Chapter 18

Developmental and Functional Assays to Study Murine and Human γδ T Cells

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Abstract

The key roles played by gamma-delta (γδ) T cells in immunity to infection and tumors critically depend on their differentiation into effectors capable of secreting cytokines (such as interferon-γ or interleukin-17), and killing infected or transformed cells. Here we detail the main methods used to investigate the differentiation of γδ T cells from murine or human origin. We describe developmental assays, such as thymic organ cultures (TOCs) and coculture of progenitors cells with OP9-DL1 stomal cells, as well as functional assays typically employed to evaluate γδ T cell cytotoxicity and cytokine production.

Key words γδ T cells, Thymic development, Thymic organ culture (TOC), Effector differentiation, Cytotoxicity, Cytokine production, Intracellular FACS staining

1 Introduction

γδ T cells are multifaceted unconventional T cells that share innate and adaptive features and functions. They are endowed with potent cytotoxicity and produce large amounts of immunomodulatory cytokines, including interferon-gamma (IFN-γ), tumor necrosis factor-alpha (TNF-α), interleukin (IL)-17, IL-10, IL-13, TGF-β, and GM-CSF [1–6]. γδ T cells sense changes in their microenvironment and respond to a variety of stress-inducible or pathogen-associated proteins or metabolites via their T cell receptor (TCR), cytokine receptors and natural cytotoxicity receptors (NCR) [7]. For all these reasons, they have been conceptualized as the first lymphoid line of immune defense [8, 9].

Mouse and human γδ T cells share many developmental and functional properties. However, whereas murine γδ T cells exit the thymus already predetermined to produce either IFN-γ or IL-17 upon activation [2, 10], human γδ thymocytes are immature and selectively acquire their type 1/cytotoxic functions upon stimulation with IL-2 or IL-15 [11]. Infections of individuals leads to peripheral differentiation and circulating γδ T cells are mostly

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mature effector cells, highly prone to produce IFN-γ and TNF-α, and notably cytotoxic against viral-infected and transformed cells [12, 13]. The mechanisms regulating IL-17 production by human γδ T cells lags behind our understanding of the differentiation of mouse IL-17$^+$ γδ T cells. Similarities and differences between mouse and human are important to decipher, since in both species γδ T cells play critical role(s) in many aspects of immune responses including infection, cancer and tissue homeostasis and repair. In particular, given their potent antitumor properties [14] and the recent finding that intra-tumoral γδ T cells are the most significant favorable prognostic immune population [15], γδ T cells are increasingly attractive mediators of cancer immunotherapy [16]. However, such potent cells are also involved in pathogenic situations, often because they are a major source of IL-17 in animal models of autoimmune disorders including collagen-induced arthritis (CIA) and experimental autoimmune encephalomyelitis (EAE), as well as in human psoriasis [17], bacterial meningitis [18], and colon cancer [19]. Therefore, a better understanding of the mechanisms involved in the regulation of their activation and functional differentiation towards IFN-γ- or IL-17-producers would greatly support the design of new clinical protocols to improve γδ T cell-mediated treatments [20].

The absence of genetic tools for selective targeting of γδ T cells in vivo has prevented the manipulation of molecular pathways in this specific cell lineage. Thus, studies on γδ T cell development and functional differentiation depend heavily on in vitro experiments. In this chapter we describe the main in vitro methods used to assess γδ T cell development and effector functions. We detail methods to study the development of γδ T cells from mouse and human thymocytes progenitors in either 3D thymic organ cultures or 2 D co-cultures with a mouse bone marrow stromal cell line (OP9-DL1). In addition, we present methods to assess the main effector functions of γδ T cells, i.e., cytokine production and cytotoxicity (as measured by their ability to kill tumor cell lines), either starting from murine or human material (γδ T cells or their thymic progenitors).

2 Materials

All approaches require basic laboratory equipment including laminar flow hood, table centrifuge, pipettes, and plasticware.

2.1 Sample Preparation anf γδ T Cell Isolation

1. Dissection kit: regular dissection forceps, surgical scissors, needles.
2. Dissection microscope.
3. Flow cytometry activating cell sorter (FACS).
4. Petri dishes.

5. Falcon tubes.
6. 70 μm cell strainers.
7. Nylon mesh.
8. Culture media: RPMI and DMEM completed with 10% fetal calf serum, 1 mM sodium pyruvate, nonessential acids amino acids, 10 mM Hepes, 100 IU/ml penicillin, 100 μg/ml streptomycin, 50 μg/ml gentamycin, 50 μM β-mercaptoethanol.
9. Heparin (stock concentration 5000 IU/ml).
10. Red blood cell lysis buffer.
11. Ficoll solution.
12. 0.8 μM Isopore membranes.
13. MACS TCRγ/δ+T Cell Isolation Kit (Miltenyi Biotec).

2.2 Developmental Assays

1. Bone marrow stroma cell line expressing Notch Ligand Delta-Like 1: OP9-DL1.
2. Falcon tubes.
3. Culture flasks.
4. 96-well plates.
5. 0.25% trypsin solution.
6. Irradiator.

2.3 Functional Assays

1. FACS analyzer.
2. FACS tubes.
3. FACS buffer: PBS, 0.1% (w/v) sodium azide, 1% FCS, 2 mM EDTA.
4. 2 mg/ml phorbol 12-myristate 13-acetate (PMA) stock solution.
5. 2 mg/ml ionomycin stock solution.
6. 2 mg/ml brefeldin A stock solution.
7. CellTrace Far Red DDAO-SE.
8. Recombinant mouse cytokines: IL-1β, IL-23, IL-7.
9. Recombinant human cytokines: IL-2, IL-7, IL-15, Flt3-L.
10. Fc Block solution of anti-Fc Receptor 24G2 rat antibody.
11. 10× Annexin V buffer (eBioscience).
12. Annexin V-FITC.
13. Anti human TCRγδ MicroBead Kit (Miltenyi).
14. Foxp3 eBioscience kit.
15. Murine antibodies: anti-TCRδ (GL3), anti-CD3ε (145-2C11), anti-CD4 (RM4-5), anti-CD8 (53-6.7), anti-CD25 (PC61 5.3), anti-CD28 (37-51), anti-IFNγ (XMG1.2), anti-IL-17A (TC11-18H10; 17B7).

16. Human antibodies: anti-PanTCRγδ (SA6E9), anti-CD3 (UCHT1), anti-CD4 (RPA-T4), anti-CD8 (HIT8a), anti-IFNγ (B27).

3 Methods

Carry out all procedures at room temperature unless otherwise specified.

3.1 Preparation of Single Cell Suspensions of γδ T Cells

All procedures are processed in the laminar flow hood in sterile conditions. γδ T cells are harvested either from thymus (γδ thymocytes) or from secondary lymphoid organs. In both cases manipulation is similar.

3.1.1 Isolation of γδ T Cells from Murine Organs

1. Remove thymus or secondary lymphoid organs. The later include spleen, and the following lymph nodes popliteal, inguinal, axillary, brachial, cervical, mesenteric, periaortic. Process thymus or secondary lymphoid organs separately.
2. Put organs in a 70 μm cell strainer placed in a Petri dish containing complete RPMI.
3. Make single cell suspensions for lymph nodes and spleen separately. Use 1 ml plastic syringe tip to dilacerate the organs with repetitive circular movements (~20×) through the mesh of the strainer.
4. For thymus or lymph nodes filter on a new 70 μm cell strainer to obtain a cell suspension. Centrifuge at 453 × *g* for 5 min. Discard supernatant and resuspend in complete RPMI.
5. For spleen, filter on a new 70 μm cell strainer to obtain a cell suspension. Centrifuge at 453 × *g* for 5 min. Discard supernatant and resuspend in red blood cell lysis (500 μl per spleen). Centrifuge at 453 × *g* for 5 min. Discard supernatant and resuspend in complete RPMI.
6. Mouse γδ thymocytes are isolated from thymus while peripheral γδ T cells are isolated from spleen and lymph nodes, in both cases by FACS as CD3+ TCRδ + cells. Refer to your facility specialist to perform FACS sorting.

3.1.2 Isolation of γδ T Cells from Human Thymus

1. Human thymic specimens are collected when excision of this organ is unavoidable in children undergoing corrective cardiac surgery (*see* **Note 1**). In a sterile Petri dish, cut the organ using sterile #7 forceps and scissors into pieces of ~5 mm^3.
2. Place a nylon cell strainer (70 μm) atop a 50 ml Falcon tube and transfer tissue and media (complete RPMI) directly onto strainer.

3. With the wide tip a plastic plunger (from a 5 ml syringe), gently but firmly press thymic tissue with a circular motion (~30×) through the strainer.
4. Pass 20 ml additional media through the screen while continuing to mash with plunger; this will dislodge any cells caught in the strainer.
5. Centrifuge at 453×*g* for 10 min. Discard supernatant and resuspend in complete RPMI.

3.1.3 Isolation of γδ T Cells from Human Peripheral Blood

1. Collect human peripheral blood on a tube containing heparin (final concentration 50 IU/ml). If using human thymocytes, proceed directly to **step 3**.
2. Gently mix anticoagulated blood with an equal volume of PBS.
3. With a sterile pipet, place 10 ml of the Ficoll solution into a 50 ml Falcon tube.
4. Slowly layer the diluted blood or thymocyte cell suspension over the Ficoll solution by gently pipetting the sample down the side of the tube.
5. Centrifuge 30 min at 500×*g*, RT, without brake.
6. Using a sterile Pasteur pipet, carefully remove the mononuclear cells, located at the interface between the plasma or media (upper layer) and the Ficoll (bottom).
7. Transfer the aspirated mononuclear cells to a 15 ml conical tube. Add 10 ml complete RPMI and mix thoroughly. Centrifuge at 453×*g* for 10 min.
8. Discard the supernatant and repeat wash with complete RPMI.
9. In both cases, (i.e., cell suspension from human blood or thymus), purify γδ T cells using the MACS TCRγ/δ+T Cell Isolation Kit (Miltenyi Biotec), following the manufacturer's recommendations.

3.2 Preparation of γδ T Cells for Thymic Organ Cultures (TOCs)

All procedures should be performed in sterile conditions in a cell culture hood. Wash surgical material (regular dissection #7 forceps and surgical scissors) in 70% ethanol and keep in complete RPMI.

3.2.1 Sample Collection from Murine Embryos

1. Prepare 100-mm sterile petri dishes, each containing 20 ml of culture.
2. Sacrifice timed pregnant mice (at gestational age E14.5 or E15.5: *see* **Note 2**) by CO_2 asphyxiation.
3. Wipe abdomens with 70% ethanol and make an abdominal incision using scissors and forceps, remove fetus-filled uteri.
4. Transfer uteri to an empty petri dish.
5. Using scissors and forceps, remove fetuses from the uteri and transfer them to a new dish containing complete RPMI.

6. Wash out the blood by transferring fetuses to new dishes containing fresh complete RPMI.
7. Place a dissecting microscope in the cell culture hood.
8. Prepare a surgery dish by wetting a sterile compress in a petri dish.
9. Place a fetus in the supine position in a petri dish under the microscope.
10. Gently open the chest (*see* **Note 3**) and locate the two lobes of the thymus.
11. The thymus lobes are removed from the body by raising them with sterile #7 forceps so that the whole lobes are lifted. The isolated lobes are placed in a 1.5 ml eppendorf filled with complete RPMI.

3.2.2 Sample Collection from Human Thymic Biopsies

1. Prepare a petri dish filled with complete RPMI on ice under a dissecting microscope.
2. Cut thymic tissue into 1–2 mm^3 pieces in complete RPMI using sterile #7 forceps and surgical scissors.
3. Collect TOCs in a U-bottom 96-well plate with 150 µl/well of complete RPMI.

3.2.3 TOCs

1. Prepare a 6-well plate with 3 ml/well of complete RPMI.
2. Place 3–4 0.8 µM Isopore membranes (bright side up) in each well of a 6-well plate, floating on the media (*see* **Note 4**).
3. By using forceps, transfer the murine fetal thymuses or human thymic pieces (up to 5/membrane) (*see* **Note 5**).
4. Place the culture plate in a 37 °C, 5 % CO_2 incubator for 7–14 days.
5. Replace half the medium every 3–4 days.
6. Apply a drop of the suspension buffer (100 µl) at the center of the lid of a 30 mm dish.
7. Transfer thymus lobes into the drop with #7 forceps.
8. Place a small (approximately 5 mm × 5 mm) piece of nylon mesh on the drop.
9. Attach 26-gauge needle to 1-ml syringe. Using forceps, bend the tip (top 5 mm, 90° angle) of needles.
10. Gently tease the lobes under the small piece of nylon mesh by softly pressing them with the needle. This procedure will release the thymocytes.
11. Transfer the cell suspension to a plastic tube and determine the cell number. Use the cell suspension for further examination of γδ T cell development by flow cytometry analysis.

3.3 γδ T Cell Differentiation on a OP9-DL1 Monolayer

3.3.1 OP9-DL1 Culture

1. Culture OP9-DL1 cells in a T25 flask in complete DMEM. The cells should be kept in the pre-confluent (up to 80%) condition, by the passage of one-fifth cells every 3 days (*see* **Note 6**).
2. To split the cells, discard culture medium and wash the flask with 4 ml of PBS.
3. Discard the PBS.
4. Trypsinize the cells with 4 ml of 0.25% trypsin solution, and incubate the cells for 5 min at 37 °C.
5. Disaggregate the cells from the plastic by pipetting them up and down, and transfer the cell suspension into a 15 ml Falcon tube containing 6 ml of complete DMEM.
6. Centrifuge the cells at 453 × *g* for 5 min. Discard the supernatant, resuspend the pellet in 10 ml of complete DMEM and transfer one-fifth of the cell suspension into a new T25 flask filled with complete DMEM.

3.3.2 γδ Progenitors/OP9-DL1 Cocultures

1. One day prior to the seeding of progenitors cells, trypsinize and wash the OP9-DL1 cells as mentioned in Subheading 3.3.1, **step 4**.
2. Count cells and resuspend at 2×10^4 cells/ml in a Falcon tube.
3. Irradiate at 1000 rad.
4. Culture 10^4 OP9-DL1 cells in 500 μl of complete DMEM/well of a 48-well plate.
5. The following day (*see* **Note 7**), isolate γδ thymocyte progenitors by flow cytometry, as follows: murine as $CD3^-$ $CD4^-$ $CD8^-$ $\gamma\delta^-$ $CD25^+$ (DN2/3) thymocytes; human as $CD3^-$ $CD4^-$ $CD8^-$ $\gamma\delta^-$ thymocytes.
6. Seed 100,000–200,000 γδ thymocyte progenitors in 500 μl of complete DMEM/well containing the OP9-DL1 monolayers. Add Flt-3 ligand and IL-7 solutions to a final concentration of 5 ng/ml each.
7. Incubate the culture at 5% CO_2 and 37 °C for 7–14 days.
8. Refresh the cocultures by transferring onto freshly prepared OP9-DL1 cells every 4–5 days. Cells can be removed by gentle pipetting and collected by centrifugation at 453 × *g* for 5 min. Use the cell suspension for further examination of γδ T cell development, by flow cytometry analysis.

3.4 Polarization of Mouse IFN-γ-Producing γδ T Cells

1. Prepare a coating solution of anti-CD3ε (145.2C11) plus anti-CD28 mAb (37.51) (both at 2 μg/ml) in PBS. Distribute 50 μl per well of in 96-well plate (round bottom) and incubate at 4 °C overnight (*see* **Note 9**).
2. Remove the coating solution and wash each well with 200 μl of complete RPMI.

3. Add 50,000 (*see* **Note 10**) purified γδ T cells in 200 µl of complete RPMI and incubate at 37 °C for a period of 16–72 h.

3.5 Polarization of Mouse IL-17-Producing γδ T Cells

1. Prepare a cytokine solution of murine IL-1β (100 ng/ml) plus IL-23 (100 ng/ml) in complete RPMI (*see* **Note 11**).
2. Distribute 100 µl of the cytokine solution in 96-well plate (round bottom), then top up with 100 µl containing 50,000 γδ T cells and incubate at 37 °C for a period of 16–72 h.

3.6 Polarization of Human IFN-γ-Producing γδ Thymocytes (See Note 12)

1. Culture isolated γδ T cells at 10^6 cells/ml in complete RPMI with IL-2 or IL-15 (10 ng/ml each) in round bottom 96-well plates during 15–20 days.
2. Monitor cell density and pass cells when necessary (typically one third cells every 3–4 days).

3.7 Cytotoxicity Assay

The culture of target cell line(s) (*see* **Note 13**) should be started at least 1 week prior to the experiment. Monitor cell density and perform regular passage to avoid confluency.

1. Activate mouse or human γδ T cells in the culture condition promoting IFN-γ production, as mentioned above.
2. Count γδ T cells after culture.
3. Wash target cells twice in PBS.
4. Resuspend at 2×10^6 of target cells in 200 µl of PBS (10×10^6/ml). Add 200 µl of 2 µM CellTrace Far Red 7-hydroxy-9H-(1,3-dichloro-9,9-dimethylacridin-2-one-succinimidyl ester, DDAose, diluted in PBS).
5. Incubate for 10 min at 4 °C in the dark.
6. Wash twice in complete RPMI.
7. Count target cells.
8. Mix targets with γδ T cells at defined titrated ratios in complete RPMI, in 96-well plates (round bottom). Keep a control condition without γδ T cells to check for the background of spontaneous death.
9. If required, add anti-CD3 (1 µg/ml) to promote redirected lysis.
10. Incubate at 5 % CO_2 and 37 °C for 4 h.
11. Wash the cells in PBS.
12. Resuspend in 100 µl of Annexin V buffer.
13. Add 2 µl of Annexin V-FITC.
14. Incubate for 15 min at room temperature, in the dark.
15. Without washing, transfer samples into FACS tubes and add 200 µl of Annexin buffer.
16. Analyze by flow cytometry.

4 Notes

1. The human biopsies are from 0- to 5-year-old donors. They can be kept in complete RPMI at 4 °C until processing, up to 3 days post-surgery.
2. Timed pregnancies in mice are achieved as follows: place two females and one male in a cage in the evening (7 p.m.), and separate them in the morning (9 a.m.). Gestational age is designated by assigning the day when mice are separated as E0.5, and is confirmed on the day of the experiment according to the size and developmental features of fetuses. Generally, eight fetuses are expected from a pregnant C57BL/6 mouse.
3. To locate embryonic thymus may require practice. Fetuses and fetal thymuses are easiest to handle at gestational age E15.5.
4. It is important to make sure that: (a) the membrane is floating, (b) the samples are not in contact with each others, and (c) the samples are not covered by a drop of medium.
5. Determination of the signaling pathways participating in γδ T cell development can be assessed and manipulated at this stage by adding blocking antibodies or selective drugs.
6. The OP9-DL1 culture should be started ~1 week prior to initiating cocultures. It is important to keep cells less than 80–90 % confluent. Monitor cell density to avoid exceeding this level of confluency, otherwise cells will stop proliferating and will start differentiating into adipocytes. Although the presence of some adipocytic cells will not affect the culture, large numbers will have a negative impact.
7. OP9-DL1 can be plated at least 6 h before being seeded with isolated progenitors.
8. It is important to choose different fluorochromes to purify gd T cells and detect cytokines.
9. Alternatively, it is possible to coat wells with anti-CD3ε and anti-CD28 Abs by incubating the antibodies solution for at least 4 h at 37 °C. However, for comparable results between experiments it is best to utilize always the same protocol.
10. 20,000 cells/well in 200 μl is the minimum number of γδ T cells to be added.
11. Another alternative to stimulate IL-17-producing γδ T cells is the combination of plate-bound anti-CD3ε plus anti-CD28 antibodies in the presence of 50 ng/ml IL-7 [21].
12. Circulating γδ T cells show a typical type 1/cytotoxic differentiated profile [13].
13. The P815 cell line is commonly used to assess murine γδ T cell cytotoxicity, in the redirected lysis condition. A panel of 20

human γδ T cell-susceptible and -resistant tumor cell lines of hematopoietic origin has been screened and described previously in the following review [20].

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