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Proliferation and Directional Differentiation of iNKT Cells Derived from DBA/1 Mice Thymus

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ABSTRACT

The rates of invariant natural killer T (iNKT) cells in vivo are very low, and the amounts of cells obtained directly from the body are hard enough to fulfill their potential in clinical application.

To overcome this problem, we subcutaneously injected alpha-galactosylceramide (α-GalCer) into DBA/1 mice and thymic single cells were isolated and cultured in vitro. Fluorescence-activated cell sorting was used to detect the iNKT cells and their subsets in the thymus after the injection of α-GalCer by different methods. In addition, in vitro changes of single-cell suspensions and their cytokines in culture supernatants were assessed.

Compared with the α-GalCer multiple subcutaneous injection group, the rates of iNKT cells in the α-GalCer single subcutaneous injection group were markedly higher at each time point, while the highest levels of iNKT1 and iNKT2 cells were observed on day 4 and 8, respectively. In α-GalCer single subcutaneous injection for 8 days and thymic mononuclear cell cultured for 14 days group, the expansion rate of iNKT cells was significantly faster than the other groups, while it reached a peak for iNKT1 cells. Interferon-gamma was consistent with the development of iNKT1 cells, however no difference was found between the cultured iNKT cells in vitro and the natural iNKT cells in vivo in terms of cytokine production.

Herein, we introduced a method in which antigenic stimulation in vivo and directed induction in vitro yielded high levels of iNKT cells with specific functions.

Keywords: Alpha-galactosylceramide; Cell differentiation; Thymus gland

INTRODUCTION

Invariant natural killer T (iNKT) cells are a population of specialized immune cells that express the characteristics receptor of both NK and T cells. The iNKT cells bear an invariant TCR (Vα24-Jα18/Vβ11 in humans, and Vα14-Jα18/Vβ8, Vβ7, or Vβ2 TCR in mice). They can recognize lipid antigenic

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as nonpolymorphic major histocompatibility complex (MHC) class I-like antigen-presenting molecule. Thus, they are considered to be able to "bridge innate and adaptive immunity".[1-6]

In the thymus, iNKT cell development describes a linear progression through four states, including stages 0 (CD24+CD44-NK1.1-), 1 (CD24-CD44-NK1.1-), 2 (CD24-CD44+NK1.1-), and 3 (CD24-CD44+NK1.1+). Depending upon the expression of distinct transcription factors and secretion of different cytokines, iNKT cells develop into three major terminally differentiated and functionally iNKT cell subsets. iNKT1 cells express the transcription factor T-bet and predominantly secrete IFN-γ; iNKT2 cells express the high levels transcription factor GATA-3 and promyelocytic leukemia zinc finger (PLZF) and secrete IL-4 and IL-13; iNKT17 cells express intermediate levels of PLZF and are positive for RAR-related orphan nuclear receptor gamma (RORγt) expression. They also secrete IL-17. iNKT2 and iNKT17 cells primarily differentiate in the thymus.[7-9] iNKT1 cells differentiate and mature in three stages before distribution, which mainly occurs in peripheral immune organs.[7-11] iNKT cells are most commonly found in the liver, bone marrow, thymus, spleen, and visceral adipose tissue,[12] while the distribution of iNKT cell subsets in different tissues is quite different. iNKT cells account for about 0.5% of thymocytes in the thymus, and about 5% of bone marrow T cells are iNKT cells. iNKT cells in mouse liver account for about 40% of lymphocytes,[13] mainly iNKT1 cells. Spleen iNKT cells account for about 2.5% of T cells, of which iNKT1 cells are located in the red pulp area and iNKT2 cells in the T cell area. Mesenteric lymph nodes are rich in iNKT2, and iNKT17 cells are mainly found in other peripheral lymph nodes.[14-15]

iNKT cells regulate immune cell differentiation and immune responses, with dual immunomodulatory functions. They exert a cytotoxic role in infections and tumors and have immunosuppressive functions in organ transplant rejection and a series of diseases such as type I diabetes, encephalomyelitis (EAE), multiple sclerosis (MS), and systemic lupus erythematosus (SLE).[16-19] Adoptive transfusion of iNKT cells in cellular immunotherapy could become a new strategy for treating tumors, infections, and various immune diseases. Although iNKT cells have important roles in immune regulation, their low in vivo levels limit their potential in clinical application.[20-23] In this research, iNKT cells were activated and expanded in the mouse thymus by subcutaneous injection of α-GalCer on the tail root and advanced directional induction in vitro, aiming to obtain large amounts of iNKT cells with a specific function and providing a basis for their use in immunotherapy.[23-25]

MATERIALS AND METHODS

Experimental Animals

A total of 90 male DBA/1 mice (6 weeks old; 16±1g), reared in a specific pathogen-free (SPF) environment, 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 20-25°C with 40–60% relative humidity and free access to water and food (regular diet). Under a 12 h/12 h light/dark cycle for at least 7 days before the experiment, animal activities were monitored daily to ensure that the animals met the experimental requirements. All experiments were approved by the Animal Welfare and Ethical Committee of Hebei University (IACUC-2017009).

Materials

KRN7000 (α-GalCer) was manufactured by ENZO Life Sciences (Farmingdale, NY, USA). BD Cytometric Bead Array (CBA) Mouse Cytokine Kit (560485), FITC-hamster anti-mouse TCR β chain (553170), PerCP-CyTM5.5-mouse anti-T-bet (561316), and Alexa Fluor 647-mouse anti-PLZF (563490) 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). KBM-551 media were from Corning Corporation. Fetal calf serum (FCS) was bought from Gibco. Penicillin and streptomycin were purchased from Invitrogen Corporation (USA). 2-mercaptoethanol was acquired from Amresco Corporation (Radnor, PA, USA). Human IL-2 was from Miltenyi Biotech (Miltenyi Biotech, GmbH, Germany); Recombinant Murine IL-4 was obtained from Peprotech (Rocky Hill, NJ 08553 USA); Bricyte E6 flow cytometry was purchased from Mindray (Mahwah, Mindray North America).

Experimental Grouping

A total of 90 DBA/1 mice were randomly divided into three groups, including the healthy control (27 mice, subcutaneous injection of 300 μL PBS at the base of the tail), α-GalCer I (45 mice, single subcutaneous injection at the tail of 2 μg α-GalCer) and α-GalCer II (18 mice, a total of three times, each time 2 μg α-GalCer, each 48 hours interval) group. In addition to detecting the changes of iNKT cells and their subgroups in the thymus, the α-Galcer I group was divided into several subgroups for direct induction of iNKT cells in vitro, including injection for 4, 6, 8 days (each subgroup 9 mice; the thymic single-cell suspension was obtained with a single injection of α-GalCer for 4, 6, 8 days, respectively) groups. The healthy control group also contained a subgroup (9 mice; the thymic single-cell suspension was obtained with subcutaneous injection of PBS for cultured in vitro). The experimental schematic is shown in Figure 1.

Isolation of Thymus Single-cell Suspensions and Direct Induction In vitro

Mice were anesthetized with an intraperitoneal injection of 50mg sodium pentobarbital per kilogram of body weight and humanely sacrificed by cervical dislocation while under deep anesthetic conditions. The skin surface was sterilized with 75% (vol/vol) ethanol. Mouse thymus was isolated using sterile surgical instruments in a sterile environment. The thymus was placed into a 200 mesh cell filter, which was immersed with 5 ml PBS solution in a petri dish. The thymus was dissected using a sterile scalpel, and the thymus fragment was gently mashed through the cell filter by a 2 mL syringe plunger and centrifuged at 1000 rpm for 5 min at 4°C. The thymic mononuclear cell was washed twice with PBS. Thymic mononuclear cells were resuspended with PBS to obtain a thymic single-cell suspension.

Thymus single-cell suspensions were prepared from mouse thymus harvested when a single injection of α-GalCer was given for 4, 6, and 8 days, respectively, plated in six-well plates. Cultured system: α-GalCer (100 ng/mL), IL-2 (100 IU/mL), IL-4 (25 IU/mL), IL-7 (5 ng/mL), vitamin C (1 μmol/mL) and β-mercaptoethanol (0.05 mmol/mL) were added and placed in a 5% CO2 incubator at 37°C. Every 4-5 days, cells were counted, readjusting cell density to 2×10^6 cells/ml. A fresh complete medium of KBM-551 (NK cell-specific, high protein content) containing penicillin, streptomycin, 15% FBS, as well as α-GalCer, IL-2, IL-4, and β-mercaptoethanol (as above) was added for further culture.

Fluorescence-activated Cell Sorting (FACS) for iNKT Cells and Subset Detection in the Thymus

iNKT cells were labeled as previously reported.[23-24] α-GalCer at 1 mg/mL was diluted to 200 μg/mL with 0.5% Tween-20 and 0.9% NaCl. Then, 5 μL of diluted α-GalCer per 100 μL of CD1d tetramer solution was added for incubation at room temperature for 12 hours. After α-GalCer injection, mouse thymus samples were obtained at 2, 4, 6, 8, 10, 12 days, respectively, for single-cell suspension preparation by homogenization. Lymphocytes were isolated with a lymphocyte separation medium and washed twice with PBS. Then, 1×10^6 cells were placed in each flow cytometry tube and treated with anti-TCR β- FITC and α-GalCer bound CD1d tetramer labeled by PE (each 2μLin 50μLPBS reaction system) for 30 minutes in the dark. After washing with PBS twice, the cells were resuspended in 500 μL PBS and assessed by FACS.

After incubation with anti-TCR β-FITC and α-GalCer bound CD1d tetramer labeled by PE, Foxp3/Transcription Factor Staining Buffer was added to cells for permeabilization and fixation.[24] Then, 5μLof PerCP-CyTM5.5 mouse anti-T-bet and 5 μL of Alexa Fluor 647-mouse anti-PLZF were added for incubation at room temperature in the dark for at least 30 minutes. After 2 washes with PBS, the cells were resuspended in 500 μL PBS and assessed by FACS. Each test analyzed 1×10^5 cells as the denominator for calculating the percentages of iNKT cells.

FACS Detection of In-vitro Changes of iNKT Cells and Subset Rates in Single-cell Suspensions from the Mouse Thymus

iNKT cells were collected at 7, 14, and 21 days when cultured and induced in vitro respectively, and washed twice with PBS. They were placed into flow cytometry tubes at 1×10^6 cells each. The detailed detection method was the same as above. After being stained and washed, the cells were finally resuspended in 500 μl PBS and assessed by FACS.

Cytokines Production was Detected by FACS

iNKT cells were cultured and induced for 14 days in vitro. They were transferred to fresh RPMI 1640

medium containing α-GalCer incubate for 48 hours. Culture supernatants were collected, and cytokines were detected (IFN-γ, TNF-α, IL-4, IL-6) by CBA Kit.

Statistical Analyses

All data are expressed as mean±SEM. Groups were compared by one-way analysis of variance (ANOVA) followed by the LSD test for pairwise comparisons. The unpaired t-test was to compare cytokine secretion between two groups. Data were analyzed using SPSS version 19.0. (SPSS Inc., Chicago, IL, USA). A *p* value of <0.05 was considered to be statistically significant.

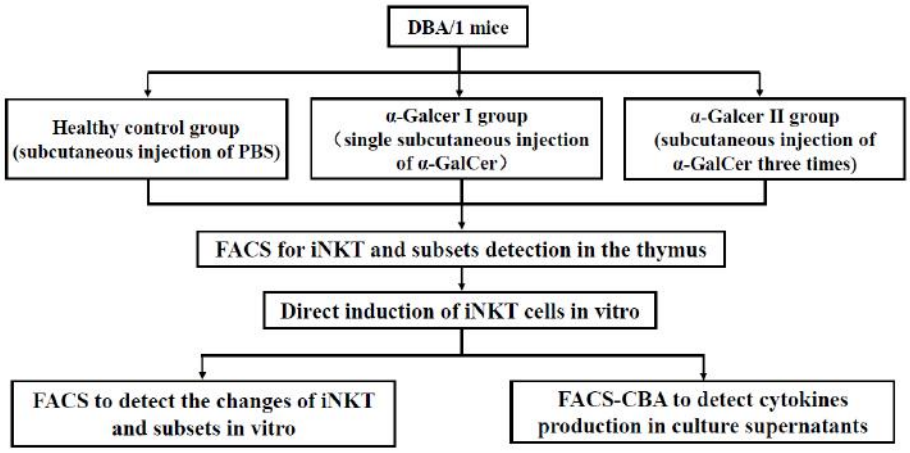

Figure 1. The experimental procedure

RESULTS

Changes of iNKT Cells in the Mouse Thymus after Subcutaneous Injection of α-GalCer

The iNKT cells frequency of α-GalCer subcutaneous injection groups was higher than the control group (p<0.05, 95% CI) (Figure 2, Table 1). The rate of iNKT cells in the mouse thymus gradually increased from day 2 to day 8 after a single subcutaneous injection of α-GalCer, reaching the peak at the 8th day (16%). After multiple subcutaneous injections of α-GalCer, the rates of iNKT cells in the thymus reached the peaked within 2 days (10%) (Figure 2, Table 1). Compared with the α-GalCer II group, the rates of iNKT cells in the α-GalCer I group were markedly higher at each time point (p<0.05, 95% CI) (Figure 2).

Table 1. Rates of invariant natural killer T (iNKT) cells in the mouse thymus (%)

Groups	Control	α-GalCerI	α-GalCerII
2d	3.89±1.06	9.02±0.88*	–
4d	4.07±0.94	12.18±1.38*$^{\#}$	–
6d	3.54±0.84	15.12±1.07*$^{\#}$	10.40±0.81*
8d	4.17±1.02	16.47±1.00* $^{\#}$	8.15±0.89*
10d	3.69±0.77	16.09±1.05* $^{\#}$	7.70±0.41*
12d	3.17±1.01	14.44±1.26*$^{\#}$	6.55±0.68*
14d	–	–	6.59±0.97*
16d	–	–	5.48±0.66*

*p<0.05 versus control group; $^{\#}p$<0.05 versus α-GalCer II group.

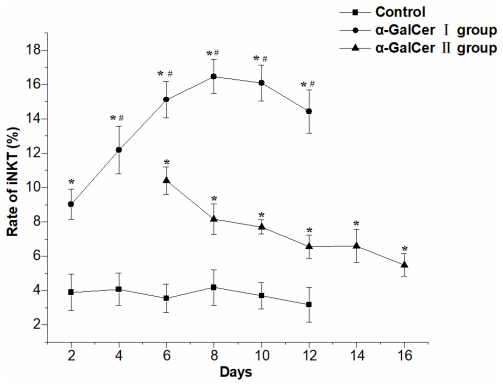

Figure 2. Changes of invariant natural killer T (iNKT) cell rate in the thymus of the alpha-galactosylceramide (α-GalCer) I, α-GalCer II, and control groups (each group n=18). α-GalCer I group, a single injection of α-GalCer, iNKT cells were detected after 2, 4, 6, 8, 10, and 12 days of α-GalCer injection, respectively. The α-GalCer II group was injected multiple times for a total of three injections, each time at 48 hours apart. iNKT cells were detected after 2, 4, 6, 8, 10, and 12 days of the third α-GalCer injection, respectively. It was possible to detect iNKT cells following the third injection of α-GalCer after 48 hours (equivalent to the sixth day of the experiment). *P<0.05 versus control group, #P<0.05 versus α-GalCer II group.

Changes of iNKT1 and iNKT2 Cells Subsets in the Thymus after Single-subcutaneous Injection of α-GalCer

The rates of iNKT1 cells in the thymus significantly increased from 2 to 4 days after a single subcutaneous injection of α-GalCer, and subsequently decreased gradually from day 4 to day 8, reaching the minimum at the 8th day (0.31%) and increasing from day 8 to day 12. Compared with the control group, the α-GalCer I group showed significantly increased rates of iNKT1 cells in the thymus at 2, 4, and 6 days, respectively (p<0.05, 95% CI). The iNKT1 cells subpopulation in the mouse thymus at 8, 10, and 12 days showed significantly lower rates (Figure 3A-C, Table 2). The rates of iNKT2 cells gradually increased from day 2 to day 8, and decreased from day 8 to day 12, reaching a peak at the 8th day (about 88%). In the α-GalCer I group, the percentage of iNKT2 cells was significantly increased at each time point compared with the control group (p<0.05, 95% CI) (Figure 3A, B, D, Table 2). Compared with the control group, the ratio of iNKT1/iNKT2 cells in each time point showed a marked increase in α-GalCer I group on day 2, day 4 (p<0.05, 95% CI) and decrease on day 8, day 10, and day 12 (p<0.05, 95% CI) (Figure 4).

Table 2. Rates of invariant natural killer T (iNKT) 1 and iNKT2 cells in the mouse thymus

Groups	Days	iNKT1(%)	iNKT2(%)
Control	2	3.77±1.13	13.15±2.82
	4	3.91±0.27	13.73±2.64
	6	4.01±0.36	12.69±2.42
	8	3.74±0.60	14.13±2.17
	10	3.95±0.88	13.79±1.77
	12	3.94±0.79	12.79±1.48
α-GalCerI	2	19.77±2.42*	21.70±2.09*
	4	26.34±0.72*	23.50±3.29*
	6	11.79±0.93*	34.41±4.06*
	8	0.31±0.52*	88.47±3.32*
	10	1.16±0.32*	22.55±3.92*
	12	2.35±0.78*	18.87±2.58*

*p<0.05 versus the control group

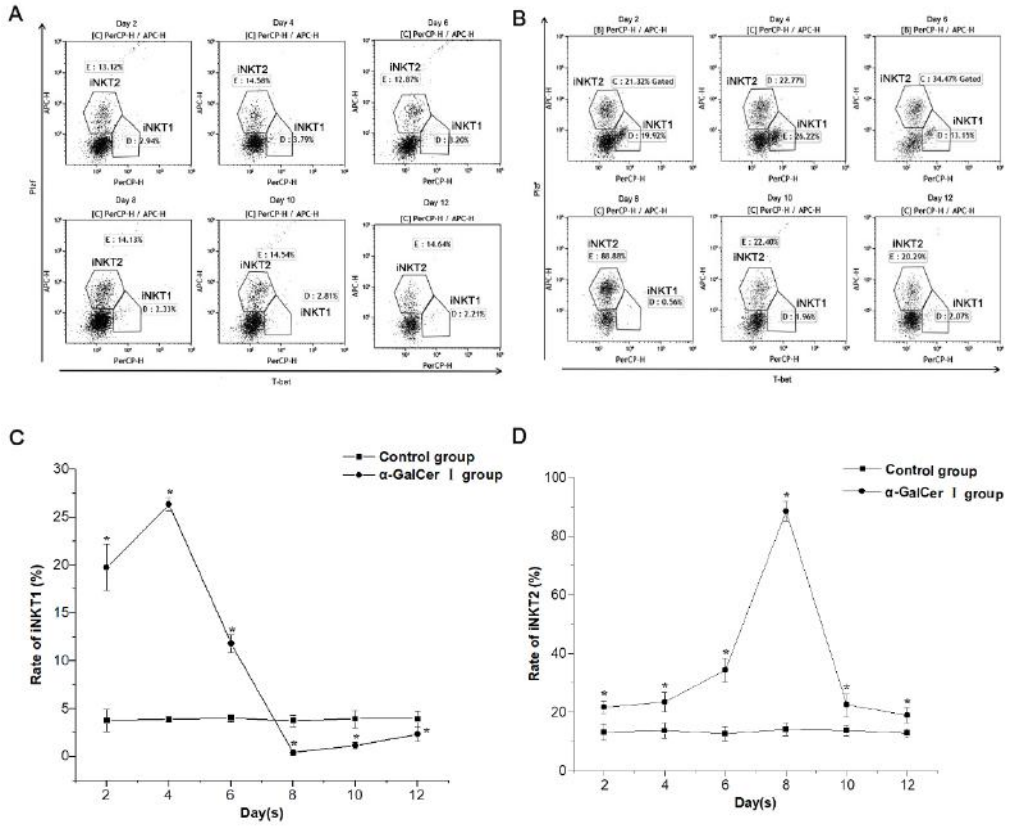

Figure 3. Changes in invariant natural killer T (iNKT) subpopulation rates in the alpha-galactosylceramide (α-GalCer) I group and control group. (A) Rates of iNKT1 and iNKT2 cells in the control group (n=18), as detected by FACS. (B) Rates of iNKT1 and iNKT2 cells in the α-GalCer I group (n=18), as detected by FACS. (C) Changes in rates of the iNKT1 cells subpopulation. (D) Changes in rates of the iNKT2 cells subpopulation. *p<0.05 versus control group; Day 2, injection of α-GalCer for 2 days; Day 4, injection of α-GalCer for 4 days; Day 6, injection of α-GalCer for 6 days; Day 8, injection of α-GalCer for 8 days; Day 10, injection of α-GalCer for 10 days; Day 12, injection of α-GalCer for 12 days. Data represent one of three experiments with similar results.

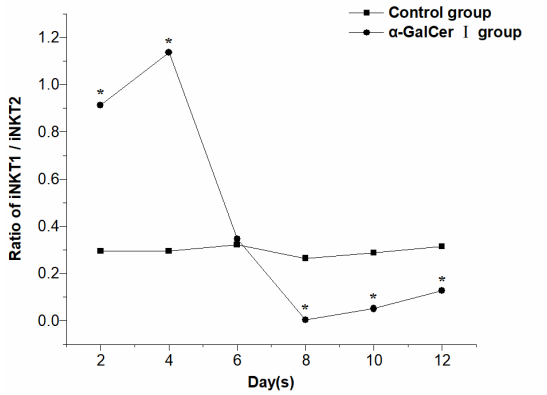

Figure 4. Ratios of iNKT1 to iNKT2 cells subpopulations in the mouse thymus between the alpha-galactosylceramide(α-GalCer) I group (n=18) and healthy controls (n=18). *p<0.05 versus control group;

Changes of iNKT Cells Cultured and Direct Induction In vitro

The rates of iNKT cells in each group all increased from day 0 to day 14 and decreased from day 14 to day 21, reaching a peak on day 14. The rates in each group were all significantly higher compared with the control group (p<0.05, 95% CI) (Figure 5, Table 3). In the group that was injected with α-GalCer for 8 days, and thymic mononuclear cells were cultured in vitro for 14 days, the rates of iNKT cells expanded significantly higher compared to the groups that were injected for 4 and 6 days (p<0.05, 95% CI) (Figure 5, Table 3).

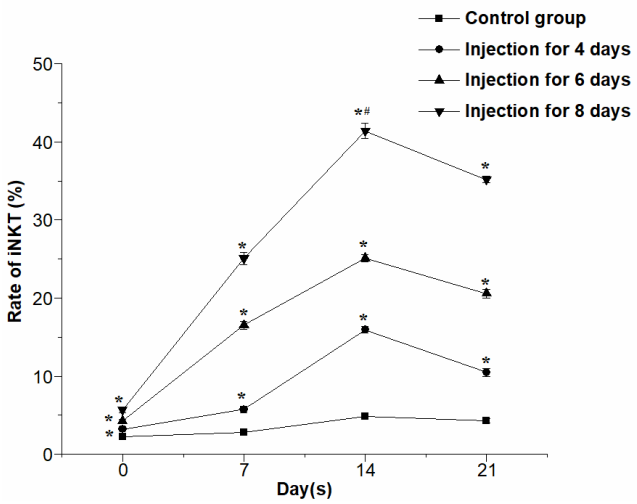

Figure 5. Changes of invariant natural killer T (iNKT) cells rates (%) after culture of mouse thymocytes in the single injection of alpha-galactosylceramide (α-GalCer) group (each subgroup n=12) and control group (n=12). *p<0.05 versus control group; $^{\#}p$<0.05 versus other groups and other incubation time groups

Table 3. Rates of invariant natural killer T(iNKT) cells in the mouse thymus, cultured in vitro (%)

Groups	Before cultured	Cultured for 7 days	Cultured for 14 days	Cultured for 21 days
control	2.24±0.37	2.85±0.08	4.87±0.28	4.28±0.27
Injection for 4 days	3.20±0.20*	5.74±0.46*	15.95±0.38*	10.49±0.49*
Injection for 6 days	4.29±0.24*	16.51±0.51*	25.12±0.50*	20.59±0.55*
Injection for 8 days	5.67±0.34*	25.09±0.77*	41.40±0.95* #	35.17±0.39*

*p<0.05 versus control group; $^{\#}p$<0.05 versus other groups and other incubation time groups.

Changes of iNKT1 and iNKT2 Cells Cultured and Direct Induction In vitro

The rates of iNKT cells were increased in the injection of the α-GalCer group compared with the control group at each time point when the thymic mononuclear cell was cultured for 14 days in vitro (p<0.05, 95% CI) (Figures 6 and 7). Consistent with the trend of iNKT cells, the percentages of iNKT1 cells in the injection of the α-GalCer group all reached a peak on day 14. The rates of iNKT1 cells in the injection of α-GalCer for 8 days group were significantly higher compared to other groups (about 94%) (p<0.05, 95% CI) (Figures 6, 7 and Table 4). At the same time, the levels of iNKT2 cells decreased in all groups, approaching zero and showing an irregular change.

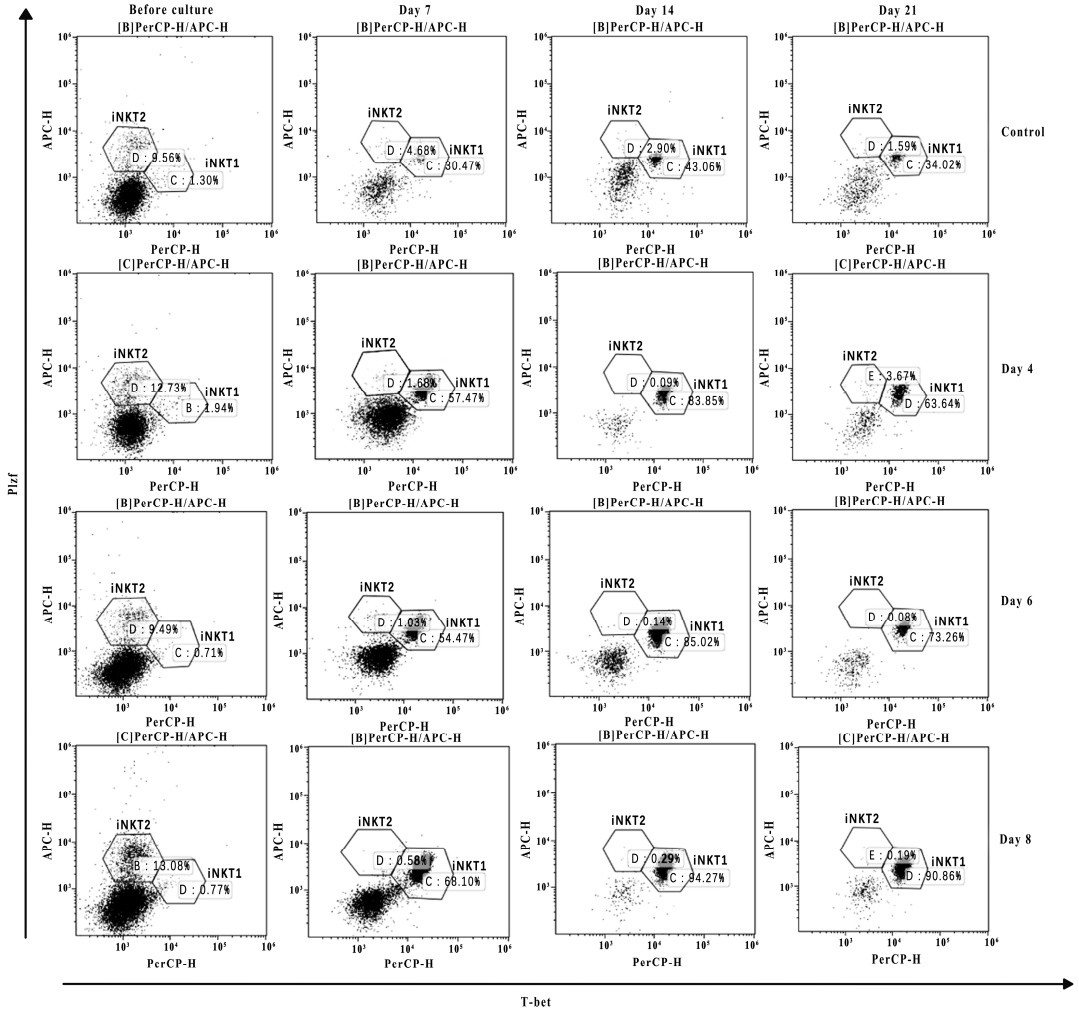

Figure 6. Flow cytometry analysis of invariant natural killer T (iNKT) cells subsets cultured in vitro in alpha-galactosylceramide (α-GalCer) single injection groups (each subgroup n=12) and control group (n=12). Day 4, injection of α-GalCer for 2 days; Day 6, injection of α-GalCer for 6 days; Day 8, injection of α-GalCer for 8 days; Day 7, cultured in vitro for 7 days; Day 14, cultured in vitro for 14 days; Day 21, cultured in vitro for 21 days; Data represent one of three experiments with similar results.

Table 4. Changes in rates of invariant natural killer T (iNKT1) cells of mouse thymocytes in the alpha-galactosylceramide (α-GalCer) single injection groups (each subgroup n=12) and control group (n=12) (%).

Groups	Before cultured	Cultured for 7 days	Cultured for 14 days	Cultured for 21 days
Control group	1.23±0.08	29.33±1.04	44.32±0.96	35.87±1.45
Injection for 4 days	1.80±0.13*	57.04±1.67*	81.95±1.40*	61.93±1.43*
Injection for 6 days	0.76±0.06*	53.00±0.92*	86.37±0.68*	71.10±1.32*
Injection for 8 days	0.87±0.06*	74.28±2.08*	94.70±0.69* #	88.36±1.50*

*p<0.05 versus the control group; #p<0.05 versus other groups and other incubation time groups.

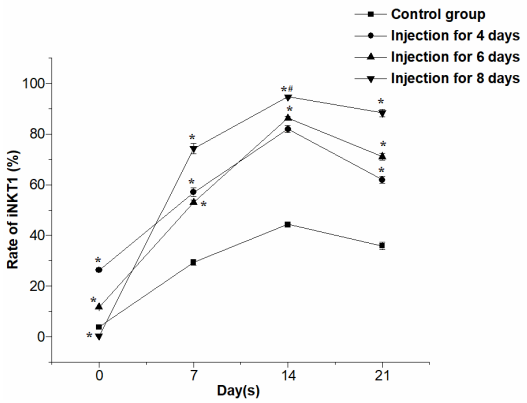

Figure 7. Changes in rates (%) of invariant natural killer T (iNKT1) cells subsets after culture of mouse thymocytes in the alpha-galactosylceramide (α-GalCer) and control groups. *p<0.05 versus control group; $^{\#}p$<0.05 versus other groups and other incubation time groups

Cytokines Detected by FACS

To confirm whether iNKT cells (injection of α-GalCer for 8 days group) cultured in vitro have biological functions, we detected cytokines derived from the culture supernatant of iNKT cells cultured in vitro for 14 days. The results of the cytokines experiment showed no difference in function between the cultured iNKT cells in vitro and the natural iNKT cells in vivo. Production of IFN-γ was consistent with the level of iNKT1 cells. The above data confirmed that the combination of α-GalCer stimulating iNKT cells in vivo with induced iNKT cells in vitro increased iNKT cells' levels and induced iNKT cells transformation into specific iNKT1 cells (Figure 8).

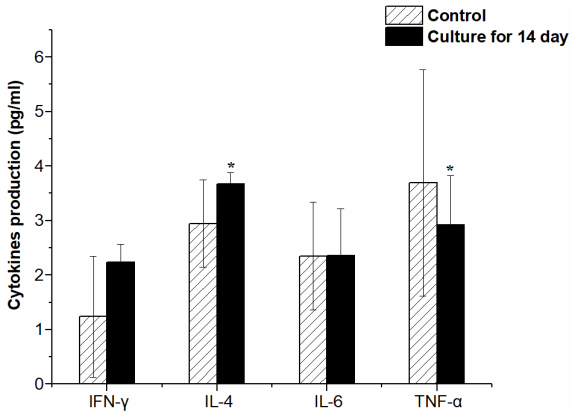

Figure 8. Cytokines of cultured supernatant were detected by CBA Kit. *p<0.05 versus the control group.

DISCUSSION

iNKT cells interact with other immune cells via direct contact, and through the secretion of different cytokines, they regulate the immune status of the body about the effect of the function of other immune cells. Therefore, iNKT cells are the center of the immune regulatory network.[16] Many experimental data have indicated that iNKT cells are associated with the development of rheumatoid arthritis, systemic lupus erythematosus, and allergic asthma. In addition, iNKT cells can control or improve related immune diseases, such as diabetes, tumors, and organ transplantation associated with complications. These contradictory

experimental results are presumed to be associated with iNKT cell function in different subpopulations.[26-31]

Our data showed that a single subcutaneous injection of α-GalCer had a better effect than multiple injections, which may be because α-GalCer affects dendritic cells (DC) maturation after multiple subcutaneous injections. On the other hand, over-activated iNKT cells would be in an inactive state for a long time. The changes of iNKT cells subsets in the thymus after a single subcutaneous injection of α-GalCer might be explained by the fact that iNKT cells were stimulated by α-GalCer and are restricted by the thymic microenvironment, whereas the iNKT2 cell subpopulation proliferates in large numbers and the iNKT1 cells subpopulation does not proliferate or migrate to settle in the periphery. Therefore, large amounts of iNKT2 cells could be obtained from the thymus at 8 days after a single subcutaneous injection of α-GalCer. Changes of iNKT cells cultured and direct induction in vitro showed that stimulating the proliferation and activation of iNKT cells in mouse thymus by injecting α-GalCer in vivo may positively influence the induction and expansion of iNKT cells in vitro. The rates of iNKT cells in the subgroup injected with α-GalCer for 8 days were significantly higher than in other subgroups, which is consistent with the trend of iNKT cells peaking at 8 days after single subcutaneous injection of α-GalCer. This suggested that 8 days after a single injection of α-GalCer is optimal for inducing iNKT cells proliferation and amplification in vitro. Thus, injecting α-GalCer for 8 days, isolating thymic mononuclear cells, then culturing and inducing expansion in vitro for 14 days can generate an optimal number of iNKT cells. Consistent with Parekh,[32] we obtained single-cell suspensions dominated by iNKT2 cells from mouse thymus after a single injection of α-GalCer, which transformed into cell populations dominated by iNKT1 cells after direct induction in vitro. We guess that iNKT2 cells at the developmental stage 2 when further cultured developed into stage 3 cells in vitro; another reason may be subpopulation transformation of iNKT2 into iNKT1 cells subpopulation due to α-GalCer. On the other hand, the iNKT2 cell subpopulation loses its proliferative capacity without the thymic microenvironment during culture in vitro, while mature iNKT1 cells at developmental stage 3 maintain theirs. The detailed mechanisms should be further investigated. Our study demonstrated that a higher number of iNKT cells could be obtained from the thymus after a single injection of α-GalCer for 8 days. The extracorporeal expansion level of iNKT cells was maximized on the 14th day after isolation of the thymocyte single-cell suspension.

Unlike previous studies that induced iNKT cell expansion, we first used α-GalCer stimulation in vivo to obtain most iNKT2 cell suspension in the thymus. However, when iNKT cells were further induced to proliferation in vitro, we found they turned into iNKT1 cells with specific functions. The expression of IL-4 and IL-4 receptors is a necessary condition for the development and function of NKT cells.[33-34] As a reductant, β-mercaptoethanol can reduce the damage of oxygen and promote cell proliferation.[35-36] Therefore, besides giving α-GalCer and IL-2, we also added IL-4 and β-mercaptoethanol in vitro culture system.

Govindarajan et al isolated spleen iNKT cells and purified them for in vitro culture, which could be amplified about 70-fold on day 18.[23] Chiba et al used spleen iNKT cells of Va14 T cell receptor (TCR) transgenic (Tg) mice to rapidly generate iNKT cell lines, which could be amplified 105-fold by repeated stimulation with α-GalCer within 8 weeks.[37] Although a large number of iNKT cells can be harvested, the operation is complicated and time-consuming. iNKT cells can be amplified more than 90 times by combining in vivo stimulation with in vitro induction, with levels of iNKT1cells improving from 0.3% to 94%. Both Johan W. Molling and Asako Chiba used dendritic cells to present α-GalCer, the exchange of information between cells contributed to the proliferation of iNKT cells.[37-38] Depending on detecting cytokines in the culture supernatant, iNKT cells induced by our method have the same biological functions as nature iNKT cells in normal mice, as they can secrete Th1 and Th2 type cytokines and have complete biological functions, which is extremely important for future applications of iNKT cells.

In this experiment, high levels of iNKT cells with specific functions were obtained from the mouse thymus by a combination of α-GalCer stimulation in vivo and induced amplification in vitro. We provided a very important research idea and research approach for the large-scale acquisition of iNKT cells in vitro and a possibility for treatment of related diseases by adoptive immunotherapy of iNKT cells.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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