Luminex

Application Note

Analysis of STAT Protein Phosphorylation in CD4+ T Cells Using the Amnis® CellStream® Flow Cytometer

Christine Probst, Raymond Kong, Peter Rhein, Stephanie Brunelle, and Phil Morrissey

Introduction

Cytokine-induced protein phosphorylation is a key cell signaling event within the immune system that modulates diverse functions, including proliferation, differentiation, apoptosis, and migration. The degree of protein phosphorylation is determined by both the cytokine dose, as well as the number of cytokine-specific receptors on the cell surface, which are differentially expressed across immune cell populations.

Flow cytometry is a powerful method to study the orchestrated response of immune cell subsets to stimulation by combining immunophenotyping markers with fluorescent antibodies specific to phosphorylated signaling molecules. However, because many of the phosphorylation events occur on weakly expressed proteins and with low stoichiometry, flow cytometers must have high sensitivity to track important changes in signaling magnitude. Flow cytometers with sensitive detection, such as the Amnis® systems, can be used to study phosphorylation events that are key to understanding the processes required for vaccine and immunotherapy development.

In this application note, we used the Amnis® CellStream® Flow Cytometer to monitor the phosphorylation of signal transducer and activator of transcription (STAT) proteins—transcription factors that migrate to the nucleus upon phosphorylation and alter gene expression. STAT3, STAT5, and STAT6, which have been associated with prognosis in cancer and other malignancies, were simultaneously monitored in CD4+ T cells.

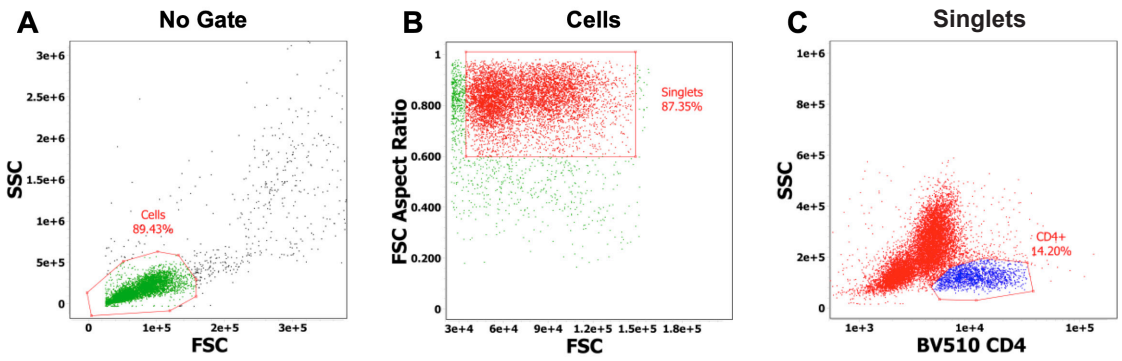
Methods

Stock aliquots of IL-2, IL-4, and IL-6 (4 µg/mL) were prepared in 1 mg/mL FBS/PBS (BD Biosciences) and frozen at -80°C until the day of the experiment. Fresh whole blood collected from healthy human donors was received the day of the experiment. 200 µL blood was stimulated using 15 µL of each interleukin (IL-2, IL-4, IL-6), as well as a mixture of IL-2, IL-4, and IL-6 for 15 min at 37°C (25 ng/mL final concentration in blood). Red blood cells were lysed and leukocytes were fixed using 1X Phosflow™ Lyse/Fix Buffer (BD Biosciences) for 8 minutes at 37°C. Cells were washed and then permeabilized using BD Phosflow™ Perm Buffer III for 30 minutes on ice. Cells were washed and then stained with a panel of fluorescently labeled antibodies against CD4 (BV510), pSTAT6 (AF488), pSTAT3 (AF647), and pSTAT 5 (PE) for 30 minutes at RT. Cells were then washed and resuspended in 300 µL of 2% FBS/PBS and data was collected on a CellStream® Flow Cytometer within four hours. Forward scatter (FSC) and side scatter (SCC) illumination were both set to 25% laser power, and 405 nm, 488 nm, 561 nm, and 642 nm lasers were all set to 100%. 20,000 single cell events were collected in Slow Mode to maximize sensitivity. Compensation controls for each fluorescent antibody were prepared using antibody capture beads, and 5,000 events were collected for each compensation control. Compensation was calculated and data analysis was performed using CellStream Analysis Software.

Results

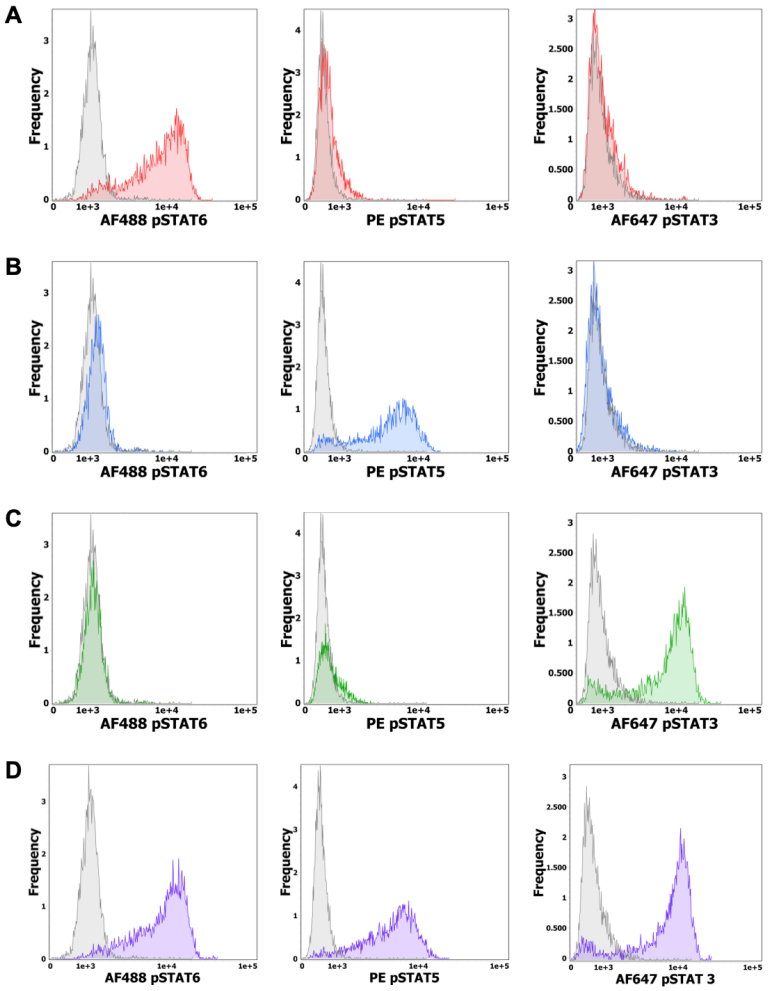
To identify CD4+ cells within human whole blood, cells were selected using FSC vs. SSC (**Figure 1A**). Single cells were identified using a plot of FSC vs. FSC Aspect Ratio (**Figure 1B**). CD4+ T cells were identified in a plot of BV510 CD4 vs. SSC (**Figure 1C**).

Figure 1. Gating strategy for the identification of cells (**A**), single cells (**B**), and CD4+ T cells (**C**).



CD4+ cells undergo differential phosphorylation of STAT proteins in response to IL-4 (**Figure 2A**, red), IL-2 (**Figure 2B**, blue), IL-6 (**Figure 2C**, green), or a mixture of IL-2/IL-4/IL-6 (**Figure 2D**, purple). Unstimulated blood was used as a negative control and is overlaid in gray on each plot. As expected, cytokine-specific phosphorylation was observed for STAT6 in response to IL-4, STAT5 in response to IL-2, and STAT3 in response to IL-6. Co-stimulation with IL-2/IL-4/IL-6 resulted in the phosphorylation of all three STAT proteins.

Figure 2. Phosphorylation of STAT6, STAT5, and STAT3 in CD4+ cells in response to stimulation with IL-4 (**A**), IL-2 (**B**), IL-6 (**C**), and IL-2/IL-4/IL-6 (**D**). Unstimulated blood is overlaid in gray.



Summary

Flow cytometry is a powerful method to monitor protein phosphorylation signaling cascades within the context of cell type, and has facilitated a mechanistic understanding of the choreographed response of immune cell subsets to cell activation. Instruments with high sensitivity are needed to reliably measure low-abundance and phosphorylation events in canonical cellular signaling pathways. In this study, the CellStream Flow Cytometer was used to simultaneously monitor the selective phosphorylation of STAT6, STAT5, and STAT3 in response to cytokine stimulation. The CellStream System achieved robust detection of multiple phosphorylation markers simultaneously, demonstrating its capability in evaluating weakly expressed antigens or subdominant epitopes as required for the development of vaccines and immunotherapies.

Luminex®
complexity simplified.

For more information, please visit: luminexcorp.com/cellstream-flow-cytometers/

luminexcorp.com

HEADQUARTERS
UNITED STATES
+1.512.219.8020
info@luminexcorp.com

EUROPE
+31.73.800.1900
europe@luminexcorp.com

CANADA
+1.416.593.4323
info@luminexcorp.com

CHINA
+86.21.8036.9888
infocn@luminexcorp.com

JAPAN
+81.3.5545.7440
infojp@luminexcorp.com

FL249514