Characterization of Immune Cell Populations in White Adipose Tissue of Wild Type and Bovine Growth Hormone Transgenic Mice

A thesis presented to the faculty of the College of Health Sciences and Professions of Ohio University

In partial fulfillment of the requirements for the degree Master of Science

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December 2012

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This thesis titled
Characterization of Immune Cell Populations in White Adipose Tissue of Wild Type and
Bovine Growth Hormone Transgenic Mice

by

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Abstract

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Characterization of Immune Cell Populations in White Adipose Tissue of Wild Type and Bovine Growth Hormone Transgenic Mice

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White adipose tissue (WAT) is a complex tissue composed mostly of adipocytes, but contains other cell types, including a variety of immune cells such as macrophages, T cells, Natural Killer T cells (NK), and depot specific differences in cellular composition and endocrine output. Growth hormone (GH) has been shown to impact immune function as well as adiposity. The immune cell population, within WAT of bGH mice has not previously been assessed. Therefore, the purpose of this study was to characterize the WAT depot specific differences in immune cell populations in bGH mice. Stromal vascular fraction (SVF) was collected from three adipose depots. Total SVF cells were significantly increased in all bGH WAT depots as compared to controls. Flow cytometry was used to examine the specific immune cell populations within the SVF. bGH WAT had greater regulatory T (Treg) cell infiltration most likely due to decreased adiposity. CD45 leukocyte stained populations, which represent the global leukocyte cell population, and CD36-F4/80 positive immune cells, which represent M2 macrophages, were significantly increased in bGH subcutaneous and mesenteric depots compared to controls. In conclusion, the significant decrease in adiposity caused by excess GH leads to marked increases in infiltration of immune cell populations in a depot specific manner as well as increased numbers of other non-immune SVF cells.
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<td>ATM</td>
<td>adipose tissue macrophages</td>
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<tr>
<td>BAT</td>
<td>brown adipose tissue</td>
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<td>bGH</td>
<td>bovine growth hormone transgenic</td>
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<tr>
<td>CCR2</td>
<td>chemokine (c-c motif) receptor 2</td>
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<td>DIO</td>
<td>diet induced obesity</td>
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<tr>
<td>ECW</td>
<td>extracellular water</td>
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<tr>
<td>FACS</td>
<td>fluorescent activated cell sorting</td>
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<td>FFA</td>
<td>free fatty acid</td>
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<td>GH</td>
<td>growth hormone</td>
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<td>GHR</td>
<td>growth hormone receptor</td>
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<td>GHRH</td>
<td>growth hormone releasing hormone</td>
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<tr>
<td>HDL</td>
<td>high density lipoprotein</td>
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<tr>
<td>HF</td>
<td>high fat</td>
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<tr>
<td>hGH</td>
<td>human growth hormone</td>
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<tr>
<td>IGF-1</td>
<td>insulin-like growth factor-1</td>
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<tr>
<td>JAK-2</td>
<td>Janus kinase-2</td>
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<tr>
<td>LDL</td>
<td>low density lipoprotein</td>
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<tr>
<td>LF</td>
<td>low fat</td>
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<td>MDSC</td>
<td>myeloid derived suppressor cells</td>
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<tr>
<td>MIF</td>
<td>migration inhibitory factor</td>
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<td>NK</td>
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<tr>
<td>Acronym</td>
<td>Term</td>
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<td>NMR</td>
<td>nuclear magnetic resonance</td>
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<td>PL</td>
<td>placental lactogen</td>
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<td>PLTP</td>
<td>phospholipid transfer protein</td>
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<td>PRL</td>
<td>prolactin</td>
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<tr>
<td>STAT</td>
<td>signal transducers and activators of transcription</td>
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<td>TAG</td>
<td>triacylglycerol</td>
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<tr>
<td>TBW</td>
<td>total body water</td>
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<tr>
<td>VLDL</td>
<td>very low density lipoprotein</td>
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<td>WAT</td>
<td>white adipose tissue</td>
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<td>wild type</td>
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Chapter 1: Introduction

Adipose tissue is a heterogeneous type of connective tissue providing insulation, mechanical support, and fuel storage for the body. Adipose tissue function is dependent on location and morphology. The two prominent types of adipose tissue include brown and white adipose tissue. Brown adipose tissue (BAT) has a primary role in heat production through oxidative phosphorylation in mitochondria (Poulos, Hausman, & Hausman, 2010), where white adipose tissue (WAT) has historically been considered as the primary site of fat energy storage. Adipose tissue has been shown to function as an endocrine organ, secreting hormones that function as metabolic signals, altering insulin sensitivity, appetite, immune function, and energy expenditure. WAT is a multifaceted organ composed of adipocytes as well as assorted cells within the stromal-vascular fraction (SVF), which include macrophages, lymphocytes, endothelial cells, and fibroblasts. Collectively, these cells contribute not only to the endocrine function of the tissue but also to the unique physiological and metabolic properties of the tissue. Adding to the complexity of adipose tissue are clear depot-specific differences in cellular complexity and endocrine output.

Adipose tissue has the ability to produce and react to many different endocrine compounds. A major endocrine hormone that has a significant role in metabolism is growth hormone (GH). Primary actions of GH include promoting longitudinal bone growth (Rosenfeld & Hwa, 2009), increasing circulating free fatty acids (FFA) through stimulation of lipolysis and lipid oxidation (Moller & Jorgensen, 2009), increasing lean mass through protein synthesis (Fryburg, Louard, Gerow, Gelfand, & Barrett, 1992), and
modulation and differentiation of immune cells (Smaniotto, Martins-Neto, Dardenne, & Savino, 2011). GH action is modulated through a complex feedback system. Secretion of GH is controlled by growth hormone releasing hormone (GHRH) and somatostatin acting directly on the somatotrophs of the anterior pituitary gland. In addition to direct effects of GH, some metabolic and physiological functions of GH are carried out through its ability to increase the secretion of insulin-like growth factor-1 (IGF-1 (Isaksson, 2004).

GH is critical to adequate development, metabolism, and cellular function. As such, dysfunction in secretion results in unique body composition and metabolic profiles. Acromegaly is a condition characterized by hypersecretion of GH, with classical symptoms including bone overgrowth specifically seen in hands, feet, and facial bones, reduced adiposity, increased lean mass, and insulin resistance (Katznelson, 2009; Moller, Butler, Antsiferov, & Alberti, 1989; Moller et al., 1992), which contribute to premature mortality. To examine the disease state more closely, mouse models have been developed to mimic the human condition. For example, the bovine growth hormone (bGH) transgenic mouse exhibits hypersecretion of GH resulting in significantly decreased adiposity and increased lean mass. The bGH model also exhibits insulin resistance, hyperinsulinemia, and decreased lifespan, similar to the characteristics in the human condition (Olsson et al., 2005; Wolf et al., 1993). GH action is not limited to the circumstances previously mentioned; GH has been found to act on the immune system, effecting immune cell proliferation, modulation, activity, and antibody synthesis (Kelley, 1990). Important to this study, the analysis of the immune cells in bGH mice has not been carefully assessed.
Immune cells are known to be present in adipose tissue, but only recently has evidence been reported that the immune cells present in this tissue may be important for the overall metabolism and physiology of the organism. Adipose tissue immune cells affect tissue inflammation and the homeostatic mechanisms of the tissue (Sun, Ji, Kersten, & Qi, 2012). Resident and infiltrating adipose tissue immune cells vary in cell type and population size, which ultimately contribute to the chronic low-grade inflammation seen in obesity. The level of adiposity results in changes in the resident and recruited immune cell populations, which contribute to or diminish tissue inflammation (Rothenberg & Hogan, 2006). Understanding the relationship between immune cells and the physiology of the tissue is important to better characterize the pathogenesis of obesity and its comorbidities and may lead to potential targets for obesity treatment.

While previous research has shown the significant role of adipose tissue on glucose metabolism, insulin regulation, and endocrine function, the contributions of different immune cell types and depot specific differences to these processes remain unclear. The influence of GH on these parameters has not been explored.

**Statement of Problem**

Very little is known about the immune cells that exist in adipose tissue and the factors that contribute to their presence or activation state. A complete understanding of the significance and function of the diverse populations of immune cells present in each individual adipose depot is necessary and challenging. To date, no one has explored the effect of GH on immune cells in adipose tissue although limited data are available for GH’s effect on immune function in spleen and blood serum. Thus, using the bGH animal
model may prove to be a valuable tool with which to explore the immune cell properties of adipose tissue. Ultimately, this model could help us understand the important factors within the tissue that establish adipose tissue immune cell populations and that contribute to its related disease states, such as type 2 diabetes. Clarifying the relationship between immune cells and adipose tissue will provide greater insight into the identification of new and effective therapies based on adipose tissue inflammation.

Research Questions

In this study, male bGH transgenic mice and littermate controls of the C57Bl/6J background strain were used to examine the immune cell profile in three adipose tissue depots. Research questions addressed were:

1. Will the total stromal vascular fraction (SVF) and SVF per gram of tissue differ between genotype (bGH and littermate controls) and depot (mesenteric, epididymal, inguinal or retroperitoneal)?

2. What are the differences in immune cell populations (macrophages, T helper cells, regulatory T cells, cytotoxic T cells, and NK T cells) found in the depots (mesenteric, epididymal, inguinal, or retroperitoneal) of the two phenotypes (bGH and littermate controls)?

Hypotheses

1. Due to the excess GH, SVF and SVF/g in bGH may be higher compared to WT.

2. Due to the effect of GH and the resulting heightened lipolysis in the bGH model, the adipose tissue will be compromised leading to an inflamed state with greater infiltration of immune cells. More specifically, bGH mice will possess:
a. A decrease in T regulatory cells with a corresponding increase in Cytotoxic T cells, NKT cells, and NK cells.

b. A shift in macrophage populations with a decrease in the M2 population.

Significance

No research on the relationship between GH and immune cells within adipose tissue exists. While previous studies have examined immune function in spleen and blood serum of bGH mouse models, no studies have examined the immune cells within adipose tissue. The purpose of this study is to examine the immune cell populations present in WAT depots (inguinal, epididymal, and mesenteric depots). The use of three distinct depots is imperative considering substantial data show that individual depots respond differently to GH’s actions. Identification of the resident immune cell populations in the C57Bl/6J wild type and bGH mice will help characterize the inflammatory profile of WAT and help elucidate fundamental depot differences, which ultimately is important in understanding the pathogenesis of diseases involving excesses or deficiencies in adipose tissue.

Limitations/Delimitations

1. There are errors of measurement due to specific equipment, nuclear magnetic resonance (NMR) measuring body composition and pipetting, which cannot be eliminated completely.

2. There is stress to the animals, which is inevitable during body composition measurements and just prior to sacrifice. Data can be affected although actions were taken to minimized stress.
3. There are errors of measurement due to human interaction with equipment through sample processing.

4. The existence of species differences accounts for the inability to apply conclusions from the data directly to the human condition.

5. The bGH animals exhibit signs of premature aging. Thus, mice of the same chronological age will be of different biological age, which needs to be considered when interpreting results.

6. Inadequate or variations in cell counts for flow cytometry analysis can alter statistical analyses and the resulting significance.

7. For flow cytometry, we make choices regarding the specific cell types to analyze by the choice of antibodies employed. Thus, not all immune cells and their activation states can be determined but are limited to the immune cell populations chosen prior to the study.

**Definition of Terms**

*Acromegaly.* A condition caused by excess GH in the body resulting in abnormal bone growth, reduced fat mass with corresponding increased lean mass, respiratory distress, and insulin resistance, ultimately contributing to mortality (Bengtsson, Brummer, Eden, & Bosaeus, 1989; Katzenelson, 2009; Liu et al., 2003).

*Adipose tissue.* Connective tissue comprised primarily of adipocytes, the lipid-laden cell of the tissue. Other cell types present include fibroblasts, endothelial cells, and macrophages. Provides mechanical support, insulation, in addition to fuel storage (Ailhaud, Grimaldi, & Negrel, 1992; Sethi & Vidal-Puig, 2007).
Adipokine. An adipose tissue secreted hormone. Can act on adipose tissue in addition to many other tissues and organs.

Antibody. The proteins of high molecular weight that are produced normally by specialized B cells after stimulation by an antigen and act specifically against the antigen in an immune response (Sun et al., 2012).

Bovine growth hormone transgenic (bGH) mice. bGH mice produce excess levels of GH resulting in similar characteristics seen in the human condition of acromegaly. The mice are giant, lean, insulin insensitive, and short-lived (Berryman et al., 2004).

Fluorescent-activated cell sorting (FACS). The process of separating cells by fluorescent emissions associated with compatible antibodies. FACS provides information on leukocytes, fibroblasts, and other cells present in the same relatively small sample (Brake & Smith, 2008; Shaul, Bennett, Strissel, Greenberg, & Obin, 2010).

Growth hormone. A vertebrate polypeptide hormone, which is a member of the class I cytokine hormone family that is secreted by the anterior lobe of the pituitary primarily regulating growth (Roby et al., 1993).

Inflammation. A local response to cellular injury that is marked by capillary dilation leukocytic infiltration, redness, heat, pain, swelling, and often loss of function that serves as a mechanism initiating the elimination of noxious agents and of damaged tissue (Sun et al., 2012).

Insulin-like growth factor I. Compound secreted by peripheral tissues that modulate GH action. Produced in liver, bone, muscle, heart, kidney, and fat tissue (Daughaday, 2000; Isaksson, 2004).
**Insulin resistance.** A disruption of insulin action resulting in the inability to balance glucose availability and physiological demands (Samuel & Shulman, 2012).

**Subcutaneous.** Being, living, or made under the skin; includes inguinal depot when referring to adipose tissue (Cinti, 2005).

**Intra-abdominal.** Located with the abdominal cavity; in terms of adipose tissue, include epididymal, retroperitoneal, and mesenteric depots when referring to adipose tissue (Cinti, 2005).

**Stromal vascular fraction.** The separation of cell types in adipose tissue sample from the adipocytes (Lumeng, Bodzin, & Saltiel, 2007).
Chapter 2: Review of Literature

Because nutrient availability and caloric demands constantly change, the body has developed complex integrated mechanisms to maintain homeostasis through balancing anabolic and catabolic energy pathways. Recent environmental changes in caloric availability and limited expenditure have resulted in physiological compromises. Obesity, which is now endemic, contributes to the increased prevalence of obesity-associated diseases like cardiovascular disease, diabetes, cancers, musculoskeletal disorders such as osteoarthritis, and premature death. These diseases significantly impact societal health, quality of life, and health system resources. Thus, there is an urgent need for better understanding of the mechanisms contributing to obesity and its associated diseases, which can lead to the development of successful therapeutic approaches and better inform economic and health policy.

The primary tissue altered in obesity is adipose tissue and has become the focus of pivotal research, providing a greater understanding of tissue complexity and function. Adipose tissue has historically been considered a site of energy storage but also acts as an endocrine organ, reacting to and secreting various hormones contributing to tissue complexity. More recently, studies have shown changes in immune cells within adipose tissue in obese states. Understanding the role of the immune system in adipose tissue will help characterize the pathogenesis of obesity and the associated comorbidities. This literature review examines adipose tissue physiology, growth hormone, and immune function, and explains current understanding of their relationship.
**Adipose Tissue**

Adipose tissue is a type of connective tissue and is largely comprised of adipocytes in addition to other cell types including macrophages, fibroblasts, endothelial cells, and immune cells (Ailhaud et al., 1992). The various cell types are critical for function and compound secretion of the tissue. Normal physiological functions of adipose tissue include mechanical support, insulation, and fuel storage. Energy from food in the diet can be stored in muscle, liver, and fat tissue, with fat tissue possessing the greatest tissue storage capacity. In times of excess energy expenditure or low intake, utilization of stored triglycerides within adipocytes occurs through lipolysis resulting in release of glycerol and free fatty acids. Periods of excess food intake or decreased energy expenditure result in efficient triglyceride deposition in the adipocytes (Sethi & Vidal-Puig, 2007).

**Endocrine function.** Adipose tissue is recognized as a potent endocrine organ secreting numerous hormones and growth factors that function as metabolic signals, altering insulin sensitivity, appetite, inflammation, immune function, and energy expenditure (Kershaw & Flier, 2004; Trayhurn & Wood, 2004). Adipose tissue derived hormones and cytokines have been termed ‘adipokines’ originating from the many cell types found within the tissue. Prominent adipose tissue hormones as well as the two most studied include adiponectin and leptin. Leptin has autocrine and paracrine effects within adipose tissue, in addition to having far reaching effects in the body. It acts as a metabolic signal of energy sufficiency by altering appetite and energy expenditure (Kershaw & Flier, 2004). In comparison, adiponectin is known for its anti-inflammatory, insulin
sensitizing, and anti-atherosclerotic properties (Brochu-Gaudreau et al., 2010; Ohashi et al., 2010; Yamauchi et al., 2001). With an increase in adiposity, circulating serum levels of adiponectin decrease (Brochu-Gaudreau et al., 2010). In addition, tumor-necrosis factor-α, interleukin-6, and nerve growth factor are examples of the expanding list of secreted adipokines. Production of these adipokines tends to be elevated in an obese state (Wajchenberg, Giannella-Neto, da Silva, & Santos, 2002), demonstrating that obesity influences more than just the mass of the adipocyte. Thus, the metabolic state of adipose tissue influences endocrine function. Moreover, adipose tissue has the ability to respond to endocrine compounds, which is apparent in the development of insulin resistance and the onset of type 2 diabetes associated with obesity.

**White adipose tissue and brown adipose tissue.** Adipose tissue is not a homogeneous tissue. Two general types of adipose tissue are recognized, white and brown, which vary in location, morphology, and function. White adipose tissue is more abundant compared to brown adipose tissue. White adipose tissue is the primary site of energy storage in large unilocular lipid droplets, which is a unique characteristic of white adipose tissue adipocytes. In comparison, brown adipose tissue displays multilocular lipid droplets and functions in heat production by the uncoupling of oxidative phosphorylation in mitochondria (Poulos et al., 2010). The heat production of brown adipose tissue and its capacity for thermal manipulation has been found to be important for newborn humans as well as other mammals (Sethi & Vidal-Puig, 2007). A majority of the brown adipose tissue capacity was thought to be lost during adulthood due to the development of additional thermoregulatory methods (Sethi & Vidal-Puig, 2007). However, brown
adipose tissue has been identified in adults, and its inverse relationship to glucose levels and body weight suggests a significant role in metabolism and energy homeostasis (Lee et al., 2011; Lee, Greenfield, Ho, & Fulham, 2010). Strategies to harness and stimulate brown adipose tissue activity could lead to pharmacological applications for the treatment and management of obesity. Because this study focuses on white adipose tissue only, the remainder of the review of literature will focus solely on white adipose tissue.

**Tissue depots.** In mammals, white adipose tissue is regionally dispersed and can be classified into two categories: subcutaneous and intra-abdominal or visceral depots. Defining depots of intra-abdominal or visceral origin have led to inconsistencies in the literature. Some authors classify visceral depots as those that drain into the portal vein, which includes only omental and mesenteric depots, in comparison to simplified definitions, which include all depots in the intra-abdominal or visceral area (Cinti, 2005). The depots commonly used are shown in Figure 1. In rodents, the subcutaneous depots are located superficially beneath the skin in the anterior and posterior regions. The posterior depot, also called the inguinal depot, is anatomically less complex and more often studied. Intra-abdominal depots are located in the thoracic and abdominal cavities and include retroperitoneal, mesenteric, and perigonadal (epididymal in males and paraovarian in females) depots all of which are the utilized in this study. The retroperitoneal depot is located on the posterior abdominal wall behind the kidneys and next to the spine. The mesenteric depot, the only true visceral fat pad in terms of drainage, is outlined by the intestines and contributes to the folding of the large and small intestines within the abdominal cavity. The perigonadal depot is associated with the
reproductive organs. In males, it is found lining the epididymis and in females it primarily surrounds the ovaries and a portion of the uterus and bladder. There is no comparable human fat pad.

Figure 1. Adipose tissue depots. The adipose organ is made up of two subcutaneous and several intra-abdominal depots. The depots examined in this study are shown. The mesenteric depot (top left) is associated with the intestines, is an intra-abdominal depot, and is the only definitive visceral depot. Inguinal (bottom left), is a subcutaneous depot lying underneath the skin. Retroperitoneal (top right), is located behind the kidney (K), and is considered to be intra-abdominal but not visceral. Epididymal (bottom right), is associated with the testes and in the image has been moved outside of the abdominal cavity. This fat depot is considered intra-abdominal but not visceral is using the stricter definition of drainage into the portal vein. Adapted from “Growth Hormone and Adipose Tissue: Beyond the Adipocyte,” by D. E. Berryman, E. O. List, L. Sackmann-Sala, E. Lubbers, R. Munn, and J. J. Kopchick, 2011, *Growth Hormone & IGF Research, 21*, p. 115. Copyright 2011 by Elsevier. Reprinted with permission.
**Depot specific differences.** White adipose tissue depots are metabolically distinct contributing to the heterogeneity of the tissue. In humans, visceral or intra-abdominal adiposity is associated with increased risk of cardiovascular disease, hypertension, type 2 diabetes, and an increase in mortality (Wajchenberg, 2000; Wajchenberg et al., 2002).

A number of depot-specific differences in metabolism and structure have been reported. Intra-abdominal depots are less responsive to the lipogenic effects of insulin and more responsive to glucocorticoids through greater receptor expression, which relates to higher fatty acid turnover and lipolysis. Drainage of the mesenteric and omental (not found in mice) depots into the portal system of the liver is thought to influence insulin sensitivity and gluconeogenesis as a result of increased fatty acid oxidation (Wajchenberg et al., 2002). However, the magnitude of the effect visceral depots has on insulin sensitivity lacks supporting evidence. Subcutaneous depots are often larger than intra-abdominal depots and may have a greater contribution to the development of insulin resistance (Wajchenberg et al., 2002). Adipocyte size varies between depots and between normal and obese states; that is, visceral adipocytes are found to be larger relative to subcutaneous adipocytes in obese mice (Gollisch et al., 2009; Sackmann-Sala, Berryman, Munn, Lubbers, & Kopchick, 2012). Protein expression of lipoprotein lipase, leptin, and pro and anti-inflammatory factors are affected by adipocyte size (Skurk, Alberti-Huber, Herder, & Hauner, 2007; Vidal, 2001). Intra-abdominal adipose depots are also known to produce less leptin and adiponectin, and have higher production of cortisol and angiotensinogen than subcutaneous depots (Wajchenberg et al., 2002). However, other proteins are also altered including human phospholipid transfer protein (PLTP), a protein
involved in lipid redistribution, chemokine (c-c motif) receptor 2 (CCR2), and macrophage migration inhibitory factor (MIF). All of these molecules have increased expression in visceral depots compared to subcutaneous adipose depots (Alvehus, Buren, Sjostrom, Goedecke, & Olsson, 2010; Linder, Arner, Flores-Morales, Tollet-Egnell, & Norstedt, 2003). A study examining white adipose tissue depot specific differences in mice, found variations in glucose and lipid metabolism, protein content, blood flow, and adipocyte size (Sackmann-Sala et al., 2012). Differences in the mesenteric depot are related to the higher degree of vasculature and larger blood supply in the tissue (Sackman-Sala et al., 2012). Overall, it is important to recognize that the differences in white adipose tissue depots are not limited to anatomical location. Some even suggest that the individual differences be considered “mini-organs.”

**Growth Hormone**

A pituitary growth factor was first hypothesized when enlarged pituitary glands were observed in acromegalic patients (Marie, 1886). Additional studies using extracts of bovine anterior pituitary gland showed a factor that was able to promote growth in normal rats and restore growth in hypophysectomized rats (Evans & Long, 1921; Li, Evans, & Simpson, 1945; Smith, 1927). This finding suggested that growth is regulated via a growth factor, which was later isolated and referred to as growth hormone (GH). Further research led to the discovery of the growth hormone receptor (GHR), and later the three-dimensional structure of GH and GHR (Abdel-Meguid et al., 1987; Leung et al., 1987). Despite almost a century of work on GH, there remain many unanswered questions, especially its specific effects on individual tissues.
**Growth hormone gene and protein structure.** GH is a member of the large class I cytokine hormone family including prolactins (PRLs), placental lactogens (PLs), and recently identified members PRL-like protein B, PLP-C, and somatolactin (Roby et al., 1993). Conservation of cysteine amino acid residues within PRLs, PLs, and GH protein sequences demonstrates structural similarity within the hormone family (Nicoll, Mayer, & Russell, 1986). GH is synthesized as a precursor molecule and reaches its final form upon cleavage and secretion (Lewis et al., 1980). GH is 191 amino acids in length and has two isoforms, a 20 kDa and 22 kDa protein (Abdel-Meguid et al., 1987; de Vos, Ultsch, & Kossiakoff, 1992), the latter being the most abundant in circulation (Lewis et al., 1980). Significant homology exists between mammalian species including bovine, human, and rat (Nicoll et al., 1986), which have provided tremendous research opportunities in the field of endocrinology.

**Growth hormone regulation and secretion.** GH regulation and secretion is controlled through a complex and dynamic feedback system. Growth hormone releasing hormone (GHRH) and somatostatin have opposing actions on the somatotrophs of the pituitary gland to regulate the secretion of GH (Brazeau et al., 1973; Tannenbaum, 1991; Tannenbaum & Ling, 1984). The pulsatile secretion of GH is initiated by GHRH in humans and other mammals such as rats (Anderson & Scanes, 2012; Tannenbaum, Epelbaum, & Bowers, 2003). In humans, the largest pulse of GH occurs in the early stages of sleep (Spiegel et al., 2000). Metabolic and somatotropic actions of GH are mediated by GH as well as by another protein, insulin-like growth factor one (IGF-1), whose expression is markedly increased by GH (Isaksson, 2004; Muller, Rigamonti, &
Cella, 2003). Circulating IGF-1 has been found to exert long-loop feedback action on GH secretion and synthesis at the pituitary gland (Anderson & Scanes, 2012; Roith, Scavo, & Butler, 2001). A visual representation of GH secretion and action can be seen in figure 2.
Figure 2. Summary of growth hormone secretion and action. The figure displays the two hypothalamic secretagogues, Growth Hormone Releasing Hormone (GHRH) and Somatostatin (SST), acting on the anterior pituitary regulating GH secretion. Stimulation or suppression of GH secretion from the anterior pituitary is displayed by the positive (+) and negative (-) symbols. The pulsatile secretion of GH is visible in the figure, with the largest pulses occurring during sleep. GH secretion and action on various tissues in the body including liver, bone, muscle, heart, kidney, and fat, which results in the production of IGF-1 initiating a negative feedback loop that ultimately leads to decreased GH secretion. Adapted from “Growth Hormone (GH), Growth Hormone Receptor, and Signal Transduction,” by J. J. Kopchick and J. M. Andry, 2000, Molecular Genetics and Metabolism, 71, p. 295.
GH Signaling and Function

GH action begins with the binding of GH to the GH receptor (GHR). GHR is a member of the class I cytokine/growth hormone/prolactin receptor superfamily (Kopchick & Andry, 2000). These receptors contain an extracellular region and a hydrophobic transmembrane region. The GHR is located on the cell membrane with varying concentrations depending on the tissue. Binding of GH to the GHR results in receptor activation of intra and intercellular signaling pathways. Two GHRs bind to two sites on the GH molecule, resulting in a conformational change and a rotation of the transmembrane domain. This rotation activates Janus Kinase (JAK2) through transphosphorylation (Brown et al., 2005; Kopchick & Andry, 2000). JAK2 activation, in turn, results in signal transducers and activators of transcription (STATs) phosphorylation. STATs translocate to the nucleus of the cell and ultimately inhibit or promote transcription of a variety of genes (Rosenfeld & Hwa, 2009). One well-characterized gene that is upregulated via this pathway is IGF-1, as mentioned previously. Other signaling pathways, such as mitogen activated protein kinase (MAPK), and phosphotidylinositol 3’ kinase (PI3K) are also activated via GH binding to its receptor and regulate the transcription of GH-responsive genes (Piwien-Pilipuk, Huo, & Schwartz, 2002). Figure 3 demonstrates the complexity of GH-GHR signal transduction pathway.

The complexity of the GH-GHR signal transduction pathway is shown by the crosstalk between GH and insulin signaling. As described above, GH binding to the receptor results in activation of JAK2, and many pathways including STATs, PI3K, and the extracellular signal-regulated kinases (Erk1/2), which includes MAPK. Upon insulin binding to the insulin receptor (IR) activation of the PI3K and Erk1/2 pathways occurs (Xu & Messina, 2009). Due to the importance of both GH and insulin in metabolism, the signaling crosstalk can interfere or enhance activation of various pathways and result in
altered gene and protein expression, further demonstrating the complexity of the actions of GH.

GH has a significant role in metabolism and acts on an assortment of tissues. A well-recognized function of GH is as a regulator of longitudinal growth in bone (Kassem, Mosekilde, & Eriksen, 1994; Kasukawa, Miyakoshi, & Mohan, 2004). GH also impacts adipose tissue, altering lipid metabolism via stimulating lipolysis and lipid oxidation and increasing circulating free fatty acids (FFA) (Moller et al., 1990; Moller & Jorgensen, 2009). Decreased fat mass and the lipolytic action of GH are apparent in humans with acromegaly, a condition characterized by GH hypersecretion, as those individuals are very lean (Katznelson, 2009). The lean physique is characteristic of the effect of GH on protein metabolism; muscle protein synthesis increases with no effect on muscle protein breakdown (Fryburg et al., 1992; Moller & Jorgensen, 2009). GH also affects glucose metabolism but has a time dependent effect. Initial actions are similar to insulin, promoting glucose utilization and lipogenesis; followed by antagonistic actions of insulin, including lipolysis and hyperinsulinemia (Moller & Jorgensen, 2009). GH action is not limited to the functions previously presented. Renal function, immune cell differentiation and modulation, are influenced by GH, which further demonstrates the dynamic endocrine role of GH in growth, metabolism, and cellular function.

Acromegaly. Acromegaly is a condition characterized by hypersecretion of GH (Katznelson, 2009). Overgrowth results in the classic symptoms of enlarged hands, feet, and facial bones, in addition to organ comorbidities including hypertrophic cardiomyopathy and respiratory distress (Katznelson, 2009). GH has a significant role in
modulating body composition, which is apparent in the acromegaly. The excess GH in acromegaly is associated with volume expansion, including increased Total Body Water (TBW) and Extracellular Water (ECW) (Bengtsson et al., 1989). Individuals with chronic or acute excess GH also have significantly reduced fat mass related to the lipolytic effects of GH and a corresponding increase in lean mass due to the anabolic actions on skeletal muscle (Bengtsson et al., 1989; Liu et al., 2003). Metabolically, acute and chronic GH exposure leads to insulin resistance as a result of increased endogenous glucose production and decreased glucose disposal in muscle tissue (Moller et al., 1989, 1992). Following treatment for acromegaly and upon normalization of GH levels, fat mass and insulin sensitivity increase (Bengtsson et al., 1989). The anabolic actions of GH in bone produce the distinctive skeletal changes seen in acromegaly through increased proliferation and differentiation of bone marrow precursor cells resulting in increased bone turnover (Kassem et al., 1994; Nishiyama et al., 1996). These abnormal physiological circumstances contribute to premature death. With effective biochemical control of GH and IGF-1, mortality of those with acromegaly can be reduced (Kauppinen-Makelin et al., 2005; Sheppard, 2005). Acromegaly is a unique physiological condition that contradicts the common assumption of a healthy state is one with less fat. Further examination of this disease state in animal models has led to greater understanding of the intrinsic role of GH in many tissues.

**Bovine growth hormone transgenic mouse model.** Bovine growth hormone (bGH) mice are valuable tools to study acromegaly. These mice overproduce bovine GH, which mimics the acromegalic disease state seen in humans including gigantism, reduced
fat mass, and increased lean mass (Wolf et al., 1993). Body composition of bGH mice is dependent on age and gender. Before 6 weeks of age, bGH mice have a larger fat mass than wild type (WT) mice. Between male and female mice, changes in fat mass and lean mass occur later in life of female mice (Palmer et al., 2009). All fat depots are impacted in the bGH mouse model with the fat mass of subcutaneous, retroperitoneal, mesenteric, and epididymal being significantly lower in bGH compared to WT mice (Berryman et al., 2004; Palmer et al., 2009). Organomegaly is characteristic of acromegaly and is seen in the bGH mouse model with almost all organs except adipose tissue having a greater weight (Berryman et al., 2004; Palmer et al., 2009). Many other features have been reported for bGH mice; Table 1 provides a summary of the phenotype of the bGH mouse model.
Table 1

*Summary of bGH Phenotype Characteristics*

<table>
<thead>
<tr>
<th>Characterized phenotype</th>
<th>Conclusions</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Longevity</td>
<td>- bGH mice have a significantly reduced lifespan</td>
<td>Wolf et al., 1993</td>
</tr>
</tbody>
</table>
| Dietary manipulation    | - Resistant to diet-induced obesity  
                          - Develop hyperphagia on HFD  
                          - Become dyslipidemic and diabetic on HFD  
                          - bGH had increased fat mass but less relative to controls | Berryman et al., 2004;  
                                                                      Berryman et al., 2006;  
                                                                      Olsson et al., 2005; |
| Lipoprotein profile     | - Decrease in levels of VLDL, FFA, and hepatic TAG  
                          - Increase in levels of HDL, LDL, and cholesterol | Frick et al., 2001;  
                                                                      Olsson et al., 2005 |
| Glucose and insulin sensitivity | - bGH mice on HFD and LFD have increased insulin levels, hyperinsulinemia, and insulin resistance  
                                  - slightly impaired or normal glucose tolerance | Berryman et al., 2006;  
                                                                      Olsson et al., 2005 |
| Body composition in aging and gender differences | - bGH had greater BW compared to controls  
                                                      - Young bGH mice have greater fat mass than controls, but do not have the increase in fat mass seen in control mice with advancing age; thus, bGH mice become significantly leaner than controls at 4mo and 6mo for male and female mice, respectively | Palmer et al., 2009 |
Table 1 (Continued)

<table>
<thead>
<tr>
<th>Characterized phenotype</th>
<th>Conclusions</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adipokine levels</td>
<td>- Leptin and adiponectin levels are lower in bGH mouse</td>
<td>Berryman et al., 2004</td>
</tr>
<tr>
<td>Energy expenditure</td>
<td>- Increased food consumption</td>
<td>Berryman et al., 2004;</td>
</tr>
<tr>
<td></td>
<td>- Increased expenditure</td>
<td>Olsson et al., 2005</td>
</tr>
<tr>
<td>Disease incidence</td>
<td>- Developed retinal dysfunction</td>
<td>Bielohuby et al., 2009;</td>
</tr>
<tr>
<td></td>
<td>- Developed cardiac interstitial fibrosis</td>
<td>Bohlooly-Y et al., 2001;</td>
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<tr>
<td></td>
<td>- Endothelial vascular dysfunction, and increased aldosterone resulted in hypertension</td>
<td>Heilbronn &amp; Campbell, 2008;</td>
</tr>
<tr>
<td></td>
<td>- Developed arthritic disorders</td>
<td>Miquet et al., 2011;</td>
</tr>
<tr>
<td></td>
<td>- Enhanced tumorigenesis</td>
<td>Ogueta et al., 2000</td>
</tr>
</tbody>
</table>

*Note.* HFD: high fat diet; LFD: low fat diet; VLDL: very low density lipoproteins; FFA: free fatty acids; TAG: triacylglycerides; HDL: high density lipoproteins; LDL: low density lipoproteins; BW: body weight.
Immune System: Innate and Adaptive Immunity

The immune response is composed of two cooperative and tightly linked systems, the innate or natural immunity followed by acquired or adaptive immunity (Janeway & Medzhitov, 2002). Innate immunity is a nonspecific and fast response to infectious agents. The adaptive response is one that is highly specific for antigens, and possesses memory so in the event of a subsequent encounter a robust and quicker response will occur. An overview of the innate and adaptive immune response characteristics can be seen in Figure 4.

**Figure 4.** Summary of the characteristics of the innate and adaptive immune responses. The innate response is characterized by the immediate, non-antigen specific response when exposed to a foreign antigen. Examples of innate immunity include mucus membranes, which trap foreign particles and prevent entrance into the body; and cough reflex to expel foreign matter. The acquired or adaptive immune response is characterized by the delayed, antigen specific response. The acquired response has the ability to develop memory, which provides protection when exposed to the same antigen. Understanding the acquired immune response had led to the development and efficacy of vaccines through the use of antigen memory.
The innate response includes actions of macrophages, mast cells, neutrophils, dendritic cells, natural killer (NK) cells, and eosinophils (Sun et al., 2012). The adaptive response is mediated by T or B lymphocytes, including subpopulations of CD4+, CD8+, NK and T cells with highly specialized antigen specific receptors for many different pathogens. It is important to note that the adaptive response is one that is delayed, requiring several days for clonal expansion, differentiations, and activation of lymphocytes (Sun et al., 2012). A visual representation of the different immune cell populations can be found in Figure 5.
Figure 5. Immune cells of innate and adaptive immunity. The innate response is the fast first line of defense with a diverse cellular composition including macrophages, mast cells, dendritic cells, and natural killer cells. The adaptive response is slower but possesses increased antigen specificity and memory. It primarily composed of B cells, CD4+ and CD8+ T cells. Certain cell types including subpopulations of T cells and Natural Killer T cells bridge the gap between the innate and adaptive response. Adapted from “Cytokines in Cancer Pathogenesis and Cancer Therapy,” by G. Dranoff, 2004, Nature Reviews: Cancer, 4, p. 13. Copyright 2004 by Nature Publishing Group. Reprinted with permission.

Growth Hormone and Immunity

GH and IGF-1 can act on the immune system and influence many functional properties. Growth hormone receptors have been detected in the thymus, bone marrow, spleen, lymph nodes, and lymphocytes (Campbell & Scanes, 1995). GH has been found to impact leukocyte function including antibody synthesis, T cell activity, and cytokine production (Kelley, 1990). Understanding the significance of the effects of GH on immune function is important due to the use of human GH for treatment of GH
deficiencies and its function as an anti-obesity agent. To better understand the relationship between GH and immune function, transgenic mouse models with varied levels of GH action have been examined. A summary of the current understandings of immune function for the bGH mouse models can be found in Table 2.
Table 2

*Summary of Immune Function in bGH Transgenic Mouse Models*

<table>
<thead>
<tr>
<th>Reference</th>
<th>Model</th>
<th>Gender</th>
<th>Age</th>
<th>Tissue</th>
<th>Immune function</th>
</tr>
</thead>
</table>
| Dialynas, Brown-Borg, & Bartke, 1999 | MT-bGH  ♂️ | 4-5mo  | Spleen | - Thymus and spleen weight are heavier in bGH compared to controls  
- Percentage of CD3+ T cells, CD8+, CD4+ subset are decreased in mice with excess GH  
- Number of B cells not altered in transgenic mice |
| Esquifino et al., 2002 | bGH gene-PEPCK  ♂️ | 2mo 6mo | Spleen | - Splenic lymphocytes are greater in bGH than controls at both ages  
- NK activity is lower in bGH; lowest at 6mo compared to controls  
- bGH has lower T cell percentages at both ages compared to controls, but in 2mo bGH CD8+ is increased  
- Excess GH results in age-dependent decreased T cell function |
<table>
<thead>
<tr>
<th>Reference</th>
<th>Model</th>
<th>Gender</th>
<th>Age</th>
<th>Tissue</th>
<th>Immune function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hall, Bartke, &amp; Martinko, 2002</td>
<td>bGH-PEPCK</td>
<td>♂</td>
<td>4mo</td>
<td>Blood serum</td>
<td>- Injections of saline or TT are used to induce Ab production</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>- bGH mice produce significantly less TT-specific Ab compared to controls</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>- CD4+ and CD8+ cell populations significantly decreased in bGH compared to controls</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>- No significant difference in B cells</td>
</tr>
</tbody>
</table>

*Note.* MT-bGH: metallothionein I-bovine growth hormone; bGH-PEPCK: bovine growth hormone phosphoenolpyruvate carboxykinase promoter; mo: month; NK: natural killer t cells; TT: tetanus toxoid; Ab: antibody.
Immunity and Adipose Tissue

As previously mentioned, adipose tissue is more than a site for energy storage. It is a complex tissue with paracrine, autocrine, and endocrine function acting on itself and many peripheral tissues. Recent studies have demonstrated the relationship between adipose tissue, obesity, and immune cell function. In obesity, increased immune cell infiltration is observed in adipose tissue and promotes inflammation of the tissue. The diversity of immune cell populations present in adipose tissue and their contributions to obesity, metabolic syndrome, and type 2 diabetes must be identified in order to adequately understand the pathogenesis of obesity and associated comorbidities.

Inflammation is the result of the body’s response to infection or injury, and is the major line of defense to restore tissue function and homeostasis. The magnitude of the inflammatory response varies depending on the physiological circumstance. In cancer, tissue injury, and pathogen infection, the inflammatory response tends to be acute and greater in magnitude, compared to the state of inflammation that is found in obesity, which is low-grade and chronic in nature due to increased infiltration of immune cells. The effects of chronic low-grade inflammation may alter the homeostatic set point by shifting metabolic and immune systems of the tissue, resulting in declining function of the tissue as a whole (Sun et al., 2012). These circumstances and the resulting inflammation are due to the presence of immune cells, their activation status, and production of various cytokines, which demonstrates the relationship between immune cell presence and potential inflammation. Thus, it is important to understand the immune response mechanisms and their significance in adipose tissue.
**Adipose tissue immune cells.** The resident immune cells in adipose tissue are proportionally unique, contribute to homeostatic mechanisms of the tissue, and help identify the state of inflammation. In comparison, the recruited immune cells from systemic circulation to the tissue reflect infection, inflammation, or tissue damage, which can enhance or diminish the immune response. Figure 6 summarizes the cell lineages for adipose tissue immune cells, followed by Table 3 below, which summarizes the existing data regarding the immune cells present in adipose tissue, their function, and role in adipose tissue.
**Figure 6.** Cell lineages for adipose tissue immune cells. Hematopoietic stem cells are multipotent cells found within the bone marrow and can give rise to the myeloid cell lineage including macrophages of phenotypes M1 (pro-inflammatory) and M2 (anti-inflammatory), eosinophils, mast cells, and dendritic cells; the lymphoid cell lineage including B cells, Natural Killer T cells (NKT), cytotoxic T cells (CD8+), T regulatory cells, and CD4+ T cells, which are associated with recruitment of immune cells to the area via TH1, TH2, and TH17 cytokine secretion profiles.
Table 3

*Adipose Tissue Immune Cells*

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Function</th>
<th>Role in adipose tissue</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Macrophages</td>
<td>- Phagocytosis of nonself antigens and cellular debris</td>
<td>- Impact systemic insulin sensitivity</td>
<td>Chawla, Nguyen, &amp; Goh, 2011; Donath &amp; Shoelson, 2011; Olefsky &amp; Glass, 2010; Weisberg et al., 2003</td>
</tr>
<tr>
<td></td>
<td>- Act as APCs to activate the adaptive response</td>
<td>- In obese state, macrophage phenotype ratios shift in adipose tissue</td>
<td></td>
</tr>
<tr>
<td></td>
<td>- Tissue-resident macrophages have a role in tissue homeostasis; clearing cell debris and dying cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eosinophils</td>
<td>- Engulf and kill bacteria and parasites</td>
<td>- Eosinophil levels are negatively correlated with adiposity and obesity</td>
<td>Rothenberg &amp; Hogan, 2006</td>
</tr>
<tr>
<td>Mast cells</td>
<td>- Secrete many proinflammatory, immunomodulatory chemicals, cytokines, and chemokines</td>
<td>- Mast cell levels increase in the obese state</td>
<td>Liu et al., 2009</td>
</tr>
<tr>
<td></td>
<td>- Involved in allergic responses, tissue homeostasis, and remodeling</td>
<td>- Affect weight gain, glucose homeostasis, and energy expenditure</td>
<td></td>
</tr>
<tr>
<td>Cell type</td>
<td>Function</td>
<td>Role in adipose tissue</td>
<td>Reference(s)</td>
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</tbody>
</table>
| Myeloid-derived suppressor cells      | - Induction of activation upon inflammation and result in immune suppression through: Treg cell development, block NK cell cytotoxicity, decrease CD8+ T cell activity | - Levels increase in obesity  
- Shown to decrease inflammation and maintain inflammatory homeostasis  
- Found to shift macrophage phenotypes towards the insulin-sensitizing phenotype | Gabrilovich & Nagaraj, 2009;  
Ostrand-Rosenberg & Sinha, 2009;  
Xia et al., 2011 |
| CD4+ T cells                         | - Subpopulation of T cells  
- Recognize polypeptides of MHC-II on APCs  
- Release cytokines that recruit immune cells to the area resulting in increased inflammation  
- Subpopulations include T_{H1}, T_{H2}, and T_{H17} | - Found to control the progression of obesity-associated inflammation through cytokine secretion | Sun et al., 2012 |
| Treg cells                           | - Subpopulation of T cells  
- CD4+ T cell primarily involved in immunosuppression | - In obese state, reduced levels of Treg cells in abdominal adipose tissue but not subcutaneous tissue  
- Upregulation of Treg cells in an obese state results in improved glucose tolerance, insulin sensitivity, and increased anti-inflammatory cytokines | Feuerer et al., 2009 |
<table>
<thead>
<tr>
<th>Cell type</th>
<th>Function</th>
<th>Role in adipose tissue</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD8+ T cells</td>
<td>- Subpopulation of T cells</td>
<td>- A significant increase in CD8+ T cell population are observed in adipose tissue in the obese state</td>
<td>Rausch, Weisberg, Vardhana, &amp; Tortoriello, 2008; Winer et al., 2009</td>
</tr>
<tr>
<td></td>
<td>- Mediators of the adaptive immune response</td>
<td>- An increase in CD8+ T cells result in an increase in macrophage recruitment</td>
<td></td>
</tr>
<tr>
<td></td>
<td>- Recognize poly peptides of MHC-I</td>
<td>- CD8+ T cells has a modest to no effect on glucose homeostasis or insulin sensitivity</td>
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<tr>
<td></td>
<td>- Produce large amounts of cytokines and chemokines to induce cytolysis</td>
<td></td>
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</tr>
<tr>
<td></td>
<td>of target cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>- Most prominent subpopulation are cytotoxic T lymphocytes</td>
<td></td>
<td></td>
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<tr>
<td>NKT cells</td>
<td>- Subpopulation of T cells</td>
<td>- NKT cells are abundant in lean adipose tissue in mice</td>
<td>Kawano et al., 1997; Lynch et al., 2009; Yoshimoto, Bendelac, Watson, Hu-Li, &amp; Paul, 1995</td>
</tr>
<tr>
<td></td>
<td>- dependent on MHC-I molecule CD1d which binds and presents lipids</td>
<td>- In humans, NKT cells are reduced in obese omental tissue</td>
<td></td>
</tr>
<tr>
<td></td>
<td>- Aid in clearance of bacterial infections</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>- Secrete T_H1 and T_H2 cytokines</td>
<td></td>
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</tbody>
</table>
Table 3 (Continued)

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Function</th>
<th>Role in adipose tissue</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B cells</td>
<td>- Major component of the humoral immune response developing antibodies and immunoglobulins for specific antigens - Activation on B cells upon binding to nonself antigens leads to activation of other immune cells including T cells resulting in clearance of the nonself antigens</td>
<td>- B cells promote insulin resistance and glucose intolerance by T cell and macrophage modulation through the production of the IgG antibody</td>
<td>Winer et al., 2011</td>
</tr>
</tbody>
</table>

**Note.** APCs: antigen presenting cells; MHC-II: class II major histocompatibility complex; TH1 and TH2: T helper cells classified by cytokine secretion; MHC-I: class 1 major histocompatibility complex.
Immune Function and Obesity

Adipose tissue is a heterogeneous endocrine organ with the capacity to produce many different compounds that are affected by lipid accumulation and adipocyte hypertrophy leading to tissue dysfunction. The resulting dysregulation of adipokines is associated with an imbalance of pro and anti-inflammatory adipokines characterizing obesity as a state of chronic low-grade inflammation (Kalupahana, Moustaid-Moussa, & Claycombe, 2012). Immune cell populations can be significantly altered in the obese state. A visual representation of the changes in immune cell populations can be seen in Figure 7.
Figure 7. Changes in immune cell populations in adipose tissue with obesity. Lean adipose tissue contains a higher proportion of anti-inflammatory macrophages. It also contains a large number of regulatory T cells, which are an immunosuppressive anti-inflammatory cell type. Obesity and adipocyte hypertrophy leads to adipocyte necrosis and an increase in pro-inflammatory macrophages. This change in immune cell populations gives rise to pro-inflammatory cytokine secretion from adipose tissue and increased systemic inflammation, which contributes to the development of insulin resistance. Adapted from “Immunity as a Link Between Obesity and Insulin Resistance,” by N. S. Kalupahana, N. Moustaid-Moussa, and K. J. Claycombe, 2012, Molecular Aspects of Medicine, 33, p. 29. Copyright 2012 by Elsevier. Reprinted with Permission.

To examine the pathogenesis of obesity, high fat diet (HFD) feeding in various mouse models has been used to induce obesity and mimic the human condition. Specialized HFD with greater than 60 percent of the kilocalories from fat, results in significantly increased adipocyte size and triacylglycerol (TAG) content, in addition to advancing hepatic insulin resistance (Kleemann et al., 2010; Kraegen et al., 1991; Li, Yu, Pan, & Unger, 2002; Samuel et al., 2004). Short-term HFD feeding alters gene function and expression of inflammatory response genes, which can result in an acute inflammatory response in adipose tissue that occurs within the first 3 days of HFD
feeding (Kleemann et al., 2010). Long term HFD feeding significantly increases body and tissue weight, development of hyperinsulinemia, hyperlipidemia, severe glucose intolerance, and insulin resistance. The homeostatic balance of anti-inflammatory and pro-inflammatory cytokines is affected by long term HFD and results in changes in immune cell composition. With increased adiposity, macrophages, T cells, B cells, and mast cells have been found to infiltrate adipose tissue. In comparison, myeloid-derived suppressor cells (MDSC), eosinophils, and Treg cells have been found to decrease with increased adiposity (Rothenberg & Hogan, 2006; Sun et al., 2012). These changes in tissue function, cellular composition, and cytokine secretion affect the inflammatory state of adipose tissue in obesity. The magnitude of infiltration in obesity can be seen in figure 8, comparing healthy and unhealthy adipose tissue samples.
Figure 8. Healthy and unhealthy AT. Image (A) represents a stain of a healthy epididymal fat depot with densely packed adipocytes and limited immune cell infiltration. Image (B) displays a stain of an unhealthy ob/ob (obese transgenic mouse, characterized by hyperphagia and extreme obesity) epididymal fat depot, containing a high level of immune cell infiltration (blue areas). Scale bars: 50 μm. Adapted from “Adipose Tissue Remodeling and Obesity,” by K. Sun, C. M. Kusminski, and P. E. Scherer, 2011, Journal of Clinical Investigation, 121, p. 2097. Copyright 2011 by the American Society for Clinical Investigation.

**Macrophages in adipose tissue.** The most commonly studied innate immune cell in adipose tissue is the macrophage. Recently, it has been determined that there are two populations of adipose tissue macrophages (ATMs) that exist within adipose tissue; each type has a very different impact on the tissue. The M2 phenotype consists of resident macrophages, which have anti-inflammatory properties; thus, these are considered beneficial. In contrast, the M1 phenotype includes infiltrating macrophages that are
recruited to adipose tissue. The M1 phenotype is considered to be primarily responsible for producing pro-inflammatory molecules; thus, M1 macrophages are considered a main contributor to the inflammation associated with obesity (Heilbronn & Campbell, 2008). Various factors, including diet and hormonal status, influence the number of macrophages. Previous research on diet modification of wild type (WT) mice has shown that high fat diets lead to significantly more ATMs in adipose tissue compared to normal diet mice (Shaul et al., 2010). Additionally, gene expression of M1 ATMs is higher in high fat diet mice compared to normal diet, signifying the increased inflammatory capacity of M1 macrophages (Lumeng et al., 2007). These observations support the importance of M1 macrophages in promoting the inflammatory process in specific adipose depots (Bourlier & Bouloumie, 2009; Weisberg et al., 2003).

**T cells in adipose tissue.** Recent research has shown the presence of adaptive immune cells in inflamed adipose tissue. In diet-induced obese mice, there is visible infiltration of T cells in visceral adipose tissue, which correlates to the development of insulin resistance (Kintscher et al., 2008). Additional research has shown insight into the role of T cells in inflammation modulation. High fat diet mice show an increase in CD8 effector T cells with a decrease in regulatory T cells (Treg) and CD4 helper T cells (Nishimura et al., 2009). In addition, Treg cell populations are decreased in white adipose tissue of obese mice compared to lean mice (Kalupahana et al., 2012). As previously mentioned, the adaptive immune response is not the immediate reaction to injury or antigens, but in the case of adipose tissue, the infiltration of T cells occurs before macrophage infiltration. With the onset of obesity, it is thought that a change in Treg cell
number influences CD8+ and CD4+ T cell populations altering the cytokine profile of the tissue. The resulting profile is most likely proinflammatory and aids in determining the M1 and M2 phenotypes of infiltrating adipose tissue macrophages. More recent research has shown the role of B cells in glucose metabolism promoting activation of pro-inflammatory macrophages and T cells, and increased production of pathogenic antibodies. The presence of these antibodies induces glucose intolerance and insulin resistance in mice, demonstrating the possible existence of a unique antibody profile in obese insulin resistant humans (Winer et al., 2011). Establishing this relationship could suggest different therapeutic approaches for disease management.

**Summary**

In conclusion, adipose tissue is a heterogeneous and complex organ. The diverse cellular composition of WAT contributes to the endocrine and immune function of the tissue. The level of adiposity effects cellular composition and endocrine output, altering the physiological functions of the tissue. WAT has the ability to produce and react to different endocrine compounds, and is significantly affected by GH. GH is primarily recognized for its effect on growth, but also influences body composition with high levels of GH decreasing adiposity and increasing lean mass. The bGH transgenic mice display the physiological consequences of excess GH. The bGH mice are giant, lean, insulin insensitive, and short-lived, simulating the condition in humans known as acromegaly. GH also influences immune system function. Cell proliferation and differentiation can be affected resulting in inflammatory response dysfunction. Another factor influencing immune cell function and modulation is the level of adiposity. Pro and anti-inflammatory
immune cells and immunosuppressive cell populations fluctuate based on the level of adiposity and the resulting inflammation. Characterization of the immune cell populations in adipose tissue of a mouse model with different levels of GH action can lead to a better understanding of the role of GH in immune cell function and of GH in adipose tissue. A better understanding of this relationship can be beneficial in developing therapeutic approaches to obesity and obesity-associated diseases. Thus, the purpose of this study is to investigate the differences in immune cell populations in male bGH mice as compared to WT controls.
Chapter 3: Materials and Methods

Previous studies have examined immune cell populations in WAT of wild type mice or various obese strains. The primary aim of this study was to examine immune cell populations in multiple white adipose tissue depots of wild type and bovine growth hormone transgenic (bGH) mice at 5 months of age. Measurement of body weight, body composition, and excised adipose tissue weight were recorded before preparation for fluorescent-activated cell sorting (FACS).

Animals

All procedures were approved by the Ohio University Institutional Animal Care and Utilization Committee. Mice were of the C57BL/6J background strain and were bred in the Edison Biotechnology Institute of Ohio University. The breeding of bGH mice has been previously described by (Berryman et al., 2004). Briefly, the bGH mice were generated by a metallothionein transcription regulatory element for the bGH cDNA (complementary DNA), which was then injected into the pronucleus of C57BL/6J mouse embryos. To determine genotype, polymerase chain reaction (PCR) on tail samples was used as previously described (Berryman et al., 2004). Six male bGH mice and littermate controls at five months of age were used for the two groups: Wild Type (WT) and bGH transgenic (bGH). Two to four mice were housed per cage in a room with controlled light cycles (12 hour light/dark) and temperature (22 ± 2 °C) and provided food and water ad libitum.

Body weight and body composition measurement. Body weight and body composition measurements were taken two weeks prior to sample collection. Body weight
measurements were taken using a Mettler Toledo PL 202-S balance. Body composition was assessed using a quantitative nuclear magnetic resonance (NMR) apparatus (Minispec, Bruker Optics, Billerica, MA), as previously described by (Palmer et al., 2009). Mice were placed in the minispec restrainers and inserted vertically into the NMR apparatus. Body composition measurements for fat, free body fluid, and lean tissue values were collected.

**Adipose tissue depots.** The common white adipose tissue depots studied in mice include inguinal, gonadal, and mesenteric. This study used samples of these three separate fat depots (one subcutaneous, two intra-abdominal), which were dissected from the male mice. The intra-abdominal fat depots included mesenteric (surrounding the intestines), and epididymal (surrounding the testes) as described in Figure 2. The day of dissection, mice were bled and sacrificed by cervical dislocation. Adipose tissue samples were dissected, weighed, and immediately placed in Krebs-Henseleit Buffer solution on ice for further processing.

**Stromal Vascular Fractionation (SVF)**

In order to develop an effective protocol, several variations of SVF isolation were tested before a final protocol was established and used in this study. A detailed protocol for optimizing the stromal vascular fractionation procedure can be found in Appendix A. Briefly, adipose tissue samples were treated with 1.1 mg of collagenase type I per 1 gram of adipose tissue (Worthington BioChemicals, Lakewood, NJ) to digest the tissue separating the adipocytes from the SVF. Following incubation, filtration and centrifugation were completed to further isolate the SVF cells from the adipocytes within
the tissue samples. The final SVF pellet was resuspended in Krebs-Henseleit Buffer solution on ice and transported to the Academic Research Center of Ohio University to prepare for flow cytometry.

**Fluorescent-Activated Cell Sorting**

SVF cells were incubated with Fc block (BD Biosciences) in fluorescent activated cell sorting (FACS) buffer (PBS 2% FBS, 0.05% sodium azide) for 20 minutes on ice to prevent nonspecific antibody staining. Cells were then incubated with specific fluorochrome-conjugated monoclonal antibodies at a 1/100 dilution to determine cell populations present in the SVF sample. A list of the specific antibodies used and their sources can be found in Table 4. The immune cell identification and staining scheme is shown in Table 5. An additional staining chart can be found in Appendix B.
Table 4

Antibodies for FACS Stainings

<table>
<thead>
<tr>
<th>Source</th>
<th>Catalog number</th>
<th>Antibody</th>
</tr>
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<tbody>
<tr>
<td>AbD Serotec, Raleigh, North Carolina</td>
<td>MCA2234A488</td>
<td>CD206 AF488</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ly-6C PerCP-Cy5.5</td>
</tr>
<tr>
<td>eBiosciences, San Jose, California</td>
<td>45-5932-80</td>
<td>MHC-II biotin</td>
</tr>
<tr>
<td></td>
<td>13-5321-81</td>
<td>F4/80 PeCy7</td>
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<tr>
<td></td>
<td>25-4801-82</td>
<td>CD36 APC</td>
</tr>
<tr>
<td></td>
<td>17-0361-80</td>
<td>CD11b AF700</td>
</tr>
<tr>
<td></td>
<td>56-0112-80</td>
<td>NK1.1 FITC</td>
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<td></td>
<td>11-5941-81</td>
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<td></td>
<td>13-0441-81</td>
<td>CD3 PeCy7</td>
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<td>25-0031-81</td>
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<td></td>
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<td></td>
<td>45-0042-81</td>
<td>CD45 APCCy7</td>
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<tr>
<td>BD Biosciences, San Diego, California</td>
<td>557659</td>
<td>CD45 APCCy7</td>
</tr>
<tr>
<td></td>
<td>550082</td>
<td>NKT PE</td>
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<tr>
<td></td>
<td>560514</td>
<td>CD62L APCCy7</td>
</tr>
<tr>
<td>R&amp;D Systems Inc., Minneapolis, Minnesota</td>
<td>FAB5538P</td>
<td>CCR2 Phycoerythrin MAb</td>
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</table>

Note. All antibodies used were anti-mouse.
### Table 5

*Immune Cell Identification Schematic*

<table>
<thead>
<tr>
<th>Antibody marker</th>
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</tr>
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<tbody>
<tr>
<td><strong>Stain 1</strong></td>
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<tr>
<td>CD45</td>
<td>Leukocytes</td>
</tr>
<tr>
<td>CD11b F4/80 CD45</td>
<td>Monocytes, macrophages</td>
</tr>
<tr>
<td>CD36 CD206 F4/80 CD45</td>
<td>M2 macrophages</td>
</tr>
<tr>
<td>CCR2 CD11B Ly6C CD45</td>
<td>Monocytes, M1 macrophages</td>
</tr>
<tr>
<td>MHC-II</td>
<td>Activation of macrophages</td>
</tr>
<tr>
<td><strong>Stain 2</strong></td>
<td></td>
</tr>
<tr>
<td>CD45</td>
<td>Leukocytes</td>
</tr>
<tr>
<td>CD45 CD3</td>
<td>T cells</td>
</tr>
<tr>
<td>CD45 CD3 CD4</td>
<td>T helper cells</td>
</tr>
<tr>
<td>CD45 CD3 CD4 CD25</td>
<td>Treg cells</td>
</tr>
<tr>
<td>CD45 CD3 CD4-</td>
<td>Cytotoxic T cells</td>
</tr>
<tr>
<td>CD44 CD62L</td>
<td>Differentiates between effector and memory T cells</td>
</tr>
<tr>
<td>NK1.1 CD3-</td>
<td>NK cells</td>
</tr>
<tr>
<td>NKT CD3</td>
<td>NKT cells</td>
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</table>

After incubation with specific antibodies, samples were subjected to multi-color flow cytometry on a FACSARia flow cytometer using FACSDiva software (Becton Dickinson, San Jose, CA). Output data was recorded by using the FACSDiva software and was further investigated by using the FlowJo flow cytometry analysis software. A
total of 10,000 to 100,000 events were collected per sample. A detailed protocol of FACS staining can be found in Appendix C.

**Statistical Analysis**

The retroperitoneal depot was excluded from data analysis due to the lack of tissue weight and resulting low cell counts for all samples. All measurement data including body weight, body composition, tissue weight, total SVF cells, total SVF cells per gram of tissue, and depot specific immune cell population quantification were analyzed by the Statistical Package for the Social Sciences Software (PASW version 18.0, Chicago, IL). All the variables and group means were calculated as mean ± the standard error of the mean (SEM). An independent t test was used to examine differences in body weight and body composition. Two-way ANOVA was used to identify the effect of genotype and depot for tissue weights, and a factorial ANOVA (3-depots x 2-genotypes) with Tukey’s Post Hoc test for total SVF, SVF per gram of tissue, and the identified immune cell populations. Statistical significance was found at a \( p < 0.05 \).
Chapter 4: Results

This study examined the impact of overexpression of GH on the immune cell profile of WAT in bGH transgenic mice relative to littermate controls. Body weight and body composition measurements were taken 2 weeks prior to sample collection. Mice were sacrificed at 5 months of age and tissue weights recorded. FACS was used to determine the various immune cell populations present in the WAT depots. Due to limited cell volumes found in the retroperitoneal adipose tissue samples, this depot was not used for data analysis; thus, three depots were analyzed: subcutaneous, epididymal, and mesenteric.

Body Weight

The difference in average body weights of bGH and WT mice are shown in Figure 9. As expected, bGH mice weighed significantly more than littermate controls.

Figure 9. Body weights of male WT (n = 6) and bGH (n = 6) mice at 5 months of age. Data are expressed as mean ± standard error of the mean (SEM). Means with a common letter do not significantly differ; p < 0.05.
Body Composition

Differences in body composition are shown in Figure 10. Data are presented in total grams (figure 10a) as well as a percent of body weight (Figure 10b) for fat mass, lean mass, and fluid mass. As expected, bGH mice displayed a two-fold increase in absolute lean mass compared to WT controls, with no significant change in total fat mass. However, when normalized to body weight, there was no increase in percent lean mass suggesting that the increased absolute lean mass is proportional to the larger body weight of the bGH mouse. A similar finding was noted for the fluid mass. Fat mass normalized to body weight revealed a dramatic and significant decrease in adiposity of the bGH mouse.
Figure 10. (a) Absolute body composition values in grams for male WT and bGH mice at 5 months of age. (b) Normalized (body composition as percentage of body weight) body composition values for male WT and bGH mice at 5 months of age. Data are expressed as mean ± SEM.
**Adipose Tissue Weight**

Absolute adipose tissue weight and tissue weight normalized to body weight for the three adipose tissue depots are shown in Figure 11(a) and 11(b) below. Within each genotype, subcutaneous and mesenteric adipose depots were significantly smaller in absolute and normalized weight compared to epididymal adipose. Absolute tissue weight for epididymal and mesenteric were significantly decreased in the bGH mice compared to littermate controls. When expressed as a percent of body weight, the difference in tissue weight between genotypes became more pronounced, with significant decreases seen in all fat pads in bGH mice.
Figure 11. Adipose tissue depot weight for male WT and bGH mice at 5 months of age. (a) Tissue weight expressed as values in grams of male WT and bGH mice at 5 months of age. (b) Tissue weight as percentages normalized to body weight for male WT and bGH mice at 5 months of age. Data are expressed as mean ± SEM. Means within a strain or depot with a common letter do not significantly differ; $p < 0.05$. 
Stromal Vascular Fractionation

Total adipose tissue for each depot was used to isolate the SVF. Significant differences were found between genotypes for total SVF cells and SVF cells per gram of tissue in the subcutaneous and mesenteric depots (see Figure 12). That is, for both depots, bGH mice had a greater number of SVF cells in a given adipose tissue mass as compared to WT tissue. Differences among the depots were found only within the bGH mice depots, with the epididymal values of total SVF cells and SVF cells per gram significantly lower compared to subcutaneous and mesenteric depots.
Figure 12. (a) Total SVF cells from adipose tissue depots of male WT and bGH mice at 5 months of age. (b) Total SVF cells per gram of adipose tissue for male WT and bGH mice at 5 months of age. Data are expressed as mean ± SEM. Means within a strain or depot with a common letter do not significantly differ; $p < 0.05$. 
Identification of Immune Cell Populations

To determine immune cells present in adipose tissue, the following gating strategy was used to identify positively stained cells within the sample. The subset of the total cells identified through flow cytometry was defined through a gate. The gate is a graphical or numerical boundary that defines the cells to include in further analyses. In this study, adipose tissue SVF samples contained multiple cell populations, and in order to identify specific cell types, we had to first restrict analysis to the CD45+ leukocyte population. Data for events within the gate are displayed in subsequent plots to determine the percentages of various subpopulations. The gating strategy was used for all samples and various antibodies to identify specific cell types.
Figure 13. Gating strategy. Gating strategy of leukocytes selected to be CD45\(^+\). Forward versus side scatter of isotype control (left); forward versus side scatter with gated subset of a WT mesenteric depot sample (center); CD45 versus side scatter with CD45\(^+\) gated population (right). This strategy was applied to each sample in order to identify the CD45\(^+\) cell population; further analysis of the CD45\(^+\) leukocyte gated population was completed to identify specific cell populations.
**Leukocytes.** Total leukocyte populations were identified by cells positively stained for CD45\(^{+}\), a transmembrane protein that aids in distinguishing leukocytes from non-hematopoietic cells. Significant differences between genotypes were seen in the mesenteric and epididymal depots with levels increased and decreased in bGH SVF, respectively. Within the bGH genotype, the mesenteric depot contained two times and eight times the amount of leukocytes compared to subcutaneous and epididymal depots, respectively. In comparison, WT mesenteric and epididymal depots had significantly lower levels of total leukocytes compared to the subcutaneous depot.

![Total Leukocytes (CD45+)](image)

*Figure 14.* Total leukocyte population. Gated as cells positively identified as CD45\(^{+}\) normalized to total SVF of each depot for male WT and bGH mice at 5 months of age. Data are expressed as mean ± SEM. Means within a strain or depot with a common letter do not significantly differ; \( p < 0.05 \).
Adipose tissue macrophages. To assess the effect of GH on macrophages in adipose tissue, CD11b+ F480+ ATMs were quantified by flow cytometry and found to represent a larger proportion of total SVF in bGH subcutaneous and mesenteric depots compared to littermate controls (see Figure 15). Depot specific differences were seen among depots of the bGH mice; the subcutaneous and mesenteric depots had a significantly higher fraction of ATMs compared to the epididymal depot. No significant differences were found between depots of WT controls.

Activation status of macrophages was evaluated by identification of cells expressing the MHC-II complex, which is a component in antigen presentation. Figure 16 displays the quantification of MHC-II positive ATMs as a percentage of total ATMs. Significant differences were found between genotypes in the subcutaneous, mesenteric,
and epididymal depots with levels increased in subcutaneous and mesenteric depots and epididymal decreased in bGH mice compared to controls. Depot specific differences were found in the bGH mice with the epididymal depot displaying significantly lower levels of ATM activation compared to the subcutaneous and mesenteric depots.

Figure 16. Activation of ATMs is indicated by cells positively identified for F4/80+ and MHC-II normalized to total ATMs of each depot for male WT and bGH mice at 5 months of age. Data are expressed as mean ± SEM. Means within a strain or depot with a common letter do not significantly differ; $p < 0.05$.

To examine the possibility that ATM subtypes may change with differences in GH action independent of total ATM content, CD206+ and CD36+ macrophages were quantified to identify the M2 or resident macrophage population compared to total ATMs of the tissue. Figure 17 shows the significant increase of type 2 ATMs in the bGH mouse compared to littermate controls, with the greatest increase in type 2 ATMs in the subcutaneous and mesenteric depots as compared to the epididymal depot in the bGH mice.
Figure 17. Quantification of M2 ATMs as cells positively identified for CD206+ and CD36+ normalized to total ATMs of the CD45+ leukocyte population of each depot for male WT and bGH mice at 5 months of age. Data are expressed as mean ± SEM. Means within a strain or depot with a common letter do not significantly differ; \( p < 0.05 \).

**Adipose tissue T cells.** Analysis of adipose tissue T cells by flow cytometry showed no significant differences between genotypes or among depots (see Figure 18). Within the adipose tissue T cell populations, T helper, Treg, and cytotoxic T subpopulations were quantified (see Figure 19). T helper cells were significantly increased in the bGH mice compared to WT mice of the subcutaneous depot only. Treg cells were significantly increased in the subcutaneous and mesenteric depots in the bGH mice compared to littermate controls. Genotype differences in cytotoxic T cell populations were found within the epididymal and mesenteric depots; that is, a significant increase in cytotoxic T cells was observed in the bGH epididymal depot, and a significant decrease was observed in the bGH mesenteric depot compared to their littermate controls.
Depot specific differences in T cell subpopulations were also observed in the bGH mice, but interestingly, no difference were seen in WT mice. Treg cells were significantly increased in both the subcutaneous and mesenteric depots but decreased in the epididymal depot within the bGH genotype. Cytotoxic T cells were significantly increased in the epididymal fat depot of the bGH mice, compared to the mesenteric depot.

Figure 18. Total T cells positively identified for CD45$^+$ and CD3$^+$ normalized to total CD45$^+$ leukocytes of each depot for male WT and bGH mice at 5 months of age. Data are expressed as mean ± SEM. Means within a strain or depot with a common letter do not significantly differ; $p < 0.05$. 
Figure 19. Quantification of T cell subpopulations. T helper cells: CD45+, CD3+, CD4+; Treg cells: CD45+, CD3+, CD4+, CD25+; Cytotoxic T cells: CD45+, CD3+, CD4-. All values normalized to total T cells (CD45+, CD3+) of each depot for male WT and bGH mice at 5 months of age. Data are expressed as mean ± SEM. Means within a strain or depot with a common letter do not significantly differ; \( p < 0.05 \).

Natural killer T (NKT) cell populations were also assessed (see Figure 20). No significant differences were found between bGH and controls. Depot specific differences were only significant between mesenteric and subcutaneous depots of the bGH mouse, with mesenteric having higher levels. No significant differences between depots were found in the WT controls.

Figure 20. Total NKT cells positively identified for NK1.1+ and CD3- normalized to total CD45+ leukocytes of each depot for male WT and bGH mice at 5 months of age. Data are expressed as mean ± SEM. Means within a strain or depot with a common letter do not significantly differ; \( p < 0.05 \).
Natural Killer (NK) cells are depicted in Figure 21 below. A significant difference was found between bGH and WT in the epididymal depot only. A three-fold difference was seen in WT epididymal NK cells compared to bGH. Depot specific differences exist between mesenteric and epididymal of the bGH genotype with mesenteric having a greater number than epididymal. Ironically, the reverse was true for control animals.

*Figure 21.* Total NK cells positively identified for NK1.1\(^+\) and CD3\(^-\) normalized to total CD45\(^+\) leukocytes of each depot for male WT and bGH mice at 5 months of age. Data are expressed as mean ± SEM. Means within a strain or depot with a common letter do not significantly differ; \(p < 0.05\).
Chapter 5: Discussion

The study was designed to investigate immune cell populations present in WAT of bGH and WT mice. Other studies have examined immune system function and cell populations in bGH mice. These studies reveal splenic and serum immune cell dysfunction along with an inhibition of antibody production and lower T cell populations in bGH mice relative to controls (Hall, Bartke, & Martinko, 2002). This study is unique as it is the first to examine immune cells present in bGH WAT. The major findings from our study were that excess GH results in depot specific changes in ATM content with polarization towards the M2 phenotype, increased T helper, Treg, and NKT cell populations, and decreased cytotoxic T cell and NK cell populations in bGH mice. This discussion will examine the genotype, cell type, and depot specific differences in immune cell populations of three WAT depots: subcutaneous (inguinal), epididymal, and mesenteric.

Body Weight and Body Composition

GH is known to have effects on body composition with overexpression of GH typically resulting in increased body weight and decreased fat mass compared to littermate controls (Berryman et al., 2004; Palmer et al., 2009). In this study, the bGH mice are larger mainly due to significant increases in the amount of absolute lean mass despite decreases in fat mass compared to littermate controls as reported previously.

Adipose Tissue Weights

Mice with excess GH have been shown to have reduced fat depots but vary in the degree by which the depots are reduced. Not all adipose tissue depots were equally
impacted by the high circulating levels of GH. That is, mesenteric had the greatest reduction in mass followed by epididymal. Subcutaneous did not demonstrate a significant reduction in tissue weight in the bGH mice. The normalization of adipose tissue weight to body weight exemplified the diminished adiposity associated with increased GH displaying significant differences in all three depots, with epididymal having the greatest difference of all depots. Berryman et al. (2004) found a similar trend with epididymal the most dramatically decreased depot in bGH mice. A summary of genotypic and depot differences can be seen in table 6 below. Depot specific differences in tissue weight between epididymal, subcutaneous, and mesenteric depots may be attributed to the variability in cellularity, metabolic activity, and endocrine function of the adipose tissue depots (Gollisch et al., 2009; Sackmann-Sala et al., 2012; Skurk et al., 2007; Vidal, 2001; Wajchenberg et al., 2002).
Table 6

*Summary of Genotypic and Depot Specific Differences in bGH Model*

<table>
<thead>
<tr>
<th></th>
<th>bGH</th>
<th>SUBQ</th>
<th>EPI</th>
<th>MES</th>
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</thead>
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<tr>
<td>Body weight</td>
<td>↑</td>
<td></td>
<td></td>
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<tr>
<td>Body composition (g,%)</td>
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<tr>
<td>Fat mass</td>
<td>¬, ↓</td>
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<td></td>
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<tr>
<td>Lean mass</td>
<td>↑, ¬</td>
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<td></td>
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<tr>
<td>Fluid mass</td>
<td>¬,</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Depot differences</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tissue weight (g,%)</td>
<td>¬, ↓</td>
<td>↓, ↓</td>
<td>↓, ↓</td>
<td></td>
</tr>
<tr>
<td>Total SVF</td>
<td>↑</td>
<td>¬</td>
<td>↑</td>
<td></td>
</tr>
<tr>
<td>Total SVF cells/g of tissue</td>
<td>↑</td>
<td>¬</td>
<td>↑</td>
<td></td>
</tr>
</tbody>
</table>

*Note.* SVF: Stromal Vascular Fraction; (¬) represents no change.

**Stromal Vascular Fraction**

Adipose tissue is primarily comprised of adipocytes in addition to many other cell types including immune cells, fibroblasts, and endothelial cells that comprise the SVF (Ailhaud et al., 1992; Bourlier & Bouloumie, 2009) and that are important to help maintain normal physiological functions of the tissue. The adipose tissue cells can thus be split into two major fractions, the mature adipocytes and SVF. The cells of the SVF vary in size, granulosity, and differentiation potential (Prunet-Marcassus et al., 2006). The
SVF fraction is a non-restrictive source of various hematopoietic derived cells and displays great plasticity due to variations in gene expression, metabolic, and endocrine function of the tissue (Prunet-Marcassus et al., 2006). Depot specific differences of SVF have been examined in subcutaneous and epididymal depots of young 6 to 8 week old C57Bl/6 mice. In this study, subcutaneous WAT has a larger SVF compared to epididymal WAT (Prunet-Marcassus et al., 2006), and these authors suggest this leads to greater plasticity of this depot. As this thesis reports SVF values from much older mice, age of the mice could contribute to the differences seen in SVF values. Regardless, the significant increase in SVF of the bGH mice compared to controls demonstrates the unique physiology and heterogeneity of cell types in bGH adipose tissue and could be a result of increased immune cell infiltration, presence of brown adipocytes, preadipocytes, or fibroblasts, some of which were not assessed in this study.

**Adipose Tissue Immune Cells**

To date, no studies have examined the effect of GH on adipose tissue immune cells. We hypothesized that excess GH may modify adipose tissue macrophage (ATM) and T cell content in fat, with depot specific differences. A challenge working with adipose tissue immune cells in WT mice on a normal chow diet is the limited ability to acquire and identify significant immune cell populations in the adipose tissue depots. In order to see significant changes in immune cell populations, challenging the system through diet modifications, caloric restriction, and physical activity results in a more robust and measurable outcome. So it is important to remember that many of the cell types examined are more commonly observed in the obese state, and that the models used
in this study are not obese but rather extremely lean. To identify specific cell populations, leukocytes were first identified and display significantly increased levels in the mesenteric depot and significantly decreased levels in the epididymal depot of the bGH mouse model. In the subcutaneous and mesenteric depots, lymph nodes are found. They are strategically located lymph tissues that contribute to the innate and adaptive immune responses by accommodating macrophages, B cells, and T cells, which could be related to the SVF and variations in cellular composition of the SVF (Altintas et al., 2012; Kim et al., 2008; Trayhurn, 2005; von Andrian & Mempel, 2003).

**Adipose tissue macrophages.** ATMs, resident and infiltrating macrophages, perform an impressive variety of functions in maintaining tissue homeostasis in response to infection and injury via phagocytosis and repairing damaged tissue (Sun et al., 2012). Additionally, macrophages serve as a bridge between the innate and adaptive immune system. In this study, macrophages account for approximately 30 percent of total CD45+ leukocytes in control adipose tissue, which is comparable to previous studies (Weisberg et al., 2003). In adipose tissue from bGH mice ATMs comprise a greater percentage of the total CD45+ leukocytes in several depots, which can lead to the conclusion that GH may regulate macrophage infiltration in adipose tissue. This phenomenon is not novel as others have shown the importance of GH signaling in macrophages function and cytokine production using macrophage specific GH receptor gene disrupted mice and the effect on adipocyte function and differentiation (Lu, Kumar, Fan, Sperling, & Menon, 2010).

**M2 adipose tissue macrophages.** The majority of ATMs have previously been reported to be of the M2 phenotype in healthy adipose tissue (Zeyda & Stulnig, 2007).
Interestingly, bGH mice had a notable increase in M2 macrophages compared to WT adipose tissue. The role of M2 macrophages in adipose tissue remodeling is important in facilitating adipose tissue expansion (Bourlier & Bouloumie, 2009). Data have suggested that high levels of circulating free fatty acids through increased lipolysis (Kosteli et al., 2010), influences ATM accumulation, specifically promoting the M2 phenotype. Since GH is well known to increase lipolysis (Johannsson et al., 1997), the increase in M2 macrophages might be expected in the bGH mice.

An important function of the M2 macrophages is tissue remodeling primarily through altering the extracellular matrix within the tissue. Interestingly, there are preliminary data to suggest that there is an increase in extracellular matrix deposition within adipose tissue in bGH mice (unpublished results), indicating the potential for an increase in fibrosis or M2 macrophages in this tissue. However, Spencer et al. (2010) showed that excess adipose tissue, as seen with obesity, appears to be more susceptible to fibrosis and M2 macrophage content. In contrast, bGH mice are very lean with low levels of adipose tissue yet have signs of fibrosis, and with the data presented in this thesis, evidence for an increase in M2 macrophages. Potentially, the link is in insulin resistance. Spencer et al., did show that insulin-resistant states, which are typically associated with obesity, result in increased fibrosis and M2 macrophages; bGH mice are insulin resistant despite being lean. An additional study by Spencer et al. (2011) examined the role of the extracellular matrix and adipocyte macrophage interactions, which demonstrated the relationship between increased collagen production by adipocytes co-cultured with M2 macrophages, further supporting the existence of a relationship between fibrosis and the
presence of M2 macrophages. Another mechanism could be attributed to the direct effect of GH on collagen synthesis and deposition altering the extracellular matrix in tissues that include adipose tissue (Bakillah, Guillot, Urios, Grigorova-Borsos, & Sternberg, 2011). Based on conclusions from these studies, an explanation for the differences in M2 ATMs in the bGH mouse could be a result of possible fibrosis in the adipose tissue and the diabetogenic nature of the whole bGH mouse.

**Adipose tissue T cells.** The interplay between T cells and adipocytes is a multifaceted relationship. Recently, T cells have been implicated in the development of obesity related insulin resistance (Kintscher et al., 2008). It has been found that in obese humans, visceral WAT contains more T cells compared to subcutaneous WAT, which is most likely due to increased recruitment or expansion within the tissue. No studies have yet examined T cells present in bGH mice and their role in adipose tissue.

**T helper cells.** CD4+ T cells include T helper cells that can be stimulated by and produce a variety of cytokines. There are three primary lineages of T helper cells: T_{H1}, T_{H2}, and T_{H17}, which are recognized by the cytokines they respond to and produce. T_{H1} and T_{H2} subpopulations are found in WAT and vary in number based on depot and the state of obesity (Lee et al., 2011; Rocha et al., 2008; Strissel et al., 2010; Winer et al., 2009). The T_{H1} and T_{H2} orchestrate cytokine production from macrophages and participate in the adaptive immune response, yet the mechanisms of T_{H1} and T_{H2} T cells and their cytokines in adipose tissue inflammation and obesity are unknown (Rocha et al., 2008). The involvement of CD4+ T cells in tissue homeostasis and insulin sensitivity is apparent during transfer of isolated CD4+ cells to adipose tissue, which reversed an
increase in body weight and insulin resistance in DIO mice (Winer et al., 2009). The potential insulin sensitizing action of CD4+ cells in WAT is interesting considering bGH mice are considered insulin resistant. Possibly the significant increase in the T helper cell population in the bGH mouse model stalls the development of overt diabetes. Since the imbalance in the TH1, TH2 cells present in WAT may contribute to the pathogenesis of obesity and insulin resistance it is important to further characterize the different populations of T helper cells in future studies using the bGH model.

**Regulatory T cells.** Regulatory T cells (Treg), a T cell subpopulation characterized by CD4+/CD25+ cells, are thought to have a significant role in obesity associated inflammation and insulin sensitivity. A study by Feuerer et al., demonstrated the relationship between Treg cells and metabolic function in normal WT mice. By depleting these cells, an accompanying decrease in insulin-stimulated insulin receptor in fat is observed. Additionally, insulin levels in the Treg depleted mice are significantly elevated resulting in insulin resistance. Treg cell populations are also significantly reduced in adipose tissue of three obese mouse models: leptin deficient mice, heterozygous mice for the yellow spontaneous mutation, and mice on a HFD which all displayed insulin resistance (Anstee & Goldin, 2006). The negative correlation between Treg cell population size and adiposity or insulin insensitivity can be applied to the bGH mouse model. That is, the significant decrease in adiposity and the diabetogenic state of the bGH mouse could account for the increase in Treg cells of WAT. In addition, a relationship exists between macrophages and Treg cells. The activation status of macrophages through MHC-II is important to determine macrophage function and can
prompt differentiation of T cells from the naïve phenotype to $T_{H1}$, $T_{H2}$, $T_{H17}$, or regulatory (Do, Valujskikh, Vignali, Fairchild, & Min, 2012). Thus, the significant increase in activated ATMs in bGH mice could influence the Treg cell population.

**Cytotoxic T cells.** Another cell type examined in this study was cytotoxic T cells. Current research has observed a positive relationship between cytotoxic T cell content, obesity, and tissue hypoxia. For example, a study by Rausch et al. (2008) shows that the WAT in DIO mice and ob/ob obese mice display hypoxic areas with an increase in macrophage and cytotoxic T cell infiltration compared to lean controls. The increase in cytotoxic T cells in the subcutaneous and epididymal depots of the bGH mice may be a reflection of insulin resistance of the whole bGH mouse. In addition, fibrosis is commonly associated with tissue hypoxia, and the fibrotic nature of the bGH adipose tissue could enhance cytotoxic T cell infiltration into the depots, demonstrating the dynamic relationship between adiposity and the ability of the tissue to properly function.

**Natural killer T cells.** Natural Killer (NK) T cells are critical therapeutic targets in many disease models, but their role in obesity and development of associated comorbidities is not well understood. The NKT cells possess similar characteristics of the CD4+ T cells in that they can produce $T_{H1}$ and $T_{H2}$ cytokines. NKT cells interact with CD1d related lipid and glycolipid antigens, which may provide a link between obesity and inflammation in adipose tissue (Borg et al., 2007; Mallevaey et al., 2011). Recent studies have demonstrated that NKT cells may contribute to obesity through the use of NKT deficient mice on a HFD. These mice had better glucose tolerance and decreased macrophage infiltration compared to controls (Ohmura et al., 2010). However, opposite
effects of NKT cell function have been observed in the transplantation of NKT cells to obese adipose tissue and result in mitigation of the metabolic dysfunction of the tissue (Elinav et al., 2006). A recent study examined the relationship between NKT cells, macrophages, and glucose tolerance. They report that activation of NKT cells by a lipid agonist enhanced macrophage polarization towards the M2 phenotype, this, in turn improved glucose homeostasis in animals in different stages of obesity (Ji et al., 2012). The increased polarization of M2 macrophages seen in the bGH mice could be enhanced by the presence of NKT cells as a result of the insulin resistance of the animal. Therefore, suggesting that the role of NKT cells and other immune cell types in adipose tissue function and the development of obesity is very complex and still controversial.

**Natural killer cells.** Few studies have examined adipose tissue Natural Killer (NK) cell frequency in obesity. O’Rourke et al., examined the NK cell populations in subcutaneous and visceral adipose tissue of obese humans and found a significant increase in NK cell populations compared to non-obese adipose tissue. These cells were thought to be a possible source of inflammatory cytokines that induce M1 macrophage inflammation in obesity (O'Rourke et al., 2009). The lower levels present in bGH WAT could be a result of the higher M2 macrophage population and decreased adiposity.

**Depot specific differences.** Depot specific differences in adipose tissue have been examined to a great extent. Differences include adipocyte size, insulin sensitivity, adipocyte differentiation, plasticity, adipokine production, and glucose and lipid metabolism are some of the many differences found between adipose tissue depots (Gollisch et al., 2009; Sackmann-Sala et al., 2012; Skurk et al., 2007; Vidal, 2001;
Wajchenberg et al., 2002). Table 7 presents a summary of depot specific differences in immune cell populations identified in the WT and bGH mouse models.

Table 7

*Summary of Depot Differences in Immune Cell Populations*

<table>
<thead>
<tr>
<th>Immune cells</th>
<th>SUBQ</th>
<th>EPI</th>
<th>MES</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total leukocytes (CD45+)</td>
<td>–</td>
<td>↓</td>
<td>↑</td>
</tr>
<tr>
<td>ATMs (F4/80+, CD11b+)</td>
<td>↑</td>
<td>–</td>
<td>↑</td>
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<tr>
<td>MHC-II (MHC-II+)</td>
<td>↑</td>
<td>↓</td>
<td>↑</td>
</tr>
<tr>
<td>M2 ATMs (CD206+, CD36+)</td>
<td>↑</td>
<td>↓</td>
<td>↑</td>
</tr>
<tr>
<td>Total T cells (CD3+)</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>T helper</td>
<td>↑</td>
<td>–</td>
<td>–</td>
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<tr>
<td>T regulatory</td>
<td>↑</td>
<td>↓</td>
<td>↑</td>
</tr>
<tr>
<td>Cytotoxic T</td>
<td>–</td>
<td>↑</td>
<td>↓</td>
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<tr>
<td>Natural killer T (NKT+, CD3+)</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Natural killer (NK1.1, CD3-)</td>
<td>–</td>
<td>↓</td>
<td>–</td>
</tr>
</tbody>
</table>

*Note.* ATMs: Adipose Tissue Macrophages; (−) represents no change.

The more dynamic depots are the subcutaneous and mesenteric depots, which contain lymph nodes contributing to the larger SVF and greater infiltration of immune cells as lymph nodes accommodate various immune cell populations including macrophages and T cells (von Andrian & Mempel, 2003). Further, differences seen in the
mesenteric depot tend to be associated with the greater degree of vasculature in the depot (Sackmann-Sala et al., 2012).

To date, few studies have examined depot specific differences in either WT or bGH adipose tissue immune cells. Individual depots, most commonly the epididymal depot, have been examined for immune cell content in relation to age and diet modification (Kalupahana et al., 2012; Rausch et al., 2008; Weisberg et al., 2003). However, it is clear from this and other studies, that each depot is very different. In this thesis, subcutaneous and mesenteric depots in the bGH mouse display a greater increase in ATMs and M2 macrophages than WT controls. bGH depot specific differences in total ATM and M2 ATM content are similar to those seen in HFD mice, with subcutaneous and mesenteric depots having an increase in M2 macrophages (Oh, Morinaga, Talukdar, Bae, & Olefsky, 2012). This phenotypic shift is similar to that seen in mice on an extended HFD, due to extensive tissue remodeling and exposure to micro-environmental cues such as lipids, cytokines, and hypoxia that results in the mixed phenotype of macrophages (Shaul et al., 2010). The similarities of bGH mice to an obese HFD mouse model was unexpected. Overall, it can be concluded that the metabolic actions and the microenvironment of the tissue are critical components in understanding macrophage polarization and further examination of bGH adipose tissue is necessary.

Depot specific differences for T cells have been examined in obese human and mouse adipose tissue. Obese visceral and subcutaneous adipose tissue of human and mice have been found to contain greater levels of NK cells and cytotoxic T cells, which demonstrates the relationship between obesity, immune cell infiltration, and resulting
tissue inflammation and remodeling (O'Rourke et al., 2009; Rausch et al., 2008). The depot expressing the greatest increase in cytotoxic T cells was the epididymal depot in bGH compared to subcutaneous and mesenteric, an opposite trend was observed with NK cells. In contrast, T cells that have do not respond vigorously to an increase in adiposity and obese adipose tissue are Treg and NKT cells. The lean nature of the bGH model demonstrates this relationship that with decreased adiposity Treg cell and NKT populations increase. Although this was not uniform among the depots, the differences can be attributed to variations in vasculature, nutrient supply, and variation in cellularity specifically the presence of macrophages in the tissue (Gollisch et al., 2009; Sackmann-Sala et al., 2012; Skurk et al., 2007; Vidal, 2001; Wajchenberg et al., 2002).

Conclusions

1. Excess GH signaling results in increased body weight and altered body composition with significantly increased lean mass and decreased fat mass when normalized to body weight as reported previously (Berryman et al., 2004, 2006; Palmer et al., 2009).

2. bGH mice have an increase in total SVF compared to littermate controls, with subcutaneous and mesenteric depots displaying the greatest increase in SVF. The increase in SVF can be related to an increase in alternative cell populations due to excess GH action and depot specific differences including the presence of adipose tissue lymph nodes in the subcutaneous and mesenteric depots.
3. Excess GH contributes to an increase in macrophage infiltration. There is a dynamic shift in macrophage phenotype towards M2 in bGH mice, which could be contribute to tissue remodeling, insulin sensitivity, and increased lipolysis.

4. Treg and NKT cells are shown to have an inverse relationship with increased adiposity, this relationship was apparent in the bGH model. That is, with significantly decreased adiposity of bGH mice, there was an increase in Treg cells that is depot specific.

5. The role of immune cells, obesity, and adipose tissue inflammation is complex and dynamic. The differences in levels of the cytotoxic T cells and NK cells found in the bGH WAT could be attributed to the adiposity of the bGH mouse and the diabetogenic state which is developed with exposure to excess GH.

6. Depot specific differences in immune cell populations of WT and bGH transgenic mice are attributed to differences in adipocyte size, insulin sensitivity, adipocyte differentiation, plasticity, adipokine production, glucose and lipid metabolism, lymph node presence, and cellularity of the tissue (Gollisch et al., 2009; Sackmann-Sala et al., 2012; Skurk et al., 2007; Vidal, 2001; von Andrian & Mempel, 2003; Wajchenberg et al., 2002).

This descriptive study was the first to address the impact of GH on immune cell populations present in WAT using bGH transgenic mice and WT controls. The bGH model demonstrates a unique immune profile, which shares characteristics of other obese and lean mouse models. Cell types associated with decreased adiposity and immunosuppressive functions, such as Treg cells and M2 macrophages, were found to be
elevated in the bGH adipose tissue. The diabetogenic nature of the bGH mouse was important to consider when examining the immune cell populations because the cell types contribute differently to the physiological functions of adipose tissue and contribute to the development and onset of diabetes in the mouse. The variations in levels of immune cells demonstrated the potential relationship between glucose homeostasis, insulin sensitivity, and whole tissue homeostasis that may provide greater insight into potential therapeutic targets for obesity and diabetes.

**Future Directions**

Below are potential avenues for future projects. Additionally, technical issues raised in this study should be addressed in the future, specifically the difficulty of identifying significant immune cell populations in adipose tissue of a young mouse on a normal chow diet.

- This study focused on the SVF population of cells in bGH mice. Other cells within the SVF should be examined because they could impact the function of the tissue. For example, the presence of brown adipocytes and alternative cell types such as fibroblasts could provide more insight to the cell populations that exist as well as the cytokine profile of the tissue.

- To better understand the role of GH in immune cell infiltration in adipose tissue, incorporating other genotypes might be useful. For example, GHA and GHR-/- mice have a decrease or absence of GH action, respectively, yet are both obese, and may display similar trends to DIO mouse models with an increase in inflammation and alternative immune cell populations including
M1 macrophages and cytotoxic T cells (Rausch et al., 2008; Shaul et al., 2010).

- Many of the current publications observing immune cell function and adipose tissue have challenged the system via high fat diet, low fat diet, or exercise. The GH mouse models mentioned above have been challenged with a HF diet and demonstrated variations in susceptibility to DIO. The bGH mice showed similar weight gain, a small increase in fat mass, and an increase in lean mass, which suggests these mice are protected from DIO (Berryman et al., 2006). Examining immune cell populations in WAT of these mice on HF or LF diets could provide more information about the development of obesity and the role of GH.

- To build on this possible project, observing the immune profile at multiple age points would also be beneficial to understanding immune cell function in adipose tissue. Recent publications have shown an increase T cell content of WAT in C57Bl/6 mice with age (Lumeng et al., 2011), but a limitation of this study is the use of the epididymal fat depot only. Since depot specific differences have been identified, including the other depots and examining additional immune cell types would be beneficial. Further, as bGH mice represent a mouse model of premature aging, the immune profile of bGH adipose tissue could reflect the role of immune cells in the aging process.

- As stated in the discussion, there is a dynamic relationship between immune cell content and function, and the development of fibrosis in adipose tissue.
Further examination of the extracellular matrix and microenvironment of adipose tissue in WT and bGH mice is necessary to better understand immune cell infiltration and their role in tissue physiology. More specifically, examine the relationship between immune cells present in adipose tissue of bGH mice and the changes in collagen to determine if the immune cells present in the tissue are trying to combat the inflammatory actions of fibrosis or alter macrophage phenotype polarization to improve the physiological state of the tissue.
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Appendix A: Stromal Vascular Fractionation Protocol

Protocol adapted from Shaul et al. (2010) and Brake & Smith (2008).

Protocol: Stromal Vascular Fraction Isolation
- This protocol can be used to isolate the SVF from adipose tissue (cells found in AT not including adipocytes), cells from liver, and splenocytes.
- Before beginning, it is a good habit to have the mice weighed and have their body composition data (if that is required)
- In order to improve the success of cell isolation, mice should be bled through the eye in order to remove as much blood as possible from the tissue.
- Prepare all tubes! Most often you will need two sets of tubes, and some cases three.
  o 1: 15 ml conical tube with KHB solution on ice, that the tissue will be placed in immediately post dissecting out from the mouse
  o 2: 15 ml (or 50 ml conical tube with BD falcon clip in filters for spleen and liver) conical tubes for adipose tissue to filter in the collagenase mixture
  o 3: *for spleen and liver, transfer the solution in 50 ml to the smaller tube
- ** for adipose tissue, in order to filter the tissue which can be found in the protocol below, you can use 10 or 5 ml cut off syringes, with 20 micron mesh that is attached to the cut off end.

Tissue Collection:
1. Asphyxiate mice with CO2, bleed, and cervically dislocate.
2. Dissect fat pads/liver/spleen and weigh immediately.
3. Transfer to Krebs-Henseleit Buffer (Sigma Aldrich) solution in 15 ml conical tubes on ice.

Krebs Henseleit Buffer:
- **K3753 Sigma Aldrich**
- KREBS-HENSELEIT BUFFER MODIFIED With 2000 mg/L glucose, without calcium chloride and sodium bicarbonate, powder, suitable for cell culture
  - K3753-10x1L $22.20
  - this solution provides 10 small containers that can be reconstituted in 1L of distilled water
  - In order to properly make the solution CALCIUM CHLORIDE and SODIUM BICARBONATE must be added to the solution
  - Steps:
    o CaCl2 solution: for 250 ml stock solution: add 7.35 grams of CaCl2 to 250 ml of distilled H2O. This stock solution can be used any subsequent time the KHB solution is made.
o Add contents of 1 container of KHB powder to 900 ml of distilled water
o Add 10 ml of CaCl2 stock solution
o Add 2.1 g of NaHCO3 sodium bicarbonate to the solution
o Add remaining distilled water to reach 1000 ml in the 1L glass container
o Check the pH* The pH must be around 7.4, therefore you must adjust with
NaOH and HCl if it is not close. This may take some time and be very
careful, use very small increments with the Pasteur glass pipettes that are
found in the chemical room to add small drops at a time. It is good to
consider using a stirring rod to place in the solution and put it on the
stirring plate while making pH adjustments.

**This solution must be made the day of! It has to be used within 24 hours
of being prepared! It does go bad! So I usually made it early in the
morning before the dissection, in total it takes about 15-30 minutes

Stromal Vascular Fraction Isolation:
1. Prepare the 15 ml conical tubes:
   a. For collagenase which is used later in the protocol it is recommended to
      use 280U/ml KHB. The collagenase type 1 CLS 1 by Worthington
      chemicals that we have here is 260U. Therefore (280U/mL) x (1mg
      collagenase/260U) = 1.1 mg collagenase/ mL of KHB solution in a 15 ml
      conical tube.
   b. With spleen use 2 ml KHB per tube (2.2-3 mg collagenase per tube)
   c. With Adipose tissue use 3-4 ml KHB per tube (4 mg collagenase per tube)
   d. The collagenase can be weighed out previously into the increments and
      placed in the refrigerator for the dissection, this will allow for greater
      efficiency during the dissection.
   e. Remember the collagenase needs to be kept cold! So if you are weighing it
      out before you must keep it on ice, and place the aliquots immediately
      back in the refrigerator! Once warm, it becomes very active! And we want
      that when we are processing our samples.
2. The tissue is placed in 15 ml conical tube with KRB solution that was previously
   weighed and recorded,
3. Mince tissue. Mince well to increase surface area to increase the efficacy of
   collagenase. Mice tissue by placing in small weight boat, and by using small
   scissors and tweezers, continuously cut the tissue into a slurry (the best you can
   do!)
4. Add minced tissue back to 15ml conical tube.
5. Add collagenase and gentle mix solution by swirling, a gentle vortex could be
   used as well.
6. Digest the tissue for 40-45 minutes at 37 °C, shaking at 80 cpm (depending on
   shaking incubator, 200-300rpm) use the shaking incubator in the room with PCR
and ice machine on the back wall. Make sure to check the temperature before placing samples in incubator.
   a. Use a timer.
   b. Continually check the temperature to make sure it is consistent and does not decrease or increase dramatically.

7. When time has elapsed, pour the solution into the 10 ml cut off syringe for adipose tissue with the attached mesh on the open end. As seen below in the first picture! Yes very scientific with the rubber band and all but this works best! If you can find clip in filters that are larger then go for it! The mesh is around 20 microns or so to allow for the cells to get through.

Or the 50 ml conical tube with BD falcon filter for spleen. As seen below in the second picture.

8. Collect the liquid into a new labeled conical tubes
   a. For spleen they are the 50 ml tubes as shown above
   b. For adipose tissue they are 15 ml conical tubes that the syringe rests in, as shown in the image below.
9. Rinse the mesh **twice** with the same amount KHB solution that was originally used in the 15 ml conical tubes, so if it was 2 ml, wash with 2 ml, if it was 4 ml, wash with 4 ml. You will use the larger pipetters to do this!

10. Separate adipocytes (or splenocytes from solution) by centrifuging solution at 500g for 5 minutes.
   a. Use the centrifuge found in the chemical room, it is larger and you can process many of the samples at once.

11. After the first centrifuge, pipette off the supernatant, and for adipose samples make sure you remove all the adipocytes that are floating on the top of the solution first! Then remove the remainder of the solution.

12. Add 1 ml of KHB solution to each tube and resuspend the pellet but continually pumping the pipette.

13. Centrifuge the tubes again for 5 minutes.

14. Remove tubes from centrifuge, remove the supernatant again.

15. SVF pellet is resuspended in 0.5 ml of cold PBS or KHB buffer for cell counting.

* you can check to see if there is a high cell count by using the hemocytometer and trypan blue stain, this is optional*
## Appendix B: FACS Staining

<table>
<thead>
<tr>
<th>Staining</th>
<th>FITC</th>
<th>PE</th>
<th>PE-Texas Red</th>
<th>Perpep/Perpep cy5.5</th>
<th>PeCy7</th>
<th>APC</th>
<th>APCCy7</th>
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<tbody>
<tr>
<td>1</td>
<td>CD206</td>
<td>CCR2</td>
<td>MHC-II biotin</td>
<td>Ly6C</td>
<td>F4/80</td>
<td>CD36</td>
<td>CD45</td>
<td>CD11b</td>
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<tr>
<td>2</td>
<td>NK1.1</td>
<td>NKT</td>
<td>CD44 biotin</td>
<td>CD4</td>
<td>CD3</td>
<td>CD25</td>
<td>CD62L</td>
<td>CD45</td>
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<td>3</td>
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**Staining 1**
- CD45: pan leukocyte staining
- CD11b F4/80 CD45: monocyte/macrophages
- CD36 CD206 F4/80 CD45: M2 macrophages
- CCR2 F4/80 CD45: M2 macrophages
- CCR2 CD11bLy6C CD45: differentiates monocyte populations
- MHC-II: indicates activation status of macrophages

**Staining 2**
- CD45: pan leukocyte staining
- CD45 CD3: T cells
- CD45 CD3 CD4: T helper cells
- CD45 CD3 CD4 CD25: Treg cells
- CD45 CD3 CD4-: cytotoxic T cells
- CD44 and CD62L helps differentiate between memory and effector T cells.
- NK1.1 CD3-: NK cells
- NKT CD3: NKT cells

**Staining 3:** Isotype controls: were prepared by a pooled strategy, where a small amount of each sample was included in control for each tissue type to create a universal isotype per tissue depot.
Appendix C: FACS Staining Protocol

Fluorescent Activated Cell Sorting (FACS) Staining Protocol
This protocol will be used to stain live cells (or cells that were frozen in appropriate medium for survival and thawed) with antibodies that can identify specific cell populations.

With spleen, liver, or adipose tissue samples it is easiest to use a 96-round bottom well plate in order to process all the samples.

There are many different ways the samples can be plated, you will have to come up with a configuration that best fits your experiment!
To provide an example: 2 stainings, stain 1 = macrophages 8 antibodies so 8 different colors, stain 2 = T cells 8 antibodies again, stain 3 = controls. 4 groups (A,B,C,D) n=6 in each group, For spleen (S) and IP (intraperitoneal wash)
In each well, the code is S or IP for the cells, the Group ABCD, and sample number 1-6 so SC3 is spleen group C sample #3.

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<td>IPD3</td>
<td>IPD4</td>
<td>IPD5</td>
<td>IPD6</td>
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</table>
Controls can be individual stains for each sample, or pooled based on all the samples. Pooling is much easier and will require usually 1 tube if you are running one tissue type like spleen or for example if using fat, multiple control tubes depending on the number of depots used. So if you use all 4 depots you will have 4 control tubes SubQ, Epi, Mes, Retro. Each tube will contain cells from each sample in the experimental group.

Steps:

1. After recovering the cells from cultures/tissues/organs, they are currently suspended in 500 uL of KHB or cold PBS solution.
2. Transfer 50 uL of a sample to a well, if there are multiple stains, such as the example provided above, then 50 uL of sample will go in a well for stain 1, and 50 uL of a sample will go in a well for stain 2. SEE THE EXAMPLE SET UP ABOVE!
3. Wash the samples with 100 uL of FACS buffer solution; by adding 100uL of FACS buffer to each well, you can use a multichannel pipette (like the ones used for ELISAS)
4. Centrifuge the plate at 1200 rpm for 10 minutes.
5. Discard the supernatant, you can do this by gently shaking off the supernatant into the sink.
6. Resuspend in FACS blocking buffer 100 uL per well, use the same method as stated in step 3 with the multichannel pipette.
   a. FACS Blocking Buffer = FACS buffer plus 10% horse serum.
7. Incubate 20 minutes on ice.
8. Prepare the antibody staining mixtures while the plate is incubating on ice.
   a. Consider a concentration of 0.25 to 0.5 mg/ml of antibody per sample
   b. So if we are using a concentration of 0.35 in order to determine the amount of antibody necessary to add to the master solution we must take into consideration the number of samples we have.
   c. For stain 1: we have 24 samples for spleen and 24 samples for IP, which results in 48 samples total. To have a concentration of 0.35 we must take 0.35uL x 48 = 16.8 uL of each antibody must be added to the master solution of stain 1. The equation is [desired antibody concentration] x # of samples = Amount of each antibody added to master solution. The same number is also used for stain 2 and controls as well.
   d. Since the mixture of antibodies alone will not be enough to fill all 48 wells per stain, and a 96 well plate holds maximum of 5 mLs and since stain 1 uses half, 2.5mL, we must add enough FACS buffer to the antibody mix to equal 2.5mL total in order to be able to fill all the wells with 50 uL of stain solution each. So in the example state above, we are using 8 antibodies per stain.
      i. 8 x 16.8 = 134.4 uL of antibody in master solution
ii. $2500\text{uL} - 134.4\text{uL} = 2365.6\text{uL}$ of FACS buffer added to equal 2.5ml total in the master solution

iii. So the equation is $\# \text{of antibodies used in stain (1-8)} \times \text{amount of each depending on concentration} = \text{total antibody in master solution}$

iv. Depending on the $\# \text{wells used per stain on the plate}$ the $\text{mL of 5mL} - \text{amount of antibody in master} = \text{amount of FACS buffer need to maximize the solution}$

9. Add 50uL of antibody mixture to each well for the desired stain.

10. Incubate on ice for 40 minutes in the dark, so cover it! With a Styrofoam lid or foil!

11. After incubation, add 100uL of FACS buffer to each well, centrifuge, for 5 minutes at 1200 rpm.

12. Discard the supernatant, add 200uL of FACS buffer/well and centrifuge again for 5 minutes at 1200 rpm.

13. After this centrifuge, discard the supernatant and resuspend in 50 uL of FACS buffer.

14. Prepare a 1/300 or 1/400 dilution of streptavidin PE-Texas red in FACS buffer
   a. So we need 50uL of this solution per well... $50\text{uL} \times 96 \text{ wells} = 4800\text{uL}$
   b. $4800\text{uL} \times (1/300)$ Streptavidin = $16\text{uL}$ of streptavidin added to $4800\text{uL}$ of FACS buffer
   c. This is added to each well so you can use the multichannel pipette for this procedure.

15. Add 50uL of this solution to each well, incubate on ice covered with aluminum foil for 30 minutes.

16. After incubation, add 100uL of FACS buffer to each well, centrifuge, for 5 minutes at 1200 rpm.

17. Discard the supernatant, add 200uL of FACS buffer/well and centrifuge again for 5 minutes at 1200 rpm.

18. After this centrifuge, discard the supernatant and resuspend in 50 uL of Cytofix.
   a. This is a formalin fixing solution that will allow us to store the samples for a long time! Which is nice because you can come back another time and run the samples in the cytometer!

19. Place the lid on the plate, label, and wrap with parafilm just to make sure the lid doesn’t slip off or if it is bumped or moved in the refrigerator all the samples are okay!

20. ****REMEMBER YOU HAVE TWO CONTROL TUBES! Which for each antibody color which is determined by the lasers that are available in the cytometer, we will have isotype controls. Therefore you follow the exact same steps taking into account only two control tubes. Making the antibody solutions are the same, and incubation will follow exactly the same! If you choose to include a control stain for each sample it is possible to set up the plate like so…
stain 1 = macrophages 8 antibodies so 8 different colors, stain 2 = T cells 8 antibodies again, stain 3 = controls

Samples are grouped by column so stain 1 for the 4 groups in columns 1-4, stain 2 for 4 groups is 5-8, a control stain for each sample is columns 9-12
** But this can only be used for one tissue type, so this would work for 4 adipose tissue depots, or 4 groups of spleen samples, or 4 groups of IP samples. This is not like the previous plate that had spleen and IP on the same plate, with pooled controls but it is another option.

SO… when it comes time to run your samples! You have all of them in a 96 well plate!
Steps to run on the cytometer:
1. Get the 15 ml round bottom BD falcon tubes (those are available in the ARC, in Dr. Benencia’s lab)
2. Label the tubes according to stain, tissue, and sample number, you can use the same system for the 96 well plate
3. Transfer the sample in a well to the corresponding tube and add 200 uL of PBS.
4. Vortex the solution
5. Repeat for each sample.
## Appendix D: ANOVA Main Effects

Table 8. Main Effects of Two-Way ANOVA

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Significant Effects Observed</th>
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<tbody>
<tr>
<td>Tissue Weight (g)</td>
<td>Genotype (P=0.024)</td>
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<tr>
<td></td>
<td>Depot (P&lt;0.05)</td>
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<td></td>
<td>Genotype x depot (P=0.619)</td>
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<tr>
<td>Tissue Weight/g Body Weight (%)</td>
<td>Genotype (P&lt;0.05)</td>
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<tr>
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<td>Depot (P&lt;0.05)</td>
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<tr>
<td></td>
<td>Genotype x depot (P=0.099)</td>
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<tr>
<td>Total SVF Cells</td>
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<td>Depot (P=0.138)</td>
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<tr>
<td></td>
<td>Genotype x depot (P=0.008)</td>
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<tr>
<td>Total SVF Cells/g of Tissue (%)</td>
<td>Genotype (P&lt;0.05)</td>
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<td>Depot (P&lt;0.05)</td>
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<td></td>
<td>Genotype x depot (P=0.008)</td>
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<tr>
<td>Total Leukocytes (CD45+)</td>
<td>Genotype (P=0.437)</td>
</tr>
<tr>
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<td>Depot (P=0.001)</td>
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<tr>
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<td>Genotype x depot (P=0.012)</td>
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<tr>
<td>Adipose Tissue Macrophages (F4/80+, CD11b+)</td>
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<td>Genotype x depot (P=0.012)</td>
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<td>Activated Macrophages (MHC-II)</td>
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<td>Genotype x depot (P&lt;0.05)</td>
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<td>M2 ATMs (CD206+, CD36+)</td>
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<td>Depot (P=0.002)</td>
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Note. Significance at $p<0.05$. 