity and effector function (268). Following a secondary challenge with influenza virus, memory $CD8^+$ T cells in the lung airways respond to initial viral loads by generating signals, such as IFN-γ, to induce the antiviral response (364,365). The protective value of $CD8^+$ T cell memory cells is strongly correlated to the ability to exert effector function at the site of infection (366). Reduction in the relative number of memory T cells in the lung airways has been correlated to a decline in the overall recall response to viral challenge (346,367). DIO mice had decreased overall IFN-γ mRNA expression in the lung, as well as decreased percentage and overall number of influenza-specific effector memory T cells producing IFN-γ post secondary challenge. These data indicate a significant reduction in the ability of influenza-specific T cells in the lungs of obese mice to respond to a heterogeneous viral strain.

The decreased production of IFN-γ by memory T cells during secondary infection in DIO mice may be due to impairment in DC function, as we previously demonstrated during primary influenza infection in DIO mice (200). Interestingly, we found that in contrast to a primary influenza infection, DC from DIO mice were able to efficiently present antigen to lean and obese memory T cells. There is some evidence that DC interaction is important for activating the $CD8^+$ memory response; however, requirements for costimualtion by DC are

considerably lower that that of naïve precursors which may explain why obese DC were able to stimulate lean memory T cells (302,370). Additionally, reduction in the number of influenza-specific T cells may indicate an impairment of memory T cells to migrate to the obese lung microenvironment. Tissue-specific homing of memory cells allows for increased efficiency of adaptive response by providing increased numbers of antigen-specific T cells at the anatomical site most likely for reinfection (360). A similar situation could be associated with the reduction in the number of DC from the obese lung versus lean controls.

Although the mechanistic link between obesity and diminished immune memory is not clear, a main factor which ties together obesity, inflammation and immune cell function is leptin resistance associated with the obese state. Leptin is recognized as an important mediator of immune function (434) and leptin expression is increased proportionately with adipose tissue mass, resulting in increased circulating levels in obesity (435). This chronic elevation appears to cause a state of leptin resistance in obese mice where leptin signaling is attenuated despite increased circulating levels (436). Leptin signaling is important for virtually all parts of the innate immune response including antiviral (IFNα/β) cytokine expression, proinflammatory IL-6 and TNFα expression, and activation and stimulation of monocytes, DC and macrophages (150,437). Leptin also modulates adaptive immunity by increasing DC function and stimulatory capacity, promoting a T_H1 response and enhancing T cell survival and proliferation (76,153,438,439). Leptin resistance has been found to negatively impact T cell responses as well as DC maturation and survival in obese mice (75,438,440). In this experiment, we have observed effects consistent with leptin resistance. Indeed, serum leptin was significantly increased in obese mice before secondary challenge and did not show an infection associated response as seen in the lean controls. Therefore, leptin resistance in obese mice may contribute to the reduced protective capacity of the memory response to secondary influenza infection.

The ability to generate functional memory T cells, either during primary infection or by vaccination, has proven to be protective against potentially lethal influenza strains exhibiting completely different surface antigens (268). The CDC and WHO have declared the currently circulating novel H1N1 influenza strain to be pandemic and obesity appears to be an independent risk factor for illness severity (205). Here we have demonstrated that increased morbidity and mortality during a secondary influenza infection in DIO mice is due to impairment in the ability to generate and maintain functional influenza-specific memory T cells. This finding, if applicable to a human obese population, has significant public health implications including current vaccine strategies which may be less effective in an increasingly obese population.

Figure 2.1. Morbidity and mortality in lean and obese mice during memory response to influenza infection. Following primary infection with influenza X-31, lean and obese mice were challenged with a dose of influenza PR8 normally lethal to lean, naïve mice. (*A*) Lean mice had 100% survival whereas obese mice had a 25% mortality rate by d 7 pi. (*B*) Obese mice fail to regain weight following secondary infection (n=12-24/group) ± SEM. *p<0.05, lean versus obese.

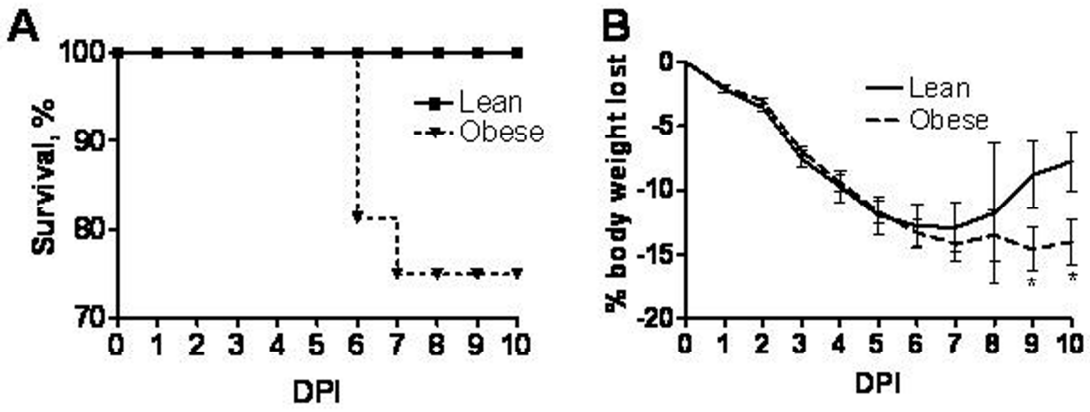


Figure 2.2. **Obese mice have increased viral titer and pathology post secondary infection**. (*A* and *B*) Obese mice had increased lung pathology at d 10 pi (n=10/group). Data are expressed as mean ± SEM. *p<0.05, lean versus obese. (*C*) Obese mice had a greater than 10 fold increase in viral titer in the lungs at d 1, and 3 post infection following secondary PR8 challenge. No virus was detected (ND = not detected) before PR8 infection (d 0). The dashed line indicates the limit of detection for the assay. Data are representative of 3 separate experiments and are expressed as $TCID_{50}$/mg lung tissue (n=3-4/group) ± SEM on a Log_{10} scale. *p<0.05, lean versus obese.

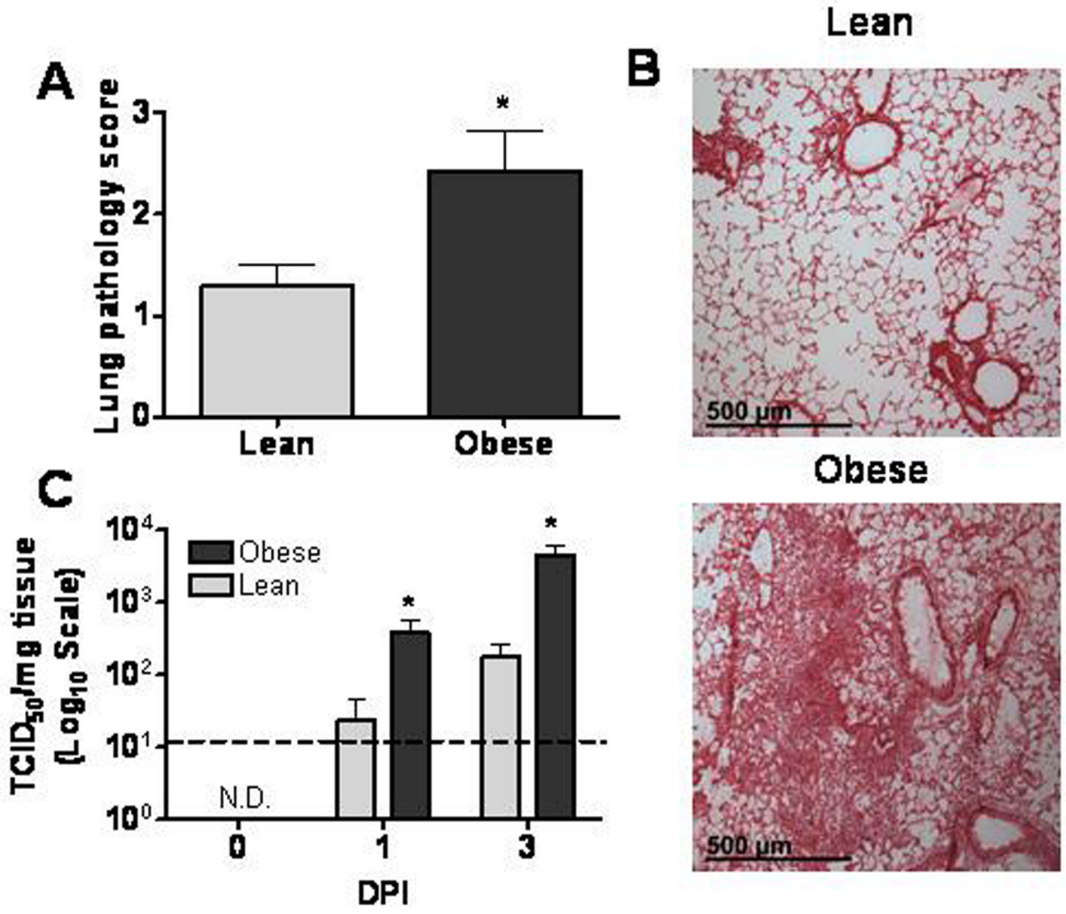


Figure 2.3. **Antiviral cytokine mRNA expression in the lung following secondary infection**. mRNA expression of (*A*) IFNα and (*B)* IFNβ were measured in the lungs of lean and obese mice following secondary infection. Data are expressed by normalization to G3PDH values then to lean value at d 0 pi (n = 6-8 per group) ± SEM. There were no significant differences in G3PDH values. *p<0.05, lean versus obese. a – p<0.05 versus day 0 of individual diet group.

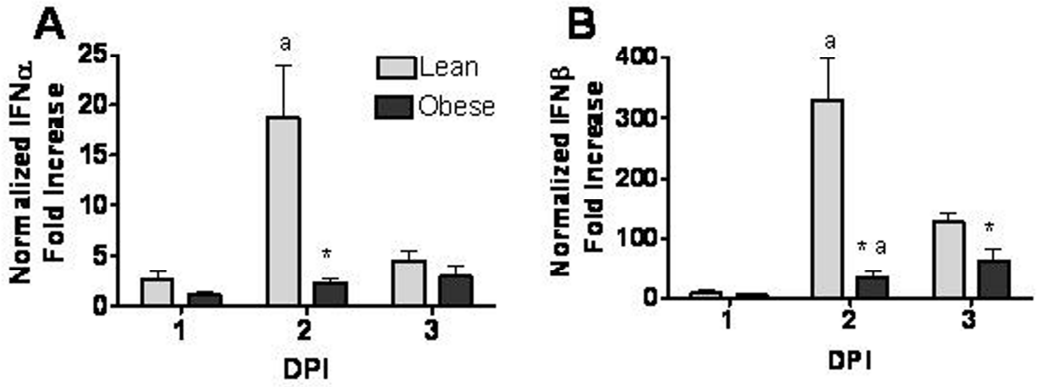


Figure 2.4. Inflammation-associated cytokine mRNA expression in the lung prior to and following secondary infection. (*A*) mRNA expression of IL-6 and TNFα were measured in the lungs of lean and obese mice before (d31 post X-31 infection) secondary infection. Data are expressed by normalization to G3PDH values (n = 6-8 per group) ± SEM. mRNA expression of (*B*) IL-6 and (*C*) TNFα were measured in the lungs of lean and obese mice following secondary infection. Data are expressed by normalization to G3PDH values then to lean value at d 0 pi (n = 6-8 per group) ± SEM. There were no significant differences in G3PDH values. *p<0.05, lean versus obese.

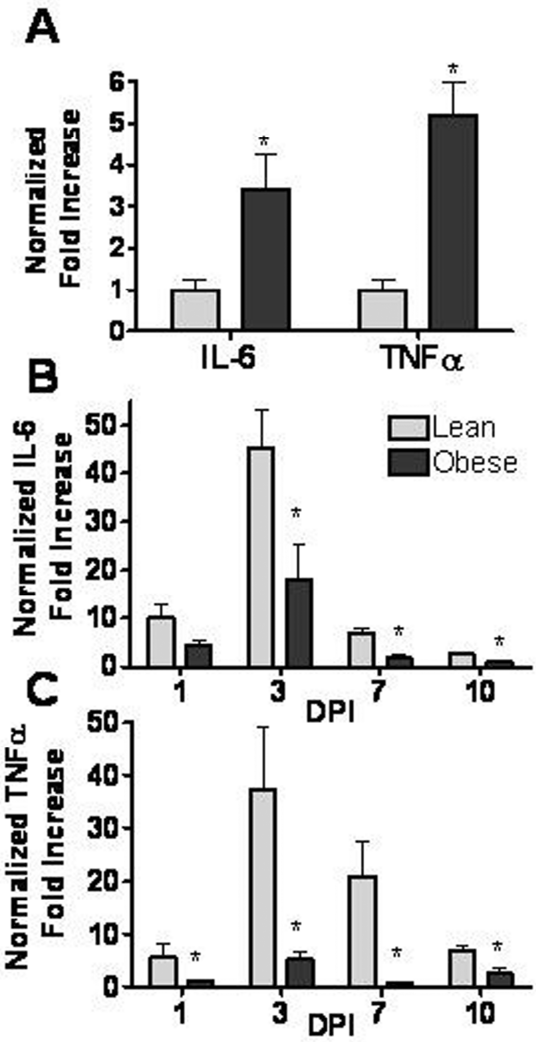


Figure 2.5. **IFN-γ expression in the lung following secondary infection is reduced in obese mice.** (*A*) mRNA expression of IFN-γ was measured in the lungs of lean and obese mice following secondary infection using quantitative real time-PCR. Data are expressed by normalization to G3PDH values then to lean value at d 0 pi (n = 6-8 per group) ± SEM. There were no significant differences in G3PDH values. *p<0.05, lean versus obese. (*B*) Number of influenza-specific effector memory T cells ($CD8^+/D^bNP_{366-374}{}^+/CD44^+/CD62L^-$) following secondary infection. (*C*) Percent of influenza-specific memory T cells at day 5 and (*D*) number of influenza-specific effector memory T cells producing IFN-γ. (*E*) Amount of IFN-γ produced by each cell using flow cytometry. Cells are gated as effector memory T cells ($CD8^+/D^bNP_{366-374}{}^+/CD44^+/CD62L^-$) unless otherwise noted. No significant differences were observed in number of total cell numbers and total $CD8^+$ T cell numbers between the lungs of lean and obese mice. Data are expressed as number or percent cells of total cells isolated and mean fluorescence intensity (n = 4-6 per group) $^+$/- SEM. *p<0.05, lean vs obese.

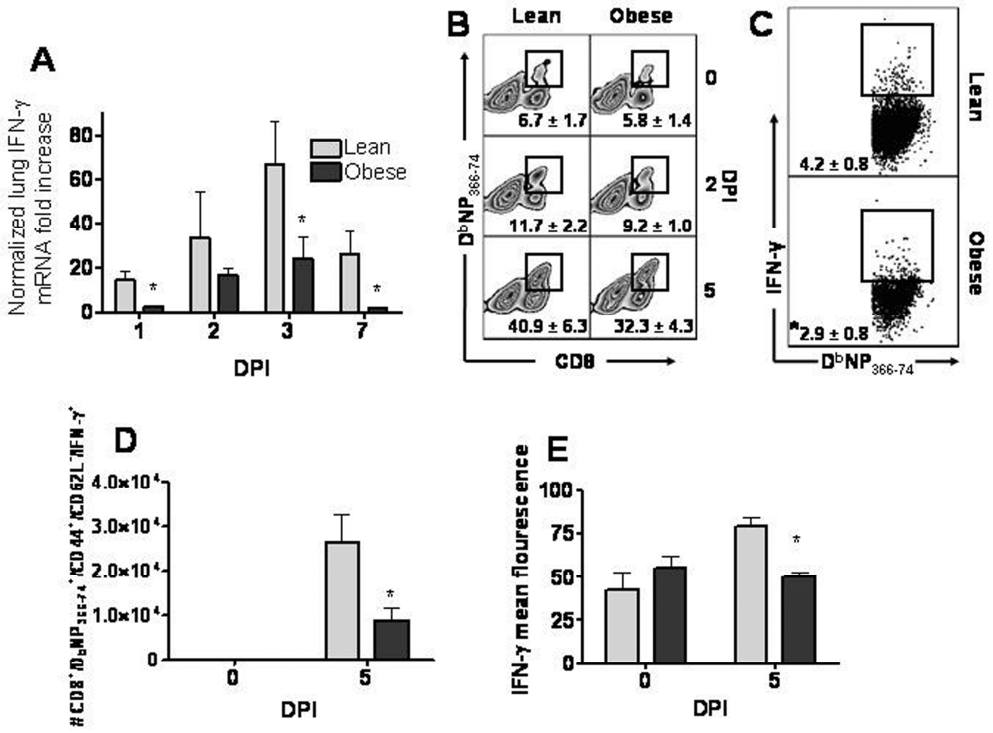


Figure 2.6. Impairment in function of memory T cells from the lungs of obese mice. (*A*) Percentage before secondary infection and (*B*) number of dendritic cells in the lungs of lean and obese mice following secondary challenge. Data are expressed as percentage and number of $CD11b^{+}/CD11c^{int}$ cells of total cells isolated (n=6/group) ± SEM. *p<0.05, lean versus obese. (*C*) Ability of lean and obese DC to present antigen to lean T cells and obese T cells from lungs of influenza-experience mice. DC and T cells were isolated from lean and obese mice at d0 (d31 post X-31 infection). Data are expressed as # of $CD3^{+}/CD8^{+}$ cells producing IFN-γ^{+} as measured by flow cytometry (n=3/group/pool) ± SEM. *p<0.05, lean versus obese.

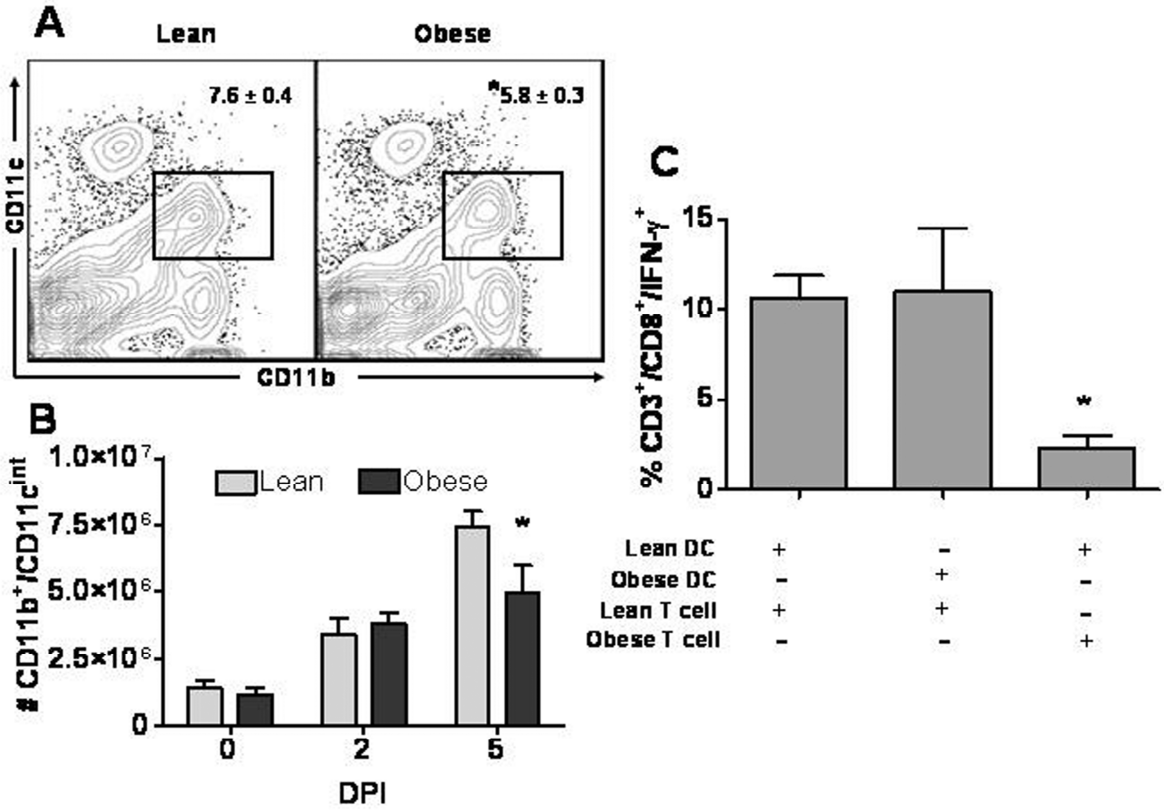
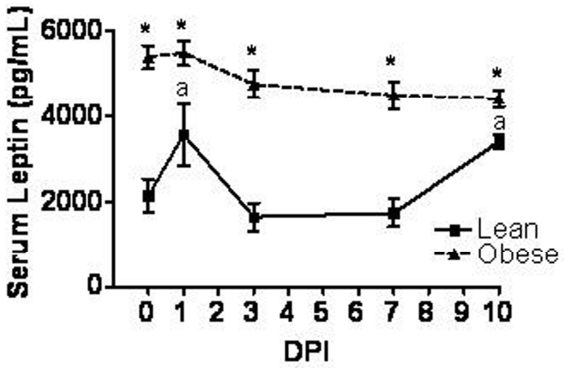


Figure 2.7. Serum leptin concentrations for lean and obese mice following secondary challenge. Serum leptin levels measured by ELISA. Data as expressed as pg/mL (n=6-8 per group) ± SEM. *p<0.05, lean versus obese. a – p<0.05 versus day 0.



CHAPTER III

INFLUENCE OF DIET-INDUCED OBESITY ON MAINTENANCE OF INFLUENZA-SPECIFIC CD8$^+$ MEMORY T CELLS

Authors: Erik A. Karlsson, Patricia A. Sheridan, Melinda A. Beck

Submitted: Journal of Nutrition

A. ABSTRACT

Obesity has been associated with increasing the risk for type 2 diabetes and heart disease, but its influence on the immune response to viral infection is under studied. Memory T cells generated during a primary influenza infection are important for protection against subsequent influenza exposures. Previously, we have demonstrated that diet-induced obese mice have increased morbidity and mortality following secondary influenza infection compared with lean mice. To determine if the problem resided in a failure to maintain functional, influenza-specific CD8$^+$ memory T cells, diet-induced obese (DIO) and lean mice were infected with influenza X-31. At 84 days post infection (p.i.), DIO mice had a 10% reduction in memory T cell numbers. This reduction may be due to significantly reduced memory T cell expression of IL-2Rβ (CD122), but not IL-7Rα (CD127), which are both required for memory cell maintenance. Peripheral leptin resistance in the DIO mice may be a contributing factor to the impairment. Indeed, leptin receptor mRNA expression was significantly reduced in the lungs of obese mice while suppressor of cytokine signaling (SOCS)1 and SOCS3 mRNA expression were increased. It is imperative to understand how the obese state alters memory T cells as impairment in maintenance of functional memory responses has significant implications for vaccine efficacy in an obese population.

B.INTRODUCTION

Obesity is a major global public health problem. Although the humans are fairly well adapted to periods of reduced food intake, they are poorly adapted to overnutrition (8). Obesity can lead to serious health consequences and, subsequently, increases in health care requirements and economic burden. Caused by a change in energy balance of increased caloric intake versus expenditure (9), obesity has been linked to numerous health problems and chronic diseases (10,11). These co-morbidities associated with obesity have been attributed to hormonal and metabolic changes related to increases in adipose tissue mass (14,15). Obesity is an independent risk factor for cardiovascular disease, type 2 diabetes, hypertension, arthritis, sleep apnea and cancer (17); however, its effects on susceptibility to infection are poorly understood. In a hospital setting, obese patients are more likely to develop secondary infections and obese individuals are at increased risk for community-related respiratory tract infections (166,169,170,172). Studies in diet-induced obese animal models have shown obese mice had lower levels of mitogen-induced interleukin (IL)-2, although interferon (IFN)-γ and IL-4 production was increased (195). Additionally, obese mice have impaired dendritic cell (DC) function and altered T cell responsiveness (180-182,192,196,441,442). Very recently, the Centers for Disease Control and Prevention have declared that obese individuals are at a greater risk of morbidity and mortality from infection with pandemic novel influenza H1N1 strain. (205). At this time, it is unclear how obesity can result in a greater risk from H1N1 infection.

Influenza is a highly contagious, seasonal respiratory illness caused by the influenza virus. In any given year, 5-15% of the world population is infected with influenza virus resulting in 3-5 million cases of severe illness and 500,000 deaths from influenza and influenza-related complications (201,202). Influenza viruses undergo continual mutation (drift) in surface antigens and occasional gene reassortment (shift) resulting in heterologous

strains with serologically distinct surface proteins. Therefore, a vaccine which promotes a robust memory B cell antibody-based response to the surface proteins of one strain of influenza could be ineffective for a strain encountered the next season expressing different surface proteins (267). By comparison, memory T cells generated during primary influenza infection can target internal proteins common and less variable among influenza strains, making them more effective against encounters with heterologous virus stains (268).

Previously, we have shown that diet-induced obesity in mice significantly reduced the memory T cell response to secondary viral challenge resulting in increased morbidity and mortality (443). In order to understand how obesity contributes to a poor response to secondary viral challenge, we examined the ability of diet-induced obese mice to maintain influenza-specific memory $CD8^+$ T cells.

C. MATERIALS AND METHODS

Animals.

Weanling, male C57BL/6J mice were obtained from Jackson Laboratories (Bar Harbor, ME, USA). All mice were housed at the University of North Carolina Animal Facility, which is fully accredited by the American Association for Accreditation of Laboratory Animal Care. Animals were housed 4/cage under pathogen-free/viral Ab-free conditions and maintained under protocols approved by the Institutional Animal Use and Care Committee. Mice were randomized to receive either a low-fat diet (D12329) or a high-fat/high-sucrose diet (D12331) for 20 wk. The diets, previously described by Surwit et al. (50,409), were obtained from Research Diets (New Brunswick, NJ, USA). Previous studies in our lab (199) and others (410,411) have confirmed that these diets result in significant diet-induced obesity (increased body weight, increased body fat mass) in these mice.

Influenza viruses and infection.

Mouse-adapted influenza virus strain X-31 (H3N2) (a generous gift from David Woodland, Trudeau Institute, Saranac Lake, NY, USA) was grown in the allantoic fluid of embryonated hen's eggs. Created by Edward Kilbourne (1969), influenza X-31 is a mouse-adapted recombinant influenza virus consisting of the external hemagglutinin (HA) and neuramidase (NA) proteins of A/Aichi/2/68 (H3N2) and the internal proteins of A/Puerto Rico/8/34 (H1N1) (412). The X-31 strain is sublethal and efficient at producing memory T cells which are able to prevent a lethal infection with a secondary A/PR/8 infection (413). For infection, following 20 weeks on the diets, lean and obese mice were anesthetized i.p. with ketamine/xylazine and subsequently inoculated intranasally with 300 EID_{50} live X-31 virus in 0.03 mL sterile PBS. Mice were maintained on the diets. By day 33 pi, no virus was detected in the lungs of either lean or obese mice, demonstrating clearance of the infection (data not shown).

Quantitation of lung and spleen mRNA cytokine levels

Lung and spleen samples were collected on d 33 and 84 p.i. and total RNA was isolated using the TRIzol method. Reverse transcription was carried out with Superscript II First Strand Synthesis kit (Invitrogen, Carlsbad, CA, USA) using oligo (dT) primers. Following previously described methods for quantitative real time polymerase chain reaction (qRT-PCR)(199), mRNA levels for murine interleukin (IL)-6, and TNFα and G3PDH were determined using previously described primer/probe sets and mRNA levels for IL-15 (Mm00434210_m1), IL-7 (Mm00434291_m1), CD122 (Mm00434264_m1), ObR (Mm00440181_m1), SOCS1 (Mm00782550_s1), SOCS3 (Mm00545913_s1) and β-Actin (Mm01205647_g1) were determined using TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA).

Isolation of cells from the lungs, spleen and draining lymph node

As previously described (200), lungs from lean and obese mice were removed and digested in HBSS (with Ca and Mg) supplemented with 160 U/mL Collagenase type 1 (Worthington, Lakewood, NJ, USA). Spleen and draining lymph node (mediastinal) cells were isolated in unsupplemented HBSS. Samples were processed into single-cell suspensions by mechanical agitation of a Stomacher (Seward, West Sussex, UK) and strained through a 40-μm nylon filter. Cells were subjected to red blood cell lysis using ACK lysis buffer for 5 min at room temperature, washed, counted then analyzed by flow cytometry.

Flow Cytometry

At least 1 x 10^6 cells were stained with fluorescein isothiocyanate (FITC)-anti-CD44, Pacific Blue-anti-CD62L (eBioscience, San Diego, CA, USA) and peridinin-chlorophyll-protein complex (PerCP)-anti-CD8α (BD Biosciences, San Jose, CA, USA). $CD8^+$ T cells specific for the major epitope of the A/PR/8 nucleoprotein were identified using a phycoerythrin (PE)-labeled $D^bNP_{366-374}$ tetramer. Non-specific tetramer staining was analyzed using an irrelevant tetramer towards herpes simplex virus. Samples were analyzed on a Cyan ADP flow cytometer (Beckman Coulter, Fullerton, CA, USA) and data was analyzed using FlowJo software (TreeStar, San Jose, CA, USA).

D. RESULTS

Diet-induced obese mice have significantly increased weight compared to lean controls.

At d 33 post infection DIO mice weighed approximately 20 g more than their lean counterparts. At d 84 pi, lean mice had gained ~5 g of weight from d 33; however, lean mice still weighed approximately 15 g less than their DIO counterparts (Figure 1).

Influenza-specific central memory T (T_{CM}) cells increase over time in the lung, spleen.

Following a primary influenza infection, a small population of influenza specific T_{CM} cells remain in secondary lymphoid tissues, which includes the bronchiole-associated lymphoid tissue (BALT) of the lungs. In the lungs of both lean and obese mice, percent (Figure 2A) but not number (Figure 2B) of T_{CM} cells increased over time. No difference was observed between the lean and obese groups. In contrast, while percent of T_{CM} cells increased in the spleen of both groups over time (Figure 2C), at d 84 pi obese mice had a significantly increased number of T_{CM} in the spleen compared to lean controls (Figure 2D).

DIO mice maintain significantly reduced numbers of influenza-specific $CD8^+$ effector memory T (T_{EM}) cells in the lungs.

Following primary influenza infection, a population of influenza-specific T_{EM} cells is maintained in the lung to more rapidly respond to the next encounter with an influenza virus. At d 33 post X-31 infection, both lean and obese mice had populations of influenza-specific T_{EM} cells in the lung (Figure 3A). In both the lean and obese mice, population percent and number of effector memory T cells declined over time; however, the DIO mice had a greater percent loss of T_{EM} cell numbers resulting in a significant decrease in T_{EM} numbers in the lungs of obese mice compared to lean controls at d 84 pi (Figure 3B and 3C). In the lymph

node and spleen, the number of T_{EM} decreased over time in both the lean and obese mice; however, no difference was observed between the two dietary groups (data not shown).

CD122 (IL-2Rβ) expression is significantly reduced on influenza-specific T_{EM} cells in the lungs of obese mice.

In both lean and obese mice, percentage of T_{EM} cells expressing CD122 increased over time; however, at d 33 pi, percentage of T_{EM} cells expressing CD122 was significantly reduced in the lungs of obese mice compared to lean controls (Figure 4A and 4B). In addition to reduced percentage of cells expressing CD122, mean fluorescence intensity of CD122 was also significantly reduced on T_{EM} cells in the lungs of obese mice (Figure 4C). No differences were observed in the mRNA expression of CD122 in the lungs of obese mice versus lean controls (data not shown).

Obese mice have altered expression of IL-15 and IL-7 mRNA in the lungs post primary infection.

IL-7 and IL-15 are the cytokines responsible for the homeostasis and survival of memory T cells. In the lungs of obese mice, IL-15 mRNA expression at d 84 pi was approximately 3 times greater versus lean controls (Figure 5A). In contrast, expression of IL-7 mRNA was decreased by 50% at d 33 in the lungs of obese mice versus lean controls. Interestingly, in both lean and obese mice expression of IL-7 mRNA significantly increased over time (Figure 5B).

ObR expression is reduced and SOCS 1 and 3 expression is increased in the lungs of obese mice.

In the lungs of obese mice, leptin receptor (ObR) mRNA expression was significantly less than in lean mice at both d33 and 84 p.i. ObR expression increased in the lungs of lean

mice by d 84 post X-31 infection; however, expression was not found to increase in the lungs of obese mice (Figure 6A). Additionally, expression of SOCS1 (Figure 6B) and SOCS3 (Figure 6C) was significantly increased in the lungs of obese mice at d 84 pi.

The lungs of obese mice have significantly increased inflammation in the absence of viral infection.

Following clearance of the X-31 infection at d 33 pi, mRNA expression of both IL-6 and TNFα was significantly increased in the lungs of obese mice compared to lean controls. mRNA expression was still significantly increased at d 84 pi.

E. DISCUSSION

A vaccine which promotes a robust memory B cell antibody-based response to the surface proteins of one strain of influenza could be ineffective for a strain encountered the next season expressing different surface proteins (267). However, there are several internal viral proteins that are highly conserved among influenza viruses, and therefore do not experience the mutations seen with the external proteins. Although these internal proteins do not generate an effective antibody response because of their lack of accessibility to antibodies, the cell-mediated immune response can recognize these proteins. Therefore, memory T cells generated during a primary influenza infection can target these internal proteins common to influenza strains, making them effective against encounters with heterologous virus stains. The ability to generate functional memory T cells, either during a primary infection or by vaccination, has proven to be protective against potentially lethal influenza strains exhibiting completely different surface antigens (268). Following viral clearance, contraction of activated $CD8^+$ T cells results in influenza-specific memory cells representing ~5 to 10% of effector $CD8^+$ T cells found at the peak of expansion during

primary infection (276,277).These long-lived memory cells can rapidly respond to a secondary infection with influenza virus.

Although the exact definitions are still controversial, memory T cells established after infection can be divided into two subsets: T_{CM} which preferentially localize to the lymphoid tissues, and T_{EM} found mainly in peripheral sites of infection (343,431). Numbers of T_{CM} in lymphoid sites appear to remain relatively constant, if not slightly increased, while the number of T_{EM} at peripheral sites steadily decreases in the months after pathogen clearance. Although both lean and obese mice demonstrated the expected increase in % T_{CM} and decrease in T_{EM}, the T_{EM} in the lungs of the obese animals declined at a significantly faster pace resulting in a 10% reduction in the numbers of T_{EM} present versus the lean controls. This reduction in the numbers of influenza-specific T_{EM} in the lungs of obese mice has significant health implications since the protective capacity from a secondary infection can be directly linked to the number of pathogen-specific memory T cells present in the tissue prior to secondary infection (367) Therefore, as we have already observed a decrease in protective capacity in influenza-specific memory T cells in obese mice (443), reduction in numbers of these cells could decrease protection even further.

Memory cells persist under normal conditions by undergoing intermittent cell division, called bystander proliferation, about once every two weeks (444). Originally, the maintenance and survival of memory T cells was thought to be mediated by signals arising from contact with depots of persistent antigens; however, memory cells have been shown to survive not only in the absence of antigen but also in the absence of MHC molecules (445,446). Survival of memory cells now appears to be maintained by members of the common gamma chain (γ_c) family of cytokines, IL-15 and IL-7.

IL-15 is essential for the regulation of slow, intermittent basal homeostatic proliferation of antigen-specific $CD8^+$ memory T cells (379). Viral challenge of $IL15^{-/-}$ and IL-$15R\alpha^{-/-}$ mice show that IL-15 is dispensable for the generation of antigen-specific $CD8^+$

memory T cells; however, the memory T cell pool decreased over time (386,387), as bystander proliferation fails to occur. Wildtype memory $CD8^+$ T cells transferred into IL-$15^{-/-}$ mice fail to proliferate and disappear rapidly (388). Overexpression of IL-15 increased the numbers of memory $CD8^+$ cells (389,390). In the lean mice, IL-15 mRNA expression was maintained at a constant level; however, expression of mRNA for IL-15 in the obese animals. was approximately 30 times greater at d 84 pi compared d 33. This increase in IL-15 mRNA did not result in increased T_{EM}. In fact, fewer TEM were found in the obese lung. Reasons for this discrepancy may be due to reduced IL-15 receptor (IL-15R) or a blockade of IL-15 signaling.

The IL-15R consists of high-affinity IL-15Rα, IL-2/15Rβ (CD122) and γ_c subchains that form a heterotrimeric receptor complex. Memory $CD8^+$ T cells express high levels of CD122. Obese, but not lean, Zucker rats have significantly decreased expression of mRNA for the γ_c and CD122 subunits of the IL-15 receptor while IL-15Rα remained unchanged (406). While the mRNA expression of CD122 and IL-15Rα in the lungs of lean and obese mice were not found to be different (data not shown), the numbers of T_{EM} expressing CD122 were significantly reduced in the lungs of diet-induced obese mice 33 days post primary infection. In addition, mean fluorescence intensity of CD122 was also significantly reduced on T_{EM} expressing the receptor, indicating a decreased amount of expression on these cells. However, by day 84, there was no difference in CD122 expression between lean and obese mice.

Leptin resistance can also play a role in decreased T_{EM} cells in the lungs of obese mice. Elevated levels of leptin in the obese state results in attenuated central leptin signaling, with the possibility of decreased signaling in the periphery (119). Leptin, IL-7 and IL-15 share structural homology and signal through the Janus Kinase (JAK)-Signal Transducer and Activator of Transcription (STAT) pathway. In the lungs of obese mice,

leptin receptor (ObR) mRNA expression was significantly decreased at d 33 pi and did not increase over time as was found in the lean controls, suggesting that leptin signaling was likely impaired.

Characteristic of leptin resistance, mRNA expression of SOCS-1 and SOCS-3, potent inhibitors of JAK-STAT signaling, were significantly increased in the lungs of obese mice. The participation of SOCS proteins, particularly SOCS-3 but also SOCS-1, as negative-feedback regulators of leptin signaling has been suggested as a causal factor for central leptin resistance (119,124,447). Indeed, SOCS-3 deficiency elevates leptin sensitivity and confers resistance to diet-induced obesity (142,448). While SOCS3- has not yet been directly linked to IL-15 signaling, it has been observed that SOCS-1 expression can directly regulate IL-15 driven homeostatic proliferation and SOCS-1$^{-/-}$ mice accumulate CD44^{+}/CD8^{+} memory phenotype T cells which express elevated levels of CD122 (449,450). We observed a decrease in the numbers of CD44^{+}/CD8^{+} memory T cells in obese mice despite an increase in IL-15 mRNA expression. Therefore, the increased SOCS expression in the lungs due to leptin resistance could be responsible for the failure of IL-15 to signal and thereby preventing the bystander proliferation required for memory T cell maintenance and resulting in a significantly reduced, lung-resident T_{EM} population over time.

Obesity has also been associated with chronic inflammation and, indeed, significant increases in IL-6 and tumor necrosis factor (TNF) α mRNA expression were seen in the lungs of obese mice at d 33 and d 84 pi. This increased inflammation has been associated with leptin resistance, as these cytokines could contribute or be resultant of upregulation of SOCS proteins (451).

While maintenance of memory T cell populations is regulated by IL-15 and IL-7, other factors could contribute to the reduction in the numbers of influenza-specific T_{EM} in the lungs of obese mice over time. Both IL-15 and IL-7 increase the resistance of memory T cells to apoptosis, allowing them to persist for long periods of time. Interestingly, leptin

signaling can also result in reduction of T cell apoptosis as leptin treatment has been found to prevent the decline of T cell numbers in the fasted state (452). If memory T cells in the lungs of obese mice are leptin resistant, then memory cells in the obesigenic environment would be at increased sensitivity to inflammation-induced apoptosis.

Diet-induced obese mice are able to generate influenza-specific memory $CD8^+$ T cells; however, the functional capability of these cells (443) and their ability to be maintained in the lung is significantly reduced. The understanding of memory T cell maintenance is still in its infancy; however, it is clear that IL-15 and IL-7 and their subsequent signals are important for maintaining the memory cell population over time. Our study has shown, for the first time, that diet-induced obesity can affect the maintenance of influenza-specific memory T cell populations in the lungs. This decrease in memory T cell numbers may be due to peripheral leptin resistance in the obesigenic lung microenvironment affecting IL-15 function. Overall, decreased numbers of T_{EM} over time, coupled with the decreased functional capability of these cells, suggests that obesity presents an even greater risk to increased morbidity and mortality from infection with a heterologous pandemic strain. In addition, vaccine strategies that promote the generation of memory T cells may be less effective in an obese population.

Figure 3.1: Diet-induced obese mice weigh more than lean controls at all time points post infection. Mice were weighed at d 33 and d 84 pi. Data is expressed as mean weight (n=6 per group) $^{+}$/- SEM, *p < 0.05, lean versus obese, § p< 0.05 , d 33 versus d 84.

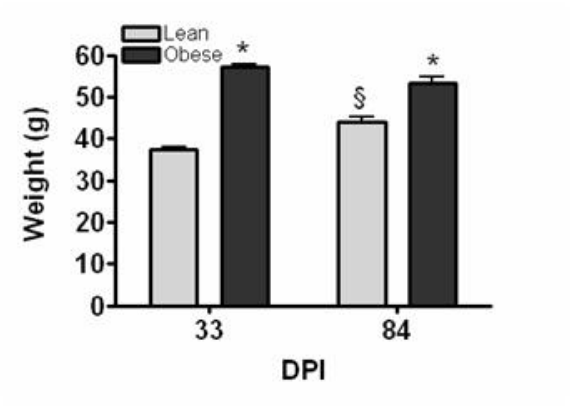


Figure 3.2. Influenza-specific central memory T cells (T_{CM}) increase over time in the lung and spleen. T_{CM} ($CD8^+/D^bNP_{366-74}{}^+/CD44^+/CD62L^+$) were identified in the lung (**A**, **B**) and spleen (**C, B**) using flow cytometry. Data are expressed as percent and total number of cells (n= 6 per group) ± SEM. *p < 0.05, lean versus obese, § p< 0.05 , d 33 versus d 84.

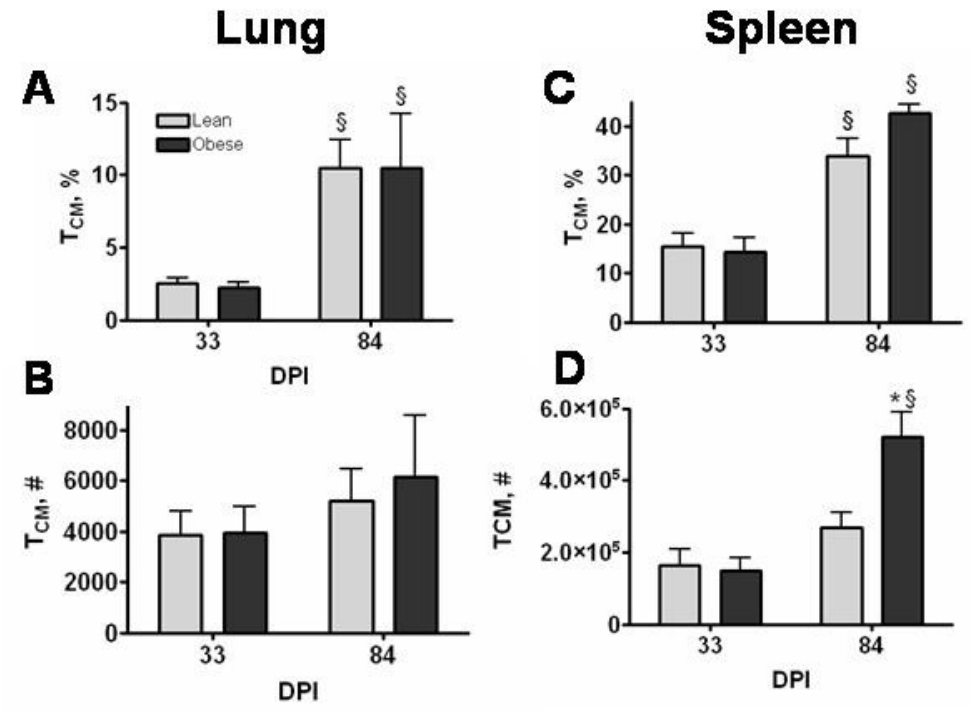


Figure 3.3. DIO mice maintain significantly reduced numbers of influenza-specific CD8$^+$ effector memory T cells (T_{EM}) in the lungs. Effector memory T cells (CD8$^+$/D^{b}NP$_{366-74}$$^+$/CD44$^+$/CD62L$^-$) were identified in the lung using flow cytometry. Percent (**A, B**) and total numbers (**C**) were calculated. Data are expressed as percent and total number of cells (n= 6 per group) ± SEM. *p < 0.05, lean versus obese, § p< 0.05 , d 33 versus d 84.

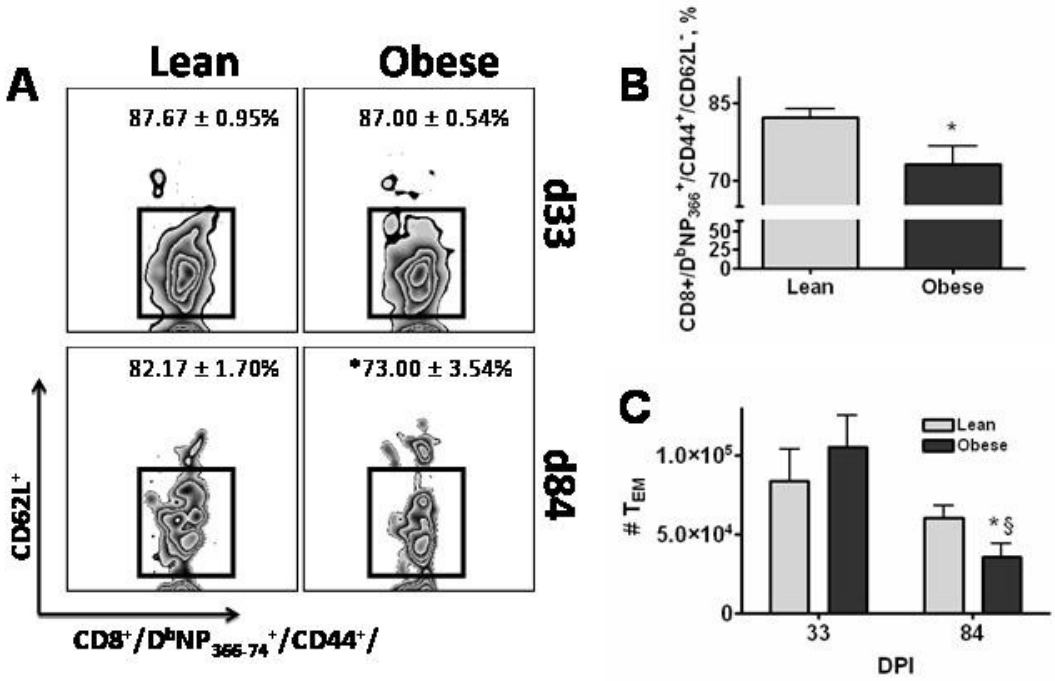


Figure 3.4. CD122 expression on influenza-specific T_{EM} cells is minimally downregulated in the lungs of obese mice. CD122 expression on effector memory T cells ($CD8^+/D^bNP_{366-74}{}^+/CD44^+/CD62L^-$) was identified in the lung using flow cytometry. Percent (**A, B**) and mean fluorescence intensity (**C**) were analyzed. Data are expressed as percent and mean fluorescence intensity of CD122 expression (n= 6 per group) ± SEM. *p < 0.05, lean versus obese, § p< 0.05 , d 33 versus d 84.

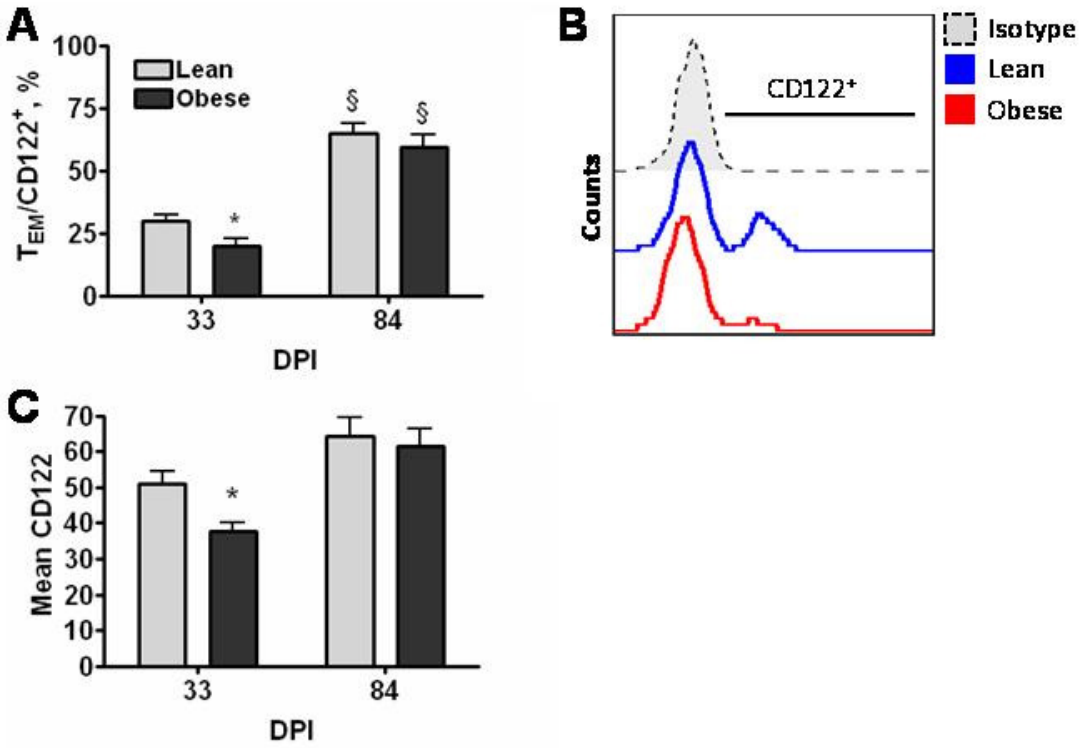


Figure 3.5. Elevated and blunted mRNA levels for murine IL-15 and IL-7, respectively, were detected in the lungs of obese mice. qRT-PCR was performed and β-actin expression was determined for all samples and used to normalize the gene expression levels for each cytokine. mRNA levels of IL-15 (**A**) and IL-7 (**B**) were tested. Data are expressed by normalization to β-actin values (n = 5-6 per group) ± SEM. *p < 0.05, lean versus obese, § p< 0.05 , d 33 versus d 84.

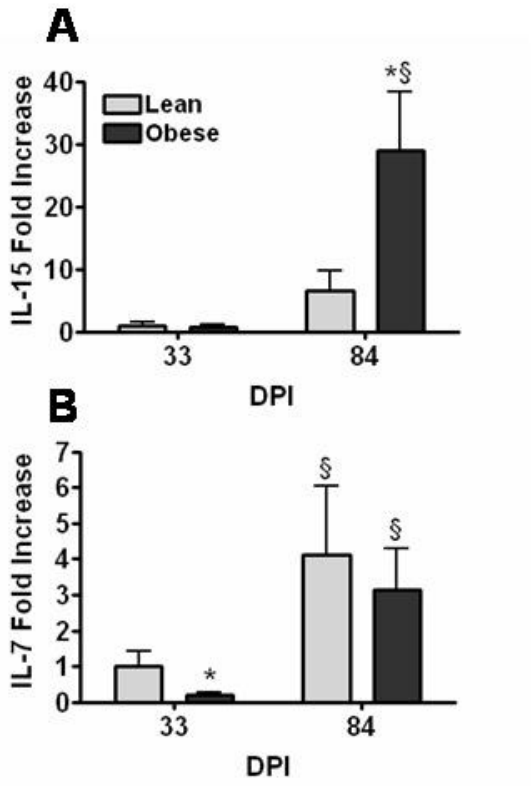


Figure 3.6. mRNA levels for murine leptin receptor (ObR) are blunted while inhibitors of leptin signaling were elevated in the lungs of obese mice. qRT-PCR was performed and β-actin expression was determined for all samples and used to normalize the gene expression levels for each cytokine. mRNA levels of ObR (**A**) as well as SOCS1 (**B**) and SOCS3 (**C**) were tested. Data are expressed by normalization to β-actin values (n = 5-6 per group) ± SEM. *p < 0.05, lean versus obese, § p< 0.05 , d 33 versus d 84.

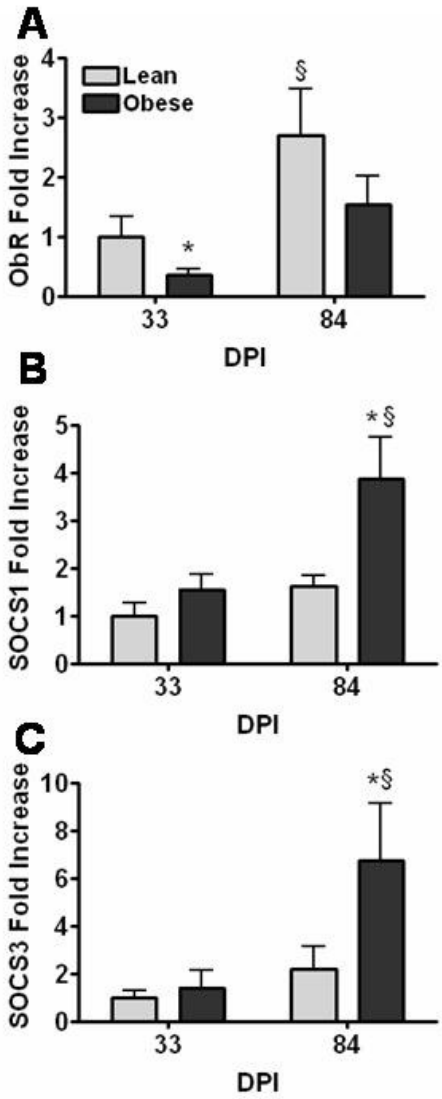
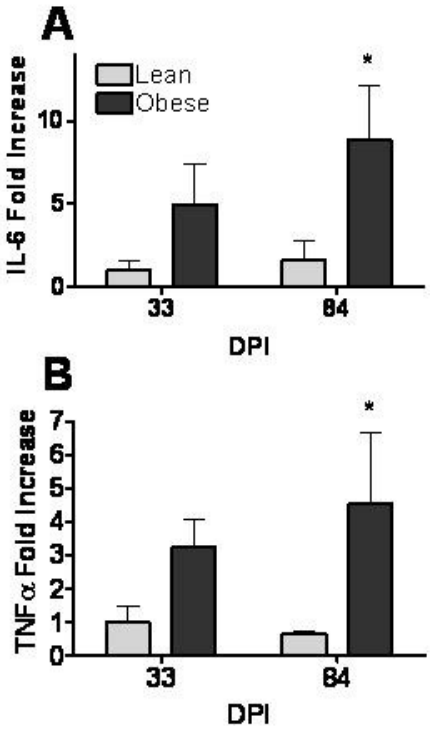


Figure 3.7. mRNA levels for murine TNFα and IL-6 were greatly increased in the lungs of obese mice. qRT-PCR was performed and G3PDH expression was determined for all samples and used to normalize the gene expression levels for each cytokine. mRNA levels of IL-6 (**A**) and TNFα (**B**) were tested. Data are expressed by normalization to β-actin values (n = 5-6 per group) ± SEM. *p < 0.05, lean versus obese.



CHAPTER IV

GENERATION OF INFLUENZA-SPECIFIC MEMORY T CELLS IN A DIET-INDUCED OBESE HOST DURING A PRIMARY INFLUENZA INFECTION

Authors: Erik A Karlsson, Patricia A Sheridan, Melinda A Beck

A. ABSTRACT

Antigen-specific memory T cells generated during a primary infection confer protection against heterologous viral strains. Previous studies in our laboratory have shown that function and maintenance of influenza-specific memory $CD8^+$ T cells is significantly impaired in the lungs of obese mice. Since development of these memory T cells is dependent on the primary response to influenza infection, we observed the development of influenza-specific memory T cells in the lungs of lean and obese mice during a primary influenza infection. Following infection with influenza X-31, obese mice had increased morbidity and increased viral load in the lungs starting at d 1 p.i. In addition, antiviral cytokine expression was severely reduced. As a result of the increased viral load, obese mice displayed increased numbers of influenza-specific $CD8^+$ T cells responding to infection in the lungs. These cells expressed significantly increased levels of the primary effector-associated transcription factors T-bet and Blimp-1 while expression of the memory formation-associated Eomesodermin was significantly reduced. Therefore, obese mice had increased severity of infection due to inability to prevent early viral replication in the respiratory epithelium. The resultant viral load then prompted the $CD8^+$ T cells response to

generate increased numbers of influenza-specific effector $CD8^+$ T cells required for viral clearance and to drive the differentiation program away from memory T cell formation.

B. INTRODUCTION

Obesity has become a worldwide epidemic; however, it's influence on the response to viral infection is still poorly understood. In the United States alone, 68.0% of the US population has a BMI ≥ 25 meaning that 2 out of every 3 people are overweight or obese (6). Obesity can be considered an over accumulation of white adipose tissue (WAT) due to increased caloric intake versus caloric expenditure (9,62). WAT is now considered an important part of body regulation and can act as an endocrine organ, secreting numerous factors which can affect several metabolic pathways. These "adipokines" participate in a wide variety of physiological or physiopathological processes including food intake, insulin sensitivity and inflammation. In addition, many of the adipokines have been found to play an intricate role in various aspects of the innate and adaptive immune response (63-66). Obesity has been linked to numerous health problems and chronic diseases (10,11) and obese individuals have been found to have a greater risk of infection in the hospital setting (168,171). With adipose acting as an endocrine organ, it is not surprising that local, obesity-driven changes in adipokine secretion can have a systemic impact on several branches of the immune system (14,15,63,66). However, the effects of these changes on the response to a viral infection are poorly understood.

One such viral infection which poses a significant threat to global public health is influenza. Influenza is a highly contagious, seasonal respiratory illness caused by the influenza virus. Influenza viruses undergo continual mutation (drift) in surface antigens and occasional gene reassortment (shift) resulting in heterologous strains with serologically distinct surface proteins. Therefore, a vaccine which promotes a robust memory B cell

antibody-based response to the surface proteins of one strain of influenza could be ineffective for a strain encountered the next season expressing different surface proteins (267). By comparison, memory T cells generated during primary influenza infection can target internal proteins common and less variable among influenza strains, making them more effective against encounters with heterologous virus stains (268).

Development of functional, fully protective influenza-specific memory $CD8^+$ T cells is highly dependent on the primary encounter with the influenza virus. While the exact mechanism which determines the development of memory T cells is still under debate, current theory suggests that both initial antigen contact and environmental effects play a role in memory cell generation. The balance of these signals is very important and there appears to be a fine tightrope walk between development of long-lived, protective memory T cells and enough effector cells to be able to clear the infection (275,295,296). Previous studies in our laboratory have shown that diet-induced obesity in mice reduces both the protective capacity of influenza-specific memory T cells and their ability to be maintained in the obese lung microenvironment (443) (See Chapter II and Chapter III). Since these factors are highly dependent on the primary response to influenza infection, we examined the development of influenza-specific memory T cells in diet-induced obese mice during a primary influenza infection.

C. MATERIALS AND METHODS

Animals.

Weanling, male C57BL/6J mice were obtained from Jackson Laboratories (Bar Harbor, ME, USA). All mice were housed at the University of North Carolina Animal Facility, which is fully accredited by the American Association for Accreditation of Laboratory Animal Care. Animals were housed 4/cage under pathogen-free/viral Ab-free conditions and 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 20 wk. The diets, previously described by Surwit et al. (50,409), were obtained from Research Diets (New Brunswick, NJ, USA). Previous studies in our lab (199) and others (410,411) have confirmed that these diets result in significant diet-induced obesity (increased body weight, increased body fat mass) in these mice.

Influenza viruses and infection.

Influenza virus strain X-31 (H3N2) (a generous gift from David Woodland, Trudeau Institute, Saranac Lake, NY, USA) was grown in the allantoic fluid of embryonated hen's eggs. Created by Edward Kilbourne (1969), influenza X-31 is a mouse-adapted recombinant influenza virus consisting of the external hemagglutinin (HA) and neuramidase (NA) proteins of A/Aichi/2/68 (H3N2) and the internal proteins of A/Puerto Rico/8/34 (H1N1) (412). The X-31 strain is sublethal and efficient at producing memory T cells which are able to prevent a lethal infection with a secondary A/PR/8 infection (413). For infection, following 20 weeks on the diets, lean and obese mice were anesthetized i.p. with ketamine/xylazine and subsequently inoculated intranasally with 300 EID_{50} live X-31 virus in 0.03 mL sterile PBS. Mice were maintained on the diets over the course of the infection.

Quantitation of lung and spleen mRNA cytokine levels:

Lung and spleen samples were collected on d 33 and 84 p.i. and total RNA was isolated using the TRIzol method. Reverse transcription was carried out with Superscript II First Strand Synthesis kit (Invitrogen, Carlsbad, CA, USA) using oligo (dT) primers. Following previously described methods (199), mRNA levels for murine G3PDH, IL-6, TNFα, MCP-1, MIP-1α, Blimp-1, Eomes and ObR were determined using quantitative real time polymerase chain reaction (qRT-PCR).

Isolation of cells from the lungs, spleen and draining lymph node:

As previously described (200), lungs from lean and obese mice were removed, digested in HBSS (with calcium and magnesium) supplemented with 160 U/mL Collagenase type 1 (Worthington, Lakewood, NJ, USA). Samples were processed into single-cell suspensions by mechanical agitation of a Stomacher (Seward, West Sussex, UK) and strained through a 40-µm nylon filter. Cells were subjected to red blood cell lysis using ACK lysis buffer for 5 min at room temperature, washed, counted then subjected to analysis by flow cytometry.

Flow Cytometry:

At least 1 x 10^6 cells were stained with fluorescein isothiocyanate (FITC)-anti-CD44, Pacific Blue-anti-CD62L, phycoerythrin (PE)-anti-Eomes, Allophycocyanin (APC)-anti-IFNγ, Alexa Flour-647-anti-Tbet (eBioscience, San Diego, CA, USA), peridinin-chlorophyll-protein complex (PerCP)-anti-CD8α (BD Biosciences, San Jose, CA, USA), and APC-Cy7-anti-CD3 (BioLegend, San Diego, CA, USA). $CD8^+$ T cells specific for the major epitope of the PR8 nucleoprotein were identified using a PE-labeled $D^bNP_{366-374}$. Non-specific tetramer staining was analyzed using an irrelevant tetramer towards herpes simplex virus. Samples were analyzed on a Cyan ADP flow cytometer (Beckman Coulter, Fullerton, CA, USA) and data was analyzed using FlowJo software (TreeStar, San Jose, CA, USA).

D. RESULTS

Diet-induced obese mice have increased percentage of body weight lost post influenza infection compared to lean mice (Figure 1). As expected, mice placed on a high fat diet gained significant amounts of weight compared to lean controls (Fig. 1A). Following infection with influenza X-31, consistent with illness, both groups lost weight; however, the infection was more severe in the obese mice as indicated by increased percent body weight lost early in infection and delayed weight regain (Fig. 1B).

Obese mice have significantly increased viral load in the lungs post influenza infection (Figure 2). Following X-31 infection, cells of the respiratory epithelium are infected with virus which then replicates and spreads to other lung cells (221). In the lean mice, virus first appeared at d 3 p.i., peaked at d 7 and was undetectable at d14. In contrast, significant levels of viral mRNA were detected in the lungs of obese mice at d 1 p.i. and were still detectable by d 14. By d 21, obese mice had cleared the virus.

Antiviral cytokine mRNA expression is altered in the lungs of obese mice (Figure 3). Respiratory epithelial cells are a primary target of influenza virus and play an important role in the pathogenesis of influenza infection. Infection of respiratory epithelial cells induces the production of the antiviral cytokines, IFN-α and IFN-β (418-422). Lean mice had an increase in IFNα mRNA expression by d 3 post X-31 infection; however, obese mice did not increase IFNα mRNA expression until d 7 p.i. (Fig. 3A). For IFNβ, both lean and obese mice increased mRNA expression at d 3 and d 7 p.i. (Fig. 3B).

Obese mice have significantly increased number of influenza-specific $CD8^+$ T cells in their lungs post influenza infection (Figure 4). Resolution of a viral infection relies on the

induction of pathogen-specific $CD8^+$ T cells (241). Following X-31 infection, both lean and obese mice had a large infiltrate of inflammatory cells into the lung (Fig 4A), most of which was lymphocytes (Fig. 4B). Interestingly, obese mice had approximately 2x the cellularity versus lean controls at d 7 (Fig. 4A, B). Percentage of activated, influenza-specific $CD8^+$ T cells significantly increased in the lungs of lean and obese mice starting at d 10 p.i. with a decreased percentage in the lungs of obese mice at d 10 (Fig. 4C). Of note, the number of influenza-specific T cells in the lungs of obese mice peaked at d 7 post infection; however, peak numbers were observed at d 10 in the lungs of lean mice (Fig, 4D).

Increased IFN-γ expressing cells in the lungs of obese mice post X-31 infection (Figure 5). $CD8^+$ T cells act to inhibit viral replication by destroying virally infected cells through the secretion of IFN-γ and perforin/granzyme B as well as Fas/FasL pathway (246). Although IFN-γ is not directly involved in the lysis of infected cells, IFN-γ secretion enhances the development of cell-mediated immunity, activates macrophages, and increases antigen presentation (250,251). IFN-γ mRNA expression was significantly increased in the lungs of lean and obese mice at d 7 and d 14 post infection (Fig. 5A). At d 7 p.i., obese mice had a significant increase in the number of influenza-specific $CD8^+$ T cells producing IFN-γ compared with lean mice.

Increased proinflammatory mRNA expression in the lungs of obese mice (Figure 6). Obesity has been associated with a chronic, low-grade inflammatory state resulting from altered secretion of adipokines and cytokines from adipose tissue (14,91). Before X-31 infection, mRNA levels of IL-6, TNF-α, MCP-1 and MIP-1α were 2 to 5 fold higher in the lungs of obese versus lean mice (Fig 6A). Following infection, approximately 3x greater IL-6 mRNA expression was observed in the lungs of obese mice at d3 p.i. (Fig. 6B). No

differences in mRNA expression of TNFα, IL-12, MCP-1 or MIP-1α between lean and obese mice were seen following influenza challenge (data not shown).

Obese mice have altered expression of memory T cell precursor transcription factors in the lungs post X-31 infection (Figure 7). Following influenza infection, antigen contact, inflammation and costimulation can stimulate the inherent and environmental programming of effector $CD8^+$ T cells which may control the expression of key transcription factors important for memory T cell generation such as T-bet, Eomesodermin (Eomes) and Blimp-1 (275,285,296,331). Following influenza challenge, obese mice had approximately double the number of $CD8^+$ effector cells expressing T-bet at d 7 post infection (Figure 7A). In addition, Blimp-1 mRNA expression was almost double in the lungs at d 14 p.i. (Figure 7B) Perhaps most strikingly, mRNA expression of eomesodermin mRNA was significantly decreased at d 7 pi in the lungs of obese mice versus lean controls (Figure 7C). In concordance the percentage of $CD8^+$ cells expressing Eomes was significantly decreased at d 3 and d 7 post infection as measured by flow cytometry (Figure 7D).

Obese mice have decreased leptin receptor (ObR) mRNA expression in the lung (Figure 9). Leptin signaling has been associated with the ability to alter the immune response and obesity has been associated with a state of leptin resistance. ObR mRNA was found to be significantly decreased in the lungs of obese mice prior to influenza infection and expression did not increase at d 1 pi as seen in the lean mice. Additionally, ObR mRNA expression remained low in the lungs of obese mice following influenza challenge and was significantly decreased at d 7 pi compared to lean controls.

E. DISCUSSION

Recent studies of the generation of long-term immunity and efficacious vaccination against viral agents have begun to focus on the generation of long-lived, antigen-specific $CD8^+$ memory T cells. From these studies, it has become apparent that the generation of functional $CD8^+$ memory T cells requires a “balancing act” between memory cell potential and terminal differentiation into full effector T cells (275,282,285,296). Antigen density on the dendritic cell and time of contact with the APC, costimulatory factors and signals and levels of inflammatory cytokines could all play a role in programming the development of memory potential (283). Any alteration of these factors may result in altered generation of a protective pool of influenza-specific memory T cells.

Resolution of a viral infection often relies on the induction of a $CD8^+$ T cell response (241). $CD8^+$ T cells actively identify and eliminate virally infected cells. During an influenza virus infection, T cell responses peak between days 7-10 p.i., resulting in viral clearance by day 15 (242-245). Indeed, in our model we observe peak numbers of influenza-specific T cells at d 7 post X-31 infection. However, obese mice had almost double the number of influenza-specific cells in the lungs compared to lean controls. In accordance, viral load was greatly increased in the obese mice which may have stimulated the observed increase in number of influenza-specific $CD8^+$ T cells in order to handle the increased viral load.

$CD8^+$ T cells are required for resolution of a viral infection and to clear virally infected cells. These functions are achieved through the secretion of IFN-γ and perforin/granzyme B as well as Fas/FasL pathway (246). While IFN-γ mRNA expression in the lungs of lean and obese mice did not differ between groups, the number of influenza-specific $CD8^+$ T cells producing IFN-γ was significantly increased in the lungs of obese mice versus lean controls, again, likely in response to the increased viral load.

Following viral infection, respiratory epithelial cells produce antiviral and inflammatory cytokines in order to limit viral infectivity and to activate and attract the immune cells to the site of infection. IFNα and IFNβ are major antiviral cytokines and can mediate direct antiviral activity of cells. In addition, these cytokines also have antiproliferative and immunomodulatory function (229). Obese mice displayed a trend towards decreased and delayed expression of these antiviral signals which may have resulted in the significantly increased viral load seen in the lungs of obese mice.

Recent evidence points out that inflammatory signals received during the primary infection can also determine the size and function of the memory cell pool. Inflammation can act as a "third signal" in the determination of the balance of effector and memory differentiation. Increased inflammation is associated with increased primary effectors but decreased memory precursors and differentiation into long-term memory. Reduced inflammation leads to reduced effector $CD8^+$ T cells and T cell contraction as well as greater numbers of $CD8^+$ memory T cells (281). mRNA expression of proinflammatory cytokines IL-6, MCP-1 and MIP-1α was greatly increased in the lungs of obese mice before X-31 infection and IL-6 mRNA expression was increased at d3 post X-31 infection compared to lean mice. This increased inflammation may have contributed to the increased number of activated $CD8^+$ T cells responding to the infection in the lungs of obese mice.

Several transcription factors have been shown to coordinate and regulate the balance between memory T cell and short lived effector T cell formation (282,327,332). The transcription factor T-bet is the master regulator of type I effector differentiation (290,328) and inflammation-induced T-bet can control effector and memory fate decisions in $CD8^+$ T cells. Increased T-bet leads to differentiation towards primary effector $CD8^+$ T cells and away from memory precursors. Inflammation has been shown to induce T-bet expression and promote effector T cell generation (290,295). Indeed, in the obese mice, the number of effector cells expressing T-bet was significantly increased in the lungs of obese mice at d 7

post infection suggesting a drive towards primary effector $CD8^+$ T cell generation and away from memory T cell formation. Another transcription factor, Blimp-1, is also thought to be involved enhancing the formation of short-lived effector cells versus memory precursors. Indeed, Blimp-1 deficiency promotes the formation of memory precursors (MPEC) (330,331). In the obese mice, Blimp-1 mRNA expression was significantly increased at d 14 post infection. Increased expression of both T-bet and Blimp-1 indicates a greater propensity for $CD8^+$ T cells to be short-lived effector T cells at the expense of generating MPEC to later form a pool of memory T cells. A third transcription factor, Eomesodermin (Eomes) is proposed to promote memory formation (327). Eomes mRNA expression was found to be reduced in the lungs of obese mice following influenza infection with reduced numbers of $CD8^+$ T cells being positive for Eomes. Thus, the increase in T-bet and Blimp-1 and decreased Eomes expression all point towards an increase in primary, short-lived effector T cell formation and a decrease in the number of cells forming the influenza-specific memory T cell pool. These data indicate that there may be a decrease in memory precursor formation in the obese mice versus the lean controls.

One factor that may tie together all of these changes is the leptin resistance associated with the obesigenic state. Leptin levels act as a general signal of energy reserves and to modulate food intake and, therefore, leptin concentrations increase proportionately to adipose mass (which results in high circulating levels in obese individuals (104-108). Obesity has been associated with a state of central leptin resistance, and, possibly, a state of peripheral leptin resistance due to an overaccumulation of suppressor of cytokine signaling (SOCS) proteins (136,137). Expression of the type I interferons, IFNα and IFNβ, can also be repressed by SOCS proteins. Therefore, leptin resistance in the respiratory epithelium may account for the reduced antiviral response seen in the obese lung and the subsequent increase in viral load (132,453,454). Leptin receptor (ObR) mRNA expression was found to be significantly decreased in the lungs of obese mice prior to

influenza challenge and did not increase following influenza infection as was seen in the lungs of the lean mice. This reduction of ObR could indicate a state of leptin resistance in the lungs of the obese mice. Indeed, elevated leptin levels have also been observed in the serum of our obese mice during previous studies (443).

Taken together, this study indicates that memory T cell generation during a primary response to influenza infection is impaired in obese mice. The increased inflammation and decreased and unresponsive leptin receptor expression in the lungs of obese mice may contribute to an inability of lung epithelial cells to respond to viral infection and may prevent the expression of antiviral IFNα and IFNβ. This decrease in antiviral IFN expression then resulted in an increased replication of virus in the epithelium resulting in increased viral loads in the obese mice at d 1 and delayed clearance of the virus at d 14 p.i. Subsequently, increased viral loads and inflammation during infection result in increased number of influenza-specific effector T cells needed to clear the infection. This increase could be tipping the balance between effector and memory T cell generation with increased primary effector T cells that will eventually go through activation-induced cell death and reduced numbers of MPEC. These conclusions are supported by the transcription factor data indicating increased T-bet and Blimp-1 expression (indicators of primary effectors) and reduced Eomes expression (indicators of MPEC) in responding $CD8^+$ T cells in the lungs of obese mice. These data help to understand why we have observed reduced functionality and decreased maintenance of influenza-specific memory T cells in diet-induced obese mice. In order to fully understand how generation of memory T cells is affected by the obesigenic environment, these studies must be repeated with a larger number of samples and more time points. In addition, ex vivo studies on activation and function of $CD8^+$ T cells are necessary to understand how their intrinsic programming and environmental experience affect their ability to develop into bona fide memory T cell precursors.

Figure 4.1. Morbidity in lean and obese mice during primary response to influenza infection. Mice placed on a high fat diet gained significant amounts of weight compared to lean controls (Fig. 1A). Following infection with influenza X-31, both groups lost weight; however, the infection was more severe in the obese mice as indicated by increased percent body weight lost early in infection and delayed weight regain (Fig. 1B). Data expressed as g of weight of percentage of body weight lost (n=12-24/group) ± SEM. *p<0.05, lean versus obese.

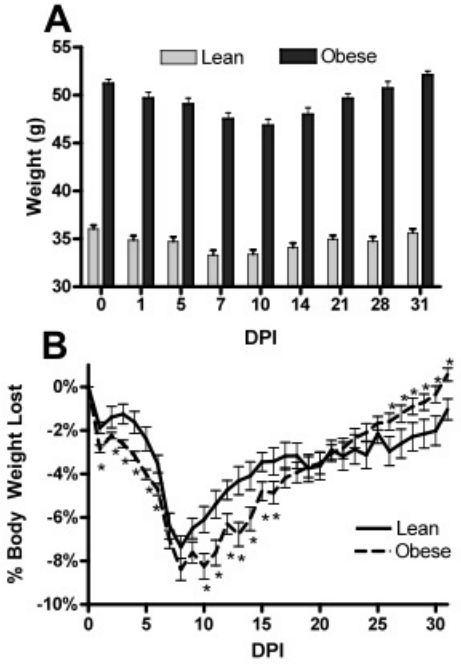


Figure 4.2. Increased viral load in the lungs of obese mice. Obese mice had greater viral mRNA expression in the lungs at d 1, 3 and 14 post infection. No virus was detected before X-31 infection (d 0). Data are expressed as change in M1 mRNA threshold cycle over uninfected controls (n=3-4/group) ± SEM. *p<0.05, lean versus obese.

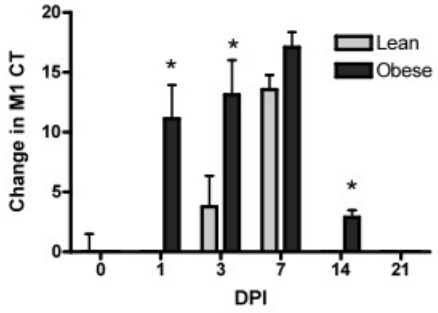


Figure 4.3. Antiviral cytokine mRNA expression in the lungs following X-31 infection. mRNA expression of (Fig. 1A) IFNα and (Fig. 1B*)* IFNβ were measured in the lungs of lean and obese mice following primary X-31 infection. Data are expressed by normalization to G3PDH values (n = 3-4 per group) ± SEM. There were no significant differences in G3PDH values.

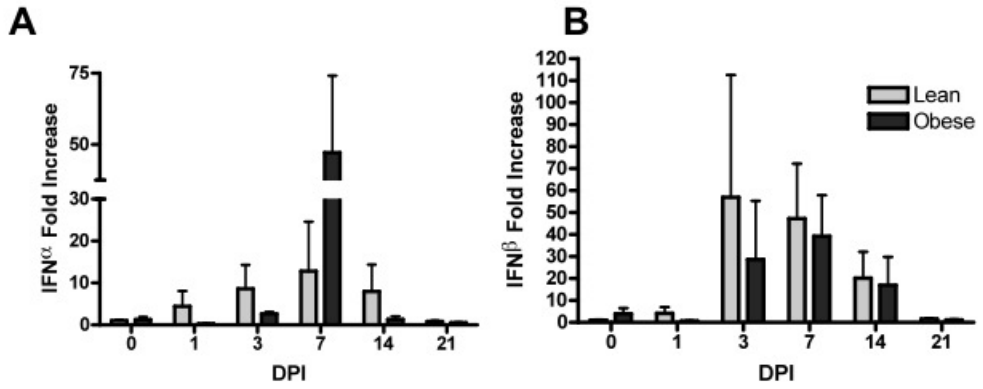


Figure 4.4. Number of responding influenza-specific CD8$^+$ T cells in increased in the lungs of obese mice. Number of total cells (Fig. 4A), number of lymphocytes (Fig. AB) and percentage (Fig. 4C) and number (Fig. 4D) of influenza-specific effector T cells (CD8$^+$/CD44$^+$/D$_b$NP$_{366-74}$$^+$) following X-31 infection were measured by flow cytometry. Data are expressed as number or percent cells of total cells isolated (n = 3-4 per group) $^+$/- SEM. *p<0.05, lean vs obese. a – p<0.05 versus d0

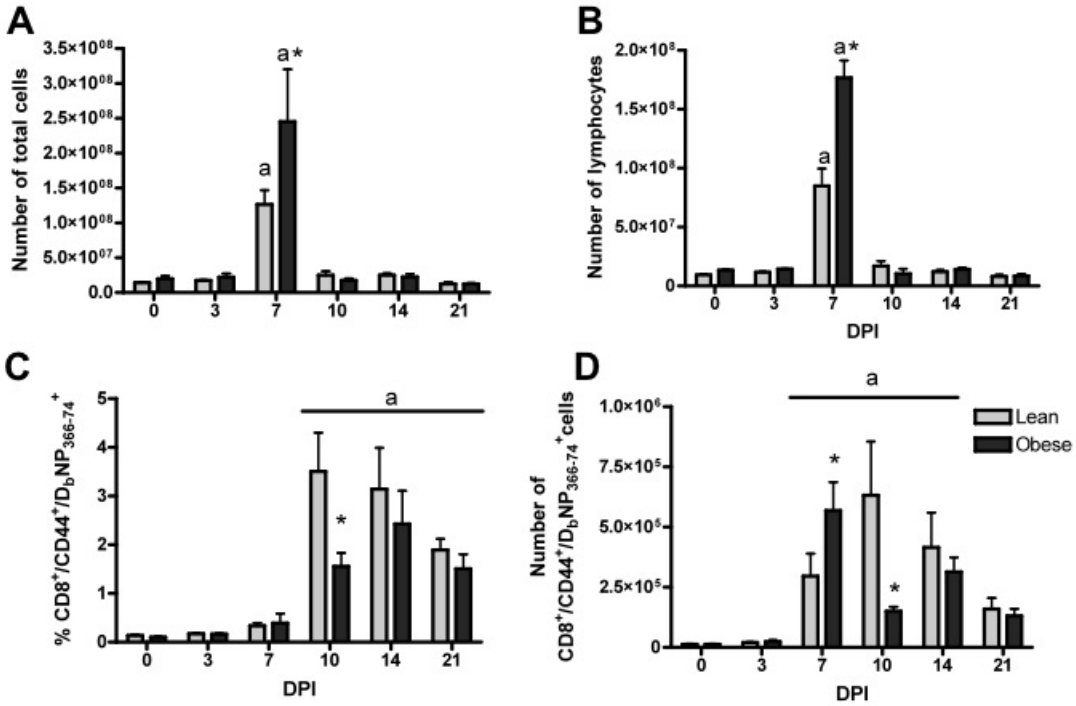


Figure 4.5. **Obese mice have increased influenza-specific T cells producing IFN-γ following infection.** mRNA expression of IFN-γ (Fig. 5A) was measured in the lungs of lean and obese mice following X-31 infection using quantitative real time-PCR. Data are expressed by normalization to G3PDH values (n = 3-4 per group) ± SEM. There were no significant differences in G3PDH values. *p<0.05, lean versus obese. (Fig. 5B) Number of influenza-specific T cells ($CD8^+/D^bNP_{366-374}{}^+/CD44^+$) producing IFN-γ following X-31 infection. Data are expressed as number of total cells isolated (n = 3-4 per group) $^+$/- SEM. *p<0.05, lean vs obese. a-p<0.05 versus d0

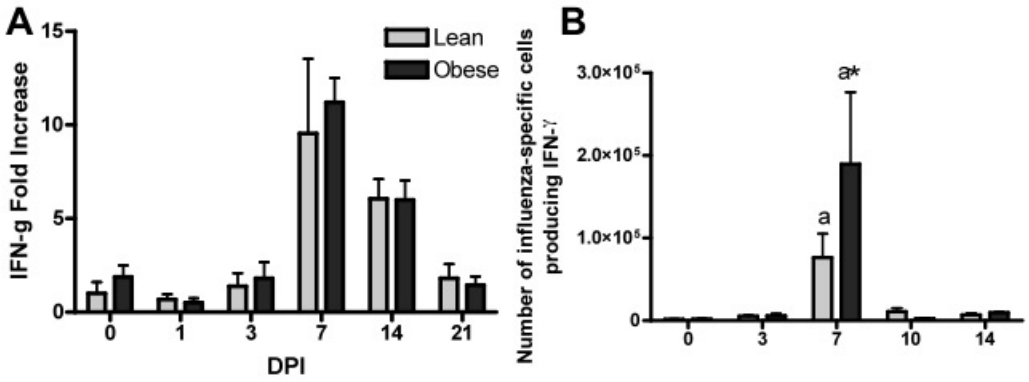


Figure 4.6. Inflammation-associated cytokine mRNA expression in the lung prior to and following primary infection. (Fig. 6A) mRNA expression of IL-6, TNFα, MCP-1 and MIP-1α were measured in the lungs of lean and obese mice before X-31 infection. (Fig. 6B) mRNA expression of IL-6 was measured in the lungs of lean and obese mice following X-31 challenge. Data are expressed by normalization to G3PDH values (n = 3-4 per group) ± SEM. *p<0.05, lean versus obese.

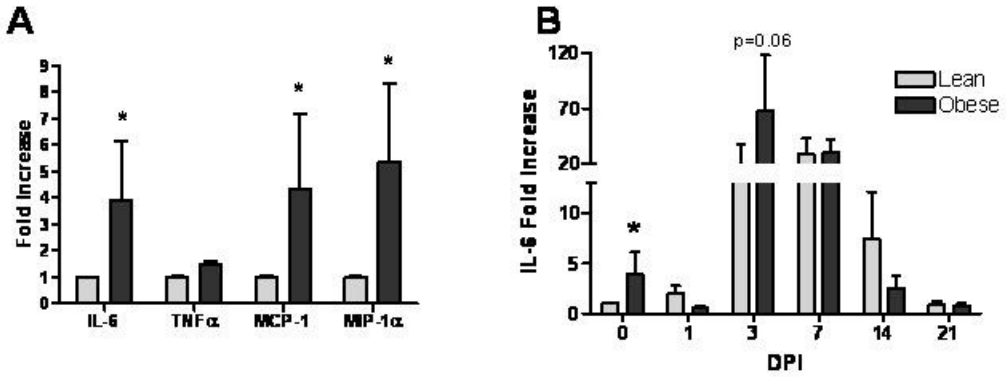


Figure 4.7. Differential expression of transcription factors associated with effector or memory formation in lean and obese mice. (Fig 7A) Number of $CD3^+CD8^+$ T cells in the lungs expressing T-bet was measured in lean and obese mice by flow cytometry. (Fig 7B) mRNA expression of Blimp-1 in the lung of obese mice. (Fig 7C) mRNA expression and (Fig 7D) percentage of $CD3^+CD8^+$ cells expressing Eomes in the lungs of lean and obese mice. Cellular data are expressed as number or percentage of total cells isolated (n = 3-4 per group) $^+$/- SEM. mRNA data are expressed by normalization to G3PDH values (n = 3-4 per group) ± SEM. *p<0.05, lean versus obese

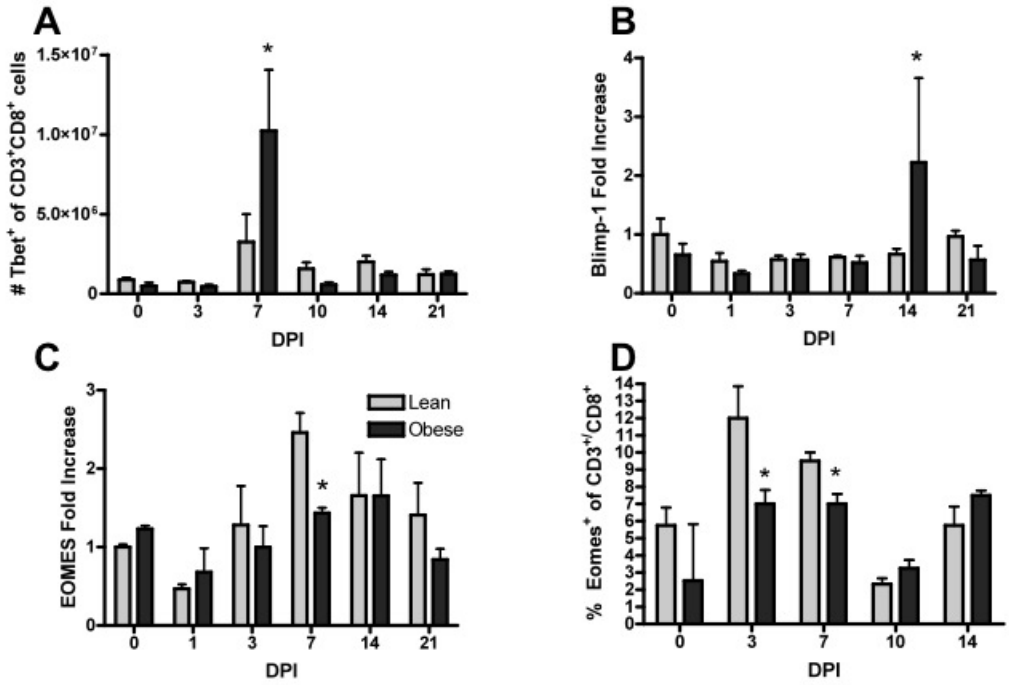
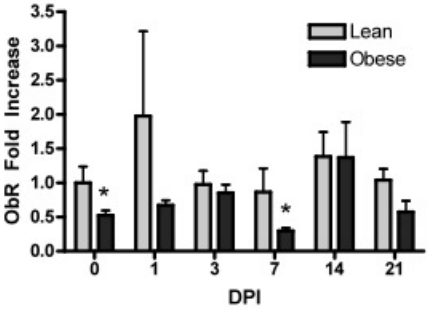


Figure 4.8. Expression of ObR mRNA in the lungs of lean and obese mice following primary influenza X-31 infection. mRNA expression of ObR was measured in the lungs of lean and obese mice following primary infection. Data are expressed by normalization to G3PDH values (n = 3-4 per group) ± SEM. There were no significant differences in G3PDH values. *p<0.05, lean versus obese



CHAPTER V

SYNTHESIS

A. Overview of Research Findings

Obesity has become a worldwide epidemic. Aside from numerous described comorbidities, obesity also appears to have a significant impact on the immune response. However, the effect of obesity on response to infection is just beginning to be understood. Infection with influenza virus remains a significant yearly cause of morbidity and mortality worldwide. Previous studies in our laboratory have demonstrated that diet-induced obesity in mice results in significant increases in morbidity and mortality versus lean controls (199,200).

Development of influenza-specific memory $CD8^+$ T cells is dependent on a balanced and effective primary response. Since we have demonstrated a significant dysregulation of the primary response to influenza infection in diet-induced obese mice, we hypothesized that obesity would also result in an impaired memory response to secondary influenza infection with a heterologous viral strain. Indeed, in this work, we have demonstrated that influenza-specific memory T cells in obese mice have a significantly reduced ability to respond to secondary challenge. In addition, we have shown that these cells are not maintained in the obesigenic environment.

B. Diet-induced obesity impairs the T cell memory response to influenza virus infection

Previous studies in our laboratory have shown that diet-induced obesity in mice significantly impairs the primary response to influenza infection resulting in increased morbidity and mortality (199,200). Since development of memory T cells depends on the primary response, we hypothesized that DIO mice would also impair a memory T cell response to a secondary influenza challenge. To test this hypothesis, lean and obese mice were first challenged with a nonlethal dose of influenza X-31. Following resolution of the primary infection and ensuring viral clearance, mice were then challenged with a lethal dose of influenza PR8. The memory response was then analyzed using a variety of methodology including qRT-PCR and flow cytometry.

In our study, priming with influenza X-31 resulted in protection against the lethal dose of a heterologous PR8 strain of influenza with 100% survival in lean mice; however, obesity reduced this protective capacity resulting in ~25% mortality. Consistent with infection, both lean and obese mice lost weight following PR8 challenge, although the infection was more severe in the obese mice as indicated by the lack of weight regain after day 7 post infection. This increased severity of infection was confirmed by the increased lung pathology and viral titers in obese mice. Early innate antiviral cytokines IFNα and IFNβ were significantly reduced in the lungs of obese mice which may have contributed to the higher viral titers and increased infiltrate found in lungs of obese mice. Additionally, infection associated upregulation of inflammatory cytokine expression was also found to be significantly blunted in the obese mice.

Following a secondary challenge with influenza virus, memory $CD8^+$ T cells in the lung airways respond to initial viral loads by generating signals, such as IFN-γ, to induce the antiviral response (364,365). The protective value of $CD8^+$ T cell memory cells is strongly correlated to the ability to exert effector function at the site of infection (366). DIO mice had

significantly decreased overall IFN-γ mRNA expression in the lung. More specifically, obese mice were found to have decreased percentage and overall number of influenza-specific effector memory T cells producing IFN-γ post secondary challenge. Next, we tested whether production of IFN-γ by memory T cells during secondary infection in DIO mice may be due to impaired DC functionality. Interestingly, we found that in contrast to a primary influenza infection, DC from DIO mice were able to efficiently present antigen to lean and obese memory T cells; however, memory T cells from DIO mice had a significantly decreased ability to respond to presented antigen.

Although the mechanistic link between obesity and diminished immune memory is not clear, a main factor which ties together obesity, inflammation and immune cell function is leptin resistance associated with the obese state. Leptin is recognized as an important mediator of immune function (434) and leptin expression is increased proportionately with adipose tissue mass, resulting in increased circulating levels in obesity (435). This chronic elevation appears to cause a state of leptin resistance in obese mice where leptin signaling is attenuated despite increased circulating levels (436). Serum leptin was significantly increased in obese mice before secondary challenge and did not show an infection associated response as seen in the lean controls. Therefore, leptin resistance in obese mice may contribute to the reduced protective capacity of the memory response to secondary influenza infection

To our knowledge, this is the first time that diet-induced obesity has been shown to affect the memory T cell response to a viral infection. It is apparent that diet-induced obesity results in an ineffective memory T cell response to influenza infection resulting in increased morbidity and mortality. Impairment of leptin signaling may contribute to this reduced functionality.

C. Influence of diet-induced obesity on maintenance of influenza-specific $CD8^+$ memory T cells

In the previous study, we found that diet-induced obesity in mice led to decreased protection from an infection with a heterologous strain of influenza virus. This decrease was found to be a result of impaired functionality of influenza-specific memory T cells in the lungs of obese mice (443). This reduced protection was associated with the memory T cell response since the number and function of influenza-specific $CD8^+$ T cells responding to secondary infection was significantly reduced in obese mice. Following viral clearance, contraction of activated $CD8^+$ T cells results in influenza-specific memory cells representing ~5 to 10% of effector $CD8^+$ T cells found at the peak of expansion during primary infection (276,277). These long-lived memory cells can rapidly respond to a secondary infection with influenza virus. Survival of memory cells now appears to be maintained by members of the common γ_c family of cytokines, IL-15 and IL-7. As the expression of both IL-7 and IL-15 can be affected by inflammatory cytokines and both share signaling pathways with the leptin receptor, we hypothesized that the maintenance of influenza-specific $CD8^+$ memory T cells would be impaired in diet-induced obese mice compared to lean controls.

To test this hypothesis, lean and obese mice were infected with influenza X-31 and memory cell populations were assessed at days 33 and 84 post infection. Memory T cells established after primary infection can be divided into two subsets: central memory T cells (T_{CM}) which preferentially localize to the lymphoid tissues, and effector memory T cells (T_{EM}) found mainly in peripheral sites of infection (343,431). Numbers of T_{CM} in lymphoid sites appear to remain relatively constant, if not slightly increased, while the number of T_{EM} at peripheral sites steadily decreases in the months after pathogen clearance. Although both lean and obese mice demonstrated the expected increase in % T_{CM} and decrease in T_{EM}, the T_{EM} in the lungs of the obese animals declined at a significantly faster pace resulting in a

10% reduction in the numbers of T_{EM} present versus the lean controls at day 84 post X-31 challenge.

IL-15 is essential for the regulation of slow, intermittent basal homeostatic proliferation of antigen-specific $CD8^+$ memory T cells (379). In the lean mice, IL-15 mRNA expression was maintained at a constant level. However, expression of mRNA for IL-15 in the obese animals was approximately 30 times greater at d 84 pi compared to d 33. This increase in IL-15 mRNA did not result in an increase in T_{EM}. In fact, fewer T_{EM} were found in the obese lung. Reasons for this discrepancy may be due to reduced IL-15 receptor or a blockade of IL-15 signaling. While the mRNA expression of IL-15 receptor components CD122 and IL-15Rα in the lungs of lean and obese mice were not found to be different (data not shown), the numbers of T_{EM} expressing CD122 were significantly reduced in the lungs of diet-induced obese mice 33 days post primary infection. In addition, mean fluorescence intensity of CD122 was also significantly reduced on T_{EM} expressing the receptor, indicating a decreased amount of expression on these cells.

Similar to our findings in the previous experiment, leptin signaling and leptin resistance in the obesigenic state may contribute to the decreased maintenance of memory T cells. Leptin, IL-7 and IL-15 share structural homology and signal through the JAK-STAT pathway. In the lungs of obese mice, leptin receptor (ObR) mRNA expression was significantly decreased at d 33 pi and did not increase over time as was found in the lean controls, suggesting that leptin signaling was likely impaired. Consistent with leptin resistance, mRNA expression of SOCS-1 and SOCS-3, potent inhibitors of JAK-STAT signaling, were significantly increased in the lungs of obese mice which may have impaired IL-15 signaling.

The exact mechanism for the reduction of CD122 expression is not yet clear; however, several possible changes associated with diet-induced obesity could be the causal factor. Type I interferon signals (IFNα/β) appears to be very important for the production of

the basal level of IL-15. IFNα/β receptor-deficient mice have half as many CD122^{hi} memory cells versus normal wildtype controls. This is even more pronounced in STAT1-deficient mice which are unresponsive to both IFNα/β and IFN-γ (446). Previous studies in our lab have shown that IFNα/β production is significantly reduced in obese mice (199,443). Therefore, a defect in the JAK-STAT signaling in T cells may be a causal factor in the decreased memory cell maintenance in the lungs of obese mice.

One factor that could cause this decreased signaling would be peripheral leptin resistance associated with obesity. Leptin expression is increased proportionately with adipose tissue mass, resulting in increased circulating levels in obesity (435). This chronic elevation appears to cause a state of central leptin resistance in obese mice where leptin signaling is attenuated in the brain despite increased circulating levels (436,455); however, peripheral leptin resistance in cells of the immune system has also been suggested (75). Leptin, IL-7 and IL-15 share structural homology and signal through the JAK-STAT pathway. Indeed, in lean Zucker rats, lean mice and obese leptin-deficient (*ob/ob*) mice, IL-15 administration inhibited fat deposition; however, recombinant IL-15 was unable to prevent fat deposition in leptin receptor-deficient obese (*fa/fa*) Zucker rats (406). The inability of IL-15 administration to reduce fat deposition in leptin receptor-deficient rodents indicates that leptin receptor signaling can be very important for the function of IL-15. Previous studies in our lab have shown that obese mice have significantly increased serum expression of leptin and in contrast to lean mice, leptin expression was not augmented by acute infection (443). In the current study, leptin receptor mRNA expression was significantly decreased in the lungs of the obese mice at d 33 pi and did not increase over time as was found in the lean controls. The reduced expression of ObR could indicate a reduction of IL-15 signaling and, therefore, reduced IL-15 maintenance of memory T cells. The increased expression of ObR mRNA in lean mice was likely due to the weight gain over time.

From these data and that of the previous experiment, it is apparent that diet-induced obese mice are able to generate influenza-specific memory $CD8^+$ T cells; however, the functional capability of these cells (443) and their ability to be maintained in the lung is significantly reduced. The understanding of memory T cell maintenance is still in its infancy; however, it is clear that IL-15 and IL-7 and their subsequent signals are important for maintaining the memory cell population over time. This study has shown, for the first time, that diet-induced obesity can affect the maintenance of influenza-specific memory T cell populations in the lungs and that this decrease in memory T cell numbers may be due to peripheral leptin resistance in the obesigenic lung microenvironment.

D. Generation of influenza-specific memory T cells in a diet-induced obese host during a primary influenza infection

Development of functional, fully protective influenza-specific memory $CD8^+$ T cells is highly dependent on the primary encounter with the influenza virus. Current theory suggests that both initial antigen contact/interaction with an APC and environmental effects play a role in memory cell generation. The overall equilibrium of these signals is very important and it is important to be able to balance between development of long-lived, protective memory T cells and enough effector cells to be able to clear the infection (275,295,296). Alteration of any part of the immune program could result in an ineffective or diminished population of antigen-specific memory T cells. In our previous studies, we have shown both reduced functionality and reduced maintenance of influenza-specific memory $CD8^+$ T cells in the lungs of diet-induced obese mice. Therefore, in this study, we sought to observe the development of these cells during a primary infection in diet-induced obese mice versus lean controls.

Following influenza X-31 infection, diet-induced obese mice lost a greater percentage of body weight and weight regain was significantly delayed indicating increased

severity of illness. Obese mice had almost double the number of influenza-specific cells in the lungs compared to lean controls at d 7 post infection. Resolution of a viral infection often relies on the induction of a $CD8^+$ T cell response and induction of induction of effector T cells can be proportional to the level of infection (241). In accordance to increased cellularity, viral load was greatly increased in the obese mice starting at d 1 p.i. which may have stimulated the observed increase in number of influenza-specific $CD8^+$ T cells in order to handle the increased viral load. In addition, the number of influenza-specific $CD8^+$ T cells producing IFN-γ was significantly increased in the lungs of obese mice versus lean controls, again, likely in response to the need to clear a larger amount of virus.

Following viral infection, respiratory epithelial cells produce antiviral and inflammatory cytokines in order to limit viral infectivity and to activate and attract the immune cells to the site of infection. IFNα and IFNβ are major antiviral cytokines and can mediate direct antiviral activity of cells (229). Obese mice displayed a trend towards decreased and delayed expression of IFNα/β mRNA which may have resulted in the significantly increased viral load seen in the lungs of obese mice. Inflammatory signals were also altered in the lungs of obese mice which may have contributed to increased number of primary influenza-specific effector $CD8^+$ T cells seen in the obese lungs. mRNA expression of proinflammatory cytokines IL-6, MCP-1 and MIP-1α was greatly increased in the lungs of obese mice before X-31 infection and IL-6 mRNA expression was increased at d3 post X-31 infection compared to lean mice.

Increased inflammation is associated with increased primary effectors but decreased memory precursors and differentiation into long-term memory (281). Indeed, increased number of activated $CD8^+$ T cells responding to the infection could indicate a larger need for primary effectors and decreased memory T cell precursor formation. Several transcription factors have been shown to coordinate and regulate the balance between memory T cell

and primary effector formation (282,327,332). Expression of these factors in the obese lung indicated an increase in short-lived effector $CD8^+$ T cell formation and a possible decrease in memory T cell precursors. Expression of effector-promoting factors T-bet and Blimp-1 was significantly increased in the lungs of obese mice at d 7 and d 14 p.i. respectively. Expression of the memory precursor-associated factor, Eomes, was found to be significantly decreased. Thus, the increase in T-bet and Blimp-1 and decreased Eomes expression all point towards an increase in primary, short-lived effector T cell formation and a decrease in the number of cells forming the influenza-specific memory T cell pool.

The inability of lung epithelial cells in obese mice to respond to viral infection and express antiviral IFN, possibly due to leptin resistance, could result in the increased viral load and subsequent increase in number of influenza-specific effector T cells needed to clear the infection. This increase could be tipping the balance between effector and memory T cell generation with increased primary effector T cells and reduced numbers of MPEC. Taken together, this study indicates that memory T cell generation during a primary response to influenza infection is indeed impaired in obese mice. These findings correlate with our previous observation of decreased influenza-specific memory $CD8^+$ T cell function and maintenance in the lungs of obese mice.

E. Future Studies

E.1. The impact of the obesigenic lung microenvironment

The lung airways are distinct from other non-lymphoid tissue since: (i) they contain surfactant proteins that are known to block T cell proliferation and (ii) they are directly exposed to the outside environment (456). The environment in which cells reside and the ability to migrate to potential sites of infection are very important for function and protectiveness of memory cells. The microanatomy of the secondary lymphoid organs as

well as tissue-specific events are critical for the immune response to specific infectious agents (457). Obese subjects display greater incidence of asthma and pulmonary related complications (21,172,458). Additionally, increased inflammatory cytokines and chemokines during influenza challenge can result in increased cellular recruitment to the lungs causing airway occlusion and bystander damage to the lung tissue itself. These changes may cause alterations in both innate and adaptive systems which can have both helpful and detrimental effects (459). Therefore, the obese lung environment may negatively affect $CD8^+$ memory T cells.

Migration of T_{CM} from lymphoid to nonlymphoid tissue results in the conversion to a T_{EM} phenotype (361). Additionally, the lung airway environment itself has been shown to directly influence surface markers and maintenance on $CD8^+$ memory cells in the absence of antigenic exposure. Memory cells isolated from the spleen and transferred intratracheally (i.t.) into the lungs downregulate LFA-1, CD27, CD127 and Ly6C and increase CD69 while the same cells given intraperitoneally (i.p.) do not go through this change (353,362). Gene expression profiles of $CD8^+$ memory cells in lung airway and spleen generated during influenza infection reveal a “focusing” of genes to favor effector function in the airway environment (363).

It has been well established that the adoptive transfer (AT) of influenza-specific cytotoxic T lymphocytes into a naïve host is protective against influenza infection (460). Direct evidence for the protective capacity of AT lymphocytes has been proven using i.t., intravenous (i.v.) and i.p. transfer studies (352,353,362). A single adoptive transfer of cells from one experimental group to another allows for the determination of environmental effects on the transferred cells. Adoptive transfer studies utilizing DIO in congenic mouse models would greatly further our understanding of the effect of memory T cell function and maintenance in the obesigenic lung microenvironment.

E.2. The effect of DIO on antigen-specific memory T cell migration

It is very important for antigen-specific memory T cells to be able to localize to the most likely site of reinfection. Memory T cells are widely distributed throughout the body; however, the site of infection and type of tissue infected can greatly influence memory T cell distribution (461). Since T_{EM} cannot proliferate in the airways, the current paradigm is that memory $CD8^+$ populations in the lung are maintained by continual recruitment from the T_{CM} population (353). The exact mechanism for memory T cell recruitment and establishment in the lung airways is unknown. Kohlmeier et al. (2007) hypothesized that continuous exposure to inhaled antigens and microorganisms could produce a state of low level inflammation which could stimulate chemokine production and memory cell recruitment (362). Infection of the lungs by influenza virus results in markedly increased numbers of influenza-specific memory T cells to be maintained in the lungs for long periods post infection. These cells preferentially migrate to the site of original infection versus other peripheral sites which did not contain replicating virus. This “preference” of antigen-specific memory T cells to migrate to the site of primary infection allows them to be present at the site of most likely reinfection (461).

This site-specificity indicates that cell-surface molecule expression may dictate homing patterns of memory cells. Several studies have shown that specific combinations of adhesion molecules and chemokine receptors are associated with T cell migration to distinct peripheral tissues such as the skin, gut, lungs and even the central nervous system (348,462-465). The importance of integrin signaling for lung homing was demonstrated by Ray et al. (2004) in which antibody blocking or genetic deficiency of the integrin important for lung homing increased memory cell numbers in the spleen but decreased numbers in the lung, thereby increasing susceptibility to secondary influenza infection (466). We observed a similar situation in the obese mice in which T_{EM} populations were significantly reduced in the lungs whereas T_{CM} cells were significantly increased in the spleen. Therefore, maintenance

signals as well as homing signals for memory T cells may be impaired in obese mice and these studies require further investigation. Studies focusing on adhesion molecule expression and cell migration in DIO mice would help to determine the impact of obesity on T cell recruitment to the lungs during a viral infection.

E.3. T cell metabolism

Proliferation of mammalian cells, including T cells, is controlled by extrinsic signals which regulate nutrient utilization (467). Non-proliferating, quiescent T cells (naïve and memory T cells) use catabolic metabolism to fuel ATP generation (468). Following antigenic stimulation and costimulation, T cells go through a metabolic switch to glycolysis and anabolic metabolism, which is required to support their proliferation and effector functions (469,470). This switch is achieved by the activation of Akt which then promotes the mTOR pathway as well as increasing utilization of glucose and amino acids (471-474). Therefore, this switching between differing metabolic states is required for effective generation of T cell fates. Indeed, the fact that metabolism underlies the functional capacity of a T cell either to respond to infection or to remain as a memory cell suggests that alteration of metabolic parameters could greatly affect memory T cell fates (333).

Very recently, mTOR activity has also been associated with generation of memory $CD8^+$ T cells. Araki et al. (2009) and Pearce et al. (2009) have shown that blocking mTOR function by rapamycin treatment promoted memory generation during both the expansion and contraction phases of the T-cell response. Additionally, Pearce et al. (2009) showed that the anti-diabetic drug metformin, activated AMPK and enhanced memory T cell generation by inhibiting the mTOR pathway (339,475). Interestingly, dietary restriction studies which have been shown to promote lifespan in a number of organisms are thought to result in reduced mTOR activity (476). While the exact mechanisms of T cell metabolic

switching are still under study, it may be interesting to pursue how DIO can alter T cell metabolism and subsequent T cell fate. Obesity has been associated with significant alterations in insulin and glucose utilization and is a significant risk factor for the development of T2DM (411,423,477). Moreover, there have been recent implications that overnutrition directly inhibits insulin signaling in muscle at the level of IRS1 through the hyperactivation of the mTOR pathway (478). Additionally, leptin signaling also appears to alter AMPK/mTOR activation (479,480). If obesity hyperactivates mTOR, memory T cell generation may be at a significant disadvantage. Future studies will focus on the alteration of metabolism in T cells from DIO mice and the effects on memory T cell generation

E.4. Regulatory T cells in the obesigenic state

Thymus-derived, naturally occurring, regulatory T (T_{reg}) cells are a subset of T lymphocytes that constitutes about 5%–10% of peripheral $CD4^+$ T cells. T_{reg} cells constitutively express the high-affinity interleukin-2 (IL-2) receptor α chain CD25 and can inhibit effector T cell responses in vitro and in vivo. T_{reg} cells express the forkhead family transcription factor Foxp3, a key control gene for their development and function. T_{reg} cells have been shown to be important for the establishment and maintenance of self-tolerance and the prevention of autoimmune and inflammatory diseases (481,482). T_{reg} cells have also been implicated in controlling the primary and memory response to viral infection. Suvas et al. (2003) found that the magnitude of a $CD8^+$ T cell–mediated immune response to an acute viral infection is also subject to control by T_{reg}. Depletion of T_{reg} with specific anti-CD25 antibody before infection with HSV resulted in enhanced $CD8^+$ T cell response (483). Furthermore, responding $CD8^+$ T cells remained activated for a longer period of time, and the memory responses of T_{reg} –depleted animals were elevated around threefold compared to controls. In vitro studies have also shown that T_{reg} can suppress T-cell

proliferation/expansion, IFN-γ secretion, and can abrogate inflammatory signals important for T cell activation and accumulation (484,485).

To date, very little work has been done to observe the effect of diet-induced obesity on the T_{reg} population; however, leptin and leptin signaling appears to be very important for T_{reg} function. De Rosa et al. (2007) have found that human T_{reg} cells express high amounts of both leptin and the leptin receptor and that the leptin pathway can act as a negative regulator of proliferation of T_{reg} (74). Indeed, Taleb et al. (2007) found that leptin deficiency in *ob/ob* mice improves the T_{reg} immune response in these animals (486). These findings may partly explain why leptin receptor deficiency can be associated with increased susceptibility to infection (165,198,487). It may also be important in DIO since obese individuals are suggested to be in a state of leptin resistance. Leptin resistance may contribute to increased T_{reg} populations in DIO models and, therefore, decrease the $CD8^+$ T cell response to influenza and the subsequent generation of protective memory. Further studies are needed to investigate the association between DIO, T_{reg} cells and development of memory T cells in the context of an influenza challenge.

E.5. Epigenetic control of memory T cells

Lineage fate decisions in T cells requires that a cell transcribes specific sets of genes while repressing or silencing others. Most of this gene expression is not controlled by permanent alteration of primary genetic information but by changes in epigenetic differences in the genes which are expressed (488-490). In eukaryotic cells, production of biologically active proteins is under sophisticated regulation at several points such as the initiation of transcription. Accessibility to genetic information by the transcription machinery depends on the “openness” of the chromatin structure. Modifications of DNA and DNA-binding histone molecules result in different chromatin structures. Epigenetic changes in DNA, such as DNA

methylation and histone modifications, allow for structural alterations in chromatin organization resulting in permissibility of transcription machinery to initiate gene transcription (491). Although epigenetic changes are established early during development and differentiation, adaptations occur throughout life in response to intrinsic and environmental stimuli. For example, DNA demethylation occurs in the IL-2 promoter of T cells within 20 minutes of stimulation (492). Molecular differentiation processes that induce memory $CD8^+$ T cell fate and drive the development of memory $CD8^+$ T cells are poorly understood. However, cell fate decisions of T cell lineages are significantly altered in mice unable to promote the gene-silencing effect of DNA methylation. These mice have profound changes in the susceptibility and resistance to parasitic infections (493,494). Altered $CD4^+$ differentiation has also been documented by experiments using an inhibitor of methylation and through genetic abrogation of the maintenance methyltransferase, Dnmt1 (495,496).

Genetic reduction of methylation ability has been found to decrease memory T cell precursor formation and the responsiveness of the resulting memory T cell pool (497). Interestingly, diet-induced obesity has been found to alter methylation status in rats (498); however, there appear to be few studies observing the effect of dietary treatment on the epigenetic modification of immune cells. Further studies need to be conducted observing the effects of DIO on epigenetic modification of T cell fate decisions and their potential effects on memory cell generation and subsequent function in the context of viral infection.

F. Public Health Significance

The ability to generate functional memory T cells, either during primary infection or by vaccination, has proven to be protective against potentially lethal influenza strains exhibiting completely different surface antigens (268). The CDC and WHO have declared the currently circulating novel H1N1 influenza strain to be pandemic and obesity appears to be an independent risk factor for illness severity (205). The present studies provide evidence that increased morbidity and mortality during a secondary influenza infection in DIO mice is due to impairment in the ability to generate and maintain functional influenza-specific memory T cells. This finding, if applicable to a human obese population, has significant public health implications given the epidemic rates of obesity worldwide, the annual incidence of influenza infection and the threat of a global pandemic.

Overall, decreased numbers of influenza-specific effector memory T cells over time, coupled with the decreased functional capability of these cells in the obese individual, suggests that obesity presents an even greater risk from infection with a heterologous pandemic strain. Obesity has been definitively linked to a number of comorbidities such as T2D and cardiovascular disease (10,13,499). In addition, increased adiposity has also been linked with several immunomodulatory effects (70,71,500). However, this work, combined with our previous studies (199,200), strengthens the association of obesity as an independent risk factor for increased morbidity and mortality from influenza virus infection.

Traditional methods of preventing influenza infection involve the generation of antibodies against viral surface proteins using vaccination. While the efficacy of vaccination in obese individuals is outside the scope of these studies, there is evidence that vaccination may not be as efficacious in obese individuals (187-189). In the context of this study, recent interest has focused on the generation of memory T cells using specific vaccination strategies (270,296,501). If these vaccine-induced memory cells cannot function in an obese

population, then new vaccination strategies may need to be considered in order to protect an increasingly obese world population.

REFERENCES

1. World Health Organization (2006). Fact Sheet 311: Obesity and overweight.

2. Catenacci, VA, Hill, JO & Wyatt, HR. The Obesity Epidemic. Clin Chest Med. 2009;30 (3):415-444.

3. Wang, Y, Beydoun, MA, Liang, L, Caballero, B & Kumanyika, SK. Will All Americans Become Overweight or Obese? Estimating the Progression and Cost of the US Obesity Epidemic. Obesity. 2008;16 (10):2323-2330.

4. Hill, JO, Wyatt, HR, Reed, GW & Peters, JC. Obesity and the Environment: Where Do We Go from Here? Science. 2003;299 (5608):853-855.

5. Sturm, R. Increases in Clinically Severe Obesity in the United States, 1986-2000. Arch Intern Med. 2003;163 (18):2146-2148.

6. Flegal, KM, Carroll, MD, Ogden, CL & Curtin, LR. Prevalence and Trends in Obesity Among US Adults, 1999-2008. JAMA. 2010;303 (3):235-241.

7. Friedman, JM. A War on Obesity, Not the Obese. Science. 2003;299 (5608):856-858.

8. Bellisari, A. Evolutionary origins of obesity. Obes Rev. 2008;9 (2):165-180.

9. Jeffery, RW & Harnack, LJ. Evidence Implicating Eating as a Primary Driver for the Obesity Epidemic. Diabetes. 2007;56 (11):2673-2676.

10. Pi-Sunyer, FX. The Obesity Epidemic: Pathophysiology and Consequences of Obesity. Obesity Res. 2002;10 (90002):97S-104S.

11. Pi-Sunyer, FX. The Medical Risks of Obesity. Obes Surg. 2002;12 (Suppl 1):6-11.

12. Sjostrom, L. Morbidity of severely obese subjects. Am J Clin Nutr. 1992;55 (2):508S-515.

13. Schelbert, KB. Comorbidities of Obesity. Primary Care: Clinics in Office Practice. 2009;36 (2):271-285.

14. Fantuzzi, G. Adipose tissue, adipokines, and inflammation. J Allergy Clin Immunol. 2005;115 (5):911-919.

15. Pond, CM. Adipose tissue and the immune system. Prostaglandins, Leukotrienes and Essential Fatty Acids: 3rd Throne Holst Foundation Symposium. 2005;73 (1):17-30.

16. Kannel, WB. Effect of weight on cardiovascular disease. Nutrition. 1997;13 (2):157-158.

17. Li, Z, Bowerman, S & Heber, D. Health Ramifications of the Obesity Epidemic. Bariatric Surg. 2005;85 (4):681-701.

18. Willett, WC, Dietz, WH & Colditz, GA. Guidelines for Healthy Weight. N Engl J Med. 1999;341 (6):427-434.

19. Lindstrom, J, Eriksson, JG, Valle, TT, Aunola, S, Cepaitis, Z, Hakumaki, M, Hamalainen, H, Ilanne-Parikka, P, Keinanen-Kiukaanniemi, S, Laakso, M, Louheranta, A, Mannelin, M, Martikkala, V, Moltchanov, V, Rastas, M, Salminen, V, Sundvall, J, Uusitupa, M & Tuomilehto, J. Prevention of Diabetes Mellitus in Subjects with Impaired Glucose Tolerance in the Finnish Diabetes Prevention Study: Results From a Randomized Clinical Trial. J Am Soc Nephrol. 2003;14 (90002):S108-113.

20. Calle, EE, Rodriguez, C, Walker-Thurmond, K & Thun, MJ. Overweight, Obesity, and Mortality from Cancer in a Prospectively Studied Cohort of U.S. Adults. N Engl J Med. 2003;348 (17):1625-1638.

21. Koenig, SM. Pulmonary complications of obesity. Am J Med Sci. 2001;321 (4):249-279.

22. Sjostrom, L. Mortality of severely obese subjects. Am J Clin Nutr. 1992;55 (2):516S-523.

23. Manson, JE, Stampfer, MJ, Hennekens, CH & Willett, WC. Body weight and longevity. A reassessment. JAMA. 1987;257 (3):353-358.

24. Manson, JE, Willett, WC, Stampfer, MJ, Colditz, GA, Hunter, DJ, Hankinson, SE, Hennekens, CH & Speizer, FE. Body weight and mortality among women. N Engl J Med. 1995;333 (11):677-685.

25. Baik, I, Ascherio, A, Rimm, EB, Giovannucci, E, Spiegelman, D, Stampfer, MJ & Willett, WC. Adiposity and Mortality in Men. Am J Epidemiol. 2000;152 (3):264-271.

26. Ajani, UA, Lotufo, PA, Gaziano, JM, Lee, I-M, Spelsberg, A, Buring, JE, Willett, WC & Manson, JE. Body mass index and mortality among US male physicians. Ann Epidemiol. 2004;14 (10):731-739.

27. Thompson, D, Edelsberg, J, Colditz, GA, Bird, AP & Oster, G. Lifetime Health and Economic Consequences of Obesity. Arch Intern Med. 1999;159 (18):2177-2183.

28. Oster, G, Thompson, D, Edelsberg, J, Bird, AP & Colditz, GA. Lifetime health and economic benefits of weight loss among obese persons. Am J Public Health. 1999;89 (10):1536-1542.

29. Sturm, R. The Effects Of Obesity, Smoking, And Drinking On Medical Problems And Costs. Health Aff. 2002;21 (2):245-253.

30. Finkelstein, EA, Fiebelkorn, IC & Wang, G (2003). National Medical Spending Attributable To Overweight And Obesity: How Much, And Who's Paying? In: Health Aff, p. hlthaff.w3.219.

31. Andreyeva, T, Sturm, R & Ringel, JS. Moderate and Severe Obesity Have Large Differences in Health Care Costs. Obesity. 2004;12 (12):1936-1943.

32. Wolf, AM & Colditz, GA. Current estimates of the economic cost of obesity in the United States. Obes Res. 1998;6 (2):97-106.

33. Colditz, GA. Economic costs of obesity and inactivity. Med Sci Sport Ex. 1999;31 (11 Suppl 1):S663.

34. Seidell, JC. The impact of obesity on health status: some implications for health care costs. Int J Obes Relat Metab Disord. 1995;19 (Suppl 6):S13-16.

35. Casper, R, Sullivan, E & Tecott, L. Relevance of animal models to human eating disorders and obesity. Psychopharmacology. 2008;199 (3):313-329.

36. Robinson, SW, Dinulescu, DM & Cone, RD. Genetic models of obesity and energy balance in the mouse. Ann Rev Gen. 2003;34 (1):687-745.

37. Hebebrand, J, Friedel, S, Schäuble, N, Geller, F & Hinney, A. Perspectives: molecular genetic research in human obesity. Obes Rev. 2003;4 (3):139-146.

38. Speakman, J, Hambly, C, Mitchell, S & Krol, E. Animal models of obesity. Obes Rev. 2007;8 (Suppl 1):55-61.

39. Friedman, JM & Leibel, RL. Tackling a weighty problem. Cell. 1992;69 (2):217-220.

40. Inui, A. Transgenic Approach to the Study of Body Weight Regulation. Pharmacol Rev. 2000;52 (1):35-62.

41. Salton, SRJ, Hahm, S & Mizuno, TM. Of Mice and Men: What Transgenic Models Tell Us about Hypothalamic Control of Energy Balance. Neuron. 2000;25 (2):265-268.

42. Johnson, PR, Greenwood, MRC, Horwitz, BA & Stern, JS. Animal Models of Obesity: Genetic Aspects. Annu Rev Nutr. 1991;11 (1):325-353.

43. Speakman, J, Hambly, C, Mitchell, S & Krol, E. The contribution of animal models to the study of obesity. Lab Anim. 2008;42 (4):413-432.

44. Carroll, L, Voisey, J & Daal, AV. Mouse Models of Obesity. Clin Dermatol. 2004;22 (345-49).

45. Pomp, D, Nehrenberg, D & Estrada-Smith, D. Complex Genetics of Obesity in Mouse Models. Annu Rev Nutr. 2008;28 (1):331-345.

46. Farooqi, S, Rau, H, Whitehead, J & O'Rahilly, S. ob gene mutations and human obesity. Proc Nutr Soc. 1998;57:471-475.

47. Farooqi, IS & O'Rahilly, S. Monogenic Human Obesity Syndromes. Recent Prog Horm Res. 2004;59 (1):409-424.

48. Buettner, R, Scholmerich, J & Bollheimer, LC. High-fat Diets: Modeling the Metabolic Disorders of Human Obesity in Rodents. Obesity. 2007;15 (4):798-808.

49. Mercer, JG & Archer, ZA. Putting the diet back into diet-induced obesity: Diet-induced hypothalamic gene expression. Eur J Pharmacol. 2008;585 (1):31-37.

50. Surwit, R, Kuhn, C, Cochrane, C, McCubbin, J & Feinglos, MN. Diet-induced type II diabetes in C57BL/6J mice. Diabetes. 1988;37 (9):1163-1167.

51. West, DB, Boozer, CN, Moody, DL & Atkinson, RL. Dietary obesity in nine inbred mouse strains. Am J Physiol Regul Integr Comp Physiol. 1992;262 (6):R1025-1032.

52. Reifsnyder, PC, Churchill, G & Leiter, EH. Maternal Environment and Genotype Interact to Establish Diabesity in Mice. Genome Res. 2000;10:1568-1578.

53. West, DB, Waguespack, J & McCollister, S. Dietary obesity in the mouse: interaction of strain with diet composition. Am J Physiol Regul Integr Comp Physiol. 1995;268 (3):R658-665.

54. Bartness, TJ, Demas, GE & Song, CK. Seasonal Changes in Adiposity: the Roles of the Photoperiod, Melatonin and Other Hormones, and Sympathetic Nervous System. Exp Biol Med. 2002;227 (6):363-376.

55. Rousseau, K, Atcha, Z & Loudon, ASI. Leptin and Seasonal Mammals. J Neuroendocrinol. 2003;15 (4):409-414.

56. Wade, GN. Obesity without overeating in golden hamsters. Physiol Behav. 1982;29:701-707.

57. Hand, MS, Armstrong, PJ & Allen, TA. Obesity: occurence, treatment and prevention. Vet Clin North Am Small Anim Pract. 1989;19 (3):447-474.

58. West, D & York, B. Dietary fat, genetic predisposition, and obesity: lessons from animal models. Am J Clin Nutr. 1998;67 (3):505S-512.

59. Ortiz, RM, Noren, DP, Litz, B & Ortiz, CL. A new perspective on adiposity in a naturally obese mammal. Am J Physiol Endocrinol Metab. 2001;281 (6):E1347-1351.

60. Kronfeld-Schor, N, Richardson, C, Silvia, BA, Kunz, TH & Widmaier, EP. Dissociation of leptin secretion and adiposity during prehibernatory fattening in little brown bats. Am J Physiol Regul Integr Comp Physiol. 2000;279 (4):R1277-1281.

61. Kemnitz, JW. Obesity in macaques: spontaneous and induced. Adv Vet Sci Comp Med. 1984;28:81-114.

62. Wozniak, S, Gee, L, Wachtel, M & Frezza, E. Adipose Tissue: The New Endocrine Organ? A Review Article. Dig Dis Sci. 2009;54 (9):1847-1856.

63. Lago, F, Dieguez, C, Gómez-Reino, J & Gualillo, O. The emerging role of adipokines as mediators of inflammation and immune responses. Cytokine Growth Factor Rev. 2007;18 (3-4):313-325.

64. Trayhurn, P & Wood, I. Signalling role of adipose tissue: adipokines and inflammation in obesity. Biochem Soc Trans. 2005;33 (Pt 5):1078-1081.

65. Trayhurn, P & Wood, IS. Adipokines: inflammation and the pleiotropic role of white adipose tissue. Br J Nutr. 2004;92:347-355.

66. Halberg, N, Wernstedt-Asterholm, I & Scherer, PE. The Adipocyte as an Endocrine Cell. Endocrinol Metab Clin North Am. 2008;37 (3):753-768.

67. Axelsson, Heimbürger, Lindholm & Stenvinkel. Adipose tissue and its relation to inflammation: The role of adipokines. J Renal Nutr. 2005;15 (1):131-136.

68. Bluher, M. Adipose Tissue Dysfunction in Obesity. Exp Clin Endocrinol Diabetes. 2009;117 (06):241-250.

69. Juge-Aubry, CE, Henrichot, E & Meier, CA. Adipose tissue: a regulator of inflammation. Best Prac Res Clin Endocrinol Metab. 2005;19 (4):547-566.

70. Karastergiou, K & Mohamed-Ali, V. The autocrine and paracrine roles of adipokines. Molec Cell Endocrinol. 2010;318 (1-2):69-78.

71. Radin, MJ, Sharkey, LC & Holycross, BJ. Adipokines: a review of biological and analytical principles and an update in dogs, cats, and horses. Vet Clin Path. 2009;38 (2):136-156.

72. Procaccini, C, Lourenco, E, Matarese, G & La Cava, A. Leptin signaling: A key pathway in immune responses. Curr Signal Transduct Ther. 2009;4 (1):22-30.

73. Lago, R, Gómez, R, Lago, F, Gómez-Reino, J & Gualillo, O. Leptin beyond body weight regulation--Current concepts concerning its role in immune function and inflammation. Cellular Immunol. 2008;252 (1-2):139-145.

74. De Rosa, V, Procaccini, C, Calì, G, Pirozzi, G, Fontana, S, Zappacosta, S, La Cava, A & Matarese, G. A Key Role of Leptin in the Control of Regulatory T Cell Proliferation. Immunity. 2007;26 (2):241-255.

75. Papathanassoglou, E, El-Haschimi, K, Li, XC, Matarese, G, Strom, T & Mantzoros, C. Leptin Receptor Expression and Signaling in Lymphocytes: Kinetics During Lymphocyte Activation, Role in Lymphocyte Survival, and Response to High Fat Diet in Mice. J Immunol. 2006;176 (12):7745-7752.

76. Mattioli, B, Straface, E, Matarrese, P, Quaranta, MG, Giordani, L, Malorni, W & Viora, M. Leptin as an immunological adjuvant: enhanced migratory and CD8+ T cell stimulatory capacity of human dendritic cells exposed to leptin. FASEB J. 2008;22 (6):2012-2022.

77. Fantuzzi, G. Leptin: Nourishment for the immune system. Eur J Immunol. 2006;36 (12):3101-3104.

78. Lazar, MA. Resistin- and Obesity-associated Metabolic Diseases. Horm Metab Res. 2007;39 (10):710-716.

79. MacDougald, OA & Burant, CF. The Rapidly Expanding Family of Adipokines. Cell Metab. 2007;6 (3):159-161.

80. Castan-Laurell, I, Boucher, J, Dray, C, Daviaud, D, Guigné, C & Valet, P. Apelin, a novel adipokine over-produced in obesity: Friend or foe? Mol Cell Endocrinol. 2005;245 (1-2):7-9.

81. Li, Q, Chen, R, Moriya, J, Yamakawa, J, Sumino, H, Kanda, T & Takahashi, T. A Novel Adipocytokine, Visceral Adipose Tissue-derived Serine Protease Inhibitor (Vaspin), and Obesity. J Int Med Res. 2008;36:625-629.

82. Bekri, S, Gual, P, Anty, R, Luciani, N, Dahman, M, Ramesh, B, Iannelli, A, Staccini-Myx, A, Casanova, D, Ben Amor, I, Saint-Paul, M-C, Huet, P-M, Sadoul, J-L, Gugenheim, J, Srai, SKS, Tran, A & Le Marchand-Brustel, Y. Increased Adipose Tissue Expression of Hepcidin in Severe Obesity Is Independent From Diabetes and NASH. Gastroenterology. 2006;131 (3):788-796.

83. Wen, Y, Gu, J, Li, S-L, Reddy, MA, Natarajan, R & Nadler, JL. Elevated Glucose and Diabetes Promote Interleukin-12 Cytokine Gene Expression in Mouse Macrophages. Endocrinology. 2006;147 (5):2518-2525.

84. Nishikawa, T, Edelstein, D, Du, XL, Yamagishi, S-i, Matsumura, T, Kaneda, Y, Yorek, MA, Beebe, D, Oates, PJ, Hammes, H-P, Giardino, I & Brownlee, M. Normalizing mitochondrial superoxide production blocks three pathways of hyperglycaemic damage. 2000;404 (6779):787-790.

85. Dandona, P, Chaudhuri, A, Mohanty, P & Ghanim, H. Anti-inflammatory effects of insulin. Curr Opin Clin Nutr Metab Care. 2007;10 (4):511-517.

86. Eder, K, Baffy, N, Falus, A & Fulop, A. The major inflammatory mediator interleukin-6 and obesity. Inflam Res. 2009;58 (11):727-736.

87. Maury, E & Brichard, SM. Adipokine dysregulation, adipose tissue inflammation and metabolic syndrome. Mol Cell Endocrinol. 2010;314 (1):1-16.

88. Takahashi, K, Mizuarai, S, Araki, H, Mashiko, S, Ishihara, A, Kanatani, A, Itadani, H & Kotani, H. Adiposity Elevates Plasma MCP-1 Levels Leading to the Increased CD11b-positive Monocytes in Mice. J Biol Chem. 2003;278 (47):46654-46660.

89. Fain, JN, Madan, AK, Hiler, ML, Cheema, P & Bahouth, SW. Comparison of the Release of Adipokines by Adipose Tissue, Adipose Tissue Matrix, and Adipocytes from Visceral and Subcutaneous Abdominal Adipose Tissues of Obese Humans. Endocrinology. 2004;145 (5):2273-2282.

90. Fain, JN (2006) Release of Interleukins and Other Inflammatory Cytokines by Human Adipose Tissue Is Enhanced in Obesity and Primarily due to the Nonfat Cells Vitamins & Hormones. In: Interleukins, Volume 74 ed. (Litwack, G., ed.), pp. 443-477. Academic Press.

91. Yudkin, JS. Adipose tissue, insulin action and vascular disease: inflammatory signals. Int J Obes Relat Metab Disord. 2003;27 (S3):S25-S28.

92. Clement, K, Viguerie, N, Poitou, C, Carette, C, Pelloux, V, Curat, CA, Sicard, A, Rome, S, Benis, A, Zucker, J-D, Vidal, H, Laville, M, Barsh, GS, Basdevant, A, Stich, V, Cancello, R & Langin, D. Weight loss regulates inflammation-related genes in white adipose tissue of obese subjects. FASEB J. 2004;18 (14):1657-1669.

93. Weisberg, SP, McCann, D, Desai, M, Rosenbaum, M, Leibel, RL & Ferrante, AW, Jr. Obesity is associated with macrophage accumulation in adipose tissue. J Clin Invest. 2003;112 (12):1796-1808.

94. Curat, CA, Miranville, A, SengenÃ¨s, C, Diehl, M, Tonus, C, Busse, R & Bouloumi, A. From Blood Monocytes to Adipose Tissue-Resident Macrophages. Diabetes. 2004;53 (5):1285-1292.

95. Fain, JN, Bahouth, SW & Madan, AK. TNF[alpha] release by the nonfat cells of human adipose tissue. Int J Obes Relat Metab Disord. 2004;28 (4):616-622.

96. Hausberger, FX. Parabiosis and transplantation experiments in hereditarily obese mice. Anat Rec. 1959;130:313.

97. Coleman, DL. Obese and diabetes: two mutant genes causing diabetes-obesity syndromes in mice. Diabetologia. 1978;14:141-148.

98. Zhang, Y, Proenca, R, Maffei, M, Barone, M, Leopold, L & Friedman, JM. Positional cloning of the mouse obese gene and its human homologue. Nature. 1994;372:425-432.

99. Friedman, JM. The function of leptin in nutrition, weight, and physiology. Nutr Rev. 2002;60:S1-14.

100. Fantuzzi, G & Faggioni, R. Leptin in the regulation of immunity, inflammation, and hematopoiesis. J Leukoc Biol. 2000;68 (4):437-446.

101. Lord, GM, Matarese, G, Howard, JK, Baker, RJ, Bloom, SR & Lechler, RI. Leptin modulates the T-cell immune response and reverses starvation-induced immunosuppression. Nature. 1998;394 (6696):897-901.

102. Sarraf, P, Frederich, RC, Turner, EM, Ma, G, Jaskowiak, NT, Rivet, DJ, III, Flier, JS, Lowell, BB, Fraker, DL & Alexander, HR. Multiple Cytokines and Acute Inflammation Raise Mouse Leptin Levels: Potential Role in Inflammatory Anorexia. J Exp Med. 1997;185 (1):171-176.

103. Lago, F, Dieguez, C, Gomez-Reino, J & Gualillo, O. Adipokines as emerging mediators of immune response and inflammation. Nat Clin Pract Rheumatol. 2007;3 (12):716-724.

104. Myers, MG, Cowley, MA & Munzberg, H. Mechanisms of Leptin Action and Leptin Resistance. Annu Rev Physiol. 2008;70 (1):537-556.

105. Morrison, CD. Leptin resistance and the response to positive energy balance. Physiol Behav. 2008;94 (5):660-663.

106. Sahu, A. Leptin signaling in the hypothalamus: emphasis on energy homeostasis and leptin resistance. Front Neuroendocrinol. 2003;24 (4):225-253.

107. Friedman, JM & Halaas, JL. Leptin and the regulation of body weight in mammals. 1998;395 (6704):763-770.

108. Otero, M, Lago, Rcaao, Lago, F, Casanueva, FF, Dieguez, C, Gómez-Reino, JJ & Gualillo, O. Leptin, from fat to inflammation: old questions and new insights. FEBS Letters. 2005;579 (2):295-301.

109. Prodi, E & Obici, S. Minireview: The Brain as a Molecular Target for Diabetic Therapy. Endocrinol. 2006;147 (6):2664-2669.

110. Elmquist, JK, Elias, CF & Saper, CB. From Lesions to Leptin: Hypothalamic Control of Food Intake and Body Weight. Neuron. 1999;22 (2):221-232.

111. Elmquist, JK, Bjørbæk, C, Ahima, RS, Flier, JS & Saper, CB. Distributions of leptin receptor mRNA isoforms in the rat brain. J Comp Neurol. 1998;395 (4):535-547.

112. Elias, CF, Aschkenasi, C, Lee, C, Kelly, J, Ahima, RS, Bjorbæk, C, Flier, JS, Saper, CB & Elmquist, JK. Leptin Differentially Regulates NPY and POMC Neurons Projecting to the Lateral Hypothalamic Area. Neuron. 1999;23 (4):775-786.

113. Munzberg, H, Bjornholm, M, Bates, SH & Myers, MG. Leptin receptor action and mechanisms of leptin resistance. Cell Mol Life Sci. 2005;62 (6):642-652.

114. Schwartz, MW, Woods, SC, Porte, D, Seeley, RJ & Baskin, DG. Central nervous system control of food intake. Nature. 2000;404 (6778):661-671.

115. Wilding, JPH. Neuropeptides and appetite control. Diabetic Med. 2002;19 (8):619-627.

116. Arora, S & Anubhuti. Role of neuropeptides in appetite regulation and obesity - A review. Neuropeptides. 2006;40 (6):375-401.

117. Tartaglia, LA, Dembski, M, Weng, X, Deng, N, Culpepper, J, Devos, R, Richards, GJ, Campfield, LA, Clark, FT, Deeds, J, Muir, C, Sanker, S, Moriarty, A, Moore, KJ, Smutko, JS, Mays, GG, Wool, EA, Monroe, CA & Tepper, RI. Identification and expression cloning of a leptin receptor, OB-R. Cell. 1995;83 (7):1263-1271.

118. Lee, G-H, Proenca, R, Montez, JM, Carroll, KM, Darvishzadeh, JG, Lee, JI & Friedman, JM. Abnormal splicing of the leptin receptor in diabetic mice. Nature. 1996;379 (6566):632-635.

119. Frühbeck, G. Intracellular signalling pathways activated by leptin. Biochem J. 2006;393 (Pt 1):7-20.

120. Tartaglia, LA. The Leptin Receptor. J Biol Chem. 1997;272 (10):6093-6096.

121. Kloek, C, Haq, AK, Dunn, SL, Lavery, HJ, Banks, AS & Myers, MG. Regulation of Jak Kinases by Intracellular Leptin Receptor Sequences. J Biol Chem. 2002;277 (44):41547-41555.

122. Hekerman, P, Zeidler, J, Bamberg-Lemper, S, Knobelspies, H, Lavens, D, Tavernier, J, Joost, H-G & Becker, W. Pleiotropy of leptin receptor signalling is defined by distinct roles of the intracellular tyrosines. FEBS Journal. 2005;272 (1):109-119.

123. White, DW, Kuropatwinski, KK, Devos, R, Baumann, H & Tartaglia, LA. Leptin Receptor (OB-R) Signaling. J Biol Chem. 1997;272 (7):4065-4071.

124. Banks, AS, Davis, SM, Bates, SH & Myers, MG. Activation of Downstream Signals by the Long Form of the Leptin Receptor. J Biol Chem. 2000;275 (19):14563-14572.

125. Starr, R, Willson, TA, Viney, EM, Murray, LJL, Rayner, JR, Jenkins, BJ, Gonda, TJ, Alexander, WS, Metcalf, D, Nicola, NA & Hilton, DJ. A family of cytokine-inducible inhibitors of signalling. Nature. 1997;387 (6636):917-921.

126. Alexander, WS. Suppressors of cytokine signalling (SOCS) in the immune system. Nat Rev Immunol. 2002;2 (6):410-416.

127. Starr, R, Willson, TA, Viney, EM, Murray, LJL, Rayner, JR, Jenkins, BJ, Gonda, TJ, Alexander, WS, Metcalf, D, Nicola, NA & Hilton, DJ. A family of cytokine-inducible inhibitors of signalling. 1997;387 (6636):917-921.

128. Sporri, B, Kovanen, PE, Sasaki, A, Yoshimura, A & Leonard, WJ. JAB/SOCS1/SSI-1 is an interleukin-2-induced inhibitor of IL-2 signaling. Blood. 2001;97 (1):221-226.

129. Naka, T, Narazaki, M, Hirata, M, Matsumoto, T, Minamoto, S, Aono, A, Nishimoto, N, Kajita, T, Taga, T, Yoshizaki, K, Akira, S & Kishimoto, T. Structure and function of a new STAT-induced STAT inhibitor. Nature. 1997;387 (6636):924-929.

130. Shen, X, Hong, F, Nguyen, V-A & Gao, B. IL-10 attenuates IFN-[alpha]-activated STAT1 in the liver: involvement of SOCS2 and SOCS3. FEBS Letters. 2000;480 (2-3):132-136.

131. Sadowski, CL, Choi, T-S, Le, M, Wheeler, TT, Wang, L-H & Sadowski, HB. Insulin Induction of SOCS-2 and SOCS-3 mRNA Expression in C2C12 Skeletal Muscle Cells Is Mediated by Stat5. J Biol Chem. 2001;276 (23):20703-20710.

132. Dalpke, A, Heeg, K, Bartz, H & Baetz, A. Regulation of innate immunity by suppressor of cytokine signaling (SOCS) proteins. Immunobiology. 2008;213 (3-4):225-235.

133. Starr, R, Fuchsberger, M, Lau, LS, Uldrich, AP, Goradia, A, Willson, TA, Verhagen, AM, Alexander, WS & Smyth, MJ. SOCS-1 Binding to Tyrosine 441 of IFN-{gamma} Receptor Subunit 1 Contributes to the Attenuation of IFN-{gamma} Signaling In Vivo. J Immunol. 2009;183 (7):4537-4544.

134. Yasukawa, H, Sasaki, A & Yoshimura, A. Negative Regulation of Cytokine Signaling Pathways. Annu Rev Immunol. 2000;18 (1):143-164.

135. Cohney, SJ, Sanden, D, Cacalano, NA, Yoshimura, A, Mui, A, Migone, TS & Johnston, JA. SOCS-3 Is Tyrosine Phosphorylated in Response to Interleukin-2 and Suppresses STAT5 Phosphorylation and Lymphocyte Proliferation. Mol Cell Biol. 1999;19 (7):4980-4988.

136. Bjorbaek, C, El-Haschimi, K, Frantz, JD & Flier, JS. The Role of SOCS-3 in Leptin Signaling and Leptin Resistance. J Biol Chem. 1999;274 (42):30059-30065.

137. Bjørbæk, C, Elmquist, JK, Frantz, JD, Shoelson, SE & Flier, JS. Identification of SOCS-3 as a Potential Mediator of Central Leptin Resistance. Molecular Cell. 1998;1 (4):619-625.

138. Elmquist, JK, Maratos-Flier, E, Saper, CB & Flier, JS. Unraveling the central nervous system pathways underlying responses to leptin. Nature Neuroscience. 1998;1:445-450.

139. Fogteloo, A, Pijl, H, Frölich, M, McCamish, M & Meinders, A. Effects of recombinant human leptin treatment as an adjunct of moderate energy restriction on body weight, resting energy expenditure and energy intake in obese humans. Diabetes Nutr Metab. 2003;16 (2):109-114.

140. Licinio, J, Caglayan, S, Ozata, M, Yildiz, BO, Miranda, PBd, O'Kirwan, F, Whitby, R, Liang, L, Cohen, P, Bhasin, S, Krauss, RM, Veldhuis, JD, Wagner, AJ, DePaoli, AM, McCann, SM & Wong, M-L. Phenotypic effects of leptin replacement on morbid obesity, diabetes mellitus, hypogonadism, and behavior in leptin-deficient adults. Proc Nat Acad Sci. 2004;101 (13):4531-4536.

141. Wang, Z, Zhou, Y-T, Kakuma, T, Lee, Y, Kalra, SP, Kalra, PS, Pan, W & Unger, RH. Leptin Resistance of Adipocytes in Obesity: Role of Suppressors of Cytokine Signaling. Biochem Biophys Res Com. 2000;277 (1):20-26.

142. Howard, JK, Cave, BJ, Oksanen, LJ, Tzameli, I, Bjorbaek, C & Flier, JS. Enhanced leptin sensitivity and attenuation of diet-induced obesity in mice with haploinsufficiency of Socs3. Nat Med. 2004;10 (7):734-738.

143. Munzberg, H & Myers, MG. Molecular and anatomical determinants of central leptin resistance. Nat Neurosci. 2005;8 (5):566-570.

144. Archer, ZA & Mercer, JC. Brain responses to obesigenic diets and diet-induced obesity. Proc Nutr Soc. 2007;66:124-130.

145. Munzberg, H, Flier, JS & Bjorbaek, C. Region-Specific Leptin Resistance within the Hypothalamus of Diet-Induced Obese Mice. Endocrinology. 2004;145 (11):4880-4889.

146. Trayhurn, P. Biology of leptin - its implications and consequences for the treatment of obesity. Int J Obes. 2001;25:S26-S28.

147. Kimura, M, Tanaka, S-i, Isoda, F, Sekigawa, K-i, Yamakawa, T & Sekihara, H. T lymphopenia in obese diabetic (db/db) mice is non-selective and thymus independent. Life Sci. 1998;62 (14):1243-1250.

148. Kanda, T, Takahashi, T, Kudo, S, Takeda, T, Tsugawa, H & Takekoshi, N. Leptin deficiency enhances myocardial necrosis and lethality in a murine model of viral myocarditis. Life Sci. 2004;75 (12):1435-1447.

149. Mancuso, P, Gottschalk, A, Phare, SM, Peters-Golden, M, Lukacs, NW & Huffnagle, GB. Leptin-Deficient Mice Exhibit Impaired Host Defense in Gram-Negative Pneumonia. J Immunol. 2002;168 (8):4018-4024.

150. Loffreda, S, Yang, SQ, Lin, HZ, Karp, CL, Brengman, ML, Wang, DJ, Klein, AS, Bulkley, GB, Bao, C, Noble, PW, Lane, MD & Diehl, AM. Leptin regulates proinflammatory immune responses. FASEB J. 1998;12 (1):57-65.

151. Lord, GM, Matarese G, Howard JK, Baker RJ, Bloom SR, Lechler RI. Leptin modulates the T-cell immune response and reverses starvation-induced immunosuppression. Nature. 1998;394:897 - 901.

152. Martin-Romero, C, Santos-Alvarez J, Goberna R, Sanchez-Margalet V. Human leptin enhances activation and proliferation of human circulating T lymphocytes. Cell Immunol. 2000;199:15-24.

153. Bernotiene, E, Palmer, G & Gabay, C. The role of leptin in innate and adaptive immune responses. Arthritis Res Ther. 2006;8 (5):217-227.

154. Lord, GM, Matarese, G, Howard, JK, Bloom, SR & Lechler, RI. Leptin inhibits the anti-CD3-driven proliferation of peripheral blood T cells but enhances the production of proinflammatory cytokines. J Leukoc Biol. 2002;72 (2):330-338.

155. Howard, JK, Lord, GM, Matarese, G, Vendetti, S, Ghatei, MA, Ritter, MA, Lechler, RI & Bloom, SR. Leptin protects mice from starvation-induced lymphoid atrophy and increases thymic cellularity in ob/ob mice. J Clin Invest. 1999;104 (8):1051-1059.

156. Caldefie-Chezet, F, Poulin, A & Vasson, M. Leptin regulates functional capacities of polymorphonuclear neutrophils. Free Radic Res. 2003;37 (8):809-814.

157. Ottonello, L, Gnerre, P, Bertolotto, M, Mancini, M, Dapino, P, Russo, R, Garibotto, G, Barreca, T & Dallegri, F. Leptin as a Uremic Toxin Interferes with Neutrophil Chemotaxis. J Am Soc Nephrol. 2004;15 (9):2366-2372.

158. Montecucco, F, Bianchi, G, Gnerre, P, Bertolotto, M, Dallegri, F & Ottonello, L. Induction of Neutrophil Chemotaxis by Leptin. Ann NY Acad Sci. 2006;1069:463-471.

159. Farooqi, IS, Matarese, G, Lord, GM, Keogh, JM, Lawrence, E, Agwu, C, Sanna, V, Jebb, SA, Perna, F, Fontana, S, Lechler, RI, DePaoli, AM & O'Rahilly, S. Beneficial effects of leptin on obesity, T cell hyporesponsiveness, and neuroendocrine/metabolic dysfunction of human congenital leptin deficiency. J Clin Invest. 2002;110 (8):1093-1103.

160. Ozata, M, Ozdemir, IC & Licinio, J. Human Leptin Deficiency Caused by a Missense Mutation: Multiple Endocrine Defects, Decreased Sympathetic Tone, and Immune System Dysfunction Indicate New Targets for Leptin Action, Greater Central than Peripheral Resistance to the Effects of Leptin, and Spontaneous Correction of Leptin-Mediated Defects. J Clin Endocrinol Metab. 1999;84 (10):3686-3695.

161. Savino, W, Dardenne, M, Velloso, LA & Silva-Barbosa, SD. The thymus is a common target in malnutrition and infection. Br J Nutr. 2007;98 (Suppl 1):S11-S16.

162. Scrimshaw, N & SanGiovanni, J. Synergism of nutrition, infection, and immunity: an overview. Am J Clin Nutr. 1997;66 (2):464S-477.

163. Field, CJ, Johnson, IR & Schley, PD. Nutrients and their role in host resistance to infection. J Leukoc Biol. 2002;71 (1):16-32.

164. Martí, A, Marcos, A & Martínez, JA. Obesity and immune function relationships. Obes Rev. 2001;2 (2):131-140.

165. La Cava, A & Matarese, G. The weight of leptin in immunity. Nat Rev Immunol. 2004;4 (5):371-379.

166. Falagas, ME & Kompoti, M. Obesity and infection. Lancet Infect Dis. 2006;6 (7):438-446.

167. Calle, EE, Thun, MJ, Petrelli, JM, Rodriguez, C & Heath, CW. Body-Mass Index and Mortality in a Prospective Cohort of U.S. Adults. N Engl J Med. 1999;341 (15):1097-1105.

168. Choban, PS & Heckler, R. Increased incidence of nosocomial infections in obese surgical patients. American Surgeon. 1995;61 (11):1001.

169. Choban, PS & Flancbaum, L. The Impact of Obesity on Surgical Outcomes: A Review. J Am Col Surg. 1997;185 (6):593-603.

170. Canturk, Z, Canturk, NZ, Cetinarslan, B, Utkan, NZ & Tarkun, I. Nosocomial Infections and Obesity in Surgical Patients. Obesity Res. 2003;11 (6):769-775.

171. Anaya, DA & Dellinger, EP. The Obese Surgical Patient: A Susceptible Host for Infection. Surg Infect. 2006;7 (5):473-480.

172. Jubber, A. Respiratory complications of obesity. Int J Clin Pract. 2004;58 (6):573-580.

173. Rimm, EB, Chan, J, Stampfer, MJ, Colditz, GA & Willett, WC. Prospective study of cigarette smoking, alcohol use, and the risk of diabetes in men. BMJ. 1995;310 (6979):555-559.

174. Baik, I, Curhan, GC, Rimm, EB, Bendich, A, Willett, WC & Fawzi, WW. A Prospective Study of Age and Lifestyle Factors in Relation to Community-Acquired Pneumonia in US Men and Women. Arch Intern Med. 2000;160 (20):3082-3088.

175. Jedrychowski, W, Maugeri, U, Flak, E, Mroz, E & Bianchi, I. Predisposition to acute respiratory infections among overweight preadolescent children: an epidemiologic study in Poland. Public Health. 1998;112 (3):189-195.

176. Beuther, DA & Sutherland, ER. Overweight, Obesity, and Incident Asthma: A Meta-analysis of Prospective Epidemiologic Studies. Am J Respir Crit Care Med. 2007;175 (7):661-666.

177. Beuther, DA. Obesity and Asthma. Clin Chest Med. 2009;30 (3):479-488.

178. Beuther, DA. Recent insight into obesity and asthma. Cur Opin Pulm Med. 2010;16 (1):64-70.

179. Lamas, O, Marti A, Martínez JA. Obesity and immunocompetence. Eur J Clin Nutr. 2002;56:S42 - S45.

180. Lamas, O, Marti, A & Martinez, J. Obesity and immunocompetence. Eur J Clin Nutr. 2002;56 (Suppl 3):S42-45.

181. Nieman, DC, Nehlsen-Cannarella, SI, Henson, DA, Butterworth, DE, Fagoaga, OR, Warren, BJ & Rainwater, MK. Immune response to obesity and moderate weight loss. Int J Obes Relat Metab Disord. 1996;20 (4):353-360.

182. Nieman, DC, Henson, DA, Nehlsen-Cannarella, SL, Ekkens, M, Utter, AC, Butterworth, DE & Fagoaga, OR. Influence of Obesity on Immune Function. J Am Diet Assoc. 1999;99 (3):294-299.

183. Nieman, D, Nehlsen-Cannarella SI, Henson DA, Butterworth DE, Fagoaga OR, Warren BJ, Rainwater MK. Immune response to obesity and moderate weight loss. Int J Obes Relat Metab Disord. 1996;20:353-360.

184. Nieman, D, Henson DA, Nehlsen-Cannarella SL, Ekkens M, Utter AC, Butterworth DE, Fagoaga OR. Influence of obesity on immune function. J Am Diet Assoc. 1999;99:294-299.

185. Tanaka, S, Inoue S, Isoda F, Waseda M, Ishihara M, Yamakawa T, Sugiyama A, Takamura Y, Okuda K. Impaired immunity in obesity: suppressed but reversible lymphocyte responsiveness. Int J Obes Relat Metab Disord. 1993;17:631-636.

186. Tanaka, S-I, Isoda F, Ishihara Y, Kimura M, Yamakawa T. T lymphopaenia in relation to body mass index and TNF- in human obesity: adequate weight reduction can be corrective. Clin Endocrinology. 2001;54:347-354.

187. Weber, DJ, Rutala, WA, Samsa, GP, Santimaw, JE & Lemon, SM. Obesity as a predictor of poor antibody response to hepatitis B plasma vaccine. JAMA. 1985;254 (22):3187-3189.

188. Weber, DJ, Rutala, WA, Samsa, GP, Bradshaw, SE & Lemon, SM. Impaired immunogenicity of hepatitis B vaccine in obese persons. N Engl J Med. 1986;314 (21):1393.

189. Eliakim, A, Swindt, C, Zaldivar, F, Casali, P & Cooper, DM. Reduced tetanus antibody titers in overweight children. Autoimmunity. 2006;39 (2):137-141.

190. Tanaka, S-I, Isoda, F, Ishihara, Y, Kimura, M & Yamakawa, T. T lymphopaenia in relation to body mass index and TNF-alpha in human obesity: adequate weight reduction can be corrective. Clin Endocrinol. 2001;54 (3):347-354.

191. Bennett, BD, Solar, GP, Yuan, JQ, Mathias, J, Thomas, GR & Matthews, W. A role for leptin and its cognate receptor in hematopoiesis. Curr Biol. 1996;6 (9):1170-1180.

192. Tanaka, S-i, Isoda, F, Yamakawa, T, Ishihara, M & Sekihara, H. T Lymphopenia in Genetically Obese Rats. Clin Immunol Immunopathol. 1998;86 (2):219-225.

193. Moriguchi, S, Kato, M, Sakai, K, Yamamoto, S & Shimizu, E. Decreased mitogen response of splenic lymphocytes in obese Zucker rats is associated with the decreased expression of glucose transporter 1 (GLUT-1). Am J Clin Nutr. 1998;67 (6):1124-1129.

194. Samad, F, Uysal, KT, Wiesbrock, SM, Pandey, M, Hotamisligil, GS & Loskutoff, DJ. Tumor necrosis factor alpha is a key component in the obesity-linked elevation of plasminogen activator inhibitor 1. Proc Nat Acad Sci. 1999;96 (12):6902-6907.

195. Mito, N, Hosoda, T, Kato, C & Sato, K. Change of cytokine balance in diet-induced obese mice. Metabolism. 2000;49 (10):1295-1300.

196. Verwaerde, C, Delanoye, A, Macia, L, Tailleux, A & Wolowczuk, I. Influence of High-Fat Feeding on Both Naive and Antigen-Experienced T-Cell Immune Response in DO10.11 Mice. Scand J Immunol. 2006;64 (5):457-466.

197. Webb, SR, Loria, RM, Madge, GE & Kibrick, S. Susceptibility of mice to group B coxsackie virus is influenced by the diabetic gene. J Exp Med. 1976;143 (5):1239-1248.

198. Ikejima, S, Sasaki, S, Sashinami, H, Mori, F, Ogawa, Y, Nakamura, T, Abe, Y, Wakabayashi, K, Suda, T & Nakane, A. Impairment of Host Resistance to Listeria monocytogenes Infection in Liver of db/db and ob/ob Mice. Diabetes. 2005;54 (1):182-189.

199. Smith, AG, Sheridan, PA, Harp, JB & Beck, MA. Diet-Induced Obese Mice Have Increased Mortality and Altered Immune Responses When Infected with Influenza Virus. J Nutr. 2007;137 (5):1236-1243.

200. Smith, AG, Sheridan, PA, Tseng, RJ, Sheridan, JF & Beck, MA. Selective impairment in dendritic cell function and altered antigen-specific CD8+ T-cell responses in diet-induced obese mice infected with influenza virus. Immunology. 2009;126 (2):268-279.

201. Murphy, B & Webster, R (1997) Orthomyxoviruses in Fields Virology. Fields, B. 3rd ed. Lippincott-Raven, Philadelphia.

202. World Health Organization (2003). WHO factsheet 211: influenza.

203. Advisory Committee on Immunization Practices Prevention and Control of Influenza. Recommendations of the Advisory Committee on Immunization Practices (ACIP). MMWR Recomm Rep. 2006;55 (RR-10):1-42.

204. Morgan, OW, Bramley, A, Fowlkes, A, Freedman, DS, Taylor, TH, Gargiullo, P, Belay, B, Jain, S, Cox, C, Kamimoto, L, Fiore, A, Finelli, L, Olsen, SJ & Fry, AM. Morbid Obesity as a Risk Factor for Hospitalization and Death Due to 2009 Pandemic Influenza A(H1N1) Disease. PLoS ONE. 2010;5 (3):e9694.

205. CDC. Intensive-Care Patients With Severe Novel Influenza A (H1N1) Virus Infection --- Michigan, June 2009. MMWR. 2009;58(Dispatch):1-4.

206. Vaillant, L, Ruche, GL, Tarantola, A & Barboza, P. Epidemiology of fatal cases associated with pandemic H1N1 influenza 2009. Eurosurveillance. 2009;14 (33):1-6.

207. Monto, A. Epidemiology and virology of influenza illness. Am J Managed Care. 2000;6 (5):S255-264.

208. Bhat, N, Wright, JG, Broder, KR, Murray, EL, Greenberg, ME, Glover, MJ, Likos, AM, Posey, DL, Klimov, A, Lindstrom, SE, Balish, A, Medina, M-j, Wallis, TR, Guarner, J, Paddock, CD, Shieh, W-J, Zaki, SR, Sejvar, JJ, Shay, DK, Harper, SA, Cox, NJ, Fukuda, K, Uyeki, TM & the Influenza Special Investigations Team. Influenza-Associated Deaths among Children in the United States, 2003-2004. N Engl J Med. 2005;353 (24):2559-2567.

209. Potter, CW (1998) Chronicle of influenza pandemics in Textbook of Influenza. Nicholson, K. G., Webster, R. G. & Hay, A. J. Blackwell Science, Oxford.

210. Beveridge, WI. The chronicle of influenza epidemics. Hist Philos Life Sci. 1991;13 (2):223-234.

211. Neumann, G, Noda, T & Kawaoka, Y. Emergence and pandemic potential of swine-origin H1N1 influenza virus. Nature. 2009;459 (7249):931-939.

212. Fraser, C, Donnelly, CA, Cauchemez, S, Hanage, WP, Van Kerkhove, MD, Hollingsworth, TD, Griffin, J, Baggaley, RF, Jenkins, HE, Lyons, EJ, Jombart, T, Hinsley, WR, Grassly, NC, Balloux, F, Ghani, AC, Ferguson, NM, Rambaut, A, Pybus, OG, Lopez-Gatell, H, Alpuche-Aranda, CM, Chapela, IB, Zavala, EP, Guevara, DME, Checchi, F, Garcia, E, Hugonnet, S & Roth, C. Pandemic Potential of a Strain of Influenza A (H1N1): Early Findings. Science. 2009;324 (5934):1557-1561.

213. Schnitzler, S & Schnitzler, P. An update on swine-origin influenza virus A/H1N1: a review. Virus Genes. 2009;39 (3):279-292.

214. Novel Swine-Origin Influenza A (H1N1) Virus Investigation Team. Emergence of a Novel Swine-Origin Influenza A (H1N1) Virus in Humans. N Engl J Med. 2009;360 (25):2605-2615.

215. Michaelis, M, Doerr, H & Cinatl, J. An influenza A H1N1 virus revival pandemic H1N1/09 virus. Infection. 2009;37 (5):381-389.

216. Cohen, J & Enserink, M. After Delays, WHO Agrees: The 2009 Pandemic Has Begun. Science. 2009;324 (5934):1496-1497.

217. Maines, TR, Jayaraman, A, Belser, JA, Wadford, DA, Pappas, C, Zeng, H, Gustin, KM, Pearce, MB, Viswanathan, K, Shriver, ZH, Raman, R, Cox, NJ, Sasisekharan, R, Katz, JM & Tumpey, TM. Transmission and Pathogenesis of Swine-Origin 2009 A(H1N1) Influenza Viruses in Ferrets and Mice. Science. 2009;325 (5939):484-487.

218. Munster, VJ, de Wit, E, van den Brand, JMA, Herfst, S, Schrauwen, EJA, Bestebroer, TM, van de Vijver, D, Boucher, CA, Koopmans, M, Rimmelzwaan, GF, Kuiken, T, Osterhaus, ADME & Fouchier, RAM. Pathogenesis and Transmission of Swine-Origin 2009 A(H1N1) Influenza Virus in Ferrets. Science. 2009;325 (5939):481-483.

219. Cohen, J. Past Pandemics Provide Mixed Clues to H1N1's Next Moves. Science. 2009;324 (5930):996-997.

220. Lamb, RA & Krug, RM (1996) Orthomyxoviridae: the viruses and their replication in Fields virology. Fields, B. N., Knipe, R. M. & Chanock, M. S. Lippincott-Raven, Philadelphia.

221. Julkunen, I, Sareneva, T, Pirhonen, J, Ronni, T, Melén, K & Matikainen, S. Molecular pathogenesis of influenza A virus infection and virus-induced regulation of cytokine gene expression. Cytokine Growth Factor Rev. 2001;12 (2-3):171-180.

222. Cros, JF & Palese, P. Trafficking of viral genomic RNA into and out of the nucleus: influenza, Thogoto and Borna disease viruses. Virus Res. 2003;95 (1-2):3-12.

223. Lamb, RA & Choppin, PW. The Gene Structure and Replication of Influenza Virus. Annu Rev Biochem. 1983;52 (1):467-506.

224. Nayak, DP, Hui, EK-W & Barman, S. Assembly and budding of influenza virus. Virus Res. 2004;106 (2):147-165.

225. Portela, A, Zürcher, T, Nieto, A & Ortín, J. Replication of orthomyxoviruses. Adv Virus Res. 1999;54:319-348.

226. La Gruta, NL, Kedzierska, K, Stambas, J & Doherty, PC. A question of self-preservation: immunopathology in influenza virus infection. 2007;85 (2):85-92.

227. Julkunen, I, Melén, K, Nyqvist, M, Pirhonen, J, Sareneva, T & Matikainen, S. Inflammatory responses in influenza A virus infection. Vaccine. 2000;19 (Supplement 1):S32-S37.

228. Sladkova, T & Kostolansky, F. The role of cytokines in the immune response to influenza A virus infection. Acta Virol. 2006;50 (3):151-162.

229. Stark, GR, Kerr, IM, Williams, BRG, Silverman, RH & Schreiber, RD. How cells respond to interferons. Annu Rev Biochem. 1998;67 (1):227-264.

230. Sallusto, F & Baggiolini, M. Chemokines and leukocyte traffic. Nat Immunol. 2008;9 (9):949-952.

231. Baggiolini, M. Chemokines and leukocyte traffic. Nature. 1998;392 (6676):565-568.

232. Zlotnik, A & Yoshie, O. Chemokines: A New Classification System and Their Role in Immunity. Immunity. 2000;12 (2):121-127.

233. Banchereau, J & Steinman, RM. Dendritic cells and the control of immunity. Nature. 1998;392 (6673):245-252.

234. Bhardwaj, N, Seder, RA, Reddy, A & Feldman, MV. IL-12 in Conjunction with Dendritic Cells Enhances Antiviral CD8+ CTL Responses In Vitro. J Clin Invest. 1996;98 (3):715-722.

235. Nguyen, KB, Salazar-Mather, TP, Dalod, MY, Van Deusen, JB, Wei, X-q, Liew, FY, Caligiuri, MA, Durbin, JE & Biron, CA. Coordinated and Distinct Roles for IFN-{alpha}{beta}, IL-12, and IL-15 Regulation of NK Cell Responses to Viral Infection. J Immunol. 2002;169 (8):4279-4287.

236. Delves, PJ & Roitt, IM. The Immune System- First of Two Parts. N Engl J Med. 2000;343 (1):37-49.

237. Janeway, CA, Travers, P, Walport, M & Shlomchik, MJ (2005) Immunobiology: the immune system in heath and disease in 6th ed. Garland Science, New York.

238. Sallusto, F & Lanzavecchia, A. The instructive role of dendritic cells on T-cell responses. Arthritis Res. 2002;4 ((Suppl 3)):S127-S132.

239. Mescher, MF, Curtsinger, JM, Agarwal, P, Casey, KA, Gerner, M, Hammerbeck, CD, Popescu, F & Xiao, Z. Signals required for programming effector and memory development by CD8+ T cells. Immunol Rev. 2006;211:81-92.

240. Tripp, R, Sarawar, S & Doherty, P. Characteristics of the influenza virus-specific CD8+ T cell response in mice homozygous for disruption of the H-2lAb gene. J Immunol. 1995;155 (6):2955-2959.

241. Doherty, PC & Christensen, JP. Accessing Complexity: The Dynamics of Virus-Specific T Cell Responses. Annual Review of Immunology. 2000;18 (1):561-592.

242. Lawrence, CW, Ream, RM & Braciale, TJ. Frequency, Specificity, and Sites of Expansion of CD8+ T Cells during Primary Pulmonary Influenza Virus Infection. J Immunol. 2005;174 (9):5332-5340.

243. Cerwenka, A, Morgan, TM & Dutton, RW. Naive, Effector, and Memory CD8 T Cells in Protection Against Pulmonary Influenza Virus Infection: Homing Properties Rather Than Initial Frequencies Are Crucial. J Immunol. 1999;163 (10):5535-5543.

244. Lawrence, CW & Braciale, TJ. Activation, Differentiation, and Migration of Naive Virus-Specific CD8+ T Cells during Pulmonary Influenza Virus Infection. J Immunol. 2004;173 (2):1209-1218.

245. Flynn, KJ, Belz, GT, Altman, JD, Ahmed, R, Woodland, DL & Doherty, PC. Virus-Specific CD8+ T Cells in Primary and Secondary Influenza Pneumonia. Immunity. 1998;8 (6):683-691.

246. Barry, M & Bleackley, RC. Cytotoxic T lymphocytes: all roads lead to death. Nat Rev Immunol. 2002;2 (6):401-409.

247. Topham, D, Tripp, R & Doherty, P. CD8+ T cells clear influenza virus by perforin or Fas-dependent processes. J Immunol. 1997;159 (11):5197-5200.

248. Johnson, BJ, Costelloe, EO, Fitzpatrick, DR, Haanen, JBAG, Schumacher, TNM, Brown, LE & Kelso, A. Single-cell perforin and granzyme expression reveals the anatomical localization of effector CD8+ T cells in influenza virus-infected mice. PNAS. 2003;100 (5):2657-2662.

249. Grossman, WJ, Revell, PA, Lu, ZH, Johnson, H, Bredemeyer, AJ & Ley, TJ. The orphan granzymes of humans and mice. Curr Opin Immunol. 2003;15 (5):544-552.

250. Price, GE, Gaszewska-Mastarlarz, A & Moskophidis, D. The Role of Alpha/Beta and Gamma Interferons in Development of Immunity to Influenza A Virus in Mice. J Virol. 2000;74 (9):3996-4003.

251. Nguyen, KB, Cousens, LP, Doughty, LA, Pien, GC, Durbin, JE & Biron, CA. Interferon [alpha]/[beta]-mediated inhibition and promotion of interferon [gamma]: STAT1 resolves a paradox. 2000;1 (1):70-76.

252. Malmgaard, L. Induction and Regulation of IFNs During Viral Infections. J Interferon Cytokine Res. 2004;24 (8):439-454.

253. Gerhard, W, Mozdzanowska, K, Furchner, M, Washko, G & Maiese, K. Role of the B-cell response in recovery of mice from primary influenza virus infection. Immunol Rev. 1997;159 (1):95-103.

254. Couch, RB. Seasonal inactivated influenza virus vaccines. Vaccine. 2008;26 (Supplement 4):D5-D9.

255. Wood, JM & Williams, MS (1998) History of inactivated influenza vaccines in Textbook of influenza. Nicholson, K. G., Webster, R. G. & Hay, A. J. Blackwell Science, Oxford.

256. Centers for Disease Control and Prevention, C. Prevention and control of influenza. Recomendations of the Advisory committee on Immunnization Practices (ACIP). MMWR. 2007;56 (R-6):1-53.

257. Nichol, KL. Efficacy and effectiveness of influenza vaccination. Vaccine. 2008;26 (Supplement 4):D17-D22.

258. Hampson, AW (2002) Influenza virus antigens and "antigenic drift" in Influenza Perspectives in medical virology. Potter, C. W. 7th ed. Elsevier, Amsterdam.

259. Brammer, T, Murray EL, Fukuda K, Hall HE, Klimov A, Cox NJ (2002). Surveillance for Influenza-United States-1997-1998, 1998-1999, and 1999-2000 Season: In Surveillance Summaries. In: MMWR, pp. 1-31. Centers for Disease Control and Prevention, Atlanta, GA.

260. Ellebedy, AH & Webby, RJ. Influenza vaccines. Vaccine. 2009;27 (Supplement 4):D65-D68.

261. Monto, AS, Ansaldi, F, Aspinall, R, McElhaney, JE, Montaño, LF, Nichol, KL, Puig-Barberà, J, Schmitt, J & Stephenson, I. Influenza control in the 21st century: Optimizing protection of older adults. Vaccine. 2009;27 (37):5043-5053.

262. Fiore, AE, Bridges, CB & Cox, NJ. Seasonal influenza vaccines. Curr Top Microbiol Immunol. 2009;333:43-82.

263. Bouvier, NM & Palese, P. The biology of influenza viruses. Vaccine. 2008;26 (Supplement 4):D49-D53.

264. Scholtissek, C, Rohde, W, Von-Hoyningen, V & Rott, R. On the origin of the human influenza virus subtypes H2N2 and H3N2. Virology. 1978;87 (1):13-20.

265. Taubenberger, JK, Reid, AH, Krafft, AE, Bijwaard, KE & Fanning, TG. Initial Genetic Characterization of the 1918 "Spanish" Influenza Virus. Science. 1997;275 (5307):1793-1796.

266. Taubenberger, JK, Reid, AH, Lourens, RM, Wang, R, Jin, G & Fanning, TG. Characterization of the 1918 influenza virus polymerase genes. Nature. 2005;437 (7060):889-893.

267. Carrat, F & Flahault, A. Influenza vaccine: The challenge of antigenic drift. Vaccine. 2007;25 (39-40):6852-6862.

268. Thomas, PG, Keating, R, Hulse-Post, DJ & Doherty, PC. Cell-mediated protection in influenza infection. Emerg Infect Dis. 2006;12 (1):48(47).

269. Castellino, F, Galli, G, Giudice, GD & Rappuoli, R. Generating memory with vaccination. Eur J Immunol. 2009;39 (8):2100-2105.

270. Brown, LE & Kelso, A. Prospects for an influenza vaccine that induces cross-protective cytotoxic T lymphocytes. Immunol Cell Biol. 2009;87 (4):300-308.

271. Ahmed, R & Gray, D. Immunological memory and protective immunity: understanding their relation. Science. 1996;272:54-60.

272. Ahmed, R & Rouse, BT. Immunological Memory. Immunol Rev. 2006;211:5-7.

273. Bender, BS, Croghan, T, Zhang, L & Small, PA. Transgenic mice lacking class I major histocompatibility complex-restricted T cells have delayed viral clearance and increased mortality after influenza virus challenge. J Exp Med. 1992;175:1143-1145.

274. Bruder, D, Srikiatkhachorn, A & Enelow, RI. Cellular Immunity and Lung Injury in Respiratory Virus Infection. Viral Immunol. 2006;19 (2):147-155.

275. Jameson, SC & Masopust, D. Diversity in T Cell Memory: An Embarrassment of Riches. Immunity. 2009;31 (6):859-871.

276. Badovinac, VP & Harty, JT. Programming, demarcating, and manipulating CD8+ T-cell memory. Immunol Rev. 2006;211 (1):67-80.

277. Bachmann, MF, Beerli, RR, Agnellini, P, Wolint, P, Schwarz, K & Oxenius, A. Long-lived memory CD8+ T cells are programmed by prolonged antigen exposure and low levels of cellular activation. Eur J Immunol. 2006;36 (4):842-854.

278. Badovinac, VP & Harty, JT. Manipulating the Rate of Memory CD8+ T Cell Generation after Acute Infection. J Immunol. 2007;179 (1):53-63.

279. Prlic, M & Bevan, MJ. Exploring regulatory mechanisms of CD8+ T cell contraction. PNAS. 2008;105 (43):16689-16694.

280. Cannarile, MA, Lind, NA, Rivera, R, Sheridan, AD, Camfield, KA, Wu, BB, Cheung, KP, Ding, Z & Goldrath, AW. Transcriptional regulator Id2 mediates CD8+ T cell immunity. Nat Immunol. 2006;7 (12):1317-1325.

281. Badovinac, VP, Porter, BB & Harty, JT. CD8+ T cell contraction is controlled by early inflammation. Nat Immunol. 2004;5 (8):809-817.

282. Joshi, NS & Kaech, SM. Effector CD8 T Cell Development: A Balancing Act between Memory Cell Potential and Terminal Differentiation. J Immunol. 2008;180 (3):1309-1315.

283. Williams, MA, Holmes, BJ, Sun, JC & Bevan, MJ. Developing and maintaining protective CD8+ memory T cells. Immunol Rev. 2006;211 (1):146-153.

284. Kedzierska, K, Stambas, J, Jenkins, MR, Keating, R, Turner, SJ & Doherty, PC. Location rather than CD62L phenotype is critical in the early establishment of influenza-specific CD8+ T cell memory. PNAS. 2007;104 (23):9782-9787.

285. Kallies, A. Distinct regulation of effector and memory T-cell differentiation. Immunol Cell Biol. 2008.

286. Bannard, O, Kraman, M & Fearon, DT. Secondary Replicative Function of CD8+ T Cells That Had Developed an Effector Phenotype. Science. 2009;323 (5913):505-509.

287. Parish, IA & Kaech, SM. Diversity in CD8+ T cell differentiation. Curr Opin Immunol. 2009;21 (3):291-297.

288. Williams, MA & Bevan, MJ. Effector and Memory CTL Differentiation. Annu Rev Immunol. 2007;25 (1):171-192.

289. Kaech, SM & Wherry, EJ. Heterogeneity and Cell-Fate Decisions in Effector and Memory CD8+ T Cell Differentiation during Viral Infection. Immunity. 2007;27 (3):393-405.

290. Joshi, NS, Cui, W, Chandele, A, Lee, HK, Urso, DR, Hagman, J, Gapin, L & Kaech, SM. Inflammation Directs Memory Precursor and Short-Lived Effector CD8+ T Cell Fates via the Graded Expression of T-bet Transcription Factor. Immunity. 2007;27 (2):281-295.

291. Wherry, EJ, Teichgraber, V, Becker, TC, Masopust, D, Kaech, SM, Antia, R, von Andrian, UH & Ahmed, R. Lineage relationship and protective immunity of memory CD8 T cell subsets. Nat Immunol. 2003;4 (3):225-234.

292. Hikono, H, Kohlmeier, JE, Takamura, S, Wittmer, ST, Roberts, AD & Woodland, DL. Activation phenotype, rather than central- or effector-memory phenotype, predicts the recall efficacy of memory CD8+ T cells. J Exp Med. 2007;204 (7):1625-1636.

293. Hand, TW, Morre, M & Kaech, SM. Expression of IL-7 receptor Î± is necessary but not sufficient for the formation of memory CD8 T cells during viral infection. PNAS. 2007;104 (28):11730-11735.

294. Ahmed, R, Bevan, MJ, Reiner, SL & Fearon, DT. The precursors of memory: models and controversies. Nat Rev Immunol. 2009;9 (9):662-668.

295. Pearce, EL & Shen, H. Making sense of inflammation, epigenetics, and memory CD8+ T-cell differentiation in the context of infection. Immunol Rev. 2006;211 (1):197-202.

296. Obar, JJ & Lefrançois, L. Memory CD8+ T cell differentiation. Ann NY Acad Sci. 2010;1183 (The Year in Immunology 2):251-266.

297. Crotty, S, Johnston, RJ & Schoenberger, SP. Effectors and memories: Bcl-6 and Blimp-1 in T and B lymphocyte differentiation. Nat Immunol. 2010;11 (2):114-120.

298. Sigal, LJ, Crotty, S, Andino, R & Rock, KL. Cytotoxic T-cell immunity to virus-infected non-haematopoietic cells requires presentation of exogenous antigen. Nature. 1999;398 (6722):77-80.

299. Jung, S, Unutmaz, D, Wong, P, Sano, G-I, De los Santos, K, Sparwasser, T, Wu, S, Vuthoori, S, Ko, K, Zavala, F, Pamer, EG, Littman, DR & Lang, RA. In Vivo Depletion of

CD11c+ Dendritic Cells Abrogates Priming of CD8+ T Cells by Exogenous Cell-Associated Antigens. Immunity. 2002;17 (2):211-220.

300. Probst, HC & van den Broek, M. Priming of CTLs by Lymphocytic Choriomeningitis Virus Depends on Dendritic Cells. J Immunol. 2005;174 (7):3920-3924.

301. Zammit, DJ & Lefrancois, L. Dendritic cell-T cell interactions in the generation and maintenance of CD8 T cell memory. Microbes Infect. 2006;8 (4):1108-1115.

302. Zammit, DJ, Cauley, LS, Pham, Q-M & Lefrancois, L. Dendritic Cells Maximize the Memory CD8 T Cell Response to Infection. Immunity. 2005;22 (5):561-570.

303. Badovinac, VP, Messingham, KAN, Jabbari, A, Haring, JS & Harty, JT. Accelerated CD8+ T-cell memory and prime-boost response after dendritic-cell vaccination. 2005;11 (7):748-756.

304. Mostbock, S, Vidal, S, Schlom, J & Sabzevari, H. Enhanced Levels of Costimulation Lead to Reduced Effector/Memory CD8+ T Cell Functionality. J Immunol. 2007;179 (6):3524-3534.

305. Viola, A, Schroeder, S, Sakakibara, Y & Lanzavecchia, A. T Lymphocyte Costimulation Mediated by Reorganization of Membrane Microdomains. Science. 1999;283 (5402):680-682.

306. Noel, P, Boise, L, Green, J & Thompson, C. CD28 costimulation prevents cell death during primary T cell activation. J Immunol. 1996;157 (2):636-642.

307. Boise, LH, Noel, PJ & Thompson, CB. CD28 and apoptosis. Curr Opin Immunol. 1995;7 (5):620-625.

308. Okkenhaug, K, Wu, L, Garza, KM, La Rose, J, Khoo, W, Odermatt, B, Mak, TW, Ohashi, PS & Rottapel, R. A point mutation in CD28 distinguishes proliferative signals from survival signals. Nat Immunol. 2001;2 (4):325-332.

309. Shahinian, A, Pfeffer, K, Lee, KP, Kündig, TM, Kishihara, K, Wakeham, A, Kawai, K, Ohashi, PS, Thompson, CB & Mak, TW. Differential T cell costimulatory requirements in CD28-deficient mice. Science. 1993;261 (5121):609-612.

310. Kaech, SM & Ahmed, R. Memory CD8+ T cell differentiation: initial antigen encounter triggers a developmental program in naive cells. Nat Immunol. 2001;2 (5):415-422.

311. Bevan, MJ & Fink, PJ. The CD8 response on autopilot. 2001;2 (5):381-382.

312. Watts, TH. TNF/TNFR family members in costimulation of T cell responses. Annu Rev Immunol. 2005;23 (1):23-68.

313. Bertram, EM, Dawicki, W & Watts, TH. Role of T cell costimulation in anti-viral immunity. Semin Immunol. 2004;16 (3):185-196.

314. Tesselaar, K, Xiao, Y, Arens, R, van Schijndel, GMW, Schuurhuis, DH, Mebius, RE, Borst, J & van Lier, RAW. Expression of the Murine CD27 Ligand CD70 In Vitro and In Vivo. J Immunol. 2003;170 (1):33-40.

315. Lens, SMA, Tesselaar, K, van Oers, MHJ & van Lier, RAW. Control of lymphocyte function through CD27-CD70 interactions. Semin Immunol. 1998;10 (6):491-499.

316. Hendriks, J, Xiao, Y & Borst, J. CD27 Promotes Survival of Activated T Cells and Complements CD28 in Generation and Establishment of the Effector T Cell Pool. J Exp Med. 2003;198 (9):1369-1380.

317. Hendriks, J, Xiao, Y, Rossen, JWA, van der Sluijs, KF, Sugamura, K, Ishii, N & Borst, J. During Viral Infection of the Respiratory Tract, CD27, 4-1BB, and OX40 Collectively Determine Formation of CD8+ Memory T Cells and Their Capacity for Secondary Expansion. J Immunol. 2005;175 (3):1665-1676.

318. van Kooten, C & Banchereau, J. CD40-CD40 ligand. J Leukoc Biol. 2000;67 (1):2-17.

319. Bennett, SRM, Carbone, FR, Karamalis, F, Flavell, RA, Miller, JFAP & Heath, WR. Help for cytotoxic-T-cell responses is mediated by CD40 signalling. Nature. 1998;393 (6684):478-480.

320. Cella, M, Scheidegger, D, Palmer-Lehmann, K, Lane, P, Lanzavecchia, A & Alber, G. Ligation of CD40 on dendritic cells triggers production of high levels of interleukin-12 and enhances T cell stimulatory capacity: T-T help via APC activation. J Exp Med. 1996;184 (2):747-752.

321. Schoenberger, SP, Toes, REM, van der Voort, EIH, Offringa, R & Melief, CJM. T-cell help for cytotoxic T lymphocytes is mediated by CD40-CD40L interactions. Nature. 1998;393 (6684):480-483.

322. Borrow, P, Tishon, A, Lee, S, Xu, J, Grewal, IS, Oldstone, MB & Flavell, RA. CD40L-deficient mice show deficits in antiviral immunity and have an impaired memory CD8+ CTL response. J Exp Med. 1996;183 (5):2129-2142.

323. Hernandez, MGH, Shen, L & Rock, KL. CD40 on APCs Is Needed for Optimal Programming, Maintenance, and Recall of CD8+ T Cell Memory Even in the Absence of CD4+ T Cell Help. J Immunol. 2008;180 (7):4382-4390.

324. Pearce, EL & Shen, H. Generation of CD8 T Cell Memory Is Regulated by IL-12. J Immunol. 2007;179 (4):2074-2081.

325. Turner, SJ, Olivas, E, Gutierrez, A, Diaz, G & Doherty, PC. Disregulated Influenza A Virus-Specific CD8+ T Cell Homeostasis in the Absence of IFN-{gamma} Signaling. J Immunol. 2007;178 (12):7616-7622.

326. Matzinger, P. The Danger Model: A Renewed Sense of Self. Science. 2002;296 (5566):301-305.

327. Intlekofer, AM, Takemoto, N, Wherry, EJ, Longworth, SA, Northrup, JT, Palanivel, VR, Mullen, AC, Gasink, CR, Kaech, SM, Miller, JD, Gapin, L, Ryan, K, Russ, AP, Lindsten, T, Orange, JS, Goldrath, AW, Ahmed, R & Reiner, SL. Effector and memory CD8+ T cell fate coupled by T-bet and eomesodermin. Nat Immunol. 2005;6 (12):1236-1244.

328. Szabo, SJ, Kim, ST, Costa, GL, Zhang, X, Fathman, CG & Glimcher, LH. A Novel Transcription Factor, T-bet, Directs Th1 Lineage Commitment. Cell. 2000;100 (6):655-669.

329. Takemoto, N, Intlekofer, AM, Northrup, JT, Wherry, EJ & Reiner, SL. Cutting Edge: IL-12 Inversely Regulates T-bet and Eomesodermin Expression during Pathogen-Induced CD8+ T Cell Differentiation. J Immunol. 2006;177 (11):7515-7519.

330. Rutishauser, RL, Martins, GA, Kalachikov, S, Chandele, A, Parish, IA, Meffre, E, Jacob, J, Calame, K & Kaech, SM. Transcriptional Repressor Blimp-1 Promotes CD8+ T Cell Terminal Differentiation and Represses the Acquisition of Central Memory T Cell Properties. Immunity. 2009;31 (2):296-308.

331. Kallies, A, Xin, A, Belz, GT & Nutt, SL. Blimp-1 Transcription Factor Is Required for the Differentiation of Effector CD8+ T Cells and Memory Responses. Immunity. 2009;31 (2):283-295.

332. Welsh, RM. Blimp Hovers over T Cell Immunity. Immunity. 2009;31 (2):178-180.

333. Pearce, EL. Metabolism in T cell activation and differentiation. Curr Opin Immunol. 2010;In Press, Corrected Proof.

334. Wullschleger, S, Loewith, R & Hall, MN. TOR Signaling in Growth and Metabolism. Cell. 2006;124 (3):471-484.

335. Dennis, PB, Jaeschke, A, Saitoh, M, Fowler, B, Kozma, SC & Thomas, G. Mammalian TOR: A Homeostatic ATP Sensor. Science. 2001;294 (5544):1102-1105.

336. Proud, CG. Regulation of mammalian translation factors by nutrients. Eur J Biochem. 2002;269 (22):5338-5349.

337. Hall, MN. mTOR--What Does It Do? Transplant Proc. 2008;40 (10, Supplement 1):S5-S8.

338. Inoki, K & Guan, K-L. Complexity of the TOR signaling network. Trends Cell Biol. 2006;16 (4):206-212.

339. Araki, K, Turner, AP, Shaffer, VO, Gangappa, S, Keller, SA, Bachmann, MF, Larsen, CP & Ahmed, R. mTOR regulates memory CD8 T-cell differentiation. Nature. 2009;460 (7251):108-112.

340. Rao, RR, Li, Q, Odunsi, K & Shrikant, PA. The mTOR Kinase Determines Effector versus Memory CD8+ T Cell Fate by Regulating the Expression of Transcription Factors T-bet and Eomesodermin. Immunity. 2010;32 (1):67-78.

341. Marshall, DR, Turner, SJ, Belz, GT, Wingo, S, Andreansky, S, Sangster, MY, Riberdy, JM, Liu, T, Tan, M & Doherty, PC. Measuring the diaspora for virus-specific CD8+ T cells. PNAS. 2001;98 (11):6313-6318.

342. Bachmann, MF, Wolint, P, Schwarz, K, Jager, P & Oxenius, A. Functional Properties and Lineage Relationship of CD8+ T Cell Subsets Identified by Expression of IL-7 Receptor {alpha} and CD62L. J Immunol. 2005;175 (7):4686-4696.

343. Sallusto, F, Lenig, D, Forster, R, Lipp, M & Lanzavecchia, A. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. Nature. 1999;401 (6754):708-712.

344. Kreijtz, JHCM, Bodewes, R, van den Brand, JMA, de Mutsert, G, Baas, C, van Amerongen, G, Fouchier, RAM, Osterhaus, ADME & Rimmelzwaan, GF. Infection of mice with a human influenza A/H3N2 virus induces protective immunity against lethal infection with influenza A/H5N1 virus. Vaccine. 2009;27 (36):4983-4989.

345. O'Neill, E, Krauss, SL, Riberdy, JM, Webster, RG & Woodland, DL. Heterologous protection against lethal A/HongKong/156/97 (H5N1) influenza virus infection in C57BL/6 mice. J Gen Virol. 2000;81 (11):2689-2696.

346. Hogan, RJ, Usherwood, EJ, Zhong, W, Roberts, AD, Dutton, RW, Harmsen, AG & Woodland, DL. Activated Antigen-Specific CD8+ T Cells Persist in the Lungs Following Recovery from Respiratory Virus Infections. J Immunol. 2001;166 (3):1813-1822.

347. Wiley, JA, Hogan, RJ, Woodland, DL & Harmsen, AG. Antigen-Specific CD8+ T Cells Persist in the Upper Respiratory Tract Following Influenza Virus Infection. J Immunol. 2001;167 (6):3293-3299.

348. de Bree, GJ, van Leeuwen, EMM, Out, TA, Jansen, HM, Jonkers, RE & van Lier, RAW. Selective accumulation of differentiated CD8+ T cells specific for respiratory viruses in the human lung. J Exp Med. 2005;202 (10):1433-1442.

349. Maxeiner, JH, Karwot, R, Hausding, M, Sauer, KA, Scholtes, P & Finotto, S. A method to enable the investigation of murine bronchial immune cells, their cytokines and mediators. Nat Protocols. 2007;2 (1):105-112.

350. Woodland, DL & Scott, I. T Cell Memory in the Lung Airways. Proc Am Thorac Soc. 2005;2:126-131.

351. Alves, NL, van Leeuwen, EMM, Remmerswaal, EBM, Vrisekoop, N, Tesselaar, K, Roosnek, E, ten Berge, IJM & van Lier, RAW. A New Subset of Human Naive CD8+ T Cells Defined by Low Expression of IL-7R{alpha}. J Immunol. 2007;179 (1):221-228.

352. Hogan, RJ, Zhong, W, Usherwood, EJ, Cookenham, T, Roberts, AD & Woodland, DL. Protection from Respiratory Virus Infections Can Be Mediated by Antigen-specific CD4+ T Cells That Persist in the Lungs. J Exp Med. 2001;193 (8):981-986.

353. Ely, KH, Cookenham, T, Roberts, AD & Woodland, DL. Memory T Cell Populations in the Lung Airways Are Maintained by Continual Recruitment. J Immunol. 2006;176 (1):537-543.

354. Hviid, L, Odum, N & Theander, TG. The relation between T-cell expression of LFA-1 and immunological memory. Immunology. 1993;78:237-243.

355. Thatte, J, Dabak, V, Williams, MB, Braciale, TJ & Ley, K. LFA-1 is required for retention of effector CD8 T cells in mouse lungs. Blood. 2003;101 (12):4916-4922.

356. Moyron-Quiroz, JE, Rangel-Moreno, J, Kusser, K, Hartson, L, Sprague, F, Goodrich, S, Woodland, DL, Lund, FE & Randall, TD. Role of inducible bronchus associated lymphoid tissue (iBALT) in respiratory immunity. Nat Med. 2004;10 (9):927-934.

357. Moyron-Quiroz, JE, Rangel-Moreno, J, Hartson, L, Kusser, K, Tighe, MP, Klonowski, KD, Lefrancois, L, Cauley, LS, Harmsen, AG, Lund, FE & Randall, TD. Persistence and Responsiveness of Immunologic Memory in the Absence of Secondary Lymphoid Organs. Immunity. 2006;25 (4):643-654.

358. Roberts, AD, Ely, KH & Woodland, DL. Differential contributions of central and effector memory T cells to recall responses. J Exp Med. 2005;202 (1):123-133.

359. Marzo, AL, Klonowski, KD, Bon, AL, Borrow, P, Tough, DF & Lefrancois, L. Initial T cell frequency dictates memory CD8+ T cell lineage commitment. Nat Immunol. 2005;6 (8):793-799.

360. Masopust, D & Lefrancois, L. CD8 T-cell memory: the other half of the story. Microbes Infect. 2003;5 (3):221-226.

361. Marzo, AL, Yagita, H & Lefrancois, L. Cutting Edge: Migration to Nonlymphoid Tissues Results in Functional Conversion of Central to Effector Memory CD8 T Cells. J Immunol. 2007;179 (1):36-40.

362. Kohlmeier, JE, Miller, SC & Woodland, DL. Cutting Edge: Antigen Is Not Required for the Activation and Maintenance of Virus-Specific Memory CD8+ T Cells in the Lung Airways. J Immunol. 2007;178 (8):4721-4725.

363. Marshall, DR, Olivas, E, Andreansky, S, La Gruta, NL, Neale, GA, Gutierrez, A, Wichlan, DG, Wingo, S, Cheng, C, Doherty, PC & Turner, SJ. Effector CD8+ T cells recovered from an influenza pneumonia differentiate to a state of focused gene expression. PNAS. 2005;102 (17):6074-6079.

364. Hogan, RJ, Cauley, LS, Ely, KH, Cookenham, T, Roberts, AD, Brennan, JW, Monard, S & Woodland, DL. Long-Term Maintenance of Virus-Specific Effector Memory CD8+ T Cells in the Lung Airways Depends on Proliferation. J Immunol. 2002;169 (9):4976-4981.

365. Ely, KH, Cauley, LS, Roberts, AD, Brennan, JW, Cookenham, T & Woodland, DL. Nonspecific Recruitment of Memory CD8+ T Cells to the Lung Airways During Respiratory Virus Infections. J Immunol. 2003;170 (3):1423-1429.

366. Cerwenka, A, Morgan, TM, Harmsen, AG & Dutton, RW. Migration Kinetics and Final Destination of Type 1 and Type 2 CD8 Effector Cells Predict Protection against Pulmonary Virus Infection. J Exp Med. 1999;189 (2):423-434.

367. Liang, S, Mozdzanowska, K, Palladino, G & Gerhard, W. Heterosubtypic immunity to influenza type A virus in mice. Effector mechanisms and their longevity. J Immunol. 1994;152 (4):1653-1661.

368. Hikono, H, Kohlmeier, JE, Ely, KH, Scott, I, Roberts, AD, Blackman, MA & Woodland, DL. T-cell memory and recall responses to respiratory virus infections. Immunol Rev. 2006;211 (1):119-132.

369. Woodland, DL & Randall, TD. Anatomical features of anti-viral immunity in the respiratory tract. Semin Immunol. 2004;16 (3):163-170.

370. Belz, GT, Wilson, NS, Smith, CM, Mount, AM, Carbone, FR & Heath, WR. Bone marrow-derived cells expand memory CD8 T cells in response to viral infections of the lung and skin. Eur J Immunol. 2006;36 (2):327-335.

371. Cauley, LS, Cookenham, T, Hogan, RJ, Crowe, SR & Woodland, DL. Renewal of Peripheral CD8+ Memory T Cells During Secondary Viral Infection of Antibody-Sufficient Mice. J Immunol. 2003;170 (11):5597-5606.

372. Sprent, J & Surh, CD. T cell memory. Annu Rev Immunol. 2002;20 (1):551-579.

373. Boyman, O, Létourneau, S, Krieg, C & Sprent, J. Homeostatic proliferation and survival of naïve and memory T cells. Eur J Immunol. 2009;39 (8):2088-2094.

374. Boyman, O, Purton, JF, Surh, CD & Sprent, J. Cytokines and T-cell homeostasis. Curr Opin Immunol. 2007;19 (3):320-326.

375. Lau, LL, Jamieson, BD, Somasundaram, T & Ahmed, R. Cytotoxic T-cell memory without antigen. Nature. 1994;369:648-652.

376. Hammarlund, E, Lewis, MW, Hansen, SG, Strelow, LI, Nelson, JA, Sexton, GJ, Hanifin, JM & Slifka, MK. Duration of antiviral immunity after smallpox vaccination. Nat Med. 2003;9 (9):1131-1137.

377. Tan, JT, Ernst, B, Kieper, WC, LeRoy, E, Sprent, J & Surh, CD. Interleukin (IL)-15 and IL-7 Jointly Regulate Homeostatic Proliferation of Memory Phenotype CD8+ Cells but Are Not Required for Memory Phenotype CD4+ Cells. J Exp Med. 2002;195 (12):1523-1532.

378. Kim, H-R, Hwang, K-A, Park, S-H & Kang, I. IL-7 and IL-15: Biology and Roles in T-Cell Immunity in Health and Disease. Crit Rev Immunol. 2008;28 (4):325-339.

379. Osborne, LC & Abraham, N. Regulation of memory T cells by [gamma]c cytokines. Cytokine. 2009;50 (2):105-113.

380. Schluns, KS & Lefrancois, L. Cytokine control of memory T-cell development and survivial. Nat Rev Immunol. 2003;3 (4):269-279.

381. Schluns, KS, Kieper, WC, Jameson, SC & Lefrancois, L. Interleukin-7 mediates the homeostasis of naive and memory CD8 T cells in vivo. Nat Immunol. 2000;1 (5):426-432.

382. Carrio, R, Rolle, CE & Malek, TR. Non-redundant role for IL-7R signaling for the survival of CD8+ memory T cells. Eur J Immunol. 2007;37 (11):3078-3088.

383. Mazzucchelli, R & Durum, SK. Interleukin-7 receptor expression: intelligent design. Nat Rev Immunol. 2007;7 (2):144-154.

384. Kim, H-R, Hong, MS, Dan, JM & Kang, I. Altered IL-7R{alpha} expression with aging and the potential implications of IL-7 therapy on CD8+ T-cell immune responses. Blood. 2006;107 (7):2855-2862.

385. Park, J-H, Yu, Q, Erman, B, Appelbaum, JS, Montoya-Durango, D, Grimes, HL & Singer, A. Suppression of IL7R[alpha] Transcription by IL-7 and Other Prosurvival Cytokines: A Novel Mechanism for Maximizing IL-7-Dependent T Cell Survival. Immunity. 2004;21 (2):289-302.

386. Becker, TC, Wherry, EJ, Boone, D, Murali-Krishna, K, Antia, R, Ma, A & Ahmed, R. Interleukin 15 Is Required for Proliferative Renewal of Virus-specific Memory CD8 T Cells. J Exp Med. 2002;195 (12):1541-1548.

387. Schluns, KS, Williams, K, Ma, A, Zheng, XX & Lefrancois, L. Cutting Edge: Requirement for IL-15 in the Generation of Primary and Memory Antigen-Specific CD8 T Cells. J Immunol. 2002;168 (10):4827-4831.

388. Judge, AD, Zhang, X, Fujii, H, Surh, CD & Sprent, J. Interleukin 15 Controls both Proliferation and Survival of a Subset of Memory-Phenotype CD8+ T Cells. J Exp Med. 2002;196 (7):935-946.

389. Marks-Konczalik, J, Dubois, S, Losi, JM, Sabzevari, H, Yamada, N, Feigenbaum, L, Waldmann, TA & Tagaya, Y. IL-2-induced activation-induced cell death is inhibited in IL-15 transgenic mice. PNAS. 2000;97 (21):11445-11450.

390. Fehniger, TA, Suzuki, K, Ponnappan, A, VanDeusen, JB, Cooper, MA, Florea, SM, Freud, AG, Robinson, ML, Durbin, J & Caligiuri, MA. Fatal Leukemia in Interleukin 15 Transgenic Mice Follows Early Expansions in Natural Killer and Memory Phenotype CD8+ T Cells. J Exp Med. 2001;193 (2):219-232.

391. Zhang, X, Sun, S, Hwang, I, Tough, DF & Sprent, J. Potent and Selective Stimulation of Memory-Phenotype CD8+ T Cells In Vivo by IL-15. Immunity. 1998;8 (5):591-599.

392. Purton, JF, Tan, JT, Rubinstein, MP, Kim, DM, Sprent, J & Surh, CD. Antiviral CD4+ memory T cells are IL-15 dependent. J Exp Med. 2007;204 (4):951-961.

393. Macia, L, Delacre, M, Abboud, G, Ouk, T-S, Delanoye, A, Verwaerde, C, Saule, P & Wolowczuk, I. Impairment of Dendritic Cell Functionality and Steady-State Number in Obese Mice. J Immunol. 2006;177 (9):5997-6006.

394. Cottam, DR, Schaefer, PA, Shaftan, GW & Angus, LDG. Dysfunctional Immune-Privilege In Morbid Obesity: Implications and Effect of Gastric Bypass Surgery. Obes Surg. 2003;13 (1):49-57.

395. Power, C, Miller, SK & Alpert, PT. Promising New Causal Explanations for Obesity and Obesity-Related Diseases 10.1177/1099800406292674. Biol Res Nurs. 2007;8 (3):223-233.

396. Cottam, DR, Mattar, SG, Barinas-Mitchell, E, Eid, G, Kuller, L, Kelley, DE & Schauer, PR. The Chronic Inflammatory Hypothesis for the Morbidity Associated with Morbid Obesity: Implications and Effects of Weight Loss. Obesity Surgery. 2004;14:589-600.

397. Park, HS, Park, JY & Yu, R. Relationship of obesity and visceral adiposity with serum concentrations of CRP, TNF-[alpha] and IL-6. Diabetes Res Clin Pract. 2005;69 (1):29-35.

398. Dandona, P, Weinstock, R, Thusu, K, Abdel-Rahman, E, Alijada, A & Wadden, T. Tumor necrosis factor-α in sera of obese patients: fall with weight loss. J Clin Endocrinol Metab. 1998;83:2907-2910.

399. Esposito, K, Pontillo, A, Ciotola, M, Di Palo, C, Grella, E, Nicoletti, G & Giugliano, D. Weight Loss Reduces Interleukin-18 Levels in Obese Women 10.1210/jc.87.8.3864. J Clin Endocrinol Metab. 2002;87 (8):3864-3866.

400. Vozarova, B, Weyer, C, Hanson, K, Tataranni, PA, Bogardus, C & Pratley, RE. Circulating interleukin-6 in relation to adiposity, insulin action, and insulin secretion. Obesity Research. 2001;9:414-417.

401. Festa, A, D'Agostino, R, Jr, Williams, K, Karter, AJ, Mayer-Davis, EJ, Tracy, RP & Haffner, SM. The relation of body fat mass and distribution to markers of chronic inflammation. International Journal of Obesity. 2001;25:1407-1415.

402. Guldiken, S, Demir, M, Arikan, E, Turgut, B, Azcan, S, Gerenli, M & Tugrul, A. The levels of circulating markers of atherosclerosis and inflammation in subjects with different degrees of body mass index: Soluble CD40 ligand and high-sensitivity C-reactive protein. Thrombosis Research. 2007;119:79-84.

403. Nicklas, BJ, You, T & Pahor, M. Behavioural treatments for chronic systemic inflammation: effects of dietary weight loss and exercise training. CMAJ. 2005;172 (9):1199-1209.

404. Devaraj, S, Kasim-Karakas, S & Jialal, I. The Effect of Weight Loss and Dietary Fatty Acids on Inflammation. Current Atherosclerosis Reports. 2006;8:477-486.

405. Argilés, JM, López-Soriano, J, Almendro, V, Busquets, S & López-Soriano, FJ. Cross-talk between skeletal muscle and adipose tissue: A link with obesity? Med Res Rev. 2005;25 (1):49-65.

406. Alvarez, B, Carbó, N, López-Soriano, J, Drivdahl, RH, Busquets, S, López-Soriano, FJ, Argilés, JM & Quinn, LS. Effects of interleukin-15 (IL-15) on adipose tissue mass in rodent obesity models: evidence for direct IL-15 action on adipose tissue. Biochim Biophys Acta. 2002;1570 (1):33-37.

407. Maury, E, Ehala-Aleksejev, K, Guiot, Y, Detry, R, Vandenhooft, A & Brichard, SM. Adipokines oversecreted by omental adipose tissue in human obesity. Am J Physiol Endocrinol Metab. 2007;293 (3):E656-665.

408. Kennedy, MK, Glaccum, M, Brown, SN, Butz, EA, Viney, JL, Embers, M, Matsuki, N, Charrier, K, Sedger, L, Willis, CR, Brasel, K, Morrissey, PJ, Stocking, K, Schuh, JCL, Joyce, S & Peschon, JJ. Reversible Defects in Natural Killer and Memory CD8 T Cell Lineages in Interleukin 15-deficient Mice. J Exp Med. 2000;191 (5):771-780.

409. Surwit, RS, Feinglos, MN, Rodin, J, Sutherland, A, Petro, AE, Opara, EC, Kuhn, CM & Rebuffe-Scrive, M. Differential effects of fat and sucrose on the development of obesity and diabetes in C57BL/6J and A/J mice. Metabolism. 1995;44 (5):645-651.

410. Petro, AE, Cotter, J, Cooper, DA, Peters, JC, Surwit, SJ & Surwit, RS. Fat, carbohydrate, and calories in the development of diabetes and obesity in the C57BL/6J mouse. Metabolism. 2004;53 (4):454-457.

411. Parekh, PI, Petro, AE, Tiller, JM, Feinglos, MN & Surwit, RS. Reversal of diet-induced obesity and diabetes in C57BL/6J mice. Metabolism. 1998;47 (9):1089-1096.

412. Kilbourne, ED. Future influenza vaccines and the use of genetic recombinants. Bull Wld Hlth Org. 1969;41:643-645.

413. Kreijtz, JHCM, Bodewes, R, van Amerongen, G, Kuiken, T, Fouchier, RAM, Osterhaus, ADME & Rimmelzwaan, GF. Primary influenza A virus infection induces cross-protective immunity against a lethal infection with a heterosubtypic virus strain in mice. Vaccine. 2007;25 (4):612-620.

414. Nelson, HK, Shi, Q, Van Dael, P, Schiffrin, EJ, Blum, S, Barclay, D, Levander, OA & Beck, MA. Host nutritional selenium status as a driving force for influenza virus mutations. FASEB J. 2001:01-0115fje.

415. Reed, LJ & Muench, H. A simple method of estimating fifty percent endpoints. Am J Hyg. 1938;27:493-497.

416. Gonzalez-Juarrero, M & Orme, IM. Characterization of Murine Lung Dendritic Cells Infected with Mycobacterium tuberculosis. Infect Immun. 2001;69 (2):1127-1133.

417. Cleret, A, Quesnel-Hellmann, A, Mathieu, J, Vidal, D & Tournier, J-N. Resident CD11c+ Lung Cells Are Impaired by Anthrax Toxins after Spore Infection. J Infect Dis. 2006;194 (1):86-94.

418. Yoneyama, M, Kikuchi, M, Natsukawa, T, Shinobu, N, Imaizumi, T, Miyagishi, M, Taira, K, Akira, S & Fujita, T. The RNA helicase RIG-I has an essential function in double-stranded RNA-induced innate antiviral responses. Nat Immunol. 2004;5 (7):730-737.

419. Guillot, L, Le Goffic, R, Bloch, S, Escriou, N, Akira, S, Chignard, M & Si-Tahar, M. Involvement of Toll-like Receptor 3 in the Immune Response of Lung Epithelial Cells to Double-stranded RNA and Influenza A Virus. J Biol Chem. 2005;280 (7):5571-5580.

420. Diebold, SS, Montoya, M, Unger, H, Alexopoulou, L, Roy, P, Haswell, LE, Al-Shamkhani, A, Flavell, R, Borrow, P & Sousa, CRe. Viral infection switches non-plasmacytoid dendritic cells into high interferon producers. Nature. 2003;424 (6946):324-328.

421. Theofilopoulos, AN, Baccala, R, Beutler, B & Kono, DH. Type I interferons (alpha/beta) in immunity and autoimmunity. Annu Rev Immunol. 2005;23 (1):307-335.

422. Le Bon, A & Tough, DF. Links between innate and adaptive immunity via type I interferon. Curr Opin Immunol. 2002;14 (4):432-436.

423. Power, C, Miller, SK & Alpert, PT. Promising New Causal Explanations for Obesity and Obesity-Related Diseases. Biol Res Nurs. 2007;8 (3):223-233.

424. Vozarova, B, Weyer, C, Hanson, K, Tataranni, PA, Bogardus, C & Pratley, RE. Circulating interleukin-6 in relation to adiposity, insulin action, and insulin secretion. Obes Res. 2001;9:414-417.

425. Festa, A, D'Agostino, RJ, Williams, K, Karter, A, Mayer-Davis, E, Tracy, R & Haffner, S. The relation of body fat mass and distribution to markers of chronic inflammation. Int J Obes. 2001;25 (10):1407-1415.

426. Devaraj, S, Kasim-Karakas, S & Jialal, I. The Effect of Weight Loss and Dietary Fatty Acids on Inflammation. Curr Atherosclerosis Rep. 2006;8:477-486.

427. Lin, S, Thomas, TC, Storlien, LH & Huang, XF. Development of high fat diet-induced obesity and leptin resistance in C57Bl/6J mice. Int J Obes Relat Metab Disord. 2000;24 (5):639-646.

428. Guo, J, Jou, W, Gavrilova, O & Hall, KD. Persistent Diet-Induced Obesity in Male C57BL/6 Mice Resulting from Temporary Obesigenic Diets. PLoS ONE. 2009;4 (4):e5370.

429. Kolumam, GA, Thomas, S, Thompson, LJ, Sprent, J & Murali-Krishna, K. Type I interferons act directly on CD8 T cells to allow clonal expansion and memory formation in response to viral infection. J Exp Med. 2005;202 (5):637-650.

430. Thompson, LJ, Kolumam, GA, Thomas, S & Murali-Krishna, K. Innate Inflammatory Signals Induced by Various Pathogens Differentially Dictate the IFN-I Dependence of CD8 T Cells for Clonal Expansion and Memory Formation. J Immunol. 2006;177 (3):1746-1754.

431. Masopust, D, Vezys, V, Marzo, AL & Lefrancois, L. Preferential Localization of Effector Memory Cells in Nonlymphoid Tissue. Science. 2001;291 (5512):2413-2417.

432. Kedzierska, K, Venturi, V, Field, K, Davenport, MP, Turner, SJ & Doherty, PC. Early establishment of diverse T cell receptor profiles for influenza-specific CD8+CD62Lhi memory T cells. PNAS. 2006;103 (24):9184-9189.

433. Kaech, SM, Wherry, EJ & Ahmed, R. Effector and memory T-cell differentiation: implications for vaccine development. Nat Rev Immunol. 2002;2 (4):251-262.

434. Lam, QLK & Lu, L. Role of leptin in immunity. Cell Mol Immunol. 2007;4 (1):1-13.

435. Friedman, JM & Halaas, JL. Leptin and the regulation of body weight in mammals. Nature. 1998;395 (6704):763-770.

436. Lin, S, Thomas, T, Storlien, L & Huang, X. Development of high fat diet-induced obesity and leptin resistance in C57Bl/6J mice. Int J Obes Relat Metab Disord. 2000;24 (5):639-646.

437. Mattioli, B, Straface, E, Quaranta, MG, Giordani, L & Viora, M. Leptin Promotes Differentiation and Survival of Human Dendritic Cells and Licenses Them for Th1 Priming J Immunol. 2005;174 (11):6820-6828.

438. Lam, QLK, Liu, S, Cao, X & Lu, L. Involvement of leptin signaling in the survival and maturation of bone marrow-derived dendritic cells. Eur J Immunol. 2006;36 (12):3118-3130.

439. Lord, GM. Role of leptin in immunology. Nutr Rev. 2002;60 (10):S35-37.

440. Vlotides, G, Sörensen, AS, Kopp, F, Zitzmann, K, Cengic, N, Brand, S, Zachoval, R & Auernhammer, CJ. SOCS-1 and SOCS-3 inhibit IFN-[alpha]-induced expression of the antiviral proteins 2,5-OAS and MxA. Biochem Biophys Res Com. 2004;320 (3):1007-1014.

441. Tanaka, S, Inoue, S, Isoda, F, Waseda, M, Ishihara, M, Yamakawa, T, Sugiyama, A, Takamura, Y & Okuda, K. Impaired immunity in obesity: suppressed but reversible lymphocyte responsiveness. Int J Obes Relat Metab Disord. 1993;17 (11):631-636.

442. Tanaka, S-i, Isoda, F, Kiuchi, Y, Ikeda, H, Mobbs, CV & Yamakawa, T. T lymphopenia in genetically obese-diabetic wistar fatty rats: Effects of body weight reduction on T cells. Metabolism. 2000;49 (10):1261-1266.

443. Karlsson, EA, Sheridan, PA & Beck, MA. Diet-induced obesity impairs the T cell memory response to influenza virus infection. J Immunol. 2010;184 (6).

444. Tough, DF, Sun, S, Zhang, X & Sprent, J. Stimulation of memory T cells by cytokines. Vaccine. 2000;18 (16):1642-1648.

445. Gray, D. A role for antigen in the maintenance of immunological memory. Nat Rev Immunol. 2002;2 (1):60-65.

446. Surh, CD, Boyman, O, Purton, JF & Sprent, J. Homeostasis of memory T cells. Immunol Rev. 2006;211:154-163.

447. Knobelspies, H, Zeidler, J, Hekerman, P, Bamberg-Lemper, S & Becker, W. Mechanism of attenuation of leptin signaling under chronic ligand stimulation. BMC Biochem. 2010;11 (2).

448. Mori, H, Hanada, R, Hanada, T, Aki, D, Mashima, R, Nishinakamura, H, Torisu, T, Chien, KR, Yasukawa, H & Yoshimura, A. Socs3 deficiency in the brain elevates leptin sensitivity and confers resistance to diet-induced obesity. Nat Med. 2004;10 (7):739-743.

449. Ilangumaran, S, Ramanathan, S, La Rose, J, Poussier, P & Rottapel, R. Suppressor of Cytokine Signaling 1 Regulates IL-15 Receptor Signaling in CD8+CD44high Memory T Lymphocytes. J Immunol. 2003;171 (5):2435-2445.

450. Davey, GM, Starr, R, Cornish, AL, Burghardt, JT, Alexander, WS, Carbone, FR, Surh, CD & Heath, WR. SOCS-1 regulates IL-15-driven homeostatic proliferation of antigen-naive CD8 T cells, limiting their autoimmune potential. J Exp Med. 2005;202 (8):1099-1108.

451. DeLany, J. Leptin hormone and other biochemical influences on systemic inflammation. J Bodyw Mov Ther. 2008;12 (2):121-132.

452. Fujita, Y, Murakami, M, Ogawa, Y, Masuzaki, H, Tanaka, M, Ozaki, S, Nakao, N & Mimori, T. Leptin inhibits stress-induced apoptosis of T lymphocytes. Clin Exp Immunol. 2002;128:21-26.

453. Pothlichet, J, Chignard, M & Si-Tahar, M. Cutting Edge: Innate Immune Response Triggered by Influenza A Virus Is Negatively Regulated by SOCS1 and SOCS3 through a RIG-I/IFNAR1-Dependent Pathway. J Immunol. 2008;180 (4):2034-2038.

454. Alexander, WS & Hilton, DJ. The Role of Suppressors of Cytokine Signaling (SOCS) Proteins in Regulation of the Immune Response doi:10.1146/annurev.immunol.22.091003.090312. Annual Review of Immunology. 2004;22 (1):503-529.

455. Scarpace, PJ & Zhang, Y. Leptin resistance: a prediposing factor for diet-induced obesity. Am J Physiol Regul Integr Comp Physiol. 2009;296 (3):R493-500.

456. Paine III, R, Chavis, A, Gaposchkin, D, Christensen, P, Mody, C, Turka, L & Toews, G. A factor secreted by a human pulmonary alveolar epithelial-like cell line blocks T-cell proliferation between G1 and S phase. Am J Respir Cell Mol Biol. 1992;6 (6):658-666.

457. Khanna, KM & Lefrancois, L. Geography and plumbing control the T cell response to infection. Immunol Cell Biol. 2008.

458. Salerno, FG, Carpagnano, E, Guido, P, Bonsignore, MR, Roberti, A, Aliani, M, Vignola, AM & Spanevello, A. Airway inflammation in patients affected by obstructive sleep apnea syndrome. Resp Med. 2004;98 (1):25-28.

459. Goulding, J, Snelgrove, R, Saldana, J, Didierlaurent, A, Cavanagh, M, Gwyer, E, Wales, J, Wissinger, EL & Hussell, T. Respiratory Infections: Do We Ever Recover? Proc Am Thorac Soc. 2007;4 (8):618-625.

460. Yap, K, Ada, G & McKenzie, I. Transfer of specific cytotoxic T lymphocytes protects mice innoculated with influenza virus. Nature. 1978;273 (5659):238-239.

461. Woodland, DL & Kohlmeier, JE. Migration, maintenance and recall of memory T cells in peripheral tissues. Nat Rev Immunol. 2009;9 (3):153-161.

462. Abitorabi, M, Mackay, C, Jerome, E, Osorio, O, Butcher, E & Erle, D. Differential expression of homing molecules on recirculating lymphocytes from sheep gut, peripheral, and lung lymph. J Immunol. 1996;156 (9):3111-3117.

463. Sigmundsdottir, H & Butcher, EC. Environmental cues, dendritic cells and the programming of tissue-selective lymphocyte trafficking. Nat Immunol. 2008;9 (9):981-987.

464. Picker, L, Terstappen, L, Rott, L, Streeter, P, Stein, H & Butcher, E. Differential expression of homing-associated adhesion molecules by T cell subsets in man. J Immunol. 1990;145 (10):3247-3255.

465. van der Most, RG, Murali-Krishna, K & Ahmed, R. Prolonged presence of effector-memory CD8 T cells in the central nervous system after dengue virus encephalitis. Int Immunol. 2003;15 (1):119-125.

466. Ray, SJ, Franki, SN, Pierce, RH, Dimitrova, S, Koteliansky, V, Sprague, AG, Doherty, PC, de Fougerolles, AR & Topham, DJ. The Collagen Binding [alpha]1[beta]1 Integrin VLA-1 Regulates CD8 T Cell-Mediated Immune Protection against Heterologous Influenza Infection. Immunity. 2004;20 (2):167-179.

467. MacIver, NJ, Jacobs, SR, Wieman, HL, Wofford, JA, Coloff, JL & Rathmell, JC. Glucose metabolism in lymphocytes is a regulated process with significant effects on immune cell function and survival. J Leukoc Biol. 2008;84 (4):949-957.

468. Fox, CJ, Hammerman, PS & Thompson, CB. Fuel feeds function: energy metabolism and the T-cell response. Nat Rev Immunol. 2005;5 (11):844-852.

469. Frauwirth, KA & Thompson, CB. Regulation of T Lymphocyte Metabolism. J Immunol. 2004;172 (8):4661-4665.

470. Frauwirth, KA, Riley, JL, Harris, MH, Parry, RV, Rathmell, JC, Plas, DR, Elstrom, RL, June, CH & Thompson, CB. The CD28 Signaling Pathway Regulates Glucose Metabolism. Immunity. 2002;16 (6):769-777.

471. Wieman, HL, Wofford, JA & Rathmell, JC. Cytokine Stimulation Promotes Glucose Uptake via Phosphatidylinositol-3 Kinase/Akt Regulation of Glut1 Activity and Trafficking. Mol Biol Cell. 2007;18 (4):1437-1446.

472. Gingras, A-C, Raught, B & Sonenberg, N. Regulation of translation initiation by FRAP/mTOR. Genes Develop. 2001;15 (7):807-826.

473. Edinger, AL & Thompson, CB. Akt Maintains Cell Size and Survival by Increasing mTOR-dependent Nutrient Uptake. Mol Biol Cell. 2002;13 (7):2276-2288.

474. Jones, RG & Thompson, CB. Revving the Engine: Signal Transduction Fuels T Cell Activation. Immunity. 2007;27 (2):173-178.

475. Pearce, EL, Walsh, MC, Cejas, PJ, Harms, GM, Shen, H, Wang, L-S, Jones, RG & Choi, Y. Enhancing CD8 T-cell memory by modulating fatty acid metabolism. Nature. 2009;460 (7251):103-107.

476. Stanfel, MN, Shamieh, LS, Kaeberlein, M & Kennedy, BK. The TOR pathway comes of age. Biochim Biophys Acta. 2009;1790 (10):1067-1074.

477. Romao, I & Roth, J. Genetic and Environmental Interactions in Obesity and Type 2 Diabetes. J Amer Diet Assoc. 2008;108 (4, Supplement 1):S24-S28.

478. Tremblay, Fdr & Marette, A. Amino Acid and Insulin Signaling via the mTOR/p70 S6 Kinase Pathway. J Biol Chem. 2001;276 (41):38052-38060.

479. Morris, DL & Rui, L. Recent advances in understanding leptin signaling and leptin resistance. Am J Physiol Endocrinol Metab. 2009;297 (6):E1247-1259.

480. Sudarsanam, S & Johnson, DE. Functional consequences of mTOR inhibition. Curr Opin Drug Discov Devel. 2010;13 (1):31-40.

481. Shevach, EM. CD4+CD25+ suppressor T cells: more questions than answers. Nat Rev Immunol. 2002;2 (6):389-400.

482. Sakaguchi, S. Naturally Arising CD4+ Regulatory T Cells for Immunologic Self-Tolerance and Negative Control of Immune Responses. Annu Rev Immunol. 2004;22 (1):531-562.

483. Suvas, S, Kumaraguru, U, Pack, CD, Lee, S & Rouse, BT. CD4+CD25+ T Cells Regulate Virus-specific Primary and Memory CD8+ T Cell Responses. J Exp Med. 2003;198 (6):889-901.

484. Piccirillo, CA & Shevach, EM. Cutting Edge: Control of CD8+ T Cell Activation by CD4+CD25+ Immunoregulatory Cells. J Immunol. 2001;167 (3):1137-1140.

485. Chappert, P, Leboeuf, M, Rameau, P, Lalfer, M, Desbois, S, Liblau, RS, Danos, O, Davoust, JM & Gross, D-A. Antigen-specific Treg impair CD8+ T-cell priming by blocking early T-cell expansion. Eur J Immunol. 2010;40 (2):339-350.

486. Taleb, S, Herbin, O, Ait-Oufella, H, Verreth, W, Gourdy, P, Barateau, V, Merval, R, Esposito, B, Clement, K, Holvoet, P, Tedgui, A & Mallat, Z. Defective Leptin/Leptin Receptor Signaling Improves Regulatory T Cell Immune Response and Protects Mice From Atherosclerosis. Arterioscler Thromb Vasc Biol. 2007;27 (12):2691-2698.

487. Farooqi, IS, Matarese, G, Lord, GM, Keogh, JM, Lawrence, E, Agwu, C, Sanna, V, Jebb, SA, Perna, F, Fontana, S, Lechler, RI, DePaoli, AM & O'Rahilly, S. Beneficial effects of leptin on obesity, T cell hyporesponsiveness, and neuroendocrine/metabolic dysfunction of human congenital leptin deficiency. J Clin Invest. 2002;110 (8):1093-1103.

488. Wilson, CB, Makar, KW, Shnyreva, M & Fitzpatrick, DR. DNA methylation and the expanding epigenetics of T cell lineage commitment. Semin Immunol. 2005;17 (2):105-119.

489. Fitzpatrick, DR & Wilson, CB. Methylation and demethylation in the regulation of genes, cells, and responses in the immune system. Clin Immunol. 2003;109 (1):37-45.

490. Sawalha, AH. Epigenetics and T-cell immunity. Autoimmunity. 2008;41 (4):245-252.

491. Lee, C-G, Sahoo, A & Im, S-H. Epigenetic Regulation of Cytokine Gene Expression in T Lymphocytes. Yonsei Med J. 2009;50 (3):322-330.

492. Bruniquel, D & Schwartz, RH. Selective, stable demethylation of the interleukin-2 gene enhances transcription by an active process. Nat Immunol. 2003;4 (3):235-240.

493. Hutchins, AS, Mullen, AC, Lee, HW, Sykes, KJ, High, FA, Hendrich, BD, Bird, AP & Reiner, SL. Gene Silencing Quantitatively Controls the Function of a Developmental trans-Activator. Molecular Cell. 2002;10 (1):81-91.

494. Hutchins, AS, Artis, D, Hendrich, BD, Bird, AP, Scott, P & Reiner, SL. Cutting Edge: A Critical Role for Gene Silencing in Preventing Excessive Type 1 Immunity. J Immunol. 2005;175 (9):5606-5610.

495. Makar, KW, Perez-Melgosa, M, Shnyreva, M, Weaver, WM, Fitzpatrick, DR & Wilson, CB. Active recruitment of DNA methyltransferases regulates interleukin 4 in thymocytes and T cells. Nat Immunol. 2003;4 (12):1183-1190.

496. Lee, PP, Fitzpatrick, DR, Beard, C, Jessup, HK, Lehar, S, Makar, KW, Pérez-Melgosa, M, Sweetser, MT, Schlissel, MS, Nguyen, S, Cherry, SR, Tsai, JH, Tucker, SM, Weaver, WM, Kelso, A, Jaenisch, R & Wilson, CB. A Critical Role for Dnmt1 and DNA Methylation in T Cell Development, Function, and Survival. Immunity. 2001;15 (5):763-774.

497. Kersh, EN. Impaired Memory CD8 T Cell Development in the Absence of Methyl-CpG-Binding Domain Protein 2. J Immunol. 2006;177 (6):3821-3826.

498. Milagro, FI, Campión, J, García-Díaz, DF, Goyenechea, E, Paternain, L & Martínez, JA. High fat diet-induced obesity modifies the methylation pattern of leptin promoter in rats. J Physiol Biochem. 2009;65 (1):1-9.

499. Cottam, DR, Mattar, SG, Barinas-Mitchell, E, Eid, G, Kuller, L, Kelley, DE & Schauer, PR. The Chronic Inflammatory Hypothesis for the Morbidity Associated with Morbid Obesity: Implications and Effects of Weight Loss. Obes Surg. 2004;14:589-600.

500. Dixit, VD. Adipose-immune interactions during obesity and caloric restriction: reciprocal mechanisms regulating immunity and health span. J Leukoc Biol. 2008;84 (4):882-892.

501. Grebe, KM, Yewdell, JW & Bennink, JR. Heterosubtypic immunity to influenza A virus: where do we stand? Microbes Infect. 2008;10 (9):1024-1029.