International Immunopharmacology 77 (2019) 105948

Contents lists available at ScienceDirect

International Immunopharmacology

journal homepage: www.elsevier.com/locate/intimp

Subcutaneous administration of α-GalCer activates iNKT10 cells to promote M2 macrophage polarization and ameliorates chronic inflammation of obese adipose tissue

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ARTICLE INFO

Keywords:
Obesity
Adipose tissue
iNKT1/iNKT2/iNKT10
M1/M2
Cytokine

ABSTRACT

Objective: The role of iNKT cells was investigated in chronic adipose tissue inflammation in obese mice after administration of α-GalCer in different pathways.

Methods: C57BL/6J mice were fed high-fat diet (HFD) for 12 weeks to establish the obese mouse model. The pathology of adipose tissue was observed by H&E staining. The rates of iNKT cells, macrophages and cell subsets in adipose tissue were detected by FCM. Cytokine levels in serum and adipose tissue lymphocyte-stimulated supernatants were assessed with the CBA kit. The expression levels of related transcription factor in adipose tissue were detected by Western blot.

Results: The proportions of iNKT cells, iNKT10 cells and M2 macrophages were decreased, while those of iNKT1 and M1 macrophages were increased in adipose tissue of HFD-fed mice. The expression levels of the related transcriptional proteins E4BP4 and Arg-1 were decreased while iNOS expression was increased in adipose tissue. Administration of α-GalCer by subcutaneous injection resulted in increased rates of iNKT10 cells and M2 macrophages, and decreased amounts of M1 macrophages in adipose tissue of HFD-fed mice. The expression of E4BP4 and Arg-1 were up-regulated, but iNOS was down-regulated. Meanwhile, infiltration of inflammatory cells into adipose tissue was further reduced.

Conclusion: The imbalance between the proportions of iNKT1 and iNKT10 cells may be involved in the development of chronic inflammation in obese adipose tissue. Administration of α-GalCer by subcutaneous injection in HFD-fed mice activates adipose tissue iNKT10 cells, which promote M2 macrophage polarization and improve chronic inflammation in obese adipose tissue.

1. Introduction

Obesity refers to an increase in body weight caused by excessive accumulation or abnormal distribution of body fat, which is a chronic metabolic disease involving multiple factors [1,2]. YUDKIN et al. first proposed obesity as a chronic low-grade inflammation in 1999 [3]. It has been demonstrated that chronic inflammation of adipose tissue is closely related to the occurrence of insulin resistance, fatty liver, type 2 diabetes, hyperlipidemia, and cardiovascular and cerebrovascular diseases [4–6]. It is known that a variety of immune cells such as

Abbreviations: α-GalCer, α-galactosylceramide; iNKT, invariant natural killer T cells; FCM, flow cytometry; CBA, cytometric bead array; IR, insulin resistance; NK, natural killer cell; DC, dendritic cell; Treg, regulatory T cell; PLZF, promyelocytic leukemia zinc finger; ROR-γt, retinoic acid receptor-related orphan nuclear receptor gamma; GATA-3, GATA binding protein-3; E4BP4, E4 Promoter-Binding Protein 4; SFD, standard-fat diet; HFD, high-fat diet; I.p, intraperitoneal injection with α-GalCer group; S.c, subcutaneous injection with α-GalCer group; GTT, glucose tolerance test; ITT, insulin tolerance test; BSA, bovine serum albumin; FCM, flow cytometry; PMA, phorbol 12-myristate 13-acetate; PBS, phosphate buffered solution; CBA, cytometric bead array; MACS, magnetic activated cell sorting; FBS, fetal bovine serum; iNOS, inducible nitric oxide synthase; ATM, adipose tissue macrophages

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https://doi.org/10.1016/j.intimp.2019.105948
Received 7 August 2019; Received in revised form 13 September 2019; Accepted 27 September 2019
Available online 16 October 2019

macrophages, NK cells, T cells, B cells and Treg cells are involved in the chronic inflammatory process of obese adipose tissue [7–13]. Previous studies have shown that immune cell dysfunction and imbalanced proportion of immune cells in adipose tissue are associated with chronic inflammation of adipose tissue, although the trigger mechanism of obesity inflammation is still unclear.

In recent years, it has been found that invariant natural killer T cells (iNKT) play an important role in chronic inflammation of obese adipose tissue. A previous work showed that the number of iNKT cells in obese patients is reduced [7], as also found in obese mice. Thus, obesity development might be inhibited by restoring the proportion of iNKT cells in the mouse [8–10]. However, it has also been reported that iNKT cells aggravate chronic inflammation of adipose tissue by releasing inflammatory cytokines in the presence of excess lipid [11,12]. At present, it is generally believed that iNKT cells are the key regulators of inflammation. However, it is still a contradiction whether iNKT cells are ameliorating in obesity. α-GalCer is a sphingolipid that was first extracted from the marine sponge Agelas mauritianas in 1994 by chloroform extraction and HPLC purification techniques [13,14]. As a classical iNKT cell-specific activator, which effectively activates iNKT1, iNKT2, iNKT17 and iNKT10, it has been widely used in the intervention studies of models such as tumors, infections and autoimmune diseases [15–17]. Studies have shown that specific activation of iNKT cell subpopulations more effectively causes immune regulation and immunotherapeutic effects [18,19]. The type of immune response mediated by iNKT cell activation is influenced by many factors, such as the structure of the ligand (α-GalCer, PBS57, C-glycoside activates iNKT cells to mediate Th1-type immune responses; whereas OCH, acC8:0, acC20:2 activation of iNKT cells mediated Th2-type immune response) [20–22], microenvironment in which iNKT cells are located, and the type of antigen-presenting cells [23]. Whether activation of different iNKT cell subtypes and the immune response type could be affected by α-GalCer administration through different routes is rarely reported. Our previous study found that α-GalCer can selectively activate different subpopulations of hepatic iNKT cells by diverse injection routes. iNKT2 cells in the liver and spleen of mice can be effectively activated by intraperitoneal injection with α-GalCer [24], while the iNKT1 cells can be effectively activated by subcutaneous injection with α-GalCer. In addition, we also found that the third day after intraperitoneal injection with α-GalCer, the rates of activated iNKT cells in relevant immune organs (liver and spleen) are highest. Meanwhile, the proportions of activated iNKT cells in relevant immune organs peaked the fifth day after subcutaneous injection with α-GalCer.

It is unclear whether various injection routes can selectively activate different subpopulations of adipose tissue iNKT cells, thereby affecting the chronic inflammatory process in obese adipose tissue. In the current study, C57BL/6J mice were fed a high fat diet to establish an obesity model, and α-GalCer was administered by intraperitoneal or subcutaneous injection to analyze proportion changes of iNKT cells and macrophage subpopulations in adipose tissue, further clarify the role of iNKT cells in chronic inflammation of obese adipose tissue.

2. Material and methods

2.1. Mice

Four- to five-week-old male C57BL/6J mice, whose average weight was 21.4 ± 0.67 g, were provided by Beijing Vital River Laboratory Animal Technology Co., Ltd. (SCXK (Beijing) 2016-0006). The mice were maintained under specific pathogen-free conditions (23 °C ± 2 °C with 50 ± 5% relative humidity) in the animal facility at Animal Lab of Medical Experiment Center, Hebei University, and all experiments were approved by the Animal Welfare and Ethical Committee of Hebei University (IACUC-2017009). The mice were provided free access water and food (standard-fat diet). Under a 12 h/12 h light/dark cycle with for at least 7 days before the experiment. Animal activities are monitored daily to ensure that the animals meet the experimental requirements. No mice died unexpectedly except experimental requirements.

2.2. Materials

High fat feed-H10060 (SCXK (Beijing) 2016-0008, was purchased from Beijing Huafukang Biotechnology Co., Ltd.); Glucose and insulin were from Solarbio (Beijing, China); PE-T-selected-CD1d tetramer was purchased from MBL International (Japan). FITC-hamster anti-mouse TCR β chain (553170), Alexa Fluor® 647-mouse anti-PLZF (563490) and PercP-Cy TM5.5 mouse anti-T-bet (561316) were obtained from BD Pharmingen (San Diego, CA, USA). PE-F4/80 (565410), FITC-CD11b (557396), Percp-CyTM5.5-CD11c (560584), Alexa Fluor®647-CD206 (565250), APC-IL-10 (561059) were from Becton Dickinson. Foxp3 Staining Buffer Set was purchased from eBioscience (California, USA). The α-GalCer (KRN7000) used in the current study is biosynthesis and was manufactured from Enzo Life Sciences (Farmingdale, NY, USA). TranscriPtion Factor Buffer Set was acquired from BD Pharmingen (San Diego, CA, USA). Accuri C6 flow cytometry was purchased from BD Pharmingen (San Diego, CA, USA).

2.3. Method

2.3.1. Groups

Male C57BL/6J mice were randomly selected as control groups and fed with standard-fat diet (SFD) (n = 15), the remaining 45 mice were fed with high-fat diet (HFD) for 12 weeks. The HFD-fed mice were then randomly divided into three groups: HFD-fed (n = 15) as model group, intraperitoneal injection (I.p) with α-GalCer group (The mice were sacrificed on the third day after injection with α-GalCer, n = 15), and subcutaneous injection (S.c) with α-GalCer group (The mice were sacrificed on the fifth day after injection with α-GalCer, n = 15).

2.3.2. General condition assessment of mice

The body weight, activity, mental state and coat gloss of the mice was recorded. We weighed the mice in each group per week. And judged their mental state by observing the activity status of the mice (excited or less active) and eating status (exuberant, good, low, and disappearing). The gloss of the hair was judged by observing the fur of the mouse (gloss, whether it was depilated), and whether it was greasy.

2.3.3. Glucose tolerance (GTT) and insulin tolerance test (ITT)

For glucose tolerance tests, the mice were fasted for 12 h and received 2 g glucose per kilogram body weight intraperitoneally and glucose levels were measured at 0, 15, 30, 60, and 120 min. For insulin resistance tests, the mice were fasted 6 h mice received 0.75U insulin per kilogram body weight intraperitoneally and glucose levels were measured at 0, 15, 30, 60, and 120 min. The fasting blood glucose was measured as blood glucose levels at 0 min.

2.3.4. Adipose tissue index and histopathology

The mice were weighed before the experiment. All mice were anesthetized with 50 mg sodium pentobarbital per kilogram of body weight by intraperitoneal injection and humanely sacrificed by cervical dislocation while under deep anesthetic conditions. Epididymal adipose tissue was harvested from HFD-fed mice and blotted on a blotting paper and weighted. The adipose tissue index was calculated as the percentage of the adipose tissue weight to the body weight of mice. Then, adipose tissue fixed in 10% formalin for 24 h, and subjected to microtome sectioning to generate 5-μm sections. H&E staining of tissue sections (5-mm) was performed for Microscopy.

We used the ImageJ software to quantify the images of adipose tissue pathology. The percentage of inflammatory cell infiltration area in the entire micrograph as a histological score was recorded. Three visual fields were randomly selected from adipose tissue sections of

mice for quantitative analysis, representing the degree of adipose tissue inflammation of the mice. Adipose tissue sections from 5 mice randomly selected in each group were quantified to indicate the degree of adipose tissue inflammation of each group. All mice in each group were reported in a summary plot with stats.

2.3.5. Quantitation of immune cells in adipose tissue using flow cytometric analysis

The peripheral blood (about 120 μL) was added in a flow tube, followed by blocking with BSA. Then, 2 μL of FITC-labeled Anti-TCR β and PE-labeled α-GalCer/CD1d-tetramer were used in 50 μL of the PBS reaction system. Incubation was conducted in the dark for 30 min at 4 °C. Erythrocyte lysis by red blood cell lysis buffer, then washing twice with PBS, resuspension of the cells in 500 μL PBS for iNKT cells detection by FCM.

Isolation of epididymal adipose tissue from mice, dissect the adipose tissue using a sterile scalpel and digested with type II collagenase for 1 h. Place the adipose tissue into 200 mesh cell filters, which were washed with 5 mL PBS solutions in petri dish. And gently mash adipose tissue fragment through the cell filter by 2 mL syringe plungers and centrifuge at 1000 rpm for 5 min at 4 °C. Wash adipose tissue mononuclear cell twice with PBS. Lymphocytes were isolated from adipose tissue mononuclear cells using lymphocyte separation medium and washed twice with PBS.

Further, 1×10^6 cells were placed in each flow tube and treated with mouse Abs (clones) were as follows: anti-TCR-β, and α-GalCer/CD1d tetramers, anti-F4/80、anti-CD11b, anti-CD11c. They were incubated at 4 °C for 30 min in the dark and washed twice with PBS. Then, the cells were re-suspended in 500 μL of PBS and iNKT cells and macrophages detected by flow cytometry.

Next, cells were fixed and permeabilized using BD Perm/Wash™ Buffer and stained with the following fluorochrome labeled mAbs: Alexa Fluor®647-CD206. Then, incubated in the dark for 30 min, washed twice with PBS, and re-suspended in 500 μL of PBS, followed M1 macrophages and M2 macrophages were detected by FACS measurement.

Adjusting the cell density to 2×10^6 cell/ml, inoculate the 12-well plate. Cultured system: RPMI-1640 complete medium (10% fetal bovine serum, FBS), Phorbol 12-myristate 13-acetate (PMA) (50 ng/mL), ionomycin (1 μg/mL) and Monensin-containing GolgiStop (4 μL/mL culture system). The cells were then cultured for 5 h and centrifuged at 1000 rpm for 5 min at room temperature. Then, FITC labeled anti-TCR-β (2 μL) and PE-labeled α-GalCer-loaded CD1d tetramers (2 μL) were incubated in 500 μL PBS reaction systems for 30 min in the dark at 4 °C, washed twice with PBS. The cells were permeabilized and fixed according to the specific procedure of Transcription Factor Buffer Set. Then, 5 μL each of PerCP-CyTM5.5 mouse anti-T-bet and APC mouse anti-IL-10 were added at room temperature in the dark for at least 30 min. After two washes with PBS, the cells were re-suspended in 500 μL PBS and iNKT subsets were assessed by FACS. PE-conjugated α-GalCer/CD1d-tetramers were generated in our laboratory, and α-GalCer (1 mg/mL) was diluted to 200 μg/mL with 0.5% Tween-20 and 0.9% NaCl. Then, 5 μL of the diluted α-GalCer was added per 100 μL of CD1d-tetramer solution, followed by incubation at room temperature for 12 h.

2.3.6. Adipose tissue lymphocyte culture supernatant and serum cytokines

Serum and adipose tissue lymphocytes were collected. Lymphocytes were isolated from adipose tissue cell suspension of mice using lymphocyte separation medium and washed twice with PBS. Then, Lymphocytes were stimulated in vitro with PMA and ionomycin for 5 h and cytokines in lymphocyte culture supernatant were analyzed by flow cytometry. The levels of cytokines (IL-2, IL-4, IL-6, IL-10, IL-17A, IFN-γ and TNF-α) were identified by cytometric bead array (CBA) kit. Cytokine detection steps are performed according to the CBA Kit instructions.

2.3.7. Western blot

The cell lysates were extracted with an extraction reagent. Whole cell lysates of epididymal adipose tissue (0.1 g) were separated on a gradient (4–20%) polyacrylamide NuPAGE gel and transferred onto polyvinylidene fluoride and blotted with anti-β-actin, anti-T-bet/Tb21, anti-Arg-1, anti-iNOS, anti-E4BP4. Fluorescence chemiluminescence detector was used to detect the expression of T-bet, E4BP4, Arg-1 and iNOS in each group. The adipose tissue was β-actin as the internal reference. Expression intensity was quantified by Image Lab.

2.3.8. Acquisition of macrophages

Six- to eight-week-old male C57BL/6J mice were intraperitoneally injected with sterile RPMI-1640 (1 mL). After three days, the peritoneal fluid was collected. The abdominal cavity was washed twice with PBS. The washing solution was collected, centrifuged at 1000 r/min for 10 min, washed twice with PBS, centrifuged at 1000 r/min for 5 min, and the supernatant was discarded.

2.3.9. Acquisition and purification of iNKT cells

Male C57BL/6J mice (6–7 weeks old) were injected intraperitoneally with α-GalCer (100 ng/g body weight) for 3 days. Spleen lymphocytes were obtained from mice under sterile conditions, washed twice with PBS, and counted. After cell density adjustment to 1×10^7 cells/100 μL, PE-α-GalCer/CD1d Tetramer was added and incubated for 15 min at 4 °C in the dark, followed by two PBS washes. Next, the cells were incubated with anti-PE MicroBeads at 4 °C for 20 min in the dark, washed twice with PBS, and resuspended in PBS (500 μL). Purified iNKT cells were obtained by MS column separation using a MACS sorter. Cell density (2×10^5 cell/mL) was adjusted in RPMI-1640 containing 10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin, and cell were seeded in 12-well flat-bottom culture plates.

2.3.10. Co-culture of iNKT cells with macrophages in vitro

The co-culture experiment set one control group (only macrophages) and two co-culture groups (iNKT cells and macrophages) in which the ratios of iNKT cells to macrophages were 1:1 and 1:5, respectively. The cell density was adjusted to 2×10^5 cells per milliliter of RPMI-1640 culture medium, and a-GalCer (100 ng/mL) was added to activate iNKT cells. Triplicate wells were set in each experiment. The cells were cultured in a 5% CO_2 incubator at 37 °C for 48 h, and macrophage frequency and subpopulations were detected by FCM. Cytokines in supernatant were detected by CBA.

2.4. Statistical analyses

Data were analyzed using SPSS version 19.0. (SPSS Inc, Chicago, IL, USA). For all analyses, data were presented as mean ± SEM. For comparison among the three groups, one-way ANOVA or nonparametric test was applied. For comparison between the two groups, unpaired *t*-test was conducted. Differences were considered statistically significant at $P < 0.05$.

3. Results

3.1. Effects of α-GalCer on general status and body weight of obese mice

The SFD-fed mice had smooth and glossy hair, were sensitive and fit. HFD-fed mice had larger body size, less activity, a greasy appearance, and hair loss (Fig. 1A). Food and drink consumptions were normal in all experimental mice. The average body weight of HFD-fed mice for 12 weeks increased by 39.9% ($P < 0.05$) compared with SFD-fed mice, reaching the body mass level requirement of obese model mice [25]. After 12 weeks of high-fat feeding, intraperitoneal injection with α-GalCer resulted in decreasing body weight of HFD-fed mice, whereas subcutaneous injection with α-GalCer resulted in increasing body

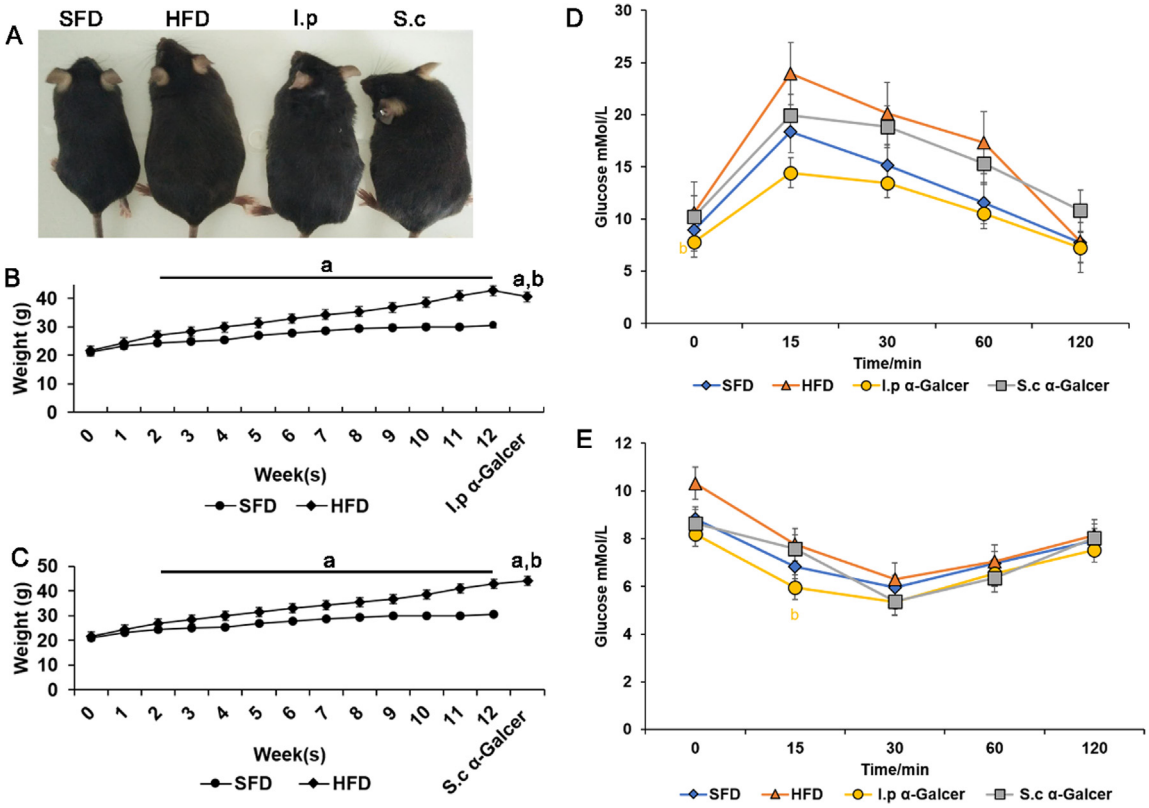

Fig. 1. The HFD-fed mice obtained more weight than SFD-fed mice, and administration of α-GalCer surely had no impact on glucose tolerance and insulin resistance in HFD-fed mice. (A): status of the mice in each group. Body weight changes in (B) SFD-fed mice, HFD-fed mice, HFD-fed mice of injected intraperitoneally with α-GalCer, (C) SFD-fed mice, HFD-fed mice and HFD-fed mice of injected subcutaneously with α-GalCer. n = 12–15 mice with three repeats. Blood glucose levels during (D) GTT and (E) ITT in mice of each group. n = 5 mice with three repeats. $^{a}P < 0.05$ vs SFD, $^{b}P < 0.05$ vs HFD.

weight of HFD-fed mice ($P < 0.05$) (Fig. 1B, C).

3.2. Effects of α-GalCer on GTT and ITT in obese mice

GTT results showed that blood glucose levels in HFD-fed mice were higher than those of SFD-fed mice at all time points, but there were no significant differences ($P > 0.05$), indicating that there was no obviously impaired glucose tolerance in HFD-fed mice for 12 weeks (Fig. 1D). The ITT results indicated that blood glucose levels in mice injected intraperitoneally with α-GalCer were lower than those of HFD-fed mice at 15 min after insulin injection ($P < 0.05$), indicating an increased sensitivity to insulin in HFD-fed mice treated with α-GalCer. After 120 min, there were no difference in blood glucose levels of mice in each group (Fig. 1E). The above results suggest that α-GalCer has no significant effect on glucose tolerance and insulin tolerance curves in HFD-fed mice.

3.3. Effects of α-GalCer on organ index and adipose tissue pathology in mice

Compared with SFD-fed mice, the HFD-fed mice had increased adipose tissue volume and adipose tissue index ($P < 0.05$). There were no significant changes in adipose tissue volume and adipose tissue index after intraperitoneal and subcutaneous treatments with α-GalCer in HFD-fed mice ($P > 0.05$) (Fig. 2A, B).

Adipocytes were increased in volume in HFD-fed mice compared with SFD-fed mice, and there was obvious inflammatory cell infiltration surrounding the adipocytes. Inflammatory cell infiltration was significantly increased following intraperitoneal α-GalCer injection in HFD-fed mice, but subcutaneous injection with α-GalCer resulted in a marked decrease in pro-inflammatory cell infiltration around the adipocytes (Fig. 2C, D). Pathological results indicated that α-GalCer injected subcutaneously in HFD-fed mice could improve chronic inflammation of adipose tissue.

3.4. Effects of α-GalCer on iNKT cells and subpopulations in adipose tissue of obese mice

3.4.1. Peripheral blood

There were no significant differences in frequency of peripheral blood iNKT cells between HFD-fed mice and SFD-fed mice ($P > 0.05$). Compared with HFD-fed mice, intraperitoneal injection with α-GalCer resulted in increasing of peripheral blood iNKT cells in HFD-fed mice ($P < 0.05$), whereas subcutaneous injection with α-GalCer has little effect on peripheral blood iNKT cells in HFD-fed mice (Fig. 3A, C).

3.4.2. Adipose tissue

The frequency of iNKT cells in adipose tissue of HFD-fed mice was significantly lower than that of SFD-fed mice ($P < 0.05$). Compared with HFD-fed mice, both intraperitoneal and subcutaneous injections with α-GalCer resulted in increasing of adipose tissue iNKT cells in HFD-fed mice ($P < 0.05$). Intraperitoneal injection with α-GalCer in HFD-fed mice resulted in increased frequency of iNKT cells in adipose tissue compared with subcutaneous injection (Fig. 3A; Fig. 3D). Further analysis of iNKT cell subsets revealed that the percentage of iNKT1 cells in adipose tissue of HFD-fed mice was significantly increased ($P < 0.05$), whereas that of iNKT10 cells was significantly decreased ($P < 0.05$) (Fig. 3B; Fig. 3E, F). The above results indicated that imbalance of the ratio of iNKT1 cells to iNKT10 cells in adipose tissue may be involved in the development of adipose tissue inflammation. After subcutaneous treatment with α-GalCer, the percentages of iNKT1 and iNKT10 cells in adipose tissue of HFD-fed mice were significantly increased ($P < 0.05$), especially iNKT10 cells. However, after

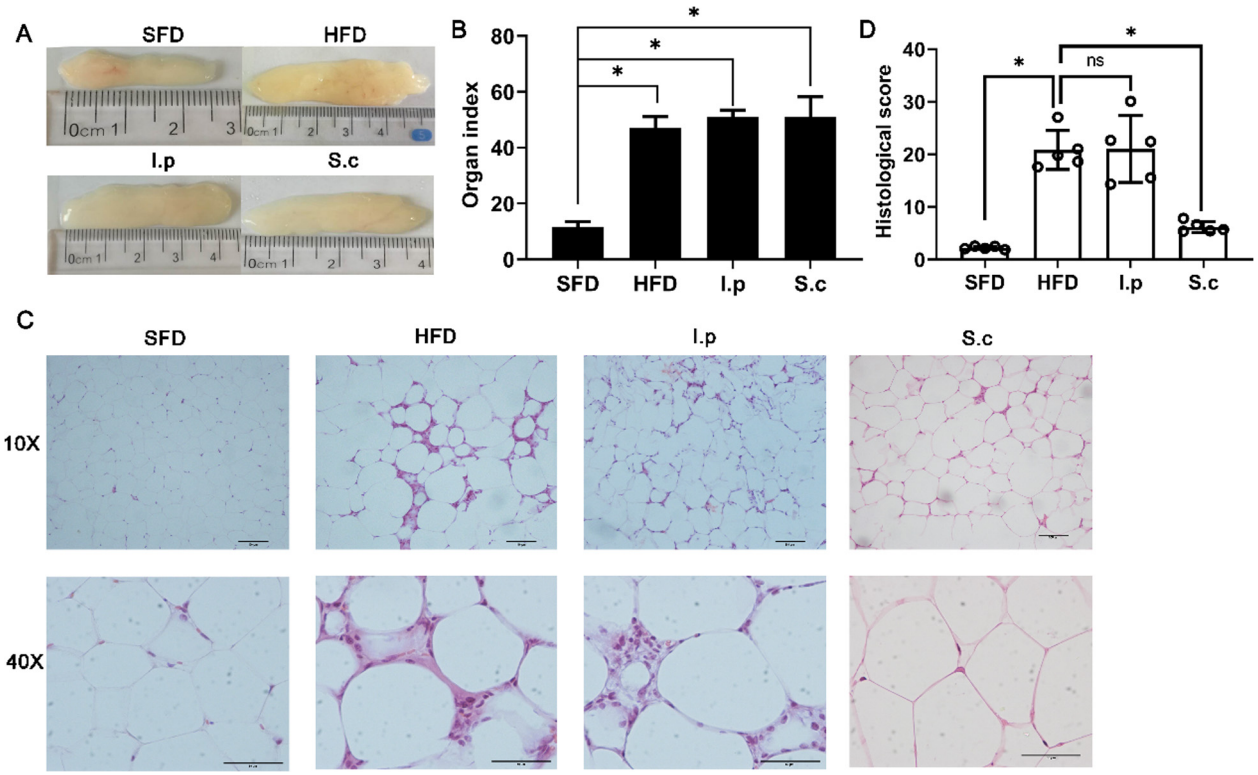

Fig. 2. The adipocytes volume of the epididymal adipose tissue in HFD-fed mice was larger than that of the other groups, and infiltration of inflammatory cell was also more serious than other groups. Adipose tissue inflammation was being improved well by subcutaneous injection with α-GalCer. (A): The appearance of adipose tissue in mice of each group. (B): Bar graph shows the comparison of organ index of epididymal adipose tissue in mice of each group. (C): Representative pictures from H&E-stained adipose tissue section of mice in each group. Photomicrographs were prepared at original magnification ×100 and ×400. (D): Bar graph showing the score of adipose tissue inflammation. Each group included five mice. Data representative of three independent experiments. *$P < 0.05$, "ns" indicates no statistical significance.

intraperitoneal injection with α-GalCer in HFD-fed mice, the proportion of iNKT1 cells in adipose tissue was decreased, while iNKT10 cells had no significant change (Fig. 3B; Fig. 3E, F). It indicated that injection with α-GalCer in different routes selectively activated iNKT cell subsets in adipose tissue, and subcutaneous injection with α-GalCer mainly activated adipose tissue iNKT10 cells.

3.5. Effects of α-GalCer on macrophage cells and subpopulations in adipose tissue of obese mice

The proportion of macrophages in adipose tissue of HFD-fed mice was increased significantly due to high-fat diet feeding for 12 weeks, especially pro-inflammatory M1 macrophages ($P < 0.05$). Administration of α-GalCer by intraperitoneal injection had no marked effects on macrophage proportion and subpopulations in adipose tissue of HFD-fed mice ($P > 0.05$). Injection with α-GalCer subcutaneously significantly reduced the proportions of total and M1 macrophages in adipose tissue, and significantly increased the proportion of M2 macrophages ($P < 0.05$) (Fig. 4A–E). These findings have important implications for improving chronic inflammation of adipose tissue. It is generally believed that α-GalCer is a specific activator of iNKT cells. Therefore, we hypothesized that subcutaneous injection with α-GalCer activates adipose tissue iNKT10 and induced polarization of M1 macrophages to M2 macrophages, thereby improving chronic inflammation of adipose tissue.

3.6. Effects of α-GalCer on secretion of cytokines in serum and adipose tissue

3.6.1. Serum

The levels of serum pro-inflammatory cytokine (IL-2, IL-17A, TNF-α and IFN-γ) were significantly increased in HFD-fed mice, whereas anti-inflammatory cytokine (IL-10 and IL-4) levels were significantly decreased compared with SFD-fed mice ($P < 0.05$). Intraperitoneal injection with α-GalCer in HFD-fed mice induced a decrease in pro-inflammatory cytokines (IL-17A and TNF-α) but an increased anti-inflammatory cytokine as IL-4 ($P < 0.05$). In addition, the level of IL-2 is further increased ($P < 0.05$). After subcutaneous injection with α-GalCer in HFD-fed mice, only serum IL-2 was significantly increased ($P < 0.05$), other cytokines were significantly decreased ($P < 0.05$). In addition to IL-10, subcutaneous injection with α-GalCer in HFD-fed mice had lower levels of cytokines (IL-2, IL-4, IL-6, IL-17A, TNF-α and IFN-γ) in serum ($P < 0.05$) than intraperitoneal injection with α-GalCer (Fig. 5A–G), these results further indicate that injected with α-GalCer subcutaneously could improve the overall inflammatory environment of obese mice.

3.6.2. Adipose tissue lymphocyte culture supernatant

The pro-inflammatory cytokines of adipose tissue in HFD-fed mice were significantly increased ($P < 0.05$), and anti-inflammatory cytokines were significantly decreased ($P < 0.05$) compared with SFD-fed mice. After intraperitoneal injection with α-GalCer in HFD-fed mice, the levels of pro-inflammatory cytokines (IL-2, TNF-α and IFN-γ) in adipose tissue were significantly increased ($P < 0.05$), while the levels of anti-inflammatory cytokines (IL-10 and IL-4) were significantly decreased ($P < 0.05$). In contrast, the pro-inflammatory cytokines (TNF-α, IL-17A and IL-6) of the adipose tissue in HFD-fed mice that subcutaneous injection with α-GalCer were significantly decreased ($P < 0.05$), while the anti-inflammatory cytokines were significantly elevated ($P < 0.05$); In addition to IL-2, subcutaneous injection with α-GalCer resulted in a significant decrease in pro-inflammatory cytokines compared with intraperitoneal injection with α-GalCer in HFD-fed mice

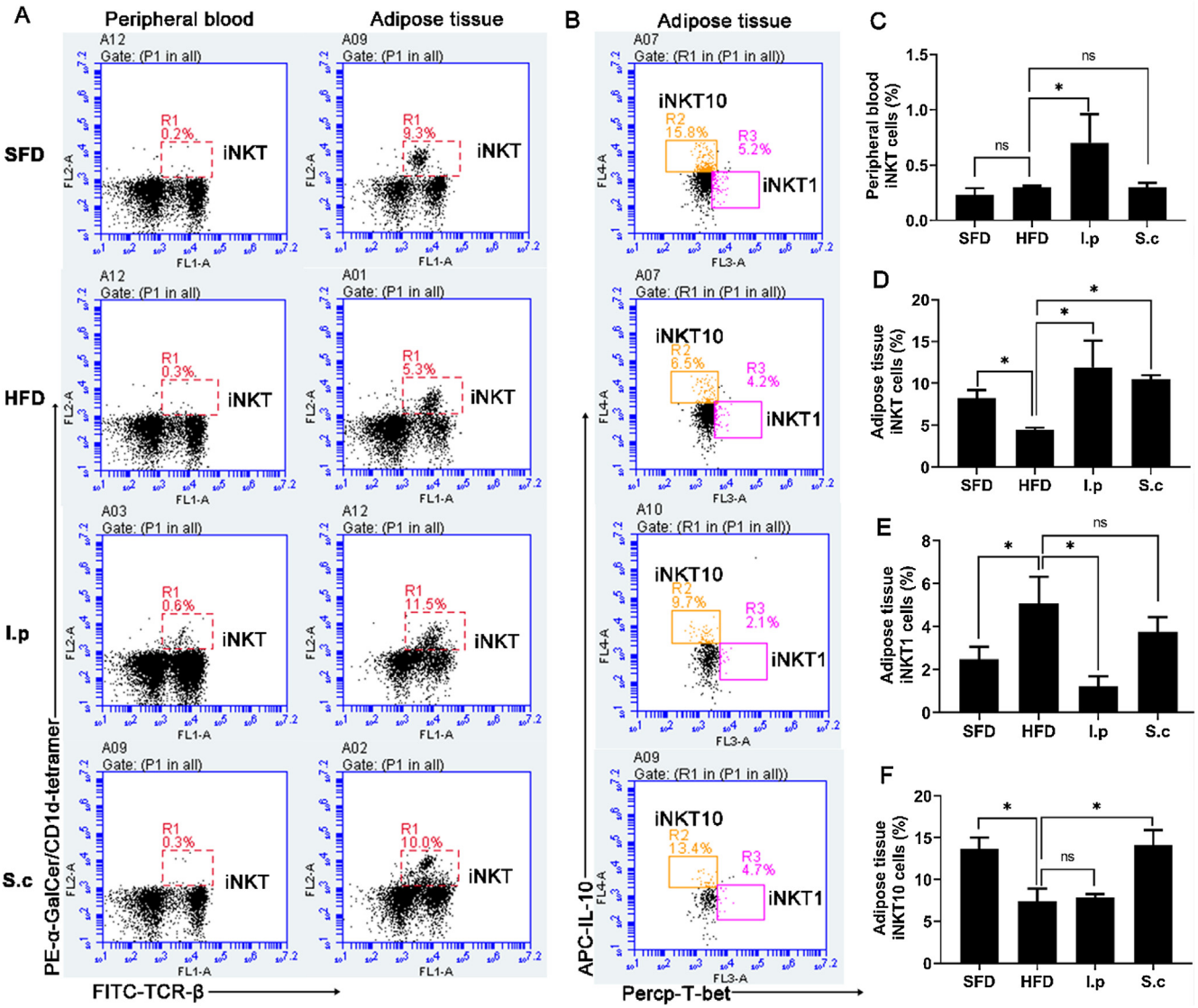

Fig. 3. Subcutaneous injection with α-GalCer in HFD-fed mice resulted in an increase in the number of iNKT10 cells in adipose tissue, whereas intraperitoneal injection with α-GalCer resulted in a decrease in the number of iNKT1 cells. Adjust the lymphocyte density to 1×10^6 per sample tube before detected by flow cytometry. For detecting the iNKT frequency, the flow cytometer was set to absorb 1×10^4 lymphocytes. For detecting the frequency of the iNKT subpopulation (iNKT1 and iNKT10), the flow cytometer was set to absorb 1×10^5 lymphocytes. iNKT cells were identified as double TCRβ- and α-GalCer/mCD1d tetramer-positive cells ($TCR\beta^+/\alpha\text{-GalCer/CD1d-tetramer}^+$) in the lymphocyte gate. Numbers on dot plots indicate percentage of iNKT cells on the lymphocyte gate and iNKT1 and iNKT10 cells on the iNKT gate. Representative dot plot of (A) peripheral blood iNKT cells, adipose tissue iNKT cells and (B) adipose tissue iNKT1 and iNKT10 cells in mice of each group. Bar graph shows the percentage of (C) peripheral blood iNKT cells, (D) adipose tissue iNKT cells, (E) adipose tissue iNKT1 cells and (F) adipose tissue iNKT10 cells in mice of each group. Each group included three mice. Data representative of three independent experiments. *$P < 0.05$, "ns" indicates no statistical significance.

($P < 0.05$), whereas anti-inflammatory cytokines were significantly elevated ($P < 0.05$) (Fig. 5H-N). It is suggestion that subcutaneous injection with α-GalCer could improve the inflammatory state in adipose tissue of obese mice.

3.7. Effects of α-GalCer on the expression of adipose tissue related transcription proteins in obese mice

The expression of transcription factor E4BP4 and Arg-1 in adipose tissue of HFD-fed mice was significantly decreased ($P < 0.05$), and expression of iNOS was significantly increased compared with SFD-fed mice ($P < 0.05$). There were no significant differences in expression of related transcription factor in adipose tissue after intraperitoneal injection with α-GalCer in HFD-fed mice ($P > 0.05$). After subcutaneous injection with α-GalCer, the expression of E4BP4 and Arg-1 was significantly increased ($P < 0.05$), while the expression of iNOS protein was significantly decreased in adipose tissue of HFD-fed mice ($P < 0.05$). The expression level of related transcriptional proteins was consistent with the trend of the flow cytometry results (Fig. 6A–E).

3.8. Effect of iNKT cells on macrophage subtype polarization

We isolated and purified spleen-derived iNKT cells for co-culture with macrophages to ensure that activated iNKT cells can influence polarization direction in macrophages. Our previous findings demonstrated that a large number of iNKT cells are obtained from the spleen in SFD-fed mice on the third day after intraperitoneal injection with α-GalCer, especially iNKT2 cells (about 58%). Mouse spleen lymphocytes were purified by MACS sorting, and the frequency of iNKT cells reached about 80%. Among these, iNKT2 cells accounted for about 94%

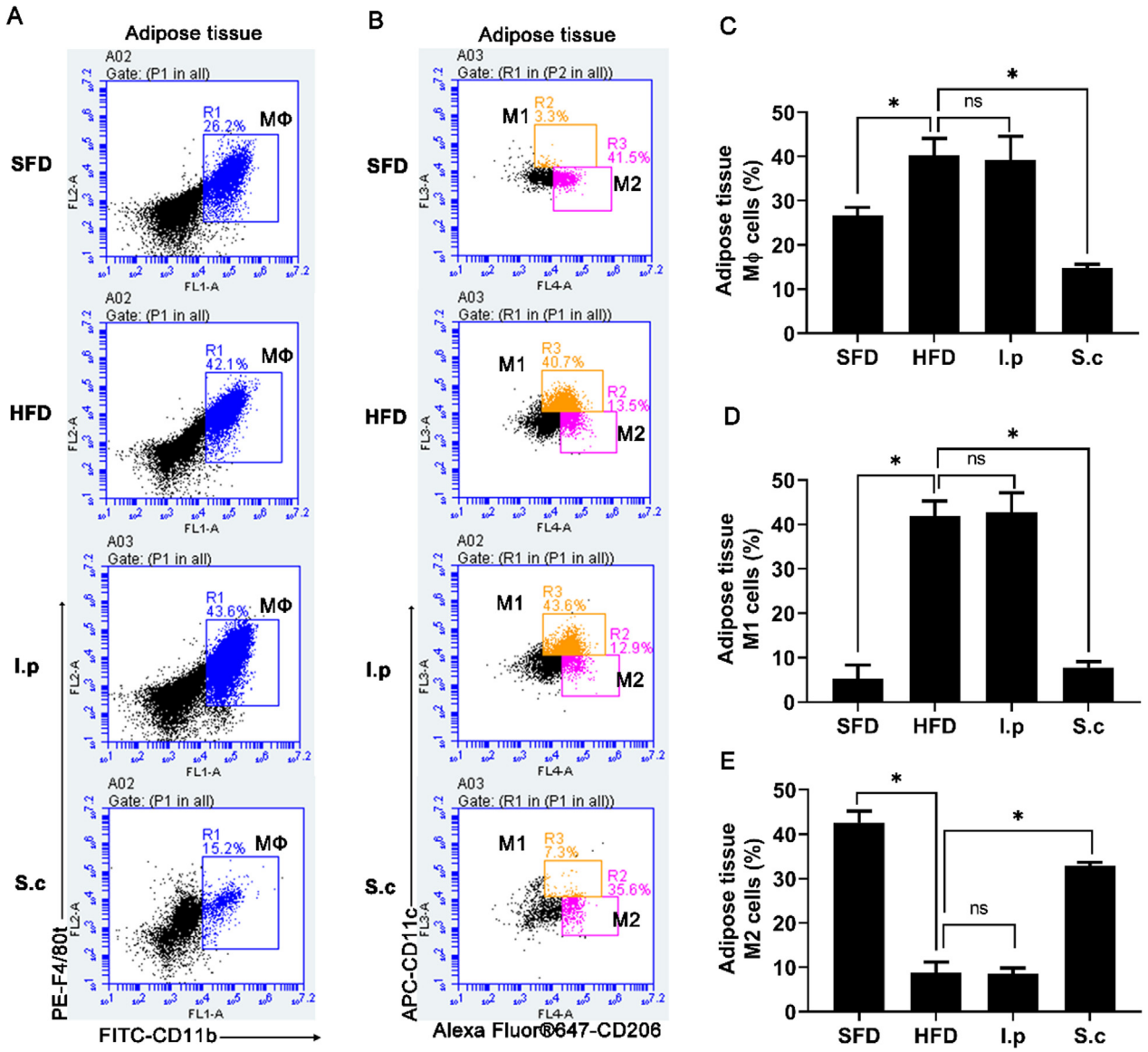

Fig. 4. Subcutaneous injection with α-GalCer resulted in decreasing of macrophages and M1 macrophages in adipose tissue of HFD-fed mice, while the frequency of M2 macrophages was increased. Macrophages were identified as double F4/80- and CD11b-positive cells ($F4/80^+CD11b^+$) in the lymphocyte gate. Meanwhile, M1 macrophages and M2 macrophages were identified as $CD11c^+CD206^-$ and $CD11c^-CD206^+$ in the macrophages gate, respectively. Adjust the lymphocyte density to 1×10^6 per sample tube before detected by flow cytometry. For detecting the macrophages frequency, the flow cytometer was set to absorb 1×10^4 lymphocytes. For detecting the frequency of the M1 macrophages and M2 macrophages, the flow cytometer was set to absorb 1×10^5 lymphocytes. Numbers on dot plots indicate the percentage of macrophages on the lymphocyte gate and M1 and M2 macrophages on the macrophages gate. Representative dot plot of (A) macrophages and (B) M1 and M2 macrophages in adipose tissue of mice. Bar graph shows the percentage of (C) macrophages, (D) M1 macrophages and (E) M2 macrophages in mice of each group. Each group included three mice. Data representative of three independent experiments. MΦ: macrophages. $^*P < 0.05$, "ns" indicates no statistical significance.

(Fig. 7A, B). Cytokines analysis of culture supernatant of purified iNKT cells showed that IL-4 was significantly more secreted than the other cytokines. (Fig. 7C).

The peritoneal macrophages (purity 96%) obtained were mainly of the M1 subtype (about 95%) and co-cultured with spleen-derived iNKT2 cells (Fig. 7D). Compared with the control group (only macrophages), M2 macrophages were significantly increased in the co-culture group (iNKT: macrophages = 1:1) ($P < 0.05$), and the frequency of M1 macrophages was significantly decreased ($P < 0.05$) (Fig. 7E, F). Cytokines analysis of the co-culture's supernatant showed that anti-inflammatory cytokines (IL-4 and IL-10) were increased, while pro-inflammatory cytokines (TNF-α, IFN-γ, IL-6) were decreased significantly ($P < 0.05$) (Fig. 7G). When iNKT cells were co-cultured with macrophages at a ratio of 1:5, macrophage subpopulations did not change significantly. This indicated that activated iNKT2 cells can regulate the polarization direction of macrophages only at certain proportions (iNKT cells: macrophages = 1:1). Therefore, we hypothesized that iNKT10 cells, as an anti-inflammatory subgroup similar to iNKT2 cells, may affect macrophage polarization in adipose tissue, thereby improving chronic inflammation in obese adipose tissue, which is consistent with *in vivo* date.

4. Discussion

Feeding mice with a high-fat diet is a common method for establishing an obesity model [26]. The above data showed that body weight and organ index in mice fed high fat diet for 12 weeks were increased significantly, and the body weight reached the obesity standard (more

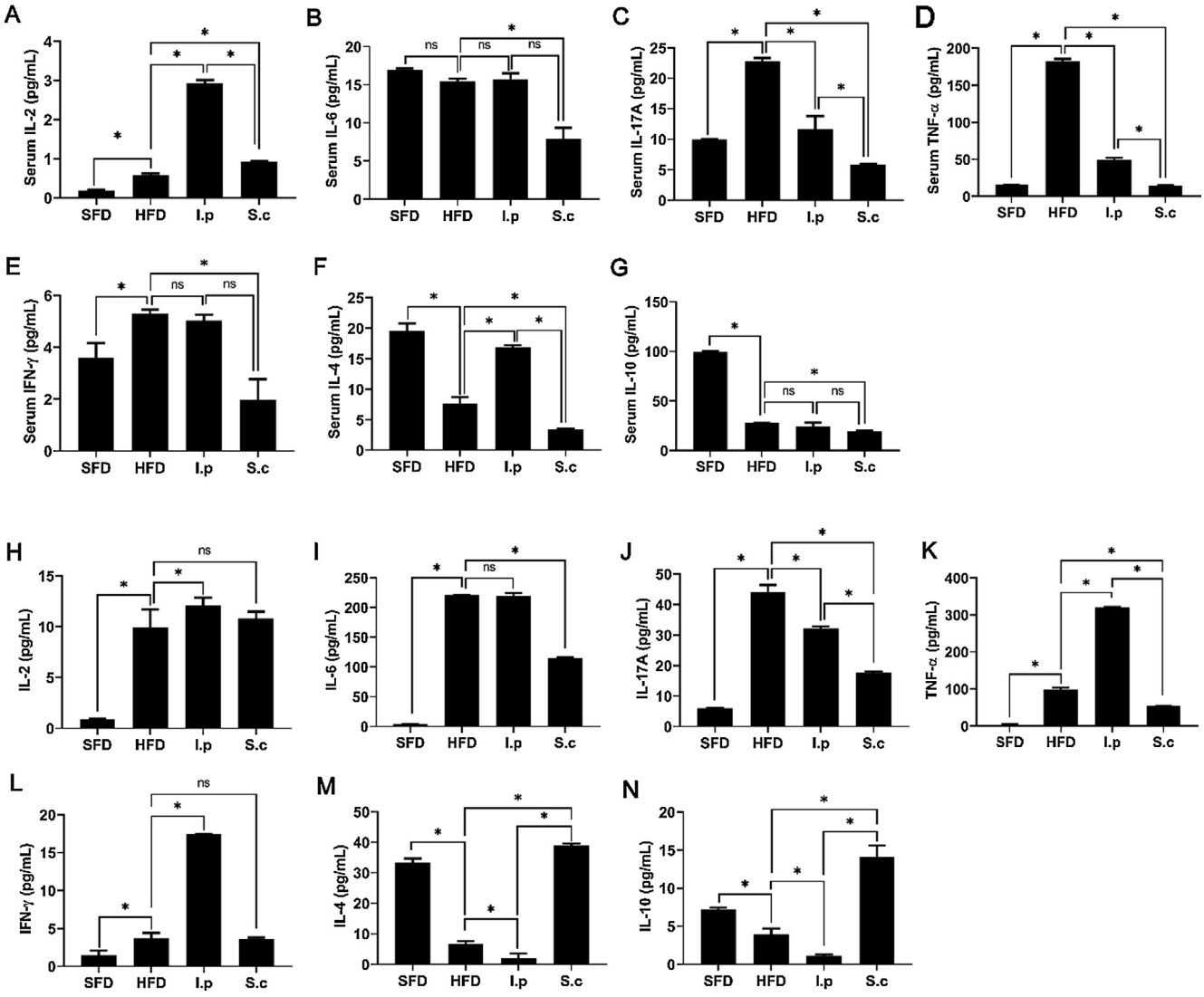

Fig. 5. Effects of injection with α-GalCer by different routes on secretion of cytokines in serum and adipose tissue. Bar graph shows the levels of serum (A) IL-2, (B) IL-6, (C) IL-17A, (D) TNF-α, (E) IFN-γ, (F) IL-4, (G) IL-10 and adipose tissue lymphocyte culture supernatant (H) IL-2, (I) IL-6, (J) IL-17A, (K) TNF-α, (L) IFN-γ, (M) IL-4, (N) IL-10 in mice of each group. Each group included three mice. Data representative of three independent experiments. *$P < 0.05$, "ns" indicates no statistical significance.

than 20% of the normal mouse body weight) [25]. A large number of inflammatory cytokines are present in serum and adipose tissue lymphocyte-stimulated supernatants, and alongside significant inflammatory cell infiltration in epididymal adipose tissue, which indicates that low-grade inflammatory status is present in high-fat-fed mice, consistent with previous reports [27,28]. HFD-fed mice did not show significantly impaired glucose tolerance and insulin resistance, which may be related to dietary composition, dietary fat content, dietary feeding duration, and experimental sites. Subsequently, we analyzed the percentage of peripheral blood iNKT cells, and the proportions of iNKT cells, macrophages and subpopulations in adipose tissue, as well as the expression levels of key transcriptional proteins in each group of mice.

Infiltration of macrophages was observed in adipose tissue of both obese patients and obese mice models [4,29–31]. Adipose tissue macrophages (ATM) are considered to be the main source of inflammatory mediators and are the most important cells that mediate inflammatory responses in obese conditions [32,33]. Recent studies have revealed increased percentage of adipose tissue macrophages in obese conditions, with accelerated transformation of anti-inflammatory M2 macrophages to pro-inflammatory M1 macrophages. Functionally, the expression levels of IL-10 and arginase-1 genes in M2 macrophages are decreased, while TNF-α and inducible nitric oxide synthase (iNOS) genes in M1 macrophages are up-regulated. This suggests that an increase in the number of macrophages is closely related to chronic inflammation of adipose tissue [32]. Our study showed that the proportions of adipose tissue macrophages and M1 macrophages were increased, whereas the rate of M2 macrophages was decreased significantly in HFD-fed mice. We could determine Arg-1 down-regulation and iNOS up-regulation at the protein level in adipose tissue of HFD-fed mice by Western blot, which is consistent with an increase in pro-inflammatory cytokines and a decrease in anti-inflammatory cytokines in adipose tissue. It can be observed that infiltration of macrophages in adipose tissue, especially the massive infiltration of the inflammatory M1 subpopulation, is a key factor in the development of obesity.

NKT cells are specific lymphocytes originally defined as the cells expressing both the characteristic T-cell marker and natural killer cell markers [34]. NKT cells exclusively recognize lipid antigens presented by the MHC-I molecule CD1d, mainly glycolipids and glycerol lipids [15,35]. iNKT rapidly responding with the secretion of large amounts of Th1 and Th2 type cytokines upon antigen recognition, including IL-2, IL-4, IL-6, IL-10, IL-17, IFN-γ, TNF-α [35–37], which regulate the

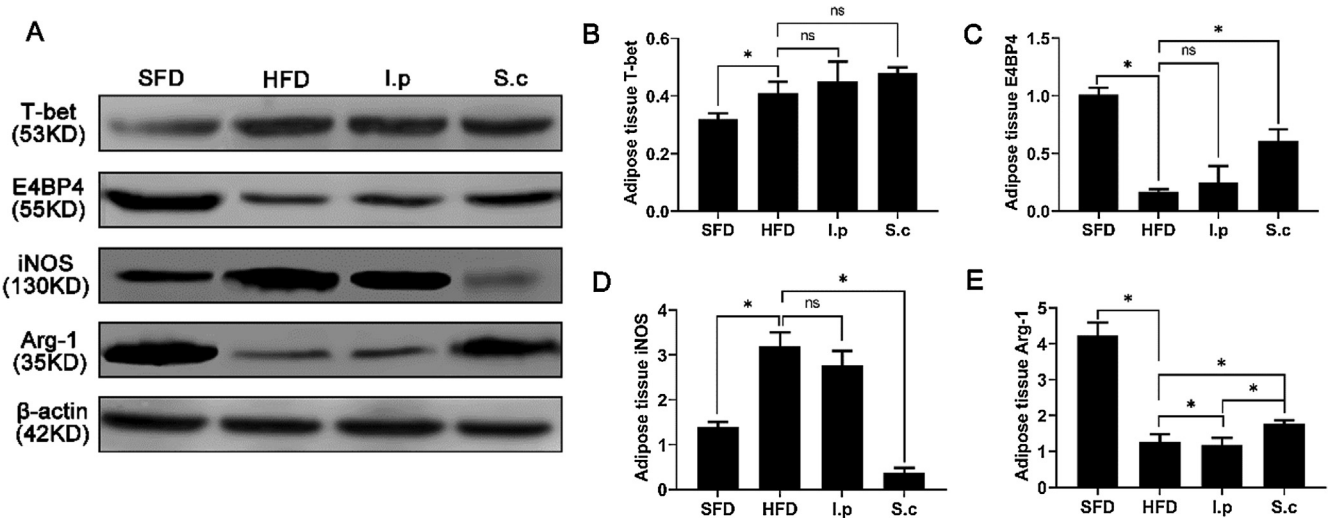

Fig. 6. Subcutaneous injection with α-GalCer in HFD-fed mice resulted in increasing of E4BP4 and Arg-1 expression and decreasing of iNOS expression in adipose tissue. (A): Expression of T-bet, E4BP4, iNOS, Arg-1 were determined in adipose tissue of mice per group by Western blot. Bar graph shows the expression of adipose tissue (B) T-bet, (C) E4BP4, (D) iNOS and (E) Arg-1 in mice of each group. Each group included three mice. Data representative of three independent experiments. $*P < 0.05$, "ns" indicates no statistical significance.

functions of dendritic cells, macrophages, B cells, T cells, and NK cells, and play a critical role in tumors, infections, and autoimmune diseases [38,39]. According to the difference in expression of transcription factor and secretion of cytokine, iNKT cells can be divided into iNKT1, iNKT2, iNKT17, and iNKT10 subpopulations [40]. iNKT cells subpopulations have tissue-specific distribution: iNKT1 express T-bet and low promyelocytic leukemia zinc finger (PLZF), secreted IFN-γ and IL-4, mainly distributed in liver and spleen. iNKT2 express GATA binding protein-3 (GATA-3) and high PLZF, secreted IL-4, mainly distributed in the lung. iNKT10 express transcription factor E4BP4, secretes IL-10, and mainly distributed in adipose tissue. iNKT17 express RORγt and inter PLZF, secreted IL-17 It is mainly distributed in lymph nodes [35,36,41,42]. It is reported that adipose tissue of mice is abundant of iNKT cells and iNKT cells account for 10–25% of adipose T cells, or 2–8% of adipose lymphocytes [8,9,43]. As a unique type of tissue-resided natural immune lymphocytes, iNKT cells may play an important role in maintaining the immune homeostasis of adipose tissue. Studies have reported that iNKT cells in adipose tissue presenting a Th2-biased cytokine profile, which is different from iNKT cells in the spleen and liver [8,9]. Similarly, cytokines of iNKT cells in the obese mice are gradually biased toward Th2-type cytokines [44]. Studies have also reported that NKT-deficient mice fed high fat diet gain more weight compared with HFD-fed (or low-fat diet) wild-type mice [11,45]. These conflicting results indicate that the role of iNKT cells in chronic inflammation of obese adipose tissue remains unclear. In this study, the frequencies of adipose tissue iNKT and iNKT10 cells were decreased, while iNKT1 cell proportion was significantly increased, indicating that decreased iNKT cell frequency in adipose tissue and imbalanced of subpopulation ratio may be involved in the development of obesity.

As a classical iNKT cell-specific activator [15], α-GalCer can effectively activate iNKT1, iNKT2, iNKT10 and iNKT17, and has been widely used in intervention studies of tumor, infection and autoimmune diseases [16,17]. Our previous experiments found that the different injection routes of α-GalCer had great differences in the activation of distinct subsets of iNKT cells in different immune organs of normal mice. To assess whether iNKT cells affect chronic inflammatory processes in obese adipose tissue, we injected mice with α-GalCer by both subcutaneous and intraperitoneal routes for immunological intervention. The results showed that injection with α-GalCer by both routes resulted in increased frequency of iNKT cells in epididymal adipose tissue, but had different effects on iNKT subpopulations. Subcutaneous injection with α-GalCer resulted in increased iNKT10 and iNKT amounts in adipose tissue. Up-regulation of the E4BP4 in adipose tissue was determined by Western blot, but there was no significant change in HFD-fed mice which intraperitoneal injection with α-GalCer. This indicates that different routes of α-GalCer injection may affect the activation levels of various subpopulations of iNKT cells in tissues and organs. The effects of these two injection routes on epididymal adipose tissue inflammation also showed very different results. After intraperitoneal injection with α-GalCer in HFD-fed mice, inflammatory cell infiltration in adipose tissue was increased significantly, which resulted in elevated pro-inflammatory cytokines in adipose tissue and decreased anti-inflammatory cytokines. However, infiltration of inflammatory cells around adipocytes was significantly reduced, while anti-inflammatory cytokines were increased and pro-inflammatory cytokines reduced in adipose tissue in HFD-fed mice after subcutaneous injection of α-GalCer. We speculate that this may be related to subcutaneous injection with α-GalCer activating iNKT10 cells in adipose tissue. Previous studies have shown that macrophages have an indispensable role in adipose tissue. To further confirm that α-GalCer-induced iNKT cells have a regulatory effect on adipose tissue macrophages, we examined the frequency of macrophages and subpopulations in epididymal adipose tissue. Intraperitoneal injection with α-GalCer had no significant effect on frequency of macrophages and subpopulations in adipose tissue, and subcutaneous injection with α-GalCer significantly reduced the proportion of macrophages and M1 macrophages in adipose tissue, and increased the proportion of M2 macrophages. We found that expression of iNOS in macrophages was decreased dramatically, while expression of Arg-1 was significantly increased. We can speculate that subcutaneous injection with α-GalCer increased the proportion of adipose tissue iNKT10 cells, which promotes polarization of M2 macrophage and improves the local inflammatory state of adipose tissue. In addition, we found the serum anti-inflammatory cytokines increased and pro-inflammatory cytokines decreased after intraperitoneal injection with α-GalCer in HFD-fed mice. This also suggests that different injection routes of α-GalCer may be a good treatment strategy for different diseases.

It has been reported that IL-4 can promote polarization of M2 macrophages. IL-10 promotes expression of IL-4Rα in IL-4-induced M2 macrophages by STAT3 pathway. Meanwhile, IL-10 enhances the expression of Arg-1 (characteristic molecule of M2 macrophages) and promotes polarization of M2 macrophages [46,47]. To further confirm the hypothesis that iNKT10 subpopulation activation promotes the polarization of M2 macrophages, we selected spleen-derived iNKT cells

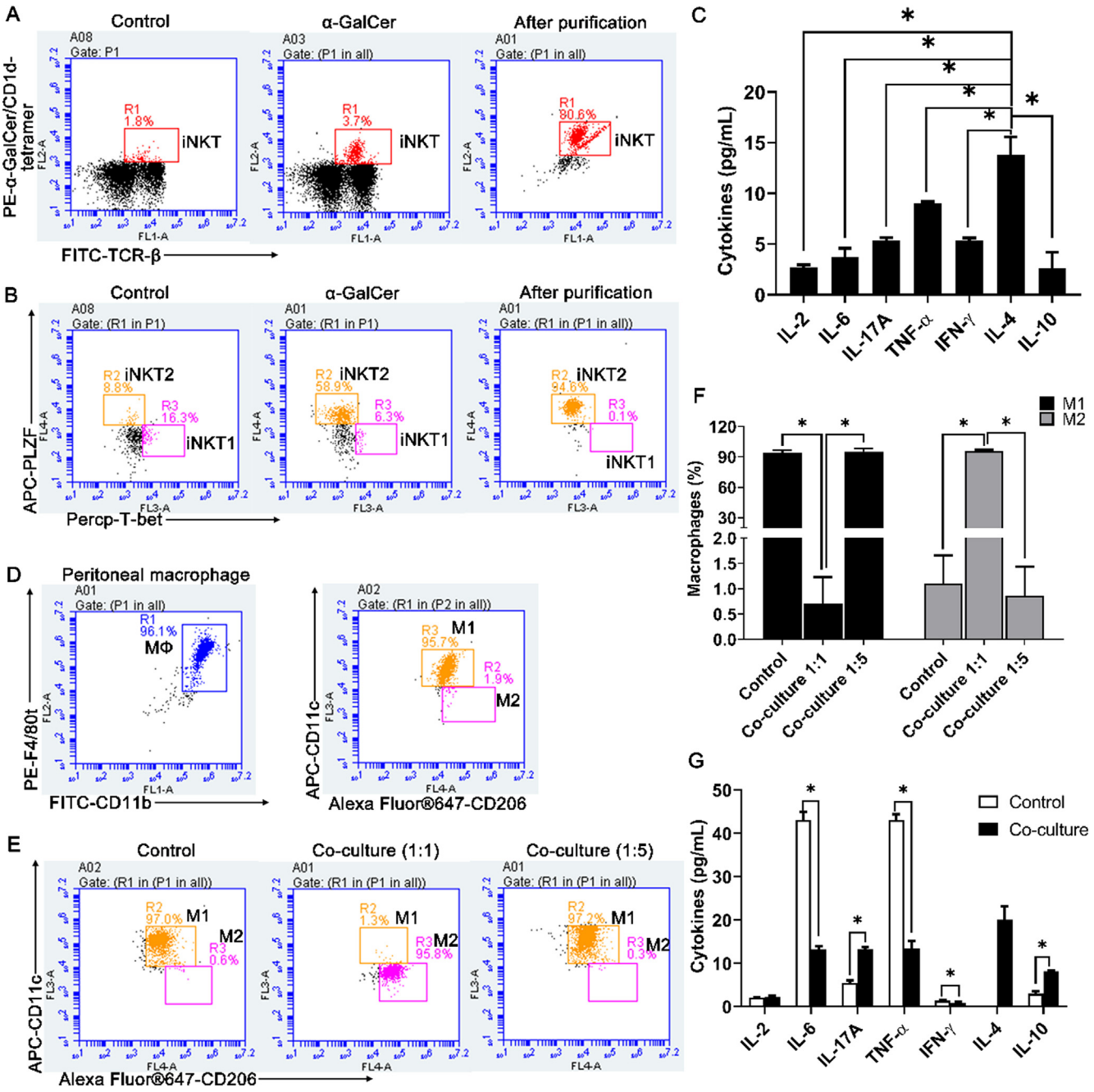

Fig. 7. iNKT cells and macrophages were co-cultured at a ratio of 1:1. iNKT2 induces polarization of M1 macrophages to M2 macrophages and increased secretion of anti-inflammatory cytokines. Representative dot plot of (A) iNKT cells and (B) iNKT subpopulations derived from spleen of SFD-fed mice. Control group: iNKT cells from spleen of SFD-fed mice; α-GalCer group: iNKT cells from spleen of SFD-fed mice, which intraperitoneal injection with α-GalCer for three days; After purification: the spleen iNKT cells was isolated and purified from the SFD-fed mice, which intraperitoneal injection with α-GalCer for three days. Bar graph shows the (C): Cytokines in the culture supernatant of purified iNKT cells were detected by CBA kit. Representative dot plot of (D) peritoneal macrophages (left) and subpopulations (right), and (E) macrophage subpopulation in co-culture experiments. Numbers on dot plots indicate the percentage of macrophages on the lymphocyte gate and M1 and M2 macrophages on the macrophages gate. Control group: only macrophage; Co-culture group: iNKT2 cells and macrophages was co-cultured at a ratio of 1:1 and 1:5, respectively. (F) Bar graph shows the percentage of M1 and M2 macrophages in each co-culture group. (G) CBA kit detects cytokines in co-culture supernatants of iNKT cells and macrophages (1:1). *$P < 0.05$, "ns" indicates no statistical significance.

(the frequency of iNKT2 cells reached 94%, similar to iNKT10 in inhibition of inflammation) co-cultured with peritoneal macrophages (M1 macrophages account for the majority) for 48 h. In the co-culture group (iNKT cells: macrophage = 1:1), the frequency of M2 macrophages increased significantly and M1 macrophages decreased dramatically. The detection of cytokines secreted in the co-culture supernatant revealed that anti-inflammatory cytokines (IL-10 and IL-4) was significantly increased. However, IL-4 was not detected in the culture supernatant of the control group, probably because the levels of IL-4 were too low to reach the minimum detection limit of the instrument. And the levels of pro-inflammatory cytokines (TNF-α, IFN-γ, and IL-6) were significantly reduced. It is worth noting that when iNKT2 cells were co-cultured with macrophages at a ratio of 1:5. No polarization of M2 macrophages was occurred. This suggests that polarization of macrophages can occur when the sufficient activated iNKT2 subsets are available at a certain proportion (iNKT cells: macrophages = 1:1).

It has been reported that intraperitoneal injection with α-GalCer in mice activates adipose tissue iNKT cells and reduces the body weight of obese mice [8,43–45,48]. Our results showed that the body weight of the mice was greatly decreased after intraperitoneal injection with α-GalCer in HFD-fed mice, but the body weight was higher than that of HFD-fed mice after subcutaneous injection with α-GalCer. Although subcutaneous injection with α-GalCer improved the inflammatory state of adipose tissue, but did not reduce the body weight of the mice.

In summary, the occurrence of chronic inflammation of obese adipose tissue is closely related to decreased iNKT cell frequency and an imbalance of iNKT subsets. Subcutaneous injection with α-GalCer effectively increased the frequencies of iNKT cells and the iNKT10 subpopulation in adipose tissue, promoted macrophage polarization towards the M2 subtype, and corrected the imbalance between pro-inflammatory and anti-inflammatory cytokines.

Funding

This work was supported by the National Natural Science Foundation of China (NSFC) [Grant numbers: 81771755], Colleges and University's science and technology key research project of Hebei province [Grant numbers: ZD2017009].

Declaration of Competing Interest

The authors declare no competing financial interests.

Acknowledgements

We are grateful to the National Natural Science Foundation of China (NSFC) (81771755), Colleges and university's science and technology key research project of Hebei province (ZD2017009) and the Animal Lab of Medical Experiment Center, Hebei University for their support.

References

[1] G.H. Goossens, The metabolic phenotype in obesity: fat mass, body fat distribution, and adipose tissue function, Obes. Facts 10 (3) (2017) 207–215, https://doi.org/10.1159/000471488.
[2] A. Engin, The definition and prevalence of obesity and metabolic syndrome, Adv. Exp. Med. Biol. 960 (2017) 1–17.
[3] J. Choi, L. Joseph, L. Pilote, Obesity and C-reactive protein in various populations: a systematic review and meta-analysis, Obes. Rev. 14 (3) (2013) 232–244, https://doi.org/10.1111/obr.12003.
[4] J. Lee, Adipose tissue macrophages in the development of obesity-induced inflammation, insulin resistance and type 2 diabetes, Arch. Pharm. Res. 36 (2) (2013) 208–222.
[5] M. Luna-Luna, A. Medina-Urrutia, G. Vargas-Alarcón, F. Coss-Rovirosa, J. Vargas-Barrón, Ó. Pérez-Méndez, Adipose tissue in metabolic syndrome: onset and progression of atherosclerosis, Arch. Med. Res. 46 (5) (2015) 392–407, https://doi.org/10.1016/j.arcmed.2015.05.007.
[6] M.C. Seelaender, M.L. Batista, Adipose tissue inflammation and cancer cachexia: the role of steroid hormones, Horm. Mol. Biol. Clin. Investig. 17 (1) (2014) 5–12, https://doi.org/10.1515/hmbci-2013-0040.
[7] L. Lynch, D. O'Shea, D.C. Winter, J. Geoghegan, D.G. Doherty, C. O'Farrelly, Invariant NKT cells and CD1d (+) cells amass in human omentum and are depleted in patients with cancer and obesity, Eur. J. Immunol. 39 (7) (2009) 1893–1901, https://doi.org/10.1002/eji.200939349.
[8] L. Lynch, M. Nowak, B. Varghese, et al., Adipose tissue invariant NKT cells protect against diet-induced obesity and metabolic disorder through regulatory cytokine production, Immunity 37 (3) (2012) 574–587, https://doi.org/10.1016/j.immuni.2012.06.016.
[9] H.S. Schipper, M. Rakhshandehroo, S.F. van de Graaf, et al., Natural killer T cells in adipose tissue prevent insulin resistance, J. Clin. Invest. 122 (9) (2012) 3343–3354, https://doi.org/10.1172/JCI62739.
[10] D. Sag, P. Krause, C.C. Hedrick, M. Kronenberg, G. Wingender, IL-10-producing NKT10 cells are a distinct regulatory invariant NKT cell subset, J. Clin. Invest. 124 (9) (2014) 3725–3740, https://doi.org/10.1172/JCI72308.
[11] L. Wu, V.V. Parekh, C.L. Gabriel, et al., Activation of invariant natural killer T cells by lipid excess promotes tissue inflammation, insulin resistance, and hepatic steatosis in obese mice, Proc. Natl. Acad. Sci. USA 109 (19) (2012) E1143–E1152, https://doi.org/10.1073/pnas.1200498109.
[12] K. Ohmura, N. Ishimori, Y. Ohmura, et al., Natural killer T cells are involved in adipose tissues inflammation and glucose intolerance in diet-induced obese mice, Arterioscler. Thromb. Vasc. Biol. 30 (2) (2010) 193–199, https://doi.org/10.1161/ATVBAHA.109.198614.
[13] T. Natori, M. Morita, K. Akimoto, et al., Agelasphins, novel antitumor and immunostimulatory cerebrosides from the marine sponge Agelas mauritianus, Tetrahedron 50 (9) (1994) 2771–2784, https://doi.org/10.1016/S0040-4020(01)86991-X.
[14] Y. Zhang, R. Springfield, S. Chen, et al., α-GalCer and iNKT cell-based cancer immunotherapy: realizing the therapeutic potentials, Front. Immunol. 6 (10) (2019) 1126, https://doi.org/10.3389/fimmu.2019.01126.
[15] L. Brossay, M. Chioda, N. Burdin, et al., CD1d-mediated recognition of an alpha-galactosylceramide by natural killer T cells is highly conserved through mammalian evolution, J. Exp. Med. 188 (8) (1998) 1521–1528, https://doi.org/10.1084/jem.188.8.1521.
[16] M. Horikoshi, D. Goto, S. Segawa, et al., Activation of Invariant NKT cells with glycolipid ligand α-galactosylceramide ameliorates glucose-6-phosphate isomerase peptide-induced arthritis, PLoS One 7 (12) (2012) e51215, , https://doi.org/10.1371/journal.pone.0051215.
[17] A. Barthelemy, S. Ivanov, M. Hassane, et al., Exogenous activation of invariant natural killer T cells by α-galactosylceramide reduces pneumococcal outgrowth and dissemination Postinfluenza, MBio 7 (6) (2016) e01440–e1516, https://doi.org/10.1128/MBIO.01440-16.
[18] G. Vahedi, Y. Kanno, Y. Furumoto, et al., Super-enhancers delineate disease-associated regulatory nodes in T cells, Nature 520 (7548) (2015) 558–562, https://doi.org/10.1038/nature14154.
[19] J.L. Matsuda, T. Mallevaey, J. Scott-Browne, L. Gapin, CD1d-restricted iNKT cells, the 'Swiss-Army knife' of the immune system, Curr. Opin. Immunol. 20 (3) (2008) 358–368, https://doi.org/10.1016/j.coi.2008.03.018.
[20] K. Miyamoto, S. Miyake, T. Yamamura, A synthetic glycolipid prevents autoimmune encephalomyelitis by inducing TH2 bias of natural killer T cells, Nature 413 (6855) (2001) 531–534, https://doi.org/10.1038/35097097.
[21] K.O. Yu, J.S. Im, A. Molano, et al., Modulation of CD1d-restricted NKT cell responses by using N-acyl variants of alpha-galactosylceramides, Proc. Natl. Acad. Sci. USA 102 (9) (2005) 3383–3388, https://doi.org/10.1073/pnas.0407488102.
[22] L. Bai, M.G. Constantinides, S.Y. Thomas, et al., Distinct APCs explain the cytokine bias of α-galactosylceramide variants in vivo, J. Immunol. 188 (7) (2012) 3053–3061.
[23] H. Zhang, R. Xue, S. Zhu, et al., M2-specific reduction of CD1d switches NKT cell-mediated immune responses and triggers metaflammation in adipose tissue, Cell Mol. Immunol. 15 (5) (2018) 506–517, https://doi.org/10.1038/cmi.2017.11.
[24] D. Chen, X. Gao, J. Wang, et al., Activation of hepatic iNKT2 cells by α-GalCer ameliorates hepatic steatosis induced by high-fat diet in C57BL/6J mice, Int. Immunopharmacol. 74 (2019) 105727, , https://doi.org/10.1016/j.intimp.2019.105727.
[25] S. Nishimura, I. Manabe, M. Nagasaki, et al., CD8+ effector T cells contribute to macrophage recruitment and adipose tissue inflammation in obesity, Nat Med. 15 (8) (2009) 914–920, https://doi.org/10.1038/nm.1964.
[26] Dong Ming, Liu Dong, Liang Yun-hai, et al., Construction of obese mouse models with high fat diet feeding: relationship between nutritional factor and metabolic syndrome, Chin. J. Tissue Eng. Res. 19 (40) (2015) 6542–6546, https://doi.org/10.3969/j.issn.2095-4344.2015.40.027.
[27] M.Y. Donath, Inflammation as a sensor of metabolic stress in obesity and type 2 diabetes, Endocrinology 152 (11) (2011) 4005–4006, https://doi.org/10.1210/en.2011-1691.
[28] Y. Ji, S. Sun, S. Xia, L. Yang, X. Li, L. Qi, Short term high fat diet challenge promotes alternative macrophage polarization in adipose tissue via natural killer T cells and interleukin-4, J. Biol. Chem. 287 (29) (2012) 24378–24386, https://doi.org/10.1074/jbc.M112.371807.
[29] O. Osborn, J.M. Olefsky, The cellular and signaling networks linking the immune system and metabolism in disease, Nat. Med. 18 (3) (2012) 363–374, https://doi.org/10.1038/nm.2627.
[30] C.K. Glass, J.M. Olefsky, Inflammation and lipid signaling in the etiology of insulin resistance, Cell Metab. 15 (5) (2012) 635–645, https://doi.org/10.1016/j.cmet.2012.04.001.
[31] J. Ide, A. Gagnon, A.S. Molgat, A. Landry, C. Foster, A. Sorisky, Macrophage-conditioned medium inhibits the activation of cyclin-dependent kinase 2 by adipogenic inducers in 3T3-L1 preadipocytes, J. Cell. Physiol. 226 (9) (2011) 2297–2306, https://doi.org/10.1002/jcp.22566.
[32] K.A. Harford, C.M. Reynolds, F.C. McGillicuddy, H.M. Roche, Fats, inflammation and insulin resistance: insights to the role of macrophage and T-cell accumulation in adipose tissue, Proc. Nutr. Soc. 70 (4) (2011) 408–417, https://doi.org/10.1017/S0029665111000565.
[33] S.U. Amano, J.L. Cohen, P. Vangala, et al., Local proliferation of macrophages contributes to obesity-associated adipose tissue inflammation, Cell Metab. 19 (1) (2014) 162–171, https://doi.org/10.1016/j.cmet.2013.11.017.
[34] Y. Makino, R. Kanno, T. Ito, K. Higashino, M. Taniguchi, Predominant expression of invariant V alpha 14^+ TCR alpha chain in NK1.1^+ T cell populations, Int. Immunol. 7 (7) (1995) 1157–1161, https://doi.org/10.1093/intimm/7.7.1157.
[35] S.H. Krovi, L. Gapin, Invariant natural killer T Cell subsets-more than just developmental intermediates, Front. Immunol. 9 (2018) 1393, https://doi.org/10.3389/fimmu.2018.01393.
[36] H. Wang, K.A. Hogquist, How lipid-specific T cells become effectors: the differentiation of iNKT subsets, Front. Immunol. 9 (2018) 1450, https://doi.org/10.3389/fimmu.2018.01450.
[37] K. Sakuishi, S. Oki, M. Araki, S.A. Porcelli, S. Miyake, T. Yamamura, Invariant NKT cells biased for IL-5 production act as crucial regulators of inflammation, J. Immunol. 179 (6) (2007) 3452–3462.
[38] V. Cerundolo, J.D. Silk, S.H. Masri, M. Salio, Harnessing invariant NKT cells in

vaccination strategies, Nat. Rev. Immunol. 9 (1) (2009) 28–38, https://doi.org/10.1038/nri2451.
[39] S. Hegde, X. Chen, J.M. Keaton, F. Reddington, G.S. Besra, J.E. Gumperz, NKT cells direct monocytes into a DC differentiation pathway, J. Leukoc. Biol. 81 (5) (2007) 1224–1235, https://doi.org/10.1189/jlb.1206718.
[40] D.I. Kwon, Y.J. Lee, Lineage differentiation program of invariant natural killer T cells, Immune Netw. 17 (6) (2017) 365–377, https://doi.org/10.4110/in.2017.17.6.365.
[41] L. Gapin, Development of invariant natural killer T cells, Curr. Opin. Immunol. 39 (2016) 68–74, https://doi.org/10.1016/j.coi.2016.01.001.
[42] L. Lynch, X. Michelet, S. Zhang, et al., Regulatory iNKT cells lack expression of the transcription factor PLZF and control the homeostasis of T(reg) cells and macrophages in adipose tissue, Nat. Immunol. 16 (1) (2015) 85–95, https://doi.org/10.1038/ni.3047.
[43] Y. Ji, S. Sun, A. Xu, et al., Activation of natural killer T cells promotes M2 Macrophage polarization in adipose tissue and improves systemic glucose tolerance via interleukin-4 (IL-4)/STAT6 protein signaling axis in obesity, J. Biol. Chem. 287 (17) (2012) 13561–13571, https://doi.org/10.1074/jbc.M112.350066.
[44] Y. Miyazaki, K. Iwabuchi, D. Iwata, et al., Effect of high fat diet on NKT cell function and NKT cell-mediated regulation of Th1 responses, Scand. J. Immunol. 67 (3) (2008) 230–237, https://doi.org/10.1111/j.1365-3083.2007.02062.x.
[45] M. Satoh, Y. Andoh, C.S. Clingan, et al., Type II NKT cells stimulate diet-induced obesity by mediating adipose tissue inflammation, steatohepatitis and insulin resistance, PLoS One 7 (2) (2012) e30568, , https://doi.org/10.1371/journal.pone.0030568.
[46] N. Makita, Y. Hizukuri, K. Yamashiro, M. Murakawa, Y. Hayashi, IL-10 enhances the phenotype of M2 macrophages induced by IL-4 and confers the ability to increase eosinophil migration, Int. Immunol. 27 (3) (2015) 131–141, https://doi.org/10.1093/intimm/dxu090.
[47] X. Huang, Y. Li, M. Fu, H.B. Xin, Polarizing macrophages in vitro, Methods Mol. Biol. 1784 (2018) 119–126, https://doi.org/10.1007/978-1-4939-7837-3_12.
[48] S. Ahn, D. Jeong, S.J. Oh, J. Ahn, S.H. Lee, D.H. Chung, GM-CSF and IL-4 produced by NKT cells inversely regulate IL-1β production by macrophages, Immunol. Lett. 182 (2017) 50–56, https://doi.org/10.1016/j.imlet.2017.01.003.