Galectin 3 in Obesity

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THESIS

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

iv

LIST OF ABBREVIATIONS

AGE   Advanced Glycation Endproduct

ALE   Advanced Lipoxidation Endproducts

ANOVA   Analysis of Variance

APN   Adiponectin

ApoE   Apolipoprotein E

APR   Acute Phase Response

BMI   Body Mass Index

CCL2   Chemokine (C-C motif) Ligand 2

CD68   Cluster of Differentiation 68

CDAA   Choline-deficient L-amino-acid-defined

cDNA   Complementary Deoxyribonucleic Acid

CHO   Chinese Hamster Ovary

CRD   Carbohydrate Recognition Domains

CRP   C Reactive Protein

DEXA   Dual-energy X-ray Absorptiometry

DIO   Diet-induced Obesity

ELISA	Enzyme-linked Immunosorbent Assay
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
GLUT4	Glucose Transporter 4
HDL	High-density Lipoprotein
HFD	High-fat Diet
IL	Interleukin
KO	Knock-out
LDL	Low-density Lipoprotein
LFD	Low-fat Diet
LPS	Lipopolysaccharide
mRNA	Messenger Ribonucleic Acid
NAFLD	Nonalcoholic Fatty Liver Disease
NASH	Nonalcoholic Steatohepatitis
OPN	Osteopontin
PAI-1	Plasminogen Activator Inhibitor-1
PAMP	Pathogen-associated Molecular Patterns
PPAR-γ	Peroxisome Proliferator-activated Receptor-γ

RAGE   Receptor for Advanced Glycation Endproduct

RT-PCR   Reverse Transcription Polymerase Chain Reaction

SAA   Serum Amyloid A

SAT   Subcutaneous Adipose Tissue

TIMP-1   Tissue inhibitor of matrix metalloprotease-1

TNF-α Tumor Necrosis Factor Alpha

VAT   Visceral Adipose Tissue

WHO   World Health Organization

WT   Wild-type

SUMMARY

Recent epidemiological studies demonstrated a rapid increase in the prevalence of obesity. The excessive fat accumulation places patients at risk of various complications, including insulin resistance, dyslipidemia, hypertension, premature heart disease, sleep apnea, Alzheimer's disease, hepatic steatosis and certain types of cancer. An increasing number of studies indicate that obesity is accompanied by low-grade systemic inflammation, with increased production of pro-inflammatory mediators in adipose tissue.

This study was designed to investigate the role of galectin 3 in regulation of adiposity and inflammation in mice. Galectin 3 is reported to stimulate adipocyte proliferation and act as a pro-inflammatory lectin. We hypothesize that galectin 3 deficiency may lead to decreased adiposity and inflammation. We assessed body composition, adiposity and inflammation in mice fed with either low-fat diet or high-fat diet.

Data show that galectin 3 deficiency leads to increased adiposity and inflammation, which negates our hypothesis. Possible reasons include the possibility that lack of galectin 3 may increase LPS-induced inflammation and adiposity, decrease alternative macrophage activation and macrophage phagocytosis, and impair removal of advanced glycation endproducts.

I.   INTRODUCTION

The prevalence of obesity has been greatly increasing during the past decades, and obesity is one of the major problems which threaten human health. Data from the World Health Organization (WHO) indicate that the prevalence of obesity worldwide has more than doubled since 1980 [1]. Studies indicate that obesity is associated with inflammation in adipose tissue, which may lead to several metabolic complications, such as insulin resistance and hypertension [2]. An increasing number of studies revealed the important role of inflammatory mediators in the development of obesity-related metabolic complications [3]. Recent studies also indicate that galectin 3 may be involved in the process of adipose tissue inflammation [4].

A. Obesity

Obesity is recognized as a global epidemic and it is the fifth leading cause of death globally. Over 2.8 million adults every year die as a result of diseases related to being overweight or obese [1]. The WHO defines obesity as a Body Mass Index (BMI, defined as a person's body weight in kilograms divided by square of the height in meters, kg/m^2) greater than or equal to 30, which includes over 200 million men and nearly 300 million women in the world [1]. The growing epidemic of obesity is also threatening the health of millions of people in the US, where 37% of adults and 17% of children and adolescents were affected by obesity in 2010 [5].

Obesity places patients at increased risk of various complications, including insulin resistance, dyslipidemia, hypertension, cardiovascular disease, certain types of cancer, Alzheimer's disease, hepatic steatosis and sleep apnea [6, 7, 8, 9]. The risk for these health complications and related mortality increases with increasing BMI. For example, in the prospective Nurse's Health Study, the mortality rate from cardiovascular disease of obese middle-aged women (BMI>32) was 4-fold higher than that of normal weight women (BMI<25). Moreover, a BMI above 32 was associated with double mortality rates from all causes (cardiovascular disease, cancer, and other causes) among the women in this study over a 16-year period [10]. On average, a BMI at 30-35 would reduce life expectancy by 2-4 years, and for the severe obese subjects whose BMI ranges from 40 to 45, the median survival is reduced by 8-10 years [11].

The growing prevalence of obesity also increases the economic burden for the obese population. In the US in 2006, an obese person spent annually $1,429 more, which is about 42% more than a normal weight person for medical expenses. The medical costs associated with obesity were estimated at $147 billion in 2008 [12].

B. Adipose tissue

Adipose tissue is a type of loose connective tissue primarily composed of adipocytes [13]. Besides adipocytes, adipose tissue also contains connective tissue matrix, fibroblasts, endothelial cells and immune cells, such as macrophages as well as T and B lymphocytes [14]. Adipose tissue is the major

storage site for triglycerides in the body, thus it has an important influence on nutrients' metabolism [15].

There are two distinct components of adipose tissue: visceral adipose tissue (VAT) and subcutaneous adipose tissue (SAT). Expansion of VAT, which is located between the inner organs inside the abdominal cavity, is associated with increased risk of type 2 diabetes, insulin resistance, cardiovascular disease and hepatic steatosis, collectively defined as metabolic syndrome [14, 16, 17, 18]. Compared to VAT, SAT is less dangerous in the development of the metabolic syndrome. Subcutaneous adipose tissue is located underneath the skin, and it is not involved in the increased risk of metabolic diseases caused by obesity. In contrast, studies show that SAT may be protective. In animal studies, transplantation of SAT into the visceral area lead to a decrease in VAT mass, increase in insulin sensitivity and improved glucose metabolism [19]. In humans, abdominal SAT is inversely associated with cardiometabolic risk factors and the metabolic syndrome in the Korean population [20].

In addition to being a fat and energy storage organ, adipose tissue is also recognized as an important endocrine organ and modulator of immune function, producing numerous adipokines that affect adipocyte proliferation, appetite, energy expenditure, insulin sensitivity, and inflammation [21]. Along with the inflammatory process associated with obesity, the adipocyte size increases through hypertrophy, and the number of adipocytes also increases through hyperplasia [22].

C. <u>Obesity and Inflammation</u>

Inflammation is part of the innate immune response to an injury or abnormal stimulation. It is a protective mechanism for the body to eliminate infectious organisms causing injury as well as tissues damaged as a consequence of such injury. During the inflammatory process, many different types of cells in the immune system secrete cytokines, which are a category of small cell-signaling molecules for cellular communication, and several other inflammatory mediators [23].

Obesity is associated with low-grade chronic inflammation. Inflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interlukin-6 (IL-6), are elevated in obese subjects [15]. Circulating level of these pro-inflammatory cytokines is directly correlated to the hyperplasia and hypertrophy of adipocytes in adipose tissue [24]. Increased adiposity is characterized by the progressive infiltration of adipose tissue by macrophages. The initial inflammatory response is mediated by classically activated macrophages (M1-polarized macrophages), which are pro-inflammatory macrophages. In contrast, the resolution of inflammation is predominantly mediated by alternatively activated macrophages (M2-polarized macrophages), which are involved in wound healing and tissue repair. In lean adipose tissue, the predominant mediators are anti-inflammatory cytokines from M2 macrophages, such as interleukin-4 and interleukin-13 [25, 26]. However, obesity leads to the recruitment and activation of M1 macrophages and T lymphocytes in adipose tissue, with elevated production of many pro-inflammatory cytokines including TNF-α and IL-6 [27].

4

1. Adiponectin

Adiponectin (APN) is an adipokine, a protein secreted from adipocytes. Plasma levels of APN are decreased in obese subjects [28]. Production of APN in adipose tissue is inversely correlated to the size of adipocytes. Studies show that large adipocytes have decreased secretion of APN, which is probably due to the inhibition by pro-inflammatory cytokines such as TNF-α [29]. In addition, some obesity-related metabolic complications, including type 2 diabetes, coronary heart disease and hypertension, are correlated with low circulating levels of APN [30, 31].

Evidence from experimental models demonstrates that APN is a protective adipokine against the metabolic dysfunctions related to obesity. First, it is reported that APN is an insulin-sensitizing adipokine. Adiponectin knock-out (KO) mice developed severe insulin resistance with a high caloric diet. Even when fed with a normal chow diet, these mice displayed insulin resistance and glucose intolerance [32]. In contrast, transgenic mice with elevated circulating levels of APN were resistant to diet-induced decrease of insulin sensitivity [33]. On the other hand, administration of APN reduced plasma free fatty acids, triglycerides and glucose after high-fat diet feeding in mice. Daily administration of APN lead to weight loss in mice fed with high-fat diet without any effect on their food intake [34]. Overall, these data indicate that APN is an insulin-sensitizing adipokine.

In addition, APN is also involved in adipose tissue's inflammation. In fact, plasma levels of the acute-phase protein C reactive protein (CRP) are negatively correlated to APN levels [35]. Evidence also indicates that APN is a modulator of macrophage function: first, APN inhibits the transformation of macrophages to

foam cells in humans [36] and APN KO mice have increased atherosclerosis [37];

second, APN suppresses production of TNF-α from macrophages [38]; third,

APN promotes phagocytosis of dead cells by macrophages, while APN KO mice

display decreased ability to phagocytose dead cells compared to wild-type mice

[39]; finally, APN KO mice have increased M1 macrophage markers and

decreased M2 macrophage markers, which indicates that APN can promote

alternative macrophage activation [40].

Thus, evidence reveals an important role for APN in regulating obesity-related

metabolic complications. Levels of APN are negatively correlated to adipose

tissue inflammation in obese subjects.

2. Leptin

Leptin is a 16-kDa protein predominantly secreted by adipose tissue. Contrary

to APN, circulating levels of leptin parallel body fat mass [41]. Serum leptin

levels parallel both the size and number of adipocytes in humans [42, 43]. In

addition, leptin production is increased following weight gain while decreased

after weight loss [42, 44]. Leptin plays a role in food intake and energy

expenditure. Daily administration of leptin reduced food intake and body weight,

but increased energy expenditure in *ob/ob* mice (leptin-deficient mice with

obesity) [45].

Besides effects on food intake and energy expenditure, leptin is also an

important modulator in inflammatory responses. Firstly, leptin can modulate T-

cell immune responses. Atrophy of the thymus was found in *ob/ob* mice,

6

indicating impaired T-cell immunity [46]. Furthermore, there was an increased T-helper cell proliferation with the administration of leptin to mice. Leptin increases Th1 (T helper lymphocyte subset, producing pro-inflammatory cytokines) and decreases Th2 (T helper lymphocyte subset, producing anti-inflammatory cytokines) cytokine production in mice [47]. Secondly, leptin directly regulates macrophage activity. There is decreased macrophage phagocytosis in *ob/ob* mice [48]. Finally, leptin is reported to affect the production of several pro-inflammatory cytokines in mice. With the administration of leptin, production of TNF-α, IL-6 and IL-12 was increased in wild-type mice [48].

Leptin is an important marker for adiposity, and its circulating level is proportional to body fat. In addition, leptin is also a powerful regulator of food intake and energy expenditure, and studies show a critical role of leptin in the immune and inflammatory response [48].

3. Tumor necrosis factor-alpha

Tumor necrosis factor-alpha (TNF-α), first described as an endotoxin-induced serum factor that causes necrosis of tumors, is a pro-inflammatory cytokine that can disturb insulin signaling [49]. Dysregulation of TNF-α production has been implicated in a variety of human diseases, including Alzheimer's disease, cancer, major depression, and inflammatory bowel disease (IBD) [50-53].

In adipose tissue, TNF-α is mainly secreted by macrophages. TNF-α can reduce mRNA expression of glucose transporter 4 (GLUT4, insulin-dependent glucose transporter) [54], and this induces insulin resistance in adipose tissue.

7

Mice lacking TNF-α or TNF-α receptors are resistant to development of insulin resistance induced by obesity [55]. In humans, TNF-α can inhibit insulin action and insulin receptor signaling in cultured adipocytes [56]. In adipose tissue, TNF-α levels are positively correlated to adipocyte size: as the cells get bigger they produce more TNF-α [57]. Production of other pro-inflammatory mediators, such as IL-6, can be stimulated by TNF-α as well [54]. Thus, TNF-α is an important pro-inflammatory cytokine leading to elevated insulin resistance and inflammation in adipose tissue.

4. Acute phase response

The acute phase response (APR) is generated during inflammation. The APR is characterized by increased number of leukocytes and changes in plasma level of acute phase proteins produced in the liver. Production of these acute phase proteins is influenced by inflammatory cytokines such as IL-6 and TNF-α. The aim of the APR is to prevent tissue damage, isolate and destroy the infectious agents and trigger the repair processes [58].

Production of several proteins is altered during the APR, including:

1) *Serum amyloid A (SAA)* belongs to the family of apolipoproteins associated with high-density lipoprotein (HDL). It is an acute-phase inflammatory protein which is predominantly produced by the liver but also by adipose tissue [59]. Levels of SAA may serve as an indicator of obesity-related inflammation [60].

2) *Plasminogen activator inhibitor-1 (PAI-1)* is highly expressed in adipose tissue and liver and is upregulated in obesity. PAI-1 can serve as a

thrombogenic mediator that leads to obesity-induced thrombogenic disorders [61].

3) *Osteopontin (OPN)* is expressed in a variety of immune cells, including macrophages, dendritic cells and lymphocytes and is also produced by the liver during inflammation. Secretion of OPN promotes recruitment of immune cells to inflammatory sites, and can also mediate cell activation and cytokine production [62].

4) *Tissue inhibitor of matrix metalloprotease-1 (TIMP-1)* is a glycoprotein which is produced mainly by activated hepatic stellate cells and Kupffer cells in the liver, and regulates various biological processes, such as cell growth, apoptosis and differentiation [63].

Levels of SAA, PAI-1, OPN and TIMP-1 are elevated during the acute-phase response. Thus, evaluation of production and expression of these mediators can serve as an indicator of the inflammatory response related to obesity.

D. <u>Galectin family</u>

Galectins are a family of soluble β-galactoside-binding lectins which share a characteristic amino acid sequence in the conserved carbohydrate recognition domains (CRDs). The 15 members can be divided into three groups depending on the different presentation of the CRD: one-CRD type, which includes galectin-1, 2, 5,7,10,11,13,14 and 15; two-CRD type, which includes galectin-4, 6, 8, 9, and 12; and a chimeric type containing a nonlectin part, galectin 3. The galectin family can bind to glycoproteins either bivalently or multivalently to cross-link cell surface

9

glycoconjugates, and in turn to trigger a cascade of transmembrane signaling pathways involved in the modulation of several cellular processes, including proliferation, differentiation, apoptosis and cytokine secretion [64].

Galectin-1 is the first member identified in the galectin family. Evidence shows that galectin-1 can function as a homeostatic agent in modulating innate and adaptive immune responses. *In vitro,* galectin-1 can control neutrophil adhesion and trafficking by impairing chemotaxis and reducing rolling and firm adhesion of neutrophils to the endothelium [65]. In addition to modulate neutrophil function, galectin-1 inhibits the antigen-presenting function and activation of macrophages. Galectin-1 also contributes to alternative activation of macrophages [66]. At high concentrations, exogenous galectin-1 decreases secretion of the pro-inflammatory cytokine IL-2 [67] and increases secretion of the anti-inflammatory cytokine IL-10 in T cells *in vitro* [68].

Galectin-9 was first cloned from Hodgkin's disease tissue. Studies demonstrate that galectin-9 induces apoptotic death of murine thymocytes [69]. Later other investigators proved that galectin-9 can induce apoptosis of Th1 cells through interaction with the Th1-specific cell-surface molecule T-cell immunoglobulin- and mucin-domain-containing molecule-3 (Tim-3) [70]. On the other hand, galectin-9 is reported to promote dendritic cell maturation [71].

Galectin-12 is expressed in adipocytes and predominantly localizes in the lipid droplets in adipose tissue. Galectin-12-deficient mice have increased lipolysis and insulin sensitivity, and reduced adiposity, with decreased number and size of adipocytes [72]. Galectin-12 is also an important mediator in adipocyte differentiation and is associated with apoptosis in adipocytes [73, 74]

Galectin-1, -9, and -12 may all be involved in adipose tissue inflammation related to obesity as our laboratory detected significantly increased levels of galectin-1 and -9 and decreased galectin-12 levels in adipose tissue during obesity [75].

E. Galectin 3

Galectin 3, a 30-kDa protein, was first identified as Mac-2, which is a cell surface antigen expressed on murine macrophages [76]. Galectin 3 is the most studied member in the galectin family. It has a single C-terminal CRD and an N-terminal domain comprising multiple repeat sequences. The flexible N-terminal domain is responsible for the formation of multimers and can cooperate with the CRD for glycan recognition [77]. As a multifunctional protein, galectin 3 is widely distributed in a variety of tissues and cells. Several studies in both animals and humans detected galectin 3 in most cell types [78]. Under *in vitro* experimental conditions, galectin 3 is predominantly detected as an intracellular protein, but it can also be found outside the cell [77]. Galectin 3 is usually present in the cytoplasm, but with certain stimuli, such as in proliferating cells and in tumor cells with apoptotic stimuli, galectin 3 can be found in the nuclear area [79, 80]. Depending on the extracellular or intracellular location, galectin 3 is involved in many biological processes, including immunomodulation, embryogenesis, host-pathogen interactions, cell migration, angiogenesis, wound healing and apoptosis [76].

11

1. Galectin 3 and inflammation

Galectin 3 is involved in many inflammatory responses. So far, most *in vitro* and *in vivo* studies indicate that galectin 3 exhibits mainly pro-inflammatory activities and triggers or amplifies inflammatory reactions in both human and animal models [77]. In animal models, no galectin 3 expression was found in normal hepatocytes in the adult rat. However, in adult rats' periportal hepatocytes with CCl_4 induced inflammation, galectin 3 expression was significantly increased [81]. In addition, galectin 3 gene inactivation reduced atherosclerotic lesions and inflammation in ApoE-deficient mice [82] and protected mice from diet-induced nonalcoholic steatohepatitis (NASH) [83]. In human studies, elevated expression of galectin 3 was detected in inflamed tissue in patients with rheumatoid arthritis and atherosclerosis [84, 85]. Galectin 3 was reported to promote neutrophil activation and act as a neutrophil adhesion molecule [86]. In galectin 3 KO mice, there was a decrease of neutrophil recruitment in the alveolar space [87]. The longevity of human neutrophils was increased after incubation with galectin 3, thus delaying spontaneous apoptosis *in vitro* [88]. In addition, evidence indicates a double role for galectin 3 in regulation of T cell function. Intracellular galectin 3 can promote T cell growth and inhibit T cell apoptosis. However, extracellular galectin 3 inhibits production of the pro-inflammatory cytokine IL-5 and cell apoptosis [89].

However, galectin 3 is also reported to have anti-inflammatory effects. There are studies indicating that galectin 3 deficiency leads to accelerated diabetic glomerulopathy [90] and diet-induced atherosclerosis in male mice [91]. Another study in mice indicates that deficiency in galectin 3 promotes hepatic injury in choline-deficient L-amino-acid-defined (CDAA) diet-induced nonalcoholic fatty liver

disease (NAFLD) [92]. Moreover, genetic delivery of galectin 3 (intratracheal instillation of plasmid DNA encoding galectin 3) suppresses airway inflammation in a model of asthma in rats [93]. In addition, galectin 3 is a negative regulator of lipopolysaccharide-mediated inflammation [94]. Macrophages from galectin 3 KO mice have decreased phagocytosis of apoptotic thymocytes [95]. Furthermore, galectin 3 is a regulator of alternative macrophage activation. Studies show that galectin 3 KO mice have reduced IL-4/IL-13-induced alternative macrophage activation. Also, recombinant galectin 3 can induce alternative activation, while IL-4-induced alternative activation is blocked by inhibitors of galectin 3 [96].

Given this evidence, galectin 3 may act as both a positive and negative regulator of inflammatory responses.

2. Galectin 3 and obesity

Serum galectin 3 is upregulated in obese subjects, and there is a positive correlation between levels of galectin 3 in blood monocytes and BMI [97]. The highest levels of galectin 3 expression were detected in type 2 diabetes patients [4]. In addition, studies also indicate that VAT may contribute to the high levels of galectin 3 observed in obese subjects [97].

Advanced glycation endproducts (AGEs) belong to the heterogeneous group of irreversible adducts [98]. Evidence shows that AGEs play a significant role in the structural and functional changes occurring in long-term diabetes and normal aging [99]. Accumulation of AGEs is enhanced with hyperglycemia in diabetes [100]. Studies using Chinese hamster ovary (CHO) cells expressing human galectin 3 proved that galectin 3 can stimulate the endocytosis of AGE and modified Low-

density Lipoprotein (LDL) [101]. In obese mice fed with a high-fat diet, serum galectin 3 was significantly increased. In addition to elevated galectin 3 levels, the AGE-binding protein galectin 3/AGE-R3 was also unregulated in the endothelium of the aorta and skeletal muscle [102]. Galectin 3 KO mice display increased glomerular accumulation of AGEs and increased oxidized-LDL when fed with an atherogenic diet [91]. Thus galectin 3 acts as a receptor for AGEs and regulates removal of AGEs.

Another study indicated that galectin 3 upregulation is associated with adipose tissue growth induced by a high-fat diet in mice, and this study also indicated that galectin 3 is mainly expressed in preadipocytes in human adipose tissue [4]. 3T3 fibroblasts can differentiate into adipocytes [103]. Both galectin 3 mRNA and protein levels were found to be increased when the quiescent 3T3-L1 cells were induced to differentiate into adipocytes [104]. Furthermore, Balb/3T3 cells with a 50% knockdown of galectin 3 had slower growth and density [105]. In addition, exogenous galectin 3 stimulated preadipocyte proliferation in primary human preadipocyte cultures [91]. All these results suggest an important role of galectin 3 in regulation of adipocyte proliferation and adiposity.

Collectively these studies indicate that increased levels of galectin 3 are associated with the increased adiposity of obesity. However, the potential role of galectin 3 in the inflammatory response and development of adiposity is still not clear.

II. AIMS AND HYPOTHESES

Specific aims and hypotheses

The main objective of this study was to determine the role of galectin 3 in the development of adiposity and inflammation in mice fed chow diet and in diet-induced obese mice.

Specific aim 1:

To compare adiposity in wild-type mice and galectin 3 KO mice.

Hypothesis 1:

Since galectin 3 stimulates preadipocyte proliferation [4] and is upregulated in adipose tissue in obese subjects [96], we hypothesize that galectin 3 deficiency would lead to decreased adiposity in mice

Specific Aim 2:

To quantify the change in levels of expression of pro- and anti-inflammatory markers in galectin 3 KO mice.

Hypothesis 2:

Since galectin 3 exhibits mainly pro-inflammatory activities and triggers or amplifies inflammatory reactions [76], we hypothesize development of reduced inflammatory responses in galectin 3 KO mice, which would result in decreased levels of pro-inflammatory markers and increased levels of anti-inflammatory markers in galectin 3 KO mice.

III. MATERIALS AND METHODS

A. <u>Animals</u>

Adult (8 weeks) male galectin 3 KO and age-matched C57BL6 wild-type (WT) mice were fed for 12 weeks with regular chow (low-fat diet, LFD, n=10 per group) or high-fat diet (HFD, containing 60% Kcal fat, from Research Diets; n=10 per group) to induce obesity (diet-induced obesity, DIO). At the end of the 12-week period, body weight was measured. Mice were then anesthetized using isoflurane and blood was collected from the retroorbital plexus. Mice were then euthanized by cervical dislocation and body composition evaluated by Dual-energy X-ray absorptiometry (DEXA).

Afterward, samples of liver, VAT and SAT were obtained and immediately frozen in liquid nitrogen until further processing. Animal studies were approved by the Animal Care and Use Committee of the University of Illinois at Chicago.

B. <u>Blood sample preparation</u>

To prepare serum, blood was collected in eppendorf tubes and allowed to clot. Samples were centrifuged at 3500 rpm for 15 minutes at 4℃. The supernatant was transferred to fresh eppendorf tubes and centrifuged again for 5 minutes. Then serum was collected, transferred again to new eppendorfs and frozen at -80℃ until use.

C. <u>Measurement of circulating mediators</u>

Serum levels of leptin, SAA, TIMP-1, OPN and APN were assessed using Enzyme-linked immunosorbent assay (ELISA) from R&D Systems and Alpco.

16

D. <u>RNA preparation</u>

1. RNA isolation

Total RNA was extracted from liver, VAT and SAT using TRIzol (Life Technologies). The tissue was homogenized in TRIzol and centrifuged at 12,000rpm for 10min at 4℃. Then the supernatant was moved to new eppendorfs and mixed with 200μL chloroform. The mixture was centrifuged at 12,000 rpm for 15 min at 4℃. The top clear layer was carefully removed to clean eppendorfs and mixed with 500 μL of isopropanol. After sitting at room temperature for 10 min (liver) or 30 min (VAT and SAT), these eppendorfs were centrifuged again at 12000 rpm for 10 min at 4℃. The RNA pellets were washed with 75% ethanol. The pellets were dried for no more than 10 min. The RNA was then diluted in 50 microliters of RNase-free water (Qiagen).

2. RNA normalization

A spectrophotometer (Nanodrop 2000, Thermo Scientific) was used to determine the RNA concentration by absorbance at 260 nanometers and to determine the purity of the RNA sample by ensuring that the A260/A280 ratio was greater than 1.8 for all samples. Samples were normalized to the same concentration of RNA depending on the lowest concentration present in each tissue. For liver, all samples were diluted to 300μL/mL. For VAT and SAT, all samples were normalized to 200μL/mL using RNAase free water.

E. Gene Expression Analysis

   Samples were analyzed by quantitative real time RT-PCR for mRNA expression

of each pro- and anti-inflammatory marker of interest. Gene expression of OPN,

SAA, TIMP-1, Galectin-1, -9, -12, CD68 and CCL2 were assessed using primers

from Applied Biosystems. TNF-α, PAI-1 and Adiponectin gene expression was

evaluated using the SYBRGREEN system. Expression of each gene was adjusted

by expression of the housekeeping gene GAPDH (SAA, OPN, TIMP-1, Galectin-1,

Galectin-9, Galectin-12, CD68 and CCL2) or β -Actin (PAI-1, TNF-α and

Adiponectin). Data obtained from RT-PCR were analyzed using the Comparative

CT method. To accurately quantify gene expression, expression of mRNA from the

gene of interest is adjusted by expression of a housekeeping gene measured in the

same sample to normalize for possible variation in the amount and quality of RNA

between different samples using the following formula:

$$\frac{X_{test}}{X_{control}} = 2^{\Delta\Delta C_T} = 2^{\left(C_{T,X} - C_{T,R}\right)_{control} - \left(C_{T,X} - C_{T,R}\right)_{test}}$$

F. Measurement of adipocyte size

   The software Image J was used to measure adipocyte size and size distribution

in VAT and SAT. For each sample, the size of 100 adjacent adipocytes in three

separate sections was collected for each tissue. Median adipocyte size was

calculated based on the values of all 100 measurements for each mouse.

G. Statistical analysis

Results are expressed as the mean±SEM. Group differences were calculated by one-way ANOVA using MedCalc software. Significance was defined as $p < 0.05$.

IV. RESULTS

A. Effects of Galectin 3 deficiency on adiposity

Serum galectin 3 levels are elevated in obese subjects [97] and galectin 3 can stimulate preadipocyte proliferation [4]. Therefore, we hypothesized that galectin 3 KO mice would have decreased adiposity due to absence of galectin 3 expression in the body, leading to reduced proliferation of preadipocytes. Body weight, % fat mass, serum leptin levels and adipocyte size in VAT and SAT were evaluated in mice fed with normal chow (lean) or HFD (DIO) for 12 weeks.

Mice fed HFD mice developed obesity and gained significantly more weight than lean mice for both WT (P<0.001) and galectin 3 KO groups (P<0.001). There were no significant differences in body weight between lean WT and lean galectin 3 KO mice. In DIO groups, galectin 3 KO mice were significantly heavier than the respective WT group (P<0.05) (Figure 1A).

The body composition was measured by DEXA and % fat mass was calculated. Mice fed with HFD had significantly elevated % fat mass compared to mice fed with regular chow (P<0.001). In lean groups, galectin 3 KO mice had 2.5-fold higher % fat mass than the diet-matched WT mice (P<0.01). In DIO groups, galectin 3 KO mice also accumulated 1.5-fold higher % fat mass than their corresponding WT mice (P<0.01) (Figure 1B).

Serum leptin levels were measured by ELISA (Figure 1C). As expected, there were significantly higher leptin levels in DIO mice compared to lean mice, both in WT and galectin 3 KO mice (P<0.001). In lean groups, galectin- 3 KO mice had about 2.4-fold higher leptin levels than diet-matched WT mice (P<0.05). In DIO groups, galectin 3 KO mice had about 1.4-fold higher leptin levels in the serum than DIO WT mice (P<0.05).

To evaluate adipocyte size, we use Image J to measure the size and size distribution of adipocytes in VAT and SAT of all mice. In SAT of galectin 3 KO groups, DIO mice had a 3.7-fold increase in median adipocyte size compared to lean galectin 3 KO mice ($P<0.001$). In WT groups, DIO mice had a 3.8-fold larger adipocyte size than lean WT mice ($P<0.001$, Figure 1D). Similar to SAT, both WT and galectin 3 KO DIO mice also had significantly increased size of adipocytes compared lean mice in VAT ($P<0.001$, Figure 1F). However, the difference between galectin 3 KO mice and the diet-matched WT mice in both VAT and SAT was not statistical significant (Figure 1D, 1F). In addition, we also evaluated adipocyte size distribution in VAT and SAT of lean and DIO mice. Results demonstrate that galectin 3 KO mice had a trend towards bigger adipocytes compared to diet-matched WT mice in SAT and VAT (Figure 1E, 1G).

In summary, both lean and DIO galectin 3 KO mice had significantly increased % fat mass and serum leptin levels compared to lean and DIO WT mice. Moreover, DIO galectin 3 KO mice had significantly increased body weight compared to DIO WT mice. Finally, there was a non-significant trend towards larger adipocytes in SAT and VAT of galectin 3 KO mice compared to WT mice. Therefore, all the data indicate that galectin 3 KO mice developed increased adiposity compared to diet-matched WT mice, which negates our initial hypothesis.
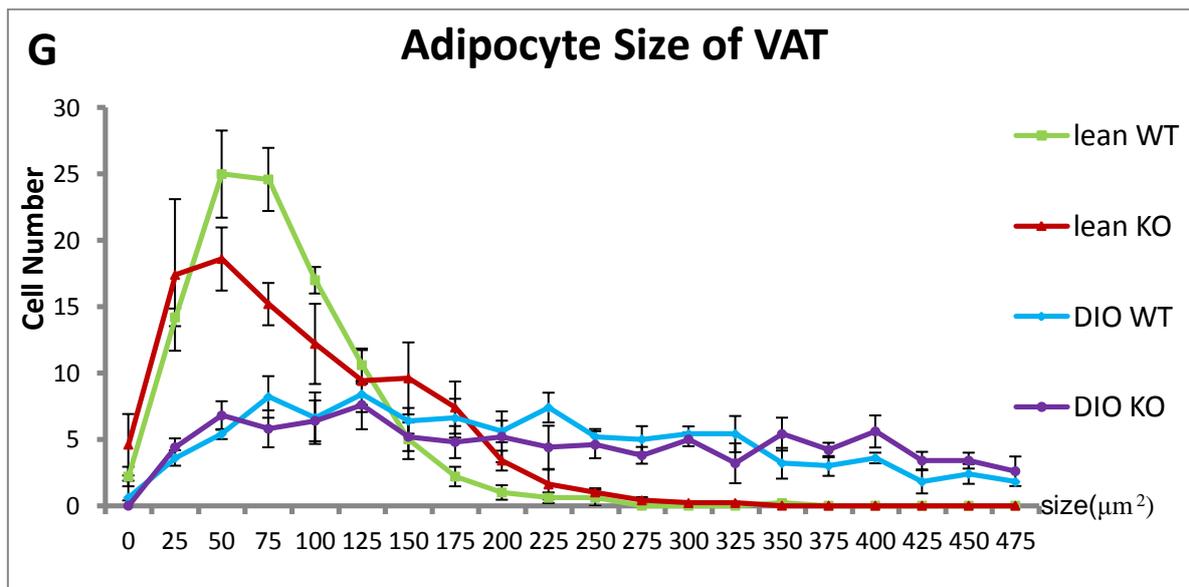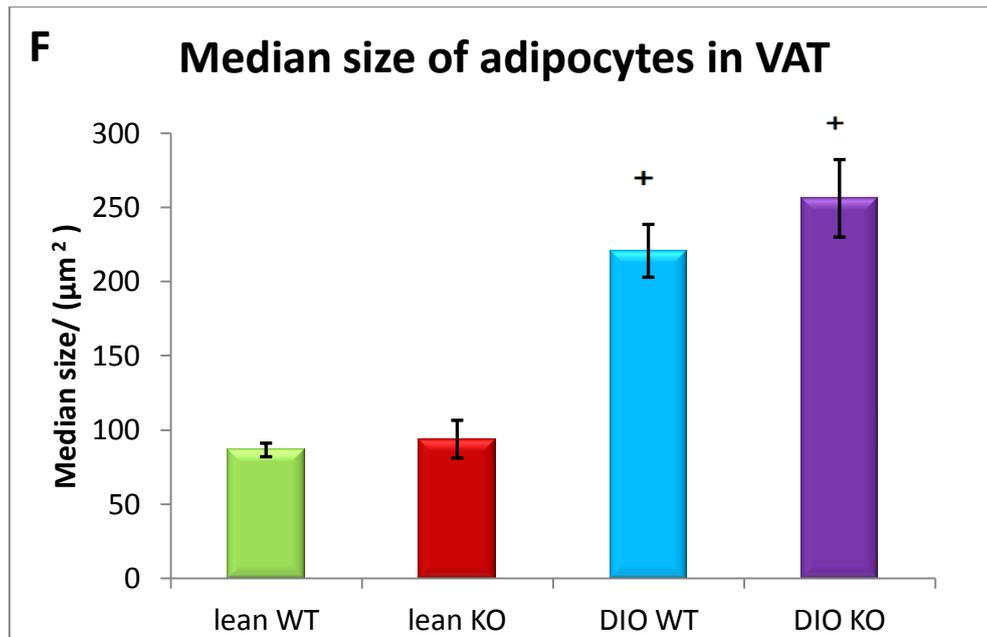
A. Body Weight
B. % Fat Mass
C. Serum Leptin

D **Median size of adipocytes in SAT**

E **Adipocyte Size of SAT**
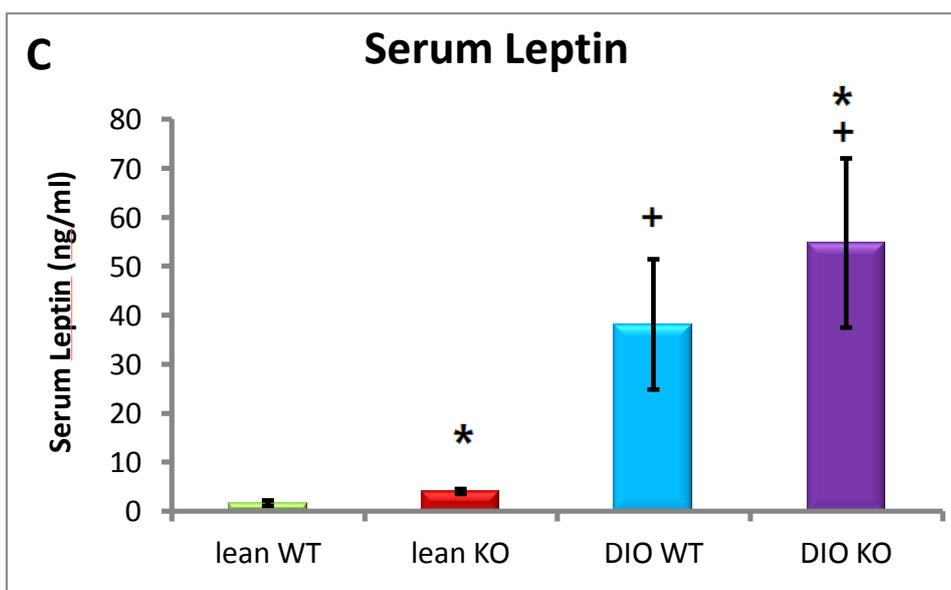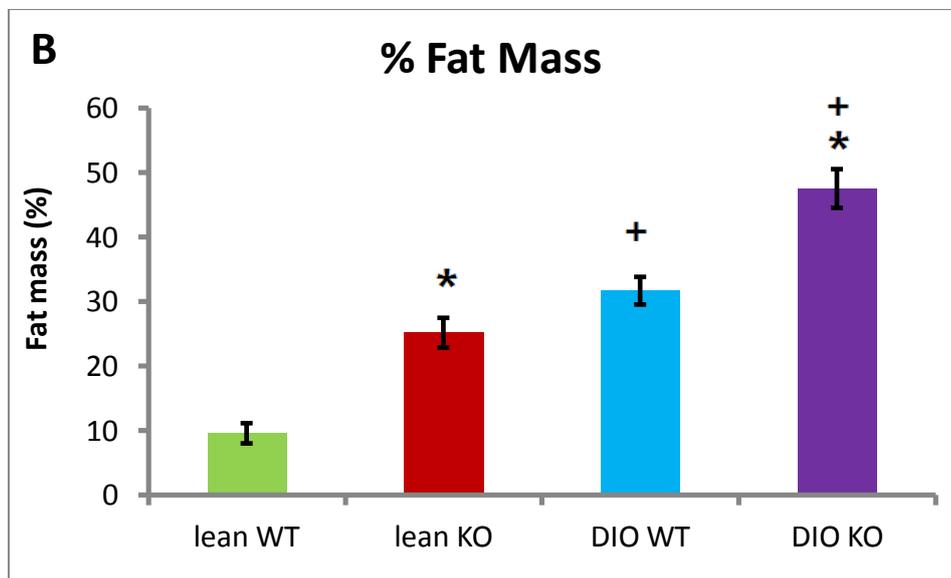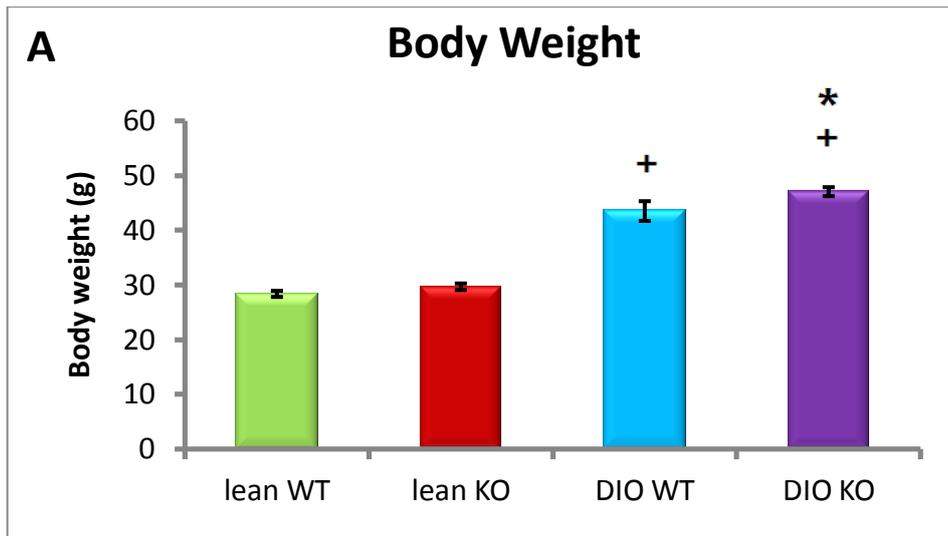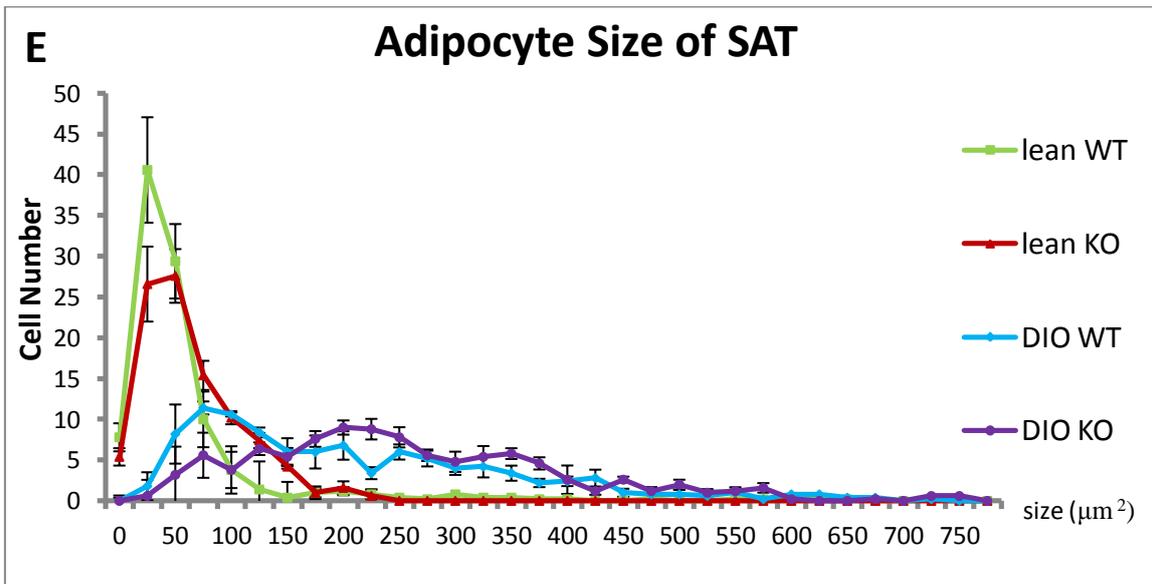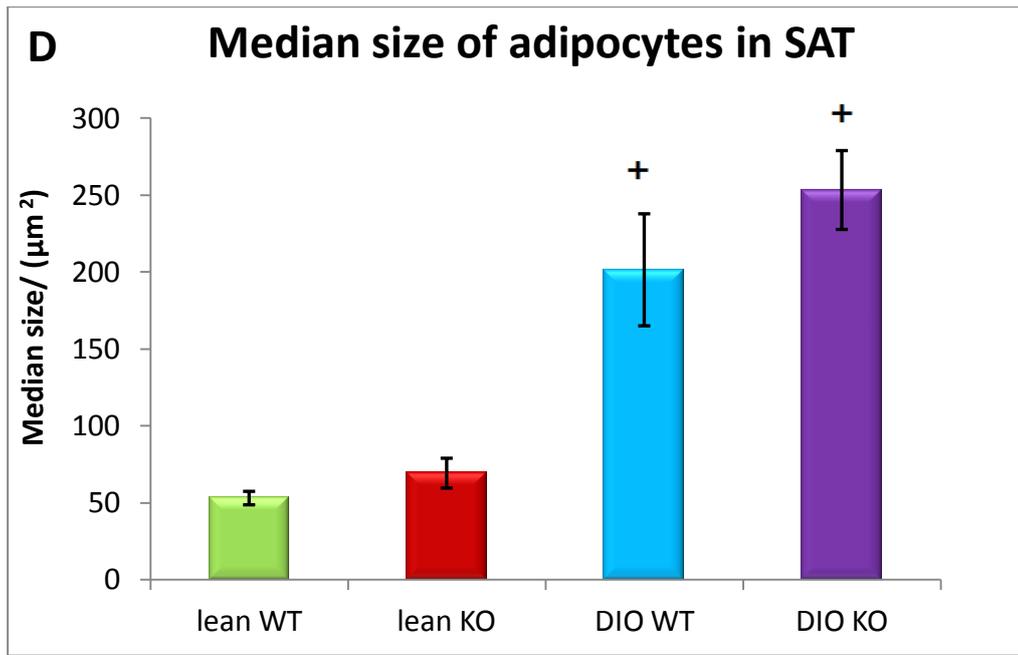
Figure. 1 Diet-induced changes of adiposity in mice. Mice were fed with regular chow (WT lean, green; galectin 3 KO lean, red) or HFD (DIO WT, blue; DIO galectin 3 KO, purple) for 12 weeks. Body weight (**A)**, % fat mass (**B**), serum leptin (**C**), median adipocyte size and size distribution of adipocytes in SAT (**D, E**) and VAT (**F, G**) were measured in WT and galectin 3 KO mice at the end of the 12-week period. Data are mean +/-SEM of 5-10 per group. *P<0.05 versus corresponding WT group; [+]P<0.05 versus lean groups by ANOVA.

B. <u>Effects of Galectin 3 KO on inflammation</u>

Most studies show that galectin 3 exhibits mainly pro-inflammatory activities and triggers or amplifies inflammatory reactions [77]. We hypothesized that galectin 3 KO mice would have a decreased inflammatory response. Serum levels of inflammatory markers were measured by ELISA. Total RNA was extracted from liver, VAT and SAT and mRNA expression was evaluated by RT-PCR.

In serum, levels of the acute phase protein SAA were significantly increased in DIO WT mice compared to the respective lean mice. In both DIO and lean groups, galectin 3 KO mice had significantly higher circulating level of SAA than WT mice (Figure 2A). There were also differences in SAA mRNA expression in the liver of the four groups. The DIO WT mice had significantly higher SAA expression in the liver than lean WT mice. However, there were no significant differences in liver SAA expression between lean and DIO galectin 3 KO mice. In both lean and DIO groups, galectin 3 KO mice showed significantly increased SAA mRNA expression compared to diet-matched WT mice (Figure 2B). Besides SAA, serum levels of APN, TIMP-1 and OPN were comparable in the four groups, although there was a trend towards higher TIMP-1 and OPN in galectin 3 lean and DIO mice compared to the respective WT groups (Table 1). Similar to the situation in serum, mRNA expression of OPN, TIMP-1 and PAI-1 trended towards an elevation in galectin 3 KO mice compared to the corresponding WT mice (Table 1).

In VAT, feeding a HFD lead to a significant increase of mRNA expression of CD68, CCL2, TNF-α and galectin-1 and reduced expression of APN and galectin-12 (Figure 2C, 2E, 2G and Table 1). Expression of CD68, which is a marker for macrophages [105], was significantly elevated in DIO galectin 3 KO mice compared

to diet-matched WT mice (Figure 2C). CCL2 is a chemokine that recruits monocytes and dendritic cells to sites of inflammation [106]. We found elevation of CCL2 mRNA expression in VAT of galectin 3 KO mice compared to their diet-matched WT group, although the difference was only significant in the DIO group (Figure 2E). Expression of APN is negatively correlated to adipose tissue inflammation [34]. In both lean and DIO mice, APN expression was significantly decreased in galectin 3 KO mice compared to diet-matched WT mice (Figure 2G). Finally, TNF-α expression was significantly increased in lean, but not in DIO, galectin 3 KO mice compared to WT mice.

Similar to VAT, mRNA expression of CD68 and CCL2 in SAT was significantly higher in DIO groups compared to lean groups (Figure 2 D, 2F). Galectin 3 KO DIO mice had significantly increased levels of CD68 and CCL2 in SAT compared to diet-matched WT mice (Figure 2D, 2F). In addition, mice in DIO groups all showed significantly higher expression of galectin-1 and galectin-9 in SAT compared to lean mice, without differences between WT and galectin 3 KO mice (Table 1).

In summary, these data indicate that galectin 3 KO mice tended to have higher inflammation in the liver, VAT and SAT compared to WT mice. Therefore, contrary to expectations, galectin 3 deficiency made the mice more inflamed in liver, VAT and SAT compared to WT mice.

A Serum SAA

B Liver SAA

C VAT CD68

D SAT CD68
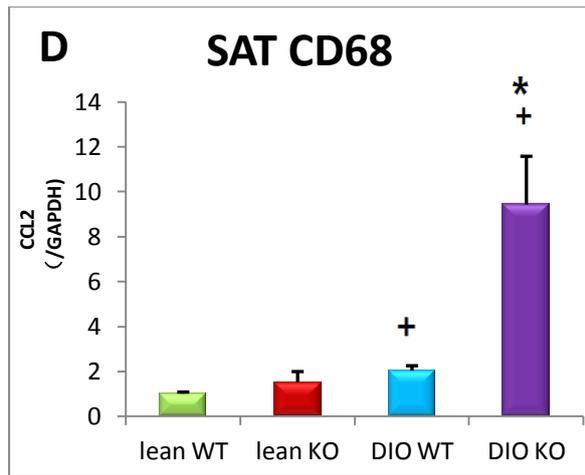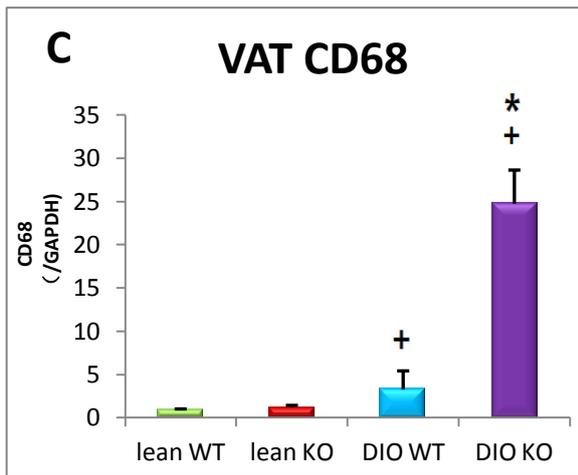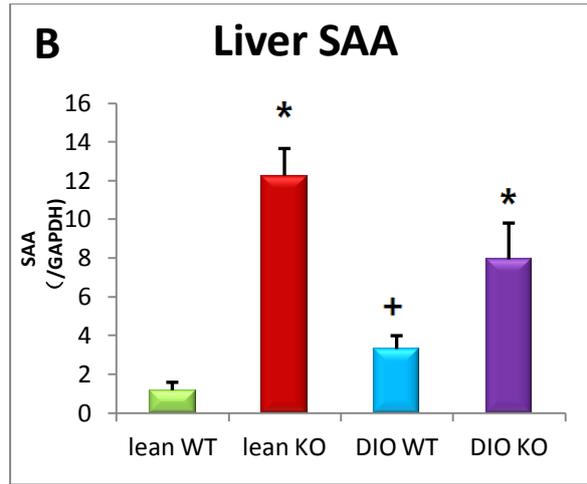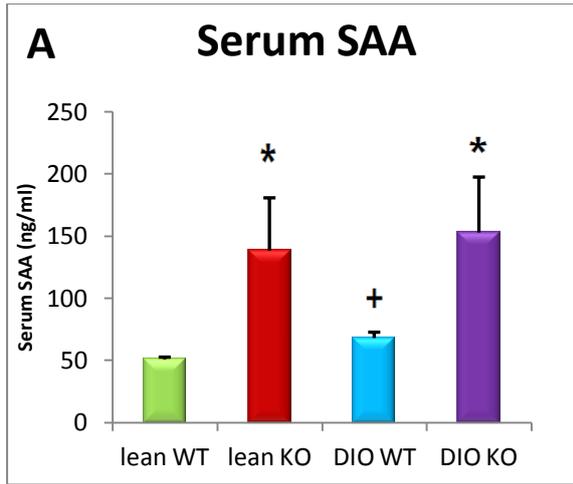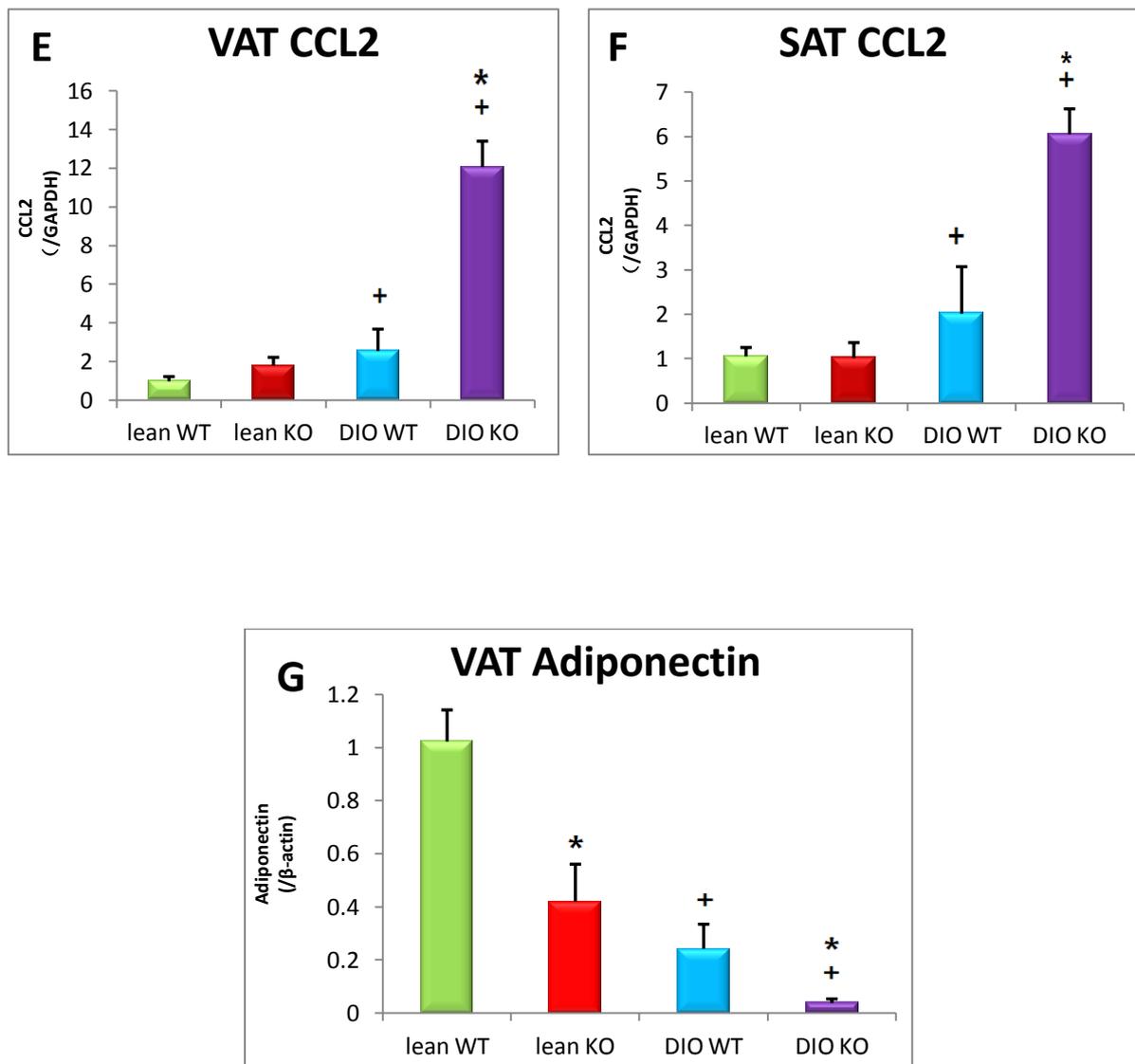
Figure. 2. Changes of inflammatory markers in serum, liver, VAT and SAT. Mice were fed with regular chow (WT lean, green; KO lean, red) or HFD (DIO WT, blue; DIO KO, purple) for 12 weeks. SAA serum protein levels (**A**) were measured by ELISA. mRNA expression of SAA in liver (**B**), CD68 (**C**, **D**) and CCL2 (**E, F**) in VAT and SAT, and Adiponectin (**G**) in VAT were evaluated by RT-PCR. Data are reported as gene expression in liver and VAT using the $\triangle\triangle^{ct}$ method. Data are mean +/-SEM of 5-10 per group. *P<0.05 versus corresponding WT group ; +P<0.05 versus lean groups by ANOVA.

Table 1. **Inflammatory markers in serum, VAT and SAT.**

	markers	lean WT	lean KO	DIO WT	DIO KO
Serum	**APN**	4.15±0.34	4.48±0.23	4.58±0.36	4.33±0.32
	TIMP-1	3.95±0.21	4.69±1.01	4.73±0.22	5.66±0.47
	OPN	57.45±5.34	78.41±15.1	47.27±15.2	66.56±12.1
Liver	**OPN**	1.10±0.24	1.06±0.24	1.09±0.16	2.07±0.73
	TIMP-1	1.31±0.22	1.24±0.20	4.21±1.84	4.27±1.88
	PAI-1	1.33±0.37	3.72±1.73	2.30±0.95	2.88±0.71
VAT	**TNF-α**	1.05±0.16	2.00±0.31*	6.96±1.65+	4.08±1.12
	Galectin-1	1.01±0.08	0.78±0.12	1.35±0.08+	1.10±0.07+
	Galectin-9	1.02±0.11	0.77±0.06	1.22±0.07	1.02±0.12
	Galectin-12	1.05±0.20	0.64±0.07	0.35±0.08+	0.24±0.07+
SAT	**TNF-α**	0.89±0.32	0.42±0.17	0.44±0.18	0.26±0.06
	Galectin-1	1.04±1.91	3.45±0.89	4.09±0.52+	5.23±0.46+
	Galectin-9	1.01±0.08	1.04±0.22	1.52±0.18+	1.74±0.12+
	Galectin-12	1.20±0.32	1.27±0.35	0.44±0.24+	0.96±0.47

Both WT and galectin 3 KO mice were fed with regular chow (lean) or HFD (DIO) for 12 weeks. Serum was obtained for measurement of APN, TIMP-1 and OPN by ELISA. RNA was extracted from liver, VAT and SAT using the TRIzol method. Expression of OPN, TIMP-1 and PAI-1 in liver, TNF-α, galectin-1, galectin-9, and galectin-12 in VAT, and TNF-α, APN, CD68, CCL2, galectin-1, galectin-9 and galectin-12 in SAT were evaluated by RT-PCR. Data are reported as gene expression in liver and VAT using the $\triangle\triangle^{ct}$ method. Data are mean +/-SEM of 5-10 per group. $^{+}P<0.05$ versus lean groups by ANOVA.

V. DISCUSSION

This study was designed to investigate the role of galectin 3 in regulation of adiposity and inflammation in mice. Galectin 3 KO mice showed altered body composition, including increased body weight, % fat mass and serum leptin levels compared to WT mice. We also found a trend towards larger adipocyte size in VAT and SAT in galectin 3 KO mice compared to the WT mice. These data demonstrate that galectin 3 deficiency changes body composition and leads to increased adiposity in mice, thus negating our hypothesis that galectin 3 deficiency would result in decreased adipose tissue accumulation.

Furthermore, we also detected increased systemic inflammation in galectin 3 KO mice compared to WT mice, as indicated by significantly higher SAA in serum and liver. Increased mRNA expression of a subset of inflammatory markers in VAT and SAT, as well as decreased APN in VAT, indicate the galectin 3 KO mice had more inflammation in adipose tissue compared to WT mice. However, mRNA expression of other markers, such as TIMP-1, PAI-1 and OPN, was not significantly elevated in galectin 3 KO mice. Nevertheless, this study demonstrates that galectin 3 deficiency leads to increased inflammation in mice, which negates our initial hypothesis that galectin 3 deficiency would decrease inflammation.

We propose three separate and non exclusive mechanisms to explain our findings:

a) The role of galectin 3 in regulating homeostasis of the intestinal barrier.

b) The role of galectin 3 in promoting M2 activation of macrophages.

c) The role of galectin 3 in binding AGE and ALE.

a) Role of galectin 3 in regulating homeostasis of the intestinal barrier. Galectin 3 levels are elevated in obese subjects [97] and studies indicate that galectin 3 stimulates proliferation of preadipocytes [4]. However, our data show that removal of galectin 3 leads to increased, rather than decreased, adiposity in mice.

Available evidence indicates that the gut microbiota and gut barrier function are related to obesity [108]. An impaired gut barrier leads to access of bacterial products (mainly LPS) from the gut microbiota into the lamina propria. When this excessive LPS reaches the liver, it may exceed the capacity of Kupffer cells to detoxify it and then enters the systemic circulation [109]. Germ-free mice fed with regular chow had 40% lower body weight than conventional mice. Once colonized with gut microbiota from conventional mice, germ-free mice gained a 57% increase in total body fat [110]. Germ-free mice were also resistant to diet-induced obesity [111]. Moreover, antibiotic treatment significantly lowered plasma LPS levels, gut permeability, and inflammation in adipose tissue as well as macrophage infiltration [112]. Furthermore, mice infused with LPS for one month to reach the same circulating levels of LPS observed in mice fed with HFD, gained the same amount of body weight and similar accumulation of intrahepatic triglyceride as mice fed a HFD [113]. These data and other evidence support the notion that LPS (and perhaps other bacterial products) from the leaky intestine lead to increased adiposity in mice. The mechanism may involve activation of the peroxisome proliferator-activated receptor-γ (PPAR-γ), which can lead to increased number of adipocytes [113], although the underlying mechanism leading to PPAR- γ activation in adipose tissue by gut-derived LPS is still not clear [115].

A recent study showed that loss of galectin 3 impairs membrane polarization of mouse enterocytes in vivo. The two characteristic markers of the brush border, β-actin and villin, were abnormally distributed on the basolateral membrane of galectin 3 KO mice, possibly leading to alterations in the gut epithelial barrier [116]. An altered epithelial barrier would increase leakage and accumulation of LPS in the lamina propria in the intestine and then the liver [117]. Evidence discussed above indicates that increased levels of LPS in the circulation lead to obesity. Thus, a possible mechanism by which galectin 3 deficiency increases adiposity may be through altered structure of intestinal epithelial cells, leading to increased leakage of LPS in the gut, which would leads to increased adiposity in galectin 3 KO mice. Furthermore, a previous study indicates that the β-galactoside carbohydrate in galectin 3 is a recognition receptor for pathogen-associated molecular patterns (PAMP) [118]. LPS is a major PAMP, which can bind to both N' and C' terminals of galectin 3 [119]. Thus, galectin 3 deficiency removes the inhibitory function of galectin 3 on LPS [94], therefore leads to increased bioactivity of LPS in galectin 3 KO mice, perhaps further increasing adipose tissue accumulation.

In addition to affecting adiposity, LPS triggers strong inflammatory responses [120]. Infusion of LPS leads to increased expression of TNF-α, IL-1, IL-6 and PAI-1 in adipose tissue and liver in mice [113]. Previous work indicates that galectin 3 KO mice showed elevated inflammatory cytokine production in response to LPS compared to WT mice, indicating that galectin 3 is a negative regulator of LPS-induced inflammation [94]. Taking these data together, the increased inflammation in galectin 3 KO mice may result from changes in the microstructure of epithelial cells, leading to increased permeability of the epithelial barrier, which

leads to greater leakage of LPS into the circulation. Without the inhibitory activity

of galectin 3 on LPS, these alterations would result in development of more

severe inflammation. Therefore, the role of galectin 3 in regulating the structure of

the epithelial barrier may explain our results both in terms of increased adiposity

and increased inflammation in the absence of galectin 3.

b) <u>The role of galectin 3 in promoting M2 activation of macrophages.</u> An

additional hypothesis for the increased inflammatory response observed in

galectin 3 KO mice involves the role of this galectin in modulating macrophage

polarization. A recent study proposed that a galectin 3 feedback loop drives

alternative macrophage activation [96]. Alternative macrophage activation is

usually associated with exposure to IL-4/IL-13 [121]. This study found that

depletion of galectin 3 or treatment with galectin 3 inhibitors can block IL-4-

mediated alternative macrophage activation. Moreover, when IL-4-mediated

alternative macrophage activation was blocked, exposure to exogenous

recombinant galectin 3 could still activate M2 macrophages [96]. In addition,

macrophages in galectin 3 KO mice were found to have defective phagocytosis of

apoptotic thymocytes [95]. With impaired phagocytosis of apoptotic cells, which

promotes inflammation, galectin 3 KO mice would develop increased

inflammation. In summary, galectin 3 KO mice may have reduced M2

macrophage activation and impaired macrophage phagocytosis of apoptotic cells,

resulting in increased inflammation in these mice.

c) <u>The role of galectin 3 in binding AGE and ALE</u>. Finally, galectin 3 acts as an

AGE receptor and also has the ability of binding modified lipoproteins and

advanced lipoxidation endproducts (ALE) [122]. Researchers found that galectin

3 KO mice developed accelerated diabetic glomerulopathy due to impaired

removal of AGEs from the renal system. They also detected increased AGE accumulation in serum of galectin 3 KO mice compared to WT mice. They indicate that galectin 3 may influence expression of other components of the AGE receptor complex and of other AGE binding proteins, in turn regulating the overall AGE receptor function [102]. In another study, galectin 3 KO mice developed accelerated lipid-induced atherogenesis, while mRNA expression of the receptor for AGE (RAGE) was higher in galectin 3 KO mice than in diet-matched WT mice [91]. Interaction of AGE with RAGE would lead to upregulation of pro-inflammatory cytokines and RAGE itself [123]. Thus, galectin 3 deficiency would impair the removal of AGE, and enhance the disposal of AGE through the pro-inflammatory pathway by binding to RAGE, as well as the accumulation of AGE, which leads to increased inflammation.

VI. CONCLUSIONS

Our studies demonstrate that galectin 3 KO mice have increased adiposity and inflammation in liver and adipose tissue. Although these results negate our initial hypotheses, this evidence indicates that galectin 3 plays an important role in the development of inflammation and obesity. To better understand the mechanisms by which galectin 3 modulates these responses, future studies should focus on the differential effects of this lectin based on its distribution and localization as well as the role of the bacterial microflora, macrophage polarization and clearance of AGE/ALE in mediating the observed effects.

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