Multiparametric assessment of CD8+ cytotoxic T-cell activation using flow cytometry

Immuno-oncology applications for the BD Accuri™ C6 Plus flow cytometer

Features

- Assess multiple indicators of T-cell activation and effector function at the single-cell level
- Multiplex measurements of T-cell proliferation and either cytokine production or degranulation
- Combine both cell surface and intracellular activation markers in the same panel

In the field of immuno-oncology, extensive studies have assessed how cytotoxic T cells can overcome tumor-induced immunosuppressive signals and become activated. Activated T cells proliferate, secrete pro-inflammatory cytokines and release lytic granules (degranulation). The granules contain proteins such as perforin and granzymes, which may cause irreversible damage to tumor cells. The multiplexing capability of flow cytometry enables simultaneous analyses of these cellular processes at the single-cell level. On the BD Accuri™ C6 Plus personal flow cytometer, with two lasers and four fluorescence parameters, you can perform these studies right on your benchtop.

Figure 1

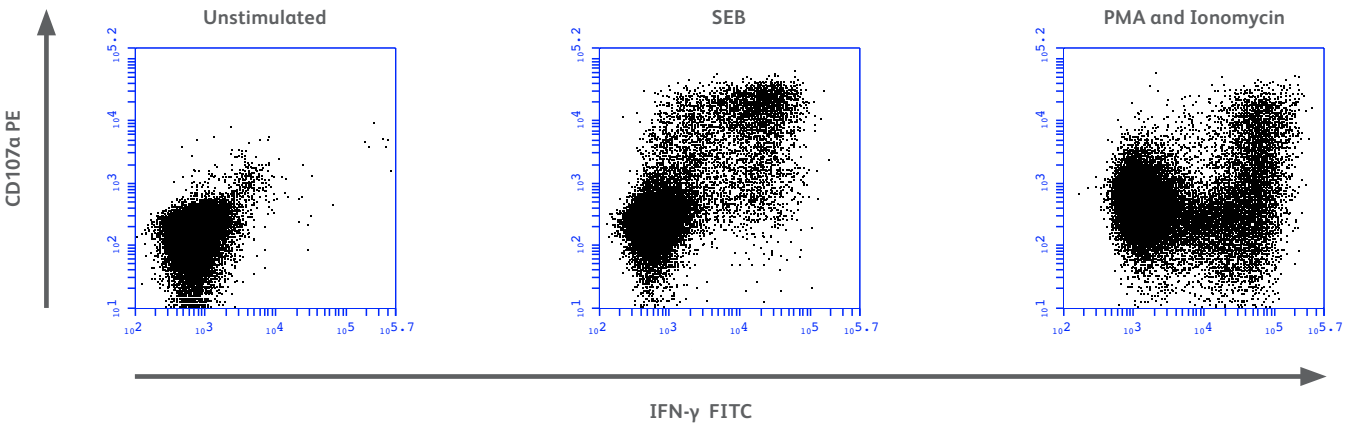

Figure 1. Activation of CD8⁺ T cells upon stimulation with polyclonal activators
PBMCs were isolated and stimulated with 1 µg/mL of SEB (Toxin Techonology) or 10 ng/mL of PMA and 1 µg/mL of ionomycin (Sigma-Aldrich) in culture media containing BD GolgiPlug™ Protein Transport Inhibitor (containing Brefeldin A), BD GolgiStop™ Protein Transport Inhibitor (containing Monensin) and BD Pharmingen™ PE Mouse Anti-Human CD107a. After 5 hours of stimulation, the cells were harvested and stained with BD Pharmingen™ APC Mouse Anti-Human CD3 and BD Pharmingen™ PerCP-Cy™5.5 Mouse Anti-Human CD8. Surface-stained cells were fixed with BD Cytofix™ Fixation Buffer and permeabilized with BD Perm/Wash™ Buffer. The cells were then stained intracellularly with BD Pharmingen™ FITC Mouse Anti-Human IFN-γ, acquired on a BD Accuri™ C6 Plus flow cytometer system and analyzed using BD Accuri™ C6 Plus software. **Results:** Cytotoxic T cells were identified by first gating on lymphocytes and then on CD3⁺ and CD8⁺ cells (not shown). CD107a was detected on the cell surface and IFN-γ was detected intracellularly in cells stimulated with SEB or PMA and ionomycin but not in unstimulated cells.

To assess cytotoxic functions of CD8⁺ T cells, peripheral blood mononuclear cells (PBMCs) were initially stimulated with polyclonal activators, either staphylococcal enterotoxin B (SEB) or phorbol 12-myristate 13-acetate (PMA) and ionomycin. Figure 1 shows that short stimulation with either SEB or PMA and ionomycin induced both degranulation and cytokine production in CD3⁺CD8⁺ T cells, as shown by the exposure of CD107a on the cell surface and increased levels of intracellular IFN-γ when compared to unstimulated cells.

Next, we employed a one-way mixed lymphocyte reaction (MLR) assay (illustrated in Figure 2) to evaluate CD8⁺ T-cell-specific response to allogeneic major histocompatibility complex (MHC) antigens. Besides giving insights into antigen-specific response, the MLR assay can provide a multifaceted window into T-cell function, including proliferation, degranulation and cytokine production. In this assay, responder CD3⁺ T cells from one donor were magnetically enriched from PBMCs and co-cultured with T-cell-depleted stimulator cells, either from the same donor (autologous) or a different donor (allogeneic). Responder cells were labeled with CFSE to measure cell proliferation as well as to facilitate separation from CFSE⁻ stimulator cells. The stimulator cells were treated with mitomycin C to inhibit their proliferation. Two four-color panels were designed for the simultaneous analysis of CFSE, CD3, CD8 and either CD107a or IFN-γ.

Figure 2. One-way MLR assay to evaluate CD8⁺ T-cell activation
Responder T cells from donor A were co-cultured with stimulator cells from either the same donor A (autologous) or a different donor B (allogeneic). T-cell responses include proliferation (measured using CFSE dilution), degranulation (exposure of CD107a on the cell surface) and cytokine production (intracellular IFN-γ expression). **Method details:** T cells were isolated from the peripheral blood of healthy donors using BD IMag™ Human T Lymphocyte Enrichment Set – DM and then labeled with BD Horizon™ CFSE for analysis of cell proliferation. The T-cell-depleted counterparts (stimulator cells) were treated with 50 µg/mL mitomycin C (Sigma-Aldrich) to cease proliferation of any remaining T cells. CFSE-labeled T cells were co-cultured with mitomycin-C-treated allogeneic or autologous stimulator cells for 4 days. Then, fresh culture media containing BD GolgiPlug Protein Transport Inhibitor (containing Brefeldin A), BD GolgiStop Protein Transport Inhibitor (containing Monensin) and BD Pharmingen PE Mouse Anti-Human CD107a was added to the cells 5 hours prior to acquisition. For the analysis of IFN-γ expression, cells were also cultured in the presence of PMA and ionomycin for 5 hours. Cells were then harvested, stained and analyzed as described in Figure 1.

Figure 2

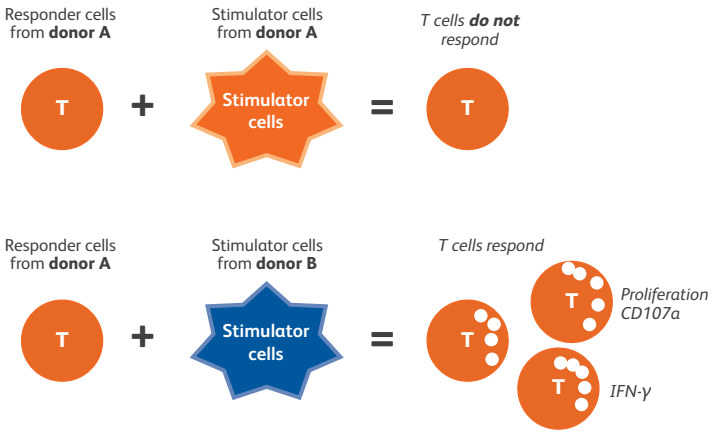

Figure 3 shows the proliferation of CFSE-labeled T-cell responders ($CD3^+CFSE^{+/low}$) in the MLR cultures. Analysis of CFSE dilution showed a modest increase in T-cell proliferation ($CFSE^{low}$ cells) in response to allogeneic stimulation as compared to SEB stimulation. The response was specific to alloantigens, as stimulation with autologous stimulator cells caused minimal proliferation, similar to unstimulated cells (Figure 3A). The cells were further gated on $CD8^+$ cells and analysis of CFSE vs CD107a revealed that allogeneic stimulation also induced degranulation of proliferating ($CFSE^{low}$) cytotoxic $CD8^+$ T cells, as indicated by the externalization of CD107a (Figure 3B).

Finally, to enhance the detection of intracellular IFN-γ in the MLR cultures, the cells were re-stimulated with PMA and ionomycin for 5 hours prior to acquisition. Proliferating $CD8^+$ T cells expressed high levels of IFN-γ when co-cultured with allogeneic stimulator cells (Figure 3C). As expected, short-term stimulation with PMA and ionomycin also induced IFN-γ expression in non-proliferating $CD8^+$ cells, albeit at a lower level. The combined results suggest that allogeneic stimulation resulted in the proliferation of functional effector cytotoxic T cells.

The BD Accuri C6 Plus can easily and simultaneously assess immune cell proliferation and activation. To learn how you could extend this assay to analyze specific T-cell subsets if you had a third laser and additional fluorescence channels, refer to our companion product information sheet for the BD FACSCelesta™ flow cytometer.

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, preoptimized detector settings and automated instrument QC result in a system that is straightforward to operate. For walkaway convenience, the optional BD CSampler™ Plus accessory offers automated sampling from 24-tube racks or multiwell plates.

Figure 3A

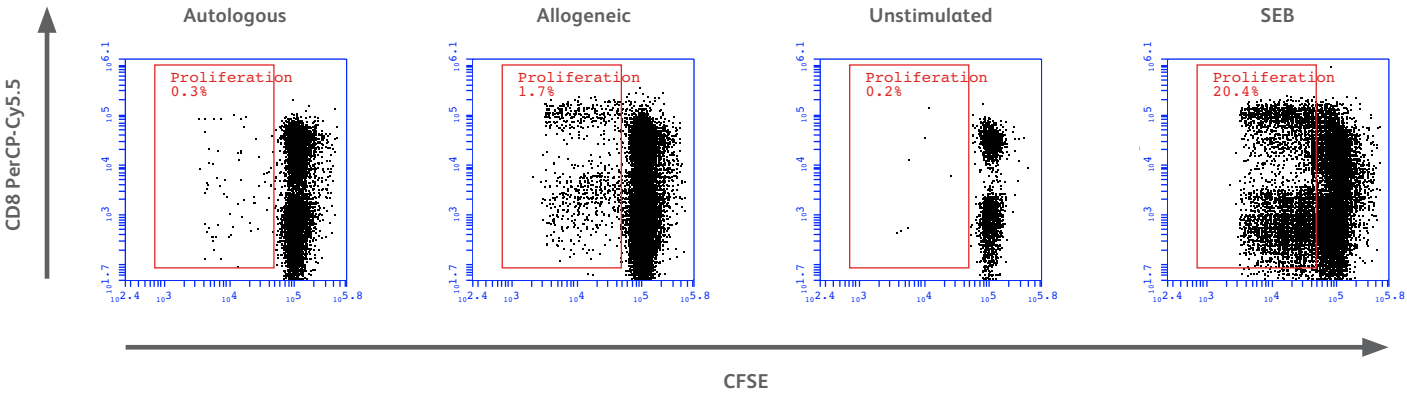

Figure 3B

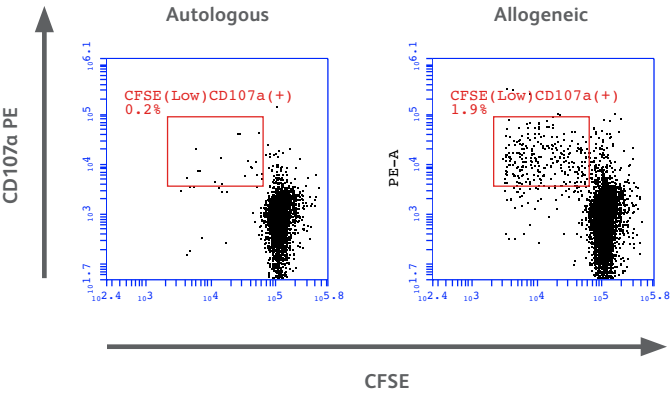

Figure 3C

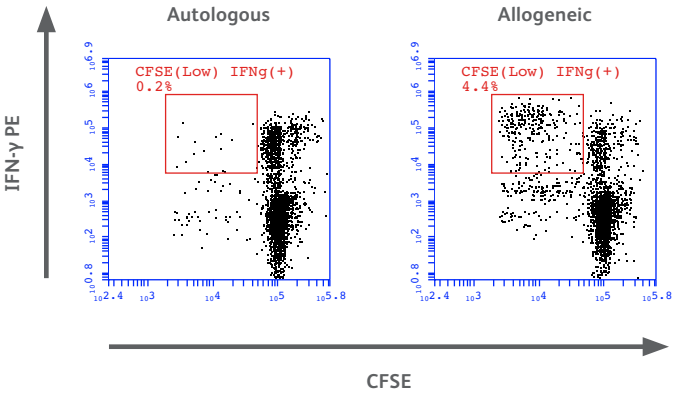

Figure 3. Activation of $CD8^+$ T cells in mixed lymphocyte reaction

CFSE-labeled T cells were cultured with allogeneic or autologous mitomycin C-treated stimulator cells and stained as described in Figures 1 and 2. Unstimulated cells and cells treated with SEB were used as negative and positive controls, respectively. Cells were gated on $CD3^+$ $CFSE^{+/low}$ responder T cells after exclusion of $CFSE^-$ stimulators (not shown). **Results: A**. Analysis of CFSE dilution showed that allogeneic stimulation induced modest, alloantigen-specific proliferation of $CD3^+$ T cells in the MLR cultures, as compared to SEB. As expected, no cell proliferation was observed upon autologous stimulation, similar to unstimulated cells. **B.** The cells were further gated on $CD8^+$ T cells to demonstrate that CD107a was detected on the surface of $CFSE^{low}$ proliferating cells. This suggested that allogeneic stimulation caused proliferation and concurrent degranulation of cytotoxic $CD8^+$ T cells. **C.** The MLR cultures were re-stimulated with PMA and ionomycin for 5 hours for analysis of intracellular IFN-γ within $CD8^+$ cells. In allogeneic co-cultures, proliferating $CD8^+CFSE^{low}$ cells expressed high levels of IFN-γ and thus developed potential cytotoxic effector functions. Note that $CFSE^{high}$ non-proliferating cells expressed lower levels of IFN-γ in both autologous and allogeneic cultures as a result of the polyclonal activation with PMA and ionomycin, as compared with the specific response against alloantigens.

Ordering information: Systems and software

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

Ordering information: Reagents

Description	Cat. No.
BD IMag™ Human T Cell Enrichment Set – DM	557874
BD Horizon™ CFSE	565082
BD GolgiPlug™ Protein Transport Inhibitor (Containing Brefeldin A)	555029
BD GolgiStop™ Protein Transport Inhibitor (Containing Monensin)	554724
BD Pharmingen™ PE Mouse Anti-Human CD107a	555801
BD Pharmingen™ FITC Mouse Anti-Human IFN-γ	552887
BD Pharmingen™ PerCP-Cy™5.5 Mouse Anti-Human CD8	565310
BD Pharmingen™ APC Mouse Anti-Human CD3	555335
BD Cytofix™ Fixation Buffer	554655
BD Perm/Wash™ Buffer	554723

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