LOCAL MACROPHAGE PROLIFERATION IN ADIPOSE TISSUE IS A CHARACTERISTIC OF OBESITY-ASSOCIATED INFLAMMATION

A Dissertation Presented

By

Shinya Ulysses Amano M.A.

Submitted to the Faculty of the

University of Massachusetts Graduate School of Biomedical Sciences, Worcester

in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

March 27, 2013

MD/PHD PROGRAM

LOCAL MACROPHAGE PROLIFERATION IN ADIPOSE TISSUE IS A CHARACTERISTIC OF OBESITY-ASSOCIATED INFLAMMATION

A Dissertation Presented By

Shinya Ulysses Amano M.A.

The signatures of the Dissertation Defense Committee signifies completion and approval as to the style and content of the Dissertation

_____
Michael Czech, Ph.D., Thesis Advisor

_____
Hardy Kornfeld, M.D., Member of Committee

_____
Stuart Levitz, M.D., Member of Committee

_____
John Harris, M.D., Ph.D., Member of Committee

_____
Gökhan Hotamisligil, M.D., Ph.D., Member of Committee

The signature of the Chair of the Committee signifies that the written dissertation meets the requirements of the Dissertation Committee

_____
Dale Greiner, Ph.D., Chair of Committee

The signature of the Dean of the Graduate School of Biomedical Sciences signifies that the student has met all graduation requirements of the school.

_____
Anthony Carruthers, Ph.D.
Dean of the Graduate School of Biomedical Sciences

MD/PhD Program
March 27, 2013

Acknowledgements

First and foremost, I would like to thank Dr. Michael Czech for bringing me into his lab and supporting me through good times and difficult times. It has been a pleasure working under his scientific and diplomatic wisdom. His unwavering optimism is infectious, and I am very thankful for his kindness. I would also like to thank Dr. Myriam Aouadi for being an excellent mentor, for encouraging me to take up this project, and for providing excellent scientific guidance and support throughout my time in the Czech lab.

I have had the pleasure of working with a great team of scientists during my time in the Czech lab. Firstly I must thank Dr. Greg Tesz for taking me under his wing when I first joined the lab. I would like to thank Dr. Jessica Cohen for her crucial and skillful assistance in nearly all aspects of this body of work, as well as Dr. Michaela Tencerova for critical help. I would also like to thank all other members of the GeRP team for their friendship and technical help: Sarah Nicoloro, Pranitha Vangala, Joe Yawe. I would also like to thank our lab guru Joe Virbasius for his scientific advice and his vegetable gardening expertise. Also, I must thank Mengxi Wang, Chang-An Guo, Dr. Adilson Guilherme, and Dr. David Pedersen for being excellent collaborators. Special thanks to Dr. Van Tran and Dr. Sophia Kogan, and thanks to all other lab members, past and present, and of course to Marty and Debbie.

I would like to acknowledge my Thesis Research Advisory Committee members, Dr. Dale Greiner, Dr. Stewart Levitz, and especially Dr. Hardy Kornfeld and Dr. John Harris for being my advisors for the past several years. Also, I am grateful to the University of Massachusetts MD/PhD program for their past and future support, and for providing me with excellent classmates who I am proud to call my friends. In particular, I must thank Timothy Chang, John Kaminski, Brian Quattrochi, and Dr. Thomas Flood.

I must thank my undergraduate and Master's thesis research advisor, Dr. Rex Pratt, for giving me an excellent foundation in scientific investigation, and my Wesleyan University Scholarship donor Donna S. Morea for sponsoring my work in research labs for all four years of my undergraduate work as well as over summers.

Most importantly, none of this would have been possible were it not for the support of my family. My mother and father made many sacrifices to provide their children with the absolute best education available, and I think about that every day. I would like to thank my father, mother, sister, and grandparents for loving and supporting me at all times. I would also like to thank my beautiful, loving wife for being there for me during this experience. Her constant support makes all goals attainable. Thank you all so much.

Abstract

Obesity and diabetes are major public health problems facing the world today. Extending our understanding of adipose tissue biology, and how it changes in obesity, will hopefully better equip our society in dealing with the obesity epidemic. Macrophages and other immune cells accumulate in the adipose tissue in obesity and secrete cytokines that can promote insulin resistance. Adipose tissue macrophages (ATMs) are thought to originate from bone marrow-derived monocytes, which infiltrate the tissue from the circulation. Much work has been done to demonstrate that inhibition of monocyte recruitment to the adipose tissue can ameliorate insulin resistance. While monocytes can enter the adipose tissue, we have shown here that local macrophage proliferation may be the predominant mechanism by which macrophages self-renew in the adipose tissue.

We demonstrated that two cell proliferation markers, Ki67 and EdU, can be readily detected in macrophages isolated from adipose tissue of both lean and obese mice. These analyses revealed that 2-4% of ATMs in lean and 10-20% of ATMs in obese mice express the proliferation marker Ki67. Importantly, $Ki67^{+}$ macrophages were identified within the adipose tissue in crown-like structures. Similarly, a 3-hour *in vivo* pulse with the thymidine analog EdU showed that nearly 5% of macrophages in epididymal adipose tissue of *ob*/*ob* mice were in the S-phase of cell division. Interestingly, obesity increased the rate of macrophage proliferation in adipose tissue but did not affect macrophage proliferation in other tissues. We also used clodronate liposomes to deplete circulating monocytes in obese mice. Surprisingly, monocyte depletion for a total of at least 80 hours did not cause a decrease in ATM content in adipose tissue. Prolonged exposure of mice to

EdU in drinking water revealed that approximately half of the ATMs in the epididymal fat pads of *ob/ob* mice had proliferated locally within 80 hours. Amazingly, these rates were the same with or without monocyte depletion, meaning that the proliferating cells were not freshly recruited monocytes.

Overall, these results suggest that local proliferation unexpectedly makes a major contribution to maintaining the large population of macrophages present in the obese adipose tissue in the steady state. This suggests that increased rates of local macrophage proliferation may also be partly responsible for the massive increase in ATM content that occurs in obesity. This information could have implications for future therapeutic strategies in the management of diabetes.

Table of Contents

List of Figures

List of Frequently Used Abbreviations

Abbreviation	Term
FFA	Free fatty acid
VEGF	Vascular endothelial growth factor
HFD	High fat diet
ND	Normal diet
ER	Endoplasmic reticulum
LDL	Low-density lipoprotein
BMI	Body mass index
TNFα	Tumor necrosis factor alpha
IL-1β	Interleukin 1 beta
IL-4	Interleukin 4
IL-6	Interleukin 6
IRS	Insulin receptor substrate
JNK	c-JUN N-terminal kinase
NFκB	Nuclear factor kappa B
IKKβ	Inhibitor of NFκB kinase subunit beta
PPARγ	Peroxisome proliferator-activated receptor-gamma
ATM	Adipose tissue macrophage
SVF	Stromal-vascular fraction
MCP-1	Monocyte chemoattractant protein 1
CCR2	CC-motif chemokine receptor 2
CLS	Crown-like structure
WT	Wild-type
GTT	Glucose tolerance test
M-CSF	Macrophage-colony stimulating factor
GM-CSF	Granulocyte-macrophage colony stimulating factor
EdU	5- ethynyl-2’-deoxyuridine
BrdU	5-bromo-2'-deoxyuridine

CHAPTER I: Introduction

In order to survive, animals must be able to store excess energy in times of plenty, and release that energy when food sources are absent. The most volume- and weight–efficient way to store excess energy is in the form of lipid[1]. Lipid is an ideal form in which to store energy due to its high energy density and a hydrophobic nature that reduces obligatory water retention, allowing animals to store as much energy in as little weight as possible. In most animals lipids are stored in distinct adipose depots[2]. Over the course of evolutionary history the adipose tissue has developed into an organ that is efficient at the uptake of excess nutrient, the conversion of that nutrient into lipid triglyceride, and the controlled release of lipid during starvation conditions[2,3]. For example, adult polar bears must endure seasonal fasting for four or more months each year and lose up to 40% of their total body weight in that time. Depending on the amount of fat stored, nearly all of that weight loss can be due to fat catabolism, while most or all lean body mass is retained[4]. Thus, the adipose tissue has the capacity to undergo enormous changes in mass in response to environmental conditions, and its proper function is critical for survival.

In humans, adipose tissue is also very efficient at storing excess nutrient and has the capacity to expand tremendously – often to a stage where greater than 50% of the total body weight is adipose tissue[5]. In the time of our ancestors, this ability to store excess energy was beneficial for survival during times of scarcity, but in the modern civilized world, human exposure to excess nutrient is perpetual. As a result, the global rates of overweight and obesity have reached epidemic levels. Obesity is typically defined as a body mass index (BMI) of over 30 kg/m^2 – a calculation based on a person's

height and weight. According to the World Health Organization more than 1 in 10 of the world's adult population was obese in 2008, and its prevalence is on the rise. Overweight and obesity were the fifth leading cause of global deaths[6]. Obesity is associated with many additional health problems, including insulin resistance, cardiovascular disease, fatty liver disease, and cancer, among several others[7,8]. Indeed, 44% of the global diabetes burden, 23% of ischemic heart disease, and a large portion of cancer are attributable to obesity[6]. In the United States, annual health care expenditures related to overweight and obesity are projected to reach nearly $1 trillion by 2030, which represents nearly 20% of total spending on health care[6,9]. In order for our society to deal with this worsening epidemic, we must advance our understanding of adipose tissue physiology and the pathogenesis of diabetes and obesity.

Mechanisms of Insulin Resistance

Adipocyte Dysfunction and Inflammation

Obesity and insulin resistance are closely associated with a state of chronic, low-grade inflammation[3,10-12]. Adipose tissue plays a central role in the development of insulin resistance. While muscle and liver are the main insulin-sensitive tissues of the body[13], adipose tissue is also highly insulin sensitive and is able to exert profound control over the function of those peripheral tissues[14]. Interestingly, the inflammatory response that occurs in obesity seems to originate in and remain within the adipose tissue itself[14]. Adipose tissue expansion places tremendous strain on the cells within the tissue, ultimately leading to adipocyte dysfunction, in which adipocytes are unable to synthesize triglyceride and instead release free fatty acid into the circulation[14]. Under these conditions, both the hypertrophic adipocytes and resident adipose tissue macrophages produce inflammatory cytokines that act to interfere with insulin signaling in adipocytes and other cells, as well as to recruit additional immune cells to the adipose tissue, which produce more cytokines in a cycle of inflammation[15]. These and other topics will be discussed below.

The Lipid Spillover Hypothesis

One prevailing theory on how obesity leads to insulin resistance is the "lipotoxicity" theory first proposed in 1995[16]. In the early stages of obesity, adipocytes remain capable of storing the increasing load of incoming triglyceride. As adipose tissue expands further, the ability of the adipocytes to store lipid decreases and excess lipid

spills over into the circulation in a phenomenon known as lipolysis. This increased rate of lipolysis in obesity results in increased plasma levels of free fatty acids (FFAs)[16,17]. In obesity, FFAs and lipid metabolites can accumulate in liver and muscle, and cause insulin resistance in these tissues[14,18]. Several lines of evidence support this view. Obesity and insulin resistance are strongly associated with high circulating FFA levels[18]. Reductions of FFA levels using anti-lipolytic compounds or by enhancement of adipose tissue activity has been shown to improve insulin sensitivity in peripheral tissues[19,20]. However, it is clear that insulin resistance can occur in the absence of elevated FFA levels, and that elevated FFA levels do not always cause insulin resistance[21]. Nonetheless, there is no doubt that elevated FFA and lipid metabolites in liver and muscle can cause insulin resistance in those tissues, as shown in humans using lipid infusions and in mice using tissue-specific lipoprotein lipase overexpression[22-24]. The importance of the ability of the adipose tissue to sequester lipids away from the rest of the body is highlighted by the hyperlipidemia and insulin resistance observed in lipodystrophic mice[25,26] and humans[27,28] that lack adipose tissue. Lipid sequestration and release represent important ways in which adipose tissue function can affect insulin sensitivity throughout the rest of the body.

Adipose Tissue Hypoxia

Another consequence of adipose tissue growth is increased oxygen demand. Adipose tissue growth is dependent on new blood vessel formation[29]. However, in obesity, the closely coupled processes of adipocyte expansion and neovascularization are

disrupted, and adipose tissue hypo-perfusion and hypoxia result[30]. Improvement of adipose tissue perfusion by overexpression of vascular endothelial growth factor (VEGF) specifically in adipose tissue was shown to improve hypoxia, adipose tissue inflammation and whole body glucose homeostasis[31]. Overexpression of VEGF in adipose tissue has also been shown to reduce adiposity[32]. A recent study has confirmed that adipose tissue vasculature depletion causes systemic insulin resistance and local adipose tissue inflammation, while overexpression of VEGF decreased adipose tissue inflammation and reduced systemic insulin resistance in a high fat diet (HFD)-fed mouse model[33]. These studies again highlight the important role adipose tissue plays in the proper metabolic functioning of the entire body. Adipose tissue hypo-perfusion may be a precipitating factor in the series of events leading to adipocyte dysfunction, adipose tissue inflammation, and total body insulin resistance.

Endoplasmic Reticulum Stress

As adipose tissue expands, tremendous strain is placed on the adipocytes to accommodate the influx of nutrient, and to process and store that nutrient as triglyceride. The surge in protein and lipid synthesis increases the synthetic demands placed on the endoplasmic reticulum (ER). When protein production exceeds the capacity of the ER to properly fold and modify nascent peptides, a cellular response takes place that slows protein production and increases the expression of protein folding chaperones[34]. It has been shown that cellular changes that occur in ER stress can inhibit insulin signaling in adipocytes in mice[35,36] and the ER stress response has been shown to be elevated in the

adipose tissue of humans with insulin resistance[37]. Genetic manipulation of the ER stress pathway can alleviate insulin resistance in mouse models of obesity[36], and treatment of obese mice with small molecules that aid in protein folding also improve glucose homeostasis[38]. It should be noted that ER stress can also contribute to insulin resistance in peripheral tissues as well[39,40]. ER stress can also initiate inflammatory signaling within the adipocyte[36,41], and may provide a link between excessive adipose tissue growth and the emergence of adipose tissue inflammation.

The progression of insulin resistance to diabetes mellitus

In the early phases of obesity and in the pre-diabetic state, the insulin resistance of peripheral tissues does not immediately result in hyperglycemia[42]. During this time, the pancreatic beta cells are able to maintan normal blood glucose levels despite insulin resistance of the peripheral tissues by increasing their mass, in a process known as beta cell compensation[42]. Beta cell mass is increased in obese non-diabetic compared to lean non-diabetic humans[43] and the same is true in rodents[44]. During this time, the beta cells also increase their expression of insulin[45]. This phase of compensatory hyperinsulinemia is followed by a decline in beta cell function[42]. If obesity progresses and insulin resistance worsens, tremendous strain in placed on the beta cells, and the ability of the beta cells to produce the compensatory hyperinsulinemia decreases[46,47]. Beta cell mass declines, and it has been shown that beta cells undergo apoptosis[43]. The details and mechanisms of beta cell failure in diabetes, which include lipotoxicity from adipose tissue lipid spillover as well as ER stress, are reviewed in Prentki and Nolan, 2006[42]. As the beta cells

progressively fail, the declining insulin levels are no longer able to overcome the insulin resistance of the peripheral tissues, and hyperglycemia ensues[48]. Clinically, a fasting plasma glucose level below 100 mg/dl is considered normal, 101-125 mg/dl is considered "impaired fasting glucose" or pre-diabetic, and diabetes mellitus is diagnosed when a patient has a fasting plasma glucose of over 126 mg/dl[49,50].

Obesity and Inflammation

Discovery of Inflammation in Obesity

Perhaps the most important factor in causing and driving adipose tissue dysfunction is inflammation. Whether induced by ER stress, hypoxia, or other factors, adipose tissue hypertrophy often results in adipocyte dysfunction. Adipocyte dysfunction, in turn, is a critical component in a cycle of inflammation within the adipose tissue[14]. Hotamisligil and colleagues and Feinstein and colleagues first discovered in 1993 that the inflammatory cytokine tumor necrosis factor alpha (TNFα) was expressed in adipose tissue and was capable of inducing systemic insulin resistance[10,51]. Importantly, TNFα expression was shown to be greatly increased in obese animals compared to lean controls. Since that time, the adipose tissue has been identified as the source of many cytokines and some even consider adipose tissue an endocrine organ[52].

Inflammation and Insulin Resistance

Since 1993, hundreds of studies have confirmed the link between inflammation and insulin resistance, and numerous excellent reviews on the subject exist[3,12,14,15], but key points will be discussed here. Studies in humans revealed that obese adipose tissue expresses TNFα constitutively, and that its expression in adipose tissue decreases after weight loss[53,54]. Similarly, TNFα concentrations were found to be elevated in the plasma of obese patients, and TNFα plasma levels decreased after weight loss[55]. Expression of the inflammatory cytokine interleukin 1 beta (IL-1β) and its receptor are increased in the

visceral adipose tissue of obese humans[56] and IL-1 and TNF have been shown to act synergistically and can increase one another's production[57]. Further evidence for obesity being an inflammatory condition came from several studies that confirmed that elevated inflammatory markers in obese patients are predictive of the development of type 2 diabetes[58-62]. Other inflammatory cytokines increased in obesity include interferon gamma, resistin, and interleukin 6 (IL-6) among many others (reviewed in Shoelson et al. 2006[12]). Importantly, TNFα infusion into healthy human subjects can quickly cause insulin resistance[63] and IL-1β has been shown to induce insulin resistance in cultured adipocytes[64]. Taken together, these and many other findings support the concept that obesity is an inflammatory condition, and inflammation itself is linked to the development of insulin resistance in humans.

Importantly, attenuation of inflammation leads to an improvement in insulin resistance. Neutralization of TNFα using a TNF receptor-IgG-fusion protein in a genetically obese rat model caused significant improvement in peripheral glucose uptake[10]. Also genetic deletion of TNFα in obese mouse models resulted in improved insulin sensitivity[65]. Unfortunately early studies which attempted to neutralize TNFα in human patients did not show a benefit in insulin sensitivity[66]. However, neutralization of TNFα has shown improvements in insulin resistance in patients with rheumatoid arthritis and ankylosing spondylitis[67,68]. Also, treatment with Interleukin-1-receptor antagonist yielded a mild improved glycaemia in human patients with type 2 diabetes[69] and also in mice[70,71]. These studies indicate that inflammatory cytokines play a critical role in the regulation of insulin sensitivity.

Inflammatory Signals Interfere With Insulin Signaling

Inflammatory cytokines interfere with the insulin signaling pathway within insulin target cells[3,12,14]. Adipocytes and other insulin target cells express the insulin receptor at their surface. The insulin receptor, when activated by insulin, phosphorylates the insulin receptor substrate (IRS) proteins within the cell on specific tyrosine residues. (For an excellent review on insulin signaling, see Taniguchi, 2006[72]). These IRS proteins are responsible for conveying the insulin signal to downstream effectors in the cell that produce the cellular response to insulin. Inflammatory signals interfere with the insulin signaling pathway at the level of the IRS proteins[3,12,14].

The stress-activated protein kinases c-Jun amino-terminal kinase (JNK) and inhibitor of nuclear factor kappa B (NFκB) kinase subunit beta (IKKβ) have proven to be critical molecules that link inflammation and insulin signaling[12,73]. Many inflammatory signals, both from extracellular and intracellular sources, produce cellular responses involving these molecules. For example, JNK and IKKβ are both activated by inflammatory cytokine signaling from outside the cell and ER stress signals from within the cell[3,12]. TNFα signaling was first shown to inhibit insulin signaling through inhibitory serine phosphorylation of IRS-1 in 1996[74]. Since that time it has become clear that TNFα and IL-1β signals activate JNK[75,76] and JNK, in turn, directly inhibits the insulin signaling cascade by inhibiting IRS-1 activity[77]. JNK accomplishes this by phosphorylating IRS-1 at Serine 307 which prevents it from being activated by the insulin receptor[77,78]. Indeed JNK has been shown to play a central role in the development of obesity and insulin

resistance in liver, muscle, adipose tissue[79], and more recently in macrophages as well [80], and genetic JNK deficiency protects mice from insulin resistance[79]. Similarly, inflammatory cytokines, including TNFα and IL-1β, also activate IKKβ[75,76]. Pro-inflammatory IKKβ activity has also been shown to increase in obesity, and inhibition of IKKβ activity, particularly in macrophages, has been shown to protect mice from the insulin resistance in obesity[81-83]. Unlike JNK however, IKKβ activity inhibits insulin signaling not through direct action on IRS-1, but by activating the transcription of a long list of inflammatory genes[84,85], which subsequently feed back on the cell and inhibit the response to insulin. This is an important way in which inflammatory signaling perpetuates a cycle of inflammation and insulin resistance.

Inflammatory Signals Also Inhibit Adipocyte Lipogenic Function

Inflammation also plays a role in generating the adipose tissue dysfunction and "lipid spillover" discussed above. In obesity, adipocytes are less able to sequester lipid and store it as triglyceride[14]. This may be partly due to the fact that enzymes responsible for de novo synthesis of fatty acids and the esterification of fatty acids intro triglyceride are significantly reduced in the adipose tissue of obese humans[86] as well as in obese mouse models[87,88]. The master regulator of these adipogenic genes, and a necessary factor in the maintenance of mature adipocyte function, is peroxisome proliferator-activated receptor-gamma (PPARγ)[89,90]. Several studies have shown that TNFα and IL-1 are able to downregulate PPARγ action in adipocytes[91-94]. Thus, inflammatory cytokines not only inhibit insulin signaling in adipocytes, they also may contribute to the inhibition of

adipocyte function and the resultant increase in lipid release from adipocytes in the obese state.

Immune Cells in Adipose Tissue

Numerous immune cell types populate the adipose tissue, and the quantities and proportions of those cell types change in obesity, contributing to inflammatory cytokine production. Macrophages are the largest population of immune cell type in the adipose tissue and produce the majority of the inflammatory cytokines that are increased in obesity[11], and will be discussed extensively below. Another large population of immune cells in adipose tissue is eosinophils, which are very numerous in lean adipose tissue, and decrease their presence in obesity[95]. Eosinophils have been shown to secrete interleukin 4 (IL-4) in the adipose tissue, which modulates macrophage function and adipose tissue insulin sensitivity in a beneficial way[95]. Similarly, regulatory T cell number in lean adipose tissue is very high, and decreases in obesity[96]. Concurrent with the decrease in regulatory T cells, an increase in CD4 T_H1 cells and CD8 T cells occurs in the adipose tissue in obesity[97,98]. Importantly, the influx of these T cells has been shown to precede macrophage accumulation in the adipose tissue, and depletion of CD8 T cells decreases macrophage content in obese adipose tissue, suggesting that T cells play a role in controlling adipose tissue macrophage content[97]. The various subsets of natural killer T cells also appear to be involved in adipose tissue inflammation and metabolism, although there are conflicting reports as to what that role may be[99-103]. B cells have also been shown to increase in their presence in adipose tissue in obesity and produce pathogenic

antibodies in the adipose tissue[98]. Interestingly, mast cells, which are normally associated with allergic responses, increase in the adipose tissue in obesity, and deficiency or stabilization of mast cells has been shown to improve insulin sensitivity in obese mice[104]. Thus, the adipose tissue is host to a variety of immune cells whose populations change with obesity and immune cell recruitment contributes to adipose tissue inflammation and obesity.

Adipose Tissue Macrophages

Macrophages Accumulate in Adipose Tissue in Obesity

Analysis of gene expression in cells isolated from obese adipose tissue has revealed that adipose tissue macrophages **(**ATMs**)** are the predominant source of the inflammatory cytokines elevated in obesity[11]. Macrophages accumulate in the adipose tissue in huge numbers in the obese state, a phenomenon first described by Weisberg et. al. and Xu et. al. in 2003[11,105]. In a lean individual, of all non-adipocyte cell types in the adipose tissue (called the stromal-vascular fraction, or SVF) only 5-10% may consist of macrophages, while macrophages can be as high as 40% of all stromal cells in obese human adipose tissue, and as high as 50% in morbidly obese mouse adipose tissue[11]. Importantly, ATM content is closely correlated with adipocyte size and body weight in mice and in humans[11,106-108]. Furthermore, macrophage accumulation in adipose tissue is temporally associated with the onset of insulin resistance[105]. Also, it is very interesting to note that in humans, not all obese individuals have insulin resistance, and inflammatory gene expression and ATM content in visceral adipose tissue has been shown to be a far better predictor of insulin resistance than simple measures of adiposity[109]. Taken together, these studies provide good evidence that under physiological conditions, macrophage content within adipose tissue is increased in obesity and is linked to the development of insulin resistance.

Alterations in Adipose Tissue Macrophage Content Affect Insulin Sensitivity

Since the initial discovery of macrophages in adipose tissue, many attempts have been made to either decrease or increase ATM content, in an attempt to determine if doing so would alter insulin sensitivity. Several groups have succeeded in demonstrating improved insulin sensitivity associated with a decrease in ATM content. For example, treatment of obese mice with the insulin-sensitizing drug rosiglitazone has been shown to decrease ATM content[105]. Surgery-induced weight loss in morbidly obese human subjects has been shown to be associated with decreased macrophage content in white adipose tissue and decreased inflammatory gene expression[110]. Diet and exercise-induced weight loss has also been found to decrease ATM content[111]. Diptheria toxin-induced cell death of macrophages within the adipose tissue in obese mice was associated with improved insulin sensitivity as well[112]. Depletion of ATMs by intraperitoneal clodronate liposome injection also improved insulin sensitivity in obese mouse models[113].

Inhibition of monocyte recruitment to the adipose tissue has, in certain cases, been found to produce a modest decrease in ATM content and was associated with improved insulin sensitivity in those cases[108,114,115]. This topic will be discussed in further detail below. Conversely, adipose tissue-specific overexpression of monocyte chemoattractant protein 1 (MCP-1) increased ATM content and decreased insulin sensitivity in both lean and obese mouse models[115,116]. Taken together, these studies support the concept that ATM content is closely correlated with insulin resistance.

The Increased Inflammatory Properties of ATMs in Obesity.

Not only do macrophage numbers increase in adipose tissue in obesity, but their

activation state is also polarized towards a pro-inflammatory phenotype[117,118]. Macrophages are a very versatile cell type, able to attain a broad range of functional states based on the cues present in their environment[119]. In lean mice, ATMs are generally characterized by an anti-inflammatory "M2-like" phenotype, as determined by their surface marker and gene expression profiles[117]. Anti-inflammatory macrophages are characterized by anti-inflammatory cytokines rather than pro-inflammatory cytokines, and are thought to prevent inflammation and promote tissue repair[120]. Anti-inflammatory "M2-like" macrophages are believed to be generated by exposure to anti-inflammatory signals such as IL-4, IL-10, IL-13, and glucocorticoids, whereas pro-inflammatory "M1-like" macrophage phenotype is believed to be a response to exposure to inflammatory cytokines such as interferon gamma and TNF-α[120]. In obesity, a significant fraction of ATMs lose that anti-inflammatory phenotype and become more pro-inflammatory, or "M1-like", expressing inflammatory cytokines that inhibit the ability of the surrounding adipocytes to respond to insulin[118]. While macrophage sub-populations in the adipose tissue may have overlapping marker expression profiles, it is generally thought that CD11c expression is characteristic of pro-inflammatory macrophage subtypes[112,118,121]. Interestingly, preventing ATMs from attaining an anti-inflammatory phenotype in obese mouse adipose tissue predisposes mice to diet-induced obesity and insulin resistance[122]. Furthermore, a recent publication reported that systemic helminth infection, a canonical M2-polarizing condition, was associated with a shift in ATM phenotype in obese mice to an entirely anti-inflammatory state, and insulin sensitivity was normalized in these mice[95]. Taken together, these reports provide strong evidence that not only increased

macrophage number, but also the increased inflammatory ATM phenotype that occurs in obesity plays an important role in the development of insulin resistance.

Inhibition of Adipose Tissue Macrophage Inflammation Improves Insulin Sensitivity

In addition to absolute decreases in macrophage number in adipose tissue, inhibition of inflammatory signaling within macrophages can also improve insulin resistance. Genetic deletion of JNK specifically in hematopoietic cells, accomplished by bone marrow transplant, was sufficient to protect mice from diet-induced insulin resistance even though the mice became just as obese as control mice[123]. This study was confirmed and extended by a recent publication showing deletion of both JNK1 and JNK2 specifically in macrophages protects mice from obesity-induced insulin resistance[80]. It is important to note that both of these studies also observed a decreased macrophage content in the adipose tissue in the JNK-deficient macrophage condition. Similarly to macrophage-specific JNK deletion, macrophage-specific IKKβ deletion also protects mice from diet-induced adipose tissue inflammation and insulin resistance without protecting from diet-induced weight gain[83]. While these studies emphasize the role of inflammatory signaling within macrophages in obesity, they have all been limited to studying macrophages throughout the body, while the question of the role of macrophages specifically within adipose tissue has been unresolved. Our group recently developed siRNA delivery technology able to specifically target macrophages within obese adipose tissue[124]. Using this technology, we were able to silence production of two inflammatory cytokines, TNFα and osteopontin, specifically in adipose tissue

macrophages, and show improved whole body glucose tolerance. This study confirms the role of inflammatory signaling specifically in macrophages, specifically in adipose tissue, as being of central importance in the development of insulin resistance in obesity.

Adipose Tissue Macrophages May Also Play a Protective Role

Despite the extensive evidence that ATMs are inflammatory and act to exacerbate insulin resistance, there is also evidence for a protective role of macrophages. Kosteli et. al. have shown that acute fasting-induced lipolysis or drug-induced lipolysis is associated with rapid recruitment of macrophages to the adipose tissue[125]. Furthermore, they showed that depletion of macrophages was associated with increased lipid release from adipose tissue during fasting conditions. These findings were confirmed by another group that directly observed that ATMs accumulate lipid in obese mice and become triglyceride foam cells even in the basal obese state[126]. Additional evidence supporting this hypothesis is that macrophages in the obese adipose tissue localize around dead and dying adipocytes, forming structures termed crown-like structures (CLS)[106,107]. When examined by electron microscopy, the macrophages in these structures were found to contain lipid-filled phagolysosomes, suggesting they sequester the lipid being released from the dying adipocytes and prevent it from entering the circulation[106]. These reports support a role for ATMs in taking up excess lipid in order to protect the rest of the body from the toxic effects of free fatty acids being released from adipocytes.

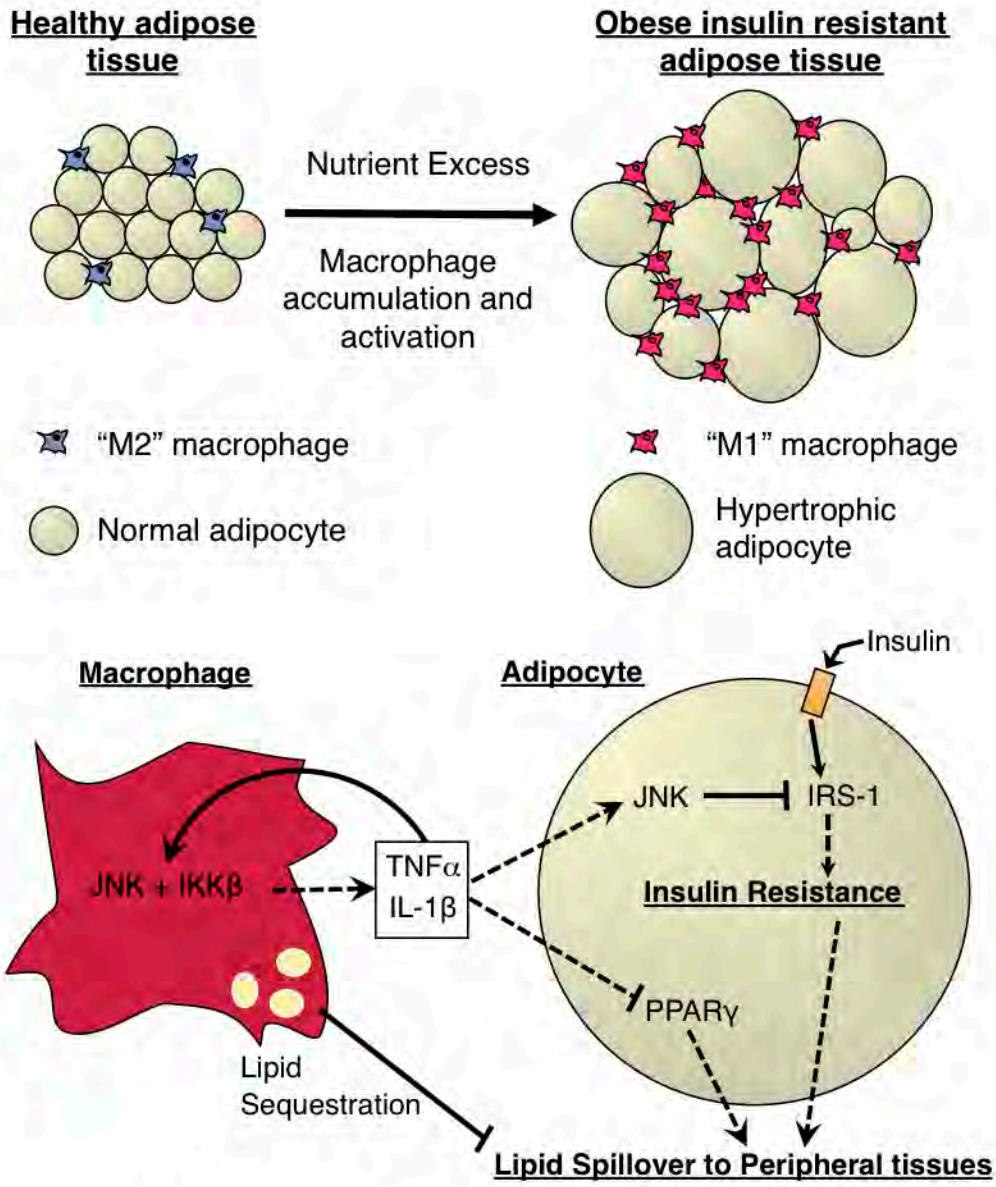


Figure 1.1 Obesity leads to inflammation and insulin resistance in adipose tissue. In healthy subjects, adipose tissue contains anti-inflammatory macrophages ("M2"). In obese subjects, adipocytes become hypertrophic, and macrophages accumulate and activate in adipose tissue. The stimulation of JNK and IKKβ induces the production of pro-inflammatory cytokines, and intereferes with adipocyte function. The increase of pro-inflammatory macrophages ("M1") in the adipose tissue further amplifies inflammation and insulin resistance. However, macrophages may also play a beneficial role in taking up excess lipids released from adipocytes. Figure adapted from Czech, MP et al. Nature Reviews Endocrinol. 2011.

The Origin of ATMs

Evidence That ATMs Are Derived From Monocytes

Much effort has been focused on determining the factors that attract macrophages to the adipose tissue in obesity. It is generally believed that myeloid precursors develop in the bone marrow, enter the circulation as blood monocytes and subsequently migrate into tissues where they terminally differentiate into macrophages[127]. Consistent with this view, early studies using total body lethal irradiation followed by bone marrow transplant concluded that ATMs were mostly bone-marrow derived[11]. Subsequent studies aimed to reduce monocyte recruitment to the adipose tissue in obesity. Monocyte chemoattractant protein 1 (MCP-1) was identified in several studies as one of the inflammatory cytokines whose expression increases in obese adipose tissue[11,128,129]. Whole body genetic deletion of MCP-1 was found to produce a modest but significant decrease in ATM content in mice fed a high-fat diet for 12-16 weeks[115]. Similarly, whole body genetic deletion of the MCP-1 receptor, CCR2, produced a modest but significant reduction in ATM content in mice fed a high fat diet[108]. This study also employed a short term drug-induced inhibition of CCR2 and observed a small but significant difference in ATM content in obese mice after 17 days of daily injection with a small molecule inhibitor of CCR2[108]. It is important to note that three other studies using MCP-1 and CCR2 knockout animal models and employing nearly identical experimental conditions failed to demonstrate any difference in ATM content in obesity[130-132].

Inhibition of osteopontin, an inflammatory cytokine and extracellular matrix protein

elevated in obesity, by either genetic deletion or injection of neutralizing antibody, was also associated with decreased macrophage content in adipose tissue in obese mice and an improvement in insulin sensitivity[133,134]. Another study employed genetic deletion of alpha-4 integrin, which is required for macrophage adhesion to endothelial cells during transmigration into tissues[114]. Myeloid-specific absence of this adhesion marker was also associated with a small but significant decrease in ATM content in diet-induced obese mouse models[114]. Likewise, a myeloid-specific knockout of Cbl-associated protein, a protein involved in macrophage migration, produced a decreased ATM content in obese adipose tissue[135]. It is critical to note that both of these studies employed total body lethal irradiation followed by bone marrow transplant in order to generate the myeloid-specific knockout models, and a recent study has shown that irradiation dramatically reduces the lifespan of resident macrophages[136].

On the other hand, as with most any tissue, monocytes can be induced to enter adipose tissue. As described above, overexpression of MCP-1 specifically in the adipose tissue was associated with an increased macrophage content in the adipose tissue, presumably due to increased monocyte recruitment from the blood[115,116]. Also described above, induction of lipolysis has been shown to attract monocytes to adipose tissue in lean mice[125]. However, a recent paper showed that the cells which infiltrate that adipose tissue during lipolysis may actually be neutrophils[137]. It is interesting to note that, unlike in lean animals, induction of lipolysis in adipose tissue of obese mice by fasting did not stimulate the recruitment of monocytes to adipose tissue[138]. However, a recent paper demonstrated that monocytes labeled with dye and adoptively transferred into obese

recipient mice do appear within the adipose tissue within 2-3 days following transfer, and more of the dye-labeled monocytes are recruited to adipose tissue in obese mice as compared to lean mice[139]. Importantly, they demonstrated that the recruitment of labeled monocytes to adipose tissue was partially dependent on MCP-1 and CCR2[139]. Overall, there is substantial evidence that monocytes can be induced to enter adipose tissue by various factors, and that the absence of certain chemokines and adhesion and migration-related molecules can attenuate the increase of macrophages in adipose tissue that occurs in obesity. However, the chemokine studies have reported varied results, and the adhesion and migration molecule knockout studies may also be interpreted as attenuation of the ability of knockout monocytes to repopulate of the adipose tissue following the destruction of resident adipose tissue macrophages by total body lethal irradiation. Therefore, we hypothesized that, in the absence of total body lethal irradiation, ATMs arise not from circulating monocytes, but from local proliferation *in situ*.

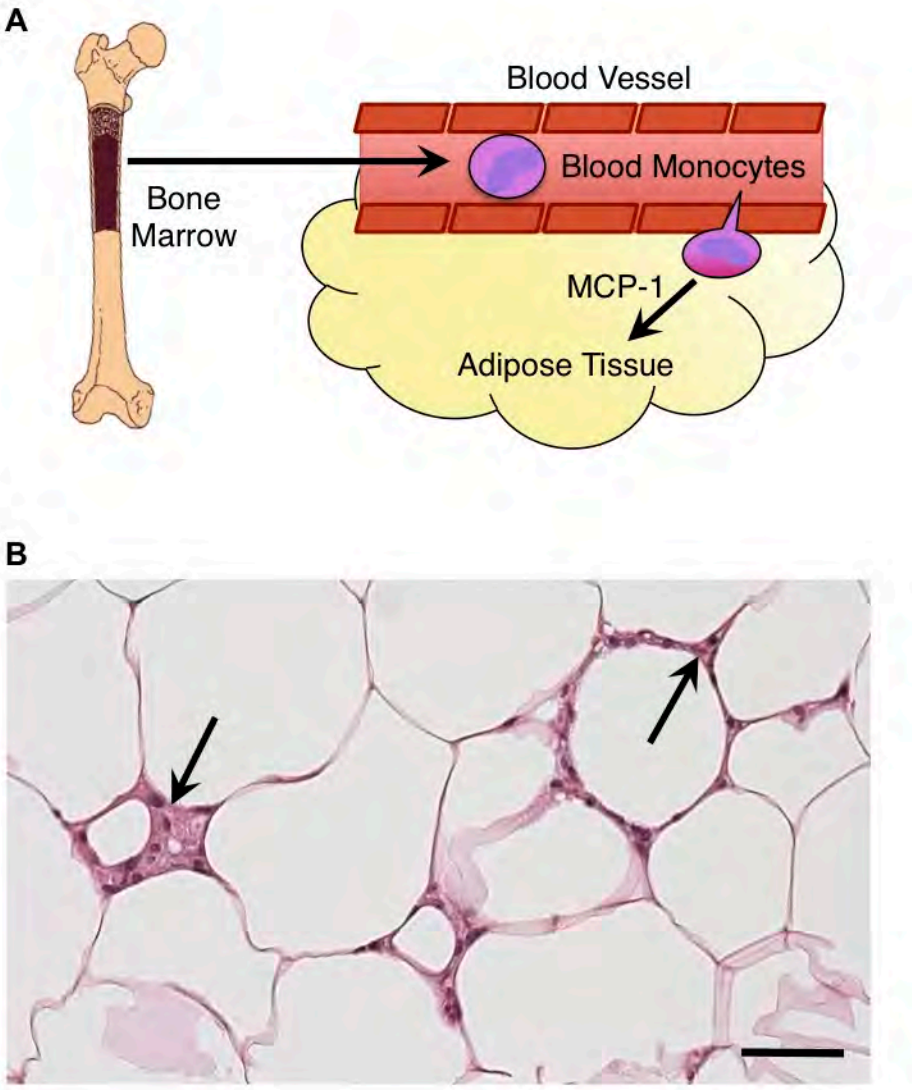


Figure 1.2 Current View of the Origin of Adipose Tissue Macrophages. (A) ATMs are currently believed to develop in bone marrow, enter the circulation as blood monocytes and then migrate into the inflamed adipose tissue, possibly due to the effects of chemoattractants such as MCP-1. (B) H&E image of an epididymal fat pad of an *ob*/*ob* mouse. 20x image, scale bar = 50 μm. Arrows indicate macrophages in crown-like structures.

Examples of Local Macrophage Proliferation

Selective Ablation of Monocytes Preserves Tissue Macrophages

Monocytes are developed from myeloid precursor cells in the bone marrow, and mature monocytes enter the bloodstream and enter the tissues where they terminally differentiate into macrophages[127,140]. It is generally thought that terminally differentiated cells are not capable of re-entering the cell cycle to proliferate. However, macrophages have long been known to be capable of proliferating *in situ* and those examples will be reviewed below. The best early evidence that tissue macrophages could proliferate locally came from experiments that involved using radioactive strontium to deplete bone marrow cells[141]. Strontium is in the same chemical group as calcium, and is stably incorporated into hydroxyapatite crystals in bone in a similar fashion to calcium[142]. Radioactive strontium-89 is a beta particle emitter with a half life of over 50 days[142]. Therefore, simply injecting mice with radioactive strontium provided an easy, targeted method to deplete bone marrow cells without affecting proliferating cells in other tissues[141]. This represents a key difference from modern day bone marrow depletion studies that employ total-body lethal irradiation, which can destroy proliferating cells not only in the bone marrow, but in many tissues of the body[136]. In the strontium study, monocytes were depleted to undetectable levels for the duration of the 10-15 day study periods[141]. Despite this profound and prolonged monocytopenia, there were no changes in total number of resident macrophages in the lung or peritoneal cavity[141]. When assayed for proliferation markers, 3% of peritoneal and 1.5% of alveolar macrophages were found to be actively

synthesizing DNA during a one hour pulse of radioactive thymidine[141]. These results suggested that resident macrophage population renewal under normal circumstances does not depend heavily on influx of circulating monocytes. In the sections below, I will review the recent studies focused on resident macrophage proliferation which have shown that, in several tissues, local proliferation plays a predominant role in maintaining tissue macrophage populations, with recruitment of monocytes playing little or no role.

Alveolar Macrophages

As described above, the 1982 radioactive strontium bone marrow depletion studies first identified that alveolar macrophages could proliferate locally. Prior to that time alveolar macrophages were believed to be derived from circulating monocytes in both mouse[143,144] and human[145,146]. However, these early studies employed total body irradiation, which inhibits the proliferation of host cells. Subsequent bone marrow transplant studies elucidated that a more gentle, fractionated irradiation regimen prevented host macrophages from destruction, and resulted in alveolar macrophages remaining of host origin for at least 45 weeks following bone marrow transplant[147]. Protection of the lungs from lethal irradiation with a lead shield prior to bone marrow transplant resulted in alveolar macrophages remaining of host origin for the lifetime of the mouse, despite reconstitution of resident macrophage populations with donor cells in tissues that received irradiation[136]. Also, in parabiotic mice which are surgically joined to share a circulatory system, alveolar macrophages were shown to self-renew with little involvement of incoming monocytes[148]. In humans who received allogeneic bone marrow

transplant and whose lungs were repopulated with macrophages of donor origin, donor macrophages recruited to lungs also proliferate *in situ*, resulting in a three-fold increase in alveolar macrophage content seven weeks after transplant[149].

In addition to proliferating locally to maintain steady-state macrophage density, alveolar macrophages also proliferate in response to inflammatory insults. Instillation of carbon particles into the lungs of mice was found to stimulate local alveolar macrophage proliferation, which accounted for an increase in macrophage content even in mice depleted of monocytes by strontium 89[150]. Similarly, a recent study demonstrated that the massive increase in macrophage content in the lung that occurs during helminth infection can arise even in the complete absence of circulating blood monocytes[151]. They also showed that this surprising result was due entirely to the local proliferation of macrophages within the lung[151]. Thus, in the lung, local macrophage proliferation is well documented and may be the predominant mechanism for macrophage population renewal in the steady state, as well as the mechanism by which macrophage populations increase during inflammatory insult.

Langerhans Cells

One of the phagocytic, antigen-presenting phagocytic cell type in the epidermis of the skin is the Langerhans cell. These cells were originally thought to originate from bone marrow-derived cells[152] but in 2002 they were shown to be self-renewing within the skin[153]. Using both bone marrow transplant and surgically joined parabiotic mouse models, Langerhans cells remained of host origin for the duration of the studies – 18 and

6 months, respectively[153]. Furthermore, this study demonstrated that Langerhans cells actively synthesize DNA in the skin, as up to 30% of the Langerhans cells incorporated BrdU after a 30 day BrdU exposure[153]. Only when resident Langerhans cells were depleted with UV irradiation or toxic chemicals did replacement of resident Langerhans cells with circulating cells of donor origin occur[153]. However, a follow-up study showed that even during the repopulation phenomenon that occurs in damaged skin, circulating monocytes enter the inflamed skin and undergo several rounds of proliferation, forming clusters of Langerhans cells[154]. Evidence in humans also supports a role for Langerhans cell self-renewal in the skin, as a case study of a human hand transplant showed that skin Langerhans cells remained of donor origin even 4.5 years after transplantation[155]. Taken together, these results indicate that under steady state conditions, skin Langerhans cells replenish themselves almost entirely through local proliferation *in situ*, and monocyte recruitment only occurs in response to inflammatory insult. Furthermore, even under inflammatory conditions, local proliferation of the freshly recruited monocytes plays an important role in populating the skin with Langerhans cells.

Kupffer Cells

Liver Kupffer cells also are widely believed to be derived from monocytes due to a study which demonstrated that Kupffer cells can be derived from blood monocytes following total body lethal irradiation[156]. However, several early publications implicated a possible role for local proliferation in the maintenance of the Kupffer cell population in mice[157-160]. Kupffer cells were subsequently shown to indeed have proliferative capacity

but that, under steady state conditions, monocyte recruitment was the predominant force in maintaining the Kupffer cell population[161]. On the contrary, one study came to the conclusion that under physiological steady state conditions, Kupffer cells are long lived and mostly self-renewing[162].

Interestingly, several groups have reported the Kupffer cells proliferate in response to inflammatory insult. Intravenous injection of zymosan has been found to stimulate robust local Kupffer cell proliferation within the liver[161]. This result was later confirmed and expanded to show that local proliferation is indeed the predominant force behind Kupffer cell hyperplasia in response to zyomsan insult[163,164]. Similarly, liver injury induced by carbon tetrachloride is accompanied by an expansion in the liver macrophage population, which has been shown to be due to both local proliferation and monocyte recruitment[165]. Further studies will be required to clarify these seemingly conflicting results, but it is clear that to some degree or other, liver macrophages are capable of proliferating *in situ* under both steady state and inflammatory conditions.

Kidney

Macrophages accumulate in the kidney in various disease states, and there they contribute to inflammatory processes that exacerbate kidney injury[166]. A surprising number of studies have reported local macrophage proliferation in kidney inflammation. For example, in rat anti-glomerular basement membrane glomerulonephropathy, local macrophage proliferation has proven to be the major mechanism by which macrophage numbers increase in the glomerulus[167]. Indeed, measurements of proliferation markers in

the macrophages in this and another related study were astoundingly high, with over 60% of all macrophages expressing the proliferation marker PCNA at any given time, and the 30-40% of those cells staining positive for BrdU incorporation after a 3-hour BrdU pulse[167,168]. High levels of local macrophage proliferation have also been documented in glomerulonephritis in human patients[169]. Importantly, proliferating macrophages were found to be concentrated in regions of severe tissue damage, suggesting that proliferating macs are an important mediator of kidney injury[169]. Local macrophage proliferation has been noted in non-immune-mediated kidney injury models as well, and the association of macrophage proliferation with tissue injury and fibrosis was clear in these models also[170]. Importantly, local macrophage proliferation is also the mechanism by which macrophages accumulate in the kidney in diabetic nephropathy in obese mice[171]. Furthermore, macrophage accumulation associated with organ rejection in allogeneic kidney transplant studies has been shown to be due to initial recruitment of circulating monocytes which then undergo local proliferation *in situ* to increase the macrophage content[172]. Taken together, there is substantial evidence that local macrophage proliferation in the kidney represents an important mechanism by which macrophage populations increase in kidney diseases.

Atherosclerosis

Macrophages are an integral cell type in the formation of atherosclerotic plaques[173], and an impressive number of studies support a role for local proliferation in the accumulation of macrophages in the growing plaque. This body of work has even

warranted recent reviews on the topic of macrophage proliferation in atherosclerosis[174,175]. Proliferating macrophages within atherosclerotic plaques were first observed in rabbit models, using microscopic examination of plaques following pulses of radiolabeled thymidine analog or growth-arresting colchicine treatment[176,177]. Around the same time, expression of genes associated with cell proliferation were found to be increased in human atherosclerotic plaques, in which macrophages were suggested to be the predominant proliferating cell type[178,179]. Myeloid-specific deletions of the tumor-suppressor genes p27 and retinoblastoma caused increased intimal macrophage proliferation and accelerated atherosclerosis development in apolipoprotein E knockout animals[180,181]. Similarly, mutation of the cyclin-dependent kinase inhibitor 2A gene in myeloid cells in low-density-lipoprotein (LDL) receptor knockout animals caused increased macrophage proliferation and exacerbated atherosclerosis[182]. While several other reports using the tumor suppressor gene p53 yielded both positive[183-185] and negative[186,187] results in regards to macrophage proliferation, it is clear that macrophage proliferation *in situ* is involved in plaque formation and the maintenance of macrophage populations in atherosclerotic plaques.

Macrophage Proliferation in Other tissues

Macrophages in several other tissues have been documented to proliferate locally to maintain macrophage populations in the steady state. For example, the spleen is one of the largest repositories of macrophages in the body, and local proliferation may account for up to half of the macrophage content in the spleen[188,189]. Microglia, a subtype of

macrophages in the central nervous system, have been shown by several groups to proliferate locally[190-192]. Peritoneal macrophages have also been shown to have proliferative capacity *in vitro* as well as *in vivo*[193-195]. Local macrophage proliferation has been documented in various other disease models including arthritis[196,197], and finally in macrophages associated with tumors[198-201]. Indeed, locally proliferating macrophages have recently been identified in high-grade breast cancers and indicate a poor prognosis[202,203].

Summary and Objectives

The adipose tissues in obese, insulin resistant mice and humans accumulate inflammatory cells, including macrophages, which secrete cytokines and other factors that can modulate adipocyte function. These adipose macrophages are thought to originate from bone marrow-derived monocytes, which infiltrate the tissue from the circulation. However, genetic deletion of molecules thought to be important in the recruitment of monocytes to adipose tissue produced either a modest decrease in adipose tissue macrophage content compared to controls, or no decrease in adipose tissue macrophage content at all.

Despite the fact that macrophages are generally thought to be a terminally differentiated cell type, there are many examples of macrophage proliferation locally within tissues. Local proliferation can be the predominant way in which tissue macrophages maintain normal density under basal conditions, and local proliferation can account for the massive increases in macrophage tissue density that occur in certain inflammatory conditions. However, the role of local macrophage proliferation in adipose tissue inflammation in the obese state has not been thoroughly investigated.

Therefore, we sought to elucidate the role of local macrophage proliferation in maintaining macrophage populations in the adipose tissue of lean and obese mice. Using both genetic and diet-induced obese mouse models, we examined macrophage populations for proliferative markers in the adipose tissue under steady-state lean and obese conditions, as well as during weight loss. To clarify the origin of adipose tissue macrophages, we studied adipose tissue macrophage population dynamics in the setting

of monocyte depletion. Finally, we made several initial efforts into elucidating the factors that may be stimulating local macrophage proliferation in the adipose tissue.

Chapter II: Local proliferation of adipose tissue macrophages in obesity-associated inflammation

Abstract

The adipose tissues in obese, insulin resistant mice and humans accumulate inflammatory cells, including macrophages, which secrete cytokines and other factors that can modulate adipocyte function. These adipose macrophages are thought to originate from bone marrow-derived monocytes, which infiltrate the tissue from the circulation. Here we show that a significant fraction of macrophages unexpectedly undergo cell division locally within adipose tissue, as detected by Ki67 expression and 5-ethynyl-2'-deoxyuridine incorporation. Adipose macrophages, but not those in other tissues, displayed dramatically increased proliferation in both genetic and diet-induced mouse models of obesity. Importantly, depletion of blood monocytes over several days had no impact on adipose tissue macrophage content. Conversely, weight loss and lipolysis specifically inhibited macrophage proliferation in the adipose tissue of obese animals. These results reveal that proliferation *in situ* is an important process by which macrophages accumulate in inflamed adipose tissue in obesity, and could have therapeutic implications for strategies to improve insulin sensitivity.

Introduction

The prevalence of insulin resistance and type 2 diabetes mellitus (T2D) has been rapidly rising worldwide over the past three decades, particularly in developing countries[204]. Obesity can induce an insulin-resistant state in adipose tissue, liver, and muscle and is a strong risk factor for the development of T2D[14,15,205-207]. In obese patients, the inability to appropriately expand subcutaneous adipose tissue leads to ectopic lipid deposition in non-adipose tissues (e.g. liver and muscle) and may be an underlying cause of insulin resistance[208-213]. Immune cells, including macrophages, accumulate in massive numbers in the adipose tissues of obese mice and human subjects[11,214-216]. It is increasingly appreciated that accumulation of macrophages in adipose tissue correlates with a chronic inflammatory state that ultimately impairs adipocyte function and may contribute to the development of insulin resistance[3,11,15,81,217,218]. Consistent with this concept, macrophage numbers in adipose tissue correlate with systemic insulin resistance even in obese human subjects that are matched for BMI[109,219-221]. Macrophages and other immune cells in adipose tissues also have beneficial effects of removing cellular debris[107] and controlling lipolytic responses in adipocytes[125].

Macrophages infiltrating into adipose tissue have been reported to be mostly derived from the bone marrow and there is evidence that monocyte recruitment to the adipose tissue increases in obesity[11,139]. Adipocytes have been shown to produce a wide range of factors, such as the chemokine monocyte chemoattractant protein 1 (MCP-1) that may recruit monocytes to adipose tissue. Both the production of MCP-1 and the expression of

its receptor CC-motif chemokine receptor-2 (CCR2) are enhanced by obesity[116,130]. However, genetic deletion of MCP-1 and CCR2 in mice produced either a modest decrease in adipose tissue macrophage (ATM) content compared to controls[108,115], or no effect that could be detected[131,132]. Perhaps this is due to other pathways of macrophage migration that operate independently of the MCP-1 pathway. However, these results might also be explained by the possibility that resident macrophages have a prolonged lifespan or proliferate *in situ* in the adipose tissue. Although terminal differentiation of mammalian cells typically results in reduced proliferative capacity, there is evidence that cells of the mononuclear phagocyte system, including macrophages, can self-replicate in the periphery both during development and in the adult[222-224]. Indeed, local macrophage proliferation has been demonstrated in kidney, atherosclerotic plaques, skin and lung in animals and humans[151,169,176,182,225].

The present study was designed to test the hypothesis that significant macrophage cell division also occurs within adipose tissue in mice, since no data are available on this important question. Here we show that a fraction of ATMs in obese mice display the proliferation marker Ki67, a protein expressed during all active phases of the cell cycle but not in non-dividing cells[226]. Genetic and diet-induced obesity greatly increased macrophage proliferation in adipose tissue, but not in other tissues studied. Furthermore, approximately 5% of macrophages within epididymal adipose tissue of obese mice were found to be in S-phase during a three-hour pulse of the more stringent cell division marker, 5-ethynyl-2'-deoxyuridine (EdU), and nearly 50% of all macrophages had

proliferated over the course of an 80 hour EdU exposure, even when circulating monocytes had been depleted. These results suggest the unexpected concept that local macrophage proliferation contributes to the overall macrophage accumulation and adipose tissue inflammation in the insulin resistant state.

Results

Adipose tissue macrophages express the cell division marker Ki67.

To study macrophage accumulation in adipose tissue of obese mice, we used 8 to 12-week old genetically obese (*ob*/*ob*) male mice and their lean control wild-type (WT) littermates. To confirm that the *ob*/*ob* mice were insulin resistant and obese, we measured body weights and performed glucose tolerance tests (GTT) (**Figure 2.1 A-B**). The average body weight of the *ob*/*ob* mice was approximately 15 g, or 50% higher than that of WT mice, and this was associated with significantly decreased glucose tolerance (**Figure 2.1 A-B**). We isolated the stromal-vascular fraction (SVF) from subcutaneous (inguinal) and visceral (epididymal) fat pads, which contains all cells of the adipose tissue except adipocytes, and analyzed it using flow cytometry. The SVF was stained with antibodies against two macrophage markers, F4/80 and CD11b. However, these proteins are also expressed in other immune cells, such as eosinophils and neutrophils[95,227]. Therefore, we also stained the SVF with antibodies against the eosinophil marker, Siglec-f, and a neutrophil marker, Gr-1. The macrophage population in the adipose tissue was defined as $F4/80^{+}/CD11b^{+}/Siglec\text{-}f^{-}/Gr\text{-}1^{-}$ (for the complete gating scheme see **Supplementary Figure 2.1**). Consistent with published studies[11,105], we observed a significant 2-3 fold increase in macrophage content in the adipose tissue of *ob*/*ob* compared to WT mice in both subcutaneous and visceral fat pads (**Figure 2.1 C-D**).

To test whether local macrophage proliferation also contributes to macrophage accumulation in the adipose tissue, we stained the SVF cells with an antibody against the

proliferation marker Ki67, which is a protein expressed during all active phases of the cell cycle[226]. Surprisingly, Ki67 signal was detected in approximately 2.3% of epididymal ATMs of lean mice, and in 10% of ATMs of *ob*/*ob* mice (**Figure 2.1 E-F**). Similar percentages of macrophages from subcutaneous adipose tissue were $Ki67^+$ as well (**Figure 1F**). We then performed immunofluorescence microscopy on plated SVF cells from epididymal fat pads of *ob*/*ob* mice. Figure 2.1 also shows images of macrophages (F4/80, red) expressing Ki67 (green) in their nuclei (DAPI, blue) (**Figure 2.1 G**). Microscopy revealed a similar percentage (approximately 10%) of $Ki67^+$ macrophages as seen with flow cytometry (data not shown). High magnification images reveal macrophages in various stages of division (**Figure 2.1 G**). Furthermore, Ki67 was often found in multinucleated macrophages (**Figure 2.1 G**). Next, AT sections from *ob/ob* mice were stained with antibodies against F4/80 (red) and Ki67 (green) and analyzed by microscopy **(Figure 2.1 H-I)**. Figure 2.1 G and H show Ki67 in F4/80 positive cells in a region of the epididymal AT rich in macrophages termed crown-like structures (CLS). Taken together, these results show by both flow cytometry and microscopic analysis that macrophages express the proliferation marker Ki67 in the AT and to a higher degree in response to obesity in mice caused by either genetic mutation or a diet high in fat.

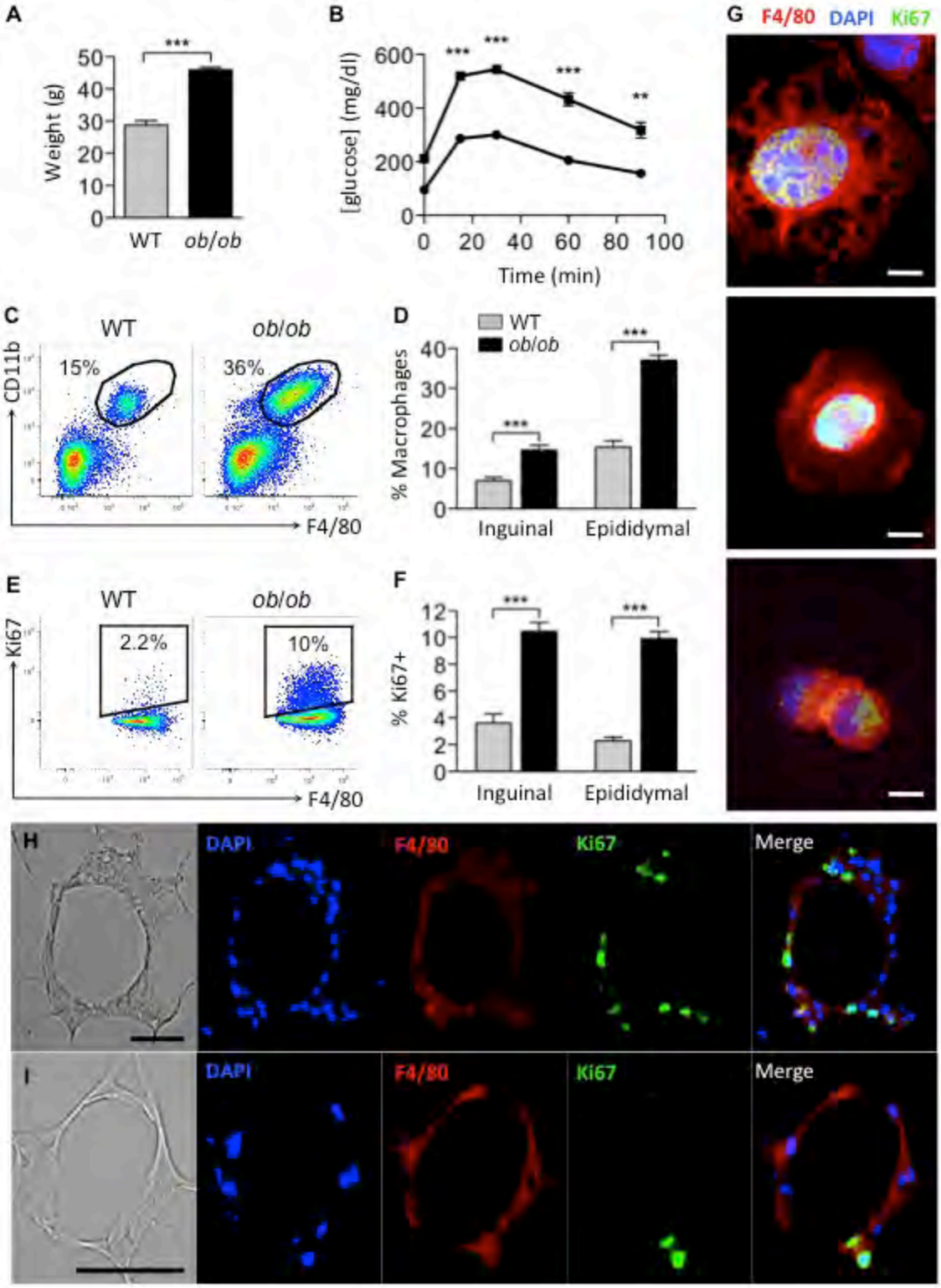


Figure 2.1. Ki67 expression in adipose tissue macrophages of lean and obese mice.

Figure 2.1. Ki67 expression in adipose tissue macrophages of lean and obese mice. (**A**) Body weights and (**B**) GTT of WT (●) and *ob*/*ob* (■) mice, n=5. (**C-F**) SVF from visceral and subcutaneous adipose tissue of WT and *ob*/*ob* mice was isolated and analyzed by flow cytometry. (**C**) Representative flow cytometry dot plots of SVF from epididymal adipose tissue. (**D**) Percentage of macrophages in SVF. (**E**) Representative flow cytometry dot plots of macrophages stained with Ki67. (**F**) Percentage of macrophages expressing Ki67. n=30-31 from 6 independent experiments for epididymal and n=10 from 2 independent experiments for inguinal fat pads. All graphs are expressed as mean ± s.e.m. Statistical significance was determined by Student's t-test. For GTT, statistical significance was determined by ANOVA and Tukey post test. **p<0.01; ***p<0.001. (**G-I**) Microscopy of plated SVF (**G**) and adipose tissue sections (**H-I**) stained with antibodies against F4/80 (red) and Ki67 (green). Nuclei were stained with DAPI (Blue). (**G**) 63x magnification image of macrophages stained with Ki67 (arrows). Scale bar = 5 μm. (**H-I**) 20x magnification images of crown-like structures from *ob/ob* mice. Scale bar = 40 μm.

Macrophages proliferate locally in adipose tissue.

To test whether macrophages undergo cell division within adipose tissue, we injected WT and *ob*/*ob* mice with the nucleoside analog to thymidine, EdU. While Ki67 is expressed during all active phases of the cell cycle, EdU is only incorporated into DNA during the S-phase. Three hours following EdU injection in mice, the SVF cells of epididymal fat pads were isolated and analyzed by flow cytometry. Approximately 1% of ATMs in lean mice and about 5% of the macrophages in the obese mice had gone through S-phase as measured during a 3-hour pulse of EdU (**Figure 2.2 A-B**). Mice were also injected with another nucleoside analog of thymidine, 5-bromo-2'-deoxyuridine (BrdU) and immunohistochemistry on epididymal adipose tissue of *ob*/*ob* mice was performed (**Figure 2.2 C**). As shown in Figure 2.2C, *ob*/*ob* adipose tissue contains regions known to be enriched with macrophages called crown-like structures (CLS; denoted by arrows). BrdU staining (denoted by arrowheads) was observed in these structures (**Figure 2.2 C**).

To test whether EdU^+ macrophages in adipose tissue derived from EdU^+ macrophages that infiltrated from the blood rather than cells propagating within the tissue, we also examined EdU incorporation in blood monocytes (**Figure 2.2 D-E**). In these same mice, blood was collected via cardiac puncture and analyzed by flow cytometry. Monocytes were positively identified by expression of CD11b, F4/80, and Ly6C (for complete blood monocyte gating scheme see **Supplementary Figure 2.2 A**). We failed to detect any EdU incorporation in blood monocytes within the 3-hour pulse, suggesting that EdU^+

macrophages seen in the adipose tissue are not recently recruited blood monocytes (**Figure 2.2 D-E**).

To further ensure that the proliferating macrophages in the adipose tissue were not EdU^+ blood monocytes, we studied the capacity of ATMs to proliferate *ex vivo*. SVF cells isolated from epididymal adipose tissue of lean and obese mice were plated and treated with EdU for three hours. The cells were then stained and analyzed by flow cytometry. Approximately 0.3% of the ATMs from lean mice were EdU^+, while greater than 2% were positive in macrophages from obese mice (**Figure 2.2 F-G**). These results suggest that the ATMs have the inherent capacity to proliferate *ex vivo*, independently of blood monocyte recruitment. Taken together, these results suggest that proliferating macrophages present in adipose tissue are not recently recruited blood monocytes that were already dividing.

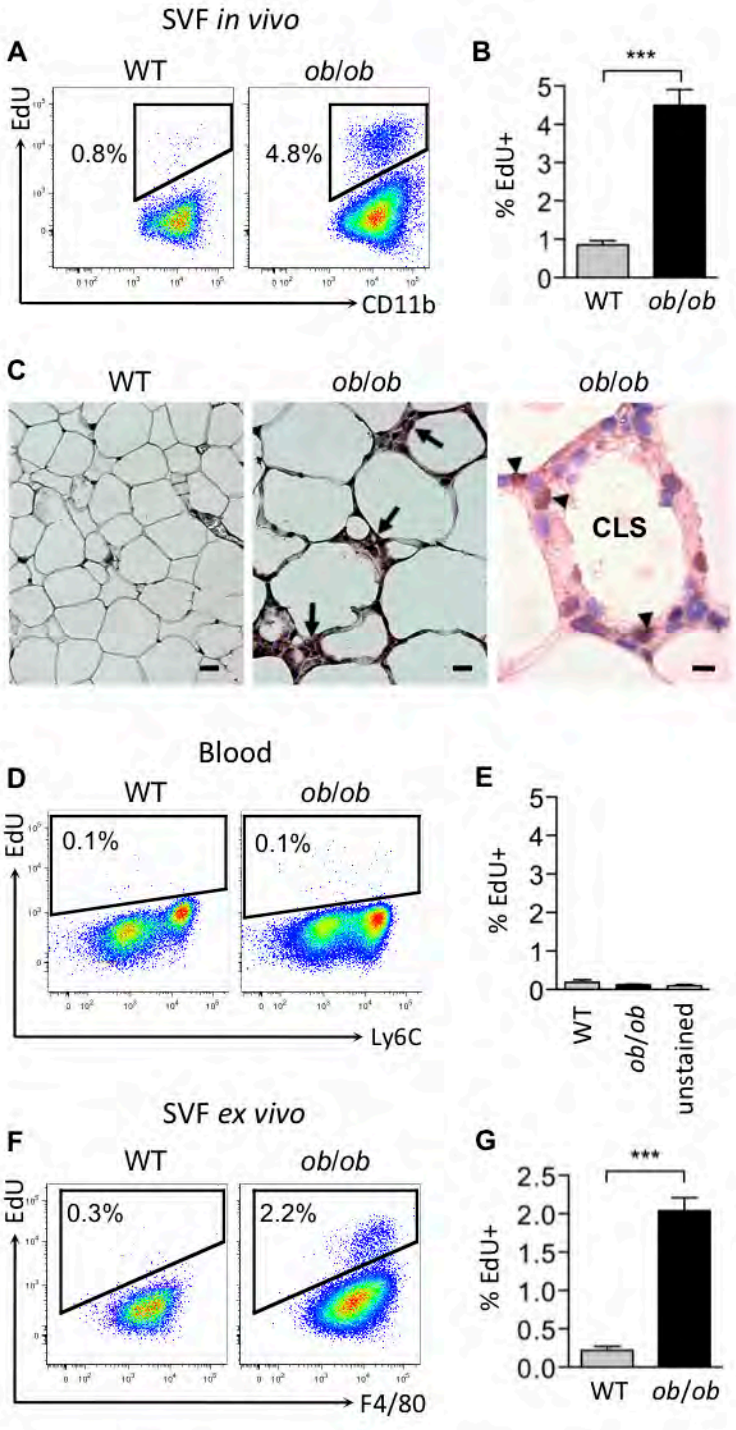


Figure 2.2. Macrophage cell division in adipose tissue detected by EdU incorporation in lean and obese mice.

Figure 2.2. Macrophage cell division in adipose tissue detected by EdU incorporation in lean and obese mice. Mice were treated with EdU and epididymal adipose tissue SVF and blood were collected 3 hours after treatment and analyzed by flow cytometry. (**A**) Representative flow cytometry dot plots of adipose tissue macrophages and (**D**) blood monocytes, and the corresponding bar graphs (**B**) and (**E**). (**C**) Left images, 20x magnification of epididymal adipose tissue of WT and *ob*/*ob* mice. Arrows denote crown-like structures (CLS). Scale bar = 50 μm. Right image, 100x magnification image of a CLS in *ob*/*ob* epididymal adipose tissue containing cells staining positive for BrdU (arrowheads). Scale bar = 10 μm. BrdU was injected 3 hours before tissue isolation. (**F-G**) Adipose tissue SVF was isolated and plated in media containing 10 μM EdU. Three hours later cells were harvested and analyzed by flow cytometry. (**F**) Representative dot plots of *ex vivo* macrophage EdU incorporation and (**G**) corresponding rates. All graphs depict mean of EdU^+ cells ± s.e.m. Statistical significance was determined by Student's t-test. For both sets of experiments n=14-15 mice/group from 3 independent experiments. ***$p<0.001$.

Obesity increases macrophage proliferation specifically in adipose tissue.

To test whether obesity stimulates macrophage proliferation in tissues other than adipose tissue, we analyzed the proliferation rate of macrophages in spleen and liver following a 3-hour pulse of EdU. The tissues were digested and, as expected, mononuclear cells analyzed by flow cytometry exhibited a higher rate of proliferation in the epididymal adipose tissue of *ob*/*ob* compared to WT mice (**Figure 2.3 A**). Interestingly, in addition to macrophages, non-macrophage ($F4/80^{-}$) cells in the adipose tissue also showed an increased rate of proliferation with obesity. The overall rate of EdU incorporation during the pulse into all adipose tissue SVF cells was approximately 0.5% in lean and 4.5% in obese mice (**Figure 2.3 A**). Conversely, we observed that less than 2% of all cells were EdU^{+} in spleen and liver, and there was no difference in EdU^{+} cells between lean and obese mice (**Figure 2.3 B-C**).

Although macrophage content increased with obesity in adipose tissue (**Figure 2.1 C**), macrophage content did not increase in spleen or liver in *ob*/*ob* mice compared to WT mice (data not shown; for spleen and liver macrophage gating schemes see **Supplementary Figure 2.3 B-C**). Figure 2.3D shows the rate of proliferation exclusively for macrophages in each tissue in lean and obese states. Importantly, while obesity was associated with a higher rate of macrophage proliferation in adipose tissue, it did not affect the EdU incorporation rate in spleen, liver or blood macrophages. These results suggest that proliferation may contribute to the increased macrophage accumulation in

the adipose tissue in obesity. Furthermore, something unique about the adipose tissue microenvironment may stimulate macrophage proliferation during obesity.

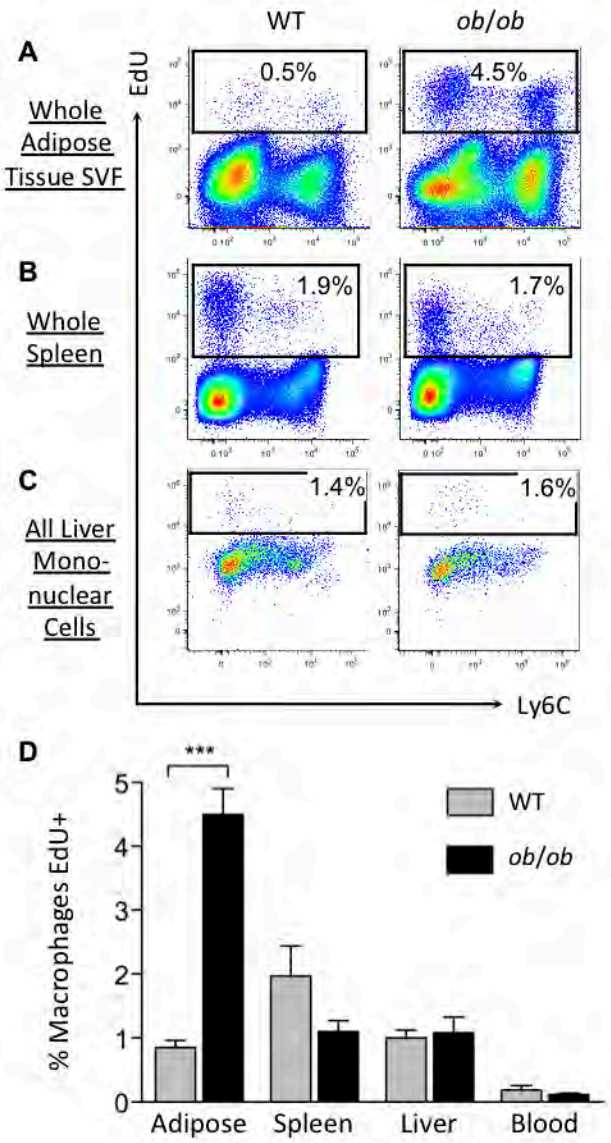


Figure 2.3. Obesity does not stimulate macrophage proliferation in liver or spleen.

Figure 2.3. Obesity does not stimulate macrophage proliferation in liver or spleen. Mice were treated with EdU and (**A**) adipose tissue, (**B**) spleen and (**C**) liver were collected and digested 3 hours after treatment. All cells were stained and analyzed by flow cytometry. Representative dot plots depict the EdU incorporation into all cells of the respective tissues. (**D**) Mean EdU incorporation rate of the macrophages of each tissue ± s.e.m.; n= 14-15 from 3 independent experiments for adipose tissue and blood, and n=9-10 from 2 independent experiments for spleen and liver. Statistical significance was determined by Student's t-test. ***$p<0.001$.

To study the contribution of monocyte recruitment in ATM accumulation during obesity, we depleted blood monocytes in *ob/ob* mice by intravenous (i.v.) injection of clodronate-loaded liposomes, which induce apoptosis once ingested by monocytes[228]. Consistent with the literature, clodronate-liposome i.v. injection depleted about 80% of blood monocytes 16 hours after injection compared to PBS-liposome injection (**Figure 2.4 A-B**). We then injected *ob/ob* mice every 16 hours with clodronate-liposomes to maintain blood monocyte depletion and measured macrophage content in the epididymal AT. Unexpectedly, ATM absolute number (50 hours, PBS-lipo 3.78 ± 0.37 *vs.* clodronate-lipo 3.88 ± 0.35; 96 hours, 4.54 ± 0.60 *vs.* clodronate-lipo 3.36 ± 0.11 ($x10^6$ cells/g of epididymal AT)) and ATM percentage in the SVF were unchanged with clodronate-liposome treatment even after prolonged blood monocyte depletion (**Figure 2.4 C**). These data raised the possibility that the increase in macrophages seen during obesity was largely the result of proliferation of the resident population. Therefore, *ob/ob* mice were given EdU in drinking water during monocyte depletion as depicted in diagram in Figure 2.3D. Thirty-two hours after EdU exposure, approximately 28% of macrophages were EdU^+ in the AT of *ob/ob* mice injected with PBS-liposomes (**Figure 2.4 E-F**). Eighty hours after EdU exposure, about half of the macrophages in the AT of *ob/ob* mice injected with PBS-liposomes had incorporated EdU (**Figure 2.4 E-F**). Importantly, depletion of blood monocyte had no effect on macrophage proliferation as observed by the EdU incorporation in the epididymal AT of *ob/ob* mice injected with clodronate-liposomes (26% after 32 hr and 46% after 80 hr) (**Figure 2.4 E-F**). These results suggest that resident macrophages proliferate independently of monocyte recruitment. However,

recently recruited macrophages may also proliferate in AT of obese mice since a recent study showed that 5% of labeled blood monocytes, transferred from donor into recipient mice, express Ki67 in the host AT two days after transfer[139].

We next analyzed the proliferative capacity of macrophages in the epididymal AT depot, where most of the macrophage expansion occurs in obesity, versus the subcutaneous AT depot. EdU incorporation by macrophages was significantly higher in the epididymal AT of *ob/ob* compared to WT mice (**Figure 2.4 G**). However, although Ki67 expression is higher (**Figure 2.1**), no difference in EdU incorporation was observed in macrophages in subcutaneous AT of *ob/ob* compared to WT mice after 80 hours of EdU exposure (**Figure 2.4 G**). Epididymal ATM proliferation rates were also higher than in subcutaneous ATMs in the PBS- and Clodronate-liposome-treated mice (**Supplementary Figure 2.3 B**). Unlike in the epididymal adipose tissue, the content of EdU^+ ATMs in the subcutaneous depots did not dramatically increase between 32 hours and 80 hours of EdU exposure, possibly indicating macrophage turnover begins to occur within this time period (**Supplementary Figure 2.3 B**). Taken together, these results suggest that macrophage proliferation plays a major role in the increase in macrophage content that occurs in the adipose tissue in obesity.

While macrophage sub-populations in the AT may have overlapping marker expression profiles, it is generally thought that CD11c expression is characteristic of pro-inflammatory macrophage subtypes[117]. Therefore, we analyzed the rate of proliferation of

pro- ($CD11c^+$) and anti- ($CD11c^-$) inflammatory macrophages (**Supplemental Figure 2.3 C**). Although proliferation of anti-inflammatory macrophages has been shown to be a signature of T_H2 inflammation in the lung[151], we failed to observe any difference in the rate of proliferation of macrophage population subtypes. This suggests that obesity increases macrophage proliferation rate independently of their inflammatory state.

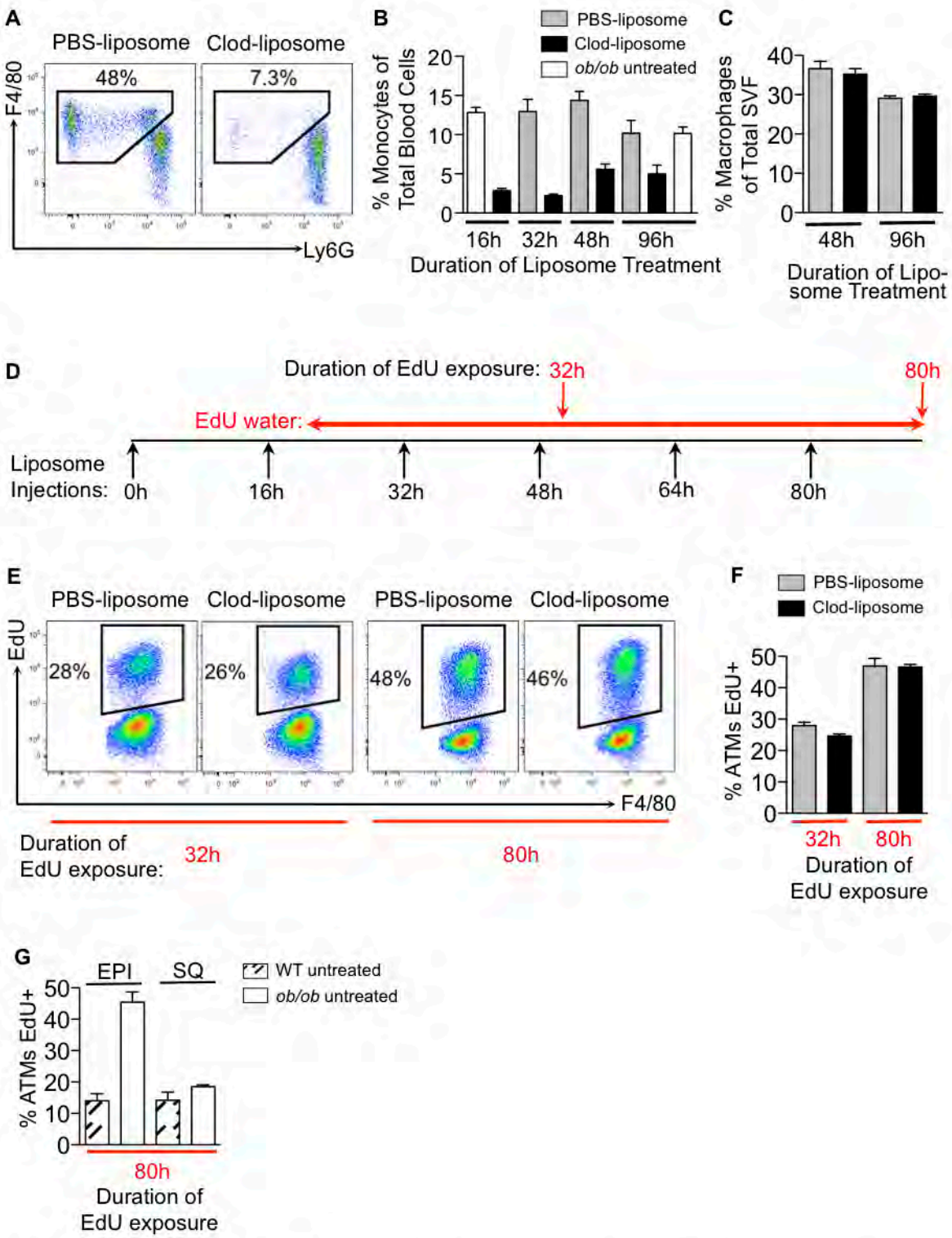


Figure 2.4. Adipose Tissue Macrophage Proliferation Occurs Independently of Blood Monocyte Recruitment.

Figure 2.4. Adipose tissue macrophages proliferate independently of monocyte recruitment. *ob*/*ob* mice were i.v. injected with either PBS-liposomes or clodronate-liposomes every 16 hours. Small amounts of blood were taken at the end of several of the 16h periods and immediately before subsequent liposome injection to ensure monocyte depletion was continuous. Untreated mice did not receive any injection. (**A**) Example flow cytometry dot-plots of $CD11b^{+}$ blood cells show depletion of monocytes, and (**B**) the quantitation of blood monocytes expressed as a percentage of total blood cells. n=10 for 16h-50h of liposome treatment and n=4-5 for the 96h time point. (**C**) Macrophage content in the AT of PBS-liposome (PBS-Lipo) and clodronate-liposome (Clod-Lipo) treated *ob*/*ob* mice; n=5 mice per group. 18 hours after initial injection, the mice were given drinking water containing EdU. (**D**) Diagram representing experimental design of treatment. (**E**) Representative flow cytograms and (**F**) quantification of EdU incorporation into ATMs during 32h and 80h of exposure to EdU drinking water in PBS-liposome-treated and monocyte-depleted clodronate-liposome-treated *ob*/*ob* mice. (**G**) Quantification of EdU incorporation into ATMs during 80h of exposure to EdU drinking water in EPI and SQ adipose depots in lean WT and *ob/ob* obese mice. n= 5 mice per group.

Differential effect of obesity on proliferation of diverse immune cell types in adipose tissue.

We next examined whether obesity modulated the proliferation of eosinophils and neutrophils, two other immune cell types thought to be involved in the development of insulin resistance[95,227]. Consistent with prior reports[95], we found that eosinophil content relative to macrophages in the adipose tissue decreased with obesity (**Supplementary Figure 2.4 A-B**). We also observed a significant decrease in the expression of a cytokine secreted by eosinophils, interleukin 4 (IL-4), in the epididymal adipose tissue of obese compared to lean mice (**Supplementary Figure 2.4 D**). Neutrophil content in adipose tissue was not significantly different between WT and *ob*/*ob* mice (**Supplementary Figure 2.4 E-F**). Importantly, neither eosinophils nor neutrophils exhibited any significant EdU incorporation (**Supplementary Figure 2.4 C and G)**, suggesting that these cells types do not proliferate in the adipose tissue. These results suggest that obesity does not stimulate proliferation of all cells in the adipose tissue, but selectively stimulates proliferation of specific cell types including macrophages.

Macrophage proliferation decreases with weight loss.

We next asked if weight loss in mice could cause a corresponding decrease in macrophage accumulation in adipose tissue. Mice were fed a high fat diet (HFD) for 7-14 weeks. At the end of the HFD period, mice had become obese and glucose intolerant (**Supplementary Figure 2.5 A**). We examined the macrophage content in the adipose tissue and the rate of macrophage proliferation. ATM content was approximately 20% of

the SVF of epididymal adipose tissue in mice fed a normal chow diet (ND) and about 50% in mice fed a HFD (**Figure 2.5 A-B)**. Flow cytometry analysis on SVF showed that 3.2% of ATMs were $Ki67^+$ in the ND-fed mice while approximately 14% of the ATMs were $Ki67^+$ in the HFD-fed mice (**Figure 2.5 C-D**). Macrophage proliferation in subcutaneous adipose tissue exhibited similar decreases to those seen in epididymal ATMs (**Supplementary Figure 2.5 E-F**). To induce weight loss, HFD-fed mice were fasted overnight. Fasted mice lost between two and three grams on average, which was equal to 5-10% of their total body weight (**Figure 2.5 F**). The macrophage content in the adipose tissue was unchanged with weight loss as determined by flow cytometry (**Figure 2.5 B and Supplementary Figure 2.5 D**). Consistently, the expression of macrophage markers (F4/80, CD11b, CD11c and TNF-α) was unchanged as measured by real time PCR (**Figure 2.5 E**). In the fasted mice, weight loss was associated with a significant decrease in ATM proliferation (**Figure 2.5 D**). A similar decrease in Ki67 staining was seen in ATMs from the subcutaneous adipose tissue of the mice that lost weight (**Supplementary Figure 2.5 F**). To test another model of weight loss, we treated *ob*/*ob* mice with the β_3-adrenoceptor agonist, CL-316,243, which is known to induce fatty acid release from adipocytes *in vivo*[229-231]. We observed that CL-316,243 treatment of wild-type animals was associated with a significant increase in $CD11b^+$ cells in the epididymal adipose tissue, but no increase was observed in *ob*/*ob* animals (**Figure 2.5 G-H)**. However, treatment of *ob/ob* animals with CL-316,243 did cause a slight but significant decrease of ATM proliferation (**Figure 2.5 I**). Taken together, these results suggest that

local ATM proliferation is a dynamic process that increases with weight gain and decreases with weight loss.

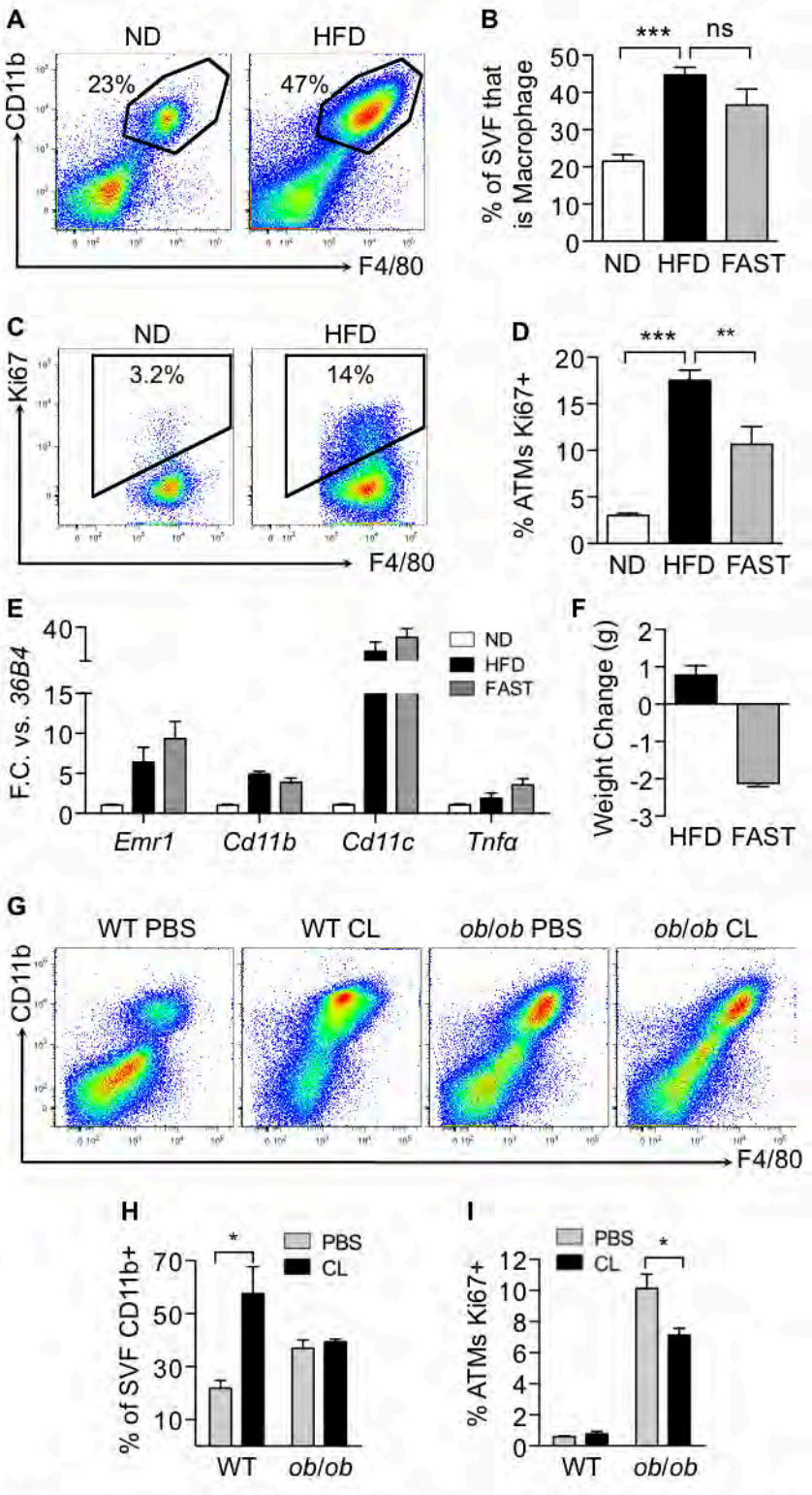


Figure 2.5. Adipose tissue macrophage proliferation decreases with weight loss.

Figure 2.5. Adipose tissue macrophage proliferation decreases with weight loss.

Mice were fed a HFD or ND for 7-14 weeks. n=17-20 mice/group from 4 independent experiments. Some HFD-fed mice were fasted overnight (FAST); n=8 mice/group from 2 independent experiments. SVF cells from epididymal fat pads were isolated and analyzed by flow cytometry. (**A**) Representative flow cytometry dot plots of the SVF F4/80 and CD11b staining. (**B**) Percentage of macrophages in SVF for ND, HFD and fasted mice. (**C**) Representative flow cytometry dot plots of Ki67 staining in ATMs. (**D**) Percentage of macrophages positive for Ki67 in ND, HFD and fasted mice. (**E**) Real-time PCR analysis of gene expression in the whole adipose tissue of the weight loss groups and of the ND control mice. (**F**) Weight change for HFD and fasted mice over the 24 hour study period. (**G**-**I**) WT and *ob*/*ob* mice were injected with PBS or CL-316,243 and epididymal adipose tissue was harvested 24 hours later. (**G**) Representative flow cytometry dot plots of SVF and (**H**) percentage of $CD11b^+$ cells in the SVF of PBS and CL-injected mice. (**I**) Percentage of macrophages positive for Ki67 in WT and *ob*/*ob* mice treated with PBS or CL. All graphs are expressed as mean ± s.e.m. Statistical significance was determined by Student's t-test. *p<0.05; **p<0.01; ***p<0.001.

Discussion

The major finding presented here is that macrophages undergo significant cell division locally within visceral and subcutaneous adipose tissues of obese mice, suggesting that *in situ* proliferation is a mechanism by which ATMs accumulate in this tissue. Using flow cytometry and immunofluorescence microscopy, we readily detected the presence of two cell proliferation markers, Ki67 and EdU, in SVF cells isolated from adipose tissue. These analyses revealed that 2-4% of ATMs in lean and 10% of ATMs in *ob*/*ob* mice express the proliferation marker Ki67 (**Figure 2.1**). Importantly, $Ki67^+$ macrophages were identified within the adipose tissue in crown-like structures (**Figure 2.1**). Similarly, a 3-hour *in vivo* pulse with the thymidine analog EdU showed that nearly 5% of macrophages in epididymal adipose tissue of *ob*/*ob* mice had gone through the S-phase of cell division (**Figure 2.2**). Importantly, EdU^+ macrophages in *ob/ob* mouse adipose tissue are not recently recruited EdU^+ blood monocytes, as no EdU staining in blood monocytes could be detected in this 3-hour period (**Figure 2.2**). Furthermore, ATMs isolated from adipose tissue continued to proliferate *ex vivo*, independently of monocyte recruitment (**Figure 2.2**). The data in Figures 2.1 and 2.2 thus document a remarkable enhancement of macrophage proliferation within adipose tissue in response to obesity in mice caused by either genetic mutation or a diet high in fat. Interestingly, the apparent rate of macrophage proliferation was lower in SVF *ex vivo* than when measured by administration of EdU *in vivo*. This suggests either that the adipose tissue environment is necessary for macrophage proliferation or that blood monocytes infiltrate the adipose tissue and then proliferate locally soon after their recruitment. This latter hypothesis is

supported by a recent study by Oh *et al*. showing that 5% of labeled blood monocytes, transferred from donor into recipient mice, express Ki67 in the host adipose tissue two days after transfer[139]. Macrophages in the skin have also been reported to be able to derive from blood monocytes that proliferate locally within the skin shortly after recruitment[154].

To determine the relative contribution of incoming monocytes versus local proliferation of macrophages to total ATM content, we depleted blood monocytes using clodronate liposomes. Over the course of 4 days, we maintained monocyte depletion at or better than 80% depletion by injecting clodronate liposomes every 16 hours. By assaying blood monocyte content at the end of each 16 hour period (immediately before the subsequent injection), we ensured that the blood monocyte content never recovered beyond what was measured. Surprisingly, despite monocyte depletion for a total of at least 80 hours, we did not see a decrease in ATM content in epididymal or subcutaneous adipose tissue (**Figure 2.5** and **Supplementary Figure 2.3**). As soon as the monocytes were depleted (beginning after the second clodronate injection), these same mice were treated with EdU in the drinking water. This allowed us to follow the proliferation of macrophages in the adipose tissue both with and without monocyte depletion. At 32 hours of EdU exposure, approximately 27% of all ATMs had proliferated, and after 80 hours of EdU exposure around 50% of all macrophages in the adipose tissue were positive for EdU. Amazingly, these rates were the same with or without monocyte depletion, meaning that the proliferating cells were not freshly recruited monocytes. This is consistent with a recent

report which noted that freshly recruited monocytes do not proliferate to an appreciable degree in the adipose tissue of obese mice[139]. Interestingly, the accumulation of EdU+ macrophages in the adipose tissue does not increase in a linear way over time. For example, 4.5% of macrophages were EdU^+ after three hours of exposure (**Figure 2.2**), and if this rate were to increase in a linear way, 100% of adipose tissue macrophages would be expected to be EdU positive after 67 hours of EdU exposure. However, it required 80 hours of exposure to reach 47% of ATMs positive for EdU (**Figure 2.4**). This effect was most pronounced in the subcutaneous adipose tissue, in which little additional accumulation of EdU^+ macrophages was seen after the first 32 hours of EdU exposure, despite the presumably constant rate of macrophage proliferation (**Figure 2.4**). This result implies that, at some point, EdU^+ macrophages are leaving the adipose tissue at the same rate as they are appearing, with the total EdU^+ macrophage content in the adipose tissue at equilibrium correlating with the average macrophage residence time in the adipose tissue. This raises the possibility that not only is macrophage proliferation increased, but macrophage death or efflux out of the adipose tissue is slowed in obesity. This is conceptually consistent with a recent report which showed that insulin resistance is associated with decreased macrophage trafficking in the adipose tissue[138]. Our results at least suggest that this is the case and that macrophage turnover is slowed particularly in epididymal adipose tissue vs. subcutaneous adipose tissue, consistent with epididymal adipose tissue being more inflamed. Additional studies will be required to elucidate the precise mechanisms by, and rates at which, macrophages leave the adipose tissue. Overall, these results suggest that local proliferation, not recruitment of circulating

monocytes, is the predominant process by which the large population of macrophages present in the obese adipose tissue is maintained in the steady state.

Recently, $CD8^+$ T-cells have been shown to have an increased rate of proliferation when co-cultured with adipose tissue of obese compared to lean mice[97]. In our experiments, some but not all cell types in adipose tissue had an increased proliferation rate in obese mice. For instance, eosinophils and neutrophils, which have been reported to play a role in the development of insulin resistance[95,227] did not proliferate within adipose tissue of either lean or obese mice (**Supplementary Figure 2.4**). While both eosinophils and neutrophils have been shown to have some proliferative capacity outside of the bone marrow[232,233], we failed to detect any EdU incorporation into these cells within adipose tissue (**Supplemental Figure 2.4**). Importantly, we observed similar rates of proliferation in macrophages expressing the marker CD11c and in those negative for CD11c (**Supplemental Figure 2.3**). While macrophage sub-populations in the adipose tissue may have overlapping marker expression profiles, it is generally thought that CD11c expression is characteristic of pro-inflammatory macrophage subtypes[112,118,121]. Although proliferation of anti-inflammatory (CD11c-) macrophages has been shown to be a signature of T_H2 inflammation in the lung[151], our results suggest that this is not the case in adipose tissue macrophages.

The data presented in Figure 2.3 reveal a striking tissue specificity of the effect of obesity on ATM proliferation since no change in cell division of macrophages could be detected in spleen or liver of obese mice. These findings imply that the adipose tissue micro-

environment in obesity is important for macrophage proliferation. A recent study showed that IL-4 secreted by eosinophils in lung stimulates macrophage proliferation during infection[151]. Interestingly, eosinophils are present in the adipose tissue and secrete IL-4[95]. Consistent with Wu *et. al.*[95], we found that eosinophil content as well as IL-4 expression in adipose tissue decrease with obesity (**Supplementary Figure 2.4**), suggesting that IL-4 may not be the signal that stimulates ATM proliferation in obesity. However, studies focusing on the role of IL-4 in ATM proliferation would require IL-4 treatment *in vivo* as well as genetic deletion of this cytokine in mice. In the macrophage cell line RAW 264.7, fatty acids have been shown to increase proliferation[234]. To test this hypothesis *in vivo*, we treated *ob*/*ob* mice with the β_3-adrenoceptor agonist, CL-316,243, which is known to induce fatty acid release from adipocytes *in vivo*[229-231]. We observed that treatment of *ob*/*ob* animals with CL-316,243 caused a slight but significant decrease of ATM proliferation, suggesting that free fatty acid may decrease proliferation *in vivo* (**Figure 2.5**). Additional investigation will be required to elucidate the mechanism by which the adipose tissue offers a unique and favorable environment for macrophage proliferation.

The origin of ATMs has previously been attributed to recruitment of blood monocytes into adipose tissue based on one study using a bone marrow chimera[11]. In the study by Weisberg, *et al.*[11], bone marrow from donor mice was injected into recipient mice after depletion of immune cells by irradiation. Six weeks later, the majority of the ATM population was composed of donor marrow-derived cells[11]. However, it has been shown that irradiation dramatically reduces the lifespan of resident macrophages[136]. In tissues

that are shielded from radiation, macrophages remain completely host-derived for at least 8 months despite reconstitution with donor marrow[136]. Jenkins *et al*. showed that shielding the lung from irradiation followed by bone-marrow transplant from donor mice generates a mixed chimerism in peripheral blood monocytes, but only a low percentage of pleural macrophages were of donor origin[151]. This demonstrated that little replacement of these cells occurred from the bone marrow under steady-state conditions[151]. This provides strong evidence that macrophages proliferate locally in tissues both to maintain the macrophage density in basal condition, and to increase it during infection. We have shown here that macrophages also proliferate locally in the adipose tissue in the basal state, and that ATM proliferation increases in the inflammatory setting of obesity.

Using mice fed a HFD we confirmed that weight gain induces a marked increase in ATM content and proliferation (**Figure 2.5**). Using overnight fasting, we tested whether weight-loss also regulates ATM proliferation. Although fasting-induced lipolysis has been shown to increase ATM content in lean mice[125], a recent study showed that fasting does not increase ATM content in obese animals[138]. Consistently, our study showed that after an overnight fast ATM content did not change in HFD-fed mice (**Figure 2.5**). Similarly, lipolysis induced by treatment with the β_3-adrenoceptor agonist, CL-316,243 failed to induce macrophage accumulation in the adipose tissue of *ob*/*ob* mice (**Figure 2.5**). In contrast, CL-316,243 treatment in lean mice induced a massive immune cell infiltration in the epididymal adipose tissue (**Figure 2.5**). It is interesting to note that both weight loss models (overnight fast and CL-316,243 treatment) were associated with a

significant decrease in the rate of ATM proliferation in obese mice, but had no effect on macrophage proliferation in lean mice (data not shown). These results suggest that macrophage proliferation is able to rapidly respond to environmental conditions in the adipose tissue.

In summary, we show here that macrophage proliferation within adipose tissue is a dynamic mechanism that increases with weight gain and decreases with weight loss. Prolonged depletion of circulating monocytes in obese mice had no effect on adipose tissue macrophage content, while EdU exposure studies revealed that at least half of the adipose tissue macrophages proliferated within an 80 hour period in the visceral adipose tissue of obese mice. Overall, these results suggest that local proliferation, not recruitment of circulating monocytes, is the predominant process by which the large population of macrophages present in the obese adipose tissue is maintained in the steady state. These results also imply that local macrophage proliferation contributes to the massive accumulation of pro-inflammatory macrophages and the inflammation of adipose tissue in obesity.

Current therapeutic strategies to decrease macrophage content in the obese adipose tissue to improve insulin sensitivity have focused on blocking the infiltration of monocytes into adipose tissue[108,115,134,235]. Our data now show that proliferation is an important process by which macrophages accumulate in adipose tissue in obesity, which could be considered in future therapeutic strategies. In this context, further investigation will be needed to

understand the mechanisms by which obesity stimulates macrophage proliferation specifically within adipose tissue and not other tissues.

Experimental Procedures

Animals, Diets and Treatments

8 to 12 week-old male wild-type C57BL/6J (WT) and B6.V-*Lepob*/*J* (*ob*/*ob*) mice were obtained from Jackson Laboratory (Bar Harbor, Maine, USA) and maintained on a 12-hour light/dark cycle. Animals were given free access to food and water. C57BL/6J wild-type mice were fed a high-fat diet (45% calories from lipids; D12451; Research Diets Inc., New Brunswick, New Jersey) for 7-14 weeks beginning at 8 weeks of age. All other mice were fed normal chow diet (Prolab 5P76 Isopro 3000, LabDiet, St. Louis, Missouri, USA). Intraperitoneal GTTs were performed as previously described[236]. Animals were weighed, sacrificed by cervical dislocation, and adipose tissues (epididymal and inguinal depots), livers and spleen were harvested at age 7-14 weeks. For the *in vivo* EdU injection studies, 8-12 week-old mice were intraperitoneally injected with 1 mg EdU in 200 μl phosphate-buffered saline (PBS) and three hours later mice were sacrificed by asphyxiation with carbon dioxide and immediately bled by cardiac puncture prior to tissue dissection. For the *in vivo* EdU drinking water studies mice were given free access to water containing 1 mg/ml EdU and 2% sucrose to offset taste aversion. For the CL-316,243 studies, mice were intraperitoneally injected with 0.5 mg/kg CL-316,243 in PBS, and sacrificed 24 hours later. Tissues to be analyzed by flow cytometry were processed immediately; other samples were frozen in liquid nitrogen and stored at –80°C prior to RNA extraction or fixed in formaldehyde prior to immunohistochemical analysis. All

procedures were performed in accordance with protocols approved by the University of Massachusetts Medical School's Institutional Animal Care and Use Committee.

Liposome Preparation

PBS and clodronate-loaded liposomes were made in a precisely scaled-down version of a method previously described[237]. Chloroform, phosphatidylcholine, cholesterol and clodronate were all purchased from Sigma. All chloroform solutions were made in glass vials and transferred with borosilicate pipettors. Solutions were filtered through 0.22 μm mixed cellulose ester filters (VWR) using a glass Hamilton syringe. 2.5 ml of phosphatidylcholine in chloroform (100 mg/ml) were added to 2.3 ml cholesterol solution (10 mg/ml in chloroform) in a 50 ml round-bottom flask. The chloroform was removed by low-vacuum rotary evaporation at 40°. After removal of chloroform, and the flask was aerated with sterile vent-filtered compressed air for up to 1 hour, until all traces of chloroform odor was removed. The phospholipid film was then dispersed in 12 ml PBS (for empty liposomes) or 0.7 M clodronate solution in PBS (for clodronate liposomes) by gentle rotation (max., 100 rpm) at room temperature (RT) for 20–30 min. The milky white suspension was kept at RT for 90 minutes and then sonicated in a waterbath for 3 min. The suspension was then kept overnight at 4°C to allow swelling of the liposomes. Before use, the liposomes were washed to remove un-encapsulated clodronate by centrifugation at 22,000*g* and 10°C for 60 min, followed by four more washes at the same speed for 25 minutes. The final liposome pellet was resuspended in sterile PBS and

adjusted to a final volume of 12 ml. Before i.v. injection the liposomes were filtered through Swinnex filters (Millipore) with 3 μm pores.

Isolation of macrophages from adipose tissue SVF.

Adipose tissue SVF cells were prepared from collagenase-digested adipose tissue, as described previously[236]. Briefly, epididymal or inguinal fat pads were mechanically dissociated using the gentleMACS Dissociator (Miltenyi Biotec) and digested with collagenase at 37°C for 45 minutes in Hank's buffered saline solution (HBSS) (Gibco, Life Technologies) containing 2% bovine serum albumin (American Bioanalytical) and 2 mg/ml collagenase (collagenase from clostridium histolyticum, Sigma). Samples were then filtered through 100 μm BD falcon cell strainers and spun at 300 *g* for 10 minutes at room temperature. The adipocyte layer and the supernatant were aspirated and the pelleted cells were collected as the SVF. The cells were then treated with red blood cell (RBC) lysis buffer, washed in PBS and then stained for flow cytometry.

Liver and spleen cell isolation

Liver cells were isolated as previously described[238]. Briefly, the livers of freshly asphyxiated mice were perfused with collagenase 4 mg/ml in 1% BSA in HBSS via the inferior vena cava and/or portal vein until livers were blanched. Small incisions at the edges of the liver were made to assist in complete blanching. After several minutes of perfusion, livers were then placed in collagenase solution at 37°C with gentle rocking for 20-30 minutes. The resulting cell digest was filtered through a 100 μm filter mesh and

several 30*g* centrifugation steps pelleted out hepatocytes. The remaining cells were pelleted by centrifugation at 500*g* and washed several times with HBSS. The cells were treated with RBC lysis buffer and then stained for flow cytometry. Spleens were manually dissociated using the plunger of a 1 ml plastic syringe in cold HBSS. The resultant cell suspension was centrifuged at 500*g* and the cell pellet was treated with RBC lysis buffer. A portion of the splenocytes were than taken for flow cytometry staining.

Flow cytometry

Cells were resuspended in PBS containing 1% BSA (FACS buffer) containing Fc block (clone 2.4G2, eBioscience, San Diego, California, USA) and allowed to block non-specific binding for 15 minutes at 4°C. Cells were then counted and incubated for an additional 20 minutes in the dark at 4°C with fluorophore-conjugated primary antibodies or isotype control antibodies. Antibodies used in these studies included: F4/80-APC (clone Cl:A3-1, AbD Serotec, Raleigh, North Carolina, USA), CD11b-PerCP-Cy5.5 (clone M1/70), Gr-1-APC-Cy7 (clone RB6-8C5), Siglec-f-PE (clone E50-2440), Ly6C-PE-Cy7 (clone AL-21), Ki67-FITC (clone B56) and IgG1κ-FITC (clone MOPC-21, BD Pharmingen San Diego, California, USA), and CD11c-V450 (clone HL-3, BD Horizon, San Jose, California, USA). For the EdU experiments, cells were surface stained according to manufacturer's instructions. Following incubation with primary antibodies, cells were washed and fixed with fixation/permeabilization buffer (eBioscience) and then permeabilized with permeabilization buffer (eBioscience). EdU was chemically conjugated to Alexa 405 or Alexa 647 fluorophore according to the

instructions of the manufacturer (Invitrogen). Sample data were acquired on a BD LSRII (BD Biosciences) and analyzed with FlowJo software (Tree Star). Sample data were initially gated on forward and side scatter, followed by a singlet cell gate, and then a gate to remove auto-fluorescent debris (see Figure S1 for complete gating scheme).

Immunohistochemistry

Adipose tissue SVF cells were plated overnight on glass coverslips in complete DMEM and then fixed for 15 min at room temperature in 4% paraformaldehyde. Cells were washed twice with PBS containing 0.3% Triton x-100 (American Bioanalytical, Natick, Massachusetts, USA) and blocked with 10% normal goat serum and were then incubated with the following antibodies: rat anti-mouse F4/80 (AbD serotec, Raleigh, North Carolina, USA; 1:50 dilution), and goat-anti-mouse Ki67 (Abcam, Cambridge, Massachusetts, USA; 1:200 dilution) overnight at 4°C. After washing, the cells were incubated with secondary antibodies (1:200 dilution) for 30 minutes at room temperature. Cells were mounted in ProLong Gold antifade reagent containing 4',6-diamidino-2-phenylindole (DAPI) (Molecular Probes, Eugene, Oregon, USA). Adipose tissue sections were blocked with 10% normal goat serum and were then incubated with anti-BrdU or Ki67 antibodies and hematoxylin stained. Images were captured on a Zeiss Axiovert 200M microscope using Axiovision software (Carl Zeiss, Thornwood, New York, USA).

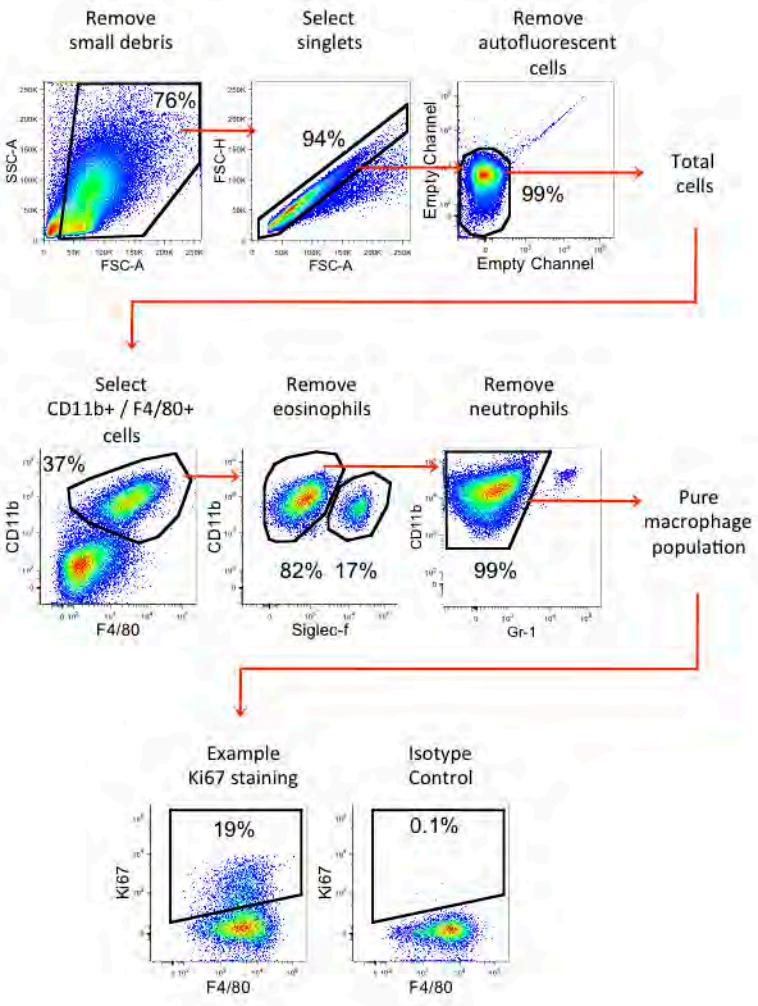
Statistical analysis

All values are shown as means ± SEM. Student's t-test for two-tailed distributions with equal variances was used for comparison between two groups; for comparisons ≥ 3 groups, two-way ANOVA was used followed by the Tukey post test. Differences $p \leq 0.05$ were considered significant. Data were entered into Microsoft Excel, and statistical analyses were performed with Graph Pad Prism 5.

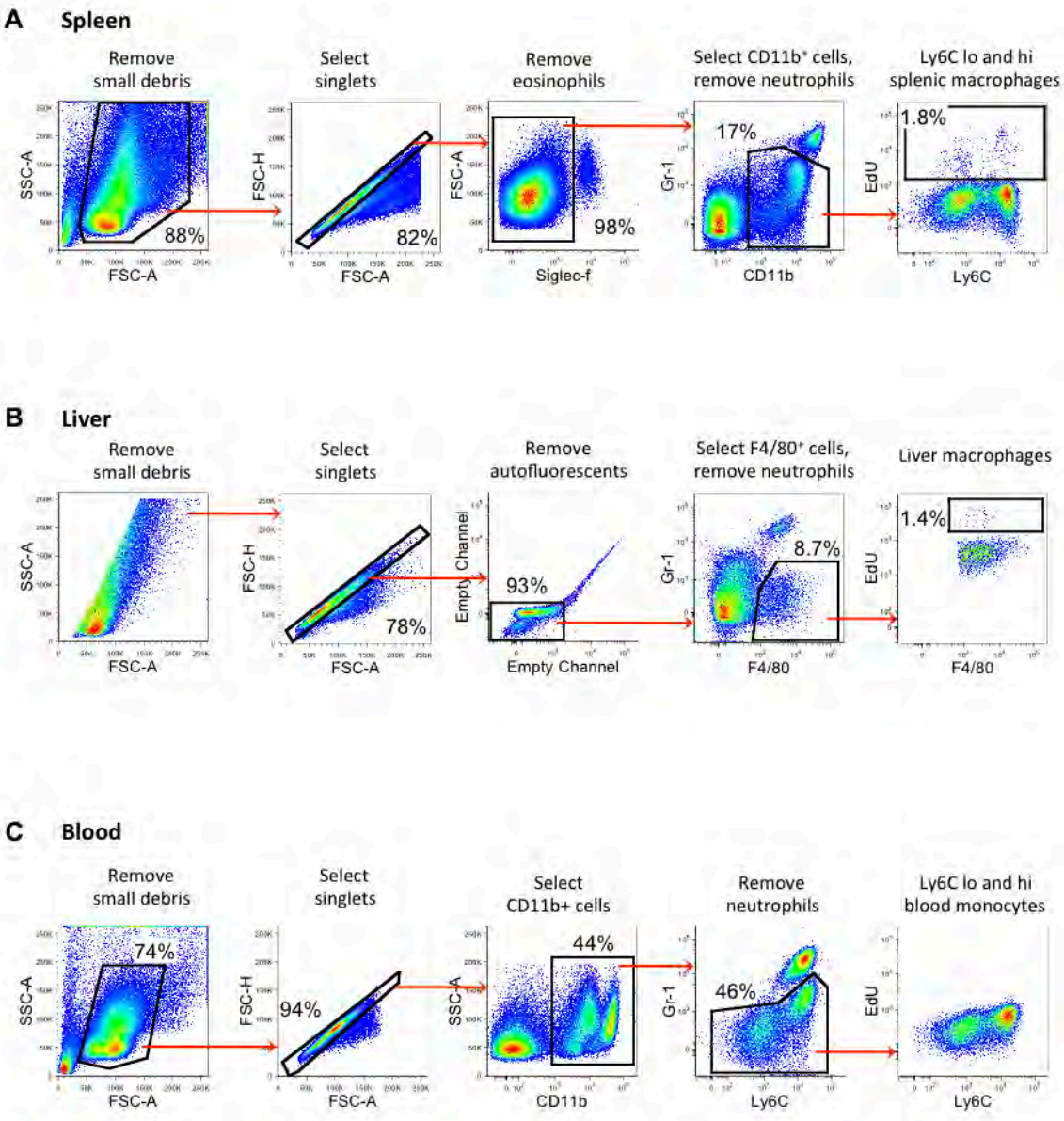
Acknowledgements

We thank Drs. Silvia Corvera, Hardy Kornfeld, Stuart Levitz, Dale Greiner, John Harris, and Joseph Virbasius and members of our laboratory group for excellent discussion of the data in this section of the thesis. We also appreciate the help of Richard Konz and the staff of Flow Cytometry Core and the Diabetes and Endocrinology Research Center Morphology Core in the University of Massachusetts. These studies were supported by grants to M.P.C. from the National Institutes of Health (DK085753, AI046629), a grant from the International Research Alliance at Novo Nordisk Foundation Center for Metabolic Research, a grant from the Juvenile Diabetes Research Foundation (17-2009-546), and by Core Facilities in the University of Massachusetts Diabetes and Endocrinology Research Center also funded by the National Institutes of Health (DK325220). The authors report that they have no conflicts of interest.

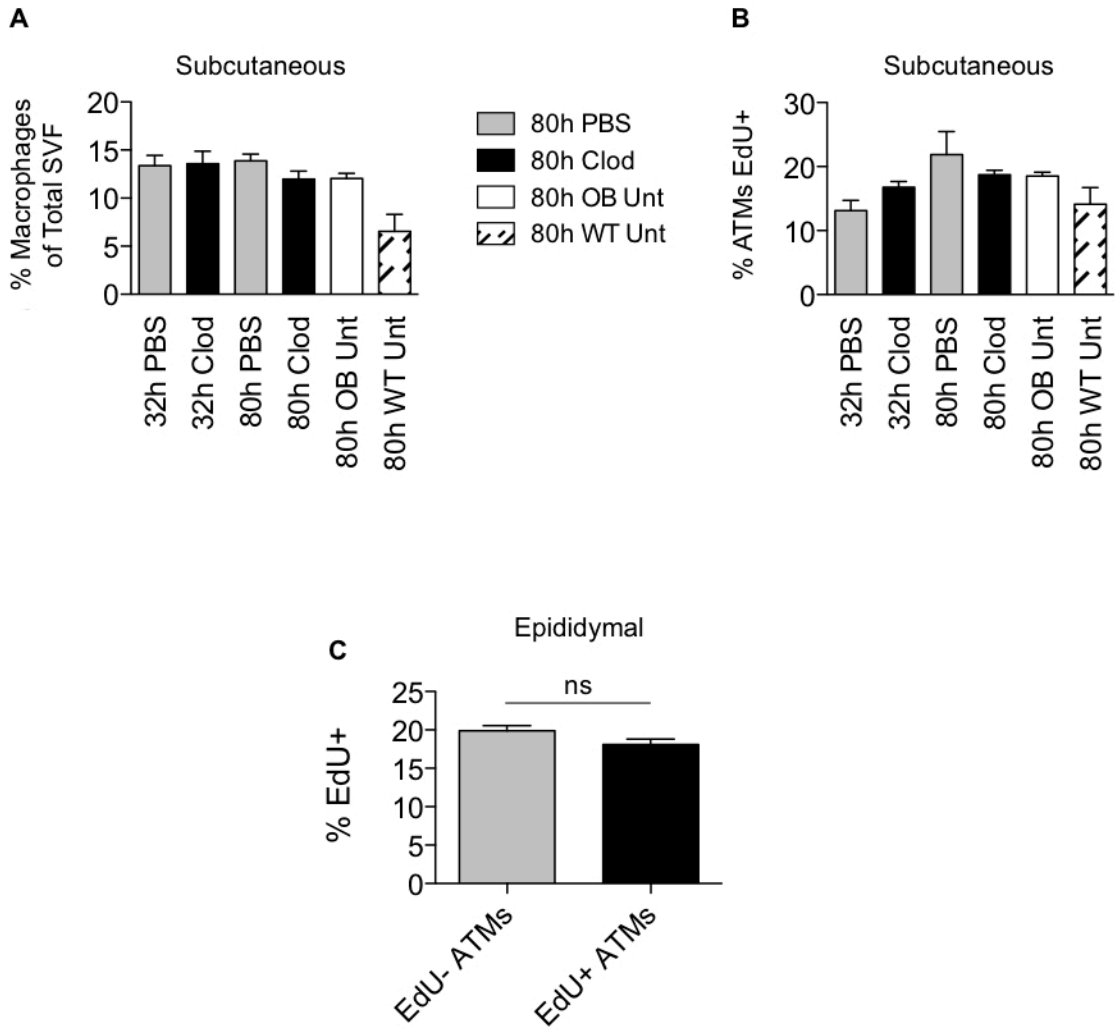
Supplemental Figures



Supplementary Figure 2.1. Flow cytometry gating scheme for identifying macrophages in adipose tissue SVF.

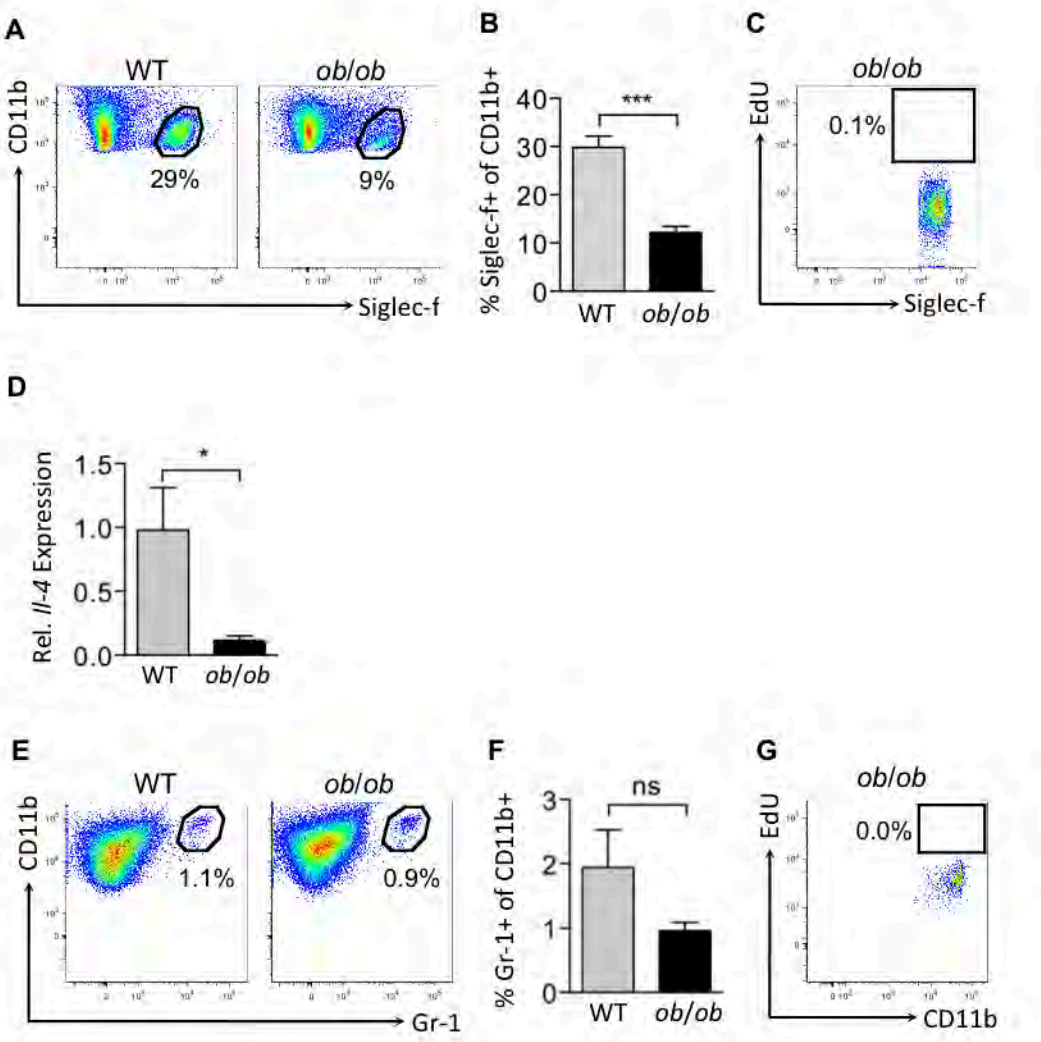


Supplemental Figure 2.2. Flow cytometry gating schemes for spleen and liver macrophages and blood monocytes.



Supplementary Figure 2.3. Adipose Tissue Macrophage Proliferation Occurs Independently of Blood Monocyte Recruitment.

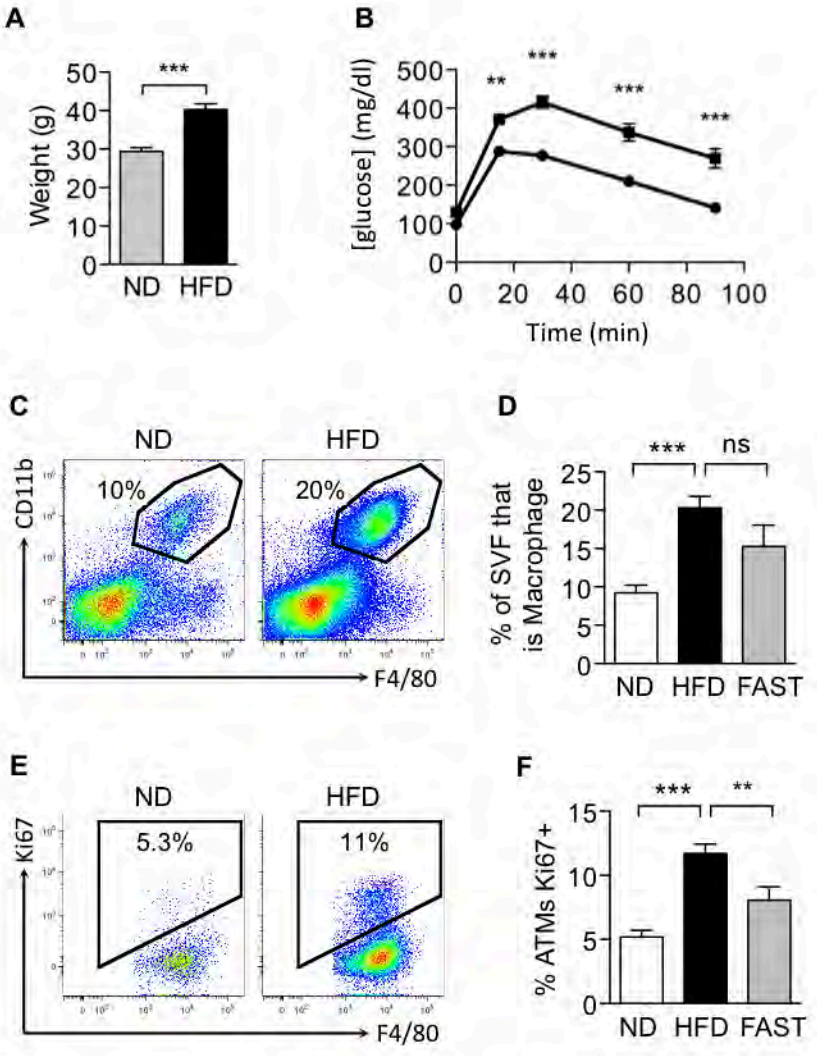
Supplemental Figure 2.3. Adipose Tissue Macrophage Proliferation Occurs Independently of Blood Monocyte Recruitment. *ob*/*ob* mice were i.v. injected with either PBS-liposomes or clodronate-liposomes every 16 hours. 18 hours after initial injection, the mice were given drinking water containing EdU. (**A**) Macrophage content in the inguinal adipose tissue of PBS-liposome-treated, clodronate-liposome-treated, untreated *ob*/*ob* mice and untreated wild-type mice; n=5 mice per group. (**B**) Quantification of EdU incorporation into ATMs during 32h and 80h of exposure to EdU drinking water in PBS-liposome-treated and monocyte-depleted clodronate-liposome-treated *ob*/*ob* mice; n=4-5 mice per group. (**C**) Percentage of macrophages that are $CD11c^+$ in macrophages that incorporated EdU (EdU^+) or not (EdU-) during an 80-hour EdU exposure in epididymal AT of *ob/ob* mice**.** n=9-10 mice/group. All graphs are expressed as mean ± s.e.m. Statistical significance was determined by Student's t-test. ns = not significant.



Supplemental Figure 2.4. Effect of obesity on proliferation of diverse immune cell types in adipose tissue.

Supplemental Figure 2.4. Effect of obesity on proliferation of diverse immune cell types within adipose tissue of *ob*/*ob* mice.

(**A**) Representative dot plots of $CD11b^+$ SVF cells stained for the eosinophil marker Siglec-f. (**B**) Percentages of $CD11b^+$ cells that are eosinophils in WT and *ob*/*ob* mice. n= 48-51 mice/group from 10 independent experiments. (**C**) Representative dot plot of SVF eosinophils 3 hours after EdU injection. (**D**) Representative dot plots of $CD11b^+$ SVF cells stained with the neutrophil marker Gr-1. (**E**) Percentages of $CD11b^+$ cells that are neutrophils. n= 14-15 mice/group from 3 independent experiments. (**F**) Representative dot plot of SVF neutrophils 3 hours after EdU injection. (**G**) Percentage of macrophages that are $CD11c^+$ in WT and *ob*/*ob* SVF. (**H**) Percentages of $CD11c^+$ and $CD11c^-$ macrophages that incorporated EdU during a 3-hour *in vivo* pulse or a 3-hour *ex vivo* pulse. n=14-15 mice/group from 3 independent experiments. All graphs are expressed as mean ± s.e.m. Statistical significance was determined by Student's t-test. ns = not significant. **p<0.01; ***p<0.001.



Supplemental Figure 2.5 Adipose tissue macrophage proliferation decreases with weight loss in mice..

Supplemental Figure 2.5 Adipose tissue macrophage proliferation decreases with weight loss in mice. Mice were fed a HFD or ND for 7-14 weeks. (**A**) Weights and (**B**) GTT of ND (●) and (■) HFD mice; n=5 mice/group. SVF cells from inguinal fat pads were isolated and analyzed by flow cytometry. (**C**) Representative flow cytometry dot plots of the SVF F4/80 and CD11b staining. (**D**) Percentage of macrophages in SVF for ND, HFD and fasted mice. (**E**) Representative flow cytometry dot plots of Ki67 staining in ATMs. (**F**) Percentage of macrophages positive for Ki67 in ND, HFD and fasted mice. All graphs are expressed as mean ± s.e.m. Statistical significance was determined by Student's t-test. **$p<0.01$; ***$p<0.001$.

CHAPTER III: Discussion and Future Directions

Obesity and diabetes are major public health problems facing the world today. Extending our understanding of adipose tissue biology, and how it changes in obesity, will hopefully better equip our society in dealing with the obesity epidemic. Macrophages and other immune cells accumulate in the adipose tissue in obesity and secrete cytokines that can promote insulin resistance. ATMs are thought to originate from bone marrow-derived monocytes, which infiltrate the tissue from the circulation. Much work has been done to demonstrate that inhibition of monocyte recruitment to the adipose tissue can ameliorate insulin resistance. While monocytes most certainly can enter the adipose tissue, we have shown here that local macrophage proliferation may be the predominant mechanism by which macrophages self-renew in the adipose tissue.

Overall, the results presented above suggest that local proliferation unexpectedly makes a major contribution to maintaining the large population of macrophages present in the obese adipose tissue in the steady state. This suggests that increased rates of local macrophage proliferation may also be partly responsible for the massive increase in ATM content that occurs in obesity. This information could have implications for future therapeutic strategies in the management of diabetes.

These findings raise a plethora of questions relating to the mechanism and consequences of macrophage proliferation within the adipose tissue. What growth factor, cytokine, or other signal is responsible for promoting the dramatic increase in macrophage proliferation in the adipose tissue in obesity? What is the origin of that factor and why is its activity increased in obesity? What intracellular signaling pathways are

required for macrophage proliferation in adipose tissue? What is the relative contribution of local macrophage proliferation vs. monocyte recruitment in maintaining the total macrophage population in obese adipose tissue? What is the average macrophage residence time in the adipose tissue and does it change in obesity? How do macrophages leave the adipose tissue? What is the metabolic implication of increasing or inhibiting macrophage proliferation specifically in the adipose tissue? I will conclude this dissertation with a discussion of a few of these issues.

What is the signal that is promoting local macrophage proliferation in adipose tissue?

In Chapter One, I reviewed the majority of known examples of local macrophage proliferation in adult mammalian tissues that exist at this time. In the lung, skin, kidney and atherosclerotic plaques, various studies have identified the factors involved in promoting local macrophage proliferation. I will review them here and discuss their implications for adipose tissue macrophage research.

In the lung, it appears that granulocyte-monocyte colony stimulating factor (GM-CSF) is critical for normal alveolar macrophage function, as GM-CSF KO mice have pulmonary pathology[239], and at least one study has linked that defect to the role of GM-CSF in both alveolar macrophage function and stimulation of alveolar macrophage proliferation[240]. Macrophage colony stimulating factor (M-CSF) has also been found to be important in alveolar macrophage content in mice younger than than 3 week old, but not adult mice[241]. Also, as discussed previously, a recent study identified eosinophil-derived

IL-4 to be the sole factor responsible for driving local macrophage proliferation in the lung during helminth infection[151].

In the skin, one study showed that young mice lacking the receptor for M-CSF completely lack Langerhans cells[154]. They also showed that even during the repopulation phenomenon that occurs in damaged skin in wild-type mice, circulating monocytes enter the inflamed skin and undergo several rounds of proliferation, forming clusters of Langerhans cells[154]. This proliferation was also dependent on the M-CSF receptor on the incoming monocytes.

In the kidney, M-CSF has been identified as the driving force in the stimulation of local proliferation in rat models of kidney injury[242] and kidney M-CSF expression is elevated in human glomerulonephritis, in which areas of high M-CSF expression levels were co-localized with high rates of local macrophage proliferation[243]. Interestingly, macrophage proliferation in allograft transplant rejection models is also dependent on M-CSF, and antibody-mediated blockade of the M-CSF receptor was found to significantly alleviate macrophage recruitment to allografted kidneys[244]. It is worth noting that inhibition of macrophage proliferation may be a promising therapeutic avenue to attenuate kidney injury in these conditions.

In atherosclerosis, numerous studies have identified oxidized LDL particles as one of the factors promoting macrophage proliferation in plaques[245-248]. Indeed, treatment of mice with cholesterol-lowering statin drugs has been shown to reduce macrophage content in atherosclerotic plaques[249,250] and at least one study has shown that statins inhibit oxidized-LDL-induced macrophage proliferation by inhibiting the mitogen

activated protein kinase (MAPK) p38 in macrophages[251]. Several reports indicate that both M-CSF and GM-CSF are critical in the macrophage proliferative response to oxidized LDL[245,247,252].

These studies on macrophage proliferation in other tissues will hopefully inform future studies of macrophage proliferation in adipose tissue. For example, it is tempting to speculate that M-CSF, GM-CSF or some form of lipid are the factors promoting macrophage proliferation in adipose tissue as they are in atherosclerotic plaques. Unfortunately, very few studies on the role of M-CSF and GM-CSF exist in the adipose tissue at this time. Weisberg et al. reported that adipose tissue macrophage content was decreased in lean mice that were deficient in M-CSF, although these mice have severe skeletal abnormalities and lack teeth, and do not become obese even on a high fat diet[11]. Another study demonstrated that mice deficient in GM-CSF do become obese on a high fat diet but were more insulin sensitive, and have decreased macrophage content and inflammatory gene expression in their adipose tissue[253]. While this may have been the result of a defect in monocyte recruitment to the adipose tissue, it is possible that GM-CSF deficiency led to an inhibition of local macrophage proliferation within the adipose tissue. For the sake of completeness, it is worth nothing that adipose-tissue and depot-specific overexpression of M-CSF in rabbits was associated with a 16-fold increase in localized adipose tissue growth compared to controls despite no change in diet[254] . The role of macrophages in stimulating the adipose tissue growth in this study is unclear, as macrophage content was not adequately documented[254].

In our lab, gene chip and RT-PCR studies have shown no increase in M-CSF or GM-CSF in obese adipose tissue at the mRNA level. However, we plan to measure the protein levels of these growth factors in the adipose tissue or in lymph draining from the adipose tissue. Preliminary experiments with M-CSF and GM-CSF injections in lean mice have yielded promising results in increasing macrophage content in adipose tissue, and inhibition of GM-CSF with neutralizing antibodies in obese mice has also yielded promising initial results in inhibiting ATM proliferation (data not shown). There may also be a role for IL-4 in ATM proliferation, but the dramatic decrease in eosinophil content and IL-4 expression that occur in the adipose tissue in obesity (Supplementary Figure 2.4) make it unlikely that IL-4 is the endogenous signal promoting ATM proliferation.

While we found that adipocyte release of free fatty acids through lipolysis actually decreased ATM proliferation in obese mice (Figure 2.5), investigation of other lipid signals may still represent a fruitful avenue of research in macrophage proliferation in adipose tissue. For example, in addition to oxidized LDL stimulation of macrophage proliferation in atherosclerosis, several other lipids have been shown to directly promote macrophage proliferation. One group has published several studies reporting that various lipids including cholesterol esters, triglycerides and several phospholipids, could accumulate in, and stimulate the proliferation of, murine peritoneal macrophage upon lipid treatment in vitro[255-259]. At least one additional lipid, ceramide 1-phosphate, has been shown to directly stimulate bone marrow-derived macrophage proliferation *in vitro*[260].

Another study showed that conditioned media from adipocytes treated with conjugated linoleic acid has been shown to increase proliferation of a macrophage cell line *in vitro*[261]. Further studies will be required to determine if lipids released from adipocytes are responsible for stimulating ATM proliferation. Insight on this subject may also come from examining macrophage proliferation in a lipodystrophic mouse model with disrupted lipid metabolism, in which macrophage content and inflammatory gene expression in adipose tissue were found to be greatly increased, to levels even higher than those seen in obesity[262]. Alternatively, induction of adipocyte apoptosis causes release of triglyceride into the circulation, rather than fatty acids, and is associated with macrophage accumulation in adipose tissue[263]. Examining the proliferation of macrophages in these mice may also provide useful insight as to the signals promoting macrophage proliferation. Investigation of the above questions will hopefully also shed light on why obesity has a specific effect on macrophage proliferation in adipose tissue but not in other tissues.

What intracellular signaling pathways are required for ATM proliferation?

As reviewed above, macrophage-specific deletion of JNK was found to decrease macrophage content in adipose tissue[80]. While most of the other studies on myeloid-specific gene knockouts employed lethal irradiation followed by bone marrow transplant, this study did not. Interestingly, the authors failed to demonstrate that the decrease in macrophage content was due to inhibition of monocyte recruitment to adipose tissue[80]. JNK activation has been shown in other studies to be critical for macrophage

development, proliferation and survival[264]. Indeed, the other MAP Kinases ERK and p38 have already been shown to mediate proliferation in atherosclerotic plaque macrophages *in vivo*[251,265]. It may be very interesting to study the rates of adipose tissue macrophage proliferation in mice with macrophage-specific MAPK deletions.

What are the relative contributions of local macrophage proliferation and monocyte recruitment to maintaining the total macrophage population in obese adipose tissue?

At least one study has directly demonstrated the recruitment of adoptively transferred labeled blood monocytes to the adipose tissue in obesity[139]. Similar experiments would need to be repeated and performed in conjunction with macrophage proliferation studies in order to answer these questions. For example, the work presented in Chapter 2 suggests that freshly recruited monocytes are not major contributors to the proliferating pool of macrophages in adipose tissue. However, prolonged EdU exposure with concurrent adoptive transfer of labeled monocytes may begin to shed light on the relative contributions of macrophage proliferation and monocyte recruitment in adipose tissue. In addition to these experiments, it would no doubt be very informative to adopt the approach taken in lungs, in which bone marrow transplant with upper body shielding revealed that, in the absence of lethal irradiation, alveolar macrophages persist for the lifetime of a mouse[136]. Therefore, experiments using bone marrow transplant of CD45.1 or GFP^+ donor marrow would reveal if and how quickly monocytes recruited from the blood replace resident tissue macs of host origin. Surgically joined parabiotic mice would

be another approach which would reveal the rate at which resident macrophages are replaced by circulating monocytes.

What is the average macrophage residence time in the adipose tissue and does it change in obesity? How do macrophages leave the adipose tissue?

Mechanisms of macrophage efflux out of adipose tissue have remained mysterious. However, the results presented in Chapter 2 suggest that in obesity in mice, complete turnover of the entire ATM population occurs somewhere in the range of once every one to two weeks (see Figure 2.4). Thus, there is no doubt that macrophages are leaving the adipose tissue at an appreciable rate. One way to investigate the question of macrophage turnover would be to use the Kaede mouse, in which cells can be photo-labeled and subsequently tracked using intravital microscopy or other methods[266]. In these experiments, a piece of adipose tissue would be exposed to light, causing the generation of a green fluorophore in the affected cells, and the disappearance of these cells from the adipose tissue would be followed over time. While technically challenging, this method does not cause any change in the behavior of cells, which is a distinct advantage over chemical or particulate dyes such as PKH26[266]. While macrophage efflux may occur, it is also entirely possible that macrophages may leave the adipose tissue by undergoing necrosis or apoptosis locally within the adipose tissue. Indeed, this has been shown to be the case in atherosclerosis (Reviewed in Andres, 2012[174]). Macrophage apoptosis markers could easily be studied in adipose tissue macrophages using microscopy and flow cytometry.

What are the metabolic implications of increasing or inhibiting macrophage proliferation specifically in the adipose tissue?

Unfortunately, there appears to be no tissue- and cell-type specific way of inhibiting macrophage proliferation exclusively in the adipose tissue at this time. Unlike in liver, where zymosan has been shown to induce macrophage proliferation[163], intraperitoneal injection of glucan particles carrying siRNA causes an inhibition of macrophage proliferation in adipose tissue (our data, not shown). In atherosclerosis, macrophage proliferation can be increased using myeloid-specific knockouts of tumor-suppressor genes (Reviewed in Andres, 2012[174]). Similar experiments may provide insight into the metabolic consequences of increasing macrophage proliferation in adipose tissue as well.

In conclusion, we have shown here that local macrophage proliferation may be the predominant mechanism by which macrophage density is maintained in the obese adipose tissue. Local macrophage proliferation may also be responsible for the increase in ATM content that occurs in obesity. Further investigation of this phenomenon, its metabolic consequences, and the other mechanisms regulating macrophage content in adipose tissue may one day contribute to improving human health.

References

1 Alberts, B. *et al. Molecular Biology of the Cell*. 4th Edition edn, (Garland Science, 2002).

2 Pond, C. in *Adipose Tissue Biology* (ed ME Symonds) Ch. 227-269, (Springer, 2012).

3 Hotamisligil, G. S. Inflammation and metabolic disorders. *Nature* **444**, 860-867, doi:10.1038/nature05485 (2006).

4 Atkinson, S. N., Nelson, R. A. & Ramsay, M. A. Changes in the Body Composition of Fasting Polar Bears (Ursus maritimus): The Effect of Relative Fatness on Protein Conservation. *Physiological Zoology* **69**, 304-316 (1996).

5 Gallagher, D. *et al.* Healthy percentage body fat ranges: an approach for developing guidelines based on body mass index. *The American journal of clinical nutrition* **72**, 694-701 (2000).

6 #311, W. F. S. WHO Fact Sheet #311. (2013).

7 Kopelman, P. Health risks associated with overweight and obesity. *Obesity reviews : an official journal of the International Association for the Study of Obesity* **8 Suppl 1**, 13-17, doi:10.1111/j.1467-789X.2007.00311.x (2007).

8 Calle, E. E. & Kaaks, R. Overweight, obesity and cancer: epidemiological evidence and proposed mechanisms. *Nature reviews. Cancer* **4**, 579-591, doi:10.1038/nrc1408 (2004).

9 Wang, Y., Beydoun, M. A., Liang, L., Caballero, B. & Kumanyika, S. K. Will all Americans become overweight or obese? estimating the progression and cost of the US obesity epidemic. *Obesity (Silver Spring)* **16**, 2323-2330, doi:10.1038/oby.2008.351 (2008).

10 Hotamisligil, G. S., Shargill, N. S. & Spiegelman, B. M. Adipose expression of tumor necrosis factor-alpha: direct role in obesity-linked insulin resistance. *Science* **259**, 87-91 (1993).

11 Weisberg, S. P. *et al.* Obesity is associated with macrophage accumulation in adipose tissue. *The Journal of clinical investigation* **112**, 1796-1808, doi:10.1172/JCI19246
112/12/1796 [pii] (2003).

12 Shoelson, S. E., Lee, J. & Goldfine, A. B. Inflammation and insulin resistance. *The Journal of clinical investigation* **116**, 1793-1801, doi:10.1172/JCI29069 (2006).

13 Biddinger, S. B. & Kahn, C. R. From mice to men: insights into the insulin resistance syndromes. *Annual review of physiology* **68**, 123-158, doi:10.1146/annurev.physiol.68.040104.124723 (2006).

14 Guilherme, A., Virbasius, J. V., Puri, V. & Czech, M. P. Adipocyte dysfunctions linking obesity to insulin resistance and type 2 diabetes. *Nature reviews. Molecular cell biology* **9**, 367-377, doi:nrm2391 [pii]
10.1038/nrm2391 (2008).

15 Olefsky, J. M. & Glass, C. K. Macrophages, inflammation, and insulin resistance. *Annual review of physiology* **72**, 219-246, doi:10.1146/annurev-physiol-021909-135846 (2010).

16 Unger, R. H. Lipotoxicity in the pathogenesis of obesity-dependent NIDDM. Genetic and clinical implications. *Diabetes* **44**, 863-870 (1995).

17 Unger, R. H. Lipotoxic diseases. *Annual review of medicine* **53**, 319-336, doi:10.1146/annurev.med.53.082901.104057 (2002).

18 Savage, D. B., Petersen, K. F. & Shulman, G. I. Disordered lipid metabolism and the pathogenesis of insulin resistance. *Physiological reviews* **87**, 507-520, doi:10.1152/physrev.00024.2006 (2007).

19 Mayerson, A. B. *et al.* The effects of rosiglitazone on insulin sensitivity, lipolysis, and hepatic and skeletal muscle triglyceride content in patients with type 2 diabetes. *Diabetes* **51**, 797-802 (2002).

20 Sugii, S. *et al.* PPARgamma activation in adipocytes is sufficient for systemic insulin sensitization. *Proceedings of the National Academy of Sciences of the United States of America* **106**, 22504-22509, doi:10.1073/pnas.0912487106 (2009).

21 Karpe, F., Dickmann, J. R. & Frayn, K. N. Fatty acids, obesity, and insulin resistance: time for a reevaluation. *Diabetes* **60**, 2441-2449, doi:10.2337/db11-0425 (2011).

22 Belfort, R. *et al.* Dose-response effect of elevated plasma free fatty acid on insulin signaling. *Diabetes* **54**, 1640-1648 (2005).

23 Boden, G. Role of fatty acids in the pathogenesis of insulin resistance and NIDDM. *Diabetes* **46**, 3-10 (1997).

24 Kim, J. K. *et al.* Tissue-specific overexpression of lipoprotein lipase causes tissue-specific insulin resistance. *Proceedings of the National Academy of Sciences of the United States of America* **98**, 7522-7527, doi:10.1073/pnas.121164498 (2001).

25 Shimomura, I., Hammer, R. E., Ikemoto, S., Brown, M. S. & Goldstein, J. L. Leptin reverses insulin resistance and diabetes mellitus in mice with congenital lipodystrophy. *Nature* **401**, 73-76, doi:10.1038/43448 (1999).

26 Shimomura, I. *et al.* Insulin resistance and diabetes mellitus in transgenic mice expressing nuclear SREBP-1c in adipose tissue: model for congenital generalized lipodystrophy. *Genes & development* **12**, 3182-3194 (1998).

27 Carr, A. *et al.* A syndrome of peripheral lipodystrophy, hyperlipidaemia and insulin resistance in patients receiving HIV protease inhibitors. *AIDS* **12**, F51-58 (1998).

28 Petersen, K. F. *et al.* Leptin reverses insulin resistance and hepatic steatosis in patients with severe lipodystrophy. *The Journal of clinical investigation* **109**, 1345-1350, doi:10.1172/JCI15001 (2002).

29 Rupnick, M. A. *et al.* Adipose tissue mass can be regulated through the vasculature. *Proceedings of the National Academy of Sciences of the United States of America* **99**, 10730-10735, doi:10.1073/pnas.162349799 (2002).

30 Hosogai, N. *et al.* Adipose tissue hypoxia in obesity and its impact on adipocytokine dysregulation. *Diabetes* **56**, 901-911, doi:10.2337/db06-0911 (2007).

31 Sun, K. *et al.* Dichotomous effects of VEGF-A on adipose tissue dysfunction. *Proceedings of the National Academy of Sciences of the United States of America* **109**, 5874-5879, doi:10.1073/pnas.1200447109 (2012).

32 Elias, I. *et al.* Adipose tissue overexpression of vascular endothelial growth factor protects against diet-induced obesity and insulin resistance. *Diabetes* **61**, 1801-1813, doi:10.2337/db11-0832 (2012).

33 Sung, H. K. *et al.* Adipose vascular endothelial growth factor regulates metabolic homeostasis through angiogenesis. *Cell metabolism* **17**, 61-72, doi:10.1016/j.cmet.2012.12.010 (2013).

34 Harding, H. P., Calfon, M., Urano, F., Novoa, I. & Ron, D. Transcriptional and translational control in the Mammalian unfolded protein response. *Annual review of cell and developmental biology* **18**, 575-599, doi:10.1146/annurev.cellbio.18.011402.160624 (2002).

35 Gregor, M. F. & Hotamisligil, G. S. Thematic review series: Adipocyte Biology. Adipocyte stress: the endoplasmic reticulum and metabolic disease. *Journal of lipid research* **48**, 1905-1914, doi:10.1194/jlr.R700007-JLR200 (2007).

36 Ozcan, U. *et al.* Endoplasmic reticulum stress links obesity, insulin action, and type 2 diabetes. *Science* **306**, 457-461, doi:10.1126/science.1103160 (2004).

37 Boden, G. *et al.* Increase in endoplasmic reticulum stress-related proteins and genes in adipose tissue of obese, insulin-resistant individuals. *Diabetes* **57**, 2438-2444, doi:10.2337/db08-0604 (2008).

38 Ozcan, U. *et al.* Chemical chaperones reduce ER stress and restore glucose homeostasis in a mouse model of type 2 diabetes. *Science* **313**, 1137-1140, doi:10.1126/science.1128294 (2006).

39 Fu, S. *et al.* Aberrant lipid metabolism disrupts calcium homeostasis causing liver endoplasmic reticulum stress in obesity. *Nature* **473**, 528-531, doi:10.1038/nature09968 (2011).

40 Oyadomari, S., Araki, E. & Mori, M. Endoplasmic reticulum stress-mediated apoptosis in pancreatic beta-cells. *Apoptosis : an international journal on programmed cell death* **7**, 335-345 (2002).

41 Urano, F. *et al.* Coupling of stress in the ER to activation of JNK protein kinases by transmembrane protein kinase IRE1. *Science* **287**, 664-666 (2000).

42 Prentki, M. & Nolan, C. J. Islet beta cell failure in type 2 diabetes. *The Journal of clinical investigation* **116**, 1802-1812, doi:10.1172/JCI29103 (2006).

43 Butler, A. E. *et al.* Beta-cell deficit and increased beta-cell apoptosis in humans with type 2 diabetes. *Diabetes* **52**, 102-110 (2003).

44 Steil, G. M. *et al.* Adaptation of beta-cell mass to substrate oversupply: enhanced function with normal gene expression. *American journal of physiology. Endocrinology and metabolism* **280**, E788-796 (2001).

45 Chen, C., Hosokawa, H., Bumbalo, L. M. & Leahy, J. L. Mechanism of compensatory hyperinsulinemia in normoglycemic insulin-resistant spontaneously

hypertensive rats. Augmented enzymatic activity of glucokinase in beta-cells. *The Journal of clinical investigation* **94**, 399-404, doi:10.1172/JCI117335 (1994).

46 Godsland, I. F., Jeffs, J. A. & Johnston, D. G. Loss of beta cell function as fasting glucose increases in the non-diabetic range. *Diabetologia* **47**, 1157-1166, doi:10.1007/s00125-004-1454-z (2004).

47 Poitout, V. & Robertson, R. P. Minireview: Secondary beta-cell failure in type 2 diabetes--a convergence of glucotoxicity and lipotoxicity. *Endocrinology* **143**, 339-342 (2002).

48 Kahn, S. E. The relative contributions of insulin resistance and beta-cell dysfunction to the pathophysiology of Type 2 diabetes. *Diabetologia* **46**, 3-19, doi:10.1007/s00125-002-1009-0 (2003).

49 Alberti, K. G. & Zimmet, P. Z. Definition, diagnosis and classification of diabetes mellitus and its complications. Part 1: diagnosis and classification of diabetes mellitus provisional report of a WHO consultation. *Diabetic medicine : a journal of the British Diabetic Association* **15**, 539-553, doi:10.1002/(SICI)1096-9136(199807)15:7<539::AID-DIA668>3.0.CO;2-S (1998).

50 Genuth, S. *et al.* Follow-up report on the diagnosis of diabetes mellitus. *Diabetes care* **26**, 3160-3167 (2003).

51 Feinstein, R., Kanety, H., Papa, M. Z., Lunenfeld, B. & Karasik, A. Tumor necrosis factor-alpha suppresses insulin-induced tyrosine phosphorylation of insulin receptor and its substrates. *The Journal of biological chemistry* **268**, 26055-26058 (1993).

52 Galic, S., Oakhill, J. S. & Steinberg, G. R. Adipose tissue as an endocrine organ. *Molecular and cellular endocrinology* **316**, 129-139, doi:10.1016/j.mce.2009.08.018 (2010).

53 Hotamisligil, G. S., Arner, P., Caro, J. F., Atkinson, R. L. & Spiegelman, B. M. Increased adipose tissue expression of tumor necrosis factor-alpha in human obesity and insulin resistance. *The Journal of clinical investigation* **95**, 2409-2415, doi:10.1172/JCI117936 (1995).

54 Kern, P. A. *et al.* The expression of tumor necrosis factor in human adipose tissue. Regulation by obesity, weight loss, and relationship to lipoprotein lipase. *The Journal of clinical investigation* **95**, 2111-2119, doi:10.1172/JCI117899 (1995).

55 Dandona, P. *et al.* Tumor necrosis factor-alpha in sera of obese patients: fall with weight loss. *The Journal of clinical endocrinology and metabolism* **83**, 2907-2910 (1998).

56 Juge-Aubry, C. E. *et al.* Regulatory effects of interleukin (IL)-1, interferon-beta, and IL-4 on the production of IL-1 receptor antagonist by human adipose tissue. *The Journal of clinical endocrinology and metabolism* **89**, 2652-2658, doi:10.1210/jc.2003-031219 (2004).

57 Dinarello, C. A. Proinflammatory cytokines. *Chest* **118**, 503-508 (2000).

58 Yudkin, J. S., Stehouwer, C. D., Emeis, J. J. & Coppack, S. W. C-reactive protein in healthy subjects: associations with obesity, insulin resistance, and endothelial

dysfunction: a potential role for cytokines originating from adipose tissue? *Arteriosclerosis, thrombosis, and vascular biology* **19**, 972-978 (1999).

59 Schmidt, M. I. *et al.* Markers of inflammation and prediction of diabetes mellitus in adults (Atherosclerosis Risk in Communities study): a cohort study. *Lancet* **353**, 1649-1652 (1999).

60 Duncan, B. B. *et al.* Low-grade systemic inflammation and the development of type 2 diabetes: the atherosclerosis risk in communities study. *Diabetes* **52**, 1799-1805 (2003).

61 Barzilay, J. I. *et al.* The relation of markers of inflammation to the development of glucose disorders in the elderly: the Cardiovascular Health Study. *Diabetes* **50**, 2384-2389 (2001).

62 Pradhan, A. D., Manson, J. E., Rifai, N., Buring, J. E. & Ridker, P. M. C-reactive protein, interleukin 6, and risk of developing type 2 diabetes mellitus. *JAMA : the journal of the American Medical Association* **286**, 327-334 (2001).

63 Krogh-Madsen, R., Plomgaard, P., Moller, K., Mittendorfer, B. & Pedersen, B. K. Influence of TNF-alpha and IL-6 infusions on insulin sensitivity and expression of IL-18 in humans. *American journal of physiology. Endocrinology and metabolism* **291**, E108-114, doi:10.1152/ajpendo.00471.2005 (2006).

64 Lagathu, C. *et al.* Long-term treatment with interleukin-1beta induces insulin resistance in murine and human adipocytes. *Diabetologia* **49**, 2162-2173, doi:10.1007/s00125-006-0335-z (2006).

65 Uysal, K. T., Wiesbrock, S. M., Marino, M. W. & Hotamisligil, G. S. Protection from obesity-induced insulin resistance in mice lacking TNF-alpha function. *Nature* **389**, 610-614, doi:10.1038/39335 (1997).

66 Ofei, F., Hurel, S., Newkirk, J., Sopwith, M. & Taylor, R. Effects of an engineered human anti-TNF-alpha antibody (CDP571) on insulin sensitivity and glycemic control in patients with NIDDM. *Diabetes* **45**, 881-885 (1996).

67 Gonzalez-Gay, M. A. *et al.* Anti-tumor necrosis factor-alpha blockade improves insulin resistance in patients with rheumatoid arthritis. *Clinical and experimental rheumatology* **24**, 83-86 (2006).

68 Kiortsis, D. N., Mavridis, A. K., Vasakos, S., Nikas, S. N. & Drosos, A. A. Effects of infliximab treatment on insulin resistance in patients with rheumatoid arthritis and ankylosing spondylitis. *Annals of the rheumatic diseases* **64**, 765-766, doi:10.1136/ard.2004.026534 (2005).

69 Larsen, C. M. *et al.* Interleukin-1-receptor antagonist in type 2 diabetes mellitus. *The New England journal of medicine* **356**, 1517-1526, doi:356/15/1517 [pii]
10.1056/NEJMoa065213 (2007).

70 Sauter, N. S., Schulthess, F. T., Galasso, R., Castellani, L. W. & Maedler, K. The antiinflammatory cytokine interleukin-1 receptor antagonist protects from high-fat diet-induced hyperglycemia. *Endocrinology* **149**, 2208-2218, doi:10.1210/en.2007-1059 (2008).

71 McGillicuddy, F. C. *et al.* Lack of interleukin-1 receptor I (IL-1RI) protects mice from high-fat diet-induced adipose tissue inflammation coincident with improved glucose homeostasis. *Diabetes* **60**, 1688-1698, doi:10.2337/db10-1278 (2011).

72 Taniguchi, C. M., Emanuelli, B. & Kahn, C. R. Critical nodes in signalling pathways: insights into insulin action. *Nature reviews. Molecular cell biology* **7**, 85-96, doi:10.1038/nrm1837 (2006).

73 Solinas, G. & Karin, M. JNK1 and IKKbeta: molecular links between obesity and metabolic dysfunction. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* **24**, 2596-2611, doi:10.1096/fj.09-151340 (2010).

74 Hotamisligil, G. S. *et al.* Uncoupling of obesity from insulin resistance through a targeted mutation in aP2, the adipocyte fatty acid binding protein. *Science* **274**, 1377-1379 (1996).

75 Chen, G. & Goeddel, D. V. TNF-R1 signaling: a beautiful pathway. *Science* **296**, 1634-1635, doi:10.1126/science.1071924 (2002).

76 Weber, A., Wasiliew, P. & Kracht, M. Interleukin-1 (IL-1) pathway. *Science signaling* **3**, cm1, doi:10.1126/scisignal.3105cm1 (2010).

77 Aguirre, V., Uchida, T., Yenush, L., Davis, R. & White, M. F. The c-Jun NH(2)-terminal kinase promotes insulin resistance during association with insulin receptor substrate-1 and phosphorylation of Ser(307). *The Journal of biological chemistry* **275**, 9047-9054 (2000).

78 Aguirre, V. *et al.* Phosphorylation of Ser307 in insulin receptor substrate-1 blocks interactions with the insulin receptor and inhibits insulin action. *The Journal of biological chemistry* **277**, 1531-1537, doi:10.1074/jbc.M101521200 (2002).

79 Hirosumi, J. *et al.* A central role for JNK in obesity and insulin resistance. *Nature* **420**, 333-336, doi:10.1038/nature01137 (2002).

80 Han, M. S. *et al.* JNK expression by macrophages promotes obesity-induced insulin resistance and inflammation. *Science* **339**, 218-222, doi:10.1126/science.1227568 (2013).

81 Yuan, M. *et al.* Reversal of obesity- and diet-induced insulin resistance with salicylates or targeted disruption of Ikkbeta. *Science* **293**, 1673-1677, doi:10.1126/science.1061620
293/5535/1673 [pii] (2001).

82 Cai, D. *et al.* Local and systemic insulin resistance resulting from hepatic activation of IKK-beta and NF-kappaB. *Nature medicine* **11**, 183-190, doi:10.1038/nm1166 (2005).

83 Arkan, M. C. *et al.* IKK-beta links inflammation to obesity-induced insulin resistance. *Nature medicine* **11**, 191-198, doi:10.1038/nm1185 (2005).

84 Barnes, P. J. & Karin, M. Nuclear factor-kappaB: a pivotal transcription factor in chronic inflammatory diseases. *The New England journal of medicine* **336**, 1066-1071, doi:10.1056/NEJM199704103361506 (1997).

85 Chen, L. W. *et al.* The two faces of IKK and NF-kappaB inhibition: prevention of systemic inflammation but increased local injury following intestinal ischemia-reperfusion. *Nature medicine* **9**, 575-581, doi:10.1038/nm849 (2003).

86 Diraison, F., Dusserre, E., Vidal, H., Sothier, M. & Beylot, M. Increased hepatic lipogenesis but decreased expression of lipogenic gene in adipose tissue in human

obesity. *American journal of physiology. Endocrinology and metabolism* **282**, E46-51 (2002).

87 Lan, H. *et al.* Gene expression profiles of nondiabetic and diabetic obese mice suggest a role of hepatic lipogenic capacity in diabetes susceptibility. *Diabetes* **52**, 688-700 (2003).

88 Nadler, S. T. *et al.* The expression of adipogenic genes is decreased in obesity and diabetes mellitus. *Proceedings of the National Academy of Sciences of the United States of America* **97**, 11371-11376, doi:10.1073/pnas.97.21.11371 (2000).

89 Imai, T. *et al.* Peroxisome proliferator-activated receptor gamma is required in mature white and brown adipocytes for their survival in the mouse. *Proceedings of the National Academy of Sciences of the United States of America* **101**, 4543-4547, doi:10.1073/pnas.0400356101 (2004).

90 Tamori, Y., Masugi, J., Nishino, N. & Kasuga, M. Role of peroxisome proliferator-activated receptor-gamma in maintenance of the characteristics of mature 3T3-L1 adipocytes. *Diabetes* **51**, 2045-2055 (2002).

91 Ye, J. Regulation of PPARgamma function by TNF-alpha. *Biochemical and biophysical research communications* **374**, 405-408, doi:10.1016/j.bbrc.2008.07.068 (2008).

92 Zhang, B. *et al.* Negative regulation of peroxisome proliferator-activated receptor-gamma gene expression contributes to the antiadipogenic effects of tumor necrosis factor-alpha. *Mol Endocrinol* **10**, 1457-1466 (1996).

93 Suzawa, M. *et al.* Cytokines suppress adipogenesis and PPAR-gamma function through the TAK1/TAB1/NIK cascade. *Nature cell biology* **5**, 224-230, doi:10.1038/ncb942 (2003).

94 Xing, H. *et al.* TNF alpha-mediated inhibition and reversal of adipocyte differentiation is accompanied by suppressed expression of PPARgamma without effects on Pref-1 expression. *Endocrinology* **138**, 2776-2783 (1997).

95 Wu, D. *et al.* Eosinophils sustain adipose alternatively activated macrophages associated with glucose homeostasis. *Science* **332**, 243-247, doi:science.1201475 [pii]
10.1126/science.1201475 (2011).

96 Feuerer, M. *et al.* Lean, but not obese, fat is enriched for a unique population of regulatory T cells that affect metabolic parameters. *Nature medicine* **15**, 930-939, doi:10.1038/nm.2002 (2009).

97 Nishimura, S. *et al.* CD8+ effector T cells contribute to macrophage recruitment and adipose tissue inflammation in obesity. *Nature medicine* **15**, 914-920, doi:nm.1964 [pii]
10.1038/nm.1964 (2009).

98 Winer, S. *et al.* Normalization of obesity-associated insulin resistance through immunotherapy. *Nature medicine* **15**, 921-929, doi:10.1038/nm.2001 (2009).

99 Ohmura, K. *et al.* Natural killer T cells are involved in adipose tissues inflammation and glucose intolerance in diet-induced obese mice. *Arteriosclerosis, thrombosis, and vascular biology* **30**, 193-199, doi:10.1161/ATVBAHA.109.198614 (2010).

100 Schipper, H. S. *et al.* Natural killer T cells in adipose tissue prevent insulin resistance. *The Journal of clinical investigation* **122**, 3343-3354, doi:10.1172/JCI62739 (2012).
101 Wu, L. *et al.* Activation of invariant natural killer T cells by lipid excess promotes tissue inflammation, insulin resistance, and hepatic steatosis in obese mice. *Proceedings of the National Academy of Sciences of the United States of America* **109**, E1143-1152, doi:10.1073/pnas.1200498109 (2012).
102 Mantell, B. S. *et al.* Mice lacking NKT cells but with a complete complement of CD8+ T-cells are not protected against the metabolic abnormalities of diet-induced obesity. *PloS one* **6**, e19831, doi:10.1371/journal.pone.0019831 (2011).
103 Satoh, M. *et al.* Type II NKT cells stimulate diet-induced obesity by mediating adipose tissue inflammation, steatohepatitis and insulin resistance. *PloS one* **7**, e30568, doi:10.1371/journal.pone.0030568 (2012).
104 Liu, J. *et al.* Genetic deficiency and pharmacological stabilization of mast cells reduce diet-induced obesity and diabetes in mice. *Nature medicine* **15**, 940-945, doi:10.1038/nm.1994 (2009).
105 Xu, H. *et al.* Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance. *The Journal of clinical investigation* **112**, 1821-1830, doi:10.1172/JCI19451
112/12/1821 [pii] (2003).
106 Cinti, S. *et al.* Adipocyte death defines macrophage localization and function in adipose tissue of obese mice and humans. *Journal of lipid research* **46**, 2347-2355, doi:10.1194/jlr.M500294-JLR200 (2005).
107 Murano, I. *et al.* Dead adipocytes, detected as crown-like structures, are prevalent in visceral fat depots of genetically obese mice. *Journal of lipid research* **49**, 1562-1568, doi:10.1194/jlr.M800019-JLR200 (2008).
108 Weisberg, S. P. *et al.* CCR2 modulates inflammatory and metabolic effects of high-fat feeding. *The Journal of clinical investigation* **116**, 115-124, doi:10.1172/JCI24335 (2006).
109 Hardy, O. T. *et al.* Body mass index-independent inflammation in omental adipose tissue associated with insulin resistance in morbid obesity. *Surgery for obesity and related diseases : official journal of the American Society for Bariatric Surgery* **7**, 60-67, doi:10.1016/j.soard.2010.05.013 (2011).
110 Cancello, R. *et al.* Reduction of macrophage infiltration and chemoattractant gene expression changes in white adipose tissue of morbidly obese subjects after surgery-induced weight loss. *Diabetes* **54**, 2277-2286 (2005).
111 Bruun, J. M., Helge, J. W., Richelsen, B. & Stallknecht, B. Diet and exercise reduce low-grade inflammation and macrophage infiltration in adipose tissue but not in skeletal muscle in severely obese subjects. *American journal of physiology. Endocrinology and metabolism* **290**, E961-967, doi:00506.2005 [pii]
10.1152/ajpendo.00506.2005 (2006).
112 Patsouris, D. *et al.* Ablation of CD11c-positive cells normalizes insulin sensitivity in obese insulin resistant animals. *Cell metabolism* **8**, 301-309, doi:S1550-4131(08)00282-9 [pii]

10.1016/j.cmet.2008.08.015 (2008).
113 Feng, B. *et al.* Clodronate liposomes improve metabolic profile and reduce visceral adipose macrophage content in diet-induced obese mice. *PloS one* **6**, e24358, doi:10.1371/journal.pone.0024358
PONE-D-11-09806 [pii] (2011).
114 Feral, C. C. *et al.* Blocking the alpha 4 integrin-paxillin interaction selectively impairs mononuclear leukocyte recruitment to an inflammatory site. *The Journal of clinical investigation* **116**, 715-723, doi:10.1172/JCI26091 (2006).
115 Kanda, H. *et al.* MCP-1 contributes to macrophage infiltration into adipose tissue, insulin resistance, and hepatic steatosis in obesity. *The Journal of clinical investigation* **116**, 1494-1505, doi:10.1172/JCI26498 (2006).
116 Kamei, N. *et al.* Overexpression of monocyte chemoattractant protein-1 in adipose tissues causes macrophage recruitment and insulin resistance. *The Journal of biological chemistry* **281**, 26602-26614, doi:M601284200 [pii]
10.1074/jbc.M601284200 (2006).
117 Lumeng, C. N., Bodzin, J. L. & Saltiel, A. R. Obesity induces a phenotypic switch in adipose tissue macrophage polarization. *The Journal of clinical investigation* **117**, 175-184, doi:10.1172/JCI29881 (2007).
118 Lumeng, C. N., Deyoung, S. M., Bodzin, J. L. & Saltiel, A. R. Increased inflammatory properties of adipose tissue macrophages recruited during diet-induced obesity. *Diabetes* **56**, 16-23, doi:10.2337/db06-1076 (2007).
119 Gordon, S. & Taylor, P. R. Monocyte and macrophage heterogeneity. *Nature reviews. Immunology* **5**, 953-964, doi:10.1038/nri1733 (2005).
120 Gordon, S. Alternative activation of macrophages. *Nature reviews. Immunology* **3**, 23-35, doi:10.1038/nri978 (2003).
121 Wentworth, J. M. *et al.* Pro-inflammatory CD11c+CD206+ adipose tissue macrophages are associated with insulin resistance in human obesity. *Diabetes* **59**, 1648-1656, doi:db09-0287 [pii]
10.2337/db09-0287 (2010).
122 Odegaard, J. I. *et al.* Macrophage-specific PPARgamma controls alternative activation and improves insulin resistance. *Nature* **447**, 1116-1120, doi:10.1038/nature05894 (2007).
123 Solinas, G. *et al.* JNK1 in hematopoietically derived cells contributes to diet-induced inflammation and insulin resistance without affecting obesity. *Cell metabolism* **6**, 386-397, doi:S1550-4131(07)00292-6 [pii]
10.1016/j.cmet.2007.09.011 (2007).
124 Aouadi, M. *et al.* Silencing genes in adipose tissue macrophages regulates whole body metabolism in obese mice. *PNAS* **In press** (2013).
125 Kosteli, A. *et al.* Weight loss and lipolysis promote a dynamic immune response in murine adipose tissue. *The Journal of clinical investigation* **120**, 3466-3479, doi:10.1172/JCI42845
42845 [pii] (2010).

126 Prieur, X. *et al.* Differential lipid partitioning between adipocytes and tissue macrophages modulates macrophage lipotoxicity and M2/M1 polarization in obese mice. *Diabetes* **60**, 797-809, doi:db10-0705 [pii]
10.2337/db10-0705 (2011).
127 Geissmann, F. *et al.* Development of monocytes, macrophages, and dendritic cells. *Science* **327**, 656-661, doi:10.1126/science.1178331 (2010).
128 Bruun, J. M., Lihn, A. S., Pedersen, S. B. & Richelsen, B. Monocyte chemoattractant protein-1 release is higher in visceral than subcutaneous human adipose tissue (AT): implication of macrophages resident in the AT. *The Journal of clinical endocrinology and metabolism* **90**, 2282-2289, doi:jc.2004-1696 [pii]
10.1210/jc.2004-1696 (2005).
129 Sartipy, P. & Loskutoff, D. J. Monocyte chemoattractant protein 1 in obesity and insulin resistance. *Proceedings of the National Academy of Sciences of the United States of America* **100**, 7265-7270, doi:10.1073/pnas.1133870100
1133870100 [pii] (2003).
130 Chen, A. *et al.* Diet induction of monocyte chemoattractant protein-1 and its impact on obesity. *Obesity research* **13**, 1311-1320, doi:10.1038/oby.2005.159 (2005).
131 Inouye, K. E. *et al.* Absence of CC chemokine ligand 2 does not limit obesity-associated infiltration of macrophages into adipose tissue. *Diabetes* **56**, 2242-2250, doi:db07-0425 [pii]
10.2337/db07-0425 (2007).
132 Kirk, E. A., Sagawa, Z. K., McDonald, T. O., O'Brien, K. D. & Heinecke, J. W. Monocyte chemoattractant protein deficiency fails to restrain macrophage infiltration into adipose tissue [corrected]. *Diabetes* **57**, 1254-1261, doi:db07-1061 [pii]
10.2337/db07-1061 (2008).
133 Kiefer, F. W. *et al.* Neutralization of osteopontin inhibits obesity-induced inflammation and insulin resistance. *Diabetes* **59**, 935-946, doi:db09-0404 [pii]
10.2337/db09-0404 (2010).
134 Nomiyama, T. *et al.* Osteopontin mediates obesity-induced adipose tissue macrophage infiltration and insulin resistance in mice. *The Journal of clinical investigation* **117**, 2877-2888, doi:10.1172/JCI31986 (2007).
135 Lesniewski, L. A. *et al.* Bone marrow-specific Cap gene deletion protects against high-fat diet-induced insulin resistance. *Nature medicine* **13**, 455-462, doi:nm1550 [pii]
10.1038/nm1550 (2007).
136 Murphy, J., Summer, R., Wilson, A. A., Kotton, D. N. & Fine, A. The prolonged life-span of alveolar macrophages. *American journal of respiratory cell and molecular biology* **38**, 380-385, doi:10.1165/rcmb.2007-0224RC (2008).
137 Roth Flach, R. J. *et al.* beta3-Adrenergic receptor stimulation induces E-selectin-mediated adipose tissue inflammation. *The Journal of biological chemistry* **288**, 2882-2892, doi:10.1074/jbc.M112.412346 (2013).

138 Asterholm, I. W. *et al.* Lack of "immunological fitness" during fasting in metabolically challenged animals. *Journal of lipid research* **53**, 1254-1267, doi:10.1194/jlr.M021725 (2012).

139 Oh, D. Y., Morinaga, H., Talukdar, S., Bae, E. J. & Olefsky, J. M. Increased Macrophage Migration Into Adipose Tissue in Obese Mice. *Diabetes*, doi:db11-0860 [pii]
10.2337/db11-0860 (2011).

140 Tacke, F. & Randolph, G. J. Migratory fate and differentiation of blood monocyte subsets. *Immunobiology* **211**, 609-618, doi:10.1016/j.imbio.2006.05.025 (2006).

141 Sawyer, R. T., Strausbauch, P. H. & Volkman, A. Resident macrophage proliferation in mice depleted of blood monocytes by strontium-89. *Laboratory investigation; a journal of technical methods and pathology* **46**, 165-170 (1982).

142 Pecher, C. Biological investigations with radioactive calcium and strontium. *Exp Biol Med* **46**, 86-91, doi: 10.3181/00379727-46-11899 (1941).

143 Godleski, J. J. & Brain, J. D. The origin of alveolar macrophages in mouse radiation chimeras. *The Journal of experimental medicine* **136**, 630-643 (1972).

144 van oud Alblas, A. B. & van Furth, R. Origin, Kinetics, and characteristics of pulmonary macrophages in the normal steady state. *The Journal of experimental medicine* **149**, 1504-1518 (1979).

145 Brunstetter, M. A., Hardie, J. A., Schiff, R., Lewis, J. P. & Cross, C. E. The origin of pulmonary alveolar macrophages. Studies of stem cells using the Es-2 marker of mice. *Archives of internal medicine* **127**, 1064-1068 (1971).

146 Thomas, E. D., Ramberg, R. E., Sale, G. E., Sparkes, R. S. & Golde, D. W. Direct evidence for a bone marrow origin of the alveolar macrophage in man. *Science* **192**, 1016-1018 (1976).

147 Tarling, J. D., Lin, H. S. & Hsu, S. Self-renewal of pulmonary alveolar macrophages: evidence from radiation chimera studies. *Journal of leukocyte biology* **42**, 443-446 (1987).

148 Sawyer, R. T. The ontogeny of pulmonary alveolar macrophages in parabiotic mice. *Journal of leukocyte biology* **40**, 347-354 (1986).

149 Nakata, K. *et al.* Augmented proliferation of human alveolar macrophages after allogeneic bone marrow transplantation. *Blood* **93**, 667-673 (1999).

150 Evans, M. J., Shami, S. G. & Martinez, L. A. Enhanced proliferation of pulmonary alveolar macrophages after carbon instillation in mice depleted of blood monocytes by strontium-89. *Laboratory investigation; a journal of technical methods and pathology* **54**, 154-159 (1986).

151 Jenkins, S. J. *et al.* Local macrophage proliferation, rather than recruitment from the blood, is a signature of TH2 inflammation. *Science* **332**, 1284-1288, doi:science.1204351 [pii]
10.1126/science.1204351 (2011).

152 van Furth, R., Nibbering, P. H., van Dissel, J. T. & Diesselhoff-den Dulk, M. M. The characterization, origin, and kinetics of skin macrophages during inflammation. *The Journal of investigative dermatology* **85**, 398-402 (1985).

153 Merad, M. *et al.* Langerhans cells renew in the skin throughout life under steady-state conditions. *Nature immunology* **3**, 1135-1141, doi:10.1038/ni852 ni852 [pii] (2002).

154 Ginhoux, F. *et al.* Langerhans cells arise from monocytes in vivo. *Nature immunology* **7**, 265-273, doi:10.1038/ni1307 (2006).

155 Kanitakis, J., Petruzzo, P. & Dubernard, J. M. Turnover of epidermal Langerhans' cells. *The New England journal of medicine* **351**, 2661-2662, doi:10.1056/NEJM200412163512523 (2004).

156 Gale, R. P., Sparkes, R. S. & Golde, D. W. Bone marrow origin of hepatic macrophages (Kupffer cells) in humans. *Science* **201**, 937-938 (1978).

157 Edwards, J. L. & Klein, R. E. Cell renewal in adult mouse tissues. *The American journal of pathology* **38**, 437-453 (1961).

158 Kelly, L. S., Brown, B. A. & Dobson, E. L. Cell division and phagocytic activity in liver reticulo-endothelial cells. *Proc Soc Exp Biol Med* **110**, 555-559 (1962).

159 Kelly, L. S. & Dobson, E. L. Evidence concerning the origin of liver macrophages. *British journal of experimental pathology* **52**, 88-99 (1971).

160 North, R. J. The mitotic potential of fixed phagocytes in the liver as revealed during the development of cellular immunity. *The Journal of experimental medicine* **130**, 315-326 (1969).

161 Crofton, R. W., Diesselhoff-den Dulk, M. M. & van Furth, R. The origin, kinetics, and characteristics of the Kupffer cells in the normal steady state. *The Journal of experimental medicine* **148**, 1-17 (1978).

162 Bouwens, L., Baekeland, M., De Zanger, R. & Wisse, E. Quantitation, tissue distribution and proliferation kinetics of Kupffer cells in normal rat liver. *Hepatology* **6**, 718-722 (1986).

163 Bouwens, L., Baekeland, M. & Wisse, E. Importance of local proliferation in the expanding Kupffer cell population of rat liver after zymosan stimulation and partial hepatectomy. *Hepatology* **4**, 213-219 (1984).

164 Bouwens, L., Knook, D. L. & Wisse, E. Local proliferation and extrahepatic recruitment of liver macrophages (Kupffer cells) in partial-body irradiated rats. *Journal of leukocyte biology* **39**, 687-697 (1986).

165 Johnson, S. J., Hines, J. E. & Burt, A. D. Macrophage and perisinusoidal cell kinetics in acute liver injury. *The Journal of pathology* **166**, 351-358, doi:10.1002/path.1711660406 (1992).

166 Van Goor, H. *et al.* Macrophages and renal disease. *Laboratory investigation; a journal of technical methods and pathology* **71**, 456-464 (1994).

167 Lan, H. Y., Nikolic-Paterson, D. J., Mu, W. & Atkins, R. C. Local macrophage proliferation in the progression of glomerular and tubulointerstitial injury in rat anti-GBM glomerulonephritis. *Kidney international* **48**, 753-760 (1995).

168 Lan, H. Y., Nikolic-Paterson, D. J., Mu, W. & Atkins, R. C. Local macrophage proliferation in the pathogenesis of glomerular crescent formation in rat anti-glomerular basement membrane (GBM) glomerulonephritis. *Clinical and experimental immunology* **110**, 233-240 (1997).

169 Yang, N. *et al.* Local macrophage proliferation in human glomerulonephritis. *Kidney international* **54**, 143-151, doi:10.1046/j.1523-1755.1998.00978.x (1998).
170 Yang, N. *et al.* Local macrophage and myofibroblast proliferation in progressive renal injury in the rat remnant kidney. *Nephrology, dialysis, transplantation : official publication of the European Dialysis and Transplant Association - European Renal Association* **13**, 1967-1974 (1998).
171 Chow, F., Ozols, E., Nikolic-Paterson, D. J., Atkins, R. C. & Tesch, G. H. Macrophages in mouse type 2 diabetic nephropathy: correlation with diabetic state and progressive renal injury. *Kidney international* **65**, 116-128, doi:10.1111/j.1523-1755.2004.00367.x (2004).
172 Le Meur, Y., Jose, M. D., Mu, W., Atkins, R. C. & Chadban, S. J. Macrophage colony-stimulating factor expression and macrophage accumulation in renal allograft rejection. *Transplantation* **73**, 1318-1324 (2002).
173 Moore, K. J. & Tabas, I. Macrophages in the pathogenesis of atherosclerosis. *Cell* **145**, 341-355, doi:10.1016/j.cell.2011.04.005 (2011).
174 Andres, V., Pello, O. M. & Silvestre-Roig, C. Macrophage proliferation and apoptosis in atherosclerosis. *Current opinion in lipidology* **23**, 429-438, doi:10.1097/MOL.0b013e328357a379 (2012).
175 Fuster, J. J. *et al.* Control of cell proliferation in atherosclerosis: insights from animal models and human studies. *Cardiovascular research* **86**, 254-264, doi:10.1093/cvr/cvp363 (2010).
176 Rosenfeld, M. E. & Ross, R. Macrophage and smooth muscle cell proliferation in atherosclerotic lesions of WHHL and comparably hypercholesterolemic fat-fed rabbits. *Arteriosclerosis* **10**, 680-687 (1990).
177 Spagnoli, L. G., Orlandi, A. & Santeusanio, G. Foam cells of the rabbit atherosclerotic plaque arrested in metaphase by colchicine show a macrophage phenotype. *Atherosclerosis* **88**, 87-92 (1991).
178 Gordon, D., Reidy, M. A., Benditt, E. P. & Schwartz, S. M. Cell proliferation in human coronary arteries. *Proceedings of the National Academy of Sciences of the United States of America* **87**, 4600-4604 (1990).
179 Rekhter, M. D. & Gordon, D. Active proliferation of different cell types, including lymphocytes, in human atherosclerotic plaques. *The American journal of pathology* **147**, 668-677 (1995).
180 Boesten, L. S. *et al.* Macrophage retinoblastoma deficiency leads to enhanced atherosclerosis development in ApoE-deficient mice. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* **20**, 953-955, doi:10.1096/fj.05-4530fje (2006).
181 Diez-Juan, A. *et al.* Selective inactivation of p27(Kip1) in hematopoietic progenitor cells increases neointimal macrophage proliferation and accelerates atherosclerosis. *Blood* **103**, 158-161, doi:10.1182/blood-2003-07-2319 (2004).
182 Kuo, C. L. *et al.* Cdkn2a is an atherosclerosis modifier locus that regulates monocyte/macrophage proliferation. *Arteriosclerosis, thrombosis, and vascular biology* **31**, 2483-2492, doi:10.1161/ATVBAHA.111.234492 (2011).

183 Guevara, N. V., Kim, H. S., Antonova, E. I. & Chan, L. The absence of p53 accelerates atherosclerosis by increasing cell proliferation in vivo. *Nature medicine* **5**, 335-339, doi:10.1038/6585 (1999).

184 Mercer, J., Figg, N., Stoneman, V., Braganza, D. & Bennett, M. R. Endogenous p53 protects vascular smooth muscle cells from apoptosis and reduces atherosclerosis in ApoE knockout mice. *Circulation research* **96**, 667-674, doi:10.1161/01.RES.0000161069.15577.ca (2005).

185 Merched, A. J., Williams, E. & Chan, L. Macrophage-specific p53 expression plays a crucial role in atherosclerosis development and plaque remodeling. *Arteriosclerosis, thrombosis, and vascular biology* **23**, 1608-1614, doi:10.1161/01.ATV.0000084825.88022.53 (2003).

186 Boesten, L. S. *et al.* Macrophage p53 controls macrophage death in atherosclerotic lesions of apolipoprotein E deficient mice. *Atherosclerosis* **207**, 399-404, doi:10.1016/j.atherosclerosis.2009.06.015 (2009).

187 van Vlijmen, B. J. *et al.* Macrophage p53 deficiency leads to enhanced atherosclerosis in APOE*3-Leiden transgenic mice. *Circulation research* **88**, 780-786 (2001).

188 van Furth, R. & Diesselhoff-den Dulk, M. M. Dual origin of mouse spleen macrophages. *The Journal of experimental medicine* **160**, 1273-1283 (1984).

189 Wijffels, J. F., de Rover, Z., Beelen, R. H., Kraal, G. & van Rooijen, N. Macrophage subpopulations in the mouse spleen renewed by local proliferation. *Immunobiology* **191**, 52-64 (1994).

190 Ajami, B., Bennett, J. L., Krieger, C., Tetzlaff, W. & Rossi, F. M. Local self-renewal can sustain CNS microglia maintenance and function throughout adult life. *Nature neuroscience* **10**, 1538-1543, doi:10.1038/nn2014 (2007).

191 de Groot, C. J., Huppes, W., Sminia, T., Kraal, G. & Dijkstra, C. D. Determination of the origin and nature of brain macrophages and microglial cells in mouse central nervous system, using non-radioactive in situ hybridization and immunoperoxidase techniques. *Glia* **6**, 301-309, doi:10.1002/glia.440060408 (1992).

192 Lawson, L. J., Perry, V. H. & Gordon, S. Turnover of resident microglia in the normal adult mouse brain. *Neuroscience* **48**, 405-415 (1992).

193 Lin, H. & Stewart, C. C. Colony formation by mouse peritoneal exudate cells in vitro. *Nature: New biology* **243**, 176-177 (1973).

194 Stewart, C. C., Lin, H. S. & Adles, C. Proliferation and colony-forming ability of peritoneal exudate cells in liquid culture. *The Journal of experimental medicine* **141**, 1114-1132 (1975).

195 van der Zeijst, B. A., Stewart, C. C. & Schlesinger, S. Proliferative capacity of mouse peritoneal macrophages in vitro. *The Journal of experimental medicine* **147**, 1253-1266 (1978).

196 Bischof, R. J., Zafiropoulos, D., Hamilton, J. A. & Campbell, I. K. Exacerbation of acute inflammatory arthritis by the colony-stimulating factors CSF-1 and granulocyte macrophage (GM)-CSF: evidence of macrophage infiltration and local proliferation. *Clinical and experimental immunology* **119**, 361-367 (2000).

197 Hamilton, J. A. & Tak, P. P. The dynamics of macrophage lineage populations in inflammatory and autoimmune diseases. *Arthritis and rheumatism* **60**, 1210-1221, doi:10.1002/art.24505 (2009).

198 Dougherty, S. T., Eaves, C. J., McBride, W. H. & Dougherty, G. J. Role of macrophage-colony-stimulating factor in regulating the accumulation and phenotype of tumor-associated macrophages. *Cancer immunology, immunotherapy : CII* **44**, 165-172 (1997).

199 Evans, R. & Cullen, R. T. In situ proliferation of intratumor macrophages. *Journal of leukocyte biology* **35**, 561-572 (1984).

200 Mahoney, K. H. & Heppner, G. H. FACS analysis of tumor-associated macrophage replication: differences between metastatic and nonmetastatic murine mammary tumors. *Journal of leukocyte biology* **41**, 205-211 (1987).

201 Stewart, C. C. Local proliferation of mononuclear phagocytes in tumors. *Journal of the Reticuloendothelial Society* **34**, 23-27 (1983).

202 Campbell, M. J. *et al.* Proliferating macrophages associated with high grade, hormone receptor negative breast cancer and poor clinical outcome. *Breast cancer research and treatment* **128**, 703-711, doi:10.1007/s10549-010-1154-y (2011).

203 Mukhtar, R. A. *et al.* Elevated levels of proliferating and recently migrated tumor-associated macrophages confer increased aggressiveness and worse outcomes in breast cancer. *Annals of surgical oncology* **19**, 3979-3986, doi:10.1245/s10434-012-2415-2 (2012).

204 Chen, L., Magliano, D. J. & Zimmet, P. Z. The worldwide epidemiology of type 2 diabetes mellitus-present and future perspectives. *Nat Rev Endocrinol*, doi:10.1038/nrendo.2011.183
nrendo.2011.183 [pii] (2011).

205 Barrett-Connor, E. Epidemiology, obesity, and non-insulin-dependent diabetes mellitus. *Epidemiologic reviews* **11**, 172-181 (1989).

206 Samuel, V. T., Petersen, K. F. & Shulman, G. I. Lipid-induced insulin resistance: unravelling the mechanism. *Lancet* **375**, 2267-2277, doi:10.1016/S0140-6736(10)60408-4 (2010).

207 Zierath, J. R. & Wallberg-Henriksson, H. From receptor to effector: insulin signal transduction in skeletal muscle from type II diabetic patients. *Annals of the New York Academy of Sciences* **967**, 120-134 (2002).

208 Hardy, O. T., Czech, M. P. & Corvera, S. What causes the insulin resistance underlying obesity? *Current opinion in endocrinology, diabetes, and obesity* **19**, 81-87, doi:10.1097/MED.0b013e3283514e13 (2012).

209 Kusminski, C. M., Shetty, S., Orci, L., Unger, R. H. & Scherer, P. E. Diabetes and apoptosis: lipotoxicity. *Apoptosis : an international journal on programmed cell death* **14**, 1484-1495, doi:10.1007/s10495-009-0352-8 (2009).

210 Lara-Castro, C. & Garvey, W. T. Intracellular lipid accumulation in liver and muscle and the insulin resistance syndrome. *Endocrinology and metabolism clinics of North America* **37**, 841-856, doi:10.1016/j.ecl.2008.09.002 (2008).

211 Lettner, A. & Roden, M. Ectopic fat and insulin resistance. *Current diabetes reports* **8**, 185-191 (2008).

212 McLaughlin, T., Lamendola, C., Liu, A. & Abbasi, F. Preferential fat deposition in subcutaneous versus visceral depots is associated with insulin sensitivity. *The Journal of clinical endocrinology and metabolism* **96**, E1756-1760, doi:10.1210/jc.2011-0615 (2011).

213 Preis, S. R. *et al.* Abdominal subcutaneous and visceral adipose tissue and insulin resistance in the Framingham heart study. *Obesity (Silver Spring)* **18**, 2191-2198, doi:10.1038/oby.2010.59 (2010).

214 Osborn, O. & Olefsky, J. M. The cellular and signaling networks linking the immune system and metabolism in disease. *Nature medicine* **18**, 363-374, doi:10.1038/nm.2627 (2012).

215 Romeo, G. R., Lee, J. & Shoelson, S. E. Metabolic syndrome, insulin resistance, and roles of inflammation - mechanisms and therapeutic targets. *Arteriosclerosis, thrombosis, and vascular biology* **32**, 1771-1776, doi:32/8/1771 [pii]
10.1161/ATVBAHA.111.241869 (2012).

216 Suganami, T., Tanaka, M. & Ogawa, Y. Adipose tissue inflammation and ectopic lipid accumulation [Review]. *Endocr J*, doi:DN/JST.JSTAGE/endocrj/EJ12-0271 [pii] (2012).

217 Goldfine, A. B. *et al.* Use of salsalate to target inflammation in the treatment of insulin resistance and type 2 diabetes. *Clin Transl Sci* **1**, 36-43, doi:10.1111/j.1752-8062.2008.00026.x (2008).

218 Lumeng, C. N. & Saltiel, A. R. Inflammatory links between obesity and metabolic disease. *The Journal of clinical investigation* **121**, 2111-2117, doi:10.1172/JCI57132
57132 [pii] (2011).

219 Bluher, M. The distinction of metabolically 'healthy' from 'unhealthy' obese individuals. *Current opinion in lipidology* **21**, 38-43, doi:10.1097/MOL.0b013e3283346ccc (2010).

220 Elbein, S. C. *et al.* Global gene expression profiles of subcutaneous adipose and muscle from glucose-tolerant, insulin-sensitive, and insulin-resistant individuals matched for BMI. *Diabetes* **60**, 1019-1029, doi:db10-1270 [pii]
10.2337/db10-1270 (2011).

221 Gauthier, M. S. & Ruderman, N. B. Adipose tissue inflammation and insulin resistance: all obese humans are not created equal. *Biochem J* **430**, e1-4, doi:BJ20101062 [pii]
10.1042/BJ20101062 (2010).

222 Daems, W. T. & de Bakker, J. M. Do resident macrophages proliferate? *Immunobiology* **161**, 204-211 (1982).

223 Gordon, S. & Mantovani, A. Diversity and plasticity of mononuclear phagocytes. *Eur J Immunol* **41**, 2470-2472, doi:10.1002/eji.201141988 (2011).

224 Hume, D. A. *et al.* The mononuclear phagocyte system revisited. *Journal of leukocyte biology* **72**, 621-627 (2002).

225 Chorro, L. *et al.* Langerhans cell (LC) proliferation mediates neonatal development, homeostasis, and inflammation-associated expansion of the

epidermal LC network. *The Journal of experimental medicine* **206**, 3089-3100, doi:jem.20091586 [pii]
10.1084/jem.20091586 (2009).
226 Scholzen, T. & Gerdes, J. The Ki-67 protein: from the known and the unknown. *J Cell Physiol* **182**, 311-322, doi:10.1002/(SICI)1097-4652(200003)182:3<311::AID-JCP1>3.0.CO;2-9 [pii]
10.1002/(SICI)1097-4652(200003)182:3<311::AID-JCP1>3.0.CO;2-9 (2000).
227 Talukdar, S. *et al.* Neutrophils mediate insulin resistance in mice fed a high-fat diet through secreted elastase. *Nature medicine*, doi:10.1038/nm.2885
nm.2885 [pii] (2012).
228 Leenen, P. J. *et al.* Heterogeneity of mouse spleen dendritic cells: in vivo phagocytic activity, expression of macrophage markers, and subpopulation turnover. *J Immunol* **160**, 2166-2173 (1998).
229 Granneman, J. G., Li, P., Zhu, Z. & Lu, Y. Metabolic and cellular plasticity in white adipose tissue I: effects of beta3-adrenergic receptor activation. *American journal of physiology. Endocrinology and metabolism* **289**, E608-616, doi:00009.2005 [pii]
10.1152/ajpendo.00009.2005 (2005).
230 Himms-Hagen, J. *et al.* Multilocular fat cells in WAT of CL-316243-treated rats derive directly from white adipocytes. *Am J Physiol Cell Physiol* **279**, C670-681 (2000).
231 Mottillo, E. P., Shen, X. J. & Granneman, J. G. beta3-adrenergic receptor induction of adipocyte inflammation requires lipolytic activation of stress kinases p38 and JNK. *Biochim Biophys Acta* **1801**, 1048-1055, doi:S1388-1981(10)00091-0 [pii]
10.1016/j.bbalip.2010.04.012 (2010).
232 Bjornson, B. H., Harvey, J. M. & Rose, L. Differential effect of hydrocortisone on eosinophil and neutrophil proliferation. *The Journal of clinical investigation* **76**, 924-929, doi:10.1172/JCI112091 (1985).
233 Yang, J. P. & Renzi, P. M. Interleukin-2 and lymphocyte-induced eosinophil proliferation and survival in asthmatic patients. *J Allergy Clin Immunol* **91**, 792-801, doi:0091-6749(93)90199-P [pii] (1993).
234 Nieves, D. & Moreno, J. J. Effect of arachidonic and eicosapentaenoic acid metabolism on RAW 264.7 macrophage proliferation. *J Cell Physiol* **208**, 428-434, doi:10.1002/jcp.20678 (2006).
235 Czech, M. P., Aouadi, M. & Tesz, G. J. RNAi-based therapeutic strategies for metabolic disease. *Nat Rev Endocrinol* **7**, 473-484, doi:10.1038/nrendo.2011.57
nrendo.2011.57 [pii] (2011).
236 Fitzgibbons, T. P. *et al.* Similarity of mouse perivascular and brown adipose tissues and their resistance to diet-induced inflammation. *Am J Physiol Heart Circ Physiol* **301**, H1425-1437, doi:ajpheart.00376.2011 [pii]
10.1152/ajpheart.00376.2011 (2011).
237 van Rooijen, N. & van Kesteren-Hendrikx, E. "In vivo" depletion of macrophages by liposome-mediated "suicide". *Methods in enzymology* **373**, 3-16 (2003).

238 Page, D. T. & Garvey, J. S. Isolation and characterization of hepatocytes and Kupffer cells. *J Immunol Methods* **27**, 159-173, doi:0022-1759(79)90262-X [pii] (1979).

239 Stanley, E. *et al.* Granulocyte/macrophage colony-stimulating factor-deficient mice show no major perturbation of hematopoiesis but develop a characteristic pulmonary pathology. *Proceedings of the National Academy of Sciences of the United States of America* **91**, 5592-5596 (1994).

240 Hawgood, S. *et al.* GM-CSF mediates alveolar macrophage proliferation and type II cell hypertrophy in SP-D gene-targeted mice. *American journal of physiology. Lung cellular and molecular physiology* **280**, L1148-1156 (2001).

241 Shibata, Y., Zsengeller, Z., Otake, K., Palaniyar, N. & Trapnell, B. C. Alveolar macrophage deficiency in osteopetrotic mice deficient in macrophage colony-stimulating factor is spontaneously corrected with age and associated with matrix metalloproteinase expression and emphysema. *Blood* **98**, 2845-2852 (2001).

242 Isbel, N. M. *et al.* Tubules are the major site of M-CSF production in experimental kidney disease: correlation with local macrophage proliferation. *Kidney international* **60**, 614-625, doi:10.1046/j.1523-1755.2001.060002614.x (2001).

243 Isbel, N. M., Nikolic-Paterson, D. J., Hill, P. A., Dowling, J. & Atkins, R. C. Local macrophage proliferation correlates with increased renal M-CSF expression in human glomerulonephritis. *Nephrology, dialysis, transplantation : official publication of the European Dialysis and Transplant Association - European Renal Association* **16**, 1638-1647 (2001).

244 Jose, M. D., Le Meur, Y., Atkins, R. C. & Chadban, S. J. Blockade of macrophage colony-stimulating factor reduces macrophage proliferation and accumulation in renal allograft rejection. *American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons* **3**, 294-300 (2003).

245 Biwa, T. *et al.* Induction of murine macrophage growth by oxidized low density lipoprotein is mediated by granulocyte macrophage colony-stimulating factor. *The Journal of biological chemistry* **273**, 28305-28313 (1998).

246 Biwa, T. *et al.* Sites of action of protein kinase C and phosphatidylinositol 3-kinase are distinct in oxidized low density lipoprotein-induced macrophage proliferation. *The Journal of biological chemistry* **275**, 5810-5816 (2000).

247 Hamilton, J. A. *et al.* Oxidized LDL can induce macrophage survival, DNA synthesis, and enhanced proliferative response to CSF-1 and GM-CSF. *Arteriosclerosis, thrombosis, and vascular biology* **19**, 98-105 (1999).

248 Matsumura, T. *et al.* Two intracellular signaling pathways for activation of protein kinase C are involved in oxidized low-density lipoprotein-induced macrophage growth. *Arteriosclerosis, thrombosis, and vascular biology* **17**, 3013-3020 (1997).

249 Rajamannan, N. M. *et al.* Atorvastatin inhibits hypercholesterolemia-induced cellular proliferation and bone matrix production in the rabbit aortic valve. *Circulation* **105**, 2660-2665 (2002).

250 Shiomi, M., Yamada, S. & Ito, T. Atheroma stabilizing effects of simvastatin due to depression of macrophages or lipid accumulation in the atheromatous plaques of coronary plaque-prone WHHL rabbits. *Atherosclerosis* **178**, 287-294, doi:10.1016/j.atherosclerosis.2004.10.024 (2005).

251 Senokuchi, T. *et al.* Statins suppress oxidized low density lipoprotein-induced macrophage proliferation by inactivation of the small G protein-p38 MAPK pathway. *The Journal of biological chemistry* **280**, 6627-6633, doi:10.1074/jbc.M412531200 (2005).

252 Biwa, T., Sakai, M., Shichiri, M. & Horiuchi, S. Granulocyte/macrophage colony-stimulating factor plays an essential role in oxidized low density lipoprotein-induced macrophage proliferation. *Journal of atherosclerosis and thrombosis* **7**, 14-20 (2000).

253 Kim, D. H. *et al.* The role of GM-CSF in adipose tissue inflammation. *American journal of physiology. Endocrinology and metabolism* **295**, E1038-1046, doi:10.1152/ajpendo.00061.2008 (2008).

254 Levine, J. A., Jensen, M. D., Eberhardt, N. L. & O'Brien, T. Adipocyte macrophage colony-stimulating factor is a mediator of adipose tissue growth. *The Journal of clinical investigation* **101**, 1557-1564, doi:10.1172/JCI2293 (1998).

255 Yui, S., Mizuno, D. & Yamazaki, M. Induction of macrophage growth by the lipid moiety of lipoprotein and its augmentation by denaturation of the lipoproteins. *Journal of leukocyte biology* **38**, 697-707 (1985).

256 Yui, S. & Yamazaki, M. Induction of macrophage growth by negatively charged phospholipids. *Journal of leukocyte biology* **39**, 713-716 (1986).

257 Yui, S. & Yamazaki, M. Induction of macrophage growth by lipids. *J Immunol* **136**, 1334-1338 (1986).

258 Yui, S. & Yamazaki, M. Relationship of ability of phospholipids to stimulate growth and bind to macrophages. *Journal of leukocyte biology* **41**, 392-399 (1987).

259 Yui, S. & Yamazaki, M. Neutral lipid accumulation in macrophages during lipid-induced macrophage growth. *Journal of leukocyte biology* **45**, 189-197 (1989).

260 Gangoiti, P. *et al.* Ceramide 1-phosphate stimulates macrophage proliferation through activation of the PI3-kinase/PKB, JNK and ERK1/2 pathways. *Cellular signalling* **20**, 726-736, doi:10.1016/j.cellsig.2007.12.008 (2008).

261 Belda, B. J., Thompson, J. T., Eser, P. O. & Vanden Heuvel, J. P. 10e12z CLA alters adipocyte differentiation and adipocyte cytokine expression and induces macrophage proliferation. *The Journal of nutritional biochemistry* **23**, 510-518, doi:10.1016/j.jnutbio.2011.02.009 (2012).

262 Herrero, L., Shapiro, H., Nayer, A., Lee, J. & Shoelson, S. E. Inflammation and adipose tissue macrophages in lipodystrophic mice. *Proceedings of the National Academy of Sciences of the United States of America* **107**, 240-245, doi:10.1073/pnas.0905310107 (2010).

263 Pajvani, U. B. *et al.* Fat apoptosis through targeted activation of caspase 8: a new mouse model of inducible and reversible lipoatrophy. *Nature medicine* **11**, 797-803, doi:10.1038/nm1262 (2005).

264 Himes, S. R. *et al.* The JNK are important for development and survival of macrophages. *J Immunol* **176**, 2219-2228 (2006).

265 Senokuchi, T. *et al.* Extracellular signal-regulated kinase and p38 mitogen-activated protein kinase mediate macrophage proliferation induced by oxidized low-density lipoprotein. *Atherosclerosis* **176**, 233-245, doi:10.1016/j.atherosclerosis.2004.05.019 (2004).

266 Tomura, M. *et al.* Monitoring cellular movement in vivo with photoconvertible fluorescence protein "Kaede" transgenic mice. *Proceedings of the National Academy of Sciences of the United States of America* **105**, 10871-10876, doi:10.1073/pnas.0802278105 (2008).