The Role of Adipose Tissue Macrophages in Obesity-Associated Insulin Resistance

by

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DEDICATION

To every Black boy and girl: the limits the world tries to impose on you do not exist.

Whatever you think you can do, you can.

In loving memory of my brothers

Stachonn Robert Nance

Daelon Christopher Locke

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TABLE OF CONTENTS

LIST OF FIGURES

Figures

LIST OF TABLES

Tables

LIST OF ABBREVIATIONS

AT = adipose tissue
ATDC = adipose tissue dendritic cell
ATM = adipose tissue macrophage
BAT = brown adipose tissue
BMI = body mass index
CCL18 = C-C motif chemokine ligand 18
CVD = cardiovascular disease
DM = Type 2 Diabetes/diabetic
EC = endothelial cell
ELISA = enzyme-linked immunosorbent assay
eWAT = epididymal white adipose tissue
iWAT = inguinal white adipose tissue
HFD = high-fat diet
HUVEC = human umbilical vein endothelial cells
IL6 = interleukin 6
i.p. = intraperitoneally
LAM = lipid-associated macrophage
Mcp1 = monocyte chemoattractant protein 1
Mcsf = macrophage colony stimulating factor
MetS = metabolic syndrome
MHO = metabolically healthy obesity
MMe = metabolically activated ATM
MUO = metabolically unhealthy obesity
MSR1 = macrophage scavenger receptor 1
ND = normal diet

NDM = non-diabetic

oWAT = omental adipose tissue

RNAseq = RNA sequencing

SAT = subcutaneous adipose tissue

scRNAseq = single-cell RNA sequencing

sn-RNAseq = single nuclear RNA sequencing

Tconv = conventional T cells

Treg = regulatory T cells

VAT = visceral/omental adipose tissue

WT = wild-type

ABSTRACT

While epidemiologic studies show strong correlations between obesity and Type 2 Diabetes (DM), there is substantial variation in measures of metabolic disease in people with obesity. Two phenotypes that have been described are 1) metabolically unhealthy obesity (MUO) and 2) metabolically healthy obesity (MHO). Individuals with MHO often have more subcutaneous adipose tissue (SAT) than visceral adipose tissue (VAT), normal insulin resistance, and adiponectin levels comparable to their non-obese lean counterparts, protecting against metabolic diseases. The MUO phenotype is described as having more VAT than SAT and increased inflammation increasing the risk for obesity-associated DM. Adipose tissue macrophages (ATMs) are a primary mediator of inflammation in adipose tissue. In the obese state, inflammatory $CD11c^+$ ATMs predominate, secreting pro-inflammatory cytokines that contribute to systemic insulin resistance. This dissertation aims to elucidate the role of ATMs in metabolic diseases and elucidate pathways that link obesity and DM by investigating two potential modulators of obesity-induced inflammation expressed in ATMs: macrophage scavenger receptor 1 (MSR1) and C-C motif chemokine ligand 18 (CCL18). Both genes were found to have higher expression in adipose tissue from obese DM individuals compared to non-DM individuals, but their potential contributions to insulin resistance are incompletely understood. In our first study, RNA sequencing of human adipose tissue (AT) from obese subjects demonstrated that *MSR1* expression is

macrophage-specific and is increased in VAT from obese individuals with DM compared to their obese, non-DM controls. Experiments in *Msr1*$^{-/-}$ and wild-type non-littermates suggested that *Msr1*$^{-/-}$ mice are protected from high-fat diet (HFD)-induced obesity. However, *Msr1*$^{-/-}$ mice were not protected from HFD-induced obesity, impaired glucose metabolism, or ATM accumulation when compared to *Msr1*$^{+/-}$ littermate controls suggesting that *Msr1* is not required for obesity-associated inflammation and insulin resistance in mice.

In our second study, single nuclear sequencing of obese human AT showed that *CCL18* is expressed in the lipid-associated macrophage (LAM) ATM subset. To elucidate the role of CCL18 in AT, human mature adipocytes from obese subjects were treated with or without CCL18 and we observed that CCL18 suppresses immune pathway genes. Experiments in C57BL6 mice treated ± CCL18 revealed that CCL18 does not impair glucose tolerance or alter leukocyte content with short-term HFD. However, in a chronic model of HFD feeding for 6 weeks, CCL18 suppressed CD11c^{+} ATM accumulation in epididymal white adipose tissue (eWAT). The accumulation of CD11c^{+} ATMs was not related to changes in proliferation rates. CCL18 treatment of obese human AT or HUVECs did not alter angiogenesis. Overall, this work demonstrated that while *Msr1* is not required for obesity-associated insulin resistance, CCL18 may have a protective role in obese adipose tissue by suppressing interleukin signaling and inflammatory ATM accumulation.

CHAPTER 1

Introduction

Portions of this chapter have been published:

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Abstract

Adipose tissue macrophages (ATMs) are a well-characterized regulator of adipose tissue inflammatory tone. Previously defined by the M1 vs M2 classification, we now have a better understanding of ATM diversity that departs from the old paradigm and reports a spectrum of ATM function and phenotypes in both brown and white adipose tissue. While the paradigm that resident ATMs predominate in the lean state and obesity leads to the accumulation of lipid-associated and inflammatory ATMs still broadly remains rigorously supported, the details of this model continue to be refined and single-cell data provide new insight into ATM subtypes and states. This review provides an updated overview of ATM activation and function, ATM diversity in humans and rodents, and novel ATM functions that contribute to metabolic homeostasis and

disease. We will also highlight the novel findings in the field of immunometabolism and ATM biology that led to the studies we have pursued in this dissertation.

Obesity, Type 2 Diabetes, & Metabolic Syndrome

Obesity is characterized by an accumulation of visceral (VAT) and subcutaneous (SAT) adipose tissue (AT) and is a potentially modifiable risk factor for Type 2 Diabetes (DM). DM is a metabolic disorder characterized by insulin resistance and hyperglycemia that affects 11% of the U.S. population, according to the Centers for Disease Control and Prevention. While the association between obesity and DM is well established, the mechanisms that link the two remain incompletely understood. Obesity is also a risk factor for Metabolic Syndrome (MetS) in both adults and children [1-3] for which a feature is excess secretion of pro-inflammatory cytokines from adipose tissue. MetS is characterized by altered glucose metabolism, reduced HDL levels, as well as elevated triglycerides, blood pressure, and waist circumference. Individuals with MetS have a higher risk of developing cardiovascular disease and DM [4].

The association between obesity and metabolic disease is variable and there is a suggestion that there are metabolically healthy and unhealthy phenotypes. Metabolically healthy obesity (MHO) phenotypes have been described where individuals with obesity based on BMI do not show evidence of cardiometabolic disease[5; 6]. While criteria for MHO vary between studies, MHO individuals typically have more SAT than VAT [7], blood pressure ≤ 140 mmHg, blood glucose ≤ 7.0mmol/L, fasting triglycerides ≤ 1.7mmol/L, HDL > 1.3 mmol/L, and no evidence of cardiovascular disease (CVD) [8-10]. Based on definitions using the number of MetS criteria and HOMA-IR, prevalence estimates are that about 50% of people with obesity can be classified as MHO in North

America and Europe.[6] In a cohort of patients with obesity undergoing bariatric surgery at Michigan Medicine and the Ann Arbor Veterans Administration, ~30% of patients have DM based on HOMA-IR. For this dissertation, we have primarily focused on the presence or absence of DM based on HOMA-IR as distinguishing MHO and MUO phenotypes. While MHO individuals may have an increase in circulating insulin and a mild risk for cardiovascular disease, and a low HOMA-IR score indicative of normal insulin sensitivity [6; 11]. The transition from MHO to metabolically unhealthy obesity (MUO) increases with age and increased waist circumference and is characterized by developing metabolic abnormalities like CVD and DM [10; 12; 13]. People with MUO have more VAT than SAT, present with muscle loss, and have increased inflammation and insulin resistance [7].

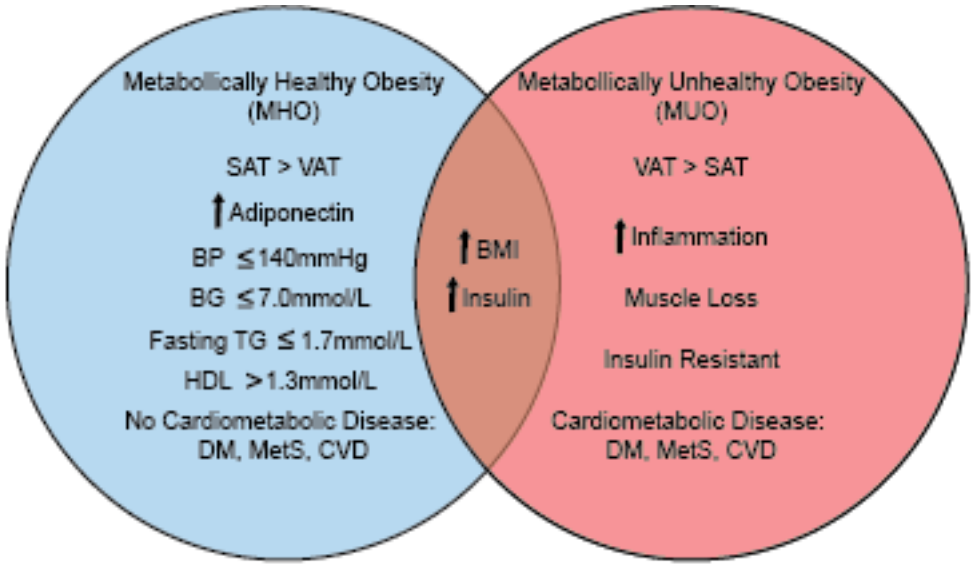

Figure 1.1. Metabolically Healthy Obesity vs Metabolically Unhealthy Obesity

A cross-sectional study of obese and non-obese adults found that there was a subset of obese individuals with elevated levels of adiponectin, indicative of a MHO

phenotype similar to their non-obese counterparts [14]. Elevated levels of adiponectin promote adipose tissue expansion, protecting against metabolic disorders [15]. This study suggests that individuals with MHO have a reduced risk for MetS than individuals with MUO; other studies define MHO as having two or fewer MetS criteria [6]. Furthermore, impaired adipose tissue function contributes to MetS risk [16].

Adipose tissue is not only a storage organ for lipids, but it also secretes adipokines and cytokines that regulate energy homeostasis and inflammation. Adipose tissue has historically been divided between adipocytes and non-adipocyte cells that compose the stromal vascular fraction (SVF) that houses preadipocytes, endothelial cells, and leukocytes like T-cells, dendritic cells, eosinophils, and adipose tissue macrophages (ATMs) [17]. Obese adipose tissue secretes excess pro-inflammatory cytokines that promote chronic inflammation, insulin resistance, and metabolic disease with many of the pro-inflammatory signals coming from the SVF [18]. Human and mouse studies have shown that increased adiposity and adipose tissue expansion are associated with ATM accumulation and crown-like structures (CLS) composed of macrophages surrounding dead and dying adipocytes [19] [20].

ATMs are a primary mediator of cytokine production in adipose tissue producing pro-inflammatory cytokines such as IL-6, TNFα [21], and IL-1β [22]. These cytokines have been shown to decrease insulin-stimulated glucose uptake in adipocytes and promote systemic insulin resistance [23]. These findings suggest a role for ATMs in the pathogenesis of obesity-induced insulin resistance. Mice fed a high-fat diet (HFD) to induce obesity have been shown to have an increased number of ATMs, $CD9^{+}$ CLS, and perilipin in their AT, suggesting that obesity induces a pro-inflammatory response

and lipid accumulation [24]. These changes are associated with quantitative and qualitative changes in the ATM content in mouse and human AT.

Adipose Tissue Macrophage Diversity

ATMs were initially described along the lines of two classical macrophage phenotypes: proinflammatory M1 and anti-inflammatory M2 based on limited markers. We now understand that this is a vast oversimplification and macrophage activation states can be defined along a spectrum as opposed to a simple two-dimensional axis [25; 26]. Treatment of human monocyte-derived macrophages with numerous stimuli identified >40 gene modules that uniquely respond to stimuli that can be used to better define co-expressed gene networks in response to stimuli outside the M1/M2 axis. In addition, features of macrophages such as developmental origin (e.g., yolk sac versus monocyte-derived), proliferative capacity, and tissue location generate a broad range of macrophage phenotypes [27; 28]. Our current understanding of ATM diversity is primarily derived from mouse models where tools for tracking and differentiating the developmental ontogeny of macrophage subtypes have been powerful tools. The expansion of single cell and single nuclear RNAseq datasets on ATMs has also revealed a greater diversity of ATMs than was previously known.

Resident ATMs in lean states have multiple developmental origins

Under normal conditions, there is a balance between anti-inflammatory cytokines and pro-inflammatory cytokines in the adipose environment to maintain metabolic homeostasis. In the lean state, resident ATMs predominate maintaining immunometabolism homeostasis. In lean mice, the majority of resident ATMs express

markers of alternatively activated M2 macrophages such as CD206 and CD163 that facilitate their identification and experimental separation [29-31]. Experimental ablation of $CD206^+$ ATMs leads to a remodeling response in adipose tissue characterized by smaller adipocytes and the proliferation of adipocyte progenitor cells [32]. Recent use of single-cell mass cytometry (CyTOF) further subdivided the lean ATMs based on markers TIM4, CD163, and MHC II (**Figure 1.2**). $CD163^+$ and $TIM4^-$ populations are dependent on *Ccr2* in mice supporting their origin from bone marrow-derived monocytes [33]. $TIM4^+$ ATMs are of embryonic origin based on lineage tracing and inducible labeling [33; 34]. These observations align with data defining $TIM4^+$ ATMs as a source of PDGFcc and regulator of AT energy storage in mice [35]. The regulation of resident ATM content is controlled independently from monocyte-derived macrophages and appears to involve mechanisms related to *in situ* proliferation. For example, early adipose tissue remodeling by HFD feeding triggers rapid ATM proliferation that expands the ATM pool in concert with adipocyte enlargement and hypertrophy (**Figure 1.2**) [28; 36].

Adipose Tissue Macrophages and Obesity

As obesity progresses, bone marrow-derived ATMs increase in quantity in adipose tissue [19] and proliferate [37] inducing chronic inflammation and increasing susceptibility for insulin resistance [25; 38-40]. Inhibition of ATM proliferation during obesity blunts AT inflammation and improves insulin sensitivity [40]. scRNA-seq of epididymal white adipose tissue (eWAT) from mice on chronic HFD revealed 14 unique clusters of leukocytes and 3 of those clusters were differentially expressed in HFD

mice.[41] Two clusters were increased with HFD: Cluster 1 contained $CD9^+$, $Trem2^+$, and $Mmp12^+$ inflammatory ATMs which are markers for lipid-associated macrophages (LAMs) discussed further below; Cluster 5 comprised $Col3a1^+$, $Ly6a^+$, $Cd34^+$ myeloid progenitor cells. One cluster was decreased (Cluster 2) and contained $Mrc1^+$, $Lyve1^+$, and $Mmp9^+$ consistent with markers of resident ATMs. Additionally, increased expression of Col1a1 and $Ly6a^+Col1a1^+$ cells in AT correlated with AT fibrosis. Overall, this demonstrated that chronic obesity promotes the infiltration of inflammatory ATMs and induces AT fibrosis which the proportion of resident ATMs decreases.

In mice, obesity induces a prominent population of ATMs expressing CD11c, CD9, and TREM2 [24; 42; 43]. Such recruited ATMs were shown to be monocyte-derived and dependent on chemokine pathways such as the CCR2/CCL2 axis for accumulation in adipose tissue in mice [44]. Over the years, $CD11c^+$ ATMs induced by obesity have been described as having metabolically activated phenotypes and lysosomally activated phenotypes that are distinct from the classic M1/M2 paradigm [26; 45]. Metabolically activated macrophages (MMes) are a phenotype induced by metabolites such as free fatty acids (FFAs), high glucose, and insulin to produce a unique inflammatory profile and express lysosomal surface proteins [46], differentiating them from classically activated M1 ATMs [26]. Cytokine expression in MMes is regulated by toll-like receptor 2 (TLR2), NADPH oxidase 2 (NOX2), and MYD88 [46]. More recently, single-cell RNAseq studies have coalesced around defining the lipid-associated macrophage (LAM) phenotype of $CD11c^+$ ATMs as being associated with TREM2 expression and with a lipid-activated profile that appears to be a common

activation pathway for many types of tissue macrophages including in adipose tissue, liver, and the brain [24; 47; 48].

LAMs in both HFD mice and humans with obesity significantly expressed CD9, Fabp5, Fabp4, CD63, CD36, and Trem2 [24]. Trem2 primarily functions in the immune response, triggering the production of constitutive cytokines. It is expressed in various tissues in both mice and humans, including adipose tissue. $TREM2^{+}$ LAM ATMs have been shown to play a functional role in adipose tissue lipid metabolism in both mouse and human VAT [24]. LAMs from *Trem2* $^{-/-}$ mice on HFD effectively store lipids and failed to accumulate in adipose tissue suggesting a requirement for *Trem2* in LAM ATM accumulation [24]. We note that studies on the metabolic role of TREM2 in mice have been somewhat mixed. *Trem2* $^{-/-}$ mice have been shown to have worse insulin resistance and hepatic steatosis related to increased serum ceramide levels [49]. Bone marrow transplantation suggested that these effects of *Trem2* deficiency were not related to immune cell TREM2 function. Overall, while TREM2 appears to be a strong marker of LAM ATMs, the functional role of TREM2 in metabolic inflammation remains not fully understood.

Surprisingly, the capacity of weight loss or calorie restriction to reverse the accumulation of LAMs in adipose tissue appears to be limited [48; 50; 51]. Weight loss interventions in mice do not restore AT immune cells to the lean state which may have implications for health and metabolic risk for individuals who were formerly obese.

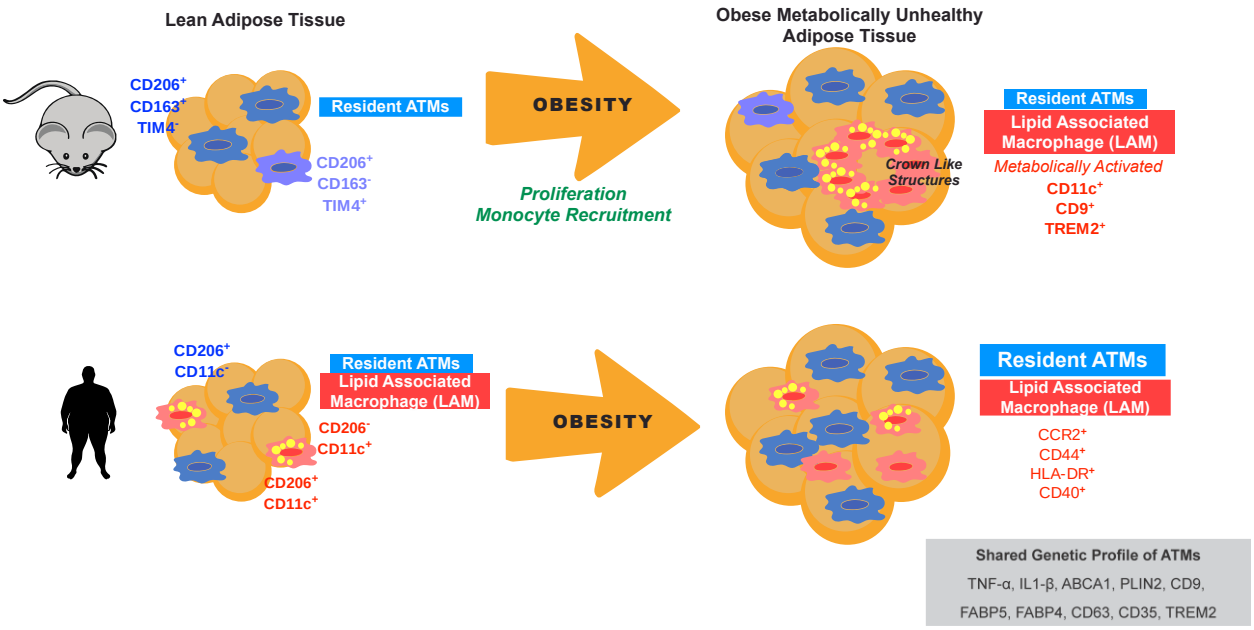

Figure 1.2. Comparison of mouse and human ATM diversity during obesity: Human vs Mouse Adipose Tissue Macrophages

There remain many gaps in our understanding of human ATM diversity and its relationship with obesity and metabolic disease. Recent RNAseq studies identify analogous ATM populations between humans and mice, however, the proportions of these cells are dramatically different in obese humans and mouse models [52; 53] (**Figure 1.2**). In general, the profound induction of LAMs in obese mice is not strongly recapitulated in human obesity. While $CD11c^+$ LAM ATMs dominate in obese and insulin resistant mice, this does not appear to be a prominent feature in human AT. Initial studies suggested that $CD206^+CD11c^+$ ATMs were increased in subcutaneous compared to visceral/omental adipose tissue depots in humans [30]. The finding of double-positive $CD206^+CD11c^+$ ATMs, a population that is less prominent in mice has been a consistent finding in human ATMs in several studies as noted below

Unlike mice where metabolic responses to HFD-induced obesity are relatively uniform, challenges remain in characterizing ATMs in the context of variation in metabolic dysfunction in obese humans [54]. $CD206^{+}CD11c^{+}$ inflammatory ATMs in SAT were reported to be increased in obese individuals with non-alcoholic fatty liver disease (NAFLD) compared to lean and obese individuals without NAFLD [55]. Macrophage concentration and Plasminogen activator inhibitor-1 (PAI-1) levels in SAT were inversely correlated with hepatic and systemic insulin sensitivity. Metabolic activation of ATMs was more robust in ATMs from obese, diabetic humans compared to obese, non-diabetic controls [56].

Using similar markers from mouse studies (CD206 and CD11c), flow cytometry analysis of human adipose tissue from obese subjects showed that VAT has significantly more M1-like ($CD206^{+}CD11c^{+}$) than M2-like ($CD206^{+}CD11c^{-}$) ATMs compared to SAT [57]. These M1-like ATMs were differentiated from M2-like ATMs by cell-surface markers CCR2, CD44, HLA-DR, and CD40, reinforcing their pro-inflammatory phenotype (**Figure 1.2**). This study observed a correlation between M1-like ATMs and the ratio of M1/M2-like ATMs in VAT with measures of insulin resistance. Additionally, human M1-like macrophages are identified by cell surface markers CD38, CD274, and CD319 [26]. While traditionally we understood that ATMs undergo a phenotypic switch from resident M2 to pro-inflammatory M1 ATMs during obesity, there is evidence that obesity produces a distinct subset of metabolically activated ATMs (MMes), ATMs isolated from obese human SAT and VAT did not express markers of classical M1 activation as expected but expressed markers for a unique subset of MMes. While both M1 ATMs and MMes produce pro-inflammatory cytokines, MMes are

differentiated from M1 ATMs by cell-surface markers ABCA1, CD36, and PLIN2, regulated by PPARγ [26].

However, many early studies did not delineate or differentiate ATMs from other myeloid cells such as adipose tissue dendritic cells which are prominent in adipose tissue and express many of the same markers such as CD11c and HLA-DR [58]. Using a more stringent separation of ATMs from dendritic cells, our group identified three primary ATM populations in human adipose tissue in both SAT and VAT depots - $CD206^{+}CD11c^{-}$, $CD206^{+}CD11c^{+}$, and $CD206^{-}CD11c^{+}$ [31]. $CD206^{-}CD11c^{+}$ dendritic cells and $CD206^{+}CD11c^{+}$ ATMs had gene expression signatures that overlapped with LAMs and were more lipid-laden. In contrast, $CD206^{+}CD11c^{-}$ resident ATMs had low lipid staining and were enriched for scavenger receptor genes. Importantly, all three populations were found in lean and obese individuals indicating that the LAMs are not dependent on obesity to reside in adipose tissue. In addition, LAM ATMs were increased in SAT compared to VAT - again a contrast from mouse models. Surprisingly, there was no significant difference in LAM-like $CD11c^{+}$ ATMs between obese and lean subjects. Instead, the quantity of $CD206^{+}CD11c^{-}$ resident ATMs were found to be increased in VAT in obese diabetic compared to lean and obese non-diabetic individuals. It is unclear if these quantitative differences contribute to metabolically unhealthy obese phenotypes or a homeostatic reaction to the obese-diabetic state.

Both human and mouse ATMs from obese AT significantly express TNFα, IL1β, ABCA1, and PLIN2. In addition, ATMs from obese VAT have greater expression of scavenger receptor CD36 compared to obese SAT in humans. A positive correlation between CD36 expression and BMI has been reported [26]. scRNA-seq of human AT

identified an uncharacterized subset of inflammatory ATMs, named IM, expressing CCL3L1, TNF, and CXCL3 that accumulate in AT of obese subjects [59]. In this report, ~30% of $CD11b^{+}CD14^{+}$ ATMs from obese WAT are IMs, which are presumably derived from a specific subset of monocytes. A wider diversity of human ATM types was reported in other single-cell studies but has not been experimentally validated [52].

Adipose Tissue Macrophage Activation & Function

The range of functions of ATMs is vast and includes their capacity to proliferate in response to adipose tissue remodeling, control of fibrotic responses and ECM production, activate adaptive immune cells such as T cells by their function as antigen-presenting cells, production of metabolic enzymes and factors that control intercellular communication in adipose tissue, modulation of adipocyte insulin sensitivity, and regulation of adipose tissue progenitor activation (**Figure 1.3**). We will highlight some of the more recent advances in identifying novel ATM functions and mechanisms of action. ATMs have a broad range of functions in different aspects of adipose tissue remodeling. Novel functions for ATMs include mitochondrial uptake which is a protective mechanism that is suppressed with obesity, induction of glycolysis, induction of phagocytic capacity, generation, and reception of extracellular vesicles (EV), and generation of inflammatory cytokines.

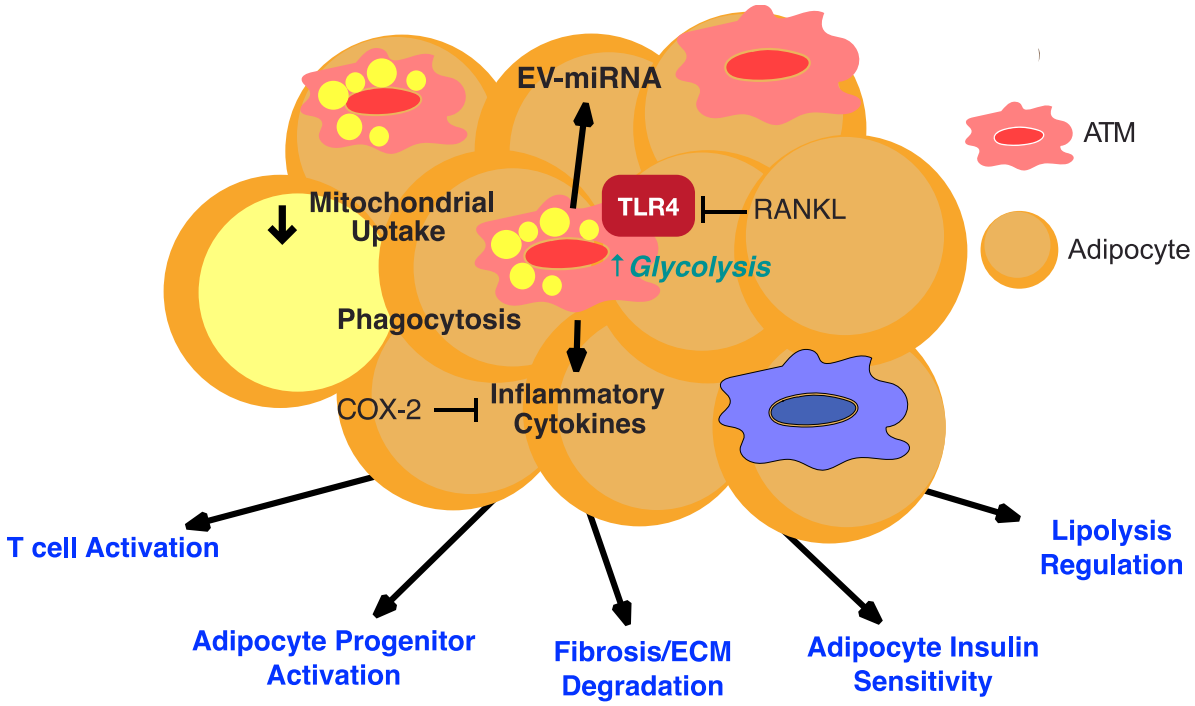

Figure 1.3. Broad functions for ATMs in the regulation of adipose tissue homeostasis.

Cytokine production and TLR4 signaling

The capacity of ATMs to generate pro-inflammatory cytokines such as TNFα and IL-6 has been reproducibly shown to be a key feature of ATM function. TLR4 activation and its downstream signaling pathways have been shown to be essential AT immune cell crosstalk and ATM activation and cytokine secretion [28; 60; 61]. Recent studies suggest inhibitory pathways in ATMs may play an important role in attempting to restrain inflammatory signals. The receptor activator of NF-κB ligand (RANKL) inhibits TLR4 activation in ATMs, subsequently crippling ATM cytokine production [62]. RANKL promotes the interaction of its receptor RANK with TRAF6, preventing TLR4 activation,

and inhibiting iNOS, TNFα, and IL1β production in ATMs [62]. This data suggests that RANKL may be a future target to limit the inflammatory response in ATMs. TLR4 signaling also regulates COX-2 expression [63], which is induced in ATMs during obesity and has been shown to limit AT inflammation [53]. Myeloid-specific deletion of COX-2 in obese mice resulted in worsened inflammation compared to WT obese mice. COX-2 deficient mice had increased ATM proliferation, pro-inflammatory cytokine production, and crown-like structures (CLS) in AT.

Phagocytic capacity as a regulator of the adipose tissue environment

One of the earliest observations is the clustering of $CD11c^+$ LAM ATMs around dead and dying adipocytes presumable to clear debris and lipid droplets. Analysis of the major subset of resident ATMs from murine epididymal white adipose tissue (eWAT) defined by TIM4 and CD163 showed that all subsets have phagocytic capacity with the $TIM4^+$ populations having the greatest endocytic potential *in vitro* and *in vivo*.[33]. The phagocytic capacity of $TIM4^+$ ATMs decreases with age but does not change with HFD. In obesity, ATMs contribute to the formation of multinucleated giant cells with high phagocytic function and the capacity to digest large particles in obese adipose tissue [64]. However, the true nature of the type of phagocytosis ATMs participate in within CLS is unclear. Studies suggest that a unique type of phagocytosis termed exophagy is the primary mechanism of CLS formation, where lysosomal enzymes are delivered to the extracellular space to facilitate the digestion of adipocyte fragments [65]. Weight loss by calorie restriction appears to activate ATM phagocytic capacity suggesting a distinct remodeling function of ATMs in settings of adipose tissue contraction [48].

Beyond the digestion of adipocytes, ATMs may participate in the uptake of other byproducts of adipocyte remodeling. Transfer of mitochondria from stressed or activated adipocytes has been shown to occur between adipocytes and macrophages in WAT and brown adipose tissue (BAT). In BAT, ATMs are responsible for removing extracellular vesicles carrying damaged mitochondria released from brown adipocytes during thermogenic activation [66]. Adipocyte to ATM mitochondrial transfer was observed to be decreased in eWAT with obesity [67]. Blockade of ATM mitochondrial transfer by inhibition of heparan sulfate biosynthesis led to increased adipose tissue mass and insulin resistance, suggesting mitochondrial clearance as a novel homeostatic function of ATMs in maintaining metabolic homeostasis. Dietary factors appear to regulate the amount of mitochondrial transfer between adipocytes and ATMs. Long-chain fatty acids suppress ATM mitochondrial capture increasing circulating mitochondrial load from adipocytes for delivery to other organs [68]. Obese mice with myeloid-specific deletion of COX-2 had decreased expression of *LAMP2*, *CD36*, and *Gas6* expression in adipose tissue, indicative of decreased phagocytotic capacity [53], suggesting that COX-2 is essential for phagocytosis by ATMs.

Extracellular vesicles and intercellular communication

Another form of intercellular communication in adipose tissue that involves ATMs is the production and uptake of extracellular vesicles (EV). Adipose tissue-derived EVs were shown to be taken up by monocytes and promote pro-inflammatory cytokine production by monocyte-derived macrophages [69]. Such EVs were shown to promote insulin resistance when injected into mice. Adipocyte-derived EVs carrying miR-34a has been

shown to be induced by obesity and transferred to ATMs where it suppressed M2 polarization pathways and promote AT inflammation and insulin resistance [70]. Beyond receiving signals through EV, ATMs have been shown to potentially modulate metabolic inflammation via the exosomes they generate. miR-690 is enriched in M2-polarized macrophage EVs that are sufficient to improve insulin sensitivity and glucose tolerance in obese mice [71]. Insulin sensitivity has also been shown to be suppressed by the delivery of miR-155-containing EVs from ATMs to adipocytes which target PPARγ. While body weight was not different between mice treated with obese ATM EV in lean mice, treated mice had impaired insulin signaling and attenuated glucose uptake in adipocytes, myocytes, and hepatocytes. Treating mice with ATM EV from lean mice repaired insulin resistance [72].

Metabolic regulation of Adipose Tissue Macrophage function

While much of the focus of immunometabolism research has centered on how ATMs alter AT and systemic metabolism, metabolic regulation of ATM function may also play a role in the response to obesity. ATMs from obese mice express gene pathways involved in glycolysis and oxidative phosphorylation, making them distinct from LPS-activated ATMs [56]. In mice, ATM activation and cytokine production are highly dependent on the bioenergetic profile of ATMs. ATMs isolated from obese AT have high glycolytic and oxidative capacity [73].

Mitochondrial function and biogenesis in AT have been shown to be impaired with obesity and Type 2 Diabetes [74; 75]. The activation state of macrophages is

known to significantly alter mitochondrial function in macrophages with classical M1 activation making macrophages more reliant on glycolysis than oxidative phosphorylation (OXPHOS) [76]. Gaps remain in our understanding of specific mitochondrial activity in ATMs in mice or humans. However, mice with reduced mitochondrial OXPHOS due to myeloid-specific deletion of CR6-interacting factor 1 (*Crif1*) had increased M1 polarization of macrophages, AT inflammation, and insulin resistance, suggesting a role for ATM mitochondrial function in metabolic dysfunction [77]. In addition, adenine nucleotide translocase 2 (ANT2) is induced in obese ATMs leading to increased mitochondrial reactive oxygen species production and damage due to modulation of free fatty acid-induced mitochondrial permeability alterations [71].

Efforts to understand the role of ATM mitochondria in cytokine production led to the testing of the near-infrared dye, IR-61, as a potential candidate to treat AT inflammation. IR-61 is a small molecule that localizes to the mitochondria of ATMs, suppressing inflammatory genes in macrophages [67]. Bone marrow-derived macrophages (BMDM) treated with IR-61 had decreased TNF, IL-6, and IL1β mRNA and protein expression upon LPS activation. This was paired with increased oxygen consumption rate and mitochondrial ATP production. Treatment of obese mice with IR-61 suppressed pro-inflammatory gene expression and reduced crown-like structures in AT accompanied by weight loss and improvements in insulin sensitivity.

Direct action of hormones altered in metabolic disease such as insulin can also shape ATM function. Insulin receptor (IR) signaling in ATMs has been shown in several studies to contribute to insulin resistance. Deletion of IR in myeloid cells led to an improvement in insulin sensitivity with obesity, related to the preservation of

IRS2/IL-4 signaling that preserves M2 activation profiles [78]. Aligned with this observation are studies showing that activation of the IR in ATMs promotes the secretion of IL-10, which has been shown to regulate hepatic glucose metabolism, suppressing gluconeogenic genes *G6pc* and *Pck* and significantly reducing glucose concentration [79]. Insulin signaling and IL-10 production in ATMs are both stunted under obese conditions [79; 80] which can promote hyperglycemia. Myeloid IR signaling may also play a role in the accumulation of ATMs and AT dysfunction with obesity. Myeloid IR deletion in obese mice decreased basal hepatic glucose production, increased insulin-stimulated glucose disposal in skeletal muscle, and blocked the accumulation of ATMs in AT with obesity [81].

A primary response to obesity and a differentiator of ATM subtypes is their accumulation and metabolism of lipids. Lipid uptake by ATMs increases with obesity in mice, with ATMs from obese mice having increased levels of triglycerides and expression of lipoprotein lipase (LPL) [36; 82]. Silencing LPL in ATMs from VAT of obese mice inhibited lipid accumulation in ATMs and increased serum FFA levels but did not affect serum triglyceride levels [83]. ATMs also express hypoxia-inducible lipid droplet associated (Hilpda), a protein that mediates lipid accumulation in AT and whose expression is increased with obesity and FFA exposure [84]. Overexpression of acyl CoA: diacylglycerol acyltransferase 1 (DGAT1) protects mice from AT inflammation suggesting that inflammatory activation and lipid storage may be decoupled in some situations [85].

BAT ATMs and remodeling with activation

The role of resident ATMs in BAT is a prominent feature of BAT remodeling upon activation. Single-cell RNA sequencing identifies dramatic remodeling of BAT macrophages and dendritic cells upon activation [86; 87]. BAT ATMs include populations of TREM2^{+} LAMs and localize to regions of new adipocyte formation suggesting a critical role for ATMs in remodeling BAT upon activation. Although the percentage of ATMs from BAT decreases with obesity [88], they have increased expression of pro-inflammatory cytokines induced by the activation of toll-like receptors [89]. Upon cold exposure, inflammatory monocytes differentiate into CD45^{+}CD11b^{+}F4/80^{+} ATMs in BAT, expressing markers of M2-like or anti-inflammatory ATMs [66].

Recent studies identified methyl-CpG binding protein 2 (*Mecp2*) as a potential regulator of BAT ATM activation. Mice with mutated nuclear transcription regulator *Mecp2* in BAT macrophages had increased weight gain, increased fat mass, and increased leptin secretion compared to controls. *Mecp2* knockout mice also exhibited decreased heat production evidenced by decreased expression of UCP-1 in BAT [90]. These findings indicate a role for ATMs from BAT in regulating adiposity and energy expenditure.

Novel Potential ATM-Derived Modulators of Obesity-Induced Changes in Adipose Tissue Immunometabolism

With obesity, there are numerous changes in AT that may provide mechanistic insight into the function of ATMs in metabolic disease and identify potentially modifiable

pathways to break the link between obesity and DM. The studies completed in this Dissertation build off of novel observation of increased expression of macrophage scavenger 1 (MSR1) and C-C motif chemokine ligand 18 (CCL18) in obese DM AT, as their role in AT has not been thoroughly studied. The premise for a focus on these inflammatory genes was based on several pieces of evidence. First, in an unbiased RNAseq evaluation of gene expression in VAT and SAT from patients with obesity, with and without DM, we identified that CCL18 and MSR1 expression is increased in VAT from obese patients with DM compared to those without DM [91; 92].

Second, both MSR1 and CCL18 have been described in the literature as highly specific to macrophages in both mice and humans. Third, while other studies have corroborated our findings of increased MSR1 and CCL18 in obese AT, few studies have provided mechanistic insight into how they may shape ATM function and contribute to adipose tissue dysfunction.

Macrophage Scavenger Receptor 1 (MSR1)

While the number of ATMs increases with obesity and insulin resistance, so does the expression of macrophage scavenger receptor (MSR1/Scavenger receptor A/CD204) suggesting that MSR1 plays a role in promoting AT inflammation. MSR1 expression in SAT has been shown to be associated with insulin resistance in non-diabetic patients [93]. Our findings show increased MSR1 expression VAT in obese diabetic patients compared to non-diabetic; however, the mechanism for this association is unknown. MSR1 is a scavenger receptor prevalent in macrophages believed to play a role in immune responses [94]; however, whether MSR1 plays a role in ATM function is not

well understood. While MSR1 expression has been shown to be associated with DM in human subjects, there is conflicting evidence on the role of MSR1 in regulating insulin resistance and glucose metabolism in mice. Using *Msr1*$^{-/-}$ mice, some studies show that MSR1 protects against obesity-induced insulin resistance in obese mice. Other studies have shown that MSR1 agonists worsen glucose tolerance [95; 96]. Additionally, MSR1 was shown to be essential for the infiltration of pro-inflammatory ATMs and maintenance of the resident ATM population, providing a rationale for their insulin-resistance phenotype [95]. However, this difference in AT inflammation was not reproduced by other groups using *Msr1*$^{-/-}$ mice [96]. Previous studies have shown that MSR1 is essential for macrophage proliferation in the peritoneum and atherosclerotic lesions [97; 98]. Our lab and others have shown that proliferation is a significant contributor to ATM dynamics for both resident and monocyte-derived ATMs [51; 99]. Together, these findings suggest a possible role for MSR1 in obesity-associated insulin resistance, but the precise mechanisms are unclear. Chapter 2 focuses on our work to try to resolve the contradictory reports in the literature using *Msr1*$^{-/-}$ mice.

C-C motif Chemokine Ligand 18

C-C motif chemokine ligand 18, or CCL18, is a chemokine secreted by macrophages that has been implicated in several cancers [100-102] and has been associated with inflammation, obesity, and insulin resistance [103; 104]. Analysis of the Type 2 Diabetes Knowledge Portal (https://DM.hugeamp.org/gene.html?gene=CCL18) demonstrates common variant gene-level associations between CCL18 and cardiomyopathy and youth onset type 2 diabetes. CCL18 has been shown to induce cytokine production and phagocytosis in cultured monocytes [105] and alter T cell content [106]. During obesity,

the formation of new blood vessels, known as angiogenesis, is essential for AT expansion. CCL18 promoted angiogenesis by inducing tube formation and promoting the migration of human endothelial cells through the CCL18 receptor, PITPNM3 [107]. While there is little known about the function of CCL18 in AT, reports show that SAT secretes CCL18 and expresses two of its receptors: GPER1, and PITPNM3. Additionally, CCL18 is highly expressed in ATMs [103]. Analysis of AT from human subjects undergoing bariatric surgery allowed us to confirm that CCL18 is enriched in ATMs and determine it differentiates obese diabetic patients from non-diabetics, suggesting a role for ATM-secreted CCL18 in obesity-associated insulin resistance. The role of CCL18 in AT has yet to be defined; however, evidence supports the rationale that CCL18 potentially acts on endothelial cells, ATMs, T cells, or adipocytes to promote insulin resistance or inflammatory activation. Importantly, orthologs for human *CCL18* are not found in mice, providing a unique opportunity to improve our understanding of a diabetes risk factor discovered in the context of human metabolically unhealthy obesity.

Dissertation Aims

While ATMs are important in regulating AT immunometabolism, our understanding of the underlying mechanisms linking macrophage biology with insulin resistance remains incomplete. This dissertation explores the role of ATMs in the pathogenesis of obesity-associated DM by investigating the function of two macrophage-specific genes and proteins associated with DM in humans: MSR1 and CCL18. The first aim (**Chapter 2**) investigates the role of MSR1 in obesity-associated inflammation and insulin resistance.

Chapter 2 addresses the effects of MSR1 on ATM infiltration and obesity-associated insulin resistance using a HFD-induced obesity mouse model with mice deficient in macrophage scavenger receptors I and II (Msr1$^{-/-}$). While these mice have not been used extensively to elucidate the downstream effects of Msr1 in adipose tissue, there are few studies that have produced conflicting evidence and did not use littermate controls. We found that MSR1 is not required for obesity-associated insulin resistance and inflammation.

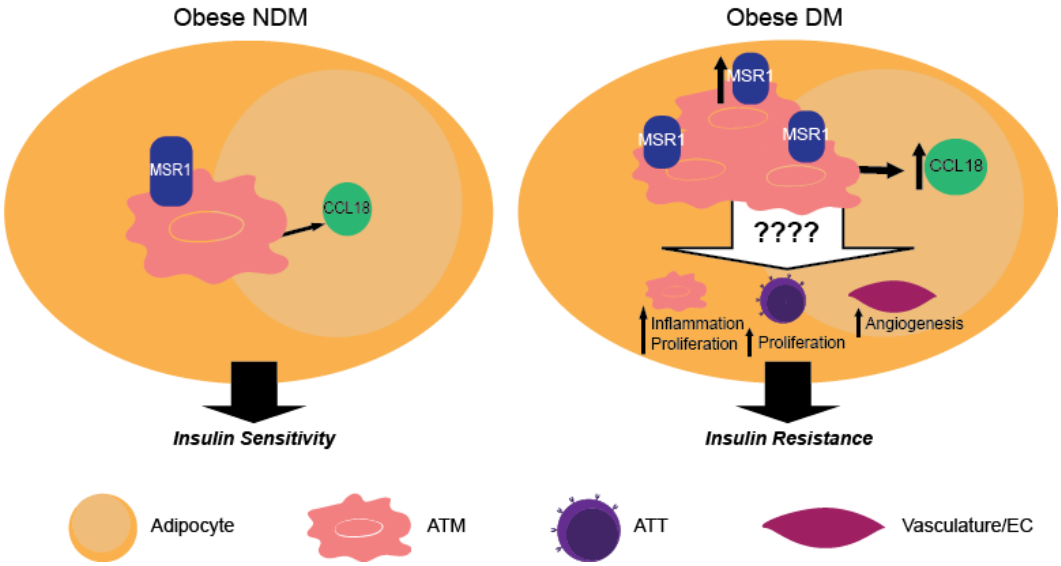

Figure 1.4. Conceptual Model of Knowledge Gaps Addressed in This Dissertation

The second aim (**Chapter 3**) of this dissertation research investigates the hypothesis that ATM-derived CCL18 promotes insulin resistance by increasing AT angiogenesis and inflammation. Chapter 3 tests this hypothesis by using both human and mouse models. Human AT explants and human endothelial cells were treated with CCL18 to assess endothelial cell tube formation and endothelial cell sprouting. Obese mice were treated with or without CCL18 to assess ATM and T-cell infiltration in the

adipose tissue. Because mice do not produce CCL18 but express their receptors in adipose tissue, this experimental design allows for gain of function experiments. This study identified some direct effects of CCL18 on human adipocytes and suggested that CCL18 treatment blunted ATM accumulation with HFD but did not alter glucose metabolism in mouse models.

CHAPTER 2

MSR1 is Not Required for Obesity-Associated Inflammation and Insulin Resistance in Mice

Portions of this chapter have been published:

Nance SA, Muir L, Delproposto, JL, Lumeng CN. MSR1 is Not Required for Obesity-Associated Inflammation and Insulin Resistance in Mice. *Scientific Reports.* 2023 Feb;13:2651.doi: 0.1038/s41598-023-29736-0. PMID: 36788340; PMCID: PMC9927046

Abstract

Obesity induces a chronic inflammatory state associated with changes in adipose tissue macrophages (ATMs). Macrophage scavenger receptor 1 (*MSR1*) has been implicated in the regulation of adipose tissue (AT) inflammation and diabetes pathogenesis; however, reports have been mixed on the contribution of MSR1 in obesity and glucose intolerance. We observed increased *MSR1* expression in VAT of obese diabetic individuals compared to non-diabetic and single nuclear RNA sequencing identified macrophage-specific expression of *MSR1* in human AT. We examined male *Msr1*$^{-/-}$ (*Msr1*KO) and WT controls and observed protection from obesity and AT

inflammation in non-littermate *Msr1*KO mice. We then evaluated obese littermate *Msr1*$^{+/-}$ (*Msr1*HET) and *Msr1*KO mice. Both *Msr1KO* mice and *Msr1HET* mice became obese and insulin resistant when compared to their normal chow diet counterparts, but there was no *Msr1*-dependent difference in body weight, glucose metabolism, or insulin resistance. Flow cytometry revealed no significant differences between genotypes in ATM subtypes or proliferation in male and female mice. We observed an increased frequency of proliferating ATMs in obese females compared to male mice. Overall, we conclude that while *MSR1* is a biomarker of diabetes status in human AT, in mice, *Msr1* is not required for obesity-associated insulin resistance or ATM accumulation.

Introduction

Type 2 Diabetes (DM) is a metabolic disorder characterized by insulin resistance and hyperglycemia and is a growing health concern. Obesity is the number one modifiable risk factor for DM [108; 109]. The association between obesity and DM is well established, but the mechanisms that link the two remain incompletely understood. Adipose tissue is a key player in regulating insulin sensitivity and nutrient storage [110]. It is composed of adipocytes and non-adipocytes, including leukocytes such as ATMs [111]. Under lean conditions, there is a balance between adipokines and anti-inflammatory cytokines to maintain metabolic homeostasis. Obesity induces AT dysfunction characterized by a state of chronic inflammation and adipocyte insulin resistance [25; 38; 39]. ATMs are a primary mediator of cytokine production in AT, producing pro-inflammatory cytokines that have been shown to inhibit glycogen synthesis [112], decrease glucose uptake [113], and impair insulin signaling [114; 115] impacting systemic insulin resistance. These findings along with animal models showing

that impaired macrophage activation improved metabolism with obesity suggest that ATMs have a direct effect on obesity-induced insulin resistance.

However, many gaps remain in understanding the beneficial and detrimental effects of ATM functions and how these functions are regulated. Along with a quantitative increase in the number of ATMs with obesity and insulin resistance, the expression of macrophage scavenger receptor (*MSR1*/Scavenger receptor A) also increases in AT [116] suggesting that *MSR1* plays a role in promoting DM. *MSR1* expression in subcutaneous adipose tissue (SAT) is associated with insulin resistance in non-diabetic patients [93]**.** GWAS studies indicate associations between SNPs in *MSR1* with waist-to-hip ratio and hip circumference adjusted for BMI [117]. The mechanism for this association is unknown.

MSR1 is a scavenger receptor prevalent in macrophages that binds many ligands, including low-density lipoproteins [118]. *MSR1* is thought to aid in phagocytosis [94; 119] and promote inflammation in macrophages [120; 121]**.** *Msr1* has been shown to play a role in macrophage proliferation in peritoneal macrophages from *Ldlr*$^{-/-}$ mice and in macrophages in atherosclerotic lesions in *Apoe*$^{-/-}$ mice [97; 98]. Similarly, in AT, both M2-like resident ATMs (CD11c^{-}) present in lean states and recruited M1-like proinflammatory ATMs (CD11c^{+}) proliferate robustly with acute and chronic high-fat diet-induced obesity [36; 99]. The persistent proliferation of CD11c^{+} ATMs contributes to the long-term effects of obesity on AT function even after weight loss [51]. The factors that regulate ATM proliferative signals are not known.

How or if *MSR1* may play a role in ATM function is not well understood. Despite the clinical association between *MSR1* expression and DM, there is evidence from

mouse studies suggesting that *Msr1* may regulate beneficial macrophage functions to protect against obesity-induced insulin resistance. *Msr1*$^{-/-}$ *ob/ob* mice were found by Zhu *et al* to be more insulin resistant and glucose intolerant compared to *Msr1*$^{+/+}$ *ob/ob* mice [95]. Similar insulin resistance was observed in *Msr1*$^{-/-}$ mice fed HFD for 16 weeks. Cavallari *et al* showed impaired glucose tolerance in obese (6-week HFD) *Msr1*$^{-/-}$ mice compared to *Msr1*$^{+/+}$ mice [96]. On the other hand, Govaere *et al* reported that obese *Msr1*$^{-/-}$ mice have increased weight gain, but are protected from obesity-induced insulin resistance, adipose tissue inflammation, and hepatic steatosis suggesting a pro-inflammatory role for *Msr1* [122].

Because obesity is associated with macrophage accumulation and chronic inflammation, Zhu et al assessed ATMs to explain their metabolic phenotype in HFD *Msr1*$^{+/+}$ and *Msr1*$^{-/-}$ mice and showed a decrease in resident CD11c^{-} ATMs and an increase in proinflammatory CD11c^{+} ATMs in *Msr1*$^{-/-}$ *ob/ob* mice [95]. However, Callivari *et al* did not note any significant differences in adipose tissue inflammation in their model and observed that administration of *MSR1* agonists worsened glucose tolerance. Conversely, Zhu *et al* suggested protective effects of Msr1 activation [95; 96]. Govaere *et al* showed that Msr1 promotes lipid accumulation and hepatic macrophage activation, suggesting a role for *Msr1* in promoting obesity-induced inflammation. Furthermore, inhibition of *Msr1* reduced fibrosis and macrophage activation in mouse and human liver, identifying *Msr1* as a potential target for treating obesity-associated inflammation and liver damage [122]. Together, these results suggest that *MSR1* may play a role in obesity-associated insulin resistance and adipose tissue inflammation, but the precise mechanisms are unclear.

We noted that a weakness of all of these studies was that littermate controls were not utilized for comparisons and female mice were not assessed. Using littermate controls is a more rigorous approach for analysis of genetically modified mice as the use of non-littermate parallel colonies can have epigenetic and environmental backgrounds that can skew results [123]. In diabetes research, the lack of littermate controls has been suggested to be a significant confounding variable in interpreting metabolic phenotypes in mice [124].

To address these gaps and evaluate the *hypothesis* that *MSR1* promotes ATM proliferation, we set out to examine the role of *Msr1* in diet-induced obesity in mice and humans. We observed associations with *MSR1* expression in visceral adipose tissue (VAT) in obese diabetic patients and confirmed that *MSR1* is highly restricted to ATMs in human adipose tissue in single nuclear RNAseq analysis. Initial studies suggested protection from diet-induced obesity in *Msr1*$^{-/-}$ mice compared to non-littermate controls. However, a comparison of littermate male and female mice did not demonstrate significant differences in obesity, glucose intolerance, or adipose tissue inflammation between genotypes. Our studies suggest that *MSR1* is a strong ATM biomarker in human adipose tissue associated with DM status, but our data do not support a functional role for *Msr1* in regulating metabolic inflammation in mice.

Methods & Materials

Animals and Diet

Msr1$^{+/-}$ (*Msr1*HET) mice were mated with *Msr1*$^{-/-}$ *(Msr1KO)* mice (Jackson Laboratory; Strain 006096) to produce experimental *Msr1*$^{-/-}$ pups and *Msr1*$^{+/-}$ littermate controls.

Pups were weaned at 21 days and assessed for sex. Mice were separated into four experimental groups: 1) *Msr1*$^{+/-}$ on normal chow diet (ND) (10% fat; Research Diets: D12450J), 2) *Msr1*$^{-/-}$ mice on ND, 3) *Ms1r*$^{+/-}$ on a high-fat diet (HFD) (60% fat; Research Diets: D12492), 4) *Msr1*$^{-/-}$ on HFD. Each group was fed a ND until 8 weeks of age at which time groups 3 and 4 were switched to HFD. All groups had free access to food and water. Weight was obtained at the final endpoint. For each time point, each group of mice was euthanized, and adipose tissue was collected for analysis. For non-littermate control experiments, *Msr1*$^{-/-}$ *(Msr1KO)* mice (Jackson Laboratory; Strain 006096) were compared with age-matched C57BL/6J male mice. For non-littermate experiments, Msr1KO and WT mice were housed in separate cages. For littermate experiments, Msr1HET and Msr1KO littermate mice were housed in the same cages. All animal protocols were approved by the University of Michigan Institutional Animal Care and Use Committee. All methods were performed in compliance with ARRIVE guidelines, the NIH Public Health Service Policy on Human Care and Use of Laboratory Animals, and the NIH Guide for the Care and Use for Animals.

Glucose & Insulin Tolerance Testing

Mice were fasted for 6 hours and injected intraperitoneally (i.p.) with 1.0g/kg of 10% glucose for the glucose tolerance test (GTT) or 1.0U/kg insulin for insulin tolerance test (ITT). Glucose levels were assessed at baseline and then every 15 minutes for 2 hours using the *Freestyle Freedom Lite* glucometer and test strips.

Flow Cytometry

Mice were euthanized and adipose tissue depots and liver were dissected at the end of the experiment. Adipose tissue was subjected to collagenase digestion to isolate the stromal vascular fraction (SVF) as described [125]. SVF was incubated with antibodies to identify macrophage subsets and proliferation and analyzed by flow cytometry on the BD Biosciences LSRFortessa™ cell analyzer as described [125]. The following conjugated antibodies were used: CD301 APC (Clone ER-MP23, BioRad, Cat# MCA2392A647), CD45 Pacific Blue (Clone 30-F11, eBiosciences, Cat# 48045182), CD64 PE (Clone X54-6/7.1.1, BD Biosciences, Cat# 558455), CD11c APC-Cy7 (Clone N418, eBiosciences, Cat# 47011482), and Ki67 PeCy7 (Clone SolA15, eBiosciences, Cat#25569882). Single-stained, isotype, and unstained SVF were used as controls.

Gene Expression

Total RNA was obtained from frozen mouse white adipose tissue via mechanical homogenization using the TissueMiser homogenizer (Fisher Scientific) and isolated using Qiagen's Rneasy® Mini Kit (Cat# 74106; Qiagen, Hilden, Germany). RNA was treated with DNase I (Cat# 79254; Qiagen, Hilden, Germany)) and cDNA was prepared using 9.8-20ng total RNA and Applied Biosciences™ High-Capacity cDNA Reverse Transcription Kit (Cat# 4368814; Thermo Fisher Scientific, Waltham, MA, USA). Transcript expression was measured using SYBR Power Green PCR Master Mix (Cat# 4367659; Thermo Fisher Scientific, Waltham, MA, USA) in a Quantstudio™ 3 real-time PCR cycler (Applied Biosystems by Thermo Fisher Scientific, Waltham, MA, USA). Target genes were compared to the geometric mean of the housekeeping gene,

Arbp using the $\Delta\Delta C_T$ method. Gene expression was analyzed in *Msr1*KO (n=5) and *Msr1*HET mice (n=3). Table 2.1 lists primers for qPCR.

Gene	Forward Sequence	Reverse Sequence
Msr1	5' – GCATGGCAACTGACCAAAGA – 3'	5' – CCATGTTCCTGGACTGACGAA – 3'
Olr1	5' – CAAGATGAAGCCTGCGAATGA – 3'	5' – ACCTGGCGTAATTGTGTCCAC – 3'
Cd36	5' – AATTAGTAGAACCGGGCCAC – 3'	5' – CCAACTCCCAGGTACAATCA – 3'
Tnfα	5' – GATCTCAAAGACAACCAACATGTG – 3'	5' – CTCCAGCTGGAAGACTCCTCCCAG – 3'
IL1β	5' - AAATACCTGTGGCCTTGGGC – 3'	5' - CTTGGGATCCACACTCTCCAG – 3'
IL-10	5' - GCTCTTACTGACTGGCATGAG – 3'	5' - CGCAGCTCTAGGAGCATGTG – 3'

Table 2.1. Primers for qRT-PCR

RNA sequencing analysis

The Genotype-Tissue Expression (GTEx) Project was supported by the Common Fund of the Office of the Director of the National Institutes of Health, and by NCI, NHGRI, NHLBI, NIDA, NIMH, and NINDS. Analysis of MSR1 expression was performed using a negative binomial regression model including sex, age, and BMI in the model in R. The data used for the GTEX described in this manuscript are available at dbGaP accession number phs000424.vN.pN on 11/12/2022.

Results

MSR1 is macrophage-specific and is increased in visceral adipose tissue from obese diabetic humans

To assess the expression of *MSR1* in human adipose tissue, we first interrogated the Genotype-Tissue Expression (GTEx) Project database for MSR1 expression in bulk

RNA sequencing data. Adjusting for sex and age, *MSR1* expression in both omentum/visceral adipose tissue (VAT) and subcutaneous adipose tissue (SAT) were significantly associated with BMI (VAT: Coefficient = 0.0280, p-value = 0.015; SAT Coefficient = 0.0370, p-value < 0.001) (**Figure 2.1a**). We have previously published an analysis of bulk RNA sequencing data from VAT and SAT from obese patients with and without diabetes mellitus (DM). Comparing non-DM (NDM) with DM patients, *MSR1* expression was significantly increased in the adipose tissue of DM patients. (**Figure 2.1b**) Further stratifying the dataset by depot identified significantly higher *MSR1* expression in SAT compared to VAT (**Figure 2.1c**). Stratifying by depot and DM status, we observed higher expression of *MSR1* in DM patients in VAT, but not SAT suggesting that MSR1 is a VAT-specific biomarker of DM in humans (**Figure 2.1d**).

To identify the cell types that express MSR1 in human adipose tissue, we interrogated a single nuclear RNAseq dataset we generated from obese human adipose tissue [126]. *MSR1* expression was exclusive to adipose tissue macrophages (MAC) and not seen in other stromal cell types (**Figure 2.1e**). Similar results were observed in the interrogation of murine and human single nuclear RNA sequencing studies by Emont *et al* [53]. Subset analysis of ATMs identified multiple subtypes including lipid-associated macrophages (LAM). MSR1 was expressed in all ATM subtypes and was not significantly enriched in one ATM type compared to others (**Figure 2.1f**). This data indicates that *MSR1* is a biomarker of all ATMs in human adipose tissue.

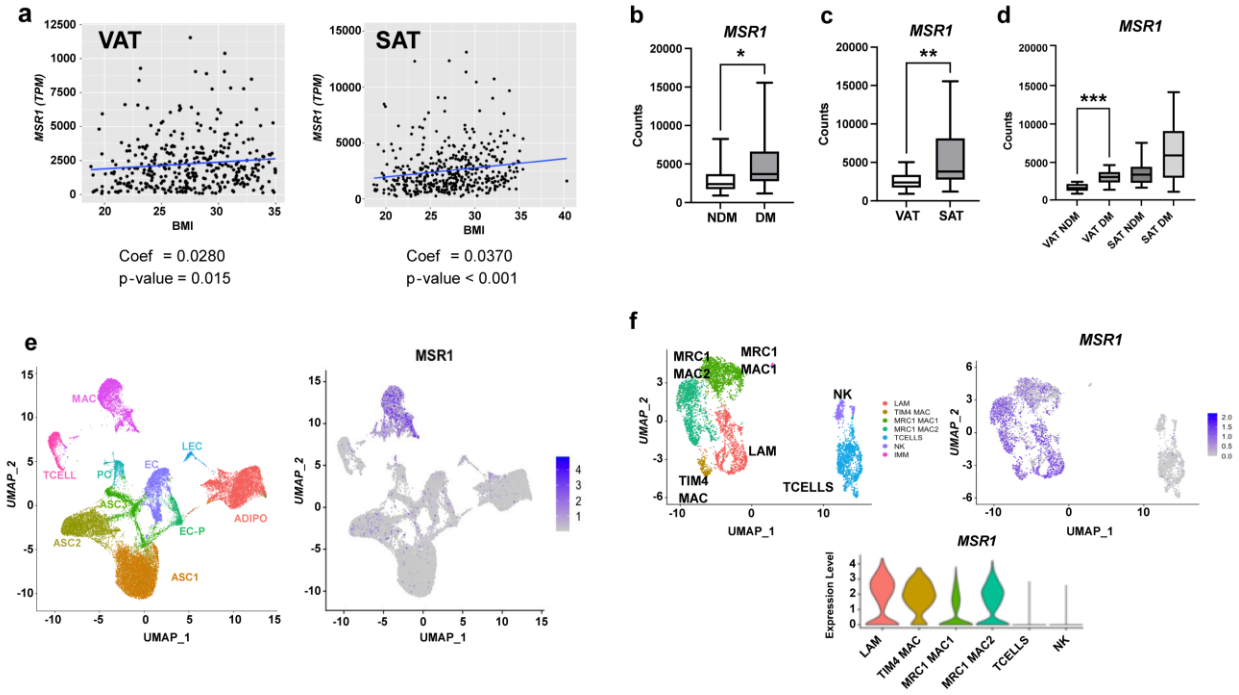

Figure 2.1. MSR1 gene expression is increased in diabetic visceral adipose tissue in humans.
a) Correlation between *MSR1* expression and BMI in omental/visceral (VAT; n=354) and subcutaneous (SAT; n=439) adipose tissue in GTEX. A negative binomial model used incorporating BMI, sex, and age in the model. b-d) *MSR1* expression from RNAseq data from an independent cohort of VAT and SAT samples (n=40). Corrected counts reported comparing b) non-diabetic (NDM) and diabetic (DM), c) VAT and SAT, and d) depots stratified by DM status. *p-value <0.05. ** p-value <0.01. ***p-value<0.001. e) UMAP plots from single nuclear RNAseq data from human VAT and SAT identifying major cell types. *MSR1* expression is restricted to macrophages (MAC). f) Subcluster analysis of macrophages and T cells from single nuclear RNAseq analysis identifying lipid-associated macrophages (LAM), TIM4 macrophages, and two types of MRC1 macrophages. MSR1 expression was identified in all ATM subtypes.

Msr1KO mice are protected from HFD-induced obesity compared to non-littermate controls

We performed experiments to compare the impact of high-fat diet-induced obesity on age-matched WT and *Msr1*KO male mice maintained as parallel lines. After 12 weeks

of HFD, *Msr1*KO mice had significantly less body weight and smaller fat pads compared to WT controls (**Figure 2.2a-b**). While fasting glucose levels were similar, insulin tolerance tests demonstrated no significant differences in insulin sensitivity in *Msr1*KO male mice compared to controls (**Figure 2.2c**). To assess whether *Msr1* was required for obesity-induced adipose tissue inflammation, flow cytometry was performed on the SVF from three fat pads: epididymal white adipose tissue (eWAT), inguinal white adipose tissue (iWAT), and omental white adipose tissue (oWAT). *Msr1*KO mice had a lower frequency of $CD45^+$ leukocytes in eWAT and iWAT, but more leukocytes were seen in *Msr1*KO oWAT (**Figure 2.2d**). *Msr1KO* mice also had fewer total $CD64^+$ ATMs in eWAT and oWAT, but not iWAT (**Figure 2.2e**). When we assessed adipose tissue dendritic cells (ATDCs) and ATM subtypes we found that *Msr1KO* mice had an increase in $CD64^-CD11c^+$ ATDCs and resident $CD64^+CD11c^-$ ATMs in eWAT compared to WT controls while inflammatory $CD64^+CD11c^+$ ATMs decreased (**Figure 2.2f-h**). The frequency of resident $CD64^+CD11c^-$ ATMs was also lower in oWAT, indicative of depot-specific differences. We observed fewer $CD45^+CD64^+Ki67^+$ proliferating ATMs in eWAT and oWAT, but not iWAT from *Msr1*KO mice after 12 weeks of HFD (**Figure 2.2i**). These results suggest that *Msr1* is necessary for weight gain with HFD-induced obesity and may be required to sustain obesity-associated inflammation. Our data also suggest a role for *Msr1* in regulating adipose tissue inflammation via promoting ATM proliferation.

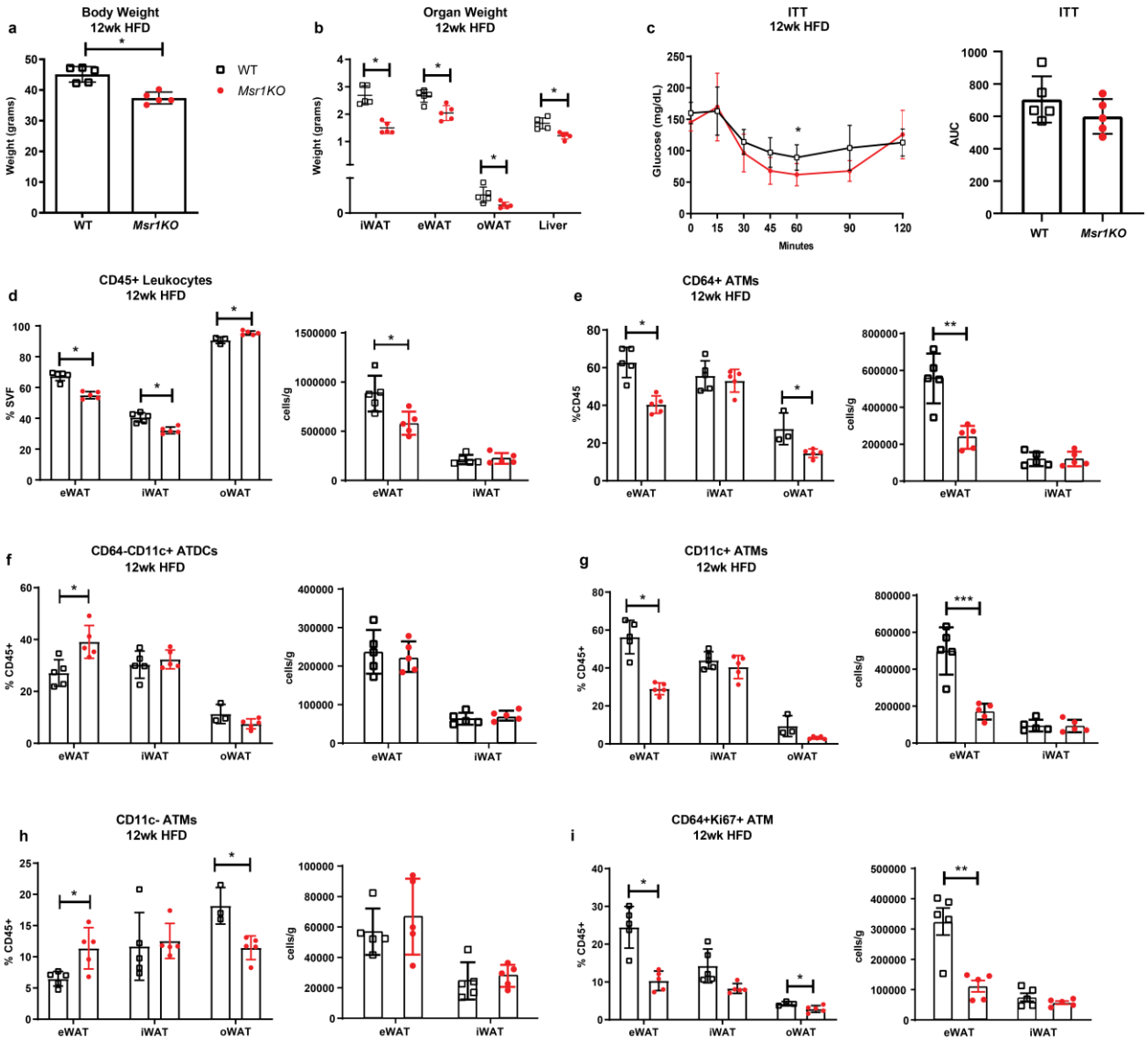

Figure 2.2. Male Msr1KO mice have decreased fat mass and inflammation compared to non-littermate wild-type controls.
a) Whole body weight for 12-week HFD fed *Msr1KO* (red) and C57BL6 wild type non-littermate controls (white). b) Organ weight for *Msr1KO* (red) and WT non-littermate controls (black). c) Insulin tolerance test (ITT) after 12 weeks of HFD. Flow cytometry analysis of d) $CD45^+$ leukocytes, e) $CD64^+$ ATMs, f) $CD64^-CD11c^+$ ATDCs, g) $CD11c^+$ ATMs, h) $CD11c^+$ ATMs, and i) $CD64^+Ki67^+$ ATMs in SVF after 12 weeks of HFD. n=5/group. *p<0.05. Statistical Analysis = T-test. Identical results were observed when the data was expressed as percent total SVF cells.

Msr1KO mice are not protected from HFD-induced obesity compared to Msr1HET littermate controls

As these results contrasted with prior reports of worse insulin resistance in *Msr1*KO mice, to improve the rigor of the study, we compared *Msr1*KO mice and littermate *Msr1*HET controls. COVID-19 pandemic restrictions did not allow us to examine littermate *Msr1HET*, *Msr1KO*, and *Msr1+/+* littermates, and the lines were subsequently lost. To justify the use of *Msr1*HET mice as a control, qRT-PCR was performed from eWAT samples demonstrating loss of *Msr1* expression in the KO mice and ~2 lower expression in HET mice compared to WT controls (**Figure 2.3a**). Adipose tissue was harvested from experimental *Msr1*KO and *Msr1*HET littermate control male mice and analyzed for *Msr1* expression, along with scavenger receptors *Olr1* and *Cd36*, and cytokines *Tnfα, IL-10,* and *IL1β* by qPCR. *Msr1KO* mice had significantly less expression of *Msr1* in eWAT than *Msr1HET* mice *(***Figure 2.3a**)*.* Gene expression of *Olr1, Cd36, Tnfα, IL-10,* and *IL1β* was not different between the two groups. This data demonstrated that *Msr1*KO mice and *Msr1*HET mice are differentiated by *Msr1* expression, making *Msr1*HET an appropriate control to explore the requirement of *Msr1* in obesity-associated inflammation and insulin resistance.

To evaluate the role of *Msr1* on obesity-associated impaired glucose metabolism and insulin resistance, 8-week-old male *Msr1KO* and *Msr1HET* mice were fed HFD to induce obesity, then assessed by glucose (GTT) and insulin tolerance tests (ITT) at 14 and 15 weeks HFD, respectively. Both HFD groups gained significantly more weight than ND mice, but there were no differences in body weight between genotypes for ND or HFD-fed mice. (**Figure 2.3b**). GTT showed that HFD-fed mice had worse glucose

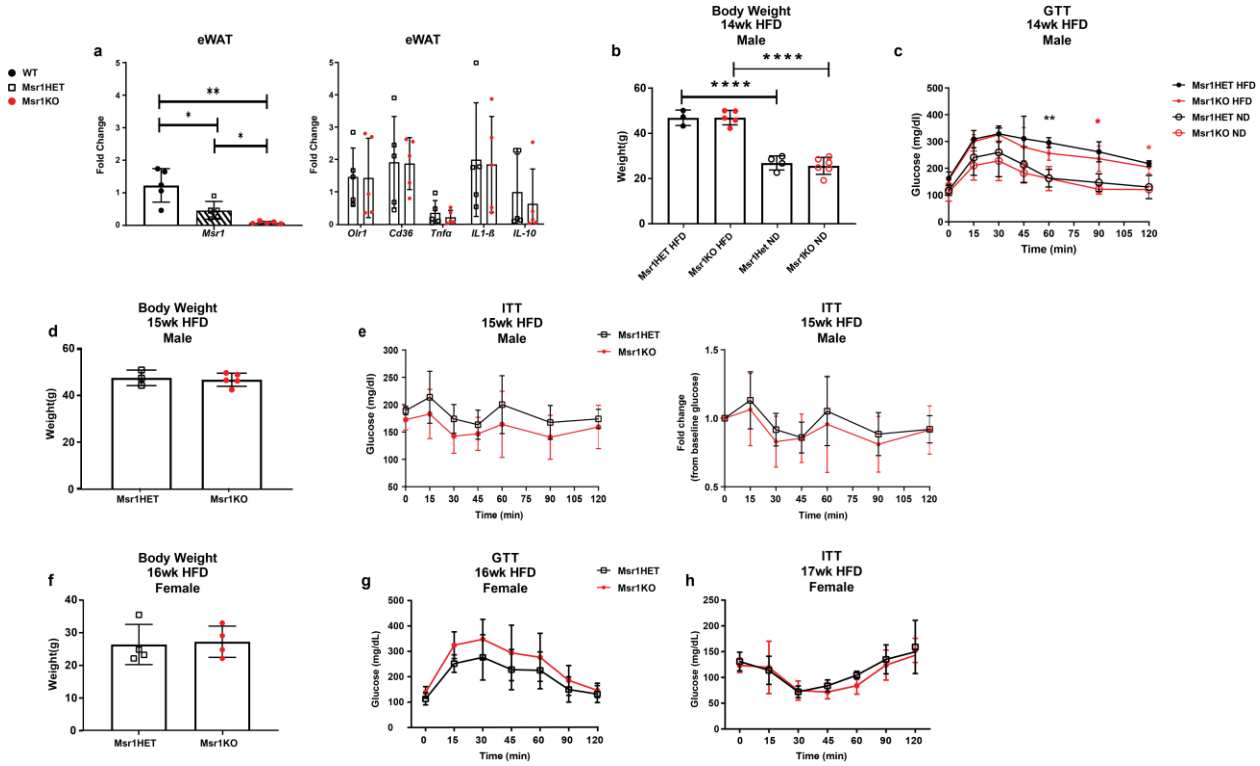

Figure 2.3. Msr1 is not required for obesity-induced changes in glucose metabolism

a) Gene expression of Msr1, scavenger receptors, and cytokines in iWAT and eWAT by qPCR from male *Msr1HET* (black) and *Msr1KO* (red) male mice. n=5/group. *p<0.05; Statistical Analysis = t-test. WT mice were non-littermate HFD-fed controls. b) Whole body weight for 14-week male ND and HFD-fed *Msr1HET* and *Msr1KO* mice at the time of (c) glucose tolerance test (GTT). d) Whole body weight for 15-week male HFD-fed mice at the time of (e) Insulin tolerance test (ITT). n=3-5 per group. f) Whole body weight for 16-week female HFD-fed *Msr1HET* (black) and *Msr1KO* (red) female mice. (g) Glucose tolerance test (GTT) and h) insulin tolerance test (ITT) after 16 and 17 weeks of HFD, respectively. HFD *Msr1HET* (black), HFD *Msr1KO* (red). n=4/group. Statistical Analysis for weight = Ordinary one-way ANOVA. ****p<0.0001 between *Msr1KO* ND and HFD groups; Statistical Analysis for GTT/ITT = 2. way ANOVA multiple comparisons. **p<0.05 between ND and HFD groups of the same genotype.

tolerance compared to ND-fed mice but there were no differences between genotypes (**Figure 2.3c**). HFD groups of mice were assessed for body weight and ITT at 15 weeks of HFD. Again, there was no difference in body weight or ITT responses between HFD *Msr1*HET and *Msr1*KO mice (**Figure 2.3d-e**).

We also assessed the response to HFD in female *Msr1*HET and *Msr1*KO mice. Both genotypes had similar weights after 16 weeks of HFD (**Figure 2.3f**). Similar to male mice, there were no significant differences in GTT or ITT between genotypes after HFD-induced obesity (**Figure 2.3g-h**). This data suggests that *Msr1* is not required for weight gain and insulin resistance during diet-induced obesity in either male or female mice.

Msr1KO is not required for ATM accumulation with HFD

We examined if *Msr1* was required for obesity-induced ATM infiltration and proliferation. After 16 weeks of HFD, the SVF was isolated from fat pads and flow cytometry was performed to quantify ATM subtypes and assess ATM proliferation. Both genotypes had similar body weights and there were no significant differences between spleen, eWAT, and liver weights between genotypes (**Figure 2.4a-b**). *Msr1*KO mice had a trend for smaller iWAT fat pads compared to *Msr1HET* (p-value = 0.078). Flow cytometry analysis of SVF revealed no differences in the quantity of CD45 cells between *Msr1*-deficient mice and littermate controls in either eWAT or iWAT (**Figure 2.4c-d**). Further analysis of the $CD45^+$ leukocytes in the SVF revealed that *Msr1KO* and *Msr1HET* mice had the same frequency of total $CD64^+$ ATMs, $CD64^+CD11c^-$ resident ATMs, $CD64^+CD11c^+$ inflammatory ATMs, and $CD64^-CD11c^+$ ATCDs (**Figure 2.4e-f**). We quantified proliferating ATMs in both genotypes by Ki67 staining. We analyzed $CD64^+Ki67^+$, $CD64^+CD11c^-Ki67^+$, and $CD64^+CD11c^+Ki67^+$ ATMs from eWAT and iWAT, and no significant differences in proliferating ATMs were observed between genotypes (**Figure 2.4g**).

We observed that there was a significantly higher proportion of proliferating $CD64^+CD11c^+Ki67^+$ ATMs in iWAT compared to eWAT in *Msr1KO*.

Similar analyses in female mice revealed no difference in body weight or organ weight after 21 weeks of HFD. (**Figure 2.5a-b**). Quantitation of ATMs by flow cytometry after 21 weeks of HFD demonstrated no differences in total $CD45^+$ leukocytes in eWAT or iWAT between genotypes (**Figure 2.5c-d**). HFD-fed female mice fail to induce significant $CD11c^+$ ATMs (**Figure 2.5e-f**) compared to male mice, but there were no differences between genotypes observed in total or subset ATM content. Similar levels of $Ki67^+$ proliferating ATMs were observed between genotypes (**Figure 2.5g**). However, when compared with male mice, HFD female mice had a greater frequency of proliferating total $CD64^+Ki67^+$ ATMs and subtypes in both iWAT and eWAT (**Figure 2.5h**). Overall, these observations indicate that *Msr1* is not required for obesity-associated adipose tissue inflammation and ATM infiltration and demonstrate that ATM proliferation is more robust in females compared to males.

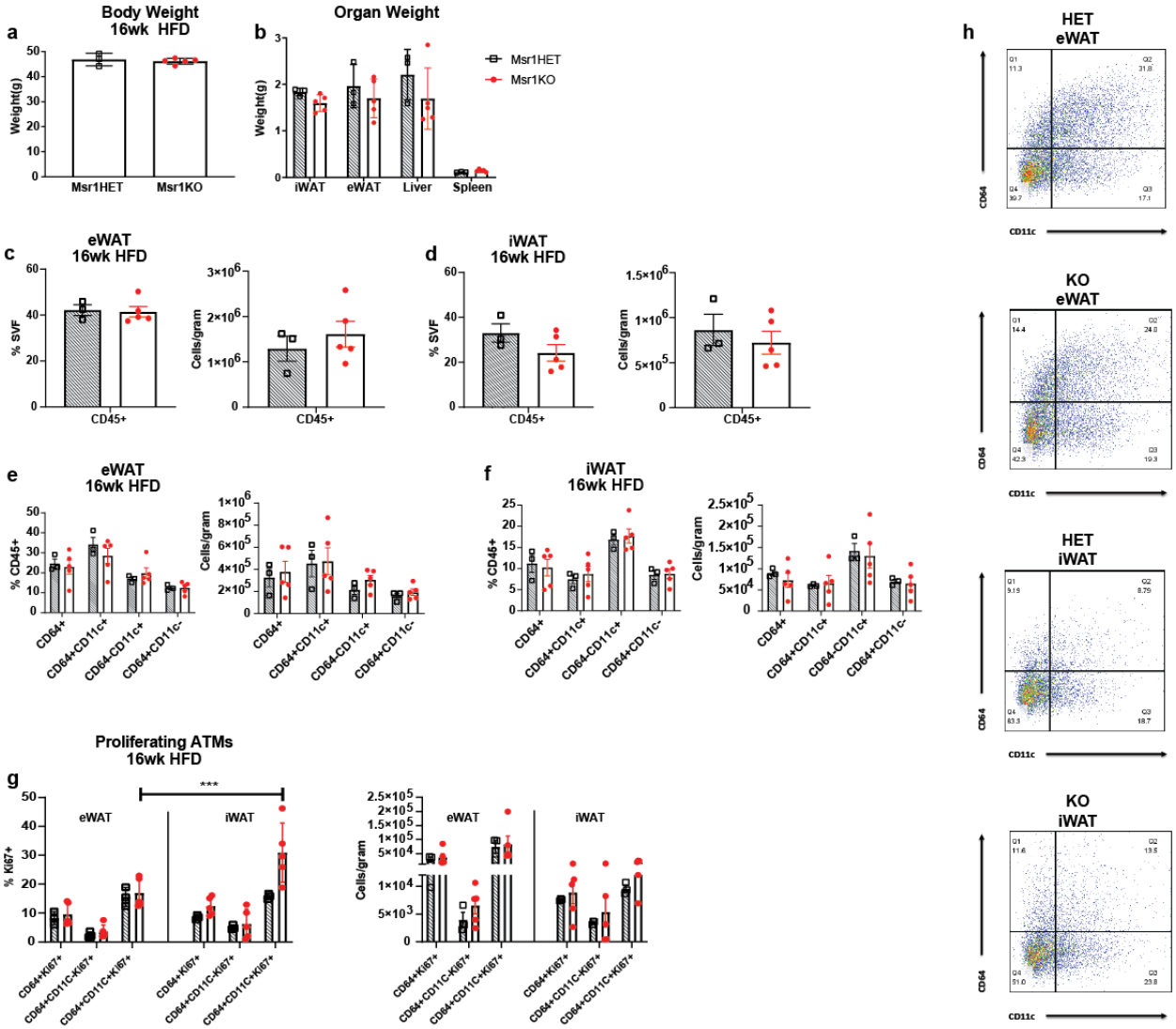

Figure 2.4. Msr1 is not required for ATM accumulation during obesity in obese male mice.

a) Whole body weight and b) organ weight for 16-week male HFD-fed Msr1HET (black) and Msr1KO (red) mice. Flow cytometry analysis of $CD45^{+}$ cells in the SVF (c-d); ATM subtypes (e-f); and proliferating ATMs (g) in eWAT and iWAT. h) Representative flow cytometry graphs. n=3-5/group. ***p=0.0005. Statistical Analysis = 2. way ANOVA multiple comparisons.

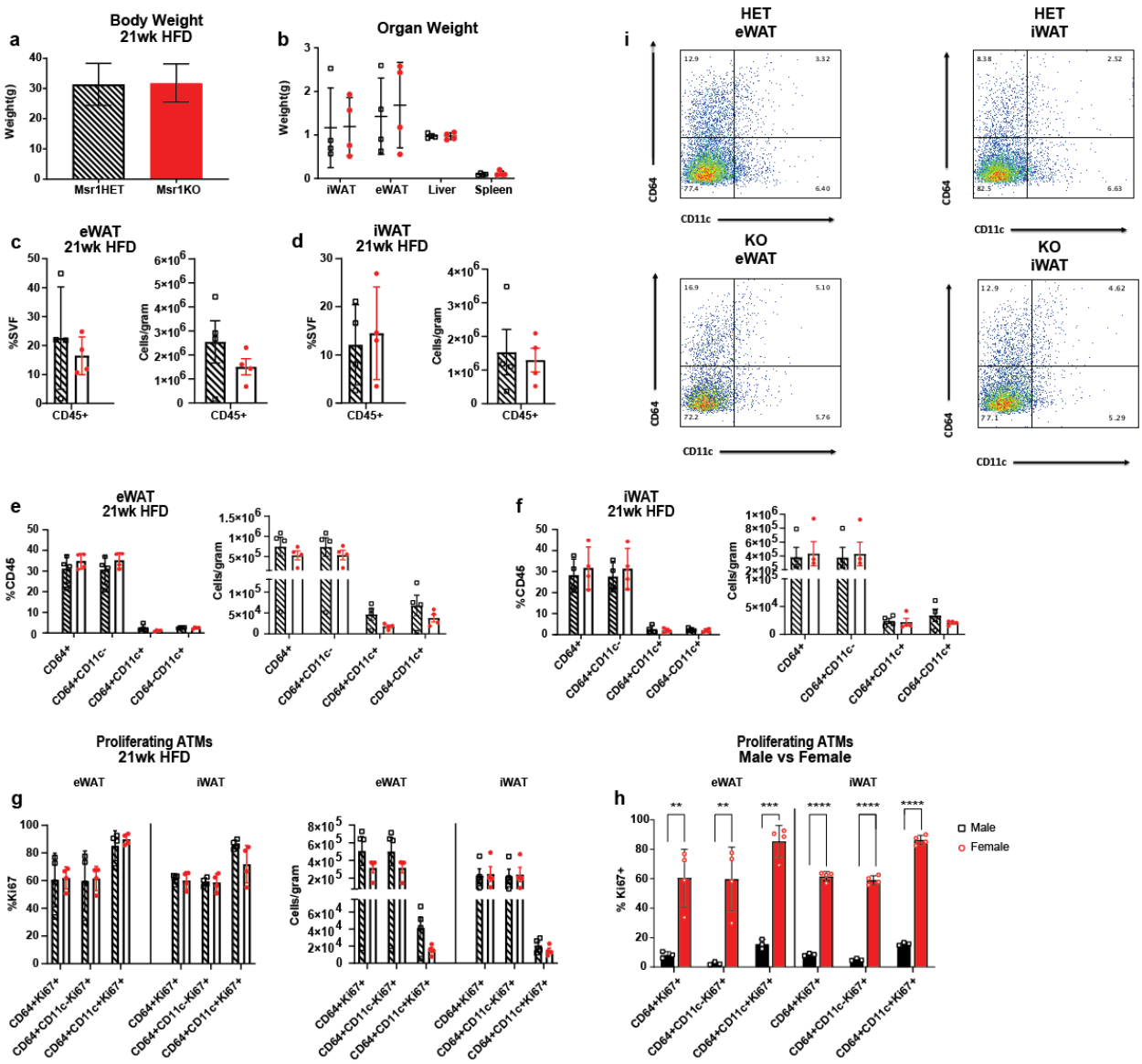

Figure 2.5. Msr1 is not required for ATM infiltration during obesity in obese female mice.

a) Whole body weight and b) organ weight after 21 weeks of HFD, at the time of euthanasia. Flow cytometry analysis showing the frequency of (c-d) $CD45^+$ cells in SVF, (e-f) %CD45 cells, and (g) proliferating ATMs in eWAT and iWAT from female *Msrt1HET* and *Msr1KO* mice. h) Quantitation of proliferating ATMs in HFD-fed male and female *Msr1HET* mice. i) Representative flow cytometry graphs for e-f. n=3-5 per group. Statistical Analysis = t-test.

Discussion

This study aimed to elucidate the role of *Msr1* in insulin resistance and adipose tissue inflammation during obesity. The premise of the study is based on our initial observations that *MSR1* is increased in the VAT of obese, diabetic humans and correlates with BMI in GTEX in both VAT and SAT. Analysis of published single nuclear RNA sequencing datasets demonstrated that *MSR1* expression is highly restricted to ATMs and may be a biomarker for total ATM content in human adipose tissue. We were able to observe increased ATM proliferation in HFD-fed female mice compared to males by flow cytometry analysis. We observed this in both subcutaneous and visceral adipose tissue depots and was related to increased proliferation in all subtypes of ATMs. This result supports previous observations of increased female ATM proliferation using Ki67 staining in adipose tissue sections with lipolysis [127]. Why female ATM proliferation is more prominent in males is unclear. It is possible that an increased proliferative capacity in female mice is related to suppression in obesity-induced adipose tissue inflammation compared to males [128].

Because we did not observe changes in ND male mice and because female mice do not exhibit the same degree of obesity as male mice [127; 129], experiments in female mice were only carried out in HFD-fed mice. Our observation of a lack of Cd11c+ inflammatory ATMs in female adipose tissue despite significant hypertrophy is consistent with prior studies showing a blunted recruitment of ATMs in female mice. While initial cohorts suggested protection from obesity and impaired ATM proliferation in male *Msr1KO* mice this was not replicated in littermate controls. We were unable to replicate prior studies suggesting that *Msr1*-deficient mice were more susceptible to

inflammation with HFD-induced obesity and instead initially observed that *Msr1*-deficient mice were protected from HFD-induced obesity and adipose tissue inflammation. The difference in weight gain observed between *Msr1KO* and WT non-littermate controls may be the primary cause for the protection from obesity-induced inflammation observed in *Msr1KO* mice. Protection from DIO is a common feature in many mouse models where macrophage genes are knocked out including other scavenger receptors such as CD36 and may be due to increased energy expenditure [130-132]. The differences we observed in obesity development in our non-littermate experiments could be attributed to differences in the microbiome as a result of the separate housing conditions.

To ensure a more rigorous experimental design, we compared *Msr1KO* mice with *Msr1HET* littermate controls. While there is no standard model for HFD feeding to induce obesity, C57BL6 mice exhibit significant weight gain and insulin resistance between 16 and 22 weeks of HFD [133]. Since we observed differences in body weight between the genotypes in the non-littermate studies, we extended the duration of HFD feeding to 16 weeks to ensure weight gain in the littermate studies. With this experimental design, we observed that both male and female *Msr1HET* and *Msr1KO* mice exhibited the same degree of HFD-induced weight gain, insulin resistance, and adipose tissue inflammation. Our findings suggest that *Msr1* does not play a significant role in controlling obesity-associated insulin resistance and inflammation.

One limitation of the studies may be the use of *Msr1*HET littermates as the control group as it is possible that *Msr1*HET mice with half the expression of WT mice. Because *Msr1*KO mice had significantly less *Msr1* expression in their adipose tissue

compared to their *Msr1*HET littermates, we still feel this rigorously tests the requirement for *Msr1* in HFD-induced obesity. However, Msr1HET may have a phenotype that differs from WT mice although no groups have reported abnormalities in *Msr1*HET mice. COVID-19 pandemic restrictions did not allow us to examine littermate *Msr1HET*, *Msr1KO*, and *Msr1*$^{+/+}$ littermates. We also attempted to use commercially available antibodies to verify Msr1 expression but were unable to get these reagents to detect Msr1 by immunofluorescence or flow cytometry (data not shown)

Using littermate controls instead of mice from a parallel but distinct colony is recommended in metabolic studies to ensure the epigenetic and environmental backgrounds are comparable [123]. Having the appropriate control is necessary to determine the phenotype of genetically modified strains and can be a significant source of variation in metabolic studies. The *Msr1KO* mice were developed from 129SV embryonic stem cells injected into C57BL/6J blastocysts and are backcrossed to C57BL/6JIco mice. Studies found that 129 X C57BL/6 mice display spontaneous autoimmunity, and in mutant mice backcrossed to this strain, the target gene is likely influenced by surrounding 129SV genes [134; 135]. Therefore, it is possible that the effects of the mixed background are significant and need to be controlled for more rigorously. However, we did conduct experiments to directly test if *Msr1HET* littermates had an altered phenotype compared to C57BL/6 WT mice however, the results we obtained in this study are all similar to our experience with HFD feeding in C57Bl/6 mice in our colony [95; 96].

Our inability to replicate findings with *Msr1KO* mice from other studies may be due to other factors that influence mouse metabolism. A recent large-scale study

comparing genetically identical mice (C57BL/6J) housed at four separate Mouse Metabolic Phenotyping Centers (MMPC) demonstrated substantial variation in the response to identical HFD feeding paradigms based on location [136]. Significant differences in site-specific metabolic rates were observed suggesting that location-specific differences in housing conditions, equipment, microbiome, or other factors may contribute to variation in responses to HFD-induced obesity. Regression analysis identified body composition, activity, photoperiod, diet, and acclimation conditions contributed the most to variance in energy expenditure. Single nuclear RNA sequencing from human adipose tissue demonstrates that *MSR1* is highly specific to ATMs. The association between *MSR1* expression and DM status may be proportional to ATM content which is increased in DM patients based on our recent results and other studies [31]. We attempted to validate MSR1 protein expression in mouse adipose tissue by immunofluorescence and flow cytometry but failed to obtain specific staining with commercially available MSR1 antibodies.

Overall, our experiments do not support a functional role for MSR1 in obesity-induced adipose tissue inflammation despite being a biomarker for ATM content. Our initial hypothesis of a role for MSR1 in adipose tissue inflammation was based on prior research supporting roles for MSR1 in the regulation of antigen presentation [137] and in lipid uptake in macrophages in the setting of fatty liver disease [122]. Given the differences between mice and humans in ATM function, future studies should evaluate potential functional roles for MSR1 activity in human ATM function.

CHAPTER 3

CCL18 Suppresses Adipose Tissue Macrophage Infiltration in Obese Mice

Abstract

C-C motif chemokine ligand 18 (CCL18) is a chemokine that has been implicated in several inflammatory diseases, including obesity-associated Type 2 diabetes mellitus (DM). Despite the associations between CCL18 and DM, the role of CCL18 in adipose tissue biology as it may relate to immunometabolism is not known. In this study, we used both human and mouse experimental models to examine the hypothesis that CCL18 contributes to adipose tissue dysfunction by altering adipogenesis, obesity-induced insulin resistance, adipose tissue inflammation, and/or angiogenesis in adipose tissue. Analysis of human adipose tissue determined that CCL18 was significantly increased in visceral adipose tissue from obese patients with DM compared to those without DM. Single-cell analysis revealed that CCL18 is primarily expressed in adipose tissue macrophages (ATMs) and both ATMs and endothelial cells (ECs) expressed its putative receptors. Furthermore, we observed that while CCL18 did not influence adipogenesis, in conjunction with TNFα, it increased the expression of macrophage colony-stimulating factor in 3T3-L1 adipocytes. RNA sequencing of human adipocytes treated with CCL18 demonstrated suppression of genes related to innate immunity and induction of genes related to cell morphogenesis. Gain of function experiments overexpressing CCL18 in mice did not alter glucose tolerance with diet-induced obesity

and treatment with early HFD feeding did not change adipose tissue leukocyte quantity. However, treatment of obese mice with CCL18 decreased the quantity of $CD11c^{+}$ proinflammatory ATMs in visceral fat depots of mice. This decrease was not associated with changes in ATM proliferation. Based on prior evidence that CCL18 can promote tumor-associated angiogenesis, 3-D angiogenesis models were used to determine if CCL18 promoted angiogenesis in human adipose tissue and HUVECs. CCL18 did not prove to promote adipose tissue angiogenesis. We conclude that while CCL18 is increased in diabetic human adipose tissue it does not promote adipose tissue inflammation or angiogenesis. However, CCL18 may have an anti-inflammatory effect in the obese state by suppressing the accumulation of ATMs in adipose tissue.

Introduction

A characteristic of obesity-associated Type 2 diabetes (DM) is the induction of pathways related to inflammation in adipose tissue in obese, diabetic patients (DM) compared to obese, non-diabetic patients (NDM). Among people with obesity, there is significant variation in metabolic disease risk indicating that the quantity of adipose tissue may not be the only determinant of metabolic health. While obesity increases the risk for DM, impaired metabolic health is a more accurate determinant of risk for morbidity from obesity-associated DM.

RNA sequencing studies from VAT from people with obesity with and without DM demonstrated enrichment of pathways in DM related to hematopoietic cell lineage, phagosomes, chemokine signaling, and cytokine–cytokine receptor interaction consistent with the link between inflammation and MUO.[91] Among the genes that are

upregulated in DM compared to NDM obese adipose tissue is C-C motif chemokine ligand 18, or CCL18 [91; 103]. CCL18 is primarily produced by myeloid cells such as dendritic cells, monocytes, and macrophages [138-140]. Analysis of human SAT revealed that gene expression of CCL18 is expressed in adipocytes and leukocytes, but most predominately in adipose tissue macrophages (ATMs)[103]. Increased circulating levels of CCL18 are associated with inflammation, obesity, and insulin resistance [103; 141]. Furthermore, CCL18 has been shown to promote macrophage activation in humans [105] and the infiltration of T cells in Balb/c mice [106] in mice. These observations suggest that CCL18 may contribute to metabolic inflammation and the risk for DM, but gaps remain in our understanding of which cells respond to CCL18 in adipose tissue.

Three human receptors for CCL18 have been previously identified: CCR8, GPER1, and PITPNM3. 4DE4 mouse pre-B cells transfected with human *CCR8 (hCCR8)* exhibited migration to CCL18 and CCL18 treatment led to an influx of intracellular calcium in these cells. Moreover, CCL18 was shown to competitively bind to hCCR8 and promote its internalization upon binding. While there are no functional orthologues of CCL18 in mice, CCL18 also activates mCcr8 [141]. Studies in primary human acute lymphocytic leukemia B cells found that CCL18 binds to GPER1, and suppresses its activation by CXCL12 [142], inhibiting the proliferation of tumor cells [100]. CCL18 binds to PITPNM3 to promote the progression of breast cancer [101], prostate cancer [143], oral cancer [102], and lung cancer [144]. Furthermore, preadipocytes and adipocytes express CCL18 receptors GPER1 and PITPNM3, but not previously identified CCR8 [103; 141].

The ability of CCL18 to influence leukocyte functions overlaps with what is known about adipose tissue leukocyte activation with obesity. In the lean state, resident M2-like $CD11c^-$ ATMs are predominant in the adipose tissue. These ATMs highly express the anti-inflammatory cytokine IL-10 which protects against obesity-associated insulin resistance [43]. Obesity induces the infiltration of classically activated M1-like ATMs that express cell-surface marker CD11c [43; 145]. In human adipose tissue, $CD11c^+$ ATM content is significantly higher in SAT compared to VAT and is associated with obesity-associated insulin resistance [30]. $CD11c^+$ ATMs are preferentially recruited to crown-like structures (CLS) in obese adipose tissue and secrete high levels of pro-inflammatory cytokines that are linked to obesity-associated insulin resistance [43]. $CD11c^+$ ATMs are derived primarily from bone marrow monocytes that are recruited to obese adipose tissue and this recruitment is the primary mechanism of their quantitative increase in obese adipose tissue. ATM content is also regulated by proliferation. Short-term high-fat diet feeding induces fat pad expansion and triggers ATM proliferation [36; 99]. Studies in mice have also shown that both $CD11c^-$ and $CD11c^+$ ATMs proliferate with chronic obesity weight gain and this effect was partially reversed with weight loss [51; 99]. The molecular mediators of ATM proliferation are not known.

For adipose tissue to expand with obesity, it requires the formation of new blood vessels, also known as angiogenesis [146]. Both VAT and SAT expand and become more angiogenic as adiposity increases [147]; however, SAT has a greater angiogenic capacity than VAT [148]. One of the stimuli for adipose angiogenesis in obesity is hypoxia. In the obese state, adipose tissue becomes hypoxic in both rodents and humans [149-151]. Studies in differentiated 3T3-F442A adipocytes showed that under

hypoxic conditions the expression of vascular endothelial growth factor (VEGF), leptin, hypoxia-inducible factor 1 alpha (HIF-1α), and matrix metalloproteinases (MMPs) is increased, suggesting hypoxia regulates adipose tissue angiogenesis [152]. Furthermore, VEGF, independent of hypoxia, can induce angiogenesis [153; 154]. Additionally, adipose tissue secretes several pro-angiogenic factors that may regulate adipose tissue angiogenesis [155]. Although the role of CCL18 in adipose tissue angiogenesis has yet to be elucidated, studies in cancer cells revealed that CCL18 from tumor-associated macrophages mediate tumor cell progression by promoting angiogenesis [107].

This study aimed to evaluate and identify the function of CCL18 in adipose tissue biology to understand the associations between CCL18 and DM status in humans. While the role of CCL18 in adipose tissue remains unclear, we hypothesized that CCL18 acts on ATMs and ECs to promote adipose tissue inflammation and angiogenesis, leading to insulin resistance during obesity. We found that CCL8 expression is increased in the adipose tissue of obese DM individuals and highly expressed in ATMs. Moreover, its receptors are expressed in both ATMs and ECs. CCL18 treatment of adipocytes decreased genes related to innate immune activation. In lean mice or mice on short-term HFD, CCL18 infusion was not sufficient to alter obesity-associated glucose resistance or change ATM content. However, in obese mice, CCL18 treatment prevented the increase in $CD11c^{+}$ ATMs in visceral adipose tissue depots independent of changes in ATM proliferation. Overall, our data suggest

that CCL18 may play an anti-inflammatory role in adipose tissue by restraining $CD11c^+$ pro-inflammatory ATM induction with obesity.

Materials and Methods

Animals and Diet

Male C57BL/6 mice were purchased from The Jackson Laboratory (Stock No: 000664) at 6 weeks of age. Mice were housed five to a cage on a 12-hours light/dark cycle with free access to normal diet chow and water. After two weeks, mice were separated into four cohorts: 1) 2-week high-fat diet (HFD) treated with CCL18 or PBS+1%BSA (PBS), 2) normal Diet (ND) treated with CCL18 or PBS that were age-matched to group 1, 3) 6-week HFD treated with CCL18 or PBS, and 4) ND treated with CCL18 or PBS that were age-matched to group 3. (n = 10 per group; 5 per treatment). All CCL18 groups received a total of 10ug of CCL18 over 2 weeks delivered through an Alzet® osmotic pump (Model No: 1002), implanted subcutaneously by the Physiology Phenotyping Core. HFD mice were fed a 60% kcal fat diet (Research Diets, Inc - D12492) compared to a 13% kcal fat diet for ND mice. All experiments were approved by the Institutional Animal Care and Use Committee at the University of Michigan.

Cell Culture

Human Preadipocytes

Primary human preadipocytes isolated from human omental adipose tissue were plated in 12-well tissue culture plates, expanded for 3 days, and differentiated into mature

adipocytes over 14 days as described [156] then treated ± TNFα (1ng/mL) or CCL18 (40ng/mL or 100ng/mL).

3T3-L1 Cells

3T3-L1 cells were plated and expanded for 3 days in 6-well tissue culture plates and differentiated into mature adipocytes for 3 days with DMI DMEM 10% FBS ± TNFα (1ng/mL) or CCL18 (20ng/mL or 100ng/mL), then Insulin DMEM 10% FBS ± TNFα (1ng/mL) or CCL18 (20ng/mL or 100ng/mL) for 3 days. Cells were maintained in DMEM 10% FBS.

Glucose Tolerance Testing

Mice were fasted for 6 hours and injected intraperitoneally (i.p.) with 1.0g/kg of 10% glucose for the glucose tolerance test (GTT). Glucose levels were assessed at baseline and then every 15 minutes for 2 hours using the *Freestyle Freedom Lite* glucometer and test strips.

Flow Cytometry Analysis

Mice were euthanized and spleen, liver, and adipose tissue depots were dissected at the end of the experiment. Adipose Tissue was subjected to collagenase digestion to isolate the stromal vascular fraction (SVF) as described [125]. SVF and splenocytes were incubated with FC Block (Invitrogen Cat# 14-0161-85) and stained with conjugated antibodies to identify macrophage and T-cell subsets and proliferation. Flow cytometry was completed on the BD Biosciences LSRFortessa™ cell analyzer as described [125]

and analyzed using FlowJo software. The following conjugated antibodies were used: Live/Dead Violet (Invitrogen Cat# L34964), CD45 PerCP Cy5.5 (Invitrogen Cat# 45-0451-82), CD64 PeCy5 (BioLegend Cat# 139331), CD11c BV605 (BioLegend Cat# 117333), CD3 APC (Invitrogen Cat# 17-0031-82), CD11b APCCy7 (Invitrogen Cat# 47-0112-82), CD4 BV650 (BioLegend Cat# 100546), CD8 FITC (Invitrogen Cat# 11-0081-85), FoxP3 PE (Invitrogen Cat# 12-4771-82), Ki67 PECy7 (Invitrogen Cat# 25-5698-82). Single-stained and unstained SVF were used as controls.

Gene Expression

Total RNA was obtained from frozen mouse white adipose tissue via mechanical homogenization using the TissueMiser homogenizer (Fisher Scientific) and isolated using Qiagen's Rneasy® Mini Kit (Cat# 74106; Qiagen, Hilden, Germany). RNA was treated with DNase I (Cat# 79254; Qiagen, Hilden, Germany)) and cDNA was prepared using 9.8-20ng total RNA and Applied Biosciences™ High-Capacity cDNA Reverse Transcription Kit (Cat# 4368814; Thermo Fisher Scientific, Waltham, MA, USA). Transcript expression was measured using SYBR Power Green PCR Master Mix (Cat# 4367659; Thermo Fisher Scientific, Waltham, MA, USA) in a Quantstudio™ 3 real-time PCR cycler (Applied Biosystems by Thermo Fisher Scientific, Waltham, MA, USA). Target genes were compared to the geometric mean of the housekeeping genes, *PPIA,* and *Arbp* using the $\Delta\Delta C_T$ method. Gene expression was analyzed in human preadipocytes and 3T3L1 cells. Tables 3.1 and 3.2 list primers for qPCR.

Gene	Forward Sequence	Reverse Sequence
IL6	5' – CAGACAGCCACTCACCTCTTC – 3'	5' – ATGTTACTCTTGTTACATGTCTCCT – 3'
FABP4	5' – CAGGAAAGTCAAGAGCACCAT – 3'	5' – CTCGTGGAAGTGACGCCTTT – 3'
PPIA	5' – CACCGTGTTCTTCGACATTG – 3'	5' – TCCTTTCTCTCCAGTGCTCAG – 3'

Table 3.1. Human primers for qRT-PCR

Gene	Forward Sequence	Reverse Sequence
Pparγ	5' – TCGCTGATGCACTGCCTATG – 3'	5' – GAGAGGTCCACAGAGCTGATT – 3'
Mcsf	5' – GACTTCATGCCAGATTGCC – 3'	5' – GGTGGCTTTAGGGTACAGG – 3'
Mcp1	5' – TTAAAAACCTGGATCGGAACCAA – 3'	5' – GCATTAGCTTCAGATTTACGGGT – 3'
Leptin	5' – GAGACCCCTGTGTCGGTTC – 3'	5' – CTGCGTGTGTGAAATGTCATTG – 3'
Il6	5' – TAGTCCTTCCTACCCCAATTTCC – 3'	5' – TTGGTCCTTAGCCACTCCTTC – 3'
Ccr8	5' – CTGCGATGTGTAAGGTGGTCTC – 3'	5' – CCTCACCTTGATGGCATAGACAG – 3'
Gper1	5' – GCCACATAGTCAACCTTGCAGC – 3'	5' – CGTCTTCTGCTCCACATAGAGC – 3'
Pitpnm3	5' – CTACCGATGTGGTCGCCTTCAT – 3'	5' – GTTCGCTTACGCAGCCACTTCT – 3'

Table 3.2. Mouse primers for qRT-PCR

Protein Expression

Media from cultured cells was harvested after differentiation and analyzed by enzyme-linked immunoassay (ELISA) to quantify protein expression. ELISA was done by the Cancer Immunology Core at the University of Michigan Medical School.

RNA sequencing analysis

Human VAT preadipocytes were differentiated into secondary mature adipocytes for 14 days as previously described [156]. Cells were treated ± TNFα (1ng/mL) or CCL18 (40ng/mL or 100ng/mL). RNA was isolated using Qiagen's Rneasy® Mini Kit (Cat# 74106; Qiagen, Hilden, Germany). RNA-Seq data was generated using the Lexogen QuantSeq 3' mRNA FWD with the UMI add-on kit and sequenced on the Illumina platform (7-8M reads per sample). Reads were Trimmed using Trim_Galore (v 0.5.0), aligned using STAR (v 2.6.0), UMI collapsed using Lexogen's supplied collapse_UMI_bam binary using GRCh38 from ENSEMBL for Reference and Annotation. Count matrices generated by Rsubread were analyzed with DESeq2 and pathway analysis was performed using GSEA v.4.2.2.

Human Adipose Tissue Samples and ex-vivo angiogenesis assay

Human adipose tissue was obtained from the Michigan Bariatric Surgery cohort. Freshly harvested samples were prepared and embedded in Matrigel as described [148] ± CCL18. Analysis of growth expansion was determined by subtracting the area of adipose tissue explant from the total area as described [157]. The area was measured using Photoshop. All samples were collected using an Institutional Review Board-approved protocol at the University of Michigan and Ann Arbor Veteran's Administration Hospital.

HUVEC endothelial cell growth assay

HUVEC-coated microbeads were incubated overnight in EGM2 media as described and then embedded in a fibrin gel matrix as described [158] ± lung fibroblasts and CCL18. Cells were fixed and stained with UEA, DAPI, and Actin to visualize ECs, nuclei, and cells using fluorescent microscopy. HUVEC-coated beads that did not interact with neighboring beads were assessed for EC sprouting by assessing network length at day 14. Tube length was quantified using the Angiogenesis Tube Formation module in Metamorph. The average tubule length of each bed for each condition was used for 2way ANOVA statistical analysis.

Results

CCL18 is expressed in LAM ATMs and endothelial cells and is increased in adipose tissue from diabetic humans

Bulk RNA sequencing (RNAseq) of adipose tissue from people with obesity revealed five differentially expressed genes related to ATMs that were increased in individuals with DM compared to age and sex-matched non-diabetic (NDM) individuals: *CCL18, CH25H, MSR1, MMP8, and SPP1* (**Figure 3.1a**). CCL18 was identified as a protein that has not been thoroughly investigated in adipose tissue. Analysis of an existing single-nuclear RNA sequencing dataset from obese human adipose tissue [126] showed that *CCL18* is predominantly expressed in ATMs (**Figure 3.1b**). Subset analysis of immune cells in human adipose tissue showed enrichment of *CCL18* expression in lipid-associated macrophages (LAMs), but not T-cells or any other immune cell populations (**Figure 3.1c**). The expression of previously identified *CCL18*

receptors *GPER1* and *PITPNM3* was primarily observed in adipocytes and adipose stromal cells (ASC), with lower but detectable expression in macrophages, ECs, and pericytes (PC) (**Figure 3.1d**). Subset analysis of endothelial cells showed high expression of *GPER1* in PCs and of *PITPNM3* in venous endothelial cells (VEC) (**Figures 3.1e**).

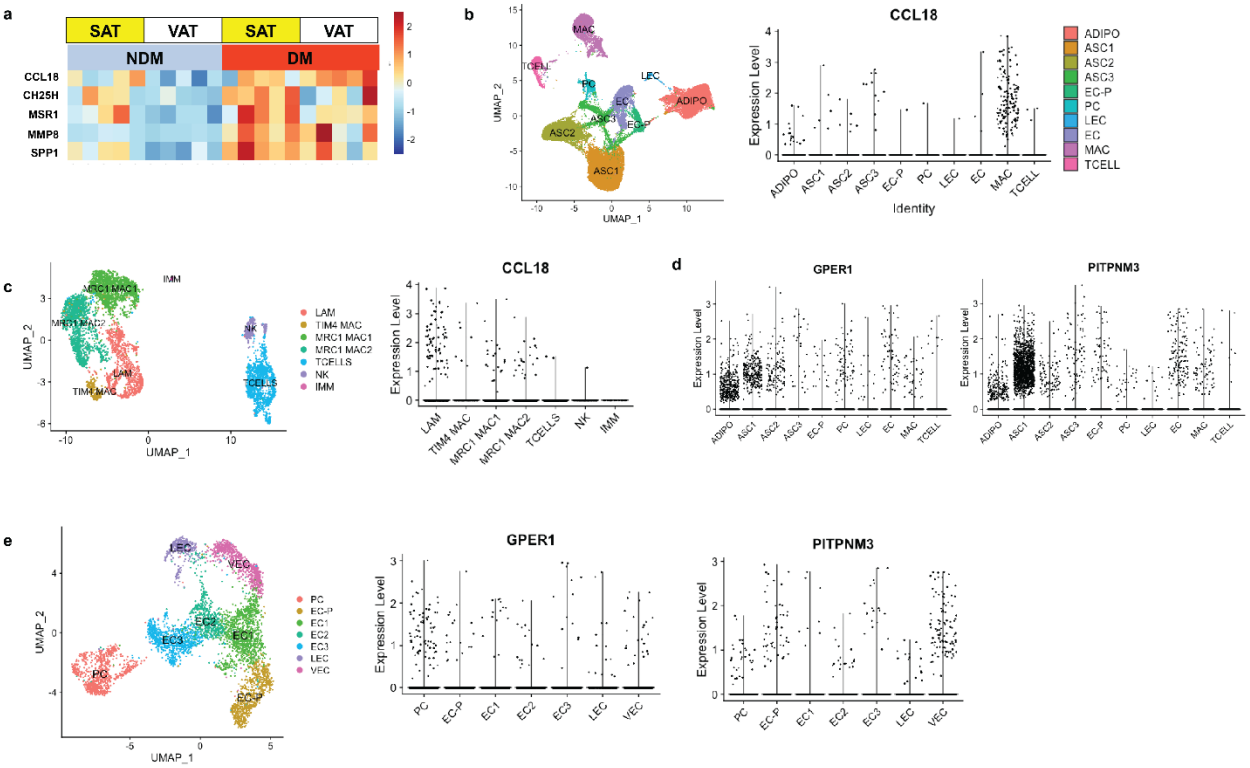

Figure 3.1. CCL18 is expressed in LAMs in human adipose tissue
a) Heatmap of significantly differentially expressed genes based on RNA sequencing of omental/visceral adipose tissue (VAT) and subcutaneous adipose tissue (SAT) from diabetic (DM) and non-diabetic (NDM) humans. b) UMAP plots of single nuclear RNA sequencing (sn-RNAseq) of human adipose tissue (n=8 samples, >35,000 nuclei) showing the major adipose tissue cell populations. Violin plot of *CCL18* in cell populations and enriched adipose tissue macrophage (MAC). c) Subcluster analysis of sn-RNAseq of human adipose tissue immune cells. Violin plots demonstrate increased CCL18 expression in lipid-associated macrophages (LAM). d) Violin plots of expression of CCL18 receptors: *GPER1* and *PITMN3* in the major human adipose tissue cell types based on sn-RNAseq. e) Subcluster analysis of endothelial cell subsets showing

expression of *GPER1* and *PITMN3*. ADIPO= adipocyte; ASC= adipose-derive stem cell; EC= endothelial cell; MAC= macrophage; PC= pericyte; TCELLS= T-cells; LAM= lipid-associated macrophage; TIM4 MAC= TIM4+ macrophage; MRC1 MAC= MRC1 macrophage; NK= natural killer cell; IMM= other immune cell; VEC= venous endothelial cell; LEC = lymphoid endothelial cell

CCL18 suppresses immune pathway genes in human adipocytes

One hypothesis is that inflammatory signals from CCL18 inhibit adipogenesis or the function of adipocytes to promote adipose tissue dysfunction. To assess this, pre-adipocytes were isolated from obese, NDM, and DM human adipose tissue and differentiated into mature adipocytes *in vitro*. Preadipocytes were treated with or without CCL18 (40 ng/mL or 100ng/mL) and tumor necrosis factor-alpha (TNFα) (1ng/mL), as a control for factors that can inhibit adipogenesis [159] during differentiation. Secretion of IL-6, MCP1, and MCSF, as well as gene expression of *IL6,* and *FABP4* were assessed to determine if CCL18 influenced inflammation and adipogenesis. TNFα induced the expression of inflammatory cytokines IL6 in both the NDM and DM subjects, but only increased MCP1 and MCSF expression in the DM subject. CCL18 did not affect inflammatory cytokine secretion in preadipocytes (**Figure 3.2a**). While TNFα induced gene expression of *IL6* in the NDM subject, neither CCL18 nor TNFα influenced the expression of *FABP4* – a marker for differentiated adipocytes (**Figure 3.2b**). These findings suggest that CCL18 does not promote an inflammatory response or alter adipogenesis in human preadipocytes.

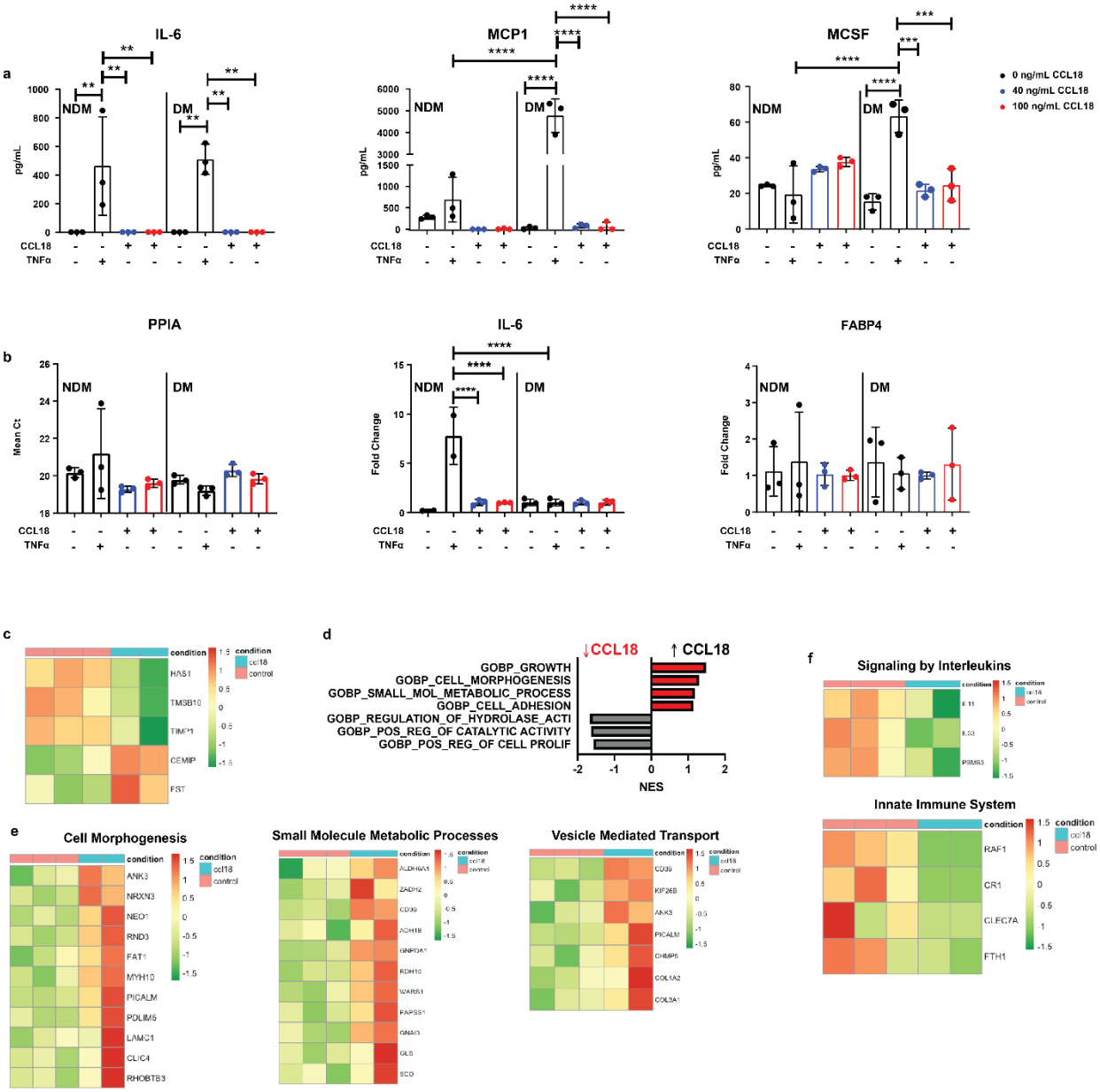

Figure 3.2. CCL18 suppresses immune pathways in human adipocytes
a) Protein expression by ELISA of IL-6, MCP1, and MCSF in mature secondary human conditioned media from adipocytes treated ± 1ng/mL TNFα, 0ng/mL CCL18 (black circles), 40ng/mL CCL18 (blue circles), or 100ng/mL (red circles) during differentiation in two human subjects. b) Gene expression by RT-qPCR of PPIA (housekeeping gene), *IL6,* and *FABP4* treated ± 1ng/mL TNFα (black circles), 40ng/mL CCL18 (blue circles), or 100ng/mL (red circles) in two human subjects. n=1 subject/group; Statistical analysis= Ordinary one-way ANOVA. **p<0.005, ***p<0.0005, ****p<0.0001. c) GSEA analysis of differentiated human adipocytes ± CCL18. n=2-3/group. c) Heatmap of significant differentially expressed genes between CCL18 and control adipocytes.

d) Significantly enriched pathways based on gene set enrichment analysis (GSEA) in adipocytes treated with CCL18. NES = normalized enrichment score. P-value <0.05 for all pathways. e) Heatmap of significant pathways induced by CCL18 treatment in human adipocytes. f) Heatmap of significant pathways suppressed by CCL18 treatment in human adipocytes.

To examine if there are direct effects of CCL18 on gene expression in human adipocytes, differentiated human adipocytes from VAT were treated with and without CCL18 before analysis by bulk RNA sequencing (n=3 for control and n=2 for CCL18 treated due to limitations in RNA quality). No significant differences in adipocyte lipid deposition were noted with CCL18 treatment (**Data not shown**). Three genes were significantly suppressed by CCL18 based on adjusted *p*-value (*HAS1, TMSB10, TIMP1*), and two genes were induced by CCL18 treatment (*CEMP* and *FST*). (**Figure 3.2c**) Based on unadjusted *p*-values, 165 genes were down-regulated, and 86 genes were upregulated. GSEA analysis was performed on these genes and identified that CCL18-induced genes that were enriched for pathways related to vesicle-mediated transport (*CD36*), growth, cell morphogenesis, and small molecule metabolic processes in adipocytes (**Figure 3.2d-e**). Pathways downregulated by CCL18 were related to pathways involved in the regulation of hydrolase activity, cell proliferation, innate immune system, and interleukin signaling (*IL33* and *IL11*) (**Figure 3.2f**). Overall, this suggests that CCL18 may downregulate pro-inflammatory signals from mature human adipocytes.

CCL18 increases TNFα induced Il6 and Mcsf expression in 3T3-L1 adipocytes

3T3-L1 cells are fibroblasts isolated from mouse embryos that can be chemically induced to differentiate into mature adipocyte-like cells [160]. To examine the hypothesis that CCL18 inhibits adipogenesis in this model, 3T3-L1 cells were expanded and treated with differentiation media ± CCL18 (20ng/mL or 100ng/mL) in the presence of absence of TNFα as a control for inhibition of differentiation. Once fully differentiated into mature adipocytes, cells were harvested and assessed for inflammatory genes and markers of differentiated adipocytes. As expected, TNFα blunted the expression of *Leptin* and *Pparγ* with or without co-treatment with CCL18 indicative of inhibition of adipogenesis [159]. CCL18 alone did not alter the expression of *Leptin* or *Pparγ,*(**Figure 3.3a-b**) suggesting CCL18 does not alter adipogenesis in murine cells. Neither CCL18 nor TNFα alone influenced the expression of inflammatory genes *Il6, Mcp1,* and *Mcsf* (**Figure 3.3c-e**). However, CCL18 in conjunction with TNFα increased the expression of *Il6* and *Mcsf*. Together these results suggest that CCL18 can potentiate TNFα induced inflammatory cytokine expression in 3TL31 adipocytes. This conflicts with the RNAseq data in obese human adipocytes, showing CCL18 suppresses the immune response.

CCL18 does not alter glucose tolerance with short-term HFD feeding

Mice do not produce endogenous CCL18; however, the known CCL18 receptors described in the literature *Ccr8, Gper1,* and *Pitpnm3* are expressed in adipose tissue (**Figure 3.4a**). While inguinal white adipose tissue (iWAT) expresses all three receptors, *Ccr8* was undetectable in epididymal white adipose tissue (eWAT). Furthermore, receptor expression did not change with CCL18 treatment *in vivo* (**Figure 3.4a**).

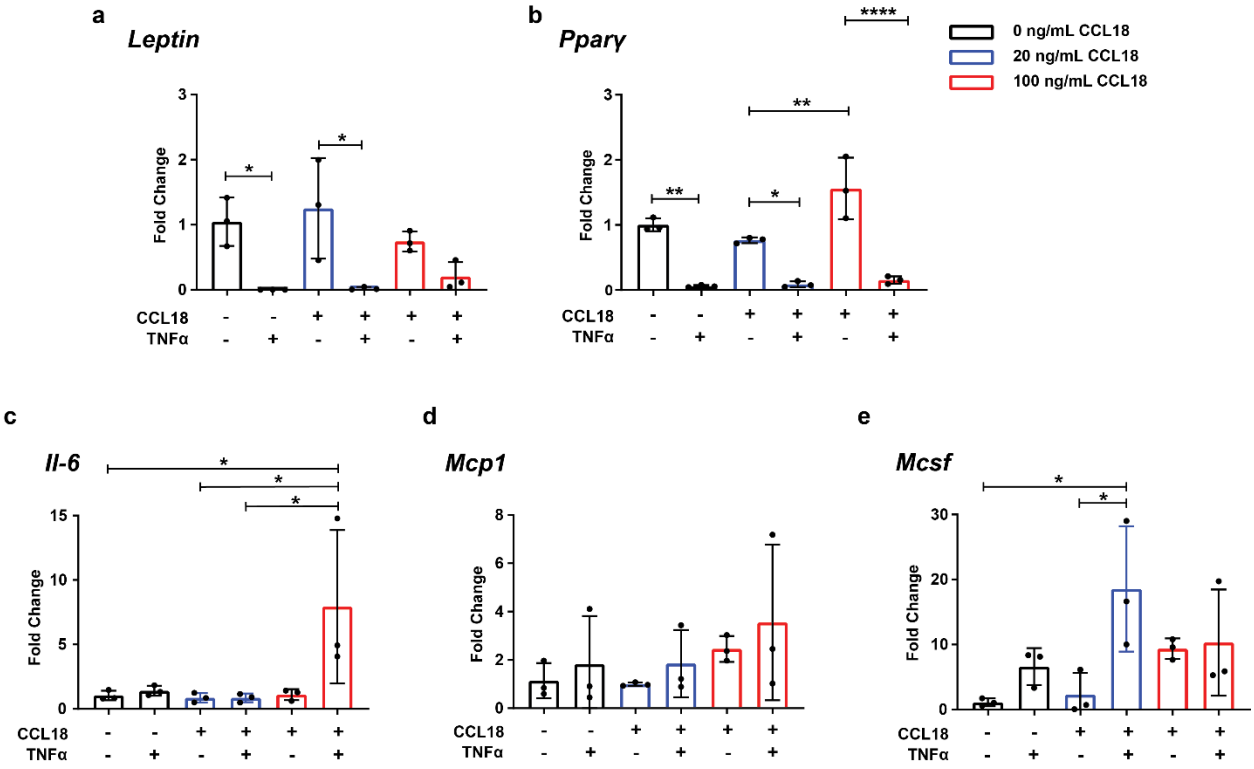

Figure 3.3. CCL18 promotes an inflammatory response in conjunction with TNFα in 3T3-L1 adipocytes
Gene expression by RT-qPCR of, a) *Leptin,* b) *Pparγ*, c) *Il6*, d) *Mcp1*, and e) *Mcsf* in differentiated 3T3-L1 cells treated ± 1ng/mL TNFα, 0ng/mL CCL18 (black circles), 20ng/mL CCL18 (blue circles), or 100ng/mL (red circles) during differentiation. n=3/group. Statistical analysis= 2way ANOVA. *p<0.05, **p<0.005, ***p<0.0005.

Because mice do not produce CCL18 but express receptors for CCL18 we designed experiments to evaluate *in vivo* gain-of-function effects of CCL18 on metabolism and metabolic inflammation.

We have observed rapid remodeling of adipose tissue leukocytes with short-term HFD feeding (2 weeks) that include induction of ATM proliferation and promotion of T cell proliferation[99]. Short-term HFD alone is sufficient to impair glucose tolerance [161] and promote alternate macrophage polarization [162]. We hypothesized that CCL18

would promote glucose intolerance and potentiate the expansion of adipose tissue leukocytes in this paradigm of short-term HFD feeding. To test this, age-matched male C57BL6 mice were implanted with Alzet® osmotic pumps to deliver CCL18 or PBS for 2 weeks and were fed either a ND or HFD during the 2-week infusion (**Figure 3.4b**). HFD mice gained significantly more weight than ND mice (**Figure 3.4c**) and there were no significant differences in body weight between CCL18 treated and control groups. Glucose tolerance testing demonstrated no significant changes in glucose tolerance between groups based on the area under the curve (**Figure 3.4d)**. HFD-fed PBS-treated animals had higher peak glucose levels compared to ND-fed PBS controls suggesting mild glucose intolerance was induced by this short-term HFD paradigm.

CCL18 does not alter adipose tissue or splenic leukocytes with short-term HFD feeding

To evaluate the hypothesis that CCL18 potentiates metabolic inflammation, SVF was isolated from eWAT, iWAT, and splenocyte controls and evaluated by flow cytometry in the short-term (2 weeks) HFD mice. HFD mice had larger fat pads and cell numbers within the fat pads compared to ND mice, but there were no differences between CCL18 treatment groups with either diet. Spleen and liver weights and splenocyte cell numbers were comparable among all groups (**Figure 3.4e-f**). When cell numbers were normalized to organ weight, there were no differences in cells/gram for eWAT, iWAT, or spleen among the groups (**Figure 3.4g**).

Flow cytometry analysis revealed no difference in the frequency of $CD45^+$ leukocytes in eWAT, iWAT, or spleen regardless of diet or treatment (**Figure 3.4h**).

However, PBS-treated HFD mice had a greater frequency of $CD11b^{+}CD3^{-}$ myeloid cells in the eWAT that was not seen in CCL18-treated mice. There was no difference in $CD11b^{-}CD3^{+}$ lymphoid cells in eWAT. (**Figure 3.4i**). Evaluating total ATMs and ATM subsets, we observed comparable total $CD64^{+}$ ATMs, $CD64^{+}CD11c^{-}$ ATMs, and $CD64^{+}CD11c^{+}$ inflammatory ATMs among all groups (**Figure 3.4j)**. HFD CCL18-treated mice had fewer $CD64^{-}CD11c^{+}$ adipose tissue dendritic cells (ATDCs) in eWAT compared to their ND counterparts. No differences in ATDCs were observed with PBS treatment. In iWAT, CCL18-treated HFD mice had a greater frequency of $CD11b^{+}CD3^{-}$ myeloid cells (**Figure 3.4k**). In iWAT, ND, and HFD, PBS mice had comparable myeloid cells, and the frequency of lymphoid cells was comparable among all groups. Like eWAT, we observed comparable total $CD64^{+}$ ATMs, $CD64^{+}CD11c^{-}$ ATMs, and $CD64^{+}CD11c^{+}$ inflammatory ATMs among all groups in iWAT, as well as ATDCs. (**Figure 3.4l**). The frequency of myeloid cells and lymphoid cells in the spleen was comparable among all groups, but with HFD, PBS control mice had a greater frequency of $CD64^{+}$ macrophages and dendritic cells compared to ND mice. HFD, CCL18 treated mice had fewer $CD64^{+}CD11c^{-}$ splenic macrophages compared to ND, CCL18 treated mice. (**Figure 3.4m-n**). Overall, CCL18 did not induce major changes in adipose tissue or splenic immune cell content in this short-term HFD paradigm.

To examine the hypothesis that CCL18 may alter ATM proliferation, we quantified proliferating ATM populations using the marker Ki67. While HFD mice had more proliferating ATMs compared to ND mice, CCL18 treatment did not alter ATM proliferation in the adipose tissue of ND or HFD mice. In the spleen, ND-CCL18-treated mice had fewer proliferating $CD64^{+}CD11c^{-}$ macrophages compared to ND-PBS mice

(**Figure 3.4o**). Together these results suggest that CCL18 does not alter the number of ATMs, independent of diet. CCL18 may suppress the proliferation of macrophages in the spleen.

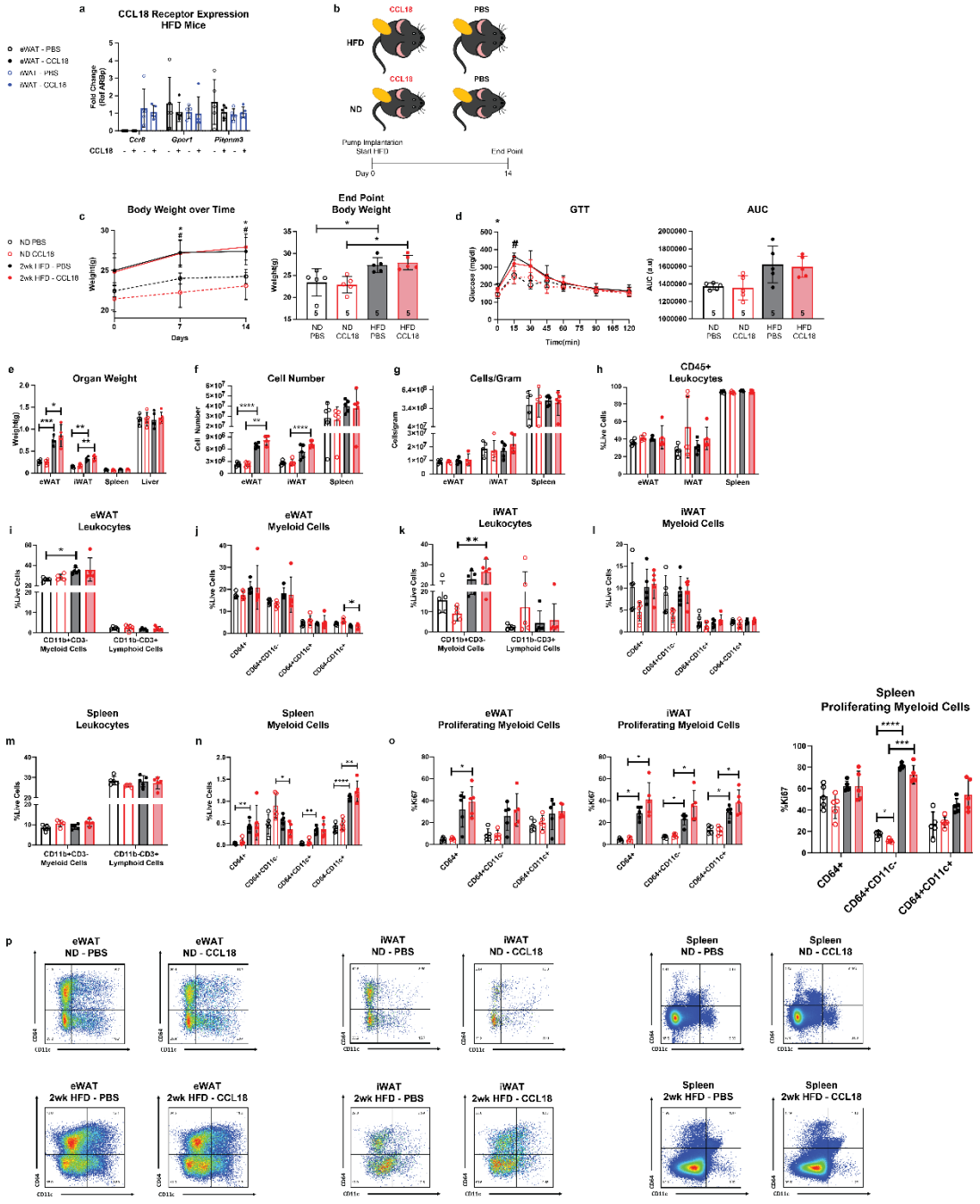

Figure 3.4. CCL18 does not alter glucose tolerance or leukocyte content with short-term HFD feeding

a) Gene expression of CCL18 receptors in the adipose tissue of C57BL6 mice ± CCL18 or PBS by RT-qPCR. Mice were injected ± CCL18 or PBS every 72 hours for 14 days. eWAT-PBS (open black circle), eWAT-CCL18 (closed black circle), iWAT-PBS (open blue circle), iWAT-CCL18 (closed blue circle). b) Experimental design and timeline of Alzet® osmotic pump experiment with acute HFD feeding. c) Body weight over time and endpoint body weight. d) Glucose tolerance test with area-under-the-curve (AUC). e)Organ weight of eWAT, iWAT, spleen, and liver. f) Cell number and g) cells per gram of eWAT, iWAT, and spleen. Flow cytometry analysis of the frequency of h) CD45+ leukocytes in eWAT, iWAT, and spleen; i) myeloid and lymphoid cells in eWAT; j) ATMs and dendritic cells (DCs) in eWAT; k) myeloid and lymphoid cells in iWAT; l) ATMs and dendritic cells in iWAT; m) myeloid and lymphoid cells in the spleen; n) macrophages and DCs in the spleen; o) proliferating ATMs/macrophages in eWAT, iWAT, and spleen; p) representative flow plots. ND-PBS (open black circle), ND-CCL18 (open red circle), 2wk HFD-PBS (closed black circle), 2wk HFD-CCL18 (closed red circle). n=3-5/group. Statistical analysis= Ordinary one-way ANOVA. *p<0.05, **p<0.005, ***p<0.0005, ****p<0.0001. For Weight over Time and GTT graphs: *p<0.05 between CCL18 groups; #p<0.05 between PBS groups.

CCL18 does not increase adipose tissue T cells with short-term HFD

CCL18 has been shown to increase T-cell populations in the peritoneal cavity of Balb/c mice [106]. To test the hypothesis that CCL18 alters adipose tissue T-cell content, we assessed T-cell populations in our short-term HFD cohort of C57BL6 ND and HFD-fed mice after 2 weeks of CCL18 treatment. CCL18 treatment did not alter the frequency of $CD8^+$, $CD4^+$, or $CD4^+CD8^+$ (DP) T-cells in eWAT (**Figure 3.5a**); however, we did observe an increase in eWAT DP T cells in HFD-fed mice. HFD-fed CCL18-treated mice had a greater frequency of $CD4^+FoxP3^+$ Tregs and fewer $CD4^+FoxP3^-$ conventional T cells (Tconv) compared to ND CCL18-treated mice (**Figure 3.5b**). In iWAT, there was not a significant difference in DP T-cells between diets (**Figure 3.5c**) and HFD mice for all treatment groups had a greater frequency of Tregs and fewer Tconv among the $CD4^+$ T cells. (**Figure 3.5d**). The increased frequency of DP T cells observed in adipose tissue was also observed in the spleen; however, HFD mice had

fewer splenic Tregs and increased Tconv compared to ND in both groups. Splenic T cells were not altered by CCL18 in this short-term paradigm (**Figure 3.5e-f**).

We evaluated T cell proliferation as a potential mechanism of these changes in adipose tissue T cell frequency. HFD feeding for 2 weeks induced the proliferation of adipose tissue T cells in eWAT, iWAT, and spleen compared to lean mice (**Figure 3.5g**). This was most prominent in Tregs compared to Tconv but did not differ between CCL18 treatment and controls. Together this data shows that CCL18 does not significantly alter adipose tissue T cell content with short-term HFD feeding.

CCL18 suppresses CD11c$^+$ ATM accumulation in eWAT with long-term HFD feeding

Since we did not observe a CCL18-dependent effect on metabolism in the paradigm of short-term HFD feeding, we examined the hypothesis that CCL18 would potentiate metabolic dysfunction in mice after the establishment of obesity in a longer-term HFD model (6 weeks) with treatment with CCL18. For these experiments, mice were fed 4 weeks of HFD or ND before implantation with Alzet® osmotic pumps ± CCL18 or PBS for 2 weeks followed by endpoint assessments (**Figure 3.6a**). To ensure the pumps were delivering CCL18 properly, serum CCL18 was measured in PBS and CCL18-treated mice. CCL18 mice had increased levels of circulating CCL18 when compared to PBS mice comparable to levels found in human serum (**Figure 3.6b**). Circulating CCL18 in humans ranges from 0-120 ng/mL [103]. After 6 weeks of HFD mice had greater weight gain and end point body weight compared to ND mice, independent of CCL18 treatment (**Figure 3.6c-d**). Consequently, HFD mice had impaired glucose

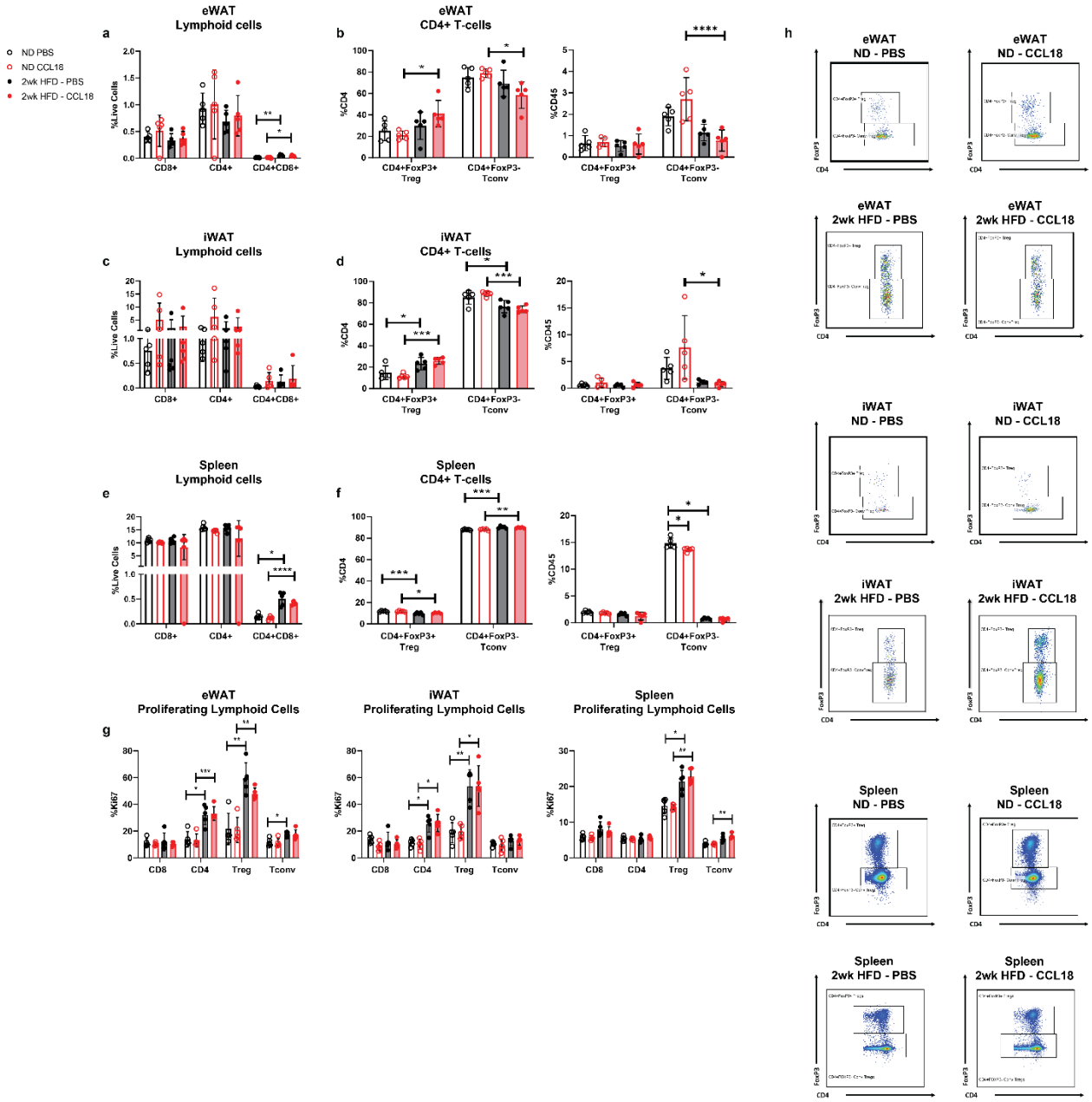

Figure 3.5. CCL18 does not increase adipose tissue T cells with short-term HFD
Flow cytometry analysis of a) lymphoid cells and b) CD4+ T cells in eWAT; c) lymphoid cells and d) CD4+ T cells in iWAT; e) lymphoid cells and f) CD4+ T cells in spleen; g) proliferating T cells in eWAT, iWAT, and spleen; h) representative flow plots. ND-PBS (open black circle), ND-CCL18 (open red circle), 2wk HFD-PBS (closed black circle), 2wk HFD-CCL18 (closed red circle). n=3-5/group. Statistical analysis= Ordinary one-way ANOVA. *p<0.05, **p<0.005, ***p<0.0005, ****p<0.0001.

tolerance compared to ND controls, however, there were no differences in glucose tolerance between the CCL18 treatment groups (**Figure 3.6e**). Although HFD mice had larger fat pads (**Figure 3.6f**), cell number and cells/gram were comparable among all groups (**Figure 3.6g-h**).

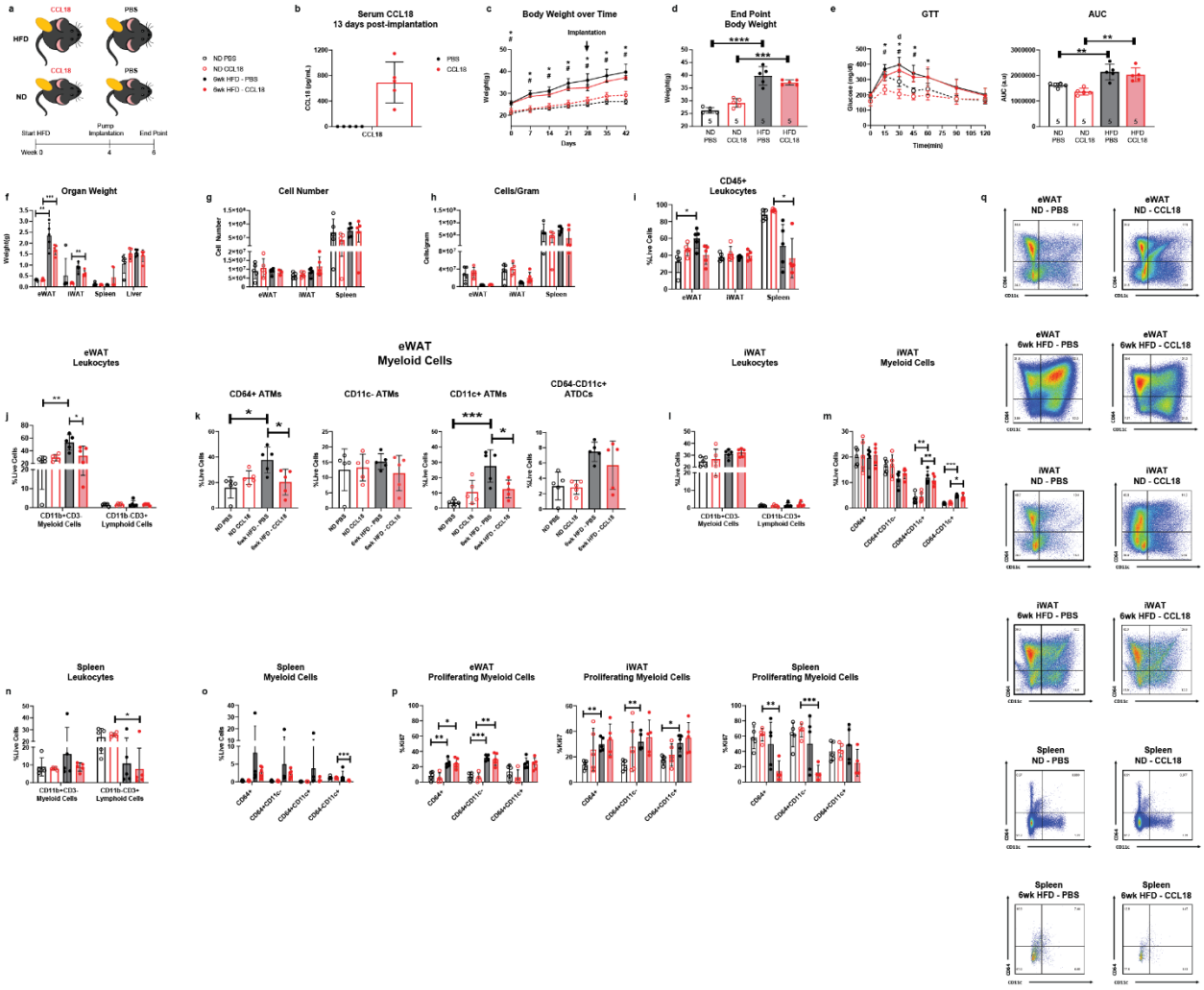

Figure 3.6. CCL18 suppresses the accumulation of CD11c⁺ ATMs in obese adipose tissue

a) Experimental design and timeline of Alzet® osmotic pump experiment with HFD feeding. b) Circulating levels of CCL18 from the serum of PBS (closed black circle) and CCL18 (closed red circle) mice by ELISA..c) Body weight over time and d) endpoint body weight. e) Glucose tolerance test with area-under-the-curve (AUC). f) Organ weight of eWAT, iWAT, spleen, and liver. g) Cell number and h) cells per gram of eWAT, iWAT, and spleen. Flow cytometry analysis of the frequency of i) CD45+ leukocytes in eWAT, iWAT, and spleen; j) myeloid and lymphoid cells in eWAT; k) ATMs and dendritic cells (DCs) in eWAT; l) myeloid and lymphoid cells in iWAT;

m) ATMs and dendritic cells in iWAT; n) myeloid and lymphoid cells in spleen; o) macrophages and DCs in spleen; p) proliferating ATMs/macrophages in eWAT, iWAT, and spleen; q) representative flow plots. ND-PBS (open black circle), ND-CCL18 (open red circle), 6wk HFD-PBS (closed black circle), 6wk HFD-CCL18 (closed red circle). n=3-5/group. Statistical analysis= Ordinary one-way ANOVA. *p<0.05, **p<0.005, ***p<0.0005, ****p<0.0001. For Weight over Time and GTT graphs: *p<0.05 between CCL18 groups; #p<0.05 between PBS groups, d<0.05 between CCL18 and PBS ND groups.

Flow cytometry analysis revealed while HFD induced an increase in $CD45^+$ leukocytes in control PBS mice, CCL18-treated HFD mice had fewer $CD45^+$ leukocytes compared to HFD control mice and were not different than CCL18 ND-fed mice (**Figure 3.6i**). This observation was due to differences in $CD11b^+CD3^-$ myeloid cells which were increased in HFD PBS-treated mice but were not induced with HFD feeding in CCL18-treated mice. In the HFD mice, CCL18-treated mice had fewer myeloid cells compared to PBS controls (**Figure 3.6j).** More specifically, while $CD64^+CD11c^+$ inflammatory ATMs in eWAT were induced by HFD in PBS-treated mice, CCL18-treated mice failed to induce accumulation of $CD11c^+$ ATMs with HFD (**Figure 3.6k**). There were no significant differences in $CD64^+CD11c^-$ resident ATMs with CCL18 treatment demonstrating a specific effect of CCL18 on $CD11c^+$ ATM content.

The changes in ATM accumulation with CCL18 were specific for eWAT as myeloid cell content did not change in iWAT with CCL18 treatment. HFD mice had increased $CD11c^+$ inflammatory ATMs in iWAT compared to ND mice independent of treatment (**Figure 3.6l-m**). No significant differences were observed between splenic myeloid cells, lymphoid cells, or macrophage subsets (**Figure 3.6n-o**). The difference in ATM content between PBS and CCL18-treated groups was not due to changes in proliferation, as ATM proliferation was induced by HFD similarly in PBS and CCL18-

treated mice (**Figure 3.6p**). In sum, CCL18 treatment after the establishment of obesity suppresses the accumulation of inflammatory $CD64^{+}CD11c^{+}$ ATMs in eWAT but does not change glucose tolerance or obesity status.

CCL18 does not alter adipose tissue T cells in long-term HFD mice

Adipose tissue T cells in mice treated with CCL18 or PBS with long-term HFD were assessed by flow cytometry. Unlike the 2-week HFD cohort, 6-week HFD mice did not display an increase in DP T cells in eWAT (**Figure 3.7a**). Similar to the 2-week HFD cohort, HFD mice had an increase in eWAT Tregs and a decrease in Tconv as a proportion of the $CD4^{+}$ adipose tissue T cells (**Figure 3.7b**) Similar to eWAT, $CD8^{+}$, $CD4^{+}$, and DP T-cells were comparable among all groups and there were no other changes in Tregs and or Tconv frequency with CCL18 treatment or diet (**Figure 3.7c-d**). There were no differences in lymphoid cells in the spleen; however, HFD mice had fewer Tconv when compared to ND mice in both treatment groups (**Figure 3.7e-f)**.

Examining T cell proliferation, we observed an induction of proliferation with HFD $CD8^{+}$ and $CD4^{+}$ T cells in eWAT in the PBS-treated group and increased proliferation with HFD $CD8^{+}$ T cells in iWAT in the CCL18-treated group. (**Figure 3.7g**). There were no significant differences in the proliferation of Tregs or Tconvs with diet or CCL18 treatment. Overall, these findings suggest that CCL18 treatment does not promote T-cell accumulation with long-term HFD feeding.

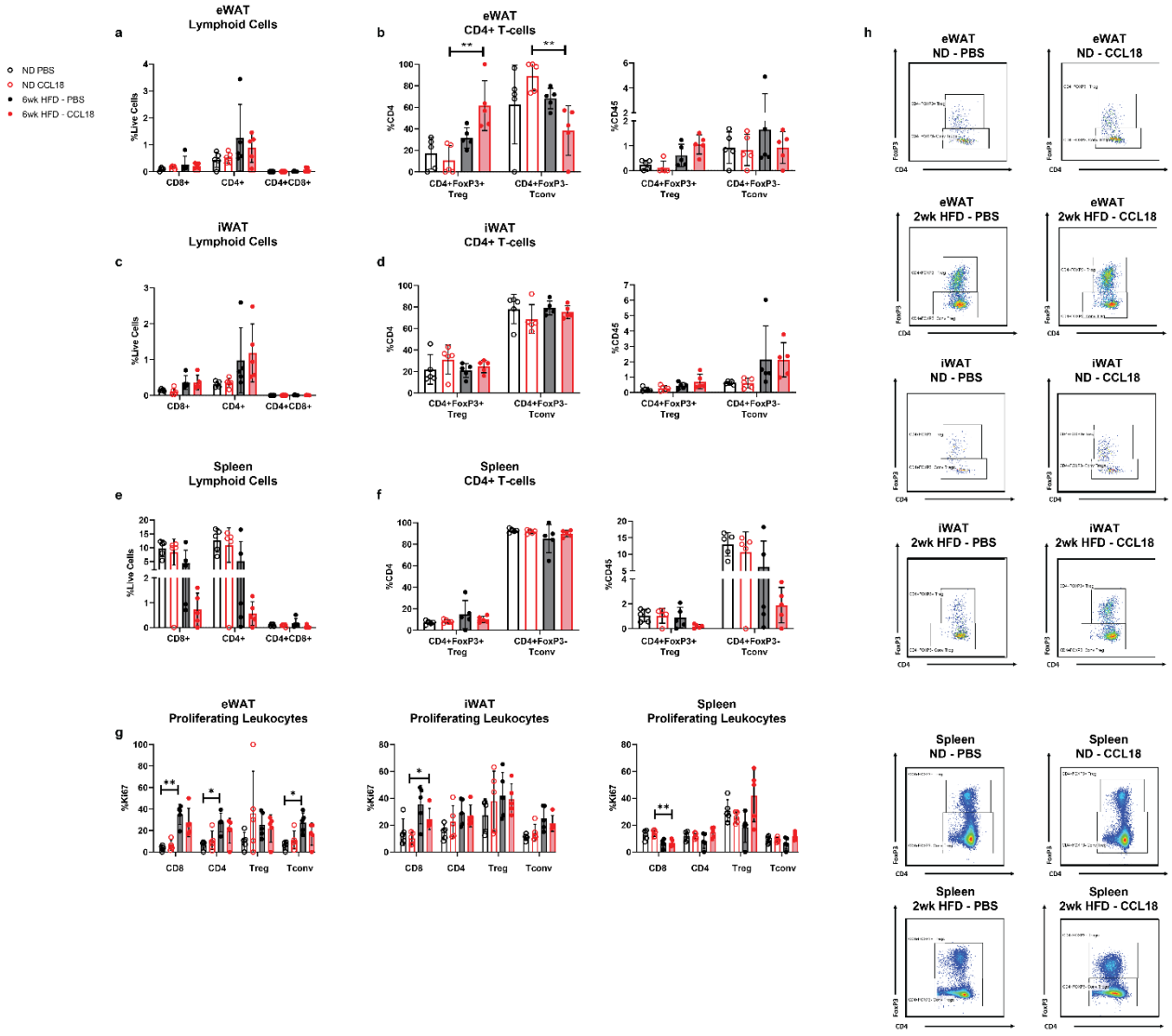

Figure 3.7. CCL18 does not alter adipose tissue T cells in long-term HFD mice
Flow cytometry analysis of a) lymphoid cells and b) $CD4^+$ T cells in eWAT; c) lymphoid cells and d) $CD4^+$ T cells in iWAT; e) lymphoid cells and f) $CD4^+$ T cells in spleen; g) proliferating T cells in eWAT, iWAT, and spleen; h) representative flow plots. ND-PBS (open black circle), ND-CCL18 (open red circle), 6wk HFD-PBS (closed black circle), 6wk HFD-CCL18 (closed red circle). n=3-5/group. Statistical analysis= Ordinary one-way ANOVA. *p<0.05, **p<0.005, ***p<0.0005, ****p<0.0001.

CCL18 does not induce angiogenesis in human adipose tissue or HUVECs

Previous studies identified CCL18 as being pro-angiogenic in both breast cancer tumors and human umbilical vein endothelial cells (HUVECs) [107]. To determine if CCL18

promotes angiogenesis in adipose tissue, angiogenesis assays were performed using explants of human VAT and SAT biopsies in Matrigel treated with growth media ± CCL18 as previously described [157] (**Figure 3.8a**). After 7 days of culture, explants were imaged, and angiogenic growth was assessed relative to the adipose tissue explant (**Figure 3.8b**). Initial experiments were performed from male VAT biopsies from NDM and DM individuals. NDM VAT treated with a high dose of CCL18 demonstrated a trend for decreased growth expansion, however, this was not seen in other samples with lower amounts of CCL18 (**Figure 3.8c**). A second set of experiments were performed in VAT and SAT explants from a female. (**Figure 3.8d**). CCL18 did not promote growth expansion in either VAT or SAT from female subjects. Consistent with other reports, SAT had a higher angiogenic capacity than VAT [148].

Because of the cellular heterogeneity of adipose tissue [163], we assessed the effect of CCL18 on angiogenesis in isolated ECs. HUVECs were embedded in a fibrin gel matrix as described [158] ± lung fibroblasts (LFs) and CCL18, and EC sprout length was assessed. While ECs with LFs had a trend for greater EC sprout length compared to ECs without LFs, CCL18 did not induce EC sprout length in either condition, independent of CCL18 concentration (**Figure 3.8e**). Together these findings suggest that CCL18 does not induce angiogenesis in human adipose tissue or HUVECs.

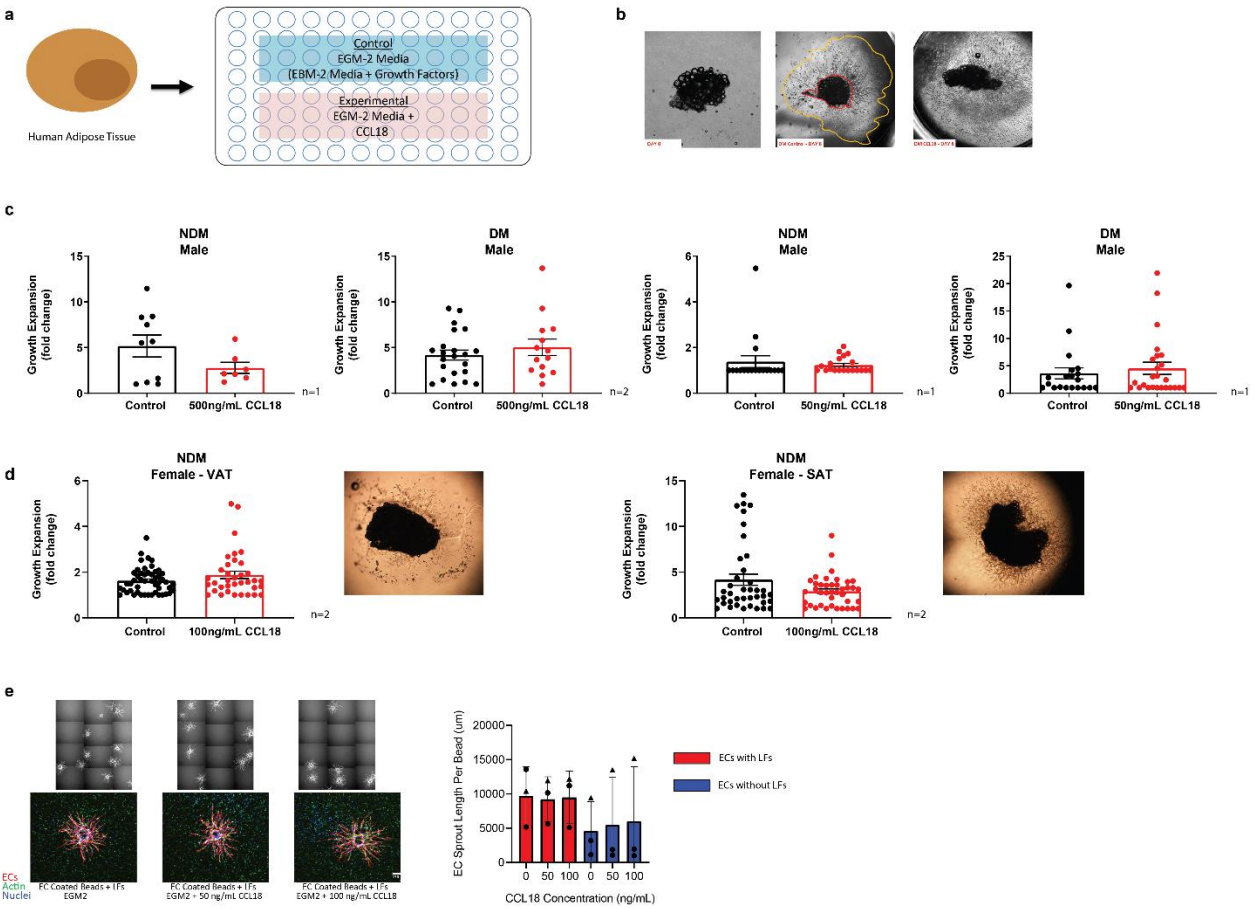

Figure 3.8. CCL18 does not promote angiogenesis in human adipose tissue or HUVECs

a) Experimental design of Matrigel angiogenesis assay ± CCL18. b) Representative images of growth expansion of adipose tissue explants at Day 0 and Day 8, as well as an illustration of the assessment of the growth expansion area. red line= adipose tissue explant area; yellow line= total area of sprouting. c) Growth expansion of male VAT ± CCL18 and d) female VAT and SAT ± CCL18. Statistical analysis= t-test; n= number of subjects; each dot represents a well; control= closed black circle; CCL18-treated= closed red circle. e) Representative images and EC sprout length of HUVECs embedded in fibril gel matrix ± CCL18. Statistical analysis= 2way ANOVA. Data from 3 independent studies; n=3/group; red bar= ECs with LFs; blue bar= ECs without LFs.

Discussion

This study aimed to examine the role of CCL18 in adipose tissue biology based on the observations that CCL18 expression is increased in the adipose tissue of people with obesity-associated DM. RNA sequencing of human adipose tissue showed that CCL18

is highly expressed in ATMs and both ATMs and ECs express identified receptors of CCL18. While the role of CCL18 in cancer progression has been studied extensively [164], there remains a gap in our understanding of the role of CCL18 in adipose tissue biology. Because there is not a mouse ortholog for CCL18 and mice possess identified receptors for CCL18, we are able to use a mouse model to study the effect of CCL18 on adipogenesis, glucose metabolism, and adipose tissue inflammation. Based on these observations and the prior literature, we examined the effects of CCL18 on several aspects related to adipose tissue stromal cell and adipocyte biology. Our primary observations are that 1) CCL18 treatment of mouse 3T3-L1 adipocytes did not alter adipogenesis, but increased the expression of *Mcsf* in conjunction with TNFα, suggesting a possible role for CCL18 in the adipocyte inflammatory response in mice, 2) CCL18 treatment of human adipocytes downregulated gene pathways related to immune responses and induced pathways related to cell morphogenesis, 3) chronic treatment of obese mice with CCL18 does not alter glucose tolerance or the obesity phenotype, 4) CCL18 treatment of obese mice decreases the accumulation of $CD11c^{+}$ inflammatory ATMs in eWAT but did not alter ATM proliferation rates, 5) CCL18 does not alter human adipose tissue angiogenesis *in vitro.*

In our *in vivo* mouse model, we observed that CCL18 did not impair glucose metabolism in mice in either short or long-term HFD models. We used two distinct experimental paradigms to evaluate the hypothesis that CCL18 shapes adipose tissue inflammation during the onset of obesity (short-term 2 weeks of HFD feeding) and in established obesity (6 weeks of HFD). Flow cytometry analysis of adipose tissue of

mice treated with or without CCL18 revealed that CCL18 did not alter ATM or T cell content with short-term HFD feeding.

In the longer-term HFD feeding model, we observed that CCL18 treatment blunted the increase in total ATMs seen with obesity primarily due to fewer $CD11c^+$ ATMs in eWAT. ATM proliferation was not changed suggesting that the CCL18 effect is independent of decreasing ATM proliferation. $CD11c^+$ ATMs are primarily monocyte-derived suggesting that CCL18 may suppress the recruitment of monocyte-derived macrophages to adipose tissue. However, in this context, there were no changes or improvements in glucose tolerance with this model. Overall, while CCL18 is associated with DM status in humans, our studies suggest that CCL18 may have regulatory functions that try to suppress ATM accumulation and adipose tissue inflammation in the obese state. This observation suggests that the anti-inflammatory functions of CCL18 may be more prominent than their pro-inflammatory functions. This is consistent with other studies showing that CCL18 inhibits the chemotaxis of chemokines that mediate the trafficking of leukocytes to sites of inflammation [165].

Because synthetic CCL18 has been shown to increase the frequency of $CD4^+$ and $CD8^+$ T cells in the peritoneal cavity of BALB/c mice [106], we hypothesized that CCL18 may alter adipose tissue T cell populations. We did not observe changes in the total numbers of $CD4^+$ or $CD8^+$ T cells in adipose tissue or spleen between treatment groups in either short or long-term HFD cohorts. However, in both studies, we observed a relative increase in the proportion of $CD4^+FoxP3^+$ Tregs and a relative decrease in $CD4^+FoxP3^-$ Tconv with HFD. Tregs regulate adaptive immunity by suppressing inflammatory signals and are decreased with long-term HFD feeding. Our inability to

reproduce the findings from Guan, et al that CCL18 increased $CD4^+$ and $CD8^+$ T cells may be attributed to the difference in genetic background between C57BL6 mice and BALB/c mice or fundamental differences in activation profiles between peritoneal and adipose tissue T cells. Studies have shown that C57BL6 and BALB/c mice have significantly different immune responses, with BALB/c mice having a more severe immune response than C57BL6 mice [166-168]. We confirmed that mice implanted with CCL18-filled pumps had circulating CCL18 2 weeks after implantation, however, additional experiments are required to confirm the biochemical activity of CCL18 in adipose tissue.

Preadipocytes have only been shown to express CCL18 receptor *CCR8* in human preadipocytes [103]; therefore, we wanted to determine if CCL18 mediated the function of mature adipocytes. Because 3T3L1 cells can differentiate into mature adipocyte-like cells, we measured the gene expression of markers of mature adipocytes after CCL18 and TNFα treatment and found that CCL18 had no effect on the differentiation of preadipocytes into mature adipocytes, but in conjunction with TNFα increased the expression of *Mcsf,* indicating a possible role for macrophage recruitment in adipocytes. *Mcsf* mediates the differentiation of hematopoietic stem cells into macrophages [169]. While CCL18 was not found to promote adipogenesis in 3T3L1 cells, this finding suggests that CCL18 promotes the differentiation of monocytes into macrophages as described previously [105]. Additional studies in monocytes would need to be carried out to confirm this. Studies in human mature adipocytes revealed an anti-inflammatory role for CCL18 by suppressing the immune response, which is not observed in our 3T3L1 experiments. This anti-inflammatory role of CCL18 seems to be

obesity-specific, as CCL18 also suppressed ATM accumulation in obese mouse adipose tissue. These findings shed light on two possible roles for CCL18 in adipose tissue: promoting monocyte differentiation in the lean state and protecting against inflammation in the obese state. Based on findings in 3T3L1 cells it is worth exploring whether CCL18 acts on human adipocytes to promote monocyte differentiation.

Since there is evidence that CCL18 is pro-angiogenic in HUVECs via the PITPNM3 receptor [107], we examined the hypothesis that CCL18 has a pro-angiogenic role in adipose tissue. Although we observed trends in CCL18 treatment and angiogenesis in human adipose tissue, we were unable to confirm a role for CCL18 in adipose tissue angiogenesis or HUVECs, as previously described [107]. The higher dose of CCL18 showed a trend for decreased growth expansion in NDM male VAT and a trend for increased growth expansion in DM male VAT, suggesting a diabetic status-dependent effect of CCL18 on angiogenesis. However, that concentration of CCL18 is not physiologically relevant; circulating CCL18 in humans ranges from approximately 5ng/mL (insulin sensitive) to 125ng/mL (insulin resistant) [103]. Because of the variability of human adipose tissue between individuals and the limited number of patient samples, additional samples need to be analyzed to determine if CCL18 promotes angiogenesis in adipose tissue. Overall, our findings do not support a role for CCL18 in obesity-induced glucose intolerance although it can blunt ATM accumulation in the obese state.

CHAPTER 4

Summary

The purpose of the studies outlined in this dissertation was to elucidate the role of adipose tissue macrophages (ATMs) in the pathogenesis of obesity-associated insulin resistance. While there are strong epidemiologic associations between obesity and metabolic disease, there is significant individual variation in the degree of metabolic dysfunction in people with obesity. There are two described phenotypes of obesity: metabolically healthy obesity (MHO) and metabolically unhealthy obesity (MUO). MUO can be distinguished from MHO by metabolic abnormalities like CVD, DM, and MetS [6; 10; 11]. Furthermore, MUO is characterized by increased inflammation [7]. Inflammatory ATMs accumulate in adipose tissue (AT) with obesity [24] and previous studies revealed inflammation as a distinct characteristic of obesity-associated DM in human AT [91].

In summary, the purpose of this work was to understand the role of ATMs in the pathogenesis of obesity-associated insulin resistance. Our experiments focused on two genes identified to be significantly increased in obese DM human AT – *MSR1* and *CCL18*. While there is limited knowledge of these genes in the context of AT in the literature, we were able to add to the field, concluding that *MSR1* is not required for obesity-induced insulin resistance and AT inflammation in mice. We also found that

CCL18 may have a role in suppressing the induction of $CD11c^+$ ATMs in obese mice, independent of proliferation.

Chapter 2

MSR1 is a known scavenger receptor that aids in phagocytosis [94; 119; 121] and promotes macrophage inflammation [120; 121] and proliferation [97; 98]. While *MSR1* has been correlated with obesity-associated DM in humans, our knowledge of its role in ATMs is limited and previous studies provide contradicting findings. Some findings suggest *Msr1* protects against obesity-induced insulin resistance [95; 96] and AT inflammation [95] in mice, while others showed *MSR1* mediates insulin resistance and inflammation [122]. These findings were limited to the use of non-littermate wild-type (WT) controls.

In our experiments, we confirmed that *MSR1* expression is increased in human VAT and utilized obese *Msr1*$^{-/-}$ mice and WT non-littermate controls, as well as obese *Msr1*$^{-/-}$ mice and *Msr1*$^{+/-}$ littermate controls to elucidate the underly mechanisms of *MSR1* in AT and insulin resistance. We confirmed *Msr1* deletion in the eWAT of *Msr1*$^{-/-}$ mice and WT and *Msr1*$^{+/-}$ littermate mice had significantly increased levels of Msr1 expression, serving as sufficient controls for our in vivo studies. We demonstrated that Msr1$^{-/-}$ mice had lower body weight, fat mass, inflammatory ATM content, and a trend for lower glucose levels compared to WT non-littermate mice, indicating that Msr1 may play a role in sustained AT inflammation and impaired glucose metabolism with obesity like previous findings [122]. On the other hand, we did not observe this phenotype in Msr1$^{-/-}$ mice compared to Msr1$^{+/-}$ littermate mice. Because littermate experiments control

for variation in genetic background, it is the most rigorous of the experiments [123]; therefore, we conclude that *Msr1* is not required for obesity-induced insulin resistance and AT inflammation in mice.

Our initial findings in *Msr1*$^{-/-}$ and non-littermate WT mice suggested a role for *Msr1* in AT inflammation via ATM proliferation; however, we were unable to replicate this finding in the *Msr1*$^{-/-}$ and Msr1$^{+/-}$ littermate cohort. A limitation of this study is the use of *Msr1*$^{+/-}$ mice as littermate controls. While we observed significant *Msr1* expression in *Msr1*$^{+/-}$ mice compared to *Msr1*$^{-/-}$ littermates, we also observed a significant difference in *Msr1* expression between WT and *Msr1*$^{+/-}$ mice. Although the WT mice were not littermates, this sheds light on a possible difference among the three genotypes. The use of littermate controls in metabolic studies is important to control for variations in genetic background [123].

Chapter 3

CCL18 is a chemokine produced by myeloid cells, including human ATMs [103] that is associated with inflammatory diseases such as cancer [100-102; 143; 144], obesity, and insulin resistance [103; 141]. CCL18 has also been shown to induce angiogenesis [107]. Like *MSR1, CCL18* expression is significantly increased in the AT of obese DM compared to obese NDM [91]. There isn't a mouse ortholog for *CCL18* making this a prime model to carry out gain-of-function experiments to elucidate the underlying mechanisms of CCL18 in AT. Previous studies in mice found that CCL18 increased T-cells in Balb/c mice [106] and CCL18 activates the mouse CCR8 receptor [141]. To understand the role of CCL18 in AT and glucose metabolism, we utilized a

combination of in vitro human and mouse experiments as well as an in vivo mouse model. We confirmed that CCL18 is significantly expressed in DM AT; single nuclear analysis of that AT demonstrated that CCL18 is highly expressed in lipid-associated macrophages (LAMs) and endothelial cells. Furthermore, CCL18 suppressed immune pathways in mature human adipocytes from individuals with obesity. Experiments in 3T3L1 adipocytes demonstrated that CCL18 promotes an inflammatory response in conjunction with TNFα. This is the opposite of what we observed in obese human adipocytes, indicating an obesity-specific response.

We confirmed CCL18 receptor expression in the AT of C57BL6 mice and treated ± CCL18 via an osmotic mini-pump to further characterize the role of CCL18 in vivo. We observed that CCL18 did not alter glucose metabolism with short-term HFD feeding or in obese mice. However, in the short-term HFD paradigm, CCL18 did not alter T cell or leukocyte content. In obese mice, however, CCL18 suppressed the accumulation of inflammatory $CD11c^+$ ATMs but still did not affect T cell content. These findings implicate an obesity-specific response to CCL18 on AT inflammation, specific to $CD11c^+$ ATMs, independent of proliferation. To determine if CCL18 was pro-angiogenic in AT, human AT explants, and HUVECs were treated ± CCL18 in a 3D matrix, and growth expansion was assessed. These experiments demonstrated that CCL18 did not promote angiogenesis in human adipose or HUVECs. Our findings suggest that CCL18 may play a protective role in obesity by suppressing ATM induction in AT.

Our in vitro and in vivo conclusions of CCL18 in AT provided an obesity-specific effect. While the 2-week HFD-fed maintained normal metabolism and no changes in leukocyte content independent of treatment, in the obese mice (6wk HFD), we did

observe that CCL18 suppressed the infiltration of ATMs that is typically observed with HFD [24; 145]. This is supplemented by our finding in obese adipocytes that CCL18 suppresses genes related to interleukin signaling and the innate immune system. This finding implicates CCL18 as a potential anti-inflammatory agent in obese AT. This anti-inflammatory effect of CCL18 is in alignment with a previous report in mouse pre-B lymphoma cells showing that CCL18 inhibits the activity of other chemokines that mediate the trafficking of leukocytes [165]. Moreover, we were unable to replicate earlier findings that CCL18 treatment in mice increased the quantity of $CD4^+$ and $CD8^+$ T cells in AT [106]. This discrepancy is likely attributed to the different genetic backgrounds of Balb/c mice used in their experiments compared to our C57BL6 mice, as Balb/c mice are known to have a more severe immune response [166-168]. We utilized C57BL6 mice in our studies as they are preferred to study metabolic disorders, like obesity and DM [170]. Additionally, differences in peritoneal macrophages and ATMs may contribute to this discrepancy. ATMs have a distinct metabolic profile during obesity compared to peritoneal macrophages, with increased expression of genes involved in lipid metabolism [56].

Angiogenesis experiments in human AT and HUVECs were unable to confirm any angiogenic effect of CCL18 that was previously reported [107]. The main limitation is the lack of biological samples from human subjects, as we observed significant variability between patients as well as between treated explants. Not all treated explants displayed growth, this may be due to the lack of ECs in a specific explant. Additional experiments to quantify ECs in each explant are needed to gain a better understanding of why some explants from one subject grew while others did not. Moreover, additional

patient samples need to be analyzed and additional HUVEC experiments need to be carried out to determine if CCL18 affects AT angiogenesis.

Overall, we demonstrated that neither Msr1 nor CCL18 are required for obesity-associated insulin resistance in mice, but CCL18 may have an anti-inflammatory effect in obese AT by suppressing the immune response.

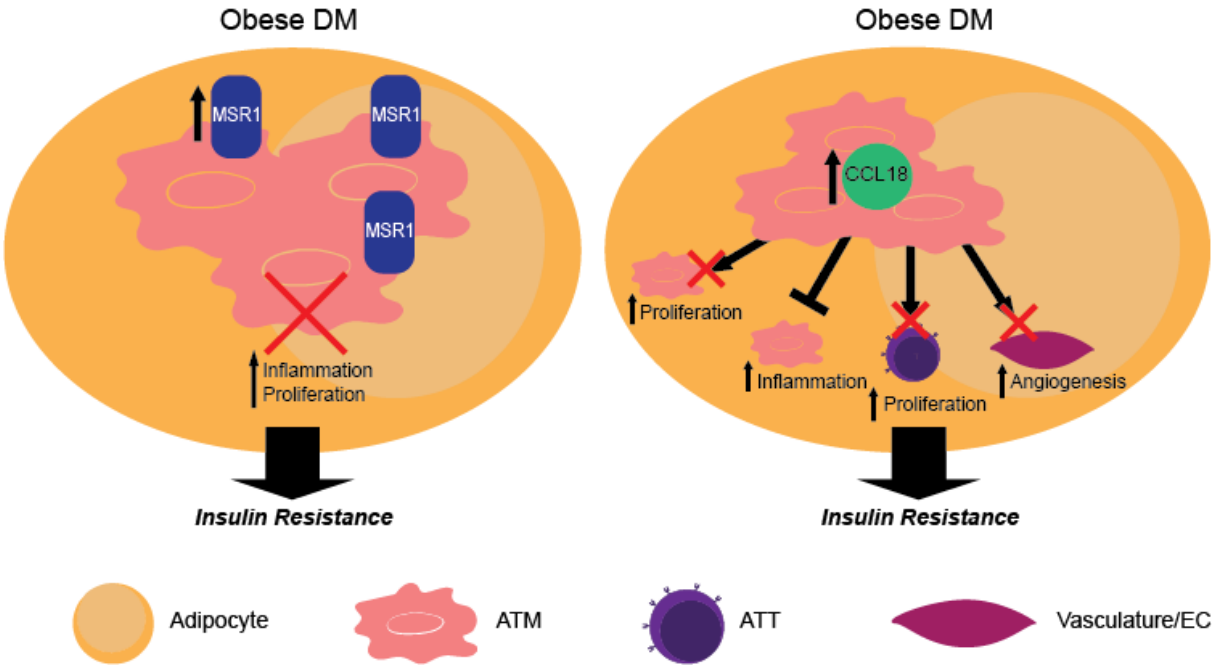

Figure 4.1 Model of MSR1 and CCL18 in obese adipose tissue

Clinical Implications and Future Directions

Although our findings did not implicate *MSR1* as a link between obesity and DM in mice, its increased expression in human AT suggests *MSR1* could be a novel clinical biomarker for MUO and DM progression in individuals with obesity. We observed contradictory results in our *Msr1*$^{-/-}$ vs WT non-littermate experiments compared to our *Msr1*$^{-/-}$ vs *Msr1*$^{+/-}$ littermate experiments. Although we agree that our littermate

experimental design more rigorously tested our hypothesis that *Msr1* contributes to obesity-associated insulin resistance and adipose inflammation through ATM proliferation, additional experiments must be carried out to determine if there is a difference in phenotype between WT and *Msr1*$^{+/-}$ littermate mice. This will allow us to determine the most suitable littermate control for future experiments. This requires a breeding strategy mating *Msr1*$^{+/-}$ male mice and *Msr1*$^{+/-}$ female mice to produce *Msr1*$^{+/+}$ (WT), *Msr1*$^{+/-,}$ and *Msr1*$^{-/-}$ littermates. Additionally, experiments confirming Msr1 protein expression in AT would contribute to confirming phenotypic differences. Moreover, using known Msr1 agonists such as low-density lipoproteins [118] and advanced glycation end products [171] in both mouse and human AT would provide insight into understanding the role of *Msr1* activation in obese AT and if it's a suitable drug target to combat obesity-associated DM.

Like *MSR1*, our findings in human AT implicate CCL18 as a novel clinical biomarker for MUO and obesity-associated DM. Moreover, we identified CCL18 as a potential link between obesity and AT inflammation. Our findings suggest that CCL18 suppresses the immune response by blocking the accumulation of CD11c^{+} ATMs in obese adipose, but not lean adipose tissue. The suppression of CD11c^{+} ATMs in obese AT was independent of proliferation. One potential mechanism for this observation is impaired CD11c^{+} ATM recruitment into AT. Multiple factors have been implicated in the recruitment of ATMs including adipose tissue dendritic cells (ATDCs), MCP-1 [172], and chemokine receptors CCR1 and CCR5 [173]. We did not see a reduction in ATDCs or MCP-1 with CCL18 treatment in mouse adipocytes. Therefore, I hypothesize that the impaired CD11c^{+} ATM recruitment observed with CCL18 treatment in obese AT is

attributed to the inhibition of CCR1 and CCR5-mediated chemotaxis as previously reported [165]. This can be tested by treating isolated obese AT ± CCL18 and ligands for CCR1 and CCR5 and carrying out a chemotaxis assay. Additionally based on findings in 3T3L1 cells that CCL18 induces *Mcsf* expression in conjunction with TNFα, it is worth exploring the direct effect of CCL18 on monocyte/macrophage differentiation. This can be done by treating cultured monocytes ± CCL18 and Mcsf in vitro and assessing macrophage subsets via flow cytometry as well as differential gene expression via RNAseq. Confirming this would provide insight into the underlying mechanism of suppressed ATM recruitment. Furthermore, future flow cytometry analyses should include CCR5 to determine if $CCR5^{+}$ ATMs are also suppressed as CCR5+ macrophages accumulate in AT with obesity and are required for ATM accumulation [174]. While we were able to confirm the presence of circulating CCL18 in our mouse cohorts, future studies are required to confirm the biochemical activity of CCL18 in adipose tissue as well as identify any mouse receptors it binds in both mouse and human AT. Biochemical activity of CCL18 can be determined by Western Blot analysis to quantify total and phosphorylated-Akt (p-Akt) in adipose tissue treated ± CCL18; p-Akt is expected to increase with CCL18 [175]. Co-immunoprecipitation experiments will determine if CCL18 binds to the three previously identified receptors in adipose tissue. To confirm if there is a role for CCL18 in AT angiogenesis, additional human AT samples must be obtained, and these experiments repeated.

In sum, while MSR1 and CCL18 gene expression increases with obesity and DM, there is no evidence that they contribute mechanistically to obesity-associated DM. This has implications towards development of therapies for DM directed towards anti-

inflammatory targets. Observing an association between DM and biomarkers such as CCL18, for example, may not indicate that CCL18 is mechanistically contributing to DM pathogenesis. In fact, our data suggests that CCL18 may be protective and may be induced in ATMs to try to dampen adipose tissue inflammation. This has implications towards the development of anti-inflammatory therapeutics for DM where ways to enhance CCL18 signaling may be more preferential as a treatment strategy as opposed to inhibition.

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