

GALECTIN-3 DELETION ENHANCES VISCERAL ADIPOSE TISSUE INFLAMMATION AND DYSREGULATES GLUCOSE METABOLISM IN MICE ON A HIGH-FAT DIET

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DELECIJA GALEKTINA-3 POSPEŠUJE INFLAMACIJU U VISCERALNOM MASNOM TKIVU I NARUŠAVA HOMEOSTAZU GLUKOZE U MIŠEVA NA ISHRANI BOGATOJ MASTIMA

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ABSTRACT

Obesity and type 2 diabetes mellitus (T2DM) constitute major health problems worldwide. Increased visceral adiposity enhances the risk of insulin resistance and type 2 diabetes. The mechanisms involved in obesity-associated chronic inflammation in metabolic tissues (metaflammation) that lead to insulin resistance and dysregulated glucose metabolism are incompletely defined. Galectin-3 (Gal-3), a β -galactoside-binding lectin, modulates immune/inflammatory responses and specifically binds to metabolic danger molecules. To dissect the role of Gal-3 in obesity and diabetes, Gal-3-deficient (LGALS3^{-/-}) and wild-type (WT) C57Bl/6 male mice were placed on a high-fat diet (HFD, 60% kcal fat) or a standard chow diet (10% kcal fat) for 6 months and metabolic, histological and immunophenotypical analyses of the visceral adipose tissue were performed. HFD-fed LGALS3^{-/-} mice had higher body weights and more body weight gain, visceral adipose tissue (VAT), hyperglycaemia, hyperinsulinemia, insulin resistance and hyperlipidemia than diet-matched WT mice. Compared to WT mice, the enlarged VAT in obese LGALS3^{-/-} mice contained larger adipocytes. Additionally, we demonstrate enhanced inflammation in the VAT of LGALS3^{-/-} mice compared with diet-matched WT mice. The VAT of LGALS3^{-/-} mice fed a HFD contained more numerous dendritic cells and proinflammatory F4/80⁺CD11c⁺CD11b⁺ and F4/80^{high} macrophages. In contrast to WT mice, the numbers of CXCR3⁺ and CD8⁺ T cells were increased in the VAT of Gal-3-deficient mice after 6 months of high-fat feeding. We provide evidence that Gal-3 ablation results in enhanced HFD-induced adiposity, inflammation in the adipose tissue, insulin resistance and hyperglycaemia. Thus, Gal-3 represents an important regulator of obesity-associated immunometabolic alterations.

Keywords: Galectin-3, obesity, hyperglycaemia, insulin resistance, metaflammation

SAŽETAK

Gojaznost i tip 2 diabetes mellitus (T2DM) predstavljaju veliki svetski zdravstveni problem. Uvećanje visceralnog masnog tkiva u gojaznosti je povezano sa većim rizikom za nastanak insulinske rezistencije i T2DM. Molekularni mehanizmi povezani sa hroničnom inflamacijom u metabolički aktivnim tkivima (metaflamacijom) u gojaznosti koji leže u osnovi insulinske rezistencije i narušene homeostaze glukoze nisu do kraja razjašnjeni. Galektin-3 je multifunkcionalni lektin sa značajnom ulogom u imunoregulaciji i metaflamaciji. Sa ciljem da se ispita uloga galektina-3 u nastanku gojaznosti i T2DM, galektin-3 deficijentni miševi (LGALS3^{-/-}) i miševi divljeg soja (WT) stavljani su na ishranu sa visokim sadržajem masti (60% kcal od masti) ili standardnu ishranu (10% kcal od masti) u trajanju od 6 meseci nakon čega su ispitivani metabolički parametri, morfologija visceralnog masnog tkiva i fenotipske karakteristike infiltrirajućih ćelija. LGALS3^{-/-} miševi na ishrani sa visokim sadržajem masti imali su veću telesnu težinu, veću količinu visceralnog masnog tkiva, hiperglikemiju, hiperinsulinemiju, izraženiju insulinsku rezistenciju i hiperlipidemiju u poređenju sa WT miševima na istom režimu ishrane. Adipociti iz uvećanog visceralnog masnog tkiva LGALS3^{-/-} miševa imali su veći dijametar u poređenju sa adipocitima u WT miševa. Izražena je veća zastupljenost dendritičnih ćelija, proinflammatory F4/80⁺CD11c⁺CD11b⁺ i F4/80^{high} makrofaga, CD3⁺CXCR3⁺ i CD8⁺ T limfocita u poređenju sa WT miševima na istom režimu ishrane. Dobijeni rezultati ukazuju na značajnu protektivnu ulogu galektina-3 u nastanku gojaznosti, insulinske rezistencije i T2DM.

Ključne reči: Galektin-3, gojaznost, hiperglikemija, insulinska rezistencija, metaflamacija





INTRODUCTION

Obesity and type 2 diabetes mellitus (T2DM) constitute a major public health problem worldwide, particularly in developing countries (1). Obesity, mainly abdominal adiposity, is linked to various metabolic alterations that increase the risk for T2DM (2). T2DM is a metabolic disorder characterized by insulin resistance (IR) followed by pancreatic β -cell dysfunction (3). Pancreatic β -cells normally compensate for obesity-associated reduced insulin sensitivity by secreting more insulin to maintain glucose homeostasis. Hyperinsulinemia occurs early in the disease progression and before the β -cell function becomes impaired, leading to late-stage (insulin-dependent) T2DM (4).

Obesity-induced diabetes is associated with low-grade inflammation in the visceral adipose tissue (VAT). Inflammation in the VAT plays an important role in metabolic abnormalities such as IR (5). Antigen-presenting cells, including dendritic cells (DCs) and macrophages, are increased in the VAT in obesity, where they potentiate both innate and adaptive immune/inflammatory responses. Numerous studies have shown the increased infiltration of the visceral adipose tissue by macrophages during obesity (6,7). Macrophages may be derived from blood monocytes and are classified as classically (M1) and alternatively (M2) activated macrophages (8). Recent studies have shown that macrophages are key cells that mediate obesity-induced metabolic abnormalities. In obesity, a shift from alternatively activated M2 to classically activated M1 macrophages, characterized by elevated F4/80, CD11b and CD11c expression, has been demonstrated (9,10). Moreover, the ablation of CD11c-positive cells normalizes insulin sensitivity in obese insulin-resistant animals (11). Adipocytes damaged by high lipid intake produce signals that promote a proinflammatory Type 1 immune response. The number of T cells is increased in the enlarged VAT during obesity in response to adipose tissue-specific factors (12). Adipose tissue-associated regulatory T cells (Tregs) protect against (13), while the recruitment of Type 1 CD4 and CD8⁺ T lymphocytes precedes, metabolic alterations in obesity (14,15). The chemokine receptor CXCR3 is highly expressed in activated T cells and is involved in the regulation of T cell trafficking and maturation. Additionally, it has been shown that CXCR3-positive cells play an important role in the modulation of obesity-induced visceral adipose tissue inflammation and systemic insulin resistance (16).

Galectin-3 (Gal-3) is a β -galactoside-binding lectin that is expressed in different tissues and cells and plays an important role in obesity, T2DM and inflammation (17,18,19). Gal-3 regulates inflammation and adipogenesis, and this lectin is expressed in adipocytes and infiltrating immune cells in the adipose tissue (20). Additionally, Gal-3 regulates adipocyte cell proliferation and differentiation (21) and has a variety of regulatory roles in the innate and adaptive immune response, depending on the disease con-

ditions (18,22,23). However, the role of Gal-3 in type 2 diabetes remains incompletely understood. The Gal-3 levels are increased in the sera of obese subjects and negatively correlate with the levels of glycosylated haemoglobin (24). In contrast with these data, Okhura et al. reported that low serum Gal-3 levels are associated with insulin resistance and T2DM (25). However, controversial results have been reported regarding the effects of Gal-3 ablation in experimental models of diabetes. It has been shown that Gal-3-deficient mice were relatively resistant to diabetogenesis in streptozotocin-induced diabetes (26). On the other hand, Pejnovic et al. reported that obese Gal-3-deficient mice had enhanced adiposity, hyperglycaemia, hyperinsulinemia, IR and systemic inflammation in comparison with their diet-matched wild-type controls (18). Moreover, in a study by Peng et al. (27), Gal-3-null mice fed a HFD had increased adiposity and dysregulated glucose metabolism. In addition, the same authors found that young Gal-3-deficient mice fed a standard diet exhibit altered glucose homeostasis, thus suggesting the modulation of glucose metabolism and possibly β -cell function by Galectin-3 (Gal-3) independently of obesity and inflammation.

To investigate the role of Gal-3 in high-fat diet-induced obesity, we used Gal-3-deficient mice on a C57Bl/6 background. We examined inflammation in the visceral adipose tissue and metabolic abnormalities following long-term HFD exposure in Gal-3-deficient and wild type (WT) mice. We report here that Gal-3 deletion enhanced high-fat diet (HFD)-induced obesity and visceral adiposity, amplified inflammation in the visceral adipose tissue and led to dysregulated glucose metabolism characterized by hyperglycaemia and insulin resistance.

MATERIALS AND METHODS

Experimental mice and study design

Gal-3-deficient mice on a C57BL/6 background and their littermate controls were obtained from the University of California Davis (Davis, CA; by courtesy of D.K. Hsu and F.T. Liu) and accommodated in our animal facilities under standard laboratory conditions in a temperature-controlled environment with a 12 h light/darkness cycle. Male 2-month-old wild type (WT) and Gal-3-deficient (LGALS3^{-/-}) mice were fed either normal chow or a 60% fat/kcal diet (Mucedola, Italy) *ad libitum*. After 6 months, the mice were sacrificed and blood samples and visceral adipose tissue were collected for analyses. All animal procedures were approved by the ethical committee of the Faculty of Medical Sciences, University of Kragujevac (Permit Number 01-2759/2).

Body weight and glucose metabolism analyses

Body weights and fasting blood glucose levels were measured once per month and after 6 months on a standard or



HFD. The mice were fasted for 4 h, and their glucose levels (mmol/L) were determined using the Accu-Chek Performa glucometer (Roche Diagnostics, Mannheim, Germany). The serum concentrations of total cholesterol and triacylglycerol were measured using the Olympus AU600 Chemistry Immuno Analyzer (Olympus, Tokyo, Japan) and the fasting insulin was measured using an Insulin ELISA kit (Alpco, Salem, NH, USA). The homeostasis model assessment of insulin resistance (HOMA-IR) was calculated using the following formula: $\text{HOMA-IR} = [(\text{Glucose mmol/L} \times \text{Insulin mU/L})] / 22.5$.

Histological analysis of visceral adipose tissue

Visceral adipose tissue (VAT), including the epididymal, mesenteric and renal fat pads, was dissected and weighed as visceral fat content. After weighing, part of the epididymal fat was fixed in 10% buffered formalin. The mean adipocyte size of the visceral fat pad was determined by computer-assisted image analysis of paraffin-embedded adipose tissue sections (5 μm) stained with haematoxylin and eosin (H&E). The adipocyte size was measured from a total of 50 cells per mouse in three separate fields using a light microscope (BX51; Olympus) equipped with a digital camera and *ImageJ* software. Analyses were performed in a blinded fashion by two independent observers. The data are expressed as the mean adipocyte diameter (μm) for each tissue in each animal.

Isolation of visceral adipose tissue stromal vascular fraction cells

Total visceral adipose tissue (VAT) was subjected to the isolation of stromal vascular fraction (SVF) cells. Collagenase digestion (1 mg/ml collagenase type II and 2% BSA (Sigma-Aldrich, St. Louis, MO)) was used to separate the SVF from the adipocytes of the VAT, as previously described (18). SVF was used for flow cytometric analysis, as described below.

Flow cytometric analyses

Adipose tissue SVF cells were stained with the following fluorescence-tagged monoclonal antibodies: anti-mouse CD45, CD3, CD4, CXCR3, CD8, CD11b, Lineage cocktail (BD Biosciences, San Jose, CA), Sca-1, NK 1.1, F4/80 and CD11c (BioLegend, San Diego, CA) or isotype-matched controls (BD Biosciences). The cells were analysed using a FACSCalibur flow cytometer (BD Biosciences) and FlowJo software (Tree Star).

Statistical analyses

Statistical analysis was performed using SPSS 13.0. The data are presented as the means \pm SEM. Statistical significance was determined by an independent-sample Stu-

dent's t test and, where appropriate, a Mann-Whitney U test. Statistical significance was assumed at $p < 0.05$.

RESULTS

Galectin-3 ablation accelerated HFD-induced obesity and obesity-related metabolic alterations

After 6 months, HFD increased the body weight and weight gain in both genotypes of mice, and these parameters were higher in HFD-fed LGALS3^{-/-} mice than in diet-matched WT mice (Fig. 1A). HFD feeding increased the visceral fat mass in both genotypes of mice, which was more pronounced in HFD-fed LGALS3^{-/-} mice than in diet-matched WT mice (Fig. 1B). The serum total cholesterol and triglyceride levels were significantly higher in obese LGALS3^{-/-} mice than in WT mice (Fig. 1C).

Galectin-3 deletion modulates HFD-induced adipocyte morphology

In view of the fact that Gal-3 is expressed in the adipose tissue and regulates adipocyte cell proliferation and differentiation, we analysed adipose tissue morphology and adipocyte size. The HFD significantly increased the adipocyte size in both genotypes of mice compared with mice on a standard diet. A significantly higher amount of total visceral adipose tissue was accompanied by larger adipocytes in LGALS3^{-/-} mice on a HFD than in diet-matched WT animals (Fig. 1D).

Gal-3 ablation accelerated HFD-induced hyperglycaemia, hyperinsulinemia and obesity-associated insulin resistance

In addition to accelerated HFD-induced obesity, the fasting blood glucose, insulin and HOMA-IR were significantly higher in obese LGALS3^{-/-} mice than in WT mice (Fig. 2). Additionally, the fasting blood glucose levels, insulin and insulin resistance were significantly higher in LGALS3^{-/-} mice than in WT mice on a standard diet (Fig. 2).

Increased dendritic cells and proinflammatory macrophages in the VAT in LGALS3^{-/-} mice on a HFD

Because obesity-induced diabetes is strongly associated with adipose tissue inflammation, we first analysed the innate immune cells in the visceral adipose tissue in both genotypes of mice after 6 months on a HFD. DCs and macrophages are known to be increased in the visceral adipose tissue during obesity. The VAT from HFD-fed LGALS3^{-/-} mice contained higher numbers of total CD11c⁺ ($p=0.034$) and CD11c⁺F4/80⁻ ($p=0.043$) DCs than the VAT from HFD-fed WT mice (Fig. 3A). In addition, the subsets of mature proinflammatory F4/80^{hi} ($p=0.034$) and triple-pos-

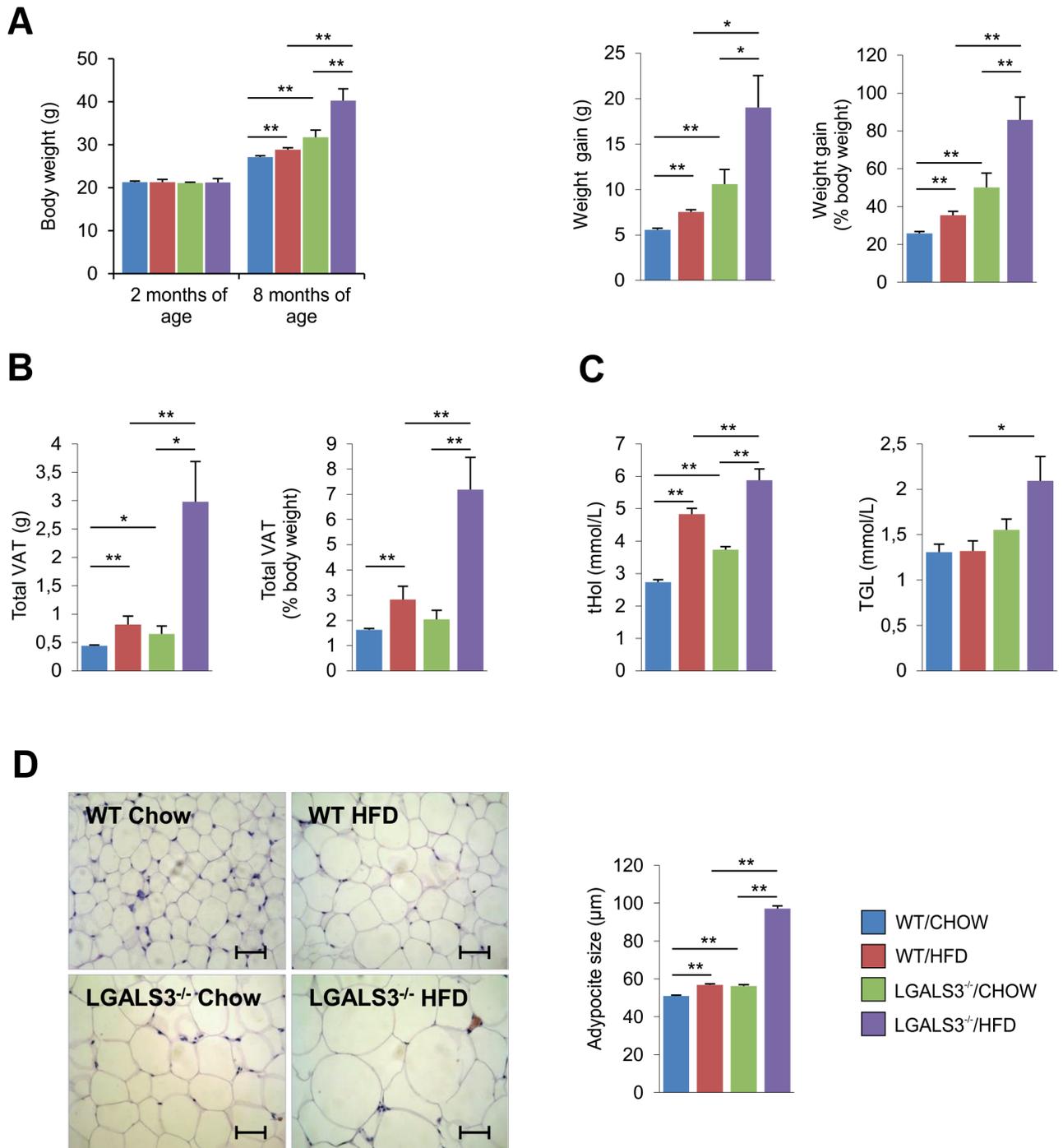


Figure 1. Galectin-3 ablation accelerated HFD-induced obesity and adipocyte hypertrophy

Male 2-month-old wild type (WT) and Gal-3-deficient (LGALS3^{-/-}) mice were fed either standard chow or a HFD *ad libitum* for 6 months. Metabolic parameters after 6 months on the HFD or standard diet are shown. (A) Body weight and weight gain were higher in HFD-fed LGALS3^{-/-} mice than in diet-matched WT mice. (B) Total VAT and visceral fat mass (% body weight) were significantly higher in LGALS3^{-/-} mice fed a HFD than in diet-matched WT animals. (C) Serum lipid levels were significantly higher in obese LGALS3^{-/-} mice than in WT mice. (D) Representative images depicting the larger adipocyte size in LGALS3^{-/-} mice on a HFD than in diet-matched WT animals (original magnification 40x, scale bar = 50 µm). The results are shown as the means ± SEM (n=5-6 mice/group), *P<0.05, **P<0.01.

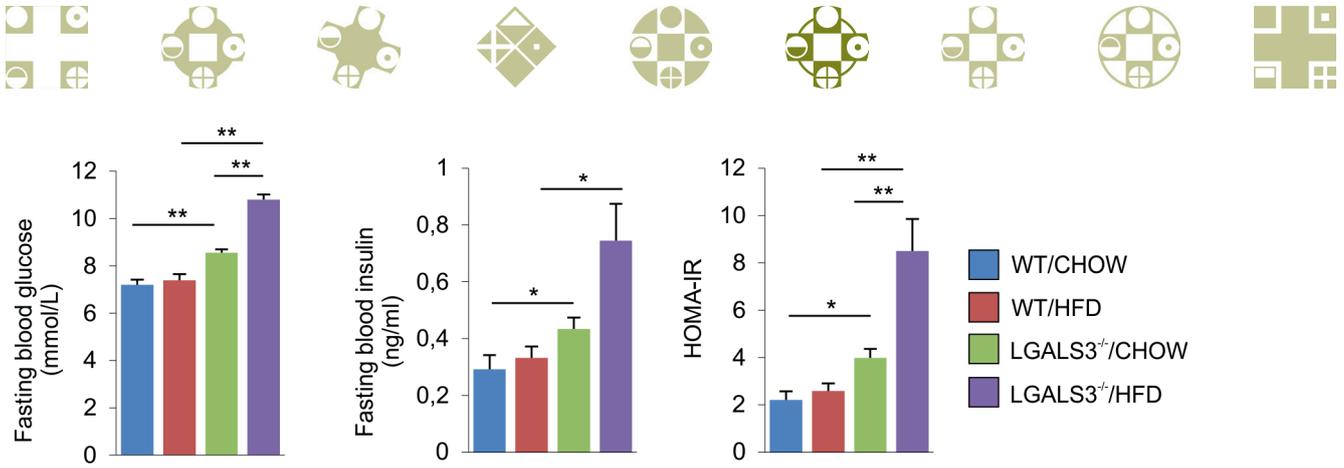


Figure 2. Galectin-3 ablation accelerated HFD-induced hyperglycaemia, insulinemia and insulin resistance
 The fasting blood glucose, insulin and HOMA-IR were significantly higher in LGALS3^{-/-} mice than in WT mice after 6 months on a HFD. The results are shown as the means ± SEM (n=5-6 mice/group), *P<0.05, **P<0.01.

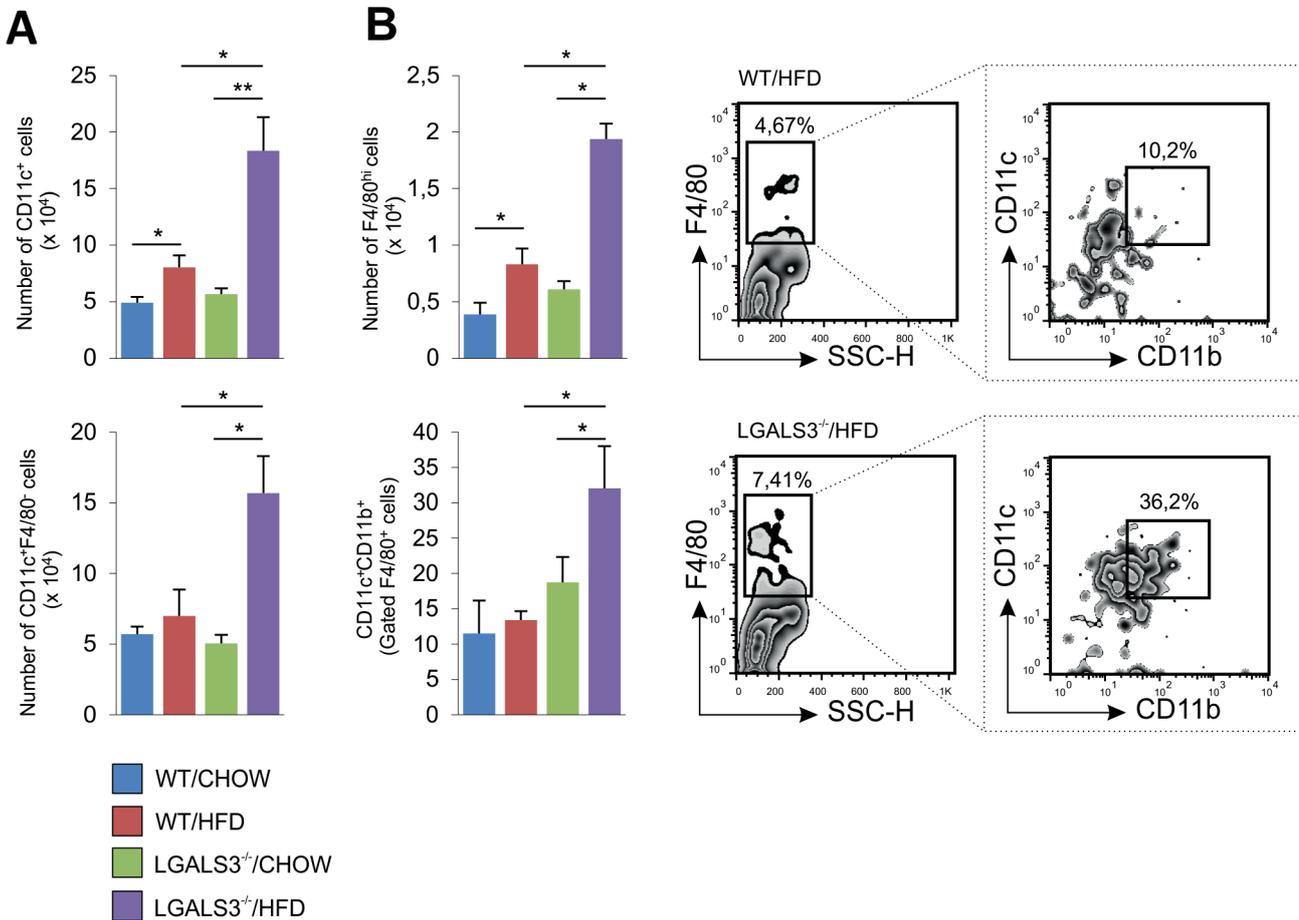


Figure 3. Increased dendritic cells and proinflammatory macrophages in the visceral adipose tissue of LGALS3^{-/-} mice on a HFD
 Representative images and flow cytometric analysis of VAT stromal vascular fraction cells from WT and LGALS3^{-/-} mice after 6 months on a HFD or standard diet. (A) Dendritic cell numbers were increased significantly in the VAT in obese LGALS3^{-/-} mice. (B) Proinflammatory macrophages were significantly increased in LGALS3^{-/-} mice fed a HFD compared with diet-matched WT mice. Representative FACS plots are shown. The results are shown as the means ± SEM (n=5-6 mice/group), *P<0.05, **P<0.01.

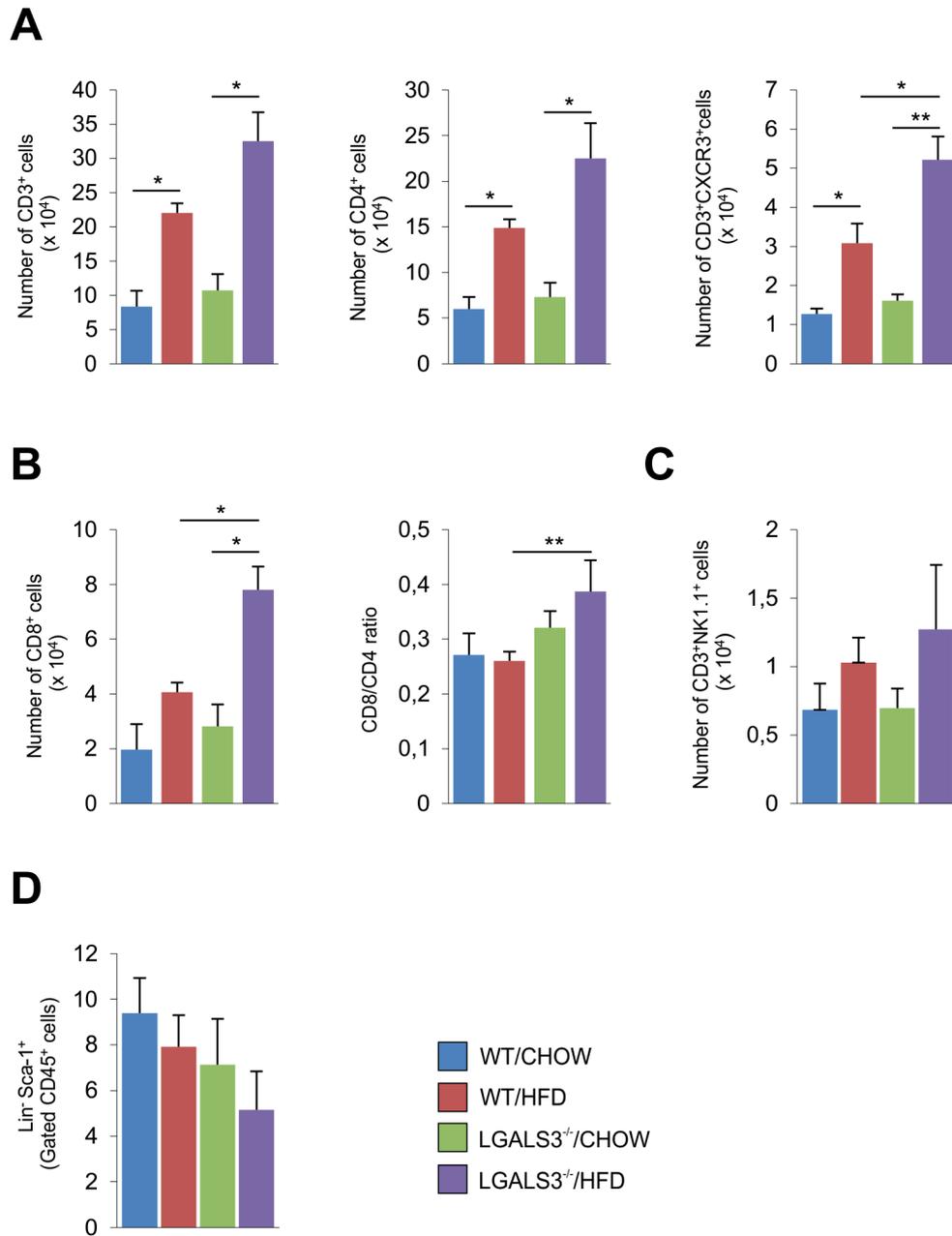


Figure 4. Increased CXCR3⁺ and CD8⁺ T lymphocytes in the visceral adipose tissue of LGALS3^{-/-} mice on a HFD

Flow cytometric analysis of VAT stromal vascular fraction cells from WT and LGALS3^{-/-} mice after 6 months on a HFD or standard diet. (A) CD3⁺, CD4⁺ and CD3⁺CXCR3⁺ T lymphocytes in the VAT. (B) The number of CD8⁺ T lymphocytes and the CD8/CD4 ratio were higher in Gal-3-deficient mice on a HFD than in diet-matched WT mice. (C) NKT lymphocytes in the VAT (D) Lin⁻Sca-1⁺ innate lymphoid cells in the VAT. The results are shown as the means ± SEM (n=5-6 mice/group), *P<0.05, **P<0.01.

itive F4/80⁺CD11b⁺CD11c⁺ macrophages (p=0.034) were significantly higher in the VAT of obese LGALS3^{-/-} mice than in WT mice (Fig. 3B).

Increased CXCR3⁺ and CD8⁺ T cells in the VAT in LGALS3^{-/-} mice on a HFD.

Increased Type 1 T cells and cytotoxic CD8⁺ T cells in the VAT are hallmarks of obesity-induced diabetes and

are strongly associated with adipose tissue inflammation. Therefore, we performed phenotypic analyses of T lymphocytes in the visceral adipose tissue in both genotypes of mice after 6 months on a HFD.

The HFD significantly increased the number of CD3⁺ and CD4⁺ T lymphocytes in both genotypes of mice compared with mice on a standard diet. There was no significant difference in the number of CD3⁺ and CD4⁺ T cells between the genotypes on a HFD (Fig. 4A). The HFD sig-



nificantly increased the number of CD3⁺CXCR3⁺ T cells in both genotypes of mice compared with mice fed a standard diet. However, the number of CD3⁺CXCR3⁺ cells ($p=0.031$) was higher in the VAT from LGALS3^{-/-} mice than in the VAT from WT mice, both on a HFD (Fig. 4A). Moreover, the number of CD8⁺ ($p=0.034$) T lymphocytes and the CD8-to-CD4 ratio (Fig. 4B) were higher in LGALS3^{-/-} mice fed a HFD than in diet-matched WT mice ($p=0.014$). There was no difference in the number of NKT cells (Fig. 4C) or the percentage of CD45⁺Lin⁻Sca-1⁺ innate lymphoid cells between the two genotypes of mice on both diets (Fig. 4D).

DISCUSSION

In this report, we demonstrate increased obesity, visceral adipose tissue inflammation and dysregulated glucose metabolism in LGALS3^{-/-} mice on a long-term high-fat diet. These effects appear to be mediated by both the metabolic and the immunoregulatory effects of Gal-3. After 6 months on a HFD, the body weight, weight gain and amount of total visceral tissue were significantly higher in LGALS3^{-/-} mice than in diet-matched WT mice (Fig. 1A and 1B). HFD feeding in Gal-3-deficient mice resulted in increased weight and visceral fat mass followed by significantly higher serum total cholesterol and triglyceride levels (Fig. 1C). Additionally, the fasting blood glucose and insulin levels, as well as the HOMA-IR, were significantly higher in obese and lean LGALS3^{-/-} mice than in WT mice (Fig. 2).

There is increasing evidence that Gal-3 plays an important role in obesity and T2DM (18). However, controversial results have been reported regarding the effects of Gal-3 in obese patients and experimental models. Our results are in agreement with the study reported by Okhura et al., which demonstrated that low serum Gal-3 levels are associated with insulin resistance in T2DM patients (25). It has been recently demonstrated that obese LGALS3^{-/-} mice have increased fasting blood glucose and insulin levels compared with diet-matched WT animals (18). Additionally, Pejnovic et al. (18) reported significantly increased IFN- γ -producing Type 1 T/NKT cells and proinflammatory M1 macrophages and reduced T regulatory cells and alternatively activated M2 macrophages in the VAT of LGALS3^{-/-} mice fed a HFD compared with diet-matched WT animals. Pang et al. (27) reported that Gal-3-deficient mice fed a HFD for 12 weeks develop increased adiposity and systemic inflammation. In addition, the same authors showed that despite the increased adiposity in Gal-3-deficient mice, there was no significant difference in the size of the adipocytes (27). In this study, we demonstrated that LGALS3^{-/-} mice fed a HFD for 6 months developed visceral adiposity, hyperglycaemia and IR. Increased adiposity in Gal-3-deficient mice on a HFD was associated with adipocyte hypertrophy (Fig. 1D).

Inflammation in the VAT during obesity plays a central role in metabolic abnormalities such as IR. We demon-

strate that a long-term HFD induced innate and adaptive immune cell infiltration in the VAT (Fig. 3 and 4). It has been reported that DCs, as professional antigen-presenting cells, have an important role in obesity-induced VAT inflammation (28). The obesity-associated increase of CD11c⁺ cells in the adipose tissue suggests that DCs play a role in macrophage recruitment and activation (29). The VAT from HFD-fed LGALS3^{-/-} mice contained higher numbers of total CD11c⁺ and CD11c⁺F4/80⁺ DCs than HFD-fed WT animals (Fig. 3A). Additionally, the expression of CD11c is one of the key characteristics of proinflammatory (M1) macrophages in addition to specific markers such as F4/80 and CD11b. Triple-positive F4/80⁺CD11b⁺CD11c⁺ macrophages (30) were more numerous in the VAT of LGALS3^{-/-} mice fed a HFD (Fig. 3B) and were recently described as a proinflammatory macrophage subset (10). This macrophage population is increased in the adipose tissue in obese vs. lean mice (10). In addition, the subset of proinflammatory F4/80^{hi} macrophages (30) was significantly higher in obese LGALS3^{-/-} mice than in WT mice (Fig. 3B). This result is in agreement with the reported data of increased proinflammatory F4/80⁺CD11c⁺CD206⁺ macrophage numbers in the VAT of LGALS3^{-/-} after 11 weeks on a HFD (18). Type 1 T cells have a major role in obesity-associated chronic inflammation (31). The HFD increased the number of CD3⁺ lymphocytes in the VAT of both genotypes of mice compared with chow-fed mice (Fig. 4A). CXCR3, a chemokine receptor that is highly expressed on activated T cells, is involved in T cell trafficking and activation (32). CD3⁺ cells expressing CXCR3 were higher in the VAT from LGALS3^{-/-} mice than in WT mice, both on a HFD (Fig. 4A). Despite the overlapping expression of chemokine receptors, CXCR3-positive cells represent Th1 lymphocytes. Th1 lymphocytes expressing CXCR3 produce more Th1-type cytokines such as IFN- γ and enhance the activity of CD8⁺ T cell effectors *in vitro* (33). Obesity is associated with increased CD8⁺ cells in the VAT (33). In our study, the number of CD8⁺ cells and the CD8/CD4 ratio (Fig. 4B) in the VAT was increased in LGALS3^{-/-} mice fed a HFD compared with diet-matched WT mice, suggesting the role of cytotoxic CD8⁺ cells in VAT inflammation and related metabolic abnormalities.

In summary, we provide evidence that Gal-3 deletion enhanced long-term HFD-induced adiposity, visceral adipose tissue inflammation, insulin resistance and hyperglycaemia. These data contribute to better understanding of the role Gal-3 in obesity, metabolic inflammation and T2DM.

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