Effect of Anti-Cancer Drugs on Expression of Intracellular Phosphoproteins in Patient Samples from Phase I/II Clinical Trials

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ABSTRACT

Understanding the signaling events mediated by intracellular protein phosphorylation in both normal lymphocytes and cancer cells will shed light on the mechanisms of immune regulation and aid in the development of novel immune regulatory and anti-cancer agents/drugs. Phospho-specific flow cytometry provides a powerful technique for rapid, sensitive, multiparameter analyses of intracellular phosphorylated signal proteins in immune cells at a feasible, cost-effective, high-throughput and robust manner.

We have developed and qualified a flow-based platform to analyze the intracellular phosphoproteins involved in cell signaling events in primary immune cells using either whole blood (WB), peripheral blood mononuclear cells (PBMCs), bone marrow (BM), or leukemic cancer cells. The assay development and optimization includes technical considerations for antigen accessibility/expression, stability of the phosphorylated epitope, fluorophore selection, surface phenotype integrity, and antibody suitability for detecting the surface and intracellular proteins in fixed and permeabilized cells. Using our phosphoflow assay, we have evaluated the effects of immune checkpoint inhibitor (Drug A) or a dual kinase inhibitor (Drug B) on the expression of intracellular phosphorylated proteins, namely, pCREB, pSYK, pERK, pSTAT3 in T and B lymphocytes. This *in vitro* immune monitoring assay for detection of phosphoproteins in immune cells enables the analysis of the effect of drug compounds and helps indicate their potential use as anti-cancer agents. The method was also used to detect pSTAT5 and pS6 expression in leukemic AML cells, and a candidate dual kinase inhibitor (Drug C) was found to decrease the expression of pSTAT5 and pS6 intracellular cell signaling proteins.

The phosphoflow-based platform developed in our lab offers a powerful technique to study intracellular signal transduction events in immune cells and supports the clinical development of anti-cancer drug compounds. Furthermore, this phosphoflow method has been qualified in compliance with GxP guidelines for use with both preclinical and clinical samples.

INTRODUCTION

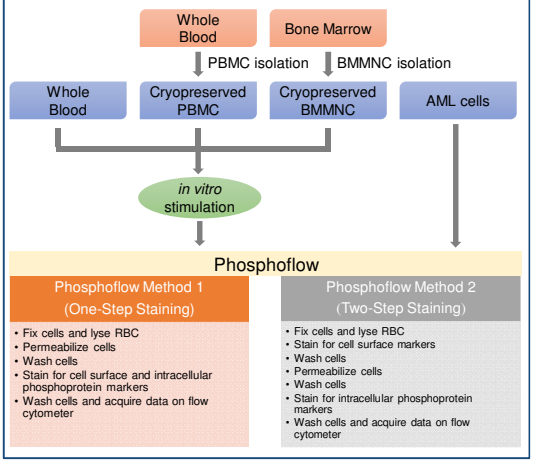
The phosphorylation of intracellular proteins in response to external stimulation is critical for the control of T and B cell signals for various cellular events, including cell activation, cell growth, apoptosis and cell cycle. The emerging phosphoflow method, used for the detection of these intracellular phosphoproteins, demonstrate numerous advantages over other techniques and provides a much more powerful tool with a larger dynamic range of data collection, rapid protocols, analysis of multiple epitopes in a single cell, and analysis in complex cell populations of peripheral blood, bone marrow or solid tissue samples.

However, there are technical challenges in the development of phosphoflow assays, especially when these assays are required for analysis of clinical study samples. The main challenges include:

a. Protein phosphorylation is a transient phenomenon, and cells need to be fixed quickly to maintain the phosphorylated state of the protein (phosphoepitope) in a cell.
b. The method requires intracellular staining of the cells after permeabilization to expose the phosphoepitopes and allow fluorescent conjugated antibodies to get into cells. The process of fixation and permeabilization may cause denaturation of some target proteins, especially cell surface markers. These technical requirements necessitate strenuous efforts to screen different antibodies and permeabilization buffers to optimize an assay method which involves the detection of defined biomarkers of interest.

We report here the development and optimization of a flow cytometry approach to detect key intracellular phosphoproteins like pCREB, pSYK, pS6, pSTAT3, and pSTAT5 in T and B immune cells and in tumor (AML) cells. These qualified flow-based assays have been used to analyze and monitor the effect of drug compounds (checkpoint drug inhibitors or dual kinase inhibitors) on immune cells from patients enrolled in Phase I/II clinical study programs. Detection of key phosphoprotein biomarkers enables the sensitivity of tumor cells to specific drug compounds to be discerned.

METHODS



PHOSPHO-SPECIFIC FLOW CYTOMETRY

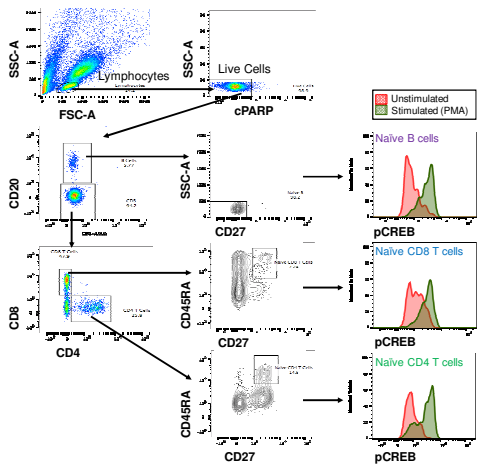


Figure 1.
Multiparametric FACS analysis of cell surface and intracellular phosphoprotein biomarkers. The FACS plots illustrate an example of the gating strategy to identify the different immune cells of interest (based on the expression and detection of cell surface markers). The phosphorylated intracellular protein expression is detectable for each of the defined immune T and B cell subsets.

DRUG A: IMMUNE CHECKPOINT INHIBITOR

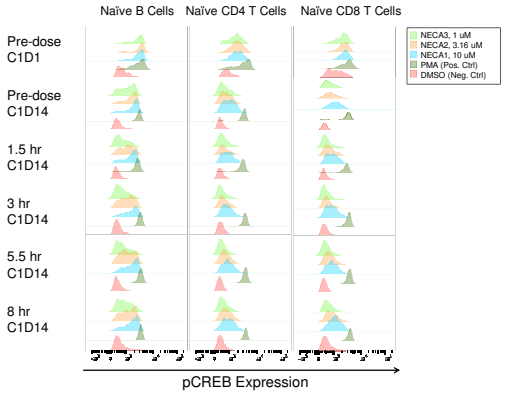


Figure 2.
Inhibition of pCREB expression in naïve B cells, CD4+ T cells and CD8+ T cells in whole blood samples from a tumor patient after treatment with Drug A at varying points in the clinical study.
The fluorescence peaks shift to the left, indicating decreased expression of the phosphoprotein, pCREB on Day 14 compared to baseline expression on Day 1.

DRUG B: DUAL KINASE INHIBITOR

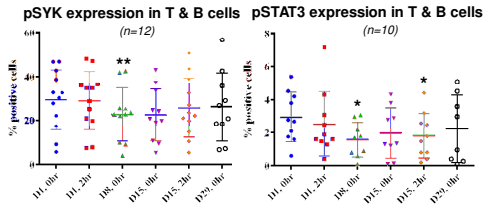


Figure 3.
Inhibition of pSYK and pSTAT3, in whole blood samples obtained from tumor patients enrolled in Phase I/II clinical study, in response to treatment with Drug B. The detection and expression of the two phosphoproteins is significantly decreased on Day 8 samples, compared to baseline expression of the markers, after drug administration *in vivo*. (pSYK, n=12, $p < 0.01$; pSTAT3, n=10, $p < 0.05$)

DRUG C: DUAL KINASE INHIBITOR

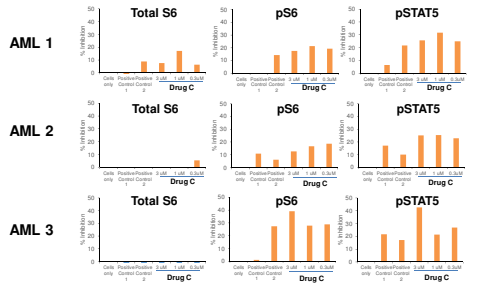


Figure 4.
Detection of phosphoproteins in immune cells from three different AML patients. Whole blood samples were treated *in vitro* with Drug C, a dual kinase inhibitor, and expression of the defined biomarkers monitored. Inhibition of pS6 and pSTAT5 in whole blood samples from three different AML patient samples. Each patient responds to drug with varying degree of inhibition, as determined by the expression of the signaling proteins pS6 and pSTAT5.

DISCUSSION AND CONCLUSIONS

- A qualified and robust phosphoflow assay platform has been developed to detect the expression of phosphorylated proteins pCREB, pSYK, pS6, pSTAT3, and pSTAT5 in immune cells and leukemic cells. The assay is used to monitor detection and expression of these phosphoproteins from patient blood samples to help determine the efficacy of signaling pathway-specific drugs *in vivo* or characterize aberrant signaling events in tumor cells.
- Immune checkpoint inhibitor Drug A decreased pCREB expression in both B cells and T cells from patients enrolled in Phase I clinical study.
- Dual kinase inhibitor Drug B inhibited the expression of pSTAT3 and pSYK in T cells and B cells.
- Dual kinase inhibitor Drug C inhibited pSTAT5 and pS6 expression in immune cells from AML patients.
- The phosphoflow-based assay developed and qualified in our lab is used to analyze and monitor immune cell sensitivity to checkpoint drug inhibitors or dual kinase inhibitors in patients enrolled in clinical trials designed for novel cancer drug compounds.

ACKNOWLEDGMENTS

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