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## Activation of Invariant Natural Killer T Cell Subsets in C57BL/6J Mice by Different Injection Modes of $\alpha$ -galactosylceramide

Ming Meng<sup>1,2</sup>, Shengde Chen<sup>1,2</sup>, Xiang Gao<sup>1,2</sup>, Huifang Liu<sup>1,2</sup>, Huijuan Zhao<sup>1,2</sup>, Jingnan Zhang<sup>1,2</sup>, Jinku Zhang<sup>3</sup>, and Dongzhi Chen<sup>1,2</sup>

<sup>1</sup> Medical School of Hebei University, Baoding, Hebei Province, China

<sup>2</sup> Key Laboratory of Pathogenesis Mechanism and Control of Inflammatory-autoimmune Diseases in Hebei Province, Baoding, China

<sup>3</sup> Department of Pathology, The First Centre Hospital of Baoding, Baoding, Hebei Province, China

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### ABSTRACT

Whether different injection modes of  $\alpha$ -galactosylceramide ( $\alpha$ -GalCer) affect the activation of different subsets of invariant natural killer T (iNKT) cells in different tissues and organs of mice is unclear.

This study included healthy control, subcutaneous injection, and intraperitoneal injection groups (n=10 in each group). The subcutaneous and intraperitoneal injection groups were injected with  $\alpha$ -GalCer (0.1 mg/kg weight), and then the changes in thymus, spleen, and liver iNKT cell frequencies and subsets were observed.

The intraperitoneal injection of  $\alpha$ -GalCer could increase the frequency of splenic iNKT cells, but the subcutaneous injection did not affect the frequency. Neither injection had any effect on the frequency of iNKT cells in the thymus and liver. The subcutaneous injection of  $\alpha$ -GalCer increased the rate of iNKT2 subsets in the thymus but did not affect the rate of iNKT1 subsets. However, the intraperitoneal injection of  $\alpha$ -GalCer did not affect thymus iNKT1 and iNKT2 subsets. Interestingly, the subcutaneous injection of  $\alpha$ -GalCer significantly increased the proportion of iNKT1 in the spleen and liver but did not significantly change the proportion of iNKT2. The intraperitoneal injection of  $\alpha$ -GalCer significantly increased the rate of iNKT2 in spleen and liver but decreased the rate of iNKT1.

Subsets of iNKT1 or iNKT2 cells in the spleen and liver were selectively activated by the subcutaneous or intraperitoneal injection of  $\alpha$ -GalCer. It provides a valuable means for treating tumors and certain autoimmune diseases. Further exploration of the activation mechanism may provide new ideas about the development of related vaccines.

**Keywords:** Cytokines; Invariant natural killer T cells; Intraperitoneal injection; Subcutaneous injection;  $\alpha$ -galactosylceramide

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**Corresponding Authors:** Dongzhi Chen, MD, PhD;  
Medical School of Hebei University, Baoding, Hebei Province,  
China. Tel: (+86 312) 5079 024, E-mail: chendzz@163.com  
Jinku Zhang, PhD;  
Department of Pathology, The First Centre Hospital of Baoding,  
Baoding, Hebei Province, China. E-mail: 843561234@qq.com

### INTRODUCTION

Invariant natural killer T (iNKT) cells are a group of special immune cells with characteristics of both natural killer (NK) and T cells.<sup>1</sup> iNKT cells have an

invariant T cell receptor (TCR) chain (human Va24-Ja18/Vβ11; murine Va14-Ja18/Vβ8.2,7,2),<sup>2,3</sup> activated by glycolipid antigens. The TCR chain is presented by CD1d molecules of nonclassical major histocompatibility complex (MHC) I.<sup>4</sup> Activated iNKT cells rapidly secrete a large number of Th1- and Th2-type cytokines (such as Interleukin (IL)-2, IL-4, IL-6, IL-10, IL-17, interferon (IFN)-γ, and tumor necrosis factor (TNF)-α).<sup>5-6</sup> They regulate the functions of dendritic cells, macrophages, B cells, T cells, and NK cells and play an important role in diseases such as tumors, infections, and autoimmune diseases.<sup>7-10</sup> iNKT cells are positively selected in the thymic cortex and migrate to the medullary area, where they undergo four stages (stage 0, stage 1, stage 2, and stage 3) and finally differentiate into three different subsets: iNKT1 (mainly secreting IFN-γ), iNKT2 (mainly secreting IL-4), and iNKT17 (mainly secreting IL-17), distributed in the thymus, spleen, liver, lymph nodes, lungs, fat, and other tissues and organs.<sup>11-14</sup>

As a classic iNKT cell-specific activator, α-GalCer is a galactosylceramide found in marine sponge extracts, which stimulates CD1d-restricted iNKT cell activation,<sup>4,15</sup> effectively activating iNKT1, iNKT2, and iNKT17. α-GalCer has been widely used in intervention research on tumors, infections, and autoimmune diseases.<sup>16-18</sup> Recent studies showed that the specific activation of iNKT cell subsets could play a more effective role in immune regulation and immunotherapy.<sup>19,20</sup> The type of immune response mediated by iNKT cells after activation is affected by many factors, for example, ligand structure (α-GalCer, PBS57, c-glycoside-activated iNKT cell-mediated Th1 immune response; OCH, acC8:0, acC20:2-activated iNKT cell-mediated Th2 immune response),<sup>21-23</sup> local environment in which iNKT cells are located, and type of antigen-presenting cells.<sup>24</sup> However, different injection routes of α-GalCer activating different iNKT cell subsets, thus affecting the type of immune response, were rarely reported. In this study, C57BL/6J mice were injected with α-GalCer subcutaneously and intraperitoneally to observe the changes in the frequency, subset proportion, and function of iNKT cells in the thymus, liver, and spleen. The findings might provide a new strategy for immunotherapy targeting iNKT cells.

## MATERIALS AND METHODS

### Experimental Animals

Thirty 6-8 week old healthy male C57BL/6J mice

(17.0±1.5 g), reared in an SPF environment, were provided by Beijing Vital River Laboratory Animal Technology Co., Ltd. (License No. SCXK (Beijing) 2015-0003). The mice were provided drinking water and rodent chow ad libitum and grouped after a week of adaptation. The mice were maintained under specific pathogen-free conditions 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 (approval number IACUC-2017012).

### Reagents and Instruments

The PE-T-selected-CD1d tetramer was purchased from MBL International (Japan). BD Cytometric Bead Array (CBA) Mouse Cytokine Kit (560485), FITC-hamster anti-mouse TCR β chain (553170), PerCP-Cy<sup>TM</sup>5.5-mouse anti-T-bet (561316) and Alexa Fluor 647-mouse anti-PLZF (563490) were obtained from BD Pharmingen (San Diego, CA, USA). Foxp3 Staining Buffer Set was purchased from eBioscience (California, USA). α-GalCer (KRN7000) was manufactured by ENZO Life Sciences (Farmingdale, NY, USA). An Accuri C6 flow cytometer (BD) was used as well.

### Experimental Grouping

The 30 male C57BL/6J mice were randomly divided into 3 groups, including the control (10 mice; no treatment), subcutaneous injection of α-GalCer (10 mice; subcutaneous injection of α-GalCer at the tail root; 0.1 mg/kg weight, the lymphocytes from the thymus, spleen, and liver were isolated on day 8) (unpublished data) and intraperitoneal injection of α-GalCer (10 mice; peritoneal injection of α-GalCer, 0.1 mg/kg weight, the lymphocytes from the thymus, spleen, and liver were isolated on day 3 (unpublished data)) groups.

### FCM Detection of iNKT Cell Frequency in Thymus, Spleen, and Liver

For the pre-treatment of CD1d tetramers, 1 mg/ml α-GalCer was diluted to 200 μg/mL with 0.5% Tween-20 and 0.9% NaCl, and 5 μL of the resulting solution was added to 100 μL of the CD1d tetramer solution. The mixture was incubated for 12h at room temperature and placed at 4°C until use. On the 3th (or 8th) day after α-GalCer injection, the mice were executed by intraperitoneal injection of 1% sodium pentobarbital (5 μg/g weight) under deep anesthesia, the thymus, spleen, and liver lymphocytes were respectively prepared,

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washed twice with phosphate-buffered saline (PBS) and placed in flow cytometry tubes ( $1 \times 10^6$  cells/tube). Then, FITC-labeled anti-TCR  $\beta$  (2  $\mu$ L) and PE-labeled  $\alpha$ -GalCer-loaded CD1d tetramers (2  $\mu$ L) were incubated in 500 $\mu$ L PBS reaction systems for 30 min in the dark, washed twice with PBS, and resuspended in 500 $\mu$ L PBS for FCM detection, with the CFlow software (BD) for analysis.

### FCM Detection of Subsets of iNKT Cells in Thymus, Spleen, and Liver

As described in 2.4, after incubation with anti-TCR  $\beta$  and PE-labeled  $\alpha$ -GalCer-loaded CD1d tetramer for extracellular markers, the cells were permeabilized and fixed according to the specific procedure of Foxp3/Transcription Factor Staining Buffer. Then, 5 $\mu$ L each of PerCP-Cy<sup>TM</sup>5.5 mouse anti-T-bet and Alexa Fluor 647 mouse anti-PLZF were added at room temperature in the dark for at least 30 minutes. After two washes with PBS, the cells were resuspended in 500 $\mu$ L PBS and assessed by FCM.

### Detection of Cytokines in Supernatant of Splenic iNKT Cells

The spleen lymphocytes of the C57BL/6J mouse control and the intraperitoneal injection groups were

collected, and the iNKT cells were purified by magnetic-activated cell sorting (MACS). Then, iNKT cell densities were adjusted to  $2 \times 10^6$ /mL (culture volume was 1.5 mL), which cultured with PMA (50 ng/mL) and (ionomycin) IO (1  $\mu$ g/mL) for 5 h, and then the supernatant was collected. The supernatant levels of IL-2, IL-4, IL-6, IL-10, IL-17A, IFN- $\gamma$  and TNF- $\alpha$  were detected with the CBA cytokine kit, in strict accordance with the manufacturer's instructions. Briefly, 50  $\mu$ L of each sample was assessed, and serum cytokine contents were detected on an Accuri C6 flow cytometer, using the FCAP software (BD).

### Statistical Analysis

The experimental data were analyzed by SPSS 24.0, which were expressed by mean  $\pm$  standard deviation ( $\bar{x} \pm s$ ), and the differences between groups were compared by analysis of variance. First, the one-factor analysis of variance (ANOVA) is used, if the variance is satisfied, the least significant difference (LSD) test is used for further comparison. If the variance is not uniform, the nonparametric test is used, the Kruskal-Wallis H test is used for further comparison, and significance was established at  $p < 0.05$ .

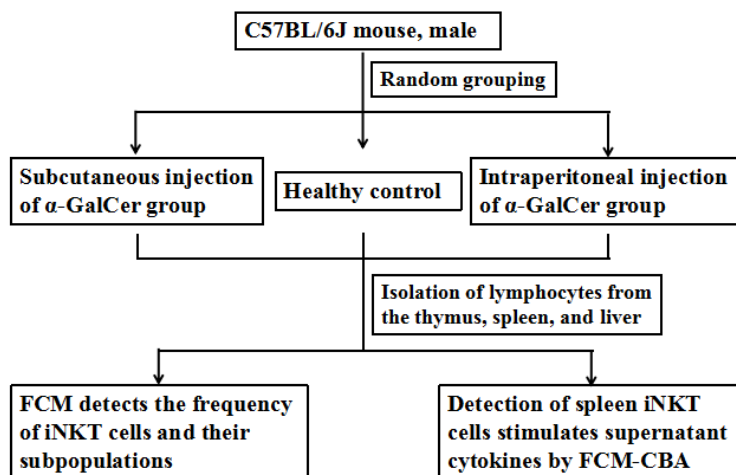


Figure 1. The figure of the experimental protocol

RESULTS

**Intraperitoneal Injection of  $\alpha$ -GalCer Could Significantly Increase the Frequency of Splenic iNKT Cells**

The frequency of iNKT cells of the subcutaneous injection group in the thymus, spleen, and liver was not significantly different from that of the control group ( $p>0.05$ ). Also, no significant difference was found in the frequency of iNKT between the thymus and liver in the intraperitoneal injection group ( $p>0.05$ ). However, the frequency of iNKT cells in the spleen significantly increased in the intraperitoneal injection group ( $p<0.05$ ), compared with the control group (Figure 2).

**Different Injection Routes of  $\alpha$ -GalCer Had Different Effects on iNKT Cell Subsets in the Thymus, Spleen, and Liver of Mice**

***Subcutaneous Injection of  $\alpha$ -GalCer Increased the Proportion of Thymus iNKT2 Subsets in Mice***

The observation of iNKT subsets in the thymus of C57BL/6J mice showed no significant difference in the rate of iNKT1 subsets in the subcutaneous injection group compared with the control group ( $p>0.05$ ). However, the rate of iNKT2 subsets increased significantly ( $p<0.05$ ). Also, no significant difference was observed in the rates of iNKT1 and iNKT2 subsets in the intraperitoneal injection group compared with the control group ( $p>0.05$ ) (Figure 3).

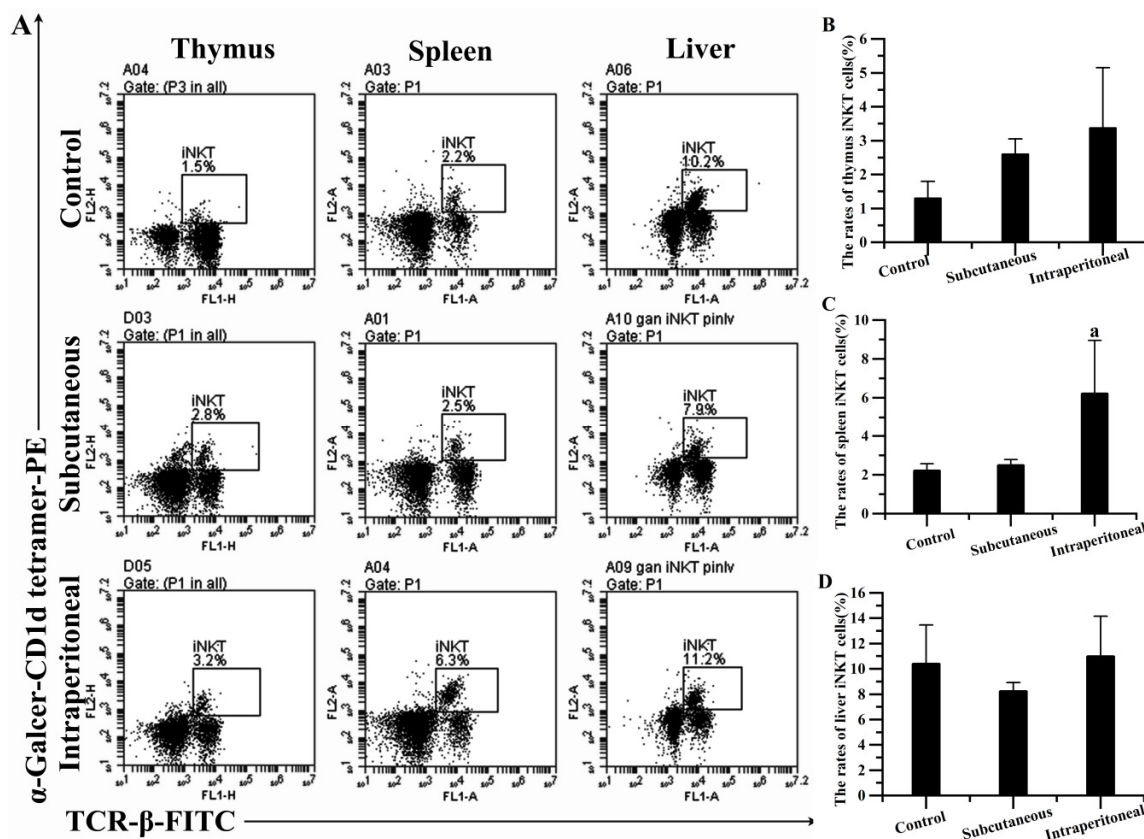
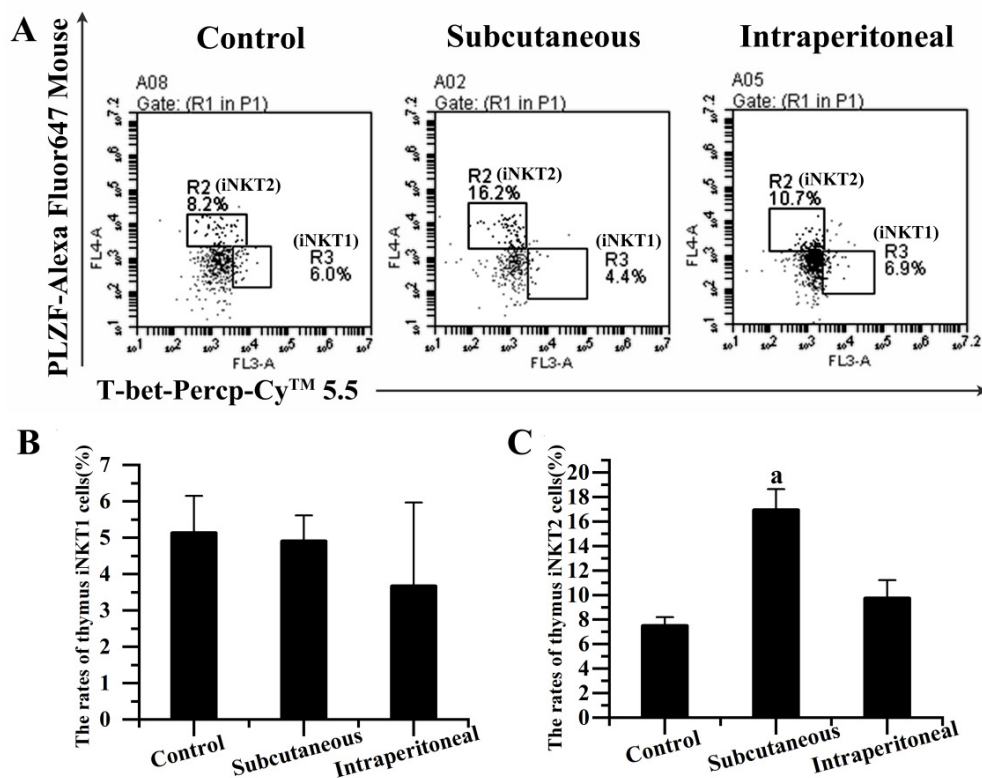


Figure 2. Rates of iNKT cells in various organs of the C57BL/6J mouse. To investigate the activation of iNKT cells by  $\alpha$ -GalCer, we detected the iNKT frequency of thymus, spleen, and liver in C57BL/6J mice. A, flow cytograms of the frequency of thymus, spleen, and liver iNKT cells after intraperitoneal or subcutaneous injection of  $\alpha$ -GalCer. B, C, and D are quantitative analysis of A, which showed that the frequency of iNKT in the spleen increased after intraperitoneal injection of  $\alpha$ -GalCer, and the difference was statistically significant (<sup>a</sup> $p<0.05$  versus the control group. n=10).



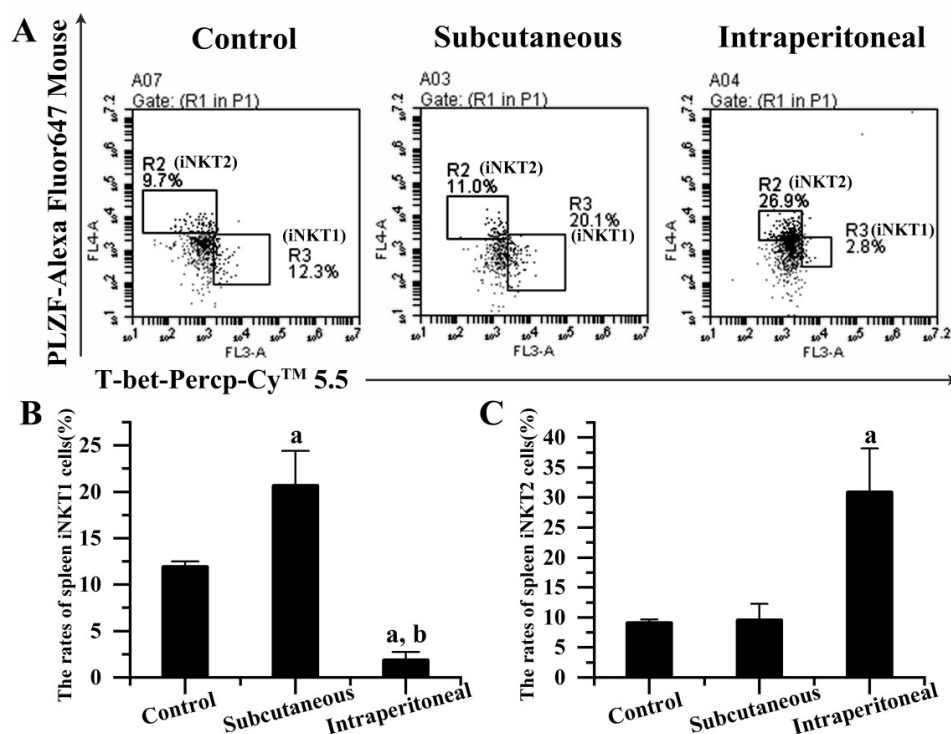
**Figure 3.** Rates of iNKT subsets in the C57BL/6J mouse thymus. To determine the activation of  $\alpha$ -GalCer on thymus iNKT1 and iNKT2 cells, we detected the ratio of thymus TCR  $\beta^+$  CD1d-Tetramer + T-bet + iNKT (iNKT1) cells and TCR  $\beta^+$  CD1d-Tetramer + PLZF+ iNKT (iNKT2) cells in different groups of mice, showing subcutaneous injection of  $\alpha$ -GalCer significantly increased the proportion of iNKT2 in the thymus ( $p < 0.05$  versus the control group,  $n = 10$ ). A, flow cytograms of iNKT subsets in the mouse thymus after intraperitoneal or subcutaneous injection of  $\alpha$ -GalCer. B and C are the quantitative analysis of A.

***Subcutaneous Injection of  $\alpha$ -GalCer Increased the Proportion of iNKT1 Subsets in Mouse Spleen, while Intraperitoneal Injection Significantly Decreased the Proportion of iNKT1 Subsets and Increased the Proportion of iNKT2 Subsets***

The observation of iNKT subsets in the spleen of C57BL/6J mice showed that the rate of iNKT1 subsets significantly increased in the subcutaneous injection group compared with the control group ( $p < 0.05$ ), with no significant difference in the rate of iNKT2 subsets ( $p > 0.05$ ). The rate of iNKT1 subsets significantly decreased ( $p < 0.05$ ) and the rate of iNKT2 subsets significantly increased in the intraperitoneal injection group compared with the control group ( $p < 0.05$ ). The rate of iNKT1 subsets was significantly lower in the intraperitoneal group compared with the subcutaneous group ( $p < 0.05$ ) (Figure 4).

***Subcutaneous Injection of  $\alpha$ -GalCer Increased the Proportion of iNKT1 Subsets in Mouse Liver, while Intraperitoneal Injection Significantly Increased the Proportion of iNKT2 Subgroup***

The observation of iNKT subsets in the liver of C57BL/6J mice showed that the rate of iNKT1 subsets significantly increased in the subcutaneous injection group compared with the control group ( $p < 0.05$ ), with no significant difference in the rate of iNKT2 subsets ( $p > 0.05$ ). The rate of iNKT1 subsets decreased, but with no significant difference ( $p > 0.05$ ), and the rate of iNKT2 subsets significantly increased in the intraperitoneal injection group compared with the control group ( $p < 0.05$ ) (Figure 5).



**Figure 4.** Rates of iNKT subsets in the C57BL/6J mouse spleen. We detected the proportion of iNKT1 and iNKT2 cells in the spleen. A, flow cytograms of iNKT subsets in the spleen after intraperitoneal or subcutaneous injection of  $\alpha$ -GalCer. B and C are the quantitative analysis of A, which showed that the proportion of iNKT1 in the spleen increased after subcutaneous injection of  $\alpha$ -GalCer, while the proportion of iNKT1 in the spleen decreased and the proportion of iNKT2 increased after intraperitoneal injection. (<sup>a</sup> $p < 0.05$  versus the control group. <sup>b</sup> $p < 0.05$  versus the subcutaneous group.  $n = 10$ ).

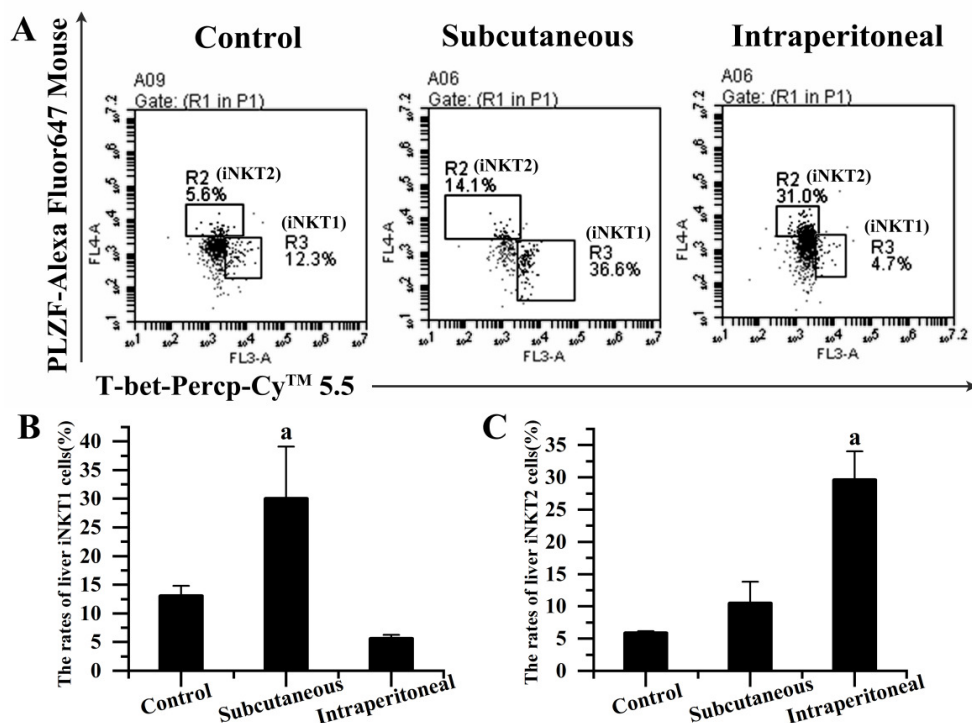
#### After Intraperitoneal Injection of $\alpha$ -GalCer, the Levels of Inflammatory Factors Secreted by the Spleen of C57BL/6J Mice Decreased and the Levels of Anti-Inflammatory Factors Increased

The levels of inflammatory cytokines, such as IFN- $\gamma$ , TNF- $\alpha$ , IL-17A, and IL-6, significantly decreased

( $p < 0.05$ ) and the levels of anti-inflammatory cytokines, such as IL-4, significantly increased ( $p < 0.05$ ) in the intraperitoneal group compared with the control group (Table 1). The ratio of IFN- $\gamma$ /IL-4 was significantly lower in the intraperitoneal injection group than in the control group ( $p < 0.05$ ).

**Table 1.** The changes of cytokines in the culture supernatant of iNKT cells of each group

Cytokines		Control	Intraperitoneal injection
Proinflammatory Cytokines	IL-17A	15.37 $\pm$ 0.16	2.62 $\pm$ 0.47*
	TNF- $\alpha$	33.42 $\pm$ 0.49	0.77 $\pm$ 0.22*
	IFN- $\gamma$	15.57 $\pm$ 0.27	1.87 $\pm$ 0.03*
Anti-inflammatory Cytokines	IL-6	41.21 $\pm$ 0.27	11.94 $\pm$ 0.41*
	IL-4	61.18 $\pm$ 1.02	110.09 $\pm$ 0.55*
	IL-10	20.08 $\pm$ 0.24	20.50 $\pm$ 0.23
IFN- $\gamma$ / IL-4		0.25 $\pm$ 0.01	0.017 $\pm$ 0.005*



**Figure 5.** Rates of iNKT subsets in the C57BL/6J mouse liver. **A**, flow cytograms of iNKT subsets in the liver after intraperitoneal or subcutaneous injection of  $\alpha$ -GalCer. **B** and **C** are the quantitative analysis of **A**. We detected the frequency of liver iNKT1 and iNKT2 cells to demonstrate the selective activation of  $\alpha$ -GalCer to the iNKT subsets. Subcutaneous injection of  $\alpha$ -GalCer increased the proportion of iNKT1 subsets in mouse liver, while intraperitoneal injection significantly increased the proportion of iNKT2 subgroup (<sup>a</sup> $p < 0.05$  versus the control group,  $n = 10$ ).

### DISCUSSION

The iNKT1 cells are a unique group of tissue-resident natural immune lymphocytes. Unlike other lymphocytes, iNKT cells are usually not involved in blood circulation. They play an important role in maintaining the immune homeostasis of local tissues.<sup>25-27</sup> They are defined as iNKT1, iNKT2, iNKT17, and iNKT10 subsets according to transcription factor and cytokine secretion bias.<sup>28,29</sup> The iNKT1 cells have a high expression level of transcription factor T-bet, secrete IFN- $\gamma$  and IL-4, and are distributed mainly in the liver and spleen. The iNKT2 cells have a high expression level of transcription factors GATA binding protein 3 (GATA3) and promyelocytic leukemia zinc-finger protein (PLZF), secrete IL-4, and are distributed mainly in the lung. The iNKT17 cells express transcription factor retinoid-related orphan nuclear receptor (ROR)- $\gamma$ t, secrete IL-17, and are distributed

mainly in the lymph nodes.<sup>30-32</sup> The iNKT10 cells express the transcription factor E4BP4, do not express PLZF, secrete IL-10, and are distributed in the adipose tissue.<sup>26</sup>

The iNKT cells are currently considered to be important immunoregulatory cells whose immunomodulatory function depends on the type of cytokine secreted.<sup>33</sup> Specific activation of different subsets of iNKT cells is the best way to exert immunomodulatory and immunotherapeutic effects. In the present study, C57BL/6J mice were injected subcutaneously and intraperitoneally with  $\alpha$ -GalCer at a dose of 0.1 mg/kg, which is the optimal dose determined by previous effective and safe dose experiments.<sup>34-38</sup> This was consistent with that reported by Horikoshi et al.<sup>16</sup> Then, the changes in the frequency and subpopulation of iNKT cells in mouse thymus, liver, and spleen were observed.

The results showed that neither of the two injection

methods affected the frequency of thymus and hepatic iNKT cells. The frequency of spleen iNKT cells increased after intraperitoneal injection but did not change after subcutaneous injection. The observation of iNKT cell subsets further revealed that subcutaneous injection of  $\alpha$ -GalCer increased the rate of thymus iNKT2 subsets, but had no effect on the iNKT1 subset. However, the intraperitoneal injection of  $\alpha$ -GalCer did not affect thymus iNKT1 and iNKT2 subsets. Surprisingly, after the subcutaneous injection of  $\alpha$ -GalCer, the iNKT1 subset in the spleen and liver increased significantly, while the iNKT2 subset had no significant difference. After intraperitoneal injection of  $\alpha$ -GalCer, the iNKT2 subset in the spleen and liver significantly increased, but the iNKT1 subset in the spleen significantly decreased. Therefore, it was believed that the subcutaneous and intraperitoneal injections had little effect on the frequency of iNKT cells in the thymus, liver, and spleen in C57BL/6J mice, which mainly changed the rate of iNKT1 and iNKT2 subsets. Especially in the liver and spleen, the iNKT2 subsets were activated mainly by the intraperitoneal injection of  $\alpha$ -GalCer, while the iNKT1 subsets were activated mainly by the subcutaneous injection of  $\alpha$ -GalCer. This revealed novel observations for the treatment of tumors and some autoimmune diseases. It was hypothesized that  $\alpha$ -GalCer entered the body in different ways and was captured by different antigen-presenting cells. It might also be that  $\alpha$ -GalCer entered the body in different ways to change the molecular structure of the antigen. The mechanism needs further research. It is believed that the study of the activation mechanism may provide new ideas for the development of related vaccines.

To further understand the function of iNKT cells predominantly of specific subtype activated by  $\alpha$ -GalCer, the iNKT cells (mainly iNKT2 subpopulations) were isolated and purified from the spleen with an intraperitoneal injection of  $\alpha$ -GalCer, and the CBA kit was used to detect cytokines in culture supernatants. The expression levels of pro-inflammatory cytokines significantly decreased, the levels of anti-inflammatory cytokines significantly increased, and the ratio of IFN- $\gamma$ /IL-4 was significantly lower compared with the control group. This result also suggested that the iNKT cells had the characteristics of tissue- and organ-specific distribution. The iNKT cells of the spleen (mainly iNKT1 or iNKT2) can be obtained by subcutaneous or intraperitoneal injection of  $\alpha$ -GalCer.

It can be used as a new method of adoptive infusion therapy for some autoimmune diseases or tumors and vaccine development.

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