Application Note

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Analysis of Phosphorylated STAT Protein Signaling in Lymphocytes Using Flow Cytometry

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Abstract

This application note examines the phosphorylation of signal transducer and activator of transcription (STAT) proteins in response to cytokine stimulation using a flow cytometry–based *phosphoflow* technique and the Agilent NovoCyte flow cytometer. The results demonstrate the efficacy of this approach for the rapid, quantitative, scalable, and multiparameter detection of phosphorylated protein in single cells.

Introduction

Protein phosphorylation is the biological process of transferring a phosphate group to a substrate protein, which primarily occurs on tyrosine, serine, and threonine residues. Protein phosphorylation can cause conformational changes, changes in protein activity, or protein-protein interactions. This event can also initiate a phosphorylation signaling cascade leading to a sequence of protein phosphorylation events.

This produces an assortment of kinases and phosphatases, which regulate intracellular protein phosphorylation. Protein phosphorylation is crucial for many cellular events including T and B cell signaling, cell metabolism, cell growth, apoptosis, and other processes.

Cytokines are a group of small secreted proteins important for immune cell-to-cell communication, immune cell activation, differentiation, and migration towards the site of inflammation/infection. Cytokines bind to receptors on the cell surface and activate intracellular cascades, such as the Janus kinase (JAK)/STAT pathway. STATs are the final phosphorylated protein in this cascade; they translocate to the nucleus and alter gene expression. There are many methods for detecting intracellular protein phosphorylation, including radiometric kinases assays, western blotting, and mass spectrometry. Western blotting is the most commonly used but has shortcomings; it is semiquantitative, time-consuming, and requires a large amount of starting material. Also, cell separation may be required to isolate a pure population of a cells from a heterogeneous mixture.

Analysis of phosphorylated proteins by flow cytometry or phosphoflow was first described in the early 2000s. By combining a phosphoflow methodology with cell surface antibody staining, rare cell populations can be studied in the presence of other cell types. Multiple cell types can also be analyzed in one sample.

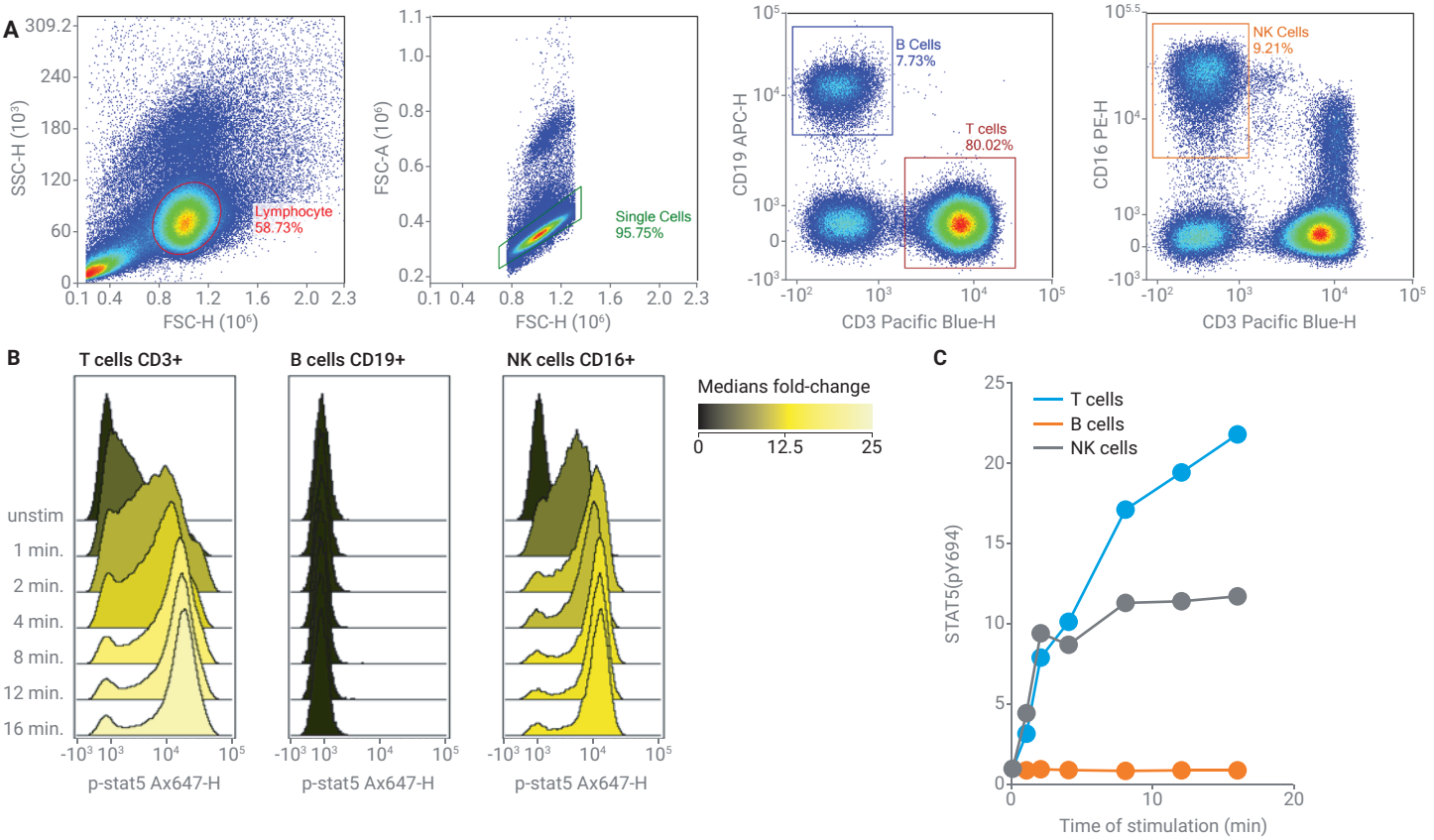

Figure 1. Time-dependent differences in IL-2–induced STAT5 phosphorylation among lymphocyte subsets. PBMCs were isolated from the blood of a healthy donor. Cells were stained with αCD19 FITC antibody before IL-2 stimulation. PBMCs were then stimulated with 100 ng/mL IL-2 for 0 to 16 minutes at 37 °C. After cytokine stimulation, cells were fixed in 1.5% paraformaldehyde for 10 minutes at room temperature and pelleted by centrifugation. Thereafter, cells were permeabilized with ice cold 100% methanol for 15 minutes. Finally, cells were stained with αCD16 PE, αCD3 Pacific Blue, and α-p-STAT5 AlexaFluor647 for one hour. The main cell population was identified by FSC-H and SSH-H. Single cells were than identified in the FSC-H and FSC-A plot to eliminate doublets. Cell surface stains identified the lymphocyte populations: CD19 B cells, CD3 T cells, and CD16 NK Cells. Data were analyzed using the CytoBank website for histogram overlay and heat map analysis (B). Changes in p-STAT5 expression were graphed as the fold change/time compared to the unstimulated sample for each lymphocyte population (C).

IL-2 induces potent p-STAT5 signaling in T and NK but not B lymphocyte cell populations

The phosphorylation of STAT5 in peripheral blood mononuclear cells (PBMCs) when induced by IL-2 was investigated to examine B, T, and NK lymphocyte cell populations. The degree of phosphorylation was determined by phosphoflow, in which a phosphorylated protein-specific antibody is used to determine the levels of p-STAT5 (Y694). PBMCs were either stimulated with IL-2 or IL-4 for a varying amount of time or concentration of cytokine. Following a typical fixation/permeabilization protocol for phosphoflow, cells were first fixed in 1.5% paraformaldehyde to stabilize the cellular structure. This was followed by permeabilization with 100% methanol to allow the phospho-specific antibodies to stain inside the cell. Surface stains identified specific population of lymphocytes: CD19 for B cells, CD3 for T cells, and CD16 for Natural Killer (NK) cells as shown in the sample gating plots (Figure 1). Since the CD19 epitope was destroyed upon exposure to formaldehyde, the PBMCs were stained with αCD19 antibody before cytokine stimulation.

PBMCs were treated with 100 ng/mL of IL-2 from 0 to 16 minutes, stained for p-STAT5, and then analyzed on the NovoCyte flow cytometer (Figure 1). p-STAT5 levels varied greatly in T, B, and NK cell populations. Both T and NK cells responded, while B cells did not show any indications of response, even when measured at the longest time point. Both T cells and NK cells responded rapidly, showing a 4.5- and 3.2-fold increase, respectively, in p-STAT5 levels within one minute of IL-2 addition.

Although time-dependent increases were sustained in T cells, no further increases in p-STAT5 were seen in NK cells after eight minutes. This result can be explained by the differential expression of IL-2 receptors in lymphocyte populations, as B cells express relatively few IL-2 receptors compared to T and NK cells. IL-2 receptor expression is especially high in activated T cell populations that are particularly reliant on IL-2 signaling for cell survival and proliferation.

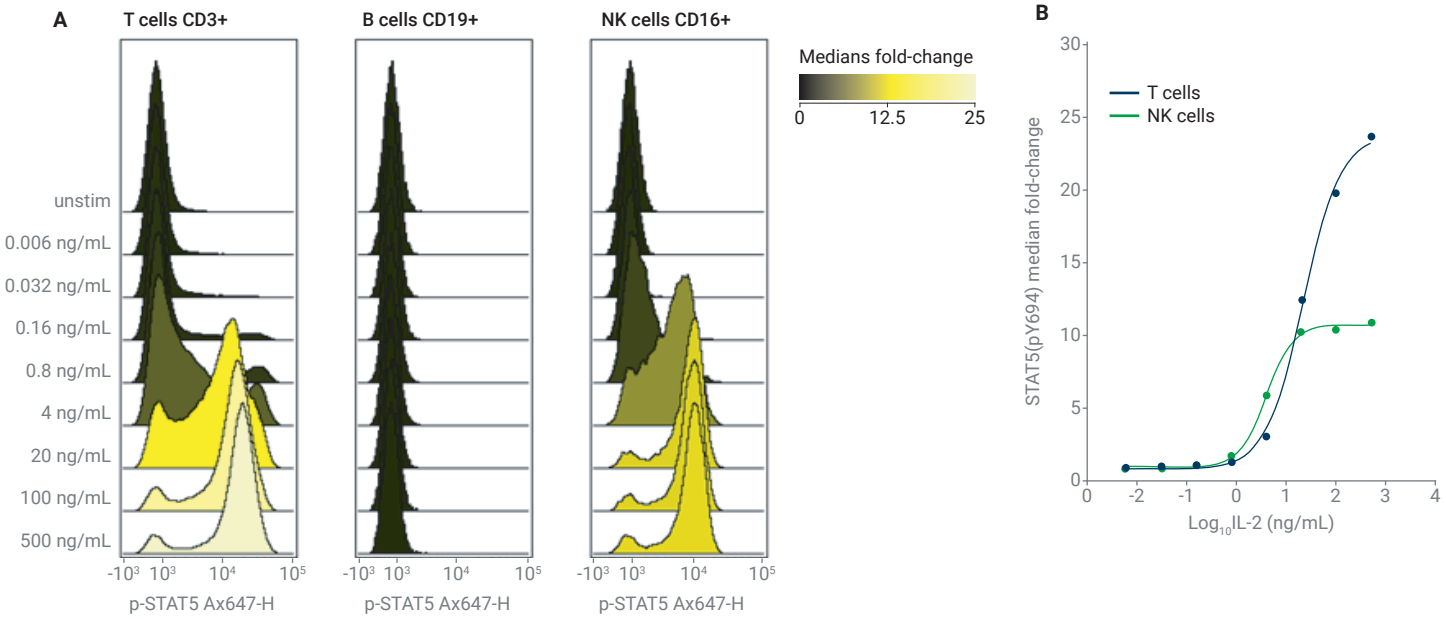

Figure 2. Dose-response analyses reveal differences in IL-2–induced STAT5 phosphorylation among lymphocyte subsets. PBMCs were isolated and stained with αCD19 FITC, as described in Figure 2. Cells were stimulated with IL-2 at a concentration of 0 to 500 ng/mL for 15 minutes. PBMCs were then fixed and permeabilized, as described in Figure 2. Data was analyzed using the tools provided by the CytoBank website for histogram overlay and heat map analysis of p-STAT5 phosphorylation results (A). Changes in p-STAT5 expression were then recorded as fold change compared to unstimulated sample, with increasing concentration of IL-2 for the T and NK cell population (B). EC50 values of STAT5 phosphorylation of T and NK cells were determined by Prism 6 software. B cells were not graphed as they did not respond to IL-2 stimulation.

Next, the phosphorylation of STAT5's dose-dependent response to IL-2 in PBMCs was investigated. Cells were treated with 0 to 500 ng/mL of IL-2 for 15 minutes followed by p-STAT5 analysis in each lymphocyte subset (Figure 2). A stronger dose-dependent response for T cells was observed than that of NK cells. While NK cells did not respond to increases in IL-2 after a concentration of 20 ng/mL, increases in p-STAT5 levels in T cells were seen at the highest concentration tested, 500 ng/mL; the calculated EC50 values of STAT5 phosphorylation of IL-2 stimulated T cells and NK cells were 21.57 ng/mL and 3.89 ng/mL, respectively (Figure 2B). The observed by p-STAT5 analysis by flow cytometry showed that T cells respond faster and stronger to IL-2 stimulation than the other lymphocyte cell subsets.

IL-4 induces robust p-STAT6 signaling in all lymphocyte cell populations

IL-4 is another important cytokine in the immune response vital for T cell differentiation, immunoglobulin class switching in B cells, and alternative macrophage activation. IL-4 is primarily secreted by activated T cells, basophils, and mast cells. IL-4 signaling results in a rapid increase in phosphorylation of STAT6 and plays an important role in the adaptive immune response of helper T cells and B cells.

In this next experiment, we examined the dose-dependent p-STAT6 response of lymphocyte populations to IL-4. PBMCs were treated with 0 to 500 ng/mL of IL-4 for 15 minutes, followed by analysis of p-STAT6 expression at the Y641 residue in T, B, and NK cell populations (Figure 3). All lymphocyte populations responded to IL-4, but B cells were the most sensitive, displaying a significant increase in p-STAT6 with as little as 0.16 ng/mL of IL-4. No significant increase was seen in T and NK cells until IL-4 concentration reached 4 ng/mL.

The p-STAT6 response in B cells also became saturated at a much lower concentration of IL-4 (20 ng/mL), while in T and NK cells, the saturation of p-STAT6 abundance was observed up to 100 ng/mL. This was underscored by the EC50 values calculated for the lymphocyte populations: B cells 2.58 ng/mL, T cells 17.51 ng/mL, and NK cells 19.39 ng/mL (Figure 3B). Using p-STAT6 as a readout for IL-4 stimulation showed B cells much more sensitive than T and NK cells.

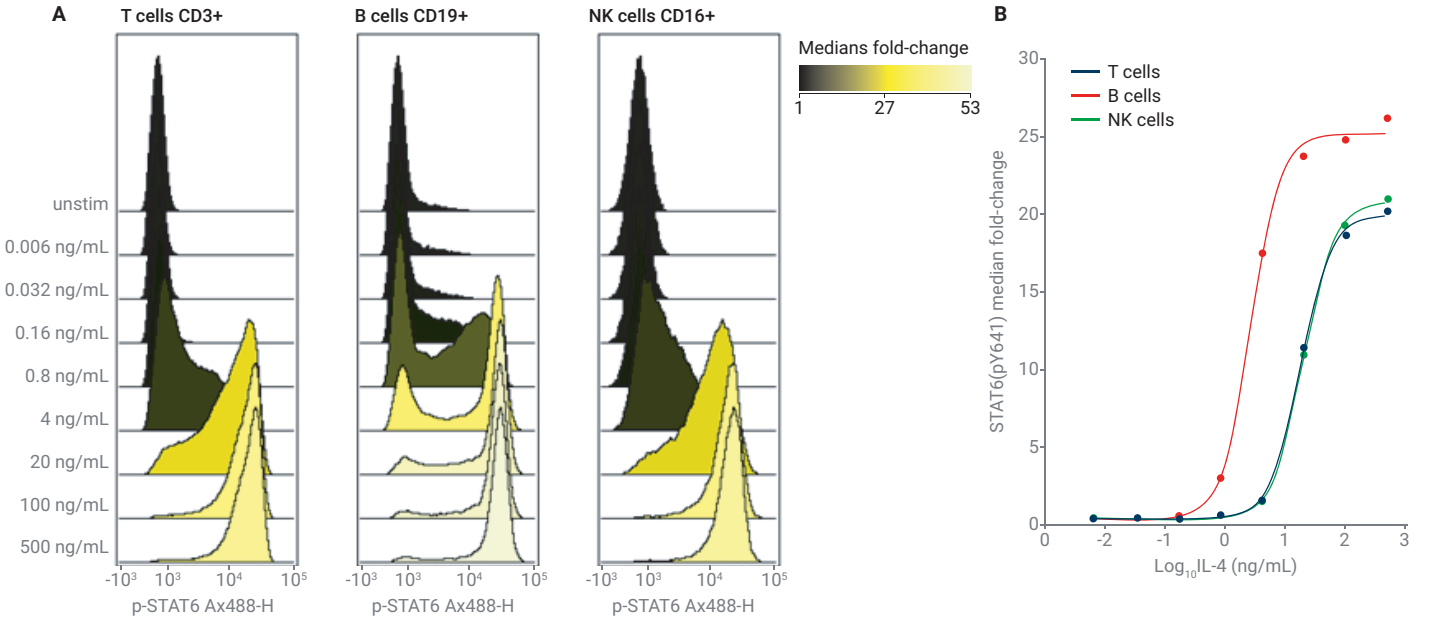

Figure 3. Dose-response analyses reveal differences in IL-4-induced STAT6 phosphorylation among lymphocyte subsets. PBMCs were isolated from the blood of a healthy donor. Cells were stained with αCD19 APC antibody before IL-4 stimulation. PBMCs were then stimulated with 0 to 500 ng/mL IL-4 for 15 minutes at 37 °C. After cytokine stimulation, cells were rapidly fixed in 1.5% paraformaldehyde for 10 minutes at room temperature and then pelleted by centrifugation. Thereafter, cells were permeabilized with ice cold 100% methanol for 15 minutes. Finally, cells were stained with αCD16 PE, αCD3 Pacific Blue, and α-p-STAT6 AlexaFluor 488 for one hour. Cells were washed to remove excess antibody and then analyzed on the NovoCyte flow cytometer. Data were analyzed using the tools provided by the CytoBank website for histogram overlay and heat map analysis of p-STAT6 phosphorylation results (A). Changes in p-STAT6 expression were recorded as fold change compared to unstimulated sample for each lymphocyte population (B). EC50 values of STAT6 phosphorylation were determined by Prism 6 software.

Tips for optimal phosphoflow staining

Phosphoflow requires a specialized protocol for intracellular antibody staining that differs from those typically performed. Optimization may be required. Attention to the following considerations will help you determine the best protocol for your experiment. Since the proteins are not on the cellular surface, fixation and permeabilization of the cell is required. Many phospho-specific antibodies are not compatible with detergent-based permeabilization methods most commonly used for intracellular staining. Therefore, special attention is necessary to determine the best fix/perm method for your specific phospho-specific antibody.

Typically, cells need to be rapidly fixed to avoid dephosphorylation and stronger permeabilization methods may be needed to permeabilize the nuclear membrane. The method in the following experiments uses 1.5% paraformaldehyde for fixation, and 100% methanol for permeabilization. Although this method will not work for every phospho-specific antibody, it is the most prevalent fix/perm method and works for many antibodies. Most phosphorylated protein stains are coupled with surface stains to identify various cell populations in a heterogeneous sample. Special consideration must be taken for the sensitivity of these epitopes to fixation because some are destroyed when exposed to fixative. The sample may require staining with certain specific surface markers before fixation, as shown in the experiments described here (all samples were prestained with αCD19 antibody before cytokine stimulation). Finally, if a surface stain is required before fixation, it is important to use antibodies conjugated to methanol-resistant fluorochromes. Many common fluorochromes, such as PE and APC, are destroyed when exposed to methanol. By keeping these factors in mind, labs can achieve optimal phosphoflow staining.

Conclusion

Using flow cytometry for analysis of phosphorylated protein levels in PBMCs is easy, rapid, and allows labs to quantitate the phosphorylation levels of T, B, and NK lymphocytes in single cells, all in the same sample. Phosphoflow is an excellent methodology for the analysis of various cell types, including primary cells and cell lines. Phosphoflow allows the quantification of complex cell signaling events and has been widely accepted in labs for drug screening, efficacy analysis, and the study of altered signal transduction in disease research, as well as other basic and clinical research. Including phosphoflow analysis to your investigations allow further understanding of relevant cell signaling pathways at the single cell level, which is not possible with traditional assays measuring phosphorylation.

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