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Chaohong Liu *Editor*

Invariant Natural Killer T-Cells

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Edited by

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Preface

The invariant natural killer T-cells (iNKT cells) contribute to homeostasis and autoimmunity and also can cause various pathological responses such as allergy, infection, excessive autoimmune response, and cancer. Without antigen stimulation or requiring clonal expansion, iNKT cells directly produce a large number of cytokines when activated. They differentiate into at least three effector subsets NKT1, NKT2, and NKT17 in thymus and express an invariant T-cell receptor (TCR), α-actin, and variable (TCR) β-chain. iNKT cells are presented by MHC class I-like molecule CD1d and express TCRs with a unique Vα14-Jα18 rearrangement, which is special in recognizing glycolipid antigens including alpha-galactosylceramide (α-Galcer). When stimulated by agonistic lipid α-Galcer, the continuous expression of cytokines enables iNKT cells to engage in innate and adaptive immune response.

During the past few years, the diversity of iNKT cell functional subsets is not unveiled like other lymphocytes because of limitations in techniques. Nowadays, with the development of all kinds of high-resolution microscopy, flow cytometry, and the advance of gene analysis technology, the function of iNKT cells and the technique of analyzing have been improved gradually as well as with other lymphocytes. This volume on *Invariant Natural Killer T-Cells* focuses on various aspects of iNKT cells. Chapters 1 and 2 focus on the analysis of genes and their ligands of iNKT cells. Chapter 3 shows protocols to learn the interactions between iNKT cells and viruses. Chapters 4–7 reveal methods of iNKT cell identification and isolation in different organs of mice and humans. Chapters 8–10 outline the procedures to study iNKT cell activation and to study the activation and transformation of iNKT cells by using flow cytometry. Chapters 11–13 establish the procedures to study iNKT cell proliferation and differentiation. Chapter 14 uses intravital confocal microscopy to observe the dynamic activities of invariant natural killer T-cells in the liver of CXCR6$^{GFP/+}$ transgenic mice. Chapters 15 and 16 focus on α-GalCer transformation technology to observe in vivo cytotoxicity and the usage of α-GalCer/CD1d-scFv fusion protein technology to redirect iNKT cells for antitumor purposes. Chapter 17 characterizes the dynamic changes in metabolic profiles associated with iNKT cell development.

I have tried to give you an idea about the current advanced protocols that help with the study of iNKT cells. This volume might not provide a big picture of current research progress in iNKT cells. From my point of view, we can still make greater progress in studying iNKT cells. These years of rapid progress in this field are just the beginning. Further research on iNKT cells will help us have deeper knowledge of the human immune system. I hope the protocols published in this volume can help those immunologists who devote themselves to studying iNKT cells. I am very thankful to all contributors for their time and experience, as well as their great suggestions, in finishing this volume. The series editor John Walker has provided great help for publishing these protocols. Finally, the staff from Springer and Han Li also provided great help for editing this volume.

Wuhan, China ***Chaohong Liu***

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Chapter 1

Genetic Analysis of *i*NKT Cell Development and Function

Julie Sahler, Orchi Anannya, Candice Limper, Brian Imbiakha, Tim Pierpont, and Avery August

Abstract

Natural killer T (NKT) cells are among the immediate and early responding immune cells and are important players in autoimmune diseases and tumor immunity. This unique subset of T cells shares properties of natural killer cells and T cells. Proper identification and characterization of NKT cell subsets is essential to understand the function and involvement of these understudied immune cells in various diseases. This review aims to summarize the known methods for identifying and characterizing NKT cells. NKT cells are divided into Type I (or invariant) and Type II, with either limited or broad TCR repertoires, respectively, that generally respond to glycolipids presented on the nonclassical MHC, CD1d. Type I NKT cells or invariant NKT cells (*i*NKT) are the most well studied and can be further subdivided into NKT1, NKT2, or NKT17 populations, classified based on their functional capacity. Conversely, less is known about Type II NKT cells because they have a more diverse TCR repertoire which make them hard to identify. However, genetic analyses have shed light on the development and function of all NKT subsets, which aids in their characterization. Further exploration of the role of NKT cells in various diseases will reveal the intricacies and importance of their novel functions.

Key words α-Galactosyl ceramide, Natural killer T cells, CD1d, CD1d/α-galactosyl ceramide tetramers

1 Introduction

Natural killer T (NKT) cells are found everywhere that conventional T cells are found. In mice, NKT cells represent ~30% of the total lymphocytes in the liver (~50% of α/β T cell receptor (TCR)$^{+}$ T cells), ~20% of the αβ T cells in the bone marrow, ~3% of the αβ T cells in the spleen, and very small percentage in the lymph node [1–3]. The most striking effector function of an activated NKT cell is

Julie Sahler, Orchi Anannya, Candice Limper, Brian Imbiakha, Tim Pierpont, and Avery August contributed equally to this work.
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their ability to rapidly produce large amounts of IL-2, IL-4, IFN-γ, and TNF-α. NKT cells appear to be pivotal for immediate responses to infection, prevent certain autoimmune diseases, and the immune response to cancer. NKT cells can respond to glycolipid ligands such as sulfatide, β-galactosyl ceramide, phosphatidylglycerol, phosphatidylinositol, and diphosphatidylglycerol presented by the MHC-1-like molecule CD1d. While the natural ligand presented to NKT cells by CD1d in vivo has previously been unclear, and thought to be a glycosylphosphatidylinositol, isoglobotrihexosylceramide (iGb3) have been reported as an endogenous ligand for these cells [4, 5].

Since NKT cells have a variety of lineages, each with their own developmental process, markers, and functions, accurate identification and assessment of function is essential to determine the role of NKT cell in disease. The aim of this review is to provide a brief description of the main NKT cell populations, describe how they are extracted, define identification markers, and highlight important genes expressed during their development and activation. Since much of the work on NKT cells has been performed in mice, the information presented here will be referring to this model unless otherwise specified.

NKT cells are divided into two primary groups: Type I and Type II, respectively, carrying limited or diverse Vα chain variants of their TCR. Type I cells (also known as *i*NKT) are the most well studied and can be further subdivided into NKT1, NKT2, or NKT17 populations, each with different effector functions. Regardless of their differences, they all utilize their TCRs to respond to a limited array of lipids, including a particular synthetic glycolipid α-galactosyl ceramide (α-GalCer), which is not recognized by conventional T cells [6]. This molecule, extracted from marine sponges, has been identified as a specific ligand for both mouse and human NKT cells and has been used in complex with the CD1d as tetramers to identify *i*NKT cells. While Type II NKT cells can respond to a variety of ligands presented on CD1d, they express a more diverse range of TCRs and so are not identified by α-GalCer/CD1d tetramers and therefore are less well studied. Here we describe how to extract and identify these NKT subsets from mice.

1.1 General Development of NKT Cells

In addition to the invariant TCR, most NKT cells express NK1.1 (NKRP1C in human), and DX5, and mature NKT cells express Ly49 and are $CD44^{Hi}/CD69^{+}/CD49b^{-/Lo}$ and produce large amounts of IL-4 and IFN-γ upon activation [7, 8]. NKT cells, like conventional T cells, arise from thymocyte progenitor cells. Currently it is unclear how early prior to TCR expression progenitor cells become committed to the NKT lineage, since they are identified by their TCR specificity. Following TCR expression, CD1d restricted cells can be identified, based on the ability to bind α-GalCer/CD1d tetramers, even prior to the expression of

NK1.1 [9]. Based on expression of the cell surface marker DX5, developing CD1d-reactive NKT cells have been divided into four stages. The first two stages are immature, with the most immature stage lacking both DX5 and NK1.1. These cells express $CD4^+$, as do the cells in the next stage, which are $DX5^+/NK1.1^-$. Mature NKT cells are divided almost equally into $DX5^+/NK1.1^+$ and $DX5^-/NK1.1^+$ [7]. In contrast to conventional T cells, which can develop in the fetus and are present at birth, NKT cells are not found until approximately 5 days postpartum in mice [10].

1.2 Type I NKT Cells or *i*NKT Cells

Type I or *i*NKT cells express an invariant TCR α chain Vα14Jα18 and a β chain with limited variability (mostly Vβ8.2, Vβ7 or Vβ2) and recognize the prototypical antigen α-GalCer [11]. In humans, these cells express Vα24-JαQ, paired with a Vβ11 β-chain, and are also referred to as Vα14 NKT cells [2]. In the mouse where *i*NKT cell development has been the most studied, developing *i*NKT cells are positively selected from the CD4/CD8 double-positive cells by the MHC molecule CD1d, which presents glycolipid complexes [12]. A number of genes, including transcription factors, signaling molecules, and receptors, have been identified as being important for the development of *i*NKT cells. Many of these genes regulate both NK and T cell development and function, confirming the view that NKT cells branch from the developmental pathway of conventional T cells at the $CD4^+CD8^+$ stage following the emergence of those carrying the Vα14Jα18 TCR [13]. These genes include CD132 (the common gamma chain receptor for IL-2, IL-4, IL-7, and IL-15) mutation of which results in reduced numbers of B, T, NK, and NKT cells. Mutations in CD132 are also responsible for X-linked severe combined immunodeficiency in humans [13]. Mutations in other genes such as Rag1/2, Notch, or the NF-κB axis (RelB or NIK), as well as genes for lymphotoxin, CD122/IL-15 axis, and LFA-1, SLAMf1, and SLAMf6, also directly affect NKT cell numbers and function [13, 14]. Targeted deletion of the genes for CD1d and the cytokine GM-CSF also result in an almost complete loss of NKT cells [13]. Targeted mutations in the signaling molecules Itk, Fyn, SAP, and Vav1 affect NKT cell differentiation and function [7, 14]. Knockout of the genes for transcription factors Ets, Mef, Irf1, and Ikaros, E, and Id proteins, or the transcriptional repressor NKAP, indicates that they are important in *i*NKT subset differentiation [13, 15–17]. The ability to identify specific genes associated with NKT cell development and function allow the use of specific approaches to identify the developmental stages.

*i*NKT cells undergo positive selection by homotypic interaction of self-lipids presented by CD1d with the TCR, along with the co-stimulation of SLAM family of receptors [18, 19]. The high signaling that results from the CD1d-TCR interaction in presence of SLAM receptor ligation allows high expression of transcription

factor Egr2, thereby enhancing transition into post-positive selection stage 0 *i*NKT cells ($CD24^{Hi}$, $CD69^{Hi}$) [20–23]. The increased expression of Egr2 subsequently allows expression of the TF PLZF, defined as the stage 1 *i*NKT cell ($CD24^{Lo}$ $CD44^{Lo}$) [24–28]. Further rounds of cell division and expansion leads to transition into stage 2 *i*NKT cells ($CD24^{Lo}$ $CD44^{Hi}$ $NK1.1^{-}$) and stage 3 ($CD24^{Lo}$ $CD44^{Hi}$ $NK1.1^{+}$) [26–28]. Stage 2 and 3 of *i*NKT cells can be exported to the periphery where they terminally differentiate into *i*NKT (PLZF ^{Lo}), *i*NKT ($PLZF^{int}$), and *i*NKT ($PLZF^{Hi}$) lineages, although there is plasticity within these subsets [26–28]. The stage 2 *i*NKT cells can differentiate into *i*NKT2 and *i*NKT17 subsets characterized by expression of the TFs GATA3 and RORγt, respectively [28–30]. The stage 3 *i*NKT cells differentiate into the *i*NKT1 subset characterized by expression of the TF Tbet, although an alternate mechanism for *i*NKT1 cells include direct differentiation from CD4/CD8 double negative CD25/CD44 double negative (DN4) thymocytes [28–30]. Similar to their conventional T cell counterparts, *i*NKT1 cells produce IFNγ, *i*NKT2 cells produce IL-4 and IL-13, while *i*NKT17 cells produce IL-17 and IL-22 [28–30]. The transcriptional regulation of these sublineages has been increasingly investigated to identify additional transcription factors that regulate their development and function. These studies have shown that Id2/3, ThPOK, Pak2 and Hdac3 are important for differentiation into *i*NKT1, and that the function of LEF1 and NKAP are important for the differentiation of *i*NKT2 and *i*NKT17 cells respectively [15–17, 28, 31–36]. Furthermore, an inverse relationship in expression of select transcription factors were observed in these *i*NKT cell subsets such that while *i*NKT1 cells are $Id2^{Hi}$ $Id3^{Lo}$ $PLZF^{Lo}$, *i*NKT2 cells are $Id2^{Lo}$ $Id3^{Hi}$ $PLZF^{Hi}$, and *i*NKT17 cells are intermediate for these transcription factors [15–17, 28, 31–36] (Fig. 1).

1.3 Type II NKT Cells

Like Type I *i*NKT cells, Type II NKT cells are CD1d restricted [37, 38]; however, unlike Type I *i*NKT cells, Type II NKT cells do not have a unique marker (nor do their TcRs bind to CD1d/α-GalCer tetramers that allow identification, like the Type I *i*NKT cells) capable of defining the cell subsets. Thus, studies of Type II NKT cells use an indirect approach to determine the presence or role of these cells in immunity. $J\alpha 18^{-/-}$ and $CD1d^{-/-}$ mice have been used as models to study the effects of type II NKT cells on infections [39]. $J\alpha 18^{-/-}$ mice are not able to generate a Vα14-Jα18 TCR chain, thus they lack Type I NKT cells while $CD1d^{-/-}$ mice lack both Type I and II NKT cells. Differences in the phenotypes in these mice following infections help infer the role of Type II NKT cells. As discussed previously, a unique feature that allows for the detection of Type I *i*NKT cells is that they bind to the α-GalCer-loaded CD1d tetramer [38]. While Type II NKT cells do not respond to α-GalCer presented on CD1d, different subsets

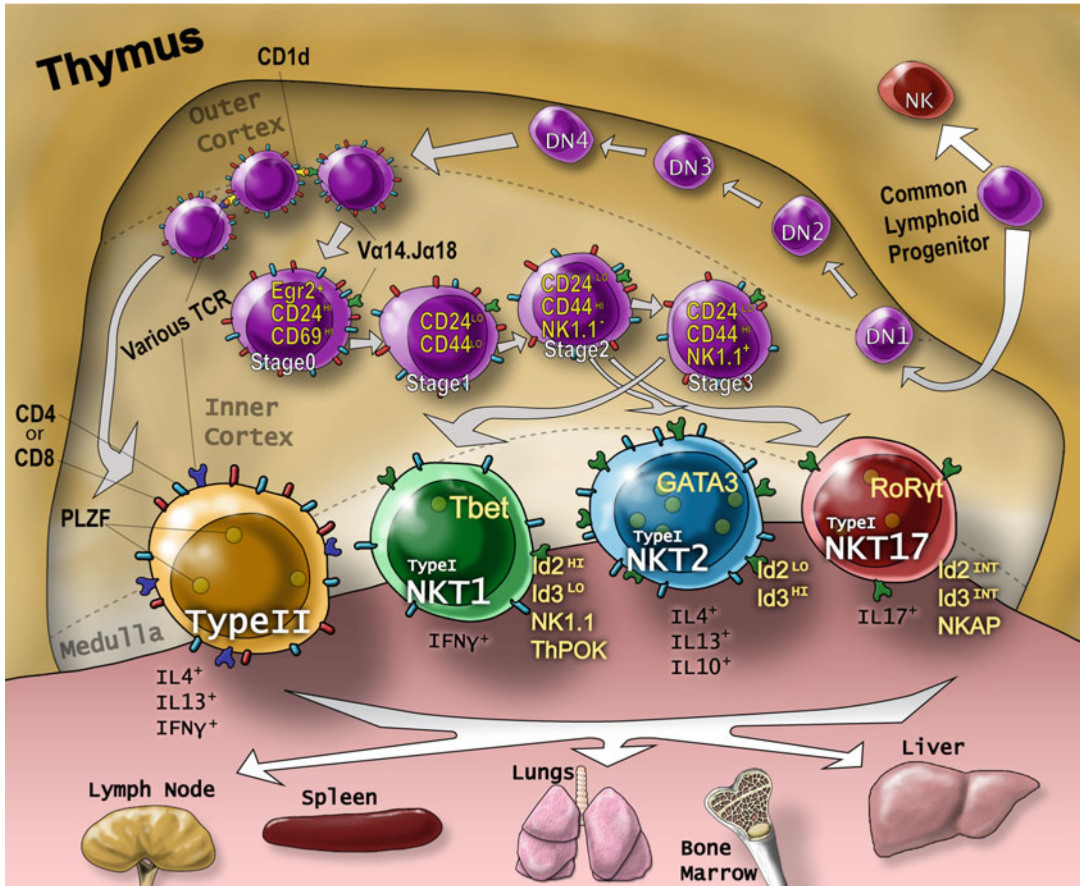

Fig. 1 NKT cells develop from common lymphoid progenitor cells that enter the thymus and undergo canonical DN1-DN4 maturation. At the $CD4^+/CD8^+$ double-positive (DP) cell stage, T cells expressing specific TCR variants undergo activation and selection by nonclassical MHC CD1d-expressing DP cells presenting endogenous glycolipids, rather than by dendritic cells as is the case for conventional T cells. DP cells expressing the invariant TCR (Mouse) [(V)Vα14-Jα18 (J)Vβ8.2, Vβ7, Vβ2] (Human) [(V)Vα24-JαQ, (J)Vβ11] become stimulated to differentiate toward **Type I NKT** cells which are defined as either **NKT1** ($PLZF^{Lo}$, $Id2^{Hi}$, $Id3^{Lo}$, $Tbet^+$, $CD4^{Lo}$, $TCRB^{Lo}$, $IFN\gamma^+$, $NK1.1^+$, $CD122^+$, $IL\text{-}4^+$), **NKT2** (PLZ^{Hi}, $Id2^{Hi}$, $Id3^{Lo}$, $GATA3^+$, $CD4^+$, $TCRB^{Hi}$, $IL\text{-}4^+$, $IL\text{-}13^+$, $IL\text{-}10^+$, $PD\text{-}1^+$), or **NKT17** ($PLZF^{int}$, $Id2^{int}$, $Id3^{int}$, $ROR\gamma t^+$, $CD4^-$, $TCRB^{int}$, $IL\text{-}17^+$, $CD138^+$, $CCR6^+$) through a common progenitor, stage 2 iNKT ($CD24^{Lo}$, $CD44^{Hi}$, $NK1.1^-$). DP cells expressing a diverse range of CD1d-restricted TCRs can be similarly stimulated to become **Type II NKT cells** ($CD4^{+/-}$, $CD8^+$, $IL\text{-}4^+$, $IL\text{-}13^+$, $IFN\gamma^+$)

react to different ligands including sulfatide, β-galactosylceramide, phosphatidylglycerol, phosphatidylinositol, and diphosphatidylglycerol presented on CD1d [40, 41]. Type II NKT cells are also characterized by the production of IL-4 and IL-13 and the expression of PLZF [42]. These $IL\text{-}4^+$, $IL\text{-}13^+$, $PLZF^{int}$ Type II NKT cells have been suggested to differentiate from the $IL\text{-}4^+$, $IL\text{-}13^+$ $PLZF^+$ Type I NKT2 subset.

1.4 Detection of NKT Cells

There are a number of different markers used to determine the number, percent, and functional characteristics of murine NKT cells. The following protocol can be used to detect Type I and II. Regardless of the NKT cell type of interest, the majority of the procedure will remain the same. However, there will be some variation in the "cell collection" section because lymphocyte extraction varies by organ and tissue type. Carry out the following procedures on ice unless otherwise specified.

2 Materials

RPMI Media.

- Heat-inactivated FBS (65 °C for 30 min).
- Sodium pyruvate.
- Non-essential amino acids.
- 0.5% HEPES.
- L-glutamine.
- Penicillin/streptomycin.
- 6 well plates.
- 96-well V-bottom plate.
- 15 mL tubes.
- Bucket containing ice.
- Dissection board.
- Lympholyte-M.
- 23-gauge needle.
- PBS.
- Collagenase A.
- Dispase II.
- gentleMACS tubes.
- 100 μM cell strainer.
- 50 mL tubes.
- 5 mL serological pipettes.
- Pipette controller.
- Pins.
- Tweezer.
- Scissors.
- 70% ethanol.
- Spray bottle.
- 70 μm cell strainer.
- 10 mL syringe (for the plunger).
- Centrifuge (able to spin to 1000 RCF).
- Flow cytometer tubes.
- Flow cytometer.
- Aspirator.
- Antibodies (*see* Table 1).

Table 1
List of antibodies used to detect various developmental stages of *i*NKT cells and types based on identifiable surface markers, transcription factors, and cytokine profiles for respective tissues

Natural killer cell		Surface markers	Transcription factors	Cytokines	Tissues	References
Early development stage(s)	0	$CD4^+8^+$ $CD24^{Hi}$ $CD69^{Hi}$			Thymus	[20–23]
	1	$CD4^+8^+$ $TCR\beta^+$ $CD24^{Lo}$ $CD44^{Lo}$	$EGR2^+$ $PLZF^+$		Thymus	[24–28]
	2	$CD4^+8^+$ $TCR\beta^+$ $CD24^{Lo}$ $CD44^{Hi}$ $NK1.1^-$			Thymus	[26–28]
	3	$CD4^+8^+$ $TCR\beta^+$ $CD24^{Lo}$ $CD44^{Hi}$ $NK1.1^+$			Thymus	[26–28]
Type I						
NKT1		CD1d $tetramer^+$ $CD4^+$ NK1.1	$PLZF^{Lo}$ $Tbet^+$ $Id2^{Hi}$ $Id3^{Lo}$ $ThPOK^+$	$IFN\gamma^+$ $IL4^+$	Liver, spleen, thymus	[8, 9, 15–17, 28, 31–36] [28–30]
NKT2		CD1d $tetramer^+$ $CD4^+$	$PLZF^{Hi}$ $GATA3^+$ $Id2^{Lo}$ $Id3^{Hi}$	$IL4^+$ $IL13^+$ $IL10^+$	LN	[9, 28–30] [9]
NKT17		CD1d $tetramer^+$ $CD4^-8^-$ $CD138^+$ $CCR6^+$	$PLZF^{Int}$ $Id2^{Int}$ $Id3^{Int}$	$IL17^+$ $IL22^+$	Lung, LN	[9, 28–30] [15–17, 28, 31–36]
Type II		$CD4^{+/-}$ $CD8^+$	$PLZF^{Int\text{-}Hi}$	$IL4^+$ $IL13^+$ $IFN\gamma^+$	Unclear	[42]

3 Methods

3.1 Prepare Solutions

1. Make media solution to be used for resuspension of isolated single cells from specific: RPMI base, 10% heat-inactivated FBS (65 °C for 30 min), 1 mM sodium pyruvate, 1 mM non-essential amino acid, 0.5% HEPES, 1 mM L-glutamine, and 100 U/mL penicillin/streptomycin.
2. Insert one 70 μM filter per well of a 6 well plates.
3. Add 2 mL of media to each well (*see* **Note 1**).
4. Required antibodies (*see* Table 1).

3.2 Cell Collection

1. Place 6 well plate in ice bucket.
2. Euthanize the mouse according to IACUC-approved method (this usually entails the use of carbon dioxide and/or cervical dislocation).
3. Pin mouse paws to a dissection board with ventral side facing up.
4. Sterilize mouse fur using 70% ethanol with a spray bottle.
5. Lift up the mouse skin with tweezers with your nondominant hand and make an incision with your scissors and cut all the way up to the lower jaw and pin the skin down (*see* **Note 2**).
6. Isolate lymphocytes from organ(s):
   (a) Thymus.
      - The thymus is above the heart; it appears white and has two lobes. Pluck the thymus and immediately place it in a well of the 6 well plate on ice.
      - Isolate lymphocytes from the thymus by gently dissociating it through a 70 μm cell strainer using the seal portion of a 10 mL plunger.
      - Use the ice bucket to slant the plate at a 10° so the media pools preferentially at the bottom of the well.
      - With a 1000 μL pipette, pull 1000 μL of the cell suspension and wash the well to collect the majority of cells.
      - After most of the cells are pooled, filter them through the filter three times to remove large tissues.
      - Collect the samples into a 15 mL tube.
      - Bring the volume of each sample to 2 mL with complete media and count the number of cells.

   (b) Liver.
      - Obtain 10 mL syringe and 23-gauge needle and flip the liver lobes.

- Inject 10 mL of PBS in the hepatic vein; this will cause it to turn white.
- With the same needle, inject 2–5 mL 0.05% collagenase A and dispase II.
- Place the liver in gentleMACS tube with 3 mL of complete medium and dissociate tissue with gentleMACS dissociator machine or follow procedure as aii-iv and then go to step bvi using a 100 μM filter.
- Place 100 μM filter on top of a 50 mL tube and filter homogenized tissue through the filter and rinse the filter with 5 mL of 2% FBS in PBS.
- Spin tubes at 400 RCF, for 5 min, and then aspirate supernatant.
- Resuspend the pellet in 10 mL PBS.
- Separate the cell suspension by equal parts into two 15 mL tubes.
- Slowly add 5 mL of lympholyte M to the bottom of each tube with a serological pipette and pipette controller while slowly pulling out of the liquid as it is being dispended.
- Centrifuge at 1200 RCF at room temperature for 20 min, 5 accelerate, and 0 decelerate settings.
- Extract the interface layer (around 5 mL) with a 1000 μL pipette and transfer into a 15 mL tube until the cloudy layer has been completely removed.
- Centrifuge 800 RCF for 10 min and aspirate the buffer while not disturbing the cell pellet.
- Resuspend the pellet into 1 mL of complete media and count the number of cells.

7. In a 96-well V-bottom plate and add desired cell number per well, plate extra wells for single cells controls (one well for each antibody color).
8. Make surface staining master mix: most antibodies can be stained at 1 μL:200 μL (antibody:PBS), but this may vary depending on the source of product. Make enough antibody mixture so that each sample will have 35 μL per well. The single cell controls can be stained with 100 μL of PBS with 0.5 μL antibody stock.
9. Pellet cells while in the plate in a centrifuge with 1000 RCF for 1 min.
10. Remove the media while being careful not to disrupt the cell pellet.
11. Aliquot 35 μL of the antibody master mix to each well. Resuspend the cells in the staining mixture.

12. Incubate the cells for 20–30 min at room temperature and away from light (*see* **Note 3**).
13. Resuspend cells with 200 μL of 1× PBS and centrifuge 1000 RCF for 1 min.
14. Remove the media while being careful not to disrupt the cell pellet.
15. Aspirate media off and add 250–300 μL to each well.
16. Resuspend cells in 250–300 μL of 1:1 ratio of PBS and media.
17. Transfer the cells into a flow cytometer tube and run the samples within 2 days' time (*see* **Note 4**).

4 Notes

1. The volume of media you will add will vary depending on the organ of tissue of interest.
2. The initial incision cut may vary depending on your organ or tissue of interest.
3. Make sure to stain the cells at the same time point between various experiments.
4. After cells are extracted from the mouse, the quality of the cells decrease over time, impacting the staining and subsequent analyses. It is best to run samples the same day of collection and wait no longer than the second day.

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Chapter 2

Genetic Studies of Natural Glycosphingolipid Ligands for NKT Cells

Dapeng Zhou, Chengfeng Xia, Peng G. Wang, Zhiyuan Li, Wenpeng Zhang, Guanghui Ni, and Jiaxi Cheng

Abstract

Glycosphingolipids (GSL) are natural ligands of NKT cells. Several laboratories have reported the in vitro activity of isoglobotriosylceramide (iGb3) in stimulating NKT cells. However, the knockout mice of iGb3 synthase showed no deficiency in development and function of NKT cells. There is a lack of knowledge on the genetics of redundant natural glycosphingolipid ligands. We have identified additional glycosphingolipid with stimulatory activity to NKT cells, including fucosyl lactosylceramide (H antigen). Here we describe the procedures to generate mice with deficiencies in Fut1, Fut2, and Sec1 genes to deplete H antigen through BAC engineering for the generation of ES cell-targeting construct, as well as the mice with deficiency of both blood group H-GSL ligand and isoglobotriosylceramide.

Key words Blood group H, α-2-Fucosyltransferase, Isoglobotriosylceramide, Natural killer T cells

Abbreviations

Blood group A	GalNAc α3 Fuc α2 Gal β
Blood group B	Gal α3 Fuc α2 Gal β
Blood group H	Fuc α2 Gal β
CFG	Consortium of functional glycomics
Fut1	α-2 fucosyltransferase I
Fut2	α-2 fucosyltransferase II
NKT	natural killer T cells
Sec1	α-2 fucosyltransferase III

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1 Introduction

Invariant natural killer T (iNKT) cells restrictedly recognize glycolipid antigens presented by the major histocompatibility complex class I-like protein CD1d [1–4]. The iNKT cells express natural killer receptors and a conserved, semi-invariant T-cell antigen receptor (TCR) composed of an invariant variable (Vα14)-joining (Jα18) chain (Vα24-Jα18 in human) combined with a limited but not invariant TCRβ repertoire dominated by Vβ8.2, Vβ7, or Vβ2 (Vβ11 in human) [5]. The distinguishing feature of iNKT cells is their capacity to rapidly secrete copious cytokines within minutes to hours of antigenic stimulation. Because of this characteristic release of both T helper 1 (Th1) and Th2 cytokines, including interferon-γ (IFN-γ), interleukin-4 (IL-4), IL-10, IL-12, and IL-13, iNKT cells play a unique role in protection against infection, suppression of autoimmunity, and increase of antitumor immunity and a significant role in allergic airway inflammation and some other immune system-related diseases and symptoms [6, 7].

The glycosphingolipid α-galactosylceramide (α-GalCer, Fig. 1) was originally discovered in an extract from a marine sponge [8, 9] and was found to strongly activate iNKT cells at very low concentrations. The salient structural feature that distinguishes α-GalCer from mammalian glycoceramides is the α-linkage between the

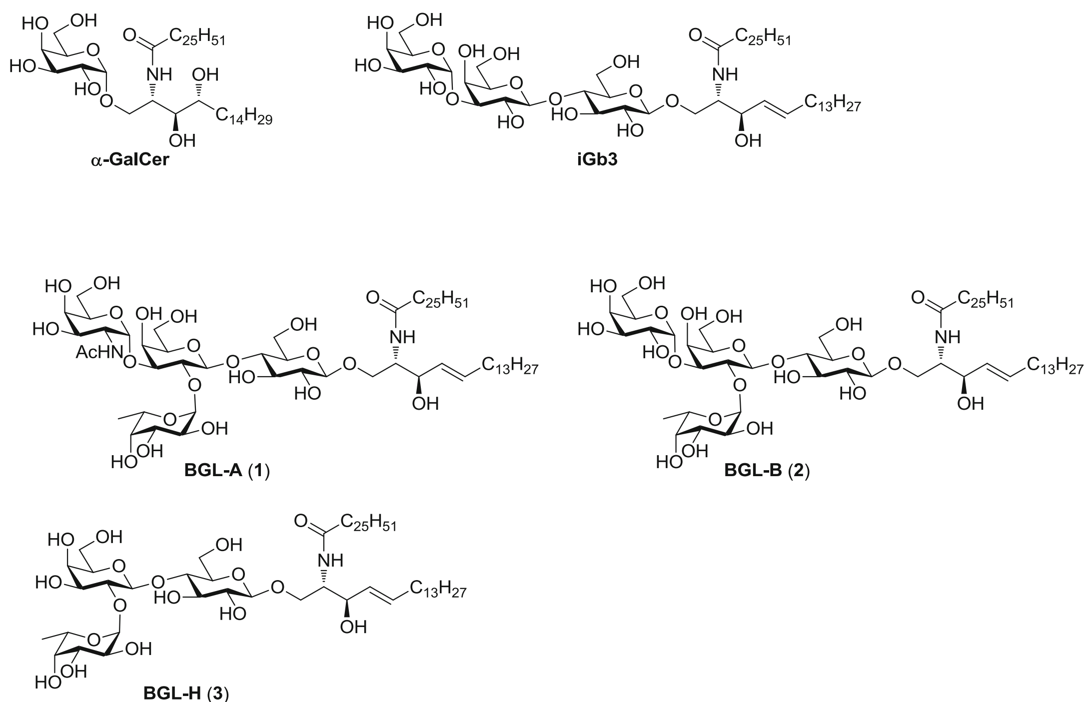

Fig. 1 Structures of α-GalCer, iGb3, and blood group glycosphingolipids

saccharide and ceramide moieties. When this molecule is presented to iNKT cells by antigen-presenting cells, it triggers a rapid, transient, and massive response of iNKT cells, causing accumulation of Th1 and Th2 cytokines and initiation of downstream immunological cascades, including responses to pathogens, tumors, tissue grafts, allergens, and other non-self-agents.

Efficient development of iNKT cells from the T-cell precursor pool in the thymus requires presentation of an endogenous antigen by CD1d to the randomly generated TCR. Although α-GalCer and its analogs from bacterial origin [10–12] exhibits strong iNKT cell stimulation leading to secretion of various cytokines, this glycolipid is not a natural product of mammalian cells. The structures of the mammalian glycolipids that serve as endogenous antigens in the thymus for iNKT cells are still unknown. Identification of these endogenous antigens is essential for understanding the process of iNKT cell maturation in the thymus and the mechanism of its activation. It will also help in exploration of endogenous ligands with different iNKT cell stimulation profiles.

Isoglobotrihexosylceramide (iGb3; Fig. 1) was the first endogenous lysosomal glycolipid reported by several groups to activate iNKT cells from mice and humans, albeit weakly [13]. However, it has been suggested that iGb3 is not a principal endogenous iNKT cell antigen [14, 15]. Gröne et al. generated mice that cannot produce iGb3 because they are deficient in iGb3 synthase (*iGb3S*, also known as α1,3-galactosyltransferase 2 [A3galt2]) [14]. The *iGb3S*$^{-/-}$ mice showed normal numbers of iNKT cells in the thymus, spleen, and liver with selected TCR Vβ chains identical to controls. Upon administration of α-GalCer, activation of iNKT cells and dendritic cells was similar in *iGb3S*$^{-/-}$ and *iGb3S*$^{+/-}$ mice. These results strongly suggest that iGb3 is not required for selection of iNKT cells in the thymus. The presence of iGb3 in mouse and human thymuses remains a controversy, mainly because of conflicting results due to different analytical methods [16–18]. Speak et al. could not detect iGb3 in mouse and human thymuses by high-performance liquid chromatography [16]. Li et al. used ion-trap mass spectrometry to analyze thymic lipids and detected both iGb3 and iGb4 [17, 18].

Thus, even though it is not disputed that iGb3 is a mammalian agonist ligand for iNKT cells, other redundant endogenous antigens that control iNKT cell development must exist. A β-glucosylceramide synthase-deficient cell line, GM95, was shown to be defective in iNKT cell stimulation [19]. β-glucosylceramide was reported as a ligand that activates peripheral NKT cells when co-stimulatory signals to NKT cells are present [20], although there is no evidence that it selects NKT cells during the positive selection stage. β-glucosylceramide does not stimulate autoreactive NKT clones, unlike ligands that mediate positive selection of NKT cells [21]. In thymic organ culture experiments, inhibition of

β-glucosylceramide synthesis abolished development of NKT cells but not conventional $CD4^+$ and $CD8^+$ T cells, suggesting the role of β-glucosylceramide-derived glycosphingolipids in mediating positive selection of NKT cells [16]. β-glucosylceramide is catalyzed by lactosylceramide synthase to give β-lactosylceramide [22], which is then further extended to different series of glycosylceramides such as the lacto and neolacto series, ganglio series, isoglobo series, and globo series.

During the search for NKT ligands, several groups including ours reported an iGb3-related structure, fucosylated iGb3 (Gal α3 [Fuc α2] Gal β4 Glc β1 Cer), in pig cells [23, 24]. Its precursor Fuc α2 Gal β4 Glc β1 Cer was also identified [24, 25]. This series of findings beg the question whether this iGb3-related structure, which is a type of B blood group glycosphingolipid antigen in human ABO blood group system, is an agonist ligand for NKT cells.

The human ABO blood group system, which is based on the presence or absence of blood group antigens A and B, comprises four major blood groups. Individuals of all four blood groups synthesize blood group glycolipid H (BGL-H), which is the precursor of BGL-A and BGL-B. Individuals of blood group AB catalyze the biosynthesis of both BGL-A and BGL-B, while blood group A individuals and blood group B individuals catalyze only the synthesis of BGL-A or BGL-B, respectively. Blood group O individuals catalyze the synthesis of neither BGL-A nor BGL-B, only BGL-H (Fig. 2). The blood group carbohydrate structures A, B,

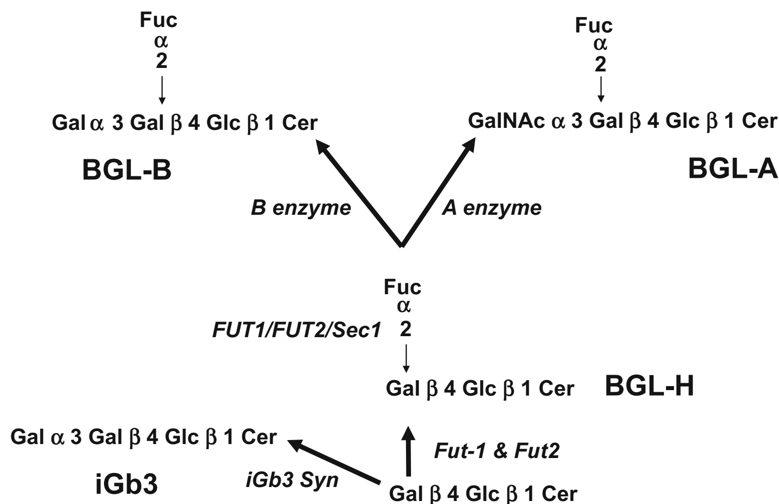

Fig. 2 Genetics of iGb3-related blood group glycosphingolipids. Fut1, α 1,2 fucosyltransferase 1 (EC 2.4.1.69); Fut2, α 1,2 fucosyltransferase 2 (EC 2.4.1.69); iGb3 Syn, α 1,3 galactosyltransferase 2 (EC 2.4.1.87); A enzyme, α 1,3 N-acetylgalactosaminyltransferase (EC 2.4.1.41); B enzyme, beta-D-galactosyl-1,4-N-acetyl-D-glucosaminide α 1,3 galactosyltransferase (EC 2.4.1.87)

and H are found at the termini of oligosaccharide chains of glycolipids and glycoproteins on the surface of erythrocytes and of endothelial and most epithelial cells [26, 27]. Pigs have a blood group AO system comparable to the ABO system in humans [28], and two of the prospective antigens, BGL-A and BGL-H, have been characterized in pigs by the Breimer group [25]. Mice, in contrast to pig and human, have only a cisAB blood group, which is encoded by a cisAB enzyme that has both α3-galactosyltransferase and α3-acetylgalactosaminyltransferase enzyme activities [29]. We have conducted in vitro experiments aimed at revealing the activities of these blood group lipids as stimulatory ligands for NKT cells.

The biosynthesis of blood group ABO structures is initiated by α-2-fucosyltransfearse (EC: 2.4.1.3441). Three members have been cloned for mouse α-2-fucosyltransfearse gene family. Enzyme activities were reported for mouse Fut1 and Fut2, while no enzyme activity has been reported for Sec1 [30, 31].

The Fut1 and Fut2 gene are located in the same chromosome with a 30 kb distance (Fig. 3a). This short genetic distance prevents the homologous recombination process required for generating double knockout mice. In this study, we sequentially depleted Fut1 gene and Fut2/Sec1 gene in ES cells and generated triple knockout mice with total deficiency of α1,2-fucosyltransferase enzyme. We also generated mice deficiency of all blood group H ligands and iGb3, which serve as a tool for further in vivo assays of endogenous ligands for NKT cells.

2 Materials

2.1 Mice and Embryonic Stem (ES) Cells

1. Fut1 KO mice, B6.129-Fut1^{tm1Sdo}/J, Jackson Laboratory.
2. Fut2 KO mice, B6.129X1-*Fut2*^{tm1Sdo}/J, Jackson Laboratory.
3. ES cell clone iTL BA1 (C57BL/6 x 129/SvEv), Ingenious Targeting Laboratory, Ronkonkoma, NY.

2.2 GSLs (Chemically Synthesized, Note 1)

1. Isoglobotriosylceramide (iGb3).
2. Blood group H GSL, Fuc α2 Gal β4 Glc β1 Cer.
3. Blood group A, GalNAc α3 Fuc α2 Gal β4 Glc β1 Cer.
4. Blood group B, Gal α3 Fuc α2 Gal β4 Glc β1 Cer.

2.3 NKT Cell Hybridoma Cells (Note 2)

The DN32.D3 [21], representing iNKT cells, were from Dr. Albert Bendelac (University of Chicago). These hybridomas were maintained in RPMI1640 medium containing 10% fetal calf serum (FCS), 2 mM sodium pyruvate, 2 mM L-glutamine, and 50 μM 2-mercaptoethanol.

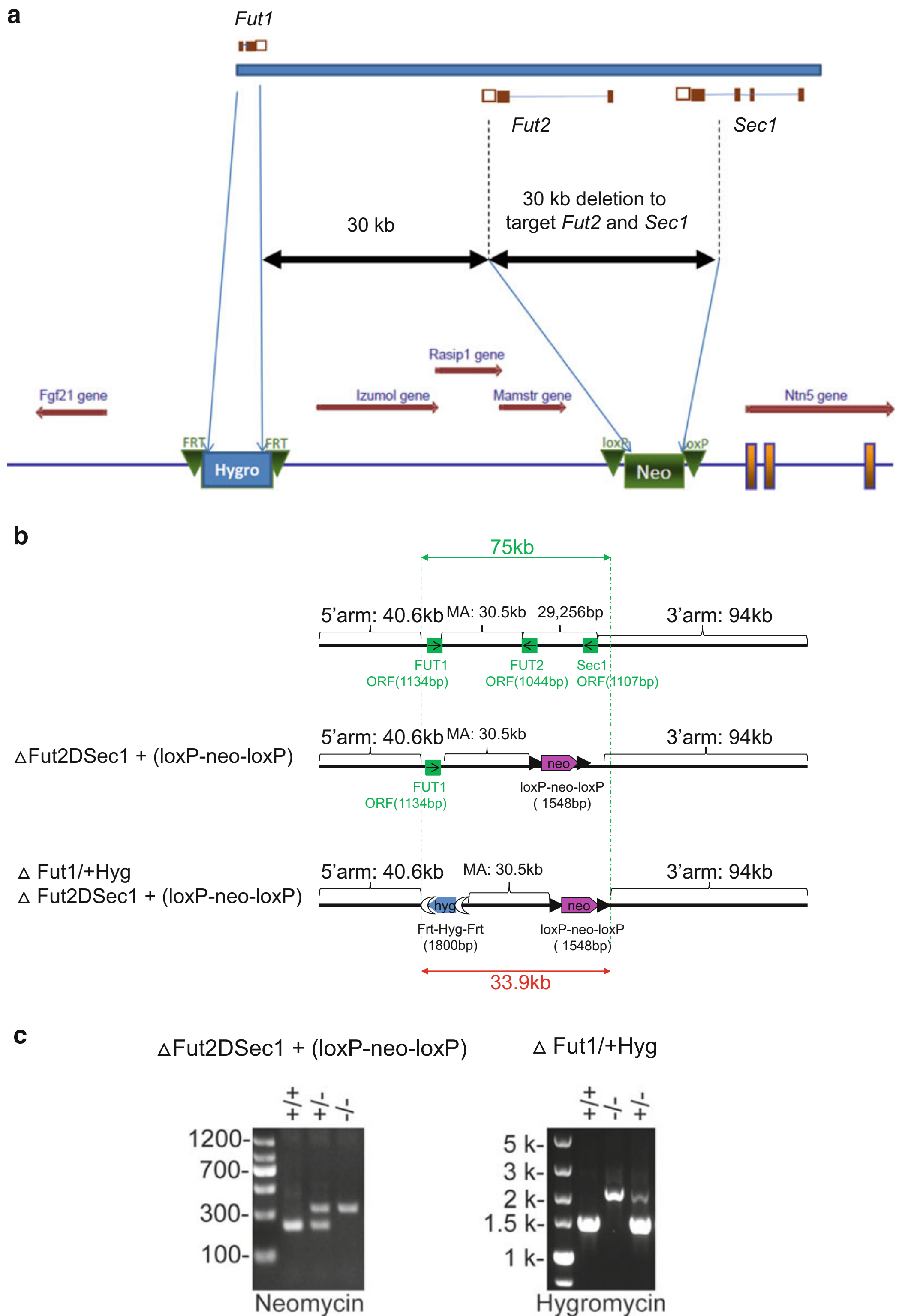

Fig. 3 Generation of Fut1/Fut2/Sec1 triple KO mice. (**a**) Two-step strategy to replace the protein coding regions (marked as empty boxes) of Fut1, Fut2, and Sec1 by targeting constructs containing neomycin and hygromycin selection markers; (**b**) scheme of genetic locus for wild-type ES cell line, △Fut2DSec1 + (loxP-neo-loxP) ES cell line, and △ Fut1/+Hyg △ Fut2DSec1 + (loxP-neo-loxP) ES cell line; (**c**) PCR results of the product lengths of wild-type allele, △ Fut1/+Hyg allele, and △Fut2DSec1 + (loxP-neo-loxP) allele

2.4 Antibodies and Glycolipid Tetramers

The PBS57-loaded mouse CD1d tetramer was from NIAID tetramer facility [32].

The anti-mouse CD3 antibody was from BioLegend.

3 Methods

3.1 Generation of Mice Deficient of FUT1, FUT2, and Sec1 (Note 3)

1. Engineering of Bacmid

   The Fut1, Fut2, and Sec1 loci are located in chromosome 7 in a 77 kb region (Table 1). A two-step knockout strategy was used to generate the mice with protein-coding regions of all three genes mutated. Firstly, a LoxP-neomycin-LoxP cassette was used to replace a 30 kb region which contains protein coding regions of both Fut2 and Sec1. Secondly, a FRT-hygromycin-FRT cassette was used to replace the protein coding region of Fut1. BAC clone RP23-223G1 was purchased from Children's Hospital Oakland Research Institute BACPAC Resources Center, and mutated constructs were made by Red/ET recombination technology [33] (Fig. 3b).

2. Generation of Fut1/Fut2/Sec1 Triple KO Mice

   Mutated constructs were electroporated into iTL BA1 (C57BL/6 × 129/SvEv) by Ingenious Targeting Laboratory, NY. Hybrid embryonic stem cells and ES clones bearing mutated loci were selected by neomycin and hygromycin resistance sequentially. Targeted iTL BA1 (C57BL/6 × 129/SvEv) hybrid embryonic stem cells were microinjected into C57BL/6 blastocysts. Resulting chimeras with a high percentage agouti coat color were mated to wild-type C57BL/6 N mice to generate F1 heterozygous offspring. Tail DNA was analyzed as described below from pups with agouti or black coat color. Primers F1385hygS and R1385hygS (Table 2, Data S1) were used to determine the mutation of Fut1 locus (replaced by FRT-hygromycin-FRT cassette). Primers Wt1, R1385neoS, and N2 were used to determine the mutation of Fut2 and Sec1 locus (replaced by LoxP-neomycin-LoxP cassette).

   Triple knockout mice with deletion of Fut1, Fut2, and Sec1 were genotyped by PCR (Fig. 3c, Table 2).

Table 1
Location of Fut1, Fut2, and Sec1 in mouse genome

Gene	Chromosome	Location	Centimorgan	Strand
Fut1	7	45617289- 45621059	29.39	+
Fut2	7	45648591- 45666394	29.41	–
Sec1	7	45677686-45694402	29.43	–

Table 2
Primers used for genotyping for Fut1/Fut2/Sec1 KO mice

Loci	Primer	Sequence	PCR product length
Hygromycin	F1385hygS R1385hygS	5′- CTG TGA GGT TCC CAG AAG GC -3′ 5′- GCT TAC AGT GCT GAC TTG GG -3′	1.47 kb for WT 1.47 kb and 2.14 kb for heterozygous 2.14 kb for homozygous
Neomycin	wt1 N2 R1385neoS	5′- AGA GGT AGA AGG TGG AGA GG -3′ 5′- TTC CTC GTG CTT TAC GGT ATC G -3′ 5′- AGT AAG ACC CAT CAC GTT G -3′	231 bp for WT 333 bp and 231 bp for heterozygous 333 bp for homozygous

3. PCR Reaction to Amplify the Hygromycin Cassette

   Tail DNA was extracted by TransDirect mouse genotyping kit (Transgen, Beijing, China). To amplify the hygromycin cassette, tail DNA samples from correctly targeted mice were amplified with primers F1385hygS and R1385hygS. F1385hygS is located upstream of the Hygro cassette, and R1385hygS is located downstream of the Hygro cassette. F1385hygS/R1385hygS amplifies a wild-type fragment of 1.47 kb in length. Hygro cassette presence is indicated by a band 2.14 kb long. PCR for Hygro cassette was performed by Expand High Fidelity PCR System (Roche catalog # 04738276001), in a 25 μL mixture containing the following:
   1.5 μL DNA (100 ng).

   200 μM dNTP.

   4% DMSO.

   1 μM Primers F1385hygS/R1385hygS.

   2.5 μL PCR buffer with 15 mM MgCl2.

   Add ddH_2O to final volume 25 μL.

   After a 10 min hot start at 99 °C, 0.125 μL of Taq polymerase was added to each PCR sample followed by a layer of two drops of mineral oil. Then the reaction was at 94 °C for 5 min, 95 °C × 30 s followed by 58 °C × 1 min (annealing step), and 72 °C for 2 min and 10 s (elongation step), for 35 cycles. The PCR product was run on a 0.8% gel with a 1 KB ladder as reference.

4. PCR Reaction to Amplify the Neomycin Cassette

   Tail DNA samples from correctly targeted mice were amplified with primers wt1, R1385neoS, and N2. N2 is located inside the Neo cassette, R1385neoS is located downstream of the Neo cassette, and wt1 is located on the targeted wild-type sequence replaced with the Neo cassette. Primer set wt1/R1385neoS amplifies a wild-type band 231 bp in length,

and N2/R1385neoS amplifies a fragment of 333 bp in length indicating Neo presence. PCR for neomycin cassette was performed by the Exo-PCR System (Roche catalog# 04738420001) in a 25 μL mixture containing the following: 1.5 μL DNA.

200 μM dNTP.

20% GC rich solution.

1 μM Primers wt1, R1385neoS, and N2.

2.5 μL PCR buffer with 15 mM MgCl2.

Add ddH_2O to final volume 25 μL.

After a 5 min hot start at 99 °C, 0.125 μL of Taq polymerase was added to each PCR sample followed by a layer of two drops of mineral oil. Then the reaction was at 94 °C for 5 min, 94 °C × 30 s followed by 60 °C × 1 min (annealing step), and 72 °C for 1 min (elongation step), for 30 cycles. The PCR product was run on a 2% gel with a 100 bp ladder as reference.

3.2 Generation of Mice Deficient of iGb3 Synthase (A3galt2)

A3galt2 knockout mice were generated by CRISPR/Cas9-based approach.

1. Two sgRNAs (ACTGAGCTGGACAACCCTTGGGG GTGT GGGAACCCCAATACCT AGG) were designed by CRISPR design tool (http://crispr.mit.edu) to target either a region upstream of the exon 5 or 3′UTR (Fig. 4) and then were screened for on-target activity using a Universal CRISPR Activity Assay (UCATM, Biocytogen Inc., Beijing, China) [34].
2. T7 promoter sequence was added to the Cas9 or sgRNA templates by PCR. Cas9 mRNA and sgRNAs were prepared and co-injected into the cytoplasm of fertilized oocytes (one cell stage, C57BL/6 strain).
3. The injected zygotes were transferred into oviducts of pseudo-pregnant females (Kunming strain, Biocytogen Inc., Beijing, China) to generate F0 mice. F0 mice with expected genotype were confirmed by PCR analysis of tail genomic DNA. Two

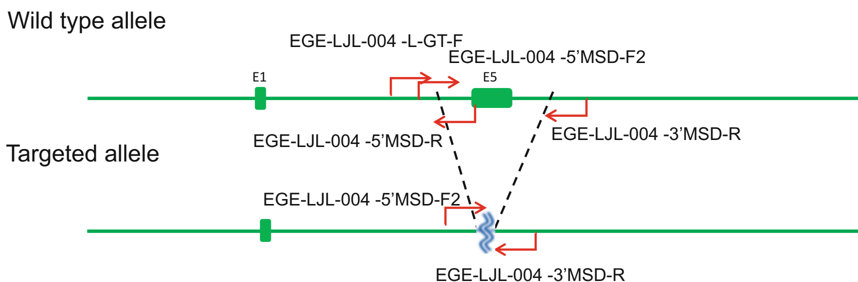

Fig. 4 Generation of A3galt2 KO mice

Table 3
Primers used for genotyping of A3galT2 KO mice

Primer	Sequence (5′-3′)	PCR product (bp)
EGE-LJL-004-5′MSD-F	CAAGGGTGGACAGACCCTTG TAGG	552 for mutant allele, 1822 for wild type allele
EGE-LJL-004-5′MSD-R	GTGTTCCAGGTACTTCTCCAGG TAC	
EGE-LJL-004-3′MSD-F	GCCAACATCTTTGGCACTACC TTAAG	598 for wild type allele
EGE-LJL-004-3′MSD-R	GTAACA TGAAGGAAGAGCCAGCATAGC	

pairs of primers were designed to amplify the exon 5-deleted mutant allele and the wild-type allele, respectively (Fig. 4, Table 3). F0 mice were mated with C57BL/6 mice to establish germline-transmitted F1 heterozygous mice.

4. PCR reaction to amplify the wild-type allele: Tail DNA samples from correctly targeted mice were amplified with primers EGE-LJL-004-L-GT-F and EGE-LJL-004-5'MSD-R, which generate a 598 bp product in wild-type allele but no signal in mutant allele. PCR for wild-type allele was performed in 10 μL volume of mixture containing the following:
   1 μL tail DNA (100 ng).

   1 μM Primers EGE-LJL-004-L-GT-F and EGE-LJL-004-5'MSD-R.

   5 μL Easy Mix (from Transgen, Beijing, China).

   Add ddH_2O to 10 μL.

   The PCR reaction was at 94 °C for 5 min, 94 °C × 30 s followed by 62 °C × 30 s (annealing step), and 72 °C for 45 s (elongation step), for 35 cycles. The PCR product was run on a 2% gel with a 100 bp ladder as reference.

5. PCR reaction to amplify the A3galt2 mutant allele: Tail DNA samples from correctly targeted mice were amplified with primers EGE-LJL-004-5'MSD-F2 and EGE-LJL-004-3'MSD-R, which generate a 552 bp product in mutant allele and 1822 bp for the wild-type allele. PCR for mutant allele was performed in 10 μL volume of mixture containing the following:
   1 μL tail DNA (100 ng).

   1 μM Primers EGE-LJL-004-5'MSD-F2 and EGE-LJL-004-3'MSD-R.

   5 μL Easy Mix (from Transgen, Beijing, China).

   Add ddH2O to 10 μL.

The PCR reaction was at 94 °C for 5 min, 94 °C × 30 s followed by 62 °C × 30 s (annealing step), and 72 °C for 45 s (elongation step), for 35 cycles. The PCR product was run on a 2% gel with a 100 bp ladder as reference.

3.3 Generation of Mice Deficient of FUT1/FUT2/Sec1/A3galt2

1. FUT1/FUT2/Sec1/A3galt2$^{-/-}$ mice were generated by breeding FUT1/FUT2/Sec1$^{-/-}$ mice and A3galt2$^{-/-}$ mice.
2. The development of NKT cells in FUT1/FUT2/Sec1/A3galt2$^{-/-}$ mice was measured by PBS57/CD1d tetramer staining (*see* **Note 4**).

4 Notes

1. Chemical synthesis of blood-group-related GSL ligands. The GSLs were synthesized as described in Data S4 and S5.
2. Stimulation of NKT cell hybridoma by GSL ligands. The NKT cell hybridomas were stimulated by mouse bone marrow-derived dendritic cells as described in Data S2.
3. This strain has been deposited in Jackson laboratory as B6.Cg-*Fut1*^{tm1Dzhou} Del(7Fut2-Sec1)1Dzhou/J).
4. The development of NKT cells in mice deficient of Fut1/Fut2/Sec1 and A3galt2 (iGb3 synthase) was measured by PBS57/CD1d tetramer staining as described in Data S3.

Acknowledgments

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Chapter 3

Retroviral Transduction of NKT Hybridoma Cells

Ke Wang, Rong Jin, and Qing Ge

Abstract

Natural killer T (NKT) cells have been shown to bridge innate and adaptive immunity. However, the rare population and hard-to-transfect of primary NKT cells slow down our understanding of cellular and molecular mechanisms of NKT development and function. To overcome these drawbacks, NKT hybridomas, especially DN32.D3 cells, are applied to study NKT cells in vitro and becoming a valuable tool. Here, we describe the method in the genetic manipulation of DN32.D3 cells by retrovirus, including the generation and concentration of retrovirus, retroviral transduction of DN32.D3 cells, and evaluation of transduction efficiency.

Key words iNKT, NKT hybridomas, DN32.D3, Retroviral transduction, Vector

1 Introduction

Natural killer T (NKT) cells are a type of innate-like unconventional αβ T lymphocytes that recognize self and exogenous glycolipid antigens and hydrophobic peptides presented by β2 microglobulin (β2m)-associated non-polymorphic MHC class I-like molecule CD1d [1, 2]. NKT cells share many features of both T cells and NK cells, thus orchestrating or even participating in innate and adaptive immune responses to protect against a variety of tumors and pathogens and contribute to tissue homeostasis and in some cases tissue damage [3–6]. There are two types of NKT cells according to their differences in TCR usage. Type I or invariant NKT (iNKT) cells express an invariant germline TCR α-chain (Vα14-Jα18 (TRAV11-TRAJ18) in mice, Vα24-Jα18 (TRAV10-TRAJ18) in humans) paired with a limited array of non-germline TCR β-chain (Vβ8.2/7/2 in mice and Vβ11 in humans) [7–9]. iNKT cells in mice represent 1–3% of T cells in most tissues and up to 50% of T cells in the liver and adipose tissue [10, 11]. In human, however, iNKT cells account for less than 1% of T cells in the blood and liver while up to 50% in adipose tissue [7, 11–13]. Type II NKT cells express polyclonal TCRαβ and are more

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abundant in humans while much less in mice [14]. Compared with iNKT cells, type II NKT cells are much less studied largely due to technical limitations to specifically identify them [15].

DN32.D3 cells are derived from a murine $CD4^{-}CD8^{-}$ (DN) iNKT hybridoma expressing TCR-Vα14/Vβ8.2 [16]. Like primary iNKT cells, DN32.D3 cells produce IL-2 upon stimulation [17, 18]. This characteristic has been used to identify iNKT-specific ligands [17, 19]. In addition, DN32.D3 cells are used for testing the capacity of lipid presentation of CD1d-expressing cells based on their IL-2 production [20–22]. DN32.D3 cells also express IFN-γ, IL-4, IL-10, and several genes critical for iNKT cell development and function, such as *Egr2* and *Zbtb16* (encoding PLZF) [23, 24]. Therefore, a number of groups used DN32.D3 cells as iNKT cells in their studies [25–27]. Furthermore, DN32.D3 cells can be used to investigate the molecular mechanisms of iNKT cells as genetic modifications can be easily performed in these cells [25, 28, 29].

Retroviral transduction is a fast and efficient method to introduce genetic materials into the murine primary T cells [30], hematopoietic stem cells [31], and any actively dividing cells [32]. Retroviral vectors provide a stable genetic modification in the infected cells because they integrate into the genome of the target cells. The gene(s)-of-interest harboring retroviral backbone needs Gag, Pol, and Env proteins for packaging [33]. Gag proteins form viral core structural proteins; Pol proteins are enzymes involved in viral replication; and Env proteins function as viral envelope glycoproteins [34]. These three elements are cotransfected with retroviral backbone in an independent vector (e.g., pCL-Eco helper vector [33]) or provided in a retroviral packaging cell line (e.g., Platinum-E cell line [35]). The Env proteins determine the host range of a retrovirus [36]. The retrovirus produced by cotransfection with pCL-Eco helper vector that we used in this protocol can infect murine and rat cells but not human cells and therefore can be safely used.

2 Materials

1. DN32.D3 cells, a kind gift from Dr. Li Bai at the University of Science and Technology of China, are used for retroviral transduction. HEK 293T cells are used for retroviral package.
2. DN32.D3 growth medium: RPMI 1640, 10% fetal bovine serum, 1% penicillin/streptomycin, 2 mM L-glutamine, and 0.1 mM β-mercaptoethanol. HEK 293T growth medium: DMEM, 10% fetal bovine serum, 1% penicillin/streptomycin, 2 mM L-glutamine, and 0.1 mM β-mercaptoethanol.

3. Plasmids: retroviral backbone pMSCV-ubc-EGFP and helper vector pCL-Eco are kind gifts from Dr. Zhongjun Dong at Tsinghua University.
4. Lipofectamine 2000 transfection reagent is used for HEK 293T transfection with retroviral package plasmids.
5. Opti-MEM I is used for diluting Lipofectamine 2000 and plasmids.
6. PBS: 8.0 g NaCl, 0.20 g KCl, 3.63 g $Na_2HPO_4{\cdot}12H_2O$, and 0.24 g KH_2PO_4 are dissolved in 1000 ml of H_2O, and the pH is adjusted to 7.20 using HCl. PBS is filtered through 0.22 μm pore size syringe filter.
7. 4 mg polybrene is dissolved in 1 ml of sterile PBS. 4 μg/ml of polybrene is used during retroviral transduction.
8. 0.45 μm pore size syringe filter is used for filtering medium containing retrovirus.

3 Methods

3.1 Retroviral Package in HEK 293T

1. One day before transfection, seed HEK 293T cells (*see* **Note 1**) in 10 cm dish with 15 ml of DMEM growth medium to make sure cells will be 70–90% confluent at the time of retroviral plasmids transfection.
2. At the day of seeding HEK 293T cells, thaw a vial of frozen DN32.D3 cells used for step "Transduction of DN32.D3 by retrovirus," culture them in RPMI 1640 growth medium, and passage them every day at split ratio of 1:2 (*see* **Note 2**).
3. At the day of transfection, dilute 10 μg of pMSCV-ubc-EGFP and 20 μg of pCL-Eco with Opti-MEM I to a volume of 1.5 ml and mix them gently. Dilute 60 μl of Lipofectamine 2000 with Opti-MEM I to a volume of 1.5 ml and mix them gently. Incubate them for 5 min at room temperature (*see* **Note 3**).
4. After the incubation, mix the diluted plasmids with the diluted Lipofectamine 2000 gently and incubate for 20 min at room temperature.
5. Add the 3 ml of complexes to different sites of 10 cm dish containing HEK 293T cells. Shake the dish gently.
6. Incubate the cells at 37 °C in 5% CO_2 incubator. Change the medium with 15 ml of pre-warmed fresh DMEM growth medium very gently (*see* **Note 4**) after 8 h of transfection.
7. After 24 h of transfection, collect the supernatant containing retrovirus. Add 15 ml of pre-warmed fresh DMEM growth medium into 10 cm dish very gently (*see* **Note 4**). Filter the collected supernatant through a 0.45 μm pore size syringe filter and store at 4 °C.

8. After 48 h of transfection, collect the supernatant containing retrovirus. Filter the collected supernatant through a 0.45 μm pore size syringe filter and store at 4 °C. Dispose of 10 cm dish containing HEK 293T cells properly.

3.2 Concentration of Retrovirus by Centrifugation (See Note 5)

1. Combine the retrovirus-containing supernatant collected from above steps. Aliquot them to 1 ml into 1.5 ml tube. Centrifuge (*see* **Note 6**) for 2 h at 17,700 × *g* (*see* **Note 7**) at 4 °C.
2. After centrifugation, the white retrovirus pellet appears at the bottom of 1.5 ml tube. Transfer 900 μl of supernatant very gently and discard. Save the rest of 100 μl of supernatant containing retrovirus pellet (*see* **Note 8**). Vertex for 10 s to resuspend the pellet and combine the aliquots into two tubes. Store at 4 °C.

3.3 Transduction of DN32.D3 by Retrovirus (See Note 9)

1. Use one tube of concentrated retrovirus-containing supernatant immediately after the harvest to prepare the mixture for the transduction. Add 5% of fetal bovine serum (*see* **Note 10**) and 4 μg/ml of polybrene (*see* **Note 11**) into concentrated retrovirus-containing supernatant. Mix them gently.
2. Collect and count DN32.D3 cells. Seed 1.8×10^5 cells per well of 48-well plate with 500 μl of transduction mixture. Centrifuge the 48-well plate for 2 h at 1500 × *g* at 32 °C (*see* **Note 12**).
3. After centrifugation, incubate the cells at 37 °C in 5% CO_2 incubator. Change the medium with 500 μl of pre-warmed fresh RPMI 1640 growth medium after 1 h of incubation.
4. After 24 h of transduction, proceed to the second round of transduction. Repeat **step 1–3** by using another tube of concentrated retrovirus-containing supernatant.
5. After 24 h of the second transduction, passage the infected cells at split ratio of 1:2.
6. After 48 h of the second transduction, proceed to downstream of experiments depending on the following applications. The infected DN32.D3 cells can be used for flow cytometry and western blot.

3.4 Determination of Transduction Efficiency by Flow Cytometry (See Note 13)

1. The transduction efficiency of infected DN32.D3 cells can be determined after 48 h of the second transduction. Collect the cells and wash with 1 ml of PBS containing 1% FBS once. Centrifuge for 5 min at 450 × *g* at 4 °C.
2. Resuspend the cell pellet with 300 μl of PBS containing 1% FBS and transfer into 5 ml flow cytometry tube.
3. Run sample on flow cytometer. As shown in Fig. 1, the percentage of positive infected cells of one experiment is 94.5% (*see* **Note 14**).

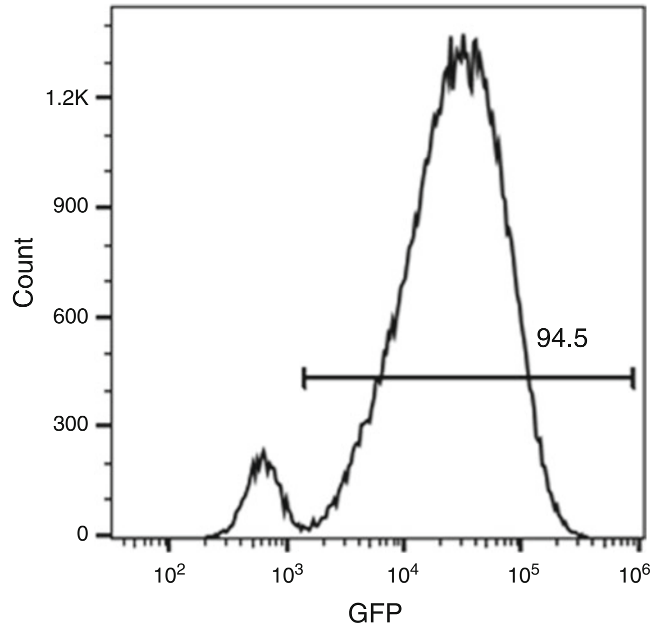

Fig. 1 Flow cytometry analysis of infected DN32.D3 cells

4 Notes

1. Use less than ten generations of HEK 293T cells after thawing to acquire high transfection efficiency.
2. Thawing DN32.D3 cells at this time point can make them ready for retroviral transduction immediately after harvesting concentrated retrovirus-containing supernatant.
3. Proceed to next step within 25 min.
4. Changing the medium for HEK 293T cells should be very gentle. Add pre-warmed fresh DMEM growth medium from side of 10 cm dish slowly and avoid disturbing the cells.
5. This concentration step improves the transduction efficiency of DN32.D3 cells.
6. Mark the position of 1.5 ml tube, and the pellet can be easily found at the bottom of the side away from the center of centrifuge.
7. 17,700 × *g* is the maximum speed of Kitman-T24 micro centrifuge (TOMY); this speed may be adjusted depending on different centrifuge. Our protocol for retrovirus concentration can be done without having ultracentrifuge.
8. The retrovirus pellet is tiny and incompact. Save 100 μl of supernatant at the bottom of the tube instead of discarding all of supernatant to decrease the loss of retrovirus.

9. Always use freshly made retrovirus to achieve high transduction efficiency.
10. The transduction is performed in the presence of additional FBS to improve the viability of cells.
11. The addition of polybrene, a cationic polymer, can enhance the efficiency of retroviral transduction.
12. Pre-warm the centrifuge to 32 °C before using.
13. The retroviral backbone pMSCV-ubc-EGFP contains a fluorophore gene. The infected cells express EGFP as an indicator of transduction efficiency, which can be detected by flow cytometry.
14. We usually achieve 94.0–99.8% of transduction efficiency with this protocol.

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Chapter 4

Methods for Studying Mouse and Human Invariant Natural Killer T Cells

Yang Zhou, Yan-Ruide Li, Samuel Zeng, and Lili Yang

Abstract

Invariant natural killer T (iNKT) cells are a unique subset of T lymphocytes that recognize lipid antigens presented by nonpolymorphic major histocompatibility complex (MHC) I-like molecule CD1d. iNKT cells play essential roles in regulating immune responses against cancer, viral infection, autoimmune disease, and allergy. However, the study and application of iNKT cells have been hampered by their very small numbers (0.01–1% in mouse and human blood). Here, we describe protocols to (1) generate mouse iNKT cells from mouse mononuclear cells or from mouse hematopoietic stem cells engineered with iNKT T cell receptor (TCR) gene (denoted as mMNC-iNKT cells or mHSC-iNKT cells, respectively), (2) generate human iNKT cells from human peripheral blood mononuclear cells or from human HSC cells engineered with iNKT TCR gene (denoted as hPBMC-iNKT cells or hHSC-iNKT cells, respectively), and (3) characterize mouse and human iNKT cells in vitro and in vivo.

Key words Invariant natural killer T (iNKT) cell, CD1d, T cell receptor (TCR), Alpha-galactosylceramide (α-GalCer), Glycolipid, Gene engineering, Hematopoietic stem cell (HSC), Cancer immunotherapy

1 Introduction

Invariant natural killer T (iNKT) cells are a unique subpopulation of innate T lymphocytes that express both natural killer (NK) cell markers and a restricted αβ T cell receptor (TCR). The restricted TCR is comprised of a canonical invariant TCRα chain (Vα14-Jα18 in mice; Vα24-Jα18 in human) paired with a semi-variant TCRβ chain (mostly Vβ8.2 in mice; mostly Vβ11 in human) [1, 2]. The early developmental stages of iNKT cells are similar to classical MHC-restricted $CD4^+$ and $CD8^+$ conventional T (Tc) cells [2, 3]. Lymphoid precursor cells arising from hematopoietic stem

Yang Zhou and Yan-Ruide Li contributed equally to this work.

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cells (HSCs) migrate to the thymus, undergo rearrangement of the TCRβ chain, and develop into CD4 and CD8 double-positive (DP) thymocytes. DP thymocytes then randomly rearrange their TCRα loci to generate intact TCR complexes expressed on the cell surface. However, unlike Tc cells that are selected by peptides presented on MHC-I or MHC-II of thymic epithelial cells, iNKT DP precursors are positively selected by glycolipids presented on CD1d expressed by DP thymocytes themselves [4]. The iNKT TCR-glycolipid-CD1d interaction, along with signals through the signaling lymphocytic activated molecules (SLAM) receptor family, provides co-stimulation for the further development of iNKT cells [3]. Owing to their unique developmental path, iNKT cells exit the thymus expressing a memory T cell phenotype. They further mature in the periphery through upregulating their expression of NK cell markers [5].

Functionally mature iNKT cells are powerful modulators of the immune response [4, 6–8]. Their most notable function is secreting copious amounts of cytokines upon stimulation, including T helper (Th)1-like (IFN-γ), Th2-like (IL-4, IL-13), Th17-like (IL-17, IL-22), and regulatory (IL-10) cytokines [4]. What cytokines are produced depends on the mechanism of cell activation, the location, and the iNKT cell subsets. iNKT cells also produce cytolytic proteins such as perforin and granzyme B and surface molecules involved in cytotoxicity such as Fas Ligand (FasL) and tumor necrosis factor α (TNF-α)-related apoptosis-inducing ligand (TRAIL) [9, 10]. Collectively, iNKT cells can profoundly influence many other immune cells, including dendritic cells, macrophages, neutrophils, NK cells, T cells, and B cells, thereby orchestrating the immune responses during infection, autoimmune disease [11], allergy [12], and cancer [9, 13, 14].

However, the extremely low number of iNKT cells, particularly in human peripheral blood (0.01–1% in healthy humans; 0.001–0.1% in cancer patients), is a significant obstacle for studying iNKT cell biology and developing iNKT cell-based therapies [5]. In our lab, we have developed methods to effectively generate large numbers of mouse and human iNKT cells through genetic engineering of HSCs (denoted as mHSC-iNKT cells and hHSC-iNKT cells, respectively [15–17]); we also routinely expand mouse iNKT cells from mouse mononuclear cells (denoted as mMNC-iNKT cells) and expand human iNKT cells from human peripheral blood mononuclear cells (denoted as hPBMC-iNKT cells) following established protocols with certain modifications [15, 17]. Here, we share our lab protocols on (1) generating mMNC-iNKT and mHSC-iNKT cells; (2) generating hPBMC-iNKT and hHSC-iNKT cells; (3) characterizing mouse and human iNKT cells with in vitro and in vivo assays.

2 Materials

Prepare all media in sterile hood (unless otherwise indicated).

2.1 Generation of Mouse iNKT Cells

2.1.1 Isolate and Expand mMNC-iNKT Cells

1. Mice: C57BL/6J (B6-WT) mouse.
2. Fetal bovine serum (FBS).
3. Phosphate-buffered saline (PBS).
4. 33% Percoll: 33% (vol/vol) of 100% Percoll, 67% (vol/vol) of 1× PBS solution.
5. 40% Percoll: 40% (vol/vol) of 100% Percoll, 60% (vol/vol) of 1× PBS solution.
6. 60% Percoll: 60% (vol/vol) of 100% Percoll, 40% (vol/vol) of 1× PBS solution.
7. Anti-mouse CD16/32 Fc block.
8. Anti-mouse CD19 microbeads.
9. Anti-mouse CD1d-Tetramer-PE.
10. Anti-PE microbeads.
11. 70 μm cell strainer.
12. Mouse iNKT culture medium: C10 medium supplemented with recombinant mouse IL-2 (final concentration 10 ng/ml) and IL-12 (final concentration 1 ng/ml).
13. Equipment: Magnetic beads separator (MACS), MACS Columns (LS column, LD column), water bath, sonicator, irradiator, pH meter.

2.1.2 Generate mHSC-iNKT Cells

1. Mice: C57BL/6J (B6-WT) mice.
2. Mouse iNKT TCR sequence [15].
3. 5-Fluorouracil (*see* **Note 1**).
4. Mouse iNKT retrovirus (*see* **Note 2**).
5. Human embryonic kidney cell line HEK293.T.
6. Polybrene.
7. Recombinant mouse IL-3.
8. Recombinant mouse IL-6.
9. Murine stem cell factor (SCF).
10. Antibiotics: sulfamethoxazole and trimethoprim.
11. Other reagents, material, and equipment were described in Subheading 2.1.

2.2 Generation of Human iNKT Cells

2.2.1 Isolate and Expand hPBMC-iNKT Cells

1. Human blood from healthy donors.
2. Ficoll-Paque Plus.
3. Anti-human iNKT MicroBeads.
4. Recombinant human IL-7.
5. Recombinant human IL-15.
6. Tris-buffered ammonium chloride buffer (TAC buffer or red blood cell lysis buffer): 0.16 M NH_4CL, 0.17 M Tris, ddH_2O.
7. MACS sorting buffer: phosphate-buffered saline (PBS), 0.5% bovine serum albumin (BSA), and 2 mM EDTA.
8. C10 medium: RPMI1640 supplemented with 10% (vol/vol) FBS, 1% (vol/vol) penicillin/streptomycin/glutamine, 1% (vol/vol) MEM NEAA, 10 mM HEPES, 1 mM sodium pyruvate, and 50 μM β-ME.
9. Human iNKT culture medium: C10 medium supplemented with recombinant human IL-7 (final 10 ng/ml) and human IL-15 (final concentration 10 ng/ml).
10. α-GalCer medium: C10 medium supplemented with α-galactosylceramide (final concentration 5 μg/ml).

2.2.2 Generate hHSC-iNKT Cells

1. Mice: NOD.Cg-Prkdc^{SCID}Il2rg^{tm1Wjl}/SzJ (NOD/SCID/IL-2Rγ$^{-/-}$, NSG) mice.
2. Human CD34$^+$ hematopoietic stem cells (CD34$^+$ HSCs) (*see* **Note 3**).
3. Human fetal thymus tissues.
4. Human iNKT TCR sequences [17].
5. RetroNectin.
6. 2% BSA.
7. X-VIVO-15 serum-free medium.
8. Carprofen.
9. Recombinant human IL-3.
10. Human Flt3-Ligand (Flt3-L).
11. Human stem cell factor (hSCF).
12. Human thrombopoietin (TPO).
13. 6-well non-tissue culture treated plates.
14. Other reagents, materials, and equipment were described in Subheading 2.1.

2.3 Characterization of Mouse or Human iNKT Cells

2.3.1 Phenotype Analysis of Mouse or Human iNKT Cells

1. Antibodies (*see* Table 1).
2. Phorbol-12-myristate-13-acetate (PMA): 1 mg PMA dissolved in 400 μl DMSO.
3. Ionomycin: 1 mg ionomycin dissolved in 400 μl DMSO.
4. GolgiStop.
5. BD fixation/permeabilization solution kit.
6. Recombinant mouse and human IFN-γ (ELISA, standard).
7. Recombinant mouse and human IL-4 (ELISA, standard).
8. Anti-mouse and human IFN-γ (ELISA, capture).
9. Anti-mouse and human IFN-γ (ELISA, detection).
10. Anti-mouse and human IL-4 (ELISA, capture).
11. Anti-mouse and human IL-4 (ELISA, capture).
12. Nunc-Immuno ELISA plate.
13. ELISA coating buffer: 325 ml of 0.1 M $NaHCO_3$, 50 ml of 0.1 M Na_2CO_3, PH = 9.4, store at room temperature (RT).
14. ELISA borate buffered saline (BBS) dilution buffer: 6.07 g H_3BO_3 (0.1 M), 7.32 g NaCl (0.012 M), 20 g 2% BSA, 1 L ddH_2O, PH = 8, store at 4 °C.
15. ELISA wash buffer (20×): 1 M Tris (PH = 8.0), 163.5 g NaCl, 10 ml Tween-20.
16. Tetramethylbenzidine (TMB).
17. Streptavidin-HRP conjugate.
18. TMB reaction stop solution (1 M H_3PO_4): 68.2 ml 85% phosphoric acid, 1 L ddH_2O, store at RT.

2.3.2 Function Analysis of Mouse or Human iNKT Cells

1. Human CD14 microbeads.
2. NK isolation kit.
3. Mouse melanoma cell line B16.F10.
4. Human multiple myeloma cell line MM.1S.
5. Human multiple myeloma cell line MM.1S-FG and MM.1S-hCD1d-FG (*see* **Note 4**).
6. Human chronic myelogenous leukemia cancer cell line K562.
7. Human chronic myelogenous leukemia cancer cell line K562-FG (*see* **Note 5**).
8. Human melanoma cell line A375.
9. Human melanoma cell line A375-A2-Eso-FG (*see* **Note 6**).
10. D-luciferin.
11. Isoflurane.
12. Zeiss Stemi 2000-CS microscope (Carl Zeiss AG).
13. IVIS 100 imaging system (Xenogen/PerkinElmer).

Table 1
List of antibodies

Antibodies		
Anti-human CD45 (Clone H130)	BioLegend	CAT#304026, RFID: AB_893337
Anti-human TCRαβ (Clone I26)	BioLegend	CAT#306716, RRID: AB_1953257
Anti-human CD4 (Clone OKT4)	BioLegend	CAT#317414, RRID: AB_571959
Anti-human CD8 (Clone SK1)	BioLegend	CAT#344714, RRID: AB_2044006
Anti-human CD45RO (Clone UCHL1)	BioLegend	CAT#304216, RRID: AB_493659
Anti-human CD161 (Clone HP-3G10)	BioLegend	CAT#339928, RRID: AB_2563967
Anti-human CD69 (Clone FN50)	BioLegend	CAT#310914, RRID: AB_314849
Anti-human CD56 (Clone HCD56)	BioLegend	CAT#318304, RRID: AB_604100
Anti-human CD62L (Clone DREG-56)	BioLegend	CAT#304822, RRID: AB_830801
Anti-human CD14 (Clone HCD14)	BioLegend	CAT#325608, RRID: AB_830681
Anti-human CD1d (Clone 51.1)	BioLegend	CAT#350308, RRID: AB_10642829
Anti-mouse TCRβ (Clone 1B3.3)	BioLegend	CAT#156305, RRID: AB_2800701
Anti-mouse NK1.1 (Clone PK136)	BioLegend	CAT#108710, RRID: AB_313397
Anti-mouse CD62L (Clone MEL-14)	BioLegend	CAT#104411, RRID: AB_30566881
Anti-mouse CD44 (Clone IM7)	BioLegend	CAT#103012, RRID: AB_312963
Anti-mouse CD4 (Clone GK1.5)	BioLegend	CAT#100412, RRID: AB_312697
Anti-mouse CD8 (Clone 53-6.7)	BioLegend	CAT#100712, RRID: AB_312751
Anti-mouse CD3 (Clone 17A2)	BioLegend	CAT#100236, RRID: AB_2561456
Anti-mouse IFN-γ (Clone XMG1.2)	BioLegend	CAT#505809, RRID: AB_315403
Anti-mouse IL-4 (Clone 11B11)	BioLegend	CAT#504103, RRID: AB_315317
Anti-mouse CD1d (Clone K253)	BioLegend	CAT#140805, RRID: AB_10643277
Anti-human CD34 (Clone 581)	BD Biosciences	CAT#555822, RRID: AB_396151
Anti-human TCR Vα24-Jβ18 (Clone 6B11)	BD Biosciences	CAT#552825, RRID: AB_394478
Anti-human Vβ11	Beckman-Coulter	CAT#A66905
Human Fc Receptor Blocking Solution (TrueStain FcX)	BioLegend	CAT#422302
Mouse Fc Block (anti-mouse CD16/32)	BD Biosciences	CAT#553142, RRID: AB_394657
LEAF purified anti-human CD1d antibody (Clone 51.1)	BioLegend	CAT#350304
LEAF purified Mouse IgG2b, k isotype ctrl (Clone MG2b-57)	BioLegend	CAT#401212
Mouse Fluorochrome-conjugated mCD1d/PSC-57 tetramer	NIH Tetramer Core Facility	

3 Methods

3.1 Generation of mMNC-iNKT Cells

3.1.1 Prepare MNCs from Mouse Spleen and Liver (Fig. 1a)

1. Euthanize mice by CO_2.
2. Clean the skin by spraying with 70% ethanol. Dissect the mouse and collect the spleen, located on the left flank. The liver is preferably flushed of circulating blood prior to collection. To do so, shift the intestines away from the body to uncover the inferior vena cava, and use a 5 ml syringe to push approximately 5 ml of PBS through it. The liver should turn yellow/white as a result.
3. Harvested spleen and liver should be collected in separate tubes containing 3–5 ml sterile C10 medium (*see* **Note 7**).
4. In a sterile hood, disperse the liver and spleen into single cell suspensions by placing each tissue in a 70 μm cell strainer and mashing with a plunger from a 3 ml syringe. Rinse the plunger and cell strainer with C10 medium and transfer the cell suspension into a new 15 ml conical tube.
5. Liver and spleen samples are processed differently. **Steps 6–11** refer to processing the liver, while **steps 12** and **13** refer to processing the spleen.
6. Add 3 ml of 60% Percoll into a new 15 ml tube.
7. Spin down (600 × *g*) the liver cells and resuspend the cell pellet in 3 ml of 40% Percoll in PBS (*see* **Note 8**).
8. Gently layer the 40% Percoll cell suspension on top of the 60% Percoll. To do this, tilt the conical tube until it is almost horizontal and add the 40% suspension drop by drop. If performed correctly, one should see a sharp demarcation between the 40% and 60%.
9. Spin at 800 × *g* for 30 min with no brakes at RT (*see* **Note 9**).
10. Aspirate the floating debris. There should be a thin layer of cells around the 3 ml line. Collect those cells while avoiding the red blood cells found at the bottom of the tube.
11. Add 5 ml C10 medium to the collection and mix well. Spin down for 5 min at 4 °C. Aspirate the supernatant and resuspend in 5 ml C10 medium.
12. Spin down (600 × *g*) the spleen cells and resuspend in 5 ml TAC buffer at room temperature for 10–20 min to lyse the red blood cells.
13. Add an additional 5 ml C10 medium to neutralize the buffer, spin down (600 × *g*). Aspirate the supernatant and resuspend the pellet with 5 ml C10 medium. Cells clumps may be observed. Filter through a 70 μm cell strainer to remove dead cell clumps.

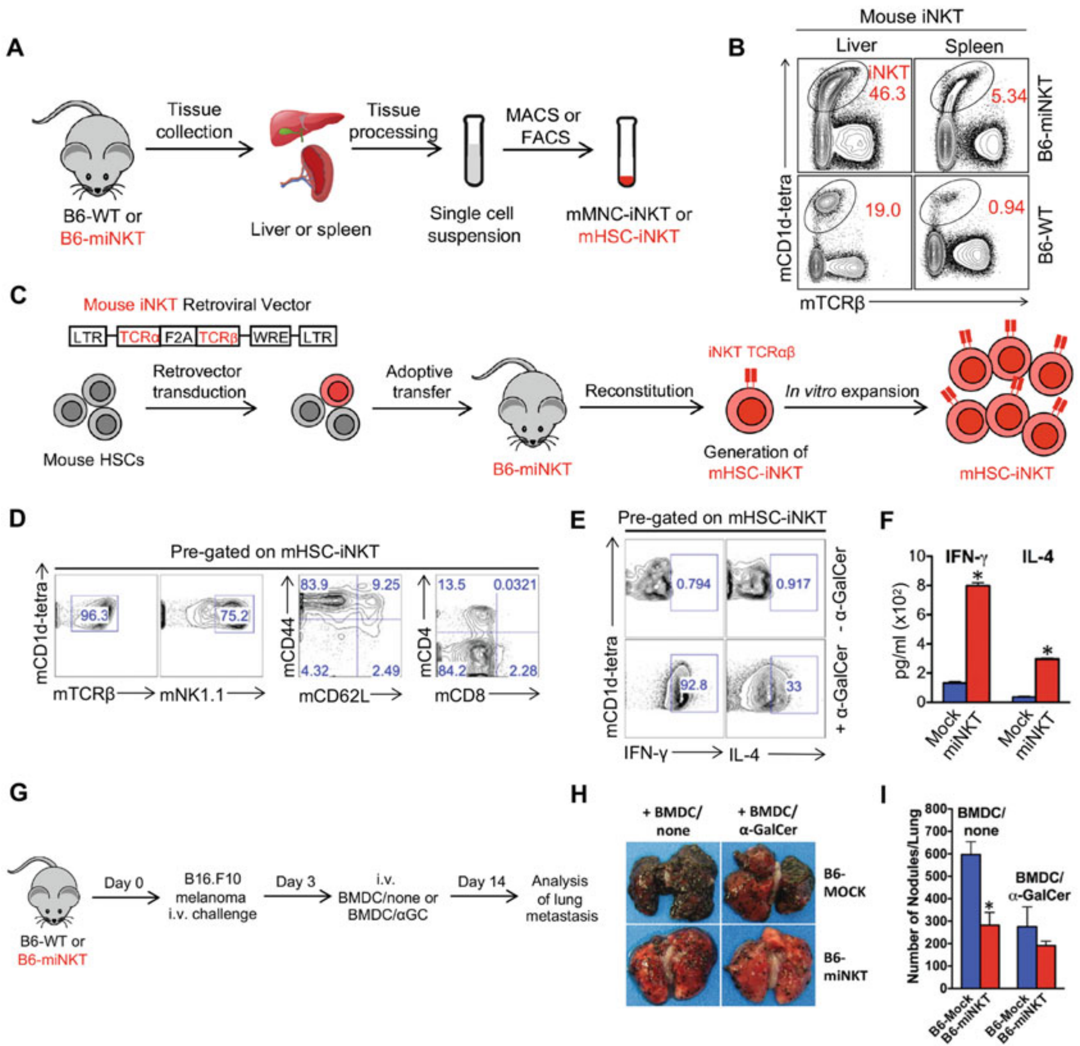

Fig. 1 Generation and characterization of mouse iNKT cells. (**a**) Diagram depicting the isolation of mMNC-iNKT cells and mHSC-iNKT cells. (**b**) FACS detection of mouse iNKT cells in liver and spleen of B6-miNKT mice or B6-WT mice. (**c**) Experimental design for generating mHSC-iNKT cells in a B6-miNKT mouse model. (**d**) FACS detection of the surface markers of mHSC-iNKT cells. These iNKT cells were detected in the liver of B6-miNKT mice for up to 6 months after HSCs adoptive transfer. (**e**, **f**) Functionality of mHSC-iNKT cells tested in vitro. Spleen cells collected from B6-miNKT mice were cultured in vitro in the presence of α-GalCer (100 ng/ml) (**e**) FACS detection of intracellular cytokine production in mHSC-iNKT cells 3 days post α-GalCer stimulation. (**f**) ELISA analysis of cytokine production of mHSC-iNKT cells in the cell culture medium 3 days post α-GalCer stimulation. (**g**–**i**) Study in vivo antitumor efficacy of mHSC-iNKT cells using an B16.F10 melanoma lung metastasis mouse model. (**g**) Experimental design. (**h**) Photos of lung tumor nodules. Representative of two experiments. (**i**) Enumeration of lung tumor nodules. Data were presented as the mean ± SEM. *$P < 0.01$, by Student's *t* test. (Note that **d**–**i** were reproduced from Ref. 15 with permission from NAS, copyright (2015) National Academy of Sciences)

14. Count live cell numbers. Cell resuspension can be kept at 4 °C, ready for sorting or FACS staining (*see* **Note 10**).

3.1.2 Magnetic Separation of mMNC-iNKT Cells

1. Resuspend MNCs from Subheading 3.1.1 in 2% FBS/PBS buffer.
2. Centrifuge the cell mixture, aspirate the supernatant, and resuspend in 100 μl of 2% FBS/PBS.
3. Add 10 μl of anti-mouse CD16/CD32 mAb to block nonspecific binding to Fcγ receptors and incubate for 5 min at 4 °C.
4. Add 20 μl of anti-mouse CD19 microbeads and incubate for 15 min at 4 °C.
5. Meanwhile, prepare a LD column and equilibrate with 2 ml of 2% FBS/PBS buffer.
6. Wash the cells with 2% FBS/PBS buffer and centrifugation at 600 × *g* for 5 min at 4 °C.
7. Resuspend cells in 500 μl of buffer and add to the LD column.
8. Collect the $CD19^{-}$ fraction into a clean 15 ml conical tube. Wash the column twice with 1 ml of 2% FBS/PBS each time and keep collecting the flow through.
9. Spin down the $CD19^{-}$ fraction and stain with 20 μl of anti-mouse CD1d-Tetramer-PE in 30 μl of 2% FBS/PBS for 20 min on ice.
10. Wash the cells with 2% FBS/PBS buffer and centrifugation at 600 × *g* for 5 min at 4 °C.
11. Stain with 20 μl of anti-PE microbeads in 100 μl of 2% FBS/PBS for 15 min on ice.
12. Meanwhile, prepare another LD column and equilibrate with 2 ml of 2% FBS/PBS.
13. Wash the cells with 2% FBS/PBS buffer and centrifuge at 600 × *g* for 5 min at 4 °C. Resuspend cells in 500 μl of buffer.
14. Load cell suspension into LD column. Mouse iNKT cells will bind to the column by positive selection. Wash the column twice with 1 ml of 2% FBS/PBS each time. The flow through can be discarded but can also be kept for troubleshooting.
15. Remove the LD column from the magnetic field and elute the cells from column.
16. The purity of the iNKT cells can be checked by FACS staining and can be further improved via FACS sorting (Fig. 1b).

3.1.3 In Vitro Expansion of mMNC-iNKT Cells

1. Count the number of mMNC-iNKT cells from Subheading 3.1.2.
2. Seed cell at 2×10^6 cells per well in the C10 medium, with or without the addition of α-GalCer (final concentration 100 ng/ml) for 5 days (*see* **Note 11**).
3. On day 3 and day 5, collect cells and run assays for mMNC-iNKT cell expansion using flow cytometry.

3.2 Generation of Mouse HSC-iNKT Cells (mHSC-iNKTs)

3.2.1 Generate HSC-iNKT Mouse (See **Note 12**) (Fig. 1c)

1. Day 0, treat B6 mice with 5-fluorouracial (250 μg per gram body weight).
2. Day 5, harvest bone marrow (BM) cells from mouse and culture the cells for 4 days in BM cell culture medium containing recombinant mouse IL-3 (20 ng/ml), IL-6 (50 ng/ml), and SCF (50 ng/ml).
3. Day 7 and 8, BM cells were spin-infected with retroviruses (*see* **Note 13**) supplemented with 8 μg/ml of polybrene, at 770 × *g*, 30 °C for 90 min.
4. Day 9, BM cells were collected and intravenously injected into B6 recipients that had received 1200 rads of total body irradiation (~1–2 × 10^6 transduced BM cells per recipient) (*see* **Note 14**).
5. The BM recipient mice were maintained on the combined antibiotics sulfamethoxazole and trimethoprim oral suspension in a sterile environment for 6–8 weeks until analysis or use for subsequent experiments.

3.2.2 Isolate and Expand mHSC-iNKT Cells (Refer Subheading 3.1)

1. Purify mHSC-iNKT cells following the steps of magnetic separation of mMNC-iNKT cells (refer Subheading 3.1.2).
2. Count the number of mHSC-iNKT cells, seed cells at 2 × 10^6 cells per well in the C10 medium, with or without the addition of α-GalCer (final concentration 100 ng/ml) for 5 days.
3. On day 3 and day 5, collect cells and run assays for mHSC-iNKT cell expansion using flow cytometry.

3.3 Generation of hPBMC-iNKT Cells

3.3.1 Isolate PBMCs from Human Peripheral Blood (Fig. 2a)

1. Obtain peripheral blood from healthy donors in blood collection tubes with heparin 1000 U/ml.
2. Centrifuge at 400 × *g* for 15 min with no brakes at RT.
3. Aspirate the supernatant and resuspend the cell pellet with PBS (10–12 ml/tube).
4. Transfer the mixture to a 50 ml conical tube. Wash once more using RT PBS (10–12 ml/tube).
5. Aspirate the supernatant and resuspend with 14 ml PBS.
6. Gently layer 14 ml of room temperature Ficoll on top of the mixture, using a 25 ml pipette (*see* **Note 15**).
7. Centrifuge at 970 × *g* for 20 min with no brakes at RT.
8. Aspirate the upper PBS layer.
9. Using a 5 ml pipette, carefully collect the PBMCs at the interface.
10. Wash with 10–20 ml PBS and centrifuge at 400 × *g* for 7 min at RT.

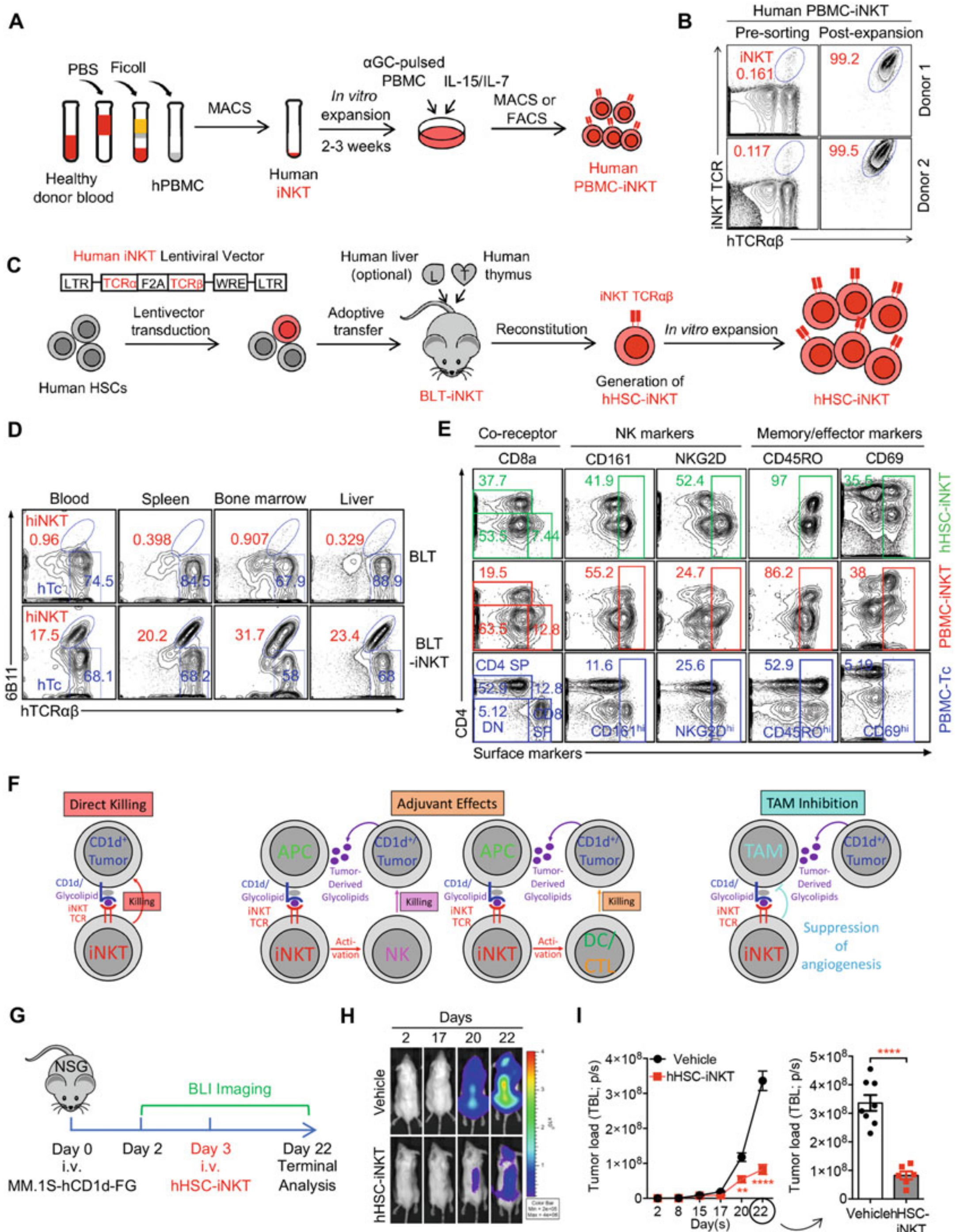

Fig. 2 Generation and characterization of human iNKT cells. (**a**) Diagram depicting the isolation and in vitro expansion of hPBMC-iNKT cells. (αGC: α-GalCer.). (**b**) FACS detection of hPBMC-iNKT cells before or after MACS sorting using anti-human iNKT microbeads. (**c**) Experimental design to generate hHSC-iNKT cells in a bone marrow-liver-thymus (BLT) humanized mouse model. (**d**) FACS detection of hHSC-iNKT cells in control BLT and BLT-iNKT mice tissues, at week 20 post-HSC transfer. Control BLT mice were generated by

11. Aspirate the supernatant and resuspend the PBMC pellet in 10 ml TAC buffer for 10–20 min at RT.
12. Centrifuge at 600 × *g* for 5 min and remove the supernatant.
13. Wash PBMCs with C10 medium once and resuspend cells in 10 ml of C10 medium.
14. Count live cell numbers.

3.3.2 Magnetic Separation of hPBMC-iNKT Cells

1. Count the number of cells in PBMC sample (*see* **Note 16**).
2. Centrifuge cell suspension at 300 × *g* for 10 min.
3. Aspirate supernatant completely and resuspend cell pellet in 400 μl of MACS sorting buffer per 1×10^8 total cells.
4. Add 100 μl of anti-human iNKT microbeads per 1×10^8 total cells. Mix well and incubate for 15 min in the refrigerator (2–8 °C).
5. Wash cells by adding 1–2 ml of buffer per 1×10^8 cells and centrifuge at 300 × *g* for 10 min. Aspirate supernatant completely and resuspend up to 1×10^8 cells in 500 μl of buffer.
6. Place column in the magnetic field of a suitable MACS separator.
7. Equilibrate column by rinsing with the appropriate amount of buffer (LS: 3 ml).
8. Apply cell suspension into the column. Collect flow-through containing unlabeled cells (*see* **Note 17**).
9. Wash column with the appropriate amount of buffer. Collect unlabeled cells that pass through and combine with the effluent (LS: 3 × 3 ml).
10. Remove column from the separator and place it on a clean 15 ml conical tube.
11. Pipette 3 ml of buffer onto the column. Immediately flush out the magnetically labeled cells by firmly pushing the plunger into the column (*see* **Note 18**).
12. Check the purity of human iNKT cells with FACS staining.

Fig. 2 (continued) adoptively transferring mock-transduced human HSCs into NSG mice engrafted with human thymus. (**e**) FACS detection of the surface markers of hHSC-iNKT cells isolated from the spleen of BLT-iNKT mice. Human PBMC-iNKT cells and human PBMC-derived conventional αβ T (PBMC-Tc) cells were included as controls. (**f**) Diagram showing the possible mechanisms used by human iNKT cells to attack tumor cells. APC, antigen presenting cell; NK, natural killer cell; DC, dendritic cell; CTL, cytotoxic T lymphocyte; TAM, tumor-associated macrophage. (**g–i**) Study in vivo antitumor efficacy of hHSC-iNKT cells using an MM.1S-hCD1d-FG human MM xenograft NSG mouse model. (**g**) Experimental design. (**h**) BLI images showing tumor burden in experimental mice over time. Representative of three experiments. (**i**) Quantification of (**h**) ($n = 6$–8). Data were presented as the mean ± SEM. ****$P < 0.0001$, by Student's *t* test. (Note that **d–i** were reproduced from Ref. 17 with permission from Elsevier, copyright (2019) Elsevier)

3.3.3 In Vitro Expansion of hPBMC-iNKT Cells

1. Count the number of PBMC-iNKT cells from magnetic separation. (Usually around 5×10^8 PBMC will yield $0.5–2 \times 10^6$ iNKT cells.)
2. Load a portion of the negative fraction ($1–2 \times 10^8$ per 5 ml) with α-GalCer (5 μg/ml) (*see* **Note 19**) and irradiate (~70% yield, 6000 rads); freeze down the remaining negative portion.
3. Seed cells at 1×10^6 iNKT: 2×10^6 α-GalCer-pulsed PBMC per 3 ml C10 medium per well of a 6-well plate. Add IL-7 and IL-15 at 10 ng/ml to the culture.
4. Monitor cell growth daily. As cells reach saturation, add C10 medium containing human IL-7 and IL-15 at 10 ng/ml and split cultures.
5. Cells can expand five- to tenfold and reach ~80% iNKT cell confluency during the first week. At day 7, cells can be restimulated (repeat **steps 2** and **3**). Cells can expand around tenfold per stimulation.
6. Take a small aliquot of iNKT culture for FACS staining (Fig. 2b).
7. The culture reaches >95% iNKT cell during the second week. iNKT cells can be frozen down and kept in liquid nitrogen for long-term storage (*see* **Note 20**).

3.4 Generation of Human HSC-iNKT Cells

*3.4.1 Generate HSC-iNKT Humanized Mouse (Fig. 2c) (See **Note 21**)*

1. Day 1, thaw and prestimulate $CD34^+$ PBSCs.
   (a) Coat 6-well non-tissue culture treated plates with RetroNectin (RN, 20 μg per vial in PBS) at RT for 2 h.
   (b) Aspirate and replace with 1 ml of 2% BSA for 30 min at RT.
   (c) Aspirate and replace with 2 ml PBS (*see* **Note 22**).
   (d) Thaw $CD34^+$ PBSC using X-VIVO-15 medium, spin at $300 \times g$ for 7 min.
   (e) Aspirate supernatant and resuspend in 5 ml of X-VIVO-15 medium and count cell number.
   (f) Spin down at $300 \times g$ for 7 min and aspirate supernatant.
   (g) Prepare 10 ml of X-VIVO-15 medium supplemented with hSCF (50 ng/ml), hFLT3L (50 ng/ml), hTPO (50 ng/ml), and hIL-3 (10 ng/ml).
   (h) Resuspend $CD34^+$ cells in X-VIVO-15/hSCF/hFLT3L/hTPO/hIL-3 medium (1×10^6 cells/ml).
   (i) Aspirate PBS in RN-coated well and seed the cells (1×10^6 per well).
   (j) Incubate at 37 °C, 5% CO_2.
2. Day 2, transduce $CD34^+$ PBSCs with lentivirus (*see* **Note 23**).

(a) Thaw concentrated virus supernatant on ice.

(b) Pipet thawed supernatant and add directly to well (*see* **Note 24**). Rock plate gently to mix.

(c) Incubate cells at 37 °C, 5% CO_2 for 24 h.

3. Day 3, prepare thymus pieces and intravenously inject transduced PBSCs to NSG mice.

   (a) Prepare fetal thymus fragments and irradiate with 500 rads (*see* **Note 25**).

   (b) Incubate irradiated thymus in C10 medium with antibiotics until surgery. Make sure to wash thymus thoroughly and keep on ice until surgery.

   (c) Irradiate NSG mice with 270 rads.

   (d) Harvest and count transduced human $CD34^+$ cells 24 h post transduction, and then resuspend in X-VIVO-15 medium (*see* **Note 26**).

   (e) Implant thymus pieces under the kidney capsule of pre-irradiated NSG mouse. Additionally, give retro-orbital injections of transduced PBSCs to each mouse.

   (f) Suture and staple the incision.

   (g) Subcutaneously inject 300 μl of 1:100 carprofen diluted in PBS (*see* **Note 27**).

4. Day 4, 5, daily injection with 300 μl of 1:100 carprofen diluted in PBS.

5. Day 7, remove the staples and monitor the conditions of BLT-iNKT mice.

6. Starting from week 6 post injection, bleed mice monthly and check human cell reconstitution by FACS staining (*see* **Note 28**).

3.4.2 hHSC-iNKT Cells from Humanized Mouse (Fig. 2d)

1. Euthanize mice by CO_2.

2. Place mouse in dorsal recumbency and clean the skin by spraying with 70% ethanol.

3. Cut the skin and expose both the abdomen and chest.

4. Puncture the heart with 26-gauge on 1 ml syringe to collect blood in a heparin-coated collection tube.

5. Collect the lung, liver, spleen, and bone marrow from humanized mouse and store the tissues on ice.

6. Disperse tissues into mononuclear cell suspension:

   (a) For blood, incubate in 5 ml TAC buffer for 20 min at RT; spin down to remove supernatant and resuspend in 1 ml C10 medium. Store at 4 °C. Sample is ready for staining (refer Subheading 3.5.1).

(b) For spleen, lung, and liver, mash tissues in C10 medium through a 70 μm cell strainer using plungers from 5 ml syringes. Collect the single cell suspension in a 15 ml conical tubes, spin down to remove supernatant, and resuspend in 14 ml 33% Percoll in PBS (spleen samples can skip Percoll separation and directly proceed to TAC lysis). Spin at 800 × *g* for 30 min with no brakes at RT. Aspirate the supernatant and resuspend the pellet in 5 ml TAC buffer. Incubate for 10 min at RT, and then add additional C10 medium and filter through cells strainer. Spin down and resuspend in fresh C10 medium. Store samples at 4 °C. Samples are ready for staining or cryopreservation (refer to Subheading 3.5.1).

(c) For bone marrow, use forceps to hold leg bones over a 15 ml conical tube and flush with C10 medium using 25-gauge needle fitted onto a 10 ml syringe. Spin down to remove the supernatant and resuspend in 10 ml TAC buffer. Incubate for 10 min at RT, and then filter through a 70 μm cell strainer. Spin down, aspirate the supernatant, and wash with 2 ml C10 medium. Resuspend the sample in C10 medium, and store at 4 °C. Sample is ready for staining or cryopreservation (refer Subheading 3.5.1).

3.4.3 In Vitro Expansion of hHSC-iNKT Cells

1. Healthy donor PBMCs were loaded with α-GalCer (by culturing 1×10^8 PBMCs in 5 ml C10 medium containing 5 μg/ml α-GalCer for 1 h in 6-well TC plate), irradiated at 6000 rads, and then used to stimulate iNKT cells.
2. To expand iNKT cells, pooled hHSC-iNKT humanized mouse tissue cells were mixed with α-GalCer-pulsed PBMCs (ratio 1:1 or 1:1.5; e.g. 1×10^6 iNKT tissue cells were mixed with 1.5×10^6 irradiated α-GalCer-pulsed PBMCs) and cultured in C10 medium for 7 days. Cells were plated in 6-well plate (2.5×10^6/ml, 3 ml/well). Recombinant human IL-7 (10 ng/ml) and IL-15 (10 ng/ml) were added to cell cultures starting from day 2. Cells were split 1:2 once confluent (about every 2–3 days).
3. On day 7, cell cultures were collected and iNKT cells were sorted out using flow cytometry (identified as $hCD45^+hTCR\alpha\beta^+6B11^+$ cells).
4. The sorted iNKT cells (>99% purify based on flow cytometry analysis) were expanded further with α-GalCer-pulsed PBMCs and human IL-7/IL-15 for another 7 to 14 days (*see* **Note 29**).
5. Expanded iNKT cells were aliquoted and frozen in LN storage tanks (e.g., 1×10^7 cells per vial for in vitro assay and 1×10^8 cells per vial for in vivo assay).

3.5 Characterization of Mouse or Human iNKT Cells

3.5.1 iNKT Cell Phenotype Analysis

1. Surface and intracellular marker staining (Figs. 1d, e, and 2e):
   - (a) Aliquot cells into labeled FACS tubes.
   - (b) Wash cells with 1 ml C10 media, spin down, and aspirate the supernatant.
   - (c) Wash cells with 1 ml PBS, spin down, and aspirate the supernatant.
   - (d) Resuspend cells in 50 μl PBS with human FcR Block and Fixable Viability Dye e506 (*see* **Note 30**).
   - (e) Incubate cells at 4 °C for 15 min shielded from light.
   - (f) Wash cells with 1 ml PBS, spin down, and aspirate the supernatant.
   - (g) Resuspend cells in 50 μl PBS with antibody cocktails.
   - (h) Incubate cells at 4 °C for 15 min shielded from light.
   - (i) Wash cells with 1 ml PBS, spin down, and aspirate the supernatant.
   - (j) For surface staining, resuspend the cell pellet in 100–200 μl PBS for flow cytometry.
   - (k) For intracellular staining, add 250 μl of BD fixation/permeabilization buffer to the cell pellet.
   - (l) Incubate cells at 4 °C for 20–30 min, shielded from light.
   - (m) Spin at 600 *g* for 5 min, and aspirate the supernatant.
   - (n) Wash twice with BD washing buffer.
   - (o) Spin and resuspend the cell pellet in 50 μl intracellular antibodies cocktail.
   - (p) Incubate cells at 4 °C for 30 min shielded from light.
   - (q) Wash cells twice with 1 ml wash buffer.
   - (r) Resuspend cells in 100–200 μl PBS for flow cytometry.
2. Stimulate cytokine production (PMA/Ionomycin stimulation):
   - (a) Resuspend cells at 1×10^6/ml in C10 medium containing PMA (final concentration 50 ng/ml) and ionomycin (final concentration 500 ng/ml).
   - (b) Transfer 1 ml of cells into capped FACS tubes.
   - (c) Add GolgiStop to cells (4 μl GolgiStop per 6 ml of C10 medium) and tightly close caps on FACS tubes.
   - (d) Incubate at 37 °C, 5% CO_2 for 4–6 h.
   - (e) Samples are ready for FACS staining (refer Subheading 3.5.1, **step 1**).
3. ELISA (following standard protocol from BD bioscience) (Fig. 1f).

(a) Coat Nunc Immunoplates with purified capturing antibody diluted in ELISA coating buffer. Add 50 μl/well and incubate for 2 h at 37 °C or overnight at 4 °C.

(b) Wash plate four times with ELISA wash buffer.

(c) Block plate with 100 μl/ well of ELISA BBS buffer. Incubate for 30 min at 37 °C or overnight at 4 °C.

(d) Wash plate four times with ELISA wash buffer.

(e) Add samples at 25 μl or 50 μl per well. Incubate for 3 h at 37 °C or 4 °C overnight.

(f) Wash plate four times with ELISA wash buffer.

(g) Add 50 μl of the biotinylated detection antibody diluted in BBS solution buffer. Incubate for 45 min at RT.

(h) Wash plate four times with ELISA wash buffer.

(i) Add 50 μl/ well of the streptavidin-HRP, diluted 1:1000 in BBS dilution buffer. Incubate for 30 min at RT, shielded from light.

(j) Wash plate eight times with ELISA wash buffer.

(k) Mix the TMB developing solution and add 50 μl/ well. Incubate at RT.

(l) Monitor the blue color change and stop reaction by adding 50 μl/well of TMB reaction stop solution.

(m) Read absorbance at 450 nm within 30 min.

3.5.2 iNKT Cell Function Analysis

1. mHSC-iNKT cell in vivo antitumor efficacy study—mouse B16 melanoma lung metastasis mouse model [15] (Fig. 1g–i):
   (a) C57BL/6 J (B6) mice received intravenous (i.v.) injection of 0.5–1 × 10^6 B16.F10 melanoma cells to model lung metastasis over the course of 2 weeks.
   (b) On day 3 post tumor challenge, the experimental mice received i.v. injection of 1 × 10^6 bone marrow-derived dendritic cells (BMDCs) that were either unloaded or loaded with α-GalCer.
   (c) On day 14, mice were humanely euthanized, and their lungs were collected and analyzed for melanoma metastasis by counting tumor nodules.
2. hHSC-iNKT cell tumor-attacking mechanism study [17] (Fig. 2f):
   (a) In vitro direct tumor cell killing assay. Human multiple myeloma cell line MM.1S was used. MM.1S-FG or MM.1S-hCD1d-FG tumor cells (5–10 × 10^3 cells per well) were co-cultured with hHSC-iNKT cells (ratio 1:1, 1:2, 1:5, and 1:10) in Corning 96-well clear bottom black plates for 24–48 h, in X-VIVO™ 15 medium with or

without the addition of α-GalCer (100 ng/ml). At the end of culture, live tumor cells were quantified by adding 150 mg/ml of D-luciferin to cell cultures and reading out luciferase activities. In order to verify CD1d-dependent tumor killing mechanism, we blocked CD1d by adding 10 mg/ml LEAF™ purified anti-human CD1d antibody or LEAF™ purified mouse IgG2b κ isotype control antibody to tumor cell cultures at least 1 h prior to adding hHSC-iNKT cells. At the end of culture, live tumor cells were quantified by adding D-Luciferin to cell cultures and reading out luciferase activities.

(b) In vitro NK adjuvant effect assay. Primary human NK cells were isolated from healthy donor PBMCs through an NK Cell Isolation Kit according to the manufacturer's instructions. K562-FG cells (5×10^4 cells per well) were co-cultured with NK cells and hHSC-iNKT cells (at ratio of 1: 2: 2) in Corning 96-well clear bottom black plates for 24 h, in C10 medium with or without α-GalCer-pulsed irradiated PBMCs as antigen-presenting cells (APCs). Live tumor cells were quantified by adding D-luciferin (150 mg/ml) to the cell cultures and reading out luciferase activities.

(c) In vitro dendritic cells (DC)/cytotoxic T lymphocyte (CTL) adjuvant effect assay. $CD1d^+$/HLA-$A2^+$ human monocyte-derived dendritic cells (MoDCs) were generated by isolating $CD14^+$ monocytes from HLA-$A2^+$ healthy donor PBMCs using anti-human CD14 beads, followed by a 4-day culture in R10 medium supplemented with recombinant human GM-CSF (100 ng/ml) and IL-4 (20 ng/ml). The NY-ESO-1 specific $CD8^+$ human CTLs were co-cultured with $CD1d^+$/HLA-$A2^+$ MoDCs in C10 medium for 3 days, with or without hHSC-iNKT cells (cell ratio 1:1:1) and α-GalCer (100 ng/ml). Tumor-killing potential of ESO-T cells was measured by adding A375-A2-ESO-FG tumor cells (1:1 ratio to input ESO-T cells) to the ESO-T/MoDC co-culture 24 h post co-culture setup and quantifying live tumor cells by luciferase activity reading in another 24 h (*see* **Note 31**).

(d) In vitro macrophage inhibition assay. $CD14^+$ monocytes were isolated from healthy donor PBMCs, followed by co-culturing with hHSC-iNKT cells (ratio 1:1) for 24–48 h in C10 medium with or without the addition of α-GalCer (100 ng/ml). At the end of culture, cells were collected for flow cytometry analysis.

3. hHSC-iNKT cell in vivo antitumor efficacy study—MM.1S human multiple myeloma xenograft NSG mouse model [17] (Fig. 2g–i):
   - (a) NSG mice were pre-conditioned with 175 rads of total body irradiation and inoculated with 0.5–1 $\times$ 10^6 MM.1S-hCD1d-FG or MM.1S-FG cells intravenously (day 0) to develop multiple myeloma over the course of about 3 weeks.
   - (b) Three days post-tumor inoculation (day 3), mice received i.v. injection of vehicle (PBS) or 1 $\times$ 10^7 hHSC-iNKT cells. Recombinant human IL-15 was intraperitoneally injected to experimental animals to support the peripheral maintenance of hHSC-iNKT cells twice per week starting from day 3 (500 ng per animal per injection).
   - (c) The tumor burden was monitored twice per week by bioluminescence (BLI) measurement.
   - (d) At around week 3, mice were humanely euthanized (refer to Subheading 3.4.2), and tissues (peripheral blood, spleen, liver, and bone marrow) were collected for flow cytometry analysis.

4 Notes

1. 5-Fluorouracil (5-FU) is a chemotherapy drug used to treat cancer. 5-FU can inhibit thymidylate synthetase function during pyrimidine synthesis. The carcinogenicity and acute toxicity of 5-FU require proper handling from lab personnel.
2. The generation of mouse iNKT TCR gene delivery retroviral vector was described in our previous publication [15].
3. Human $CD34^+$ HSCs are commercially available from HemaCare Corporation (Northridge, California, USA).
4. Human multiple myeloma (MM) cell line MM.1S, human chronic myelogenous leukemia cancer cell line K562, and human melanoma cell line A375 were all purchased from the American Type Culture Collection (ATCC) (Manassas, Virginia, USA) and cultured in ATCC recommended media. The stable MM.1S-FG and MM.1S-hCD1d-FG tumor cell lines were engineered by transducing the parental MM.1S cell line with the lentiviral vectors encoding the intended gene(s) to overexpress human CD1d and/or firefly luciferase and enhanced green fluorescence protein (GFP) dual-reporters (FG). $CD1d^+$ and/or GFP^+ cells were sorted by flow cytometry 72 h post viral transduction to generate stable cell lines.
5. The stable K562-FG tumor cell line was engineered by transducing the parental K562 cell line with lentiviral vectors

encoding FG. GFP^+ cells were sorted by flow cytometry post 72 h of virus transduction to generate stable cell line.

6. The stable A375-A2-Eso-FG tumor cell line was engineered by transducing the parental A375 cell line with lentiviral vectors encoding human HLA-A2.1, human NY-ESO-1, and FG.
7. Keeping tissues on ice during processing improves the viability of cells.
8. 33%, 40%, and 60% Percoll can be prepared beforehand and stored long term at 4 °C. However, Percoll must be warmed back to RT before usage.
9. Centrifuge must be equilibrated to RT before starting and the centrifuge brakes must be turned off. Excessive deceleration can remix the separating layers.
10. Cells from spleen and liver can be combined for in vitro expansion if needed. Cells from multiple mice can be combined if needed.
11. mMNC-iNKT cells may be expanded and cultured in vitro for up to 3 weeks using repetitive stimulations with anti-CD3ε and anti-CD28 every 7–8 days [18]. α-GalCer stimulation leads to apoptosis and is not suitable for long-term expansion of mouse iNKT cells [18].
12. We have established a B6-miNKT mouse model through genetic engineering of hematopoietic stem cells [15] to produce large numbers of iNKT cells. Compared to B6-WT mice, B6-miNKT mice provide a significantly higher yield of iNKT cells.
13. Spin infections on two sequential days increase retrovirus transduction rate. Preferably, the second infection should be performed within 12–15 h after the first one to infect cells at different stages of the cell cycle.
14. For a secondary BM transfer, fresh whole BM cells harvested from the primary BM recipients are intravenously injected into secondary B6 recipient mice that had received 1200 rads of total body irradiation (~1×10^7 total BM cells per recipient). Details were described in previous publications [15, 16].
15. Tilt the tube and bring the pipette close to surface of the blood/PBS mixture. Slowly pipette out 1–2 ml. Then detach the pipette to allow gravity to dispense the remainder of the Ficoll. When the flow stops, reattach the pipette to push out any remaining Ficoll before removing the pipette from the conical tube. Be extremely careful at this step to make sure that the interface is not disturbed.
16. Choose the right column to use based on the sample cell number. For example, if there are ~2×10^8 total PBMCs, use one LS column.

17. Do not let the column dry out and avoid adding bubbles into the column. Bubbles inside the column can interfere with the sample and decrease selection efficacy.
18. To increase the purity, the eluted fraction can be enriched over a second column. Repeat the magnetic separation procedure as described if needed.
19. α-GalCer glycolipid and DMSO are immiscible at RT. To prepare the stock α-GalCer (1 μg/μl), add the proper volume of DMSO into the α-GalCer powder and heat it at 80 °C in a water bath for 10 min, followed by 10 min of sonication at 50 °C. Then, vortex the vial for full 2 min until the solution turns clear. Aliquot it into glass vials and store them in a −20 °C freezer. To prepare the α-GalCer working solution (5 μg/ml), heat the aliquot at 80 °C for 5 min followed by 5 min of sonicating at 50 °C. Then vortex the aliquot for a full 60 s and add 200 μl of pre-warmed C10 medium. Sonicate for another 5 min and vortex for 60 s. Add the rest of the pre-warmed C10 medium to make the final concentration 5 μg/ml. α-GalCer aliquots from −20 °C are single-used. Do not refreeze after diluting with media.
20. Cell expansion fold is donor-dependent. iNKT cells from different donors can be in vitro expanded for up to 3 weeks.
21. Standard BLT (human bone marrow-liver-thymus engrafted NOD/SCID/$\gamma c^{-/-}$) humanized mouse is established by co-implanting human fetal liver and thymus pieces under the renal capsule of NSG mouse together with intravenous injection of human $CD34^{+}$ HSCs. In our modified approach, only thymus pieces are placed under the renal capsule together with intravenous injection of engineered $CD34^{+}$ HSC.
22. RN-coated plate with PBS can be left for several hours in the hood.
23. Pre-stimulate $CD34^{+}$ cells for 12–18 h before transduction. One option is to coat the plate in the late afternoon and seed cells at around 6 pm. The next morning, add the virus for transduction.
24. The generation of human iNKT lentivirus is described in our prior publication [15]. Do not vortex, just very gently mix the concentrated virus. If necessary, adding poloxamer and PEG-2 can improve virus transduction rate [19].
25. Both fresh and frozen fetal thymus can be used for implantation. Fetal thymus should be pre-cut into 1 mm^3 cube size. Each mouse can be implanted with one to two pieces of thymus fragments.
26. Keep small portions of un-transduced and transduced $CD34^{+}$ cells in X-VIVO-15 media supplemented with cytokines post

virus transduction for a 72-h culture. Collect cells and perform intracellular staining of Vβ11 to detect the virus transduction efficacy.

27. Carprofen works as painkiller to relieve the pain from surgery. It can be substitute with other analgesics based on institution recommendation.
28. The viral transduction rate, the quality of human fetal thymus, and the quality of surgery will all contribute to the quality of HSC reconstitutions. BLT-iNKT mice can live around 6 months to 1 year.
29. Sorted iNKT cells are expected to expand tenfold in the first week and another tenfold in the second week.
30. Optimize antibody dilution beforehand.
31. NY-ESO-1 specific $CD8^+$ human cytotoxic T lymphocytes (CTLs, or ESO-T cells) were generated through engineering human $CD34^+$ HSCs with a TCR gene encoding a 1G4 TCR (HLA-A2- restricted, NY-ESO-1 tumor antigen-specific) and differentiating the TCR gene-engineered HSCs into CD8+ CTLs in an artificial thymic organoid (ATO) culture [17].

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Chapter 5

Isolation and Detection of Murine iNKT Cells in Different Organs

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Abstract

The invariant NKT (iNKT) cells are innate-like lymphocytes that share phenotypic and functional characteristics with NK cells and T cells, playing an important role in both human and mouse physiology and disease and bridging the gap between the innate and adaptive immune responses. The frequency and subtypes of iNKT cells in major immune organs are different, which also determines the regional immune characteristics of iNKT cells. Here, we report a protocol about the isolation of iNKT cells in the thymus, spleen, and liver of C57BL/6, CD1d$^{-/-}$, and Jα18$^{-/-}$ mice.

Key words iNKT cells, Spleen, Thymus, Liver

1 Introduction

1.1 The Characteristics of iNKT Cells

The invariant NKT (iNKT) cells are innate-like lymphocytes that are conserved in mice and humans and share phenotypic and functional characteristics with NK cells and T cells, connecting the innate and adaptive immune responses [1, 2]. iNKT cells have relatively constant TCR chains (Vα24-Jα18/Vβ11 in humans, Vα14-Jα18/Vβ8.2, Vβ7, Vβ2 in mice), which recognize glycolipid antigens and can be efficiently activated through recognition of α-galactosylceramide (α-GalCer) in the context of CD1d, a monomorphic MHC class I-like molecule [3–5]. After the activation, NKT cells produce large amount of Th1 and Th2 cytokines (IL-2, IL-4, IL-6, IL-10, IL-17, IFN-γ, and TNF-α), enabling them to act as powerful regulators of the immune system [1, 6], including modulating Th1/Th2 immune balance, and affecting the function of other immune cells such as T cells, B cells, DCs, and

Mengqing Cong and Xiang Li contributed equally to this work.

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macrophages [7]. iNKT cells also express typical markers of NK cells, including inhibitory and activated killer receptors, such as NK1.1, CD16, and CD122, exerting cytotoxic effects in certain condition [8].

1.2 The Function of iNKT

iNKT cells play a crucial role in a number of immune-related diseases. They not only help the antitumor immunity and anti-infection effect but also induce immunosuppression in a series of autoimmune diseases and organ transplantation rejection, such as type 1 diabetes mellitus, autoimmune encephalomyelitis (EAE), multiple sclerosis (MS), systemic lupus erythematosus (SLE), and rheumatoid arthritis (RA) [8–11]. In addition, iNKT cells play an indispensable role in immune and metabolic-related diseases, including obesity, type 2 diabetes, and cardiovascular diseases (CVD) [12].

1.3 iNKT Cells Development

iNKT precursor cells are derived from thymus $CD4^{+}CD8^{+}$ double-positive cells and escaped the traditional T cell development pathway [13]. However, they still need to go through positive selection and negative selection to obtain appropriate, semi-invariant and CD1d-dependent TCR and self-tolerance [14, 15]. In addition to positive selection and negative selection, iNKT cells then mature through the following four stages based on the different expression of cell surface proteins CD24, CD44, and NK1.1, including stage 0 ($CD24^{+}CD44^{lo}NK1.1^{lo}$), stage 1 ($CD24^{-}CD44^{lo}NK1.1^{lo}$), stage 2 ($CD24^{-}CD44^{hi}NK1.1^{lo}$), and stage 3 ($CD24^{-}CD44^{hi}NK1.1^{+}$) [16, 17]. During the process of maturation, some iNKT cells begin to leave thymus and migrate into peripheral organs for further maturation at stage 2 [18, 19].

1.4 The Subtypes of iNKT Cells

According to the different transcription factors and cell functions, iNKT cells are distributed in the peripheral tissues in the form of at least three major subpopulations (NKT1, NKT2, and NKT17 cells) and play various roles by releasing different cytokines (inflammatory and anti-inflammatory) [20–24]. The frequency and subtypes of iNKT cells in different tissues and organs of mice are variable greatly, which also determines the regional immune characteristics of iNKT cells. Different organs have different isolation methods. Here, we describe a modified version of this protocol about isolating iNKT cells from the spleen, liver, and thymus, based on previous report [25, 26]. We compared the percentage and number of total iNKT cells in the thymus, spleen, and liver of C57BL/6, $CD1d^{-/-}$, and $J\alpha18^{-/-}$ mice by using CD1d tetramers and TCRβ to gate iNKT cells.

2 Materials

2.1 Mice

1. Wild-type C57BL/6J mice: Purchase from Charles River Laboratories.
2. CD1d$^{-/-}$ mice: On the C57BL/6 background were provided by Prof. Albert Bendelac.
3. Jα18$^{-/-}$ mice: On the C57BL/6 background were provided by Prof. Albert Bendelac.

2.2 Materials and Solutions

1. 75% alcohol.
2. 10× PBS: 80 g NaCl, 2 g KCl, 29 g $Na_2HPO_4 \cdot 12H_2O$ and 2 g KH_2PO_4 in 1 L ddH_2O.
3. 1× PBS: Diluted from 10× PBS by ddH_2O.
4. Hemolysis buffer: Beyotime, Cat #: C3702-120 Ml.
5. The stock solution of Percoll: GE Healthcare, Lot: 10276722.
   100% Percoll: Use Percoll stock solution with 10*PBS in accordance with volume ratio 9:1.

   40% Percoll: Diluted from 100% Percoll by 1× PBS.

   70% Percoll: Diluted from 100% Percoll by 1× PBS.
6. Antibodies:
   Purified anti-mouse CD16/CD32 antibody: BioLegend, Cat#: 101302, clone: 93, working dilution 1/200.

   PerCP/Cyanine5.5 anti-mouse CD45: BioLegend, Cat #: 103132, clone: 30-F11, working dilution 1/100.

   FITC anti-mouse TCR β chain antibody: BioLegend, Cat #: 109206, clone: H57-597, working dilution 1/100.

   PE anti-mouse CD24: BioLegend, Cat #: 138504, clone: 30-F1, working dilution 1/100.

   PerCP/Cyanine5.5 anti-mouse/human CD44 Antibody: BioLegend, Cat #: 103032, clone: IM7, working dilution 1/100.

   CD1d-tetramers: Provide by NIH, working dilution 1/200.

   PE/Cyanine7 anti-mouse NK-1.1 Antibody: BioLegend, Cat #: 108714, clone: PK136, working dilution 1/300.
7. DAPI: Sigma-Aldrich, Cat #: D9542, working concentration: 2 μg/mL.
8. 200 mesh iron gauze net (70 μm strainer): Purchase from Consumables Company in China.
9. 200 mesh nylon mesh (70 μm strainer): Purchase from Consumables Company in China.

10. Scissors and tweezers: Purchase from Consumables Company in China.
11. 1 mL syringe plunger: Purchase from Consumables Company in China.
12. 1.5 mL Eppendorf tube: Purchase from Consumables Company in China.
13. 15 mL and 50 mL centrifuge tube: NEST company.
14. Hemocytometer chamber: Purchase from Consumables Company in China.
15. FACS tubes: BD, cat#: 352008-Falcon.

2.3 Equipment

1. Cryogenic Centrifuge: Xiangyi, L535R.
2. Vortexer: DLVB.
3. FACS DIVA: BD FACSVerse.

2.4 Software

1. FlowJo™ 10 software.
2. GraphPad Prism 7.04.

3 Methods

3.1 Preparation of Tissue Samples from C57BL/6, CD1d$^{-/-}$, and Jα18$^{-/-}$ Mice

1. 8 to 12 weeks male C57BL/6, CD1d$^{-/-}$, or Jα18$^{-/-}$ mice are used as experimental mice (5 mice in each group).
2. Mice are euthanized via cervical dislocation, and the abdominal fur of mice is disinfected with 75% alcohol, the liver and spleen in abdominal cavity and thymus in the chest are carefully harvested by dissection tools, and put them in 1 x PBS on ice for later use.

3.2 Isolation of Splenocytes

1. Put 200 mesh iron gauze net (70 μm strainer) on the 50 mL centrifugal tube, cut the spleen separated in Subheading 3.1 step into several small segments with scissors and apply the spleen on it, rinse the strainer with 1× PBS, splenocytes are collected in PBS and gently homogenize through a 70 μm cell strainer using the hard end of a syringe plunger, and keep rinsing the strainer with PBS while homogenizing the spleen until all the residual tissues are white connective tissue (*see* **Note 1**). Centrifuge at 650 × *g* for 10 min at 4 °C.
2. After centrifugation, remove the supernatant, and add 1 mL hemolysis buffer; incubate 5 min at room temperature to lyse redundant erythrocytes (*see* **Note 2**).
3. After RBCs fully lysis, 10 mL 1× PBS is added to terminate the lysis and then passed through a 70 μm cell strainer to obtain a single cell suspension. Centrifuge at 650 × *g* for 10 min at 4 °C,

and collect cell pellet at the bottom of the tube, washing cells with 1× PBS again.

4. The cells at the bottom of the tube are splenocytes. Resuspend cells with an appropriate volume of 1× PBS, cells are counted using a hemocytometer chamber, and stored at 4 °C for subsequent FACS analysis.

3.3 Isolation of Thymocytes

1. Put 200 mesh iron gauze net (70 μm strainer) on the top of the 50 mL centrifuge tube, cut the thymus separated in Subheading 3.1 step into several small segments with scissors, rinse the gauze with 1× PBS, thymocytes are collected in PBS and gently homogenized through a 70 μm cell strainer using the hard end of the syringe plunger, and keep rinsing the strainer with PBS while homogenizing the thymus until all the residual tissues are white connective tissue (*see* **Note 1**). Centrifuge at 650 × *g* for 10 min at 4 °C.
2. Cells pellet at the bottom of the tube are thymocytes. Resuspend cells with an appropriate volume of 1× PBS. Cells are counted using a hemocytometer chamber and stored at 4 °C for subsequent FACS analysis.

3.4 Harvest Lymphocytes from the Liver

1. Experimental preparation: the stock solution of Percoll is mixed with 10× PBS at 9:1 ratio to acquire 100% Percoll, which is further diluted to 40% Percoll with 1× PBS (*see* Subheading 2.2, **item 5**).
2. Put 200 mesh iron gauze net (70 μm strainer) on the top of the 50 mL centrifuge tube, cut the liver separated in Subheading 3.1 into several small segments with scissors, rinse the gauze with 1× PBS, liver single cells are collected in PBS and gently homogenize through a 70 μm cell strainer using the hard end of the syringe plunger, and keep rinsing the strainer with PBS while homogenizing the liver until all the residual tissues are connective tissue (*see* **Note 1**).
3. Add 1× PBS up to 50 mL, centrifuge at 50 × *g* for 2 min at 4 °C, and only keep the cell supernatant.
4. Repeat **step 3** for 2–3 times to remove hepatocytes to the most extent, and only keep the cell supernatant, then centrifuge at 650 × *g* for 10 min at 4 °C, and collect cells at the bottom of the tube.
5. Cells are resuspended with 3–4 mL 40% Percoll solution and then added on 3 mL 70% Percoll along the wall of centrifugal tube carefully; keep the interface between 40% and 70% Percoll clear and no shaking as far as possible (*see* **Notes 3** and **4**). Centrifuge at 1260 × *g* for 30 min at room temperature, with the speed increased by 6 and decreased by 2.

6. After centrifugation, cells at the interface between 40% and 70% Percoll layer are retained and resuspended with 1× PBS, centrifuge at 650 × *g* for 10 min at 4 °C (*see* **Note 5**). Cells at the bottom of the tube were collected and washed again with 1× PBS.
7. Cells pellet at the bottom of the centrifuge tube are liver lymphocytes, which are resuspended with 1× PBS, counted using a hemocytometer chamber and stored at 4 °C for subsequent FACS analysis.

3.5 Detection iNKT Cells by Flow Cytometry

1. Take the spleen, thymus, and liver lymphocytes prepared in Subheadings 3.2, 3.3, and 3.4, respectively. Adjust the number of cells per test ranging from 2×10^5 to 1×10^6 cells in 100 μL PBS each tube.
2. Purified anti-mouse CD16/CD32 antibody was added to each sample in **step 1** (dosage see antibodies in Subheading 2.2, **item 6**), which blocks antibodies unspecific binding sites on cells, and incubated at 4 °C for 15 min.
3. The combined flow cytometric antibodies (dosage see antibodies in Subheading 2.2, **item 6**) were added into each tube. The antibodies used in the study are listed here: PerCP/Cyanine5.5 anti-mouse CD45, FITC anti-mouse TCR β chain antibody, PE anti-mouse CD24, PerCP/Cyanine5.5 anti-mouse/human CD44 Antibody, CD1d-tetramers, and PE/Cyanine7 anti-mouse NK1.1 Antibody. Antibodies are added into each sample in **step 2** and incubated in dark at 4 °C for 45 min (*see* **Note 6**).
4. Wash with 1 mL 1× PBS, and then centrifuge at 650 × *g* for 10 min at 4 °C. Collect cells at the bottom of the tube, and resuspend with 200 μL 1× PBS. Cells are filtered into the flow tube through 200 mesh nylon mesh and placed on the ice for recording by flow cytometry.
5. 5 min before flow cytometry record, DAPI (used to distinguish dead and living cells) is added and mixed well. Then record cells by FACS DIVA.
6. All flow cytometry data are analyzed with FlowJo™ 10 software and GraphPad Prism 7.04 software.

3.6 Representative Results

We detected the percentage and number of total iNKT cells in the thymus, spleen, and liver of C57BL/6, CD1d$^{-/-}$, and Jα18$^{-/-}$ mice by using CD1d-tetramers and TCR β to label iNKT cells. The scheme of the iNKT gating in the liver, spleen, and thymus of C57BL/6, CD1d$^{-/-}$, and Jα18$^{-/-}$ mice is shown in Fig. 1, CD1d-tetramer$^+$ TCRβ$^+$ cells are considered as iNKT cells. The detailed proportions and numbers of total iNKT cells in the liver,

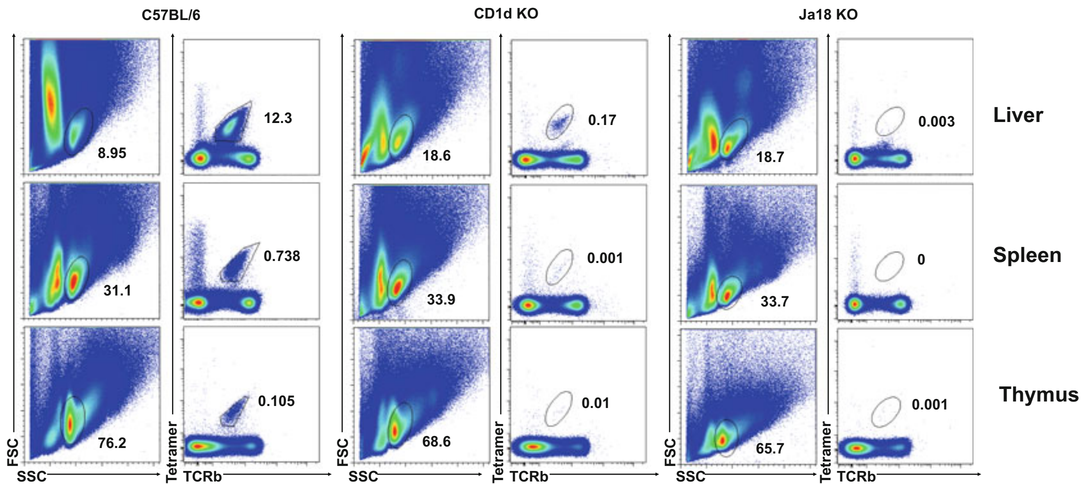

Fig. 1 Scheme of the iNKT gate in the liver, spleen, and thymus of C57BL/6, CD1d$^{-/-}$, and Jα18$^{-/-}$ mice. Five male C57BL/6, CD1d$^{-/-}$, or Jα18$^{-/-}$ mice aged from 8 to 12 weeks were sacrificed, removing the liver, spleen, and thymus to harvest the single cell suspension. iNKT cells were gated on CD1d-tetramer^{+} TCRβ^{+} by FACS

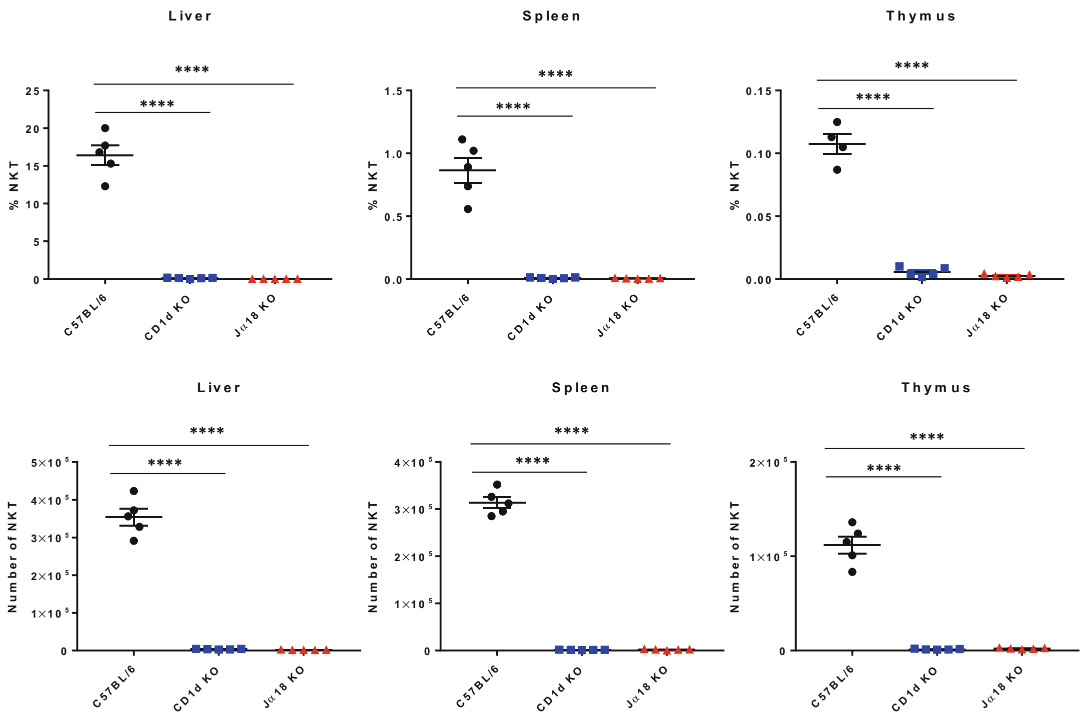

Fig. 2 The proportion and number of total iNKT cells in the liver, spleen, and thymus of C57BL/6, CD1d$^{-/-}$, and Jα18$^{-/-}$ mice. The liver, spleen, and thymus were harvested, and cells were stained directly ex vivo by CD1d tetramers and TCRβ, showing the proportion and number of iNKT cells in CD45^{+} cells. Symbols represent individual animals. Data are shown by mean plus SEM. Significant was determined by unpaired *t*-test ($n = 5$ mice per group). * $p < 0.05$,*** $p < 0.001$, *** $p < 0.001$,**** $p < 0.0001$

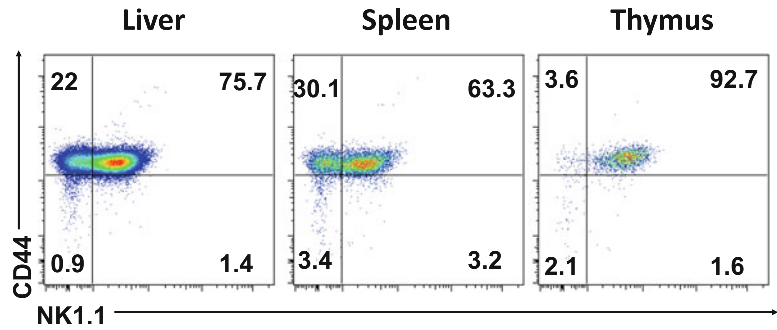

Fig. 3 The stage of iNKT cell development in the liver, spleen, and thymus of C57BL/6 mice. C57BL/6 mice aged from 8 to 12 weeks were sacrificed, removing the liver, spleen, and thymus to harvest the single cell suspension and detecting the developmental stage of iNKT cells by FACS according to using anti-mouse CD24, CD44, and NK1.1 antibody. NKT stage 0 and 1, $CD44^{lo}$ $NK1.1^{lo}$; NKT stage 2, $CD44^{hi}$ $NK1.1^{lo}$; NKT stage 3, $CD44^{hi}NK1.1^{+}$

spleen, and thymus of C57BL/6, $CD1d^{-/-}$, and $J\alpha18^{-/-}$ mice are shown in Fig. 2. Moreover, we also detected the stage of iNKT cell development in the liver, spleen, and thymus of C57BL/6 mice which divided into four stages according to the different expression of cell surface proteins CD24, CD44, and NK1.1: stage 0 ($CD24^{+}CD44^{lo}NK1.1^{lo}$), stage 1 ($CD24^{-}CD44^{lo}NK1.1^{lo}$), stage 2 ($CD24^{-}CD44^{hi}NK1.1^{lo}$), and stage 3 ($CD24^{-}CD44^{hi}NK1.1^{+}$). Due to very few iNKT cells in these organs, we did not detect the iNKT cells development in $CD1d^{-/-}$ and $J\alpha18^{-/-}$ mice (Fig. 3).

3.7 Discussion

The invariant NKT (iNKT) cells bridge the gap between the innate and adaptive immune responses and played an important role in both human and mouse physiology and disease. iNKT was distributed variously in different tissues, which also determines the regional immune characteristics of iNKT cells. Here, we detect the proportions, numbers, and developmental stage of iNKT cells in the thymus, spleen, and liver of C57BL/6, $CD1d^{-/-}$, and $J\alpha18^{-/-}$ mice. Our results showed that the proportions and numbers of iNKT were abundant in the liver of C57BL/6 mice, occupying around 20% of total immune cells, while that in spleen and thymus is only about 1% (Figs. 1 and 2). Moreover, in the liver and spleen of C57BL/6 mice, iNKT is mainly at stage 2 and stage 3, indicating most iNKT cells are mature in these organs. In the thymus, almost all iNKT cells in the thymus are at stage 3, rather than other immature stages (Fig. 3). During the development of iNKT, TCR rearrangement and positive and negative selection are required, which Jα18 and CD1d play an indispensable role [27]. Once lost the gene of Jα18 or CD1d, iNKT cells fail to

reach maturity and go through death. Our results also confirm this result, because in CD1d$^{-/-}$ or Jα18$^{-/-}$ mice, it is hard to detect iNKT cells in the liver, spleen, and thymus (Fig. 3).

Notably, considering the inconvenient of liver perfusion and there was no significant difference between the effect of traditional grinding liver and liver perfusion on iNKT separation, we prefer to use the traditional liver grinding method to isolate liver iNKT cells.

In summary, we provide a modified protocol and basic data about NKT cells distribution in different organs for further NKT research.

4 Notes

1. During homogenizing the spleen, thymus, and liver through a 70 μm cell strainer using the hard end of a syringe plunger, pay attention to vertical grinding up and down, rather than grinding left and right, and wash the isolated single cells into the centrifuge tube in time to prevent the secondary damage to the cells by grinding (*see* Subheading 3.2, **step 1**, Subheading 3.3, **step 1**, and Subheading 3.4, **step 2**).
2. When lysis RBCs, keep in mind of homogenizing the cells by vortex immediately to ensure sufficient lysis of red blood cells, where the cell suspension change from turbid to clear (*see* Subheading 3.2, **step 2**).
3. In order to have an effective separation of lymphocytes, Percoll should be kept at room temperature; otherwise, low temperature will affect the density of Percoll (*see* Subheading 3.4, **step 5**).
4. In order to keep the interface between 40% and 70% Percoll clear and no shaking, the centrifuge tube should be tilted, and 40% Percoll should be added on 70% Percoll at a constant speed, which is beneficial to the effective separation of lymphocytes (*see* Subheading 3.4, **step 5**).
5. Collecte the cells at the edge of the interface of 40% and 70% percoll layer, and dilute the collected cell suspension with 1 x PBS as much as possible and mixing it well, which can prevent the loss of target cells as much as possible (*see* Subheading 3.4, **step 6**).
6. In order to reduce the operation error between samples, mix the antibodies as much as possible, and then add it to each sample evenly (*see* Subheading 3.5, **step 3**).

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Chapter 6

Identifying, Isolation, and Functional Use of Human Liver iNKT Cells

Wenjing He, Dongmei Ye, and Yifang Gao

Abstract

It is widely accepted that iNKT cells are abundant in the liver and play a role in various liver disorders. In here, we describe an optimized protocol in identifying and isolating invariant natural killer T (iNKT) cells by magnetic beads to further use in functional assays.

Key words iNKT, Proliferation, Flow cytometry

1 Introduction

In recent years, innate T cells have been increasingly recognized as an important cell group in regulating immune responses. Three major populations within the innate T cell group are known, namely, invariant NKT cells (iNKT cells), mucosal associated invariant T cells (MAIT cells), and gamma delta T cells (γδ T cells) [1]. Unlike their conventional counterparts, these cells rapidly recognize foreign pathogen signals and manifest immediate effector functions post-activation. This allows innate T cells to perform effector immune responses much earlier than conventional T cells.

Variations in the frequency of these cells have been found in numerous disease compared to healthy controls. For example, iNKT cells are one of the most well-studied innate T cell populations; their frequencies are significantly reduced in multiple sclerosis, systemic lupus erythematosus, rheumatoid arthritis, and cancer, compared with healthy controls [2]. Studies also found a lower percentage of gamma delta T cells in various cancer states [3]. As the newest member in this family, MAIT cells were found to be decreased in varies infectious disease including HIV and HCV [4, 5].

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It is widely accepted that iNKT cells are abundant in liver and highly conserved in mammals. The percentage of these innate T cells could direct or indirectly influence the outcome of immunotherapy for cancer and/or autoimmunity. [6–8] The number of certain innate T cells, i.e., iNKT cells, has become an entry criteria for certain trials [9–11]. Hence, an efficient and reliable analysis of percentage of these innate T cells is important. So that it can be used for monitoring the number of these cells in patient using limited amount of sample.

Multi-parameter flow cytometry base assay has been the main tool to study the innate T cells. Analysis of these cells is particularly challenging since their frequency among peripheral blood T cells is relatively low, i.e., the mean percentage of iNKT cells is around 0.1% (ranging from less than 0.01% to 1%). In current practice, iNKT cells are identified with either TCR markers Vα24/Vβ11 or CD1d tetramer, whereas MAIT cells are identified with Vα7.2/CD161, Vα7.2/IL-18R, or MR1 tetramer [12]. In here, we described an optimized protocol in isolating invariant natural killer T(iNKT) cells by magnetic beads to further use in functional assays.

2 Materials

1. Human iNKT cell isolation kit (6B11; Miltenyi Biotec).
2. Human recombinant protein IL-2 (PeproTech).
3. MACS isolation buffer (Miltenyi Biotec).
4. KRN7000(Avanti).
5. PMA (Sigma)/ionomycin (Sigma).
6. Brefeldin A (Invitrogen).
7. Ficoll-Paque (GE Healthcare).
8. Phosphate-buffered solution(Sigma, 0.01 M phosphate buffer + 0.0027 M potassium chloride + 0.137 M sodium chloride, pH 7.2–7.6).
9. Collagenase from *Clostridium histolyticum* (Sigma).
10. CellBanker 2 (Zenoaq).
11. FACS lysing solution (BD).
12. Antibodies: 20 μl Vα24 FITC (C15) (Beckman Coulter, USA); 20 μl Vβ11 PE (C21) (Beckman Coulter, USA); 20 μl CD161 APC (DX21) (BD, USA); 20 μl Vα7.2 PE (3C10) (BioLegend, USA); 5 μl of TCRγδ Pacific Blue (B1) (BD, USA); 20 μl of CD3 PerCP (SK7) (BD, USA); 5 μl CD8β APC (2ST8.5H7) (BD, USA); 5 μl CD8α AmCyan (SK1) (BD, USA);5 μl CD4 PE-Cy7 (RPA-T4) (BD, USA); Purified anti-human CD3(BD, USA); Purified anti-human CD28(BD, USA).

13. 70 μm nylon mesh, sterile (Sorfa).
14. FACSCanto with FACS Diva software (BD).
15. BD Multicolor CompBeads for compensation (BD).

3 Methods

3.1 Sample Collection and Preparation

Obtain human liver specimens from surgical specimens. Liver specimens should be stored at room temperature for a maximum of 4 h before performing the assay. For analysis of the stability of the marker, blood samples can be stored at CellBanker 2 solution at 4 °C and process as soon as possible.

3.2 Liver Cells Preparation

Process liver specimens as described previously [3].

1. Perfuse tissues with 1 mg/ml collagenase at 37 °C for 20 min to dissociate collagen fibrils in connective tissues (Fig. 1).
2. Mechanically dissociate undigested tissues by passing through a 70 μm mesh.

3.3 Isolation of Mononuclear Cells from Liver Sample

The mononuclear cells from liver specimens can be isolated by density gradient as follows:

1. Add Ficoll-Paque (15 ml) to a 50 ml centrifuge tube.
2. Warm up RPMI1640 medium at 37 °C.
3. Mix the meshed liver cells with 30 ml RPMI1640, and carefully layer the diluted sample (30 ml) on Ficoll-Paque (*see* **Note 3**).
4. Centrifuge at 400 × *g* for 25–30 min at 18–20 °C.
5. Carefully remove the sample from centrifuge without disturbing the layers and collect the interphase from each sample (cloudy layer with clear layer below and straw layer above) into 50 ml (Falcon) tubes.

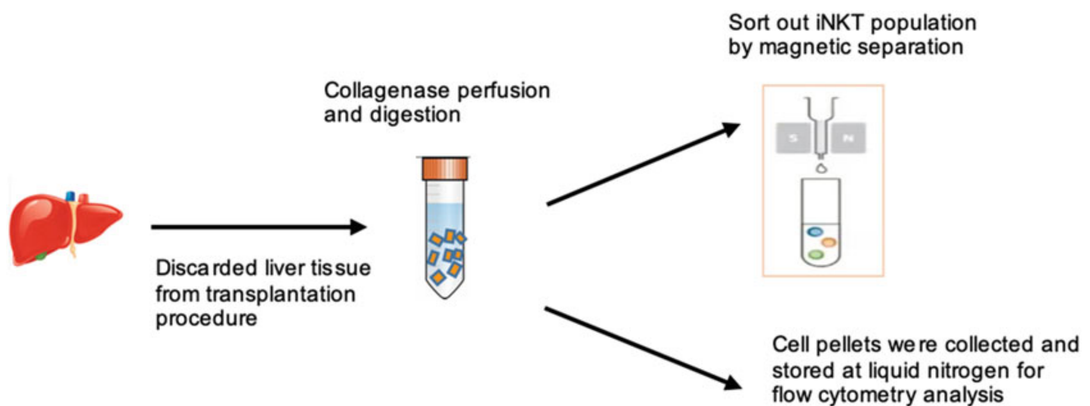

Fig. 1 Schematic diagram of liver iNKT separation

6. Add PBS (phosphate buffered solution) to make up to 50 ml. Spin at 300 × *g* for 5 min, discard the supernatant.
7. Resuspend the cells by adding pre-warmed RPMI1640.
8. Isolate iNKT cells.
   (a) Suspend the cells in 1 ml cold MACS isolation buffer, and isolate the iNKT cells using the human iNKT cell isolation kit, according to the manufacturer's protocols (*see* **Note 2**).
   (b) Centrifuge the purified iNKT cells for 5 min at 400 × *g* and resuspend in 2 ml RPMI1640 medium; count the cells and use for functional assay.

3.4 One Tube Innate T Cells for Identifying iNKT Cells and MAIT Cells

1. Add a minimum of 10^5 cells into 5 ml polystyrene FACS Tubes.
2. Stain samples with the anti-human antibodies for 15 min at room temperature per recommended by the manufacturer:

   20 μl Vα24 FITC (C15) (Beckman Coulter, USA); 20 μl Vβ11 PE (C21) (Beckman Coulter, USA); 20 μl CD161 APC (DX21) (BD, USA), 20 μl Vα7.2 PE (3C10) (BioLegend, USA); 5 μl of TCRγδ Pacific Blue (B1) (BD, USA); 20 μl of CD3 PerCP (SK7) (BD, USA); 5 μl CD8β APC (2ST8.5H7) (BD, USA); 5 μl CD8α AmCyan (SK1) (BD, USA); and 5 μl CD4 PE-Cy7 (RPA-T4) (BD, USA). As a parallel comparison, tubes containing only iNKT cells (CD3/Vα24/Vβ11/CD161/CD4/CD8) and MAIT cells (CD3/Vα7.2/CD161/CD4/CD8) identification can also be labeled.
3. Add 3 ml of FACS lysing solution (BD) for an additional 15 min at 4 °C.
4. Wash samples twice with FACS buffer and analyze straight away.

3.5 Gating Strategy and Sample Analysis

1. Run samples on a FACSCanto with FACS Diva software.
2. For quality control, set up compensation using BD CompBeads for each experiment.
3. Identify lymphocyte population with FSC and SSC.
4. Gate to acquire 100,00 CD3 positive T cells, and then calculate the number of iNKT cells by gating on CD3+TCRγδ-Vα24+-Vβ11+, and it is recorded as percentage of alpha beta T cells.
5. Create a "NOT GATE" to further identify the MAIT cells.

   The number of MAIT cells can be calculated on the population of non-iNKT αβ T cells by gating on CD3+TCRγδ-Vα7.2+CD161+ T cells.
6. Analyze the further phenotype of all innate T cells (iNKT cells and MAIT cells) with CD4 and CD8 markers.

3.6 Cytokine Production by Innate T Cells

1. Incubate whole blood cultures with anti-CD3 (1 μg/ml)/CD28(1 μg/ml) or PMA (100 ng/ml)/ionomycin (100 ng/ml) for 6 h at 37 °C with 5% CO_2.
2. Add Brefeldin A (BFA, 5 μg/ml) after an hour initial activation.
3. Wash the cells with FACS washing buffer and label with monoclonal antibodies to surface molecules: CD3 PerCP; Vα7.2PE; Vβ11PE; CD161APC; IFNγ PE-Cy7; TCRγδ Pacific Blue; and CD8 AmCyan for 10 min at room temperature.
4. Lyse erythrocytes and fix leukocytes using FACS lysing solution at 4 °C for 10 min simultaneously.
5. Then wash cells with FACS wash buffer and permeabilize with permeabilization solution 2 to 10 min at room temperature.
6. Wash cells again with FACS wash buffer and stain for intracellular cytokine IFN-γ and iNKT TCR Vα24-FITC for 10 min at room temperature in the dark.
7. Wash cells two times with FACS wash buffer and proceed for acquisition.

3.7 Functional Assay of iNKT Cells

1. Suspend 5×10^5 LMC in 1 ml of complete RPMI1640 culture medium with 100 IU/ml of recombinant human IL-2 and 100 ng/ml of the KRN7000 (*see* **Note 1**).
2. Incubate cells at 37 °C with 5% CO_2 for 7 days.
3. Assess proliferation of iNKT cells at day 7 using the FACS panel. Proliferation index can be calculated by comparing the total number of iNKT cells pre-proliferation to the total number of iNKT cells post-proliferation.

4 Notes

1. The high concentration of IL-2 and KRN7000 are critical for the expansion of iNKT cells. Otherwise, more exhausted iNKT cells will be found in the system.
2. The number of iNKT cells in the liver specimen varied from case to case. In a typical liver tissue, it can make up around 10–20% of the liver mononuclear cells. However, the number will be much lower in certain liver disorders. It should pay attention that the purity of iNKT cells will be much lower in the tissues with low numbers of iNKT cells. In this case, FACS sorting can further increase the purity of the sorting.
3. If performing cell to cell co-culture in liver system, Percoll methods is preferred compared to Ficoll. Percoll gradient will allow isolate hepatic non-parenchymal cells. Cells could perform in various densities.

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Chapter 7

Isolation and Characterization Methods of Human Invariant NKT Cells

Liu Rui and Wang Hua

Abstract

Natural killer T cells (NKT) are abundant in the hepatic sinuses and account for about 20–50% of rat liver lymphocytes. Type I or invariant NKT cells (iNKT) exert a powerful pro-inflammatory effect when activated, while type II NKT cells are more heterogeneous and mainly play an immunomodulatory role. Here we mainly introduced the isolation and characterization methods of human invariant NKT cells. Through immunomagnetic beads and flow cytometry, iNKT cells can be isolated specifically, and that explains functional analysis can be further established.

Key words Invariant NKT cells (iNKT), Isolation, Characterization, Immunomagnetic beads, Flow cytometry

1 Introduction

Conventional $CD4^+$ and $CD8^+$ T cells are the most studied T cell subgroups, but the nonconventional T cells have become a hot topic in recent years, and their number and influence are more abundant than previously thought [1, 2]. The main subpopulations of unconventional T cells include natural killer T (NKT) cells, mucosal-associated invariant T (MAIT) cells and invariant T cells; in general, these T cells account for about 10% of circulating T cells, usually they are the majority of T cells in tissues such as liver and intestinal mucosa [3]. Natural killer T cells (NKT) are abundant in the hepatic sinuses and account for about 20–50% of rat liver lymphocytes [4].

In recent research, iNKT cells have been shown to play an important role in inflammation, fibrosis, tissue repair, viral infection, and tumor immunology. For example, iNKT cells can directly kill tumor cells through antigen recognition or enhance the antitumor response by depleting tumor-associated macrophages (TAM) and promoting the cytotoxic T lymphocytes (CTLs) and

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natural killer (NK) cells activation [5]. iNKT are mostly $CD4^+$ or $CD4^-$ $CD8^-$ ("double negative"), although a few $CD8^+$ iNKT can be found in some humans, and they are innate-like CD1d-restricted T cells that express the invariant T cell receptor (TCR) composed of Vα24 and VB11 in humans [6]. In this article, we detail the isolation, in vitro expansion, and functional characterization methods of human iNKT cells.

2 Materials

Use ultrapure water (prepared by purified deionized water, with a sensitivity of 18 MΩ-cm at room temperature) and analytical grade reagents to prepare all solutions. Prepare and store all reagents (unless otherwise noted) at 4 °C. When disposing of waste, all waste disposal regulations must be carefully observed. Sodium azide is not added to the reagents.

2.1 Isolation of Invariant NKT Cells by Immunomagnetic Beads

1. Human blood (heparinized blood).
2. Ficoll-Hypaque solution.
3. Phosphate-buffered saline (PBS).
4. PBS/EDTA: PBS with 2 mM EDTA.
5. FcR-blocking reagent (Human IgG).
6. Binding buffer: PBS (APPENDIX 2A) with 2 mM EDTA and 2% (v/v) human serum.
7. α-GalCer [7].
8. DMSO freezing mixture: 90% FBS/10% DMSO.
9. T cell medium (*see* **Note 1**): RPMI-1640 supplemented with 10% fetal bovine serum (FBS), 15 mM HEPES, 1× non-essential amino acids, 1× essential amino acids, 4 mM glutamine final (including additional 2 mM from standard medium), 5.5×10^{-5} M 2-mercaptoethanol, 10μg/ml gentamicin, 100 IU/ml human IL-2, 4 °C once IL-2 has been added.
10. Unconjugated or PE-conjugated anti-Vα24 mAb (Coulter).
11. Unconjugated or PE-conjugated 6B11 anti-invariant TCRα mAb.
12. Goat anti-mouse IgG or anti-PE microbeads (Miltenyi Biotec).
13. MS columns (for up to 10^8 starting cells; Miltenyi Biotec).
14. LS columns (for up to 10^8 starting cells; Miltenyi Biotec).
15. Magnetic separation device (Miltenyi Biotec).

    The above reagent can be replaced by direct 6B11-conjugated microbeads/iNKT kit (Miltenyi Biotec). Alternatives to α-GalCer are PHA-P (Difco) or mitogenic CD3 mAb.

2.2 Isolation of Invariant NKT Cells by Flow Cytometry

See Subheading 2.1 (**steps 1–9**); for additional materials, see below:

1. Conjugated anti-Vα24 mAb (clone C15B2, PE, or FITC conjugate).
2. Conjugated 6B11 anti-invariant TCR mAb or α-GalCer (or its stable analog such as PBS-57)-loaded CD1d tetramer.
3. Conjugated anti-Vβ11 mAb (clone C21D2, Coulter FITC, or PE conjugates).
4. Conjugated isotype matched control mAbs (Coulter, PharMingen).
5. IgG1 isotype control for 6B11 or unloaded CD1d tetramer control conjugate.
6. FACS buffer: PBS with 1% human serum and 1% FBS.
7. Fluorescence-activated cell sorting (FACS) instrument.

2.3 Recognition and Quantitation of Invariant NKT Cells

See Subheading 2.2; for additional materials, see below:

1. Flow cytometry buffer (FC buffer): PBS (APPENDIX 2A) with 1% human serum, 1% FBS, 0.1% sodium azide.
2. PBS with 4% (w/v) paraformaldehyde.

3 Methods

3.1 Isolation of Invariant NKT Cells by Immunomagnetic Beads

Immunomagnetic beads can be used to isolate Vα24$^+$ or 6B11$^+$ T Cells [8, 9] from small amount of peripheral blood. Although this method is suitable for small samples, it is more efficient when the sample size is larger. The method described below can upregulate or downregulate the sample size based on samples of 10^8 PBMC cells.

1. Put fresh heparinized blood into a 15/50 ml conical centrifuge tube, add an equal volume of room temperature PBS, and mix well.
2. Centrifuge at 200 × *g* for 15 min at room temperature, and then remove the supernatant suspension containing platelets and cell debris. (When isolating cells from a leukapheresis donor, dilute blood with PBS (1:4 blood/PBS).)
3. Add an equal volume of PBS at room temperature.
4. Insert the tip of the pipet containing Ficoll-Hypaque deep into the bottom of the sample tube, and slowly inject the Ficoll-Hypaque solution at the bottom of the cell mixture (*see* **Note 2**).
5. Centrifuge at 2000 rpm (900 × *g*) for 20 to 30 min at room temperature with a deceleration rate of 0.

6. Remove the upper layer containing the plasma and most of the remaining cell platelets. Transfer the mononuclear lymphocyte cell layer (white turbid band between the plasma and Ficoll-Hypaque layer) to another centrifuge tube.
7. Wash the PBMCs twice, each time in 15 ml PBS/EDTA (*see* **Note 3**), 1000 × *g* centrifugation for 6–10 min, room temperature or chilled, resuspend in binding buffer at 10^8 cells/ml, and place on ice for 15 min.
8. Add Vα24 or 6B11 monoclonal antibody (*see* **Note 4**) to the remaining cells at a concentration of approximately 10μg/ml, and incubate on ice for 20 to 30 min.
9. Wash the cells twice with 1.5 ml PBS/EDTA, 1000 × *g* each time, centrifuge at room temperature or chilled for 6–10 min, and resuspend 10^8 cells in 0.8 ml binding buffer. Add 0.2 ml goat anti-mouse IgG beads, incubate for 20–30 min, and shake on ice.
10. Pre-wash the Miltenyi column with 3 ml binding buffer and then assemble on magnet. Wash the cells twice with PBS/EDTA, resuspend in 1 ml binding buffer, and spread the cells on the column. Wash the column three times, using 3 ml PBS/EDTA each time, and collect the eluate (containing unbound cells). Equal portions of unbound PBMCs (10^7) are set aside for irradiation and use as feeder cells (**step** 7).
11. Remove the column from the magnet and elute bound cells with 3 ml PBS/EDTA. Wash the cells in 1.5 ml T cell culture medium, centrifuge at 1000 × *g* room temperature or chilled for 6–10 min, and resuspend in 0.1 ml T cell culture medium. Count viable cells in iNKT-enriched cell preparation. The recovered cells can be examined directly by flow cytometry to determine the success of the separation (*see* **Note 5**).
12. Add the same amount of irradiated autologous PBMCs (set aside in **step 5** as feeder cells) and purified iNKT-containing cell preparations to the wells of the 96-well plate (*see* **Note 6**). Add 20 ng α-GalCer and use T cell culture medium (final 100 ng/ml α-GalCer) to bring the final volume to 200μl/well. Autotrophic cells are preferred because they should not stimulate allogenic responses.
13. Without changing the medium, culture in a CO_2 incubator containing T cell culture medium for about 2 weeks. Then gently remove about 150μl and replace with fresh T cell culture medium. This step should be updated every 2 to 3 days until most of the hole is occupied. Cells could then be divided into 96-well plates in a ratio of 1:2, or if the growth rate is very wide, cells from the 96-well plate can be transferred to one of the 24-well plate.

14. Cultured by the above method, the cells will continue to proliferate for several weeks. When cell proliferation slows down or stops, cells can be restimulated selectively or with polyclonal mitogens. Or, if stationary cells need to be produced for functional assays, they can be switched to a low-IL-2 (10 U/ml) T-cell medium. In this case, IL-2 concentration should be gradually moved from 100 to 50 to 20 and gradually moved to 10 U/ml after the last week or so to avoid cell death.

3.2 Isolation of Invariant NKT Cells by Flow Cytometry

Flow cytometry sorting is an alternative method to immunomagnetic beads. FACS can also set multiple parameters, while magnetic beads usually can only use a single sort or sequence of negative and positive selection. This section introduces the application of FACS system to isolate iNKT cells. Reliable detection of very small Numbers of cells using flow cytometry requires the use of at least two antibodies and attention to blocking the nonspecific background.

1. Separate PBMCs from 50–100 ml of blood (see above) by Ficoll-Hypaque to obtain at least 10^8 cells. Set aside half for irradiation and use as feeder cells.
2. Centrifuge the remaining cells at $1000 \times g$ in 15 ml PBS/EDTA for 6–10 min at room temperature or chilled, resuspend at 10^7 to 10^8 cells/ml in FACS buffer, and place them on ice for 15 min (*see* **Note 4**).
3. Take out a part of the cells (10^6 cells in 0.1 ml), and then add 1μg of control FITC-bound mAb. Add FITC-Vα24 mAb to the remaining cells at a concentration of 10μg/ml and incubated on ice for 30 min.
4. Identify the $V\alpha24^+$ population and gate on these cells for sorting. In order to maximize cell viability, cells are sorted directly into a tube containing TCM.
5. Wash the sorted cells twice in T cell medium and culture with feeder cells set previously and α-GalCer as described above for the beads purification.

3.3 Recognition and Quantitation of Invariant NKT Cells

The content of iNKT cells in human peripheral blood is relatively low. Flow cytometry is usually used for identification and quantitative analysis of iNKT in human peripheral blood. CD3 mAb can most directly differentiate T cell populations and is included in the laser channel of multicolor FACS. 6B11mAb can detect Vα24-Jα18 CDR3 independently of TCRβ, so it is more selective than Vα24 or Vβ11. However, 6B11 may also have weak nonspecific binding to other monocytes that are mainly FcR^+. In this case, two specific labeled antibodies are of great significance for explicit recognition. Another staining scheme that can accurately identify iNKT is the double staining of Vα24 and Vβ11 mAB [10]. Vα24mAb can specifically recognize iNKT, as well as all $V\alpha24^+$ T cells, and the

combination with Vβ11 greatly improves the specificity. The experimental methods of the two staining schemes are basically the same, except that different binding antibodies are replaced. The following takes the 6B11mAb staining scheme to describe the specific steps.

1. Separate PBMCs from 50 to 100 ml of blood (see above) by Ficoll-Hypaque to obtain at least 10^8 cells. Set aside half for irradiation and use as feeder cells.
2. Centrifuge at $1000 \times g$, room temperature or chilled for 6–10 min, wash a total of 1×10^6 PBMCs twice with PBS, and then resuspend in the FC buffer for1×10^7 cells/ml. Incubate on ice for at least 15 min.
3. Divide 100μl of cell suspension in a special tube for flow cytometry.
4. Add PE-conjugated Vα24 and FITC-conjugated 6B11 and compatibly conjugated CD3 (*see* **Note 4**) and/or other mAbs to a 0.1 ml total volume. (The control should include a single isotype-matched non-specific monoclonal antibody and a monoclonal antibody that binds to each specific monoclonal antibody.)
5. Wash once with FC buffer and resuspend in 0.5 ml FC buffer for flow cytometry.
6. Analyze with flow cytometry within a few hours or fix the cells with 4% paraformaldehyde in PBS to preserve the sample.

In addition, there are still CD4, CD8, CD56, CD161, and NKG2D [11–13] which are often used to further define the markers of iNKT cells. These protocols are similar to the flow cytometric identification of iNKT cells mentioned above, and different combinations of markers can be used for personalized analysis.

4 Notes

1. TCM can be stored at 4 °C for a month and for a longer time without glutamine, 2-mercaptoethanol, or IL-2.
2. Use 3 ml Ficoll-Hypaque per 10 ml blood/PBS mixture. Use up to 10 ml Ficoll-Hypaque per 40 ml white blood cell/RBC/PBS mixture.
3. The second washing with FcR blocking agent may reduce background, especially when washing PBMC.
4. The concentration of antibody is determined according to the relevant instructions of the purchased manufacturer and product. Antibodies at the appropriate dilution should be filter sterilized through a 0.22μm filter just prior to use.

5. After cell isolation, flow cytometry should be performed to determine cell purity, proportion, and viability, especially when the expected iNKT cell line cannot be produced in subsequent culture.
6. Up to 10^4 per well in a round-bottomed 96-well plate, up to 10^5 per well in a flat bottom plate. In the absence or presence of autogenous APC, allogeneic cells can be used as a source of $CD1d^+$ APC due to the considerable specificity provided by α-GalCer.

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Chapter 8

Detection of Mouse Type I NKT (iNKT) Cells by Flow Cytometry

Vibhuti Joshi and Masaki Terabe

Abstract

Flow cytometry is an effective tool in immunology that uses laser as a light source to yield scattered and fluorescent light signals read by photomultiplier tubes or photodiodes for detection. Flow cytometry allows immunophenotyping using fluorescently conjugated antibodies for the identification of subgroups of immune cells at a single-cell level. Natural killer T (NKT) cells are CD1d-restricted T cells, which recognize lipid antigens, unlike conventional T lymphocytes that recognize peptide antigens presented by class I or class II MHC. The unique T cell receptor (TCR) of type I NKT or invariant natural killer T (iNKT) cells are comprised of an invariant α-chain that pairs with a limited repertoire of β-chains. Type I NKT cells play an essential role in the orchestration of the innate and adaptive immune responses against various diseases. Here, we will review the process of identifying mouse type I NKT cells by flow cytometry, which serves as a foundational technique for studying these cells.

Key words Spleen, Thymus, Lymph node, NKT cells, Flow cytometry, CD1d tetramers, Antibody

1 Introduction

Natural killer T (NKT) cells are unconventional T cells that recognize lipid antigens presented by a monomorphic nonclassical class I like MHC molecule, CD1d [1]. There are two main groups of NKT cells currently known: designated as type I or invariant (iNKT) and type II NKT cells. Type I NKT cells are characterized by a semi-invariant T cell receptor (TCR) α chain that is comprised of Vα14Jα18 gene segments in the majority of cells paired with Vβ8, 7, or 2 in mice and Vα24Jα18 with Vβ11 in humans. In type II NKT cells, both the α chain and β chain of the TCR are variant/diverse [2]. In mice, type I NKT cells have a frequency of approximately 0.5% of the T cell population in the blood and peripheral lymph nodes, 0.5% of T cells in the thymus, and 2.5% of T cells in the spleen, mesenteric, and pancreatic lymph nodes, and up to 30% of T cells in the liver [3]. Although NKT cells make up a small

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fraction of T cells, they play an important role in immune regulation of metabolic disorders, inflammation, infections, and cancer in part to their ability to quickly produce a wide range of cytokines upon activation leading to the regulation of innate and adaptive immune cell functions [4–7]. In this chapter, we will be focusing on the detection of the type I NKT cells.

Type I NKT cells can be classified based on surface marker expressions, cytokine productions, and transcription factor expressions. In mice, type I NKT cells mostly consist of $CD4^{+}$ single-positive and $CD4^{-}CD8^{-}$ double-negative (DN) cells [6]. Mouse studies have defined functional subsets based on the expression of the transcription factors PLZF, T-bet, and RORγ-t, produced from common progenitor cells which express transcription factor PLZF [8, 9]. Functional subsets of NKT cells, NKT1 (T-bet^{+}), NKT2 ($PLZF^{hi}$), and NKT17 (RORγ-t^{+}) cells, generally correspond to Th1, Th2, and Th17 of $CD4^{+}$ helper T cell subsets, respectively [9]. Additionally, IL-10 producing $E4BP4^{+}NKT10$ [10, 11], IL-21 producing Bcl-$6^{+}NKT_{FH}$ [12, 13], and $Foxp3^{+}NKT_{reg}$ cells have also been reported [14]. More recently, a high-dimensional single-cell analysis of thymic type I NKT cells has shown developmental type I NKT trajectories. They showed various type I NKT cell subsets with unique biology (designated iNKT0, iNKT17, iNKT2a, iNKT2b, iNKT1a, iNKT1b, iNKTb, and iNKTc) and, notably, did not identify NKT10 and NKT_{FH} subsets that have been previously identified in peripheral organs [7].

Detection of type I NKT cells by flow cytometry is an essential requirement for studying these cells. Although various methods have been used to identify type I NKT cells, the development of CD1d tetramers loaded with α-GalCer or its analogs has significantly enhanced the specificity of type I NKT cell detection through flow cytometry [15, 16]. Here, we will describe a method for identifying type I NKT cells from the mouse tissues by using flow cytometry. It is difficult to detect NKT10, NKT_{reg}, and NKT_{FH} subsets without specific stimulation, so in this chapter, we are only focusing on the detection of NKT1, NKT2, and NKT17 subsets of type I NKT cells.

2 Materials

2.1 Mouse Tissue Experiment Materials

2.1.1 Mice

C57BL/6 mice and BALB/c mice (7–8 weeks old) were used to perform all the protocols. Mouse lymphoid organs (spleen, thymus, and lymph nodes) were used to detect type I NKT cells in this chapter.

2.1.2 Media and Buffers

1. RPMI-1640. Store at 4 °C.
2. RPMI-1640 with 10% FBS. Store at 4 °C.

3. Phosphate-buffered saline (1x PBS). Store at room temperature.
4. Ammonium-chloride-potassium (ACK) lysis buffer. Store at room temperature.
5. Flow cytometry (FACS) buffer: Hank's buffered salt solution (HBSS) with 0.05% bovine serum albumin (BSA), 0.05% sodium azide (NaN_3). Store at 4 °C.
6. True-Nuclear™ Transcription Factor Buffer Set (BioLegend). Store at 4 °C.

2.1.3 Instruments and Plasticwares

1. Hemocytometer or cell counter.
2. Flow cytometer (we used MACS Quanta Analyzer 16 (Miltenyi Biotec) to obtain the data presented in this chapter).
3. High-speed table-top centrifuge.
4. 15 mL tubes.
5. 50 mL tubes.
6. Transfer pipette.
7. FACS tubes.
8. Cell strainer or nylon membrane (40μm pore size).
9. Petri dish (60 × 15 mm).

2.1.4 Antibodies, Staining Kit, and Other Fluorochrome-Labeled Reagents

1. LIVE/DEAD™ Fixable Aqua Dead Cell Stain Kit (Thermo Fisher Scientific). Store at −20 °C.

   Note: Reagents used for the staining should be titrated prior to the experiment.
2. Antibodies and other fluorochrome reagents used are summarized in Table 1.
3. Staining panels used in this experiment are shown in Table 2.

   Note: Fluorescence Minus One (FMO) control, which is the same as the full staining panel except there is one reagent removed, is frequently used to identify cells expressing a desired marker. Having this type of control is extremely important in studying a low frequency cell population like iNKT cells. However, since antibodies for intracellular or intranuclear staining frequently create a significant amount of signals in cells that do not express the marker, we did not use FMO in this experiment. Instead, we used Panel A which does not include reagents to stain intranuclear proteins to check the background signal level of gated type I NKT cells. Another useful approach to reduce a noise or background signal level is to use a cocktail of antibodies with the same fluorochrome against cell surface molecules that are known not to be expressed on iNKT cells such as anti-CD19, anti-CD11c, and CD11b.

Table 1
Antibodies and other fluorochrome-labeled reagents required for the detection of mouse type I NKT cells by flow cytometry

	Antibody	Fluorochrome	Clone	Company
1	Anti-CD3	FITC	17A2	BioLegend
2	Anti-CD4	Alexa Fluor 700	GK1.5	BioLegend
3	Anti-CD8α	PerCP-Cy5.5	53.6.7	BioLegend
4	Anti-T-bet	BV421	4B10	BioLegend
5	Anti-RORγ-t	PE	AFKJS-9	eBioscience
6	Anti-PLZF	PE-Cy7	9E12	BioLegend
7	PBS57/CD1d-tetramer	APC		NIH-tetramer Core Facility
8	TruStain FcX (anti-CD16/32)	–	93	BioLegend

Table 2
Staining panels for mouse type I NKT cell detection by flow cytometry

Panel A		
	Reagent	Fluorochrome
1	anti-CD3	FITC
2	anti-CD8α	PerCP-Cy5.5
3	PBS57/CD1d-Tetramer	APC
4	anti-CD4	Alexa Fluor 700
5	LIVE/DEAD	Aqua
Panel B (full staining panel)		
	Reagent	Fluorochrome
1	anti-CD3	FITC
2	anti-CD8α	PerCP-Cy5.5
3	PBS57/CD1d-tetramer	APC
4	anti-CD4	Alexa Fluor 700
5	LIVE/DEAD	Aqua
6	anti-RORγ-t	PE
7	anti-PLZF	PE-Cy7
8	anti-T-bet	Brilliant Violet 421

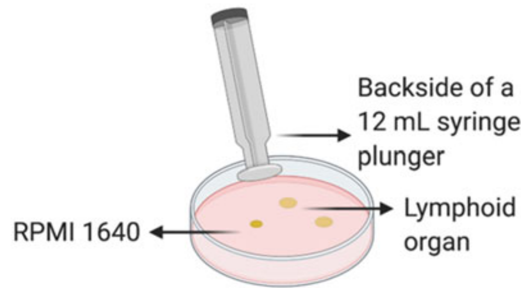

Fig. 1 Illustration of tissue mashing method. The figure depicts the method to mash lymphoid organs by using the backside of the plunger for preparing a single-cell suspension

3 Methods

3.1 Detection of Type I NKT Cells from Mouse Tissue by Flow Cytometry

3.1.1 Preparation of Single-Cell Suspension from a Mouse Spleen

1. Euthanize the mouse.
2. Harvest the spleen into the 60 × 15 mm petri dish filled with RPMI 1640 (2 mL) and mash it with the backside of the 12 mL syringe plunger (Fig. 1).
3. Loosen the cell aggregates by pipetting the cell suspension. After passing the cell suspension through a cell strainer using a transfer pipette, transfer the suspension into a fresh 15 mL tube.
4. Centrifuge at 300 × *g*, for 5 min at room temperature.
5. Discard supernatant and loosen the cell pellet.
6. Add 1–3 mL ACK lysis buffer by using a 5 mL pipette and mix gently 8–10 times; quickly add the fresh RPMI 1640 to make the total volume 15 mL.
7. Centrifuge at 300 × *g*, for 5 min at room temperature.
8. Discard supernatant, loosen the cell pellet well, and resuspend the cells in 10 mL of RPMI 1640.
9. Pass through a cell strainer.
10. Centrifuge at 300 × *g*, for 5 min at room temperature.
11. Discard supernatant, loosen the cell pellet well, and resuspend the cells in 10 mL of RPMI 1640.
12. Centrifuge at 300 × *g*, for 5 min at room temperature.
13. Repeat **steps 11** and **12**.
14. After discarding supernatant and loosening the cell pellet, resuspend the cells in an appropriate volume of RPMI 1640 with 10% FBS.
15. Count the cells.

3.1.2 Preparation of Single-Cell Suspension from the Thymus and Lymph Nodes

1. Euthanize the mouse and harvest the thymus and lymph nodes (subiliac and axillary) into two separate 60 × 15 mm petri dishes with approximately 2 mL of RPMI 1640 each. Mash the tissue with the backside of the 12 mL syringe plunger.

   Note: Approximately 1 × 10^6 cells will be obtained from one lymph node. Combining multiple lymph nodes is recommended to obtain an adequate number of cells if necessary.

   We used accessory axillary lymph nodes and subiliac lymph nodes [17] in the present analysis.

2. Loosen the cell aggregates by pipetting the cell suspension. After passing the cell suspension through a cell strainer using a transfer pipette, transfer the suspension into a fresh 15 mL tube.

   Note: Collect thymus cells in a 50 mL tube to minimize the damage of cells during centrifuge.

3. Centrifuge at 300 × *g*, for 5 min at room temperature.
4. Discard supernatant, loosen the cell pellet well, and resuspend the thymocytes and lymph node cells in RPMI 1640 with 10% FBS.
5. Count the cells.

3.1.3 Staining Dead Cells

The staining procedure is the same for all cell types. In the present protocol, we used LIVE/DEAD™ Fixable Aqua Dead Cell Stain Kit (Thermo Fisher Scientific); other similar types of dyes can be used to stain dead cells.

Thaw DMSO of the kit (Component B) while preparing cells.

1. Aliquot 1–3 × 10^6 cells into FACS tubes.
2. Add 1–2 mL of FACS buffer to each tube. Centrifuge at 300 × *g*, for 5 min at 4 °C, and discard supernatant.
3. Loosen the pellet and resuspend the cells in 1–2 mL PBS.
4. Centrifuge at 300 × *g*, for 5 min at 4 °C. Discard supernatant and loosen the pellet well.
5. Add 50μL of DMSO (Component B of the kit) to a vial of LIVE/DEAD dye powder (Component A) as recommended in the manufacturer's protocol. Mix well.

   It is recommended to use as soon as possible.

6. Add LIVE/DEAD dye to the cells and mix well. Incubate for 30 min at room temperature in the dark.
7. Add 1–2 mL FACS buffer and centrifuge at 300 × *g*, for 5 min at 4 °C.
8. Discard supernatant and loosen the pellet well.

3.1.4 Cell Surface Protein Staining

1. Add 1μg of TruStain FcX™ (anti-mouse CD16/32) antibody in each sample tube to block a Fcγ receptor that prevents nonspecific binding of IgG to cells.
2. Incubate for at least 10 min at 4 °C in the dark.
3. Without washing, add CD1d tetramers and incubate for 30 min at 4 °C in the dark.
4. Add antibodies to stain cell surface markers (anti-CD3, anti-CD4, anti-CD8α) and incubate again for 30 min at 4 °C in the dark.
5. After incubation, add 1–2 mL FACS buffer and centrifuge at 300 × *g*, for 5 min at 4 °C. Discard supernatant and loosen the pellet well.
6. Repeat **step 5** two more times.

3.1.5 Intranuclear Transcription Factor Staining

Different subsets of type I NKT cells in mice are detected by using various transcription factors. Staining of these transcription factors require special processing of samples with the True-Nuclear Transcription Factor buffer set from BioLegend.

It is critical to have well loosened the cell pellet before adding True-Nuclear™ 1× Fix buffer.

1. Add 1 mL of True-Nuclear™ 1× Fix buffer to each tube, vortex gently, and incubate for 60 min at room temperature in the dark. Follow manufacture's protocol to prepare True-Nuclear™ 1× Fix buffer.

   Note: We can suspend the protocol at this step by using Cyto-Last™ Buffer (BioLegend) and storing at 4 °C in the dark up to 12–18 h. After completion of **step 1**, centrifuge at 300 × *g*, for 10 min at room temperature. Loosen the pellet and resuspend the cells with 0.5 mL/tube Cyto-Last™ buffer. Store tubes at 4 °C in the dark up to 12–18 h. To resume the staining, centrifuge at 300 × *g*, for 10 min at room temperature and discard Cyto-Last™ buffer before proceeding to **step 2**.
2. Add 2 mL of the True-Nuclear™ 1× Perm Buffer to each tube. Follow manufacture's protocol to prepare True-Nuclear™ 1× Perm buffer.
3. Centrifuge at 300 × *g*, for 10 min at room temperature. Discard supernatant and loosen the pellet well.
4. Add 2 mL of the True-Nuclear™ 1× Perm Buffer to each tube. Centrifuge at 300 × *g* for 10 min at room temperature and discard supernatant.
5. Resuspend the cell pellet in 100μL of True-Nuclear™ 1× Perm buffer.

6. Add antibodies against transcription factors diluted in True-Nuclear™ 1× Perm buffer (anti-T-bet, anti-PLZF and anti-RORγ-t).
7. Incubate the tubes for 30 min at room temperature in the dark.
8. Add 2 mL of the True-Nuclear™ 1× Perm Buffer, and centrifuge tubes at 300 × *g*, for 10 min at room temperature.
9. Discard the supernatant and add 2 mL of FACS buffer.
10. Centrifuge the sample tubes at 300 × *g*, for 10 min at room temperature, and discard the supernatant.
11. Loosen the cell pellet and resuspend in 400μL of FACS buffer to acquire the data on a flow cytometer.

    Note: Always vortex the tube before acquiring a data on flow cytometer.

3.1.6 Data Acquisition

Data acquisition procedures vary among flow cytometers used to acquire data from the samples. We used MACS Quanta Analyzer 16 (Miltenyi Biotec), after performing the recommended calibration to check laser settings and cleaning procedure.

3.1.7 Data Analysis

The data was analyzed by the FlowJo software (Becton Dickinson and Company).

Type I NKT Cell Identification Gating Strategy

For type I NKT cell identification, first, gate on live cells by using the signals from LIVE/DEAD aqua and forward scatter area (FSC-A) channels. Then, select singlets among live cells by using forward scatter height (FSC-H) and FSC-A channels, and select lymphocytes among live singlets by using side scatter area (SSC-A) and FSC-A channels. Next, gate on type I NKT cells (iNKT) as PBS57-loaded CD1d tetramer positive and CD3 intermediate cells (Fig. 2).

The frequency of type I NKT cells in each lymphoid organ is different. It is also known that the frequencies are different among inbred strains of mice [8]. Here, we show a representative data from BALB/c and C57BL/6 mice organs (Fig. 3).

Identification of Type I NKT Cell Subsets

CD4 expression is one way to identify subsets of type I NKT cells. In contrast to conventional T cells, which can be identified as either $CD4^{+}CD8^{-}$ or $CD4^{-}CD8^{+}$, most mouse NKT cells are either $CD4^{-}CD8^{-}$ double-negative (DN) or $CD4^{+}$ (Figs. 2 and 3).

The three major functional subsets of type I NKT cells are NKT1, NKT2, and NKT17.

They can be identified by using combinations of three transcription factors PLZF, T-bet, and RORγ-t. NKT1, NKT2, and NKT17 cells are identified as $\text{T-bet}^{+}\text{ROR}\gamma\text{-t}^{-}$ PLZF^{low}, $\text{PLZF}^{hi}\text{ROR}\gamma\text{-t}^{-}\text{T-bet}^{-}$, and PLZF^{int} $\text{ROR}\gamma\text{-t}^{hi}$ T-bet^{-}, respectively (Figs. 2 and 4). The levels of PLZF expression are frequently

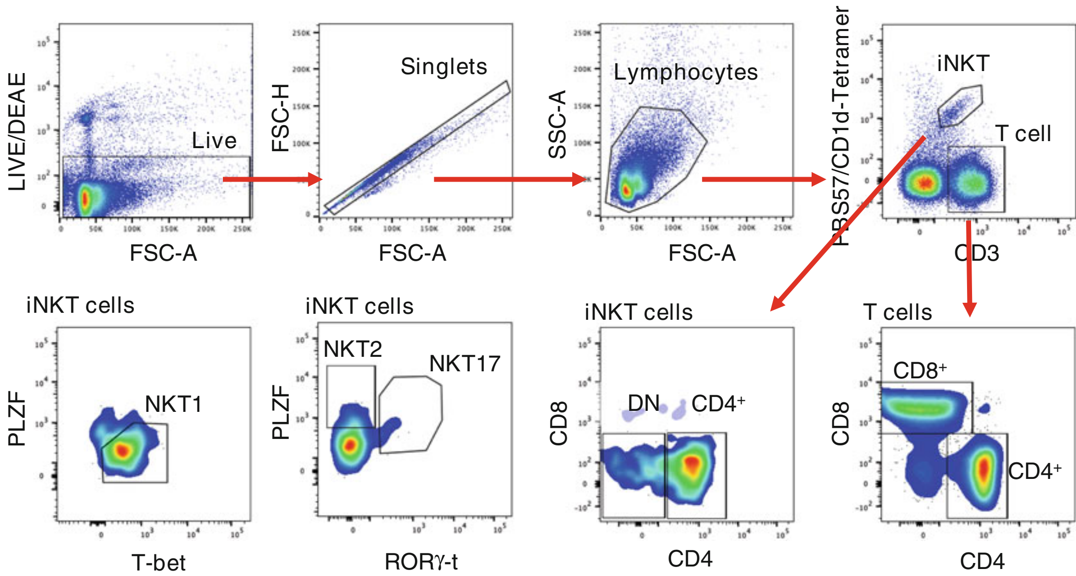

Fig. 2 A gating strategy of type I NKT cells and their functional subsets

observed as overlapping. Thus, it is not recommended to define the subsets simply based on PLZF expression levels. Here, we defined NKT2 and NKT17 by using PLZF and RORγ-t as $PLZF^{+}ROR\gamma\text{-}t^{-}$ and $PLZF^{int}ROR\gamma\text{-}t^{+}$ cells, respectively. NKT1 is better defined by using a two-dimensional plot with PLZF and T-bet as $PLZF^{lo}T\text{-}bet^{+}$ cells (Figs. 2 and 4).

Similar to the frequencies of type I NKT cells, the frequencies of NKT1, NKT2, and NKT17 are also different among lymphoid organs and among strains of mice (Fig. 4) [8, 9].

4 Notes

1. Titrating reagents for staining is highly recommend in order to use the optimal concentration of each reagent. Fluorochrome-labeled reagents used in this protocol have been optimized before the experiment.
2. PBS57 loaded on CD1d tetramers is an analog of α-GalCer which has been shown to have similar activity with α-GalCer (KRN7000) [18].
3. Prepare appropriate single strain control by cells or by beads to compensate fluorescence signals.
4. Loosening the cell pellet well after centrifuging is critical for all reagents to work optimally.
5. It is highly recommended to use 50 mL tubes for thymus cells centrifugation in order to reduce damage to cells.

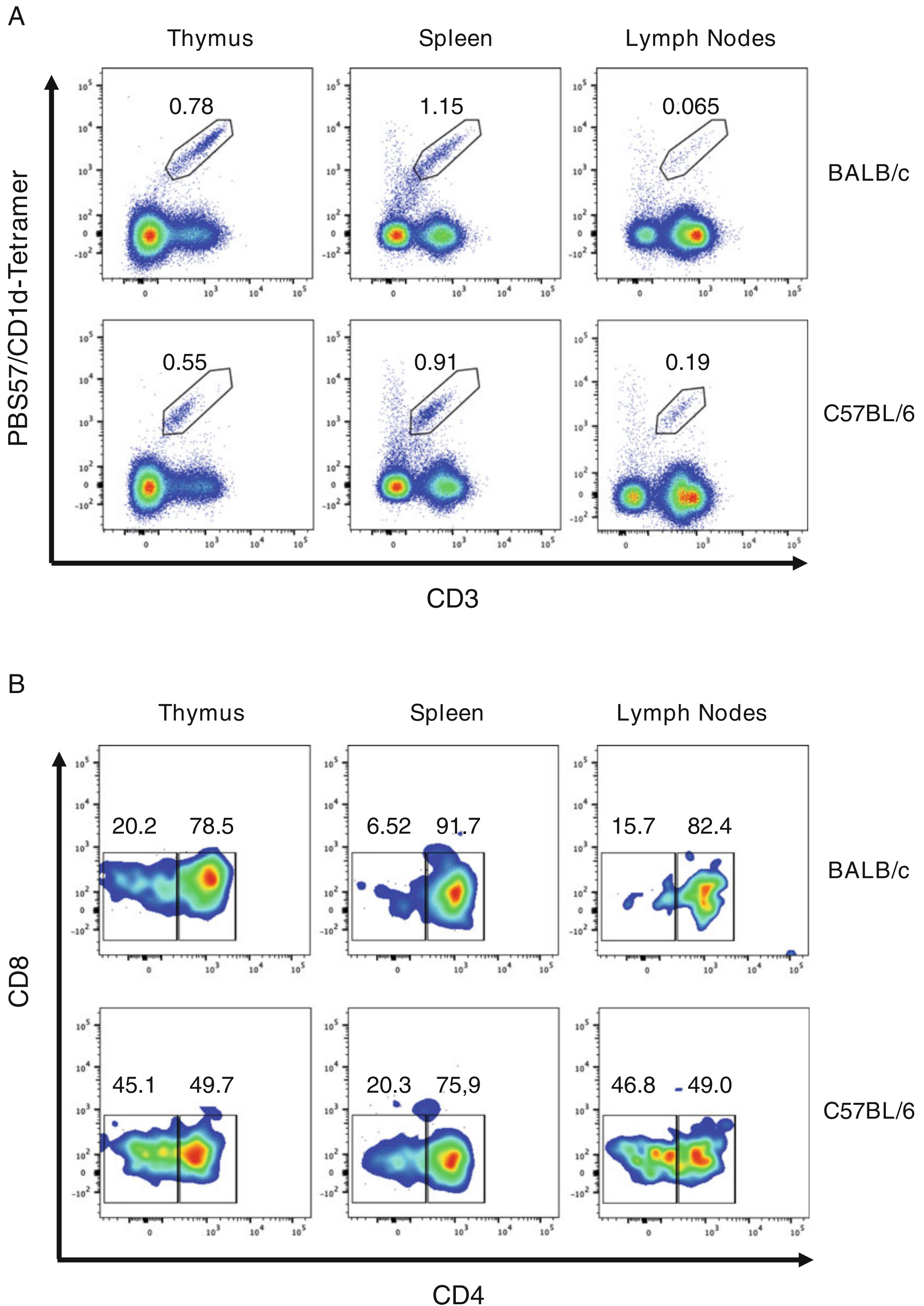

Fig. 3 Frequencies of type I NKT cells in different lymphoid organs from different strains of mice. (**a**) Frequencies of type I NKT cells in the thymus, spleen, and lymph nodes from BALB/c and C57BL/6 mice. (**b**) Frequencies of $CD4^{+}CD8^{-}$ and $CD4^{-}CD8^{-}$ DN subsets of type I NKT cells in thymus, spleen, and lymph nodes of BALB/c and C57BL/6 mice

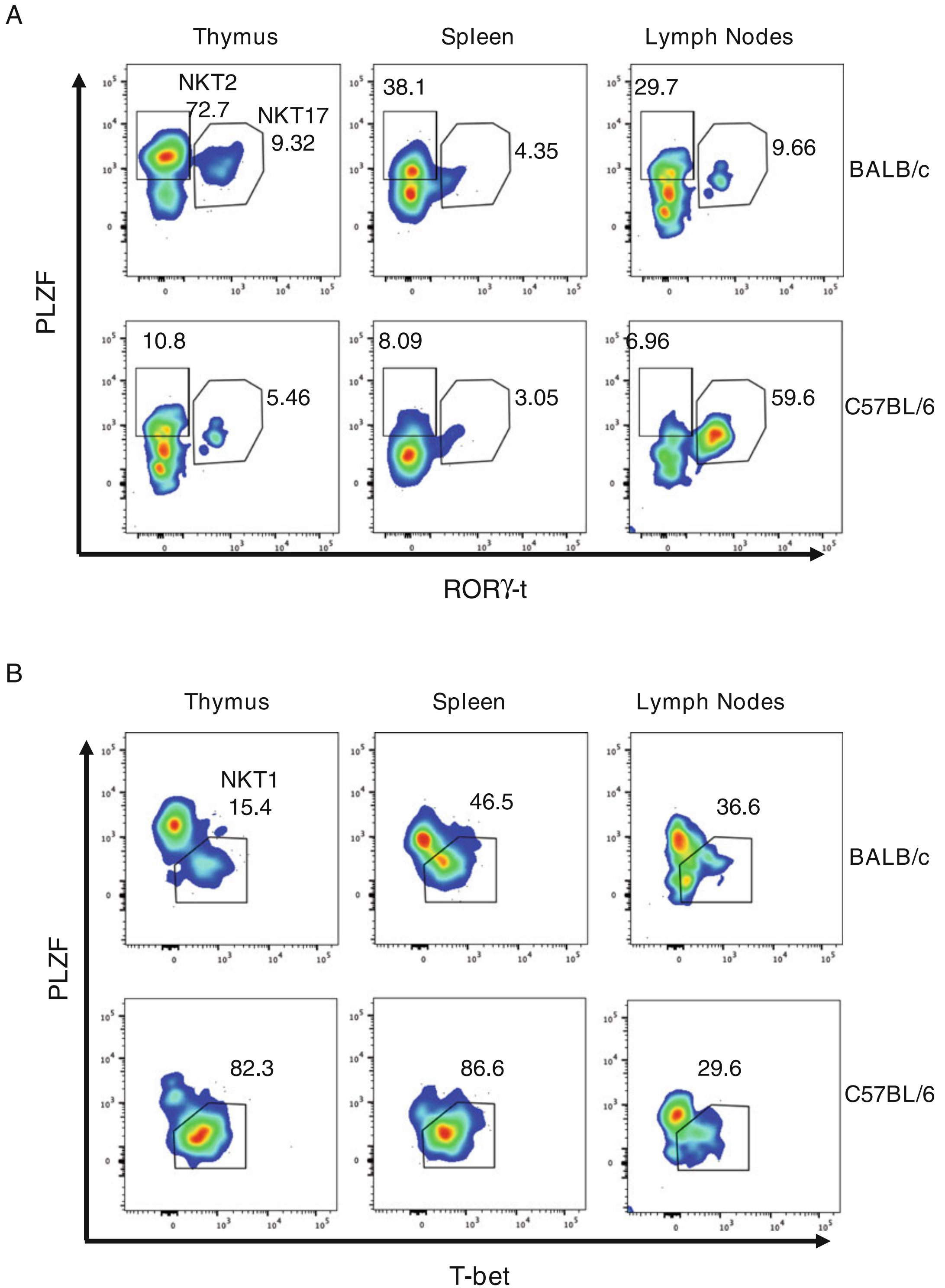

Fig. 4 Frequencies of type I NKT functional subsets in different lymphoid organs from different strains of mice. (**a**) Frequencies of NKT2 and NKT17 in the thymus, spleen, and lymph nodes from BALB/c and C57BL/6 mice. (**b**) Frequencies of NKT1 in the thymus, spleen, and lymph nodes from BALB/c and C57BL/6 mice

6. Combining 2 to 5 lymph node is necessary for detection of NKT cells as single lymph node do not provide enough number of cells for analysis.
7. Prepare buffers by following manufacturer's instructions.
8. It is optimal to acquire data as soon as samples are prepared.

Acknowledgments

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Chapter 9

Identification of Rare Thymic NKT Cell Precursors by Multiparameter Flow Cytometry

Jihene Klibi and Kamel Benlagha

Abstract

Mouse invariant natural killer T (NKT) cells are a subset of T lymphocytes which have been shown to play a significant role in innate and adaptive immune responses. Features of innate responses are attributed to these cells because they can be stimulated simultaneously with the same ligand to produce quickly and in large amount cytokines without prior immunization. Because these characteristics could be exploited for clinical applications, NKT cells have attracted considerable interest. Many studies have investigated the molecular mechanisms through which they are selected and differentiate. These studies are based on developmental models that serve as a scaffold to understand the specific roles played by various factors and to identify checkpoints during cellular development. Analysis of NKT cell precursors at the HSA^{high} stage, stage 0, can reveal potential selection defects, whereas analysis of NKT cells at the HSA^{low} stage can shed light on defects in the maturation/differentiation of the different NKT cell subsets (NKT1, 2, and 17). Unlike HSA^{low} NKT cell subsets, HSA^{high} NKT cell precursors are not accurately identified by flow cytometry because of their extreme rarity. Here, we describe an NKT cell enrichment strategy to identify unambiguously NKT cell precursors at the HSA^{high} stage that can be used to assess their distribution and characteristics by multicolor flow cytometry.

Key words NKT cells, Flow cytometry, Early precursors, Thymocyte development

1 Introduction

Flow cytometry is a powerful technique to count and measure cell properties [1]. This technique makes it possible to measure and analyze, simultaneously, and on a large number of cells, several constituents of the same element defined by several parameters: size (FSC, forward scatter), graininess or texture (SSC, side scatter), and fluorescence (FL). The technology involves cells carried in a liquid vein passing in front of a laser beam. The cells are thus aligned, one behind the other, and can be analyzed by combining an optical system with electronics (photomultipliers), to record their light scattering and fluorescent parameters. In addition to

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being a multiparametric technology, flow cytometry can be used to analyze cell subpopulations, even when represented in small proportions, within a mixture of several cell types on the condition that they differ by at least one parameter. Subpopulation identification and counting can be useful to help us understand disease pathogenesis.

The immune cell types represented at low levels in lymphoid organs include invariant natural killer T (NKT) cells. Conventional mature HSA^{high} T cells express either CD4 or CD8, whereas mature HSA^{high} NKT cells in mice express only CD4 or neither of these receptors (DN). These cells represent around 0.5% of the cells in the thymus; conventional CD4 and CD8 T cells represent around 15% of thymic cells [2]. NKT cells express a restricted TCR repertoire which contrasts with the polyclonal repertoire of alpha beta (αβ) TCRs expressed on conventional T cells. The NKT receptor is composed of an invariant Vα14 alpha chain paired with Jα18, Vα14-Jα18, and a variable beta chain essentially consisting of Vβ8, Vβ7, or Vβ2 [3]. Functionally, there are also differences between NKT and conventional T cells; the latter recognize peptides presented by MHCI and MHCII molecules, whereas NKT cells recognize glycolipids presented by the nonclassical MHCI molecule CD1d [2]. The study of NKT cells benefited greatly from the generation of CD1d tetramers and the identification of the NKT cell ligand α-galactosylceramide [4]. For example, CD1d tetramers loaded with α-galactosylceramide can be used to track mature HSA^{low} NKT cells based on their TCR specificity rather than their expression of NK or activation markers, such as NK1.1 and CD44 (*see* **Note 1**) [5]. Ontogeny studies using CD1d tetramers revealed that before reaching the final $NK1.1^{+}CD44^{+}$ stage, or stage 3 (whereafter they are termed NKT1 cells), NKT cells passed through a transitional stage, stage 2, when they express CD44 but not NK1.1. These CD44-positive stage 2 NKT cells derive from stage 1 NKT cells, which express neither NK1.1 nor CD44. Based on these observations, a linear developmental model was proposed for mature HSA^{low} NKT cells, which was subsequently used in most studies investigating the specific role of the factors controlling NKT cell development (Fig. 1a) [5].

In terms of cytokines, NKT1 cells produce mainly IFN-γ, but NKT cells producing IL-17 (called NKT17) and IL-4/IL-13 (called NKT2) have also been described [6–8]. These subpopulations mirror the Th cell subgroups described for mainstream T cells, in that they produce Th1, Th17, and Th2 cytokines, respectively. Analysis of the intracellular expression patterns for the transcription factors controlling cytokine production, such as T-bet, RORγt, GATA3, and PLZF—the master gene necessary for the development of all subsets—was used to distinguish these NKT cell subsets. Thus, three groups were defined: NKT1, NKT17, and NKT2 [9]. Based on these data, an alternative NKT lineage

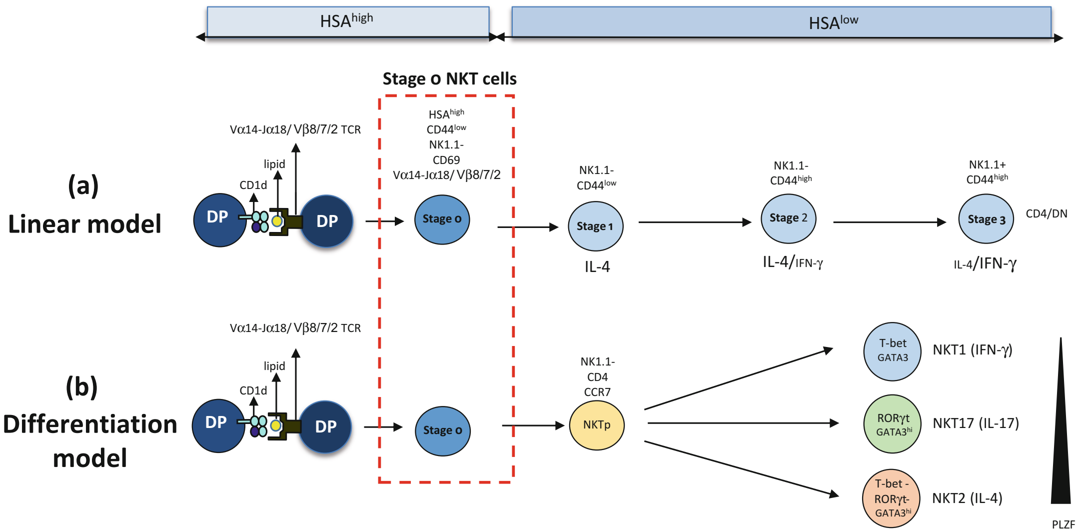

Fig. 1 Linear and differentiation models of NKT cell development. (**a**) The linear development model describes three steps through which NKT cell progress during their development, based on the differentiation markers CD44 and NK1.1. Stage 1 and 2 cells undergo massive expansion before acquiring the phenotypic and functional markers related to the NK lineage characterizing developmental stage 3 cells. (**b**) The differentiation model relies on intracellular staining patterns for lineage-specific transcription factors such as T-bet, GATA3, PLZF, and RORγt. Three functional iNKT subsets are distinguished in the thymus, designated as iNKT1, iNKT2, and iNKT17. The NKTp population represents precursors of NKT cell subsets and is defined by its CCR7 expression pattern. The HSA^{high} CD44^{low} stage 0 NKT cells highlighted by the dashed red box are common to both models and represent the earliest NKT cell precursors detected, the closest to thymic positive selection. These cells are quiescent and show a bias in Vβ8 usage like that described for their later HSA^{low} derivatives. This population expresses the early activation marker CD69, reflecting a strong TCR signal transmitted during their agonist selection. DP: double positive; NKTp: natural killer T cell precursors; HSA: heat-stable antigen

differentiation model was proposed for HSA^{low} NKT cells, in which terminally differentiated cells produce several cytokines derived from common precursors (Fig. 1b) [9].

The earliest precursors of NKT cells were detected at the HSA^{high} stage [10]. This stage is called stage 0 and is common to both the linear and the differentiation models (Fig. 1a and b). These cells represent the NKT cell developmental stage that is closest to positive selection, and the original study describing them proposed that HSA^{high} stage 0 cells could represent a branch point between NKT and conventional T lineages [10]. These cells are rare and difficult to unambiguously detect because they are not cycling like the cells at developmental stages 1 and 2. Several studies have investigated the proportion and absolute numbers of stage 0 NKT cells in the thymus. However, many of these studies do not use appropriate strategies and controls to determine whether the stage 0 cells identified are bona fide NKT cells [11]. Stage 0 cells can only be unambiguously identified without recourse to intracellular staining by using a minimum of six parameters: expression of TCRβ, HSA,

and Vβ8; non-expression of NK1.1 and CD44; and binding CD1d tetramers [10]. A bias in their beta chain usage, skewed toward Vβ8 expression, demonstrates that the cells analyzed are bona fide NKT cells (*see* **Note 2**). However, because stage 0 cells are rare, enrichment strategies are necessary before these cells can be characterized by flow cytometry. This enrichment step is important and necessary because HSA^{high} cells analyzed without appropriate enrichment will include contaminant cells and thus provide skewed results [11]. In this chapter, we provide a protocol to enrich NKT cells before polychromatic flow cytometry to identify these cells at the earliest HSA^{high} stage 0 of their development (Fig. 2).

2 Materials

1. FACS solution (for cell harvesting and staining): 1× PBS, 5% fetal calf serum (FCS), 1% sodium azide, 25 mM HEPES.
2. 1.5-mL Eppendorf tubes for tissue collection.
3. Plunger from a 1 mL syringe for tissue dissociation.
4. 40-μm-pore cell strainer.
5. 50-mL Falcon tube for recovery of cell suspension from dissociated tissue.
6. MACS buffer: 1× PBS, 0.5% bovine serum albumin, 25 mM HEPES.
7. NIH-prepared CD1d tetramers loaded with PBS57 (CD1d-PBS57).
8. Anti-fluorochrome (APC) microbeads (Miltenyi Biotec).
9. 15 mL Falcon tubes and 5 mL FACS tubes for cell labeling.
10. Fluorochrome-conjugated antibodies specific for the appropriate surface markers (Table 1).
11. Viability dye.

2.1 Equipment

1. AutoMACS Pro separation system (Miltenyi Biotec).
2. Flow cytometer for data collection.

3 Methods

3.1 Staining Design

To design appropriate multicolor panels for cell staining, it is necessary to have information on the cytometer and fluorochromes that will be used. It is important to know how the flow cytometer works, and how fluorescence is generated, and detected as this information is critical to design an appropriate polychromatic panel (*see* **Note 3**) [12]**.** The antibodies used in the panel should be titrated using equivalent numbers of cells to the number that will be used in the experimental samples.

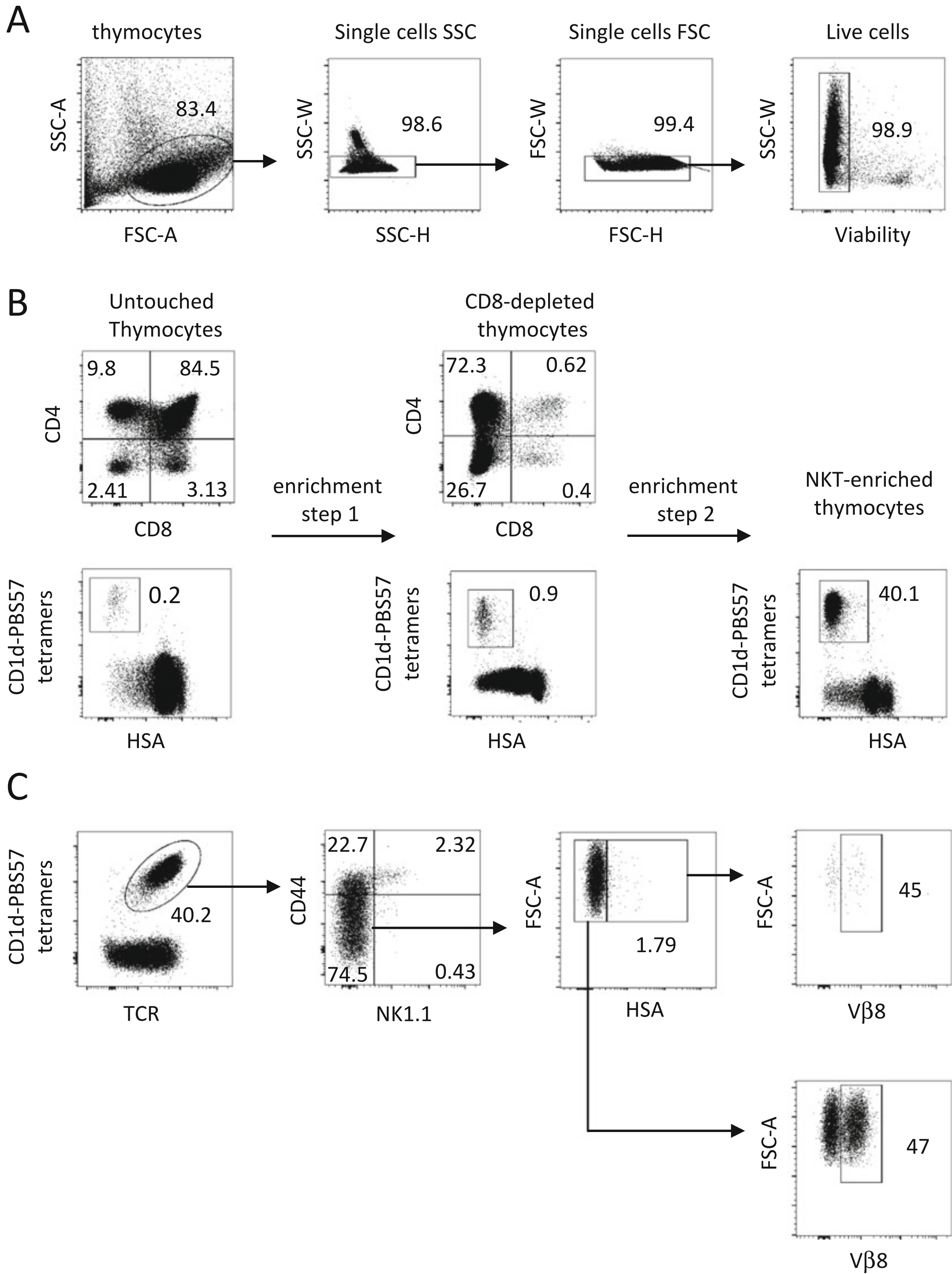

Fig. 2 NKT enrichment steps and identification of stage 0 NKT cells. CD1d-PBS57 tetramer-positive thymocytes from 2-week-old C57BL/6 x BALB/c F1 mice (*see* **Note 1**) were enriched prior to analysis. Expression of the markers outlined in Table 1 was analyzed. (**A**) Dot plots indicate the gating strategy used to identify live single cells. (**B**) NKT cell enrichment by depletion of CD8-positive cells (step 1) then by positive selection of NKT cells from CD8-depleted thymocytes using CD1d-PBS57 tetramers (step 2). The efficiency of

3.2 Isolation of Thymic NKT Cells

Due to their rarity, identification of stage 0 NKT cells requires an enrichment step. The standard method used to reveal populations present at such low levels is to enrich for thymic NKT cells. The enrichment protocol outlined below uses a two-step magnetic bead-based enrichment procedure: the first step is to deplete CD8-positive cells, and the second pulls down NKT cells from the CD8-depleted thymocytes using CD1d-PBS57 tetramers, a variant of α-galactosylceramide [13] (*see* **Note 4**). The protocol was optimized for the Miltenyi AutoMACS Pro Separator using the recommended programs that come as standard on that system.

3.2.1 Preparing the Thymocyte Suspension and Enriching for NKT Cells by Depleting CD8-Positive Cells

1. To preserve cell viability, pre-cool all buffers. In addition, throughout the procedure, cells should be kept cold on ice or in the fridge at 4 °C, unless otherwise specified.
2. Place a 40-μm-pore cell strainer on the top of a 50 mL Falcon tube.

Table 1
Sample staining panel to characterize stage 0 NKT cells

Laser	LP	BP	Fluorophore	Antigen	Clone
Red (640 nm)	750	780/60	APC-Cy7	Viability dye	
	None	670/14	APC	TCRβ	H57-597
Violet (405 nm)	505	525/50	BV510	CD24 (HSA)	M1/60
	690	710/50	BV711	CD4	GK1.5
	600	610/20	BV605	CD8	H1.2F3
	None	450/50	BV421	NK1.1	PK136
UV (355 nm)	690	740/35	BUV737	CD44	IM7
Blue (488 nm)	505	530/30	FITC	Vβ8.1/.2	KJ16-133
Yellow-green (561 nm)	570	586/15	PE	CD1d-PBS57 tetramers	NIH[a]
FC block			Purified	CD16/32	2.4G2

Flow cytometry panel to characterize stage 0 thymic NKT cells post-selection, designed for analysis on a BD LSRFortessa X-20 equipped with five lasers. Numbers in the first column indicate the wavelength emitted by the lasers. The wavelengths of light detected are also indicated, for long-pass (LP) and bandpass (BP) filters, for each channel used
[a]Provided by the NIH tetramer facility

Fig. 2 (continued) CD8 depletion and NKT cell enrichment is assessed based on CD4/CD8 and HSA/CD1d-PBS57 tetramer expression. (**C**) Identification of stage 0 NKT cells. Positively selected NKT cells from enrichment step 2, defined as expressing TCR and binding PBS57-CD1d tetramers, comprise stage 0 NKT cells defined as HSA-expressing $CD44^{low}NK1.1^{-}$ cells. These cells show a bias in Vβ8 expression akin to that of their HSA^{low} derivatives, which indicates that they represent bona fide NKT cells

3. Recover the thymus into a 1.5 mL Eppendorf tube containing 1 mL FACS buffer.
4. Pour the contents of the Eppendorf tube (thymus + buffer) onto the 40-μm-pore cell strainer and gently dissociate tissue by teasing the thymus apart by applying rotary movements with a 1 mL Syringe plunger. Rinse the strainer with two 10 mL volumes of FACS buffer. Remove the strainer and make up the volume of the suspension to 40 mL with FACS buffer (*see* **Note 5**).
5. Centrifuge the cell suspension at 300 × *g*-force for 10 min. All centrifugation steps should be performed at 4 °C to preserve cell viability.
6. Resuspend the cell pellet in 10 mL of MACS buffer and count cells.
7. Remove an aliquot of 3×10^6 cells to assess CD4/CD8, and NKT/HSA expression within the whole thymus. This aliquot may be reserved on ice for later staining, in parallel to the NKT-enriched fractions.
8. Centrifuge cell suspension at 300 × *g*-force for 10 min and resuspend cells at 5×10^7/mL.
9. Transfer cells to a 15-mL Falcon tube.
10. Add Fc-receptor block (1/500), and incubate for 10 min, followed by APC-conjugated anti-CD8 antibodies (1/200) to label cells for 20 min. Incubation should be performed on ice and in the dark.
11. Wash cells by adding 10 mL of FACS buffer and centrifuge at 300 × *g*-force for 10 min.
12. Resuspend cells in 10 mL MACS buffer.
13. Centrifuge cell suspension at 300 × *g*-force for 10 min.
14. Resuspend cell pellet in 80μL of MACS buffer per 10^7 total thymocytes.
15. Add 10μL of anti-APC microbeads per 10^7 total thymocytes (*see* **Note 6**), and incubate for 20 min at 4 °C (*see* **Note 7**).
16. Wash cells by adding 10 mL of MACS buffer to cells and centrifuging at 300 × *g*-force for 10 min (wash step may be repeated, if desired).
17. Prepare thymocytes for separation by resuspending them in MACS buffer at a concentration of 10^8 cells per 500μL. Using the AutoMACS Pro Separator and the DEPLETES program (appropriate for normal or strong antigen expression, which is the case for CD8 expression on thymocytes), separate the CD8-positive cells from the negative fraction. The separator will deposit each fraction in separate collection tubes.

18. Count cells in the CD8-depleted fraction (negative fraction or flow through).
19. Centrifuge at 300 × *g*-force for 10 min.
20. Resuspend the cell pellet at 5×10^6 cells per 100μL.

3.2.2 Enriching NKT Cells from CD8-Depleted Thymocytes by Positive Selection

1. Label the CD8-depleted thymocytes with PE-conjugated CD1d tetramers loaded with PBS57 (PE CD1d-PBS57). Incubate for 20 min at 37 °C or 45 min at 4 °C (*see* **Note 8**).
2. Wash cells by adding 10 mL of MACS buffer to cells, and remove an aliquot of 1×10^6 cells to allow assessment of CD4/CD8 and NKT/HSA cell expression (*see* **Note 9**). This aliquot may be reserved on ice with the whole-thymus aliquot and stained later alongside the NKT-enriched fraction.
3. Centrifuge the remaining cells at 300 × *g*-force for 10 min.
4. Resuspend the cell pellet in 80μL of MACS buffer per 10^7 total thymocytes.
5. Add 10μL of anti-PE microbeads per 10^7 total thymocytes and incubate for 20 min at 4 °C (*see* **Note 10**).
6. Wash cells by adding 10 mL of MACS buffer and centrifuging at 300 × *g*-force for 10 min (wash step may be repeated, if desired).
7. Prepare thymocytes for separation by resuspending them at 10^8 cells per 500μL in MACS buffer. Using the AutoMACS Pro Separator and the POSSELD program (appropriate for normal or strong antigen expression and for a frequency of selected cells <5–10%, which is the case for NKT cells in the CD8-depleted thymus fraction), separate the tetramer-positive cells from the negative fraction. The separator will deposit each fraction in separate collection tubes.
8. Count cells in the positive fraction and pellet them by centrifugation. Resuspend them in FACS buffer at 0.5×10^6 cells per 100μL. Transfer the cell suspension to a 5-mL FACS tube for subsequent staining steps.

3.2.3 Surface Staining

All steps should be performed at 4 °C unless otherwise specified. Samples should be protected from light to prevent photobleaching and to maintain the integrity of the fluorophores.

1. Antibodies for surface staining should be prepared as a 10× master mix. Prepare enough mix to stain each sample with 10μL of the 10× stock mixture, including one extra stain volume (10μL) to allow for pipetting loss. Antibodies should be diluted in FACS buffer to the appropriate concentration, as determined previously by titration. Remember to prepare a 10× single stain solution, containing only one of each

antibody, for every fluorochrome in the panel. These single stains should be applied to samples of thymocytes with no prior staining.

2. Add 10μL 10× master mix to the 100μL samples and incubate for 20–25 min at 4 °C.
3. Wash cells by adding 2 mL FACS buffer and centrifuging at 300 × *g*-force for 5 min (wash step may be repeated, if desired).
4. Resuspend cell pellets in 100μL FACS buffer and add 1μL of fixable viability dye Zombie NIR. Incubate for 10 min at room temperature in the dark (*see* **Note 11**).
5. Wash cells by adding 2 mL FACS buffer cells and centrifuging at 300 × *g*-force for 5 min (wash step may be repeated, if desired).
6. Resuspend cell pellets in 200μL of FACS buffer and analyze by flow cytometry (*see* **Note 12**).

4 Notes

1. Mature NKT cells express phenotypic and functional features of NK cells, e.g., they express NK1.1 and produce IFN-γ [2]. However, not all mouse strains express the NK1.1 marker (e.g., BALB/c mice). In these cases, other differentiation markers such as CD69 and CD122 could be used instead. NK markers such as NKG2D, expressed only by a fraction of NK1.1-positive cells, could also be used.
2. Mainstream T cells express a variable repertoire of 21 functional TCR β chain [14]. In the case of NKT cells, the Vβ repertoire is composed mainly of Vβ8, Vβ7, or Vβ2 chains as a result of bias [2]. As a consequence, the frequency of Vβ8-, Vβ7-, or Vβ2-expressing cells is higher in NKT cells compared to mainstream T cells. Thus, around 15% of mainstream T cells express Vβ8, compared to around 50% for NKT cells (using anti-Vβ8.1.2 mAbs) [11]. Thus, the Vβ8 expression pattern can be efficiently used to identify true NKT cells when analyzing rare stage 0 NKT cells or unidentified cells.
3. Multiple parameters can be analyzed on each cell thanks to the possibility to measure a large number of colored dyes simultaneously. As a general rule, the type of lasers and detectors available in the cytometer used will determine the fluorochromes that can be deployed. It is important to mention that the brightness of a fluorochrome will differ depending on the instrument used. The intensity and relative brightness of a fluorochrome used should also be considered. The brightest fluorochromes should be reserved for dim antigens or markers of rare populations. In our panel, PE (coupled to

CD1d-PBS57 tetramers) and APC (coupled to TCRβ), considered among the brightest fluorochromes, are used to label NKT cells. The colors selected for our panel use dyes excited by five different lasers. The excitation and emission spectra for these dyes, and the filters used to detect the fluorescence emitted by these dyes are shown in Table 1. Because of the spectral overlap, each fluorochrome will contribute signal to several detectors, therefore the contribution in detectors not assigned to that fluorochrome must be subtracted from the total signal through a process termed compensation.

4. Stage 0 NKT cells are exceedingly rare, and their proportions have been reported to represent between 0.2% and 0.5% of NKT cells in 2–5-week-old C57BL/6 mice [10]. The quiescent state of HSA^{high} stage 0 NKT explains their low frequency compared to their expanding HSA^{low} NKT cell progeny at subsequent developmental stages, stages 1 and 2 [10]. The rationale behind depleting CD8 thymocytes to enrich NKT cells is related to the fact that mature HSA^{low} NKT cells are reported not to express CD8, rather they are CD4^{+} or CD4^{-}CD8^{-} (DN). Hence, the HSA^{high} cells that we detect using the proposed protocol will not be CD4^{+}CD8^{+} cells. It is worth mentioning that strategies detecting stage 0 NKT cells by direct pull-down without prior CD8 depletion result in the detection of 50% CD4^{+}CD8^{+} NKT cells; these cells are false positives [15, 16].
5. The thymus should be teased apart gently until the capsule becomes clear in the wet filter (the filter will be wetted by the MACS buffer poured with the thymus from the tissue collection tube). To avoid clogging of the filter and cell loss upon centrifugation due to high cell density, do not use more than one thymus per filter. This is particularly important when working with young thymuses which contain up to 250×10^{6} thymocytes. To increase cell viability during this relatively lengthy protocol, we included HEPES buffer in the MACS and FACS buffers to stabilize the pH throughout the prolonged processing.
6. Miltenyi recommends using 20μL of MACS beads per 10^{7} cells. We performed titrations with these beads and found that 10μL was sufficient for this first step of NKT cell enrichment using anti-CD8 mAbs and separation equipment.
7. The CD8 depletion step could also be performed using Mouse Depletion Dynabeads from Invitrogen, that are superparamagnetic polystyrene beads (4.5μm diameter) coated with a polyclonal sheep anti-rat IgG antibody, in conjunction with rat monoclonal antibodies binding to mouse CD8, and Invitrogen DynaMag magnets. These magnets instantly pull the

Dynabeads-bound target to the tube wall allowing recovery of the untouched cell suspension containing enriched NKT cells. We have tested this technique and found it to give equivalent results to those obtained with anti-fluorochrome (APC) microbeads and the AutoMACS Pro Separator system (Miltenyi Biotec).

8. Preparation of tetramers may vary depending on the source. NIH-prepared CD1d tetramers loaded with PBS57 (CD1d-PBS57) come at a concentration of approximately 1 to 1.5 mg/mL. They should be titrated before use (http://tetramer.yerkes.emory.edu).
9. When staining total thymocytes, we stain 3×10^6 cells per sample to compensate for the low frequency of NKT cells, which is more accentuated in 2–3-week-old mice as NKT cells accumulate with age [6]. As the CD8-depleted fraction is enriched in NKT cells, we stain only 1×10^6 of these cells. Titrations to select optimal working dilutions of antibodies should be performed on the number of cells that will be stained during the experiment.
10. Miltenyi recommends using 20μL of MACS beads per 10^7 cells. We performed titrations with these beads and found that 10μL was sufficient for this second step in the enrichment of NKT cells using CD1d-PBS57 tetramers and separation equipment.
11. Like fluorescence-labelled antibodies, viability dye should be titrated using the number of cells that will be stained in the experiment. This is doubly important in the case Zombie NIR, as this dye will react with and binds to some proportion of proteins present in FACS buffer (serum proteins in our case), and thus a higher amount may be required.
12. Cells could be fixed and stored in FACS buffer for analysis the next day or up to 5 days after fixation. Remember to also fix single-stained cell suspensions for compensation adjustment, and store them in parallel.

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Chapter 10

iNKT Cel Transfer: The Use of Cell Sorting Combined with Flow Cytometry Validation Approach

Marcella Cipelli, Theresa Ramalho, Cristhiane Favero de Aguiar, and Niels Olsen Saraiva Camara

Abstract

Natural killer T (NKT) cells are an innate-like T cell subset that recognize lipid antigens presented by CD1d-expressing antigen presenting cells (APCs), such as dendritic cells, macrophages, and B cells. They can be subdivided into two different subsets according to the variation in αβ TCR chains: type I and type II NKT cells. Type I, also called invariant NKT cells (iNKT), express restricted TCRs with an invariant α-chain (Vα24-Jα18 in humans and Vα14-Jα18 in mice) and limited β-chains. Here we have established a protocol in which iNKT cells are isolated from a donor wild-type mouse and transferred into iNKT KO (Jα18$^{-/-}$) mouse. Below we will explore the methods for cell sorting of splenic iNKTs, iNKT cells transfer, and detection of transferred cells into the liver using flow cytometry technique.

Key words iNKT cell, Cell sorting, Cell transfer, Flow cytometry, Jα18$^{-/-}$ mice, Retro-orbital injection, Isolation of splenocytes, Liver lymphocytes isolation

1 Introduction

Natural killer T (NKT) cells are an innate-like T cell subset that has lipid antigens as ligands. CD1d-expressing antigen presenting cells (APCs), such as dendritic cells, macrophages, and B cells, present lipid antigens to NKT cells through the CD1 molecule [1]. CD1 is a major histocompatibility complex (MHC) class I-like molecule adapted for self- and non-self-glycolipid antigens [1]. The CD1d lipid-enriched antigens that NKT T cell receptor (TCR) recognizes include isoglobotrihexosylceramide, a mammalian glycosphingolipid, as well as microbial α-glycuronyl ceramides found in the cell wall of Gram-negative lipopolysaccharide-negative bacteria [2]. -α-Galactosylceramide (α-GalCer), a bioactive compound with anti-

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tumoral activity found in marine sponges, is the most potent KNT TCR ligand yet described [2]. α-GalCer potentially activates both human and mouse NKT cells. Also, due to a high degree of conservation, α-GalCer presented by a human CD1d is able to activate murine NKT cells and vice versa [3].

NKT cells can be subdivided into two different subsets according to the variation in αβ TCR chains: type I and type II NKT cells. Type I, also called invariant NKT cells (iNKT), expresses a restricted TCRs with an invariant α-chain (Vα24-Jα18 in humans and Vα14-Jα18 in mice) and limited β-chains [4]. Type I iNKT cells include two subpopulations: a $CD4^{+}$ and a $CD4^{-}CD8^{-}$ double-negative (DN) population. Yet, type II, also referred as noninvariant NKT (niNKT), expresses Vα3.2-Jα9/Vβ8, Vα8/Vβ8, and other TCRs. The TCR repertoire of type II niNKTs varies considerably between human and mice [5].

It is known if distinct subsets of NKT cells mediate different responses specifically. However, it is clear that such subsets exerts regulatory and/or protective immune functions through tissue-specific stimuli [4]. In the context of antitumoral response, studies have suggested that type I iNKTs subset mediate antitumoral responses in mice [6], yet type II niNKT cells downregulate such antitumoral immunosurveillance [7]. In addition, it has been reported autoreactive responses of the type II subset to endogenous myelin-derived glycolipid sulfatide in mice [8]. Studies in human has proposed that DN NKT cells mediate tumor rejection since they predominantly secrete Th1 cytokines. In contrast, CD4 NKT cells produce Th2 cytokines mostly. In this sense, it has been suggested that distinct NKT populations may regulate themselves, similarly to the regulatory interplay of Th1/Th2 cells [4].

In addition to Th1 and Th2 cytokines, NKT cells also produce IL-17, contributing to Th17 responses [9]. This subtype, called NKT17, rapidly produces IL-17 dependently of IL-7 in response to IL-23 RORγT activation [10]. Contrastingly, it has been reported that strong activation of iNKTs with α-GalCer induces a regulatory state, far from deactivated or anergic, which secretes IL-10 and expresses PD-1 [11]. This subtype is called NKT10 [11]. Moreover, depending on the context, iNKT cells also secrete granulocyte-macrophage colony-stimulating factor (GM-CSF) and chemokines, such as RANTES and MIPs [12–14]. In addition, iNKT cells exert cytotoxic function due to their capability to express and secrete cytolytic proteins, such as granzyme and perforin, and membrane ligands, such as FasL [12, 15].

Since NKTs have crucial importance in regulatory, protective, and harmful immune responses, there is a need to explore deeply the role of these cells using different tools. Murine models of iNKT cell deficiency have unravel the importance of NKT cells in host survival in the context of infections, antitumoral response, transplant tolerance, and autoimmunity [4]. iNKT cell-deficient mouse

models include CD1d$^{-/-}$ and Jα281KO (Jα18$^{-/-}$ or Traj18$^{-/-}$) mice. The CD1d$^{-/-}$ mouse strain is not able to efficiently induce the positive selection of iNKT cells during thymus differentiation. Yet Jα18$^{-/-}$ mice that lack the *Traj18* gene segment does not develop the invariant TCRα chain which is essential for iNKT development [16].

Here we have stablished a protocol in which iNKT cells are isolated from a donor wild-type mouse. Once isolated, the cells are prepared to be transferred into iNKT KO (Jα18$^{-/-}$) mice. Below we will explore the methods for cell sorting of splenic iNKTs, iNKT cells transfer, and detection of transferred cells into selected tissues using flow cytometry. Such protocol was stablished by our group for a study in which we investigated how iNKT cells regulate intestinal homeostasis and gut microbiota [17]. Also, our protocol can be used in donor mouse models with fluorescent NKTs cells [18] whose fluorescence can be detected in a recipient mouse.

2 Materials

2.1 Mouse Strains

Use mouse strains C57BL/6 and Jα18$^{-/-}$ (iNKT KO) male mice, aged 8–12 weeks. Jα18$^{-/-}$ mice have a specific deletion of the Jα281 gene segment, which codes for the iNKT-specific joining region of the TCR; thus, the invariant Vα14-Jα18 TCR is not expressed in these mice and consequently iNKT cells do not develop. Animal experiments must always be performed following guidelines of the corresponding Ethics Committee for Animal Experimentation.

2.2 General Reagents

1. Isoflurane.
2. Sterile phosphate-buffered saline 1× (PBS 1×): 137 mM NaCl, 10 mM Phosphate, 2.7 mM KCl, pH 7.4.
3. PBS 1× + 2% fetal bovine serum (FBS).
4. Sorting medium: RPMI 1640 medium + 3% FBS.
5. RPMI 1640 medium + 10% FBS.
6. ACK Lysis Buffer: 0.15 M NH_4Cl, 10 mM $KHCO_3$, 0.1 mM Na_2EDT, adjust PH to 7.2–7.4) (*see* **Note 1**).
7. APC-conjugated anti-mouse TCRβ antibody (BioLegend, clone H57-597).
8. PE-labeled mouse CD1d-PBS57 tetramer (kindly provided by NIH tetramer core facility).
9. PerCP-conjugated anti-mouse CD45 antibody (BioLegend, clone 30-F11).
10. Digestion solution: collagenase VIII (Sigma) 0.5 mg/mL and 10 U/mL DNase I, grade II (Roche) diluted in PBS 1× + 2% FBS.

11. Percoll solution: make a 1.5 M solution of NaCl, and then to 9 parts of Percoll (Sigma) add 1 part of 1.5 M NaCl. For example, for a 100 mL final solution, mix 10 mL of 1.5 M NaCl solution in 90 mL of Percoll. This solution is equivalent to Stock Isotonic Percoll (SIP) or 100% Isotonic Percoll.
12. 40% and 70% Isotonic Percoll solutions: to make 40% and 70% Isotonic Percoll solutions, add 4 mL of Isotonic Percoll in 6 mL of PBS 1× and 7 mL of Isotonic Percoll in 3 mL of PBS 1×, respectively.

2.3 Equipment

1. Scissors.
2. Forceps.
3. Cell strainer 70 μm and 100 μm.
4. Disposable syringe 1 mL plunger.
5. Syringe needles gauge 26G.
6. 15 and 50 mL conical centrifuge tubes.
7. 5 mL round-bottom polystyrene tubes.
8. Professional adjustable-volume micropipette (1000, 200, 20 μL).
9. Micropipette tips (1000, 200, 20 μL).
10. Heating pad or other warming device.
11. Precision laboratory balance.
12. Thermal incubator with rotation unit.
13. Cell culture centrifuge.
14. BD FACS Aria III.
15. BD FACS Canto II.
16. Software package for analyzing flow cytometry data (FlowJo v10).

3 Methods

3.1 Isolation of Splenocytes from WT Mice (Donor) and iNKT Cells Staining

1. Euthanize WT mice using isoflurane and cervical dislocation as a secondary means to assure death. After euthanasia, open the peritoneal cavity using scissors and forceps and remove the spleen.
2. Place the organs on a Petri dish and mash through it using a syringe plunger and PBS 1× solution. Filter cell suspension in a 70 μm cell strainer over a 50 mL conical centrifuge tube and then centrifuge (400 × *g*, 5 min, 4 °C), discarding the supernatant after that (Fig. 1).
3. Add 1 mL per spleen of ACK lysing buffer to cells and homogenize. Incubate for 5 min at room temperature, add three times

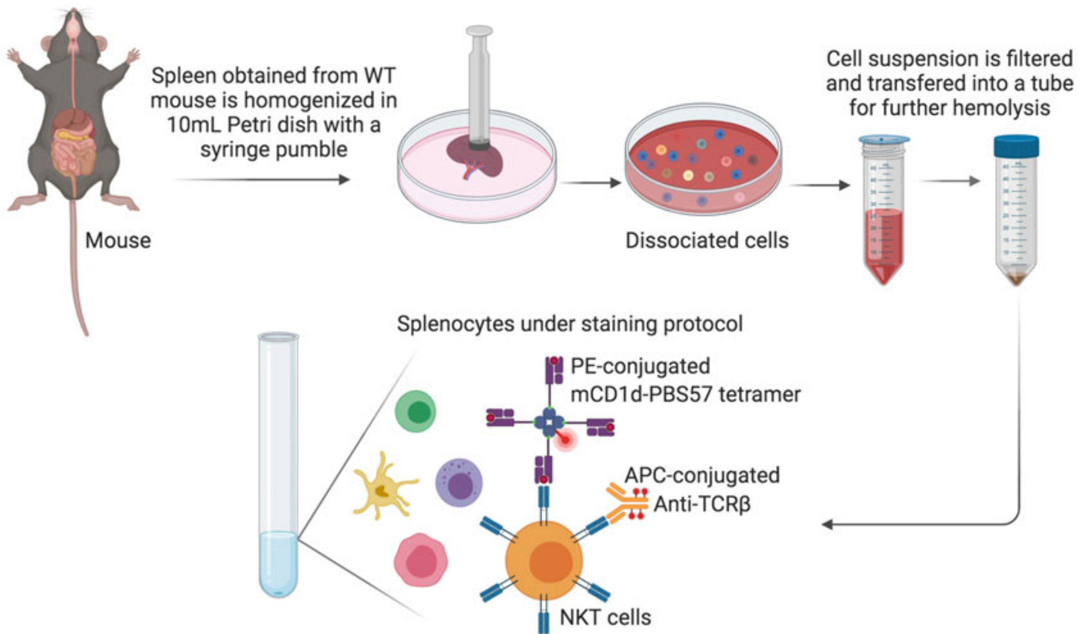

Fig. 1 Schemes of isolation of splenocytes from wild-type mice (donor) followed by staining. In this stage, the cell suspension is obtained from the whole organ and proceeded into iNKT molecules staining using antibodies conjugated to fluorochromes

the initial volume of PBS 1× to stop the reaction, and centrifuge (400 × *g*, 5 min, 4 °C).

4. Resuspend the pellet with PBS 1× + 2% FBS and transfer all the volume into a 15 mL conical centrifuge tube.
5. Add 100 μL PBS 1× + 2% FBS containing APC-conjugated anti-mouse TCRβ antibody and PE-labeled mouse CD1d-PBS57 to stain cells for 30 min at 4 °C, protected from light (Fig. 1).
6. After that time, add excess PBS 1× + 2% FBS to wash unbound antibody and centrifuge the cells.
7. Discard the supernatant and resuspend the pellet in RPMI medium + 3% FBS to proceed to cell sorting (Fig. 1).

3.2 WT iNKT Cell Sorting and Cell Transfer into $J\alpha 18^{-/-}$ Mice (Recipient)

1. Run the cell suspension obtained in the previous step in a BD FACS Aria III cell sorter, selecting iNKT cells as $TCR\beta^{int}$/$mCD1dPBS57^{+}$ population (*see* **Note 2**).
2. Collect sorted cells in a 15 mL conical centrifuge tube containing sterile RPMI + 10% FBS and count cells (*see* **Note 3**).
3. Centrifuge (400 × *g*, 10 min, 4 °C) and resuspend in sterile PBS 1× for injection of around 2–3 × 10^5 iNKT cells in a final volume of 150 μL per mouse.

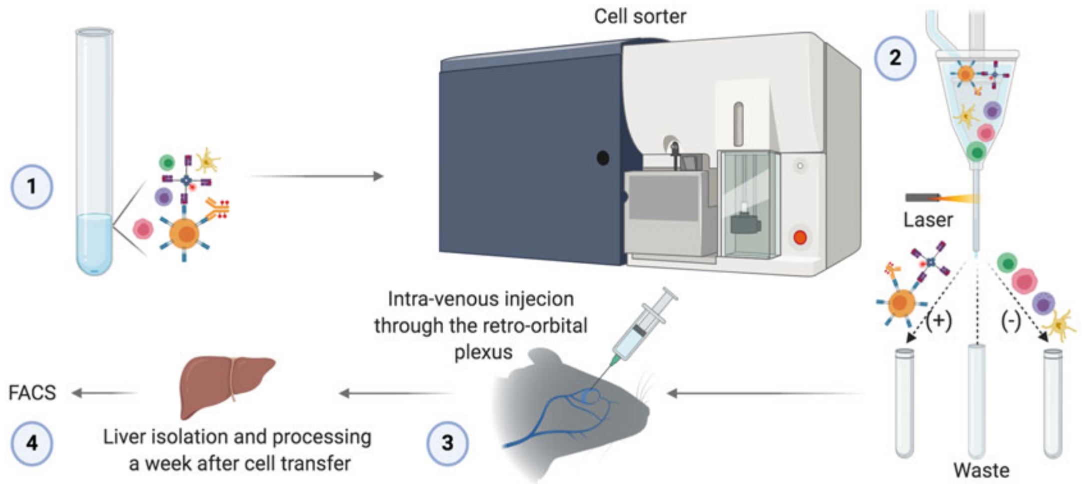

Fig. 2 Cell sorting and iNKT transfer scheme. After staining with antibodies (1), the cells are sorted under aseptic conditions by BD FACS Aria III, selecting TCRβ^{int}/mCD1dPBS57^{+} population (2). Then, a cell suspension containing 2–3 × 10^5 iNKT cells in a final volume of 150 μL PBS 1× are prepared and injected intravenously through the retro-orbital sinus of Jα18$^{-/-}$ mouse (3). One week after injection, the mouse is euthanized and liver is harvested. Afterward, liver cells are isolated and proceeded with staining of iNKT cells, followed by flow cytometry detection (4)

4. Inject intravenously (*see* **Note 4**) the cell suspension through the retro-orbital sinus using a 1 mL disposable syringe with needle gauge 26G into isoflurane-anesthetized Jα18$^{-/-}$ mouse placed on a heating pad covered with a protective layer of paper toweling or gauze to prevent any thermal injury (Fig. 2).

3.3 iNKTs Detection by Flow Cytometry in Liver of Jα18$^{-/-}$ Mice After WT Cells Transference

1. One week after iNKT cell transfer, euthanize Jα18$^{-/-}$ mice, open the peritoneal cavity using scissors and forceps, and remove the liver.
2. For liver digestion: Place and chop (using scissors) the organs in a 15 mL conical centrifuge tube containing 3 mL per liver of digestion solution and incubate at 37 °C with agitation (200 rpm) for 20 min. After that time, filter the digested organ suspension through a 100 μm cell strainer, mashing the remaining pieces with a syringe plunger and wash with excess PBS 1× to stop the reaction (Fig. 3).
3. Centrifuge the liver cells suspension (400 × *g*, 5 min, 4 °C) and discard the supernatant.
4. Put 3 mL 70% Isotonic Percoll solution in the 15 mL centrifuge conical tube.
5. Resuspend cells in 5 mL of 40% Isotonic Percoll solution and overlay on the 70% Isotonic Percoll solution previously placed in the tube.
6. Centrifuge the tube at 500 × *g* for 25 min at room temperature, without break.

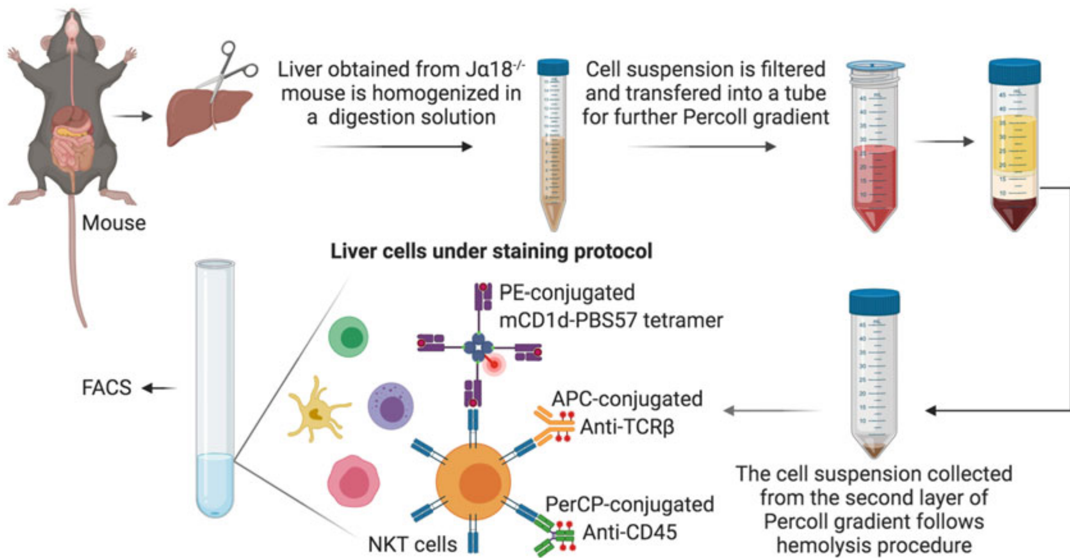

Fig. 3 Liver processing for further detection of transferred iNKT cells in Jα18$^{-/-}$ mouse liver. Liver is collected and processed in a digestion solution. After hemolysis, cell suspension follows a staining protocol with PE-conjugated mCD1d-PBS57 tetramer, APC-conjugated Anti-TCRβ, and PerCP-conjugated anti-CD45 antibodies. The fluorescence of the antibodies is later detected by BD FACSCanto II representing the detection of iNKT cells

7. Collect the cells that remained at the interface between the two solutions, transfer to a new tube, centrifuge (400 × *g*, 5 min, 4 °C) and then discard the supernatant (Fig. 3).
8. After isolation of lymphocytes from liver, add 1 mL of ACK lysis buffer in the pellet, homogenize, and incubate for 3 min at room temperature. Add 3 volumes of PBS 1× to stop the reaction and centrifuge (Fig. 3).
9. Resuspend the pellet in 100 μL PBS 1× + 2% FBS containing the staining antibodies and tetramer: anti-CD45 (PerCP), anti-TCRβ (APC), and mCD1dPBS57 tetramer (PE). After 30 min of incubation at 4 °C protected from light, wash the cells by adding excess PBS 1× + 2% FBS and centrifuge (Fig. 3).
10. Resuspend the pellet in 200 μL PBS 1× each and run the cells in BD FACSCanto II flow cytometer. Analyze data using FlowJo v10 to create the dot plots (Fig. 4). Perform debris and doublets from your data selecting exactly the population of FSC-H x FSC-A gate. Select the iNKT cell population (TCRβ^{+} mCD1d-PBS57^{+}) from CD45^{+} cells gate.

4 Notes

1. Used for the lysis of red blood cells in samples containing white blood cells.
2. To avoid contamination is necessary to perform an aseptic sort.

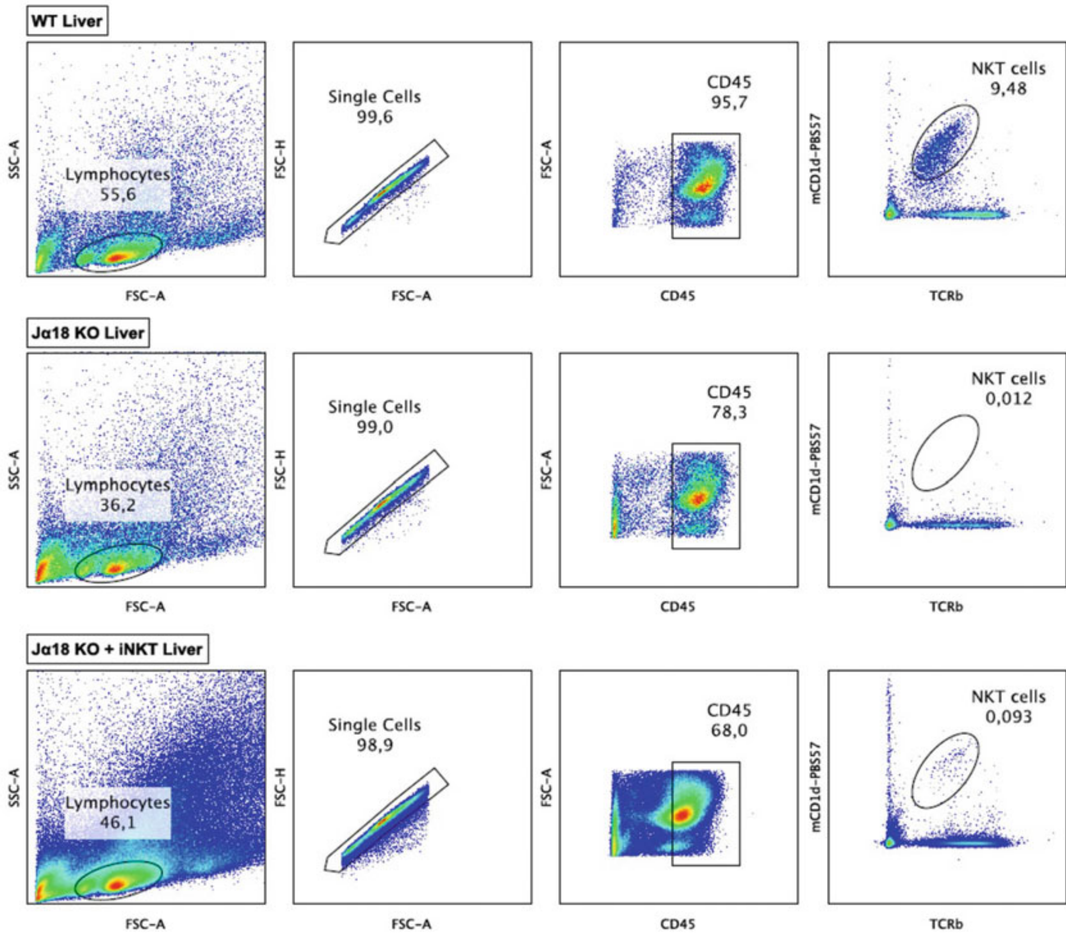

Fig. 4 Gate strategy to detect iNKT cells in liver of WT donor and Jα18$^{-/-}$ recipient mice. After liver digestion and lymphocytes isolation by Percoll gradient technique, the cell suspension is stained with the antibodies, and frequency of iNKT cells can be observed in WT, Jα18$^{-/-}$ and Jα18$^{-/-}$ + iNKT mice 1 week after iNKT cell transfer by flow cytometry

3. In all the steps of iNKT cells injections, preparation is necessary for the use of sterile reagents and perform all the procedures in a laminar flow hood previously cleaned with 70% alcohol and 15 min of UV sterilization.
4. Carefully put the anesthetize mouse laterally lying with its head facing to the right side. Protrude the mouse's eyeball applying a very delicate pressure to the skin in the dorsal and ventral directions to the eye. Do not apply excessive pressure, because this could obstruct the blood flow and hamper the injection. It is also very important not to apply exacerbated pressure to the trachea, because this could hinder mouse's breathing. Carefully introduce the needle at an angle of approximately 30°, into the

medial eye angle, following the edge of the eyeball down until the needle tip is at the base of the eye. Then, slowly injects the cell suspension and do not aspirate before injection. After that, withdraw the needle carefully and slowly. The injection procedure takes only a short time, then the mouse can be gentle placed in the cage without the heating pad, and within 45 s to 1 min after the injection, it will be moving normally.

Acknowledgments

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Chapter 11

Expansion of Human iNKT Cells Ex Vivo

Jing Wang, Chen Zhao, and Jianqing Xu

Abstract

Invariant natural killer T (iNKT) cells are credited with antitumor activity by preclinical studies and clinical trials. Efficient expansion of iNKT cells ex vivo is essential for their translational usage. The culturing procedure described here provides an optimized method for ex vivo expansion of iNKT cells using recombinant human IL-15 (rhIL-15) and recombinant human IL-12 (rhIL-12), which results in cell products with enhanced cytokine secretion and cytotoxicity while maintaining the purity and viability of iNKT cells.

Key words rhIL-15, rhIL-12, Antigen-presenting cells, α-GalCer, Cytokine secretion, Cytotoxicity

1 Introduction

iNKT cells are characterized by their exclusive usage of invariant Vα14Jα18 antigen receptor, which mostly paired with Vβ8.2 in mice and Vβ11 pair in human to recognize lipid antigens presented by CD1d molecule [1, 2]. The importance of iNKT cells in mediating protection against tumors is highlighted by several findings [3–5]. Efficient expansion of iNKT cells ex vivo is essential for their translational usage. A synthetic glycolipid, α-galactosylceramide (-α-GalCer), was identified as the prototypic ligand for iNKT cells [6]. Upon ligand ligation, iNKT cells are activated and elicited effector cell functions by releasing cytokines, functioning as a bridge between innate and adaptive immune responses [7]. The activities of iNKT cells are regulated by cytokines produced by the immune cells they interact with. One of the best-known cytokine regulators of iNKTs is IL-12, which is produced by mature dendritic cells (DCs) that have been activated through Toll-like receptors [8]. The IL-12-triggerd iNKT cells mainly release IFN-γ, which provides an adjuvant effect by inducing activation and expansion of NK cells and other immune cells, including neutrophils, DCs, or macrophages in the innate immune system and CD4+ Th1

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or CD8+ T cells in the adaptive immune system. In contrast, when iNKT cells are engaged with marginal zone B cells or regulatory DCs, which produce IL-10 instead of IL-12, they no longer produce IFN-γ, but rather produce IL-10 to mediate regulatory responses [9, 10].

With an improved knowledge of iNKT cells, it has been widely accepted that both ligand recognition and cytokine-mediated stimulation are required for ex vivo expansion of iNKT cells. Along this line, a classic culturing method of iNKT cells first described by Masaru Taniguchi et al. [11] employed α-GalCer presented by autologous DCs in combination with the cytokine rhIL-2. Here we presented an effective iNKT cell culturing method modified from the original α-GalCer-rhIL-2 approach, which is optimized to enhance the functionality of the cell products. It should be noted that this method is primarily used for culturing human iNKT cells ex vivo from human PBMCs without enrichment.

2 Materials

2.1 Preparation of Human PBMCs from Human Peripheral Blood

Ficoll-Paque plus.

Sterile phosphate buffer saline (PBS).

Serum-free cell medium suitable for culturing cells. RPMI 1640 medium with 10% FBS (V/V) can be used for research purpose.

2.2 Expansion of iNKT Cells

α-GalCer, rhIL-2, rhIL-7, rhIL-15, and rh IL-12. Prepare the solution following the manufacturer instruction, divided into small aliquots prior to storage at −20 °C to avoid repeated freezing and thawing.

Serum-free cell medium is suitable for culturing cells. RPMI 1640 medium with 10% FBS (V/V) can be used for research purpose.

2.3 Expansion of DCs

rhIL-4 and GM-CSF. Prepare the solution following the manufacturer instruction, divided into small aliquots prior to storage at −20 °C to avoid repeated freezing and thawing.

Serum-free cell medium suitable for culturing cells. RPMI 1640 medium with 10% FBS (V/V) can be used for research purpose.

2.4 Assessment of the Purity of iNKT Cells in the Cell Products

Staining buffer (PBS, 2% FBS, V/V).

Antibodies: PE-conjugated anti-human Vα24 TCR and fluorescence-conjugated anti-human Vβ11 TCR.

2.5 Measurement of Cytokine Secreted by iNKT Cells

Detection kit of enzyme-linked immunosorbent assay (ELISA) or cytometric bead array (CBA) for human IFN-γ and human IL-4.

3 Methods

3.1 Preparation of Human PBMCs from Human Peripheral Blood

1. Transfer 10 mL of anti-coagulated human peripheral blood into a plastic tube (*see* **Note 1**).
2. Dilute the whole blood with two volumes of PBS.
3. Place 15 mL of Ficoll-Paque plus in a 50 mL tube. Carefully lay up 30 mL of the diluted blood sample without disturbing the interface.
4. Centrifuge at 600 × *g* for 30 min at room temperature (RT) with acceleration at level 3 and deceleration at level 2.
5. Human PBMCs should be visible at the PBS/Ficoll-Paque plus interface. Carefully remove the upper PBS layer and harvest PBMCs at the interface by a pipette.
6. Wash twice with 30 mL of PBS. Between wash, pellet the PBMC by centrifugation at 800 × *g* for 5 min at RT without limitation on acceleration or deceleration.
7. Resuspend PBMCs with 20 mL of serum-free culture medium and count live cell by Trypan blue staining.
8. Keep a fraction of PBMCs in liquid nitrogen as antigen-presenting cells (APCs) for restimulation of human iNKT cells during the culture period.

3.2 Culture of Human iNKT Cells

1. Wash 1×10^7 isolated PBMCs twice with serum-free cell medium by centrifugation at 800 × *g* for 5 min at RT. Resuspend the cell pellet in serum-free medium to a final density of 2×10^6 cells/mL.
2. Plate the cells in T25 flask, add α-GalCer to make a final concentration of 100 ng/mL, and culture in the 5% CO_2 incubator for 7 days. Refeed the cells with serum-free medium supplemented with 100 ng/mL α-GalCer on day 3.
3. Add recombinant human IL-2 (rhIL2) and recombinant human IL-7 (rhIL-7) into the culture medium at the concentration of 100 IU/mL and 10 ng/mL, respectively, on day 7.
4. Add a-GalCer-pulsed autologous DCs on day 7 to restimulate iNKT cells.

   For preparation of APCs containing DCs from peripheral blood, quickly thaw autologous PBMCs stored in liquid nitrogen, plate in flask, and keep in the cell incubator for 1 h (*see* **Note 2**). Loosen non-adherent cells by swirling the plate and aspirate the medium. Add serum-free medium containing 50 ng/mL of recombinant human granulocyte-macrophage colony-stimulating factor (rhGM-CSF) and 100 ng/mL of recombinant human IL-4 (rhIL-4) to the flask. Keep in the

cell incubator for 7 days. Pulse the cell culture with 200 ng/mL of a-GalCer for 24 h. Collect the cells by centrifugation, wash twice with serum-free medium, resuspend in serum-free medium supplemented with α-GalCer, rhIL-2, and rhIL-7, and then add into the iNKT culture.

5. Replenish the iNKT culturing with α-GalCer-pulsed autologous DCs in the presence of a final concentration of 10 ng/mL of rhIL-15 on day 14.
6. Add rhIL-12 to the iNKT culturing flask to a final concentration of 10 ng/mL on day 20.
7. Refeed the cells every 3–4 days (*see* **Note 3**). Put off the addition of rhIL-12 if the cell counting on day 20 is low. IL-12 could be added into the culturing medium 24–48 h before harvest (*see* **Note 4**).

Anticipated Results.

Human iNKT cells can be expanded by culturing human PBMCs over a period of 3–4 weeks (*see* **Note 5**). The approximate yield of iNKT cells is 10^8 per 10^7 human PBMCs without enrichment.

3.3 Assessment of iNKT Cell Population by FACS

For FACS-based assessment, iNKT cells can be stained with α-GalCer/human CD1d dimer, anti-human αβ TCR, or a combination of anti-human Vα24 TCR anti-human Vβ11 TCR [11]. The procedure described below uses PE-conjugated anti-human Vα24 TCR and FITC-conjugated anti-human Vβ11 TCR as an example (*see* **Note 6**).

1. Resuspend 1×10^6 cells in 50μL staining buffer containing 1μg human IgG; incubate for 10 min at 4 °C to block nonspecific binding to Fcg receptors (FcγRs).
2. Wash the cells twice with 50μL staining buffer. Between the washes, the cells are pelleted by centrifugation at 800 × *g* for 5 min at 4 °C.
3. Resuspend the cells in 50μL staining buffer containing PE-conjugated anti-human Vα24 TCR and FITC-conjugated anti-human Vβ11 TCR mAbs; incubate for 30 min at 4 °C in the dark. For the negative control, cells are stained with isotype-matched control antibody.
4. Wash the cells with staining buffer by centrifugation at 800 × *g* for 5 min at 4 °C and resuspend in FACS buffer. Keep the cells on ice in the dark before being analyzed on flow cytometer.
5. iNKT population is identified as double Va24- and Vb11-positive cells (Fig. 1a).

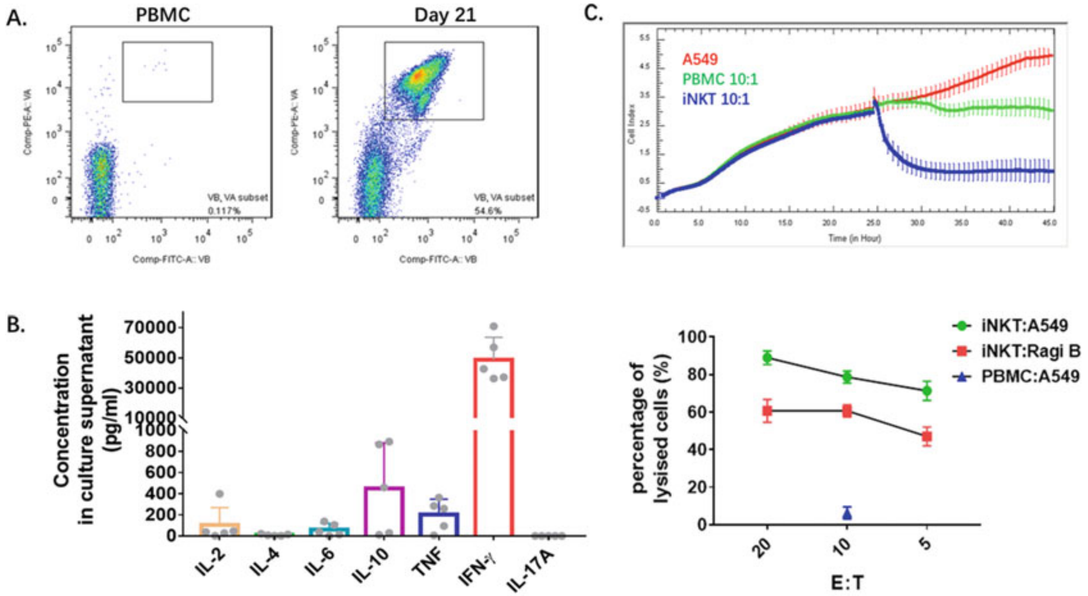

Fig. 1 Characterization of iNKT cell product. The ex vivo-expanded iNKT cells from five health volunteers were evaluated for the abundance of iNKT cells and their functionality. (**a**) Example of FACS-mediated assessment of iNKT subpopulation, identified as TCR Vα24 + Vβ11+ cells, in PBMC and the derived 21-day culture. (**b**) Quantification of the cytokines in the medium of 21-day culture using cytometric bead array. (**c**) Cytotoxicity of iNKT cells against the model cell lines of A549 and Raji B assessed by real-time cell analysis system. Upper panel: raw picture. Lower panel: analyzed data

Anticipated Results.

Generally, iNKT cells are less than 0.5% of human PBMCs. There are significant variations among individuals in the percentages of iNKT cells in human PBMCs. As shown in Fig. 1a, our method has the capacity to expand the concentration of iNKT cell population by hundred-to-thousand-fold.

3.4 Assessment of the Antitumor Functionality of iNKT Cells

1. Measurement of cytokines secreted by iNKT cells.

   The ratio of IFN-γ/IL-4 has been regarded as an effective indicator to the antitumor activity of iNKT cells [12]. Cytokines can be quantitatively detected by either enzyme-linked immunosorbent assay (ELISA) or cytometric bead array (CBA) following manufacturer protocol (Fig. 1b).

2. Cytotoxicity of iNKT cells.

   The antitumor cytotoxicity of iNKT cells can be evaluated by measuring the ratio of cytolyzed tumor cells after co-incubation with the iNKT cells being assayed. This measurement would be facilitated by machine that can perform real-time cell analysis, allowing a convenient monitoring of killing dynamics (Fig. 1c).

4 Notes

1. Although PBMCs used here were buffy coat which are separated from whole blood, it is feasible to use leukapheresis from the donor.
2. Monocytes are separated here by using their differentiated capacity of attachment to the bottom of the flask. It is also feasible to use CD16+ monocyte isolation kit to separate monocyte from other cell populations.
3. Cell density is a key impactor to the purity of iNKT cells in the final cell product. Seed 2×10^6 cells/mL PBMCs to the flask at the very beginning of cell culture, and keep the density at 1×10^6 cells/mL when refeed the culture system with fresh medium.
4. The culture procedure could be separated into three periods with different cytokines added. In the first 7 days, iNKT cells are enriched with sole α-GalCer added into the medium. Viability and cell count decreases, while debris increases in the system. The proliferation curve begins to go up after the addition of IL-2, and IL-7 are added on day 7. Cells proliferate quickly, especially iNKT cells, which were activated with α-GalCer before. In the last 7 days, IL-15 and IL-12 are added to enhance the secretion of IFN-γ and cytotoxicity of iNKT cells.
5. Cytokines are added according to the anticipant concentration based on the volume of medium refeeded into the system, other than the total volume of the culture.
6. TCR-Vα24+TCR-Vβ11+ cells are regarded as iNKT cells by using flow cytometry here. CD1d-dimer can replace anti-TCR-Vα24 antibody to detect iNKT cells with anti-TCR-Vβ11 antibody.

Acknowledgments

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Chapter 12

The Expansion and Cytotoxicity Detection of Human iNKT Cells

Xue Cheng, Xiaosheng Tan, Rui Dou, Xiongwen Wu, and Xiufang Weng

Abstract

Invariant natural killer T (iNKT) cell is a type of innate-like T cell subsets with both T and NK cell phenotype and functions. They recognize lipid antigens presented by CD1d molecules and can be specifically activated by alpha-galactosylceramide (α-GalCer) in vitro. After activation, iNKT cells expand efficiently and exert direct killing effects. Based on it, we mainly introduce the protocols of detection of human iNKT cell functions in vitro, including in vitro expansion and their cytotoxicity to tumor cells.

Key words iNKT cells, Expansion, Cytotoxicity

1 Introduction

Compared with conventional T cells, iNKT cells are more limited in the diversity of T cell receptors, which usually use Vα24-Jα18 paired Vβ11 in humans and Vα124-Jα18 paired Vβ8.2, 7 or 2 in mouse [1]. iNKT cells are abundant in the liver and also found in peripheral blood, lung, intestine, thymus, bone narrow, spleen, and adipose tissue [2]. iNKT cell recognize lipid antigens presented by CD1d, a non-polymorphic major histocompatibility complex class I-like antigen-presenting molecule [3, 4]. The alpha-galactosylceramide (α-GalCer), a synthetic lipid of a chemical purified from the deep sea sponge *Agelas mauritianus* [5], can activate iNKT cells specifically in vitro. Upon activation, iNKT cells can release a large number of cytokines instantly, expand efficiently [6], directly kill tumor cells, and cross-talk with other immune cells to activate both innate and adaptive immune responses [3, 4, 7]. Although they are relatively low frequent in humans, the unique characteristics make them essential for the immune responses in pathological conditions including autoimmunity, infection diseases, and cancers [2, 3,

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8]. Here, we mainly introduce methods of detection of human iNKT cell functions in vitro, including α-GalCer-induced iNKT cell expansion and their direct cytotoxicity to tumor cells.

2 Materials

2.1 Expansion of Human iNKT Cells In Vitro

1. Plastic tubes: 1.5 ml tube, 15 ml tube, 50 ml tube.
2. Flat-bottom 96-well culture plates.
3. Hemocytometer.
4. Ultrasonic cleaner.
5. Phosphate-buffered saline (PBS) without calcium and magnesium.
6. RPMI medium 1640 basic (1×).
7. Complete RPMI1640 culture medium: RPMI1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin, 100 mg/ml streptomycin, 10 mM HEPES buffer solution, 0.1 mM MEM nonessential amino acids, 1 mM sodium pyruvate, and 5.5 mM 2-mercaptoethanol (2-ME).
8. Ficoll-Paque Plus.
9. α-GalCer.
10. Human recombinant interleukin-2 (IL-2).
11. Trypan blue staining solution, 0.4% (wt/vol).
12. Allegra X-15R centrifuge.
13. Flow cytometer.

2.2 Isolation of Human iNKT Cells

1. Phosphate-buffered saline (PBS) without calcium and magnesium.
2. Bovine serum albumin (BSA) albumin fraction V.
3. Separation buffer: PBS, PH7.2, 0.5% BSA, and 2 mM EDTA.
4. APC-labeled PBS57/CD1d-tetramer: provided by the Tetramer Core Facility of the National Institutes of Health, USA (http://research.yerkes.emory.edu/tetramer core/MR1-Tetramers.html).
5. Anti-APC MicroBeads.
6. MS Column.
7. OctoMACS Separator.
8. Hemocytometer.
9. Allegra X-15R centrifuge.
10. Flow cytometer.

2.3 Cytotoxicity Assay of Human iNKT Cells

1. CD1d transfectant of HepG2 cell line (HepG2-tmCD1d) with highly surface CD1d expression.
2. Phosphate-buffered saline (PBS) without calcium and magnesium.
3. Fetal bovine serum (FBS).
4. Tag-it Violet™ Proliferation and Cell Tracking Dye.
5. FITC Annexin V Apoptosis Detection Kit I.
6. Hemocytometer.
7. Incubator.
8. Allegra X-15R centrifuge.
9. Flow cytometer.

3 Methods

3.1 Human iNKT Cells Expansion from Human Peripheral Blood Mononuclear Cells (PBMCs)

3.1.1 Preparation of Human Peripheral Blood Mononuclear Cells (PBMCs)

1. Dilute the peripheral blood with equal volume of 1 × PBS.
2. Add 5 ml Ficoll-Paque Plus to the 15 ml centrifuge tube, and add the diluted blood onto the Ficoll-Paque Plus along the wall of the centrifuge tube carefully to prevent break of the separation interface.
3. Centrifuge at 900 × *g* for 25 min at room temperature, where the accelerating and decelerating rates are both 4 (*see* **Note 1**).
4. After centrifugation, the liquid is divided into four layers from top to bottom, including the plasma layer, the ring-shaped milky white cell layer, the transparent separation liquid layer, and the red blood cell layer, respectively.
5. Carefully draw the cell layer into another 15 ml centrifuge tube, add at least twice the volume of 1 × PBS, mix well, and centrifuge at 500 × *g* for 10 min.
6. Discard the supernatant, add 5 ml 1 × PBS to resuspend the cell pellet, and centrifuge at 500 × *g* for 10 min.
7. Discard the supernatant and resuspend the cell pellet in complete RPMI 1640 culture medium.
8. Check cell viability by Trypan blue staining, and count the amounts of live cells on a hemocytometer under an optical microscope.

3.1.2 Human iNKT Cells Expansion in Response to α-GalCer/IL-2 In Vitro

1. Adjust the cell density of PBMCs to 5×10^6/ml.
2. Seed the cells into 96-well culture plate (100 μl/well).
3. Add 100 μl complete RPMI 1640 culture medium containing 400 ng/ml α-GalCer with or without 100 U/ml IL-2. α-GalCer should be dissolved by ultrasonic for 30 min at room temperature.

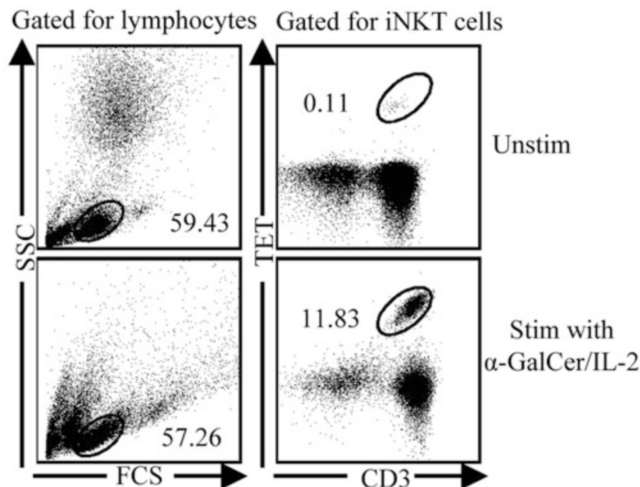

Fig. 1 Human iNKT cells expand from PBMCs upon α-GalCer/IL-2 stimulation PBMCs from heathy donor were stimulated with α-GalCer and IL-2 for 7 days, followed by flow cytometry detection with anti-CD3 antibody and PBS57/CD1d tetramer. Representative plots are shown

4. Mix well and incubate at 37 °C in the incubator with 5% CO_2.
5. Collect 100 μl supernatant, and then supply 100 μl complete RPMI 1640 culture medium containing 50 U/ml IL-2 on the third day of cell culture.
6. Collect the cells and detect the expansion ratio of CD3+PBS57-CD1d-TET+iNKT cells by flow cytometry on the seventh day.
7. The result shows iNKT cells are efficiently expanded with higher ratio (Fig. 1).

3.2 Cytotoxicity Assay of Human iNKT Cells

3.2.1 Enrichment of Human iNKT Cells

1. Collect the expanded cells and centrifuge at 500 × *g* for 6 min.
2. Aspirate supernatant completely, resuspend cell pellet (<5 × 10^7) in 200 μl complete RPMI 1640 culture medium with 1 μl APC-labeled PBS57/CD1d-tetremer, mix well, and incubate for 30 min at 4 °C in the dark.
3. Wash the cells with 5 ml separation buffer by centrifuging at 500 × *g* for 6 min.
4. Aspirate supernatant completely, resuspend cell pellet in 90 μl complete RPMI 1640 culture medium and 10 μl anti-APC microbeads, mix well, and incubate for 30 min at 4 °C in the dark (*see* **Note 2**).
5. Wash the cells with 5 ml separation buffer by centrifuging at 500 × *g* for 6 min.
6. Resuspend the cells with 2 ml separation buffer.
7. Insert MS Columns to OctoMACS™ (*see* **Note 3**).

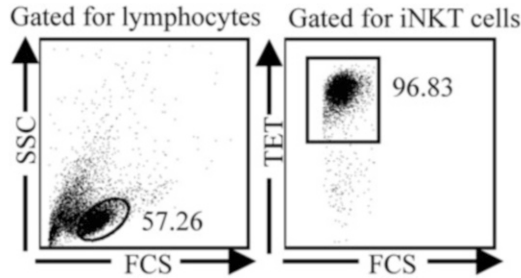

Fig. 2 Purity of enriched iNKT cells. The purity of enriched iNKT cells was detected by flow cytometry with PBS57/CD1d tetramer. Representative plots are shown

8. Prepare MS Columns by rinsing with separation buffer.
9. Apply cell suspension onto the prepared column twice and wash for three times with 1 ml separation buffer.
10. Remove the column from the magnet and place it on a suitable collection tube.
11. Pipette 2 ml separation buffer onto the MS column. Immediately flush out the retained cells within the column with the magnetically labeled cells by firmly pushing the plunger into the column.
12. Collect the cells by centrifuging at 500 × *g* for 6 min and resuspend with 1 ml complete RPMI 1640 culture medium.
13. Check the cells viability by Trypan blue staining, count live cell amounts on a hemocytometer under an optical microscope, and check the purity of isolated cells by flow cytometry.
14. The purity of enriched iNKT cells is shown in Fig. 2.

3.2.2 Prepare of Targets

1. Collect HepG2-tmCD1d cells and wash three times with PBS by centrifuging at 400 × *g* for 5 min.
2. Prepare a 5 μM working solution by diluting 1 ul of 5 mM Tag-it Violet™ stock solution in 1 ml RPMI 1640 (*see* **Note 4**).
3. Resuspend the cells at 1×10^7 to 1×10^8/ml in 5 μM Tag-it Violet™ working solution, mix well, and incubate for 20 min at 37 °C in the dark.
4. Quench the staining by adding half of the original staining volume of FBS, and incubate at 37 °C for 5 min in the dark.
5. Wash three times with PBS by centrifuging at 400 × *g* for 5 min.
6. Resuspend the cells with 1 ml complete RPMI 1640 culture medium.
7. Check the cells viability by Trypan blue staining, and count live cell amounts on a hemocytometer under an optical microscope.

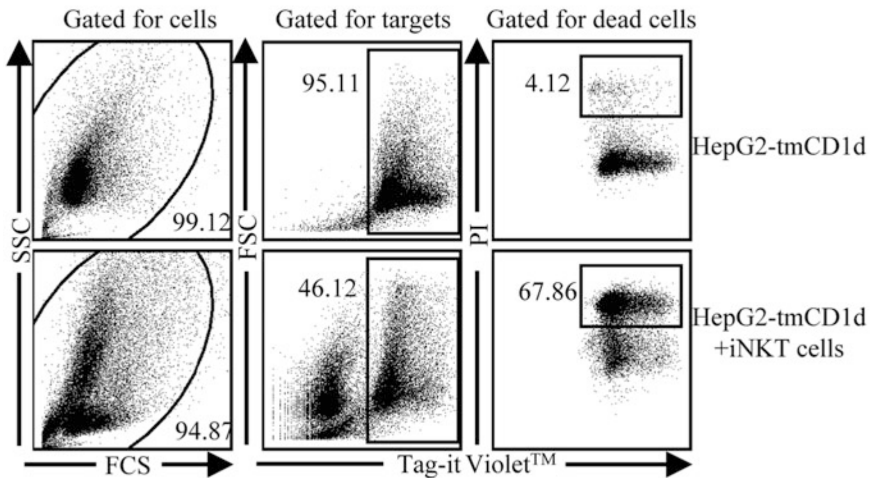

Fig. 3 Killing activity of iNKT cells against HepG2-tmCD1d. The enriched iNKT cells were co-cultured with Tag-it Violet™-labeled HepG2-tmCD1d for 24 h, followed by PI staining. Representative plots for PI positively stained targets are shown

3.2.3 Cytotoxicity Assay

1. Adjust the cell concentration of targets to 5×10^5/ml, and then seed the cells to 96-well culture plates (100 μl/well).
2. Adjust the cell concentration of effectors to 1×10^6/ml, and then seed the cells to 96-well culture plates (100 μl/well).
3. Mix well and incubate at 37 °C for 24 h in an incubator with 5% CO_2.
4. Detect the PI-positive stained targets using FITC Annexin V Apoptosis Detection Kit I by flow cytometry.
5. Figure 3 shows the ratios of PI positively stained dead cells (PI+ %) in indicated groups. Killing activity (% lysis) was calculated by subtracting PI+% in control group (HepG2-tmCD1d) from that in experiment group (HepG2-tmCD1d+iNKT cells).

4 Notes

1. The centrifugal force and time can be adjusted appropriately according to the blood sample volume to achieve the best separation effect.
2. The antibody and microbeads dosage need to be adjusted appropriately according to the numbers of total cells.
3. It is necessary for choosing an appropriate MACS column and MACS Separator depending on the number of total cells and magnetically labeled cells.
4. Adjust the appropriate dosage of Tag-it Violet™ according to the number of total cells.

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Chapter 13

Ex Vivo Expansion of Th2-Polarizing Immunotherapeutic iNKT Cells from Human Peripheral Blood

Natasha K. Khatwani, Kelly J. Andrews, and Asha B. Pillai

Abstract

iNKT cells, classified as innate lymphocytes with invariant TCRs, have been highlighted as a putative, "off-the-shelf" cellular immunotherapeutic strategy for the treatment of malignant and nonmalignant diseases. However, their paucity in human blood limits their immunotherapeutic applications. Herein we describe a rigorously optimized 21-day ex vivo expansion method to achieve log-fold increases in immunotherapeutic human iNKT cells.

Key words iNKT cells, NKT cells, Immunotherapy, Cancer immunotherapy, Adoptive cellular therapy, Hematopoietic stem cell transplantation, Bone marrow transplantation, Transplantation, Cytotoxicity, Leukemia

1 Introduction

Human invariant natural killer T (iNKT) cells are a specialized subset of αβ T lymphocytes that express an invariant TCR α-chain (Vα24Jα18), paired with the TCR β-chain, Vβ11 [1–3]. Unlike conventional αβ T lymphocytes, iNKT cells neither recognize peptide antigens nor are restricted by conventional polymorphic MHC/HLA molecules; instead, they recognize glycolipid antigens, such as the synthetic glycolipid, α-galactosylceramide (-α-GalCer), presented on the non-polymorphic glycoprotein molecule CD1d [4–7]. Once stimulated by CD1d-presented glycolipids, iNKT cells can rapidly secrete both Th1 and Th2 cytokines. iNKT cells have been extensively studied for their potent role in immunoregulation, given their ability to promote direct and indirect tolerogenic effects [7–11]. After allogeneic transplantation, for example, iNKT cells have been found to regu-

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late graft-versus-host disease (GvHD), while simultaneously maintaining potent graft-versus-tumor (GVT) effects. This separation of undesirable GvHD from the salutary GVT driven by allogeneic T cells is considered the "holy grail" of hematopoietic stem cell transplantation (HSCT) [12–15]. However, the advancement of iNKT cellular immunotherapies is significantly hindered by their low frequency in peripheral blood (< 0.01%).

To address this need, we have developed a highly reproducible and robust 21-day method for the ex vivo expansion of human peripheral blood iNKT cells. Our protocol incorporates concepts from conventional three-step signaling mechanisms in T cell activation [16] tailored specifically for optimal iNKT cell expansion, namely, the use of α-GalCer (**signal 1:** antigen-iTCR engagement) presented on PBMC-derived APCs (**signal 2:** co-stimulation) and supplemented with recombinant human (rh)IL-2 and (rh)IL-7 (**signal 3:** cytokines/growth factors) to achieve a striking 320-fold (median) increase of iNKT cells from day 0 to day 21 [17]. Additionally, we show via the ^{51}Cr release assay, that a single-step signal 1/signal 2 activation of expanded and sorted iNKT cells can significantly enhance their cytotoxicity against the CD1d-expressing Jurkat (T-ALL) cell line as compared to expansion alone.

2 Materials

2.1 iNKT Cell Expansion and Culture

1. Human PBMCs: may be purchased or isolated from single-donor blood products by Ficoll-Paque Plus® density gradient (*see* **Note 1**).
2. iNKT Media: RPMI 1640® w/ L-glutamine, 10 mM HEPES, 0.02 mg/mL gentamicin, 10% human AB serum (*see* **Note 2**). Store at 4 °C and warm to 37 °C prior to use in culture.
3. Sterile 0.45 μm CA membrane vacuum filtration unit (*see* **Note 3**).
4. Cytokines and growth factors: 100 U/mL rhIL-2, 0.4 ng/mL or 4 ng/mL rhIL-7, and 100 ng/mL α-galactosylceramide (α-GalCer) (*see* **Note 4**).
5. Stimulating antibody: 1 μg/mL anti-CD3 monoclonal antibody (MoAb).
6. Irradiator (*see* **Note 5**).
7. 0.1% Trypan blue.
8. Hemocytometer or automated cell counter.
9. T-75 sterile cell culture flasks (*see* **Note 6**).

Table 1
Recommended human iNKT sort panel

Sort Panel		
Antibody target	**Fluorophore**	**Clone**
CD3	APC	HIT3a
Vα24	PE-Cy7	6B11
Live/dead	PI or DAPI	–

Table 2
Recommended human iNKT flow cytometry panel

Flow cytometry panel		
Antibody target	**Fluorophore**	**Clone**
CD3	APC	HIT3a
Vα24	PE-Cy7	6B11
Vβ11	PE	REA559
CD4	APC-Cy7	RPA-T4
CD8	eF450	SK1
Live/dead	BV510 (ghost 510®)	–

2.2 Flow Cytometry and Cell Sorting

1. Cell sorter.
2. Cell analyzer.
3. Antibodies (*see* Tables 1 and 2).
4. FACS buffer: 1× PBS (Ca^{2+}, Mg^{2+} free), 1% human AB serum (*see* **Note 2**), 0.5 mM EDTA, 0.1% NaN_3.
5. Sort buffer: 1× PBS (Ca^{2+}, Mg^{2+} free), 2% human AB serum (*see* **Note 2**).
6. 2% Paraformaldehyde (PFA).
7. Round-bottom polystyrene FACS tubes.
8. 15 mL sterile conical polypropylene tube.

2.3 Functional Assay: Antitumor Cytotoxicity

1. Jurkat cell line.
2. Jurkat growth media: RPMI 1640® w/ L-glutamine, 10% FBS (*see* **Note 2**), 1% penicillin–streptomycin. Store at 4 °C and warm to 37 °C prior to use.
3. Day 21 expanded, sorted human iNKT cells.

4. iNKT media: RPMI 1640® w/ L-glutamine, 10 mM HEPES, 0.02 mg/mL gentamicin, 10% human AB serum (*see* **Note 2**). Store at 4 °C and warm to 37 °C prior to use.
5. 50 ng/mL α-galactosylceramide, α-GalCer.
6. Chromium-51 radionuclide, ^{51}Cr (0.1 mCi) (PerkinElmer).
7. Anti-CD2/3/28 T Cell Activation/Expansion Bead Kit (Miltenyi Biotec).
8. 96-well round bottom plate.
9. LumaPlate™ (Thermo Fisher Scientific).
10. Liquid scintillation counter.
11. Triton-X (SIGMA).

3 Methods

Carry out all procedures at room temperature in a sterile BSL2 biosafety cabinet, and follow standard sterile cell culture technique unless otherwise specified.

3.1 iNKT Expansion

1. On day 0, plate single-donor human PBMCs in T-75 flasks at a concentration of 2×10^6 cells/mL in iNKT media. (iNKT media should be filtered, warmed to 37 °C, and supplemented with 100 U/mL rhIL-2, 0.4 ng/mL rhIL-7 and 100 ng/mL α-GalCer prior to use.) Incubate T-75 flasks for 7 days in 37 °C, 5% CO_2 (*see* **Notes 3** and 7).
2. On day 7, stain cells and sort for live CD3$^+$, Vα24$^+$ (clone 6B11, eBioscience 50-112-3387) iNKT cells using the sort panel shown in Table 1. Collect sorted cells in chilled iNKT media in a 15 mL conical polypropylene tube. *See representative day 7 pre-sort and post-sort flow plots*, Fig. 1.

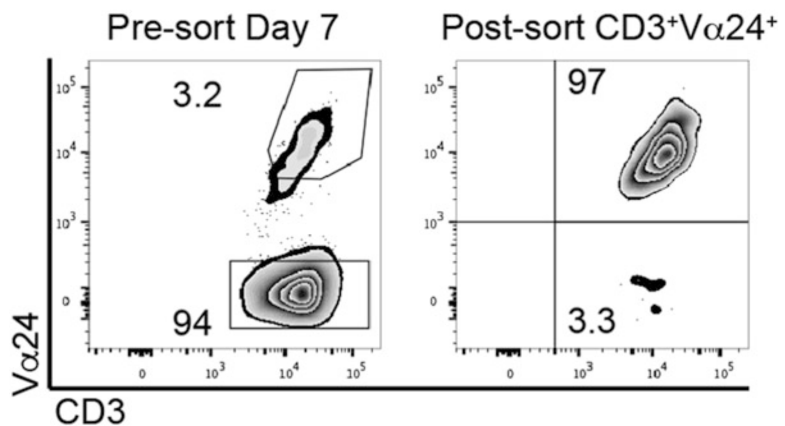

Fig. 1 Representative flow plots showing CD3 and Vα24 expression pre- and post-sort on gated CD3$^+$ cells on day 7. (Reprinted with permission from [17])

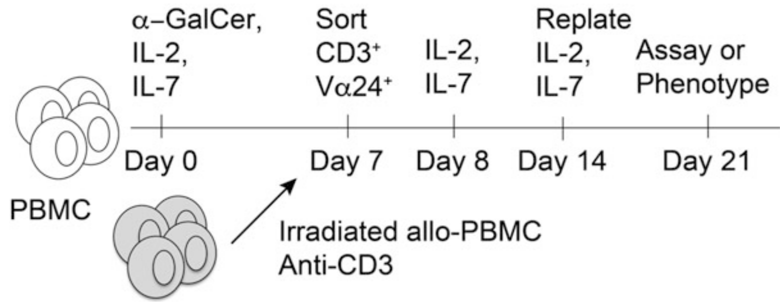

Fig. 2 Human iNKT ex vivo expansion timeline. (Reprinted with permission from [17])

3. Count sorted day 7 $CD3^+V\alpha24^+$ iNKT cells using trypan blue exclusion, and replate in a T-75 (*see* **Note 6**) at a concentration of 1×10^3 to 5×10^4 iNKT cells/mL of iNKT media with allogeneic PBMC a ratio of sorted iNKT cells to irradiated allogeneic PBMC of 1:50 (*see* **Note 8**). Supplement iNKT media with 1 μg/mL anti-CD3 MoAb, 100 U/mL rhIL-2 and 4 ng/mL rhIL-7 (*see* **Note 9**). Incubate culture flasks, standing flasks upright to enhance cell-cell contact, for an additional 14 days (through day 21), replacing fresh iNKT media supplemented with 100 U/mL rhIL-2 and 4 ng/mL rhIL-7 on days 14 and 21.

   On day 21, iNKT + feeder cells may be cryopreserved for subsequent surface phenotyping and/or functional assays, or they can be stained with antibodies (*see* Table 2) and $CD3^+V\alpha24^+$ iNKT cells re-sorted for immediate use. Collect sorted cells in chilled iNKT media in a 15 mL conical polypropylene tube, and count using trypan blue exclusion. *See expansion protocol timeline in* Fig. 2.

3.2 iNKT Yield and Phenotype Assessment by Flow Cytometry

Harvested samples may be further handled under nonsterile conditions. Keep samples on ice at all times to preserve viability and function.

1. Assess and compare iNKT expansion yield and phenotypes by flow cytometry at days 0, 7 (pre-sort), 14, and 21, by harvesting at least 100,000 cells/sample at relevant time points.
2. Wash each sample with 1–2 mL of FACS buffer and centrifuge for 5 min at 400 × *g*. Carefully decant supernatant and repeat wash step.
3. Using the panel shown (*see* Table 2), stain samples and relevant compensation controls for 30 min in the dark, on ice.
4. Wash each sample using 100–200 μL of FACS buffer and centrifuge for 5 min at 400 × *g*. Carefully decant supernatant and repeat this wash step two times further.

5. Fix the stained cells with 100 μL of 2% PFA for 10 min in the dark, on ice. Wash with 100–200 μL of ice cold FACS buffer and centrifuge for 5 min at 800 × *g*. Carefully decant supernatant and repeat this wash step two times further.
6. For immediate flow cytometry analyses, resuspend cell pellets in 200–300 μL of fresh, ice-cold FACS buffer, and transfer to round-bottom FACS tubes to run samples. For future analysis, store cell pellets in the dark at 4 °C until time of analysis (*see* **Note 10**).
7. Analyze data using FlowJo® v10. *See iNKT expansion yield comparisons between days 0, 7, 14, and 21 in Fig. 3 and CD4/CD8 phenotypic comparisons between days 0, 7, and 21 in Fig. 4.*

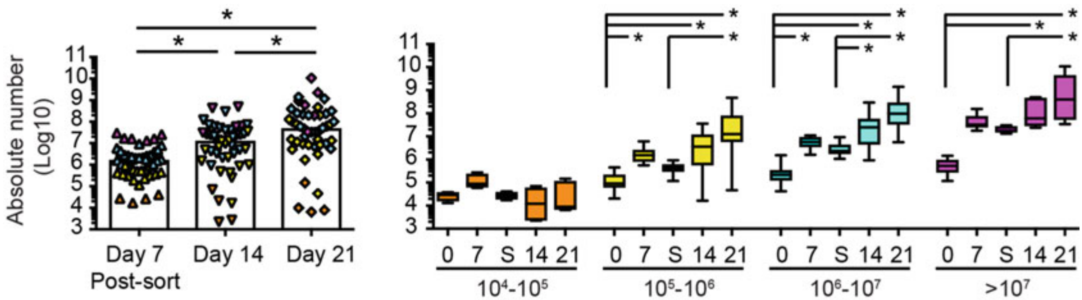

Fig. 3 *Left panel:* median absolute number CD3$^+$Vα24$^+$ iNKT cells at day 7, day 14, and day 21 stratified by day 7 sort yield categories 10^4 to 10^5 (orange, $N = 4$), 105-106 (yellow, $N = 16$), 10^6 to 10^7 (cyan, $N = 21$), and $> 10^7$ (magenta, $N = 6$). *Right panel:* sub-analysis of expansion data by day 7 sort categories showing median and IQR ± range absolute number CD3$^+$Vα24$^+$ iNKT cells at specified time points in the expansion protocol (S, day 7 post-sort). (Reprinted with permission from [17])

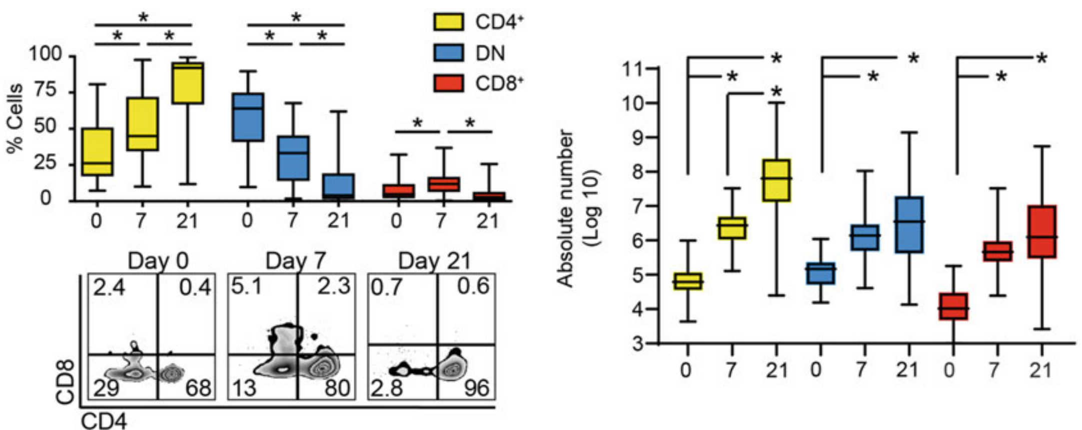

Fig. 4 *Left panel:* Median and IQR ± range percentage *(top)* with representative flow plots *(bottom)* of CD4 and CD8 expression in gated CD3$^+$Vα24$^+$ cells on days 0, 7, and 21 ($N = 36$). *Right panel*: Median and IQR ± range absolute number of CD4$^+$, DN, and CD8$^+$ CD3$^+$Vα24$^+$ iNKT cells on days 0, 7, and 21 (left panel; $N = 36$). $P < 0.05$ is represented by *. (Reprinted with permission from [17])

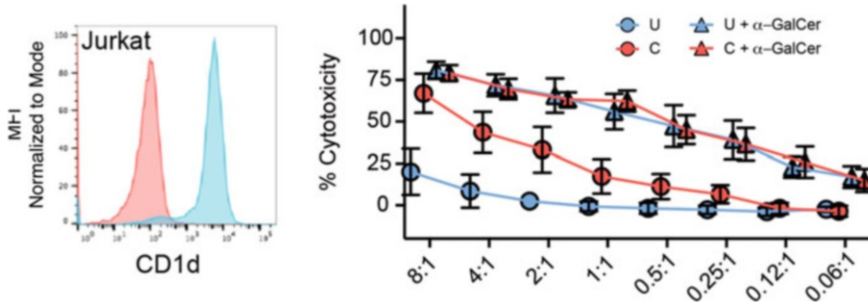

Fig. 5 *Left Panel:* Representative histogram showing CD1d expression, normalized to mode, on Jurkat cells. *Red*, isotype control; *blue*, anti-CD1d. *Right Panel:* Mean percent cytotoxicity ± SEM of day 21 sorted iNKT effectors (E) against Jurkat targets (T) at the indicated E:T target ratios (*x* axis) via ^{51}Cr release assay ($N = 4$). *Blue circles*, iNKT + unloaded beads; *blue triangles*, iNKT + unloaded beads + 50 ng/mL α-GalCer; *red circles*, iNKT + anti-CD2/3/28 loaded beads; *red triangles*, iNKT + anti-CD2/3/28 loaded beads + 50 ng/mL α-GalCer. (Reprinted with permission from [17])

3.3 iNKT Functional Assay: 51Cr Release Antitumor Cytotoxicity Assay

1. In a 24-well plate, stimulate day 21 iNKT cells (effectors, ***E***) (either freshly sorted or previously sorted, cryopreserved and re-thawed) using anti-CD2/CD3/CD28 loaded beads, control beads, or media only (*see* **Note 11**). Incubate for 24 h at 37 °C, 5% CO_2.
2. Label Jurkat T-ALL human cell line (targets, ***T***) with 0.1 mCi ^{51}Cr in 200 μL of pre-warmed Jurkat media. Incubate for 24 h at 37 °C, 5% CO_2 (*see* **Notes 12** and **13**).
3. After 24 h, wash ^{51}Cr-labeled Jurkat cells three times with Jurkat media (*see* **Note 13**), and plate in triplicate in 96-well round-bottom plate at 10^5 cells/well, either with or without 50 ng/mL α-GalCer.
4. After 24 h, add stimulated iNKT cell effectors (***E***) over ^{51}Cr-labeled Jurkat targets (***T***) in the 96-well plate holding the target cell number constant and varying the effector numbers to achieve **E:T ratios** of 8:1 to 0.0625:1. Incubate plates for 18 h at 37 °C in 5% CO_2.
5. After 18 h of co-culture, centrifuge the 96-well plate at $400 \times g$ for 5 min and transfer the supernatant to a LumaPlate™.
6. Place the LumaPlate™ in a liquid scintillation counter and quantitate beta-emission (*see* **Note 14**). *See Fig. 5 for representative cytotoxicity data across varying E:T ratios.*

4 Notes

1. Two allogeneic donors are needed per single-donor expansion (one is the iNKT expansion product and the other is the allogeneic feeder source, co-cultured with sorted iNKT cells from day 7 onward). Autologous PBMCs may also be used as

feeders in place of allogeneic PBMCs. PBMCs may be either freshly isolated prior to expansion or previously cryopreserved and thawed for expansion.

2. Human AB serum and fetal bovine serum (FBS) must have complement heat-inactivated for 30 min at 56 °C.

3. We recommend filtering formulated iNKT media through a 0.45 μm CA membrane filtration unit before using for culture.

4. 0.4 ng/mL rhIL-7 (1×) is used for the first 7 days of expansion, prior to the first sort. 4 ng/mL rhIL-7 (10×) is used for the remainder of the expansion period, i.e., on day 7/8 post-sort and day 14.

5. For feeder irradiation, either a standard orthovoltage source irradiator or a ^{137}Cs (cesium-137) radioisotope source irradiator may be used.

6. Depending on donor-specific iNKT yield variations on day 7 post-sort, T-25 flasks, 6-well, 12-well, 24-well, or 48-well plates may be needed to accommodate lower iNKT yields. For example, if day 7 post-sort yield is ~8000 total iNKT cells ($<10^4$), then the number of irradiated allo-PBMCs needed is 50×8000 cells = 400,000 irradiated allo-PBMCs. Thus, the total number of cells to plate is 400,000 allo-PBMCs + 8000 day 7 iNKTs = 408,000 total cells. Plate these cells in a 48-well plate (1 well), in ~400–500 μL media.

7. Our data shows that higher iNKT day 7 yields correlate with higher iNKT yields at the end of 21 days expansion (*see* Fig. 3). Therefore, it is advised to start with at least 2×10^8 PBMCs per donor. Given the recommended PBMC culture concentration of 2×10^6 PBMCs/mL iNKT media, 10 mL/flask of PBMC-iNKT media suspension and a total of 10 T-75 flasks are required for 2×10^8 starting PBMCs.

8. Irradiate the required number of allogeneic or autologous feeder human PBMCs at 5000 cGy. Feeder cells may be either fresh or previously cryopreserved and thawed.

9. 100 U/mL rhIL-2 and 4 ng/mL rhIL-7 may be added as early as day 7, immediately after sorting, or as late as day 10.

10. It is highly recommended to analyze samples within 1 week of staining and fixing.

11. Prepare stimulation beads (loaded with antibodies or unloaded beads) as per manufacturer's instructions. Stimulate iNKT cells using a 1:2, bead:iNKT cell ratio in 1 mL of iNKT media.

12. Harvest Jurkat cell line at 80–90% confluence for use in cytotoxicity assay.

13. ^{51}Cr presents an external dose hazard via its gamma emissions. Users must minimally follow the "*Safe Handling Guide: Chromium-51 Handling Precautions*" provided by the

manufacturer and are encouraged to consult their institutional radiation safety specialist to tailor safety precautions to the user's specific needs and/or applications.

14. Use the following formula to calculate % cytotoxicity from counts per minute (CPM) data collected from liquid scintillation counter:

$$\frac{\left(CPM_{experimental} - CPM_{spontaneous}\right)}{\left(CPM_{maximum} - CPM_{spontaneous}\right)} \times 100 = \%Cytotoxicity$$

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Chapter 14

Intravital Microscopy Imaging of Invariant Natural Killer T-Cell Dynamics in the Liver Using CXCR6-eGFP Transgenic Mice

Zhou Hong, Zeng Zhutian, and Wang Fei

Abstract

The immune response in the liver is a highly dynamic process involving the recruitment of many types of immune cells. As a powerful imaging technique, intravital microscopy has been widely used for real-time observation and quantification of cell movements in living animals. Here we describe the use of an in vivo half-dissociated preparation method combined with intravital confocal microscopy to observe the dynamic activities of invariant natural killer T cells in the liver of CXCR6$^{GFP/+}$ transgenic mice. We believe that this method will enable researchers to explore the dynamics of many other types of immune cells in the liver.

Key words Intravital microscopy, Live cell imaging, Invariant natural killer T cells, Liver, CXCR6$^{GFP/+}$ transgenic mice

1 Introduction

Natural killer T (NKT) cells are a heterogeneous group of T cells that share properties of both T cells and natural killer cells. These cells can efficiently recognize the self and foreign lipid and glycolipid antigens presented by the major histocompatibility complex class I-like molecule CD1d [1, 2]. NKT cells constitute approximately 1% of all peripheral blood T cells. These cells are involved in removing pathogens or tumor cells from the body, as well as in the development of allergic reactions and autoimmune diseases. Invariant NKT (iNKT) cells represent a major population of NKT cells expressing a unique invariant TCRα chain (Vα14Jα18 in mice or Vα24Jα18 in humans) and a limited number of variable TCRβ chains. iNKT cells are mainly distributed in the liver, thymus, adipose tissue, spleen, lymph nodes, and peripheral blood [3, 4]. iNKT cells are particularly enriched in the murine liver and form a nexus between innate and adaptive immunities; therefore,

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these cells play an important role in mediating immune responses under inflammatory conditions in the liver [5, 6].

Intravital microscopy (IVM) imaging has become a cutting-edge technique for tracking the behaviors of living cells in real time while providing remarkable insight into cellular dynamics and pathophysiological processes within tissues. Liver half-dissociated fixation is a process in which the mouse's liver is partially separated from the body and stabilized onto an observation board without damaging the hilar structure (Fig. 1). With this method, cellular activity of iNKT cells in the liver could be examined in real time by confocal microscopy.

In CXCR6$^{GFP/+}$ transgenic mice, one allele of the murine *CXCR6* gene is replaced with the gene encoding green fluorescent

Fig. 1 (**a**) Expose the abdominal cavity of the experimental mouse, tie the free end of the sternum stem with surgical thread, and pull it upward and fix it; (**b**) use forceps to lift the cholecyst, and carefully dissect the falciform ligament; (**c**) transfer the mouse to the observation board, fix the traction line, remove other organs in the abdominal cavity, and wrap them with moist gauze; (**d**) lift the stomach and carefully dissect the patogastric ligament; (**e**) use multiple air-laid papers to pull the liver out of the abdominal cavity, and fix it on the transparent glass area of the observation board; (**f**) after the surgery, the size and position of the free liver can be viewed from the bottom of the observation board

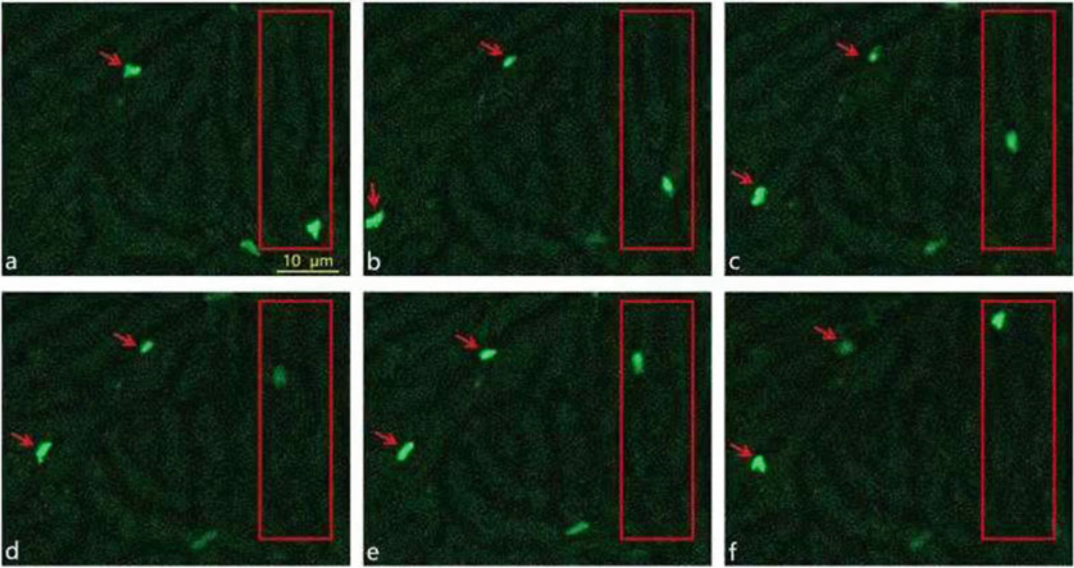

Fig. 2 Migratory trajectories of iNKT cells in murine liver using IVM. The recording time was 20 min, with an interval of 2 min for each capture (**a**–**f**). The fields of view show the movement track of green iNKT cells in the liver tissue of CXCR6$^{GFP/+}$ transgenic mice

protein (GFP), enabling GFP to be specifically expressed by CXCR6-expressing cells that are mainly found in the liver and spleen. These transgenic mice can be used as an ideal model to visualize the cellular behaviors of hepatic iNKT cells, as more than 70% of GFP-expressing cells in the liver are iNKT cells. Here we established a gentle preparation and stable fixation method for observing the migratory trajectories of iNKT cells in the liver of anesthetized CXCR6$^{GFP/+}$ transgenic mice using intravital microscopy (Fig. 2).

2 Materials

2.1 Mice

CXCR6$^{-/GFP}$ transgenic mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA). Mice between 8 and 10 weeks of age were preferred for this procedure. All animal experimental protocols were performed in accordance with the requirements of experimental animal ethics and national animal care guidelines.

2.2 Reagents

1. 0.9% sodium chloride, normal saline.
2. 75% ethyl alcohol solution.
3. 1.25% avertin solution.

2.3 Equipment

1. Confocal microscope.
2. Pet electric clipper.
3. Chemical hair removal cream.
4. Sterile cotton swabs.
5. Microscope cover slips, 24 × 50 mm.
6. Sterile cotton gauze, 8.5 × 5 cm.
7. 1 mL and 20 mL syringes.
8. Dissecting scissors (straight), length 9 cm.
9. Dissecting scissors (curved), length 9 cm.
10. Graefe serrated forceps (straight), length 10 cm.
11. Graefe serrated forceps (curved), length 10 cm.
12. Microdissecting scissors.
13. Graefe tissue forceps.
14. Gemini cautery system (*see* **Note 1**).
15. Millex GS filter unit, 0.22 μm.
16. Sterile surgical gloves.
17. Imaging board.
18. Medical tape.
19. Air-laid paper.
20. 4-0 Nonresorbable polypropylene sutures.
21. Autoclave.
22. Heating pad.

2.4 Reagent and Equipment Setup

1.25% avertin solution: Dissolve 2.5 g of tribromoethanol in 5 mL of *tert*-Amyl alcohol, add 200 mL of normal saline in it, mix thoroughly, and then filter-sterilize the solution through a 0.22 μm filter. This solution should be stored at 4 °C, which remains stable up to a month.

Custom-made imaging board: Position the 24 × 50 cover slip on a viewing window and fix it with a tape.

3 Methods

Disinfect the working space using 75% ethanol and all surgical instruments by autoclaving for 1.5 h. Prepare all the reagents at room temperature (20–24 °C) in the laboratory and perform filter sterilization.

3.1 Preoperative Preparation of Mouse

Timing ~20–30 min

1. Weigh the mouse and intraperitoneally inject an appropriate dose of 1.25% avertin using a 1 mL syringe (*see* **Note 2**).
2. Place the anesthetized mouse in dorsal recumbency, and fix all the four legs with medical tape on a heating pad to maintain its body temperature during the surgery (*see* **Note 3**).
3. Thoroughly shear the hair over the ventral side of the mouse using an electric clipper. Apply a layer of chemical hair removal cream over the shaved region with a cotton swab. Next, use a sterile gauze to wipe off the cream toward the head (opposing the direction of the fur) after 1–2 min (*see* **Note 4**).
4. Sterilize the shaved area with 75% ethanol, which also helps remove residual hair and the chemical hair removal cream.

3.2 Surgery for Liver IVM

Timing ~30–50 min

1. Hold the skin of the mouse's abdomen with serrated forceps, and make a 1.5 cm single midline incision through the skin using sterile scissors, extending down from the xiphoid process. Carefully incise the linea alba to separate the fascia located between the skin and underlying muscle and open the abdomen. Make a 1 cm lateral incision from the end of the midline incision to the left and right sides while carefully exposing and avoiding the large vessels in the abdominal wall. Cauterize all visible vessels on one side and remove the skin. Repeat the same procedure on the other side (*see* **Note 5**).
2. Lift the peritoneal wall with smooth forceps, make an incision longitudinally using sterile scissors, and separate the peritoneum from the both sides by cautery system, thereby exposing the proximal liver to the mid-axillary line (*see* **Note 6**).
3. Tie the xiphoid process at the end of the sternum stem with a surgical suture, pull this suture up to the top of the mouse's head, and tape it down to the heating plate. Grab the cholecyst with smooth forceps with maximum precaution. The falciform ligament can be observed between the diaphragm, cholecyst, and liver wall. Cut the ligament downward to the suprahepatic inferior vena cava to dissociate the liver from the diaphragm (*see* **Note** 7).
4. Clean the visible glass area in the center of the observation board using an air-laid paper. Thereafter, place a sterile gauze on the right side of the observation board, and drip normal saline onto the gauze to moisten the gauze on the left side.
5. Move the mouse to the observation board. Tape the xiphoid traction thread on the front of the mouse's head. The large and small intestine, spleen, and stomach should be sequentially removed from the abdominal cavity with two cotton swabs

and then were placed on the lower limbs. Lift the stomach with forceps and carefully dissect the hepatogastric ligament. Wrap all the exposed organs of the mice with a gauze (*see* **Note 8**).

6. By moving and squeezing other tissues or organs of the mouse, the liver viscera can be exposed to the field of vision facing upward, and the largest lobe of the liver is attached to the optical window made of glass. Cover the surface of the liver lobe with air-laid paper, and slowly pull the paper to move the liver lobe out of the abdominal cavity. Use additional air-laid paper to cover and move the liver as described above if necessary (*see* **Note 9**).
7. Adjust the position of the liver by pulling two pieces of air-laid paper to ensure that a large area of the liver is firmly attached to the visible glass area of the heating plate. Next, fix the position of the air-laid paper with tape (*see* **Note 10**) (Fig. 1).

3.3 IVM Observation

Timing ~40 min to several hours

Turn on the confocal microscope and apply appropriate laser and filter settings. The exposed liver lobe should be firmly attached onto the glass area of the heating plate and be visible through the microscope slot (*see* **Note 11**). The excitation light should be focused directly on the exposed area of the liver (*see* **Note 12**). Use acquisition software for tracking and recording, and set the time interval for time-lapse imaging (*see* **Note 13**) (Fig. 2).

4 Notes

1. During cauterization, do not always maintain constant heating. Heat the knife, release the switch, and quickly perform electrocoagulation of the blood vessels or cut the tissues.
2. The dosage of 1.25% avertin working solution is calculated according to the body weight of the mouse at 400 μL/20 g. During injection into the abdominal cavity, fix the mouse upside down with the head of the animal facing downward. This prevents mouse organs, particularly the liver, getting damaged by injection. After waiting for 5–10 min after anesthetization, the mouse can be lightly clamped with ophthalmic forceps to determine the anesthesia state. If the anesthesia is effective, fix the mouse's limbs with tape, and then proceed further.
3. Do not overheat the mouse and keep the heating pad at 36–37 °C, as this enhances the depth of the anesthetic and may be lethal to mice by causing respiratory failure.

4. Hair is strongly autofluorescent; therefore, it is essential to remove as much hair as possible or use mineral oil to prevent it from sticking to the abdominal cavity or liver surface and hampering imaging.
5. For blunt separation, it is best to use Graefe tissue forceps. If bleeding occurs, quickly cauterize the vessels to stop bleeding.
6. As the peritoneum layer is thin and blood vessels are abundant, the procedure starting from infliction of the wound should be performed with an electrocoagulation knife. Expose the lower edge of the chest cavity and xiphoid process on both sides of the mid-axillary line and down to the lower abdominal area.
7. Do not touch the liver during surgery. While lifting the gallbladder, precautions should be taken to avoid puncturing of the gallbladder and bile outflow, as this can obscure the visible area under the microscope. While dissecting the falciform ligament, precautions should be taken to avoid damage to the diaphragm (including diaphragmatic veins), abdominal aorta, or inferior vena cava.
8. The gauze used to wrap the exposed organs of the mouse must first be wetted with normal saline and wrapped around the lower body of the mouse. During the experiment, ensure that the gauze is moistened with normal saline to avoid dehydration and death of the mouse.
9. Observe the diaphragm surface of mouse's liver under a confocal microscope.
10. Do not touch the liver with instruments or cotton swab throughout the procedure except while using air-laid paper.
11. Procedures involving movement and handling of the mouse should be performed with caution.
12. Because the excitation light of the inverted confocal microscope is emitted from the bottom, when the liver is placed in the best observation area, it nearly blocks the beam of excitation light.
13. After debugging under an eyepiece, the shooting mode can be selected on a computer to capture the live images of the liver. Sequentially recorded images from the same field of view can be exported as a time-lapse video.

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Chapter 15

In Vivo Cytotoxicity by α-GalCer-transactivated NK Cells

Patrick T. Rudak and S. M. Mansour Haeryfar

Abstract

Invariant natural killer T (*i*NKT) cells are innate-like, lipid-reactive T lymphocytes known for their potent immunomodulatory properties. In addition to expressing and utilizing cytolytic effector molecules of their own against certain target cells, *i*NKT cells can be stimulated with α-galactosylceramide (α-GalCer) to augment the cytotoxic capacity of natural killer (NK) cells. Herein, we describe a flow cytometry-based in vivo killing assay that enables examination of α-GalCer-promoted cytotoxicity against β2 microglobulin knockout ($\beta 2M^{-/-}$) target cells, which mimic tumor and virus-infected cells displaying little to no MHC class I molecules on their surface. Using an anti-asialo GM1 antibody, which depletes NK cells but not *i*NKT cells, we confirmed that the increased clearance of $\beta 2M^{-/-}$ cells in α-GalCer-primed recipients was mediated by NK cells. The protocol detailed here can be leveraged to assess the functional fitness of *i*NKT cells and their crosstalk with NK cells and to further our understanding of α-GalCer-promoted cytotoxicity in preclinical immunotherapeutic applications.

Key words *i*NKT cells, NK cells, α-galactosylceramide, Cell-mediated cytotoxicity, In vivo killing assays, Flow cytometry, CFSE, Anticancer immunity, Antiviral immunity, Immunotherapy

1 Introduction

Invariant natural killer T (*i*NKT) cells are innate-like T lymphocytes harboring a canonical Vα14-Jα18 gene rearrangement in their T cell receptor (TCR) α chain that is paired with a limited number of Vβ chain options [1, 2]. *i*NKT cells recognize and respond rapidly to glycolipid molecules typified by α-galactosylceramide (α-GalCer) [3]. Upon activation, *i*NKT cells release T helper (T_H)1-, T_H2-, or T_H17-type cytokines, thus shaping the nature of immune responses in various contexts [4], including in cancer immune surveillance and antimicrobial host defense.

α-GalCer-stimulated mouse *i*NKT cells are known to transactivate and augment the cytotoxic function of natural killer (NK) cells [5]. This functional axis relies largely on *i*NKT cells' ability to secrete mediators such as IFN-γ [6] and on reciprocal interactions

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between *i*NKT cells and dendritic cells (DCs), which lead to DC maturation and their upregulated expression of NK cell-activating ligands [5]. α-GalCer-transactivated NK cells are indispensable for the antimetastatic effects of α-GalCer in B16 melanoma, EL4 lymphoma, and other mouse tumor models [7, 8], as well as for the resolution of infections with hepatitis B virus and murine cytomegalovirus [9, 10].

α-GalCer-based immunotherapies have been tested in clinical trials for malignancies and viral diseases [11, 12]. Once optimized, they will offer tempting modalities that should work in genetically diverse patient populations since α-GalCer and similar glycolipid antigens are presented to *i*NKT cells by a non-polymorphic molecule called CD1d [13, 14]. Furthermore, *i*NKT cells' recognition mode is evolutionarily conserved [15]. This is to the extent that human *i*NKT cells bind mouse glycolipid-loaded CD1d and vice versa [16]. Therefore, experimental methods that enable examination of mouse *i*NKT cell activation by α-GalCer and its downstream effects should be informative for understanding *i*NKT cell functions and therapeutic potentials in clinical settings.

In vivo killing assays offer a sensitive and well-controlled approach to studying cell-mediated cytotoxicity within the intact architecture of lymphoid tissues [17]. Therefore, they are superior to traditional in vitro cytotoxicity assays in which the effector phase takes place in test tubes. In vivo killing assays can be employed to assess the cytolytic effector function of tumor-specific $CD8^+$ T cells [17–19], virus-specific $CD8^+$ T cells [20, 21], NK cells [22], *i*NKT cells [23], and alloantibodies [24]. We recently optimized and used a version of these assays for the measurement of α-GalCer-transactivated NK cells [25, 26]. This method was adapted from a previous report describing the clearance of β2 microglobulin knockout ($\beta2M^{-/-}$) target splenocytes, which simulate MHC class $I^{nil/low}$ tumor or virus-infected cells [27], in wild-type (WT) mice [22]. Syngeneic naïve WT splenocytes, which are co-injected into the recipient, serve as control and should remain viable. Labeling control and $\beta2M^{-/-}$ target cells with different concentrations of 5-(and-6)-carboxyfluorescein diacetate *N*-succinimidyl ester (CFSE) before their co-transfer, in equal numbers, into α-GalCer- or vehicle-primed recipients allows for their accurate detection by flow cytometry in multiple locations, including the spleen, liver, and lungs. Percent in vivo killing of $\beta2M^{-/-}$ target cells can be taken as a measure of NK cell-mediated cytotoxicity. By pre-treating the recipients with an anti-asialo GM1 antibody, which depletes NK cells but not *i*NKT cells [28], we have confirmed the enhancement of $\beta2M^{-/-}$ target cell lysis in α-GalCer-primed animals to be largely NK cell-dependent.

The method described here can be employed to assess the crosstalk established between *i*NKT and NK cells following in vivo treatment with α-GalCer or its analogs [11, 12]. When combined with other tools, such as gene knockout mice,

neutralizing or blocking monoclonal antibodies (mAbs), and pharmacological agents, it can shed light on pathways that govern the above crosstalk and the ultimate act of killing. Moreover, this assay should help in testing the preclinical efficacy of new or modified *i*NKT cell-based treatments for cancer and viral diseases. Among information that cannot be deduced from the data are the exact number of killing cycles before the effector cells rest and recycle and a global picture of all tissue sites in which target cells are detected and destroyed.

In this chapter, we provide a step-by-step assay protocol, covering treatment procedures, tissue processing, target cell labeling and injection, data acquisition and analysis, and the formula we use to calculate percent in vivo cytotoxicity. We offer several technical points to ensure assay precision and the high quality of data to be generated.

2 Materials

2.1 General Instruments, Plasticware, and Buffers

1. Certified class II biological safety cabinet (BSC).
2. Centrifuge.
3. Vortex mixer.
4. Water bath.
5. Ice bucket.
6. Hemocytometer or an automated cell counter.
7. Flow cytometer with data acquisition software. The panels described in this chapter are compatible with a two-laser (488 nm and 633 nm), eight-color BD FACSCanto II flow cytometer equipped with BD FACSDiva software.
8. 15-mL and 50-mL conical centrifuge tubes.
9. 1.5-mL microcentrifuge tubes.
10. 5-mL polystyrene round-bottom tubes for flow cytometry.
11. 1× phosphate-buffered saline (PBS), pH 7.4.
12. Staining buffer for flow cytometry: 1× PBS supplemented with 2% heat-inactivated fetal bovine serum (FBS).

2.2 Mice

1. Target cell donors: Adult WT C57BL/6 mice and adult β2M$^{-/-}$ mice on a C57BL/6 background (*see* **Notes 1** and **2**).
2. Target cell recipients: Adult WT C57BL/6 mice (*see* **Note 3**).

2.3 Materials and Reagents for Treatment Procedures

1. 1-mL syringes with 28-gauge needles (*see* **Note 4**).
2. KRN7000/α-GalCer (Funakoshi Co., Ltd.): to be dissolved in ultrapure water containing 5.6% sucrose, 0.75% L-histidine, and 0.5% Tween-20, heated for 10 min in an 80 °C water bath, and subsequently stored in 4–8 μg aliquots at −80 °C.

3. Vehicle for KRN7000 containing 5.6% sucrose, 0.75% L-histidine, and 0.5% Tween-20 in ultrapure water to be heated for 10 min at 80 °C and then stored in small aliquots at −80 °C.
4. Rabbit anti-mouse asialo GM1 polyclonal antibody (Cedarlane), if applicable: to be reconstituted in ultrapure water following the manufacturer's instructions and stored in aliquots of desired quantity at 4 °C until use.
5. Normal rabbit serum (NRS), if applicable: to be reconstituted in ultrapure water according to the manufacturer's instructions and stored in aliquots of desired volume at −20 °C until use.

2.4 Instruments and Materials for Tissue Processing

1. Autoclaved surgical instruments: scissors and tweezers.
2. Single-edge razor blades.
3. 15-mL Wheaton Dounce glass homogenizer.
4. 37 °C humidified incubator containing 6% CO_2.
5. MACSMix tube rotator (Miltenyi Biotec).
6. 100 mm × 15 mm polystyrene Petri dishes.
7. 10-mL syringe plungers.
8. 70-μm nylon mesh cell strainers compatible with 50-mL conical centrifuge tubes.
9. Parafilm.
10. 70% ethanol in a spray bottle.
11. Type IV collagenase from *Clostridium histolyticum* (MilliporeSigma).
12. Percoll PLUS density gradient media (GE Healthcare).
13. RPMI 1640 medium.
14. 10× Dulbecco's PBS.
15. Filter-sterilized water suitable for cell culture.
16. ACK (ammonium-chloride-potassium) lysis buffer containing 150 mM ammonium chloride, 10 mM potassium bicarbonate, and 0.1 mM ethylenediaminetetraacetic acid (EDTA) in ultrapure water.

2.5 Labeling and Injection of Target Cells

1. 1-mL syringes with 29-gauge needles (*see* **Note 4**).
2. CFSE reconstituted to a concentration of 5 mM in dimethyl sulfoxide, aliquoted and stored at −80 °C until use.
3. Heat-inactivated FBS.

2.6 Immunophenotyping (If Applicable)

1. Fc γ receptor blocking reagent: unconjugated rat anti-mouse CD16/CD32 mAb (clone 2.4G2).
2. Fluorescent dyes, fluorochrome-conjugated CD1d tetramers, and fluorochrome-conjugated mAbs (Table 1).

Table 1
Reagents and cytofluorimetric settings used to confirm the efficacy of anti-asialo GM1 Ab treatment in C57BL/6 mice

Marker	Reagent or mAb clone	Fluorochrome[a]	Excitation laser (nm)	Emission filter (nm)	Source
Live/dead	Fixable viability dye	eFluor 780	633[b]	780/60	Thermo Fisher Scientific
*i*NKT cell TCR	PBS-57-loaded CD1d tetramer	APC	633[b]	660/20	NIH Tetramer Core Facility
*i*NKT cell TCR (negative control)	Unloaded CD1d tetramer	APC	633[b]	660/20	NIH Tetramer Core Facility
TCR-β	H57-597	FITC	488[c]	530/30	Thermo Fisher Scientific
NK1.1	PK136	PE-Cy7	488[c]	780/60	Thermo Fisher Scientific

[a]If necessary or desired, alternative fluorochromes for each reagent are available from the sources listed (or other sources)
[b]Red laser
[c]Blue laser

3 Methods

3.1 General Considerations

1. Prior to commencing mouse experiments, ensure that the procedures have been approved by your institutional animal ethics committee (*see* **Note 5**).
2. During donor tissue processing, use sterile instruments and materials and perform all procedures inside a certified class II BSC.
3. During tissue processing, keep tissues/cell suspensions on ice whenever possible, use ice-cold buffers, and perform all centrifugation steps at 4 °C unless otherwise indicated.

3.2 Treatment of Recipient Mice

1. Thaw α-GalCer (2 μg for each mouse to be injected) and the corresponding vehicle at room temperature (*see* **Note 6**).
2. Dilute α-GalCer or vehicle with PBS to a final volume of 200 μL per injection. Prepare each mixture in 1.5-mL microcentrifuge tubes. Keep tubes on ice until injection.
3. Draw up each dose of α-GalCer or vehicle into a syringe with a 28-gauge needle. Avoid drawing air into the syringe as much as possible.

4. With the needle pointing upward, gently flick the top of the syringe and depress the plunger slightly to allow any air bubbles to escape.
5. Inject WT mice intraperitoneally (i.p.) with α-GalCer or vehicle (*see* **Notes 7** and **8**).
6. Return all mice to home cages for 24 h.

3.3 Isolation of Splenocytes from Donor Mice

1. Euthanize the required number of naïve WT and $\beta 2M^{-/-}$ donor mice (*see* Subheading 3.3, **step 15**, and Subheading 3.4, **steps 26–27**) following your approved animal use protocol.
2. Spray the abdominal area of each carcass liberally with 70% ethanol.
3. For each carcass, use tweezers to pull a portion of skin around the center of the abdomen upward, and use scissors to make a small incision in the area. Avoid deep cuts not to puncture or pierce the peritoneum.
4. Pull gently on either end of the incision to peel the skin away from the abdominal region and fully expose the peritoneum.
5. Cut the peritoneum with scissors to access the peritoneal cavity.
6. Locate the spleen toward the left side of the mouse. Gently pull the spleen upward with tweezers while removing as much surrounding connective tissue and fat as possible with scissors.
7. Place WT and $\beta 2M^{-/-}$ spleens into separate 50-mL conical centrifuge tubes containing 10 mL PBS. Transfer the content into a 15-mL glass homogenizer.
8. Grind the spleen several times until it is disrupted into a single-cell suspension (*see* **Note 9**). Transfer the homogenate into separate 50-mL conical tubes. Fill the remainder of each tube with PBS.
9. Centrifuge tubes at 400 × g for 5 min.
10. Discard the supernatant and wash cells once more in PBS.
11. Remove erythrocytes by resuspending cells in 4 mL of ACK lysis buffer for 3 min at room temperature. Immediately afterward, dilute the ACK lysis buffer with 10 mL of PBS.
12. If multiple WT and $\beta 2M^{-/-}$ mice are needed to obtain adequate cell yields, pool cell suspensions together in separate tubes designated for WT and $\beta 2M^{-/-}$ target splenocytes.
13. Filter each cell suspension through a 70-μm cell strainer into a new 50-mL conical tube.
14. Centrifuge tubes at 400 × g for 5 min, discard the supernatant, and resuspend cells in 5 mL of PBS.
15. Using a hemocytometer or an automated cell counter, count splenocytes and confirm their viability (of at least 95%) by

trypan blue dye exclusion. Be as accurate as possible. We typically obtain 5–10 × 10^7 cells/spleen with ≥99% viability depending on the donor's age.

16. Isolated splenocytes from WT and β2M$^{-/-}$ mice are to be used as control target cells and NK target cells, respectively (*see* **Note 10**).

3.4 Labeling and Injection of Target Cells

1. Transfer all the splenocytes from the preparation containing the lower number of cells (either WT or β2M$^{-/-}$ cell preparation) into a 15-mL conical centrifuge tube. Transfer an equal number of cells from the other preparation to a separate 15-mL conical tube (*see* **Note 11**). Be as precise as possible while pipetting.
2. Fill the remainder of both tubes with PBS.
3. Spin the tubes at 400 × *g* for 5 min.
4. During the centrifugation step above, prepare 7 mL of a 2 μM solution of CFSE in PBS. Upon adding the stock solution of CFSE to PBS, vortex thoroughly. Next, in a separate tube, add 1 mL of the above solution (2 μM CFSE) to 9 mL of PBS to prepare a 0.2 μM solution of CFSE. Vortex thoroughly.
5. Discard most of the supernatant from each tube containing splenocyte suspensions, leaving behind approximately 50–100 μL of PBS on top of the pelleted cells (*see* **Note 12**). Be as consistent as possible with the amount of PBS left in each tube. Make sure both tubes contain visually similar PBS levels.
6. Resuspend cells thoroughly in the remaining PBS in each tube by gently pipetting up and down several times. Ensure that the resulting suspension is completely homogenous.
7. Add 5 mL of 0.2-μM and 2-μM CFSE solutions to WT and β2M$^{-/-}$ splenocyte suspensions, respectively, while shaking the tubes by hand at a moderate pace (*see* **Note 13**).
8. Incubate samples in a 37 °C water bath for 15 min. At 5-min intervals, mix tubes gently by hand to ensure that the cells remain in suspension.
9. Immediately afterward, add 3 mL of FBS to each tube. Mix well by inversion and/or gentle shaking by hand.
10. Fill the remainder of each tube with PBS.
11. Centrifuge tubes at 400 × *g* for 5 min.
12. Discard the supernatant and wash cells once more in PBS.
13. Resuspend cells thoroughly in 3 mL of PBS.
14. Into a single 5-mL polystyrene tube containing 500 μL of PBS, carefully pipet 10 μL, each, of WT and β2M$^{-/-}$ splenocyte suspensions (*see* **Note 14**).

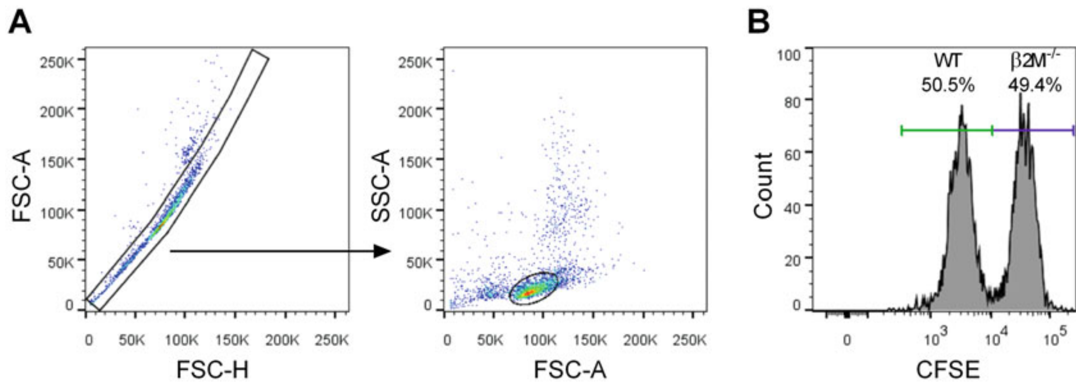

Fig. 1 Confirming equal numbers of WT and β2M$^{-/-}$ splenocytes present in cell mixtures. (**a**) Gating strategy used to define singlets (left panel) followed by lymphocytes (right panel) during cytofluorometric analyses. (**b**) After applying the pre-gate described in (**a**), the frequencies of CFSE^{low} and CFSE^{high} cells after being mixed at a 1:1 ratio are determined as shown in the representative histogram

15. With the CFSE channel open (488 nm excitation laser, 530/30 nm emission filter), acquire 5,000–10,000 events from the preliminary cell mixture using a flow cytometer.
16. After excluding doublets based on FSC-A and FSC-H properties, gate on lymphocytes using FSC-A and SSC-A characteristics (Fig. 1a).
17. Using histograms, draw CFSE^{low} and CFSE^{high} gates corresponding to WT and β2M$^{-/-}$ target cells, respectively.
18. Calculating the frequencies of CFSE^{low} and CFSE^{high} events will allow for subtle volume adjustments to cell suspensions to be made, if necessary, before they will be mixed again for injection into the recipients. The final cell mixture should contain equal or near-equal numbers of CFSE^{low} and CFSE^{high} cells (*see* **Note 15**).
19. Into a new 5-mL polystyrene tube containing 500 μL of PBS, carefully add adjusted volumes of WT and β2M$^{-/-}$ splenocyte suspensions, no more than 10 μL each, based on the above frequency ratio calculation.
20. Confirm by flow cytometry that the new cell mixture contains the desired CFSE^{low} and CFSE^{high} cell frequencies (50 ± 2% of total CFSE^{+} cells for each population).
21. Once the required volume of each cell suspension to prepare a target cell mixture with a ~1:1 ratio of WT to β2M$^{-/-}$ splenocytes is known, transfer the highest possible volume of each splenocyte preparation into a single 15-mL conical tube.
22. Pipet 10 μL of the above cell mixture into a 5-mL polystyrene tube containing 500 μL of PBS.
23. Set the CFSE^{low} population as the "stopping gate" and record 2,000 events by flow cytometry (Fig. 1b).

24. Fill the remainder of the 15-mL conical tube containing the cell mixture with PBS.
25. Centrifuge the tube at 400 × *g* for 5 min, discard the supernatant, and resuspend cells in 1 mL of PBS.
26. Count the number of cells in the mixture and assess their viability by trypan blue dye exclusion. Use PBS to adjust the mixture to a concentration of 4–6 × 10^7 cells/mL (*see* **Note 16**).
27. Inject each recipient mouse that had received either α-GalCer or vehicle 24 h earlier (*see* Subheading 3.2) with 200 μL of the above target cell mixture intravenously (i.v.).

3.5 Processing of Recipient Mouse Tissues

1. Euthanize recipient mice 2 h after being injected with donor cell suspensions (*see* **Note 17**).
2. For each recipient, access the peritoneal cavity according to Subheading 3.3, **steps 2–5**.
3. Excise the liver by gently pulling the organ upward with tweezers while using scissors to detach it from the diaphragm, intestines, and other surrounding tissues (*see* **Note 18**). Ensure that all hepatic lobes are collected. Non-parenchymal liver mononuclear cell (MNC) isolation is described in Subheading 3.5.1.
4. Remove the spleen and prepare a splenocyte suspension as described for donor mice in Subheading 3.3, **steps 6–14**. During the final step, resuspend splenocytes in staining buffer.
5. Cut into the thoracic cavity with scissors to locate the lungs. Harvest the lungs by grasping them firmly with tweezers while cutting away the trachea and connective tissue with scissors. Once outside the carcass, separate the lungs from unwanted surrounding organs including the heart and the thymus. Lung MNC isolation is described in Subheading 3.5.2.

3.5.1 Liver MNC Isolation

1. Place each liver in a Petri dish containing PBS and shake it with tweezers to wash away any excess blood.
2. Transfer each liver to a new Petri dish containing PBS. Use a razor blade to chop each liver into fine pieces.
3. Pour each sample in PBS into a 15-mL glass homogenizer. Grind the pieces several times until they are disrupted into a single-cell suspension (*see* **Note 19**).
4. Transfer each homogenate into a separate 50-mL conical tube. Fill the remainder of each tube with PBS.
5. Centrifuge tubes at 400 × *g* for 5 min.
6. Discard the supernatant before washing cells once more with PBS.

7. During the centrifugation step above, prepare 12.5 mL of 33.75% Percoll in PBS per liver by mixing 14.06 mL of filter-sterilized water, 2.5 mL of 10X PBS, and 8.44 mL of Percoll PLUS. Use reagents stored at room temperature for mixing.
8. Resuspend cells in 12.5 mL of 33.75% Percoll solution.
9. With the brake off, centrifuge tubes at 700 × *g* for 12 min at room temperature.
10. Carefully siphon off the top layer containing the hepatic parenchymal cell fraction before aspirating the remainder of the supernatant.
11. Resuspend cells in 3 mL of ACK lysis buffer for 3 min at room temperature. Immediately afterward, dilute the ACK lysis buffer with 10 mL of PBS.
12. Filter each cell suspension through a 70-μm cell strainer and into a new 50-mL conical tube.
13. Centrifuge tubes at 400 × *g* for 5 min, discard the supernatant, and resuspend cells in 1 mL of staining buffer.

3.5.2 Lung MNC Isolation

1. Place each set of lungs into a 1.5-mL microcentrifuge tube containing 750 mL of PBS.
2. Within each tube, cut the lungs into fine pieces with small scissors. Between lungs, clean the scissors extensively with 70% ethanol.
3. Transfer each lung sample into a separate 15-mL conical tube containing 4 mL of RPMI medium supplemented with 0.5 mg/mL collagenase IV.
4. Wrap parafilm around the cap of each tube to prevent leakage and incubate samples at 37 °C in a MACSMix tube rotator (or a similar instrument) for 1 h.
5. Filter each lung sample through a 70-μm cell strainer and into a 50-mL conical tube.
6. With the cell strainer still attached to the 50-mL conical tube, gently break apart the unfiltered lung tissue into finer fragments using the rubber end of a 10-mL syringe plunger.
7. Wash the cell strainer extensively with PBS to let the remaining cells into the tube underneath.
8. Centrifuge tubes at 400 × *g* for 5 min.
9. Discard the supernatant and wash cells once more with PBS.
10. Resuspend cells in 3 mL of ACK lysis buffer for 3 min at room temperature. Immediately afterward, dilute the ACK lysis buffer with 10 mL of PBS.
11. Centrifuge tubes at 400 × *g* for 5 min, discard the supernatant, and resuspend cells in 3 mL of staining buffer.

3.6 Data Acquisition and Analysis

1. Transfer 500 μL of recipient liver MNC, lung MNC, and splenocyte suspensions to separate 5-mL polystyrene tubes for cytofluorometric analysis (*see* **Note 20**). Keep cells at 4 °C or on ice until their cytofluorometric interrogation.
2. Define the gates for singlets, lymphocytes, $CFSE^{low}$ donor cells, and $CFSE^{high}$ donor cells as described in Subheading 3.4, **steps 15–17** (*see* **Note 21**).
3. With the $CFSE^{low}$ population set as the stopping gate, record 2,000 events from each sample.
4. Export all of the recorded data from the experiment as Flow Cytometry Standard (FCS) files.
5. Import all of the FCS files into FlowJo (BD Biosciences) or similar software for data analysis.
6. After applying the same gating strategy utilized during data acquisition, use histograms for CFSE to visualize the differences in the frequencies of remaining $CFSE^{high}$ ($\beta 2M^{-/-}$) cells between recipients (*see* **Note 22**). A maximum *y*-axis value, which entails a count of typically between 80 and 120, will need to be applied manually to adequately visualize $CFSE^{+}$ populations (Fig. 2a).
7. Export the number of events recorded for $CFSE^{low}$ and $CFSE^{high}$ populations from every recipient and the initial donor cell mixture to a spreadsheet software such as Microsoft Excel.
8. For each recipient, calculate cytotoxicity against target cells using the following formula: percent specific killing = {1 − [(# of $CFSE^{high}$ events in recipient ÷ # of $CFSE^{low}$ events in recipient) ÷ (# of $CFSE^{high}$ events pre-injection ÷ # of $CFSE^{low}$ events pre-injection)]} × 100. The events recorded during Subheading 3.4, **step 23** serve as "pre-injection" data.
9. Plot the summary data for each group using GraphPad Prism (La Jolla, CA) or a similar graphing software (Fig. 2b).

3.7 Verifying the Contribution of NK Cells to Enhanced $\beta 2M^{-/-}$ Target Cell Clearance in α-GalCer-Treated Recipients

1. Dilute the recommended, lot-specific dose of NK cell-depleting anti-asialo GM1 polyclonal antibody in PBS to a total volume of 200 μL. Dilute an equivalent volume of NRS with PBS.
2. Draw up each solution into a syringe with a 28-gauge needle.
3. Inject WT mice with diluted anti-asialo GM1 antibody or NRS i.p.
4. Three days later, sacrifice mice for their splenocytes (*see* Subheading 3.3, **steps 6–14**), liver MNCs (*see* Subheading 3.5.1), and lung MNCs (*see* Subheading 3.5.2).
5. Seed 1–10 × 10^{5} cells from each sample into a separate 5-mL polystyrene tube. Include one additional tube per sample for negative control staining of *i*NKT cells.

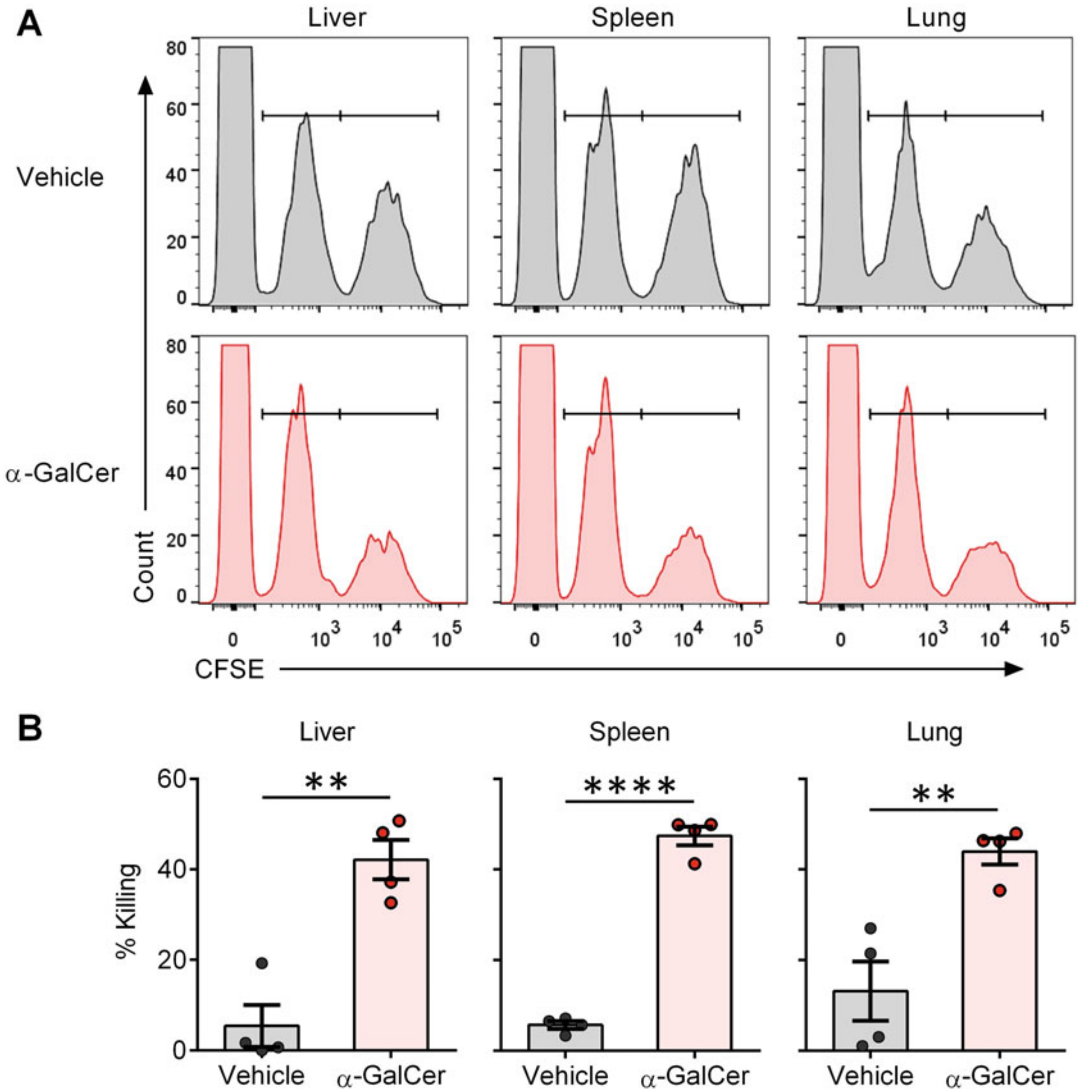

Fig. 2 α-GalCer treatment augments cytotoxicity against β2M$^{-/-}$ target cells. WT C57BL/6 mice received 2 μg α-GalCer or vehicle 24 h before they were injected with equal numbers of CFSE^{low} WT and CFSE^{high} β2M$^{-/-}$ splenocytes. Representative histograms (**a**) and summary data (**b**) illustrate the magnitude of cytotoxicity toward β2M$^{-/-}$ cells in indicated tissues after 2 h. ** and **** denote statistically significant differences with $p < 0.01$ and $p < 0.0001$, respectively, using unpaired Student's *t*-tests

6. Stain cells with Fixable Viability Dye (Thermo Fisher Scientific) and wash according to the supplier's instructions.
7. Resuspend cells at a concentration of 1–10 × 10^6 cells/mL in cold staining buffer.
8. Prepare 5 μg/mL of an Fcγ receptor blocking reagent. Add 20 μL directly to each cell suspension. Incubate cells on ice for 10 min.

9. Stain cells with the fluorochrome-conjugated tetramers/antibodies listed in Table 1 (except for unloaded CD1d tetramer), each at a 1:200 dilution, for 30 min at 4 °C. In separate tubes, include negative control staining for *i*NKT cells using cells stained with unloaded CD1d tetramer instead of PBS-57-loaded CD1d tetramer.
10. Wash cells with 3 mL of cold staining buffer.
11. Centrifuge tubes at 400 × *g* for 5 min at 4 °C.
12. Wash cells twice more with 3 mL of cold staining buffer.
13. Resuspend cells in 100–200 μL of cold staining buffer. Keep cells at 4 °C or on ice until their cytofluorimetric interrogation.
14. To account for spectral overlap between fluorochromes, calculate and apply a compensation matrix on the cytometer using cells stained with each of the reagents listed in Table 1 alone (except for unloaded CD1d tetramer).
15. Record 1–5 × 10^5 total events from each sample on the cytometer.
16. Export the data as raw FCS files into FlowJo (or similar software). For each tissue in each mouse, analyze the frequencies of NK cells and *i*NKT cells among lymphocytes after excluding nonviable cells and doublets (Fig. 3a, b).
17. Once NK cell depletion has been confirmed, use the effective dose of anti-asialo GM1 antibody and an equal volume of NRS as per Subheading 3.7, **step 1**.
18. Inject separate cohorts of WT mice i.p. with anti-asialo GM1 antibody or NRS.
19. Two days later, inject mice with α-GalCer or vehicle i.p. (*see* Subheading 3.2).
20. After 24 h, perform in vivo killing assays and analyze their results as described in Subheadings 3.3–3.6 (Fig. 3c, d).

4 Notes

1. The β2M$^{-/-}$ mice used to generate data for this chapter were provided by Dr. Anthony Jevnikar (Western University, London, ON) and bred in-house. They are commercially available for purchase, for example, from the Jackson Laboratory (catalog #002087).
2. WT and β2M$^{-/-}$ mice on other genetic backgrounds (e.g., on a BALB/c background) can be used as applicable.
3. Donor and recipient mice should be sex-matched and closely age-matched.

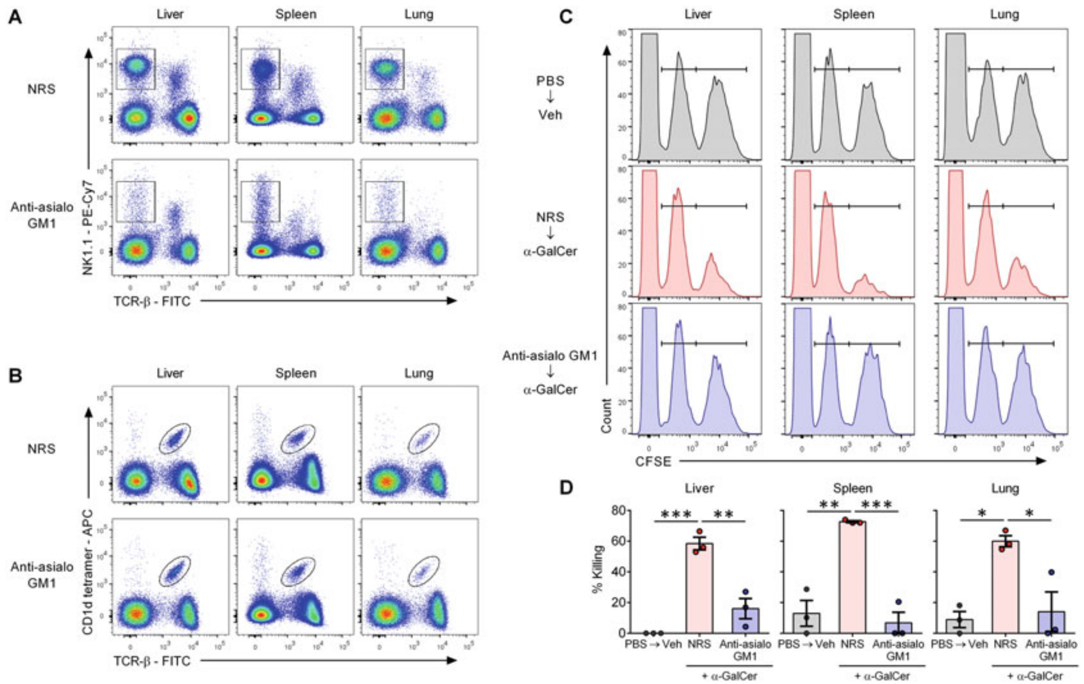

Fig. 3 NK cells are required for the enhanced clearance of β2M$^{-/-}$ target cells in α-GalCer-treated recipients. (**a** and **b**) WT C57BL/6 mice were injected with 50 μL of anti-asialo GM1 antibody or an equivalent volume of normal rabbit serum (NRS). Three days later, the presence or absence of NK1.1^{+}TCR-β^{-} NK cells (**a**) and PBS-57-loaded CD1d tetramer^{+}TCR-β^{+} *i*NKT cells (**b**) in indicated tissues was assessed. Data from representative samples (two mice per group) are illustrated. (**c**, **d**) Separate cohorts of mice were injected with anti-asialo GM1 antibody or NRS 2 days before they were given 2 μg α-GalCer. A control cohort received PBS followed by vehicle (for α-GalCer) instead. Twenty-four hours later, mice received equal numbers of CFSE^{low} WT (control) and CFSE^{high} β2M$^{-/-}$ splenocytes. Percent specific killing of CFSE^{high} β2M$^{-/-}$ cells in recipient mice was calculated using a formula described in the text under Data Acquisition and Analysis. Representative histograms (**c**) and summarized data (**d**) are depicted. *, **, and *** denote differences with $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively, using one-way ANOVA

4. We routinely use insulin syringes for i.v. and i.p. injections. This minimizes the amount of leftover suspensions or reagents after the plunger is depressed.
5. The data presented in this chapter were collected in compliance with Animal Use Protocol #2018-093 approved by the Animal Care Committee of Animal Care and Veterinary Services at Western University.
6. Out of an abundance of caution and to dissolve precipitates possibly forming during the freezing process, heat each thawed aliquot of α-GalCer in an 80 °C water bath for 10 min before preparing the solution to be injected.
7. Ideally, group comparisons should be made between cage-mates.

8. Other glycolipid agonists of *i*NKT cells, such as a T_H1-polarizing α-GalCer analog called α-C-GalCer [29, 30], can be tested for NK cell transactivation using in vivo killing assays.
9. The presence of some connective tissue during homogenization may be inevitable. However, removing non-lymphoid tissue pieces as much and as early as possible will reduce clumping in subsequent steps and will ultimately improve cell yield.
10. Splenocytes are often chosen as ideal target cells due to their abundance in adult mice and the relative simplicity of their isolation. As potential alternatives, numerous other types of hematopoietic cells can be found in the lungs, thymus, and bone marrow of donor mice. Of note, the activation and maturation states of target cells may impact the rate of killing in recipients [22].
11. As an example, if the total number of WT splenocytes recovered is 5×10^7 and the total number of $\beta2M^{-/-}$ splenocytes recovered is $>5 \times 10^7$, use only 5×10^7 cells from each sample.
12. For smaller pellet sizes (i.e., splenocytes obtained from one donor), leave ~50 μL behind. For larger pellets (i.e., splenocytes obtained from more than one donor) leave closer to ~100 μL behind.
13. While adding CFSE, concurrent shaking of splenocyte suspensions will prevent cells from clumping and/or settling. Failure to do so may result in cell populations that will generate a wider distribution of $CFSE^+$ events in flow cytometry, which could hinder the interpretation of assay results.
14. After drawing each sample into a pipette tip, clean the external surface of the tip before transferring the content in order to minimize pipetting errors.
15. As an example, if $CFSE^{low}$ and $CFSE^{high}$ events represent 55% and 45% of splenocytes, dividing 45 by 55 computes a WT-to-$\beta2M^{-/-}$ cell sample ratio of 0.818:1. Therefore, mix 8.18 μL of the WT cell suspension with 10 μL of the $\beta2M^{-/-}$ suspension.
16. This concentration range accounts for $8–12 \times 10^6$ cells being injected per mouse. If cell yield is limited, injecting a lower number of cells is possible but not ideal. Doing so will necessitate the acquisition of more total events from recipient mice (*see* Subheading 3.6), which may lead to overwhelming background interference when attempting to visualize distinct $CFSE^+$ populations.
17. Longer assay times may be required to observe greater cytotoxicity.
18. To maintain optimal hepatic MNC viability, we recommend removing the gall bladder with scissors before harvesting the liver.

19. If a glass homogenizer is used to grind the liver, a "loose" pestle is recommended.
20. Whether NK cell-mediated cytotoxicity can be observed in other vascularized tissues that could conceivably be accessed by donor cells (e.g., lymph nodes, gut associated lymphoid tissue) remains to be validated in our laboratory.
21. A large CFSE^{-} population corresponding to recipient splenocytes should be easily distinguishable from injected donor cells during data acquisition.
22. Minor gating adjustments may be required between samples obtained from different tissue locations. However, always apply identical gates to samples obtained from the same tissue, which will be compared directly.

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Chapter 16

Redirecting iNKT Cell Antitumor Immunity with α-GalCer/CD1d-scFv Fusion Proteins

Lianjun Zhang and Alena Donda

Abstract

Invariant natural killer T (iNKT) cells display important properties that could bridge the innate and adaptive immunity, and they have been shown to play key roles in cancer immunotherapy. However, administration of iNKT cell agonist αGalCer fails to induce sustained antitumor immunity due to the rapid anergy induction after an initial strong activation. To this end, we have designed a recombinant CD1d protein that is fused to an anti-TAA scFv, which is able to recruit iNKT cells to the tumor site and induce tumor regression. Importantly, recombinant CD1d fusion proteins loaded with α-GalCer demonstrated sustained activation of iNKT cells upon repeated injections and superior tumor control, as compared to α-GalCer treatment.

Key words iNKT cell, CD1d-antitumor scFv, α-Galactosylceramide (αGalCer), Tumor-associated antigen, Anergy

1 Introduction

Invariant natural killer T (iNKT) cells represent a unique lineage of innate T cells which recognize glycolipid antigens presented on MHC like molecule CD1d [1, 2]. Interestingly, iNKT cells usually express certain NK-specific receptors and demonstrate strong cytotoxicity [1]. Importantly, iNKT cells have been well shown to promote antitumor immunity, at least in part via crosstalk with dendritic cells and transactivation of NK cells, or even killing of tumor-associated macrophages in the tumor microenvironment [3–5]. Recently, iNKT cells are used to carry chimeric antigen receptor (CAR) against ganglioside GD2 for immunotherapy of neuroblastoma [6].

The iNKT/CD1d agonist α-galactosylceramide (αGalCer) triggers a very strong iNKT cell activation associated with antitumor effects [3]. However, this initial massive iNKT cell activation is followed by a hyporesponsive state to subsequent free α-GalCer

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treatments [7]. Therefore, iNKT cell anergy is a hurdle for efficient iNKT cell-mediated immunotherapy [3, 4, 8]. To this end, we have generated CD1d-antitumor fusion proteins to induce sustained iNKT cell activation by fusing CD1d with antitumor-associated antigen (TAA) scFv [8–11] (Fig. 1). In this regard, the recombinant αGalCer-loaded CD1d-scFv fusion protein will allow specific tumor targeting, and we previously showed that repeated treatments also lead to prolonged antitumor immunity [8, 9, 11]. Of note, iNKT cells activated by the α-GalCer/CD1d-scFv fusion showed similar PD-1 up-regulation, but they still produced IFNγ upon repeated injections, suggesting that PD-1 is not sufficient to mediate the iNKT cell anergy [9, 11]. We also noticed that, in contrast to free α-GalCer ligand, recombinant α-GalCer/CD1d-scFv fusion protein induce more rapid production of IFNγ and TNFα, indicating a mechanistic difference in the activation of iNKT cells depending on whether it occurs via endogenous CD1d expressed by antigen-presenting cells or via the recombinant α-GalCer/CD1d-scFv fusion protein. Another advantage of the α-GalCer/CD1d-scFv fusion proteins resides in the tumor targeting via its anti-TAA scFv, which allows redirecting the immune response to the tumor [9–11]. We have shown that the specific tumor targeting of the CD1d fusion, as compared to the non-targeted CD1d fusion protein, resulted in a higher reactivity of iNKT cells upon several stimulations, as shown by the increased cytokine production associated with better antitumor effects in mice. Yet, development of more stable covalent conjugates of αGalCer-CD1d-scFv may represent more effective cancer immunotherapeutic approaches [12].

2 Materials

2.1 Recombinant CD1d Fusion Proteins

The α-GalCer analog KRN7000 (Alexis Biochemicals Corp.) is dissolved in PBS-0.5% Tween-20. Genetic fusion of mouse β2-microglobulin (β2m) with the soluble part of mouse CD1d (sCD1d) and the anti-TAA scFv are performed by overlapping PCR, and a His Tag is added at the C-term for purification. Recombinant CD1d fusion proteins are produced by transient transfection of the HEK293-EBNA (Cellular Biotechnology Laboratory, EPFL, Switzerland), and supernatants are affinity purified on the Sartobind His-Tag membrane adsorbers for exchange chromatography (Sartorius AG, Germany). The recombinant fusion proteins are loaded with α-GalCer before administration in vivo.

2.2 CD1d Tetramer

The CD1d tetramer is developed by engineering a BirA consensus sequence at the C-terminus of the soluble mouse CD1d protein. The CD1d monomer is biotinylated by the BirA enzyme (Avidity, Denver, CO), and after loading with αGalCer, it is tetramerized on streptavidin-PE (Invitrogen) using a molar ratio of 4:1.

2.3 Antibodies Used for Flow Cytometry Analysis

The following antibodies are used to characterize iNKT cells: Fixable Viability Dye eFluor™ 506 (eBioscience 65-0866-14), α-GalCer/CD1d tetramer-PE (home-made), anti-mouse CD3-Alexa 700 (17A2, BioLegend 100216), anti-mouse CD4-FITC (RM4-5, BioLegend 100510), anti-mouse NK1.1-PerCPCy5.5 (PK136, BioLegend 108728), anti-mouse CD8a-PE-Texas Red (Ly2, Thermo Fisher Scientific MCD0817), and anti-mouse B220 FITC (RA3-6B2, BioLegend 103206), anti-mouse IFN-γ-PerCP/Cy5.5 (XMG1.2, BioLegend 505821), and anti-mouse TNFα-Pacific Blue (MP6-XT22, BioLegend 506318).

2.4 Buffers

T cell complete media: RPMI 1640 basal medium (Gibco No. 11875-093) supplemented with 10% fetal bovine serum (FBS), non-essential amino acids, penicillin-streptomycin (10,000 U/ml, Gibco No. 15140-114), L-glutamine (Gibco No. 25030-024), non-essential amino acids (NEAA, Gibco No. 11140-035), sodium pyruvate (Gibco No. 11360-039), and 50 μM β-mercaptoethanol (Sigma No. M-7522).

Fixation buffer (BioLegend Cat. No. 420801).

Intracellular staining perm wash buffer (BioLegend Cat. No. 421002).

RBC lysis buffer (BioLegend Cat. No. 420301).

FACS buffer: Phosphate-buffered saline (PBS) with 2% FBS plus 1 mM EDTA and 0.05% sodium azide.

3 Methods

3.1 Administration of α-GalCer/CD1d-scFv Fusion Proteins

1. Inject i.v. with 0.4 μg αGalCer or equimolar amounts of α-GalCer/CD1d-scFv fusion proteins (40 μg) (Fig. 1).
2. Sacrifice the animals 2 h after the last injections of α-GalCer or α-GalCer/CD1d-scFv fusion proteins.

3.2 Preparation of Single-Cell Suspension

1. Harvest the spleens and smash through a 70 μm filter.
2. Add 2 ml 1× Red Blood Cell Lysis Buffer (*see* **Note 1**) and incubate for 5 min at room temperature.

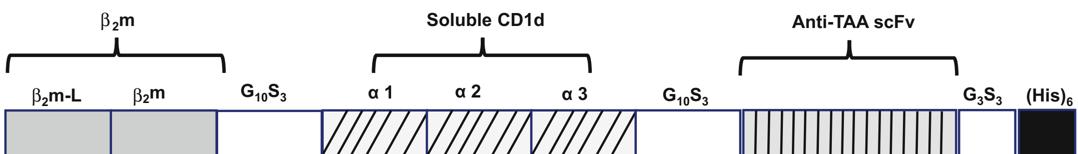

Fig. 1 Schematic representation of the design of CD1d/anti-TAA scfv fusion protein

3. Stop the RBS lysing by adding 10 ml T cell complete media and centrifuge at 400 × *g* for 5 min and discard the supernatant.
4. Resuspend the cell pellet in 5 ml T cell complete media and keep on ice.

3.3 Surface Staining

1. Transfer 3–5 million splenocytes/well to a round-bottom 96-well plate.
2. Centrifuge the plate at 400 × *g* and discard the supernatant.
3. Wash once in and then resuspend in 100 μl of 1:250 dilution of Aqua Dead (*see* **Note 2**) and incubate for 20 min at room temperature in the dark.
4. Centrifuge the plate at 400 × *g* and discard the supernatant.
5. Block the Fc receptors with 50 μl of rat anti-mouse CD16/32 2.4G2 hybridoma supernatant for 15 min.
6. Add 150 μl FACS buffer to each well, centrifuge the plate at 400 × *g*, and discard the supernatant.
7. Add 50 μl of αGalCer/CD1d-PE tetramer to each well and incubate for 25 min on ice.
8. Add 150 μl FACS buffer to each well, centrifuge the plate at 400 × *g*, and discard the supernatant.
9. Add 50 μl surface antibody cocktails (*see* **Note 3**) or isotype controls (*see* **Note 4**) at recommended concentrations and further incubate for 25 min on ice (Fig. 2).
10. Add 150 μl FACS buffer to each well, centrifuge the plate at 400 × *g*, and discard the supernatant.

3.4 Fixation and Permeabilization

1. Resuspend the cell pellet in 100 μl fixation buffer. Incubate for 20 min on ice.
2. Add 100 μl 1× Intracellular Staining Permeabilization Wash Buffer (dilute 10× stock solution with ddH_2O), and centrifuge for 5 min at 400 × *g*. Discard the supernatant.
3. Wash again with 200 μl 1× Intracellular Staining Permeabilization Wash Buffer, and centrifuge for 5 min at 400 × *g*. Discard the supernatant.

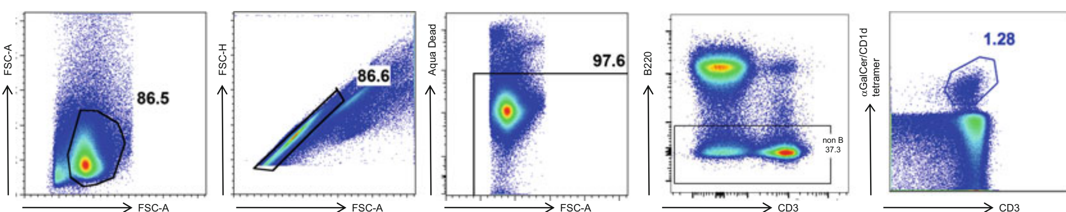

Fig. 2 Gating strategy of iNKT cells from mouse spleen

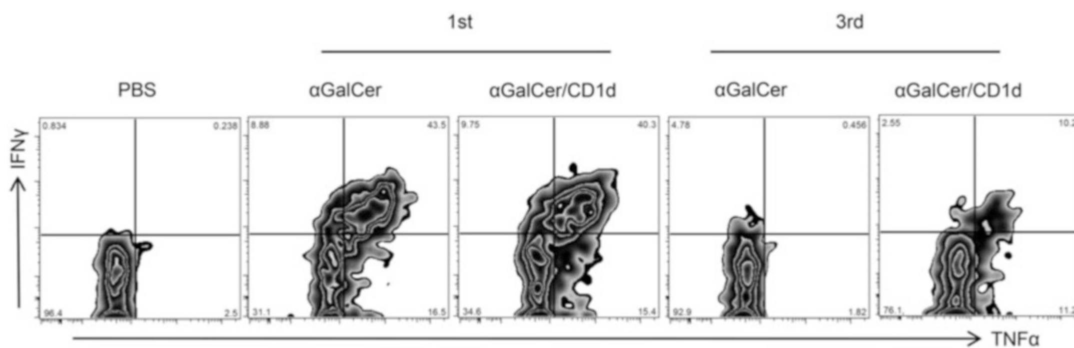

Fig. 3 Flow cytometric analysis of IFNγ and TNFα production by spleen iNKT cells upon the first or third injection of free α-GalCer or α-GalCer-loaded CD1d-scFv fusion protein

3.5 Intracellular Staining

1. Stain the cell pellet with the appropriate amounts of antibodies IFNγ-PerCP/Cy5.5 and TNFα-Pacific Blue diluted in 1× Intracellular Staining Permeabilization Wash Buffer (Fig. 3).
2. Incubate for 30 min on ice.
3. Wash 1× with 200 μl 1× Intracellular Staining Permeabilization Wash Buffer (*see* **Note 5**) and centrifuge at 400 × *g* for 5 min.
4. Repeat **step 3**.
5. Resuspend the cell pellet in 200 μl FACS buffer prior to acquisition.

4 Notes

1. Dilute the 10× Red Blood Cell Lysis Buffer to 1× working concentration with ultrapure water.
2. Aqua Dead should be diluted with PBS.
3. It is important to gate out B cells with anti-mouse B220 antibody to exclude nonspecific staining of α-GalCer/CD1d tetramer.
4. Appropriate isotype controls are needed to carry out flow cytometric analysis.
5. Dilute 10× Intracellular Staining Permeabilization Wash Buffer to 1× with ultrapure water.

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Chapter 17

Investigating the Dynamic Changes in iNKT Cell Metabolic Profiles During Development

Jana L. Raynor and Hongbo Chi

Abstract

Emerging research has highlighted the importance of metabolic pathways and metabolites in dictating immune cell lineage decisions during thymocyte development. Here, we discuss several complementary approaches, including flow cytometry, metabolic flux, and transcriptome analyses, to characterize the dynamic changes in metabolic profiles associated with invariant natural killer T cell development.

Key words Metabolism, iNKT cell, Glycolysis, Oxidative phosphorylation, Flow cytometry, Seahorse, Bioinformatics

1 Introduction

Cellular metabolic processes are broadly categorized as anabolic or catabolic, characterized by the building up or breaking down of macromolecules that either use or generate energy, respectively. It is now well established that peripheral T cell homeostasis, activation, differentiation, and function are associated with dynamic changes in cellular metabolism [1, 2]. However, emerging studies highlight that these alterations in cellular metabolism are not limited to peripheral immune cells. We recently showed that metabolic signals driven by mechanistic target of rapamycin (mTOR) complex 1-dependent glycolysis regulate the lineage decisions of αβ and γδ T cells during development in the thymus [3]. Further, invariant natural killer T (iNKT) cells, a specialized subset of innate-like αβ T cells, dynamically remodel glycolysis and mitochondrial homeostasis and oxidative phosphorylation (OXPHOS) during thymic development, and these changes correlate with quiescence entry [4]. Metabolic pathways may also direct the emergence of iNKT effector subsets during thymocyte development [5], as these subsets have varied metabolism-associated transcriptional profiles and

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requirements for Opa1-driven mitochondrial fusion [4]. These recent studies highlight the important role of immunometabolism in dictating T cell developmental fate and function.

There are several unique challenges of studying immunometabolic processes during thymocyte development. First, while several in vitro and emerging in vivo systems are available to study the metabolic state of peripheral T cell populations [6–8], the thymic microenvironment provides unique signals that cannot be fully recapitulated in vitro. Thus, there is a need to establish integrative approaches that reliably assess the metabolic state of thymic cell populations in vivo. Second, thymic populations, including iNKT cells, are relatively more rare than conventional T cell subsets, which makes it difficult to perform conventional profiling assays, such as metabolomics or CRISPR-based screening approaches, to assess the metabolic requirements of these cells in vivo. In this chapter, we provide an overview of a recently established comprehensive approach to profile the metabolic state of developing iNKT cells [4], which can be applied to both developmental and effector subsets. First, we describe a flow cytometry-based approach for profiling of mitochondrial parameters and glucose uptake as a surrogate for glycolysis. Next, we discuss how to apply Seahorse metabolic flux analysis on these cells. Finally, we detail how to perform transcriptome-based predictions of metabolic state using gene set enrichment analysis (GSEA).

2 Materials

2.1 Preparation of an Enriched Single-Cell Suspension from the Mouse Thymus

1. Hanks' balanced salt solution (HBSS) (Gibco, #14170–112). Add 2% heat inactivated fetal bovine serum (FBS). Store at 4 °C.
2. 10 cm polystyrene petri dish (Kord-Valmark, #2900).
3. 155 μm nylon mesh (Component Supply).
4. 3 ml syringe (BD, #309657).
5. 15 ml conical tube (available from multiple vendors).
6. Centrifuge (e.g., Thermo Fisher Scientific Sorvall ST Plus Series).
7. $CD8^+$ T Cell Isolation Kit (Milteyni Biotec, #130-117-044).
8. LS columns (Milteyni Biotec, #130-042-401) and magnet (Milteyni Biotec, #130-091-051).
9. Hemocytometer and microscope for cell counting.

2.2 Flow Cytometric Assessment of iNKT Cell Metabolism

1. Flat-bottom 96-well plate (Falcon, #353072).
2. FACS buffer. Prepare using 1× PBS (Gibco, #14190-144) with the addition of 2% FBS. Store at 4 °C.

3. Metabolic dyes to assess mitochondrial quantity, membrane potential, cellular or mitochondrial reactive oxygen species (ROS), and glucose uptake. Prepare the dyes in FACS buffer immediately prior to use according to the below final working concentrations.
   (a) MitoTracker™ Deep Red or Green (Invitrogen, #M22426 or #M7514). Working concentration = 10 nM.
   (b) TMRM (ImmunoChemistry Technologies, #9105). Working concentration = 10 nM.
   (c) CellROX™ Deep Red (total cellular ROS indicator) (Invitrogen, #C10422). Working concentration = 2.5 μM.
   (d) MitoSOX™ Red (mitochondrial superoxide indicator) (Invitrogen, #M36008). Working concentration = 5 μM.
   (e) CM-H2DCFDA (general ROS indicator) (Invitrogen # C6827). Working concentration = 1 μM.
   (f) 2-NBDG (Thermo Fisher Scientific, # N13195). Working concentration = 30 μM.
4. 37 °C incubator. Either a 5% CO_2 or a non-CO_2 incubator can be used.
5. Mouse CD1d tetramer loaded with the glycolipid PBS-57 and conjugated to the R-phycoerythrin (PE) or Allophycocyanin (APC) fluorophore (mCD1d-PBS-57-PE or mCD1d-PBS-57-APC) (NIH Tetramer Core Facility).
6. Anti-TCRβ antibody conjugated to the Pacific Blue (PB) fluorophore (anti-TCRβ-PB; clone H57-597, available from multiple vendors).
7. Flow cytometer (e.g., BD LSRFortessa).

2.3 Agilent Seahorse Metabolic Flux Analysis of iNKT Cells

1. HBSS containing 2% FBS.
2. mCD1d-PBS-57-PE tetramer and anti-TCRβ-PB.
3. 5 ml polypropylene tube (Falcon, #352063).
4. 50 μm cell strainers (Sysmex CellTrics, #04-004-2327).
5. Complete Click's medium (FUJIFILM Irvine Scientific, #9195) containing 10% FBS, 1× penicillin/streptomycin/glutamine (Gibco, #10378-016), and 63 μM β-mercaptoethanol (Sigma-Aldrich M6250).
6. Agilent Seahorse Dulbecco's modified essential medium (DMEM; Gibco, #103575-100). The morning of the Seahorse assay, aliquot 48 ml of DMEM and supplement with the below reagents and adjust the pH of the medium to 7.4 (*see* **Note 1**).
   (a) 1 ml of d-(+)-glucose (Sigma-Aldrich, #G8644). Working concentration = 10 mM.

(b) 0.5 ml of 100 mM sodium pyruvate (Gibco, #11360–070). Working concentration = 1 mM.

(c) 0.5 ml of 200 mM L-glutamine (Gibco, #25030-149). Working concentration = 2 mM.

7. Agilent Seahorse XFe 96 instrument.
8. Agilent Seahorse FluxPak including 96-well cartridge plate, cell culture plate, and XF calibrant (#102601-100).
9. Ultrapure water (Invitrogen, #10977-015).
10. Poly-L-lysine (Sigma, #P8920).
11. Drugs for the Agilent Seahorse mitochondrial stress test (*see* **Note 1**). Stock solutions of these drugs should be prepared in supplemented DMEM Seahorse medium immediately prior to running the Seahorse assay. Working concentrations listed below are the final drug concentrations after injection.
    (a) Oligomycin (Tocris Bioscience, #4110). Working concentration = 1 μM.
    (b) Carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP) (Sigma-Aldrich, #C2920). Working concentration = 1.5 μM.
    (c) Rotenone (Sigma-Aldrich #R8875) (*see* **Note 2**). Working concentration = 0.5 μM.
12. 37 °C dry, non-CO_2 incubator (e.g., Labnet mini incubator).

2.4 Transcriptome-Based Bioinformatics to Assess iNKT Cell Metabolism

1. Gene expression data from microarray (e.g., Affymetrix Mouse Gene 2.0 ST Array) or RNA-sequencing generated in-house or downloaded from a public data repository [e.g., NCBI Gene Expression Omnibus (GEO)].
2. GSEA software from Broad Institute. This software is free to download from https://www.gsea-msigdb.org/gsea/index.jsp.
3. Data files necessary to input into GSEA (*see* **Note 3**).
    (a) annotation.chip—File contains the annotation of each probe set for each gene based on the microarray expression chip used and is typically provided by the manufacturer. If it is not available within the GSEA software, it can be uploaded manually.
    (b) phenotypelabels.cls—The purpose of this file is to inform the GSEA software how to annotate the samples in the expression.gct file. The file can be prepared in Excel and saved as a tab deliminated text file with the extension .cls.
    (c) expression.gct—This is the expression dataset file and contains the gene identifiers and sample expression data. It can be prepared in Excel and saved as a tab deliminated text file with the extension .gct or .txt.

3 Methods

3.1 Preparation of iNKT Cells from the Thymus for Flow Cytometry and Cell Sorting

1. Harvest the thymus from one mouse into 1 ml of HBSS containing 2% FBS in a 10 cm petri dish (*see* **Note 4**).
2. Set a piece of 155 μM nylon mesh over the thymus, and crush the tissue with the flat end of a 3 ml syringe (*see* **Note 5**).
3. Transfer the single-cell suspension into a 15 ml conical tube.
4. Wash the petri dish with 3 ml of HBSS containing 2% FBS and transfer into the 15 ml tube.
5. Centrifuge the cells at 600 × *g* for 5 min.
6. Resuspend the cell pellet in 500 μl of HBSS containing 2% FBS (*see* **Note 6**).
7. Enrich the iNKT cells by depleting $CD8^+$ T cells using Milteyni Biotec $CD8^+$ T Cell Isolation Kit per the manufacturer's instructions (*see* **Note 7**).
8. Centrifuge the cells at 600 × *g* for 5 min.
9. Resuspend the cell pellet in HBSS containing 2% FBS at a concentration of 20×10^6 cells/ml. The cells are now ready to stain with fluorophore-conjugated antibodies and mouse CD1d tetramer to identify the iNKT cells by flow cytometry. The protocols for staining for flow cytometric analysis and cell sorting are described in the following sections.

3.2 Flow Cytometric Assessment of iNKT Cell Metabolism

1. Aliquot 2×10^6 cells isolated from the thymus into one well of a flat-bottom 96-well plate (*see* **Note 8**).
2. Centrifuge the cells at 600 × *g* for 5 min and then remove the supernatant.
3. Dilute metabolic dyes in FACS buffer (*see* **Note 9**).
4. Add 50 μl of diluted metabolic dye to 2×10^6 cells.
5. Incubate the cells in a 37 °C incubator for 45 min for 2-NBDG or 30 min for other dyes.
6. Wash the cells by adding 100 μl of FACS buffer.
7. Centrifuge the cells at 600 × *g* for 5 min and then remove the supernatant.
8. For surface staining, add 20 μl of FACS buffer containing mCD1d-PBS-57-PE and anti-TCRβ-PB at a 1:200 dilution (i.e., 0.1 μl of tetramer or antibody stock into 20 μl of FACS buffer) (*see* **Note 10**).
9. Incubate the cells at room temperature (RT) for 40 min.
10. Wash the cells with 100 μl of FACS buffer.
11. Centrifuge the cells at 600 × *g* for 5 min and then remove the supernatant.

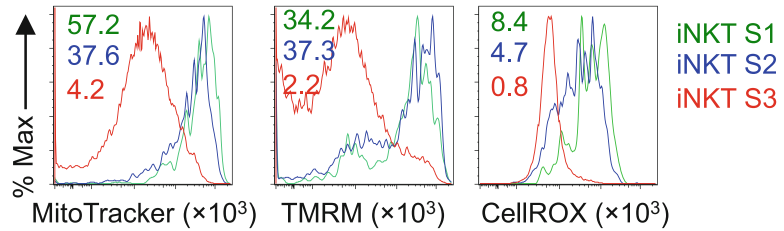

Fig. 1 Flow cytometric analysis of MitoTracker™ (left), TMRM (middle), and CellROX™ (right) in thymic iNKT developmental stage 1 (S1), stage 2 (S2), and stage 3 (S3). These data indicate a reduction in mitochondrial mass (MitoTracker™) and membrane potential (TMRM), as well as reduced cellular ROS (CellROX™), in S3 iNKT cells. Numbers indicate the mean fluorescence intensity (MFI). (Similar observations have previously been published [4, 10, 11])

12. For flow cytometry analysis, resuspend 2 × 10^6 cells in 50–100 μl of FACS buffer.
13. Analyze the iNKT cells on a flow cytometer (*see* **Note 11**). *See* Fig. 1 for representative MitoTracker™, TMRM, and CellROX™ staining from thymic iNKT developmental stages.

3.3 Agilent Seahorse Analysis of iNKT Cell Glycolysis and OXPHOS

The evening prior to the Seahorse assay:

1. Coat the wells of the 96-well cell culture plate with poly-L-lysine. To do so, add 50 μl of poly-L-lysine diluted 1:10 in ultrapure water per well. Incubate the plate overnight at 4 °C.
2. Hydrate the calibrant plate by adding 200 μl of ultrapure water per well, and place the plate in the non-CO_2 37 °C incubator overnight.

The day of the Seahorse assay (see ***Note 12****):*

In the morning, prepare the DMEM Seahorse medium. Place the medium and cell culture plate coated with poly-L-lysine in a 37 °C non-CO_2 incubator to warm. Seahorse analysis requires >95% pure population of live thymic iNKT cells; therefore, cells should be sorted the morning of the Seahorse assay.

1. Isolate and enrich cells from the thymus for iNKT cell sorting (*see* **steps 1–7** in Subheading 3.1).
2. Stain the enriched cells from one thymus in 500 μl of HBSS containing 2% FBS, mCD1d-PBS-57-PE (1:200 dilution) and anti-TCRβ-PB (1:200 dilution) (*see* **Note 13**).
3. Incubate the cells at RT for 40 min.
4. Wash the cells with 2 ml of HBSS containing 2% FBS.
5. Centrifuge the cells at 600 × *g* for 5 min and then remove the supernatant.
6. Resuspend the cell pellet at a concentration of 15–20 × 10^6 cells/ml in HBSS containing 2% FBS.

7. Filter the cells through a 50 μm cell strainer to remove any remaining cell clumps and transfer the cells into a 5 ml polypropylene tube for cell sorting. Sort cells into collection tubes containing complete Click's medium.
8. While cells are sorting, remove the water from the calibrant plate and replace with 200 μl of XF calibrant per well. Place the plate back into the non-CO_2 37 °C incubator. This step should be done at least 1 h prior to running the Seahorse assay.
9. Count the cell number obtained from the sort (*see* **Note 14**).
10. Wash cells into 37 °C pre-warmed DMEM Seahorse medium supplemented with glucose, sodium pyruvate, and L-glutamine.
11. Resuspend cells in DMEM Seahorse medium at a concentration of 5×10^6 cells/ml.
12. Count the cells a second time to confirm the cells are at a concentration of 5×10^6 cells/ml (*see* **Note 15**).
13. Wash the Agilent Seahorse cell plate to remove the poly-L-lysine with 200 μl/well of 1× PBS. Dump the 1× PBS from the plate into the sink, and blot the plate onto a paper towel to remove excess liquid. Repeat this step one more time.
14. Immediately plate the cells by adding 50 μl of 5×10^6 cells/ml single cell suspension per well of an Agilent Seahorse 96-well cell culture plate (=250,000 iNKT cells into each well). Be sure to add the cells slowly to obtain an even cell distribution on the bottom of the well. It is best to have enough cells to plate 4 technical replicate wells per sample (*see* **Note 16**).
15. Centrifuge the plate at $200 \times g$ for 10 s, with the centrifuge acceleration and deacceleration set at medium speed. Rotate plate 180° and spin again at $200 \times g$ for 10 s. Check the cells under a microscope to confirm that the cells adhered as a single monolayer and are 90–95% confluent.
16. Incubate the cell plate in a 37 °C non-CO_2 incubator for 0.5–1 h. After the incubation is complete, add the remaining DMEM Seahorse medium to bring the final volume per well equal to 175 μl.
17. While the cell plate is incubating, prepare the drugs (oligomycin, FCCP, and rotenone) for the mitochondrial stress test.
18. Add 25 μl of the drugs into the appropriate port of the Seahorse cartridge:
    (a) Oligomycin (8× stock solution)—Port A.
    (b) FCCP (9× stock solution)—Port B.
    (c) Rotenone (10× stock solution)—Port C.
19. After the drugs are added, load the cartridge into the Agilent Seahorse XFe 96 instrument and initiate the run. Set up the

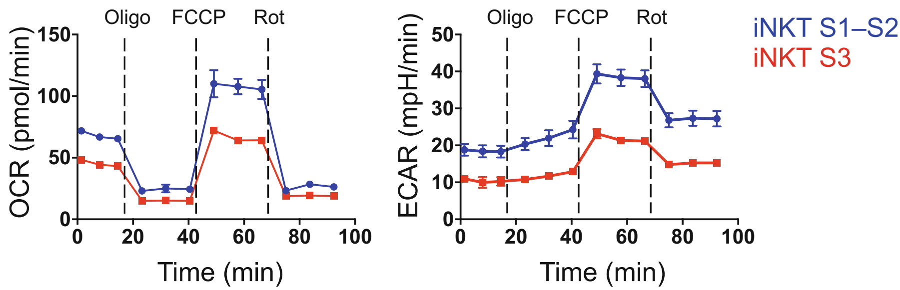

Fig. 2 iNKT developmental stages 1–2 (S1–S2) and stage 3 (S3) were sorted from the thymus of C57BL/6 mice and analyzed by Seahorse. Oxygen consumption rates (OCR, left) and extracellular acidification rates (ECAR, right) were measured before and after the sequential addition of mitochondrial inhibitors (Oligo, oligomycin; FCCP, carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone; and Rot, Rotenone). These data demonstrate that S3 iNKT cells have reduced OCR, an indicator of OXPHOS, and ECAR, an indicator of glycolysis, compared to S1–S2 iNKT cells. (Similar observations have previously been published [4])

template for the run, including the drugs and ports being used and the cell plate map. Parameters should be as follows: cycles, 3; mix, 3 min; wait, 2 min; and measure, 3 min. Select run assay to begin. **Critical**: Be sure to remove the lid of the cartridge plate before loading into the instrument.

20. After the Agilent Seahorse XFe 96 instrument completes the initiation (~15–20 min), it will prompt you to add the cell plate. Follow the prompts provided by the instrument. **Critical**: Be sure to remove the lid of the cell plate before loading into the instrument.
21. The Agilent Seahorse XFe 96 instrument will indicate when the run is complete. Unload the cell plate and export the data for analysis. *See* Fig. 2 for representative oxygen consumption rates (OCR), an indicator of OXPHOS, and extracellular acidification rates (ECAR), an indicator of glycolysis, from thymic iNKT developmental stages.
22. Before discarding the cell plate, confirm that the cell monolayer is still intact by examining under a microscope. Inconsistent cell adherence during the Seahorse assay may account for variability in the OCR and ECAR data.

3.4 Transcriptome-Based Bioinformatic Assessment of iNKT Cell Metabolism

1. Open the GSEA analysis software.
2. Under the "Load data" tab, add the annotation.chip, phenotypelabels.cls, and expression.gct files, and then select "Load these files."
3. After the files are loaded, go to the "Run Gsea" tab and fill out the following boxes under "Required fields":
   (a) Expression dataset—Select the expression dataset. If the dataset is not listed, it has not been loaded properly under the "Load data" tab.

(b) Gene set database—Select the curated gene sets of interest available in Molecular Signatures Database (MSigDB) (*see* **Note 17**).

(c) Number of permutations—Specify the number of permutations to assess the statistical significance of the enrichment score. GSEA recommends 1000 permutations.

(d) Phenotype labels—Select the directionality of comparison (e.g., knockout_versus_wild-type).

(e) Collapse dataset to gene symbols—Select "true."

(f) Permutation type—Select "gene_set." The "gene_set" option is recommended when there are fewer than seven samples within one group. "Phenotype" is the recommended method when there are seven or more samples in each group.

(g) Chip platform—From the pop-up window, click on the "Chips (local chip)" tab and select the annotation.chip file uploaded. Alternatively, click on the "Chips (from website)" tab and select the chip file used to generate your data. If the chip file is not available within the software, it is necessary to upload an annotation.chip file obtained from the manufacturer.

4. Select the "Show" button next to "Basic fields." Here you can change the name of the analysis and select the directory where the analysis will be saved.
5. Select the "Show" button next to "Advanced fields." Here you can modify the number of plots you want GSEA to generate under the "Plot graphs for the top sets of each phenotype" field.
6. Select "Run" to execute the GSEA analysis. *See* Fig. 3 for an example of the enrichment plots generated by GSEA.

4 Notes

1. Supplements for Seahorse DMEM medium (glucose, sodium pyruvate, L-glutamine), and drugs (oligomycin, FCCP, rotenone) can also be purchased from Agilent.
2. 1 μM Antimycin A can be used in addition to rotenone.
3. For more information on how to format the phenotypelabels.cls and expression.gct files, refer to the User Guide provided by GSEA (https://www.gsea-msigdb.org/gsea/doc/GSEAUserGuideFrame.html).
4. This protocol can be applied for secondary lymphoid tissues, such as the spleen and peripheral lymph nodes.

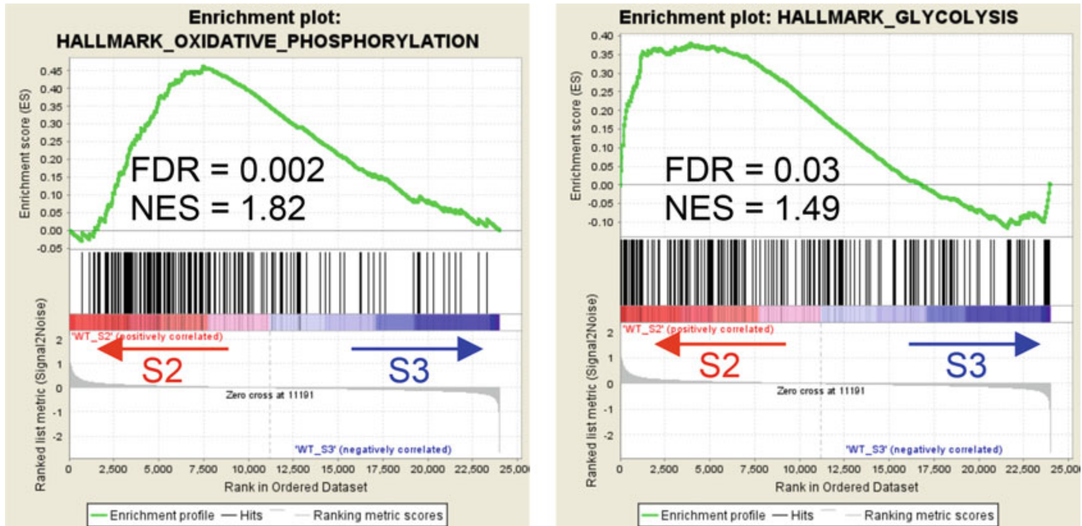

Fig. 3 GSEA enrichment plots of two HALLMARK gene sets, oxidative phosphorylation (OXPHOS) (left), and glycolysis (right), from the pairwise comparison of thymic iNKT developmental stage 2 (S2) and stage 3 (S3). Results show increased expression of genes related to OXPHOS and glycolysis in S2 relative to S3 iNKT cells. Gene expressions in iNKT developmental stages were determined using the Affymetrix Mouse Gene 2.0 ST Array. FDR, false discovery rate. NES, normalized enrichment score. (Similar observations have previously been published [4])

5. Grind the tissues gently in order to reduce mechanical damage and maintain viability of the lymphocytes. It is also important to keep tissues and cells on ice during processing.
6. If isolating cells from the spleen, an additional red blood cell lysis step is required. To do so, centrifuge the splenic cells ($600 \times g$ for 5 min) and remove the supernatant. Add 1 ml of ACK lysis buffer (Gibco, #A1049201) per spleen for 1 min at RT. Add 9 ml of HBSS containing 2% FBS, centrifuge ($600 \times g$ for 5 min), remove the supernatant, and resuspend cells in HBSS containing 2% FBS.
7. This step is optional, but it will decrease the amount of time needed for sorting and sample acquisition on the flow cytometer.
8. If more than 2×10^6 cells are required for flow cytometry analysis, scale up the number of cells and volumes for metabolic dyes and antibody mixes accordingly (e.g., 10×10^6 cells can be stained in 250 μl of metabolic dye mix and 100 μl of antibody mix).
9. Two dyes can be combined to minimize the number of staining sets. We typically combine MitoTracker™ Green (FITC channel) with CellROX™ Deep Red (APC channel) and TMRM (PE channel) with 2-NBDG (FITC channel).

10. If analysis of iNKT development stages is required, include the following antibodies in the staining set at this step: anti-CD24 (M1/69), NK1.1 (PK136), and CD44 (1M7). Stage 1 iNKT thymocytes are $CD24^{-}NK1.1^{-}CD44^{-}$. Stage 2 iNKT thymocytes are $CD24^{-}NK1.1^{-}CD44^{+}$. Stage 3 iNKT thymocytes are $CD24^{-}NK1.1^{+}CD44^{+}$.
11. Cells should be collected on a flow cytometer within 1–3 h of staining with metabolic dyes.
12. General tips for Seahorse:
    (a) Only use a non-CO_2 37 °C incubator for warming cell plates and medium.
    (b) Maintain reagents at 37 °C as much as possible to minimize temperature fluctuations.
13. To sort the iNKT developmental stages, also stain the cells with anti-CD24, NK1.1, and CD44 antibodies and sort the subsets as outlined in **Note 10**. If iNKT effector subsets need to be sorted, reporter mice are the best tools [5], although combinations of surface markers have been described to faithfully subset iNKT effector cell populations [9].
14. Typical iNKT cell yield from the thymus of one 5–6-week-old C57BL/6 mouse: 150,000–250,00 cells.
15. If the cell concentration has changed between the first and second count, adjust the volume added per well to plate 250,000 cells/well.
16. 200,000 cells/well is the lower limit for iNKT cells.
17. We have found the following molecular signature database collections available in MSigDB (https://www.gsea-msigdb.org/gsea/msigdb) to contain informative metabolic gene signatures:
    (a) H: HALLMARK gene sets.
    (b) C2: curated gene sets (including the KEGG and BIOCARTA subsets).
    (c) C5: ontology gene sets, specifically the Gene Ontology (GO) gene sets.

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