DISSERTATION

VISCERAL ADIPOSITY AND PRO-INFLAMMATION: CONTRIBUTIONS AND CONSEQUENCES OF IMMUNITY

Submitted by

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ABSTRACT

VISCERAL ADIPOSITY AND PRO-INFLAMMATION: CONTRIBUTIONS AND CONSEQUENCES OF IMMUNITY

Obesity among Americans has reached a strikingly prevalent rate with ~36% of adults falling into this category. As this rate continues to rise, so do the number of individuals with chronic diseases associated with excessive adipose tissue accumulation. Dysregulation of adipose tissue is a fundamental driver of obesity associated comorbidities. In particular, deposition of adipose tissue within the visceral cavity is especially deleterious, while subcutaneous adipose tissue has been identified as being metabolically protective. Consequently, visceral adiposity is demonstrated to be more highly associated with increased pro-inflammation. This inflammation has been demonstrated to be the causal link between obesity and its associated comorbidities. The purpose of this research was to investigate why visceral adiposity is so detrimental.

We propose that the lymphatic system is central to the heightened inflammatory potential associated with adipose tissue deposition in the visceral cavity. The visceral lymph node serves as the immune nexus between the visceral adipose tissue and the small intestines and is thus impacted by alterations in these tissues as a consequence of poor diet and obesity. In these studies we utilized mouse models of diet induced obesity (DIO) to elucidate the relationships between these tissues that potentially contribute to heightened pro-inflammatory potential of visceral adiposity relative to subcutaneous. We fed mice a high fat westernized diet (HFD) for 7 or 13 weeks, and then analyzed immune cell populations in the visceral and subcutaneous lymph

nodes and adipose tissue and the small intestine, adipose tissue inflammatory markers, and lymph node morphology. Visceral adipose tissue had an inherently greater potential for pro-inflammation based on immune cell populations in lean controls relative to subcutaneous adipose tissue. Seven weeks of DIO was associated with decreased potential for immune suppression in the visceral adipose tissue, small intestines and visceral lymph nodes and elevated pro-inflammatory immune cell subsets. Consistent with this, pro-inflammatory cytokines were significantly elevated in the visceral adipose tissue. In association with this, visceral lymph nodes demonstrated hypertrophy, increases in total viable immune cells and immune cell subsets indicative of an adaptive like immune response. These changes were specific to the visceral cavity. Thirteen weeks of DIO caused a suppression of total viable immune cells and pro-inflammatory subsets in both the visceral adipose tissue and lymph nodes. Subcutaneous tissues were relatively unaffected. Alterations in immune cells were associated with significant fibrosis specific to the visceral lymph nodes. Increased incidence of comorbidities and immune related pathologies associated with visceral adiposity is likely, at least in part, a consequence of heightened pro-inflammation in the visceral lymph node as a result of chronic immune stimulation from both the visceral adipose tissue and small intestines. We propose that lymph node fibrosis is a result of chronic inflammation associated with obesity. In turn, this contributes to immune suppression that is associated increased susceptibility to pathogen infection and disease morbidity.

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As I sat reflecting on my time spent in pursuit of a PhD I came across a quote that rang especially true to me, “Families are the compass that guides us. They are the inspiration to reach great heights, and our comfort when we occasionally falter” –Brad Henry. I am truly blessed to have a family that has been all of these things for me. Without them none of this would have been possible.

To my daughter, Madeleine, you have been my inspiration to reach great heights. It is because of you that I have had the determination to push through hard times and achieve my goals. Your bright smile, laughter, and love for everything that you do always serve to remind me to enjoy the little things in life. I dedicate this work to you. Dare to dream big, as I know you will do great things.

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CHAPTER 1: SPECIFIC TOPIC REVIEW

Introduction: current obesity statistics and trends for the United States

Obesity has become epidemic in the United States. Current statistics estimates that over two thirds of the total American population is overweight and half of those individuals are obese [1]. This is especially concerning because 10 years ago in 2007, adult obesity rates were estimated to be one fourth (~26%) of the adult population [2]. This represents a 1 percent increase per year over the last 10 years. The increase in the obesity epidemic is further supported by the fact that in 1990, no state had an obesity rate great than 15%. It wasn't until 1997 that a single state surpassed obesity rates of greater than 20% [3]. Thus, in a period from 1985 to 1997, 12 years, only one state had rates reach a total increase in obesity trends of 5%. In a 10 year period from 1997 to 2007 rates increased another 5%, and then in a 10 year period from 2007 to 2017 a 10% increase. Currently, the number of obese people within the United States is roughly equal to the number of normal weight, healthy individuals. This represents a significant health care crisis for the United States. As of 2016, a meta-analysis of healthcare data indicated that total annual medical spending attributable to obese individuals was 149.4 billion dollars [4]. Overall, obesity rates continue to increase yearly, subsequently this is associated with increases in comorbidities associated with increases in adiposity.

Obesity and disease risk: metabolic disease and comorbidities

Obesity is known to be associated with a number of comorbidities. These include, but are not limited to, heart disease, type 2 diabetes, hypertension, liver inflammation, and immune deficiencies [5-11]. In particular, visceral adipose tissue accumulation is highly associated with the comorbidities of obesity whereas subcutaneous adipose tissue deposition is not [12-15]. The

concept that visceral adipose tissue accumulation best relates to metabolic disease links to adipose tissue distribution studies. For example, a study including 930 obese men and women support that visceral adiposity, compared with lower body adiposity, has a greater risk profile for the comorbidities of obesity [16]. The study also supports that men have greater a propensity for visceral adipose deposition than women. However, women characterized with visceral adipose deposition had metabolic risk similar to those demonstrated in men [16]. Furthermore, studies indicate that visceral adiposity is associated with the increased incidence of specific diseases. It has been demonstrate that every 1% increase in visceral fat increases the odds ratio of liver inflammation and fibrosis to ~2.4 and 3.5 respectively [17, 18]. Indeed, regression analysis support that visceral adiposity remains a strong predictor of steatohepatitis when other obesity related factors such as insulin resistance are controlled for [17]. As stated previously, type 2 diabetes mellitus is highly associated with visceral adiposity. Both waist and waist to hip circumference are stronger predictors of the incidence of type 2 diabetes than Body Mass Index (BMI) [7]. Rodent models further support that visceral adipose tissue is more detrimental, and in fact subcutaneous adipose tissue is metabolically protective. One such study demonstrated that both autologous (subcutaneous from same mouse) and hetero (subcutaneous from obese donor mouse) transplantation of subcutaneous adipose tissue to the visceral depot in diet induced obese mice improved metabolic measures. Removal of the subcutaneous adipose tissue however exacerbated metabolic detriments [19]. Human research supports that visceral deposition of adipose tissue is particularly harmful, yet the reasons as to why this depot links to greater deleterious outcomes remains to be elucidated.

Obesity and inflammation: visceral and subcutaneous differences

The visceral and subcutaneous adipose tissue depots are inherently different in a number of ways. These include, but are not limited to, depot/adipocyte-specific variations in lipid metabolism, lipolytic activity, insulin action, adipokine expression, and proximity to vital organs [20-22]. The close proximity of visceral adipose tissue to organs such as the kidneys, intestines and liver is of particular importance as it surrounds, attaches and shares vasculature with them. This is proposed to be particularly detrimental within the liver as drainage into the portal vein directly exposes hepatocytes to secretory products such as pro-inflammatory cytokines as well as metabolites released by visceral adipocytes [23-25]. Excessive visceral adipose tissue deposition results in chronic exposure of the liver to elevated levels of free fatty acids that lead to ectopic lipid deposition and the development or exacerbation of insulin resistance [26, 27]. Specific characteristic of the adipocytes themselves play a role in this as visceral adipocytes tend to be larger and as a consequence are insulin resistant and thus are more highly lipolytic and secreting elevated levels of free fatty acids [21, 22]. Elevated free fatty acids have also been demonstrated to influence inflammation [28, 29]. As such dysregulated visceral adipose tissue is also more highly associated with inflammation than that of subcutaneous adipose tissue and is linked with increased levels of circulating pro-inflammatory factors such as TNF-α, IL-6, and MCP-1 [30-33]. Visceral adipose tissue has also been shown to be more highly infiltrated by immune cells such as mast cells and macrophages that promote the release of highly pro-inflammatory cytokines such as TNF-α, IL-6 and IL-1β [34, 35]. Consequently, this results in exposure of organs in the visceral cavity to elevated levels of pro-inflammation with deleterious consequences. In the liver, IL-6 has been demonstrated to induce insulin resistance [36, 37]. TNF-α has been demonstrated to promote fibrosis in the lungs and liver [38, 39]. Thus, specific

characteristics of dysregulated adipose tissue are key drivers in the pathogenesis of obesity associated comorbidities.

Obesity and inflammation: lymphatics and the immune system

Central to induction of inflammation is the immune system. This system is comprised of the innate and adaptive immune response that work together to remove an inflammatory stimulus and promote tissue repair [40-42]. Lymph nodes are the primary site for development of regional immunity. These structures serve as facilitators in the interactions between immune cells (monocytes, macrophages, dendritic cells, and lymphocytes) and antigens [43-45]. Innate immunity is the first line of defense that responds to numerous foreign stimuli and elicit a rapid and non-specific immune response [46, 47]. In turn, antigen presenting cells (i.e. dendritic cells and macrophages) then serve to bridge together the innate response with adaptive immunity [40, 48, 49]. These interactions serve to develop protective immune responses to pathogen infection and tissue damage. Lymph nodes are almost exclusively embedded within adipose tissue [50]. They collect effluent from the surrounding adipose tissue, thus they are in a unique position to constantly survey pathogen infection, tissue damage and potentially harmful metabolites [51, 52]. In obesity, adipose tissue metabolic derangements serve as a signal to the immune system that there is tissue injury [53-55]. This elicits an immune response in an attempt to resolve the inflammation and promote tissue repair. However, obesity is defined as a state of chronic low grade inflammation. In this situation adipose tissue dysregulation is not resolved and chronic persistent inflammatory stimulus continually engages immune cells [56, 57]. In turn, lymph nodes are likely exposed to chronic elevated levels of pro-inflammatory stimuli via the effluent of the inflamed adipose tissue.

Obesity is also linked with compromised immunity. Indeed numerous human epidemiologic studies support this. In a clinical setting, observations have been made to suggest that obese patients are at an increased risk for numerous secondary pathogen infections including but not limited to sepsis, pneumonia, and bacteremia [58, 59]. Similarly, patients with a high BMI are demonstrated to be more likely to suffer increased incidence of surgical site infections [60]. These observations are not limited to secondary infections within a clinical setting. Recent data from the 2009 H1N1 flu pandemic also indicate that obesity was a risk factor for hospitalization and death [61]. Obesity is not only a risk factor associated with infection but has also been linked to poor vaccine efficacy in both humans and rodent models [10, 62-65]. Numerous rodent models have generated data implicating multiple mechanisms by which impaired immunity may be occurring. Significant differences between genetic and diet induced obese models have also been demonstrated. These include but are not limited to impaired cellular metabolism [66-68], dysregulated leptin signaling [69, 70], and impaired dendritic cell functionality [71, 72]. Overall, impaired immune function has been demonstrated in response to a small number of infections with varying mechanisms being identified. The overall systemic impact has of obesity in relation to impaired immunity remains to be fully investigated.

Conclusions

Adipose tissue has been demonstrated to be more than just a site for storage of fat. In fact it is a highly active endocrine organ. In the context of obesity dysregulation of adipose tissue depots is closely associated with disease and co-morbidities. Visceral adipose tissue is especially detrimental and is more highly associated with increased disease and mortality. This is likely a result of a greater capacity for pro-inflammation than the subcutaneous adipose tissue as well as its close proximity to vital organs. Of significant importance is the fact that lymph nodes, an

important staging ground for protective immunity, are almost exclusively located within adipose tissue depots. This placement allows for a unique relationship under chronic inflammatory conditions for adipose tissue lymph node crosstalk to uniquely alter the inflammatory milieu. This is likely particularly important in the visceral adipose tissue depot as it has been demonstrated to be more highly associated with chronic inflammation and disease incidence.

CHAPTER 2: OBESITY-INDUCED CHRONIC LOW GRADE INFLAMMATION: GASTROINTESTINAL AND ADIPOSE TISSUE CROSSTALK[1][2][3]

Summary

The prevalence of overweight/obese adults and children continues to rise and with it the incidence of many secondary health consequences. Inflammation is pivotal in the manifestation of the comorbidities of obesity and is a product of dysregulated adipose tissue. Hence, obesity is a state of fat-induced prolonged inflammation that causes a decrease in immune defense. It is well established that central/visceral fat accumulation (the "apple" shape) is a risk factor for many adverse inflammatory metabolic outcomes associated with obesity, whereas peripheral or subcutaneous fat (the "pear" shape) is associated with a reduced risk. Considerable gains have been made in characterizing these regional differences in adipose tissue inflammation-induced metabolic dysregulation, but very little is known about the role of the immune system despite obesity being an inflammatory disease. The aim of this review is to elucidate how the lymphatic system, that drains tissues such as inflamed adipose tissue, may play a role in obesity-induced inflammation. Particular focus is placed on how the gastrointestinal tract that is connected to

[1]A modified version of this chapter is published as A Magnuson, J Fouts, A Booth, M Foster. Obesity-induced chronic low grade inflammation: Gastrointestinal and adipose tissue crosstalk. Integrative Obesity and Diabetes. 2015.

[2]My contributions to this work include the writing of several sections as well as overall review for accuracy.

visceral adipose tissue via the lymphatic system may play a role in differential metabolic outcomes of distinct adipose tissue distributions. Overall, to accurately understand the pathogenic mechanisms that lead to the prolonged state of inflammation induced by obesity the role of the lymphatics must be elucidated.

Introduction

Obesity is a growing epidemic. In 2014 the National Center for Health Statistics reported that ~70% of adults in the United States were overweight and that half of the overweight individuals were also obese [73]. Obesity, as traditionally measured by body mass index (BMI), is a risk factor for several diseases including, but not limited to, dyslipidemia, hypertension [74], non-alcoholic fatty liver disease (NAFLD) [75], atherosclerosis, cardiovascular disease (CVD) [76], and type-2-diabetes [77]. Consequently this condition is also a strong predictor of mortality [78]. However, exclusive markers of obesity such as positive energy balance and BMI are not accurate predictors of metabolic dysregulation. Rather adipose tissue distribution is the best determinant of an individual's risk towards the development metabolic disease. It is common to investigate the differential aspects of adipose tissue distribution and its relation to metabolic risk via comparison of two different body types, the apple shape (visceral/central adipose tissue accumulation) or the pear (lower body subcutaneous adipose tissue distribution). Visceral adipose tissue accumulation occurs within the intra-abdominal cavity among vital organs such as the liver, pancreas, gut and kidneys whereas accumulation of lower body subcutaneous adipose tissue occurs between the muscle and skin (hypodermis) of the thighs, buttocks and lower stomach.

The concept that central obesity produces metabolic outcomes that are distinct from peripheral obesity is well established [79, 80]. Numerous epidemiological studies demonstrate that excessive accumulation of visceral adipose tissue is associated with pathological conditions such

as hypertension, dyslipidemia [81-83] (increased circulating triglycerides, free fatty acids, LDL to HDL ratio), cardiovascular disease, type-2 diabetes [84-88] and non-alcoholic fatty liver disease [89]. In opposition, studies propose that peripheral adipose tissue is protective, functioning as a "metabolic sink" that protects against lipid accumulation in non-adipose tissues. Consistent with this subcutaneous adipose tissue accumulation is associated with cardioprotection [90]. In particular, when compared with visceral adiposity, individuals with subcutaneous adiposity had lower circulating triglycerides and higher HDL cholesterol [90]. This relation between subcutaneous adipose tissue deposition and metabolic protection, however, is best associated with gluteofemoral subcutaneous adiposity. Truncal/abdominal subcutaneous adiposity is implicated in the pathogenesis of insulin resistance [91-93], atherosclerosis and cardiometabolic risk [94, 95], whereas gluteofemoral adiposity is associated with insulin sensitivity, higher HDL and decreased risk for type-2-diabetes and metabolic syndrome [96-101]. We have begun to elucidate the mechanisms that causally relate the different metabolic outcomes of adipose tissue distribution [102-105] and have demonstrated that the strong link between visceral obesity and adverse metabolic outcomes is both anatomic location, proximity to the hepatic portal vein, and adipose depot specific physiology. Adipose depot specific characteristics, however, are also influenced by outside (extrinsic) factors such as, but not limited to, the immune (lymphatic) system. Hence below we discuss the postulate that the lymphatic system plays an important role in central/visceral obesity-mediated metabolic impairments. In addition, we will discuss how the gastrointestinal tract may prime visceral adiposity to be more detrimental than subcutaneous.

Adipose tissue

Visceral adipose tissue has the greatest association with pathogenesis of obesity-related disease despite its relatively low amount which constitutes 10% of total adiposity, whereas

subcutaneous adipose tissue is ~85% [106]. Despite this the visceral adipose depot is proposed to play a deleterious role in obesity-induced health consequences because, as previously mentioned, where it is located. Hence, previous investigations primarily focus on visceral adipose tissue and liver interactions because effluent from visceral adipocytes containing metabolites, secretory products and inflammatory cytokines to the portal vein can be detrimental to insulin-sensitive hepatocytes [107-109]. These factors have major influence on hepatic processes such as glycogenesis, gluconeogenesis and very low-density lipoproteins (VLDL) synthesis [110-115]. Although the liver does play a large role in the exacerbation of obesity-induced metabolic disease, visceral adipose tissue surrounds, covers, attaches and shares vasculature with numerous other abdominal organs, yet these tissues are less investigated.

Adipose tissue is a complex endocrine organ with fundamental metabolic and immune regulatory roles. Adipocytes are characterized to release numerous hormones and signaling molecules, adipokines or adipocytokines that work in an autocrine and paracrine fashion or peripherally in an endocrine fashion. Adipocytokines regulate numerous physiological processes. In particular the role adipocytokines play in chronic pro-inflammation during obesity is gaining attention. Specifically, obesity associated adipocyte hypertrophy and hyperplasia dysregulates the sensitive microenvironment within adipose depots and alters their physiological processes, subsequently this can lead to changes in whole body homeostasis. Obesity-induced inflammation evoked during this process has been identified to be pivotal in the manifestation of the metabolic comorbidities and is identified to be a product of dysregulated adipose tissue. Hence, the short-term inflammation in adipose tissue that is a principal defense in response to acute injury becomes dysregulated during prolonged inflammation, as occurs in obesity, and is no longer beneficial [116]. Overall, obesity is a state of fat-induced prolonged inflammation.

Inflammation, induced by excessive adipose tissue accumulation, appears to link obesity to disease risk [117-119]. It is well established that central/visceral fat accumulation is a risk factor for many adverse inflammatory metabolic outcomes associated with obesity, whereas peripheral or subcutaneous fat is associated with a reduced risk. Hence, the increased propensity for individuals with visceral obesity to experience comorbidities appears to be linked to the increased capacity of this depot to induce inflammation [120]. Numerous studies demonstrate that visceral fat from obese individuals is characterized by increased macrophage infiltration, cytokine and chemokine production and other supportive immune cell types compared with non-visceral adipose tissue [121-125]. Despite considerable gains in characterization of regional differences in adipose tissue inflammation-induced metabolic dysregulation, very little is known about the role of the actual immune system in obesity-induced inflammation.

Lymphatic system and lymph nodes

The health and regulation of adipose tissue is primarily determined by the lymphatic system. Lymphatic system functions include; 1.) interstitial fluid removal, 2.) white blood cell transport, 3.) absorption and transportation of lipids and 4.) activation and initiation of immune cells and response. Overall, this system, which openly circulates throughout the body, is the conduit for immune cells that serves to produce an immune response to pathogen invasion and tissue injury. Lymph nodes are lymphatic system structures that are the primary site for the development of protective immune responses, including antibody responses and cellular immune responses that work via filtering and releasing immune cells to and from tissues. Regardless of location, lymph nodes are primarily characterized to occur embedded in adipose tissue depots [50]. Lymph nodes embedded in and attached to adipose tissue continuously survey and monitor exposure of adipose tissue to potentially harmful pathogens and metabolites [51, 52]. Various

immune cells within lymph nodes can be recruited and activated to defend adipose tissue against tissue damage, toxicity or impaired function [126]. Despite this association the relation between the adipose tissue and lymph node regulation is underemphasized. To accurately understand the pathogenic mechanisms that lead to the prolonged state of inflammation induced by obesity the role of the lymphatic system and adipose tissue depot crosstalk must be elucidated.

Though research is not yet extensive in lymph node and adipose tissue crosstalk, seminal studies indicate that obesity-induce immune dysregulation, chronic pro-inflammation, is likely due to fundamental alterations that occur in lymph node morphology, immune cell populations and immune response. Kim *et al.* demonstrate that the adverse inflammatory profile specific to visceral adipose tissue accumulation, but not subcutaneous, may be due to site-specific dysregulation of lymph nodes. First, they demonstrate that diet-induced adipose tissue accumulation reduces the weight of mesenteric, but not subcutaneous, lymph nodes [127]. The decrease in visceral lymph node mass is associated with a decrease in lymphoid cell number which is a result of activated T cell death, a measure the was ~4 fold higher in high fat diet fed mice than control [127]. This group proposed that various obesity-induced mediators (*e.g.* free fatty acids, oxidative stress, and inflammatory cytokines) that are released at a greater rate from mesenteric adipose tissue than subcutaneous cause greater immune cell apoptosis within this region [127]. They further propose, but do not test, that metabolic endotoxemia-induced lymphocyte death, increases in circulating lipopolysaccharide (LPS) due to enhanced gut permeability, could mediate metabolic consequences associated with differential adiposity distributions. This topic, gut-adipose tissue crosstalk, will be discussed more below. Weitman *et al.* also provide studies that support the postulate that impairment of the lymphatic system may be involved in the pathology associated with obesity. They examine if obesity impairs lymphatic fluid transport and consequently dendritic

cell migration. They analyzed lymphatic flow by microlymphangiography via dermal lymphatic vessels and determine lymphatic flow to be delayed ~3 fold in obese mice compared with lean [52]. In addition, overall uptake, drainage and transport of interstitial fluid is decreased in these obese mice [52]. Taken together these alterations are associated with lymphatic fluid stasis, a condition of inhibited lymph flow that subsequently exacerbates disease risk due to decreased immune defense. Consistent with this dendritic cell migration was decreased ~5 fold in obese mice [52], indicating that compared with lean animals obese mice are prone to dysregulated cell-mediated immunity because of disturbances between dendritic and T cell populations. This, in turn, can exacerbate the deleterious cycle of chronic low grade inflammation because a proper immune response cannot be produced if antigen-presenting cells (dendritic cells) cannot interact with T cells that tailor immune responses to those antigens. Unlike Kim *et al.* the Weitman group did not investigate regional differences in lymphatic flow, hence it remains unknown if these changes are exacerbated within the visceral cavity and play a role in the immune dysfunction associated with visceral obesity. However, Weitman *et al.* replicated that obese mice have smaller lymph nodes and further extended previous studies by establishing these smaller nodes are associated with abnormal morphology [52]. Specific mechanisms for obesity-induced lymph node atrophy remain unclear.

These above studies suggest that the lymphatic system, that drains tissues including, but not limited to, inflamed adipose tissue, plays a role in obesity-induced inflammation. These studies also suggests that diet-induced obesity can differentially influence lymph node regulation according to location. Diet-induced obesity affects lymphoid tissues which, in turn, will also directly impact the development of immunity, including immune responses to pathogens, infections, cancer and vaccines in obese individuals. Specifically, the inability to alleviate

inflammation, as occurs in obesity, has been show to play a role in chronic diseases such as such as type 2 diabetes [128], immune-related diseases [129-132], and reduced effectiveness of vaccines [133]. Obesity impairs the ability to mitigate inflammation, however, the factors involved in this impairment are poorly understood and to our knowledge, the interaction between obesity and lymphatic system immune responses has received very little attention in the field of obesity research.

Obesity is not only considered a precursor for a number of chronic diseases but is also associated with an increased risk for poorer prognosis in many immune-mediated conditions [129-132]. Numerous studies in humans demonstrate that obesity attenuates host defense [133-136], thus increasing susceptibility to infections. In particular, data collect during the 2009 influenza outbreak demonstrated that obese individuals were at greater risk for infection caused by morbidity and mortality [137]. Obese individuals are also characterized by an impaired responsiveness to influenza vaccination [133]. Hence, it is important to understand how the immune system, specifically the lymphatics, contributes to obesity-induced inflammation because it will begin to address why obese individuals are more susceptible to disease.

Gastrointestinal tract

Traditionally, inflammation originating from adipose tissue was thought to be the fundamental initiator of the metabolic disease associated with obesity, but emerging evidence suggests the intestines contributes greatly to the development of metabolic disease [138]. The small intestine is the first interface between the body and diet, consequently this region is the first exposed to excessive and detrimental nutrients ingested such as excessive sugar and fat intake that leads to increased adiposity. Because of this the gastrointestinal tract is now emerging as initiator of the events that contribute to obesity-associated systemic inflammation. The underlying

mechanisms for this connection are currently being elucidated and thus far are contributed to Western diet-induced alterations in gut microbiota and epithelial barrier disruptions, increase gut permeability, that increase endotoxin release.

Reduced microbial diversity and altered bacteria phylum ratios are associated with the metabolic syndrome phenotype [139]. However, research is just now distinguishing if changes in gut microbiota prompt the low grade chronic inflammation associated with obesity or if alterations in gut microbiota are a secondary consequence of obesity. Research thus far demonstrates that gut microbiota contributes to obesity-induced inflammation via regulation of adipose depot stores. Specifically, the gut microbiota is a factor that regulates adipose tissue storage via enhance lipid release from the liver and adipocyte lipid uptake [140] as well as increased energy harvested from diet driven by microbiota derived metabolites [141]. Therefore, high fat diet-induced alterations in gut microbiota play a role in obesity-induced inflammation via exacerbation of adipose tissue accumulation. In addition, emerging research demonstrates that high fat diet-induced alterations in gut microbial composition play a role in inflammation of the intestine that likely leads to inflammation associated with obesity. More specifically, obesity prone rats exhibit alterations in microbiota, explicitly an increase in enterobacteriales (lipopolysaccharide associated species), along with increases in activation of toll-like receptor 4 (TLR4) and intestinal inflammation compared with obese resistant rats [142]. The previous factors where demonstrated to be the necessary initiators for the development of the obese-prone phenotype [142]. This study and others [143] support the postulate that alterations in gut microbiota leading to increased intestinal inflammation and bacterial translocation are fundamental in the development of chronic low grade inflammation associated with obesity.

Overall, there is a strong link between high fat-induced alterations in gut microbiota, increases in harmful gut bacteria, gut inflammation and inflammation observed in obesity. Gut microbiota component lipopolysaccharide (LPS) an endotoxin, the main constituent of the outer membrane of Gram negative bacteria, plays a fundamental role in obesity-induced chronic low grade inflammation because it causes immune system activation. LPS is a major inducer of the inflammatory response and is increased in blood circulation following chronic fat ingestion because, as described above, diets high in fat cause increased gastrointestinal permeability and bacterial translocation (increased gut leakage) [144]. In obesity adipose tissue endotoxaemia triggers an innate response that causes the release of proinflammatory cytokines, hence exacerbating chronic low grade inflammation already produced by excessive adipose tissue accumulation.

Taken together, obesity-induced chronic low grade inflammation development is attributed to and exacerbated by the deleterious cycle between dysregulated gut and adipose tissue. As discussed above it is recognized that high fat diet-induced alterations in gut microbiota, altered gut metabolite release, gut inflammation and increased gastrointestinal permeability are fundamental drivers of the dysregulation. However, research suggests that high fat diet-induced intestinal inflammation does not just manifest chronically due to altered gut microbiota, but can also occur acutely due to direct effects of the diet. First, it has been demonstrated in rat epithelial cells that nutrient absorption of long chain fatty acids modulates intestinal mucosal immunity via enhancing cytokine release specific for neutrophil migration [145]. Thus excessive exposure of long chain fatty acids cause intestinal epithelial cells to release certain cytokines that cause migration of first responder phagocytes, neutrophils, to defend against the insult and activate other immune cells. Being persistently immunologically challenged, the epithelial cells of the gastrointestinal tract are

integrated with immune cells. As such, the gut is characterized to contain 70% of the body's immune cells which play a role in maintaining homeostasis of the gut [146]. These immune cells found along the epithelium of the intestine, intraepithelial lymphocytes (IEL), and within peyer's patches, aggregated lymphoid nodules, release afferent lymph to the mesenteric lymph nodes that are surrounded by visceral adipose tissue (Figure 1). Much like intestinal epithelial cells, immune cells of the gastrointestinal tract are proposed to be capable of modifying immune function of the intestinal mucosa when directly exposed to fatty acids. *In vitro* long chain free fatty acids are demonstrated to alter interferon-Ɣ release from IELs collected from BALB/c mice [147] and suppress antigen presentation and chemotactic ability of dendritic cells [148]. *In vivo* fat absorption, specifically long chained fatty acids, in rats is demonstrated to activate intestinal mucosal mast cells and associated mediators [149]. In mice, a diet high in cholesterol causes accumulation of myeloid cells within the intestine within hours of exposure [150]. Overall, both intestinal mucosal epithelial cells and resident antigen cells are capable of directly detecting and inducing inflammatory responses to lipids such as cholesterol and long chain fatty acids. Because these intestinal immune populations drain to the mesenteric lymph nodes it is likely regional differences in lymph node immune cell can be driven by direct diet effect on the intestines and may play a role in the relation of adipose tissue distribution to metabolic disease.

Conclusion

Visceral adipose tissue surrounds, covers, attaches and shares vasculature with numerous abdominal organs, thus location of this depot is proposed to play a fundamental role in the co-morbidities associated with obesity, whereas subcutaneous adiposity is not. The increased link to obesity comorbidities appears to be due to the increased capacity of this depot to induce inflammation [120]. The next major advance in understanding the obesity epidemic will involve

an appreciation that not all fat pads are created equally and an understanding of the fundamental processes that regulate inflammation. Visceral adipose tissue surrounds and attaches to the mesenteric lymph nodes that collect lymph fluid and immune cells effluent from the gastrointestinal tract. Although lymph nodes are the nexus of the gastrointestinal tract and visceral adipose tissue, its role is often underemphasized despite being fundamental. In particular, little is known about regional lymph node induction of adipose tissue inflammation. We postulate that obesity-mediated changes in lymph node immune cells within adipose depots determine the extent and negative consequences associated with obesity-induced inflammation. The contribution of intestinal immune cell populations and cytokine release to regional adiposity risk has yet to be investigated. The lymphatic connection between the gastrointestinal tract and visceral adipose tissue permits early diet direct immune alterations in the intestine to be detected within mesenteric lymph nodes. Resulting changes in mesenteric lymph nodes can directly affect the adipose depot it resides in. Hence, high-fat diet that modulates immune functions in the intestinal mucosa produces gut-derived pro-inflammatory cytokines and associated immune cells, which will then directly contribute to alterations in cell populations within the visceral adipose depot. This location specific lymph node regulation likely drives the differences between the associated metabolic risk of the "apple" and "pear" shape. Figure 2 is summary of proposed mechanisms of visceral obesity-induced chronic low grade inflammation.

Figures

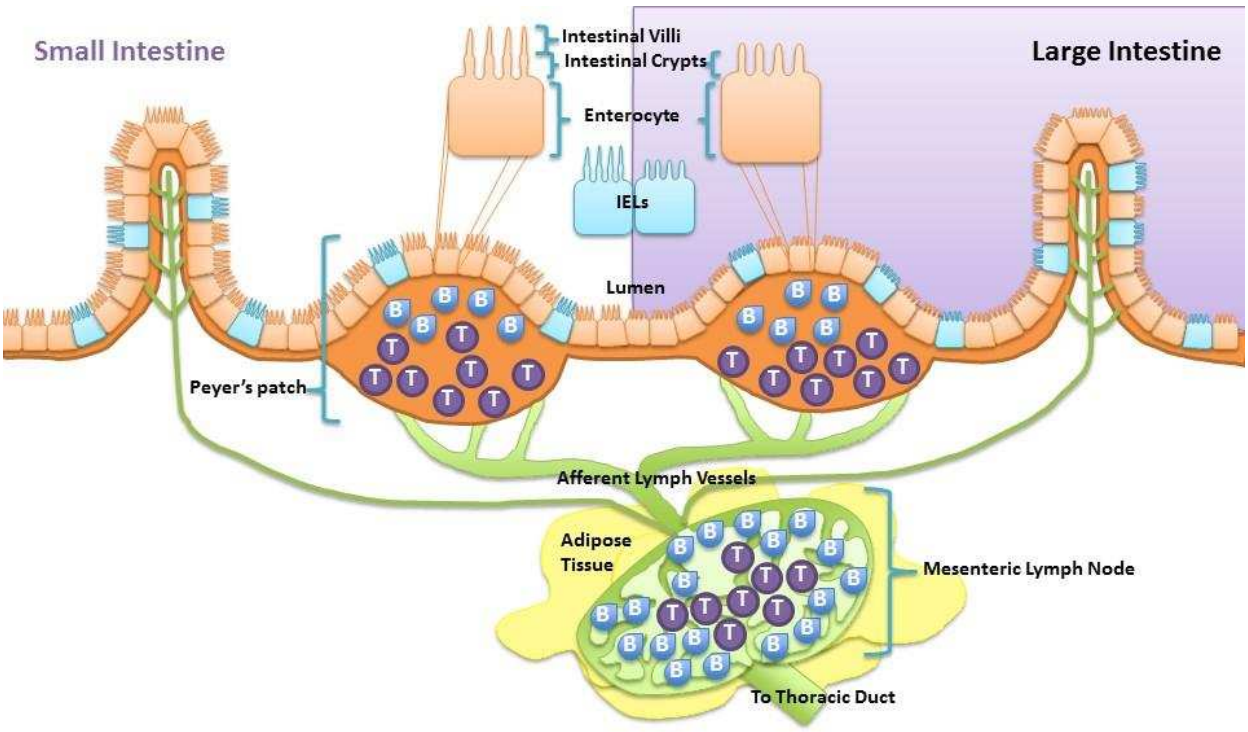


Figure 2.1 - Intraepithelial lymphocytes (IELs), Peyer's Patches and Mesenteric Lymph Nodes. Peyer's patches and IELs are present in both the small and large intestine and serve to monitor incoming food particles and microbes in the lumen of the gut. The cells present in these locations are a mixture of B, T and granulocyte cells (not shown). These cells through their monitoring of the gut environment serve to both coordinate and suppress immune responses depending upon the presence and or absence of pathogenic bacteria or inflammatory molecules. The lymphocytes that have encountered inflammatory stimuli can then be trafficked to the mesenteric lymph node housed in the peritoneal cavity to further educate the immune system and effect or suppress and immune response. This relationship between the IELs and immune cells from the Peyer's Patches have been implicated in the pathogenesis of obesity and its associated co-morbidities via priming the immune system for a chronic inflammatory response in the persistent state of what the body perceives as an injury. Subcutaneous lymph nodes are not associated with the same detrimental effects as the visceral lymph nodes due to the fact that they are not in direct connection with the gut.

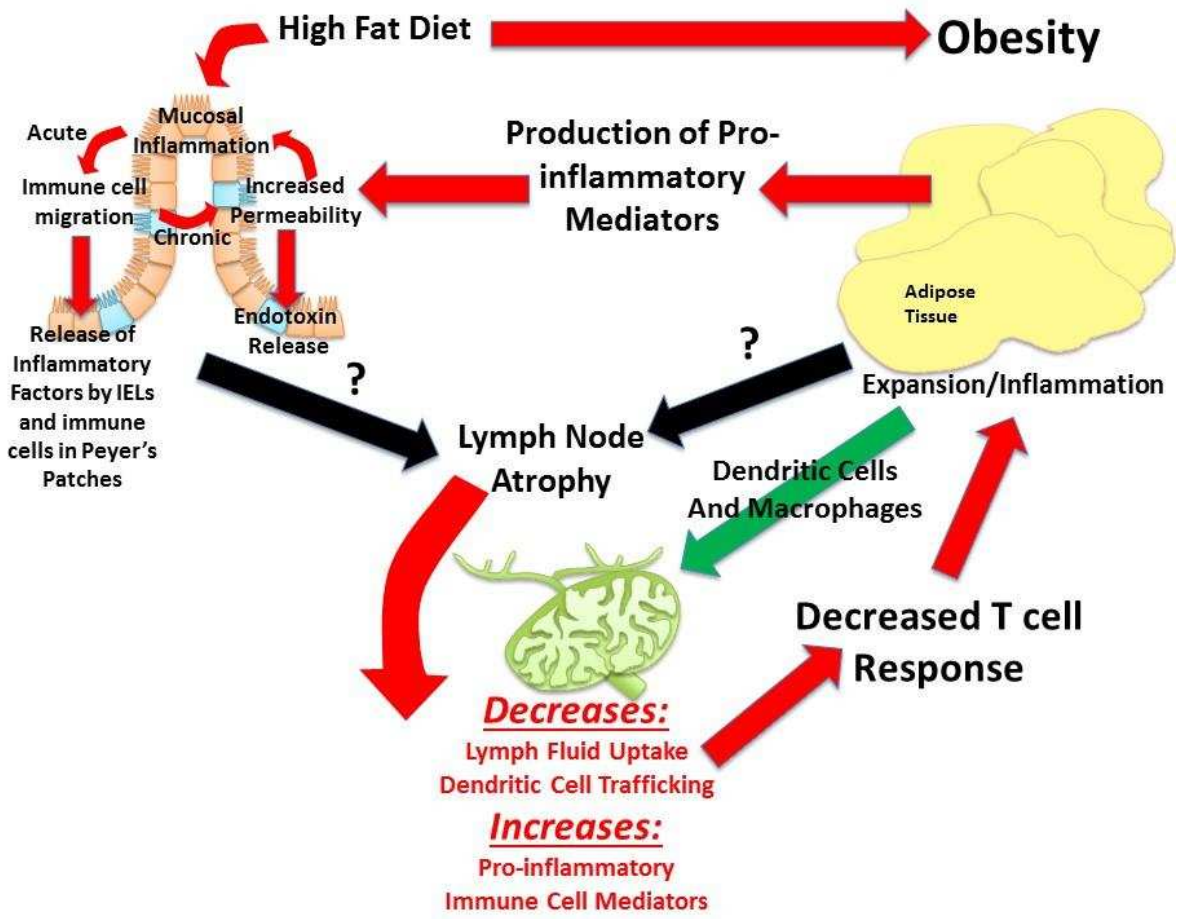


Figure 2.2 - Effects of inflammation on gut-adipose-lymph node system. Acute response to fats in the diet are the direct induction of inflammation within intestinal epithelial cells, IELs and other immune cells. Inflammation within gut cell populations likely alters immune cell response within mesenteric lymph nodes and consequently can alter immune response in visceral adipose tissue. Chronic response includes alterations in gut microbiota leading to increased gut permeability and LPS endotoxaemia-induced inflammation. As adipose tissue expands it becomes inflamed releasing pro-inflammatory mediators that are proposed to further influence gut permeability negatively. This increased permeability causes a release of endotoxin from the gut into the peritoneal cavity. The endotoxin in conjunction with inflammatory factors from the adipose tissue may drive lymph node atrophy. Other factors that may contribute to this not show in the diagram are hypoxia and free fatty acids. The atrophy of the lymph node impairs ability to absorb extracellular fluids and receive cells, such as dendritic cells and macrophages, trafficked from the site of a perceived injury, in this case the inflamed adipose tissue. These dendritic cells and macrophages are immune mediators and prime other cells, mainly T cells to respond to the injury. However, due to the decreased immune cell uptake during lymph node atrophy, the immune systems response is blunted allowing for the injury to persist and the inflammatory cycle to continue. The endotoxin from the gut can also prime IELs and Peyer's Patches to release more inflammatory factors that can negatively impact other organ systems

CHAPTER 3: OMENTECTOMY AS A PROCEDURE FOR THE CO-MORBIDITIES OF OBESITY[4][5]

Summary

Adipose tissue distribution is an important determinant of obesity-related comorbidities. It is well established that central obesity (intra-abdominal/visceral adipose tissue accumulation) is a risk factor for many adverse metabolic outcomes such as dyslipidemia, hypertension, insulin resistance and type-2-diabetes. Research demonstrates a highly associated link between intra-abdominal (visceral) adipose tissue inflammation and metabolic syndrome, thus the omentum has become an adipose depot of key interest. Indeed, it has been proposed and demonstrated that resection of the omentum, omentectomy, ameliorates metabolic dysregulation, but the results in humans are currently controversial. Despite these controversial results, there is a lack of knowledge about the basic physiological alterations that may occur with removing the omentum in obese or non-obese individuals. It is well established that metabolic improvements following omentum resection are, in part, due to alterations in adipokines (adipocyte hormones that act via autocrine, paracrine, and/or endocrine mechanisms) that were previously dysregulated. Yet, the omentum also contains lymphatic tissue that directly communicates with adipocytes. The role, however, of the lymphatic system/lymph node alterations are underemphasized in omentum resection in obese individuals. In addition to adipose tissue storage the omentum is proposed to function as a

[4] A modified version of this chapter is published as Magnuson, A., A. Booth, and M. Foster, *Omentectomy as a procedure for the co-morbidities of obesity*. 2014. 1-29.

[5] My contributions to this work include the writing of several sections as well as overall review of the article for accuracy.

peritoneal surveillance for disease and absorber of foreign particulates in peritoneal fluid. For this reason, the inflammatory basis of metabolic syndrome is emerging as interplay between immunological/lymph and adipose tissue. This overview will discuss obesity-induced dysregulation of adipose tissue and lymphatics as well as omentectomy/visceral lipectomy as it pertains to human and rodent research with specific focus on visceral adipose tissue and lymphocyte regulation.

Obesity-induced adipose tissue dysregulation

Following a harmful stimulus, acute inflammation is the first line of defense in the body's healing process, which consists of a protective immune response to remove damaged cells. It is most commonly characterized by redness, swelling, heat and pain and typically lasts a few days to a week. If the initial inflammatory insult is not resolved, the condition becomes chronic and can often lead to tissue injury. Obesity is characterized as a state of chronic low-grade systemic inflammation occurring as a consequence of intrinsic adipose tissue immune system activation that promotes production and release of pro-inflammatory cytokines [1]. More specifically, the adipose depot, comprised of adipocytes, stromal vascular pre-adipocytes and immune cells, is a dynamic tissue that quickly responds to alterations in nutrient intake (*i.e.* fasting and overfeeding) [2]. Although adipocyte hypertrophy and hyperplasia are typical responses to nutrient overload, accelerated growth or accumulation is recognized as a harmful stimulus and propagates an inflammatory response [3, 4]. This response is perpetuated by dysregulation of adipocytokines [5, 6]. Obesity-induced inflammation initiates locally within the adipose tissue depot, but spreads systemically to other organs in the body (*i.e.* liver, kidneys, muscle, etc.) [6]. Accordingly, obesity-induced low grade inflammation, in part, is a proposed link to numerous obesity associated co-morbidities such as type-2 diabetes, insulin resistance, dyslipidemia, non-

alcoholic fatty liver disease (NAFLD) and cardiovascular disease [7]. The inciting etiology of adipose tissue inflammation and subsequent dysfunction remains to be elucidated. Overall, evidence supports the role of an adipose tissue inflammatory response in the association between obesity and the pathogenesis of chronic metabolic diseases.

Adipose tissue hypoxia, insufficient oxygen supply to expanding and/or proliferating adipocytes, is a proposed inciter of obesity-induced inflammation. During seminal adipocyte expansion, angiogenic factors are increased and subsequent processes in vasculature formation/remodeling are enhanced. The extended vascular network allows uninterrupted delivery of oxygen and nutrients to expanding regions of the adipose depot. Excessive adipose tissue expansion, however, exceeds the availability of angiogenic factors and vasculature development resulting in a reduction of adipose tissue blood flow [8, 9]. Because oxygen diffusion within the adipose depot is limited, decreased blood flow results in hypoxia; this condition occurs in obesity-induce adipocyte growth and proliferation [10]. Insufficient capillary growth/decreased oxygen availability leads to adipocyte dysfunction comprising upregulation of injurious adipokine genes (e.g. TNFα, IL-6, Resistin) and downregulation of beneficial adipokine genes (e.g. adiponectin) and oxidative stress, adipose tissue fibrosis, and eventual cell death/apoptosis [10]. Overall obesity-induced alterations in vasculature function and availability influences adipose tissue homeostasis.

Data supports the hypoxia-induced adipose tissue inflammation postulate, but the direct mechanisms of these responses are not well understood. It has been proposed that adipose tissue hypoxia incites adipocyte dysfunction because it initiates the process of pro-inflammatory cytokine production and immune cell infiltration [11-13]. During obesity-induced inflammation, adipocytes secrete inflammatory cytokines that play a role in maintenance of metabolic

dysfunction. Major contributors include monocyte chemoattractant protein 1 (MCP-1), tumor necrosis factor-α (TNFα), and interleukin-6 (IL-6) [8]. MCP-1 serves as the primary recruiter of monocytes from blood vessels surrounding adipose tissue. These monocytes, at maturation are M1 macrophages that produce oxidative metabolites and pro-inflammatory cytokines in preparation for adipocyte defense [14]. Increased levels of TNFα, as occurs in obesity, are associated with dysregulation of glucose homeostasis and induction of insulin resistance [15, 16]. TNFα also has an inverse relationship to endothelial lipoprotein lipase activity thus it is associated with alterations in lipid metabolism that consequently results in dyslipidemia [17]. Lastly, studies demonstrate TNFα production mediates the production of some adipokines such as leptin and adiponectin [1, 16]. Adipose tissue-derived IL-6 is estimated to make up a third of the total circulating IL-6 in blood, secreted by both adipocytes and macrophages [18]. Unlike TNFα, which acts in an autocrine or paracrine fashion, IL-6 is an endocrine cytokine and plasma concentrations correlate positively with increased lipolysis and insulin resistance [19, 20]. Increased circulating levels of IL-6 are responsible for increased production of C-reactive protein (CRP) by the liver, a strong marker of metabolic risk linked to cardiovascular disease and type-2 diabetes [1].

Dysfunctional lipid metabolism is also an associated co-morbidity of obesity. Lipid dysregulation occurs when requirement for lipid storage exceeds the quantity of adipocyte space, thus perpetuating a milieu favoring inflammation. When lipids exceed adipocyte storage capacity dyslipidemia develops and is characterized by increased levels of triglyceride associated with lipoproteins in the blood [21]. Circulating excess lipid is then transported and ectopically deposited into tissues not designed for lipid storage, such as the liver, kidneys, and/or muscle. In addition, obesity is accompanied by a decrease in insulin signaling which affects multiple tissues

including adipocytes by reducing the anti-lipolytic effect of insulin [6]. Consequently, these adipocytes are less effective at storing dietary fatty acids and exacerbate the leak of lipids into the blood. Macrophage infiltration and inflammation-related gene expression, however, occur before the development of insulin resistance in animal models [13, 22-24]. Therefore, adipose tissue lipid dysregulation is not the causal basis of obesity-induced inflammation, but rather, a consequence that contributes to its perpetuation.

Obesity-induced adipocyte dysregulation also leads to disturbances in endocrine regulation of appetite and energy balance. The adipose depot releases biologically active molecules such as adipokines, chemokines, hormone-like factors and numerous other mediators [25]. Adipokines have been demonstrated to affect appetite and satiety, glucose and lipid metabolism, inflammation and immune functions as well as blood pressure [25]. The expression and release of a number of adipokines are increased in the adipose depot with obesity, thus adiposity is connected to dysregulated appetite and metabolism [26]. For example, leptin, a lipostatic signal whose expression directly relates to the proportion of adiposity, is increased with fat accumulation and serves as an anorexigenic signal indicating food intake can be decreased because of positive energy stores [27]. In obesity, leptin receptors in the brain become resistant to the anorexigenic effect of leptin, thus dysregulating appetite and satiety [28]. Other adipokines that play a role in obesity-induced alterations in food intake and regulation of body weight include resistin, visfatin, adipsin and retinal binding protein-4 [29]. In contrast, adiponectin acts as an insulin enhancer and has anti-atherogenic properties. When bound to its receptors, signaling pathways are activated that lead to insulin-sensitization, thus low levels are detrimental to normal glucose function [30]. Circulating adiponectin is decreased in obese individuals and

reduced adiponectin has been associated with dyslipidemia and atherosclerosis marked by an increase in CRP, linking the adipose signaling hormone to the inflammatory process [31, 32].

Regional adipocyte differences

Traditionally the role of adipose tissue was thought to be limited to storage and insulation. It is now well established that adipose tissue is an immune and endocrine organ that plays a pivotal role in energy homeostasis. The important function of the integrated adipose depot is emphasized by adverse metabolic consequences induced by excessive lipid accumulation. Obesity-related adverse health consequences occur in individuals with predominant upper body adipose distribution [33-37]. This adipose tissue accumulation specifically occurs in the abdominal cavity among organs and is associated with metabolic disorders such as dyslipidemia [38], hypertension [39, 40], insulin resistance and type-2-diabetes [41, 42]. In contrast, increased lower body subcutaneous adipose tissue is associated with a reduced risk of metabolic complications [43] as is upper body subcutaneous fat [44-46]. The mechanisms for this depot distribution and metabolic outcome connection remain to be elucidated, however the proposed mediators commonly attributed to adipocyte dysregulation are excessive accumulation (as previously discussed), location and/or adipose depot intrinsic characteristics.

Intra-abdominal adipose tissue, also known as visceral, includes two distinct depots the mesenteric and omental (Figures 3.1 and 3.2). These two central depots are presumed to predispose individuals to adverse health consequences due to their anatomical site that permits venous drainage to the liver via the portal vein; *i.e.,* insulin-sensitive hepatocytes are directly exposed to the metabolites and secretory products released by visceral adipocytes [47-49]. Hence, an increased volume of visceral fat, and subsequent release of fatty acids, glycerol and

lactate in addition to numerous adipokines and pro-inflammatory cytokines deposited directly into the portal vein would be expected to have a major influence on these hepatic processes.

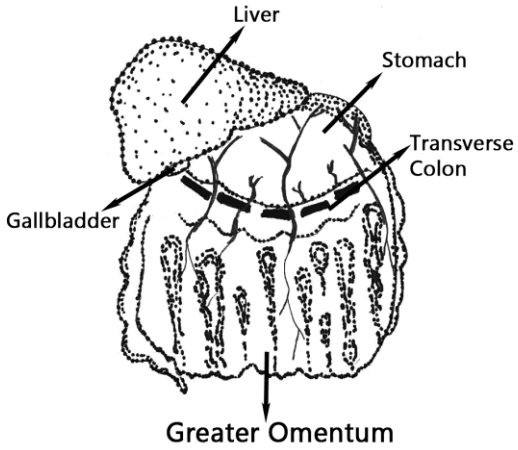


Figure 3.1 - Human Omentum - Omental adipose depot connects the stomach to adjacent organs. The greater omentum, described as “an apron”, is suspended from the greater curvature of the stomach and proximal duodenum and extends to the small bowel. The dashed bars represent area of excision for full omentectomy removal.

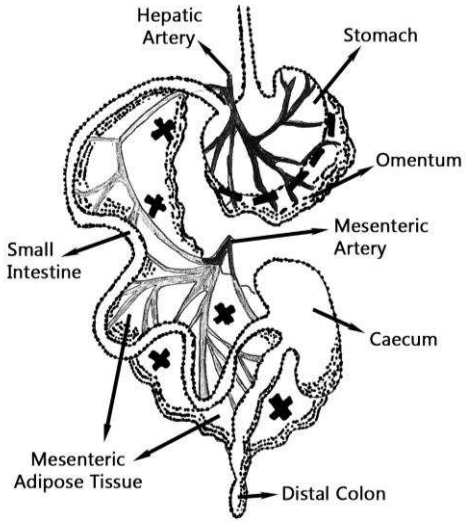


Figure 3.2 - Rodent Visceral Depots (Mesenteric and Omental) – The omentum, suspended from the greater curvature, is relatively small in rodent models and does not connect organs within the abdominal cavity. The mesenteric adipose depot is contained within the mesentery peritoneum that encloses the jejunum and ileum and connects the intestines to the dorsal abdominal wall. The dashed bars represent area of excision for full omentectomy removal, while crosses represent specific sites of mesenteric adipose tissue excision that avoid nerve innervation, vasculature and lymph nodes.

It is well established that lipid influx is an important factor in visceral effluent that influences hepatic processes. Indeed, primary development of insulin resistance is a proposed consequence

of enhanced fatty acid release, following excessive visceral adipose tissue accumulation, to the liver via portal circulation. Elevated free fatty acid flux to the liver promotes liver gluconeogenesis [49, 50], reduces enzymes involved in fatty acid oxidation and increases fat storage and synthesis in the liver [51-53] and insulin resistance [53] while decreasing hepatic insulin binding and degradation [54]. These alterations result in systemic hyperinsulinemia [55], attenuation of insulin suppression of hepatic glucose production (*i.e.* hepatic insulin resistance) [56] and ultimately facilitate hepatic glucose production by providing a continuous source of energy and substrate [56]. Fatty acid-induced dysregulation of insulin-regulated pathways ultimately perpetuates the metabolic effects of obesity by increasing dyslipidemia [57] and ectopic lipid accumulation in the liver [55, 58]. Overall, this suggests that marked central adiposity is one of the main determinants of insulin resistance. This is the basis of Randle's portal/visceral hypothesis which states that increased adiposity, particularly in visceral depots, leads to greater fatty acid flux and inhibition of insulin action in insulin-sensitive tissues [59]. Adipose tissue depots are inherently distinct in structure and metabolic characteristics and these factors create another delineation between the metabolic effects of native adipocytes within the visceral and lower body subcutaneous adipose depots. Adipose tissue depots exhibit differential regulation of triglyceride [60] and fatty acid turnover [61]. These differences are driven by lipolysis rate and adipocyte responsiveness to insulin. When compared to subcutaneous, visceral adipocytes have higher rates of catecholamine-induced lipolysis [62, 63] and express higher numbers of beta adrenergic receptors [64, 65]. Visceral adipocytes are also less responsive to the anti-lipolytic effect of insulin than subcutaneous [66-68] with visceral fat having a lower binding affinity for insulin [69] and reduced insulin receptor substrate (IRS)-1 protein expression [67].

Overall, the lack of sensitivity to insulin can further enhance fatty acid flux to the liver in individuals with visceral obesity.

The differentiation capacity of adipocyte precursor cells varies regionally. Total adipocyte number is regulated via the ratio of apoptosis and development of new cells. As with FA turnover, adipocytes also display a depot-specific susceptibility to apoptosis. More specifically, visceral adipose tissue *in vitro* is more susceptible to tumor necrosis factor (TNF-α), an apoptotic stimulus, than subcutaneous fat [70]. Conversely inhibitors of TNF-α mediated cell death is also expressed at higher levels in visceral than in subcutaneous adipocytes [71]. Visceral adipose tissue also exhibits differences in preadipocyte cell number and differentiation capacity. For example, preadipocytes in confluent cultures from subcutaneous adipose tissue have greater differentiation capacity than those from visceral depots [72]. In sum, visceral fat is characterized by a reduced capacity for differentiation [72] and increased susceptibility to apoptotic stimuli [70] compared with subcutaneous which are factors that could exacerbate an inflammatory environment.

Other intrinsic differences between visceral and subcutaneous adipose tissue include adipokine and cytokine expression, depot configuration and extrinsic factors. More specifically, gene expression [73-75] and release [76, 77] of leptin and adiponectin (adipokines previously discussed) is higher in subcutaneous adipose tissue than visceral. In opposition, the visceral depot exhibits an enhanced inflammatory profile with increased cytokine expression, specifically IL-6, IL-8, PAI-1, MCP-1 and Visfatin, compared with subcutaneous (for a review see ref [78]). Differences in depot characteristics also extend to adipocyte architecture, connective tissue, and additional cells such as macrophages and immune and stromovascular cells within the depot [79, 80]. Extrinsic factors including, but not limited to, vasculature/angiogenic capacity,

innervation/lipolysis drive and lymphatic/immune stimulation are once again higher in the visceral depot, which is likely due to intestinal/gut proximity [81, 82].

Omentectomy as a treatment for obesity

Visceral adipose tissue consists of two depots, the mesenteric and the omental. The mesenteric adipose depot is contained within the mesentery peritoneum that encloses the jejunum and ileum and connects the intestines to the dorsal abdominal wall. The omentum peritoneum (Figure 1) that encases the omental adipose depot connects the stomach to adjacent organs. Peritoneal extensions of this region are divided into greater and lesser portions. The lesser peritoneal connects the lesser curvature of the stomach and proximal duodenum with the liver, whereas the greater peritoneal is suspended from the greater curvature of the stomach and proximal duodenum and extends to the small bowel. Unlike the lesser omentum and mesenteric peritoneal, the greater omentum, often described as “an apron”, is mobile and freely moves around the peritoneal cavity. This abdominal sheet is composed primarily of adipose tissue, but also contains gastroepiploic and lymphatic vessels.

As previously discussed visceral adipose tissue accumulation, mesenteric and omental, is highly associated with the co-morbidities of obesity unlike lower body subcutaneous adipose deposition which is suggested to be “protective”. Hence visceral removal in obese subjects could attenuate obesity co-morbidities such as insulin resistance, inflammation and glucose intolerance. The mesenteric depot in humans, however, cannot be surgically removed without risk of complications [83]. In contrast, the omentum because of its anatomical location can be removed with ease. The omentum, previously thought to perform no important functions, plays a role in energy regulation by influencing glucose and fatty acid metabolism.

Omentectomy (*i.e.* removal of all or part of the greater omentum and its constituent fat) (Figure 1) has unclear physiological implications in humans. Whereas some human studies demonstrate omental fat removal improves insulin action [83, 84] in obese patients, others observe no alterations [85, 86]. The conflicting results are likely due to inherent experimental limitations and individual variation between humans. For example, the omentectomy procedure is rarely individually executed and is often associated with gastric bypass or banding or intestine removal [83-86]. Thus the accelerated weight loss due to the additional procedures could mask the effects of omental fat removal. Other factors that may contribute to these contradictions include variations in pre- and post-operative BMI or body mass, amount of adipose tissue removed, age, sex, and duration of study.

In rodents, selective reduction in intra-abdominal adipose tissue improves metabolic profile. Specifically, intra-abdominal lipectomy (adipose tissue removal) reverses insulin resistance and glucose intolerance in obese, aged and young rodents [87-92]. Most rodent studies, however, are an inadequate representation of human omentectomy. First, in rodent models, the removed intra-abdominal adipose depot is attached to the reproductive organs rather than to portal drainage to the liver [93] and no human equivalent. Second, the rodent omentum is not equivalent in size to the human omentum; it is minuscule, often negligible in lean animals. Still, visceral lipectomy in rodents is a useful tool to investigate whether visceral obesity is a consequence or a contributing factor of the co-morbidities of obesity. Despite the omental difference, human omentectomy can be mimicked in the rodent by excising the whole omentum and portions of the mesenteric depot (Figure 2) [94]. In the latter, care must be taken to avoid removal of vasculature, lymph drainage or nerves that innervate the intestine. Visceral adipose tissue (*i.e* omental and mesenteric) removal in rodents improves glucose tolerance and reduces

liver triglyceride storage [94]. Deleterious effects of an expanding visceral depot are proposed to be a result of the visceral effluent, such as free fatty acids and adipocytokines, which affect insulin-sensitive hepatocytes [47-49]. Despite the location of intra-abdominal adipose tissue removal, these studies demonstrate that free fatty acids and adipo/cytokines play prominent roles in fat removal-induced improvements in insulin signaling and glucose homeostasis [87-92]. The long-term impact, however, of visceral adipose tissue removal in rodents and humans is currently unknown.

Although omentectomy is a procedure of interest for obesity treatment it is most commonly utilized in many cancer-staging procedures (pathological evaluation to determine the severity of cancer). These include, but are not limited to, primary peritoneal, ovarian, fallopian tube and gastric cancers [95-97]. Despite the vast use of omentectomy in abdominal cancer procedures or its investigational use as a potential treatment for obesity the long-term post-operative consequences remain unknown. Although necessity may outweigh the potential negative consequences of greater omentum removal in cancer patients it is essential to understand effects of this surgery in relation to the co-morbidities of obesity.

The omentum and immunity

Visceral adipose tissue accumulation is highly associated with greater risk for metabolic and cardiovascular disease, thus its removal may reverse these pathological conditions. Omentectomy, however, removes more than just visceral adipocytes. The omentum, which is bathed in the peritoneal fluid, is proposed to be a “peritoneal surveillance system” which serves to shield and limit abnormalities in the intra-abdominal cavity such as infectious and inflammatory processes and neoplastic intraperitoneal dispersion. This protective role, however, is often underemphasized in obesity treatment omentectomy where the outcome measurements

focus primarily on glucose tolerance, insulin sensitivity, circulating lipids, weight/BMI and adipocytokines. There has been little consideration to the long-term effects of omentum removal on immunity homeostasis.

The omentum is a physiologically dynamic tissue with therapeutic potential. It predominantly consists of adipose tissue, but also contains blood vessels and lymphatic/cellular tissues that are an integral part of the immune system defending the peritoneal cavity. The omental lymphatic system helps to clean and maintain proper function of organs in the abdominal cavity via absorbing edema fluids and removing metabolic waste and toxic substances (i.e. bacteria or other contaminants that enter the cavity from the small intestine and colon) [98]. Within the fatty omenta are capsule encased lymph nodes and numerous secondary lymphoid opaque structures called milky spots [99]. The lymph node is split into three compartments; the cortex, paracortex, and medulla. Housed within these three compartments are lymphocytes, B and T cells, antigen presenting cells and macrophages (Figure 3) [100]. Here the naive B and T cells become mature and undergo clonal expansion after interacting with antigen presenting cells and subsequently defend against infection and/or neoplastic intraperitneal dispersion [100]. Mature B-lymphocytes primary action is the release of antibodies (IgG, IgM, IgA, IgE, and IgD) that bind foreign antigens; each B cell produces a single programmed recognition reaction out of hundreds of millions of possibilities [40]. T-cells, unlike B cells, do not recognize full antigens, but rather fragments of antigens present on the surface of damaged cells (*i.e.* those infected by virus or cancerous cells). T-cells also differ from B-cells by their ability to enter cells infected or infiltrated by an infectious agent [40]. The T-cell population can be further subdivided relative to primary function. For example, there are those that direct immune response such as the T-lymphocytes and T-helper cells and others that directly attack infected or mutated cells like

cytotoxic T-lymphocytes and natural killer cells. A host of other cells are also involved in the generalized immune response such as granulocytes and mast cells [40]. Immune cells contained within lymph nodes are distributed throughout the body via lymphatic veinules, a bidirectional transport network carrying lymph fluid out and inflammatory mediators, antigenic materials and antigen presenting cells in [100].

As previously mentioned the omentum also contains secondary lymphoid structures called milky spots [99, 101]. These spots primarily consist of macrophages (immune cells that ingest or phagocytize antigenic material), but also include T-lymphocytes, stromal and mast cells and a site-specific subset of B lymphocytes [102, 103]. Hence the populations of cells within the milky spots differ from those that are found in the lymph nodes. Milky spots, regarded as a main source of peritoneal macrophages [104], primary function is to clear particles, bacteria and tumor cells from the peritoneal cavity [105-107]. Macrophages within milky spots, yet to encounter foreign antigenic material, remain quiescent until an antigen is introduced. Once activated these macrophages present the antigens to naïve lymphocytes to initiate maturation. Accordingly the lymph node is the site of maturation for lymphocytes that interact with antigen presenting cells, thus macrophages present antigens that can then be recognized by lymphocytes in the immune response [65, 66]. Once these interactions occur, the lymphocytes travel out of the lymph node and circulate throughout the body and peritoneal cavity [99]. In general milky spots are a collection area for antigens that allows fast exchange of quarantined foreign material and initiation of immune response. They differ from lymph nodes in development, morphology and immunological properties [99, 108] and are specifically suited for migration of leukocytes and rapid movement of fluids.

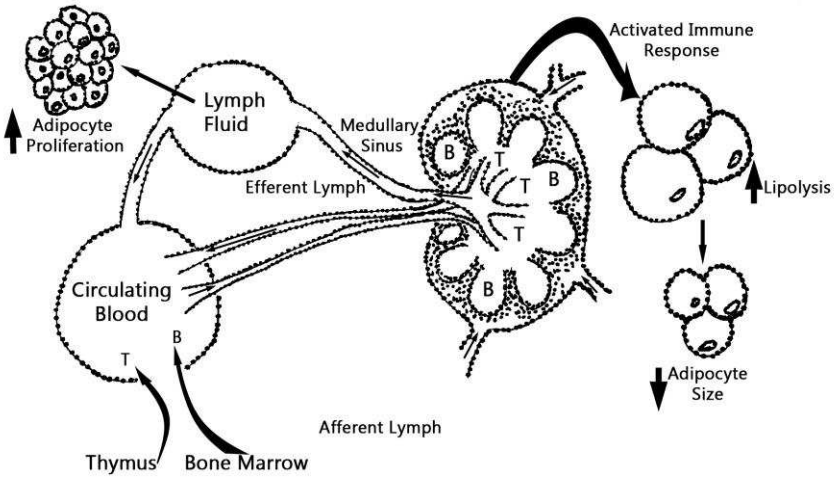


Figure 3.3 - Adipose Tissue-Lymph Node Crosstalk – T and B-lymphocytes produced in the thymus and bone marrow enter peripheral lymph tissue via blood circulation. Once in the lymph node naive B-lymphocytes reside in the primary follicles of the cortical lymphoid follicles, whereas T cells reside mainly in the paracortical area. In the presence of an activated immune response energy is derived from free fatty acids released from adipocytes following an increase in adipose lipolysis. This ultimately results in a decrease in perinodal adipocyte cell size. Lymph fluid has been demonstrated to increase adipocyte proliferation.

Adipose tissue - lymph node crosstalk

As previously discussed virtually all mammalian lymphatic vessels and nodes are in close spatial association with adipose tissue [109]. Adipocytes in close proximity to lymph nodes, termed “perinodal”, are found roughly within a 2mm radius [110]. Adipocytes further out are designated “middle”, 5-10mm from node, or “outside” at the distance of 10mm or greater. Perinodal adipocytes have been demonstrated to be a reservoir of energy for lymphatic vessels and structures needed to deploy an immune response [111]. Both lymphocytes and tissue-derived dendritic cells utilize this energy from fatty acid metabolism (Figure 3.3). Dendritic cells that increase in number following chronic immune stimulation/inflammation [112] are a specific set of antigen presenting cells that function to activate specific immune responses involved with T-cells [113]. Studies demonstrate that chronic inflammation of lymph nodes, immune stimulation, increases lipolysis in perinodal adipocytes and subsequently decreases adipocyte cell size [109]. This local interaction with adipocyte liberated lipids, free fatty acids, subsequently nourish

and/or regulate lymphocytes. In contrast, immune stimulation has also been demonstrated to increase perinodal adipogenic activity. Indeed, chronic inflammation of lymph nodes in rats increases the number of adipocytes surrounding the nodes [114]. Lymph fluid can also promote differentiation of preadipocytes [115]. For example, abnormal lymph leakage due to disruption in lymphatic vascular integrity promotes ectopic growth of adipose tissue due to increased lipid storage in adipocytes and increased differentiation of preadipocytes [116]. Another study observed ectopic adipose growth in edematous regions of individuals with chronic lymphedema [117]. Overall, adipocytes that surround lymph nodes are actively involved in the transient immune response, explaining the spatial relationship of adipose tissue to lymph vessels.

The healing omentum

The role of the omentum in peritoneal defense is, in part, due to immunity, but also a supportive role in repairing tissue damage. The omentum has the ability to adhere to sites of inflammation on adjacent organs and subsequently destroy pathogens and repair damaged tissue [98, 102]. Indeed, the omentum is a source of growth factors, neurotransmitters, neurotrophic factors and inflammatory mediators that help to promote tissue regeneration [98, 118]. The healing qualities are due to stem cell production within this tissue permitting subsequent tissue regeneration of surrounding organs after damage occurs [118]. Based on its inherent defense mechanisms and its angiogenic ability to help seal off and repair damaged or infected tissues the omentum has potent healing properties and for this reason has been used by surgeons for some time. Clinically, cells from the omentum have been used for healing and tissue regeneration in procedures such as, but not limited to, gastrointestinal [119], neuro- [120], cardiothoracic [121], gynecological [122] and urological [123] surgeries. Omental fixation or wrapping to cover or surround tissue is an effective method of minimizing post-operative complications, surgical

morbidity or infection while enhancing vascularization and tissue restoration. Omentum interposition has wide therapeutic application in many branches of surgery.

Implications of omentum removal in obesity

The omentum is a versatile organ demonstrated to play a role in peritoneal defense by way of antigen removal and tissue repair. The importance of this role, however, is underemphasized in obesity-treatment surgeries (*i.e.* bariatric surgery) with concomitant omentum removal, instead the primary focus is placed on adipocyte dysregulation. Although adipose tissue hypoxia is proposed to incite obesity co-morbidities, growing evidence suggest the small intestine, the first interface between the body and diet, contributes greatly to the development of metabolic disease [124]. Indeed, the contribution of gut microbiota, microorganisms in the digestive tract, is gaining increased recognition. Gut microbiota component lipopolysaccharide (LPS), the main constituent of the outer membrane of Gram-negative bacteria, causes immune system activation. LPS, an endotoxin, is a major inducer of the inflammatory response and is increased in blood circulation following chronic fat ingestion because diets high in fat cause increased gastrointestinal permeability (increased gut leakage) [125]. In the peritoneal cavity endotoxaemia triggers an innate response that causes the release of pro-inflammatory cytokines that subsequently interferes with whole body glucose and lipid metabolism. Although lymph nodes are the nexus of the gastrointestinal tract and adipose tissue, its role is often underemphasized despite being fundamental.

As previously discussed, central obesity is highly associated with metabolic dysregulation. Proposed fundamental obesity-induced triggers or aggravators include adipose depot increases in deleterious adipokines, cytokines, free fatty acid leakage and inflammation. Recent studies also demonstrate peritoneal lymphatics play a role in the development of obesity-

associated co-morbidities. For example, diet-induced and genetic obesity increases lymph node associated T-lymphocytes [126, 127], mast cells (immune cell with granules that contain histamine and heparin) and immune cell apoptosis [128]. Some, however, demonstrate obesity induces decreases in T-lymphocytes while exacerbating immune responses by enhancing T-cell activation [129]. Increased lymph node mast cell density may further incite exacerbation by activating lymph nodes [130] and facilitating the recruitment of T-lymphocytes [131]. These chronic changes ultimately lead to apoptosis of crucial cells that play a role in immune homeostasis. Indeed, lymphoid cellularity of diet-induced obese mice declines as mesenteric adipose tissue accumulates resulting in atrophy of lymph node structures [132]. Alterations of lymphatic tissue and associated cells are proposed to be initiated by the surrounding microenvironment that is dictated by adjacent adipocytes and peritoneal fluid.

In obese humans, omentectomy with and perhaps without bariatric surgery, may reverse metabolic co-morbidities in a multifaceted way. First, omentum removal decreases the amount of hypoxic visceral adipose tissue. Consequently, deleterious factors released from impaired adipocytes (*e.g.* adipocytokines and free fatty acids) within the omentum depot are no longer part of the effluent to the liver. Second, omentectomy also includes removal of dysregulated lymphatics, milky spots and lymph nodes, responsible for exacerbating the cycle of immune injury. Therefore, the removal of this one visceral depot alone may be adequate in reversing the co-morbidities of obesity, but success of this procedure may be dependent upon appropriate food intake and maintenance of a healthy weight. Specifically, if the omentum is dysregulated it is likely the other visceral depot, mesenteric, is too. Hence, if the omental depot is removed, but energy excess and need for adipose tissue storage continues, energy surplus will likely be shunted to the mesenteric depot. The mesenteric depot is susceptible to obesity-induced

alterations and like the omentum contains lymph nodes that can become impaired [133], however the progression of these events in human mesenteric is unknown. Yet, it is plausible that omentectomy without subsequent weight loss or reduction in calories would not attenuate the co-morbidities of obesity in the long-term because the mesenteric depot remains intact. The beneficial effects of omentectomy would be greater if combined with gastric bypass, assuring weight loss occurs and is maintained. Current human research is unclear about omentectomy as a procedure for the co-morbidities of obesity. Follow-up studies should investigate effects of adiposity distribution, specifically discerning the quantity of omental and mesenteric adipose tissue, and systematically observe mesenteric alterations (*e.g.* quantitiy) and circulating adipocytokine and endotoxin concentration following omental depot removal.

CHAPTER 4: DIET-INDUCED OBESITY CAUSES VISCERAL, BUT NOT SUBCUTANEOUS, LYMPH NODE HYPERPLASIA VIA INCREASES IN SPECIFIC IMMUNE CELL POPULATIONS[6][7][8]

Summary

Objectives

The spatial proximity of adipose depots to secondary lymph nodes allows a unique relation between the two systems. Obesity, predominately-visceral adiposity, links to numerous diseases, hence we postulate that secondary lymphatics within this region contributes to disease risk.

Material and Methods

Male C57BL/6 mice were fed standard chow (18% kcal fat) or Western diet (45% kcal fat) for 7 weeks. Visceral and subcutaneous lymph nodes and associated adipose depots they occupy were excised. Lymph node morphology and resident immune cell populations were characterized via histopathology, immunofluorescence, and flow cytometry. Adipose tissue immune cell populations were also characterized.

Results

Obesity caused lymph node expansion, increased viable cell number, and deviations in immune cell populations. These alterations were exclusive to visceral lymph nodes. Notably,

[6] This chapter includes the complete published manuscript for this research titled *Diet induced obesity causes visceral, but not subcutaneous, lymph node hyperplasia via increases in specific immune cell populations,* A.M. Magnuson, D.P. Regan, J.K. Fouts, S.W. Dow, M.T. Foster, *Cell Proliferation*, 2017. 50(5). My contributions to this publication included the bulk of the analysis, statistics, and significant contribution to laboratory procedures.

[7] Reuse of this article, both print and electronic, has been granted by John Wiley and Sons under license number 4219520782376, Oct. 31 2017.

[8] Table and figure numbers have been modified to reflect that they are specific to this chapter, e.g. Figure 1 is now Figure 4.1. Only minimal modifications were made to meet formatting requirements. No other modifications were made.

pro-inflammatory antigen presenting cells and regulatory T cells increased in number in the visceral lymph node. Obesity, however, reduced T regulatory cells in visceral lymph nodes. The visceral adipose depot also had greater reactivity toward HFD than subcutaneous, with a greater percent of macrophages, dendritic and CD8+ T cells. Immune cell number, in both the visceral and subcutaneous, however decreased as adipose depots enlarged.

Conclusion

Overall, HFD has a greater influence on visceral cavity than the subcutaneous. In the visceral lymph node, but not subcutaneous, HFD-induced obesity decreased cell populations that suppressed immune function while increasing those that regulate/activate immune response.

Introduction

It is currently estimated that ~70% of adults in the United States are overweight and a striking half of those are further categorized as obese [73]. Obesity is a precursor for a number of chronic diseases and increases the risk of poorer prognosis in many immune-mediated conditions [129-132]. Adipose tissue dysregulation is a fundamental driver of the comorbidities associated with obesity [116]. In particular, the dysregulation in and accumulation of visceral adipose tissue (fat stored in the intra-abdominal cavity among the visceral organs that drains to the portal vein) [128, 151] has a greater association with obesity-related comorbidities than subcutaneous adipose tissue (fat stored between the muscle and skin) [90, 101, 152]. Inflammation, induced by excessive adipose tissue accumulation, appears to link obesity to disease and immune risk [117-119]. Hence, the increased propensity for individuals with visceral obesity to experience comorbidities may be linked to the increased capacity of this depot to induce inflammation [120]. This would indicate that distinct adipose tissue depots might differentially contribute to processes that regulate obesity-induced inflammation.

The health and regulation of adipose tissue is primarily maintained by the lymphatic system [153-155]. The lymphatic system serves as the conduit for immune cells (e.g. dendritic cells, monocytes, neutrophils and other leukocytes). These cells serve as the responders to tissue injury or pathogen invasion and are fundamental for the development of protective immune responses, including antibody and cellular immune responses. Lymph nodes are predominately embedded in adipose tissue depots [50], thus are in a proximal location to continuously survey and monitor exposure of adipose tissue to potentially harmful pathogens and metabolites [51, 52]. Immune cells within lymph nodes can be recruited and activated to defend adipose tissue against damage, toxicity or impaired function [126]. In terms of immunity, obesity is characterized as a state of chronic low-grade inflammation caused by an inability to alleviate inflammation within adipose tissue. Hence, the lymphatic system is likely greatly impacted by this chronic inflammation, given the intimate association and cross-talk between adipose and lymphoid tissues. Therefore, any disease process that affects lymphoid tissues will also directly influence the development of immunity, including immune responses to pathogens, infections, cancers and vaccines. Overall, components of the lymphatic system must also contribute to obesity-induced comorbidities, yet there is very little understanding of the role the lymphatic system plays in obesity linked disease manifestation.

Despite the spatial association and immune communication between adipose depots and lymph nodes, there remains critical knowledge gaps in our understanding of the molecular and cellular relationship between these two tissues. Given the continuous exposure of visceral draining lymph nodes to soluble inflammatory mediators released from immune cells infiltrating adipose tissue depots, we hypothesize that visceral lymph nodes represent an important sentinel of immune cell changes and subsequent dysregulation secondary to high fat diet-induced obesity.

In the present study, we examined how high fat diet-induced obesity influences lymph node micro-architecture and resident immune cell populations. In addition, we also investigated whether lymphatic response to diet-induced obesity is different between visceral and subcutaneous lymph nodes, given that visceral adiposity is highly associated with inflammation and metabolic disease [128, 151], while subcutaneous is not [90, 101, 152, 156, 157]. We hypothesized that the immune cell populations within lymph nodes will be fundamentally different between those residing in visceral versus subcutaneous adipose tissue.

Methods

Animals and diet

Male C57BL/6 mice (Jackson Laboratory, Bar Habor, Maine) (2-3months, ~24g) were single housed under controlled conditions (12:12 light-dark cycle, 50–60% humidity, and 25° C) and allowed one week of acclimation before experiment start. Following acclimation mice were given free access to water and a standard chow or Western high fat diet until termination at 7 weeks. Standard CHOW diet (CHOW:Harlan Teklad LM485, Madison, WI) was 3.1 kcal/g with 18% kcal from fat (6% by weight of diet). The fats in the diet (from wheats, meal and animals fats) consisted of monounsaturated, polyunsaturated and saturated fatty acids (2.4%, 1.2% and 2.1% by weight of diet). The Western high fat and sugar diet (Harlan Teklad, TD.08811, Madison, WI;) was 4.7 kcal/g and contained 45% kcal from fat,(23% by weight of diet). The Western diet consisted of monounsaturated, polyunsaturated and saturated fatty acids from milk fat and soybean oil (7.19%, 1.87% and 14.15% by weight of diet). Carbohydrates including sucrose, corn starch, maltodextrin and cellulose represented 40% of total kcal. For simplicity and because the majority of the calories of the Western diet are fat, this group will be called high fat

diet, HFD. Body mass and food intake were recorded weekly. Procedures were reviewed and approved by the Colorado State University Institutional Animal Care and Use Committee.

Terminal procedures (blood collection and tissue harvesting)

Termination occurred after mice were on respective diets for 7 weeks. Systemic blood was collected from anesthetized mice and serum was stored at - 80°C. Visceral and inguinal adipose tissue and lymph nodes were excised and immediately stored in Hank's balanced salt solution (HBSS) or fixative. Inguinal adipose tissue is a subcutaneous fat depot located between external oblique and skin whereas the visceral (mesenteric) adipose depot is located in the peritoneal cavity among the intestines and drains into the portal vein [158]. Bilateral subcutaneous lymph nodes were excised from the inguinal adipose depots and visceral, mesenteric, lymph nodes were excised from the visceral depot. The spleen and thymi were also collected. Spleens were placed in (HBSS) or fixative and thymi where placed in fixative only.

Separation of immune cells from stromal tissues

Following euthanasia and tissue harvest, lymph nodes and spleens were briefly stored in HBSS + 5% fetal bovine serum (FBS) on ice, prior to filtering through a 40 μm cell strainer. Cell suspensions in HBSS + 5% FBS were centrifuged at 1200 rpm at 4°C for 5 minutes, supernatants were discarded, and cells from lymph nodes were re-suspended in FACS (1% BSA, 0.1% sodium azide, and PBS) buffer for immunostaining. Spleen cells were first re-suspended in ammonium-chloride-potassium (ACK) buffer (0.5% phenol red solution, ammonium chloride, potassium bicarbonate, disodium EDTA, and distilled water) to lyse red blood cells, prior to resuspension in FACS buffer for immunostaining. For adipose depots, tissue was minced and digested with 2X collagenase D (Roche, Fisher cat # 50-720-3639) at 37°C for 30 minutes. The

collagenase reaction was stopped with HBSS + 5% FBS. Tissue was triturated, filtered and washed with HBSS + 5% FBS. Cell suspension was centrifuged and re-suspended in FACS.

Flow cytometry

Total and viable cell counts were obtained from cell suspensions labeled with trypan blue exclusion dye (ThermoFisher Scientific, Waltham, MA) and counted via the Cellometer (Nexcelom, Lawrence, MA). 5 x 10^5 or 1 x 10^6 cells were plated per well. After washes with FACS buffer, plates were spun and supernatant removed. Cells were re-suspended in normal mouse serum to block non-specific binding and incubated at room temperature for 5 minutes prior to addition of the primary antibodies. All primary antibodies were diluted 1:200 and included the following rat anti-mouse clones: CD8a (clone 53-6.7, cat. no. 13-0081-85, eBioscience), CD4 (clone RM4-5, cat. no. 48-0042-82, eBioscience), CD3 (clone 145-2c11, cat. no. 11-0031-82 eBioscience), intracellular Foxp3 (clone FJK-16a, cat. no. 12-5773-82, eBioscience), B220 (clone RA3-6B2, cat. no. 17-0452-82, eBioscience), CD11b (clone M1/70, cat. no. 48-0112-82, eBioscience), CD11c (clone N418, cat. no. 417-0114-82, eBioscience), F4/80 (clone BM8, cat. no. 25-5931-82, eBioscience), and MHCII (clone M5/114.15.2, cat. no. 17-5321-81, eBioscience). After incubation and washes with FACS, streptavidin-pacific orange (cat. no. 532365, Invitrogen) was added at a dilution of 1:500 for detection of biotinylated primary antibodies. In addition, a permeabilization kit (cat. no. 00-5521, eBioscience) was used in accordance with kit instructions for FoxP3 intracellular staining. After cell surface labeling, cells were fixed in 4% PFA, washed and stored in FACS buffer until flow analysis. Flow cytometry of immune cell populations were analyzed using a MoFlo Legacy Cell Analyzer and Sorter (Beckman Coulter, Indianapolis, IN). A 100 μM flow tip was used for sample uptake. Data were analyzed using Summit software version 4.2 (Beckman Coulter, Indianapolis, IN).

Adipokine/cytokine assay

Serum insulin, leptin, resistin, interleukin 6 (IL-6), monocyte chemotactic protein-1 (MCP-1) and plasminogen activator inhibitor 1 (PAI 1 total) concentration was determined using commercial kits (EMD Millipore Corporation, Billerica, MA) and analyzed on a Luminex instrument (LX200; Millipore, Austin, TX).

Histology and immunocytochemistry

Visceral and subcutaneous lymph nodes, spleen, and thymus were removed and immersion-fixed in 10% neutral buffered formalin for 24 hours prior to paraffin embedding and sectioning for routine hematoxylin and eosin (H&E) staining. Another subset of lymph nodes were fixed in a 1% periodate-lysine-paraformaldehyde (PLP) solution at 4C overnight, placed in 30% sucrose the following day for 24 hours, and then rinsed and embedded in OCT (Tissue-Tek, VWR cat. no. 25608-930) for immunofluorescence (IF). All frozen tissues were sectioned on cyrostat at 10µm for IF staining. Lymph node area for an individual sample was the sum of four sections sliced across the largest mid-region portion of the lymph node. Thymus and spleen measures were taken through the largest cross section of the tissue. For lymph node IF staining, directly conjugated primary antibodies, B220 (eBioscience Ref. 53-0452-80), and CD3 (ebioscience Ref. 50-0032-80), were added to slides at a 1:100 dilution. DAPI counterstain (Millipore 50-874-10001) was used to visualize cells. Slides were coverslipped with Fluoromount-G (eBioscience 00-4958-02) before being imaged and analyzed with CellSens Dimension software (Version 1.15)

Statistics

Data are expressed as mean ± standard error of the mean (SEM). Statistical analysis of total lymph node area measures, but not ICC measures, were performed using two-way ANOVA

between-subjects analysis of variance (ANOVA) (IBM SPSS for Windows; SPSS, Chicago, IL) with diet (CHOW and HFD) and location (Visceral and Subcutaneous) as the factors. Post-hoc tests for two-way ANOVA were made using LSD test. Data analyzed by Two-way ANOVA were further analyzed via One-way ANOVA for significance values among individual data points. Post-hoc tests of individual groups were made using LSD tests. Food intake, body weight, lymph node ICC, spleen cell percent and viable counts, thymus area, inflammatory protein factors, adipokine protein and insulin concentration comparisons were analyzed with a one-way ANOVA. Post-hoc tests for these measures were made using LSD tests. Although a two-way ANOVA would be suitable for lymph node and adipose tissue cell percent and viable number, one-way ANOVA results were solely reported. In the results section, analysis from the one-way ANOVA was applied to first discuss anatomical differences among CHOW animals only (Ex. Visceral CHOW vs. Subcutaneous CHOW differences) without confounds of including diet in the main effect that would occur with a 2-way. Second, one-way ANOVA data was used to analyze how diet changes immune cells within a specific tissue (Ex. Visceral lymph node CHOW vs. Visceral lymph node HFD) without the confound of a tissue main effect which would include subcutaneous samples. Post-hoc tests for these measures were made using LSD tests.

Results

Food intake, body weight and circulating factors

Cumulative kcals ingested over the 7 week experiment was significantly higher in HFD (n = 5) mice compared with CHOW (n = 5) (Table 4.1, one-way ANOVA, LSD post-test; P = 0.0001). The increased intake was associated with a significant increase in body mass (Table 4.1, CHOW vs. HFD; P = 0.045) and total adiposity (Table 4.1, CHOW vs. HFD; P = 0.009). Compared with control, HFD fed mice had significantly larger epididymal (CHOW vs. HFD; P =

0.05), perirenal (CHOW vs. HFD; P = 0.05), dorsal (CHOW vs. HFD; P = 0.05) and inguinal (CHOW vs. HFD; P = 0.01) white adipose tissue (Table 4.1). The visceral depot was increased with HFD, but did not reach significance (Table 4.1; P = 0.063). Glucose response to glucose injection was significantly different between diet groups with an increased area under the curve (AUC) for HFD animals compared with CHOW (Table 4.1, CHOW vs. HFD; P = 0.024). Consistent with increased adiposity, leptin (CHOW vs. HFD; P = 0.001) and insulin (CHOW vs. HFD; P = 0.012) concentration and several markers of systemic inflammation (interleukin-6; IL-6, P = 0.018, monocyte chemotactic protein-1; MCP-1 P = 0.011, and plasminogen activator inhibitor 1; PAI 1, P = 0.025) were significantly elevated in HFD fed mice (Table 4.1). Resistin was also elevated in HFD mice.

Lymph node histology

HFD in general increased lymph node size (Figure 4.1E, two-way ANOVA, LSD post-test; Main effect diet; P = 0.0001). This was primarily due to increases in the visceral lymph node. Hence, mean area of the visceral lymph node was significantly increased, ~2 fold, in HFD mice (Figure 4.1B; n=5) compared with CHOW controls (Figure 4.1A; n=5) (Figure 4.1E, one-way ANOVA, LSD post-test; P = 0.004). HFD did not significantly change subcutaneous lymph node size. Immunofluorescent labeling for pan T (CD3) and B cell (B220) markers was then utilized to further examine the relative contributions of specific immune cell types and their respective microanatomical regions to the observed increases in lymph node area. Again, 7 weeks of HFD significantly increased the total area of the visceral lymph nodes (Figure 4.2E, one-way ANOVA, LSD post-test; P = 0.011, CHOW (n = 5) vs. HFD (n = 5)). Specifically, HFD-induced increases in LN area were the result of enlargement of both the T cell zone (Figure 4.2A-B) (CD3=red) and B cell follicles (B220, green) in the cortex. However, the T cell zone

was the only region which showed a statistically significant increase in mean area (Figure 4.2F; CHOW vs. HFD, P = 0.017). Of note, HFD did not result in changes in lymph node size or T cell zone or B cell follicles of subcutaneous lymph nodes (Data not shown).

Adipose tissue cell viability and immune population frequency

The visceral adipose depot has the greatest disposition for HFD-induced inflammation. Numerous previous studies in rodents (For Review See: [159]) typically investigate epididymal adipose tissue, which drains into the systemic circulation [160], not directly to the liver via hepatic portal vein [161-163]. Hence, although located in the abdominal cavity, epididymal adipose tissue is not the same as visceral that releases direct effluent into the liver. In addition, adipose tissue depots are inherently different in numerous ways [164-167], thus the current study examines differences between immune cell populations of the true portal draining visceral adipose depot and the inguinal subcutaneous depot. In addition, this study extended the examination of depot differences to include evaluation of immune cell differences between lymph nodes embedded within the visceral and subcutaneous adipose depots.

The percentage of distinct immune cell populations were inherently different between the visceral and subcutaneous adipose depots, and furthermore, were differentially affected by HFD (n = 7 per group). Specifically, in CHOW fed mice, the visceral adipose depot contained a significantly higher percentage of $CD3^+$ T cells (Table 4.2, one-way ANOVA, LSD post-test, CHOW Vis fat vs. CHOW SQ fat; p = 0.0001) than the subcutaneous depot. This increase in CD3+ T cells was predominately due to elevations in $CD4^+$ helper T cells, which were also present in a significantly greater percentage in the visceral depot as compared to the subcutaneous (Figure 4.3A, one-way ANOVA, LSD post-test, CHOW Vis Fat vs. CHOW SQ fat; p = 0.008). Surprisingly, the visceral adipose depot contained a significantly lower

percentage of $CD11b^+/F4/80^+$ macrophages (Figure 4.4A, CHOW Vis fat vs. CHOW SQ fat p = 0.015), and $CD4^+/Foxp3^+$ regulatory T cells (Tregs) (Figure 4.3B, CHOW Vis fat vs. CHOW SQ fat p = 0.016) as compared to the subcutaneous depot.

Absolute numbers of distinct immune cell subsets were determined by multiplying percent of representative cell populations by the total viable cell count. Because adipose tissue depots were different sizes relative to location and diet, viable cell number reported was normalized (number of cells/per gram of adipose tissue). First, total viable cell count was not different between CHOW visceral (n=7) and subcutaneous (n=7) adipose depots (Figure 4.5A). Despite this, viable numbers of distinct cell types were significantly different between the two depots. Specifically, in CHOW fed animals total number of B220+ (Table 4.2; one-way ANOVA, CHOW Vis fat vs. CHOW SQ fat; p = 0.02), CD3+ (Table 4.2; CHOW Vis fat vs. CHOW SQ fat, p = 0.0001), $CD3^+CD4^+$ (Figure 4.6B; one-way ANOVA, CHOW Vis fat vs. CHOW SQ fat, p = 0.0001) and $CD3^+CD8^+$ (Figure 4.6D; CHOW Vis fat vs. CHOW SQ fat, p = 0.015) cells within the visceral depot were significantly greater than those in the subcutaneous. In contrast, $F4/80^+CD11b^+$ macrophage (Figure 4.6A, CHOW Vis fat vs. CHOW SQ fat, p = 0.0001) and $CD4^+Foxp3^+$ Treg (Figure 4.6C, Location main effect; p = 0.0001) cell numbers were significantly lower in the visceral depot compared with subcutaneous.

Immune cell populations within the visceral and subcutaneous adipose depots were differentially altered by HFD. Specifically, the visceral depot had a greater tendency toward HFD-induced immune cell alterations. Compared with controls, HFD significantly increased visceral adipose tissue percentage of $F4/80^+CD11b^+$ (Figure 4.4A; CHOW Vis fat vs. HFD VIS fat, p = 0.0001), $CD3^+CD8^+$ (Figure 4.3C; CHOW Vis fat vs. HFD VIS fat, p = 0.0001) and $CD11c^+CD11b^+$ (Figure 4.4B; CHOW Vis fat vs. HFD VIS fat, p= 0.001) cells, but significantly

lower percentage of $CD4^+Foxp3^+$ (Figure 4.3B; CHOW Vis fat vs. HFD VIS fat, p = 0.002) cells. HFD only changed $CD4^+Foxp3^+$ cells in the subcutaneous depot, decreasing cell population percentage compared with CHOW mice (Figure 4.3B; CHOW SQ fat vs. HFD SQ fat p = 0.002).

Compared with respective controls, HFD caused a significant 60% decrease in normalized (per gram adipose tissue) total viable cell number in both the visceral and subcutaneous adipose depots (Figure 4.5A; Chow vs. HFD - Vis fat, p =0.018, SQ fat; p = 0.008). HFD-induced increases in adipose depot size were not associated with a concomitant proportional increase in immune cell numbers. In the visceral adipose depot HFD significantly reduced B220+ (Table 4.2; one-way ANOVA, CHOW Vis fat vs. HFD Vis fat; p = 0.05), CD3+ (Table 4.2; CHOW Vis fat vs. HFD Vis fat; p = 0.0001) and $CD3^+CD4^+$ (Figure 4.6B; CHOW Vis fat vs. HFD Vis fat; p = 0.003) immune cells. HFD also significantly reduced immune cell populations in the subcutaneous adipose depot, this included $F4/80^+CD11b^+$ (Figure 4.6A; CHOW SQ fat vs. HFD SQ fat; p = 0.0001), $CD3^+CD4^+$ (Figure 4.6B; CHOW SQ fat vs. HFD SQ fat; p = 0.003), $CD4^+Foxp3^+$ (Figure 4.6C; CHOW SQ fat vs. HFD SQ fat; p = 0.0001), $CD3^+CD8^+$ (Figure 4.6D; CHOW SQ fat vs. HFD SQ fat; p = 0.004) and $CD11b^+$ $CD11c^+$ (Figure 4.6E; CHOW SQ fat vs. HFD SQ fat; p = 0.045).

Lymph node cell viability and immune population frequency

We postulated that, similar to the visceral and subcutaneous adipose depots, immune cell populations within the regional draining lymph nodes would be inherently different from one another, and be reflective of the observed immune cell changes in the surrounding adipose tissue depots. Indeed, there were significant differences in numerous immune cell subsets between the visceral and subcutaneous lymph nodes. Visceral lymph nodes of CHOW mice contained a

significantly greater percent of CD3+ T cells (Table 4.2; one-way ANOVA, CHOW Vis LN vs. CHOW SQ LN; $p = 0.001$), F4/80+CD11b$^+$ macrophages (Figure 4.8A; one-way ANOVA, LSD post-test, CHOW Vis LN vs. CHOW SQ LN $p = 0.004$) and CD4$^+$Foxp3$^+$ regulatory T cells (Figure 4.8C, CHOW Vis LN vs. CHOW SQ LN; $p = 0.0001$) compared with subcutaneous lymph nodes. However, CD3$^+$CD4$^+$ helper T cell percent (Figure 4.8B, CHOW Vis LN vs. CHOW SQ LN; $p = 0.012$) was lower in the visceral lymph nodes.

In mice, the visceral lymph node is much larger than subcutaneous, hence the visceral lymph node inherently encapsulates a greater number of viable immune cells than the subcutaneous. Therefore all distinct viable immune cell populations in the visceral lymph node were significantly increased compared with the subcutaneous, thus viable number comparisons are not discussed relative to location.

HFD caused significant alterations in the visceral, but not subcutaneous, lymph node immune cells. The most prevalent visceral lymph node differences occurred among viable immune cell number. For example, HFD only significantly decreased the percent of CD4$^+$Foxp3$^+$ regulatory T cells (Figure 4.8C, CHOW VLN vs. HFD VLN; $p = 0.002$) in the visceral lymph node. The percent of other immune cell groups remained unchanged by diet. Diet, however, significantly changed the viable immune cell number. As previously stated, HFD increased visceral, but not subcutaneous, lymph node area (Figure 4.1). In HFD mice increased visceral lymph node size was positively associated with a significant increase in total visceral lymph node viable cell number (Figure 4.5, CHOW VLN vs. HFD VLN; $p = 0.011$). Cell populations that increased in number within the visceral lymph node following 7 weeks of HFD included F4/80$^+$CD11b$^+$ (Figure 4.7A, CHOW VLN vs. HFD VLN; $p = 0.0001$), CD3$^+$ (Table 4.2, CHOW VLN vs. HFD VLN; $p = 0.02$), CD3$^+$CD4$^+$ (Figure 4.7C, CHOW VLN vs. HFD

VLN; p = 0.003) and $CD11c^{+}CD11b^{+}$ (Figure 4.7E, CHOW VLN vs. HFD VLN; p = 0.023) cells. HFD mice, however, had a decrease visceral lymph node number of $CD4^{+}Foxp3^{+}$ (Figure 4.7B, CHOW VLN vs. HFD VLN; p = 0.0001) cells compared with the CHOW group. HFD-induced obesity did not change immune cell frequency or number in the subcutaneous depot. Overall, there was a differential immune cell response between the visceral and subcutaneous lymph nodes to HFD-induced obesity.

Thymus and spleen

HFD caused a significant, ~50%, decrease in thymus area (Figure 4.9A-B, one-way ANOVA, CHOW vs. HFD; P = 0.014), which on histopathological evaluation appeared secondary to generalized atrophy of both the cortex and medulla (Figure 4.9). HFD also induced changes in splenic histo-morphology (Figure 4.10A), without altering overall area of the spleen (Figure 4.10B). While HFD significantly decreased the overall splenic lymphoid follicle number by ~50% compared with CHOW fed animals (Figure 4.10C, CHOW vs. HFD; P = 0.002), the total follicular area was unchanged (Figure 4.10D). Lymphoid follicles in HFD animals were secondary lymphoid follicles, which contained prominent germinal centers and expanded mantle and marginal zones, consistent with reactive follicular hyperplasia. Secondary follicles were increased in number by ~2 fold as compared to CHOW animals (Figure 4.10E, CHOW vs. HFD; p = 0.021).

Spleen cell viability and immune population frequency

Because HFD-induced significant morphological alterations to normal splenic architecture, we further examined changes in the absolute numbers as well as relative percentages of normal immune cell subsets within the spleen. Compared with control, CHOW-fed animals, HFD-fed mice displayed a significant increase in number of splenic viable

$F4/80^+CD11b^+$ macrophages (CHOW 6.48E+04 ± 8.21E+03 vs. HFD 1.07E+05 ± 1.65E+04; p = 0.041), but not percent. HFD also significantly increased the percentage (Chow 7.09 ± 0.73 vs. HFD 9.94 ± 0.77; p = 0.02) and number (CHOW 7.24E+05 ± 9.60E+04 vs. HFD 1.27E+06 ± 3.56E+04; p = 0.001) of $CD3^+CD4^+$ T helper cells. No significant differences were observed in percent or total values for T cells, cytotoxic T cells, regulatory T cells, or B cells (Data not shown).

Discussion

Obesity, predominately-visceral adiposity, links to numerous diseases including insulin resistance, diabetes and cardiovascular disease [128, 151]. Fundamental factors linking central adiposity, but not subcutaneous, to disease risks include heightened adipocyte associated inflammation with a higher infiltration of immune cells [168]. Proper defense and health maintenance of organ systems, including adipose tissue, depends on the coordination of innate and adaptive immune responses that occur predominately in the lymphatic system. The spatial proximity of adipose depots to secondary lymph nodes allows a unique relation between the two systems. However, this subject has received little attention, despite the conceivable negative consequences of increased adiposity on lymph node function. To examine the relation between increased adiposity and secondary lymph node morphology and immune cell populations we utilized a Westernized diet to induce obesity in mice. Obesity caused reactive lymphoid hyperplasia, a change that was most prominent in visceral lymph nodes. Increased visceral lymph node size was associated with increased viable cell number, which included increased numbers of pro-inflammatory antigen presenting cells, including but not limited to macrophages, dendritic and T helper cells. However, T regulatory cells were reduced in visceral lymph nodes. Immune cell alterations in subcutaneous lymph nodes were limited solely to reductions in T

regulatory cells. The visceral adipose depot also had greater reactivity toward HFD than subcutaneous. Principal HFD-induced changes in visceral adipose tissue occurred via shifts in relative immune cell populations leading to a greater percentage of macrophages, dendritic cells and CD8+ T cells. However, HFD-induced increases in adiposity decreased viable immune cells in both visceral and subcutaneous depots. Taken together, HFD alterations have a greater association with visceral lymph nodes than subcutaneous where cell populations that suppress immune function were decreased while those that are pro-inflammatory and typically serve to activate immune responses were increased.

Seven weeks of HFD increased body mass by ~20%, total adiposity by ~90%, and individual visceral and subcutaneous adipose depots by ~125%. Consistent with this, markers of metabolic dysregulation were increased including systemic glucose, insulin, leptin and plasminogen activator inhibitor (PAI-1; contributor to obesity and inducer of thrombi in vasculature) concentration. Circulating inflammatory cytokines interleukin 6 (IL-6) and monocyte chemotactic protein 1 (MCP-1) were also doubled in HFD fed mice. IL-6 release may originate from several cell types including macrophages, fibroblast, epithelial cells, endothelial cells and T and B cells [169]. Major targets of IL-6 include auxiliary populations of T and B cells and results in activation and proliferation of immune cells and stimulation of inflammatory responses. MCP-1, a chemotactic factor for monocytes, is produced in numerous cell types including macrophages, endothelial cells and adipocytes [170]. Increases in MCP-1, as occurs in obesity, recruits monocytes/macrophages via high endothelial venules of draining lymph nodes, and hence is associated with enhanced recruitment of pro-inflammatory macrophages [171]. Taken together these measures indicate seven weeks of HFD was sufficient to induce metabolic

dysregulation and concomitant release of factors that increase stimulation of monocytes and lymphocytes.

HFD-induced obesity had a greater effect on visceral lymph node size (~100% increase) than subcutaneous. These secondary lymphoid organs are the principal location for T- and B-cell responses to pathogens. The lymph node consists of distinct compartments including the B cell zone, paracortex that includes the T cell zone and the medulla consisting of efferent lymphatic vessels where naïve and activated lymphocytes leave. In general, lymph node swelling is a sign of adaptive immunity. Infection or immunization causes an expansion of afferent lymphatic vessels which allows the enhanced recruitment of antigen presenting cells from the periphery [172] while also transiently restricting lymphocyte exit through efferent vessels [173]. Subsequently the size of the draining lymph node expands considerably as result of influx and trapping of naive lymphocytes which enhances interactions with antigen presenting cells, this further drives hypertrophy with additional proliferation of antigen-specific lymphocytes [174]. Although inflammation incited by obesity is unlike traditional infections, we demonstrated here that lymph node hypertrophy is associated with adiposity-induced chronic low-grade inflammation. Our findings, however, are in opposition to previous studies demonstrating that obesity results in lymph node atrophy [52, 175]. In these studies, HFD caused a 50% or greater decrease in visceral lymph node size. We propose that differential outcomes result from longer diet durations (~10 weeks on HFD) [52, 175] and/or higher percent of fat in diet (~60%) [52].

HFD-induced increases in visceral lymph node size were associated with expansion of B cell zones and T cell areas within the cortex, hence we additionally characterized a broad assortment of immune cell populations. This included cells from the visceral and subcutaneous lymph node as well as adipose depots (VWAT and IWAT) that encapsulated them. Previous

studies demonstrate that obesity alters immune cell populations within adipose tissue, especially fat within the visceral cavity (For Review See: [116]). The macrophage, part of the innate immune response, is among the best-characterized immune cell within adipose tissue and can both initiate immune response and assist in resolution [176]. Immune cells with cell surface expression of $F4/80^+$ [177], either alone or in conjunction with $CD11b^+$ [178, 179] are traditionally characterized as macrophages. Previous reports demonstrate that obesity increases the number of macrophages in adipose tissue [29, 180-183] including $F4/80^+CD11b^+$ cells [178]. $F4/80^+CD11b^+$ is a general macrophage marker that includes both M1 and M2 types. During obesity-induced inflammation we postulate that these macrophages within adipose depots are predominately of an M1/pro-inflammatory phenotype [183-186]. However, in this study we did not asses the functional status of these cells. Future studies aimed at further phenotyping these cells with functional markers such as iNOS or Arginase I, or evaluating changes in cytokine gene expression are warranted. HFD increased the proportion of $F4/80^+CD11b^+$ cells in visceral, but not subcutaneous adipose tissue. Like previous studies [34, 187] visceral adipose tissue had greater HFD-induced macrophage infiltration than subcutaneous. However, the visceral depot initially had a lower proportion of macrophages than the subcutaneous of CHOW mice. HFD-induced an increase in macrophage percent in the visceral lymph node, but subcutaneous remained unchanged. In CHOW mice, we postulate that the larger proportion of macrophages in the subcutaneous adipose depot, compared with visceral, may play a part in the protective role of this adipose tissue. It is likely that alternatively activated M2 macrophages predominate in CHOW mice, hence these cells block inflammatory response and promote tissue repair [188]. T regulatory percent difference between the adipose depots of CHOW mice further supports this prediction. Although HFD did not modify macrophage percent within the subcutaneous depot, it

is again plausible that it promoted a change in their activation state from M2 to a more pro-inflammatory M1 phenotype. HFD also increased antigen presenting $CD11b^+CD11c^+$ myeloid dendritic cell percent in the visceral adipose tissue [183, 189, 190]. This cell type is characterized to play a fundamental role in T-cell accumulation within adipose tissue and is positively associated with obesity and insulin resistance. Similar to adipose tissue depots, lymph nodes have a differential response to HFD-induced obesity. Visceral lymph nodes have greater reactivity to HFD than subcutaneous. $F4/80^+CD11b^+$ and $CD11b^+CD11c^+$ cell number only increased in the visceral lymph node, similar to the depot that it resides in. Hence, increased infiltration of antigen presenting cells was specific to tissues in the visceral cavity. This supports and extends previous data associating visceral obesity with pro-inflammation [33].

In obesity macrophages are major producers of inflammatory mediators in adipose depots [191, 192]. Numerous lymphocytes, however, also reside in adipose tissue and play a role in inflammation and metabolic dysregulation. These include, but are not limited to, $CD4^+Foxp3^+$ (T regulatory cells) [193], $CD3^+CD4^+$ (T helper cells) [194], $CD3^+CD8^+$ (cytotoxic T Cells) [195] and $B220^+$ (B cells) [196]. Similar to the innate immune response, adaptive immune cell populations changed in the adipose tissue and lymph nodes of HFD obese mice. Consistent with previous literature HFD increased the cytotoxic T cell population in visceral adipose tissue. Cytotoxic T cells increase in adipose tissue during obesity and precede accumulation of macrophages; hence these cells initiate and propagate adipose tissue inflammation [195]. This cell type is a critical mediator of metabolic dysfunction and links the visceral adipose depot to metabolic risks [195]. Cytotoxic T cell percent, however, did not change within the visceral lymph node during obesity, but viable number of $CD3^+CD4^+$ T helper cells were increased. We propose that antigen presenting macrophages and dendritic cells within and from dysregulated

adipose tissue present antigen peptides to naïve $CD3^+CD4^+$ cells which causes activation and subsequent cell division and differentiation to effector T cells. This process occurs while antigen presenting cells are traveling to draining lymph nodes via afferent lymphatics [197]. Hence, the increased lymph nodes size of obese animals, in part, is likely due to the expansion of helper T cells. $CD3^+CD4^+$ cell subsets where not further characterized in the current study, however we would predict that pro-inflammatory cells represent the greatest portion of this population. Neither cytotoxic or helper T cells changed in the subcutaneous adipose depots or lymph nodes. This data supports that fat accumulation in the visceral cavity is more pro-inflammatory than that in the subcutaneous region and likely leads to the differential outcomes of specific types of fat accumulation.

Actions of T helper and cytotoxic T cells are mediated by Tregs, $CD4^+Foxp3^+$ cells (for review see: [198]), which impede activation and subsequent pro-inflammatory reactions. Numbers of viable Tregs were vastly different between tissues collected from the visceral (intra-abdominal) and subcutaneous regions. When normalized by weight, the visceral adipose depot of CHOW mice contained ~9,000 Tregs whereas the subcutaneous depot from CHOW mice contained ~70,000. Others demonstrate depot distinct differences in Tregs with epididymal adipose tissue (another intra-abdominal depot) having a higher frequency than subcutaneous [199], however the epididymal depot should not be considered a visceral depot. Visceral adipose tissue accumulation links to metabolic disease and is highly associated with greater pro-inflammatory potential and immune cell infiltrates than subcutaneous [200]. We postulate the lower numbers of Tregs in visceral adipose tissue of CHOW mice contribute to increased inflammatory capacity in this depot, especially in HFD-induced obesity. HFD-induced obesity causes Treg depletion in intra-abdominal depots, epipdidymal and visceral, and is positively

correlated to markers of innate and adaptive immune activation [201]. Hence, less Tregs associate with a higher number of activated effector T cell populations. In the current study, HFD-induced obesity reduced Tregs in both visceral and subcutaneous adipose tissue, but macrophages and cytotoxic t-cells only increased in the visceral depot. Macrophage polarization, M1/M2, may also contribute to the differential adaptive immune response between the two depots. The current study, however, did not characterize specific macrophage polarization. Unlike the visceral adipose depot, the visceral lymph node contained the greatest number of Tregs, ~110,000, compared with subcutaneous, ~6,000. This depot difference is likely due to the proximity and connection of the visceral lymph node to the gastrointestinal tract. The visceral (mesenteric) lymph node is the key site of oral tolerance induction functioning as a firewall to preserve systemic ignorance to commensal organisms and food proteins [202]. Specifically, the Tregs within the visceral lymph node mediate oral tolerance to help prevent autoimmune and allergic responses and control the magnitude and duration of inflammatory responses [203, 204]. We propose the higher number of Tregs in the visceral lymph node of CHOW mice are pertinent to maintaining systemic health by preventing unwanted priming reactions [202]. HFD-induced decreases in visceral lymph node Tregs is likely associated with the increased macrophages and Thelper cells.

B cells also contribute to obesity-induced pathophysiology. High fat feeding increases the infiltration of B cells in the epididymal depot before significant accumulation of adipose tissue mass and likely precedes infiltration of T cell types [205]. B cell infiltration drives obesity related co-morbidities, such as insulin resistance, through cytokine production, T cell modulation and antibody production (For review see: [206]). We support previous studies indicating that HFD increases B cells within adipose tissue [205] and extend these results by demonstrating

considerably greater increases in the visceral depot (increased ~75%) than subcutaneous (increased ~25%). Similarly and consistent with our immunocytochemistry, HFD increased B cell numbers in the visceral lymph node by 45%, but no change occurred in the subcutaneous lymph node. We postulate that the increase in B cells within the visceral lymph node is due to proximity and connection to the gastrointestinal tract. B and plasma cell hyperplasia occur in response to antigenic stimulus that requires antibody production [207]. Such factors that trigger B cell population and number increases include contact with microbiota [208], bacterial peptidoglycan and endotoxins, i.e. lipopolysaccharide, [209]. Gut permeability increases during obesity, which permits bacterial translocation from the gut lumen [210, 211]. This could contribute to enhance B cell numbers in the visceral lymph nodes of HFD mice.

In addition to lymph nodes we also examined larger lymphatic tissues, the thymus and the spleen. Unlike the lymph nodes, 7 weeks of HFD decreased the size of the thymus by ~50%. This, however, is consistent with previous studies that report 3-9 months of HFD (66% fat) to reduce thymocyte counts and increase apoptosis of T cells [212]. Here we extend this study by demonstrating a shorter diet duration with less fat content results in thymic involution. HFD-induced obesity did not change overall size of the spleen, but did cause a change in morphology of structures contained within. HFD caused a 50% reduction in splenic follicle number; yet total follicle area was not different between the diets. HFD significantly increased the ratio of Secondary:Primary lymphoid follicles compared with CHOW. An increase in secondary lymphoid follicles with germinal centers is characteristic of induction of antigen stimulation. These changes are consistent with follicular hyperplasia and are indicative of a rise in small B cells generated to bind antigen, interact with T cells and rapidly differentiate to long-lived plasma cells or memory B cells while exiting the germinal center (For review see: [213]).

In conclusion, central adiposity is demonstrated to highly associate with metabolic disease whereas subcutaneous adiposity is linked with metabolic protection. Here we postulated that disparities among differing deposition is not solely due to adipose tissue itself, but is also driven, in part by the secondary lymphatics embedded within. Here we support and extend previous studies by demonstrating that legitimate visceral adipose tissue that drains to the liver and visceral lymph nodes have greater reactivity to HFD than subcutaneous adipose tissue and lymph nodes. We postulate the greater sensitivity to dietary changes arise from programming within the abdominal cavity with close proximity to the gut. Indeed, visceral lymph nodes are connected to the gastrointestinal tract and play an integral role in suppression of immune response to antigens ingested. Visceral lymph nodes also drain visceral adipose tissue effluent, thus can directly react to immune or metabolic reactions that occur within adipose tissue. This could include reactivity to metabolites, cytokines, adipose derived immune cells and other chemotactic stimulus. It remains to be defined if immune regulation within the visceral lymph node initiate HFD-induced inflammation within the abdomen or if adipose tissue dysregulation evokes lymph node dysregulation. We predict visceral lymph nodes permit inflammation and increases in adiposity exacerbate it.

Limitation

Diets used in the current study were selected to induce significant body mass differences among the CHOW and HFD (Western) group. Hence, diet-induced obesity was fundamental to evaluating immune system alterations. Although this was accomplished, it is still worthwhile to note that a limitation of the study was the variations in macro and micro dietary composition among the two diets types (Ex. Protein amount). Studies in the future should utilize purified

diets that allow for a better comparison between CHOW and HFD (Western) diets that best matches for macro- and micro-nutrients between experimental diets.

Tables

Table 4.1 - Cumulative food intake (kcals), total body weight (g), individual adipose depot mass, total adiposity (g), and glucose response to glucose tolerance test (area under the curve). Systemic circulating measures; insulin, leptin, resistin, IL-6, MCP1, and PAI1. Cumulative kcals ingested over 7 weeks was significantly higher in HFD mice compared with CHOW and was associated with a significant increase in total body mass, and total adiposity (* ≤ 0.045). Individual adipose tissue depots of HFD fed mice were significantly larger (epididymal, perirenal, dorsal, and inguinal) compared to CHOW controls (* ≤. 0.05) The visceral depot increased, but did not reach statistical significance. Glucose tolerance was significantly different between HFD and CHOW mice as measured by area under the curve and is greater in HFD mice (* ≤ 0.024). Markers associated with increased adiposity, leptin and insulin, and markers of systemic inflammation (IL-6, MCP-1, and PAI-1) were significantly increased in HFD mice compared to controls (* ≤ 0.025).

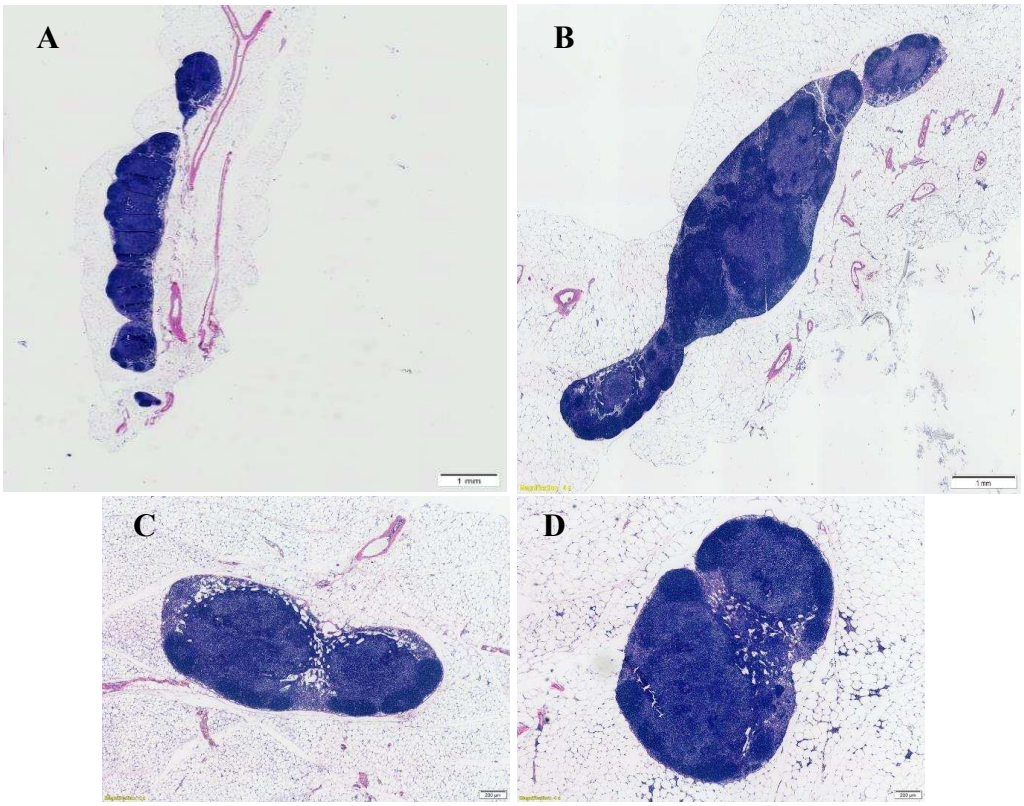
	Food Intake (kcals)	Body Weight (g)	VWAT	IWAT	Total Adiposity	GTT AUC x 10^4
Chow	1024 ± 31.80	33.16 ± 0.911	0.43 ± 0.08	0.61 ± 0.12	3.46 ± 0.52	1.2 ± 0.2
HFD	1292 ± 26.03*	40.4 ± 2.63*	0.97 ± 0.21	1.41 ± 0.18*	6.72 ± 0.70*	2.3 ± 0.3*

	Insulin	Leptin	Resistin	IL-6	MCP1	PAI 1 Total
Chow	606.6 ± 143	5887 ± 1535	751 ± 126	3.04 ± 0.4	4.83 ± 0.9	756 ± 192
HFD	2503 ± 658*	22514 ± 2439*	973 ± 63	6.21 ± 1.1*	9.175 ± 0.8*	3767 ± 1072*

Table 4.2 - Percent and total viable B220+ and CD3+ T cells with interactions for diet (CHOW vs HFD), area (Visceral vs Subcutaneous), and diet by area. In CHOW fed mice the visceral adipose tissue contained a significantly higher percentage of CD3+ T cells than the subcutaneous depot (* $p = 0.0001$). In CHOW fed animals total number of B220+ and CD3+ cells were significantly higher in the visceral compared to subcutaneous adipose tissue depots (* ≤ 0.02). In the visceral adipose tissue depot HFD significantly decreased viable B220+ and CD3+ cells compared to CHOW (* ≤ 0.05).

Cell Type	Visceral Adipose Tissue		Subcutaneous Adipose Tissue		P value		
	CHOW	HFD	CHOW	HFD	Diet	Area	Diet* Area
% B cells	1.81 ± 0.25	2.50 ± 0.20	2.31 ± 0.31	2.63 ± 0.61	—	—	—
Viable B cells	$5.92x10^4 \pm 9.93x10^3$	$4.60x10^4 \pm 8.67x10^3$ *	$3.68x10^4 \pm 4.97x10^3$ **	$1.89x10^4 \pm 5.51x10^3$ b	0.004	0.05	—
% CD3 T cells	6.08 ± 0.60	7.34 ± 0.91	2.82 ± 0.43 **	2.47 ± 0.32	—	0.0001	—
Viable CD3 T cells	$1.10x10^5 \pm 7.31x10^3$	$5.83x10^4 \pm 5.63x10^3$ *	$2.17x10^4 \pm 2.05x10^3$ **	$1.27x10^4 \pm 3.09x10^3$	0.0001	0.001	0.017
	Visceral Lymph Node		Subcutaneous Lymph Node				
% B cells	38.32 ± 6.40	42.93 ± 4.91	38.72 ± 4.32	42.12 ± 3.18	—	—	—
Viable B cells	$5.13x10^5 \pm 1.18x10^5$	$1.17x10^6 \pm 1.85x10^5$ *	$3.68x10^5 \pm 8.32x10^4$	$3.69x10^5 \pm 7.00x10^4$	0.01	0.003	0.039
% CD3 T cells	33.64 ± 2.1	36.8 ± 2.24	51.7 ± 4.24**	46.59 ± 3.73	—	0.001	—
Viable CD3 T cells	$6.33x10^5 \pm 6.58x10^4$	$9.07x10^5 \pm 6.41x10^4$ *	$4.46x10^5 \pm 6.08x10^4$	$4.92x10^5 \pm 9.98x10^4$	0.049	0.001	—

Figures



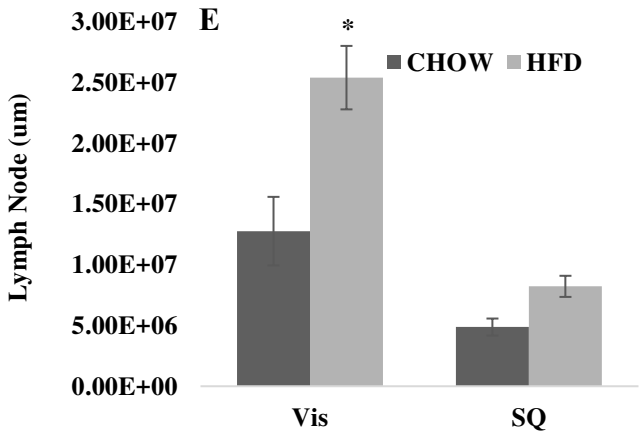


Figure 4.1 - Average lymph node area (μm^2). Visceral lymph node of **A.)** CHOW and **B.)** HFD mouse. Subcutaneous lymph node of **C.)** CHOW and **D.)** HFD mouse. Lymph node area for an individual sample was the sum of four sections sliced across the largest mid-region portion of the lymph node. Lymph node area for an individual sample was the sum of four sections sliced across the largest mid-region portion of the lymph node. **E.)** HFD significantly increased mean lymph node size of visceral lymph nodes (* = 0.004) but not subcutaneous compared to CHOW controls.

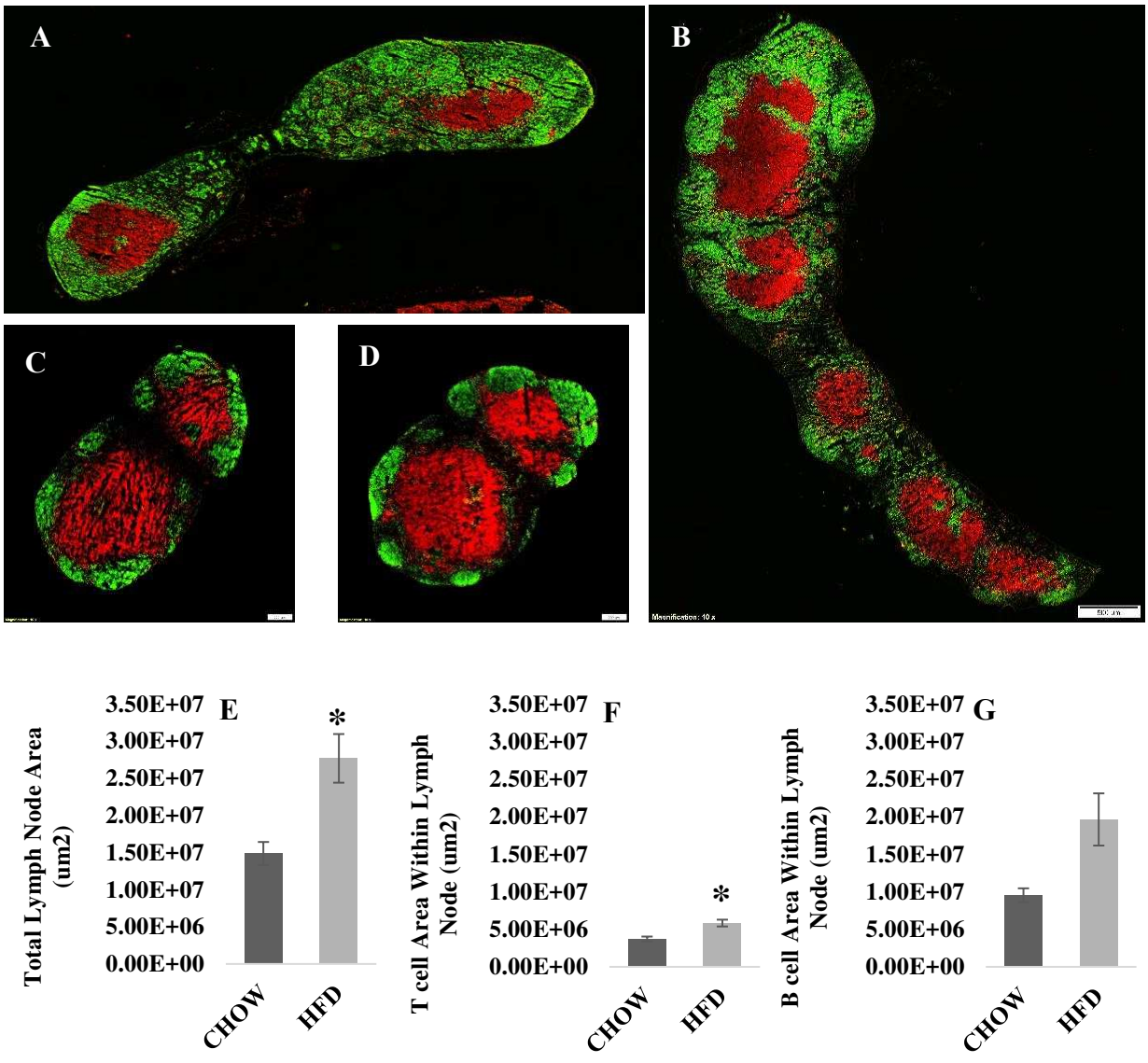


Figure 4.2 - Lymph node immunocytochemistry (ICC) of B220+ (green) and CD3+ T cells (red). Visceral CHOW **A.)** and HFD **B.)** lymph node. Subcutaneous CHOW **C.)** and HFD **D.)** lymph node. HFD significantly increased the total area of the visceral lymph nodes (* = 0.011) **E.),** with specific enlargement in both the T cell zone (* = 0.017; CD3=red) **F.)** and B cell follicles (B220, green) **G.)** in the cortex.

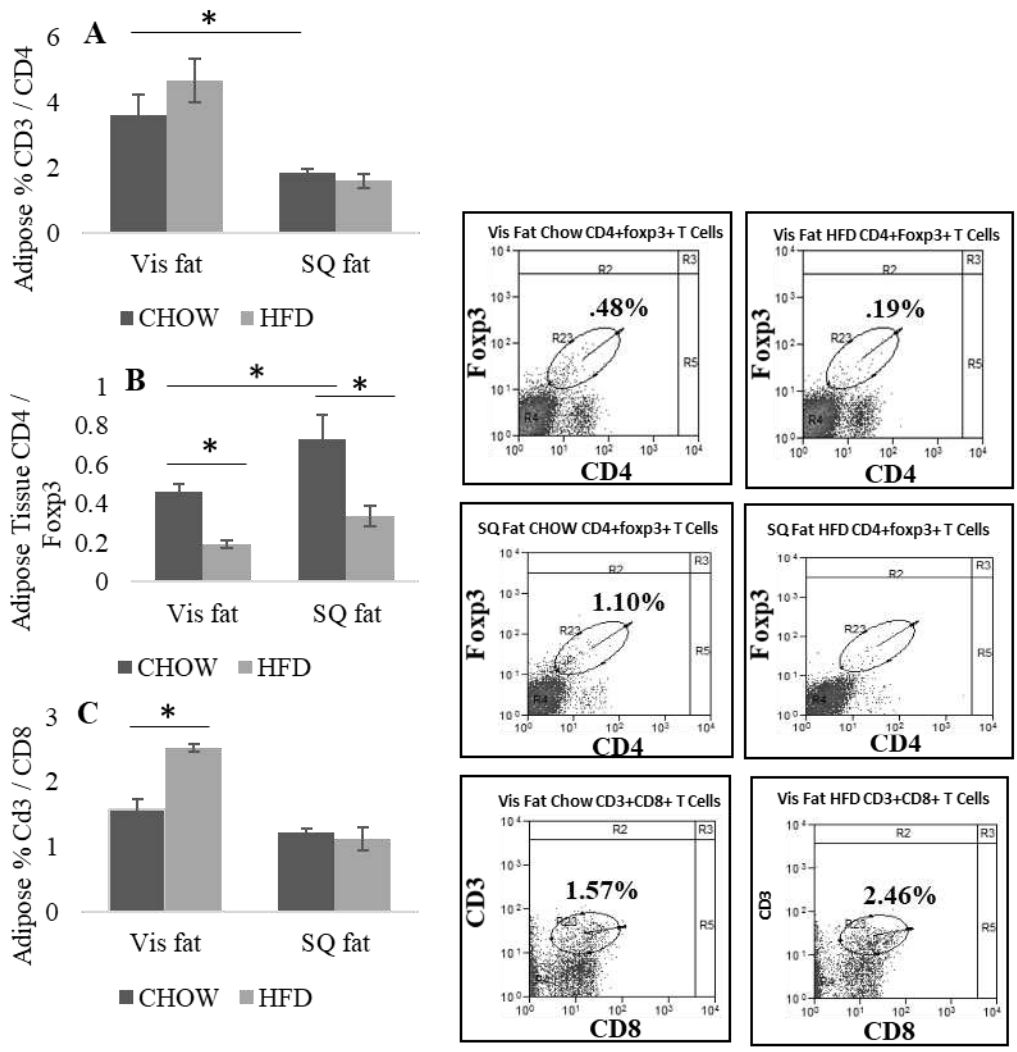


Figure 4.3 - Immune cell percent in visceral and subcutaneous adipose tissue depots. In CHOW mice the visceral adipose depot contained a significantly lower percent of CD4+Foxp3+, regulatory T cells (* = 0.016) **B.)**, compared to the subcutaneous depots from CHOW animals. In opposition, CD3+CD4+ T helper cells (* = 0.008) **A.)** were significantly increased in the visceral adipose tissue depot of CHOW mice compared with respective subcutaneous adipose depots. HFD altered immune cell populations within lymph nodes. In the visceral lymph node HFD significantly increased CD3+CD8+, Cytotoxic T cells (* = 0.0001) **C.)** but significantly decreased CD4+Foxp3+, regulatory T cells (* = 0.002) **B.).** HFD also significantly decreased regulatory T cells in the subcutaneous adipose depot (* = 0.002) **B.).** Right Column, respective scatter plots of significant diet differences.

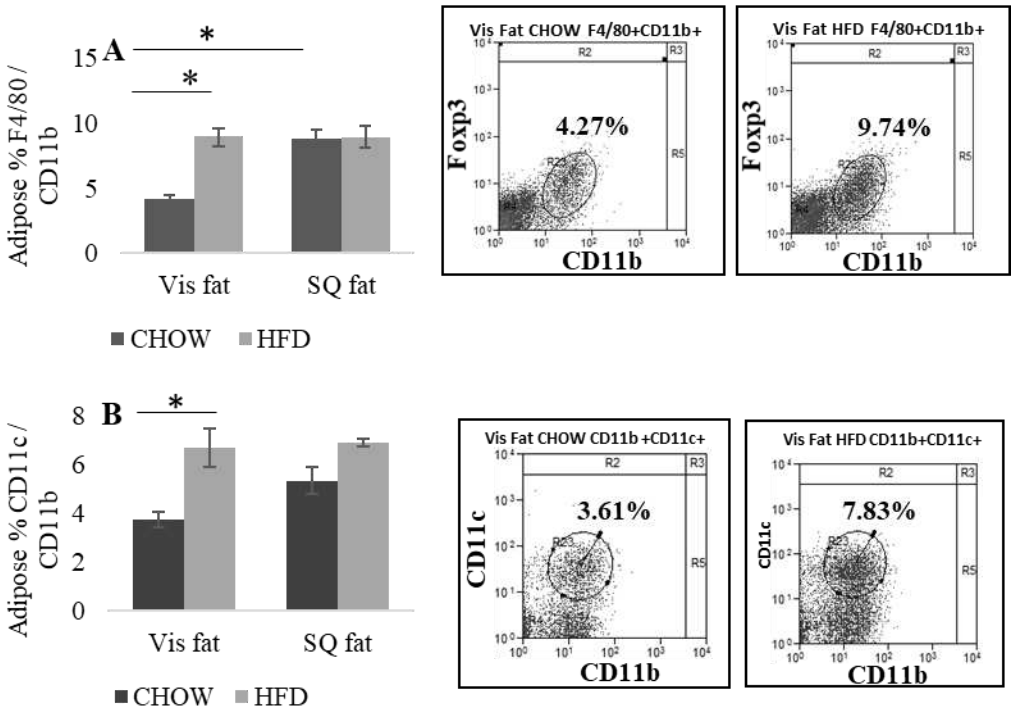


Figure 4.4 - Immune cell percent in visceral and subcutaneous adipose tissue depots. In CHOW mice the visceral adipose depot contained significantly lower percent of CD11b+F4/80+ macrophages (*=0.015) **A.)**. HFD altered immune cell populations within the lymph nodes. In the visceral lymph node HFD significantly increased CD11b+F4/80+ macrophages (*=0.0001) **A.)** and CD11c+CD11b+ dendritic cells (*=0.001) **B.)**.

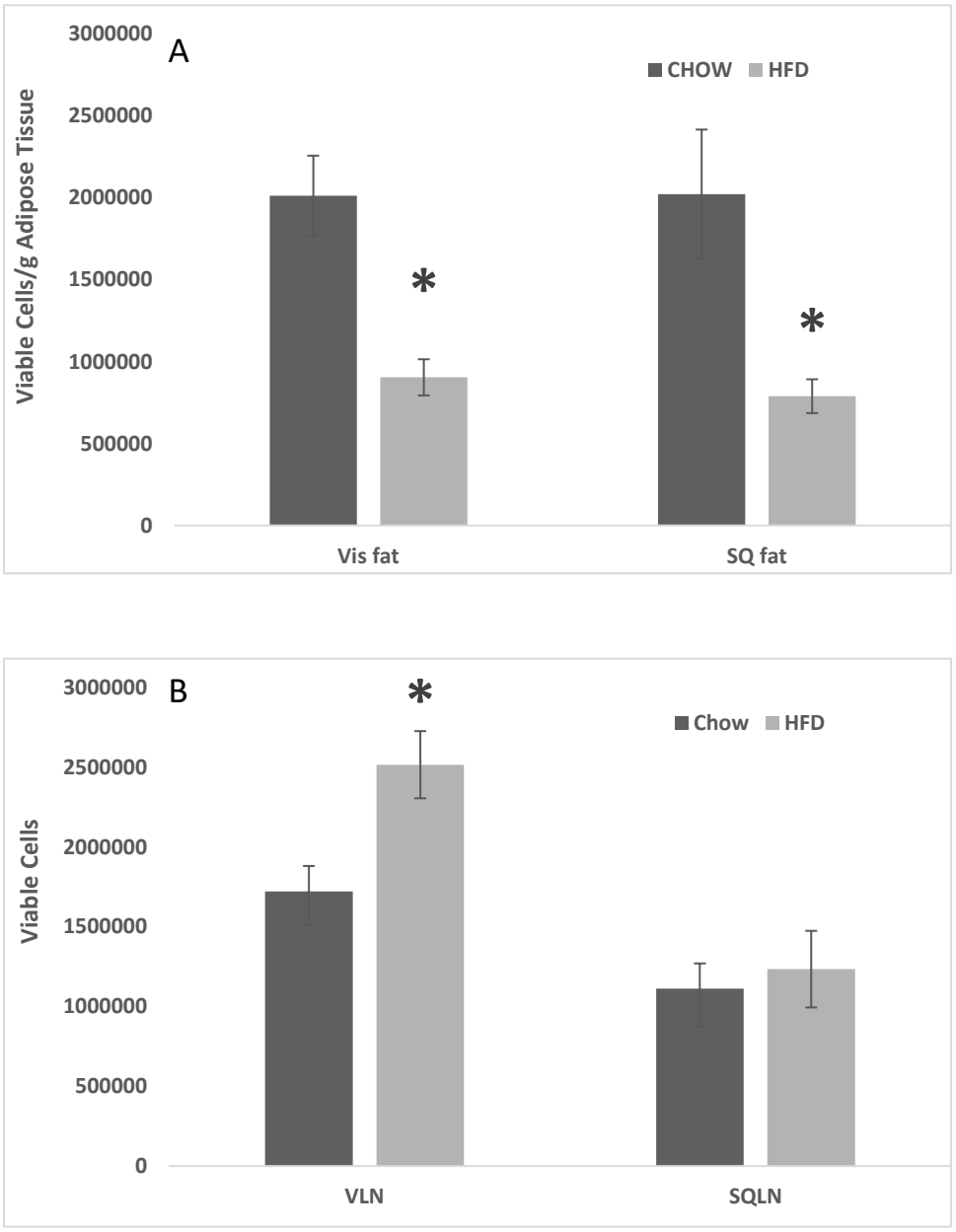


Figure 4.5 - Total viable cells of CHOW and HFD animals in visceral and subcutaneous adipose tissue (cells/g) and visceral and subcutaneous lymph nodes. Total viable cell count was not different between CHOW visceral and subcutaneous adipose depots **A.)**. Compared with controls, HFD caused a significant ~60% decrease in normalized (per gram adipose tissue) total viable cell number in both the visceral and subcutaneous adipose depots (* = 0.008). In the visceral only, HFD significantly increased lymph node viable cell number (* =0.01) **B.)**

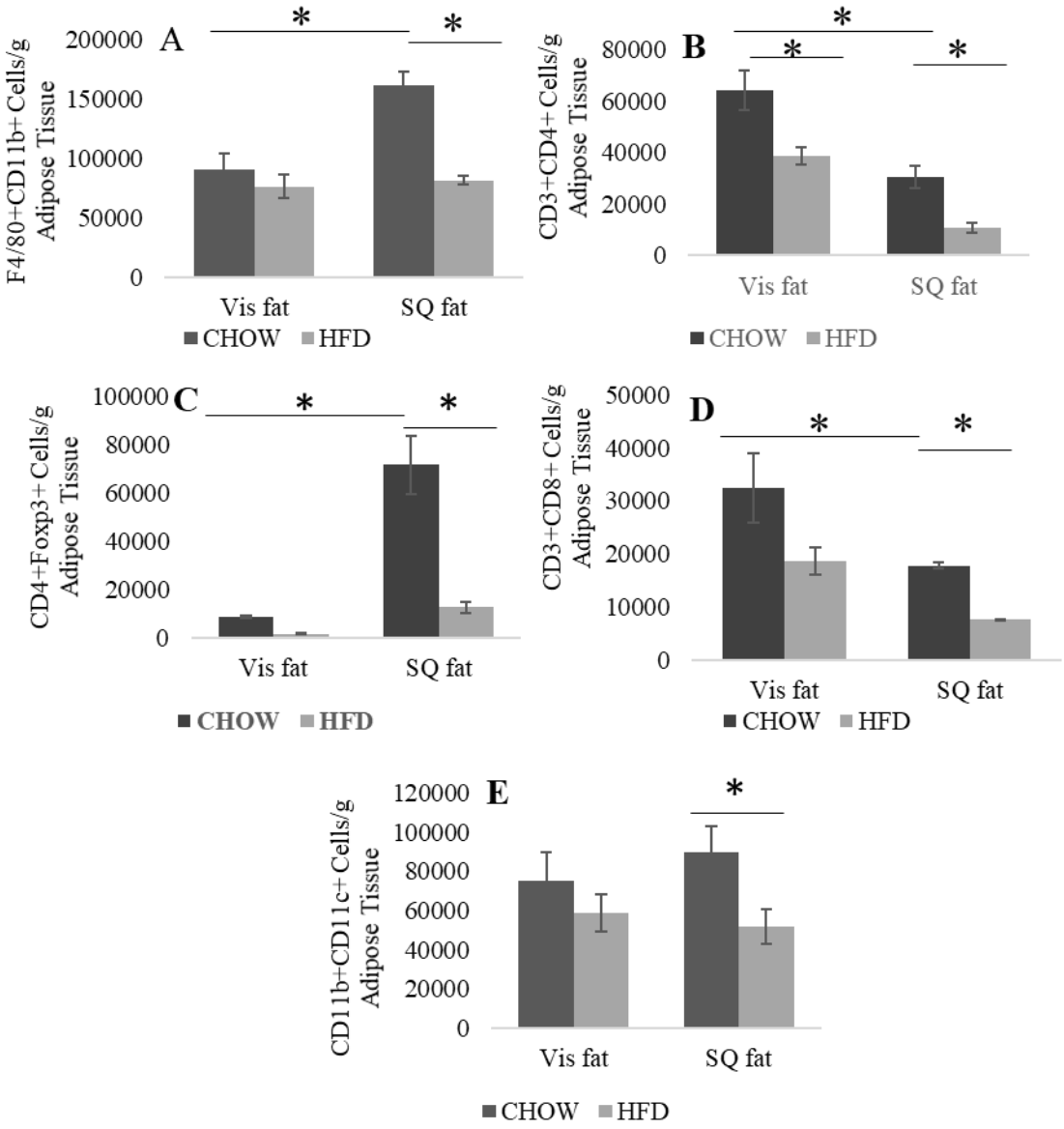


Figure 4.6 - Total viable immune cell numbers in visceral and subcutaneous adipose tissue (# cells/gram). In CHOW mice the total number of $CD3^+CD4^+$, T helper cells, (* = 0.0001) **B.)** and $CD3^+CD8^+$, cytotoxic T cells, (* = 0.015) **D.)** cells within the visceral adipose depot were significantly greater than those in the subcutaneous. $F4/80^+CD11b^+$, macrophages, (* = 0.0001) **A.)** and $CD4^+Foxp3^+$, T regulatory cell, (* = 0.0001) **C.)** number was significantly lower in the visceral adipose depot of CHOW mice compared with CHOW subcutaneous. HFD significantly reduced the viable number of $CD3^+CD4^+$, T helper cells, (* = 0.003) **B.)** in the visceral adipose depot and $F4/80^+CD11b^+$, macrophages, (* = 0.001) **A.)**, $CD3^+CD4^+$, T helper cells, (* = 0.003) **B.)**, $CD3^+CD8^+$, cytotoxic T cells, (* = 0.004) D) and $CD11b^+$ $CD11c^+$, Dendritic cells, (* = 0.045) **E.)** in the subcutaneous adipose depot relative to CHOW controls.

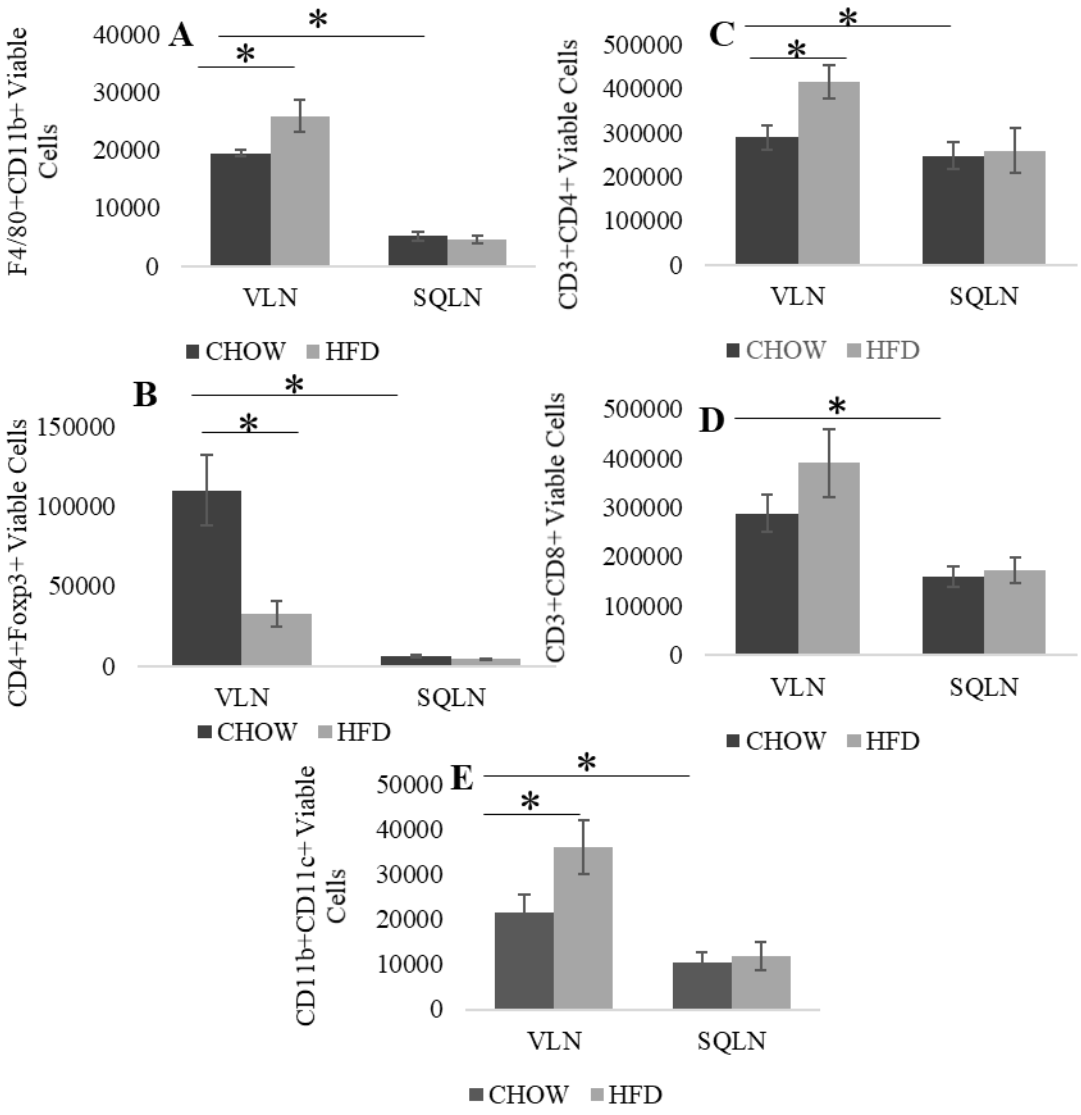


Figure 4.7 - Total viable immune cell numbers in visceral and subcutaneous lymph nodes. HFD significantly increased viable number of visceral lymph node F4/80^{+}CD11b^{+}, macrophages, (* = 0.0001) **A.)**, CD3^{+}CD4^{+}, T helper cells (* = 0.003) **C.)** and CD11c^{+}CD11b^{+}, dendritic cells (* = 0.23) **E.)** compared with respective CHOW controls. Visceral lymph node number of CD4^{+}Foxp3^{+}, T regulatory cells (* = 0.0001) **B.)** was significantly decreased in HFD mice compared with the CHOW controls. HFD did not change immune cell number in the subcutaneous lymph node.

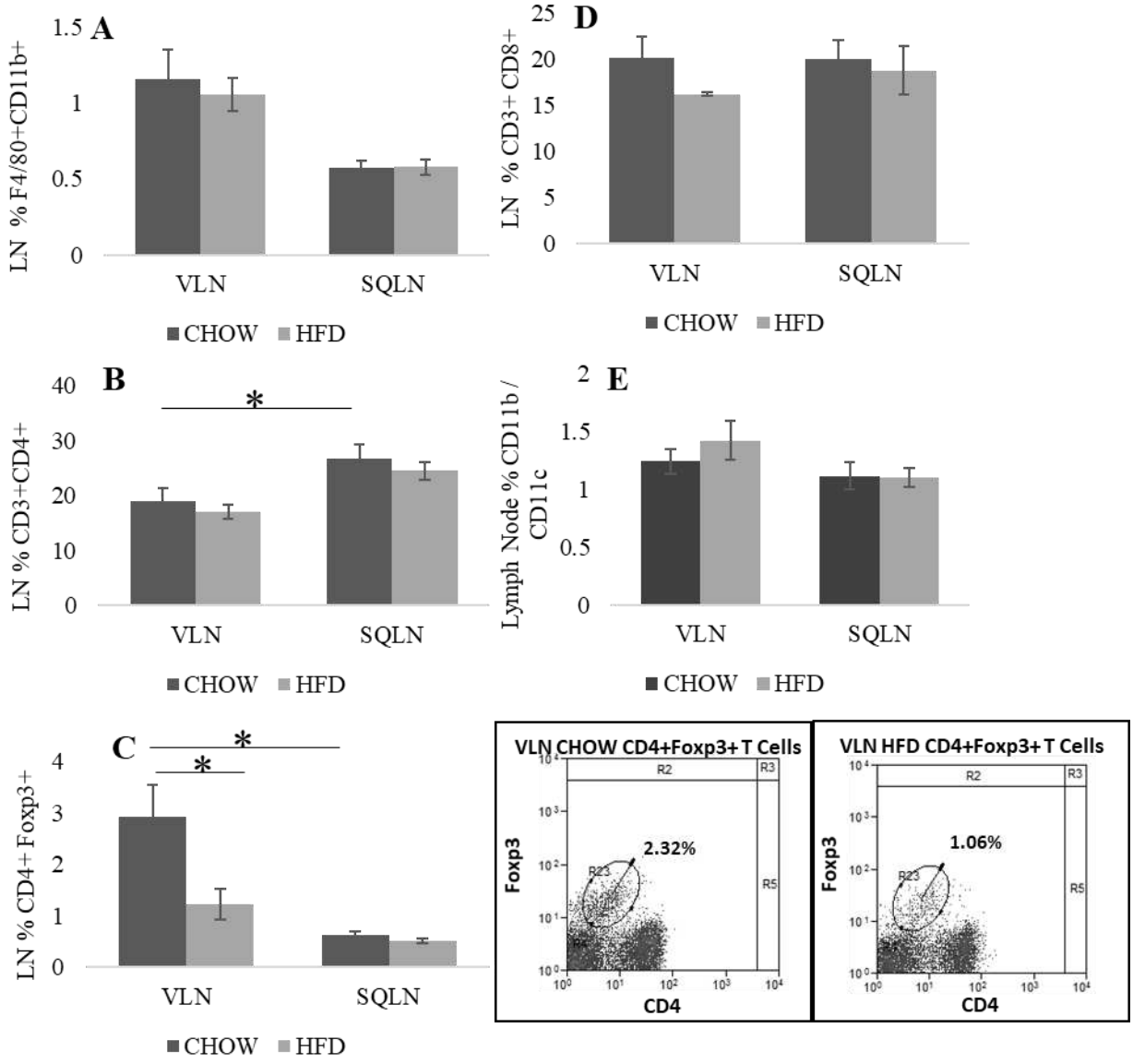


Figure 4.8 - Immune cell percent in visceral and subcutaneous lymph nodes. Visceral lymph nodes of CHOW mice contained a significantly greater percent of F4/80+CD11b^{+} macrophages (* = 0.004) **A.)** and CD4^{+}Foxp3^{+} regulatory T cells (* = 0.0001) **C.)** compared with CHOW subcutaneous lymph nodes. CD3^{+}CD4^{+} helper T cell percent (* = 0.012) **B.)**, however, was significantly lower in the visceral lymph nodes of CHOW mice. HFD significantly decreased the percent of CD4^{+}Foxp3^{+} regulatory T cells (* = 0.002) **C.)** in the visceral lymph node only.

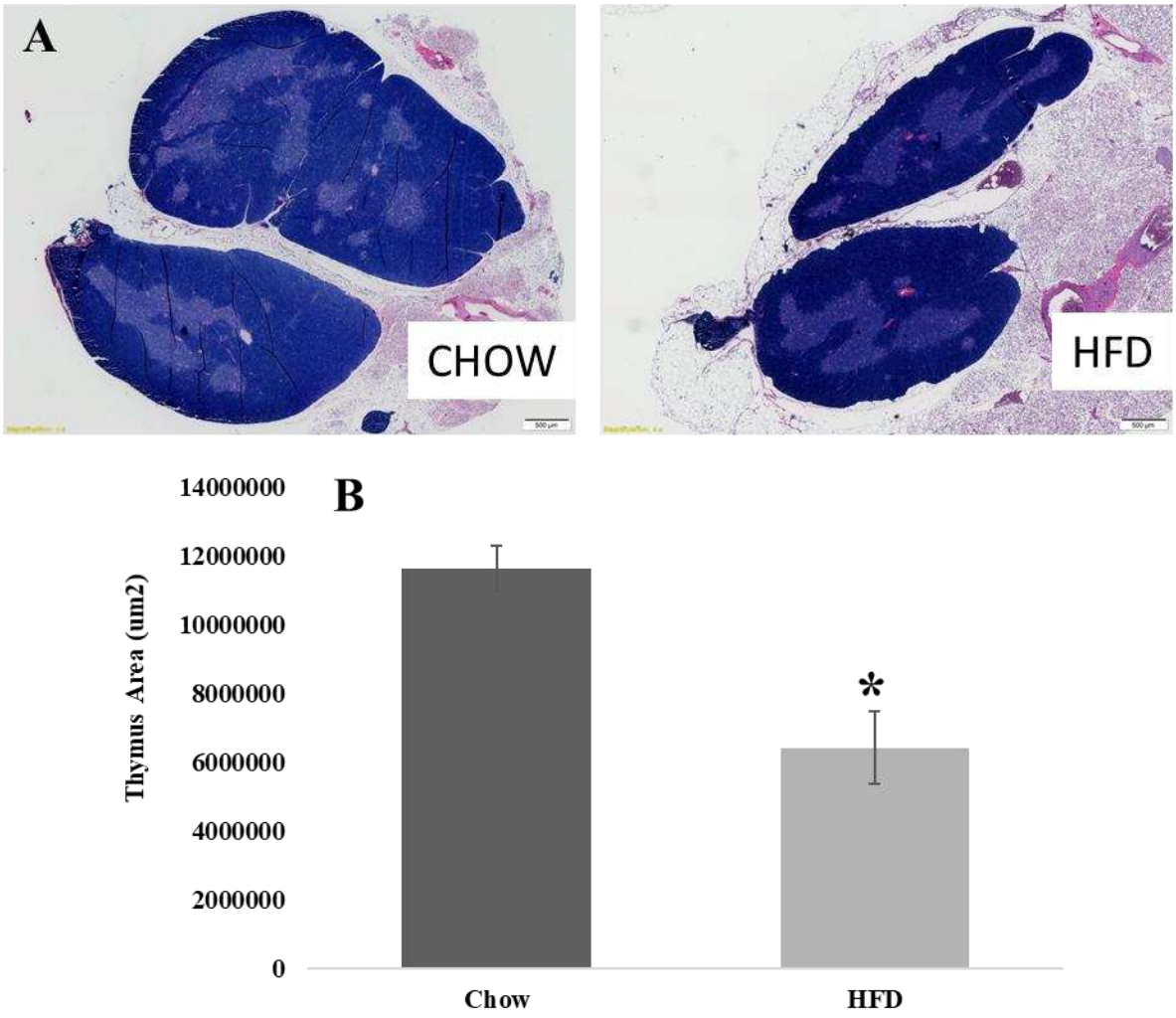


Figure 4.9 - Thymus area (µm^2) of CHOW and HFD fed animals. H&E stained CHOW and HFD **A.)** mouse thymus. HFD caused a significantly decrease, ~50%, in thymus area compared with CHOW (* = 0.014) **B.).** This reduction appeared secondary to generalized atrophy of both the cortex and medulla.

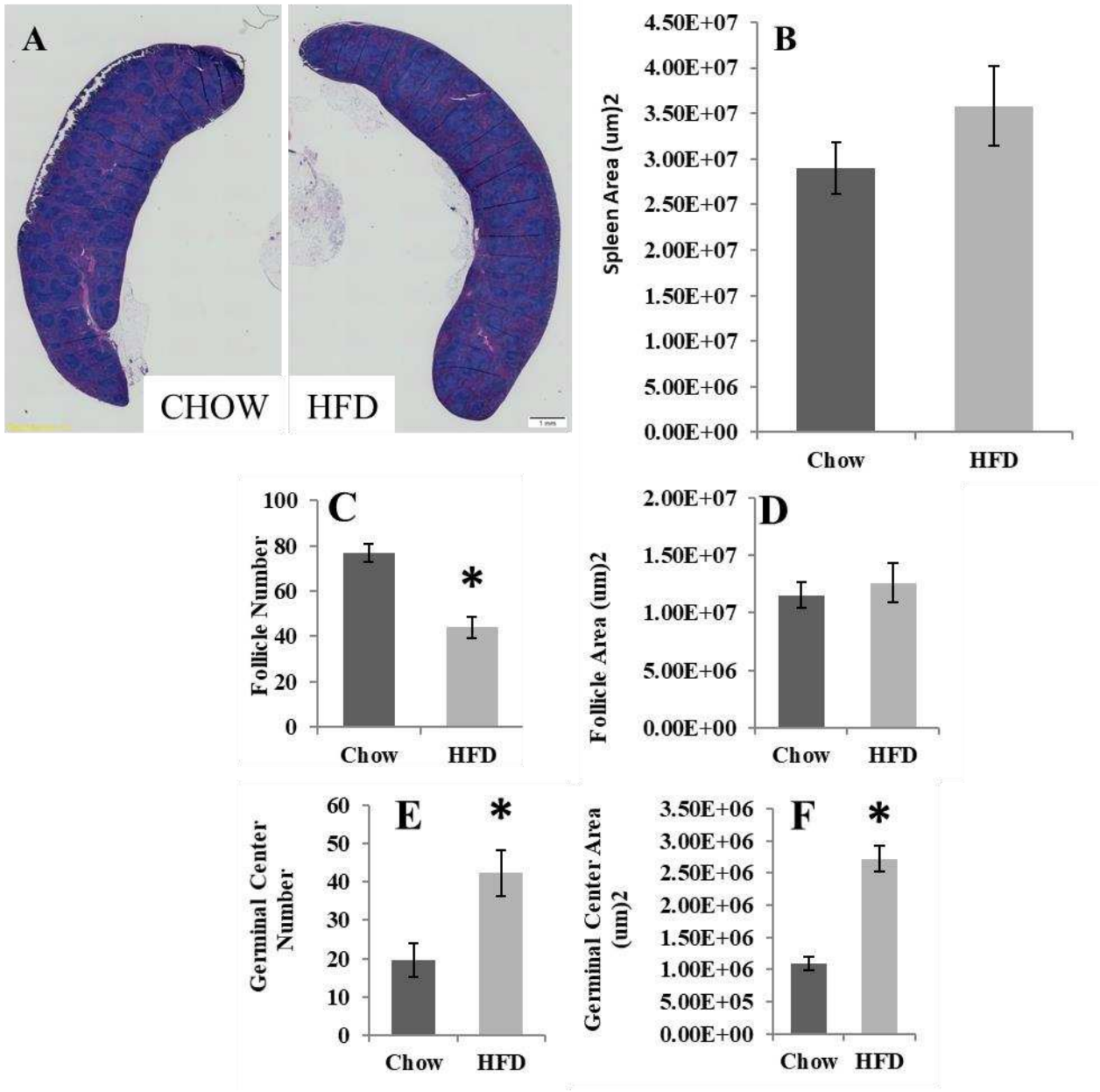


Figure 4.10 - Spleen area (µm^2) of CHOW and HFD fed animals. H&E stain of CHOW and HFD mouse spleen **A.)** HFD altered spleen architecture **A.),** without altering the total area of the spleen **B.)**. HFD significantly decreased overall spleen lymphoid follicle number, ~50%, compared with CHOW (* = 0.002) **C.)**. In HFD animals, the total follicle area was not changed compared with control **D.)**, but secondary lymphoid follicles in HFD animals significantly increased approximately 2 fold in number (* = 0.021) **E.)**. Secondary follicle total area of HFD mice was also significantly increased ~2 fold (* = 0.001) compared with CHOW animals **F.)**.

CHAPTER 5: VICSERAL ADIPOSE TISSE INFLAMMATION IS ASSOCIATED WITH LYMPH NODE FIBROSIS AND IMPAIRED IMMUNITY[9][10]

Summary

Obesity associated comorbidities and disease pathologies occur predominantly in people with greater visceral adipose tissue deposition compared to subcutaneous. This is due to a greater degree of inflammation associated with the visceral depot. We have previously demonstrated in a HFD mouse model that adipose tissue inflammation was associated with an immune like response including expansion of pro-inflammatory immune cell subsets and hypertrophy specific to the visceral lymph node. In contrast to these findings, others have demonstrated that 8-10 weeks of HFD induced obesity is associated with suppression of immunity that is specific to the visceral lymph node including a decrease in pro-inflammatory immune cell subsets associated with adaptive immunity. The purpose of this current experiment was to reproduce previous studies that demonstrate HFD suppressed immune function in the visceral lymph node as well as elucidate a potential mechanism that drives lymph node atrophy and immune suppression. Male C57BL/6 mice were fed a CHOW or high fat diet (HFD) for 7 or 13 weeks. At termination, the visceral and subcutaneous adipose tissues and lymph nodes were collected. Immune cell populations in these tissues were determined by flow cytometry, collagen deposition with in the lymph nodes was determined my massons trichrome staining, and proliferative capacity of lymph node T cells determined by mitogen stimulation assay. At 7 weeks CHOW visceral tissues

[9] My contribution to this chapter included the bulk of the analysis, statistics, and interpretation of all data. I wrote the bulk of the manuscript and created all tables and figures.

[10] Table and figure numbers have been modified to reflect that they are specific to this chapter, e.g. Figure 1 is now Figure 5.1.

were demonstrated to have a greater potential for pro-inflammation and reactivity to HFD. 13 weeks HFD was associated with a significant decrease in total and specific subsets of viable immune cells specific to the visceral and adipose tissue and lymph nodes. Alterations in immune cells were associated with significant collagen deposition within the visceral lymph node but not subcutaneous. Consistent with reductions of viable immune cell populations, mitogen stimulation of T cells from the HFD visceral lymph node showed a reduced proliferative capacity. Overall the reductions in viable immune cell subsets specific to the visceral cavity are likely, in part, a result of lymphatic tissue fibrosis. We propose that this fibrosis is a consequence of visceral adipose tissue inflammatory signals.

Introduction

Obesity among Americans has become strikingly prevalent reaching a high of ~36% of adults [1, 214]. As the rate of obesity rises, so do the number of individuals with chronic diseases associated with excessive adipose tissue accumulation [215]. Dysregulation of adipose tissue is a fundamental driver of obesity-associated co-morbidities [216, 217], specifically accumulation of visceral adipose tissue (fat stored within the peritoneal cavity among visceral organs) [218, 219]. In opposition, subcutaneous adipose tissue (fat that is stored between the muscle and the skin) accumulation is associated with metabolic protection [220, 221]. Adipose tissue induced pro-inflammation is the casual link toward increased incidence of chronic disease and immune associated pathologies [222, 223]. Hence, the increased risk of disease associated with excessive adipose tissue accumulation in obesity is likely linked to an increased capacity for pro-inflammation within the visceral depot [20, 224]. We propose to examine how visceral adipose tissue pro-inflammation uniquely contributes to processes that regulate and drive obesity associated inflammation.

Inflammation is regulated by the immune system in response to injury resulting from stimuli including, but not limited to, tissue/cell damage, pathogens, and metabolites [225]. Lymph nodes, secondary lymphatic tissues, are important components of the immune system as a staging ground for immune responses to pathogen infection [226]. Lymph nodes are predominately embedded within adipose tissue [227]. This means lymph nodes and immune cells incased within are constantly exposed to adipose depot effluent and are continuously surveying and monitoring adipose for exposure to noxious stimulus such as harmful metabolites [52, 126, 228]. During obesity the close proximity of immune system lymph nodes and adipose tissue may be particularly deleterious because of the pro-inflammatory cytokine release coming from the dysregulated depot.

In a HFD mouse model we previously demonstrated a relation between elevated visceral adipose tissue cytokines associated with metabolic disease and (i.e. 1l-1β, IL-6, TNFα and MCP-1) and lymph node morphology and function (In press). Specifically, 7 weeks of HFD intake caused visceral lymph node swelling, ~2x larger [229], which is indicative of an adaptive immune response that typically involves an increase in immune cells [230]. Consistent with this we demonstrated HFD-induced lymph node swelling was due to an increase in total viable immune cell populations contained within [229]. Lymph node alterations associated with obesity were specific to the visceral lymph node because subcutaneous adipose tissue and lymph nodes did not show significant changes in response to HFD [231]. Together, these observations indicate that visceral adiposity is indeed associated with heightened inflammation and greater propensity to alteration by HFD induced obesity relative to the subcutaneous depot.

In contrast to our findings in 7 week HFD mice, other studies indicate that HFD is associated with decreased immune function. In particular, others show that a HFD consisting of

60% fat for for 8-10 weeks, induces a 3.4 fold reduction in the size of lymph node, specifically the subcutaneous, and inhibited dendritic cell migration from adipose tissue into the lymph node [52]. This study, however, did not evaluate changes to the visceral lymph node, which we postulate to have a greater association with metabolic disease associated with obesity. A study by Kim et al also found that 10 weeks of HFD consisting of 45% fat induced mesenteric lymph nodes atrophy, reducing weight by ~50% relative to CHOW controls [232]. This was associated with a reduction in total number of immune cells contained within the visceral lymph node. Specific immune cell subsets decreased included, CD4 and CD8 positive t cells, regulatory T cells, macrophages, and dendritic cells. The authors conclude that HFD-induced lymph node alterations were responsible for obesity-related immune dysfunction [232]. As previously stated our previous study opposed the above findings.

The purpose of the current experiment is twofold. First we aim to recapitulate previous studies that demonstrate HFD induces a decline in lymph node function. Second, we propose to elucidate the mechanisms that drive lymph node atrophy and the decline of immune cells incased within. Since obesity links to fibrosis in numerous tissue [233-237] we suspect it may be playing a role in lymph node pathophysiology. The most relevant example is adipose tissue fibrosis, which is induced by increased levels of hypoxia as fat pads expand in obesity. This leads to activation of pro-inflammatory pathways and increased deposition of collagen in the extracellular matrix (ECM). Subsequently, pro-inflammatory signaling is activated that recruits immune cells to the adipose tissue. This further potentiates the adipose tissue inflammation driving fibrosis. To examine this mice will be fed HFD 45% fat for 13 weeks. Visceral and subcutaneous adipose tissue and lymph nodes will be collected to evaluate immune cell subset response.

Methods

Animals and diet

Male C57BL/6 mice (Jackson Laboratory, Bar Harbor, Maine) (n=10 per group, 2-3 months, 27.7±0.22 g) were single housed under controlled conditions (12:12 light-dark cycle, 50%-60% humidity, and 25 C). Mice were allowed 1 week of acclimation prior to experiment start. Mice were given free access to water and a standard CHOW (Harlan Teklad LM485, Madison, WI, USA, 3.1 kcal/g, 18% from fat) or Western high-fat diet (HFD: Harlan Teklad, TD.08811, Madison, WI, USA, 4.7 kcal/g, 45% kcal from fat) until termination at 13 weeks. Bi-weekly measures of body mass and food intake were taken. All procedures underwent review and approval by the Colorado State University Institutional Animal Care and Use Committee.

Terminal procedures (blood collection and tissue harvesting)

Mice were anesthetized, systemic blood was collected and serum was stored at -80 C. Adipose tissue and lymph nodes were collected from the visceral and inguinal depots. Tissues were immediately stored in either Hank's balanced salt solution (HBSS) or fixative. The visceral adipose tissue depot is located within the peritoneal cavity among the intra-abdominal organs including the gastrointestinal tract. The inguinal adipose tissue depot (lower body subcutaneous adipose tissue) is located between the muscle and the skin directly above the femoral muscle of the leg. The lymph node within the visceral cavity is a nodule chain whereas the subcutaneous nodes are singular bilateral structures. Other tissues collected include the spleen, placed in both HBSS and fixative.

Separation of immune cells and stromal tissues

Immune cell isolation was done as previously described [229]. Briefly, lymph nodes and adipose tissue were stored on ice in HBSS_5% fetal bovine serum (FBS) and then passed

through a 40 µm cell strainer. Bilateral subcutaneous lymph nodes were processed together. Single cell suspensions were centrifuged and then re-suspended in Fluorescence-activated cell sorting (FACS) buffer for immunostaining. Spleen cells were treated using Ammonium-chloride-potassium (ACK) lysis buffer to lyse red blood cells prior to re-suspension in FACS buffer. Adipose tissue was processed using 2X collagenase D (Roche, Fisher cat no. 50-720-3639, Roche Molecular Diagnostics, Pleasonton, CA, USA), triturated and washed with HBSS+5% FBS to digest the extra cellular matrix prior to re-suspension in FACS buffer.

Flow cytometry

Total and viable cell counts were obtained via trypan blue exclusion dye and counted using a Cellometer (Nexcelom, Lawrence, MA, USA). Based on total and viable cell count results, cells were plated for immunostaining between $5x10^5$ and $5x10^6$ cells per well in round bottom 96 well plates. The actual number of cells plated was dependent upon the total number of viable cells that were recovered from the tissue. The following rat-anti mouse clones were used for immunostaining at a dilution of 1:200 for all primary antibodies: (APC panel) Major Histocompatibility complex II (MHCII) (clone M5/114.15.2. cat. No. 17-5321-81; eBioscience), F4/80 (clone BM8, cat. No. 25-5931-82; eBioscience), CD11c (clone N418, cat. No. 417-0114-82; eBioscience), CD11b (clone M1/70, cat. no. 48-0112-82; eBioscience), (T and B cell panel) B220 (clone RA3-6B2, cat. no. 17-0452-82), intracellular Foxp3 (clone FJK-16a, cat. no. 12-5773-82; eBioscience), CD3 (clone 145-2c11, cat. no. 11-0031-82; eBioscience), CD8a (clone 53-6.7, cat. no. 13-0081-85; eBioscience), and CD4 (clone RM4-5, cat. no. 48-0042-82; eBioscience). Biotinylated primary antibody detection was done using secondary streptavidin-pacific orange (cat. no. 532365; Invitrogen, Carlsbad, CA, USA) at a 1:500 dilution. Intracellular detection of Foxp3 was done utilizing a permeabilization (cat. no. 00-5521; eBioscience) in

accordance with kit instructions. After staining, cells were fixed using 4% paraformaldehyde (PFA), then washed and stored in FACS buffer until analysis. Flow analysis was done as outlined in a previous study [229].

Histology

Histology was performed on both the visceral and subcutaneous lymph nodes. After excision, nodes were fixed in 10% neutral buffered formalin for 24 hours and then embedded in paraffin wax for haematoxylin and eosin (H&E) staining as well as Trichrome staining (Sigma Aldrich) for collagen. Trichrome stain was used in accordance with the manufacturer protocol. The area of lymph nodes was assessed by the sum of 4 random, yet evenly spaced, sections from the largest mid-region portion of each separate node. Quantification was blinded. Stained lymph node sections were analyzed using CellSens Dimension software (Version 1.15, Olympus, Shinjuku, Tokyo, Japan). Collagen area within lymph nodes was analyzed using imageJ. Trichrome stains collagen blue, as such, these locations were manually selected and counted across 4 representative images in order to quantify the total amount of collagen in the sample.

Cytokine assay

Inflammatory protein factors were assessed using a pre-designed cytokine assay for Mesoscale (Rockville, Maryland) and a single IL-13 Mesoscale assay (Rockville, Maryland). Visceral and subcutaneous lymph nodes and adipose tissues were excised from euthanized mice and immediately frozen using liquid nitrogen for storage. Prior to analysis, cytokines were extracted from frozen tissues via sonication per manufacturer's instructions. Both Mesocale assays were run according to the kit protocols.

Statistics

Data are expressed as a mean ± standard error of the mean (SEM). Means with different letters are significantly different. One way analysis of variance (ANOVA) (IBM SPSS for Windows; SPSS, Chicago, IL, USA) was used to analyze food intake, body weight, collagen area of the lymph nodes and lymph node and adipose tissue immune cell percent and viable counts. Post hoc analysis for these tests was done using Least Significant Difference (LSD) tests. In the results, one-way ANOVA data were applied to first discuss anatomical differences among CHOW animals only (Ex. Visceral CHOW vs Subcutaneous CHOW differences) and then to analyze how diet changes immune cells within a specific tissue (Ex. Subcutaneous lymph node CHOW vs Subcutaneous lymph node HFD). These tests were performed separately for each 7 and 13 week time point. Statistical analysis of lymph node area measures using two-way ANOVA between subjects with location (visceral and subcutaneous) and diet (CHOW and HFD) as the factors. Post hoc analysis for two-way ANOVA were done utilizing LSD tests.

Results

Food intake and body weight

Cumulative Kcals ingested over 7 weeks was significantly higher in mice fed a HFD (n=5) compared with CHOW (n=5) (Table 5.1, p= 7.3E-5, one-way ANOVA, LSD post-test). Increased intake of kcals was associated with a significant increase in body mass (Table 5.1, p= 1.67E-9). Consistent with this, individual white adipose tissue depots, visceral, epididymal, perirenal and inguinal, and total adiposity (previous adipose tissue depots added together) were significantly increased in the HFD group (data not shown).

Cumulative Kcals ingested over 13 weeks was significantly higher in mice fed a HFD (n=5) compared with CHOW (n=5) (Table 5.1, p=7.3E-5), one-way ANOVA, LSD post-test).

Body mass (Table 5.1, p=3.21E-9, one-way ANOVA, LSD post-test) and total adiposity (Table 5.1, p=2.17E-10, one-way ANOVA, post-test LSD) were also increased in HFD mice compared with CHOW.

7 Week lymph node histology

7 week HFD mice had an increase in visceral lymph node size (~2 fold) (Figure 5.1C p=), but not subcutaneous, compared to CHOW controls. The visceral lymph node of HFD mice also had an increase in fibrosis primarily located in the sub-capsular region (Figure 5.1A, CHOW Vs HFD; P=0.002) compared to CHOW controls. HFD was also associated with a significant increase in fibrosis to the sub-capsular region of the subcutaneous lymph node (figure 5.1B, CHOW SQ Sub Cap VS HFD SQ Sub Cap; P=0.014) but not the center region of the node.

7 week adipose tissue and lymph node immune cells

Adipose tissue immune cell percent CHOW differences

The visceral and subcutaneous adipose tissue depots had inherent differences in populations of distinct immune cells subsets; this is demonstrated in respective CHOW control mice. Specifically, the visceral adipose depot of CHOW fed mice had a significantly greater percent of CD3+ T cells sets (Figure 5.3D, CHOW VIS fat VS CHOW SQ fat; P=0.022) including CD3+CD4+ helper T cells (Figure 5.3B, one-way ANOVA, LSD post-test, CHOW Vis fat VS CHOW SQ fat; P=0.02) and CD3+CD8+ cytotoxic T cells (Figure 5.3C, CHOW Vis fat VS CHOW SQ fat; P=0.002) compared with the respective subcutaneous depot. In contrast, the CHOW visceral depot contained a significantly lower percent of CD4+FoxP3+ regulatory T cells (Figure 5.3A, CHOW VIS FAT VIS CHOW SQ FAT; P=7.8E-7) compared with the subcutaneous depot.

Adipose tissue immune cell percent HFD alterations

Immune cells within the visceral and subcutaneous adipose depots, however, differentially responded to 7 weeks of HFD intake. In particular, in the visceral adipose tissue HFD intake significantly decreased the percent of CD3+ T cells (Figure 5.3D, CHOW Vis fat VS HFD Vis fat; P=0.043), specifically CD3+CD4+ helper T cells (Figure 5.3B CHOW Vis fat vs HFD Vis fat; P=0.004), compared with control. In the subcutaneous depot, however, CD4+Foxp3+ Tregs percent (Figure 5.3A, CHOW SQ fat VS HFD SQ fat; P=7.7E-6) was significantly decreased with HFD intake.

Adipose tissue viable cell CHOW differences

Adipose tissue viable cell numbers were normalized (number of cells per gram of adipose tissue) to account for immune number differences that may be driven by adipose depot size. When normalized the visceral adipose of CHOW mice contained the same number of viable immune cells as the subcutaneous depot (Figure 5.2A). Total number of CD3+CD8+ cytotoxic T cells (Figure 5.3G, CHOW Vis VS CHOW SQ; P=0.013) and CD3+ T cells (Figure 5.3H, CHOW Vis VS CHOW SQ; P=0.002) were significantly higher in visceral adipose tissue than the subcutaneous adipose tissue of CHOW fed mice. In contrast, CD4+Foxp3+ regulatory T cells (Figure 5.3E, CHOW Vis vs CHOW SQ; P=7.03E-8) were significantly lower in the visceral adipose tissue relative to subcutaneous of CHOW mice.

Adipose tissue viable cell HFD alterations

Significant decreases in several viable immune cell subsets were observed in response to HFD. In the visceral adipose depot the only HFD alteration was a significant reduction in CD3+CD4+ helper T cells (Figure 5.3F, CHOW Vis Fat VS HFD Vis fat; P=0.004). In the

subcutaneous adipose depot, HFD diet significantly reduced CD3+CD4+ helper T cells (Figure 5.3F, CHOW SQ fat VS HFD SQ fat; P=0.044) and CD3+CD8+ cytotoxic T cells (Figure 5.3G, CHOW SQ fat VS Vis fat; P=0.016) as well as CD4+Foxp3+ Tregs (Figure 5.3E, CHOW SQ fat VS HFD SQ fat; P=3.0E-7).

Lymph node immune cell percent

Unlike the adipose tissue, there were no inherent differences in the frequency of immune cell populations within the visceral and subcutaneous lymph nodes of CHOW mice.

Lymph node immune cell percent HFD alterations

7 weeks of HFD differentially altered several subsets of immune cells in both the visceral and subcutaneous lymph nodes. Within the visceral lymph node, HFD resulted in a significant increase in the frequency of CD3+CD8+ cytotoxic T cells (Figure 5.5C, CHOW VLN VS HFD VLN; P=0.001) and significant decreases in CD11b+F4/80+ macrophages (Figure 5.6A, CHOW VLN VS HFD VLN; P=0.015) relative to CHOW controls. Within the subcutaneous lymph node the frequency of CD3+CD4+ helper T cells (Figure 5.5B, CHOW SQLN VS HFD SQLN; P=0.001) was increased while frequency of CD11b+F4/80+ macrophages (Figure 5.6A, CHOW SQLN VS HFD SQLN; P=0.018) and CD11b+CD11c+ dendritic cells (Figure 5.6B, CHOW SQLN VS HFD SQLN; P=0.01) were decreased relative to CHOW controls.

Lymph node viable cell HFD alterations

In mice, the visceral lymph node is inherently larger than the subcutaneous. Hence, distinct viable immune cell subsets within the visceral lymph node were always significantly greater than those in the subcutaneous, thus comparisons among location will not be discussed.

Within the visceral lymph node, 7 weeks of HFD increased total viable immune cell populations, however this was not significant, (Figure 5.2B). Consistent with this several viable

immune subsets were altered in HFD mice. In the visceral lymph node HFD intake significantly increased CD3+ T cells (Figure 5.5H, CHOW VLN VS HFD VLN; P=0.001), including CD3+CD4+ helper (Figure 5.5F, CHOW VLN VS HFD VLN; P=1.67E-5) and CD3+CD8+ cytotoxic T cells (Figure 5.5G, CHOW VLN VS HFD VLN; P=0.001). HFD intake, however, significantly decreased CD11b+CD11c+ dendritic cells compared with CHOW (Figure 5.6D, CHOW VLN VS HFD VLN; P=0.013). Subcutaneous lymph node total viable immune cell numbers were similar between CHOW and HFD groups. The only HFD diet alteration to occur in the subcutaneous lymph nodes was an increased CD3+CD4+ helper T cells (Figure 5.5F, CHOW VLN VS HFD VLN; P=0.013) compared to CHOW controls.

Lymph node histology 13 weeks

HFD increased visceral lymph node, but not subcutaneous, area by ~2 fold (Figure 5.8, CHOW VLN VS HFD VLN; P=4.27E-6) compared to CHOW controls. Total fibrosis in the visceral lymph node was significantly greater in HFD mice compared with CHOW in the sub-capsular (Figure 5.7A, CHOW VLN Sub Cap VS HFD VLN Sub Cap; P= 2.33E-4) and center regions (Figure 5.7A, CHOW VLN Center VS HFD VLN Center; P=0.001). These areas included fibrosis across the cortical, paracortical and medullary areas. HFD intake, however, did not significantly alter collagen deposition in the subcutaneous lymph node relative to CHOW controls (Figure 5.7B). Figure 5.7 C-F are representative images of collagen deposition in CHOW visceral (Figure 5.7C-D) and HFD visceral (Figure 5.7E-F) lymph nodes.

13 week adipose tissue and lymph node immune cells

Adipose tissue immune cell percent CHOW differences

The visceral and subcutaneous adipose tissue depots of 13 week HFD mice had inherent differences in populations of distinct immune cell subsets, this is demonstrated in respective

CHOW control mice. Specifically, visceral adipose tissue contained significantly greater populations of CD3+CD4+ helper T cells (Figure 5.9B, CHOW Vis fat VS CHOW SQ fat; P=0.036), and CD3+CD8+ cytotoxic T cells (Figure 5.9C, CHOW Vis fat VS CHOW SQ fat; P=0.04) compared with CHOW subcutaneous adipose tissue. In contrast, the visceral depot contained a significantly smaller percent of CD4+Foxp3+ Tregs (Figure 5.9A, CHOW Vis fat VS CHOW SQ fat; P=0.017).

Adipose tissue immune cell percent HFD alterations

Immune cell populations within the visceral and subcutaneous adipose tissue depots were minimally altered by HFD. Compared with CHOW controls, HFD significantly decreased the frequency of only CD3+CD4+ helper T cells (Figure 5.9B, CHOW Vis fat VS HFD Vis fat; P=0.022) in the visceral adipose tissue. In subcutaneous adipose tissue only the frequency of CD3+CD4+ helper T (Figure 5.9B, CHOW SQ fat VS HFD SQ fat; P=0.024) cells was increased relative to CHOW controls with no decreases to any of the immune cell subsets.

Adipose tissue viable cells CHOW differences

The chow visceral adipose tissue contained a significantly greater number of total viable immune cells than the subcutaneous (Figure 5.11A, CHOW Vis fat VS CHOW SQ fat; P=7.7E-6). Consistent with this several viable cell subset populations within the CHOW visceral adipose tissue were significantly greater than the subcutaneous CHOW. Specifically, CD3+CD4+ helper T cells (Figure 5.9F, CHOW Vis fat VS CHOW SQ fat; P=0.001), CD3+ CD8+ cytotoxic T cells (Figure 5.9G, CHOW Vis fat VS CHOW SQ fat; P=3.43E-4), CD11b+CD11c+ dendritic cells (Figure 5.10D, CHOW Vis fat VS CHOW SQ fat; P=0.009), and CD3+ T cells (Figure 5.9H, CHOW Vis fat VS CHOW SQ fat; P=0.015) were all elevated in the visceral compared to subcutaneous adipose tissue.

Adipose tissue viable cells HFD alterations

Compared with respective controls, HFD caused a significant 63.5 and 52.4 percent decrease in total viable cells (per gram of adipose tissue) in both the visceral and subcutaneous adipose tissue depots (Figure 5.11A, CHOW Vis fat VS HFD Vis fat; P=9.21E-7 and CHOW SQ fat VS HFD SQ fat; P=0.018). As a result multiple immune cell subsets were decreased in the visceral adipose depot of HFD mice relative to CHOW controls. Specifically, HFD resulted in decreases to CD4+Foxp3+ Tregs (Figure 5.9E, CHOW Vis fat VS HFD Vis fat; P=0.046), CD3+CD4+ helper T cells (Figure 5.9F, CHOW Vis fat VS HFD Vis fat; P=3.9E-4), CD3+CD8+ cytotoxic T cells (Figure 5.9G, CHOW Vis fat VS HFD Vis fat; P=1.68E-4), CD11b+CD11c+ dendritic cells (Figure 5.10D, CHOW Vis fat VS HFD Vis fat; P=0.002), and CD3+ T cells (Figure 5.9H, CHOW Vis fat VS HFD Vis fat; P=0.002). The only viable immune cell alteration to occur within HFD subcutaneous adipose depot was a decrease in the number of CD3+Foxp3+ Treg cells (Figure 5.9E, CHOW Vis fat VS HFD Vis fat; P=0.046) relative to CHOW controls.

Lymph node immune cell percent CHOW differences

Frequency of immune cell subsets were not strikingly different within the visceral and subcutaneous lymph nodes of CHOW mice. Specifically, the only significant difference among the CHOW animals was an elevation in the percent of CD4+Foxp3+ Tregs in the visceral lymph nodes compared to subcutaneous of CHOW mice (Figure 5.12A, CHOW VLN Vs CHOW SQLN; $P=3.85E^{-5}$).

Lymph node immune cell percent HFD alterations

13 weeks of HFD resulted in few alterations to immune cell frequency in the visceral or subcutaneous lymph nodes. In the visceral lymph node of HFD mice there was only a significant

decrease in CD4+Foxp3+ Tregs (Figure 5.12A, CHOW VLN VS HFD VLN; P=1.31E-5) compared with CHOW controls. In the subcutaneous lymph node the only significant shift to immune cell frequency was a significant increase in CD3+CD8+ cytotoxic T cells (Figure 5.12C, CHOW SQLN VS HFD SQLN; P=0.017).

Lymph node viable cell HFD alterations

As previous discussed differences between lymph node immune cell numbers will not be discuss due to inherent size differences between the visceral and subcutaneous lymph nodes. Despite few alterations to immune cell frequency, there were numerous significant changes in viable populations in both the visceral and subcutaneous lymph nodes. First, 13 weeks of HFD was associated with a significant decrease in total viable cells within the visceral but not subcutaneous lymph node compared to chow controls (Figure 5.11B, CHOW VLN VS HFD VLN; P=0.019). Consequently numbers within immune cell subsets were also altered by HFD. Specifically, HFD caused significant decreases to visceral lymph node CD4+Foxp3+ Tregs (Figure 5.12E, CHOW VLN VS HFD VLN; P=4.73E-8), CD3+CD4+ helper T cells (Figure 5.12F, CHOW VLN VS HFD VLN; P=0.015) and CD11b+F480+ macrophages (Figure 5.13C, CHOW VLN VS HFD VLN; P=0.002) compared with CHOW controls. Within the subcutaneous lymph node the only HFD-induced shift in viable immune cell population was a significant increase to CD3+CD8+ cytotoxic T cells (Figure 5.12G, CHOW SQLN VS HFD SQLN; P=0.017).

13 week cell viability

A cell culture assay was used to determine if 13 weeks of HFD inhibited T cell proliferation induced by ConA, a T-cell stimulator. Immune cells collected from HFD visceral lymph node showed significantly reduced total (Figure 5.14A, CHOW VLN Vs HFD VLN; P=

6.83E-7) and viable (Figure 5.14B, CHOW VLN Vs HFD VLN; P= 0.004) numbers after 72 hours of cell culture compared with CHOW controls. Cells cultured from within the HFD subcutaneous lymph node showed no difference in total or viable cells at 72 hours compared with chow controls.

Discussion

Dysregulation of adipose tissue endocrine function and excessive production of deleterious pro-inflammatory cytokines is suggested to be a significant driver of numerous obesity associated diseases and co-morbidities [12, 238-241]. It is well established that the comorbidities of diet induced obesity have a greater association with visceral adipose tissue expansion than subcutaneous [242, 243]. Lymph nodes, which are predominately embedded in adipose tissue, are fundamental barriers and staging areas for immune cell response toward tissue injury or pathogens. Adipose tissue dysregulation, such as excessive pro-inflammation, could have deleterious effects on lymph node function. However this area of research has received little attention. In the present study, we demonstrate that the visceral adipose tissue has greater pro-inflammatory potential than the subcutaneous and a greater reactivity towards HFD. This data supports that immunity within the visceral lymph node is plastic and dynamic where earlier time points suggest a robust response to HFD, whereas later time points indicate exhaustion with declines in immune cell number. Overall, a chronic noxious stimulus such as obesity can lead to lymph node pathophysiology that hinders immune cell communication.

In our previous publication [229], we demonstrated that 7 weeks of HFD feeding caused lymph node expansion, increased viable cell number, and deviations in immune cell populations. We further demonstrated that the previous alterations were exclusive to visceral lymph nodes. Notably, pro-inflammatory antigen presenting cells and regulatory T cells increased in number in

the visceral lymph node. In addition, the visceral adipose depot also had greater reactivity toward HFD than subcutaneous, with a greater percent of macrophages, dendritic and CD8+ T cells. These findings, however, are controversial because others demonstrated that 8-10 weeks of HFD feeding resulted in a reduction in the size of lymph node and an associated decrease in immune cell number [52, 232]. The current experiment sought to recapitulate previous studies that demonstrate HFD induces a decline in lymph node function and elucidate the mechanisms that drive lymph node atrophy and the decline of immune cells incased within.

Previous studies demonstrate that at 10 weeks of HFD induced obesity, signs of immune suppression are evident by decreases in immune cell populations, total viable immune cells, and lymph node atrophy [232]. Specific immune cell subsets also demonstrate increased levels of apoptosis and reduced proliferative capacity. By extending our timeline of DIO to 13 weeks we were able to replicate these findings. However, there are a few key differences. Within the previously published study, the authors did not assess changes in the immune cell populations of adipose tissue depots that housed the visceral or subcutaneous lymph nodes. Much like Kim et al. we observed that 13 weeks of HFD caused a decline in total viable immune cell number and consequently a decrease to viable populations of subsets involved with adaptive immunity. Furthermore, within the adipose tissue of the visceral lymph node there were also significant decreases to total viable immune cell subsets involved in adaptive immunity. Together these data replicate the previous findings that the adaptive immune response is impaired within the visceral lymph node and extend this to demonstrate that this is also occurring within the surrounding visceral adipose tissue. Immune system dysfunction was further demonstrated by a decreased capacity for proliferation in HFD immune cells from the visceral lymph node. These alterations in immune capacity were not observed in either the subcutaneous lymph nodes or adipose tissue.

This supports our previous findings that the visceral lymph nodes and adipose tissue are more highly associated with immune alterations in response to DIO. We also demonstrated that 13 weeks of DIO was associated with significant deposition of collagen in both the sub-capsular space and center, specific to the visceral lymph node. Collagen deposition in lymph nodes has been demonstrated to be associated with suppressed immunity in models of chronic infectious disease. Thus this is likely directly contributing to the decline in immunity and increased risk to pathogen infection.

Chronic inflammation is associated with fibrosis in a number of disease states including, but not limited to, liver in non-alcoholic steatohepatitis, kidney disease, cardiac fibrosis, pulmonary fibrosis, and the adipose tissue itself [233-237]. Of particular importance is the role of fibrosis in adipose tissue pathophysiology, especially as demonstrated during obesity. It is proposed that rapid expansion of the adipose tissue depot initially leads to a hypoxic environment as vasculature does not keep up with depot growth. This leads to an increase in the protein expression of HIF1α that initiates collagen deposition as well as promotes the induction of pro-inflammation [244]. The process begins with production of cytokines and chemotactic factors that promote macrophage infiltration both in response to HIF1α [245, 246] as well as a response to necrotic adipocytes [247]. We have previously characterized visceral adipose tissue and lymph node immune cell alterations in 7 week HFD mice. We extend that previous study to evaluate relative visceral immune cell population toward visceral lymph node fibrosis. Both 7 and 13 weeks of HFD intake increase lymph node fibrosis, however the extent of fibrosis was directly associated with duration of HFD feeding. We propose that this is a consequence of prolonged exposure to inflammatory stimuli. Both Il-1β and IL-6 have been demonstrated to be involved promoting production of collagen in human fibroblasts. Stimulation of these fibroblasts

by IL-1β was demonstrated to promote pro-collagenic functions of fibroblasts by increasing the synthesis of IL-6 that in turn acted in an autocrine manner to promote collagen deposition. Thus IL-6 that is also elevated in response to hypoxia can directly promote collagen deposition [248]. TNF-α has become increasingly implicated in the induction of pathologic fibrosis in many disease states. Numerous studies have demonstrated that anti-TNF treatment or TNF-r blockade significantly reduce both expression and production of TGF-β in several cell types including fibroblasts, adipocytes, and human proximal tubular cells [249-252]. This has significant implications for the pathogenesis of fibrosis in adipose tissue as M1 macrophages are significant producers of TNF-α and are highly enriched in obese adipose tissue.

Interestingly, several observations within this study are consistent with those of models of chronic infectious disease. First, significant fibrosis is induced at 13 weeks and is associated with HFD induced obesity. In infectious disease, chronic immune activation due to viral persistence is associated with fibrosis in lymph nodes [253, 254]. This persistent chronic immune activation is similar to what we see occurring with obesity due to HFD. Second, multiple shifts in immune cell subsets reflect those observed in end stage pathogenesis of chronic infectious disease. One of the hallmarks of this lymph node fibrosis, is a decrease in CD4+ helper T cells [254]. We observed this in both the visceral adipose tissue and lymph nodes of mice at 13 weeks of diet induced obesity. It has been demonstrated that these CD4+ T cells are essential in maintaining the health of the fibroblastic reticular cell (FRC) network that is essential to the interaction of antigen presenting dendritic cells with T cells in order to elicit an immune response. Interestingly, these FRCs are also important in providing necessary support to these T cells in the form of IL-7 [255, 256]. Consequently, as collagen deposition progresses in the extra cellular matrix, these cells are prohibited from the essential interactions that are need to sustain

the health and function of both populations. It has also been previously demonstrated that in obese mice, TGF-β levels are actually elevated in the visceral adipose tissue [257]. Effluent from the visceral adipose tissue is drained by the visceral lymph node and therefore could be chronically exposed to TGF-β via adipose tissue effluent. As previously discussed, we identified a number of inflammatory factors within obese adipose tissue that can influence the expression and production of TGF-β. TNF-α has been identified as one of the pro-inflammatory cytokines that may influence the production of TGF-β [249-252]. Stimulation of adipocytes in culture with TNF-α resulted in increases of TGF-β in in cell supernatants [258]. However, we did not measure the levels of TGF-β in this study. Preliminary data that we collected using a TGF-β inhibitor demonstrated that collagen deposition within the visceral lymph node was reversible upon blockade of this particular cytokine. Therefore, it is plausible that chronic inflammatory stimulation likely leads to long-term exposure of FRCs to TGF-β and over time fibrosis leads to the impairment of the FRC network that is critical in the support and activation of adaptive immune responses [259].

In conclusion, visceral adiposity is initially associated with a pro-inflammatory immune like response that is specific to the visceral lymph nodes. We support previous findings by demonstrating that 7 weeks of DIO is associated with an increase in inflammatory immune cell subsets within the visceral lymph node. We extend this by recapitulating previously published results in the literature showing that long term exposure to HFD induced obesity and chronic inflammation is associated with an impaired immune response. We extend these previous findings by demonstrating that fibrosis of the lymph node is associated with the decreased immunity. This is a novel finding in the context of obesity, that models close with those of chronic pathogen infection. We postulate that exposure to chronic inflammatory stimuli is

driving this fibrosis and as obesity persists long term it leads to immune suppression within the visceral lymph nodes. It remains to be defined the specific pro-inflammatory signals that are driving this pathogenic fibrosis. Similarly, it is possible that fibrosis promoting cytokines are coming from the adipose tissue and that cells within the lymph node are also producing these signals causing a deleterious loop.

Tables

Table 5.1 - Cumulative food intake (kcals), total body weight (g), and total adiposity (g). Cumulative kcals ingested over 7 and 13 weeks was significantly higher in HFD mice compared with CHOW and was associated with a significant increase in total body mass and total adiposity (*=P<0.05).

	7 Weeks		P value	13 weeks		P value
	CHOW	HFD		CHOW	HFD	
Cumulative Kcals	387.6±9.19	555.9±8.75 *	P=7.30E-5	1012.1±15.1	747.6±11.5 *	P=9.04E-10
Body Mass (grams)	28.9±0.67	39.39±0.77 *	P=1.67E-9	31.01±0.43	44.3±0.67 *	P=3.21E-9
Total Adiposity (grams)	2.74±0.48	5.34±0.63 *	p≤ 0.05	1.76±0.12	5.65±0.21 *	P=2.17E-10

Figures

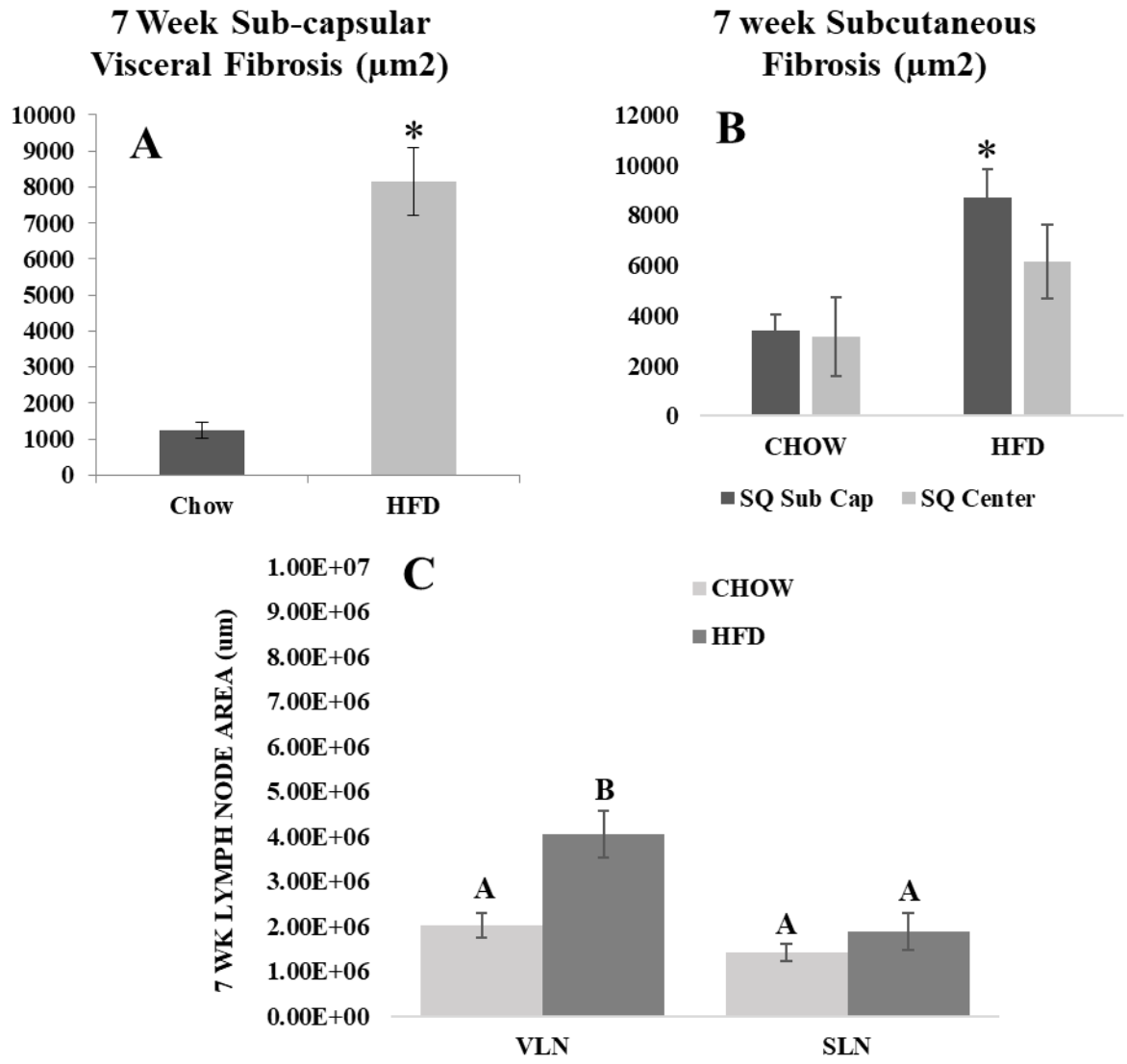


Figure 5.1 - 7 week lymph node histology. Values are represented as a mean value ±SEM. Means with different letters are significantly different (one-way ANOVA, post-hoc analysis LSD, P<0.05). 7 week visceral and subcutaneous collagen deposition in the sub-capsular and center (cortex, paracortex and medullary zones) of the visceral and subcutaneous lymph nodes. HFD feeding was associated with significant collagen deposition in the sub-capsular regions of both the visceral (**A,** *=0.002) and subcutaneous (**B,** *=0.014). HFD resulted in a significant increase in visceral (C, CHOW VLN Vs HFD VLN, P=) but not subcutaenous lymph node area.

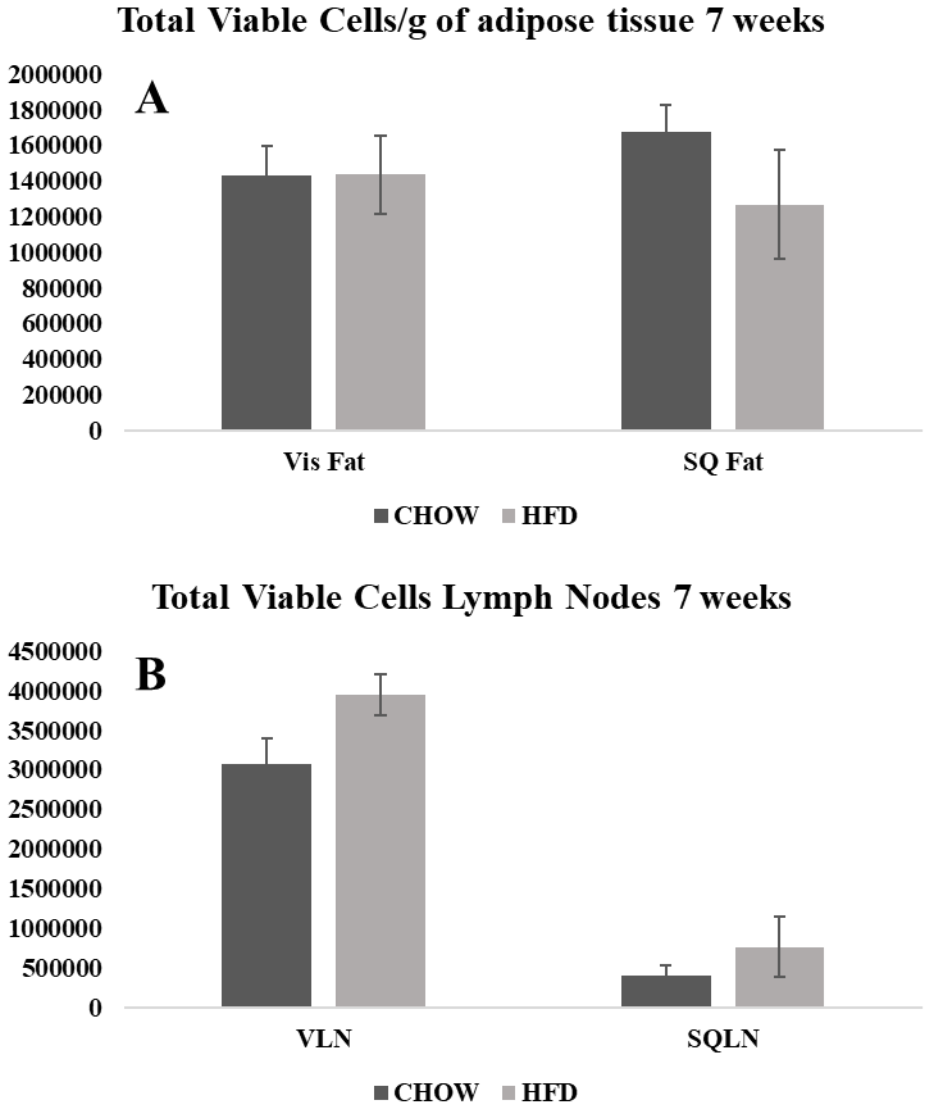


Figure 5.2 - 7 week adipose tissue and lymph node percent and total viable cells: There were no significant differences in total viable cells within any adipose tissue depot at 7 weeks. Total viable cells between lymph nodes are not compared due to inherent size differences between the two depots. HFD did not significantly alter total viable cells in either the visceral or subcutaneous lymph nodes.

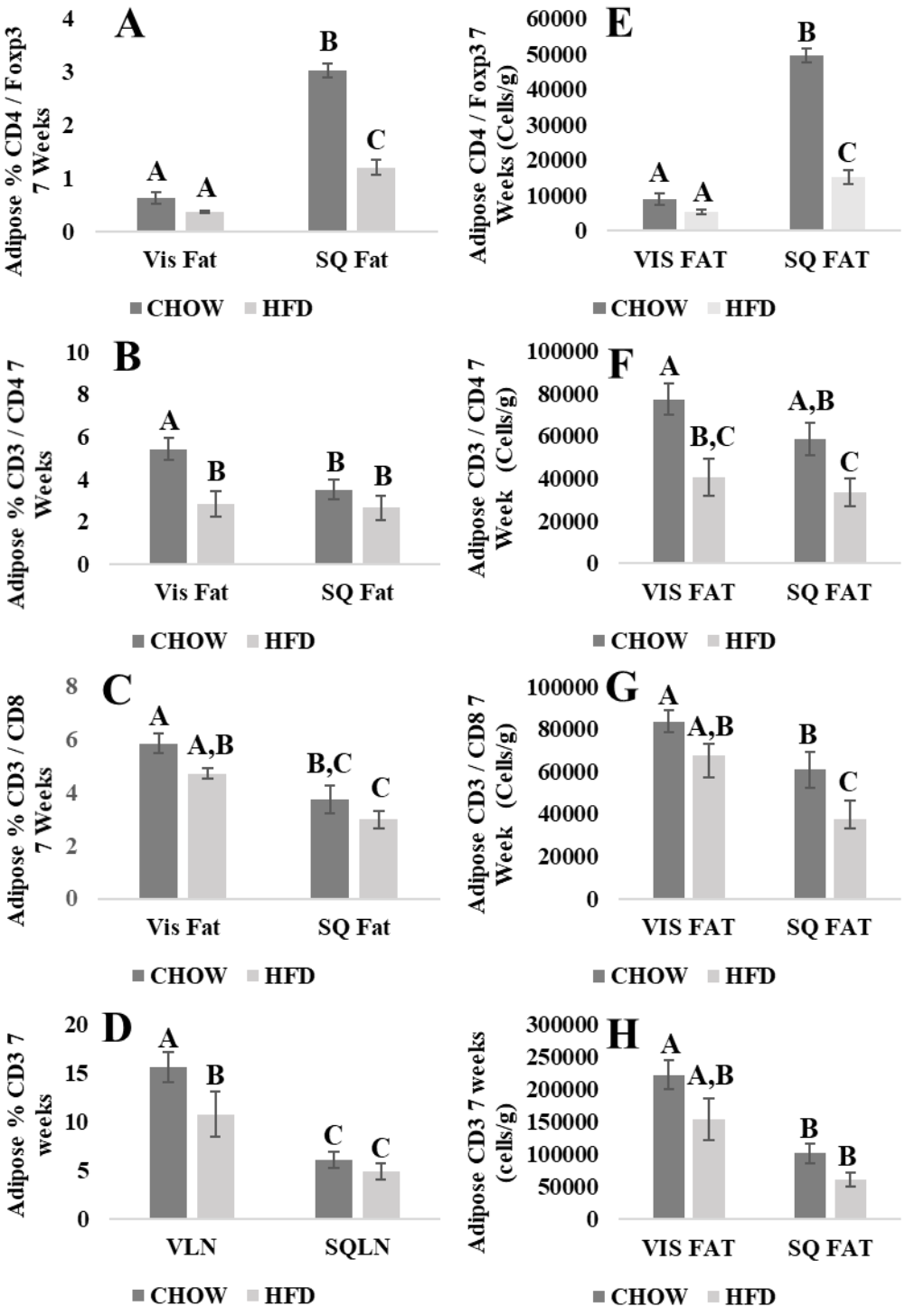


Figure 5.3 - 7 week percent and viable adipose tissue T cells. <u>Location Differences in CHOW mice:</u> In CHOW mice the visceral depot had significantly greater percent populations of CD3+CD4+ helper T cells (**<u>B,</u>** CHOW VIS FAT Vs CHOW SQ FAT, P=0.02), CD3+CD8+ cytotoxic T cells **(<u>C,</u>** CHOW VLN Vs HFD SQLN, P=0.002) and CD3+ total T cells (**<u>D,</u>** CHOW VIS FAT Vs HFD SQ FAT, P=0.022), but a significantly lower percent of CD4+Foxp3+ Tregs (**<u>A,</u>** CHOW VIS FAT Vs CHOW SQ FAT, P=7.87E^{-7}) compared with the subcutaneous CHOW

controls. **HFD induced alterations in immune cell frequency:** In visceral fat HFD significantly lowered frequency of CD3+CD4+ helper T cells (**B,** CHOW VIS FAT Vs HFD VIS FAT, P=0.004) and CD3+ total T cells (**D,** CHOW VIS FAT Vs HFD VIS FAT, P=0.043) compared with CHOW controls. In the subcutaneous depot, HFD only significantly decreased CD4+Foxp3+ regulatory T cells (A, CHOW SQ FAT Vs HFD SQ FAT, P=7.76E^{-6}) compared with respective CHOW controls. **Location Differences in CHOW mice:** CHOW visceral fat contained significantly greater total viable CD3+CD8+ cytotoxic T cells (**G,** CHOW VIS FAT Vs CHOW SQ FAT, P=0.013) and CD3+ total T cells (**H,** CHOW VIS FAT Vs CHOW SQ FAT, P=0.002) compared with subcutaneous CHOW. **HFD induced alterations in immune cell viable number:** HFD resulted in a significant decrease to total viable CD3+CD4+ helper T cells (**F,** CHOW VIS FAT Vs HFD VIS FAT, P= in the visceral adipose tissue and total viable CD3+CD4+ helper T cells(**F,** CHOW SQ FAT Vs HFD SQ FAT, P=0.044), CD3+CD8+ cytotoxic T cells (**G,** CHOW SQ FAT Vs HFD SQ FAT, P=0.016), and CD4+Foxp3+Treg cells (E, CHOW SQ FAT Vs HFD SQ FAT, P=3.0E^{-7}) in the subcutaneous adipose tissue compared with respective CHOW controls.

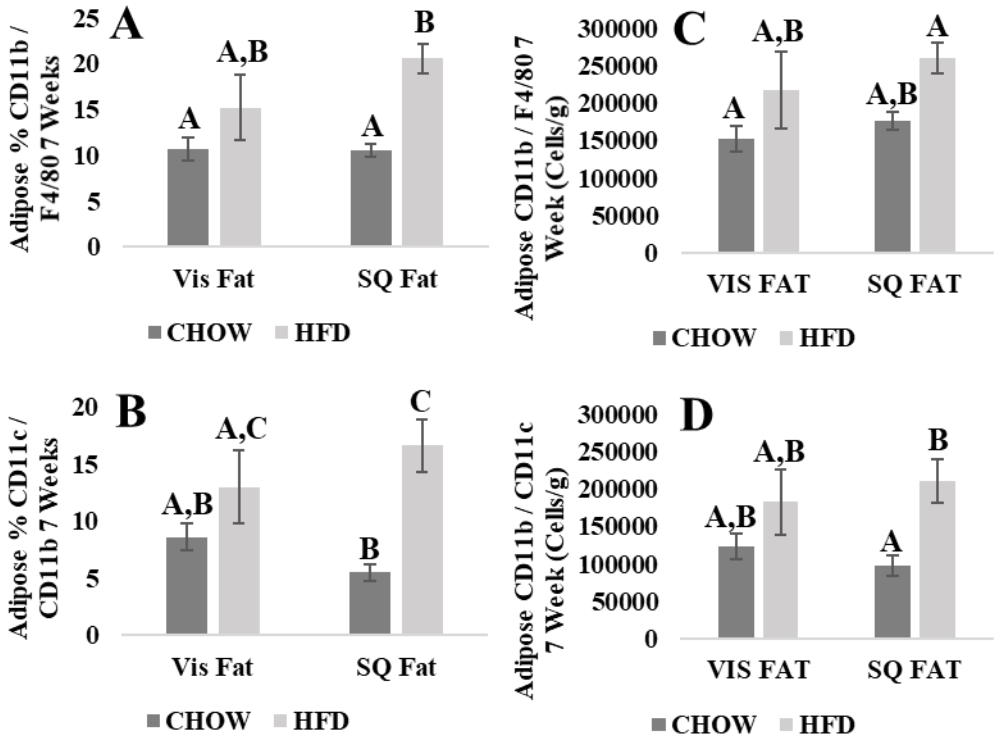


Figure 5.4 - 7 week adipose tissue percent and viable antigen presenting cells: Immune cell percent and number were not different between adipose tissue depots. **HFD induced changes in immune cell frequency and viable numbers:** HFD did not significantly alter percent or viable immune cells in the visceral adipose tissue. In the subcutaneous adipose tissue HFD resulted in a significant percent increase to CD11b+F4/+ macrophages (**A,** CHOW SQLN Vs HFD SQLN, P=0.007) and CD11c+CD11b+ dendritic cells (**B,** CHOW SQLN Vs HFD SQLN, P=0.004) relative to CHOW controls. However, only the CD11b+CD11c+ dendritic cells (D, CHOW SQLN Vs HFD SQLN, P=0.016) had a significant increase in viable cell numbers.

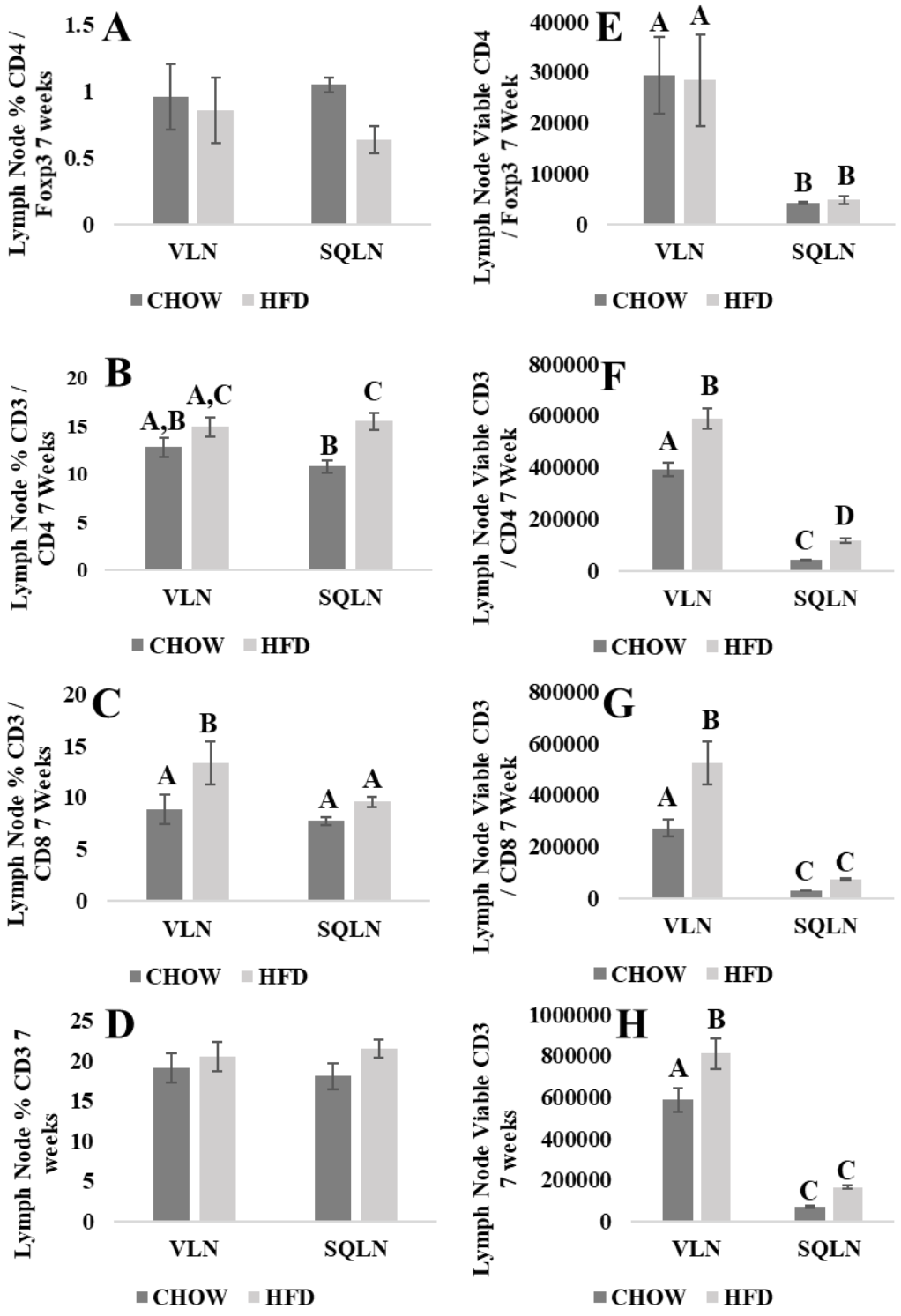


Figure 5.5 - 7 Week lymph node percent and viable T cells: 7 week visceral and subcutaneous CHOW lymph nodes did not have significant percent differences. **HFD induced changes in immune cell frequency:** HFD resulted in significant increases in the frequency of CD3+CD8+

cytotoxic T cells (**C,** CHOW VLN Vs HFD VLN P=0.001) in the visceral lymph nodes relative to CHOW controls. In the subcutaneous lymph node HFD significantly increased CD3+CD4+ Helper T cells (**B,** CHOW SQLN Vs HFD SQLN, P=0.001) relative to CHOW controls. **HFD induced changes in immune cell viable numbers:** Within the visceral lymph node there were significant increases to both CD3+CD4+ helper T cells (**F,** CHOW VLN Vs HFD VLN, P=1.67E-5), CD3+CD8+ cytotoxic T cells (**G,** CHOW VLN Vs HFD VLN, P=0.001), and CD3+ total T (**H,** CHOW VLN Vs HFD VLN, P=0.001) cells relative to CHOW controls. In subcutaneous lymph nodes the only HFD induced change was a significant decrease in CD3+CD4+ helper T cells (**F,** CHOW SQLN Vs HFD SQLN, P=0.013).

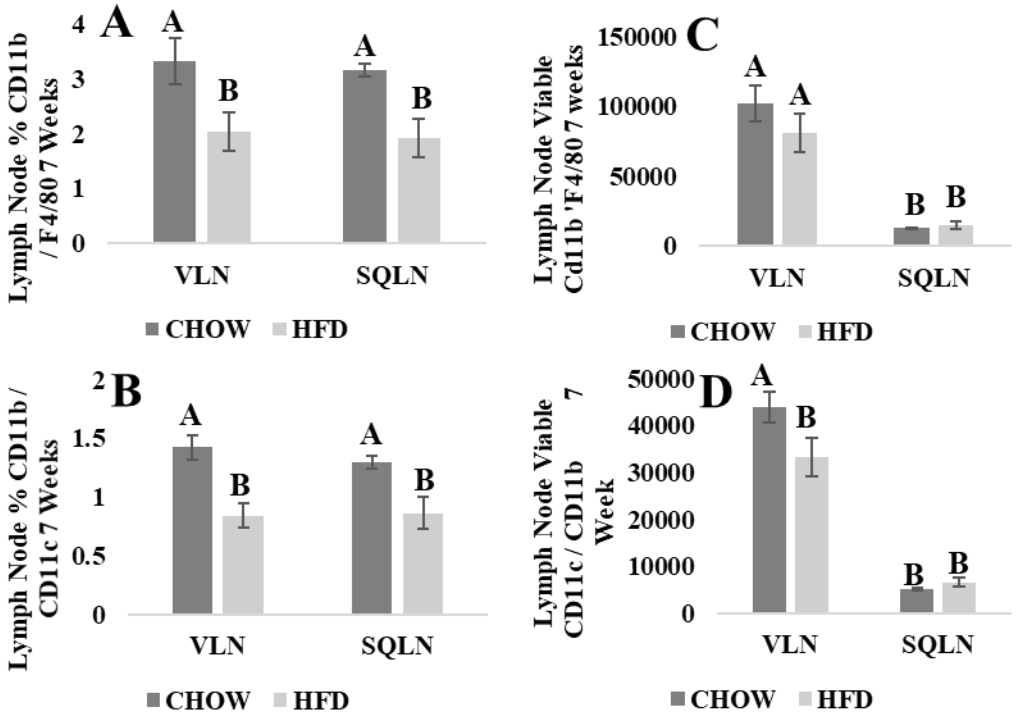


Figure 5.6 - lymph node percent antigen presenting cells: No significant differences in frequency of immune cell numbers were observed between the CHOW visceral and subcutaneous lymph nodes. **HFD induced changes in immune cell frequency:** HFD resulted in significant percent decreases in CD11b+/F4/80+ macrophages (**A,** CHOW VLN Vs HFD VLN, P=0.015) and CD11b+CD11c+ dendritic cells (**B,** CHOW VLN Vs HFD VLN, P=0.001) in the visceral lymph node relative to CHOW controls. Similarly, HFD decreased both CD11b+/F4/80+ macrophages (**A,** CHOW SQLN VS HFD SQLN; P=0.018) and CD11b+CD11c+ (**B,** CHOW SQLN Vs HFD SQLN, P=0.01) dendritic cells in the subcutaneous lymph node relative to CHOW controls. **HFD induced changes in viable immune cells:** The only shift in viable cell populations as a result of HFD was a decrease in CD11b+CD11c+ (**D,** CHOW VLN Vs HFD VLN, P=0.013) dendritic cells in the visceral lymph node relative to CHOW controls.

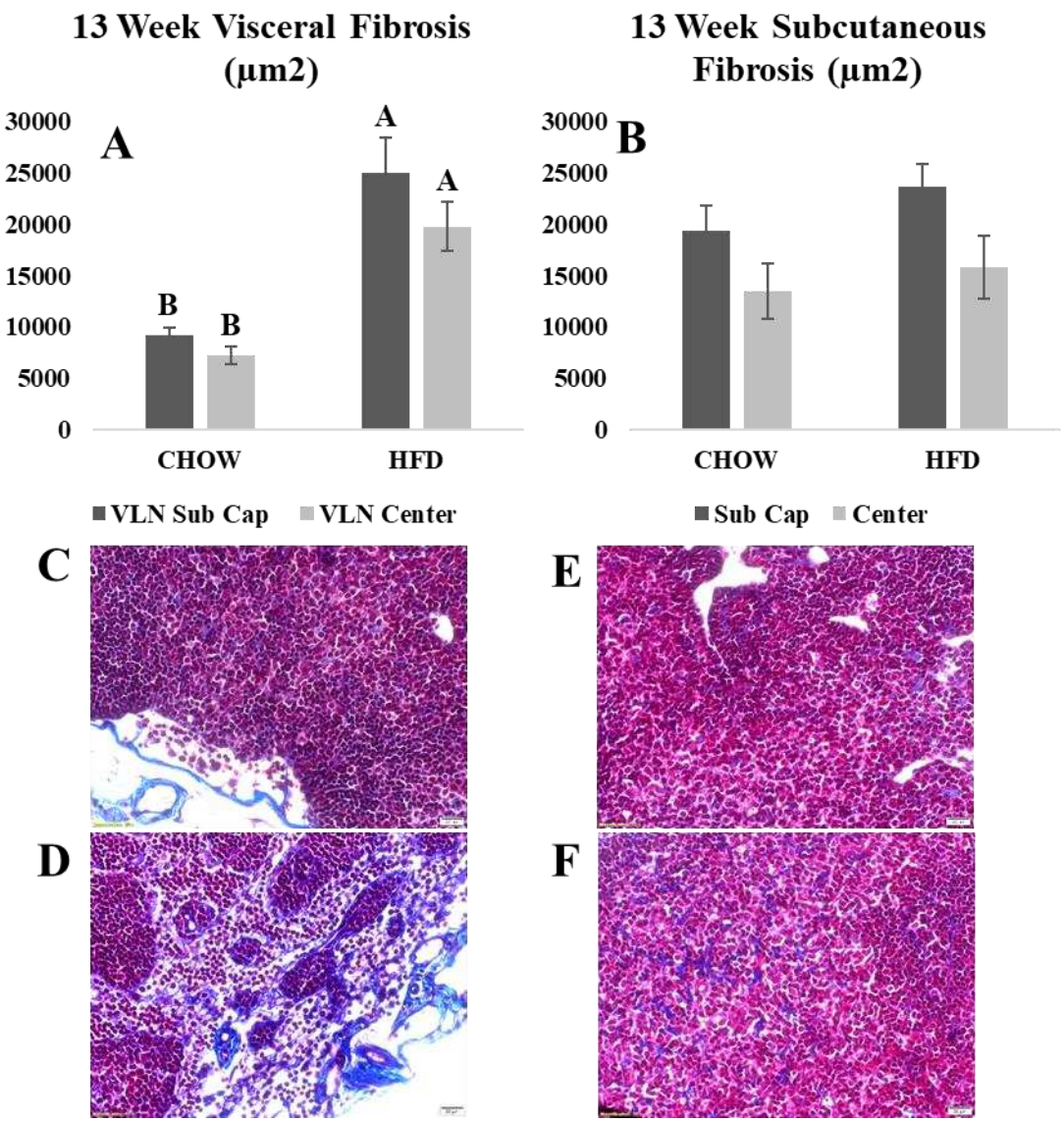


Figure 5.7 - 13 week lymph node fibrosis: Total fibrosis was greater in HFD visceral lymph nodes in both the sub-capsular (**A,** CHOW VLN Sub Cap Vs HFD VLN Sub Cap, $P=2.33E^{-5}$) and center (**A,** CHOW VLN Center Vs HFD VLN Center, P=0.001) regions relative to CHOW controls. HFD did not significantly alter collagen deposition in the subcutaneous lymph nodes relative to CHOW controls. Representative images demonstrating collagen deposition in the CHOW sub-capsular region **(C),** HFD sub-capsular region **(D),** CHOW center region **(E),** and HFD center region **(F)** in the visceral lymph nodes (All images at 20X magnification).

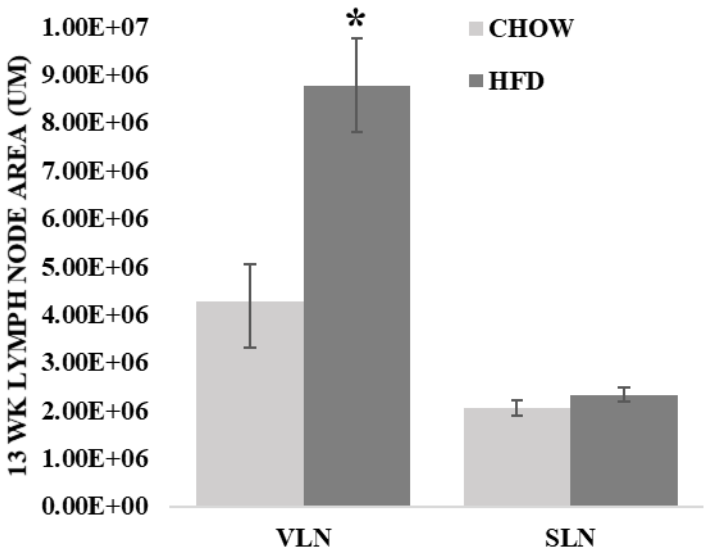


Figure 5.8 - 13 week lymph node size: 13 weeks of HFD resulted in a significant increase in size of roughly 2 fold (CHOW VLN Vs HFD VLN, *P=4.27E^{-6}) to the visceral lymph node compared with CHOW controls.

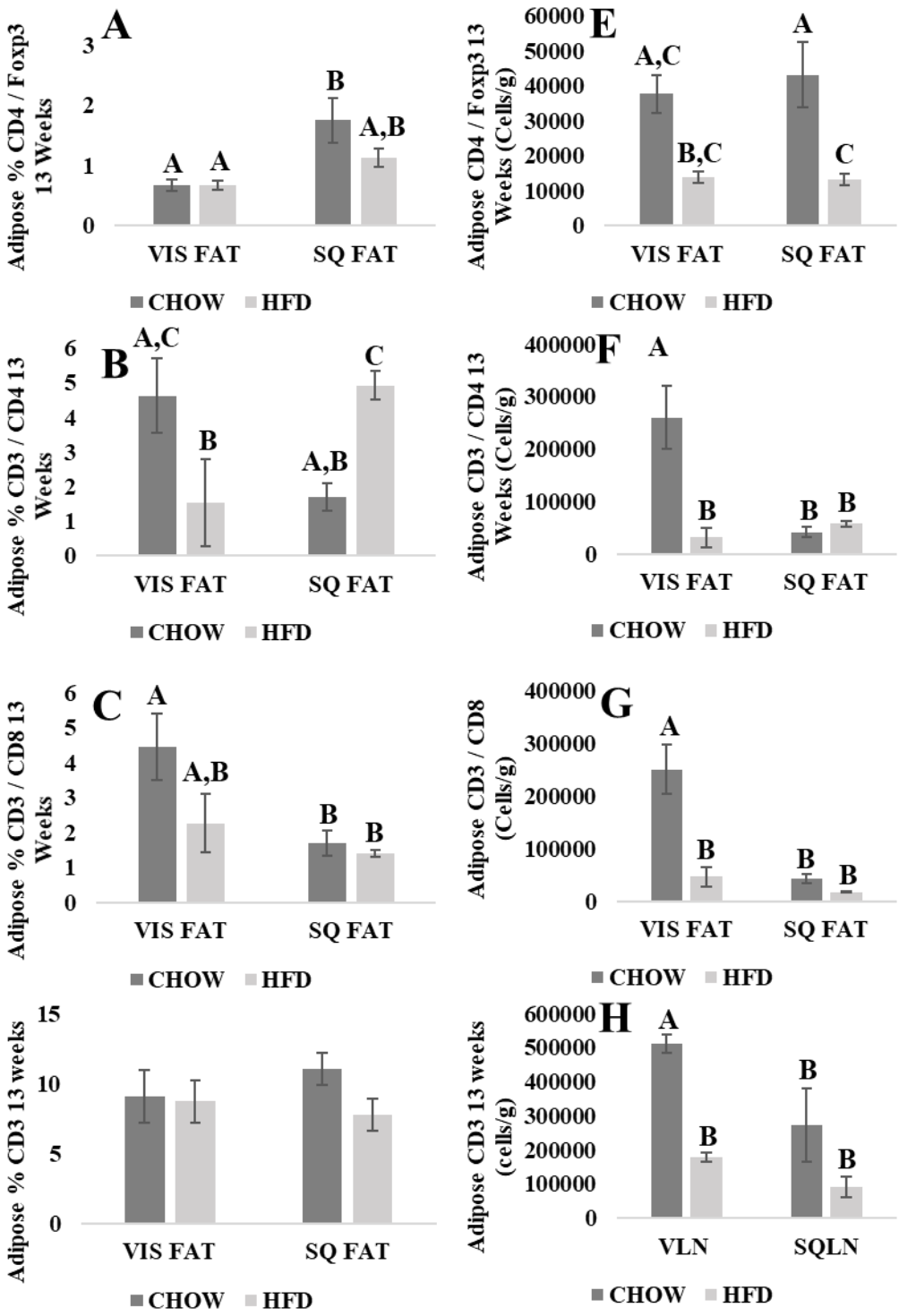


Figure 5.9 - 13 week adipose tissue T cells percent: <u>Location differences in CHOW mice immune cell frequency:</u> 13 weeks resulted in distinct differences between CHOW visceral and subcutaneous adipose tissue. In visceral adipose tissue there were significantly greater percent populations of CD3+CD4+ helper T cells (**<u>B,</u>** VIS FAT Vs SQ FAT, P=0.036) and CD3+CD8+ cytotoxic T cells (**<u>C,</u>** CHOW Vis Fat Vs CHOW SQ FAT, P=0.04) and a significantly smaller

percent of CD3+FoxP3+ regulatory T cells (**A,** CHOW VIS FAT Vs CHOW SQ FAT, P=0.022) compared with CHOW subcutaneous. **HFD induced changes in immune cell frequency:** Within the visceral adipose tissue, HFD only caused a percent decrease in CD3+CD4+ helper T cells (**B,** CHOW VIS FAT Vs HFD VIS FAT, P=0.022) relative to CHOW controls. Similarly, in the subcutaneous adipose tissue there was only a significant increase in CD3+CD4+ helper T cells (**B,** CHOW SQ FAT Vs HFD SQ FAT, P=0.024) relative to CHOW controls. Viable cells were also inherently different between CHOW depots. **Location differences in CHOW mice viable immune cells:** CHOW visceral adipose tissue contained significantly greater numbers of CD3+CD4+ Helper T cells (**F,** CHOW VIS FAT Vs Chow SQ FAT, P=0.001), and CD3+CD8+ (**G,** CHOW VIS FAT Vs CHOW SQ FAT, P=3.43E-4) cytotoxic T cells relative to CHOW subcutaneous. **HFD induced changes in immune viable immune cells:** HFD resulted in significant decreases in the visceral adipose tissue to CD3+Foxp3+ regulatory T cells (**E,** CHOW VIS FAT Vs HFD VIS FAT, P=0.046), CD3+CD4+ Helper T cells (**F,** CHOW VIS FAT Vs HFD VIS FAT, P=3.94E-4), CD3+CD8+ cytotoxic T cells (**G,** CHOW VIS FAT Vs HFD VIS FAT, P=1.68E-4), and CD3+ total T cells (H, CHOW VIS FAT Vs HFD VIS FAT, P=0.002) relative to CHOW controls. Within the subcutaneous adipose tissue HFD resulted in a decrease in CD3+Foxp3+ regulatory T cells (**E,** CHOW SQ FAT Vs HFD SQ FAT, P=0.046) relative to CHOW controls.

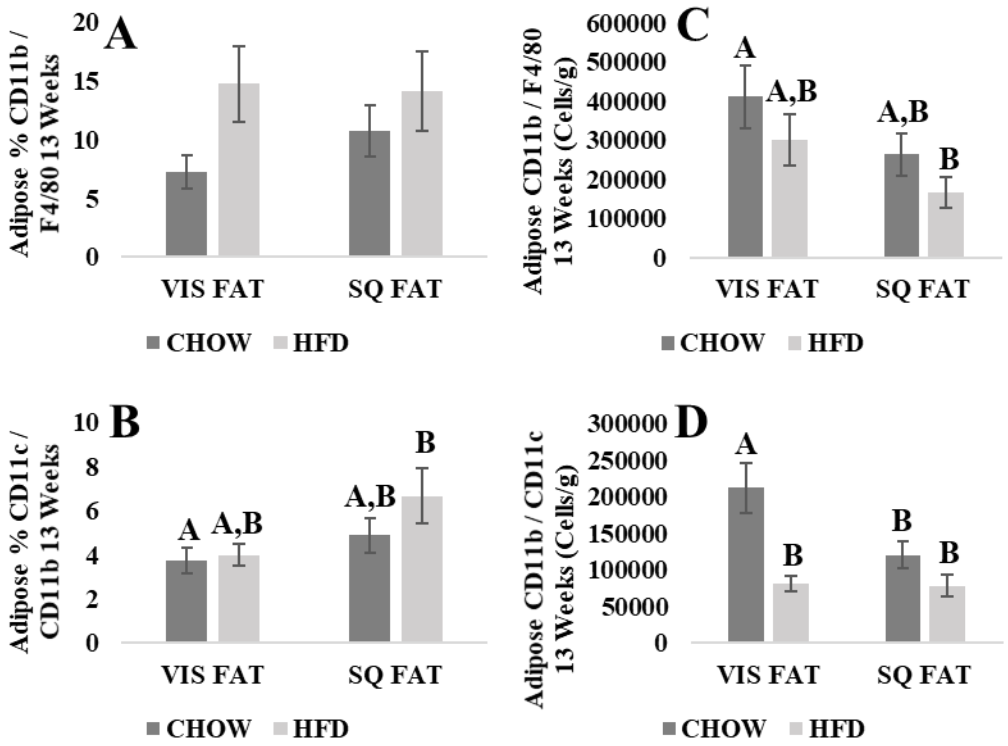


Figure 5.10 - 13 week adipose tissue antigen presenting cells percent: There were no significant differences in immune cell frequency. **Location differences in CHOW mice viable immune cells:** The visceral fat contained a significantly greater number of viable CD11b+CD11c+ dendritic cells (**D,** CHOW VIS FAT Vs CHOW SQ FAT). **HFD induced**

changes in viable immune cells: HFD only resulted in a significant decrease in viable CD11b+/CD11c+ dendritic cells in the visceral fat (**D,** CHOW VIS FAT Vs HFD VIS FAT, P=0.002).

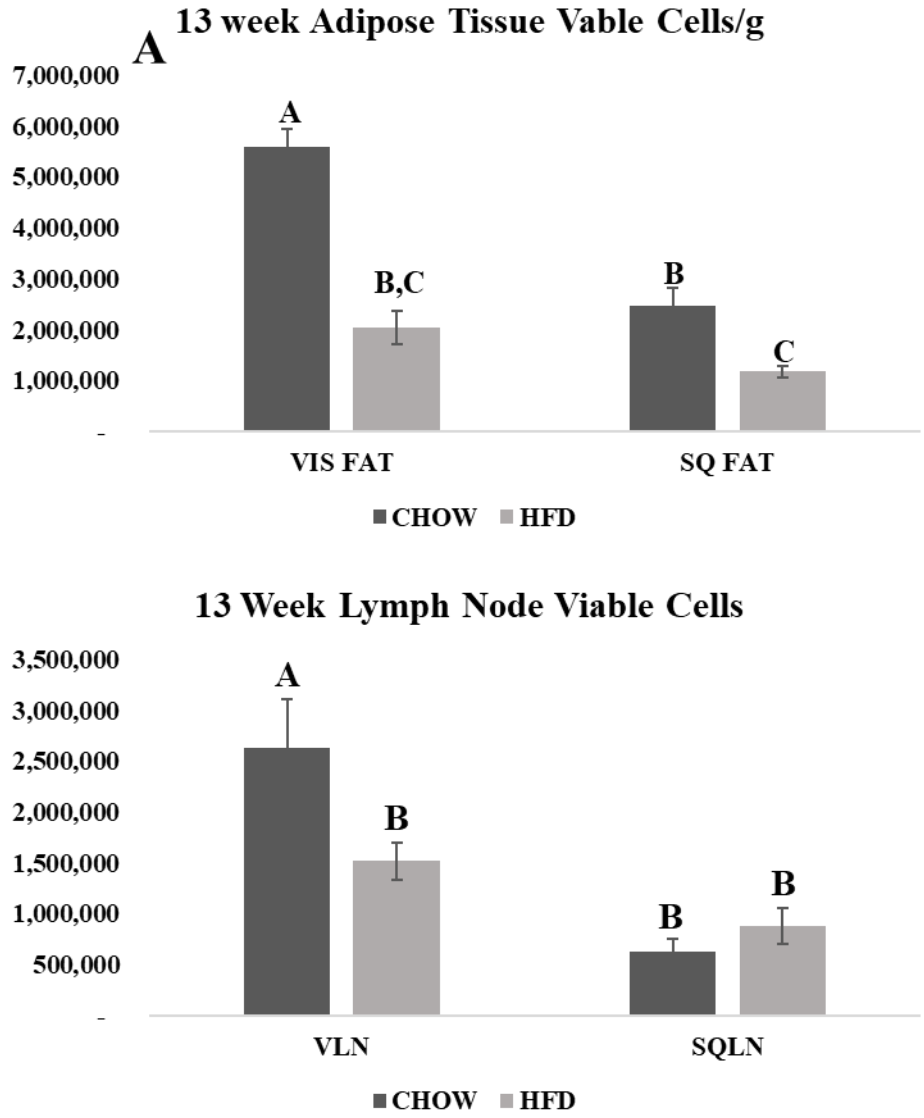


Figure 5.11 - 13 week adipose tissue and lymph node viable cell counts: The CHOW visceral adipose tissue contained a significantly greater number of total viable immune cells (**A,** CHOW VIS FAT Vs CHOW SQ FAT, P=7.7E-6) relative to subcutaneous CHOW. HFD resulted in a significant decrease in total viable cells in both the visceral (**A,** CHOW VIS FAT Vs HFD VIS FAT, P=9.21E-7) and subcutaneous (**A,** CHOW SQ FAT Vs HFD SQ FAT, P=0.018) adipose tissue depots relative to CHOW controls. HFD resulted in a significant decrease in total viable cells in the visceral (**B,** CHOW VLN Vs HFD VLN, P=0.019) but not subcutaneous lymph node relative to CHOW controls.

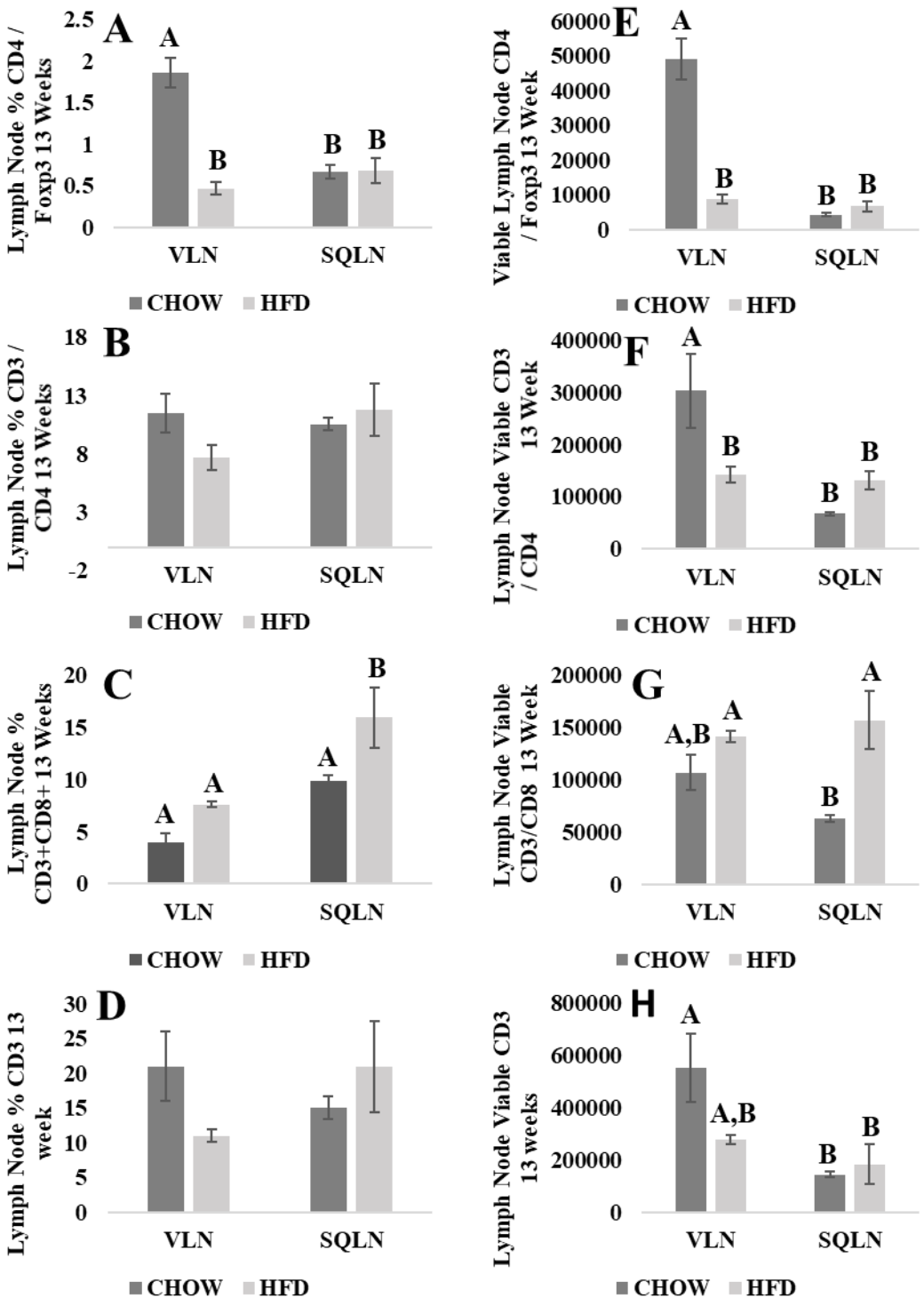


Figure 5.12 - 13 week lymph node percent and viable T cells: <u>Location differences in CHOW mice immune cell frequency:</u> CHOW visceral lymph nodes contained a greater percent of CD4+Foxp3+ regulatory T cells (**<u>A</u>,** CHOW VLN Vs CHOW SQLN, $P=3.85E^{-5}$) relative to CHOW subcutaneous. **<u>HFD induced changes in immune cell frequency:</u>** 13 Weeks of HFD resulted in a significant decrease in CD4+Foxp3+ regulatory T cells (**<u>A</u>,** VLN CHOW Vs VLN

HFD, P=1.31E-5) within the visceral lymph node compared with CHOW controls. In the subcutaneous lymph node HFD resulted in a significant increase in the percent of CD3+CD8+ cytotoxic T cells (**G,** CHOW SQLN Vs HFD SQLN, P=0.017) relative to CHOW controls. **HFD induced changes in viable immune cells:** HFD resulted in significant decreases in CD3+Foxp3+ regulatory T cells (**E,** CHOW VLN Vs HFD VLN, P=4.73E-8), and CD3+CD4+ helper T cells (**F,** P=0.015) in visceral lymph nodes compared with CHOW controls. Within the subcutaneous lymph node the only HFD shift was a significant increase in CD3+CD8+ cytotoxic T cells (**G,** CHOW SQLN Vs HFD SQLN, P=0.017) relative to CHOW controls.

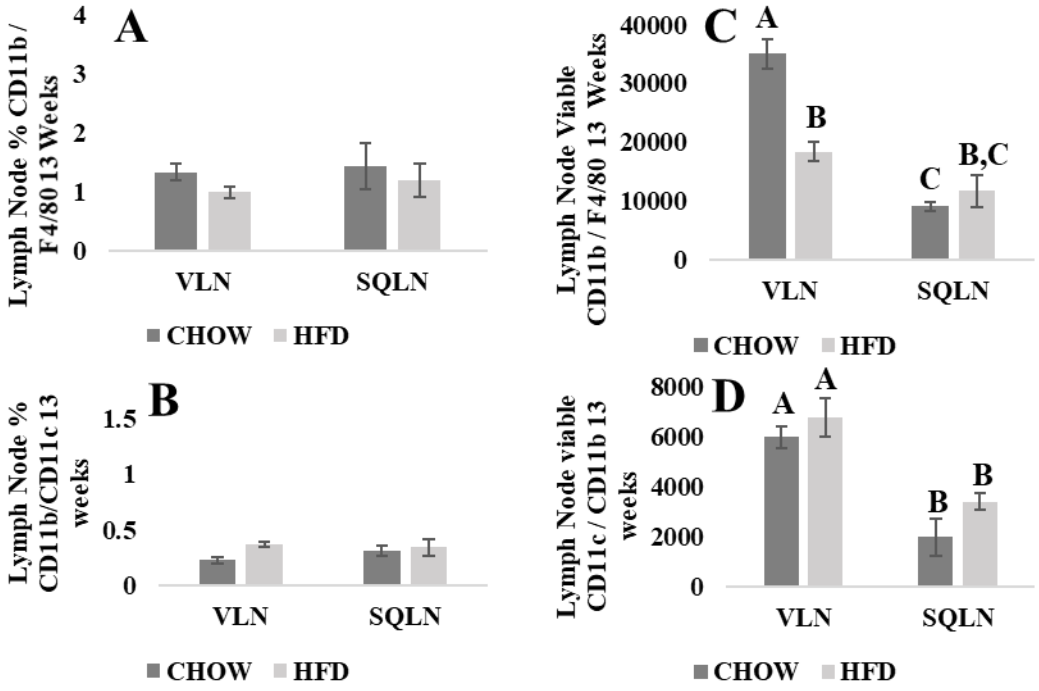


Figure 5.13 - 13 week lymph node percent and viable antigen presenting cells: There were no differences in frequency of antigen presenting cells in the visceral or subcutaneous lymph nodes. **HFD induced changes in viable immune cells:** 13 weeks HFD resulted in a significant decrease in CD11b+F4/80+ macrophages in the visceral lymph node relative to CHOW controls (**C,** CHOW VLN Vs. HFD VLN, P=0.002).

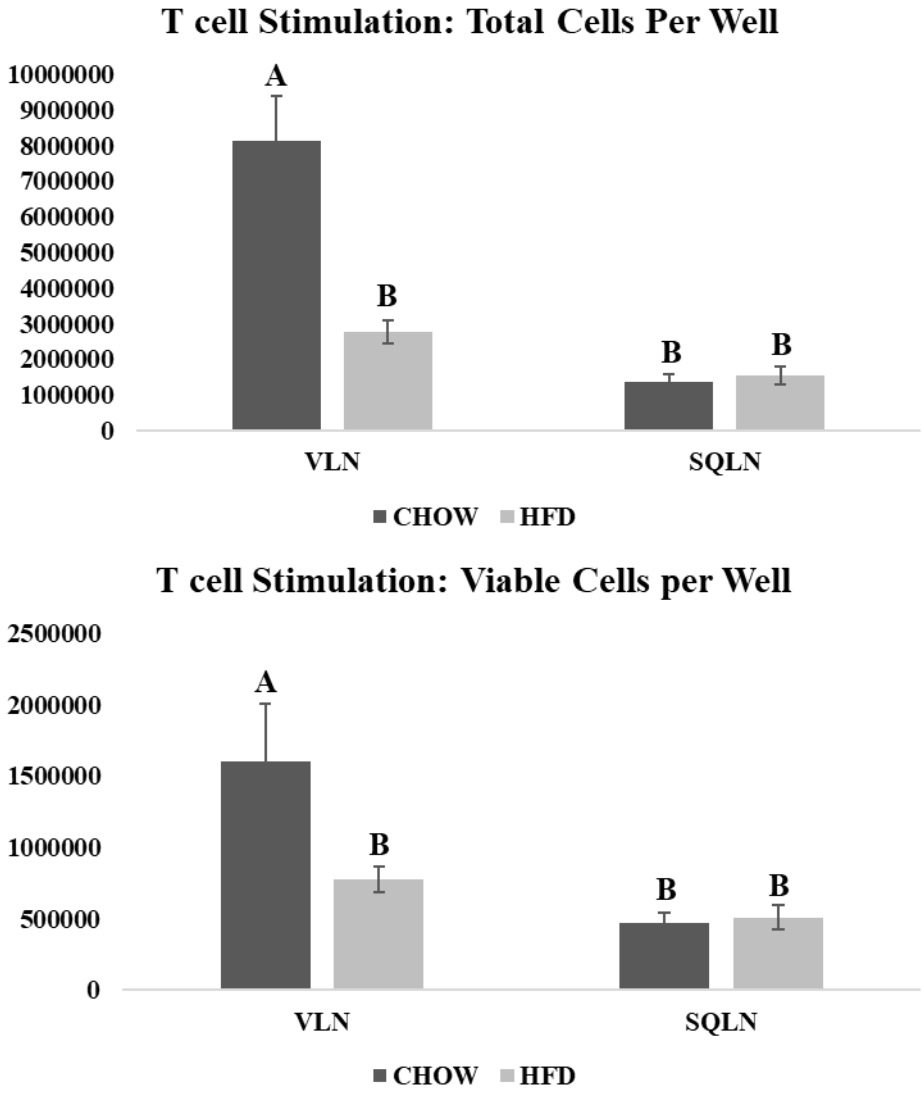


Figure 5.14 - T cell stimulation assay: Stimulation of T cells from the visceral and subcutaneous lymph nodes of both CHOW and HFD fed animals with Concanavalin A resulted in markedly different total and viable cell counts after 72 hours. Total and viable cells were significantly lower from HFD visceral lymph nodes (**A,** CHOW VLN Vs HFD VLN, P= $6.37E^{-7}$; **B,** CHOW VLN Vs HFD VLN, P= 0.004) compared to CHOW controls. Subcutaneous lymph node cells were unaffected by HFD.

CHAPTER 6: ADIPOSE TISSUE EXTRINSIC FACTOR: OBESITY-INDUCED INFLAMMATION AND THE ROLE OF THE VISCERAL LYMPH NODESUMMARY[11][12]

Summary

Obesity-related adverse health consequences occur predominately in individuals with upper body fat distribution commonly associated with increased central adiposity. Visceral adipose tissue accumulation is described to be the greatest driver of obesity-induced inflammation, however evidence also supports that the intestines fundamentally contribute to the development of obesity-induced metabolic disease. The visceral adipose depot shares the same vasculature and lymph drainage as the small intestine. We hypothesize that the visceral lymph node, which drains adipose tissue and the gastrointestinal tract, is central to the exacerbation of systemic pro-inflammation. Male C57BL/6 mice were fed CHOW or high fat diet (HFD) for 7 weeks. At termination the mesenteric depot, visceral lymph node and ileum, jejunum and Peyer's patches were collected. Cytokine concentration was determined in adipose tissue whereas immune cell populations where investigated in the visceral lymph node and intestinal segments by flow cytometry. Visceral adipose tissue and the gastrointestinal tract mutually influence immune cells enclosed within the visceral lymph node. HFD increased visceral lymph node immune cell number. This likely resulted from 1.) an increase in immune cells migration from the small intestines likely from activated dendritic cells that travel to the lymph node and 2.)

[11] A modified version of this chapter is under review at *Physiology and Behavior:* Magnuson AM, Fouts JK, Regan DP, Booth AD, Dow SW, Foster MT. (2017) with the title *Adipose Tissue Extrinsic Factor: Obesity-Induced Inflammation and the Role of the Visceral Lymph Node*. My contributions to this publication included a significant portion of the laboratory procedures, statistics and the bulk of the analysis. I helped to generate all figures except figure 1.

[12] Table and figure numbers have been modified to reflect that they are specific to this chapter, e.g. Figure 1 is now Figure 6.1. Only minimal modifications were made to meet formatting requirements. No other modifications were made.

cytokine effluent from visceral adipose tissue that promoted expansion, survival and retention of pro-inflammatory immune cells. Overall, the visceral lymph node, the immune nexus of visceral adipose tissue the small intestines, likely plays a fundamental role in exacerbation of systemic pro-inflammation by HFD-induced obesity.

The research of Tim Bartness greatly enhanced the understanding of adipose tissue regulation. Studies from his laboratory significantly contributed to our awareness of extrinsic factors that influence body fatness levels. Specifically, the work he produced eloquently demonstrated that adipose tissue was more complex than an insulating storage center; it was connected to our brains via the sympathetic and sensory nervous system. Mapping studies demonstrated that adipose tissue both receives and sends information to the brain. Further, his lab demonstrated that nervous system connections contributed to lipolysis, thermogenesis and adipocyte proliferation and growth. The work of Tim Bartness will continue to influence adipose tissue research. As such, Tim Bartness directly inspired the following research. Adipose tissue extrinsic factors are not limited to the peripheral nervous system. The lymphatic system is an additional extrinsic factor that cross talks with adipose tissue, however its role in this context is under emphasized. Here we begin to elucidate how the lymphatic system may contribute to the comorbidities associated with visceral adipose tissue accumulation.

Introduction

Obesity increases the risk of disease comorbidities including, but not limited to, cardiovascular disease [76], liver inflammation [75], insulin resistance and type-2-diabetes [77]. Systemic low-grade chronic inflammation is commonly described to drive tissue impairments that exacerbate disease risks [151, 260-263]. Excessive adipose tissue accumulation in obesity is associated with regional inflammation, which subsequently drives systemic inflammation that

contributes to deleterious health outcomes [116-119]. In particular, evidence supports that obesity-related adverse health consequences occur predominately in individuals with upper body fat distribution commonly associated with increased central adiposity [128, 151, 264, 265]. Here we question; why is visceral adiposity so detrimental?

Adipose tissue can be modified by both intrinsic and extrinsic factors. One extrinsic factor capable of adipose tissue cross talk is the lymphatic system. This association is particularly important in inflammatory processes. Specifically, lymph nodes are predominantly embedded in adipose tissue [50], and are the primary site for the development of regional protective immune responses. Here lymph nodes serve as conduits that facilitate interactions between immune cells (e.g. monocytes, macrophages, dendritic cells, neutrophils and lymphocytes) and antigens. The visceral lymph node located within the intra-abdominal cavity collects effluent from the visceral adipose depot. In adipose tissue, immune cells within the lymph nodes serve as the responders to tissue injury or pathogen invasion and are fundamental for the development of protective immune responses. Hence, lymph nodes serve as posts that continuously survey and monitor exposure of adipose tissue to potentially harmful pathogens and metabolites [51, 52]. Immune cells within lymph nodes can be recruited and activated to defend adipose tissue against tissue damage, toxicity or impaired function [126]. The link between obesity and impaired immune function has been known for some time, yet there is very little understanding of how excessive adipose tissue deposition alters the lymphatic system. We hypothesize that chronic adipose tissue-driven inflammation influences lymphatic tissue function, in part, by changes in resident immune cell populations. This, in turn, can affect systemic immunity and heighten disease risk. Obesity-mediated inflammation is therefore a disease state that involves adaptations in the extrinsic lymphatic system.

Human and rodent studies suggest the greatest driver of obesity-induced inflammation is visceral adipose tissue accumulation. As it accumulates and dysregulates during obesity, free fatty acids, adipokines and pro-inflammatory cytokines increase in circulation subsequently effecting insulin sensitive tissues such as the liver [266, 267]. However, the visceral adipose depot shares the same vasculature and lymph drainage as the small intestine. Hence, evidence also supports that the intestines fundamentally contribute to the development of obesity-induced metabolic disease [138] by way of inflammation.

The gastrointestinal (GI) tract is the first line of protection from antigens and immunomodulatory agents consumed from our diet or localized within our gut. The GI tract must continuously distinguish beneficial nutrients and commensal bacteria from harmful pathogens [268, 269]. Maintenance of immunologic defense and intestinal homeostasis engages ~70% of the body's immune cells [270], hence the intestine has considerable influence on systemic immune function. Consequently, the gastrointestinal immune system within the small intestine must maintain physiological gut homeostasis despite continual exposure to varying lumen contents. Within the small intestine, this is accomplished by a semi-hierarchical organization of immune regions. Immune response can occur at the epithelial surface of the lumen and lamina propria, where regional immune cells attempt to eliminate noxious pathogens instantaneously (For review see:[271]). These same immune cells also release signaling molecules that can prompt activation of a greater immune response. Gut-associated lymphoid tissue (GALT), a network of lymphoid tissue containing immune cells (for review see: [272, 273]), also defends against pathogens. GALT associated immune cells play a critical role in the protection from and inhibition against the penetration of gut-derived pathogens. Overall, gut immune homeostasis is fundamental to the protection of systemic immunity.

Much like adipose tissue, a poor diet high in fat can cause inflammation of the gastrointestinal tract. Acute or chronic treatment/intake of lipids can shift small intestine immunity toward pro-inflammation, this is best demonstrated in rodent models. For example, high fat diet (HFD) feeding increases tumor necrosis factor α (TNFα) expression and nuclear factor κβ (NF- κβ) activation, markers of pro-inflammation, in the small intestines of mice [274]. Consistent with this others demonstrate that HFD increases TNFα concentration within the lamina propria of the small intestines as well as other pro-inflammatory markers such as interleukin 6 and 17 (IL6 and 17) [275]. Epithelial cells also release IL6, as well as growth-related oncogene/cytokine-induced neutrophil chemoattractant-1 (GRO/CINC-1; stimulates locomotion and activation of neutrophils) in the presence of long chain fatty acids [145]. HFD-induced pro-inflammation in the small intestine likely results from epithelial cell injury, damaged morphology, that occurs as lipid transverses the enterocyte from the lumen [276]. Disruption of small intestine homeostasis by lipids causes epithelial and stroma cells of the small intestines to engage and activate lymphoid cells in attempt to sustain immunity, mediate inflammation and eventually incite resolution [277]. Overall, chronic inflammation, as occurs with intake of saturated fat diets, disrupts the mucosal barrier enhancing permeability of pathogens, which subsequently exacerbates pro-inflammation [278, 279]. Small intestines pro-inflammation, ensuing from acute or chronic exposure to HFD, influences systemic physiological and pathological processes.

We postulate that both visceral adipose tissue accumulation and the gastrointestinal tract influence systemic immunity during obesity. Both tissues are likely responsible for the comorbidities associated with obesity. We hypothesize that the visceral lymph node, which drains adipose tissue and the gastrointestinal tract, is central to the exacerbation of systemic pro-

inflammation. A limitation of previous studies in this area include specific focus on one distinct gastrointestinal region. To examine comprehensively HFD-induced alterations of the small intestines requires distinction between different regions contained within. There are inherent anatomical and physiological differences between the duodenum, jejunum, and ileum (For review see:[271]). Primary differences that may influence immune regulation include differing populations of epithelial cells and absorption capacity. Therefore, characteristics of immunity may be specific to anatomical location among the gut. In addition, Peyers's patches, organized lymphoid nodules located throughout the jejunum and ileum, play a fundamental role in the maintenance of intestinal immune homeostasis, yet the role of these nodules is often underemphasized. In this study, we used a Westernized diet to induce obesity in mice to investigate if diet-induced obesity differentially changed immune cell populations in the Peyer's patches and distinct segments of the small intestines. Along with this we describe immune cell changes in the visceral lymph node, a structure proposed to control immigration of immune cells from the gut and enhance tolerance to gut derived pathogens [202]. Last, adipose tissue cytokine concentration was used to determine if visceral lymph node immune cell alterations are related to visceral adipose tissue signaling.

Methods

Animals

Male C57BL/6 mice (n=10, aged 2-3 months, 27.7±0.22 g) from Jackson Laboratory, Bar Habor, Maine, were single housed under controlled conditions (12:12 light-dark cycle, 50–60% humidity, 25°C) and allowed one week of acclimation before experiment start. Following acclimation, mice were given free access to water and a standard CHOW (CHOW:Harlan Teklad LM485, Madison, WI; 3.1 kcal/g with 18% kcal from fat (6% by weight of diet)) or Westernized

diet (HFD) (Harlan Teklad, TD.08811, Madison, WI; 4.7 kcal/g with 45% kcal from fat,(23% by weight of diet)) until termination at 7 weeks. Body mass and food intake were recorded weekly. Procedures were reviewed and approved by the Colorado State University Institutional Animal Care and Use Committee.

Terminal procedures (blood collection and tissue harvesting)

Termination occurred after mice were on respective diets for 7 weeks. Mice were anesthetized with isoflurane and terminated by decapitation. Adipose depots were collected and weighed and the mesenteric (visceral) depot was stored at -80°C. The visceral lymph node embedded inside the visceral depot was collected. Peyer's patches (n = 5 mice, 4 collected Peyer's Patches/mouse) were first collected from the small intestines (along the ileum and jejunum). Next 30mm portions of ileum and jejunum (n = 5 each), without visible Peyer's patch portions, were collected. Intestine tissues and the visceral lymph node were placed into Hank's balanced salt solution (HBSS) until further processed.

Adipose tissue cytokines

Visceral adipose tissue Interleukin -1β, -5, -6 and -13, as well as KC GRO (a.k.a. chemokine (C-X-C motif) ligand 1 (CXCL1) and tumor necrosis factor α (TNFα) protein concentration was measure by Mesoscale (Rockville, Maryland).

Separation of immune cells from stromal tissues

Ileum, jejunum and Peyer's patches samples were removed from HBSS, placed in collagenase solution (2 mL/sample, Sigma Aldrich, Cat#C9891) and minced. Tissues were placed in an incubator at 37°C for 25-30 minutes then removed and samples were triturated with 18 gauge needles on 3mL syringes. Tissues and collagenase solution were passed through a 40µm cell strainer, and rinsed with 10mL HBSS to deactivate collagenase. Visceral lymph nodes

were dissociated and filtered through a 40 μm cell strainer without the use of collagenase. All cell suspensions were centrifuged at 2000 rpm at 4°C for 5 minutes. Supernatant was poured off and samples were re-suspended in 400μL of FACS buffer (1% BSA, 0.1% sodium azide, and PBS) for immediate cell count (see below: Cell Counting) then temporarily stored.

Cell counting – total and viable

Tissue cell suspensions were pipetted onto 96-well plates and mixed with trypan blue exclusion dye marker (ThermoFisher Scientific, Waltham, MA). Total and viable cell counts were evaluated on a Cellometer (Nexcelom, Lawrence, MA).

Flow cytometry

Between 5 x 10^5 and 1 x 10^6 cells from intestinal suspensions were plated per well. Cells were washed, spun and supernatant was removed and discarded. To prevent non-specific binding of antibodies cells were re-suspended in normal mouse block and briefly incubated. Primary antibodies were added at a 1:200 dilution in two different panels. Panel 1 included: anti-CD8 (clone 53-6.7, cat. no. 13-0081-85, eBioscience), anti-CD4 (clone RM4-5, cat. no. 48-0042-82, eBioscience), anti-CD3 (clone 145-2c11, cat. no. 11-0031-82 eBioscience), and intracellular Foxp3 (clone FJK-16a, cat. no. 12-5773-82, eBioscience) for T cell differentiation; anti-B220 (clone RA3-6B2, cat. no. 17-0452-82, eBioscience) for B cell identification; anti-NKG2D (clone CX5, cat. no. 13-5882-82, eBioscience) for natural killer cells. Panel 2) included: anti-CD11b (clone M1/70, cat. no. 48-0112-82, eBioscience), anti-CD11c (clone N418, cat. no. 417-0114-82, eBioscience), anti-F4/80 (clone BM8, cat. no. 25-5931-82, eBioscience), and anti-MHCII (clone M5/114.15.2, cat. no. 17-5321-81, eBioscience) for antigen presenting cell differentiation. After incubation and washes with FACS buffer secondary antibody streptavidin-pacific orange (cat. no. 532365, Invitrogen) was added at a dilution of 1:500. In addition, a

permeabilization kit (cat. no. 00-5521, eBioscience) was used in accordance with kit instructions for FoxP3 intracellular staining. Last, stained cells were fixed in 4% PFA, washed and stored in FACS buffer for analysis the following day. Flow cytometry of immune cell populations were analyzed using MoFlo Legacy Cell Analyzer and Sorter (Beckman Coulter, Indianapolis, IN). Data were analyzed using Summit software version 4.2 (Beckman Coulter, Indianapolis, IN).

Statistics

Data are expressed as mean ± standard error of the mean (SEM). Food intake, body weight, intestine tissue cell percent and viable counts were analyzed with a One-way ANOVA for significance values among individual data points. Post-hoc tests of individual groups were made using LSD tests. Lymph node immune cell percent and viable number as well as adipose tissue cytokines were analyzed by T-test. For all experiments, differences among groups were considered statistically significant if $p \leq 0.05$.

Results

Food intake and body weight

Cumulative kcal intake was significantly higher in HFD mice compared with Chow (Chow 387.54 kcals ± 9.19, HFD 555.9 kcals ± 8.75; p = 7.3E-5) following 7 weeks of intake. As a result 7 weeks of HFD intake significantly increased body mass (Chow 28.9 ± 0.67, WD 39.39 ± 0.77; p = 1.67E-9) compared with Chow controls. Consistent with this individual adipose tissue, epididymal, visceral, perirenal and inguinal white adipose tissue, and total adiposity (previous adipose tissue depots added together) were significantly increased in the HFD group (Table 6.1; $p \leq 0.05$).

Adipose depot cytokines

Adipose tissue accumulation, especially visceral (central) adiposity, is associated with increases in cytokines that contribute to pro-inflammation. Cytokine concertation was measured in the visceral depot to associate adipose depot changes with extrinsic lymph node immune cell signaling. Interleukin (IL) proteins were analyzed because of their role in regulating immune response. In particular, these ILs include proteins which generally contribute to pro-inflammation IL-1β and IL-6 and those that are demonstrated to be higher in lean animals IL-5 and IL-13. IL-1β, IL-5 and IL-6 (Figure 6.1 A-C) concentrations were increased in the visceral adipose depots of HFD mice, both IL-5 (Figure 6.1B; $p = 0.05$) and -6 (Figure 6.1C; $p = 0.003$) were significantly increased compared with controls. IL-13, however, decreased in the visceral depot of HFD animals (Figure 6.1 D). In addition, KC GRO (a.k.a. chemokine (C-X-C motif) ligand 1 (CXCL1) and tumor necrosis factor α (TNFα) protein concentration was measured. KC GRO is thus far characterized to be increased during pro-inflammation and obesity and aids as a chemoattractant for neutrophils [280]. TNFα is a protein involved in systemic inflammation. Both KC GRO (Figure 6.1E; $p = 0.018$) and TNFα (Figure 6.1F; $p = 0.019$) were significantly increased in HFD mice compared with control.

Visceral lymph node cell cumber and flow cytometry

7 weeks of HFD significantly increased the number of viable cells within the visceral lymph node (Figure 6.2A; $p = 0.02$). In general, HFD did not shift immune cell percent within the visceral lymph node, with the exception of $CD4^{+}Foxp3^{+}$ regulatory T cells (Tregs) immune cells that play a role in suppression of immune response. The percent of Tregs were significantly decreased in HFD mice compared with CHOW control (Figure 6.2B; $p = 0.039$). Absolute numbers of distinct immune cell subsets were determined by multiplying percent of

representative cell populations by the total viable cell count. This data is represented in Figure 6.2C. HFD intake did not cause vast shifts in immune cell frequencies, but did cause multiple significant changes in viable visceral lymph node immune cell numbers. Specifically, mice fed HFD had significantly higher numbers of F4/80+CD11b^{+} macrophages ($p = 0.022$), CD11b+CD11c+ dendritic cells ($p = 0.017$), CD3^{+}CD4^{+} helper T cells ($p = 0.024$), CD3+ general T cells ($p = 0.02$) and B220+ B cells ($p = 0.002$) within the visceral lymph node compared with CHOW control (Figure 6.2C). In opposition, CD4^{+}Foxp3^{+} regulatory T cells viable number was decreased in HFD mice (Figure 6.2C; $p = 0.011$).

Intestine cell number and flow cytometry

7 weeks of HFD did not alter total viable cell number in ileum, jejunum or Peyer's patch samples (Data not shown). A limitation of this study was the processing of whole intestinal sections. This method of collection interferes with the determination of viable immune cell populations because of the numerous different cells types located within the intestines that are a similar size to immune cells of interest. Hence, viable cell number for the intestines would not be reflective of the actual immune cell number in the intestine. Therefore, only percent frequency will be reported for the ileum, jejunum and Peyer's patches.

Immune cell frequency was evaluated in several distinct intestine regions because of the inherent anatomical and physiological differences between intestine segments (For review see:[271]). Differences in epithelial cell number, absorption capacity and numerous other factors can influence immune regulation. In general, changes in immune cell frequency where similar between intestine regions. First, in the ileum HFD caused significant decreases (Figure 6.3A) in percent of CD4^{+}Foxp3^{+} regulatory T ($p = 0.011$), NKG2D+ ($p = 0.002$) and NKG2D+CD4+ ($P = 0.014$) immune cells while significantly increasing percent of CD11c+MCHII+ dendritic cells (p

= 0.016) compared with CHOW. Similarly, in the jejunum the percent of $CD4^+Foxp3^+$ regulatory T (p = 0.015) and NKG2D+CD4+ (P = 0.034) immune cells were significantly decreased and CD11c+MCHII+ dendritic cells (p = 0.008) frequency was significantly increased in HDF mice. Additionally, HFD significantly decreased the percent of jejunum CD3+CD4+ helper T cells (Figure 6.4A; p = 0.011) compared with CHOW. Last, Peyer's patches located along the ileum and jejunum (Figure 6.5) of HFD mice had significant decreases in percent of $CD4^+Foxp3^+$ regulatory T (p = 0.011), NKG2D+ (p = 0.002), NKG2D+CD4+ (P = 0.014) and CD11b+GR1+ myeloid suppressor (p = 0.042) immune cells. Similar to the ileum and jejunum CD11c+MCHII+ dendritic cells (p = 0.005) in Peyer's patches were significantly increased in HFD mice compared with CHOW (Figure 6.5).

Discussion

Obesity induces chronic low-grade inflammation. It is suggested that obesity associated inflammation is incited and exacerbated by excessive dysregulated adipose tissue deposition. Dysregulation of adipose tissue endocrine function, pro-inflammatory cytokines and deleterious cytokines are implicated in pathophysiology of numerous diseases. Visceral adipose tissue accumulation, in particular, is highly associated with adverse metabolic profiles [90]. Emerging evidence additionally supports a relation between dysregulated visceral adipose tissue and gut dysfunction [281]. Specifically, visceral adipose tissue pro-inflammation causes gastrointestinal leakiness [282, 283] and gut inflammation drives adipose tissue accumulation [284], both factors together exacerbate systemic pro-inflammation. Because the gastrointestinal tract and visceral adipose tissue commonly drain to visceral lymph node, we proposed to investigate the associated changes between the three intra-abdominal tissues during obesity. Here we demonstrated that both the visceral adipose depot and the gastrointestinal tract likely influence

immune cells enclosed within the visceral lymph node. This occurred by HFD-induced increases in visceral adipose tissue pro-inflammatory cytokines and a reduction in the tolerant milieu of the intestines needed to suppress unnecessary immune reaction to commensal bacteria and beneficial nutrients.

Visceral adipose tissue accumulation is highly associated with metabolic disease [266, 267]. Deleterious effects of excessive visceral depot expansion, in part, are due to increased production and release of pro-inflammatory cytokines [285] that circulate directly to the liver and systemically. Cytokine release from adipose tissue originate from both adipocytes and immune cells contained within the depot in response to the changing milieu induced by HFD. Consistent with previous studies, we demonstrate that HFD-induces deleterious cytokine release from visceral adipose tissue. Il-1 plays a fundamental role in immune response. These molecules comprise a family of pro-inflammatory cytokines that induce a complex network of additional pro-inflammatory cytokines/mediators via interaction with leukocytes. Specifically, IL-1b in adipose tissue is known as mononuclear cell factor and a lymphocyte activating factor that originates from macrophages and is activated by adipocyte-specific caspase-1 [286]. Specifically, release of IL-1b, in a situation such as obesity, enhances antigen-mediated expansion of pro-inflammatory cell types such as Th1 and Th17 T cells [287]. IL-1b also promotes the recruitment and retention of macrophages [288]. Similar to IL-1b, IL-6 is a fundamental regulator of T cells. Of the numerous immune-modulating cytokines increased in obesity, IL-6 dysregulation significantly contributes to pro-inflammation and metabolic dysregulation [289, 290]. Release of Il-6 causes numerous events to occur that allow for T cell survival. This includes promoting T cell development [291] and activation and tissue invasion [292, 293]. In addition, Il-6 permits effector T cells to overcome suppression by regulatory T

cells [293, 294] while correspondingly inhibiting immune suppression T cells [295]. In opposition are interleukins associated with anti-inflammation that are typically decreased in obesity including IL-5 and IL-13. IL-5 plays a role in maintaining immune cell populations that contribute to metabolic homeostasis including glucose tolerance, and protection against diet-induced obesity in adipose tissue [296, 297]. Similarly, IL-13 is also associated with conservation of glucose homeostasis [298]. In response to a pathogen TNFα is typically the first cytokine released [299]. Within tissues TNFα recruits cells towards pro-inflammatory stimuli by inducing vasodilation, which allows increased infiltration of immune cells such as lymphocytes, neutrophils and monocytes [300]. TNFα also induces the release of KC GRO (CXCL1) from immune cells such macrophages and neutrophils [301, 302], this further facilitates pro-inflammatory immune cell recruitment. Obesity increases both TNFα [303] and CXCL1 release [301]. Overall, our data demonstrates that 7 weeks of HFD increases pro-inflammatory cytokines while decreasing immune-suppressing cells types. This subsequently creates a signal in adipose tissue that permits increased infiltration of pro-inflammatory cells types. We propose effluent from visceral adipose tissue into the visceral lymph node influence immune cells incased within.

The visceral lymph node, however, is not limited to visceral adipose depot effluent. Intestine health can also influence the visceral lymph node. The gastrointestinal (GI) tract is the first line of protection from antigens and immunomodulatory agents consumed from our diet or localized within our gut. Hence, it is likely that immune dysregulation within the gut can directly influence immune cells encased within the visceral lymph node. In the present study, we comprehensively investigated how HFD altered immune cell populations within the small intestines by comparing distinct intestinal regions with inherent anatomical and physiological differences. The jejunum and ileum as well as Peyer's patches contained throughout these two

regions were evaluated for differential response to HFD. Distinct intestine sections were evaluated to determine if regional differences in specialized epithelial cells, receptor types, antigen uptake or absorption capacity could disparately influence HFD outcomes. Although there were some minor differences between gut regions, most changes among the three sections evaluated were similar.

First this includes HFD-induced decrease in regulatory T cells (Tregs: CD4+foxp3+) in all three intestine tissues. These regulatory cells are fundamental in maintaining homeostasis within the gut as demonstrated in numerous inflammatory disease models (For review see: [304]). Generally, Tregs are upregulated during homeostatic conditions and consequently downregulated in order to elicit an immune response to pathogens or other inflammatory stimuli [305]. This is the first indication that HFD induced obesity is potentially driving a shift towards the pro-inflammatory environment associated with obesity. As previously discussed, this shift in Treg cells is well characterized within the ileum and jejunum in obesity as well as other inflammatory bowel disorders [304] but to the best of our knowledge, this is the first time this has been documented within Peyer's patches in response to diet induced obesity. Second are the dendritic cells (DCs: CD11c+MHCII+) which are antigen presenting cells. Within the intestines, dendritic cells function to extend cellular extensions between epithelial cells to sample pathogens or commensal factors of the intestinal lumen. Dendritic cell percent was increased in all three HFD intestinal samples. Research demonstrates that harmful pathogens can trigger dendritic cells to elicit a pro-inflammatory cascade that includes 1.) both attracting and stimulating maturation and differentiation of B and T lymphocytes to mature into effector cells [306, 307], 2.) sustainability of recirculating T lymphocytes [308], and 3.) stimulation of antibody production from B cells by IL-12 secretion [309]. We would speculate these changes in immune system

milieu would lead to a shift in percent increase of effector immune cells that play a role in pro-inflammation, yet this did not occur within the small intestine. Hence, the third common change to occur among our three tissue regions was decreased NKG2D+ immune cells, in particular those that where NKG2D+CD4+. The role of CD4 T cells expressing the NKG2D receptor is best described in Crohn's disease [310-312]. NKG2D+CD4+ cells in the lamina propria of patients presenting with Crohn's disease were associated with a pro-inflammatory cytokine profile [310] with a high expression of both IFN-y, TNF-α and increased cytotoxic capability [310]. Taken together, this demonstrates that NKG2D+CD4+ cells may play an important role in shaping the pro-inflammatory profile of the small intestine during HFD induced obesity and inflammation. However, we observed a decrease in NKG2D+CD4+ cells. Similarly, but specific to the Jejunum, there was also a decrease in small intestine CD3+CD4+ effector T cells, this again was contradictory to our prediction. Last, studies demonstrate that myeloid derived suppressor cells (MDSCs), characterized as CD11B+GR1+, induce intestinal tolerance by suppressing T-cell [313]. In Peyer's patches only, HFD decreased MDSCs compared with CHOW. Taken together, in the intestines HFD induces release of suppressive immune cell types typically needed for toleration of commensal bacteria and variable lumen components. Release of this suppression caused a pro-inflammatory environment with an increase in antigen presenting dendritic cells that present to and activate effector cells. Hence, an increase in dendritic cell migration to the intestine increases antigen presentation and consequently T and B cell response [314]. However, there was not a corresponding increase in the percent of effector cells within the intestine instead there was a decrease. We propose this decrease in immune cell frequency may be due to immune cell migration from the gut.

Data supports that gastrointestinal immunity and systemic tolerance is predominately mediated by the visceral lymph node [202]. Gastrointestinal tolerance is the ability of immune cells to resist an unnecessary pro-inflammatory response to harmless food antigens and commensal bacteria and remain in a homeostatic state (for review see: [315]). The visceral lymph node serves as a conduit that continuously surveys and monitors exposure of the small intestine to potentially harmful pathogens and metabolites [51, 52]. Within this lymph node immune cells can be recruited and activated to defend against damage, pathogens or impaired function [126]. A fundamental role of the visceral lymph node is to create tolerance to beneficial nutrients and commensal bacteria. This process is prompted by anti-inflammatory antigen presenting dendritic cells that constitutively traffic from the intestinal epithelium and Peyer's patches to the visceral lymph node where T cell tolerization can occur [316-318]. Consequently, this creates a protective barrier within the lymph node, via enrichment in tolerant T cells, which prevents unnecessary systemic pro-inflammatory priming reactions [319-321]. HFD, however, releases immune tolerance leading to enhanced pro-inflammation. As we have demonstrated, HFD-induced obesity is associated with an increase in dendritic cells within the intestines, we propose these cells are primed for immune response and not tolerance (pro-inflammatory dendritic cells). As such, we would expect an associated increase in dendritic activated immune cells that defend against antigen presented to them (effector cells). This effector immune response did not occur within the intestine. The visceral lymph node, however, swelled in size as a response to HFD intake. The increase is size was due to an influx/proliferation of immune cells. In general, there was not a change in percent distribution of immune cell type within the visceral lymph node, besides decreased percent of T regs, but there was an increase dendritic cells, macrophages and effector T and B cells types. Previous studies demonstrate that immune

cells migrate from PPs [322, 323] to lymphatics in the mesenteric lymph nodes [323]. The movement of lymphocyte populations is described to occur in reaction to mobile antigen presenting cells, such as dendritic cells (DCs) [324, 325]. Upon antigen/pathogen presentation dendritic cells mature and release cytokines while migrating from PPs to the mesentery. The release of cytokines is described to attract recently activated T lymphocytes [324].
We propose the diet-induced increase in the percent of dendritic cells and release of immune tolerance in the intestine causes immune cell expansion in the visceral lymph node. Here the visceral lymph node functions as a primary barrier that, in the case of HFD-induced inflammation, attempts to prevent the spread of regional pathogens.

Taken together this data demonstrates that visceral adipose tissue and the gastrointestinal tract mutually influence immune cells enclosed within the visceral lymph node. During HFD-induced obesity, excessive adipose tissue deposition leads to a shift in deleterious immune cell types subsequently leading to increased release of several cytokines. This effluent is taken up by the visceral lymph node. Within the visceral lymph node adipose derived pro-inflammatory cytokines aid in the expansion, survival and retention of pro-inflammatory T cells (For example Th1 and Th17) and macrophages. Gastrointestinal dysfunction also contributes to this diet-induce metabolic dysregulation. The gastrointestinal tract and visceral adipose tissue likely cause an additive pro-inflammatory response to HFD-induced obesity in the visceral lymph node. Gastrointestinal tolerance is the ability of immune cells to resist an unnecessary pro-inflammatory response to harmless food antigens and commensal bacteria. The visceral lymph node contributes to gastrointestinal tolerance while also protecting systemic immunity by providing a barrier to prevent spread of gastrointestinal pathogens. However, diet-induced obesity leads to an inhibition of gastrointestinal tolerance, which consequently promotes

migration of immune reactive cells to the visceral lymph node in an attempt to stimulate an immune barrier to protect systemic immunity. We propose, however, that chronic effluent of pro-inflammatory cytokines from excessive adipose tissue expansion will eventually disrupt this process and gratuitously exacerbate lymph node immune cell expansion. We predict in obesity, this can result in immune cell dysfunction, exhaustion and immunosuppression within the intra-abdominal cavity.

Tables

Table 6.1 - Total and individual adipose tissue weight depots in grams. Total adiposity is significantly greater in HFD fed mice compared with CHOW (p= .011). All individual depot weights are significantly greater in HFD fed mice compared with CHOW (≤.05).

	7 week		*p* value
	Chow	HFD	
Total	2.74 ± 0.48	5.34 ± 0.63	**0.011**
Epididymal	1.10 ± 0.16	1.87 ± 0.09	**0.003**
Visceral	0.32 ± 0.02	1.25 ± 0.08	**0.001**
Perirenal	0.59 ± 0.11	1.08 ± 0.15	**0.030**
Inguinal	0.61 ± 0.14	1.41 ± 0.20	**0.010**

Figures

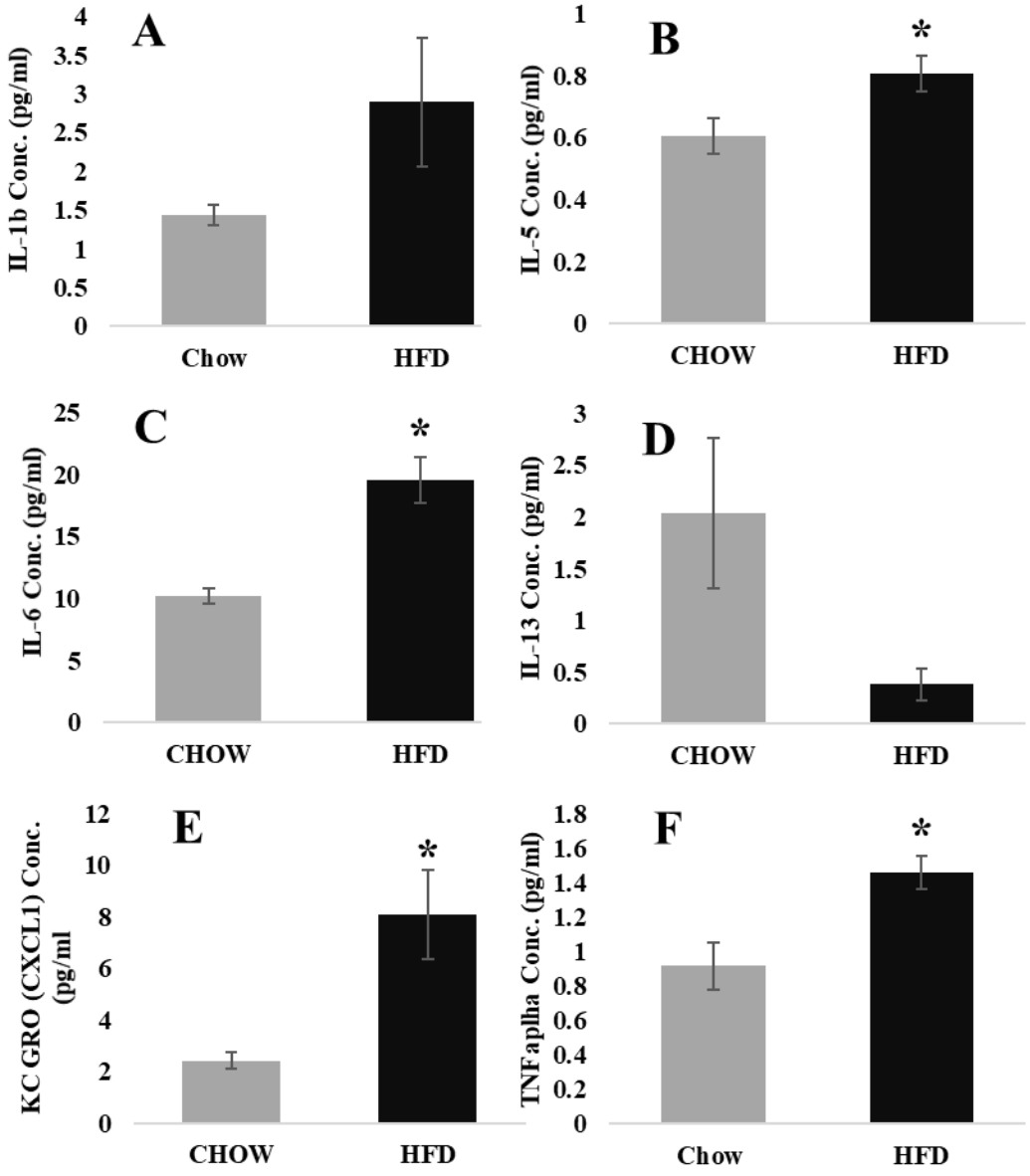


Figure 6.1 - Visceral adipose tissue cytokine concentrations (pg/ml). Compared with CHOW, HFD increased the concentration of pro-inflammatory interleukins IL-1b **A.)** and IL-6 (* = 0.003) **C.)**. The anti-inflammatory IL-5 **B.)** was also significantly increased (* = 0.05) whereas the other IL-13 was decreased **D.)**. Additionally pro-inflammatory cytokines that were significantly increased include KC GRO (* = 0.018) **E.)** and TNFα (* = 0.019) **F.)**.

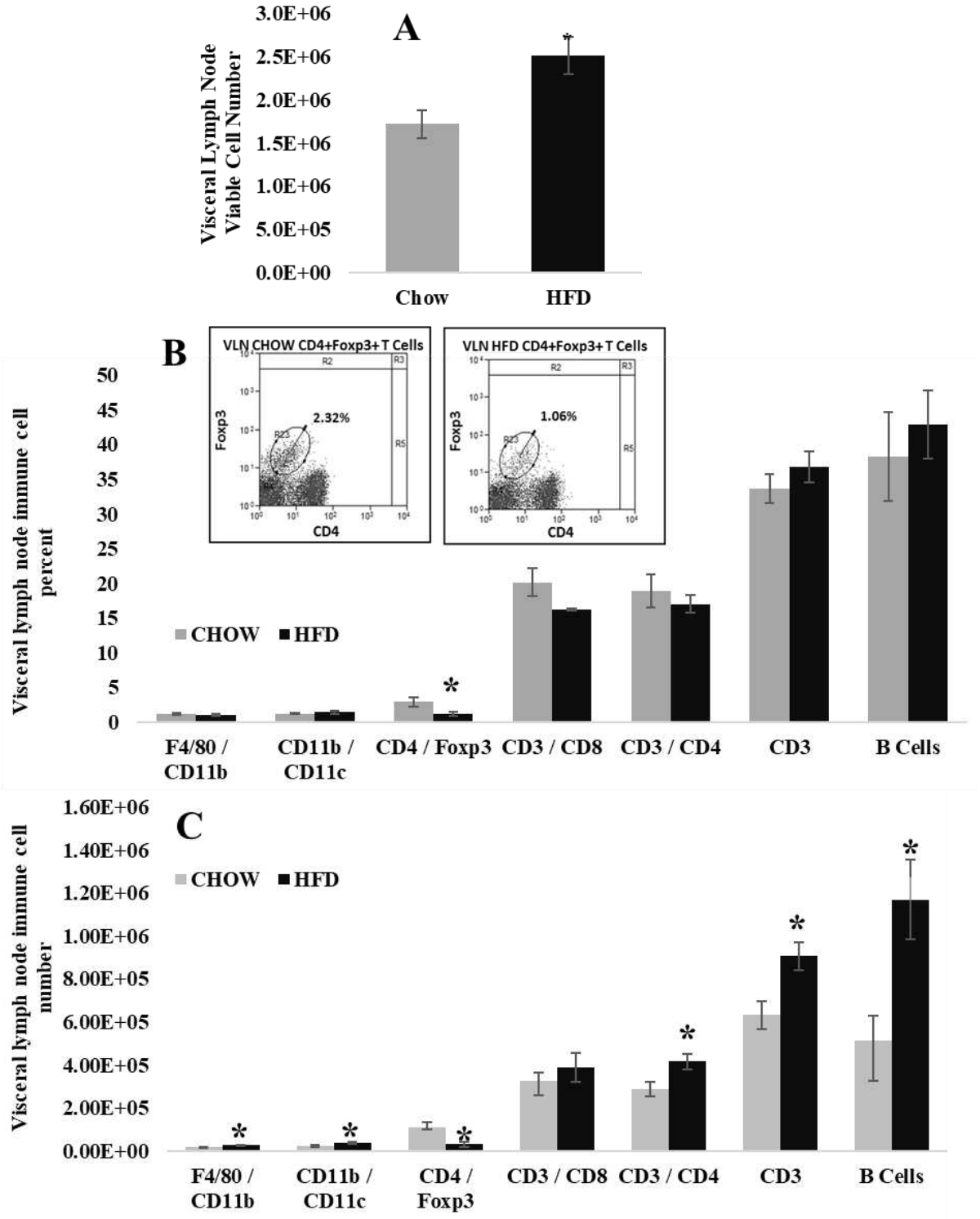


Figure 6.2 - visceral lymph node immune cell number and frequency. A.)Total visceral lymph node viable cells number - HFD significantly increased total number of immunes cells within the visceral lymph node compared with CHOW (* = 0.02). **B.) Percent frequencies (%) of individual immune cell populations** – $CD4^{+}FoxP3^{+}$ cells (regulatory T) were the only immune

cells to be significantly decreased in HFD fed mice compared with CHOW (* = 0.039). The inset represents scatter plots of the significant population enclosed within the circle. **C.) Viable immune cells for distinct populations -** F4/80^{+}CD11b^{+}macrophages (* = 0.022), CD11b^{+}CD11c^{+} dendritic cells (* = 0.017), CD3^{+}CD4^{+}helper T cells (* = 0.024), CD3^{+} pan T cell (* = 0.02), and B220^{+} B cells (* = 0.002) were increased in the visceral lymph node of HFD fed mice compared with CHOW. CD4^{+}FoxP3^{+} regulatory T cells, however, were decreased in HFD mice (** = 0.011)

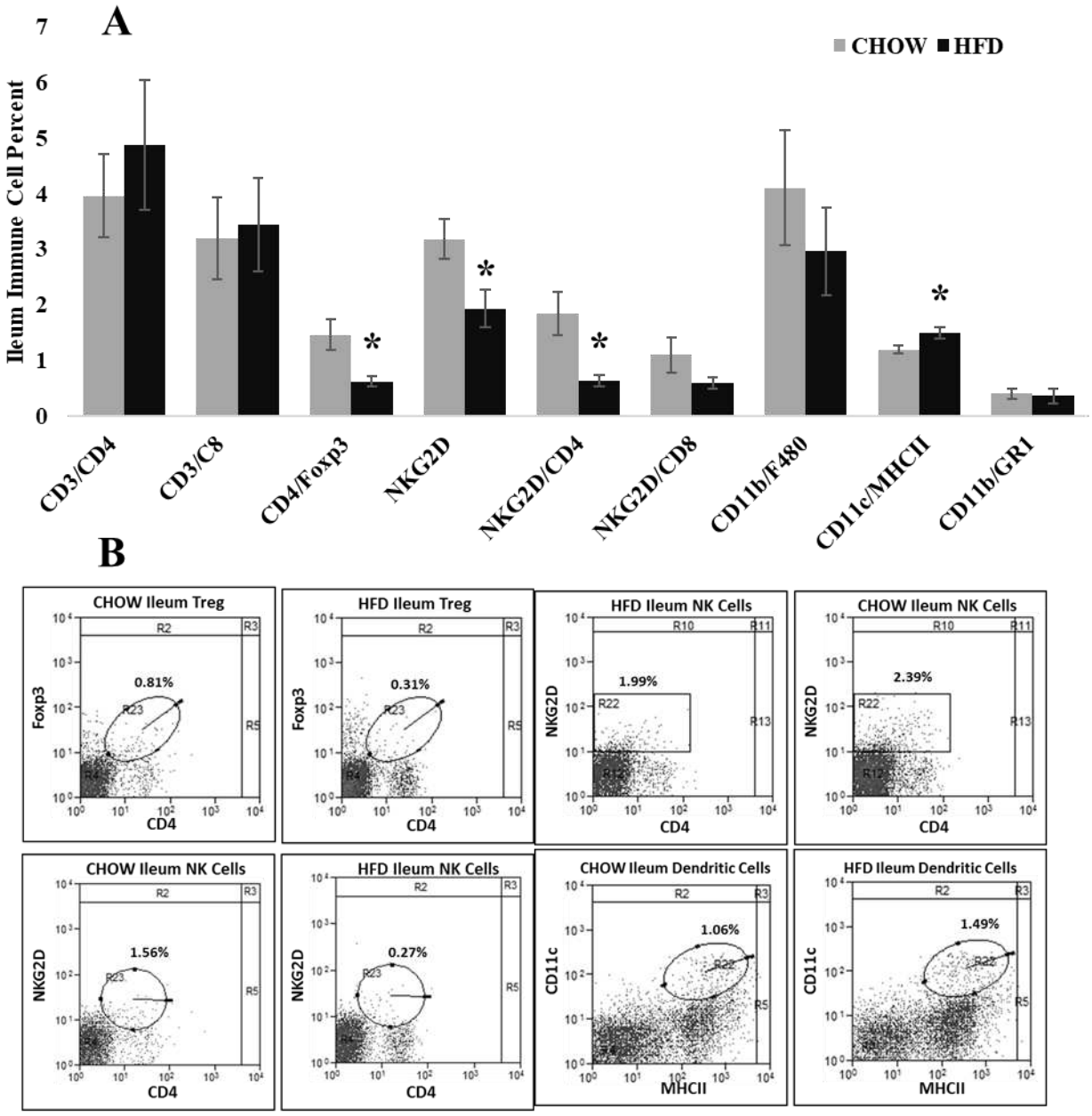


Figure 6.3 - Immune cell frequency (%) for specific populations within the ileum.
A.) CD4^{+}FoxP3^{+} regulatory T (* = 0.011), NKG2D^{+} (* = 0.002) and NKG2D^{+}CD4^{+} (* = 0.014) immune cells were significantly decreased in HFD fed mice compared with CHOW, whereas CD11c^{+}MHCII^{+}dendritic cells (* = 0.014) were significantly increased. **B.)** Representative scatter

plots of immune cell frequency for populations significantly altered by HFD. Circles indicate double positive populations while the square represents all cells positive for a single marker, NKG2D.

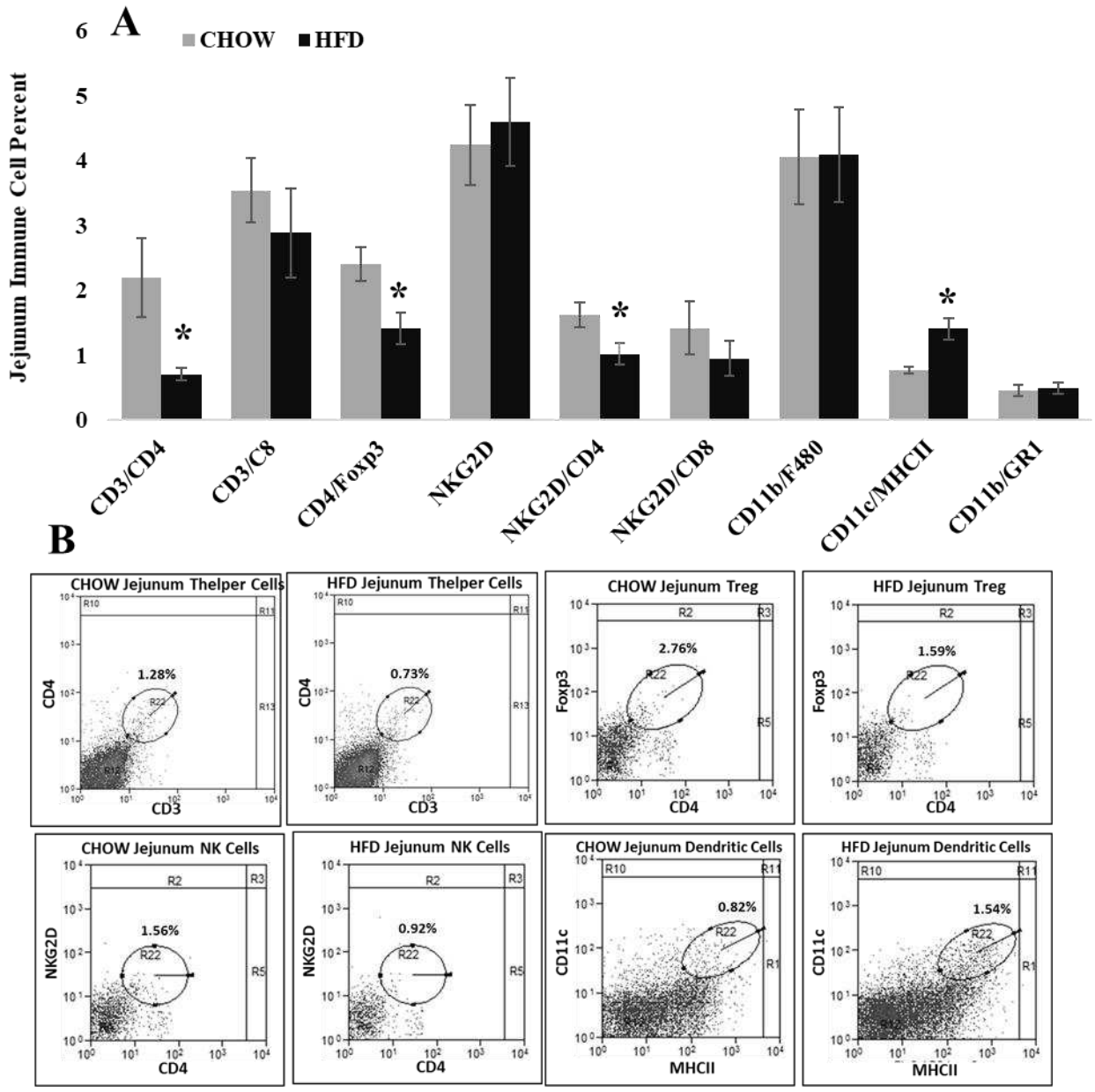


Figure 6.4 - Immune cell frequency (%) for specific populations within the jejunum.
A.) $CD4^+FoxP3^+$ regulatory T (* = 0.015), $NKG2D^+CD4^+$ (* = 0.034), and $CD3^+CD4^+$ helper T cells (* = 0.011) were significantly decreased in HFD fed mice compared with CHOW, whereas $CD11c^+MHCII^+$dendritic cells (* = 0.008) were significantly increased. **B.)** Representative scatter plots of immune cell frequency for populations significantly altered by HFD. All populations are double positive and indicated by circles.

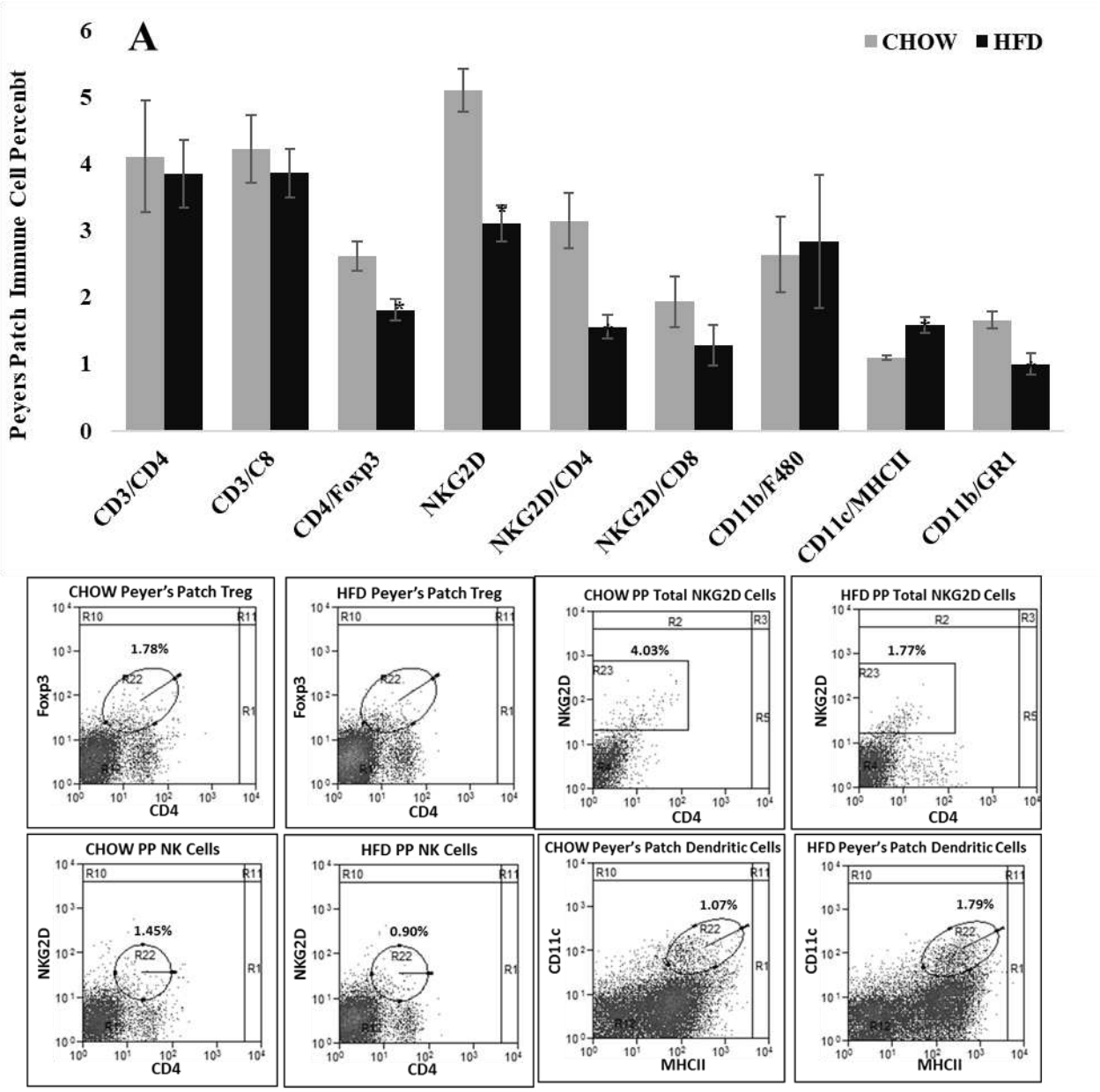


Figure 6.5 - Immune cell frequency (%) for specific populations within the Peyer's patches collected along the Ileum and jejunum. **A.)** Significant decreases occurred in $CD4^+FoxP3^+$ regulatory T (* = 0.011), $NKG2D^+$ (*= 0.002), $NKG2D^+CD4^+$ (p= 0.014) and $CD11b^+Gr1^+$ Myeloid suppressor (* = 0.042) immune cells of HFD mice compared with CHOW. The frequency of $CD11b^+MHCII^+$ dendritic cells, however, were significantly increased in HFD mice (* = 0.005). **B.)** Representative scatter plots of immune cell frequency for populations significantly altered by HFD. The circles indicate double positive populations while the square indicates all cells positive for a single marker NKG2D.

CHAPTER 7: CONCLUSIONS AND FUTURE DIRECTIONS

Obesity is associated with a host of comorbidities including, but not limited to, liver inflammation, type 2 diabetes, cardiovascular disease, insulin resistance, cancers and increased susceptibility to pathogen infection [12, 239-241, 326]. Obesity induced inflammation is proposed to be a key factor driving the development of these co-morbidities. Visceral adiposity is demonstrated to be especially deleterious as it is more highly associated with pro-inflammation, whereas the subcutaneous adipose tissue is demonstrated to be protective [13-15]. Here we addressed why visceral adiposity is so detrimental relative to subcutaneous. We proposed that the high association of visceral adipose tissue accumulation to metabolic disease may be due to extrinsic factors such as the lymphatic system. Data within this dissertation support that the visceral lymph node, embedded within the visceral adipose depot, was highly reactive to HFD-induced obesity. The subcutaneous lymph node, however, was not as susceptible to HFD-induced pro-inflammation. Previous data supports that the visceral lymph node acts as an intestinal immunologic barrier that protects system immune homeostasis. Therefore, we proposed visceral adiposity links to disease risk because of effluent that runs to the lymph node. In support of this the present dissertation identifies several significant findings. First, the visceral lymph node is observed to have a greater capacity for immune suppression by vastly elevated levels of regulatory T cells when compared to the subcutaneous lymph node. Second, the immune cell profiles of the visceral adipose tissue are such that this depot has a greater potential for pro-inflammation by significant elevations of specific immune cell subtypes relative to the subcutaneous adipose tissue. Third, HFD induced obesity results in a decrease in immunosuppressive capacity and an increase to inflammatory cell subsets that is specific to the

visceral adipose tissues and lymph nodes compared with subcutaneous as well as the small intestine. Finally, long term diet induced obesity (DIO) results in immune suppression specific to the visceral adipose tissue and lymph node that is associated with significant visceral lymph node fibrosis.

In order to elucidate the relationships between these tissues (visceral adipose tissues and lymph nodes and intestines) that contribute to heightened pro-inflammatory potential of visceral adiposity, when compared to subcutaneous (subcutaneous adipose tissue and lymph nodes), we utilized mouse models of diet-induced obesity (DIO). We examined HFD induced outcomes on immune profiles of lymph nodes, subcutaneous and visceral adipose tissue and the small intestines utilizing a variety of methods. First, flow cytometric analysis was used to assess how specific immune cell subsets changed in response to diet induced obesity. Analysis of systemic and adipose tissue cytokines served as markers for inflammatory signals that shape the inflammatory immune cell profile. Lastly, histology was performed to visualize how lymph node architecture was influenced by diet-induced obesity (DIO).

Inflammatory potential of visceral lymph nodes and adipose tissue

Immune cell profiles of CHOW-fed (control) animals indicated that even in the context of a healthy immune system that is void of excessive pro-inflammation, the visceral adipose tissue has an inherently greater potential for pro-inflammation compared to the subcutaneous depot. Regulatory T cells are vitally important in the suppression of inflammation as well as promoting oral and systemic tolerance [327]. Here we demonstrated that visceral adipose tissue contains a significantly lower number of regulatory T cells than the subcutaneous tissue. This observation is of particular importance as this has significant implications for the greater pro-inflammatory potential of the visceral adipose tissue depot. A significantly lower number of

these regulatory T cells would indicate that there is a lesser ability to inhibit inflammation in response to a pro-inflammatory stimulus such as those that are present in response to rapid expansion of adipose tissue depots (i.e. hypoxia and necrotic adipocytes) when compared to the subcutaneous adipose tissue. In support of this, HFD resulted in increased pro-inflammatory cell subsets as well as cytokines within the visceral, but not subcutaneous adipose tissue when compared to control animals. This pro-inflammatory visceral adipose tissue response was also associated with significant alterations to the morphology and immune cell profile of the visceral lymph node. This included the swelling of the visceral lymph node, which resulted from increases in total viable immune cells, including the expansion of potentially pro-inflammatory subsets such as antigen presenting cells and helper T cells. These changes did not occur in the subcutaneous lymph node, we predict lack of response was due low reactivity of the subcutaneous adipose depot to HFD. It is well documented in the literature that adipose tissue inflammation is associated with significant infiltration of immune cell subsets into the adipose tissue that exacerbate pro-inflammation [195, 216, 328]. We demonstrate that increased pro-inflammation within the visceral adipose tissue is associated with visceral lymph node pro-inflammation and expansion of immune cells. Again, this response is not observed within the subcutaneous lymph node that is housed within the subcutaneous adipose tissue. This difference is an important point of distinction that helps to identify a potential factor that drives the elevated pro-inflammatory potential of the visceral adipose tissue resulting in a greater association with the co-morbidities of obesity.

Previous data supports that differential immune responses to pathogen challenge between subcutaneous and visceral adipose tissue can be driven by lymph nodes embedded within. For example, peripheral lymph nodes (axillary or brachial) transplantation into visceral adipose

tissue significantly reduces immune response to an oral pathogenic challenge of cholera toxin compared with sham surgery mice [329]. This study highlights a potential way to assess the contributions of the visceral lymph node to elevated visceral adipose tissue pro-inflammation when compared to the subcutaneous adipose tissue. Utilizing the defined transplantation model, subcutaneous lymph nodes can be transplanted to the visceral adipose tissue and visceral lymph nodes to the subcutaneous adipose tissue prior to HFD feeding to induce obesity. This would allow us to assess if inherent differences in the capacity for immune responses between the two lymph nodes are indeed partly responsible for the increased pro-inflammatory potential of the visceral adipose tissue relative to the subcutaneous. One potential pitfall of this study is the inherent size differences between the visceral and subcutaneous lymph nodes. To account for this, lymph node portions could be weighed before transplantation to normalize the amount of secondary lymphoid tissue that is being transplanted between the depots. Also, as the inguinal lymph nodes are bi-lateral (one lymph node located on each side of the body), multiple subcutaneous lymph nodes could be transplanted.

A surprising observation within our study was that the subcutaneous adipose tissue of CHOW mice contained a significantly greater proportion of macrophages than that of CHOW visceral adipose tissue. Despite the fact that we did not characterize macrophage polarization in this current study, we speculate that this may play an important role in protecting the subcutaneous adipose tissue from the inflammatory consequences of obesity relative to the visceral adipose tissue. Macrophage polarization has been demonstrated to be an important aspect of obesity induced inflammation. In general, two broad categories of macrophage polarization have been identified that include the classically activated M1 pro-inflammatory and M2 alternatively activated anti-inflammatory macrophages. M1 macrophages produce cytokines

such as IL-1β and TNF-α and are involved in immune responses to pathogen infection and noxious stimuli. Alternatively activated M2 anti-inflammatory macrophages produce cytokines such as 1L-10 and TGF-β. Resident macrophages within subcutaneous adipose tissue have been characterized to be predominately M2 alternatively activated phenotype that produce anti-inflammatory cytokines such as IL-10 [330]. Furthermore, at 7 weeks of DIO, there was a significant increase in the frequency of macrophages within the visceral adipose tissue that was not observed in the subcutaneous. It has also been demonstrated that the shift in macrophage polarization to a predominate M1 phenotype is due to the recruitment of macrophages to the adipose tissue and not phenotypic switching of macrophages that are resident [330]. Taken together we can postulate that differences in macrophage infiltration and polarization between the visceral and subcutaneous adipose tissue may partially play a role in the differences in inflammatory potential between the two depots. It has also been demonstrated that transplantation of obese subcutaneous adipose tissue to the visceral cavity of obese mice ameliorates metabolic disturbances associated with obesity in mice [19]. Taken together, these observations highlight the potential for a future study to assess macrophage polarization in the obese subcutaneous adipose tissue and their potential to suppress obesity associated inflammation. In order to do this, macrophages that mimic M2 polarization demonstrated in subcutaneous depot could be injected into obese visceral adipose tissue. Subsequently, metabolic outcomes and inflammatory potential of the visceral adipose tissue could be assessed by measuring changes to immune cell populations as well as the resultant cytokine milieu. We hypothesize that by increasing the M2 macrophage population within the visceral adipose tissue, this depot would subsequently be protected from pro-inflammation associated with DIO and would maintain a similar profile to that of the subcutaneous adipose tissue.

Intestinal inflammation and the visceral lymph node

As previously discussed, the gastrointestinal tract effluent drains into the visceral lymph node. Therefore we also examined how HFD induced obesity altered immune cells within the gut to associate relative changes to those demonstrated in the visceral lymph node. A significant limitation of this research was that we did not separate the layers of the intestines in an attempt to isolate immune cells from distinct layers of the tissue. Due to the numerous cell types within the intestine that are of similar size to lymphocytes we were unable to assess changes to the total viable number and were limited to observations solely on the shift in frequency of immune cell subsets. With HFD, the small intestine showed a significant decrease in subsets of immune cells that work to suppress inflammation, such as regulatory T cells and myeloid derived suppressor cells. These cell populations are responsible for development of oral tolerance to food derived antigens and commensal microbiota [331, 332]. There was also an increase in antigen presenting dendritic cells. Although we observed an increase in the frequency of antigen presenting dendritic cells (DCs) we did not characterize their phenotype. We speculate that these DCs are activated and represent a pro-inflammatory subset that samples antigen in the dysregulated gut environment and traffic to the visceral lymph node to elicit an immune response in an attempt to protect from systemic inflammation. These observations suggest that HFD and obesity promote a pro-inflammatory shift within the small intestines.

It is well documented that HFD and obesity are associated with intestinal inflammation and immune cell activation [274, 333, 334]. Numerous studies have demonstrated that HFD feeding changes the microbial community within the gut that in turn drives adipose tissue inflammation [335-337]. In these studies, it has been demonstrated that changes in the microbiota precede adipose tissue deposition and inflammation. It is postulated that HFD feeding

triggers intestinal pro-inflammation by reducing several bacterial species associated with protection against, these include Bifidobacteria [338, 339] and Akkermansia [340, 341]. Decreases in these species link to a decline in barrier function of the intestine [342] and subsequently increases in circulating levels of bacterial endotoxin (LPS) [343]. A significant limitation to our studies is that we did not characterize changes to the microbiota. We can speculate that with increased inflammation we would likely see a similar reduction to both Bifidobacteria and Akkermansia species. In support of a reduction in Akkermansia, this particular bacteria is positively associated with regulatory T cells, hence reductions in Akkermansia are associated with a reduced number of regulatory T cells within the gut [344]. Consistent with this observation, regulatory T cells in the small intestine were reduced in 7 week HFD mice. This indicates that changes to specific bacterial species can influence specific immune cell populations. Therefore, characterization of the microbiota in response to HFD would give great insight into how distinct bacteria may influence the specific populations of immune cells. Saturated fatty acids have also been identified as a potential activator of intestinal pro-inflammation and may represent another mechanism by which visceral lymph nodes may become reactive during HFD feeding [345, 346]. Hence, another potential future study would be to assess the contribution of microbiota to shifts in immune cell populations and pro-inflammation independently of a high saturated fat diet. In order to do this, transplantation of the intestinal microbiome of obese mice into healthy mice to induce obesity could be utilized [347]. We hypothesize that obesity induced by microbiome transplant would result in a lesser inflammatory response compared to DIO initially. However, as obesity persists we would expect that the combination of visceral adipose tissue dysregulation and gut inflammation would

ultimately result in similar elevations to metabolic disturbances and co-morbidities that are associated with visceral adiposity.

Interestingly, we also observed a decrease in the percent of potentially pro-inflammatory cell subsets, including helper T cells and NKG2D+ CD4+ T cells, which have been demonstrated to be important in pro-inflammation associated with Crohn's disease [348]. Pro-inflammation within the gut is generally associated with an increase in pro-inflammatory cell subsets. However, at least one study has demonstrated that significant pro-inflammatory shifts within the small intestine are not observed until a significantly later time-point in DIO [349]. Based on the observed immune response within the visceral lymph node we hypothesize that activated immune cells from the gut are trafficking to the lymph node where they prime effector subsets to protect systemic immunity from gut-derived pathogens and noxious stimuli.

It has been suggested that the visceral lymph node plays an important role in protecting the body from systemic inflammation by acting as a barrier to the intestines [350, 351]. We observed that within the CHOW lymph nodes of healthy mice there is a vastly larger viable population of regulatory T cells when compared to the subcutaneous lymph node. As regulatory T cells are essential in maintaining tolerance and suppressing unnecessary inflammatory responses, this supports the idea that the visceral lymph node is protecting against systemic inflammation generated by signals derived from the gastrointestinal tract. HFD feeding drastically reduced this suppressive population. This supports our observation that there is indeed a priming of the immune system within the visceral lymph node that is associated with diet-induced obesity. Taken together, we have demonstrated that there is a release from immune suppression in both the visceral adipose tissue and the small intestine after prolonged exposure to a HFD. Pro-inflammatory cell subsets likely traffic from the gut to the VLN to prime an immune

response in an attempt to protect from systemic inflammation. However, the visceral adipose tissue is also providing pro-inflammatory stimuli via its effluent to the visceral lymph node. This exacerbates the expansion of immune cell populations and likely disrupts the ability of the visceral lymph node to act as a protective barrier to the intestines. This is likely a significant contributor to the development of obesity associated co-morbidities and disease risk that are especially prevalent in visceral adiposity. In order to better define the sequence in which these events occur, fluorescent labeling of antigen presenting cells would provide insight into traffic from the intestine to the visceral lymph node. Assessing cell trafficking from the gut to the visceral lymph node as well as the shifts in the immune cell population within would help to determine the progression of inflammation among tissues. Specifically, if increases in pro-inflammatory potential of immune cell populations within the visceral lymph node precede adipose tissue expansion and subsequent dysregulation and increased inflammation. We speculate that time course studies assessing changes before our 7 week time point would show that an immune response is occurring early within the visceral lymph node to serve as protection from systemic pro-inflammation. As diet duration continues and adipose tissue becomes dysregulated and begins to accumulate pro-inflammatory macrophages that promote pro-inflammation, we would expect to see exacerbation of the immune response occurring within the visceral lymph node. This promotes a cycle in which pro-inflammation from the gut and adipose tissue both serve to elevate inflammation in the visceral cavity relative to subcutaneous adipose tissue and lymph nodes.

Obesity and immune suppression

It has also been demonstrated that obese individuals are more susceptible to pathogen infection and respond poorly to vaccination [10, 61, 62, 352], suggesting that these individuals

are immunocompromised. Some literature suggests that HFD-induced obesity is in fact associated with lymph node atrophy, reductions in visceral lymph node viable immune cells, specific immune cell subsets, and impaired proliferative capacity of immune cells [232]. Therefore, we extended the timeline of our HFD feeding in order to exacerbate the exposure of lymph nodes to chronic pro-inflammatory signals derived from the gut and adipose tissue. Thirteen weeks of HFD feeding was associated with significant decreases to viable immune cell populations specific to the visceral cavity, in both visceral adipose tissue and lymph nodes. Specific subsets including effector and cytotoxic T cells as well as macrophages and dendritic cells were decreased. This is a sign of immune suppression. Interestingly, the visceral lymph node did not atrophy, but rather remained in a hypertrophied state. We speculate that this continued hypertrophy may be a result of dysfunction of the fibroblastic reticular cells (FRCs) and the reticular cell network. It has been demonstrated that DC interaction with FRCs to control contractility of these cells as well as the tensile ability of the actomyosin cytoskeleton [353]. Following an immune challenge, tension of the actomyosin cytoskeleton is released as activated DCs that express CLEC-2 alter the activity of podoplanin (PDPN) and result in reduced tension of the cytoskeleton allowing for expansion of the lymph node. Dysregulation of these interactions as well as deposition of collagen may inhibit the ability of the cytoskeletal networks to expand and contract.

We also demonstrated that in response to a pathogen-like stimulus, cells taken from HFD visceral lymph nodes showed significantly reduced proliferative capacity relative to CHOW controls in a cell culture model. Based on these results we characterized the morphology of the lymph nodes in order to identify potential reasons for the observed decrease in viable immune cell populations and reduced proliferative capacity. By use of Masons Tri-chrome staining we

observed that there was significant deposition of collagen within the visceral lymph node but not the subcutaneous. In models of chronic infectious disease, collagen deposition within the lymph nodes is associated with immune-suppression [253, 354, 355]. Thus, we have extended previous observations of compromised immunity in obesity by demonstrating a potential mechanism by which this occurs. A significant limitation to this work is that we did not measure the levels of TGF-β either systemically or within specific tissues.

In chronic viral infection, it has been demonstrated that increased fibrosis within lymph nodes is a result of increased levels of regulatory T cells and a subsequent increase in the production of TGF-β [356]. Contrary to this, we have demonstrated that there is a decrease in the number of regulatory T cells in both the visceral lymph node and the surrounding adipose tissue. It is plausible that the remaining population of regulatory T cells are producing TGF-β and that upon long term chronic exposure results in fibrosis of the lymph node. Utilizing flow cytometric cell sorting to isolate regulatory T cells from the obese visceral lymph nodes and subsequently measuring TGF-β production in cell culture relative to CHOW controls would help to elucidate the potential contribution of this cell subset to visceral lymph node fibrosis. It has also been demonstrated that obese adipose tissue has higher levels of TGF-β [257]. Therefore, because the visceral lymph node drains effluent from the surrounding adipose tissue, it is also plausible that cytokines produced within the adipose tissue could be partly responsible for the observed fibrosis. To assess this anti-TGF-β antibodies injected into the visceral adipose tissue of obese mice to limit the exposure of the visceral lymph node to this cytokine from the surrounding adipose tissue could be utilized. Alternatively, it has been demonstrated that pro-inflammatory cytokines can influence the production of collagen from fibroblasts. Specifically, TNF-α has been shown to increase fibrosis in several fibrotic diseases [250, 252, 357] by upregulating TGF-

β and subsequent collagen production in fibroblasts. To test if TGF-β production from fibroblasts themselves is in part responsible for lymph node fibrosis we could utilize a lymph node tissue specific knockout of TGF-β in fibroblastic reticular cells. Specific knockout of TGF-β has been demonstrated in lung fibroblasts [358].

As previously stated, it has been demonstrated that there are inherent differences between the visceral and subcutaneous lymph nodes in response to pathogenic stimulus in transplantation models. It was identified that this may be due to inherent differences in the phenotypes of fibroblastic reticular cells that comprise the fibroblastic reticular cell networks within the visceral and subcutaneous lymph nodes [329]. In order to test if these differences in the fibroblasts between the lymph nodes is in part responsible for elevated fibrosis specific to the visceral lymph nodes in our model of diet induced obesity a transplantation model could be used. Transplantation of inguinal subcutaneous lymph nodes into the visceral adipose tissue depot and visceral lymph nodes into the subcutaneous depot prior to HFD feeding could be utilized to assess whether exposure of the inguinal lymph node to the pro-inflammatory visceral environment elicits similar changes in lymph node morphology. Specific characteristics of how the immune environment have been identified. However, the characteristics defining maintenance of the extra cellular matrix have not been defined in this context. Expression levels of collagen proteins and markers for activation of fibroblasts could be measured to assess if differences in these cells between locations may be responsible for difference levels of collagen deposition.

In conclusion, we have significantly contributed to the field of adipose tissue biology and obesity research by adding to the understanding of why visceral adiposity is so much more detrimental than subcutaneous adipose tissue deposition. It is well characterized that the immune

system plays a central role in the development of obesity associated chronic low grade inflammation within adipose tissue. However, the role that lymphatic tissues, and the immune cells that are housed within them, play have received little attention. In demonstrating that the visceral lymph node is a central component in chronic low grade inflammation that is associated with visceral obesity we have identified a potential target for the development of future therapeutic approaches to address obesity-associated deleterious metabolic outcomes and co-morbidities. As obesity has been demonstrated to be associated with impaired immunity and reduced vaccine efficacy this presents a significant public health challenge, particularly as obesity and its consequences are affecting an increasingly younger population. By identifying obesity related fibrosis in lymph nodes as a potential mechanism that influences immune suppression we have identified an area of future research to help address this significant public health challenge.

REFERENCES

1. Ogden, C.L., et al., *Prevalence of obesity among adults and youth: United States, 2011-2014*.
2. Control, C.f.D. and Prevention, *State-specific prevalence of obesity among adults--United States, 2007.* MMWR. Morbidity and mortality weekly report, 2008. **57**(28): p. 765.
3. Control, C.f.D. and Prevention, *Obesity trends among US adults between 1985 and 2010.* Atlanta, GA: Department of Health and Human Services, Centers for Disease Control and Prevention, 2010.
4. Kim, D.D. and A. Basu, *Estimating the Medical Care Costs of Obesity in the United States: Systematic Review, Meta-Analysis, and Empirical Analysis.* Value in Health, 2016. **19**(5): p. 602-613.
5. Eckel, R.H. and R.M. Krauss, *American Heart Association call to action: obesity as a major risk factor for coronary heart disease.* Circulation, 1998. **97**(21): p. 2099-2100.
6. Hubert, H.B., et al., *Obesity as an independent risk factor for cardiovascular disease: a 26-year follow-up of participants in the Framingham Heart Study.* Circulation, 1983. **67**(5): p. 968-977.
7. Wang, Y., et al., *Comparison of abdominal adiposity and overall obesity in predicting risk of type 2 diabetes among men.* The American Journal of Clinical Nutrition, 2005. **81**(3): p. 555-563.
8. Hall, J.E., et al., *Obesity-associated hypertension. Hyperinsulinemia and renal mechanisms.* Hypertension, 1992. **19**(1 Suppl): p. I45.
9. Morgan, O.W., et al., *Morbid Obesity as a Risk Factor for Hospitalization and Death Due to 2009 Pandemic Influenza A(H1N1) Disease.* PLOS ONE, 2010. **5**(3): p. e9694.
10. Kim, Y.-H., et al., *Diet-induced obesity dramatically reduces the efficacy of a 2009 pandemic H1N1 vaccine in a mouse model.* Journal of Infectious Diseases, 2011. **205**(2): p. 244-251.
11. Kehrl, J.H., et al., *Production of transforming growth factor beta by human T lymphocytes and its potential role in the regulation of T cell growth.* The Journal of Experimental Medicine, 1986. **163**(5): p. 1037.
12. Björntorp, P., *Metabolic implications of body fat distribution.* Diabetes care, 1991. **14**(12): p. 1132-1143.
13. Kissebah, A.H. and G.R. Krakower, *Regional adiposity and morbidity.* Physiological reviews, 1994. **74**(4): p. 761-812.
14. Vague, J., *The degree of masculine differentiation of obesities a factor determining predisposition to diabetes, atherosclerosis, gout, and uric calculous disease.* The American journal of clinical nutrition, 1956. **4**(1): p. 20-34.
15. Björntorp, P., *" Portal" adipose tissue as a generator of risk factors for cardiovascular disease and diabetes.* Arteriosclerosis, Thrombosis, and Vascular Biology, 1990. **10**(4): p. 493-496.
16. Krotkiewski, M., et al., *Impact of obesity on metabolism in men and women. Importance of regional adipose tissue distribution.* Journal of Clinical Investigation, 1983. **72**(3): p. 1150-1162.

17. van der Poorten, D., et al., *Visceral fat: A key mediator of steatohepatitis in metabolic liver disease.* Hepatology, 2008. **48**(2): p. 449-457.
18. van der Poorten, D., et al., *Visceral fat: a key mediator of steatohepatitis in metabolic liver disease.* Hepatology, 2008. **48**(2): p. 449-457.
19. Foster, M.T., et al., *Subcutaneous adipose tissue transplantation in diet-induced obese mice attenuates metabolic dysregulation while removal exacerbates it.* Physiological Reports, 2013. **1**(2).
20. Ibrahim, M.M., *Subcutaneous and visceral adipose tissue: structural and functional differences.* Obesity Reviews, 2010. **11**(1): p. 11-18.
21. Mårin, P., et al., *The morphology and metabolism of intraabdominal adipose tissue in men.* Metabolism, 1992. **41**(11): p. 1242-1248.
22. Misra, A. and N.K. Vikram, *Clinical and pathophysiological consequences of abdominal adiposity and abdominal adipose tissue depots.* Nutrition, 2003. **19**(5): p. 457-466.
23. Pologe, L., et al., *IRS-1-mediated inhibition of insulin receptor tyrosine kinase activity in TNF-ac-and obesity-induced insulin resistance.* Science, 1996. **271**: p. 665.
24. Bergman, R., *Non-esterified fatty acids and the liver: why is insulin secreted into the portal vein?* Diabetologia, 2000. **43**(7): p. 946-952.
25. Williamson, J.R., R. Kreisberg, and P. Felts, *Mechanism for the stimulation of gluconeogenesis by fatty acids in perfused rat liver.* Proceedings of the National Academy of Sciences, 1966. **56**(1): p. 247-254.
26. Boden, G., *Role of fatty acids in the pathogenesis of insulin resistance and NIDDM.* Diabetes, 1997. **46**(1): p. 3-10.
27. Heilbronn, L., S. Smith, and E. Ravussin, *Failure of fat cell proliferation, mitochondrial function and fat oxidation results in ectopic fat storage, insulin resistance and type II diabetes mellitus.* International journal of obesity, 2004. **28**(S4): p. S12.
28. Tripathy, D., et al., *Elevation of free fatty acids induces inflammation and impairs vascular reactivity in healthy subjects.* Diabetes, 2003. **52**(12): p. 2882-2887.
29. Nguyen, M.A., et al., *A subpopulation of macrophages infiltrates hypertrophic adipose tissue and is activated by free fatty acids via Toll-like receptors 2 and 4 and JNK-dependent pathways.* Journal of Biological Chemistry, 2007. **282**(48): p. 35279-35292.
30. Pou, K.M., et al., *Visceral and subcutaneous adipose tissue volumes are cross-sectionally related to markers of inflammation and oxidative stress.* Circulation, 2007. **116**(11): p. 1234-1241.
31. Despres, J.-P. and I. Lemieux, *Abdominal obesity and metabolic syndrome.* Nature, 2006. **444**(7121): p. 881-887.
32. Tsigos, C., et al., *Circulating tumor necrosis factor alpha concentrations are higher in abdominal versus peripheral obesity.* Metabolism, 1999. **48**(10): p. 1332-1335.
33. Fontana, L., et al., *Visceral fat adipokine secretion is associated with systemic inflammation in obese humans.* Diabetes, 2007. **56**(4): p. 1010-3.
34. Altintas, M.M., et al., *Mast cells, macrophages, and crown-like structures distinguish subcutaneous from visceral fat in mice.* Journal of lipid research, 2011. **52**(3): p. 480-488.
35. Weisberg, S.P., et al., *Obesity is associated with macrophage accumulation in adipose tissue.* Journal of Clinical Investigation, 2003. **112**(12): p. 1796-1808.
36. Senn, J.J., et al., *Interleukin-6 induces cellular insulin resistance in hepatocytes.* Diabetes, 2002. **51**(12): p. 3391-3399.

37. Klover, P.J., et al., *Chronic exposure to interleukin-6 causes hepatic insulin resistance in mice.* Diabetes, 2003. **52**(11): p. 2784-2789.
38. Wree, A., et al., *NLRP3 inflammasome driven liver injury and fibrosis: Roles of IL-17 and TNF.* Hepatology, 2017.
39. Zhang, K., et al., *TNF-alpha-mediated lung cytokine networking and eosinophil recruitment in pulmonary fibrosis.* The Journal of Immunology, 1997. **158**(2): p. 954-959.
40. Hoebe, K., E. Janssen, and B. Beutler, *The interface between innate and adaptive immunity.* Nature immunology, 2004. **5**(10): p. 971-974.
41. Schwartz, M., et al., *Innate and adaptive immune responses can be beneficial for CNS repair.* Trends in neurosciences, 1999. **22**(7): p. 295-299.
42. Epelman, S., P.P. Liu, and D.L. Mann, *Role of Innate and Adaptive Immunity in Cardiac Injury and Repair.* Nature reviews. Immunology, 2015. **15**(2): p. 117-129.
43. Butcher, E.C., et al., *Lymphocyte Trafficking and Regional Immunity*, in *Advances in Immunology*, F.J. Dixon, et al., Editors. 1999, Academic Press. p. 209-253.
44. Miller, M.J., et al., *Two-Photon Imaging of Lymphocyte Motility and Antigen Response in Intact Lymph Node.* Science, 2002. **296**(5574): p. 1869-1873.
45. Pape, K.A., et al., *The Humoral Immune Response Is Initiated in Lymph Nodes by B Cells that Acquire Soluble Antigen Directly in the Follicles.* Immunity, 2007. **26**(4): p. 491-502.
46. Medzhitov, R., *Toll-like receptors and innate immunity.* Nature reviews. Immunology, 2001. **1**(2): p. 135.
47. Akira, S., S. Uematsu, and O. Takeuchi, *Pathogen Recognition and Innate Immunity.* Cell, 2006. **124**(4): p. 783-801.
48. Medzhitov, R. and C.A. Janeway, *Innate immunity: impact on the adaptive immune response.* Current Opinion in Immunology, 1997. **9**(1): p. 4-9.
49. Akira, S., K. Takeda, and T. Kaisho, *Toll-like receptors: critical proteins linking innate and acquired immunity.* Nature immunology, 2001. **2**(8): p. 675.
50. Pond, C.M. and C.A. Mattacks, *Interactions between adipose tissue around lymph nodes and lymphoid cells in vitro.* J Lipid Res, 1995. **36**(10): p. 2219-31.
51. Arngrim, N., et al., *Reduced adipose tissue lymphatic drainage of macromolecules in obese subjects: a possible link between obesity and local tissue inflammation?* Int J Obes (Lond), 2013. **37**(5): p. 748-50.
52. Weitman, E.S., et al., *Obesity impairs lymphatic fluid transport and dendritic cell migration to lymph nodes.* PloS one, 2013. **8**(8): p. e70703.
53. Vandanmagsar, B., et al., *The NLRP3 inflammasome instigates obesity-induced inflammation and insulin resistance.* Nat Med, 2011. **17**(2): p. 179-188.
54. Nijhuis, J., et al., *Neutrophil Activation in Morbid Obesity, Chronic Activation of Acute Inflammation.* Obesity, 2009. **17**(11): p. 2014-2018.
55. Halberg, N., et al., *Hypoxia-inducible factor 1α induces fibrosis and insulin resistance in white adipose tissue.* Molecular and cellular biology, 2009. **29**(16): p. 4467-4483.
56. Buckley, C.D., et al., *Fibroblasts regulate the switch from acute resolving to chronic persistent inflammation.* Trends in Immunology, 2001. **22**(4): p. 199-204.
57. Cildir, G., S.C. Akıncılar, and V. Tergaonkar, *Chronic adipose tissue inflammation: all immune cells on the stage.* Trends in Molecular Medicine, 2013. **19**(8): p. 487-500.
58. Calle , E.E., et al., *Body-Mass Index and Mortality in a Prospective Cohort of U.S. Adults.* New England Journal of Medicine, 1999. **341**(15): p. 1097-1105.

59. Choban, P.S., et al., *Increased incidence of nosocomial infections in obese surgical patients.* Am Surg, 1995. **61**(11): p. 1001-5.
60. Choban, P.S. and L. Flancbaum, *The Impact of Obesity on Surgical Outcomes: A Review.* Journal of the American College of Surgeons, 1997. **185**(6): p. 593-603.
61. Morgan, O.W., et al., *Morbid obesity as a risk factor for hospitalization and death due to 2009 pandemic influenza A (H1N1) disease.* PloS one, 2010. **5**(3): p. e9694.
62. Park, H.-L., et al., *Obesity-induced chronic inflammation is associated with the reduced efficacy of influenza vaccine.* Human Vaccines & Immunotherapeutics, 2014. **10**(5): p. 1181-1186.
63. Smith, A.G., et al., *Diet-induced obese mice have increased mortality and altered immune responses when infected with influenza virus.* The Journal of nutrition, 2007. **137**(5): p. 1236-1243.
64. Karlsson, E.A., P.A. Sheridan, and M.A. Beck, *Diet-Induced Obesity Impairs the T Cell Memory Response to Influenza Virus Infection.* The Journal of Immunology, 2010. **184**(6): p. 3127-3133.
65. Louie, J.K., et al., *A novel risk factor for a novel virus: obesity and 2009 pandemic influenza A (H1N1).* Clinical Infectious Diseases, 2011. **52**(3): p. 301-312.
66. MacIver, N.J., et al., *Glucose metabolism in lymphocytes is a regulated process with significant effects on immune cell function and survival.* Journal of leukocyte biology, 2008. **84**(4): p. 949-957.
67. Fox, C.J., P.S. Hammerman, and C.B. Thompson, *Fuel feeds function: energy metabolism and the T-cell response.* Nature reviews. Immunology, 2005. **5**(11): p. 844.
68. Frauwirth, K.A., et al., *The CD28 signaling pathway regulates glucose metabolism.* Immunity, 2002. **16**(6): p. 769-777.
69. Martín-Romero, C., et al., *Human leptin enhances activation and proliferation of human circulating T lymphocytes.* Cellular immunology, 2000. **199**(1): p. 15-24.
70. Lord, G.M., et al., *Leptin inhibits the anti-CD3-driven proliferation of peripheral blood T cells but enhances the production of proinflammatory cytokines.* Journal of leukocyte biology, 2002. **72**(2): p. 330-338.
71. Macia, L., et al., *Impairment of dendritic cell functionality and steady-state number in obese mice.* The Journal of Immunology, 2006. **177**(9): p. 5997-6006.
72. Smith, A.G., et al., *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): p. 268-279.
73. *Health, United States, 2014: With Special Feature on Adults 55-64.* 2014, Center for Disease Control and Prevention.
74. Brown, C.D., et al., *Body mass index and the prevalence of hypertension and dyslipidemia.* Obes Res, 2000. **8**(9): p. 605-19.
75. Miyake, T., et al., *Body mass index is the most useful predictive factor for the onset of nonalcoholic fatty liver disease: a community-based retrospective longitudinal cohort study.* J Gastroenterol, 2013. **48**(3): p. 413-22.
76. Lamon-Fava, S., P.W. Wilson, and E.J. Schaefer, *Impact of body mass index on coronary heart disease risk factors in men and women. The Framingham Offspring Study.* Arterioscler Thromb Vasc Biol, 1996. **16**(12): p. 1509-15.
77. Sanada, H., et al., *High body mass index is an important risk factor for the development of type 2 diabetes.* Intern Med, 2012. **51**(14): p. 1821-6.

78. Haffner, S.M., *Relationship of metabolic risk factors and development of cardiovascular disease and diabetes.* Obesity (Silver Spring), 2006. **14 Suppl 3**: p. 121S-127S.
79. Despres, J.P., et al., *The insulin resistance-dyslipidemic syndrome: contribution of visceral obesity and therapeutic implications.* Int J Obes Relat Metab Disord, 1995. **19 Suppl 1**: p. S76-86.
80. Fox, C.S., et al., *Abdominal visceral and subcutaneous adipose tissue compartments: association with metabolic risk factors in the Framingham Heart Study.* Circulation, 2007. **116**(1): p. 39-48.
81. Mamo, J.C., et al., *Postprandial dyslipidemia in men with visceral obesity: an effect of reduced LDL receptor expression?* Am J Physiol Endocrinol Metab, 2001. **281**(3): p. E626-32.
82. Chan, D.C., H.P. Barrett, and G.F. Watts, *Dyslipidemia in visceral obesity: mechanisms, implications, and therapy.* Am J Cardiovasc Drugs, 2004. **4**(4): p. 227-46.
83. Zambon, A., M. Marchiori, and E. Manzato, *[Dyslipidemia in visceral obesity: pathophysiological mechanisms, clinical implications and therapy].* G Ital Cardiol (Rome), 2008. **9**(4 Suppl 1): p. 29S-39S.
84. Anjana, M., et al., *Visceral and central abdominal fat and anthropometry in relation to diabetes in Asian Indians.* Diabetes Care, 2004. **27**(12): p. 2948-53.
85. Hayashi, T., et al., *Visceral adiposity and the risk of impaired glucose tolerance: a prospective study among Japanese Americans.* Diabetes Care, 2003. **26**(3): p. 650-5.
86. Wei, M., et al., *Waist circumference as the best predictor of noninsulin dependent diabetes mellitus (NIDDM) compared to body mass index, waist/hip ratio and other anthropometric measurements in Mexican Americans--a 7-year prospective study.* Obes Res, 1997. **5**(1): p. 16-23.
87. Dowse, G.K., et al., *Abdominal obesity and physical inactivity as risk factors for NIDDM and impaired glucose tolerance in Indian, Creole, and Chinese Mauritians.* Diabetes Care, 1991. **14**(4): p. 271-82.
88. Araneta, M.R. and E. Barrett-Connor, *Ethnic differences in visceral adipose tissue and type 2 diabetes: Filipino, African-American, and white women.* Obes Res, 2005. **13**(8): p. 1458-65.
89. Speliotes, E.K., et al., *Fatty liver is associated with dyslipidemia and dysglycemia independent of visceral fat: the Framingham Heart Study.* Hepatology, 2010. **51**(6): p. 1979-87.
90. Porter, S.A., et al., *Abdominal subcutaneous adipose tissue: a protective fat depot?* Diabetes Care, 2009. **32**(6): p. 1068-75.
91. Abate, N., et al., *Relationships of generalized and regional adiposity to insulin sensitivity in men.* J Clin Invest, 1995. **96**(1): p. 88-98.
92. Goodpaster, B.H., et al., *Subcutaneous abdominal fat and thigh muscle composition predict insulin sensitivity independently of visceral fat.* Diabetes, 1997. **46**(10): p. 1579-85.
93. Abate, N., et al., *Relationship of generalized and regional adiposity to insulin sensitivity in men with NIDDM.* Diabetes, 1996. **45**(12): p. 1684-93.
94. Ferreira, I., et al., *The metabolic syndrome, cardiopulmonary fitness, and subcutaneous trunk fat as independent determinants of arterial stiffness: the Amsterdam Growth and Health Longitudinal Study.* Arch Intern Med, 2005. **165**(8): p. 875-82.

95. Wildman, R.P., et al., *Subcutaneous adipose tissue in relation to subclinical atherosclerosis and cardiometabolic risk factors in midlife women.* Am J Clin Nutr, 2011. **93**(4): p. 719-26.
96. Snijder, M.B., et al., *Low subcutaneous thigh fat is a risk factor for unfavourable glucose and lipid levels, independently of high abdominal fat. The Health ABC Study.* Diabetologia, 2005. **48**(2): p. 301-8.
97. McLaughlin, T., et al., *Preferential fat deposition in subcutaneous versus visceral depots is associated with insulin sensitivity.* J Clin Endocrinol Metab, 2011. **96**(11): p. E1756-60.
98. Koster, A., et al., *Body fat distribution and inflammation among obese older adults with and without metabolic syndrome.* Obesity (Silver Spring), 2010. **18**(12): p. 2354-61.
99. Gallagher, D., et al., *Adipose tissue distribution is different in type 2 diabetes.* Am J Clin Nutr, 2009. **89**(3): p. 807-14.
100. Yim, J.E., et al., *Femoral-gluteal subcutaneous and intermuscular adipose tissues have independent and opposing relationships with CVD risk.* J Appl Physiol (1985), 2008. **104**(3): p. 700-7.
101. Manolopoulos, K.N., F. Karpe, and K.N. Frayn, *Gluteofemoral body fat as a determinant of metabolic health.* Int J Obes (Lond), 2010. **34**(6): p. 949-59.
102. Foster, M.T., et al., *Transplantation or removal of intra-abdominal adipose tissue prevents age-induced glucose insensitivity.* Physiol Behav, 2010. **101**(2): p. 282-8.
103. Foster, M.T., et al., *Removal of intra-abdominal visceral adipose tissue improves glucose tolerance in rats: role of hepatic triglyceride storage.* Physiol Behav, 2011. **104**(5): p. 845-54.
104. Foster, M.T., et al., *Transplantation of non-visceral fat to the visceral cavity improves glucose tolerance in mice: investigation of hepatic lipids and insulin sensitivity.* Diabetologia, 2011. **54**(11): p. 2890-9.
105. Foster, M.T., et al., *Subcutaneous Adipose Tissue Transplantation in Diet-Induced Obese Mice Attenuates Metabolic Dysregulation While Removal Exacerbates It.* Physiol Rep, 2013. **1**(2).
106. Klein, S., et al., *Waist circumference and cardiometabolic risk: a consensus statement from shaping America's health: Association for Weight Management and Obesity Prevention; NAASO, the Obesity Society; the American Society for Nutrition; and the American Diabetes Association.* Diabetes Care, 2007. **30**(6): p. 1647-52.
107. Hotamisligil, G.S., et al., *IRS-1-mediated inhibition of insulin receptor tyrosine kinase activity in TNF-alpha- and obesity-induced insulin resistance.* Science, 1996. **271**(5249): p. 665-8.
108. Bergman, R.N., *Non-esterified fatty acids and the liver: why is insulin secreted into the portal vein?* Diabetologia, 2000. **43**(7): p. 946-52.
109. Williamson, J.R., R.A. Kreisberg, and P.W. Felts, *Mechanism for the stimulation of gluconeogenesis by fatty acids in perfused rat liver.* Proc Natl Acad Sci U S A, 1966. **56**(1): p. 247-54.
110. Nemecz, M., et al., *Acute effect of leptin on hepatic glycogenolysis and gluconeogenesis in perfused rat liver.* Hepatology, 1999. **29**(1): p. 166-72.
111. Borba-Murad, G.R., et al., *Comparative acute effects of leptin and insulin on gluconeogenesis and ketogenesis in perfused rat liver.* Cell Biochem Funct, 2005. **23**(6): p. 405-13.

112. Bassil, M.S., et al., *Acute effect of leptin and ghrelin injection on postprandial glycogen and lipid synthesis in rats.* Ann Nutr Metab, 2007. **51**(1): p. 14-21.
113. Gerner, R.R., et al., *Metabolic inflammation: role of cytokines in the crosstalk between adipose tissue and liver.* Can J Physiol Pharmacol, 2013. **91**(11): p. 867-72.
114. Neuschwander-Tetri, B.A., *Hepatic lipotoxicity and the pathogenesis of nonalcoholic steatohepatitis: the central role of nontriglyceride fatty acid metabolites.* Hepatology, 2010. **52**(2): p. 774-88.
115. Topping, D.L. and P.A. Mayes, *Insulin and non-esterified fatty acids. Acute regulators of lipogenesis in perfused rat liver.* Biochem J, 1982. **204**(2): p. 433-9.
116. Mraz, M. and M. Haluzik, *The role of adipose tissue immune cells in obesity and low-grade inflammation.* J Endocrinol, 2014. **222**(3): p. R113-27.
117. Thijssen, E., A. van Caam, and P.M. van der Kraan, *Obesity and osteoarthritis, more than just wear and tear: pivotal roles for inflamed adipose tissue and dyslipidaemia in obesity-induced osteoarthritis.* Rheumatology (Oxford), 2014.
118. Guarner, V. and M.E. Rubio-Ruiz, *Low-grade systemic inflammation connects aging, metabolic syndrome and cardiovascular disease.* Interdiscip Top Gerontol, 2015. **40**: p. 99-106.
119. Esser, N., et al., *Inflammation as a link between obesity, metabolic syndrome and type 2 diabetes.* Diabetes Res Clin Pract, 2014. **105**(2): p. 141-50.
120. Ghigliotti, G., et al., *Adipose tissue immune response: novel triggers and consequences for chronic inflammatory conditions.* Inflammation, 2014. **37**(4): p. 1337-53.
121. Cartier, A., et al., *Plasma soluble tumour necrosis factor-alpha receptor 2 is elevated in obesity: specific contribution of visceral adiposity.* Clin Endocrinol (Oxf), 2010. **72**(3): p. 349-57.
122. Fried, S.K., D.A. Bunkin, and A.S. Greenberg, *Omental and subcutaneous adipose tissues of obese subjects release interleukin-6: depot difference and regulation by glucocorticoid.* J Clin Endocrinol Metab, 1998. **83**(3): p. 847-50.
123. Lemieux, I., et al., *Elevated C-reactive protein: another component of the atherothrombotic profile of abdominal obesity.* Arterioscler Thromb Vasc Biol, 2001. **21**(6): p. 961-7.
124. Malavazos, A.E., et al., *Proinflammatory cytokines and cardiac abnormalities in uncomplicated obesity: relationship with abdominal fat deposition.* Nutr Metab Cardiovasc Dis, 2007. **17**(4): p. 294-302.
125. Curat, C.A., et al., *Macrophages in human visceral adipose tissue: increased accumulation in obesity and a source of resistin and visfatin.* Diabetologia, 2006. **49**(4): p. 744-7.
126. von der Weid, P.Y. and K.J. Rainey, *Review article: lymphatic system and associated adipose tissue in the development of inflammatory bowel disease.* Aliment Pharmacol Ther, 2010. **32**(6): p. 697-711.
127. Kim, C.S., et al., *Visceral fat accumulation induced by a high-fat diet causes the atrophy of mesenteric lymph nodes in obese mice.* Obesity (Silver Spring), 2008. **16**(6): p. 1261-9.
128. Kissebah, A.H. and G.R. Krakower, *Regional adiposity and morbidity.* Physiol Rev, 1994. **74**(4): p. 761-811.
129. Versini, M., et al., *Obesity in autoimmune diseases: not a passive bystander.* Autoimmun Rev, 2014. **13**(9): p. 981-1000.
130. Kim, S.I., et al., *Impact of underweight after treatment on prognosis of advanced-stage ovarian cancer.* J Immunol Res, 2014. **2014**: p. 349546.

131. Schmitz, K.H., et al., *Impact of obesity on cancer survivorship and the potential relevance of race and ethnicity.* J Natl Cancer Inst, 2013. **105**(18): p. 1344-54.
132. Novosad, S., et al., *Role of obesity in asthma control, the obesity-asthma phenotype.* J Allergy (Cairo), 2013. **2013**: p. 538642.
133. Sheridan, P.A., et al., *Obesity is associated with impaired immune response to influenza vaccination in humans.* Int J Obes (Lond), 2012. **36**(8): p. 1072-7.
134. Milner, J.J. and M.A. Beck, *The impact of obesity on the immune response to infection.* Proc Nutr Soc, 2012. **71**(2): p. 298-306.
135. Bochicchio, G.V., et al., *Impact of obesity in the critically ill trauma patient: a prospective study.* J Am Coll Surg, 2006. **203**(4): p. 533-8.
136. Yaegashi, M., et al., *Outcome of morbid obesity in the intensive care unit.* J Intensive Care Med, 2005. **20**(3): p. 147-54.
137. Mancuso, P., *Obesity and respiratory infections: does excess adiposity weigh down host defense?* Pulm Pharmacol Ther, 2013. **26**(4): p. 412-9.
138. de Wit, N.J., et al., *The role of the small intestine in the development of dietary fat-induced obesity and insulin resistance in C57BL/6J mice.* BMC Med Genomics, 2008. **1**: p. 14.
139. Ley, R.E., et al., *Obesity alters gut microbial ecology.* Proc Natl Acad Sci U S A, 2005. **102**(31): p. 11070-5.
140. Backhed, F., et al., *The gut microbiota as an environmental factor that regulates fat storage.* Proc Natl Acad Sci U S A, 2004. **101**(44): p. 15718-23.
141. Turnbaugh, P.J., et al., *An obesity-associated gut microbiome with increased capacity for energy harvest.* Nature, 2006. **444**(7122): p. 1027-31.
142. de La Serre, C.B., et al., *Propensity to high-fat diet-induced obesity in rats is associated with changes in the gut microbiota and gut inflammation.* Am J Physiol Gastrointest Liver Physiol, 2010. **299**(2): p. G440-8.
143. Fei, N. and L. Zhao, *An opportunistic pathogen isolated from the gut of an obese human causes obesity in germfree mice.* ISME J, 2013. **7**(4): p. 880-4.
144. Teixeira, T.F., et al., *Potential mechanisms for the emerging link between obesity and increased intestinal permeability.* Nutr Res, 2012. **32**(9): p. 637-47.
145. Yoshida, H., et al., *Fatty acids enhance GRO/CINC-1 and interleukin-6 production in rat intestinal epithelial cells.* J Nutr, 2001. **131**(11): p. 2943-50.
146. Castro, G.A. and C.J. Arntzen, *Immunophysiology of the gut: a research frontier for integrative studies of the common mucosal immune system.* Am J Physiol, 1993. **265**(4 Pt 1): p. G599-610.
147. Hara, Y., et al., *Exposure to fatty acids modulates interferon production by intraepithelial lymphocytes.* Immunol Lett, 2003. **86**(2): p. 139-48.
148. Tsuzuki, Y., et al., *Differential modulation in the functions of intestinal dendritic cells by long- and medium-chain fatty acids.* J Gastroenterol, 2006. **41**(3): p. 209-16.
149. Ji, Y., et al., *Activation of rat intestinal mucosal mast cells by fat absorption.* Am J Physiol Gastrointest Liver Physiol, 2012. **302**(11): p. G1292-300.
150. Progatzky, F., et al., *Dietary cholesterol directly induces acute inflammasome-dependent intestinal inflammation.* Nat Commun, 2014. **5**: p. 5864.
151. Bjorntorp, P., *Metabolic implications of body fat distribution.* Diabetes Care, 1991. **14**(12): p. 1132-43.

152. Snijder, M.B., et al., *Trunk fat and leg fat have independent and opposite associations with fasting and postload glucose levels: the Hoorn study.* Diabetes Care, 2004. **27**(2): p. 372-7.
153. Kuan, E.L., et al., *Collecting lymphatic vessel permeability facilitates adipose tissue inflammation and distribution of antigen to lymph node-homing adipose tissue dendritic cells.* J Immunol, 2015. **194**(11): p. 5200-10.
154. Ryan, T.J., *Lymphatics and adipose tissue.* Clin Dermatol, 1995. **13**(5): p. 493-8.
155. Pond, C.M., *Adipose tissue, the immune system and exercise fatigue: how activated lymphocytes compete for lipids.* Biochem Soc Trans, 2002. **30**(2): p. 270-5.
156. Snijder, M.B., et al., *Larger thigh and hip circumferences are associated with better glucose tolerance: the Hoorn study.* Obes Res, 2003. **11**(1): p. 104-11.
157. Snijder, M.B., et al., *Associations of hip and thigh circumferences independent of waist circumference with the incidence of type 2 diabetes: the Hoorn Study.* Am J Clin Nutr, 2003. **77**(5): p. 1192-7.
158. Rytka, J.M., et al., *The portal theory supported by venous drainage-selective fat transplantation.* Diabetes, 2011. **60**(1): p. 56-63.
159. Mathis, D., *Immunological goings-on in visceral adipose tissue.* Cell Metab, 2013. **17**(6): p. 851-9.
160. Harris, R.B. and R.L. Leibel, *Location, location, location.* Cell Metab, 2008. **7**(5): p. 359-61.
161. del Mar Romero, M., et al., *Site-related white adipose tissue lipid-handling response to oleoyl-estrone treatment in overweight male rats.* Eur J Nutr, 2009. **48**(5): p. 291-9.
162. Matsuzawa, Y., et al., *Pathophysiology and pathogenesis of visceral fat obesity.* Ann N Y Acad Sci, 1995. **748**: p. 399-406.
163. Matsuzawa, Y., et al., *Pathophysiology and pathogenesis of visceral fat obesity.* Diabetes Res Clin Pract, 1994. **24 Suppl**: p. S111-6.
164. Bjorntorp, P., *The regulation of adipose tissue distribution in humans.* Int J Obes Relat Metab Disord, 1996. **20**(4): p. 291-302.
165. Jensen, M.D., *Health consequences of fat distribution.* Horm Res, 1997. **48 Suppl 5**: p. 88-92.
166. Yu, R., et al., *Mesenteric adipose tissue-derived monocyte chemoattractant protein-1 plays a crucial role in adipose tissue macrophage migration and activation in obese mice.* Obesity (Silver Spring), 2006. **14**(8): p. 1353-62.
167. Romero Mdel, M., et al., *Different modulation by dietary restriction of adipokine expression in white adipose tissue sites in the rat.* Cardiovasc Diabetol, 2009. **8**: p. 42.
168. Harman-Boehm, I., et al., *Macrophage infiltration into omental versus subcutaneous fat across different populations: effect of regional adiposity and the comorbidities of obesity.* J Clin Endocrinol Metab, 2007. **92**(6): p. 2240-7.
169. Sebba, A., *Tocilizumab: the first interleukin-6-receptor inhibitor.* Am J Health Syst Pharm, 2008. **65**(15): p. 1413-8.
170. Kanda, H., et al., *MCP-1 contributes to macrophage infiltration into adipose tissue, insulin resistance, and hepatic steatosis in obesity.* The Journal of clinical investigation, 2006. **116**(6): p. 1494-1505.
171. Palframan, R.T., et al., *Inflammatory Chemokine Transport and Presentation in HEV A Remote Control Mechanism for Monocyte Recruitment to Lymph Nodes in Inflamed Tissues.* The Journal of experimental medicine, 2001. **194**(9): p. 1361-1374.

172. Angeli, V., et al., *B cell-driven lymphangiogenesis in inflamed lymph nodes enhances dendritic cell mobilization.* Immunity, 2006. **24**(2): p. 203-215.
173. Tan, K.W., et al., *Expansion of cortical and medullary sinuses restrains lymph node hypertrophy during prolonged inflammation.* The Journal of Immunology, 2012. **188**(8): p. 4065-4080.
174. Yang, C.-Y., et al., *Trapping of naive lymphocytes triggers rapid growth and remodeling of the fibroblast network in reactive murine lymph nodes.* Proceedings of the National Academy of Sciences, 2014. **111**(1): p. E109-E118.
175. Kim, C., et al., *Visceral Fat Accumulation Induced by a High-fat Diet Causes the Atrophy of Mesenteric Lymph Nodes in Obese Mice.* Obesity, 2008. **16**(6): p. 1261-1269.
176. Murray, P.J. and T.A. Wynn, *Protective and pathogenic functions of macrophage subsets.* Nat Rev Immunol, 2011. **11**(11): p. 723-37.
177. Austyn, J.M. and S. Gordon, *F4/80, a monoclonal antibody directed specifically against the mouse macrophage.* European journal of immunology, 1981. **11**(10): p. 805-815.
178. Xu, H., et al., *Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance.* The Journal of clinical investigation, 2003. **112**(12): p. 1821-1830.
179. Bain, C.C., et al., *Constant replenishment from circulating monocytes maintains the macrophage pool in the intestine of adult mice.* Nature immunology, 2014. **15**(10): p. 929-937.
180. Bassaganya-Riera, J., et al., *PPAR γ is highly expressed in F4/80 hi adipose tissue macrophages and dampens adipose-tissue inflammation.* Cellular immunology, 2009. **258**(2): p. 138-146.
181. Weisberg, S.P., et al., *CCR2 modulates inflammatory and metabolic effects of high-fat feeding.* The Journal of clinical investigation, 2006. **116**(1): p. 115-124.
182. Brake, D.K., et al., *ICAM-1 expression in adipose tissue: effects of diet-induced obesity in mice.* American Journal of Physiology-Cell Physiology, 2006. **291**(6): p. C1232-C1239.
183. Lumeng, C.N., J.L. Bodzin, and A.R. Saltiel, *Obesity induces a phenotypic switch in adipose tissue macrophage polarization.* The Journal of clinical investigation, 2007. **117**(1): p. 175-184.
184. Fujisaka, S., et al., *Telmisartan improves insulin resistance and modulates adipose tissue macrophage polarization in high-fat-fed mice.* Endocrinology, 2011. **152**(5): p. 1789-1799.
185. Shaul, M.E., et al., *Dynamic, M2-like remodeling phenotypes of CD11c+ adipose tissue macrophages during high-fat diet–induced obesity in mice.* Diabetes, 2010. **59**(5): p. 1171-1181.
186. Zeyda, M., et al., *Newly identified adipose tissue macrophage populations in obesity with distinct chemokine and chemokine receptor expression.* International journal of obesity, 2010. **34**(12): p. 1684-1694.
187. Lesna, I.K., et al., *Characterisation and comparison of adipose tissue macrophages from human subcutaneous, visceral and perivascular adipose tissue.* Journal of Translational Medicine, 2016. **14**(1): p. 208.
188. Gordon, S. and P.R. Taylor, *Monocyte and macrophage heterogeneity.* Nat Rev Immunol, 2005. **5**(12): p. 953-64.
189. Ohtani, M., et al., *Cutting edge: mTORC1 in intestinal CD11c+ CD11b+ dendritic cells regulates intestinal homeostasis by promoting IL-10 production.* The Journal of Immunology, 2012. **188**(10): p. 4736-4740.

190. Wu, H., et al., *CD11c expression in adipose tissue and blood and its role in diet-induced obesity.* Arteriosclerosis, thrombosis, and vascular biology, 2010. **30**(2): p. 186-192.
191. Weisberg, S.P., et al., *Obesity is associated with macrophage accumulation in adipose tissue.* The Journal of clinical investigation, 2003. **112**(12): p. 1796-1808.
192. Lumeng, C.N., et al., *Increased inflammatory properties of adipose tissue macrophages recruited during diet-induced obesity.* Diabetes, 2007. **56**(1): p. 16-23.
193. Feuerer, M., et al., *Lean, but not obese, fat is enriched for a unique population of regulatory T cells that affect metabolic parameters.* Nature medicine, 2009. **15**(8): p. 930-939.
194. Zeyda, M., et al., *Inflammation correlates with markers of T-cell subsets including regulatory T cells in adipose tissue from obese patients.* Obesity, 2011. **19**(4): p. 743-748.
195. Nishimura, S., et al., *CD8+ effector T cells contribute to macrophage recruitment and adipose tissue inflammation in obesity.* Nature medicine, 2009. **15**(8): p. 914-920.
196. Winer, D.A., et al., *B cells promote insulin resistance through modulation of T cells and production of pathogenic IgG antibodies.* Nature medicine, 2011. **17**(5): p. 610-617.
197. Randolph, G.J., et al., *Differentiation of phagocytic monocytes into lymph node dendritic cells in vivo.* Immunity, 1999. **11**(6): p. 753-761.
198. Sakaguchi, S., *Naturally arising CD4+ regulatory T cells for immunologic self-tolerance and negative control of immune responses.* Annu. Rev. Immunol., 2004. **22**: p. 531-562.
199. Feuerer, M., et al., *Lean, but not obese, fat is enriched for a unique population of regulatory T cells that affect metabolic parameters.* Nat Med, 2009. **15**(8): p. 930-9.
200. Monteiro, R. and I. Azevedo, *Chronic inflammation in obesity and the metabolic syndrome.* Mediators Inflamm, 2010. **2010**.
201. Deiuliis, J., et al., *Visceral adipose inflammation in obesity is associated with critical alterations in tregulatory cell numbers.* PloS one, 2011. **6**(1): p. e16376.
202. Macpherson, A.J. and K. Smith, *Mesenteric lymph nodes at the center of immune anatomy.* J Exp Med, 2006. **203**(3): p. 497-500.
203. Curotto de Lafaille, M.A., et al., *Adaptive Foxp3+ regulatory T cell-dependent and -independent control of allergic inflammation.* Immunity, 2008. **29**(1): p. 114-26.
204. Weiner, H.L., et al., *Oral tolerance.* Immunol Rev, 2011. **241**(1): p. 241-59.
205. Duffaut, C., et al., *Unexpected trafficking of immune cells within the adipose tissue during the onset of obesity.* Biochem Biophys Res Commun, 2009. **384**(4): p. 482-5.
206. Winer, D.A., et al., *B Lymphocytes in obesity-related adipose tissue inflammation and insulin resistance.* Cell Mol Life Sci, 2014. **71**(6): p. 1033-43.
207. Elmore, S.A., *Histopathology of the lymph nodes.* Toxicol Pathol, 2006. **34**(5): p. 425-54.
208. Wu, H.J. and E. Wu, *The role of gut microbiota in immune homeostasis and autoimmunity.* Gut Microbes, 2012. **3**(1): p. 4-14.
209. Dziarski, R., *Preferential induction of autoantibody secretion in polyclonal activation by peptidoglycan and lipopolysaccharide. II. In vivo studies.* J Immunol, 1982. **128**(3): p. 1026-30.
210. Bergheim, I., et al., *Antibiotics protect against fructose-induced hepatic lipid accumulation in mice: role of endotoxin.* J Hepatol, 2008. **48**(6): p. 983-92.
211. Thuy, S., et al., *Nonalcoholic fatty liver disease in humans is associated with increased plasma endotoxin and plasminogen activator inhibitor 1 concentrations and with fructose intake.* J Nutr, 2008. **138**(8): p. 1452-5.
212. Yang, H., et al., *Obesity accelerates thymic aging.* Blood, 2009. **114**(18): p. 3803-12.

213. Allen, C.D., T. Okada, and J.G. Cyster, *Germinal-center organization and cellular dynamics.* Immunity, 2007. **27**(2): p. 190-202.
214. Statistics, N.C.f.H., *Health, United States, 2014: with special feature on adults aged 55–64.* 2015.
215. Bjorntorp, P., *Obesity and adipose tissue distribution as risk factors for the development of disease. A review.* Infusionstherapie, 1990. **17**(1): p. 24-7.
216. Mraz, M. and M. Haluzik, *The role of adipose tissue immune cells in obesity and low-grade inflammation.* Journal of Endocrinology, 2014. **222**(3): p. R113-R127.
217. Blüher, M., *Adipose tissue dysfunction contributes to obesity related metabolic diseases.* Best Practice & Research Clinical Endocrinology & Metabolism, 2013. **27**(2): p. 163-177.
218. Lafontan, M. and M. Berlan, *Do regional differences in adipocyte biology provide new pathophysiological insights?* Trends in Pharmacological Sciences, 2003. **24**(6): p. 276-283.
219. Harman-Boehm, I., et al., *Macrophage Infiltration into Omental Versus Subcutaneous Fat across Different Populations: Effect of Regional Adiposity and the Comorbidities of Obesity.* The Journal of Clinical Endocrinology & Metabolism, 2007. **92**(6): p. 2240-2247.
220. Neeland, I.J., et al., *Associations of visceral and abdominal subcutaneous adipose tissue with markers of cardiac and metabolic risk in obese adults.* Obesity, 2013. **21**(9): p. E439-E447.
221. Goossens, G.H., *The Metabolic Phenotype in Obesity: Fat Mass, Body Fat Distribution, and Adipose Tissue Function.* Obesity Facts, 2017. **10**(3): p. 207-215.
222. Esser, N., et al., *Inflammation as a link between obesity, metabolic syndrome and type 2 diabetes.* Diabetes Research and Clinical Practice, 2014. **105**(2): p. 141-150.
223. Bluher, M., *Adipose tissue dysfunction contributes to obesity related metabolic diseases.* Best Pract Res Clin Endocrinol Metab, 2013. **27**(2): p. 163-77.
224. Kintscher, U., et al., *T-lymphocyte Infiltration in Visceral Adipose Tissue.* A Primary Event in Adipose Tissue Inflammation and the Development of Obesity-Mediated Insulin Resistance, 2008. **28**(7): p. 1304-1310.
225. Licastro, F., et al., *Innate immunity and inflammation in ageing: a key for understanding age-related diseases.* Immunity & Ageing, 2005. **2**(1): p. 8.
226. Willard-Mack, C.L., *Normal Structure, Function, and Histology of Lymph Nodes.* Toxicologic Pathology, 2006. **34**(5): p. 409-424.
227. Yoffey, J.M. and F.C. Courtice, *Lymphatics, lymph and the lymphomyeloid complex.* Lymphatics, lymph and the lymphomyeloid complex., 1970.
228. Arngrim, N., et al., *Reduced adipose tissue lymphatic drainage of macromolecules in obese subjects: a possible link between obesity and local tissue inflammation[quest].* Int J Obes, 2013. **37**(5): p. 748-750.
229. Magnuson, A., et al., *Diet-induced obesity causes visceral, but not subcutaneous, lymph node hyperplasia via increases in specific immune cell populations.* Cell proliferation, 2017. **50**(5).
230. Soderberg, K.A., et al., *Innate control of adaptive immunity via remodeling of lymph node feed arteriole.* Proceedings of the National Academy of Sciences of the United States of America, 2005. **102**(45): p. 16315-16320.
231. Magnuson, A.M., et al., *Diet-induced obesity causes visceral, but not subcutaneous, lymph node hyperplasia via increases in specific immune cell populations.* Cell Proliferation, 2017. **50**(5): p. e12365-n/a.

232. Kim, C.S., et al., *Visceral Fat Accumulation Induced by a High-fat Diet Causes the Atrophy of Mesenteric Lymph Nodes in Obese Mice.* Obesity, 2008. **16**(6): p. 1261-1269.
233. Cavalera, M., J. Wang, and N.G. Frangogiannis, *Obesity, metabolic dysfunction, and cardiac fibrosis: pathophysiological pathways, molecular mechanisms, and therapeutic opportunities.* Transl Res, 2014. **164**(4): p. 323-35.
234. Eschalier, R., et al., *Features of cardiac remodeling, associated with blood pressure and fibrosis biomarkers, are frequent in subjects with abdominal obesity.* Hypertension, 2014. **63**(4): p. 740-6.
235. Ratziu, V., et al., *Liver fibrosis in overweight patients.* Gastroenterology, 2000. **118**(6): p. 1117-1123.
236. Sharma, K., *Obesity, oxidative stress, and fibrosis in chronic kidney disease.* Kidney International Supplements, 2014. **4**(1): p. 113-117.
237. Sun, K., C.M. Kusminski, and P.E. Scherer, *Adipose tissue remodeling and obesity.* The Journal of clinical investigation, 2011. **121**(6): p. 2094.
238. O'neill, S. and L. O'driscoll, *Metabolic syndrome: a closer look at the growing epidemic and its associated pathologies.* Obesity reviews, 2015. **16**(1): p. 1-12.
239. De Pergola, G. and F. Silvestris, *Obesity as a major risk factor for cancer.* Journal of obesity, 2013. **2013**.
240. Bilecik¹, N.A., et al., *Prevalence of metabolic syndrome in women with rheumatoid arthritis and effective factors.* International journal of clinical and experimental medicine, 2014. **7**(8): p. 2258.
241. Lee, C.G., et al., *Visceral abdominal obesity is associated with an increased risk of irritable bowel syndrome.* The American journal of gastroenterology, 2015. **110**(2): p. 310.
242. Kuk, J.L., et al., *Visceral fat is an independent predictor of all-cause mortality in men.* Obesity, 2006. **14**(2): p. 336-341.
243. Harman-Boehm, I., et al., *Macrophage infiltration into omental versus subcutaneous fat across different populations: effect of regional adiposity and the comorbidities of obesity.* The Journal of Clinical Endocrinology & Metabolism, 2007. **92**(6): p. 2240-2247.
244. Halberg, N., et al., *Hypoxia-Inducible Factor 1α Induces Fibrosis and Insulin Resistance in White Adipose Tissue.* Molecular and Cellular Biology, 2009. **29**(16): p. 4467-4483.
245. Ye, J., et al., *Hypoxia is a potential risk factor for chronic inflammation and adiponectin reduction in adipose tissue of ob/ob and dietary obese mice.* Am J Physiol Endocrinol Metab, 2007. **293**(4): p. E1118-28.
246. Hosogai, N., et al., *Adipose tissue hypoxia in obesity and its impact on adipocytokine dysregulation.* Diabetes, 2007. **56**(4): p. 901-11.
247. Cinti, S., et al., *Adipocyte death defines macrophage localization and function in adipose tissue of obese mice and humans.* J Lipid Res, 2005. **46**(11): p. 2347-55.
248. Duncan, M.R. and B. Berman, *Stimulation of collagen and glycosaminoglycan production in cultured human adult dermal fibroblasts by recombinant human interleukin 6.* J Invest Dermatol, 1991. **97**(4): p. 686-92.
249. Samad, F., et al., *Tumor necrosis factor alpha is a key component in the obesity-linked elevation of plasminogen activator inhibitor 1.* Proc Natl Acad Sci U S A, 1999. **96**(12): p. 6902-7.
250. Phillips, A.O., et al., *Induction of TGF-β1 synthesis in D-glucose primed human proximal tubular cells by IL-1β and TNFα.* Kidney International, 1996. **50**(5): p. 1546-1554.

251. Sullivan, D.E., et al., *TNF-α induces TGF-β(1) expression in lung fibroblasts at the transcriptional level via AP-1 activation.* Journal of Cellular and Molecular Medicine, 2009. **13**(8b): p. 1866-1876.
252. Samad, F., et al., *Tumor necrosis factor α is a key component in the obesity-linked elevation of plasminogen activator inhibitor 1.* Proceedings of the National Academy of Sciences, 1999. **96**(12): p. 6902-6907.
253. Schacker, T.W., et al., *Lymphatic tissue fibrosis is associated with reduced numbers of naive CD4+ T cells in human immunodeficiency virus type 1 infection.* Clinical and Vaccine Immunology, 2006. **13**(5): p. 556-560.
254. Paiardini, M. and M. Muller-Trutwin, *HIV-associated chronic immune activation.* Immunol Rev, 2013. **254**(1): p. 78-101.
255. Niu, N. and X. Qin, *New insights into IL-7 signaling pathways during early and late T cell development.* Cell Mol Immunol, 2013. **10**(3): p. 187-189.
256. Schacker, T.W., et al., *Lymphatic Tissue Fibrosis Is Associated with Reduced Numbers of Naïve CD4(+) T Cells in Human Immunodeficiency Virus Type 1 Infection.* Clinical and Vaccine Immunology, 2006. **13**(5): p. 556-560.
257. Samad, F., et al., *Elevated expression of transforming growth factor-beta in adipose tissue from obese mice.* Molecular medicine, 1997. **3**(1): p. 37.
258. Samad, F., et al., *Elevated expression of transforming growth factor-beta in adipose tissue from obese mice.* Molecular Medicine, 1997. **3**(1): p. 37-48.
259. Estes, J., et al., *Collagen deposition limits immune reconstitution in the gut.* J Infect Dis, 2008. **198**(4): p. 456-64.
260. O'Neill, S. and L. O'Driscoll, *Metabolic syndrome: a closer look at the growing epidemic and its associated pathologies.* Obes Rev, 2015. **16**(1): p. 1-12.
261. De Pergola, G. and F. Silvestris, *Obesity as a major risk factor for cancer.* J Obes, 2013. **2013**: p. 291546.
262. Bilecik, N.A., et al., *Prevalence of metabolic syndrome in women with rheumatoid arthritis and effective factors.* Int J Clin Exp Med, 2014. **7**(8): p. 2258-65.
263. Lee, C.G., et al., *Visceral abdominal obesity is associated with an increased risk of irritable bowel syndrome.* Am J Gastroenterol, 2015. **110**(2): p. 310-9.
264. Vague, J., *The degree of masculine differentiation of obesities: a factor determining predisposition to diabetes, atherosclerosis, gout, and uric calculous disease.* Am J Clin Nutr, 1956. **4**(1): p. 20-34.
265. Bjorntorp, P., *"Portal" adipose tissue as a generator of risk factors for cardiovascular disease and diabetes.* Arteriosclerosis, 1990. **10**(4): p. 493-6.
266. Nielsen, S., et al., *Splanchnic lipolysis in human obesity.* J Clin Invest, 2004. **113**(11): p. 1582-8.
267. van der Poorten, D., et al., *Visceral fat: a key mediator of steatohepatitis in metabolic liver disease.* Hepatology, 2008. **48**(2): p. 449-57.
268. Mowat, A.M., *Anatomical basis of tolerance and immunity to intestinal antigens.* Nat Rev Immunol, 2003. **3**(4): p. 331-41.
269. Helander, H.F. and L. Fandriks, *Surface area of the digestive tract - revisited.* Scand J Gastroenterol, 2014. **49**(6): p. 681-9.
270. Vighi, G., et al., *Allergy and the gastrointestinal system.* Clin Exp Immunol, 2008. **153 Suppl 1**: p. 3-6.

271. Mowat, A.M. and W.W. Agace, *Regional specialization within the intestinal immune system.* Nat Rev Immunol, 2014. **14**(10): p. 667-85.
272. Kunisawa, J., Y. Kurashima, and H. Kiyono, *Gut-associated lymphoid tissues for the development of oral vaccines.* Advanced Drug Delivery Reviews, 2012. **64**(6): p. 523-530.
273. Wershil, B.K. and G.T. Furuta, *4. Gastrointestinal mucosal immunity.* J Allergy Clin Immunol, 2008. **121**(2 Suppl): p. S380-3; quiz S415.
274. Ding, S., et al., *High-fat diet: bacteria interactions promote intestinal inflammation which precedes and correlates with obesity and insulin resistance in mouse.* PloS one, 2010. **5**(8): p. e12191.
275. Novotny Nunez, I., et al., *Lactobacillus casei CRL 431 administration decreases inflammatory cytokines in a diet-induced obese mouse model.* Nutrition, 2015. **31**(7-8): p. 1000-7.
276. Kvietys, P.R., et al., *Jejunal mucosal injury and restitution: role of hydrolytic products of food digestion.* Am J Physiol, 1991. **261**(3 Pt 1): p. G384-91.
277. Miura, S., et al., *Modulation of intestinal immune system by dietary fat intake: relevance to Crohn's disease.* J Gastroenterol Hepatol, 1998. **13**(12): p. 1183-90.
278. Lam, Y.Y., et al., *Increased gut permeability and microbiota change associate with mesenteric fat inflammation and metabolic dysfunction in diet-induced obese mice.* PLoS One, 2012. **7**(3): p. e34233.
279. Lam, Y.Y., et al., *Effects of dietary fat profile on gut permeability and microbiota and their relationships with metabolic changes in mice.* Obesity (Silver Spring), 2015. **23**(7): p. 1429-39.
280. Oliveira, M.C., et al., *Acute and sustained inflammation and metabolic dysfunction induced by high refined carbohydrate-containing diet in mice.* Obesity (Silver Spring), 2013. **21**(9): p. E396-406.
281. Lam, Y.Y., et al., *Role of the gut in visceral fat inflammation and metabolic disorders.* Obesity (Silver Spring), 2011. **19**(11): p. 2113-20.
282. Cani, P.D., et al., *Changes in gut microbiota control metabolic endotoxemia-induced inflammation in high-fat diet-induced obesity and diabetes in mice.* Diabetes, 2008. **57**(6): p. 1470-81.
283. Everard, A., et al., *Cross-talk between Akkermansia muciniphila and intestinal epithelium controls diet-induced obesity.* Proc Natl Acad Sci U S A, 2013. **110**(22): p. 9066-71.
284. Gambero, A., et al., *Mesenteric adipose tissue alterations resulting from experimental reactivated colitis.* Inflamm Bowel Dis, 2007. **13**(11): p. 1357-64.
285. Booth, A., A. Magnuson, and M. Foster, *Detrimental and protective fat: body fat distribution and its relation to metabolic disease.* Horm Mol Biol Clin Investig, 2014. **17**(1): p. 13-27.
286. Stienstra, R., et al., *The inflammasome-mediated caspase-1 activation controls adipocyte differentiation and insulin sensitivity.* Cell Metab, 2010. **12**(6): p. 593-605.
287. Ben-Sasson, S.Z., et al., *IL-1 acts directly on CD4 T cells to enhance their antigen-driven expansion and differentiation.* Proc Natl Acad Sci U S A, 2009. **106**(17): p. 7119-24.
288. Rider, P., et al., *IL-1alpha and IL-1beta recruit different myeloid cells and promote different stages of sterile inflammation.* J Immunol, 2011. **187**(9): p. 4835-43.
289. Pickup, J.C., et al., *NIDDM as a disease of the innate immune system: association of acute-phase reactants and interleukin-6 with metabolic syndrome X.* Diabetologia, 1997. **40**(11): p. 1286-92.

290. Kern, P.A., et al., *Adipose tissue tumor necrosis factor and interleukin-6 expression in human obesity and insulin resistance.* Am J Physiol Endocrinol Metab, 2001. **280**(5): p. E745-51.
291. Atreya, R., et al., *Blockade of interleukin 6 trans signaling suppresses T-cell resistance against apoptosis in chronic intestinal inflammation: evidence in crohn disease and experimental colitis in vivo.* Nat Med, 2000. **6**(5): p. 583-8.
292. McLoughlin, R.M., et al., *IL-6 trans-signaling via STAT3 directs T cell infiltration in acute inflammation.* Proc Natl Acad Sci U S A, 2005. **102**(27): p. 9589-94.
293. Nish, S.A., et al., *T cell-intrinsic role of IL-6 signaling in primary and memory responses.* Elife, 2014. **3**: p. e01949.
294. Yoshida, H., et al., *Anti-IL-6 receptor antibody suppressed T cell activation by inhibiting IL-2 production and inducing regulatory T cells.* Eur J Pharmacol, 2010. **634**(1-3): p. 178-83.
295. Bettelli, E., et al., *Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells.* Nature, 2006. **441**(7090): p. 235-8.
296. Wu, D., et al., *Eosinophils sustain adipose alternatively activated macrophages associated with glucose homeostasis.* Science, 2011. **332**(6026): p. 243-7.
297. Molofsky, A.B., et al., *Innate lymphoid type 2 cells sustain visceral adipose tissue eosinophils and alternatively activated macrophages.* J Exp Med, 2013. **210**(3): p. 535-49.
298. Jiang, L.Q., et al., *Autocrine role of interleukin-13 on skeletal muscle glucose metabolism in type 2 diabetic patients involves microRNA let-7.* Am J Physiol Endocrinol Metab, 2013. **305**(11): p. E1359-66.
299. Beutler, B.A., *The role of tumor necrosis factor in health and disease.* J Rheumatol Suppl, 1999. **57**: p. 16-21.
300. Arango Duque, G. and A. Descoteaux, *Macrophage cytokines: involvement in immunity and infectious diseases.* Front Immunol, 2014. **5**: p. 491.
301. Nunemaker, C.S., et al., *Increased serum CXCL1 and CXCL5 are linked to obesity, hyperglycemia, and impaired islet function.* J Endocrinol, 2014. **222**(2): p. 267-76.
302. Griffin, G.K., et al., *IL-17 and TNF-alpha sustain neutrophil recruitment during inflammation through synergistic effects on endothelial activation.* J Immunol, 2012. **188**(12): p. 6287-99.
303. Weisberg, S.P., et al., *Obesity is associated with macrophage accumulation in adipose tissue.* J Clin Invest, 2003. **112**(12): p. 1796-808.
304. Sun, M., et al., *Regulatory immune cells in regulation of intestinal inflammatory response to microbiota.* Mucosal Immunol, 2015. **8**(5): p. 969-978.
305. Josefowicz, S.Z., et al., *Extrathymically generated regulatory T cells control mucosal TH2 inflammation.* Nature, 2012. **482**(7385): p. 395-9.
306. Adema, G.J., et al., *A dendritic-cell-derived C-C chemokine that preferentially attracts naive T cells.* Nature, 1997. **387**(6634): p. 713-7.
307. Ghiringhelli, F., et al., *Activation of the NLRP3 inflammasome in dendritic cells induces IL-1beta-dependent adaptive immunity against tumors.* Nat Med, 2009. **15**(10): p. 1170-8.
308. Brocker, T., *Survival of mature CD4 T lymphocytes is dependent on major histocompatibility complex class II-expressing dendritic cells.* J Exp Med, 1997. **186**(8): p. 1223-32.
309. Dubois, B., et al., *Dendritic cells enhance growth and differentiation of CD40-activated B lymphocytes.* J Exp Med, 1997. **185**(5): p. 941-51.

310. Allez, M., et al., *CD4+NKG2D+ T cells in Crohn's disease mediate inflammatory and cytotoxic responses through MICA interactions.* Gastroenterology, 2007. **132**(7): p. 2346-58.
311. Pariente, B., et al., *Activation of the receptor NKG2D leads to production of Th17 cytokines in CD4+ T cells of patients with Crohn's disease.* Gastroenterology, 2011. **141**(1): p. 217-26, 226 e1-2.
312. Camus, M., et al., *Oligoclonal expansions of mucosal T cells in Crohn's disease predominate in NKG2D-expressing CD4 T cells.* Mucosal Immunol, 2014. **7**(2): p. 325-34.
313. Haile, L.A., et al., *Myeloid-derived suppressor cells in inflammatory bowel disease: a new immunoregulatory pathway.* Gastroenterology, 2008. **135**(3): p. 871-81, 881 e1-5.
314. Kerjaschki, D., et al., *Lymphatic neoangiogenesis in human kidney transplants is associated with immunologically active lymphocytic infiltrates.* J Am Soc Nephrol, 2004. **15**(3): p. 603-12.
315. Maloy, K.J. and F. Powrie, *Intestinal homeostasis and its breakdown in inflammatory bowel disease.* Nature, 2011. **474**(7351): p. 298-306.
316. Huang, F.P., et al., *A discrete subpopulation of dendritic cells transports apoptotic intestinal epithelial cells to T cell areas of mesenteric lymph nodes.* J Exp Med, 2000. **191**(3): p. 435-44.
317. Worthington, J.J., et al., *Intestinal dendritic cells specialize to activate transforming growth factor-beta and induce Foxp3+ regulatory T cells via integrin alphavbeta8.* Gastroenterology, 2011. **141**(5): p. 1802-12.
318. Sun, J.B., C. Czerkinsky, and J. Holmgren, *Sublingual 'oral tolerance' induction with antigen conjugated to cholera toxin B subunit generates regulatory T cells that induce apoptosis and depletion of effector T cells.* Scand J Immunol, 2007. **66**(2-3): p. 278-86.
319. Hadis, U., et al., *Intestinal tolerance requires gut homing and expansion of FoxP3+ regulatory T cells in the lamina propria.* Immunity, 2011. **34**(2): p. 237-46.
320. Worbs, T., et al., *Oral tolerance originates in the intestinal immune system and relies on antigen carriage by dendritic cells.* J Exp Med, 2006. **203**(3): p. 519-27.
321. Schulz, O., et al., *Intestinal CD103+, but not CX3CR1+, antigen sampling cells migrate in lymph and serve classical dendritic cell functions.* J Exp Med, 2009. **206**(13): p. 3101-14.
322. von Andrian, U.H. and T.R. Mempel, *Homing and cellular traffic in lymph nodes.* Nat Rev Immunol, 2003. **3**(11): p. 867-78.
323. Koboziev, I., F. Karlsson, and M.B. Grisham, *Gut-associated lymphoid tissue, T cell trafficking, and chronic intestinal inflammation.* Ann N Y Acad Sci, 2010. **1207 Suppl 1**: p. E86-93.
324. Sallusto, F. and A. Lanzavecchia, *Mobilizing dendritic cells for tolerance, priming, and chronic inflammation.* J Exp Med, 1999. **189**(4): p. 611-4.
325. Cella, M., F. Sallusto, and A. Lanzavecchia, *Origin, maturation and antigen presenting function of dendritic cells.* Curr Opin Immunol, 1997. **9**(1): p. 10-6.
326. Miura, S., et al., *Modulation of intestinal immune system by dietary fat intake: relevance to Crohn's disease.* Journal of gastroenterology and hepatology, 1998. **13**(12): p. 1183-1190.
327. Sakaguchi, S., et al., *Regulatory T cells and immune tolerance.* Cell, 2008. **133**(5): p. 775-787.

328. Weisberg, S.P., et al., *Obesity is associated with macrophage accumulation in adipose tissue.* Journal of clinical investigation, 2003. **112**(12): p. 1796.
329. Ahrendt, M., et al., *Stromal cells confer lymph node-specific properties by shaping a unique microenvironment influencing local immune responses.* The Journal of Immunology, 2008. **181**(3): p. 1898-1907.
330. Lumeng, C.N., et al., *Phenotypic Switching of Adipose Tissue Macrophages With Obesity Is Generated by Spatiotemporal Differences in Macrophage Subtypes.* Diabetes, 2008. **57**(12): p. 3239-3246.
331. Hadis, U., et al., *Intestinal tolerance requires gut homing and expansion of FoxP3+ regulatory T cells in the lamina propria.* Immunity, 2011. **34**(2): p. 237-246.
332. Gabrilovich, D.I. and S. Nagaraj, *Myeloid-derived-suppressor cells as regulators of the immune system.* Nature reviews. Immunology, 2009. **9**(3): p. 162.
333. Calder, P.C., *Fatty acids and immune function: relevance to inflammatory bowel diseases.* International reviews of immunology, 2009. **28**(6): p. 506-534.
334. Calder, P.C., *Fatty acids and inflammation: the cutting edge between food and pharma.* European journal of pharmacology, 2011. **668**: p. S50-S58.
335. Cani, P.D., et al., *Metabolic endotoxemia initiates obesity and insulin resistance.* Diabetes, 2007. **56**(7): p. 1761-1772.
336. Cani, P.D., et al., *Changes in gut microbiota control metabolic endotoxemia-induced inflammation in high-fat diet–induced obesity and diabetes in mice.* Diabetes, 2008. **57**(6): p. 1470-1481.
337. Cani, P.D., et al., *Selective increases of bifidobacteria in gut microflora improve high-fat-diet-induced diabetes in mice through a mechanism associated with endotoxaemia.* Diabetologia, 2007. **50**(11): p. 2374-2383.
338. Griffiths, E.A., et al., *In vivo effects of bifidobacteria and lactoferrin on gut endotoxin concentration and mucosal immunity in Balb/c mice.* Digestive diseases and sciences, 2004. **49**(4): p. 579-589.
339. Wang, Z., et al., *The role of bifidobacteria in gut barrier function after thermal injury in rats.* Journal of Trauma and Acute Care Surgery, 2006. **61**(3): p. 650-657.
340. Everard, A., et al., *Cross-talk between Akkermansia muciniphila and intestinal epithelium controls diet-induced obesity.* Proceedings of the National Academy of Sciences, 2013. **110**(22): p. 9066-9071.
341. Shin, N.-R., et al., *An increase in the Akkermansia spp. population induced by metformin treatment improves glucose homeostasis in diet-induced obese mice.* Gut, 2013: p. gutjnl-2012-303839.
342. Cani, P.D., et al., *Changes in gut microbiota control inflammation in obese mice through a mechanism involving GLP-2-driven improvement of gut permeability.* Gut, 2009. **58**(8): p. 1091-1103.
343. Kim, K.-A., et al., *High fat diet-induced gut microbiota exacerbates inflammation and obesity in mice via the TLR4 signaling pathway.* PloS one, 2012. **7**(10): p. e47713.
344. Derrien, M., et al., *Modulation of mucosal immune response, tolerance, and proliferation in mice colonized by the mucin-degrader Akkermansia muciniphila.* Frontiers in microbiology, 2011. **2**.
345. Milanski, M., et al., *Saturated fatty acids produce an inflammatory response predominantly through the activation of TLR4 signaling in hypothalamus: implications for the pathogenesis of obesity.* Journal of Neuroscience, 2009. **29**(2): p. 359-370.

346. Lee, J.Y., et al., *Saturated fatty acids, but not unsaturated fatty acids, induce the expression of cyclooxygenase-2 mediated through Toll-like receptor 4.* Journal of Biological Chemistry, 2001. **276**(20): p. 16683-16689.
347. Turnbaugh, P.J., et al., *An obesity-associated gut microbiome with increased capacity for energy harvest.* nature, 2006. **444**(7122): p. 1027-131.
348. Allez, M., et al., *CD4+ NKG2D+ T cells in Crohn's disease mediate inflammatory and cytotoxic responses through MICA interactions.* Gastroenterology, 2007. **132**(7): p. 2346-2358.
349. Luck, H., et al., *Regulation of Obesity-Related Insulin Resistance with Gut Anti-inflammatory Agents.* Cell Metabolism, 2015. **21**(4): p. 527-542.
350. Huang, F.-P., et al., *A Discrete Subpopulation of Dendritic Cells Transports Apoptotic Intestinal Epithelial Cells to T Cell Areas of Mesenteric Lymph Nodes.* The Journal of Experimental Medicine, 2000. **191**(3): p. 435-444.
351. Macpherson, A.J. and K. Smith, *Mesenteric lymph nodes at the center of immune anatomy.* The Journal of Experimental Medicine, 2006. **203**(3): p. 497-500.
352. Karlsson, E.A. and M.A. Beck, *The burden of obesity on infectious disease.* Experimental Biology and Medicine, 2010. **235**(12): p. 1412-1424.
353. Acton, S.E., et al., *Dendritic cells control fibroblastic reticular network tension and lymph node expansion.* Nature, 2014. **514**(7523): p. 498-502.
354. Fletcher, A.L., S.E. Acton, and K. Knoblich, *Lymph node fibroblastic reticular cells in health and disease.* Nature reviews. Immunology, 2015. **15**(6): p. 350-361.
355. Zeng, M., et al., *Cumulative mechanisms of lymphoid tissue fibrosis and T cell depletion in HIV-1 and SIV infections.* The Journal of clinical investigation, 2011. **121**(3): p. 998.
356. Estes, J.D., et al., *Simian immunodeficiency virus-induced lymphatic tissue fibrosis is mediated by transforming growth factor β1-positive regulatory T cells and begins in early infection.* The Journal of infectious diseases, 2007. **195**(4): p. 551-561.
357. Sullivan, D.E., et al., *TNF-α induces TGF-β1 expression in lung fibroblasts at the transcriptional level via AP-1 activation.* Journal of cellular and molecular medicine, 2009. **13**(8b): p. 1866-1876.
358. Von Pfeil, A., et al., *Irradiated homozygous TGF-beta1 knockout fibroblasts show enhanced clonogenic survival as compared with TGF-beta1 wild-type fibroblasts.* Int J Radiat Biol, 2002. **78**(5): p. 331-9.