Assessing IL-2-Induced STAT5 Phosphorylation in Fixed, Permeabilized Foxp3+ Treg Cells by Multiparameter Flow Cytometry

1. Preparing single cell suspension (30 min)
   - Thymus → Pipetting → Filtering
   - LNs → Pipetting → Filtering

2. IL-2 stimulation (2 hrs)
   - Ghost dye staining (4°C, 20 min)
   - Pre-incubation (37°C, 30 min)
   - IL-2 incubation (37°C, 30 min)

3. Staining (4 hrs)
   - Foxp3 staining (20–25°C, 30 min)
   - pSTAT5 staining (20–25°C, 40 min)
   - Surface markers staining (20–25°C, 20 min)

4. Data collection and analysis (3 hrs)
   - FACS → CD25
   - IL-2 (ng/mL) → Tregs
   - Conventional CD4 T cells

Assessing IL-2-induced phospho-STAT5 (pSTAT5) content can reveal the cytokine responsiveness of individual T cells. Identifying distinct T cell subsets by nuclear transcription factors, such as Foxp3, and concurrently quantifying intracellular pSTAT5, however, has been technically challenging. Conventional Foxp3 staining buffers quench pSTAT5 signals while commonly used pSTAT5 staining protocols fail to detect Foxp3. The current protocol resolves these issues by describing a procedure to assess IL-2-induced pSTAT5 contents in Foxp3+ CD4 Treg cells using multiparameter flow cytometry.
Protocol

Assessing IL-2-Induced STAT5 Phosphorylation in Fixed, Permeabilized Foxp3+ Treg Cells by Multiparameter Flow Cytometry

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SUMMARY
Assessing IL-2-induced phospho-STAT5 (pSTAT5) content can reveal the cytokine responsiveness of individual T cells. Identifying distinct T cell subsets by nuclear transcription factors, such as Foxp3, and concurrently quantifying intracellular pSTAT5, however, has been technically challenging. Conventional Foxp3 staining buffers quench pSTAT5 signals, while commonly used pSTAT5 staining protocols fail to detect Foxp3. The current protocol resolves these issues by describing a procedure to assess IL-2-induced pSTAT5 contents in Foxp3+ CD4 Treg cells using multiparameter flow cytometry.

For complete details on the use and execution of this protocol, please refer to Waickman et al. (2020).

BEFORE YOU BEGIN

© Timing: 30 min

Note: The day before analysis, design the staining chart for the experiment (see an example of staining chart in Table 1). The single-color staining tubes are necessary for setting up compensation. Use unstimulated cells for preparing compensation tubes, but use cells stimulated with the highest concentration of IL-2 for preparing the pSTAT5 fluorescence compensation tube.

1. Set up water bath to 37°C.
2. Precool cell centrifuge to 10°C.
3. Chill HBSS and PBS solutions to 4°C.
4. Place 40 mL of 4% paraformaldehyde solution (4% PFA) on ice.
5. Prewarm 25 mL of RPMI-1640 medium (serum-free) in the 37°C water bath.
6. Place 50 mL of 90% methanol into −80°C freezer.
7. Label the FACS tubes according to the staining chart.

KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Antibodies          |        |            |
| Anti-mouse Foxp3 eFluor 660 (FJK-16a) | eBioscience | Cat#: 50-5773-82 \nRRID: AB_11218868 |

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| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Anti-mouse CD8α Pacific Blue (SH10) | eBioscience | Cat#: MCD0828 RRID: AB_10372364 |
| Anti-mouse CD4 PE-Cyanine7 (GK1.5) | Tonbo Biosciences | Cat#: 60-0041-U100 RRID: n/a |
| Anti-mouse CD25 PE (PC61.5) | eBioscience | Cat#: 12-0251-83 RRID: AB_465608 |
| Anti-mouse phospho-STAT5 (pY694) Alexa Fluor 488 (47/Stat5 (pY694)) | BD Biosciences | Cat#: 612598 RRID: AB_399881 |
| Anti-mouse IgG1 κ isotype control Alexa Fluor 488 (MOPC-21) | BD Biosciences | Cat#: 557782 RRID: AB_396870 |
| Anti-mouse TCRb Alexa Fluor 594 (H57-597) | Biolegend | Cat#: 109238 RRID: AB_2563324 |
| Anti-mouse CD16/32 (2.4G2) | BD Biosciences | Cat#: 553141 RRID: AB_394656 |

### Chemicals, Peptides, and Recombinant Proteins

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Recombinant human IL-2 protein | R&D Systems | Cat#: 202-IL-050/CF |
| Ghost dye Violet 510 | Tonbo Biosciences | Cat#: 13-0870-T500 |
| Paraformaldehyde 16% solution (16% PFA) | Electron Microscopy Sciences | Cat#: 15710 |
| Methanol | Sigma-Aldrich | Cat#: M1775 |
| Acetic acid | Sigma-Aldrich | Cat#: A6283-500ML |
| Sodium azide | Sigma-Aldrich | Cat#: S2002-500G |
| Modified Cohn fraction V bovine serum albumin powder (BSA) | Equitech-Bio | Cat#: BAC65 |
| Defined fetal bovine serum | HyClone | Cat#: SH30070.03 |
| Standard fetal bovine serum | HyClone | Cat#: SH30088.03 |
| 0.4% trypan blue | Gibco | Cat#: 15250061 |
| PBS | Gibco | Cat#: 10010023 |
| HBSS | Gibco | Cat#: 14175095 |
| RPMI-1640 medium | Gibco | Cat#: 21870076 |
| Water (molecular biology grade) | Quality Biological | Cat#: 351-029-131 |

### Critical Commercial Assays

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Fixation/permeabilization concentrate | Invitrogen | Cat#: 00-5123-43 |
| Fixation/permeabilization diluent | Invitrogen | Cat#: 00-5223-56 |
| Permeabilization buffer (10X) | Invitrogen | Cat#: 00-8333-56 |

### Experimental Models: Organisms/Strains

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Mouse: C57BL/6 (C57BL/6NCrl) | Charles River Laboratories | n/a |

### Software and Algorithms

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| FlowJo software | FlowJo LLC | https://www.flowjo.com |
| GraphPad Prism 7 software | GraphPad | https://www.graphpad.com |
| Canvas X software | Canvas GFX | https://www.canvasgfx.com |

### Other

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| 60 µm nylon filters | Merck Millipore | Cat#: NY6000010 |
| 100 µm nylon filters | Merck Millipore | Cat#: NY1H00010 |
| 0.22 µm vented Millex-GV filters | Merck Millipore | Cat#: SLGV255F |
| Cell centrifuge | Thermo Fisher | Model: Legend XTR |
| Cell culture CO2 incubator | Thermo Fisher | Model: 370 |

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MATERIALS AND EQUIPMENT

Recombinant IL-2 Stock (100 μg/mL)

Solution A (100 mM acetic acid): To make solution A, add 57 μL of acetic acid to 2.5 mL water (molecular biology grade). Adjust the final volume of the solution to 10 mL with water (molecular biology grade).

Solution B (1% BSA): To make solution B, dissolve 250 mg BSA into 25 mL PBS and sterilize using 0.22 μm vented Millex-GV filter.

Solution C (0.1% BSA in 100 mM acetic acid): This solution is prepared by adding 1.1 mL solution B to 10 mL solution A.

100 μg/mL IL-2 stock: Add 5 mL solution C to 500 μg recombinant IL-2 protein pellet, and mix well until completely dissolved. Aliquot 100 μL per tube and store at −20°C until further use.

Note: The IL-2 stock solution (100 μg/mL) can be kept at 4°C for no more than 2 months after opening. Avoid freeze/thaw cycle.

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Water bath          | Fisher Scientific | Cat#: FSGPD2S |
| Hemocytometer counting chamber with V-slash | Paul Marienfeld GmbH | Cat#: 0650030 |
| Standard 25 microscope | ZEISS | n/a |
| BD FACS LSRII flow cytometer | BD Biosciences | n/a |

Table 1. Example of an IL-2 Stimulation and Staining Chart for LN Cells and Thymocytes

| Tube # | Condition | Antigens |
|--------|-----------|----------|
|        | IL-2 stimulation | Alexa Fluor 488, PE, PE-Cy7, Pacific Blue, eFluor 660, Alexa Fluor 594, Violet 510 |
| 1      | 1 ng/mL Isotype Ctrl | CD25, CD4, CD8x, Foxp3, TCRβ, Ghost dye |
| 2      | 1 ng/mL pSTAT5 | CD25, CD4, CD8x, Foxp3, TCRβ, Ghost dye |
| 3      | 0.3 ng/mL Isotype Ctrl | CD25, CD4, CD8x, Foxp3, TCRβ, Ghost dye |
| 4      | 0.3 ng/mL pSTAT5 | CD25, CD4, CD8x, Foxp3, TCRβ, Ghost dye |
| 5      | 0.1 ng/mL Isotype Ctrl | CD25, CD4, CD8x, Foxp3, TCRβ, Ghost dye |
| 6      | 0.1 ng/mL pSTAT5 | CD25, CD4, CD8x, Foxp3, TCRβ, Ghost dye |
| 7      | 0.03 ng/mL Isotype Ctrl | CD25, CD4, CD8x, Foxp3, TCRβ, Ghost dye |
| 8      | 0.03 ng/mL pSTAT5 | CD25, CD4, CD8x, Foxp3, TCRβ, Ghost dye |
| 9      | Medium Isotype Ctrl | CD25, CD4, CD8x, Foxp3, TCRβ, Ghost dye |
| 10     | Medium pSTAT5 | CD25, CD4, CD8x, Foxp3, TCRβ, Ghost dye |
| 11     | Medium | – |
| 12     | 1 ng/mL pSTAT5 | – |
| 13     | Medium | CD25 – |
| 14     | Medium | CD4 – |
| 15     | Medium | CD8x – |
| 16     | Medium | – Foxp3 |
| 17     | Medium | – TCRβ |
| 18     | Medium | – |

Continued...
### FACS buffer: store at 4 °C.

| Reagent             | Final Concentration | Amount   |
|---------------------|---------------------|----------|
| BSA                 | 0.1%                | 0.5 g    |
| Sodium azide        | 0.1%                | 0.5 g    |
| HBSS                | n/a                 | 500 mL   |
| Total               | n/a                 | 500 mL   |

### 4% PFA: store at room temperature (RT, 20 °C–25 °C) in the dark.

| Reagent | Final Concentration | Amount |
|---------|---------------------|--------|
| 16% PFA | 4%                  | 10 mL  |
| PBS     | n/a                 | 30 mL  |
| Total   | n/a                 | 40 mL  |

### 90% methanol: store at room temperature (RT, 20 °C–25 °C).

| Reagent                            | Final Concentration | Amount |
|------------------------------------|---------------------|--------|
| Methanol                           | 90%                 | 45 mL  |
| Water (molecular biology grade)    | n/a                 | 5 mL   |
| Total                              | n/a                 | 50 mL  |

### Harvest medium: store at 4 °C.

| Reagent                              | Final Concentration | Amount |
|--------------------------------------|---------------------|--------|
| Standard fetal bovine serum          | 10%                 | 50 mL  |
| RPMI-1640 medium                     | n/a                 | 450 mL |
| Total                                | n/a                 | 500 mL |

### 10% FCS-HBSS: store at 4 °C.

| Reagent                            | Final Concentration | Amount |
|------------------------------------|---------------------|--------|
| Defined fetal bovine serum         | 10%                 | 50 mL  |
| HBSS                               | n/a                 | 450 mL |
| Total                              | n/a                 | 500 mL |

### 0.08% trypan blue: store at room temperature (RT, 20 °C–25 °C).

| Reagent | Final Concentration | Amount |
|---------|---------------------|--------|
| 0.4% trypan blue | 0.08%       | 10 mL  |
| PBS     | n/a                 | 40 mL  |
| Total   | n/a                 | 50 mL  |

### Foxp3 Fix/Perm solution: prepare freshly and keep on ice until further use.

| Reagent                              | Final Concentration | Amount |
|--------------------------------------|---------------------|--------|
| Fixation/Permeabilization concentrate | 25%             | 10 mL  |
| Fixation/Permeabilization diluent    | n/a                 | 30 mL  |
| Total                                | n/a                 | 40 mL  |

### 1× Permeabilization buffer: prepare freshly, put on ice.

| Reagent                            | Final Concentration | Amount |
|------------------------------------|---------------------|--------|
| Permeabilization buffer (10×)      | 10%                 | 5 mL   |
| Water (molecular biology grade)    | n/a                 | 45 mL  |
| Total                              | n/a                 | 50 mL  |
**STEP-BY-STEP METHOD DETAILS**

**Preparation of Lymphocyte Single-Cell Suspension**

- **Timing:** 30 min

This section describes how to process the thymus and lymph nodes (LN) into single-cell suspensions that will be used for *in vitro* IL-2 stimulation and fluorescent antibody staining.

1. Preparing the thymocyte suspension
   a. The thymus is surgically removed from C57BL/6 mice and placed on gauze to remove blood and residual connective tissues using tweezers.
   b. Thymus is placed into a 10 cm petri dish, and then gently disrupted and processed into small pieces (around 2–3 mm³) using tweezers.
   c. Resuspend the thymus tissues with 10 mL harvest medium by pipetting up and down with a 10 mL pipette (more than 15 times) to generate a thymocyte suspension.
      - **CRITICAL:** Avoid generating air bubbles during the pipetting. Air bubbles result in increased cell death and loss of cells.
   d. Filter the cell suspension into 15 mL conical tubes by passing through 100 μm nylon filters and place the cells on ice until further use.

2. LN dissection
   a. The LNs are surgically removed and placed on gauze to remove residual connective tissues.
   b. Place two frosted glass slides in 10 cm petri dish.
   c. Add 10.5 mL harvest medium into the petri dish and wet the frosted sides of two glass slides.
   d. Put the LNs on one of frosted glass slide and press weakly while rubbing the glass slides in circular motions to rupture the LNs.
   e. Resuspend the LN suspension by pipetting up and down more than 15 times using 10 mL pipettes to make a well-mixed cell suspension.
      - **CRITICAL:** Avoid generating air bubbles during the pipetting. Air bubbles cause increased cell death and loss of cells.
   f. Filter the cell suspension into 15 mL conical tubes through 100 μm nylon filters, and place the filtered cell suspension on ice until further use.
      - **CRITICAL:** Cell suspension should be kept on ice to ensure maximum cell viability.

3. Counting of cell numbers
   a. Add 10 μL single-cell suspension to 190 μL 0.08% trypan blue (1: 20 dilution).
   b. Take 10 μL of the cell suspension from step 3a and count cells using a hematocytometer.

   - **Note:** We routinely use trypan blue and a hematocytometer to count the number of viable cells. If the viability is less than 80%, it is critical to use live/dead discrimination dyes prior to IL-2 stimulation to obtain accurate results.

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### Fc block solution: store at 4°C.

| Reagent                  | Final Concentration | Amount |
|--------------------------|---------------------|--------|
| Anti-mouse CD16/32 (2.4G2) | 12.5 μg/mL          | 25 μL  |
| FACS buffer              | n/a                 | 975 μL |
| Total                    | n/a                 | 1 mL   |

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In Vitro IL-2 Stimulation

@ Timing: 2 h

This section describes how to stimulate lymphocytes with recombinant IL-2 to trigger STAT5 phosphorylation. IL-2 stimulation and Foxp3 staining are performed in the same tube, and then split into two tubes for isotype control antibody staining and anti-phospho-STAT5 staining.

4. Live/dead dye (Ghost dye Violet 510) staining (Troubleshooting 1)

Note: If the cell viability is greater than 90%, live/dead dye staining can be omitted, and experiment can proceed directly to step 5.

a. Transfer 4 million LN cells into each FACS tube and fill with 2 mL PBS.

Note: For thymocytes, transfer 8 million cells into each FACS tube. The frequency of Foxp3+ Tregs is much lower among thymocytes than among LN cells. Therefore, it is necessary to use more thymocytes than LN cells to obtain sufficient number of cells for Foxp3+ Treg cell analysis (see Limitations).

b. Centrifuge tubes at 1,500 rpm (500 x g) for 7 min at 4°C.

c. Discard the supernatant and add 1 μL Ghost dye Violet 510 to each tube according to the staining chart, mix well and incubate the cells at 4°C for 20 min in the dark by covering the tubes with aluminum foil.

Note: After discarding the supernatant, the cell pellet should be resuspended in the remaining buffer (in this case PBS) which is usually around 50 μL. In case that there is less residual buffer leftover, PBS can be added up to 50 μL.

d. Fill the tubes with 2 mL RPMI-1640 medium, centrifuge at 1,500 rpm (500 x g) for 7 min at 10°C to wash out excess dye.

a. Discard the supernatant and add 200 μL of prewarmed RPMI-1640 medium to each tube. Vortex the tubes gently and incubate cells in a 37°C water bath for 30 min.

△ CRITICAL: This step is necessary to allow the cells to dephosphorylate STAT5 from in vivo signaling and let the pSTAT5 return to basal levels.

b. During the incubation, make a serial dilution of the 100 μg/mL IL-2 stock into each 2 mL of 2 ng/mL, 0.6 ng/mL, 0.2 ng/mL, and 0.06 ng/mL of IL-2 using RPMI-1640 medium. Also, prepare a tube of 4 mL RPMI-1640 medium without IL-2 as medium control. Prewarm the 5 tubes in a 37°C water bath.

c. Add each 250 μL of the 2 ng/mL, 0.6 ng/mL, 0.2 ng/mL, 0.06 ng/mL IL-2 solution to stimulation tubes (final concentration of IL-2 is 1 ng/mL, 0.3 ng/mL, 0.1 ng/mL, and 0.03 ng/mL, respectively). Add 250 μL RPMI-1640 medium to the medium control tubes.

d. Incubate the cell suspension in a 37°C water bath for 30 min.

Note: The RPMI-1640 media that is used in the IL-2 stimulation should be serum-free.

Antibody Staining of Fixed and Permeabilized Cells

@ Timing: 4 h

This section describes how to stain cells for intranuclear Foxp3, intracellular pSTAT5, and surface markers.

6. Foxp3 staining

a. Remove tubes from 37°C water bath and place them immediately on ice.
b. Add 3 mL of cold Foxp3 Fix/Perm solution to cells, and incubate the tubes on ice for 20 min.

△ CRITICAL: Because adding the Foxp3 Fix/Perm solution terminates IL-2 signaling, this step should be carefully timed.

c. Centrifuge the tubes at 1,500 rpm (500 × g) for 7 min at 10°C.

d. Discard the supernatant, add 1 mL 1× Permeabilization buffer to each tube, and centrifuge at 1,500 rpm (500 × g) for 7 min at 10°C.

Pause Point: The cell suspensions can be stored overnight (16–18 h) at 4°C after adding 1 mL 1× Permeabilization buffer to the cell pellet from step 6d.

e. Repeat step 6d to wash the cells again.

f. While the FACS tubes are spinning in the centrifuge, dilute eFluor 660-Foxp3 antibodies (1:10) in 1× Permeabilization buffer into working concentration.

g. Discard the supernatant, and add 5 μL Fc block solution to each tube to a final concentration of 1.25 μg/mL, including the single-color compensation tubes.

h. Add 10 μL of diluted eFluor 660-Foxp3 antibody to each sample tube and to the single-color compensation tubes for Foxp3 staining.

Note: When using twice the number of cells for staining, such as for thymocytes staining, add twice the amount of Fc block solution (10 μL) and diluted eFluor 660-Foxp3 antibodies (20 μL) to each tube, because there are 8 million instead of the usual 4 million cells in each tube.

i. Gently vortex the FACS tubes and incubate cells at room temperature (RT, 20–25°C) for 30 min.

j. Fill the FACS tubes with 1 mL ice-cold HBSS, and spin down cells at 1,500 rpm (500 × g) for 7 min at 10°C.

k. Discard the supernatant, and repeat step 6j to wash the cells again.

7. Phospho-STAT5 and isotype control staining

a. Resuspend pelleted cells from step 6k in 500 μL ice-cold HBSS.

b. Add 500 μL 4% PFA (final concentration is 2% PFA) to each tube, mix gently, and vortex the tubes (Troubleshooting 2).

c. Incubate FACS tubes on ice for 30 min.

d. Add 2 mL of ice-cold HBSS to each tube, and then centrifuge at 1,500 rpm (500 × g) for 7 min at 10°C.

e. Discard the supernatant and add 2 mL of ice-cold HBSS to each tube to wash the cells again, followed by centrifugation at 1,500 rpm (500 × g) for 7 min at 10°C.

f. Discard the supernatant and add 1 mL of prechilled 90% methanol to each tube.

△ CRITICAL: Make sure to prechill the 90% methanol at −80°C and keep it cold because warmed up methanol can disrupt the cell membrane. The repeated fixation and permeabilization steps are critical to achieve efficient permeabilization of the nuclear membrane while not losing intracellular pSTAT5 signals.

g. Incubate the tubes on ice for 30 min.

h. Add 2 mL of ice-cold 10% FCS-HBSS to cells, and spin down cells at 1,500 rpm (500 × g) for 7 min at 10°C.

i. Discard the supernatant and repeat step 7h to wash the cells again.

j. Resuspend cells in 170 μL of FACS buffer and split each sample into two tubes (around 110 μL for each tube).

k. Add 2 mL of cold FACS buffer to each tube, then spin down cells at 1,500 rpm (500 × g) for 7 min at 10°C.

l. Discard the supernatant and add 10 μL of Alexa Fluor 488-conjugated isotype control antibodies or 10 μL of Alexa Fluor 488-conjugated pSTAT5 antibody to sample tubes, including the single-color compensation tube.
m. Gently mix the cells and incubate FACS tubes at room temperature (RT, 20°C–25°C) for 40 min.

8. Surface marker staining
a. While cells are incubating in step 7m, dilute the surface marker antibodies in FACS buffer according to the dilution factors as indicated below.

| Antibody                  | Dilution for Working Concentration |
|---------------------------|-----------------------------------|
| CD25-PE                   | 1:10                               |
| CD4-PE-Cy7                | 1:10                               |
| CD8α-PacBlue              | 1:10                               |
| TCRβ-Alexa Fluor 594      | 1:40                               |

**Note:** Antibodies conjugated with tandem dyes, such as PE-Cy7, should be always freshly diluted because of the possibility of degradation. On the other hand, antibodies conjugated with non-tandem dyes can be diluted to a working solution in advance and kept at 4°C for further use.

b. Use 10 μL of each of the diluted antibodies to make a master mix for surface marker antibodies according to the number of samples. Make extra amounts of the master mix (calculate for 2 or 3 more samples than necessary) to secure sufficient amounts of antibodies and to compensate for pipetting errors.

c. Add 40 μL of the antibody master mix to each tube. For the single-color compensation tubes, add 10 μL of the diluted antibody according to the staining chart.

d. Incubate tubes at room temperature (RT, 20°C–25°C) for 20 min.

e. Add 2 mL of cold FACS buffer to each tube, and then spin down cells at 1,500 rpm (500 x g) for 7 min at 10°C.

f. Discard the supernatant and repeat step 8e to wash the cells again.

g. Discard the supernatant and resuspend pellet in 150 μL cold FACS buffer.

h. Filter the cells into new FACS tubes using 60 μm nylon filters and place the tubes on ice until flow cytometric analysis.

**Note:** Filter the cell suspension through 60 μm nylon filters to remove cellular aggregates. To prevent reaggregation, cells should be not filtered earlier than 30 min before the start of data acquisition.

**Sample Collection and Data Analysis**

**Timing:** 3 h

This section describes how to collect samples using a flow cytometer and which software to use for data analysis.

9. Use unstained cells and single-color control staining to set appropriate PMT voltages and compensations for each parameter.

10. At least 0.5 million cells should be collected per tube at a flow rate of 6,000 events/s or less. A representative voltage setting for acquisition would be as follows: FCS: 565, SSC: 330, Alexa Fluor 488: 500, PE: 500, PE-Cy7: 630, PacBlue: 390, eFluor 660: 460, Alexa Fluor 594: 510, Violet 510: 375.
Note: The voltage of each parameter needs to be adjusted using unstained compensation tubes and single stained compensation tubes for each experiment.

11. The fcs format files were analyzed using FlowJo with linear scales for FCS-H and FCS-W, and log scales for fluorochrome parameters. Statistical analyses were performed using GraphPad Prism 7, and the figures for publication were created in Canvas X.

EXPECTED OUTCOMES

IL-2 stimulation will result in increased levels of pSTAT5 in Foxp3+ CD4 T cells, but not in Foxp3-negative conventional CD4 T cells. As previously reported (Cho et al., 2010; Keller et al., 2020; Waickman et al., 2020), conventional CD4 T cells express only low levels of IL-2Rβ and they are poor responders to IL-2. Foxp3+ Treg cells, on the other hand, express large amounts of IL-2 receptors and they are highly effective in responding to IL-2. Consequently, it is expected that Treg cells will show substantial phosphorylation of STAT5 upon IL-2 stimulation whereas conventional CD4 T cells will not.

Because it is difficult to isolate Treg cells in sufficient numbers to directly assess their IL-2 response, the IL-2 stimulation is conducted with total LN cells. Foxp3+ Treg cells are then identified by intranuclear staining of Foxp3 proteins. The gating strategy to identify Treg cells and conventional CD4 T cells among LN cells is shown in Figure 1. IL-2-induced STAT5 phosphorylation is then assessed on both Foxp3+ Treg cells and conventional CD4 T cells. The results are displayed either as fold-induction of pSTAT5 with increasing amounts of IL-2 compared to medium-incubated cells (Figure 2A) or as the percentage of pSTAT5-positive cells upon IL-2 stimulation (Figure 2B).

The frequency of Treg cells differs depending on the tissue (Park et al., 2018). The fraction of Foxp3+ Treg cells in the thymus is substantially diminished compared to that in the LN. Thus, assessing IL-2 signaling in thymic Foxp3+ Treg cells is further complicated because of their low numbers. We found that staining multiple tubes and appending/merging their data can provide a solution to this problem. On another note, thymic Foxp3+ Treg cells contain a significant population of Treg progenitor cells that are identified as Foxp3loCD25− CD4 T cells (Owen et al., 2019; Tai et al., 2013). Alternatively, Foxp3+CD25+ CD4 T cells were also found to harbor Treg progenitor cells (Lio and Hsieh, 2008; Owen et al., 2019), and it has not been clear whether IL-2 responsiveness differs between these two alternative Treg precursor populations. Using this protocol, we now assessed the IL-2 responsiveness in these populations, for which a gating strategy as indicated in Figure 3 was applied. In the thymus samples, IL-2 stimulation showed that the fold increase in pSTAT5 was prominent for mature Treg cells but minimal for both the Foxp3loCD25− and Foxp3+CD25+ progenitor populations (Figure 4A). Importantly, the same data can be reanalyzed based on the frequency of pSTAT5-positive cells in IL-2-stimulated cells (Figure 4B). This way, it is evident that the Foxp3+CD25+ progenitor population is significantly more responsive to IL-2 than the Foxp3loCD25− progenitor populations. On the other hand, Foxp3+CD25+ mature Treg cells are
substantially more efficient than any other CD4 thymocytes population for IL-2 signaling. Thus, alternative methods of data analysis can reveal differences in IL-2 signaling that are not immediately visible upon data acquisition.

QUANTIFICATION AND STATISTICAL ANALYSIS

The relative abundance of intracellular pSTAT5 is most effectively visualized by determining the fold increase of pSTAT5 in IL-2-treated cells over medium-treated cells. To this end, first, the difference in the Mean Fluorescence Intensity (MFI) of anti-pSTAT5 staining and the MFI of isotype control staining is assessed for each condition. This value is commonly referred to as ΔMFI. The ΔMFI for each IL-2 stimulating condition is then divided by the ΔMFI of medium-treated cells which provides the fold increase of STAT5 phosphorylation in IL-2-treated cells. As an example from an actual experiment, the pSTAT5 MFI of IL-2 (1 ng/mL)-treated Foxp3+ Treg cells was found to be 122. Isotype control staining of the same cells under the same condition resulted in an MFI of 10.3. Thus, the ΔMFI for pSTAT5 would be 111.7. Next, the pSTAT5 ΔMFI of medium-treated Foxp3+ Treg cells was determined in the same manner, which was 8.54 and which would correspond to background pSTAT5 levels. Finally, the pSTAT5 ΔMFI of IL-2 (1 ng/mL)-treated Foxp3+ Treg cells was divided by the pSTAT5 ΔMFI of medium-treated Foxp3+ Treg cells, which showed that IL-2 stimulation at a concentration of 1 ng/mL resulted in a 16.8 fold increase of pSTAT5 abundance (Table 2). The fold increase is then further determined for other IL-2 dilutions (i.e., 0.3 ng/mL, 0.1 ng/mL etc.) and then plotted as a line graph (Figure 2A). Usually, three or more independent experiments are performed to obtain sufficient statistical power, and data are then shown as mean ± SEM of multiple analyses (Figure 2A).
Notably, not all IL-2-responsive T cells are equally potent in inducing pSTAT5, so that the degree of STAT5 phosphorylation can be heterogeneous. In such cases, the same data can be also visualized as the percentage of pSTAT5-positive cells among IL-2-stimulated cells. As shown in Figure 2B, without IL-2 stimulation, there are minimal numbers of pSTAT5-positive cells among both Foxp3+CD25+ Treg cells and conventional CD4 T cells (Figure 2B, top). However, the frequency of pSTAT5-positive cells dramatically increases in Foxp3+CD25+ Treg cells upon stimulation with increasing concentrations of IL-2. Conventional CD4 T cells, on the other hand, remain unresponsive to IL-2, as previously described (Cho et al., 2010; Waickman et al., 2020). The percentages of pSTAT5-positive cells are then determined for each IL-2 dilution (i.e., 1 ng/mL, 0.3 ng/mL etc.) and then plotted as a line graph (Figure 2B). To obtain sufficient statistical power, usually, three or more independent experiments are performed, and data are then shown as mean ± SEM of multiple analyses (Figure 2B).

LIMITATIONS

Because this staining protocol requires multiple fixation and washing steps, there is a substantial loss in cell numbers during the staining. We found that a minimum number of 4 million cells per tube is required to recover sufficient numbers of cells for flow cytometric analysis. This problem is exacerbated when the frequency of Foxp3+ Treg cells is very low (<5%), such as in the case of thymocytes. In tissues like the small intestine epithelium where the fraction of Foxp3+ Treg cells is even further reduced (Park et al., 2018), the loss of cells numbers significantly impairs the ability to reliably identify Foxp3+ Treg cells. One way to overcome this limitation is to prepare duplicate tubes in the experiment, and then append the acquired FACS data into one data file for analysis.

Another limitation of this protocol is the potential decrease in surface staining intensity which is caused by the repeated fixation steps that result in the loss of antibody-staining epitopes on surface molecules. Because the paraformaldehyde fixation and the methanol fixation can destroy some of the antibody binding sites, we found that some of the antibodies show dramatically reduced staining intensities compared to unfixed cells. Anti-TCRβ staining, for example, is markedly diminished on thymocytes that were fixed using this protocol, and it requires careful analysis and gating to identify mature T cells. CD8 coreceptor expression is also significantly affected by the repeated fixation and permeabilization steps. Therefore, designing the staining panel needs to consider these limitations to achieve optimal staining.

TROUBLESHOOTING

Problem 1
Damaged or apoptotic cells in the samples.
If the single-cell suspension used for IL-2 stimulation contains a large fraction of damaged cells, this can lead to false positive cells for pSTAT5 because of auto-fluorescence or non-specific antibody binding to apoptotic cells.

Potential Solution
The use of fixable live/dead discrimination dye, such as Ghost dye Violet 510, can identify damaged or dead cells so that they can be excluded from analysis. We chose to use Ghost dye Violet 510 because its emission spectrum was compatible with our antibody staining panel. We did not test other viability dyes for this protocol. Of note, the use of live/dead discrimination dye adds an additional step for pSTAT5 staining. Therefore, live/dead discrimination dyes can be omitted when cell viability is acceptable for analysis.

Table 2. Example of pSTAT5 MFI Values upon IL-2 Stimulation of LN Cells

| Sample | Condition | Foxp3⁺ CD25⁺ CD4 T cells | Conventional CD4 T cells |
|--------|-----------|--------------------------|--------------------------|
| -      | IL-2 stimulation | Anti-pSTAT5 | Isotype Ctrl | ΔMFI | Anti-pSTAT5 | Isotype Ctrl | ΔMFI |
| 1      | 1 ng/mL    | 122          | 10.3         | 111.7 | 16.8 | 8.26 | 8.54 |
| 2      | 0.3 ng/mL  | 108          | 9.54         | 98.46 | 15.9 | 7.62 | 8.28 |
| 3      | 0.1 ng/mL  | 88           | 9.28         | 78.72 | 15.2 | 7.74 | 7.46 |
| 4      | 0.03 ng/mL | 33.9         | 9.76         | 24.14 | 15.5 | 8.11 | 7.39 |
| 5      | Medium     | 16           | 9.35         | 6.65  | 14.3 | 7.74 | 6.56 |
Problem 2

Weak or inconsistent intracellular staining for pSTAT5 in IL-2-stimulated cells.

This is a common problem that is caused by imperfect cell permeabilization so that intracellular staining is performed at suboptimal conditions.

Potential Solution

With time, paraformaldehyde degrades into formalin which affects the efficiency of cell fixation and subsequent permeabilization. We recommend discarding 4% PFA that has been prepared more than 4 weeks ago. Replacing the stock formaldehyde solution and making a new working solution of 4% PFA can solve the problem of weak or inconsistent intracellular staining.

RESOURCE AVAILABILITY

Lead Contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Jung-Hyun Park (parkhy@mail.nih.gov).

Materials Availability
No new materials were generated.

Data and Code Availability
All flow cytometry data acquired during this study are available upon request. No new dataset was generated.

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AUTHOR CONTRIBUTIONS

C.L. performed the experiments, analyzed data, and wrote the manuscript. J.-H.P. supervised the project and wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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