



Characterization of Mouse $\gamma\delta$ T Cell Subsets in the Setting of Type-2 Immunity

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Abstract

Accumulating evidence indicates that $\gamma\delta$ T cells are a critical component of type-2 immunity. However, the role of these cells in type-2 immune responses seems to be divergent. $\gamma\delta$ T cells are heterogeneous lymphocytes that can be further divided into TCR-V γ/δ definable subsets. Different subsets have distinct and sometimes opposite function during immune responses. In this chapter, we describe the detailed protocol for characterization of $\gamma\delta$ T cell subsets in a mouse model of ovalbumin (OVA)/alum-induced type-2 immunity. Our protocol includes identifying $\gamma\delta$ T cell subsets by flow cytometry, functionally inactivating individual subsets in vivo, purifying $\gamma\delta$ T cell subsets, and using adoptive cell transfer to explore the role of individual subsets in OVA/alum-induced IgE responses.

Key words Type-2 immunity, $\gamma\delta$ T cells, TCR-V γ/δ , Subsets, OVA/alum, IgE, Flow cytometry, Adoptive cell transfer

1 Introduction

Discovered 31 years ago, $\gamma\delta$ T cells are one of the three types of lymphocytes that express clonally distributed antigen receptors [1–3]. Unlike $\alpha\beta$ T cells and B cells whose antigen receptors recognize MHC-peptide complex and intact protein, respectively, $\gamma\delta$ T cells recognize a wide array of antigens including peptides [4], proteins [5, 6], phosphoantigens [6], and lipids [7] through their TCR, which consists of paired single γ and δ chains. The diversity of $\gamma\delta$ TCR repertoire results from the recombination of variable (V), diversity (D), and joining (J) gene segments. This recombination joins one of each V, D, and J segments (δ locus) or V and J segments (γ locus) to generate the variable region of each $\gamma\delta$ TCR [8]. The mouse genome has seven V γ gene segments for which different nomenclatures of these segments are used [9, 10]. Of note, we follow the Heilig and Tonegawa nomenclature in this chapter [9].

$\gamma\delta$ T cells expressing specific V γ segments have distinct developmental kinetics, anatomic location, and function. $\gamma\delta$ T cells can thus be divided into V γ -defined subsets [11]. Some V δ chains are preferentially paired with specific V γ chains. For example, V $\delta 6.3/4$ and V $\delta 6\lambda 12$ are more frequently used by V $\gamma 1^+$ cells than V $\gamma 4^+$ cells. Other V δ chains are shared across several V γ -defined subsets. For example, the usage of V $\delta 4$ and V $\delta 5$ chains is similar between V $\gamma 1^+$ and V $\gamma 4^+$ cells, and V $\gamma 5^+$ cells in the skin and V $\gamma 6^+$ cells in the reproductive track use the same V $\delta 1$ chain. By using monoclonal antibodies against individual TCR-V γ/δ chains (*see* Tables 1 and 2), we are able to identify and purify these subsets for investigating their function in immune responses [12–21].

Type-2 immunity is crucial in host defense against parasitic infection, but it also initiates inappropriate allergic responses. Early evidence indicated that $\gamma\delta$ T cells can contribute to type-2 immune responses in mouse models of parasitic infection [22], allergic airway inflammation [23], as well as allergen-induced IgE production [24]. With the fact that $\gamma\delta$ T cells are heterogeneous and capable of producing both Th1 and Th2 cytokines, it is not surprising to find that the role of $\gamma\delta$ T cells in aforementioned models varies, which might be attributable to functionally distinct subsets [25]. To explore the precise function of individual $\gamma\delta$ T cell subsets during type-2 immune responses, we used the following strategy. Firstly, we examined the changes of $\gamma\delta$ T cell subsets during these immune responses by flow cytometry. Secondly, we used subset knockout mouse models or antibody-induced cell inactivation systems to evaluate the influence of different subsets on type-2 immune responses. Finally, we confirmed the function of each subset by adoptive cell transfer [26–29]. In this chapter, we will focus on OVA/alum-induced IgE responses, in which a single intraperitoneal (i.p.) injection of OVA protein together with the adjuvant aluminum hydroxide (OVA/alum) induces a robust anti-OVA IgE response. The methods of identifying V γ/δ -defined $\gamma\delta$ T cell subsets, antibody-induced cell inactivation, purification of $\gamma\delta$ T cell subsets from mouse spleen and lung, adoptive cell transfer, and detecting OVA-specific IgE response are discussed in detail. These methods can be used to evaluate $\gamma\delta$ T cells in other models of type-2 immunity as well.

2 Materials

2.1 Animals (*See Note 1*)

1. Wild-type C57BL/6 mice (B6).
2. B6.TCR- $\delta^{-/-}$ mice (deficient in all $\gamma\delta$ T cells).
3. B6.TCR-V $\gamma 4^{-/-}$ /V $\gamma 6^{-/-}$ mice (deficient in V $\gamma 4^+$ and V $\gamma 6^+$ cells).
4. B6.TCR-V $\gamma 1^{-/-}$ mice (deficient in V $\gamma 1^+$ cells).

Table 1

Currently used numbering systems for mouse $V\gamma$ genes and monoclonal antibodies that recognize the $V\gamma$ proteins

IMGT	Heilig and Tonegawa [8]	Garman [9]	Monoclonal antibodies		Refs.
			Clone	Comments	
TRGV1	$V\gamma 1$	$V\gamma 1.1$	2.11	Produced by immunizing Armenian hamsters with irradiated $V\gamma 1^+$ 1T3.13.1 hybridoma cells. 2.11 mAb recognizes $V\gamma 1$ -C $\gamma 4$ protein and does not cross-react with $V\gamma 2$, $V\gamma 4$, $V\gamma 5$, $V\gamma 6$, and $V\gamma 7$ chains	[12]
TRGV2	$V\gamma 2$	$V\gamma 1.2$	4B2.9	Generated using the same protocol as with 2.11 mAb, 4B2.9 mAb binds to both $V\gamma 1^+$ and $V\gamma 2^+$ cells	[13]
TRGV3	$V\gamma 3$	$V\gamma 1.3$	–	C $\gamma 3$ is a pseudo gene	
TRGV4	$V\gamma 4$	$V\gamma 2$	UC3-10A6	Produced by immunizing Armenian hamsters with irradiated $V\gamma 4^+$ G8 hybridoma cells. UC3-10A6 mAb recognizes $V\gamma 4$ protein and has no cross-reactivity with $V\gamma 1$, $V\gamma 2$, $V\gamma 5$, $V\gamma 6$, and $V\gamma 7$ chains	[14]
TRGV5	$V\gamma 5$	$V\gamma 3$	536	Produced by immunizing golden Syrian hamster with $V\gamma 5^+$ dendritic dermal cell line 7–17	[15]
TRGV6	$V\gamma 6$	$V\gamma 4$	17D1	An IgM antibody that binds to both $V\gamma 5$ -V $\delta 1^+$ and $V\gamma 6$ -V $\delta 1^+$ cells, the latter happens only when the cells are first reacted with an anti-C δ antibody	[16, 17]
TRGV7	$V\gamma 7$	$V\gamma 5$	GL1.7 or F4.67	F4.67 is an IgG2a mAb specific for $V\gamma 7$ chain	[18]

Table 2

Monoclonal antibodies that recognize individual $V\delta$ chains

$V\delta$ chains	mAbs	Comments	Refs.
V $\delta 4$	GL-2	Hamster IgG	[19]
V $\delta 5$	F45.152	Mouse IgG2a	[18]
V $\delta 6.3/6.4$	17-C	Reacts to V $\delta 6.3$ in C57BL6 and V $\delta 6.4$ in DBA2	[20]
V $\delta 6\lambda 12$	F4.22	Mouse IgG2a	[18]
V $\delta 8$	B20.1	Rat IgG reacts to V $\alpha 2$ chains as well	[21]

5. B6.TCR-V γ 1 transgenic mice (express a specific V γ 1⁺ γ chain).
6. B6.TCR- $\beta^{-/-}$ mice (deficient in all $\alpha\beta$ T cells).
7. B6.TCR- $\beta^{-/-}$ /IFN- $\gamma^{-/-}$ mice (deficient in all $\alpha\beta$ T cells and IFN- γ).

2.2 OVA/Alum Solution and Intraperitoneal Injection

The following solutions should be made in a biosafety cabinet.

1. Saline: 0.9% NaCl.
2. 100 μ g/mL OVA: Dissolve 10 mg of endotoxin-free chicken ovalbumin (OVA) in 10 mL of saline (0.9% NaCl). Sterilize by filtering through a 0.22 μ m syringe filter, and then make a 1:10 dilution to make a final concentration of 100 μ g/mL before use.
3. Aluminum hydroxide (Alum Inject; *see Note 2*).
4. OVA/alum solution: 50 μ g/mL OVA; add equal volume of aluminum hydroxide drop by drop to 100 μ g/mL of OVA. The final concentration of OVA will be 50 μ g/mL. Mix it thoroughly for 20 min at room temperature to achieve a homogeneous solution before intraperitoneal (i.p.) injection.
5. 1 mL plastic syringe and 18-gauge (G) needle.

2.3 OVA Inhalation

1. 1% OVA solution: Dissolve 5 g of endotoxin-free chicken ovalbumin (OVA) in 500 mL of saline (0.9% NaCl). Sterilize by filtering through a 0.22 μ m membrane filter. Aliquot and store at -80°C for up to 12 months.
2. Ultrasonic nebulizer (Lumiscope).
3. Plastic chamber for nebulization.

2.4 Intravenous Injection of Anti-TCR-- γ/δ Monoclonal Antibodies

1. 20 $\times$ buffered salt solution (BSS) stock A: Dextrose 20.0 g, KH_2PO_4 1.2 g, Na_2HPO_4 3.8 g. Add ddH $_2$ O to bring volume to 1 L.
2. 20 $\times$ buffered salt solution (BSS) stock B: $\text{CaCl}_2\cdot 2\text{H}_2\text{O}$ 3.8 g, KCl 8.0 g, NaCl 160.0 g, $\text{MgCl}_2\cdot 6\text{H}_2\text{O}$ 4.0 g, MgSO_4 2.0 g. Add ddH $_2$ O to bring volume to 1 L.
3. 1 $\times$ BSS: Add 50 mL of stock A and 50 mL of stock B to 900 mL of ddH $_2$ O. Sterile by filtration into 200 mL flasks.
4. Anti-TCR-C δ antibody solution: 1 mg/mL unconjugated hamster pan anti-TCR-C δ (clone GL3) in BSS.
5. Anti-V γ 1 antibody solution: 1 mg/mL unconjugated hamster anti-V γ 1 (clone 2.11) in BSS.
6. Anti-V γ 4 antibody solution: 1 mg/mL unconjugated anti-V γ 4 (clone UC3-10A6) in BSS.
7. Isotype antibody solution: 1 mg/mL unconjugated hamster IgG isotype in BSS.
8. 1 mL plastic syringe and 30 G needle.

2.5 Single-Cell Suspension of the Spleen and Lung

1. Scissors.
2. Forceps.
3. Dissection board.
4. Syringe plunger.
5. 35 × 10 mm round petri dish.
6. 70 μ m nylon filter.
7. 5 mL and 10 mL disposable pipette.
8. Pasteur pipette.
9. Portable Pipet-Aid™ pipette controller
10. 15 and 50 mL conical centrifuge tube.
11. 37 °C incubator.
12. 1–10 μ L pipette tips, 20–200 μ L pipette tips, 100–1000 μ L pipette tips.
13. Vacuum aspirator.
14. BSS.
15. Gey's RBC lysis solution.
NH₄Cl 0.83 g.
KHCO₃ 0.1 g.
0.5% phenol red 0.1 mL.
Add ddH₂O to bring volume to 100 mL.
16. 70% ethanol (v/v).
17. Lung digestion solution: 0.125% dispase II (Roche), 0.2% collagenase II (Sigma-Aldrich), and 0.2% collagenase IV (Sigma-Aldrich) in BSS.

2.6 FACS Staining for $\gamma\delta$ T Cell Subsets

1. Staining buffer: 2% FCS and 0.05% sodium azide in 1× PBS.
2. Fixation buffer: 2% PFA in 1× PBS.
3. 96-well round bottom culture plate.
4. FACS machine (LSR II).
5. Fc receptor blocking reagent: In every 100 μ L of staining buffer, add 1 μ L of anti-mouse CD16/CD32 antibody (clone 2.4G2; 1 mg/mL) and 2 μ g of hamster or rat IgG. Fc receptor blocking reagent must be made shortly before use.
6. TCR-specific antibodies.
PE-Cy7-labeled anti-CD3 ϵ (clone 145.2-C11).
PE-Cy5-labeled anti-TCR-C β (clone H57.157).
eFluor450-labeled pan anti-TCR-C δ (clone GL3).
FITC-labeled anti-V γ 1 (clone 2.11).
PE-labeled anti-V γ 1/2 (clone 4B2.9).
APC-labeled anti-V γ 4 (clone UC3-10A6).
Biotinylated anti-V γ 7 (clone GL1.7).
APC-Cy7-conjugated streptavidin.

Biotinylated anti-V δ 4 (clone GL-2).

Anti-V δ 5 (clone F45.152).

Anti-V δ 6.3 (clone 17-C).

Anti-V δ 6 λ 12 (F4.22).

Anti-V δ 8 (clone B20.1).

7. First-step reagent for V γ subsetting: 1:100 dilution of $\gamma\delta$ -eFluor 450, V γ 1-FITC, V γ 1/2-PE, and V γ 7-biotin antibody in staining buffer (*see Note 3*).
8. Second-step reagent V γ subsetting: 1:100 dilution of $\alpha\beta$ -PE-Cy5, CD3 ϵ -PE-Cy7, V γ 4-APC, and APC-Cy7-streptavidin in staining buffer (*see Note 4*).
9. First-step reagent for V δ subsetting: 1:100 dilution of a distinct biotinylated anti-V δ antibody (V δ 4, V δ 5, V δ 6.3, V δ 6 λ 12, V δ 8) in staining buffer.
10. Second-step reagent for V δ subsetting: 1:100 dilution of V γ 1-FITC, V γ 1/2-PE, $\alpha\beta$ -PE-Cy5, CD3 ϵ -PE-Cy7, V γ 4-APC, and APC-Cy7-streptavidin in staining buffer.

2.7 Nylon Wool Enrichment of T Cells and MACS Purification of $\gamma\delta$ T Cells

1. Nylon wool.
2. Phosphate buffered saline (PBS).
3. BSS.
4. 6 mL syringe with cap.
5. 23 G needle.
6. Several rubber stoppers.
7. 37 °C water bath.
8. MS magnetic column (Miltenyi Biotec).
9. MACS separator (Miltenyi Biotec).
10. Pre-Separation Filter (Miltenyi Biotec).
11. Nylon wool incubation buffer: Sterile 5% fetal calf serum (FCS) in BSS.
12. MACS buffer: Sterile 0.5% bovine serum albumin (BSA) in 1 $\times$ PBS.
13. Streptavidin-conjugated magnetic beads (Miltenyi Biotec).
14. Biotinylated anti-TCR-C δ (clone GL3) antibody.
15. Anti-V γ 1 (clone 2.11) antibody.
16. Anti-V γ 4 (clone UC3-10A6) antibody.

2.8 Biotinylation of OVA and ELISA for Detecting Total and OVA-Specific IgE

1. Coating buffer: 1 $\times$ PBS.
2. Blocking buffer: 10% FCS in 1 $\times$ PBS.
3. Washing buffer: 0.05% Tween-20 in 1 $\times$ PBS.
4. TMB solution (BD biosciences).
5. Stop solution: 2 N HCl.

6. Rat anti-mouse IgE coating antibody (clone R35–72, 0.5 mg/mL): Make a 1:250 dilution to a final concentration of 2 μ g/mL before use.
7. Biotinylated rat anti-mouse IgE detecting antibody (clone R35–118, 0.5 mg/mL): Make a 1:250 dilution to a final concentration of 2 μ g/mL before use.
8. Streptavidin-conjugated horseradish peroxidase (HRP).
9. 0.1 M borate buffer (PH 8.4): Used for labeling OVA with biotin.
Stock A: 0.2 M sodium carbonate (21.2 g anhydrous/L)
Stock B: 0.2 M sodium bicarbonate (16.8 g/L)
0.1 M sodium bicarbonate; add stock A to B until the pH value is 8.4 and the final concentration is 0.1 M.
10. Dialysis tubing.
11. *N*-hydroxysuccinimidobiotin: 1 mg/mL in DMSO.
12. Biotinylated OVA: 1 mg/mL biotinylated OVA, 10% FCS in PBS.
13. Biotinylated OVA solution: 5 μ g/mL biotinylated OVA in PBS.
14. VERSAmax tunable microplate reader.
15. SoftMax Pro 4.7.1 software.

3 Methods

3.1 Immunization of Different Mouse Strains with OVA/Alum

1. Bring enough 1 mL plastic syringes, 18-G needles, and fresh OVA/alum solution (50 μ g/mL) to the animal facility. Mix it thoroughly before i.p. injection (*see Note 5*).
2. Fill the syringe with 200 μ L of OVA/alum solution.
3. Gently but securely restrain the mouse with ventral abdomen exposed and head pointed slightly downward. Inject 200 μ L of OVA/alum solution into the caudal abdomen. Pull back on syringe plunger to make sure the needle has not penetrated the bladder or intestines (*see Note 6*). Each mouse will get 10 μ g of OVA in alum.

3.2 Repeatedly Sensitize Mice with OVA Aerosol to Induce OVA-Specific Tolerance

1. Add 1% OVA solution to the container of the ultrasonic nebulizer.
2. Gently place up to eight mice in the plastic chamber used for nebulization (*see Note 7*).
3. Connect the nebulizer to the inlet of the plastic chamber.
4. Turn on the nebulizer, and set the pressure to 5 psi. Allow mice in the chamber exposed to OVA aerosol for 30 min.
5. Repeat this procedure daily, 5 days/week for 2 weeks, and subsequently once per week. Since mice are exposed to

aerosolized OVA for at least 10 consecutive days, they are designated as 10 N treated mice and lose their ability to mount an IgE response upon i.p. OVA/alum injection. These mice will be used as $\gamma\delta$ T cell donors for adoptive cell transfer.

3.3 Intravenous Injection of $\gamma\delta$ T Cell-Specific mAbs to Functionally Inactivate $\gamma\delta$ T Cells or Their Subpopulation

1. Four days before i.p. immunization with OVA/alum solution, use antibody solutions (Subheading 2.4) that are specific for $\gamma\delta$ T cells, V γ 1, or V γ 4 subsets to deplete cells according to the experimental design. For example, to functionally inactivate all $\gamma\delta$ T cells, anti-TCR-C δ antibody solution will be used, and the isotype antibody solution will be injected into a mouse in the same cage to serve as a control. For inactivating V γ 1 subsets, the anti-V γ 1 antibody solution will be used. For inactivating V γ 4 subsets, the anti-V γ 4 antibody solution will be used.
2. Inject 200 μ L of antibody solutions intravenously via the tail vein to the mouse strain of interest.

3.4 Prepare Single-Cell Suspension from the Spleen and Lung

1. Bring precold BSS, 15 mL conical centrifuge tubes, forceps, scissors, and 70% ethanol to the animal facility.
2. Sacrifice the mouse by placing it in a CO₂ euthanasia chamber; observe the animals continuously during the euthanasia procedure. Verify death by lack of cardiac pulse and fixed and dilated pupils. Confirmation of euthanasia by cervical dislocation may be needed in some cases.
3. Wet the mouse with 70% ethanol to reduce the chance of contamination.
4. Place the mouse on a dissection board in a left side lateral position. Make a 1-in.-incision at the left of the peritoneal wall with surgical scissors. Remove the spleen quickly, and transfer it to a 15 mL conical tube filled with cold BSS. Keep on ice until use.
5. Place the mouse in a supine position, and cut open the thoracic cavity with the incision beginning at the xiphoid and extending to the neck with surgical scissors. Perfuse the lung by injecting into the heart with BSS. Sufficient perfusion will result in the loss of RBC and a color change of the lung to white. Transfer lung lobes to a 35 mm petri dish, and chop it into small digestible pieces using a razor blade.
6. Transfer grounded lung tissue into a 50 mL conical tube containing 20 mL of lung digestion buffer. Keep on ice until all samples have been collected.
7. Incubate the 50 mL conical tube with teased lung tissue at 37 °C for 90 min.
8. During the lung digestion incubation, prepare the single-cell suspensions of the spleen by cutting the spleen into small

pieces and transferring tissue to a 70 μm nylon filter placed on the top of a 50 mL conical centrifuge tube.

9. Mash the spleen tissue with the top of a 5 mL syringe plunger while adding the BSS slowly. Then fill the tube with BSS, and centrifuge at $200 \times g$ for 8 min at 4 °C. Aspirate and wash again with BSS followed by resuspension of cell pellet with Gey's RBC lysis buffer.
10. Prepare the single-cell suspension of digested lung tissue by following the same protocol as described above for spleen tissue.
11. Incubate spleen and lung cells in 3 mL of Gey's RBC lysis buffer at room temperature for 5 min to eliminate the remaining RBCs. Fill the tube with 10 mL of cold BSS, and centrifuge at $200 \times g$ for 8 min at 4 °C. Aspirate and wash again with BSS followed by resuspension of cell pellet with appropriate volume of buffer for FACS staining (*see* Subheading 3.5) or nylon wool T cell enrichment (*see* Subheading 3.6).

3.5 FACS Staining of Single-Cell Suspension from the Spleen and Lung

Since some fluorochrome-labeled antibodies are not commercially available, we use a seven-color, two-step staining protocol with the first step using staining reagents containing one homemade biotinylated mAb and the second step using fluorochrome-labeled streptavidin (*see* **Note 8**).

1. Make two-step staining reagents for detecting TCR- γ -defined subsets (*see* Fig. 1; **Notes 3** and **4**).
2. Make two-step staining reagents for detecting TCR- δ -usage in TCR- γ -defined subsets (*see* Fig. 2): In total, you will need six wells to characterize $\gamma\delta$ subsets (1 for V γ and 5 for V δ).
3. Resuspend cell pellet with staining buffer, and adjust the concentration to $5 \times 10^7/\text{mL}$.
4. Plate cells to a 96-well round bottom culture plate (1×10^6 cells, 20 μL per well) according to the experimental design.
5. Add 100 μL of Fc receptor blocking solution per well using a multichannel pipettor, and incubate for 10 min at 4 °C.
6. Centrifuge at $200 \times g$ for 5 min, dump media, and remove excess liquid by blotting on paper towels.
7. Gently vortex the plate, and add 20 μL of staining buffer per well (*see* **Note 9**).
8. Add 80 μL of the first-step reagent per well to make the final volume of 100 μL per well. Incubate in a 4 °C refrigerator for 30 min.
9. Add 100 μL of staining buffer per well, and centrifuge at $200 \times g$ for 5 min. Dump media, and remove excess liquid by blotting on paper towels.

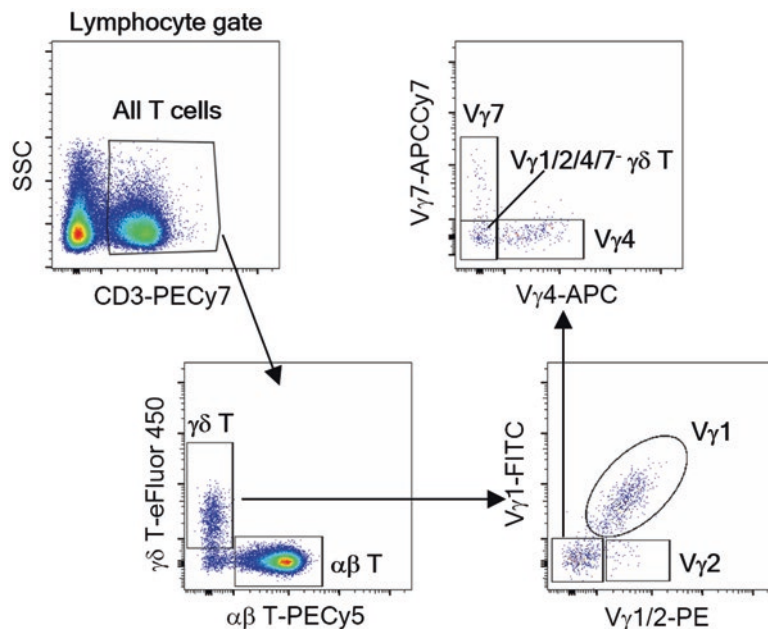


Fig. 1 Gating strategy used for determining TCR- γ -defined subsets. Live lymphocytes from the spleen were plotted as CD3 versus side scatter, and CD3⁺ T cells were gated. Gated T cells were then plotted as $\alpha\beta$ T versus $\gamma\delta$ T, and $\gamma\delta$ T⁺ $\alpha\beta$ T⁻ cells were gated as $\gamma\delta$ T cells for further analysis by plotting as V γ 1/2 versus V γ 1. The double-positive cells were gated as V γ 1⁺ cells and V γ 1/2⁺ V γ 1⁻ single-positive cells as V γ 2⁺ cells. The double-negative cells were therefore gated as V γ 1/2⁻ and were subsequently plotted as V γ 4 versus V γ 7. Single-positive cells were gated as V γ 4⁺ and V γ 7⁺ cells, respectively. The double-negative cells were determined as V γ 1/2/4/7⁻ $\gamma\delta$ T cells

10. Gently vortex the plate, and add 20 μ L of staining buffer per well.
11. Add 80 μ L of the second-step reagent per well to make the final volume of 100 μ L per well. Incubate in a 4 $^{\circ}$ C refrigerator for 30 min.
12. Add 100 μ L of staining buffer per well, and centrifuge at $200 \times g$ for 5 min. Dump media, and remove excess liquid by blotting on paper towels.
13. Gently vortex the plate, and resuspend cells with 200 μ L of fixation buffer (*see Note 10*).
14. Transfer cells to FACS tubes using a multichannel pipettor, and analyze the cells using BD LSR II flow cytometer (*see Note 11*).

3.6 Nylon Wool Enrichment of T Cells

1. Prepare sterile nylon wool columns. Weigh 0.3 g of processed nylon wool, and insert it into a 6 mL syringe with cap. Nylon wool column should be autoclaved and kept sterile before use.

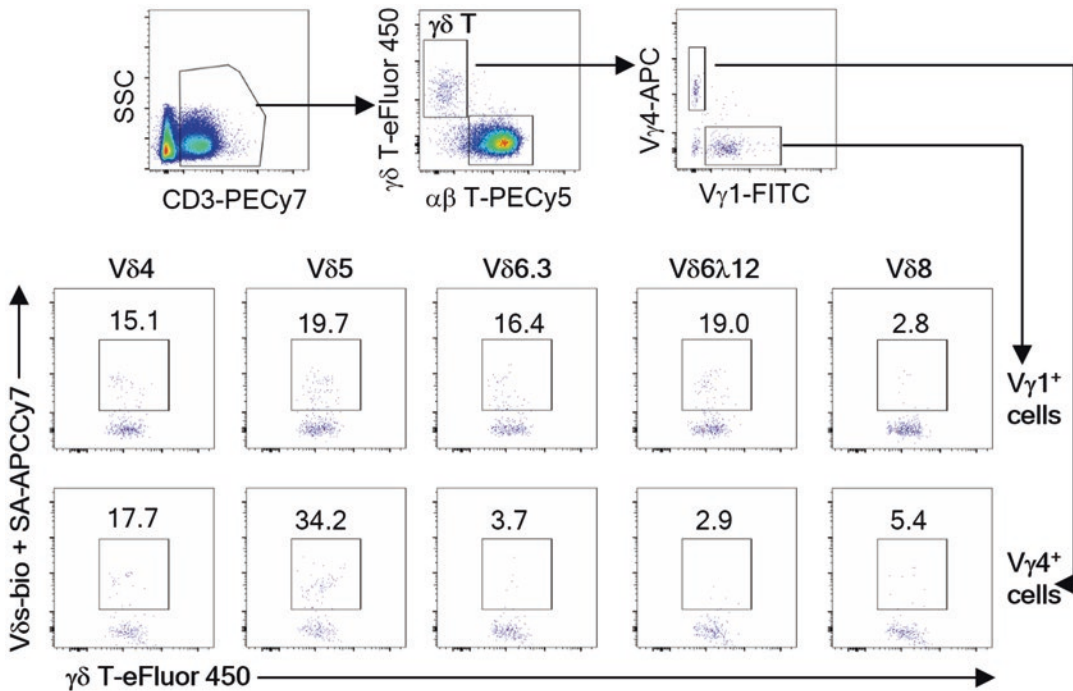


Fig. 2 Gating strategy used for determining TCR- δ -usage in TCR- γ -defined subsets. Splenic $\gamma\delta$ T cells were gated as described in Fig. 1 and then plotted as V γ 1 versus V γ 4. The TCR- δ -usage in V γ 1⁺ and V γ 4⁺ cells (the two most frequent cell $\gamma\delta$ T cell populations in the spleen) were plotted as $\gamma\delta$ T versus V δ 4, V δ 5, V δ 6.3, V δ 6 λ 12, and V δ 8

2. Warm nylon wool incubation buffer in a 37 °C water bath; keep them at 37 °C throughout the procedure.
3. Attach a 23 G needle to a nylon wool column in a biosafety cabinet. Place this column on the top of a 15 mL conical centrifuge tube; equilibrate the column by running 10 mL of BSS through the column. Aspirate the column for approximately 30 s using vacuum to remove any trapped air bubble.
4. Attach rubber stopper to the needle of the column, and add 3 mL of incubation buffer to cover the nylon wool. Incubate the column in a 37 °C, 5% CO₂ humidified incubator for 40 min.
5. Count the cells with a Coulter counter, and adjust the cell concentration to 5–10 × 10⁷/mL with volume of 2 mL.
6. Remove the rubber stopper, and aspirate the column for approximately 30 s using vacuum.
7. Attach the rubber stopper to the needle of the column. Transfer 2 mL of single-cell suspension to the column.
8. Incubate the column in an upright position in a 37 °C, 5% CO₂ humidified incubator for 40 min.

9. Fill the column with 5 mL of incubation buffer, then remove the rubber stopper, and start collecting the effluent cells. Fill the column as needed with 10 mL of incubation buffer.
10. Collect 14 mL of effluent. Centrifuge at $200 \times g$ for 8 min at 4°C , and resuspend cells with MACS buffer.

3.7 MACS Purification of $\gamma\delta$ T Cells or Their Subsets and Adoptive Cell Transfer

1. Count the cells, and adjust the cell concentration to 2×10^7 /mL with cold MACS buffer. Add $1\ \mu\text{g}$ of Fc-blocking reagents followed by incubating in a $2\text{--}8^\circ\text{C}$ refrigerator for 15 min.
2. Add $1\ \mu\text{g}$ of biotinylated anti-TCR-C δ , anti-V γ 1, or anti-V γ 4 mAbs for purifying total $\gamma\delta$ T, V γ 1 $^+$, and V γ 4 $^+$ cells, respectively. Incubate in a $2\text{--}8^\circ\text{C}$ refrigerator for 15 min.
3. Fill the tube with 4 mL of cold MACS buffer, and then centrifuge at $200 \times g$ for 8 min at 4°C .
4. Wash the cells twice with 4 mL of MACS buffer. Resuspend cell pellet in $90\ \mu\text{L}$ of MACS buffer per 10^7 cells.
5. Add $10\ \mu\text{L}$ of streptavidin beads per 10^7 cells. Incubate in a $2\text{--}8^\circ\text{C}$ refrigerator for 15 min.
6. Wash cells twice with 4 mL of MACS buffer. Resuspend cell pellet with MACS buffer at a concentration no more than 2×10^8 /mL.
7. Place MS columns in the MACS separator magnet. Equilibrate each column with $500\ \mu\text{L}$ of MACS buffer.
8. Place a Pre-Separation Filter on the top of each column and a collecting tube under the column. Add cell suspensions onto the filter.
9. Rinse three times by applying $500\ \mu\text{L}$ of MACS buffer each time (*see Note 12*).
10. Place a 5 mL collecting tube in the upright position. Remove the column from the magnet, and place it in the collecting tube.
11. Add 1 mL of MACS buffer. Immediately insert a plunger into the column, and flush out the buffer containing the positively selected cells.
12. Wash cells twice with BSS by centrifuging at $200 \times g$ for 8 min at 4°C . Resuspend the cell pellet with BSS, and determine the purity by staining cells with streptavidin-APC and anti- $\gamma\delta$ TCR-PE (*see Fig. 3*). Of note, cells are usually purified from naive or 10 N treated B6.TCR- $\beta^{-/-}$ mice (*see Subheading 3.2 for preparing 10 N treated mice*).
13. Adjust the concentration to 1.5×10^7 /mL according to the cell number and purity. Bring several 1 mL syringes and 30-G needle to the animal facility. Inject recipient mice with $200\ \mu\text{L}$

of cell suspension per mouse intravenously via the tail vein shortly before the OVA immunization. Each recipient will get 3×10^4 cells. The control mice instead receive 200 μ L of BSS with no cells.

3.8 Biotinylation of OVA

1. Place 1 mg/mL of OVA in PBS in pre-wetted dialysis tubing, and incubate at 4 °C overnight in borate buffer (pH 8.4).
2. Incubate 1 mL of dialyzed OVA with 150 μ L of *N*-hydroxysuccinimidobiotin in DMSO for 4 h at room temperature.
3. Place biotinylated OVA in a fresh dialysis tubing; perform overnight dialysis against PBS at 4 °C.
4. Sterilize the biotinylated OVA by filtering through a 0.22 μ m syringe filter. Store in 10% FCS at -20 °C until use.

3.9 Measure Serum OVA-Specific or Total IgE Antibody by ELISA

1. Harvest sera on day 14 after the i.p. immunization with OVA/alum.
2. Coat the plate with 100 μ L/well of 2 μ g/mL rat anti-mouse IgE coating antibody in coating buffer. Cover the plate, and incubate overnight at 4 °C.
3. Wash the plate six times with 300 μ L/well of washing buffer. Dump media quickly, and remove excess liquid by blotting on paper towels.
4. Block the plate with 200 μ L/well of blocking buffer. Cover the plate, and incubate at room temperature for 30 min.
5. Wash three times, add 100 μ L of serum samples or standards to each well, and incubate at room temperature for 1 h (*see Note 13*).
6. Wash the plate six times. Dump media quickly, and remove excess liquid by blotting on paper towels. Add 100 μ L/well of biotinylated OVA solution or anti-IgE capture antibody solution to the wells followed by incubation for 1 h at room temperature.
7. Repeat the washing step to remove unbound biotinylated proteins. Add 100 μ L of 1:1000 diluted streptavidin-conjugated HRP to each well. Cover the plate, and incubate at room temperature for 30 min.
8. Repeat the washing step. Add 100 μ L/well of TMB substrate solution to each well, and incubate at room temperature in a drawer for 15 min.
9. Repeat the washing step. Add 100 μ L/well of stop solution to stop the reaction. Read the plate immediately using a VERSAmax tunable microplate reader, and process using a SoftMax Pro 4.7.1 software.

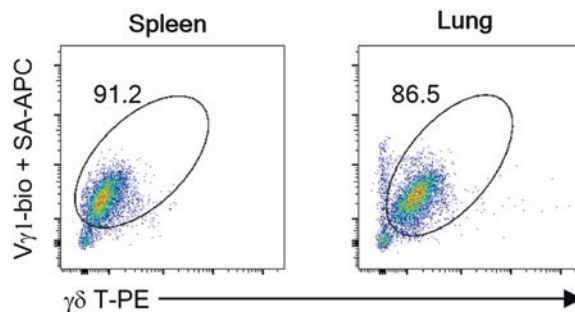


Fig. 3 The purity of $V\gamma 1^+$ cells from the spleen and lung after MACs purification. Single-cell suspensions from the spleen and lung of a B6.TCR $\beta^{-/-}$ mouse were enriched for T cells by passing through the nylon wool column. The nylon wool nonadherent cells were subsequently purified for $V\gamma 1^+$ cells by MACS. Purified cells were then stained with streptavidin-APC and $\gamma\delta$ T-PE. Non-specific staining was observed in lung cells and might be caused by the presence of endogenous biotin

4 Notes

1. Only female mice are used in all experiments at 6–12 weeks of age.
2. Aluminum hydroxide should be shaken well before use.
3. The 2.11 mAb reacts to $V\gamma 1$ chain, whereas 4B2.9 reacts to both $V\gamma 1$ and $V\gamma 2$ chains. The double-positive cells are therefore $V\gamma 1^+$ cells, and the single-positive cells (4B2.9) are $V\gamma 2^+$ cells. These two mAbs have to be added at the same step. Using 2.11 precede 4B2.9 will result in a very dim staining profile of 4B2.9.
4. This combination works pretty well for the spleen. The addition of CD3e-PECy7 and $\alpha\beta$ T-PECy5 can further exclude the contamination of $\alpha\beta$ T cells and thus gives us a better separation of $\gamma\delta$ T cells. However, due to the higher autofluorescence of lung cells, FITC channel should be empty and set as “autofluorescence channel.” Therefore, we switch $\alpha\beta$ T to FITC, $V\gamma 1$ to APC, and $V\gamma 4$ to PECy5 (Percp-eFluor 710) to get a better staining.
5. Calculate the amount of OVA/alum solution required for each experiment; always prepare and bring excess volume of OVA/alum solution to the animal facility. Make sure the solution is homogenous before filled in the syringe. Remove air bubble in a needle by flicking it and squirting a little.
6. To reduce discomfort caused by the injection and the risk of any injection site infection, a new needle should be used for

each mouse. Warming up the OVA/alum solution with your hands will be helpful in preventing the animal from discomfort if the outdoor temperature is too low. After injection, observe the mouse for bleeding or any signs of irritation and pain.

7. Placing more than eight mice in the same chamber will impede the effectiveness of nebulization. Observe the ventilation, and refill 1% OVA to the container if needed.
8. The presence of endogenous biotin in lung tissue might cause some background staining when biotinylated antibodies and fluorochrome-labeled streptavidin are used for staining. Set blank control for each step; single-color and fluorescence minus one (FMO) controls should be run for each experiment to minimize this effect.
9. Stain cells in a 96-well plate make handling multiple samples much easier. However, it will take a while to add different antibodies to multiple wells. To prevent cells from drying out, we recommend adding 20 μ L of staining buffer to each well immediately after aspirating the plate and then keeping it on ice before adding antibodies.
10. Stained cells can be stored in fixation buffer for up to 3 days in the dark at 4 °C.
11. Lung cells tend to clump together, which will cause the formation of doublets and even clog the FACS machine; it's better adding additional 200 μ L of fixation buffer to lung cells and filtering through a 70 μ m nylon filter shortly before analyzing.
12. The effluent contains unlabeled cells that may be useful in some experiments.
13. The standard for OVA-specific IgE is obtained from pooled sera of hyperimmunized BALB/c mice (10 mice), which is arbitrarily assigned to equal 1000 ELISA U. Purified mouse IgE is used for total IgE standard. Make eight two-fold serial dilutions for both standards with OVA-specific sera starting from straight sera and total IgE standard from 500 μ g/mL.

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