MODULATION OF CELL-MEDIATED IMMUNITY TO PREVENT HIGH-FAT DIET-INDUCED OBESITY AND INSULIN RESISTANCE

by

LINNA YAN

(Under the Direction of Dexi Liu)

ABSTRACT

Since several lines of evidence point to high-fat diet-induced obesity accompanied with chronic low-grade inflammation, we hypothesized that immune modulators could prevent the development of high-fat diet-induced obesity. To address this issue, we selected three commonly used immune modulators: cyclosporine A, fingolimod and laquinimod to explore their roles in preventing of high-fat diet-induced obesity by twice weekly *i.p*. injection. Our results showed that cyclosporine A, fingolimod and laquinimod treatment protected the mice from development of insulin resistance and maintained glucose sensitivity with a high-fat diet. Also, real-time PCR analysis demonstrated that the expression levels of inflammatory related genes were down-regulated on fingolimod and laquinimod treatment mice. These beneficial effects were associated with blockade of macrophage infiltration and suppression of immune responses. Therefore, cyclosporine A, fingolimod and laquinimod may be the potential therapies in preventing of high-fat diet-induced obesity.

INDEX WORDS: Cyclosporine A, Fingolimod, Laquinimod; High-fat diet-induced obesity, Inflammation, Insulin resistance

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LINNA YAN

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LINNA YAN

Major Professor: Dexi Liu

Committee: Shelley B. Hooks
Eileen J. Kennedy

Electronic Version Approved:

Maureen Grasso
Dean of the Graduate School
The University of Georgia
May 2014

DEDICATION

This thesis is dedicated to my parents, Zihou Yan and Chunhui Zhou, for giving birth to me and for their endless love and supporting me economically and spiritually throughout my life.

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CHAPTER 1. INTRODUCTION

1.1. Obesity and obesity-associated diseases

Obesity is defined as a medical condition in which excess body fat accumulates in adipose tissue due to imbalanced energy intake and energy expenditure. There are several indexes used to assess obesity. The most common measure of obesity is body mass index (BMI), which is weight in kilograms divided by height in meters squared. In the United States, a BMI greater than 30 is considered obese. The prevalence of obesity worldwide has reached epidemic proportions. In 2012, the Centers for Disease Control and Prevention (CDC) reported that more than 34% of adults and approximately 17% (or 12.5 million) of children and adolescents between the ages of 2-19 years were obese in the United States (Ogden, Carroll *et al.* 2014). To draw the attention of the public, policy makers and the medical community to the severity of the obesity epidemic, the American Medical Association recognized obesity as a disease in 2013.

Obesity has been linked to many severe diseases. The most closely related is diabetes, especially type-2 diabetes. According to the World Health Organization, more than 90% of diabetes patients worldwide have type-2 diabetes. Being overweight or obese contributes to the development of diabetes by making cells more resistant to the effects of insulin. Endoplasmic reticulum (ER) stress response is abnormally active in livers of obese individuals, where it contributes to the development of hyperglycemia, or high blood glucose levels. Matthew Watt *et al.* has discovered that fat cells release a protein called pigment epithelium-derived factor. Release of this protein serves as a key link leading to the development of type-2 diabetes. When this novel protein is released in the bloodstream, it causes the liver and muscles to become

desensitized to insulin. Insulin triggers cells to absorb glucose from the bloodstream, store it and convert it to energy for the body. When cells become insulin resistant due to excess fat, more insulin is produced by the pancreas to counteract the resistance, thereby overworking the pancreas and eventually impairing insulin secretion.

Hypertension, or high blood pressure, is another disease with a known connection to obesity. About 75 million people in the US suffer from high blood pressure, a major risk factor for heart disease. Blood pressure tends to increase with weight gain and age. Though the reason obesity is a major cause of high blood pressure is unknown, research has shown that obese patients display an increase in blood volume and arterial resistance (Kotsis, Stabouli *et al.* 2010). In fact, obesity induces a high secretion of insulin due to efforts in decreasing excessive sugar concentration in blood. This insulin secretion is significantly high compared to non-obese subjects. Moreover, insulin is responsible for many modifications in the body, including inducing a thickening of the vessels, which causes an increase in their rigidity. Excess insulin secretion can also decrease the reabsorption of water and salt by the kidneys, which increases blood volume, therefore, increasing the blood pressure. In addition, obesity can cause a high sensitivity to sodium, resulting in an increased rigidity of the peripheral arteries.

According to the American Heart Association, obesity is a major risk factor in the development of coronary heart disease, which can lead to a heart attack or stroke. Atherosclerosis, the main underlying cause of cardiovascular disease, gradually hardens the walls of the arteries, causing them to lose their elasticity and narrow, impairing blood flow. Those who are overweight are at a greater risk of heart attack before age 45. Obese adolescents have a greater chance of heart attack before age 35 than non-obese adolescents. High cholesterol levels are considered one of the leading causes of heart attacks. One study found that for every

kilogram (2.2 pounds) of weight a person loses, high density lipoprotein levels increase by 0.35 mg/dL (Bray 2008).

A study by the American Heart Association shows that the odd of developing cancer increases by 50 percent when overweight. In women, the risk of developing cancer increases if they are more than 20 pounds overweight. Several possible explanations have been suggested to explain the association between obesity and increased risks of certain cancers. For example, fat tissue produces excess amounts of estrogen, high levels of which have been associated with the higher risks of breast, endometrial, and other cancers (Kaaks, Lukanova *et al.* 2002). Adipocytes can produce hormones, called adipokines, which may stimulate or inhibit cell growth (Greenberg and Obin 2006). For example, leptin, which is more abundant in obese people, may promote cell proliferation, whereas adiponectin, which is less abundant in obese people, may have anti-proliferative effects (Rodríguez, Mastronardi *et al.* 2013). Adipocytes may also have direct and indirect effects on other tumor growth regulators, including mammalian target of rapamycin (mTOR) and AMP-activated protein kinase (Towler and Hardie 2007).

1.2. Obesity and the pathological characteristics

Obesity is associated with chronic, low-grade inflammation in adipose tissue, particularly in the case of high fat diet-induced obesity. The chronic inflammatory response has its origin in the links existing between adipose tissue and the immune system. With excessive energy overload, adipose tissue mass expands and the adipocyte size increases. This expansion alters the structure of adipose tissue, which increases the release of free fatty acids into circulation and decreases oxygen delivery to adipocytes (Ye, Gao *et al.* 2007). It further causes micro-hypoxia in regional areas and induces the expression of hypoxia-inducible factor-1 (HIF-1) and its downstream target genes. Combined with excessive nutrients, the expression level of genes

involved in inflammation and ER stress are up-regulated in hypoxic adipose tissue (Hosogai, Fukuhara *et al.* 2007, Wang, Wood *et al.* 2007). This can lead to activation of immune inflammatory responses, and therefore, the apoptosis of adipocytes. Consequently, activation of these responses results in recruitment of macrophages into adipose tissue, forming the crown-like structure (CLS) around adipocytes (Cinti, Mitchell *et al.* 2005, Strissel, Stancheva *et al.* 2007) and releasing pro-inflammatory cytokines. Since these macrophages are in an M1 activation state, once recruited, they further activate the intracellular pro-inflammatory pathway and amplify inflammation state (Rodriguez, Catalán *et al.* 2007). In a feed-forward circle, these activations promote macrophage recruitment and additional macrophages infiltrate into adipose tissue. This is the pathway shown to contribute to the development of obesity from energy overloading.

Studies have demonstrated that inflammatory macrophages are responsible for obesity-associated inflammation and disease. In a lean state, M2 macrophages and Treg cells help prevent inflammation in adipose tissue and maintain metabolic homeostasis. Eosinophil-derived *IL-4* and *IL-13* promote the maintenance of M2 macrophages in fat, and *IL-10*, secreted by both Treg cells and M2 macrophages, limits local inflammation. Approaches stimulating M2 macrophages can dampen inflammation in adipose tissue and improve metabolic function. Furthermore, several mechanisms have been implemented in shifting the M1/M2 balance in favor of M1 in diet-induced obesity. Free fatty acids promote inflammatory macrophage activation.

While these findings put macrophages at the center stage of obesity-induced inflammation and insulin resistance, they are not the only important participants (Nikolajczyk, Jagannathan-Bogdan *et al.* 2011). It is believed that T cells also play a critical role in the

regulation of fat-induced inflammation. Although macrophages can activate T cells, changes in T cells have been reported to proceed the changes in adipose tissue macrophages during obesity. T cell polarization instigates obesity-induced tissue inflammation. Major histocompatibility complex II (MHCII) pathway is crucial to the antigen presentation process. Antigens presented on MHCII molecules are recognized by the T cell receptor on $CD4^+$ T-helper (Th) cells and, in conjunction with co-stimulatory signals, trigger their polarization to inflammatory Th1, anti-inflammatory Th2 depending on the cytokine milieu. Th1 helper cells are the host immunity effectors against intracellular bacteria and protozoa. They are activated by *IL-12*, *IL-2* and their effector cytokine like IFN-γ (Mosmann, Cherwinski *et al.* 2005). The main effector cells of Th1 immunity are macrophages. The key Th1 transcription factors are STAT4 and T-bet (Mosmann, Cherwinski *et al.* 2005). Th2 helper cells are the host immunity effectors against multicellular helminths. They are triggered by *IL-4* and their effector cytokines are *IL-4*, *IL-5*, and *IL-13*. The key Th2 transcription factors are STAT6 and GATAs (Mosmann, Cherwinski *et al.* 2005).

Previous studies indicate that the ratio of Th1/Th2 cells, determined by cytokine environment, is a key determinant of tissue inflammation. In a diet-induced obese state, Th1 cell levels are elevated, while Th2 cells remain stable (Winer, Chan *et al.* 2009). Also, lymphocyte-free RAG^{null} mice reduced weight gain and improved insulin sensitivity predominately through Th2 cells after $CD4^+$ T cell transfer into DIO (Winer, Chan *et al.* 2009). Deng *et al* has reported that MHCII up-regulation on adipocytes was observed as early as 2–3 weeks following a high fat diet, suggesting that MHCII-mediated activation of Th1 cells is an early event in triggering adipose tissue inflammation. Following this activation, induction of cytokine/chemokines may recruit macrophages that amplify the tissue inflammation.

Insulin resistance (IR) is a common consequence of obesity where cells fail to respond to the normal actions of the hormone insulin. After insulin is produced, cells become resistant to insulin and are unable to use it effectively, leading to hyperglycemia. Beta cells in the pancreas subsequently increase production of insulin, further contributing to hyperinsulinemia. The pancreas is an important organ secreting insulin and glucagon, both of which are necessary in glucose homeostasis. Obesity-induced inflammatory activity in pancreatic islets plays a critical role in the development of type-2 diabetes (Donath, Böni-Schnetzler *et al.* 2010).

The liver, a major metabolic organ regulating gluconeogensis, glycogen storage, lipogenesis, cholesterol synthesis and secretion, is also affected by obesity (Hotamisligil 2006). Obesity activates inflammation events in liver cells by activation of Kupffer cells (Baffy 2009), and increase hepatic lipogenesis, leading to nonalcoholic fatty liver diseases (NAFLD). Hepatic inflammation is a strong risk factor of insulin resistance, nonalcoholic steatohepatitis and dyslipidemia (Kremer, Hines *et al.* 2006, Baffy 2009).

Muscle, another tissue playing a critical role in glucose homeostasis, is closely linked to glucose uptake and energy expenditure. Obesity-induced overproduction of inflammatory cytokines in muscle may be linked to macrophage infiltration in obese animals (Hong, Ko *et al.* 2009). In addition, obesity-induced inflammation, occurring in adipose tissue, may directly lead to adipose tissue dysfunction. Adipose tissue dysfunction is a combination of all these complications. Recent studies demonstrate that adipose tissue is not only a depot for fat storage, but is also widely recognized as a complex endocrine and paracrine organ, in which several inflammatory cytokines and bioactive mediators, such as interleukin-6 (*IL-6*) and tumor necrosis factor-α (*TNF-α*), were produced to influence body weight and insulin resistance (Van Gaal, Mertens *et al.* 2006).

1.3. Causes of obesity

Obesity tends to run in families, suggesting that it may have a genetic cause. However, family members share not only genes, but also diet and lifestyle that may contribute to obesity. Separating these lifestyle factors from genetic ones is often difficult. Still, growing evidence points to heredity as a strong determining factor in obesity. In a study on adults who were adopted as children, researchers found that their adult weights were closer to their biological parents' weights than their adoptive parents', suggesting that the environment provided by the adoptive family had less influence on the development of obesity than genetic makeup (Silventoinen, Rokholm *et al.* 2010).

Although genes are an important factor in many cases of obesity, environment also plays a significant role. Environment includes lifestyle and behaviors, such as food intake and exercise. Diet is one of the main environmental factors contributing to obesity. Americans tend to have high-fat diets, often prioritizing taste and convenience over nutritional content when choosing meals. High-fat diets usually involve more energy intake than the body requires, eventually causing obesity if accompanied by inadequate physical excise.

Psychology is another factor that influences eating habits and obesity. Many people eat in response to negative emotions such as boredom, sadness, or anger. While most overweight people have no more psychological disturbances than people at normal weights, about 30 percent of those who seek treatment for serious weight issues have problems with binge eating. During a binge eating episode, people eat large quantities of food, while feeling they can't control how much they are eating. Those with the most severe binge eating problems are considered to have a binge eating disorder. These people may have more difficulty losing weight and keeping the weight off than people without binge eating problems. Some will need special help, such as

counseling or medication, to control their binge eating before they can successfully manage their weight.

Other factors are believed to contribute to obesity as well. For example**,** illnesses can lower metabolism or trigger an increased appetite, causing obesity. These include hypothyroidism, Cushing's syndrome, depression, and certain neurological problems (Chokka, Tancer *et al.* 2006, Kola, Christ-Crain *et al.* 2008). Certain drugs, such as steroids and some antidepressants, could cause excessive weight gain through the same mechanisms (Chokka, Tancer *et al.* 2006).

1.4. Current treatment of obesity

Although the prevalence of obesity worldwide has reached epidemic proportions, the number of anti-obesity drugs available on the market is limited. So far, orlistat (Alli) is the only drug approved by the US Food and Drug Administration (FDA) for long-term use against obesity. It can block the absorption of intestinal fat by inhibiting pancreatic lipase activity (Ballinger and Peikin 2002, Hutton and Fergusson 2004, Thurairajah, Syn *et al.* 2005, Drew, Dixon *et al.* 2007). An additional anti-obesity drug, rimonabant (Acomplia) was approved in Europe, but not approved by the US FDA due to safety concerns (Moreira and Crippa 2009). Lorcaserin hydrochloride (Belviq®) and Qsymia™ were approved by FDA in 2012 for adults who have a BMI of 30 or greater. (Qsymia is a combination of two FDA-approved medicines: phentermine and topiramate.)

Unfortunately, current anti-obesity drugs have multiple downfalls preventing them from being used in large-scale clinically. First, weight-loss effects of these drugs require controlled diet and active exercises from the patients to reach ideal clinical effects (Avenell, Broom *et al.* 2004, Nicklas, Ambrosius *et al.* 2004, Després, Golay *et al.* 2005, Shyh and Cheng-Lai 2014).

The side effects of these drugs are severe, including steatorrhea, cardiovascular diseases, nephropathy and liver failure (Thurairajah, Syn *et al.* 2005, de Simone and D'Addeo 2008, Karamadoukis, Shivashankar *et al.* 2009).

The use of diet supplements has been popular in general public in dealing with obesity epidemics. For example, Ephedra (also called Ma-Huang) is a natural product obtained from plants, the active ingredient of which is ephedrine (Shekelle, Hardy *et al.* 2003). Ephedra causes short-term weight loss, but has serious side effects, including high blood pressure and heart stress. Ephedra was sold as dietary supplement in the past, but a total ban on ephedra–containing substance was placed in 2004. Another example is Chromium, a mineral sold for reducing body fat. Unfortunately, no weight-loss benefit from Chromium has been confirmed by studies, while a few serious side effects were reported (Anderson 1998). Diuretics and herbal laxatives are products that cause the patient to lose water weight instead of fat weight. They can also lower the body's potassium levels, which may cause heart and muscle problems (Kassirer and Harrington 1977). Additional example includes Hoodia, a cactus native to Africa. It is sold in pill form as an appetite suppressant. However, no firm evidence shows that Hoodia has significant weight-loss activity. No large-scale research has been done on humans to show whether Hoodia is effective or safe.

Weight-loss surgery is the most effective procedure for people who suffer from extreme obesity (BMI of 40 or more), especially when other treatments have failed. Weight-loss surgery also is an option for people who have a BMI of 35 or more and life-threatening conditions, such as severe sleep apnea (a condition in which patients have one or more pauses in breathing or shallow breaths while sleeping), obesity-related cardiomyopathy or severe type-2 diabetes. Two

common weight-loss surgeries include banded gastroplasty and Roux-en-Y gastric bypass (Brolin, Robertson *et al.* 1994).

In gastroplasty, a band or staples are used to create a small pouch at the top of the stomach (Mason 1982). This surgery limits the amount of food and liquids the stomach can hold, which results in limited food intake. In a gastric bypass, a small stomach pouch is created with a bypass around part of the small intestine where most of the calories are absorbed. The procedures involve less influence on food intake but effectively reduce body absorption of calories. Weight-loss surgery can effectively decrease body weight and improve the health of seriously obese patients. However, the surgery can be risky, depending on the overall health of the patients. Gastroplasty has few long-term side effects, but the patients must limit the food intake dramatically. Gastric bypass has more side effects, including nausea, bloating, diarrhea, and faintness. These side effects are all part of a condition called dumping syndrome. After a gastric bypass surgery, multivitamins and minerals are needed to prevent nutrient deficiencies. Lifelong medical follow up is needed for both surgical procedures, which present a burden for patients both psychologically and economically.

1.5. Objectives of the study

Several lines of evidence show that obesity could alter metabolic signaling, leading to the activation of innate and adaptive immune responses. Obesity can also cause impairment of immune functions and metabolic homeostasis (Marti, Marcos *et al.* 2001, Gregor and Hotamisligil 2011). It has been reported that an increasing number of immune cells infiltrating into metabolic tissue is a feature of the inflammatory state of obesity. We and others have previously shown that blocking macrophage activation can block the development of high-fat diet-induced obesity (Bu, Gao *et al.* 2013, Gao, Ma *et al.* 2013). The current study was designed

to extend our previous research by focusing on the involvement of T-cells and lymphocytes in high fat diet –induced obesity and insulin resistance. The goal of the study was to search for an immune modulator to suppress activity of cell-mediated immune responses as a way to block diet-induced obesity. The study involves direct assessment of 3 compounds with known activity in modulating cell-mediated immune responses. The common properties of each of the 3 compounds are listed in Table 1.

Cyclosporin A (CsA) is a common immune modulator clinically used in solid organ transplantation patients to prevent immune rejection. The mechanism of CsA-mediated the immunosuppressive activity is to inhibit signal transduction pathways (Ho, Clipstone *et al.* 1996). CsA has a few protein targets, including cytosolic cyclophilins (CyP), an immunophilin family that possesses peptidyl-prolyl *cis-trans* isomerase activity (PPIase) and may serve as ATP-independent molecular chaperone proteins. The CsA-CyP complexes can create composite surfaces that specifically block the activity of Ca(2+)- and calmodulin-dependent protein phosphatase calcineurin (CaN). Inhibition of the action of calcineurin blocks translocation of the cytosolic component of the nuclear factor of activated T cells (NF-AT), resulting in a failure to activate the genes regulated by the NF-AT transcription factor necessary for T-cell proliferation (Zav'yalov, Denesyuk *et al.* 1995).

CsA reduces the activity of the immune system by interfering with the activity and growth of T cells (Flechner 1983). Also, a later study showed that CsA decreased *IL-6* synthesis at the post-transcriptional level on human macrophage (García, López *et al.* 1999). In addition, it has been documented that CsA treatment could reduce inflammation by down-regulating production of several pro-inflammatory cytokines. Furthermore, the study by Jiang *et al.* showed that CsA was able to improve glucose metabolism, resulting in remarkably attenuated food

intake and body weight gain in the obese mice (Jiang, Wang *et al.* 2013). As CsA is a traditional immune modulator with anti-inflammatory features by the regulation of T cells, we hypothesized that CsA might possess anti-inflammatory effects to obesity.

Fingolimod is a novel immune modulator used as the first-line treatment for relapsing forms of multiple sclerosis (Kappos, Antel *et al.* 2006). It can prolong allograft transplant survival in numerous models by inhibiting lymphocyte emigration from lymphoid organs (Budde, Schütz *et al.* 2006). The mechanism of fingolimod-mediated immunosuppression is thought to be carried out through the suppression of lymphocyte infiltration (Mansoor and Melendez 2008). *In vivo*, fingolimod is phosphorylated to form fingolimod-phosphate, which resembles naturally occurring lysophospholid sphingosine 1-phosphate (S1P), an extracellular lipid mediator whose major effects are mediated by cognate G protein-coupled receptors. There are at least 5 S1P receptor subtypes, known as S1P subtypes 1-5 (S1P1-5), 4 of which bind fingolimod-phosphate. S1P has a higher concentration in body fluids than in lymphoid tissues, creating a concentration gradient between the lymphoid and the circulatory system. Lymphocytes are guided from the lymphoid tissues into the circulatory system by the binding between the surface S1P receptors and the S1P1 along the concentration gradient. Fingolimod-phosphate is a high affinity agonist of S1P1 receptor that activates lymphocyte S1P1 via high-affinity receptor binding. Therefore S1P1 is down-regulated, leading to the inhibition of lymphocyte infiltration (Chun and Hartung 2010).

Fingolimod is a structural analogue of sphingosine, an important amino alcohol in the synthesis of sphingolipids and the integrity of cell membranes. A previous study has demonstrated that fingolimod decreases plasma concentrations of pro-inflammatory cytokines in renal transplant patients, such as *IFN-γ*, *TNF-α*, *IL-6* and *IL-12* (Sommerer, Giese *et al.* 2008).

Also, fingolimod normalized MCP-1 renal gene expression and significantly down-regulated the activity of M1 and M2 macrophages in rats, which was beneficial in tissue remodeling and repair (Schaier, Vorwalder *et al.* 2009). Although immune modulation effects of fingolimod have been extensively studied, other effects of fingolimod are still unclear. Here we describe a novel role of fingolimod in the prevention of obesity, involving the regulation of lipogenesis, lipolysis and chronic inflammation *in vivo*.

Laquinimod is an experimental immune modulator used for treating multiple sclerosis. It has been reported that laquinimod prevented multiple sclerosis relapses by lowering the infiltration of CD4+ and CD8+ T cells in the central nervous system (Jolivel, Luessi *et al.* 2013). Valerie Jolivel's study also showed that laquinimod treatment led to decreased chemokine production by both murine and human dendritic cells. This is associated with decreased monocyte chemo-attraction (Jolivel, Luessi *et al.* 2013). In addition, laquinimod suppresses production of inflammatory cytokine including *IFN-γ*, *TNF-α*, *IL-12* or *IL-17* and enhances release of *IL-4* and *TGF-b*, suggesting a down-regulation of Th1 and Th17 immune responses (Jolivel, Luessi *et al.* 2013). Another study also demonstrated that laquinimod could down-regulate cytokine production by the activation of microglia and macrophages (Brück and Wegner 2011). The mechanism of action for laquinimod in immunosuppression is believed to involve inhibition of the infiltration of T lymphocytes and macrophages, the molecular process of which, however, is still not fully understood (Brunmark, Runstrom *et al.* 2002). The T_H1 cytokines are recognized as pro-inflammatory and represent the driving force in the T-cell-induced inflammatory process. Laquinimod can trigger a shift from T_H1 type immune response to T_H2/T_H3 immune response, together with the inhibition of IL-12 and TNF-α, and the enhancement of IL-4, IL-10, and TGF-β (Zou, Abbas *et al.* 2002, Yang, Xu *et al.* 2004) which is

the prime cytokine of the T_H3 type of T regulatory cells (Weiner 2001). IL-10 can inhibit T cells by inhibiting IL-12 production via antigen-presenting cells and the blockade of IFN-γ synthesis via differentiated T_H1 cells. With multiple pathways inhibited or enhanced, laquinimod can change the cytokine balance in favor of T_H2/T_H3 cytokines IL-4, IL-10 and TGF-β. Therefore laquidnimod can suppress the infiltration of T cells, leading to the immunosuppressive effects, the reason that I selected laquinimod for the study to explore its potential in blocking high fat diet-induced obesity.

1.6. Experimental design

The experiment design involves maintaining mice continuously on a fat diet for 15 weeks. Twice weekly injections of immune modulators were performed on days 1 and 4 and the control animals received vehicle solution. Body weight and fat content of each animal was measured weekly. Food consumption was estimated by measuring the amount of food reduced from the amount of previous day. Glucose intolerance was performed on week 12 with 6hr fasting. The same animals were allowed a one-week recovery before the insulin sensitivity test. Animals were sacrificed by the end of week 15, internal organs collected and histological, cell biological, biochemical and molecular biological examinations performed in selected tissue samples. Five animals were used for each of the data point collected.

Table 1: Information of selected immune modulators

	Cyclosporine A	Fingolimod	Laquinimod
Function	An immunosuppressant drug widely used in organ transplantation to prevent rejection	An immunomodulating drug for treating multiple sclerosis	An experimental immunomodulator, being investigated for treatment of multiple sclerosis
Pharmacokinetic data	Metabolized by CYP3A4, Half-life: about 26 hours	Metabolized by CYP4F2, Half-life: 6-9 days	Metabolized by CYP3A4, Half-life: about 80 hours
Mechanism of action	The exact mechanism of action of cyclosporine is not known. Experimental evidence suggests that the effectiveness of cyclosporine is due to specific and reversible inhibition of immunocompetent lymphocytes in the G0- or G1-phase of the cell cycle. T-lymphocytes are preferentially inhibited. The T-helper cell is the main target, although the T-suppressor cell may also be suppressed. Cyclosporine also inhibits lymphokine production and release including interleukin-2 or T-cell growth factor (TCGF)	Fingolimod is metabolized by sphingosine kinase to the active metabolite, fingolimod-phosphate. Fingolimod-phosphate is a sphingosine 1-phosphate receptor modulator, and binds with high affinity to sphingosine 1-phosphate receptors 1, 3, 4, and 5. Fingolimod-phosphate blocks the capacity of lymphocytes to egress from lymph nodes, reducing the number of lymphocytes in peripheral blood. The mechanism by which fingolimod exerts therapeutic effects in multiple sclerosis is unknown, but may involve reduction of lymphocyte migration into the central nervous system.	Laquinimod reduces leukocyte migration into the central nervous system by down-regulation of VLA-4-mediated adhesiveness, inhibiting Th17- proinflammatory responses and also by modulating the cytokine balance in favour of Th2/Th3 cytokines interleukin (IL)-4, IL-10 and transforming growth factor (TGF)-β. It also increases levels of brain-derived neurotrophic factor.
Major side effects	Hyperkalemia, kidney and liver dysfunction, hypertension (due to renal vasoconstriction and increased sodium reabsorption), increases the risk of squamous cell carcinoma and infections.	Headache, flu-like symptom, diarrhea, back pain, liver transaminase elevations and cough.	Infection (urinary tract infection); nervous system disorder (headache); musculoskeletal disorder (back pain)

CHAPTER 2. MATERIALS AND METHODS

2.1. Materials

Cyclosporine A (CsA), Fingolimod (fingolimod) and Laquinimod were purchased from Selleckchem (Houston, TX). High-fat diet (60% calories from fat, 20% from carbohydrates and 20% from proteins) was purchased from Bio-serve (Frenchtown, NJ). Dimethyl sulfoxide (DMSO), hydrochloric acid and ammonia were purchased from Sigma-aldrich (St. Louis, MO). TRIZOL was purchased from Invitrogen (Grand Island, NY). Chloroform and isopropanol were purchased from Mallinckrodt Baker (Phillipsburg, NJ). Ethanol was purchased from Decon Labs (King of Prussia, PA). Insulin (Humulin) was purchased from Eli Lilly (Indianapolis, IN). Xylene and permount were purchased from Fisher Scientific (Pittsburgh, PA). Oil-red O solution and fluorogel with Tris buffer were purchased from Electron Microscopy Science (Hatfield, PA). The glucose meters and test strips were purchased from LifeScan (Milpitas, CA). RNeasy lipid tissue mini kits for mRNA extraction from adipose tissues were purchased from QIAGEN (Valencia, CA). ELISA kit for serum insulin detection was purchased from Mercodia (Winston Salem, NC). Commercial kit for H&E staining was purchased from BBC Biochemical (Washington, DC). Commercial kits for determination of serum level of triglycerides, cholesterol and non-esterified fatty acids were purchased from Thermo Scientific (Middletown, VA). All primers for PCR were synthesized at Sigma (St. Louis, MO).

2.2. Instrumentation

EchoMRI-100 from Echo Medical Systems (Houston, TX) was used for the analysis of body composition. Opsys MR from DYNEX Technologies (Chantilly, VA) was used for

determination of serum concentration of insulin. Genesys 10S UV-Vis spectrophotometer from Thermo Scientific (Middletown, VA) was used for determination of serum concentrations of triglycerides, cholesterol and NEFA. Dremel 300i from Dremel (Racine, WI) was used for tissue homogenization. Thermal cycler (model 2720) and StepOnePlus Real-Time PCR System from Life Technologies (Grand Island, NY) were used for the analysis of gene expression. Manual rotary microtome from Leica (model RM2235) (Buffalo Grove, IL) was used for tissue section and H&E staining. Cryostat from Leica (model CM1850) (Buffalo Grove, IL) was used for cutting tissue samples for Oil-red O staining. ECLIPSE-Ti optical microscope from Nikon (Melville, NY) was used for examination of tissue section on slides. NIS-Elements AR software from Nikon (Melville, NY) was used for measurements of the size of adipocytes on slides.

2.3. Animals and treatment

Twenty male C57BL/6 mice were purchased from Charles River (Wilmington, MA). All animals were acclimatized for 3 days before experiments and housed with a 12 h light-dark cycle. Mice were divided into 4 groups (n=5) and fed with high fat-diet (60% calories from fat, 20% from carbohydrates, and 20% from proteins). All animals were intraperitoneally (*i.p.*) injected twice a week with CsA, fingolimod or laquinimod solubilized in DMSO. Group one mice were injected with DMSO. Group two mice were injected with CsA (50 mg/kg). Group three mice were injected with fingolimod (0.04 mg/kg). Group four mice were injected with laquinimod (2.5 mg/kg). Body weight and food intake were measured weekly. Body compositions were analyzed at the end of treatment using EchoMRI-100. The animal protocol was approved by the Institutional Animal Care and Use Committee of the University of Georgia (Protocol Number, A2011 07-Y2-A3).

2.4. Intra-peritoneal glucose tolerance test (IPGTT) and insulin sensitivity test

Intraperitoneal glucose tolerance tests were conducted in mice fasted for 6 hours. Animals were administrated with 1.5 g/kg body weight of glucose in 10% phosphate buffer saline (pH 7.4) at 0 min. Blood glucose levels were measured using glucose test strips and glucose meter at 0, 30, 60 and 120 min after glucose injection.

For insulin sensitivity tests, animals were fasted for 4 h and administrated with insulin (*i.p.,* 0.75 U/kg) in 10% phosphate buffer saline (pH 7.4). Blood glucose levels were measured at 0, 30, 60 and 120 min after injection.

2.5. Gene expression analysis

Liver, white adipose and brown adipose tissue samples were freshly collected and frozen at -80 °C immediately until use. Total mRNA from the liver was isolated using the TRIZOL reagent from Invitrogen. Approximately 100 mg of liver tissue sample and 1 mL TRIZOL were transferred into falcon tubes. The mixture was homogenized with a Dremel 300i rotating blade homogenizer to generate a homogeneous suspension, which was transferred into eppendorf tubes and left at room temperature for 5 min. To the tissue homogenate, 200 µL of chloroform was added followed by vortex for 15 sec. After being left at room temperature for 3 min, the samples were centrifuged at 12,000 × g for 15 min at 4 °C. After centrifugation, there were three phases visible within the tube, while the top phase was transferred to a fresh tube, the bottom phases discarded. Isopropanol (500 µL) was added to the sample collected and incubated at room temperature for 10 min. Then, the samples were centrifuged at 12,000 × g for 10 min at 4 °C, after which the supernatant was removed. The RNA pellet was washed with 500 µL ethanol (80% EtOH), followed by centrifugation at 7,500 × g for 10 min at 4°C and the supernatant removed. Same procedure was repeated one more time to wash the pellet. Then, the tubes were

transferred to a 70 °C heat block for 3 min until complete dryness of the samples. Finally, the pellet was reconstituted in 81 μL of water and the resulting solutions were kept in -80°C freezer until use.

The mRNA from WAT and BAT were isolated using QIAGEN commercial kit. WAT or BAT (100 mg) and 1 mL of QIAzol were mixed in falcon tubes and homogenized. After 5 min of incubation at room temperature, 200 μL of chloroform was added into the homogenate and vigorously vortexed for 15 sec. The samples were centrifuged at 12,000 × g for 15 min at 4 °C after 3 min of incubation at room temperature. Then the top aqueous phase was transferred into a new tube and vortexed with 1 mL of 70% ethanol. Up to 700 μL of the mixture was transferred to a RNeasy Mini spin column in 2 mL collection tube and centrifuged at 8,000 × g at room temperature for 15 sec. RW1 Buffer (700 μL) was added to the RNeasy column and centrifuged at 8,000 × g for 15 sec. Then 500 μL of RPE Buffer was added to the RNeasy column and centrifuged at 8,000 × g for 15 sec. Another 500 μL of RPE Buffer was added to the column again and centrifuged at 8,000 × g for 2 min. Finally, the RNeasy column was placed in a new 1.5 mL eppendorf tube. After addition of 50 μL RNease-free water, the column-tube assembly was centrifuged at 8,000 × g for 1 min. The samples were stored in -80 °C freezer until use.

The isolated RNA (2 μg) was reverse-transcripted into first strand cDNA using the SuperScript III First-Strand Synthesis System. After being mixed and briefly centrifuged, 2 μg of total RNA, 1 μL of 50 μM oligo(dT_{20}) primer and 1 μL of annealing buffer were combined and added with RNase/DNase-free water to reach 8 μL of final solution. The mixture was incubated in a thermal cycler at 65 °C for 5 min and then immediately placed on ice for at least 1 min. The contents of the tube were collected by brief centrifugation and left on ice, to which 10 μL of 2X First-Strand Reaction Mix and 2 μL of SuperScript III/RNaseOUT Enzyme Mix were added. The

mixture was then vortexed and centrifuged briefly, followed by an incubation at 50 °C for 50 min. The reaction was terminated at 85 °C for 5 min and then chilled at 4 °C. The synthesized cDNA was stored at -20 °C until use.

Real-time quantitative PCR was performed using SYBR Green as detection reagent on the ABI StepOne Plus Real-Time PCR system. The data were normalized to glyceraldehyde 3-phosphate dehydrogenase (GADPH) mRNA as an internal control and analyzed using the ΔΔCt method. All primer sequences used are listed in Table 2.

Table 2. Primer sequences for qRT-PCR analysis for gene expression

Gene name	Forward sequence (F)	Reverse sequence (R)
F4/80	TGACTCACCTTGTGGTCCTAA	CTTCCCAGAATCCAGTCTTTCC
IL-6	TAGTCCTTCCTACCCCAATTTCC	TTGGTCCTTAGCCACTCCTTC
TNF-α	CCCTCACACTCAGATCATCTTCT	GCTACGACGTGGGCTACAG
MCP-1	TTAAAAACCTGGATCGGAACCAA	GCATTAGCTTCAGATTTACGGGT
CD68	TGTCTGATCTTGCTAGGACCG	GAGAGTAACGGCCTTTTTGTGA
FAS	GGAGGTGGTGATAGCCGGTAT	TGGGTAATCCATAGAGCCCAG
ACC-1	ATGGGCGGAATGGTCTCTTTC	TGGGGACCTTGTCTTCATCAT
SCD-1	TTCTTGCGATACACTCTGGTGC	CGGGATTGAATGTTCTTGTCGT
G6P	CGACTCGCTATCTCCAAGTGA	GTTGAACCAGTCTCCGACCA

2.6. H&E staining

For hematoxylin and eosin (H&E) staining, tissue samples were freshly collected from euthanized mice. Liver, WAT and BAT samples were fixed in 10% neutralized buffered formalin (NBF), dehydrated using gradient ethanol and embedded in paraffin. The tissue samples were soaked in 70% EtOH for 60 min, 85% EtOH for 30 min, 95% EtOH for 30 min, 100% EtOH for 60 min twice and xylene for 60 min twice at room temperature. Then the tissue samples were soaked in paraffin overnight.

Tissue sections were cut at 6 μm in thickness and dried at 37 °C for 120 min. H&E staining was performed following the instruction of a commercial kit from BBC Biochemical. The tissue section slides were soaked in 100% xylene for 3 min three times, 100% EtOH for 3 min three times and 95% EtOH for 3 min twice. Then they were rinsed using distilled water for three times and soaked in hematoxylin for 8 min. The slides were rinsed under running tap water until the water is no longer colored. Then the slides were dunked three times in acidified alcohol (1% HCl in 70% EtOH) until the sections turned pink. After 5 min of rinsing under tap water, the slides were dunked in 0.1% ammonia until the color darkened noticeably. After another 5 min of rinsing under tap water, the slides were soaked in eosin Y for 1 min and rinsed again under running tap water for 4 times. Then the slides soaked in 95% EtOH for 2 min twice, 100% EtOH for 2 min twice and xylene for 2 min twice. Finally, the slides were cover-slipped using Permount. Slides were examined using a Nikon ECLIPSE-Ti optical microscope. Pictures and size measurements were taken using Nikon NIS-Elements AR software.

2.7. Oil-red O staining

For Oil-red O staining, liver samples were freshly collected and immediately frozen in liquid nitrogen. Tissue sections were cut at 8 μm in thickness using cryostat Leica CM1850. Tissue sections were placed on slides and washed with 60% isopropanol. Next, the slides were stained with freshly prepared Oil-red O working solution for 15 min and then rinsed with 60% isopropanol. Furthermore, the slides were lightly stained with alum haematoxylin for 5 dips and then rinsed with distilled water. Finally, the slides were mounted using glycerine jelly. Slides were examined using a Nikon ECLIPSE-Ti optical microscope and pictures were taken using Nikon NIS-Elements AR software.

2.8. Biochemical analysis of blood samples

After animals were euthanized, blood samples were collected from heart cavities immediately and centrifuged to collect serum at 4,000 × g for 5 min. Triglycerides, cholesterol and non-esterified fatty acid (NEFA) concentrations were measured following the instruction from the manufacture of the test kits from Thermo Scientific. Insulin levels in blood were determined using a commercial ELISA kit from Mercodia Development Diagnostics.

(a). Determination of serum concentration of triglycerides: Two μL of serum sample and calibrator were added into 200 μL of freshly prepared reagent. Samples and calibrator were incubated for 5 min and analyzed using spectrophotometer with wavelength set at 500 nm. The triglyceride concentrations were calculated using the following equation:

*[Triglycerides] = (Absorbance of sample/Absorbance of calibrator) * [Calibrator] (mmol/dL)*

(b). Cholesterol concentration determination: Two μL of serum sample and calibrator were added into 200 μL of freshly prepared reagent. Samples and calibrator were analyzed using spectrophotometer with wavelength set at 505 nm. The cholesterol concentrations were calculated using the following equation:

*[Cholesterol] = (Absorbance of sample/Absorbance of calibrator) * [Calibrator] (mmol/dL)*

(c). Measurement of serum concentration of NEFA: Three and one half μL of serum sample was added into 150 μL of reagent 1 of commercial kit from Thermo Scientific. The mixture was incubated for 3 min and then added with 75 μL of reagent 2. After 4.5 min of incubation, the solution was analyzed using spectrophotometer with wavelength set at 546 nm.

(d) Determination of serum concentration of insulin: First, 10 μL of sample, control or calibrator, respectively, were added into each well. Then, 100 μL of enzyme conjugate (1X solutions) were added into each well. The mixture was mixed on a plate shaker at room temperature for 2 h. After mixing, all wells were washed for 6 times using buffer (1X solution)

before adding 200 μL substrate chromogen reagent (TMB). After 15 min of incubation, 50 μL of stop solution (acidic solution) was added and shaken for 5 sec to ensure mixing. The samples were analyzed using spectrophotometer with wavelength set at 450 nm.

2.9. Statistics

Statistical analyses were performed by one-way analysis of variance (ANOVA). A difference of $p<0.05$ was considered significant. The data were presented as the mean ± standard deviation (SD).

CHAPTER 3. RESULTS

3.1. CsA, fingolimod and laquinimod treatment attenuates body weight gain of C57BL/6 mice.

I first measured body weight to examine whether selected immune modulators could affect body weight gain of C57BL/6 mice fed a high fat-diet. CsA, fingolimod and laquinimod were given intra-peritoneally at doses 50, 0.040 and 2.5 mg/kg twice weekly for 15 weeks, respectively, according to previous studies (Moon, Jeong *et al.* 2012, Jiang, Wang *et al.* 2013). Body weights of all the treated mice were significantly ($P< 0.05$) lower than that of control animals at the end of treatment (Figure 1). The average body weight of CsA treated animals was ~34% less than that of control animals. As shown in Figure 1a, CsA treated mice increased their body weight at a much lower rate than that of control mice. The average body weight of animals treated with fingolimod or laquinimod was ~16% or ~9.2% less than the control at the end of the experiment. Their body weight gain slowed down compared to control animals from week 7, although they were at the same body weight level in the first 7 weeks. Laquinimod appears to have a least impact on weight gain (Figure 1a). Intra-peritoneal injection of modulators did not affect food intake of animals (Figure 1b). These results indicated that CsA, fingolimod and laquinimod attenuated body weight gain of mice fed a HFD, showing activities against high fat-induced obesity.

An analysis of body composition (to determine fat mass) by magnetic resonance proton spectroscopy (EchoMRI) was performed to investigate the effect of immune modulators on fat accumulation. Results shown in Figure 1c revealed that the average fat mass of CsA and

fingolimod treated mice was significantly lower ($p < 0.05$) than the control by ~60.8% and ~22.2%, respectively. However, there was no significant impact of laquinimod treatment comparing to control mice (Figure 1c). The treated animals showed the same lean mass to that of the control, suggesting that these immune modulators did not affect the lean mass of treated mice (Figure 1c). The lowered body weights of treated mice were due to lower fat mass.

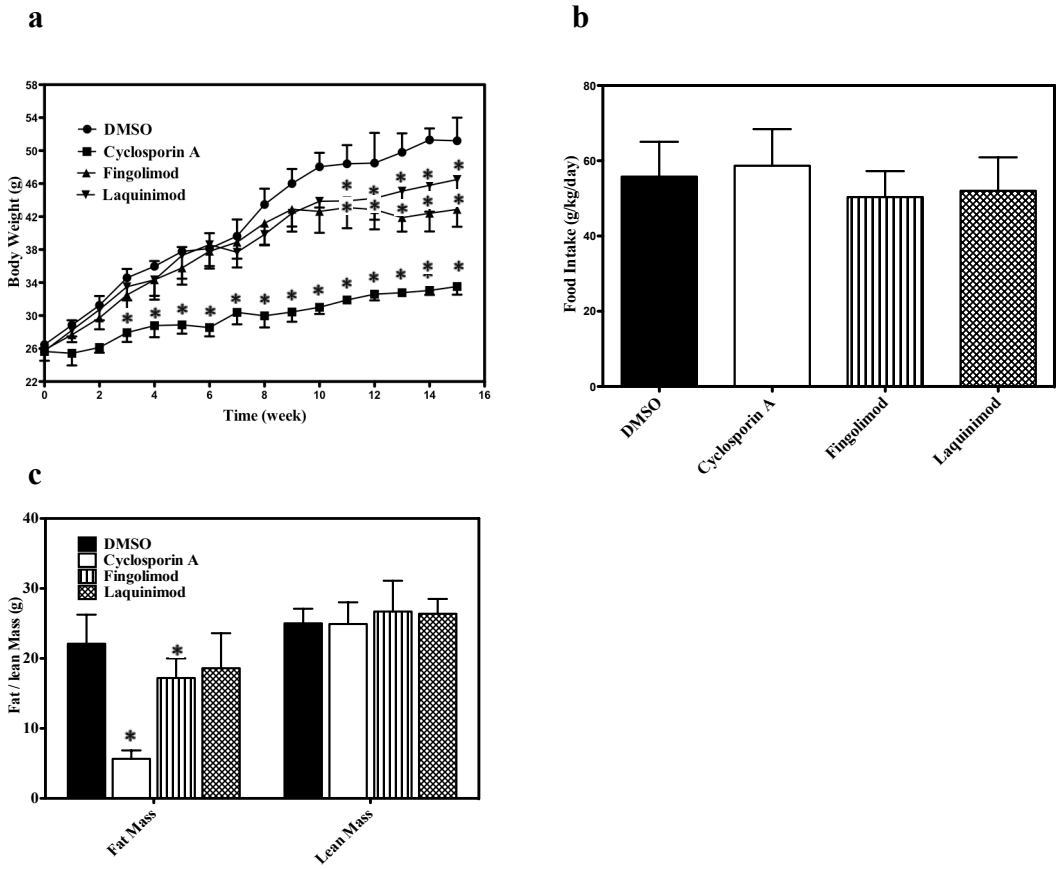

Figure 1. Effect of cyclosporine A, fingolimod and laquinomod on body weight and food-intake of animals fed with a high-fat diet. Time-dependent body weight growth curve (n=5, **P*<0.10 compared with mice *i.p.* injected with DMSO control vehicle) (a). Average daily food intake (b). Mouse fat and lean mass (n=5, **P*<0.05 compared with mice *i.p.* injected with DMSO control vehicle) (c).

3.2. CsA, fingolimod and laquinimod prevented HFD-induced hypertrophy in adipocytes.

To gain further insight about alteration of body composition, CsA, fingolimod or laquinimod treated and control mice fed a HFD for 15 weeks were dissected to collect WAT and

BAT. Visual inspection revealed a difference in WAT size between immune modulator treated animals and control animals. Moreover, we performed H&E staining on WAT and BAT from HFD-fed mice dosed with these immune modulators and DMSO as control to measure the alteration on morphology of adipocytes.

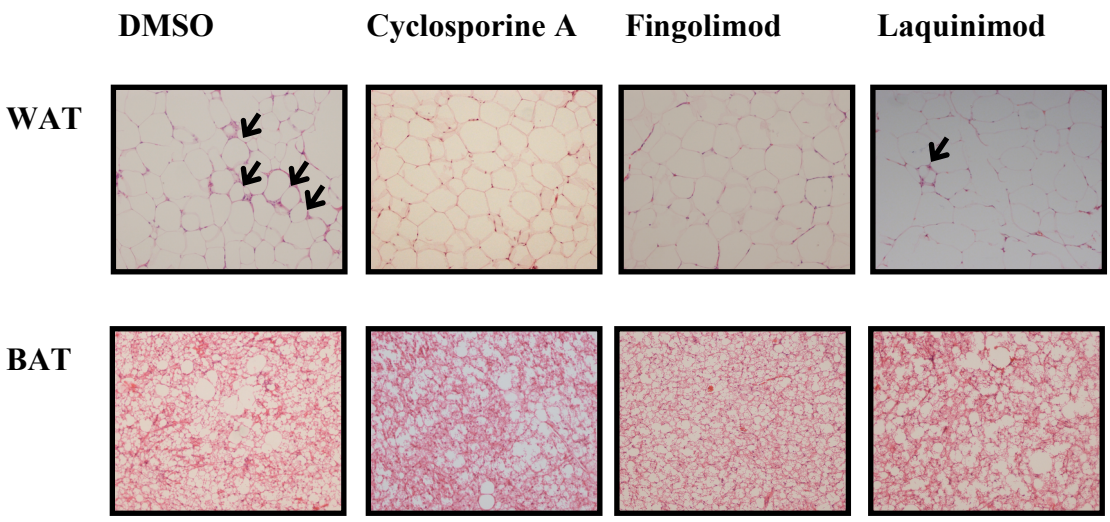

Figure 2. CsA, fingolimod and laquinimod prevented HFD-induced hypertrophy in adipocytes. Images of H&E staining on white adipose tissue (WAT) (arrows point to crown-like structures) (upper pannel) and brown adipose tissue (BAT) (lower pannel).

Based on our previous data, WAT from HFD-fed mice treated with DMSO showed an increased number of crown-like structure with H&E staining, which was a sign of macrophage activation in adipose tissues. This indicated that HFD-feeding increased immune cell infiltration and caused apparent hypertrophy in adipocytes of WAT. Adipocyte hypertrophy leads to adipose tissue hypoxia, a potential cause of adipose tissue inflammation and cell death, which can be shown in H&E staining experiments as crown-like structures around the adipocytes (Ye, Gao *et al.* 2007). According to the results of the H&E staining, the number of crown-like structures was reduced after the treatment of CsA, fingolimod and laquinimod, indicating these three immune

modulators decreased immune cell infiltration. It also suggested that the immune modulators blocked the development of hypertrophy induced by HFD (Figure 2 upper pannel).

Aggrandized lipid accumulation in BAT was observed in HFD-fed mice receiving DMSO administration as the control. Compared to the control group, the average size of adipocytes was smaller in animals treated with CsA, fingolimod or laquinimod, respectively (Figure 2 lower pannel). These results suggest that immune modulators attenuated HFD-induced hypertrophy of BAT.

3.3. CsA, fingolimod and laquinimod modulated the transcription of genes involved in chronic inflammation in WAT and BAT.

To investigate the possible mechanism underlying the effect of CsA, fingolimod and laquinimod on formation of crown-like structure in WAT and lipid accumulation in BAT, we measured the mRNA levels of genes involving in chronic inflammation in WAT and BAT. F4/80 is a marker for mature mouse macrophages (M1 macrophage) (Austyn and Gordon 1981). TNF-α is a prototypical pro-inflammatory cytokine found from macrophages infiltration into adipose tissue in obese mice and activates various signal transduction cascades to inhibit insulin action (Hotamisligil 2006). It provides the first clues between inflammation and insulin resistance (Fantuzzi 2005). IL-6 is a gene encoding a pro-inflammatory cytokine secreted by T cells and macrophages. MCP-1 is a member of the C-C chemokine family recruiting monocytes, memory T cells, and dendritic cells to the sites of inflammation. CD68 is a glycoprotein binding to low density lipoprotein. Also, it is a marker expresses on monocytes/macrophages. Altogether, the expression level of *F4/80, MCP-1, TNF-α, IL-6* and *CD68* are associated with chronic inflammation (Table 3).

Table 3. Genes analyzed in the study

Gene name	Function
F4/80	A biomarker, transmembrane protein presented on the cell-surface of macrophages
IL-6	Interleukin-6, a cytokine secreted by T cells and macrophages, stimulate immune response (Bendtzen 1988)
TNF-α	Tumor necrosis factor alpha, a pro-inflammatory adipokine produced by activated macrophages (M1) involved in systemic inflammation, a member of a group of cytokines that stimulate the acute phase reaction (Strieter, Kunkel *et al.* 1993)
MCP-1	The monocyte chemoattractant protein-1, a member of the C-C chemokine, and a potent chemotactic factor for monocytes, a key molecule mediating macrophage infiltration (Deshmane, Kremlev *et al.* 2009)
CD68	A glycoprotein that binds to low-density lipoprotein, a marker expressed on monocytes/macrophages (Holness and Simmons 1993)
FAS	Fatty acid synthase, a multi-enzyme protein that catalyzes fatty acid synthesis
ACC-1	Acetyl-CoA carboxylase-1, a regulator for fatty acids metabolism
SCD-1	Stearoyl-CoA desaturase-1, a key enzyme in fatty acid metabolism, responsible for forming a double bond in Stearoyl-CoA (Zhang, Ge *et al.* 1999)
G6P	Glucose 6-phosphate, a key enzyme in glucose metabolism

The mRNA levels of *F4/80, TNF-α, CD68* and *MCP-1* genes were measured in WAT in control group as well three treated groups. As demonstrated in previous studies, the total WAT for the expression of macrophage-specific marker genes, *F4/80* and *CD68* were markedly increased in control animals, indicating HFD increased adipose tissue macrophage infiltration. HFD also increased the expression of M1 (pro-inflammatory) macrophage marker genes, including *TNF-α* and *IL-6*. As shown in Figure 3a, fingolimod treatment significantly reduced the mRNA levels of *F4/80, TNF-α, CD68* and *MCP-1* by ~62%, ~59%, ~77% or ~60%, respectively, compared to those of the vehicle control group, which suggesting that fingolimod

decreased obesity-induced adipose tissue macrophage infiltration and reduced the number of HFD-induced M1 macrophage. Laquinimod treatment significantly decreased the mRNA level of *CD68* by ~31% and *MCP-1* by ~40%; while no significantly modulated mRNA levels of *F4/80* and *TNF-α* were observed against those of vehicle control group. CsA treatment up-regulated the mRNA level of *F4/80* by ~84%, *TNF-α* by ~180%, *CD68* by 31% and *MCP-1* by 120%, which indicated that CsA is lack of effect on reduction of number of macrophage.

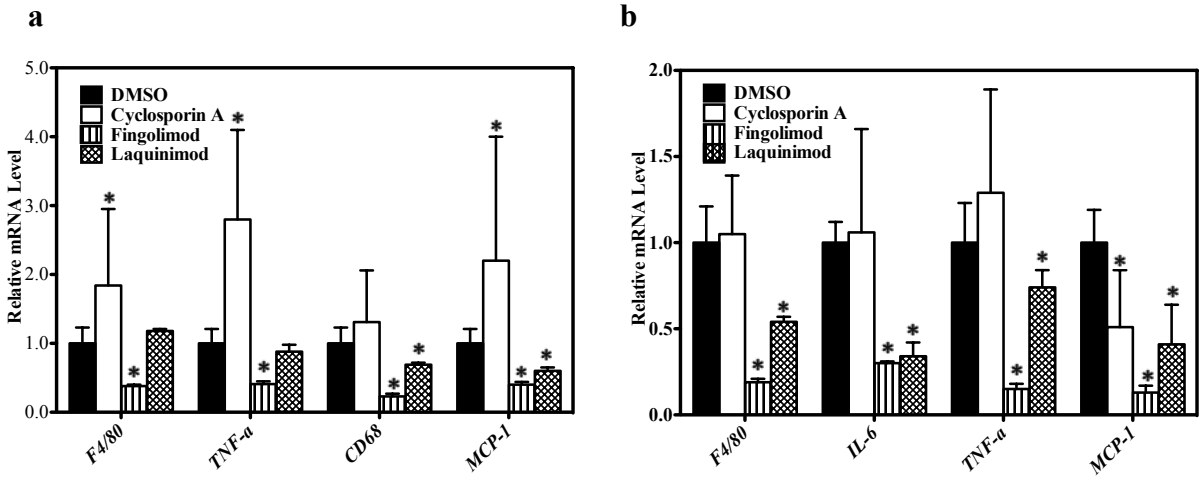

Figure 3. **CsA, fingolimod and laquinimod modulated the transcription of genes involved in chronic inflammation in WAT and BAT.** Relative mRNA level of inflammatory-related genes (*F4/80, TNF-α, CD68* and *MCP-1*) in white adipose tissue (n=5, **P*<0.05 compared with mice *i.p.* injected with DMSO control vehicle) (a). Relative mRNA level of inflammatory-related genes (*F4/80, IL-6, THF*-α and *MCP-1*) in brown adipose tissue (n=5, **P*<0.05 compared with mice *i.p.* injected with DMSO control vehicle) (b).

Furthermore, we measured mRNA level of *F4/80, IL-6, TNF-α* and *MCP-1* by RT-qPCR in BAT. As shown in Figure 3b, similar to the result in WAT, fingolimod treatment significantly down-regulated the mRNA level of *F4/80, IL-6, TNF-α* and *MCP-1* by ~81%, ~70%, ~85% and ~87% compared to those of vehicle control group. Laquinimod treatment also markedly lowered

the mRNA level of *F4/80, IL-6, TNF-α* and *MCP-1* by ~46%, ~66%, ~26% and ~59%, respectively, compared to those of vehicle control group. However, no significant different mRNA levels of *F4/80, IL-6* and *TNF-α* was observed between the CsA treated and control group with exception of *MCP-1* gene which was down-regulated by ~59%.

3.4. CsA and fingolimod blocked HFD-induced fat accumulation in liver without altering serum lipid levels.

After 15 weeks of HFD-feeding, the weight of liver dramatically increased in vehicle control mice due to fat accumulation in the liver. H&E staining of liver sample from mice treated with CsA and fingolimod showed less lipid accumulation (white spots) than the control but laquinimod showed no significant effects (Figure 4a upper pannel). Similarly, in the Oil-red O staining experiments, less fat (red spots) was observed in the liver tissue from CsA and fingolimod treated mice; while no significant difference was noticed in the laquinimod treated group (Figure 4a lower pannel). Both experiments showed that CsA and fingolimod treatment prevented HFD-induced fat accumulation in the liver but laquinimod did not. Meanwhile, serum concentrations of triglycerides, cholesterol and free fatty acids remain the same with the exception of fingolimod treated animals which showed an approximately 20% reduction in serum level of cholesterol (Figure 4b).

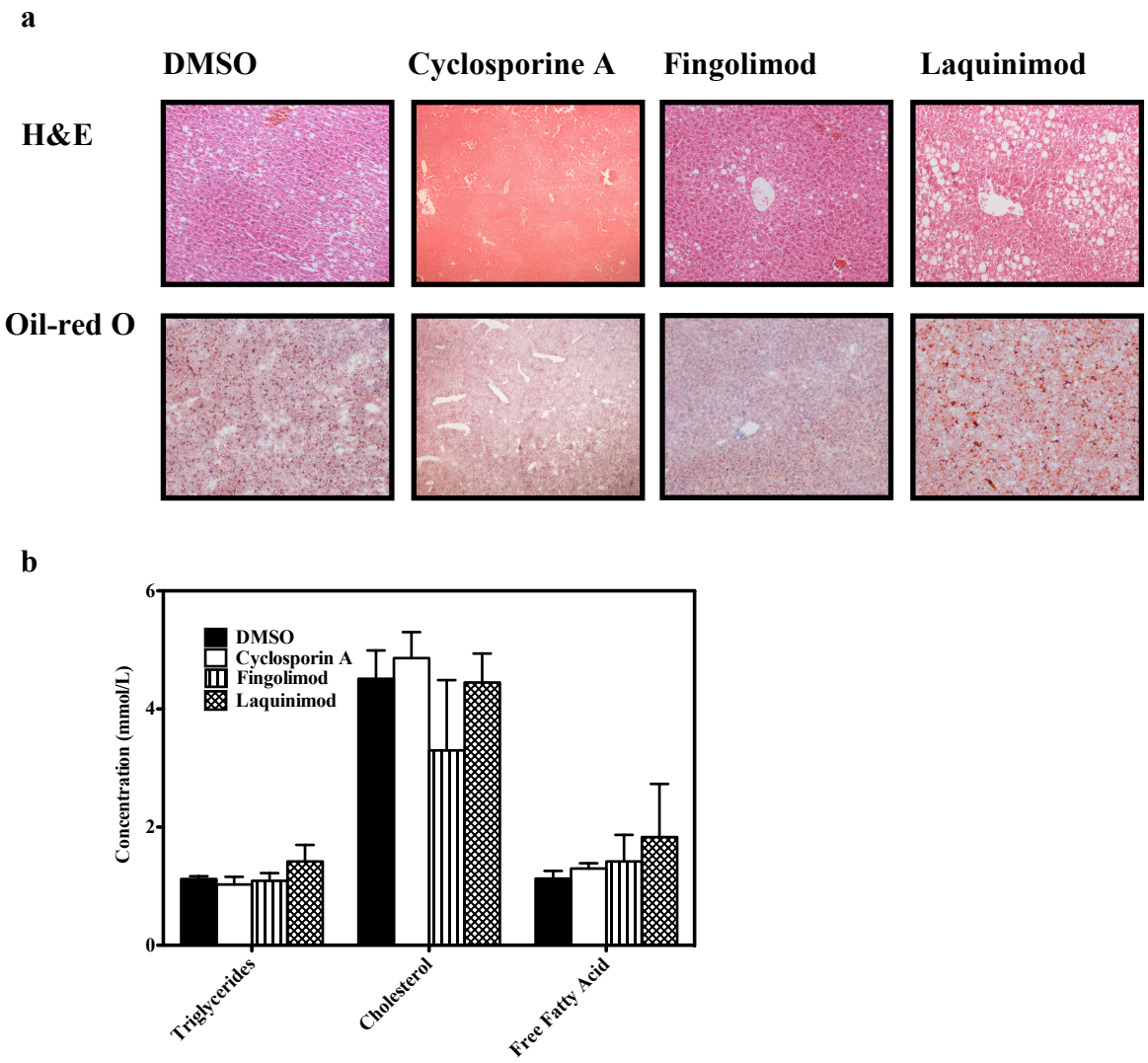

Figure 4. CsA, fingolimod and laquinimod blocked HFD-induced fatty liver. Representative images of H&E staining on liver (upper pannel) and Oil-red O staining on liver sections (lower pannel) (a). Serum concentration of triglycerides, cholesterol and free fatty acid (n=5, **P*<0.05 compared with mice *i.p.* injected with DMSO control vehicle) (b).

3.5. CsA, fingolimod and laquinimod modulated the transcription of genes involved in chronic inflammation and nutrient metabolism in liver.

The data above showed that CsA, fingolimod and laquinimod treatment could cause a significant decline in liver lipid accumulation in the C57BL/6 mice, raising a possible role of CsA, fingolimod and laquinimod against lipogenesis. To address this issue, we measured

expression levels of genes involved in liver lipogenesis, including *FAS, ACC-1, SCD*-1 and *G6P*. *ACC-1* and *SCD-1* are responsible for the metabolism of fatty acid. *FAS* regulated lipid transfer. G6P is a key enzyme regulating glucose metabolism (Table 2). Thus the expression levels of *ACC-1, SCD-1, FAS* and *G6P* are related to nutrient metabolism. Quantitative PCR was performed on total RNAs extracted from liver. All the mice treated with immune suppressor showed different levels of induction of lipogenic gene, compared to HFD-fed vehicle control mice (Figure 5b). To be more detailed, CsA significantly raised the expression levels of *FAS* by ~3.1 folds, *ACC-1* by ~2.1 folds and *SCD-1* by ~3.1 folds, except *G6P* that was not affected. Similarly, fingolimod largely up-regulated the expression levels of *FAS* by ~6.2 folds, *ACC-1* by ~7.8 folds and *SCD-1* by ~4.6 folds, but not *G6P*. Meanwhile, laquinimod showed a different gene modulating profile, up-regulating *SCD-1* by ~2.3% and *G6P-1* by ~1.8 folds and leaving *FAS* and *ACC-1* unchanged.

Since HFD-induced fatty liver is always accompanied with chronic inflammation, we measured the mRNA levels of several inflammatory genes, including *F4/80, IL-6, TNF-α* and *MCP-1* (Figure 5a). The results showed that the treatment with fingolimod significantly down-regulated *IL-6, TNF-α* and *MCP-1* by ~91%, ~81% and ~85%, respectively; while *F4/80* was slightly up-regulated. Similarly, laquinimod markedly down-regulated *IL-6, TNF-α* and *MCP-1* by ~92%, ~76% and ~64% respectively, and slightly up-regulated *F4/80*. This slight increase of *F4/80* in both treated groups was not statistically significant. However, treatment with CsA showed a very different result, in which *F4/80*, *IL-6* and *TNF-α* increased ~1.8, ~2.3 or ~2.4 folds, respectively. The expression level of *MCP-1* was slightly lowered without statistical significance.

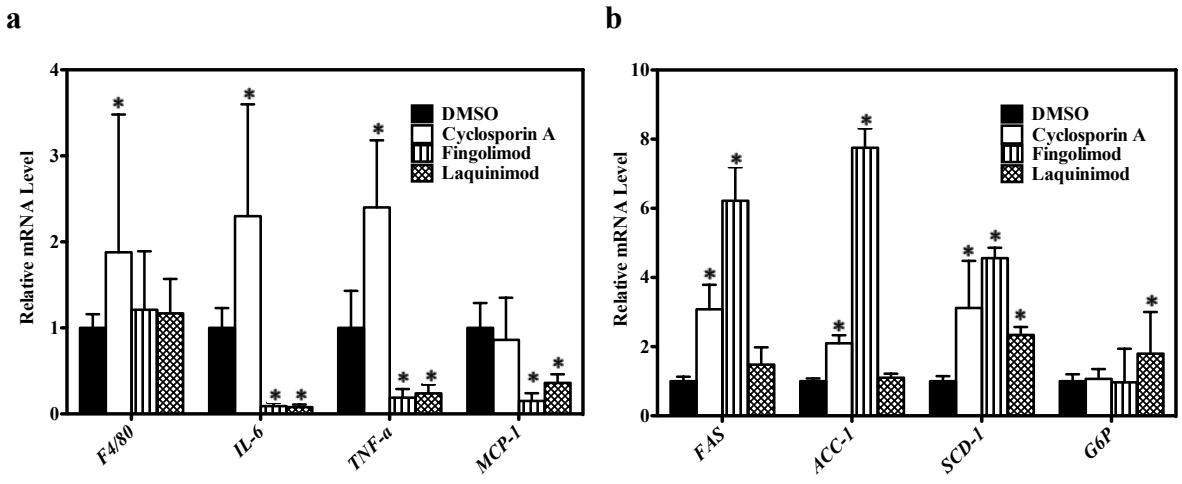

Figure 5. CsA, fingolimod and laquinimod modulated the transcription of genes involved in chronic inflammation and nutrient metabolism in liver. Relative mRNA level of inflammatory-related genes (*F4/80, IL-6, TNF-α* and *MCP-1*) (n=5, **P*<0.05 compared with mice *i.p.* injected with DMSO control vehicle) (a). Relative mRNA level of nutrient metabolic-related genes (*FAS, ACC-1, SCD-1* and *G6P*) (n=5, **P*<0.05 compared with mice *i.p.* injected with DMSO control vehicle) (b).

3.6. CsA, fingolimod and laquinimod improved insulin sensitivity in mice fed a HFD.

An important consequence of obesity is the development of insulin resistance. Since CsA, fingolimod and laquinimod have shown to attenuate body weight gain in C57BL/6 mice, we reasoned that CsA, fingolimod and laquinimod might prevent mice from insulin resistance, keeping pancreas function well. In order to identify whether CsA, fingolimod and laquinimod treatment prevented mice from high fat-diet induced insulin resistance, intraperitoneal glucose tolerance tests were performed at the 14th week of the experiment. After 6 hour of fasting, we intraperitoneally injected glucose (1.5 g/kg) and determined glucose levels at 0, 30, 60 and120 min after injection by commercial glucose test strips and meters. Blood glucose levels under fasting conditions prior to glucose load (0 min) were similar among CsA, fingolimod and laquinimod treated mice and DMSO control mice. Furthermore, blood glucose levels were

elevated in the control mice, indicating impaired glucose tolerance in these mice (Figure 6a). However, the blood glucose levels were lowered in CsA, fingolimod and laquinimod treated mice, compared to that of control mice (Figure 6a). AUC calculations make this point clearer that CsA, fingolimod and laquinimod treatment lead to ~26.6%, ~24.6% and ~28.6% decrease compared to the control animals (Figure 6b).

The insulin sensitivity test was examined 1 week later with an insulin tolerance test (ITT) on the same mice. After 4 h of fasting, we intra-peritoneally injected insulin (0.75 U/kg) and determined glucose levels at 0, 30, 60 and 120 min after injection using commercial glucose test strips and meters. The glycemic drop following insulin administration were more pronounced for CsA, fingolimod or laquinimod treated mice than DMSO control mice. This indicated that HFD caused an apparent insulin resistance, as shown in Figure 6c, an increase of blood glucose level in the control group dosed with DMSO. However, CsA, fingolimod and laquinimod treatments effectively blocked this resistance by attenuating the rise of blood glucose levels after the insulin injections with a better insulin sensitivity (Figure 6c). Moreover, the difference in blood glucose levels remained between DMSO control animals and immune modulator treated animals for the duration of the experiment (120 min).

In addition, we measured serum levels of insulin using commercial kits. The serum insulin concentrations of CsA and fingolimod treated mice were significantly decreased compared to those of the vehicle control (Figure 6d); while the laquinimod treated group was not significantly different from the control. This result confirms that CsA and fingolimod improved insulin sensitivity in mice fed a HFD.

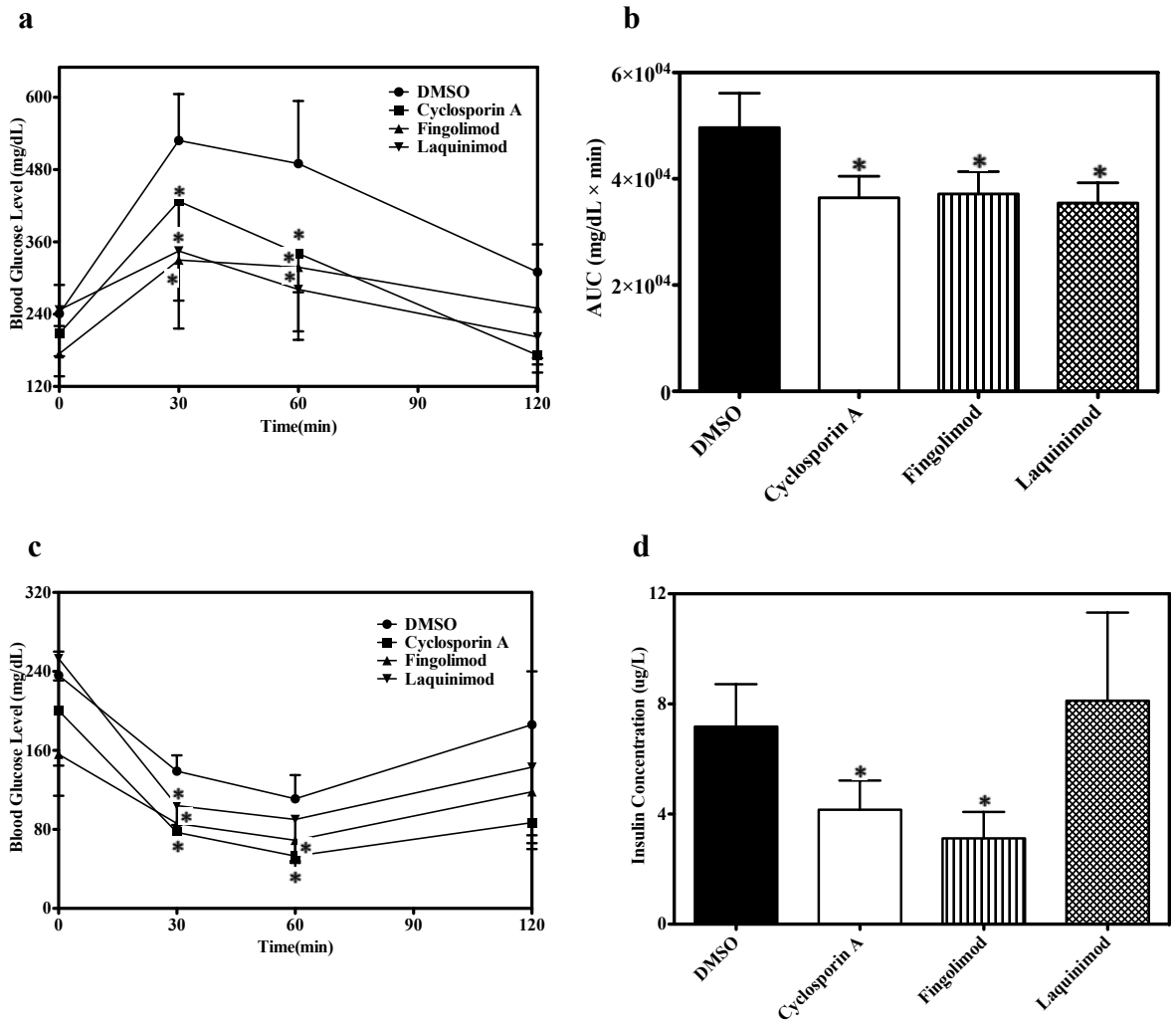

Figure 6. CsA, fingolimod and laquinimod improved insulin sensitivity in mice fed HFD. Glucose profile in IPGTT (n=5, **P*<0.05 compared with mice *i.p.* injected with DMSO control vehicle) (a). AUC of IPGTT (b). Glucose profile of ITT (n=5, **P*<0.05 compared with mice *i.p.* injected with DMSO control vehicle) (c). Serum concentration of fasting insulin level in mice (n=5, **P*<0.05 compared with mice *i.p.* injected with DMSO control vehicle) (d). IPGTT: intra-peritoneal glucose tolerance test, AUC: area under curves, ITT: insulin tolerance test.

CHAPTER 4. DISCUSSION

In this study, I demonstrated that CsA, fingolimod and laquinimod blocked HFD-induced weight gain without affecting food intake (Figure 1). The H&E and Oil-Red O staining results showed that three immune modulators decreased immune cell infiltration and blocked the development of adipocyte hypertrophy (Figures 2 and 4). Specifically, CsA and fingolimod treatment prevented HFD-induced macrophage infiltrations and hypertrophy in adipocytes, and blocked fat accumulation in liver. Results from real-time RT-PCR analysis revealed that fingolimod and laquinimod treatment significantly suppressed the HFD-induced elevation of transcriptional elevation of *F4/80, TNF-α, CD68 and MCP-1*. Fingolimod and laquinimod treatment down-regulated expression of genes involving in chronic inflammation in adipose tissue and liver (Figure 5). In contrast, CsA up-regulated expression of the same set of genes. The results from IPGTT and insulin sensitivity tests confirmed the above results that CsA, fingolimod and laquinimod treatment prevented HFD-induced obesity and insulin resistance (Figure 6). Due to difference in activity and mechanisms of action for the three compounds employed in this study, their effects on HFD-induced obesity and development of fatty liver are discussed separately as follows.

4.1. CsA

Gene expression experiments focused on inflammation were used to study the mechanism behind the anti-obese activity of CsA. CsA significantly up-regulated the transcription level of *F4/80*, *TNF-α, MCP-1* and *CD68* in WAT, BAT and liver. Since *F4/80*, *TNF-α, MCP-1* and *CD68* are specific-markers for macrophages, the increased levels of these

genes in the CsA dosed mice indicated that macrophage response was activated by CsA. Based on the fact of activated macrophage response and the up-regulated inflammation-related genes, it is possible that the activated inflammatory response was a result of compensatory macrophage activation. The mechanism of its anti-obese effect may relate to other immune cells, among which T cells have been proposed as the major target by multiple publications. T helper (Th) lymphocytes can differentiate into two distinct subsets, Th1 and Th2, as defined by various functions and cytokine characterizations. The functional differences between Th subsets are explained primarily through the activities of the cytokines they secrete. *IFN-γ* is the characteristic cytokine of Th1 cells, which also produce *IL-2* and *TNF-β*. *IL-4* is the corresponding characteristic cytokine of Th2 cells, which also secrete *IL-5, IL-6, IL-9* and *IL-13*. The T-box transcription factor (T-bet) was first described as the transcription factor that is responsible for the development of Th1 CD4+ T cells. T-bet induces the production of *IFN-γ* and regulates the Th1 cell-migratory program by regulating the expression of chemokines and chemokine receptors such as CCL3 and CCL4. By inducing *IFN-γ* in CD4+ T cells, T-bet regulates inflammation and immune-regulation factors. T-bet expression in Th1 cells contributes to chronic inflammatory disease. Th2 can elevate the expression of anti-inflammatory cytokines, such as *IL-4* and *IL-13*, which can increase thermogenic gene expression, fatty acid metabolism and energy expenditure (Nguyen, Qiu *et al.* 2011). Therefore, we think CsA may suppress Th1 differentiation and promote Th2 differentiation, demonstrating anti-inflammatory and anti-obese activities. Since Th1 differentiation is mediated by STAT4 and T-bet transcription factors, we hypothesized CsA could suppress Th1 differentiation by inhibiting STAT4 or T-bet.

Additionally, the IPGTT and insulin sensitivity tests indicated CsA could improve insulin sensitivity and therefore protect mice from insulin resistance. A previously published research

indicated that T-bet could modulate metabolic physiology and obesity-associated insulin resistance by using mice deficient in the T-bet (T-bet$^{-/-}$) (Stolarczyk, Vong *et al.* 2013). T-bet deficiency causes a defect in the development of the Th1 subset, with a significant reduction of *IFN-γ*, but a significant increase in *IL-4* production. Furthermore, T-bet deficiency reduced the Th1 response by shifting the Th1/Th2 balance in favor of Th2 phenotype. Our understanding of how T-bet function to regulate the Th1 cell fate decision mostly stems from the murine *Ifng*, which encodes the key Th1 cytokines. Lineage-specific expression of this gene is dependent on a series of distal regulatory elements that act together to form domains segregated from surrounding chromatin. These regulatory elements have been characterized by sequence conservation and DNaseI hypersensitivity and a number of them exhibit enhancer or insulator activity (Hatton, Harrington *et al.* 2006, Lee, Kim *et al.* 2006). During Th1 differentiation, T-bet directly binds to regulatory elements at the *Ifng* locus, leading to changes in histone modification and *Ifng* transcription. T-bet also binds with the insulator/boundary element binding protein CTCF and the cohesin complex to conserved distal elements flanking the Ifng locus, forming intra-chromosomal loops between these elements and the *Ifng* gene. In Th1 cells, T-bet binds to the *Il4* promoter and a silencer element downstream. Accordingly, CsA might improve insulin sensitivity by suppressing T-bet, which is consistent with the hypothesis in the last paragraph. Further investigation is needed to reveal effects of CsA on T-bet.

4.2. Fingolimod

Similar to CsA, both physiological and morphological experiments suggested that fingolimod is an effective anti-obese drug, which could significantly block body weight gain and fat mass accumulation. Based on experiments at the cellular level, fingolimod inhibited the formation of crown-like structures around adipocytes, indicating that fingolimod could

suppresses macrophage recruitment and therefore decrease the level of local inflammation response. At the molecular level, the expression level of genes related to chronic inflammation were down-regulated by fingolimod administration, which also indicated that fingolimod could play an anti-inflammatory role by suppressing the activation of macrophages. Therefore, we think that the anti-obese activity of fingolimod originates from its inhibitory effects against macrophage immune response. Monocytes can differentiate into macrophages and dendritic cells. All these three immune cells can secrete *MCP-1*, which could further recruit more monocytes and activate the macrophage infiltration. Since *MCP-1* is a key factor in the process of macrophage infiltration, it is highly possible that *MCP-1* is the target of fingolimod. By inhibiting the generation or activity of *MCP-1*, fingolimod could block the cascade of macrophage infiltration and related immune response. Therefore fingolimod demonstrated anti-obese activity by the suppression of inflammatory response.

In the mean time, fingolimod dosed mice demonstrated higher glucose tolerance in the IPGTT experiments, compared to the control group. It also improved the insulin sensitivity, suggesting the dosed mice could effectively respond to high blood glucose levels, which was consistent with the higher glucose tolerance. The activity of facilitating the body to clear blood sugar of fingolimod might also originate from its possible target *MCP-1*. *MCP-1* is a critical cytokine on regulation of insulin sensitivity. Over-expression of *MCP-1* in adipose tissue can reduce insulin sensitivity in damaged or inflamed tissues. By inhibiting the *MCP-1* expression, fingolimod can effectively enhance the insulin sensitivity and therefore boost the hypoglycemic ability of the body, indirectly contributing to its anti-obese activity.

4.3. Laquinimod

In body weight gain experiment, laquiminod demonstrated a weaker inhibitory activity effects than that of CsA and fingolimod. In body composition analysis, the decrease of fat mass by laquinimod was not significant. In the Oil-red O staining experiment, laquinimod did not demonstrate a significant activity of inhibiting fat deposit in the liver. All these results were consistent with each other, suggesting laquinimod had a significantly lower anti-obese activity at the physiological and histological levels.

Both H&E staining and inflammation-related gene expression experiments showed that laquinimod had some anti-inflammatory activity, which was similar to but weaker than that of fingolimod. We think that the mechanism of anti-inflammatory and anti-obese activities of laquinimod was similar to that of the fingolimod, which was majorly fulfilled by inhibiting macrophage infiltration. Accordingly, laquinimod may also target *MCP-1* though with a lower potency.

In conclusion, data collected from this study demonstrated that CsA, fingolimod and laquinimod treatment protected C57BL/6 mice from high-fat diet-induced obesity, insulin resistance and hepatic steatosis at different levels. Among three modulators examined, CsA is more effective than fingolimod and laquinimod. These beneficial effects were associated with regulation of inflammatory immune responses and blood glucose adjustment. These results suggest that CsA, fingolimod and laquinimod are promising drugs for prevention of HFD-induced obesity, inflammation and related complications. Strategically, immune modulation could be an attractive approach for preventing high fat diet-induced obesity.

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