Expression of Immune Checkpoint Receptors on Peripheral Blood Immune Cells Using a 10-Color Assay on the BD FACSLyric™ Flow Cytometer

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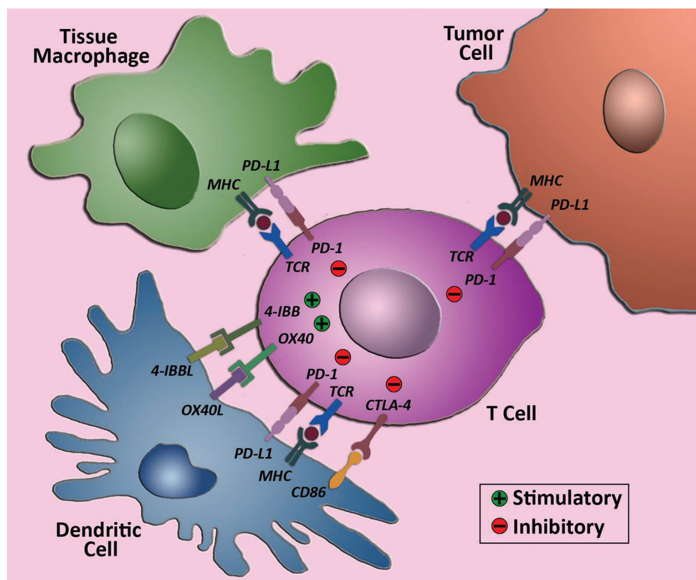
Abstract

The use of multicolor flow cytometry has the potential to transform patient diagnosis and disease monitoring for blood and solid cancers. As the total number of parameters that can be simultaneously measured on an individual cell increases, the capability and impact of clinical flow applications also expands dramatically. In this application note, we highlight the multiparameter capability of the 10-color BD FACSLyric™ platform. Using the BD FACSLyric system and BD FACSuite™ software, we analyzed changes in the expression of a panel of immune checkpoint receptors on peripheral blood mononuclear cells (PBMCs) from healthy donors. Assessment of immune checkpoint marker expression may help to inform candidate therapy selection or monitor efficacy in patients being treated with immunomodulatory drugs. We show that, following ex vivo stimulation, PBMCs exhibited robust increases in immune checkpoint receptor expression levels in both CD3^{+} T cells and CD3^{-} lymphocytes. The observed changes were time- or concentration-dependent. Taken together, this work suggests that the BD FACSLyric enables users to design useful 10-color assays and acquire data with a high degree of informational content.

Introduction

Clinical flow cytometry was first used in the 1980s for the management of patients with the human immunodeficiency virus.[1-4] Since that time, the capability and the healthcare impact of multicolor immune profiling studies have evolved dramatically. Key advances in instrument and fluorophore performance, an expanding range of clinical applications, and cutting-edge computational techniques for data analysis, now allow the user to routinely and rapidly acquire patient data with a high degree of informational content.[5-7] The application of multicolor flow cytometry as an aid for the diagnosis or management of cancer patients has been particularly successful. For example, the EuroFlow Consortium has developed clinically validated and standardized 8-color antibody panels to use as diagnostic aids in the classification of patients with leukemia or lymphoma.[8] Multicolor reagent panels may also be useful for tracking minimal residual disease in patients with acute myeloid leukemia or chronic lymphocytic leukemia.[9-11]

Evasion of the immune response is a hallmark of cancer.[12] Neoplastic cells may use multiple mechanisms to suppress the T-cell response against antigens expressed in the tumor microenvironment.[13, 14] For example, chronic and persistent tumor-antigen stimulation upregulates the inhibitory programmed cell death 1 receptor (PD-1, also known as CD279) on the surface of activated CD8$^+$ T cells infiltrating neoplastic tissue. Expression of the PD-1 ligand PD-L1 (or CD274) by malignant cells suppresses effector T-cell activity, thereby attenuating the antitumor immune response.[15] Therapeutic blockade of the PD-1:PD-L1 interaction has elicited durable clinical responses and tumor regression in melanoma patients.[16-18] In addition to the PD-1:PD-L1 interaction, there are a number of other inhibitory and stimulatory pathways that collectively determine the reactivity, or conversely, the lack of reactivity of a given T cell within the context of an immune response. Some of these pathways are summarized in **Figure 1**.



The availability of high-performance flow cytometers that can detect 10 or more parameters on individual cells has helped to drive early success in this field of cancer immunotherapy. For example, multicolor immune profiling of patient melanoma samples has shown that increased frequency of tumor-infiltrating, PD-1^{high}CD8$^+$ T cells correlates with the response to anti-PD-1 monotherapy and increased progression-free survival.[14] In another study, anti-PD-1 treated melanoma patients showed increased frequency of CD8$^+$ T cells in tumor biopsies relative to patients being treated with targeted therapy.[20] Despite these successes, only a minority of patients appear to respond to anti-PD-1 monotherapy. Taken together, these studies suggest that there is an unmet need for reliable biomarkers that can accurately select the most appropriate immunotherapy or predict a patient's clinical response to immune checkpoint blockade.[21]

In this application note, we present a 10-color BD antibody panel for the characterization and quantitation of immune checkpoint receptors on activated peripheral T cells (see **Table 1**). PBMCs from pooled healthy donors were stimulated with phorbol 12-myristate 13-acetate (PMA) + ionomycin, anti-CD3 + anti-CD28 antibodies (CD3 + CD28), or phytohemagglutinin (PHA). The data was acquired and analyzed using BD FACSuite software. We show that immune checkpoint receptor expression is regulated in part by time-in-culture or by stimulatory conditions. We observed that PMA + ionomycin appeared to induce the most robust changes in immune checkpoint receptor expression when compared to PHA or CD3 + CD28. Application of this model assay for 10-color immune profiling of checkpoint receptor expression in tumor infiltrates may help to inform candidate selection or monitor efficacy in patients being treated with immunomodulatory drugs.

Figure 1. The relative immune responsiveness of a given T cell is dictated by a combination of inhibitory and stimulatory signals that are generated through cell-to-cell interactions with tissue macrophages, antigen-presenting cells (such as dendritic cells) and tumor cells.

(Figure adapted from Shin et al[19])

Methods

Healthy Donor PBMC Stimulation and Cell Culture Conditions

Ninety-six-well plates were coated with 250 µL per well of phosphate-buffered saline (PBS as a nonstimulated control) or anti-CD3 epsilon (antibody clone UCHT1; BD Biosciences Catalog No. 555329) at concentrations of 0.5 µg/mL (low), 2.0 µg/mL (intermediate or int), or 8.0 µg/mL (high). Coated plates were incubated at 37°C, 5% CO_2 for 2 hours and then placed in 4°C refrigeration overnight. The following day, all wells were rinsed twice with PBS. Fresh PBMCs from healthy donors was collected using BD Vacutainer® CPT™ Cell Preparation Tubes. PBMCs were pooled from three individuals and seeded into nonstimulated control or CD3-coated wells at a concentration of 3×10^5 cells/250 µL per well suspended in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal calf serum. After seeding with pooled PBMCs, the wells containing anti-CD3 antibody were treated with 2 µg/mL of anti-CD28 antibody. Parallel wells containing the pooled PBMCs were incubated with PBS plus PHA at 1 µg/mL (low), 3 µg/mL (int), or 5 µg/mL (high), or with PBS plus 500 ng/mL ionomycin plus PMA at 5 ng/mL (low), 50 ng/mL (int), or 500 ng/mL (high). Plates containing pooled PBMCs were then incubated at 37°C and 5% CO_2 for 12 or 24 hours. For all experimental conditions, wells were prepared in triplicate. Cells from each set of triplicate wells were pooled prior to staining.

Acquisition and Data Analysis on the BD FACSLyric Platform

After the incubation periods described previously were complete, replicate wells in each plate were pooled and the cells were transferred to 12 x 75-mm, 5-mL tubes, such that each condition in the plate was represented by one staining tube. Cells were pelleted via centrifugation (7 min at 300*g*) and washed twice using PBS. Washed cells were then stained with the 10-color antibody panel shown in **Table 1** and incubated at room temperature, under low-light conditions, for 30 minutes in BD Horizon™ Brilliant Stain Buffer (BD Biosciences Catalog No. 659611). After the staining period was complete, PBMCs were washed twice with PBS and centrifuged for 7 min at 300*g*. The cell pellets were resuspended in staining buffer and acquired on a 10-color BD FACSLyric flow cytometer with the 4-3-3 configuration, using BD FACSuite software. Instrument setup was performed using modified lyse/wash settings. Data acquisition and analysis was performed with BD FACSuite software.

Results

We designed a panel of 10 antibody-fluorochrome conjugates (all from BD Biosciences) specific for immune checkpoint receptors expressed by activated T cells. As shown in **Table 1**, the panel includes the leucocyte receptor CD45, the T-cell co-receptor CD3, the major histocompatibility complex class II receptor HLA-DR, the co-stimulatory receptor CD28, and the immune checkpoint receptors cytotoxic T-lymphocyte antigen CTLA-4 (or CD152), OX40 (or CD134), 4-1BB (or CD137), PD-1 (or CD279), B7-2 (or CD86), and PD-L1 (or CD274).

	Target Antigen	Alternate Name	Clone	Fluorophore	BD Catalog No.
1	CD45	PTPRC	2D1	APC-H7	560178
2	CD3	n/a	SK7	PerCP-Cy™5.5	340949
3	HLA-DR	n/a	G46-6	BD Horizon BV510	563083
4	CD28	n/a	CD28.2	PE-Cy™7	560684
5	CD134	OX40	ACT35	PE	555838
6	CD137	4-1BB	4B4-1	APC	550890
7	CD274	PD-L1	M1H1	FITC	558065
8	CD86	B7-2	2331	Alexa Fluor® 700	561124
9	CD152	CTLA-4	BNI3	BD Horizon BV421	562743
10	CD279	PD-1	EH12.1	BD Horizon BV605	563245

Table 1. A 10-color antibody panel for the characterization and quantitation of immune checkpoint receptors on activated T cells.

Effector T-cell function is regulated by multiple receptor-ligation-induced signaling pathways. These pathways may be either stimulatory or inhibitory. For example, the ligation of CD134/OX40 or CD137/4-1BB promotes T-cell proliferation, survival, and functional activity (see **Figure 2**). Conversely, cross-linking CTLA-4/CD152 or PD-1/CD279 dampens activated T-cell-effector function by promoting apoptosis, anergy, or functional exhaustion. Taken together, this complexity suggests that selecting a diverse panel of immune checkpoint receptors (both agonistic and antagonistic) that are expressed by activated T cells may help identify biomarkers that will predict a patient's response to immune checkpoint blockade.

The proposed gating strategy for the 10-color immune checkpoint receptor panel is shown in **Figure 3**. Briefly, $CD45^{+}$ lymphocytes were separated into $CD3^{+}$ T cells (orange contours) and $CD3^{-}$ cells (blue contours). The expression of CD134, CD137, PD-L1/CD274, HLA-DR, CD86, CD152, and PD-1/CD279 was then quantified in both cell populations.

As shown in **Figure 3**, PMA plus ionomycin-stimulated lymphocytes demonstrated increased expression of CD134, CD137, PD-L1/CD274, HLA-DR, CD86, CD152, and PD-1/CD279 relative to the unstimulated control, data that is consistent with published results.[32] For example, $CD3^{+}$ lymphocytes (orange contours) showed increased expression of CD134 following stimulation with an intermediate (int) concentration of PMA (50 ng/mL) plus ionomycin (41.8% positive) relative to the low concentration of PMA (5 ng/mL) plus ionomycin or the nonstimulated control (20.3% and 0.6% positive, respectively) conditions. In comparison, activation-induced upregulation of CD134 was muted in $CD3^{-}$ (blue contours) lymphocytes (int PMA = 9.9% positive, low PMA = 1.9%, and nonstimulated = 0.6%). Similarly, PD-L1/CD274 expression was strongly upregulated in $CD3^{+}$ T cells (int PMA = 43.7% positive, low PMA = 20.2% positive, and nonstimulated = 2.6% positive) but not in $CD3^{-}$ lymphocytes (int PMA = 24.5%, low PMA = 16.6%, and nonstimulated = 16.6%). Finally, both $CD3^{+}$ T cells and $CD3^{-}$ lymphocytes showed a similar pattern of activation-induced CD137 expression (73.9% and 77.8% respectively in high PMA conditions). CD28 expression did not change (data not shown).

OX40/CD134

Ligation of the T-cell costimulatory receptor CD134 has been shown to improve the T-cell response after activation by promoting T-cell proliferation, survival, and functional activity.[22] Agnostic anti-CD134 antibodies can promote an antitumor immune response in patients.[23] CD134 is expressed by activated T cells and activated neutrophils.[24]

4-1BB/CD137

Ligation of the co-stimulatory receptor CD137 enhances T-cell proliferation, cytokine productions, cytokine activity, and survival.[25] CD137 is expressed on activated T cells, dendritic cells, NK cells, and granulocytes. 4-1BB agonists can promote an antitumor immune response in patients.[26]

PD-L1/CD274

PD-L1/CD274 is expressed on activated T cells, NK cells, macrophages, dendritic cells, and B cells. CD274 ligation inhibits the proliferation of activated T cells and decreases their production of effector cytokines.[27]

HLA-DR

HLA-DR is an MHC class II cell-surface receptor expressed on monocytes, B cells, dendritic cells, and activated T cells. The primary function of HLA-DR is to present peptide antigens to T cells.[28]

B7-2/CD86

The costimulatory receptor CD86 is expressed on antigen-presenting cells and activated T cells.[29] CD86 is the ligand for CD28 and CTLA-4, two receptors expressed by resting and activated T cells, respectively.[31]

CTLA-4/CD152

CD152 is expressed by activated T cells and it functions as a negative regulator of T-cell activation.[31] CTLA-4 is also expressed by NK cells and granulocytes.[30] Monoclonal antibodies that block CTLA-4 ligation are used for treatment of melanoma and lung cancer.[31]

PD-1/CD279

CD279 inhibits the activation of T cells by promoting apoptosis, anergy, or functional exhaustion.[27] PD-1 is expressed on the surface of activated T cells, B cells, and monocytes.

Figure 2. Immune checkpoint receptors expressed by activated T cells.

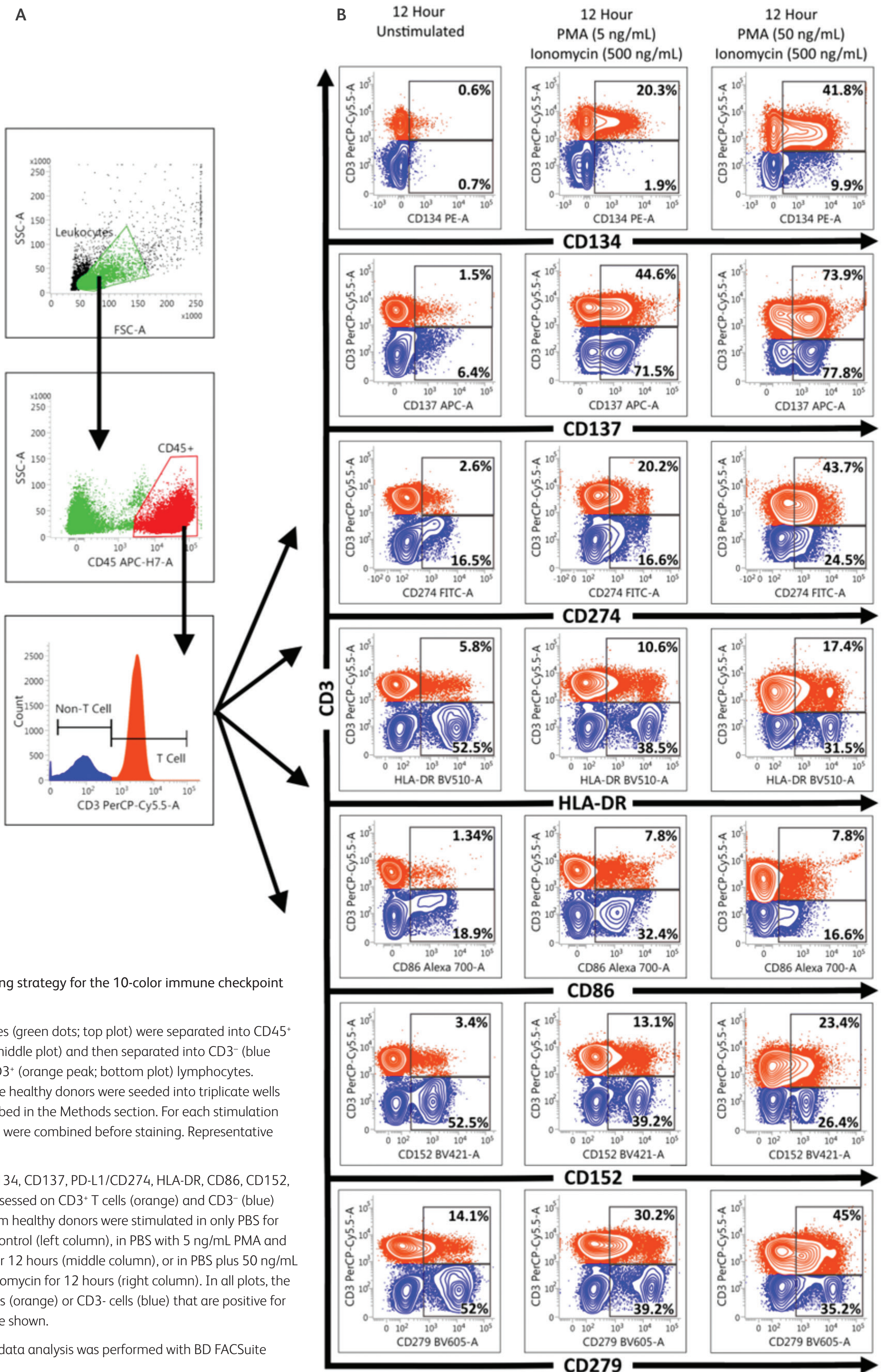


Figure 3. Proposed gating strategy for the 10-color immune checkpoint receptor panel

A. $SSC^{low}FSC^{high}$ leucocytes (green dots; top plot) were separated into $CD45^+$ lymphocytes (red dots; middle plot) and then separated into $CD3^-$ (blue peak; bottom plot) or $CD3^+$ (orange peak; bottom plot) lymphocytes. PBMCs pooled from three healthy donors were seeded into triplicate wells and stimulated as described in the Methods section. For each stimulation condition, triplicate wells were combined before staining. Representative data is shown.

B. The expression of CD134, CD137, PD-L1/CD274, HLA-DR, CD86, CD152, and PD-1/CD279 was assessed on $CD3^+$ T cells (orange) and $CD3^-$ (blue) lymphocytes. PBMCs from healthy donors were stimulated in only PBS for 12 hours as a negative control (left column), in PBS with 5 ng/mL PMA and 500 ng/mL ionomycin for 12 hours (middle column), or in PBS plus 50 ng/mL PMA and 500 ng/mL ionomycin for 12 hours (right column). In all plots, the percentages of $CD3^+$ cells (orange) or CD3- cells (blue) that are positive for the indicated markers are shown.

All data acquisition and data analysis was performed with BD FACSuite software.

We observed that intermediate concentrations (50 ng/mL) of PMA plus ionomycin appeared to induce robust upregulation of CD134, CD137, PD-L1/CD274, HLA-DR, CD152, and PD-1/CD279, but not CD86, in CD3⁺ T cells. We next expanded our 10-color BD FACSLyric study to include additional stimulation conditions. As shown in **Figure 4**, PBMCs from healthy donors were cultured in PBS alone to serve as the untreated control (unt) or under three treatment conditions: anti-CD3 plus anti-CD28 antibodies (CD3 + CD28, blue lines), PHA (red lines), or PMA plus ionomycin (green lines). For each of the three activating stimuli, we varied the concentration of the stimulating agent (low, int, or high). PBMCs were stimulated for either 12 or 24 hours.

In addition, upregulation of CD134, PD-L1/CD274, CD152, and PD-1/CD279 was similar in CD3⁺ T cells activated with either PMA plus ionomycin or PHA for 12 hours. Conversely, PMA plus ionomycin strongly upregulated CD137 at low and int concentrations when compared to PHA-stimulated CD3⁺ T cells at 12 hours. Finally, we observed that the percent positivity of HLA-DR, CD86, CD152, and PD-1/CD279 was highest in CD3⁺ T cells that were stimulated with the intermediate (50 ng/mL) concentration of PMA plus ionomycin for 24 hours. For most of the receptors that we measured, activation-induced upregulation following treatment with CD3 + CD28 was blunted when compared to PMA plus ionomycin or PHA.

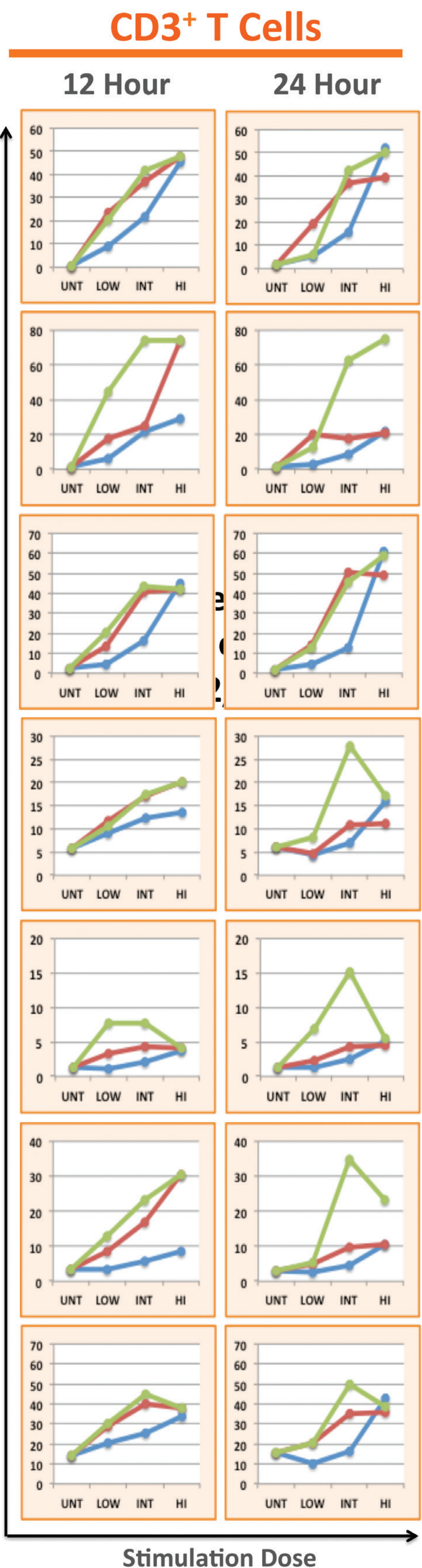


Figure 4. Expression of checkpoint markers following prolonged stimulation

PBMCs from healthy donors were transferred to 96-well plates coated with anti-CD3 antibody at three concentrations: 0.5 µg/mL (low), 2.0 µg/mL (int), or 8.0 µg/mL (high). Anti-CD28 antibody was added at a concentration of 2 µg/mL. In parallel wells, PBMCs were incubated with PHA at three concentrations: 1 µg/mL (low), 3 µg/mL (int), or 5 µg/mL (high). In a third set of wells, PBMCs were incubated with PMA at concentrations of 5 ng/mL (low), 50 ng/mL (int) or 500 ng/mL (high). 500 ng/mL of ionomycin was then added to all wells containing PMA. Nonstimulated (unt) PBMCs were used as controls. CD3⁺ T cells are shown.

Conclusions

Immune checkpoint therapy blocks the receptor pathways that suppress T-cell activity targeting the cells in the tumor microenvironment. Unlike immunohistochemistry, multicolor immune profiling enables investigation of both phenotype and function for an in-depth characterization of the activation state of T cells. Since anti-PD-1 or anti-CTLA-4 monotherapy appears to benefit only a fraction of treated individuals, there is a clear need to identify reliable predictive biomarkers to help inform patient selection or monitor treatment effectiveness. Our data suggests that combined with BD FACSuite software, the BD FACSLyric enables users to design useful 10-color assays and generate meaningful data with a high degree of informational content. The results presented in this study may also provide a useful method for measuring therapeutic efficacy of various therapies during the development of immune checkpoint-inhibiting drugs.

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