The Development of Polymer Constructs for Adipose Tissue Engineering Applications

Kendall Murphy

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THE DEVELOPMENT OF POLYMER CONSTRUCTS FOR ADIPOSE TISSUE ENGINEERING APPLICATIONS

by

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ABSTRACT

The adipose tissue functions as the body’s main energy reservoir and plays a central role in maintaining whole body energy homeostasis. The ability to modulate this tissue’s inherent endocrine and metabolic functions has promising implications in treating disease associated with adipose tissue dysfunction. This work revolves around two diseases where adipose tissue inflammation and metabolic dysfunction drive the disease, obesity and cachexia. Both diseases impact a significant population of U.S. adults and substantially reduce patient quality of life.

In this study, we first demonstrate the use of novel therapeutic platforms engineered to specifically target adipose tissue inflammation and lipid catabolism through localized drug delivery for the treatment of obesity. Specifically, we developed poly(lactide-co-glycolide) scaffolds loaded with resveratrol, a small molecule with promising anti-obesity and anti-inflammatory properties, but one that suffers from poor bioavailability. Implant into the epididymal fat of lean mice indicates that resveratrol augments an anti-inflammatory environment established by PLG scaffolds without drug. Furthermore, this strategy protected against inflammatory stimuli, such as mice fed a high fat diet and adipocytes treated with pro-inflammatory cytokines. Additionally, mice pre-treated with resveratrol loaded scaffolds and then fed a high fat diet gained significantly less body weight and adipose tissue mass compared to mice that received scaffolds without the drug.
Collectively, this shows that PLG scaffolds are a promising platform for the treatment of metabolic diseases.

Secondly, we characterize the impact of chemotherapy treatment on adipose tissue remodeling as a model for cancer associated cachexia. Here we report for the first time that a clinically relevant bolus of doxorubicin significantly reduces animal body weight and induces fibrosis in subcutaneous adipose tissue in female rats. Similar to cachexic patients, this response was associated with an increase in collagen 1 and a marker of activated fibroblasts. Finally, we indicate that the subcutaneous adipose tissue exhibited greater fibrosis compared to visceral adipose tissue. This work is expected to provide greater understanding of doxorubicin’s potential role in promoting cancer-associated cachexia and provide insight for the development of future strategies to sustain adipose tissue health during chemotherapy treatment.
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CHAPTER 1
BACKGROUND AND SIGNIFICANCE

1.1 Obesity Epidemic

Obesity is a metabolic disease defined as excessive fat accumulation\(^1\). A patient is clinically considered obese when his or her body mass index (BMI), calculated as weight (kg) / height (m\(^2\)), is greater than or equal to 30 and severely obese when his or her BMI is greater than or equal to 40\(^2\). The Center for Disease Control and Prevention’s recent report states that 42.4% of U.S. adults (age 18 and older) are considered obese and 9.2% are considered severely obese based on the previously stated definitions\(^2\). In 2000, the prevalence of obesity and severe obesity in U.S. adults were 30.5% and 4.7%, respectively, indicating a striking increase in the percent of both populations over the last two decades\(^2\). Therefore, a significant portion of the U.S population deals with this disease and its associated diseases, as obesity is a key risk factor for several chronic and life-threatening diseases, including heart disease, stroke, type 2 diabetes, and cancer\(^3\). As the prevalence of obese patients increase, it is likely that the prevalence of these comorbidities will increase as well. Relevant to the current global public health crisis, obesity was determined to be a risk factor for more severe outcomes during the influenza A H1N1 pandemic of 2009 and therefore, is likely to also be a risk factor for COVID-19\(^4,5\). In addition, not only does obesity have a negative impact on patient quality of life in terms of mobility, productivity, and mental state, but also has a significant economic impact as well. For example, the
healthcare costs of obese patients are approximately 30% higher than patients with a normal weight. Furthermore, annual medical cost from treating obesity-related disorders in the U.S. is projected to increase by $28 billion per year by 2020 and $66 billion per year by 2030 due to the increase in prevalence of obesity and obesity-related disorders. Collectively, continued increase in obesity prevalence and the disease’s overwhelming health and economic burdens signify the need for continued research activities to further our understanding of the biological events driving the disease and to develop novel therapeutic platforms.

1.2 Adipose Tissue Function

Obesity is associated with excess accumulation of fat, or adipose tissue, which is the main organ involved in the progression of this disease. This tissue functions as the body’s main energy reservoir and plays a central role in maintaining whole body energy homeostasis. Adipocytes, the tissue’s parenchymal cell, accomplish this by storing excess caloric energy in the form of triglycerides in specialized lipid droplets. As other organs in the body require energy, triglycerides are broken down into fatty acids, via a process called lipolysis, released into the blood stream and are ultimately taken up by the energy demanding organs.

Decades of research indicate a key function of adipose tissue as a storage site for lipids; however, in the last two decades, it has become well accepted that this tissue also functions as an endocrine organ. Indeed, the adipose tissue regulates metabolism, immunity, reproduction, and many other processes required for homeostasis through the hormones, cytokines, and other factors it secretes. For example, the adipose tissue modulates whole body metabolic systems through the release of adipokines from
adipocytes which travel to distant organs via the blood stream to promote certain responses critical to metabolic health. Commonly studied adipokine examples include adiponectin, an adipokine involved in increasing glucose uptake and lipid metabolism\textsuperscript{13}, and leptin, which controls satiety\textsuperscript{10}.

The adipocyte is not the only endocrine acting cell in the adipose tissue. In fact, the adipose tissue is comprised of several other cell types, such as immune cells and stromovascular cells, that secrete various factors that can signal in both an endocrine and paracrine manner, impacting distant tissues as well as local adipocytes. Macrophages, an innate immune cell, make up 5\% of the total cells within the adipose tissue and is the largest immune cell population residing within the tissue\textsuperscript{14}. Under lean conditions, adipose tissue resident macrophages possess an anti-inflammatory phenotype in which they produce anti-inflammatory cytokines, lipid buffering, and promote insulin sensitivity\textsuperscript{15}. In addition, adipose tissue macrophages also play a key role in immune surveillance and removal of dying cells via efferocytosis within the tissue\textsuperscript{14,16}. Furthermore, macrophages and their secreted factors control adipocyte metabolic function, including lipolysis and differentiation\textsuperscript{16}. For example, anti-inflammatory macrophages enhance mitochondrial activity in adipocytes in vitro via both direct-contact and non-contact mechanisms, suggesting the role of soluble factors\textsuperscript{17}. Undoubtedly, adipose tissue function is inextricably linked to the immune cells present within it and their phenotype. The following section will further elaborate on the role macrophages play during obesity induced adipose tissue dysfunction.
1.3 Adipose Tissue Dysfunction During Metabolic Disease

Looking at lipid metabolism through the eyes of a chemical engineer, one should imagine an energy balance, where the amount of energy taken into the system (i.e. eaten) should balance with the amount of energy needed by the system to function (energy expenditure). In the case of chronic overnutrition, there is a higher amount of energy going into the body than what the body requires; therefore, this energy must be stored. Adipocytes store this energy in the form of lipids in specialized droplets. Therefore, during obesity, lipid droplets become larger and in turn increase the size of the adipocytes, termed hypertrophy. However, the adipose tissue has limited storage capacity; therefore, chronic overnutrition can lead to an accumulation of lipid metabolites in other tissues, such as the liver and skeletal muscle.

Lipid accumulation in the adipose tissue leads to local inflammation as the number of macrophages are significantly increased in the adipose tissue during obesity, making up to 40% of all adipose tissue cells in obese mice. Environmental changes that occur in the adipose tissue during obesity development, such as an increase in lipid exposure and hypoxia, activates resident macrophages and adipocytes to secrete pro-inflammatory chemokines and cytokines, such as monocyte chemoattractant protein-1 (MCP-1), TNF-α, and IL-6. Pro-inflammatory cytokine signaling can significantly modulate adipocyte metabolism. For example, TNF-α signaling leads to reduced glucose uptake, increased lipolysis, and potentially reduced adipocyte differentiation and lipogenesis. Through the release of these soluble factors, the increase in the number of proinflammatory macrophages in the tissue has been suggested to contribute to local and systemic insulin resistance, potentially leading to a type 2 diabetic state.
1.4 Current Obesity Treatment Strategies

Current methods to mitigate this disease include lifestyle changes, pharmaceuticals, and bariatric surgery. Each of these strategies pose challenges that hinder their effectiveness. First, diet and exercise alone do not typically result in long term outcomes for those with severe obesity. For example, severely obese patients who participated in a dietary and physical activity intervention plan and group support sessions lost approximately 9% of their body weight within the first year; however, these participants gained almost half of their loss back by year four.\(^22\)

There are several FDA approved medications that are prescribed to patients to be taken in combination with lifestyle interventions as a weight loss regimen. Some of these medications work to make patients feel satiated faster, such as Lorcaserin, and others work to reduce the amount of fat absorbed after eating, such as Orlistat\(^23\). In fact, patients who supplement their lifestyle intervention regimen with FDA approved weight loss drugs lose 3-9% more of their starting weight compared to those patients who do not\(^24\). However, the severe side effects associated with these drugs, including increased heart rate and diarrhea among several others, deters their use. Furthermore, in February 2020, the FDA requested to withdraw Lorcaserin from the market due to an increase in potential cancer risk\(^21\). Therefore, these drugs are considered effective in achieving weight loss; however, their negative effects likely out ways their benefits.

Finally, bariatric surgery, an option for the morbidly obese, is highly invasive and comes with high risks of post-operative or chronic complications\(^25\). For example, sleeve gastrectomy is a common bariatric surgery where the patient’s overall stomach size is reduced by 25%. One in every 5 sleeve gastrectomy patients develop gastroesophageal
reflux disease (GERD), which is associated with frequent and severe heartburn, vomiting, and difficulty swallowing, leading to maladaptive eating and possible weight gain\textsuperscript{25}. If GERD does not improve naturally, medications are used to manage the severe symptoms\textsuperscript{26}. Due to the ineffectiveness and inconveniences of the current obesity management strategies, it is quite evident that novel therapeutic strategies must be developed. These strategies should be minimally invasive, prevent unwanted side effects associated with systemically administered drugs and bariatric surgeries, and designed to also be combined with diet and exercise.

\section{1.5 Biomaterials for Tissue Engineering Applications}

Tissue engineering is a sophisticated collaboration between engineering, life sciences, pharmaceutical science, and material science. The primary goal of tissue engineers is to use a combination of biocompatible materials, drugs, and biological products, such as cells and proteins, to repair injured tissue or direct tissue function\textsuperscript{27}. Generally, biomaterials developed for these applications are designed to allow for cell mobility and extracellular matrix deposition in order to enhance cell survival and integrate the material with the host tissue\textsuperscript{28}. Therefore, a common construct for tissue engineering applications is in the form of porous scaffolds fabricated from either biological or synthetic materials which can be loaded with drugs, proteins, cells, or a combination thereof to promote desired tissue events\textsuperscript{29}. Undoubtedly, one of the most commonly used synthetic polymers for this application is poly(lactide-co-glycolide) (PLG), likely due to its biocompatibility, biodegradability, and previous FDA approval\textsuperscript{30}. One example of an FDA approved PLG construct is Zoladex, a PLG matrix loaded with goserelin acetate that is
used as a prostate cancer treatment\textsuperscript{31}. A central focus of this dissertation is the use of porous PLG scaffolds for adipose tissue engineering.

Polymer scaffolds have been successfully utilized as platforms for adipose tissue engineering. For example, they have been supplemented with adipose derived stem cells, immune cells, and/or a host of different stimuli to generate healthy adipose tissue \textit{in vivo} as a repair mechanism for soft tissue defects as well as to develop 3-dimensional \textit{in vitro} models to better study adipose tissue biology and potential obesity therapeutics in the dish\textsuperscript{32–34}. Furthermore, polymer scaffolds have extensively been used to enhance the survival of endocrine cell (islets) grafts upon implant into the adipose tissue\textsuperscript{28,35–37}. However, to our knowledge, little efforts have been taken to use these devices to directly repair injured adipose tissue or impact its inherent metabolic or endocrine functions. Furthermore, little research has tested the benefit of these scaffolds as drug delivery vehicles to directly target the adipose tissue. We propose that polymer scaffolds could be a very convenient strategy to deliver promising anti-obesity drugs whose success may otherwise be hindered by poor bioavailability or harsh side effects. Furthermore, while scaffolds have been implanted into the adipose tissue previously, their effects on the adipose tissue immune compartment and lipid metabolism remains uncharacterized.

1.6 Host Response to Biomaterial Implants

A central aim for tissue engineers is to understand the immune response to biomaterial implants and develop strategies to mitigating or direct this response that ensues upon implant of foreign materials. The immune response to biomaterial implants within the adipose tissue remains largely uncharacterized; however, studies of subcutaneous biomaterial implant indicates a cascade of events that make up an acute inflammatory
phase, chronic inflammatory phase, and wound healing phase. Upon implant, injury disrupts homeostasis within the tissue and the adsorption of proteins on the surface of the material induces the acute inflammation phase. During this phase, proinflammatory cytokines such as tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) as well as chemokines, such as CXCL1 and monocyte chemotactic protein 1 (MCP-1), are secreted to recruit neutrophils and monocytes from the bloodstream and into the implant site. If the material is not fully degraded during the acute phase, the chronic inflammation phase follows and is characterized by the presence of macrophages, monocytes, lymphocytes, such as natural killer (NK) cells, T cells, and B cells at the implant site. The wound healing process begins with the resolution of inflammation through the increase of anti-inflammatory cytokines, such interleukin-10 (IL-10), interleukin-4 (IL-4) and interleukin-13 (IL-13), downregulation of inflammatory mediators, and apoptosis of immune cells. IL-4 and IL-13 stimulate macrophages to fuse together to form multinucleated giant cells at the biomaterial interface. Furthermore, anti-inflammatory macrophages stimulate extracellular matrix remodeling and fibroblast activation resulting in the encapsulation of the material. In terms of developing implantable devices to modulate adipose tissue function for the treatment of metabolic disease, it would be beneficial to limit inflammatory cytokine production upon implant while simultaneously enhancing anti-inflammatory cytokines that may improve adipose tissue health. This is particularly important since excess adipose tissue inflammation characterized by inflammatory immune cell infiltration and inflammatory cytokine production is associated with metabolic disease progression.
1.7 Fundamentals of PLG Drug Delivery Devices

1.7.1 Fabrication of PLG Microparticles and Scaffolds

PLG is an attractive polymer to use in the development of drug delivery devices due to its biodegradability, biocompatibility, and its easily tailored characteristics, such as molecular weight and lactide:glycolide ratio (composition)\(^4\). In addition, it is easily fabricated into drug delivery devices\(^2\). Extensively used PLG drug delivery constructs are in the forms of microparticles and scaffolds\(^1\). Our work revolves around loading drugs into PLG microparticles via a single emulsion (oil in water) solvent evaporation process\(^3\).

Briefly, PLG and drug of choice are dissolved in an organic solvent and then this mixture is emulsified using a homogenizer in the presence of an emulsifier (such as polyvinyl alcohol). This mixture is added to water in which the organic solvent is extracted thus hardening the oil droplets into particles. The particles are washed and removed via centrifugation and subsequent lyophilization. Certainly, one can directly inject these particles for various applications, but in the case of our work, we then mix these particles with a sacrificial porogen, salt, to form our porous drug loaded PLG scaffolds. Briefly, the particle salt mixture is pelleted under a ton of pressure in a die of specific dimensions and this pellet is then placed in a custom-made pressure vessel exposed to high-pressure CO\(_2\) gas. In this environment, the glass transition temperature of PLG is reduced, therefore liquifying the polymer surrounding the salt crystals\(^4\). After a pressurization and depressurization sequence, the particles return to a solid state fused around the salt crystals\(^4\), which is leached from the scaffold after washing in deionized water leaving a porous drug loaded matrix. Once fabricated, scaffolds are dissolved in an organic solvent and measured against a standard curve using a spectrophotometer to determine initial drug
loading. Furthermore, drug release kinetics are determined by incubating drug loaded scaffolds in deionized water at 37 °C and 5% CO$_2$ and then measuring the amount of drug in the scaffold after certain time frames. Several polymer properties can be modified prior to fabrication that will impact drug release from the scaffold. PLG degradation fundamentals and how polymer properties alter degradation are further discussed in the next section.

1.7.2 Factors that Effect Release Kinetics

PLG is deemed biodegradable as it degrades in water into lactic acid and glycolic acid via hydrolysis of its ester linkages$^{45}$. Degradation highly depends on bulk erosion such that water penetrates the matrix and hydrolyzes the polymer into oligomers and then monomers$^{41}$. A biphasic drug release profile is commonly observed using PLG devices where an initial burst of drug is followed by a stable release phase. The initial burst is due to drug on the surface as well as initial penetration of water that begins to decrease the molecular weight of the polymer, but has not hydrolyzed the polymer to the point of monomer production$^{41}$. In the second phase, drug diffuses through the polymer layer as water hydrolyzes the polymer into oligomers and monomers$^{41}$. Polymer degradation can be modified by altering polymer characteristics, such as composition, molecular weight, and matrix size and geometry$^{42}$. For example, degradation rate increases with the glycolic portion of the polymer composition; therefore, a 65:35 lactic acid to glycolic acid ratio degrades faster than a 75:25 ratio$^{41}$. Furthermore, higher molecular weight polymers generally exhibit slower degradation rates. Lastly, the size and shape of the matrix will impact the surface area to volume ratio, which increases the degradation rate$^{42}$. Therefore,
PLG is a suitable material to achieve desired delivery kinetics of various drugs as the degradation kinetics can be easily altered by changing polymer properties.

1.8 Resveratrol

Resveratrol (trans-3,5,4′-trihydroxystilbene) is a naturally occurring polyphenol found in various plants, such as grape skins and peanuts, that, in plants, protects the plant against ultraviolet rays, pathogens, injury, and abiotic stresses\(^\text{46}\). Since the “French paradox” was termed, a proposal that wine consumption protected French people from heart disease despite their consumption of a high fat diet, resveratrol has established a reputation of providing health benefits\(^\text{47}\). Relevant to our overall work, this molecule has been extensively studied for its anti-inflammatory and anti-obesity properties\(^\text{48}\). For example, cultured adipocytes treated with conditioned media from resveratrol and LPS treated RAW 264.7 macrophages protected cells from elevated levels of TNF-α and IL-6 and inflammation-related changes in adipokine gene expression\(^\text{49}\). In addition, oral resveratrol treatment significantly reduced body weight and adipose tissue mass in high fat diet fed and genetically obese rodents\(^\text{50–53}\). Much research indicates that resveratrol enhances activity of proteins that lead to lipid mobility via lipolysis and lipid catabolism via fatty acid oxidation\(^\text{54–59}\). For example, resveratrol has shown to increase the expression of adipose triglyceride lipase (ATGL)\(^\text{52,54,55,60}\) and carnitine palmitoyl transferase 1 (CPT1)\(^\text{56–58,61}\), the rate limiting enzymes involved in lipolysis and fatty acid oxidation, respectively\(^\text{18}\). Furthermore, these effects are likely a result of resveratrol activation of AMP kinase (AMPK)\(^\text{54,59}\). Therefore, through its cellular actions in adipocytes and macrophages, resveratrol has achieved pre-clinical success as an obesity therapeutic.
However, resveratrol clinical trials have resulted in modest or no effects on metabolic parameters in obese and type 2 diabetic patients\textsuperscript{62,63}. A significant shortcoming of resveratrol’s use as an anti-obesity drug that has hindered its clinical success is its poor bioavailability\textsuperscript{64}. When taken orally, sulfate conjugation occurs rapidly in the intestine and liver, which appears to be the rate-limiting step in resveratrol’s bioavailability\textsuperscript{65}. We propose that resveratrol’s clinical effectiveness as an anti-obesity drug could be improved by locally delivering the drug to the target tissue. Therefore, resveratrol is an appropriate candidate for delivery via PLG scaffold directly to the adipose tissue in order to bypass this rapid metabolism that occurs when the drug is taken orally.

1.9 Resveratrol Loaded Polymer Scaffolds for Adipose Tissue Engineering

A central hypothesis of this dissertation is that resveratrol loaded PLG scaffolds promote favorable immune and metabolic responses upon implant into adipose tissue compared to PLG scaffolds without a drug payload. A combination of animal and cell culture studies are utilized in this project to understand how scaffold-based delivery of resveratrol modulates the immune response to PLG scaffolds and enhances local lipid metabolism after implant into adipose tissue. The results from this work aims to demonstrate that this technology is a promising novel strategy for the treatment of obesity and lays the foundation for future studies using biomaterials as a strategy to direct the adipose tissue’s inherent endocrine and metabolic functions.

1.9.1 Aim 1: Characterize the immune response to resveratrol loaded poly(lactide-co-glycolide) scaffolds after implant into adipose tissue

As biomaterial therapies are used to address adipose tissue dysfunction, the immune response to these systems must be established. This is especially important as the immune
compartment of adipose tissue can drive metabolic disease. Therefore, the first aim of this work to test the hypothesis that resveratrool delivery from PLG scaffolds would limit inflammation following implant into visceral adipose tissue compared to PLG scaffolds without drug. In this aim, we established that PLG scaffolds implanted into adipose tissue of healthy C57BL/6 mice promote an anti-inflammatory environment characterized by low levels of pro-inflammatory cytokines compared to unmanipulated fat. Resveratrool delivery augments this anti-inflammatory environment by decreasing monocyte and lymphocyte numbers at the implant site and increasing expression of anti-inflammatory cytokines. To investigate therapeutic relevancy, we investigated resveratrool delivery to induce an anti-inflammatory response in proinflammatory environments such as adipocytes challenged with TNF-α and the fat pads of mice challenged with a high fat diet. This aim results in the conclusion that PLG scaffolds are a promising platform for the treatment of adipose tissue inflammation that drives metabolic disease as the polymer alone induces an anti-inflammatory environment and that it can be easily engineered to deliver drugs that can further augment this response.

1.9.2 Aim 2: Assess the efficacy of resveratrool loaded scaffolds to enhance adipose tissue lipid metabolism

As a strategy to overcome resveratrool’s poor bioavailability, resveratrool was incorporated into the matrix of PLG scaffolds for direct delivery to the adipose tissue. The second aim of this project assessed the ability of resveratrool loaded scaffolds to enhance local lipid catabolism compared to scaffolds with no drug. Studies in this aim investigate adipocyte size and expression of proteins involved in lipid catabolism after implant in lean mice led to the proposal that resveratrool scaffolds decrease adipocyte size because
resveratrol increases lipid utilization in scaffold-infiltrating immune cells, possibly through elevating CPT1 levels or activity. Furthermore, mice pre-treated with resveratrol scaffolds and then fed a high fat diet gained significantly less total body weight and epididymal adipose tissue mass compared to mice that received scaffolds containing only polymer. Importantly, this scaffold-based strategy required a single administration compared to previous animal studies indicating that oral resveratrol delivery requires daily dosing. This work indicates that localized delivery of metabolism modulating agents to the adipose tissue may overcome issues with bioavailability and that the role of biomaterials should be further investigated as a therapeutic strategy for metabolic disease.

1.10 Understanding Adipose Tissue Remodeling in Response to Chemotherapy

A key goal of this dissertation is to develop drug loaded scaffolds to protect adipose tissue from inflammatory stimuli, such as high fat diet induced obesity. Like obesity, another instance where adipose tissue inflammation may promote the disease is cachexia. Cachexia is a condition associated with involuntary adipose tissue atrophy resulting in considerable weight loss that affects 50-80% of cancer patients and is responsible for approximately 20% of cancer-related deaths. This debilitating symptom of cancer progression significantly reduces patient quality of life and correlate with worsened prognosis. This condition may be, in part, related to the use of chemotherapies, such as doxorubicin, an extensively used chemotherapeutic agent for the treatment of a wide variety of cancers. Treatment with this chemotherapy is associated with toxicity of various organs, including heart, kidneys, and liver, in addition to other severe and unpleasant side effects. Doxorubicin’s negative effect on adipose tissue function may
play a role in cachexia development; however, only few studies have pre-clinically characterized the impact of doxorubicin treatment on adipose tissue function\textsuperscript{71–73}.

Biopsies from cachexic cancer patients have presented with subcutaneous adipose tissue architectural remodeling, such as decreased adipocyte area, increased inflammatory cell infiltration, and extracellular matrix deposition, all of which have significant negative consequences to adipose tissue function and systemic processes controlled by this tissue\textsuperscript{74,75}. Studies have investigated doxorubicin’s impact on adipocyte lipid metabolism; however, few studies have aimed to understand doxorubicin’s role in promoting adipose tissue remodeling. Furthermore, there are several critical, unanswered questions that should be addressed regarding the role of doxorubicin administered dose, dosing schedule and the gender of the studied population in pre-clinical studies. Working to answer these questions will provide greater understanding of doxorubicin’s potential role in promoting cancer-associated cachexia and provide insight for the development of future strategies to sustain adipose tissue health during chemotherapy treatment.

\textit{Aim 3: 1.10.1 Characterize the impact of a clinically relevant, chronic bolus of doxorubicin on adipose tissue remodeling in female rats}

To date, most in vivo work studying doxorubicin’s effect on adipose tissue has been tested in male rodents by injecting approximately 2 mg/kg multiple times over several weeks or once for few days\textsuperscript{71,73}. However, this does not accurately model clinical dosages of most cancer patients, which is approximately 20 mg/kg once every 3 weeks. In addition, there is a significant need to study the effect in female rodents since doxorubicin is regularly used to treat several female cancers, such as breast and ovarian cancer. Therefore, the third aim of this project assessed the effect of a single, 20 mg/kg doxorubicin bolus on
adipose tissue inflammation 9 days after injection, a time point 6 days longer than other studies using this clinically relevant dose, in female rats. Body weight and fat pad weights were assessed at the end of the study as a measure of administered doxorubicin induced cachexia. Furthermore, remodeling was assessed by histological means, such as measuring adipocyte area and number, as well as staining for extracellular matrix. A possible mechanism of fibrosis was also developed. Visceral and subcutaneous adipose tissue depots were analyzed to determine fat pad anatomical location as a factor involved in the extensiveness of doxorubicin induced inflammation. This work results in a better understanding of how doxorubicin effects visceral and subcutaneous adipose tissue inflammation in female rats.
CHAPTER 2
RESVERATROL DELIVERY FROM POROUS POLY(LACTIDE-CO-
GLYCOLIDE) SCAFFOLDS PROMOTES AN ANTI-INFLAMMATORY
ENVIRONMENT WITHIN VISCERAL ADIPOSE TISSUE¹

2.1 Introduction
While the adipose tissue is a common site for biomaterial supported delivery of endocrine cells (i.e., islets), the field has yet to investigate biomaterial strategies to modulate this tissue’s inherent endocrine function²⁸,³⁵–³⁷,⁷⁶. Indeed, the adipose tissue regulates metabolism, immunity, reproduction, and many other processes required for homeostasis through the factors it secretes⁸,¹¹,¹²,⁷⁷. Therefore, an ability to control adipose secretory function is a promising avenue to treat metabolic diseases. In devising platforms to modulate the adipose tissue, it is important to consider that (i) biomaterial implant causes local changes in immune cell populations⁷⁸ and (ii) adipose tissue function is inextricably linked to the immune cells resident within it⁷⁹,⁸⁰. Furthermore, excessive adipose tissue inflammation characterized by immune cell accumulation and inflammatory cytokine production is associated with metabolic disease¹⁵,⁸¹,⁸². Thus, from a tissue engineering perspective, it is important to understand how the adipose tissue immune environment

responds to biomaterial implants and develop strategies to modulate this response, if necessary.

The immune response to biomaterial implants within the adipose tissue remains largely uncharacterized; however, studies of subcutaneous biomaterial implant indicates there are three phases: acute inflammation, chronic inflammation, and wound healing\textsuperscript{78}. During the acute inflammation phase, proinflammatory cytokines such as tumor necrosis factor-alpha (TNF-\(\alpha\)) and interleukin-6 (IL-6), as well as chemokines, such as CXCL1 and monocyte chemotactic protein 1 (MCP-1), are secreted to recruit neutrophils and monocytes from the bloodstream and into the implant site\textsuperscript{40,78}. Chronic inflammation follows this phase and is characterized by the presence of macrophages, monocytes, lymphocytes, such as natural killer (NK) cells, T cells, B cells, and foreign body giant cells at the biomaterial interface\textsuperscript{40,78}. The wound healing process begins with the resolution of inflammation through the increase of anti-inflammatory cytokines, such as interleukin-10 (IL-10), interleukin-4 (IL-4), and interleukin-13 (IL-13), down regulation of inflammatory mediators, and apoptosis of immune cells\textsuperscript{40}. In terms of developing implantable devices to modulate adipose tissue function, especially for treatment of metabolic diseases, it would be beneficial to limit inflammatory cytokine expression upon device implant while simultaneously enhancing anti-inflammatory cytokines that might improve adipose tissue health.

In this study, we investigated resveratrol release from porous poly(lactide-co-glycolide) (PLG) scaffolds that integrate with the adipose tissue\textsuperscript{28}. Resveratrol, a naturally occurring polyphenol, was selected because it protects mice and primates from diet induced obesity, in part, through its metabolic effects on adipose tissue\textsuperscript{50,53,83–85}. In addition,
resveratrol decreases inflammation associated with biomaterial implant in bone, cartilage, and the vasculature\textsuperscript{86–91}. However, scaffold-based delivery of resveratrol to modulate the immune response to biomaterial implant in the adipose tissue has not been investigated. Therefore, we performed the following studies to investigate whether resveratrol delivery from PLG scaffolds would limit inflammation and speed its resolution following implant into visceral adipose tissue. We chose to deliver resveratrol directly from the scaffold instead of oral delivery because resveratrol suffers from poor bioavailability and requires frequent large doses to address adipose tissue inflammation\textsuperscript{50,53,65,83}. To investigate therapeutic efficacy of our strategy, we determined whether resveratrol delivery from scaffolds could induce an anti-inflammatory response in proinflammatory environments including adipocytes challenged with TNF-\textalpha and the fat pads of mice fed a high fat diet.

2.2 Materials and Methods

2.2.1 Particle Fabrication

Particles were produced by an oil-in-water emulsion-solvent evaporation technique. The oil phase consisted of a 3:1 mixture of dichloromethane (Sigma) and ethanol (Sigma) containing 6\% w/w PLG (purchased from Evonik, 75:25 mole ratio lactide to glycolide, 0.76 dL/g) and 10 mg/mL of resveratrol (Sigma). The aqueous phase consisted of 1\% w/v PVA (Sigma) dissolved in ultrapure water. The oil phase was homogenized with the aqueous phase using 1:7 volume ratio with a benchtop homogenizer. The emulsion was then immediately added to ultrapure water and stirred for 5 hour to allow particles to harden and organic solvents to evaporate. Particles were collected via centrifugation, washed with ultrapure water, and lyophilized.
2.2.2 Scaffold Fabrication

Scaffolds were fabricated by mixing PLG particles with 250-500 µm NaCl particles in a 1:30 ratio, and the mixture was then pelleted in a die. Scaffolds were gas-foamed using 800 psi CO\textsubscript{2} at room temperature in a pressure vessel. The salt porogen was removed by washing in ultrapure water. Complete salt removal was confirmed by scaffold weight and microscopy. Scaffolds that did not contain resveratrol were fabricated from particles produced using an oil phase that did not contain resveratrol or ethanol.

2.2.3 Scanning Electron Microscopy

Particles or scaffolds were added to carbon adhesive tape on aluminum stubs and gold sputtered three times for 1 minute with a Denton Desk II vacuum sputter coater. Compressed air was used to ensure a monolayer of particles prior to sputtering. Images were taken on a TESCAN Vega3 scanning electron microscope at 10 kV.

2.2.4 Measuring Resveratrol Loading Capacity and Encapsulation Efficiency in PLG particles and Scaffolds

Particles and scaffolds were weighed and then dissolved in a defined volume of DMSO (Sigma) and analyzed for absorbance at 330 nm, which is the maximum absorbance wavelength for these solutions as determined by a wavelength scan (Fig 2.1). PLG in the solution did not affect resveratrol’s absorbance spectrum (data not shown). Absorbance values were then compared to a nine-point standard curve allowing for the interpolation of the unknown resveratrol concentration in the sample. Standard curves were highly reproducible between experiments (Fig 2.2). Particle loading capacity (PLC) and scaffold loading capacity (SLC) was determined using equations 1 and 2, respectively.
\[
\text{PLC} \left( \frac{\mu g}{mg} \right) = \frac{M_{P-RSV} (\mu g)}{M_p (mg)} \tag{1}
\]

\[
\text{SLC} \left( \frac{\mu g}{mg} \right) = \frac{M_{S-RSV} (\mu g)}{M_S (mg)} \tag{2}
\]

\(M_{P-RSV}\) and \(M_{S-RSV}\) is the mass of resveratrol measured in particles and scaffolds, respectively. \(M_p\) and \(M_S\) is the mass of particles and scaffold analyzed. To investigate the effect of storage on resveratrol loading capacity, particles or scaffolds were analyzed for resveratrol at 2 and 52 weeks after fabrication.

Resveratrol encapsulation efficiency was calculated for particles (\(EE_p\)) and scaffolds (\(EE_s\)) using eqs 3 and 4, respectively.

\[
EE_p (%) = \left( \frac{M_{P-Recovered} (mg) \ast \text{PLC} \left( \frac{\mu g}{mg} \right)}{M_{RSV-Emulsion} (mg)} \right) \ast 100 \tag{3}
\]

\[
EE_s (%) = \left( \frac{M_S (mg) \ast \text{SLC} \left( \frac{\mu g}{mg} \right)}{M_{P-Scaffold \ Fabrication} (mg) \ast \text{PLC} \left( \frac{\mu g}{mg} \right)} \right) \ast 100 \tag{4}
\]

\(M_{P-Recovered}\) is the mass of particles recovered after particle fabrication, \(M_{RSV-Emulsion}\) is the mass of resveratrol added to the emulsion, and \(M_{P-Scaffold \ Fabrication}\) is the mass of particles used in scaffold fabrication.

2.2.5 3T3-L1 Cell Culture

3T3-L1 fibroblasts (ATCC) were differentiated into adipocytes following ATCC’s protocol, with slight modifications. Briefly, 3T3-L1 cells were seeded in 6-well plates at
80000 cells/well or 24-well plates at 20000 cells/well and cultured in DMEM (Corning Cellgro) supplemented with 10% Super Calf Serum (GemCell) and 1% Pen/Strep (Fisher). Forty-eight hours after confluence was reached, media was exchanged with differentiation media consisting of DMEM supplemented with 10% FBS (Fisher), 10 µg/mL bovine insulin (Sigma), 0.25 µM dexamethasone (Sigma), and 0.5 mM isobutylmethylxanthine (Sigma). Forty-eight hours after differentiation media was added, media was exchanged for DMEM supplemented with 10% FBS and 10 µg/mL bovine insulin. After another 48 hours, media was exchanged for DMEM supplemented with 10% FBS. Cells were then cultured until treatment with particles or scaffolds, which occurred 9 days after exposure to differentiation media.

2.2.6 Particle Treatment of 3T3-L1 Adipocytes and Oil Red O Assay

On day 9 of differentiation, 3T3-L1 adipocytes in 6 well plates were treated with 300 µg/mL of PLG particles, PLG+RSV particles 2 weeks after fabrication, or PLG+RSV 52 weeks after fabrication. As a positive control, 3T3-L1 adipocytes were treated with 60 µM resveratrol. Forty-eight hours later, cells were washed and then fixed using 10% formalin in PBS. Immediately before the experiment, particles were washed in 35% ethanol in water and then suspended in DMEM media.

An Oil Red O assay was performed on fixed cells to visualize their lipid content. Cells were washed with ultrapure water followed by 60% isopropanol. Cells were then incubated with Oil Red O (Sigma) for 10 min at room temperature followed by four washes with ultrapure water to ensure removal of unbound Oil Red O. Images were taken on Nikon Eclipse Ci microscope with a 4x objective. To quantify the staining, Oil Red O was extracted from the cells by incubating them in 100% isopropanol for 10 min at room
temperature. Absorbance of the isopropanol solution was then measured at 500 nm using a spectrophotometer. Absorbance was normalized to that of untreated differentiated cells.

2.2.7 Resveratrol In Vitro Release Assay

To investigate resveratrol release, scaffolds were weighed and then incubated under ultrapure water at 37 °C for 0.25, 1, 3, 7, or 14 days. At the end of the assay, scaffolds were retrieved from the incubator and stored in a desiccator until dry. Dryness was confirmed by scaffold weight. The amount of resveratrol remaining in the scaffold was then measured as described in Section 2.2.4.

2.2.8 Animal Care and Scaffold Implant

All animal procedures were approved by the University of South Carolina Institutional Animal Care and Use Committee. Six week-old male C57BL/6 mice were purchased from Jackson Laboratories. Mice were allowed to acclimate for 2 weeks. Prior to implant, all scaffolds were washed in 70% ethanol and then rinsed in sterile PBS. Mice were anesthetized with isoflurane, and the surgical site was shaved and disinfected with betadine and alcohol. Following a lower abdominal midline incision one scaffold was wrapped into each epididymal fat pad (i.e., each mouse received two scaffolds). The abdominal wall was then closed with a running stitch and the skin was closed with wound clips. Epididymal fat pads were collected for analysis 3, 7, or 14 days after implant.

2.2.9 Flow Cytometry

Seven and 14 days after implants, epididymal fat pads were harvested following euthanasia and washed in ice cold PBS (Sigma). Fat pads were minced, digested in collagenase (Liberase TL, Roche), and passed through a 100 μm filter. The stromal vascular fraction was harvested by centrifugation, washed in MACS buffer (PBS, 0.5 mM
EDTA, 30% BSA) and incubated with anti-CD16/32 prior to adding an antibody cocktail against extracellular antigens. The following antibodies were purchased from Biolegend: anti-CD45 clone 30-F11, anti-Ly6G clone 1A8, anti-F4/80 clone BM8, anti-NK1.1 clone PK136, anti-CD19 clone 6D5, anti-CD11b clone M1/70, anti-CD3 clone 17A2, and Trustain fcX (anti-CD16/32) clone 93. The following isotype controls were also purchased from Biolegend: mouse IgG2a clone MOPC-173, rat IgG2a clone RTK2758, rat IgG2b clone RTK4530. After antibody incubation, cells were washed, fixed, and analyzed using a FACS Aria flow cytometry (BD Biosciences). The number of CD45 cells in each flow cytometry sample was calculated using Bang’s Laboratories Flow Cytometry Absolute Count Standard, which was added prior to data acquisition. FlowJo software (Treestar) was utilized to compensate and analyze data. FMOs with isotype controls were used to determine specific antibody signal. The gating scheme used in the flow cytometry analysis is depicted in Figure 2.3.

2.2.10 Enzyme Linked Immunosorbent Assay (ELISA)

Following euthanasia, epididymal fat pads were harvested at 3, 7, or 14 days after scaffold implant, washed in ice cold PBS, frozen on dry ice, and stored at -80°C until homogenization. Prior to homogenization, tissues were weighed and then homogenized in a defined volume of RIPA buffer containing Halt Protease Inhibitor Cocktail (Thermo Fisher Scientific), PhosStop (Sigma), and phenylmethylsulfonyl fluoride (PMSF) protease inhibitor (Thermo Fisher Scientific) using a benchtop homogenizer. Insoluble materials was removed by centrifugation. Whole tissue homogenate samples were aliquotted and stored at -80°C until use.
All cytokines were measured in whole tissue homogenate using Duoset ELISA kits (R&D Systems); however, several modifications were made to the manufacturer’s instructions to improve sensitivity. A high binding 96-well plate was incubated with capture antibody overnight at 4°C. The plate was then blocked with 10% BSA for 3 hours at room temperature. Samples were incubated on the plate overnight at 4°C. Detection antibody incubation lasted for 3 hours at room temperature before incubation with streptavidin-HRP for 2 hours at room temperature. After incubation with the substrate for 20 minutes, stop solution was added to stop the enzymatic reaction, and absorbance was measured at 450 nm. Background was removed by measuring absorbance at 540 nm. A standard curve was used to determine each cytokine concentration in each sample.

2.2.11 Western Blotting

Whole tissue homogenate was obtained as stated in the previous section. Total protein in the sample was quantified using the Pierce BCA protein assay kit (Thermo Fisher Scientific). 30-50 µg of protein was separated by 8-15% SDS polyacrylamide gel electrophoresis, depending on targeted molecular weight, and transferred to 0.2 µm nitrocellulose membranes. The membranes were blocked with 5% nonfat dry milk in Tris-buffered saline containing Tween-20 (TBST) for 1 hour at room temperature. Primary antibodies for Bax, Mcl-1, and IL-6 were purchased from Cell Signaling Technologies, CD206 was purchased from Abcam, and arginase-1 was purchased from Santa Cruz Biotechnology. Each primary antibody was added to each blot at 1:1000 dilution, with the exception was arginase-1 which was added at 1:100 dilution, in 5% nonfat dry milk in TBST overnight at 4°C. Each membrane was then washed with TBST before incubation with a polyclonal secondary antibody (Abcam) or, in the case of arginase-1, a monoclonal
secondary antibody (Biorad), conjugated to horseradish peroxidase for 1 hour at room temperature. The blots were then developed with SuperSignal enhanced chemiluminescent substrate solution (Pierce). To verify equal loading of samples, blots were incubated under stripping buffer for 20 minutes and washed with TBST. The blot then underwent the same protocol as previously stated to probe for GAPDH (Cell Signaling Technologies). ImageLabs software was used to analyze the relative intensity of each protein.

2.2.12 High Fat Diet Feeding

Mice were allowed to acclimate for 2 weeks prior to being placed on a 60% high fat diet (Research Diets D12492) while a control group was kept on a normal chow diet. The high fat diet was initiated 1 week prior to scaffold implant, and the mice remained on the diet until the end of the study. Five weeks after scaffold implantation, mice were sacrificed under anesthesia, and epididymal fat pads were collected for analysis. Fat pads were homogenized and analyzed for cytokine expression using ELISA.

2.2.13 In Vitro Model of 3T3-L1 Adipocyte Inflammation

On day 9 of differentiation, PLG or PLG+RSV scaffolds were introduced into wells containing 3T3-L1 adipocytes. Prior to addition, all scaffolds were washed in 70% ethanol and then rinsed in sterile PBS. After 72 hours of pretreatment with the scaffold, 10 ng/mL TNF-α was then added to each well and allowed to incubate for an additional 24 hours. Media was collected at this time point for further analysis. Differentiated cells not treated with TNF-α served as a control. Cell media collected at the end of the experiment was analyzed for MCP-1 using a Duoset ELISA kit (R&D Systems).
2.2.14 Statistics

Comparisons between two or more groups over time were conducted with a two-way ANOVA followed by Tukey’s multiple comparisons test. The p-values calculated by the two-way ANOVA which addresses the effect of resveratrol, time, and the interactions between the two are reported as $p_{\text{drug}}$, $p_{\text{time}}$, and $p_{\text{interaction}}$, respectively. Comparisons between three groups at a single time point were made with a one-way ANOVA followed by Tukey’s multiple comparisons test. Comparisons between two groups at a single time point were made with an unpaired t test. The number of samples analyzed and the statistical analysis performed are detailed in each figure legend. All analyses were completed using GraphPad Prism. In all figures, the error bars denote standard error of the mean (SEM).

2.3 Results

2.3.1 Characterization of Resveratrol Scaffolds

Resveratrol loaded PLG particles (PLG+RSV) were prepared using a single emulsion and solvent evaporation technique. The morphology of these particles and particles containing only the polymer (PLG) is shown in Figure 2.4 A-B. The images show that the morphology of PLG+RSV particles are irregular in shape compared to PLG particles, which are spherical. These particles were then used to fabricate scaffolds using a gas foaming and particulate leaching method. The structure of PLG+RSV and PLG scaffolds are shown in Figure 2.4 C-D. Both scaffolds contain a similar porous structure indicating that particle morphology does not have an impact on scaffold structure. During the gas foaming process, the polymer is saturated with high pressure CO$_2$ (800 psi) which leads to the nucleation and growth of gas pores in the polymer particles$^{44}$. The polymer particles expand and fuse around the salt particles, resulting in a continue matrix$^{44}$. It is
likely that the gas foaming process is the reason why particle morphology does not impact the final scaffold structure.

The amount of resveratrol in particles and scaffolds was measured by dissolving the polymer in DMSO and measuring the absorbance of the solution at 330 nm. Resveratrol encapsulation within PLG particles is reproducible, with three representative batches containing 54±2, 61±2, and 59±1 µg resveratrol/mg particles (Figure 2.5A). Scaffold fabrication from these particles is also reproducible. After salt leaching, scaffolds made from the particles in Figure 2.5A contained 35±1, 36±3, and 36±3 µg resveratrol/mg scaffold (Figure 2.5B) and weighed 2.29±0.02, 2.48±0.17, and 2.43±0.07 mg (Figure 2.5C). The amount of resveratrol in a scaffold can be calculated by multiplying the loading capacity by the scaffold weight, which was between 80 and 90 µg. Collectively, this data demonstrates we can fabricate resveratrol loaded scaffolds with reproducible drug payloads.

The drug encapsulation efficiency in the particles was 20.3 ± 0.7% (Table 2.1). This encapsulation efficiency was impacted by the mass of PLG particles we were able to recover following the single emulsion/solvent evaporation fabrication procedure. The mass yield for particles was typically 40%. Drug loading capacity dropped from ~60 µg/mg for particles to ~35 µg/mg for scaffolds, giving the scaffolds a drug encapsulation efficiency of 59.6 ± 3.1% (Table 1). Inspection of our fabrication procedure suggests that resveratrol is lost during the salt porogen removal step because scaffolds assayed immediately after gas foaming have a drug encapsulation efficiency of nearly 100% (data not shown).

Resveratrol stability, which is indicated by its absorption at 330 nm, was studied by analyzing a fourth batch of particles and scaffolds shortly after fabrication and one year
later (Figure 2.6 A-B). When particles were analyzed immediately after fabrication, they contained 65 ± 0.5 µg resveratrol/mg particles and when this same batch of particles were analyzed one year later, they contained 61 ± 6 µg resveratrol/mg particles (Figure 2.6A). When scaffolds analyzed directly after fabrication, they contained 37 ± 0.6 µg resveratrol/mg scaffold, and when scaffolds from the same batch were analyzed one year later, they contained 36 ± 0.4 µg resveratrol/mg scaffold (Figure 2.6B). These results indicate that resveratrol activity is not lost after one year when resveratrol particles or scaffolds are kept in dry conditions and protected from light.

To confirm that resveratrol was active after one year of encapsulation in PLG, we investigated the ability of PLG+RSV particles to induce lipolysis in differentiated 3T3-L1 adipocytes by measuring lipid content with an Oil Red O assay. We found that PLG+RSV particles stored for either 2 or 52 weeks significantly decreased lipid content of the adipocytes to a similar extent, while lipid content was not affected by the PLG particles (Figure 2.6C). Representative microscopic images were used to validate the decrease in lipid staining in the PLG+RSV and RSV treated groups (Figure 2.6D). Collectively, these results demonstrate that resveratrol remains bioactive after its encapsulation in PLG and that it remains active for at least one year.

The resveratrol release profile of scaffolds over 14 days was characterized \textit{in vitro} (Figure 2.7A). At each time point scaffolds were removed from the assay and tested for resveratrol content. The initial amount of resveratrol in the scaffold was taken to be the average amount of resveratrol measured in three scaffolds from the same batch immediately after salt leaching. Resveratrol exhibited a biphasic release pattern (burst followed by a plateau), with ~50% of the drug released in the first 3 days. The amount of
resveratrol released during the plateau phase accounted for ~10% of the initial resveratrol in the scaffold. The release profile indicated that there was ~30% of the resveratrol present within the scaffold after 14 days. Importantly, this burst release of resveratrol could be beneficial in decreasing the acute inflammatory phase which occurs 1-5 days after injury. Minimal scaffold degradation (~5% mass loss) was required for drug release over this time period (Figure 2.7B).

2.3.2 Effect of Resveratrol on Immune Cell Infiltration Following Scaffold Implant

To quantify the effect of resveratrol on immune cell infiltration into adipose tissue following scaffold implant, we employed flow cytometry. Total immune cell numbers were measured based on CD45 expression 7 and 14 days after implant (Figure 2.8A). As expected, PLG scaffold implant increased immune cell numbers at day 7. Contrary to our expectations, resveratrol did not decrease immune cell numbers at this time point relative to PLG scaffolds. Surprisingly, immune cell numbers significantly decreased from day 7 to day 14 for both scaffold groups, and we could not detect differences between any of the groups at day 14 by Tukey’s multiple comparison’s test. The data indicate immune cell infiltration following PLG implant into fat largely resolves within 2 weeks without intervention. While not significant, immune cell numbers in the PLG+RSV group tended to be higher at day 7 and lower at day 14 compared to the PLG group.

To better understand these trends, 7 immune cell populations were measured at 7 and 14 days: macrophages (F4/80), neutrophils (Ly6G), monocytes (CD11b), NK cells (NK1.1), T cells (CD3), NKT cells (NK1.1 CD3), and B cells (CD19) (Figure 2.8B-C). For ease of interpretation, immune cell numbers for the scaffold groups are normalized by the naïve controls. At day 7, resveratrol delivery resulted in a significant increase in
neutrophils with a concomitant decrease in T cells, NK cells, and NKT cells (Figure 2.8B). At 14 days, all populations exhibited a trend of decrease, but only monocytes, NK cells, and B cell numbers were significantly lower in resveratrol groups (Figure 2.8C). The data suggest that even though resveratrol dose not decrease the total number of immune cells, it does have an impact on particular immune cell populations.

Flow cytometry revealed that macrophage number was not different between PLG and PLG+RSV at either time point; however, we hypothesized that resveratrol may modify macrophage phenotype. To identify changes in macrophage phenotype, two molecules associated with M2 polarization, CD206 and arginase-1\textsuperscript{93}, were measured in scaffold homogenates 7 days after implant using Western blot (Figure 2.9A). At this time point, CD206 was unchanged in both the PLG and PLG+RSV scaffold groups compared to naïve fat (Figure 2.9B). In contrast, arginase-1 was \(~35\) fold higher in both PLG and PLG+RSV groups compared to naïve fat (Figure 2.9C). The data indicate that resveratrol delivery does not impact CD206 or arginase-1 expression, but scaffold implant elevates arginase-1 expression above naïve levels.

2.3.3 Effect of Resveratrol on Cytokine Levels Following Scaffold Implant

Cytokines control immune cell infiltration, residence, and survival in the tissue. Thus, we investigated the effect of resveratrol delivery on protein levels of proinflammatory cytokines MCP-1, TNF-\(\alpha\), IL-6, and CXCL1 as well as anti-inflammatory cytokines IL-4, IL-10, and IL-13 in whole tissue homogenates 3, 7, and 14 days after scaffold implant. There was no significant interaction (\(p_{\text{interaction}}\)) between drug treatment and time detected by two-way ANOVA for the analyses performed in Figure 2.10H. MCP-1 exhibited the largest increase following PLG scaffold implant of all cytokines measured,
but was not affected by resveratrol (Figure 2.10A). MCP-1 expression significantly decreased by day 14 as determined by Tukey’s multiple comparisons test. Interestingly, scaffold implant, irrespective of drug payload, did not increase whole tissue levels of TNF-α, IL-6, or CXCL1 compared to naïve levels (Figure 2.10 B-D). We did detect a significant effect of resveratrol on TNF-α ($p_{\text{drug}}=0.0147$) and CXCL1 ($p_{\text{drug}}=0.0168$) by two-way ANOVA (Figure 2.10 H), but drug delivery largely served to bring expression up toward naïve levels. To validate these findings, Western blot was used to detect IL-6 in all groups. We were not able to detect IL-6 in any tissues by this method; however, IL-6 was detected in Raw 264.7 cells after incubation with LPS and Brefeldin A (Figure 2.11).

In terms of anti-inflammatory cytokines, scaffolds, irrespective of drug payload, did not modulate IL-4 expression (Figure 2.10E). On the other hand, there was a significant effect of resveratrol on IL-10 ($p_{\text{drug}}=0.0065$) and IL-13 ($p_{\text{drug}}=0.0023$), with resveratrol increasing expression of both these proteins. In addition, expression of both these molecules increased over time (Figure 2.10F-G). Together, these results suggest that resveratrol’s effect on IL-10 and IL-13 may be responsible for decreases in immune cell numbers at day 14 as measured by flow cytometry (Figure 2.8B-C).

### 2.3.4 Effect of Resveratrol on Bax and Mcl-1 Following Scaffold Implant

Apoptosis is increased in tissues during resolution of inflammation, reflecting the clearance of immune cells that are no longer needed. In addition, resveratrol increases immune cell apoptosis, especially in neutrophils. Thus, we measured the expression of Bax, a proapoptotic protein, and Mcl-1, an antiapoptotic protein, in whole tissue protein homogenates 7 and 14 days after implant (Figure 2.12A). Bax and Mcl-1 proteins were present in naïve fat (Figure 2.12A), but only Bax levels increased with scaffold implant
(Figure 2.12A); Mcl-1 levels were largely unaffected (Figure 2.12B). There was no significant interaction between treatment and time detected by two-way ANOVA for Figure 2.12B or Figure 2.12C. There was a trend for resveratrol to increase Bax levels, but it was not significant ($p_{\text{drug}} = 0.0508$); however, over time Bax expression did decrease ($p_{\text{drug}} = 0.0040$). Collectively, these results suggest that scaffold implant into the fat may increase rates of apoptosis since proapoptotic Bax increase and antiapoptotic Mcl-1 is relatively unchanged; however, resveratrol’s ability to alter Bax expression, if indeed it does, was difficult to detect.

2.3.5 Ability of Resveratrol to Induce Anti-Inflammatory Responses in Inflammatory Environments

An important application of PLG+RSV scaffolds would be to induce an anti-inflammatory response in inflamed adipose tissue. To investigate the ability of PLG+RSV scaffolds to achieve this, we utilized a common model of adipose tissue inflammation in which mice are fed a high fat diet. Mice received PLG or PLG+RSV scaffolds in both epididymal fat pads 1 week after being fed a high fat diet. The mice were then fed the diet for an additional 5 weeks prior to harvest of the epididymal fat (Figure 2.13A). Cytokine analysis was conducted using ELISA to measure MCP-1, IL-10, and IL-13 in the epididymal fat. High fat diet feeding resulted in an increase in proinflammatory cytokines MCP-1 and a decrease in anti-inflammatory cytokines IL-10 and IL-13 (Figure 2.13B). The results demonstrate that PLG+RSV scaffolds, but not PLG scaffolds, significantly decreased MCP-1 expression in the epididymal fat. Interestingly, PLG+RSV scaffolds did not increase the expression of IL-10 and IL-13.
To investigate resveratrol’s anti-inflammatory effect on adipocytes, we utilized an *in vitro* model of adipocyte inflammation. On day 9 after differentiation, cells were treated with PLG scaffolds or PLG+RSV scaffolds for 72 hours and then treated with 10 ng/mL TNF-α for 24 hours (Figure 2.13C). Media was collected and analyzed for MCP-1 using ELISA. Differentiated cells that were treated with TNF-α secreted ~2.5 fold more MCP-1 compared to untreated differentiated cells (Figure 2.13D). PLG scaffolds had no effect on MCP-1 secretion; however, PLG+RSV scaffolds significantly decreased MCP-1 compared to differentiated cells treated with only TNF-α or pretreated with PLG scaffolds prior to TNF-α treatment.

**2.4 Discussion**

This report presents the characterization of resveratrol loaded PLG scaffolds and their novel use in modulating the immune response to biomaterial implants in the adipose tissue. Porous PLG scaffolds containing resveratrol were fabricated using a gas foaming/particulate leaching method in which NaCl served as the sacrificial porogen. To our knowledge, this approach is not commonly used for the fabrication of small molecule loaded PLG scaffolds, and our results provide further insight into drug loading and release kinetics of this approach. Our scaffolds contained 37 µg of resveratrol per mg of PLG (Figure 2.5), which is at least 4-fold higher loading than previous studies using biodegradable polyester scaffolds to deliver resveratrol\textsuperscript{86,91}. However, this scaffold fabrication method was suboptimal because removal of the salt porogen led to a 40% loss of resveratrol loaded in the scaffold. To improve drug loading efficiency of scaffolds, a nonparticulate leaching method to promote scaffold porosity should be investigated. Nonetheless, resveratrol loaded scaffolds fabricated using this method exhibited a burst
and plateau release profile (Figure 2.7) and overall delivered a large amount of resveratrol over 14 days compared to sintered resveratrol encapsulated PLG nanoparticles which delivered 7 times less resveratrol in a sustained manner\textsuperscript{86}. In addition, resveratrol in our PLG scaffolds did not degrade over one year when stored at room temperature conditions as determined by measuring absorbances at the wavelength of trans-resveratrol, the active isomer of resveratrol\textsuperscript{103}. Furthermore, we confirmed this finding with a bioactivity assay that indicated both fresh PLG+RSV particles and those stored for one year induced lipolysis in 3T3-L1 adipocytes and to the same extent (Figure 2.6). The ability to maintain bioactivity while stored at room temperature is an advantage of small molecule delivery compared to other immunomodulatory strategies that require temperature-controlled storage. For example, proteins and viral vectors are kept at freezing temperatures to maintain activity, posing challenges during manufacturing, storage, and transportation once in the market.

Initially, \textit{in vivo} studies investigated resveratrol delivery as a way to modulate the immune environment within adipose tissue following implant of PLG scaffolds. Resveratrol impacted the prevalence of monocytes and several lymphocyte populations over the time course of our experiments (Figure 2.8). Monocytes and lymphocytes play key roles in adipose tissue inflammation during obesity\textsuperscript{104}; therefore, the ability to modulate their number is significant to future therapies. Monocytes and lymphocytes are recruited to sites of inflammation by inflammatory cytokines. Resveratrol delivery increased expression of the anti-inflammatory cytokines IL-10 (Figure 2.10), which inhibits inflammatory cytokine production and signaling in many cell types, and this effect may be responsible for the decrease in monocytes and lymphocytes recruited to the scaffold.
implant site. IL-10 is produced by macrophages (among other cells), with M2 polarized macrophages expression IL-10 at higher levels than proinflammatory macrophages\(^{105}\). Thus, we hypothesized that resveratrol delivery increased macrophage polarization toward an M2 phenotype within the implant site. Surprisingly, we were not able to detect elevation of CD206 or arginase-1, markers of M2 macrophage polarization, following resveratrol delivery (Figure 2.9). However, there was a 35-fold increase in arginase-1 due to the PLG scaffold implant and it is likely that further upregulation via resveratrol was not possible (Figure 2.9). In addition to IL-10, resveratrol delivery increased expression of IL-13 (Figure 2.10), which is not produced by macrophages, but by TH2 lymphocytes, mast cells, basophils, eosinophils, and IL2 cells. In particular eosinophils and IL2 cells are present in the adipose tissue and have the ability to shape immune responses favoring immune resolution rather than inflammation\(^{106}\).

An ability to modulate the cytokine milieu in the adipose tissue would be useful for treating metabolic diseases associated with adipose tissue inflammation. Therefore, we investigated the efficacy of PLG+RSV scaffolds in mitigating adipose tissue inflammation that occurs in high fat diet fed mice. We found that resveratrol delivery decreased expression of MCP-1 in the adipose tissue of mice fed a high fat diet for 6 weeks (Figure 2.13). High fat diet elevates MCP-1 after 1 week\(^{107}\) and promotes inflammatory macrophage infiltration into the adipose tissue, which drives systemic insulin resistance\(^{108}\). Therefore, decreasing this cytokine is an important benchmark for treating adipose tissue inflammation. We also found that pretreatment with PLG+RSV scaffolds prevented TNF-\(\alpha\) induced MCP-1 secretion from differentiated 3T3-L1 adipocytes (Figure 2.13), suggesting that the decrease in MCP-1 \textit{in vivo} was mediated, at least in part, by resveratrol.
acting on adipocytes. In contrast to healthy adipose tissue (Figure 2.9), PLG+RSV scaffolds did not increase IL-10 and IL-13 expression in adipose tissue from mice fed the high fat diet; however, scaffold implant did not further decrease the expression of these anti-inflammatory proteins either, which were decreased by the high fat diet. Because adipose tissue expression of IL-10 and IL-13 protects mice against the detrimental effects of high fat diet, future studies should aim to quantify small molecules that increase these factors when released from scaffolds implanted into the adipose tissue.

We hypothesize that the potential mechanism for resveratrol’s protection against MCP-1 elevation is through its effects on the NF-κB pathway, a regulator of cytokine production. Resveratrol inhibits the DNA binding activity of the NF-κB complex through activation of Sirt-1, which inhibits NF-κB transcription in 3T3-L1 adipocytes. Interestingly, upon degradation, PLG produces lactic acid, a bioactive molecule that abrogates the proinflammatory response of macrophages stimulated with LPS via downregulation of the NF-κB pathway through GPR82 receptor. This is a plausible mechanism as to why PLG scaffold without drug loading does not produce proinflammatory cytokine production upon implant into adipose tissue (Figure 2.9). Furthermore, delivery of lactate via PLG implant accelerates the wound healing response. Therefore, the delivery of resveratrol from a degrading PLG scaffold may synergistically promote an anti-inflammatory environment through the combinatorial actions of resveratrol and lactic acid on adipocytes and macrophages. Furthermore, PLG degradation creates an acidic environment. Resveratrol is most stable at pH < 6. Therefore, this acidic environment could have beneficial effects on drug stability.
Notable was our finding that PLG scaffold implant did not increase expression of TNF-α and IL-6 in the epididymal adipose tissue relative to naïve fat, and in fact, these cytokines appeared to decrease. Generally, the literature reports that biomaterial implant increases these factors and an important metric for anti-inflammatory drug delivery is the ability of the delivered factor to decrease their expression. However, we note from an extensive literature search that rarely are the experimental groups receiving the “empty” biomaterial and the “drug-loaded” biomaterial compared to an unmanipulated tissue\textsuperscript{37,118–124}. Thus, in these cited studies it is possible that the “drug-loaded” group actually decreased inflammatory gene expression below what we call “ naïve” levels. On the other hand, we may have uncovered an attribute specific to adipose tissue. In a healthy state, the adipose tissue is biased toward anti-inflammation\textsuperscript{77}; therefore, we hypothesize that upon implant the biomaterial invokes a series of signaling pathways that downregulate inflammatory mediators such as TNF-α and IL-6. In support of this hypothesis, we find that scaffold implant upregulated arginase 35-fold (Figure 2.10), an enzyme that inhibits production of TNF-α and IL-6\textsuperscript{125}.

A limitation of the study is that we did not conduct a dose response. To minimize animal use, we chose to deliver the maximal amount of resveratrol we could in a 2.5 mg scaffold, which we knew from previous work is well tolerated by the mouse\textsuperscript{126}. While resveratrol ultimately had anti-inflammatory effects, the spike in neutrophils at the 7 day time point that occurred with its delivery was likely counterproductive. As we believe the neutrophil influx was the result of local resveratrol concentrations that were too high, future studies should investigate not only scaffolds with lower payloads of resveratrol, but also scaffolds with high payloads designed to release resveratrol more slowly.
In conclusion, resveratrol can be encapsulated into PLG particles using a single emulsion solvent evaporation technique and these particles can be formed into porous PLG scaffolds using gas foaming followed by particulate leaching with NaCl as the sacrificial porogen. This method results in scaffolds with high resveratrol loading and drug release with a burst phase followed by plateau. In healthy tissue, resveratrol delivery augments an anti-inflammatory environment established by PLG scaffolds, characterized by high arginase-1 and low TNF-α and IL-6, through increased expression of IL-10 and IL-13. In terms of therapeutic applications, PLG+RSV scaffolds decrease MCP-1 expression in the fat of mice fed a high fat diet, an inflammatory molecule that spurs the chronic inflammation associated with obesity’s comorbidities. Thus, we conclude that PLG scaffolds are a promising platform for the treatment of adipose tissue inflammation that drives metabolic disease, since the polymer alone induces an anti-inflammatory response and the scaffold is easily engineered for drug and cell delivery. Finally, we hypothesize that further investigation into the anti-inflammatory effects of PLG in the adipose tissue will lead to new drug targets and therapeutic strategies for metabolic disease and other syndromes associated with chronic inflammation.

### 2.5 Tables

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CHAPTER 3
MODULATION OF ADIPOCYTE SIZE AND FAT PAD WEIGHT VIA RESVERATROL RELEASING SCAFFOLDS IMPLANTED INTO THE EPIDIDYMAL ADIPOSE TISSUE

3.1 Introduction
The adipose tissue functions as the body’s main energy reservoir and plays a central role in maintaining whole body energy homeostasis. Adipocytes, the tissue’s parenchymal cell, accomplish this by storing excess lipids in the form of triglycerides in specialized lipid droplets. As other organs in the body require energy, triglycerides are broken down into fatty acids (via lipolysis), released into the bloodstream and are ultimately taken up by the energy demanding organ. However, the adipose tissue has a limited storage capacity; therefore, chronic overnutrition can lead to an accumulation of lipid metabolites in other tissues. Furthermore, lipid accumulation in the adipose tissue has significant consequences including adipose tissue inflammation and dysregulated insulin signaling, which are associated with metabolic diseases such as obesity and type 2 diabetes.

Current methods to mitigate this lipid accumulation include lifestyle changes, pharmaceuticals, and bariatric surgery. Each of these strategies pose challenges that

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hinder their effectiveness. Diet and exercise alone do not typically result in long term outcomes for those with severe obesity and FDA approved drugs that improve lipid storage in the adipose tissues, for example thiazolidinediones, are complicated by off-target effects including cardiotoxicity. Finally, bariatric surgery is highly invasive and comes with high risks of post-operative or chronic complications. Therefore, a strategy that enhances lipid metabolism in the adipose tissue and prevents the side effects associated with systemically administered drugs that can also be combined with diet and exercise would be highly beneficial to combat lipid accumulation.

Biomaterials have been extensively studied to repair injured skin, bone, and muscle; however, only in recent years have researchers addressed adipose tissue with tissue engineering strategies. Our recent studies indicate that, upon implant into the epididymal adipose tissue, scaffolds made from poly(lactide-co-glycolide) (PLG) initiate remodeling of the host tissue that shares many characteristics of wound healing, including recruitment of immune cells and extracellular matrix deposition. This remodeling serves to degrade and integrate the scaffold with the host tissue. To our knowledge, no one has studied the metabolic consequences on the adipose tissue after biomaterial implant; however, we anticipate that this response is energetically demanding, and fueled, in part, by the metabolism of lipids. In fact, our previously published results indicate that the host response to PLG scaffolds is responsible for enhancing local lipid utilization as evidenced by a decrease in epididymal fat weight in both healthy mice and mice challenged with a high fat diet.

In the current study, we investigated the hypothesis that the scaffold’s effects on local lipid levels could be enhanced through the incorporation of resveratrol into the
scaffold polymer matrix. Resveratrol, a natural polyphenol, was chosen because it enhances activity of proteins that lead to lipid mobility via lipolysis and lipid catabolism via fatty acid oxidation. For example, resveratrol has shown to increase the expression of adipose triglyceride lipase (ATGL), and carnitine palmitoyltransferase (CPT1), the rate limiting enzymes involved in lipolysis and fatty acid oxidation, respectively. In addition, the literature indicates that these effects are a result of resveratrol’s activation of AMP kinase (AMPK). AMPK activation has shown to regulate ATGL expression and inhibit acetyl-coA-carboxylase (ACC) leading to an increase in CPT1 activity.

To study resveratrol scaffold’s effect, we first characterized the tissue remodeling induced by resveratrol loaded PLG scaffolds after implant into the epididymal adipose tissue. We hypothesized that resveratrol’s impact on metabolic programs would decrease lipid accumulation, as observed by adipocyte area, compared to PLG implants without a drug payload 28 days after implant, a time point 2 weeks longer than our previously published report. Specifically, we measured resveratrol’s effect on the expression of ATGL and CPT1 as well as AMPK activation. Furthermore, to study the ability of resveratrol loaded scaffolds to prevent onset of high fat diet induced weight gain, mice received either PLG scaffolds without a drug payload or resveratrol loaded PLG scaffolds and were then challenged with a high fat diet containing 60% calories from fat, a mouse model of diet induced obesity. The epididymal fat pad was chosen as the implant site because this fat pad expands more during the initial stages of high fat diet feeding compared to other fat depots; therefore, decreasing this fat pad’s mass would be a significant achievement during the study time frame. While scaffolds have been used to deliver
resveratrol to modulate inflammation for several tissue engineering applications\cite{86,89,91,141}, we are the first to investigate scaffold-based delivery of resveratrol to modulate metabolic programs in the adipose tissue.

3.2 Materials and Methods

3.2.1 Scaffold Fabrication

Scaffolds were fabricated from PLG microparticles using a gas foaming, particulate leaching technique. Particles containing resveratrol (Sigma) were fabricated as previously described\cite{142}. Briefly, a 1:3 volume ratio of dichloromethane (Sigma) and ethanol (Sigma) containing 6% w/w PLG (75:25 mol ratio lactide to glycolide 0.76 dL/g, Evonik) and 10 mg/mL resveratrol was added to an aqueous solution of 1% w/v PVA (Sigma) in a 1:7 volume ratio and homogenized. The emulsion was then added to ultrapure water and stirred for 5 hours to remove the dichloromethane and promote solidification of the polymer and encapsulation of resveratrol. Particles were washed with ultrapure water and collected via centrifugation. Particles were lyophilized and stored in aluminum foil in a desiccator to ensure particles were protected from light and moisture. Particles that did not contain resveratrol were fabricated in a similar fashion except the oil phase contained only dichloromethane and 6% w/w PLG.

To fabricated scaffolds, polymer particles were mixed with a sacrificial porogen, NaCl (250-500 µm particles), in a 1:30 mass ratio and pelleted in a die. The resulting tablet was gas foamed using 800 psi CO$_2$ at room temperature in a custom-made pressure vessel and the salt porogen was removed by washing in ultrapure water. Complete salt removal was verified by scaffold weight and microscopy. Scaffolds containing resveratrol were made with resveratrol loaded PLG particles and scaffolds that did not contain the drug were
made with particles containing only PLG. Scaffolds were fabricated so that they weighed 2.08 mg ± 0.06 mg (mean ± SEM). From here on, scaffolds that contain resveratrol are referred to as “RSV scaffolds” and scaffolds containing only the polymer are referred to as “PLG scaffolds.”

3.2.2 Characterizing Scaffold Structure and Pore Size

Scaffolds were added to carbon adhesive tape on aluminum stubs and gold sputtered two times for 1 minute using a Denton Desk II vacuum sputter coater. Images were taken on a Tescan Vega3 scanning electron microscopy at 5kV. The face of the scaffolds was imaged to give a top view observation. Scaffolds were also imaged using a Nikon Eclipse Ci microscope at 4x magnification. ImageJ was used to quantify the average pore diameter in both scaffold groups.

3.2.3 Measuring Resveratrol Loading in Scaffolds

Resveratrol loading in scaffolds was measured as previously described. Briefly, after leaching, scaffolds were weighed and dissolved in a defined volume of DMSO (Sigma). The solution was then analyzed for absorbance at 330 nm and absorbance values were compared to a nine-point standard curve allowing for the interpolation of the unknown resveratrol concentration in the sample. Scaffold loading was calculated as the amount of resveratrol measured in the scaffold (µg) normalized by the mass of the scaffold analyzed (mg).

3.2.4 In Vitro Release Assay

Resveratrol release from scaffolds over time was determined as previously described. Briefly, scaffolds were weighed and incubated in ultrapure water at 37 °C for 0.25, 1, 3, or 7 days. At each time point, scaffolds were removed from the incubator and
stored in a desiccator until dry, which was confirmed by scaffold weight. The amount of resveratrol remaining in the scaffold was measured as described in the previous section.

3.2.5 Animal Care and Scaffold Implant

All animal procedures were approved by the University of South Carolina Institutional Animal Care and Use Committee and NIH guidelines for the care and use of laboratory animals were observed. Six-week-old male C57BL/6 mice were purchased from the Jackson Laboratory and, upon arrival, acclimated for 2 weeks prior to the scaffold implant procedure. Prior to implant, scaffolds were washed in 70% ethanol and rinsed in sterile PBS. Mice were anesthetized with isoflurane and their abdomens were shaved and prepped with betadine and ethanol. A lower abdominal midline incision was made, and one scaffold was wrapped into each epididymal fat pad (i.e. each mouse received two PLG or RSV scaffolds, one in each epididymal fat pad). The intraperitoneal cavity was closed with a running stitch and the skin was closed with wound clips.

3.2.6 Animal Study Design

To investigate the effect of RSV scaffolds on adipocyte size and adipose tissue gene expression, three mice per group (RSV or PLG) received a scaffold into each epididymal fat pad and were then euthanized 28 days later. One fat pad was frozen for western blot, and one fat pad was fixed and embedded in paraffin for histology. In a second study designed to investigate adipose tissue gene expression at an earlier time point, 4 mice per group were implanted with one scaffold per epididymal fat pad. Fourteen-days later mice were euthanized and epididymal fat pads were harvested and frozen for western blot.

To assess the effect of RSV scaffolds on mice fed a high fat diet, five mice per group (RSV or PLG) received a scaffold into each epididymal fat pad. Twenty-eight days
after scaffold implant, mice were placed on a 60% high fat diet (Research Diets D12492) and euthanized 28 days after induction of the diet. Fat pads were collected for protein and histological analysis. Mice were allowed access to the diet ad libitum during the entire study except when they were fasted prior to body weight measurements.

3.2.7 Fasting Weight Measurements

On days 14 and 28 after initiation of the high fat diet, mice were fasted for 6 hours beginning at 7AM with access to water. Weight measurements were obtained by placing the mouse into a beaker tared on an Ohaus digital scale.

3.2.8 Histological Analysis

After collection, fat pads were washed in sterile PBS, placed in 4% paraformaldehyde for 24 hours, and then paraffin embedded. Serial, 5 µm sections were cut from the paraffin blocks and stained with hematoxylin and eosin (VWR). Sections were imaged using a Nikon Eclipse Ci microscope at 4x or 40x magnifications. ImageJ software was used to quantify adipocyte area from 3 random 40x images per section. Three sections were quantified from 3 mice per group. All adipocytes within the field of view were quantified.

3.2.8 Western Blotting

Collected fat pads were washed in sterile PBS, frozen on dry ice and stored at -80°C. Tissues were homogenized in a defined volume of RIPA buffer (Fisher Scientific) containing Halt Protease Inhibitor Cocktail (Thermo Fisher Scientific), PhosStop (Sigma), and PMSF (Thermo Fisher Scientific) using a benchtop homogenizer. Insoluble material was removed by centrifugation and whole tissue homogenate samples were aliquoted and stored at -80°C until use.
Total protein in the homogenate was quantified using the Pierce BCA protein assay kit (Thermo Fisher Scientific). Twenty or 30 µg protein was separated by 8 or 10% polyacrylamide gel electrophoresis based on target protein molecular weight. Separated proteins were then transferred to 0.2 µm nitrocellulose membranes. The membranes were blocked with 5% nonfat dry milk in Tris-buffered saline containing Tween-20 (TBST) for 1 hour at room temperature. Primary antibody for phosphorylated AMPK-T172 (Cell Signaling Technologies, CST), ATGL (CST), phosphorylated ACC-S79 (CST), and CPT1 (Abcam) was added to each blot at 1:1000 dilution in 5% BSA in TBST and allowed to incubate overnight at 4°C before incubation with a polyclonal secondary antibody conjugated to horseradish peroxidase (Abcam) for 1 hour at room temperature. The blots were then developed with SuperSignal enhanced chemiluminescent substrate solution (Thermo Fisher Scientific). To verify equal loading, blots were incubated with stripping buffer for 25 minutes and washed with TBST. The blot then underwent the same protocol as previously stated for total AMPK, total ACC, or GAPDH (all purchased from CST). Blots probed for AMPK were stripped a second time and underwent the same protocol to probe for GAPDH. ImageLabs software was used to analyze the relative intensity of each protein.

3.2.9 Immunohistochemical Staining

Serial tissue sections from each mouse, 5 µm in thickness, were deparaffinized, rehydrated, and processed for antigen retrieval in citrate buffer using a vegetable steamer. Sections were blocked with a TBS solution containing Tween-20, BSA, goat serum, and anti-CD16/32 (Biolegend) and incubated with an anti-CPT1 antibody (Abcam). Sections were treated with hydrogen peroxide prior to incubation with an HRP labelled polymer
conjugated to goat anti-rabbit immunoglobulins (EnVision+, Dako). HRP was detected using the Liquid DAB+ Substrate Chromogen system (Dako). Mayer's hematoxylin (Dako) was used to counterstain. Three sections from each mouse were stained and then imaged using a Nikon Eclipse Ci microscope. Analysis included 3 mice per group.

3.2.10 Raw 264.7 Cell Culture and Resveratrol Treatment

Raw 264.7 macrophages (ATCC) were cultured at 37°C with 5% CO₂ in DMEM media (Fisher Scientific) supplemented with 1 mM sodium pyruvate (Fisher Scientific), 1500 mg/L sodium bicarbonate (VWR), 10% fetal bovine serum (Fisher Scientific), and 1% penicillin/streptomycin (Fisher Scientific) at 57,000 cells/cm² in 6 well plates. Twenty-four hours after seeding, cells were treated with 0, 10 or 25 µM resveratrol dissolved in ethanol. Twenty-four hours later, cells were removed via trypsin 0.25% EDTA (Fisher Scientific) and a cell scraper. Cells were lysed in RIPA buffer supplemented with protease inhibitors via 21G needle and syringe. Protein fraction was collected after centrifugation at 10,000 xg for 10 minutes and stored in -20 °C. Western blot analysis of CPT1 in treated macrophages was conducted as previously stated using 10 µg of total protein content which was determined via BCA assay and β-actin (Sigma) served as the loading control.

3.2.11 Statistical Analysis

Statistically significant differences between PLG and RSV implanted groups were evaluated using an unpaired student’s t-test. Statistically significant differences between three groups were determined using a one-way ANOVA with a Tukey’s multiple comparison test. Statistical significance was set to a p value less than 0.05. The number of samples analyzed is detailed in each figure legend. All analyses were completed using GraphPad Prism. In all figures, data indicates mean ± standard error of the mean (SEM).
3.3 Results

3.3.1 Resveratrol Scaffold Characterization

Scaffold structure was observed using scanning electron microscopy and light microscopy and these images, shown in Figure 3.1 A-D, indicate that both scaffolds contained similar porous structure. Indeed, ImageJ analysis of light microscopy images, as representatively shown in Figure 1 B and D, determined the average pore diameter was 454±31.8 µm and 434±7.45 µm in PLG and RSV scaffolds, respectively, and therefore, were not significantly different (Figure 3.1E). Resveratrol scaffolds used in this study contained approximately 48±2 µg RSV/mg scaffold; therefore, delivering approximately 100 µg resveratrol total per 5mm x 2mm scaffold (Figure 3.1F-G). Release kinetics of resveratrol from scaffolds used in this study is depicted in Figure 3.2. One-week in vitro release characterization indicates a burst release profile where approximately 50% of the resveratrol initially loaded in the scaffold is released in the first 3 days with approximately 30% of the initial resveratrol remaining in the scaffold after 7 days.

3.3.2 Adipose Tissue Composition after Scaffold Implant

Tissue sections from epididymal fat 28 days after PLG or RSV scaffold implant revealed extensive cellular infiltration and protein deposition not present in unmanipulated fat (Figure 3.3A,D, unmanipulated fat not shown). This tissue remodeling was localized around irregular shape voids indicating pieces of scaffold removed during histological processing (Figure 3.3 B,E; specific areas highlighted by black boxes in Figure 3.3 A,D). There were no obvious qualitative differences in tissue remodeling as indicated by H&E staining. Interestingly, however, from a metabolic perspective, adipocytes surrounding RSV scaffolds looked to be smaller in area compared to those surrounding PLG scaffolds.
(Figure 3.3 C,F; specific areas highlighted by black boxes in Figure 3.3 A,D). Indeed, quantification of all adipocytes surrounding RSV scaffolds were approximately 1000 µm$^2$ compared to those surrounding PLG scaffolds which measured approximately 1500 µm$^2$ (Figure 3.3G). Taken together, scaffolds induce local tissue remodeling in the adipose tissue, while resveratrol delivery from scaffolds decreases adipocyte area compared to scaffolds with no drug payload.

### 3.3.3 Expression of Key Proteins Involved in Lipid Metabolism Following Scaffold Implant

To further investigate a possible mechanism for resveratrol’s significant impact on adipocyte area, proteins involved in lipid regulation were measured via western blot in whole tissue homogenate. Adipose triglyceride lipase (ATGL), carnitine palmitoyltransferase 1 (CPT1), phosphorylated acetyl-co-A-carboxylase (pACC), and phosphorylated AMP kinase (pAMPK) levels were quantified (Figure 3.4). Interestingly, we did not observe a significant difference in the expression of these proteins between scaffold groups at the 28-day timepoint. In addition, a large amount of variability was observed when pACC and ACC were measured. We then looked at protein expression 14 days after implant to determine if any differences were present at an earlier time point. Interestingly, at 14 days after scaffold implant, phosphorylated AMPK levels were significantly higher in the RSV scaffold group compared to PLG (Figure 3.5E).

Since CPT1 is the rate limiting enzyme involved in fatty acid oxidation, we were interested in how its spatial distribution was altered after RSV scaffold implant; therefore, we stained fat pads collected 28 days after implant with CPT1 using immunohistochemistry (Figure 6). We found that CPT1 was most highly expressed in the new tissue surrounding both scaffolds (Figure 3.6A,B). Looking more closely at the scaffold environment
indicated by black boxes, there is evidence indicating that CPT1 is expressed by the giant cells, which are formed following macrophage fusion \(^{143}\), located at the interface of the tissue and material (highlighted by yellow arrows), as well as other non-adipocyte cells in the new tissue (highlighted by red arrows) (Figure 3.6C,D).

**3.3.4 Expression of CPT1 in Resveratrol Treated RAW 264.7 Macrophages**

The histology presented above indicated that macrophages in the scaffold environment are CPT1 positive; however, we did not detect an effect of resveratrol on CPT1 expression using westerns, which was surprising. It is possible that carrying out the measurements in whole tissue homogenates kept us from detecting changes that were occurring in the scaffold recruited cells. To determine if it is possible for resveratrol to modulate CPT1 levels in macrophages, we treated RAW 264.7 macrophages with resveratrol and measured CPT1 protein levels via western blot (Figure 3.7A,B). Twenty-four hours after initial seeding, the macrophages were treated with 10 or 25 µM resveratrol for 24 hours. Western blot indicated that 25 µM resveratrol increased CPT1 expression by 30% relative to vehicle control (Figure 3.7B). No difference was detected between cells treated with 10 µM and untreated cells indicating the importance of dose.

**3.3.5 Protective Effect of Resveratrol Scaffolds in Mice Challenged with a High Fat Diet**

To determine if resveratrol loaded scaffolds could prevent diet induced obesity compared to PLG scaffolds, lean mice were pre-treated with RSV scaffolds for 4 weeks prior to being placed on a high fat diet for an additional 4 weeks, as illustrated in Figure 3.8A. Body weight measurements indicate that 14 days after switching to the high fat diet, mice that received RSV scaffolds gained significantly less weight than mice that received PLG scaffolds (Figure 3.8B). This effect remained after 28 days on the high fat diet (Figure
Additionally, at the end of the study, epididymal fat pads collected from mice that received RSV scaffolds weighed significantly less than those from the PLG scaffold group (Figure 3.8C).

Histological sections of the epididymal fat pad collected at the end of this study were analyzed similarly to those in the previously discussed in the lean animal study. Adipocytes in the RSV scaffold group appeared smaller than those from the PLG scaffold group (Figure 3.9A) and, indeed, image analysis supported this observation. In fact, adipocyte area in the RSV scaffold group was 30% smaller compared to the PLG group (Figure 3.9B). Similar to results observed in the lean animal study, this observation was not accompanied by whole tissue modifications in ATGL, CPT1, pACC, or pAMPK in the RSV scaffold group (Figure 3.10).

3.4 Discussion

Chronic overnutrition leads to lipid accumulation that has detrimental consequences such as adipose tissue inflammation and dysregulated insulin signaling resulting in the progression of metabolic diseases such as obesity and type 2 diabetes. Therefore, we are pursuing an approach to combat metabolic disease by enhancing lipid catabolism in the adipose tissue. Extensive research shows that resveratrol has the ability to increase lipid catabolism; however, the molecule’s poor bioavailability proved to be a great obstacle during clinical trials. As a strategy to overcome this shortcoming, we incorporated resveratrol into a scaffold for direct delivery to the adipose tissue and investigated its ability to modulate fat pad size. Mice pre-treated with resveratrol scaffolds and then fed a high fat diet gained significantly less total body weight and epididymal adipose tissue mass compared to mice that received scaffolds containing only polymer. The
effect on total body weight of this localized therapy might not be surprising because the epididymal fat pad is large, expands quickly, and contributes significantly to total body weight gain in this model\textsuperscript{140}. Importantly, this scaffold-based strategy required a single administration compared to previous studies indicating that oral resveratrol delivery requires daily dosing\textsuperscript{50,52,61}. Furthermore, it is possible that this scaffold-based strategy could be easily combined with lifestyle interventions, such as calorie restriction and physical activity, which could further enhance its potential as a promising therapy for metabolic diseases.

In both lean and high fat diet fed mice, resveratrol scaffolds reduced adipocyte area compared to PLG scaffolds indicating a reduction in lipid content since adipocyte area and lipid content are positively correlated\textsuperscript{146}. We propose that resveratrol scaffolds promote the utilization of lipids within the implant site and that this effect is likely due to the combination of the host response to biomaterial implant and the release of resveratrol from the scaffold. The host response exhibited here, characterized by local cellular infiltration and protein deposition, is similar to wound healing which depends on cellular activity that is largely fueled by fatty acid metabolism\textsuperscript{147–149}. Furthermore, oral delivery of resveratrol to animals as well as treatment of cultured cells impacts the activity of several proteins involved in lipid metabolism, as described in several review articles\textsuperscript{58,150,151}. For example, treatment of cultured 3T3-L1, porcine, and human adipocytes with resveratrol led to an increase in lipolysis as a result of elevated ATGL levels\textsuperscript{52,54,55,60,152}. In addition, resveratrol increased CPT1 mRNA levels in adipose tissue from pigs and rats fed a resveratrol supplemented diet and cultured adipocytes treated with resveratrol\textsuperscript{56–58,61,51}. Contrary to our initial hypothesis, scaffold-based resveratrol delivery did not significantly increase
expression of either ATGL or CPT1 when measured in whole tissue homogenate collected 14 or 28 days after implant. We must consider that measuring these proteins in whole tissue may have decreased our sensitivity to see significant changes. For example, CPT1 is found on the mitochondrial membrane; therefore, it may be difficult to measure with high sensitivity in whole tissue homogenate. It is also possible we did not see differences in these proteins between scaffold groups at day 28 because the resveratrol payload was exhausted at that time point. However, we did see increased levels of activated AMPK at day 14 in the tissue. Literature indicates that resveratrol activates AMPK and resveratrol’s effect on ATGL and CPT1 are consequences of AMPK activation. Thus, it is possible that the decreased adipocyte size at day 28 was due to increase in ATGL and CPT1 enzyme activity at earlier time points.

Remarkably, resveratrol scaffolds protected mice from high fat diet induced weight gain and epididymal fat accumulation 8 weeks after initial implant, while the in vitro release profile indicates that approximately 70% of the initial resveratrol is released from the scaffold in the first week. We speculate that as pieces of the scaffold are encapsulated by the immune system, resveratrol release is slowed, extending its resident time in the tissue. These potential areas of high resveratrol concentration would more readily impact cells that are in direct contact. Therefore, we believe that macrophages are a particularly interesting population for further study as they are recruited to the implant site and fuse together to form multinucleated giant cells that are in direct contact with the scaffold. Interestingly, we show that CPT1 is more highly expressed in the newly formed tissue surrounding the scaffold compared to surrounding adipocytes, and the giant cells were CPT1 positive. This data is further supported by work that demonstrated that CPT1 is more
highly expressed in adipose tissue macrophages compared to adipocytes. However, spatial information of CPT1 expression was similar in both resveratrol and PLG scaffolds; therefore, to further study resveratrol’s potential effect on macrophages, we treated RAW 264.7 macrophages with resveratrol. We found that resveratrol treatment increased CPT1 expression. It has been shown that resveratrol decreases lipid levels in RAW 264.7 macrophages. Our work suggests that resveratrol decreases lipid levels in RAW 264.7 macrophages through increasing CPT1. In conclusion, we hypothesize that the mechanism by which resveratrol scaffolds decrease fat pad size relative to blank scaffolds is that upon scaffold implant, cells are recruited to the implant site, including macrophages, which can fuse into giant cells, and the resveratrol released increases fatty acid utilization in these cells leading to decreased adipocyte lipid content reflected in decreased adipocyte size.

While this work demonstrates that resveratrol releasing scaffolds decrease fat pad size in mice fed a high fat diet compared to scaffolds with no drug, this study does have limitations. First, we focused on demonstrating a protective effect, which lays a promising foundation for the technology, but is not translatable to humans as this strategy will likely be used as an obesity intervention, not a prophylactic. Secondly, we limited the scope of the study to RSV scaffold’s impact on the epididymal fat pad. Due to differences in composition and metabolic activity in visceral and subcutaneous fat depots, it remains unclear if this scaffold-based strategy would achieve similar results in other fat depots. Furthermore, there is a question of if this strategy would need to target multiple fat depots simultaneously to observe a significant therapeutic effect in humans. In addition, this study does not address if the use of scaffolds to enhance lipid metabolism would work in other metabolically relevant tissues, such as skeletal muscle; however, this is the focus of future
work. Finally, we must consider that the extensive remodeling that occurred as a result of
the implant may have negative effects on adipose tissue. Future work is focused on
developing injectable technologies that would not induce extensive remodeling but would
rather individually target immune cells to modulate gene programs involved in lipid
catabolism.

In conclusion, implant of resveratrol releasing scaffolds into epididymal fat of lean
mice decreased adipocyte size, a surrogate for lipid content, compared to PLG scaffolds
without a drug payload. Interestingly, the expression of CPT1 and ATGL, key proteins
involved in fatty acid catabolism, whose elevated expression is associated with decreased
adipocyte size, were unchanged in the epididymal fat at 14 or 28 days after scaffold
implant. However, AMPK activation was increased at 14 days, which indicates that ATGL
and CPT1 activity levels could have been elevated, providing a plausible explanation for
the decreased adipocyte size. Histology indicated that scaffold-recruited immune cells
expressed CPT1 at higher levels than other cells in the fat pad and resveratrol increased
CPT1 in cultured macrophages by 30%. Thus, it is possible that our study of whole tissue
homogenates limited our ability to detect increases in CPT1 because it was occurring in a
small population of cells. Taken together, we hypothesize that increases in CPT1 levels
and/or activity in macrophages within the scaffold play a role in resveratrol’s effect on
adipocyte size. We also demonstrate that mice implanted with RSV scaffolds are more
resistant to fat gain during 4 weeks of high fat diet compared to mice that received scaffolds
with no drug. We also demonstrate that mice implanted with resveratrol scaffolds are more
resistant to fat gain during 4 weeks of high fat diet compared to mice that received scaffolds
with no drug. Importantly, this effect was realized with a single administration of scaffolds,
which is considerably less than the daily dosing regimen usually required for resveratrol’s weight loss effects on animals fed a high fat diet. We conclude that direct delivery of resveratrol to the fat tissue overcomes issues with bioavailability and that tissue engineering scaffolds can be used to achieve this goal. This work motivates further study into the use of biomaterials for localized delivery of metabolism modulating agents to key metabolic cells in adipose tissue as a treatment for metabolic disease secondary to over-expanded adipose tissue.
3.5 Figures

**Figure 3.1: Scaffold characterization.** (A-D) Scanning electron microscopy and bright field microscopy images of PLG (A-B) and RSV (C-D) scaffolds. Scale bar represents 500 µm for B and D. (E) Quantification of pore diameter using ImageJ analysis of images depicted in B and D. Data was collected from three scaffolds per group. Statistics were determined using an unpaired t-test. (F-G) Top (F) and side (G) profile of scaffold implants measuring approximately 5 mm in diameter and 2 mm in height.
Figure 3.2: Resveratrol release profile. One-week *in vitro* release profile of resveratrol loaded PLG scaffolds. Data are from 3-8 scaffolds per time point. Error bars denote SEM.
Figure 3.3: Epididymal adipose tissue remodeling after resveratrol scaffold implant. (A-F) Histological sections of epididymal fat pads 28 days after implant of PLG (A-C) or RSV (D-F) scaffolds at 4x (A,D) or 40x (B,C,E,F) magnification. Scale bar represents 500 µm for A and D and 50 µm for B, C, E, and F. (G) Quantification of adipocyte area. Data was collected from 3 40x images per section, with 3 sections per mouse and 3 mice per group. Statistics were calculated by an unpaired t-test. * p<0.05 compared to PLG.
Figure 3.4: Effects of resveratrol scaffolds on key lipolysis and fatty acid oxidation protein expression 28 days after implant in lean mice. (A) Representative western blots of ATGL, CPT1, phosphorylated ACC (Ser79), total ACC, phosphorylated AMPK (Thr172), and total AMPK protein expression in adipose homogenates 28 days after implant. (B-E) Quantification of (B) ATGL, (C) CPT1, (D) ACC, and (E) AMPK expression. Data is from 3 mice per group. Statistics were calculated using an unpaired t-test.
Figure 3.5: Effects of resveratrol scaffolds on key lipolysis and fatty acid oxidation protein expression 14 days after implant in lean mice. (A) Representative western blots of ATGL, CPT1, phosphorylated ACC (Ser79), total ACC, phosphorylated AMPK (Thr172), and total AMPK protein expression in adipose homogenates 14 days after implant. (B-E) Quantification of (B) ATGL, (C) CPT1, (D) ACC, and (E) AMPK expression. Data is from 4 mice per group. Statistics were calculated using an unpaired t-test. * indicates p<0.05 compared to PLG.
Figure 3.6: Spatial analysis of CPT1 in the epididymal fat pad after scaffold implant. (A-D) CPT1 stained histological sections of epididymal fat pads 28 days after implant of PLG (A,C) or RSV (B,D) scaffolds at 4x (A,B) or 40x (C,D) magnification. Scale bar represents 500 µm for A-B and 50 µm for C-D. Data is representative of 3 sections per mouse with 3 mice per group.
Figure 3.7: Effect of resveratrol on CPT1 expression in RAW 264.7 macrophages. Representative western blot of CPT1 protein expression in RAW 264.7 macrophages treated with 0, 10, or 25 µM RSV for 24 hours. (B) Quantification of CPT1 expression. Data is from three 6 well plates with 2 wells per group on each plate. Statistics were calculated using a one-way ANOVA with a Tukey’s multiple comparison test. *indicated p<0.05 compared to untreated (0 µM).
Figure 3.8: Impact of scaffolds on weight gain and epididymal fat pad mass in mice challenged with a high fat diet. (A) Timeline of experiment. (B) Percent increase in fasting body weight relative to day 0 when the high fat diet began. (C) Epididymal fat pad weight 28 days after high fat diet challenge. N=5 mice per group. Statistics were conducted using an unpaired t-test. *indicates p<0.05 compared to PLG.
Figure 3.9: Effects of resveratrol scaffolds on epididymal fat adipocyte size after high fat diet challenge. (A-B) Histological sections of epididymal fat pads that received (A) PLG or (B) RSV scaffolds 28 days after high fat diet challenge taken at 40x magnification. Scale bar represents 50 µm. (C) Quantification of adipocyte area. Data collected from 3 images per section, with 3 sections per mouse and 3 mice per group. Statistics were calculated using an unpaired t-test. *indicates p<0.05 compared to PLG.
Figure 3.10: Effects of resveratrol scaffolds on key lipolysis and fatty acid oxidation protein expression after high fat diet challenge. (A) Representative western blots of ATGL, CPT1, phosphorylated ACC (Ser79), total ACC, phosphorylated AMPK (Thr172), and total AMPK protein expression in adipose homogenates after high fat diet challenge. (B-E) Quantification of (B) ATGL, (C) CPT1, (D) ACC, and (E) AMPK expression. Data is from 5 mice per group. Statistics were calculated using an unpaired t-test. * indicates p<0.05 compared to PLG.
CHAPTER 4

SINGLE BOLUS DOXORUBICIN ADMINISTRATION INDUCES FIBROSIS IN SUBCUTANEOUS ADIPOSE TISSUE: A THERAPEUTIC OPPORTUNITY

4.1 Introduction

Cachexia is a condition associated with involuntary skeletal muscle and adipose tissue atrophy resulting in weight loss that affects 50-80% of cancer patients and is responsible for approximately 20% of cancer-related deaths. This debilitating symptom of cancer progression significantly reduces patient quality of life and correlates with worsened prognosis. Patients that receive cytotoxic chemotherapy treatments often present with changes in body mass and composition. Additionally, ovarian cancer patients have a worse survival rate when they lose skeletal muscle and adipose tissue mass while undergoing chemotherapy treatment. Therefore, this condition may be, in part, related to the use of chemotherapies, such as doxorubicin. Doxorubicin is an extensively used chemotherapeutic agent for the treatment of a wide variety of cancers, including leukemia, stomach, breast, and ovarian. Treatment with this chemotherapy is associated with cardiotoxicity, nephrotoxicity, and hepatotoxicity, in addition to other severe and unpleasant side effects. Doxorubicin’s negative effects on adipose tissue function may

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Murphy, K.P., Patterson, A.T., Montalvo, R. Hall, H.E., Carter, G.J., Smuder, A.J., Gower, R.M. To be submitted to Experimental Physiology.
Adipose tissue is most notably known for its role as a lipid storage site, but this tissue also controls metabolism, immunity, reproduction, and many other processes required for homeostasis through the factors it controls ⁸,¹²,¹⁵⁸,¹⁵⁹. Therefore, dysfunction of this tissue can lead to a host of systemic complications, such as chronic systemic inflammation and insulin resistance, which contribute to a type 2 diabetic like state. Biopsies from cachexic cancer patients have presented with subcutaneous adipose tissue architectural remodeling, such as decreased adipocyte area, increased inflammatory cell infiltration, and fibrosis ⁷⁴. Fibrosis is defined as the excessive deposition of extracellular matrix proteins, including collagen 1, which is a key provider of the major structural framework of adipose tissue ¹⁶⁰. During obesity related fibrosis, macrophages recruited to the site of injury are known to be the key regulator of fibrosis ⁷⁵. These cells produce soluble factors, such as TGF-B1, that activate fibroblasts already within the tissue as well as factors that recruit new fibroblasts ⁷⁵. Activated fibroblasts possess an increase in α-smooth muscle actin and lay down extracellular matrix proteins, such as collagen 1 ¹⁶¹. Fibrosis has significantly negative consequences to adipose tissue function and as a result to systemic processes controlled by the adipose tissue ⁷⁵. Fibrosis limits cell to cell communication required for proper function and induces a rigidness that reduces flexibility, resulting in decreased lipid storage capacity and consequently lipid deposition in distant tissues ⁷⁵. This rigidity is also likely to be uncomfortable for the patient leading to a decreased quality of life. Furthermore, fibrosis alters the adipose tissue secretome by reducing the secretion of health promoting factors, such as adiponectin, which promotes insulin resistance ⁷⁵.
There is limited literature investigating doxorubicin’s impact on adipocyte lipid metabolism\textsuperscript{71–73,162,163}; however, even fewer studies have aimed to understand doxorubicin’s role in promoting adipose tissue remodeling similar to that observed in cachexic cancer patients\textsuperscript{71}. Furthermore, there are several critical, unanswered questions that should be addressed regarding the role of doxorubicin administered dose, dosing schedule, and the gender of the studied population. To date, most in vivo work studying doxorubicin’s effect on adipose tissue has been tested in male rodents by injecting small dosages multiple times over several weeks. However, this does not accurately model clinical dosages of most cancer patients. A common patient dose of doxorubicin is 60 mg/m\textsuperscript{2} administered once every 21 days, therefore, when scaled to rodents this is equivalent to a 20 mg/kg dose\textsuperscript{164}. However, it is unknown how adipose tissue responds to this larger bolus after 72 hours, since only few have assessed larger dose at early time points\textsuperscript{165}. Furthermore, there is a significant need to study the effect of this chemotherapy on adipose tissue in female rodents, as to our knowledge this response has never been reported. Since doxorubicin is used to treat several female cancer, such as breast and ovarian cancer\textsuperscript{157}, and adipose tissue wasting is associated with worsened prognosis in ovarian cancer patients\textsuperscript{156}, it is imperative that doxorubicin’s role to promote adipose tissue dysfunction in females is investigated.

Therefore, to begin to address these gaps in literature, the overall aim of this manuscript was to characterize the effects of a clinically relevant bolus of doxorubicin on adipose tissue fibrosis in female rats. Visceral and subcutaneous adipose tissue depots were studied to determine if anatomical location was a factor involved in the extensiveness of doxorubicin induced fibrosis, a response not yet investigated. This work is expected to
provide greater understanding of doxorubicin’s potential role in promoting cancer-associated cachexia and provide insight for the development of future strategies to sustain adipose tissue health during chemotherapy treatment.

4.2 Materials and Methods

4.2.1 Animal Study Design

The Animal Care and Use Committee of the University of South Carolina approved these experiments. Adult four-six-month old female Sprague-Dawley rats were maintained on a 12:12 h reverse light-dark cycle and provided rat chow and water ad libitum. Figure 4.1A illustrates the timeline of the study. A cohort of Sprague-Dawley female rats were weighed on day 0 and then randomly assigned to either receive a bolus of doxorubicin hydrochloride (20 mg/kg body weight) via intraperitoneal injection or equal volumes of saline. Animals were monitored daily. Due to a decline in animal health, the number of animals actually studied in each group was reduced. Furthermore, the end point of the study was changed from 14 to 9 days due to animal health. At the end of the study, 5 animals that had received doxorubicin and 6 animals that received equal volumes of saline were studied.

On day 9, animals were weighed and then acutely anesthetized with sodium pentobarbital (60 mg/kg intraperitoneally). After reaching a surgical plane of anesthesia, the heart was removed for mechanistic studies conducted by the Smuder group. Following euthanasia, approximately 200-400 mg of the ovarian or inguinal fat pads were submerged in 4% paraformaldehyde for histological analysis. The rest of the ovarian and inguinal fat pads were flash frozen in liquid nitrogen and stored at -80°C for protein or triglyceride analysis.
4.2.2 Histological Analysis

Ovarian and inguinal fat was fixed in 4% paraformaldehyde, embedded in paraffin, and serially cross-sectioned into 5 µm sections. Sections were stained with hematoxylin and eosin (H&E) for morphological analysis. Using a Nikon Eclipse ci microscope, three random images were taken at 20x magnification of four sections per fat pad per animal. Image J software was used to quantify adipocyte area from 4 sections per tissue from 4 saline injected animals and 5 Dox treated animals. All adipocytes within the field of view were quantified. Adipocyte number in each field of view is also reported.

4.2.3 Triglyceride Quantification

Triglycerides in ovarian and inguinal fat were quantified using the Triglyceride Colorimetric Assay kit purchased from Cayman Chemical. The measurements were made according to the manufacturer’s instructions. Briefly, frozen fat pads (150 mg) were homogenized in 750 µL of diluted NP40 Substitute Assay Reagent containing Halt Protease Inhibitor Cocktail (Thermo Fisher Scientific) using a benchtop homogenizer. Samples were centrifuged at 10,000 x g for 10 minutes at 4 °C and the supernatant was collected and stored at -80°C until analyzed. Samples were added to a 96-well plate and a coupled enzymatic reaction was initiated to hydrolyze triglycerides in the samples to produce glycerol and fatty acids. After a 15-minute incubation, the plate was analyzed at 540 nm using a spectrophotometer. A standard curve was used to extrapolate triglyceride concentration in mg/dl in the unknown samples. Triglyceride content was reported as mg triglyceride per g homogenized tissue.
4.2.4 Protein Quantification

Frozen fat pads (approximately 500 grams) were homogenized in a defined volume of RIPA buffer (Fisher Scientific) containing Halt Protease Inhibitor Cocktail (Thermo Fisher Scientific), PhosStop (Sigma), and PMSF (Thermo Fisher Scientific) using a benchtop homogenizer. Centrifugation was used to removal all insoluble material and the supernatant was collected, aliquoted, and stored at -80 °C. Total protein in the homogenate was quantified using the Pierce BCA protein assay kit (Thermo Fisher Scientific). Total protein is reported as mg protein per gram of homogenized tissue.

4.2.5 Western Blotting

Ten-thirty µg protein was separated by 8% polyacrylamide gel electrophoresis and then transferred to 0.2 µm nitrocellulose membranes. Membranes were then blocked with 5% nonfat dry milk in Tris-buffered saline containing Tween-20 (TBST) for 1 hour at room temperature. Primary antibody for anti-collagen 1 (Abcam) and anti-α smooth muscle actin (Cell Signaling Technologies) was added to each blot at 1:1000 dilution in 5% BSA in TBST and allowed to incubate overnight at 4°C before incubation with a polyclonal secondary antibody conjugated to horseradish peroxidase (Abcam) in 5% milk for 1 hour at room temperature. The blots were developed with SuperSignal enhanced chemiluminescent substrate solution (Thermo Fisher Scientific). To verify loading, blots were incubated with stripping buffer for 25 minutes and washed with TBST. The blot underwent the same protocol as previously stated for β-actin (Sigma) using a monoclonal secondary antibody conjugated to horseradish peroxidase (Biorad). ImageLabs software was used to analyze the relative intensity of each protein. Results were reported as intensity of the primary antibody relative to the intensity of β-actin.
4.2.6 Statistical Analysis

Statistically significant differences between saline and Dox treated groups were evaluated using an unpaired student’s t-test. Statistical significance was set to a p value less than 0.05. The number of samples analyzed is detailed in each figure legend. All analyses were completed using GraphPad Prism. In all figures, data is presented as individual data points from each rat analyzed with mean and standard deviation.

4.3 Results

4.3.1 Effect of Doxorubicin on Animal Body Weight and Fat Pad Mass

As anticipated, a 20 mg/kg intraperitoneal Dox bolus significantly reduced the body weight of treated animals compared to the saline group 9 days after administration (Figure 4.1B). Additionally, Dox reduced animal body weight by approximately 20% when comparing body weight measured prior to injection and before euthanasia on day 9 (Figure 4.1C). However, to our surprise, the weight of the ovarian (Figure 4.1D) and the inguinal (Figure 4.1E) fat pads normalized to body weight did not significantly change 9 days after Dox treatment compared to saline injected controls. Additionally, there is significant variability between the Dox treated animals in body weight and fat pad weights that is not observed in the saline control group. For example, the body weight of Dox treated animals changed anywhere from losing 33% of their body weight to gaining 2% of their body weight (Figure 4.1B).

4.3.2 Adipose Tissue Remodeling in Doxorubicin Treated Animals

Since cancer induced cachexia patients present with changes in adipose tissue remodeling such as extracellular matrix deposition, cellular infiltration, and reduced adipocyte area, both the ovarian and inguinal fat pads were histologically assessed 9 days
after Dox treatment. Figure 4.2 shows representative tissue sections of saline and Dox treated ovarian (Figure 4.2A-B) and inguinal (Figure 4.2 C-D) stained with hematoxylin and eosin. We did not detect major differences between ovarian fat collected from saline rats compared to healthy ovarian fat (data not shown). Furthermore, we observed a slight increase in cellular infiltration (purple arrow), extracellular matrix deposition (yellow arrow), and vessel formation (red arrow) surrounding adipocytes in Dox treated ovarian fat (Figure 4.2B). Compared to the ovarian fat, the inguinal fat inherently has less adipocytes, larger intercellular space, increased blood vessels (red arrow), and an inherent abundance of collagen fibers (yellow arrows) as seen in inguinal fat collected from saline treated rats (Figure 2.4 C). Doxorubicin treatment enhanced the abundance of extracellular matrix proteins within the inguinal fat compared to saline control (Figure 4.2D compared to C). Furthermore, Dox treatment established a more severe fibrotic response in the inguinal fat compared to the ovarian fat, as supported by a significant increase in extracellular matrix deposition in Figure 4.2D compared to Figure 4.2B.

As a quantitative, surrogate method to measure the extent of fibrosis in both tissues as a function of Dox treatment, total protein content in both fat pads was measured (Figure 4.3). Total protein was not significantly altered with Dox treatment compared to saline in either ovarian or inguinal fat, but there was an upward trend observed in both tissues. As observed in the weight measurements, there was large variability in this measurement between the Dox treated animals. Similarly, the extent to which Dox induced fibrosis in the inguinal fat as observed histologically was highly variable. The image shown in Figure 4.2D is deemed as the average extent of fibrosis and is associated with the individual data point closest to the average total protein content in Figure 4.3B. Large variability was not
observed histologically between Dox treated ovarian fat pads, likely because Dox induced inflammation was not as severe in this fat pad compared to the inguinal.

Upon this observation, we investigated a possible mechanism for the severe fibrosis that occurred in inguinal fat pads by measuring collagen 1 and α-smooth muscle actin (α-SMA) within inguinal adipose tissue homogenate using western blot (Figure 4.4A). Interestingly, both collagen 1 (Figure 4.4B, p=0.042) and α-SMA (Figure 4.4C, p=0.054) relative to β-actin were significantly increased in inguinal fat pads collected from Dox treated animals as determined by an unpaired student’s t-test. This data suggests a possible role of activated fibroblasts in this fibrotic response induced by Dox treatment that warrants further dissection.

4.3.3 Adipocyte Morphological Changes in Doxorubicin Treated Animals

Adipose tissue function is highly dictated by adipocyte function; therefore, we then investigated how Dox treatment altered adipocyte area and number in ovarian and inguinal fat pads by further investigating H&E stained sections shown in Figure 4.2. Adipocytes in ovarian fat pads collected from Dox treated animals looked to be smaller (Figure 4.5A compared to B); however, upon quantification using ImageJ, adipocytes were not significantly smaller in area compared to the saline treated group (Figure 4.5E), but there was a decreasing trend observed. However, there were significantly more adipocytes within each frame indicating a potential state of hyperplasia (Figure 4.5F). Similarly, adipocytes in inguinal fat pads collected from Dox treated animals also appeared to be smaller in stained sections (Figure 4.5C compared to D); however, upon quantification there was not a significant difference in adipocyte area or number between groups. Once again, we observed large variability in the quantification between Dox treated animals, especially in
the inguinal fat analysis. The histological image in Figure 4.5D is associated with the individual data point in Figure 4.5G and H closest to the mean. To test the hypothesis that Dox treatment reduced triglyceride content as suggested by a reduction in adipocyte area, we measured total triglyceride content in each tissue (Figure 4.6). In ovarian and inguinal fat, triglyceride content was not significantly reduced with Dox treatment, and in fact trended upward compared to saline injected animals. To our surprise, the animals that exhibited smaller adipocyte area measured to have more triglycerides within the tissue.

4.4 Discussion

Cachexia is a significant symptom of cancer progression that leads to approximately 20% of cancer-related deaths and a major reduction in the patient’s quality of life. Because patients that receive cytotoxic chemotherapy treatments often present with changes in body mass and composition\(^{155}\), it is likely that chemotherapy plays a role in progressing this condition. Doxorubicin is a widely used chemotherapeutic with severe cytotoxic effects on the heart, liver, and kidney\(^ {157}\); however, the role of doxorubicin administration on adipose tissue wasting remains unclear. In this report, we demonstrated that a 20 mg/kg bolus of Dox significantly reduced body weight, but not adipose tissue weight, and differentially promoted fibrosis of adipose tissue depots 9 days after injection in female rats. To our knowledge, these results are the first to suggest that Dox induced adipose tissue fibrosis also occurs in female rodents and varies based on adipose tissue anatomical location.

Our results indicate a significant importance of Dox administration, such as dose and study duration, on adipose tissue function. A single 20 mg/kg doxorubicin bolus was chosen for this study because it represents a relevant clinical human dose scaled to rodents.
This dose has been used to evaluate Dox effects on skeletal muscle\textsuperscript{166} and heart\textsuperscript{165}; however, the effect of Dox on adipose tissue at this dose has yet to be investigated. Our results indicate 9 days after injection, Dox significantly reduced animal body weight, which is a similar response demonstrated by studies that administered this dose, but ended the study 24-72 hours after injection\textsuperscript{165,167}. Furthermore, our results did not indicate a significant reduction in ovarian (perigonadal) or inguinal (subcutaneous) fat or adipocyte area in either tissue. We hypothesize that body weight changed due to Dox induced changes in other organs such as skeletal muscle\textsuperscript{166}. Contrary to our results, when 2.5 mg/kg Dox was administered twice per week for two weeks, rodents maintained their body weight, significantly lost perigonadal and subcutaneous adipose tissue mass, and observed a significant reduction in adipocyte area in subcutaneous tissue\textsuperscript{71}. However, when administering Dox as either a single, large bolus or multiple small injections, fibrosis occurred in subcutaneous tissue and was associated with an increase in collagen\textsuperscript{71}. This leads to the conclusion that dose and administration schedule may have an impact on how Dox impacts adipose tissue.

Pre-clinical studies have determined that Dox induces fibrosis and inflammation in several organs, such as the liver\textsuperscript{168} and heart\textsuperscript{169}, but there is limited knowledge on Dox’s fibrotic effect on adipose tissue. Similar to a previous report\textsuperscript{71}, we demonstrated that a Dox bolus induced fibrosis in subcutaneous adipose tissue. Dox induced adipose tissue fibrosis was associated with an elevation in collagen 1 and α-smooth muscle actin expression, a marker of activated fibroblasts\textsuperscript{161}. Therefore, we propose the mechanism of this response may be two-fold: 1) Dox stimulates the infiltration of macrophages that in turn activate fibroblasts to synthesize extracellular matrix proteins via secreted soluble factors and/or 2)
Dox directly activates fibroblasts to synthesize extracellular matrix proteins. This hypothesis is supported by literature suggesting that Dox treatment increases infiltration of macrophages at injured site, as observed in Dox induced nephropathy\textsuperscript{170}, and also activates cardiac fibroblasts which led to an elevation in \(\alpha\)-smooth muscle actin expression and collagen 1 expression\textsuperscript{171}. Additionally, this fibrotic response was similar to that in subcutaneous adipose tissue biopsies from cancer cachexic patients characterized by an elevation in collagen 1, \(\alpha\)-smooth muscle actin\textsuperscript{74,172}. Furthermore, cachexic patient biopsies also indicated altered TGF-\(\beta\) signaling, suggesting a possible role for TGF-\(\beta\) in Dox induced adipose tissue fibrosis, which should be a focus of future studies.

Furthermore, in this report, we established for the first time that Dox more severely induces a fibrotic response in inguinal fat, a subcutaneous fat pad, compared to ovarian fat, a visceral fat pad, in female rats. This is particularly interesting because in this study Dox was administered intraperitoneally, where the ovarian fat was bathed in the drug upon injection. We hypothesize that inherent differences in composition and function between visceral and subcutaneous fat pads may play a role in this altered effect. One such difference is that subcutaneous fat has higher basal levels of PPAR\(\gamma\), a nuclear receptor involved in adipocyte differentiation and is also more responsive to PPAR\(\gamma\) ligands, such as thiazolidinediones\textsuperscript{34,173}. If this tissue is more responsive to PPAR\(\gamma\) ligands, then it’s possible that it could be more sensitive to agents that inhibit PPAR\(\gamma\), such as Dox\textsuperscript{174}. Additionally, activation of PPAR\(\gamma\) can block TGF-\(\beta\) signaling and thus fibrosis\textsuperscript{175}. Therefore, the subcutaneous fat may be more fibrotic due its heightened sensitivity to PPAR\(\gamma\) inhibition after Dox leading to the enhanced TGF-\(\beta\) induced fibrosis.
The data set reported here exhibited large variability associated with the Dox treated animals. It is hypothesized that this large variability is associated with the use of female rats in the study. This is the first time that adipose tissue dysfunction has been reported in female rats; however, in a study using male rodents, there doesn’t appear to be large variances in data collected from adipose tissue, even when using a small sample size\(^7\). Sexual dimorphism has been established in Dox induced toxicities as female rodents have shown to be protected against Dox induced cardiotoxicity compared to males\(^176\). Therefore, some animals appeared to be more resistant than others to Dox treatment potentially due to differences associated with the fact that they are female. Furthermore, fluctuations in female reproductive hormones in response to estrous cycles could complicate the system and potentially be a source of data variability between Dox treated animals. Fluctuations in sex hormones plays a significant role in adipose tissue metabolic function; for example, a decrease in estrogen is associated with an increase in fat pad weight\(^177\). Further investigation is warranted on sexual dimorphism and the role of female sex hormones on Dox induced adipose tissue dysfunction. This is especially important to understand for ovarian cancer patients, a population in which chemotherapy induced adipose tissue wasting is associated with lower survival\(^156\).

Since adipose tissue wasting is a main component of cancer associated cachexia, and is in part due to toxic chemotherapies, there is a significant need for novel therapies to sustain adipose tissue health and metabolic function during chemotherapy treatment. We propose in future studies to utilize particle delivery systems that are easily injected into the subcutaneous fat pad to target macrophages as a strategy to prevent doxorubicin induce adipose tissue fibrosis. The results presented here indicate that a chronic Dox bolus maybe
an appropriate therapeutic testing model for the development of adipose tissue fibrosis in female cancer patients; however, to maintain 100% animal survival, further investigation into adipose tissue dysfunction of clinically relevant dosage and schedules is needed.

4.5 Figures

Figure 4.1: Study timeline, body weight change, and fat pad mass 9 days after Dox administration. (A) Study timeline. (B-C) Body weight (B) and body weight percent change (C). (D-E) Ovarian (D) and inguinal (E) adipose tissue mass. Data is from n=5 Dox treated animals and n=6 saline controls. Data is presented as individual data points with mean ± standard deviation.
Figure 4.2: OWAT and IWAT histological analysis. (A-D) Histological sections of ovarian (A,B) and inguinal (C,D) fat pads collected 9 days after saline (A,C) and Dox (B,D) injection stained with hematoxylin and eosins. Scale bar represents 500 µm.

Figure 4.3: Effect of Dox administration on total protein content 9 days after injection. Protein content measured in (A) ovarian and (B) inguinal fat pads. Data is collected from n=6 saline injected rats and n=5 Dox treated rats. Statistics determined using an unpaired student’s t-test.
Figure 4.4: Effect of Dox administration on profibrotic proteins. (A) Representative western blots of collagen 1 and α-smooth muscle actin expression in inguinal adipose homogenate 9 days after treatment. (B-C) Quantification of (B) collagen 1 and (C) α-smooth muscle. Data is collected from n=6 saline injected rats and n=5 Dox treated rats. Statistics determined using unpaired student’s t-test. * indicates p<0.05 compared to saline.

Figure 4.5: OWAT and IWAT adipocyte area and number 9 days after Dox administration. (A-D) Histological sections of ovarian (A,B) and inguinal (C,D) fat pads collected 9 days after saline (A,C) and Dox (B,D) injection stained with hematoxylin and eosin. Scale bar represents 100 µm. (E-H) Quantification of adipocyte area (E,G) and number (F,H) from ovarian (E,F) and inguinal (G,H) fat pads. Data was collected from 3 random 20x images per section, with 4 sections per fat pad, and 4-5 rats per group. Statistics determined using an unpaired student’s t-test. * indicates p<0.05.
Figure 4.6: Effect of Dox administration on triglyceride content 9 days after administration. Triglyceride content was measured in (A) ovarian and (B) inguinal fat pads. Data is collected from n=6 saline injected rats and n=5 Dox treated rats. Statistics determined using an unpaired student’s t-test.
CHAPTER 5
CONCLUDING REMARKS AND FUTURE DIRECTIONS

5.1 Concluding Remarks

Once known as an inert site for lipid storage, the adipose tissue has recently become well accepted as an endocrine organ that plays a key role in whole body homeostasis. The ability to directly modulate its inherent endocrine and metabolic functions has promising implications in treating diseases associated with adipose tissue dysfunction. One such example is obesity, in which a significant portion of the United States adult population has been diagnosed, a trend that continues to rise. Since obesity is a critical risk factor for numerous severe and chronic diseases, this epidemic has become an ever increasing economic and health burden. With the current interventions possessing substantial challenges, novel therapeutic platforms engineered to specifically target the adipose tissue’s metabolic and endocrine function are warranted. One central objective of this work was to test the effectiveness of scaffold-based delivery of resveratrol, a promising anti-obesity drug that suffers from poor bioavailability, to reduce inflammation and lipid accumulation upon implant into the adipose tissue and thus establish its potential as an anti-obesity therapeutic platform.

The first aim accomplished this objective by further understanding how the immune compartment within the adipose tissue was altered after poly(lactide-co-glycolide) scaffold implant and how the incorporation of resveratrol modulated this response. This was particularly important to understand as inflammation drives metabolic disease within the
adipose tissue. Resveratrol was successfully incorporated into the polymer matrix with high resveratrol loading and exhibited a drug release with a burst followed by a plateau phase. In addition, resveratrol loaded materials maintained their loading and activity for at least a year. The \textit{in vivo} results of this aim, outlined in Chapter 2, demonstrated that the incorporation of resveratrol augments an anti-inflammatory environment established by PLG scaffolds, characterized by low levels of pro-inflammatory cytokines and increased expression of anti-inflammatory cytokines upon implant in lean mice. More therapeutically relevant were the results that treatment with resveratrol scaffolds decreased inflammatory cytokines in mice challenged with a high fat diet and adipocytes treated with an inflammatory stimulus. This work demonstrates to the tissue engineering community that PLG scaffolds are a promising platform for the treatment of adipose tissue inflammation, which drives metabolic disease, due to their ability to promote an anti-inflammatory environment and their feasibility to be engineered as drug delivery vehicles.

The goal of the second aim, described in Chapter 3, was to investigate resveratrol scaffold-based delivery to enhance lipid catabolism within the adipose tissue. Implant of resveratrol scaffolds in lean mice decreased adipocyte size relative to scaffolds with no drug payload, which is a surrogate measurement of lipid load within the cells. Furthermore, the results support the hypothesis that resveratrol scaffolds decrease adipocyte size because resveratrol increases lipid utilization in scaffold-infiltrating immune cells, potentially by elevating expression or activity of the rate limiting enzyme in fatty acid oxidation. Importantly, mice pre-treated with resveratrol scaffolds prior to high fat diet feeding exhibited decreased weight gain, adipose tissue expansion, and adipocyte hypertrophy compared to scaffolds without a drug payload. This was the first study of its kind to indicate
that a single administration of resveratrol via PLG scaffold delivery was as successful in preventing weight gain compared to the daily regiment generally needed for oral delivery. These results indicate that localized delivery of metabolism modulating agents directly to the tissue of interest may overcome poor bioavailability. Taken together with the results described in Chapter 2, this work demonstrates to the tissue engineering field that PLG scaffolds are not only a suitable platform for adipose tissue regeneration and in vitro model development, but also a promising platform for local drug delivery to the adipose tissue. Furthermore, this strategy has the potential to be a promising therapeutic anti-obesity strategy through its ability to direct adipose tissue inflammatory and metabolic programs.

In addition to obesity, cachexia is a debilitating disease that is associated with adipose tissue wasting and inflammation and decreases the quality of life for a significant number of cancer patients. In the work described in chapter 4, we aimed to establish how a doxorubicin bolus impacted adipose tissue remodeling in female mice, a response that had yet to be reported, but is significant since a large portion of doxorubicin treated patients are women. Doxorubicin treatment significantly reduced body weight, but treatment did not decrease adipose tissue mass or adipocyte area. Furthermore, significant fibrosis occurred in subcutaneous adipose tissue to a greater extent than visceral that was associated with an increase in expression of a fibroblast activation marker and collagen, a response similarly found in human cachexia patients. These results provide further understanding of how chemotherapy can potentially promote cachexia, a disease that leads to 20% of cancer deaths. Furthermore, this work may provide insight for the development of future platforms to sustain adipose tissue health during chemotherapy treatment.
5.2 Future Directions

While Chapters 2 and 3 undoubtedly demonstrate that resveratrol loaded scaffold delivery is a promising strategy for directing adipose tissue function, there are several challenges that this strategy must overcome in order to increase its future translatability. Overcoming these challenges should be a major focus of future work. The first challenge is that as it has been studied so far, the implantation process requires an invasive surgical procedure in which the abdominal cavity is opened. This could be alleviated in two ways: 1) target a subcutaneous fat pad or 2) develop an injectable system that would not require an implant surgery. Targeting a subcutaneous fat pad would allow for the scaffold to be implanted via a small incision and a trocar. However, this strategy has not been studied in subcutaneous fat pads; therefore, we do not know if this strategy will have the same beneficial effects in subcutaneous fat due to differences in composition and metabolic activity in visceral and subcutaneous fat depots. Additionally, developing an injectable system would bypass the need for surgical procedures and would allow for targeting of any fat depot.

Secondly, this strategy is associated with extensive remodeling where the immune system encapsulates the material due to its inability to remove the material. As elaborated on in Chapter 4, this fibrotic response can have negative effects on adipose tissue function. Therefore, future work should focus on developing an injectable technology that would also not induce extensive remodeling. Literature suggests that a promising strategy would be to develop a particle system that would rather individually target immune cells to modulate gene programs involved in inflammation and metabolism.
Finally, the release kinetics of resveratrol from the scaffold should be improved. The current fabrication technique results in a scaffold that is loaded with a large amount of resveratrol that releases in a burst phase followed by a plateau phase. This was strategic when desiring to modulate the acute inflammatory phase which occurs in the first few days following implant; however, it is likely that extended release over longer periods of time will enhance its effectiveness as a therapeutic for metabolic disease. Furthermore, even though we saw an increase in anti-inflammatory cytokines in Chapter 2, an initial increase in neutrophils occurred that was likely counterproductive and potentially due to the burst release of resveratrol from the scaffold. Fabrication parameters that could be tailored in order to extend this release are polymer molecular weight and the amount of resveratrol loaded within the scaffold. Increasing the polymer molecular weight and decreasing the amount of resveratrol loaded is likely to reduce the burst release and extend the release from the scaffold over time.

In Chapter 4, the effect of Doxorubicin to induce adipose tissue fibrosis in female rats was demonstrated. In the model used in this study, the dose and study timeframe were associated with decreased animal survival. Therefore, further understanding of the adipose tissue fibrosis that results after i.p. injection of a clinically relevant dose given for a shorter time is likely necessary to maintain animal survival. Furthermore, there is a therapeutic opportunity to use biomaterials to sustain adipose tissue health during chemotherapy treatment. Therefore, a particle drug delivery system could be designed to inhibit fibrosis by locally delivering a potent anti-inflammatory molecule or targeting macrophage inflammatory function. This doxorubicin induced fibrosis model could be utilized to test the efficacy of these therapeutic systems in future studies. Collectively, the future
improvements and studies discussed could lead to the development of potentially life-saving therapeutic strategies for adipose tissue dysfunction associated with obesity and cachexia.
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APPENDIX A

BEST PRACTICES FOR WESTERN BLOTTING

Western blotting is a technique that is extensively used in biological research to determine relative expression of specific proteins in the sample. There are several online resources that one can take advantage of to learn this technique (doi:10.4103/1947-2714.100998, 10.1111/sms.12702, and Biorad’s Introduction to Western Blotting webpage). However, over the course of my Ph.D., I have analyzed lysate from cell culture experiments and collected adipose tissue from animal experiments and the techniques that I have learned while running these experiments are detailed below.

A.1 Sample collection and storage

When analyzing protein from either cells or tissues, the first thing that must be done is to isolate the protein fraction from your sample. There is a specific protocol for cells collected from cell culture and tissues collected from animal studies. The homogenization buffer consisting of RIPA buffer, 100x PhosStop, 100x PMSF, and 100x protease inhibitor cocktail is the same for both samples. To lyse RAW 264.7 cells, 100 µL of the homogenization buffer should be added to each sample for experiments where more than 200,000 cells were seeded. I would suggest decreasing the cell volume to 75 µL for experiments where fewer than 200,000 Raw 264.7 cells were seeded. When using 3T3-L1 cells, experiments with 80,000 cells per well or greater can be homogenized in 100 µL. Cells are then lysed using a needle and syringe. Do not create bubbles during lysing. The homogenized cells are then centrifuged and the supernatant (protein fraction) is collected.
from the top. Be careful not to disturb the pellet at the bottom. Supernatant is aliquoted into appropriately labeled tubes and stored in the -80°C freezer.

For tissues, the tissues are first weighed (usually this is done upon collection from the animal prior to freezing) and placed into a 2 mL round bottom tube. It is important that they are placed in a round bottom tube because the homogenizer tip can fully reach the bottom of the tube during homogenization, which it cannot when using a 1.7 mL conical tube. The samples are to be kept on ice at all times. Homogenization buffer is added to the tissue at a volume (µL) that is 4 x tissue mass (mg). If the tissue is larger than 250 mg (addition of 1mL buffer), I suggest splitting the tissue into multiple 2 mL round bottom tubes. This will ensure that the liquid does not overflow during homogenization. A pair of surgical scissors is then used to finely mince each tissue and the samples are then placed on the rocker in the fridge for 45 minutes. Be sure that when they are placed on the rocker, they are placed longways and ensure that the solution is moving up and down within the tube. The large homogenizer tip used for particle fabrication should be exchanged for the smaller tip and cleaned with ethanol and water prior to homogenization. Be sure to have reserved the homogenizer on the google calendar or talked with lab mates prior to planning the homogenization. Each sample will be homogenized for 3x 1min at 10,000 rpm. I suggest homogenizing each sample in one experimental group once and then repeating all of them 2 more times. This will ensure each sample stays as cold as possible. The homogenizer must be cleaned between each sample using water and ethanol. The microcentrifuge should be “fast temped” to 4°C prior to centrifugation of samples at 18,000 xg for 45 minutes. Supernatant is collected from samples, aliquoted into appropriately labeled tubes and stored in -80°C freezer. Be sure not to disturb the pellet at the bottom. For fatty tissues,
such as adipose tissue, there will also be an insoluble fatty cake on the top. I found that the easiest way to deal with this layer is to push it to the side or even out using a 1000 µL pipette tip.

A.2 Total protein analysis

In order to appropriately compare the result from each sample, the amount of total protein analyzed must be the same for each sample. Therefore, a BCA assay is used to determine the amount of total protein in each sample. A 2 mg/mL bovine serum albumin aliquot is diluted in homogenization buffer to create a standard curve to which the unknown samples will be compared. Usually, each sample is added to the 96 well plate using a 1:5 or 1:2 ratio of sample to homogenization buffer based on the concentration of the sample and where it falls on the standard curve. Be sure to add each standard and sample on the plate in duplicate, at least. Solution A and B provided in the kit are mixed in a 1:50 ratio of solution B to A and 200 µL of the mixture is added to each well. I would recommend using a 300 µL multichannel pipette to do this as quickly and uniform as possible. This is a light sensitive reaction; therefore, be sure to cover the plate and place it in aluminum foil on the slow rocker. After 30 minutes, the plate is ran at 562 nm on the Spectramax located in Catawba. Since the volume of each well is important when reading the absorbance, be careful when transporting the plate across the street. Generally, I have found that there is 2-3 mg/mL protein isolated from epididymal fat collected from chow fed healthy male C57Bl/6 mice, 4.5 mg/ml protein per 1x10^6 Raw 264.7 cells, 5 mg/ml protein per 1x10^6 3T3-L1 undifferentiated cells, and 13 mg/ml protein per 1x10^6 3T3-L1 differentiated cells when cells were lysed in 100 µL of buffer.
Once you have the absorbance values for the plate, upload them in Excel. The standard curve will be plotted with the x axis as absorbance and y axis as concentration. Be sure to average the absorbance values for each standard and subtract the background (0 mg/mL) from each prior to plotting. Add a linear trendline and equation to the graph. The $R^2$ value should be greater than 0.98. If it isn’t or you see that duplicate samples are not similar, then I suggest repeating the assay and be sure to watch out for any pipetting errors. The equation can then be used to solve for protein concentration (mg/mL) in the unknown samples by plugging in the average absorbance value for each unknown (after subtracting the background).

The amount of protein that you need to load to the gel will be dependent on the protein of interest that you are analyzing. I suggest conducting an optimization western blot where you analyze one sample from each group at different protein amounts loaded to the gel in order to determine the appropriate loading amount. I suggest adding a positive control in this optimization experiment as well. I had much success contacting Cell Signaling for free positive controls for several antibodies. Most proteins that I have analyzed in adipose tissue and cell lysate have been successful when adding 10-30 µg to the gel.

To determine the volume of each sample needed to add to the gel (µL) that will provide the appropriate amount of protein, divide the amount of protein that should be added to the gel (µg) by the total concentration (µg/µL). The protein concentration and the amount of protein desired to load will determine the total volume that should be added to the gel. If it is greater than 25 µL, then the 10 well comb should be used during gel casting as it can accommodate up to 45 µL. If 25 µL or less can be loaded and you have more than
10 samples, the 15 well comb can be used during gel casting. The amount of sample buffer (2X Biorad sample buffer + β mercaptoethanol) should be half of the total volume. Homogenization buffer should be added to make up the difference between the total volume, sample, and sample buffer.

A.3 Gel preparation

The molecular weight of the protein of interest will dictate the percentage of acrylamide in the gel that is used, such that the molecular weight and acrylamide percentage are inversely proportional. When probing for higher molecular weight proteins (100-250) kDa, I suggest casting an 8% acrylamide gel to ensure the greatest separation between the top three standards. However, most of the proteins that we probe for will be successful using a 10% gel (25-100 kDa). For smaller proteins, I suggest using a 15% acrylamide gel. The separation gel is first made at the appropriate acrylamide percentage using the recipe found on the google drive. Once the casting apparatus is set up, I suggest adding the comb to the top to ensure you are aware how high to pour the stacking gel (it should not be poured higher than the comb will allow). Methanol is added to the stacking gel solution once it is poured using a 1 mL pipette so to remove any air bubbles. It normally takes approximately 25 minutes for the solution to gel, which can be determined by observing the leftover solution. Once it is gelled, the extra solution on the top is poured out and the stacking gel solution is poured on the separating gel. The stacking gel is casted the same way for every gel, no matter the acrylamide percentage. The comb of choice as determined by experimental design must be added to the gel solution quickly after pouring. Be sure the Biorad emblem on the comb is facing you when you slide it into the apparatus. If it is backwards and you try to push it in it will break the glass cast. Furthermore, be sure
there are no air bubbles in the comb as this can have a negative impact during well loading and electrophoresis.

A.4 Buffer preparation

The main buffers needed to successfully run these experiments are running buffer, transfer buffer, and tris buffered saline (TBS) and the recipes for each buffer is found on the google drive. I suggest making a 5X stock of running buffer that can be diluted when needed. Prior to making this stock, make a 10% solution of sodium dodecyl sulfate (SDS) using the heated stir plate that you should then use to add the appropriate amount of SDS to the 5X stock. Do not add solid SDS to the 5X running buffer stock as it is extremely difficult to dissolve and stocks made with solid SDS have been associated with electrophoresis issues. Transfer buffer is usually made the day of the experiment. Be sure to pH the buffer prior to adding the appropriate amount of methanol. Most proteins that we probe for in our lab require transfer buffer that contains 20% methanol; however, high molecular weight proteins (>150 kDa) it is suggested to use transfer buffer containing 10% methanol to prevent these proteins from precipitating out. I suggest making a 5X solution of TBS using the recipe. To make TBST, the wash buffer, dilute this stock and add 10% tween.

A.5 Sample preparation

In order to reduce proteins so that they can be ran on gel electrophoresis, samples must be mixed with Laemmli sample buffer supplemented with β-mercaptoethanol and heated at 100°C for 5 minutes. Turn the hot plate on first thing to allow ample time to reach 100°C. To make the sample buffer, first determine the total volume you need for your experiment as determined in section A.2 and then make a solution that is 5% β-
mercaptoethanol in 2x Laemmli sample buffer supplied from Biorad. According to the calculations conducted in section A.2, mix the appropriate volumes of protein lysate, RIPA buffer, and sample buffer and vortex to mix thoroughly. Make a hole in the cap of each sample and place on the heating block for 5 minutes. During these 5 minutes, set up your gel electrophoresis station.

**A.6 Gel Electrophoresis**

To set up your station, put the previously made gels in the gel electrophoresis cell with the gel combs facing inward. If you are only running one gel, replace the second gel with the running plate to ensure that running buffer will remain within the inner cell. Add running buffer to the inner cell all the way to the top of the gel casts and then add running buffer to the outside cell until it reaches the two-gel line. Carefully, remove the combs from the gel cast and top off the inner cell with running buffer. Once the samples are removed from the heating block, vortex the samples thoroughly in order to thoroughly break the disulfide bonds. Quickly spin down the samples to collect all of the volume at the bottom. I suggest putting the samples in the order in which you plan to load them into the gel as this will make it easier to ensure they are in the proper order upon loading. Be careful with this because once your samples are loaded, you have no way of confirming which sample is in which lane. Add approximately 3 µL of Biorad ladder to the first lane in each well and then add the appropriate volume of sample to each lane. Be careful to pipette the same volume of each sample as this can cause differences in loaded protein content. Once you are finished loading, place the electrophoresis cell lid on top ensuring that the black and red rods are inserted into the black and red holes on the lid. Turn the powerpack on and set it to 90V for the first 30 minutes and then increase the voltage to 125V until the ladder has...
separated the entire length of the gel. Bubbles will indicate that the gel is successfully running. The time necessary for the gel to run is approximately 1.5 hours for a 10% gel; however, this will change based on the acrylamide percentage in the gel.

A.7 Transfer

In my opinion, transfer can be the most difficult aspect of the experiment to perfect; therefore, it may take a few trials to become comfortable with this part. First, transfer buffer should be made fresh as stated in the protocol. Most proteins require transfer buffer that contains 20% methanol; however, extremely high molecular weight proteins (>250), for example, acetyl co-A-carboxylase (280 kDa), should be transferred using transfer buffer containing 10% methanol, as methanol can cause high molecular weight proteins to precipitate out during transfer. Prior to removing the gels from the running tank, place the transfer tank in a Styrofoam box, pack ice around the tank, and place an icepack in the transfer tank. Then gather the appropriate number of transfer cassettes (red and black) (1/gel), black sponges (2/gel), filter paper (2/gel), nitrocellulose membrane (1/gel) and transfer container. Be sure to cut the nitrocellulose membrane to as similar size to the gel as possible and submerge in transfer buffer. Submerge the filter paper and black sponges in the transfer buffer and then arrange the transfer cassette where the assembly is completed on the black portion of the cartridge (i.e. black is closest to you). Once the protein is sufficiently separated, gels can be removed from the running tank. Gently crack the gel open, scrape off the wells and transfer the gel from the cast into transfer buffer until ready to load into the transfer cassette. Then assemble the sponges, gel, membrane, and filter paper in the transfer cassette as follows: black sponge, filter paper, gel, nitrocellulose membrane, filter paper, and black sponge. Be sure to roll out any air bubbles between each
layer to ensure protein transfer. The proteins will transfer from left to right; therefore, when you place the assembled cassette in transfer tank it is important for the red side to face to the right so that the protein can travel from the gel into the membrane. Add transfer buffer until the fill line and run the apparatus at 25V overnight (approximately 17 hours). If running multiple gels, be sure to mark down which slot which gel was transferred in. Additionally, smaller proteins may over transfer; therefore, for low molecular weight proteins (>20 kDa), I suggest transferring for 3 hours instead of overnight. Once the transfer is complete, remove the gel and nitrocellulose membrane and use the gel outline to cut any excess membrane. Then remove the membrane, place it in a labeled plastic blot container with TBST, and wash for 5 minutes to remove any residual transfer buffer. From this point, if the membrane dries out it is unusable; therefore, ensure that the blot is always submerged.

A.8 Immunoblotting

In order to block nonspecific binding, add 5% nonfat milk to each blotting container and allow to incubate at room temperature on the rocker for 1 hour. Remove the milk and wash with TBST for 1 minute to remove all excess milk. Most primary antibodies that we purchase from Abcam or Cell Signaling are used at 1:1000 dilution in 5% BSA. Add the primary antibody solution to all blots and allow to rock in the refrigerator overnight. The next day, remove the primary antibody and wash in TBST for 20 minutes and repeat 2 more times. Primary antibodies will either be grown in rabbit or mouse; therefore, will require an anti-rabbit or anti-mouse secondary antibody. Both antibodies are used at 1:2000 dilution in 5% milk supplemented with 1:5000 dilution of the Biorad ladder secondary.
Blots are incubated at room temperature on the rocker for 1 hour and then washed with TBST for 4 times 15 minutes.

*A.9 Imaging blot using ChemiDoc*

The western blot imager is found in Catawba. When turning the machine on be sure to turn on the imager and camera a few minutes prior to turning the computer on to ensure a connection. Wipe down the imager before and after use with 70% ethanol. Upon opening the ImageLab software, take the following actions:

- New protocol
- Application -> Select -> Blots -> Chemi hi resolution
- Imaging Area -> Bio-Rad Criterion Gel
- Set the appropriate exposure time

To develop the signal, mix a 1:1 ratio of each component of the chemiluminescent substrate. Then exchange the TBST on the blot for chemiluminescent substrate and incubate for 2 minutes while rocking the blot. Be sure to do this in the dark to prevent photobleaching. Using the forceps, gently place the membrane on the imager and ensure its proper positioning by selecting position gel on the software. Once appropriately placed, expose the blot for the appropriate amount of time determined by the saturation of the band of interest. I suggest using the exposure setting where you set how many images the camera takes in a certain time period. Imaging exposure times will vary depending on protein of interest. Most proteins that I have measured use an exposure time between 5-60 seconds. If the blot needs to be stripped and probed for a different protein, place the blot back in the plastic container submerged in TBST to ensure the blot doesn’t dry out. If not, the blot can be disposed.
A.10 Probing for second protein

Wash the blot in TBST for 10 minutes to remove any residual chemiluminescent substrate that may still be on the blot and then incubate the blot in 4 mL Biorad stripping buffer for 25 minutes at room temperature on the rocker. This stripping buffer contains β-mercaptoethanol; therefore, it is necessary to excessively wash the blot in TBST to remove any residual β-mercaptoethanol that may interfere in the immunoblotting process. I recommend washing the blot at least 4 times in TBST for 5 minutes each time. Continue the immunoblotting and analysis process as previously stated for the next protein. Generally, this is GAPDH or β-actin, which are both incubated at 1:5000 dilutions in 5% BSA. GAPDH requires the rabbit secondary antibody and β-actin requires the mouse secondary antibody.

A.11 Analyzing intensity of bands using Image Lab

ImageLab software can be downloaded for free directly from the Biorad website so that you can analyze the intensity of your band on your own computer. Once you have opened the file, select Lane and Bands in the Analysis Toolbox, and then select to manually indicate the lanes. Indicate the number of lanes of samples that you will analyze and then manipulate the lanes so that each lane contains only one band and is the appropriate width for that band. In the newest version of ImageLab, to change the width of the lane, you need to select Bands from the Lane and Bands toolbox, select a lane and then return to Lanes. Once you have the lanes in the proper place, select Bands, Add and then click on your bands on interest. Clicking Adjust in the toolbox will allow you to ensure that the height of each band contains the entire band and that all heights are the same. Once this is accomplished, click Analysis Table from the top toolbar and a table will be displayed on
the bottom of the screen. Copy the adjusted intensity for each lane into a new excel spreadsheet and normalize the intensity of the protein of interest by the total protein if looking at a phosphorylation site or the housekeeping protein (i.e. GAPDH or β-actin). The intensity of the housekeeping protein should be approximately the same between each sample on the gel.
APPENDIX B

BEST PRACTICES FOR FLOW CYTOMETRY EXPERIMENTS

Flow cytometry is a powerful technique that uses cell surface markers to measure the prevalence of different cell populations within a parent cellular suspension. During my time in the lab, I used flow cytometry to determine the number of different immune cell populations found within the adipose tissue after PLG scaffold implant. Additionally, I used this tool to determine the purity of porcine derived adipose tissue stem cells and bone marrow derived macrophages. This section will go into detail of best practices to ensure while conducting flow cytometry experiments.

B.1 Designing flow cytometry panels and controls

When designing a flow cytometry experiment, it is necessary to first determine the purpose of the experiment, the cell surface markers needed, and the capabilities of your flow cytometer. For this example, the purpose of your experiment is to quantify the number of macrophages in the adipose tissue. We must first consider the capabilities of our equipment. We use the BD FACS Aria II flow cytometer located at the University of South Carolina Medical School. This machine contains 488 and 640 lasers with filters for FITC, PE, PE-Texas Red, and PE-Cy7 on the 488 laser and APC and APC-Cy7 on the 640 laser. Therefore, your staining cocktail can only contain a maximum of six markers; however, we determined that PE-Texas Red and PE signal significantly overlap; therefore, I suggest using only FITC, PE, PE-Cy7, APC and APC-Cy7 labeled antibodies in the development of your panel. The commonly used surface markers for macrophages is CD11b and F4/80.
However, you determine that you need to first isolate the immune cells within the tissue; therefore, you also need CD45 as a global immune cell marker. Therefore, you need three antibodies each labeled with a different fluorochrome that can be analyzed by the flow cytometer. If you had decided to quantify several immune cell populations including macrophages, neutrophils, monocytes, T cells, B cells and natural killer cells you would need antibodies for the surface markers for each cell population, including CD45 (total immune cells), CD11b (monocytes, macrophages), Ly6G (neutrophils), F4/80 (macrophages), CD3 (T cells), CD19 (B cells), and NK1.1 (natural killer cells). The flow cytometer, however, does not have the capabilities to analyze all seven antibodies. To overcome this, you can split the sample in half and run either a lymphocyte panel or myeloid panel. Each panel would incorporate a dump gate, where all markers for the other cell types would be added with the same fluorochrome label. For example, all myeloid or lymphocyte lineage markers would be stained with one color and the other lineage markers would be stained with individual fluorochromes.

The concentration of each antibody and the recommended amount to use for staining is usually found on the supplier’s website. Most of our flow cytometry antibodies are purchased from Biolegend. For example, CD45-FITC comes at a 0.5 mg/ml concentration and is recommended to use less than or equal to 0.25 µg per $10^6$ cells. Use these values to back calculate the volume of antibody that needs to be added to stain each sample. However, it is suggested that you titrate the antibody to determine the concentration that provides the largest difference in stained and unstained sample. In my experience, using half of the recommended antibody will be appropriate for staining.
There are specific controls that must be incorporated into your flow cytometry experimental design in addition to the samples that will receive the master mix (i.e. the staining cocktail). To remove any emission spectra overlap between different fluorochromes, single fluorochrome compensation controls must be included. These controls are developed when you stain either a small portion of your sample or beads with each fluorescently labeled marker individually. The main thing to consider here is that whatever you stain must be as fluorescently bright as your mastermix stained samples in order to compensate appropriately. Additionally, you will also need an unstained sample to use for compensation. These blank samples will also help you determine where your negative populations are as well as determine the level of potential autofluorescence. Finally, the use of fluorescent minus one control samples will aid in determining the appropriate gating scheme. To develop these controls, you will stain a small portion of your sample with a mastermix including all fluorescently labeled markers except for one which will be the isotype control of that particular antibody labeled with the same fluorochrome as the marker you are using in the mastermix. You will have as many isotype control labeled samples as the number of markers in your panel design. The isotype control information for each antibody is also found on the supplier’s website.

B.2 Preparing samples for staining

The main type of cells that we have analyzed via flow cytometry have been isolated from whole blood, tissues, or cell cultures. Whole blood and cells collected from cultures do not need any additional processing prior to staining; however, tissues must be digested prior to staining. To digest tissues, inject Liberase, which is a combination of collagenase I and collagenase II, directly into the tissue. The tissue should become clearer as you inject
indicating that the cells are being removed from the tissue. Then mince the tissue into tiny pieces and incubate in the oven for 20 minutes to activate the liberase. The addition of EDTA will stop the reaction for the samples to then be strained through a 100 µm filter to remove any undissociated pieces. Cells can then be collected, washed via centrifugation, and transferred to polypropylene flow tubes for subsequent staining. All samples should be kept in ice until analyzing on the flow cytometer unless otherwise stated. If the tissue is a highly vascularized tissue such as the liver, the red blood cells also need lysed using ACK buffer. If multiple people are dissociating samples from the same experiment, be sure to keep track of who prepared which samples in case there is any variability. Additionally, a small experiment was conducted in 2017 that aimed to compare adipose tissue dissociation using liberase or collagenase 1 and the results indicated that the number of total immune cells was not significantly different in tissues isolated using either digestion method. However, I suggest that this experiment be repeated if this question still remains because the variability using collagenase 1 was high.

B.3 Staining samples

Various immune cell populations including macrophages, monocytes, B cells, and granulocytes express FC receptors, CD16 and CD32, on their surface. In order to prevent non-specific binding to these FC receptors, cells are first incubated with anti-mouse CD16/32 antibody that is not fluorescently labeled. Samples are then incubated with the appropriate antibody cocktail as determined during your panel design. From this point on be sure to keep stained samples in the dark to prevent photobleaching. The unbound antibodies are then removed via centrifugation. Fixing the tissues using fixation buffer purchased from Biolegend will allow for the samples to be kept overnight if you aren’t able
to analyze the samples on the flow cytometer until the next day. If they are kept in the dark at 4°C, I suggest analyzing them on the flow cytometer within 24-48 hours maximum. If the sample is whole blood, the red blood cells can be lysed and fixed simultaneously using Biolegend’s Lyse/Fix buffer. Samples are then washed thoroughly and transferred to the filter cap polystyrene flow cytometry tubes to remove any debris. Lastly, if using counting beads to calculate the number of each cell population, the exact volume of the sample to be analyzed on the flow cytometer must be recorded. I suggest pipetting the sample into a new polypropylene flow cytometry tube to determine the volume. The samples are prepared to be analyzed on the flow cytometer. Carefully, transport the samples on ice, counting beads, 100 µL pipette and tips to the medical school.

B.4 Running the samples on the flow cytometer

The BD FACS Aria II flow cytometer and cell sorter is located at the University of South Carolina Medical School and is housed in the Instrument Resource Facility. Kia Zellers is the point of contact for any questions that you may have regarding the machine and is responsible for flow cytometer maintenance, such as refilling the sheath fluid or emptying the waste. You will need to contact her for proper flow cytometer training that will then allow you to independently use the machine. Like all IRF equipment, the flow cytometer must be scheduled prior to use and can be done so on the IILabs solutions website. I highly suggest scheduling the flow cytometer for longer than you anticipate.

Turn the flow cytometer and laser on using the buttons on the left side of the flow cytometer. Open the BD FACSDiva software on the computer and begin setting up your experiment. Under the “Gower Lab” folder, create a new experiment and label it appropriately. Then add a specimen underneath this experiment and label appropriately.
You can add multiple “specimens” to the same experiment folder if you are running several similar experiments in a short period of time. Then under the specimen, add a sample for each sample that you have prepared to run on the flow cytometer and rename each appropriately. Next turn the flow stream on by clicking the red X on the top of the stream and wait until the drops are even. If these samples have been ran before, then you most likely will have a good idea of the fluorochrome settings that should be used and the dot plots that need to be created; however, if this is the first time that you are running these samples, I suggest making an extra mastermix stained sample to use to determine the appropriate fluorochrome settings (under cytometer settings, parameters). Fluorochromes fluorescent intensity is on a log scale and forward and side scatter are on a linear scale. Knowing which cell populations you are interested in will allow you to initially create dot plots and target populations within those dot plots. For example, if you are analyzing the number of macrophages within the sample, you know those cells are positive for CD11b and F4/80. Therefore, you first make an FSC versus SSC plot and gate out any debris likely located at the origin. You can then plot that population on the fluorochromes that CD11b and F4/80 are labeled and gate the double positive population. Once you have created the dot plots for all the target cells, vortex and then load the extra sample to begin acquisition. Additionally, I suggest running this extra sample at a low flowrate (< 3), located on the bottom toolbar, in order to have enough time and sample to determine the settings. Once acquisition begins, you will see dots collecting on your dot plots, indicating cells. Alter the settings to ensure that all of your cells are located and appropriately spread out on the FSC versus SSC dot plot and that each fluorochrome positive cells are located at approximately $10^3$ on the log scale. Be sure to also record forward scatter height and width (FSC-H, FSC-
W) and side scatter height and width (SSC-H, SSC-W) in order to remove any doublet cell populations in the analysis. The other settings that are normally changed are found on the bottom toolbar, including number of events shown in your dot plots on the right panel, number of events recorded either total or in a particular population, and the flow rate. Events are equivalent to the number of cells that have passed through the laser and it is good practice to record as many events in your sample as possible. I suggest recording a minimum of 5,000 events in each target cell population if possible. I normally select to show 1,000,000 so that all of the events will show on the dot plots. Additionally, I do not suggest running the sample at a flow rate higher than 5, as running the samples at a high flow rate can increase the variability due to the position of the cells within the laser (i.e. more groups and less individual cells). Furthermore, by adding a population statistics table, the software will tell you the number of events that are in each population as you run your sample.

Once you have determined your settings and created your dot plots and populations, you can start recording your various samples. After loading the sample and the event rate is stable (approximately 5 seconds), click record to record your data. If you do not click record, your data will not be saved. You have the liberty to analyze any sample in any order; however, I suggest starting with your FMO controls to ensure that your gating scheme is appropriate and then move on to your mastermix stained samples. If you are using counting beads, add the beads prior to vortexing and then load the sample. Counting beads are fluorescent in all channels; however, the best gate that I have found for locating the counting beads is located on SSC versus APC. Finally, record the blank and compensation samples. All samples must be analyzed using the same settings. Collect the
data by right clicking the specimen and exporting onto your flash drive. I always save both the 2.0 and 3.0 versions just in case; however, I use the 2.0 version during the analysis.

**B.5 Analyzing samples using FlowJo**

To analyze data using FlowJo, drag and drop files into FlowJo. Because you may have a large number of files to analyze, I suggest first organizing the data based on sample groupings (i.e. mastermix stained, blanks, compensation controls, and isotype controls) by clicking Create New Groups of Samples and dragging and dropping the files into the appropriate groups. Then using your compensation samples, gate on your positive and negative populations for the fluorochrome used to stain that sample. Ensure that your positive populations are as bright in each channel as your sample. If they aren’t, you will need to repeat the compensation controls using a different sample with the same settings that was used to analyze the main sample. To develop a compensation matrix that can then be applied to the rest of your samples, click Platform, Compensate Samples, then Define New Matrix. Drag and drop your positive and negative gates for the appropriate fluorochrome into the table and then click Compute & Close. To apply the compensation matrix to the rest of your samples, select all samples then Platform, Compensate Samples, and the title of your compensation matrix. You should see a colored bar to the left of each sample denoting that it was compensated.

Next, use your isotype controls to develop the appropriate positive gates that you will then apply to your mastermix stained samples. Use forward scatter vs side scatter plot to determine the main cell population as there maybe cell debris located at the origin that should be gated out. Then gate out doublets by gating side scatter width versus side scatter height. Looking back at our macrophage panel example, one isotype control will be stained
with the isotype control for CD45, CD11b, or F4/80. Use the sample stained with the CD45 isotype control to determine where the positive gate for CD45 should be located. Complete this analysis for the samples stained with the CD11b or F4/80 isotype control as well. Then copy and paste the gating scheme developed using the isotype controls onto the mastermix stained samples.

There are different methods of reporting flow cytometry data that are commonly used. Dot plots are a convenient way to illustrate significant and obvious differences between groups. An easy way to compare dot plots between groups is to use the layout function to compare representative samples from each group side by side. Histograms are also a common method to depict how mean fluorescent intensities shift between groups. The layout function will also allow you to overlay histograms from different samples so that they can be compared on a single plot. Furthermore, the number of cells in each cell population can be used along with the counting bead concentration and sample volume to calculate the number of total cells in each population in the total sample. This cellular quantification allows for statistical analysis between groups. Finally, the average mean fluorescent intensity of each sample can be determined using the statistic function and can then be averaged and statistically compared between groups. This is particularly useful when investigating the presence of a marker on a particular cell type.
APPENDIX C

MANUSCRIPT COPYRIGHTS

Figure C.1: Screenshot of permission from ACS Journals to reuse published work in this dissertation.