Analysis of Checkpoint Marker Expression on Immune Cells Using a 12-Color Assay on the BD FACSLyric™ Flow Cytometer

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Abstract

Introduction: Modulation of the inhibitory pathways that dampen the immune response may represent a major advance in modern cancer treatment. Antibodies that block ligation of immune checkpoint receptors, such as the programmed cell death protein 1 (PD-1) or CD274, have demonstrated acceptable toxicity and a durable antitumor response in some patients with advanced melanoma. Despite this success, only a subset of patients benefits from immune checkpoint blockade. The clinical impact of immune checkpoint blockade may be increased by careful assessment of checkpoint receptor expression patterns in patients, that may inform candidate selection or help to monitor clinical efficacy and adverse events in patients being treated with immunomodulatory drugs. Here, we demonstrate the potential of a comprehensive 12-color immune checkpoint panel using the BD FACSLyric™ platform and stimulated peripheral blood mononuclear cells (PBMCs).

Methods: Immune checkpoint receptor expression was evaluated using a 12-color antibody panel and the BD FACSLyric flow cytometer. PBMCs from healthy donors were cultured ex vivo with or without stimulation and the kinetics of immune checkpoint receptor expression measured. Results: Following ex vivo stimulation, PBMCs exhibited robust increases in immune checkpoint marker expression levels in both the T-cell compartment and the non-T-cell compartment. Our work suggests that the 12-color BD FACSLyric platform may be useful for characterizing and quantifying immune checkpoint receptor expression.

Conclusions: As immune therapeutic strategies in oncology advance, it will be important to accurately characterize, at the single-cell level, the dynamic immunophenotypic changes relevant to treatment success.

Multiparater flow cytometry allows detailed immune cell subsetting in patient diagnosis and disease monitoring. Here, we highlight the potential of the 12-color BD FACSLyric flow cytometer. We show that the BD FACSLyric enables users to acquire results with a high degree of informational content.

Methods

PBMC Culture Setup and Stimulation

Ninety-six-well plates were seeded with PBMCs isolated via density-gradient Ficoll separation at a concentration of 1 x 10^6 cells per well suspended in complete MMEM culture media. PBMCs were set up from three different donors (all normal). MMEM culture media were either left untreated (unstimulated control) or were supplemented with phylemughagglutinin (PHA) at concentrations of 3 or 6 μg/mL. Wells were set up in triplicate, and were incubated at 37°C, 5% CO2 for 24 hours and 48 hours (data not shown).

Flow Cytometry

After incubation for each of the designated time periods (24 and 48 hours), the cells in each well of the plates were collected and transferred to standard 12 x 75 mm tubes. The cells were washed twice with phosphate-buffered saline (PBS) and recovered via centrifugation (7 minutes at 300g). Cells were then stained for 30 minutes with the 12-color antibody panel shown in Table 1 below along with BD Horizon™ Brilliant Stain Buffer (Cat. No. 659611). BD Horizon Brilliant™ Violet (BV) and BD Horizon Brilliant™ Blue (BB) dyes were used in this study. After staining, cells were again washed twice with PBS and recovered via centrifugation (7 minutes at 300g). Samples were then analyzed on a preoperated 12-color (4-3-5) BD FACSLyric system, and data analysis was performed using BD FACSuite™ software.

Results (1)

Gating strategy: BD FACSLyric 12-color immune checkpoint marker assay

Figure 1. 12-color checkpoint panel-gating strategy

Cells were cultured for 24 and 48 hours. At the conclusion of each time point, cells were stained with the 12-color flow cytometry panel. Dead cells, B cells (CD19+), and monocytes (CD14+) were excluded from the analysis using a "dump" gating strategy (APC-Fluor 123). Lymphocytes were then identified based on FCS vs SSC. CD3 expression within the lymphocyte population identified T cells and CD68 expression identified monocytes. T cells were further divided into CD4+ T cells and CD8+ T cells. Expression of CD123 (OKB4), CD273 (PD-L2), CD274 (PD-L1), CD279 (PD-1), CD223 (LAG-3), CD366 (TIM-3), and CD252 (CTLA-4) was measured.

Figure 2. Expression of checkpoint markers following prolonged stimulation

Cells were stimulated with PHA at concentrations of 3 μg/mL and 6 μg/mL. Stimulated cells were compared against unstimulated controls. Expression levels of all six of the seven checkpoint markers measured showed activation induced increases in expression in at least one of the three target cell types measured. CD273 (PD-L2) demonstrated no measurable increases after stimulation and is not shown here. The 48-hour time point data (not shown), showed even higher levels of expression across all checkpoint markers, however there was also a large amount of activation-induced cell death. Each plot shows the percentage of each given cell type (CD4+ or CD8+ T cells or NK cells) that stained positive for a given checkpoint marker. These donors were tested: donor A (red line), donor B (blue line), and donor C (green line).

Table 3: 12-Color checkpoint panel

<table>
<thead>
<tr>
<th>CD4+ T Cells</th>
<th>CD8+ T Cells</th>
<th>NK Cells</th>
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<tbody>
<tr>
<td>CD274 (PD-L1)</td>
<td>CD123</td>
<td>CD279 (PD-1)</td>
</tr>
<tr>
<td>CD223 (LAG-3)</td>
<td>CD274 (PD-L1)</td>
<td>CD279 (PD-1)</td>
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<tr>
<td>CD366 (TIM-3)</td>
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<tr>
<td>CD252 (CTLA-4)</td>
<td>CD274 (PD-L1)</td>
<td>CD279 (PD-1)</td>
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References


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