Characterization and Functional Analysis of Mouse Invariant Natural T (iNKT) Cells

Natural T (NKT) cells are innate lymphocytes that have immunoregulatory properties (reviewed in Bendelac et al., 2001 and Van Kaer, 2005). Mouse NKT cells are a heterogeneous subset, amongst which the Va14Ja18 T cell receptor (TCR) α-chain-positive T (iNKT) lymphocytes predominate. iNKT cells express an invariant Va14Ja18 TCR α-chain that predominantly pairs with Vb8.2 β-chain, and sometimes with Vb7 and Vb2 β-chains (Lantz and Bendelac, 1994). Current evidence suggests that iNKT cells recognize self (Brigl et al., 2003; Wu et al., 2003; Zhou et al., 2004b), as well as foreign (Fischer et al., 2004; Kinjo et al., 2005; Mattner et al., 2005; Sriram et al., 2005), lipid antigens presented by MHC-like, CD1d molecules. When activated in vivo, they secrete both T helper (Th1- and Th2-type) cytokines (Van Kaer, 2005) and, hence, can regulate a variety of immune responses ranging from immunosuppression to immunoagression. Activated iNKT cells can prevent autoimmune diseases, maintain immune privilege, and support engraftment of transplanted tissues. Furthermore, iNKT cells can mediate adjuvant activities and consequently enhance tumor immunity and immune responses to pathogens (Van Kaer, 2005).

Human and mouse CD1d-restricted glycolipid antigens and the iNKT cell functions they elicit are highly conserved (Van Kaer, 2005), whereby, making the mouse an excellent animal model for understanding iNKT cell biology in vivo. iNKT cells represent ~1% of total leukocytes in the mouse thymus, spleen, bone marrow, and blood (Takahama et al., 1991; Bendelac et al., 1994; Ohteki and MacDonald, 1994; Eberl et al., 1999). In the liver, they represent ~20% to 30% of total leukocytes. Conspicuously, however, iNKT cells are rarely found in the lymph nodes (Ohteki and MacDonald, 1994), but they can traffic through there upon immune challenge (Martin-Fontecha et al., 2004). This means that there are, at most, ~10^6 iNKT cells in each mouse lymphoid organ, which makes analysis of this rare population extremely challenging. Thus, major adaptations of classical immunological methods described elsewhere are needed to make studies of iNKT cell biology feasible. This unit introduces basic protocols for the characterization and functional analyses of murine iNKT cells. Basic Protocol 1 describes four- and six-color flow cytometry to identify, quantify, and phenotype small numbers of iNKT cells. Assays for probing iNKT cell functions in vivo and in vitro are included in Basic Protocols 2 and 3, respectively. Basic Protocol 4 entails maintenance and functional use of Va14⁺ NKT hybridomas. This unit also contains support protocols describing enrichment of iNKT cells using immunolabeled magnetic separation (see Support Protocol 1), generation of CD1d tetramer (see Support Protocol 2), and lipid antigen loading on cell-bound (see Support Protocol 3) or soluble (see Support Protocol 4) CD1d.

### IDENTIFICATION, QUANTIFICATION, AND PHENOTYPING OF iNKT CELLS BY FLOW CYTOMETRY

Identification of this rare cell subset among other cells requires at least two criteria: positive staining with anti-CD3ε (or anti-TCRβ) mAb and antigen-loaded CD1d tetramers. α-Galactosylceramide (αGalCer) is a marine sponge–derived synthetic glycolipid antigen recognized by almost all Va14Ja18⁺ iNKT cells when presented by CD1d (Kawano et al., 1997; Burdin et al., 1998). Fluorochrome-conjugated, αGalCer-loaded CD1d-tetrameric molecule (CD1d-tetramer) is currently the most specific and sensitive reagent...
for the identification of iNKT cells (Benlagha et al., 2000; Matsuda et al., 2000). In-depth characterization of a small iNKT cell population requires careful prevention of nonspecific background staining and exclusion of CD1d-tetramer-negative cells during analysis. Thus, basic identification and quantification of iNKT cells depends on three- to four-color flow cytometry, while detailed phenotyping of iNKT subsets at distinct maturation stages requires at least six-color flow cytometric analysis (Stanic et al., 2004).

**NOTE:** All solutions and equipment coming in contact with cells must be sterile, and proper sterile technique must be employed.

### Materials

- Mouse lymphoid organs (*UNIT 1.9*)
- Complete RPMI-1640 (*APPENDIX 2A*) supplemented with 10% FBS, cold
- Flow cytometry buffer (FACS buffer; see recipe)
- Purified anti-mouse CD16/CD32 mAb (Fc block, BD Biosciences)
- Conjugated anti-mouse CD3ε mAb or conjugated anti-mouse TCRβ mAb (BD Biosciences)
- Conjugated anti-mouse CD8α mAb (BD Biosciences)
- Conjugated anti-mouse B220 mAb (BD Biosciences)
- PE- or APC-conjugated CD1d-tetramer (see Support Protocol 2; NIAID tetramer facility)
- 2% (w/v) paraformaldehyde (see recipe)
- Conjugated mAbs to other cell surface proteins of interest (as available, BD Biosciences or Molecular Probes)
- 15-ml polypropylene tubes
- 5-ml polystyrene FACS tubes or 96-well round-bottom polystyrene microtiter plates
- Sorvall RT7 centrifuge (or equivalent)
- Four-, six-, or more-color flow cytometer (FACSCalibur, FACSCanto, or LSRII)
- Analysis software for FACS data (e.g., FlowJo, Treestar; Chapter 5)
- Additional reagents and equipment for isolating mouse lymphoid organs (*UNIT 1.9*), preparing single-cell suspension (*UNIT 3.1*), and FACS analysis (Chapter 5)

1. Collect mouse lymphoid organs (*UNIT 1.9*) from freshly sacrificed animals in a 15-ml polypropylene tube containing 5 ml cold RPMI-1640/10% FBS. Prepare an erythrocyte-free single-cell suspension (*UNIT 3.1*).

   *Routine analyses of mouse iNKT cells are performed on leukocytes isolated from thymus, spleen, and liver, as these organs contain the highest numbers of iNKT cells (~10⁶).*

   *Keep the single-cell suspension on ice at all times during the procedure. Proceed to FACS staining and analysis immediately since iNKT cells may die within several hours following preparation of single-cell suspension of mononuclear cells. Alternatively, when intact organs are stored on ice in RPMI-1640/10% FBS, iNKT cells can survive up to 24 hr. Unless necessary, this is not recommended since iNKT cell yields may be reduced in some organs (e.g., liver). This property might be useful for acquisition of organs for pilot experiments from a long-distance collaborator.*

2. Determine the number of cells in the suspension (e.g., by using a hemacytometer; *APPENDIX 3A*). Resuspend cells at 1 x 10⁷ cells/ml in RPMI-1640/10% FBS. Pipet 200 µl of cells into FACS tubes or a 96-well round-bottom microtiter plate. Wash two times with FACS buffer.

   *Use 4 ml FACS buffer when staining in tubes or 0.2 ml FACS buffer when staining in microtiter plates. Centrifuge FACS tubes for 5 min at 200 x g or microtiter plates for 2 min at 670 x g, 4°C. FACS staining in microtiter plates is highly recommended for*
3. Resuspend cells in 50 µl FACS buffer containing 0.2 µg anti-CD16/CD32 mAb. Incubate 15 min on ice.

   *This step is necessary to block Fc receptors and reduce nonspecific mAb binding.*

   *The use of 0.05% sodium azide in FACS buffer is essential to prevent antibody or CD1d-tetramer-induced capping of the iNKT cell receptor. For this reason, staining with CD1d-tetramers at room temperature is not recommended.*

4. Wash two times with FACS buffer. Resuspend in 50 µl of FACS buffer containing 0.2 µg conjugated anti-CD3ε (or anti-TCRβ) mAb, anti-CD8α mAb (for thymus), anti-B220 mAb (for spleen and liver), and 0.1 µg CD1d-tetramer. For best results, incubate protected from light for 2 hr on ice.

   *It may be useful to titrate each mAb in initial experiments and adjust the amount needed for optimal staining.*

   PE- or APC-conjugated CD1d-tetramer and PerCP.Cy5.5- or PE-conjugated anti-CD3ε or (anti-TCRβ) mAb are recommended for good separation of iNKT cells. Use FITC-conjugated mAb, to electronically gate out the major CD1d-tetramer-negative population during analysis (i.e., CD8α+ cells in the thymus and B220+ cells in the spleen and liver).

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**Figure 14.13.1** Lineage-specific phenotyping of thymic, splenic, and hepatic iNKT cells using six-color flow cytometry. Thymic iNKT cells were enriched by depleting CD8α+ cells (see Support Protocol 1). iNKT cells were identified as TCRβ+CD1d-tetramer+ cells within residual CD8αLOW/CD24LOW population (upper panel). Splenic iNKT cells were identified as CD3ε+CD1d-tetramer+ cells within B220LOW population (lower panel). Six-color flow cytometry is performed on an LSRII flow cytometer; data are collected and compensated using FACSDiva software (Beckton Dickinson), and analyzed with FlowJo (Treestar). Phenotypic analysis of iNKT cells involves a six-color, eight-channel strategy; one channel each on two different lasers is utilized to gate on CD3ε+CD1d-tetramer+ cells, one “dump” channel to enhance specificity and three channels to evaluate the phenotype of iNKT cells (e.g., DX5 vs NK1.1 staining; see Basic Protocol 1).
Independent samples should be stained with conjugated isotype–matched mAbs and APC- or PE-conjugated “empty” (non-αGalCer-loaded, or another non-antigenic glycolipid-loaded) CD1d-tetramer (highly recommended when working with new vials of antibodies or CD1d-tetramer preparation).

As CD1d-tetramer is prepared from biotinylated monomers, simultaneous use of biotinylated mAbs in the same reaction tube is not recommended.

5. Wash two times with FACS buffer (as in step 2). Resuspend in 0.3 ml FACS buffer if samples will be analyzed immediately. For later analysis, store cells in FACS buffer containing 2% paraformaldehyde and keep 4 to 7 days at 4°C protected from light until analyzed.

6. Analyze by flow cytometry: collect at least 5–10 × 10^5 events. Identify iNKT cells as CD3ε^+CD1d-tetramer^+ cells within CD8^α^LOW thymocytes or B220^LOW^ splenocytes and liver mononuclear cells (Fig. 14.13.1).

   Calculate absolute numbers of iNKT cells from % CD3ε^+ CD1d-tetramer^+ cells identified within the particular organ determined in step 6 and total leukocyte numbers determined in step 2.

7. (Optional) Further characterize iNKT cells using conjugated mAbs specific for different CD1d-tetramer^+ subsets or different developmental stages (e.g., Vb2, Vb7, Vb8.1/2, DX5, CD24, CD25, CD44, CD69, CD122, CD127, CD161, Ly49A/D, Ly49C/I). For these analyses, use six- or more-color flow cytometry and collect at least ~1 × 10^6 events. 

   See Table 14.13.1 for the authors’ recommendations of clones and colors for each mAb for reliable multicolor analyses.

### Table 14.13.1 Recommended mAbs for iNKT Cell Multicolor Analysis

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Fluorochrome</th>
<th>Clone</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3ε</td>
<td>PE, PerCP-Cy5.5, APC, PE-Cy7</td>
<td>145-2C11</td>
</tr>
<tr>
<td>CD4</td>
<td>FITC, PE, PerCP-Cy5.5</td>
<td>RM4-4</td>
</tr>
<tr>
<td>CD5</td>
<td>FITC</td>
<td>53-7.3</td>
</tr>
<tr>
<td>CD8α</td>
<td>FITC, PerCP-Cy5.5</td>
<td>53-6.7</td>
</tr>
<tr>
<td>CD8α</td>
<td>APC-Cy7</td>
<td>RM2214</td>
</tr>
<tr>
<td>CD24</td>
<td>FITC</td>
<td>M1/69</td>
</tr>
<tr>
<td>CD25</td>
<td>PerCP-Cy5.5</td>
<td>PC61</td>
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<tr>
<td>CD44</td>
<td>FITC</td>
<td>IM7</td>
</tr>
<tr>
<td>CD69</td>
<td>FITC</td>
<td>H1.2F3</td>
</tr>
<tr>
<td>CD122</td>
<td>PE</td>
<td>TM-β1</td>
</tr>
<tr>
<td>CD127</td>
<td>PE</td>
<td>SB/14</td>
</tr>
<tr>
<td>B220</td>
<td>FITC, PerCP-Cy5.5, APC-Cy7</td>
<td>RA3-6B2</td>
</tr>
<tr>
<td>NK1.1</td>
<td>PE, PerCP-Cy5.5, APC, APC-Cy7</td>
<td>PK136</td>
</tr>
<tr>
<td>DX5</td>
<td>PE, APC</td>
<td>DX5</td>
</tr>
<tr>
<td>Ly49A/D</td>
<td>PE</td>
<td>12A8</td>
</tr>
<tr>
<td>Ly49C/I</td>
<td>FITC</td>
<td>SE6</td>
</tr>
<tr>
<td>IL-4</td>
<td>PE</td>
<td>11B11</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>PE</td>
<td>XMG1.2</td>
</tr>
<tr>
<td>IL-2</td>
<td>PE</td>
<td>JES6-5H4</td>
</tr>
</tbody>
</table>
To enhance specificity and to collect maximal number of CD1d-tetramer+ cells, especially when working with genetically altered animals with reduced iNKT cell numbers, enrichment of live iNKT cells by immunolabeled magnetic bead separation (see Support Protocol 1) is highly recommended.

To enhance specificity and to open as many channels as possible for phenotyping, use only one “dump” channel to gate out all non-iNKT cell populations. This will allow one “dump” channel, two channels to gate for CD3ε+ and CD1d-tetramer+ cells, and three (or more) channels for phenotyping of iNKT cells.

**iNKT CELL FUNCTIONAL RESPONSES TO ACTIVATION IN VIVO**

Of the several cellular lipids that activate iNKT cells in vitro, only a few do so in vivo. αGalCer is recognized by almost all iNKT cells, and, hence has been extensively used to probe their physiological roles (Carnaud et al., 1999; Singh et al., 1999; Fujii et al., 2002; Bezbradica et al., 2005b). Administration of αGalCer, either intravenously (i.v.) or intraperitoneally (i.p.), leads to specific presentation of this glycolipid by CD1d and the rapid activation of iNKT cells (Carnaud et al., 1999). iNKT cell activation can be quantified directly by measuring iNKT-specific cellular changes that occur immediately (within 60 to 90 min) after exposure to an activation signal or indirectly by measuring changes that occur later through transactivation of other immune cells. Thus, hallmarks of iNKT cell activations are: (1) rapid and robust production of a wide variety of Th1- and Th2-type cytokines (within 60 to 90 min post-αGalCer administration); (2) upregulation of activation markers on iNKT cells (e.g., CD69, 2 to 3 hr post-injection); (3) iNKT cell receptor downmodulation (~6 hr post-activation); (4) trans-activation of other immune cells; e.g., dendritic cells (DC), B cells, NK cells, and T cells (4 to 6 hr post-injection); (5) expansion (2 to 3 days); and (6) homeostatic recovery (7 days post-injection) of iNKT cell numbers (reviewed in Van Kaer, 2005). Thus, iNKT cell activation can be tracked experimentally by: (1) intra- and extracellular flow cytomery (detection of intracellular cytokines and cell-surface activation markers on iNKT and other leukocytes) and (2) ELISA (detection of cytokines secreted into the serum by iNKT and other leukocytes). This protocol describes how lipid antigens are prepared for in vivo administration, and outlines basic procedures for detection of early and late iNKT cell-mediated immune responses ex vivo.

**NOTE:** All solutions and equipment coming in contact with cells must be sterile, and proper aseptic techniques are essential if cell culture is intended.

**Materials**

- αGalCer (obtained for research purposes from Kirin Brewery or Axxora) or other antigenic lipids (as available)
- Vehicle (see recipe)
- Mice, age matched (~6 to 10 weeks old)
- Complete RPMI-1640 (APPENDIX 2A)/10% (w/v) FBS, ice cold
- Brefeldin A (Sigma)
- FACS buffer (see recipe)
- Cytofix/Cytoperm fixation/permeabilization buffer for intracellular flow cytometry (BD Biosciences)
- Perm/Wash staining buffer for intracellular flow cytometry (BD Biosciences)
- Conjugated anti-IL-2 mAb, anti-IL-4 mAb, or anti-IFN-γ mAb (BD Biosciences)
- 2% (w/v) paraformaldehyde (see recipe)
- ELISA blocking buffer: PBS/10% (w/v) FBS
- 80°C water bath sonicator
- 1-ml syringes fitted with 25- or 30-G needles
- 15-ml polypropylene tubes
- 1.5-ml microcentrifuge tubes
Additional reagents and equipment for anesthetizing animals (UNIT 1.4); euthanizing animals (UNIT 1.8); removal of mouse lymphoid organs (UNIT 1.9); blood collection from mouse (UNIT 1.7); preparation of erythrocyte-free single-cell suspension (UNIT 3.1); enzyme-linked immunosorbent assays (ELISA; UNIT 2.1); and flow cytometry (see Basic Protocol 1 and Chapter 5)

1. Make a primary stock of αGalCer at 200 µg/ml in vehicle. Sonicate primary stock 10 min in a heated 80°C water bath sonicator.

2. For each mouse to be injected, make working solution of αGalCer by diluting 5 µg (25 µl) of αGalCer from primary stock in vehicle to a final volume of 200 µl for i.p. and 100 µl for i.v. injections (UNIT 1.6).

   *For optimal activation, injecting 5 to 10 µg of αGalCer/mouse is recommended. Other lipid antigens can be tested using this protocol. It may be useful to titrate each antigen in initial experiments and adjust the amount needed for optimal iNKT cell activation. When testing other lipids, inject one mouse with the same amount of αGalCer as a positive control. This will allow you to compare the potency of the test lipid antigen with that of αGalCer. To date, αGalCer is the most potent iNKT cell antigen discovered. Note that other lipids may require other vehicles (e.g., DMSO). It is important that the vehicle used to deliver lipid antigen neither be toxic to the mouse nor antigenic to iNKT cells. Thus, in each experiment, a control mouse must be injected with the same concentration of vehicle alone.*

3. Inject αGalCer working solution, or the same concentration of vehicle alone, using a 1-ml syringe fitted with a 25- or 30-G needle. Inject at least two mice with αGalCer and two with vehicle alone. Analyze one of each at 2 hr post-injection, and the others at 6 hr post-injection (this will allow the measurement of immediate, as well as, late effects of iNKT cell activation in vivo).

   *iNKT cell responses to antigenic stimulation can be detected ~30 min earlier by i.v. antigen delivery compared to i.p. delivery.*

   *Alternatively, αGalCer may be delivered to the animals directly loaded onto CD1d at the cell surface of the particular antigen presenting cell type (APC; for details see Support Protocol 3).*

   *In all experiments, use mice deficient in glycolipid antigen presentation and/or iNKT cells, e.g., CD1d10/0, N-acetylhexosaminidase B (HexB)0/0 or Ja180/0 mice, as negative controls. This is essential to dissect specific, iNKT cell–mediated immune responses from non-iNKT cell–dependent immune reactions. Negative controls are particularly important when doing ELISA to measure cytokines secreted in the serum upon administration of αGalCer in vivo. Unlike intracellular flow cytometry, ELISA can not identify the cellular source of cytokines. Thus, when working with iNKT cells, using a combination of several complementary, independent techniques to address the biological question at hand is highly recommended.*

4. Perform cardiac puncture (UNIT 1.7) and collect blood in 1.5-ml microcentrifuge tubes. Euthanize mice (UNIT 1.8) and collect lymphoid organs (UNIT 1.9). Let blood sit at 4°C for several hours to overnight to separate the serum. Proceed to step 9 for further serum analyses.

   *Spleen and liver are routinely used to study early iNKT cell activation, as these peripheral organs contain the highest number of iNKT cells (~10⁶).*

   *Collect blood in 1.5-ml microcentrifuge tubes and let sit for several hours to overnight at 4°C to separate the serum. Proceed to step 9 for further serum analyses.*

5. Place organs immediately in a 15-ml polypropylene tube containing 5 ml of ice-cold complete RPMI-1640/10% FBS and 1 µg/ml Brefeldin A.
Brefeldin A is an inhibitor of intracellular protein transport. It enhances detection of intracellular cytokines. Because it is a reversible inhibitor, it is important that all buffers used for extra- or intracellular staining and washing contain ∼1 µg/ml of Brefeldin A.

6. Prepare erythrocyte-free single-cell suspension (UNIT 3.1) and determine the number of cells in the suspension (e.g., by using a hemacytometer; APPENDIX 3A). Proceed with four-color extracellular FACS analysis as described in Basic Protocol 1. Use at least three colors to identify the cell population of interest and leave the fourth color open for intracellular cytokine detection. Alternatively, use the fourth color to identify activation-induced cell surface markers.

iNKT cell activation can be quantified directly by measuring iNKT-specific cellular changes that occur immediately (2 hr) after exposure to an activation signal or indirectly by measuring changes that occur later through transactivation of other immune cells. iNKT cells are activated 2 hr after αGalCer injection. This can be tracked by the upregulation of the early activation marker, CD69, on the surface of CD3ε⁺CD1d-tetramer⁺ cells. Furthermore, by 2 hr, activated iNKT cells synthesize high levels of IL-2, IL-4, IFN-γ, and Csf-2, which can be detected by intracellular flow cytometry. Of these, IL-2, IL-4, and Csf-2, but not IFN-γ, are detected at 2 hr post-injection in the serum by ELISA. At 6 hr post-injection, iNKT cells downregulate their TCR and, thus, become undetectable by direct flow cytometry. However, other immune cells, e.g., B cells, DC, T cells, or NK cells, are transactivated during this time. This feature is routinely used in biological assays as an indirect measure of iNKT cell activation. Transactivated cells upregulate co-stimulatory markers and early activation markers (e.g., CD69) and/or secrete cytokines, which are detectable by extra- and intracellular flow cytometry, respectively. See Table 14.13.2 for identification of different cell types by flow cytometry, the kinetics of their activation, and cytokine secretion. Recommended clone numbers and fluorochromes for individual mAbs are listed in Table 14.13.1.

7. After extracellular staining, wash the unbound mAbs one time with 4 ml FACS buffer or Perm/Wash buffer if staining in tubes or 0.2 ml FACS or Perm/Wash buffer if staining in microtiter plates. Decant the supernatant and resuspend cells in Cytofix/Cytoperm buffer. Incubate 10 min on ice. Wash cells one time with Perm/Wash buffer.

After each centrifugation, discard FACS buffer and gently vortex the tube/microtiter plate to disperse the cell pellet.

Although basic principles of intracellular FACS staining apply to iNKT cell staining, many of the commonly used buffers and techniques are not compatible with the CD1d-tetramer staining. Thus, the use of Cytofix/Cytoperm, as the fixation/permeabilization buffer, and Perm/Wash, as the staining buffer, are highly recommended for intracellular flow cytometry. Both buffers should contain 1 µg/ml Brefeldin A.

8. Add 50 µl Perm/Wash buffer containing either 0.2 µg anti-IL-2, anti-IL-4, or anti-IFN-γ mAbs. Incubate 1 hr on ice protected from light.

Cytokines listed above are most commonly used to measure iNKT cell activation. Other surface markers/cytokines of interest can be used following the same protocol. Perform pilot experiments to determine their expression/secretion kinetics and adjust the protocol accordingly.

9. Wash two times with Perm/Wash buffer (as in step 7) to remove unbound mAbs. Wash once with FACS buffer to re-seal permeabilized cells. If samples will be analyzed immediately, resuspend cells in 0.3 ml FACS buffer. For later analysis, store cells in FACS buffer containing 2% paraformaldehyde and keep 4 to 7 days at 4°C until analyzed.

See Figure 14.13.2 for an example of electronic gating and intracellular cytokine detection within iNKT cells 2 hr post-αGalCer injection.
Table 14.13.2  Cellular and Molecular Markers Commonly Used to Track iNKT Cell Functional Responses In Vivo

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Change</th>
<th>Time post-injection</th>
<th>How to detect the change</th>
</tr>
</thead>
<tbody>
<tr>
<td>iNKT</td>
<td>CD69 upregulation</td>
<td>2 hr</td>
<td>FACS staining of CD69 on CD3ε⁺CD1-tetramer⁺ cells</td>
</tr>
<tr>
<td>iNKT</td>
<td>Intracellular cytokine</td>
<td>2 hr</td>
<td>Intracellular FACS staining within CD3ε⁺CD1-tetramer⁺ cells</td>
</tr>
<tr>
<td>iNKT</td>
<td>TCR dowmodulation</td>
<td>6 hr</td>
<td>FACS staining of CD3ε⁺CD1-tetramer⁺ cells</td>
</tr>
<tr>
<td>iNKT</td>
<td>Cytokine secretion</td>
<td>2 hr</td>
<td>ELISA detection of serum cytokines</td>
</tr>
</tbody>
</table>

Indirect effects on transactivated cells

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Change</th>
<th>Time post-injection</th>
<th>How to detect the change</th>
</tr>
</thead>
<tbody>
<tr>
<td>DC</td>
<td>CD80, CD86 upregulation</td>
<td>6 hr</td>
<td>FACS staining of CD80 and CD86 on CD11c⁺ cells</td>
</tr>
<tr>
<td>B cells</td>
<td>CD69, CD86 upregulation</td>
<td>6 hr</td>
<td>FACS staining of CD69 and CD86 on B220⁺ cells</td>
</tr>
<tr>
<td>T cells</td>
<td>CD69 upregulation</td>
<td>6 hr</td>
<td>FACS staining on CD3ε⁺CD8α⁺ or CD3ε⁺CD4⁺ T cells</td>
</tr>
<tr>
<td>NK cells</td>
<td>Intracellular IFN-γ production</td>
<td>6 hr</td>
<td>Intracellular FACS staining within CD3ε⁻DX5⁺ cells</td>
</tr>
</tbody>
</table>

Figure 14.13.2  Flow cytometric analysis of iNKT cell activation. C57BL/6 mice were adminis-tered 5 µg αGalCer or vehicle i.p. Two hr later, CD3ε⁺CD1d-tetramer⁺ cells were electronically gated within B220LOW splenocytes and IFN-γ expression was monitored by flow cytometry. Histograms represent IFN-γ detected within iNKT cells of vehicle (gray histograms) or αGalCer (open histograms)-treated mice (see Basic Protocol 2).

10. When serum separates above coagulum, microcentrifuge tube 5 min at maximum speed. Carefully collect the serum (0.5 to 1 ml if possible) and store for up to 12 months at −20°C or use immediately for ELISA.

   *discard blood coagulum in biohazard waste.*

11. For ELISA, make serum dilution 1:1, 1:10, and 1:20 in ELISA blocking buffer.
Dilution is recommended, because the amounts of some cytokines in the original serum samples may be beyond the linear range of the assay. Account for the dilution factor when performing the calculations.

The amount of cytokine secreted upon αGalCer administration in vivo is directly proportional to the number of iNKT cells. As iNKT cell numbers change with age, always use age-matched animals. Normalize the amount of cytokines detected by ELISA against the absolute numbers of iNKT cells detected by flow cytometry (as described in Basic Protocol 1).

### BASIC PROTOCOL 3

**iNKT CELL FUNCTIONAL RESPONSES TO ACTIVATION IN VITRO**

iNKT cells can be activated by a variety of agents, including pharmacological agents (PMA/ionomycin), polyclonal stimulators (anti-CD3ε and anti-CD28 crosslinking mAbs), and CD1d-restricted lipid antigens. Of these, only CD1d-restricted antigens (e.g., αGalCer) selectively activate iNKT, but not other T cells. Thus, αGalCer has been extensively used to specifically probe iNKT cell functions in vivo and in vitro (Carnaud et al., 1999; Singh et al., 1999; Fujii et al., 2002; Bezbradica et al., 2005b). αGalCer-mediated iNKT cell activation results in cytokine synthesis and secretion as well as proliferation of activated iNKT cells. In vitro functional assays are used to measure αGalCer-induced iNKT cell proliferation and establish dose-response curves, or to test iNKT cell responses to antigen in the absence of one or more cell types that can be easily depleted from the culture using magnetic cell sorting. Advantages of in vitro assays are: (1) several different experimental conditions can be tested with a minimum number of animals; and (2) iNKT cells can be enriched prior to analysis (see Support Protocol 1). This enrichment step can significantly increase the sensitivity of the assay. However, if feasible, in vitro experiments should be used only for screening, or as a complementary assay performed prior to or in parallel with in vivo analyses of iNKT cell function (see Basic Protocol 2).

**NOTE:** All solutions and equipment coming in contact with cells must be sterile, and proper aseptic techniques are essential if cell culture is intended.

**NOTE:** All culture incubations should be performed in a humidified 37°C, 5% CO2 incubator unless otherwise specified.

#### Materials

- Mouse lymphoid organs *(UNIT 1.9)*
- Complete RPMI-1640 *(APPENDIX 2A)* supplemented with 10% (w/v) FBS, ice cold and 37°C
- 5-(and -6)-carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes)
- αGalCer (obtained for research purposes from Kirin Brewery or Axxora) or other antigenic lipid (as available)
- Vehicle (see recipe)
- 15-ml polypropylene tubes
- 96-well round-bottom polystyrene microtiter plates with lids
- 37°C, 5% CO2 humidified cell culture incubator
- 80°C water bath sonicator

Additional reagents and equipment for removal of mouse lymphoid organs *(UNIT 1.9)*; preparation of erythrocyte-free single-cell suspensions *(UNIT 1.3)*; CFSE labeling *(UNIT 4.9)*; anesthetizing animals *(UNIT 1.4)*; euthanizing animals *(UNIT 1.8)*; enzyme-linked immunosorbent assays (ELISA; *(UNIT 2.1)*); and flow cytometry (see Basic Protocol 1 and Chapter 5)
1. Collect mouse lymphoid organs from freshly sacrificed animals in 15-ml polypropylene tubes containing 5 ml ice-cold RPMI-1640/10% FBS (UNIT 1.9). Prepare erythrocyte-free single-cell suspensions (UNIT 3.1).

Routine in vitro assays are performed on freshly isolated splenocytes because the spleen is the peripheral lymphoid organ that contains the highest numbers of mature iNKT cells (∼10^6).

Upon harvesting organs, proceed with the experiment immediately since iNKT cells may die within several hours of single-cell suspension preparation.

2. Determine the number of cells in the suspension (e.g., by using a hemacytometer; APPENDIX 3A). Resuspend cells at 5 × 10^6 cells/ml in RPMI-1640/10% FBS. Label the cells using CFSE (UNIT 4.9).

Perform the following steps in the dark as CFSE is extremely photosensitive. Label 5−10 × 10^6 cells in 1 ml of 1 µM CFSE working solution. Labeling should be performed in a 15-ml conical tube for exactly 8 min at room temperature while gently rocking. These indicated adjustments to the protocol given in UNIT 4.9 are necessary due to the high sensitivity of iNKT cells to the toxic effect of CFSE.

3. Pipet 100 µl of CFSE-labeled cell suspension (5 × 10^5 cells) into individual wells of a 96-well round-bottom microtiter plate. Keep cells in incubator while preparing the antigen.

Skip wells between samples to avoid cross-well contamination. Aim to keep a final culture volume of 200 µl per well.

4. Make primary stock of αGalCer at 200 µg/ml in vehicle. Sonicate primary stock in a heated (80°C) water bath sonicator for ten min.

5. From primary stock, make 2× working solution of αGalCer in pre-warmed (37°C) RPMI-1640/10% FBS. Start with a concentration of 20 µg/ml and make serial dilutions 1:10 (eight tubes total). For controls, add same volume of vehicle to pre-warmed (37°C) RPMI-1640/10% FBS.

Other lipid antigens can be tested using this protocol. When testing other lipids, use the same amounts of αGalCer as a positive control. This will allow comparison between the potency of the test lipid antigen and that of αGalCer. Note that other lipids may require other vehicles (e.g., DMSO). It is important that vehicle is neither toxic nor antigenic to iNKT cells. Thus, in each experiment, control wells should contain the same concentration of vehicle alone.

6. Add 100 µl of 2× αGalCer or vehicle working solution to the responder cells from step 3. Cover the plate and incubate for 5 days.

Alternatively, αGalCer may be delivered to the responder culture directly loaded on CD1d on the cell surface of a particular APC (see Support Protocol 3). If doing so, responder culture must be depleted (using autoMACS, see Support Protocol 1) of the CD1d^+ APC.

7. Collect culture supernatant and measure secreted cytokines by ELISA (UNIT 2.1). Analyze the amount of cytokines detected against the amount of antigen added in the well to create a dose-response curve.

To eliminate intra-experimental variations, perform all stimulations in triplicate.

8. Collect the cells and analyze proliferation of CD3ε^+ CD1d-tetramer^+ iNKT cells by CFSE dye dilution assay (UNIT 4.9).

Alternatively, proliferation can be measured by determining incorporation of [3H]thymidine into DNA (UNIT 7.10).
Because cell death might occur after 5 days in culture, which would interfere with the interpretation of the data, electronic elimination of dead cells by using 7AAD or propidium iodide staining is highly recommended.

Small aliquots of culture supernatants can be collected before day 5, and analyzed for secreted cytokines every 24 hr. The CFSE dilution assay, however, can be done only after 4 to 5 days because iNKT cells are not detectable at early time points due to activation-induced TCR down modulation.

As negative controls, use splenocytes or liver MNC isolated from mice deficient in glycolipid antigen presentation and/or iNKT cells, e.g., CD1d10/0, HexB0/0 or Ja180/0 mice. This is essential to distinguish specific, iNKT cell–mediated immune responses from non-iNKT cell–dependent immune reactions.

MAINTENANCE AND FUNCTIONAL USE OF NKT CELL HYBRIDOMAS

Mouse-derived Va14+ NKT hybridomas express the invariant Va14Ja18 TCR and share CD1d-restriction and antigen specificity of primary iNKT cells (Bendelac et al., 1995; Gui et al., 2001). Although functional outcomes of their stimulation are somewhat different (hybridomas secrete only IL-2 while primary iNKT cells secrete a wide variety of Th1 and Th2 cytokines), in the absence of an adequate strategy for obtaining 100% pure, nonactivated primary iNKT cells, NKT hybridomas are currently the best model system used to screen invariant TCR reactivity towards newly discovered lipid antigens. This system successfully avoids the interference of other non-iNKT cells with data interpretation, a common problem currently associated with in vivo and in vitro iNKT cell functional assays. Additionally, NKT hybridomas are used to test CD1d expression and function on the surface of different cell types (e.g., CD4+8+ thymocytes, DC, B cells, macrophages). Because Va14Ja18 iNKT cells recognize a CD1d-restricted self-glycolipid antigen that resides within the late endosomes/lysosomes (Zhou et al., 2004b), if CD1d assembly and antigen loading paths are intact in a particular cell type, it will activate NKT hybridomas when co-cultured in proper ratios (Stanic et al., 2003). This protocol describes ways to measure NKT hybridoma activation using a variety of stimuli. Culture conditions and examples of reagents to induce this activation are provided. Monitoring IL-2 secretion following stimulation quantifies hybridoma activity.

Materials

- Va14+ NKT hybridomas
- Complete OPTI-MEM medium (see recipe)
- CD1d+ stimulator cell lines (made in individual laboratories) or primary cells (e.g., thymocytes, UNIT 1.9, or DC, UNIT 3.7)
- Lipid-loaded soluble mouse CD1d (see Support Protocol 4)
- Purified anti-CD3ε mAb (BD Biosciences)
- PBS (APPENDIX 2A)
- ELISA blocking buffer: PBS (APPENDIX 2A)/10% (w/v) FBS
- 100-mm tissue culture plates
- 96-well flat-bottom polystyrene microtiter plates with lids
- 37°C, 5% CO₂ humidified cell culture incubator

Additional reagents and equipment for enzyme-linked immunosorbent assays (ELISA; UNIT 2.1)

NOTE: All solutions and equipment coming in contact with cells must be sterile, and proper aseptic techniques are essential if cell culture is intended.

NOTE: All culture incubations should be performed in a 37°C, 5% CO₂ humidified incubator, unless otherwise specified.
1. Culture NKT hybridomas in 100-mm tissue culture plates in complete OPTI-MEM medium for at least 3 to 4 days before beginning the assay.

   *This allows for the stabilization of cellular metabolic processes in freshly thawed cell lines and results in optimal responsiveness to stimulation.*

   **NKT hybridomas are optimal for functional assays for up to 6 weeks. After this time, their responsiveness is dramatically reduced, and new cell culture needs to be initiated from a frozen stock (APPENDIX 3G).**

   **NKT hybridomas grow in suspension, divide very rapidly, and should be split 1:5. Cells should not be overgrown at any time, or split at the day of the experiment, as this affects their responsiveness to stimulation significantly. Viability of cells used for experiment should be in the range of 90% to 100% for optimal response (APPENDIX 3B).**

2. Harvest hybridomas into 15-ml tubes and centrifuge 5 min at 200 \( \times \) g, room temperature. Decant supernatant and resuspend hybridomas at 5 \( \times \) 10^5 cells/ml in complete OPTI-MEM medium. Pipet 100 \( \mu \)l (5 \( \times \) 10^4 cells)/well of a 96-well flat-bottom microtiter plate.

   *Leave three wells with NKT hybridomas in medium alone, containing no stimulus, to account for the basal IL-2 secretion.*

3. Stimulate hybridomas in one of the following ways:

   a. **For CD1d\(^{+}\) cell lines carrying endogenous NKT cell antigen:** Add 100 \( \mu \)l of stimulator cell line to wells containing hybridomas in a 1:1 stimulator-to-responder ratio.

   b. **For CD1d\(^{+}\) cell lines pulsed with the lipid that is being screened for NKT cell receptor reactivity (see Support Protocol 3):** Add 100 \( \mu \)l of lipid-loaded stimulator cell line to wells containing hybridomas in a 1:1 stimulator/responder ratio.

   c. **For plate-bound CD1d-pulsed with the lipid that is being screened for NKT cell receptor reactivity (see Support Protocol 4):** Add 5 \( \times \) 10^4 hybridomas (resuspended in 200 \( \mu \)l complete OPTI-MEM medium) over wells containing lipid-loaded CD1d.

   d. **For CD1d\(^{+}\) primary mouse cells carrying endogenous iNKT cell antigen. (e.g., CD4\(^{+}\)8\(^{+}\) thymocytes, DC; see UNIT 3.7 for DC):** Add 100 \( \mu \)l of primary stimulator cells to wells containing hybridomas in a 10:1 stimulator/hybridoma ratio.

   e. **For crosslinking anti-CD3\(\varepsilon\):** Use 10 \( \mu \)g/ml soluble anti-CD3\(\varepsilon\) for 5 \( \times \) 10^4 hybridoma cells in 200 \( \mu \)l complete OPTI-MEM medium. Alternatively, bind anti-CD3\(\varepsilon\) mAb to wells of an ELISA plate at 2 \( \mu \)g/ml in PBS overnight at 4\(^{\circ}\)C. Wash away unbound Ab and add 5 \( \times \) 10^4 hybridomas (diluted in 200 \( \mu \)l complete OPTI-MEM medium).

   Some cell lines are maintained under antibiotic selection. Because hybridomas carry no antibiotic-resistance genes, it is essential to wash out all antibiotics from stimulator cell line prior to mixing them with hybridomas. For this, resuspend both hybridomas and stimulators in complete OPTI-MEM medium.

   **Anti-CD3\(\varepsilon\) stimulation can be used as a positive control in all NKT hybridoma functional assays described in this unit.**

   *Aim to keep a final culture volume of 200 \( \mu \)l/well. Perform all stimulations in triplicate to account for intra-experimental variability.*

4. Co-culture hybridomas with the stimulator in microtiter plates with lid for \( \sim \)18 hr.

5. Centrifuge plates 5 min at 670 \( \times \) g, room temperature, and harvest 100 \( \mu \)l of supernatant from each well for sandwich IL-2 ELISA ([UNIT 2.1](#)).

6. For ELISA, dilute supernatant at 1:1, 1:10, and 1:20 in ELISA blocking buffer.
Dilation is recommended because the amount of IL-2 secreted in the original sample may be above the linear range of the assay. Account for the dilution factor when performing the calculations.

ENRICHMENT OF iNKT CELLS FROM MOUSE LYMPHOID ORGANS USING MAGNETIC CELL SEPARATION

Specific cell-surface antigens are commonly used as a tool to separate one cell population from a pool of cells by using either immunolabeled magnetic bead separation (MACS; UNIT 3.5) or fluorescence-activated cell sorting (FACS; Chapter 5). Many of the iNKT cell-surface antigens are known. To date, however, the only surface antigen common to all Va14Ja18\(^+\) iNKT cells and not shared with other leukocytes is their invariant TCR. iNKT cell receptor can be specifically identified using CD1d-tetramer (Benlagha et al., 2000; Matsuda et al., 2000). CD1d-tetramer-based sorting of iNKT cells, however, has several limitations. (1) Separation may result in iNKT cell activation and activation-induced cell death (AICD). (2) iNKT cells that remain intact upon CD1d-tetramer-based sorting are useful for phenotypic analysis, but not for functional analyses since the iNKT cell receptor may be occluded by the tetramer and, hence, may be refractive for further receptor-mediated stimulation. Thus, in the absence of a better approach, this protocol outlines a standard MACS separation strategy for iNKT cell enrichment prior to performing in vitro functional assays or phenotypic analysis. Using this strategy, iNKT cells can be enriched up to 20% to 25% from the thymus and up to 10% to 15% from the spleen of wild-type animals. Cells remain viable, intact, and responsive to stimulation and can be used for further functional analyses. Enriched fractions are free of CD8\(^+\), B220\(^+\), CD11c\(^+\), and CD11b\(^+\) cells. Non-iNKT cells in enriched fractions include CD4\(^+\) T cells, \(\gamma\delta\) T cells, and NK cells. Using MHC class II–deficient animals (Cosgrove et al., 1991), which lack CD4\(^+\) T cells but have wild-type levels of iNKT cells (Bendelac et al., 1994), the interference of the major contaminant, CD4\(^+\) T cells, in enriched iNKT cell cultures may be overcome using the same protocol.

NOTE: All solutions and equipment coming in contact with cells must be sterile, and proper aseptic techniques are essential if cell culture is intended.

NOTE: Do not use sodium azide at any step during the enrichment procedure, because this impairs subsequent cell growth and responsiveness to activation.

NOTE: When using autoMACS, follow the manufacturer’s instructions on proper installation, use, and care of the machine.

Materials

Mouse lymphoid organs (UNIT 1.9)
Complete RPMI-1640/10% FBS, cold
MACS running buffer (see recipe)
MACS rinsing buffer (see recipe)
Anti-mouse B220-coated magnetic beads (Miltenyi Biotec)
Anti-mouse CD8\(^+\)-coated magnetic beads (Miltenyi Biotec)
Anti-mouse CD11c-coated magnetic beads (Miltenyi Biotec)
Anti-mouse CD11b-coated magnetic beads (Miltenyi Biotec)
15-ml polypropylene tubes
5-ml capped polystyrene MACS tubes
AutoMACS sorter (Miltenyi Biotec)
40-µm filter

Additional reagents and equipment for isolating mouse lymphoid organs (UNIT 1.9), preparing single-cell suspension (UNIT 3.1), and FACS analysis (Chapter 5); for immunomagnetic separation (UNIT 3.5)
1. Collect mouse lymphoid organs (*UNIT 1.9*) from freshly sacrificed animals in a 15-ml polypropylene tube containing 5 ml cold RPMI-1640/10% FBS. Prepare erythrocyte-free single-cell suspensions (*UNIT 3.1*).

Routine enrichment of mouse iNKT cells are performed on leukocytes isolated from thymus and spleen.

The single-cell suspension should be kept on ice at all times during the procedure. Perform MACS sorting immediately since iNKT cells may die within several hours of single-cell suspension preparation from lymphoid organs. Alternatively, intact organs stored on ice in complete RPMI-1640/10% FBS maintain functional integrity. Unless necessary, this is not recommended since iNKT cell yields may be reduced in some organs (e.g., liver). Notwithstanding that, this property may be useful for acquisition of organs for pilot experiments from a long-distance collaborator.

2. Determine the number of cells in the suspension (e.g., by using a hemacytometer; *APPENDIX 3A*). Resuspend at a concentration of 1–1.5 × 10⁸ cells/ml in cold MACS running buffer in capped, sterile 5-ml polystyrene MACS tubes.

3. Wash two times with cold MACS running buffer. For each wash use 4 ml wash buffer centrifuge tubes 5 min at 200 × g, 4°C. After each centrifugation, discard supernatant and gently vortex the tube to disperse cell pellet.

4. Add 100 µl microbeads/ml of MACS running buffer (use anti-CD8α-microbeads for thymocytes or anti-B220-coated microbeads for splenocytes). Incubate 30 min on ice protected from light.

Keep reaction protected from light, as beads are photosensitive.

Cells might pellet during incubation. To increase the efficiency of binding, mix the tube gently by inversion several times during the 30-min incubation period.

5. During incubation, turn on the autoMACS system. Run the clean program according to manufacturer’s instructions.

Starting the system and clean program takes up to 10 min. Failure to prepare the machine for separation in advance will prolong the incubation time in step 4 and might increase nonspecific binding.

6. Wash the cells once with MACS running buffer as in step 3. Resuspend 1 × 10⁸ cells/ml in MACS running buffer. Filter the cells through a 40-µm filter.

This washing step will remove excess microbeads, which is essential to prevent saturation of MACS columns.

It is critical to filter cells to prevent the columns from clogging. The cells are very concentrated and rinsing the filter with a small volume of MACS running buffer will increase recovery.

7. Remove labeled cells using “depletes” program on the autoMACS sorter (*UNIT 3.5*). Collect the negative fraction.

The thymic negative fraction contains non-CD8α⁺ cells and is ready for phenotyping (see Basic Protocol 1) or in vitro functional analyses (see Basic Protocol 3). The splenic negative fraction contains non-B220⁺ cells and is ready to proceed with step 8.

8. Resuspend B220⁻ splenocytes at 1–1.5 × 10⁸ cells/ml in MACS running buffer. Add 100 µl anti-CD8α-coated, 80 µl anti-CD11c-coated, and 80 µl anti-CD11b-coated microbeads/ml in MACS running buffer. Incubate 30 min on ice protected from light.

Because B220⁺ cells represent ~60% of the total splenocytes, it is important to perform a stepwise enrichment strategy in the spleen: deplete B220⁺ cells first and then deplete the other cells (CD11c⁺, CD11b⁺, and CD8α⁺) later. This will prevent the saturation of
the autoMACS columns and will increase the percentage of iNKT cells collected in the negative fraction. Alternatively, use MHC class II–coated beads to deplete B cells, DCs, and macrophages all at once.

To increase the efficiency of binding, mix the tube gently by inversion several times during the 30-min incubation period.

9. Repeat steps 6 and 7. Collect the negative fractions containing enriched splenic iNKT cells.

Between sorts, rinse autoMACS with MACS rinsing buffer by choosing “Rinse” program from the instrument’s menu and follow manufacturer’s instructions.

These cells are now ready for in vitro functional studies (see Basic Protocol 3).

10. Analyze a small fraction of collected cells by flow cytometry to determine the percentage of iNKT cells in the enriched fraction.

SUPPORT PROTOCOL 2

PREPARATION OF CD1d-β2M TETRAMERS

Direct and specific identification of iNKT cells from differing strains of mice is currently feasible only with the use of CD1d-αGalCer tetramer (Benlagha et al., 2000; Matsuda et al., 2000; Stanic et al., 2003). Furthermore, use of CD1d-tetramer complexed to other lipids permits flow cytometric determination of subset reactivities exhibited by the restricted, yet somewhat heterogenous, TCR repertoire of iNKT cells isolated from mice. Thus, CD1d-tetramer is an essential reagent used for: (1) identification of iNKT cells within complex leukocyte mixtures (e.g., thymic or splenic single-cell suspensions); (2) study of iNKT cell reactivity to glycolipid antigens; and (3) study of iNKT cell receptor avidity for different glycolipid antigens by flow cytometry. This protocol describes growth of insect cell lines expressing soluble, β2m-complexed CD1d tagged with BirA substrate (biotinylation sequence) peptide at its C-terminus; purification of such complexes and loading with the prototypical glycolipid antigen αGalCer. As noted earlier in this unit, investigators may also obtain CD1d-tetramers from the NIAID tetramer facility in Atlanta, GA.

NOTE: All solutions and equipment coming in contact with cells must be sterile, and proper aseptic techniques are essential if cell culture is intended.

NOTE: Insect cells grow optimally at 27°C without CO2; they also grow at room temperature, but slowly.

Materials

High-Five (Invitrogen) cells transfected with pIZT-mouse β2m and pIB-sCD1d1BSP His-6 (Stanic et al., 2003)
Complete Express Five serum-free medium (complete Express Five SFM; see recipe)
Blasticidin S HCl (Invitrogen)
Zeocin selection reagent (Invitrogen)
0.05% (w/v) sodium azide
PBS (APPENDIX 2A).
HisTrap kit (Amersham Biosciences) containing:
0.1 M NiSO₄
Buffer A
Phosphate buffer, pH 7.4
2 M imidazole, pH 7.4
HisTrap columns
5-ml syringe
10 mM Tris-Cl, pH 8.0
Micro BCA protein assay kit (Pierce)
BirA enzyme kit (Avidity) containing:
10× Biomix-A: 0.5 M bicine buffer, pH 8.3
10× Biomix-B: 100 mM ATP, 100 mM MgOAc, 500 µM d-biotin
BirA enzyme
Additional d-biotin
10 µM αGalCer (or other glycolipids of choice per glycolipid-specific protocol)
1 mg/ml streptavidin-APC or streptavidin-PE (Molecular Probes)
150-ml tissue culture flasks
1-liter sterile, polystyrene bottles
Centramate tangential-flow concentration system with a 30,000 MWCO Omega membrane (Pall Filtron)
Slide-A-Lyzer dialysis cassettes (3000 MWCO; Pierce)

Grow High-Five cells expressing soluble CD1d-β2m complexes

1. Thaw High-Five cells containing sCD1d1-BSP His-6 and mouse β2m plasmids into Express Five SFM.
   
   *The authors' follow the protocols for growth and maintenance of insect cell lines from Invitrogen (http://invitrogen.com/content/sfs/manuals/insectman.pdf).*

2. Maintain cells without selection for 24 hr at 27°C without CO2.

3. Prepare complete Express Five SFM with 10 µg/ml blasticidin S HCl and 300 µg/ml Zeocin selective medium.

   Medium containing blasticidin S HCl is effective for <2 weeks at 4°C as blasticidin S HCl is not stable for long-term storage upon dilution. Blasticidin S HCl should not be subjected to freeze-thaw cycles, therefore, upon receipt, working stocks should be prepared at a concentration of 10 mg/ml and frozen at −80°C.

4. Carefully remove spent culture medium from the flask and discard, and add complete Express Five SFM without detaching the cells from the plate.

   Use 30 or 50 ml medium for 175- or 225-cm² flasks, respectively.

   High Five cells are loosely adherent, and this adherence to the flask is an important determinant of culture viability. Critically, when passaging cells into new flasks, remove medium with selection (complete ExpressFive SFM supplemented with blasticidin S HCl and Zeocin), replace with complete Express Five medium without selection, and store at 4°C, transfer into new flasks without selection and allow cells to attach for ~30 min at 27°C, before removing nonselective medium and adding selective medium.

5. After reaching a culture size of ~300 ml (e.g., in five 150-ml tissue culture flasks) decrease Zeocin concentration to 150 µg/ml.

   An easy method to determine the appropriate expression of pIZT-mouse β2m vector is to monitor the expression of GFP (encoded on the pIZT plasmid) using a standard fluorescence microscope. Cells expressing β2m will appear green.

6. Grow at least 2 to 5 liters of culture prior to purification (~2 weeks of cell culture).

   Be very careful when growing High Five cells, as splitting at a ratio of more than 1:4 may result in slow growth of the cell culture and/or alteration of the cellular phenotype. Additionally, never allow cells to grow beyond confluence (split approximately every 36 to 48 hr), as (1) selection antibiotics will become ineffective and (2) High Five cells will alter their phenotype and cease providing high-level expression. In the authors' experience, spinner cultures (in >250-ml bottles) yield mediocre protein levels, therefore, growth in multiple 150-ml tissue culture flasks is recommended.
7. Harvest supernatants of entire culture, centrifuge 5 min at 460 × g, 4°C.

Be very careful not to include the supernatants of any flasks in which cells appear overgrown, unhealthy, or dying; visually inspect using a standard phase-contrast microscope. These supernatants will contain cellular debris and proteases that will degrade soluble CD1d-β2m complexes prior to purification.

8. Carefully remove supernatant without disturbing the cellular pellet and repeat steps 7 and 8.

9. Collect entire cell- and cell fragment–free recombinant High Five cell culture supernatant in 1-liter sterile plastic bottles. Supplement collected supernatant with 0.05% sodium azide and store at 4°C. 

In the authors’ experience, storage of collected supernatants should not be extended beyond ∼1-week period, as soluble CD1d-β2m begins to precipitate, and/or digested by trace proteases found in High Five cell culture supernatants (see Critical Parameters and Troubleshooting).

Purify soluble CD1d-β2m complexes

10. Using a Centramate tangential-flow diafiltration system fitted with a 30,000 MWCO Omega membrane under 2 psi of pressure (∼3 hr of close monitoring), concentrate 5 to 10 liters of culture supernatant to ∼200 ml and exchange with PBS.

The 30,000 MWCO concentrate result in an ∼50 to 200 ml volume because this is the dead-space of the filtration circuit.

11. Once retentate volume approaches 200 ml, add 800 ml of PBS, pH 7.0, and continue concentration until retentate is again 200 ml.

12. Repeat step 11 three additional times to exchange 99.84% of cell culture medium for PBS.

Express Five medium contains chelating agents that will strip the HisTrap column of Ni, which is essential for purification of His-6-tagged proteins, resulting in loss of soluble CD1d-β2m complexes in the effluent. Consequently, >99% exchange of cell culture medium for PBS is essential for successful purification of CD1d-β2m complexes.

Purify soluble CD1d-β2m complexes immediately from the concentrated supernatants

13. Purify soluble CD1d-β2m complexes by using the HisTrap kit according to the manufacturer’s instructions. Use the following concentrations and volumes for washing and elution:

a. Wash new column with 10 ml of sterile water.
b. Charge column with 1 ml of 0.1 M NiSO₄.
c. Equilibrate column with 10 ml of buffer A (1 ml/min, 4°C).
d. Run entire concentrate (∼50 to 200 ml) at 1 ml/min at 4°C.
e. Wash column with 5 ml PBS, pH 7.4 (sterile, degassed).
f. Wash column with 5 ml PBS, pH 7.4, containing 5 mM imidazole.
g. Wash column with 5 ml PBS, pH 7.4, containing 10 mM imidazole.
h. Wash column with 5 ml PBS, pH 7.4, containing 20 mM imidazole.
i. Wash column with 5 ml PBS, pH 7.4, containing 25 mM imidazole.

j. Elute soluble CD1d-β2m complexes with 20 ml PBS, pH 7.4, containing 300 mM imidazole. Collect and label individual 1-ml fractions for SDS-PAGE analysis of purified protein.
14. Take ~2 to 5 µl of each eluted fraction and analyze by 15% SDS-PAGE (UNIT 8.1) and Coomassie-based staining (UNIT 8.9).

Soluble CD1d-β2m needs to be >95% pure for successful tetramerization.

15. Dialyze 1-ml purified fractions containing soluble CD1d-β2m complexes using a Slide-A-Lyzer 3000 MWCO membrane cassettes against 1 liter of 10 mM Tris-Cl, pH 8.0, to prepare fractions for biotinylation. Use at least three changes of buffer to remove all PBS.

High-NaCl concentrations found in PBS inhibit biotinylation reactions.

Do not filter using spin columns (e.g., Centricon) because soluble CD1d-β2m binds to membranes.

16. Determine protein content using a Micro BCA protein assay kit according to manufacturer’s instructions.

17. Biotinylate 1 mg of pure soluble CD1d-β2m monomer in 10 mM Tris-Cl, pH 8.0, using the BirA enzyme kit following manufacturer’s instructions. Incubate 12 to 16 hr at 30°C.

18. Dialyze using a Slide-A-Lyzer 3000 MWCO against 1 liter PBS, pH 7.4, with five buffer changes.

Full exchange of biotin-containing solution for PBS is essential to remove all free biotin, which would otherwise bind streptavidin used for tetramerization and occupy spots that should normally be occupied by monomeric soluble, biotinylated CD1d-β2m. Thus, the persistence of any biotin will generate heterogeneous CD1d-β2m-streptavidin that are not tetramers, but rather, lower-order complexes.

19. Determine protein content with the Micro BCA protein assay kit as in step 16.

**Prepare streptavidin-PE and streptavidin-APC soluble CD1d-β2m tetramers**

20. Incubate desired volume of biotinylated soluble CD1d-β2m monomer with 10 µM αGalCer (or other glycolipids of choice per glycolipid-specific protocol) for 16 hr at room temperature, and then an additional 2 to 4 hr at 37°C.

The authors find it useful to add 0.05% (w/v) sodium azide to purified soluble CD1d-β2m monomers to prevent potential bacterial contaminant growth during incubation at 37°C and long-term storage.

Glycolipid-loaded CD1d can be stored at −80°C for extended periods of time. However, do not subject to freeze-thaw cycles, as CD1d-β2m complexes will precipitate.

21. To each 100-µg aliquot of glycolipid-loaded soluble CD1d-β2m monomer, add 75 µg streptavidin-APC or 140 µg of streptavidin-PE to achieve a 4:1 molar ratio necessary for tetramerization.

The molecular mass of four molecules of CD1d-β2m is ~200 kD, while streptavidin-APC and streptavidin-PE are 168 kD and 310 kD, respectively. Best results are obtained when CD1d-β2m monomers are mixed with streptavidin in a molar ratio of 4:1 to 5:1.

22. Allow complexing to occur for at least 12 hr at 4°C prior to experimental use.

23. Test prepared tetramers using C57BL/6 thymocytes and splenocytes per Basic Protocol 1.

For studies involving kinetics of CD1d-tetramer/TCR interactions, further purify CD1d-tetramer by ion-exchange chromatography to ensure high purity of the preparation.
LIPID LOADING ON CD1d AT THE CELL SURFACE OF ANTIGEN-PRESENTING CELLS

In mice, CD1d is expressed by CD4+8+ thymocytes, hepatocytes, B lymphocytes, macrophages, and DC (Mandal et al., 1998). Therefore, each of these cell types has the potential to present lipids and activate iNKT cells. This protocol describes lipid-antigen loading on the CD1d+ APC. Lipid-loaded CD1d+ cells can be adoptively transferred into recipient mice and used to probe iNKT cell functions in vivo (Fujii et al., 2002; Bezbradica et al., 2005b; see Basic Protocol 2). Alternatively, CD1d+ cells can be used to probe iNKT cell functions in in vitro co-cultures with either primary iNKT cells (see Basic Protocol 3) or NKT hybridomas (see Basic Protocol 4). The same protocol can be used for lipid loading on CD1d+ cell lines.

NOTE: All solutions and equipment coming in contact with cells must be sterile, and proper aseptic techniques are essential if cell culture is intended.

NOTE: All culture incubations should be performed in a 37°C, 5% CO2 humidified incubator, unless otherwise specified.

Materials

MACS-purified mouse CD1d+ APC (e.g., DC, B cells, macrophages: UNITS 3.5 & 3.7) or CD1d+ cell lines
Complete RPMI-1640 (APPENDIX 2A) supplemented with 10% FBS, room temperature and 37°C
αGalCer (obtained for research purposes from Kirin Brewery or Axxora) or other antigenic lipids (as available)
Vehicle (see recipe)
PBS (APPENDIX 2A)
Complete OPTI-MEM medium (see recipe)
Low-binding 96-well flat-bottom polystyrene microtiter plates with lids or 5-ml capped FACS tubes
80°C water bath sonicator with heater
1-ml syringes fitted with 25- or 30-G needles
15-ml polypropylene tubes
Additional reagents and equipment for cell sorting (UNITS 3.5 & 3.7)

1. MACS purify (UNITS 3.5 & 3.7) APC of interest from freshly sacrificed animals. Collect positive fractions of sorted DC, B cells, and macrophages determined by CD11c-, B220-, and CD11b-coated magnetic beads, respectively.

2. Resuspend APC at 1 × 10^7 cells/ml in complete RPMI-1640/10% FBS and pipet 100 µl (1 × 10^6 cells) in each well of a 96-well microtiter plates. Keep cells in incubator while preparing the antigen.

   Because DC and macrophages are highly adherent cells, use low-binding microtiter plates for lipid loading. Alternatively, for larger cell numbers (e.g., B cells) load 0.5 ml cell suspension per 5 ml FACS tubes.

3. Prepare a primary stock of αGalCer at 200 µg/ml in vehicle. Sonicate primary stock 10 min in an 80°C water bath sonicator.

4. From primary stock, make 2 × αGalCer working solution at 200 ng/ml in pre-warmed (37°C) complete RPMI-1640/10% FBS. For control wells, add same volume of vehicle to pre-warmed (37°C) complete RPMI-1640/10% FBS.
Other lipid antigens can be loaded onto CD1d using this protocol. Note that other lipids may require other vehicles (e.g., DMSO). It is important that the vehicle is neither toxic nor antigenic to iNKT cells. Thus, in each experiment, control wells should contain the same number of cells cultured with the same concentration of vehicle alone.

Some lipids might require the presence of detergent (e.g., 0.01% to 0.05% Triton-X100) or lipid transfer proteins (e.g., saposins, GM1 activator) for optimal loading of CD1d (see Critical Parameters and Troubleshooting).

5. Add 100 µl of 2× αGalCer or vehicle working solution to the cell culture from step 2. Cover and incubate 18 hr.

Aim to keep a final culture volume of 200 µl/well of the 96-well microtiter plates or 0.5 ml for FACS tubes.

Alternatively, the kinetics of lipid loading can be faster if higher concentrations are used (e.g., 1 µg/ml), but is not recommended. Freshly sorted CD1d+ APC can be stressed and stress may activate cellular lipid metabolism leading to enhanced expression of endogenous antigens on the cell surface and undesired activation of iNKT cells.

6. Wash the antigen-pulsed cells four to six times with prewarmed (37°C) complete RPMI-1640/10%FBS to remove free αGalCer.

7. Resuspend cells in appropriate medium (see below) and immediately use for in vivo or in vitro iNKT cell stimulation assays.

   a. For in vivo iNKT cell stimulation: Resuspend αGalCer- or vehicle-pulsed APC in 100 µl PBS and transfer i.v. into recipient mice. Proceed with Basic Protocol 2, step 4.

      For optimal results, transfer ∼6 × 10^5 DC or 25 × 10^6 B cells per recipient and measure iNKT cell responses 2 and 24 hr later, respectively. See Critical Parameters and Troubleshooting for details.

   b. For in vitro iNKT cells activation: Resuspend αGalCer- or vehicle-pulsed APC in 100 µl complete RPMI 1640/10% FBS and add to 100 µl responder cell suspension. Use 1:1 to 1:100 stimulator/responder ratios. Using 1:100 for DC and 1:50 for B cells is recommended. Proceed with Basic Protocol 3, step 3.

      Responder culture should be MACS-depleted of CD1d+. When working with highly enriched iNKT cells, reduce recommended stimulator/responder ratios by ten-fold.

   c. For stimulation of NKT hybridomas: Resuspend αGalCer- or vehicle-pulsed APC in 100 µl complete OPTI–MEM medium and add to 100 µl responder cell suspension at 10:1 stimulator/responder ratio. Proceed with Basic Protocol 4, step 3.

**SUPPORT PROTOCOL 4**

**LIPID LOADING ON PLATE-BOUND SOLUBLE CD1d**

Recombinant soluble CD1d, lacking the transmembrane and cytoplasmic domains, is secreted as a monomer from mammalian (Joyce et al., 1998) or insect cell lines (Benlagha et al., 2000; Matsuda et al., 2000). Monomeric soluble CD1d can be used in functional assays. NKT hybridomas can be successfully activated by the cell-free lipid antigen presented by plate-bound CD1d without requiring additional costimulation (Stanic et al., 2003). This assay is routinely used to test CD1d-restriction and NKT cell receptor reactivity of novel lipid antigens (Mattner et al., 2005). The following protocol describes binding of soluble CD1d to the bottom of ELISA plates and describes conditions for loading of lipid antigens onto plate-bound CD1d.

**NOTE:** All solutions and equipment must be sterile, and proper aseptic techniques are essential if use with lipid-loaded CD1d in cell culture is intended.
Materials

Soluble CD1d monomers (Joyce et al., 1998 and see Support Protocol 2)
PBS (APPENDIX 2A), room temperature and 37°C
5 µg/ml BSA in PBS
αGalCer (obtained for research purposes from Kirin Brewery or Axxora) or other antigenic lipids (as available)
Vehicle (see recipe)
96-well ELISA microtiter plates with lid
80°C water bath sonicator
Sorvall RT7 rotor (or equivalent)

1. Dilute purified, soluble CD1d monomers to 5 µg/ml in PBS and pipet 50 µl in one-half of all wells of a 96-well ELISA microtiter plate. As a negative control, coat the remaining half with 5 µg/ml BSA in PBS.

   CD1d preparation should be sodium azide–free, because sodium azide impairs subsequent cell growth and responsiveness to activation.

2. Leave 18 hr at 4°C to allow CD1d and BSA to bind to the plate.

3. Gently wash plate with PBS equilibrated to room temperature to remove unbound CD1d.

   Soluble CD1d does not adhere to the plate as well as mAbs to classical ELISA plates. Therefore, gently invert the plate over a waste receptacle to remove the existing fluid, restore to upright position, then slowly add PBS to each well by placing pipet tip on the wall of the well, being careful not to disturb the monolayer or cross contaminate the wells. Repeat the dump-and-wash procedure two times. Use of a microtiter plate washer for this protocol is not recommended.

   PBS wash buffer should not contain Tween-20, as residual detergent could affect cellular viability and/or responsiveness to stimulation.

4. While washing, make primary stock of 200 µg/ml αGalCer in vehicle. Sonicate primary stock 10 min in an 80°C water bath sonicator.

5. From primary stock, make 10 µM αGalCer working solution in vehicle.

   Other lipid antigens can be loaded onto CD1d using this protocol when prepared as 10 µM solutions. Note that other lipids may require other vehicles (e.g., DMSO). Some lipids might require the presence of other detergents (e.g., 0.01% to 0.05% Triton X-100) or lipid transfer proteins (e.g., saposins, GM2 activator) for optimal loading on CD1d (see Critical Parameters and Troubleshooting).

6. Add 100 µl of 10 µM αGalCer or vehicle working solution to CD1d- or BSA-coated wells and incubate covered for 18 hr at room temperature or 4 to 6 hr at 37°C.

7. Wash the plates gently as described in step 3 four to six times with pre-warmed (37°C) PBS to remove excess lipid.


REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2A; for suppliers, see APPENDIX 5.

Complete Express Five serum-free medium

Express Five serum-free medium (Invitrogen) containing:
20 mM L-glutamine
100 U/ml penicillin

continued
100 µg/ml streptomycin sulfate
Filter through a 0.2-µm filter and store sterile up to 6 months at 4°C

**Complete OPTI-MEM medium**

OPTI-MEM medium (GIBCO) containing:
- 4% (v/v) heat-inactivated (30 min at 56°C) FBS
- 2 mM L-glutamine
- 2.4 mg/ml sodium bicarbonate
- 50 µM 2-mercaptoethanol (*APPENDIX 2A*)
- 100 U/ml penicillin
- 100 µg/ml streptomycin sulfate
Filter through a 0.2-µm filter and store sterile up to 1 month at 4°C

**FACS buffer**

PBS (*APPENDIX 2A*), pH 7.0, containing:
- 2% (v/v) heat-inactivated (30 min at 56°C) FBS
- 0.05% (w/v) sodium azide
Filter through a 0.2-µm filter and store sterile up to 12 months at 4°C

**MACS rinsing buffer**

PBS (*APPENDIX 2A*), pH 7.0, containing:
- 4 ml 0.5 M EDTA (*APPENDIX 2A*), pH 8.0 (final 2 mM)
Filter through a 0.2-µm filter and store sterile up to 12 months at 4°C

**MACS running buffer**

PBS (*APPENDIX 2A*), pH 7.0, containing:
- 4 ml 0.5 M EDTA (*APPENDIX 2A*), pH 8.0 (final 2 mM)
- 2% (v/v) heat-inactivated (30 min at 56°C) FBS
Filter through a 0.2-µm filter and store sterile up to 12 months at 4°C

**Paraformaldehyde, 2% (w/v)**

PBS, pH 7.0, (*APPENDIX 2A*) containing:
- 20 g/liter paraformaldehyde (final 2% w/v)
Heat and stir in fume hood until dissolved
Filter through a 0.2-µm filter and store sterile up to 12 months at 4°C

**CAUTION:** Do not inhale because paraformaldehyde is carcinogenic.

**Vehicle for αGalCer**

PBS (*APPENDIX 2A*), pH 7.0
- 0.05% Tween 20
Filter through a 0.2-µm filter and store sterile up to 1 to 2 months at 4°C

**COMMENTARY**

Human and mouse CD1d-restricted glycolipid antigens and the iNKT cell functions they elicit are highly conserved (Van Kaer, 2005), therefore, making the mouse an excellent animal model for understanding iNKT cell biology in vivo. Mouse iNKT cells are a heterogeneous subset, amongst which the Va14Ja18 TCR α-chain-positive T (iNKT) lymphocytes predominate. iNKT cells express an invariant Va14Ja18 TCR α-chain that predominantly pairs with Vb8.2 β-chain, and sometimes with Vb7 and Vb2 β-chains (Lantz and Bendelac, 1994). iNKT cell ontogeny proceeds through the very same early stages (CD3−4−8− triple-negative, TN1–4) as do the developing conventional T lymphocytes. The commitment towards iNKT lineage occurs at the CD4+CD8+ stage (Gapin et al., 2001; Benlagha et al., 2005; Bezbradica et al., 2005a; Egawa et al., 2005) and relies on invariant TCR interaction with CD1d expressed by CD4+8+ thymocytes (Bendelac, 1995). Positively selected, immature iNKT cells express the invariant TCR and undergo maturation-dependent
expression of NK1.1 and DX5 yielding two precursors (DX5−NK1.1− and DX5+NK1.1+) of mature (DX5+NK1.1+ and DX5−NK1.1+) iNKT cells (Gadue and Stein, 2002). Phenotypic maturation in the thymus is coupled with functional programming of iNKT cells.

iNKT cells are innate lymphocytes that have immunoregulatory properties (reviewed in Bendelac et al., 2001; Van Kaer, 2005). They recognize self (Brigl et al., 2003; Wu et al., 2003; Zhou et al., 2004b) as well as foreign (Fischer et al., 2004; Kinjo et al., 2005; Mattner et al., 2005; Sriram et al., 2005) lipid antigens presented by MHC-like, CD1d molecules. When activated in vivo, they secrete both Th1- and Th2-type cytokines (Van Kaer, 2005), therefore, can regulate a variety of immune responses ranging from immunosuppression to immunoagression. Activated iNKT cells can prevent autoimmune diseases, maintain immune privilege, and support engraftment of transplanted tissues. Furthermore, iNKT cells can mediate adjuvant activities and consequently enhance tumor immunity and immune responses to pathogens (Van Kaer, 2005). Developing experimental models to understand when and how iNKT cells develop and acquire their unique immunoregulatory function, as well as how this function is regulated in vivo, is essential for understanding iNKT cell biology and rational design of iNKT cell–based immunotherapies.

There are at most ∼106 iNKT cells in each of the mouse lymphoid organs. Low iNKT cell numbers and shared surface phenotype with other immune cells (e.g., NK cells, CD8+ T cells and CD4+ T cells) made early analyses of this rare population extremely challenging and somewhat nonspecific. A major breakthrough in identifying iNKT cells as a distinct population and understanding their biology was the discovery of the unique CD1d-restricted reactivity to the synthetic glycolipid αGalCer (Kawano et al., 1997; Burdin et al., 1998) and the subsequent generation of fluorochrome-conjugated αGalCer-loaded CD1d-tetramer (Benlagha et al., 2000; Matsuda et al., 2000). The recent discovery of natural endogenous (Brigl et al., 2003; Wu et al., 2003; Zhou et al., 2004b), as well as foreign (Fischer et al., 2004; Kinjo et al., 2005; Mattner et al., 2005; Sriram et al., 2005), lipid antigens will hopefully facilitate further improvement of the techniques described in this unit and lead to better understanding of the biological significance of iNKT cells.

Critical Parameters and Troubleshooting

Major adaptations of classical immunological methods are needed to make studies of iNKT cell biology feasible. Thus, critical points to consider have already been emphasized in the individual protocols in this unit, some of which are summarized below.

Identification, quantification, and phenotyping of iNKT cells by flow cytometry

Because there are very few iNKT cells in lymphoid organs, identification of this cell population is challenging and requires careful prevention of nonspecific background staining and exclusion of CD1d-tetramer-negative cells during analysis. To enhance specificity and to identify the maximal number of CD1d-tetramer-positive cells, especially when working with genetically altered animals with reduced iNKT cell numbers, the authors highly recommend: (1) MACS enrichment of live iNKT cells prior to analysis, and (2) six- or more color flow cytometric analysis. This will allow sorting out and excluding major non-iNKT cell populations and will significantly enhance both sensitivity and specificity of iNKT cell detection. As iNKT cells are very prone to apoptosis, all experiments should be done immediately after collecting lymphoid organs from freshly sacrificed animals.

iNKT cell functional responses to activation in vivo

Tracking iNKT cell activation in vivo requires a combination of two methods: flow cytometry and ELISA. As a more specific assay, the authors recommend flow cytometric detection of early iNKT cell activation. At 6 hr post-activation, iNKT cells downregulate the TCR, therefore, becoming undetectable for further direct flow cytometric analyses. Other immune cells, however, e.g., B cells, DC, T cells, or NK cells, are transactivated by this time (see Table 14.13.2). The authors recommend using this feature as an indirect measure of iNKT cell activation. Transactivated cells upregulate co-stimulatory molecules and early activation markers (e.g., CD69) and/or secrete cytokines, which can be detected by extra- and intracellular flow cytometry and ELISA. It is essential, when using indirect assays, to use mice deficient in glycolipid antigen presentation and/or iNKT cells, e.g., CD1d10/0, HxB10/0, or Ja1810/0 mice as negative controls. This will dissect specific, iNKT cell mediated–immune responses from non-iNKT cell–dependent immune reactions.
iNKT cell functional responses to activation in vitro

iNKT cell activation–induced proliferation can be measured in vitro by CFSE-dye dilution assay. As iNKT cells are very sensitive to toxic effects of CFSE, the following adaptation of a protocol described in UNIT 4.9 is recommended for success of this experiment. Label 5–10 × 10^6 enriched iNKT cells in 1 ml of 1 µM CFSE working solution, freshly prepared from CFSE stock. Labeling should be performed in a 15-ml conical tube for exactly 8 min at room temperature while rocking gently protected from light. Block reaction with 1 ml ice-cold PBS/50% FBS and 12 ml RPMI-1640/10% FBS. Wash two times with ice-cold RPMI-1640/10% FBS and immediately put iNKT cells in culture containing either antigen or iNKT cell growth factors (e.g., 10 ng/ml recombinant human IL-7 and 100 ng/ml IL-15). Every 24 hr, collect small aliquots of culture supernatant before day 5 and analyze for secreted cytokines by ELISA. The CFSE dye dilution assay, however, can be done only after 4 to 5 days because iNKT cells are not detectable at earlier time points due to activation-induced TCR down-modulation.

Maintenance and functional use of NKT cell hybridomas

An NKT hybridoma viability of 95% to 100% is required for success of the assay described in Basic Protocol 4, therefore, (1) do not use hybridomas after they have been cultured for >6 weeks; (2) as NKT hybridomas grow in suspension and divide very rapidly, split them in 1:5 ratio; and (3) cells should not be overgrown at any time or split on the day of the experiment.

Different CD1d^+ cells have distinct CD1d surface expression levels. To avoid saturation of NKT hybridomas while performing functional assays with different CD1d^+ stimulators, setting multiple cultures containing different stimulator/responder ratios is highly recommended.

Enrichment of iNKT cells from mouse lymphoid organs using magnetic cell separation

iNKT cells can be enriched through depletion of non-iNKT cells. As their phenotype partially overlap with the phenotype of other immune cells, it is currently not feasible, using the depletion strategy, to obtain pure iNKT cell populations. Notwithstanding that, enrichment of iNKT cells will enhance sensitivity of other assays and will allow detection of very low functional responses to certain lipid antigens.

A freshly enriched iNKT cell population will not remain viable for longer than a few hours. Thus, resuspend cells in RPMI1640/10% FBS containing either antigen or iNKT cell growth factors (e.g., 10 ng/ml recombinant human IL-7 and 100 ng/ml IL-15) and use them immediately or place cells in a 37°C, 5% CO_2 humidified incubator. Note that iNKT cells could be enriched up to 80% from TCRαβ^+; Va14^+ mice (Bendelac et al., 1996). These mice do not develop conventional T cells due to the lack of TCR α-chain but develop up to 20-fold higher number of iNKT cells due to existing rearranged iNKT cell receptor. However, Va14ja18 TCR transgenic iNKT cells, owing to the timing of their TCR expression during ontogeny, might have altered functional behavior compared to wild-type iNKT cells.

Preparation of CD1d-tetramers

The production of CD1d monomers in sufficient amounts for tetramer preparation using the insect cell system may take up to several weeks. Improper handling of High Five cells at any point during the culture process can alter their phenotype and cease high-level expression. Thus, it is essential to perform a regular control of CD1d expression level and integrity in collected supernatants. Take ~5 to 10 ml of collected supernatants every 2 to 4 days and purify His-tagged CD1d using Ni-chelating Sepharose beads. Separate proteins using 15% SDS-PAGE and analyze using Coomassie-based staining. CD1d needs to be >95% pure with heavy chain/β2m ratio of ~1:1 for successful tetramerization. To prevent CD1d degradation, it may be useful to add protease inhibitors to the collected High Five culture supernatants containing CD1d. A combination of 0.1 mM AEBSF, 1 µM phosphoramidon, 1 µM phosphoramidase, 1 µM pepstatin A, 1 µM bestatin, and 1 µM E-64 (Sigma) is recommended to inhibit proteases in the harvested supernatants.

Lipid loading on CD1d at the cell surface of APC

Lipid-loaded CD1d^+ APC can be adoptively transferred into recipient mice to probe the roles of different APC in eliciting immune responses to lipid antigens. Thus, DC from different donors loaded with different lipids can be transferred into recipient mice conditionally depleted of DC (Bezbradica et al., 2005b). hDTRtg mice, which lack a functional endogenous diphtheria toxin (DT) receptor (DTR), express human DTR-GFP (green fluorescent protein) under the control of the murine
CD11c enhancer promoter cassette that restricts CD11c expression to DC (Jung et al., 2002). DT administration (4 ng DT in PBS per gram of body weight, i.p.) to hDTR<sup>−</sup> mice selectively depletes DC within 12 hr and maintains a DC-deficient state until 36 hr (Jung et al., 2002). DT is neither toxic to mice lacking the hDTR transgene nor does it affect macrophages, B and T lymphocytes in hDTR<sup>−</sup> and non-transgenic animals (Jung et al., 2002; Bezbradica et al., 2005b). Most importantly, DT treatment does not alter iNKT cell number in hDTR<sup>−</sup> mice (Bezbradica et al., 2005b).

Thus, transferring ~600,000 lipid-loaded DC into recipients treated with DT 24 hr earlier is recommended. DC-mediated iNKT cell responses can be detected in recipients as early as 2 hr post-transfer. Importantly, described specificity of DT is achieved only if animals are maintained as hemizygotes for the transgene. When homozygotes were treated with DT, it was partially toxic for CD8<sup>+</sup> T cells (Jung et al., 2002 and authors’ unpub. observ.) and, in some experiments, for iNKT cells as well (unpub. observ.). Similarly, mice genetically deficient in B cells (μMT mice; Kitamura et al., 1991) can be used to probe the roles of B cell roles in iNKT cell activation in vivo (Bezbradica et al., 2005b). When transferring B cells, use ~25–30 x 10<sup>6</sup> B cells per recipient and allow ~24 hr for B cells to home peripheral organs. To eliminate interference of endogenous B cells, use mice genetically deficient in B cells (μMT mice) as recipients.

**Lipid loading on plate-bound CD1d**

NKT hybridomas can be successfully activated by cell-free lipid antigen presentation by plate-bound CD1d without requiring additional costimulation (Benlagha et al., 2000; Stanic et al., 2003). Although αGalCer efficiently loads onto plate-bound CD1d when dissolved in vehicle, other lipids may not. Thus, use 0.01% to 0.05% Triton X-100 in PBS as an alternative vehicle. When doing so, it is important to wash away all Triton X-100 before adding hybridomas, as the detergent may disrupt the cell membrane and cause cell death.

Saposins are endosomal lipid transfer proteins, and their presence may be required in culture for efficient loading of the lipids generated in the endosomal/lysosomal compartment (Zhou et al., 2004a). Thus, the addition of 5 µg/ml of individually purified saposins (saposin A, B, C, and D) is recommended during the lipid loading step in Support Protocol 4 when loading iGb3 or other endosomal lipids onto CD1d.

**Anticipated Results**

By using a combination of subset enrichment protocols and six-color flow cytometry in which at least ~1 x 10<sup>6</sup> events are collected, as many as 14,000 iNKT cells can be identified (Stanic et al., 2004). This is important in studies that utilize genetically altered animals that develop extremely low iNKT cell numbers. iNKT cell function can be tested in vitro with as few as 250,000 splenocytes. Note that iNKT cells isolated from some genetically altered animals might be nonfunctional or dysfunctional. This could impact the feasibility of some experiments described in this unit, however, needs to be empirically determined. From a highly productive High Five cell culture, ~1 mg pure CD1d can be isolated from 1 liter of supernatant, although results may vary.

**Time Considerations**

The analysis of iNKT cells by flow cytometry requires ~4 to 6 hr, as will iNKT cell enrichment from freshly isolated mouse organs. iNKT cell activation in vitro takes up to 5 days, while activation of hybridoma followed by ELISA requires 3 days. Lipid loading onto soluble plate-bound CD1d lasts ~1 day. The most time-consuming protocol in this unit is the generation of soluble CD1d and the preparation of CD1d-tetramers. Depending on how much CD1d is needed, this process might take 2 to 5 weeks.

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