Adipose Tissue T Cell Function and Diversity in Obese Mice and Humans

by

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DEDICATION

To my parents, for Sunday evening phone calls and a lifetime of encouragement

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ABSTRACT

Adipose tissue induced chronic low-grade inflammation is a critical link between obesity and insulin resistance. Despite studies implicating adipose tissue T cells (ATT) in the initiation and persistence of adipose tissue inflammation, fundamental gaps in knowledge regarding ATT function impedes progress towards understanding their role in type II diabetes. This dissertation explores the mechanisms of ATT maintenance via interactions with antigen presenting cells (APC), the tissue specific characteristics of ATTs, and how obesity regulates ATT inflammatory function upon activation. We find that the network between ATTs and peptide-majorhistocompatability complex II (pMHCII) presented on the cell surface of adipose tissue dendritic cells (ATDC) is required for ATT maintenance. Knocking down MHCII on dendritic cells with CD11cCre x $MHCII^{fl/fl}$ (M11cKO) mice reconfigures ATT composition, but does not significantly impact systemic glucose or insulin sensitivity. To address gaps in the understanding of how obesity alters ATT function, we employed an *in-vitro* activation assay to analyze T cell activation in visceral adipose. Our findings challenge the dogma that ATTs acquire pro-inflammatory potential in obese fat, and show that measures of ATT activation based on cellular markers, proliferation, and cytokine secretion are impaired in obese mice and humans. Chronic antigen presentation or soluble mediators from obese stromal vascular cells are sufficient for driving ATT impairment. Single cell RNA sequencing of mouse $CD3^{+}$ ATTs shows diversity in T cell subsets and gene expression dependent upon adipose tissue depot of origin and obesity. There are nine distinct subsets of ATTs and 12 weeks of high fat diet (HFD) feeding influences

their proportions. In visceral adipose tissue depots where functional ATT impairment was observed, we see HFD induces genes enriched in models of T cell exhaustion. Further studies are required to pinpoint the specific mechanisms leading to ATT exhaustion and to determine whether ATT inflammatory impairment influences insulin resistance in the context of obesity.

Chapter 1 - Introduction

This dissertation is comprised of three primary studies that aim to address critical questions regarding adipose tissue T (ATT) cell activation in the context of obesity-induced chronic low-grade inflammation. Chapter 1 will focus on introducing obesity and its burden on public health by describing its epidemiology and associated co-morbidities. I will discuss the importance of adipose tissue in its capacity to regulate systemic metabolism in health, and how adipose tissue dysfunction develops when forced to compensate for excess energy intake. I will highlight the importance of adipose tissue-derived inflammation and its regulation of systemic metabolism in obese individuals. Although adipose tissue is a complex heterogeneous niche for a range of leukocytes that regulate inflammation in obesity, I will focus on what is know about the network of antigen presenting cells, how they communicate with T cells, and the critical gaps in knowledge that exist. Then I will describe murine and human models used to study ATT cells and the scope of the projects undertaken in this dissertation. Chapter 2 will detail ATT regulation via adipose tissue dendritic cells and the importance of major histocompatibility complex II-mediated antigen presentation in maintaining ATTs. Chapter 3 will characterize tissue-specific properties of ATTs and their function in lean and obese adipose tissue. I will focus on describing a novel finding of ATT inflammatory impairment in obese adipose tissue. Chapter 4 will use single cell RNA sequencing to profile the range of ATT subsets, accounting for the adipose depot of origin and identify signaling pathways differentially regulated between lean and obese mice. Finally, I will discuss the contribution of this work to the understanding of obesity-induced inflammation and type II diabetes in Chapter 5.

Defining obesity and its effects on health

Epidemiological studies indicate that 18% of all deaths among Americans 40-85 years of age result from obesity-related diseases (1). However, the emergence of obesity is historically recent and its contribution to severe cardiometabolic morbidities is still vastly underexplored. Up until the 1990s, obesity was not recognized as public health burden in the United States; however, 30 years later it is evident that obesity has become incredibly prevalent. According to the latest CDC statistics collected in 2018, 42.4% of US constituents are obese (2). Although obesity is often emphasized in the United States population, obesity is a global phenomenon due in part to production and consumption of more processed, affordable, and effectively marked food (3, 4). Therefore, understanding the biology of overweight and obese individuals will be vital for developing therapeutics for a growing population of patients with metabolic diseases.

Obesity is commonly measured by body mass index (BMI), where: $BMI = \frac{persons\ body\ weight\ (kg)}{height^2\ (m)}$

The Centers for Disease Control groups people as underweight, healthy weight, overweight, obese, and class 3 obese based upon body mass index (BMI) measurements (See Table 1A).

Table 1A – Weight status based on BMI

BMI	Weight Status
Below 18.5	Underweight
18.5 - 24.9	Normal/Healthy Weight
25 - 29.9	Overweight
30 – 34.9	Obese – Class I

35 – 39.9	Obese – Class II
40 and Above	Obese – Class III

Although BMI correlates with metabolic disease outcomes like glucose intolerance and type II diabetes, BMI is a poor surrogate for body fat content and does not distinguish lean mass from and fat mass (5-7). Therefore, high BMI can either indicate that an individual has higher than average lean mass or fat mass, particularly in children. Techniques like dual energy x-ray absorptiometry (DXA) and BODPOD are better determinants of fat mass (8, 9).

Increased body fat percentage is caused by an imbalance in energy intake and energy expenditure. Fat mass and body weight are maintained when energy input is equal to energy expenditure. Weight loss occurs when energy expenditure exceeds energy input, while weight gain occurs when energy intake exceeds energy expenditure (10). Therefore, obesity most usually occurs due to over-consumption of foods that contain macronutrients with high energy density and/or a sedentary lifestyle. Some groups are more prone to developing obesity than others. Gene/environment interactions contribute to obesity (11). Socioeconomic status can also determine environmental conditions which influences chronic weight gain (12). Since the steep increase in obesity is predicted to continue, it is essential to be able to define obesity and understand the physiological underpinnings of increased fat mass.

Comorbidities of obesity

Rising prevalence of obesity is a major public health concern because people with obesity have an increased chance of developing an array of metabolic and non-metabolic morbidities. Increased adiposity is tightly associated with metabolic syndrome, an umbrella term

encompassing metabolic disorders such as fatty liver disease, atherosclerosis, hypertension, stroke, and Type II diabetes (13-16). Type II diabetes occurs due to insulin resistance, which results in hyperglycemia (17). This occurs due to the inability of cells to sense insulin in the periphery and upregulate glucose transporter proteins that are required to shuttle glucose from blood into cells. When glucose is unable to be utilized by cells, it remains in the blood and over time results in end-stage diabetic outcomes like diabetic retinopathy and poor wound healing (18, 19). Several mechanisms are responsible for mediating insulin resistance in obese conditions. Hormones that regulate hunger, increased adipose tissue mass, increased lipolysis, and adipose tissue mediated inflammation all play a role in pathogenesis of obesity-associated morbidities. Each of these factors will be described later in this chapter.

In addition to cardiometabolic diseases, obesity is a risk factor for a spectrum of diseases that are influenced by immune cells outside of adipose tissue. Studies have shown that obese individuals have a higher risk of developing cancer (20). The International Agency for Research on Cancer has determined that colon, postmenopausal breast, endometrial, kidney, and esophageal cancer are associated with obesity (21). It has been hypothesized that hyperinsulinemia, sex steroid hormones, adipokine secretion, and chronic low-grade inflammation induced by obesity can all individually contribute to cell proliferation and dysregulation of the cell cycle, creating cancerous cells (22). Studies have also shown that immune responses against cancer cells are impaired in obese states, but these cells are also more easily targetable by immunotherapies than T cells from lean cancer patients (23).

Consistent with the observations of immune dysfunction in people with obesity, obesity is also associated with an increased risk for more severe morbidity associated with viral infections. Both bacterial and viral pulmonary infections including tuberculosis, influenza, and

coronaviruses have been shown to cause more severe morbidity and mortality in people with obesity (24-27). While the exact mechanisms are not yet fully understood as to why obesity and diabetes are risk factors for adverse outcomes with viral infections, both metabolic and immune functions are hypothesized to play a role. Hyperglycemia and hyperinsulinemia impair immune cell function because ATP utilization is required for pro-inflammatory responses (27). People with obesity may also upregulate receptors needed for viral entry into cells and result in increased viral load compared to lean subjects (26). Overall, it can be hypothesized that chronic low-grade inflammation dampens the immune response required for an activating stimulus (28). In addition to worsened outcomes in cancer and infectious diseases, obese diabetic individuals also have a poor response to vaccines (29).

Therefore, people with obesity have paradoxical regulation of the immune system that both contribute to chronic low-grade inflammation in adipose tissue, and result in inadequate inflammatory responses to caner and viral infections in non-adipose tissue locations. Understanding the mechanisms that mediate these differences and reconciling how both pro- and anti-inflammatory responses can result from obesity is a big picture question that we set to address in this dissertation. The following sections will focus on the basics of adipose tissue and immune cell function and how each is regulated by the development of obesity.

Adipose tissue structure and function

While adipose tissue was initially overlooked as a simple connective tissue that cushions and insulates the body, adipose tissue is now known to be a dynamic and important physiologic modulator. Since adipose tissue can regulate lipid storage, de novo lipogenesis, thermodynamics, and secretion of inflammatory mediators and hormones, it has the capacity to induce major

systemic changes in nutrient availability and storage (30-33). Adipocytes, the parenchymal cells of adipose tissue, are the primary mediators of energy storage and release. In times of energy excess, nutrients are converted into neutral lipids and stored in adipocytes, so that in times of nutrient deficit (e.g. fasting) lipids can be broken down and released in a process called lipolysis (34). This results in the release of free fatty acids into the bloodstream, which can be utilized for energy in peripheral tissues. Once free fatty acids are released into the blood stream, they become available substrate for ATP production. Being able to sense systemic states of energy excess or depletion make adipose tissue a "rheostat" for regulating systemic metabolism.

In addition to lipid storage and release, adipocytes are important in regulating appetite via communication with the brain. Adipose tissue mediates the production and release adipocytokines (adipokines), which are hormones that regulate hunger and inflammation (35). Two of the most studied are leptin and adiponectin. In lean individuals, leptin is a satiety hormone with pro-inflammatory properties that signals through the hypothalamus and indicates energy intake is not needed (36). Leptin secretion is directly proportional to the body fat content of an individual, where more secretion occurs with increased body fat thereby acting as a negative feedback mechanism to maintain a healthy weight (37). Adiponectin is another adipokine secreted by adipose tissue but has opposite properties of leptin. Like leptin, adiponectin plays an important role in systemic metabolism, specifically through the modulation of glucose and lipid metabolism in insulin-sensitive tissues. However, high adiponectin levels in the circulation are seen in lean individuals and have anti-inflammatory properties (38). Insulin resistant individuals have much lower adiponectin than lean individuals, and increasing adiponectin levels improves systemic metabolism and decreases inflammation (39, 40).

However, adipose tissue function is also dependent upon the proper functioning of non-parenchymal cells, which includes the endothelium, neurons, stem cell-like preadipocytes, and immune cells. Obese adipose tissue is associated with damage induced by hypoxia due to inadequate vascularization of adipose tissue as it expands to accommodate excess lipid (see *Adipose tissue expansion*). Innervation and neuronal regulation of adipocytes are also vital for thermogenesis and brown adipose tissue function (described in *Distribution of Adiposity*). Pre-adipocytes are crucial mediators of hyperplasia and metabolically healthy adipose tissue expansion (see *Adipose tissue expansion in response to nutrient excess).*

Adipose tissue expansion in response to nutrient excess

While we know that obesity is associated with the risk of metabolic syndrome, not all obese people become diabetic. Both lean and obese people can be metabolically healthy or unhealthy (41). It is apparent that the quantity of adipose tissue isn't the most important aspect of obesity, and the quality of obese adipose tissue drives metabolic syndrome (42). Adipose tissue undergoes major changes to accommodate chronic energy imbalance and prolonged periods where energy intake exceeds energy expenditure. One of the primary functions of adipocytes is to store excess energy in the form of lipid. Although adipocytes can expand their capacity for lipid storage, there is a limit to their storage capacity. When lipid content exceeds adipose tissue storage capacity, excess lipid overflows and is deposited in organs in which lipid deposition is detrimental (43). Metabolic organs like the liver and muscle often accumulate lipid after adipose tissue can no longer store excess energy, which causes lipotoxicity and tissue damage.

Adipocyte remodeling is an important feature of adipose tissue expansion to accommodate increased lipid storage. Adipocytes undergo hyperplasia, which is the production of new adipocytes and hypertrophy, the enlargement of existing adipocytes, in order to increase

lipid storage capacity. It is metabolically beneficial for adipose tissue to increase its volume as much as possible (44). Since adipocytes have limited ability to increase their size, enhanced hyperplastic ability is metabolically beneficial. Extra cells provide excess storage without stressing adipocytes to store lipids to a point where their capacity is exceeded. Even though hyperplasia results in increased fat mass, it prevents ectopic lipid deposition. Unhealthy adipocyte expansion also leads to increased basal lipolysis, which results in increased release of FFA into the bloodstream. Therefore, increased fat tissue in the absence of excess hypertrophy and lipolysis is metabolically beneficial as it prevents lipid deposition and damage in metabolic tissues like the liver and smooth muscle (43).

Adipose tissue distribution creates functional heterogeneity among depots

Unlike most organs, adipose tissue is not a contiguous tissue and is divided into depots that are distributed throughout the body. Adipose tissue exists in many localized fat pads both subcutaneously and viscerally. Each depot is classified based upon anatomic location. Environmental signals vary throughout the body corresponding to various organ systems and associated depots (renal, mesenteric, gonadal, subcutaneous, etc). Depots are very heterogeneous based upon adipocyte function, and stromal vascular composition/function (45). Broadly, adipose tissue can be divided into 3 subsets of depots depending on their primary functions. Brown, beige, and white adipocytes all have different developmental origins, locations, and functions.

White adipose tissue (WAT) is known for its lipid storage/release, and hormonal regulation described previously. Brown adipose tissue (BAT) is typically only found in subscapular regions of infants and serves as a thermogenic organ before the development of skeletal muscle needed for shivering thermogenesis seen in adults (46). Heat generation in brown

adipose tissue has been attributed to the expression of uncoupling protein 1 (UCP1), a transmembrane protein in mitochondria responsible for decreasing proton gradient that is generated by oxidative phosphorylation (47). UCP1 moves protons from the inter-membrane space into the mitochondrial matrix in a fast substrate oxidation process that generates heat instead of ATP. Beta-3 adrenergic signaling and cold exposure are known to induce UCP1 and heat generation in brown adipose tissue depots (48, 49).

Adults retain properties of brown fat in depots that are called beige or brite (the portmanteau of brown and white). Subcutaneous depots have properties of white adipose tissue when in thermoneutrality, but can transform by upregulating expression of UCP1. Therefore, beige adipose tissue has properties of thermogenic tissue in times of prolonged cold exposure. Although brown and brite adipose tissue is responsible for maintaining temperature homeostasis in times of cold, scientists have been interested in utilizing UCP1 as an energy sink that burns through excess protons in mitochondria (50). This would allow for the rapid consumption of excess energy stores in obese individuals. Significant research aims to harness brite fat in this process to burn off excess energy in a safe manner.

In addition to function of adipocytes, WAT location and aggregation also have important implications in obesity co-morbidities. Enlargement of visceral adipose tissue depots around the abdominal organs is associated with metabolically unhealthy obesity while increased subcutaneous fat is less detrimental (51). Subcutaneous fat expansion is more common in females, while males are prone to storing lipid in visceral depots (52). This correlates with increased risk for men developing type II diabetes and cardiovascular disease. In humans, the omentum is the predominant visceral adipose tissue depot and covers the abdominal cavity, underneath the peritoneum (53). There are also renal, epicardial, intramuscular depots, and fat

surrounding lymph nodes and blood vessels. All of these depots can lead to dysregulated homeostasis of associated organs in the context of obesity. When they forced to store excess that exceeds the capacity of associated adipose tissue depots, ectopic lipid accumulation can occur once associated depots lose the ability to expand and store excess lipid.

Adipose tissue resident leukocytes have specialized functions

Many obesity-associated morbidities such as type 2-diabetes and metabolic syndrome are related to insulin resistance, which is mechanistically linked to adipose tissue dysfunction and inflammation (54-56). Clinical and preclinical studies have identified chronic low-grade inflammation as a critical link between obesity and insulin resistance and have coined the term "metainflammation" (57-60). Adipocytes and adipose tissue macrophages in individuals with obesity produce elevated levels of TNFα, resulting in impaired insulin receptor signal transduction and lipolysis in adipocytes (57, 61, 62). Adipocytes in obese tissue also secrete pro-inflammatory factors like CCL2, which result in monocyte migration and trafficking into adipose tissue where they are polarized into inflammatory macrophages (63). This immune regulation directly regulates metabolism within white adipose tissue where crosstalk between immune cells and adipocytes contributes to the development of insulin resistance (64, 65).

One study that highlights this, is an RNA-seq analysis performed by our lab used to identify pathways in adipose tissue that are features of human diabetes in an unbiased manner (66). Pathway analysis indicated that the top five pathways distinguishing diabetic adipose tissue from the metabolically healthy samples were immune-related. Studies have shown that when adipose tissue inflammation is abrogated using techniques like immunotherapy, metabolic parameters are improved and glucose intolerance can be reversed (67). Therefore, our overall

objective is to diminish adipose tissue inflammation as a method of alleviating disease in obese patients independent of their fat mass.

Adipose tissue inflammation occurs as adipose tissue changes to accommodate chronic energy imbalance and increase its lipid storage capacity. In lean tissue, immune cells are mostly tolerogeneic and contain predominantly anti-inflammatory cells such as Tregs and resident adipose tissue macrophages (ATMs) (68, 69). With chronic energy imbalance, the NFκB signaling pathway is activated in adipocytes resulting in pro-inflammatory chemokine and cytokine release (64). MCP-1 secretion results in the recruitment of monocytes, which differentiate into pro-inflammatory ATMs (68). However, blocking NFκB signal transduction is not an option for improving chronic low-grade inflammation, since downstream IKKβ is critical for preventing apoptosis of adipocytes and exacerbated macrophage infiltration (70). In addition to ATMs, it has been reported that pro-inflammatory dendritic cells, T cells, B cells expand, while anti-inflammatory cells such as Tregs, and natural killer T (NKT) cells decrease (71-75). These changes in leukocyte number and function result in increased secretion of IFNγ, IL1β, and MCP-1 into circulation. These cytokines then influence insulin-sensitive tissues such as the liver and smooth muscle, by activating the NFκB pathway, downregulating the insulin depending on glucose transport protein Glut4, thereby reducing insulin sensitivity and increasing blood glucose levels (See Figure 1-1).

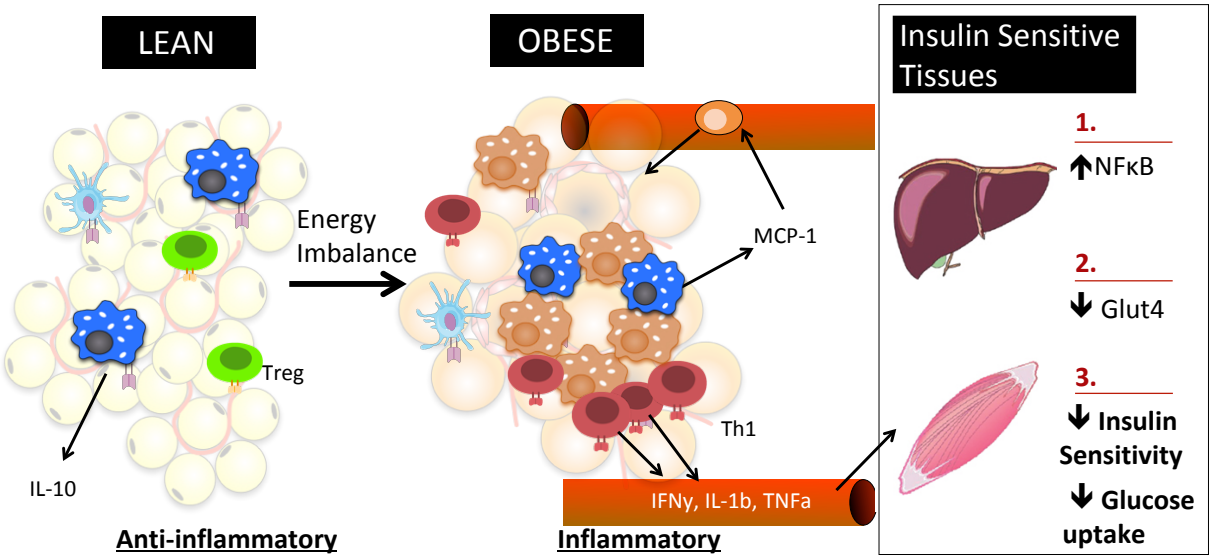

Figure 1-1 – Pro-inflammatory cytokine secretion from obese adipose tissue inhibits insulin signaling

The molecular mechanisms of immune-mediated insulin resistance are mediated by cytokine signaling that directly inhibits insulin signaling transduction pathways. In homeostatic conditions, insulin – insulin receptor ligation leads to signaling through the IRS/PI3K/AKT pathway, resulting in translocation of Glut4 to the cell surface (76). This allows for glucose transport from the bloodstream, which is required for energy generation. However, inflammatory conditions lead to inhibition of this signaling pathway via induction of JNK. Negative feedback proteins, like suppressor of cytokine signaling proteins (SOCS), are induced by cytokine signaling to maintain cellular homeostasis and prevent hyper-inflammatory states. However, SOCS are also are responsible for IRS inhibition and prevention of Glut4 translocation and resulting glucose import (77) (See Figure 1-2).

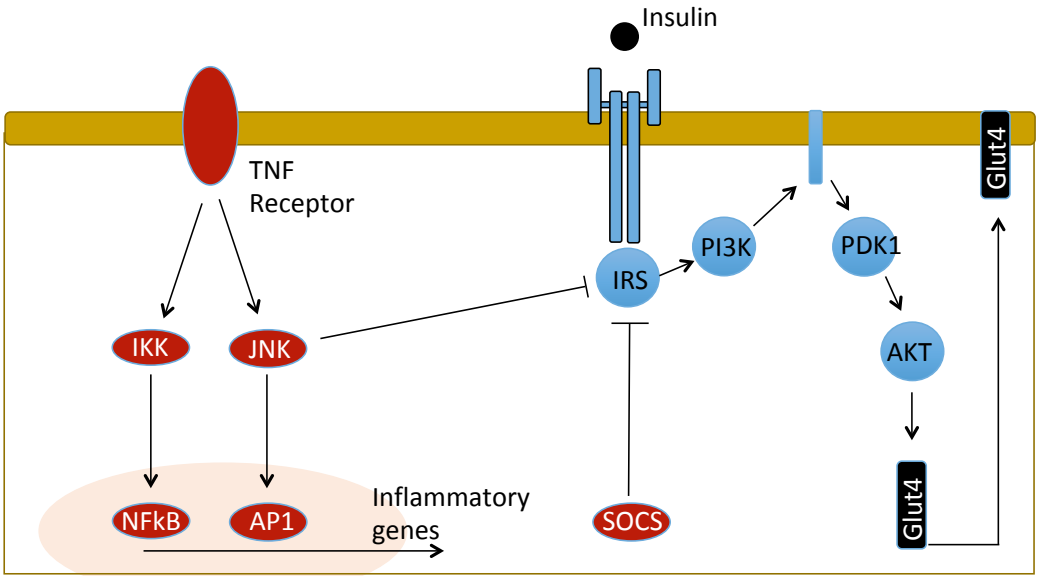

Figure 1-2 – Integration of inflammatory and insulin signal transduction pathways
Insulin signaling occurs via IRS/PI3K/AKT signal transduction, resulting in translocation of glucose transporter Glut4 to the cell surface. However, inflammatory signaling cascades are responsible for modulating the insulin-signaling cascade. In inflammatory environments, cytokine signaling via TNFα induces phosphorylation of IKK and JNK, which in turn activate transcription factors NFκB and AP1. Signal transduction through this pathway leads to transcription of additional inflammatory genes, and negative feedback signaling proteins, likes SOCS. In addition to AP1 induction, JNK directly inhibits IRS and prevents Glut4 translocation.

Since JNK and SOCS induction is conserved in signaling cascades of multiple pro-inflammatory cytokines, inhibiting a single cytokine would be ineffective at improving insulin sensitivity in states of chronic low-grade inflammation. Therefore understanding the processes that initiate cytokine secretion in locations like obese adipose tissue is important for potential targets for diabetes therapeutics.

Both innate and adaptive immune cells are responsible for pro-inflammatory cytokine secretion in obese adipose tissue. However, these cells are not regulated independently and network with each other to regulate inflammation. This dissertation focuses on one of these leukocyte networks that have been implicated in obesity-induced inflammation: antigen presenting cell (APC) activation of adipose tissue T cells (ATT). Understanding how ATTs are activated and function lean and obese environments will elucidate potential targets for reducing

metabolically driven inflammation. Although studies have detailed the process by which APCs induce T cell activation in the lymphatic system, immune cell properties depend upon their local environments. Leukocyte function can have a vastly different function depending upon their surroundings and environmental factors, and T cell activation and function in adipose tissue are incompletely understood.

Antigen presentation and T cell activation

Canonical antigen presentation in lymphoid tissues

Antigens, or antibody generators, are foreign non-self proteins that are inducers of an adaptive immune response. B cells and T cells, also known as lymphocytes, are the primary mediators of the adaptive immune response and target removal of antigens via antibody production and cytotoxic destruction of infected host cells. In the context of infections, antigens can be either intracellular (viruses), or extracellular (bacteria) and specific immune responses can be mounted against both cell endogenous and exogenous insults. Even though immune responses are described in the terms of infections, inflammatory responses can also occur due to autoimmune reactions, transplantation, or in response to microbial products form the microbiome (78). Antigens induce this targeted inflammation is via processing and presentation by innate immune cells – called antigen presenting cells (APCs). APCs phagocytose exogenous antigens or process endogenous antigens; break them down into peptides and present peptides on majorhistocompatabiliy complex (MHC) molecules.

Antigen presenting cells in adipose tissue

Three distinct immune cell populations mediate exogenous antigen presentation and are known as professional antigen presenting cells: dendritic cells, macrophages, and B cells. Each is capable of employing phagocytosis to ingest extracellular proteins. Adipose tissue macrophages (ATM) are important regulators of pro-inflammatory T cell activation in obese adipose tissue (79). Studies using LysMCre x MHCIIfl/fl transgenic mice fed high fat diet have decreased CD4 T cell content and improved insulin sensitivity and glucose tolerance (80). However, dendritic cells are the primary professional APCs in lymphoid organs and canonically phagocytose antigen and drain to lymph nodes where they present peptide to T cells.

Adipose tissue dendritic cells (ATDC) studies have been limited because of the difficulty of distinguishing macrophages from dendritic cells. It has been suggested that CD11c+ ATDCs are responsible for ATM infiltration and contribute to the inflammatory milieu in the context of obesity (81). A high proportion of ATMs in obese tissue also express CD11c, which is an important marker for ATDC. However CD64 cleanly separates ATMs from ATDC, making terminal analyses of these cells possible (73). ATDC content is increased due to CCR7 chemoattraction in the context of obesity (73). Zbtb46 has also been identified as an ATDC specific protein that distinguishes these cells from ATMs. Using Zbtb46-GFP mice, functional implications for ATDCs in lean and obese adipose tissue has been elucidated. Adipocyte mediated release of PPARβ and β-catenin in lean tissue maintain tolerogenic capacity of ATDCs, but as secretion of these factors are decreased in obese adipose tissue, ATDCs begin to mediate an inflammatory response (82). Despite these studies, little is known about the process adipose ATDC mediated antigen presentation. The ability to bridge the innate and adaptive immune response via peptide-MHCII is the major role of dendritic cells in classic immune reactions. Myeloid-derived dendritic cells canonically undergo endocytosis antigen and migrate to

lymphoid organs to survey a large pool of T cells. ATDCs do not drain to lymph nodes, yet they are capable of forming MHCII – TCR interactions within adipose tissue. Since ATDCs express high levels of MHCII, they could be important modulators of T cell activation in WAT.

Canonical T lymphocyte function

T cells remain quiescent until they are activated through their T cell receptors (TCR) and co-receptors. TCRs are rearranged during development to recognize a single distinct peptide, which is only recognized when it is presented on an MHC complex. Co-stimulation of CD28 from surface receptors on antigen presenting cells (e.g CD80, CD40), and cytokine signals in the local environment (e.g. IL-12, TGFβ) (83-85) must also accompany cognate peptide TCR interactions. The result of this antigen-specific signaling engagement is the activation of T cells and a directed inflammatory response to the antigen it recognizes. T cells proliferate in a clonal manner and secrete additional effector cytokines. Although activated effector T cells have heterogeneous inflammatory functions depending upon polarizing cytokines in their environment at the time of activation, they are all characterized by expression of IL-2Ra (CD25) on the cell surface and IL-2 secretion (86).

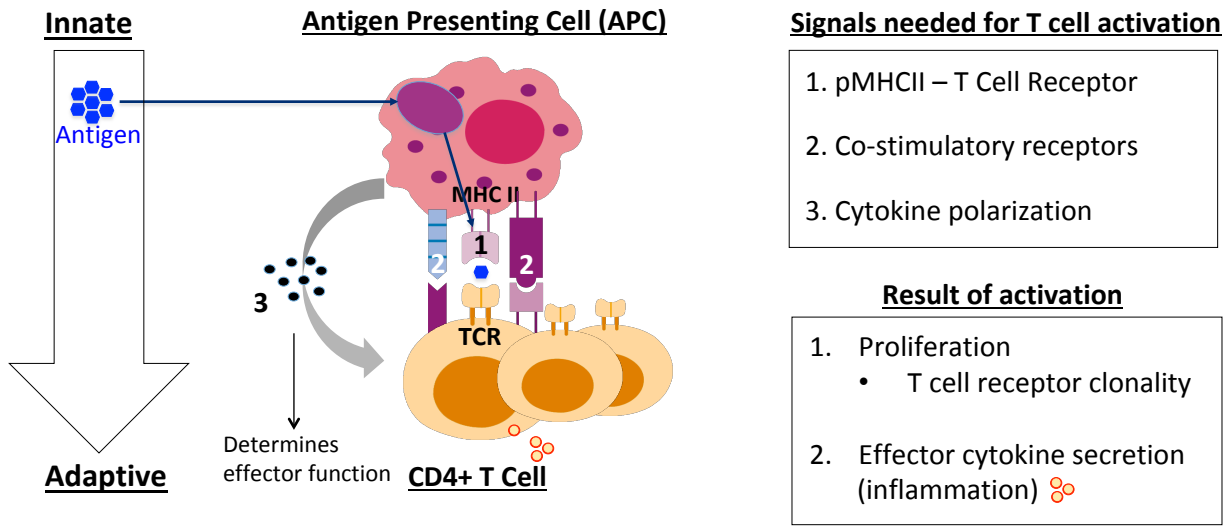

Figure 1-3 – Antigen presenting cells facilitate T cell activation and function

T cells also have negative feedback mechanisms that prevent prolonged inflammation after recognition of peptide MHC complexes and removal of the foreign antigen that induces activation. After activation and initiation of inflammatory responses, co-receptors are decreased on the cell surface of T cells. Instead co-inhibitory receptors like PD-1, Lag3^{+}, and CTLA-4 are expressed (87, 88). Co-inhibitory receptor engagement results in inhibition of Ras signaling in addition to AKT and subsequent survival/metabolism signaling pathways. NFkB pathways are also induced downstream of TCR signaling engagements, and subsequent SOCS production also serves as a negative feedback mechanism to dampen inflammation after an initial inflammatory burst. After effector T cell activation occurs and the cognate antigen is cleared from the system, ~90% of the T cells that had expanded in response to pMHCII recognition undergo apopotosis. The remaining cells survive and return to a quiescent state, but retain memory to the antigen it responded to previously. These memory cells are capable of enacting a quick response (within 2 days instead of 7) after a second encounter with their cognate antigen, and are responsible for memory to previous infections.

Functional T cell impairments: exhaustion, senescence, and anergy

There are several mechanisms by which T cells can fail to elicit an inflammatory response when they come into contact with their cognate antigen. Exhaustion, senescence, and anergy are all well described processes by which T cell hyporesponsivenes can occur. The result of T cell impairment is decreased proliferation, and secretion of granzyme and/or effector cytokines.

Exhaustion occurs when T cells are subjected to chronic activation due to constant exposure to their cognate antigen (87). This usually occurs in the context of chronic viral infections, cancer, and autoimmune environments. Molecular mechanisms responsible for exhaustion include increased and lasting expression co-inhibitory TCR receptors such as PD1 and CTLA-4 (89). Inhibition of signaling pathways downstream of the TCR including PI3K/AKT is also a hallmark of exhaustion and result in inflammatory dysfunction (90). Additionally, dysfunction in the context of T cell exhaustion can occur due to inefficient metabolic activity (91)

Senescence is associated with aging and is marked by shortened telomeres, cell cycle arrest, and loss of co-activating receptor expression (CD28) (92, 93). Although senescence can naturally occur with ageing, it is also associated with autoimmune diseases and chronic infections after prolonged proliferation. This phenotype is usually identified by increased expression of cell cycle inhibition proteins like P16, p21, and p53, in addition to Tim3 and CD57.

Anergy is another form of induced inflammatory impairment, but is induced by incomplete activation and signaling through the TCR and associated receptors. Usually cognate

TCR –MHCII engagement occurs in the absence of co-receptor engagement. This is thought to be a mechanism that prevents excessive T cell inflammation and exits as a peripheral means to prevent autoimmunity and induce tolerance to self-antigens (94).

Although T cell exhaustion, senescence, and anergy can all be beneficial negative feedback mechanisms that prevent excessive inflammation and cytokine storms, when there are chronic infections, persistent antigen exposure, or enduring proliferation, these mechanisms can prevent resolution of infections, autoimmunity, chronic inflammation, or cancer.

Diversity of T cell subsets and functions

T cells are divided according to their response to exogenous or endogenous origin of peptide presentation. Dendritic cells, macrophages, and B cells are known as professional antigen presenting cells that phagocytose antigens from the extracellular space and present peptides on MHCII molecules. MHCII specifically interfaces with CD4 T helper cells. CD4 is a glycoprotein expressed on the surface of T cells, and its engagement with MHCII results in phosphorylation of intracellular Lck, which is required for TCR signal transduction in addition to the 3 primary signals indicated above. CD4 cells can either have inflammatory characteristics or anti-inflammatory properties and are known as T conventional (Tconv) and T regulatory (Treg) subsets, respectively. Tconv cells can be further subdivided based upon signal 3 - the cytokine signaling they receive during activation. Different cytokines result in polarization of T cells and direct them to secrete cytokines necessary to resolve the antigenic stimulus (85). See Table 1B for effector CD4 Tconv subpopulations.

Table 1B – Identifying Tconv effector T cell subsets

Effector Subset	Polarizing cytokine	Effector cytokine	Transcription factor
Th1	IL-12, IFNy	IFNy, IL-2	T-bet
Th2	IL-4	Gata3	IL-4, IL-5, IL-13
Th17	TFGb, IL-6	RORyt	IL-17
Treg	TFGb, IL-2	FoxP3	TGFb, IL-10
Tfh	IL-6, IL-21	Bcl6	IL-21, IL-17

Additionally, all nucleated cells are capable of presenting endogenous antigens that are processed and loaded onto MHC class I molecules. MHCI associates with CD8 glycoproteins on cytotoxic T cells which results in perforin/granzyme mediated death of the endogenous APC. Similar to CD4, CD8 associates with MHCI and is responsible for Lck phosphorylation and TCR signaling, activation, and inflammation/perforin generation.

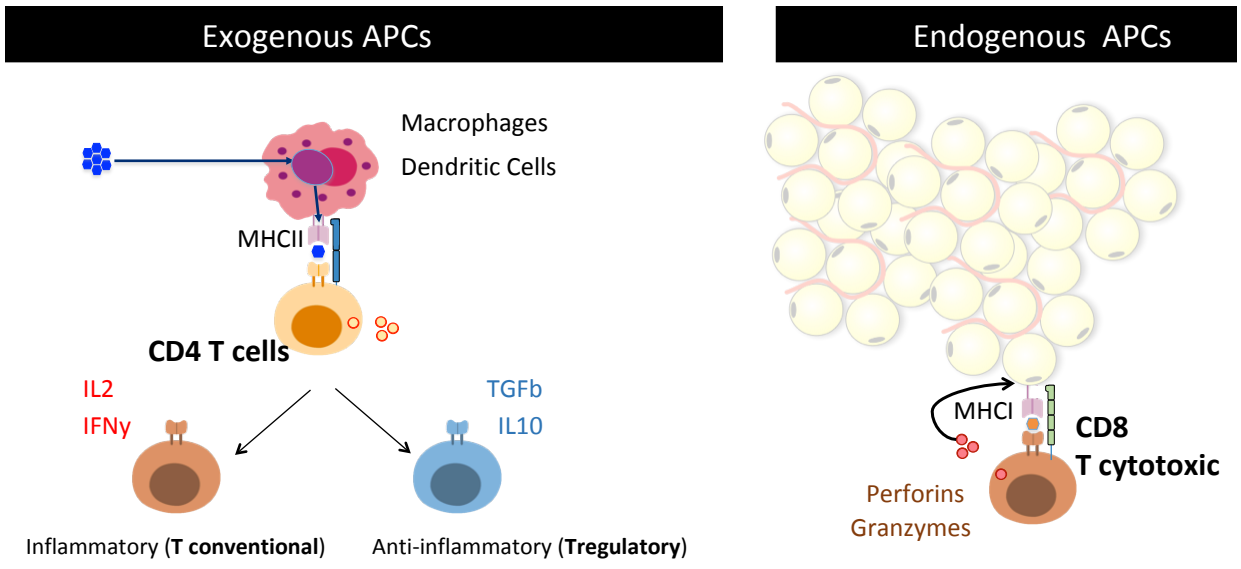

Figure 1-4 – Exogenous and endogenous antigens are responsible for CD4 and CD8 T cell activation

We know each of these 3 subsets (Tconv, Treg, and $CD8^+$ cytotoxic) T cells reside within adipose tissue and are regulated by the development of obesity. However, the exact function of the cells and their tissue-specific characterization after activation is unknown.

Innate Lymphoid Cells mediate inflammation and metabolism in adipose tissue

Innate lymphoid cells (ILC) have functional similarities to Tconv and CD8+ effector cells, but lack Rag2 mediated TCR rearrangement and adaptive immune cell characteristics. Although they do not express TCRs or recognize specific antigens presented by MHCII, they are known to amplify inflammatory signals found in their environment, maintain immune surveillance, and contribute to metabolic homeostasis. There are three groups of ILCs: ILC1, ILC2, and ILC3 which secrete effector cytokines that correspond with Th1, Th2, and Th17 Tconv. Natural killer (NK) cells are also considered a subset of ILCs, which possess cytotoxic function similar to $CD8^+$ T cells. Instead of recognizing antigen, ILCs are responsive to innate immune activating signals like PAMPS via pattern recognition receptors and cytokines that drive T cell function like IL-1β and TGFβ. Although ILCs are usually associated with mucosal surfaces, they have also been identified as important mediators of adipose tissue inflammation. ILC2 cells, prominent in lean individuals, have been shown to regulate adipocyte differentiation and promote the development of beige fat that has thermogenic characteristics (65). However, non-adipose tissue NK cells in have reduced cytolytic capacity in subjects with obesity and contribute to increased risk for cancer (95). Again this highlights the paradox between decreased inflammatory capacity of immune cells in outside of adipose tissue in obesity, while immune cells within adipose tissue mediate chronic low grade inflammation.

Adipose tissue T lymphocytes (ATTs) have unique tissue resident properties

There are multiple subsets of ATTs that either maintain inflammatory homeostasis in lean conditions (Tregs) or contribute adipose tissue inflammation in the context of obesity (Tconv, CD8+, γδ T cells).

Anti-inflammatory Tregs (ATTreg) act to maintain homeostasis in lean adipose tissue (96). In lean adipose tissue, resident Tregs (ATTregs) comprise ~40% of total $CD4^+$ T cells, 2-3x more than is seen in lymphoid organs like the spleen (96). Fat resident Tregs are derived from the thymus and have a distinct transcriptional profile which features upregulation of PPARγ (69, 75). With obesity, the frequency of ATTregs is reduced and their anti-inflammatory functions are inhibited (96). ATTregs have a distinct clonal T cell receptor (TCR) repertoire suggesting they may be maintained by antigen(s) present in adipose tissue (96). Since TCR-antigen specificity is required for T cell proliferation, this finding indicates that there could be a clonal expansion of Tregs in lean WAT. Therefore, finding the antigens within WAT that stimulate proliferation and activation of Tregs is highly sought-out due to their potential influence on meta-inflammation therapy.

With obesity, $CD4^+$ Th1 conventional ATT cells and $CD8^+$ cells are expand and contribute to insulin resistance in mice and humans (67, 97). There are several studies that indicate Tconv and CD8+ ATTs contribute to chronic low-grade inflammation. Neutralizing ATTs in obese adipose tissue using αCD3 antibody injections improve glucose sensitivity (67). CD8+ ATTs accumulate in mice fed HFD prior to pro-inflammatory macrophage recruitment and contribute to insulin resistance themselves (97). The literature describing Tconv cells are indicates these cells also contribute obesity mediated inflammation and insulin resistance. Obese macrophage specific MHCII knockout mice (LsyMCre x $MHCII^{fl/fl}$) have improved glucose and

insulin sensitivity compared to wild type controls. However, more recent studies indicate that Tconv inflammation is not due to canonical T cell activation and effector cytokine secretion. Tconv in obese adipose tissue have markers of cell cycle arrest and senescence, and secrete high levels of osteoponin instead of IL2 and IFNγ (98). Adipose tissue Th1 Tconv cells have restricted TCR repertoires, indicating they are proliferating in response to one or several antigens (99, 100). Strikingly, the TCR repertoires in lean and obese adipose tissue are distinct suggesting that different antigenic signals control their appearance, proliferation and activation. Once activated, Th1 cells release IFNγ, potentiating inflammatory profiles of ATMs and subsequent insulin resistance (101). In obese conditions, Th1 TCR repertoires are also restricted, but to a different set of antigens than adipose tissue Tregs (99). Since the proliferation of T cells classically occurs in an antigen-specific manner requiring peptide presentation by antigen presenting cells (APCs), it appears that Tconv are activated in response to antigens found exclusively in obese fat.

Mouse models of obesity

There are several ways to model obesity using animal models. Mice are the choice of immunologist because there are abundant reagents available to assess and manipulate DNA, RNA, and proteins in mice. We use the C57Bl/6J mouse strain because male mice are susceptible to robust adipose tissue expansion, adipose tissue immune cell activation, and the development of insulin resistance. Other common strains like BALBc mice are resistant to weight gain and obesity-induced morbidities (102, 103). C57Bl/6J mice are used for diet-induced obese models (DIO) when placed on high fat diets (HFD) starting at 6 weeks of age. The standard HFD chow used in the field is composed of 5.24kcal/gm 20% protein, 60% fat, 20% carbohydrate. However, there is some criticism that 60% HFD this does not model diet of obese

humans. Therefore, diets composed of 40% fat and increased carbohydrates are used in studies that are interested in modeling diet. Normal diet (ND) chow maintains lean and metabolically healthy C57Bl/6J mice and is composed of 4.09kcal/gm 29.8% protein, 13.4% fat, 56.7% carbohydrate.

Several transgenic mouse strains are also commonly used in obesity studies. Lep^{ob} (*Ob/Ob*) mice have a spontaneous mutation in their leptin gene. Without leptin signals, satiety is never achieved and animals continuously feel hungry, causing hyperphagia, hyperglycemia, glucose intolerance, and elevated plasma insulin. Another leptin related mutant mouse that is used for obesity research are *Db/Db* mice, which have a spontaneous mutation in the leptin receptor. Metabolic disease in these mice is more severe than DIO mice and occurs independently of high fat diet feeding. However, leptin signaling plays an important role in leukocyte function, therefore *Ob/Ob* and *Db/Db* models have limitations in immunologic studies.

Weight loss and bariatric surgery

Weight loss is recommended to all obese individuals because it is a proven way to improve metabolic syndrome and alleviate insulin resistance. Despite metabolic improvements, adipose tissue macrophage accumulation is not reduced with weight loss in mice (104). Whether this has long-term implications on adipose tissue health, or whether exercise mediated weight loss would result in the reversal of ATM infiltration is yet to be determined. Increasing energy expenditure over energy consumption results in weight loss and is achieved through both diet and exercise. However, it is difficult for obese individuals to lose weight and maintain reduced body weight. In morbidly obese individuals bariatric surgeries can aid in starting and maintain weight loss.

When obesity causes severe co-morbidities or it is difficult to lose weight via diet and exercise, bariatric surgery is considered. Most people considered for these surgeries have a BMI over 40. Bariatric surgery candidates have to undergo intense screening and make lifestyle changes that promote healthy eating and exercise habits. Surgeries result in permanent changes to the digestive system and healthy habits need to be established. By decreasing the volume of the stomach, the absorption of nutrients through the gut, or both bariatric surgeries aid in reducing calorie consumption. Roux-en-Y surgeries are the most commonly performed. These procedures entail reducing the stomach with a normal capacity of 3 pints down to a 1-ounce pouch by sealing off the top of the stomach. Additionally, the duodenum (beginning of the small intestine) is bypassed and the jejunum is connected to the re-sized stomach to reduce nutrient absorption. Sleeve gastrectomy is another method of reducing the absorption and volume of food that can be consumed. This method removes around 80% of the stomach leaving a tube-shaped pouch. Reduced stomach size reduces food intake and can also result in decreased secretion of the appetite-stimulating hormone, ghrelin.

Bariatric surgeries also allow us the opportunity to collect visceral adipose tissue from humans. Since abdominal fat requires invasive procedures to acquire, these surgeries are vital for studying human adipose tissue-resident immune cells. Unlike murine studies where lean animals can be used as normal controls, all bariatric surgery patients are obese. However, metabolically unhealthy and unhealthy patients can be grouped and compared. Blood is collected from each bariatric surgery patient along with omental biopsies. Hemoglobin A1c (HbA1c) is an indicator of long-term blood glucose levels and can be a method for determining whether an individual is diabetic without having to perform glucose tolerance tests. Measurements below 5.6 are considered normal, 5.7-6.4 is Pre-diabetic, and diabetic patients are 6.5 or higher.

Scope of the dissertation

There are significant gaps in knowledge regarding how immune cells interact with each other to influence homeostatic maintenance of lean adipose tissue, and chronic low-grade inflammation in obese metabolically unhealthy individuals. One critical interconnection between innate and adaptive immune responses in adipose tissue is the activation of CD4 T cells by antigen presenting cells like macrophages and dendritic cells. This specific network between innate and adaptive immune cells is particularly interesting because there is evidence that Tconv cells in obese adipose tissue have restricted TCR repertoires (99). Therefore ATTs are activated in an antigen-specific manner. However, it remains to be determined the specific antigens that are required for ATT activation and downstream inflammation. Therefore, the goal of this dissertation was to assess both sides of the connection between antigen presenting cells and T cells to best understand this autoimmune-like component of type II diabetes.

Context for Chapter 2- Adipose tissue dendritic cell signals are required to maintain T cell homeostasis and obesity-induced expansion

Although dendritic cell-mediated T cell activation has been well described in the context of lymphoid organs, both APCs and T cells within tissues have specific properties depending upon the environment in which they reside. Although ATMs have been shown to mediate T cell activation via peptide MHCII presentation in obese adipose tissue, the role of ATDCs has not been explored (80). The major gap in the field and question I sought to answer in this chapter is: how do professional antigen-presenting cells communicate and contribute to ATT cell function in obesity?

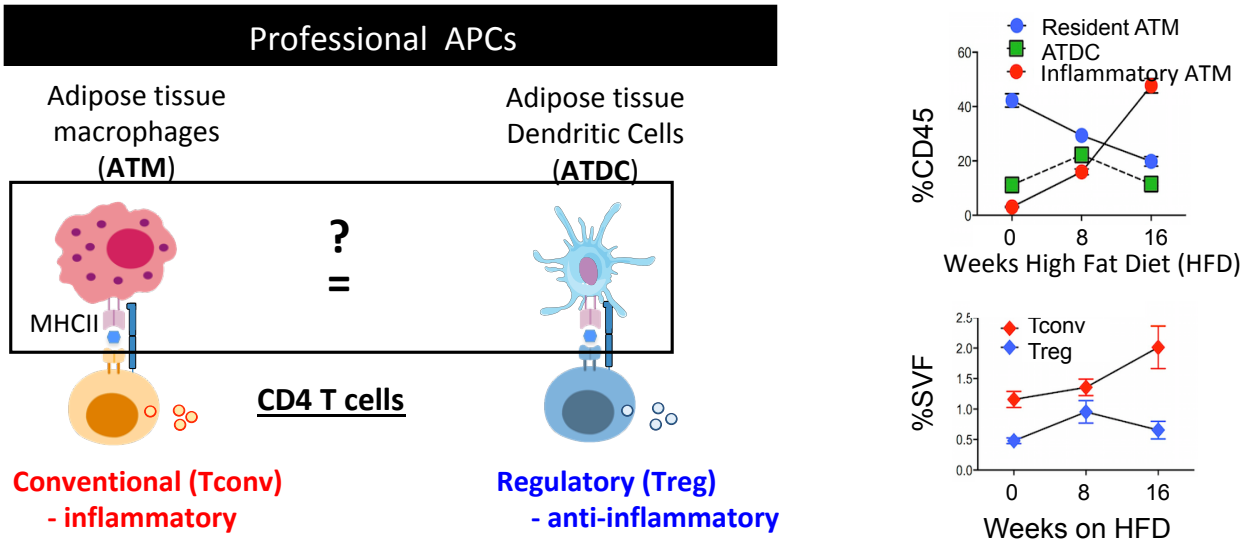

Figure 1-5 – Hypothesis: ATMs and ATDCs differentially regulate T cell polarization and function

The first step to answering this question was to distinguish whether there are differences in T cell activation via MHCII signaling by adipose tissue macrophages and dendritic cells. Since a cognate TCR interaction with a peptide-loaded MHCII has been identified as the mechanism that activates T cells in adipose tissue, further investigation of non-macrophage antigen presentation in WAT warranted scrutiny.

Lean tissue contains higher frequencies of ATDCs than obese WAT. Interestingly, frequencies of ATDCs do not progressively decrease over time. Instead, their frequency increases during short-term high fat diet exposure, and decrease after pro-inflammatory macrophage infiltration occurs. ATDC population dynamics mirror those of WAT Tregs, while $CD11c^{+}$ ATMs correspond with Th1 expansion (Figure 1-5). Therefore, we hypothesize that ATDCs and ATMs differentially regulated ATT polarization and function. If ATDCs and ATMs secrete different cytokines during T cell interactions, APCs may be responsible for the type of adaptive immune response elicited in WAT. Determining whether these APCs can differentially

activate T cells could significantly advance the understanding of adipose-specific immune health, and how it is modulated in different nutrient environments.

Such a task proved to be difficult since ATMs and ATDCs share a common stem cell progenitor. Many of their cell surface markers and functions are similar, making experimental alterations difficult to produce. However, if ATDCs and ATMs are identified as differential regulators of T cell proliferation, it would narrow the search for antigens activating Tregs and Th1 cells in their respective environments. Since antigen identification is so difficult to pursue with given biomedical technology, isolating separate APCs would be an important first step in this process. Ultimately, determining antigens responsible for inflammation in WAT would provide a target that would suppress $CD11c^+$ recruitment and pro-inflammatory cytokine production. Such a reversal would provide a significant breakthrough in creating therapies for type II diabetes.

Context for Chapter 3 - Adipose tissue T cells have impaired inflammatory capacity in obese mice and humans

It is unknown whether ATTs have the same response to cognate antigen presentation as T cells in lymphoid organs. The major gap in the field and question I sought to answer in this chapter was: what are the characteristics of activated ATT cells?

There are several critical gaps in our understanding of ATT cells and their function in insulin resistance. A few crucial and feasible questions that need to be addressed include: (1) What cytokines are secreted by $CD4^+$ and $CD8^+$ ATT cells upon activation and how does this change with obesity status and depot of origin? (2) What are the properties of human $CD4^+$ and $CD8^+$ ATT cells in relation to diabetes status in humans? (3) Which features of ATT cell

activation and maintenance in obesity contribute to metabolic dysfunction? Very little is known about the consequences of ATT cell activation in different adipose tissue depots and how this is changed with obesity. Even less is known about human ATT cell activation and how it relates to diabetes status. The objective of this aim is to evaluate the *hypothesis* that ATT cell activation profiles will have differences depending upon depot of origin and that in obese mice would have enhanced pro-inflammatory functions than ATT in lean tissue, contributing towards chronic low-grade inflammation.

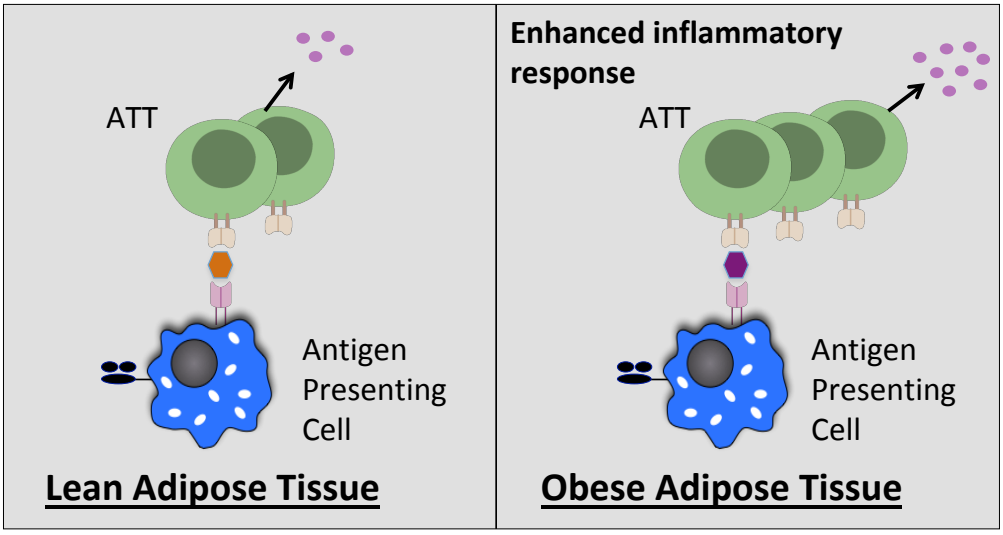

Figure 1-6 – Hypothesis: ATTs in obese adipose tissue have enhanced inflammatory responses

Previous limitations in studying ATTs have been insufficient substrate because ATTs are a rare cell population and difficult to maintain viability in-vitro. Therefore, a new assay was developed to perform basic functional assessments. Murine ATTs from two visceral depots (oWAT and eWAT) and human biopsies from normal and diabetic bariatric surgery patients were used to collect primary ATTs and test their function in response to an activating stimulus.

Context for Chapter 4- Heterogeneity of adipose tissue T cell subtypes

Immune cell functional capacity is often grouped by protein expression of transcription factors and cell surface receptors. However, defining cell populations based on known protein expression can be limiting and lead to a reductionist view of immune cell subsets. In the past decade, single-cell RNA sequencing has become more widely accessible. Improved analysis of ATTs using this technology can be achieved because of several important factors. First, each cell is sequenced independently of the others and can be clustered due to the similarity of transcriptional expression of thousands of genes instead of 10-20 proteins that can be detected with techniques like flow cytometry.

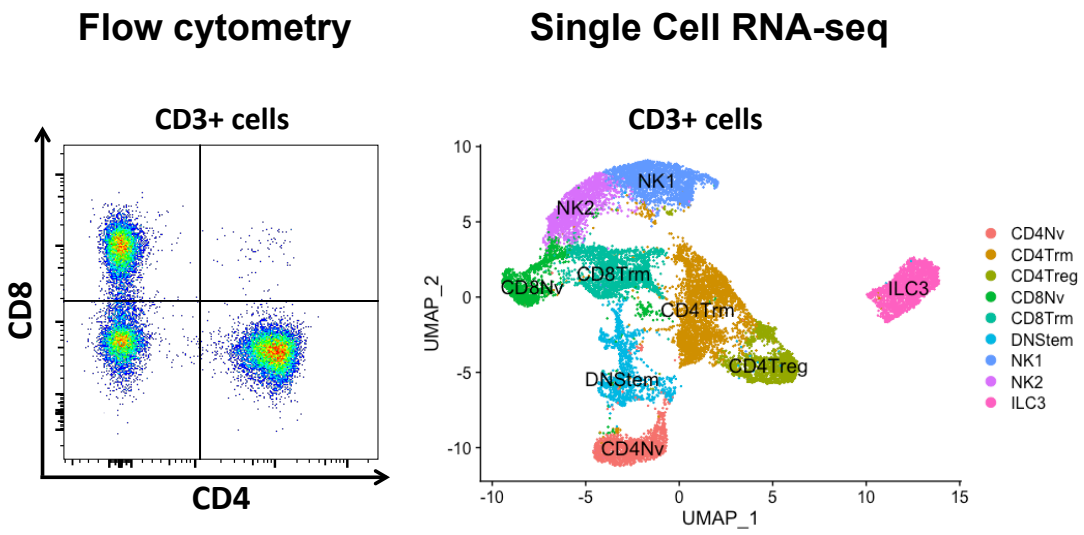

Figure 1-7 – Interrogating ATT heterogeneity with single cell RNA-seq

An additional benefit of SC RNA sequencing is detailed intracellular gene expression, which can be analyzed with pathway sequencing databanks like Gene Set Enrichment Analysis (GSEA) and protein annotation through evolutional relationship (PATHER). Rare cell

populations can be difficult to assess with conventional assays because sufficient protein or RNA cannot be obtained. Therefore, SC RNAseq allows us to assess potential mechanisms and functional differences between different ATT clusters, differences in clusters between depots, and modifications due to HFD feeding.

Overall objectives

The major question in the field, and overall objective of this dissertation was to make steps towards identifying adipose tissue antigens responsible for ATT activation in the context of obesity. Not only are these antigens a target for reducing ATT mediated metabolic inflammation, their presence and inflammatory induction have the potential for re-framing type II diabetes as an adipose tissue centric autoimmune disease. Interrogating questions regarding ATDC mediated ATT maintenance and activation, tissue-specific characteristics, diet-mediated changes in ATT functionality, and heterogeneity of $CD3^+$ ATTs has resulted in significant novel information contributing to the field of adipose tissue inflammation. We have closed several gaps in what we know about ATTs in obesity-induced diabetes that inform next steps for identifying the activating antigens in question. However, equally important novel findings challenge the dogma that ATTs chronically produce pro-inflammatory mediators and suggest obesity induces ATT inflammatory impairment. Further studies will be required to determine the physiological implications for this finding in the context of metabolic diseases, and associated increased morbidities related to infections and cancer development in obese individuals.

Chapter 2: Adipose Tissue Dendritic Cell Signals are Required to Maintain T Cell Homeostasis and Obesity-Induced Expansion

This chapter has been published:

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Author Contributions: BFZ bred transgenic mice, EP performed experiments in Figure 2-5, CEP and JLD both performed experiments in Figures 2-1, 2-2, 2-3, 2-6. CEP performed experiments in Figure 2-4. CEP and CNL conceived of experiments, wrote, and edited the manuscript.

Abstract

Adipose tissue derived chronic inflammation is a critical component of obesity induced type II diabetes. Major histocompatibility complex II (MHCII) mediated T cell activation within adipose tissue is one mechanism that contributes to this phenotype. However, the contribution of dendritic cells as professional antigen presenting cells in adipose issue has not previously been explored. Using $Itgax^{Cre}$ x $MHCII^{fl/fl}$ (M11cKO) mice we observed adipose tissue specific changes in adipose tissue leukocytes. While there was a complete knockout of MHCII in dendritic cells, MHCII was also absent on the majority of macrophages. This resulted in reduction of TCR expression in $CD4^+$ T cells in obese adipose tissue, and an increase in $CD8^+$ and $CD4^+$ $CD8^+$ double positive T cells with decreased $CD4^+$ T cells independent of diet type. Increased $CD8^+$ cells were not observed in the spleen, suggesting adipose tissue T cell regulation is tissue specific. In vitro studies demonstrated more potent antigen presentation function in

adipose tissue dendritic cells compared to macrophages. Obese M11cKO mice had decreased $CD11c^+$ adipose tissue macrophages. Despite the changes of immune cellularity in adipose tissue, M11cKO largely did not change inflammatory gene expression in adipose tissue and did not demonstrate differences in glucose and insulin intolerance. Overall MHCII expression on $CD11c^+$ cells is important for maintaining $CD4^+$ and $CD8^+$ adipose tissue T cells, but these cellular changes fail to alter inflammatory output and systemic metabolism.

Introduction

Type II diabetes is widely prevalent in the United States. The latest CDC statistics indicate that 9.4% of the entire population has diabetes, and that 87.5% of this group is overweight or obese (105). Obesity induced diabetes is characterized by insulin resistance, which is mechanistically linked to adipose tissue dysfunction and chronic inflammation (54-56, 106). Preclinical and clinical studies have shown chronic low-grade inflammation is a critical link between obesity and insulin resistance, and inhibiting adipose tissue inflammation improves insulin sensitivity and metabolic capacity (57-60). Therefore understanding the mechanisms inducing adipose tissue inflammation and developing methods to reverse this phenotype are critical steps in developing diabetes treatments.

Components of both the innate and adaptive immune system reside within adipose tissue and their inflammatory output shifts with in- creased adiposity. Lean fat predominantly contains cells with anti-inflammatory phenotypes, such as arginase expressing adipose tissue macrophages (ATMs), group 2 innate lymphoid cells (ILC2) and regulatory T cells (Tregs), which maintain metabolic homeostasis (65, 68, 96). However with obesity, cross-talk between adipocytes and tissue resident leukocytes leads to adipose tissue inflammation characterized by qualitative and quantitative changes in adipose immune cells. With obesity, both $CD4^+$ Th1 and $CD8^+$ adipose tissue T cells (ATTs) are activated and contribute to insulin resistance in obese conditions (67, 97). $CD4^+$ T conventional (Tconv) ATTs have restricted TCR repertoires in obese adipose tissue, indicating that they are being activated and proliferating by in response to specific antigens residing within the adipose tissue (99). Adipose tissue Tregs (ATTregs)

comprise ~40% of $CD4^+$ T cells in lean mice, have a distinct transcriptional profile, and have a restricted TCR repertoire that contributes to metabolic homeostasis (75, 96). The quantity of ATTregs is diminished with obesity-induced inflammation contributing to the pro-inflammatory environment.

MHC class II and the antigen presenting cells (APCs) control ATT cell content and activation, and appear to be diverse (69, 73, 107). Adipocytes have been implicated in antigen presentation, but professional phagocytes are also prominent features of adipose tissue (107). An extensive resident ATM population exists in lean mice, and with obesity activated $CD9/CD11c^+$ macrophages accumulate in conjunction with pro-inflammatory $CD4^+$ Th1 cells (72, 108). ATMs have potent APC capacity required for ATT activation in obesity. Using $Lyz2^{Cre}$ x $MHCII^{fl/fl}$ mice, MHCII expression in ATMs was shown to be required for the obesity-induced generation of $CD4^+$ conventional Th1 ATTs, but not the maintenance of adipose tissue $CD4^+$ cells on normal diets (80). This mouse model also targeted some adipose tissue dendritic cells (ATDC), which have been shown to contribute to the inflammatory response to obesity in visceral adipose tissue depots and may explain the ability to maintain normal $CD4^+$ ATT numbers (73, 82, 109)

It is currently not well understood if ATDCs and ATMs have redundant functions in activating ATT cells, or whether they possess unique activation or polarization of ATTs in obese states. Since ATDC are the main $CD11c^+$ cell in lean adipose tissue (73), we generated mice with knockout of MHCII (H2-Ab1) in CD11c expressing cells using $CD11c(Itgax)^{cre}$ drivers crossed to $H2\text{-}Ab1^{fl/fl}$ transgenic mice (M11cKO). We hypothesized that inhibiting ATDC

antigen presentation using $CD11c^{Cre}$ would disrupt Treg populations and subsequently worsen glucose tolerance in lean mice. We observed that M11cKO mice had fewer $CD4^+$ adipose tissue T cells along with increase in $CD8^+$ ATT independent of diet type. Additionally M11cKO had impaired ATT TCR expression on $CD4^+$ Treg and Tconv in obese settings. This phenotype was related to loss of MHCII in ATDC as well as most ATMs and contributed to a slight decrease in the accumulation of $CD11c^+$ ATMs in obese mice. In vitro studies demonstrated that ATDC had stronger APC function than ATMs explaining the requirement for MHCII in ATDC for $CD4^+$ cell maintenance. Overall our results suggest differential regulation of ATT cell maintenance and activation by ATDC and ATM.

Materials and Methods

Animal Studies

C57Bl/6J, CD11c^{Cre} (JAX stock 008068, B6.Cg-Tg(Itgax-cre)1- 1Reiz/J (110) and MHCII$^{fl/fl}$ (B6.129X1-H2-Ab1$^{tm1Koni/J}$) mice were obtained from the Jackson Laboratory. MHCII$^{fl/fl}$ x CD11c^{Cre} (M11cKO) mice were generated by breeding Itgax/Cd11c^{Cre} with MHCII$^{fl/fl}$ mice. Cre-negative and MHCII$^{fl/+}$ littermates were used as controls. Male mice were fed ad libitum either a normal diet (LabDiet PicoLab 5L0D 4.09kcal/gm 29.8% protein, 13.4% fat, 56.7% carbohydrate) or a high fat diet (HFD; Research Diets D12492, 5.24kcal/gm 20% protein, 60% fat, 20% carbohydrate) beginning at 6 weeks of age. All mouse procedures were approved by the University Committee on Use and Care of Animals at the University of Michigan and were conducted in compliance with the Institute of Laboratory Animal Research Guide for the Care and Use of Laboratory Animals.

Metabolic evaluations

Glucose tolerance tests (GTT) and Insulin Tolerance Tests (ITT) were performed after a 6 hour fast. For GTTs, mice were injected IP with D-glucose (0.7 g/kg). For ITTs, mice were injected IP with human insulin (Humulin 1 U/kg). For both GTTs and ITTs, blood glucose concentrations (mg/dL) were measured at 0, 15, 30, 45, 60, 90, and 120 min after injection from tail nick with a glucometer.

Isolation of adipose tissue SVF and flow cytometry analysis

The stromal vascular fraction (SVF) was isolated from whole adipose tissue as previously

described (111). Briefly, adipose tissue depots were dissected and weighed. Tissue was then mechanically disrupted by mincing, and chemically digested by rocking tissue in 1 mg/ml collagenase IV (Sigma Aldrich) at 37°C for 30 min. Cells were then quenched with RPMI media and filtered through 100 nm mesh prior to RBC lysis and subsequent filtering with 70 nm mesh filters.

Cells were incubated in Fc Block for 5 min on ice and stained with indicated antibodies for 30 min at 4°C: Anti-Mouse CD45 eFluor 450 [48-0451-82], Anti-Mouse CD8a FITC [11-0081-82], Anti-Mouse MHC Class II (I-A/I-E) PE-Cy7 [25-5321-82], Anti-Mouse CD4 APC [17-0041-82], Anti-Mouse CD11c APC-eFluor® 780 [47-0114-80], Anti-Mouse CD25 APC [17-0251-82] from eBioscience, anti-mouse CD3ε PerCP/ Cy5.5 [145-2C11] from Biolegend, and anti-Mouse CD64 a and b Alloantigens PE [558,455] from BD Pharmingen.

Stained cells were washed twice with FACS buffer and fixed for intracellular staining with Anti-Mouse Foxp3 PE [12-4771-82] from eBioscience, using a FoxP3 transcription kit (BD Biosciences). Analysis was performed on a FACSCanto II Flow Cytometer and analyzed with Flow Jo software (Treestar).

Gating strategy

Representative flow gating of eWAT was performed as the following: cells were gated on Singlets (FSC–W x FSC-H) → Scatter (FSC-A x SSC-A) → $CD45^+$ → and then separated by $CD3^+$ and $CD3^-$. $CD3^+$ cells were further sub-gated into specific T cell populations (CD4 by CD8). The $CD4^+$ population was further divided into $FoxP3^+$ (Treg) and $FoxP3^-$ (Tconv) groups. $CD3^-$ cells were used to assess APCs. ATDCs are $CD11c^+$ $CD64^-$, and ATMs are $CD64^+$.

Gene expression analysis

RNA was extracted from tissue by homogenizing epididymal white adipose tissue (eWAT) in Trizol, followed by phase separation with chloroform. The aqueous fraction was then processed using RNeasy Midi Kit purification (Qiagen) following standard protocol. cDNA was generated from 0.5 μg total RNA using high-capacity cDNA reverse transcription kits (Applied Biosystems). Power SYBR Green PCR Master Mix (Applied Biosystems) and the StepOnePlus System (Applied Biosystems) were used for real-time quantification PCR. Acidic ribosomal phosphoprotein P0 (Arbp) expression was used as an internal control for data normalization. Samples were assayed in triplicate, and relative expression was determined using the $2^{-\Delta\Delta CT}$ method.

Adipocyte sizing

Adipose tissue was fixed with 10% formalin, and imaged with an immunofluorescence microscope at 10X. Adipocyte circumference was measured using ImageJ.

CFSE staining and T cell proliferation assays

APCs. SVF was extracted and stained with CD45, CD11c, CD64, and CD3 flow antibodies (listed in section 2.3). A FACS sort was performed to isolate ATMs, ATDCs, CD11c- $CD64^{-}$ $CD45^{+}$, and $CD45^{-}$cell populations using a Sony MA900 sorter. $CD3^{+}$ cells were excluded. Each of the 4 cell populations was plated overnight, before being pulsed with 10 μg/ml of OVA or BSA for 6 hours. $CD3^{+}$ cells were isolated from the lymph nodes and spleen of an OTII mouse. These cells were then stained with CFSE Cell Trace Proliferation Kit (Life Technologies) and co-cultured with APCs at a 1:10 ratio. After 4 days cells were taken and stained for flow

cytometry analysis. Gating strategy is as follows: Singlets (FSC–W x FSC-H) → Scatter (FSC-A x SSC-A) → Live cells (Live/Dead Fixable Violet Dead Cell Stain Kit, Invitrogen) → $CD4^+$ → $FoxP3^-$ → $CD25^+$ $CFSE^-$ (antibodies listed in section 2.3).

Hepatic triglyceride content

Livers were weighed, snap frozen in liquid nitrogen, and stored at −80 °C. 50–100 mg of frozen liver was cut and homogenized in buffer containing NP-40, Tris-Hcl, and NaCl. Chloroform was added, vortexed, and samples dried overnight in a speed vacuum. Another round of chloroform extraction was performed on dried samples before residual lipid was dissolved in butanol. Triglycerides were measured using the Infinity Triglyceride Assay Kit (Sigma), and normalized to the initial mass of tissue homogenized.

Statistical analysis

All values are reported as mean ±SEM. Differences between groups were determined using unpaired, two-tailed Students t-Test or two-way ANOVA with Tukey post hoc tests with Graph Pad Prism 7 software. P values less than 0.05 were considered significant.

Results

MHCII$^{fl/fl}$ x CD11c^{Cre} (M11cKO) deletes MHCII expression in ATDCs and most ATMs and decreases CD11c^{+} ATMs in obese mice

In lean mice, the primary CD11c^{+} adipose tissue myeloid cells are ATDC (Cho et al., 2016). Therefore, we sought to eliminate MHCII expression in ATDC by generating conditional dendritic cell knockout mice by crossing MCHII$^{fl/fl}$ x CD11c^{Cre} (Itgax^{cre}) mice (M11cKO). Al- though transgenic lines such as Zbtb46^{Cre} are more specific for targeting conventional DCs, off target effects like colitis that prevent weight gain and illicit off target gut inflammation limit their utility for obesity studies centered on adipose tissue biology (112, 113). M11cKO and littermate controls were fed either normal diet (ND) chow or high fat diet (HFD) for 15 weeks. Body weight of control and M11cKO mice on a ND did not differ significantly. After HFD feeding, both genotypes gained significant weight, but HFD fed M11cKO weighed slightly less than littermate controls (**Figure 2-1A**). Organ weight at termination demonstrated no significant differences in non-adipose tissues such as liver and spleen. Epididymal (eWAT) and inguinal (iWAT) adipose tissue mass in HFD fed M11cKO mice were mildly, but significantly, decreased compared to littermate controls (**Figure 2-1B**). When normalized to total body weight, fat mass in M11cKO mice was not significantly different than controls (**Supplemental Figure 2-1A**).

Adipose tissue leukocytes in eWAT were assessed by flow cytometry (Strategy shown in **Supplemental Figure 2-1B**). In both lean and obese mice, the quantity of total ATDCs (CD45^{+}CD64^{-}CD11c^{+}) and ATMs (CD45^{+} CD64^{+}) were not different between M11cKO and

controls. The frequency of ATDCs as percentage of immune cells was not significantly changed by diet or genotype. As expected, the frequency of total ATMs from the HFD fed cohorts was significantly elevated compared to ND controls. Obese M11cKO mice had a trend towards less total ATM content. $CD11c^{-}$ $CD64^{-}$ cells were unchanged by genotype, but decreased in HFD-fed M11cKO compared to ND controls (**Figure 2-1C**). Using flow cytometry, we verified that M11cKO mice had absent MHCII expression on ATDCs. While nearly all ATDCs from controls expressed MHCII, almost all ATDC from Mc11KO mice had lost MHCII expression (2.27% ±0.58 of M11cKO ATDC expressed MHCII) (**Figure 2-1D**). M11cKO mice also lacked MHCII expression on the majority of ATMs in ND and HFD fed mice (12.2%± 3.14 of the ATMs retained MHCII expression in M11cKO mice) (**Figure 2-1E**).

Since obesity is known to induce $CD11c^{+}$ ATMs, we quantified $CD11c^{+}$ and $CD11c^{-}$ ATMs in control and M11cKO mice (**Figure 2-1F**). M11cKO and controls did not differ in the proportion of $CD11c^{-}$ or $CD11c^{+}$ ATMs in lean mice fed ND. M11cKO mice fed HFD had fewer $CD11c^{+}$ ATMs compared to controls. There were no differences in $CD11c^{-}$ ATMs in HFD fed M11cKO mice. Given the significant reduction in MHCII expressing $CD64^{+}$ ATMs, we assessed MCHII expression on both $CD11c^{+}$ and $CD11c^{-}$ subtypes in the M11cKO and controls (**Figure 2-1G**). Surprisingly, both $CD11c^{+}$ and $CD11c^{-}$ ATMs had reductions in MHCII expression in M11cKO mice. This suggests that there may be transient CD11c expression in the $CD11c^{-}$ population or precursors that give rise to $CD11c^{-}$ ATMs. Overall, M11cKO mice lack MHCII in all ATDCs and also have lost MHCII in the most, but not all, ATMs. This contrasts with $Lyz2^{Cre}$ x $MCHII^{fl/fl}$, which targets all ATMs, and most, but not all,

ATDC (80, 111). A summary of similarities and differences between Lyz2^{Cre} and CD11c^{Cre} are summarized in (**Table 2A**).

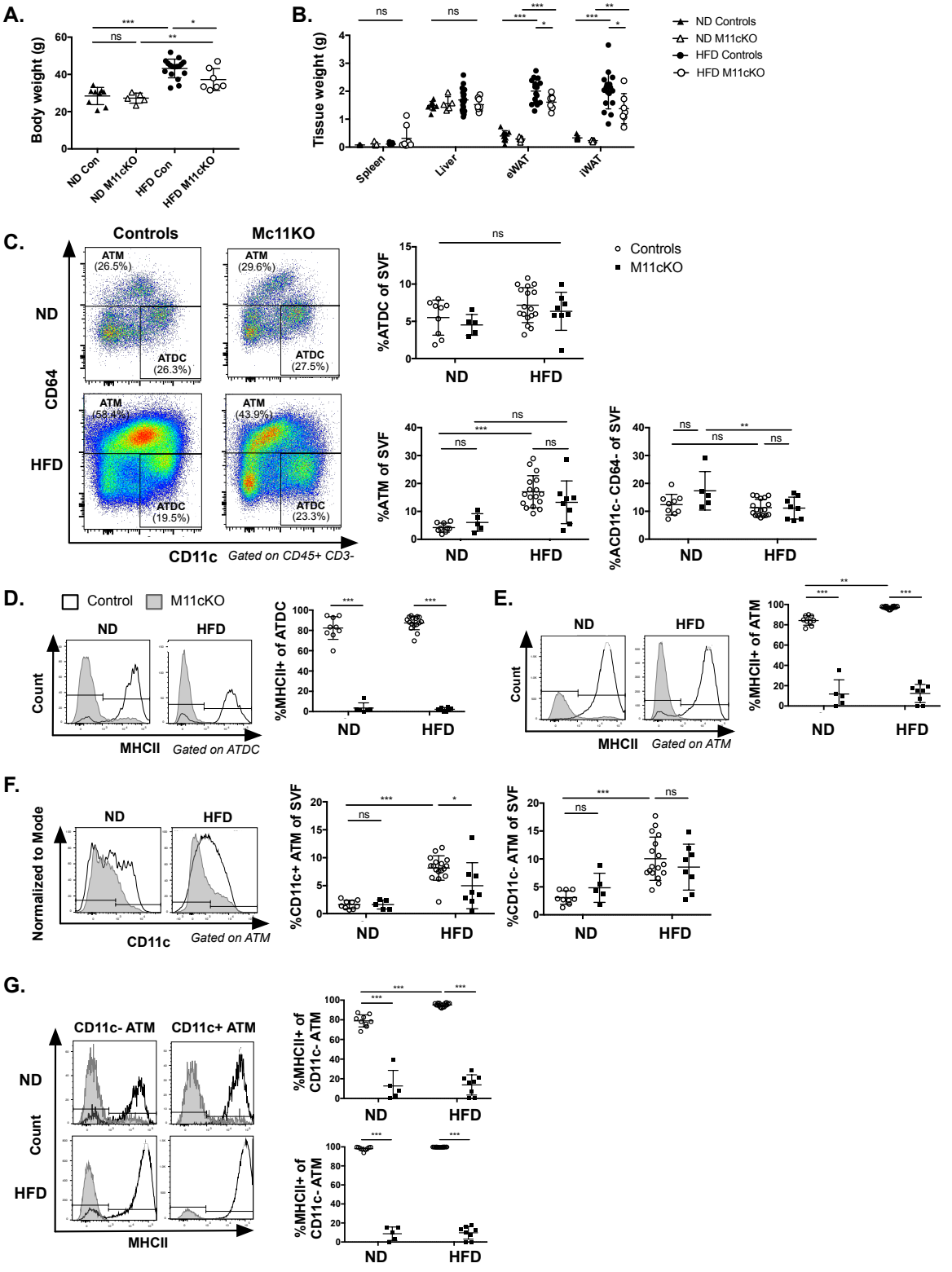


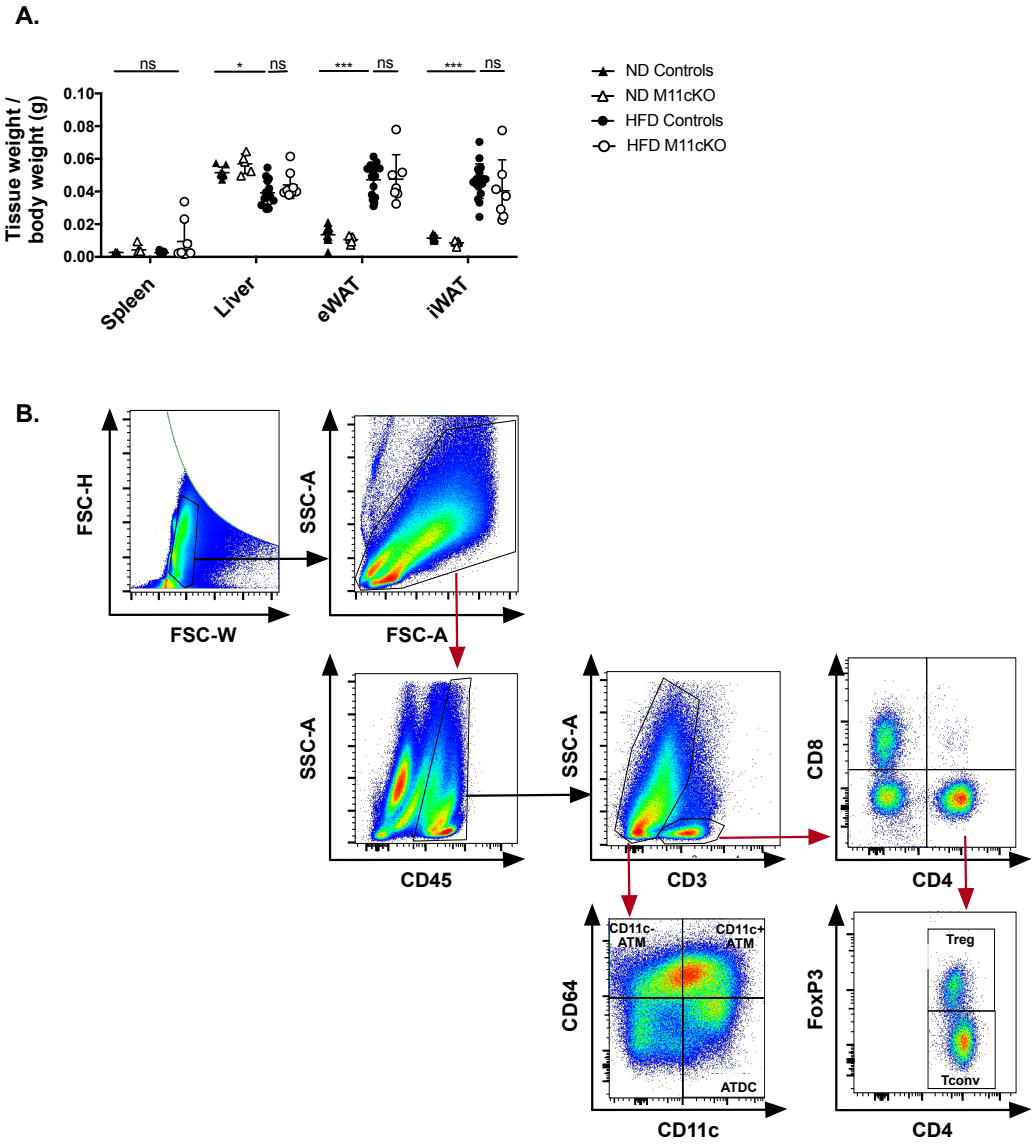
Figure 2-1- CD11cCre Reiz drives MHCII knockdown in both adipose tissue dendritic cells and macrophages

(A) Terminal body weight of transgenic M11cKO mice and littermate controls on ND or HFD after 15 weeks of feeding.

(B) Tissue weights of spleen, liver, eWAT, and iWAT at time of termination,

(C) Representative flow plot illustrating ATDC, ATM and CD11c- CD64- populations gated from CD45+ CD3- cells. Frequencies of each group are measured as a percentage of SVF shown on the right.
(D) Representative flow cytometry histograms and frequencies of MHCII expression on ATDCs,
(E) ATMs,
(F) CD11c+ and CD11c- ATMs as a percentage of SVF.
(G) Representative historgrams showing MHCII expression and frequencies of MHCII expression on Cd11c= and CD11c+ ATMs.

*p < 0.05, **p < 0.01, ***p < 0.0001. n = 5-17 mice/group



Supplemental Figure 2-1: Additional data related to Figure 2-1

(A) Tissue weights normalized to body weights. * p< 0.05, ** p <0.01, *** p < 0.0001

(B) Representative flow gating of eWAT. Cells were fated on Singlets → Scatter → CD45+ → CD3- cells were taken for antigen presenting cell assessment. **ATM** = CD64+, **ATDC** = CD64- CD11c+. CD3+ cells were taken and subdivided by CD4 and CD8 expression. **Treg** and **Tconv** populations were determined by FoxP3 expression on cells within the Cd4 population.

Table 2A – ATDC v ATM MHCII contribution to systemic metabolism and inflammatory properties in ND and HFD-fed mice

	ND		HFD	
	M11cKO v WT Con (ATDC)	MMKO v WT Con (ATM)	M11cKO v WT Con (ATDC)	MMKO v WT Con (ATM)
Systemic Metabolism				
GTT AUC	Unchanged	Unchanged	Unchanged	Improved
ITT AUC	Unchanged	Unchanged	Unchanged	Improved
Inflammatory Properties				
ATM CD11c+ expression	Unchanged	Unchanged	Decreased	Decreased
ATDCMHCII+ expression	Complete KO	Majority KO	Complete KO	Majority KO
ATM MHCII + expression	Majority KO	Complete KO	Majority KO	Complete KO
Splenic Tconv	Decreased	Unchanged	Decreased	Unchanged
Splenic Treg	Decreased	Unchanged	Decreased	Unchanged
Splenic $CD8^+$	Unchanged	Unchanged	Increased	Unchanged
Splenic DPT	Unchanged	Unchanged	Increased	Unchanged
eWAT Tconv	Decreased	Unchanged	Decreased	Decreased
eWAT Treg	Unchanged	Unchanged	Decreased	Decreased
eWAT $CD8^+$	Increased	Increased	Increased	Unchanged
eWAT DPT	Increased	Unchanged	Increased	Unchanged

M11cKO mice have increased adipose tissue $CD8^+$ and decreased $CD4^+$ T cells

Previous studies have shown that deletion of MHCII in ATMs using $Lyz2^{Cre}$ results in normal $CD4^+$ levels in lean mice and a reduction of conventional $CD4^+$ ATT cells in obese mice with subsequent metabolic improvement (80, 111). We phenotyped ATT cells by flow cytometry in M11cKO mice and controls fed ND and HFD (**Figure 2-2A**). In both, lean and obese mice M11cKO mice had fewer $CD4^+$ ATTs compared to WT controls suggesting that ATDC MHCII is required for maintenance this population (**Figure 2-2B**). Analysis of Tconv and Treg within the $CD3^+$ population showed that Tconv were significantly de- creased in lean and obese M11cKO mice while Treg were decreased only in the HFD fed M11cKO mice (**Figure 2-2C and 2-2D**). However, when Tregs are assessed as the frequency of total $CD4^+$ T cells, neither diet nor genotype changed the frequency of this population (**Supplemental Figure 2-2A**). This decrease in $CD4^+$ ATT was associated with a significant increase in $CD8^+$ cells (**Figure 2-2E**) and an increase in $CD4^+$ $CD8^+$ double positive T (DPT) cells (**Figure 2-2F**), while $CD4^-$ $CD8^-$ double negative T cells remained unchanged (**Figure 2-2G**) in both ND and HFD mice. Since an increase in DPT cells is an aberration when found outside of the thymus, we assessed the thymus (**Supplemental Figure 2-2B**) and bone marrow T cells (**Supplemental Figure 2-2C**). M11cKO mice did not have increased DPT cells in either compartment and similar CD8 and CD4 single positive content, suggesting that these cells are not escaping thymic regulation, but may be forming in adipose tissue. When expressed as a percentage of the total SVF, there were no significant differences in total $CD3^+$ T cell content in ND mice, but HFD fed M11cKO mice had a significant increase in total $CD3^+$ T cells (**Figure 2-2H**). This increase was attributed to $CD8^+$

ATT cells as $CD8^+$ cells are significantly increased as a percentage of the SVF in both ND and HFD fed M11cKO compared to littermate controls (**Figure 2-2I**).

Because of the increase in $CD8^+$ ATTs in an environment that has reduced MHCII expression, we assessed H2-kb (MHCI) gene expression to see if it was changed in M11cKO mice to compensate for decreased MHCII. However, MHCI expression was unchanged by diet or M11cKO in whole eWAT (**Figure 2-2J**). To examine any qualitative differences in $CD4^+$ T cells in the M11cKO mice, CD3/TCR expression was quantified by flow cytometry. While $CD8^+$ ATTs had similar CD3 expression in control and M11cKO mice (Fig. 2K), both $CD4^+$ Tconv (**Figure 2-2L**) and Treg (**Figure 2-2M**) had a reduction in CD3 expression as quantified by median fluorescence intensity (MFI) in HFD fed mice. This suggests that lack of MHCII stimulus in M11cKO lead to decreased expression of the TCR on both $CD4^+$ Tconv and Treg. In sum, MHCII expression on ATDC is required for the normal and high fat diet induced homeostasis of $CD4^+$ ATTs and results in an increased in $CD8^+$ ATT cells in lean and obese conditions.

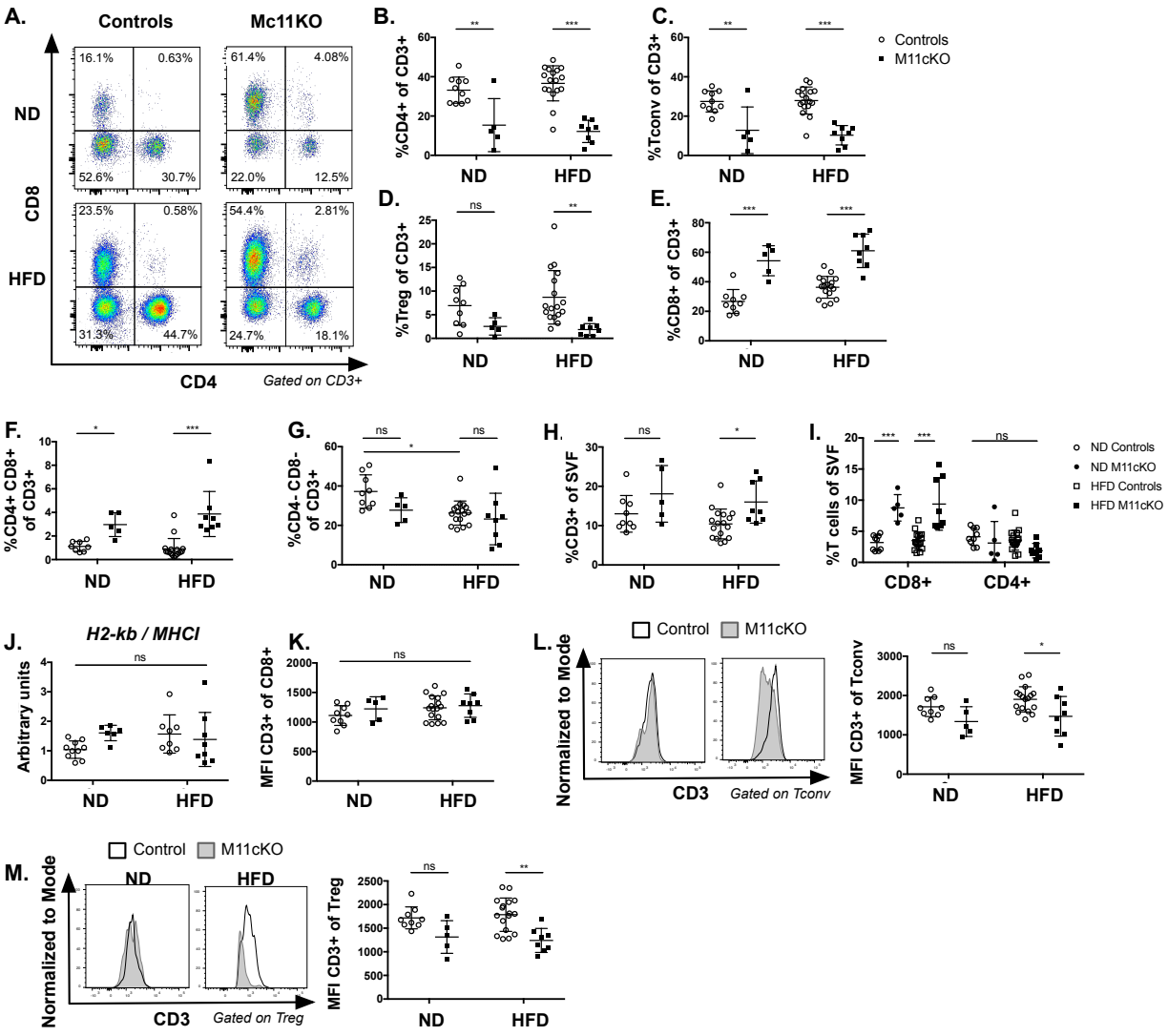
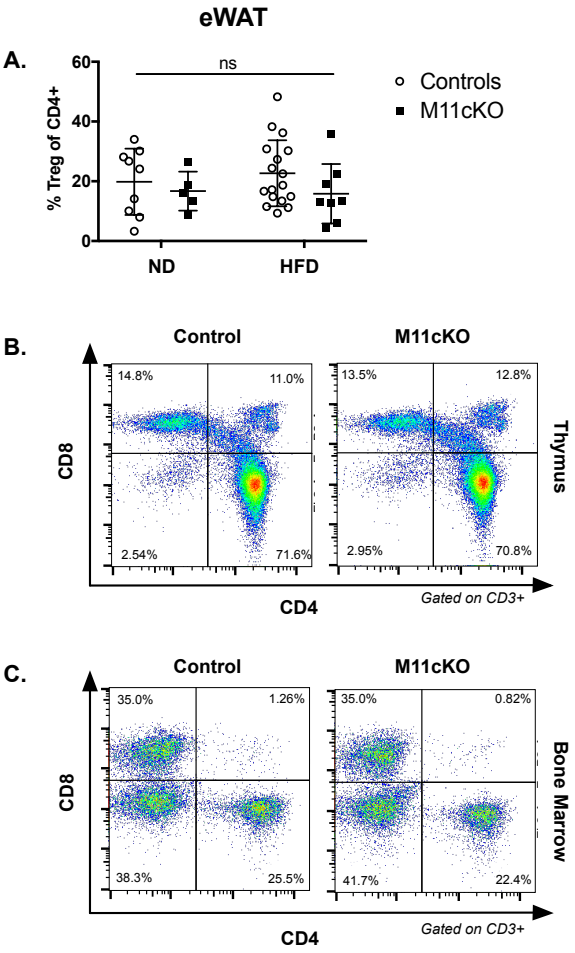


Figure 2-2 – M11cKO decreases CD4+ and increases CD8+ ATTs in eWAT

(A) Flow plots illustrating diffrences in CD4 and CD8 expression on Cd3+ cells**.**
(B) Frequency of total CD4+ of CD3+ T cells.
(C) Frequency of FoxP3- CD4+ (Tconv) of CD3+
(D) Frequency of FoxP3+ CD4+ (Treg) of CD3+ T cells,
(E) Frequency of CD8+ of CD3+ Tcells
(F) Frequency of CD4+ CD8+ double positive T cells (DPT) as a percentage of total CD3+ T cells,
(G) Frequency of CD4- CD8- double negative cells as a percentage of CD3+ T cells,
(H) Frequency of CD3+ of SVF,
(I) Frequency of CD8+ and CD4_ T cell populations as a percentage of total SVF
(J) RT-qPCR analysis of H2-kB (MHCI) gene expression in eWAT
(K) Median fluorescence intensity (MFI) of CD3 in CD8+ population
(L) MFI of CD3 in the Tconv population
(M) MFI of Cd3 in the Treg population

*p < 0.05, **p < 0.01, ***p < 0.0001. n = 5-17 mice/group



Supplemental Figure 2-2 – Additional data related to Figure 2-2

(A) Frequency of FoxP3+ of CD4+ ATT cells from eWAT.
(B) Representative plots of CD4+ and CD8+ thymic T cells from control and M11cKO mice, gated on CD3+cells.
(C) Representative plots of CD4+ and CD8+ bone marrow T cells from control and M11cKO mice, gated on CD3+ cells.

Splenic T cell phenotype in M11cKO indicates ATT maintenance is adipose tissue specific

We evaluated spleens of the M11cKO mice to determine if the T cell changes observed in adipose tissue were fat specific (**Figure 2-3A**). Similar to adipose tissue, splenic total $CD4^+$,

Tconv, and Treg (**Figures 2-3B–2-3D**) cells were significantly decreased as a percentage of T cells in M11cKO in- dependent of diet type – although the magnitude of the decrease was not as significant as seen in adipose tissue. When expressed as percentage of the $CD3^+$ population, $CD8^+$ T cells were unchanged in ND M11cKO mice, but HFD led to an increased frequency of $CD8^+$ T cells in the M11cKO mice (**Figure 2-3E**). Splenic DPT cells were increased in frequency only in HFD fed M11cKO mice (**Figure 2-3F**). Overall, the quantity of DPT cells in the spleen was half of that observed in M11cKO eWAT. M11cKO mice had an increase in $CD4^-$ $CD8^-$ double negative T cells only in ND fed mice (**Figure 2-3G**). As a percentage of all splenocytes, lean ND fed M11cKO mice had significantly decreased $CD3^+$ T cells com- pared to littermate controls, however no differences in splenic $CD3^+$ cells were seen in HFD fed mice (**Figure 2-3H**). Unlike ATTs, splenic cells showed decreased $CD4^+$ cells as calculated by frequency of splenocytes in the M11cKO mice, while there were no differences in $CD8^+$ T cells (**Figure 2-3I**). The decrease in $CD4^+$ was not attributable to specific de- creases in splenic Treg or Tconv in HFD fed mice (Fig. 3J). Unlike adipose tissue, CD3/TCR expression on splenic $CD8^+$ (**Figure 2-3K**), Tconv (**Figure 2-3L**), and Tregs (**Figure 2-3M**) are all unchanged by either diet or genotype. Overall, our data demonstrates ATT specific regulation in M11cKO mice compared to the spleen, thymus and bone marrow, and suggests there is environment specific signals in WAT that drive $CD8^+$ accumulation.

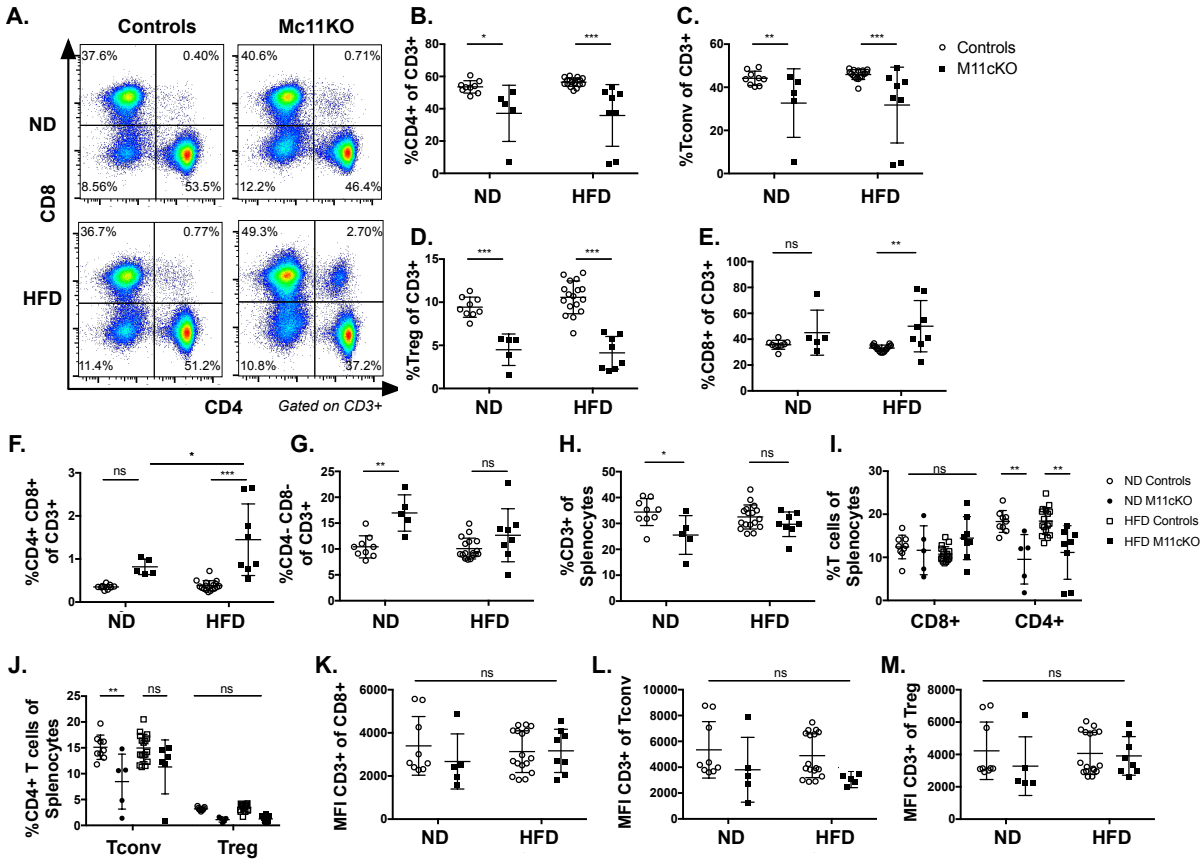


Figure 2-3 – M11cKO decreases CD4+ T cells in the spleen.

(A) Flow plots illustrating differences in CD4 and CD8 expression on CD3+ cells,
(B) Frequency of total CD4+ of CD3+ T cells,
(C) Frequency of FoxP3- CD4+ (Tconv_ of CD3+ T cells
(D) Frequency of FoxP3+ CD4+ (Treg) of CD3+ T cells
(E) Frequency of Cd8+ of CD3+ T cells,
(F) Frequency of CD4+ CD8+ double positive T cells (DPT) of CD3+ T cells,
(G) Frequency of CD4- CD8- double negative of CD3+ T cells,
(H) Frequency of CD3+ cells as a percentage of splenocytes
(I) Frequency of CD8+ and CD4+ T cells as percentage of splenocytes
(J) Frequency of Tconv and Treg Cd4+ T cells as a percentage of splenocytes,
(K) Median fluorescence intensity (MFI) of CD3 in Cd8+ cells,
(L) MFI of CD3 on Tconv cells,
(M) MFI of CD3 of Treg cells

*p < 0.05, **p < 0.01, ***p < 0.0001. n = 5-17 mice/group

ATDCs are more efficient antigen presenting cells than ATMs

Despite the ability of $Lyz2^{Cre}$ and $CD11c^{Cre}$ drivers to delete MHCII in both ATMs and ATDC, there are striking differences between T cell regulation in these mice as $Lyz2^{Cre}$ x $MHCII^{fl/fl}$ have normal number of $CD4^{+}$ cells on a normal diet. $Lyz2^{Cre}$ x $MHCII^{fl/fl}$ mice have residual expression of MHCII on ATDC, while M11cKO mice have residual expression of MHCII in ATMs (~12%). We wanted to assess whether these differences could be due to different antigen presentation capabilities of ATDCs and ATMs. We hypothesized that ATDCs are more potent APCs than ATMs to explain the significant loss of $CD4^{+}$ ATT cells in M11cKO mice. To test this, we assessed FACS sorted ATDCs, ATMs, $CD45^{+}$ $CD11c^{-}$ $CD64^{-}$ cells (containing B cells), and $CD45^{-}$ non-immune cells (used as a negative control). Sorted populations were used in an antigen specific reaction to assess their ability to induce splenic OTII T cell proliferation after OVA or BSA treatment. After 4 days of co-culture, $CD4^{+}$ cells were significantly increased in OVA pulsed ATDCs, but not other adipose tissue cell types (**Figure 2-4A**). ATDC and ATMs were able to induce Tregs when pulsed with OVA, however this was not seen with other leukocytes or $CD45^{-}$ cells (**Figure 2-4B**). In contrast, only ATDC were able to induce Tconv in response to OVA (**Figure 2-4C**). Overall, this suggests that differences in $Lyz2^{Cre}$ v $CD11c^{Cre}$ mediated MHCII-KO may be due to the efficiency of the residual population of ATDC or ATM ex- pressing MHCII in these models.

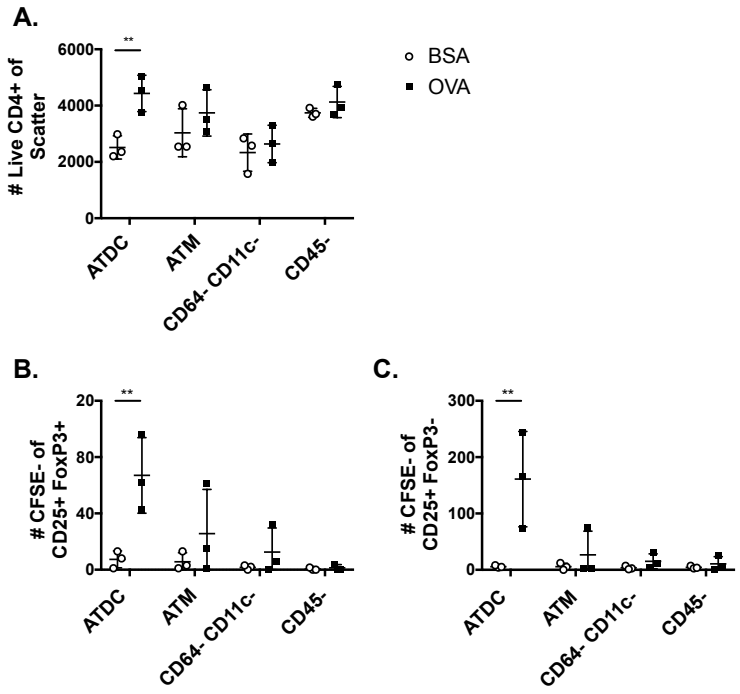


Figure 2-4 – ATDC are more potent antigen presenting cells than ATMs.

4 adipose tissue cell populations were FACS sorted from pooled WAT to assess their antigen presenting capacity. Cells were pulsed with OVA and co-cultured with CFSE stained CD3+ OT-II splenic T cells. CD45-, CD45+ CD64+ (ATM), CD45+ CD11c_ CD64- (ATDC) and CD45+ CD11c- CD64- populations were compared side-by-side.

(A) Number of Live CD4+ cells co-cultured with the APCs pulsed with BSA or OVA,

(B) Number of CFSE- CD25+ FoxP3+ (Treg) cells,

(C) Number of CFSE- CD25+ FoxP3- (Tconv) cells

*p < 0.05, **p < 0.01, *** < 0.0001. n = 3 biological replicates

Adipose tissue inflammatory gene expression is unchanged in M11cKO mice

We assessed pro-inflammatory cytokine gene expression in M11cKO mice. Despite reduced quantity of pro-inflammatory $CD11c^{+}$ ATMs in M11cKO mice, Tnfa expression trends towards an increase in control HFD fed eWAT, and gene expression was significantly increased in HFD fed M11cKO mice compared to ND controls (**Figure 2-5A**). Ccl2/Mcp1 expression was significantly increased in obese eWAT and did not differ between M11cKO and WT mice

(**Figure 2-5B**). T cell related cytokine genes were assessed. In lean M11cKO mice, there was increased expression of Ifng compared to controls. In obese mice, there was no significant difference in expression in M11cKO mice (**Figure 2-5C**). Il2 expression was significantly decreased between ND and HFD fed M11cKO groups (**Figure 2-5D**). Collectively this data suggests that M11cKO do not have significant alterations in inflammatory gene expression compared diet matched littermate controls.

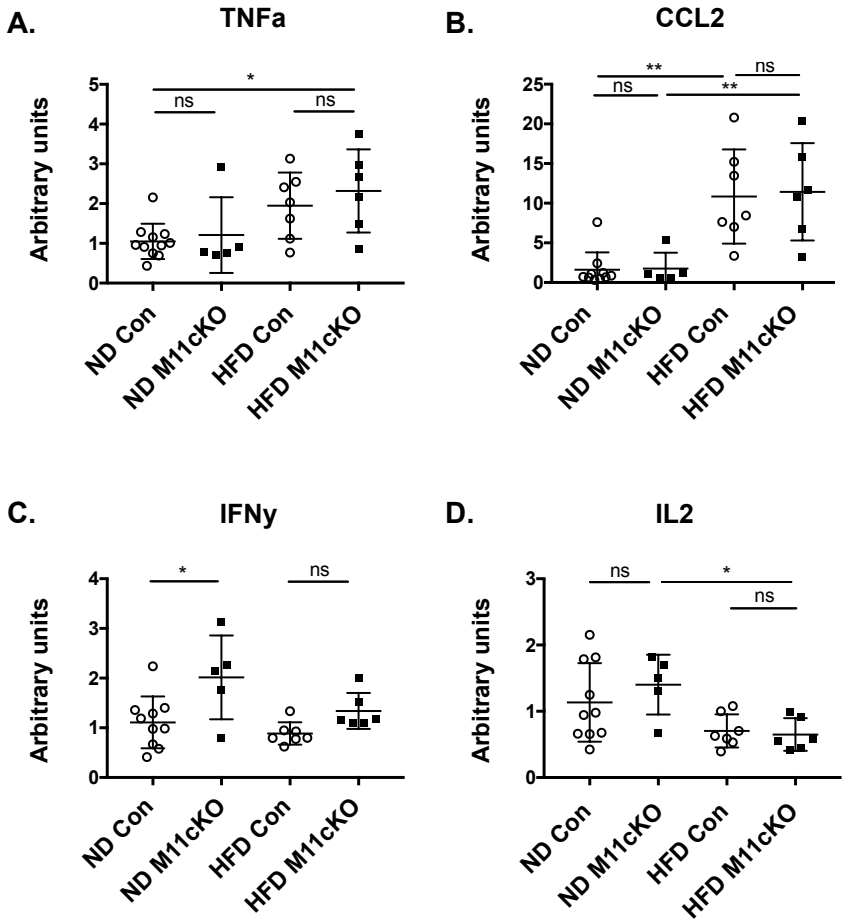


Figure 2-5 – M11cKO does not change inflammatory gene expression in eWAT

Gene expression of whole snap frozen eWAT as assessed by RT-qPCR. Calculations were performed by normalizing to housekeeping gene Arbp, using $2^{\Delta\Delta}$CT.

(A) TNFα, **(B)** MCP1/CCl2, **(C)** IFNγ, **(D)** IL2. *p < 0.05, **p < 0.01, ***p < 0.0001. n = 5-11 mice/group.

M11cKO mice have normal glucose and insulin tolerance

To determine whether antigen presentation by ATDCs influences systemic metabolism, we assessed glucose tolerance in ND and HFD fed mice. The ND fed controls and M11cKO mice showed no significant differences in GTT. With HFD feeding, both genotypes showed similar degrees of fasting hyperglycemia and glucose intolerance with similar area under the curve (AUC) measures (**Figure 2-6A**). Similar to the GTT, while HFD feeding induced insulin resistance based on ITT, there were no significant differences between M11cKO mice and controls (**Figure 2-6B**). Although metabolic parameters were not significantly different in M11cKO mice, frequency of CD11c + ATMs positively correlates with insulin resistance (**Supplemental Figure 2-3A**), but not glucose intolerance (**Supplemental Figure 2-3B**), of both control and M11cKO mice. Histologic analysis of eWAT suggested architectural differences between HFD fed M11cKO and controls. Analysis of adipocyte size distribution showed an increase in small adipocytes in M11cKO mice (**Figure 2-6C**). Although M11cKO mice had decreased lipid content in adipose tissue, triglyceride storage was not preferentially shuttled to the liver. HFD feeding increased liver triglyceride, but M11cKO did not significantly change triglyceride content per gram of tissue (**Figure 2-6D**). Collectively this data shows a mild protection from HFD induced obesity in M11cKO mice associated with decreased fat mass that does not alter glucose or insulin tolerance.

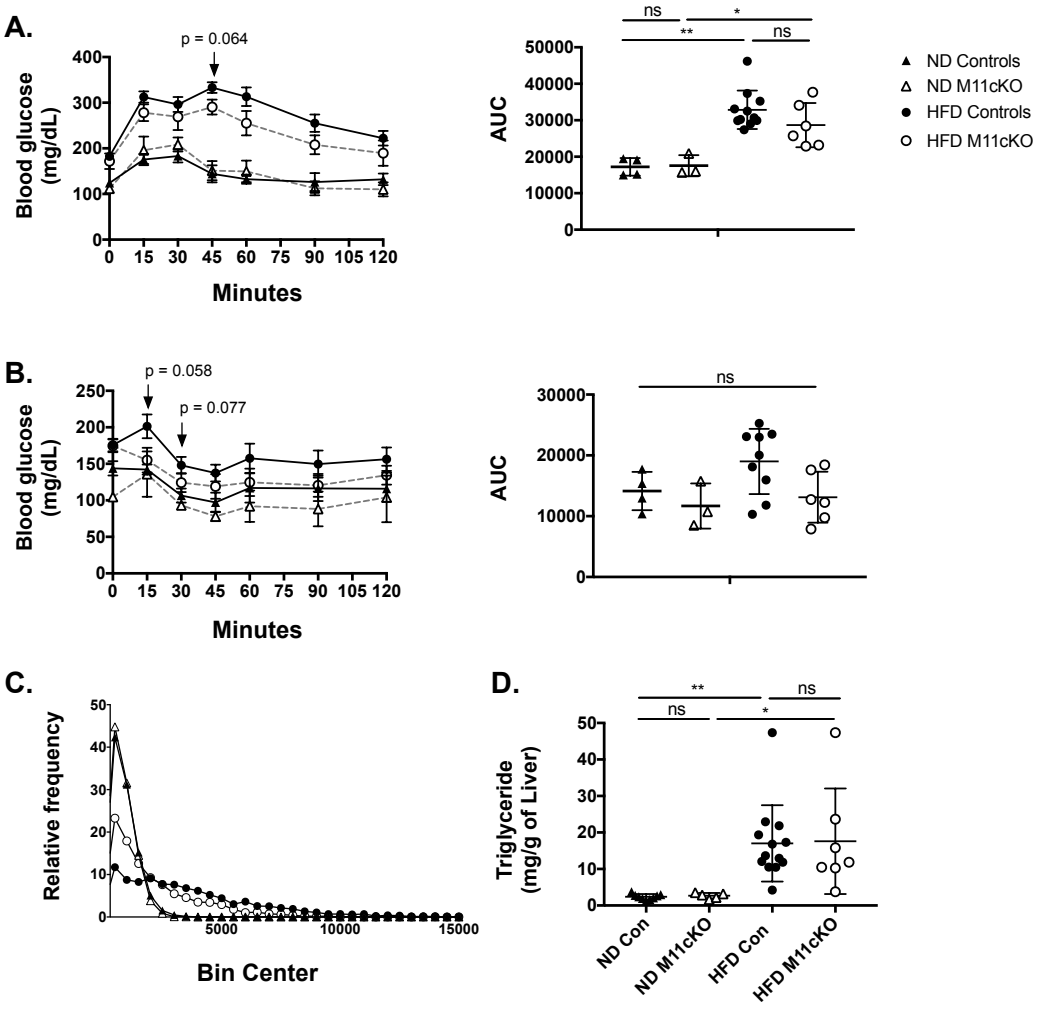
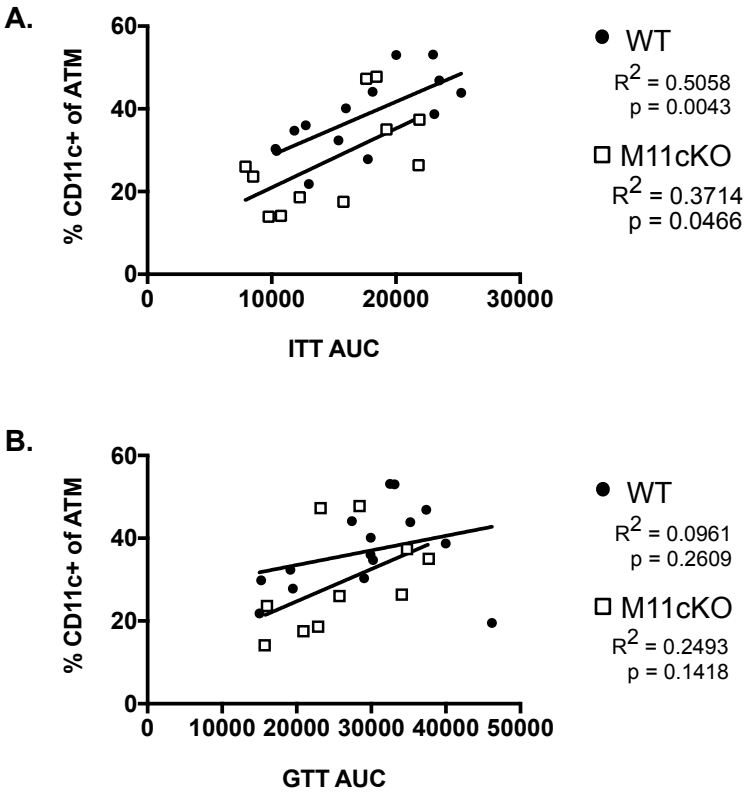


Figure 2-6 – M11cKO does not alter glucose or insulin tolerance

(A) Glucose tolerance test of mice at 12 weeks of age and area under the curve (AUC) quantifications,
(B) Insulin tolerance test of mice at 13 weeks of age and AUC quantifications
(C) Distribution of adipocytes circumferences, smaller bin size indicates smaller adipocyte size.
(D) Trigylceride content measured from snap frozen liver samples.

*p < 0.05, **p < 0.01, ***p < 0.0001. n = 3-9 mice/group



Supplemental Figure 2-3 – Additional data relates to Figure 2-6.

(A) Area under the curve (AUC) of insulin tolerance tests v ATM frequency in control and M11cKO groups,
(B) AUC of insulin tolerance tests v ATM frequency in control and M11cKO groups. R2 and p-values were calculated in linear regression analyses.

Discussion

This study has several primary findings relevant to the study of immunometabolism. ATDC are more potent APCs compared to ATMs and other non-leukocyte cells. ATDC derived MHCII signals were required for $CD4^+$ ATT cell maintenance as M11cKO mice had decreased $CD4^+$ cells, and more $CD8^+$ ATT cells in lean and obese settings. The dependency of $CD4^+$ cells on DC derived MHCII was much more prominent in adipose tissue compared to other lymphoid organs such as the spleen.

Similar to other studies, we observed that targeting myeloid cells with $CD11c^{Cre}$ mice expands beyond DC (114). $CD11c^{Cre}$ deleted MHCII expression on all ATDC and ~88% of $CD11c^+$ and $CD11c^-$ ATMs leaving a residual $MHCII^+$ population of ATMs. This suggests that Itgax is transiently expressed in ATMs or ATM precursors regardless of their CD11c surface expression at the time of harvest. This observation may also be related to "leaky" expression of the transgene. We note that use of another Itgax-cre-EGFP mouse (110) provided similar results of deletion of MHCII in both ATMs and ATDC (data not shown). In contrast to the M11cKO mice, $Lyz2^{Cre}$ targets all ATMs such that $Lyz2^{Cre}$ x $MHCII^{fl/fl}$ have a residual $MHCII^+$ population of ATDCs and result in significant phenotypic differences (80, 111).

While $Lyz2^{Cre}$ driven MHCII-KO resulted in no changes in T cells in lean mice and decreased Tconv in HFD fed mice, M11cKO led to different results (summarized in Table 2A). Most notably ATT populations were dysregulated in M11cKO mice independent of diet type. The absence of ATDC MHCII expression resulted in a reduction in $CD4^+$ Tconv and Tregs and

expansion of $CD8^+$ cytotoxic T cells and $CD4^+$ $CD8^+$ DPT cells. An increase in $CD8^+$ and DPT cells was adipose tissue specific in M11cKO mice. In this setting, the small number of $MHCII^+$ ATMs was not sufficient to maintain normal $CD4^+$ ATT cell numbers. In the spleen, we observed what would be considered canonical T cell dysregulation in the absence of DC mediated antigen presentation, as there was a decrease in Tconv $CD4^+$ cells. Since Tconv cell maintenance requires low affinity "tonic" TCR and MHCII interactions, loss of antigen presentation by dendritic cells may have driven this decrease in the $CD4^+$ T helper cell population (115). In addition, there was a significantly reduced expression of the TCR in $CD4^+$ ATT cells in HFD-fed M11cKO mice. TCR expression is dynamically regulated to tune inflammatory capacity of T cells. Since TCR expression regulates T cell functionality, it is possible that although the Tconv and Treg cells in HFD M11cKO are still present, that they are functionally impaired and unable to mount a typical inflammatory response (116). CD3/TCR downregulation is observed in T cells after cognate pMHCII activation in acute/subacute models of antigen dependent inflammation (117-121). Our observation that obesity decreases CD3/TCR expression in ATT cells in KO mice suggest that either there is an increase in pMHCII signals from other cells in AT or that our chronic model results in a downregulation of the TCR due to a lack of tonic signaling. We have examined residual $MHCII^+$ cells in the KO mice and have not observed an increase in surface MHCII expression in any cell type. Therefore, we hypothesize that in the absence of MHCII, CD3/TCR downregulation is due to a loss of tonic signals from APCs in adipose. Expansion of $CD8^+$ ATT cells with MHCII manipulation is consistent with other reports. MHCII-KO leads to an influx of $CD8^+$ T cells into tumors of cancer-laden mice, leading to increased inflammation and smaller tumor burden (122). Although it is unknown

whether the increase in $CD8^+$ T cells in M11cKO adipose tissue is due to recruitment due to adipose tissue secreted chemoattractants, the increased skewing of ATT towards $CD8^+$ production compared to spleen suggest that local ATDC dependent signals generate this observation. In addition to work showing the majority of ATTs are of the T resident memory phenotype, work has also shown that tissue resident lymphocytes often have unique characteristics (123, 124)

The appearance of ATT DPT cells was also an unusual and intriguing finding. DPTs are usually only found in the thymus during T cell development, but DPT cells appear in several dysregulated autoimmune and chronic inflammatory diseases (125). The significant increase in this population seems to be an ATT specific adaptation to loss of MHCII expression by ATDCs. Since the presence of DPT was not significantly increased globally in M11cKO mice, we speculate that $CD8^+$ T cells may have some plasticity allowing them to assume a Tconv phenotype. This could be a compensatory mechanism that occurred due to decreased TCR expression on Tconv and Treg ATTs. The dependence of $CD4^+$ ATT cells on MHCII expression in all ATDC (M11cKO) but not all ATMs ($Lyz2^{Cre}$ x MHCIIfl/fl) suggested that ATDC are stronger APC than ATMs or other potential APCs in adipose tissue. Our studies in FACS sorted population's show that ATDCs are superior at inducing activation and proliferation of T cells compared to ATMs and other $CD11c^-$ $CD64^-$ leukocytes containing B cells. It is unclear if MHCII expression on B cells in adipose tissue may play a role in ATT maintenance or activation and should be considered in future studies. Some studies have suggested that preadipocytes express MHCII and have downstream effects on ATTs (107), however we did not see evidence for this in our functional assay using $CD45^-$ cells. Since these studies were performed with

splenic OTII T cells, further studies need to be performed assessing ATT specific characteristics in lean and obese adipose tissue.

Like $Lyz2^{Cre}$ x $MHCII^{fl/fl}$ mice, M11cKO mice have a reduction in pro-inflammatory $CD11c^{+}$ ATMs in HFD fed mice compared to the controls, which would be suggestive of improved metabolic inflammation in adipose tissue. Despite this, glucose and insulin tolerance was not altered in M11cKO mice, while $Lyz2^{Cre}$ x MHCIIfl/fl mice had improved metabolism. This may be related to the sustained increase in $CD8^{+}$ ATT in M11cKO mice, which have been shown to participate in obesity-induced adipose tissue inflammation (97). Indeed, analysis of inflammatory gene expression in eWAT showed that M11cKO mice had similar levels of Tnfa, Ccl2, and Il2 expression. In fact, Tnfa and Ifng were higher in M11cKO mice than controls, which could be mediated by the increased $CD8^{+}$ ATT cells.

The body weights of HFD-fed M11cKO mice were significantly lower than the diet-matched littermate controls. Body weight discrepancy results in smaller fat pad mass in the M11cKO mice, but not spleen or liver weights. Liver triglyceride content is not significantly different in M11cKO mice compared to their diet matched controls, indicating that lipid storage capacity of adipocytes is not dysregulated. Similar to whole body MHCII-KO mice (80, 111), lean mass of M11cKO mice may be decreased compared to controls. Regardless of the net adipose tissue weight, the fat mass is not significantly smaller when it is normalized to body weight. This suggests that net adipose tissue weight may not be a clear predictor of metabolism or adipose tissue inflammation. There are several possible explanations for the different metabolic phenotypes observed in the $Lyz2^{Cre}$ v $CD11c^{Cre}$ driven MHCII knockouts. It has been shown that inhibiting dendritic cell mediated antigen presentation in the gut induces

changes in the microbiome and associated inflammation (113). Consequently, even if there is attenuated inflammation in the adipose tissue, chronic inflammation from the gut could lead to sustained cytokine release and systemic inflammation, and subsequent metabolic disorder. MHCII may contribute to processes like beta-oxidation, lipolysis, and mitochondrial production, however it is likely that impairment of nutrient absorption via gut inflammation may underlie the weight differences.

Overall diet independent and adipose tissue specific changes in M11cKO mice give insight into the importance of ATDC mediated antigen presentation signals. $CD8^+$ and DPT ATT cells increase in ND and HFD fed M11cKO mice. However, $CD11c^+$ ATMs are decreased and TCR expression is decreased on $CD4^+$ ATTs in HFD fed obese M11cKO mice. These cellular changes did not significantly improve the inflammatory output of obese adipose tissue and also did not improve glucose or insulin tolerance. M11cKO shows that ATM and ATDCs have cell type specific regulation of ATTs and resultant inflammatory and metabolic phenotype of obese mice.

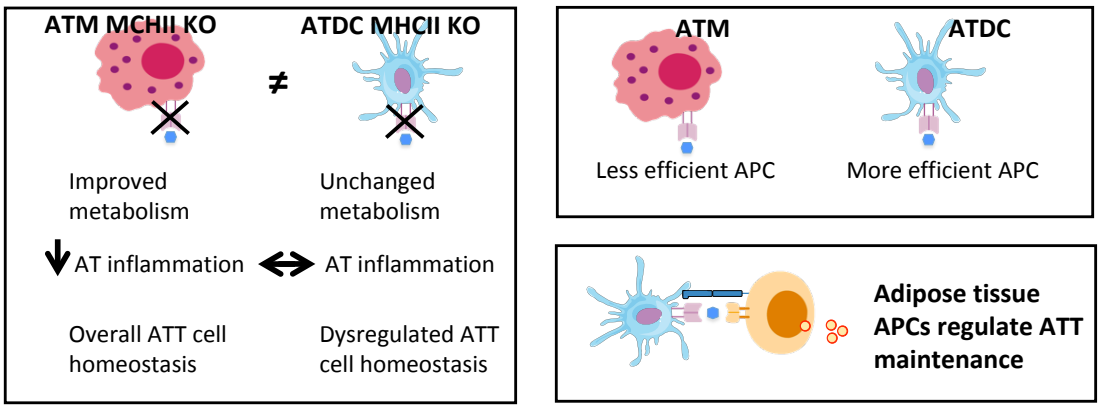


Figure 2-7 – Chapter 2 conclusions

Chapter 3: Adipose Tissue T Cells Have Impaired Inflammatory Capacity in Obese Mice and Humans

This chapter is under revision at JCI Insight:

Porsche CE, DelProposto JL, Geletka L, O'Rourke RO, Lumeng CN. Adipose tissue T cells have impaired inflammatory capacity in obese mice and humans.

Author Contributions: CEP performed experiments for each figure shown in this chapter. JLB helped perform mouse sacs needed for ATT activation assays and flow cytometry. LG extracted cells from human adipose tissue. ROO facilitated human sample acquisition. CEP and CNL conceived of experiments, wrote, and edited the manuscript. MDRC Chemistry Core performed luminex assays, and the Cancer Center Immunology Core performed ELISA assays.

Abstract

Adipose tissue induced chronic low-grade inflammation is a critical link between obesity and insulin resistance. Despite studies implicating adipose tissue T cells (ATT) in the initiation and persistence of adipose tissue inflammation, fundamental gaps in knowledge regarding ATT function impedes progress towards understanding their role in diabetes pathogenesis and complications. Little is known about the antigens that activate ATT cells, functional differences between fat depots, or effector cytokine profiles of activated ATT populations in obesity. We hypothesized ATT activation and function would be dependent upon anatomic location and that high fat diet induced obesity would potentiate their inflammatory properties. We assessed ATT activation and inflammatory potential in mouse and human stromal vascular fraction (SVF). Surprisingly, ATTs from obese epididymal white adipose tissue (eWAT) had senescent characteristics. Stimulation failed to induce CD25 upregulation, ATT proliferation, or heightened

IFNγ and IL-2 secretion, which is seen in spleen, omentum, and lean eWAT samples. Human ATTs collected from bariatric surgery patients also have impaired inflammatory activation correlating with increased HbA1c levels. Both environmental and cell-intrinsic factors are implicated in ATT dysfunction. Co-culture of obese SVF in transwell plates inhibits ATT activation of lean samples. This suggests soluble factors from obese SVF diminish the inflammatory potential of ATTs. Signaling through the T cell receptor is necessary for induction of ATT impairment, but is independent of upregulated PD1 expression on the cell surface. In sum, this work suggests that obesity-induced ATT impairment is dependent upon localized signals from soluble factors and cell-to-cell interactions in adipose tissue, which blunt signaling pathways proximally downstream of the T cell receptor.

Introduction

Obesity-associated morbidities such as type II diabetes are characterized by insulin resistance, which is mechanistically linked to adipose tissue dysfunction and inflammation (54-56). Clinical and pre-clinical studies have identified chronic low-grade inflammation as a critical link between obesity and insulin resistance (57). Immune regulation and systemic metabolism are interconnected within white adipose tissue. Lean fat has a largely anti-inflammatory immune environment containing a predominance of resident adipose tissue macrophages (ATM) with anti-inflammatory properties and $CD4^+$ T regulatory cells (Treg) (96). However, as excess lipid storage capacity is needed and adipocyte hypertrophy occurs, qualitative and quantitative changes in immune cells also arise. Obese diabetic individuals have a predominance of activated macrophages, pro-inflammatory $CD4^+$ T conventional (Tconv) cells, and an influx of $CD8^+$ cytotoxic T cells in their adipose tissue, accompanied by systemic metabolic dysfunction (72, 97, 126).

Conventional views of adipose tissue T cell (ATT) functionality indicate that they contribute to chronic low-grade inflammation and insulin resistance in obese subjects. Initial studies assessing ATTs in obese adipose tissue observed improved insulin sensitivity with the administration of neutralizing $CD3^+$ antibody injection (67). The functions of cytotoxic $CD8^+$, $CD4^+$ Tconv, and T regulatory (Treg) ATTs have also been shown to play a critical role in adipose tissue inflammation. $CD8^+$ cytotoxic ATTs are required for the development of insulin resistance and infiltrate adipose tissue of high-fat diet (HFD)-fed mice before macrophages (97). Antigen-presenting cells in obese adipose tissue have also been implicated in Tconv induced inflammation, which can be reversed with conditional major histocompatability complex II

(MHCII) knockout in ATMs but not adipose tissue dendritic cells (80, 127). Largely, ATTs with anti-inflammatory phenotypes (Treg, Th2) decrease (96, 128), and ATTs with pro-inflammatory phenotypes increase ($CD8^+$ and Th1/$Tbet^+$ Tconv) in murine and human obese adipose tissue (72, 97, 128). Tregs comprise around 40% of $CD4^+$ ATTs in lean tissue, 2-3x more than is seen in lymphatic tissues (96). Adipose tissue Tregs have tissue-specific characteristics and depend upon the master regulator of lipid metabolism, PPARγ (75). Additionally, they have a distinct clonal T cell receptor (TCR) repertoire, which suggests they are maintained by antigens in lean adipose tissue (69). Tconv also have tissue-specific characteristics. Lean fat contains Tconv with a predominantly Trm phenotype (123). As HFD is given, effector memory adipose tissue Tconv cells with restricted TCR repertoire accumulate, suggesting recognition of antigens specific to obese fat (99). Strikingly, the TCR repertoire in lean and obese adipose tissue is distinct. However, the antigens regulating Tregs and Tconv in lean and obese tissue remain unknown.

More recent studies call into question the activation state and function of Tconv cells in obese adipose tissue. Obese adipose tissue contains more $PD1^+$ T cells, which have been described as having senescent characteristics (98). Despite impaired IL2 and IFNγ production, $PD1^+$ ATTs secrete increased osteopontin, contributing to inflammation independent of classical T cell activation and inflammation. However, understanding the disruption of canonical Th1 T cell functionality in obese individuals is equally important. Lymphatic T cells in obese subjects have less potent inflammatory responses, making viral infections such as influenza and COVID-19 more dangerous in individuals with metabolic dysfunction (25, 26). Fewer $CD69^+$ $granzyme^+$ cytotoxic T cells and $CD69^+$ $IFN\gamma^+$ T helper cells were found in obese individuals receiving influenza immunizations compared to lean controls (25). Additionally, preclinical and clinical studies highlight that obesity results in more severe outcomes to influenza infection (28, 129).

More clarification regarding tissue specific functionality of ATTs is needed to understand how they contribute to chronic low-grade inflammation and may provide insight to mechanisms governing global T cell responses in obese patients.

Studies have also been limited by a primary focus on murine white adipose tissue (eWAT) with limited studies assessing functional characteristics of human ATTs. Omental white adipose tissue (oWAT) is the prominent visceral fat depot in humans, residing in the peritoneum and covering most abdominal organs (130, 131). In addition to lipid storage, the oWAT plays a substantial role in immunologic surveillance of the peritoneum (132). ATT and B cells are enriched within oWAT and aggregate in structures called fat associated lymphoid clusters (FALCs; milky spots), which can process and induce an immune response against antigens (133). Understanding how type II diabetes influences human ATT function is of interest for both obesity-induced diabetes and response to foreign peritoneal antigens.

Fundamental gaps in knowledge regarding tissue-specific characteristics of $CD4^+$ Tconv and $CD8^+$ adipose tissue T cell (ATT) function impedes progress towards understanding their role in diabetes. Many gaps remain in our understanding of how adipose tissue T cells (ATT) function relates to insulin resistance. We do not know tissue-specific characteristics of ATTs in mice or humans, including which cytokines they secrete and how they proliferate when they receive an activating stimulus. How obesity influences ATT inflammatory potential has not yet been fully elucidated. We sought to answer these questions and hypothesized that HFD feeding would potentiate inflammatory potential in ATTs. However, our findings indicate that obesity induces impairment of ATT inflammatory potential in obese mice and diabetic humans.

Materials and Methods

Animal Studies

C57BL/6J (000664), B6.BKS(D)-Lepr^{db}/J (000697), and MHCII$^{fl/fl}$ (B6.129X1-H2-Ab1^{tm1Koni}/J) x *LysM*^{Cre} (B6.129P2-Lyz2^{tm1cre1fo}/J) mice were obtained from the Jackson Laboratory. MHCII$^{fl/fl}$ x *Lzy2*^{Cre} (MMKO) mice were generated by breeding *Lzy2*^{Cre} with MHCII$^{fl/fl}$ mice. Cre-negative littermates were used as controls. Male mice were fed ad libitum either a normal diet (ND; LabDiet PicoLab 5L0D 4.09kcal/gm 29.8% protein, 13.4% fat, 56.7% carbohydrate) or a high fat diet (HFD; Research Diets D12492, 5.24kcal/gm 20% protein, 60% fat, 20% carbohydrate) beginning at 6 weeks of age. All mouse procedures were approved by the University Committee on Use and Care of Animals at the University of Michigan and were conducted in compliance with the Institute of Laboratory Animal Research Guide for the Care and Use of Laboratory Animals.

Isolation of Murine Adipose Tissue SVF and Flow Cytometry Analysis

The stromal vascular fraction (SVF) was isolated from whole adipose tissue as previously described (111). Briefly, adipose tissue depots were dissected and weighed. Tissue was then mechanically disrupted by mincing, and chemically digested by rocking tissue in 1mg/ml collagenase IV (Sigma Aldrich) at 37°C for 30 mins. Cells were then quenched with RPMI + FBS media and filtered through 100nm mesh prior to RBC lysis and subsequent filtering with 70nm mesh filters.

Cells were incubated in Fc Block for 5 minutes on ice before staining with indicated antibodies for 30 minutes at 4°C.

α-mouse antibodies used: AF488-CD4 [100423], APCcy7-CD8 [100713], Brilliant Violet 605-CD279 (PD1) [135219], PE/Cy7-CD28 [102125], APC-TCR-b [109211] from Biolegend, PerCpcy5.5-CD3 [45-0031-82] APC-CD25 [17-0251-82], PE-FoxP3 [12-4771-82], PEcy7-Ki67 [25-5698-82] from eBioscience, and Live/dead Fixable Dead Cell Violet Stain Kit [L34955] from Invitrogen.

Stained cells were washed twice with FACS buffer and fixed for intracellular staining using a FoxP3 transcription kit (BD Biosciences). Analysis was performed on an LSR Fortessa Flow Cytometer and analyzed with Flow Jo software (Treestar).

Human samples

Visceral adipose tissue was collected from male bariatric surgery patients with Institutional Review Board approval (HUM00074075) from the University of Michigan and Ann Arbor Veterans Administration Hospital. Tissue was finely minced using surgical scissors (DR Instruments, Bridgeview, IL USA; 4SB), then digested in 3mg/ml collagenase II (Life Technologies (Thermo Fisher), Waltham, MA USA; 17101015) for 30 minutes. Digested tissue was then processed in the same manner as digested murine adipose tissue to obtain single-cell suspensions of SVF.

Cells were incubated in Fc Block for 5 minutes on ice before staining with indicated antibodies for 30 minutes at 4°C.

α-human antibodies used: AF488-CD4 [317419], PerCPcy5.5-CD3 [300429], APC-CD25 [356109], APCcy7-CD8 [344713], APC-PD1 [329907], PE-FoxP3 [320107] from Biolegend, and Live/Dead Fixable Dead Cell Violet Stain Kit [L34955] from Invitrogen.

Adipose tissue T cell Activation Assay

SVF was extracted from murine eWAT or human oWAT as described in the two previous subsections. Splenocytes were also collected and isolated by crushing and rinsing through 70um mesh. SVF and splenocytes were treated with red blood cell lysis buffer (NH4Cl in Tris-HCl buffer) for 5 mins at room temperature, and then quenched with complete RPMI. Cells were counted and $4x10^5$ SVF or splenocytes were plated in 96 well round-bottom plates and rested overnight. The next morning, αCD3/CD28 dynabeads (Life Technologies) were added to the cultures at a 1:4 bead to cell ratio (as determined by titration experiments). No cytokines or growth factors were added to the media to polarize T cells or stimulate cells otherwise. After 3 days of culture in complete RPMI medium with dynabeads, 96 well plates were spun down at 450xg for 7 minutes. Supernatants were aspirated and used for Luminex or ELISA analysis. Cells and dynabeads were collected in FACS buffer ($PBS^{-/-}$ + 0.5%BSA) and transferred to FACS tubes, which were inserted into EasySep magnets. Cells were transferred into new tubes, while magnetic beads were selectively removed using the magnet. Cells were then washed with 1x PBS before staining with viability dye and extracellular FACS antibodies. Cells were then washed and stained/fixed with intracellular markers as described above.

PMA/Ionomycin T cell activation

Splenocytes and SVF were purified from spleen and eWAT and resuspended in a single cell suspension. Cells were plated at $4x10^5$ cells/ well in a 96 well round-bottom plate and rested overnight. The next morning, 10ug/ml of Brefeldin A was added to cultures to block protein secretion from the Golgi apparatus. After 1 hour, SVF was stimulated with 10ng/ml of PMA and 250ng/ml of Ionomycin. After stimulation for 4 hours, cells were collected, washed, and stained

for flow cytometry to assess live ATT subpopulations and intracellular IFNγ (Alexa Fluor 647 IFN-γ [505814[from Biolegend).

Cytokine Analysis

Luminex assays were performed on supernatants collected after 3 days of culture. Murine samples were analyzed with EMD Millipore's Milliplex MCYTOMAG-70k kit. Human samples were analyzed using EMD Millipore's Milliplex HCYTOMAG-60k kit. Subsequent cytokine secretion assays were performed on IL2, IFNγ, and MCP1 using ELISAs performed in the University of Michigan's Cancer Center Immunology Core.

In-vivo PD1 Blockade

At 6 weeks of age, C57Bl/6J mice were fed ND or HFD for 18 weeks to induce ATT impairment. At 24 weeks of age, mice were given injections of α-mouse PD-1 (CD279) Clone RMP1-14 (BioXCell), or IgG2a isotype control, anti-trinitrophenol, Clone 2A3 (BioXCell) at $10mgkg^{-1}$ every third day for 4 total injections. The day after the last injection, systemic metabolism was assessed by glucose tolerance test (GTT). After re-feeding and resting overnight, mice were sacrificed and a fraction of fresh SVF was stained for flow cytometry analysis to assess basal T cell differences and ensure the PD1 antibody entered adipose tissue and blocked PD1. The following antibodies were used: (AF488-CD4 [100423] and APCcy7-CD8 [100713] from Biolegend, PerCPcy5.5-CD3 [45-0031-82], PE-FoxP3 [12-4771-82], and Alex Fluor 647 anti-rat IgG2a [407511] from eBioscience. The remaining SVF was used for an ATT activation assay (described above) to assess whether PD1 blockade restored ATT inflammatory potential.

Metabolic Evaluations

Glucose tolerance tests (GTT) were performed after a 6-hour fast. Mice were injected IP with D-glucose (0.7g/kg) and blood glucose concentrations (mg/dL) were measured at 0, 15, 30, 45, 60, 90, and 120 mins after injection from tail nick with a glucometer.

Transwell ATT Activation Assays for Soluble Factor Analysis

To test whether soluble factors in HFD eWAT SVF contribute to ATT impairment, co-culture experiments in transwell plates were performed. ND SVF was isolated and plated in the bottom of transwell plates. 96-well 0.4um pore transwell chambers were seeded with ND or HFD SVF. Bottom wells and top chambers were cultured separately O/N at 37°C. The next day, upper chambers were co-cultured with ND SVF for 24 hours prior to αCD3/CD28 dynabeads stimulation ND SVF. Cells were stimulated and kept in co-culture for 3 days, at which point supernatants and cells were taken for ATT activation analysis as described above.

Statistical Analysis

All values are reported as mean $\pm$ SEM. Differences between groups were determined using unpaired, two-tailed Students t Test or two-way ANOVA with Tukey post hoc tests with Graph Pad Prism 5 software. P values less that 0.05 were considered significant.

Results

ATT activation and inflammatory capacity is decreased with obesity

Previous limitations in assessments of ATT function are related to poor viability of ATTs in-vitro and the small number of cells that can be collected from a single mouse (134). To overcome these limitations, we devised a culture system where all stromal vascular cells from epididymal white adipose tissue (eWAT) were collected and cultured. ATTs were assessed within this heterogeneous fraction. This paradigm allowed for flow analysis of ATTs following *in-vitro* experimentation and retention of environmental signals from other leukocytes, pre-adipocytes, and endothelial cells. After 3 days of culture with or without αCD3/CD28 dynabeads, supernatants were collected and cells were assessed by flow cytometry (**Figure 3-1A**). Since Tconv and cytotoxic $CD8^+$ T cells upregulate CD25 after receiving stimulus from an antigen-presenting cell (86), IL-2Ra (CD25) frequency (**Figure 3-1B**) and MFI (**Supplementary figures 3-1A and 3-1B**) was measured on Tconv, $CD8^+$, and Treg cells from spleen and eWAT from mice fed normal diet (ND) or HFD for 18 weeks. We noted significant differences in T cell activation depending upon the tissue of origin and diet consumed. Tconv and cytotoxic $CD8^+$ T cells from the spleen both increased frequency and MFI of CD25 when stimulated with dynabeads, as expected. HFD feeding resulted in more robust CD25 expression on $CD8^+$ T cells when activated compared to stimulated ND control splenic T cells. However, ATTs had marked differences in their capacity for stimulation. Unstimulated ATTs had higher basal levels of CD25 expression and dynabead stimulation failed to induce CD25 of ND ATT cells to the same capacity as those in the splenocyte cultures. Additionally, ATTs from HFD fed mice are resistant to CD25 upregulation. Unlike Tconv and CD8+ T cells, CD25 expression is constitutively

expressed on Tregs, as described previously (135, 136). However, CD25 expression was decreased on HFD eWAT Tregs independent of stimulation. Ki67 expression measured by frequency (**Figure 3-1C**) and MFI (**Supplemental Figures 3-1C and 3-1D**) in Tconv and $CD8^+$ T cells were also dependent upon tissue of origin. In bead stimulated splenocyte cultures, Ki67 was significantly increased in Tconv and $CD8^+$ cells independent of diet type. However, ATTs were resistant to Ki67 induction in $CD8^+$, Tconv, and Tregs in response to dynabead stimulus. Total live T cell numbers in cultures also indicated significant differences in proliferative capacity of splenic and adipose tissue T cells (**Supplemental Figures 3-1E and 3-1F**). While splenic T cell numbers significantly increased with TCR stimulus, cell numbers of ATTs present in in-vivo cultures remain unchanged with or without dynabead stimulation.

Cytokine secretion data also indicated impaired HFD ATT inflammatory response to TCR stimulation (**Figure 3-1D**). Supernatants from ATT cell activation assays were collected for assessment by multiplex Luminex assays. T cell effector cytokines were secreted following similar trends as CD25 upregulation. In splenocyte fractions, TCR stimulation significantly increased cytokine secretion, and HFD feeding enhanced effector T cell inflammatory cytokine release of IIL2, IFNy, IL-17, and IL4 (**Figure 3-1E**). However, HFD has the opposite effect on ATT inflammatory cytokine secretion. Unlike ND SVF, which induces a significant increase of Th1, Th2, and Th17 cytokine release with dynabead stimulation, HFD ATTs fail to induce the same level of cytokine secretion in HFD SVF fractions. ND Rag1KO SVF was used for ATT activation assays to ensure dynabead stimulus wasn't inducing effector T cell cytokines in the absence of ATTs (**Supplemental Figure 3-1G**). Overall, this data shows that obesity induced by

18 weeks of HFD feeding impairs murine eWAT T cell activation and T cell cytokine production, but has minimal effects on splenic T cells function.

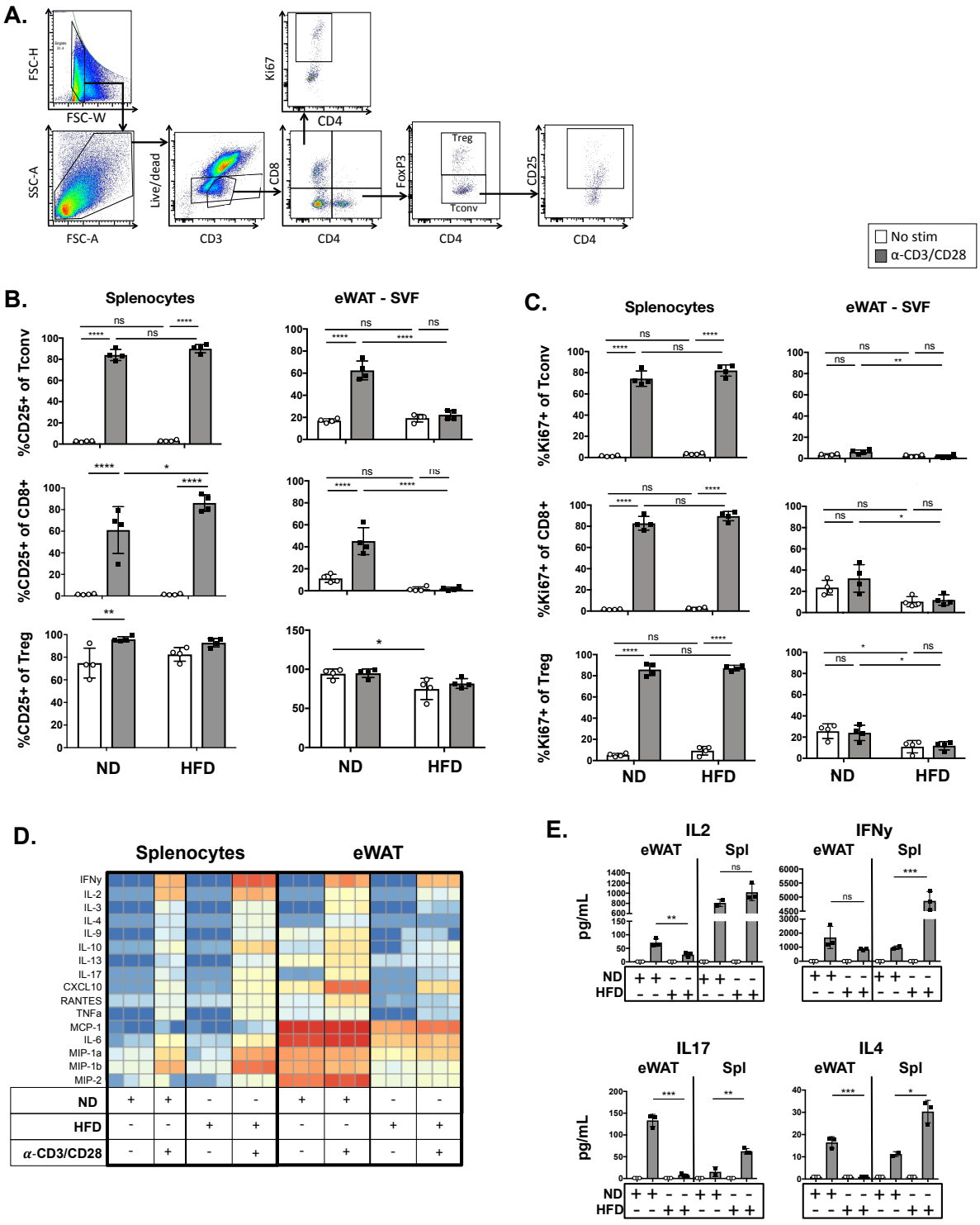
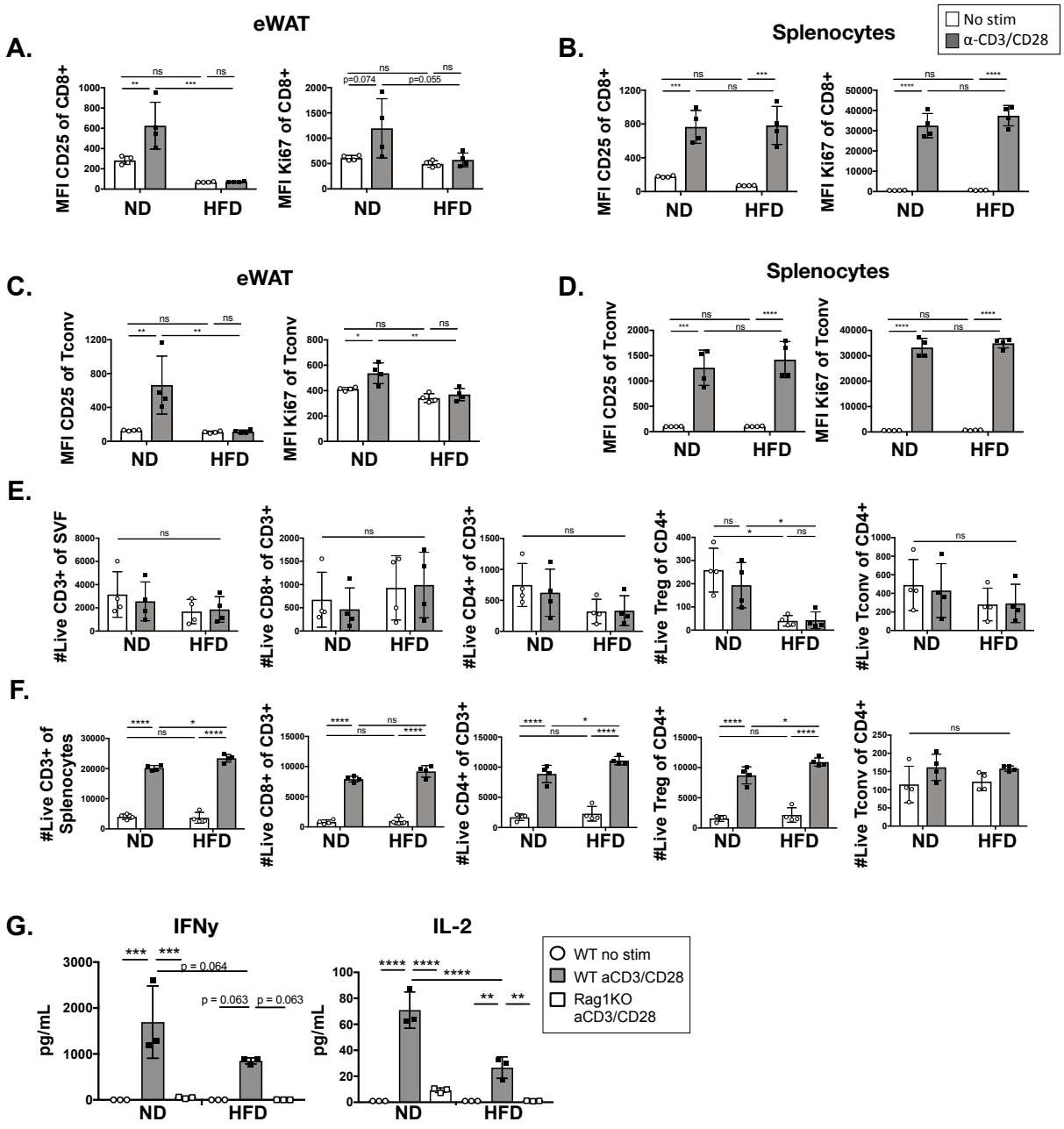


Figure 3-1 – ATT activation capacity is suppressed with HFD feeding.

(A) Flow cytometry-gating strategy used for ATT activation assays. Representative plot shows eWAT SVF after 3 days of co-culture with αCD3/CD28 dynabeads.

(B) Frequency of CD25 expression on Tconv, CD8+, and Tregs after ATT activation assays. T cells from splenocytes and eWAT SVF cultures taken from ND and HFD-fed mice after 18 weeks of feeding.
(C) Frequency of Ki67 expression on Tconv, CD8+, and Tregs from splenocytes and eWAT after T cell activation assays. Cells fractions were assessed with or without αCD3/CD28 dynabead co-culture for 3 days.
(D) Luminex assessment of supernatants taken from T cell activation cultures after 3 days.
(E) Luminex data represented by bar graphs to show cytokines secretion of Th1, Th2, and Th17 specific cytokines.



Supplemental Figure 3-1 – Additional data for ATT activation assays.

(A) MFI of CD25,
(B) MFI of Ki67 in CD8+ and Tconv ATT cells from eWAT after ATT activation assays.
(C) MFI of CD25,
(D) MFI of Ki67 on CD8+ and Tconv cells from splenocytes after ATT activation assays,
(E) Number of eWAT cells,
(F) Number of spenocytes seen in Live CD3+, total CD4+, Tconv, Treg, and CD8+ subpopulations in ATT activation assays.
(G) Frequency of live cells in ATT activation cultures from eWAT and splenocytes,

(H) IFNγ and IL2 secretion from WT v Rag1KO SVF with αCD3/CD28 dynabead stimulation
(I) Bar graphs depicting heat map data in Figure 3-1D.

ATT activation potential is decreased in diabetic humans

Increased Th1 polarized $CD4^+$ ATTs have been reported in obese diabetic humans (128). However, our murine culture system indicates ATTs from obese diabetic tissue have functionally impaired inflammatory properties. Therefore, we assessed human ATTs using omentum biopsies from BMI matched bariatric surgery patients (**Table 3A**). HbA1c levels were used to classify patients as non-diabetic (NDM; <5.7) or diabetic (DM; >6.5). ATT activation and inflammatory potential were then measured using the same ATT activation assay used for murine cells. We observed decreased CD25+ upregulation in DM ATTs after 3 days of stimulation with αCD3/CD28 dynabeads (**Figure 3-2A**). T cell specific inflammatory cytokine release was also significantly lower in cells taken from DM patients. Both IL2 and IFNγ were significantly increased in culture supernatant from stimulated NDM ATTs, but ATTs from DM human samples were unable to secrete these cytokines to the same degree (**Figure 3-2B**). However, MCP1, a myeloid derived cytokine was not significantly different. We performed a Luminex assay to broadly assess effector cytokines from DM v NDM human SVF cultures (**Figure 3-2C**). With ATT simulation, SVF cells from DM humans had a diminished capacity to secrete pro-inflammatory effector T cell cytokines compared to obese NDM controls. Overall, ATTs from DM visceral human adipose tissue have an impaired inflammatory phenotype upon TCR stimulation similar to obese diabetic mice.

Table 3A – Bariatric Surgery Enrollment Data

	NDM (average)	DM (average)	t Test
# / Sex	7/males	3/males	P = N/A
Age	51.1	58.7	P = 0.04
BMI	44.2	55	P = 0.133
HbA1c	5.75	8.23	$P = 5.29 \times 10^{-6}$
Blood glucose	94.0	165.3	P = 0.0004
Pre-op Cholesterol	155.4	169.3	P = 0.613
HDL	39.00	47.67	P = 0.673
LDL	89.00	82.67	P = 0.839

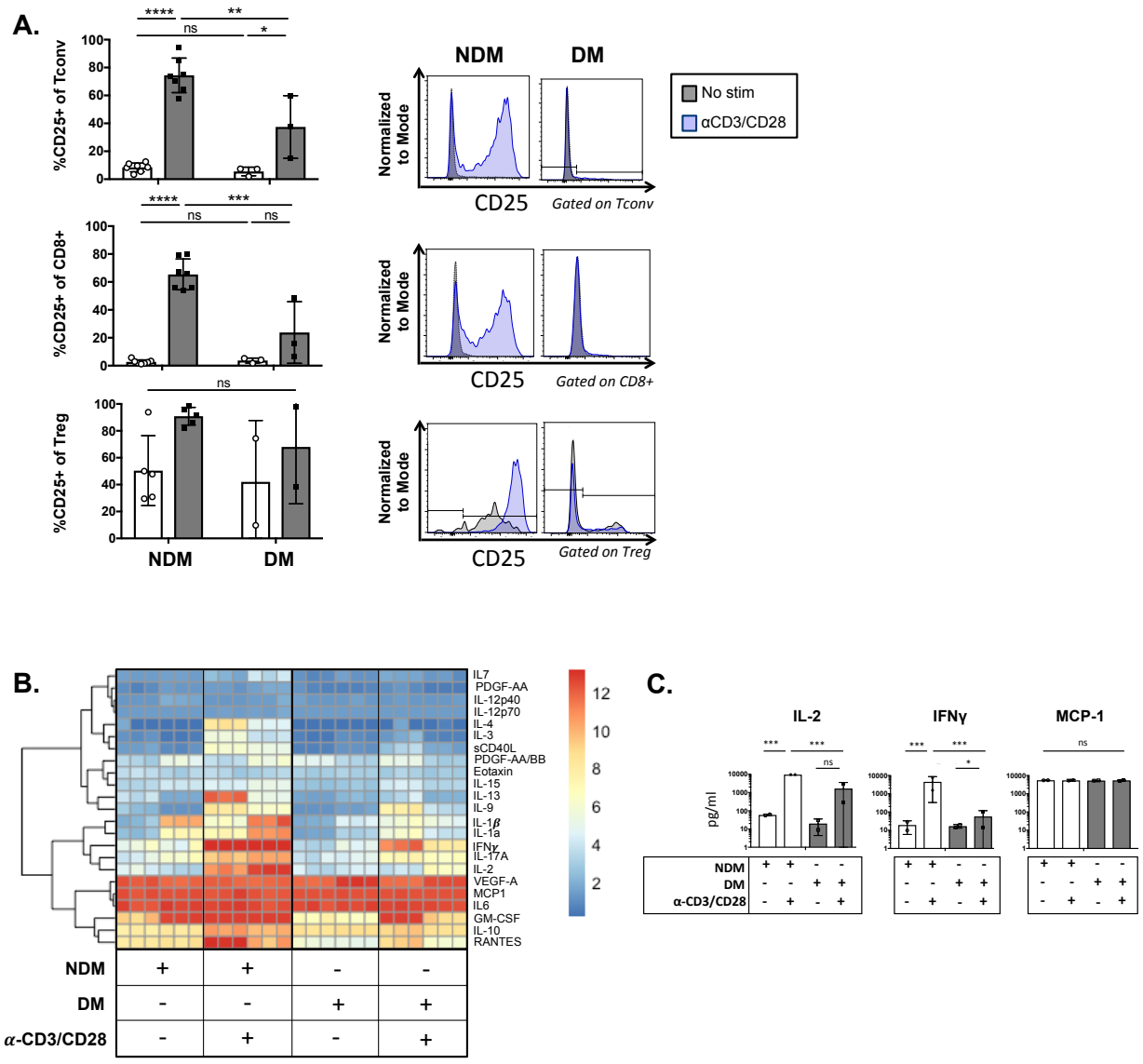


Figure 3-2 – Human ATT activation capacity is impaired in diabetic patients.

(A) Frequency of CD25 expression of NDM and DM human oWAT ATTs after activation assays with αCD3/CD28 dynabeads.

(B) Luminex assessment of supernatants taken from ATT activation cultures. N = 2 biological replicates/group, 3 technical replicates.

(C) IL2, IFNγ, and MCP1 cytokines in culture supernatants from human ATT activation assays.

*p < 0.05, **p < 0.01, *** < 0.0001

ATT dysfunction measured by CD25 expression and cytokine secretion is independently regulated with chronic HFD feeding.

Since diabetic mice and humans have ATTs that are unable to elicit robust inflammatory responses with TCR activation, we examined the kinetics of ATT dysfunction over the course of 18 weeks of HFD feeding. ATT activation assays were used to assess CD25 expression and cytokine secretion from mice fed HFD for 1, 6, 12, and 18 weeks beginning at 6 weeks of age. Following 1 week of HFD feeding, CD25 expression was induced in HFD Tconv (**Figure 3-3A, Supplemental Figure 3-2A**) and $CD8^+$ ATT (**Figure 3-3B, Supplemental Figure 3-2B**) to a similar capacity as age-matched ND controls. After 6 weeks of HFD feeding, basal CD25 expression was increased compared to other time points. Although CD25 was increased in ND and HFD ATTs after stimulation, the difference in expression between untreated and TCR stimulated groups becomes less pronounced. By 12 weeks of HFD feeding, CD25 induction failed to occur after TCR stimulation, and the same trend persisted through 18 weeks. The impairments in ATT cytokine secretion did not correlate with CD25 expression over the time course. IL2 and IFNγ were measured as indicators of effector T cell cytokine secretion, and MCP1 were evaluated as a control for innate immune cell function (**Figure 3-3C, Supplemental Figure 3-2C and 3-2D**). IL2 and IFNγ secretion was impaired at 6 weeks of HFD feeding compared to age-matched stimulated controls and persisted through the later time points. MCP1 secretion was not significantly changed based on diet or stimulus, as expected. In sum, ATT dysfunction was not observed with acute HFD feeding and requires an extended period of HFD feeding.

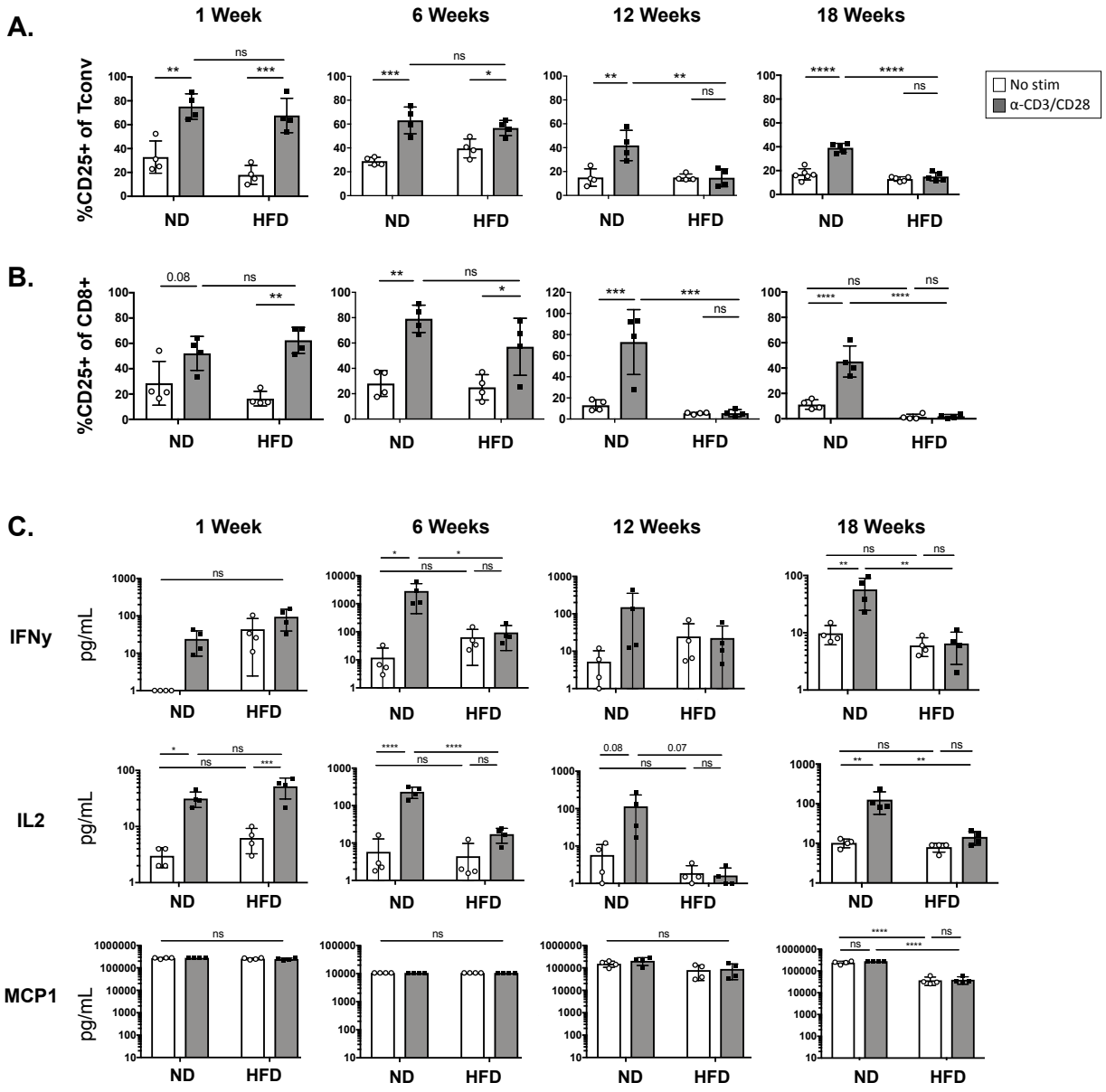


Figure 3-3 – Kinetics of ATT dysfunction

(A) Frequency of CD25 expression on eWAT Tconv and

(B) Frequency of CD25 expression on eWAT CD8+ T cells after ATT activation assays. Assays were performed after 1, 6,12, and 18 weeks of HFD-feeding.

(C) IL2, IFNγ, and MCP1 concentration in supernatants of ATT activation assays at 1, 6, 12, and 18 weeks of HFD feeding.

*p < 0.05, **p < 0.01, ***p < 0.0001. n = 4 biological replicates/group

Macrophage MHCII signals are required for ATT activation impairment

We hypothesized that impairment in TCR signaling pathways may explain impaired T cell activation capacity with chronic HFD feeding. First, Phorbol 12-myristate 13-acetate (PMA)/Ionomycin stimulation was used to activate ATTs independent of the TCR. In contrast to αCD3/CD28 stimulation, PMA/Ionomycin efficiently induced IFNγ production in HFD $CD8^+$ and Tconv ATTs (**Figure 3-4A**). HFD $CD8^+$ ATTs had significantly greater IFNγ production than stimulated ND ATTs. PMA/Ionomycin stimulation experiments suggest that HFD ATTs maintain their ability to produce pro-inflammatory cytokines when activated independent of the TCR. To test whether chronic TCR stimulation is necessary for ATT impairment (much like exhausted T cells in tumor microenvironments), we examined ATTs from $MHCII^{flfl}$ xLysMCre (MMKO) mice where MHCII is absent on a majority of macrophages and dendritic cells in adipose tissue (**Supplemental Figure 3-3A**) (80). MMKO mice and WT controls were placed on ND or HFD diet for 12 weeks before analysis of T cell activation (**Figure 3-4B**). HFD fed MMKO mice gained significantly less weight than controls, and have decreased proinflammatory ATMs (**Supplemental Figures 3-4B and 3-4C**). While MMKO mice ATTs from ND mice were activated to a similar capacity in WT and MMKO cultures based on CD25 expression, IL2, and IFNγ secretion. However, HFD induced impairment of CD25, IL-2, and IFNγ induction was absent in MMKO mice. Macrophage MHCII deletion seemed to only impact adipose tissue, as splenic T cell inflammatory capacity was unchanged in ND or HFD fed mice (**Figure 3-4C**). Overall, this data suggests that ATT impairment in HFD-fed mice requires insulin resistance, reaching a significant weight gain, or macrophage-derived MHCII signals.

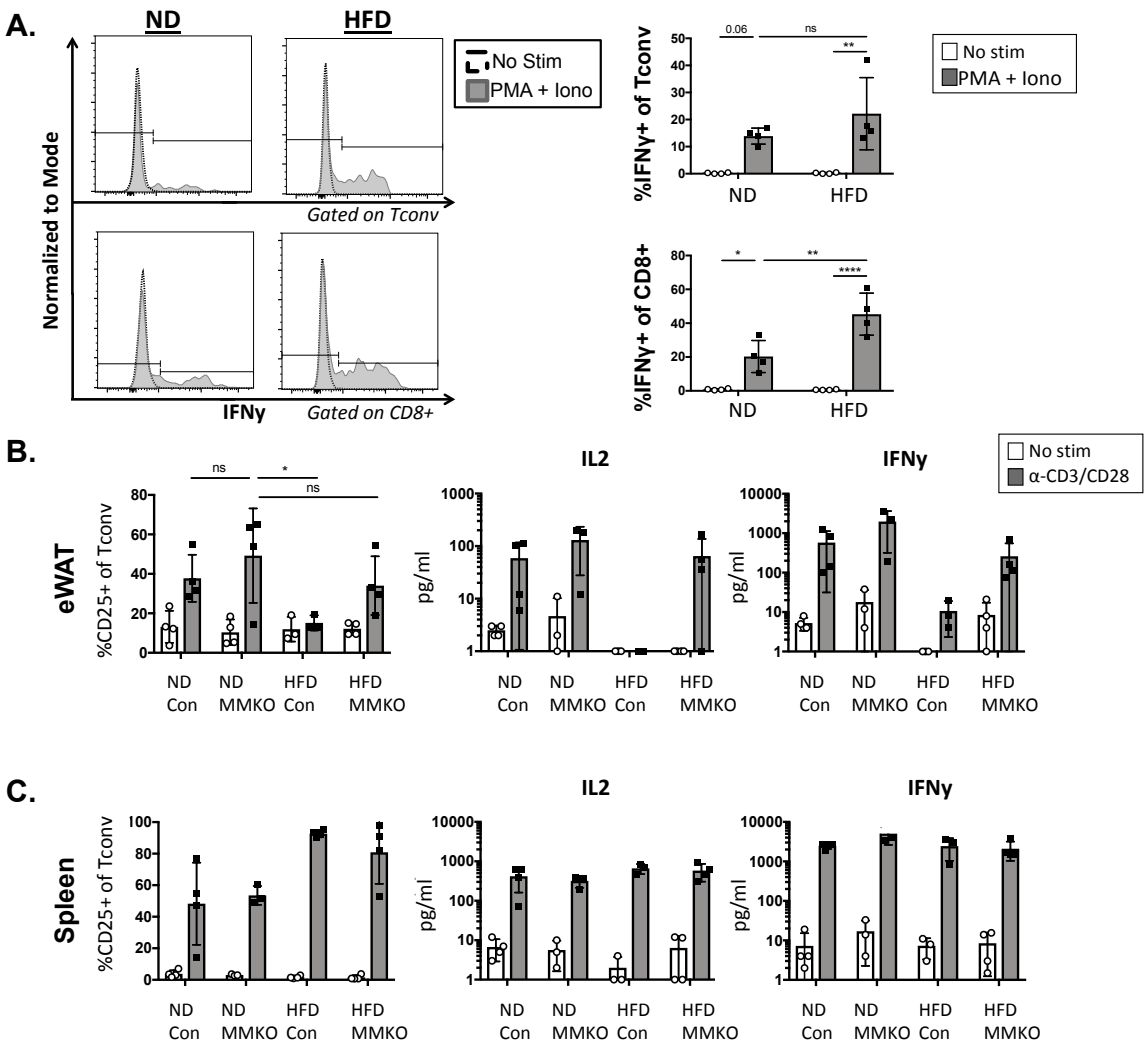


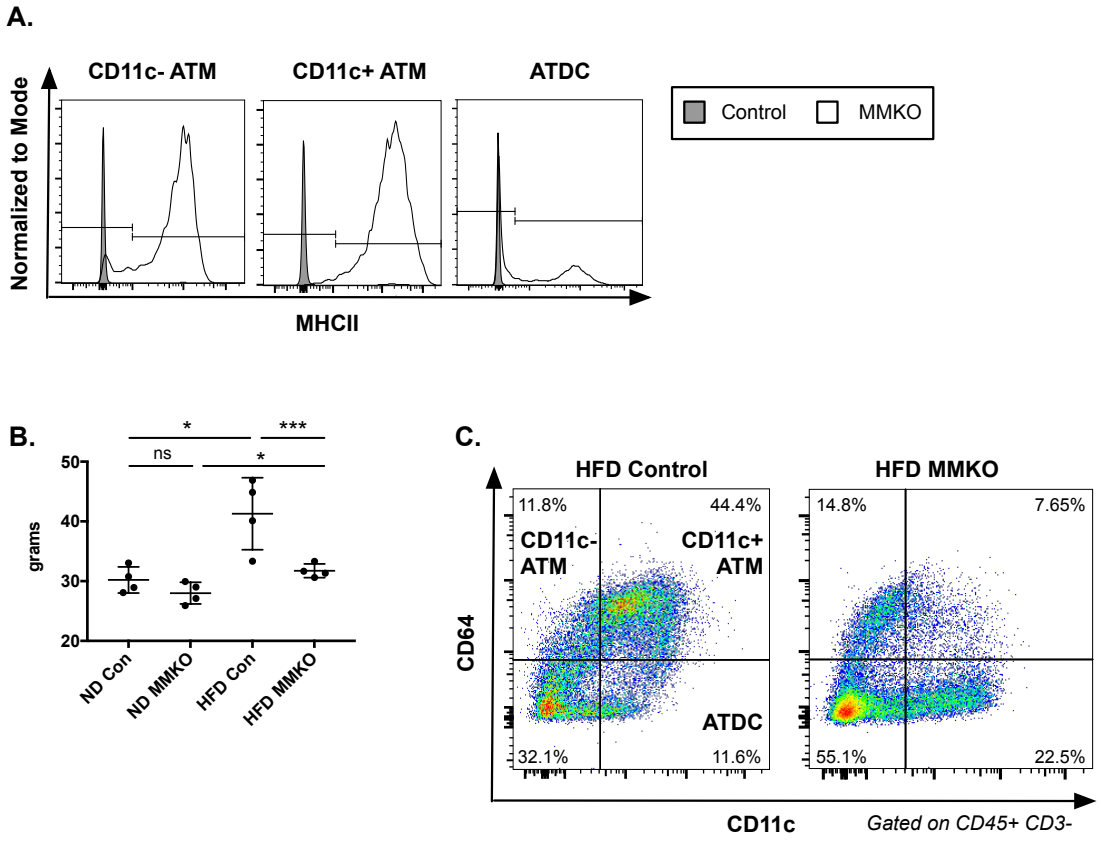
Figure 3-4 – TCR signaling is required for obesity induced ATT dysfunction

(A) Intracellular IFNγ in Tconv and CD8+ ATT cells after stimulation with PMA and Ionomycin for 4 hours. HFD was administered for 12 weeks. Representative histograms are shown on the left, and biological replicates shown on the right.

(B) LsyMCre x MHCIIfl/fl (MMKO) mice and age-matched littermate controls were fed ND or HFD for 12 weeks and used for ATT activation assays.

(C) MMKO splenocytes assessed for cell surface CD25 expression and supernatants for Il2 and IFNγ concenrations after 3 days of αCD3/CD28 dynabead stimulation.

*p < 0.05, **p < 0.01, ***p < 0.0001. n = 3-4 biological replicates/group



Supplemental Figure 3-2 – Metabolic and immunologic phenotype of HFD MMKO mice

(A) Flow plots illustrating MCHII knockout in the primary antigen presenting cells in HFD adipose tissue

(B) Body weights of MMKO mice

(C) Flow plots illustrating antigen presenting cell subsets in HFD MMKO adipose tissue after 12 weeks.

*p < 0.05, **p < 0.01, ***p < 0.0001. n = 3-4 biological replicates/group

T cell receptor and co-activating receptors are differentially expressed in obese mice and humans

Given the impairment in obese ATT inflammatory capacity in the presence chronic peptide-MHCII, we assessed the whether lack of TCR and co-activating receptors expression was responsible. Median fluorescence intensity of TCRβ and co-activating receptor CD28 was measured by flow cytometry on freshly extracted eWAT and splenic Tconv and $CD8^+$ T cells (**Figure 3-5A**). In mice fed HFD diet for 12 weeks, TCRβ was more highly expressed on Tconv and $CD8^+$ cells in eWAT, and CD28 was more highly expressed in eWAT Tconv. However, TCRβ was not changed by HFD feeding in splenic T cells and CD28 was significantly decreased on splenic $CD8^+$ cells. TCR co-inhibitory receptors were assessed by flow cytometry including CTLA-4 on eWAT ATTs (**Supplemental Figure 3-4A**) and splenic T cells (**Supplemental Figure 3-4B**). Although eWAT ATTs had higher CTLA-4 expression compared to splenic T cells, expression did not change with HFD feeding. PD1 was more highly expressed on eWAT ATTs than splenic T cells, but the expression on splenic T cells and ATT Tconv cells was not changed by HFD feeding (**Figure 3-5B**). However, PD1 was significantly increased in HFD $CD8^+$ ATTs. PD1 expression was also assessed on ATTs extracted from human oWAT (**Figure 3-5C**). Even though the frequency of PD1 expressing ATTs from human oWAT was substantial, it was not correlated with HbA1c or diabetic status. Overall, these results suggest that loss of TCR and co-activating receptor expression is not the mechanism of ATT impairment with obesity.

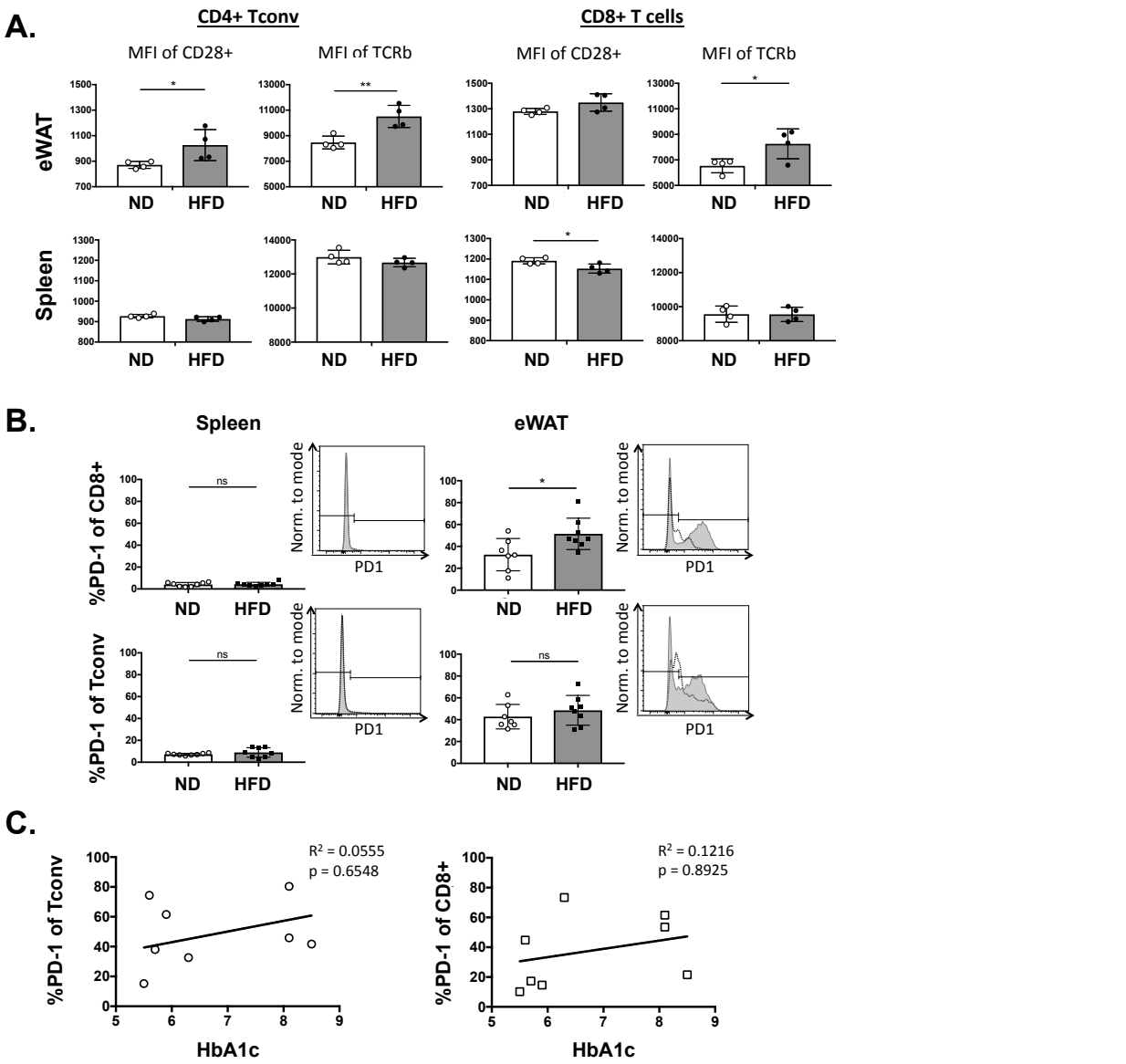


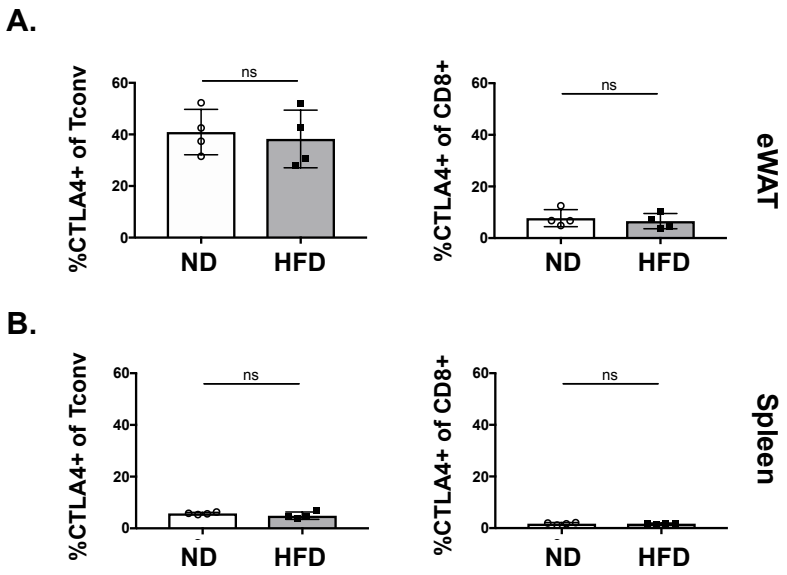
Figure 3-5 – T cell receptor and co-receptor components are differentially expressed in obese mice.

Surface receptor expression was assessed on mice fed HFD for 12 weeks and age matched ND controls.

(A) CD28 and TCRβ expression on freshly isolated Tconv and CD8+ T cells. n = 4 biological replicates/group

(B) Frequency of PD1 expression on CD8+ T cells and Tconv from freshly isolated eWAT and splenocytes. Representative histograms of PD1 expression are shown on the right of each group. n = 7-8 biological replicates/group

(C) Correlation of PD1 expression on Tconv and CD8+ ATTs taken from human omentum and HbA1c. n = 3-7 biological replicates/group



Supplemental Figure 3-3 – CTLA4 expression on eWAT and splenic T cells

(A) Frequency of CTLA-4 expression on freshly isolated Tconv and CD8+ eWAT ATTs and,
(B) Spleen, measured by flow cytometry.

***In-vivo* PD1 blockade fails to reverse ATT impairment or induce metabolic changes in obese mice.**

PD1 is highly expressed in eWAT ATTs, PDL1 is highly expressed on adipocytes (137), and HFD significantly increased PD1 expression in $CD8^+$ cytotoxic T cells. Therefore we performed an in-vivo PD1 blockade to evaluate the hypothesis that PD1 expression was required for impaired ATT inflammatory capacity with obesity. Obese mice (18 weeks of HFD) were treated with a PD1 blocking regimen or IgG2a control for 2 weeks (**Figure 3-6A**). PD1 blockade did not induce significant difference in body or organ weights (**Supplemental Figures 3-5A**). Glucose tolerance was assessed and obese mice had elevated fasting glucose levels and exhibited glucose intolerance (**Figure 3-6B**). However, PD1 blockade did not significantly change glucose tolerance compared to diet matched IgG2a controls. At the terminal endpoint, eWAT derived SVF and splenocytes were isolated and fractions were taken to assess basal T cell composition

and binding of αPD1 to ATTs (**Supplemental Figure 3-5B**). PD1 antibody injections did not alter frequencies of Tconv, Treg, or $CD8^+$ ATTs (**Supplemental Figure 3-5C**). However, the PD1 blocking antibody penetrated into adipose tissue and effectively bound to ATT cells. ATT activation assays were also performed to assess the inflammatory potential of cells after the PD1 blockade. CD25 expression was increased in stimulated ND eWAT $CD8^+$ ATT that received PD1 blockade compared to stimulated isotype control. The same increase in CD25 expressing cells were not seen in ND Tconv populations. More importantly, PD1 blockade did not rescue CD25 expression/upregulation with dynabead stimulation on HFD $CD8^+$ or Tconv cells (**Figure 3-6C**). However, PD1 blockade increased CD25 expression on stimulated ND and HFD splenic $CD8^+$ T cells, and Tconv from ND mice (**Figure 3-6D**). Since CD25 and effector T cell cytokine regulation seems to be regulated independently, we also assessed cytokine secretion in culture supernatants. IL2 and IFNγ secretion were not rescued in stimulated ATTs from HFD mice given αPD1. PD1 blockade also did not increase cytokine secretion in activated ATT cells from ND mice (**Figure 3-6E**). Additionally, PD1 blockade didn't increase IL2 or IFNγ secretion in ND or HFD splenic T cells (**Figure 3-6F**). Overall, PD1 blockade can effectively penetrate adipose tissue and bind to PD1 expressing ATT cells. However, PD1 blockade did not rescue inflammatory impairment of ATT cells in obese mice.

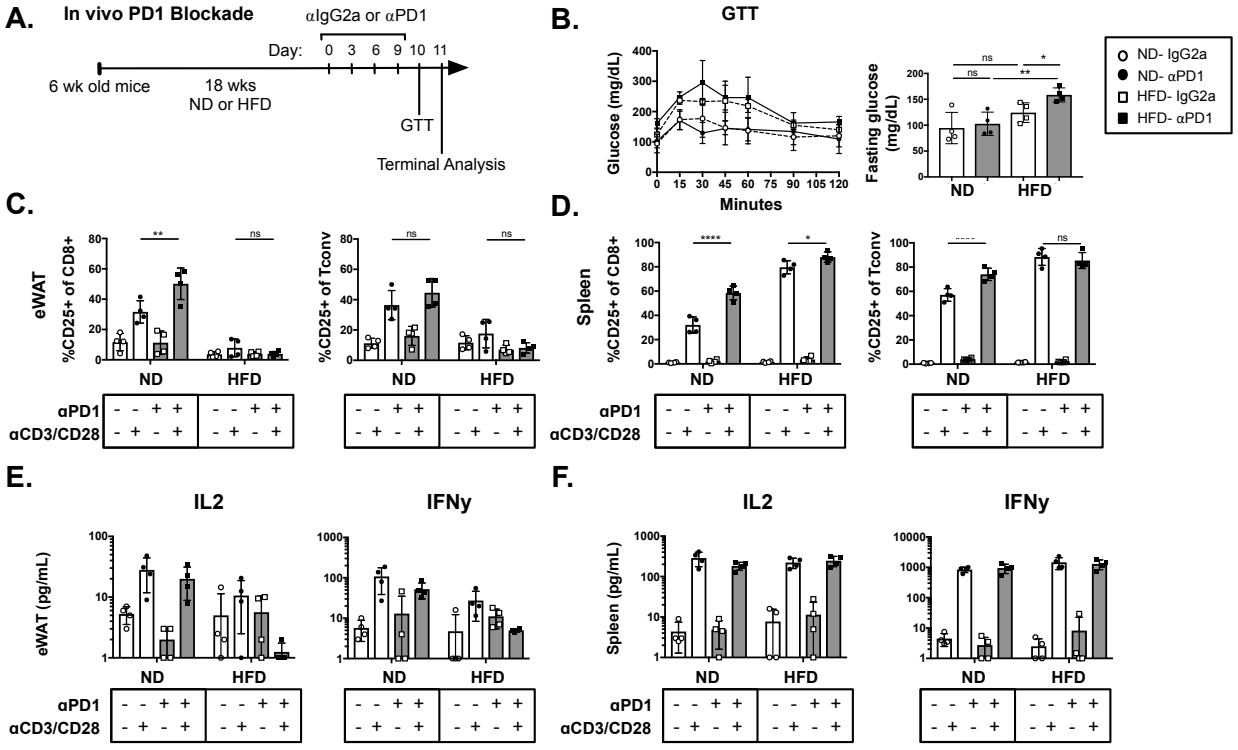


Figure 3-6 – PD1 blockade does not reverse ATT impairment after 18 weeks of HFD feeding.

(A) Illustration of *in-vitro* PD1 blockade timeline and interventions.

(B) Glucose tolerance test of ND and HFD-fed mice after administration of αPD1 or IgG2a isotype infections (left) and glucose levels after fasting for 6 hours (right).

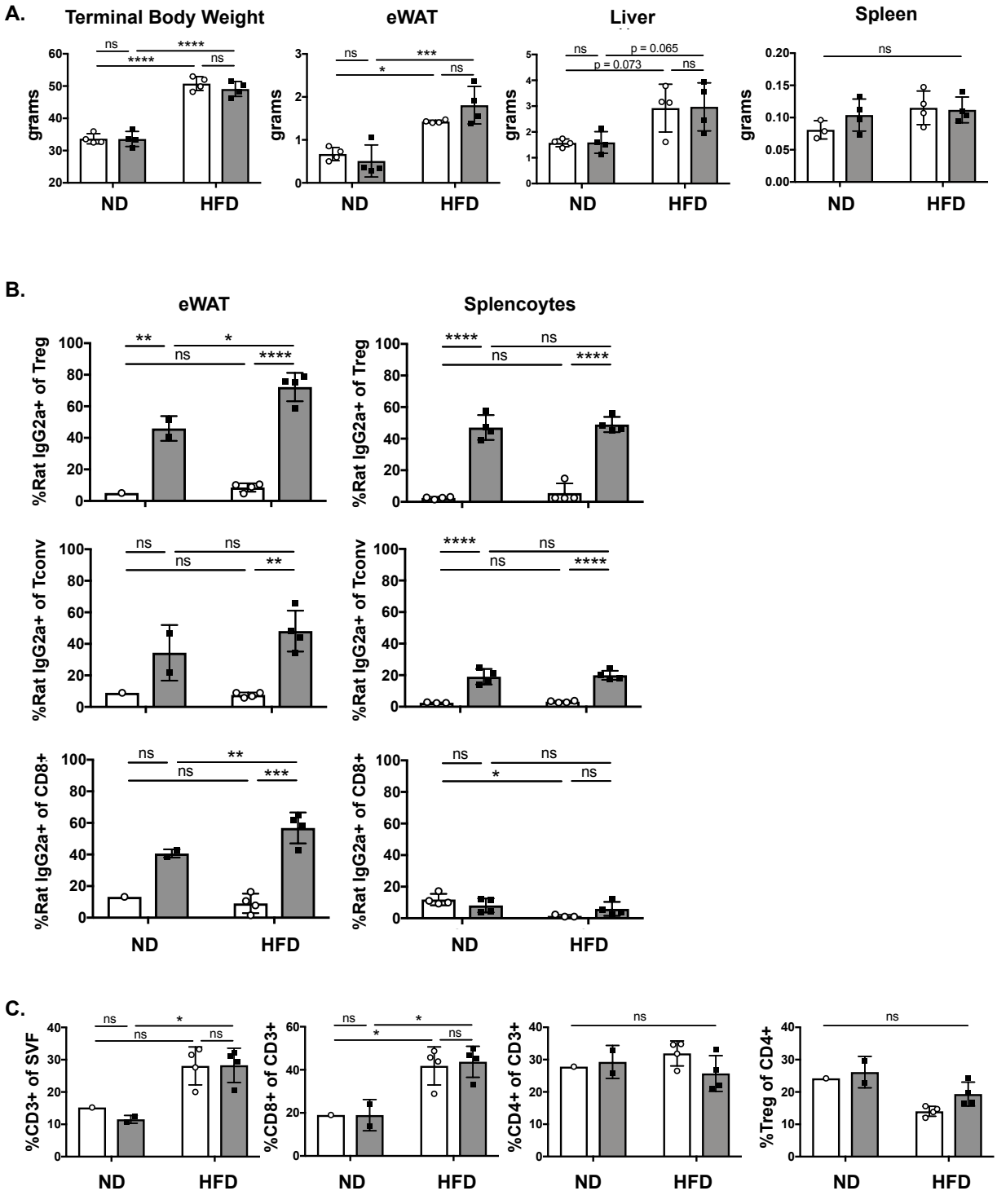
(C) ATT Activation assays were performed on eWAT SVF and,

(D) Splenocytes after PD1 blockade regimen. Frequency of CD25 expression on CD8+ T cells and Tconv measured 3 days after dynabead stimulation.

(E) IL2 and IFNγ concentrations in culture supernatants from PD1 blockade ATT activation assays of eWAT SVF and,

(F) Splenocytes.

*p < 0.05, **p < 0.01, ***p < 0.0001. n = 3-4 biological replicates/group



Supplemental Figure 3-4 – PD1 blockade phenotyping data of mice fed HFD for 18 weeks.

(A) Terminal body and organ weights of PD1 blockade mice after IgG2a or PD1 antibody injections.
(B) Flow cytometric analysis of fresh non-cultured ATT cells after PD1 blockade.
(C) Flow cytometric measurement of T cells bound by Rat IgG2a (*in-vivo* PD1 antibody).

Soluble factors from obese eWAT impair ATT inflammatory capacity

To test whether HFD eWAT SVF contains soluble factors that decrease ATT inflammatory potential, transwell co-culture studies were employed. ND eWAT SVF was cultured in the bottom wells, while the upper chambers were filled with media only, ND eWAT SVF, or HFD eWAT SVF at 1:1 cell ratio and co-cultured for 24 hours before stimulation of the ND SVF with aCD3/CD28 dynabeads for 3 days. Co-culture with ND SVF did not significantly alter CD25 upregulation after stimulation on $CD8^+$ or Tconv. However, HFD eWAT SVF significantly decreased CD25 expression on both cell types demonstrating that soluble factors contributed to impairment in T cell activation (**Figure 3-7A**). Also, while MCP1 secretion was not impacted by co-culture, IL2 and IFNy were decreased when ND eWAT ATTs were co-cultured with HFD SVF, but not ND SVF (**Figure 3-7B**). Since leptin has been reported to contribute to T cell impairment in obesity, we tested if leptin signaling in T cells was necessary for ATT dysfunction (23). ATTs from leptin receptor-deficient *Db/Db* mice and *Db/+* controls were assessed using the ATT activation assay. CD25 upregulation was not rescued in *Db/Db* mice (**Figure 3-7C**). IFNy secretion also failed to be restored in obese *Db/Db* mice, while MCP1 was secreted to similar quantities as diet-induced obese mice (**Figure 3-7D**). In sum, soluble factors from HFD eWAT SVF mediate ATT inflammatory potential, and leptin is not a critical mediator.

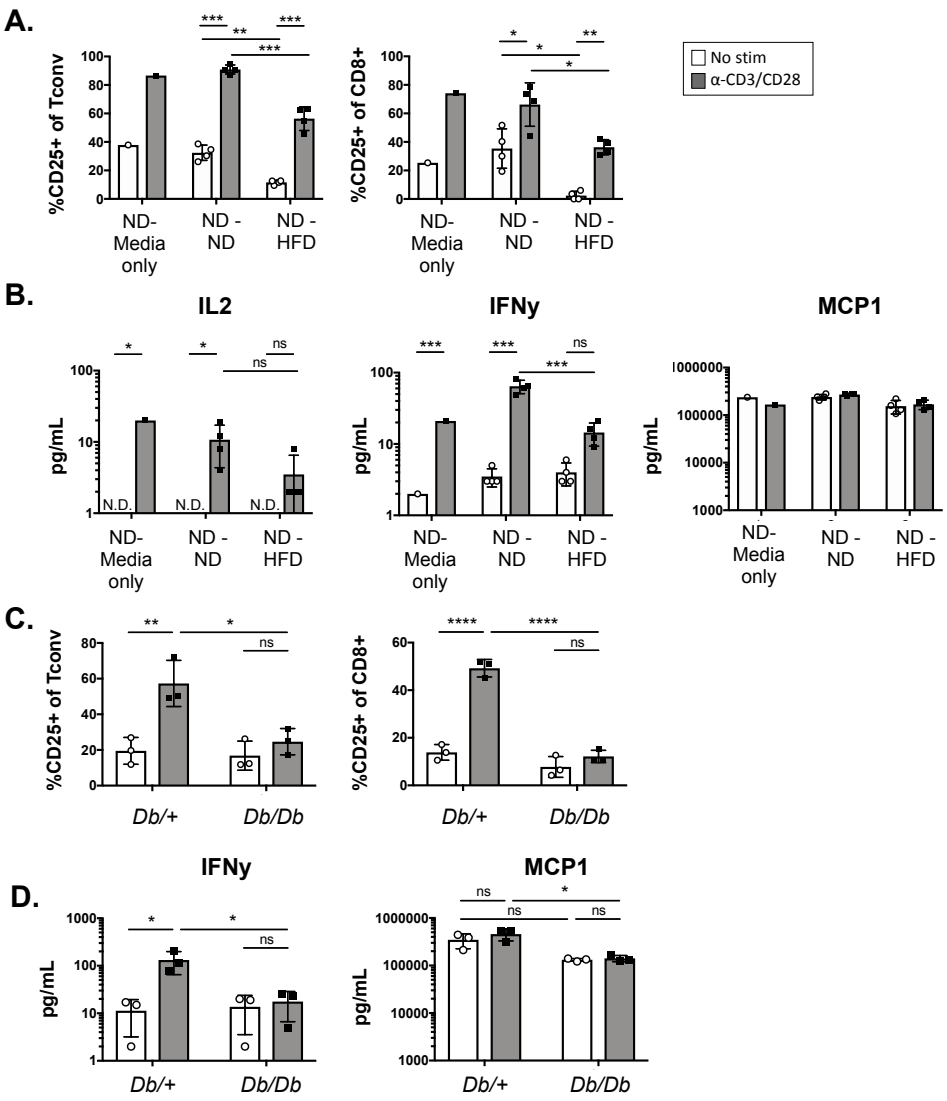


Figure 3-7 – Soluble factors in HFD eWAT inhibit inflammatory capacity of ND ATTs

(A) Frequency of CD25 expression on Tconv and CD8+ ATT from ND-media only, ND-ND (ND SVF top chamber), and ND-HFD (HFD SVF top chamber) were copared after ATT activation assays.

(B) IL2, IFNγ, and MCP1 concentration in culture supernatants from co-culture ATT activation assays of ND eWAT.

(C) Frequency of CD25 expression on Tconv and CD8+ ATTs after ATT activation assay performed on eWAT SVF of 8 weeks on *Db/+* and *Db/Db* mice.

(D) IFNγ and MCP1concentration in supernatants from *Db/Db* ATT activation assay cultures.

*p < 0.05, **p < 0.01, ***p < 0.0001

Discussion

This study has uncovered several novel findings regarding ATT biology in the context of obesity-induced inflammation. To address gaps in the field, we developed an *in-vitro* culture method to allow us to assess adipose tissue depot specific characteristics of ATT inflammatory potential. We found that HFD dampens the inflammatory capacity of Tconv and $CD8^+$ ATTs, which lose their ability to respond to a TCR stimulus. Upon stimulation with αCD3/CD28 dynabeads, eWAT ATTs from both ND and HFD tissue fail to induce Ki67 expression, unlike splenic T cells. Additionally, ATTs from HFD lose their ability to increase CD25 expression on the cell surface or secrete pro-inflammatory cytokines including IFNy and IL2. One limitation of our study is that our *in-vitro* model cannot account for tissue architecture and adipocyte derived signals found in adipose tissue. However, we found that culturing ATTs within a heterogeneous SVF fraction allowed us to maintain necessary environmental signals that differentiate ATT cells from obese and lean mice. Previous studies have demonstrated that ATTs are contributors to chronic low-grade inflammation in obese adipose tissue (67, 72, 97). However, more recent studies have suggested $CD153^+$ $PD1^+$ $CD4^+$ ATTs are induced in obese tissue, express markers cellular senescence, and have decreased classical effector cytokine production after stimulation (98). While this study defines a subset of tissue resident memory $CD4^+$ cells (using CD153) with senescent characteristics, the authors conclude these ATTs still mediate chronic low-grade inflammation due to osteopontin secretion. We performed a comprehensive analysis of effector cytokines secreted after T cell activation, which align with previous finding of decreased canonical T cell cycokine secretion in ATTs from obese mice. Instead of focusing on CD153+ Tconv cells, we decided to assess entire Tconv and CD8+ ATT populations since decreased

effector cytokine secretion after TCR simulation could be due to chronic antigen exposure and exhaustion. Shirakawa et al did not assess functional characteristics of Tconv activation such as proliferation, cell markers of activation, or perform a comprehensive panel of effector Tconv cytokines. While a fraction of Tconv cells may be senescent, we thought a more comprehensive functional assessment of ATTs would be beneficial.

We also assessed ATT activation from the human omentum, the major visceral adipose tissue organ. Adipose tissue was collected from a cohort BMI matched bariatric surgery patients that were non-diabetic (NDM; HbA1c <5.7) or diabetic (DM, HbA1c>6.5). Although we hypothesized that obesity and diabetes would result in ATTs with enhanced pro-inflammatory characteristics as seen in previous studies by McLaughlin et al (128), we found that ATTs had decreased inflammatory response to αCD3/CD28 stimulus from metabolically unhealthy patients. Further collection of human biopsies will be helpful for making assessments regarding associations between ATT inflammatory response and systemic metabolism (HbA1c), blood glucose, and circulating lipid contents. These correlations may be able to help determine whether adipose tissue functions, like lipolysis, or systemic metabolic impairments play a role in ATT function.

ATT impairment in diabetic human omentum and murine eWAT led us to explore this phenotype further. Since TCR repertoires are restricted on Tconv ATTs in obese mice, we hypothesized that ATTs were becoming exhausted due to continuous exposure to their cognate antigens, similar to T cells in tumor microenvironments (87, 99, 100). Therefore, we performed a timecourse comparison of ATT inflammatory potential after dynabead stimulation to assess ATT inflammatory capacity of ATTs after acute and chronic HFD. We found that 1-week short-term HFD diet failed to inhibit ATT inflammatory potential, indicating that ATT dysfunction is not an

acute response to diet. However, after 6 weeks of feeding, ATTs are unable to secrete increased IFNy or IL2 when stimulated with αCD3/CD28 dynabeads. However, they retain their ability to upregulate CD25 expression. This indicates that CD25 expression and cytokine secretion are regulated independently of each other. ATT dysfunction also occurs before macrophage infiltration into WAT, but after glucose sensitivity starts to change (138, 139). This suggests that ATT dysfunction does not correlate with systemic glucose insensitivity and impairment is not dependent on glucose availability alone. It also implies that influx of proinflammatory ATMs is not required for ATT dysfunction.

Since chronic HFD feeding is required for ATT impairment to occur, we hypothesized that ATTs were becoming functionally exhausted during prolonged periods of exposure to their activating antigen. Unlike dynabead stimulation assays where HFD ATTs have reduced inflammatory potential, activating signals from PMA/Ionomycin that bypass the TCR resulted in higher IFNy production than ND ATTs. Previous studies implicating ATTs in chronic low-grade inflammation have assessed ATT phenotype using PMA/Ionomycin stimulation, which explains the previous discrepancies between ATT inflammation and senescence (72, 128). Therefore, we tested whether chronic TCR signaling in obese adipose tissue was required for decreased response to αCD3/CD28 activation after 12 weeks of HFD feeding. $MHCII^{flfl}$ x LysMCre knock out mice (MMKO) were used to substantially decrease antigen presentation to ATTs during 12 weeks of HFD feeding. HFD MMKO mice had restored ATT inflammatory capacity upon αCD3/CD28 dynabead stimulus, which did not occur in WT obese controls. This suggests that prolonged signaling through the TCR could be required for ATT impairment in obese mice. However systemic metabolism could also play an important role in ATT activation capacity, considering HFD MMKO mice have improved insulin and glucose tolerance compared to HFD

WT controls. Additionally, HFD MMKO mice fail to gain as much weight as the WT HFD group, which is a major limitation to this experiment. Establishing whether TCR signaling or improved insulin sensitivity in MMKO mice result in restored ATT inflammatory capacity needs to be explored in further studies.

Since signaling through the TCR is required for ATT impairment, we assessed TCRβ, co-activating, and co-inhibitory receptors on ATTs from lean and obese mice. TCRβ and CD28 were expressed more highly on ATTs from HFD-fed eWAT than ND controls, indicating that impairment is not due to inadequate TCR expression. Co-inhibitory expression is a hallmark of T cell exhaustion, so PD1 and CTLA-4 expression were also assessed. Both proteins were expressed on a larger frequency of ATTs than splenic T cells. However, HFD only increased PD1 expression on $CD8^+$ and not Tconv ATTs. Nevertheless, we decided to perform an *in-vivo* PD1 blockade to assess whether PD1 signaling was responsible for reduced ATT activation capacity in ND ATTs compared to splenic T cells and whether $CD8^+$ ATTs in obese mice would have restored inflammatory capacity following a blockade. We found that PD1 blockade did not influence the GTT of ND or HFD mice and that T cell inflammatory potential was not reversed. Further studies will be needed to pinpoint the mechanism responsible for ATT impairment, but signaling pathways proximally downstream of the TCR are inhibited. Distal signaling pathways seem intact, since PMA/Ionomycin induces PKC signaling and calcium influx, and IFNγ production occurs in HFD ATTs with this stimulation (140, 141). We attempted to perform phospho-flow experiments to assess phosphorylation of proteins like Zap70, Lat, and Erk to pinpoint where TCR signal transduction is impaired. Unfortunately we had difficulty with this technique and alternative methods like western blots are also difficult to perform on rare cell populations where substrate is limited. Further studies utilizing techniques like single cell RNA

sequencing will allow us to explore multiple potential mechanisms driving ATT inflammatory impairment.

Since ND and HFD SVF have different environmental signals, we also assessed whether soluble factors in the culture conditions influenced ATT activation. Using transwell plates we determined that soluble factors in the HFD SVF are sufficient to decrease inflammatory potential of ND ATTs. We started by assessing leptin as a potential candidate since it has been implicated in T cell exhaustion associated with obese cancer patients (23). We utilized the leptin receptor mutant *Db/Db* mice, to assess whether leptin signaling was necessary or sufficient for inducing ATT impairment in obese conditions (142). ATTs from *Db/Db* mice had an impaired inflammatory phenotype similar to ATTs from diet-induced obese mice. Since leptin is produced by adipose tissue and secretion is increased with obesity, this finding implies that ATT dysfunction is independent of leptin. This is an interesting finding and suggests that ATT inflammatory impairment occurs due to a mechanism distinct from T cell exhaustion associated with cancer in people with obesity. Further studies will be required to determine which soluble factors from obese SVF reduce effector cytokine secretion after TCR stimulus. Determining whether these inhibitory factor(s) are derived from immune cells, pre-adipocytes, or endothelial cells will be the immediate next step.

It is still unknown whether ATT impairment in obese mice and humans is advantageous or inhibits the resolution of chronic low-grade inflammation in adipose tissue. On one hand, decreased secretion of Th1 and Th17 associated cytokines would reduce the inflammatory tenor of obese individuals. Alternatively, unresolved T cell stimulation through the TCR would result in a lack of inhibitory signals to macrophages that would reduce their inflammatory response. It has been shown that weight loss restores T cell function, and this coincides with improved

insulin sensitivity (104). Further studies are needed to determine which soluble factors are responsible for ATT impairment and the precise point proximally downstream of the TCR where signaling is impaired. This will be of significant importance even if T cell impairment is not essential for obesity-induced chronic low-grade inflammation and impairment is beneficial for metabolic diseases. Since lymphatic T cells in obese individuals also seem to have impaired inflammatory characteristics that lead to increased cancer risk, decreased response to viral infections such as influenza, and decreased vaccine immunogenicity (29, 143, 144). Therefore, finding the soluble factors that lead to ATT cell impairment in obese individuals may be important information for resolving T cell-mediated impairments outside of adipose tissue in obese patients.

Chapter 4: Adipose Tissue T Cell Heterogeneity Revealed by Single Cell RNA Sequencing

Portions of this chapter is under revision at JCI Insight:

Porsche CE, DelProposto JL, Geletka L, O'Rourke RO, Lumeng CN. Adipose tissue T cells have impaired inflammatory capacity in obese mice and humans.

Author Contributions: CEP and JLB acquired and FACS sorted cells for sequencing. CEP and CNL performed sequencing analysis, conceived of experiments, wrote, and edited the manuscript. University of Michigan Advanced Genomics Core performed single cell sequencing.

Abstract

Determining the compositional and functional diversity of ATT cells will be important for understanding their contribution to homeostasis or inflammation in lean and obese conditions. Different subtypes of ATTs such as Tregs or Tconvs could influence the inflammatory tenure of adipose tissue. Additionally, localized environmental factors could be responsible for gene expression, function, and heterogeneity of ATTs. To assess these measurements of heterogeneity, we used single cell RNA sequencing of sorted $CD3^+$ ATT cells from three distinct white adipose tissue depots. We found that within a single depot, there are multiple ATT cell subsets that expand beyond Tconv, Treg, and $CD8^+$ groups described in Chapter 3. Not only can we define naïve and memory subsets, we identified ILC3s, progenitors, and natural killer cells within the total $CD3^+$ fraction. The frequency of each of these populations depended upon the depot in which they reside, and whether they were derived from mice fed ND or HFD. Gene expression analysis suggests ATTs in lean eWAT and iWAT are basally activated

to a greater extent than oWAT. HFD feeding also led to differential gene expression from cells in ND depots. CD4 T cell subsets from obese eWAT were enriched for $CD8^+$ T cell exhaustion associated with chronic LCMV and terminal exhaustion after performing GSEA pathway analysis. We identified *Btla, Nlrc3, Dusp6, and Dicer1* as genes that could be contributing to exhaustion via co-inhibitory receptor expression, inhibition of TCR signaling, and translation regulation. Assessing these mechanisms of impairment further will be important for understanding ATT dysfunction and their influence on systemic metabolism.

Introduction

Several factors could contribute to ATT inflammatory impairments described in Chapter 3, however, limitations in ATT cell number and substrate impeded our progress towards understanding the mechanistic underpinnings of dysfunction. One hypothesis is that localized environmental signals may influence ATT function. Adipose tissue depots have structural and function differences some of which depend upon anatomical location. Since visceral depots (eWAT and oWAT) are responsible for obesity mediated chronic low-grade inflammation while subcutaneous (iWAT) expansion is protective. Therefore, investigating depot differences in ATT function lean and obese states may provide insight into mechanistic differences in eWAT ATTs.

In addition to depot differences, obesity is known to regulate the frequency of immune cell populations and their functions. However, ATT Activation assays performed in Chapter 3 did not clearly distinguish in ATT subpopulations frequencies between lean and obese mice. Recent studies have used single cell RNA sequencing technology to assess the composition of adipose tissue leukocytes in relation to obesity and insulin sensitivity in humans and mice. TREM2+ lipid-associated macrophages were identified as induced in HFD fed mice suggesting a specific ATM phenotype that is distinct form the M1/M2 axis (145). CD9+ ATMs were identified in mouse and human adipose tissue as potential mediators of diabetes and obesity (108). Single cell analysis of stromal cells from obese humans identified numerous myeloid and lymphoid populations that differ based on diabetes status (146).

Most of these studies focus on total stromal cell populations of which T cells are a small proportion (10-25%), which decreases the power by which subtypes of ATT's can be identified.

Several studies have focused on the diversity of T cells using single cell technology that have provided insight into molecular diversification of tissue resident Tregs. This study shows that genes expression of tissue Tregs originating in eWAT, colon, or muscle is significantly different (147). Therefore, localized signals from tissue of origin can influence gene expression of resident immune cells. SC RNAseq analysis has also focused on ATTs after short-term ketogenic diet feeding. One week of ketogenic diet changed almost doubled the frequency of ATT and B cell populations. Additionally, ketogenic diet increased transcription of *PPARα*, *S1pr1*, *Itgae*, and *Lgals3* in γδT cells, which implicates changes in pro-longevity, cell trafficking, migration, and cell adhesion (148). Despite the depth of information that can be extracted from these SC RNAseq analyses, there are several gaps that we wanted to address with our own big data collection and analysis. We wanted to assess how different white adipose tissue depots influence the ATT immune compartment, how chronic HFD feeding for 12 weeks influences gene expression and subpopulation composition, and acquire a depth of sequencing to provide insight into intracellular mechanisms that could be contributing to ATT impairment in both Tconv and CD8+ populations.

Cellular and functional heterogeneity of T cells has been demonstrated in lymphatic and adipose tissues. We hypothesized that regulation of these populations with HFD feeding could be contributing to the ATT impairment phenotype. Although we separately assessed Tconv, $CD8^+$, and Treg cells in Chapter 3, each of these groups are probably composed on several smaller subsets of ATTs. Each of these three populations could also contain γδ T or innate lymphoid cells (ILC). γδ T cells are usually found in mucosal or barrier tissue sites and protect the host from infection, however these cells have also been found to play an important role in adipose tissue homeostasis. Adipose tissue γδ T cells in visceral fat and are important for maintaining

Treg populations and are responsible for maintaining core body temperate after cold exposure (149). Innate lymphoid cells (ILC) are another subset of T cells found in adipose tissue, including natural killer (NK) cells. Interestingly, adipose tissue NK cells lose their cytotoxic killing strength in obese adipose tissue similar to defects in inflammatory capacity we observed in $CD8^+$ ATTs (150). They lack TCR expression and do not provide adaptive immune responses to specific antigens. However, their transcription factors and effector cytokines and cytolytic mediators similar Tconv and CD8+ cells. ILC1, ILC2, and ILC3, and NK cells are smaller subsets of ILCs with properties that mirror Th1, Th2, and Th17 Tconv and CD8+ cells, respectively. Natural killer T cells (NKT) have been found in adipose tissue and are protective against obesity induced chronic low grade inflammation (151). Instead of TCRs, invariant NKT cells recognize lipid antigens presented on Cd1d molecules instead of MHCII.

In addition to heterogeneity of T cell subsets, adipose tissue depots have their own distinct functional differences due to local environmental signals. We hypothesized that white adipose tissue ATTs would have disparate compositions and gene expression depending upon anatomical locations from which the depot was collected. We decided to assess differences in three white adipose tissue depots. Omentum (oWAT) and eWAT were assessed to determine potential differences between visceral WAT. Additionally, ATTs from iWAT were collected to determine differences in ATT function subcutaneous adipose tissue. We found that sorting CD3+ cells increased the depth of sequencing in order for us to assess 9 separate clusters of ATTs. Diet and depot also regulated gene expression and pathway analysis, and ultimately led to specific targets of impairment in obese eWAT ATTs.

Despite evidence for multiple subsets of ATTs in adipose tissue, few studies have performed expression analysis on lean or obese mice. Although adipose tissue immune cells have

been analyzed using SC RNAseq, previous studies have failed to obtain a high enough proportion of cells in single cell datasets needed to distinguish these smaller populations from gross CD8+ and CD4+ cells. Differences in state of activation are also of interest for true Tconv and CD8+ subsets. Although it has been shown that ATTs mostly have a memory phenotype, with few naïve cells, confirming this with an unbiased analysis would be of interest and could contribute to differences in ATT inflammatory capacity seen with HFD feeding (80). In order to achieve deeper sequencing of ATT populations, we decided to sort CD3+ cells from SVF prior to single cell RNA sequencing analysis. This analysis allowed us to identify 9 distinct clusters of ATTs. Diet and depot also regulated gene expression and pathway analysis, and ultimately led to specific targets of impairment in obese eWAT ATTs.

Materials and Methods

Mice

Male mice on a C57B/6J background were purchased from Jackson Laboratory (000664). At 6 weeks of age, mice were either kept on normal diet (ND; LabDiet PicoLab 5L0D 4.09kcal/gm 29.8% protein, 13.4% fat, 56.7% carbohydrate) or switched to high fat diet (HFD; Research Diets D12492, 5.24kcal/gm 20% protein, 60% fat, 20% carbohydrate) for 12 weeks.

ATT cell acquisition for sequencing

The stromal vascular fraction (SVF) was isolated from whole adipose tissue as previously described (Chapter 2 Materials and Methods). $CD3^+$ cells were sorted from 3 adipose tissue depots (eWAT, oWAT, and iWAT) from lean and obese mice. ATT cells were stained for FACS sorting using a Sony MA900 (see Table 4A for antibody details).

Table 4A: Antibodies used for CD3+ ATT cell sorting.

Antigen/Target	Fluorophore	Clone	Company	Catalog #
Live/dead	Brilliant Violet-450	N/A	Invitrogen	L34955
CD45	APC	30-F11	eBioscience	47-0451-82
CD3	PE	145-2C11	eBioscience	12-0031-82

Single Cell RNA sequencing

$CD3^+$ cells were collected in duplicate from ND and HFD eWAT, oWAT, and iWAT. Samples

were taken to the University of Michigan Advanced genomics core for library construction with the 10x Genomics platform (V2.0) and sequencing (Next-Seq).

Data analysis

Count matrixes were generated with Cellranger (10x Genomics) with post-processing with Seurat (v3) (152). Ribosomal and mitochondrial genes were removed prior to analysis and cells were filtered to remove cells with <200 unique genes identified. Following transformation of the data, integrated analysis of all the depots and conditions was performed. ATT clusters were defined by identifying genes significantly enriched in each cluster compared to the other cells independent of diet and depot and comparing with gene profiles in the Immunological Genome Project (ImmGen) (153). Differentially expressed (DE) genes were identified using Wilcoxon rank sum test adjusting for false discovery rate. DE genes were defined as adjusted *p*-value of <0.05 and log2 Fold changes >0.3. Gene pathway enrichment was analyzed using the biological pathways PATHER gene ontology (GO) database. Data sets were then separated by depot of origin and DE genes for ND and HFD ATTs were assessed for each cluster and each depot. Additional packages used for data analysis to try to remove contaminants include Cellbender, Liger, and SoupX (154-156).

Btla analysis

The stromal vascular fraction (SVF) was isolated from whole eWAT as previously described (Chapter 2 Materials and Methods). $CD3^+$ cells were sorted from four lean and obese mice. ATT cells were stained for FACS analysis and sorting using a Sony MA900. Btla, CD4, and CD8 antibodies were added into the sorted cells so BTLA expression could be analyzed on subpopulations of $CD3^+$ ATTs. Sorted $CD3^+$ cells were taken for RNA extraction using a Qiagen

RNA micro kit. cDNA was generated from RNA using high-capacity cDNA reverse transcription kits (Applied Biosystems). Power SYBR Green PCR Master Mix (Applied Biosystems) and the StepOnePlus System (Applied Biosystems) were used for real-time quantification PCR

Results

Adipose tissue T cells are comprised of 9 distinct subsets

SC RNAseq analysis shows that ATTs can be divided into more granular subpopulations than $CD4^+$ Tconv, $CD4^+$ Treg, CD8+, and double negative (DN) populations that we typically define by flow cytometry (**Figure 4-1A**). 12,447 cells from ND (n = 4 mice pooled) and 4,309 cells from HFD (n = 2 mice pooled) were collected for SC RNAseq analysis. This allowed a much more detailed analysis of ATTs than previous SC RNAseq analysis on bulk CD45+ cells from adipose tissue. We defined each subset of $CD3^+$ cells based upon the genes that were most enriched in each cluster using cell type specific genes delineated by literature and ImmGen (**Figure 4-1B**). A population of cells expressing markers of proliferation (*Stmn1* and *Mki67*) was identified that expressed low levels of *Cd4* and *Cd8a* consistent with their identification as double negative cells with immature/stem like properties (DNstem). CD4 Tregs (*Foxp3*), natural killer (NK) cells (*Klra7*), and ILCs (*Tnfsf11*) were identified (**Figure 4-1C**). CD4Trm, CD8Trm, and NK1 cells expressed *Tbx1*/Tbet consistent with a Th1 polarized T cells. ILCs were also further defined by Rorc expression consistent with identification as ILC3s. Markers of activation, like pro-inflammatory cytokines and proliferation were used to gauge basal function of these subsets. CD4Trm, CD8Trm and NK1 cells expressed the highest levels of pro-inflammatory cytokine genes (*Ifng, Il2*). The NK2 subcluster has characteristics of naïve T cells including expression of *Sell* and *Dapl* in the absence of *Ifng* and *Il2*. DNstem cells were the only population and proliferating in basal conditions (**Figure 4-1F**).

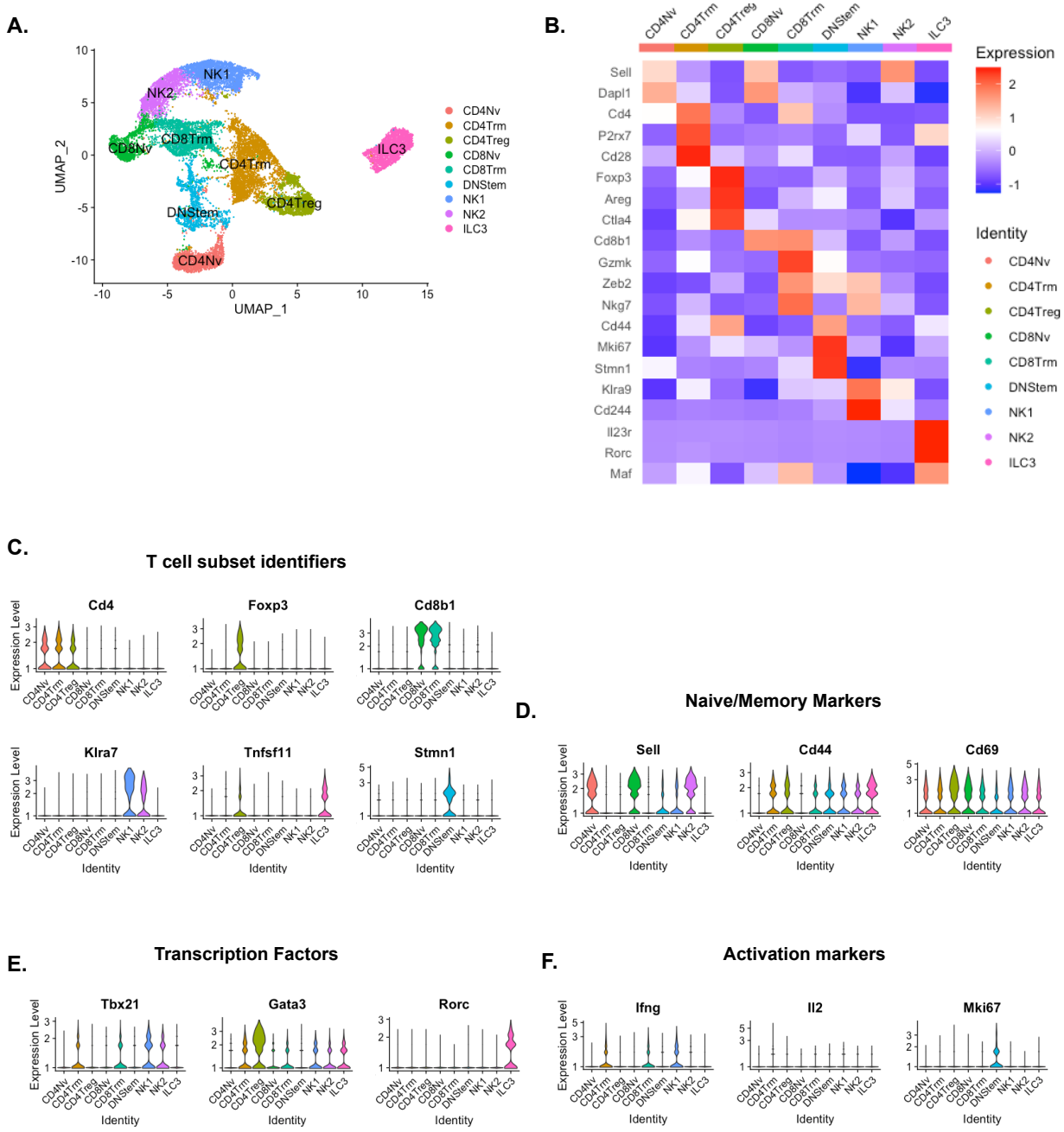


Figure 4-1 – SC RNAseq of CD45+ CD3+ cells reveals 9 distinct ATT subsets.

(A) TSNE plot of different ATT clusters based upon similarity of gene expression.
(B) Each cluster was compared and enriched genes for each group were used to determine the identity of each group. This heatmap shows gene of interest used to determine each cluster is shown.
(C) Violin plots showing gene expression markers associated with T cell and ILCs.
(D) Violin plots of naïve and memory T cell markers
(E) Violin plots of transcription factors used to distinguish Tconv and ILC subsets
(F) Violin plots of effector cytokines and markers of proliferation used to classify T cell activation and inflammation of ATT subsets.

Proportions of ATT subsets are dependent upon depot and diet

ATTs from eWAT, iWAT, and oWAT all contained the 9 ATT clusters defined above (**Figure 4-2A**). We wanted to assess how diet regulated the proportion of ATT subtypes (**Figure 4-2B**). We have previously assessed total CD4 Tconv, CD4 Treg, total $CD8^+$, and DN populations in eWAT of ND and HFD fed mice using flow cytometry. In HFD mice, we see increased frequency of total $CD3^+$ ATTs, with increased $CD8^+$, decreased Treg, and unchanged total CD4+ Tconv and DN populations (**Figure 4-2C**). We see similar trends in eWAT ATT subpopulations defined by SC RNAseq of CD3+ cells (**Figure 4-2D**). In eWAT from HFD mice, the proportion of CD4Treg decrease and CD8Trm increase relative to ND mice. DN populations like NK1 and NK2 are not changed with HFD, while ILC3s decrease. Naïve cell populations also decrease, consistent with previous studies (80). The proportion of CD4Trm cells slightly decreased in HFD mice and DNstem cells significantly increase.

Subpopulation changes with HFD are also dependent upon depot of origin. Notably, iWAT has fewer naïve cells (CD4Nv and CD8Nv) and ILC3s compared to eWAT, while CD4Trm and NK1 populations are more prominent in iWAT. The decreases in CD4Tregs and increases in CD8Trm observed in eWAT are not seen in iWAT. The ATT subtypes in oWAT differ significantly from both iWAT and eWAT. oWAT is enriched for both CD4Nv and CD8Nv ATT types and diet type has less pronounced changes in the frequencies of the oWAT subpopulations.

Gene expression differences in ATT subsets suggest iWAT and eWAT have higher gene expression of inflammatory cytokines like Ifng and Il2 than oWAT. (**Figure 4-2F**). Overall oWAT ATTs seem to be in a quiescent state compared to the other depots. eWAT and iWAT have higher expression of cytokine (*Il2*, *Ifng*, *Il10*) and chemokine (*Ccl4*), genes as well as genes

downstream of the TCR signaling cascade (*Jun*). However, ILC3 cells in oWAT may be more active in homeostatic conditions than those in eWAT and iWAT since they have higher expression of *Jun*, *Ccr6*, and *Cd83*.

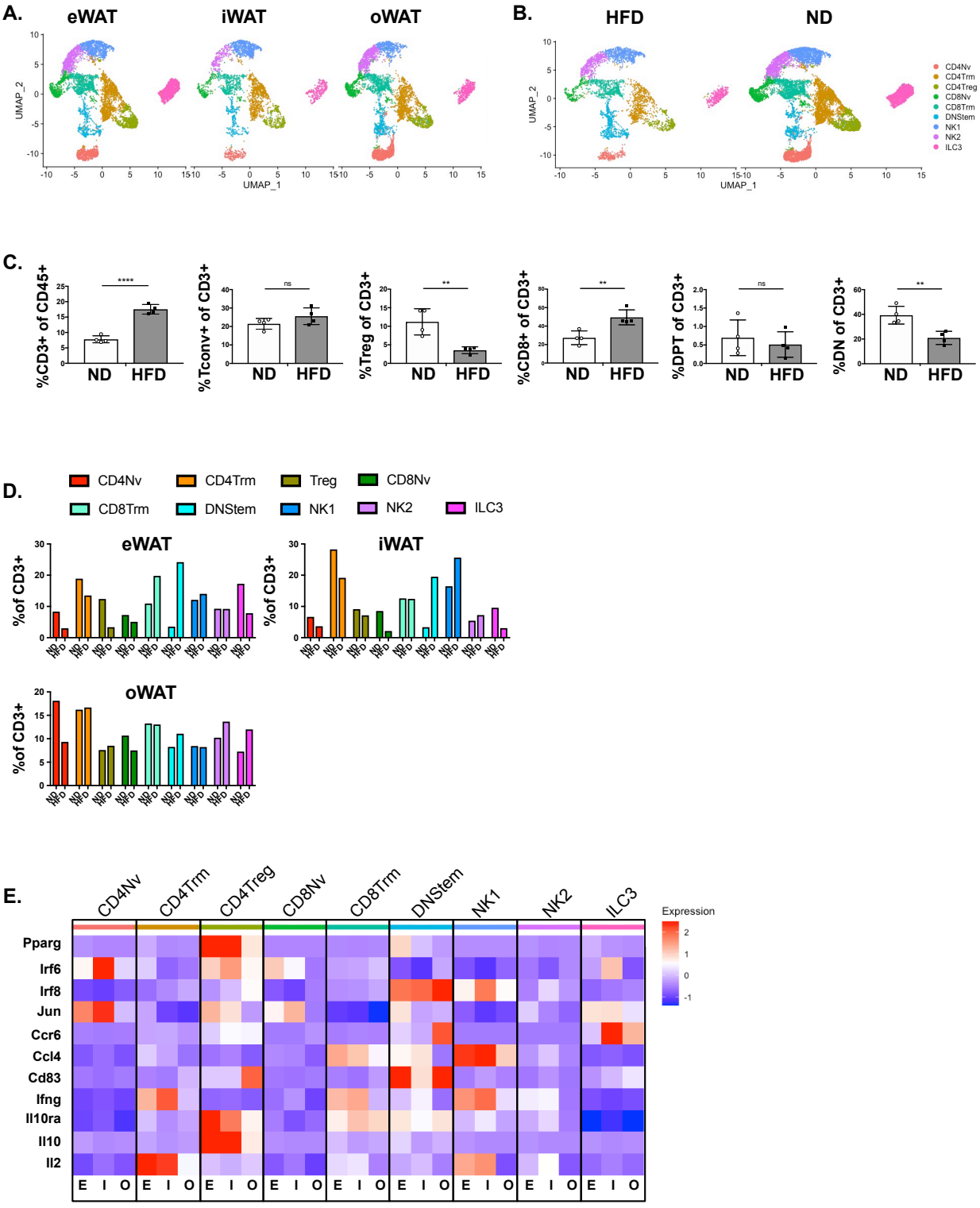


Figure 4-2 – Depot and diet influence compostion and function of ATTs.

(A) TSNE plots of ATTs from eWAT, iWAT, and oWAT

(B) TSE plots of ATTs from ND and HFD (12weeks of feeding) from eWAT

(C) Flow cytometry assessment of ATT cell composition with 12 weeks of HFD feeding
(D) SC RNAseq assessment of ATT cell composition in ND and HFD fed mice after 12 weeks of feeding.
(E) Heatmap illustrating gene expression differences in each ATT cluster depending upon depot of origin. E = eWAT, I = iWAT, O =oWAT

Gene expression of $CD4^+$ and $CD8^+$ ATTs are differentially regulated by depot of origin

DE genes from eWAT, oWAT, and iWAT CD8Trm cells were assessed by pathway enrichment analysis to identify functional differences between depots. Compared with iWAT and eWAT, oWAT depots had few DE genes to generate statistically significantly upregulated pathways suggesting that the oWAT signals may not influence the function of CD8Trm specifically. Differences between iWAT and eWAT provided insight into their function. We were able to assess 158 genes enriched in iWAT and 1,329 enriched genes in eWAT when comparing the CD8Trm clusters, which yielded significantly differentiated signaling pathways in PANTHER. CD8Trm cells from eWAT were enriched in signaling pathways suggesting suppressed inflammation. Regulation of chromatin silencing and regulation of T cell differentiation were enriched in eWAT CD8Trm, whereas T helper differentiation, catalytic activity, and intracellular signal transduction were enriched in iWAT CD8Trm (**Figure 4-3A**).

A similar trend towards anti-inflammatory/suppressed eWAT phenotype relative to iWAT was observed for CD4Trm cells. The top 6 most significantly enriched pathways in eWAT included regulation of type 2 immune responses, peptide-tyrosine dephosphorylation, and negative regulation of DNA recombination. Pathways enriched in iWAT included positive regulation of JUN kinase, hormone-mediated signaling pathway, and response to cytokine demonstrating activation of TCR signaling pathways and hormone responses (**Figure 4-3B**).

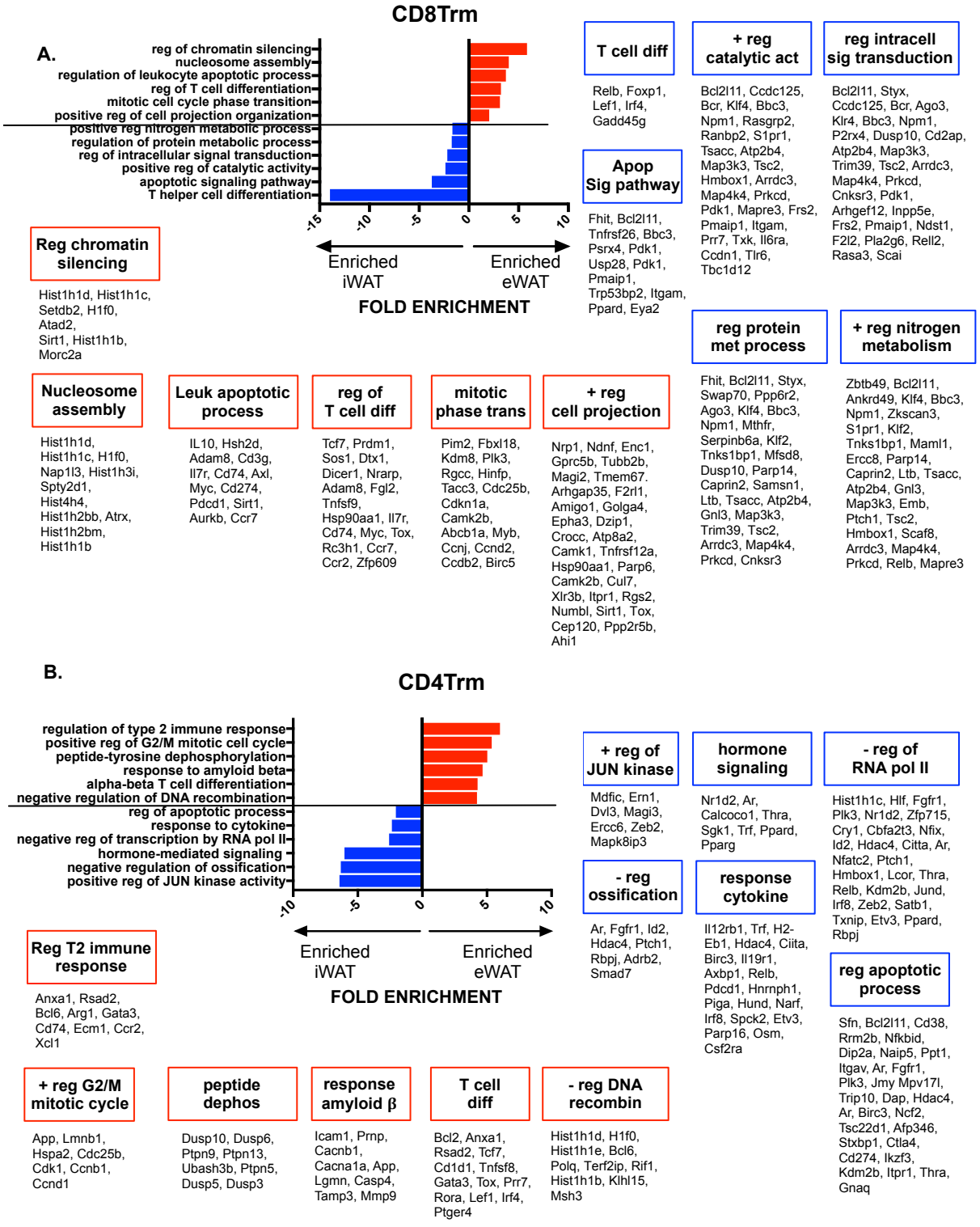


Figure 4-3 – Memory cell pathway enrichment differences in eWAT and iWAT

(A) Top 6 DE pathways in CD4Trm cells from iWAT and eWAT determined by PANTHER biological processes.
(B) Top 6 DE pathways in CD8Trm cells from iWAT and eWAT determined by PANTHER biological processes.

Differences in CD4Nv cells were also of interest and provide insight into the functional capacity naïve ATT cells. In eWAT, CD4Nv cells have enriched expression of glycolipid metabolic process, and negative regulation of RNA polymerase II. iWAT CD4Nv cells once again were enriched for pathways indicating a more active pro-inflammatory pathways such as cellular response to cytokine stimulus, positive regulation of transcription, and positive regulation of molecular function (**Figure 4-4A**). Finally, CD8Nv cells from eWAT and iWAT both suggested suppressed inflammatory capacity, however they are potentially related to separate mechanisms (**Figure 4-4B**). In eWAT CD8Nv histone H3-K4 trimethylation and chromosome condensation are enriched, while in iWAT CD8Nv negative regulation of response to stimulus, regulation of RNA polymerase II, and negative regulation of metabolic processes are enriched. In sum, differences in CD4 and CD8 subsets indicates that eWAT ATTs have suppressed inflammatory characteristics compared to iWAT resident ATTs.

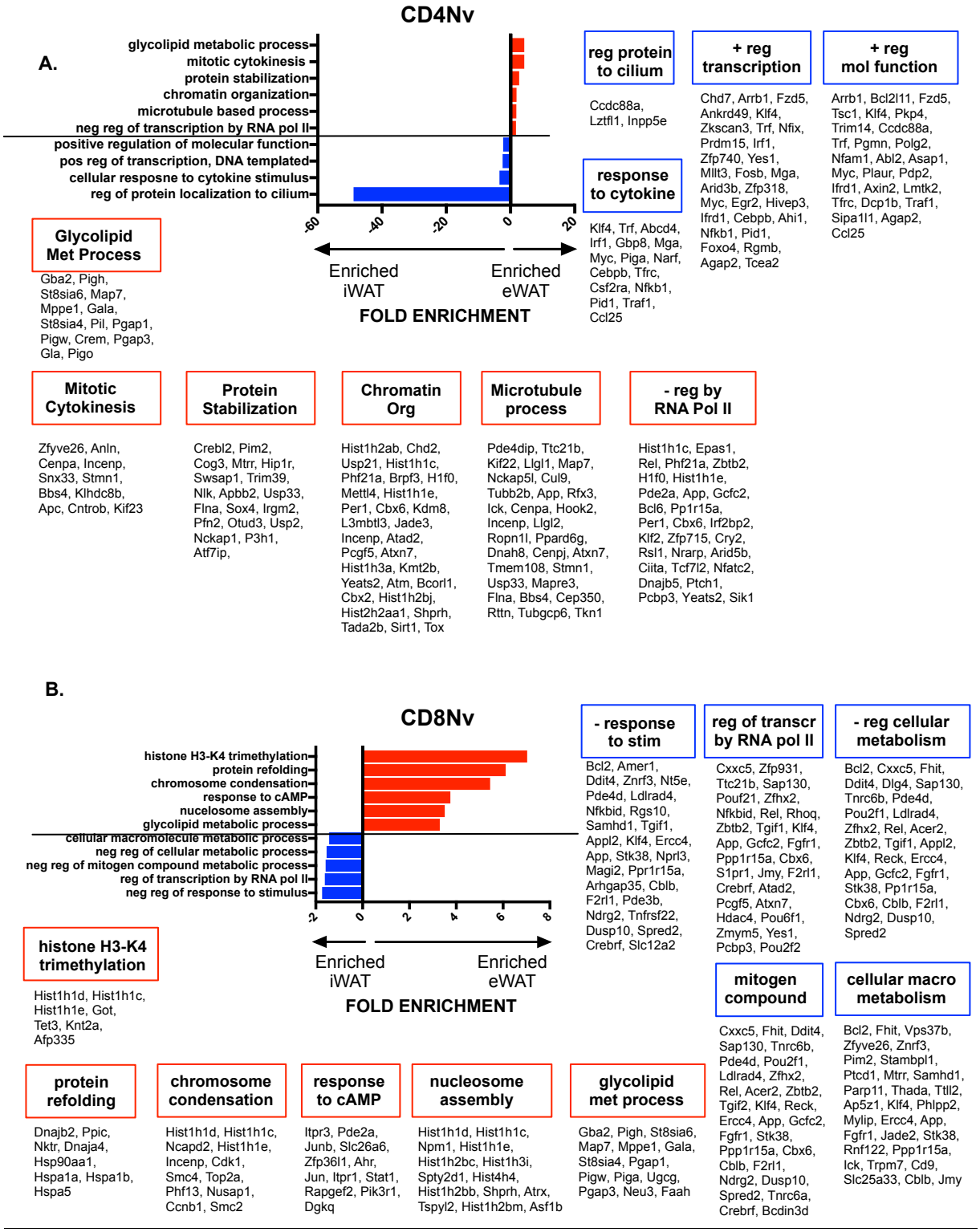


Figure 4-4 - Naïve cell pathway enrichment differences in eWAT and iWAT

(A) Top 4-6 DE pathways in CD4Nv cells from iWAT and eWAT determined by PANTHER biological processes.

(B) Top 5-6 DE pathways in CD8Nv cells from iWAT and eWAT determined by PANTHER biological processes.

Obesity mediated CD4Trm ATT impairment is associated with T cell exhaustion

In addition to gene expression differences between depots, differences between ND and HFD mice was of vital interest. Understanding mechanisms responsible for ATT impairment seen in Chapter 3 was explored by comparing each of the nine ATT subsets in ND and HFD eWAT. DE genes in ND eWAT CD8Trm cells indicated enrichment of inflammatory and proliferation pathways such as IL1b production, positive regulation of T cell proliferation, and positive regulation of GTPase activity (**Figure 4-5A**). CD4Trm cells in ND eWAT also are enriched for genes related to T cell activation and inflammation including cellular response to GM-CSF, activation of MAPKKK activity, and T cell co-stimulation genes (**Figure 4-5B**). Similar to other reports, our ability to detect significant DE genes induced in HFD ATT subsets was limited by significant ambient contamination of the HFD datasets with macrophage specific genes (e.g. *Cd84, Lyz2*) (148). This in combination with fewer HFD ATTs that were sequenced resulted in very few DE genes enriched in HFD ATTs. For example, in the CD4Trm cluster where ND v HFD cells were compared, there were 1,578 DE genes in ND cells and 34 DE genes in HFD ATTs where significance was defined by an adjusted p-value of <0.05. However, comparing 2 clusters within a single diet yielded better results. We decided to compare CD4Trm v CD4Nv to differentiate function differences between memory and naïve ATTs in eWAT (**Figure 4-5C**). We identified CD4Trm and CD8Trm cells in HFD eWAT resembled exhaustion of CD4+ and CD8+ T cells as defined in the context of chronic LCMV infection, respectively (157). Several individual genes enriched in HFD ATTs were significantly enriched and point to potential mechanisms of dysfunction. We identified specific genes of interest that could contribute to exhaustion including: *Btla, Nlrc3, and Dicer1*. Each gene would contribute to exhaustion using different cellular mechanisms and caused by disparate triggers including

intracellular TCR signaling regulation, co-inhibitory receptor expression, and mRNA regulation (**Figure 4-5D**). Additionally, genes responsible for signaling downstream of the TCR, such as Map4k3, Nfkb1, Akt3, and Ifng were all enriched in ND ATT subsets. We sorted $CD3^+$ ATTs from ND and HFD eWAT and performed qPCR on candidate genes that could be contributing to T cell exhaustion. We observed an increase of Btla expression in HFD derived ATTs, and expression was not detected in ND derived cells (**Figure 4-5E**). Additionally, BTLA expression was assessed by flow cytometry, where increased frequency was observed in total $CD4^+$ ATT and DN populations. In sum, diet influences gene expression differences in eWAT derived CD4Trm and CD8Trm clusters obesity inducing T cell exhaustion signatures in CD4Trm and Cd8Trm cells.

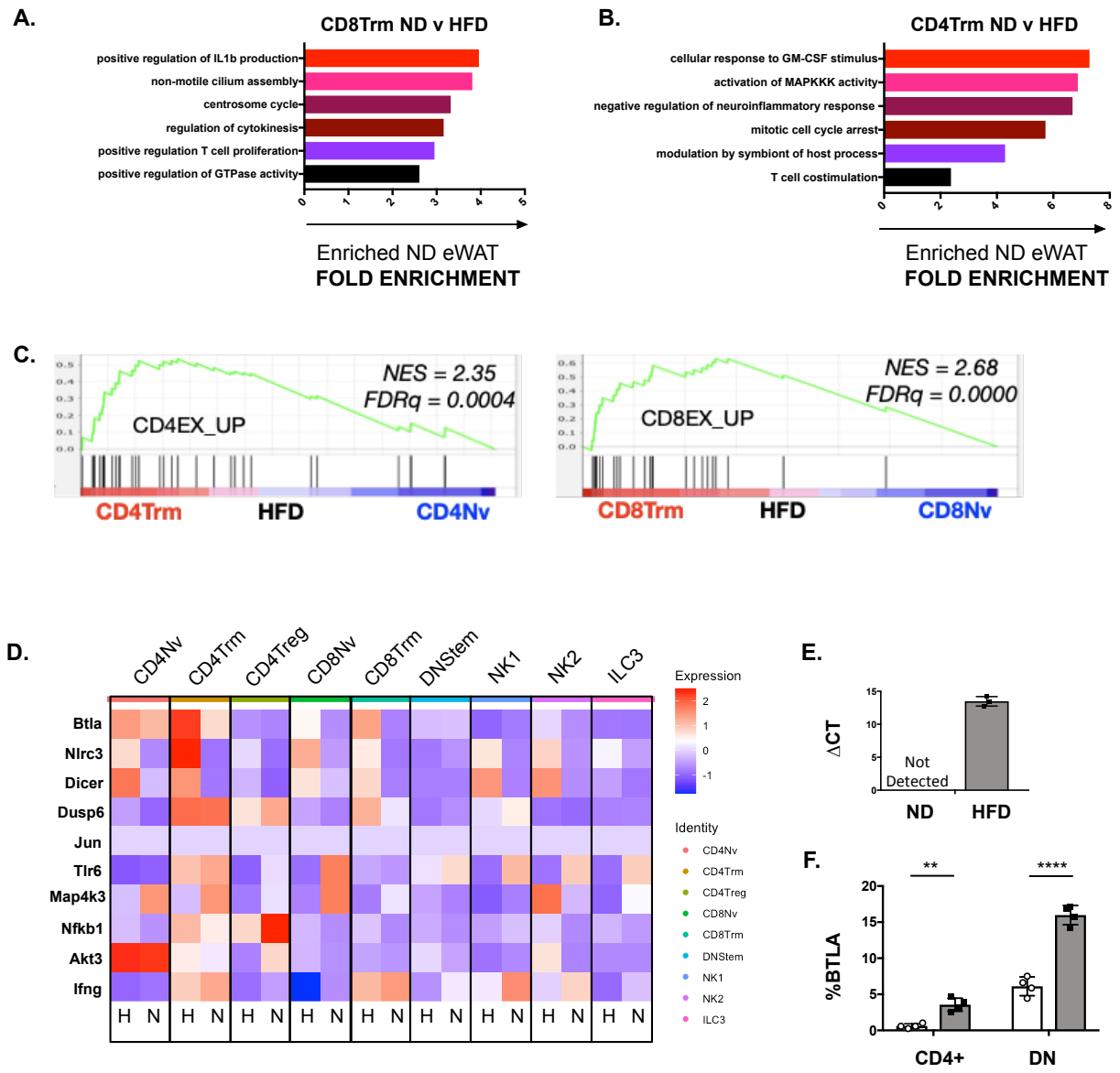


Figure 4-5 – HFD induces an exhaustion phenotype in CD4Trm and CD8Trm cells

(A) Top 6 PANTHER pathways enriched in ND v HFD CD8Trm,
(B) Top 6 PANTHER pathways enriched in ND v HFD CD4Trm,
(C) GSEA analysis of HFD CD4Trm and CD9Trm gene expression compared to previously published transcriptomes of exhausted CD4+ and CD8+ T cells respectively,
(D) Gene expression of potential target genes responsible for T cell exhaustion and canonical downstream TCR proteins in ND and in HFD ATTs. H = HFD, N = ND
(E) qPCR of *Btla* from FACS sorted $CD3^+$ eWAT ATTs,
(F) Flow cytometry analysis of BTLA expression on $CD4^+$ and DN eWAT ATTs.

Discussion

SC RNA sequencing has provided us multiple insights into the heterogeneity of adipose tissue stromal cells. In this chapter, we focus on the diversity of ATTs based on depot and chronic HFD feeding in mice, which has not been the focus of previous studies in the field. Previous reports have evaluated changes in ATT after 1 week of ketogenic diet where pronounced expansion of NK, ILC, and γδ T cells and reduced *IL1b* expression was observed (148). Although early expansion of ATTs is metabolically beneficial after 1 week of ketogenic diet, chronic feeding results in insulin resistance similar to HFD. However, SC RNA analysis was not used to evaluate the composition or gene expression of ATTs during an insulin resistant state.

By sorting $CD3^+$ ATTs from mice fed HFD for 12 weeks and age matched ND controls we were able to sequence more than 15,000 cells and gain in depth insight into this rare cell population. We were able to identify 9 subsets of ATTs that overlap with populations that have been implicated in obesity mediated chronic low-grade inflammation literature. Although there were some limitations in analyzing the sequencing data, such as macrophage gene contamination and decreased depth of sequencing in HFD samples, we were still able to make conclusions regarding the composition and gene signatures of $CD3^+$ ATTs.

Using basic glycoprotein and transcription factor expression, we were able to broadly identify $CD4^+$ Tconv, $CD4^+$ Treg, $CD8^+$, and double negative (DN) cell groups that we have observed with flow cytometry. However we observed multiple subsets of cells reside within these larger classifications. *Sell*, *Cd44*, and *Cd69* identified naïve and tissue resident memory

cells within both $CD4^+$ Tconv and $CD8^+$ positive subsets. In addition, we observed several populations of cells that comprise the DN compartment, including ILCs. We were surprised to ILC subsets in our analysis since they canonically do not express CD3. Whether there was a problem with our sort, or there are ILC-like cells in adipose tissue that have similar gene expression is something that will need to be assessed in later studies. Furthermore, previous studies have implicated adipose tissue ILC2 cells as important mediators of homeostasis in lean tissue, but few studies implicate ILC3 (158). Interestingly, DE genes in this cluster also resembled γδ T cells when top 200 genes were run through ImmGen. Therefore γδ T cells may also be integrated in this cluster and further subdivision would reveal both ILC2 and γδ T cells in this subcluster (149). Two NK populations were observed, however they lacked expression of the invariant NKT marker *Cd1d*. The NK1 cluster has higher expression of inflammatory cytokines like *Ifng* and *Il2* while NK2 cells seem to be either quiescent or inactivated based on the expression of naïve T cell markers like *Sell* and *Dapl.* ILC3s also seem to comprise the DN subpopulation and were more transcriptionally different from the other clusters. In addition to these known ATT subpopulations, we identified another population that has yet to be explored. DNstem population contained stem cell like properties and expressed *Stmn1*. This cluster was only population that displayed proliferative properties without stimulation and expressed *Mki67*. It was also observed in ketogenic diet studies where it was identified as a multi-lineage proliferative population (148).

Diet and depot differences were also prominently found with the SC RNAseq analysis. ND and HFD ATTs were sequenced from eWAT, iWAT, and oWAT. The proportion of each 9 subclusters was calculated in each depot from ND and HFD mice. The regulation of each eWAT population was compared to analysis performed using flow cytometry of ND and HFD mice fed

for 12 weeks to determine whether our SC RNAseq analysis was consistent with previous studies. Flow and SC RNAseq of eWAT ATT analysis remained consistent for some populations. For example, CD8Trm cells increase and Treg decrease with HFD feeding in both flow and sequencing analyses. Additionally, CD4Nv and CD8Nv clusters decrease with HFD in SCRNA as has been shown in previous flow analysis (80). However, there were also some inconsistencies. Tconv cells in flow analysis are not significantly changes in flow analysis. Since we know that CD4Nv decrease, it was surprising to see that CD4Trm also decreased in the SC RNAseq analysis. Clarifying whether net decrease of Tconv cells seen in SC RNAseq is biologically accurate, or whether subsets other than CD4Nv and CD4Trm contribute total CD4 ATT will need to be determined in future studies. The DN cells are also more difficult to interpret. While NK1 and NK2 populations were unchanged with HFD feeding, ILC3s decreased, but DNstem increased. Having a better understanding of DN stem and their activity in ND and HFD mice will be important for understanding ATT regulation in the future. It is also unclear where double positive T cells (DPT) observed in flow analysis fit into the clusters delineated using SC RNAseq. We hypothesize that DNstem have the capacity to develop into DPTs and $CD4^+$ cells in obese mice, and the function of this population should be explored further.

Differences in subpopulation regulation were also distinct depending upon depot of origin. Compared to eWAT, iWAT had fewer Tregs and HFD did not significantly change their composition. There were also fewer iWAT ILC3s and CD8Trm were not increased with HFD feeding in iWAT feeding. oWAT differed significantly from eWAT and iWAT. There were significantly more CD4Nv and CD8Nv cells in oWAT than the other depots. There were also fewer changes to ATT subpopulation composition with HFD. oWAT is known to have a higher

density of fat associated lymphoid clusters (FALCs), lymph node-like structures where T cells and B cells aggregate around APCs (132). These structures network with the lymphatic system, making access to naïve T cells more abundant that depots like eWAT where FALCs are scarce. eWAT has also been shown to be a reservoir for memory T cells developed against viral infections (123). Distribution and regulation of ATT subpopulations correlate with functional differences in eWAT and oWAT. ATT activation assays performed on ND and HFD ATTs indicated that HFD induced ATT impairment was specific to eWAT Tconv and CD8+ cells, since oWAT ATTs were able to upregulate $CD25^+$ after co-stimulation with αCD3/CD28. Exploring gene expression differences between each cluster and depot could explain these functional differences. Basal gene expression analysis showed that ATTs from oWAT did not express genes indicating activation, such as *Ifng, Il2, Il10, and Jun*. Since there are more naïve cells in oWAT, and inflammatory gene expression is low, it seems that maintaining quiescence helps maintain ATT activation in HFD conditions.

We assessed CD8Trm, CD8Nv, CD4Trm, and CD4Nv populations in more depth, since these groups are most likely the bulk of $CD8^+$ and $CD4^+$ Tconv cells were studied in chapter 3. DE genes from eWAT and iWAT CD8Trm were analyzed using PATHER biological processes analysis to determine pathway enrichment for each group. eWAT CD8Trm cells have properties of suppressed inflammation including regulation of chromatin silencing and regulation of T cell differentiation. In iWAT, pathways related to T helper differentiation, catalytic activity, and intracellular signal transduction were enriched, suggesting CD8Trm have pro-inflammatory properties. An anti-inflammatory/suppressed eWAT phenotype was observed in CD4Trm cells, however significantly enriched pathways were different than CD8Trm cells. In CD4Trm cells, pathways related to the regulation of type 2 immune responses, peptide-tyrosine

dephosphorylation, and negative regulation of DNA recombination were observed in eWAT, while pathways related to the positive regulation of JUN kinase, hormone-mediated signaling pathway, and response to cytokine consistent with activation of TCR signaling pathways were observed in iWAT. This suggests impairment of HFD CD4 and CD8 ATTs seen in obese eWAT may be induced by separate mechanisms. Naïve cell populations also provide insight into differences due to depot. CD8Nv cells from eWAT were enriched for histone H3-K4 trimethylation and chromosome condensation, while in iWAT negative regulation of response to stimulus, regulation of RNA polymerase II, and negative regulation of metabolic processes. Since these cells are naïve, it would be expected that they would be quiescent and not upregulate pathways involved in activation or inflammation. However, enrichment of anti-inflammatory pathways observed in naïve cells was disparate from those seen in CD8Trm from eWAT. Whether inflammatory suppression in naïve cells is due to quiescent states, and whether differential suppression is due to inherent cellular characteristics or environmental surroundings would be interesting to address.

Finally, we compared gene expression of ND and HFD ATTs from eWAT to gain insight into potential mechanisms of inflammatory impairment. We were particularly interested in assessing clusters of cells that compose Tconv and CD8+ populations that we tested in Chapter 3, and focused on CD8Trm and CD4Trm since they are prominent in HFD adipose tissue. ATTs in obese tissue had few genes differentially regulated compared to ND ATTs. Since there were fewer HFD ATTs sequenced, limited DE genes could be due to decreased depth of sequencing. To overcome this limitation we compared CD4Trm and CD4Nv cells in eWAT of ND and HFD adipose tissue. When DE genes upregulated in HFD CD4Trm compared to ND CD4Trm, and HFD CD8Trm to ND CD8Trm, we observed enrichment for GSEA pathways related to $CD4^+$

and $CD8^+$ T cell exhaustion, respectively. HFD CD4Trm and CD8Trm relationship to T cell exhaustion was seen when compared to gene sets related to chronic LCMV infection (157). The hallmarks of T cell exhaustion include overexpression of cell surface inhibitory markers, downregulation of gene transcription of molecules involved in signaling from TCR and cytokine receptors, and transnationally, metabolic and bioenergetics deficiencies (159). Specific genes that could be responsible for T cell exhaustion include *Btla, Nlrc3*, Dusp6, *and Dicer1 (160-162)*. With qPCR of sorted eWAT ATTs from ND and HFD mice, we show that *Btla* is significantly upregulated in ATTs from HFD mice. Flow cytometry confirmed that HFD $CD4^+$ and DN ATTs have significantly increased expression of BTLA in these subpopulations. Investigating whether BTLA blockade could reverse ATT exhaustion should be investigated further. Additionally, other predicted mechanisms and their influence on TCR signaling should be performed in future studies to elucidate the mechanisms responsible for ATT exhaustion. Further studies exploring whether other ATT clusters from obese mice have similar exhaustion phenotypes and if clusters in iWAT or oWAT have similar characteristics will also provide insight into functional heterogeneity of ATTs.

Chapter 5: Conclusions and Future Directions

Summary

Prior to starting this work, we had the goal of resolving several gaps in knowledge related to ATT biology including the nature of activating signals from myeloid cells, the capacity of ATT to respond to activating signals, and diversity of ATTs dependent upon depot of dietary conditions. The results of the work presented in this dissertation fills these gaps and challenges current dogma that adipose tissue T cells (ATTs) are activated in terms of cytokine secretion and proliferation in the context of obesity. In chapter 2, we found that there were profound systemic metabolic and inflammatory differences in transgenic mouse models depending on whether MHCII is deleted from ATMs or ATDCs expresses MHCII. Previous studies have shown that LysMCre x $MHCII^{fl/fl}$ mice that lack MHCII expressing ATMs have improved GTT, ITT, and reduced pro-inflammatory CD11c+ ATMs with HFD feeding. However, with loss of MCHII on ATDCs there is a gross dysregulation of ATT cells, and unchanged systemic GTT and ITT in obese mice. Although systemic metabolic dysfunction may be the result of MHCII deficiency in organs other an adipose tissue, this study suggests that ATDCs are required for providing signals to maintain CD4+ and CD8+ ATT homeostasis. ATDCs are more efficient APCs and loss of MHCII signals required for tonic signaling is also detrimental to chronic low grade inflammation and insulin sensitivity in obese mice.

In Chapter 3, we hypothesized that ATTs cells have tissue resident functions independent of adipose tissue APCs and influence obesity mediated inflammation. ATTs are regulated in the

complex heterogeneous niche of white adipose tissue, and obesity regulates their function. Unlike previous findings, we show that activation in terms of cellular markers, proliferation, and cytokine secretion is impaired in obese murine eWAT and human oWAT. Antigen presentation is required for ATT impairment to occur over the course of 12 weeks of HFD feeding, and soluble mediators from obese SVF drive impairment phenotypes of ATT activation. Although the T cell exhaustion marker PD1 was increase on CD8+ ATTs in obese adipose tissue, PD1 blockade failed to reverse ATT inflammatory impairment.

In Chapter 4, we utilized single cell RNA sequencing to evaluate potential signaling pathways and gene expression regulation to determine possible mechanisms of dysfunction. Since obesity influences cellular metabolism and inflammation, there are multiple pathways that could influence obesity induced ATT impairment. In addition to finding several gene targets of ATT impairment in murine eWAT, we found ATT context is important, as oWAT show no impairments in ATT activation and inflammation. However, in eWAT we identified there were several possible mechanisms to explain ATT impairment. CD4Trm and CD8Trm in obese mice are enriched for $CD4^+$ and $CD8^+$ exhaustion pathways identified in the context of chronic LCMV infection. We confirmed with both transcriptional and protein levels that BTLA is increased in HFD ATTs, and perusing BLTA blockade will be an interesting target to peruse in the future. Additionally, inflammasome and mRNA regulating proteins like *Nlrc3* and *Dicer1* will be interesting targets to investigate further. Verifying *Nlrc3, or Dicer1* upregulation and potential transgenic mouse models will help assess whether ATT impairment in obese adipose tissue is physiologically beneficial or detrimental.

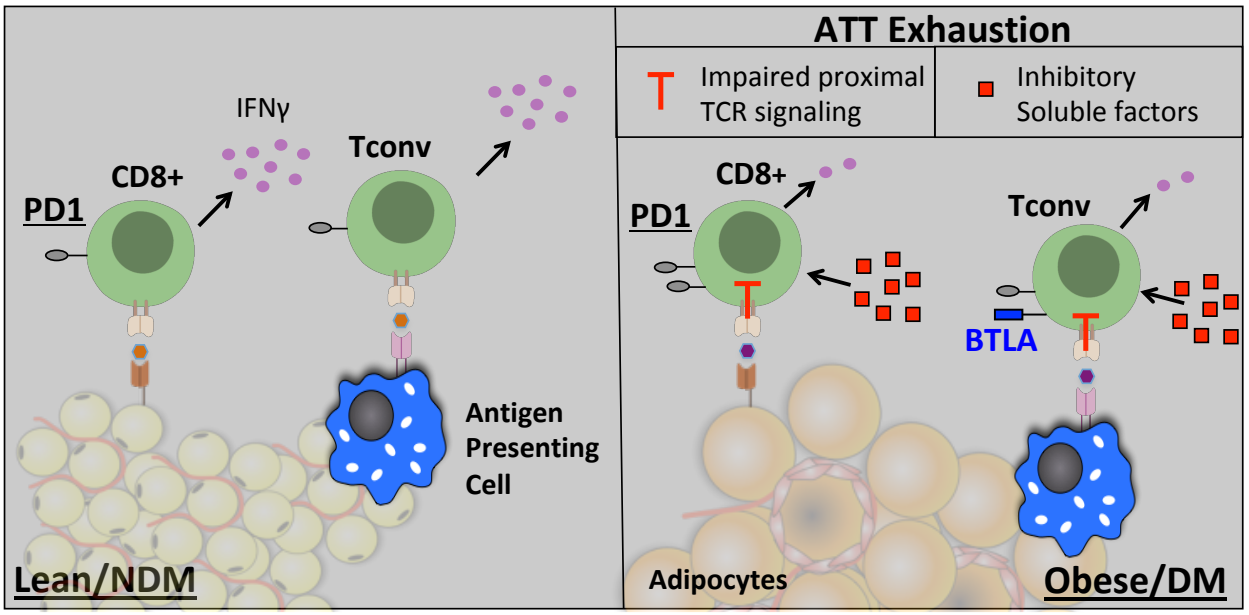


Figure 5-1 – Concluding model of obesity induced ATT exhaustion

Objectives, Major Findings, and Implications for Chapter 2

ATM mediated antigen presentation has been implicated in ATT activation, inflammation, and insulin resistance in obese adipose tissue. This suggests the network between innate and adaptive immune systems is important in regulation of chronic low-grade inflammation. (80). However, dendritic cells, not macrophages, are the primary antigen-presenting cell subtype in lymphoid organs while their antigen presenting capabilities was unknown in adipose tissue. Therefore, our objective was to assess ATDC to ATT networks and determine whether ATDC are responsible for ATT mediated inflammation as is seen with ATMs, or whether they maintain anti-inflammatory signals in the context of obesity. We hypothesized that ATDCs and ATMs were responsible for activation and maintenance of Tregs and pro-inflammatory Tconv cells, respectively.

Our findings showed that ATMs and ATDCs have distinct differences in ATT maintenance, however differential polarization of CD4 T cells does not seem to be a primary mechanism. Instead, it seems that ATDCs are more efficient APCs and are better at activating T cells with small concentrations of antigen. These findings have several important implications for interpreting previous studies of MHCII mediated ATT activation and future directions. While reducing antigen presentation with the $MHCII^{fl/fl}$ x LysMCre (MMKO) does not significantly regulate ATT homeostasis, $MHCII^{fl/fl}$ x CD11cCre (M11cKO) results in gross Tconv, Treg, and $CD8^{+}$ dysregulation. Therefore, in MMKO mice, MHCII presentation by ATDCs is required for maintaining tonic signals needed to maintain ATT homeostasis. However knocking out MHCII presentation by highly efficient ATDCs, as seen in M11cKO, removes signals needed to maintain $CD4^{+}$ Tconv and Treg ATTs. Therefore, breaking the network connecting innate and adaptive immune systems through antigen presentation is effective at reducing obesity induced inflammation and improving systemic metabolism only when ATDC MHCII can maintain tonic signals required fro CD4 homeostasis.

This study adds to previous literature implicating ATDCs as contributors to chronic low-grade inflammation in obese adipose tissue. ATDC mediated MHCII expression does not significantly alter glucose or insulin tolerance in ND or HFD mice. This suggests that T cell homeostasis may not be essential for adipose tissue inflammation, factors like CCR7 mediated ATDC trafficking and signals from PPARy by are more critical components of chronic low-grade inflammation.

There are several important limitations of using $MHCII^{fl/fl}$ x CD11cCre (M11cKO) mice. Although it was our intention to assess ATDC mediated ATT inflammation and insulin resistance in obese mice, it is clear that MCHII signals are also knocked down in other cell types

like macrophages. Additionally disruption of dendritic cell mediated antigen presentation in other tissues like the gut makes conclusions regarding systemic inflammatory and metabolic measures unclear. While gene expression of *Il1b* and *Ccl2* in adipose tissue is not reduced in HFD M11cKO mice, it is unclear whether adipose tissue specific inflammation contributes to statistically unchanged GTT and ITT results in HFD WT and M11cKO cohorts. Dendritic cell mediated antigen presentation is important for gut homeostasis, and M11cKO resulted in rectal prolapse and GI inflammation that makes interpreting systemic inflammation and metabolism difficult (113). Experimentally, there are several studies that would help strengthen this study. Evaluating T cell specific inflammatory cytokines such as IL2 and IFNy from in the blood and gut would make it clear which tissues contribute to inflammation in M11cKO mice. Additionally, assessing intracellular cytokine production/polarization and proliferation of ATTs would help determine function differences in WT and M11cKO mice. Unfortunately, there are not technologies that allow us to knockdown MHCII specific to adipose tissue, but SC RNAseq may reveal better Cre drivers for ATDC specific MHCII knockout mice in the future.

This study raises important questions that should be addressed in the future including:

1) What mechanism(s) drives increase of CD8+ and CD4+ CD8+ double positive ATTs in M11cKO mice? We hypothesize that DNstem cells identified in Chapter 4 may be responsible for ATT expansion, and understanding their function may be important.
2) Does the inflammatory capacity of M11cKO ATTs differ from WT ATTs in lean and obese mice? Determining whether inflammatory capacity of M11cKO ATTs remains intact (like MMKO mice in Chapter 3) would be of interest.
3) What is the function of DN and DPT ATT cells in lean and obese adipose tissue? Although some studies have explored function of ILC cells that could comprise the DN

population, the complexity of this population is not well understood. Defining how DN and DPT cells arise in AT, and how obesity regulates their inflammatory function will be next steps in understanding ATTs.

Addressing these questions will be important next steps in understanding how ATTs are regulated in adipose tissue, and their contribution to chronic low-grade inflammation.

Objectives, Major Findings, and Implications for Chapter 3

Although understanding adipose tissue leukocyte networks like APCs and ATTs are important for comprehending obesity mediated adipose tissue mediated chronic low-grade inflammation, basic knowledge of cell function is also required. Our objective for this chapter was to determine ATT specific characteristics after activation via TCR and CD28 specific activation. We *hypothesized* that there would be function differences compared to splenic T cells, and that ATTs would have enhanced inflammatory output in obese environments.

Despite previous studies indicating ATTs contribute to obesity-mediated inflammation, our findings have led us to challenge this dogma. ATTs were cultured within the stromal vascular fraction from adipose tissue and stimulated in a T cell specific with αCD3/CD28 dynabeads. We found activation in terms of CD25 (IL-2rα) cell surface expression and cytokine secretion were blunted in ATTs from obese adipose tissue compared to lean ATT and splenic T cell controls. LysMcre x $MHCII^{fl/fl}$ mediated antigen presentation is required for development of ATT impairment, and soluble factors secreted by HFD SVF can also decrease inflammatory capacity of ATTs. Similar to what is known about T cell exhaustion after chronic antigen stimulation in other settings (e.g. tumor infiltrating lymphocytes), we observed HFD induced

impairments in T cell proliferation, cytokine production, and expression of activation markers in both mice and humans.

Recent studies from other groups find phenotypes that are similar to ours, but come to different conclusions. Shirakawa et al. determined that there is a higher frequency of $PD1^+$ CD4 Tconv cells in obese eWAT that have reduced IFNy and increased osteopontin (*Spp1*) production (98). Ultimately, they conclude that CD4Tconv in obese eWAT are senescent but contribute to chronic low-grade inflammation via osteopontin secretion. Although we both see canonical inflammatory impairment in ATTs from obese tissue, senescence and exhaustion are disparate modalities for impairment. Senescence does not account for chronic exposure to activating antigens or the consequences for impaired canonical T cell inflammatory properties. Furthermore, our SC RNAseq analysis of CD4Trm and CD8Trm subsets did not detect enrichment of senescence markers described by Shirakawa (*Cdkn1a, Cdkn2b, Satb1, Eef1a1*) in HFD groups. However, there are reports that show obesity induces T cell exhaustion in the spleen and liver of mice fed HFD for 10 months (23). They determine that although obesity induces $PD1^+$ expression, T cell exhaustion, and increases the risk for cancer, obese mice are also more responsive to PD1 blockade and recover with immunotherapy treatment. This suggests unlike ATTs, obesity does not result in terminal exhaustion in in T cells outside of adipose tissue. Whether this is due to different environmental signals or activating antigens will be interesting to explore in future studies.

Although these findings are provocative, there are several limitations that should be considered. First, ATT activation assays required immune and stromal cells from adipose tissue to maintain viability of the cells over the course of 3 days. Non-T cells in this culture could influence the cytokine secretion and proliferation data. Since the composition of ND and HFD

SVF are different, there could be factors in the culture conditions that contribute to our observation of ATT inflammatory impairment. This could be viewed as a benefit, since it models signals ATTs would receive in vivo, however, it also does not cleanly distinguish ATT cell function. Therefore it is difficult to determine to pinpoint whether ATTs or surrounding cells are responsible for reduced ATT functionality in obese tissue. Although there are multiple cell types within SVF that could influence ATT function, we remove adipocytes. Adipose tissue parenchymal cells are potentially important mediator of $CD8^+$ ATT function since they expression MHCI and PDL1. Additionally, it is possible that differences in lipid composition in ND and HFD SVF could influence ATT functional outcomes. However complex, each of these possible contributions to ATT impairment are testable and could lead to a mechanistic driver of ATT impairment in future studies.

There are several important questions regarding ATT impairment in obese adipose tissue that should be explored in the future. Determining the mechanisms for ATT impairment is essential and was a primary goal investigated further in chapter 4. Unraveling whether ATT impairment is due to metabolic dysregulation such as insulin resistance, co-inhibitory receptor expression (other than PD1), or impairments in intracellular signaling mechanisms are important to understand. Importantly, CD8 and Tconv cell impairments may be due to discrete mechanisms, which will be interesting to dissect in the future. If constant antigen exposure is necessary for Tconv impairment, focusing on antigens from the SVF or gut may be of interest. However, if CD8+ ATTs have characteristics of exhaustion, adipocyte composition presentation of endogenous antigens by MHCI should be investigated.

Once mechanisms of ATT impairment have been established, we will be able to evaluate whether reduced inflammatory capacity is beneficial in the context of chronic low-grade

inflammation. Currently we can imagine that either reduced ATT inflammatory capacity is beneficial because it limits pro-inflammatory cytokine secretion like IFNγ and IL2. However, reduced ATT inflammatory capacity may also prevent maintenance of adipose tissue homeostasis. Additionally, honing in on kinetics of *in-vivo* ATT activation with HFD will be important. *In-vivo* activation occurs before 6 weeks of HFD feeding where we first saw impairments in cytokine secretion. Pinpointing when ATTs first recognize their cognate obesity antigen(s) responsible for Tconv activation will critical for studying the antigens responsible for Tconv activation. Once adipose tissue antigens are found, we will have the ability to study obesity as an autoimmune disease of the adipose tissue. Additionally, we will be able to determine whether stopping ATT activation prior to 6 weeks of HFD feeding will abrogate chronic low-grade inflammation with chronic HFD feeding.

Objectives, Major Findings, and Implications for Chapter 4

This study was the first to perform in depth SC RNAseq on ATTs from mice receiving a chronic 12-week regimen of HFD. Previous studies by the Dixit Lab have performed SC RNAseq on ATTs from mice fed a ketogenic diet for 1 week, revealing increased ATT cell composition with decreased *Il1b* expression (148). As we observed in Chapter 3, there are functional differences in ATTs depending upon duration of HFD feeding. Understanding the diversity of ATTs in the context of cellular subsets, depot differences, and diet induced gene expression differences was our overall objective of Chapter 4. Each of these three factors helped us comprehend the function of ATTs and how they are regulated by localized environmental signals and obesity. We found nine distinct ATT subtypes in SC RNAseq analysis of murine eWAT, oWAT, and iWAT. While most of the ATT cell subsets have been identified previously,

our comprehensive analysis of composition and gene expression in lean and obese mice provide us with extensive data to explore.

We decided to focus on ATT clusters we believed to comprise the CD4+ Tonv and CD8+ groups where we observed inflammatory ATT impairment in Chapter 3. Comparing resident memory (CD4Trm) to naïve (CD4Nv) within ND and HFD groups allowed us to assess a sufficient number of DE genes to perform pathway analysis for each group. Strikingly, we saw CD4Trm and CD8Trm cells from HFD eWAT were enriched for $CD4^+$ and $CD8^+$ T cell exhaustion observed in chronic LCMV infections (157). An exhausted phenotype observed in freshly isolated CD4Trm and CD8Trm explains functional findings of ATT inflammatory impairment observed in Chapter 3. Exhaustion results in inhibition of T cell proliferation, IL-2R expression, and cytokine secretion that cannot be reversed with PD1 blockade, reflecting results observed in eWAT ATTs. We have verified BTLA is upregulated in HFD cells and is a good target to investigate in immunotherapy moving forward. We have also pinpointed specific genes like *Nlrc3, Dusp6, and Dicer1*, which could mediate molecular aspects of T cell exhaustion such as inhibition of TCR signaling pathways, and translation defects. Each of these mechanisms will also be explored further in the future.

Data from this analysis also provides information about $CD3^+$ ATT subsets of which we did not perform in depth analysis in this dissertation. The function and identity of DNStem cells in SC RNAseq and have been of interest. There is not currently literature that explores the function or development of these cells in lean or obese states. Since they possess gene expression signatures indicating progenitor function, it would be interesting to assess whether they are responsible for increases in T cell compartments observed after 1 week of ketogenic diet observed by the Dixit group (148). We also hypothesize these progenitor-like cells may develop

into the $CD4^+$ $CD8^+$ double positive T (DPT) cells observed in M11cKO mice from Chapter 2. We did not have sufficient information or substrate to investigate the origin of DPT cells in Chapter 2, but further analysis of DNStem may lead to some hypotheses regarding their ability to transform into canonical T cell subtypes.

While SC RNA seq of ATTs from 3 depots in lean and obese mice provides a wealth of data that will need to be explored in more detail in future studies, there are several limitations that should be noted. Since ND and HFD ATT sorting and sequencing were done separately, there were fewer ATTs sequenced from the obese adipose tissue. The $CD3^+$ cells from HFD mice also had universal contamination with macrophage genes, which is a problem that has been seen previously in the field. There were also some questionable findings in regards to cell types found after the $CD3^+$ sort. Why there were NK and ILC3 cells that are known lack CD3 expression is unknown. Future studies can investigate whether there was a technical mistake with our sort, or there are CD3+ subsets of ATTs with gene expression similar to ILCs and NK cells can be assessed in flow cytometry.

Final Thoughts

Obesity induced ATT exhaustion is a novel finding that can reshape the way we think about chronic low-grade inflammation. While previous studies suggested that antigen presentation via MHCII contributes to ATT inflammation and insulin resistance in the context of obesity, it appears that chronic exposure to antigen ultimately results in decreased inflammatory potential of T cells. Understanding the juxtaposition of chronic low-grade inflammation with T cell exhaustion will be important for understanding how obesity can simultaneous induce

inflammatory mediated insulin resistance and result in impairment of T cell inflammatory responses resulting in increased morbidity and mortality in the context of infections and cancer.

Moving forward, identifying the antigen(s) responsible for ATT activation and their ultimate exhaustion still need to be identified. This will allow us to understand whether ATTs play an important role in initiating inflammation via mechanisms like the recruitment of innate immune cells and the metabolic result of exhaustion. Alternatively, it is possible that ATTs do not play an essential role in adipose tissue induced chronic low-grade inflammation and subsequent insulin resistance.

However, understanding ATT exhaustion could be critical for understanding obesity-induced morbidity outside the scope of type II diabetes. People with obesity have a higher risk for worsened outcomes to viral infections and are more prone to cancer, two diseases that are highly dependent upon antigen specific responses from T cells. Further exploration of mechanisms responsible for exhaustion will be important to detail and investigate in lymphatic T cells. If T cell impairments associated with obesity are systemic and occur with similar kinetics, it will be important to investigate the gut as a possible source of antigen. However, if ATT exhausting is unique, adipocytes may be important to peruse.

Ultimately, regardless of the source of antigen, mechanisms governing ATT exhaustion, and the role of ATTs in chronic low-grade inflammation, future studies will provide us with information that will help shape our strategies for treating both metabolic and immune morbidities associated with obesity.

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