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# Activation of hepatic iNKT2 cells by α-GalCer ameliorates hepatic steatosis induced by high-fat diet in C57BL/6J mice

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ABSTRACT

The existence of association between the subpopulation of iNKT cells with different functions and nonalcoholic fatty liver disease has not been confirmed. To investigative the role of iNKT cells in the pathogenesis of non-alcoholic fatty liver disease, we established a non-alcoholic fatty liver model by feeding C57BL/6J mice for 12 weeks with a high-fat diet and injecting α-GalCer through different routes to activate hepatic iNKT cells. The liver of the mice fed a high-fat diet (HFD) had severe hepatic steatosis appearance, elevated pro-inflammatory cytokines and reduced anti-inflammatory cytokines in the liver, and high serum levels of TC, LDL, HDL, and ALT. Our results showed that the percentage of iNKT cells in the liver of the HFD-fed mice was lower than that of the control mice. The expression levels of the related transcription factor of T-bet increased but that of GATA-3 decreased in the HFD-fed mice. The administration of α-GalCer by intraperitoneal injection resulted in increasing of hepatic iNKT and iNKT2 cells but decreasing of hepatic iNKT1 cells, and the expression of GATA-3 and anti-inflammatory cytokine (IL-4) was increased in the liver, and hepatic steatosis was ameliorated in the HFD-fed mice. The administration of α-GalCer by subcutaneous injection resulted in a decrease in hepatic iNKT and iNKT2 and an augmentation of hepatic iNKT1 cells. However, hepatic steatosis was not significantly improved. We concluded that the intraperitoneal injection with α-GalCer effectively improved hepatic steatosis, according to increasing the number of hepatic iNKT2 cells. The precise mechanism requires further exploration.

## 1. Introduction

Nonalcoholic fatty liver disease (NAFLD) refers to the clinical pathological syndrome characterized by diffuse hepatic alveolar steatosis [1]. NAFLD is a collective name that encompasses various stages of liver diseases, which clinical spectrum ranging from non-alcoholic fatty liver (NAFL), non-alcoholic steatohepatitis (NASH), to fibrosis and cirrhosis, eventually developed into hepatocellular carcinoma (HCC) [2]. Numerous factors are involved in the pathogenesis of NAFLD. Obesity, low-grade inflammation associated with obesity and insulin resistance (IR) is considered the most important risk factors for liver lesions, and the infiltration of inflammatory cells and secretion of cytokine accelerates the further deterioration of NAFLD [3,4].

Invariant natural killer T cells (iNKT cells) are a population of specialized immune cells that express the characteristics receptor of both natural killer cells (NK) and T cells [5]. iNKT cells are activated by the glycolipid antigen α-galactosylceramide (α-GalCer), which is presented by the non-classical MHC-I molecule CD1d, interacting with TCRs, expressed in iNKT cells [6,7]. Activated iNKT cells rapidly secrete a large number of Th1, Th2 type cytokines (such as IL-2, IL-4, IL-6, IL-10, IL-17, IFN-γ, TNF-α) [7], which have regulatory role on dendritic cells (DC), macrophages($M_\Phi$), B lymphocyte, T lymphocyte and NK cells

*Abbreviations:* NAFLD, nonalcoholic fatty liver disease; iNKT, invariant natural killer T cells; α-GalCer, α-galactosylceramide; NAFL, nonalcoholic fatty liver; NASH, nonalcoholic steatohepatitis; HCC, hepatocellular carcinoma; IR, insulin resistance; NK, natural killer cell; DC, dendritic cell; PLZF, promyelocytic leukemia zinc finger; ROR-γt, retinoic acid receptor-related orphan nuclear receptor gamma; GATA-3, GATA binding protein-3; RD, regular diet; HFD, high-fat diet; CDD, choline-deficient diet; BD, BD biosciences; I.p, intraperitoneal injection with α-GalCer group; S.c, subcutaneous injection with α-GalCer group; GTT, glucose tolerance test; ITT, insulin tolerance test; AST, aspartate aminotransferase; ALT, acid aminotransferase; LDL, low density lipoprotein; HDL, high density lipoprotein; TC, total cholesterol; TG, triglyceride; CBA, cytometric bead array

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and play an important role in diseases such as cancer, infection and autoimmune diseases [8–10]. iNKT cells are divided into iNKT1, iNKT2, iNKT10, and iNKT17 based on the expression of different transcription factors and the secretion of different cytokines [11]. iNKT cell subpopulations have tissue-specific distribution, such as: iNKT1 expresses T-box 21 (T-bet) and low promyelocytic leukemia zinc finger (PLZF), mainly reside in liver and spleen. iNKT2 expresses high PLZF and GATA binding protein-3 (GATA-3), mainly reside in the lung. iNKT10 expresses transcription factor E4BP4 and low PLZF, mainly reside in adipose tissue. iNKT17 expresses retinoic acid receptor-related orphan nuclear receptor gamma (ROR-γt) and inter PLZF, mainly reside in lymph node [12–16]. Each of these subpopulations is uniquely involved in the regulation role in the immune environments of different tissues.

In recent years, iNKT cells have been confirmed to be critically involved in the development of NAFLD. A range of studies have revealed iNKT cell reduction in the liver of NAFLD mouse models induced by high-fat diet (HFD) [17,18] or choline-deficient diet (CDD) [19]. The research by Lynch and Nowak indicated that adoptive transfer iNKT cells from normal mice into HFD-fed ob/ob mice or activating iNKT cells with α-GalCer in HFD-fed ob/ob mice can improve hepatic steatosis and the inflammatory environment [20]. However, high-fat diet has been reported to induce liver iNKT cell activation to secrete IFN-γ, which induces liver damage, whereas the liver type II NKT cells exert the opposite effect, inhibiting iNKT cell-mediated liver injury [21]. Wu et al. reported that α-GalCer specifically activated iNKT cells, leading to Th1-type responses, which exacerbated hepatic steatosis in HFD-fed mice [22]. These conflicting results may be associated with different subpopulations of iNKT cells that were activated at different NAFLD stages. Another reason could have been the use of different species for the development of animal models. In our previous study, we found that intraperitoneal or subcutaneous injection with α-GalCer selectively activates different subpopulations of hepatic iNKT cells. Hepatic iNKT2 cells were selectively activated by intraperitoneal injection with α-GalCer, whereas hepatic iNKT1 cells were selectively activated by subcutaneous injection with α-GalCer. We used both intraperitoneal and subcutaneous injection with α-GalCer to selectively activate hepatic iNKT subpopulations in HFD-fed mice for further exploration of the role of different subpopulations of iNKT cells in the development of NAFLD and its mechanism.

## 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 purchased from Beijing Hua Fu Kang Biotechnology Co., Ltd. (SCXK(Jing)2016-0006). The animals were maintained in the Animal Laboratory of Medical Experiment Center, Hebei University, at 23 °C ± 2 °C with 50 ± 5% relative humidity and free access water and food (regular 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. Animal studies were approved by the Animal Welfare and Ethical Committee of Hebei University (IACUC-2018019) and were carried out in strict accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals.

### 2.2. Materials

High fat diet (H10060) was manufactured by Beijing Hua Fu Kang Biotechnology Co., Ltd. (SCXK(Jing)2016-0006). Glucose and insulin were purchased from Solarbio (Beijing, China). 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-T-selected-CD1d tetramer was purchased from MBL International (Japan). Foxp3 Staining Buffer Set was purchased from eBioscience (California, USA). KRN7000 (α-GalCer) was manufactured by 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

C57BL/6J mice were randomly selected as control groups and were fed with regular diet (RD) (n = 10). The model group was fed with high-fat diet for 12 weeks (n = 30). The HFD-fed mice were then randomly divided into three groups, including HFD-fed group (n = 10), a group with intraperitoneal injection (I.p) with α-GalCer (n = 10), and a group with subcutaneous injection (S.c) with α-GalCer (n = 10). Intraperitoneal or subcutaneous injection with α-GalCer (100 ng/g weight) was administered in mice after they were HFD-fed for 12 weeks. Then, in the intraperitoneal injection group, the mice were sacrificed on the third day, whereas those in the subcutaneous injection group were sacrificed on the fifth day after injection with α-GalCer.

#### 2.3.2. General condition assessment of mice

The weight, activity, mental state and coat gloss of the mice was dynamically monitored.

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

Six animals were randomly selected from each group. The mice were fasted for 12 h and fasting blood glucose was measured as blood glucose levels at 0 min. The mice were injected intraperitoneally with 2 g of glucose (GTT) or 0.75 U of insulin (ITT) per gram of body weight. The blood glucose levels were detected at 15, 30, 60, and 120 min, respectively. Detection of blood glucose levels at various time points by blood glucose test strips. Blood sampling from the tail vein.

#### 2.3.4. Biochemical indicators in serum

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. The blood of the mice was collected, allowed to stand at room temperature for 30 min, centrifuged at 3000*g* for 10 min at 4 °C, and the serum was separated. The levels of serum aspartate aminotransferase (AST), acid aminotransferase (ALT), low density lipoprotein (LDL), high density lipoprotein (HDL), total cholesterol (TC), triglyceride (TG) was measured by an automatic biochemical analyzer.

#### 2.3.5. Liver histopathology

Mice were humanely sacrificed by cervical dislocation while under deep anesthetic conditions and disinfected in 75% alcohol. Liver tissue was harvested from HFD-fed mice, 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.

#### 2.3.6. Detection of $CD4^+$T, $CD8^+$T, and iNKT cells frequencies in the mouse liver

Further, $1 \times 10^6$ cells were placed in each flow cytometry tube and treated with mouse Abs (clones) were as follows: anti-CD3, anti-CD4, anti-CD8, anti-TCR-β, and α-GalCer/CD1d tetramers. Next, they were incubated at 4 °C for 30 min in the dark and washed twice with PBS. Then, the cells were resuspended in 500 μL of PBS and detected by flow cytometry. PE-conjugated α-GalCer/CD1d tetramers were next 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.

*2.3.7. Detection of iNKT cell frequency in mouse peripheral blood*

A volume of 120 μL of peripheral mouse blood was withdrawn in a flow tube, and the non-specific staining was blocked with BSA. Then, 2 μL of FITC-labeled Anti-TCR β and PE-labeled α-GalCer-loaded CD1d tetramer were used in 50 μL of the PBS reaction system. Incubation was conducted in the dark for 30 min, followed by washing twice with PBS, resuspension of the cells in 500 μL PBS, and measurement with FACS.

*2.3.8. Detection of mouse liver iNKT cell subsets*

The FITC-labeled-anti-TCR-β and PE-labeled α-GalCer/CD1d tetramer antibody were incubated with cells. Further, the cells were stained for cell surface markers and then fixed and permeabilized using Foxp3/TranscriPtion Factor Staining Buffer and stained with the following fluorochrome labeled mAbs: PerCP-CyTM5.5 Mouse anti-T-bet (5 μL) and Alexa Fluor® 647 Mouse Anti-PLZF (5 μL). Then, they were incubated in the dark for 30 min, washed twice with PBS, and resuspended in 500 μL of PBS, followed by FACS measurement.

*2.3.9. Liver lymphocyte culture supernatant and serum cytokines*

Serum and liver lymphocyte were collected. Lymphocytes were isolated from liver mononuclear cells of mice using lymphocyte separation medium and washed twice with PBS. Then, lymphocytes were stimulated in vitro with PMA and ionomycin for 5 h in the presence of GolgiPlug and cytokines in lymphocyte culture supernatant were analyzed by flow cytometry. Following strictly the instructions, the levels of cytokines, including IL-2, IL-4, IL-6, IL-10, IL-17A, IFN-γ, and TNF-α, were detected by cytometric bead array (CBA) kit. Next, the standard sample was prepared and mixed, and the microspheres were captured. Then, 50 μL of the capture microspheres was added to each tube, add 50 μL of the standard was diluted to each standard tube. Next, 50 μL of the sample was transferred into each sample tube, and 50 μL PE detection reagents was added to all the tubes. The specimens were incubated at room temperature for 2 h in the dark. Then, 1 mL of washing solution was added to each tube, followed by centrifugation at 200 × *g*, for 5 min. Further, the supernatant was discarded, 300 μL of lotion was added into each tube, and detection was performed by a flow cytometer, followed by data analysis.

*2.3.10. Western blot*

The cell lysates were extracted with an extraction reagent. Whole cell lysates of liver tissue (50 mg) were separated on a gradient (4–20%) polyacrylamide NuPAGE gel and transferred onto polyvinylidene fluoride and blotted with anti-T-bet/Tb21, anti-GATA3, anti-GAPDH, and anti-β-actin. Fluorescence chemiluminescence detector was used to detect the expression of T-bet and GATA-3 in each group. The liver was GAPDH as the internal reference. Expression intensity was quantified by Image Lab.

*2.4. Statistical analyses*

Data were analyzed using SPSS version 24.0. (SPSS Inc., Chicago, IL, USA). For all analyses, data were presented as mean ± SEM. For comparison among the three groups, one-way ANOVA with Tukey correction 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. General observation of mice*

The hair of RD-fed mice was smooth, whereas that of the HFD-fed mice were greasy and easily prone to hair loss. The RD-fed mice were shiny, responsive, and in good spirits, whereas the HFD-fed mice had a larger body size, lower activity; they were apathetic and with a slow response (Fig. 1).

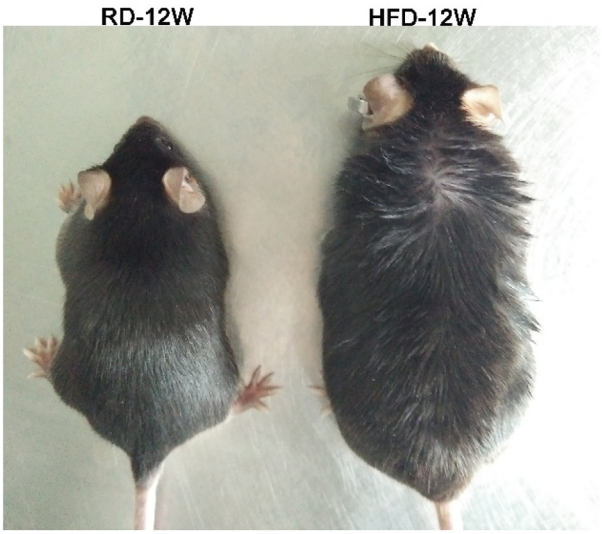


**Fig. 1.** Status of the mice in the control group and HFD-fed group. Comparison of RD-fed control mice (left side) and obese mice fed a high-fat diet for 12 weeks (right side).

*3.2. Effect of α-GalCer intervention on the body weight of the HFD-fed mice*

We found that the HFD-fed mice gained more weight than the RD-fed mice (weight increased by 39%), reaching the standard of obesity (BMI > 20%) [23]. Administered with α-GalCer by intraperitoneal injection resulted in decreasing in weight of HFD-fed mice, whereas administered with α-GalCer by subcutaneous injection resulted in increasing in weight in the HFD-fed mice compared with HFD-fed mice (Fig. 2A–B).

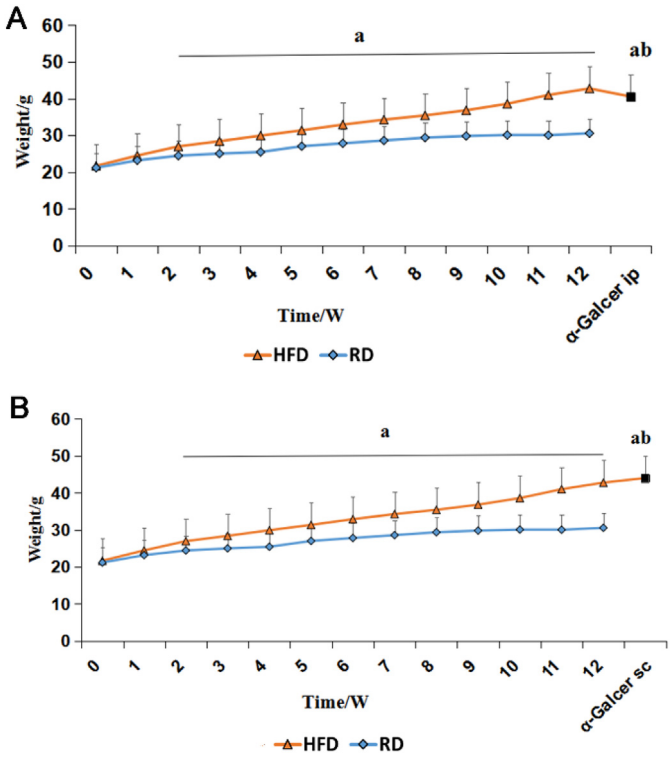


**Fig. 2.** Body weight of mice fed a high fat diet for 12 weeks was increased compared with that of RD-fed mice. Body weight changes in (A) RD-fed mice, HFD-fed mice, HFD-fed mice of injected intraperitoneally with α-GalCer, (B) RD-fed mice, HFD-fed mice and HFD-fed mice of injected subcutaneously with α-GalCer. $^{a}P < 0.05$ vs RD; $^{b}P < 0.05$ vs HFD.

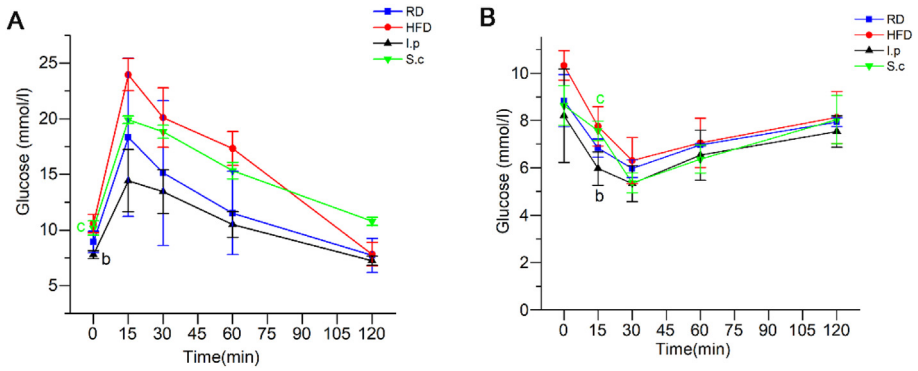


**Fig. 3.** No significant impairment in insulin resistance and glucose tolerance of administration of α-GalCer in HFD-fed mice. Blood glucose levels during (A) GTT and (B) ITT in mice of each group. $^{a}P < 0.05$ vs RD, $^{b}P < 0.05$ vs HFD, $^{c}P < 0.05$ vs I.p.

### 3.3. Effect of α-GalCer on GTT and ITT in high-fat-fed mice

The insulin resistance and glucose tolerance of the mice were tested after 12-week HFD feeding. The blood glucose levels of the HFD-fed mice were higher than those of the RD-fed mice at each time point, but no significant difference was present. This result suggested the lack of obviously impaired glucose tolerance and insulin resistance in the HFD-fed mice. No significant improvement in the overall glucose tolerance and insulin tolerance curves was established after the administration of α-GalCer by intraperitoneal or subcutaneous injection (Fig. 3A, B).

### 3.4. Effect of α-GalCer on the liver status of HFD-fed mice

In the RD-fed mice, we established a dark red liver color, smooth surface, sharp edges, and tough texture. The liver of mice fed with high-fat diet for 12 weeks showed a large volume. It's the appearance became yellowish or creamy yellow, slightly white. The surface was fine, grainy, swollen, and brittle, and the edges were blunt (Fig. 4A).

In the RD-fed mice, the liver sinus was clearly visible after H&E staining of the liver slides was performed. The hepatic cord of these mice was neatly arranged, the liver cells were free of steatosis, the cell structure was clear, and the cytoplasm was abundant. However, severe hepatic steatosis occurred in the liver of HFD-fed mice, which was effectively attenuated by the intraperitoneal injection with α-GalCer, although the subcutaneous injection with α-GalCer exerted no beneficial effects on the hepatic steatosis in HFD-fed mice (Fig. 4B).

### 3.5. Effect of α-GalCer on hepatic $CD4^+T$ and $CD8^+T$ cells in HFD-fed mice

The frequency of $CD4^+$T and $CD8^+$T cells was significantly decreased in the HFD-fed mice ($P < 0.05$). The administration of α-GalCer lowered the numbers of $CD4^+$T cells in the HFD-fed mice. Hepatic $CD8^+$T cells were significantly decreased in the HFD-fed mice of intraperitoneal injection with α-GalCer and significantly increased in the mice of subcutaneous injection with α-GalCer ($P < 0.5$) (Fig. 5A–C).

### 3.6. Effect of α-GalCer on iNKT cells in the peripheral blood and liver of HFD-fed mice

#### 3.6.1. Peripheral blood

There was no significant difference in frequency of iNKT cells between RD-fed mice and HFD-fed mice ($P > 0.05$). The frequency of iNKT cells was increased in mice after intraperitoneal injection with α-GalCer ($P < 0.05$), and no significant change was observed after the subcutaneous injection with α-GalCer ($P > 0.05$) (Fig. 6A, C).

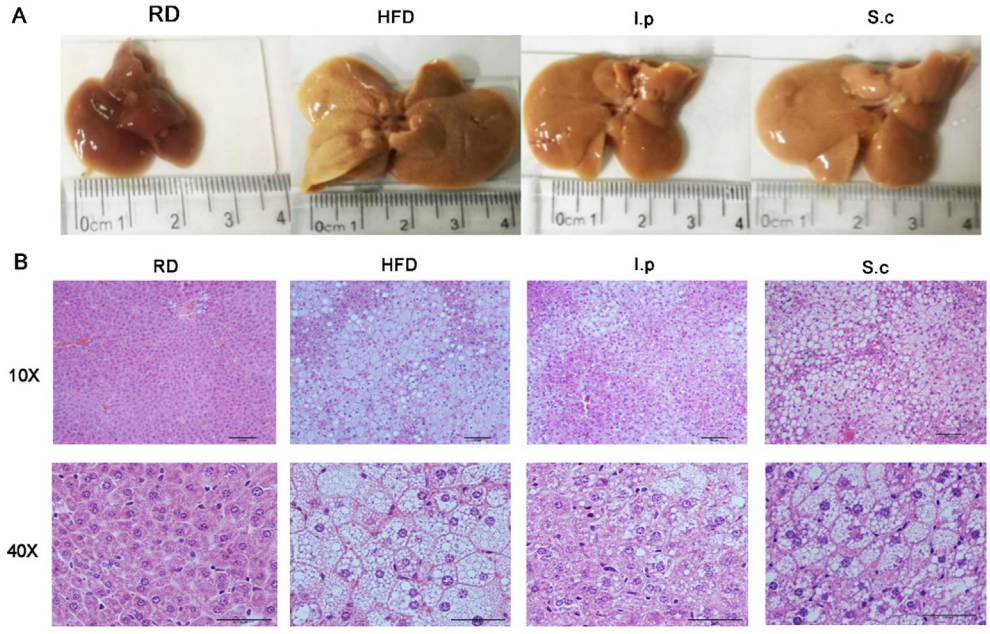


**Fig. 4.** Intraperitoneal injection of α-GalCer in HFD-fed mice instead of subcutaneous injection of α-GalCer can improve hepatic steatosis induced by high-fat diet. (A): The appearance of liver in mice of each group; (B): representative pictures from H&E-stained liver section of RD-fed mice, HFD-fed mice, HFD-fed mice of intraperitoneal and subcutaneous injection with α-GalCer.

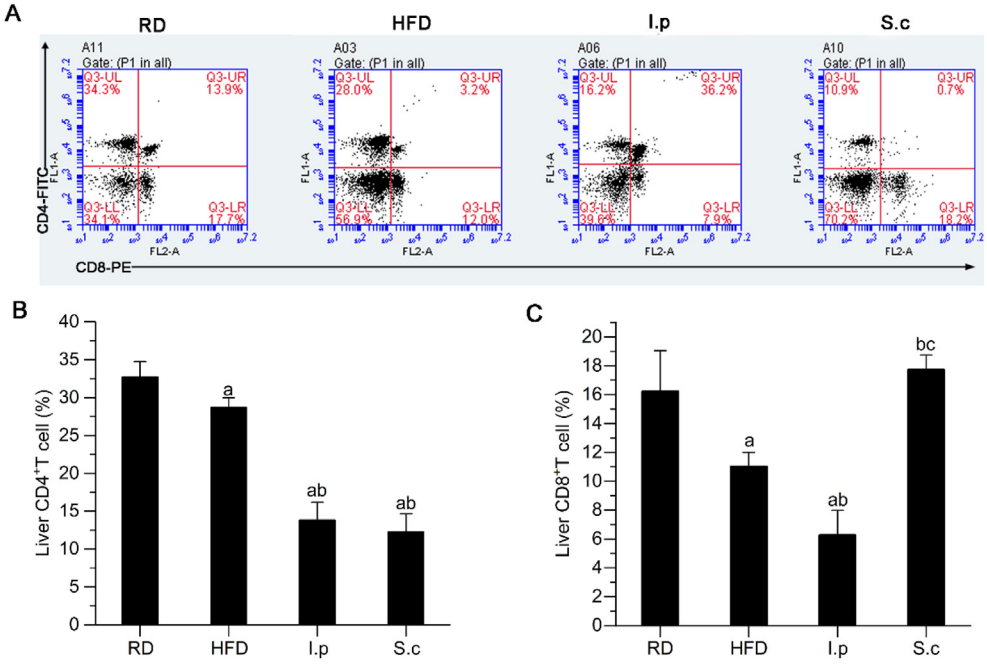


**Fig. 5.** Flow cytometry analysis the percentage of $CD4^+$T and $CD8^+$T cells in the liver of mice. (A): Representative dot plot of hepatic $CD4^+$T and $CD8^+$T cells in mice of each group. Numbers on dot plots indicate percentage of $CD4^+$T and $CD8^+$T cells on the lymphocyte gate. Bar graph shows the percentage of (B) $CD4^+$T cells and (C) $CD8^+$T cells in the liver of mice. $^aP < 0.05$ vs RD, $^bP < 0.05$ vs HFD, $^cP < 0.05$ vs I.p.

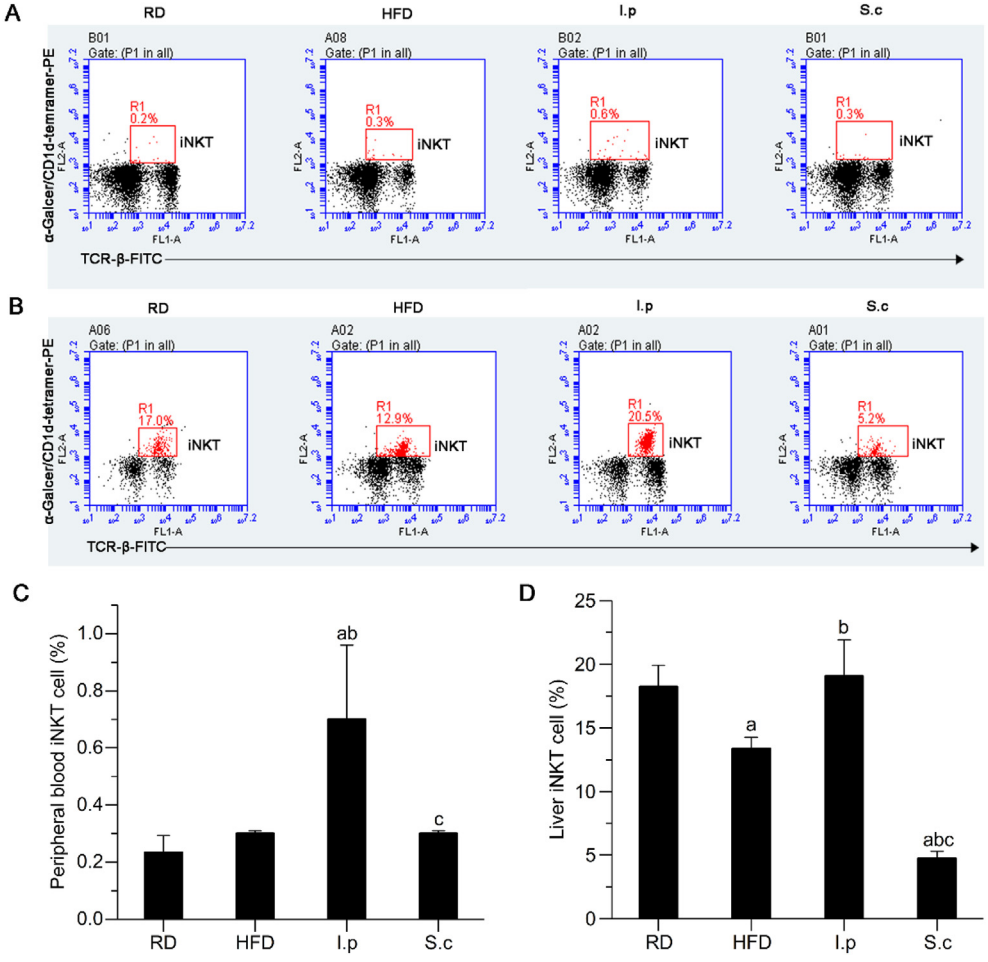


**Fig. 6.** Flow cytometry analysis the percentage of iNKT cells in the peripheral blood and liver. iNKT cells were identified as double TCRβ- and α-GalCer/mCD1d tetramer-positive cells ($TCR\beta^+$/α-GalCer/CD1d-$tetramer^+$) in the lymphocyte gate. Representative dot plot of iNKT cells in (A) peripheral blood and (B) liver of mice in each group. Numbers on dot plots indicate percentage of iNKT cells on the lymphocyte gate. Bar graph shows the percentage of iNKT cells of (C) peripheral blood and (D) liver in mice of each group. $^aP < 0.05$ vs RD, $^bP < 0.05$ vs HFD, $^cP < 0.05$ vs I.p.

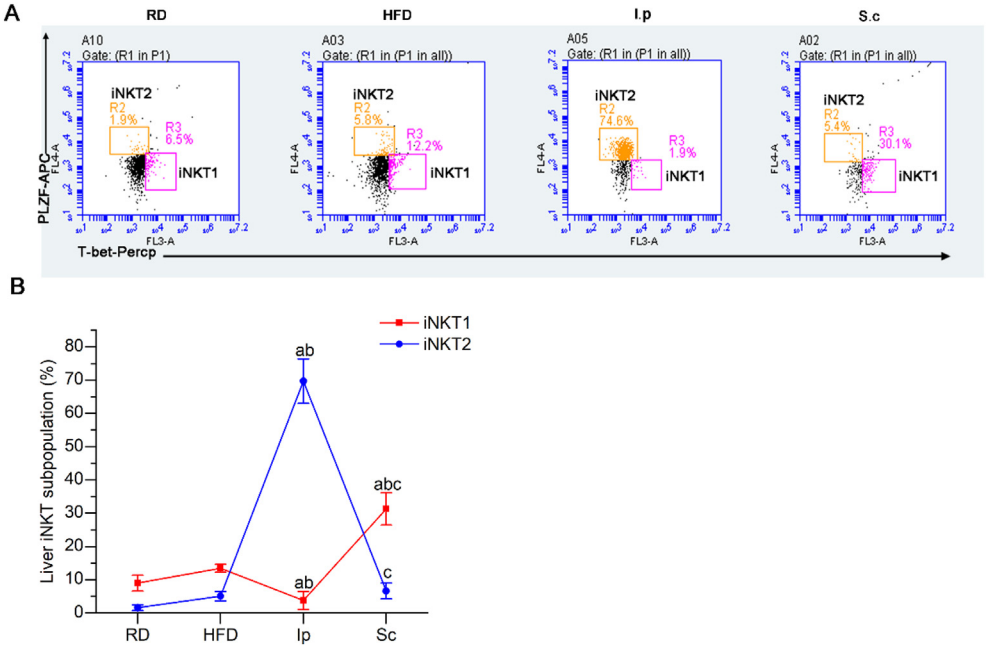


**Fig. 7.** Intraperitoneal injection of α-GalCer in HFD-fed mice resulted in increasing of hepatic iNKT2 but subcutaneous injection of α-GalCer resulted in increasing of hepatic iNKT1. (A): Representative dot plot of hepatic iNKT1 and iNKT2 cells were detected by Flow cytometry. (B): Line chart shows iNKT1 and iNKT2 cells in liver of mice in each group. $^{a}P < 0.05$ vs RD, $^{b}P < 0.05$ vs HFD, $^{c}P < 0.05$ vs I.p.

*3.6.2. Liver*

The frequency of iNKT cells in the HFD-fed mice was lower than that in the RD-fed mice. The quantity of iNKT cells was significantly higher in the mice which administration of an intraperitoneal injection of α-GalCer. Administration of α-GalCer by subcutaneous injection resulted in reduction in the frequency of iNKT cells in HFD-fed mice (Fig. 6B, D).

*3.7. Effect of α-GalCer on the hepatic iNKT subsets in HFD-fed mice*

Previous studies have shown that the iNKT1 subpopulation is predominant in the liver of normal mice. In the present study, we found no difference in the frequency of iNKT1 cells and iNKT2 cells between HFD-fed and RD-fed mice ($P > 0.05$). After the application of an intraperitoneal injection with α-GalCer, the frequency of iNKT1 cells was significantly decreased, and that of iNKT2 cells was significantly increased in HFD-fed mice ($P < 0.05$). However, the abundance of iNKT1 cells was significantly higher ($P < 0.05$) in HFD-fed mice treated with a subcutaneous injection of α-GalCer, and there was no significant change in iNKT2 cells ($P > 0.05$) (Fig. 7A, B). The above results indicate that different hepatic iNKT subpopulations can be activated by various routes of injection α-GalCer.

*3.8. Effect of α-GalCer on cytokines in the liver lymphocyte culture supernatant of HFD-fed mice*

The levels of pro-inflammatory cytokines, such as IL-6, IL-17A, TNF-α, and IFN-γ, were increased in the liver lymphocyte culture supernatant of HFD-fed mice ($P < 0.05$), whereas that of the anti-inflammatory cytokine (IL-4) decreased significantly ($P < 0.05$). The intraperitoneal injection with α-GalCer induced a decrease in inflammatory cytokines (such as IL-2, IL-6, IL-17A, TNF-α, and IFN-γ) ($P < 0.05$) but an increased those of anti-inflammatory cytokine (IL-4, IL-10) ($P < 0.05$) in the HFD-fed mice. Subcutaneous injection with α-GalCer resulted in decreasing of inflammatory mediators (such as IL-2, IL-6, IL-17A, and TNF-α) ($P < 0.05$) and significantly increasing only of anti-inflammatory cytokine IL-10 ($P < 0.05$) (Fig. 8A–G).

*3.9. Effects of α-GalCer on the expression of hepatic GATA-3 and T-bet in the liver of HFD-fed mice*

The expression of the hepatic transcription factor T-bet was significantly higher but that of GATA-3 was lower in the HFD-fed mice than in the RD-fed mice ($P < 0.05$). A difference was detected in the between the data obtained after the intraperitoneal and subcutaneous injection with α-GalCer in the HFD-fed mice. After the intraperitoneal injection with α-GalCer, the expression of hepatic T-bet decreased and GATA-3 increased ($P < 0.05$). Subcutaneous injection with α-GalCer can increase expression of hepatic T-bet, and no effect on expression of GATA-3 ($P < 0.05$) (Fig. 9A–C).

*3.10. Effect of α-GalCer by intraperitoneal injection on serum biochemical parameters in HFD-fed mice*

We further tested serum biochemical indicators to confirm the improvement of NAFLD by intraperitoneal injection with α-GalCer. The serum levels of TC, HDL, LDL and ALT in the HFD-fed mice were significantly higher ($P < 0.05$) than those in the RD-fed mice. The serum levels of TC, HDL, and LDL were significantly lower ($P < 0.05$) in the HFD-fed mice treated with intraperitoneal injection with α-GalCer than in the HFD-fed mice, but LDL showed no significant change ($P > 0.05$) (Fig. 10A, B).

## 4. Discussion

Feeding mice with high-fat diet is a common method for establishing an NAFLD model [17,24]. In this study, we used a high-fat diet to feed C57BL/6 male mice to establish an animal model of NAFLD. The weight, liver histopathology, GTT, and ITT were observed in the HFD-fed mice. Our results showed that the weight of the HFD-fed mice significantly increased. Additionally, the liver volume also increased significantly and obtained a yellowish or creamy yellow color. The surface was fine and grainy, and the skin was swollen and brittle. The results of liver pathology revealed considerable accumulation of fat, a large number of vacuoles in the liver cells, and severe fatty degeneration. Moreover, the serum TC, LDL, HDL, and ALT levels were markedly elevated in the HFD-fed mice. The above results indicated that the NAFLD model had been clearly established in the HFD-fed mice for 12 weeks. van der Heijden et al. reported that significant glucose tolerance and insulin tolerance in HFD-fed C57BL/6J mice for 24 and 40 weeks [25]. The investigation of Martin-Murphyyanj observed that HFD-fed CD1d$^{-/-}$ mice were more prone to glucose tolerance and insulin resistance [26]. Our findings demonstrated no significant

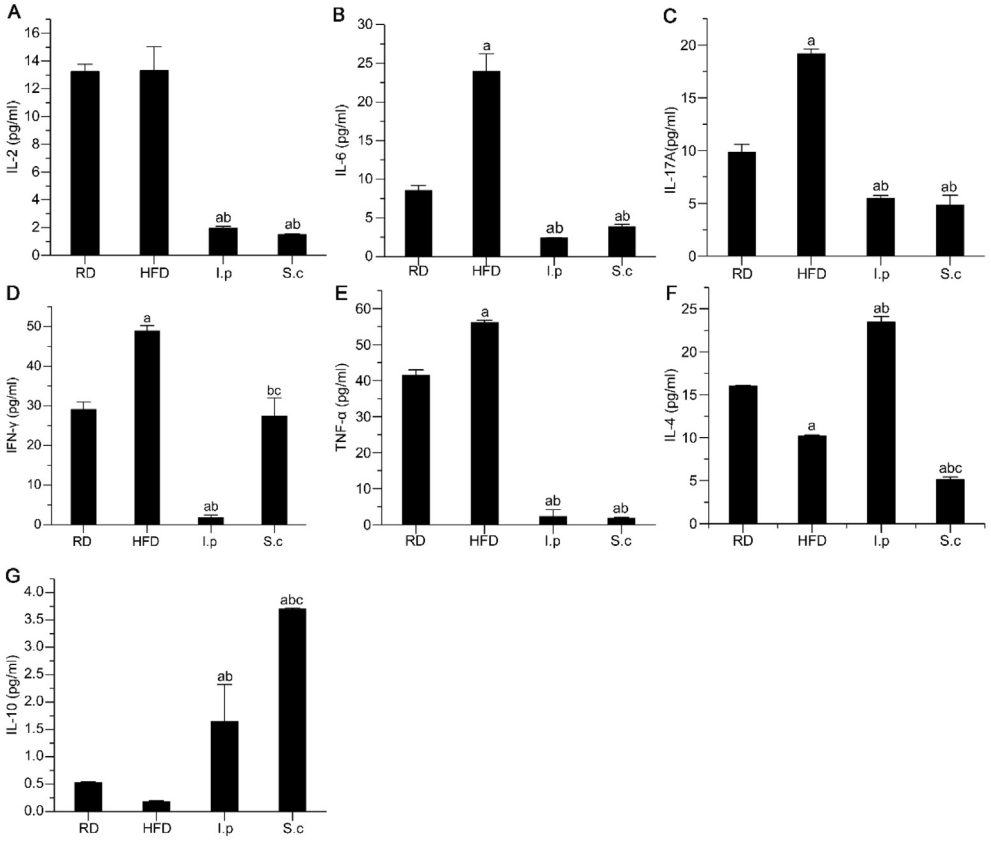


**Fig. 8.** Effects of injection with α-GalCer by different routes on secretion of hepatic cytokines. Bar graph shows the levels of hepatic (A) IL-2, (B) IL-6, (C) IL-17A, (D) IFN-γ, (E) TNF-α, (F) IL-4, (G) IL-10 in mice of each group. $^{a}P < 0.05$ vs RD, $^{b}P < 0.05$ vs HFD, $^{c}P < 0.05$ vs I.p.

glucose tolerance and insulin resistance, which may be related to the different high-fat feeding time or high-fat diet formulation. Subsequently, we examined the frequency of $CD4^{+}$T, $CD8^{+}$T cells, iNKT cell, and iNKT cells subpopulations in the liver and peripheral blood. The secretion of cytokines in liver tissue culture supernatants and the expression of T-bet and GATA-3 were determined.

The results of Bhattacharjee indicated that accumulation of $CD8^{+}$ T cells occurred in the liver of a mouse model of NAFLD, induced by the high-fat and high-carbohydrate diet [27]. The study by Ma and Kesarwala also revealed that $CD8^{+}$T cells did not obviously change in mice after the application of methionine-deficient diet [28]. Here, we found that the abundance of $CD4^{+}$T and $CD8^{+}$T cells was reduced in the mice that were HFD-fed for a period of 12 weeks. That finding is not completely consistent with the ones of previous studies, but the causes and underlying mechanisms need to be further explored.

NAFLD can be categorized histologically into non-alcoholic fatty liver (NAFL) and non-alcoholic Steatohepatitis (NASH). NAFL is defined as the presence of at least 5% of hepatic steatosis and hepatocyte ballooning, without hepatocellular injury. On the other hand, NASH is defined as the presence of at least 5% hepatic steatosis and inflammation with liver injury, with or without fibrosis [29,30]. NASH is a transitional period from simple hepatic steatosis to cirrhosis and even liver cancer [31]. Infiltration of inflammatory cells and production of inflammatory cytokines further promote the development of NAFLD. Detection of cytokines in liver tissue culture supernatants revealed that pro-inflammatory cytokines (IL-6, IL-17A, TNF-α, and IFN-γ) were significantly increased and anti-inflammatory cytokines IL-4 were significantly reduced in HFD-fed mice. In the HFD-fed mice, the expression of the Th1-type transcription factor T-bet was significantly higher in the liver tissue, whereas that of the Th2-type transcription factor GATA-3 was significantly lower. Combined with elevated serum ALT levels, these data indicated that there was a certain level of inflammation in the liver tissue of NAFLD, induced by the high-fat diet, although no obvious inflammatory cell infiltration was found in liver pathology.

Abundant iNKT cells and iNKT cells accounting for 20%–30% of the liver T cells were earlier reported to be present in mouse liver tissue [32]. As a unique tissue-localized natural immune lymphocyte, iNKT cells play an important role in maintaining the immune homeostasis of the liver. Studies by Martin-Murphy showed that $CD1d^{-/-}$ mice (deficient in NKT cells) following high-fat diet were more prone to gain body weight and hepatic steatosis [26]. We detected the frequency of iNKT cells in the peripheral blood and liver of HFD-fed mice.

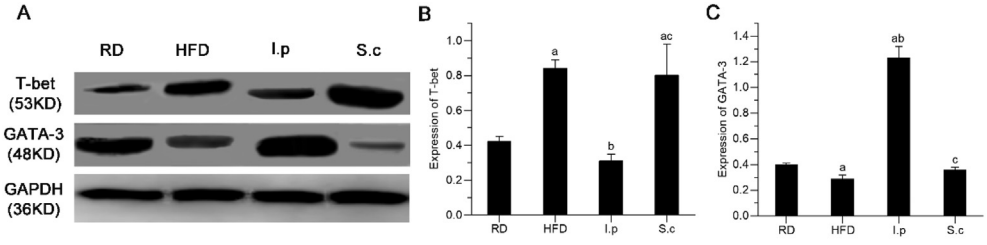


**Fig. 9.** Expression of hepatic T-bet is decreased and GATA-3 is increased in HFD-fed mice that injected intraperitoneally with α-GalCer instead of injected subcutaneously with α-GalCer. (A): Western blot of T-bet and GATA-3 protein expression in liver homogenates from mice of each group. Bar graph shows the quantification of the ratio of (B) T-bet- and (C) GATA-3-positive area in the indicated groups. $^{a}P < 0.05$ vs RD, $^{b}P < 0.05$ vs HFD, $^{c}P < 0.05$ vs I.p.

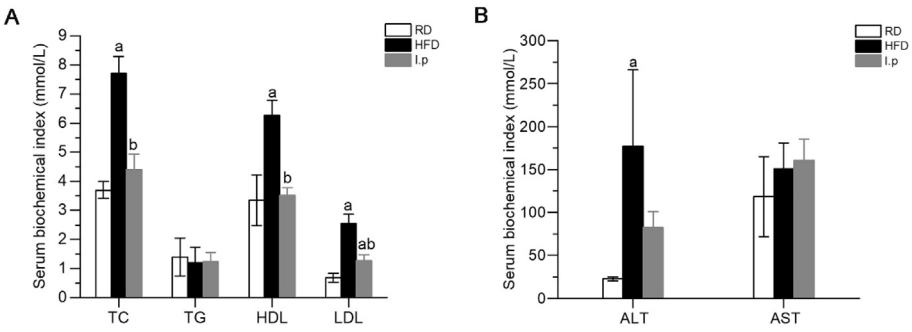


**Fig. 10.** Analysis of serum biochemical indicators by automatic biochemical analyzer. The operation process is the same as the section of method 2.3.4. (A) Serum TC, TG, HDL, and LDL levels of the mice of each of the groups. (B) Serum ALT and AST levels of the mice of each of the groups. $^{a}P < 0.05$ vs RD, $^{b}P < 0.05$ vs HFD, $^{c}P < 0.05$ vs I.p.

Consistently with the most commonly reported conclusions [17–19], we found no significant changes in the frequency of iNKT in peripheral blood between RD-fed mice and HFD-fed mice, whereas the frequency of hepatic iNKT cells obviously decreased in the HFD-fed mice. These results suggest that the decrease in the number of hepatic iNKT cells may be involved in the occurrence of NAFLD, induced by high-fat diet.

α-GalCer is a classical iNKT cell-specific activator extracted from sponge [6]. It has been widely used in the intervention of tumor, infection and autoimmune diseases [33,34]. Previous studies have revealed that the specific activation of iNKT cell subtypes can more effectively exert its immune regulation and immunotherapy [35]. Our previous experiments showed that an injection with α-GalCer by different routes caused substantial differences in the activation of different subsets of iNKT cells in different immune organs of normal mice. Subcutaneous injection with α-GalCer activates mainly the iNKT1 subpopulation in the liver and spleen, while intraperitoneal injection with α-GalCer mainly activates the iNKT2 subpopulation in the liver and spleen. Therefore, we used both subcutaneous and intraperitoneal injection with α-GalCer immunological intervention for high-fat diet-induced NAFLD. The data obtained demonstrated that the intraperitoneal injection with α-GalCer reduced in the weight, lowered the serum TC, HDL, and LDL levels, and significantly improved hepatocyte lipid accumulation. However, the animals in the group treated with a subcutaneous injection of α-GalCer did not show improvement in their hepatic steatosis. Further analysis in the frequency of hepatic iNKT and subpopulations in HFD-fed mice found that the intraperitoneal injection with α-GalCer significantly increased the frequency of iNKT cells, whereas it was significantly decreased in the HFD-fed mice treated with subcutaneous injection of α-GalCer. The frequency of hepatic iNKT1 cells was significantly decreased, that of iNKT2 cells was significantly increased after the application of the intraperitoneal injection of α-GalCer. The subcutaneous injection of α-GalCer markedly increased the frequency of hepatic iNKT1 cells but that of hepatic iNKT2 cells was not significantly changed. Moreover, the administration of intraperitoneal injection of α-GalCer reduced the levels of inflammatory cytokines (IL-2, IL-6, IL-17A, TNF-α, and IFN-γ) in the HFD-fed mice and increased their anti-inflammatory cytokines (IL-4 and IL-10) levels. Additionally, the subcutaneous injection of α-GalCer caused an increase in the levels of IL-10, whereas those of other cytokines significantly decreased. Further detection of the related transcription factors revealed that the expression of hepatic T-bet was decreased and the expression of hepatic GATA-3 was increased in HFD-fed mice by the application of α-GalCer administered by intraperitoneal injection. Different from intraperitoneal injection results, the expression of hepatic T-bet was increased and the expression of GATA-3 protein was not significantly changed in HFD-fed mice of α-GalCer by subcutaneous injection.

The aforementioned results showed a difference in the effects of α-GalCer administered by different routes on NAFLD, induced by high-fat diet, which is closely associated with activation of different iNKT cells subpopulations. Hepatic iNKT2 cells can be selectively activated by intraperitoneal injection with α-GalCer while improving hepatic steatosis. This also suggests that the use of different a-GalCer injection routes may be a good treatment strategy for different diseases. We hypothesize that the activation of hepatic iNKT2 cells by intraperitoneal injection with α-GalCe and secretion of anti-inflammation cytokines IL-4 affect the hepatocyte-related receptors involved in the lipid metabolism [36]. An improvement in the local inflammatory environment in the liver is also possible by immunomodulating other immune cell responses and the secretion of cytokines, indirectly affecting the liver lipid metabolism. Nonetheless, an improved comprehension of the detailed mechanism requires further investigation.

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## Declaration of Competing Interest

The authors declare no competing financial interests.

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