**Abstract**

Cancer immunotherapies that modulate the immune system and improve T-cell function have shown great promise. In non-hematological malignancies (e.g., Melanoma, NSCLC), novel investigational therapies with checkpoint inhibitors, bispecific antibodies or combination therapies can benefit from measuring immune cell subsets that correlate with treatment outcome in easily accessible peripheral blood. Balance of T-cell subsets (Naive vs. Memory), expression of checkpoint inhibitors (e.g., PD-1, TIM-3, LAG-3) prior to treatment or an increase in CD8+ T-cell proliferation post-treatment have been individually suggested to be predictors of clinical response. However, simultaneous measurement of all of these immune cell subsets in both pre- and post-treatment patient specimens has been technically challenging. We have developed a high-complexity (14-color), fit-for-purpose flow cytometry panel for concurrent detection of several crucial T-cell subsets and assessed its utility in metastatic melanoma patients treated with immunotherapies.

**Method Development**

1) Creation of specimens expressing all target biomarkers:
   a) PBMCs stimulated in vitro with aCD3, aCD8 and IL-2 (“PBMC-T”)
   b) PBMCs stimulated in vitro with IL-15 (“PBMC-15”)

2) Selection of optimal antibody clones, titrations and fluorochrome combinations:

3) Verification of assay specificity and gating strategy on internal positive and negative control populations:

**Validation Results Summary**

<table>
<thead>
<tr>
<th>Analytical Sensitivity (LLOQ)</th>
<th>Inter-assay Pass</th>
</tr>
</thead>
<tbody>
<tr>
<td>Precision Assessment (&lt; 25% CV above LLOQ)</td>
<td>Inter-Assay Pass</td>
</tr>
</tbody>
</table>

**Clinical Utility/Verification**

Clinical Specimens: PBMCs from melanoma patients (n=10) enrolled in a phase 2 clinical trial of high dose IL-2 treatment with or without Aflibercept (soluble decoy VEGF receptor).

**Results:** Compared to baseline, post-treatment specimens showed a measurable increase in proliferating T-cells: e.g. CD8+PD-1+Ki-67+ (< 25% CV above LLOQ)

**Conclusions & References**

We have successfully validated a novel and high-complexity (14-color) flow cytometry assay for concurrent assessment of multiple T-cell phenotypes in a single tube.

Implementation of the assay in clinical trials showed robust increases in multiple subsets of proliferating T-cells post-treatment, demonstrating the immediate clinical utility of the assay in human trials investigating T-cell modulating agents.