MACRONUTRIENT ACTIVATION OF ENDOTHELIUM DEPENDENT LEUKOCYTE TRAFFICKING: METABOLIC IMPLICATIONS

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ABSTRACT

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Obesity and insulin resistance are characterized by elevated pro-inflammatory proteins in the blood and immune cell accumulation in the visceral adipose tissue. Resident leukocytes release tumor necrosis factor α (TNFα) and other inflammatory cytokines which stimulate adipocyte lipolysis, recruit leukocytes to adipose tissue, promote pro-inflammatory immune cell polarization, facilitate oxidative stress, and activate intracellular kinases which dull insulin signaling cascades in metabolic tissues. Immune cell mediated dysregulation of stromal and parenchymal cells has raised suspicion that insulin resistance is an immune disorder initiated by activated white blood cells with over-nutrition. Efforts to improve pathological metabolism by reducing inflammation have yielded mixed results in humans and animal models. The role of inflammation and immune cell accumulation in the visceral fat (VF) in the progression of insulin resistance remains presently debated. There is, however, a consensus that identifying the triggers for obesity and impaired insulin signaling is of the utmost importance. The goal of this report is to identify dietary fat absorption as a key initiator of inflammatory action and insulin desensitization which may be dampened by reducing immune cell accumulation in adipose tissue.

To explore how lean, healthy organisms become obese and insulin resistant, we examined the inflammatory consequences of isocaloric but variable macronutrient loads in the VF of lean mice. Mice were administered single liquid meals composed of low-fat (10% fat) or high-fat (60% fat) diet and observed by intravital microscopy to quantify leukocyte-endothelium interactions in mesenteric postcapillary venules (MPCV) 1, 2, 3, and 4 hours after oral gavage. Leukocyte rolling and leukocyte adhesion were transiently elevated within 1 hour after feeding and returned to baseline levels 4 hours later. Endothelial cell surface expression of P-selectin (Psel), a rapidly activated cell adhesion molecule (CAM), confirmed that high-fat feeding induced Psel dependent leukocyte rolling through the VF microcirculation. Furthermore, leukocyte accumulation in the VF was modestly increased by a single high-fat meal (HFM). Repetitive high-fat diet (HFD) consumption for 24 hours prolonged elevated leukocyte-endothelium interactions and promoted neutrophil accumulation in the VF. The neutrophilic enzyme myeloperoxidase (MPO), a producer of the chlorinating agent hypochlorous acid, increased in abundance and activity in the VF of HFM fed mice. Elevated leukocyte-endothelium interactions, leukocyte infiltration, and MPO activity in VF were not observed in Psel deficient ($Psel^{-/-}$) mice following lipid overload. To ascertain if MPO is required for sustained endothelial activation, leukocyte-endothelium interactions and leukocyte infiltration were monitored in high-fat fed MPO deficient ($MPO^{-/-}$) mice. Similar to the $Psel^{-/-}$ mice, $MPO^{-/-}$ mice were protected from the inflammatory effects of high-fat feeding. Our data supports postprandial hyperlipemia as an inducer of transient and Psel dependent inflammatory reactions that are sustained by prolonged HFD consumption.

To study whether early phase inflammatory interventions granted late phase metabolic improvements, wild-type (WT), Psel deficient (Psel$^{-/-}$), and MPO deficient (MPO$^{-/-}$) C57BL/6 mice were given *ad libitum* access to LFD (10% fat) or HFD (60% fat) for 12-16 weeks. All mouse groups given HFD became obese. Prolonged HFD consumption sustained elevated leukocyte-endothelium interactions in MPCVs and was accompanied by increased local and systemic TNFα in WT mice. High-fat fed WT mice were hyperglycemic, hyperinsulinemic, glucose intolerant, and insulin resistant compared to LFD fed controls. Psel$^{-/-}$ mice were protected from leukocyte-endothelium interactions as well as local and systemic TNFα accumulation despite extended HFD consumption. Surprisingly, high-fat fed Psel$^{-/-}$ mice were equally hyperglycemic, hyperinsulinemic, glucose intolerant, and insulin resistant as the inflamed, high-fat fed WT mice. MPO$^{-/-}$ mice were also protected from elevated systemic TNFα and gained slightly less weight than the other high-fat fed groups. While MPO$^{-/-}$ mice were hyperglycemic and glucose intolerant, they did have improved insulin stimulated glucose clearance.

The data presented in this report demonstrates the pro-inflammatory nature of postprandial hyperlipemia and the insulin desensitizing nature of prolonged HFD consumption. Ablation of VF immune cell accumulation by Psel deletion is not sufficient for improving insulin signaling or glycemic control, which is consistent with prior reports. Deletion of MPO, however, did result in slightly less obesity and marginally improved insulin signaling. We conclude that while immune cell accumulation in the VF contributes to the progression of insulin resistance, it is not a prerequisite for metabolic pathology development.

DEDICATION

This dissertation is dedicated to the people who saw me through it:
Ashley, Mom, and Dad

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LIST OF ABBREVIATIONS

AGE	Advanced glycation end product
AP-1	Activator protein-1
BAT	Brown adipose tissue
BMI	Body mass index
DIO	Diet-induced obesity
eCAMs	Endothelial cell adhesion molecules
Esel	E-selectin
FACS	Fluorescence-activated cell sorting
FFA	Free fatty acid
GTT	Glucose tolerance test
HFD	High-fat diet
HFM	High-fat meal
ICAM	Intercellular adhesion molecule
IKKβ	IκB kinaseβ
IL	Interleukin
i.p.	Intraperitoneal
IRAK	IL-1 receptor associated kinase
IRS-1	Insulin receptor substrate-1
ITT	Insulin tolerance test
JNK	c-Jun N-terminal kinase
LFA-1	Lymphocyte function-associated antigen-1
LFD	Low-fat diet

LFM	Low-fat meal
Lsel	L-selectin
MAP	Mean arterial pressure
MCP-1	Monocyte chemoattractant-1
MPCV	Mesenteric postcapillary venule
MPO	Myeloperoxidase
$MPO^{-/-}$	Myeloperoxidase deficient
MUFA	Monounsaturated fatty acid
NF-κB	Nuclear factor-κB
NO	Nitric oxide
PI3K	Phosphoinositide 3-kinase
Psel	P-selectin
$P\text{-}sel^{-/-}$	P-selectin deficient
PSGL-1	P-selectin glycoprotein ligand-1
PUFA	Polyunsaturated fatty acid
ROS	Reactive oxygen species
SAT	Saturated fatty acid
SF	Subcutaneous fat
TNFα	Tumor necrosis factor α
UCP-1	Uncoupling protein-1
VF	Visceral fat
WT	Wild-type

CHAPTER 1: INTRODUCTION

Background and Significance

Healthy, physiological tissue remodeling necessitates immune cell activity. Macrophages are required for the clearance of stromal cells and extracellular matrix during the reshaping of adipose tissue during growth (O'Brien et al 2012) while depletion of macrophages impairs angiogenesis in expanding fat (Han et al 2011). Weight loss, a key endeavor in the fight against obesity, is dependent upon macrophage accumulation in adipose tissue (Kosteli et al 2010). Immune cell function during adipose tissue expansion, like many other necessary physiological phenomena, can fall into dysregulation, resulting in a pathological state. The coupling of obesity and metabolic dysfunction is a pathological setting in which adipose tissue and immune cell dysregulation encourage one another in a positive feedback loop.

Obesity, in its simplest form, is the result of an imbalance in energy intake and expenditure. The consumption of energy dense foods in conjunction with a sedentary lifestyle are the most commonly reported determinants driving obesity development. During fat mass accumulation, several changes in the adipose organ occur. As adipocytes store increasingly greater amounts of triglyceride, they hypertrophy, growing in volume, which, in turn, lengthens the distance oxygen must travel from the blood to its intracellular targets, causing regional intracellular hypoxia (Hosogai et al 2007, Regazzetti et al 2008). Hypoxia lowers insulin sensitivity in adipose tissue (Halberg et al 2009, Copps et al 2009). Additionally, excess intracellular lipid induces both endoplasmic reticulum and oxidative stress in adipocytes, activating insulin desensitizing kinases (Ozcan et al 2004, Gao et al 2002). Adipose tissue dysregulation is also characterized by imbalanced lipid secretion

and storage; elevations in systemic lipids contribute to the insulin desensitizing of metabolic tissues like skeletal muscle and the liver (Guilherme et al 2008). Immune cell accumulation within adipose tissue is another hallmark of obesity. Under the physiological circumstances, leukocytes promote healthy growth and tissue remodeling (Sun et al 2011). In the case of obesity, however, adipose tissue macrophages have pro-inflammatory autocrine and paracrine effects on surrounding adipocytes and stromal cells, recruiting even more local immune cell invasion (Gregor & Hotamisligil 2011). This inflammatory phenotype is often referred to as a "chronic state of low-grade inflammation" and is an indicator of obesity related metabolic and cardiovascular diseases. The cumulative effect of obesity associated stressors is diminished local and systemic insulin sensitivity. **The degree to which each factor contributes to the progression of insulin resistance is presently unknown.**

Our individual and national size matters. Body mass index (BMI) is a mathematical model (kg/m^2) used to quantify population fatness in which BMI scores are stratified into categories of varying health. A BMI quotient in the 25-30 range is considered overweight while a value greater than 30 is categorically obese. Overweight and obesity, leading risk factors for cardiovascular disease and type II diabetes, afflicts 68.5% of adults in the United States, with 34.9% considered obese (Ogden 2014). Annually, approximately 25% of deaths in the US are caused by nonspecific cardiovascular disease, which is intimately linked to obesity and metabolic dysregulation. Diabetes mellitus afflicts nearly 10% of the American adult population. Of those patients, between 90-95% have type II diabetes, characterized by hyperglycemia coupled with insulin resistance. In 2011, the estimated national bill for cardiovascular diseases was $320.1 billion (Mozaffarian et al 2015).

Perhaps more alarming than the incidence of obesity and insulin resistance in adults is the growing population of young obese patients. About 17% of children between 2-19 years old are overweight or obese (Ogden 2014) while roughly 3 out of 1000 young Americans (>20 years old) have been diagnosed with diabetes mellitus (Dabelea 2014). Incidence of type I and type II diabetes diagnosis in the young population has risen by 20 and 30% respectively between 2001 and 2009 (Dabelea 2014). Additionally, gestational diabetes is suspected to diminish insulin sensitivity and increase affinity for obesity in offspring (Ma et al 2015). Without radical reductions in the obesity and insulin resistance on the American population, quality of life will continue to deteriorate while mortality and healthcare costs rise.

Adipose Tissue and Inflammation

Adipose tissue is not the singular, dormant fuel storage organ it was once regarded as. Fat function varies depending on the organ's cell anatomy, macronutrient exposure, and location. These factors determine metabolic, enzymatic, and endocrine function. Brown adipocytes are unique to mammals and make up brown adipose tissue. This fat expresses the enzyme uncoupling protein-1 (UCP-1), which enables the generation of heat instead of adenosine triphosphate during mitochondrial β-oxidation of fatty acids (Enerback 2009, Cannon & Nedergaard 2004). Brown adipose tissue makes up only a small percentage of total adipose tissue; the vast majority of fat is white adipose tissue. White adipose tissue is composed of white adipocytes that contain a single, large lipid droplet that occupies ~90% of the cell volume (Lee et al 2013). The white adipose tissue's primary function, energy storage, is dependent upon the adipocyte's ability to transfer lipids to and from the blood. In the fasted state, beta-adrenergic stimulation causes lipolysis: the hydrolysis of

triglycerides into free fatty acids (FFAs) and glycerol in a series of lipase mediated reactions. FFAs enter the blood and bind albumin, where they are transported through the vasculature (Rosen & Spiegelman 2006). In the fed state, insulin secreted by pancreatic β-cells binds white adipse tissue insulin receptors, causing lipoprotein lipase driven release of FFAs from lipoproteins. FFAs enter the adipocyte and are stored as triglyceridess esterified to glycerol. Additionally, insulin stimulates white adipose tissue glucose transporter mediated glucose uptake from the blood. Thus, insulin promotes fat accumulation (Frayn et al 1994, Dimitriadis et al 2011).

When a person becomes fat, it is their white adipose tissue that has expanded. While all fat people have increased white adipose tissue mass, not all fat people are hampered with the metabolic and cardiovascular complications associated with obesity. Metabolically healthy obese patients make up approximately 25% of the total adult obese patient pool (Denis & Obin 2013). Comparisons between the metabolically healthy obese population and the sick obese population has provided insights into mechanisms and characteristics of adipose tissue dysregulation. Individuals who accumulate subcutaneous fat (SF) have better health outcomes than those who accumulate visceral fat (VF), indicating regional differences in fat function. Adipose tissue synthesis of hormones called adipokines is critical for metabolic regulation and is altered in obesity. SF depots such as superficial abdominal, gluteal, and limb fat produce leptin, a hormone that increases energy expenditure and suppresses hunger through central nervous system modulation (Halaas et al 1995, Friedman & Halaas 1998). During obesity, central leptin sensitivity is impaired and leptin's anti-obesity effects are diminished (Ozcan et al 2009).

The VF includes adipose depots which lie within the abdominal cavity, local to the gastrointestinal tract. VF secretions strongly influences liver health because blood flow from the VF drains directly into the portal circulation, perfusing the liver. During VF lipolysis, therefore, the liver is washed in lipid rich blood, increasing the likelihood of fat deposition in the liver, disrupting hepatocyte function. VF adipokines include adiponectin which protects endothelial function and is inversely correlated with obesity and adipose dysregulation. VF adipocytes also produce monocyte chemoattractant-1 (MCP-1), interleukin (IL)-6, and TNFα (Bruun et al 2005, Fried et al 1998, Hotamisligil et al 1995). VF derived pro-inflammatory signals have autocrine, paracrine, and endocrine effects, recruiting immune cells to the adipose tissue. Infiltrating immune cells then release their own pro-inflammatory signals. The inflammatory action of VF growth promotes immune cell accumulation and VF dysregulation in a positive feedback loop.

Comparisons between the sick obese and metabolically healthy patient populations have revealed that sick patients have substantially more MCP-1 and TNF-α in serum (O'connell et al 2011) and increased VF adipose tissue macrophages (Kloting et al 2010) compared to metabolically protected obese individuals. Macrophage plasticity allows for environmental induction of phenotypic shifts between a pro-inflammatory, or M1 and anti-inflammatory, or M2 phenotype. While research of inflammation in fat has historically focused on macrophages, we now know that mast cells (Liu et al 2009), natural killer T cells (Ohmura et al 2010), and B cells (Winer et al 2011) contribute to the progression of obese adipose tissue inflammation. Innate and adaptive immune cells residing in fat secrete pro-inflammatory cytokines like MCP-1, TNF-α, and interferon gamma that counteract anti-inflammatory IL-4 and IL-10 (Nishimura et al 2009, Schroder et al 2004) and activate

serine kinases such as c-Jun N-terminal kinase (JNK), IκB kinaseβ (IKKβ), and IL-1 receptor-associated kinase (IRAK). These kinases block insulin stimulation of the phosphoinositide 3-kinase (PI3K)/Akt pathway by serine phosphorylating insulin receptor substrate-1 (IRS-1) (Hirosumi et al 2002, Gao et al 2002, Kim et al 2005). Nuclear factor-κB (NF-κB) and activator protein-1 (AP-1), strong inflammatory transcription factors, are up regulated, exacerbating the inflammatory situation. The pro-inflammatory phenotype of obesity simultaneously inhibits anti-inflammatory action while promoting a chronic inflammatory state.

The crosstalk between inflammation and metabolism in adipose tissue is well established; immune cell accumulation and insulin resistance are closely correlated. Reducing the pro-inflammatory state in the VF of obese animals or high fat fed animals has been shown to have insulin sensitizing effects. An early discovery reported not only do obese, insulin resistant rodents have elevated TNFα levels in their VF but disrupting TNFα signaling improved insulin sensitivity (Hotamisligil 1993). TNFα impairs insulin action directly by promoting serine phosphorylation of IRS-1 and indirectly by stimulating lipolysis. Lipolysis, a physiological process which is dysfunctional in obesity, was stimulated in cultured adipocytes treated with TNFα, IL-1, and several members of the interferon family of cytokines (Feingold et al 1992). More recent evidence has demonstrated that shifting immune cell phenotype from pro-inflammatory to anti-inflammatory may improve insulin resistance and even obesity. Immunotherapy was used in obese rodents to shift the T cell population in the VF from a Th1 phenotype to several anti-inflammatory T cell phenotypes, including the Th2 phenotype (Winer et al 2009). Mice with anti-inflammatory T cells showed improvements in insulin sensitivity.

The Endothelium: Physiology and Pathology

The endothelium is the single layer of cells lining the luminal surface of lymphatic and blood vessels. Endothelial cells are present in all tissues receiving blood flow; the health of nearly all tissues, therefore, is dependent on proper endothelial cell function. Everything that requires transport into or out of the blood must traverse the endothelium. The endothelium provides nutrients, hormones, and immune cells with an arena for interaction.

Regional vascular endothelial cell populations are charged with unique responsibilities determined by their anatomical location. All endothelial cells synthesize nitric oxide (NO), but the NO produced by the arterial and arteriolar endothelium regulates blood pressure and blood flow by relaxing local smooth muscle. Capillaries consist of only a single layer of endothelial cells, facilitating plasma protein exchange with extravascular tissues. Leukocyte trafficking is mediated by endothelial cells in postcapillary venules. Under resting conditions, venular endothelial cells minimally express cell adhesion molecules (CAMs), allowing leukocytes to flow freely in blood (Pober & Sessa 2007). Endothelial activation causes a transient shift in endothelial cell phenotype to enable immune cell passage out of the blood.

The activation of the endothelium sets in motion the necessary cellular processes required for endothelial CAM (eCAM) expression, resulting in leukocyte extravasation (**Figure 1** from Hickey & Kubes 2009). This process, diapedesis, is well characterized (Kolaczkowska & Kubes 2013). First, a pro-inflammatory signal, such as histamine or lipopolysaccharide (LPS), binds endothelial cells. Endothelial expressed Psel and E-

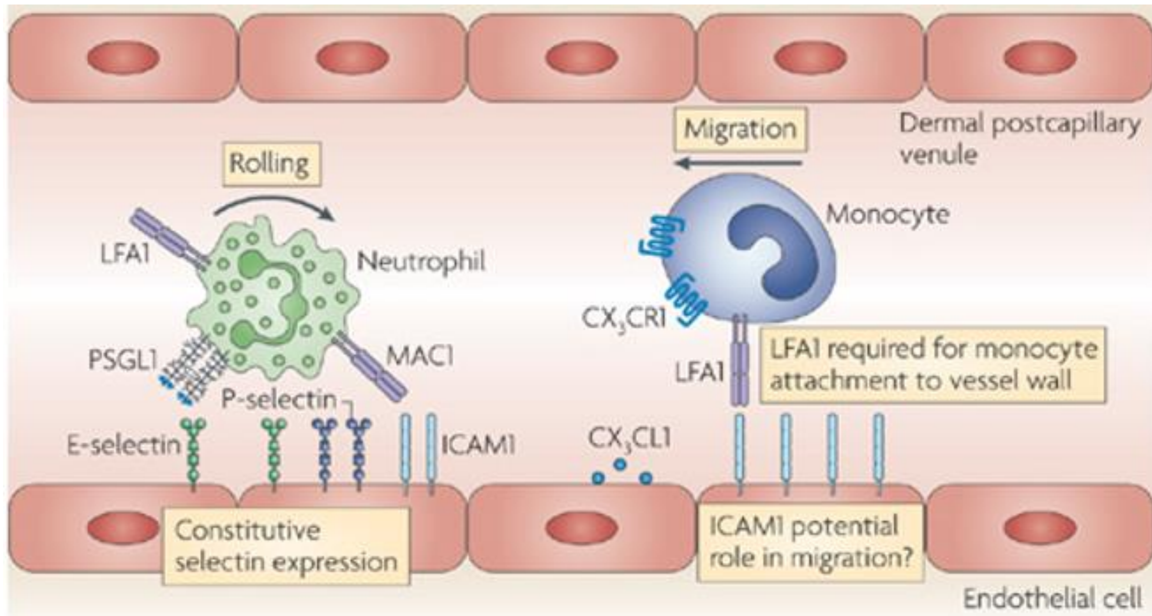

Figure 1: Steps of leukocyte extravasation

selectin (Esel) are transported to the luminal surface of vessels, extending into the blood. Psel is constitutively stored in cytosolic Weibel-Palade Bodies, making it the fastest eCAM responder. Esel must be *de novo* synthesized with each unique inflammatory response, making it the slower of the two endothelial selectins. Both selectins tether leukocytes to the endothelium by forming reversible bonds with leukocyte expressed ligands, such as Psel glycoprotein ligand-1 (PSGL-1). The simultaneous forming and breaking of selectin bonds with leukocyte ligands causes leukocytes to roll along the vessel wall. This process is referred to as leukocyte rolling. When immune cells are slowed by selectins, they are more likely to firmly adhere to endothelial cells. Integrin binding mediates leukocyte adhesion to the endothelium. The endothelial integrins intercellular adhesion molecule (ICAM)-1 and ICAM-2 bind the constitutively expressed leukocytic integrins lymphocyte function-associated antigen-1 (LFA-1) and Mac1. Gaps between endothelial cells are opened by the "loosening" of tight junction regulating proteins like platelet-endothelial cell adhesion molecule-1. Leukocytes bound to endothelial cells pass through these open

junctions, through the basement membrane, and into extravascular tissues. Typically, the endothelium returns to the resting state after the pro-inflammatory stimulus is removed.

It is important to distinguish endothelial activation from endothelial dysfunction. Endothelial activation is a physiological prerequisite for combatting infections and healing wounds. Endothelial dysfunction is the loss of resting state endothelial processes, primarily due to diminished NO bioavailability (Davignon & Ganz 2004). Anti-coagulant, vasomotor, and other functions of the resting endothelium are impaired by reduced NO activity. NO bioavailability is compromised by diminished NO production by endothelial cells and inactivation of NO by reactive oxygen species (ROS), resulting in nitrotyrosine formation (Deanfield et al 2007). Prolonged endothelial activation facilitates a transition to endothelial dysfunction.

Over-nutrition impairs endothelial cell function and insulin sensitivity through shared mechanisms. Hyperglycemia and hyperlipemia each overload mitochondria causing ROS production (Brownlee 2005). ROS inhibit the glycolytic enzyme glyceraldehyde-3 phosphate dehydrogenase, further increasing intracellular glucose and promoting the formation of advanced glycation end product (AGE) precursors and diacylglycerol. AGE precursors are capable of modifying intracellular proteins and binding extracellular AGE receptors, producing pro-inflammatory cytokines and eCAM expression (Du et al 2003). Diacylglycerol activates the protein kinase C family of pro-inflammatory cytokines, increasing NF-κB expression (Itani et al 2002). Downstream of these events, serine kinases JNK, IKKβ, and IRAK serine phosphorylate IRS-1, desensitizing the insulin stimulated PI3K pathway (Hirosumi et al 2002, Gao et al 2002, Kim et al 2005). Plasma nutrients

drive intercellular generation of metabolic intermediates that interfere with insulin signaling and promote inflammation.

Being Lean: The Forgotten Step in Becoming Fat

Valuable information about the relationships between obesity, insulin resistance, and inflammation has been collected from studying sick humans and animal models of disease. The intense search for a trigger that initiates these disease states continues, however, and few conclusions can be drawn without close examination the first phase: normal weight and metabolism. When facing problems of quantity such as hyperglycemia or NO bioavailability, two therapeutic strategies are available: altering rate of production or rate of removal. By studying how lean, healthy mammals become fat, unhealthy mammals, we hope to slow the production rate of new obese and insulin resistant patients.

Considering the deleterious effects nutrients may have on inflammatory and oxidative cellular processes, the postprandial phases may be regarded as repetitive moments in an organism's life that strongly influence health. The reaction of healthy tissue to nutrients must be known so that we might understand how healthy tissue falls into dysregulation. In 2011, a hypothesis was described that posed the very act of eating as the pro-inflammatory trigger that initiates obesity (**Figure 2** from Gregor & Hotamisligil 2011). More specifically, that nutrients are innately pro-inflammatory and cause transient, pulsatile inflammatory responses in metabolic tissues such as skeletal muscle, liver, and adipose tissue. Over time, these inflammatory responses become amplified and recruit immune cells locally, impairing normal metabolism and resulting in further immune cell accumulation. Alternatively, it is possible that strictly excess nutrient absorption is pro-inflammatory. In this case, excess nutrients may bind and activate innate immune receptors

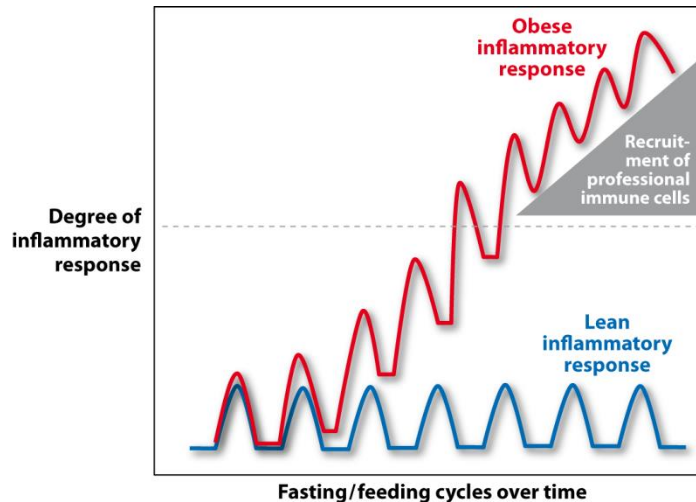

Figure 2: Pulsatile inflammatory responses to meals

on immune (Shi et al 2006), endothelial (Mudaliar et al 2014), and metabolic cell types (Eguchi et al 2012, Holland et al 2011).

To characterize how nutrients drive leukocyte infiltration into healthy fat, the selectin family of CAMs must be further described. Selectins, Ca^{2+} dependent, transmembrane glycoproteins, are responsible for initiating the adhesion of leukocytes to the vascular endothelium by tethering immune cells to the venular wall (McEver 2015). The selectin family is comprised of L-selectin (Lsel), Esel, and, finally, Psel, which will be the focus of this work. Lsel is constitutively expressed on the surface of neutrophils, monocytes, and certain T cells, B cells, and natural killer cells. Lsel serves as a ligand for both Psel and Esel, promoting the interactions with platelets and endothelial cells (Kansas 1996). Leukocyte-leukocyte bonds are formed by Lsel ligation of PSGL-1. Antibody blockade of Lsel hinders leukocyte rolling along the endothelium *in vivo* (Ley et al 1993). Esel is strictly localized to endothelial cells and requires transcription and translation upon stimulation from a pro-inflammatory signal prior to activation. TNFα, IL-1, and LPS are

known to induce Esel synthesis, a process which takes several hours (Bevilacqua et al 1989). Esel has several well characterized ligands including PSGL-1, Lsel, CD44, and neutrophilic ESL-1 (Zarbock et al 2011). Functional blockade Esel diminishes leukocyte rolling on the endothelium, however, antibody blockade of Esel in early phases of leukocyte-endothelium interactions does not reduce leukocyte rolling (Hwang et al 2004). Psel is stored preformed in secretory α-granules and Weibel-Palade Bodies of platelets and endothelial cells, respectively (McEver et al 1989). This enables the rapid induction of platelet and endothelial cell pro-adhesive phenotypes in response to acute inflammatory signals. Psel is translocated to the plasma membrane within minutes of stimulation, quickly mounting the immune response. The predominant ligand for Psel is PSGL-1 but Lsel ligation has also been reported (Zarbock et al 2011). Like Esel, Psel synthesis is stimulated by pro-inflammatory signals, making Psel important for long term inflammatory reactions in addition to its short term action.

Selectin-ligand binding induces different signaling pathways in neutrophils (Zarbock et al 2011). CD44 ligation by Esel has been shown to redistribute neutrophil CAMs, specifically Lsel and PSGL-1, in a p38 MAPK dependent pathway (Green et al 2004, Hidalgo et al 2007). Ligation of neutrophilic PSGL-1 by Psel and Esel has been shown to activate the integrin LFA-1, which binds endothelial ICAM-1 resulting in slower neutrophil rolling (Kuwano et al 2010, Miner et al 2008).

Neutrophils have been identified as the first immune cell population to infiltrate adipose tissue upon high fat diet consumption (Elgazar-Carmon et al 2008, van Oostrom et al 2003) and a recent study has implicated MPO, a neutrophilic heme peroxidase, as a potential contributor to insulin resistance in the diet induced obese (DIO) mouse models of

obesity (Wang et al 2014). MPO synthesizes the potent chlorinating agent HOCL from hydrogen peroxide and CL^- ions which interacts with both foreign and host substrates (Lau & Baldus 2006). Because MPO is highly cationic, it quickly localizes to the negatively charged endothelium upon release. Endothelial cells internalize MPO via endocytosis of MPO bound to albumin within caveolae (Tiruppathi et al 2004). In the subendothelial space, MPO interferes with extracellular matrix function by tyrosine nitrating fibronectin (Baldus et al 2001) in the absence of neutrophilic extravasation. MPO accumulation in the subendothelial space facilitates reductions in NO bioavailability for smooth muscle cells. MPO reduces NO bioavailability in a HOCL independent manner, resulting in the formation of peroxynitrite (Eiserich et al 2002). Diminished NO bioavailability has consequences on vascular relaxation, CAM expression, platelet aggregation (Lau & Baldus 2006). In the postprandial phase, elevated MPO activity and markers for endothelial cell apoptosis have been reported in healthy male volunteers (Spallarossa et al 2008). Similar experiments in women have produced conflicting results, however, and the role of MPO in the postprandial phase remains unclear (Schindhelm et al 2008).

The development of insulin resistance appears to be a consequence of immune cell accumulation in metabolic tissues during obesity. The goal of this dissertation is to determine whether intervention in the early stages of immune cell infiltration of adipose depots may protect insulin sensitivity in the face of fat accumulation. Therefore, we examine the effects of deleting Psel, an initiator of diapedesis, and MPO, which is a key enzyme of neutrophils, the immune system's earliest responding white blood cell population, on metabolic health in the acute and chronic stages of high-fat feeding.

CHAPTER 2: FAT CONSUMPTION DRIVES INFLAMMATION

Abstract

Whether immune cell infiltration of fat occurs before, during, or after adipose tissue expansion is still debated. Human studies have recently demonstrated postprandial lipemia induces inflammatory action and endothelial dysfunction in lean subjects. More specifically, oral fat challenge resulted in significant neutrophil recruitment and elevated eCAM expression, but evidence of extravasation was not examined (van Oostrom et al 2003). The endothelial activation following lipid absorption is rapid, indicating upregulation of Psel, the endothelium's fastest responder to inflammatory stimuli. The temporal relationship and intercellular mechanisms between lipids and immune responses are unknown. We hypothesized that a single HFM is sufficient for the swift induction of Psel dependent leukocyte infiltration into the VF.

To characterize normal immune responses to acute lipid overload, WT C57BL/6 mice were given either a low fat (10% fat) or a high fat (60% fat) liquid meal by gavage. FFA analysis after oral gavage revealed that a single HFM causes postprandial hyperlipemia. All mice were studied 1, 2, 3, and 4 hours post gavage by intravital microscopy to measure postprandial kinetics of leukocyte rolling and leukocyte adhering in MPCVs. Single HFMs activated Psel and increased leukocyte-endothelium interactions leukocyte-endothelium interactions in the MPCVs as early as 1 hour but not later than 4 hours after exposure. Mice given 24 hour *ad libitum* access to HFD had a prolonged elevation in leukocyte-endothelium interactions as determined by intravital microscopy. Identification of VF infiltrating immune cell populations determined by fluorescence-activated cell sorting (FACS) revealed primarily neutrophils infiltrate VF in the early phase. The neutrophilic enzyme MPO, a producer of oxidative stress, was more abundant

and active in high fat fed mice. Psel deletion prevented high-fat feeding induced leukocyte-endothelium interactions, leukocyte infiltration, and MPO activity in the VF. To ascertain if MPO is required for sustained endothelial activation, leukocyte-endothelium interactions and leukocyte infiltration were monitored in high-fat fed $MPO^{-/-}$ mice. Surprisingly, $MPO^{-/-}$ mice were protected from the inflammatory effects of high-fat feeding.

Taken together, these data demonstrate the rapid, upregulation of Psel in the endothelium in response to lipid overload. Furthermore, repetitive high-fat feeding prolongs an otherwise transient immune response to hyperlipemia. Postprandial hyperlipemia in lean mammals promotes leukocyte accumulation in the VF which is known to encourage fat accumulation and diminish insulin sensitivity. The role of MPO in this process requires further investigation.

Introduction

Consumption of HFMs produces spikes in serum lipid levels regardless of weight and metabolic health (Miller et al 2003). Even in normal subjects, consumption of HFMs transiently elevates postprandial lipids in the range of those found in fasting obese, insulin resistant patients (Moreton 1947, Schaefer et al 2001). These episodes of transient postprandial hyperlipemia in healthy individuals are accompanied by transient increases in inflammatory action (van Oostrom et al 2003, de Vries et al 2014a). The consumption of a fatty meal by healthy volunteers induces rapid changes in the quantity and activation state of circulating white blood cells (van Oostrom et al 2004, Gower et al 2011), especially neutrophils (van Oostrom et al 2003). Postprandial responses to lipids are exacerbated in patients with metabolic syndrome whom experience much higher peak concentrations of plasma triglycerides than healthy individuals following fat absorption. The increased

plasma triglycerides in metabolically compromised subjects are accompanied by amplified postprandial peak inflammatory markers (Schauren 2014, Perez-Martinez 2014).

Hence, it has been proposed that metabolic organs, such as the adipose tissue, experience a pulsatile inflammatory response during the feeding/fasting cycles. Thus, lean individuals experience mild inflammatory peaks after feeding which are resolved after the consumed nutrients are metabolized. With consistent overfeeding, though, this pulsatile inflammatory response is more intense and of longer duration, which ultimately leads to the unresolved inflammation associated with obesity (Gregor & Hotamisligil 2011). As evidence for the mechanisms regulating the engagement of nutrients with pathogen or immune sensors is still under investigation, two main questions remain unanswered: 1) how do nutrients initiate pulsatile inflammatory responses; 2) how do nutrients communicate with immune cells? Answering these questions will shed new light on the overall pathogenesis of insulin resistance by clarifying the process that links dietary nutrients to inflammation and associated metabolic and vascular complications.

Interestingly, postprandial hyperlipemia is also associated with endothelial activation. Postprandial increases in serum endothelial microparticles (Ferreira et al 2004) and reduced vascular compliance (Blendea et al 2005) coupled with increased serum nitrotyrosine (Ceriello et al 2002) have been documented in normal weight, insulin sensitive patients after a lipid overload. Impaired flow-mediated dilation and increased nitrotyrosine formation are evidence for oxidative stress, a likely contributor to insulin desensitization (Brownlee 2005). Additionally, healthy and diabetic patients both undergo elevations in serum concentrations of soluble cell adhesion molecules, more specifically, eCAMs, following a lipid challenge (Nappo et al 2002, Ceriello et al 2004). Endothelial

cell activation is characterized by the expression of integrins and selectins of which only Psel is constitutively stored in cytosolic Weibel-Palade bodies. This enables rapid Psel translocation to the vascular lumen during endothelial activation. Taken together, these data suggest a role for the vascular endothelium of the microcirculation in the initiation of deleterious outcomes associated with postprandial hyperlipemia. We hypothesized that fatty meals induce a rapid expression of the selectin family of eCAMs in the VF microcirculation and that this process initiates the trafficking of pro-oxidative inflammatory cells into VF depots. Accordingly we studied the impact of HFMs on leukocyte-endothelium interactions, leukocyte infiltration, and leukocyte enzymatic activity within the adipose tissue microcirculation. We demonstrate that consumption of a HFM causes postprandial upregulation of endothelial expressed Psel with subsequent infiltration and enzymatic activity of innate immune cells in visceral adipose depots, but not in subcutaneous ones. We also establish that genetic ablation of Psel or MPO prevents postprandial leukocyte trafficking in response to HFMs.

Materials and Methods

Animal Models

All experiments were approved by Institutional Animal Care and Use Committee (IACUC) at Temple University and were in accordance with approved protocols. Male C57BL/6J (Jackson Laboratory Stock #000664, Bar Harbor, ME) mice were used to study normal physiological responses to dietary challenges. Psel deficient (Psel$^{-/-}$) mice (Jackson Laboratory Stock #002289) were acquired to determine the role of Psel in tissue homeostasis in the face of lipid overload. MPO$^{-/-}$ mice (Jackson Laboratory Stock #004265) were acquired to determine whether MPO activity is required for sustained

endothelial activation in the adipose tissue microcirculation. All mice were aged 8-12 weeks and had an average body weight of 25 g at the time of experiments. The mice were maintained on a 12 hour light-dark cycle in the animal facility at Temple University.

Diets Administered

Detailed diet composition information is listed in Table 1 and Table 2. Food was delivered to mice in ***liquid*** form or ***chow*** form. ***Liquid Diet***: Low-fat (Research Diets Inc. D12450BL, 10% fat) and high-fat (Research Diets Inc. D12492L, 60% fat) powder food was suspended in water, warmed, and shaken until homogenous before feeding. Isocaloric (1.5kcal), 0.7mL liquid meals were administered by oral gavage. A 1.5kcal load is the caloric equivalent of a small snack considering that C57BL/6 mice eat approximately 12-13 kcals per day (Albanes 1987, Petro et al 2004). The 0.7mL volume was selected due to the stomach volume of the mice (Wolfensohn & Lloyd 1994) and to keep the liquid meal viscosity low. ***Chow Diet***: Mice were given ad libitum access to water and low-fat diet (LFD) (Research Diets Inc. D12450B 10% fat) or HFD (Research Diets Inc. D12492 60% fat) in pellet form. There was no difference in macronutrient composition between the liquid or pellet forms of the LFD or HFD.

Time Course Studies

Mice were administered a ***single meal*** or ***repeated meals***. ***Single meal***: Single, liquid meals were administered to healthy mice to examine purely macronutrient-induced changes in endothelial cell and immune cell behavior. Mice were examined 1, 2, 3, and 4 hours after oral gavage. These early time points provide insight into the function of endothelium expressed selectin family proteins: rapidly translocated Psel and *de novo* synthesized Esel. The preparation of mice for intravital microscopy required an hour of work, making 1 hour

Table 1

LFD macronutrient and fatty acid profile

Nutrient	Mass (mg) in 1.5 kcal meal	Kcals (%)
Total Protein	74.88	20.00
Total Carbohydrate	262.47	70.00
Sucrose	131.24	35.00
Total Fat	16.77	10.00
SATs	3.81	2.28
Palmitic acid	2.49	1.50
Stearic acid	1.19	0.71
MUFAs	5.01	3.01
Palmitoleic acid	0.12	0.07
Oleic acid	4.84	2.90
PUFAs	7.95	4.77
Linoleic acid	7.02	4.21

Table 2

HFD macronutrient and fatty acid profile

Nutrient	Mass (mg) in 1.5 kcal meal	Kcals (%)
Total Protein	75.98	20.00
Total Carbohydrate	76.27	20.00
Sucrose	27.08	6.80
Total Fat	101.21	60.00
SATs	32.39	19.43
Palmitic acid	19.84	11.91
Stearic acid	10.70	6.42
MUFAs	36.33	21.80
Palmitoleic acid	1.35	0.81
Oleic acid	34.44	20.66
PUFAs	32.39	19.43
Linoleic acid	29.07	17.44

post-gavage the earliest time point observable. ***Repeated meals***: Mice were given *ad libitum* access to water and chow diet for 24 hours to study the effects of repeat exposure to lipid overload in healthy animals. Chow diet was delivered in the morning.

Plasma FFA Analysis

Plasma samples were collected from WT mice 3 hours following a single liquid meal. Frozen samples were sent to the Vanderbilt University Mouse Metabolic Phenotyping Center for FFA quantification according to standard protocols.

Leukocyte-endothelium Interactions Quantification By Intravital Microscopy

Mesenteric VF: Following intraperitoneal (i.p.) administration of 80mg/kg pentobarbital, mice were prepared for intravital microscopy studies as previously described (England et al 2013). A polyethylene catheter was inserted in the right carotid artery to monitor mean arterial blood pressure (MAP). Four distal loops of ileal tissue, exteriorized through a midline laparotomy, were superfused with 37ºC Krebs-Henseleit buffer in an intravital microscopy Plexiglas chamber attached on the stage of an Eclipse FN1 Physiostation Microscope (Nikon Corp., Japan). A relatively straight, unbranched segment of postcapillary venule with a length >100µms and a diameter between 25 and 40µms was randomly studied in each mouse. Leukocytes were stained with Rhodamine 6G and illuminated with a mercury bulb light source. Observation of the mesenteric microcirculation was made with 20x salt water-immersion lens. Images were projected by high-resolution, intensified video cameras (XR Mega-10 EX ICCD; Stanford Photonics INC, CA) onto a high-resolution, color video monitor (Multiscan 200-sf, Sony), and the image recorded on A WIN XP Imaging Workstation. All data were analyzed using computerized imaging software (Micro-Manager, Vale Lab UCSF, CA). Leukocyte rolling

and leukocyte adhesion were studied in MPCVs. Leukocyte rolling is defined as the number of leukocytes rolling past a fixed point per minute; leukocyte adhesion is defined as the number of leukocytes firmly adhered to 100μm length of endothelium for at least 30 seconds.

Subscapular SF: Following anesthesia administration and right carotid artery canulation, the subcutaneous subscapular fat was exposed. Subscapular postcapillary venules were identified by the same criteria as MPCVs. Leukocyte rolling and leukocyte adhesion was quantified using equal standards to quantification in the VF. Exposed subcutaneous tissues were superfused with 37ºC Krebs-Henseleit buffer.

Endothelial Psel Activation

Immunohistochemical localization of Psel was determined using mAb PB1.3 as previously reported (Scalia et al 1997). To determine if high-fat feeding induced leukocyte accumulation within adipose depots is a Psel dependent phenomena, WT mice were gavaged with LFD or HFD. Following oral gavage, mice were perfused with co-staining antibodies to endothelial cell marker CD31 and PB1.3, an antibody specific to surface expressed, active Psel. Epididymal VF and subscapular SF depots were excised and prepared using a whole mount staining assay.

Invading Immune Cell Differentiation Analysis

Epididymal VF samples and subscapular SF samples were collected from mice 2 and 4 hours following single meals and after 24 hours of ad libitum access to LFD or HFD to quantify leukocyte infiltration into adipose depots. Samples were finely minced then further digested in a collagenase type 1 solution. The stromal vascular fraction was isolated by centrifugation and fluorescently stained with antibodies to determine the presence of

CD11B positive innate immune cells (Becton Dickinson, Franklin Lakes, NJ) and CD4 positive lymphocytes (Becton Dickinson). VF samples were also stained with antibodies specific to Ly6g positive neutrophils (Becton Dickinson) and F4/80 positive macrophages (Abcam, Cambridge, UK). Samples were loaded into a Becton Dickinson FACScan machine and quantified using Cell Quest Pro software. Data analysis was performed using FlowJo 8.7 (FlowJo, LLC, Ashland, Oregon).

Identification of Fat Infiltrated Immune Cell Location

To determine where leukocytes were taking up residency in adipose tissue, mice were given *ad libitum* access to low-fat or high-fat chow diet for 3 days. Mice were anesthetized with an intraperitoneal dose of sodium pentobarbital and canulated via the right carotid artery. Mice were perfused with 4% paraformaldehyde (PFA). Epididymal VF was excised and submerged in 4% PFA overnight in 4ºC. Samples were dehydrated and embedded in paraffin wax. Tissue slices were stained using fluorescent antibodies specific to Ly6g positive neutrophils (Becton Dickinson) and caveolin (Becton Dickinson). Slides were viewed using a Nikon FN1 Eclipse microscope and a Nikon Coolpix 4500 camera. Images were analyzed using ImageJ software (National Institutes of Health, Bethesda, MD).

MPO Quantification

To measure MPO accumulation in fat tissue, epididymal VF and subscapular SF samples were excised from mice 2 hours after a single liquid meal. Fat samples were minced, digested in a collagenase type 1 solution, and centrifuged. The stromal vascular fraction was loaded onto polyacrylamide gels for western blot analysis. An antibody against the MPO dimer (Santa Cruz, sc-16129) was used for quantification of MPO protein

and an antibody against actin (Cell Signaling, 8H10D10) was used as a loading control. Densitometry was determined using ImageJ software.

MPO activity within the epididymal visceral adipose tissue was quantified. Isolated stromal vascular fractions were collected then suspended in a 10% w/v HTAB buffer as previously described (Mulane et al 1985). Samples were homogenized 4 times for 30 seconds and put through 3 freeze-thaw cycles using liquid nitrogen and a 37 ºC water bath. Homogenized samples were then centrifuged at 21,000 g for 30 minutes at 4ºC. The volume of the supranatant, which contains the MPO enzyme, was measured and the pellet discarded. MPO activity was assayed by mixing 10 µL of sample (supranatant) with 190 µL of ODI solution and 0.0005% H_2O_2. The absorbance at 450nm was measured every 15 seconds for 2 minutes with a Bio-Tek Instruments FLX 800 Microplate Fluorescence Reader and KC4 software spectrophotometer.

Statistics

Data are represented as mean ± SEM. The Student's T test was used to analyze plasma FFAs, leukocyte-endothelium interactions, percent of fluorescent staining in venules, MPO accumulation, and MPO activity. Two-way ANOVA was used to analyze immune cell infiltration. Statistical analysis was set such that *=$p<0.05$ **=$p<0.01$ ***=$p<0.001$.

Results

A Single HFM Increases Plasma FFAs

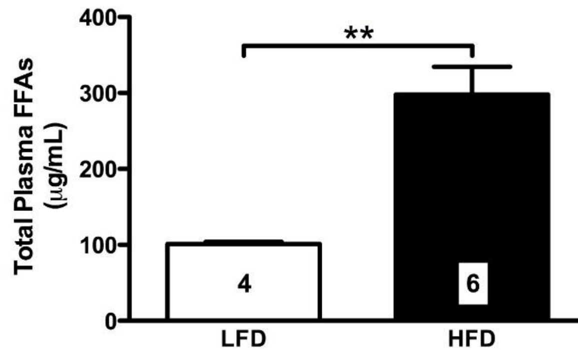

Figure 3: Single HFMs cause postprandial lipemia

Plasma FFA quantification verified that a single HFM induces postprandial lipemia. Blood samples were collected from mice 3 hours after a single liquid meal. Plasma was isolated and lipid analysis was performed by gas chromatography. A single HFM caused a 3-fold increase in mouse plasma FFA concentrations compared to low-fat gavaged controls (**Figure 3**). Thus, the high-fat liquid meal composition used in our study significantly increases postprandial lipid levels in the blood.

Single HFMs Transiently Increase Leukocyte-Endothelium Interactions in MPCVs

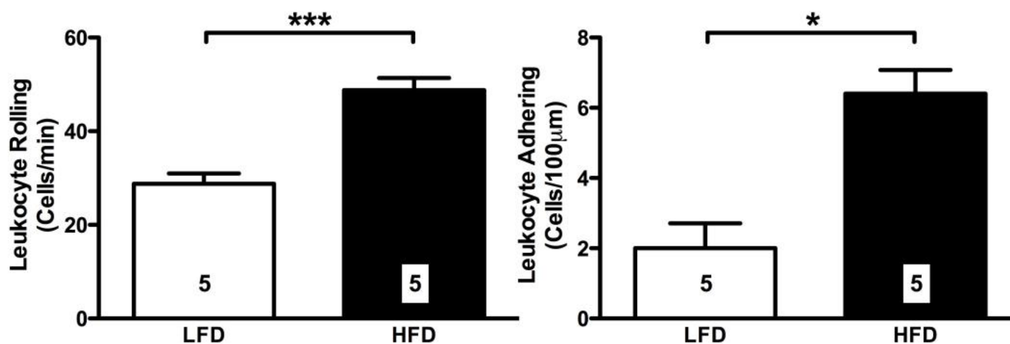

Figure 4: Lipid overload rapidly increases leukocyte-endothelium interactions in MPCVs

A single HFM induces a rapid increase in VF microcirculation leukocyte-endothelium interactions. WT mice were administered a single liquid meal prior to

leukocyte-endothelium interaction quantification by intravital microscopy in the MPCVs. One hour following a single HFM, leukocyte rolling increased 2-fold (**Figure 4, left**) and leukocyte adhesion increased 3-fold (**Figure 4, right**) from baseline. Administration of a LFM did not increase in leukocyte-endothelium interactions. The high-fat liquid meal rapidly activates endothelial cells within the VF microcirculation.

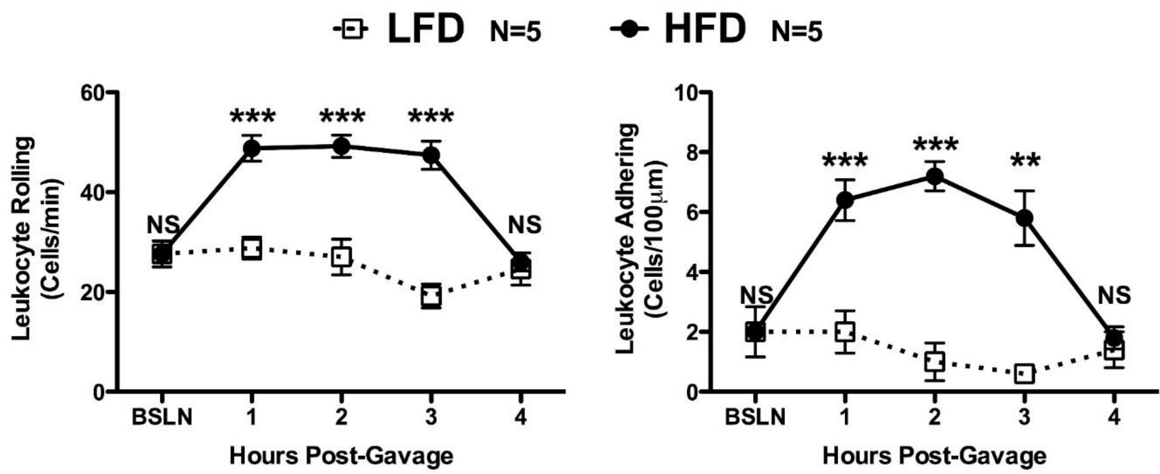

Figure 5: A single HFM transiently elevates leukocyte-endothelium interactions in MPCVs

The inflammatory response to acute lipid overload is transient. Leukocyte-endothelium interactions in the MPCVs were quantified in mice without dietary challenge to establish baseline leukocyte rolling and leukocyte adhesion values. Immune cell kinetics were then quantified in mice 1, 2, 3, and 4 hours following administration of single liquid meals to establish the temporal relationship between nutrient absorption and the pro-adhesive phenotype of the VF endothelium. Rolling and adhering leukocytes were quantified 1, 2, 3, and 4 hours after a single liquid meal. The number of both rolling (**Figure 5, left**) and adhering (**Figure 5, right**) leukocytes remained at peak levels 2 and 3 hours following administration of a HFM. The elevated leukocyte rolling and leukocyte adhesion counts returned to at baseline levels 4 hours after high-fat gavage. The LFM did not

increase leukocyte-endothelium interactions, verifying that the mechanical stress of the liquid load did not cause elevations in leukocyte trafficking.

Repetitive High-fat Feeding Prolongs Lipid-Induced Leukocyte-Endothelium Interactions in MPCVs

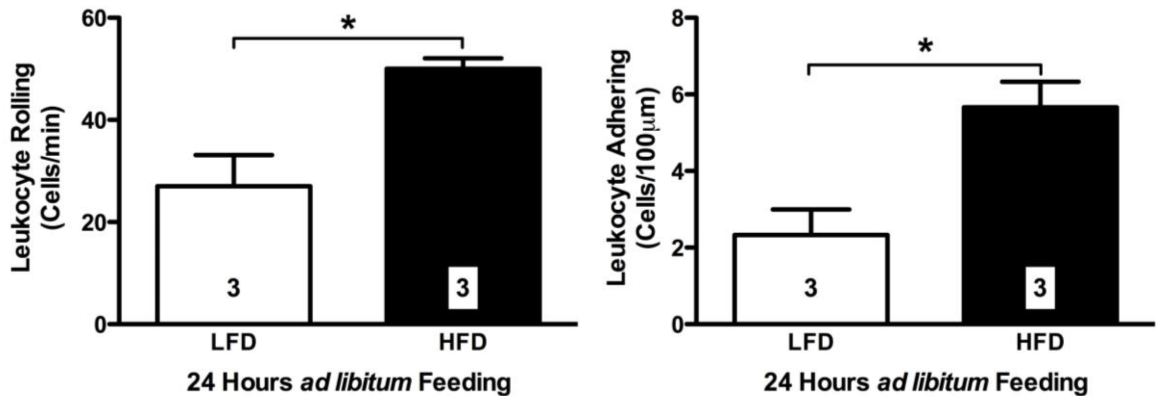

Figure 6: Repetitive high-fat feeding maintains elevated leukocyte-endothelium interactions in MPCVs

Elevated leukocyte-endothelium interactions are sustained by repetitive HFD consumption. Mice given *ad libitum* access to low or high-fat chow diet for 24 hours were examined by intravital microscopy. Rolling (**Figure 6, left**) and adhering (**Figure 6, right**) leukocytes increased 2-fold in the MPCVs of high-fat fed mice compared to low-fat fed controls. Interestingly, prolonged exposure to HFD did not increase leukocyte-endothelium interactions above the number recorded in mice fed single HFMs (**Figure 5**), suggesting a limit to hyperlipemia induced leukocyte trafficking through the VF microcirculation.

Consecutive HFMs result in elevated leukocyte-endothelium interactions in the mouse MPCVs. Mice were administered two, consecutive high-fat oral gavages with a 5 hour delay between gavages. We chose to stagger administration of the high-fat loads by 5 hours to allow for leukocyte-endothelium interactions in the MPCVs to return to baseline values (**Figure 5**). WT mice gavaged twice with HFD showed increased leukocyte-

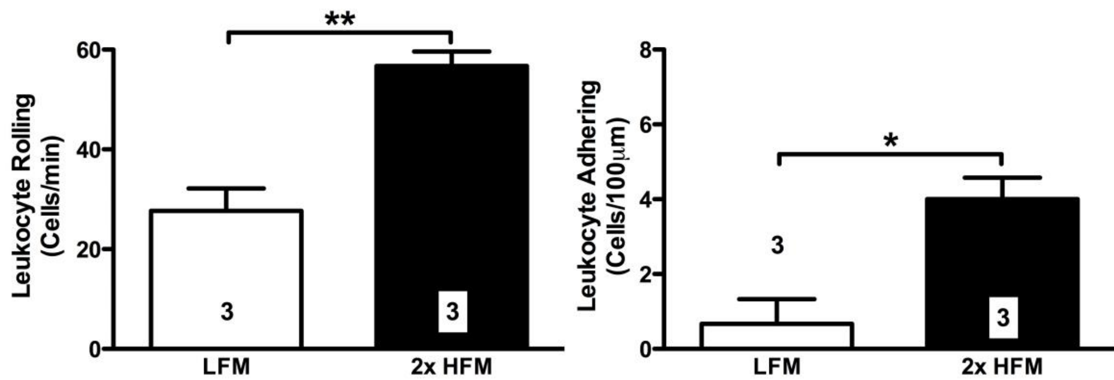

Figure 7: Consecutive lipid overloads sustain elevated leukocyte-endothelium interactions in MPCVs

endothelium interactions in the VF microcirculation equal to their single gavaged or 24 hour chow fed counterparts (**Figure 7**). Thus, repeated exposures to high-fat diet cause repeated inflammatory responses.

Lipid Overload Causes VF but Not SF Low-grade Immune Responses

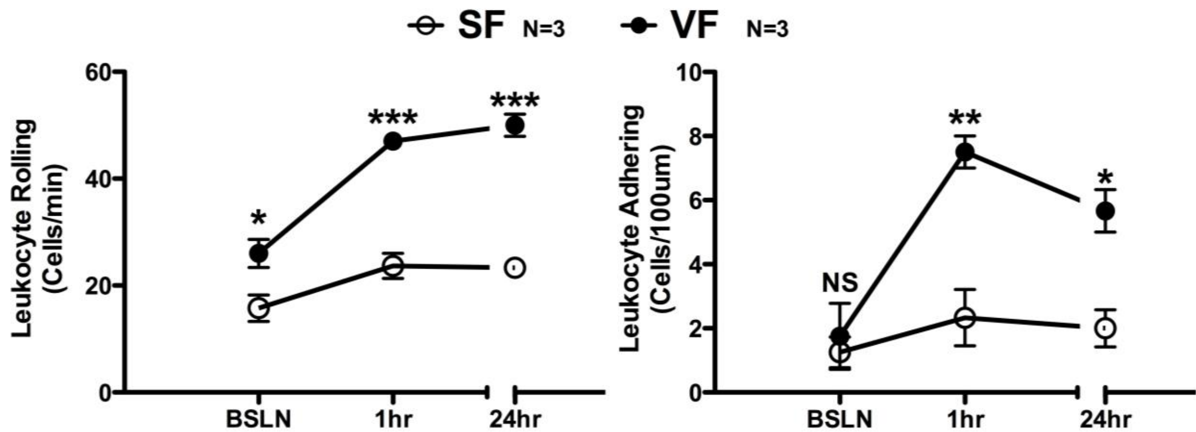

Figure 8: Lipid overload increases leukocyte-endothelium interactions in the VF but not the SF

Lipid overload does not significantly increase leukocyte-endothelium interactions in the SF. WT mice were prepared for intravital microscopy observation of the subscapular adipose depot to establish baseline leukocyte rolling and leukocyte adhesion values in the SF. This experiment was repeated in mice 1 hour after a single HFM and after 24 hours of

high-fat feeding. Postprandial lipemia did not induce significant changes in leukocyte rolling (**Figure 8, left**) or leukocyte adhesion (**Figure 8, right**) within the SF microcirculation. Acute, HFD-induced increases in leukocyte-endothelium interactions are local to the VF but not the SF.

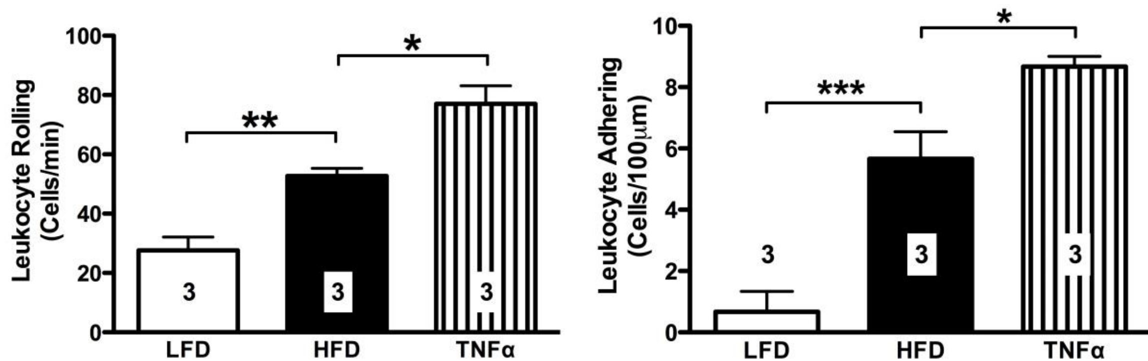

Figure 9: Lipid overload causes a low-grade immune response

HFD is not the strongest pro-inflammatory stimulus in the VF. WT mice were i.p. injected with 20ng/g TNFα 4 hours before recording leukocyte-endothelium interactions in the MPCVs by intravital microscopy. A 4 hour gap between TNFα delivery and quantification of leukocyte rolling and leukocyte adhesion was chosen to allow for transcription induced increases eCAMs (Kansas 1996). The number of leukocyte rolling and leukocyte adhesion was significantly higher in the TNFα injection group than the single HFM group (**Figure 9**). HFD exposure elicits a relatively low inflammatory response in the VF compared to a potent pro-inflammatory cytokine.

Acute Lipid Overload Causes Psel Translocation in VF But Not SF

Psel is rapidly expressed on the luminal surface of the VF microcirculation but not the SF microcirculation following lipid overload. Mesenteric VF samples were excised from mice administered a single liquid meal for whole mount co-staining with the

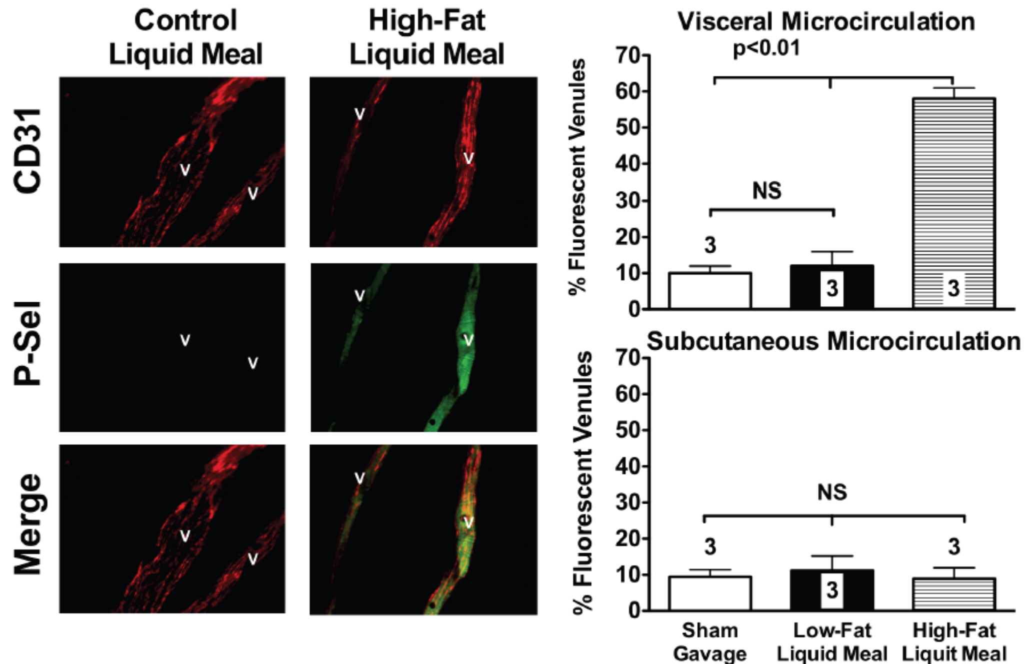

Figure 10: VF Psel is activated by lipid overload

endothelial cell marker CD31 and mAb PB1.3, an antibody specific to surface expressed, active Psel. Within the VF microcirculation, dietary fat exposure caused a 6-fold increase in Psel activity in comparison with low-fat gavaged mice (**Figure 10, top histogram**). No HFM-induced changes in Psel activation were recorded in the subcutaneous adipose tissue (**Figure 10, bottom histogram**). These data provide insight into why we see a robust increase in VF leukocyte-endothelium interactions and no change in SF immune cell activity (**Figure 8**). Why Psel does not respond to lipid overload in SF is currently unknown and further studies are necessary to understand how nutrient overload affects the microcirculation in different fat depots.

Lipid Overload Causes Neutrophil Accumulation in the VF

HFD exposure increases leukocyte infiltration of the VF. Stromal vascular fractions were isolated from epididymal and subscapular adipose depots excised from WT mice to

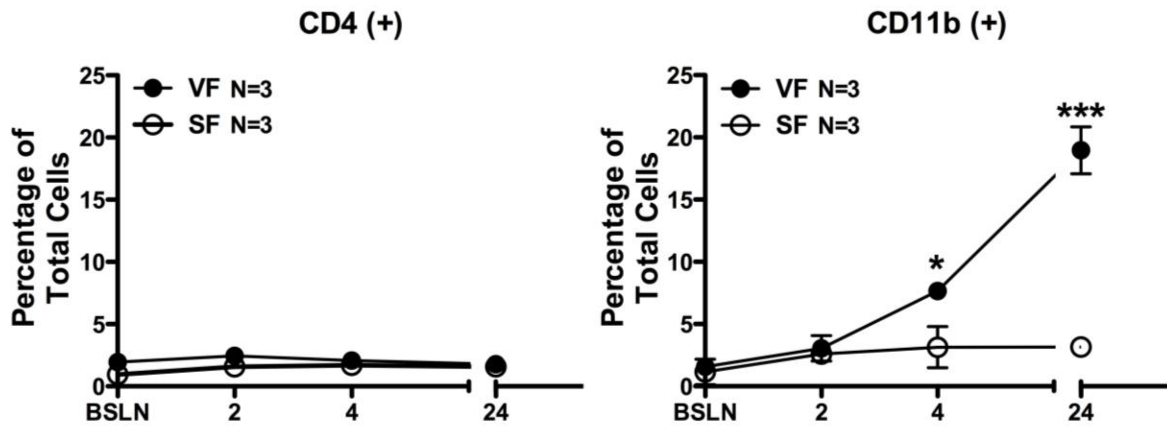

Figure 11: Innate immune cells selectively infiltrate VF but not SF after lipid overload

establish local baseline lymphocyte and leukocyte populations. Changes in immune cell infiltration were then checked 2 and 4 hours after a single HFM and after 24 hours of *ad libitum* high-fat feeding. Lymphocyte infiltration did not change in the VF or the SF (**Figure 11, left**). Leukocyte infiltration was significantly elevated in the VF 4 hours after a single HFM compared to baseline and continued to increase with repetitive HFD consumption (**Figure 11, right**). High-fat feeding increases innate immune cell accumulation in the VF.

Neutrophils are the primary VF infiltrating leukocyte following HFD consumption. Stromal vascular fractions isolated from the VF of WT mice were stained with antibodies specific to monocytes and neutrophils to identify the specific leukocytes invading VF following HFD consumption. No significant increases in monocyte staining were recorded in the VF within 24 hours of HFD exposure (**Figure 12, left**). Neutrophil staining in the stromal vascular fraction rose with prolonged HFD exposure, reaching significance at 4 hours after a single HFM and continuing to increase with 24 hours of *ad libitum* access to HFD (**Figure 12, right**). Thus, acute lipid overload causes neutrophil infiltration of the VF but not the SF.

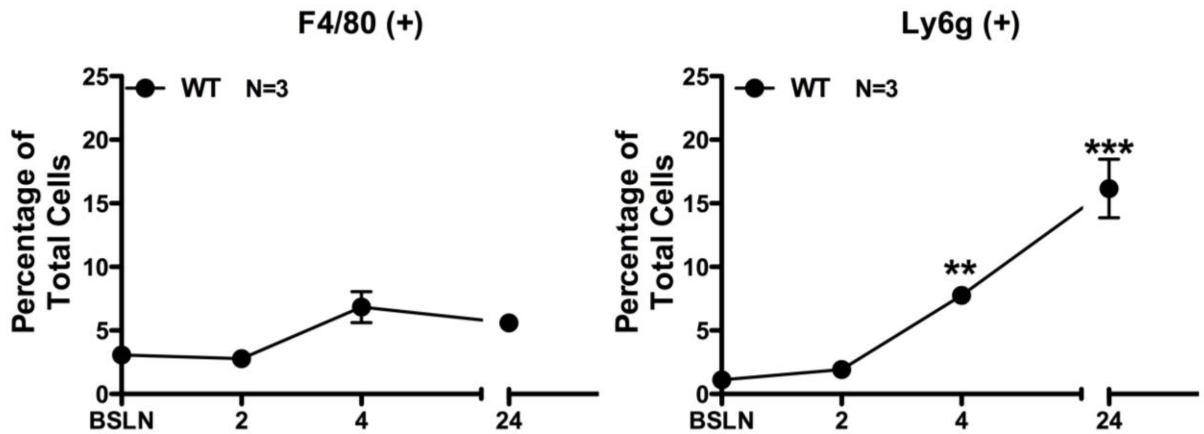

Figure 12: Neutrophil accumulation in the VF begins after a single HFM

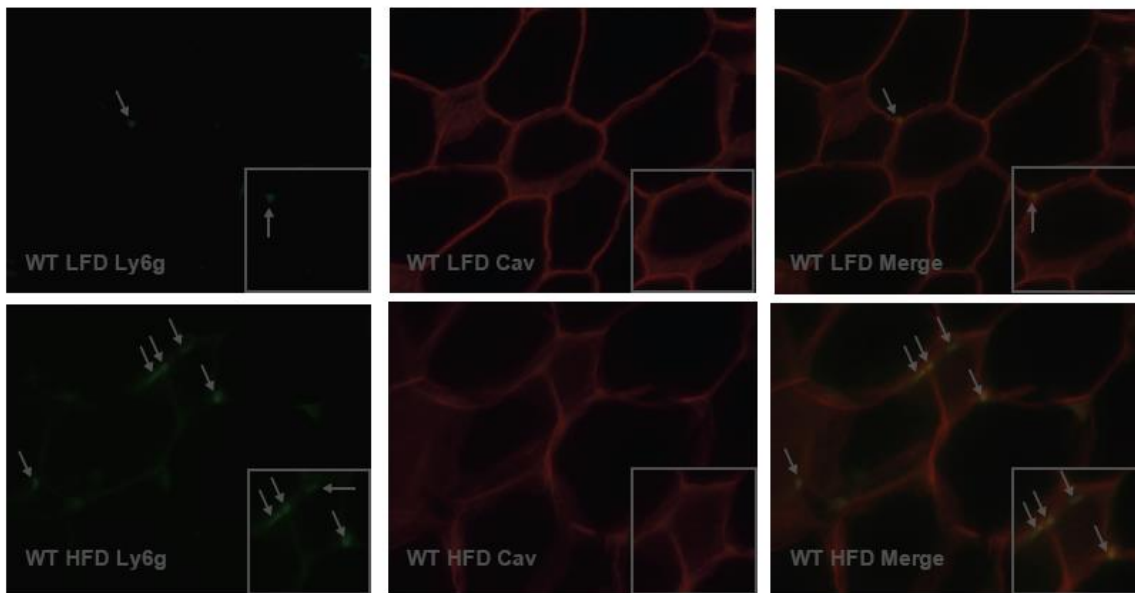

Figure 13: Neutrophils accumulate in extracellular spaces among adipocytes in VF

Neutrophils take up residency in the extracellular spaces between VF adipocytes following HFD consumption. WT mice were given ad libitum access of LFD or HFD for 3 days before epididymal VF samples were collected for immunofluorescence studies. LFD fed mice showed nearly no neutrophil staining in the VF (**Figure 13, top row**). HFD fed mice had neutrophil infiltration in the extracellular spaces between adipocytes in the VF

(**Figure 13, bottom row**). These results corroborate previous reports that leukocytes claim residency in the extracellular spaces forming "crown-like structures" (Cinti et al 2005).

Lipid Overload Increases the Accumulation and Activity of MPO

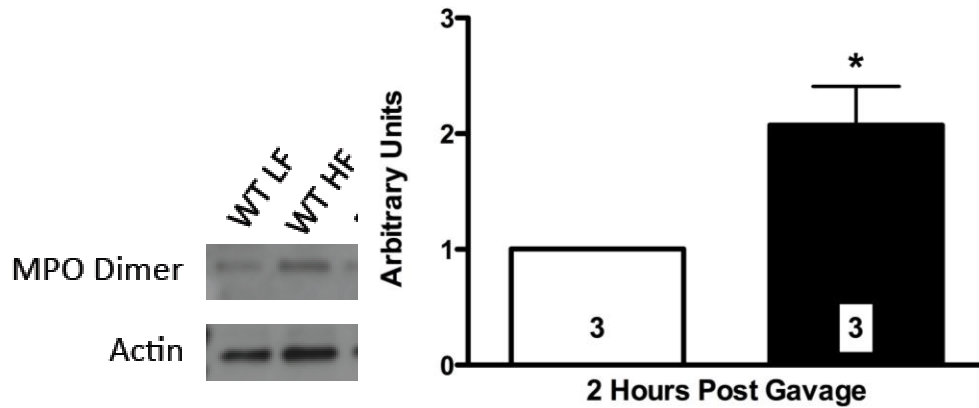

Figure 14: MPO accumulates in the VF following a single HFM

Neutrophilic MPO accumulates in the VF stromal vascular fraction following a single HFM. VF samples were excised from WT mice 2 hours after oral gavage. Stromal vascular fractions were analyzed by western blot and an MPO dimer antibody was used to quantify accumulation of MPO. Administration of the high-fat liquid meal caused a 2-fold increase in MPO accumulation in the VF stromal vascular fraction as compared to the isocaloric low-fat liquid meal (**Figure 14**). Interestingly, MPO accumulation in the stromal vascular fraction occurs earlier than significant neutrophil accumulation in the VF (**Figure 12, right**).

Single lipid overloads induce elevated MPO activity in the VF stromal vascular fraction of WT mice. The stromal vascular fraction was isolated from excised VF of mice administered a single liquid meal. MPO activity was significantly increased 2 hours following lipid overload in the VF as compared to low-fat gavaged controls (**Figure 15**). The timing of increased MPO activity compliments our reported increase in MPO

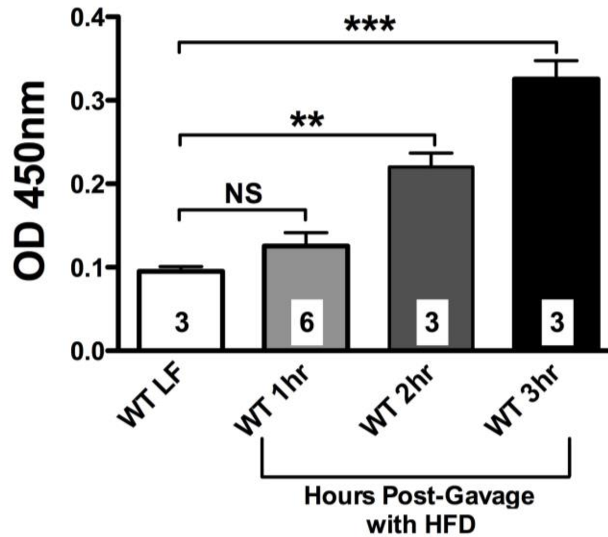

Figure 15: MPO activity increases in the VF following a single HFM

accumulation (**Figure 14**). Thus, acute lipid overload increases the accumulation and activity of the chlorinating enzyme MPO.

FFA Superfusion of the Mesentery Causes a Rapid Increase in Leukocyte-Endothelium Interactions

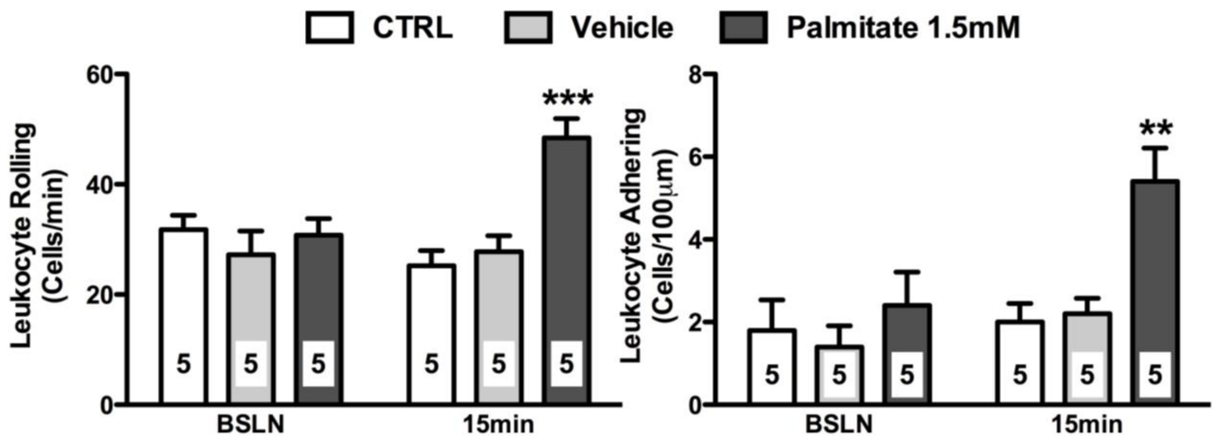

Figure 16: Leukocyte-endothelium interactions are increased in MPCVs after only 15 minutes of lipid superfusion

Palmitate superfusion causes activation of MPCV endothelial cells within 15 minutes. WT mice were prepared for intravital microscopy without dietary perturbation. Baseline leukocyte rolling and leukocyte adhesion values were recorded immediately

before superfusion of a palmitate solution over the mesenteric vasculature. Leukocyte-endothelium interactions in the MPCV were significantly increased 15 minutes after introduction of the palmitate complex compared to vehicle superfused controls (**Figure 16**). These *in vivo* results support previously published *in vitro* data which demonstrate Psel translocation requires mere minutes upon perturbation (Geng et al 1990). Presently, only palmitate induction of leukocyte-endothelium interactions has been tested.

Psel Deletion Blocks Lipid-Induced Leukocyte-Endothelium Interactions in MPCVs

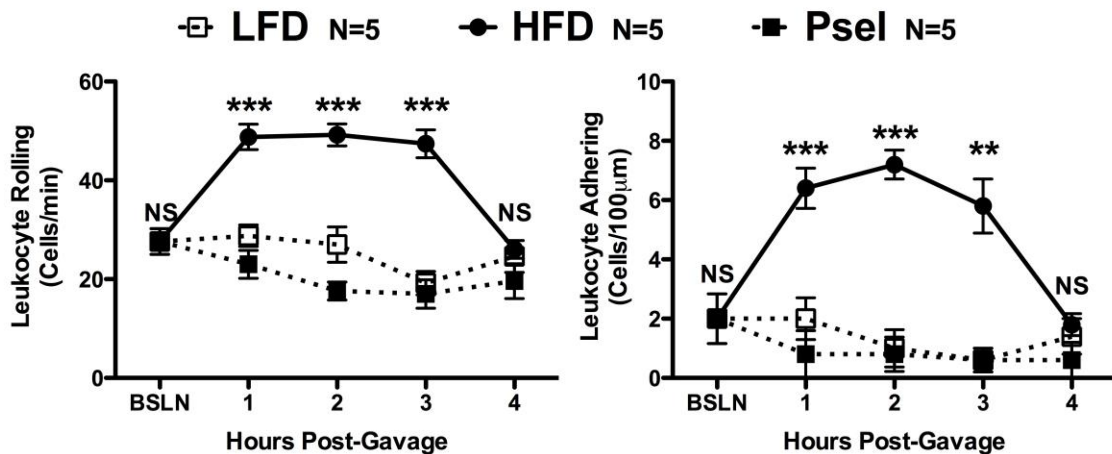

Figure 17: Psel deletion prevents single HFM induced leukocyte-endothelium interactions in the VF

Psel deletion protects mice from single HFM induced increases in MPCV leukocyte-endothelium interactions. Leukocyte rolling and leukocyte adhesion were quantified by intravital microscopy in the MPCV of Psel$^{-/-}$ mice administered single HFMs by oral gavage. Psel deletion prevented lipid induced increases in MPCV leukocyte-endothelium interactions within 4 hours of fat overload (**Figure 17**). These data suggest that the fast increase in leukocyte rolling and leukocyte adhesion following HFD consumption is a Psel mediated response.

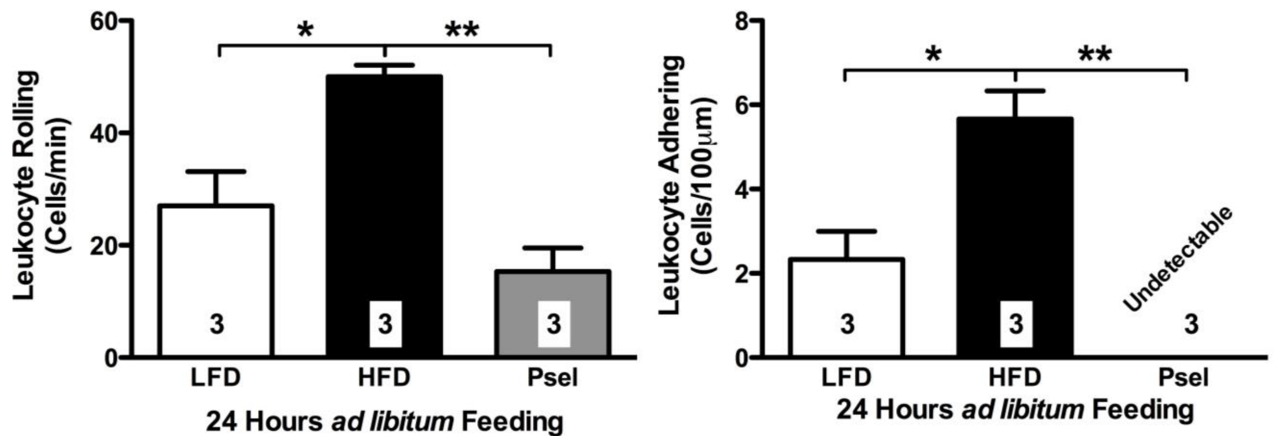

Figure 18: Psel deletion prevents repetitive high-fat feeding induced leukocyte-endothelium interactions in the VF

Ablation of Psel blocks elevations in leukocyte-endothelium interactions in the face of repetitive high-fat feeding. Leukocyte-endothelium interactions were quantified in the MPCV of Psel$^{-/-}$ mice following 24 hours of *ad libitum* access to HFD by intravital microscopy. Psel$^{-/-}$ mice were protected from prolonged HFD consumption-induced leukocyte-endothelium interactions (**Figure 18**). Psel deletion offers protection from the repetitive high-fat feeding induced inflammatory reactions. The expression of eCAMs other than Psel (e.g. Esel) was not quantified.

Psel Deletion Prevents Lipid-Induced Leukocyte Accumulation in the VF

Psel deletion prevents lipid overload induced leukocyte infiltration of the VF. Stromal vascular fractions isolated from the VF of Psel$^{-/-}$ mice were stained with antibodies specific to monocytes and neutrophils to identify the specific leukocytes invading VF following HFD consumption. No changes in lipid induced leukocyte accumulation were recorded in Psel$^{-/-}$ mice (**Figure 19**). Thus, leukocyte infiltration of the VF in response to lipid overload is a Psel dependent process.

Psel deletion does not influence where infiltrated neutrophils reside in VF. Psel$^{-/-}$ mice given 3 day *ad libitum* access to HFD before epididymal VF samples were excised

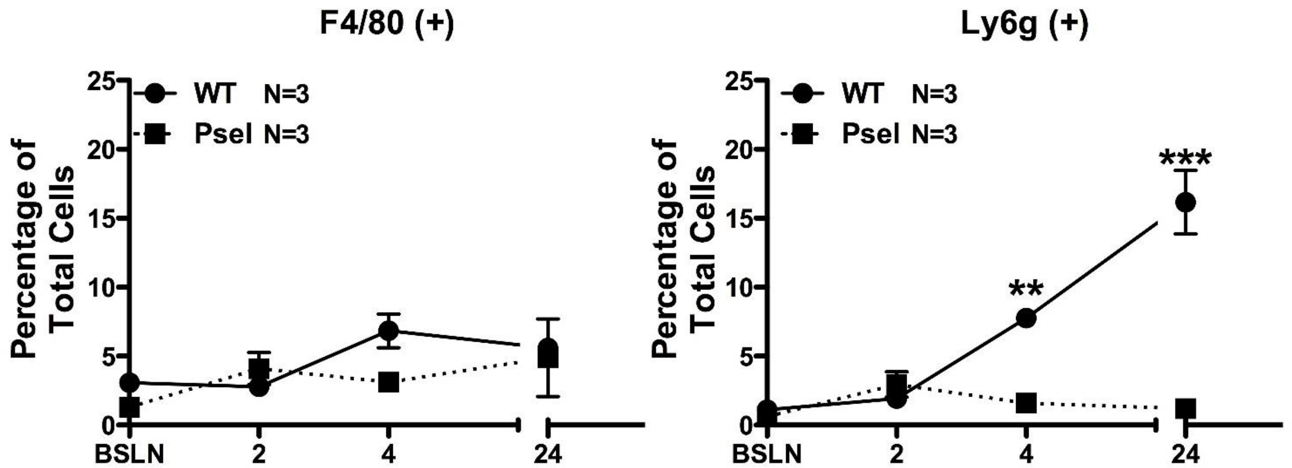

Figure 19: Psel deletion prevents lipid overload induced immune cell infiltration in VF

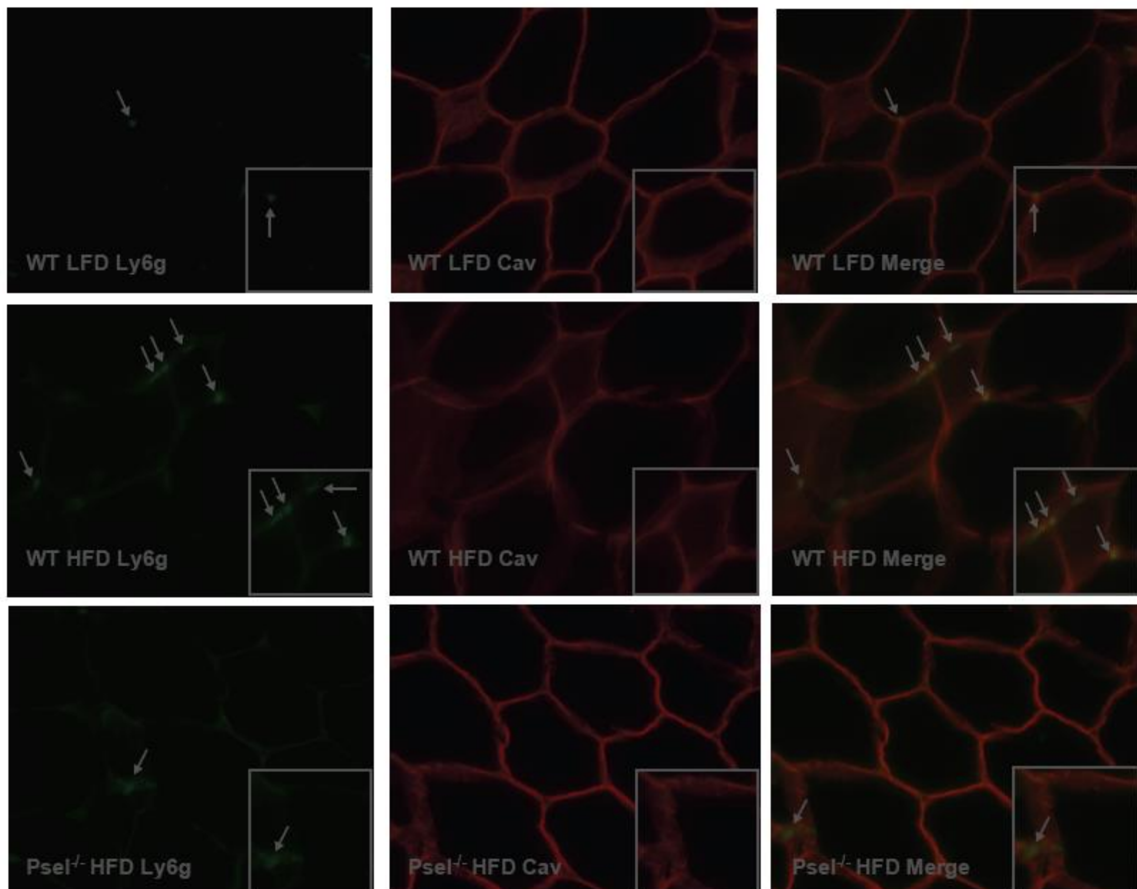

Figure 20: Neutrophil residency in VF extracellular spaces is Psel independent

for immunofluorescence studies. Neutrophil staining in the VF of high-fat fed Psel$^{-/-}$ mice, while diminished, still appeared in the extracellular spaces among adipocytes (**Figure 20,**

bottom row). Psel impacts the number of infiltrating leukocytes, not where invaded immune cells reside.

Psel Deletion Prevents Lipid Induced Increases in MPO Accumulation and Activity

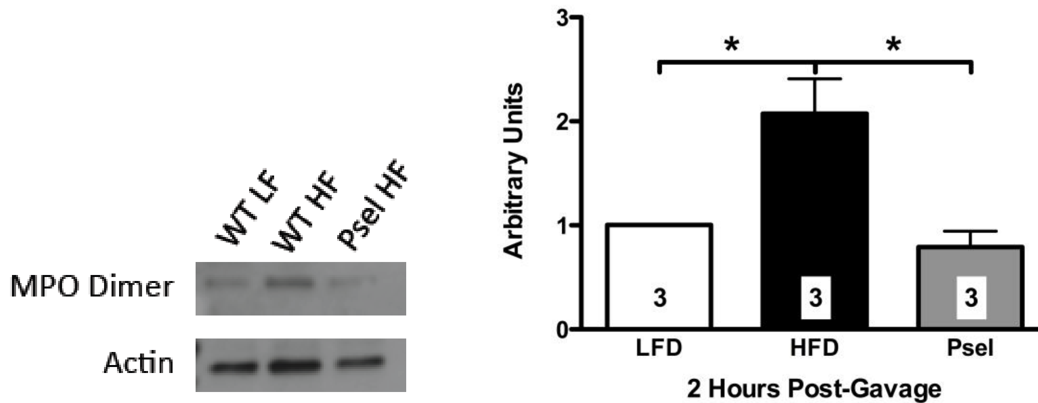

Figure 21: Psel deletion blocks HFM induced increases in VF MPO accumulation

Psel deletion blocks the accumulation of MPO in the VF stromal vascular fraction following a single HFM. Stromal vascular fractions of $Psel^{-/-}$ mice were analyzed by western blot and an MPO dimer antibody was used to quantify accumulation of MPO. Lipid overload failed to increase MPO accumulation above control levels following HFD in $Psel^{-/-}$ mice (**Figure 21**). These data, in conjunction with a previous report (Lorant et al 1993), suggest that Psel plays a key role in granulocyte exocytosis of stored enzymes.

Ablation of Psel prevents lipid overload induced activation neutrophilic MPO. The stromal vascular fraction was isolated from excised VF of $Psel^{-/-}$ mice administered a single liquid meal. MPO activity was diminished below basal levels by Psel deletion (**Figure 22**). A direct relationship between CAMs and MPO function is currently unknown.

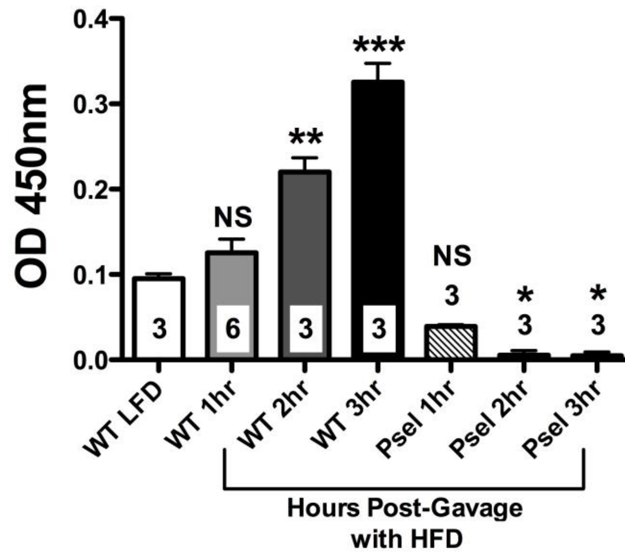

Figure 22: Psel deletion prevents basal MPO activity in the VF

MPO Deletion Prevents Immune Responses to Lipid Overload in the VF

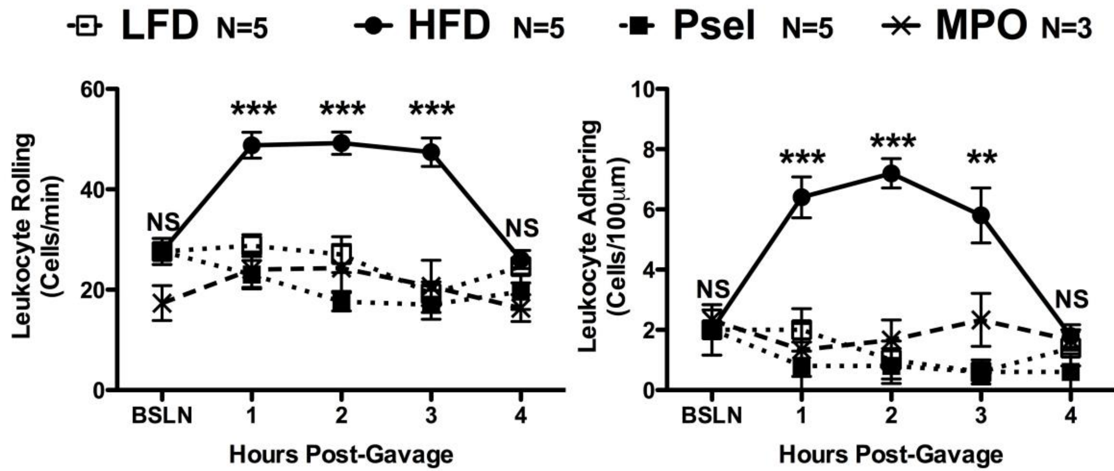

Figure 23: MPO deletion protects the VF from single HFM induced leukocyte-endothelium interactions

Deletion of the neutrophilic enzyme MPO prevents lipid overload induced leukocyte-endothelium interactions in the VF. Leukocyte rolling and leukocyte adhesion were quantified by intravital microscopy in the MPCV of $MPO^{-/-}$ mice administered single HFMs by oral gavage. MPO deletion prevented lipid induced increases in MPCV leukocyte-endothelium interactions within 4 hours of fat overload (**Figure 23**). MPO has

been implicated as a contributor to endothelial dysfunction (Golubinskaya et al 2014) but the relationship between MPO and eCAM expression has not been thoroughly examined. Further studies are required.

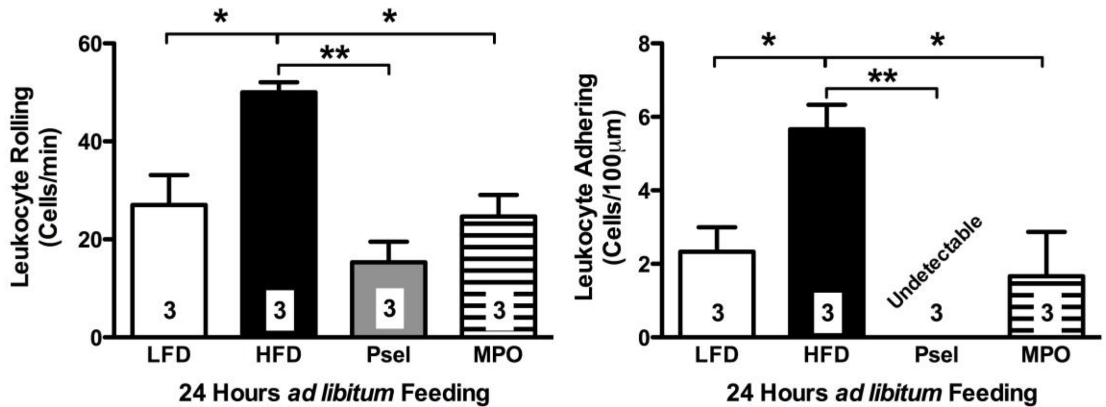

Figure 24: MPO deletion protects the VF from repetitive high fat feeding induced leukocyte-endothelium interactions

Ablation of MPO blocks elevations in leukocyte-endothelium interactions in the face of repetitive high-fat feeding. Leukocyte-endothelium interactions were quantified in the MPCV of $MPO^{-/-}$ mice following 24 hours of *ad libitum* access to HFD by intravital microscopy. $MPO^{-/-}$ mice were protected from repetitive HFD consumption-induced leukocyte-endothelium interactions (**Figure 24**). These results were unexpected as no direct link between MPO and the pro-adhesive phenotype of activated endothelial cells has been established.

Repetitive HFD consumption for 24 hours did not increase leukocyte infiltration in the VF of $MPO^{-/-}$ mice. Stromal vascular fractions isolated from the VF of $MPO^{-/-}$ mice were stained with antibodies specific to monocytes and neutrophils to identify the specific leukocytes invading VF following HFD consumption. No changes in lipid induced leukocyte accumulation were recorded in $MPO^{-/-}$ mice (**Figure 25**). The mechanism behind

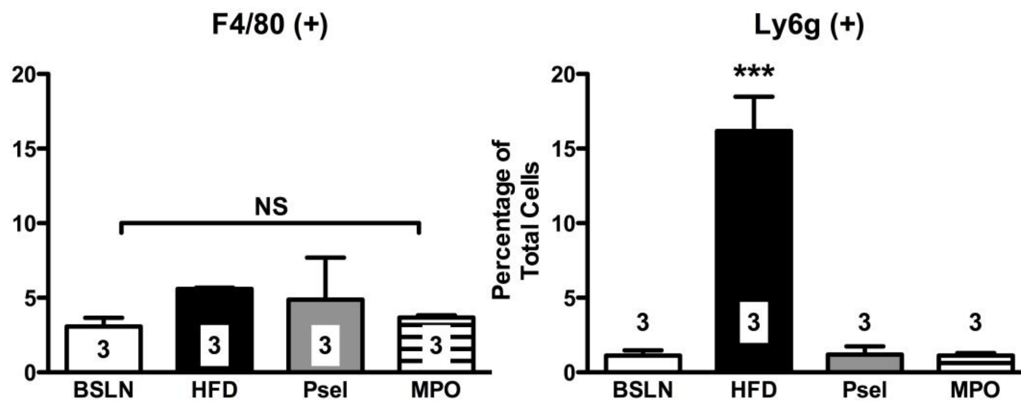

Figure 25: MPO deletion prevents lipid overload induced immune cell infiltration in VF

MPO disruption of leukocyte diapedesis is presently unknown. These results imply a novel role for MPO in the initial stages of immune cell extravasation. Further studies must be conducted before any conclusion can be drawn.

Discussion

We have characterized the direct relationship between nutrients and inflammation in healthy mice. By studying lean animals, our observations are not complicated by the multitude of metabolic changes associated with chronic models of obesity and insulin resistance. Our data corroborates human trials which describe the inflammatory nature of postprandial lipemia (de Vries et al 2014b). Following a single HFM, WT mice experience hyperlipemia (**Figure 3**) initiating a Psel dependent (**Figure 10**), rapid, but transient elevation in leukocyte-endothelium interactions (**Figure 5**) in the VF but not the SF (**Figure 8**). Indeed, a single HFM proved sufficient for modest increases in the accumulation of leukocytes in the VF (**Figure 11**). The prolonged inflammatory response reported in our double-gavage and 24 hour *ad libitum* feeding data strongly suggest that the influx of FFAs associated with HFD consumption is one pro-inflammatory trigger which activates the VF microcirculation (**Figures 6 & 7**). Repetitive consumption of HFD

caused a robust increase in neutrophil accumulation in the VF (**Figure 12**). The importance of Psel in hyperlipemia induced leukocyte infiltration is best demonstrated by the total lack of leukocyte influx and enzymatic activity in the VF of Psel$^{-/-}$ mice (**Figures 17-22**). Our data suggests a novel role for MPO in the initial stages of immune cell recruitment to the VF as well (**Figures 23-25**).

Whether nutrients are inherently inflammatory or an excess of nutrients is required to elicit an immune response is presently debated (Gregor & Hotamisligil 2011). Based on our single, liquid meal data, we determine that nutrients can be pro-inflammatory in the absence of caloric excess. While the kcal/gm of the low-fat and high-fat powders were unequal, an absolute 1.5kcal mass was measured and suspended in equal volumes of water. The difference in viscosity of the fluid was negligible. The isocaloric low-fat and high-fat liquid meals differed only in macronutrient composition. It should also be noted that a 1.5kcal load is not an especially large challenge and is certainly far from caloric excess, even in mice (Petro et al 2004). The different inflammatory responses to the two diets was robust and definitively one-sided: the HFM was pro-inflammatory while the LFM was immunologically inert. More examination is required to determine the various macronutrient thresholds for inflammation.

Two modes of endothelial activation have been described (Pan et al 1998). Short type activation relies on the ligation of g-protein coupled receptors by pro-inflammatory agents like such as histamine or thrombin. Cytosolic Weibel-Palade bodies which store Psel are rapidly translocated to the plasma membrane in a matter of minutes (Kansas 1996, Zimmerman et al 1985). After roughly 20 minutes, g-protein coupled receptors become desensitized to their agonist and the inflammatory signal is diminished (Gainetdinov et al

2004). Longer states of endothelial activation are achieved through cytokine induction of pro-inflammatory transcription factors. More specifically, leukocyte derived TNFα and IL-1 activate NF-κB and AP-1, resulting in the synthesis of eCAMs such as Esel, ICAM-1, and VCAM-1 in a process which can take hours before eCAMs are active (Pober & Sessa 2007). While we have not collected signaling data, the haste of leukocyte-endothelium interaction induction and the speed of leukocyte-endothelium interaction resolution in our WT mice fed a single HFM (**Figure 5**) and the equally quick surface expression of Psel (**Figure 10**) suggest that high-fat feeding results in a fast type inflammatory response in lean mice. The lipid superfusion experiment, in which leukocyte-endothelium interactions were elevated only 15 minutes after lipid exposure, supports this conclusion (**Figure 16**). Sustained low leukocyte-endothelium interaction values after 24 hours of HFD consumption in $Psel^{-/-}$ mice also indicate a lack of other eCAM expression (e.g. Esel), however this was not verified experimentally.

In recent years, neutrophils have been identified as important contributors to the early phases of fat feeding induced inflammation (Elgazar-Carmon et al 2008, Talukdar et al 2012). Independent groups demonstrated that neutrophil infiltration of WT mouse VF peaks no later than 3 days after initiating HFD consumption and that neutrophilic enzyme activity is also elevated. We have shown that neutrophils respond to high-fat feeding much faster than 3 days. A single HFM modestly increases neutrophil infiltration of the VF (**Figure 12**) and these neutrophils release their chlorinating enzyme MPO (**Figures 14 & 15**). After only 24 hours of *ad libitum* HFD consumption, WT mice had a robust increase in VF neutrophil accumulation (**Figure 12**). Several reports have argued a large contribution from neutrophils and neutrophilic enzymes like MPO (Wang et al 2014) and

elastase (Talukdar et al 2012, Mansuy-Aubert et al 2013) in promoting an inflammatory, insulin resistant phenotype. The metabolic consequences of neutrophilic enzyme activity in adipose tissue have been demonstrated in the chronic stage of high-fat feeding; our data demonstrate neutrophils exert their influence in healthy mammals following a single HFM, long before diet-induced obesity is established.

The timing mechanism of neutrophil degranulation and MPO release is still unclear. Previous reports have established that 70% of leukocytes observed by intravital microscopy are neutrophils (Hwang et al 2004). Also, ligation of neutrophilic β2 integrins by coupled Psel and platelet activating factor increases neutrophilic intracellular calcium concentrations, priming neutrophils for degranulation (Lorant et al 1993). Leukocyte-endothelium interactions were elevated 1 hour after a high-fat gavage (**Figure 4**). MPO activity in the VF stromal vascular fraction, however, was not significantly increased until 2 hours after a single HFM (**Figure 15**). The gap in time between elevated leukocyte-endothelium interactions and increased MPO activity suggests that neutrophil degranulation is dependent upon neutrophil rolling along the endothelium. FFA ligation of innate immune cell Toll receptors has been described (Shi et al 2006) but our data indicates that the FFAs associated with postprandial lipemia do not drive systemic neutrophil degranulation without CAM ligation. It must also be noted that MPO accumulation (**Figure 14**) and activity in the VF stromal vascular fraction is increased before significant neutrophil infiltration in the VF (**Figure 12**). It was previously asked how a distinction can be made between MPO released by neutrophils *in vivo* and MPO freed by neutrophil destruction during tissue digestion. We report elevations in VF MPO (**Figures 14 & 15**) that precede increased neutrophil infiltration in the VF (**Figure 12**).

The transient nature of the inflammatory response to a single HFM may have implications for food behavior. A single HFM was sufficient to cause a mild inflammatory response (**Figure 9**) with minor increases in neutrophil accumulation in the VF. A hopeful interpretation of the transient immune response is that occasionally eating pro-inflammatory (junk) food is safe if paired with a regular healthy diet. What was not examined was the length of time required for immune cell clearance after either a single HFM or 24 hours of *ad libitum* high-fat feeding. Interspersing regular chow diet consumption with occasional HFMs may prevent the cumulative growth of immune cell populations and subsequent insulin resistance in metabolic tissues. The quick resolution of HFM-induced inflammation also may provide insights into why time restricted feeding is beneficial. Time restricted feeding is the practice of condensing food consumption to a limited time during the day without reducing daily caloric intake. A recent study demonstrated that mice given *ad libitum* access to HFD for 8 hours/day then fasted for 16 hours/day for a period of 12 weeks consumed equal calories to mice with 24 hour *ad libitum* access to HFD while being protected from obesity, inflammation, and glucose intolerance (Hatori et al 2012). Protection from the deleterious effects of extended HFD consumption provided by time restricted feeding has so far been much stronger than the protection accompanying reductions in immune cell accumulation (Russo et al 2010, Sato et al 2011). How time restricted feeding influences the pulsatile inflammatory reactions associated with macronutrient-induced disease progression are unknown and warrant examination.

Many questions remain. The absence in leukocyte-endothelium interactions and immune cell infiltration into the SF (**Figures 8 & 11**) is at least partly explained by the SF's inert Psel (**Figure 10**) but why Psel remains silent is unknown. Recent reports suggest

a lower density of lipoprotein lipase and, therefore, a decreased capacity for lipid transport in the SF microcirculation as compared to the VF microcirculation (Koutsari et al 2011, Scalia 2013). Thrombin and histamine are known activators of Psel; WT mice could be challenged with either and then observed by intravital microscopy to help verify this discrepancy in Psel activity. Why repetitive HFD consumption does not cause increased leukocyte-endothelium interactions or neutrophil infiltration in the VF of Psel$^{-/-}$ mice is unclear (**Figure 18 & 19**). In future experiments, expression of other eCAMs such as Esel and ICAM-1 should be measured in Psel$^{-/-}$ mice challenged with HFD. Additionally, leukocyte rolling and leukocyte adhesion should be observed by intravital microscopy in Psel$^{-/-}$ mice challenged with TNFα or IL-1 in an effort to stimulate the synthesis of other eCAMs.

Finally, the lack of leukocyte-endothelium interactions and neutrophil infiltration in the VF of MPO$^{-/-}$ mice was especially puzzling. Neutrophilic β2 integrin ligation by coupled Psel and platelet activating factor increases intracellular calcium in neutrophils, priming neutrophils for degranulation (Lorant et al 1993). The lack of MPO accumulation and activity in high-fat fed Psel$^{-/-}$ mice (**Figures 21 & 22**) supports the notion that MPO release requires Psel dependent leukocyte rolling. The absence of increased leukocyte-endothelium interactions in on MPO$^{-/-}$ mice suggests MPO is required for the induction of neutrophil rolling (**Figures 23 & 24**). A study on MPO$^{-/-}$ mice fed a chronic HFD was recently published (Wang et al 2014) but no early phase data was reported. Before carrying on any studies with MPO$^{-/-}$ mice, the author would first examine the neutrophil biology of this model. The state of neutrophil CAM expression and degranulation capability should be characterized by isolating neutrophils from these mice and treating them with pro-

inflammatory cytokines. Performing a bone marrow transplant study in which WT mice and $MPO^{-/-}$ mice have their respective MPO synthesizing neutrophil production capacities switched would also improve our understanding of the role MPO plays in leukocyte extravasation.

CHAPTER 3: FAT CONSUMPTION DRIVES INSULIN RESISTANCE

Abstract

Inflammation is closely correlated with the metabolic complications associated with obesity. A robust elevation of pro-inflammatory markers in the blood of obese, insulin resistant patients has been well characterized. It has also been reported that the metabolically obese, normal weight patients have an equal level of inflammatory cytokines in their blood when compared to obese insulin resistant individuals, suggesting inflammation as a key regulator of metabolic health. Anti-inflammatory pharmaceutical interventions in diabetic patients have yielded mixed results. Animal model studies have demonstrated that shifts in immune cell polarization and reductions in immune cell accumulation within fat are capable of boosting metabolic health with widely varying degrees of success. This has raised suspicion that inflammation plays a causal role in the development and progression of metabolic dysfunction. Whether decreased immune cell infiltration in fat depots preserves metabolic health in obesity remains though unclear. Accordingly, we studied the role of endothelial expressed Psel in insulin sensitivity in mice with DIO.

Our experiments reveal that Psel$^{-/-}$ and MPO$^{-/-}$ mice are protected from short term high-fat feeding induced inflammation. To elucidate whether early phase inflammatory interventions granted late phase metabolic improvements, WT, Psel$^{-/-}$, and MPO$^{-/-}$ C57BL/6 mice were given *ad libitum* access to LFD (10% fat) or HFD (60% fat) for 12-16 weeks. Prolonged HFD consumption sustained elevated leukocyte-endothelium interactions in MPCV and was accompanied by increased local and systemic TNFα in WT mice. DIO WT mice were hyperglycemic, hyperinsulinemic, glucose intolerant, and insulin

resistant compared to LFD fed controls. DIO $Psel^{-/-}$ mice were protected from leukocyte-endothelium interactions as well as local and systemic TNFα accumulation despite extended HFD consumption. Surprisingly, DIO $Psel^{-/-}$ mice were equally hyperglycemic, hyperinsulinemic, glucose intolerant, and insulin resistant as the inflamed, DIO WT mice. $MPO^{-/-}$ mice were also protected from elevated systemic TNFα. While DIO $MPO^{-/-}$ mice were hyperglycemic and glucose intolerant, they did have improved insulin stimulated glucose clearance. All mouse groups given HFD became obese although $MPO^{-/-}$ mice gained slightly less weight than the other high-fat fed groups.

Taken together, these data indicate that Psel and MPO deletion each reduce the inflammation associated with HFD consumption but fail to improve glycemic regulation. These data suggest that blockade of immune cell accumulation in visceral adipose depots alone may not reduce insulin resistance associated with obesity.

Introduction

Inflammation, obesity, and insulin resistance are tightly correlated and the search for a causal relationship between these factors goes on. Obese, insulin resistant patients are characterized by higher circulating white blood cell counts, immune cell accumulation in metabolic tissues, and high levels of pro-inflammatory cytokines such as TNFα, MCP-1, and IL-6 in the blood (Hotamisligil et al 1995, Fried et al 1998). A recent report demonstrated that metabolically obese, normal weight patients have nearly equal pro-inflammatory markers in their blood compared to classical obese and metabolically sick individuals (Di Renzo et al 2010). This suggests that even in the absence of obesity inflammation may still drive insulin resistance. The relationship between inflammation and metabolic health, though, remains unclear. Efforts to improve metabolism by lowering

inflammation has met with mixed results. Administration of etanercept, a TNFα blocking antibody, to diabetic patients did not improve glycemic control but did lower several inflammatory cytokines (Bernstein et al 2006). Salicylates, however, have been shown to improve glucose regulation by interfering in the IKKβ/NF-κB pathway (Yuan et al 2001, Goldfine et al 2010).

Animal models have also been used to better define the relationship between insulin resistance and inflammation. Different methods for improving insulin sensitivity and glucose control by interfering in inflammation are being developed. Disruption of pro-inflammatory signaling events was one of the first approaches to show the potential benefits of inflammatory reduction for metabolic health. Interference in TNFα signaling in DIO rodent models improves insulin sensitivity (Hotamisligil et al 1993) but, as previously mentioned, does not work in humans (Bernstein et al 2006). More recent efforts focus on a key source of pro-inflammatory cytokines- immune cells. The revelation of white blood cell polarization has led groups to race to identify mechanisms that regulate immune cell phenotype. Attention has been directed to M2 macrophage phenotype induction by overexpressing the key immuno-metabolism integrator STAMP2 (Han et al 2013), administration of interferon tau (Ying et al 2014), and activation of the transcription factor interferon regulatory factor 4 (Eguchi et al 2013). Immunotherapy has been suggested as a potential technique for promoting anti-inflammatory T cell phenotypes (Winer et al 2009).

Instead of changing immune cell behavior within adipose tissue to improve insulin sensitivity, other groups have attempted to prevent leukocyte accumulation altogether. Diet induced and genetic obese models of PSGL-1 deficient mice have recently been studied. PSGL-1 is a leukocyte expressed ligand for the selectin family of CAMs. Endothelial

expressed Esel and Psel each bind PSGL-1, making PSGL-1 blockade a significant hindrance to immune cell extravasation. Two independent studies reported PSGL-1 deletion successfully prevented immune cell accumulation in the VF (Sato et al 2011, Russo et al 2010). The Sato group reported reductions in hyperinsulinemia without changes to hyperglycemia while the Russo group reported no changes in metabolism when comparing DIO PSGL-1 deficient mice with DIO WTs.

With the relationship between inflammation and insulin sensitivity still uncertain, we endeavored to study the role of Psel and MPO in the development of insulin resistance. We hypothesized that Psel is required for HFD induced immune cell accumulation in the VF and that MPO, a producer or oxidative stress, is necessary to sustain the inflammatory response to HFD. Furthermore, as a result of dampened inflammatory action, Psel$^{-/-}$ and MPO$^{-/-}$ mice would be metabolically protected from HFD consumption. Accordingly, we studied the effects of extended HFD diet consumption on the inflammatory state and metabolic health of WT, Psel$^{-/-}$, and MPO$^{-/-}$ C57BL/6 mice. We demonstrate that Psel$^{-/-}$ and MPO$^{-/-}$ mice are indeed protected from HFD induced local and systemic inflammation. Despite ablation of inflammatory action, Psel$^{-/-}$ mice are not metabolically protected. Our MPO$^{-/-}$ mice remain hyperglycemic but exhibit improved insulin stimulated glucose clearance. Taken together, these data indicate that Psel and MPO deletion each reduce the inflammation associated with HFD consumption. However, blockade of immune cell accumulation in visceral adipose depots alone may not prevent the metabolic complications associated with obesity.

Materials and Methods

Animal Models

All experiments were approved by Institutional Animal Care and Use Committee (IACUC) at Temple University and were in accordance with approved protocols. Male C57BL/6J (Jackson Laboratory Stock #000664, Ann Arbor, MI) mice were used to study normal physiological responses to dietary challenges. Psel$^{-/-}$ mice (Jackson Laboratory Stock #002289) were acquired to determine the role of Psel in tissue homeostasis in the face of extended lipid overload. MPO$^{-/-}$ mice (Jackson Laboratory Stock #004265) were acquired to determine whether MPO activity is required for sustained endothelial activation in the adipose tissue microcirculation. All mice were aged 8-12 weeks and had an average body weight of 25 g when put on diet. Mice were given *ad libitum* access to water and low fat diet (LFD) (Research Diets Inc. D12450B 10% fat) or HFD (Research Diets Inc. D12492 60% fat) in pellet form for 12-16 weeks. Detailed diet composition information is listed in Table 1 and Table 2.The mice were maintained on a 12 hour light-dark cycle in the animal facility at Temple University.

Leukocyte-Endothelium Interaction Quantification by Intravital Microscopy

Following intraperitoneal administration of 80mg/kg pentobarbital, mice were prepared for intravital microscopy studies as previously described in rats (Scalia et al 2011). A polyethylene catheter was inserted in the right carotid artery to monitor mean arterial blood pressure. Four distal loops of ileal tissue, exteriorized through a midline laparotomy, were superfused with 37ºC Krebs-Henseleit buffer in an intravital microscopy Plexiglas chamber attached on the stage of an Eclipse FN1 Physiostation Microscope (Nikon Corp., Japan). A relatively straight, unbranched segment of postcapillary venule

with a length >100μms and a diameter between 25 and 40μms was randomly studied in each mouse. Leukocytes were stained with Rhodamine 6G and illuminated with a mercury bulb light source. Observation of the mesenteric microcirculation was made with 20x salt water-immersion lens. Images were projected by high-resolution, intensified video cameras (XR Mega-10 EX ICCD; Stanford Photonics INC) onto a high-resolution, color video monitor (Multiscan 200-sf, Sony), and the image recorded on A WIN XP Imaging Workstation. All data were analyzed using computerized imaging software (Micro-Manager). Leukocyte rolling and leukocyte adhesion were studied in MPCVs. Leukocyte rolling is defined as the number of leukocytes rolling past a fixed point per minute; leukocyte adhesion is defined as the number of leukocytes firmly adhered to 100μm length of endothelium for at least 30 seconds.

Total Body Weight and Adiposity

Total body weight was measured in grams after 16 weeks of *ad libitum* diet consumption. Epididymal, subscapular, and mesenteric fat depots were excised and weighed individually to determine changes in adiposity between experimental groups.

Plasma Glucose

Unfasted plasma glucose was measured following 12-16 weeks of *ad libitum* diet consumption. Blood was collected with a tail vein prick and blood glucose was measured using an Alpha Trak 2 glucose meter.

Fasting Plasma Insulin

Mice were fasted overnight following 12-16 weeks of ad libitum diet consumption. Blood was collected by cardiac puncture and the plasma isolated by centrifugation. Plasma

samples were flash frozen in liquid nitrogen and then mailed to the Case Western University Mouse Metabolic Phenotyping Center. Plasma insulin was measured by ELISA.

Glucose Tolerance Test

Mice were fasted overnight following 12-16 weeks of *ad libitum* diet consumption before glucose tolerance test (GTT). Tail tips were clipped and fasting blood glucose measured using an Alpha Trak II glucose meter. After baseline blood glucose was recorded, a bolus dose of dextrose (1g/kg) was delivered by intraperitoneal injection. Blood glucose was measured 15, 30, 45, 60, 90, and 120 minutes after glucose injection.

Insulin Sensitivity

Insulin tolerance tests (ITT) were administered to measure insulin stimulated glucose clearance. Mice were fasted for 4 hours following 12-16 weeks of *ad libitum* diet consumption. Tails tips were clipped and blood glucose measured using an Alpha Trak II glucose meter. After baseline blood glucose was recorded, a bolus dose of insulin at (0.7U/kg) was delivered by i.p. injection. Blood glucose was measured 15, 30, 45, 60, 90, and 120 minutes after insulin challenge.

A linear regression of the ITT glucose curve was calculated. Change in glucose over change in time was calculated for the first 30 minutes of the ITT. The slope of the curve is considered change in glucose over change in time.

TNFα ELISA

Cardiac punctures were performed on mice fed either LFD of HFD for 12-16 weeks. Blood samples were centrifuged to isolate plasma. Plasma samples were sent to the Case Western University Mouse Metabolic Phenotyping Center for TNFα ELISA analysis.

Reported VF TNFα ELISA values are from an old data set generated by Dr. Scalia.

Statistics

Data are represented as mean ± SEM. The Student's T test was used to analyze leukocyte-endothelium interactions, total body weight, plasma glucose, plasma insulin, local TNFα, plasma TNFα, and Δ[Glucose]/Δtime. Two-way ANOVA was used to determine statistical significance of adiposity, GTT, and ITT. Statistical analysis was set such that *=p<0.05 **=p<0.01 ***=p<0.001.

Results

Extended HFD Consumption Causes Inflammation

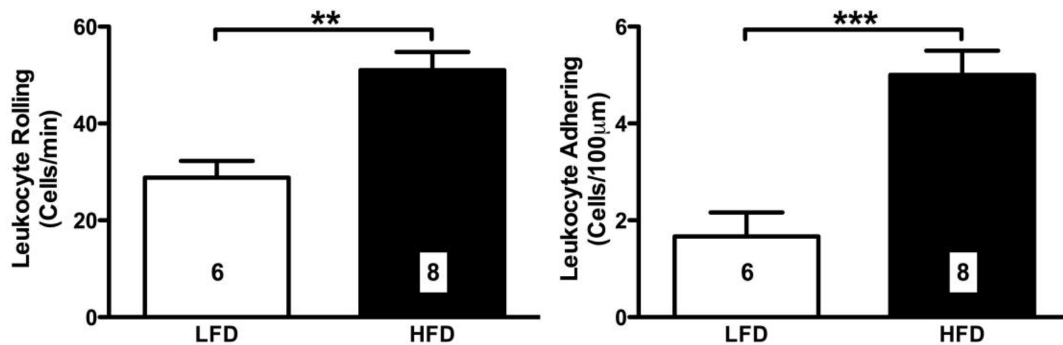

Figure 26: Prolonged HFD consumption sustains elevated leukocyte-endothelium interactions in MPCV

Chronic HFD consumption sustains elevated leukocyte-endothelium interactions in the VF of WT mice. WT mice consumed LFD or HFD for 12 weeks before quantification of leukocyte-endothelium interactions in MPCVs by intravital microscopy. HFD fed mice had significantly higher leukocyte rolling (**Figure 26, left**) and leukocyte adhesion (**Figure 26, right**) than LFD fed counterparts. Immune cell trafficking through the VF microcirculation does not diminish as long as HFD feeding persists.

Pro-inflammatory cytokine expression is increased in the VF of HFD fed WT mice. ELISA analysis of VF TNFα expression demonstrated that chronic HFD consumption

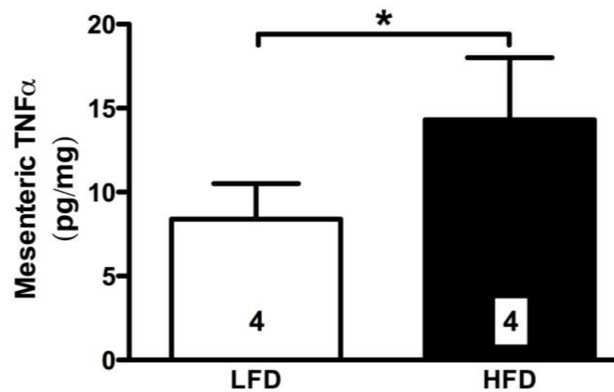

Figure 27: VF TNFα concentrations are increased by prolonged HFD consumption

significantly increases accumulation of local TNFα compared to LFD fed controls (**Figure 27**). Our data does not indicate whether the TNFα was released by leukocytes or adipocytes. Chronic HFD consumption results in a pro-inflammatory phenotype in the VF.

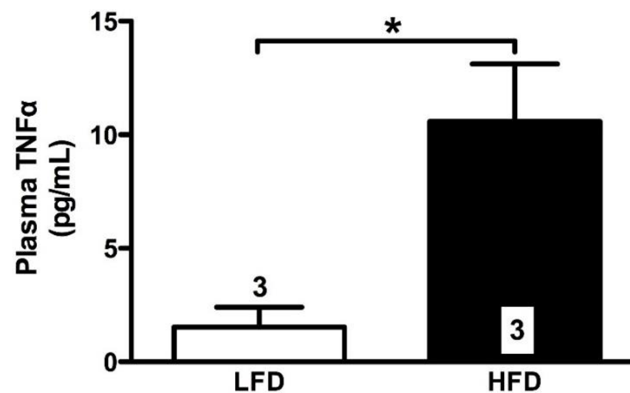

Figure 28: Plasma TNFα concentrations are increased by prolonged HFD consumption

Chronic HFD consumption increases systemic pro-inflammatory cytokine concentrations. ELISA analysis of plasma TNFα expression demonstrated that chronic HFD consumption increased TNFα concentration in the blood 2-fold compared to LFD fed controls (**Figure 28**). Thus, chronic consumption of HFD by WT mice causes local and systemic inflammation.

Extended HFD Consumption Increases Fatness

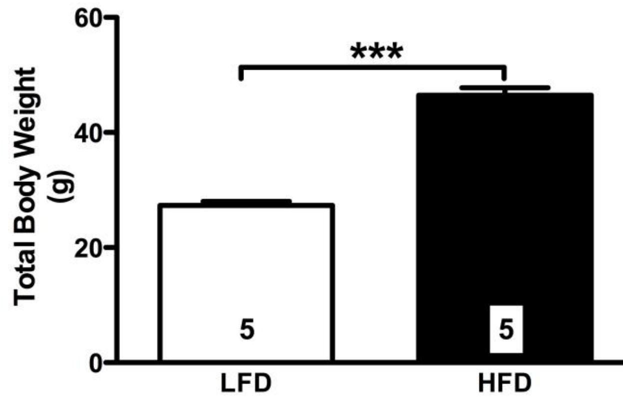

Figure 29: Prolonged HFD consumption increases total body weight

Chronic HFD consumption causes obesity in WT mice. Total body weight measurement of WT mice fed LFD or HFD for 12 weeks demonstrated HFD increases body mass significantly more than LFD (**Figure 29**). WT mice fed HFD are obese.

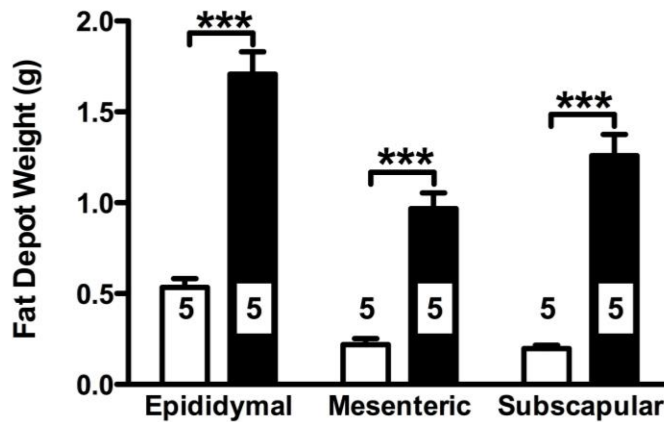

Figure 30: Prolonged HFD consumption increases adiposity

Chronic HFD consumption increases adiposity in WT mice. Measurement of epididymal, subcutaneous, and mesenteric fat pads demonstrated all fat depots expand during extended high-fat feeding (**Figure 30,** black bars). Thus, 12 weeks of HFD consumption causes DIO in WT mice.

DIO Increases Metabolic Markers

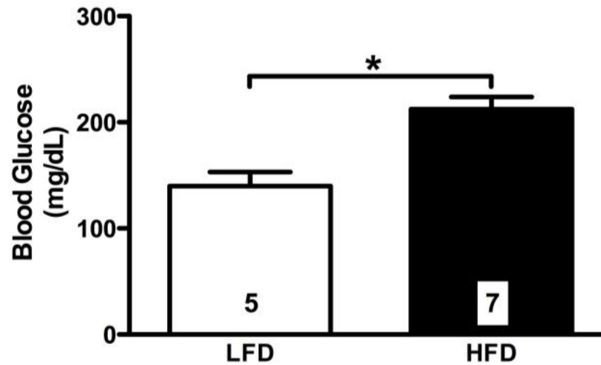

Figure 31: Prolonged HFD consumption increases plasma glucose

DIO WT mice are hyperglycemic. Blood glucose measurement in WT mice fed LFD and HFD for 12 weeks demonstrated high-fat feeding causes hyperglycemia in WT mice (**Figure 31**). DIO WT mice have diminished glycemic regulation.

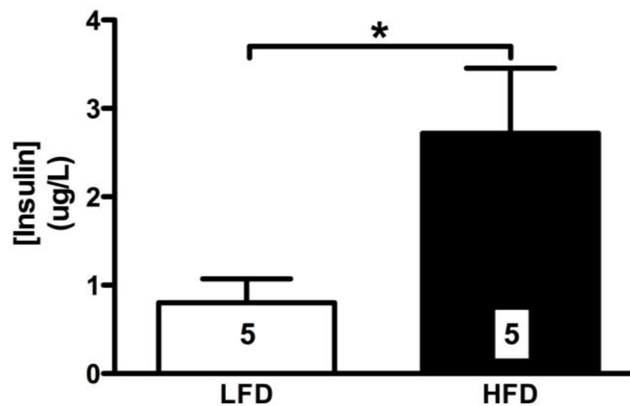

Figure 32: Prolonged HFD consumption increases fasting plasma insulin

DIO WT mice are hyperinsulinemic. Fasting plasma insulin ELISA analysis in WT mice fed LFD and HFD for 12 weeks demonstrated high-fat feeding causes hyperinsulinemia in WT mice (**Figure 31**). DIO WT mice have impaired insulin regulation. The combination of hyperglycemia and hyperinsulinemia is an indicator of insulin resistance.

DIO Impairs Glucose Homeostasis

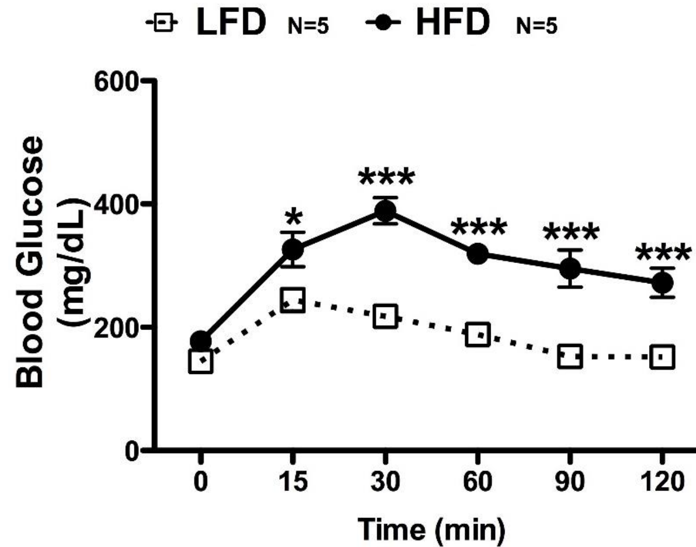

Figure 33: Prolonged HFD consumption causes glucose intolerance

DIO WT mice are glucose intolerant. The GTT was administered to WT mice after 16 weeks of LFD or HFD consumption. HFD fed mice had a significantly higher blood glucose concentration than their LFD fed counterparts following bolus dextrose i.p. injection (**Figure 33**). Chronic HFD consumption causes glucose intolerance in DIO WT mice.

DIO WT mice have impaired insulin stimulated glucose clearance. The ITT was administered to WT mice after 16 weeks of LFD or HFD consumption. HFD fed mice had a significantly higher blood glucose concentration than their LFD fed counterparts following bolus insulin i.p. injection (**Figure 34**). Chronic HFD consumption reduces insulin responsiveness in DIO WT mice. Diminished insulin-induced glucose clearance is indicative of impaired insulin signaling, a hallmark of type II diabetes.

Chronic HFD consumption impairs insulin clearance of glucose over time in WT mice. A linear regression of the ITT curve during the first 30 minutes following insulin

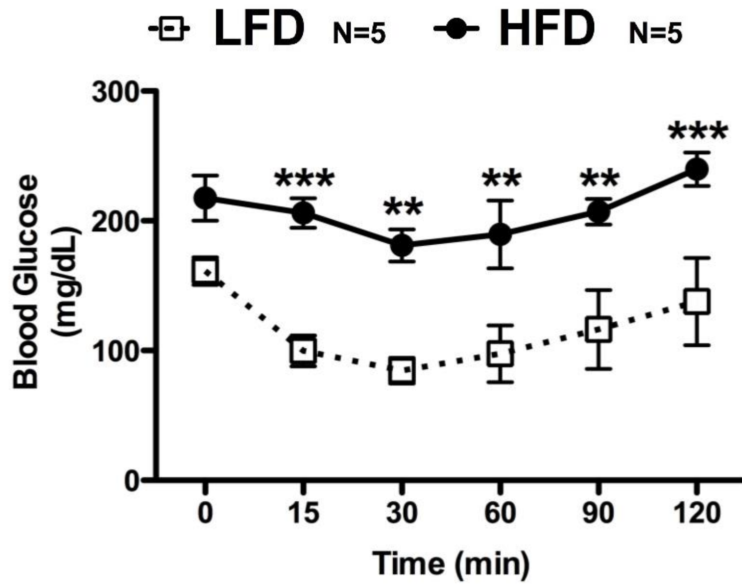

Figure 34: Prolonged HFD consumption impairs insulin responsiveness

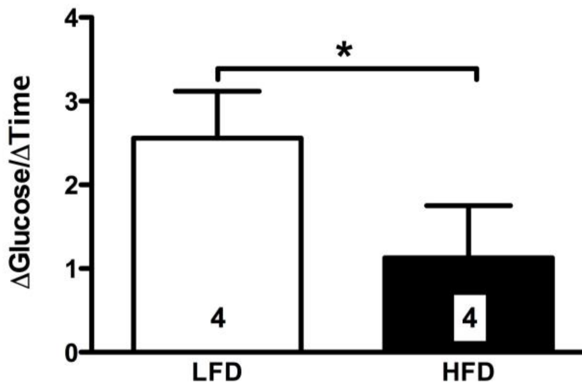

Figure 35: Prolonged HFD consumption impairs glucose disposal rate

injection was generated. The magnitude of the slope was plotted representing Δ[Glucose]/Δtime. DIO WT cleared glucose at approximately half the rate of the LFD WT mice during the first 30 minutes after insulin injection (**Figure 35**). Thus, chronic consumption of an obesogenic HFD by WT mice reduces insulin sensitivity.

Psel Deletion Prevents HFD Induced Inflammation

Psel deletion protects mice from chronic HFD consumption-induced elevations in leukocyte-endothelium interactions in the VF of Psel$^{-/-}$ mice. Psel$^{-/-}$ mice consumed HFD for 12 weeks before quantification of leukocyte-endothelium interactions in MPCVs by

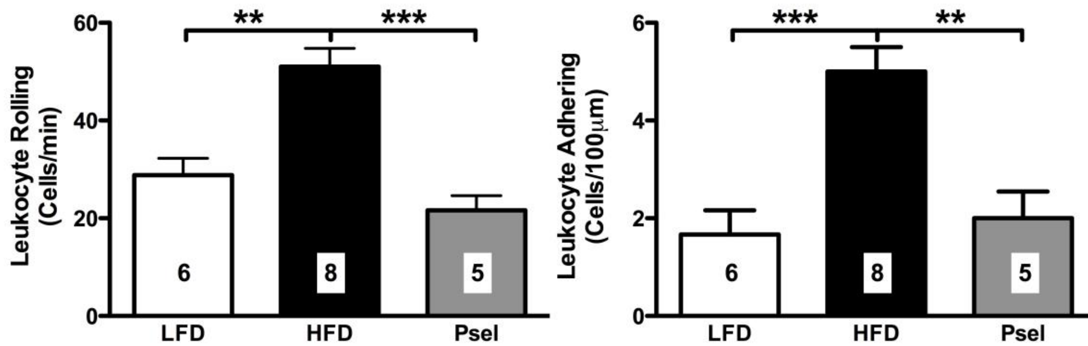

Figure 36: Psel deletion prevents prolonged HFD exposure induced leukocyte-endothelium interactions

intravital microscopy. HFD fed Psel$^{-/-}$ mice had significantly lower leukocyte rolling (**Figure 36, left**) and leukocyte adhesion (**Figure 36, right**) compared to HFD fed WT mice. Psel deletion prevents a chronic HFD consumption-induced pro-adhesive phenotype in the MPCV endothelium.

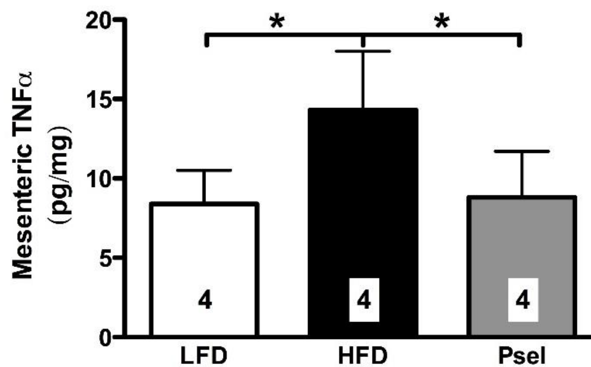

Figure 37: Prolonged HFD consumption does not increase VF TNFα in Psel$^{-/-}$ mice

Chronic HFD consumption does not increase VF pro-inflammatory cytokine concentrations in Psel$^{-/-}$ mice. ELISA analysis of VF TNFα expression demonstrated that Psel$^{-/-}$ mice are have significantly lower concentrations of TNFα compared to HFD-fed WT mice (**Figure 37**). Psel deletion prevents local HFD-induced accumulation of TNFα in the visceral adipose tissue.

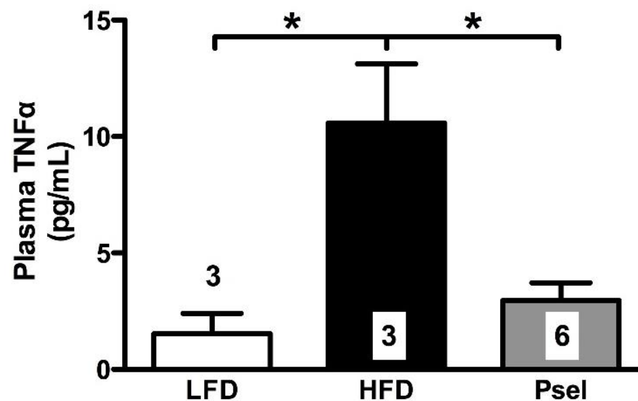

Figure 38: Prolonged HFD consumption does not increase plasma TNFα in Psel$^{-/-}$ mice

Chronic HFD consumption does not increase systemic pro-inflammatory cytokine plasma concentrations in Psel$^{-/-}$ mice. ELISA analysis of plasma TNFα expression demonstrated that Psel$^{-/-}$ mice are protected from chronic HFD-induced increases in plasma TNFα concentration (**Figure 38**). Thus, Psel$^{-/-}$ mice are protected from chronic HFD-induced local and systemic inflammation.

Psel Deletion Does Not Reduce Fat Accumulation

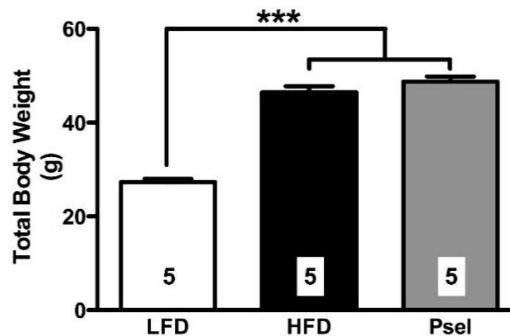

Figure 39: Prolonged HFD consumption causes weight gain in Psel$^{-/-}$ mice

Chronic HFD consumption causes obesity in Psel$^{-/-}$ mice. Total body weight measurement of Psel$^{-/-}$ mice fed HFD for 12 weeks demonstrated HFD significantly increases body mass compared to LFD fed WT mice. (**Figure 39**). Psel deletion does not reduce the obesogenic effects of chronic HFD consumption.

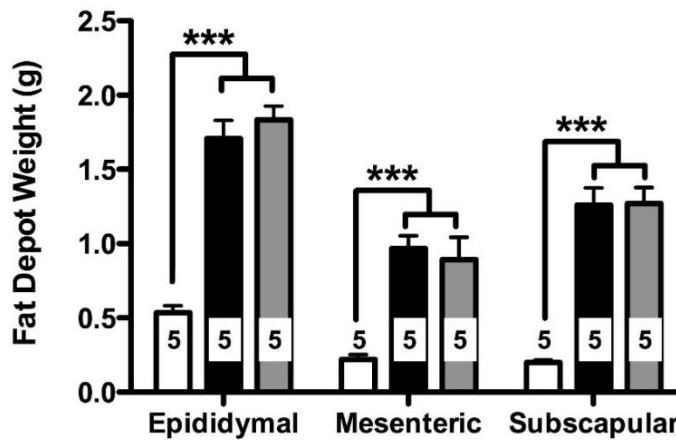

Figure 40: Prolonged HFD consumption increases adiposity of Psel$^{-/-}$ mice

Chronic HFD consumption increases adiposity in Psel$^{-/-}$ mice. Measurement of epididymal, subcutaneous, and mesenteric fat pads demonstrated all fat depots expand during extended high-fat feeding (**Figure 40**). Thus, 12 weeks of HFD consumption causes DIO in Psel$^{-/-}$ mice.

Psel Deletion Does Not Protect Metabolic Markers

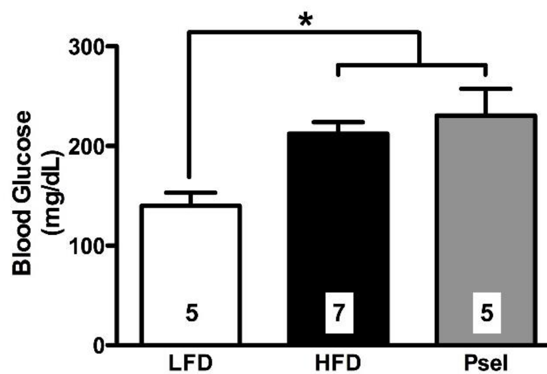

Figure 41: Psel$^{-/-}$ mice are hyperglycemic following prolonged HFD consumption

DIO Psel$^{-/-}$ mice are hyperglycemic. Blood glucose measurement in Psel$^{-/-}$ mice fed HFD for 12 weeks demonstrated high-fat feeding causes hyperglycemia in Psel$^{-/-}$ mice (**Figure 41**). DIO Psel$^{-/-}$ mice have diminished glycemic regulation.

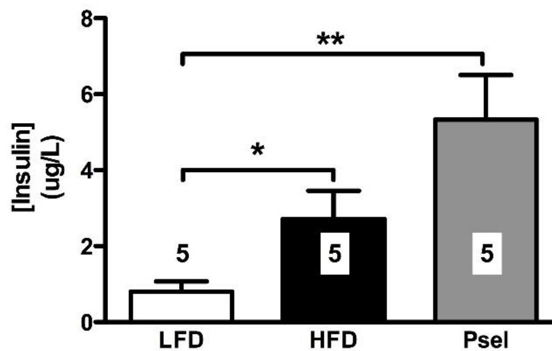

Figure 42: Psel$^{-/-}$ mice are hyperinsulinemic following prolonged HFD consumption

DIO Psel$^{-/-}$ mice are hyperinsulinemic. Fasting plasma insulin ELISA analysis in Psel$^{-/-}$ mice fed HFD for 12 weeks demonstrated high-fat feeding causes hyperinsulinemia in Psel$^{-/-}$ mice (**Figure 42**). DIO Psel$^{-/-}$ mice have impaired insulin regulation. The combination of hyperglycemia and hyperinsulinemia is an indicator of insulin resistance.

Psel Deletion Does Not Improve DIO Glucose Regulation

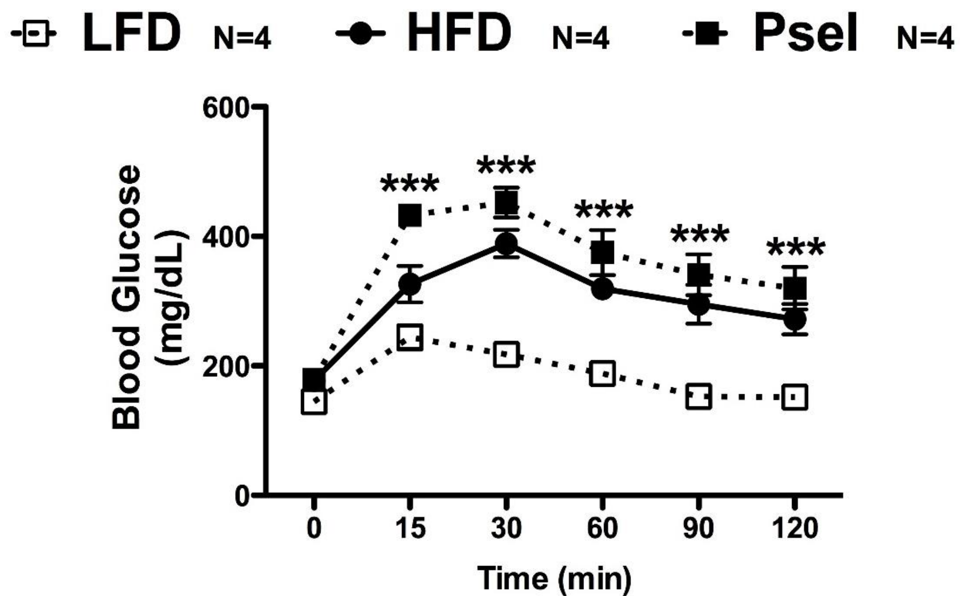

Figure 43: DIO Psel$^{-/-}$mice are glucose intolerant

DIO Psel$^{-/-}$ mice are glucose intolerant. The GTT was administered to Psel$^{-/-}$ mice after 16 weeks HFD consumption. HFD fed Psel$^{-/-}$ mice had significantly higher blood

glucose concentrations than LFD fed WT mice following bolus dextrose i.p. injection (**Figure 43**). Chronic HFD consumption causes glucose intolerance in DIO Psel$^{-/-}$ mice.

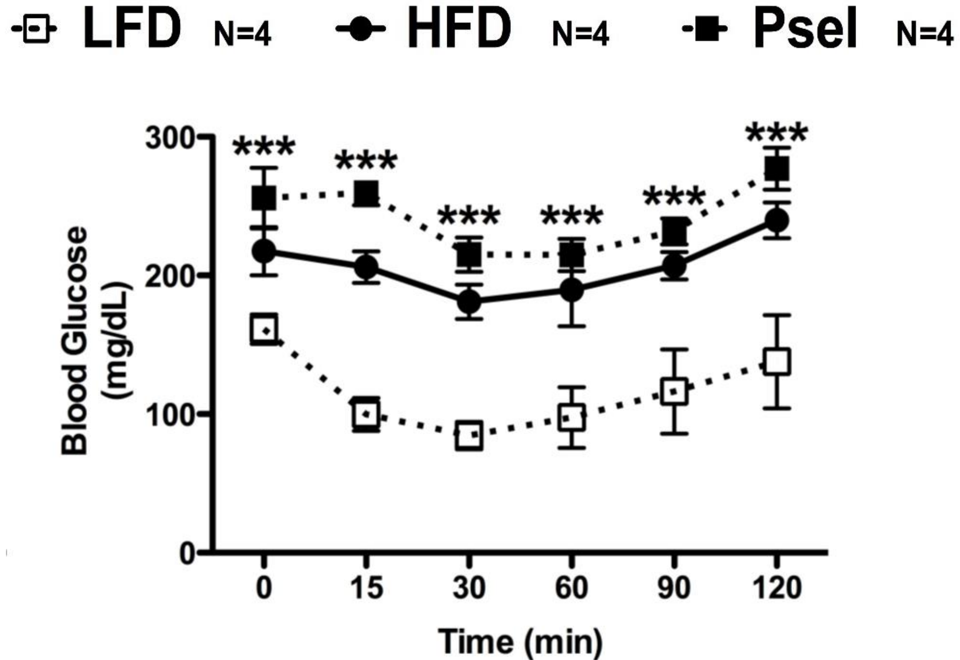

Figure 44: DIO Psel$^{-/-}$mice have impaired insulin responsiveness

DIO Psel$^{-/-}$ mice have impaired insulin stimulated glucose clearance. The ITT was administered to Psel$^{-/-}$ mice after 16 weeks HFD consumption. HFD fed Psel$^{-/-}$ mice had a significantly higher blood glucose concentration than LFD fed WT mice following bolus insulin i.p. injection (**Figure 44**). Psel deletion did not improve insulin stimulated glucose clearance in DIO Psel$^{-/-}$ mice. Diminished insulin-induced glucose clearance is indicative of impaired insulin signaling, a hallmark of type II diabetes.

Psel deletion does not improve insulin stimulated glucose clearance over time following chronic HFD consumption. Linear regression analysis of the ITT curve during the first 30 minutes after insulin injection demonstrated HFD consumption dulls insulin mediated glucose disposal (**Figure 45**). Thus, Psel$^{-/-}$ mice are not protected from the metabolic complications associated with chronic HFD consumption.

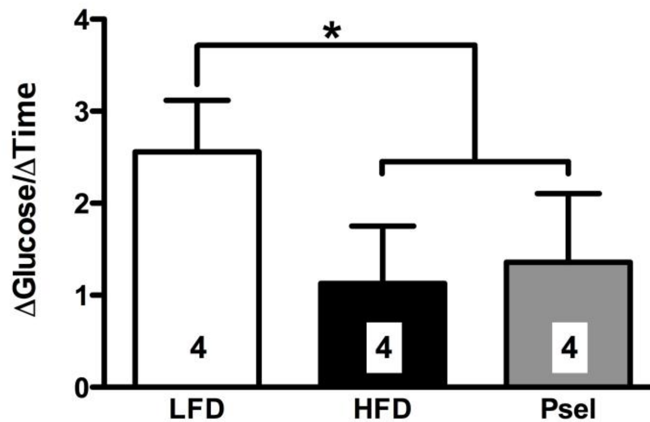

Figure 45: DIO Psel$^{-/-}$ mice have diminished glucose disposal rates

MPO Deletion Ameliorates DIO Induced Inflammation

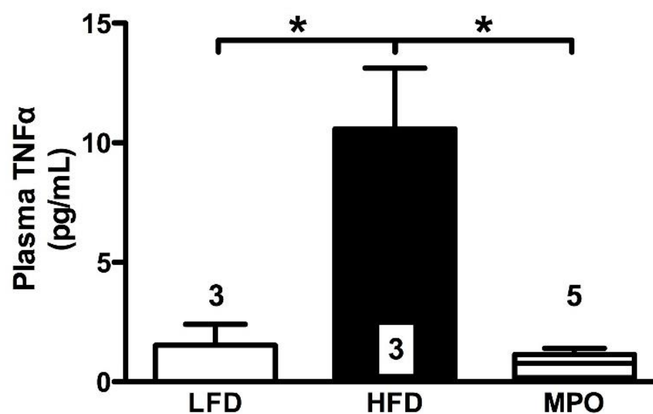

Figure 46: MPO deletion prevents prolonged HFD induced increases in plasma TNFα

Chronic HFD consumption does not increase systemic pro-inflammatory cytokine plasma concentrations in MPO$^{-/-}$ mice. ELISA analysis of plasma TNFα expression demonstrated that MPO$^{-/-}$ mice are protected from chronic HFD-induced increases in plasma TNFα concentration (**Figure 46**). These results are consistent with a recent report which demonstrated DIO MPO$^{-/-}$ mice are protected from inflammation in the VF (Wang et al 2014). Thus, MPO$^{-/-}$ mice are protected from chronic HFD-induced local and systemic inflammation.

MPO Deletion Slightly Reduces Fat Accumulation

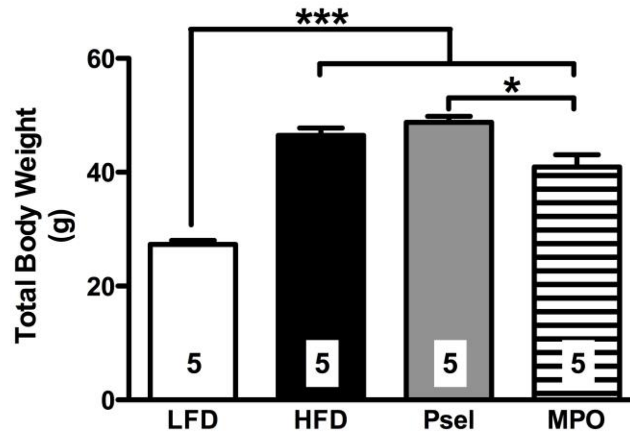

Figure 47: Prolonged HFD consumption causes weight gain in $MPO^{-/-}$ mice

Chronic HFD consumption causes obesity in $MPO^{-/-}$ mice. Total body weight measurement of $MPO^{-/-}$ mice fed HFD for 12 weeks demonstrated HFD significantly increases body mass compared to LFD fed WT mice. (**Figure 47**). Notably, $MPO^{-/-}$ mice gained significantly less weight than $Psel^{-/-}$ mice. These results are consistent with a previous report (Wang et al 2014).

$MPO^{-/-}$ Mice Are Hyperglycemic

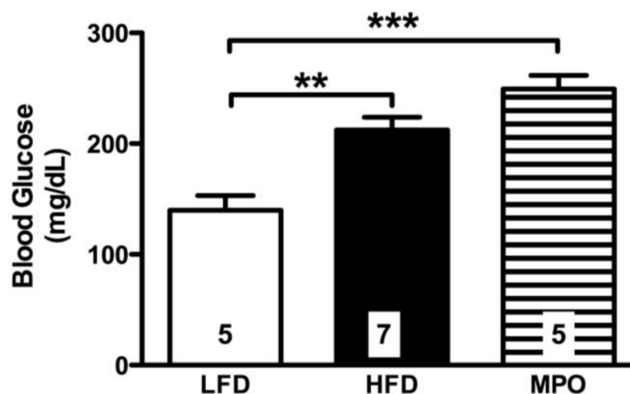

Figure 48: DIO $MPO^{-/-}$ mice are hyperglycemic

DIO $MPO^{-/-}$ mice are hyperglycemic. Blood glucose measurement in $MPO^{-/-}$ mice fed HFD for 12 weeks demonstrated high-fat feeding causes hyperglycemia in $MPO^{-/-}$ mice

(**Figure 48**). DIO MPO$^{-/-}$ mice have diminished glycemic regulation.. Our results match those recently reported (Wang et al 2014).

MPO Deletion Does Not Improve Glucose Tolerance

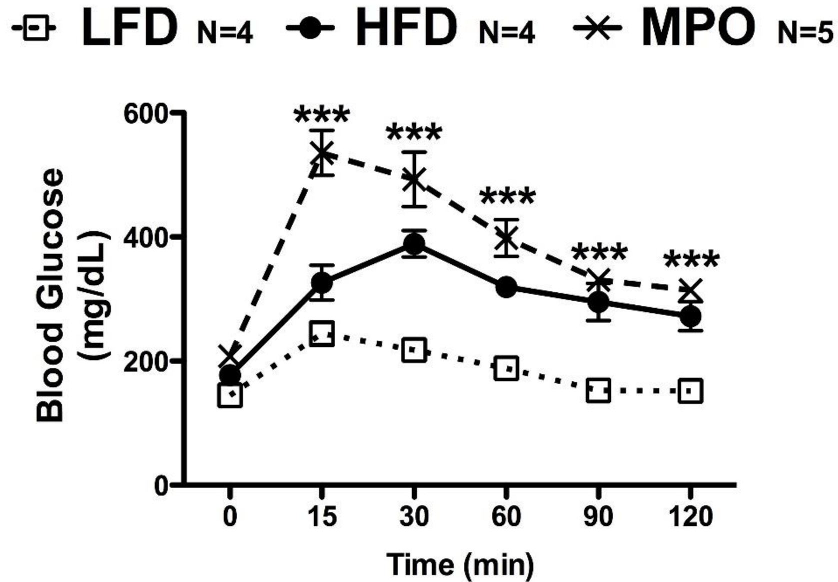

Figure 49: Prolonged HFD fed MPO$^{-/-}$ mice are glucose intolerant

DIO MPO$^{-/-}$ mice are glucose intolerant. The GTT was administered to MPO$^{-/-}$ mice after 16 weeks HFD consumption. Bolus dextrose injection caused the highest spike in blood glucose in HFD fed MPO$^{-/-}$ mice (**Figure 49**), however, the MPO$^{-/-}$ mice did have plasma glucose levels near DIO WT mice 2 hours after glucose injection, which required significant glucose clearance. Nevertheless, chronic HFD consumption causes glucose intolerance in DIO MPO$^{-/-}$ mice.

Insulin stimulated glucose clearance is improved in DIO MPO$^{-/-}$ compared to DIO WT mice. The ITT was administered to MPO$^{-/-}$ mice after 16 weeks of HFD consumption. The hyperglycemia of HFD fed MPO$^{-/-}$ mice was ameliorated by an i.p. injection of insulin

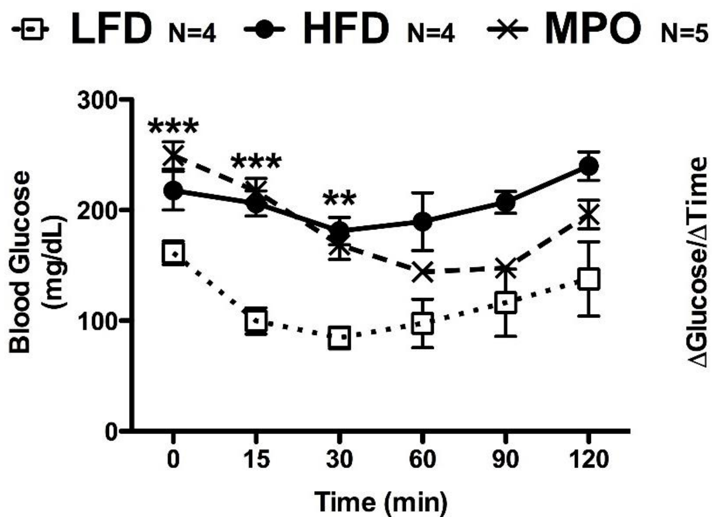

Figure 50: MPO deletion protects insulin responsiveness during prolonged HFD

and eventually reached glucose levels near those in WT LFD fed mice (**Figure 50**). MPO deletion improved insulin stimulated glucose clearance in DIO MPO$^{-/-}$ mice.

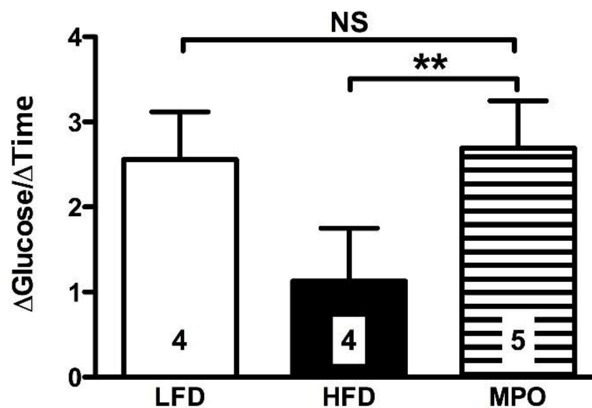

Figure 51: MPO deletion protects glucose disposal rates

MPO deletion improves insulin stimulated glucose clearance following chronic HFD consumption. Linear regression analysis of the ITT curve during the first 30 minutes after insulin injection demonstrated HFD consumption fails to impair insulin mediated glucose disposal in MPO$^{-/-}$ mice (**Figure 51**). Thus, MPO$^{-/-}$ mice are protected from the insulin resistance associated with chronic HFD consumption despite having impaired glucose regulation.

Discussion

We have demonstrated that reducing immune cell accumulation in the adipose tissue of high-fat fed mice does not necessarily improve metabolic health outcomes. HFD induced recruitment of leukocytes into visceral adipose tissue has been well characterized as a contributor to insulin resistance (Weisberg et al 2003, Talukdar et al 2012). Our obese, inflamed, and metabolically impaired WT mice fed HFD are a classic manifestation of the DIO mouse model. Like other anti-inflammatory genetic mouse models (Sato et al 2011), our $Psel^{-/-}$ and $MPO^{-/-}$ mice became obese with prolonged HFD exposure (**Figure 47**). And again, like other gene knockout models, Psel and MPO deletion each significantly reduced inflammatory markers (**Figures 36, 37, & 46**). Despite the ablation of measured inflammatory signals, $Psel^{-/-}$ and $MPO^{-/-}$ mice were each hyperglycemic and glucose intolerant (**Figures 48 & 49**). $Psel^{-/-}$ mice had impaired insulin induced glucose clearance, which is consistent with results found in PSGL-1 deficient mice (**Figure 43**). Hyperglycemia notwithstanding, $MPO^{-/-}$ mice did show improved glucose clearance following bolus insulin delivery when compared to high-fat fed WT and $Psel^{-/-}$ mice (**Figures 50 & 51**). Taken together, this data indicates that robust amelioration of VF immune cell accumulation alone may not be a strong enough intervention to prevent the development of metabolic dysfunction.

Immune cell infiltration of metabolic tissues is considered a contributing factor to diminished insulin signaling and glycemic control. For example, adipose tissue macrophages release pro-inflammatory cytokines like TNFα and MCP-1 which have paracrine effects on nearby cells and recruit more leukocytes to the tissue. In addition to the evidence that the invading immune cells worsen the metabolic situation, there is also

evidence that reductions in inflammation improve metabolic health with varying degrees of success (Mansuy-Aubert et al 2013). Of all of the attempts to improve metabolic health by reducing immune cell invasion, two examples are especially relevant to our findings in the Psel$^{-/-}$ mice (Sato et al 2011, Russo et al 2010). The deletion of PSGL-1, a leukocytic ligand for CAMs, Esel and Psel in particular, has been shown twice to dramatically reduce immune cell accumulation in fat and marginally improve metabolic health in DIO mouse models, if any benefit is reported at all. It must be noted that PSGL-1 is constitutively expressed by several leukocyte populations and binds CAM ligands on multiple cell types, such as other white blood cells or endothelial cells. PSGL-1 deletion, therefore, mutes the function of any PSGL-1 binding CAM, including all of the selectins. With that in mind, and considering the scant improvements in metabolic health provided by PSGL-1 deletion, it is unsurprising, that the deletion of a single eCAM, Psel, is not sufficient for improving metabolic health in the face of constant HFD consumption.

The metabolic improvements in our MPO$^{-/-}$ mice are mostly consistent with a recent report (Wang et al 2014). In the Wang publication, DIO MPO$^{-/-}$ mice were shown to have decreased leukocyte accumulation in the VF, lower inflammatory markers, and improved glycemic regulation. Our data set includes decreased plasma TNFα (**Figure 46**), slightly decreased weight gain (**Figure 47**), and improved insulin responsiveness (**Figures 50 & 51**) in DIO MPO$^{-/-}$ mice. Further examination of glucose tolerance in these mice is required. Wang et al reported increased fatty acid oxidation, showing higher UCP-1 expression and rectal temperatures as an explanation for the decreased fat accumulation. We took no data to investigate thermogenesis and cannot comment on mitochondrial function in these mice.

How MPO deletion interferes with leukocyte-endothelium interactions and immune cell infiltration of VF remains unclear.

Granting that MPO ablation does reduce immune cell accumulation in adipose tissue, the question of why we see improved insulin signaling in the $MPO^{-/-}$ mice but not the $Psel^{-/-}$ mice, who also demonstrate reduced immune cell accumulation, remains. When released from neutrophils, MPO synthesizes HOCL, a strong oxidant, from chloride ions and hydrogen peroxide. MPO decreases NO bioavailability two ways: catalyzing superoxide binding with NO to form peroxynitrite and uncoupling eNOS, thereby decreasing NO production. Loss of NO promotes oxidative stress, a suspected major contributor to the progression of insulin resistance. Oxidative stress, which can develop in endothelial cells and many other immune and non-immune cell types, initiates intracellular serine kinase activation which deactivates IRS-1. Therefore, in addition to reducing immune cell accumulation in metabolic tissues, MPO ablation likely prevents MPO dependent mechanisms of insulin desensitization, including oxidative stress. Furthermore, the Wang group poses that, in addition to generating oxidative stress, hypochlorous acid is responsible for the nitration of the insulin signaling molecules IRS-1, Akt, and insulin receptor (IR)-β (Wang et al 2014). We demonstrated that the activity of MPO in VF stromal vascular fractions occurs before neutrophil infiltration of the VF (**Figures 12 & 15**). Thus, MPO activity is capable of increasing in the absence of immune cell accumulation in the VF. We did not measure MPO activity in $Psel^{-/-}$ mice after extended HFD consumption so we are uncertain whether MPO contributed to the insulin resistance in the $Psel^{-/-}$ mice. That said, since both $Psel^{-/-}$ and $MPO^{-/-}$ mice are protected against immune cell accumulation in

the VF, the $MPO^{-/-}$ mice may retain some insulin sensitivity because of protection from MPO's enzymatic activity.

Several experiments are required to better understand our results. We quantified VF and plasma levels of TNFα to understand the local and systemic inflammatory state. While TNFα is a classic pro-inflammatory cytokine, other inflammatory markers can be tested. Levels of IL-1, MCP-1, and KC, a murine homolog of IL-8 which signals neutrophil recruitment (Hol et al 2010) should be quantified in plasma and metabolic tissues like adipose tissue, skeletal muscle, and the liver. Whether HFD induced local inflammation in metabolic tissues like the skeletal muscle and liver, which we did not examine, occurs despite Psel and MPO deletion is uncertain. The status of signaling proteins downstream of insulin, such as IRS-1, should be determined in the metabolic tissues. Oxidative stress is a potential contributor to the progression of insulin resistance in the absence of leukocyte infiltration. Quantification of nitrotyrosine or eNOS uncoupling would provide insights into whether oxidative stress was present despite the absence of inflammation.

CHAPTER 4: DISCUSSION AND FUTURE DIRECTIONS

The evidence that immune cell accumulation promotes insulin resistance is very strong. Observational studies have firmly established the overabundance of pro-inflammatory agents in the blood and metabolic tissues of obese, insulin resistant patients. Moreover, both obese and lean insulin resistant patients have significantly higher serum inflammatory markers than people who are obese but metabolically healthy (Di Renzo et al 2010). These observations provide us with correlative data that draws our attention to pathological mechanisms that may drive disease progression. To that end, we have learned that infiltrating immune cells contribute to the pathological state in various ways. Resident immune cells have paracrine influence over surrounding cell types; when leukocytes and lymphocytes release inflammatory signals, they cause dysfunction (Lee et al 2013). Immune cells have been implicated in initiating premature cell death (Spallarossa et al 2008), damaging extracellular matrix proteins (Baldus et al 2001), generating ROS (Roebuck 1999), and recruiting more immune cells to the metabolic tissues (Petri et al 2008). These events are insulin desensitizing and propagate the inflammatory state. Despite abundant evidence for the ability of white blood cells to cause metabolic tissue dysfunction, the causal role of inflammation in developing insulin resistance is still debated.

Our data suggests that immune cell accumulation is not necessarily a prerequisite for insulin resistance. By blocking Psel and MPO expression in separate mouse strains, we have significantly reduced markers for inflammation with small gains, if any, in metabolic health. Past attempts at improving metabolic health have employed far harsher genetic manipulations and met with only marginal success. PSGL-1 deletion greatly reduced immune cell accumulation in the VF of high-fat fed mice. The metabolic benefit was lightly

reduced serum insulin concentrations; these mice were still hyperglycemic and had equal A1C values to their high-fat fed WT controls (Sato et al 2011). The second group using DIO PSGL-1 deficient mice reported no difference in fasting glucose or insulin compared to DIO WT controls (Russo et al 2010). The evidence for immune cell accumulation in the VF as the engine driving insulin resistance is progressively less convincing.

The effects of nutrients on postprandial inflammation and metabolic health are receiving more attention (de Vries et al 2014a, Klop et al 2012, Lacroix et al 2012). Our acute feeding data demonstrates the ability of nutrients to induce modest leukocyte infiltration of VF in lean, healthy rodents **(Figure 12**). Repetitive and regular consumption of pro-inflammatory foods results in continued leukocyte infiltration (**Figure 11**), and eventually, insulin resistance (**Figures 34 & 35**). This data roughly outlines a series of small inflammatory reactions to HFD consumption which eventually evolve into heavily inflamed VF (**Figures 26 & 27**) and loss of glycemic control (**Figure 33**). There has been speculation about this process in the past (Gregor & Hotamisligil 2011) but experimental evidence has so far been lacking.

We have shown that Psel deletion protects mice from short term HFD feeding induced neutrophil accumulation in the VF (**Figure 20**). This protection is evidently sustained during chronic HFD consumption (**Figure 36**) and reduces local and systemic expression of pro-inflammatory cytokines (**Figures 37 & 38**). Despite an improved inflammatory phenotype, $Psel^{-/-}$ mice are as metabolically impaired as inflamed controls (**Figures 43 & 45**). Similarly, MPO deletion reduced postprandial lipemia induced inflammatory action (**Figures 23-25**) and lowered systemic TNFα levels in DIO mice (**Figure 46**). Consistent with a recent report, MPO deficient mice had slightly less weight

gain and mildly improved insulin sensitivity (**Figure 47 & 51**) (Wang et al 2014). Interestingly, $MPO^{-/-}$ mice were glucose intolerant but highly responsive to insulin (**Figure 51**) which is more characteristic of type I diabetes phenotype than a type II phenotype. We did not measure β-cell function or signaling events downstream of insulin such as phosphorylation of IRS-1 and IR-β. Determining why $MPO^{-/-}$ mice are more sensitive to insulin than $Psel^{-/-}$ mice requires further investigation. Because both groups experience dampened immune cell trafficking into the VF following extended HFD exposure, it is unlikely that leukocyte accumulation accounts for the difference in metabolic health.

This thesis does not exonerate infiltrating immune cells in metabolic tissues. None of our data negates any of the previously described repercussions associated with pathological immune cell accumulation in fat. We have simply demonstrated that interference in immune cell accumulation does not sufficiently improve glycemic regulation. One or more different mechanisms must contribute to the desensitization of insulin. Immune cell accumulation in the VF, therefore, appears to be a symptom of HFD consumption which also acts as a secondary contributor to insulin resistance. Leukocyte invasion of VF may be a strong facilitator of insulin resistance but not a prerequisite.

Understanding how $Psel^{-/-}$ mice become insulin resistant without immune cell accumulation in VF will improve our knowledge of how insulin resistance develops and potentially provide clinicians with more therapeutic targets and strategies. In our examination of the acute effects of HFD consumption, nearly all variables tested are reliant upon Psel activation. In the acute phase (Chapter 2), the inflammatory phenotype of high-fat fed $Psel^{-/-}$ mouse data closely resembles that of the WT low-fat fed mice (**Figure 17**). However, in the chronic phase, the metabolic phenotype of high-fat fed $Psel^{-/-}$ mice better

reflects the DIO WT mouse data (**Figures 43 & 45**). By only examining leukocyte trafficking in response to acute lipid overload, which is ablated in Psel$^{-/-}$ mice, we have limited knowledge about how Psel$^{-/-}$ mice respond HFMs. Leukocyte-endothelium interactions are a good measure of endothelial activation in mice with the eCAMs required for leukocyte-endothelium interactions, not Psel$^{-/-}$ mice. Therefore, other markers of endothelial dysfunction and oxidative stress must be quantified. Measuring nitrotyrosine formation or eNOS function in Psel$^{-/-}$ mice fed HFD would permit insights into whether Psel$^{-/-}$ mice experience and develop endothelial dysfunction and oxidative stress in response to high-fat feeding. These pathological phenotypes drive insulin resistance in the absence of immune cell accumulation. A recent report concluded that endothelial function is preserved in DIO PSGL-1 deficient mice (Wang et al 2012). As previously stated, PSGL-1 deletion is a much stronger intervention than Psel deletion, and endothelial function in DIO Psel$^{-/-}$ mice must be explored.

The MPO$^{-/-}$ mice need to be better characterized as well. Deletion of MPO prevents acute HFM induced leukocyte-endothelium interactions (**Figures 23 & 24**) and extravasation (**Figure 25**) but the mechanism is unclear. Specifically, we cannot determine if endothelial or neutrophilic function is impaired. Again, without a marker of endothelial activation aside from leukocyte-endothelium interactions, our understanding of the acute reaction to high-fat feeding is limited. Quantification of nitrotyrosine formation, eCAM expression, and eNOS function would help characterize the endothelial biology of these mice. To better determine neutrophil function, neutrophils can be isolated from these mice and incubated with known neutrophil activators like LPS or TNFα. Quantification of other neutrophil primary granule enzymes like elastase would help determine whether

neutrophils deficient in MPO degranulate normally. It is possible MPO plays a role in the initiation of leukocyte rolling; it is also possible that genetic deletion of MPO impairs neutrophil biology in a presently unknown way.

Consideration must be given to what factors may drive metabolic dysfunction in the absence of immune cell accumulation in VF. Our obesogenic, HFD caused significant weight gain in WT, Psel deficient, and MPO deficient mice (**Figure 47**). Adipocyte hypertrophy is a characteristic of obesity which drives tissue hypoxia (Hosogai et al 2007, Halberg et al 2009). Adipose tissue hypoxia diminishes local insulin sensitivity (Regazzetti et al 2008, Copps et al 2009). DIO Psel$^{-/-}$ and MPO$^{-/-}$ mice may experience adipose tissue hypoxia, causing desensitization to insulin without inflammatory cell action. Hypoxia-inducible factor, a transcription factor activated under hypoxic stress, serves as a marker for hypoxia. Measuring hypoxia-inducible factor in the VF of DIO Psel$^{-/-}$ and MPO$^{-/-}$ mice may provide insight into how insulin sensitivity deteriorates in the absence of leukocyte infiltration.

Hyperglycemia and hyperlipemia have each been shown to overload mitochondria, increasing electron donor formation which ultimately leads to oxygen reduction and the generation of superoxide (Ceriello & Motz 2004). ROS are a powerful source of cell dysfunction that promote inflammatory signaling. In the presence of ROS, endothelial NO is scavenged by superoxide to form the strong oxidant peroxynitrite. Uncoupling of eNOS further decreases NO bioavailability and increases endothelial dysfunction (Giugliano et al 1997). In pancreatic β-cells, ROS interfere with insulin secretion, resulting in β-cell dysfunction and subsequent diminished glycemic control (Krauss et al 2003), which perpetuates hyperglycemia. ROS induces protein kinase C activation leading to

downstream serine phosphorylation of IRS-1 by JNK, IKKβ, and IRAK in adipose tissue (Itani et al 2002). The deleterious effects of ROS on metabolism are more complex and numerous than described here. It must be noted, however, that each of these ROS dependent mechanisms are independent of immune cell activity and may contribute to the metabolic pathological state found in DIO mice in the absence of immune cell accumulation in metabolic tissues. Inflammatory immune cell action, however, perpetuates ROS formation.

There is substantial evidence that inflammation is beneficial in obesity. Insulin drives the expansion of fat tissue; by reducing adipose tissue sensitivity to insulin, leukocytes limit insulin's ability to drive lipid accumulation in adipocytes (Saltiel 2012). In addition, leukocytes stimulate adipocyte lipolysis, further attenuating adipocyte expansion (Ye & McGuinness 2012). Inflammation, therefore, may be considered a protective mechanism against further increases in fat mass. Interestingly, our DIO Psel$^{-/-}$ mice, which are protected from VF inflammation, gained slightly more weight than DIO WT mice (**Figure 39**).

Inflammation also influences fat expansion by increasing energy expenditure (Ye & McGuinness 2012). TNFα receptor deficient mice have lowered metabolic rates and increased adiposity compared to WT mice when fed normal chow, indicating the importance of TNFα signaling in energy homeostasis (Pamir et al 2009). IL-1 receptor antagonist, an anti-inflammatory protein secreted by adipose tissue, interferes with the activity of the pro-inflammatory IL-1 (Juge-Aubry et al 2003). Mice deficient in IL-1 receptor antagonist have increased IL-1 activity, increased energy expenditures, and protection from DIO and insulin resistance (Matsuki et al 2003, Somm et al 2005). To

further demonstrate the anti-obesogenic effects of IL-1, IL-1 receptor deletion causes a 2-fold increase in adiposity coupled with hyperglycemia and insulin resistance in mice fed LFDs (4% fat) (Garcia et al 2006). Similarly, IL-6 deficiency causes obesity and insulin resistance in mice fed a non-obesogenic diet (Wallenius et al 2002). The overexpression of adiponectin, an anti-inflammatory adipokine, causes exaggerated fat accumulation without increased inflammation in mice (Kim et al 2007) These data suggest that anti-inflammatory methods of metabolic protection hinder the benefits inflammation provides to energy balance, causing a state of "inflammation resistance" which promotes weight gain (Ye & McGuinness 2012).

One limitation of this report is the lack of variability in dietary compositions examined; we have compared how consumption of one LFD and one HFD affects inflammation and metabolic health. The LFD is moderately high (Sakomoto et al 2012) in sucrose, which makes up 35% of the total calories of the diet (**Table 1**). A previous study has demonstrated that isocaloric LFDs composed of either corn starch or sucrose are not obesogenic in mice (Surwit et al 1995). Postprandial hyperglycemia, however, activates the endothelium and impairs brachial artery dilation in healthy volunteers (Mah et al 2011). The differential effects of acute sucrose, fructose, and glucose loads on postprandial leukocyte-endothelium interactions and Psel activation remain unknown.

The unique abilities of fatty acids to elicit or inhibit inflammatory action are well described: SATs are widely regarded as pro-inflammatory while MUFAs are considered anti-inflammatory (van Dijk et al 2009, Oh et al 2010). The fat portion of the HFD utilized in this report is evenly composed of saturated fatty acids, monounsaturated fatty acids, and polyunsaturated fatty acids (**Table 2**). Identification of which fatty acids in our HFD were

responsible for inflammatory action and metabolic complications, therefore, could not be made. We demonstrated that direct exposure of MPCVs to palmitic acid, a precursor to ceramide, induces leukocyte-endothelium interactions (**Figure 16**). We chose to examine palmitic acid because it is a well-established pro-inflammatory agent (Benoit et al 2009, Eguchi et al 2012, Shi et al 2006), but palmitic acid is only one of several fatty acids that makes up a large portion of the HFD. Pre-incubation of endothelial cells with oleic acid significantly reduces the expression of VCAM-1, Esel, and ICAM-1 following TNFα, IL-1, and LPS challenges (Carluccio et al 1999). Skeletal muscle incubation with palmitic acid increased diacylglycerol and the protein kinase C pathway while incubation with oleic acid did not (Coll et al 2008). Furthermore, co-incubation of the two fatty acids together reduced diacylglycerol formation, decreased inflammatory gene expression, protected insulin sensitivity, and increased expression of genes regulating mitochondrial β-oxidation (Coll et al 2008). Interestingly, oleic acid is more abundant in our HFD, which had pro-inflammatory effects in the acute and chronic phases (**Figures 5 & 26**), than palmitic acid (**Table 2**). The protective effects of oleic acid *in vitro* were not preserved in our *in vivo* model. The individual effects of the fatty acids included in our composite HFD on leukocyte-endothelium interactions following acute exposure should be further studied.

In conclusion, we have described a novel characterization of the relationship between metabolism and inflammation in healthy mice. Macronutrient composition determines inflammatory reactions to food consumption independent of caloric excess. A single episode of postprandial hyperlipemia stimulates modest leukocyte flux into the VF during a transient immune response. Repetitive and prolonged HFD consumption sustains inflammatory action after mice become obese and insulin resistant. Reducing immune cell

accumulation in the VF by genetic deletion of Psel does not improve metabolic health. Deletion of MPO, which also reduces immune cell accumulation and may have a secondary insulin sensitizing function, improves insulin sensitivity without lowering hyperglycemia. These data indicate that immune cell accumulation and subsequent inflammatory state in the VF is not the primary driver of insulin resistance. The identification of inflammation independent mechanisms which develop insulin resistance may shift focus to new therapeutic targets and strategies for curtailing the progression of obesity and metabolic disorders.

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