

Analysis of immuno-oncology biomarkers using personal flow cytometry

Cell and cancer biology applications for the BD Accuri™ C6 Plus flow cytometer

Features

- Assess T-cell activation in heterogeneous cell populations
- Design multicolor panels with advanced bright dyes to detect and resolve low-density antigens
- Monitor the levels of multiple cytokines in one sample simultaneously
- The BD CSampler™ Plus option offers easy-to-use automation for walkaway convenience

Immuno-oncology has become the subject of intense scientific and therapeutic interest, as researchers probe the mechanisms by which cancer cells inhibit T-cell activation and escape T-cell attack, and explore methods of circumventing this inhibition. Because these research questions are inherently multivariate, involving the complementary push-and-pull signaling of activation and inhibition, they are ideal for multicolor flow cytometry, which can assess multiple parameters in heterogeneous cell populations at the single-cell level. On the BD Accuri™ C6 Plus personal flow cytometer, you can perform these studies right on your benchtop.

Co-inhibitory receptors on immune cells are crucial regulators of T-cell activation. This inhibition mechanism is a normal physiological process to attenuate T-cell activation, but it is also exploited by cancer cells to escape immune response. These receptors, including PD-1 (CD279) and LAG-3 (CD223), are upregulated on activated T cells. Both antigen-presenting cells and cancer cells can inhibit T-cell activation by binding PD-1 ligands, such as PD-L1 (CD274), to the PD-1 receptor.



Thus, PD-1 and PD-L1 are important targets for research on new cancer drugs. One research challenge is that both PD-1 and PD-L1 are variably expressed prior to cell activation, and may require bright dyes to detect and resolve clearly when expression is low.

Figure 1A shows levels of PD-1 and LAG-3 over selected intervals after stimulation of peripheral blood mononuclear cells (PBMCs) with anti-CD3 and anti-CD28. One would expect these activated cells to upregulate both molecules, and as shown in both the top and bottom panels, they did increasingly upregulate LAG-3 (paired with the bright fluorochrome PE) in each time interval. The results for PD-1, however, depended on the panel design. When PD-1 was paired with PerCP-Cy^{5.5} (lower plots), only marginal upregulation was observed. When PD-1 was paired with the bright new polymer dye BD Horizon Brilliant™ Blue 700 (BB700) (upper plots), the expected upregulation was clearly detected. BB700 was designed as a brighter alternative to PerCP-Cy^{5.5}, yet is detected in the same fluorescence channel (FL3).

Besides evaluating activation markers on the cell surface, T-cell effector function can be assessed by analyzing the cytokine content secreted by activated cells. Bead-based flow cytometric immunoassays such as BD™ Cytometric Bead Array (CBA) are powerful methods for quantifying cytokines because they allow researchers to multiplex many analytes with very little sample. For example, the BD™ CBA Human Th1/Th2/Th7 Cytokine Kit can quantitate IL-2, IL-4, IL-6, IL-10, TNF, IFN-γ and IL-17A simultaneously with a two-color assay. On the BD Accuri C6 Plus, the beads are excited by the red laser and detected in FL4, while the PE reporter is excited by the blue laser and detected in FL2. A free downloadable BD Accuri™ C6 Plus software template simplifies setup and acquisition.

Figure 1B shows a BD CBA cytokine analysis of PBMC culture supernatants, confirming T-cell activation by increased secretion of some of the cytokines analyzed: IFN-γ, TNF, IL-2, IL-10 and IL-6. The experiment was facilitated and streamlined by using the BD CSampler Plus accessory for automated analysis of multiple samples.

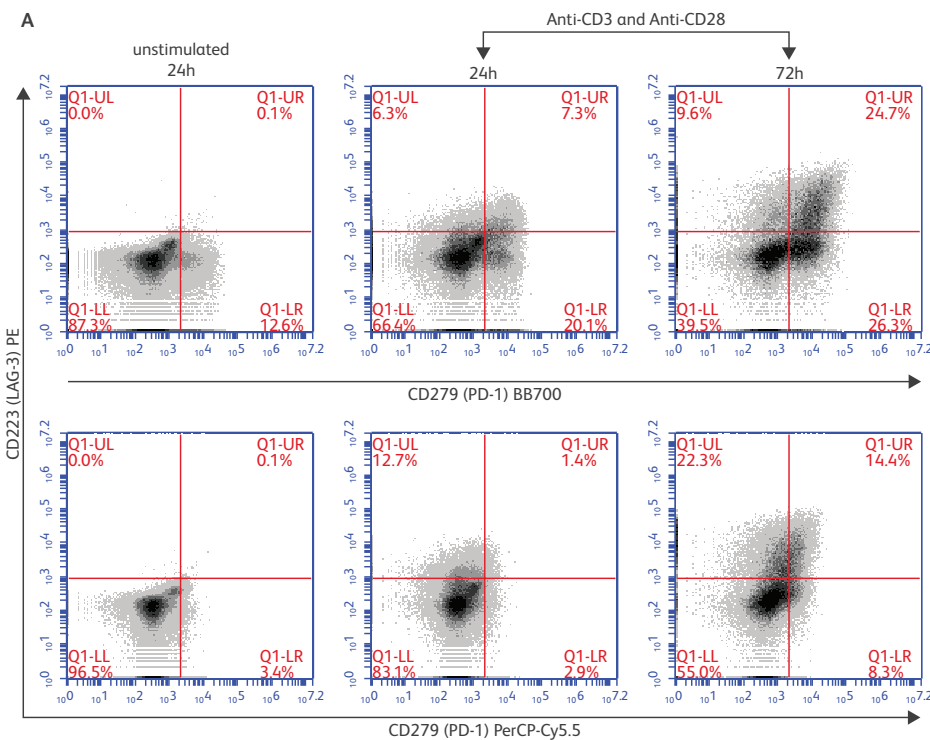
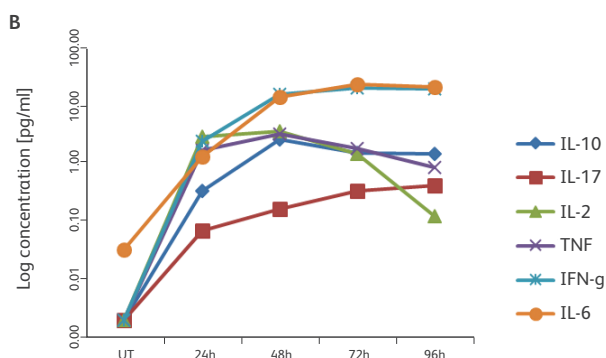


Figure 1. Assessment of human T-cell activation upon in vitro stimulation

Human PBMCs were stimulated with anti-CD3 and anti-CD28 for 96 hours. The cells and cell culture supernatants were harvested at the indicated time points for flow cytometric analysis. **A.** Cells were stained with BD Pharmingen™ PE Mouse Anti-Human LAG-3 (CD223) (Cat. No. 565616) and with either BD Horizon BB700 or BD Pharmingen™ PerCP-Cy5.5 Mouse Anti-Human CD279 (PD-1) (Cat. Nos. 566460 and 561273) at the indicated time points. Cells were also stained with BD Via-Probe™ Red Nucleic Acid Stain (Cat. No. 565804) for live/dead cell discrimination, and the two-color dot plots were gated on live lymphocytes (data not shown). Upregulation of both LAG-3 and PD-1 was observed in stimulated cells when the bright dyes PE and BB700, respectively, were used (upper plots). However, PD-1⁺ cell populations were not fully resolved in samples stained with PerCP-Cy5.5 anti-PD-1. **B.** Supernatants from the PBMC cultures were used for simultaneous analysis of several cytokines using the BD CBA Human Th1/Th2/Th17 Cytokine Kit (Cat. No. 560485). The graph shows increased, differential concentrations over time of six different cytokines in stimulated cultures when compared to unstimulated cultures (UT). Samples were collected using the BD CSampler Plus and the BD CBA kit template, and data was analyzed using FACSArray™ software v4.0.



Two of these cytokines, IFN- γ and TNF, are known to increase the expression levels of PD-1 ligands in antigen-presenting cells as well as in certain tumor cells. These ligands can be assessed on cancer or antigen-presenting cells using flow cytometry.

Figure 2A shows that cell staining with BD Horizon Brilliant™ Blue 515 (BB515) PD-L1 yielded better resolution than FITC PD-L1. Again, BB515 was designed as a brighter alternative to FITC, yet is detected in the same fluorescence channel (FL1).

Figure 2B demonstrates that either conditioned medium from activated PBMCs (red) or a combination of rhIFN- γ and rhTNF cytokines (blue) induced PD-L1 expression in the MDA-MB-468 breast-cancer cell line. However, a similar induction was not observed when treating the cells with conditioned medium from unstimulated PBMCs (green), further confirming the specific role played by secreted cytokines in activating cancer cells.

Figure 3 shows that stimulation of immature dendritic cells (iDCs, involved in tumor antigen presentation) with either conditioned medium (24h supernatant, plot C) or recombinant cytokines (hIFN- γ and rhTNF, plot B) resulted in both cell maturation (demonstrated by upregulation of CD83) and induction of PD-L1. However, adding anti-IFN- γ and anti-TNF blocking antibodies to the conditioned medium prevented upregulation of CD83 and PD-L1, and ultimately maturation of the DCs (plot D).

In these experiments, PerCP-Cy5.5 and FITC were not bright enough to optimally resolve PD-1 and PD-L1. Of course, these popular fluorochromes are still perfectly satisfactory for many if not most flow cytometry experiments. But for challenging work, the panel design principle of pairing bright fluorochromes with low-density antigens can be helpful. With the advent of BD Horizon Brilliant dyes, there is now a bright dye for every fluorescence channel of the BD Accuri C6 Plus: BB515 (FL1), PE (FL2), BB700 (FL3) and APC (FL4).

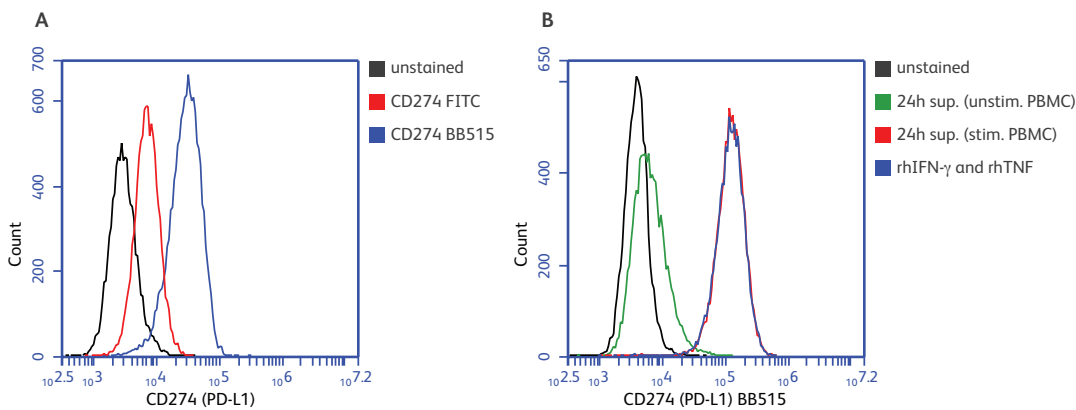


Figure 2. PD-L1 upregulation in cancer cells following different stimulation treatments

A. MDA-MB-468 breast cancer cells were stimulated with of BD Pharmingen™ rhIFN- γ (100 ng/mL; Cat. No. 554617) and stained with either BD Horizon BB515 or BD Pharmingen™ FITC Anti-Human CD274 (PD-L1) (Cat. Nos. 564554 and 558065). BB515 staining resulted in stronger fluorescence signal and better separation between stained and unstained cells. **B.** Robust upregulation of PD-L1 was observed when cells were cultured overnight with either conditioned medium from stimulated PBMCs (24h supernatant) or a combination of recombinant cytokines rhIFN- γ (100 ng/mL) and BD Pharmingen™ rhTNF (15 ng/mL; Cat. No. 554618), whereas very low levels of PD-L1 expression were detected in cells cultured with supernatants from unstimulated PBMCs.

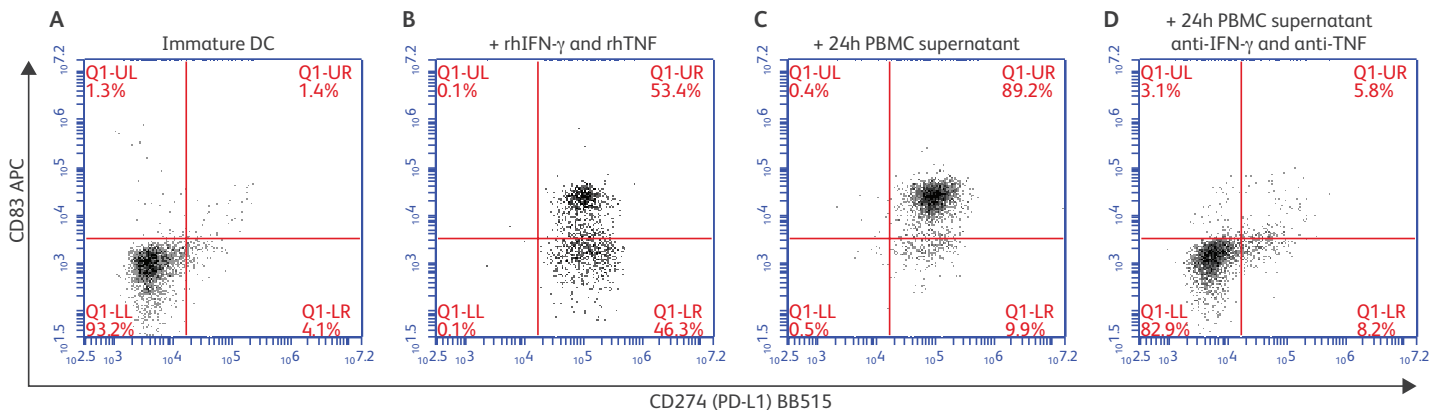


Figure 3. PD-L1 and CD83 expression following induction and blockage of DC maturation

Human monocyte-derived DCs were differentiated using BD Pharmingen™ rhGM-CSF (50 ng/mL; Cat. No. 550068) and BD Pharmingen™ rhIL-4 (50 ng/mL; Cat. No. 554605). **A.** Immature DCs obtained on day 6 of culture did not express CD83 (BD Pharmingen™ APC Mouse Anti-Human CD83) or PD-L1 surface markers. **B–C.** Upregulation of these markers was observed upon maturation induced by three-day stimulation with rhIFN- γ and rhTNF or conditioned medium (24h supernatant) from activated PBMC cultures. **D.** Cells were also stimulated with the same conditioned medium in the presence of BD Pharmingen™ Purified NA/LE Mouse Anti-Human IFN- γ (10 μ g/mL; Cat. No. 554698) and TNF (10 μ g/mL; Cat. No. 554508) blocking antibodies, which resulted in a significant inhibition of maturation, as indicated by the lack of CD83 and PD-L1 expression.

Easy to use, simple to maintain and affordable, the BD Accuri C6 Plus personal flow cytometer is equipped with a blue laser, a red laser, two light scatter detectors and four fluorescence detectors. A compact and transportable design, fixed laser alignment, pre-optimized detector settings and automated instrument QC result in a system that is simple to use.

For walkaway convenience, the optional BD CSampler Plus accessory offers automated sampling from 24-tube racks or multiwell plates. This option maintains cells in suspension while providing the convenience of preparing, staining and running your samples in the same plate.

Ordering information

Description	Cat. no.
BD Accuri™ C6 Plus Flow Cytometer System	660517
BD Accuri™ C6 Plus Workstation and Software	661391
BD CSampler™ Plus Automated Sampling System (optional)	660519

Class 1 Laser Product.

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