

Galectin-3 in Obesity and Endotoxin-Induced Inflammation

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THESIS

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Chapter V and VI are unpublished manuscripts which have been submitted for review. I designed the study, collected and analyzed all data, generated all figures, and wrote the manuscripts. Dr. Giamila Fantuzzi helped design the study, analyze the data, edit the manuscripts, and provided all materials.

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LIST OF ABBREVIATIONS

AA	African-American
AGE	Advanced Glycation Endproducts
ALE	Advanced Lipoxidation Endproducts
ANOVA	Analysis of Variance
APN	Adiponectin
APR	Acute-Phase Response
APP	Acute-Phase Protein
BMI	Body Mass Index
BMM	Bone Marrow-derived Macrophages
CCL-2	Chemokine (C-C motif) Ligand 2
CD	Cluster of Differentiation
cDNA	Complementary Deoxyribonucleic Acid
CRD	Carbohydrate Recognition Domain
CRP	C-Reactive Protein
CT	Cycle Threshold
CTL	Control
CVD	Cardiovascular Disease
DEXA	Dual-energy X-ray Absorptiometry
ELISA	Enzyme-linked Immunosorbent Assay
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
HFD	High-fat Diet
IFN- γ	Interferon- γ

i.p.	Intraperitoneal
IL	Interleukin
IQR	Interquartile Range
JAK	Janus Kinase
KO	Knock-out
LBP	Lipopolysaccharide Binding Protein
LPS	Lipopolysaccharide
mRNA	Messenger Ribonucleic Acid
NF- κ B	Nuclear Factor Kappa B
N-Lac	N-acetyllactosamine
OPN	Osteopontin
PAI-1	Plasminogen Activator Inhibitor-1
PCOS	Polycystic Ovary Syndrome
PPR	Pattern-Recognition Receptor
RAGE	Receptor for Advanced Glycation Endproducts
ROS	Reactive Oxygen Species
RT-PCR	Reverse Transcription Polymerase Chain Reaction
SAA	Serum Amyloid A
SAS	Statistical Analysis System
SAT	Subcutaneous Adipose Tissue
SEE	Standard Error of Estimate
SEM	Standard Error of Mean
SOCS3	Suppressor of Cytokine Signaling 3

STAT	Signal Transducer and Activator of Transcription
TCR	T Cell Receptor
TLR	Toll-like Receptor
TNF	Tumor Necrosis Factor
T2D	Type 2 Diabetes
VAT	Visceral Adipose Tissue
WT	Wild-type

SUMMARY

The rapidly increasing prevalence of obesity and obesity-associated metabolic complications has become a significant threat not only to human health but also to the global economy. Characterized as a low-grade chronic inflammatory state, obesity causes a variety of metabolic alterations, such as increased circulation of inflammatory mediators, mild but persistent endotoxemia, activation of acute-phase responses, as well as increased infiltration of immune cells in adipose tissue. Galectin-3 is an inflammatory mediator whose levels are significantly elevated in obesity.

This study was designed to investigate the association between circulating Galectin-3 levels and markers of adiposity/inflammation in a cohort of metabolically healthy, predominantly African-American women with varying cardiovascular disease risks, as well as impact of Galectin-3 on endotoxin-induced inflammation in mice. We hypothesized that Galectin-3 would positively correlate with increased adiposity and inflammation in women and that Galectin-3 would work as an anti-inflammatory protein in obesity via its inhibiting effects on endotoxin-induced inflammation. Additionally, we also investigated the regulatory effects of endotoxin on Galectin-3 levels *in vivo* and *in vitro*.

Data show that Galectin-3 positively associates with obesity and inflammation in our study cohort, while presence of cardiovascular risk may weaken such association. Moreover, the up-regulation of Galectin-3 in obesity is possibly driven by multiple factors rather than increased adiposity alone, as elevation of Galectin-3 reaches a threshold when BMI is over 30 kg/m². However, our mouse experiments indicate that Galectin-3

specifically promotes endotoxin-induced inflammation, which negates our initial hypothesis. Therefore, based on the available studies and our own findings, we proposed that the protective role of Galectin-3 in obesity may be due to regulatory effects of Galectin-3 on other biological processes rather than on endotoxin-induced inflammation. Finally, endotoxin affects Galectin-3 by regulating its production at both the protein and gene expression levels in a complex manner.

I. INTRODUCTION

The parallel sharp rise in the prevalence of obesity and obesity-associated metabolic complications is an escalating public health concern in the United States and throughout the world [1, 2]. Obesity-associated inflammation, dysregulated glucose and lipid metabolism, multi-organ insulin resistance, and impaired gut barrier function all contribute to the development of a variety of chronic metabolic and inflammatory diseases [3]. Many underlying mechanisms for development of obesity-associated metabolic abnormalities and inflammatory complications have been proposed, including alteration of inflammatory mediators, inflammation in adipose tissue, activation of acute-phase responses, as well as mild but persistent endotoxemia [3-6].

Galectin-3 (Gal3), one of the inflammatory mediators whose levels are significantly increased in obesity, has been studied for its potential regulatory effects on adiposity and obesity-associated inflammation [7-9]. The association between Gal3 and obesity has been defined in Caucasian and Asian populations but not in other races/ethnicities, including African-Americans (AA) [10-14]. Moreover, recent studies reveal regulating effects of Gal3 on lipopolysaccharide (LPS), a strong immune response activator, in mice [15]. However, controversial data exist regarding the promoting or inhibiting role of Gal3 on lethal doses of LPS-induced inflammation [16, 17]. Therefore, questions remain on the relationship between Gal3 and obesity-associated inflammation in humans of diverse races/ethnicities as well as how Gal3 regulates LPS-induced inflammation, which may work as a potential mechanism linking elevated Gal3 levels with inflammation in obesity.

II. BACKGROUND

A. Obesity

1. Obesity epidemiology

Obesity has rapidly become a worldwide epidemic during the past few decades [18]. With no sign of abating, the prevalence of obesity has nearly doubled since 1980 [1, 18]. The World Health Organization defines obesity as a Body Mass Index (BMI= body weight (kilograms) / height² (meters²), kg/m²) greater than or equal to 30, which includes more than 600 million adults and 41 million children under age five worldwide [18]. As one of the countries with the highest prevalence of obesity, nearly 36% of the United States population is obese [2]. Besides genetic causes, obesity usually results from an imbalance between calorie intake and calorie expenditure [18]. Being obese places people at high risk of developing various complications, such as insulin resistance, type 2 diabetes (T2D), hypertension, dyslipidemia, liver steatosis, cardiovascular diseases (CVD), and certain types of cancer [19-24]. Obesity not only affects the health of a large section of the population, it also causes a heavy economic burden by raising direct annual medical care costs by \$316 billion, as well as causing countless indirect costs [25, 26]. Therefore, the rising prevalence of obesity, which leads to increased hazard health impact and economic burdens, calls for immediate attention.

2. Obesity and inflammation

Inflammation is part of the body's immediate immune response to a variety of stimuli [27]. It helps the elimination of harmful substances and protects the body from subsequent damages caused by those stimuli [27]. Many organs and immune cells are

involved in inflammation, which results in altered production and circulation of cytokines, a group of small cell-signaling proteins secreted by a broad range of cells [28].

Obesity is a chronic low-grade inflammatory state characterized by excess expansion of adipose tissue with increased size and number of adipocytes (hypertrophy and hyperplasia) [5]. Adipose tissue is primarily composed of adipocytes, as well as extracellular matrix, fibroblasts, endothelial cells and immune cells [29]. Two compartments of adipose tissue have been widely studied in obesity, visceral adipose tissue (VAT), which is the fat accumulated around the internal organs, and subcutaneous adipose tissue (SAT), which is the fat located underneath the skin [29, 30]. Expansion of VAT in obesity contributes more to the increased risk of many obesity-associated metabolic complications compared to SAT [30]. In addition to serve as a storage site for excess fat, adipose tissue also works as an active endocrine and immune organ [29]. Obesity leads to inflammation in adipose tissue with progressive immune cell infiltration and dysregulation of production of inflammatory mediators in adipose tissue [31].

The alterations of obesity-related production of inflammatory mediators, including cytokines, chemokines, and adipokines, contribute to the onset and progression of obesity-related clinical complications [5, 29, 31]. In our study, we will mainly focus on the following proteins as markers of inflammation.

- 1) Interleukin-6 (IL-6) is an important inflammatory cytokine produced by various cells, which include adipocytes and immune cells [32]. Elevation of circulating IL-6 is present in both obese humans and animals [33]. Moreover, plasma IL-6 levels are positively associated with hypertrophy of adipocytes in humans [34]. However,

controversial data exist regarding the protective or deleterious role IL-6 plays in insulin resistance and glucose dysregulation, as levels of IL-6 positively correlate with increased adiposity, systemic inflammation, and insulin resistance in obese humans and animals, while disruption of IL-6 or its hepatic signaling leads to more severe insulin resistance in mice [33-36].

- 2) Tumor necrosis factor (TNF) is a pro-inflammatory cytokine mainly produced by macrophages and lymphocytes [37]. Obesity is accompanied by TNF overexpression in adipose tissue and elevation in the systemic circulation [37, 38]. TNF induces activation of mitogen-activated protein kinases and nuclear factor kappa B (NF- κ B) signaling pathways and increased downstream inflammatory protein production, including production of TNF itself and IL-6, which in turn further amplify inflammatory responses [39, 40]. By promoting serine phosphorylation and inhibiting insulin-induced tyrosine phosphorylation of insulin-receptor substrate-1, TNF impairs the downstream effectors of insulin signaling, resulting in the inability of cells to respond to insulin in insulin-sensitive tissues and organs and contributing to the development of T2D [41].
- 3) Chemokine (C-C motif) ligand 2 (CCL-2) recruits macrophages, monocytes, and dendritic cells to inflammatory sites [42]. Obesity induces up-regulation of CCL-2, which promotes infiltration of immune cells in adipose tissue, followed by increased inflammatory cytokine production from those immune cells and advanced inflammation [43].
- 4) Adiponectin (APN) is an important insulin-sensitizing and anti-inflammatory protein produced by adipocytes [44]. Significantly down-regulated APN levels are observed

in obese individuals, as well as patients with T2D and cardiovascular complications [45, 46]. Several studies have indicated reciprocal inhibiting effects of APN and TNF, as up-regulation of TNF in obesity suppresses APN secretion, while APN inhibits TNF production in macrophages and dendritic cells [47-49].

- 5) Leptin, a protein predominantly secreted by adipocytes, plays an important role in mediating the balance between food intake and energy expenditure in healthy non-obese humans and animals [50]. The high levels of leptin present in obesity are accompanied by a diminished or abolished ability of leptin to regulate the balance of energy intake and expenditure, a condition known as leptin resistance [50, 51]. Contrary to the changes of APN in obesity, elevated circulating leptin levels parallel the increased adiposity and positively correlate with the size and number of adipocytes in humans [50, 52].

Together, obesity-associated aberrant adipose tissue expansion, advanced immune cell infiltration, and dysregulated production of inflammatory mediators are all key contributors to the pathogenesis of obesity-associated metabolic and inflammatory complications [5].

3. Acute-phase response

As a central element of innate immunity, the acute-phase response is provoked in response to different stimuli, such as infection and obesity-associated inflammation [53]. During the acute-phase response, hepatic production of acute-phase proteins (APPs) is significantly up-regulated and these proteins are released into the systemic circulation [53, 54]. In our study, we will mainly focus on the following APPs for evaluation of acute-phase responses.

- 1) C-reactive protein (CRP), the first APP described, has been widely used as a prognostic marker of CVD risk [55, 56]. CRP binds to phosphocholine on the surface of damaged or dead cells, enhancing phagocytosis of those harmful particles by macrophages [56]. In response to increased inflammation and cytokine production, particularly immune cell-produced IL-6, hepatic production of CRP is significantly enhanced, which in turn leads to elevated CRP in the blood [57]. Three categories of circulating CRP have been defined to reflect CVD risk: <1 $\mu\text{g/mL}$ (low CVD risk), 1-3 $\mu\text{g/mL}$ (moderate CVD risk) and >3 $\mu\text{g/mL}$ (high CVD risk) [55].
- 2) Serum amyloid A (SAA) is a major inflammatory APP produced by the liver in response to various stimuli [58]. Hepatic mRNA expression and systemic circulation of SAA are significantly elevated in many inflammatory conditions, including obesity, bacterial infections, and atherosclerosis [59-61]. SAA performs its immunomodulatory functions by promoting the recruitment of immune cells and production of inflammatory cytokines [59]. Three isoforms of SAA are present in mice, SAA-1 and SAA-2 are primarily expressed and produced by liver, whereas SAA-3 is induced in many tissues, such as colon and adipose tissue [62, 63].
- 3) Hepcidin is predominantly produced by the liver and regulates iron homeostasis by reducing iron absorption by enterocytes as well as release of iron from macrophages [64]. Obesity-associated inflammation and bacterial and viral infections all induce increased hepatic production of hepcidin, which lowers iron availability in many inflammatory conditions [65]. There are two isoforms of hepcidin in mice [66]. Hepcidin-1 has been reported to play a more important role in regulation of iron homeostasis and inflammation than hepcidin-2 [67].

- 4) Plasminogen activator inhibitor-1 (PAI-1) production is highly up-regulated in obesity, T2D, atherosclerosis, and bacterial infections [68-70]. Synthesized mainly by adipose tissue and liver, overexpression of PAI-1 is attributed to the increase production of TNF and oxidative stress in obese humans and animals [71, 72]. PAI-1 inhibits fibrinolysis and promotes thrombogenic processes that lead to development of thrombosis in many diseases [73].

In summary, elevation of APPs is associated with increased inflammation in many diseases [53, 54]. Therefore, quantification of these proteins serves as a marker of inflammation.

4. Macrophages

Macrophages are a type of immune cell that engulf and digest foreign particles and cell debris [74]. Their precursor monocytes are produced from hematopoietic stem cells in the bone marrow and released into the circulation to seed in different tissues throughout the body, and then further differentiate into a variety of subpopulations depending on their tissue location, such as adipose tissue macrophages, Kupffer cells in the liver, osteoclasts in the bone and alveolar macrophages in the lungs [74-76]. Macrophages express various cell surface markers. Among those markers, F4/80 is expressed on most mouse tissue macrophages and widely used in the study of macrophage physiology and pathology in mice [76]. At the steady state, without activation, tissue resident macrophages mainly perform a surveillance function as they closely monitor the invasion of foreign substances as well as signs of tissue damage. Once activated, in addition to phagocytosis of harmful particles, macrophages also release a large amount

of cytokines to promote the inflammatory process, and eventually alert and activate the adaptive immune system by presenting antigens to the helper T cells [76-78].

Two subsets of macrophages are categorized based on their functions on inflammation: the classically activated macrophages, which promote inflammation and production of pro-inflammatory cytokines such as TNF and IL-6, and the alternatively activated macrophages, which help the resolution of inflammation [79]. Obesity leads to increased infiltration of macrophages in adipose tissue and greatly changes the subset composition of macrophages, as numbers of classically activated macrophages are significantly increased, while counts of alternatively activated anti-inflammatory macrophages are reduced [79, 80]. According to Weisberg et al, the accumulation of adipose tissue macrophages is positively correlated with body mass and adipocyte size in mice and with BMI in humans. Based on their estimate, in lean mice and normal weight humans there are less than 10% macrophages of total cells in adipose tissue, while this number goes up to more than 50% in leptin-deficient (*ob/ob*) mice with extreme obesity and around 40% in obese humans [81]. Along with increased percentages of macrophages, there are also significantly increased levels of cytokines released from those macrophages in adipose tissue, such as TNF, IL-6 and CCL-2, all of which are associated with increased insulin resistance and lead to impaired glucose disposal and enhanced triglyceride lipolysis in adipose tissue, resulting in development of hyperinsulinemia, hyperglycemia, and hyperlipidemia [82, 83]. Besides adipose tissue, increased numbers of islet-associated macrophages are also detected in the pancreas of T2D patients, obese mice induced by high-fat diet (HFD) feeding and diabetic leptin-receptor deficient (*db/db*) mice [84]. Taking all this evidence into

consideration, macrophages play a key role in obesity-induced inflammation and metabolic complications.

B. Lipopolysaccharide

Lipopolysaccharides (LPS) are major components of the outer membrane of gram-negative bacteria [15]. Also known as endotoxin, LPS works as a strong activator of the immune system and triggers inflammation [15, 85]. As one major type of pathogen-associated molecular pattern, LPS binds to the toll like receptor 4 (TLR4) expressed on the surface of phagocytes, such as macrophages and dendritic cells, and transduces signals via either MyD88-dependent or MyD88-independent pathways to activate the NF- κ B transcriptional system, as well as upregulates the levels of Mitogen-activated protein kinase and the transcriptional factor active protein-1 [85]. LPS-induced phagocyte activation also promotes the downstream production of oxidants and inflammatory cytokines, including IL-6, TNF and IL-1, which subsequently exacerbate the cascade of immune responses [86]. Evidence has shown that blocking activation of the NF- κ B pathway reduces production of oxidants such as reactive oxygen species, as well as the levels of inflammatory cytokines, including IL-6, TNF and IL-1, in LPS-treated macrophages and dendritic cells [87, 88]. Moreover, administration of LPS also induces hepatic acute-phase responses, which is indicated by the dramatically up-regulated gene expression of APPs, including SAA-1, PAI-1, and hepcidin-1 in response to LPS [89, 90]. Along with elevation of hepatic and circulating APPs, LPS also causes alterations of hematological parameters in peripheral blood, including leukopenia (decrease of leukocyte counts), neutrophilia (increase of neutrophil counts),

lymphopenia (decrease of lymphocyte counts), and monocytosis (increase of monocyte counts) [89].

Obesity causes a mild but persistent elevation of circulating LPS levels in both humans and rodents, which may contribute to development of low-grade inflammation and metabolic complications such as insulin resistance [91]. In rodent models, both HFD fed Wild-type (WT) mice and normal chow diet-fed *ob/ob* mice have increased circulating LPS levels compared to their control groups [91, 92]. In addition, HFD significantly changes the composition of the microbiota in the mouse gut and increases gut permeability, which favors the release of LPS from the gut into the circulation [92]. Meanwhile, antibiotic treatment of HFD mice or *ob/ob* mice significantly decreases both plasma and cecal levels of LPS and ameliorates insulin resistance [91]. Similar to rodents, both non-diabetic obese subjects and T2D patients have higher plasma LPS levels compared to healthy lean controls [93, 94]. Moreover, a long-term combined lifestyle therapy (diet, exercise and psychological interventions), which effectively reduces BMI (15%) and fat mass (33%) in obese adolescents (BMI > 95th percentile), significantly decreases their endotoxin levels by 21%, with improved insulin sensitivity as assessed by the homeostasis model assessment index for insulin resistance (HOMA-IR, 41%) [95]. Given all this evidence, obesity-induced mild and persistent elevation of circulating LPS levels may play a role in obesity-related metabolic complications.

C. Galectin-3

Galectin-3 (Gal3) belongs to a family of soluble proteins that recognize and bind to β -galactoside sugars on the cell surface [9]. As the only chimeric type in the galectin family, Gal3 has a single C-terminal carbohydrate recognition domain (CRD) and an N-terminal domain that facilitates glycan recognition by the CRD [8, 9]. As a multifunctional protein, Gal3 plays a broad spectrum of roles in regulation of many biological processes, including immunity, embryogenesis, wound healing, angiogenesis, neurogenesis, and apoptosis [7-9]. For example, Gal3 binds to endogenous glycans, specifically cell surface β -galactosides and N-acetyllactosamine (N-Lac), and modulates intracellular signaling pathways related to cell activation, proliferation, and apoptosis [7, 8]. Moreover, Gal3 also acts a scavenger molecule for glucose and lipid adducts, including advanced glycation end-products (AGEs) and advanced lipoxidation end-products (ALEs) [96].

Gal3 is detected in most tissues and cell types in both animals and humans, with presence not only on the cell surface but also in the cytoplasm and extracellular spaces and the circulation [7, 8, 97]. Gal3 can be present on the cell surface and intracellularly, both in the cytoplasm and nuclear area, and also be secreted into extracellular spaces such as systemic circulation [98, 99]. Depending on the localization, Gal3 may perform different functions. For example, cytoplasmic Gal3 is a well-defined anti-apoptotic protein through its binding ability to the apoptosis regulation molecule Bcl-2, while, on the other hand, nuclear Gal3 promotes apoptosis in human prostate cancer cells [97, 98].

1. Galectin-3 in inflammation

Gal3 is involved in many inflammatory responses, and upregulation of this protein has been detected in various diseases in humans and experimental animals [7, 8]. For example, there is high expression of Gal3 in the inflammatory tissues of patients with rheumatoid arthritis, atherosclerosis, asthma and certain types of cancer [100-103]. Although various studies confirmed the large amount of Gal3 produced in different inflammatory states, the role Gal3 plays in inflammation is dependent on its cellular location, cell types involved, and pathophysiological conditions [97]. In general, Gal3 is considered as a powerful pro-inflammatory mediator. Exogenous Gal3 stimulates the oxidative burst and release of superoxide anions from human peripheral blood neutrophils and monocytes [97, 104]. Gal3 also promotes neutrophil activation and acts as a neutrophil adhesion molecule to laminin and endothelial cells [105]. Disruption of the Gal3 gene in mice alleviates peritoneal inflammatory responses induced by injection of thioglycollate broth, as indicated by decreased numbers of macrophages and neutrophils in the peritoneal cavity of Gal3 knock-out (KO) mice compared to their matched WT mice [106, 107]. Moreover, Gal3 deficiency leads to decreased severity of disease in rodent models of pneumococcal pneumonia and inflammatory bowel disease [108, 109]. Pharmacological inhibition of extracellular Gal3 by N-Lac, which inhibits Gal3 by binding to its CRD with high affinity, protects rodents against hypertensive nephropathy and myocardial fibrosis [110, 111].

While more and more evidence points to the pro-inflammatory functions of Gal3, there are some studies revealing that Gal3 may also work as an anti-inflammatory mediator. Genetic delivery of cDNA encoding Gal3 suppresses airway inflammation in a model of

asthma in rats [112]. Moreover, Gal3 KO mice are more susceptible to intraperitoneal infection with the protozoan parasite *Toxoplasma gondii* compared to WT mice [113]. Our laboratory also detected increased systemic inflammation and adipose tissue inflammation in HFD Gal3 KO mice compared to their matched WT groups [114]. In fact, controversial data exist in several rodent models of obesity, atherosclerosis, renal and liver diseases regarding the pro- and anti-inflammatory roles Gal3 plays (**Table I**). Thus, given this evidence, Gal3 may play both pro- and anti-inflammatory roles in inflammatory responses.

TABLE I. RESPONSE OF GALECTIN-3 KO MICE IN MODELS OF METABOLIC DISEASE

Model	Strains and treatments	Outcome of Gal-3 KO mice compared to WT mice	Reference
Obesity and insulin resistance	WT and Gal3 KO mice on chow or HFD	Increased adiposity and inflammation; Impaired glucose metabolism	[114, 115]
Atherosclerosis	WT and Gal3 KO mice on atherogenic diet	Increased severity	[116]
	Apo-E KO and Apo-E/Gal3 KO on chow or high cholesterol diet	Decreased severity	[117, 118]
Renal disease	WT and Gal3 KO mice with age-, chemical- or diet-induced glomerular lesions	Increased severity	[119-122]
	WT and Gal3 KO mice with ischemia-reperfusion renal injury	Decreased severity	[123]
Liver disease	WT and Gal-3 KO mice with age- or diet-related NAFLD/NASH	Increased severity	[124-126]
	WT and Gal3 KO mice with age-related NAFLD/NASH	Decreased severity	[127]

2. Galectin-3 and endotoxin

In 1996, Mey's group first proved that different strains of LPS could bind to Gal3. For example, LPS from *Klebsiella pneumoniae* binds to the CRD part of Gal3 via its β -galactoside-containing side-chains, while LPS from *Salmonella minnesota* interacts with the N-terminal domain of Gal3 via its lipid A/inner core region [128].

As mentioned before, LPS is a strong activator of immune responses and activates a variety of immune cells, such as neutrophils, monocytes and macrophages [89].

Fermino et al. reported that, by interacting with LPS via both the N- and C-terminal parts, Gal3 can greatly increase the binding efficacy of LPS to the neutrophil surface and result in enhanced neutrophil activation *in vitro*. Also, pre-treatment with Gal3 increases the ability of LPS to induce septic shock in Balb/C mice [16]. These data indicate that Gal3 serves as a promoter in LPS-mediated neutrophil activation and inflammatory responses.

Macrophages are the major Gal3-producing cells and they play a significant role in both innate and adaptive inflammatory responses [7, 78]. Several studies regarding the role Gal3 plays in LPS-stimulated inflammation specifically in macrophages were also conducted. Li et al. demonstrated that Gal3 is a negative regulator of LPS-mediated inflammation in murine bone-marrow derived macrophages (BMM), supported by data indicating that, in response to LPS, there is increased production of cytokines (IL-6, IL-12 and TNF) and reduced NF- κ B signaling in cells from Gal3 KO mice compared to those from WT mice. Also, exogenous Gal3 reduces LPS-induced cytokine production. Li et al. also found that Gal3 KO mice in a C57BL6 background are more susceptible to LPS-induced endotoxin shock compared to WT mice [17].

Thus, due to the opposite results obtained when using different cell types *in vitro* and mouse strains *in vivo*, the question of whether Gal3 works as a negative or positive regulator of LPS-induced inflammation is still unsettled.

While Gal3 mediates LPS-induced inflammation, administration of LPS also affects Gal3 levels both *in vivo* and *in vitro*. Several studies have been done to investigate regulation of Gal3 production in response to LPS stimulation [17, 89, 105, 129]. Data have shown that administration of LPS affects circulating Gal3 levels in WT mice and released Gal3 levels from murine BMM *in vitro*, with peak levels of Gal3 at 2h-post LPS treatment and then decreasing with time [17, 89], while surface expression of Gal3 in mouse peritoneal macrophages is down-regulated in response to LPS [105]. Moreover, co-treatment with Interferon (IFN)- γ and LPS decreases Gal3 expression in BMM lysates [129]. Thus, the direction of LPS-induced alteration of Gal3 production may rely on the localization of Gal3 and the phenotype of macrophages.

Moreover, both humans and animals with obesity-related chronic inflammation have slightly elevated but mild and persistent LPS levels in the circulation [91, 93, 130]. In HFD WT mice, the circulating level of LPS is about 0.02 ng/mL. However, the two *in vivo* studies regarding the regulatory role of Gal3 on LPS-induced inflammation both chose to use high lethal doses of LPS (23 mg/Kg body weight and 6.2-25 mg/Kg body weight) to induce septic shock in mice, which may not represent the mild endotoxemia of obesity [16, 17]. Therefore, to better understand the regulation and functions of Gal3 in a low-grade chronic inflammatory state with mild LPS elevation similar to those observed in obesity, experiments of low dose and repeated administration of LPS should be conducted.

3. Gal3 in obesity and obesity-associated metabolic complications

Both murine and human adipose tissue synthesize Gal3 [131]. Compared to SAT, higher Gal3 protein level is detected in VAT, which contributes more to development of obesity-related metabolic complications [12, 132]. Recombinant human Gal3 stimulates the proliferation of primary human preadipocytes *in vitro* [131]. Cellular Gal3 levels in monocytes isolated from blood of overweight and T2D donors are significantly elevated compared to those from normal weight subjects, with the highest levels detected in T2D donors' cells. Treatment with the antidiabetic protein APN reduces Gal3 levels in monocytes from normal weight donors, but not in cells from T2D patients [133].

In addition, various animal studies have provided solid evidence that Gal3 plays a key role in modulating the pathophysiological changes during obesity [114, 115, 132]. In the HFD-induced obese mouse model, Gal3 KO mice develop accelerated adiposity and significantly increased adipose tissue mass with higher leptin compared to their matched WT mice, while no differences of food intake are observed between the two genotypes [114]. Moreover, impaired glucose metabolism and hyperglycemia were also confirmed in both lean and obese Gal3 KO mice [114, 115]. Finally, Gal3 KO mice exhibit pronounced systemic inflammation and pancreatic inflammation versus their WT counterparts when fed a HFD [114, 115]. All these observations indicate a significant role of Gal3 in modulating obesity and obesity-associated metabolic complications in mice.

Several human studies have been conducted to investigate circulating levels of Gal3 in different populations. Increased serum levels of Gal3 are present in obese and T2D subjects as compared to non-obese healthy individuals [10, 12]. As shown in **Table II**,

evidence from those studies indicates that Gal3 levels are associated with adiposity (BMI and Waist/hip ratio) and markers of inflammation (CRP and IL-6). However, these associations were lost in populations with pathological conditions such as T2D and polycystic ovary syndrome (PCOS) [11]. Obesity increases risk of developing cardiovascular complications [20]. As one of the proteins significantly elevated in obesity, the association between Gal3 and CVD has also been widely studied and Gal3 has emerged as a new biomarker for CVD risk [13, 14, 134]. In a Caucasian population, plasma Gal3 levels are correlated with a variety of CVD risk factors, including blood pressure, serum lipids, and renal function [14]. In addition, adverse outcomes in CVD patients are associated with elevation of Gal3 in the circulation [135].

In summary, current studies confirm the association between Gal3 levels and obesity in certain populations, and pathological conditions such as T2D and PCOS may disturb this association. However, most studies conducted so far only included Caucasian and Asian populations. Thus studies focusing on the association between Gal3, obesity, and inflammation in subjects with racial/ethnic diversity are missing.

D. Summary

Collectively, up-regulation of Gal3 is present in both obesity-associated and LPS-induced inflammation, while development of obesity is accompanied with mild but persistent elevation of LPS. Therefore, Gal3 may perform modulatory effects in the progression of obesity-associated inflammation via its regulation of LPS-induced inflammation.

TABLE II. HUMAN STUDIES OF CIRCULATING GALECTIN-3 LEVELS IN OBESITY AND OBESITY-ASSOCIATED METABOLIC COMPLICATIONS

Study	Study design	Ethnicity	Gender	Results	Ref
Weigert et al. 2009	Normal weight (NW, N=23); Overweight (OW, N=30); Type 2 Diabetes (T2D, N=30)	Caucasian	Male	1. Serum Gal3 was significantly higher in T2D and OW subjects compare to NW subjects; 2. Serum Gal3 was comparable between the T2D and OW subjects; 3. Serum Gal3 was positively correlated with BMI, waist/hip ratio, IL-6 and leptin, but not with fasting blood glucose, levels of CRP, LDL or APN.	[12]
	T2D (N=100)	Caucasian	Male (55%) Female (45%)	1. Serum Gal3 was similar between males and females; 2. Serum Gal3 was not correlated with BMI, levels of CRP, IL-6, LDL, hemoglobin duration of diabetes; 3. Serum Gal3 was negatively correlated with HbA1c.	[12]
Boer et al. 2011	General population from Prevention of RENal and Vascular ENd-stage Disease cohort, with median follow-up of 10 years, measurements were taken at baseline (N=7968)	Caucasian	Male (49.8%) Female (50.2%)	1. Plasma Gal3 was positively correlated with BMI, waist/hip ratio, renal functions, fasting blood glucose, levels of CRP, LDL and triglycerides, while negatively correlated with HDL levels; 2. Female subjects have significantly higher Gal3 levels compared to the male subjects; 3. The percentages of population who developed T2D were significantly different between Gal3 quintiles, with highest number in the top Gal3 quintile (Q5).	[14]
Yilmaz et al. 2014	Polycystic ovary syndrome (PCOS, N=56) Healthy control (N=41)	Not specified, Most likely Caucasian	Female	1. Women with PCOS have significantly higher serum Gal3 compared to the healthy controls; 2. Serum Gal3 was correlated with fasting blood insulin and HOMA-IR, but not with BMI or CRP levels.	[11]
Jin et al. 2013	T2D (N=284)	Asian	Male (58%) Female (42%)	Serum Gal3 levels were associated with increased odds of heart failure, nephropathy, and peripheral arterial disease.	[10]

III. AIMS AND HYPOTHESES

A. Rationale

Elevation of Gal3 at both circulating and tissue gene expression levels is present in obesity [12, 114, 132]. Current available human studies have only provided evidence that Gal3 associates with obesity and obesity-related metabolic complications in the Caucasian and Asian populations [10, 12]. Therefore, a study focusing on a population composed of different races/ethnicities should be conducted to fill this gap.

Moreover, a mild but persistent elevated circulating level of endotoxin is associated with obesity and obesity-related metabolic complication [91, 93]. Gal3 binds to LPS and regulates LPS-induced inflammation, while LPS affects Gal3 levels [16, 17, 89, 105, 129]. Hence, the increased Gal3 of obesity may work as an adaptive mechanism to counteract obesity-associated metabolic complications via its regulatory effects on LPS-induced inflammation. However, many questions remain regarding the role Gal3 plays in LPS-induced inflammation. Controversial data exist regarding the promoting or inhibiting effects of Gal3 on endotoxin shock in mice, [16, 17, 105, 129]. Furthermore, the effects of Gal3 on acute inflammation induced by non-lethal doses of LPS in mice has not been studied yet. Additionally, no data are currently available regarding the role of Gal3 in regulating inflammation caused by repeated injection of ultra-low dose LPS, which represents the actual concentration of LPS observed during endotoxemia in obesity. Finally, available studies indicate that administration of LPS significantly up-regulates circulating Gal3 levels in mice *in vivo* and released Gal3 protein levels from murine BMMs *in vitro* [17, 89]. However, LPS-induced alteration of Gal3 gene

expression *in vivo* and *in vitro* have not been studied. Therefore, studies should be conducted to elucidate: 1) The effects of Gal3 deficiency on non-lethal doses of LPS-induced inflammation *in vivo* and *in vitro*; 2) The regulatory effects of LPS on Gal3 protein and mRNA expression levels; 3) The effects of Gal3 deficiency on repeated administration of ultra-low dose of LPS-induced inflammation in mice.

B. Specific aims and hypotheses

The main objective of this study was to investigate the relationship between Gal3 and obesity-associated inflammation in humans and the role Gal3 plays in LPS-induced inflammation in mice.

Specific aim 1:

To investigate the changes of circulating Gal3 levels in obesity as well as the association between levels of Gal3 and markers of adiposity and inflammation in a cohort of predominantly African-American and mostly obese healthy women.

Hypothesis 1: Women with higher BMI will have higher serum levels of Gal3.

Hypothesis 2: Women with higher circulating levels of the inflammatory markers IL-6 and CRP will have higher serum levels of Gal3.

Specific aim 2:

To investigate the reciprocal regulation of Gal3 and LPS in mice.

Hypothesis 1: Since Gal3 KO mice are more susceptible to endotoxin shock, and BMMs from Gal3 KO mice produce more cytokines compared to BMMs from WT mice [17], we hypothesize that deficiency of Gal3 will aggravate acute inflammation induced by non-

lethal doses of LPS in mice, as Gal3 KO mice will have higher markers of inflammation in the circulation with increased hepatic acute-phase responses.

Hypothesis 2: Since LPS administration up-regulates circulating Gal3 *in vivo* and released levels from mouse BMM [17, 89], we hypothesize that LPS alters production of Gal3 at both the mRNA and the protein levels in mice *in vivo* and mouse peritoneal macrophages *in vitro*.

Specific aim 3:

To investigate the effect of Gal3 deficiency in the response to repeated injections of ultra-low doses of LPS in mice.

Hypothesis: Since Gal3 KO mice showed increased severity and mortality in response to LPS-induced septic shock compared to WT mice [17], and Gal3 deficiency augments obesity and obesity-associated inflammation in mice [114], we hypothesize that higher inflammation and more severe metabolic abnormalities will be present in Gal3 KO mice in response to repeated injections of ultra-low dose LPS that mimics the mild endotoxemia of obesity .

IV. RELATIONSHIP OF GALECTIN-3 WITH OBESITY, IL-6, AND CRP IN WOMEN

(Previously published as Pang, J., V.T. Nguyen, D.H. Rhodes, M.E. Sullivan, C. Braunschweig, and G. Fantuzzi, *Relationship of galectin-3 with obesity, IL-6, and CRP in women*. J Endocrinol Invest, 2016.)

Abstract

Purpose: To evaluate the association of Galectin-3 (Gal3) with obesity and inflammatory status in a cohort of metabolically healthy, predominantly African American women with varying cardiovascular disease (CVD) risk as determined by CRP levels.

Methods: We assessed the association between BMI and serum levels of Gal3, IL-6, CRP and adiponectin in metabolically healthy women (N=97) to determine the overall association between Gal3, obesity and inflammation in groups at different CVD risk.

Results: Obese women had significantly higher serum Gal3 compared to non-obese participants ($P=0.0016$), although Gal3 levels were comparable among different classes of obesity. BMI ($R^2=0.1406$, $P=0.0013$), IL-6 ($R^2=0.0689$, $P=0.035$) and CRP ($R^2=0.0468$, $P=0.0419$), but not adiponectin, positively predicted the variance of Gal3 levels in the total study population. However, the predicting effect of BMI ($R^2=0.2923$, $P=0.0125$) and inflammation ($R^2=0.3138$, $P=0.038$) on Gal3 was only present in women at low/moderate risk of CVD ($CRP \leq 3 \mu\text{g/mL}$).

Conclusions: Gal3 is positively correlated with obesity and inflammation in women, while the presence of elevated CVD risk may disturb the strength of Gal3 as a biomarker of inflammation.

Introduction

The prevalence of obesity has nearly doubled worldwide since 1980 [136, 137]. The World Health Organization defines obesity as Body Mass Index (BMI) equal to or greater than 30 kg/m^2 with three sub-categories: obese class I (BMI: 30.00 kg/m^2 - 34.99 kg/m^2), obese class II (BMI: 35.00 kg/m^2 - 39.99 kg/m^2) and obese class III (BMI $\geq 40.00 \text{ kg/m}^2$) [136]. More than one third of US adults are obese, with 6.6% categorized as obese class III [137]. Obesity increases risk of developing various conditions, including insulin resistance and diabetes, dyslipidemia, liver steatosis, hypertension, cardiovascular disease (CVD) and certain types of cancer [5, 138-140]. The World Health Organization estimates that being overweight (BMI: 25.00 kg/m^2 ~ 29.99 kg/m^2) or obese contributes to causing approximately 44% of diabetes, 7–41% of certain cancers and 23% of ischemic heart disease worldwide [141].

Obesity is characterized by excess expansion of adipose tissue, with increased size and number of adipocytes and a progressive immune cell infiltration into adipose tissue [29]. Production of many cytokines and adipokines is altered in obesity, contributing to metabolic complications and diseases, at least in part through induction of a chronic low-grade inflammatory state [142]. For example, reduction of adiponectin (APN) and elevation of leptin production by adipocytes is likely a key contributor to the pathogenesis of insulin resistance and Type 2 Diabetes (T2D) in obesity [143]. Moreover, adipocyte hypertrophy is negatively correlated with APN levels and positively associated with circulating levels of inflammatory cytokines, including interleukin-6 (IL-6) and tumor necrosis factor in obese subjects [34]. In response to increased production of inflammatory cytokines – particularly IL-6 - from the immune cells that infiltrate adipose

tissue, circulating levels of the hepatic acute-phase reactant C-reactive protein (CRP) are elevated in obese subjects [56]. Numerous studies confirm the role of CRP as a prognostic marker of CVD risk [144]. Ridker et al. defined three levels of CRP as predictors of CVD risk: <1 $\mu\text{g/mL}$ (low CVD risk), $1\text{--}3$ $\mu\text{g/mL}$ (moderate CVD risk) and >3 $\mu\text{g/mL}$ (high CVD risk) [55]. In summary, altered production of adipokines, cytokines, and other mediators during obesity leads to development of chronic inflammation, which in turn contributes to development of obesity's co-morbidities.

Galectins are a family of soluble β -galactoside-binding lectins that share an amino acid sequence in the conserved carbohydrate recognition domain [145]. Galectin-3 (Gal3), a unique member of the galectin family with a single C-terminal carbohydrate recognition domain and an N-terminal domain, is present in most tissues and cell types, with wide distribution on the cell surface and the cytoplasm as well as release into extracellular spaces and the circulation [9, 99]. A multifunctional protein, Gal3 is involved in many biological processes, including immunomodulation, embryogenesis, host-pathogen interactions, cell migration, angiogenesis, wound healing, and apoptosis [8].

A significant elevation of Gal3 levels is present in humans and experimental animals with various metabolic disturbances and pathologies, such as obesity, T2D, rheumatoid arthritis, atherosclerosis, asthma, and certain types of cancer [97, 100-103]. So far, most *in vitro* and *in vivo* studies indicate that Gal3 exhibits mainly pro-inflammatory activities and triggers or amplifies inflammatory reactions in several animal models, including experimental heart failure, renal diseases, and autoimmune disorders [110, 123, 146]. However, some researchers also reported anti-inflammatory effects of Gal3 in models of atherosclerosis, non-alcoholic fatty liver disease, and asthma [112, 116,

125]. Given this evidence, depending on the pathology and the precise experimental conditions, Gal3 may act as either a positive or a negative regulator of inflammatory responses.

Both murine and human adipose tissue synthesizes Gal3, with predominant expression in the stromovascular fraction compared to mature adipocytes [131]. Increased serum and monocyte-associated levels of Gal3 are present in obese and T2D subjects and experimental animals as compared to non-obese controls [12, 114, 132, 133]. In men, Gal3 levels positively correlate with adiposity (BMI and waist/hip ratio) and markers of inflammation (IL-6 and CRP) [12, 133]. In addition, recombinant Gal3 stimulates proliferation of human preadipocytes *in vitro*, suggesting a potential role for Gal3 in adipose tissue expansion during obesity [131].

In a study conducted in a Caucasian population, plasma Gal3 levels were not only correlated with BMI and CRP, but also associated with a variety of CVD risk factors, including blood pressure, serum lipids, and renal function [14]. This study also revealed gender differences in serum Gal3 levels, with women having higher Gal3 levels than men and the association between Gal3 and CVD risk factors being stronger in women than in men [14]. In addition, a negative correlation between Gal3 levels and diastolic function has been reported in an obese class III cohort [13]. Finally, serum Gal3 levels are also excellent prognostic markers in patients with heart failure [134]. However, studies focusing on the association between Gal3, obesity, and inflammation in subjects of diverse races/ethnicities are still missing.

In this study, we assessed the association between Gal3, obesity, markers of inflammation and CVD risk as defined by CRP levels in a cohort of predominantly

African American and mostly obese healthy women. We hypothesized that circulating Gal3 levels would positively correlate with adiposity and inflammatory markers in this population.

Methods

1. Study population

Data were obtained from premenopausal women (N=97) included in the “Vitamin D, inflammation and relations to insulin resistance in morbidly obese pre-menopausal women” study (n=76) [147] and the “Modulation of the IL-6 system and STAT-3 activation in lymphocytes of lean and obese women” study (n=23). These studies were conducted at the University of Illinois at Chicago and approved by the relevant Institutional Review Board. Informed consent was obtained from all individual participants included in the study. All serum assays reported in the present study were measured by our laboratory or obtained from participants’ medical records. Women were eligible if they were premenopausal, free of diabetes, significant medical or inflammatory conditions, not pregnant and English-speaking.

Demographic and medical information including sex, race/ethnicity, age, date of serum sample, height, weight, serum levels of IL-6, CRP, and APN were obtained from the primary investigators of the original studies. The subjects’ height was assessed using a fixed stadiometer. Weight was measured using an electronic scale and accurate to 0.1 kg. BMI was calculated using the average of height and weight triplicate readings following the Quetelet’s index: weight (kg)/height (m²) [148].

2. Quantification of biomarkers

Blood samples were collected from overnight-fasted subjects as part of the original studies; samples were centrifuged and serum collected and stored –at 80°C until retrieval for analysis. Serum concentration of Gal3, IL-6, CRP, and APN was evaluated in duplicate using ELISA kits from R&D Systems, Minneapolis, MN and Alpco, Salem, NH.

3. Statistical Analysis

Descriptive statistics were used to describe basic demographic, biochemical and anthropometric measures. All continuous and categorical variables were expressed as number of subjects and percentage, respectively. Variables were explored for outliers (mean \pm 3SD). Normal distribution was determined by the Shapiro-Wilk test and the normality probability plot. Normally and non-normally distributed data are expressed as mean \pm SD or median (IQR), respectively. Square root transformation was used to achieve normality in non-normally distributed variables (Gal3, IL-6, and CRP).

Spearman correlation test was conducted to explore the association between all continuous variables. After adjustment for age and race, ANOVA and analysis of covariance were performed to evaluate the differences of anthropometric characteristics and biomarkers in women categorized in three obesity groups: non-obese (BMI<30kg/m²), obese class I&II (BMI: 30-40kg/m²) and obese class III (BMI \geq 40kg/m²). To evaluate the influence of adiposity and inflammation on Gal3 levels, multiple linear regression models were built using Maximum R-Square selection.

The study population was then dichotomized into low/moderate versus high CVD risk

groups based on serum CRP levels: low/moderate risk of CVD (CRP ≤ 3 $\mu\text{g/mL}$) and high risk of CVD (CRP > 3 $\mu\text{g/mL}$). Differences in anthropometric characteristics and biomarker levels between groups of low/moderate and high risk of CVD were assessed by independent Student's t-test (continuous variables) and Chi-square test (categorical variables). Additional correlation tests and multiple linear regression modeling were conducted to evaluate the association between Gal3, IL-6, and CRP with all other anthropometric characteristics and biomarker levels.

A value of $P < 0.05$ was considered statistically significant. All statistical analyses was performed using SAS 9.3 (SAS Inc., Cary, NC, USA).

Results

Anthropometric characteristics and levels of biomarkers of the women meeting eligibility criteria (N=97) are presented in Table III. The average subject was 36.4 ± 8.8 years old, with a BMI of 45.0 ± 12.0 kg/m^2 . Most women were categorized as class III obesity (N=68, 70.1%) and AA (N=60, 61.8%). Median (IQR) Gal3 level was 5.3 (2.3-10.0) ng/mL , which is comparable with values reported in the literature [12, 14].

Correlations between serum levels of Gal3 and anthropometric characteristics and biomarkers were examined in the whole study cohort. Gal3 positively correlated with BMI ($r=0.40$, $P=0.0003$), CRP ($r=0.22$, $P=0.04$), and IL-6 ($r=0.26$, $P=0.012$). However, Gal3 levels were not significantly different among race/ethnicity groups and no association was noted with APN (data not shown).

Table III ANTHROPOMETRIC CHARACTERISTICS AND BIOMARKERS OF THE STUDY COHORT

Variable	Mean \pm SD /Median (IQR) (N=97)
Age (years)	36.4 \pm 8.8
Age distribution [n (%)]	
< 30 y	23 (23.7)
30 y to 40 y	39 (40.2)
\geq 40 y	35 (36.1)
BMI (kg/m²)	45.0 \pm 12.0
BMI category [n (%)]	
< 30 non-obese	10 (10.3)
30 to < 40 Obese Class I & II	19 (19.6)
\geq 40 Obese Class III	68 (70.1)
Race/Ethnicity [n (%)]	
Caucasian	12 (12.4)
African American	60 (61.8)
Hispanic	19 (19.6)
Other	6 (6.2)
IL-6 (pg/mL)	3.3 (2.0 – 4.6)
CRP (μg/mL)	4.3 (2.7 – 6.7)
APN (μg/mL)	3.9 (2.5 – 6.4)
Galectin-3 (ng/mL)	5.3 (2.3 – 10.0)

Mean \pm SD (normally distributed variables), median and Interquartile Range (IQR) (non-normally distributed variables) were assessed for continuous variables.

We then categorized our study cohort into three groups based on obesity status (Table IV). Overall there were significantly more African American (AA) obese than lean women ($P=0.0107$), and women in the obese class III group were younger than those in the non-obese and obese class I&II groups ($P=0.0004$). The inflammatory markers IL-6,

CRP, and Gal3 were all significantly higher in obese class I&II and class III groups as compared to the non-obese group. However, levels of CRP and Gal3 were comparable between the two obese classifications, while IL-6 was significantly elevated in women with class III obesity compared to those in the class I&II group ($P<0.0001$). Due to the significant differences in age and race (AA) between the three obesity status groups, ANOVA analysis adjusted for age and race (AA) was also conducted; differences in IL-6, CRP and Gal3 levels between groups were maintained ($P<0.0001$, $P=0.0067$ and $P=0.0093$ for IL-6, CRP and Gal3, respectively).

Table IV ANTHROPOMETRIC CHARACTERISTICS AND BIOMARKERS OF WOMEN WITH DIFFERENT OBESITY STATUS¹

Variable	Non-obese (BMI<30kg/m ²)	Obese Class I&II (BMI: 30-40kg/m ²)	Obese Class III (BMI≥40kg/m ²)	P
N	10	19	68	
Age (years)	42.8 ± 12.2	41.0 ± 8.6	34.2 ± 7.4	0.0004 ^{b,c}
BMI (kg/m ²)	22.2 ± 4.1	36.4 ± 3.2	50.8 ± 8.4	<0.0001 ^{a,b,c}
Race [n (%)]				0.0107 ²
Non-AA	2 (20)	8 (42)	21 (31)	
AA	8 (80)	11 (58)	47 (69)	
IL-6 (pg/mL)	0.8 (0.4-1.1)	2.6 (1.4-2.8)	4.0 (2.9-5.1)	<0.0001 ^{a,b,c}
CRP (µg/mL)	0.8 (0.4-2.4)	4.7 (2.3-7.2)	4.8 (2.9-7.0)	0.0023 ^{a,b}
APN (µg/mL)	5.4 (3.6-7.4)	3.1(2.2-6.0)	3.8 (2.5-6.4)	0.7707
Gal3 (ng/mL)	0.5 (0.4-2.8)	3.4 (2.1-9.0)	7.4 (4.1-10.4)	0.0016 ^{a,b}

¹: Non-normally distributed variables have been transformed to normal for one-way ANOVA; AA: African American

^a: Non-obese vs Obese Class I&II,

^b: Non-obese vs Obese Class III,

^c: Obese Class I&II vs Obese Class III;

²: Chi-square test was used for the categorical variable Race (AA).

Due to the high collinearity between BMI, IL-6, and CRP levels, separate multiple regression models were utilized to explore the relationship between Gal3 and adiposity (BMI) as well as between Gal3 and inflammation (IL-6 and CRP). All models were adjusted for age and race (AA). Adiposity and inflammation significantly predicted Gal3 level, with BMI explaining 14.06% of the variance of serum Gal3 levels ($P=0.0013$). On the other hand, the inflammatory markers IL-6 and CRP accounted for 6.89% ($P=0.035$) and 4.68% ($P=0.0419$) of the variance of Gal3, respectively (Table V).

TABLE V MULTIPLE LINEAR REGRESSION MODELS FOR EVALUATION OF POTENTIAL CONFOUNDERS PREDICTING CIRCULATING GAL3 LEVELS IN THE STUDY COHORT¹

Models*	β (SEE)	P	R²
Gal3 = BMI		0.0008	0.1478
Intercept	1.28 (0.89)		
BMI (kg/m ²)	0.04 (0.01)	0.0013	0.1406
Gal3 = IL-6		0.0235	0.1028
Intercept	2.23 (0.81)		
IL-6 (pg/mL)	0.54 (0.25)	0.035	0.0689
Gal3 = CRP		0.0225	0.0826
Intercept	2.83 (0.70)		
CRP (μ g/mL)	0.35 (0.17)	0.0419	0.0468

*All models include age and race (AA).

¹: Non-normally distributed variables have been transformed to normal for evaluation of the multiple linear regression analysis.

β , parameter estimate; SEE, standard error of estimate.

Table VI presents results with participants dichotomized into low/moderate risk of CVD (CRP \leq 3 μ g/mL, N=32) versus high risk of CVD (CRP>3 μ g/mL, N=65). The groups did not differ by race. However, not surprisingly, BMI was significantly higher (P=0.0002) and there were significantly more class III obese women (P=0.0038) in the high compared to the low/moderate CVD risk group. Also, significantly higher levels of IL-6 (P=0.0001) and lower levels of the anti-diabetic adipokine APN (P=0.0063) were present in the high risk group. However, Gal3 levels were not significantly different between the two groups.

TABLE VI ANTHROPOMETRIC AND METABOLIC CHARACTERISTICS OF WOMEN AT LOW/MODERATE AND HIGH RISK OF CVD¹

Variable	Low/Moderate risk of CVD CRP \leq 3 μ g/mL	High risk of CVD CRP > 3 μ g/mL	P
N	32	65	
Age (year)	37.5 \pm 11.8	35.9 \pm 6.8	0.4715
BMI (kg/m ²)	38.6 \pm 12.3	48.2 \pm 10.7	0.0002
BMI category [n(%)]			0.0038²
< 30 non-obese	8 (25)	2 (3)	
30 to < 40 Obese Class I & II	5 (16)	14 (22)	
\geq 40 Obese Class III	19 (59)	49 (75)	
Race/Ethnicity [n(%)]			0.3945 ²
Caucasian	6 (19)	6 (9)	
African American	18 (56)	42 (65)	
Hispanic	5 (16)	14 (21)	
Other	3 (9)	3 (5)	
IL-6 (pg/mL)	2.2 (1.0-3.7)	3.9 (2.7-4.8)	0.0001
CRP (μ g/mL)	2.1 (0.7-2.6)	3.3 (2.3-5.2)	<0.0001
APN (μ g/mL)	5.5 (3.5-7.9)	3.3 (2.3-5.2)	0.0063
Gal3 (ng/mL)	4.7 (2.3-8.7)	7.0 (2.4-10.2)	0.2687

¹: Comparison of differences between the two groups was calculated by independent t test for continuous variables;

²: Chi-square test was performed for comparison of categorical variables.

Next, the correlation between serum Gal3 and each of the other parameters in the two CVD risk groups were explored separately. Similar to the findings in the entire study population, Gal3 was positively correlated with BMI ($r=0.65$, $P<0.0001$), IL-6 ($r=0.51$, $P=0.0034$) and CRP ($r=0.45$, $P=0.0120$) in the group at low/moderate risk of CVD. However, when women had high risk of CVD, the correlation between Gal3 and inflammation (IL-6 and CRP) was lost, while the strength of the correlation with BMI was diminished ($r=0.28$, $P=0.0318$). When adjusted for age and race (AA), multiple linear regression models indicated that Gal3 was significantly predicted by adiposity ($R^2=0.2923$, $P=0.0125$) and inflammation ($R^2=0.3138$, $P=0.038$) only in the group of women at low/moderate risk of CVD but not in the group with high CVD risk (Table VII).

TABLE VII MULTIPLE LINEAR REGRESSION MODELS FOR EVALUATION OF POTENTIAL CONFOUNDERS PREDICTING CIRCULATING GAL3 LEVELS IN WOMEN AT LOW/MODERATE AND HIGH RISK OF CVD¹

	Low/Moderate risk of CVD CRP ≤ 3 $\mu\text{g/mL}$ N=32			High risk of CVD CRP > 3 $\mu\text{g/mL}$ N=65		
Models*	β (SEE)	P	R ²	β (SEE)	P	R ²
Gal3 = BMI		0.0067	0.3591		0.24	0.0935
Intercept	-0.33 (0.75)			2.09 (1.38)		
BMI (kg/m^2)	0.05 (0.02)	0.0125	0.2923	0.03 (0.02)		
Gal3 = CRP		0.038	0.3138		0.46	0.0801
Intercept	0.76 (1.09)	0.49		2.47 (1.34)		
Age (years)	-0.02 (0.02)	0.17		-0.04 (0.03)		
CRP ($\mu\text{g/mL}$)	0.91 (0.52)	0.09		0.37 (0.33)		
APN ($\mu\text{g/mL}$)	0.11 (0.07)	0.1		0.23 (0.30)		
AA	0.70 (0.42)	0.11		0.06 (0.39)		

*All models include age and race (AA).

¹: Non-normally distributed variables have been transformed to normal for evaluation of the multiple linear regression analysis.

β , parameter estimate; SEE, standard error of estimate; AA, African American.

The CVD risk and inflammatory marker CRP showed a comparable pattern of correlation as Gal3 did, with BMI ($R^2=0.5130$, $P<0.0001$) and IL-6 ($R^2=0.3557$, $P=0.0016$), significantly and positively predicting serum CRP levels only in women at low/moderate risk of CVD but not in those with high CVD risk (Table VIII). Additionally, BMI significantly affected IL-6 levels regardless of CVD risk status ($R^2=0.4947$, $P<0.0001$ for women at low/moderate risk of CVD, and $R^2=0.3223$, $P<0.0001$ for women at high risk of CVD). Among women at low/moderate CVD risk, inflammation positively predicted serum IL-6 levels ($R^2=0.3557$, $P=0.0016$), whereas it had no effect in those at high CVD risk (Table IX). Finally, we did not find any significant correlation between APN and other parameters in either group (data not shown).

TABLE VIII MULTIPLE LINEAR REGRESSION MODELS FOR EVALUATION OF POTENTIAL CONFOUNDERS PREDICTING CIRCULATING CRP LEVELS IN WOMEN AT LOW/MODERATE AND HIGH RISK OF CVD¹

Models*	Low/Moderate risk of CVD CRP ≤ 3 $\mu\text{g/mL}$ N=32			High risk of CVD CRP > 3 $\mu\text{g/mL}$ N=65		
	β (SEE)	P	R^2	β (SEE)	P	R^2
CRP = BMI		<0.0001	0.5713		0.64	0.0597
Intercept	-0.21 (0.33)			2.12 (0.59)		
BMI (kg/m^2)	0.03 (0.01)	<0.0001	0.5130	0.3e-03(0.01)		
CRP = IL-6		0.0047	0.4733		0.39	0.0711
Intercept	0.77 (0.29)	0.0137		1.97 (0.47)		
IL-6 (pg/mL)	0.15 (0.04)	0.0016	0.3557	0.03 (0.04)		

*All models include age and race (AA).

¹: Non-normally distributed variables have been transformed to normal for evaluation of the multiple linear regression analysis.

β , parameter estimate; SEE, standard error of estimate.

TABLE IX MULTIPLE LINEAR REGRESSION MODELS FOR EVALUATION OF POTENTIAL CONFOUNDERS PREDICTING CIRCULATING IL-6 LEVELS IN WOMEN AT LOW/MODERATE AND HIGH RISK OF CVD¹

Models*	Low/Moderate risk of CVD CRP ≤3 µg/mL N=32			High risk of CVD CRP > 3 µg/mL N=65		
	β (SEE)	P	R ²	β (SEE)	P	R ²
IL-6 = BMI		0.0001	0.5327		<0.0001	0.4025
Intercept	-1.78 (0.84)	0.0466		-0.21 (1.02)		
BMI (kg/m ²)	0.11 (0.02)	<0.0001	0.4947	0.11 (0.02)	<0.0001	0.3223
IL-6 = CRP		0.0012	0.5348		0.45	0.0812
Intercept	-0.01 (1.23)			3.45 (1.84)		
CRP (µg/mL)	2.16 (0.61)	0.0016	0.3557	0.37 (0.45)		

*All models include age and race (AA).

¹: Non-normally distributed variables have been transformed to normal for evaluation of the multiple linear regression analysis.

β, parameter estimate; SEE, standard error of estimate.

Discussion

Our study revealed an association between circulating Gal3 levels and markers of adiposity and inflammation in a cohort of predominantly obese and African American metabolically healthy women. Similar to studies evaluating Gal3 in Caucasian populations [12, 14], we found that Gal3 levels were significantly higher in obese women compared to non-obese participants. Additionally, increased adiposity (BMI) and inflammation (IL-6 and CRP) had a positive and significant influence on Gal3 levels in our study population. However, when dichotomized by CVD risk, adiposity and inflammation only positively predicted Gal3 levels in subjects at low/moderate risk of CVD.

Studies conducted in the Caucasian population indicate Gal3 levels are positively correlated with BMI or waist/hip ratio and inflammation [12, 14]. We now extend these findings by demonstrating a significant correlation between Gal3 and BMI, as well as between Gal3 and the inflammatory markers IL-6 and CRP, in a multi-racial cohort of metabolically healthy but for the most part obese women. Multiple linear regression models further confirmed adiposity (BMI) and inflammation (IL-6 and CRP) as positive predictors on the variance of serum Gal3 levels. To our best knowledge, our study is the first to provide evidence of a correlation between Gal3 and obesity in women of diverse racial/ethnic backgrounds. Although we did not observe differences in Gal3 levels among race/ethnic groups, our study was statistically underpowered to detect such differences, preventing us from drawing definitive conclusions on this aspect of the results.

Gal3 levels were higher in women with any degree of obesity compared to non-obese subjects. However, women in obesity class I&II had comparable levels of Gal3 compared to those in obesity class III, though the former had a lower degree of inflammation, as indicated by their lower circulating IL-6 levels. These findings indicate that Gal3 levels do not differ between women with different degrees of obesity. Indeed, Gal3 levels appear to plateau at a BMI of 30, after which no further upregulation of Gal3 would be expected with increased adiposity in obese women without metabolic complications. A previous study conducted in Caucasian men also failed to detect significant differences in serum Gal3 levels between overweight subjects and T2D patients, even though individuals with T2D had a higher BMI compared to the healthy overweight group [12]. Collectively, these findings suggest that the elevation of

circulating Gal3 levels in obesity or obesity-related metabolic complications may be driven by multiple factors rather than a simple increase in adiposity.

In recent years, Gal3 has emerged as a biomarker for CVD risk [135, 149]. Indeed, elevated Gal3 is associated with CVD risk factors such as serum lipids, blood pressure, and renal function, while also correlating with adverse outcomes in patients with heart failure [13, 14, 134, 135]. In our study, we separated the whole cohort into two CVD risk groups based on CRP levels. Since more obese class III women fell into the high CVD risk group, this group had higher BMI and inflammatory status compared to the women in the low/moderate CVD risk group. However, serum Gal3 levels were comparable between the two CVD risk groups. This is likely due to both groups being comprised of obese subjects, especially obese class III and, as mentioned above, Gal3 levels not being significantly different by obesity class.

In addition, we evaluated the correlation between Gal3 and various parameters in the two CVD risk groups separately. In the low/moderate CVD risk group, correlations between Gal3 and BMI, IL-6 and CRP were similar to those found for the entire sample, suggesting Gal3 acts as an excellent marker of obesity and obesity-related inflammation in women at low/moderate CVD risk and relatively low inflammatory status. However, Gal3 levels were no longer associated with either adiposity (BMI) or inflammation (IL-6 and CRP) in women at high CVD risk, suggesting a potential inflammation-induced dysregulation of circulating Gal3 levels in these women and/or a plateau effect on Gal3 once the threshold level of 3 µg/ml. Weigert et al. and Yilmaz et al. also reported loss of correlation between Gal3 and parameters of adiposity and inflammation in Caucasian

individuals with T2D or polycystic ovary syndrome [11, 12], indicating that the presence of significant metabolic, hormonal, and/or inflammatory complications disrupts the association between obesity and circulating Gal3.

To better characterize the inflammatory status in women at high CVD risk, we further evaluated two additional biomarkers, IL-6 and CRP. We found IL-6 positively associated with adiposity (BMI) but not with inflammation (CRP) in women at high CVD risk. However, CRP correlations were similar to those we found for Gal3. Indeed, among women with high CRP, and therefore high risk of CVD, CRP completely lost its association with both adiposity (BMI) and inflammation (IL-6).

Finally, we did not find any significant correlation between circulating levels of APN and Gal3, in agreement with the study performed in Caucasian men [12]. However, a previous *in vitro* study reported downregulation of monocytic Gal3 levels by APN in cells obtained from normal-weight donors [133]. This disagreement may result from different localization of Gal3 (monocytic *versus* serum Gal3) as well as the possibility that APN and Gal3 may interact differently *in vitro* and *in vivo*.

Our study has several strengths. First, this is the first study focusing on the association between Gal3, obesity, and inflammation in women who are predominantly African American. Second, our study included various parameters of inflammation, presenting comprehensive evidence linking Gal3 with the increased inflammation of obesity. Third, our study is the first to provide evidence for a possible adiposity-related threshold of Gal3 increase in women. Finally, we present novel information of how Gal3 associates with obesity and inflammation in metabolically healthy women at different risks of CVD.

Our study also has limitations. First, the study was restricted to women, and most subjects were in the obese class III category and metabolically healthy. Therefore, our results are not generalizable to men or to the general population of women. Second, BMI was used to assess adiposity; better measures, such as waist circumference or body composition analysis would have strengthened our findings. Finally, only a single marker - CRP- was used to separate the cohort into low/moderate and high CVD risk groups. Other factors, including serum lipid levels, B-type natriuretic peptide levels, and blood pressure reflect risk of CVD [13] and their inclusion might have strengthened or altered our findings.

In summary, the results of our study indicate that circulating Gal3 levels positively associate with BMI and inflammation in metabolically healthy, mostly obese and African American women, while the increase in Gal3 levels may reach a threshold at a BMI of 30; therefore Gal3 levels do not differ between women with different degrees of obesity. In addition, we reported altered associations between adiposity and the inflammatory markers Gal3, IL-6, and CRP in women at high risk of CVD, with a potential Gal3 plateau once circulating CRP reaches a threshold of 3 µg/ml. Our findings suggest that Gal3 may be used as a potential biomarker for evaluation of obesity and obesity-related inflammation in women who are at low/moderate risk of CVD.

Ethical approval: All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national

research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Conflict of Interest: The authors declare that they have no conflict of interest.

V. THE RECIPROCAL REGULATING EFFECTS OF LIPOPOLYSACCHARIDE AND GALECTIN-3 IN MICE

Abstract

The presence of the bacterial product lipopolysaccharide (LPS) in the systemic circulation contributes to increased risk and progression of many chronic pathologies. Galectin-3 (Gal3) interacts with LPS and regulates LPS-induced inflammation, while administration of LPS affects Gal3 production both *in vivo* and *in vitro*. However, several open questions remain about the reciprocal regulating effects of LPS and Gal3. We investigated the effects of Gal3 deficiency on acute inflammation induced by non-lethal doses of LPS in mice, as well as the alteration of Gal3 production in response to LPS. *In vivo*, LPS induced lower elevation of circulating cytokines and chemokines, as well as lower induction of hepatic acute-phase protein mRNA, but more severe hematological abnormalities, in Gal3 KO compared to WT mice. However, inhibition of extracellular Gal3 by N-acetyllactosamine did not replicate the alterations mentioned above, which were specific for LPS (TLR4)-induced inflammation, as we observed opposite outcomes in Poly I:C (TLR3)-injected mice. Absence of Gal3 also caused lower LPS-induced up-regulation of cytokine release *in vitro* in mouse splenocytes, but not in peritoneal macrophages, compared to cells from WT mice. Finally, LPS differentially affected protein production *versus* gene expression of Gal3 both *in vivo* and *in vitro*. In conclusion, Gal3 promotes LPS-induced acute inflammation in mice, while LPS influences production of Gal3 in a complex pattern.

Introduction

Lipopolysaccharide (LPS), also known as endotoxin, is the major outer membrane component of gram-negative bacteria [15]. Endotoxemia can not only cause endotoxic shock, which is related to at least 200,000 deaths annually in United States [150], but also contributes to increased risk and progression of several chronic pathologies, including obesity, type 2 diabetes, atherosclerosis, inflammatory bowel disease, and chronic heart failure [93, 94, 151-153].

Through the transmembrane pattern-recognition receptor (PRR) TLR4, LPS transduces intracellular signals that activate inflammatory pathways, primarily the nuclear factor kappa B (NF- κ B) transcriptional system. Activation of these pathways induces production of inflammatory cytokines, including interleukin (IL) -1, IL-6, and tumor necrosis factor (TNF), which perpetuate and amplify the inflammatory cascade [15]. Several intra- and extra-cellular mediators modulate the ability of cells to respond to LPS [15, 154, 155]. A better understanding of these modulating factors may contribute to developing treatments for endotoxemia.

Galectins are a group of β -galactoside-binding proteins that contain conserved carbohydrate-recognition domains (CRDs) [9]. Galectin-3 (Gal3), the only chimeric member of the family, contains a single C-terminal CRD and a flexible N-terminal domain. The N-terminal domain allows formation of Gal3 multimers while it cooperates with the CRD for glycan recognition [97]. A broad spectrum of roles has been attributed to Gal3, including modulation of host-pathogen interactions, cell migration, angiogenesis, wound healing, apoptosis, and promotion of both cell-cell and cell-extracellular matrix adhesion [9, 97]. Depending on its cellular location, the cell types

involved, and the pathological conditions or disease models examined, Gal3 can have either pro- or anti-inflammatory properties in its ability to modulate a variety of innate and adaptive immune responses [8, 97, 99, 103].

Lacking a typical secretion signal peptide, Gal3 is mainly located intracellularly, in both the cytoplasm and the nucleus. However, Gal3 is also present in extracellular fluids, including the systemic circulation [97, 99]. Gal3 binds to endogenous glycans, specifically cell surface β -galactosides and N-acetyllactosamine (N-Lac) [7, 156].

Pharmacological inhibition of extracellular Gal3 by N-Lac, which inhibits Gal3 by binding with high affinity to its CRD, protects rodents from hypertensive nephropathy and myocardial fibrosis [110]. In addition to its ability to interact with endogenous glycans, Gal3 also binds to glycans present on the surface of bacteria, viruses, and fungi [157]. Indeed, Gal3 can directly interact with several TLR ligands, including different strains of LPS [128].

Administration of LPS increases circulating levels of Gal3 in mice [89], while exposure of bone marrow-derived macrophages (BMM) to LPS *in vitro* increases Gal3 release [17]. However, surface expression of Gal3 in mouse peritoneal macrophages is down regulated in response to LPS [105], while co-treatment with IFN- γ and LPS decreases Gal3 expression in BMM lysates [129]. Thus, convincing evidence indicates that LPS regulates levels of Gal3, but the direction of change may depend on the location of Gal3 (extracellular vs cell-associated).

While LPS regulates Gal3, Gal3 in turn modulates the response to LPS. Li et al found that Gal3 negatively regulates LPS-induced inflammation. Indeed, these authors showed that LPS-stimulated BMM from Gal3-deficient mice produce higher levels of

inflammatory cytokines and have increased LPS-induced signaling compared to cells from WT mice. *In vivo*, Gal3-deficient mice are more susceptible than WT mice to LPS-induced shock [17]. In contrast to these findings, Fermino et al. demonstrated that Gal3 promotes, rather than inhibits, LPS binding to neutrophils and enhances neutrophil activation *in vitro*. At variance with the results of Li et al., the Fermino group also found that pre-incubation of LPS with Gal3 leads to more severe LPS-induced shock in mice, suggesting that Gal3 potentiates LPS-induced inflammation *in vivo* [16]. Although differences in the strain of mice and the dose of LPS may account for the sharp discrepancy between the results of these two studies, current evidence nevertheless highlights the potential importance of Gal3 in the modulation of LPS-induced inflammation, possibly through direct interactions between Gal3 and LPS. However, both groups chose relatively high doses of LPS to induce endotoxic shock, which may not represent the actual concentration of LPS observed during endotoxemia and/or chronic conditions [16, 17].

To help fill some of the knowledge gaps and resolve the controversy on the reciprocal regulating effects of LPS and Gal3, in the present study we asked whether Gal3 modulates the response to a non-lethal dose of LPS and whether LPS alters production of Gal3 at both the mRNA and the protein level in mice. We also investigated whether Gal3 is involved in modulating the response to TLR ligands other than LPS.

Materials and Methods

1. Animals and treatments

Gal3 KO and WT mice on a C57BL6 background (The Jackson Laboratory, Bar Harbor, ME) were housed in the animal facility of the University of Illinois at Chicago with free access to standard chow diet and water. Adult (5-6 months) age- and sex-matched female and male Gal3 KO and WT mice (5-10 per group) received one intraperitoneal (i.p.) injection of LPS (*E. coli* O111:B1, Sigma Chemical Co., St. Louis, MO) at either 10 µg/mouse (high-dose experiment) or 100 ng/mouse (low-dose experiment). Control mice received an injection of PBS. At 2h, 6h and 16h post LPS administration, and at 6h post PBS, mice were anesthetized using isoflurane and blood was collected from the retro-orbital plexus in heparin tubes. Mice were then euthanized by cervical dislocation. After evaluation of hematologic parameters using the HV950FS (Drew Scientific, Inc, Waterbury, CT), 50 µL of blood were used for flow cytometry analysis as described below. The remaining blood samples were centrifuged at 3500 rpm for 15 minutes at 4°C. The plasma was then transferred to fresh Eppendorf tubes and frozen at -80°C for future use. The liver was frozen immediately in liquid nitrogen until further processing. To inhibit extracellular Gal3, WT male and female mice received two i.p. injections of N-Lac (5 mg/kg body weight) or vehicle at 24h and 30min prior to administration of LPS (10 µg/mouse). Mice were then sacrificed at 6h post LPS administration; blood and tissues were collected as described above. The dose and schedule of N-Lac administration were chosen according to Yu et al. [110].

For Poly I:C challenge, age-matched female Gal3 KO and WT mice (n=3 per group) were injected with 100 µg Poly I:C i.p. Mice were euthanized, and blood and tissues collected at 6h post injection.

All animal studies were approved by the Animal care and Use Committee of the University of Illinois at Chicago.

2. Peritoneal macrophage and splenocyte stimulation

Peritoneal macrophages were collected from Gal3 KO and WT mice by peritoneal lavage immediately following euthanasia. Cells were washed, counted, and plated in 24-well cell culture plates at a concentration of 2 million cells/well. The cells were washed again after 2h to remove non-adherent cells. The remaining adherent cells were treated with either 1 µg/mL LPS or RPMI (control) overnight at 37°C in 5% CO₂. Cells were then harvested for evaluation of cytokine mRNA expression. The culture supernatant was collected for measurement of released levels of Gal3 and cytokines. In a separate experiment, peritoneal macrophages from WT mice were collected and stimulated with 1 µg/mL LPS as described above, then cells and the culture supernatant were collected at 1.5, 3, 6, 12, 24, and 48 h to assess the time course of LPS-induced alterations of Gal3 gene expression and released levels.

Single-cell suspensions of splenocytes were obtained by pressing the spleen from Gal3 KO and WT mice through a 100 µm cell strainer with 10 mL PBS. Then, cells were washed, re-suspended, counted, and cultured in 24-well cell culture plates at the concentration of 5 million cells/well and treated with either LPS (1 µg/mL) or RPMI overnight at 37 °C with 5% CO₂. Cell culture supernatants were collected for measurement of released levels of cytokines.

3. Measurement of biomarkers

Levels of Gal3, IL-6, Interferon- γ (IFN- γ), chemokine (C-C motif) ligand 2 (CCL2), TNF, and osteopontin were measured using ELISA kits from Life Technologies and R&D Systems. Blood glucose was measured using a glucometer.

4. RNA expression analysis

Total RNA was extracted from liver and cultured peritoneal macrophages using TRIzol (Life Technologies) and reverse transcribed. Gene expression of Serum amyloid A-1 (SAA-1), Plasminogen Activator Inhibitor-1 (PAI-1), Hepcidin-1, and Gal3 was assessed by real-time RT-PCR using either the SYBRGreen or the TaqMan system. Expression of each gene was adjusted by expression of the housekeeping gene GAPDH. Data obtained from RT-PCR were analyzed using the Comparative CT method.

5. Flow cytometry

Splenocytes and whole blood collected from mice from the high-dose LPS experiment at 6h post LPS were surface-stained with antibodies against Ly6B, Ly6C (BD Bioscience), Ly6G, and CD3 (eBioscience). Whole blood was then lysed with RBC lysis buffer to remove erythrocytes.

Peritoneal macrophages collected from WT mice were pre-incubated with anti-CD16/CD30 antibody (eBioscience) to block the Fc receptor prior to further staining. Cells were first surface-stained with anti-F4/80 antibodies (eBioscience), then fixed in IC fixation buffer followed by incubation with permeabilization buffer (eBioscience), and intracellularly stained with anti-Gal3 antibody (eBioscience).

All samples were analyzed on a C6 Accuri cytometer (BD Bioscience).

6. Statistical analysis

Data are expressed as mean \pm SEM. Statistical significance of differences was evaluated by independent t test or ANOVA. A value of $P < 0.05$ was considered statistically significant. All statistical analysis was performed using SAS 9.3 (SAS Inc., Cary, NC, USA).

Results

1. Effect of Gal3 deficiency on LPS-induced cytokine production

To assess the effect of Gal3 deficiency on LPS-induced cytokine production, age-matched female Gal3 KO and WT mice received a non-lethal high-dose of LPS i.p. (10 $\mu\text{g}/\text{mouse}$).

As expected, LPS induced a significant increase in circulating cytokine and chemokine levels in all mice at 2h and 6h post injection. However, as shown in Figure 1A-C, plasma levels of IL-6, TNF and CCL-2 were significantly lower in Gal3 KO compared to WT mice at both 2 and/or 6h post LPS. Plasma levels of these mediators returned to baseline at 16h in all mice, with no differences due to genotype. Additionally, peak levels of IFN- γ (6 h) were also significantly lower in Gal3 KO mice compared to WT mice, with no difference at either 2h or 16h post-LPS (Figure 1D). Moreover, circulating levels of the anti-inflammatory cytokine IL-10 were significantly elevated in response to LPS injection in both Gal3 KO and WT mice, with a peak value observed at 2h. At 6h post-LPS injection, plasma IL-10 declined in both groups, with comparable levels in Gal3 KO and WT mice. However, IL-10 slightly increased at 16h compared to 6h in WT mice, while no significant changes were observed in Gal3 KO mice, leading to

significantly lower plasma IL-10 levels in Gal3 KO compared to WT mice at 16h (Figure 1E). Finally, levels of the chemotactic and pro-inflammatory molecule osteopontin were significantly elevated in response to LPS administration in both mouse genotypes, but about 55% lower in Gal3 KO compared to WT plasma at 16 h (Figure 1F).

These results were confirmed in male mice. Similar to what we observed in female mice, male Gal3 KO mice also had about 75% lower IL-6, 40% lower IFN- γ , 30% lower IL-10, and 20% lower CCL-2 compared to their WT counterparts at 6h post-LPS (data not shown).

In summary, Gal3 KO mice had a significantly lower LPS-induced elevation of circulation cytokines and chemokines compared to WT mice, which suggests that Gal3 deficiency protects mice from LPS-induced inflammation by inhibiting production of pro-inflammatory mediators.

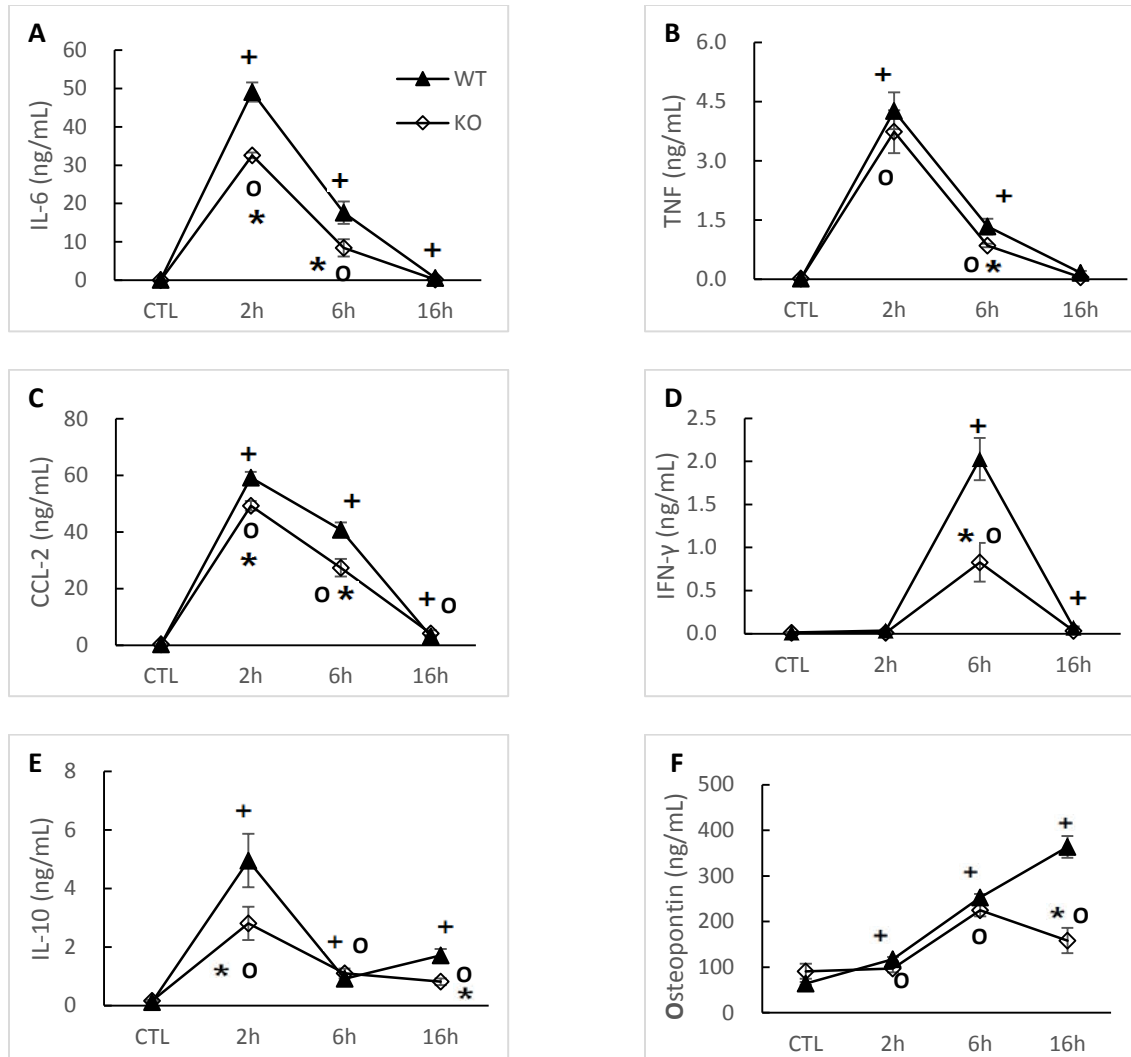


Figure 1 LPS-induced changes of circulating cytokine and chemokine levels in female Gal3 KO and WT mice.

Female mice were injected with 10 μ g LPS or sterile PBS as control (CTL). Plasma was obtained at 2h, 6h, and 16h after LPS administration for measurement of circulating levels of IL-6 (A), TNF (B), CCL-2(C), IFN- γ (D), IL-10 (E), and osteopontin (F) by ELISA. Data are mean \pm SEM of 5-10 per group. * P <0.05 versus corresponding WT group; + P <0.05 versus control group in WT mice; ° P <0.05 versus control group in Gal3 KO mice by one-way ANOVA.

To further investigate the association between Gal3 and LPS-induced inflammation and the cellular source of Gal3 during the inflammatory process, we designed *in vitro* experiments to investigate the effects of Gal3 deficiency on cytokine production from two types of cells involved in LPS-induced inflammatory responses, peritoneal macrophages and splenocytes.

Adherent peritoneal macrophages collected from WT and Gal3 KO mice were stimulated with 1 µg/mL LPS and cultured overnight; released cytokine levels were then quantified. As shown in Figure 2A-2C, stimulation with LPS significantly increased levels of IL-6, TNF, and IL-10 in the supernatant of cells obtained from both WT and KO mice. However, in contrast with the results of *in vivo* experiments, we did not find any significant differences between WT and KO mice when we measured cytokine production by peritoneal macrophages *in vitro*.

The results were different when we assessed *in vitro* cytokine production by splenocytes. In this case, splenocytes isolated from Gal3 KO mice released significantly lower levels of IL-6 and TNF, but similar levels of IL-10, compared to cells from WT mice (Figure 2F).

In summary, Gal3 deficiency did not affect LPS-induced cytokine production from mouse peritoneal macrophages, but lead to lower pro-inflammatory -- but not anti-inflammatory -- cytokine production from mouse splenocytes.

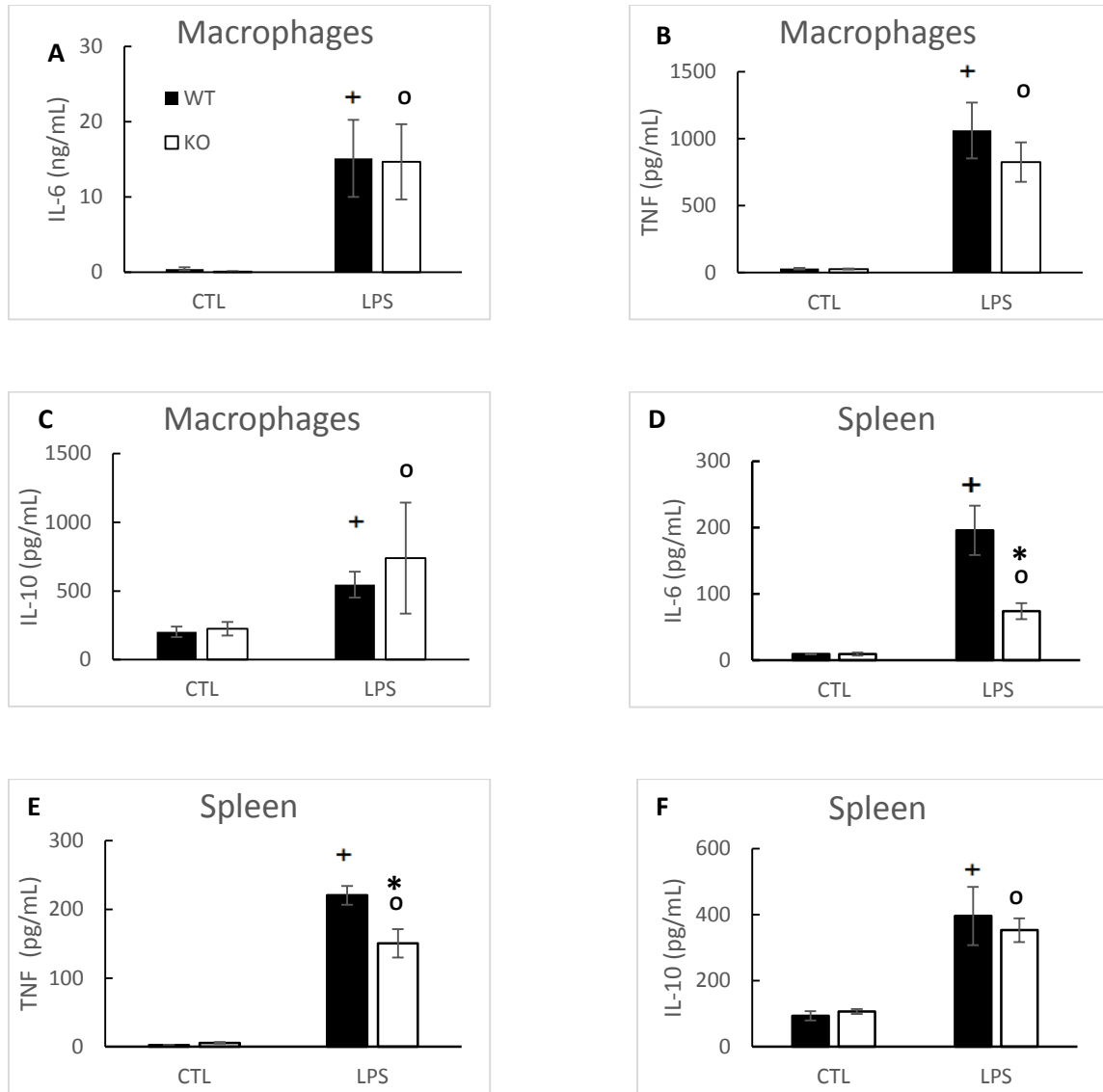


Figure 2 LPS-induced changes of cytokine production in peritoneal macrophages and splenocytes from Gal3 KO and WT mice in vitro.

Mouse peritoneal macrophages and splenocytes were cultured overnight with LPS (1 $\mu\text{g/mL}$) or RPMI (CTL). Levels of IL-6 (**A, D**), TNF (**B, E**), and IL-10 (**C, F**) were measured by ELISA in the culture supernatant. Data were obtained as the average from at least three independent experiments. Data are mean \pm SEM. * $P < 0.05$ versus corresponding WT group; + $P < 0.05$ versus control group in WT mice; ° $P < 0.05$ versus control group in Gal3 KO mice by one-way ANOVA.

2. Effect of Gal3 deficiency on LPS-induced acute phase proteins and hematological alterations

Exposure to LPS leads to induction of the hepatic acute phase response [89]. We examined the potential involvement of Gal3 in this response by comparing gene expression of three acute-phase proteins – SAA-1, PAI-1, and hepcidin-1 -- in the liver of WT and Gal3 KO mice exposed to LPS.

As shown in Figure 2A, administration of 10 µg/mouse of LPS (a high but non-lethal dose) led to a 2,000-3,000 fold up-regulation of hepatic SAA-1 mRNA expression in both WT and Gal3 KO mice, with no significant differences due to genotype at any time point. However, hepatic SAA-1 mRNA expression trended towards a plateau at 16h in Gal3 KO mice while it kept increasing in WT mice (Figure 3A). We reasoned that such a robust acute phase reaction could possibly overwhelm a potential modulating role of Gal3. Therefore, to better understand the effect of Gal3 deficiency on LPS-induced acute phase responses, we repeated the study using a lower dose of LPS (100 ng/mouse). This low dose of LPS induced a significantly lower SAA-1 hepatic mRNA expression in Gal3 KO compared to WT mice at 6h, despite still leading to ~1000-fold induction in each group (Figure 3B). We also observed non-significant trends towards lower induction of hepatic mRNA expression of PAI-1 and hepcidin-1 in Gal3 KO compared to WT in response to both the high and low doses of LPS (data not shown). In summary, absence of Gal3 lowered the hepatic acute-phase response induced by LPS, particularly when considering SAA-1, though with less efficiency than observed when measuring circulating cytokine and chemokine levels.

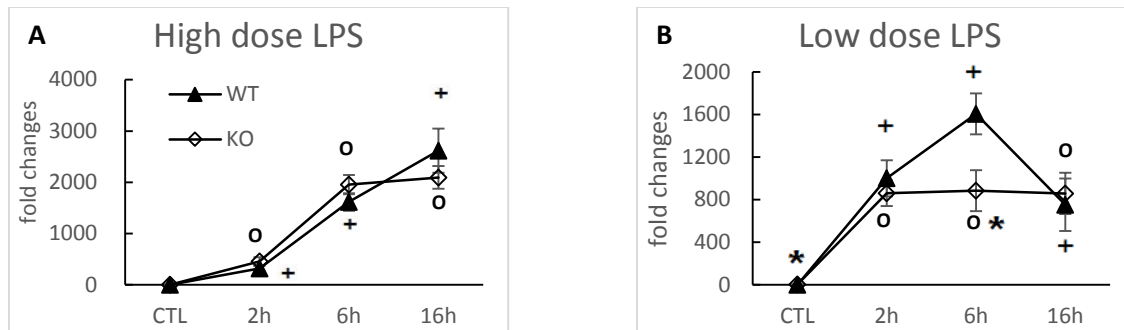
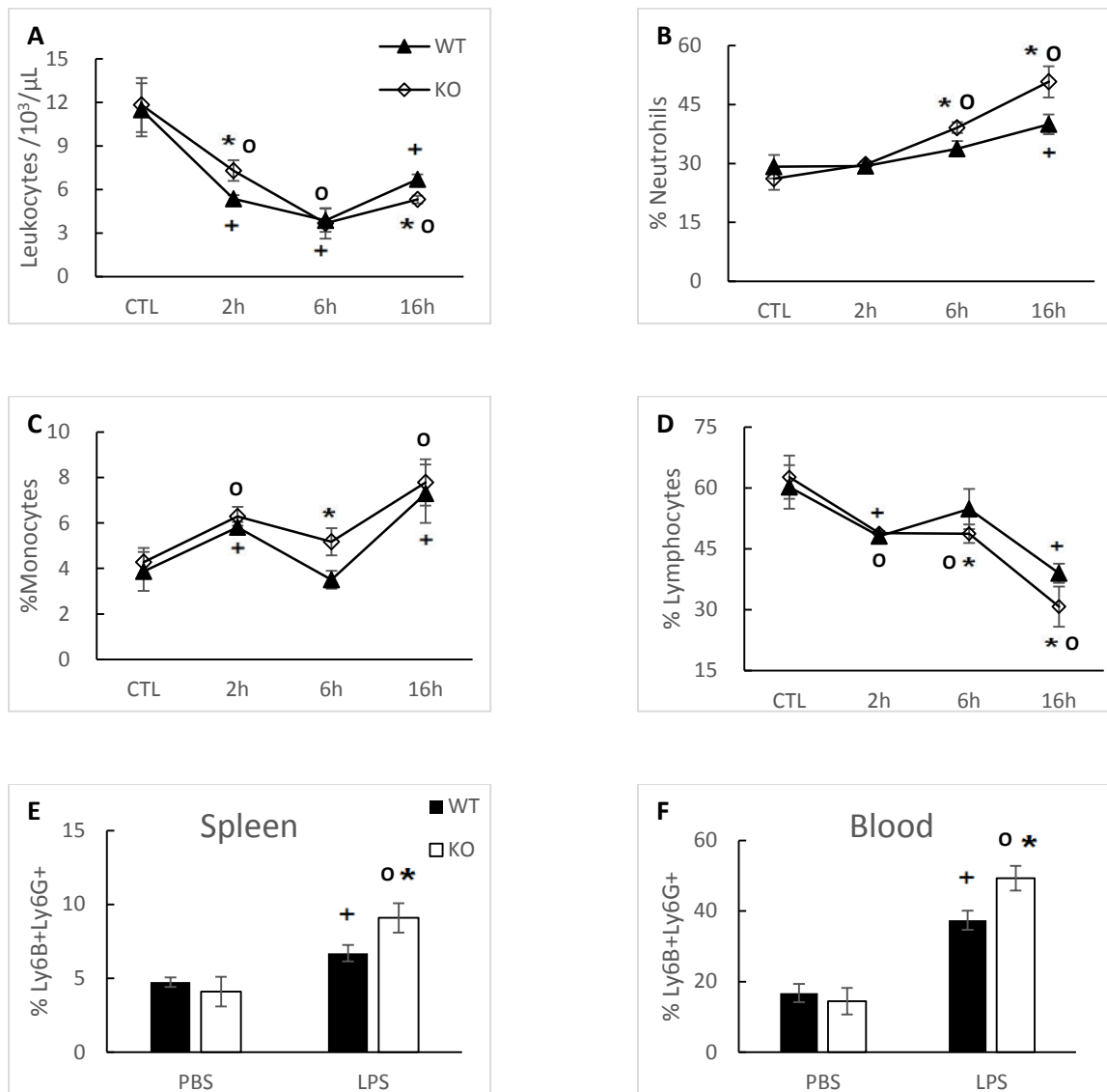


Figure 3 Hepatic SAA-1 mRNA expression in Gal3 KO and WT mice in response to high (10 µg) and low (100 ng) doses of LPS.

Female Gal3 KO and WT mice received either a high (10 µg) or a low dose (100 ng) of LPS; mice in the control groups (CTL) received 100 µL of sterile PBS. Hepatic mRNA expression of SAA-1 was evaluated in both high (A) and low (B) dose experiments. Data are reported as gene expression in liver using the $\Delta\Delta\text{ct}$ method. Data are mean \pm SEM of 5-10 per group. * $P < 0.05$ versus corresponding WT group; + $P < 0.05$ versus control groups in WT mice; ° $P < 0.05$ versus control groups in KO mice by one-way ANOVA.

Next, we evaluated hematologic parameters in peripheral blood, as markers of systemic inflammation. As shown in Figure 4A, all LPS-injected mice developed significant leukopenia, with a nadir at 6h, compared to control mice that received PBS. The decline in leukocyte counts was less rapid, but the recovery was also slower, in Gal3 KO mice compared to their WT counterparts, resulting in significant differences between genotypes at both 2h and 16h post-LPS. Leukopenia was associated with relative neutrophilia, which was more severe at 6h and 16h post-LPS in Gal3 KO mice compared to WT mice (Figure 4B). Relative monocytosis and lymphopenia were also more severe in Gal3 KO mice compared to WT mice (Figure 4C and 4D). Flow cytometry analysis confirmed these findings. In fact, Gal3 KO mice had significantly higher percentages of Ly6B⁺Ly6G⁺ neutrophils (Figure 4E, 4F) and

Ly6C⁺Ly6B⁺ monocytes in both the peripheral circulation and the spleen compared to WT mice (Figure 4G, 4H), while CD3⁺ T cell were comparable between the two genotypes (data not shown). Cumulatively, these data indicate that Gal3 deficiency significantly affected both the kinetics and the magnitude of hematological abnormalities in mice exposed to LPS.



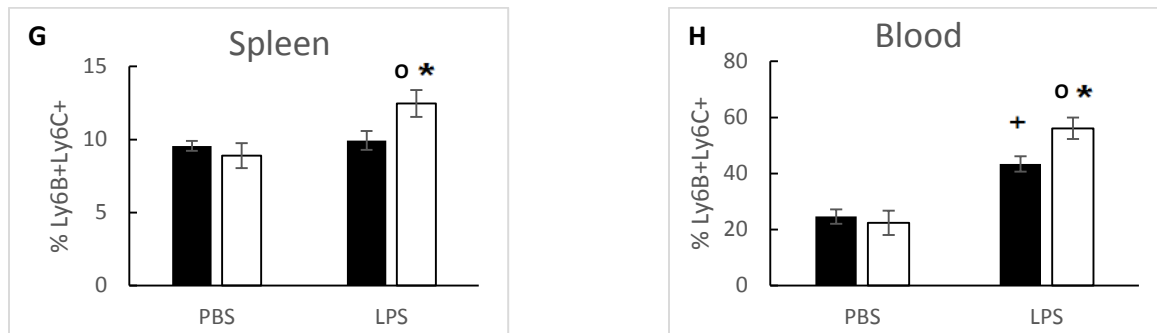


Figure 4 LPS-induced alteration of hematological parameters in blood and spleen.

Blood was obtained at 2h, 6h, and 16h after administration of 10 μ g LPS or at 6h after administration of sterile PBS as control (CTL) for evaluation of total leukocytes (**A**), % neutrophils (**B**), % monocytes (**C**), and % lymphocytes (**D**) counts in female Gal3 KO and WT mice. At 6h post 10 μ g LPS or 100 μ L sterile PBS injections % Ly6B⁺Ly6G⁺neutrophils (Spleen: **E**; Blood: **F**) and % Ly6B⁺Ly6C⁺ monocytes (Spleen: **G**; Blood: **H**) were evaluated by flow cytometry in male Gal3 KO and WT mice. Data are mean \pm SEM of 5-10 per group. *P<0.05 versus corresponding WT group; +P<0.05 versus control groups in WT mice; ^oP <0.05 versus control groups in KO mice by one-way ANOVA.

3. Effect of Gal3 deficiency on Poly I:C-induced inflammation

To understand whether Gal3 specifically affects only LPS-, and thus TLR4-, induced inflammation, we injected WT and Gal3 KO mice with the TLR3 activator Poly I:C and evaluated their inflammatory status at 6h post injection. Circulating levels of IL-6 and CCL-2 were significantly increased in response to Poly I:C administration, with Gal3 KO mice trending towards higher plasma levels compared to WT mice (Figure 5B, 5C). Moreover, Gal3 KO mice developed more severe leukopenia compared to their WT counterparts in response to Poly I:C (Figure 5D), with comparable magnitude of neutrophilia and lymphopenia between the two genotypes (data not shown).

Administration of Poly I:C also significantly reduced blood glucose levels in both Gal3 KO and WT mice, with Gal3 KO mice suffering from more severe hypoglycemia compared to their WT counterparts (Figure 5A). Together, these results indicate that Gal3 deficiency aggravates Poly I:C-induced inflammation in mice, which contrasts with the results we obtained when evaluating LPS-induced inflammation.

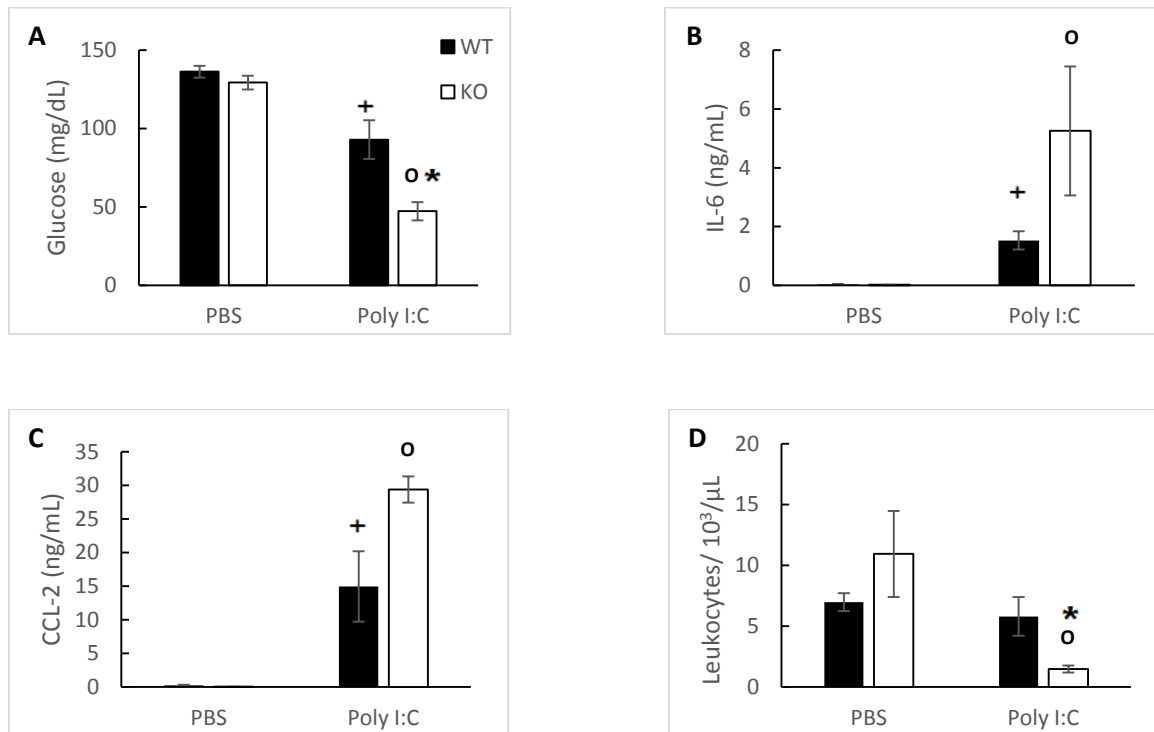


Figure 5 Poly I:C-induced inflammation in Gal3 KO and WT mice.

Female Gal3 KO and WT mice received i.p. injections of 100 μg Poly I:C or 100 μL sterile PBS as control. At 6h post injection, blood glucose (A), plasma levels of IL-6 (B) and CCL-2 (C), as well as total leukocyte counts (D) were evaluated. Data are mean \pm SEM of 5-10 per group. * $P < 0.05$ versus corresponding WT group; ⁺ $P < 0.05$ versus control groups in WT mice; ^o $P < 0.05$ versus control groups in KO mice by one-way ANOVA.

4. Effect of inhibition of extracellular Gal3 on LPS-induced inflammation

Gal3 performs contrasting functions depending on its cellular localization. For example, cytoplasmic Gal3 is a well-defined anti-apoptotic protein while extracellular Gal3 promotes apoptosis of T cells [7, 99]. Previous animal studies have shown N-Lac binds to the CRD domain of Gal3 with high affinity [9]. Administration of N-Lac inhibits extracellular Gal3-mediated effects on cardiac remodeling in rats and mice and myocardial fibrosis in human dermal fibroblasts [110]. To understand the effects of Gal3 cellular localization on LPS-induced inflammation, we pretreated WT male mice with the Gal3 inhibitor N-Lac or with vehicle. Inflammatory status was evaluated at 6h post-LPS administration.

Significant elevation of circulating IL-6 and CCL-2 levels was observed in all LPS-treated mice, with comparable levels in mice treated or not with N-Lac (Figure 6A, 6B). As expected, LPS-injected mice also developed leukopenia, neutrophilia, and lymphopenia. However, inhibition of extracellular Gal3 by N-Lac did not alleviate or aggravate those LPS-induced hematological alterations (Figure 6C-6E). These results were confirmed in female mice (data not shown).

In summary, inhibition of extracellular Gal3 by administration of N-Lac prior to LPS did not alter the response of WT mice to LPS.

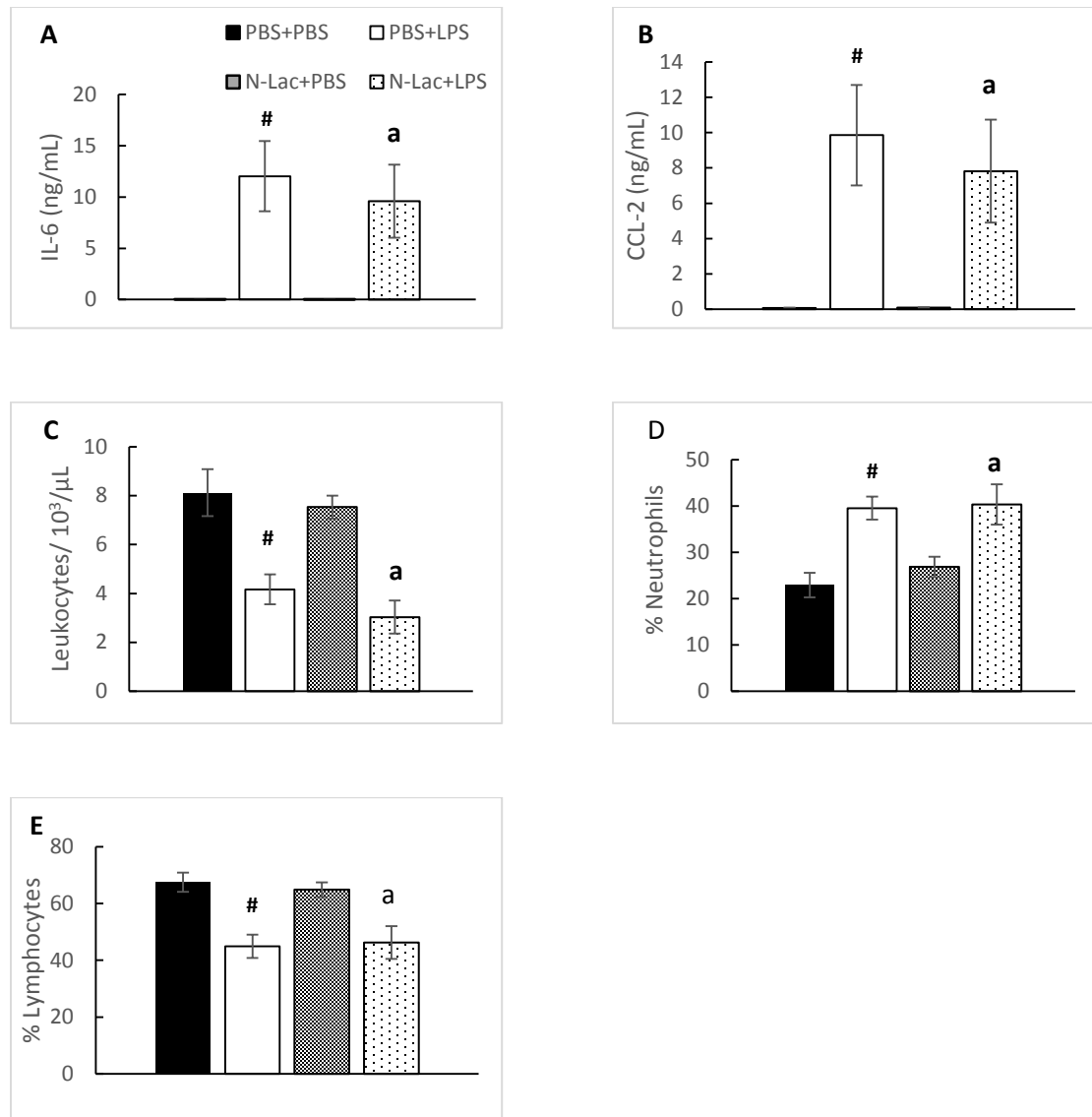


Figure 6 Effect of inhibition of extracellular Gal3 on LPS-induced inflammation.

WT male mice received two i.p. injections of N-Lac (5 mg/kg body weight) or sterile PBS at 24h and 30min prior to administration of LPS (10 μg). Mice were sacrificed at 6h post LPS administration for evaluation of plasma levels of IL-6 (**A**) and CCL-2 (**B**) and well as total leukocytes (**C**), % neutrophils (**D**), and % lymphocytes (**E**) in whole blood. Data are mean \pm SEM of 5-10 per group. [#]P<0.05 versus corresponding PBS+PBS group; ^aP<0.05 versus N-Lac+PBS groups by one-way ANOVA.

5. LPS regulates Gal3 production and gene expression *in vivo* and *in vitro*

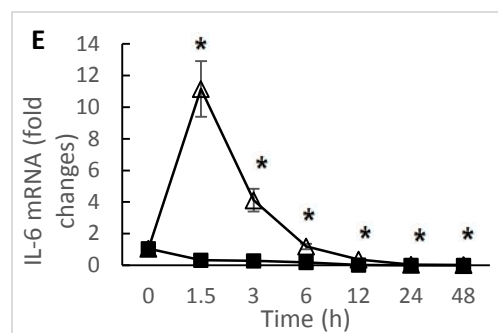
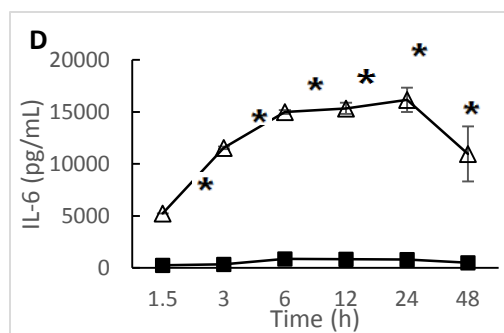
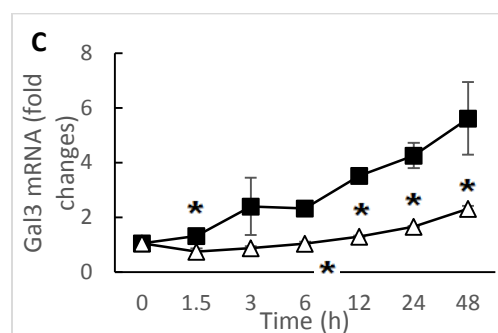
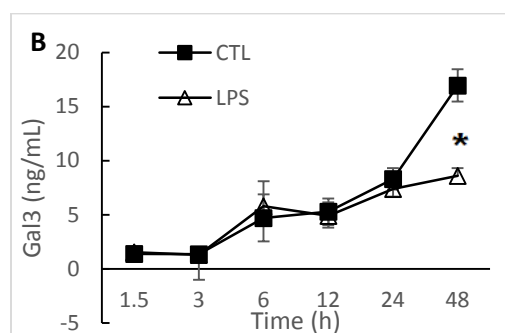
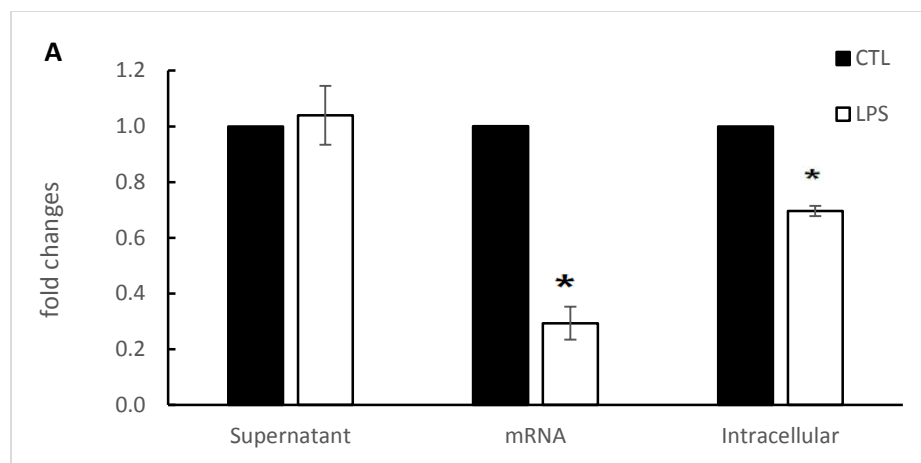
In addition to regulating LPS-induced inflammation, Gal3 may also work as an inflammatory modulator that is itself affected by LPS [16, 105, 129]. Thus, we evaluated the time course of LPS-induced alterations of Gal3 production and gene expression both *in vivo* and *in vitro*.

Gal3 is predominantly produced by macrophages [7]. To evaluate the effect of LPS on Gal3 production *in vitro*, we isolated mouse peritoneal macrophages and stimulated them with 1 µg/mL LPS for different amounts of time. As shown in Figure 7A, overnight incubation with LPS significantly down-regulated both mRNA expression and intracellular levels of Gal3 in F4/80⁺ macrophages, while released Gal3 levels in the cell culture supernatant were similar between LPS-treated and untreated cells.

Next, we performed an extended time-course to understand the kinetics of Gal3 gene expression and released protein in macrophages in response to LPS. As expected, LPS activated macrophages, significantly increasing IL-6 protein levels beginning at 1.5h and all the way up to 48h, while IL-6 mRNA expression reached a peak at 1.5h and declined thereafter (Figure 7C, 7D). However, Gal3 protein release and mRNA expression showed a very different pattern of change compared to those of IL-6. In unstimulated cells, levels of released Gal3 progressively increased within the full time course up to 48h; stimulation with LPS did not significantly alter the amount of Gal3 released into the supernatant until 48h, at which time significantly lower levels of Gal3 were present in the supernatant of LPS-stimulated cells compared to non-stimulated control cells (Figure 7B). Moreover, LPS suppressed Gal3 mRNA expression in macrophages as early as 1.5h, and suppression was consistent throughout the full time course (Figure 7C).

When WT mice received an injection of 10 µg of LPS, circulating Gal3 levels significantly increased, doubling at 2h compared to the levels of PBS-injected mice. Plasma Gal3 then declined to levels comparable to those of PBS-injected mice, but significantly rose again by 16h. Interestingly, hepatic Gal3 mRNA expression showed a pattern of change that was opposite to that of circulating Gal3. Indeed, hepatic Gal3 gene expression reached a nadir at 2h, with a 40% reduction compared to expression in the liver of PBS-injected mice. Next, a rapid elevation of hepatic Gal3 mRNA expression was observed, with a 2-fold peak at 6h, followed by return to levels comparable to those of control mice by 16h (Figure 7F). Such specular changes of circulating protein levels and hepatic mRNA expression of Gal3 were confirmed in mice receiving the lower dose (100 ng) of LPS (data not shown).

In summary, LPS significantly suppressed Gal3 gene expression *in vitro* in macrophages, while inducing time-related alternating changes *in vivo* in the liver. Furthermore, whereas circulating Gal3 levels were temporarily up-regulated in response to LPS administration *in vivo*, LPS did not increase released Gal3 in the supernatant of macrophage cultures, actually suppressing levels of extracellular Gal3 at 48h. Finally, circulating levels and hepatic gene expression of Gal3 showed a specular pattern of change in response to LPS stimulation *in vivo*.



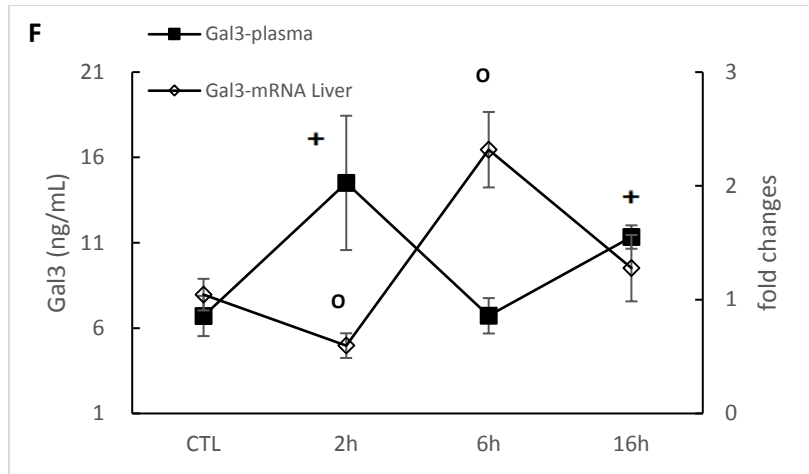


Figure 7 . LPS-induced alterations of Gal3 levels *in vitro* and *in vivo*.

Adherent mouse peritoneal macrophages were cultured with 1 $\mu\text{g/mL}$ LPS or RPMI (CTL) overnight. Intracellular and released Gal3 protein as well as Gal3 mRNA expression was evaluated in F4/80⁺ macrophages (A). In a separate experiment, mouse peritoneal macrophages were cultured with 1 $\mu\text{g/mL}$ LPS or RPMI (CTL). Levels of released Gal3 (B) and IL-6 (D) as well as mRNA expression of Gal3 (C) and IL-6 (E) were evaluated at 1.5, 3, 6, 12, 24, and 48h post LPS stimulation. Hepatic mRNA expression and plasma levels of Gal3 were measured at 2h, 6h, and 16 h post LPS (10 μg) LPS and 6h post PBS (CTL) in female WT mice . * $P < 0.05$ versus corresponding RPMI control cells; ⁺ $P < 0.05$ versus hepatic Gal3 mRNA expression in control groups; ^o $P < 0.05$ versus plasma Gal3 levels in control groups by one-way ANOVA.

Discussion

In the present report, we investigated the effects of Gal3 deficiency on acute inflammation induced by non-lethal doses of LPS in mice, as well as the alteration of Gal3 production in response to LPS. We demonstrate that Gal3 specifically promotes LPS (TLR4)-induced acute inflammation in mice. Meanwhile, LPS differentially affected protein production and gene expression of Gal3 both *in vivo* and *in vitro*.

As a multi-functional protein, Gal3 plays a key role in the inflammatory processes that underlie a variety of pathological conditions, including infectious diseases [7, 8, 157]. Previous studies have shown that Gal3 binds to LPS and regulates LPS-induced inflammation in mouse models of endotoxic shock that used lethal doses of LPS [16, 17, 128]. Here, for the first time, we report that Gal3 KO mice develop lower systemic inflammation compared to their WT counterparts in response to non-lethal doses of LPS, as indicated by the significantly lower circulating cytokines and chemokines levels and reduced acute-phase response of Gal3 KO mice compared to WT mice. As a strong immune stimulator, LPS triggers massive inflammatory responses by activating the NF- κ B signaling pathway and up-regulating downstream production of mediators such as IL-6 and TNF [15, 154, 155]. In a previous study, investigators observed attenuated thioglycollate-induced peritoneal inflammation, with significantly reduced NF- κ B responses, in Gal3 KO mice compared to WT mice, suggesting a potential role of Gal3 as an inflammatory response amplifier involved in the activation of NF- κ B signaling pathways [106]. This evidence is supported by another study indicating that Gal3 promotes NF- κ B activation in colonic epithelial cells [158]. Therefore, Gal3 deficiency might protect mice from LPS-induced acute inflammation by inhibiting LPS-triggered NF- κ B activation, thus reducing downstream cytokine production.

Severe leukopenia is usually present after LPS administration in mice [159]. Gal3 deficiency altered both the kinetics and the magnitude of this response. In fact, our study showed a slower reduction of peripheral blood leukocyte counts in Gal3 KO mice compared to WT mice, indicating a slower progress and lower systemic inflammation in the absence of Gal3. These results are in agreement with the lower circulating

inflammatory mediators and hepatic acute responses of Gal3 KO mice discussed above. However, Gal3 KO mice developed more severe neutrophilia from 6h post LPS injection compared to WT mice. A previous study demonstrated that Gal3 promotes adhesion of neutrophils to endothelial cells [160]. Therefore, adhesion defects may account for the more severe neutrophilia of mice that lack Gal3. Moreover, absence of Gal3 suppresses infiltration of macrophages and neutrophils into the peritoneal cavity in response to injections of thioglycollate [106], a finding that may explain the higher levels of neutrophils and monocytes we observed in the spleen of Gal3 KO mice compared to spleens of WT mice.

The TLR4 agonist LPS is not the only PRR ligands with which Gal3 can interact [157]. To test whether the modulating effect of Gal3 deficiency is specific for LPS/TLR4, we compared the response of Gal3 KO and WT mice to the TLR3 agonist Poly I:C. At variance with the decreased inflammation induced by LPS, Gal3 KO mice responded to Poly I:C with exacerbated acute inflammation. Therefore, contrary to the results obtained when examining LPS-induced inflammation, Gal3 deficiency actually aggravated Poly I:C (TLR3)-induced acute inflammation mice. At variance with all the other TLRs that mostly signal via MyD88-dependent pathways, TLR3 utilizes the TRIF-dependent pathway [161]. Hence, Gal3 may be involved in both MyD88- and TRIF-dependent pathways but performing differential regulatory functions on their downstream responses.

Various cell types and tissues express and produce Gal3 [7, 8, 99]. However, the main source of Gal3 involved in regulation of systemic inflammation remains unidentified. Thus, we designed *in vitro* experiments using two types of cells known to be involved in

LPS-induced inflammation [162, 163]. Absence of Gal3 did not alter cytokine production in LPS-stimulated mouse peritoneal macrophages, whereas splenocytes lacking Gal3 released less cytokines into the culture supernatant in response to LPS stimulation when compared to WT splenocytes. This discrepancy suggests that the spleen and splenic Gal3 production, rather than peritoneal macrophages, may be more important in modulating LPS-induced inflammation *in vivo*.

Gal3 is predominantly expressed in the cytoplasm but also exists in the extracellular space, including the systemic circulation. Different localizations determine different functions of Gal3 [7, 99]. To investigate whether extracellular or intracellular Gal3 affects LPS-induced inflammation, we treated mice with the Gal3 inhibitor N-Lac, which binds to with high affinity to Gal3's CRD [7, 9, 110]. However, we did not detect any difference in cytokine production or severity of hematological abnormalities between mice treated or not with N-Lac. These data suggest that the intracellular, but not the extracellular form of Gal3 plays a key role in regulating LPS-induced inflammation. Moreover, our *in vitro* experiment using mouse peritoneal macrophages demonstrated that LPS significantly down-regulated Gal3 levels only intracellularly, as both intracellular protein levels and gene expression of Gal3 were significantly lower in LPS-stimulated cells compared to non-stimulated ones. In contrast, levels of Gal3 released from the cells into the culture supernatant were comparable between cells treated or not with LPS throughout the time course, from 1.5h to 24h. These results suggest the relevant interaction between Gal3 and LPS take place inside the cell, indicating that Gal3 modulates LPS-induced inflammation at the level of intracellular signaling pathways rather than through direct binding to LPS in the extracellular space.

Many inflammatory conditions are associated with upregulation of Gal3 [7, 8, 103]. Several studies reported that LPS affects Gal3 levels both *in vivo* and *in vitro* [17, 89, 105]. Here, we evaluated the full time-course of LPS-induced Gal3 alterations at both the protein and mRNA expression levels *in vivo* and *in vitro*. Consistent with our previous findings, LPS increased Gal3 protein levels in the systemic circulation, with a peak at 2h followed by a nadir at 6h. Interestingly, the pattern of Gal3 gene expression in the liver in response to LPS was opposite to that observed in the circulation. In fact, hepatic Gal3 gene expression reached a nadir at 2h after LPS, but then peaked at 6h. In an attempt to understand this puzzling pattern, we performed *in vitro* studies that, however, did not help us reach definitive conclusions. In fact, we found reduced Gal3 mRNA expression in peritoneal macrophages treated with LPS when compared with unstimulated cells, while LPS did not alter the level of Gal3 released into the cell culture supernatant until the 48h time point. Our results are in agreement with data indicating that exposure to IFN- γ /LPS decreases Gal3 expression *in vitro* [129], but they are also at variance with the results Li et al. obtained in mouse BMMs [17]. In summary, LPS differentially regulates Gal3 mRNA expression vs Gal3 released protein both *in vivo* and *in vitro*. More studies will be necessary to conclusively tease out the details of the complex regulation of Gal3 production and release.

Together with several strengths, that include a combination of *in vivo* and *in vitro* experiments as well use of both genetic deletion and pharmacologic inhibition of Gal3, our study also has some limitations. These include lack of evaluation of the efficiency of N-Lac inhibition of Gal3 *in vivo*, lack of measurements of direct NF- κ B activation in

response to LPS stimulation, as well as not using littermate groups of Gal3 KO and WT mice.

In summary, we demonstrated that Gal3 promotes acute inflammation induced by non-lethal doses LPS in mice. In addition, the promoting effects of Gal3 are specific for LPS -- and thus TLR4 -- signaling, since we obtained opposite findings when using a TLR3 agonist. Moreover, it is likely that intracellular, rather than extracellular, Gal3 plays the key role in modulating LPS-induced inflammation. Finally, LPS affects Gal3 by regulating its production at both the protein and the gene expression level in a complex manner. Given that up-regulation of Gal3 and mild but persistent elevation of LPS are both present in many diseases, our study contributes to a better understanding of the interactions between Gal3 and LPS that characterize the pathogenesis of many clinical conditions.

VI. EFFECT OF GALECTIN-3 DEFICIENCY IN THE RESPONSE TO REPEATED ULTRA-LOW DOSES OF LPS IN MICE

Abstract

A mild but persistent elevation of circulating lipopolysaccharide (LPS) is associated with many chronic metabolic and inflammatory diseases. Galectin-3 (Gal3), a protein whose levels are increased in many pathologies, binds to LPS and regulates inflammation. In the present study, we repeatedly injected WT and Gal3 KO mice with ultra-low doses of LPS that mimic the mild endotoxemia of chronic diseases. We found that WT mice responded to LPS with a trend towards increased adiposity, development of mild hyperglycemia, and induction of the hepatic acute-phase response. These LPS-induced alterations were either absent, less marked, or even reversed in Gal3 KO mice. Therefore, deficiency of Gal3 protects mice from repeated injections of ultra-low dose LPS, suggesting that Gal3 promotes LPS-induced inflammation in mice.

Introduction

Numerous pathologies -- including obesity, diabetes, chronic heart failure, gastrointestinal diseases, and more -- are associated with chronic inflammation [164-167]. These same conditions present with alterations in the gut microbiota and an impaired gut barrier [4, 168, 169]. Impairment of the gastrointestinal epithelial barrier allows bacterial products from the gut into the lamina propria and the blood [170]. Indeed, these pathologies are associated with a mild but persistent elevation of circulating lipopolysaccharide (LPS), one of the main components of the outer membrane of gram-negative bacteria [93, 94, 151-153]. For example, significantly elevated circulating LPS levels are present in both high-fat diet (HFD)-fed obese mice and genetically obese *ob/ob* mice compared to non-obese mice [91, 92]. Feeding a HFD also induces changes in the microbiota that favor release of LPS into the circulation, whereas administration of antibiotics reduces both cecal and plasma levels of LPS in obese mice [91]. Because LPS is a strong activator of the innate immune response [85], a persistent -- even if mild -- elevation of LPS in the systemic circulation can lead to sustained inflammation.

Galectin-3 (Gal3), a unique chimeric galectin family member, has been widely studied as a modulator of inflammation in both humans and rodents [7]. Levels of Gal3 are significantly elevated both in the systemic circulation and in tissues during inflammation in both humans and experimental animals [7, 8]. However, the specific role Gal3 plays in modulating inflammation depends on the cell type that produces it, the specific cellular localization of this galectin as well as the exact pathology involved [8, 99]. Deficiency of Gal3 leads to increased disease severity in several murine models,

including increased adiposity, systemic inflammation, and impaired glucose metabolism in a HFD-induced obesity model [114], as well as accelerated glomerular injury and exacerbated accumulation of advanced glycation end-products in models of diabetes [119, 120, 122]. A potential mechanism by which Gal3 may control inflammation is by performing maintenance of the epithelial gut barrier. In fact, lack of Gal3 impairs membrane polarization of mouse enterocytes *in vivo* [171], which may favor entrance of bacterial products, including LPS, into the systemic circulation. The ability of Gal3 to directly bind to LPS and to modulate LPS-induced inflammation are additional potential mechanisms linking this galectin to obesity and chronic pathologies.

All together, available evidence led us to formulate the hypothesis that elevation of Gal3 serves as an adaptive mechanism to counteract increased inflammation in many pathological conditions, at least in part via Gal3's regulatory effects on LPS-induced inflammation. To test this hypothesis, in the present study we administered repeated injections of ultra-low doses of LPS to WT and Gal3 KO mice to elevate systemic LPS to a level consistent with that observed in obesity and in chronic pathologies characterized by mild endotoxemia. We then evaluated the effect of Gal3 deficiency on adiposity, circulating levels of inflammatory cytokines and hematological parameters, as well as mRNA expression of inflammatory markers in liver and adipose tissue.

Materials and Methods

1. Animals and treatment

Age-matched female WT and Gal3 KO mice (The Jackson Laboratory, Bar Harbor, ME) on a C57BL6 background (4-6 months, 5-12 per group) received repeated i.p. injections

of LPS (*E. coli* O111:B1, Sigma Chemical Co., St. Louis, MO) at 5 ng/Kg body weight (BW) every 3 days from Day 1 to Day 10, a previously reported model [172] . Control mice received injections of PBS. Body weight was measured before each injection and at sacrifice. On Day 11 (16h after the last injection), all mice were fasted for 3h, then anesthetized using isoflurane and blood was collected. After cervical dislocation, liver and perigonadal fat were collected, weighed, and immediately frozen in liquid nitrogen until processing. Hematological parameters were evaluated on peripheral heparin-treated blood using HV950FS (Drew Scientific, Inc., Waterbury, CT). The remaining blood samples were then centrifuged and plasma was collected in clean Eppendorf tubes and frozen at -80°C for future cytokine measurements.

All animal studies were approved by the Animal care and Use Committee of the University of Illinois at Chicago.

2. Miscellaneous measurements

Blood glucose was measured from a tail nick immediately before sacrifice using a glucometer. Circulating levels of Leptin, Adiponectin, IL-6, chemokine (C-C motif) ligand 2 (CCL-2) and osteopontin were measured by ELISA using kits from Life Technologies and R&D Systems.

3. Gene expression analysis

Total RNA was isolated from liver and perigonadal fat using TRIzol (Life Technologies) and reverse transcribed. Gene expression of Adiponectin, CCL-2, Hepcidin-1, Leptin, LPS Binding Protein (LBP), Peroxisome proliferator-activated receptor gamma

coactivator 1-alpha (PGC-1 α), Plasminogen Activator Inhibitor-1 (PAI-1), Serum Amyloid A-1 (SAA-1), and Suppressor of cytokine signaling 3 (SOCS3) was assessed by real-time RT-PCR using either then SYBRGreen system or the TaqMan system. Relative expression was calculated using the $\Delta\Delta^{CT}$ method after normalizing for expression of GAPDH.

4. Statistical analysis

Data are expressed as mean \pm SEM. Statistical significance of differences was evaluated by independent t test or ANOVA. Differences were considered statistically significant at a value of $P < 0.05$. All statistical analysis was performed using SAS 9.3 (SAS Inc., Cary, NC, USA).

Results

1. Effect of repeated LPS administration on body weight, adiposity, adipokines and glycaemia in WT and Gal3 KO mice

Age-matched WT and Gal3 KO mice received 3 injections of LPS at 5 ng/Kg body weight or PBS, as detailed in the Methods section. Body weight was monitored throughout the whole treatment. As shown in Figure 8A, regardless of LPS administration, Gal3 KO mice had a significantly higher body weight compared to WT mice. Repeated administration of LPS did not significantly affect the weight of either Gal3 KO or WT mice.

Along with higher body weight, control Gal3 KO mice also had higher % perigonadal fat mass compared to control WT mice, while fat mass was comparable between the two

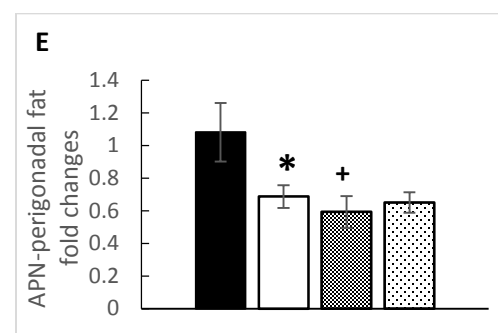
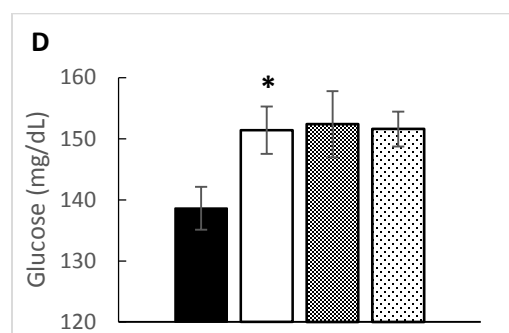
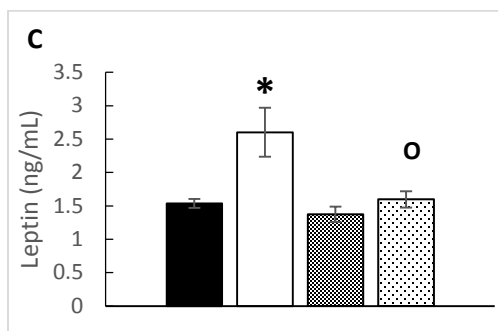
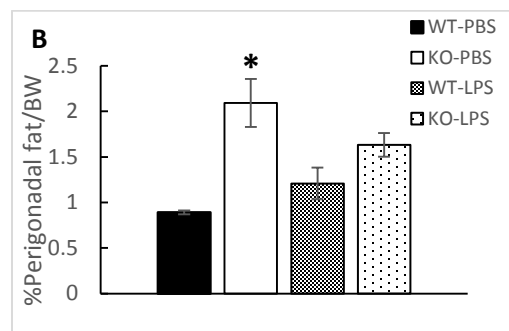
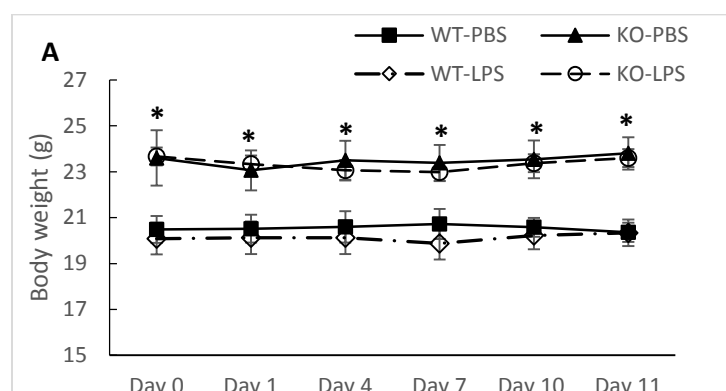
genotypes after LPS administration. Interestingly, in response to LPS WT mice trended towards a higher % perigonadal fat mass (~35% increase) when compared to their own control group, while Gal3 KO mice trended towards a lower fat percentage (~22% decrease; Figure 8B). In agreement with differences in fat mass, and as we previously demonstrated [114], control Gal3 KO mice had significantly higher circulating leptin compared to their WT counterparts. Administration of LPS significantly down-regulated circulating leptin levels in Gal3 KO but not in WT mice (Figure 8C).

We observed significantly higher fasting glucose levels in control Gal3 KO mice compared to the control WT group, again in agreement with previous studies [114]. Repeated administration of LPS led to a trend towards higher glucose levels in WT mice, while no changes were observed in Gal3 KO mice. Therefore, the differences in blood glucose between Gal3 KO and WT mice seen in the control groups were no longer present in LPS-injected groups (Figure 8D).

Perigonadal fat mRNA expression of adiponectin was 32% lower in control Gal3 KO mice compared to control WT mice, reflecting differences in both adiposity and glucose levels observed between the two genotypes and in line with our previous observations [114]. Administration of LPS significantly reduced adiponectin expression only in WT mice, thus eliminating the differences between the two genotypes (Figure 8E). Finally, circulating adiponectin levels were comparable in Gal3 KO and WT mice. However, LPS administration significantly down-regulated plasma levels of adiponectin only in Gal3 KO mice but not in WT mice (Figure 8F).

In summary, even though LPS administration did not affect body weight in either genotype, WT mice injected with LPS trended towards higher adiposity while Gal3 KO

mice reacted to LPS in the opposite direction. The significant baseline differences between the two genotypes in terms of % perigonadal fat mass, circulating leptin levels, adiponectin gene expression as well as blood glucose levels were all offset by repeated administration of ultra-low doses of LPS.



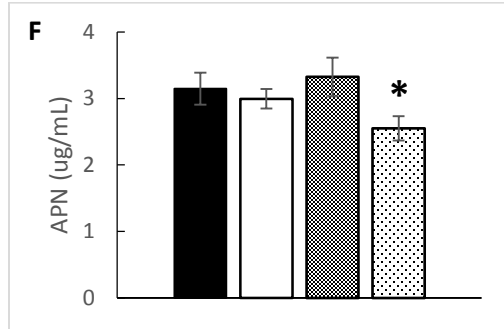


Figure 8 Effects of repeated ultra-low LPS administration on body weight, adiposity, adipokines, and glycaemia in Gal3 KO and WT mice.

Female Gal KO and WT mice received repeated i.p. injections of LPS at 5 ng/Kg body weight (BW) every 3 days from Day 1 to Day 10. Mice were sacrificed 24h post last LPS injection on Day 11. Body weight (**A**) was measured before each injections and sacrifice. Blood glucose levels (**D**) were measured before sacrifice. Plasma levels of leptin (**C**) and APN (**F**) were evaluated by ELISA. APN mRNA expression in the perigonadal (**E**) fat was examined by RT-PCR. Data are reported as gene expression in liver using the $\Delta\Delta\text{ct}$ method. Data are mean \pm SEM of 5-12 per group. * $P < 0.05$ versus corresponding WT group; $^{\circ}P < 0.05$ versus control groups in WT mice; $^{\circ}P < 0.05$ versus control groups in KO mice by one-way ANOVA.

2. Effect of repeated LPS administration on systemic and adipose tissue inflammation in WT and Gal3 KO mice

Next, we evaluated the inflammatory status of WT and Gal3 KO mice. Administration of LPS in the $\mu\text{g}/\text{mouse}$ range significantly increases circulating cytokine levels while inducing leukopenia in mice [89]. However, the ultra-low dose of LPS we used did not raise levels of the inflammatory marker IL-6 to detectable levels and did not significantly alter the relative proportion of circulating leukocyte populations (data not shown).

Similarly, circulating levels of the chemokine CCL-2 were comparable between WT and

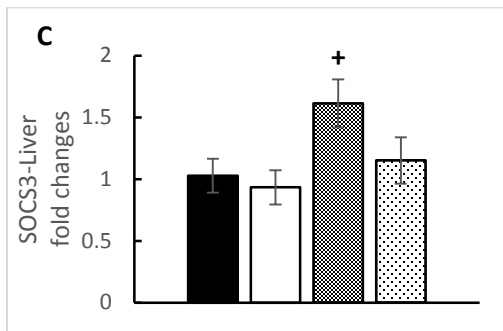
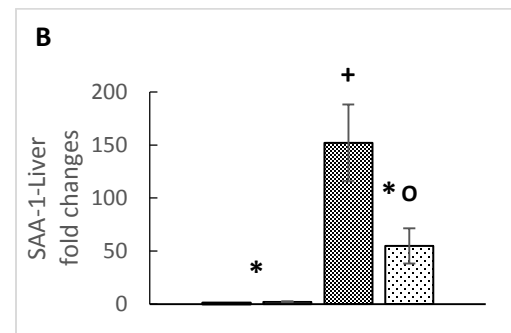
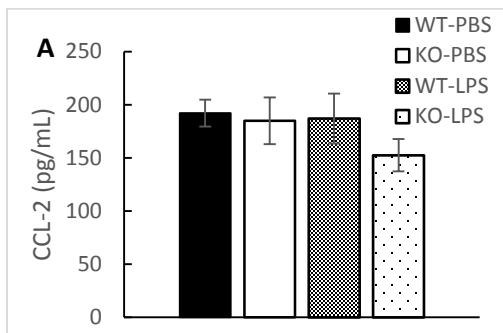
Gal3 KO mice regardless of LPS administration, with no significant changes observed in response to LPS in either genotype (Figure 9A).

Therefore, to assess the presence of systemic inflammation using a more sensitive measure, we turned to hepatic mRNA expression of the acute-phase protein SAA-1 and the signal transduction modulator SOCS-3. Baseline hepatic expression of SAA-1 was 2-fold higher in Gal3 KO compared to WT control mice (Figure 9B). Both Gal3 KO and WT mice responded to LPS with a significant elevation in hepatic mRNA expression of SAA-1, indicating induction of the acute-phase response in LPS-injected mice despite the ultra-low dose we employed. Importantly, Gal3 KO mice reacted to LPS with a significantly lower induction of SAA-1 compared to WT mice (Figure 9B). In agreement, LPS only induced a significant elevation of hepatic SOCS3 mRNA expression in WT but not in Gal3 KO mice (Figure 9C). Finally, there were no differences in hepatic gene expression levels of the acute-phase proteins PAI-1 and Hepcidin-1 or the marker of energy metabolism PGC-1 α between the two genotypes or in control *versus* LPS groups (Figure 9D).

Since Gal3 KO and WT mice reacted to LPS administration in opposite directions in terms of perigonadal fat changes, we also evaluated adipose tissue inflammation. As shown in Figure 9E, control Gal3 KO mice had significantly higher gene expression of LPS binding protein (LBP) in perigonadal fat compared to control WT mice. Injection of LPS significantly reduced LBP mRNA in Gal3 KO mice, while having no significant effect in WT mice. Similarly, LPS only down-regulated adipose tissue CCL-2 mRNA expression in Gal3 KO mice (Figure 9F). Finally, no significant alteration of TNF mRNA

expression was detected in perigonadal fat in response to LPS in either genotype (data not shown).

In summary, Gal3 KO mice responded to LPS with a reduced hepatic acute-phase response compared to their WT counterparts. However, LPS reduced markers of adipose tissue inflammation only in Gal3 KO WT mice.



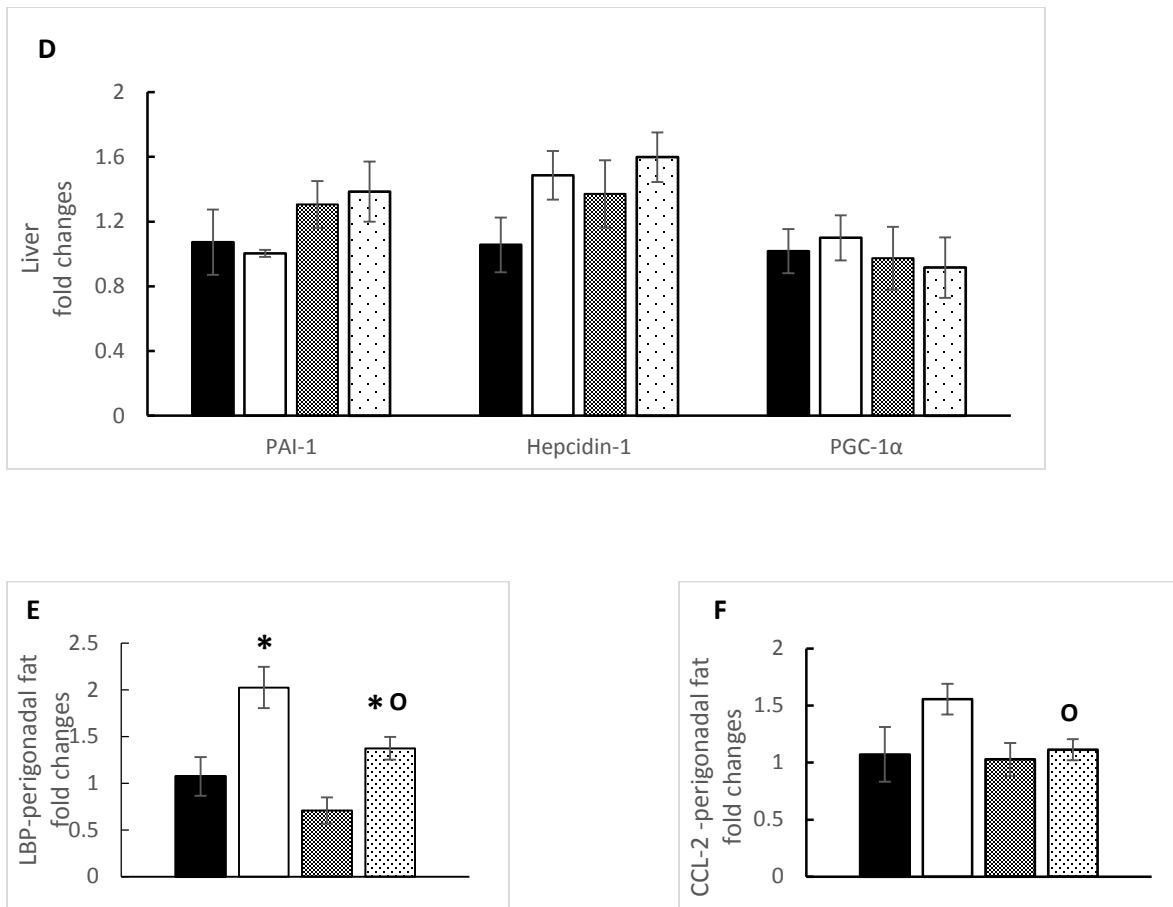


Figure 9 Effect of repeated ultra-low dose LPS administration on mRNA expression of inflammatory markers in liver and perigonadal fat in Gal3 KO and WT mice.

Inflammation was evaluated by measuring plasma level of CCL-2(A), mRNA expression levels of SAA-1 (B), SOCS3 (C), PAI-1, Hepcidin-1, PGC-1α (D) in liver, as well as LBP (E) and CCL2 (F) in perigonadal fat. Data are reported as gene expression in liver using the $\Delta\Delta ct$ method. Data are mean \pm SEM of 5-12 per group. *P<0.05 versus corresponding WT group; ^oP<0.05 versus control groups in WT mice; °P <0.05 versus control groups in KO mice by one-way ANOVA.

Discussion

In the present study we compared the response of WT and Gal3 KO mice to repeated injections of ultra-low doses of LPS to assess the contribution of Gal3 to pathologies characterized by mild endotoxemia,.

We report that WT mice responded to LPS with a trend towards increased adiposity coupled with hyperglycemia, reduced expression of adiponectin as well as induction of the hepatic-acute phase response. Our results agree with a report by Cani et al.

indicating that chronic infusion of LPS using mini-pumps increases adiposity and insulin resistance in C57BL6 mice [173], suggesting that endotoxemia alone can initiate obesity. However, in our study we used a much lower dose of LPS, which may reflect more closely the levels observed in obesity and chronic inflammatory pathologies.

Our data also confirm our previous findings demonstrating that, compared to age- and sex-matched WT mice, control Gal3 KO develop excess adiposity characterized by elevated fat mass with increased leptin and SAA-1 levels, and mild hyperglycemia, as well as reduced adipose tissue expression of adiponectin but unchanged circulating adiponectin concentrations [114]. Thus, control Gal3 KO mice present with several of the alterations that in WT mice develop only after administration of LPS. Numerous studies demonstrated the importance of impaired gut barrier function and altered microbiota in the onset and progression of many chronic inflammatory conditions, as a leaky epithelial barrier in the gut allows bacterial products to enter the lamina propria and eventually reach the systemic circulation and other organs [4, 94, 168-170].

Delacour et al. found that Gal3 KO mice had an aberrant distribution of brush border markers along their enterocyte lateral membrane, suggesting the presence of a

dysfunctional gut barrier in the absence of Gal3 [171]. Therefore, gut barrier abnormalities may lead to accumulation of bacterial products -- including the TLR4 agonist LPS and other microbial-derived molecular patterns that signal through TLR2 and TLR3 -- in Gal3 KO mice at baseline. Furthermore, our previous study showed that Gal3 KO mice have an aggravated response to TLR3-mediated inflammation compared to WT mice [174]. Moreover, Ruas et al. demonstrated that lack of Gal3 results in increased transcriptional levels of TLR2 and cytokine production in murine macrophages [175]. Therefore, all together evidence of a functional connection between Gal3 and TLR2/3 signaling contributes to explain the increased baseline inflammatory phenotype in Gal3 KO mice compared to WT mice.

Mild endotoxemia may exist in Gal3 KO mice at baseline due to an impaired gut barrier. However, repeated injections of LPS induced a general trend towards a worsened metabolic and inflammatory status in WT mice, whereas this response was much less marked – and in some cases even absent or reversed – in Gal3 KO mice, suggesting that Gal3 KO may have developed LPS tolerance, i.e., an inability to fully respond to an LPS challenge after a prior exposure to LPS [176]. Indeed, we detected that induction of the hepatic acute-phase response by LPS was significantly blunted in Gal3 KO compared to WT mice. These data are in agreement with our previous findings showing that feeding a HFD induced a much less marked relative response, in terms of inflammatory alterations, in Gal3 KO compared to WT mice [114], mostly as a result of KO mice starting out as already fatter, hyperglycemic and inflamed compared to WT mice. Furthermore, the discrepancies we observed between Gal3 KO and WT mice receiving repeated ultra-low doses LPS are also consistent with our previous study

demonstrating that Gal3 KO mice had less severe inflammation than WT mice after a single injection of a high but non-lethal dose of LPS [174]. Another group found that Gal3 works as an endogenous danger-signaling molecule to enhance the activation of Janus kinase/ signal transducers and activators of transcription (JAK/STAT) signaling pathways in mouse and rat glial cells [177]. Hence, without the augmentative effects of Gal3, defective activation of JAK/STAT may protect Gal3 KO mice from LPS-induced inflammation.

At variance with adipose tissue expression of adiponectin, which did not change in Gal3 KO mice in response to LPS, we found a significant decrease in plasma APN in LPS-injected Gal3 KO mice, with no change in WT groups. Pini et al. previously reported plasma APN levels were significantly down-regulated in response to a single injection of LPS in WT mice [89], which is inconsistent with what we found in our study. This discrepancy could result from the different dose and schedule of LPS administration used in the two studies. At variance with WT mice, we found plasma APN levels to be significantly reduced in Gal3 KO mice after LPS administration. A recent study indicated that bone marrow adipose tissue is a major source of circulating of APN [178].

Deficiency of Gal3 leads to an altered bone marrow environment in mice, affecting both the hematopoietic and non-hematopoietic compartments [179]. Therefore, the modified bone marrow microenvironment in Gal3 KO mice may result in inadequate production of APN to compensate for the inhibiting effect of LPS on adipose tissue APN expression, leading to decreased systemic APN.

In conclusion, repeated administration of ultra-low doses of LPS led to increased adiposity, mild hyperglycemia, and systemic inflammation in WT mice, while Gal3 KO

mice were relatively protected from such alterations, possibly due to their metabolic and inflammatory status already being altered at baseline. These results suggest that upregulation of Gal3 contributes to the pathophysiological alterations of conditions characterized by mild endotoxemia, which is against our initial hypothesis that upregulation of Gal3 would act as an adaptive protective mechanism. However, in agreement with previous findings, Gal3 is an important modulator of metabolic and inflammatory status under baseline conditions.

VII. DISCUSSION

Overall, in the three aims of the present study we investigated:

- 1) The association between circulating levels of Gal3 and markers of adiposity and inflammation in a cohort of predominantly obese and AA metabolically healthy women with different risks of CVD;
- 2) The reciprocal regulating effects of Gal3 and LPS in inflammation both *in vivo* and *in vitro* in mice;
- 3) The role of Gal3 in inflammation induced in mice by repeated injections of ultra-low dose LPS, which mimics the mild but persistent endotoxemia of obesity.

Collectively, we found:

- 1) Circulating levels of Gal3 were significantly higher in obese women compared to non-obese individuals. Increased adiposity and inflammation had a positive influence on circulating Gal3 levels in our study cohort. However, when we dichotomized the study population by CVD risk, adiposity and inflammation only correlated with Gal3 in women at low/moderate risk of CVD;
- 2) Gal3 specifically promoted LPS (TLR4)-induced acute inflammation in mice, as Gal3 KO mice were protected from inflammation induced by non-lethal doses of LPS. The reciprocal regulating effects of Gal3 and LPS may mainly take place in the intracellular spaces and in a complex manner.
- 3) Repeated injections of ultra-low dose LPS induced a trend towards a worsened metabolic and inflammatory status in WT mice, whereas this response was much less marked – and in some cases even absent or reversed – in Gal3 KO mice.

Together, our findings agree with the previous humans studies conducted in Caucasian and Asian populations showing that Gal3 levels are positively associated with increased adiposity and inflammation [10, 12, 14]. We extended those correlations to a multiracial cohort of predominantly AA and obese but metabolically healthy women. Moreover, we detected a plateau of Gal3 levels when BMI reaches 30, which indicates that elevation of Gal3 may be driven by multiple factors rather than just adiposity. Furthermore, the correlation between Gal3 and adiposity/inflammation was only present in women at low/moderate risk of CVD but not in those at high risk. We have two non-exclusive potential explanations for this finding: 1) In agreement with previous studies, significant metabolic, hormonal, and/or inflammatory complications disrupt the association between Gal3 and obesity [11, 12, 14]; 2) The group of women with high risk of CVD is predominantly composed of obese subjects. As we mentioned before, up-regulation of Gal3 reaches a plateau when BMI is over 30. Therefore, due to the high BMI in women at high CVD risk, the plateau of Gal3 may result in the loss of correlation between Gal3 and adiposity/inflammation.

However, the animal studies negate our initial hypothesis that the elevation of Gal3 in obesity works as an adaptive protective mechanism to counter obesity-associated inflammation by negatively regulating LPS-induced inflammation. In fact, Gal3 actually promoted both the acute and chronic inflammatory responses caused by non-lethal doses of LPS in mice. In response to a single injection of LPS, Gal3 KO mice showed lower circulating levels of cytokines and chemokines and less robust hepatic acute-phase responses compared to WT mice. LPS administration leads to activation of the NF- κ B signaling pathway and downstream cytokine production, which assists in the

amplification of the inflammatory cascade [15]. Several animal studies have shown decreased NF- κ B activation in Gal3 KO mice or in cells without Gal3 [106, 158].

Therefore, it is likely that Gal3 KO mice were protected from acute inflammation induced by non-lethal doses of LPS via reduced activation of the NF- κ B signaling pathway.

Moreover, previous studies indicate that Gal3 promotes adhesion of neutrophils to endothelial cells [160], which could account for the higher % neutrophils in the peripheral blood of Gal3 KO mice compared to that in WT mice. Additionally, i.p. injection of thioglycollate results in less immune cell counts in the peritoneal cavity in Gal3 KO mice than in WT mice [106]. This finding may explain the higher splenic % neutrophils and monocytes in Gal3 KO mice receiving LPS compared to their WT counterparts. Furthermore, Gal3 KO mice responded more to the TLR3 agonist poly I:C than WT mice did, which is opposite to what we observed with LPS administration, indicating that the promoting effect of Gal3 is specific for LPS-, and therefore TLR4-mediated signaling.

Finally, inhibition of extracellular Gal3 did not replicate the effects of genetic deletion of Gal3 in mice in response to LPS, while *in vitro* experiments using mouse peritoneal macrophages indicated that LPS only down-regulated Gal3 levels inside the cells.

Therefore, it is likely that intracellular Gal3 plays the main role in regulating LPS-induced inflammation via intracellular activation of inflammatory signaling pathways.

As we expected, repeated injections of ultra-low dose LPS induced a trend towards higher adiposity, mild hyperglycemia, and increased hepatic acute-phase responses in WT mice, while those alterations were either less marked, absent, or even reversed in Gal3 KO mice, which agrees with the effects of Gal3 deficiency in LPS-induced acute

inflammation. We can therefore conclude that Gal3 promotes both acute and chronic inflammation induced by LPS in mice.

Elevated Gal3 levels are present in the circulation of both obese humans and animals, while Gal3 KO mice exhibit more severe inflammation and advanced adiposity in response to HFD-induced obesity compared to WT mice [12, 114, 115]. This evidence reveals a potential protective role of Gal3 in obesity. Hence, up-regulation of Gal3 may work as an adaptive mechanism to counteract the detrimental effects of obesity-induced pathological changes, such as increased inflammation, aberrant expansion of adipose tissue, and ectopic lipid and glucose adducts accumulation. Moreover, Gal3 is essential for the maintenance of an intact gut barrier structure [171], which prevents leakage of bacterial products into the gut and eventually to the systemic circulation. Obesity leads to impaired gut barrier function, which allows more bacterial products, including LPS and other TLR agonists, to enter the circulation and cause inflammation [180]. Our animal studies have shown that Gal3 promotes LPS-induced inflammation in mice. Therefore, we can conclude that the protective effects of Gal3 on obesity-associated inflammation is not due to the reciprocal regulation of Gal3 and LPS. Stronger protective activities that Gal3 performs in obesity may counteract its promoting effects on the mild endotoxemia of obesity.

Numerous studies have been conducted to investigate the roles Gal3 plays in a variety of biological processes [7, 8, 103]. Based on the available studies as well as our own findings, here we propose several potential mechanisms by which Gal3 may act as a protective mediator by directly regulating obesity and obesity-associated metabolic complications.

Firstly, as we mentioned before, in contrast to the positive influence on LPS (TLR4)-induced inflammation, Gal3 inhibits TLR2 and TLR3-mediated inflammation [174, 175]. In fact, we showed that Gal3 KO mice develop more severe inflammation and hypoglycemia in response to a TLR3 agonist compared to WT mice, while another group observed increased TLR2 expression and cytokine production in murine macrophages lacking Gal3 [175]. Obesity leads to impaired gut barrier that allows more bacterial products, including TLR2 and TLR3 agonists, into the circulation [181, 182]. Therefore, the up-regulation of Gal3 in obesity may work as an inhibitor to counteract increased TLR2/3 agonist-induced inflammation due to the leaky gut.

Secondly, defective lipolysis in adipose tissue is present in obesity and contributes to abnormal triglyceride storage and free fatty acid release [183]. We have previously shown that obese Gal3 KO mice have a significantly suppressed mRNA expression of adipocyte triglyceride lipase, a rate-limiting enzyme in lipolytic pathways, indicating a possible decreased lipolysis in adipose tissue due to Gal3 deficiency [114]. In our current study, Gal3 KO mice in the control group already had significantly higher body weight with elevated % perigonadal fat mass compared to age-matched WT mice. These findings could be due to defective lipolysis in the adipose tissue of Gal3 KO mice. Therefore, Gal3 may work as a protective molecule to maintain lipolysis in obese humans and animals.

Thirdly, Pejnovic et al. found increased infiltration of pro-inflammatory macrophages and enhanced activation of T cell receptor (TCR)-mediated signaling in adipose tissue of obese Gal3 KO mice [115]. Another group indicated that extracellular Gal3 down-regulates TCR-mediated signaling pathways to dampen the uncontrolled activation of T

cells [184]. Moreover, the balance between the pro- and anti-inflammatory phenotypes of macrophages is also affected by Gal3 [129]. Therefore, Gal3 may inhibit obesity-associated inflammation by modulating immune cell infiltration and activation in adipose tissue.

Finally, accumulation of the glucose and lipid products AGE and ALE is a key detrimental factor in obesity and obesity-associated complications [185]. As a scavenger molecule, Gal3 binds to AGE/ALE and is involved in the removal of those molecules [96]. In fact, increased renal AGE accumulation has been observed in Gal3 KO mice, with accelerated diabetic glomerulopathy compared to WT mice [122]. Also, Gal3 deficiency in mice with atherosclerosis led to increased mRNA expression of the receptor of AGE (RAGE), which binds to AGE and leads to elevated pro-inflammatory cytokine production [116]. Hence, up-regulation of Gal3 in obesity may help remove the excess production of AGE and ALE and maintain the homeostasis of glucose and lipid metabolisms.

Overall, all of these Gal3-mediated regulatory effects may work together to maintain or regain homeostasis in obesity.

VIII. STRENGTHS & LIMITATIONS

A. Strengths

Our study has several strengths.

- 1) Our study is the first to focus on the association of Gal3 with obesity and obesity-associated inflammation in women who are predominantly AA, thus providing further evidence of Gal3 as an inflammatory modulator in the general population with racial/ethnic diversity.
- 2) We are the first to report a possible adiposity-related threshold of Gal3 in women, which reveals that up-regulation of Gal3 may be driven by multiple factors rather than only adiposity.
- 3) We are the first to evaluate the relationship between Gal3 and adiposity/inflammation in metabolically healthy women with different CVD risks.
- 4) We are the first to indicate the promoting effect of Gal3 in inflammation induced by non-lethal doses of LPS.
- 5) We included a combination of *in vivo* and *in vitro* experiments to investigate the reciprocal regulation of Gal3 and LPS.
- 6) We used both age-matched male and female mice to investigate the reciprocal regulation of Gal3 and LPS.
- 7) We used both genetic deletion and pharmacologic inhibition of Gal3 in mice to locate intracellular Gal3 as the major regulator on LPS-induced inflammation.

B. Limitations

Our study also has several limitations.

- 1) The study only included women, and the majority of the study subjects were in the obese class III category but metabolically healthy, which may not be generalizable to men or the general population.
- 2) We only used CRP levels to evaluate CVD risk, while inclusion of other risk factors may have either strengthened or weakened our findings.
- 3) The Gal3 KO and WT mice used in our study were sex- and age-matched but not littermates.
- 4) We did not evaluate the efficiency of N-Lac inhibition of Gal3 *in vivo*.
- 5) We did not measure activation of the NF- κ B signaling pathway in the LPS-induced acute inflammation model, or JAK/STAT activation in the repeated injections of ultra-low dose LPS model.

IX. CONCLUSIONS

Our study demonstrated that up-regulated circulating Gal3 levels associate with increased adiposity and inflammation in predominantly obese and AA metabolically healthy women, while metabolic complications disturbs this positive association. Up-regulation of Gal3 as an adaptive mechanism to counteract obesity-induced metabolic complications is not due to the reciprocal regulation of Gal3 and LPS, as Gal3 specifically promotes LPS (TLR4)-induced inflammation in mice.

Together, our study contributes to a better understanding of the interactions between Gal3 and LPS that characterize the pathogenesis of many clinical conditions, including obesity.

X. FUTURE DIRECTION

To better understand the mechanisms by which Gal3 modulates obesity and obesity-associated inflammation, further studies should be focusing on the following aspects:

- 1) Because Gal3 interacts with different TLR agonists and performs opposite regulating effects on them, more studies should be done to elucidate the possible signaling pathways by which Gal3 modulates TLR agonist-induced inflammation.
- 2) Gal3 deficiency protects mice from mild endotoxemia, with reduced inflammatory protein mRNA expression in adipose tissue, while obesity-induced adipose tissue inflammation is more pronounced in Gal3 KO mice. This discrepancy indicates Gal3 works as a multifunctional protein responding differently to various stimuli and results in diverse regulatory effects of Gal3 in many biological processes. Therefore, further studies should be conducted regarding the role Gal3 plays in several obesity-associated metabolic alterations, such as adipocyte lipolysis, immune cell infiltration and activation, as well as aberrant accumulation and removal of glucose and lipid adducts.
- 3) As a mediator of inflammation, Gal3 is involved in the pathogenesis of many diseases. Our study indicates that LPS significantly affects Gal3 at both protein and gene expression levels *in vivo* and *in vitro*, which may in turn influence the inflammatory pathways mediated by Gal3. However, the mechanism of such impact of LPS on Gal3 is still not clear. Therefore, more studies should be conducted to elucidate the underlying mechanisms of how LPS regulates Gal3.

Appendix A

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Curriculum Vitae

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PUBLICATIONS

1. **Pang, J.**, V.T. Nguyen, D.H. Rhodes, M.E. Sullivan, C. Braunschweig, and G. Fantuzzi, *Relationship of galectin-3 with obesity, IL-6, and CRP in women*. J Endocrinol Invest, 2016.
2. **Pang, J.**, D.H. Rhodes, M. Pini, R.T. Akasheh, K.J. Castellanos, R.J. Cabay, D. Cooper, M. Perretti, and G. Fantuzzi, *Increased adiposity, dysregulated glucose metabolism and systemic inflammation in Galectin-3 KO mice*. PLoS One, 2013. 8(2): p. e57915.
3. Akasheh, R.T., **Pang, J.**, York, J.M., and G. Fantuzzi, *New pathways to control inflammatory responses in adipose tissue*. Curr Opin Pharmacol, 2013. **13**(4): p. 613-7. (These authors equally contributed to this manuscript)
4. Qiang, G., H. Whang Kong, S. Xu, H.A. Pham, S.D. Parlee, A.A. Burr, V. Gil, **Pang, J.**, A. Hughes, X. Gu, G. Fantuzzi, O.A. MacDougald, and C.W. Liew, *Lipodystrophy and severe metabolic dysfunction in mice with adipose tissue-specific insulin receptor ablation*. Mol Metab, 2016. **5**(7): p. 480-90.
5. Akasheh, R.T., M. Pini, **Pang, J.**, and G. Fantuzzi, *Increased adiposity in annexin A1-deficient mice*. PLoS One, 2013. 8(12): p. e82608.

PENDING PUBLICATIONS

1. **Pang, J.**, Fantuzzi, G., *The reciprocal regulating effects of lipopolysaccharide and Galectin-3 in mice*. (Submitted: J Leukoc Biol, 2016).
2. **Pang, J.**, Fantuzzi, G., *Effect of galectin-3 deficiency in the response to repeated ultra-low doses of LPS in mice*. (Submitted: Biochem Biophys Res Commun, 2016).
3. Liew, C.W., Xu, S., Wang, X., Whang Kong, H., McCann, M., **Pang, J.**, Fantuzzi, G., O'Donnell, M., and E.D. Lewandowski, *Multiphasic metabolic regulation in the systemic and peripheral organ responses to cardiac hypertrophy*. (Submitted: Circulation, 2016)

SCIENTIFIC MEETINGS/ CONFERENCES

Cell to Community Research Day, Chicago, IL, October 2015

Oral presentation: Galectin-3 in LPS-induced inflammation

UIC Diabetes-Obesity Research Day, Chicago, IL, October 2014

American Diabetes Association Annual Meeting, Chicago, IL, June 2014

Digestive Disease Week, Chicago, IL, May 2014

UIC Cancer Center Forum, Chicago, IL, April 2014

Poster presentation: Increased serum Galectin-3 in non-diabetic obese women

Combined Annual Meeting of Clinical and Translational Research and the Midwestern Section of the American Federation for Medical Research, Chicago, IL, April 2014

Kenneth Rainin Foundation Innovations Symposium, Chicago, IL, July 2013

Keystone Symposia on Molecular and Cellular Biology, Keystone, CO, February 2013

Poster presentation: Increased adiposity, dysregulated glucose metabolism and systemic inflammation in Galectin-3 KO mice.

Cells to Community: Current Research in Integrative Pathophysiology and Health Promotion, Chicago, IL, September 2012

Oral presentation: Increased adiposity, dysregulated glucose metabolism and systemic inflammation in Galectin-3 KO mice.

Obesity 30th Annual Scientific Meeting, San Antonio, TX, August 2012

Poster presentation: Increased adiposity, dysregulated glucose metabolism and systemic inflammation in Galectin-3 KO mice.

UIC Student Research Forum, Chicago, IL, April 2012

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