Galectin-3 Deficiency Accelerates High-Fat Diet Induced Obesity and Amplifies Inflammation in Adipose Tissue and Pancreatic Islets

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ABSTRACT

Obesity-induced diabetes is associated with low-grade inflammation in adipose tissue and macrophage infiltration of islets. We show that ablation of Galectin-3, a galactoside-binding lectin, accelerates high-fat diet-induced obesity and diabetes. Obese LGALS3\(^{-/-}\) mice have increased body weight, amount of total visceral adipose tissue, fasting blood glucose and insulin levels, HOMA-IR and markers of systemic inflammation compared to diet-matched WT animals. Obese LGALS3\(^{-/-}\) visceral adipose tissue exhibited increased incidence of Type-1 T and NKT lymphocytes and pro-inflammatory CD11c+CD11b macrophages and decreased CD4+CD25+FoxP3+ Tregs and M2 macrophages. Pronounced mononuclear cell infiltrate, increased expression of NLRP3 inflammasome and IL-1β in macrophages and increased accumulation of advanced glycation endproducts (AGE) and receptor for AGE (RAGE) expression were present in pancreatic islets of obese LGALS3\(^{-/-}\) animals accompanied with elevated phosphorilated NFκB p65 and mature Caspase-1 protein expression in pancreatic and visceral adipose tissue. In vitro stimulation of LGALS3\(^{-/-}\) peritoneal macrophages with lipopolysaccharide (LPS) and saturated fatty acid palmitate caused increased Caspase-1 dependent IL-1β production and increased phosphorilation of NFκB p65 compared to WT cells. Transfection of LGALS3\(^{-/-}\) macrophages with NLRP3 siRNA attenuated IL-1β production in response to palmitate and LPS plus palmitate. Obtained results suggest important protective roles for Gal-3 in obesity-induced inflammation and diabetes.
Metabolic inflammation, ‘metaflammation’, is a chronic, low-grade adipose tissue inflammation triggered by various metabolic ‘danger’ signals during obesity that precedes the development of insulin resistance and type 2 diabetes (1). Adipose tissue-associated regulatory T cells (Tregs), Type 2 T helper cells and alternatively activated M2 macrophages protect from instigation of nutrient excess-induced inflammation (2), while the recruitment of Type 1 T helper lymphocytes and M1 macrophages and decreased Tregs in adipose tissue precede metabolic disorders (3, 4). Pro-inflammatory IL-1β and TNF-α impair insulin sensitivity, but molecular pathways that associate inflammation, diet and type 2 diabetes are not fully understood (5). It has been postulated that activation of NFκB, a family of transcription factors that regulate the expression of proinflammatory genes upon cell stimulation with various factors including hyperglycemia and free fatty acids (FFAs) is a molecular mechanism involved in insulin resistance (6). Most recently, the crucial role for NLRP3 inflammasome that consists of NLRP3 molecules, adaptor protein ASC and procaspase-1 that catalytically activates caspase-1 causing the release of IL-1β and IL-18 was demonstrated in studies in which ablation of NLRP3 inflammasome prevented obesity-induced inflammation and insulin resistance (7, 8, 9).

Galectin-3, also known as Mac-2, is a 30-kD β-galactoside-binding lectin, mainly located in the cytoplasm, but also in the nucleus, is expressed by a variety of cell types and regulates various T-cell functions and innate immune responses (10). Gal-3 plays an important disease-exacerbating role in autoimmune/inflammatory and malignant diseases (11, 12, 13, 14). Gal-3 is also one of the pattern recognition receptors (PRRs) that bind and mediate degradation of modified lipoproteins and advanced glycation end-products (AGE) (15). In contrast to other receptor for AGE (RAGE), Gal-3 acts to protect from AGE-induced tissue injury. Therefore, Gal-3 ablation accelerates AGE-induced kidney injury in diabetes (16) and enhances atherogenesis (17). Gal-3
protects β-cells from the cytotoxic effect of IL-1β in rats (18) and its increased expression was demonstrated in islet endothelial cells in obesity-induced diabetes in mice (19).

The aim of this study was to investigate the role of Gal-3 in high-fat diet-induced obesity and associated metabolic abnormalities by using mice lacking Gal-3 on a C57BL/6 background. We report here that Gal-3 ablation accelerated high-fat diet-induced obesity and amplified inflammation in adipose tissue and pancreatic islets.

RESEARCH DESIGN AND METHODS

Experimental mice

Male Galectin-3 deficient (LGALS3<sup>−/−</sup> mice) on the C57BL/6 background and their littermate controls, wild-type (WT) C57BL/6 mice (6-weeks old) obtained from University of California Davis, CA, USA (by courtesy of Dr D.K. Hsu and Prof F.T. Liu) were fed either low-fat diet (LFD) (3% fat) or high-fat diet (HFD) (60% fat) obtained from Mucedola, Milano, Italy for 11 or 18 weeks. All animal procedures were approved by the Ethical Committee (04/11) of the Faculty of Medical Sciences, University of Kragujevac, Serbia.

Metabolic parameters

Body weights and fasting blood glucose levels were measured once every two weeks. Mice were fasted for 4h and glucose levels (mmol/l) were determined using Accu-Chek Performa glucometer (Roche Diagnostics, Mannheim, Germany). Serum concentrations of lipids and HbA1c were measured using Olympus AU600 chemistry immuno analyzer (Olympus, Japan) and fasting insulin in sera using Rat/Mouse Insulin ELISA kit (Millipore Corporation, Billerica, MA, USA). HOMA-IR was calculated as described (20).
Isolation of visceral adipose tissue stromal vascular fraction (SVF) cells

Total visceral adipose tissue (VAT) was minced, placed in PBS containing 1mg/ml collagenase type II and 2% BSA (Sigma-Aldrich, St.Louis, MO, USA), incubated for 1h in water bath at 37°C and passed through a 40 µm nylon cell strainer (BD Biosciences San Jose, CA, USA) to enrich stromal vascular fraction cells (SVF).

Isolation of pancreatic mononuclear cells

Dissected pancreata were pooled, minced and digested using 2mg/ml collagenase type V in HBSS (Sigma-Aldrich) with 10% of FCS for 15 minutes in water bath at 37°C (21) and digests passed through a 40 µm cell strainer.

Isolation of splenocytes

Spleens were excised and single-cell suspensions obtained by mechanical disrupting and RBCs lysed.

Flow cytometry

Cells were labeled with fluorochrome-conjugated monoclonal antibodies: anti-mouse CD3, CD4, CD8, CD44, CD62L, CD279 (PD-1), CD11b, IFN-γ, IL-17 (BD Biosciences), NK1.1, F4/80, CD206 and CD11c antibodies (BioLegend, San Diego, CA, USA). For intracellular staining, cells were activated with PMA/ionomycin and processed as previously described (22). Primary rabbit anti-mouse NLRP3, IL-1β, NFκB (phospho S536) antibody (1µg/ml, Abcam, Cambridge, UK) and secondary Goat anti-rabbit antibody labeled with PE-Cy5.5 (1/400, Invitrogen,
Carlsbad, CA, USA) were used. Cells were analyzed with FACSCalibur Flow Cytometer (BD Biosciences) and analysis conducted with FlowJo (Tree Star).

**Pancreatic histology and insulitis scoring**

Pancreatic tissue cryostat sections were stained with H&E. Histological analysis of the distribution of inflammatory cell infiltrate in pancreatic islets was performed in blinded fashion by two independent observers. The images were captured with a light microscope (BX51, Olympus, Japan) equipped with a digital camera. Insulitis was graded and a mean insulitis score calculated (23).

**Immunofluorescent staining**

Immunofluorescent staining of pancreatic tissue cryosections (5µm) was performed using rabbit anti-mouse NLRP3 (1:200), IL-1β (1:200), IL-18 (1:200), RAGE (1:400) and AGE (1:200) antibodies (Abcam), followed by incubation with goat anti-rabbit IgG antibody PE-Cy 5.5, followed by FITC-conjugated anti-mouse insulin antibody (1:400, Abcam)). The sections were mounted with ProLong Gold antifade reagent with DAPI (Invitrogen) and analyzed at 40 X magnification using Nikon eclipse Ti-E inverted research microscope equipped with NIS-Elements Imaging software (Nikon Instruments Inc., Melville, NY, USA). Only brightness and contrast were adjusted.

**Immunohistochemistry**

Pancreatic tissue cryosections were incubated with biotinlated F4/80 antibody (Invitrogen) followed by visualization using Mouse Specific HRP/DAB Detection IHC Kit (Abcam) and incubated with rabbit anti-mouse NLRP3, IL-1β, AGE, RAGE and Gal-3 were visualized by
rabbit specific conjugate (Expose Rb specific HRP/AEC detection IHC Kit, Abcam) and photomicrographed with a digital camera mounted on light microscope (Olympus BX51, Japan), digitized and analyzed (24). Results are expressed as % positive staining in the specified region.

**Cell culture**

Macrophages were collected from peritoneal cavity of mice under sterile conditions and cultured in complete DMEM medium supplemented with 10% FCS at 37°C in 5% CO₂ incubator. After pretreatment with LPS (100 ng/ml) for 4 h, cells were stimulated with BSA alone or palmitate-BSA (100 µM), glucose (22mM), H₂O₂ (10µM) (all from Sigma-Aldrich) for 24 h. Where indicated cells were pre-incubated with Caspase-1 inhibitor Z-YVAD-FMK (10µM) (Bachem AG, Bubendorf, Switzerland).

**Caspase-1 activity assay**

For the determination of Caspase-1 activity, we used Caspase-1 Colorimetric Kit (#BF14100, R&D Systems) according to manufacturer's recommendations.

**NLRP3 knock-down with small interfering RNA (siRNA transfection)**

Peritoneal macrophages were seeded on 6-well plates (1X10⁶ cells/well), washed once with 2 ml of siRNA Transfection Medium and transfected with 60 pmols NLRP3 siRNA or with 60pmols Negative Control siRNA, and incubated for 7h according to the manufacturer’s instructions (Santa Cruz, USA).
Western blot

Pancreatic tissue lysates were prepared in a solution containing 62.5 mM Tris-HCl pH 6.8, 2% w/v sodium dodecyl sulfate (SDS), 10% glycerol, 50 mM dithiothreitol (DTT), 0.01% w/v bromophenol blue, 1 mM phenylmethylsulphonyl fluoride (PMSF), 1 µg/ml aprotinin, 2 mM EDTA, while proteins from visceral adipose tissue were isolated with TRIzol reagent (Genosys, Woodlands, TX, USA) and further processed (25). Rabbit polyclonal IgG antibodies to ASC (1:500), NLRP3 (1:500), IL-1β (1:2500), Caspase-1 (1:500), NFkB p65 (phospho S536) (1:500), NFkB p65 (1:500) (all from Abcam) and mouse anti-mouse IgG1 β-actin antibody (1:500) (Sigma-Aldrich) as internal control were used, followed by donkey anti-rabbit HRP at 1:10000 or sheep anti-mouse HRP (1:2500) (GE Healthcare, Buckinghamshire, England). Detection was performed by chemiluminescence (ECL, GE Healthcare) and photographs made by X-ray films (Kodak, USA). Scion Image Alpha 4.0.3.2 (Scion Corporation, Frederick, MD, USA) was used to calculate protein expression.

Cytokine measurement

Cytokine levels were determined using Mouse Duo Sets for IL-1β/IL-1F2, IL-6, IL-4, IL-13, IL-10, IFN-γ, IL-17 and CRP (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's recommendations.

Statistical analyses

All data are presented as means±s.e.m. Statistical significance was determined by analysis of variance (one-way ANOVA and Bonferroni post-hoc test), independent sample t-test, and where appropriate, Kruskal-Wallis and Mann-Whitney U test. Relationships between variables were
assessed using Pearson's correlation. Statistical significance was assumed at \( p<0.05 \). Statistical analyses were performed using the SPSS 13.0.

RESULTS

Lack of Galectin-3 accelerates high fat diet-induced obesity and obesity-related metabolic abnormalities

Body weight (Fig 1A) and total visceral adipose tissue weight (Fig 1B) were significantly higher in HFD-fed LGALS3\(^{-/-}\) mice compared to other experimental groups at both 11 and 18 weeks, and significantly greater in WT mice on HFD vs. LFD at 18 weeks (Fig 1A and 1B). There was no difference in food intake between LGALS3\(^{-/-}\) and WT mice (Fig 1A). Fasting blood glucose levels and insulinemia, and HbA1c were significantly increased in HFD-fed LGALS3\(^{-/-}\) mice compared to other experimental groups at both 11 and 18 weeks, while in WT mice on HFD vs. LFD at 18 weeks the differences did not reach statistical significance (Fig 1C and 1D). Hyperglycemia was noticed in LGALS3\(^{-/-}\) mice fed LFD at 18 weeks (Fig 1C). Of note, the significantly increased body weight (26.25g±0.66 vs. 22.19g±0.67, \( p=0.010 \)) and fasting glycemia (8.92mmol/l±0.47 vs. 6.82mmol/l±0.31, \( p=0.004 \)) were already noticed after three weeks of HFD in LGALS3\(^{-/-}\) mice compared to diet-matched WT mice. HbA1c (%) and fasting glycemia significantly correlated in LGALS3\(^{-/-}\) mice at 18 weeks (Fig 1C). HOMA-IR was significantly higher in HFD-fed LGALS3\(^{-/-}\) mice compared to other experimental groups at 11 and 18 weeks (Fig 1E). Total, HDL and non-HDL cholesterol serum levels increased with HFD, but were not significantly different between the genotypes and serum levels of triglycerides were similar between experimental groups at 11 weeks (data not shown).
Type-1 T/NKT cells are increased in visceral adipose tissue in obese LGALS3<sup>−/−</sup> mice

Significantly increased CD3+ T cells and CD3+NK1.1+ NKT cells were found in visceral adipose tissue in HFD-fed LGALS3<sup>−/−</sup> mice (Fig 2A) with markedly increased CD3+NK1.1+ IFN-γ+ Type 1 NKT cells, while CD3+IFN-γ+ Type 1 T cells were significantly higher compared with LFD-fed mice of both genotypes at 11 weeks (Fig 2B). The incidence of CD4+ or CD8+ T naïve, memory or effector populations and CD3+IL-17+ cells was not different between groups (data not shown). CD4+CD25+FoxP3+ Tregs were significantly lower in VAT and spleens (p=0.071) of obese LGALS3<sup>−/−</sup> mice compared with other experimental groups (Fig 2C and 2D). CD4+PD-1+ cells were significantly increased in VAT and spleens with HFD in LGALS3<sup>−/−</sup> mice (Fig 2E).

Increased pro-inflammatory and reduced alternatively activated M2 macrophages in LGALS3<sup>−/−</sup> visceral adipose tissue

We next monitored the recruitment of macrophages into adipose tissue after 11 weeks on HFD to look for potential mechanisms of glucose metabolism abnormalities in obese LGALS3<sup>−/−</sup> mice. Pro-inflammatory F4/80+CD11c+CD206+ macrophages significantly increased in adipose tissue of LGALS3<sup>−/−</sup> mice fed HFD compared to other experimental groups (Fig 3A). HFD increased splenic F4/80+CD11b+CD11c+ bone-marrow-derived dendritic cells (BMDC) in mice of both genotypes (data not shown). After 18 weeks on HFD, adipose tissue BMDCs were significantly higher in LGALS3<sup>−/−</sup> compared with WT mice (72.63±3.91 vs. 38.04±10.54, p=0.006). Alternatively activated F4/80+CD11c−CD206+ M2 macrophages were markedly reduced in VAT from both LGALS3<sup>−/−</sup> and WT mice fed HFD compared with LFD-fed WT mice at 11
weeks (Fig 2B) and further decreased in LGALS3<sup>−/−</sup> mice fed HFD for 18 weeks, being significantly lower compared with diet-matched WT mice (0.93±0.093 vs. 7.66±2.99, p=0.046).

**Body weights and fasting glycemia correlate with adipose tissue pro-inflammatory macrophages and Tregs in LGALS3<sup>−/−</sup> mice**

In LGALS3<sup>−/−</sup> mice, pro-inflammatory F4/80<sup>+</sup>CD11c<sup>+</sup>CD206<sup>+</sup> adipose tissue macrophages significantly correlated with body weight (r=0.919, p=0.001) and fasting blood glucose levels (r=0.912, p=0.002) (Fig 3C), whereas Tregs inversely correlated with body weight (r=0.633, p=0.067) and fasting glycemia (r=0.819, p=0.007) (Fig 3D) in contrast to wild-type mice (data not shown).

**Systemic inflammatory profile in obese LGALS3<sup>−/−</sup> mice**

Serum cytokine levels were measured to evaluate systemic inflammatory profile in studied mice. CRP, IL-6, and IL-4 levels significantly increased in sera of LGALS3<sup>−/−</sup> mice with HFD, while IL-13 and IL-10 levels significantly decreased compared with diet-matched WT mice at 11 weeks (Fig 4A). After 18 weeks on HFD, IL-4 and IL-1β levels were significantly higher, while IL-10 levels remained significantly lower in LGALS3<sup>−/−</sup> mice (Fig 4B) with no significant difference in serum levels of IFN-γ (Fig 4A and 4B) and IL-17 (data not shown) compared with WT mice.

**Pronounced mononuclear cell infiltration in pancreatic islets in obese LGALS3<sup>−/−</sup> mice**

Given the fact that LGALS3<sup>−/−</sup> mice exhibited obesity-related abnormalities of glucose metabolism, we performed histological analysis of the distribution of inflammatory cell infiltrate in pancreatic islets from LGALS3<sup>−/−</sup> and WT mice fed HFD or LFD for 11 weeks (Fig 5A). We found significantly increased average insulitis as expressed by insulitis score in HFD-fed
LGALS3<sup>−/−</sup> mice compared with other experimental groups (Fig 5B). The percentage of total intact islets and those with peri-vascular/peri-ductal and peri-insulitis was 58% in LGALS3<sup>−/−</sup> mice compared with 89% in WT mice, both on HFD (p<0.001). The percentage of islets with mild or severe insulitis were 24% and 17% in HFD-fed LGALS3<sup>−/−</sup> mice compared to 10% of islets with mild insulitis and none with severe insulitis in WT mice fed HFD (both p<0.001). Of note, the percentage of islets with mild or severe insulitis were 17% and 5% in LFD-fed LGALS3<sup>−/−</sup> mice compared to 2% of islets with mild insulitis (p<0.05) and none with severe insulitis in WT mice fed LFD (p<0.05). The percentage of islets with mild insulitis was significantly higher in HFD-fed WT mice compared with LFD-fed WT mice (p<0.05).

Flow cytometric analysis of mononuclear cells isolated from digested pancreata of obese LGALS3<sup>−/−</sup> mice revealed that the majority cells were F4/80+CD11b+ myeloid cells, among which the pro-inflammatory F4/80+CD11c+CD11b+ BMDCs were the predominant cell type (Fig 5C).

**Higher incidence of NLRP3 inflammasome and IL-1β expressing macrophages in islets and adipose tissue in obese LGALS3<sup>−/−</sup> mice**

We next sought to assess NLRP3 inflammasome and IL-1β expression in pancreatic islets of mice fed HFD for 11 weeks. Immunofluorescence and morphometric immunohistochemical data showed increased NLRP3 inflammasome expression in islets of HFD-fed LGALS3<sup>−/−</sup> mice (Fig 6A, upper panel) compared with HFD-fed WT mice (Fig 6A, lower panel) with significantly higher percentage of NLRP3 inflammasome positive area in islets of obese LGALS3<sup>−/−</sup> mice (Fig 6B) and prominent infiltration of F4/80+ macrophages (Fig 6C). Also, increased IL-1β expression in islets from obese LGALS3<sup>−/−</sup> mice (Fig 6D, upper panel) compared with HFD-fed WT mice (Fig 6D, lower panel) was found with significantly higher percentage of IL-1β positive
area in islets of obese LGALS3\(^{-/-}\) mice (Fig 6E and 6F). IL-18 was not detected (data not shown).

Furthermore, we show significantly higher percentages of NLRP3 inflammasome and IL-1\(\beta\) expressing macrophages derived from digested pancreatic tissue (Fig 6G) and VAT (Fig 6H) from HFD-fed LGALS3\(^{-/-}\) mice compared with diet-matched WT controls.

Western blot analyses showed similar expression of NLRP3 inflammasome in pancreata among experimental groups and significantly increased expression of ASC in HFD-fed LGALS3\(^{-/-}\) mice compared with LFD-fed LGALS3\(^{-/-}\) and WT mice (both p<0.05). Procaspase-1 was significantly increased in HFD-fed LGALS3\(^{-/-}\) mice compared to other experimental groups (all p<0.05) and mature Caspase-1 in comparison with LFD-fed LGALS3\(^{-/-}\) mice (p<0.05). ASC expression was significantly higher in HFD-fed WT mice compared to LFD-fed LGALS3\(^{-/-}\) mice (p<0.05) (Fig 6I).

In VAT, a significant increase of NLRP3 inflammasome expression was found in HFD-fed LGALS3\(^{-/-}\) compared with LFD-fed LGALS3\(^{-/-}\) mice and WT mice (both p<0.05) and increased ASC, procaspase-1 in comparison with LFD-fed LGALS3\(^{-/-}\) mice (both p<0.05). Mature Caspase-1 protein expression was significantly higher in obese LGALS3\(^{-/-}\) mice compared with other experimental groups (all p<0.05). NLRP3 and ASC expression were significantly lower in LFD-fed LGALS3\(^{-/-}\) mice compared with HFD or LFD-fed WT mice (all p<0.05). Procaspase-1 was significantly lower in LFD-fed LGALS3\(^{-/-}\) mice compared with HFD-fed WT mice (p<0.05) (Fig 6J).

**Obese LGALS3\(^{-/-}\) mice have higher AGE accumulation and RAGE expression in pancreatic islets**

As Gal-3 has a role in the uptake and removal of metabolic compounds with pro-inflammatory properties related to diabetes complications we examined the expression of advanced glycation
endproducts (AGE) and receptor for AGE (RAGE) in pancreatic islets. Immunofluorescence and IHC data showed increased accumulation of AGE (Fig 7A and 7B) and increased percentage of RAGE positive area in islets in LGALS3−/− mice fed HFD for 11 weeks compared with diet-matched WT mice (Fig 7C and 7D).

We show increased expression of Gal-3 protein in pancreatic islets of HFD-fed WT mice compared to LFD-fed WT mice at 11 weeks (Fig 7E).

**NLRP3 inflammasome expression and Caspase-1 dependent IL-1β production are increased in stimulated peritoneal macrophages in LGALS3−/− compared to WT mice**

We demonstrate the inherent genotype differences in cellular response of LGALS3−/− mice vs. WT mice as LPS (100ng/ml), palmitate-BSA (100µM), and LPS plus palmitate induced significantly higher IL-1β and IL-6 production in LGALS3−/− peritoneal macrophages (Fig 8A). IL-1β production was significantly reduced in the presence of Caspase-1 inhibitor Z-YVAD-FMK (10µM), while IL-6 production was not affected (Fig 8A). No significant difference in IL-1β production between LGALS3−/− and WT mice was observed when cells were stimulated with high-glucose or H2O2 with and without priming with LPS (data not shown).

*In vitro* stimulation of cells with LPS or palmitate significantly increased percentages of NLRP3 inflammasome expressing F4/80+ macrophages (Fig 8B) and significantly increased Caspase-1 activity (Fig 8C) in LGALS3−/− compared with WT macrophages. Transfection of LGALS3−/− peritoneal macrophages with NLRP3 siRNA resulted in a significant reduction of the NLRP3 inflammasome expression compared to cells transfected with control siRNA (15.6% vs. 3.7%) (Fig 8D). Transfection with NLRP3 siRNA, but not with control siRNA, led to a significant decrease in IL-1β production by LGALS3−/− macrophages stimulated with palmitate alone and LPS plus palmitate (Fig 8D).
Increased activation of NFκB in LGALS3−/− mice

We next tested for the differences in NFκB activation in LGALS3−/− vs. WT mice by using an antibody against the NFκB p65 subunit phosphorylated at the amino acid residue serine 536, an activated form of NFκB which is induced in response to a variety of proinflammatory stimuli, including LPS. Fig 8E represents the percentage of phospho NFκB p65 expressing peritoneal macrophages in response to LPS (1µg/ml) with and without palmitate (100µM). A significant increase of phospho NFκB p65 expressing F4/80+ macrophages in LGALS3−/− compared with WT cells in response to LPS and LPS plus palmitate was observed (Fig 8E).

Western blotting showed significantly increased expression of phospho NFκB p65 in pancreata of LGALS3−/− fed HFD for 18 weeks compared with other experimental groups (all p<0.05) (Fig 8F). Phospho/total ratio of NFκB p65 was the highest in pancreata of HFD-fed LGALS3−/− mice (0.816) compared to HFD-fed WT mice (0.413) and LFD-fed LGALS3−/− mice (0.120) or WT animals (0.345). In VAT, phospho NFκB p65 expression was significantly higher in HFD-fed LGALS3−/− mice compared with LFD-fed LGALS3−/− mice (p<0.05) (Fig 8F). Phospho/total ratio of NFκB p65 was the highest in VAT of HFD-fed LGALS3−/− mice (0.842) compared with HFD-fed WT mice (0.469) and LFD-fed LGALS3−/− mice (0.370) or WT animals (0.272).

**DISCUSSION**

This study provides evidence that knockdown of Galectin-3 results in accelerated high-fat diet-induced obesity and diabetes, as indicated by the significantly higher body weight and total visceral adipose tissue weight, hyperglycemia, increased HOMA-IR, severe insulitis and pronounced infiltration of pancreatic islets with pro-inflammatory macrophages. Ongoing inflammation in largely expanded adipose tissue in obese LGALS3−/− mice was characterized by
the increased incidence of Type 1 CD3+ T and CD3+ NK1.1+ NKT lymphocytes expressing IFN-γ. Th1 effector cells have a major role in obesity-associated chronic inflammation (26) and NKT cells depletion prevents diet-induced metaflammation and glucose intolerance (27). Reduced adipose tissue and splenic Tregs in obese LGALS3\(^{−/−}\) mice are in line with the data of depleted adipose tissue Tregs during obesity (28). High-fat diet increased the incidence of activated CD4+ PD-1+ T cells in visceral adipose tissue and spleens in LGALS3\(^{−/−}\) mice, possibly due to enhanced TCR-mediated signaling in T cells in Gal-3 deficiency (29, 30).

F4/80+CD11c+CD206+ macrophages, increased in VAT of obese LGALS3\(^{−/−}\) mice, were recently described as a novel macrophage subset to facilitate adipose tissue inflammation and insulin resistance in obese human subjects (31). Eighteen weeks of HFD in VAT of LGALS3\(^{−/−}\) mice increased pro-inflammatory F4/80+CD11b+CD11c+ BMDC, cells that have a crucial role in obesity-induced inflammation (32). Markedly reduced alternatively activated M2 macrophages in VAT of LGALS3\(^{−/−}\) mice are in line with the reported data of Gal-3 involvement in polarization of M2 macrophages (33, 34).

Accelerated HFD-induced obesity in Gal-3 deficient mice was associated with systemic inflammation. Increased IL-6 and IL-1β induced by HFD in LGALS3\(^{−/−}\) mice are in line with the data of increased production of IL-1β and IL-6 in Gal-3 deficient macrophages during infection (35). Increased systemic IL-4 might indicate enhanced T cell function during nutrient excess in LGALS3\(^{−/−}\) mice as Gal-3-deficient CD4+ T cells were shown to produce increased amounts of IFN-γ and IL-4 after T-cell receptor engagement (36). Lack of Gal-3 on dendritic cells favors lower IL-10 production and increased IFN-γ by allogeneic T cells (37). Reduced levels of IL-13 and IL-10 in sera of obese LGALS3\(^{−/−}\) mice might contribute to amplified obesity-induced inflammation. IL-10 has a protective role in type 2 diabetes by increasing insulin sensitivity in skeletal muscle (38).
The most striking finding in our study was the presence of severe insulitis in obese LGALS3\textsuperscript{-/-} mice, but also in lean LGALS3\textsuperscript{-/-} mice. Enhanced expression of Gal-3 protects rat pancreatic \( \beta \)-cells from the cytotoxic effect of IL-1\( \beta \) (18) and Gal-3 is highly expressed in islet endothelial cells in obesity-induced diabetes in mice (19), but its role in diabetes progression still remains to be elucidated. Moreover, we show upregulation of Gal-3 protein in pancreatic islets in HFD-fed WT mice. F4/80\textsuperscript{+} CD11c\textsuperscript{+}CD11b\textsuperscript{+} BMDCs was the most abundant cell type in islet infiltrates in obese LGALS3\textsuperscript{-/-} mice and higher expression of NLRP3 inflammasome and IL-1\( \beta \) in F4/80\textsuperscript{+} macrophages reflects ongoing inflammation in islets of HFD-fed LGALS3\textsuperscript{-/-} mice. Apart from macrophages \( \beta \)-cells could produce IL-1\( \beta \) when exposed to pro-inflammatory metabolic signals possibly through NLRP3 inflammasome activation (39). Most recently, type 2 diabetes has been considered as an auto-inflammatory disease with the central role for NLRP3-ASC inflammasome-mediated IL-1\( \beta \) production (40, 41). We did not find significant increase in protein content of NLRP3 inflammasome in whole tissue lysates of pancreas and VAT in HFD-fed LGALS3\textsuperscript{-/-} mice compared to HFD-fed WT mice. However, a trend towards increased ASC and NLRP3 inflammasome protein content was found in pancreas and VAT, respectively. The increased mature Caspase-1 protein expression found in VAT of obese LGALS3\textsuperscript{-/-} mice might indicate increased NLRP3 inflammasome activation in adipose tissue in Gal-3 deficiency. In fact, LGALS3\textsuperscript{-/-} macrophages produced increased amounts of IL-1\( \beta \), which was Caspase-1 dependent, had increased NLRP3 inflammasome expression and higher Caspase-1 activity in response to LPS and/or palmitate compared to WT cells. In addition, silencing of NLRP3 by siRNA attenuated IL-1\( \beta \) production by LGALS3\textsuperscript{-/-} macrophages suggestive for the enhanced NLRP3 inflammasome activation in Gal-3 deficient mice. The most recent study show that NLRP3 expression is increased by TLR agonists including LPS in murine macrophages in an NF\( \kappa \)B dependent manner (42). Moreover, LGALS3\textsuperscript{-/-} macrophages produced increased amounts
of IL-1β and IL-6 in response to LPS stimulation, which is line with the evidence of markedly elevated LPS-induced pro-inflammatory cytokines in Gal-3-deficient macrophages since Gal-3 binds LPS and prevents its activity (43). High-fat diet strongly increases intestinal permeability and increases blood levels of endotoxin (44). Endotoxin, free fatty acids and AGE, through TLRs and RAGE activate NFκB and lead to IL-1β and TNF-α production, thus promoting insulin resistance (39, 45). LGALS3−/− macrophages had increased expression of phospho NFκB p65 in response to LPS which complements the reported data (46). Increased pancreatic and also visceral adipose tissue phospho NFκB p65 expression could be related to increased accumulation of AGE and upregulation of RAGE in islets of LGALS3−/− mice fed HFD. These findings indicate that additional NFκB mediated pro-inflammatory pathways (47) might operate in amplified obesity-induced inflammation in LGALS3−/− mice.

Collectively, the amplified obesity-induced inflammation in adipose tissue and pancreatic islets in LGALS3−/− mice suggest a protective role for Gal-3 in obesity and type 2 diabetes which could be of therapeutic relevance.

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N.P. and J.P. researched data and wrote the manuscript. I.J., G.R., M.M, and I.N. researched data. N.Z. and A.Dj. researched data and contributed to discussion. N.A. reviewed/edited the manuscript. M.L. contributed to discussion and reviewed/edited manuscript.
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FIGURE LEGENDS

Figure 1. LGALS3<sup>−/−</sup> mice fed HFD have increased body weight, enhanced visceral adiposity, hyperglycemia, hyperinsulinemia and increased HOMA-IR

A. An image of the larger body size of LGALS3<sup>−/−</sup> versus WT mice fed HFD. A significant increase in body weights in HFD-fed LGALS3<sup>−/−</sup> mice compared to other experimental groups after 11 or 18 weeks, and WT mice fed HFD vs. WT mice fed LFD after 18 weeks. A food intake in LGALS3<sup>−/−</sup> and WT mice

B. Significantly increased amount of visceral adipose tissue in HFD-fed LGALS3<sup>−/−</sup> mice vs. other experimental groups after 11 or 18 weeks and WT mice on different diets after 18 weeks

C. Significant hyperglycemia of LGALS3<sup>−/−</sup> mice on HFD compared to other experimental groups after 11 or 18 weeks and LFD-fed LGALS3<sup>−/−</sup> vs. WT mice after 18 weeks. Increased HbA1c (%) of LGALS3<sup>−/−</sup> mice on HFD vs. other experimental groups which significantly correlates with fasting blood glucose levels in LGALS3<sup>−/−</sup> mice (r=0.671, p=0.048)

D. Significant increase of fasting insulin levels in sera of LGALS3<sup>−/−</sup> fed HFD vs. other experimental groups after 11 or 18 weeks

E. Significant increase of HOMA-IR in HFD-fed LGALS3<sup>−/−</sup> mice compared to other experimental groups after 11 or 18 weeks

The results are shown as the means ± s.e.m. for eight to twelve animals (11 weeks) or four to seven mice (18 weeks) per group.

r, Pearson’s correlation coefficient; *p<0.05; **p<0.001; ¶ p<0.05; § p<0.05
**Figure 2.** Increased Type 1 T and NKT cells and reduced T regs in visceral adipose tissue of HFD-fed LGALS3−/− mice

A. Increased percentages of CD3+ T and in visceral adipose tissue of LGALS3−/− mice fed HFD compared to other groups after 11 weeks. CD3+NK1.1+ NKT cells in VAT of LGALS3−/− mice fed HFD were significantly increased compared to WT mice on both diet conditions

B. LGALS3−/− mice fed HFD have significantly increased frequencies of CD3+IFN-γ+ in VAT compared to LFD-fed mice of both genotypes, while CD3+NK1.1+ NKT cells that express IFN-γ were significantly higher compared to other experimental groups

C. Adipose tissue regulatory CD4+CD25+FoxP3+ T cells are reduced, and the trend towards decrease of Tregs is observed in spleens (p=0.071) of LGALS3−/− vs. WT mice fed HFD

D. Representative FACS plot of CD4+CD25+FoxP3+ T cells in VAT from HFD-fed LGALS3−/− and WT mice

E. Significantly increased percentages of CD4+PD-1+ cells in VAT and spleen from HFD-fed LGALS3−/− mice vs. WT mice on both diet conditions

The results are shown as the means ± s.e.m. for four to six animals per group.

*p<0.05

**Figure 3.** Increased frequencies of adipose tissue F4/80+CD11c+CD206+ macrophages in obese LGALS3−/− mice correlate with body weights and blood glucose levels in LGALS3−/− mice

A. LGALS3−/− mice fed HFD for 11 weeks have significantly increased percentages of F4/80+CD11c+CD206+ macrophages in VAT compared to other experimental groups

B. LGALS3−/− fed HFD have significantly lower percentages of alternatively activated F4/80+CD11c-CD206+ M2 macrophages in VAT compared to WT mice fed LFD
C. Positive correlation between the frequencies of pro-inflammatory F4/80+CD11c+CD206+ macrophages with body weights (r=0.919, p=0.001) and fasting blood glucose levels (r=0.912, p=0.002) in LGALS3<sup>−/−</sup> mice

D. Inverse correlation between the frequencies of regulatory T cells with body weights (r=0.633, p=0.067) and blood glucose levels (r=0.819, p=0.007) in LGALS3<sup>−/−</sup> mice

r, Pearson’s correlation coefficient;

The results are shown as the means ± s.e.m. for four to six animals per group.

*p<0.05; §p<0.05

**Figure 4.** Serum cytokines levels in HFD-fed LGALS3<sup>−/−</sup> and WT mice

A. CRP and cytokine levels in LGALS3<sup>−/−</sup> and WT mice fed HFD for 11 weeks

B. CRP and cytokine levels in LGALS3<sup>−/−</sup> and WT mice fed HFD for 18 weeks

The results are shown as the means ± s.e.m. for four to six animals (11 weeks) or four to seven mice (18 weeks) per group.

* p<0.05

**Figure 5.** Histological analysis of infiltrating mononuclear cells in pancreatic islets

A. H&E staining was performed on pancreatic tissue sections, representative images of insulitis scores in LGALS3<sup>−/−</sup> mice fed HFD for 11 weeks. Magnification=40, scale bars=100 µm

B. Pancreatic islet inflammation (insulitis) was graded from 1 to 5, according to the extent of peri- and intra-islet infiltration by mononuclear leukocytes as follows: 1=no islet infiltration; 2=peri-vascular/periductal islet infiltration; 3=peri-insulitis; 4=mild insulitis (<25% islet area infiltrated); 5=severe insulitis (>25% islet area infiltrated). LGALS3<sup>−/−</sup>
mice on HFD had significantly increased percentage of islet with severe insulitis compared to other experimental groups. LGALS3<sup>−/−</sup> mice on LFD had significantly higher percentage of severe insulitis compared to WT mice on both diet conditions.

The results are shown as percentages of islets with insulitis derived from four to six mice per group.

*<i>p</i>&lt;0.05; **<i>p</i>&lt;0.001; †<i>p</i>&lt;0.05; §<i>p</i>&lt;0.05

C. FACS plots of mononuclear cells isolated from pooled pancreata (n=5) LGALS3<sup>−/−</sup> mice fed HFD. The dot plots depict FSC and SSC (left) and the CD11b<sup>+</sup>CD11c<sup>+</sup> cells among gated F4/80<sup>+</sup> cells (right). In FACS analyses mononuclear infiltrates was not found in mice from other experimental groups.

**Figure 6.** Increased expression of NLRP3 inflammasome and IL-1β in pancreatic islets of LGALS3<sup>−/−</sup> mice fed HFD

A. Immunofluorescence staining for insulin (green) and NLRP3 inflammasome (red) together with DNA staining with DAPI (blue) in pancreatic islets from representative HFD-fed LGALS3<sup>−/−</sup> (upper panel) or WT mice (lower panel)

B. Immunohistochemical analyses of the amount of NLRP3 inflammasome positive areas in islets was done on pancreatic tissue sections (4-6 mice per group) (*<i>p</i>&lt;0.05)

C. Representative IHC staining of F4/80<sup>+</sup> macrophages (brown) and NLRP3 inflammasome (red) in islets from HFD-fed LGALS3<sup>−/−</sup> or WT mice

D. Immunofluorescence staining for insulin (green) and IL-1β (red) together with DNA staining with DAPI (blue) in pancreatic islets from representative HFD-fed LGALS3<sup>−/−</sup> (upper panel) or WT mice (lower panel)
E. Immunohistochemical analyses of the amount of IL-1β positive areas in islets was done on pancreatic tissue sections (4-6 mice per group) (*p<0.05)

F. Representative IHC staining of F4/80+ macrophages (brown) and IL-1β (red) in islets from HFD-fed LGALS3−/− or WT mice

G. Representative FACS plot of F4/80+NLRP3+ and F4/80+IL-1β+ macrophages in pancreas of HFD-fed LGALS3−/− and WT mice

H. Representative FACS plot of F4/80+NLRP3+ and F4/80+IL-1β+ macrophages in VAT of HFD-fed LGALS3−/− and WT mice. Significantly increased percentage of F4/80+NLRP3+ macrophages in HFD-fed LGALS3−/− vs. WT mice (*p<0.05)

I. Western blot analyses of NLRP3, ASC, procaspase-1 and caspase-1 expression in pancreas. HFD fed LGALS3−/− mice had significantly higher ASC expression when compared to LFD-fed LGALS3−/− and WT mice, significantly higher procaspase-1 expression when compared to all experimental groups and significantly higher active caspase-1 expression when compared to LGALS3−/− mice fed LFD (all *p<0.05). ASC expression was significantly higher in HFD-fed WT mice compared to LFD-fed LGALS3−/− mice (¶ p<0.05).

J. Western blot analyses of NLRP3, ASC, procaspase-1 and caspase-1 expression in VAT. LGALS3−/− mice on HFD showed significantly increased expression of NLRP3 when compared to LFD-fed LGALS3−/− and WT mice and significantly increased expression of active caspase-1 when compared to all experimental groups (all *p<0.05). NLRP3 and ASC expression were significantly lower in LFD-fed LGALS3−/− mice compared with HFD or LFD-fed WT mice (all ¶ p<0.05). Procaspase-1 was significantly lower in LFD-fed LGALS3−/− mice compared with HFD-fed WT mice (¶ p<0.05).

The results are representative of two to three repeated experiments.
**Figure 7.** Increased expression of AGE and RAGE in pancreatic islets of HFD-fed LGALS3\(^{-/-}\) mice

A. Immunofluorescence staining for insulin (green) and AGE (red) together with DNA staining with DAPI (blue) in pancreatic islets from representative LGALS3\(^{-/-}\) (upper panel) and WT mice fed a HFD (lower panel)

B. Evaluation of % AGE positive areas was done on IHC stained tissue sections from 4-6 mice per group

C. Immunofluorescence staining for insulin (green) and RAGE (red) together with DNA staining with DAPI (blue) in pancreatic islets from representative LGALS3\(^{-/-}\) (upper panel) and WT mice fed a HFD (lower panel)

D. Evaluation of % RAGE positive areas was done on IHC stained tissue sections from 4-6 mice per group

E. Representative IHC image of Gal-3 expression in HFD or LFD-fed WT mice

The results are representative of two to three repeated experiments.

*p<0.05

**Figure 8.** Stimulated LGALS3\(^{-/-}\) peritoneal macrophages have increased NLRP3 inflammasome expression, Caspase-1 dependent IL-1\(\beta\) production and higher expression of phosphorylated NF-\(\kappa\)B p65

A. Significantly higher production of IL-1\(\beta\) by LGALS3\(^{-/-}\) vs. WT peritoneal macrophages upon simulation with LPS (100ng), palmitate-BSA (PA-BSA) (100\(\mu\)M) or LPS plus PA, while the production was significantly reduced in the presence of caspase-1 inhibitor Z-YVAD (10\(\mu\)M). LGALS3\(^{-/-}\) macrophages stimulated with LPS, PA or LPS plus PA
produce more IL-6 when compared to WT macrophages and the production was not
affected with caspase-1 inhibitor Z-YVAD

B. Significantly increased percentage of F4/80+NLRP3+ macrophages in LGALS3<sup>−/−</sup> vs. WT
mice when stimulated with LPS, PA-BSA or LPS plus PA. Representative FACS plot of
F4/80+NLRP3+ peritoneal macrophages LGALS3<sup>−/−</sup> and WT mice.

C. Significantly increased Caspase-1 activity in cell lysates of LGALS3<sup>−/−</sup> peritoneal
macrophages stimulated with LPS (100ng), PA (100µM) or LPS plus PA in comparison
with WT peritoneal macrophages

D. Significantly reduced palmitate (100µM) and LPS (100ng) plus palmitate (100µM)
stimulated IL-1β production from LGALS3<sup>−/−</sup> macrophages transfected with NLRP3
siRNA compared to cells treated with control siRNA. Representative histogram of
NLRP3 expression in macrophages transfected with NLRP3 siRNA or control siRNA

E. Significantly increased expression of phosphorilated NFκB p65 in LGALS3<sup>−/−</sup>
macrophages stimulated with LPS (1µg/ml) and LPS plus PA (100µM) when compared to
WT macrophages. Representative histograms of F4/80+ macrophages expressing NFκB
p65 (phospho S536) in LGALS3<sup>−/−</sup> and WT mice

F. WB analyses of NFκB p65 and phospho-NFκB p65 expression in pancreas and VAT.
HFD-fed LGALS3<sup>−/−</sup> mice have significantly increased expression of phosphorilated
NFκB p65 when compared to all experimental groups in pancreas and compared to LFD-
fed LGALS<sup>−/−</sup> and WT mice in VAT. All experimental groups have similar expression of
total NFκB p65 in pancreas and VAT.

The results are representative of two to three repeated experiments. *p<0.05
Diabetes

A

| score 1 | score 2 | score 3 | score 4 | score 5 |
|---------|---------|---------|---------|---------|
| normal islet | perivascular/periductal infiltration | peri-insulitis | mild insulitis | severe insulitis |

B

![Graph showing the percentage of islets in different conditions](image)

C

![Flow cytometry plots](image)

249x344mm (300 x 300 DPI)
