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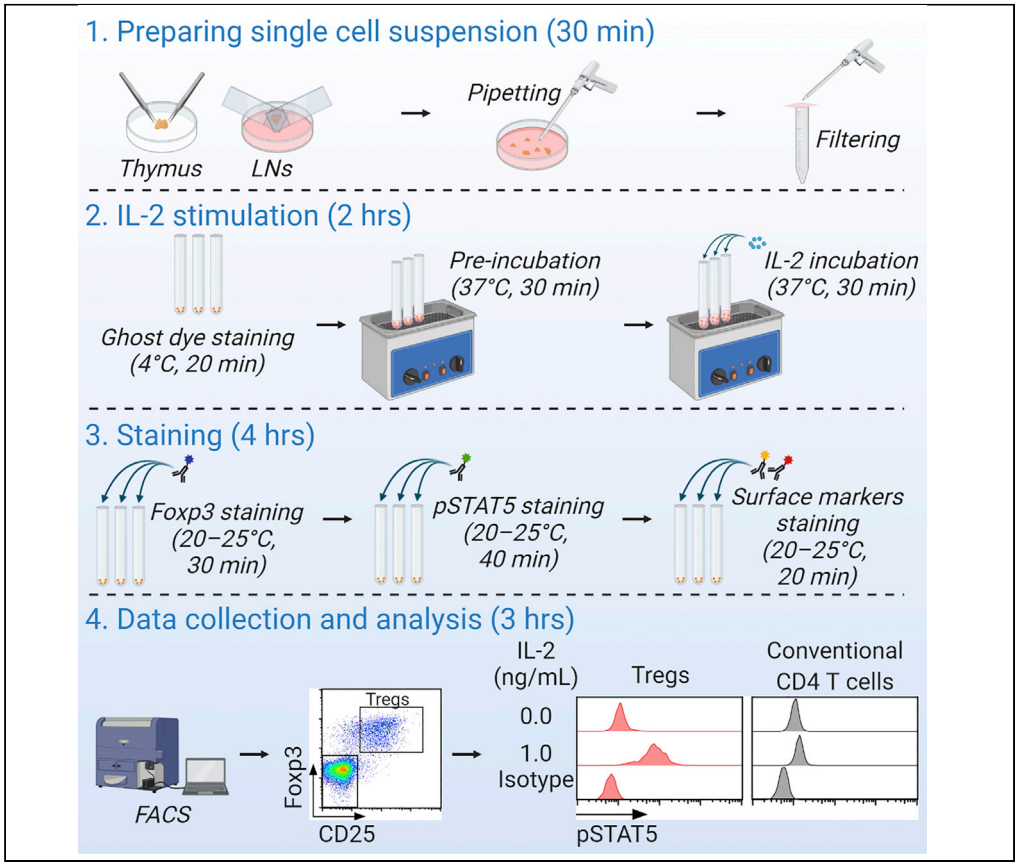
Protocol

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



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HIGHLIGHTS

Assessing IL-2-induced STAT5 phosphorylation (pSTAT5) by flow cytometry

Optimized fixation, permeabilization protocol for intracellular pSTAT5 staining

Concurrent detection of pSTAT5 and nuclear Foxp3 in the same cells

Protocol is also applicable for the detection of pSTAT5 in other T cell subsets

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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-16s)	eBioscience	Cat#: 50-5773-82 RRID: AB_11218868

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Anti-mouse CD8α Pacific Blue (5H10)	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 TCRβ 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		
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		
Fixation/permeabilization concentrate	Invitrogen	Cat#: 00-5123-43
Fixation/permeabilization diluent	Invitrogen	Cat#: 00-5223-56
Permeabilization buffer (10×)	Invitrogen	Cat#: 00-8333-56
Experimental Models: Organisms/Strains		
Mouse: C57BL/6 (C57BL/6NCrl)	Charles River Laboratories	n/a
Software and Algorithms		
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		
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#: SLGVV255F
Cell centrifuge	Thermo Fisher	Model: Legend XTR
Cell culture CO_2 incubator	Thermo Fisher	Model: 370

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Water bath	Fisher Scientific	Cat#: FSGPD2S
Hematocytometer 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

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.

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

Tube #	Condition	Antibodies						
-	IL-2 stimulation	Alexa Fluor 488	PE	PE-Cy7	PacBlue	eFluor 660	Alexa Fluor 594	Violet 510
1	1 ng/mL	Isotype Ctrl	CD25	CD4	CD8α	Foxp3	TCRβ	Ghost dye
2	1 ng/mL	pSTAT5	CD25	CD4	CD8α	Foxp3	TCRβ	Ghost dye
3	0.3 ng/mL	Isotype Ctrl	CD25	CD4	CD8α	Foxp3	TCRβ	Ghost dye
4	0.3 ng/mL	pSTAT5	CD25	CD4	CD8α	Foxp3	TCRβ	Ghost dye
5	0.1 ng/mL	Isotype Ctrl	CD25	CD4	CD8α	Foxp3	TCRβ	Ghost dye
6	0.1 ng/mL	pSTAT5	CD25	CD4	CD8α	Foxp3	TCRβ	Ghost dye
7	0.03 ng/mL	Isotype Ctrl	CD25	CD4	CD8α	Foxp3	TCRβ	Ghost dye
8	0.03 ng/mL	pSTAT5	CD25	CD4	CD8α	Foxp3	TCRβ	Ghost dye
9	Medium	Isotype Ctrl	CD25	CD4	CD8α	Foxp3	TCRβ	Ghost dye
10	Medium	pSTAT5	CD25	CD4	CD8α	Foxp3	TCRβ	Ghost dye
11	Medium	–	–	–	–	–	–	–
12	1 ng/mL	pSTAT5	–	–	–	–	–	Ghost dye
13	Medium	–	CD25	–	–	–	–	Ghost dye
14	Medium	–	–	CD4	–	–	–	Ghost dye
15	Medium	–	–	–	CD8α	–	–	Ghost dye
16	Medium	–	–	–	–	Foxp3	–	Ghost dye
17	Medium	–	–	–	–	–	TCRβ	Ghost dye
18	Medium	–	–	–	–	–	–	Ghost dye

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

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

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 mm^3) 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.

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 × *g*) for 7 min at 10°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 × *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 1× Permeabilization buffer, and spin down 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.

Note: We recommend making master mix when staining multiple antibodies to reduce variability between samples by pipetting.

   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 × *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.

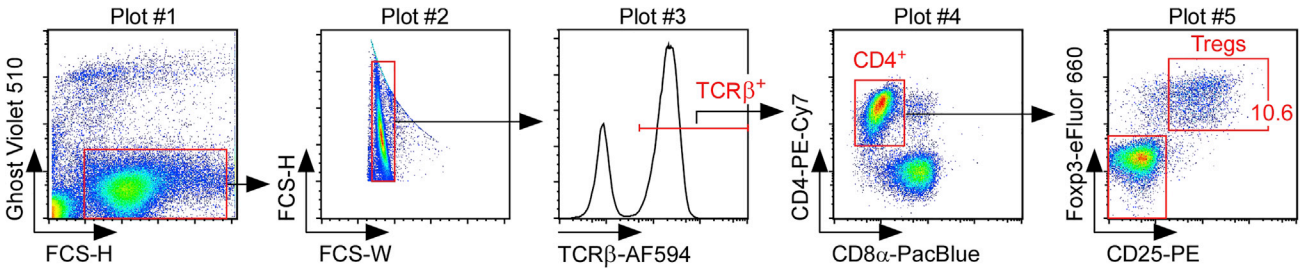

Figure 1. Gating Strategy to Identify Foxp3$^+$CD25$^+$ Treg Cells among LN CD4 T Cells

Live gate was applied on a single-cell suspension of LN lymphocytes based on Forward Scatter Height (FSC-H) and live/dead cell exclusion dye (Ghost Violet 510) (plot #1). Doublets were gated out based on Forward Scatter Width (FSC-W) and Forward Scatter Height (FCS-H) (plot #2). T cells were then identified by anti-TCRβ staining (plot #3), and CD4 versus CD8 profile was generated from TCRβ$^+$ cells (plot #4). Finally, mature Foxp3$^+$CD25$^+$ Treg cells were identified by CD25 versus Foxp3 expression among CD4 T cells (plot #5).

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 Foxp3^{lo}CD25$^-$ 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 Foxp3^{lo}CD25$^-$ and Foxp3$^-$CD25$^+$ progenitor populations (Figure 4A). Importantly, the same data can be re-analyzed 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 Foxp3^{lo}CD25$^-$ progenitor populations. On the other hand, Foxp3$^+$CD25$^+$ mature Treg cells are

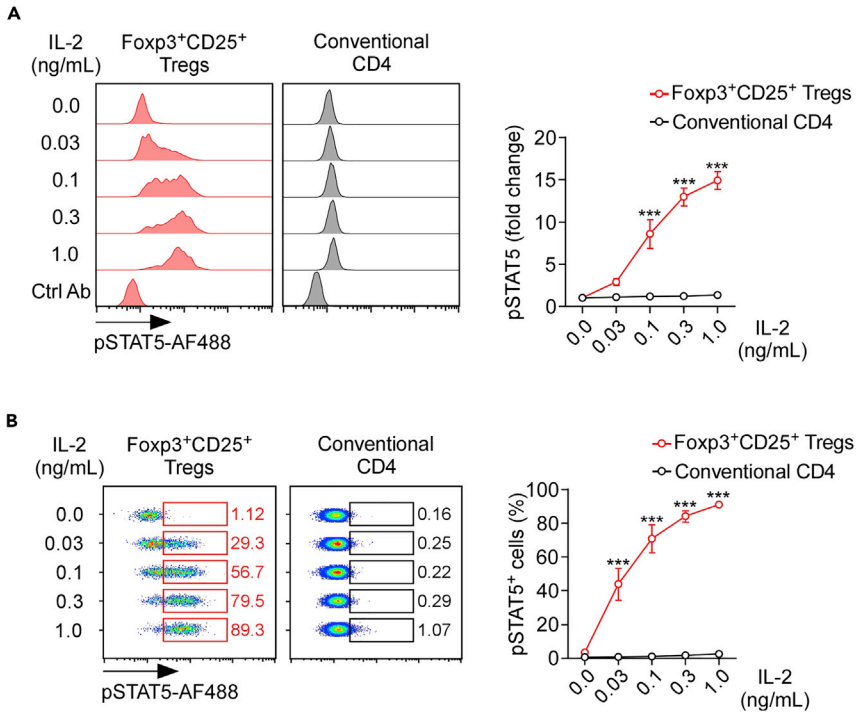

Figure 2. IL-2-Induced STAT5 Phosphorylation in LN Foxp3$^+$CD25$^+$ Treg Cells

(A) Histograms show representative pSTAT5 expression in IL-2-stimulated Foxp3$^+$CD25$^+$ Tregs and conventional LN CD4 T cells over medium control (0.0 ng/mL). Ctrl Ab indicates isotype control. Fold changes in pSTAT5 amounts are shown in the right panel. Results show summary (mean ± SEM) of three independent experiments.

(B) Frequencies of pSTAT5$^+$ cells were assessed in Foxp3$^+$CD25$^+$ Tregs and conventional LN CD4 T cells that were stimulated with increasing dosages of recombinant IL-2 (left). Numbers next to the gates are frequency of pSTAT5$^+$ cells. Percentages of pSTAT5$^+$ cells are shown in the right panel. Results show summary (mean ± SEM) of three independent experiments.

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).

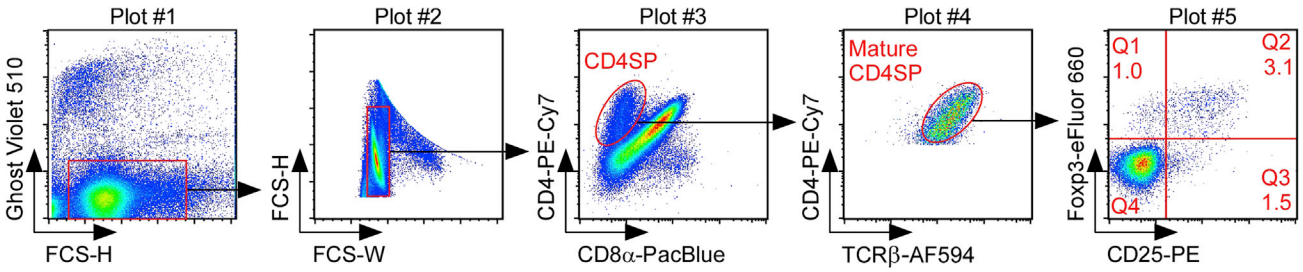

Figure 3. Gating Strategy to Identify Foxp3$^+$CD25$^+$ Treg Cells among Total Thymocytes

Live gate was applied on a single-cell suspension of total thymocytes based on FSC-H and live/dead cell exclusion dye (Ghost Violet 510) (plot #1). Doublets were gated out based on FSC-W and FCS-H (plot #2). CD4 single-positive (CD4SP) thymocytes were identified by CD4 versus CD8 coreceptor staining (plot #3), whereby CD4, CD8 double-positive (DP) cells mostly correspond to pre-selection immature thymocytes. Mature CD4SP thymocytes were further identified by high levels of TCRβ and CD4 coreceptor expression (plot #4). Finally, Foxp3^{lo} (quadrant 1, Q1) and CD25$^+$ (quadrant 2, Q2) Treg cell progenitors, mature Foxp3$^+$CD25$^+$ (quadrant 3, Q3) Treg cells, and conventional CD4 thymocytes (quadrant 4, Q4) were identified by CD25 versus Foxp3 expression among mature CD4SP thymocytes (plot #5) (Owen et al., 2019). All data were collected after proper color compensation using single-color compensation tubes.

Notably, not all IL-2-responsive T cells are equally potent in inducing pSTAT5, so that the degree of STAT5 phosphorylation can be heterogenous. 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.

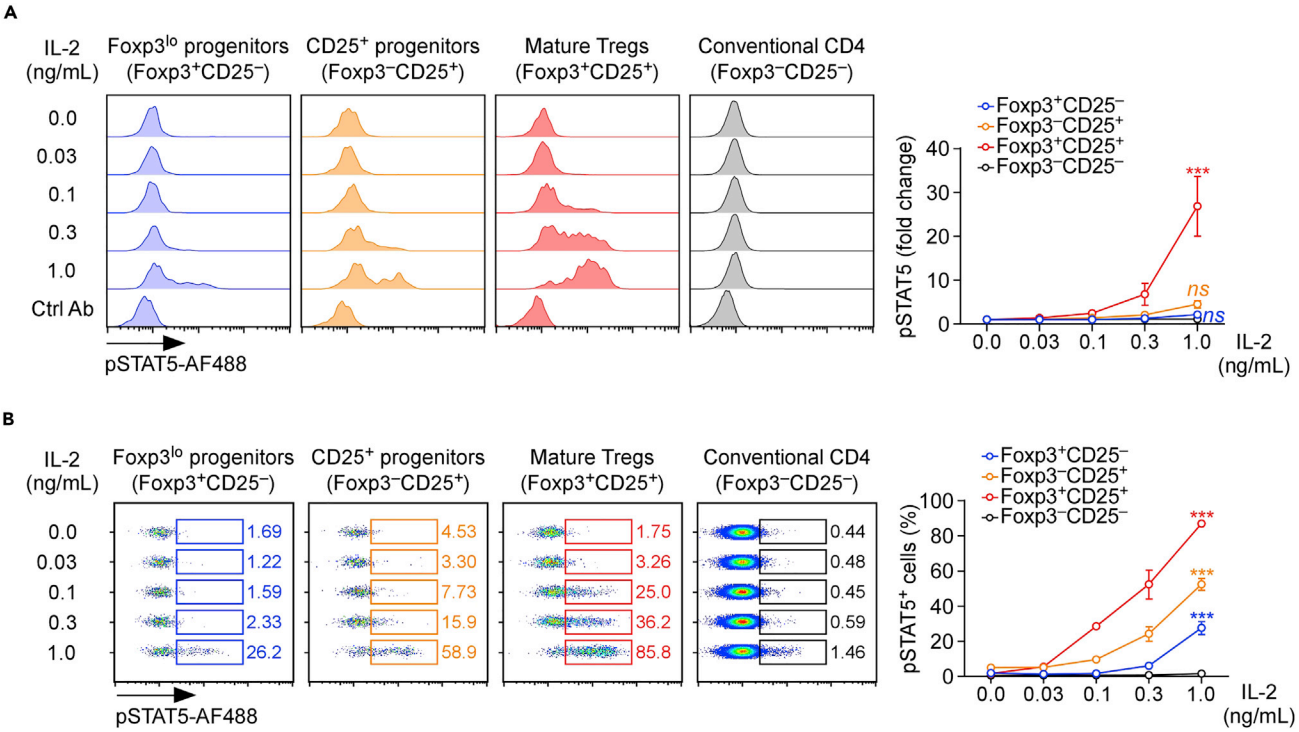

Figure 4. IL-2-Induced STAT5 Phosphorylation in Foxp3$^+$CD25$^+$ Treg Thymocytes

(A) Histograms show representative pSTAT5 expression in the indicated IL-2-stimulated mature CD4SP thymocyte subpopulations that were identified based on CD25 and Foxp3 staining. Ctrl Ab indicates isotype control. Fold changes in pSTAT5 amounts are shown in the right panel. Results show summary (mean ± SEM) of three independent experiments.

(B) Frequency of pSTAT5$^+$ cells was assessed in Foxp3^{lo} Treg progenitors, CD25$^+$ Treg progenitors, Foxp3$^+$CD25$^+$ mature Tregs, and conventional CD4 thymocytes that were stimulated with increasing dosage of recombinant IL-2 (left). Numbers next to the gates are frequency of pSTAT5$^+$ cells. Percentages of pSTAT5$^+$ cells are shown in the right panel. Results show summary (mean ± SEM) of three independent experiments.

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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