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Publisher’s note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.
Protocol to assess cell-intrinsic regulatory mechanisms using an ex vivo murine T cell polarization and co-culture system

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SUMMARY
This protocol describes an ex vivo cell culture system for simultaneously generating a mixture of CD4+ T helper lineages, including T helper 17 (Th17), RORγt+ Treg, and conventional Treg (cTreg), in proportions representative of those found in mucosal tissues in vivo. When combined with a co-culture approach, this system allows a more rapid assessment of a candidate molecule’s T cell-intrinsic and -extrinsic functions over the traditional bone marrow chimera and co-transfer approaches. For complete details on the use and execution of this protocol, please refer to Ma et al. (2022).

BEFORE YOU BEGIN
For experiments involving the use of retrovirus, it is important to follow the universal safety precautions outlined in (Coffin et al., 1997), including the use of a class II biological safety cabinet, lab coat, protective sleeves, and double gloves. Decontamination can be carried out with standard detergent or 70% ethanol (Coffin et al., 1997).

The retroviral vectors described in this protocol are detailed in Figure 1. The workflow of this protocol is outlined in Figure 2. Some steps are modified from previous reports (Eremenko et al., 2021; Flaherty and Reynolds, 2015). In this section, we describe the steps for preparing the retroviral constructs, culturing the Plat-E retrovirus packaging cell line, and obtaining the appropriate mice before the start of T cell isolation and culture.

DNA plasmid preparation

© Timing: 2–3 days

This section prepares retroviral constructs encoding expression cassettes for unique cell surface molecules that will be used for distinguishing T-cells from distinct sources in the co-culture assay.

1. Transformation and culturing of E. coli.
   a. 50 μL DH5α E. coli is transformed with 50 ng of either MSCV-IRES-Thy1.1 (Thy1.1) or MSCV-IRES-NGFR (NGFR) -encoding plasmids by heat shock at 42°C for 30 seconds.
   b. The transformed DH5α cells are evenly plated on a Luria-Bertani (LB) agar plate with 100 μg/mL ampicillin and incubated at 37°C overnight.
c. One transformed E. coli clone (single colony) is picked and grown overnight in 50 mL LB medium containing 100 μg/mL ampicillin with agitation (250 rpm) at 37°C.

d. Final spectrophotometer assessment at the 600 nm absorbance (A600) of 1–1.5 will yield \(1 \times 10^9\) Colony Forming Units (CFU)/mL.

e. Pellet bacteria following the manufacturer’s instructions for the PurelinkTM HiPure Plasmid Filter Midiprep Kit [Life Technologies, K210005] or equivalent bacterial Midiprep kit to isolate plasmids from bacterial cultures prepared in step 1.

2. DNA concentration of the purified plasmids is determined using a spectrophotometer (e.g., Nanodrop).

Establishment of Platinum-E (Plat-E) cell culture

© Timing: 1 week

The Plat-E retroviral packaging cell line was derived from the 293T cell line. Plat-E cells exhibit longer stability and produce higher yields of retroviral structure proteins than conventional NIH3T3-based cell lines. These cells already express \textit{gag}, \textit{pol}, and \textit{env} genes encoding the retroviral structure proteins, allowing retroviral packaging with transfection of a single plasmid.

3. Recovery of frozen Plat-E cell stock.
   a. Obtain a Plat-E cell stock vial from \(-80°C\) or liquid nitrogen storage.

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Figure 1. Schematic representation of the retroviral vectors described in this protocol
Top: MSCV-IRES-NGFR (NGFR, Addgene #27489). Bottom: MSCV-IRES-Thy1.1 (Thy1.1, Addgene #17442).

Figure 2. Schematic overview of the murine splenic T-cell retroviral transduction and co-culture protocol
Step 1: Production of Thy1.1 and NGFR-expression construct-carrying retroviruses in the Plat-E packaging cell line. Step 2: Isolation, activation, and transduction of splenic naïve CD4⁺ T cells from RORγt\textbf{WT} and RORγt\textbf{S182A} mice. Step 3: Co-culture, polarization, and harvest of retrovirally transduced RORγt\textbf{WT} and RORγt\textbf{S182A} CD4⁺ T cells marked by cell surface Thy1.1 or NGFR.
b. Seed cells onto a 10 cm² plate or a 75 cm² flask with 10 mL Plat-E medium pre-warmed to room temperature (RT).

c. Incubate at 37°C in a humidified 5% CO₂-containing incubator.

4. Post recovery, cells should be passaged 1–2 times when confluency reaches ~80%.

5. After the second passage, cells are now optimal for transfection and virus production.

**Mouse models**

The following protocol describes one recent application of this assay for evaluating the cell-intrinsic role of a post-translational modification on a master T cell transcription factor RORγt. For this experiment, naïve CD4⁺ T cells are obtained from 8–14 weeks old gender-matched and co-housed RORγt²WT and RORγt²S182A littermates in C57BL/6 background. “RORγt²WT” refers to wild-type mice. “RORγt²S182A” refers to a phospho-null knock-in mouse line generated by replacing the serine codon on Rorc with that of alanine using CRISPR-Cas9. For more information on the mouse models used in this protocol, please refer to (Ma et al., 2022).

**Institutional permissions**

All animal studies followed guidelines approved by the Institutional Animal Care and Use Committee (IACUC) of the University of California, San Diego. Readers wishing to follow this protocol must first obtain approval from the official animal use ethical committee of their respective institutions to perform the animal studies described herein.

**KEY RESOURCES TABLE**

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Chemicals, peptides, and recombinant proteins | | |
| DMEM | Thermo Fisher Scientific | Cat# 11960044 |
| Iscove’s Modified Dulbecco’s Medium (IMDM) | Sigma-Aldrich | Cat# I3390 |
| Opti-MEM I Reduced Serum Medium | Life Technologies | Cat# 31985062 |
| Fetal Bovine Serum (FBS) | Sigma-Aldrich | Cat# GS-5727 |
| L-Glutamine-200 mM (100X) | Life Technologies | Cat# 21985023 |
| β-mercaptoethanol | Sigma-Aldrich | Cat# G1272 |
| Gentamycin | Sigma-Aldrich | Cat# G1272 |
| Penicillin-streptomycin | Thermo Fisher Scientific | Cat# 10378016 |
| ACK Lysing Buffer | Gibco | Cat# A1049201 |
| PBS (1x) pH 7.4 | Thermo Fisher Scientific | Cat# 10010-049 |
| Polybrenne Infection / Transfection Reagent | Sigma-Aldrich | Cat# TR-1003-G |
| TransIT-²93 Transfection Reagent | Mirus | Cat# MIR2700 |
| 0.05% Trypsin-EDTA (1X), phenol red | Life Technologies | Cat# 25300054 |
| Phorbol 12-myristate 13-acetate (PMA) | Sigma-Aldrich | Cat# P8139 |
| Ionomycin | Sigma-Aldrich | Cat# I0634 |
| GolgiSTOP | BD | Cat# 554724, RRID: AB:2869012 |
| Ampicillin | Sigma-Aldrich | Cat# A0166 |
| Luria Broth Base | Invitrogen | Cat# 12795-027 |
| LB Agar | Invitrogen | Cat# 22700-025 |
| 0.5 M EDTA | Invitrogen | Cat# AM9260G |
| LIVE/DEAD Fixable Aqua Dead Cell Stain Kit, 405 nm (Dilution 1:500) | Thermo Fisher Scientific | Cat# L34957 |
| Recombinant Human TGF-beta 1 Protein | R&D | Cat# 240-B |
| Recombinant Mouse IL-6 Protein | R&D | Cat# 406-ML |
| Recombinant Mouse IL-1β Protein | R&D | Cat# 401-ML |

Critical commercial assays

| PureLink®/HiPure Plasmid Midiprep Kit | Life Technologies | Cat# K210005 |
| Naive CD4⁺ T Cell Isolation Kit, mouse | Miltenyi Biotec | Cat# 130-104-453 |
| eBioscience® Foxp3 / Transcription Factor Staining Buffer Set | Thermo Fisher Scientific | Cat# 00-5523-00 |

(Continued on next page)
### REAGENT or RESOURCE SOURCE IDENTIFIER

#### Cell lines and bacteria strain

**Human: Plat-E Retroviral Packaging Cell Line**  
Cell Biolabs, Inc.  
Cat# RV-101

**DH5α**  
Biomiga  
Cat# CC1101-01

#### Experimental models: Organisms/strains

**C57BL/6 RORγtWT and RORγtSTAT6 mice** (gender-matched 8–12-week-old littermates)  
Ma et al., (2022) N/A

#### Recombinant DNA

**MSCV-IRES-NGFR** (NGFR)  
Addgene  
RRID: Addgene_27489

**MSCV-IRES-Thy1.1** (Thy1.1)  
Addgene  
RRID: Addgene_17442

#### Antibodies

**Rabbit IgG Fraction to Hamster IgG (Whole Molecule) Antibody** (Dilution 1:20)  
MP Bio  
Cat# 0855398, RRID: AB_2334935

**PE anti-human CD271 (NGFR) Antibody** (Clone ME20.4) (Dilution 1:400)  
BioLegend  
Cat# 205251, RRID: AB_1595584

**Pacific Blue™ anti-rat CD90/mouse CD90.1 (Thy-1.1) Antibody** (clone OX-7) (Dilution 1:400)  
BioLegend  
Cat# 506939, RRID: AB_2565780

**PE/Cyanine5 anti-mouse CD4 Antibody** (clone GK1.5) (Dilution 1:400)  
BioLegend  
Cat# 100410, RRID: AB_312695

**CD3e Monoclonal Antibody** (clone 145-2C11), Functional Grade (Dilution 1:1000)  
Thermo Fisher Scientific  
Cat# 16-0031-86, RRID: AB_468849

**CD28 Monoclonal Antibody** (clone 37.51), Functional Grade (Dilution 1:4000)  
Thermo Fisher Scientific  
Cat# 16-0281-86, RRID: AB_468923

**ROR gamma (t) Monoclonal Antibody** (clone B2D), PE-eFluor 610 (Dilution 1:200)  
Thermo Fisher Scientific  
Cat# 61-6981-82; RRID: AB_2574650

**IL-10 Monoclonal Antibody** (clone JES5-16E3), Alexa Fluor 488 (Dilution 1:100)  
Thermo Fisher Scientific  
Cat# 53-7101-80; RRID: AB_469925

**FOXP3 Monoclonal Antibody** (clone FJK-16s), PE-Cyanine5.5 (Dilution 1:200)  
Thermo Fisher Scientific  
Cat# 35-5773-82; RRID: AB_2337136

**ChromPure Rat IgG** (Dilution 1:50)  
Jackson ImmunoResearch  
Cat# 012-000-013; RRID: AB_2337136

#### Software and algorithms

**FlowJo™ v10.8**  
BD  
https://www.flowjo.com/

### Other

**Biological Safety Cabinet**  
Thermo Fisher Scientific  
Cat# 1384-M

**Centrifuge**  
Eppendorf  
Cat# 5810R

**NanoDrop Lite**  
Thermo Fisher Scientific  
Cat# BZ8917251

**ZE5 Cell Analyzer**  
Bio-Rad  
Cat# 12004278

**15 mL Conical tubes**  
Coming  
Cat# 188261

**50 mL Conical tubes**  
Coming  
Cat# 227270

**24-well tissue culture plate**  
Fisher Scientific  
Cat# 353047

**6-well tissue culture plate**  
Eppendorf  
Cat# 30720113

**10 mL serological pipettes**  
Genesee Scientific  
Cat# 12-112

**5 mL serological pipettes**  
Genesee Scientific  
Cat# 12-110

**3 mL syringe**  
Fisher Scientific  
Cat# 14823436

**Millex-HV Syringe Filter Unit, 0.45 µm**  
Fisher Scientific  
Cat# SLHV033RS

**40 µm cell strainer**  
Falcon  
Cat# 352340

**Curved forceps**  
Denville  
Cat# S728685

**Delicate tip straight forceps**  
Denville  
Cat# S728696

**Micro dissecting scissors 3.5”**  
Roboz Surgical Store  
Cat# RS-5910

**Vi-Cell cell counter**  
Beckman Coulter  
Cat# A241595
**Alternatives:** In theory, all reagents and resources listed in the ‘key resources table’ can be substituted with equivalent items from other suppliers; however, it should be noted that the protocol has been calibrated to the reagents listed in the above table and alternatives have not been tested on the protocol performance.

### MATERIALS AND EQUIPMENT

#### LB medium

| Reagent              | Amount   |
|----------------------|----------|
| Luria Broth Base     | 25 g     |
| MilliQ H2O           | Up to 1 L|
| **Total**            | 1 L      |

Autoclave on a liquid cycle (20 min at 15 psi), and then store at RT for up to 3 months.

#### LB agar plate with ampicillin

| Reagent              | Amount   |
|----------------------|----------|
| Luria Broth Base     | 12.5 g   |
| Agar                 | 7.5 g    |
| MilliQ H2O           | Up to 0.5 L|
| **Total**            | 0.5 L    |

Autoclave on a liquid cycle (20 min at 15 psi). Allow the agar solution to cool to 55°C-60°C, then add 500 µL of 100 mg/mL ampicillin and swirl to mix. Pour 20 mL of LB agar per 10 cm² plate and allow plates to cool and solidify. Store at 4°C for up to 2 months.

#### Plat-E medium

| Reagent                  | Final concentration | Amount |
|--------------------------|---------------------|--------|
| Fetal Bovine Serum       | 10% (v/v)           | 5 mL   |
| L-Glutamine (200 mM)     | 2 mM                | 0.5 mL |
| DMEM                     | n/a                 | 44.5 mL|
| **Total**                | n/a                 | 50 mL  |

Prepare in a biological safety cabinet. Store at 4°C for up to one month.

#### IMDM complete medium

| Reagent                        | Final concentration | Amount   |
|--------------------------------|---------------------|----------|
| L-Glutamine (200 mM)           | 2 mM                | 1 mL     |
| β-mercaptoethanol (55 mM)      | 55 µM               | 50 µL    |
| Gentamicin (10 mg/mL)          | 50 µg/mL            | 250 µL   |
| Penicillin-streptomycin (100X) | 1 mg/mL Streptomycin, 100 U/mL Penicillin | 500 µL |
| Fetal Bovine Serum             | 10% (v/v)           | 5 mL     |
| IMDM                           | n/a                 | 43.2 mL  |
| **Total**                      | n/a                 | 50 mL    |

Prepare in a biological safety cabinet. Store at 4°C for up to two weeks.

⚠️ **CRITICAL:** β-mercaptoethanol is toxic. It may cause severe skin, eye, and respiratory tract irritation if absorbed or inhaled. Always wear personal protective equipment (PPE) when handling and use only inside a well-ventilated safety cabinet or fume hood.
T-cell polarization medium

| Reagent (stock concentration) | Final concentration | Amount |
|-------------------------------|---------------------|--------|
| anti-CD3e (1000 μg/mL)        | 0.25 μg/mL          | 2.5 μL |
| anti-CD28 (1000 μg/mL)        | 1 μg/mL             | 1 μL   |
| IL-6 (10 μg/mL)               | 20 ng/mL            | 2 μL   |
| TGFβ (2 μg/mL)                | 5 ng/mL             | 2.5 μL |
| IL-1β (10 μg/mL)              | 20 ng/mL            | 2 μL   |
| IMDM                          | n/a                 | 990 μL |
| Total                         | n/a                 | 1 mL   |

Prepare in a biological safety cabinet. Use immediately.

MACS buffer

| Reagent                        | Final concentration | Amount |
|--------------------------------|---------------------|--------|
| Fetal Bovine Serum             | 2% (v/v)            | 1 mL   |
| EDTA (500 mM)                  | 2 mM                | 0.2 mL |
| PBS (1 x)                      | n/a                 | 48.8 mL|
| Total                          | n/a                 | 50 mL  |

Prepare in a biological safety cabinet. Store at 4°C for up to one month.

Antibodies cocktail n 1 – Surface markers post-restimulation – 1× concentration

| Reagent (stock concentration) | Amount | Working dilution (in antibodies cocktail) |
|-------------------------------|--------|-------------------------------------------|
| LIVE/DEAD Fixable Aqua (500×) | 0.2 μL | 1:500                                      |
| CD4 PE-Cy5 (0.2 mg/mL)        | 0.25 μL| 1:400                                      |
| Thy1.1 Pacific Blue (0.5 mg/mL)| 0.25 μL| 1:400                                      |
| NFGR PE (0.05 mg/mL)          | 0.25 μL| 1:400                                      |
| PBS (1 x)                     | Up to 100 μL     |                                            |
| Total                         | 100 μL         |                                            |

Store at 4°C until use and protect from light.

Antibodies cocktail n 2 – Intracellular markers post-restimulation – 1× concentration

| Reagent (stock concentration) | Amount | Working dilution (in antibodies cocktail) |
|-------------------------------|--------|-------------------------------------------|
| RORγt PE-eFluor 610 (0.2 mg/mL)| 0.5 μL | 1:200                                      |
| Foxp3 PE-Cyanine5.5 (0.2 mg/mL)| 0.5 μL | 1:200                                      |
| IL-10 Alexa Fluor 488 (0.5 mg/mL)| 1.0 μL | 1:100                                      |
| IL-17A APC/Cyanine7 (0.2 mg/mL)| 1.0 μL | 1:100                                      |
| ChromPure Rat IgG* (11.6 mg/mL)| 2.0 μL | 1:50                                       |
| Permeabilization buffer** (1 x)| Up to 100 μL |                                           |
| Total                         | 100 μL |                                            |

Store at 4°C until use and protect from light.

*See CRITICAL text on step 13.
** From eBioscience™ Foxp3 / Transcription Factor Staining Buffer Set.

STEP-BY-STEP METHOD DETAILS

Retrovirus production – Days 1–4

© Timing: 4 days

This section outlines the steps for producing retroviral constructs encoding expression cassettes for Thy1.1 or NGFR in Plat-E cells.
1. Seeding Plat-E cell working culture – Day 1.

Δ CRITICAL: Ensure Plat-E culture medium does NOT contain penicillin-streptomycin before transfection.

a. Once the pre-established Plat-E cell stock culture (pre-passaged 1–2x) has been grown to ~80% confluency, gently remove culture media and wash cells with 5 mL RT sterile PBS 1x.
b. Gently apply 1–2 mL trypsin-EDTA 0.05% solution and spread across the plate or flask to ensure even coverage.
c. Incubate plate or flask in a 5% CO₂ incubator at 37°C for 3–5 min to trypsinize cells.
d. Add 5 mL RT Plat-E medium to inhibit further digestion.
e. Transfer cell suspension into a 15 mL conical tube and centrifuge at 500 x g for 5 min, RT.
f. Remove supernatant and resuspend cells with 5 mL RT Plat-E medium.
g. Determine the viable cell concentration of the resuspension using a hemocytometer or automated cell counter (e.g., Vi-cell cell counter).
h. In a 6-well tissue culture plate, seed 0.5 x 10⁶ viable Plat-E cells in 1.25 mL RT Plat-E medium into two wells:
   i. Well #1: “Thy1.1 retrovirus”—For the production of Thy1.1-encoding retroviral constructs.
   ii. Well #2: “NGFR retrovirus”—For the production of NGFR-encoding retroviral constructs.
i. Incubate at 37°C in a 5% CO₂ incubator overnight.

2. Plat-E cell transfection with plasmid DNA – Day 2.

Note: Be careful to avoid generating bubbles when mixing by pipette.

a. Make Mixture “A” by adding 250 μL of RT Opti-MEM™ reduced serum medium and 15 μL of RT Mirus transfection reagent into a 1.5 mL tube. Mix well by pipetting. Incubate at RT for 5 min.
b. Make a separate Mixture “B” for Thy1.1 or NGFR transfection each by mixing 125 μL of Opti-MEM™ with 2.5 μg of either Thy1.1- or NGFR-plasmid DNA into a separate 1.5 mL tube. Mix well by pipetting.
c. Carefully pipette 125 μL of Mixture “A” into each Mixture “B” tube and mix well by pipetting. Now there should be two transfection mixtures: one for Thy1.1 transfection and one for NGFR transfection, each containing ~250 μL.
d. Incubate the transfection mixtures at RT for 18 min.
e. Homogenize each transfection mixture by carefully pipetting up and down 3–5 times.
f. Use a P200 pipette to evenly distribute each transfection mixture dropwise onto their corresponding well:
   i. Distribute the Thy1.1 transfection mixture onto Well #1: “Thy1.1 retrovirus”.
   ii. Distribute the NGFR transfection mixture onto Well #2: “NGFR retrovirus”.
g. Gently swirl the plate to mix and incubate for 16–20 h at 37°C in a 5% CO₂ incubator overnight.

3. Culture medium change – Day 3.

a. Gently remove the transfection mixture-containing medium from each well.
b. Add 2 mL RT IMDM complete medium to each well.
c. Incubate the transfected cells for a further 24 h at 37°C in a 5% CO₂ incubator.

4. Retrovirus harvest – Day 4.

a. Aspirate the 2 mL of retrovirus-containing medium from each well into a 3 mL syringe and pass through a 0.45 μM syringe filter (we use the Thermofisher™ Millex-HV 0.45 μM Syringe Filter Unit) to remove large debris from the packaging cell line.
b. Aliquot 0.5 mL of the retrovirus-containing medium into a clean 1.5 mL tube on ice.
c. Each 0.5 mL aliquot of retrovirus-containing medium is now ready to be used immediately for a single naïve CD4+ T-cell transduction (step 9).
Note: Virus-containing tubes can be stored frozen at −80°C for later use (up to 6 months).

Note: For best transduction efficiency, opt to use freshly harvested (never frozen) retrovirus-containing medium for naïve CD4+ T-cell transduction. This protocol assumes this recommendation is followed.

Naïve CD4+ T cell isolation and activation – Day 3

© Timing: 5–6 h

In the following section, naïve CD4+ T cells are isolated from murine spleens. Cells are seeded onto a 24-well plate pre-coated with anti-hamster IgG, which will help spatially cluster the hamster anti-mouse CD3e and anti-mouse CD28 antibodies to activate naïve T cells, as described in (Ciofani et al., 2012; Ma et al., 2022). This system requires fewer antibodies for more effective T cell activation than direct coating with anti-CD3e and anti-CD28.

5. Pre-coat 24-well plate.
   a. Dilute anti-hamster IgG stock solution (1 mg/mL) at 1:20 with sterile PBS (1×).
   b. Add 240 μL diluted anti-hamster IgG (0.05 mg/mL) to four wells of a 24-well plate.
   c. Incubate the plate for 2 h at 37°C in a 5% CO2 incubator.

   Alternatives: The 24-well plate can be pre-coated in advance, sealed with parafilm (to prevent evaporation), and incubated at 4°C at least overnight for up to 1 week as needed.

6. Naïve CD4+ T cell isolation and activation.
   a. Euthanize mice according to applicable IACUC guidelines. Here, we use RORγtWT and RORγtS182A cohoused littermates (one of each) of the same gender.
   b. Take the spleen from each mouse and place it onto a 40 μM cell strainer on top of a 6-well plate with 5 mL IMDM complete medium in each well.
   c. Use a plunger from a sterile 3 mL syringe to grind each spleen into a single cell suspension.
   d. Transfer the single-cell suspension to a 15 mL falcon tube and centrifuge at 650 × g for 5 min at 4°C to pellet splenic cells.
   e. Carefully remove the supernatant and add 1 mL ACK lysis buffer to each cell pellet to eliminate red blood cells. Mix well by pipetting and incubate at RT for 1 min.

   Note: Avoid incubating cells in ACK lysis buffer for longer than 1 min to prevent death of lymphocytes.

   f. Add 9 mL MACS buffer to halt further cell lysis, and centrifuge at 650 × g for 5 min at 4°C.
   g. Decant supernatant. The cell pellet should appear white as an indication of successful red blood cell lysis and removal.
   h. Proceed to purify naïve CD4+ T cells from the cell pellet using the Naive CD4+ T Cell Isolation Kit according to the manufacturer’s instructions.

   Note: Each spleen should yield ~2–10 × 10⁶ naïve CD4+ T cells.

   i. Wash the four precoated wells of the 24-well plate (from step 5) with 500 μL sterile PBS (1×) or IMDM medium.
   j. In two wells each, seed 0.2–0.3 × 10⁶ RORγtWT or RORγtS182A naïve CD4+ T cells in 500 μL IMDM complete medium together with anti-CD3e and anti-CD28 at a final concentration of 0.25 μg/mL and 1 μg/mL, respectively.
   k. Incubate at 37°C in a 5% CO2 incubator for 24 h.
Note: Compared to RPMI, IMDM showed better performance in inducing PMA/Ionomycin-mediated activation of CD4+ T cells (Zimmermann et al., 2015). This media has been adopted by multiple recent T cell studies (Amir et al., 2018; Ciofani et al., 2012; Ma et al., 2022; Veldhoen et al., 2009).

Note: We omit the addition of IL-2 to the IMDM T-cell activation mixture as it was previously found to negatively impact the generation of Th17 cells in culture assays (Laurence et al., 2007; Liao et al., 2011).

Retroviral transduction – Day 4

© Timing: 2 h

In this section, Thy1.1 or NGFR retroviral constructs are used to transduce, and label activated CD4+ T cells with Thy1.1 or NGFR surface markers.

△ CRITICAL: Transduction of activated CD4+ T cells occurs optimally during the mitotic phase of the cell cycle, which corresponds to 24–48 h post-anti-CD3e and anti-CD28 stimulation.

7. Add 4 μg polybrene per aliquot to two 0.5 mL aliquots of Thy1.1 or NGFR retrovirus medium each (prepared in step 4). Mix well by pipetting.

Note: If the retrovirus medium was previously frozen, it must be thawed on ice for 1–2 h before the addition of polybrene.

8. Gently remove the medium from all wells seeded with RORγtWT or RORγtS182A CD4+ T cells of the 24-well plate prepared in step 6.

9. Add a 0.5 mL aliquot of Thy1.1 retrovirus medium to one well of RORγtWT CD4+ T cells and one well of RORγtS182A CD4+ T cells each. Do the same for the NGFR retrovirus medium.

10. Spin inoculates by centrifuging the plate at 900 × g for 90 min at 32°C with an acceleration rate of 3 and a deceleration rate of 1.

△ CRITICAL: Centrifugation at 32°C is crucial for transduction as previously described (Eremenko et al., 2021; Pampusch et al., 2020).

△ CRITICAL: The retrovirus-containing plate should be sealed with plastic wrap before centrifugation to avoid the release of retroviral particles into the centrifuge.

11. Gently remove the plastic wrap (if used) from the plate and incubate overnight at 37°C in a 5% CO₂ incubator.

Ex vivo T cell co-culture and polarization – Days 5–8

© Timing: 4 days

Transduced Thy1.1- or NGFR-expressing CD4+ T cells are co-cultured and polarized ex vivo as previously described (Ma et al., 2022).

12. Setup CD4+ T cell co-culture – Day 5.
   a. Add 40 μL/well of diluted anti-hamster IgG (same as described in step 5) into two wells of a 96-well plate.
b. Incubate the plate for 2 h at 37°C in a 5% CO₂ incubator.
c. Prepare the T-cell polarization medium as outlined under “materials and equipment”.

**Note:** The cytokine combination comprising the T-cell polarization medium was chosen to closely mimic the T lymphocyte populations observed in the intestinal lamina propria—namely a mixture of Th17 (Th17, RORγtFoxp3⁺), RORγt Treg (RORγtFoxp3⁺), and conventional Treg (cTreg, RORγt⁻ Foxp3⁺) lineages (Figure 4). IL-6 and TGFβ alone—without the addition of anti-IFNγ and anti-IL-4—are sufficient to effectively polarize naïve CD4⁺ T cells toward the Th17 lineage (Bedoya et al., 2013). To induce concomitant polarization towards RORγt⁺ Treg and RORγt⁻ cTreg lineages, we utilize TGFβ at 5 ng/mL, a dose known for polarizing naïve CD4⁺ T cells toward the Treg lineages in culture (Shevach et al., 2008). IL-1β (20 ng/mL) is used to mimic an inflammatory tissue environment, such as that of the colon post Dextran Sodium Sulfated (DSS) induced epithelial injury (Ma et al., 2022).

**Alternatives:** Cytokine combinations/concentrations can be adjusted as desired to obtain polarization towards specific T-helper subsets.

d. Gently remove the retrovirus-containing medium from each well of the T-cell transduction plate from step 11.
e. Use 0.5 mL T-cell polarization medium to resuspend transduced T-cells from each well by gentle pipetting and transfer to a fresh 1.5 mL tube.
f. Count cells as described in step 1.g.
g. After the 2-h incubation of the 96-well plate, remove the anti-hamster IgG solution and wash both pre-coated wells with 100 µL PBS or IMDM medium.
h. Seed Thy1.1- and NGFR-expressing cells at a 1:1 ratio into both wells for a combined cell density of 0.08–0.12 × 10⁶ cells per well (as illustrated in Figure 2):
   i. Group #1: 100 µL NGFR-transduced RORγtWT T-cells + 100 µL Thy1.1-transduced RORγtS182A T-cells.
   ii. Group #2: 100 µL Thy1.1 transduced RORγtWT T-cells + 100 µL NGFR-transduced RORγtS182A T-cells.
i. Incubate the plate for 48–72 h at 37°C in a 5% CO₂ incubator.

**Note:** To avoid overgrowth if cells need to be cultured for longer than 72 h before restimulation, cells should be split into new pre-coated wells at a 1:2–1:4 ratio after 72 h.

13. T-cell restimulation and FACS staining – Day 7 or 8.
   a. Add restimulation cocktail (final concentrations: 5 ng/mL PMA, 500 ng/mL ionomycin, and 0.625 µL/mL GolgiStop) to each well. Mix well by pipette.
b. Incubate for an additional 4–5 h at 37°C in a 5% CO₂ incubator.

**Note:** For the following steps as part of the experiment illustrated in Figures 3 and 4, we followed the manufacturer’s instructions suggested for the eBioscience™ Foxp3 / Transcription Factor Staining Buffer Set.

c. Centrifuge the cells at 600 × g for 5 min at 4°C and decant supernatant.
d. Wash the cells with 150 µL PBS 1x. Centrifuge and decant supernatant as before.
e. Add 40 µL of antibodies cocktail n°1 to each well to stain for surface markers. Mix well by pipetting.
f. Incubate the cells for 30 min at 4°C in the dark.
g. Wash the cells with 150 µL MACS buffer. Centrifuge and decant supernatant as before.
h. Fix and permeabilize the surface-stained cells with 40 µL fixation/permeabilization solution (from eBioscience™ Foxp3 / Transcription Factor Staining Buffer Set) for 30 min or overnight at 4°C in the dark.
i. Wash cells with 150 μL Permeabilization buffer 1× (from eBioscience™ Foxp3 / Transcription Factor Staining Buffer Set) per well.

j. Centrifuge the cells at 1000 × g for 5 min at 4°C and decant supernatant.

k. Repeat step 13. i-j once.

△ CRITICAL: After cell fixation/permeabilization, the centrifugation speed should be increased to 1000 × g (from step 13. j onward) to avoid cell loss after decanting of supernatant.

l. Add 40 μL of antibodies cocktail n°2 to each well to stain for intracellular transcription factors/cytokines. Mix well by pipetting.

△ CRITICAL: To reduce the potential background of RORγt staining, ChromPure Rat IgG (1:50) should be included in antibodies cocktail n°2.

m. Incubate the cells for 60 min at 4°C in the dark.

n. Wash cells with 150 μL Permeabilization buffer 1× per well.

o. Centrifuge the cells at 1000 × g for 5 min at 4°C and decant supernatant.

p. Repeat step 13. n-o once.

q. Resuspend cells in 100–200 μL of MACS Buffer or PBS 1× per well.

r. Measure the fluorescence intensity of each labeled cell using a flow cytometer (we used the Bio-Rad ZE5 Cell Analyzer).

**EXPECTED OUTCOMES**

The retroviral transduction efficiency of naïve CD4+ T cells ranges from 15%–30% (Figure 3). 72 h incubation of transduced CD4+ T cells in our T-cell polarization medium yields ~50% Th17 cells.
Ex vivo co-culture

In vivo (intestinal lamina propria)

Figure 4. Comparison of ex vivo co-culture and in vivo intestinal lamina propria T lymphocyte populations

Top: Representative flow cytometry plots of transduced RORγtWT (NGFR+) and RORγtS182A (Thy1.1+) CD4+ T cells co-cultured and polarized toward multiple T cell subsets, including Th17 (RORγtFoxp3+, blue), RORγt+ Treg (RORγtFoxp3+, red) and cTreg (RORγtFoxp3+, green). Bottom: Representative flow cytometry plots from the small intestinal and colonic lamina propria (siLP and cLP), respectively. Lamina propria T lymphocyte populations were isolated as previously described by (Lefrancois and Lycke, 2001; Valle-Noguera et al., 2020). Note that IL-10 staining on ex vivo (top) and in vivo (bottom) samples was performed with clone JES5-16E3 (Alexa Fluor 488) and JES5-16E3 (Alexa Fluor 700), respectively.

(RORγtFoxp3), ~30% RORγt+ Tregs (RORγtFoxp3+), and ~5% cTregs (RORγtFoxp3+) (Figure 4, top panel). These ex vivo differentiated T-cell subsets harbor IL-17A and IL-10 production capabilities like those obtained in vivo (Figure 4, bottom panel).

In our published study, we found that CD4+ T subsets from RORγtS182A mice displayed an increase in IL-17A production and a decrease in IL-10 production compared to the cells from RORγtWT mice. In
the co-culture assay (Figure 4), RORγt<sup>S182A</sup> T cell subsets co-cultured in the presence of RORγt<sup>WT</sup> cells also maintained an increased IL-17A production capacity and a reduced IL-10 production capacity. This suggests that RORγt<sup>S182</sup> regulates CD4<sup>+</sup> T cell effector functions in a cell-intrinsic manner (Ma et al., 2022). If the candidate molecules under investigation exert paracrine roles in driving T cell differentiation and/or function, we would instead observe co-cultured control and mutant cells displaying similar differentiation and cytokine patterns.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

Un-transduced T cells can be used as a flow gating control for identifying the retrovirus transduced T cell population. Isotype controls can be used to decipher a gating strategy for identifying transcription factor and cytokine positive populations on the flow cytometer. Summarized graphs should be presented as means ± standard deviation. Significant differences can be evaluated using the student’s t-test, paired t-test, or multiple t-tests. A two-tailed p-value of <0.05 is considered statistically significant in all experiments.

**LIMITATIONS**

Retrovirus is not suitable for the transduction of non-proliferating cells. Caution must be exercised for retrovirus transduction of mutant T-cells that have diminished or loss of proliferative capacity (Cepko and Pear, 2001; Eremenko et al., 2021). Retrovirus transduction can substantially negatively impact T-cell viability. Providing transduced CD4<sup>+</sup> T cells with recombinant IL-2 and IL-7 may increase their viability and proliferative capacity as previously described (Eremenko et al., 2021), but may negatively impact polarization towards the desired T-helper subsets. In addition, retrovirus transduction efficiency is relatively low (combined NGFR and Thy1.1 transduction efficiencies are ~40%–60%) in our system. Furthermore, the T-cell polarization conditions described above typically yield only ~10%–30% RORγt<sup>+</sup> Tregs and <10% cTreg cells. Polarization conditions need to be tailored for individual studies before performing large-scale experiments.

**TROUBLESHOOTING**

**Problem 1**
Plat-E cells fail to grow after transfection with plasmid DNA (steps 2 and 3).

**Potential solution**
Transfecting excessive amounts of plasmid DNA can negatively impact Plat-E cell growth. Reduce the amount of plasmid DNA used for transfection.

**Problem 2**
Low Plat-E transfection efficiency (steps 2 and 3).

**Potential solution**
- Do not transfet freshly thawed Plat-E cells. Cells require sufficient time to fully recover from the freeze-thaw process.
- Do not add penicillin-streptomycin into the Plat-E culture medium.
- After transfection, avoid physically disturbing the plate as this could detach Plat-E cells from the bottom. Pre-coating plates with poly-D-lysine can enhance the adherence of Plat-E cells.
- Repeat transfections can be carried out the subsequent day to further improve transfection efficiency.

**Problem 3**
Low transduction efficiency (steps 7–11).
**Potential solution**
- Adjust the transduction window of T cells, e.g., delaying the time of transduction to 48 h post-activation as described for CD8 cells (Kurachi et al., 2017).
- Increase transduction virus titers.
- Reduce the naïve T-cell seeding density in the 24-well plate.

**Problem 4**
Low CD4$^+$ T cell viability post-transduction (steps 9–11)

**Potential solution**
- Replace retrovirus-containing medium with 500 μL fresh IMDM complete medium 4–6 h post-transduction to provide optimal nutrients to the T cells.
- Avoid plating excessive numbers of T-cells, which can quickly deplete nutrients. Follow the recommended seeding density outlined in the Methods section.
- Use an optimal polybrene concentration of 6–16 μg/mL.
- Confirm centrifugation settings. Centrifugation time can be increased to 2–3 h to ensure retroviral enrichment at the bottom of the well where the cultured cells reside.

**Problem 5**
Low CD4$^+$ T cell activation/polarization (steps 12–13).

**Potential solution**
- Confirm appropriate cytokine and antibody concentrations were used.
- Avoid freeze-thaw cycles on cytokines and antibodies. Typically, stock concentrations of cytokines and antibodies are 20–100 mg/mL and 1–10 mg/mL, respectively. These should be stored in 5–20 μL aliquots in the −80°C freezer according to manufacturer recommendations.
- Ensure culture media components have not expired.

**RESOURCE AVAILABILITY**

**Lead contact**
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Wendy Jia Men Huang (wendyjmhuang@ucsd.edu).

**Materials availability**
This study did not generate new unique reagents.

**Data and code availability**
This study did not report datasets and code.

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**AUTHOR CONTRIBUTIONS**
S.M. designed, performed the experiments, and wrote the manuscript. J.E.H. and W.J.M.H. edited the manuscript.

**DECLARATION OF INTERESTS**
The authors declare no competing interests.
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