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Original Article

Immunology

Galectin-12 enhances inflammation by promoting M1 polarization of macrophages and reduces insulin sensitivity in adipocytes

Lei Wan[1,2,3,4], Hui-Ju Lin[2,5], Chi-Chun Huang[6], Ying-Chi Chen[2,3], Yu-An Hsu[7], Chia-Hung Lin[7], Hsiu-Chu Lin[2,6], Ching-Yao Chang[3], Su-Hua Huang[3], Jane-Ming Lin[5], and Fu-Tong Liu[1,6,8]

[2]School of Chinese Medicine, China Medical University, No. 91, Hsueh-Shih Road, Taichung 40402, Taiwan, [3]Department of Biotechnology, Asia University, Taichung, Taiwan, [4]Department of Gynecology, and [5]Department of Ophthalmology, China Medical University Hospital, Taichung, Taiwan, [6]Institute of Biomedical Sciences, Academia Sinica, Taipei, Taiwan, [7]Institute of Molecular Medicine, National Tsing Hua University, Hsinchu, Taiwan, and [8]Department of Dermatology, University of California, Davis, School of Medicine, 3301 C Street, Suite 1400, Sacramento, CA 95816, USA

[1]To whom correspondence should be addressed. e-mail: leiwan@mail.cmu.edu.tw; lei.joseph@gmail.com (LW), ftliu@ibms.sinica.edu.tw; fliu@ucdavis.edu (FTL)

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Abstract

Galectin-12 is a member of an animal lectin family with affinity for β-galactosides and containing consensus amino acid sequences. Here, we found that galectin-12 was expressed in macrophages and thus aimed to determine how galectin-12 affects inflammation and macrophage polarization and activation. The ablation of galectin-12 did not affect bone marrow cells to differentiate into macrophages, but reduced phagocytic activity against *Escherichia coli* and lowered the secretion of nitric oxide. The ablation of galectin-12 also resulted in the polarization of macrophages into the M2 direction, as indicated by increases in the levels of M2 markers, namely, resistin-like β (FIZZ1) and chitinase 3-like 3 (Ym1), as well as a reduction in the expression levels of a number of M1 pro-inflammatory cytokines. We found that the diminished expression of pro-inflammatory cytokines in macrophages resulting from galectin-12 deletion was due to reduced activation of IKKα/β, Akt and ERK, which in turn caused decreased activation of NF-κB and activator protein 1. The activation of STAT3 was much higher in Gal12$^{-/-}$ macrophages activated by lipopolysaccharide, which was correlated with higher levels of IL-10. Adipocytes showed higher insulin sensitivity when treated with Gal12$^{-/-}$ macrophage-conditioned media than those treated with Gal12$^{+/+}$ macrophages. We conclude galectin-12 negatively regulates macrophage polarization into the M2 population, resulting in enhanced inflammatory responses and also in turn causing decreased insulin sensitivity in adipocytes. This has implications in the treatment of a wide spectrum of metabolic disorders.

Key words: Galectin-12, inflammation, macrophage polarization

Introduction

Galectin-12 was first discovered in 2001 by two independent groups (Hotta et al. 2001; Yang et al. 2001). The protein contains two carbohydrate-recognition domains joined by a linker region in a single polypeptide chain. Galectin-12 is predominantly expressed in adipose tissue, but is also detected in low amounts in the heart, pancreas, spleen, thymus and peripheral blood leukocytes. The downregulation of galectin-12 in mouse 3T3-L1 cells impairs their differentiation into adipocytes (Hotta et al. 2001; Yang et al. 2001, 2004; Fasshauer et al. 2002; Harrison et al. 2007). Recently, Yang et al. found that galectin-12 is mostly located in large lipid droplets in adipocytes and functions as a negative regulator of lipolysis. Lipolysis is enhanced in galectin-12 knockout ($Gal12^{-/-}$) mice through increased protein kinase A activity and cAMP levels. Furthermore, galectin-12 deficiency improves insulin sensitivity and glucose tolerance in overweight animals (Yang et al. 2011). Collectively, these data show that galectin-12 is important in energy metabolism and might be a promising therapeutic target to combat metabolic disorders (Baum 2011).

Obesity-associated insulin resistance is a major factor underlying the development of type 2 diabetes. Impaired insulin sensitivity involves multiple organs, including hypertrophic adipose tissue and fatty liver (Shoelson et al. 2003, 2006; Boden et al. 2005; Zemel et al. 2008). In recent years, it has become clear that obesity is associated with chronic, low-grade inflammation, which shows that inflammation might be a potential mechanism for obesity-related insulin resistance and ultimately the pathogenesis of type 2 diabetes mellitus. During the course of obesity, macrophages infiltrate adipose tissue, and along with adipocytes, secrete a variety of pro-inflammatory cytokines and chemokines, including resistin, interleukin (IL)-6, tumor necrosis factor (TNF)-α, IL-1β and monocyte chemoattractant protein (MCP)-1. Many of these cytokines, such as TNF-α and IL-6, have been shown to induce insulin resistance, both locally and systemically. Moreover, the adipocyte-secreted chemokine MCP-1 reduces insulin sensitivity (Shoelson et al. 2003, 2006; Sun and Zemel 2008; Herrero et al. 2010; Zemel et al. 2010). In visceral fat obesity, adipose tissue is markedly infiltrated by macrophages that participate in initiating and maintaining inflammatory responses when activated in this tissue (Weisberg et al. 2003; Xu et al. 2003; Clement et al. 2004). This macrophage infiltration and expression of inflammatory genes correspond to the development of insulin resistance, which indicates that macrophages are important mediators of chronic inflammation in adipose tissue (Weisberg et al. 2003; Xu et al. 2003).

Macrophages in adipose tissue play an important role in adipose inflammation. Several reports have indicated the heterogeneity of macrophages infiltrating into adipose tissue. Generally, there are two different polarization states of macrophages: M1, or classically activated macrophages (pro-inflammatory), which can be induced by lipopolysaccharide (LPS) or the Th1 cytokine interferon (IFN)-γ; and M2, or alternatively activated macrophages (AAMs), which are promoted by when Th2 (IL-4 or IL-13) and are anti-inflammatory and IL-10-secreting (Huang et al. 2012). In obese adipose tissue, infiltrating macrophages are mainly of the M1 state, which promote the inflammatory response in adipose tissue (Odegaard et al. 2007, 2008; Kang et al. 2008; Lumeng et al. 2008). Moreover, monocytes in patients with obesity or obesity/type 2 diabetes mellitus show more M1 markers and fewer M2 markers compared with those in normal-weight controls. This phenotypic change in monocytes is associated with the deterioration of several metabolic parameters and the stiffness of arteries (Satoh et al. 2010). A diet-induced obesity animal model has shown that increasing AAMs in adipose tissue improves insulin sensitivity. Therefore, increasing the number of AAMs shows potential to be an effective method to alleviate insulin resistance (Shoelson et al. 2006; Nishimura et al. 2009; Roth et al. 2011).

Galectin-12 is expressed by adipocytes, but whether it is expressed in macrophages is currently not known. It is not known if galectin-12 knockout improves insulin sensitivity by modulating macrophage polarization. Determining how galectin-12 affects inflammation and macrophage polarization and activation is important for understanding the biological role of galectin-12, and therefore, we attempted to study this phenomenon in detail.

Discussion

In this study, we found that galectin-12 was expressed in macrophages, and the level of galectin-12 was increased in macrophages activated by LPS or palmitic acid (Figure 2). The promoter region of galectin-12 contains two binding sites for sequence-specific protein 1 (SP1) and one binding site for CCAAT/enhancer-binding protein (C/EBP) (Hotta et al. 2001). LPS and palmitic acid can activate both SP1 (Hirata et al. 2008) and C/EBP (Udofa et al. 2013) through TLR-4, which in turn promotes the expression of galectin-12 in macrophages.

Co-cultured macrophages and adipocytes significantly increased the secretion of pro-inflammatory cytokines, such as TNF-α, which modulates insulin sensitivity in adipocytes (Sell and Eckel 2010; Suganami and Ogawa 2010; Rocha and Folco 2011). TNF-α induces the lipolysis of hypertrophied adipocytes to release saturated fatty acids, which activate macrophages through TLR-4 and TLR-2 to induce the secretion of inflammatory cytokines (Suganami et al. 2005; Sell and Eckel 2010). The vicious cycle developed between saturated fatty acids and TNF-α continuously induces inflammatory responses in the adipose tissue and subsequently induces insulin resistance. Hypertrophied adipocytes can secrete MCP-1, which plays important roles in the infiltration of macrophages into adipose tissue. Inhibiting the infiltration of macrophages by pharmacological or genetic approaches (i.e., MCP-1 knockout or CCR2 knockout) improves the secretion of adipokines/inflammatory cytokines, which suppress insulin resistance. The levels of pro-inflammatory cytokines TNF-α, IL-6, MCP-1 and CXCL1 are low in LPS-treated, palmitic acid-treated, and hypertrophied adipocyte culture supernatant-activated $Gal12^{-/-}$ macrophages. In co-culture experiments with macrophages and hypertrophied adipocytes, we found low levels of TNF-α, IL-6, MCP-1 and CXCL1 in $Gal12^{-/-}$ macrophage/adipocyte interaction. Moreover, culture supernatants of LPS- and palmitic acid-activated $Gal12^{-/-}$ macrophages increased insulin-mediated glucose uptake in adipocytes.

M2 macrophages generate IL-10 and other anti-inflammatory cytokines, as well as arginase, which inhibits the activity of nitric oxide synthase (iNOS). $Gal12^{-/-}$ macrophages produce NO in low quantities. In contrast, M1 macrophages release iNOS and classical pro-inflammatory cytokines (IL-1, IL-6 and TNF-α). In addition, TLR-4 and TLR-2 are expressed in high amounts in adipocytes, pre-adipocytes, macrophages and endothelial cells and are involved in the inflammatory process (Kuo et al. 2011). Signals mediated by both cytokines/chemokines and TLR-2/TLR-4 predominantly culminate in the activation of NF-κB, an important transcription factor in the inhibition of insulin signaling. NF-κB drives the production of

numerous pro-inflammatory cytokines, including IL-1β, TNF-α and IL-6, and is central in obesity-induced inflammation. It has been shown that a high-fat diet increases NF-κB activation, which may lead to increased inflammation. The other important transcription factors involved in pro-inflammatory cytokine expression are AP1 and IRF3. The activation of AP1 is mediated by the phosphorylation and activation of MAPKs (JNK, p38 and ERK) (Figure 1). Targeted deletion or pharmacological inhibition of the NF-κB pathway (IKK) (Winer et al. 2009; Balistreri et al. 2010; Ilan et al. 2010) or MAPK pathway (JNK) (Solinas et al. 2007) restores insulin sensitivity in obese mice.

TLR-2/TLR-4 can also activate IRF3, which in turn activates the expression of type I IFNs. Type I IFNs induce receptor heterodimerization, and subsequently, the IFNAR1 subunit is phosphorylated, and it binds to STAT2. STAT2 is phosphorylated at Tyr690. The phosphorylated STAT2 binds to IFNAR2 and recruits STAT1 or STAT6 to the receptor. Subsequently, STAT1 or STAT6 is phosphorylated at Tyr701 or Tyr641, respectively, by JAK1 or Tyk2 (Figure 1) (Wan et al. 2009). STAT1, STAT2 and STAT6 work in concert to exert antiviral and antitumor activities. However, the influence of type I IFNs on insulin sensitivity has not been studied. In our results, we found that the ablation of galectin-12 inhibited the activation of NF-κB- and AP1-mediated inflammatory responses, which indicates galectin-12 is a pro-inflammatory protein (Figure 1).

TNF-α is produced by both adipocytes and macrophages. Weisberg *et al.* found that macrophages are responsible for the production of almost all TNF-α (Weisberg et al. 2003). Comparatively, macrophages, adipocytes and the non-macrophage stromal vascular fraction express approximately the same amount of IL-6. These data suggest that adipose tissue-infiltrating macrophages are the major

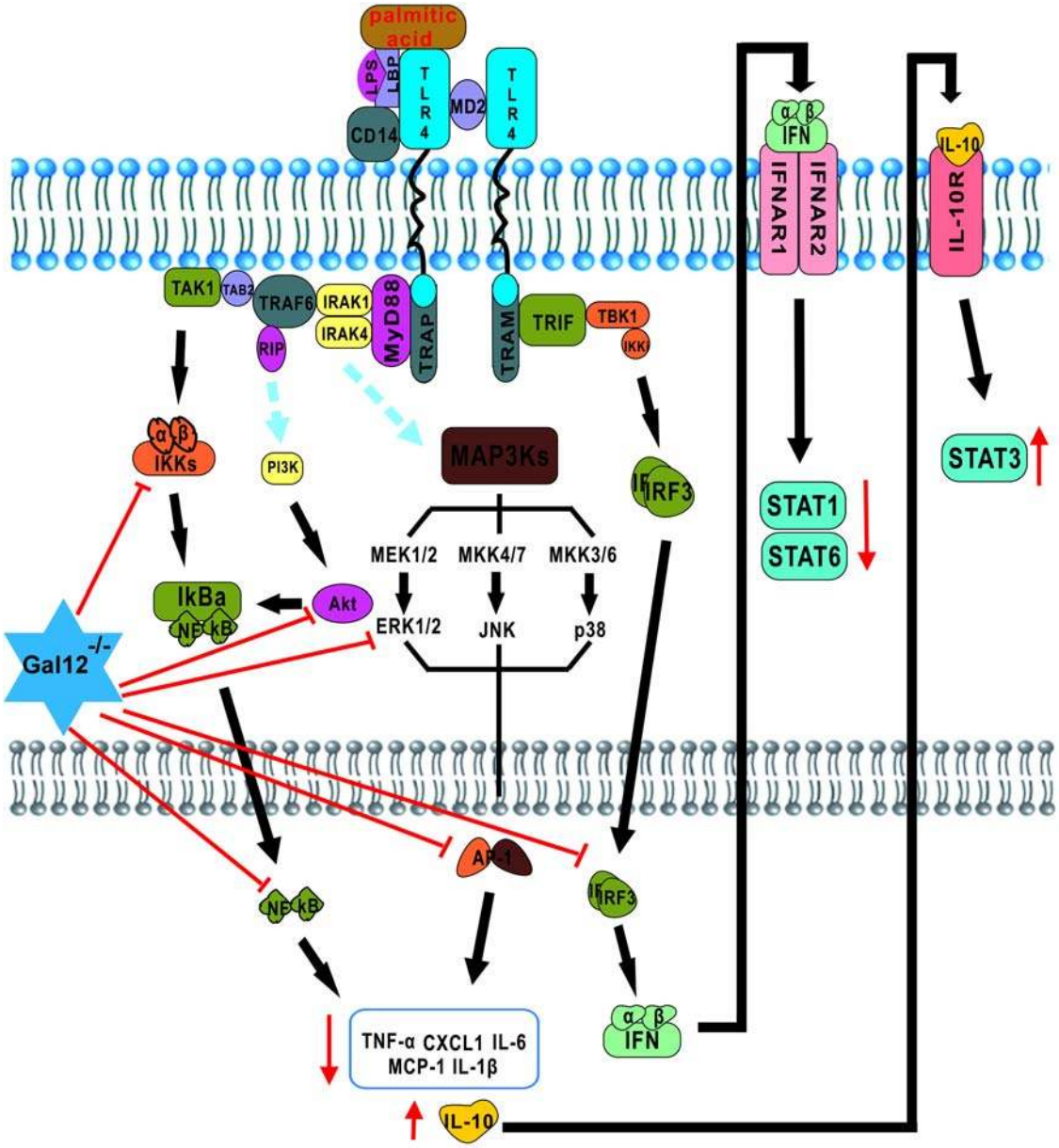

Fig. 1. Schematic presentation of how galectin-12 modulates the signaling pathway to modulate the levels of inflammatory cytokines. This figure is available in black and white in print and in color at *Glycobiology* online.

players in increased serum TNF-α and IL-6 in obese patients and are, therefore, important in inducing insulin resistance (Weisberg et al. 2003). Hereof, increasing the expression of chemokines by hypertrophied adipocytes was shown to be important in the recruitment of monocytes into adipose tissue. We found that the Gal12$^{-/-}$ mice fed a high-fat diet exhibited fewer macrophage infiltrations into adipose tissue compared with Gal12$^{+/+}$ mice (Supplementary data, Figure S5A and B). We also found that the total cholesterol and triglyceride in sera were lower in Gal12$^{-/-}$ mice fed a high-fat diet (data not shown). Since total cholesterol and triglyceride are risk factors for atherosclerosis, these data indicate inhibiting Gal12 would be a target for atherosclerosis. This was not caused by the likely differentiation of monocytes from stem cells in the Gal12$^{-/-}$ mice. We did not find any difference in the complete blood count results between Gal12$^{+/+}$ and Gal12$^{-/-}$ mice (Supplementary data, Table S1).

The level of galectin-12 is downregulated in Gal3$^{-/-}$ mice fed with or without high-fat diet, which indicates galectin-3 may alter the expression of galectin-12 to exert its biological activity (Pang et al. 2013). There are no significant differences in the galectin-3 levels between Gal12$^{+/+}$ and Gal12$^{-/-}$ macrophages (Supplementary data, Figure S6). Thus, the effect of galectin-12 in modulating macrophage polarization is not due to altered galectin-3 expression in these cells.

Galectin-12 is garnering increased attention because of its involvement in the regulation of lipid metabolism and improvement of insulin sensitivity and glucose tolerance. Insulin sensitivity is associated with chronic inflammation and the complex regulation of adipocytes and macrophages. In the present study, we found that macrophages express galectin-12 and that it influences macrophage plasticity. Furthermore, the ablation of galectin-12 caused macrophages to polarize into M2 macrophages, which reduced inflammation and increased the insulin sensitivity of adipocytes. Our findings indicate that galectin-12 is a potential target for the development of a novel galectin-12-targeted therapy for the treatment of metabolic disorders.

Materials and methods

Animals and animal care

Gal12$^{+/+}$ and Gal12$^{-/-}$ mice with a C57BL/6 background were used in this study. All animal studies were approved by the China Medical University Animal Care and Use Committee. Mice were housed in ventilated cages (4 animals/cage) in a pathogen-free facility that maintained a 12-h light/12-h dark cycle. Mice were provided with autoclaved water and pellet food ad libitum. Littermates of Gal12$^{+/+}$ and Gal12$^{-/-}$ mice at ages 6–8 weeks were used.

For studies of high-fat-induced obesity (macrophage infiltration into adipose tissue), all mice were fed a standard diet till 8 weeks of age. From 8 weeks of age onward, male littermates of Gal12$^{+/+}$ and Gal12$^{-/-}$ mice were fed diets containing either 10% kcal from fat or 60% kcal from fat for 15 weeks. Epididymal adipose tissues were collected for immunohistochemical staining of macrophages by using anti-F4/80 monoclonal antibody.

Reagents and antibodies

Lactose and sucrose Sepharose beads were prepared according to Levi and Teichberg (1981). Mouse anti-galectin-12 polyclonal antibody was obtained by immunizing Gal12$^{-/-}$ Balb/c mice with recombinant mouse galectin-12. The sera of galectin-12 immunized mice were purified and used in the experiments.

Fluorescein isothiocyanate (FITC)-anti-CD11b and PE-anti-F4/80 were obtained from Abcam. Antibodies against IKKα/β, phosphorylated IKKα/β (Ser176/180), Akt, phosphorylated Akt (Ser473), ERK, phosphorylated ERK (Thr202/Tyr204), phosphorylated p38 (Thr180/Tyr182), JNK, phosphorylated JNK (Thr183/Tyr185), NFκB p65, phosphorylated NFκB p65 (Ser536), cJun, phosphorylated cJun (Ser 63), STAT1, phosphorylated STAT1 (Tyr701), STAT3, phosphorylated STAT3 (Tyr705), STAT6, phosphorylated STAT6 (Tyr641), phosphorylated insulin receptor substrate (IRS)-1 (Ser318), phosphorylated IRS-1 (Ser473) and β-actin were from Cell Signaling Technology.

Preparation of bone marrow-derived macrophages

Bone marrow cells from Gal12$^{+/+}$ and Gal12$^{-/-}$ mice were cultured in 10-cm petri dishes at 2×10^6 cells/mL in RPMI medium (Invitrogen), supplemented with 10% fetal bovine serum containing 10 ng/mL mouse recombinant GM-CSF (Peprotech). The culture medium was replaced on Days 3 and 5 to remove non-adherent cells. On Day 7, adherent cells were used for the experiments. To determine the differentiation of macrophages, cells from 7-day-old cultures were first treated with Fc receptor blocking reagent (Miltenyi Biotec) and then stained with FITC-anti-CD11b and PE-anti-F4/80 and analyzed by flow cytometry (Becton Dickinson).

Quantitative polymerase chain reaction

Total RNA was extracted by using an RNA isolation kit (Qiagen). cDNA was obtained by using reverse transcriptase (Invitrogen). Five micrograms of total RNA were used for each reverse transcription reaction. Primers and probes used for real-time quantitative polymerase chain reaction (qPCR) were selected from the universal probe system (Roche). Glyceraldehyde 3-phosphate dehydrogenase was used as a reference to normalize the RNA levels in each sample.

Immunofluorescence staining

Macrophages plated on 16-well chamber slides were washed with Tris-buffered saline (TBS), fixed with 4% paraformaldehyde and blocked with 1% bovine serum albumin and 0.1% Triton X-100 for 1 h. Cells were incubated with anti-galectin-12 antibody for 1 h. Next, the cells were washed with TBS and incubated with the appropriate secondary antibody and 4′,6-diamidino-2-phenylindole (DAPI) DNA stain. Cells were imaged using fluorescence microscopy. All experiments were performed at least in triplicate.

Immunohistochemistry

Adipose tissues were retrieved from mice fed a normal or high-fat diet for 10 weeks and embedded in paraffin. Antigen retrieval was performed by placing the slides containing fixed tissue sections in citrate buffer (pH 6.0) at 100°C for 3 min. Macrophages were stained with anti-F4/80 antibody. The EnVision System peroxidase kit (Dako, Carpentaria, CA, USA) was used to visualize immunoreactivity.

Phagocytosis assay

FITC-conjugated *Escherichia coli* bioparticles (Life Technologies) were opsonized with *E. coli* bioparticle opsonizing reagent (Life Technologies) according to the manufacturer's protocol.

Macrophages were cultured on cover slides in 6-well plates. Macrophages were washed and placed in serum-free medium and then incubated with opsonized FITC-conjugated *E. coli* bioparticles at 37°C for 30 min. The cover slides were washed with PBS, and macrophage nuclei were subjected to DAPI staining. Cells were imaged using fluorescence microscopy. All experiments were performed at least in triplicate.

Cytokine enzyme-linked immunosorbent assay

Macrophage culture supernatants were harvested and assayed for cytokine content using the commercially available enzyme-linked immunosorbent assay reagents for TNF-α, IL-6, MCP-1, IL-12 and CXCL1 and were measured according to the manufacturer's instructions (Duoset R&D).

Nitrite measurements

Nitrite concentrations in the cell supernatants were measured using the Griess reaction according to manufacturer's instructions (Life Technologies).

Western blot

Macrophages were harvested, washed with 10 mL of PBS three times, and lysed with mammalian protein extraction reagent (Thermo Fisher Scientific) containing phosphatase and protease inhibitor cocktails (Roche Life Science). Proteins were separated by 12.5% sodium dodecyl sulfide–polyacrylamide gel electrophoresis (SDS–PAGE) and blotted onto a polyvinylidene difluoride membrane. Membranes were probed with primary antibodies and then probed with an anti-mouse IgG secondary antibody conjugated with horseradish peroxidase. Binding was detected by a chemiluminescent substrate according to the manufacturer's instructions (Thermo Fisher Scientific). Blots shown are representative of at least three individual experiments.

Glucose uptake assay

Conditioned media were collected from Gal12$^{+/+}$ and Gal12$^{-/-}$ macrophages treated with LPS from *E. coli* (L2630; Sigma) at 0.1 ng/mL or palmitic acid at 400 μM for 16 h. The media were filtered through a 0.45-μm filter before incubation with adipocytes for 24 h. Adipocytes were starved in serum-free low-glucose DMEM for 3 h before insulin stimulation. 2-Deoxyglucose uptake assays were performed according to the manufacturer's protocol (Abcam).

Statistical analysis

For each study group, data were derived from at least three independent experiments. Statistical analysis was performed using Student's *t*-test to compare differences in values between the control

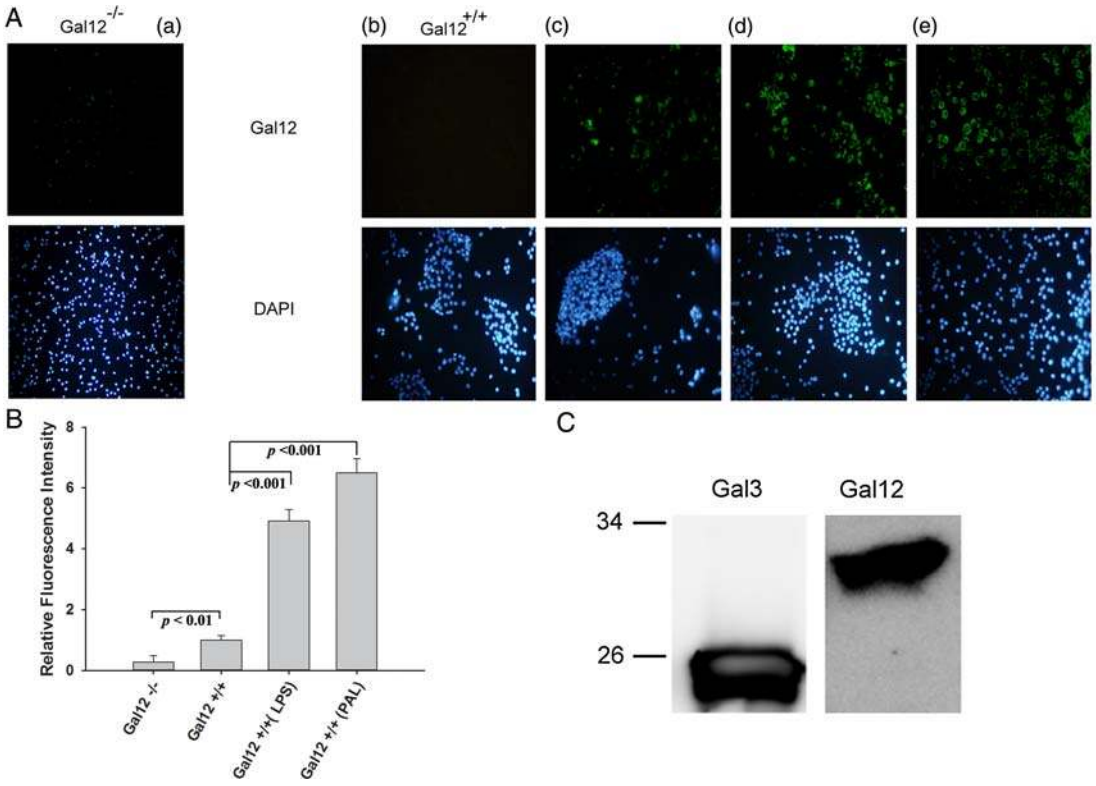

Fig. 2. Galectin-12 is expressed in macrophages. BMDMs were used to detect the expression of galectin-12. (**A**) Galectin-12 was detected by immunofluorescence staining in Gal12$^{+/+}$ and Gal12$^{-/-}$ BMDMs. Nuclei were stained with DAPI. (a) PBS-treated Gal12$^{-/-}$ BMDMs, (b) PBS-treated Gal12$^{+/+}$ BMDMs stained with anti-mouse IgG FITC-labeled antibody as a secondary control, (c) PBS-treated Gal12$^{+/+}$ BMDMs, (d) Gal12$^{+/+}$ BMDMs treated with 100 ng/mL LPS for 24 h, (e) Gal12$^{+/+}$ BMDMs treated with 400 μM palmitic acid for 24 h. (**B**) Relative fluorescence quantitation of the level of galectin-12 in (A), the fluorescence of Gal12$^{+/+}$ BMDMs was set as 1. Results are the mean ± SD of three independent experiments. (**C**) Lactose beads were incubated with 8 mg of total cell lysate of Gal12$^{+/+}$ BMDMs to pull down galectin-12 and -3. The cell lysate was incubated with 100 μL of beads for 24 h at 4°C and galectins were then dissociated from the beads by SDS–PAGE sample buffer. Twenty microliters of beads were used to perform a western blot analysis. The blot used to detect galectin-12 was stripped and re-probed with anti-galectin-3 antibody. This figure is available in black and white in print and in color at *Glycobiology* online.

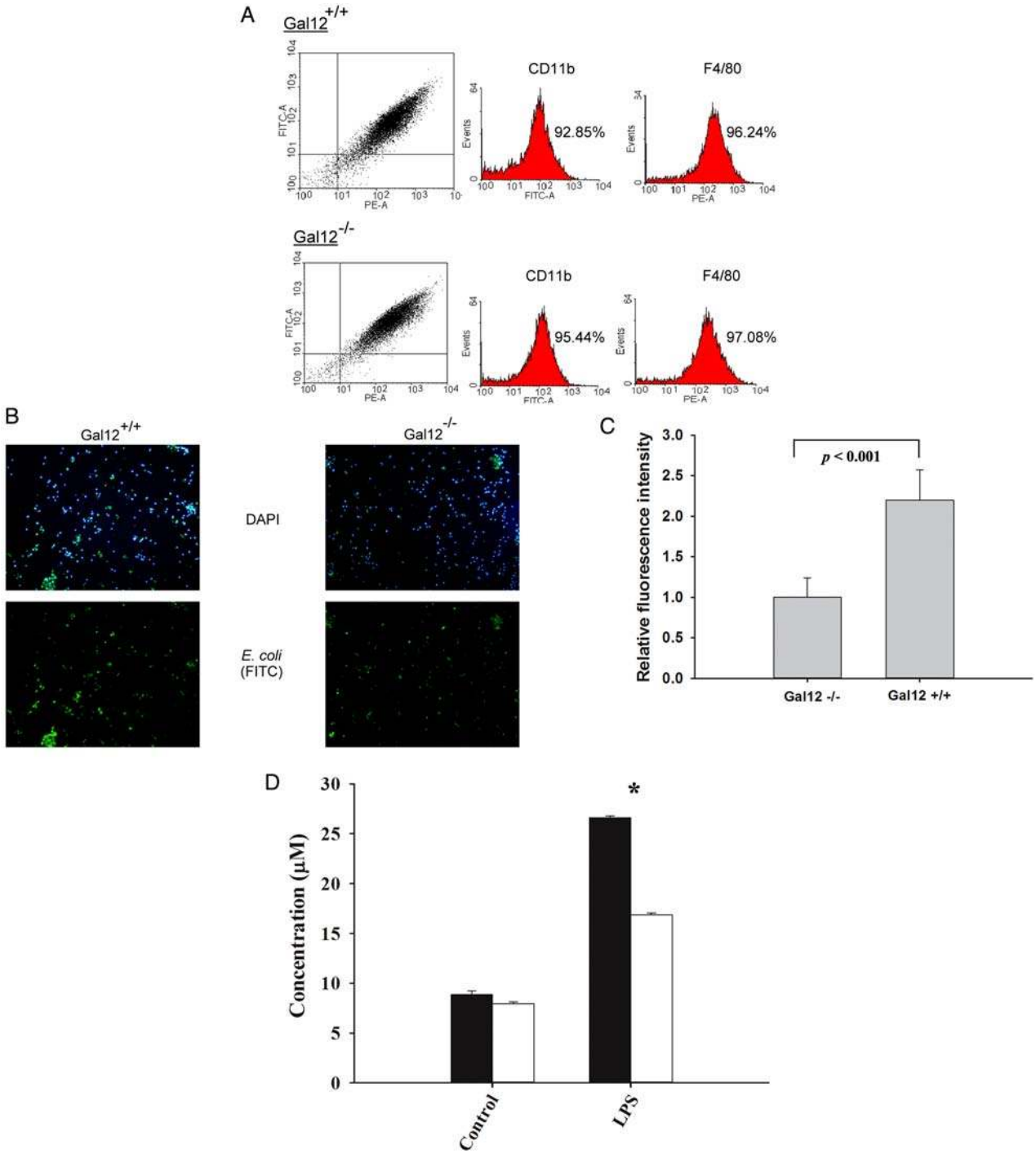

Fig. 3. Galectin-12 deficiency does not influence macrophage differentiation. (**A**) Bone marrow cells were treated with 10 ng/mL GM-CSF for 7 days to promote macrophage differentiation. Macrophages were harvested and stained with anti-F4/80 (PE) and ant-CD11b (FITC) antibodies. Percentages denote $CD11b^+$ or $F4/80^+$ cells among total cells. (**B**) Macrophages were cultured in chamber slides at 5×10^4 cells per well and incubated with 1.5×10^6 FITC-labeled *E. coli* for 30 min at 37°C. Cells were washed with phosphate-buffered saline and stained with DAPI. (**C**) Relative fluorescence quantitation of the phagocytosed FITC-labeled *E. coli* in (B), the fluorescence of $Gal12^{-/-}$ BMDMs was set as 1. Results are the mean ± SD of three independent experiments. (**D**) Macrophages were treated with 100 ng/mL LPS for 24 h, and the levels of NO in the cell culture supernatants were determined using Griess reagent. Black bars: $Gal12^{+/+}$; white bars: $Gal12^{-/-}$. Results are the mean ± SD of three independent experiments. Asterisks indicate that the calculated *p*-values for paired comparisons between control and LPS-treated samples are statistically significant. This figure is available in black and white in print and in color at *Glycobiology* online.

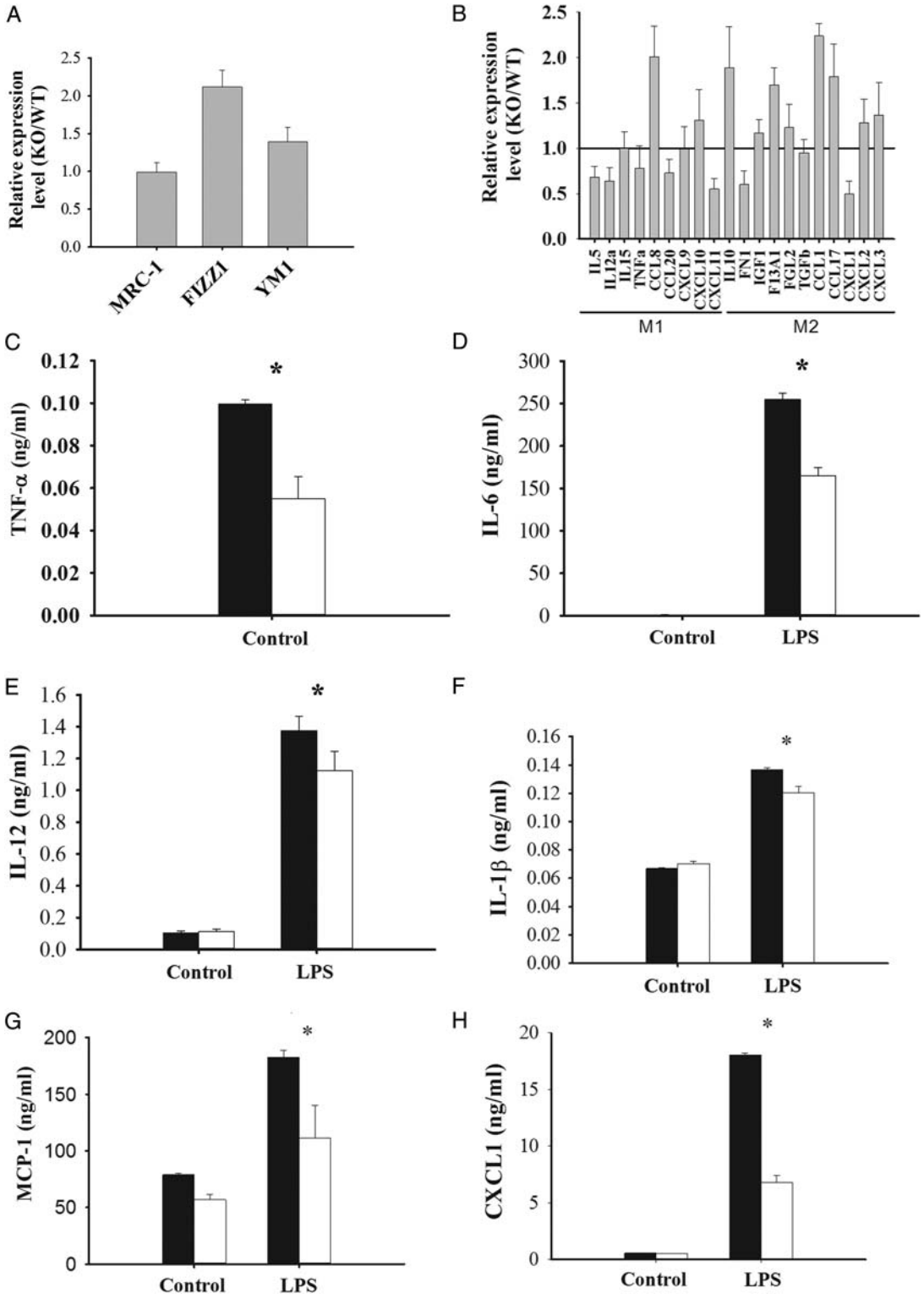

Fig. 4. Galectin-12 knockout mice exhibit M2 polarization in macrophages. (**A**) Real-time qPCR was performed to determine the levels of M2 markers in Gal12$^{+/+}$ and Gal12$^{-/-}$ BMDMs. (**B**) qPCR was performed to determine the levels of M1 or M2 cytokines and chemokines in Gal12$^{+/+}$ and Gal12$^{-/-}$ BMDMs. (**C**)–(**H**) BMDMs from Gal12$^{+/+}$ and Gal12$^{-/-}$ C57BL/6 mice were cultured in the presence of LPS for 24 h, and then, the TNF-α (C), IL-6 (D), IL-12 (E), IL-1β (F), MCP-1 (G) and CXCL1 (H) levels in the culture supernatant were determined. LPS was added at a concentration of 100 ng/mL. Black bars: Gal12$^{+/+}$; white bars: Gal12$^{-/-}$. Results are the mean ± SD of three independent experiments. Asterisks indicate that the calculated *p*-values for paired comparisons between control and LPS-treated samples are statistically significant.

and experimental groups. The results are presented as the mean ± SD. $P < 0.05$ indicates a statistically significant difference.

Results

Galectin-12 is expressed in macrophages

The expression of galectin-12 in Raw264.7 and bone marrow-derived macrophages (BMDMs) was determined. The expression of galectin-12 in the mouse macrophage line Raw264.7 was determined by pull down assay using lactose beads. We found that galectin-12 was pulled down from this cell line by lactose beads (Supplementary data, Figure S1). We could also detect galectin-3 expression in Raw264.7 macrophages (Supplementary data, Figure S1). Immunofluorescence staining was used to detect the expression of galectin-12 in BMDMs. While no signal was detected in Gal12-knockout ($Gal12^{-/-}$) macrophages (Figure 2A-a), as expected, staining was clearly observed in wild-type macrophages ($Gal12^{+/+}$, Figure 2A-c). Galectin-12 expression was upregulated by LPS (Figure 2A-d and B) and palmitic acid (Figure 2A-e and B) treatments. In Figure 2C, we used 8 mg total cell lysate of BMDMs to pull down galectin-12 and -3 by lactose beads, and it was confirmed that the galectin-12 was expressed in BMDMs. These data indicate that galectin-12 is expressed in macrophages.

Galectin-12 deficiency does not affect macrophage differentiation

Studies using bone marrow transplants have demonstrated that most macrophages in adipose tissue are derived from bone marrow (Weisberg et al. 2003). We used BMDMs to characterize the differentiation pattern of macrophages. The differentiation status between $Gal12^{+/+}$ and $Gal12^{-/-}$ macrophages was compared by using CD11b and F4/80 macrophage markers. As shown in Figure 3A, in both $Gal12^{+/+}$ and $Gal12^{-/-}$ cells, over 95% of the cells exhibited macrophage markers, indicating that galectin-12 deficiency did not influence macrophage differentiation. We then compared the phagocytic activities between $Gal12^{+/+}$ and $Gal12^{-/-}$ macrophages. $Gal12^{-/-}$ macrophages showed lower phagocytic activity against *E. coli* compared with $Gal12^{+/+}$ macrophages (Figure 3B and C). The secretion of nitric oxide (NO) was also measured (Figure 3D). The NO concentration was lower in $Gal12^{-/-}$ macrophages when activated with LPS.

Ablation of galectin-12 promotes M2 polarization of macrophages

The downregulation of NO production and reduced phagocytic activities in $Gal12^{-/-}$ macrophages suggested that galectin-12 might influence the polarization status of macrophages into M2 subtype. The levels of M2 markers (MRC-1, FIZZ1 and Ym1) were determined by qPCR. As shown in Figure 4A, the level of MRC-1 was comparable between $Gal12^{+/+}$ and $Gal12^{-/-}$ macrophages. The other two markers FIZZ1 and Ym1 were more highly expressed in $Gal12^{-/-}$ macrophages (2.12- and 1.39-fold) (Figure 4A). We also tested several cytokines/chemokines that are known to be expressed in either M1 or M2 macrophages. The levels of the M1 chemokines CCL8 and CXCL10 were 2.01- and 1.31-fold higher in $Gal12^{-/-}$ macrophages. The rest of the M1 markers had lower expression or no changes between the $Gal12^{+/+}$ and $Gal12^{-/-}$ macrophages (Figure 4B). For the M2 cytokines/chemokines, the levels of IL-10 (1.89-fold), IGF1 (1.17-fold), F13A1 (1.7-fold), FGL2 (1.23-fold), CCL1 (2.24-fold), CCL17 (1.79-fold), CXCL2 (1.28-fold) and CXCL3 (1.37-fold) were higher in $Gal12^{-/-}$ macrophages (Figure 4B). We also found that the levels of the following M1 cytokines and chemokines, TNF-α (Figure 3C), IL-6 (Figure 4D), IL-12 (Figure 4E), IL-1β (Figure 4F), MCP-1 (Figure 4G) and CXCL1 (Figure 4H) were lower in $Gal12^{-/-}$ macrophages when activated with 100 ng/mL LPS for 24 h.

Saturated fatty acids can activate macrophages through Toll-like receptor 4 (TLR-4) and promote macrophage polarization into M1 macrophages. $Gal12^{+/+}$ and $Gal12^{-/-}$ macrophages were treated with different concentrations of palmitic acid for 24 h, and the expression levels of TNF-α, IL-6, IL-12, MCP-1 and CXCL1 were determined by ELISA. We found lower expression levels of IL-6 (Supplementary data, Figure S2A), TNF-α (Supplementary data, Figure S2B), IL-12 (Supplementary data, Figure S2C) and CXCL1 (Supplementary data, Figure S2D), but not MCP-1 (Supplementary data, Figure S2E) in $Gal12^{-/-}$ macrophages. Hypertrophic adipocytes release long-chain saturated fatty acids, which activate macrophages. 3T3-L1 cells were differentiated into adipocytes, and culture supernatant was collected on Day 21. Hypertrophied adipocyte culture supernatant was used to treat $Gal12^{+/+}$ and $Gal12^{-/-}$ macrophages for 24 h. We found lower TNF-α, IL-6, IL-12, MCP-1 and CXCL1 in $Gal12^{-/-}$ macrophages (Figure 5A). $Gal12^{+/+}$ and $Gal12^{-/-}$ macrophages were co-cultured with hypertrophied adipocytes to determine the levels of these cytokines/chemokines. Their levels were lower in $Gal12^{-/-}$ macrophages (Figure 5B). Considering these findings together, we surmise that galectin-12 deficiency in macrophages results in the cells polarized more to the M2 direction when activated.

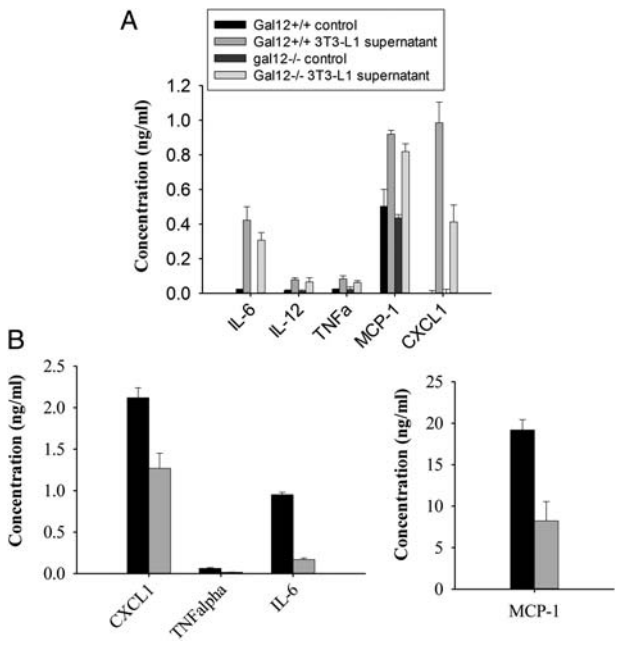

Fig. 5. $Gal12^{-/-}$ BMDMs expressed a lower level of inflammatory cytokines when activated by the culture supernatant of hypertrophied adipocytes. (**A**) 3T3-L1 cells were differentiated into adipocytes for 21 days, and the culture supernatant of hypertrophied adipocytes was collected on Day 21. 3T3-L1 culture supernatant was used to treat $Gal12^{+/+}$ and $Gal12^{-/-}$ BMDMs for 24 h, and the levels of TNF-α, IL-6, IL-12, MCP-1 and CXCL1 were determined by ELISA. (**B**) $Gal12^{+/+}$ and $Gal12^{-/-}$ BMDMs were co-cultured with 3T3-L1-derived adipocytes (Day 21) for 24 h, and the levels of TNF-α, IL-6, MCP-1 and CXCL1 were determined by ELISA. Black bars: $Gal12^{+/+}$; gray bars: $Gal12^{-/-}$.

Galectin-12 modulates NF-κB, AP-1 and IRF3 signaling pathways

To determine the molecular mechanisms of how galectin-12 regulates the polarization of macrophages, we monitored the activation of the canonical NF-κB pathway and MAPK pathway, which involves activation of NF-κB and activator protein 1 (AP1) to promote the expression of pro-inflammatory cytokines/chemokines. We found that the phosphorylation of IKKα/β and Akt was lower in Gal12$^{-/-}$ macrophages (Figure 6A and B). The lower activation of IKKα/β and Akt reduces the phosphorylation of IκB, an NF-κB inhibitor, which leads to lower NF-κB activation and eventually lower cytokine/chemokine expression. ERK activates AP1, which also increases the expression of pro-inflammatory cytokines. We found that ERK activation was lower in Gal12$^{-/-}$ macrophages (Figure 6C). The phosphorylation of p38 and JNK was not different between Gal12$^{+/+}$ and Gal12$^{-/-}$ macrophages (Figure 6D and E, respectively). The activation of NF-κB p65 and c-Jun were also significantly lower in Gal12$^{-/-}$ macrophages treated with LPS or palmitic acid (Figure 7A and B, respectively). There was no significant difference in the activation of interferon regulatory factor-3 (IRF3) between Gal12$^{+/+}$ and Gal12$^{-/-}$ macrophages; however, the level of IRF3 was significantly lower in Gal12$^{-/-}$ macrophages (data not shown).

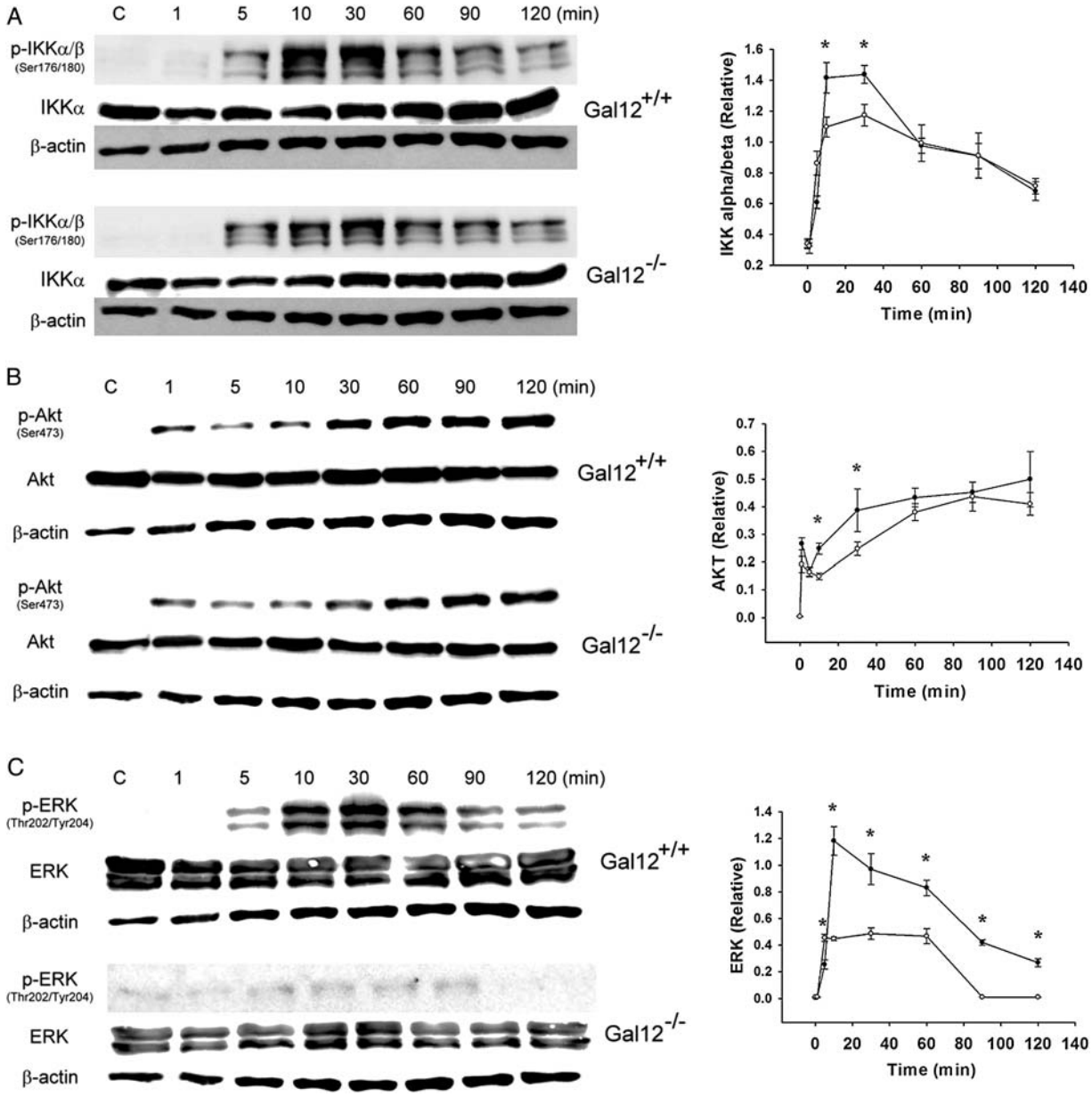

Fig. 6. Galectin-12 deficiency downregulated the activation of the NF-κB pathway and MAPK pathway in BMDMs. BMDMs were treated with 100 ng/mL LPS for the indicated times. Cells were harvested, and western blotting was performed to determine the phosphorylation of IKKα/β, Akt, JNK, ERK and p38. (**A**) IKKα/β (ser176/180), (**B**) Akt (Ser473), (**C**) JNK (Thr183/Tyr185), (**D**) ERK (Thr202/Tyr204) and (**E**) p38 (Thr180/Tyr182). The relative phosphorylation levels were determined by densitometry analysis. Western blots are one representative figure of three independent experiments. Results are the mean ± SD of three independent experiments. Asterisks indicate that the calculated *p*-values for paired comparisons between Gal12$^{+/+}$ and Gal12$^{-/-}$ are statistically significant. ●, Gal12$^{+/+}$; ○, Gal12$^{-/-}$.

Signaling through TLR-4 can also activate IRF3, which subsequently promotes the expression of type I IFN. Type I IFN then binds to the type I IFN receptor to phosphorylate STAT1 and STAT6. In Gal12$^{-/-}$ macrophages, the activation of STAT1 and STAT6 was lower compare with Gal12$^{+/+}$ macrophages (Supplementary data, Figure S3A and B, respectively). These results suggest that Gal12$^{-/-}$ macrophages secrete less type I IFNs when activated by LPS. The activation of macrophages also increases the levels of IL-10, which then phosphorylates STAT3. The activation of STAT3 was much higher in Gal12$^{-/-}$ macrophages (Figure 8).

Gal12$^{-/-}$ macrophage-conditioned medium improves insulin sensitivity

To determine whether the improve insulin sensitivity in Gal12$^{-/-}$ mice are partly due to the M2 polarization of Gal12$^{-/-}$ macrophages. Macrophage-conditioned medium was collected by treatment with 0.1 ng/mL LPS or 400 μM palmitic acid for 16 h and then added to 3T3-L1 adipocytes for 24 h. Adipocytes were then treated with insulin, and a glucose uptake assay was used to evaluate insulin responsiveness. Adipocytes exposed to conditioned media

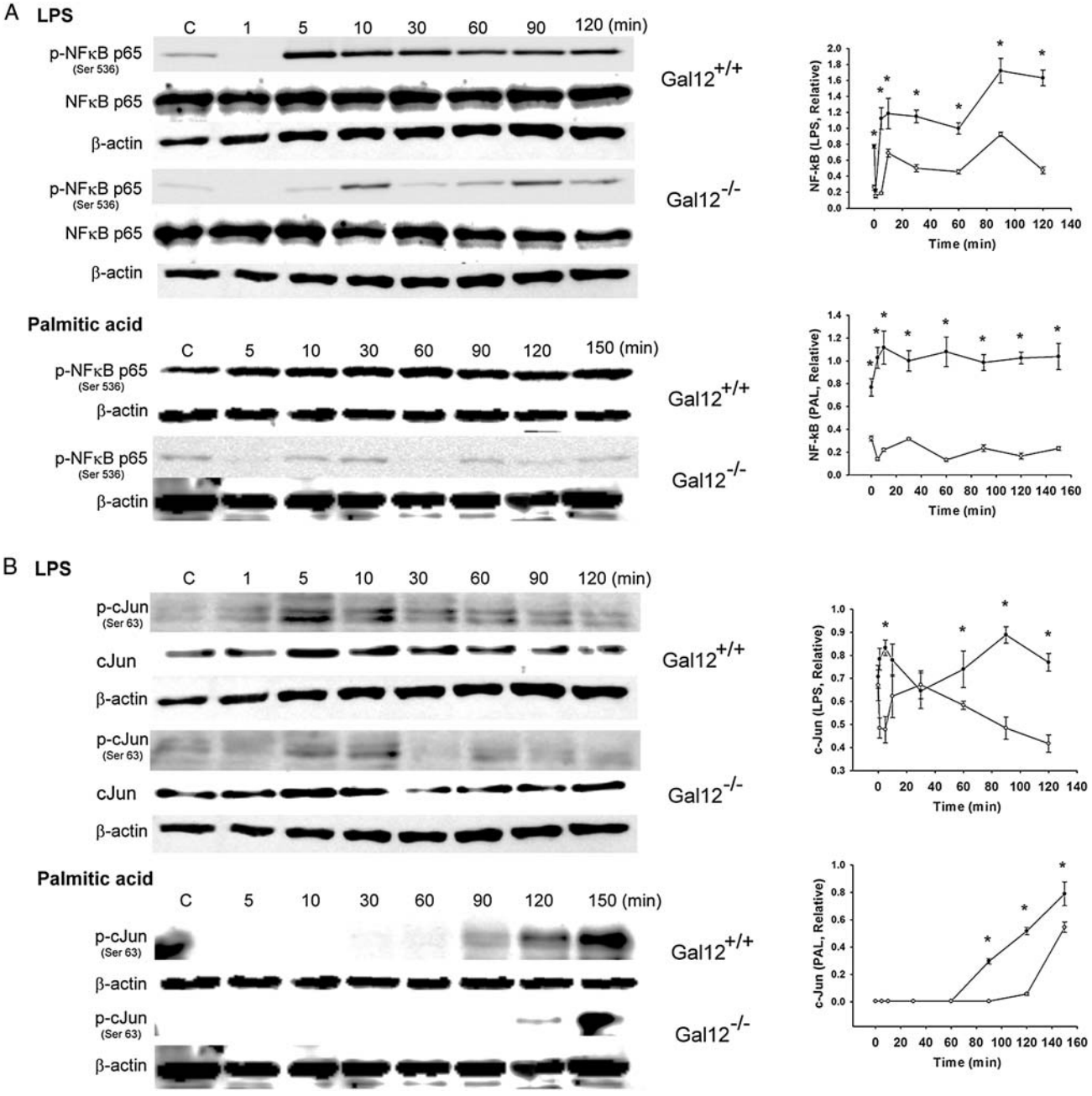

Fig. 7. Galectin-12 deficiency downregulated the activation of the NF-κB p65 and cJun in BMDMs. BMDMs were treated with 100 ng/mL LPS or 400 μM palmitic acid for the indicated times. Cells were harvested, and western blotting was performed to determine the phosphorylation of NF-κB p65 and cJun. (**A**) NF-κB p65 (Ser536) and (**B**) cJun (Ser63). Western blots are one representative figure of three independent experiments. Relative quantitation results are the mean ± SD of three independent experiments. Asterisks indicate that the calculated p-values for paired comparisons between Gal12$^{+/+}$ and Gal12$^{-/-}$ are statistically significant. ●, Gal12$^{+/+}$; ○, Gal12$^{-/-}$.

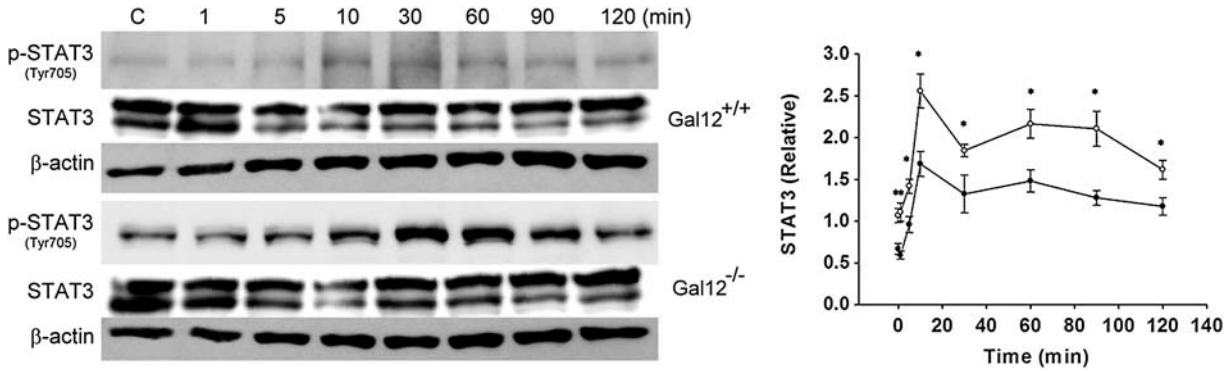

Fig. 8. Galectin-12 deficiency influences the activation of the STAT signaling pathway. BMDMs were treated with 100 ng/mL LPS for the indicated times. Cells were harvested, and western blotting was performed to determine the phosphorylation of STAT3 (Tyr705). Western blots are one representative figure of three independent experiments. Relative quantitation results are the mean ± SD of three independent experiments. Asterisks indicate that the calculated *p*-values for paired comparisons between Gal12$^{+/+}$ and Gal12$^{-/-}$ are statistically significant. ●, Gal12$^{+/+}$; ○, Gal12$^{-/-}$.

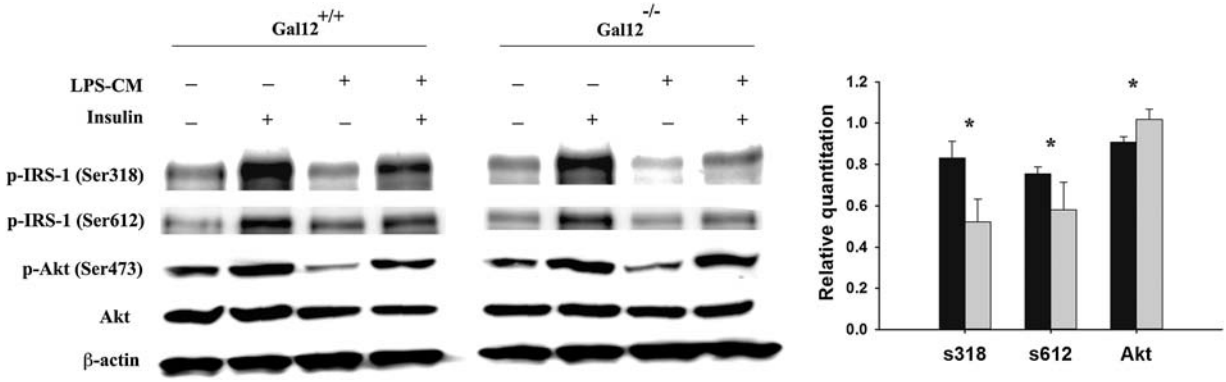

Fig. 9. Gal12$^{-/-}$ macrophage-conditioned medium (CM) enhanced insulin action in 3T3-L1 adipocytes. 3T3-L1 adipocytes were treated with or without CM for 24 h and then with or without 100 nM insulin for 30 min. Adipocyte cell lysates were harvested, and western blotting was performed to determine the phosphorylation of IRS-1 (Ser318), IRS-1 (Ser612) and Akt (Ser473). The relative phosphorylation levels were determined by densitometry analysis. Black bars: Gal12$^{+/+}$; gray bars: Gal12$^{-/-}$. Relative quantitation results are the mean ± SD of three independent experiments. Asterisks indicate that the calculated *p*-values for paired comparisons between Gal12$^{+/+}$ and Gal12$^{-/-}$ are statistically significant.

from LPS- or palmitic acid-activated Gal12$^{-/-}$ macrophages exhibited significantly higher insulin-stimulated glucose uptake compared with those exposed to that from similarly treated Gal12$^{+/+}$ macrophages (Supplementary data, Figure S4). We then examined insulin-signaling components. Adipocytes treated with Gal12$^{-/-}$ macrophage-conditioned medium had significantly lower serine phosphorylation of IRS-1 at Ser318 and Ser612, which is known to affect insulin function (Figure 9), compared with those treated with Gal12$^{+/+}$ macrophage-conditioned medium. Consistently, the former had higher Akt activation than the latter (Figure 9).

Supplementary data

Supplementary data for this article are available online at http://glycob.oxfordjournals.org/.

Conflict of interest statement

None declared.

Abbreviations

AAM, alternatively activated macrophage; Akt, v-Akt murine thymoma viral oncogene; AP1, activator protein 1; BMDM, bone marrow-derived macrophage; C/EBP, CCAAT/enhancer-binding protein; CCL, chemokine (C–C motif) ligand; CCR2, C–C chemokine receptor type 2; cJun, Jun proto-oncogene; CXCL, chemokine (C-X-C motif) ligand; DAPI, 4′,6-diamidino-2-phenylindole; ERK, extracellular signal-regulated kinases; FITC, fluorescein isothiocyanate; FIZZ1, resistin like alpha; GM-CSF, granulocyte-macrophage colony-stimulating factor; IFN, interferon; IKKα/β, i-kappa-b kinase; IL, interleukin, TNF-α, tumor necrosis factor-α; iNOS, nitric oxide synthase; IRF3, interferon regulatory factor 3; IRS-1, insulin receptor substrate-1; JAK1, Janus kinase 1; JNK, c-Jun N-terminal kinase; LPS, lipopolysaccharide; MCP-1, monocyte chemoattractant protein-1; MRC-1, mannose receptor, C type 1; NFκB, nuclear factor kappa-light-chain-enhancer of activated B cells; p38, p38 MAP kinase; SP1, sequence-specific protein 1; STAT, signal transducer and activator of transcription; TLR, toll-like receptor; TYK2, tyrosine kinase 2; Ym1, chintinase-like 3.

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