5667. High-Throughput Analysis of MAPK and JAK-STAT Signaling in CT26 Tumors Using a Combination of Immunophenotyping and Phospho-Flow Cytometry

David Draper, Alden Wong, Philip Lapinski, Sheri Barnes, Maryland Rosenfeld Franklin and Scott Wise; Labcorp Early Development Laboratories Inc., Ann Arbor, Michigan

Introduction and Background

- The development of robust platforms that enable phospho-protein-based analysis of cell signaling in the tumor microenvironment is pivotal for small molecule drug development. Currently lacking are reliable applications that can simultaneously measure multiple phospho-proteins in both tumor cells and immune cells *ex vivo*. This can be crucial because many signaling pathways are commonly involved in both pro- and anti-tumor responses in these two cellular compartments.
- Labcorp has validated a phospho-flow cytometry-based platform that measures the phospho-protein levels of multiple signaling proteins in separate and distinct subsets simultaneously, which can include solid tumor-derived immune subsets and tumor cells.
- The Labcorp phospho-flow platform can be applied to the analysis of treated cell lines and heterogeneous tissue-derived cell cultures *in vitro* as well as the *ex vivo* analysis of inflamed tissue from pharmacology studies.
- In this presentation, we demonstrate how these services can be used to analyze MAPK and JAK-STAT pathway-associated kinases *in vitro* in cultured MV-4-11 acute myeloid leukemia (AML) cells and activated murine splenocytes, as well as *ex vivo* in both tumor-derived CD8⁺ T cells and tumor cells using the CT26 model for colorectal carcinoma.

Materials and Methods

- To analyze *in vitro* treatment-induced effects on cellular phospho-protein levels, MV-4-11 cells or BALB/c splenocytes were seeded into multi-well plates and stimulated as indicated. Following incubation the cells were fixed and permeabilized, and then stained with fluorescent directly conjugated phospho-specific antibodies. For analysis that included immunophenotyping to delineate subsets, cells were stained for cell surface markers prior to fixation.
- For *ex vivo* solid tumor phospho-flow analysis, CT26 cells were implanted subcutaneously on the flank of BALB/c hosts. After enrollment into groups, animals were dosed with trametinib and a murine PD-L1 directed antibody (anti-mPD-L1) either alone or in combination as indicated (Figure 3). On the day of the last dose, tumors were harvested and processed into single cell suspensions using the gentleMACS™ system and associated reagents (Miltenyi, Germany).
- All samples were acquired on an Attune™ NxT Flow Cytometer (Thermo Fisher Scientific, Waltham, MA). Data was analyzed using Flowjo software (Treestar Inc., Ashland, OR). Where indicated a fluorescence minus one (FMO) control was included to quantitate the phospho-protein level magnitude under each condition.
- Phospho-protein analysis was quantified by group median fluorescence intensity (MFI) and standard deviation measurement. Where indicated, statistical analysis was performed using a Student's T-test (* $p<0.05$).

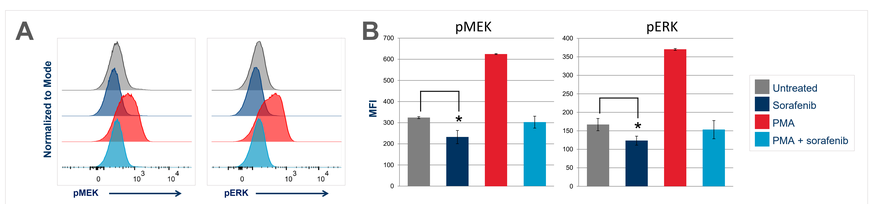

Figure 1. Phospho-flow analysis of sorafenib-induced inhibition of pMEK/ERK protein levels in MV-4-11 cells *in vitro*. Sorafenib treatment blocks the PMA-induced increase in phospho-MEK/ERK levels. A) Histogram overlays displaying data from single representative samples. B) Phospho-MEK and phospho-ERK levels from 3 independent experiments that were combined.

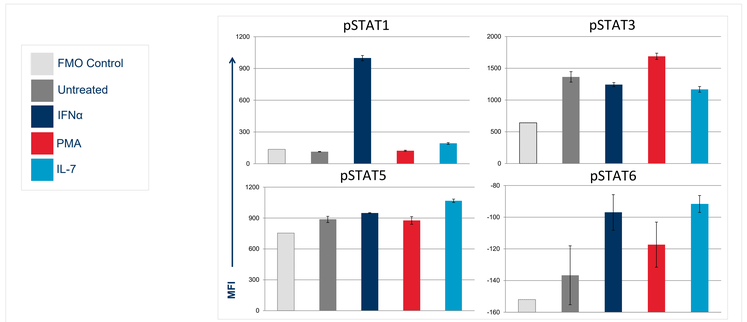

Figure 2. *In vitro* phospho-flow analysis of JAK/STAT signaling in CD8⁺ T cells. Phospho-flow analysis of treatment-induced effects on phospho-STAT signaling protein levels. Data was acquired in triplicate and is representative of two independent experiments.

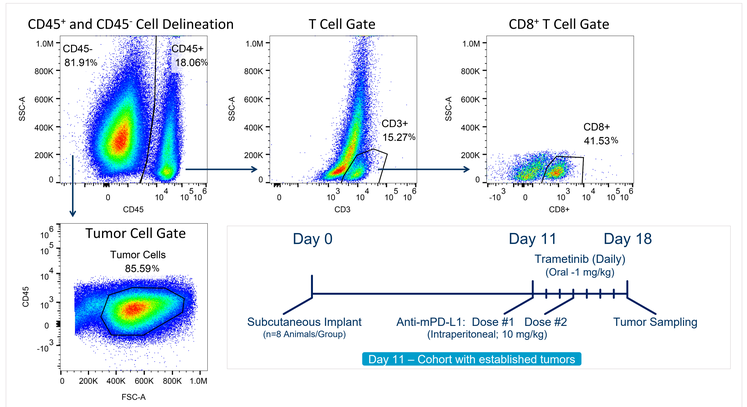

Figure 3. Delineation of CT26-derived tumor and infiltrating CD8⁺ T cells for simultaneous *ex vivo* phospho-flow analysis. The gating strategy above demonstrates the surface receptor immunophenotyping methodology that enables phospho-flow analysis in CD8⁺ T cells and tumor cells independently. The timeline illustrates the schedule for *in vivo* therapy administration and tissue sampling. Percentages indicate proportion of the parental gate.

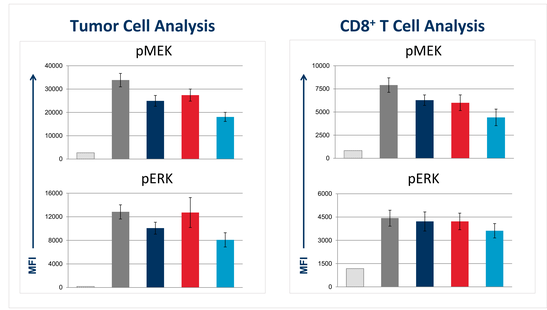

Figure 4. *Ex vivo* MAPK analysis in CT26 tumor-derived cells. *Ex vivo* analysis of phospho-MEK/ERK levels in tumor and infiltrating CD8⁺ T cells.

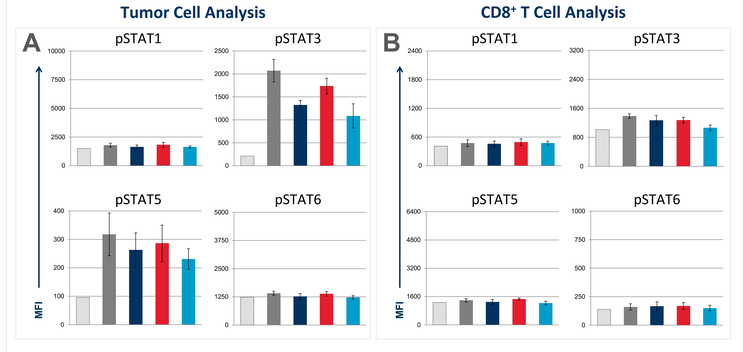

Figure 5. *Ex vivo* JAK/STAT analysis in CT26 tumor-derived cells. *Ex vivo* analysis of phospho-STAT levels in tumor and infiltrating CD8⁺ T cells.

Results and Conclusions

- The Labcorp phospho-flow platform can measure baseline phosphorylation levels of multiple signaling proteins *ex vivo* simultaneously, as well as differential effects that *in vivo* therapy has on multiple cellular compartments.
- *In vitro* phospho-flow assays produced data that is consistent and reproducible, which enables accurate measurements of small changes between groups that meet statistical significance.
- In this study we demonstrate successful phospho-protein analysis of two MAPK and four JAK-STAT proteins in a single sample.
- Reports have demonstrated that combined therapy with trametinib and checkpoint inhibition have greater efficacy than single agent treatment alone. Our results suggest that the combined effects may be attributed not only to MEK and STAT inhibition in tumor cell signaling, but also to a reduction in STAT3 phosphorylation levels in tumor infiltrating CD8⁺ T cells.

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