

Generation of Human iNKT Cell Lines

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[Abstract] Natural killer T (NKT) cells comprise an important immunoregulatory T cell subset and express cell surface proteins characteristic of both natural killer cells and T cells. Invariant NKT (iNKT) cells are activated by lipid antigen presented in the context of CD1d molecules, in contrast to classic T cell subsets, which recognize peptide antigens presented by MHC molecules. Following activation, iNKT cells rapidly secrete large amounts of cytokines and can lyse tumor cells and virally infected cells; however, iNKT cells are reduced in patients with autoimmune disease and cancer. The potential to characterize and investigate the prospective use of iNKT cells for therapeutic purposes has significantly increased with the ability to stimulate and expand human iNKT cells. In this protocol, we describe a method to generate and propagate primary human iNKT cells. Specifically, primary iNKT cells were isolated from human peripheral blood mononuclear cells (PBMC), and then expanded periodically with irradiated α -GalCer loaded autologous immature dendritic cells (DC) in the presence of human IL-2.

Materials and Reagents

1. Blood sample to collect iNKT cells
2. Ficoll-Paque Plus (GE Healthcare Biosciences, catalog number: 17-1440)
3. PE-anti-V α 24J α 18 antibody (6B11) (Biolegend, catalog number: 342904)
4. Anti-CD16/32 antibody (Biolegend, catalog number: 101320)
5. Anti-V α 24 antibody (Beckman Coulter, catalog number: IM2283)
6. Anti-CD3 antibody (Biolegend, catalog number: 300312)
7. Recombinant human GM-CSF (R&D Systems, catalog number: 215-GM)
8. Recombinant human IL-4 (R&D Systems, catalog number: 204-IL)
9. Recombinant human IL-2 (Proleukin) (BD Biosciences, catalog number: 354043)
10. Human dendritic cell isolation kit (human CD14⁺ magnetic microbeads) (MiltenyiBiotec, catalog number: 130-050-201)
11. Mitomycin C (Sigma-Aldrich, catalog number: M4287)

12. Human iNKT cell isolation kit (V α 24J α 18⁺ magnetic microbeads) (MiltenyiBiotec, catalog number: 130-094-842)
13. α -galactosylceramide (α -GalCer, KRN7000) (Enzo Life Sciences, catalog number: BML-SL232)
14. RPMI-1640 medium (Life Technologies, Gibco[®], catalog number: 11875)
15. Non-essential vitamin solution (Life Technologies, Gibco[®], catalog number: 11140-050)
16. MEM Vitamin solution (Life Technologies, Gibco[®], catalog number: 11120-052)
17. Sodium Pyruvate (Life Technologies, Gibco[®], catalog number: 11360-070)
18. 2-mercaptoethanol (Life Technologies, Gibco[®], catalog number: 21985-023)
19. Nalgene Freezing Container (Thermo Fisher Scientific, catalog number: 15-350-50)
20. Antibiotics: penicillin-streptomycin
21. Heat inactivated fetal bovine serum
22. MACS buffer (see Recipes)
23. Complete medium (see Recipes)

Equipment

1. Centrifuge with swing out rotor and capable of 300-700 $\times g$
2. BD LSR II flow cytometer (BD Biosciences)
3. Sterilized Pasteur pipettes
4. 50 ml conical tubes
5. 0.22 μ M filter
6. T-175 flask
7. T-25 flask
8. 37 °C 5% CO₂ incubator

Procedure

1. Collect peripheral blood mononuclear cells (PBMCs). For Ficoll density gradient centrifugation separation of lymphocytes from a buffy coat or leukopheresis pack, first dilute heparinized blood with an equal volume of 1x PBS at room temperature.
2. Add 15 ml of Ficoll (warmed to room temperature) to 50 ml conical tubes. Then slowly overlay 25 ml of the diluted blood mixture on top of the Ficoll. Centrifuge at 400 $\times g$ for 30 min at room temperature with the brake off.
3. Carefully remove the 2-3 ml lymphocyte interface (white ring between the media and Ficoll, please see schematic diagram in Figure 1) with a sterilized Pasteur pipette and transfer to a new 50 ml conical tube.

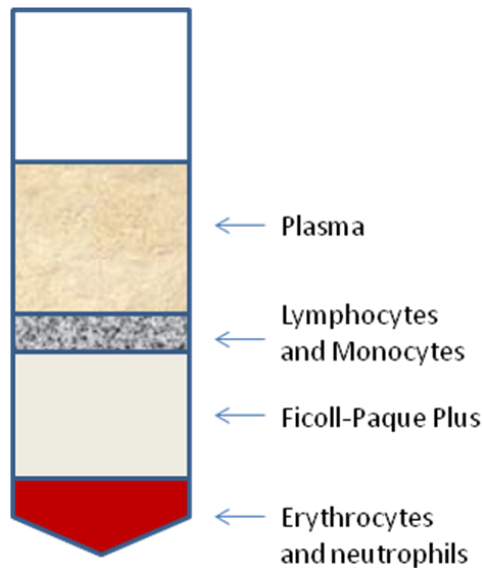


Figure 1. Schematic of Ficoll-Paque Plus separation of human peripheral blood.

4. Wash the cells by resuspending the cell pellets in 5 ml PBS, then combine all of the pellets into one 50 ml tube, and fill the tube with PBS to 50 ml and spin down cells at 400 x g for 10 min at room temperature with the brake on.
5. Discard the supernatant and combine the tubes from a single individual donor to a single tube and wash the PBMCs again with 20 ml PBS. Then count the PBMCs and resuspend at a concentration of 5×10^7 cells/ml in ice-cold MACS buffer.

Note: A leukopak is typically used for these studies and the initial volume of blood is 400-450 ml, but the product has been enriched for leukocytes and the final volume used for Ficoll separation in step 1 is around 40 ml. After the completion of step 5, it is best to continue with the rest of the protocol. If not, the cells should be cultured with complete medium at 10^7 cells/ml in T-175 flask overnight in 37 °C 5% CO₂ incubator, and must be processed into the next step in 24 h.

6. DAY 1- Isolate human CD14⁺ cells: Use human CD14-magnetic microbeads according to the manufacturer's instructions.
 - a. For CD14⁺ PBMCs, culture 10^7 cells with 8 ml complete medium and 20 ng/ml rhGM-CSF and 20 ng/ml rhIL-4 for 4-5 days in T-25 flask to generate immature dendritic cells (DCs). Freeze the extra cells for future use at 10^7 /vial).
 - b. Keep the CD14^{neg} fraction for iNKT cell isolation. In order to maintain the cells, culture in complete medium and 10 U/ml rhIL-2 at 10^7 /ml in a T-175 flask. It is not necessary to replace medium when expanding DC cells.

7. DAY 5-To expand the primary iNKT cells: Collect CD14^{neg} PBMCs and wash with 10 ml complete medium. Use the human iNKT cell isolation kit according to the manufacturer's instructions.
 - a. To isolate V α 24⁺ hNKT cells:
 - i. Collect CD14⁻ PBMC and wash with wash medium once.
 - ii. Resuspend the pellets with 30 ml ice cold MACS buffer and pass through a 70 μ m sterile cell strainer, then spin at 400 x g for 5 min.
 - iii. Resuspend pellets with 1 ml MACS buffer, add anti-V α 24 antibody, 5 μ g/10⁶ positive cells (NKT cells comprise 0.01%-0.5% of the lymphocyte population in PBMC). Incubate on ice for 30 min.
 - iv. Wash cells twice in 30 ml ice cold MACS buffer.
 - v. Resuspend pellets with 1ml MACS buffer, add anti-mouse IgG microbeads 20 μ l/10⁶ positive cells. Incubate on ice for 20 min.
 - vi. Wash cells once in 30 ml ice cold MACS buffer, while centrifuging prepare an MS column by adding 500 μ l MACS buffer.
 - vii. Resuspend the cells in 500 μ l ml MACS buffer. Then pipette the cells into the MS separating column. Make sure to avoid generating bubbles by pipetting slowly. Rinse the column by adding 500 μ l ml MACS buffer. Repeat twice. Add 1 ml fresh MACS buffer and remove column from magnet. Place column into a 15 ml conical tube. Insert plunger and push out contents to obtain purified iNKT cells. Count NKT cell enriched fraction. You should have 0.02-0.5 million cells.
8. In order to expand primary human iNKT cells:
 - a. First, pretreat the immature DCs with mitomycin C. Resuspend immature DCs at 5 x 10⁶ cells/ml in RPMI containing 0.05 mg/ml mitomycin C, incubate at 37 °C in the dark for 30 min, then wash three times with 10 ml complete media.
 - b. Pulse immature DCs by incubating the cells in complete medium (12 well plates/ 1 ml per well) containing 200 ng/ml α -GalCer and 20 U/ml rhIL-2 at 2-3 x 10⁶/ml at 37 °C for 1 h.
9. To the 12 well plates- Add 1 ml in complete medium containing 20 U/ml rhIL-2 V α 24⁺ human iNKT cells to wells containing α -GalCer-loaded DCs (DC:iNKT should be 5:1, for example 2 x 10⁶ DC cells + 0.4 x 10⁶ iNKT cells), mix gently.
10. After 2 days, collect cells with a sterilized serological pipet and transfer into 15 ml tubes. Bring volume to 10 ml with RPMI medium containing 5% FBS. Centrifuge at 300 x g for 10min with braking, discard supernatant, and resuspend cells with complete medium plus 20 U/ml rhIL-2 at 0.4 x 10⁶ cells/ml, and then culture cells in 12-well plate at 3 ml/well.
11. Culture cells for 7-10 days, and replace medium every 2 days as step 9.
12. Flow cytometric analysis of expanding iNKT cells.

Gate on lymphocytes and check purity by flow cytometric analysis, using PE-anti-Vα24Ja18 antibody (6B11) - see Figure 2.

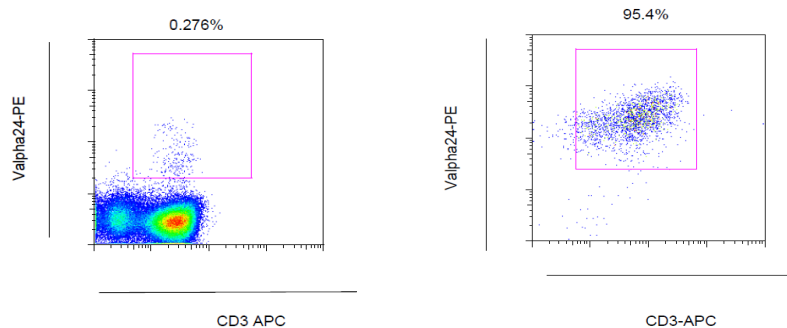


Figure 2. A. iNKT cells among human PBMCs. B. iNKT cell line isolated and expanded from human PBMCs.

Flow cytometry procedure:

- a. Collect 0.1×10^6 cells, and transfer into 1.5 ml tube, and filled with 1 ml FACS buffer (0.2% FBS in PBS).
 - b. Centrifuge cell $600 \times g$ for 5 min, and then discard supernatant.
 - c. Resuspend cells in 50 μ l FACS buffer, and add 0.5 μ l anti-CD16/32 antibody for 15 min to block non-specific binding, and then wash as step a.
 - d. Resuspend cells in 50 μ l FACS buffer, and add 5 μ l PE anti-Vα24 antibody and 5 μ l APC anti-CD3 antibody for 30 min on ice in dark, and then wash as step a.
 - e. Resuspend cells in 200 μ l PBS, and run samples on an LSRII FACS machine.
13. To maintain iNKT cells culture: Culture iNKT cells in complete medium plus 20 U/ml rhIL-2, and every 2-3 days replaced with fresh medium. When the density of viable cells is greater than 2×10^6 cells/ml, the cells should be diluted to 0.4×10^6 cells/ml with complete medium plus 20 U/ml rhIL-2. To prepare stocks of iNKT cells, iNKT cells with ice-cold RPMI medium plus 10%DMSO and 20% FBS at 5×10^6 cells/ml medium/frozen vial, using Nalgene Freezing Container.

Recipes

1. Complete medium
 - RPMI medium
 - 100 mM sodium pyruvate
 - 10 mM non-essential vitamin solution
 - 100 mM MEM Vitamin solution

- 5 x 10⁵ M 2-mercaptoethanol
- 50 U/ml penicillin-streptomycin
- 10% heat inactivated fetal bovine serum
- 2. MACS buffer
 - 1 L PBS free of Ca²⁺ and Mg²⁺
 - 5 g BSA
 - 2 mmol EDTA
 - Sterilized by passing through 0.22 µM filter

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