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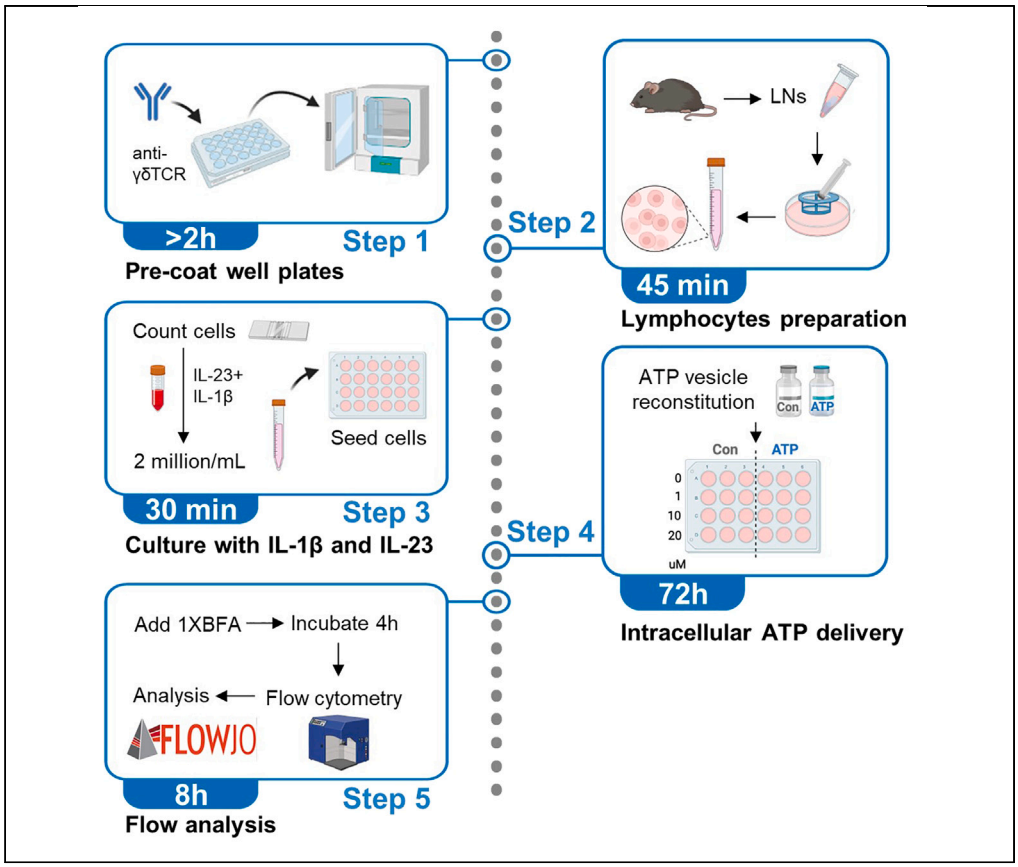
Intracellular ATP delivery to *in vitro* expanded mouse CD27^{-} γδ T cells



Intracellular ATP supports the function of γδT17 cells in mice. Here, we present a protocol for intracellular ATP delivery to *in vitro* expanded mouse CD27^{-} γδ T cells. We describe steps for pre-coating well plates, preparing lymphocytes, culturing CD27^{-} γδ T cells, and ATP delivery. We then detail functional evaluation of γδ T cells by flow cytometry. Appropriate concentrations of control and ATP vesicles are detailed for intracellular ATP delivery, which can also be applied to other immune cells.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Isolation of mouse peripheral lymph nodes

In vitro CD27^{-} γδ T cell expansion with cytokines IL-1β and IL-23

Intracellular ATP delivery to CD27^{-} γδ T cells with ATP vesicles

Functional evaluation of ATP vesicle-treated γδ T cells by flow cytometry

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Intracellular ATP delivery to *in vitro* expanded mouse CD27$^-$ γδ T cells

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SUMMARY

Intracellular ATP supports the function of γδT17 cells in mice. Here, we present a protocol for intracellular ATP delivery to *in vitro* expanded mouse CD27$^-$ γδ T cells. We describe steps for pre-coating well plates, preparing lymphocytes, culturing CD27$^-$ γδ T cells, and ATP delivery. We then detail functional evaluation of γδ T cells by flow cytometry. Appropriate concentrations of control and ATP vesicles are detailed for intracellular ATP delivery, which can also be applied to other immune cells.

For complete details on the use and execution of this protocol, please refer to Wang et al. (2023).[1]

BEFORE YOU BEGIN

This protocol below describes how to expand CD27$^-$ γδ T cells from mouse peripheral lymphocytes and increase cellular ATP levels with ATP vesicles. The low cell number of γδ T cells limits some experimental manipulations. Here, we use anti-γδTCR antibody plus cytokines IL-1β and IL-23 to establish a primary CD27$^-$ γδ T cell line. Anti-γδTCR promotes the proliferation of γδ T cells; meanwhile, IL-1β and IL-23 drive the differentiation of γδT17 cells.[2,3]

Since ATP is cell membrane impermeable, previous studies used 250 U/mL Streptolysin-O (SLO) to make pores in the membrane, and then deliver exogenous ATP through these pores.[4,5] After 4 h of recovery, cell viability returns to 50%–60%. However, SLO treatment would inevitably cause the loss of intracellular components during recovery time. Therefore, we employ ATP vesicles to deliver ATP via membrane fusion.[6,7]

Preparation of complete medium, buffers and operative instruments

Timing: 30 min

1. See "materials and equipment" for preparation of needed materials.
2. Pre-warm the complete RPMI 1640 medium and culture medium in a 37°C water bath incubator.
3. Disinfect scissors and tweezers with 70% ethanol.

Preparation of a sterile culture hood

Timing: 40 min

4. Place sterile PBS, complete RPMI 1640 medium, 15 mL and 50 mL tubes, 24-well plates, cell dishes, cell strainers, 2 mL syringes, scissors and tweezers into a culture hood.
5. Turn on the UV light and sterilize the hood for at least 30 min.

⚠ CRITICAL: All steps except for "Functional evaluation of γδ T cells by flow cytometry" part need to be conducted in a sterile culture hood.

ATP and control vesicle reconstitution

Timing: 20 min

6. Place 10 mL complete RPMI 1640 medium on ice for 10 min.
7. Add 8 mL pre-cooled complete RPMI 1640 medium into ATP or control vesicles respectively and mix well. The final concentrations are both 2 mM.

Note: The lyophilized vesicles are stored at −20°C. Fresh ATP and control vesicles should be reconstituted before use and can be stored at 4°C for up to 2 days.

Note: ATP and control vesicles are highly fusogenic liposomes (Avanti Polar Lipids, Inc). The composition of 2 mM ATP vesicles is 20 mg/mL Soy PC/DOTAP (50:1), Trehalose/Soy PC (2:1), 2 mM KH_2PO_4 and 2 mM Mg-ATP. The detailed manufacturing, compositions and diameters of vesicles are described in previous studies.[6,8]

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-mouse CD3 Percp/Cy5.5 (clone 17A2, 1:500)	BioLegend	Cat#100218
Anti-mouse γδTCR APC (clone GL3, 1:500)	BioLegend	Cat#118116
Anti-mouse CD27 FITC (clone LG.3A10, 1:500)	BioLegend	Cat#124208
Anti-mouse IL-17A PE/Cy7 (clone TC11-18H10.1, 1:500)	BioLegend	Cat#506922
Fixable Viability Dye eFluor 780	eBioscience	Cat#65-0865-14
Anti-mouse γδTCR (clone UC7, working concentration 1 μg/mL)	Home made	N/A
Chemicals, peptides, and recombinant proteins		
Fetal bovine serum (FBS)	Atlanta Biologicals	Cat#SIII50
RPMI 1640	Sigma-Aldrich	Cat#R8758
Dulbecco's phosphate-buffered saline (PBS)	Sigma-Aldrich	Cat#D8537
2-Mercaptoethanol	Gibco	Cat#21985023
Penicillin-Streptomycin solution (100×)	Corning	Cat#30-002-CI
Brefeldin A (1000×)	BioLegend	Cat#420601
Fixation buffer	BioLegend	Cat#420801
Intracellular Staining Permeabilization Wash Buffer (10×)	BioLegend	Cat#421002
Vitasol liposome/control liposome	Avanti Polar Lipids (provided by Dr Sufan Chien from the University of Louisville)	N/A
Recombinant mouse IL-23 (carrier-free, working concentration 10 ng/mL)	BioLegend	Cat# 589004
Recombinant mouse IL-1β (carrier-free, working concentration 10 ng/mL)	BioLegend	Cat# 575104
Experimental models: Organisms/strains		
Mouse: C57BL/6J (6–8 weeks, female or male)	The Jackson Laboratory	JAX:000664

(Continued on next page)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Software and algorithms		
FlowJo	BD Biosciences	https://www.flowjo.com
Other		
40 μm Cell strainers	Sorfa	Cat#251100
44 μm Nylon mesh filter	Component Supply	Cat#U-CMN-44
24-well plate	MIDSCI	Cat#TP92424
15 mL conical centrifuge tubes	Corning	Cat#430052
50 mL conical centrifuge tubes	Corning	Cat#430829
40 × 9 mm Petri dish	Sangon Biotech	Cat#F611001-0001
2 mL syringe	BD	Cat#302204
Hemacytometer, cell counting chamber	Fisher Scientific	Cat#02-671-51B
CO_2 incubator	Thermo Fisher Scientific	N/A
HFsafe-1200LCB2 biosafety cabinet	Heal Force	N/A
BD FACSCanto	BD Biosciences	N/A

MATERIALS AND EQUIPMENT

Complete RPMI 1640 medium		
Reagent	Final concentration	Amount
RPMI 1640	N/A	939 mL
Fetal bovine serum	10%	50 mL
100× Penicillin-Streptomycin solution	Penicillin 100 U/mL Streptomycin 100 μg/mL	10 mL
2-Mercaptoethanol (55 mM)	55 μM	1 mL
Total		**1000 mL**
Complete RPMI 1640 medium is stored at 4°C up to one month.		

Culture medium		
Reagent	Final concentration	Amount
Complete RPMI 1640 medium	N/A	25 mL
rmIL-23 (100 μg/mL)	10 ng/mL	2.50 μL
rmIL-1β (200 μg/mL)	10 ng/mL	1.25 μL
Total		**~25 mL**
Fresh culture medium should be prepared before experiments.		

1× Intracellular staining permeabilization wash buffer (1× wash buffer)		
Reagent	Final concentration	Amount
10× Intracellular staining permeabilization wash buffer	1 X	5 mL
ddH_2O	N/A	45 mL
Total		**50 mL**
1× Intracellular staining permeabilization wash buffer is stored at 4°C up to one month.		

Flow antibody mix preparation

Diluted Viability dye		
Reagent	Channel	Amount
Fixable Viability dye	APC/Cy7	1 μL

(*Continued on next page*)

Continued		
Reagent	Channel	Amount
PBS	N/A	49 μL
Total		50 μL

Diluted Viability dye can be stored at 4°C up to 2 days in the dark.

Mix			
Reagent	Channel	Final concentration	Amount/tube
Diluted Viability dye	APC/Cy7	1:500	0.5 μL
Anti-mouse CD3	Percp/Cy5.5	0.4 μg/mL	0.5 μL
Anti-mouse γδTCR	APC	0.4 μg/mL	0.5 μL
Anti-mouse CD27	FITC	1 μg/mL	0.5 μL
Total			2 μL

Flow antibody mix should be prepared before experiments.

STEP-BY-STEP METHOD DETAILS

Pre-coat well plates with anti-γδTCR mAb

Timing: > 2 h

This section describes how to pre-coat well plates with mouse anti-γδTCR antibody.

1. Prepare antibody mix by adding 25 μL anti-γδTCR antibody (clone UC7, 0.25 mg/mL) into 6.25 mL sterile PBS, and mix well. The final concentration is 1 μg/mL.

   Note: For subsequent experiments, we need to pre-coat 24 wells in total.

2. Add 250 μL antibody mix per well in a 24-well plate (MIDSCI).
3. Place the plate into a 37°C incubator and incubate at least 2 h or overnight.

Isolate mouse peripheral lymph nodes and prepare single-cell suspensions

Timing: 45 min

This section describes how to harvest peripheral lymph nodes from mice and followed by single-cell suspension preparation.

4. Euthanize the mouse (C57BL/6J, 6–8 weeks, female or male) according to the local ethical policy and animal welfare regulations (e.g., CO_2). Spray the mouse with 70% ethanol and fix the animal with dissecting pins in a flat styrofoam.

   Note: For subsequent experiments, we need to harvest lymph nodes from 3–4 mice.

5. Cut the skin longitudinally along the abdominal midline and open the skin along limbs with scissors.

   ⚠ CRITICAL: Keeping the peritoneum intact during processing will help operative field exposure.

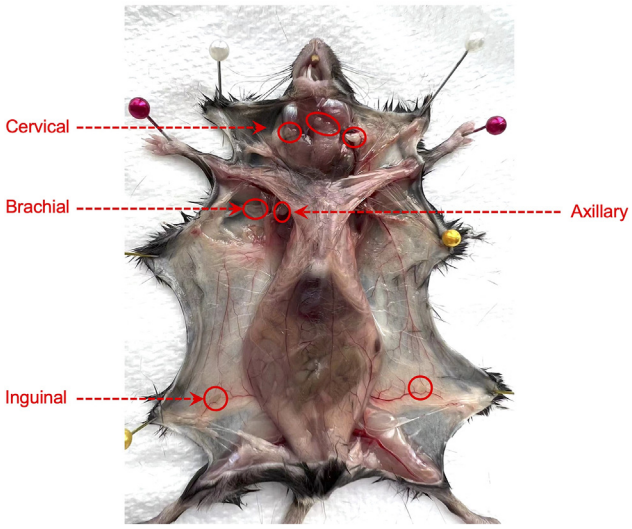

Figure 1. Locations of mouse peripheral cervical, brachial, axillary, and inguinal lymph nodes

6. Harvest the cervical, brachial, axillary and inguinal lymph nodes from the locations shown in Figure 1. Put those peripheral lymph nodes into ice-cold complete RPMI 1640 medium.
7. Place a 40 μm cell strainer in a petri dish and rinse with sterile PBS.
8. Transfer the lymph nodes into the cell strainer and grind with a syringe plunger. Use 5–10 mL RPMI 1640 medium to pass through the strainer and transfer the medium to a 15 mL tube.
9. Centrifuge at 500 × *g* for 5 min at 4°C and discard the supernatant.
10. Add 1 mL complete RPMI 1640 medium to resuspend the pellet and count the cells using hemacytometer.

Note: The estimated cell number of lymphocytes from lymph nodes of each mouse ranges from 15 to 25 million. Different strains of mice, such as BALB/c, may show different yields.

Culture lymphocytes with IL-1β and IL-23 in the pre-coated 24-well plate

Timing: 30 min

This section describes how to resuspend lymphocytes in culture medium which contains rmIL-1β (10 ng/mL) and rmIL-23 (10 ng/mL), and seed cells in the pre-coated 24-well plate.

11. Prepare culture medium as described in "materials and equipment."
12. Centrifuge at 500 × *g* for 5 min at 4°C and discard the supernatant. Adjust the cell concentration to 2 million/mL with the pre-warmed culture medium and mix well.
13. Discard the PBS from the pre-coated 24-well plate.
14. Add 1 mL of the above single cell suspension per well and gently shake the plate to make the cells evenly distributed.

⚠ CRITICAL: The primary CD27$^-$ γδ T cell line can survive for up to 7–8 days.

Intracellular ATP delivery with ATP vesicles

Timing: 3 days

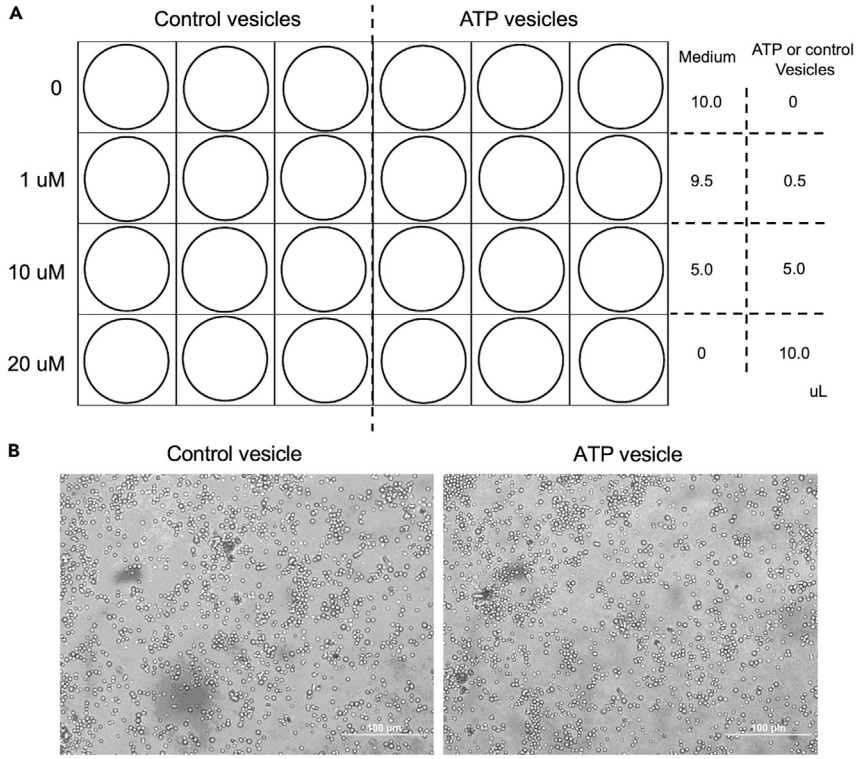

Figure 2. Intracellular ATP delivery to γδ T cells with ATP vesicles

(A) Calculation examples for ATP and control vesicle treatment.

(B) Representative images of γδ T cells treated with ATP and control vesicles under microscope. Scale bar, 100 μm.

This section describes how to reconstitute ATP and control vesicles, and treat lymphocytes with vesicles at appropriate concentrations.

15. Prepare freshly reconstituted ATP and control vesicles as described in "ATP and control vesicle reconstitution" part.

⚠ CRITICAL: After reconstitution with complete RPMI 1640 medium, ATP vesicles need to be used within 2 days. Thus, prepare it freshly before experiments.

16. Add ATP and control vesicles at indicated concentrations (0, 1, 10, 20 μM) and mix well. Example of calculations is shown in Figure 2A.

Note: Vesicles will settle to the bottom of wells and appears like white granules that cover cells (Figure 2B). The cells are bright and round at day 1. Cell aggregations will be formed over 72-h incubation.

17. Culture for 72 h in a 37°C incubator and observe the culture solution daily.

⚠ CRITICAL: The 72-h incubation time is determined with primary γδ T cells. We did not observe any phenotype change under other conditions (24–48 h). Performing incubation time kinetics is necessary for other cell types.

Functional evaluation of γδ T cells by flow cytometry

Timing: 8 h

This section describes how to evaluate IL-17 production from γδ T cells in ATP and Control vesicle treated cells with flow cytometry.

18. Add 1 μL Golgi-plug Brefeldin A (BFA, 1000×) per well to block transport processes and incubate for 4 h.
19. Collect cells from each well into 15 mL conical tubes. Centrifuge at 500 × *g* for 5 min at 4°C and discard the supernatant. Resuspend the cell pellet in 150 μL of PBS.
20. Add 1 μL Fc Blocker per tube, mix well and incubate for 10 min at 4°C.
21. During incubation, prepare diluted Viability dye and Flow antibody mix as described in the "materials and equipment."
22. Add 2 μL antibody mix per tube and incubate for 20 min at 4°C in the dark.
23. Wash by 1 mL PBS per tube. Centrifuge at 500 × *g* for 5 min at 4°C and discard the supernatant.
24. Add 250 μL Fixation Buffer per tube and mix well. Incubate for 20 min at 25°C in the dark.
25. During incubation, prepare 1× intracellular staining permeabilization wash buffer (1× wash buffer) as described in the "materials and equipment."
26. Wash twice with 1 mL 1× wash buffer per tube, centrifuge at 500 × *g* for 5 min at 4°C and discard the supernatant.
27. Resuspend the cell pellet in 150 μL of 1× wash buffer, add 0.5 μL anti-mouse IL-17 PE/Cy7 antibody per tube and mix well.
28. Incubate for more than 2 h at 4°C in the dark.
29. Wash by 1 mL 1× wash buffer per tube, centrifuge at 500 × *g* for 5 min at 4°C and discard the supernatant.
30. Add 300 μL 1× wash buffer and filter cell suspension with a 44 μm nylon mesh before analysis with Canto flow cytometer.
31. See Figure 3A for the gating strategy with FlowJo software.

EXPECTED OUTCOMES

The cell culture system of IL-23, IL-1β plus anti-γδTCR antibody exclusively supports the proliferation of CD27$^-$ γδ T cells. Cytokines IL-23 and IL-1β induce the IL-17 production of γδ T cells.[2] Anti-γδTCR triggers γδ T cell proliferation while constrains its IL-17 production,[9] hence ensuring an appropriate IL-17 baseline for subsequent experiments. The purity of *in vitro* expanded γδ T cells reaches 30%–50% on Day 3, and most of them are CD27$^-$ γδ T cells (Figure 3A). The low percentage of the first FSC-SSC gating is due to the death of other lymphocytes, such as αβT cells and B cells. Compared to Control group (Con), ATP vesicle treatment remarkably enhances the IL-17 production of γδ T cells (Figure 3B).

LIMITATIONS

The concentrations and treatment time of ATP vesicles are titrated with primary mouse γδ T cells. These conditions should be optimized for other cell types. Additionally, compared to SLO-assisted ATP delivery system, the treatment time of ATP vesicles is relatively long. Therefore, our protocol is not suitable for short-term ATP delivery.

TROUBLESHOOTING

Problem 1

Failure to find adequate peripheral lymph nodes (related to step 6).

Potential solution

- Cut the skin from anus to the chin and spread it to open along limbs. Lymph nodes appear to be translucent ashen round or long nodes. Inguinal lymph nodes are the easiest to be found in the place where blood vessels converge. Brachial and axillary lymph nodes are hidden under adipose tissues and muscles. The location of cervical lymph nodes is shown in Figure 1.

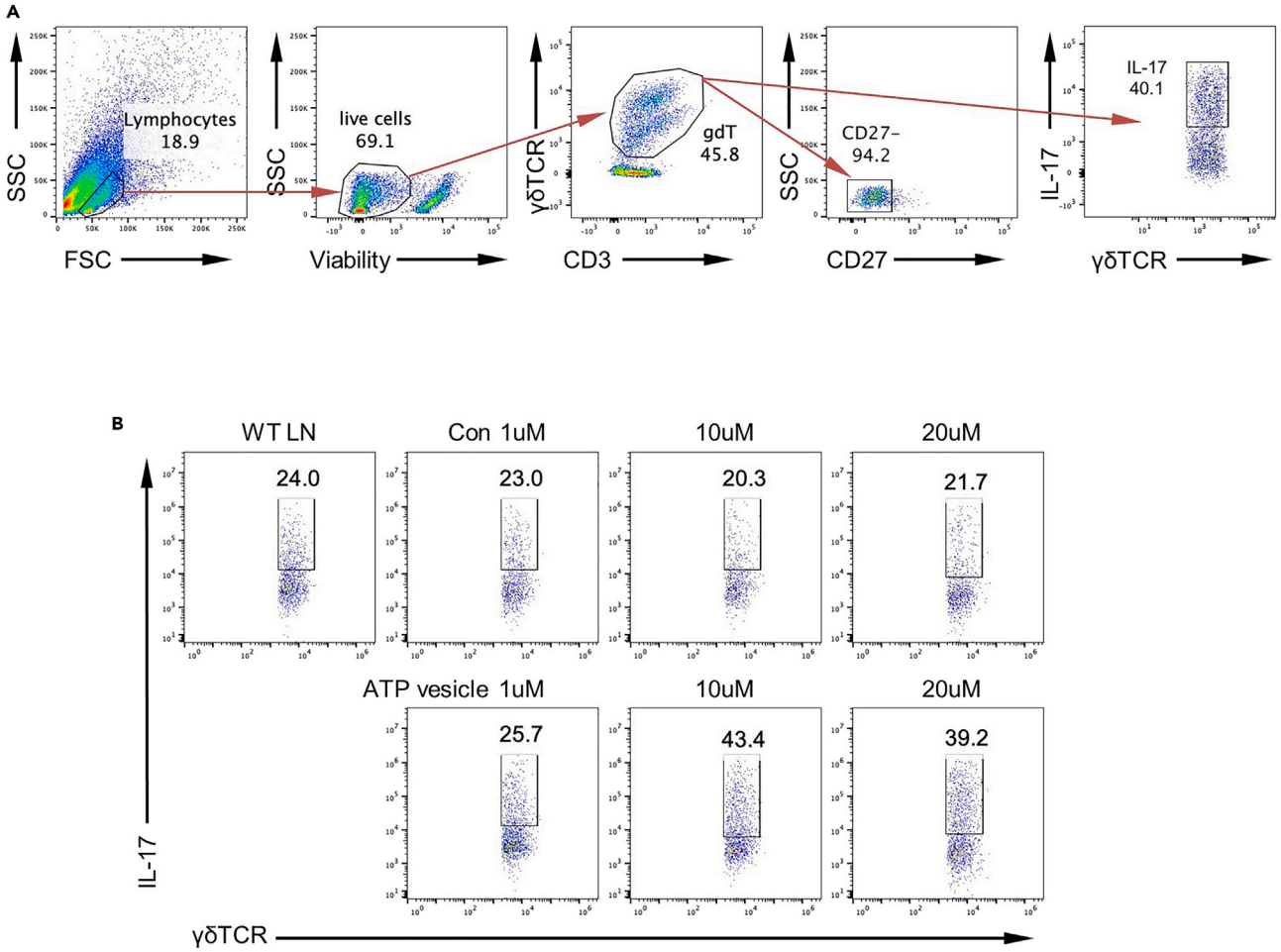

Figure 3. Functional evaluation of γδ T cells by flow cytometry

(A) Representative gating strategy of live $CD3^+CD27^-\gamma\delta TCR^+$ cells and IL-17 production in γδ T cells.

(B) Cells from adult peripheral lymph nodes were cultured with Control (Con) and ATP vesicles at indicated concentrations in the presence of IL-23 and IL-1β for 72 h and incubated with 1× BFA for 4 h. Intracellular IL-17 levels were measured by flow cytometry. Plots were gated on live $CD3^+\gamma\delta TCR^+$ cells. Panel (B) has been reported in the manuscript related to this protocol.[1]

Problem 2

Low purity of γδ T cells on Day 3 (related to "materials and equipment" and step 12).

Potential solution

- The cell purity is influenced by anti-γδTCR signal intensity and cytokine bioactivity. Therefore, we recommend titrating the concentration of anti-γδTCR antibody and using freshly prepared cytokines.
- Cell density is another key factor. If the density of 2 million cells/mL in each well fails to establish a relatively pure γδ T cell line, cell density titration is recommended for individual laboratories.

Problem 3

Low viability of cells after ATP vesicle treatment (related to step 16).

Potential solution

- High concentration of ATP vesicles can reduce cell viability. We recommend using 0–20 μM for mouse T cells. However, concentration titration is still needed for other cell types or other species.

Problem 4

The IL-17 production of γδ T cells is not increased after ATP vesicle treatment (related to step 31).

Potential solution

- First, careful titration of incubation time and cytokine concentration is needed when phenotype is not as expected. Second, make sure the basic level of IL-17 production is not very high (less than 40% γδ T cells). If IL-17 production is too high in control vesicle group, concentration titration of IL-1β and IL-23 is recommended.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Jun Yan (jun.yan@louisville.edu).

Materials availability

This protocol does not generate unique reagents.

Data and code availability

The published article includes all datasets generated or analyzed during this study.

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AUTHOR CONTRIBUTIONS

Funding acquisition and supervision, J.Y.; Writing – original draft, Y.W.; Writing – review and editing, Y.W. and J.Y. All authors have read and approved the article.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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