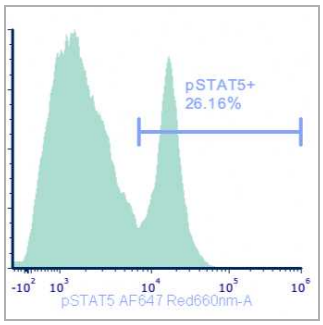

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WORKS FOR ME 1

Flow cytometric measurement of STAT5 phosphorylation to assess the activity of common γ chain cytokines in T cells

COMMENTS 0

DOI
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ABSTRACT

Common γ chain cytokines such as IL2, IL15, and IL7 activate the JAK3/STAT5 signaling cascade, resulting in rapid phosphorylation of STAT5. The assay described herein, based on the original protocol by Krutzik & Nolan, details the use of flow cytometry to quantify the levels of phospho-STAT5 after brief in vitro cytokine stimulation of T cells. This assay works for both mouse and human-derived T cell samples. This assay can be used to compare the biological activity of agonistic cytokines, verify the activity of cytokine batches, or compare T cell responsiveness under different conditions.

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KEYWORDS

T cell activation, intracellular flow cytometry, phosphoflow, cytokines, phosphoprotein

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Introduction

1 Common γ chain cytokines such as IL2, IL15, and IL7 activate the JAK3/STAT5 signaling cascade, resulting in rapid phosphorylation of STAT5. The assay described herein, based on the original protocol by Krutzik & Nolan, details the use of flow cytometry to quantify the levels of phospho-STAT5 (pSTAT5) after brief in vitro cytokine stimulation of T cells. This assay works in both mouse and human primary T cells as well as immortal IL2-dependent T cell lines. This assay can be used to compare the biological activity of agonistic cytokines, verify the activity of cytokine batches, or compare T cell responsiveness under different conditions.

The assay involves starving a given T cell sample of IL2 or other cytokines (2 days), inducing pSTAT5 by cytokine stimulation (15 min.), cell fixation by paraformaldehyde (10 min.), permeabilization by methanol (10 min.), antibody staining for pSTAT5 (30 min.), and analysis by flow cytometry. Because methanol fixation alters the performance of many antibodies, co-staining with other surface antigens will need to be optimized for your use case. We typically use a pure population of activated primary T cells (>70% CD3+) or a T cell line to reduce the background from other cell types, forgoing the necessity of co-staining for non-T cell surface antigens.

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Materials

2

- Fluorophore-conjugated Anti-Stat5 (pY694). Clone: 47/Stat5(pY694). We use an AF647 conjugate: BD Cat# 612599. This antibody recognizes both mouse and human phospho-Y694 STAT5.
- FACS Buffer: PBS free of Ca2+/Mg2+ supplemented with 0.5% BSA + 0.1% sodium azide
- PBS
- 100% Methanol kept ice-cold
- 10x 15% w/v Paraformaldehyde (PFA)
- Cytokine of interest (e.g. IL2)
- Cell sample of interest
- Culture media
- 96-well V-bottom plate
- For primary T cell activation: anti-CD3/28-coated beads or plate

Method

3 *Prepare cells of interest.* Ensure there are enough cells for the number of conditions to be tested. Plan to plate approximately $5x10^4$ - $1x10^5$ cells per well for the subsequent stimulation step.

3.1 Immortal T cell line (e.g. CTLL-2):

1. Harvest cells from culture, wash with PBS, and resuspend in fresh culture media without cytokines.
2. Incubate for 2 days at 37C

Primary T cells from PBMC:

1. Enrich T cells from PBMC using MACS or FACS
2. Activate with bead- or plate-bound anti-CD3/28 for 3 days
3. Harvest from culture, wash with PBS, resuspend in fresh culture media without cytokines and without anti-CD3/28. (Note 1)
4. Incubate for 2 days at 37C

4 *Stimulation and staining.* Cells and cytokines will be first be added in a well, and thereafter paraformaldehyde will be added directly to a final concentration of 1.5% from a 15% stock. Typically cells and cytokines are plated in 200uL total and 20uL 15% PFA is added. Appropriate controls will depend on your experiment, but typically a media/vehicle only control serves as a negative control and 300U/mL IL2 serves as a positive control.

4.1

1. Harvest IL2-starved cells and plate $5x10^4$ - $1x10^5$ cells per well in a 96-well V-bottom plate at 100uL per well.
2. Make 2x stocks of cytokines of interest or appropriate controls. Add 100ul per well of 2x stocks.
3. Incubate 37C at 15 minutes. (Note 2)
4. Add 20uL per well of 15% PFA.
5. Incubate 10 minutes at room temperature.
6. Spin plate 860xg for 4 min. at 4C

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7. Decant supernatant by flicking into waste container and dab plates on paper towels (Note 3)
8. Resuspend cells in 200uL per well of ice-cold methanol.
9. Incubate 10 minutes at 4C
10. Spin plate 860xg for 4 min. at 4C. Decant supernatant as above. Wash cells once with FACS buffer by resuspending in 200uL per well FACS buffer, spinning again, decanting supernatant and dabbing on paper towel.
11. Resuspend each well in 50uL/well staining mix: anti-pSTAT5 diluted 1:50 in FACS buffer.
12. Incubate 30 min. at room temperature protected from light.
13. Wash cells twice with FACS buffer as above. Finally resuspend cells in 200uL per well FACS buffer.
14. Acquire on a flow cytometer. Ensure FSC and SSC values are adequate, as fixed and permeabilized cells display reduced FSC and SSC.

Representative results

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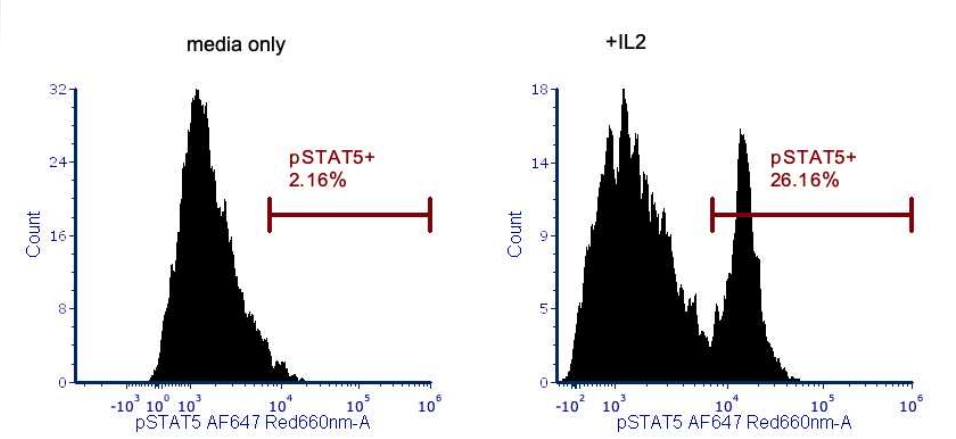

Murine CTLL-16 cells were stimulated with either media alone (left) or IL2 (right) and analyzed according to the protocol described herein.

Notes

6

1. Even though no exogeneous IL2 is added on day 0, T cells produce their own IL2 when activated, necessitating removal.
2. pSTAT5 levels peak at 15 minutes post-cytokine exposure (Bayer et al (2007)).
3. Care must be taken to reduce cell loss during this step. Cell size is reduced after PFA fixation, resulting in a more sparse cell pellet compared to that of unfixed cells. If cell loss is a major concern, dab entire plate onto a stack of paper towels on absorbent pads without decanting supernatant.

References

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