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Development of a Flow Cytometry Phospho-STAT5 Assay in Nonhuman Primate T Cells

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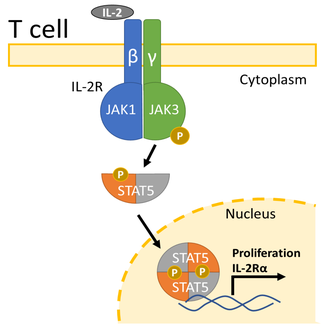
INTRODUCTION

- **Phosphorylated STAT5 is an important signal molecule in T cell activation and proliferation.** IL-2 activates T cells by binding to IL-2Rβγ, which associates with JAK tyrosine kinases. These kinases phosphorylate the receptor subunits, which subsequently recruit and phosphorylate STAT5 (pSTAT5) signal molecule. pSTAT5 is necessary for T cell activation, proliferation, and an overall effective immune response. pSTAT5 quantification has become a popular way to assess T cell proliferation and has great potential in the field of toxicology.
- **Flow cytometry has recently become a common platform for pSTAT5 detection.** Unlike more labor-intensive methods such as western blotting, flow can report endogenous pSTAT5 levels in multiple cell populations in a precise and high throughput manner. This is achieved by measuring levels of a detector fluorophore conjugated to anti-pSTAT5 antibody coupled with a panel of other immunophenotyping markers.
- **To date, a flow assay has not been developed in *Macaca fascicularis* for the purposes of a toxicological study.** Flow cytometry pSTAT5 assays have previously been described in literature in primary and secondary cell lines for discovery or diagnostic purposes.
- **This poster details the method development of a flow-cytometry-based pSTAT5 assay in nonhuman primates (NHP).** Peripheral blood (PB) from six naïve *Macaca fascicularis* was isolated and stimulated *ex vivo* with human recombinant IL-2, followed by staining with a pSTAT5 immunophenotyping panel. pSTAT5 levels were quantitated in CD4+ T cells, PD1+CD4+ T cells, CD8+ T cells, PD1+CD8+ T cells, and CD25+CD4+ T cells as relative percentage of parent.
- **Method development included:**
  1. Assay feasibility on the LSRFortessa flow cytometer
  2. Reproducibility between analysts and individual replicates
  3. Comparison of pSTAT5 activation between PB and peripheral blood mononucleated cells (PBMCs)
  4. Fluorescence minus one control (FMO) for each fluorophore



Adapted from Ross and Cantrell, *Annu Rev Immunol*, 2019

Figure 1. Overview of IL-2-Induced STAT5 Activation in T Cells
IL-2 binds to IL-2Rβγ, which associates with tyrosine kinases JAK1 and JAK3. This leads to the phosphorylation of STAT5 into pSTAT5. pSTAT5 then forms a homodimer and translocates to the nucleus, where it acts as a transcription factor for proliferative genes and IL-2Rα (CD25), a prerequisite of the higher affinity IL-2Rαβγ. This process is necessary for T cell activation and proliferation in response to disease.

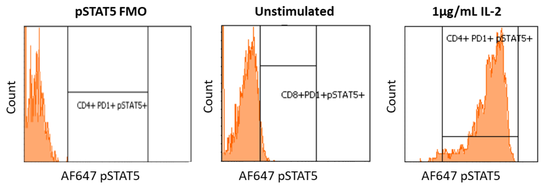


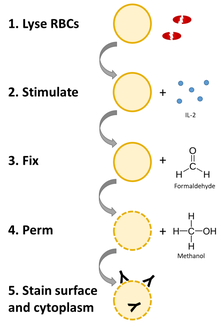
Figure 2. pSTAT5 Peak Comparison Between Controls and Stimulated Blood
In a pilot study, pSTAT5 gates were set for the FMO control (which did not have the pSTAT5-AF647 antibody), unstimulated blood, and blood stimulated with 1µg/mL of IL-2. Stimulated blood displayed a strong positive pSTAT5 peak while the FMO and unstimulated controls did not. FMOs for all fluorophores displayed <2% of positive cells for each control, indicating minimum background staining for this panel.

MATERIALS AND METHODS

Sample Collection

Flow Cytometry Analysis on Peripheral Blood (PB)	
Species	Cynomolgus (*Macaca fascicularis*) monkey
Matrix	0.5-2 mL of whole blood collected in 4 mL BD Vacutainer CPT tubes with sodium citrate 0.1 mL of commercially available PBMCs
Storage conditions	Ambient temperature

Analytical Method for Detection of pSTAT5 in PB



Antibodies	Clone
CD25-BV421	MA251
CD8-AF488	RPA-T8
PD1-PE	EH12.1
CD4-BV605	L200
pSTAT5-AF647	47/Stat5 (pY694)
NKG2A-PE-Vio770	Rea110
Live/Dead Near IR-APC-Cy7	N/A
CD3-BV785	SP34-2

Cynomolgus blood (100µL) was incubated twice with 1X ACK Lysis Buffer followed by wash with PBS-CMF and centrifugation. Cells were stained with a working concentration of Live Dead stain followed by centrifugation and wash. Cells were incubated at 37 °C with shaking for 20 minutes with 0.1-1µg/mL human recombinant IL-2 in RPMI+10% FBS or media alone for unstimulated controls. Cells were then fixed with Cytofix™ Buffer at 37°C with shaking for 10-12 minutes, followed by centrifugation and wash. Cells were permeabilized with Phosflow™ Perm III Buffer on wet ice or at 2-8 °C for 8-12 minutes, followed by centrifugation and wash. Cells were then incubated with immunophenotyping antibodies in the dark at ambient temperature for 45-60 minutes. Cells were washed with FACS Buffer, centrifuged, resuspended in FACS buffer, and analyzed by flow cytometry. The cytometer was set to acquire 300,000 events in the lymphocyte gate or for a total of 3 minutes for each sample.

Flow Cytometry Data Acquisition and Analysis

Data Acquisition

Data acquisition was performed using an LSRFortessa™ cytometer equipped with BD FACSDiva™ acquisition software. Appropriate filter sets and mirrors were used to capture BV421, AF488, PE, BV605, AF647, PE-Vio770, APC-Cy7, and BV785 fluorescence. Flow cytometer calibration was performed prior to each analysis. Fluorescence compensation was conducted automatically using UltraComp eBeads™ Compensation Beads stained with single color controls, ArC™ Amine Reactive Compensation Beads stained with Live/Dead Near IR.

Data Analysis

Data collected on the cytometer was analyzed using BD FACSDiva™ software to measure relative percentages, median fluorescence intensity (MFI), and a number of events within each gate of interest. Single cells were identified out of total cells using the following sequential gate: FSC-H vs. FSC-W followed by SSC-H vs. SSC-W. Lymphocytes and Monocytes were identified from single cells via FSC-A vs. SSC-A profile. Subsequent gating strategies to identify the population of interests are described in the Results section.

RESULTS

Immunophenotyping Strategy for pSTAT5 Detection in PB

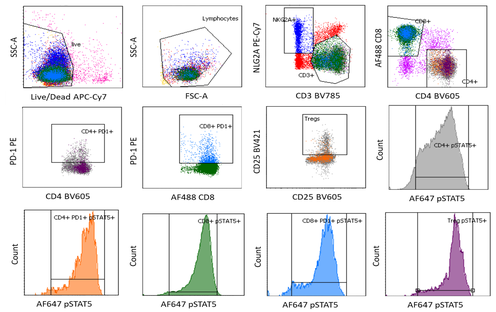


Figure 3. Gating Strategy for pSTAT5+ T Cells in Peripheral Blood
Cells were stained with a pSTAT5 Immunophenotyping panel and gated in order to identify pSTAT5+ T cells. After gating out doublets, lymphocytes were identified as FSC low and SSC low. Natural Killer cells were identified as NKG2A+CD3- and Total T cells as CD3+NKG2A-. From Total T cells, Cytotoxic T cells (CD8+CD4-) and Helper T cells (CD4+CD8-) were gated. PD1+ activated T cell subsets were identified from Cytotoxic T cells, Helper T cells, and T regulatory cells (CD25+CD4+). Finally, pSTAT5+ cells were identified on Cytotoxic T cells, T Helper cells, T regulatory cells, and PD1+ T Cytotoxic cells and PD1+ T Helper cells.

pSTAT5 Assay Feasibility by T Cell Subset in PB

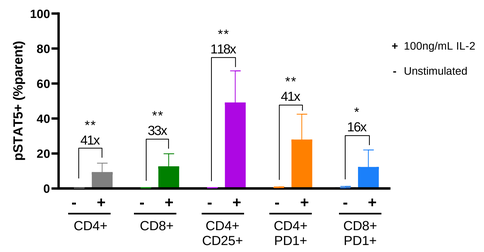


Figure 4. pSTAT5 Assay Feasibility in NHP Peripheral Blood by T Cell Subset
100µL of blood from six animals was stimulated with recombinant IL-2 for 15 minutes at 37°C and compared to its unstimulated counterpart. Addition of IL-2 to blood resulted in significant upregulation of pSTAT5 in CD4+, CD8+,CD25+CD4+, PD1+CD4+, and CD8+PD1+ T cells (Paired T Test, *p<0.05, **p<0.01). CD4+ T cells exhibited a 41-fold increase in pSTAT5, CD8+ T cells a 33-fold increase, T regulatory cells (CD25+CD4+) a 118-fold increase, PD1+CD4+ T cells a 41-fold increase, and PD1+CD8+ T cells a 16-fold increase.

pSTAT5 Assay Reproducibility Between Analysts

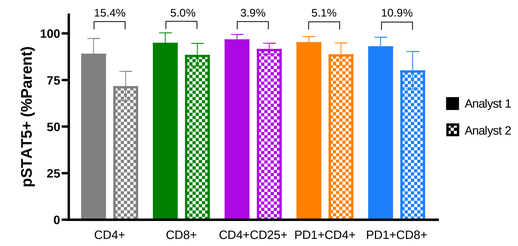


Figure 5. Inter-Analyst Comparison of %CV Between pSTAT5+ T Cells
To measure assay reproducibility, two analysts simultaneously processed blood and compared percent coefficient of variance (%CV) for each replicate. The average %CV of six animals was reported for each pSTAT5+ T cell population and met acceptance criteria of %CV≤20-30%. Total T cells, NK cells, CD4+ T cells, CD8+ T cells, T regulatory cells, PD1+CD4+ T cells, and PD1+CD8+ T cells met acceptance criteria. One of six animals had a %CV above acceptance criteria in Total T cells. These data indicate that this assay is highly reproducible between analysts in both pSTAT5+ cell populations and pSTAT5- populations.

pSTAT5 Assay FMO Controls for All Fluorophores in Panel

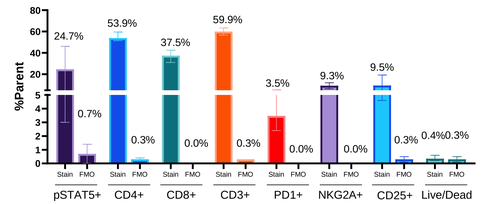


Figure 6. pSTAT5 Immunophenotyping Flow Cytometry Panel FMOs and Full Stain
To test for fluorescent background spread in the multicolor immunophenotyping panel, FMOs were tested for each antibody. Blood was pooled from multiple animals, and each FMO was tested twice. Average % parents and range is shown above. All FMOs met the internal standard of ≤2% parents, indicating low background interference in our panel.

pSTAT5 Activation in PB Versus PBMCs

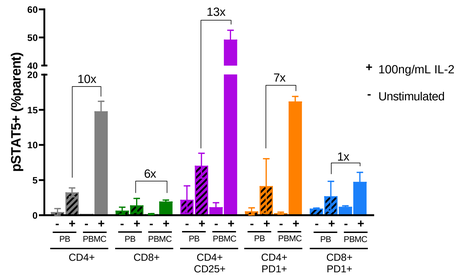


Figure 7. Comparison of pSTAT5 Activation Between PB and PBMCs
PB or commercially purchased naïve cynomolgus PBMCs were stimulated with recombinant IL-2 for 15 minutes at 37°C and compared to their unstimulated counterpart. Fold activation (%Parent$_{stimulated}$/ %Parent$_{unstimulated}$) was determined for PB and PBMCs, then fold change was determined between PB and PBMCs (Fold activation$_{PBMCs}$/Fold activation$_{PB}$). Usage of PBMCs rather than PB resulted in a 10-fold increase in CD4+ T cell activation, 6-fold increase in CD8+ T cell activation, 13-fold increase in CD4+CD25+ T cell activation, 7-fold increase in PD1+CD4+ T cell activation, and no change in PD1+CD8+ T cell activation.

CONCLUSIONS

A novel flow cytometry panel to detect pSTAT5+ T cell populations in the blood of *Macaca fascicularis* was developed. This flow cytometry panel offers a promising new way to quantify T cell proliferation and can be used for ongoing and future preclinical studies in *Macaca fascicularis*. The following were shown for this assay:

1. **Feasibility** was confirmed in PB by comparing endogenous pSTAT5+ with and without IL-2 stimulation, which indicated a significant upregulation in pSTAT5 in T cell subsets after stimulation.
2. **Reproducibility** was confirmed between separate analysts for all cell populations in the immunophenotyping panel.
3. **Panel quality** was demonstrated by little fluorescent spread between cell populations in the antibody panel.
4. **pSTAT5 activation was compared between PB and PBMCs** and showed that PBMCs had higher levels of pSTAT5 after IL-2.