A Role for Adipocyte Progenitor Cells in Obesity-Induced MCP-1 and M1 Macrophage Accumulation in Visceral Adipose Tissue

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Abstract

Obesity is one of the top preventable causes of death in the United States, as it can lead to cardiovascular disease, type II diabetes, and cancer. Visceral adipose tissue (VAT) accumulation and inflammation directly link to metabolic dysfunction and obesity-associated disease, and act as predictors of obesity-associated mortality. As such, identification of novel targets to limit diet-induced VAT accumulation has the potential to impact morbidity and mortality.

Inflammatory cells, as well as the cytokines they produce, play a large role in obesity-related diseases. High-fat diet (HFD) results in the induction of expression of proinflammatory genes in both mice and humans, such as monocyte chemoattractant protein-1 (MCP-1). MCP-1 is a potent chemotactic factor for monocytes. Once infiltrated into the adipose tissue in the advanced stages of obesity, macrophages participate in the inflammatory pathways that are activated in obese adipose tissue, including insulin signaling, toll-like receptor (TLR) activation, and the nuclear factor kappa B (NFκB) pathway. While it is evident that macrophages are the main producers of MCP-1 during later stages of obesity, it is less clear where the initial obesity-induced increases in MCP-1 are derived.

The helix-loop-helix (HLH) family of transcription factors is a highly conserved group that plays a role in the differentiation and growth of a variety of cell types.

Inhibitor of Differentiation 3 (Id3) belongs to the HLH transcription factor family, and protein levels increase in the stromavascular fraction (SVF) of visceral adipose tissue during obesity. Global deletion of Id3 attenuates HFD-induced obesity, seen by decreased body weight and attenuated expansion of VAT. However, it is yet to be determined whether Id3 plays a role in obesity-induced metabolic perturbations or inflammation within VAT.

MCP-1 intracellular staining determined that $CD45^{-}CD31^{-}Ter119^{-}$ $CD34^{+}CD29^{+}Sca\text{-}1^{+}$ adipocyte progenitor cells (AdPCs) within VAT are the first cells to produce MCP-1 after initiation of HFD. Production of MCP-1 was limited to the $CD24^{-}$ subpopulation of AdPCs, those that have been previously demonstrated to be further committed to the adipocyte lineage. 1 week of HFD results in an increase in the number of $CD24^{-}$ AdPCs, primarily via proliferation. Human AdPCs, identified as $CD45^{-}CD31^{-}CD34^{+}CD44^{+}CD90^{+}$ cells, also expressed MCP-1, with higher expression in omental adipose tissue compared to subcutaneous adipose tissue. Additionally, high surface levels of CD44 on AdPCs marked the most abundant producers of MCP-1, in both murine and human VAT.

Id3 was identified as a critical regulator of AdPC proliferation, which may be dependent on Id3-induced repression of the $p21^{Cip1}$ promoter. Id3-deficient mice had fewer $CD24^{-}$ AdPCs, as well as reduced MCP-1 levels and attenuated accumulation of M1 macrophages within the VAT. *Id3*$^{-/-}$ mice also had improved

glucose uptake during glucose tolerance tests (GTTs) as well as enhanced insulin-stimulated phosphorylation of AKT. Adoptive transfer of *Id3*$^{+/+}$ AdPCs to *Id3*$^{-/-}$ mice resulted in increased MCP-1 secretion and significantly enhanced M1/M2 macrophage ratio in VAT. Adoptive transfer also resulted in increased weight gain and worsened glucose tolerance. However, adoptive transfer of *Id3*$^{-/-}$ AdPCs to *Id3*$^{-/-}$ mice had no effect on these parameters, leading us to believe that AdPCs must express Id3 to have MCP-1-mediated increases in M1 macrophages and glucose intolerance.

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List of Abbreviations

ADD	adipocyte determination- and differentiation-dependent factor
AdPC	adipocyte progenitor cell
AKT	also known as protein kinase B (PKB)
ANOVA	analysis of variance
aP2	adipocyte protein-2, also known as FABP4
AUC	area under the curve
BAT	brown adipose tissue
bHLH	basic helix-loop-helix
BMI	body mass index
BMP	bone morphogenic protein
BrdU	bromodeoxyuridine
CD	cluster of differentiation
CDC	Centers for Disease Control and Prevention
cDNA	complementary DNA
C/EBP	CCAAT/enhancer binding protein
CLS	crown-like structures
CCL	C-C chemokine ligand
CCR	C-C chemokine receptor
CXCR	C-X-C chemokine receptor
Cre	causes recombination enzyme
DAPI	4’,6-diamidino-2-phenylindole
db/db	mouse strain lacking the leptin receptor
DC	dendritic cell
DIO	diet-induced obesity
DMEM	Dulbecco’s modified eagle medium
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid, or edetic acid
ELISA	enzyme-linked immunosorbent assay
eVAT	epididymal VAT

FAS	fatty acid synthase
F12	nutrient mixture F-12
FABP4	fatty acid binding protein-4, also known as aP2
FACS	fluorescence-activated cell sorting
FBS	fetal bovine serum
FFA	free fatty acids
FITC	fluorescein isothiocyanate
fl/fl	homozygous for the floxed allele
floxed	flanked by two lox P sites
FMO	fluorescence minus one
gDNA	genomic DNA
GLUT4	glucose transporter type 4
gMFI	mean fluorescence intensity, using geometric mean
GTT	glucose tolerance test
GWAS	genome-wide association study
HA	hyaluronic acid
HFD	high fat diet
HLH	helix-loop-helix
IBMX	3-isobutyl-1-methylxanthine
Id	inhibitor of differentiation
Id3$^{-/-}$ mouse	mouse globally null for *Id3*
IFNγ	interferon gamma
IGF	insulin-like growth factor
IL	interleukin
ILC2	innate lymphoid type 2 cell
iNOS	inducible nitric oxide synthase
i.p.	intraperitoneal
IRB	Institutional Review Board
kcal	kilocalorie
Lin	lineage
LN	lymph node

LPS	lipopolysaccharide
LTβR	lymphotoxin-beta receptor
LTo	lymphoid tissue organizer
LUC	luciferase-containing promoter construct
Ly6C	lymphocyte antigen 6C
LysM	lysozyme M
Mac	macrophage
Mac-1	macrophage-1 antigen, or CD11b
MAPK	mitogen-activated protein kinase
MCE	mitotic clonal expansion
MCP-1	monocyte chemoattractant protein-1 or monocyte chemotactic protein-1, also known as CCL2
MFI	mean fluorescence intensity
MGC	multinucleated giant cell
MIP	macrophage inflammatory protein
mRNA	messenger RNA
NCD	non-communicable diseases
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NIH	National Institutes of Health
ns	non-significant
ob/ob	mouse strain lacking leptin
OPN	osteopontin
pAKT	phosphorylated AKT
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PDGFR	platelet-derived growth factor receptor
PF	peritoneal fluid
PPARγ	peroxisome proliferator-activated receptor gamma
RBC	red blood cell
RLU	relative light unit
RNA	ribonucleic acid

SAT	subcutaneous adipose tissue
Sca-1	stem cells antigen-1
SDF-1	stromal cell-derived factor 1
SEM	standard error of the mean
siRNA	small interfering RNA
Splen.	splenocytes
SREBP	sterol regulatory element binding protein, human homolog to ADD
SVF	stromavascular fraction
T_H	T helper
TLR	toll-like receptor
TNFα	tumor necrosis factor-alpha
TUNEL	terminal deoxynucleotidyl transferase dUTP nick end labeling
VAT	visceral adipose tissue
VEGF	vascular endothelial growth factor
WAT	white adipose tissue
WHO	World Health Organization
Zfp423	zinc-finger protein 423

Chapter 1

Introduction

Pathogenesis of obesity

Body mass index (BMI), defined as the weight in kilograms divided by the height in meters squared (kg/m^2), is the most widely used measure of obesity due to its low cost and simplicity[1]. The World Health Organization (WHO) and National Institutes of Health (NIH) have defined 'overweight' individuals as having a BMI between 25.0 and 29.9 kg/m^2, and 'obesity' as an individual having a BMI of 30.0 kg/m^2 or higher [2].

According to reports by the World Health Organization (WHO) and the Center for Disease Control and Prevention (CDC), two-thirds of American adults are overweight or obese, including more than one-third (78.6 million) that are obese[3, 4]. Until recently, obesity was only seen in developed nations. However, obesity worldwide has more than doubled since 1980. In 2014, more than 1.9 billion adults were overweight, including over 600 million that were obese[5]. This results in 13% of the world's population being obese. If the number of overweight and obese individuals continues to rise, it is projected that by 2030, over half of the world's population will be overweight or obese[6].

Obesity is associated with some of the leading preventable causes of death, including heart disease, stroke, type 2 diabetes, and several forms of cancer. These are also referred to as noncommunicable diseases (NCDs). NCDs are the leading cause of death worldwide, with an estimated 17.5 million NCD deaths caused by cardiovascular disease[7]. Owing to the increase in obesity, life

expectancy in developed countries is expected to decrease for the first time in recent history[8]. Obesity-associated disease also results in an increased financial burden. Medical costs of those who are obese have been calculated to be at least $1400 higher per year than those of normal weight, resulting in annual medical costs of greater than $150 billion in the United States alone[4, 9].

Composition, distribution, and function of adipose tissue

Initially, adipose tissue was thought to have the sole purpose of lipid storage, by storing excess energy as triglycerides, and releasing fatty acids when needed[10]. Indeed, its primary function is to store and release fat in response to energy-balance needs[11]. However, it is now widely accepted that adipose tissue is a metabolically active tissue, consisting of a variety of cell types[10]. Adipose tissue is also a main endocrine organ, controlling energy homeostasis and metabolism through secretion of molecules referred to as 'adipokines'[12].

While the vast majority of adipose tissue mass is comprised of lipid-filled mature adipocytes, or 'fat cells', they account for less than half of the total cells[13, 14]. More than 90% of the adipocyte cell volume is made up of a single, spherical, lipid vacuole separated from the rest of the cytoplasm by a non-membranous barrier containing perilipin[15]. The remaining cells are referred to as the stromavascular fraction (SVF), and are a heterogeneous mixture of cells containing adipocyte precursors (also called preadipocytes), fibroblasts, leukocytes, epithelial cells, endothelial cells, and other cells comprising the

vasculature and nerve tissue[16-18]. For study of the individual cellular populations, adipose tissue can be digested, and then following dissociation and centrifugation, adipocytes float and the remaining cells pellet[19]. It was discovered that the SVF contained adipocyte precursor cells when culture of human SVF cells resulted in differentiation into lipid-laden cells after 2 months[20].

Adipose tissue comprises various discrete depots, which are located in defined positions throughout the body. These various depots develop at specific and distinguishable prenatal and postnatal times and have discrete and distinct morphologies[21]. Brown adipose tissue (BAT) forms during embryonic development, before the development of other adipose depots. In humans, white adipose tissue (WAT) development begins early in the second trimester of gestation, and by birth, both visceral and subcutaneous depots are well-developed[22]. The rate of adipogenesis surges in response to increased nutrient availability, and there is a marked postnatal expansion of adipose compartments[23]. In rodents, WAT develops mainly after birth[22].

In neonates and newborns, BAT comprises about 1% of body weight[24]. The major BAT depots in rodents are in the interscapular region embedded in and around deep back muscles[25]. A similar interscapular BAT depot has been located in human infants, which then regresses and is absent in adults[26]. As such, WAT is the main type of adipose tissue found in adults[27].

WAT depots are generally categorized as subcutaneous fat or visceral fat. Subcutaneous adipose tissue (SAT) accounts for about 80% of all body fat[28], and forms between muscle and dermis[29]. The main areas for SAT fat deposition are the femerogluteal regions, the back, and the anterior abdominal wall[28]. Visceral adipose tissue (VAT) accounts for up to 20% of all body fat[28], and forms around visceral organs, including the stomach, intestines, heart, kidneys, and other internal organs[30]. Rodent adipose tissue depots are relatively small, and contain far fewer blood vessels, connective tissue matrix, and nonfat cells than the large adipose depots of humans[31]. The main SAT depot is the inguinal depot, and the main VAT depots include epigonadal, retroperitoneal, omental, mesenteric, and epicardial[32]. While the epigonadal fat is a large, discrete VAT organ in rodents, it does not exist in human anatomy[33].

The formation of BAT was necessary for evolutionary success, as thermogenesis enhances neonatal survival[24]. The function of BAT is to transfer energy from food into heat, through nonshivering thermogenesis[34]. Brown adipocytes convert nutrients into chemical energy in the form of heat, through mitochondrial biogenesis, energy uncoupling and dissipation[35].

WAT is thought to be the main site of lipid storage by storing excess energy as triglycerides and releasing fatty acids when needed[10]. Initially, this was thought to be the sole purpose of WAT. Indeed, its primary function is to store and release fat in response to energy-balance needs[11]. However, it is now widely accepted

that adipose tissue is a metabolically active tissue, consisting of a variety of cell types[10]. Adipose tissue is also a main endocrine organ, controlling energy homeostasis and metabolism through secretion of molecules referred to as 'adipokines'[12]. WAT is also capable of massive expansion and contraction in response to chronic alterations in energy balance, accounting for as little as 5% of body mass in extremely lean athletes[36], or as much as 60% of body mass in morbidly obese individuals[37].

Adipose tissue dysfunction during obesity

Increased energy intake relative to energy expenditure results in excess accumulation of WAT, eventually leading to obesity[38]. The size of WAT is incredibly plastic, and an increased caloric intake can increase the mass by up to 30-fold[39]. Increase in WAT mass in obesity occurs from an increase in adipocyte number (hyperplasia) and/or adipocyte size (hypertrophy)[40]. Even after obese individuals undergo severe weight loss, the elevated number of adipocytes is maintained[41], indicating that childhood obesity has lifelong effects on adipose tissue homeostasis. During obesity, there is a higher rate of adipocyte turnover due to a decrease in adipocyte lifespan and an increased rate of apoptosis[42].

As adipocytes are thought to be post-mitotic[12], increase in adipocyte number is due to production of new adipocytes from adipocyte precursors[43]. It is thought that the number of adipocytes in most WAT depots is established during

childhood and adolescence[41], requiring that new adipocytes be formed from adipocyte precursors in adulthood to maintain adipocyte numbers.

When there is an increase in triglyceride accumulation during obesity, especially when obesity is induced by a fat-rich diet, adipocytes can grow in volume by 6-7-fold during hypertrophy[44]. However, adipocytes have a maximal size, and once this is reached they spontaneously undergo necrosis[45]. This 'critical size' is genetically determined and is specific for each WAT depot[46]. As obese adipose tissue can reach 60-70% of total body weight, hyperplastic growth is required for massive WAT expansion[47, 48]. Adipocyte hyperplasia is generally seen as protective against metabolic dysregulation[42], as the newly formed, small adipocytes have improved lipid uptake. Increased adipocyte size correlates with serum insulin concentrations, insulin resistance, and increased risk of developing type 2 diabetes[49-51].

During obesity, adipocyte eventually lose the capacity to efficiently store triglyceride, leading to free fatty acid release into the circulation[52]. When dysregulation of fatty-acid storage and release occurs in obesity, fatty-acid overflow into ectopic sites leads to lipotoxicity[53, 54]. Ectopic fat accumulation occurs in bone marrow, muscle, and liver, potentially contributing to additional dysfunction of these tissues[55]. Similarly, when adipocytes are unable to form lipid droplets, as seen in lipodystrophies, the resultant ectopic fat deposition leads to insulin resistance, dyslipidemia, hepatic steatosis, and type 2 diabetes[56].

Rodent models of obesity

Both genetic and diet-induced models of murine obesity are used to study adipose tissue in the laboratory setting. Common genetic models of obesity include *ob/ob* mice that lack leptin, or the 'obese gene,'[57] and *db/db* mice that lack the leptin receptor and become diabetic as well as obese[58]. Diet-induced obesity (DIO) results from switching mice from a standard low-fat chow diet to a diet in which most of the calories derived from fat, ranging from 40-60% fat[59]. Interestingly, studies have demonstrated that while *ob/ob* mice and DIO mice have increased fat mass due to both hypertrophy and hyperplasia, as seen in human fat, *db/db* mice have fat expansion only due to hypertrophy[60]. This provides another link between adipocyte hypertrophy and metabolic disease, as these mice have severe insulin resistance and metabolic dysfunction.

Depot-specific effects of obesity

In humans, the accumulation of VAT correlates with increased risk of diabetes, hyperlipidemia, cardiovascular disease and mortality[61-63]. Conversely, increased SAT fat deposition protects against certain aspects of metabolic dysfunction[64]. In rodents, while inguinal SAT is associated with improved metabolic parameters[65], the accumulation of epigonadal VAT is associated with decreased insulin sensitivity[66]. Even lean individuals with a high ratio of central to peripheral fat demonstrate reduced insulin sensitivity[67]. Removal of VAT in humans results in decreased insulin and glucose levels[68], and improved insulin responsiveness[69].

It is thought that the anatomic location of VAT contributes to abnormalities in glucose homeostasis[70]. Lipolysis of VAT triglycerides releases free fatty acids (FFA) directly into the portal vein[71], which impair insulin's ability to suppress hepatic glucose production and stimulate glucose disposal in skeletal muscle[10]. It has been estimated that up to 20% of FFA that appear in the portal vein and in the systemic circulation are derived from lipolysis of VAT fat in obese subjects[72]. Increased IL-6 concentrations in the portal vein of obese patients support the association between accumulation of VAT and metabolic dysfunction[73]. However, others hypothesize that characteristics of the individual fat depots, and not their anatomical position, play large roles in their different effects on metabolism[22, 65]. The adipocytes that make up the different VAT and SAT depots have distinct biology and secretion profiles[74]. Elegant murine reciprocal transplant experiments determined that transplantation of SAT into visceral locations improved metabolic parameters, whereas transplantation of VAT into subcutaneous locations did not[65]. Within three weeks of transplantation of SAT into visceral locations, a striking rearrangement in gene expression was shifted toward a subcutaneous signature[75].

Histologically, SAT is heterogeneous and contains mature unilocular adipocytes interspersed with small multilocular adipocytes, whereas VAT fat is more uniform and consists primarily of large unilocular adipocytes[76]. SAT depots have increased rates of adipose turnover and new adipocyte formation[50]. VAT is more

metabolically active, and more heavily vascularized and innervated, with hyperlipolytic adipocytes[33, 77].

Adipose tissue inflammation

A typical acute inflammatory response ends with a highly regulated process of resolution that allows transition to the homeostatic state. When the resolution phase cannot occur, such as in obesity, where there is continued overnutrition and weight gain, a chronic inflammatory state ensures[78]. Chronic, low-grade inflammation in VAT, mediated by increased levels of chemokines and cytokines[79, 80], is what links obesity to its associated diseases[81].

Tumor necrosis factor-α (TNFα) was the first inflammatory molecule to be identified and studied in adipose tissue. Adipose TNFα expression is increased during obesity, and contributes to insulin resistance[82]. *Ob/ob* mice that were also deficient for TNF or TNF receptors have improved insulin sensitivity compared to lean controls[83]. TNFα exerts local autocrine as well as paracrine effects, to stimulate the production of other inflammatory cytokines in adipose tissue[84, 85]. TNFα treatment of human and mouse adipocytes resulted in increased chemokine expression, an effect that was dependent on activation of NFκB[86].

Adipocyte hypertrophy is thought to play both direct and indirect roles in adipose tissue inflammation, by switching adipocyte secretion towards a proinflammatory profile[87], and by inducing a proinflammatory response in other cells. While it was

originally thought that adipocytes were the source of the altered release of cytokines during obesity[79, 88], it is now understood that most factors released by adipose tissue from obese individuals were derived from cells other than adipocytes[89].

In obesity, inflammatory cells can constitute up to 40% of the adipose tissue, cellularly[10], as demonstrated in Figure 1 below. B and T lymphocytes have been located within weeks of initiation of HFD[90, 91]. It has been suggested that B and T lymphocytes may have protective roles in adipose tissue inflammation, as mice lacking these cells accumulate more activated macrophages during obesity[54]. However, the roles of lymphocytes in obesity have been determined to be subset-dependent, with type 2 lymphocytes being associated with lean adipose and type 1 lymphocytes being associated with obese adipose[92].

Higher amounts of TNFα, IL-6, IL-8 and MCP-1 are released from the VAT depots during obesity[74, 84, 93, 94]. TNFα, IL-6 and other pro-inflammatory cytokines are produced by macrophages, and interfere with the physiology of insulin receptor signaling[95]. Adipose tissue production of IL-6 makes up to 15-30% of circulating levels[73]. Weight loss in obese individuals reduced adipose tissue expression of IL-6, IL-8 and TNFα, and increased adiponectin levels[96]. IL-6 is the major stimulatory factor for the production and secretion of acute-phase proteins by the liver[97], and correlates with in vivo insulin resistance[98].

Figure 1. Obesity-induced adipose tissue inflammation

Immune cells increase in number during obesity, and have a phenotypic switch from anti-inflammatory M2 macrophages and T_H2 T lymphocytes to pro-inflammatory M1 macrophages and T_H1 T lymphocytes. This is accompanied by an increase in the size and number of adipocytes. M1 macrophages surround dead and dying adipocytes in obese adipose tissue, and these are termed 'crown-like structures' or CLS.

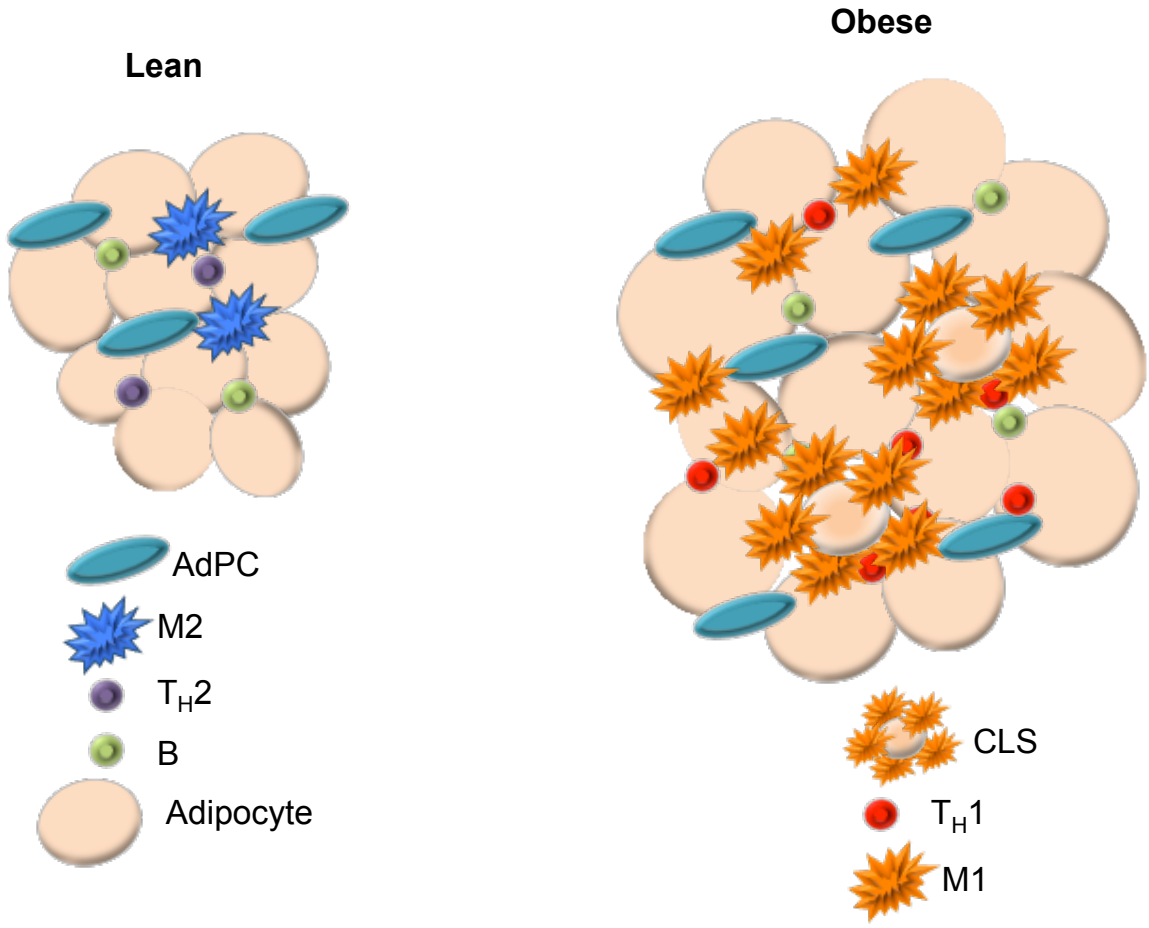

Figure 1: Obesity-induced adipose tissue inflammation

Adiponectin, possessing anti-inflammatory properties and normally present in high concentrations in the circulation, is found to be decreased in obesity[99] and increased after weight loss[100]. Additionally, adiponectin is expressed in lower amounts in VAT compared to SAT[101]. The proinflammatory state of adipose tissue can inhibit the normal differentiation of adipocyte precursors into mature adipocytes, resulting in adipocyte hypertrophy instead of hyperplasia[10]. Instead of forming new adipocytes to cope with the increased lipid accumulation, existing adipocytes become dysfunctional.

Adipose tissue macrophages

Macrophages are monocytic phagocytes that function in innate immunity and wound healing by sequestering and clearing pathogens, dead cells, and cell debris in an activation-dependent manner[102]. Adipocyte death due to excessive hypertrophy promotes infiltration of macrophages to adipose tissue, facilitating chronic inflammation during obesity[103]. Macrophages in adipose tissue of obese mice are frequently found in aggregates, with some aggregates completely surrounding the adipocytes in 'crown-like structures' (CLS)[104], as demonstrated in Figure 1, above. CLS formation can be increased up to 30-fold in obese mice compared to lean controls, and at least 90% of macrophages in WAT of obese mice are present in CLS[45]. The CLS can contain up to 15 macrophages per adipocyte, indicating that even a low frequency of adipocyte death can result in substantial adipose tissue inflammation[102]. These CLS are not seen in obese

mice and humans with hyperplastic obesity, and the individuals remain insulin sensitive[45].

Adipose tissue macrophages have been characterized into different macrophage phenotypes, including M1 and M2. M1 macrophages are 'classically activated' macrophages due to lipopolysaccharide (LPS) and interferon gamma (IFNγ) signaling[105]. M2 macrophages are 'alternatively activated' macrophages, due to IL-4, IL-10 and IL-13, are tissue-resident, and promote tissue repair[105]. In lean adipose tissue, M2 macrophages are the predominant macrophage type[106]. M1 macrophages increase in both number and percentage during obesity[107], and promote local inflammation due to expression of TNFα, IL-6 and iNOS[105, 108]. Classical $CCR2^{+}Ly6C^{hi}$ monocytes are rapidly recruited to sites of inflammation in several murine models of tissue injury, and are thought to be precursors of M1 macrophages[109].

Obesity induces a phenotypic switch from an anti-inflammatory M2 polarized state to a proinflammatory M1 state[106]. A switch in phenotype of adipose tissue macrophages from M2 to M1 is linked to the development of insulin resistance in mice[81, 106, 108, 110]. Labeling with fluorescent dye PKH26 confirmed that macrophage recruitment in response to HFD is due to increased infiltration, rather than reprogramming of M2 macrophages to M1[111]. Macrophage-secreted factors block insulin action in adipocytes, leading to a decrease in AKT

phosphorylation and impaired insulin-stimulated translocation of glucose transporters[112].

MCP-1-mediated recruitment of M1 macrophages

MCP-1 levels in adipose tissue and in the circulation are increased in mice due to both genetic- and diet-induced models of obesity[113]. MCP-1 levels are significantly reduced in obese humans after weight loss intervention[96, 114]. Overexpression of MCP-1 results in enhanced adipose tissue macrophages, insulin resistance, and increased hepatic triglyceride content[113]. Eventually, adipose tissue macrophages become the predominant source of HFD-induced MCP-1 production[84, 115].

The expression of MIP-1α, MCP-1, MAC-1, F4/80, and CD68, genes associated with macrophage function or activation, has been found to be predominantly expressed in the SVF of epididymal VAT (eVAT), rather than in adipocytes[81]. Specifically, MCP-1 production by SVF is higher than by adipocytes, and overall expression is higher in visceral adipose than in subcutaneous adipose[31, 84]. Similarly, it was determined that non-adipocytes were the main source of MCP-1 secretion from human VAT explants[85].

Omental adipose tissue has been directly linked to metabolic disease and insulin resistance through its specific MCP-1 production and macrophage infiltration[116]. MCP-1 impairs insulin-stimulated glucose uptake by cultured adipocytes in

vitro[117]. Mice null for MCP-1 have improved insulin sensitivity and glucose tolerance, decreased adipocyte size, decreased serum free fatty acids, and a 30% reduction in adipose tissue macrophages[113]. Interestingly, these mice also have defects in the production of type 2 helper (T_H2) cytokines by antigen-primed lymphocytes, as well as decreased T_H2 polarization[118]. MCP-1-deficient mice also have reduced macrophage recruitment into the peritoneal cavity[119].

CCR2, the receptor for MCP-1, is highly expressed on a subpopulation of blood monocytes[120], as demonstrated in Figure 2. Mice deficient in CCR2 have attenuated HFD-induced weight gain, with a 35% reduction in adipose tissue macrophages in epididymal adipose[104, 114, 121]. The initial studies performed on *Ccr2*$^{-/-}$ mice determined that CCR2 is the main receptor for MCP-1. In the peritoneal cavity, there was a reduction in thioglycollate-elicited cells, but no change in the number of resident macrophages[122]. Expression of CCR2 on monocytes is required for mobilization from the bone marrow[120]. *Ccr2*$^{-/-}$ mice have a severe reduction in the number of circulating Ly6C^{hi} inflammatory monocytes as a result of impaired egress from the bone marrow[120].

Therapeutic attempts to reduce obesity and adipose tissue inflammation

Most approved obesity treatments produce only moderate and temporary effects[30, 123]. Even when used in combination with dieting, none of the available

Figure 2. MCP-1-mediated recruitment of CCR2^{+} Ly6C^{hi} monocytes to adipose tissue

The MCP-1 produced in adipose tissue creates a chemotactic gradient that recruits circulating monocytes expressing high levels of Ly6C and CCR2. Once these monocytes enter the adipose tissue, they differentiate into proinflammatory M1 macrophages.

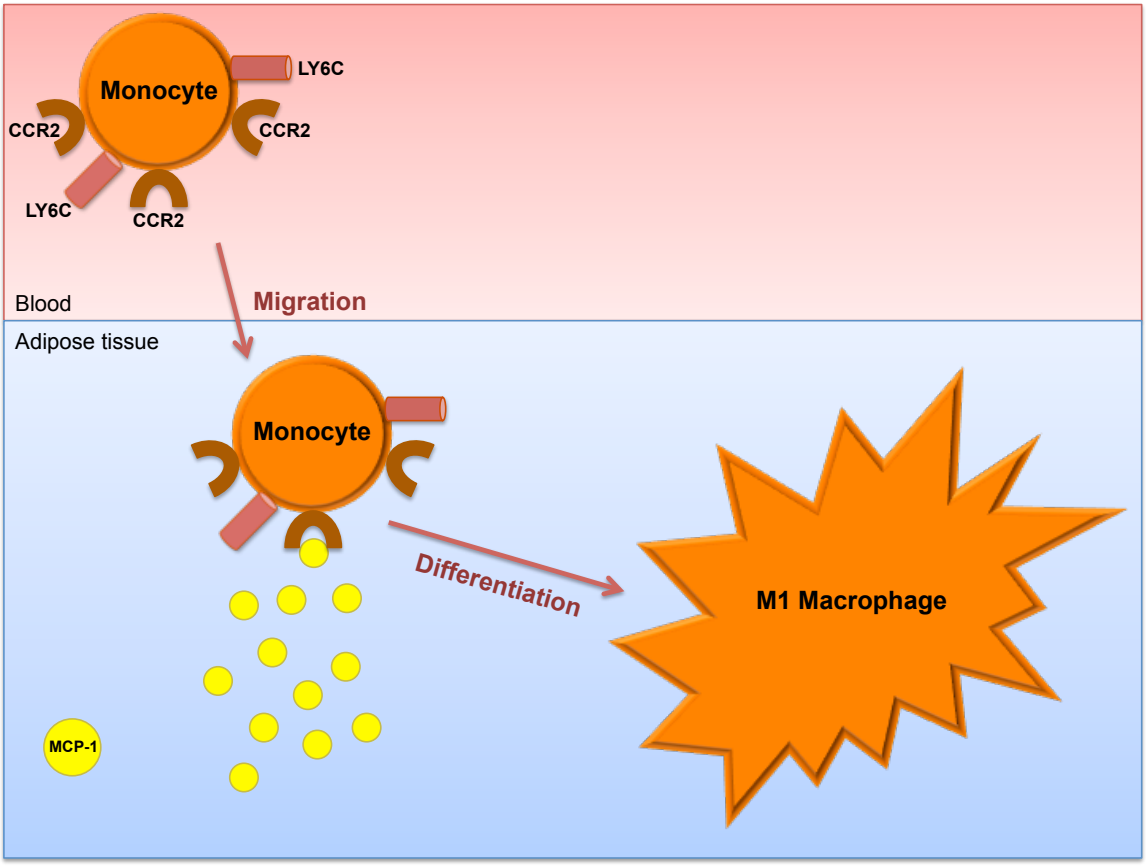

Figure 2: MCP-1-mediated recruitment of $CCR2^{+}Ly6C^{hi}$ monocytes to adipose tissue

drugs are satisfactory for long-term weight management[124]. While TNFα plays an important role in obesity and insulin resistance, attempts to neutralize TNFα have been unsuccessful in affecting insulin resistance in type 2 diabetic patients[125]. This may be partly due to the local autocrine and paracrine effects of TNFα, as it does not appear to be released by the adipose tissue into the systemic circulation in vivo[81].

Adipocyte progenitor cells

The Swiss mouse 3T3 fibroblast cell line, spontaneously immortalized in the 1970s, has been shown to accumulate lipid after being maintained at confluence in culture[126-128]. Through study of the 3T3-L1 and 3T3-F422A preadipocyte cell lines, the transcription factor cascade culminating in induction of PPARγ and adipogenesis has been well-characterized[129], providing enormous progress in adipocyte biology[130]. PPARγ is a nuclear hormone receptor[131], and drives adipogenesis through induction of genes responsible for lipid storage, lipid synthesis, and glucose sensing[132-134]. As the centerpiece of the transcriptional cascade, PPARγ is necessary and sufficient for in vitro adipogenesis[129]. PPARγ has additional roles in stem cell proliferation, self-renewal, and cell determination[135].

The steps and transcriptional cascade of adipogenesis are detailed below in Figure 3. A period of mitotic clonal expansion (MCE) occurs prior to adipocyte

Figure 3. Transcriptional cascade of adipogenesis

In the initial stages of adipogenesis, a round of proliferation occurs, referred to as mitotic clonal expansion (MCE). During this time, C/EBPβ and C/EBPδ are transiently expressed, stimulate expression of C/EBPα, which activates PPARγ. An increase in PPARγ activity stimulates additional expression of C/EBPα, which maintains the differentiated state. During this transcriptional cascade, preadipocytes commit to the adipocyte lineage, and undergo a second round of proliferation. During this time, preadipocytes begin to express adipocyte proteins, including FAS, FABP4, adipsin, GLUT4, adiponectin, and leptin. Differentiation is complete when the cells acquire a lipid droplet.

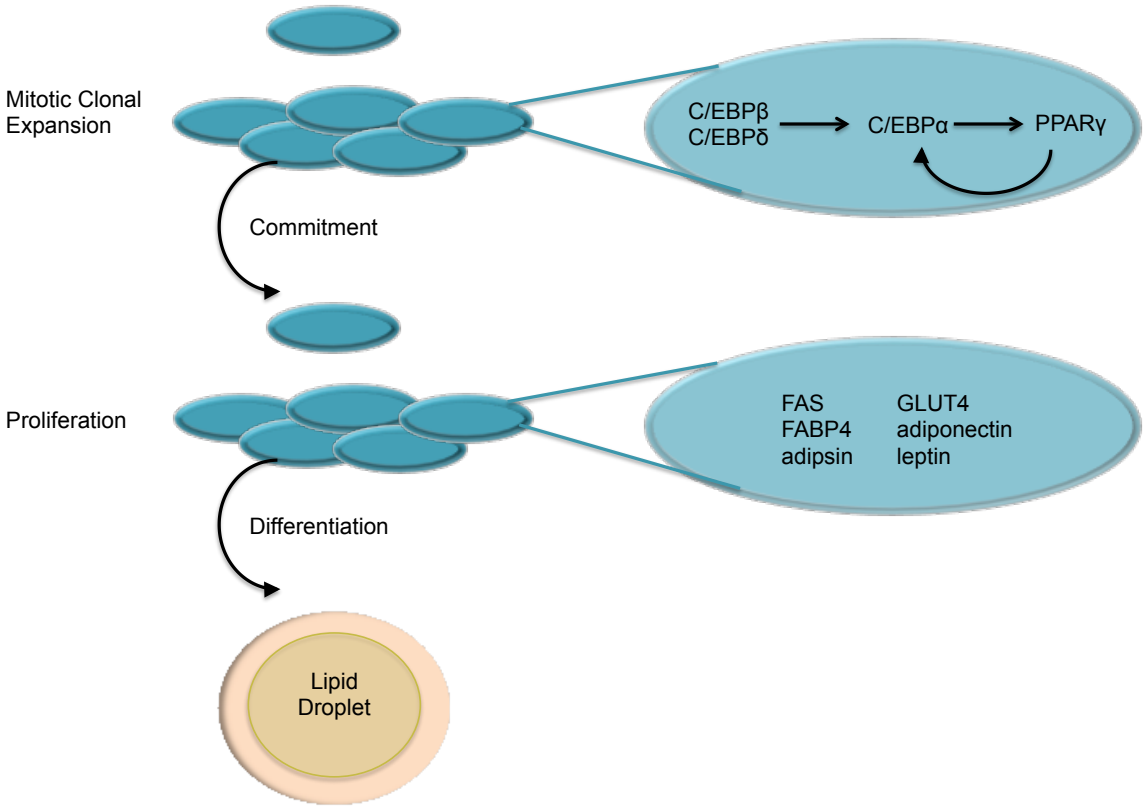

Figure 3: Transcriptional cascade of adipogenesis

commitment in the initial stages of adipogenesis[136]. During MCE, transient expression of C/EBP proteins (α, β, δ) activates expression of PPARγ[137]. PPARγ then acts to stimulate additional expression of C/EBP, to maintain the differentiated state. While cells lacking expression of C/EBPα are capable of adipocyte differentiation, albeit lacking insulin sensitivity[138], PPARγ-deficient cells are incapable of adipogenesis[139].

Once preadipocytes have committed to the adipocyte lineage, and have entered the terminal differentiation stage, they begin to express metabolic genes and adipokines, including FABP4 (also known as AP2), FAS, adipsin, GLUT4, leptin, and adiponectin[130, 134]. Cellular confluence is a requirement for in vitro adipogenesis[128], possibly in order to inhibit continued cellular proliferation due to cell contact[140]. The accumulation of lipid within mature adipocytes relies on expression of perilipin[141]. In addition to characterizing the adipogenic transcription factor cascade, cell culture studies have implicated bone morphogenic proteins (BMPs), WNTs, as well as insulin growth factors (IGFs) as regulators of adipogenesis[29, 142].

The common adipogenic cocktail for in vitro adipogenesis with mouse cell lines includes the addition of insulin, IBMX and dexamethasone[126, 143]. Adipogenesis of human primary cells and cell lines requires the addition of PPARγ agonists or indomethacin[144]. While we have learned much about adipogenesis from preadipocyte cell lines, there are a number of differences between 3T3-L1

adipogenesis and the gene programs observed in adult mouse VAT[145]. There is evidence that in vitro temporal expression of adipogenic transcription factors, such as PPARγ, occurs differently in vivo during embryonic development[146].

The first attempts to isolate primary preadipocytes selected cells that adhered to a cell culture dish; however, selection of WAT-resident SVF cells by adherence alone results in a mixed population of cells. The majority of these cells are unable to form adipocytes in vitro, even with an adipogenic cocktail, and they lack significant adipogenic activity in vivo[147]. Originally, lack of CD45 expression and possession of CD34 expression were the sole characteristics for identifying preadipocytes from a heterogeneous population[148]. Some initial studies found that the $CD31^{-}CD34^{+}$ population isolated from human WAT SVF was enriched for adipogenic activity[149].

In the past several years, elegant methods of identifying adipocyte progenitor cells in vivo have been made available[146, 147, 150]. These adipogenic cell populations have been characterized in great detail from mice using flow cytometry to isolate populations of cells based on expression of cell surface proteins[146, 147, 151-153]. The Rodeheffer group identified that $CD45^{-}CD31^{-}Ter119^{-}CD29^{+}CD34^{+}Sca\text{-}1^{+}$ cells are highly adipogenic in culture[147]. These cells can be further divided into two populations, those that express CD24 and those that do not, as shown in Figure 4. The $CD24^{-}$ population of these cells have restricted

Figure 4. Characterization of $CD24^+$ and $CD24^-$ AdPCs

As described by the Rodeheffer group, $CD24^+$ AdPCs are upstream progenitors. CD24 expression is lost during commitment to the adipocyte lineage. These cell types have different properties of adipogenesis seen *in vivo*.

CD24^{+} AdPC	CD24^{-} AdPC
Can reconstitute an entire functional WAT depot	Unable to reconstitute WAT depot
Extensive expansion prior to terminal differentiation	Limited adipogenic capacity *in vivo*
Requires WAT microenvironment for adipogenic capacity	Adipogenic capacity is not dependent on WAT microenvironment

Figure 4: Characterization of CD24^{+} and CD24^{-} AdPCs

adipogenic capacity when transplanted into fatless A-Zip mice, while the $CD24^+$ population can reconstitute an entire functional WAT depot[147]. Further characterization of these cells determined that the $CD24^+$ cells are upstream progenitors, and lose CD24 expression once they become further committed to the adipocyte lineage[154, 155]. The $CD24^+$ population is capable of extensive expansion prior to terminal differentiation, but require the WAT microenvironment for their adipogenic capacity. The $CD24^-$ preadipocyte population however has low adipogenic capacity that is not dependent on the WAT microenvironment[154]. The activated $CD24^+$ adipocyte progenitors can rapidly differentiate into $CD24^-$ preadipocytes, in as little as 24 hours[154, 155].

Sca-1, an important marker of murine adipose precursor cells, does not have a clear human homolog[156]. This indicates that human adipocyte precursors require different markers for isolation. While not as well-characterized as murine adipocyte progenitor cells, $CD45^-CD31^-CD34^+CD90^+CD44^+$ cells from human adipose tissue have been validated as functional adipocyte precursors[157, 158].

The search for the initial source of MCP-1

The search for the cell that initially produces MCP-1 in response to HFD has implicated a variety of cells within the SVF, including endothelial cells, mast cells, and $CD8^+$ T-lymphocytes[159, 160]. It has been hypothesized that mast cells appear in obese AT before the more numerous macrophages, and may even regulate the influx of macrophages via MCP-1 secretion[161].

Preadipocytes have been implicated in adipose tissue inflammation, although most studies were performed in vitro, using immortalized cell lines or the heterogeneous population of SVF cells. LPS stimulation results in secretion of numerous NFκB-regulated inflammatory cytokines, including TNFα, IL-6, IL-8 and MCP-1, from both preadipocytes and adipocytes[162]. In these studies, preadipocytes were determined to have the capacity for a greater inflammatory response in response to NFκB and MAPK signaling, as compared to mature adipocytes[162, 163].

Further analysis of 3T3-L1 cells determined that they share more characteristics of immune cells than those of human preadipocytes or adipocytes[31], indicating that the original studies of inflammatory response may not recapitulate what happens to adipocyte precursors in vivo. However, isolated $CD34^+$ cells from murine VAT expressed significant levels of MCP-1, both basal and induced by LPS[164]. This suggests that preadipocytes may indeed have a role in MCP-1 production, although this has not been properly investigated in vivo using functional adipocyte precursors.

HLH transcription factors

The basic helix-loop-helix (bHLH) family of transcription factors is a highly conserved group that plays a role in the differentiation and growth of a variety of cell types[165-167]. Class A bHLH proteins are exemplified by the E-proteins, and

are ubiquitously expressed[168]. Class B bHLH proteins are a much larger collection of tissue-specific transcription factors, such as MyoD in muscle cells[169], TAL1/LYL-1 in hematopoietic cells[170], and NeuroD in neuronal cells[171]. There are six human E-proteins that are conserved among mammals: E12 and E47, which are splice variants from the E2A gene, ITF-1, ITF-2a and ITF-2b, which are from the E2-2 gene, and HEB[172]. These factors function either as homodimers or heterodimers, with tissue-specific bHLHs. The usual configuration for E-box binding and transcriptional activation is a class A-class B bHLH heterodimer[173]. The tissue-specific bHLHs require heterodimerization with an E-protein for function activity[174].

Inhibitors of differentiation (Ids) are a unique subset of HLH factors. While the Ids possess an HLH domain, they lack the basic domain required for DNA binding. Through the HLH domain, they heterodimerize with the E-proteins and disrupt E-protein dimers, thereby inhibiting the ability of those factors to regulate transcription[175], as shown in Figure 5. Because of this mechanism of action, the Ids are considered dominant negative inhibitors of bHLH-mediated DNA binding and transcription regulation[165, 167]. There are four known mammalian Id genes (Id 1-4), whose functions are overlapping but not redundant[165, 167].

The Id proteins are broadly but not uniformly expressed. Levels tend to be high during development and in proliferating cells, while they are low in healthy adult tissues, terminally differentiated cells, and quiescent cells[167, 175]. While Id3 is

Figure 5. Paradigm of HLH function.

bHLH family members can hetero- or homodimerize through their HLH region, enabling their basic domain to bind to conserved regions on DNA termed ‘E-boxes.’ Id proteins lack the basic domain, sequestering E proteins from DNA, thereby regulating transcription.

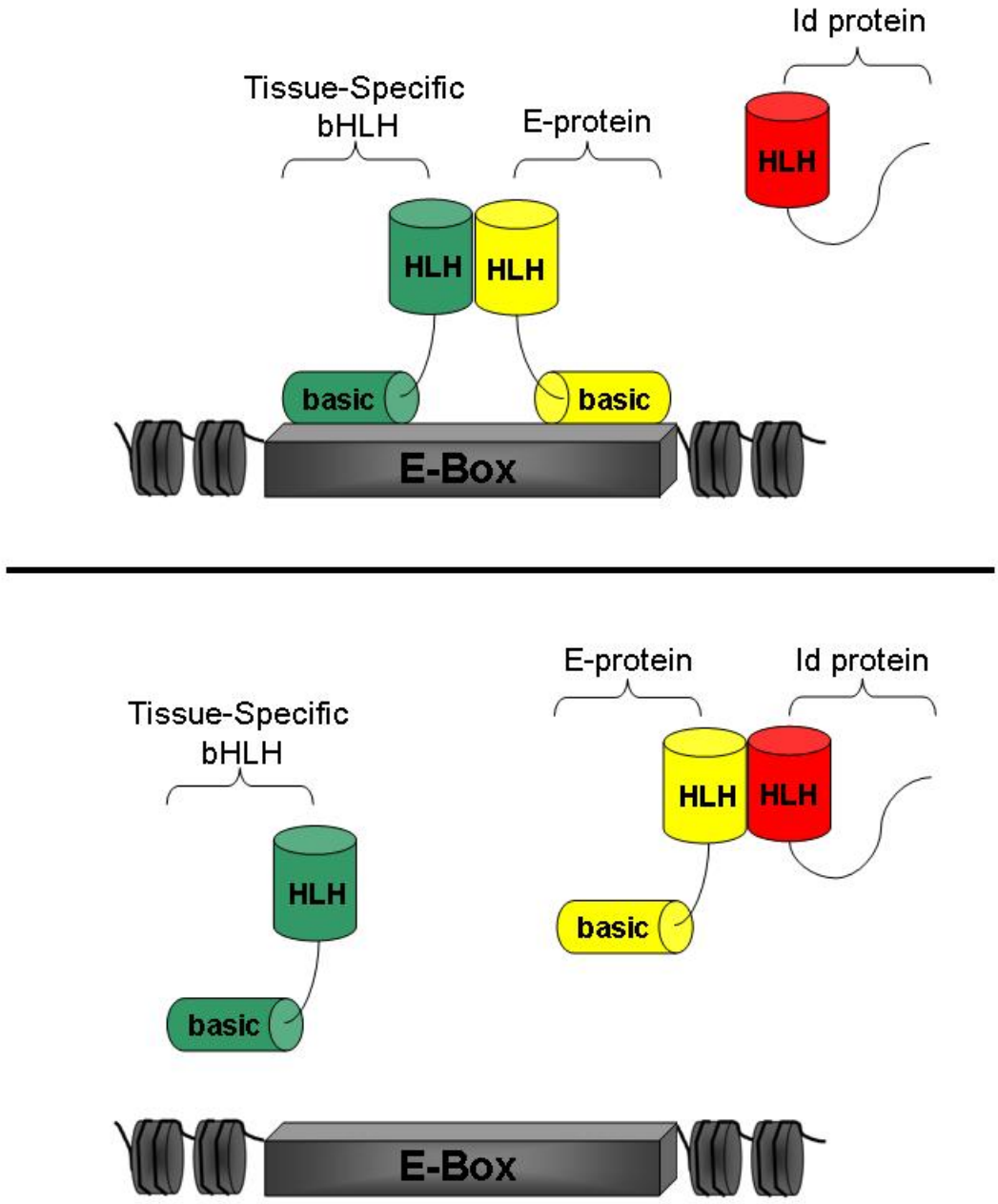

Figure 5: Paradigm of HLH function

normally only expressed during development and in lymphocytes, it can be re-expressed during disease, including cancer and vascular disease[167, 176]. Id3 has known growth-promoting effects[176-178]. Id3 is known to negatively regulate lineage commitment, cell fate decisions, and the timing of differentiation[167, 177]. Enforced ectopic expression of Id genes in a variety of cell lineages can promote cell growth and inhibit differentiation, and this is typically manifested in a delayed exit from the cell cycle under differentiation-inducing conditions[179-181].

Id3 and obesity

Recent work suggests that Id3 acts as a regulator of metabolic health in obesity[182, 183]. Global deletion of Id3 attenuates HFD-induced obesity[182]. Id3 has been linked to obesity through its regulation of adiponectin, an adipocyte-derived hormone that regulates insulin sensitivity and is reduced in obesity. Id3 antagonizes ADD1/SREBP-1c-mediated activation of the adiponectin promoter. *Id3*$^{-/-}$ mice have increased adiponectin in visceral adipose tissue and serum[184, 185]. Adipocyte determination and differentiation factor 1 (ADD1), also known as sterol regulatory element binding protein-1c (SREBP-1c), binds to both sterol regulatory elements (SREs) and E-boxes. ADD1/SREBP-1c is an adipose-restricted bHLH that controls genes involved in the cellular availability of cholesterol and fatty acid synthesis, making it essential for cholesterol metabolism[166, 186]

Project rationale and summary

At the inception of this project, the initial source of MCP-1 after initiation of HFD

was unclear, and studies that had suggested preadipocytes play a role had used rudimentary means to isolate these cells. Prior studies in the lab had demonstrated that Id3-deficient mice were partially protected from visceral adipose tissue expansion and weight gain due to a 20-week HFD. At the time, this was hypothesized to be due to Id3-mediated angiogenesis during obesity, yet it was unclear if Id3 was playing additional roles in diet-induced obesity or adipose tissue inflammation. We hypothesized that Id3 may be playing a role in the initial stages of diet-induced obesity.

In Chapter 3, we provide evidence that adipocyte progenitor cells (AdPCs) are important producers of MCP-1 in the initial stages of HFD feeding. Flow cytometry identified $CD45^{-}CD31^{-}Ter119^{-}CD34^{+}CD29^{+}Sca\text{-}1^{+}$ AdPCs as the primary producers of MCP-1 after 1 week of HFD. The production of MCP-1, as well as IL-6 was increased due to stimulation by HFD. MCP-1 secretion and expression was limited to the $CD24^{-}$ population of AdPCs, those that have been previously demonstrated to be further committed to the adipocyte lineage. In addition, in murine VAT, the number of AdPCs doubled after 1 week of HFD. Within an obese human cohort, an equivalent population of AdPCs was identified, and it was determined that these cells were also producing MCP-1. Expression of MCP-1 was specific to AdPCs expressing high levels of surface CD44, and to AdPCs within the omental adipose.

In Chapter 4, we suggest that Id3 is a critical regulator of AdPC-mediated M1

macrophage accumulation. Id3 mRNA levels were increased due to HFD in sorted AdPCs. The expression of Id3 appeared to regulate $p21^{Cip1}$ promoter activation, as demonstrated by promoter reporter assays as well as AdPCs isolated from global Id3-deficient mice. Utilizing the *Id3*$^{-/-}$ model, these mice had significantly fewer AdPCs, reduced MCP-1 levels, and attenuated M1 macrophage accumulation in response to HFD. Importantly, rescue of AdPC numbers via adoptive transfer resulted in restoration of MCP-1 levels and M1 macrophage numbers, but only when the injected AdPCs expressed Id3.

Chapter 2

Materials & Methods

Mice

The Institutional Animal Care and Use Committee of the University of Virginia has approved all animal experiments. Mice on a *C57Bl/6J* background were used in all experiments. *C57Bl/6J* mice were purchased from Jackson Laboratory (stock# 000664). *Id3*$^{-/-}$ mice were provided by Yuan Zhuang (Duke University), and were bred with *C57Bl/6J* mice to generate *Id3*$^{+/-}$ mice that were used for breeding *Id3*$^{-/-}$ mice and *Id3*$^{+/+}$ littermate controls. *LysM*$^{cre/cre}$ mice were provided by Norbert Leitinger (University of Virginia). *Id3*$^{fl/fl}$ mice were provided by Yuan Zhuang (Duke University). *Id3*$^{fl/fl}$ mice were bred to *LysM*$^{cre/cre}$ mice to generate first *Id3*$^{fl/+}$*LysM*$^{cre/+}$ mice, which were bred to each other to generate *Id3*$^{fl/fl}$*LysM*$^{cre/+}$. These mice were then bred to *Id3*$^{fl/fl}$*LysM*$^{+/+}$ mice to generate *Id3*$^{fl/fl}$*LysM*$^{cre/+}$ and littermate control *Id3*$^{fl/fl}$*LysM*$^{+/+}$ mice. The number of mice used in each experiment is provided in the figure legends. Only male mice were used for experiments, and all animals were given standard chow diet (Tekland 7012) and water *ad libitum* until they were genotyped. For high-fat feeding studies, littermates were placed on a 60% kCal fat diet (Research Diets, D12492) for the designated length of time.

Study Approval

Patients were recruited through the Bariatric Surgery Clinic at the University of Virginia. All patients were ≥18 years of age and obese (BMI ≥30), and provided informed written consent prior to participation in the study. The study was approved by the Human IRB Committee at the University of Virginia, IRB

#14180. All procedures were in accord with the declaration of Helsinki. Flow cytometry was performed on fresh omental and subcutaneous adipose tissue, collected between March and October of 2014.

Injections

BrdU Injections

150µl of BrdU (15mg/ml, BD Biosciences) was intraperitoneally injected into *Id3*$^{+/+}$ and *Id3*$^{-/-}$ mice five times over the course of 1 week. Injections were at 72, 120, 144, 150 and 164 hours post initiation of HFD.

Adoptive Transfer Injection

Donor *Id3*$^{+/+}$ and *Id3*$^{-/-}$ mice were fed a HFD for 2 weeks prior to transfer. CD45^{-} CD31^{-}Ter119^{-}CD29^{+}CD34^{+}Sca-1^{+} adipocyte progenitor cells were harvested from donor animals. *Id3*$^{+/+}$ and *Id3*$^{-/-}$ hosts received a single i.p. injection of either 5.0x10^{4} AdPCs in 250ul saline or saline alone (vehicle control). After 72 hours of recover, mice were continued on a HFD for the remainder of the experiment, except for two overnight fasts for 2 week and 6 week GTT (described below).

Metabolic Studies

Glucose Tolerance Test

Mice were fasted overnight in wood chip-lined cages. At the beginning of each experiment, a small tail snip was made and baseline blood glucose levels were determined. Mice were then injected i.p. with 1.4 g dextrose (Hospira) per kg

body weight, and blood glucose levels were measured at 10, 20, 30, 60, 90, and 120 minutes post-injection. Mice had access to water *ad libitum* throughout the experiment.

Insulin Injections for pAKT Western

Mice were fasted overnight in wood chip-lined cages. Mice were injected with 10U/kg insulin (Eli Lilly). Mice were euthanized after five minutes, and omental adipose tissue was removed and flash-frozen for later analysis (see Western blotting protocol below).

Tissue Processing

Adipose Tissue

Murine epididymal stromavascular fraction was isolated as previously described[182]. Human omental and subcutaneous adipose tissue was processed using an adapted version of published methods[187]. In brief, adipose tissue was placed in PBS supplemented with 5.5mM glucose and 50µg/ml gentamicin and was processed as soon after collection as possible. Ten g adipose tissue was minced with scissors and digested in 30ml PBS containing 1% BSA (Gemini) and 2.5g/L Collagenase II (Worthington) in a shaking 37° incubator for 15 minutes. PBS containing 0.1% BSA and 1mM EDTA was added to stop the collagenase activity. The SVF was then successively passed through 425µm and 180µm sieves (WS Tyler), and finally through a 40µm filter. The remaining SVF was stained for flow cytometry.

Peritoneal Cells

Peritoneal cells were harvested by peritoneal lavage as previously described[188, 189], 4 days after intraperitoneal injection of 3 ml of thioglycollate (BD Biosciences). Briefly, peritoneal lavage was done with DMEM media supplemented with 10% heat inactivated FBS, 2mM L-glutamine, 1X Penicillin/Streptomycin, and 2 mM EDTA (all purchased from GIBCO). 4ml media were injected through the peritoneal wall, the mouse was agitated to detach cells, then a small hole was opened in the peritoneal membrane to recollect lavage fluid. Peritoneal macrophages were isolated by macs column purification with negative selection by CD4, CD8 and CD19 microbeads, followed by positive selection by F4/80 microbeads (Miltenyi Biotech).

Spleen

Splenocytes were prepared as previously described[189]. Briefly, spleen was removed from the peritoneal cavity and placed in 70 µm cell strainers in 30 mm dishes filled with 10ml of DMEM supplemented with 10% heat inactivated FBS, 2mM L-glutamine, 1X Penicillin/Streptomycin, and 2 mM EDTA (all purchased from GIBCO). Spleens were homogenized using the plunger of a 1ml syringe and media was transferred into 15 ml falcon tubes.

Flow Cytometry

Red blood cells in the SVF were lysed if necessary with RBC lysis buffer (155mM NH_4Cl, 10mM $KHCO_3$, 0.1mM Na_2EDTA, pH 7.4). All cells were strained through 70μm filters and incubated with Fc- block (FCR-4G8, Invitrogen) for 10 minutes on ice prior to staining. Cells were stained on ice and protected from light for 20 minutes. Fc-block and antibodies were diluted in either FACS buffer (PBS containing 1% BSA and 0.05% NaN_3) for flow cytometry or sorting buffer (PBS containing 1% BSA) for cell sorting experiments. SVF cells were incubated with fluorophore-conjugated antibodies for flow cytometry. Isolated SVF cells were labeled with PKH26 (Sigma-Aldrich) according to manufacturer's instructions.

Viability was determined by either LIVE/DEAD® fixable yellow cell staining (Invitrogen) or DAPI (Sigma-Aldrich). Cells were run on a CyAn ADP (Beckman Coulter) or sorted on an Influx Cell Sorter (Benton-Dickenson). Fluorescence minus one (FMO) samples were used to set gates for all antibodies. Flow cytometry was performed at the Flow Cytometry Core Facility at the University of Virginia. Cells were quantified using CountBright counting beads (Fisher). All flow cytometry data were analyzed using FlowJo 9.7.6 software (Tree Star Inc.).

Murine Flow Cytometry Antibodies

CD11c (N418), CD19 (1D3), CD24 (M1/69), CD29 (HMb1-1), CD3ε (500A2), F4/80 (BM8), and Sca-1 (D7) were purchased from eBioscience, BrdU (B44), CD45 (30-F11), MCP-1 (2H5) and Ter119 (Ly-76) were purchased from BD

Bioscience, CD206 (MMR), CD31 (390), and CD34 (MEC14.7) were purchased from BioLegend, and CD11b (M1/70.15) was purchased from Caltag.

Human Flow Cytometry Antibodies

CD31 (WM59), CD34 (561), CD90 (5E10), CD44 (BJ18) were purchased from Biolegend and CD45 (2D1) was purchased from BD Bioscience.

Intracellular Staining

Murine SVF cells were cultured in DMEM/F12-10 supplemented with Brefeldin A (10 µg/ml, Sigma-Aldrich) immediately after collection, and were harvested after 5 hours using Cellstripper (Corning). Human SVF cells were cultured in DMEM/F12-10 overnight, and Brefeldin A (10 µg/ml, Sigma-Aldrich) was added the next morning for 5 hours prior to harvest. Cells were then fixed and permeabilized with FIX&PERM (Invitrogen) according to manufacturer's instructions.

BrdU Uptake

Cell proliferation was measured by the incorporation of BrdU into genomic DNA during the S phase of the cell cycle, using FITC BrdU Flow Kit (BD Biosciences).

FACS Sorting

Murine AdPCs were sorted based on expression of CD29, CD34 and Sca-1, and were gated from cells negative for CD45, CD31, Ter119, and live/dead marker

DAPI. Lineage positive (Lin^+) cells were gated based on expression of CD45, CD31 or Ter119, and were gated from cells negative for live/dead marker DAPI. SVF cells were gated from cells negative for live/dead marker DAPI.

Promoter-Reporter Analysis

Full length Id3 was previously subcloned into a pAdlox expression vector[176]. $p21^{Cip1}$ luciferase promoter construct[178] was a gift from Xiao-Hong Sun (Oklahoma University) and MCP-1 luciferase promoter construct was purchased from SwitchGear. 3T3-L1 and OP-9 cells were transfected with 0.9 µg of expression plasmid, using empty pAdlox vector to maintain the same amount of DNA, along with 0.1 µg of appropriate promoter. Twenty-four hours after transfection, luciferase activity was measured using the Luciferase Assay Kit (Promega) for $p21^{Cip1}$ activity and LightSwitch Luciferase Assay Kit (SwitchGear) for MCP-1 activity, as per the manufacturer's instructions.

Cell and Tissue Culture

SVF cells, sorted primary cells, and whole adipose tissue were cultured in DMEM/F12, supplemented with 10% FBS and antibiotics. Media was collected after 24 hours, and the supernatant was frozen in aliquots. Undifferentiated OP-9 cells and 3T3-L1 fibroblasts were maintained as previously described[184]. For adipogenesis, sorted primary adipocyte progenitor cells were cultured and induced to differentiate into adipocytes and stained with Oil Red O, as described elsewhere[190]. Transient transfections were performed three times in triplicate

using FuGENE HD (Promega) according to the manufacturer's instructions. Empty vectors were used to keep the total DNA transfected uniform across all wells.

Real-Time PCR

Total RNA was isolated from murine adipose tissue, isolated adipocytes, and SVF cells, reverse transcribed to cDNA, and used in real-time PCR reactions as described previously[184], with the primers listed below. Briefly, RNA was isolated from SVF cells using the RNeasy Plus Micro kit with gDNA elimination columns (Qiagen). RNA was isolated from adipose tissue and isolated adipocytes using Trizol Reagent (Invitrogen) as per the manufacturer's instructions, and genomic DNA was removed by DNase I (Invitrogen). RNA was reverse transcribed with SuperScript III First-Strand Synthesis System by Oligo d(T) tailing (Invitrogen). PCR reactions were always performed in triplicate. Total cDNA was diluted 1:5 in water and 3 µl were used for each real-time PCR reaction using the C1000 Thermal Cycler and CFX96 Real Time system (Bio-Rad) with SYBR® Green real-time PCR (SsoFast™ EvaGreen® Supermix, Bio-Rad). The relative amount of all mRNAs was calculated using the comparative threshold cycle (C_t) method. Cyclophilin mRNA was used as the invariant control.

Id3 forward TGCTACGAGGCGGTGTGCTG

Id3 reverse TGTCGTCCAAGAGGCTAAGAGGCT

$p21^{Cip1}$ forward TCTCCCATTTCTTAGTAGCAGTTG

$p21^{Cip1}$ reverse GCTTTGACACCCACGGTATT

MCP-1 forward GGTGTCCCAAAGAAGCTGTA

MCP-1 reverse TGTATGTCTGGACCCATTCC

Cyclophilin forward TGCCGGAGTCGACAATGAT

Cyclophilin reverse TGGAGAGCACCAAGACAGACA.

ELISA

Mouse MCP-1 (eBiosciences, 88-7391) levels were determined with commercial kits.

Western Blotting

Protein extracts from peritoneal macrophages and splenocytes, and western blotting were performed as previously described with antibodies against Id3 (Calbioreagents) and β-tubulin (Cell Signaling Technology). 10 mg omental adipose tissue was homogenized in 250ul protein lysis buffer (10% glycerol, 1% NP-40, 137mM NaCl, 25mM HEPES pH 7.4, 1mM EGTA) containing protease inhibitors and phosphatase inhibitors (Sigma-Aldrich) and lysed on ice for 30 minutes. Protein lysates were supplemented with 11% β-mercaptoethanol and 0.5% bromophenol blue. Samples were resolved on 4-20% Tris-glycine gels (Invitrogen) and transferred to a polyvinylidene difluoride membrane (BioRad). Western blotting was performed with antibodies against AKT (1:1000, Cell Signaling) and Thr308 pAKT (1:1000, Cell Signaling), followed by horseradish peroxidase-linked secondary antibody (Jackson). Immunoreactive bands were visualized by enhanced chemiluminescence after incubation with ECL reagent

(Amersham Pharmacia Biotech). Densitometry was analyzed with ImageJ. Relative AKT phosphorylation was determined by normalizing pAKT to total AKT in each sample.

Statistics

For mouse data, a student's t-test was performed on data with normal distribution and equal variance. If data sets had unequal variance, a t-test with Welch's correction was used. For non-Gaussian distributed data, a Mann-Whitney test was performed to determine statistical significance. For comparisons of three or more independent variables, a one-way ANOVA test was used with a Kruskal-Wallis post-hoc test. Results are displayed containing all replicated experiments, and values shown are mean ± SEM unless otherwise noted. For human clinical data, baseline characteristics are reported as mean ± standard deviation for normally distributed variables, and as median with interquartile range for non-normally distributed variables. Spearman correlation coefficients were used to determine the strength of association between variables. Data were analyzed with Prism 6.0a (GraphPad Software, Inc.) or SAS 9.3 and displayed using Prism 6.0a.

Chapter 3

Committed $CD45^{-}CD31^{-}Ter119^{-}CD29^{+}CD34^{+}Sca\text{-}1^{+}CD24^{-}$ adipocyte progenitor cells are the initial source of HFD-induced MCP-1 production

Adapted from Kaplan, J.L. et al. Adipocyte progenitor cells initiate monocyte chemoattractant protein-1-mediated macrophage accumulation in visceral adipose tissue. *Molecular Metabolism* (2015)

Abstract:

Objective: Macrophages are important producers of obesity-induced MCP-1; however, initial obesity-induced increases in MCP-1 production precede M1 macrophage accumulation in visceral adipose tissue (VAT). The initial cellular source of obesity-induced MCP-1 *in vivo* is currently unknown. Preliminary reports based on *in vitro* studies of preadipocyte cell lines and adherent stromavascular fraction cells suggest that resident stromal cells express MCP-1. In the past several years, elegant methods of identifying adipocyte progenitor cells (AdPCs) have become available, making it possible to study these cells *in vivo*. In this study, we sought to determine the initial cellular source of MCP-1.

Methods: *C57BL/6J* mice were fed either a standard chow or HFD for varying lengths of time. Flow cytometry, semi-quantitative real-time PCR, and ELISAs were used to assess the importance of AdPCs during diet-induced obesity. Flow cytometry was also performed on a cohort of 14 patients undergoing bariatric surgery.

Results: Flow cytometry identified committed $CD45^{-}CD31^{-}Ter119^{-}$ $CD29^{+}CD34^{+}Sca\text{-}1^{+}CD24^{-}$ adipocyte progenitor cells as producers of high levels of MCP-1 in VAT. High-fat diet increased AdPC numbers. Additionally, flow cytometry identified MCP-1-producing $CD45^{-}CD31^{-}CD34^{+}CD44^{+}CD90^{+}$ AdPCs in human omental and subcutaneous adipose tissue, with a higher percentage in omental adipose. Furthermore, high surface expression of CD44 marked

abundant MCP-1 producers, only in visceral adipose tissue.

Conclusions: This study provides the first *in vivo* evidence, to our knowledge, that committed AdPCs in VAT are the initial source of obesity-induced MCP-1. Inhibition of CD44 expression in AdPCs may serve as unique therapeutic targets for the regulation of adipose tissue inflammation.

Introduction:

Chronic low-grade inflammation in visceral adipose tissue (VAT) links obesity to obesity-associated disease, such as cardiovascular disease, type II diabetes, and cancer[33, 191]. Monocyte chemoattractant protein-1 (MCP-1) is a crucial mediator of chronic inflammation in VAT. Local and systemic levels of MCP-1 are increased in obese mice and humans compared to lean controls[192, 193]. Increases in local production of MCP-1 in murine VAT can occur as early as 2 days post initiation of high-fat diet (HFD)[114]. These increased local levels of MCP-1 act as a chemotactic signal to recruit $CCR2^+$ proinflammatory monocytes that differentiate into $F4/80^+CD11c^+$ M1 macrophages upon entry into adipose tissue[87, 113]. The increased ratio of proinflammatory M1 macrophages to resident $F4/80^+CD206^+$ M2 macrophages is a hallmark feature of adipose tissue inflammation in murine visceral obesity[107], and links to metabolic disease through insulin resistance[106].

In sustained obesity, M1 macrophages become the main producers of MCP-1 and provide a positive feedback signal to recruit additional M1 macrophages[115]. However, M1 macrophages are not present in large numbers in murine adipose tissue until at least 8-10 weeks of HFD[90]. Obesity-induced production of MCP-1 occurs before these macrophages have migrated to the adipose tissue[194], suggesting that another cell type is responsible for early macrophage accumulation. Preliminary reports based on *in vitro* studies of preadipocyte cell lines and adherent SVF cells suggest that resident stromal cells express MCP-1[163]. The stromal cell that initiates MCP-1-mediated macrophage accumulation in

early obesity has not been clearly identified.

The present study is the first *in vivo* evidence, to our knowledge, that the source of early obesity-induced MCP-1 in VAT is adipocyte progenitor cells (AdPCs). Results demonstrated that as little as 1 week of HFD enhanced AdPC proliferation with resultant increase in local MCP-1 production. We also provide novel evidence that AdPCs in human VAT produce MCP-1, and identify CD44 as a key marker of MCP-1-producing AdPCs in human and murine visceral adipose tissue.

Results:

CD45$^-$CD34$^+$ SVF cells are the predominant source of early HFD-induced MCP-1 production in VAT

To determine the main source of early HFD-induced MCP-1, isolated SVF cells and adipocytes from VAT of *C57Bl/6J* mice were analyzed for MCP-1 mRNA levels. There was significantly more MCP-1 mRNA in SVF cells in mice fed 1 week of HFD compared to chow-fed animals (Figure 6A). In contrast, the amount of mRNA in the adipocyte fraction did not significantly change (Figure 6B). In addition, the level of MCP-1 mRNA was higher in the SVF cells than in the adipocytes, from 1 week HFD-fed mice (Figure 6C). Consistent with this finding, analysis of the supernatant of isolated cultured SVF cells and adipocytes from mice fed 1 week of HFD demonstrated higher levels of MCP-1 secreted by SVF cells compared to adipocytes (Figure 6D).

To determine the initial SVF cell responsible for HFD-induced MCP-1 expression, intracellular MCP-1 staining via flow cytometry was performed on VAT from *C57Bl/6J* mice. Interestingly, two populations of MCP-1 positive cells were identified; those with low levels of fluorescence (MCP-1^{mid}) and those with high levels of fluorescence (MCP-1^{hi}) (Figure 6E). The gates were set based on the MCP-1 FMO (Figure 7A). The two populations, as well as the forward scatter/side scatter characteristics (Figure 7B) suggested that more than one cell type within VAT was producing MCP-1, and that the level of expression was cell-type dependent. Utilizing cell surface markers to determine cell phenotype,

Figure 6. $CD45^{-}CD34^{+}$ SVF cells in VAT express high levels of MCP-1

(A-D) SVF and adipocytes were isolated from epididymal VAT of 8-10 week old *C57BL/6J* mice fed 1 week of either chow or HFD, and were analyzed for MCP-1 production. (A-C) MCP-1 mRNA levels in SVF cells and adipocytes, represented as fold increase over chow (A, B) and comparison between populations from HFD-fed mice (C). n=10. (D) MCP-1 levels in the supernatant from SVF cells and adipocytes of 1 week HFD-fed *C57BL/6J* mice, cultured for 24 hours. n=5. (E, F) Flow cytometry analysis of SVF cells from VAT of 8-10 week old *C57BL/6J* mice, n=15. (E) Representative flow plot of intracellular MCP-1 staining in SVF. 1 = $MCP\text{-}1^{mid}$, 2 = $MCP\text{-}1^{hi}$. (F) Characterization of $MCP\text{-}1^{hi}$ and $MCP\text{-}1^{mid}$ cells using CD45, CD34, F4/80 and CD11b surface staining. 71.6±1.5% of $MCP\text{-}1^{hi}$ cells were $CD45^{-}CD34^{+}$ and 66.2±2.1% of $MCP\text{-}1^{mid}$ cells were $CD45^{+}F4/80^{+}CD11b^{+}$. Shown are mean values ± SEM, * $p<0.05$, ** $p<0.01$, **** $p<0.0001$, ns = $p>0.05$

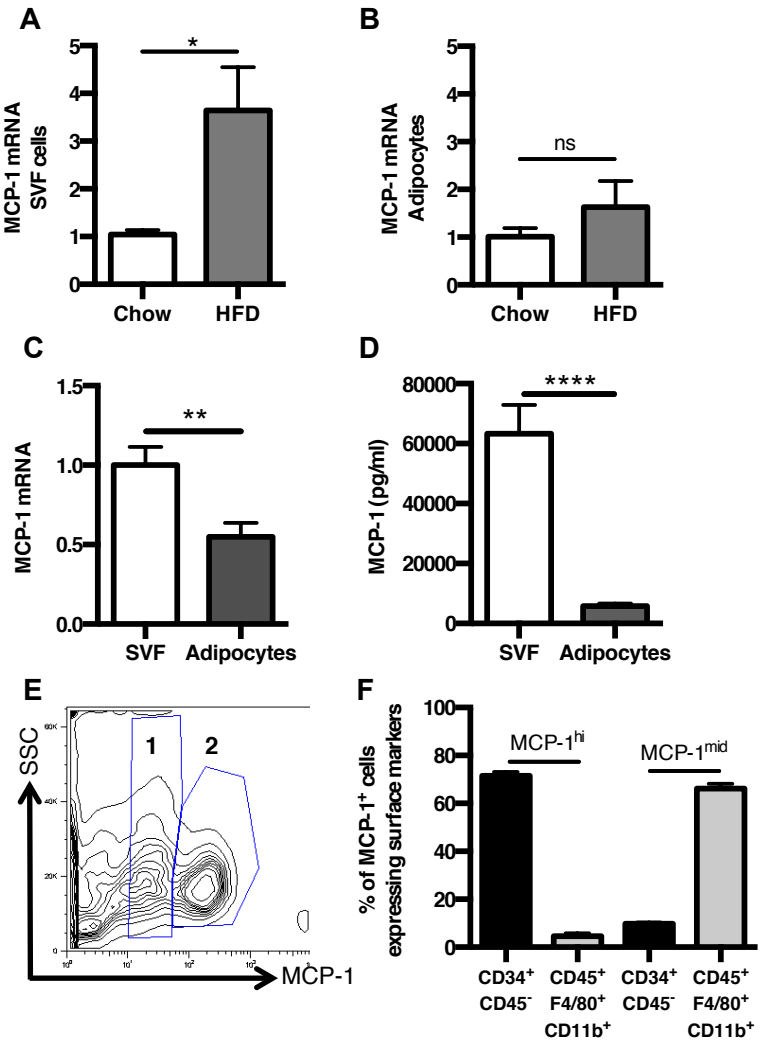

Figure 6: CD45$^-$CD34$^+$ SVF cells in VAT express high levels of MCP-1

Figure 7. Characterization of $CD45^{-}CD31^{-}Ter119^{-}CD29^{+}CD34^{+}Sca\text{-}1^{+}$ cells

SVF were isolated from epididymal VAT of 8-10 week old *C57BL/6J* mice fed either 1 week of chow or HFD. (A) Fluorescence minus one (FMO) negative control for MCP-1 intracellular staining, used to set gates #1 (MCP-1^{mid}) and #2 (MCP-1^{hi}). (B) Representative flow plot of forward scatter and side scatter characteristics from MCP-1^{hi} and MCP-1^{mid} cells. (C, D) Oil Red O staining from sort purified $CD45^{-}CD31^{-}Ter119^{-}CD29^{+}CD34^{+}Sca\text{-}1^{+}$ cells cultured under adipogenic conditions. n=3, each group including 6-8 pooled mice pooled. Oil Red O staining was visualized (C) and quantitated (D) via plate spectrophotometer, represented as fold increase over Chow-Undifferentiated. Shown are mean values ± SEM

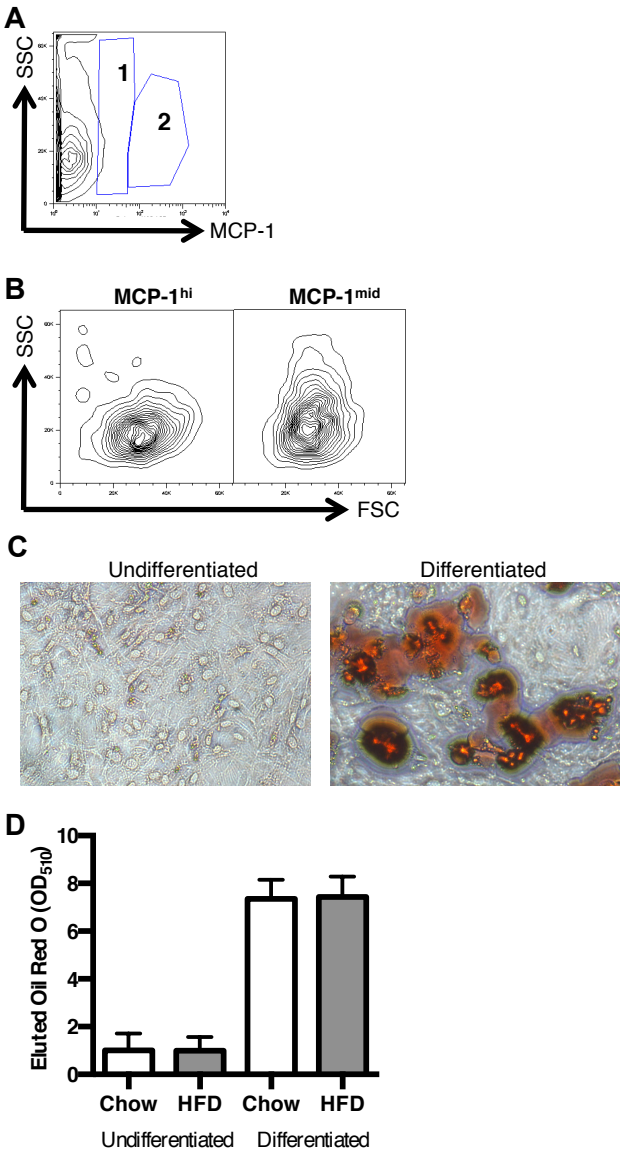

Figure 7: Characterization of $CD45^{-}CD31^{-}Ter119^{-}CD29^{+}CD34^{+}Sca\text{-}1^{+}$ cells

MCP-1^{hi} cells were identified as mostly CD45^{-}CD34^{+}, consistent with identification of a progenitor cell[148], although the type of progenitor cell could not be determined with this strategy. In contrast, the MCP-1^{mid} cells were mostly CD45^{+}F4/80^{+}CD11b^{+}, consistent with a hematopoietic macrophage-like population[195] (Figure 6F).

Characterization of CD45^{-}CD31^{-}Ter119^{-}CD29^{+}CD34^{+}Sca-1^{+} AdPCs

In the past several years, elegant methods to specifically identify adipocyte progenitor cells (AdPCs) using CD45^{-}CD31^{-}Ter119^{-}CD29^{+}CD34^{+}Sca-1^{+} have become available[147]. Utilizing this gating strategy (Figure 8A), the two MCP-1-producing cell types were further characterized. To verify that these cells were AdPCs, CD45^{-}CD31^{-}Ter119^{-}CD29^{+}CD34^{+}Sca-1^{+} cells were sorted based on surface markers and cultured under adipogenic conditions. Consistent with previous findings[147], CD45^{-}CD31^{-}Ter119^{-}CD29^{+}CD34^{+}Sca-1^{+} cells formed mature lipid-filled adipocytes as evidenced by morphology and Oil Red O staining (Figure 7C,D).

To determine what percentage of this well-defined AdPC population produced high levels of MCP-1, intracellular staining for MCP-1 in combination with surface marker characterization to identify AdPCs, was performed. The vast majority of the MCP-1^{hi} cells were characterized as AdPCs based on surface marker expression (Figure 8B). Consistent with results in Figure 6F demonstrating that the MCP-1^{mid} cells were predominantly CD45^{+}F4/80^{+}CD11b^{+}, only a small

Figure 8. Committed $CD45^{-}CD31^{-}CD29^{+}CD34^{+}Sca\text{-}1^{+}CD24^{-}$ AdPCs express and secrete high levels of MCP-1

Epididymal VAT from 8-10 week old *C57BL/6J* mice was harvested and processed for SVF cells. (A, B) Flow cytometry analysis of $CD45^{-}CD31^{-}Ter119^{-}CD29^{+}CD34^{+}Sca\text{-}1^{+}$ AdPCs with representative flow plot (A) and the percentage (B) of AdPCs with MCP-1^{hi} and MCP-1^{mid} expression. n=6 (C) MCP-1 levels as measured by ELISA in the supernatant of equivalent numbers of sort purified AdPCs, total SVF, and Lin^{+} ($CD45^{+}/CD31^{+}/Ter119^{+}$) cells. n=3, each group including 6-8 mice pooled. (D, E) Analysis of MCP-1^{hi} cells in $CD24^{+}$ and $CD24^{-}$ AdPCs with representative plots (D) and quantitation (E) of MCP-1 intracellular staining. n=15. Shown are mean values ± SEM

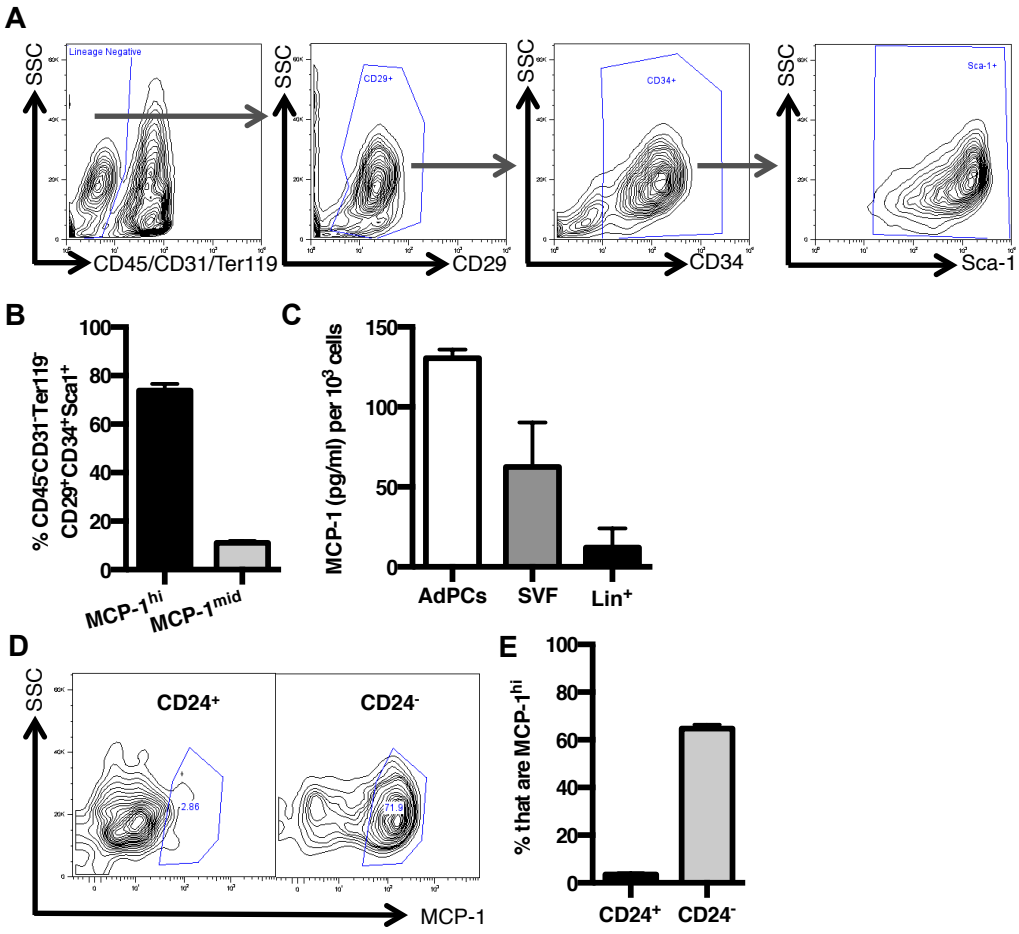

Figure 8: Committed CD45$^-$CD31$^-$CD29$^+$CD34$^+$Sca-1$^+$CD24$^-$ AdPCs express and secrete high levels of MCP-1

percentage of the MCP-1^{mid} cells expressed AdPC surface markers (Figure 8B). To determine if the AdPCs with MCP-1^{hi} intracellular staining also secreted higher levels of MCP-1 compared to total SVF cells and lineage positive cells (Lin^{+}), equal numbers of each cell type sort-purified from VAT were cultured. Of note, AdPCs secreted the highest levels of MCP-1: twice as much as the total SVF, and about 10-fold more than the Lin^{+} cells (Figure 8C).

To determine if AdPCs were expressing other inflammatory molecules besides MCP-1, AdPC-conditioned media from mice fed 1 week of HFD was assayed for 7 additional inflammatory molecules that are induced during obesity. Interestingly, the only cytokine produced at high enough levels to be detected was IL-6 (Figure 9A). To identify if HFD affects secretion of either IL-6 or MCP-1, AdPCs were isolated from chow-fed and 1 week HFD-fed mice. HFD resulted in an increase in secretion of both IL-6 and MCP-1 (Figure 9B,C). IL-6 and MCP-1 are the only two of the 8 cytokines examined to be abundantly expressed in AdPCs. In addition, their expression is induced with HFD stimulation.

AdPCs include cells at different stages of differentiation. CD24^{+} AdPCs are upstream progenitor cells, and loss of CD24 expression occurs as they become further committed to the adipocyte lineage[154, 155]. To determine if there was a relationship between AdPC commitment and MCP- 1 expression, MCP-1 intracellular staining was analyzed in both CD24^{+} and CD24^{-} AdPCs. Results demonstrated that it was the committed CD24^{-} AdPCs that expressed high levels

Figure 9. AdPCs selectively express MCP-1 and IL-6 following HFD

Epididymal VAT from 8-10 week old *C57BL/6J* mice fed 1 week of either chow or HFD was harvested and processed for SVF cells. AdPCs were cultured for 24 hours and supernatant was assayed for pro-inflammatory cytokines and chemokines. (A) Levels of individual cytokines as measured by multiplex in the supernatant of sort purified AdPCs from 1 week HFD-fed mice. (B, C) IL-6 (B) and MCP-1 (C) levels as measured by ELISA in the supernatant of sort purified AdPCs from chow- and 1 week HFD-fed mice.

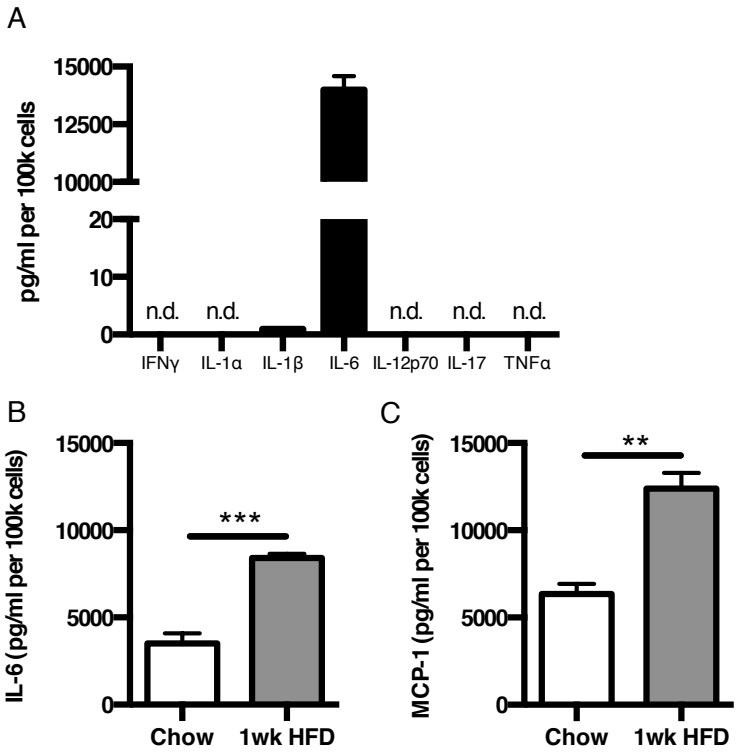

Figure 9: AdPCs selectively increase secretion of MCP-1 and IL-6 following HFD

of MCP-1, while only a small percentage of upstream $CD24^+$ progenitors expressed MCP-1, as seen in the representative flow plot and quantitation of 15 mice (Figure 8D,E). Results suggested that further commitment to the adipocyte lineage stimulates AdPCs to produce high levels of MCP-1.

HFD induces proliferation of AdPCs

To determine the early effects of HFD on MCP-1 production by AdPCs, *C57Bl/6J* mice were fed HFD for 1 week. The numbers of MCP-1^{hi} and MCP-1^{mid} cells were quantified, and results demonstrated that HFD tripled the number of MCP-1^{hi} cells, while the number of MCP-1^{mid} cells was unchanged (Figure 10A). Since MCP-1^{hi} cells are primarily AdPCs, the number of AdPCs was also quantified in VAT after 1 week of HFD. Results demonstrated that 1 week of HFD doubled the number of AdPCs (Figure 10B), indicating that the increased number of MCP-1^{hi} cells may be due to an expansion of the pool of AdPCs.

To determine if HFD-increased AdPC numbers were due to HFD-induced proliferation, a bromodeoxyuridine (BrdU) uptake assay was performed (Figure 10C). Results demonstrated a 2-fold increase in the percentage of AdPCs that incorporated BrdU into their DNA after 1 week of HFD (Figure 10D), indicating that proliferation was responsible for early increases in AdPC numbers. Of note, only $CD24^-$ AdPCs expanded in response to HFD, while there was no change in the number of $CD24^+$ AdPCs (Figure 10E). Thus, these data indicate that HFD results in an expansion of committed $CD24^-$ adipocyte progenitor cells.

Figure 10. 1 week of HFD promotes proliferation of AdPCs

(A, B, E) SVF was isolated from epididymal VAT of 8-10 week old *C57BL/6J* mice fed 1 week of either chow or HFD. n=10-11 per group. (A) Flow quantitation of MCP-1^{hi} and MCP-1^{mid} cells per mouse (paired eVAT depots). (B) Flow quantitation of total AdPCs per mouse (paired eVAT depots). (C-D) 7-8 week old male *C57BL/6J* mice were fed standard chow or HFD for 1 week, and were injected with BrdU (bromodeoxyuridine) 5 times over the course of the diet. n=11. (C) Time course of BrdU injections during 1 week of diet. (D) Percentage of BrdU uptake in AdPCs. (E) Flow quantitation of $CD24^{+}$ and $CD24^{-}$ AdPCs per mouse (paired eVAT depots). Shown are mean values ± SEM, ** $p<0.01$, ns = $p>0.05$.

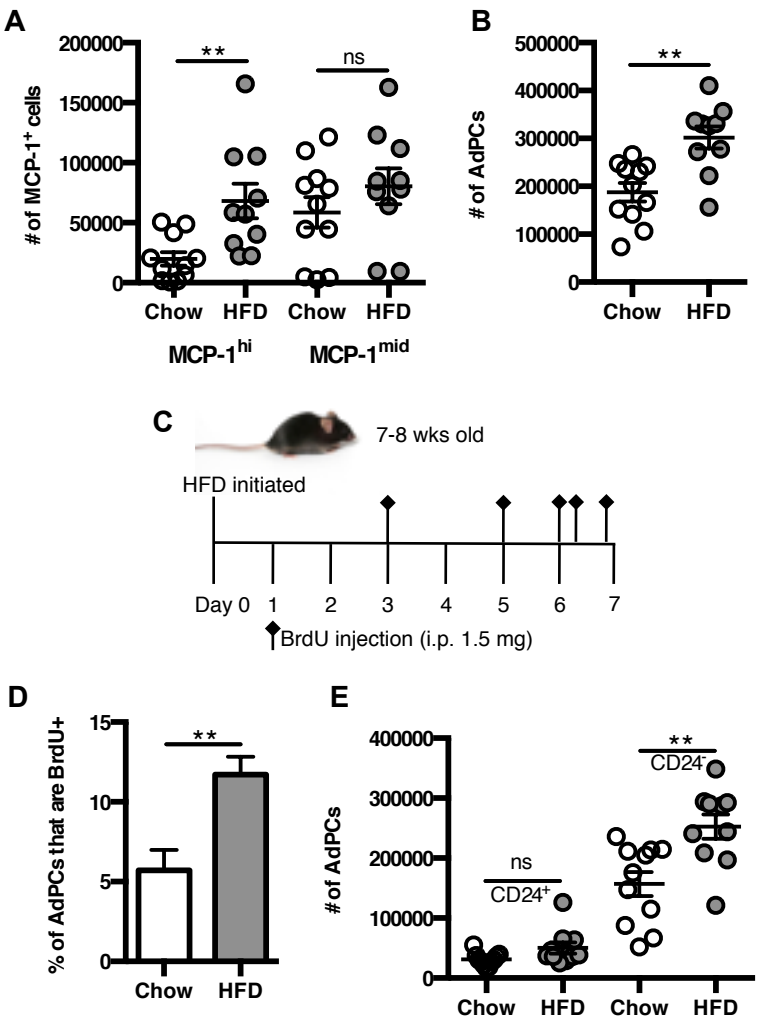

Figure 10: 1 week of HFD promotes proliferation of AdPCs

MCP-1 expression in human omental AdPCs is marked by high levels of CD44

As results in murine studies do not always reflect human disease, we evaluated omental and subcutaneous adipose tissue from a cohort of 14 obese patients undergoing bariatric surgery to determine if adipocyte progenitors in human VAT also express MCP-1. Intracellular cytokine staining and cell phenotyping via flow cytometry with markers validated to identify AdPCs in humans[157, 158] was performed in omental and subcutaneous adipose tissue. These markers identified $CD45^{-}CD31^{-}CD34^{+}$ $CD90^{+}CD44^{+}$ human AdPCs, which have been validated as functional adipocyte precursors[157]. Representative flow cytometry plots of human AdPCs in both omental and subcutaneous adipose tissue are depicted in Figure 11A,B.

Utilizing these surface markers, the percentage of SVF cells that were $CD45^{-}$ $CD31^{-}CD34^{+}$ $CD90^{+}CD44^{+}$ AdPCs in both omental and subcutaneous adipose tissue was determined, finding similar percentages in both depots (Figure 12A). Intracellular staining for MCP-1 determined that a greater percentage of AdPCs from omental adipose were positive for MCP-1 than cells from subcutaneous adipose (Figure 11C,D and Figure 12B). In addition, while most of the $CD45^{-}$ $CD31^{-}$ cells were positive for both CD34 and CD90, there appeared to be a broader range of CD44 expression within this progenitor cell population, particularly in omental adipose. Within the broad range of CD44 expression in omental AdPCs, the percentage of MCP-1^{+} cells greatly differed (Figure 12C).

Figure 11. Identification of $CD45^{-}CD31^{-}CD34^{+}CD90^{+}CD44^{+}$ adipocyte progenitor cells and MCP-1 intracellular staining

Subcutaneous and omental adipose tissue were collected during bariatric surgery from consenting human subjects, and were processed for SVF cells. (A, B) Representative plot of flow cytometry identification of $CD45^{-}CD31^{-}CD34^{+}CD90^{+}CD44^{+}$ adipocyte progenitor cells in (A) human omental SVF and (B) human subcutaneous SVF. (C, D) MCP-1 intracellular staining in representative plots from omental (C) and subcutaneous (D) $CD45^{-}CD31^{-}CD34^{+}CD90^{+}CD44^{+}$ adipocyte progenitor cells.

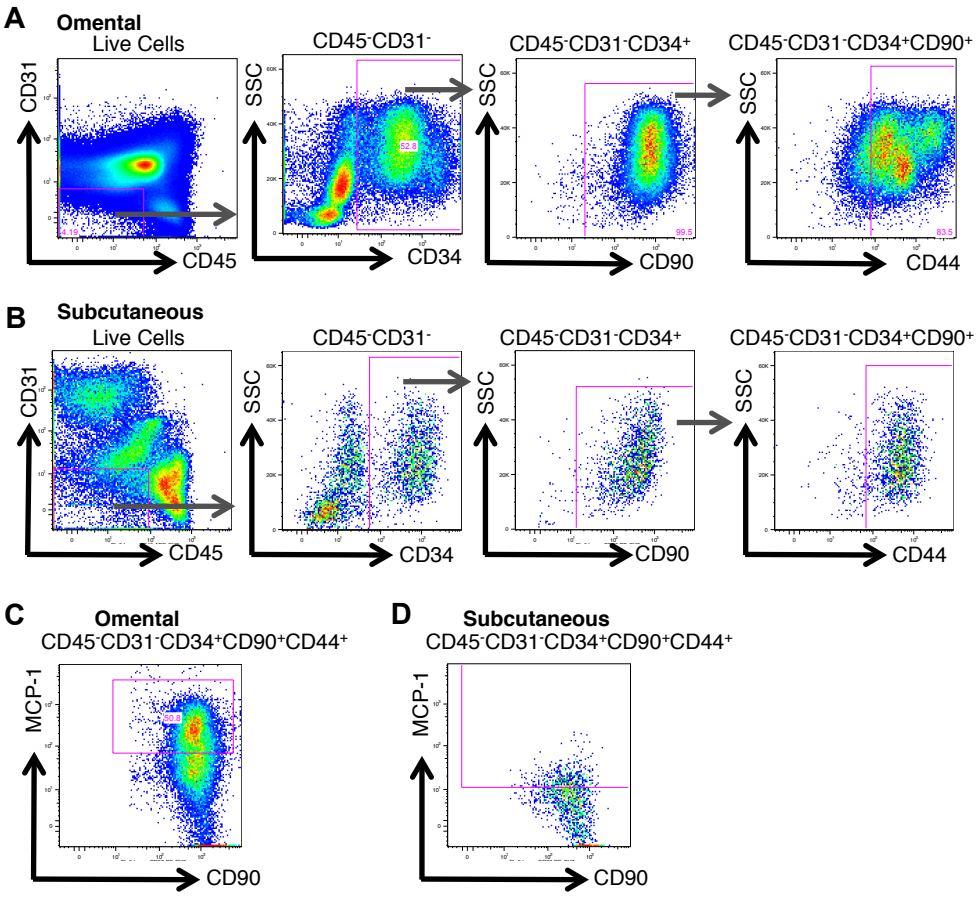

Figure 11: Identification of $CD45^{-}CD31^{-}CD34^{+}CD90^{+}CD44^{+}$ adipocyte progenitor cells and MCP-1 intracellular staining

Figure 12. Human omental adipocyte progenitor cells express abundant MCP-1: an effect marked by high levels of CD44

(A-E) Subcutaneous and omental adipose tissue were collected during bariatric surgery from consenting human subjects (n=14), and were processed to SVF cells for flow cytometry. (A) Percentage of SVF cells that were $CD45^{-}CD31^{-}CD34^{+}CD90^{+}CD44^{+}$ adipocyte progenitor cells in human omental and subcutaneous adipose tissue. (B) Percentage of AdPCs that were MCP-1^{+} in omental and subcutaneous adipose tissue. (C) Representative plots depicting the heterogeneity of CD44 staining in $CD45^{-}CD31^{-}CD34^{+}CD90^{+}$ cells from omental VAT, and MCP-1 intracellular staining in each CD44 subset. 1 = $CD44^{-}$, 2 = $CD44^{lo}$, 3 = $CD44^{hi}$ (D) Percentage of AdPCs that are MCP-1^{+} as a function of $CD44^{hi}$ and $CD44^{lo}$ status in omental and subcutaneous adipose tissue. (E) Correlation of gMFI of MCP-1 with gMFI of CD44 in $CD45^{-}CD31^{-}CD34^{+}$ $CD90^{+}$ AdPCs in both omental and subcutaneous adipose tissue. Shown are mean values ± SD. (F, G) SVF was isolated from epididymal VAT of 8-10 week old *C57BL/6J* mice fed 1 week of either chow or HFD. n=4. (F) Percentages of $CD45^{-}CD31^{-}CD34^{+}CD29^{+}Sca\text{-}1^{+}CD24^{-}$ AdPCs that are MCP-1^{hi} as a function of $CD44^{hi}$ and $CD44^{lo}$ status, in chow-fed and HFD-fed mice. (G) Correlation of gMFI of MCP-1 with gMFI of CD44 in $CD45^{-}CD31^{-}CD34^{+}CD29^{+}Sca\text{-}1^{+}CD24^{-}$ AdPCs, fed chow or HFD. Shown are mean values ± SEM, * $p<0.05$, ** $p<0.01$, **** $p<0.0001$, ns = $p>0.05$. gMFI = Mean Fluorescence Intensity, using geometric mean.

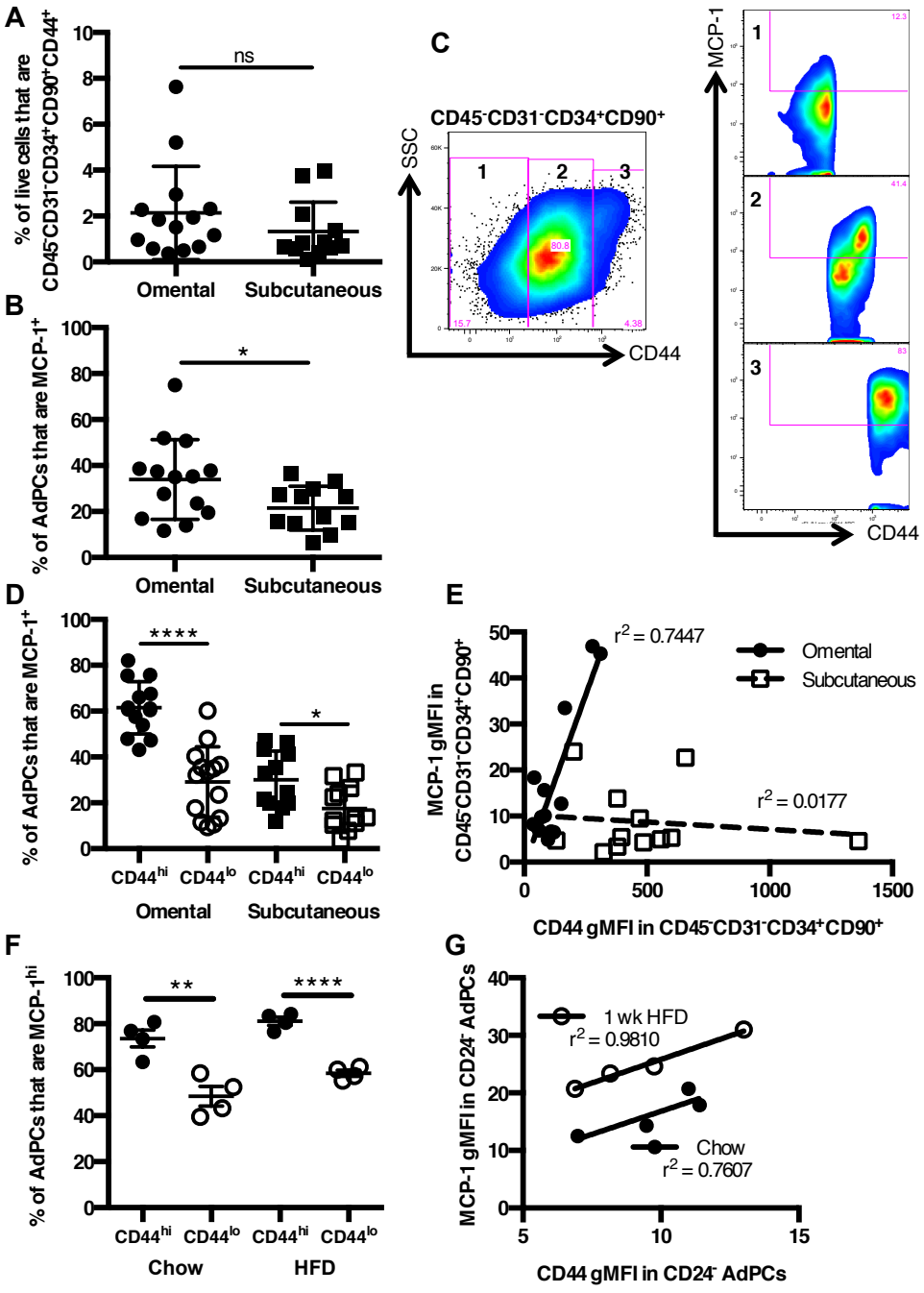

Figure 12: Human omental adipocyte progenitor cells express abundant MCP-1: an effect marked by high levels of CD44

There was a larger percentage of MCP-1^{+} cells in the CD44^{hi} population than in the CD44^{lo} population (Figure 12D). Notably, in CD45^{-}CD31^{-}CD34^{+}CD90^{+} cells from omental adipose, the geometric mean fluorescence intensity (gMFI) of CD44 highly correlated to the gMFI of MCP-1 (Figure 12E). However, there was no correlation in subcutaneous adipose, nor was there a correlation in any other population of cells within the omental adipose (data not shown).

While CD44 was not one of the markers in the murine AdPC panel, the association of CD44 with MCP-1 production in humans led us to determine if CD44^{hi} AdPCs were also abundant producers of MCP-1 in mice. MCP-1 intracellular staining was analyzed in CD24^{-}CD44^{hi} AdPCs compared to CD24^{-} CD44^{lo} AdPCs. As seen in the human VAT, there was a larger percentage of MCP-1^{hi} cells in the CD44^{hi} population than in the CD44^{lo} population in both chow-fed and HFD-fed mice (Figure 12F). Additionally, in the CD24^{-} AdPCs, the gMFI of CD44 highly correlated to the gMFI of MCP-1, seen in both chow-fed and HFD-fed mice (Figure 12G). This data provides evidence that CD44 marks AdPCs that express the highest levels of MCP-1 both in mice and humans.

Discussion:

The present study clearly identifies AdPCs as the initial source of MCP-1 in response to HFD in mice and demonstrates that AdPCs in humans also produce abundant MCP-1. Utilizing murine models, we demonstrate that the HFD-induced increase in MCP-1 is due to an increase in the number of MCP-1-producing AdPCs. MCP-1 expression within the AdPCs was limited to those that lacked surface expression of CD24 and had high expression of CD44, as demonstrated in Figure 13.

MCP-1 is one of the most well characterized inflammatory factors produced during obesity because of its action as a potent chemoattractant for M1 macrophages. When MCP-1 is deleted in mice, the number of M1 macrophages found in adipose tissue is significantly reduced[113]. Inhibition of M1 macrophage infiltration into adipose tissue leads to improvement of adipocyte function, as well as attenuation of obesity-induced insulin resistance[121]. While transcriptional regulation of MCP-1 has been very well characterized, the source of early obesity-induced MCP-1 is much less clear. Production of MCP-1 by adipocytes has been well documented[113, 121], but evidence of higher production by SVF cells indicates that adipocytes are not the main source of MCP-1 during obesity[31]. The search for the cell that initially produces MCP-1 in response to HFD has implicated cells within the SVF, including endothelial cells[159], mast cells, and $CD8^+$ T cells[160]. This study provides clear *in vivo* evidence that AdPCs are the main sources of initial HFD-induced MCP-1 production.

Figure 13. Model of MCP-1 production by AdPCs in VAT during HFD

During HFD-induced visceral adipose tissue expansion, there is an increase in the number of AdPCs, specifically, those that lack expression of CD24. These $CD24^-$ AdPCs express MCP-1, with increased MCP-1 production coming from those with high surface expression of CD44. Expression of MCP-1 by AdPCs is responsible for most MCP-1 produced in VAT during the initial stages of HFD.

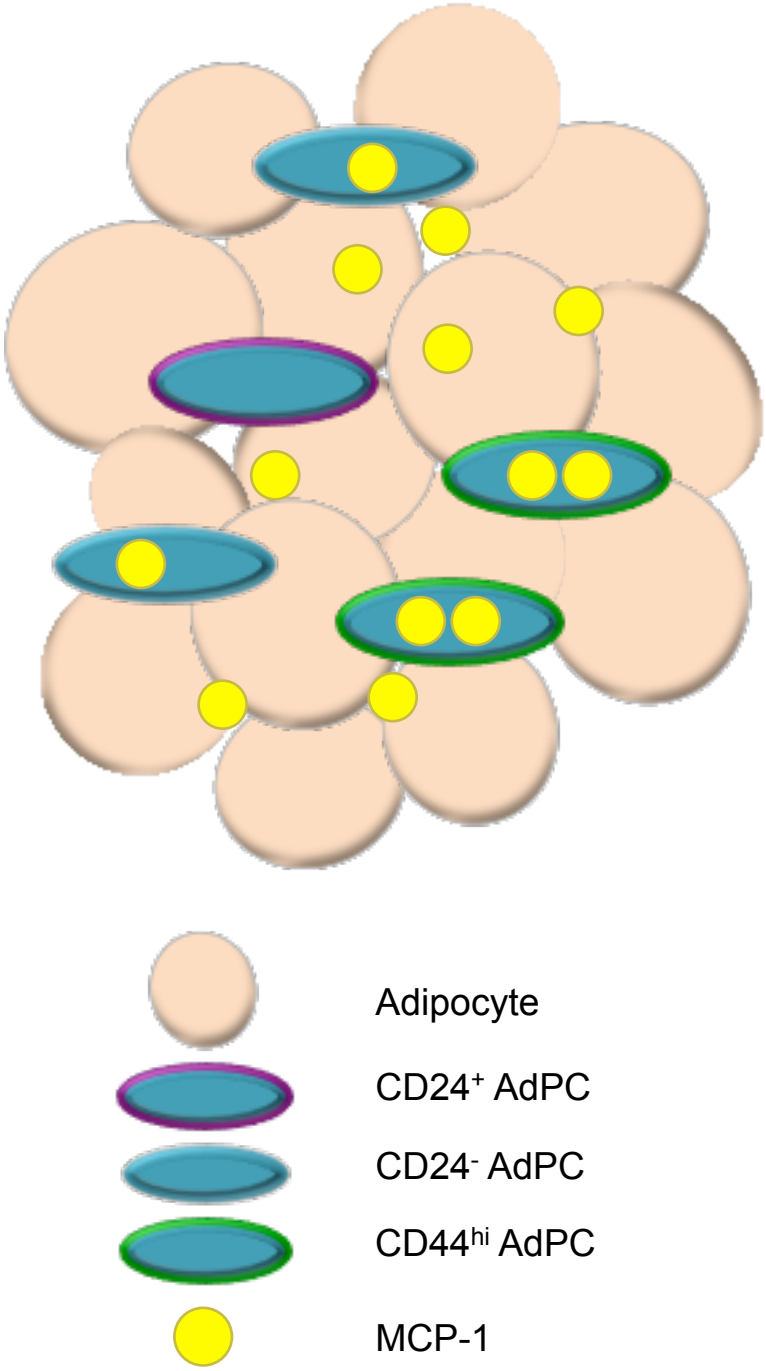

Figure 13: Model of MCP-1 production by AdPCs in VAT during HFD

Progenitor cells in tissues were originally thought to function as a reservoir of precursors poised to replenish the mature differentiated cells when needed. Yet, our data provides evidence of an important immunomodulatory function of AdPCs during times of altered tissue homeostasis as seen in disease states such as obesity, suggesting that progenitor cells have biological impact on the response to perturbation of homeostasis when in their undifferentiated state. This indicates that progenitor cells serve an additional function beyond replenishing the pool of differentiated cells.

It is well accepted that MCP-1 in adipose tissue functions to recruit inflammatory macrophages, promoting metabolic dysregulation during obesity. However, it is intriguing to speculate as to why a progenitor cell would produce a macrophage chemoattractant when perturbed by nutritional excess. As the adipose depot first starts to expand, macrophages recruited by MCP-1 may have multiple roles. An increase in blood supply is required to support the adipose tissue growth needed to accommodate the increased lipid with HFD. Macrophages support endothelial sprouting for the formation of new functional blood vessels[196], and Tie-2^{+} angiogenic macrophages are recruited by MCP-1[197]. Deletion of MCP-1 has been demonstrated to result in reduced tumor angiogenesis[198], suggesting that MCP-1 is important for new blood vessel formation to support rapidly expanding tissues. Adipocyte progenitors can reside in the adipose vasculature[146], as seen by expression of preadipocyte determination factor Zfp423 in capillary sprouts

from human adipose tissue[199], allowing them to be conveniently poised to produce MCP-1 and attract the needed macrophages to support angiogenesis in the growing adipose depot. Further studies are needed to determine if AdPC-derived MCP-1 is necessary for VAT angiogenesis, although these studies will be challenging as there is no unique marker of AdPCs that can allow for AdPC-specific deletion of MCP-1.

Our murine results demonstrated significantly greater MCP-1 expression in the $CD24^-$ AdPCs compared to the $CD24^+$ AdPCs, suggesting that CD24 may inhibit AdPC production of MCP-1. Supporting this hypothesis, global deletion of CD24 results in rapid increase in both local and systemic levels of MCP-1 in a murine cecal ligation and puncture model[200]. In addition to representing the commitment status of progenitor cells, CD24 is a glycosylphosphatidylinositol-anchored cell surface protein[201], also known as Heat Stable Antigen, with expression in a variety of cell types. Its function is poorly understood, owing to its variable glycosylation in different cell types[202] and its lack of a cytoplasmic domain, preventing intracellular signaling[203]. Whether CD24 expression in AdPCs is directly regulating MCP-1 production, or if it marks an adipocyte commitment step that promotes MCP-1 production, is unknown. Interestingly, CD24 is not used in the identification of human adipocyte progenitor cells, and it is not clear if its expression has different functions in murine versus human AdPCs.

The inflammation associated with obesity has been linked to diseases such as

atherosclerosis[204], cancer[205], and autoimmune disease[206]. Efforts have been made to diminish systemic inflammation in the hope of treating obesity-related disease, as with anti-TNFα treatment. However, it was demonstrated that systemic ablation of this proinflammatory signaling pathway resulted in dysfunctional adipogenesis, hepatic steatosis, and ectopic lipid accumulation[207]. Learning more about the inflammatory properties of AdPCs could provide a unique approach to targeting harmful adipose tissue inflammation and obesity-associated disease, while preserving immune homeostasis. The novel demonstration of MCP-1-producing AdPCs in human omental adipose underscores the potential clinical relevance of these data as it opens the door for discovery of unique approaches that could lead to strategies that would limit the obesity-induced inflammatory cascade at an early stage, preventing the amplification of inflammation seen with the chronic accumulation of inflammatory macrophages.

Human AdPCs bear a unique set of identifying surface markers from mouse AdPCs, and are identified by lack of CD45 and CD31, and expression of CD34, CD44 and CD90[157, 208]. Utilizing these markers, we identified AdPCs in obese human omental and subcutaneous adipose tissue. Consistent with visceral adipose depots harboring more inflammation than subcutaneous adipose depots, we found that AdPCs in human omental adipose produced more MCP-1 than cells from subcutaneous adipose. These data suggest that adipose depot-specific differences in inflammation may be due to depot-specific differences in

AdPCs. Indeed, previous studies have suggested that AdPCs in the different depots arise from different precursor origins, and may not share a common precursor cell[76, 209]. In addition, recent findings provide evidence that HFD-induced AdPC proliferation was limited to the VAT, and not seen in subcutaneous adipose[210]. This introduces the possibility that the greater inflammation seen in omental compared to subcutaneous adipose tissue may be, at least in part, due to greater production of MCP-1 by AdPCs. This may be quite relevant for human disease, as omental adipose tissue has been directly linked to metabolic disease and insulin resistance through MCP-1 production and macrophage infiltration[116].

Notably, results also demonstrated that CD44 marks a unique population of AdPCs that express abundant MCP-1 in VAT from both mice and humans. CD44 is a receptor for both osteopontin (OPN) and hyaluronic acid (HA), and has been shown to upregulate overall MCP-1 levels via receptor engagement with these ligands[211, 212]. Studies have demonstrated that levels of OPN[213] and HA[214] increase during obesity. CD44 levels in serum of obese human subjects positively correlated with the prevalence of insulin resistance, as well as to HbA1c, an index of glycemic control[215]. Direct targeting of CD44 in VAT AdPCs could provide a therapeutic strategy to potentially diminish adipose tissue inflammation.

Chapter 4

Id3 promotes HFD-induced MCP-1, M1 macrophages, and glucose intolerance through proliferation of CD24^{-} AdPCs

Adapted from Kaplan, J.L. et al. Adipocyte progenitor cells initiate monocyte chemoattractant protein-1-mediated macrophage accumulation in visceral adipose tissue. *Molecular Metabolism* (2015)

Abstract:

Objective: We have previously published that global deletion of transcription factor Inhibitor of Differentiation 3 (Id3) attenuates high fat diet-induced obesity, but it is unclear if Id3 plays a role in diet-induced MCP-1 production. We sought to identify molecular regulators mediating MCP-1 production.

Methods: *Id3*$^{+/+}$ and *Id3*$^{-/-}$ mice were fed either a standard chow or HFD for 1-8 weeks, as specified. Flow cytometry, semi-quantitative real-time PCR, ELISAs and adoptive transfers were used to determine the mechanism by which AdPCs expand in number during diet-induced obesity, and how modulation of AdPC numbers affect VAT macrophages and metabolic function.

Results: High-fat diet-induced increase of AdPC numbers was dependent on Id3. HFD resulted in increased Id3 mRNA levels in AdPCs, and Id3 reduced p21Cip1 promoter activation in a preadipocyte cell line. Loss of Id3 resulted in increased p21^{Cip1} levels in AdPCs and attenuated AdPC proliferation. In addition, Id3-deficient mice had reduced MCP-1 levels and fewer M1 macrophages in VAT, compared to *Id3*$^{+/+}$ littermate controls. Myeloid-specific Id3-/- mice had no difference in MCP-1 levels in VAT. AdPC rescue by adoptive transfer of 50,000 *Id3*$^{+/+}$ AdPCs into *Id3*$^{-/-}$ recipient mice increased MCP-1 levels and M1 macrophage number in VAT.

Conclusions: Helix-loop-helix transcription factor Id3 was identified as a critical

regulator of $p21^{Cip1}$ expression, AdPC proliferation, MCP-1 expression and M1 macrophage accumulation in VAT. Inhibition of Id3 and AdPC expansion may serve as unique therapeutic targets for the regulation of adipose tissue inflammation.

Introduction:

Recent work suggests that the transcription factor Inhibitor of Differentiation 3 (Id3) acts as a regulator of metabolic health in obesity[182, 183]. Id3 is a dominant negative inhibitor of the basic helix-loop-helix (bHLH) family of transcription factors, which is a highly conserved group of proteins that play a role in the differentiation and growth of a variety of cell types[165, 167]. Id3 is known to negatively regulate lineage commitment, cell fate decisions, and the timing of differentiation. Id3 has been found to promote proliferation in many models and systems, particularly in cancer, through alteration of the expression of critical cell cycle regulatory proteins, including $p21^{Cip1}$ [167, 177].

While Ids are primarily expressed during development, they are re-expressed during disease, such as diet-induced obesity[167, 176]. We have previously published that Id3 antagonizes E47-dependent activation of adiponectin promoter activity[184, 185]. We have also published that Id3 promotes adipose tissue vascularization through regulation of vascular endothelial growth factor (VEGF-A) [182, 216]. We have postulated that this mechanism may be important in diet-induced obesity, as Id3-deficient mice are protected from HFD-induced weight gain and adipose tissue expansion[182, 216]. Others have suggested a role for Id3 in preadipocyte commitment and differentiation[217]. Together, these data indicate that Id3 plays a role in diet-induced obesity, but whether Id3 plays a role in metabolic dysfunction during adipose tissue expansion and inflammation has yet to be determined.

We identify Id3 as a critical regulator of $p21^{Cip1}$ expression and proliferation in AdPCs. Consistent with these findings, mice null for Id3 had a loss of obesity-induced MCP-1 production and attenuated accumulation of M1 macrophages in VAT. Id3-deficient mice also had improved glucose uptake and insulin signaling. Importantly, adoptive transfer of $Id3^{+/+}$ AdPCs into $Id3^{-/-}$ mice significantly increased the amount of MCP-1 and the M1/M2 ratio in VAT, and perturbed glucose uptake.

Results:

Id3 promotes HFD-induced proliferation of AdPCs and represses AdPC expression of p21^{Cip1}

To determine if Id3, a known regulator of diet-induced obesity and adipose tissue expansion, plays a role in HFD-induced AdPC proliferation, we utilized the *Id3*$^{-/-}$ mouse. To determine first whether HFD led to changes in Id3 expression in AdPCs, Id3 mRNA levels were measured in sort-purified AdPCs from chow-fed and HFD-fed mice. Results demonstrated a HFD-induced increase in Id3 expression in AdPCs (Figure 14A). To determine if loss of Id3 altered proliferation of AdPCs, BrdU uptake was measured in *Id3*$^{-/-}$ mice and compared to *Id3*$^{+/+}$ littermate controls. In contrast to *Id3*$^{+/+}$ mice, *Id3*$^{-/-}$ mice had no increase in proliferating AdPCs due to HFD (Figure 14B), providing evidence that HFD-induced proliferation of AdPCs is dependent on Id3.

To determine if loss in obesity-induced AdPC proliferation in *Id3*$^{-/-}$ mice translated to actual differences in the number of committed CD24^{-} AdPCs, CD24^{-} AdPCs were quantified after 1 week of chow or HFD. In contrast to the *Id3*$^{+/+}$ mice, the CD24^{-} AdPCs from *Id3*$^{-/-}$ mice failed to expand after 1 week of HFD (Figure 14C). To confirm that the lack of increase in CD24^{-} AdPCs after HFD in the *Id3*$^{-/-}$ mice was not due to an increase in adipocyte differentiation, Oil Red O uptake was measured. Consistent with previous findings[182], results demonstrated equivalent Oil Red O uptake between genotypes (Figure 14D), providing evidence that Id3 regulation of AdPC number is not through modulated differentiation.

Figure 14. HFD reduces $p21^{Cip1}$ expression and promotes proliferation of committed $CD24^-$ AdPCs in an Id3-dependent manner

(A, E) SVF was isolated from epididymal VAT of 8-10 week old *C57BL/6J* mice fed 1 week of either chow or HFD, and $CD45^-CD31^-Ter119^-CD29^+CD34^+Sca\text{-}1^+$ cells were sort purified. n=3, each group including 6-8 mice pooled. (A) Id3 mRNA levels, represented as fold increase over chow. (B-D, G) SVF was isolated from epididymal VAT of 8-10 week old $Id3^{+/+}$ and $Id3^{-/-}$ mice fed 1 week of either chow or HFD. (B) Percentage of BrdU uptake in AdPCs, as described in Fig 3C. n=7-11. (C) Quantitation of $CD24^-$ AdPCs per mouse (paired eVAT depots). n=7-9. (D) Oil Red O staining from sort purified AdPCs cultured under adipogenic conditions. n=3, each group including 6-8 pooled mice pooled. (E) $p21^{Cip1}$ mRNA levels, represented as fold increase over chow. (F) $p21^{Cip1}$ promoter activity in OP-9 cells, transfected with plasmid encoding ID3 and $p21^{Cip1}$ luciferase-expressing promoter construct (p21-LUC), as measured by RLU (relative luminescence units). Performed in triplicate, repeated three times. (G) $p21^{Cip1}$ mRNA expression, represented as fold increase over $Id3^{+/+}$. Shown are mean values ± SEM, * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

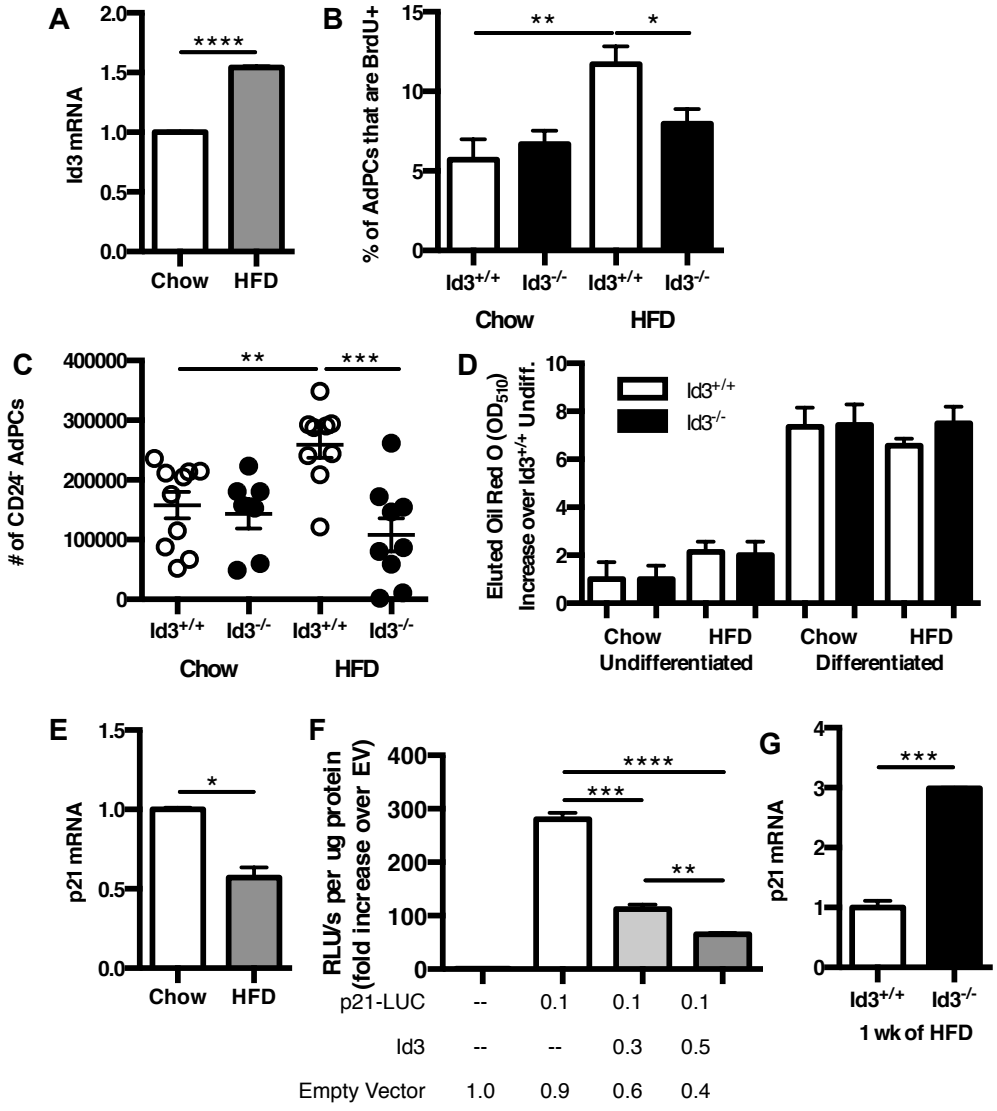

Figure 14: HFD reduces p21^{Cip1} expression and promotes proliferation of committed CD24^{-} AdPCs in an Id3-dependent manner

Id3 has previously been demonstrated to inhibit the expression of $p21^{Cip1}$ [167, 177], a cyclin-dependent kinase inhibitor that prevents entrance into S phase, keeping cells growth-arrested in G1[218]. To determine if HFD inhibits $p21^{Cip1}$ expression in AdPCs, $p21^{Cip1}$ mRNA levels were measured in sort-purified AdPCs from chow-fed and HFD-fed mice. Results showed a significant decrease in $p21^{Cip1}$ mRNA expression in AdPCs from mice fed HFD (Figure 14E). To determine if Id3 regulates $p21^{Cip1}$ promoter activation in AdPCs, an Id3 expression construct was co-transfected with a $p21^{Cip1}$ luciferase-expressing promoter construct into OP-9 and 3T3-L1 preadipocyte cell lines. Promoter reporter assays demonstrated that Id3 repressed $p21^{Cip1}$ promoter activation in a dose-dependent manner in both OP-9 (Figure 14F) and 3T3-L1 cells (data not shown). In addition, $p21^{Cip1}$ mRNA levels were measured in AdPCs from both *Id3*$^{-/-}$ mice and wild-type littermate controls. AdPCs from mice null for Id3 had significantly greater $p21^{Cip1}$ mRNA levels (Figure 14G). These data together suggest that HFD-induced Id3 inhibits $p21^{Cip1}$ expression and promotes cell cycle progression in AdPCs.

Id3 promotes adipose tissue MCP-1 levels through expansion of MCP-1^{hi} AdPCs

To determine if loss of Id3 attenuates the HFD-induced increase in MCP-1^{hi} cells, intracellular staining for MCP-1 was performed in *Id3*$^{-/-}$ mice compared to *Id3*$^{+/+}$ littermate controls. Results demonstrated a significant attenuation of obesity-induced MCP-1^{hi} cells in *Id3*$^{-/-}$ mice (Figure 15A). As with the *Id3*$^{+/+}$ mice, HFD did

Figure 15. Id3 promotes HFD-induced MCP-1 in VAT

(A, B, G) SVF was isolated from epididymal VAT of 8-10 week old *Id3*$^{+/+}$ and *Id3*$^{-/-}$ mice fed 1 week of either chow or HFD. n=7-10. Flow quantitation of MCP-1^{hi} cells (A) and MCP-1^{mid} cells (B) per mouse (paired eVAT depots). (C-E) Epididymal VAT and subcutaneous adipose tissue were harvested from 8-10 week old *Id3*$^{+/+}$ and *Id3*$^{-/-}$ mice fed 4 weeks of either chow or HFD. n=5-6. (C, D) MCP-1 mRNA levels in epididymal (C) and subcutaneous (D) adipose, represented as fold increase over *Id3*$^{+/+}$ chow. (E) MCP-1 levels as measured by ELISA in the supernatant of epididymal VAT, cultured for 24 hours. MCP-1 secretion was normalized per mouse (paired eVAT depots). (F) Weights of epididymal VAT from 8-10 week old *Id3*$^{+/+}$ and *Id3*$^{-/-}$ mice fed chow-diet or 1 or 4 weeks of HFD. (G) Correlation of quantified MCP-1^{hi} cells with epididymal VAT weight in 1 week HFD-fed *Id3*$^{+/+}$ and *Id3*$^{-/-}$ mice. (H) MCP-1 promoter activity in OP-9 cells, transfected with plasmid encoding ID3 and MCP-1 luciferase-expressing promoter construct (MCP-1-LUC), as measured by RLU (relative luminescence units). Performed in triplicate, repeated three times. (I) MCP-1 levels as measured by ELISA in the supernatant of sort purified AdPCs from 1 week HFD-fed mice. n=3, each group including 6-8 pooled mice pooled. Shown are mean values ± SEM, * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$, ns = $p>0.05$.

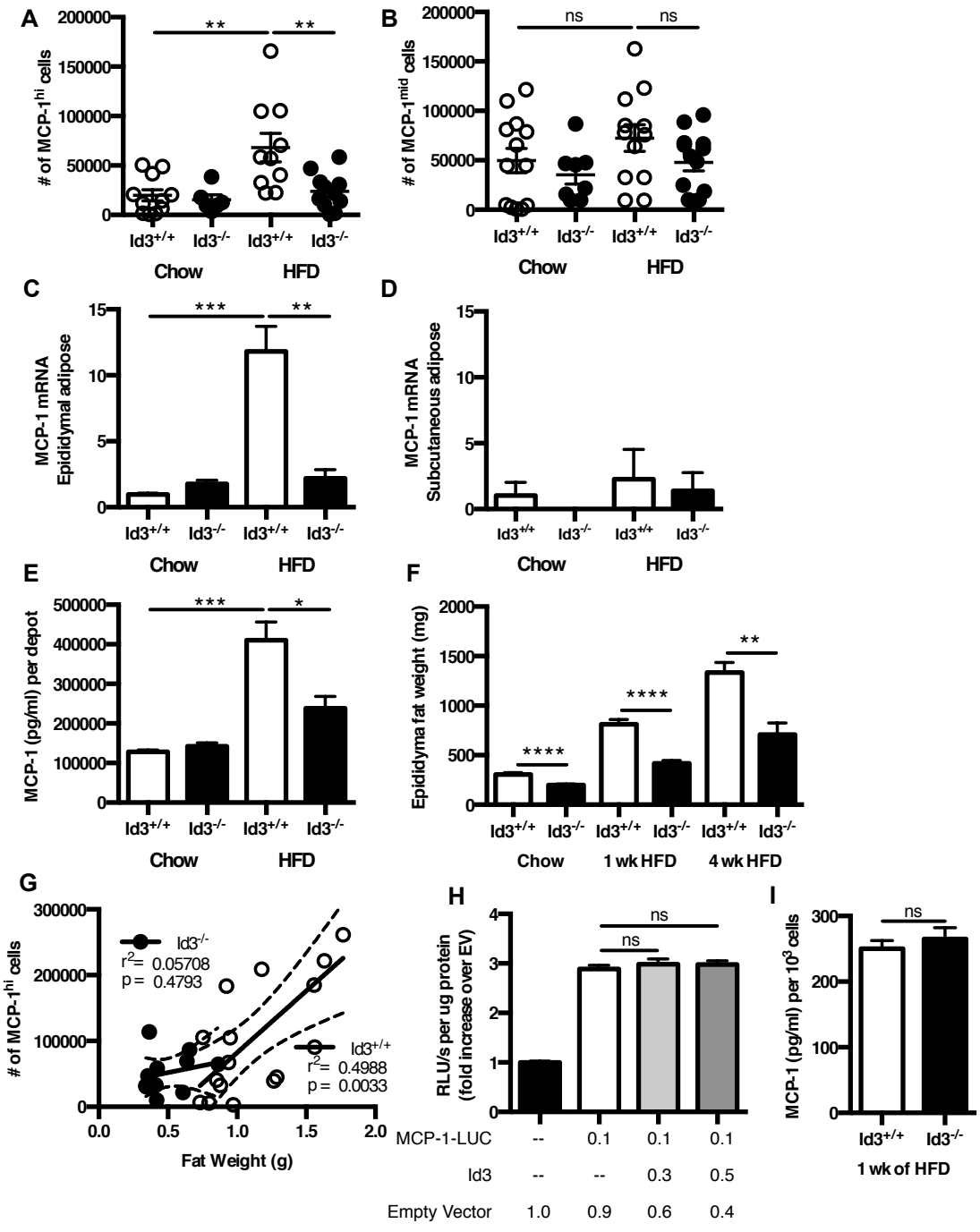

Figure 15: Id3 promotes HFD-induced MCP-1 in VAT

not change the number of MCP-1^{mid} cells in *Id3*$^{-/-}$ mice, and there was no difference due to loss of Id3 (Figure 15B). To determine if Id3 specifically promotes VAT MCP-1 expression and if early effects impact whole tissue at later time points, MCP-1 mRNA and protein levels were measured in both visceral and subcutaneous adipose tissue from *Id3*$^{+/+}$ and *Id3*$^{-/-}$ mice. *Id3*$^{+/+}$ mice had a significant increase in MCP-1 mRNA levels in VAT after 4 weeks of HFD, while this was markedly attenuated in mice null for Id3 (Figure 15C). This finding was specific to VAT, since there was no induction of MCP-1 mRNA levels in subcutaneous adipose tissue, nor were there Id3-dependent differences (Figure 15D). Consistent with the mRNA data, HFD-induced MCP-1 secretion from cultured VAT was attenuated in *Id3*$^{-/-}$ mice (Figure 15E). These data suggest that early obesity-induced MCP-1 production in VAT is Id3-dependent.

To determine if Id3 regulates MCP-1 expression or secretion from adipocytes, adipocytes were isolated from *Id3*$^{+/+}$ and *Id3*$^{-/-}$ mice, fed 1 week of HFD. Results demonstrated that there was no difference in MCP-1 mRNA or protein levels in adipocytes due to global loss of Id3 (Figure 16A,B).

As we have previously published that *Id3*$^{-/-}$ mice are partially protected from diet-induced obesity, we wanted to determine if reduced MCP-1 production in *Id3*$^{-/-}$ mice was accompanied by reduced adipose tissue expansion after 1 and 4 weeks of HFD. Indeed, while HFD induced an increase in epididymal VAT weight in both genotypes, *Id3*$^{-/-}$ mice had an attenuated effect (Figure 15F). Interestingly,

Figure 16. Loss of Id3 does not affect adipocyte expression of MCP-1

Adipocytes were isolated from 8 week old *Id3*$^{+/+}$ and *Id3*$^{-/-}$ mice fed 1 week of HFD. (A) MCP-1 mRNA levels from isolated adipocytes. (B) MCP-1 levels as measured by ELISA in the supernatant of adipocytes cultured for 24 hours. MCP-1 secretion was normalized per mouse (total adipocytes from paired eVAT depots). Shown are mean values ± SEM, ns = $p>0.05$

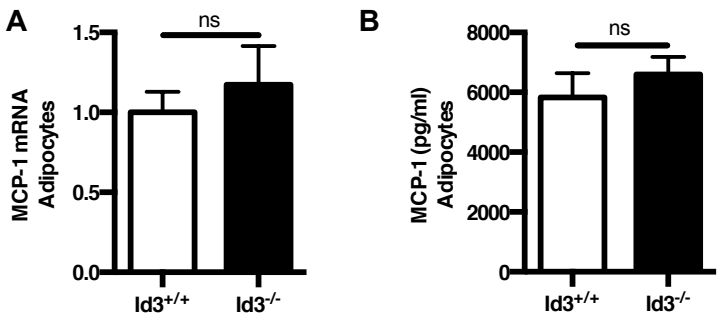

Figure 16: Loss of Id3 does not affect adipocyte expression of MCP-1

while the number of MCP-1^{hi} cells found in VAT from *Id3*$^{+/+}$ mice correlated with the size of the fat depot, there was no such correlation in *Id3*$^{-/-}$ mice (Figure 15G), suggesting that the number of MCP-1^{hi} cells is not influenced by VAT depot size in these mice.

To determine if Id3 regulates MCP-1 gene expression and protein production on a per cell basis, promoter reporter and MCP-1 protein assays were performed. OP-9 and 3T3-L1 preadipocyte cell lines were co-transfected with an MCP-1 promoter luciferase reporter construct and a plasmid encoding Id3. Results demonstrated that Id3 had no effect on MCP-1 promoter activation (Figure 15H and data not shown). Equivalent numbers of sort purified AdPCs from *Id3*$^{-/-}$ mice and *Id3*$^{+/+}$ littermate controls were cultured and the supernatant was assayed for MCP-1. Results demonstrated no genotype-dependent differences in the level of MCP-1 produced (Figure 15I).

Id3 does not regulate MCP-1 in myeloid cells

Macrophages are important producers of MCP-1[219]. To determine if loss of Id3 in macrophages affects MCP-1 expression, heterozygous transgenic mice containing Cre driven by the lysozyme promoter (*LysM*$^{Cre/+}$) were crossed with homozygous floxed Id3 mice (*Id3*$^{fl/fl}$) (Figure 17A). The resultant *Id3*$^{fl/fl}$*LysM*$^{Cre/+}$ mice were null for *Id3* specifically in myeloid cells, while myeloid cells from *Id3*$^{fl/fl}$*LysM*$^{+/+}$ littermates maintained Id3 expression. Deletion was confirmed in peritoneal macrophages (Figure 17B,C). Myeloid-specific loss of Id3 did not

Figure 17. *Id3*$^{fl/fl}$*LysM*$^{Cre/+}$ mice do not have impaired adipose tissue expansion or MCP-1 expression

(A) Generation of *Id3*$^{fl/fl}$*LysM*$^{Cre/+}$ mice. (B) Id3 protein levels in total splenocytes (splen.) and column-purified peritoneal macrophages (macs), normalized to housekeeping protein β-Tubulin. (C) Id3 mRNA levels in column-purified peritoneal macrophages. n=5. (D) Epididymal VAT was harvested from 8-10 week old Id3$^{fl/fl}$ LysM$^{+/+}$ and Id3$^{fl/fl}$ LysM$^{Cre/+}$ mice fed 4 weeks of either chow or HFD, and cultured for 24 hours. Culture supernatant was analyzed for MCP-1 levels via ELISA, and was normalized per mouse (paired eVAT depots). n=7-10. Shown are mean values ± SEM, ns = $p>0.05$

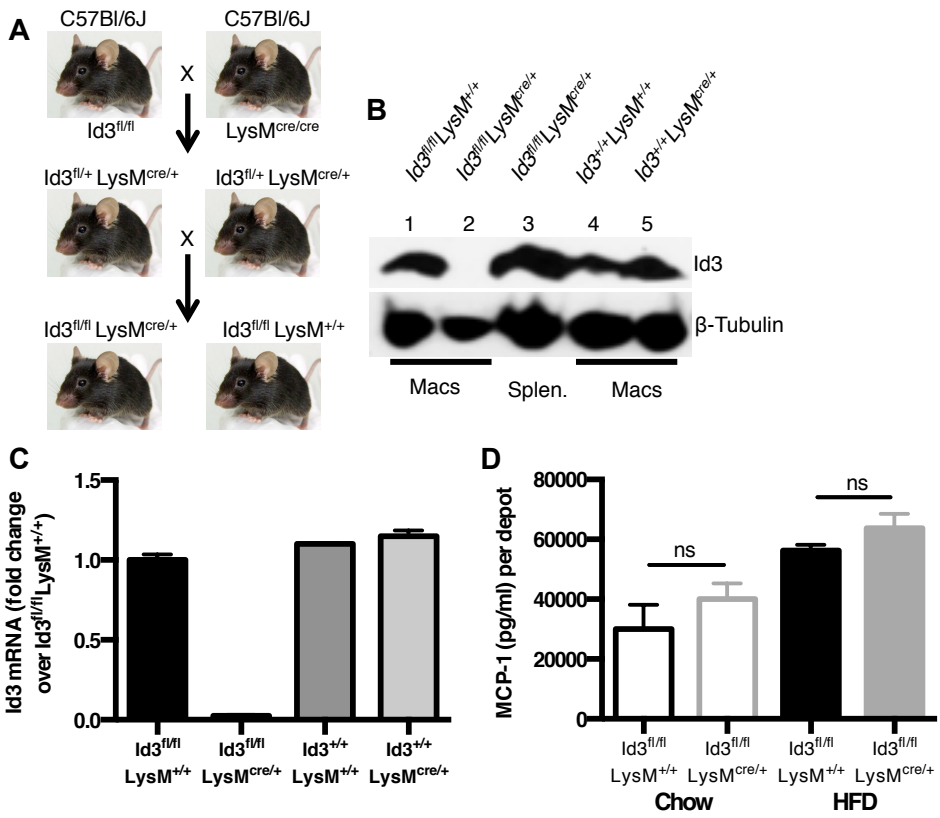

Figure 17: *Id3$^{fl/fl}$LysM$^{Cre/+}$* mice do not have impaired adipose tissue expansion or MCP-1 expression

affect HFD-induced MCP-1 secretion from VAT (Figure 17D), providing evidence that Id3 is not regulating obesity-induced MCP-1 expression through a myeloid cell.

$Id3^{-/-}$ mice have reduced inflammatory macrophage content in VAT

MCP-1 has been shown to promote macrophage infiltration into adipose tissue, and $Id3^{-/-}$ mice have a significant reduction in MCP-1. To determine whether loss of Id3 attenuates HFD-induced macrophage accumulation, flow cytometry was performed on VAT of $Id3^{+/+}$ and $Id3^{-/-}$ mice after 4 weeks of HFD. Results demonstrated that $Id3^{+/+}$ mice had a 2-3 fold obesity-induced increase in both $F4/80^{+}CD11c^{+}$ M1 and $F4/80^{+}CD11c^{-}$ M2 macrophage subsets (Figure 18A,B). In contrast, $Id3^{-/-}$ mice lacked an obesity-induced increase in M1 macrophages, resulting in a significantly reduced M1/M2 ratio compared to $Id3^{+/+}$ mice (Figure 18C). There were no differences in either macrophage subset in chow-fed animals. To determine if the reduced M1/M2 ratio in HFD-fed $Id3^{-/-}$ mice was accompanied by improved metabolic function, a glucose tolerance test (GTT) was performed in $Id3^{+/+}$ and $Id3^{-/-}$ mice after 2 weeks and 6 weeks of HFD. $Id3^{-/-}$ mice had improved glucose tolerance based on glucose readouts compared to $Id3^{+/+}$ mice, at both time points (Figure 18D-E, G-H). To determine if $Id3^{-/-}$ mice also had improved insulin signaling, western blotting for both total and phosphorylated AKT was performed after insulin injection. $Id3^{-/-}$ mice had higher relative levels of AKT phosphorylation (Figure 18F), suggesting an improvement in insulin signaling, as compared to the $Id3^{+/+}$ mice.

Figure 18. Loss of Id3 reduces HFD-induced VAT M1 macrophage accumulation

(A-C) SVF was isolated from epididymal VAT of 8-10 week old *Id3*$^{+/+}$ and *Id3*$^{-/-}$ mice fed 4 weeks of either chow or HFD. n=4-5. SVF cells were stained for flow cytometry quantitation of F4/80^{+}CD11c^{+} M1 macrophages (A), F4/80^{+}CD11c^{-} M2 macrophages (B) per gram of fat, and M1/M2 macrophage ratio (C). (D-F) 6 week-old *Id3*$^{+/+}$ and *Id3*$^{-/-}$ mice were fed 2 weeks of HFD. (D,E) Glucose tolerance test (GTT) was performed. n=6-8. (D) Blood glucose measurements, with asterisks denoting comparison at individual time points. (E) Area under the curve measurements. (F) Western blotting for insulin-stimulated pAKT in omental adipose tissue, normalized to total AKT levels. n=6-8. (G-H) 6 week-old *Id3*$^{+/+}$ and *Id3*$^{-/-}$ mice were fed 6 weeks of HFD. Glucose tolerance test (GTT) was performed. n=6-8. (G) Blood glucose measurements, with asterisks denoting comparison at individual time points. (H) Area under the curve measurements. Shown are mean values ± SEM, * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$, ns = $p>0.05$.

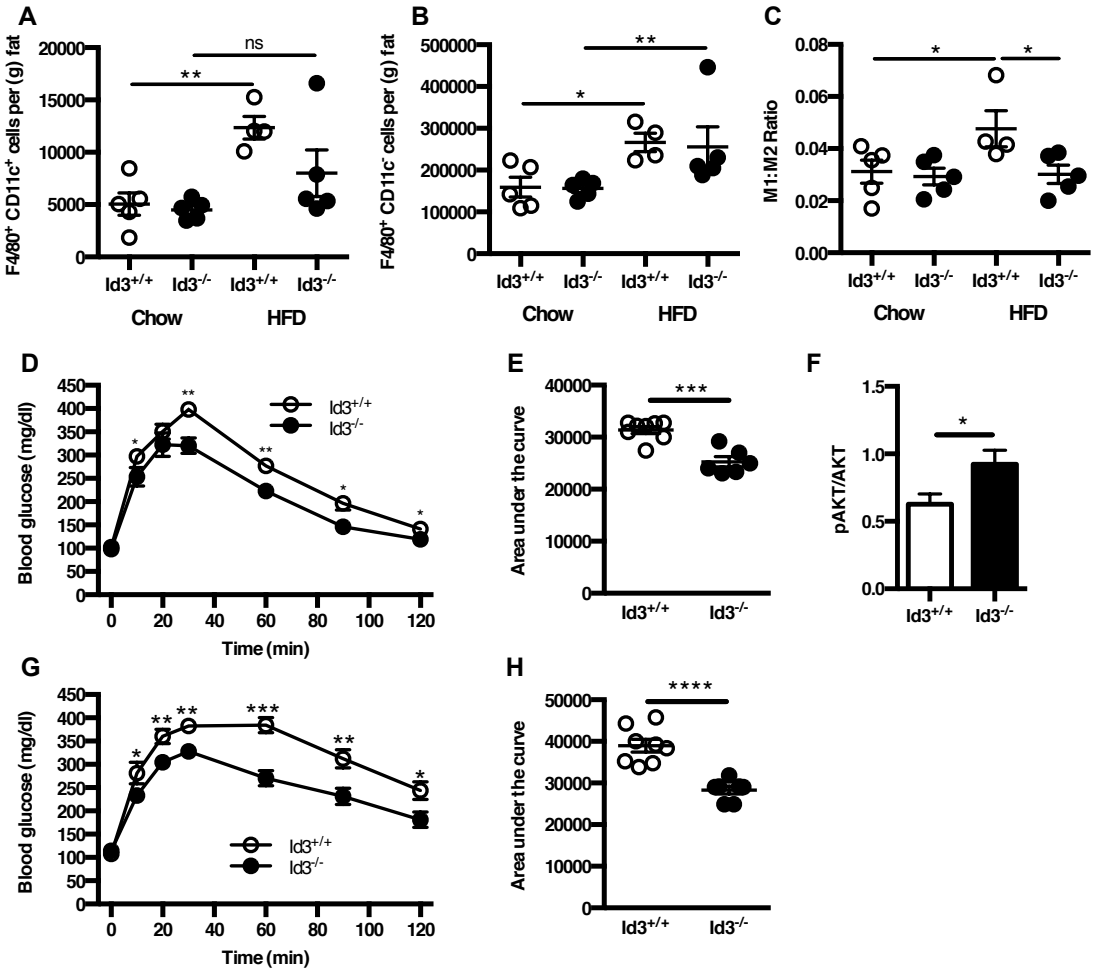

Figure 18: Loss of Id3 reduces HFD-induced VAT M1 macrophage accumulation

Adoptive transfer of *Id3*$^{+/+}$ AdPCs to *Id3*$^{-/-}$ mice enhances MCP-1 expression and M1 macrophage accumulation in VAT

To determine if rescue of the deficiency in AdPC number in *Id3*$^{-/-}$ mice increased VAT MCP-1 and M1 macrophage numbers, *Id3*$^{-/-}$ recipient mice were i.p. injected with 50,000 sorted AdPCs from *Id3*$^{+/+}$ mice or *Id3*$^{-/-}$ mice fed 2 weeks of HFD, or vehicle control, and were fed HFD for 8 weeks (Figure 19A). A pilot experiment was performed to track injected cells (Figure 20A), and confirmed that injected cells did traffic to the VAT within 1 week of injection. To determine if injection of *Id3*$^{+/+}$ AdPCs to *Id3*$^{-/-}$ mice would lead to metabolic dysfunction, GTTs were performed after 2 and 6 weeks of HFD. Indeed, *Id3*$^{-/-}$ mice receiving *Id3*$^{+/+}$ AdPCs had reduced glucose sensitivity, seen by increased glucose after glucose injection and a failure to return to baseline levels, compared to mice receiving *Id3*$^{-/-}$ AdPCs and vehicle control (Figure 19B,C and Figure 20B,C).

Interestingly, injection of *Id3*$^{+/+}$ AdPCs led to enhanced weight gain (Figure 13D) and VAT expansion (Figure 19E) in the *Id3*$^{-/-}$ mice, compared to vehicle control and injection if *Id3*$^{-/-}$ AdPCs. Injection of *Id3*$^{+/+}$ AdPCs to *Id3*$^{-/-}$ mice increased levels of MCP-1 in supernatant from VAT culture compared to mice receiving *Id3*$^{-/-}$ AdPCs and vehicle control (Figure 19F). Moreover, injection of *Id3*$^{+/+}$ AdPCs resulted in increased F4/80^{+}CD11c^{+}CD206^{-} M1 macrophages (Figure 19G), while numbers of F4/80^{+}CD11c^{-}CD206^{+} M2 macrophages were unchanged (Figure 19H), significantly enhancing the M1/M2 ratio (Figure 19I). These data provide evidence that MCP-1-producing AdPCs are key mediators of obesity-

Figure 19. Adoptive transfer of *Id3*$^{+/+}$ AdPCs restores HFD-induced MCP-1 expression and M1 macrophage accumulation in *Id3*$^{-/-}$ mice

(A) Setup of i.p. injection of vehicle or 50,000 sort-purified AdPCs from 2 week HFD-fed *Id3*$^{+/+}$ mice or *Id3*$^{-/-}$ mice into *Id3*$^{-/-}$ recipient mice. n=7. After 72 hours, recipient mice were fed HFD. GTT was performed after 6 weeks of HFD, and mice were sacrificed after 8 weeks of HFD. (B-C) GTT performed after 6 weeks of HFD. (B) Blood glucose measurements, with asterisks denoting comparison at individual time points. + signifies comparison of vehicle to *Id3*$^{+/+}$ cells and * signifies comparison of *Id3*$^{+/+}$ cells to *Id3*$^{-/-}$ cells (C) Area under the curve measurements. (D) Weight gain over 8 weeks of HFD, with asterisks denoting comparison of vehicle to *Id3*$^{+/+}$ at individual time points. (E) Epididymal weights at sacrifice. (F) MCP-1 levels as measured by ELISA in the supernatant of epididymal VAT, cultured for 24 hours. MCP-1 secretion was normalized per paired depots. (G-I) Flow quantitation of F4/80^{+}CD11c^{+}CD206^{-} M1 macrophages (G) and F4/80^{+}CD11c^{-}CD206^{+} M2 macrophages (H) per gram of fat and ratio of M1/M2 macrophages (I). Shown are mean values ± SEM, * or + $p<0.05$, ** $p<0.01$, **** $p<0.0001$, ns = $p>0.05$.

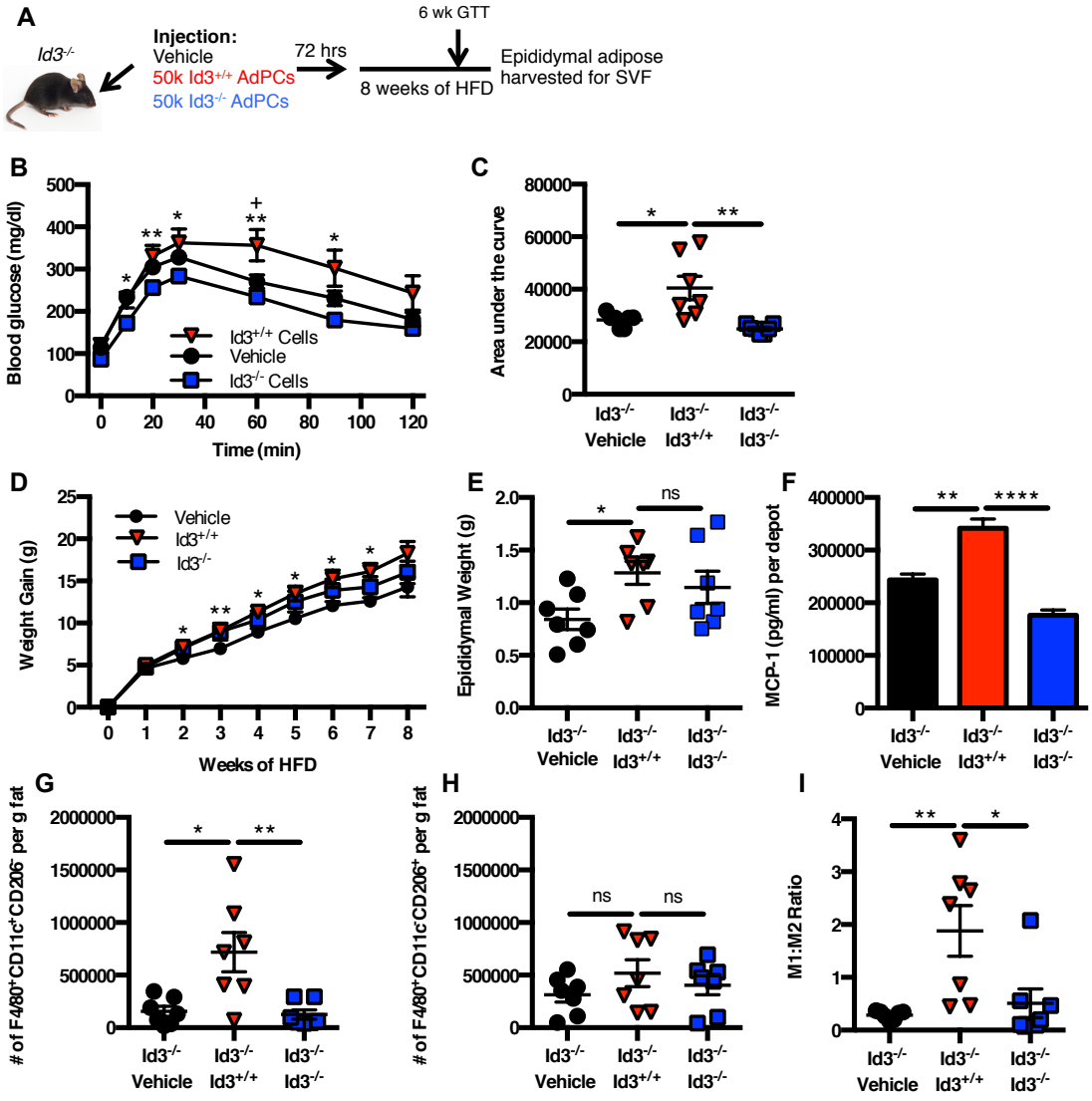

Figure 19: Adoptive transfer of *Id3*$^{+/+}$ AdPCs restores HFD-induced MCP-1 expression and M1 macrophage accumulation in *Id3*$^{-/-}$ mice

Figure 20. Intraperitoneally injected AdPCs traffic to VAT Vehicle (PBS) or 50,000 *Id3*$^{+/+}$ sort-purified AdPCs were labeled with PKH26 and i.p. injected into *Id3*$^{+/+}$ recipient mice. (A) 24-hrs, 72-hrs, and 1 week after injection, peritoneal fluid and epididymal VAT were harvested from recipient mice. Peritoneal cells and SVF cells were analyzed via flow cytometry for quantitation of PKH26^{+} cells. (B,C) Recipient mice were fed HFD for 2 weeks, and GTT was performed. (B) Blood glucose measurements, with asterisks denoting comparison at individual time points. (C) Area under the curve measurements. Shown are mean values ± SEM, * $p<0.05$, ** $p<0.01$.

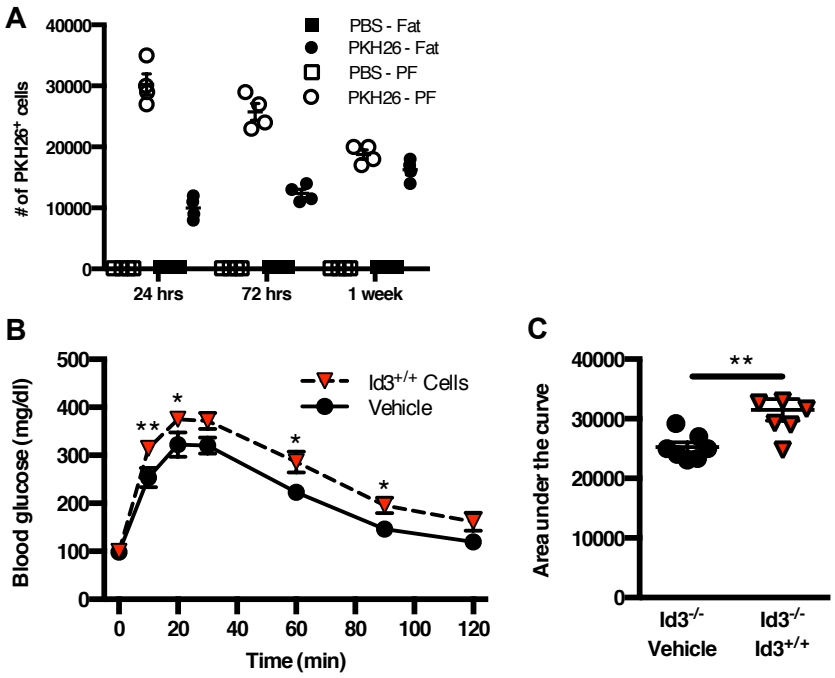

Figure 20: Intraperitoneally injected AdPCs traffic to VAT

induced M1 macrophage accumulation in VAT, and that expression of Id3 in the AdPCs is crucial for these effects.

To determine if adoptive transfer of AdPCs, either with or without expression of Id3, had additional effects on the immune population of VAT, total B lymphocytes, total T lymphocytes, and total dendritic cells (DCs) were quantified in *Id3*$^{-/-}$ recipient mice. The number of T cells and DCs were unchanged due to adoptive transfer of either *Id3*$^{+/+}$ or *Id3*$^{-/-}$ AdPCs (Table 1). Interestingly, injection of *Id3*$^{+/+}$ AdPCs resulted in a non-statistically significant increase in the number of B lymphocytes in the VAT of *Id3*$^{-/-}$ mice. Injection of *Id3*$^{-/-}$ AdPCs had the opposite effect, with a non-statistically significant decrease in the number of B lymphocytes. This resulted in a significant difference in B cells when comparing the two groups receiving AdPCs, suggesting that AdPCs may play a role in the accumulation of B cells in VAT during obesity, and that expression of Id3 may act to promote this accumulation.

As we demonstrated that addition of *Id3*$^{+/+}$ AdPCs to *Id3*$^{-/-}$ mice increased MCP-1 levels, M1 macrophage accumulation in visceral fat, enhanced diet-induced obesity, and worsened glucose tolerance, we wanted to determine if addition of *Id3*$^{+/+}$ AdPCs to Id3-sufficient mice would result in the same endpoints. With injection of 50,000 *Id3*$^{+/+}$ AdPCs, there was no significant enhancement of glucose intolerance or diet-induced weight gain (Figure 21). There also was no significant enhancement of MCP-1 production or M1 macrophage accumulation

				P values		
Injection	Vehicle	*Id3*$^{+/+}$	*Id3*$^{-/-}$	Vehicle vs Id3$^{+/+}$	Vehicle vs Id3$^{-/-}$	*Id3*$^{+/+}$ vs *Id3*$^{-/-}$
B cells per g	208615 ± 33520	293770 ± 44554	111070 ± 36635	0.1590	0.0678	**0.008**
T cells per g	222811 ± 60173	446014 ± 173587	131324 ± 24533	0.2576	0.2166	0.145
DCs per g	424511 ± 142030	377459 ± 94121	278314 ± 40042	0.7773	0.3386	0.452

Table 1: Immune Cell Numbers in VAT after Adoptive Transfer to *Id3*$^{-/-}$ mice

Figure 21. Adoptive transfer of *Id3*$^{+/+}$ AdPCs to *Id3*$^{+/+}$ recipients does not affect glucose tolerance or HFD-induced weight gain

Vehicle or 50,000 sort-purified AdPCs from 2 week HFD-fed *Id3*$^{+/+}$ mice were i.p. injected into *Id3*$^{+/+}$ recipient mice. After 72 hours, recipient mice were fed HFD. GTT was performed after 2 and 6 weeks of HFD, and mice were sacrificed after 8 weeks of HFD. (A-B) GTT performed after 2 weeks of HFD. n=4-5. (A) Blood glucose measurements, with asterisks denoting comparison at individual time points. (B) Area under the curve measurements. (C-D) GTT performed after 6 weeks of HFD. n=5-8. (C) Blood glucose measurements, with asterisks denoting comparison at individual time points. (D) Area under the curve measurements. (E) Weight gain over 8 weeks of HFD. (F) Epididymal weights at sacrifice. Shown are mean values ± SEM, ns = $p>0.05$.

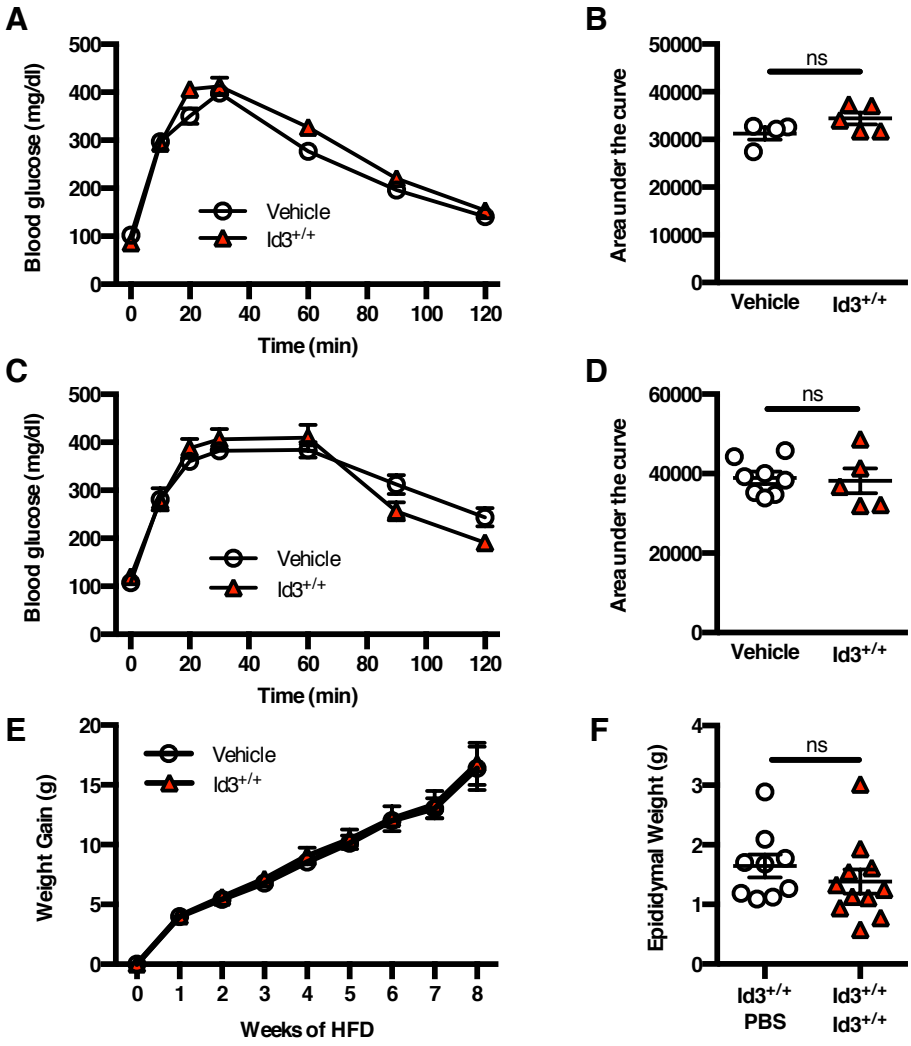

Figure 21: Adoptive transfer of *Id3*$^{+/+}$ AdPCs to *Id3*$^{+/+}$ recipients does not affect glucose tolerance or HFD-induced weight gain

in visceral adipose tissue (Figure 22). However, injection of *Id3*$^{+/+}$ AdPCs did result in an increased accumulation of B cells in the *Id3*$^{+/+}$ mice, with no change in T cells or dendritic cells (Table 2).

Finally, to determine if the number of AdPCs was still elevated after 8 weeks of HFD, both CD24^{-} and CD24^{+} AdPCs were quantified at the end of the study. Interestingly, there was no difference in the number of total AdPCs, CD24^{+} AdPCs, or CD24^{-} AdPCs, when comparing *Id3*$^{-/-}$ mice that received vehicle injection to those that received injection of *Id3*$^{+/+}$ or *Id3*$^{-/-}$ AdPCs (Figure 23A-C). In addition, there was no difference in the number of total AdPCs, CD24^{+} AdPCs, or CD24^{-} AdPCs when comparing *Id3*$^{+/+}$ mice that received vehicle injection to those that received injection of *Id3*$^{+/+}$ AdPCs (Figure 23D-F).

Figure 22. Adoptive transfer of *Id3*$^{+/+}$ AdPCs to *Id3*$^{+/+}$ recipients does not affect MCP-1 levels or adipose tissue macrophages

Vehicle or 50,000 sort-purified AdPCs from 2 week HFD-fed *Id3*$^{+/+}$ mice were i.p. injected into *Id3*$^{+/+}$ recipient mice. After 72 hours, recipient mice were fed HFD. GTT was performed after 2 and 6 weeks of HFD, and mice were sacrificed after 8 weeks of HFD. (A) MCP-1 levels as measured by ELISA in the supernatant of epididymal VAT, cultured for 24 hours. MCP-1 secretion was normalized per paired depots. (B-D) Flow quantitation of F4/80^{+}CD11c^{+}CD206^{-} M1 macrophages (B) and F4/80^{+}CD11c^{-}CD206^{+} M2 macrophages (C) per gram of fat and ratio of M1/M2 macrophages (D). Shown are mean values ± SEM, ns = $p>0.05$.

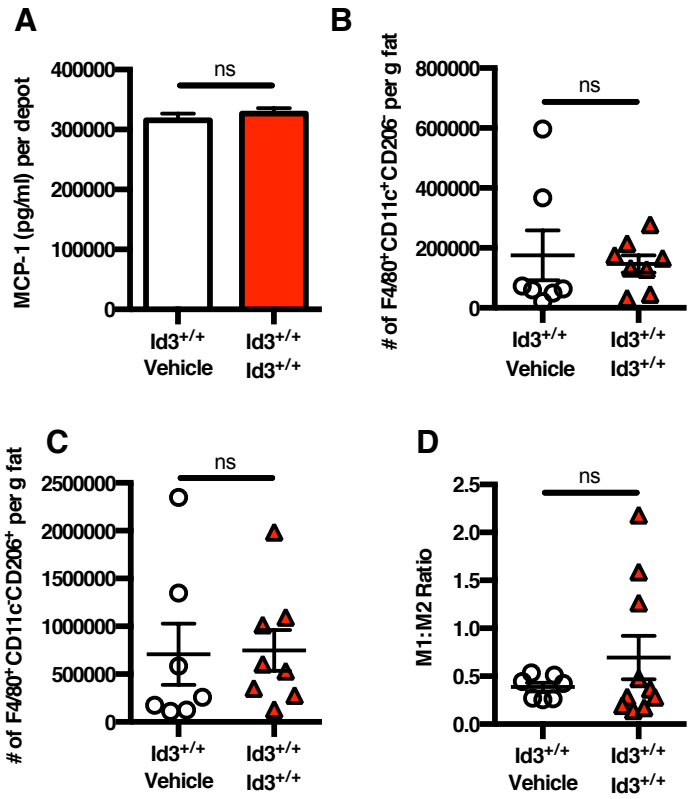

Figure 22: Adoptive transfer of *Id3*$^{+/+}$ AdPCs to *Id3*$^{+/+}$ recipients does not affect MCP-1 levels or adipose tissue macrophages

Injection	Vehicle	*Id3*$^{+/+}$	P value
B cells per g	56640 ± 12745	206951 ± 49468	**0.037**
T cells per g	100408 ± 38952	188210 ± 41773	0.163
DCs per g	421741 ± 167056	391148 ± 81967	0.861

Table 2: Immune Cell Numbers in VAT after Adoptive Transfer to *Id3*$^{+/+}$ mice

Figure 23. Adoptive transfer of AdPCs does not affect number of AdPCs after 8 weeks of HFD

Vehicle or 50,000 sort-purified AdPCs from 2 week HFD-fed *Id3*$^{+/+}$ and *Id3*$^{-/-}$ mice were i.p. injected into *Id3*$^{-/-}$ and *Id3*$^{+/+}$ recipient mice. After 72 hours, recipient mice were fed HFD, and mice were sacrificed after 8 weeks of HFD. SVF was isolated from epididymal VAT. Quantitation of (A, D) CD24^{-} AdPCs, (B, E) CD24^{+} AdPCs, and (C, F) total AdPCs per mouse (paired eVAT depots). Shown are mean values ± SEM, ns = $p>0.05$.

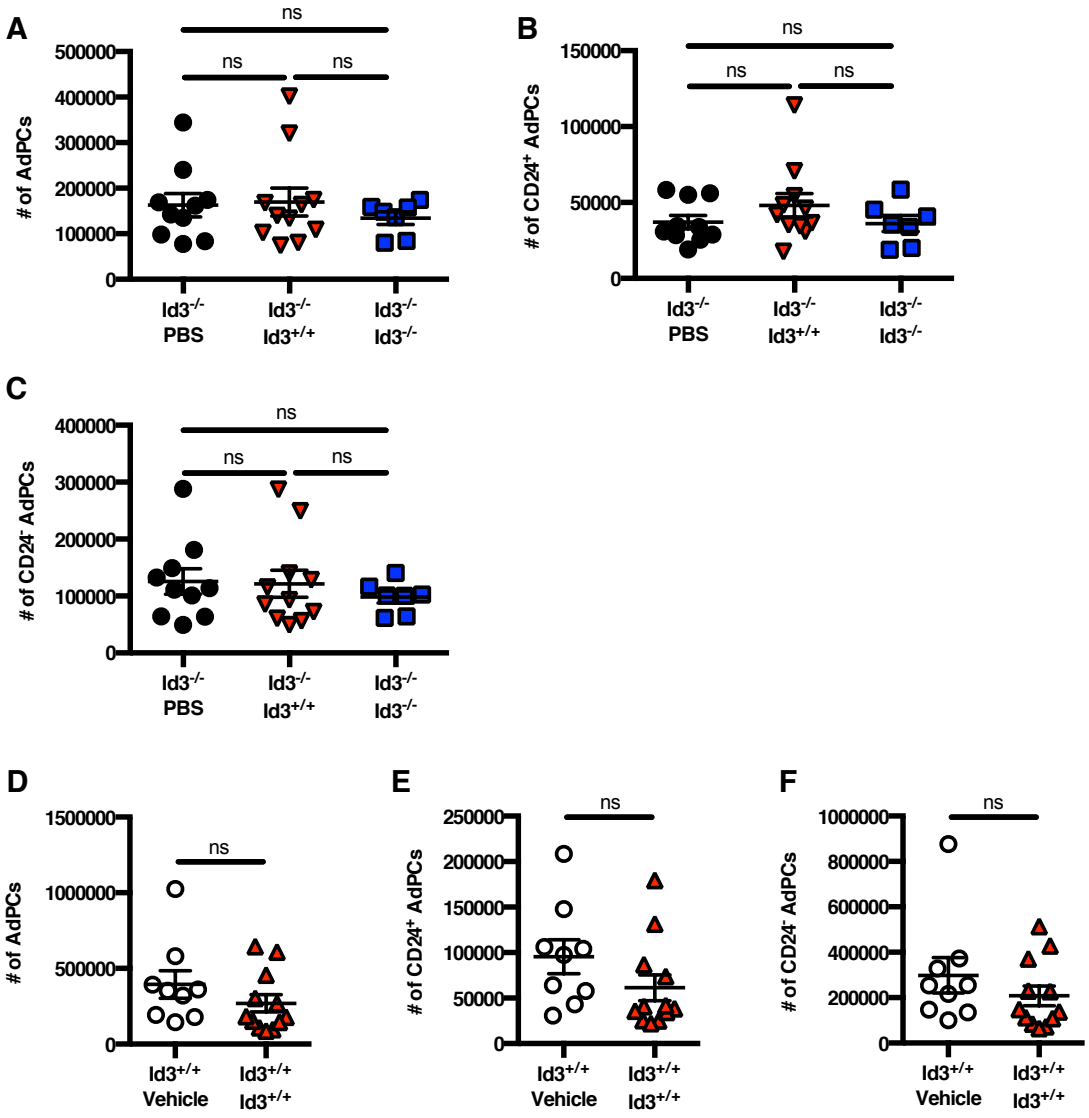

Figure 23: Adoptive transfer of AdPCs does not affect number of AdPCs after 8 weeks of HFD

Discussion:

We identify Id3 as a key mediator of HFD-induced AdPC proliferation, MCP-1 production and inflammatory macrophage accumulation in VAT.

The molecular pathways regulating normal adipose development during embryogenesis have been well-described[220, 221]. Yet, the molecular mechanisms mediating early AdPC expansion in response to HFD are incompletely understood. Recent evidence suggests that the serine threonine protein kinase AKT2 promotes early HFD-induced AdPC expansion in VAT, despite the fact that it is not essential for normal adipose development[210]. Similarly, necdin, a pleiotropic protein that possesses pro-survival and anti-mitotic properties has been demonstrated to inhibit proliferation of adipocyte progenitors, but only in response to HFD[222]. Our murine studies provide evidence that the helix-loop-helix transcription regulator Id3 promotes HFD-induced AdPC accumulation in VAT. In similar fashion to AKT2 and necdin, Id3 does not affect normal adipose tissue growth and development, as adipose tissue size and body weight of *Id3*$^{-/-}$ mice match littermate *Id3*$^{+/+}$ controls at baseline[182]. Id3, normally expressed during development and in lymphocytes, can be re-expressed during disease[167] as seen previously in SVF from HFD-fed mice[182], and in results presented in this study. A role for Id3 in preadipocyte commitment and differentiation[217] has been suggested, however, results from the present study demonstrate that loss of Id3 has no effect on AdPC differentiation to adipocytes. Instead, results provide evidence that Id3 promotes HFD-induced AdPC proliferation *in vivo*.

Furthermore, results suggest that it is this Id3-dependent AdPC expansion that is responsible for HFD-induced MCP-1 production, macrophage accumulation, and metabolic dysfunction (Figure 24).

Interestingly, adoptive transfer of *Id3*$^{+/+}$ AdPCs to *Id3*$^{+/+}$ mice did not affect MCP-1 levels, the number of M1 macrophages, or glucose tolerance. This suggests that addition of AdPCs only results in these phenotypic changes in a mouse that has an endogenous reduced population. It is possible that increased MCP-1 and resultant accumulation of M1 macrophages are dependent on proliferation of AdPCs, which is likely tightly regulated. In the setting of adoptive transfer to the *Id3*$^{+/+}$ host, the normal proliferative response to HFD might be diminished, potentially through an attenuated reduction in p21^{Cip1} levels, leaving the final number of AdPCs unchanged.

We see after 8 weeks of HFD that there are no differences in the number of total AdPCs, CD24+ AdPCs, or CD24- AdPCs. Interestingly, the number of AdPCs present at 8 weeks of HFD is similar to the number present at 1 week of HFD. This indicates that there may be a peak expansion of these cells at an intermediate time point, followed by a contraction. It is also possible that the peak expansion is at 1 week post initiation of HFD, and that these numbers are sustained throughout obesity.

The number of B lymphocytes was increased in both sets of recipient mice, due

Figure 24. Model of the role of Id3 in AdPC-mediated M1 macrophage accumulation and glucose intolerance

As seen in global *Id3*$^{-/-}$ mice, loss of Id3 results in attenuated HFD-induced weight gain and MCP-1-mediated adipose tissue inflammation. Adoptive transfer of *Id3*$^{+/+}$ AdPCs to *Id3*$^{-/-}$ recipient mice restores the HFD-induced phenotype seen in *Id3*$^{+/+}$ mice.

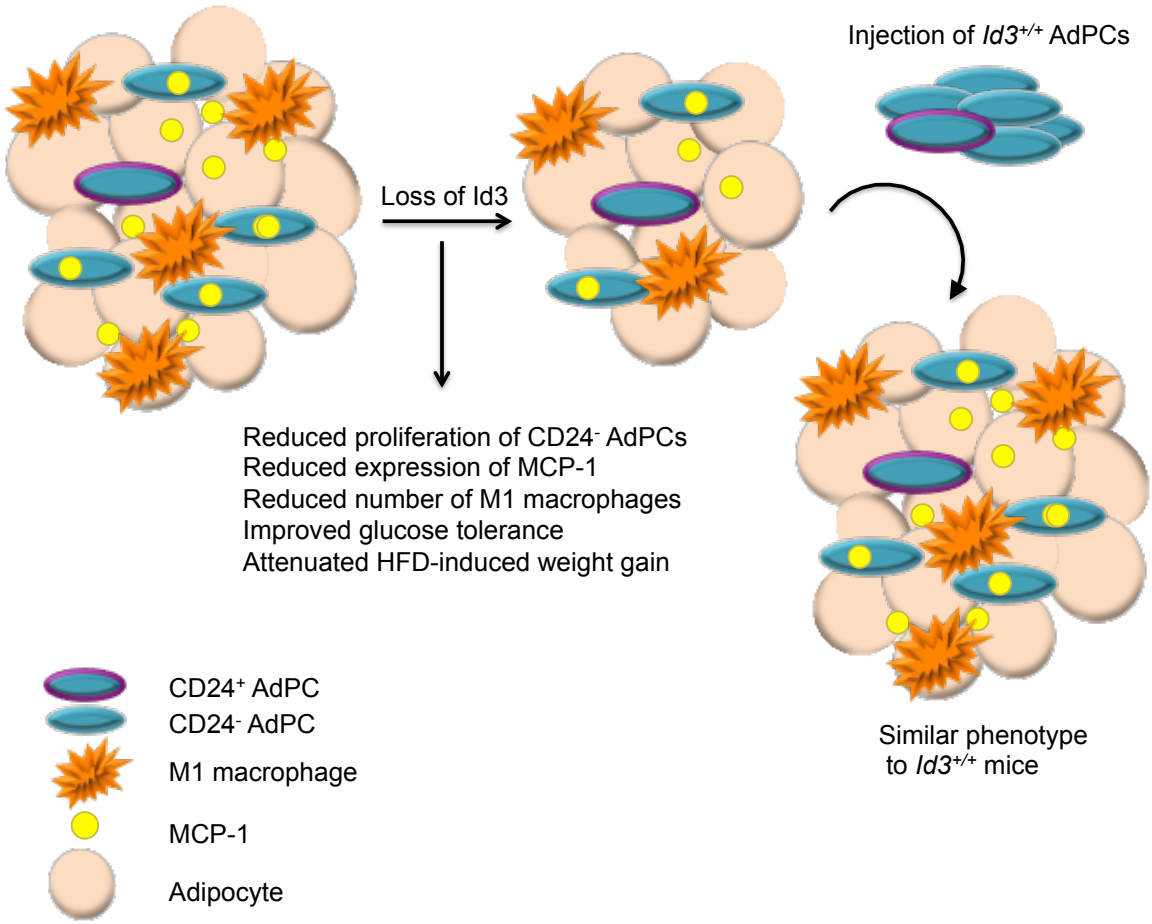

Figure 24: Model of the role of Id3 in AdPC-mediated M1 macrophage accumulation and glucose intolerance

to adoptive transfer of *Id3*$^{+/+}$ AdPCs, while they appear to be decreased due to injection of *Id3*$^{-/-}$ AdPCs in the *Id3*$^{-/-}$ recipient mice. It appears that these cells are supportive of this specific leukocyte population, and not T lymphocytes or dendritic cells, although it is not clear what subset of B cells is affected. The increased number of B cells could be due to increased survival, proliferation, or recruitment. AdPCs may be producing a chemoattractant for B cells, or a B cell mitogen to stimulate proliferation. This factor could be IL-6 or MCP-1, or could be an additional cytokine produced by AdPCs. It is also possible that AdPCs are stimulating another cell type to secrete a B cell mitogen. Another possibility is that a subset of AdPCs possesses the ability to trans-differentiate into lymphocytes. Further studies will determine the subset of B cells that are affected by AdPC adoptive transfer and the mechanism of their increased numbers. This data provides a unique connection between adipose tissue and the immune system.

We identify the cell cycle regulator p21^{Cip1} as a key Id3 target, possibly in HFD-induced AdPC proliferation. Loss of p21^{Cip1} has previously been demonstrated to attenuate HFD-induced adipocyte hyperplasia[223]. Consistent with these findings, 1 week of HFD significantly reduced p21^{Cip1} mRNA in AdPCs and mice null for Id3 have significantly more p21^{Cip1} mRNA in AdPCs in response to HFD, implicating Id3 and p21^{Cip1} as potential regulators of HFD-induced AdPC expansion.

While Id3 regulation of $p21^{Cip1}$ in $CD24^-$ AdPCs may be one mechanism whereby Id3 regulates AdPC expansion, future studies involving loss and gain of $p21^{Cip1}$ function are needed to confirm its essential role in HFD-induced AdPC expansion. Id3 may regulate AdPC numbers through other mechanisms besides proliferation. Furthermore, reduced MCP-1 production due to reduced numbers of AdPCs may not be the only mechanism whereby loss of Id3 attenuates macrophage numbers in adipose tissue and improves metabolic function. Modulation of VAT MCP-1 levels and M1 macrophages, and systemic glucose tolerance due to global loss of Id3, as well as after adoptive transfer of $Id3^{+/+}$ AdPCs into the Id3-deficient host were surprisingly dramatic. This was especially surprising due to the relative modest changes in adipose tissue macrophage recruitment and metabolic function in mouse models with deletion of MCP-1[111, 113, 119]. Id3 regulates many pathways, such as those involved in growth, differentiation, apoptosis, and chemokine and cytokine production. Further studies to identify additional mechanisms whereby Id3 in AdPCs regulates macrophage accumulation and glucose intolerance are ongoing.

Id3 has previously been implicated in the role of adipose tissue expansion and HFD-induced weight gain[182]. One potential caveat to this study could be that effects seen here are simply secondary to the reduction in diet-induced obesity. However, $Id3^{-/-}$ mice do not have significant attenuation in HFD-induced total weight gain until 16 weeks of HFD[182]. Surprisingly, we did see reduced VAT mass in $Id3^{-/-}$ mice after 1 and 4 weeks of HFD. It is not yet clear if differences in

MCP-1 are secondary to altered VAT expansion, or if increased MCP-1 in the *Id3*$^{+/+}$ mice is promoting weight gain. Additionally, while an expansion in fat mass of *Id3*$^{+/+}$ mice correlated with an increase in MCP-1^{hi} cells and AdPCs, there was no such correlation in *Id3*$^{-/-}$ mice, despite fat expansion (Figure 15G and data not shown). Importantly, as seen in the adoptive transfer experiment in Figure 19, *Id3*$^{-/-}$ mice receiving *Id3*$^{+/+}$ AdPCs had similar weights and adipose tissue expansion to those receiving *Id3*$^{-/-}$ AdPCs, but had altered M1/M2 ratio and worsened glucose metabolism. This indicates that the roles of Id3 and AdPCs extend beyond expansion of fat mass.

We have previously published that Id3 is an important regulator of HFD-induced visceral adipose expansion and microvascular blood volume[182]. While Id3 regulation of VEGF-A expression, a known angiogenic factor, was proposed as a potential mechanism mediating this effect, results of the present study raise the interesting possibility that loss of Id3 may also limit VAT microvascular blood volume and protect from HFD-induced VAT expansion by limiting AdPC proliferation and MCP-1 production.

In the context of this study, we focused on the role of AdPC expansion and production of MCP-1, but it is clear that AdPCs are playing roles in addition to MCP-1-induced M1 macrophage accumulation. MCP-1 is an important regulator of M1 macrophage accumulation in visceral adipose tissue, but it is of course not the only chemokine or cytokine involved in adipose tissue inflammation, and it is

also not the only means to expansion of adipose tissue macrophages. Future studies identifying the initial source of other chemokines, and the role of AdPCs in producing these factors, would be of interest. The AdPCs could also be altering other populations within the adipose tissue, indirectly promoting M1 macrophage accumulation as well as glucose intolerance. Future study of this unique population of cells is required to fully understand their role in diet-induced obesity.

Chapter 5

General Discussion and Future Directions

AdPCs recruit M1 macrophages through production of MCP-1

In our adoptive transfer experiments, we demonstrated that addition of *Id3*$^{+/+}$ AdPCs to *Id3*$^{-/-}$ mice enhanced the accumulation of M1 macrophages within the visceral adipose tissue. We postulated that the increased MCP-1 secreted from these cells resulted in additional chemotaxis of M1 macrophages, but did not directly prove that the additional M1 macrophages infiltrated into the fat. It is possible that the injected *Id3*$^{+/+}$ AdPCs actually differentiated into the M1 macrophages we detected in the fat, and were responsible for the increases we observed.

Others have demonstrated that preadipocytes can take on macrophage-like qualities[53, 224, 225]. In vitro studies using preadipocyte cell lines have demonstrated that during inflammatory conditions, preadipocytes can be stimulated to perform phagocytic and antimicrobial activities similar to those of specialized phagocytic cells such as macrophages[53]. Interestingly, it was demonstrated that once preadipocytes stop proliferating and begin adipogenesis, they lose all phagocytic activity[53]. In addition, Ap2 and PPARγ, proteins that have long been used to identify the adipocyte lineage, are also detected in macrophages[226]. One group used profiling analysis to define the common features shared by preadipocyte, adipocyte and macrophage populations, and determined that the preadipocyte profile was much closer to the macrophage than to the adipocyte profile[224]. There may be more similarities between these preadipocytes and macrophages than originally thought.

One group has demonstrated that preadipocytes can take on macrophage-like qualities, specifically after being injected into the peritoneal cavity, suggesting that this environment may have a role in the altered phenotype[224]. In these studies, the preadipocytes rapidly acquired high phagocytic activity and index, and expressed macrophage-specific antigens of F4/80, Mac-1, CD80, CD86, and CD45. The group demonstrated that cell-to-cell contact between preadipocytes and the peritoneal macrophages partially induced this preadipocyte phenotypic conversion[224].

To further identify whether the increase in M1 macrophages is due to increased infiltration or due to trans-differentiation of the AdPCs, we could perform the following experiments. First, we could label the AdPCs with PKH26 prior to injection, and retrieve them from the peritoneal cavity and the adipose tissue after 1 week. At this point, we would perform flow cytometry for macrophage-specific surface markers, such as F4/80, Mac-1, and CD45, and determine whether the labeled cells had gained expression of these markers. One difficulty in trying to answer these questions is that the cells are sorted based on expression of several surface markers via FACS, and these antibodies remain on the cell surface for at least 1 week, post-sorting. There is not free space in the flow cytometry panel for the addition of macrophage-specific markers. To try to free up some space in our flow panel, we have performed some minimal marker cell sorts, where we tried to capture the same population of cells without using

the extensive AdPC flow panel. We found that cells negative for lineage markers and positive for CD34 mostly recapitulated our findings with the full panel of markers, based on secretion of MCP-1 and IL-6 (Figure 25). This supports findings from the Rodeheffer group, in that 95.68% of AdPCs can be identified by $Lin^{-}CD34^{+}$ cells[190]. In addition to assessing surface marker expression on the AdPCs post-injection, functional assays could be utilized to identify any macrophage-like qualities, such as phagocytosis and anti-microbial responses.

One finding that suggests these cells do not become macrophages *in vivo* is that we don't see an increase in M1 macrophages when *Id3*$^{+/+}$ AdPCs are injected into the *Id3*$^{+/+}$ mouse. This suggests that the MCP-1 produced by the AdPCs is driving recruitment of M1 macrophages, and that addition of *Id3*$^{+/+}$ AdPCs to the *Id3*$^{-/-}$ mice helps increase the local levels of MCP-1. *Id3*$^{+/+}$ mice already have high levels of MCP-1, so there is no further increase of M1 macrophages. In order to determine if MCP-1 being produced by the AdPCs is sufficient to increase the number of M1 macrophages, AdPCs isolated from *Id3*$^{+/+}$ *Mcp-1*$^{-/-}$ mice would be injected into the *Id3*$^{-/-}$ *Mcp-1*$^{+/+}$ recipient mice. We would hypothesize that without the ability to produce MCP-1, there would no longer be an increase in the number of M1 macrophages in the VAT. Of note, it is unclear if weight gain would still be increased due to adoptive transfer of the *Mcp-1*$^{-/-}$ AdPCs. It would be interesting to see if weight gain was affected independently of MCP-1 and macrophage accumulation.

Figure 25. MCP-1 secretion from AdPCs characterized by different flow cytometry panels

Epididymal VAT from 8-10 week old *C57BL/6J* mice was harvested and processed for SVF cells. MCP-1 levels as measured by ELISA in the supernatant of equivalent numbers of sort purified AdPCs, Lin^-CD34^+ cells, and $Lin^-Sca\text{-}1^+$ cells. 8 mice were pooled for n=1. Shown are mean values ± SEM, * $p < 0.05$

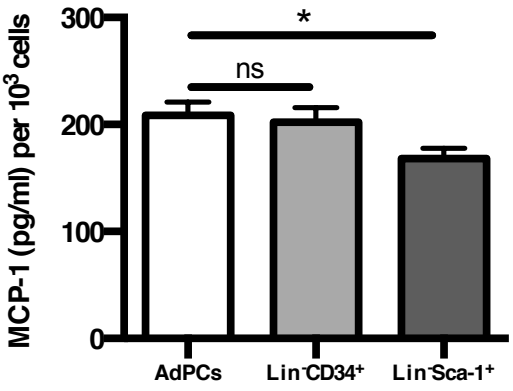

Figure 25: MCP-1 secretion from AdPCs characterized by different flow cytometry panels

We have not yet determined that the increase we see in M1 macrophages is due to recruitment. The Lumeng laboratory as well as the Randolph laboratory have utilized PKH26 as a method of distinguishing newly recruited macrophages from those that were already resident in the tissue[110, 227]. Using this technique, we could inject mice receiving *Id3*$^{+/+}$ AdPCs with PKH26, after 7 weeks of HFD. Macrophages already present in the adipose tissue at the time of injection would take up the dye, while newly recruited macrophages would be PKH26^{-}. We would then be able to identify how many macrophages infiltrated into the adipose tissue during the last week of HFD.

Finally, AdPCs could be promoting the accumulation of macrophages by secreting a factor that stimulates macrophage proliferation. Highly proliferative populations of macrophages have been identified within adipose tissue, but they are mostly within the M2 macrophage subset[228], where we do not see AdPC-dependent regulation. Additional studies have demonstrated that MCP-1 even plays a role in macrophage proliferation within the adipose tissue, in addition to the role it plays in recruiting monocytes from the circulation[229]. This proliferation was specific to macrophages within VAT, and was lost in *Mcp-1*$^{-/-}$ mice. In addition, proliferation was detected in both M1 and M2 macrophage populations. In our previous experiments utilizing BrdU uptake as a means to measure proliferation, we did not look at BrdU incorporation within the macrophage population. A future experiment would measure BrdU uptake within macrophage subsets in *Id3*$^{+/+}$ and *Id3*$^{-/-}$ mice after being fed a HFD. Additionally, BrdU uptake

could be measured post adoptive transfer, to determine if injection of *Id3*$^{+/+}$ AdPCs in the *Id3*$^{-/-}$ recipient mice affects proliferation of either macrophage subset.

We, as well as others, have demonstrated that the function and differentiation of AdPCs differ in the contexts of development and obesity[210, 222]. In our study, the donor mice were primed with 2 weeks of HFD. This was performed to both prepare the cells for the 8 week HFD the recipient mice would be placed on, as well as to increase the numbers of AdPCs sorted from the HFD-fed donor mice. It is possible that the short-term HFD programmed the AdPCs, and that the same cells isolated from chow-fed mice would have a different function. We demonstrated that HFD results in an increase in MCP-1 and IL-6 secretion from AdPCs. Perhaps if the cells were isolated from chow-fed mice, we may see an improvement in the metabolic phenotype of the mice. A future direction would be to inject both *Id3*$^{-/-}$ and *Id3*$^{+/+}$ with *Id3*$^{+/+}$ AdPCs isolated from both chow-fed and HFD-fed mice, and then to put the recipient mice on either a chow or a HFD for the course of the study, as demonstrated in Figure 26.

AdPCs from VAT undergo proliferation in response to HFD

Proliferative capacity of adipocyte progenitor cells in visceral adipose depots has been controversial. Many studies have determined that adipose-derived stem cells, SVF cells, and adipocyte progenitors derived from subcutaneous adipose tissue have increased ability to proliferate in vitro, as compared to cells derived

Figure 26. Proposed model of chow versus HFD adoptive transfer to determine if HFD priming of AdPCs and continued HFD are necessary for inflammatory effects

Setup of i.p. injection of vehicle or 50,000 sort-purified AdPCs from chow-fed or 2 week HFD-fed *Id3*$^{+/+}$ mice or *Id3*$^{-/-}$ mice into *Id3*$^{-/-}$ recipient mice. After 72 hours, mice will be placed on chow diet or HFD, and continued for 8 weeks.

Figure 26: Proposed model of chow versus HFD adoptive transfer to determine if HFD priming of AdPCs and continued HFD are necessary for inflammatory effects

from visceral adipose tissue[230-233]. However, our data, as well as data from others[210, 234], clearly demonstrates that adipocyte progenitor cells from visceral adipose tissue have a rapid proliferation-based expansion due to initiation of HFD. AKT2 signaling has been implicated in the expansion of adipocyte precursors, and this HFD-induced proliferation was limited to the visceral adipose tissue[210].

There are several scenarios that could explain this potentially confounding data. First, some of these studies were done in vitro[232], while our data, and data from the Rodeheffer lab[210] represent proliferation occurring in vivo, in response to HFD. Just as adipogenesis occurs differently in vitro from in vivo, it is possible that proliferation may also differ. Other studies have looked at later time points following initiation of HFD[233], and it is possible that they are missing the initial burst of proliferation. The enhanced proliferation in the visceral AdPCs may only be seen immediately following a HFD, and may be lost at later time points.

It is not completely clear whether an increase in AdPCs should result in enhanced or reduced weight gain with resultant metabolic dysfunction. The standard model of adipogenesis *in vitro* requires that there is a final stage of mitotic clonal expansion before preadipocytes commit to the adipocyte lineage and begin the differentiation process[136]; therefore, it is commonly thought that expansion of the precursor pool will lead to adipocyte hyperplasia. Along these same lines, previous studies have demonstrated that an increase in the

accumulation of adipocyte precursors corresponds to a reduction in diet-induced obesity[235, 236], suggesting that an increase in the progenitor pool will help create new adipocytes that remove the burden from dysfunctional hypertrophic adipocytes. However, there may be a disconnect between proliferation of AdPCs and actual adipogenesis. Our data demonstrated that an increase in AdPCs led to enhanced weight gain and metabolic dysfunction, but only in mice that had reduced AdPC numbers to start with.

It is entirely possible that the proliferation seen during early obesity might not lead to formation of new, functional adipocytes. It is likely that the adipogenic potential of the precursor cells is an important factor in their metabolic effects, and it is possible that this is linked to the expression of MCP-1. Our data suggests that increased numbers of AdPCs during obesity is detrimental to overall health, leading to expansion of WAT and decreased glucose tolerance. In support of these findings, one group has shown that ablation of the adipocyte precursor pool acts to halt growth of white adipose tissue and promote the formation of beige fat and increased energy expenditure[237], suggesting that increased numbers of AdPCs would act to support growth of WAT. This group suggests that targeting of white adipocyte progenitors could be developed as a strategy to sustained modulation of WAT metabolic activity[237].

We did not see any changes when *Id3*$^{+/+}$ AdPCs were injected into the *Id3*$^{+/+}$ recipient mouse. An increase in AdPCs in the *Id3*$^{+/+}$ mouse did not affect MCP-1

levels, M1 macrophages, or systemic glucose tolerance. It is possible that in the *Id3*$^{+/+}$ mouse, the proliferation and expansion of the AdPC pool is tightly regulated, and addition of AdPCs from donor mice would not significantly increase the final size of the pool. While there may be initial increases in the number of AdPCs due to injection into the *Id3*$^{+/+}$ mouse, it is possible that proliferation would be reduced or halted until the number of AdPCs reached equilibrium. The tight regulation of proliferation could be mediated by an increase in cell cycle inhibitor p21^{Cip1}, which we already demonstrated is regulated by Id3. Proliferation is tightly regulated in many cell types, and p21^{Cip1} has been previously demonstrated to mediate negative regulatory feedback loops to control proliferation[238]. This hypothesis could be tested using BrdU uptake assays following injection of AdPCs, to determine if proliferation was reduced due to an increase in the number of AdPCs present. In addition, p21^{Cip1} levels could be measured in AdPCs and VAT following adoptive transfer.

AdPCs may support adipose vascularization

One question derived from our findings is, why are AdPCs expressing MCP-1 in the context of HFD? We have suggested that MCP-1^{+} AdPCs may be helping to recruit macrophages that participate in angiogenesis. Tie-2^{+} macrophages are a unique subset with a noninflammatory profile that participate in angiogenesis[197, 239]. New blood vessels arise as a result of sprouting of the adjacent resident endothelial cells, called tip cells[240]. Tie-2^{+} macrophages promote vascular network expansion by supporting the sprouting growth of tip cells[196]. Adipose

tissue macrophages sit at the interface between blood vessels and adipocytes[52], putting them in the perfect position to both respond to cues from the circulation, and to play a role in angiogenesis. It has been hypothesized that highly vascularized adipose tissue is more metabolically healthy, giving newly formed adipocytes adequate access to nutrients[241].

While MCP-1 has been implicated in the recruitment of Tie-2^+ macrophages to areas that require angiogenesis, it is necessary for us to confirm that AdPC-derived MCP-1 is sufficient and required for this process in the early stages of HFD-feeding in vivo. An experiment that could be performed to address this hypothesis is to feed *C57BL/6J* mice 1 week of chow or HFD, and assess the number of F4/80^+Tie-1^+ macrophages in visceral adipose tissue. Next, *Mcp-1*$^{-/-}$ mice would receive an adoptive transfer of *Id3*$^{+/+}$ AdPCs, and the number of Tie-2^+ macrophages in the fat would be quantified. Finally, *Id3*$^{+/+}$ and *Id3*$^{-/-}$ recipient mice would receive an adoptive transfer of *Mcp-1*$^{-/-}$ AdPCs, with the same endpoints being measured. In addition, the vascular status of the fat, determined by VEGF-A levels and microvascular blood volume, would be determined before and after adoptive transfer.

It is possible that adipose tissue expansion is supported through AdPC-induced vascularization. It has been demonstrated by several groups that preadipocytes/adipocyte progenitor cells survive better and have increased adipogenesis when they are closer to capillaries and endothelial cells[146, 242].

AdPCs are found lining the outer part of developing lymph nodes (LN) and inside the LN anlage during embryogenesis[151]. In the developing fat, they are preferentially located around blood vessels. In particular, VAT is susceptible to inflammation as a result of insufficient oxygenation of hypertrophic adipocytes, resulting in lipotoxicity[243]. Subcutaneous WAT typically remains sufficiently vascularized, resulting in its improved glucose homeostasis and energy consumption, as compared to VAT[244]. Conversely, obesity and insulin resistance are both associated with reduced adipose tissue blood flow[245].

Inhibition or activation of angiogenic factors has been demonstrated to control adiposity, however, angiogenic activity in adipose tissue is context-dependent. While inhibition of VEGF-A at the initial stages of HFD resulted in enhanced weight gain and systemic insulin resistance[246], VEGF-A inhibition in mice that were already obese resulted in a reduction in body weight and fat mass, as well as an improvement in metabolic health[246]. Similarly, we hypothesize that MCP-1 production by AdPCs in the early stages of HFD and obesity will promote angiogenesis to support the growing adipose tissue, resulting in improved metabolic health. Without angiogenesis to support early adipose expansion, ectopic lipid accumulation in skeletal muscle and the liver results in systemic insulin resistance. Conversely, we believe that MCP-1 production by AdPCs long-term will result in M1 macrophage-mediated chronic inflammation. Determining the timeline of MCP-1 production and resultant angiogenesis versus macrophage infiltration would help identify the window when MCP-1 production

should be inhibited. Angiotensin II is a peptide hormone that causes vasoconstriction, and has VEGF-mediated angiogenic effects. There is evidence that Angiotensin II can enhance MCP-1 production by preadipocytes[247]. In addition, Angiotensin II-induced vascular remodeling is positively correlated with MCP-1 levels[248]. This suggests that during angiogenesis, AdPCs are stimulated to make more MCP-1 in order to facilitate continued vascular growth.

Most of this study was performed at very early time points during diet-induced obesity. We demonstrated that AdPCs are the first cells to express and secrete MCP-1 following initiation of HFD, but it is not yet clear what the timeline of response is. It is widely accepted in the literature that macrophages are the main producers of MCP-1 in established obesity[84, 115], but it is not clear when exactly the switch occurs, and if Id3 plays a role. While we hypothesize that AdPCs eventually stop producing MCP-1, the results from our human cohort indicate that this population continues to express MCP-1 during advanced obesity. It is possible that the timeline as well as the producers of murine adipose tissue MCP-1 differ from that of human adipose tissue MCP-1. Continued experiments using MCP-1 intracellular staining at different time points (4, 8, 12 and 16 weeks of HFD) could help determine the timeline of MCP-1 production by different cellular populations during obesity. Determining the timeline and relative contribution of different cell types within human adipose tissue is not as straightforward. Comparison of AdPCs from lean and obese adipose tissue could help determine the MCP-1 production at baseline, but would not give us a

timeline. While not a perfect solution, analysis of VAT from patients at varying stages of obesity could help provide us with a rough timeline. The stages of obesity could be determined by both the BMI as well as the number of years the patient was obese.

One thing that hasn't been completely characterized is why the AdPCs are producing MCP-1 as well as IL-6, especially considering that they did not produce any other inflammatory cytokines that we tested. It is possible that expression of one of these inflammatory molecules is specifically stimulating expression of the other in an autocrine manner. In THP-1 cells, MCP-1 was found to induce IL-6 expression, which then led to further increases in both MCP-1 and IL-6 due to a positive feedback loop[249]. In addition, treatment of peripheral blood mononuclear cells with IL-6 induced production of MCP-1[250], and treatment of subcutaneous preadipocytes with IL-6 induced a secretory profile more similar to visceral preadipocytes, resulting in increased chemoattraction of monocytes and macrophages[251]. Characterization of the *Mcp-1*$^{-/-}$ AdPCs to see if they are expressing and secreting IL-6 may shed some light.

Lymphotoxin-β receptor signaling may promote MCP-1 expression in AdPCs

Just as our data presents the question of why AdPCs are participating in adipose tissue inflammation, we are also left wondering how these cells are programmed to do this. AdPCs have been shown to support lymphoid structure, especially

when induced by lymphotoxin-β receptor (LTβR) signaling[151]. Under these conditions, these cells will abandon the adipocyte lineage and become lymphoid tissue organizer cells, in order to promote lymphoid tissue growth. LTβR stimulation results in a decrease in adipogenic markers and reduced differentiation into adipocytes, acting through activation of the alternative NFκB pathway to block transcriptional upregulation of PPARγ and C/EBPα[151].

It has been proposed that differentiation of adipocyte precursors into lymphoid stromal cells in the presence of proinflammatory signals is a common phenomenon that could also contribute to the remodeling of lymphoid tissue during immune response, or the formation of ectopic lymphoid tissue during chronic inflammatory disease[151]. In the context of these studies, LTβR signaling promoted development of lymphoid tissue organizer (LTo) cells from the AdPCs in subcutaneous adipose tissue. However, several visceral adipose depots, such as the epididymal depot, do not have organized lymphoid structures. It is unclear how lymphotoxin-β receptor signaling would affect AdPCs from these visceral depots. To test this, AdPCs isolated from eVAT would be treated with LTα1β2 to stimulate the LTβR. Following this treatment, adipogenesis would be induced *in vitro* using the adipogenic cocktail of insulin, IBMX, and dexamethasone. Adipogenesis, based on Oil Red O uptake, as well as morphology, would be compared to unstimulated cells. Additionally, LTβR levels would be measured in AdPCs from VAT of mice fed chow or HFD, to determine if expression of the receptor is limited to AdPCs in SAT.

It is known that signaling through the LTβR activates NFκB signaling through both classical and alternative pathways, and that this signaling upregulates expression of chemokines and chemokine receptors[192, 252]. It is possible that this signaling is what is responsible for HFD-induced MCP-1 production by AdPCs. This would suggest that by making MCP-1, adipocyte precursors are committing to a different lineage, even if they were already considered to be 'committed preadipocytes.' Does this signaling happen before or after loss of CD24 expression? I would hypothesize that CD24 expression is lost first through commitment to the adipocyte lineage, and following this commitment, LTβR signaling induces MCP-1 expression. Further studies to determine the effect of LTβR signaling on MCP-1 expression in AdPCs need to be performed. MCP-1 levels could be measured after both stimulation and neutralization of the LTβR on isolated AdPCs from both subcutaneous and visceral adipose depots.

Depot-specific differences in AdPCs

Studies have demonstrated via genome-wide expression arrays that AdPCs isolated from different adipose depots possess unique qualities, and that each depot should be treated as a separate "miniorgan" [76, 253]. It has been suggested that adipocyte precursors in different adipose depots arise from different precursor origins, and may not share a common precursor [76, 209]. We have demonstrated that AdPCs from epididymal visceral adipose tissue secrete high levels of MCP-1, as well as IL-6. One question is whether these cells have the

same role in other adipose depots, including other visceral depots (omental, retroperitoneal, epicardial), as well as subcutaneous depots. Using the same criteria for AdPCs, MCP-1 intracellular staining could be performed on multiple adipose depots from both chow-fed and HFD-fed mice, to compare MCP-1 production in this population across depots. Additionally, *Id3*$^{+/+}$ AdPCs from subcutaneous depots could be i.p. injected into *Id3*$^{-/-}$ mice, to see if they are capable of producing the same results we demonstrated in this study, in regards to MCP-1 production, M1 macrophage accumulation, and adipose tissue expansion.

Adipocyte hypertrophy versus hyperplasia is a much-debated topic, especially when comparing subcutaneous adipose tissue to visceral adipose tissue. Functional differences in SVF cells from different depots have been noted since the 1980s[254, 255]. The paradigm has been that visceral fat grows mostly by hypertrophy and subcutaneous by hyperplasia, providing a rationale for the different effects of specific adipose depots on metabolic health. However, studies using the AdipoChaser mouse for lineage tracing of the adipocyte line determined that HFD-induced adipose tissue expansion in both visceral and subcutaneous depots is mainly due to hypertrophy during the first month of HFD[253, 256]. After 1 month, hyperplasia via adipogenesis was initiated in the visceral depots, while hypertrophy remained the main source of expansion in subcutaneous adipose. This was surprising based on the accepted notion that subcutaneous adipose tissue expands primarily by hyperplasia. Adipocytes

derived from subcutaneous progenitors accumulate more lipid and express higher levels of PPARγ and C/EBPα upon differentiation compared to visceral progenitor cells[230, 231], indicating that they are perhaps better equipped for hypertrophy. This suggests that preferential expansion of subcutaneous fat still results in improved metabolic health.

Several groups have shown that while subcutaneous-derived AdPCs differentiate very well in culture, and require little or no stimulation[147], AdPCs derived from visceral adipose tissue have very little adipogenesis[190], even when induced with an adipogenic cocktail. One group demonstrated that with the addition of BMP4 to the culture of visceral AdPCs, they could rescue the apparent defect in adipogenesis[234]. It is not yet clear where the BMP4 is produced. It is possible that visceral AdPCs are producing less BMP4, or that BMP-induced activation within VAT is via paracrine signaling. High expression of BMP4 in human WAT correlates with reduced body weight and lower levels of proinflammatory cytokines such as TNFα and MCP-1. Treatment of human adipocytes with exogenous BMP4 reduced their ability to recruit monocytes through chemotaxis[257]. Additionally, cells treated with BMP4 have downregulated expression of MCP-1[257], suggesting that a more subcutaneous-like AdPC may have decreased MCP-1 expression.

Does the functional Id3 SNP affect MCP-1 in humans?

Previous studies identified single nucleotide polymorphisms (SNPs) in the human ID3 gene[258]. SNP rs11574 is the only tagged SNP found within the coding region of the ID3 gene, and was associated with subclinical atherosclerosis in the Diabetes Heart Study[258]. This nonsynonymous SNP results in a marked attenuation in the ability for ID3 to affect downstream transcription[258], therefore associating the SNP with decreased Id3 function. The allele frequency of the SNP is as high as 48% in Caucasian diabetic populations[259]. Considering the reduced number of AdPCs and reduction in MCP-1 and M1 macrophages seen in VAT from Id3-deficient mice, it would be of interest to determine whether patients with the Id3 SNP rs11574 also have reduced AdPC numbers, and a reduction in MCP-1 and M1 macrophages in omental adipose tissue. Our current cohort of 14 paired adipose depots is not adequately powered to compare patients with and without the SNP. Continued enrollment in this study would make it possible to answer these questions in the future. We have already demonstrated that omental and subcutaneous adipose in humans contain AdPCs, and that a portion of these AdPCs express MCP-1. The patients could be separated into groups based on their Id3 status, and the proportion of AdPCs that are MCP-1^{+} based on intracellular staining could be measured, as done previously.

Subset-specific characteristics of AdPCs

We demonstrated in both murine and human adipose tissue that high surface expression of CD44 marked abundant production of MCP-1. Interestingly, this

was only seen in the adipocyte progenitor cells, and did not extend to the global population of SVF cells. It is possible that CD44 is directly regulating MCP-1 production in these cells. Signaling through CD44 and its ligands has been shown in other cell types and tissues to upregulate MCP-1 production[211, 212]. CD44 levels in serum of obese human subjects positively correlated with the prevalence of insulin resistance, as well as to HbA1c, an index of glycemic control[215]. CD44 was identified as the top candidate in a GWAS study searching for genes implicated in the molecular pathogenesis of type 2 diabetes[215]. CD44-deficiency in a diabetic mouse model results in improved insulin sensitivity and reduced adipose tissue inflammation[215]. Is CD44 signaling in AdPCs necessary for MCP-1 production? *CD44*$^{-/-}$ mice could be used to determine whether expression of CD44 on the surface of AdPCs is necessary for their expression and secretion of MCP-1. In addition, CD44 signaling could be blocked as a means to try to prevent MCP-1 production in these cells. This could be accomplished by injection of a CD44-neutralizing antibody into mice, incubation of whole fat or isolated SVF cells with a CD44-neutralizing antibody, or direct incubation of AdPCs with a CD44-neutralizing antibody.

PDGFRα^{+} AdPCs can differentiate into either brown or white adipocytes, depending on the nature of inductive signals[237, 256, 260]. Interestingly, a subpopulation of PDGFRα^{+} progenitors was identified with high expression of CD44[261]. It was determined that preadipocyte proliferation at the sites of adipocyte clearance occurred almost exclusively in this subpopulation. In

response to HFD and adipocyte death, M2 tissue-resident adipose tissue macrophages release osteopontin, which recruits the PDGFRα^{+} CD44^{+} progenitors to proliferate and differentiate at sites of adipocyte clearance[261, 262]. Deletion of OPN, ligand for CD44, reduced CLS formation, and prevented the recruitment and proliferation of this subset of preadipocytes[261]. *In vivo* adipogenesis was restricted to this subset of progenitors, as levels of CD44 expression strongly correlated with lipid content in PDGFRα^{+} cells[262]. Conversely, HA, another ligand for CD44, is secreted by adipocytes throughout the differentiation process[263], as well as during adipocyte hypertrophy[214], and has been demonstrated to bind to monocytes expressing CD44[264]. These data demonstrate a unique interaction between AdPCs and adipose tissue macrophages, possibly one that is independent of MCP-1[214].

Initiation of HFD results in increased production of HA and OPN[213, 214]. It is unknown whether increased expression of these CD44 ligands acts to stimulate AdPCs, either in the role of their expression of MCP-1, or in their HFD-induced proliferative response. OPN acts as the chemoattractant in CD44-mediated macrophage recruitment, but it also activates the transcription of NFκB and AP-1[265], thereby modulating the inflammatory milieu. Expression of OPN is upregulated by up to 40-fold due to diet-induced obesity[266], and this is seen specifically in adipose tissue M1 macrophages[213]. OPN-deficient mice have reduced HFD-induced adipose tissue macrophage infiltration and inflammatory gene expression, as well as improved metabolic function[213, 267]. Importantly,

these effects are seen after just 2 weeks of HFD[268], indicating that OPN and CD44 play an important role in the early stages of diet-induced obesity and adipose tissue inflammation.

We have clearly demonstrated that only $CD24^-$ AdPCs, those that are further committed to the adipocyte lineage, are producing MCP-1. This could be due to one of two possible mechanisms. First, it is possible that CD24 is directly regulating MCP-1 production, and that downregulation of surface expression of CD24 is necessary for MCP-1 to be produced. It is also possible that MCP-1 is only produced in committed cells, and that CD24 downregulation is happening coincidentally. This could be tested in part by performing gain- and loss-of-function studies with CD24 expression. The hypothesis would be that forced expression of CD24 would prevent commitment, as well as prevent expression of MCP-1, and conversely, downregulation of CD24 would lead to either an early induction of MCP-1, or possibly higher levels of MCP-1.

The Rodeheffer group has demonstrated that only $CD24^+$ AdPCs can recapitulate adipose depots following injection into the fatless A-Zip mouse, even though the $CD24^-$ AdPCs have similar rates of *in vitro* adipogenesis[147, 155]. It is possible that CD24 expression is playing a role in the localization of these cells. Perhaps CD24 is necessary for cells to come in close proximity to the vasculature, and that without CD24, the AdPCs are left in 'undesirable' locations

within the site of injection. It is not yet clear how CD24 could be playing a role in this localization.

Interestingly, $CD24^{-}CD44^{+}$ cells have an important role in another disease process – cancer metastasis. This population of cells has more stem cell-like characteristics in human breast tumors[269], and the presence of these cells is correlated with disease severity and prognosis due to their invasive nature[270]. $CD24^{-}CD44^{+}$ cells also have increased IL-6 signaling as compared to other tumor cell types[269]. CD24 expression has been linked to metastatic progression, and it is hypothesized that CD24 facilitates interactions with the vasculature[271]. CD24-deficient mice also have inhibition of NFκB activation[272], and bone marrow-derived B cells from these mice have increased CXCR4-mediated chemotaxis[273]. These studies provide a rationale for the hypothesis that CD24 may be important in localization of AdPCs within the adipose tissue, but the exact mechanism is yet to be determined.

Is loss of Id3 specifically within the AdPC responsible for reduced MCP-1 and M1 macrophages?

In this work, we have studied the effects of loss of Id3 in every cell, and loss of Id3 in every cell but the AdPC, after adoptive transfer of $Id3^{+/+}$ AdPCs to $Id3^{-/-}$ recipients. However, we have not deleted Id3 from only the AdPC, to determine whether this is sufficient for reduced MCP-1 and M1 macrophages, and improved glucose tolerance. However, specific deletion of Id3 from AdPCs is not feasible,

due to limitations of distinguishing AdPCs both within the adipose tissue environment as well as from the rest of the tissues in the mouse. Studies that have specifically distinguished AdPCs from other cells within the adipose tissue have used markers that were found on cellular populations in other cells, making them unsuitable for a conditional knockout. AdPCs are identified via flow cytometry by a combination of markers. These markers are not individually capable of identifying these cells, as they are expressed on other cell types in different tissues.

Recently, a proteolytic cleavage fragment of decorin, the WAT7 receptor, was determined to be specifically expressed on the surface of adipocyte progenitors in WAT[237]. This receptor was absent on mesenchymal stem cells of other tissues, indicating that it may be a method to identify only AdPCs. Expression of WAT7 on AdPCs was determined through phage display technology, and a peptide ligand that targets this receptor was identified[152, 237]. This peptide could be used to target AdPCs, and to possibly delete Id3 via delivery of siRNA.

Adoptive transfer experiments comparing injection of *Id3*$^{+/+}$ AdPCs to injection of *Id3*$^{-/-}$ AdPCs into *Id3*$^{-/-}$ recipients clearly demonstrated significant differences in MCP-1 levels, M1 macrophage accumulation, and glucose tolerance. These differences were seen based on differential expression of Id3 in only the one cell type, the AdPCs that were injected. While it is certainly possible, and likely, that Id3 is playing a role in many other cell types to promote MCP-1-mediated

inflammation as well as glucose intolerance during obesity, we demonstrated that loss of Id3 in the AdPC is responsible for an attenuation in these endpoints.

AdPCs may support lymphocyte survival and/or development

Lymphedema is associated with obesity, as lymphatic malfunction due to lymph leakage and ruptured lymphatic vessels in mice results in obesity[274]. Most lymphatic vessels and lymph nodes are surrounded by white adipose tissue[275]. It has been speculated that perhaps obesity could be prevented via modulation of the lymphatic vasculature[30]. Close interactions between lymphoid cells in lymph nodes and the surrounding adipose tissue result in lymphocyte infiltration into adipose tissue during early stages of adipose tissue inflammation[276].

Injection of *Id3*$^{+/+}$ AdPCs into both *Id3*$^{+/+}$ and *Id3*$^{-/-}$ recipient mice results in an increased population of B cells in the adipose tissue, although subsetting was not performed so it is unknown if this was due to B1 or B2 cell expansion. This indicates that increased AdPCs are supporting lymphocytes within the adipose tissue. Evidence of this increase within the *Id3*$^{+/+}$ recipient mice indicates that this effect is independent of MCP-1 levels and M1 macrophages, as adoptive transfer had no effect on these in the *Id3*$^{+/+}$ hosts. Adipocyte precursors have been shown to support immune development and function[151, 224, 262]. There is evidence that AdPCs can promote survival of both $CD4^+$ and $CD8^+$ T cells[151], but studies have not been performed with B cells.

How are AdPCs promoting B cell accumulation, and is this is due to increased proliferation, survival, or recruitment? IL-5 and IL-13 are known to induce activation and proliferation of B cells[277], especially the B1 subset[278]. These cytokines are primarily produced by innate lymphoid type 2 cells (ILC2s) in adipose tissue[279]. AdPCs have not been demonstrated to produce these B cell mitogens, so either they are regulating via a different mechanism, or through an indirect manner. ILC2s have also been shown to stimulate PDGFRα^{+} AdPCs through their expression of the IL-4 receptor[280], so it is possible that AdPCs promote B cell accumulation through type 2 signaling mechanisms.

It is also unclear which B cell subset is increasing due to injection of *Id3*$^{+/+}$ AdPCs. Isolated B lymphocytes could be cultured with either isolated AdPCs, or their conditioned media, and then assayed via flow cytometry for total numbers, proliferation via BrdU uptake, and apoptosis via TUNEL staining. Flow cytometry would help determine which B cell subset is being affected by the presence of these cells. If no changes are seen due to co-culture or conditioned media, it is possible that the effects are indirect. In this case, the total SVF could be cultured, and enhanced with either increased numbers of AdPCs or addition of AdPC conditioned media. B2 cells have been previously demonstrated to have a more pro-inflammatory phenotype than B1 cells, through secretion of T_H1 polarizing cytokines, and directly contribute to insulin resistance[281]. Due to the worsened glucose tolerance seen after injection of *Id3*$^{+/+}$ AdPCs, it is possible that only B2

cells are increased in number after adoptive transfer, however, this will be determined in future experiments.

Some adipose tissue inflammation is beneficial

Adipose tissue inflammation can directly lead to insulin resistance, diabetes, and cardiovascular disease. However, not all inflammation is detrimental. The body needs to be able to sustain a normal immune response to infection or pathogens[282]. Even in the context of HFD and obesity, it is still helpful to have some inflammation. Macrophages enter adipose tissue to clean up dead and dying adipocytes[42, 45]. Even before adipocyte death, macrophages are recruited to help out with angiogenesis and vascularization[196, 239]. Impaired expression of TNFα within adipocytes resulted in ectopic lipid accumulation, increased glucose intolerance, and systemic inflammation[207]. This suggests that adipose tissue inflammation may play a role in the storage of excess nutrients. Additionally, adipose tissue inflammation plays an important role in maintaining proper intestinal barrier function to filter gut-derived endotoxin[207].

Thus, it is possible that most of the 'good' inflammation that occurs in adipose tissue is during the initial stages of obesity, and might coincide with the actions of MCP-1$^+$ AdPCs. As we have hypothesized that the MCP-1 initially produced by AdPCs could be recruiting Tie-2$^+$ angiogenic macrophages, we don't want to completely block this part of the inflammatory cascade. It is the second stage of AdPC-induced inflammation, where the MCP-1 produced is recruiting CCR2$^+$

Ly6C^{hi} inflammatory monocytes[283], and the resultant infiltration of M1 macrophages causes pathogenic inflammation. It has been demonstrated in other forms of chronic inflammation, that without resolution of inflammation, macrophages become activated and fuse to form multinucleate giant cells (MGCs). These MGCs can persist in the site of inflammation and produce proinflammatory cytokines[284].

Inflammation and adipose tissue expansion seem to go hand-in-hand, but it is unclear which causes which. We do know that not all adipose tissue becomes inflamed, as similarly, not all obese individuals develop metabolic disease. Does adipose tissue expansion itself induce adipose tissue inflammation? Or, does preexisting adipose tissue inflammation drive weight gain? It is possible that these two scenarios both exist, but at different stages of obesity. These questions have been asked in regard to the Id3 global knockout mouse. When put on a HFD, these mice are partially protected from diet-induced obesity, as they have attenuated adipose tissue expansion[182]. We have also demonstrated that these mice have reduced adipose tissue inflammation, as seen by reduced levels of MCP-1 and reduced M1/M2 macrophage ratio. Are we seeing reduced inflammation simply because the adipose tissue isn't expanding? Or, is the reduction in inflammation, which is seen at the initial stages of high-fat feeding, preventing subsequent expansion of adipose tissue? As we see similar expansion of subcutaneous adipose depots in the *Id3*$^{-/-}$ mice as we do in the *Id3*$^{+/+}$ mice[182], I would speculate that the inflammation may be driving the adipose

expansion. Adoptive transfer of MCP-1-deficient AdPCs into the *Id3*$^{-/-}$ host would help determine if increased adiposity is seen even in the absence of MCP-1-induced inflammation.

While our data has clearly demonstrated that HFD induces proliferation of AdPCs through Id3-dependent p21 mechanisms, we have not ruled out other methods of altering AdPC numbers. Crossno et al. identified that high-fat feeding promotes the trafficking of bone marrow-derived circulating progenitor cells to adipose tissue[285]. We have demonstrated that Id3 regulates chemokine receptor expression, specifically CXCR4 (unpublished data) and CCR6[189], in the context of B cells and atherosclerosis. The SDF-1/CXCR4 chemoattractant axis has been implicated in mobilization of stem cells and progenitors[286]. Endothelial cell-derived SDF-1 promotes chemotaxis and differentiation of human adipose tissue-derived progenitor cells, and contributes to the formation of the vascular network during adipose tissue development[287]. We have yet to determine if Id3 is playing a role in preadipocyte recruitment, or if MCP-1^{+} preadipocytes are recruited to VAT during HFD, either prior to or in addition to their proliferative expansion.

Chapter 6

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