Poster# B87

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

Aaron J. Middlebrook, Tri T. Le, Nihan Kara, Casey Fox, and Alice Wang. *BD Biosciences, San Jose, CA*

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 CD279), 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, data 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.

Multiparameter 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 3 x 10^5 cells per well suspended in complete DMEM culture media. PBMCs were set up from three different donors (all normal). DMEM culture media were either left untreated (unstimulated control) or were supplemented with phytohemagglutinin (PHA) at concentrations of 3 or 6 µg/mL. Wells were set up in triplicate, and were incubated at 37°C, 5% CO_2 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 two times with phosphate –buffered saline (PBS) and recovered via centrifugation (7 minutes at 300*g*). 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 two times with PBS and recovered via centrifugation (7 minutes at 300*g*). Samples were analyzed on a preproduction 12-color (4-3-5) BD FACSLyric system, and data analysis was performed using BD FACSuite™ software.

	Fluorophore	Antigen	Alt Designation	Clone	BD Cat. #
1	BV421	CD152	CTLA-4	BN13	562743
2	BV510	CD4	--	SK3	562970
3	BV605	CD366	TIM-3	7D3	742856
4	BV711	CD3	--	UCHT1	563725
5	BV786	CD8	--	RPA-T8	563823
6	BB515	CD274	PD-L1	MIH 1	564554
7	BB700	CD279	PD-1	EH12	566460
8	PE	CD223	LAG-3	T47-530	565616
9	PE-Cy7™	CD134	OX40	ACT35	563663
10	Alexa Fluor 647®	CD16	--	3GB	557710
10	Alexa Fluor 647®	CD56	--	R19-760	563443
11	APC-R700	CD273	PD-L2	MIH 18	565189
12	APC-H7	BD Horizon™ Fixable Viability Stain 780	Live/Dead	--	565388
12	APC-H7	CD14	--	MϕP9	641394
12	APC-H7	CD19	--	SJ25C1	641395

Table 1: 12-Color checkpoint panel

Results (1)

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

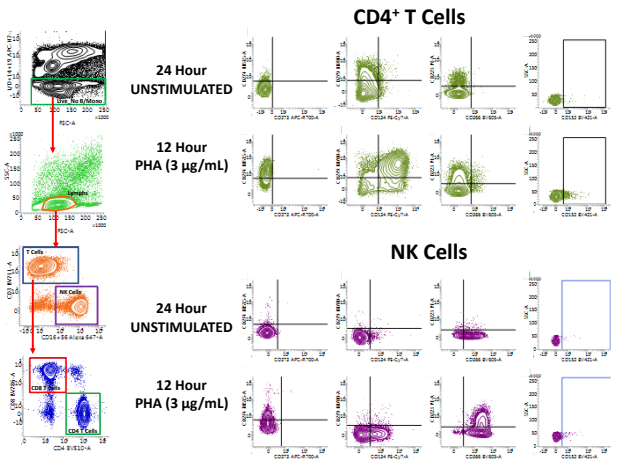

Figure 1. 12-color checkpoint panel-gating strategy
Cells were cultured for 24 and 48 hours. At the conclusion of each time point, cells were stained with the 12-color flow cytometry panel. Dead cells, B cells (CD19⁺), and monocytes (CD14⁺) were excluded from the analysis using a "dump" channel gating strategy (APC-H7). Lymphocytes were then identified based on FSC vs SSC. CD3 expression within the lymphocyte population identified T cells and CD16/CD56 expression identified NK cells. T cells were further divided into CD4⁺ T cells and CD8⁺ T cells. Expression of CD134 (OX40), CD273 (PD-L2), CD274 (PD-L1), CD279 (PD-1), CD152 (CTLA-4), CD366 (TIM-3), and CD223 (LAG-3) was enumerated on CD4⁺ and CD8⁺ T cells as well as NK cells.

Results (2)

Immune checkpoint markers: compiled results expression on CD4, CD8 T cells, and NK cells

CD274 (PD-L1)
Programmed cell death protein 1 ligand or PDL-1 (CD274) is expressed on activated T cells, NK cells, macrophages, myeloid DCs, and B cells. Ligation with PD-1 (CD279) inhibits TCR-mediated activation of IL-2 production and T-cell proliferation.[2]

CD279 (PD-1)
PD-1 (CD279) plays an important role in preventing the activation of T cells. PD-1 is expressed on the surface of activated T cells, B cells, and monocytes. PD-1 mediated inhibition is accomplished through a dual mechanism of promoting apoptosis in antigen specific T cells while reducing apoptosis in regulatory T cells (suppressor T cells).[1]

CD134 (OX40)
OX40 (CD134) ligation on T cells prevents activation-induced cell death. OX40 has a critical role in the maintenance of an immune response due to its ability to enhance survival.[2] CD134 is also expressed on activated neutrophils.[3]

CD223 (LAG-3)
Lymphocyte-activation gene 3 (CD223), also known as LAG-3 is expressed on activated T cells, B cells and NK cells. LAG-3's primary ligand is MHC class II, to which it binds with higher affinity than CD4. The protein negatively regulates cellular proliferation, activation, and homeostasis of T cells.

CD366 (TIM-3)
T-cell immunoglobulin and mucin-domain containing-3 (TIM-3; CD366). CD366 is a T-helper 1-specific type I membrane protein involved in regulating T-cell responses. It is also a known marker of NK cell maturation and may negatively regulate NK cell function.

CD152 (CTLA-4)
Cytotoxic T-lymphocyte antigen–4 (CTLA-4; CD152) is a T-cell costimulatory receptor that functions as a negative regulator of T-cell activation. Its ligands are CD86 and CD80. It is expressed by activated T cells, B cells, and granulocytes.[4]

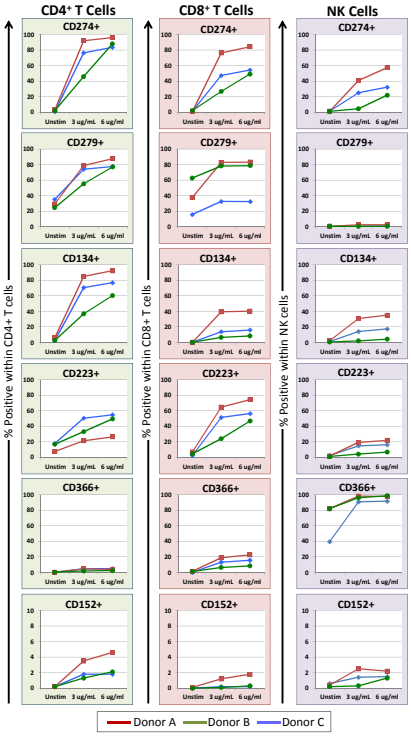

Figure 2. Expression of checkpoint markers following prolonged stimulation
Cells were stimulated with PHA at concentrations of 3 µg/mL and 6 µg/mL. Stimulated cells were compared against unstimulated controls. Expression levels of all six out of the seven checkpoint markers measured showed activation induced increases in expression in at least one of each 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. Three donors were tested: donor A (red line), donor B (blue line), and donor C (green line).

Conclusions

Unstimulated

Cell Type	CD274 (PD-L1)	CD279 (PD-1)	CD134 (OX40)	CD223 (LAG-3)	CD366 (TIM-3)	CD152 (CTLA-4)
CD4 T Cell	+	--	--	--	+	--	--
CD8 T Cell	+	--	--	--	+	--	--
NK Cell	--	--	--	--	--	+	--

Stimulated

Cell Type	CD274 (PD-L1)	CD279 (PD-1)	CD134 (OX40)	CD223 (LAG-3)	CD366 (TIM-3)	CD152 (CTLA-4)
CD4 T Cell	++	--	+++	++	+	--	+
CD8 T Cell	++	--	+++	++	++	+	+
NK Cell	++			+	++	+++	+

- We were successfully able to use a 12-color flow cytometry assay on the BD FACSLyric flow cytometer that demonstrates an accurate and reproducible method of determining expression levels of immune checkpoint markers which included CD134, CD273 (PD-L2), CD274 (PD-L1), CD279 (PD-1), CD223 (LAG-3), CD366 (TIM-3), and CD152 (CTLA-4) (Figure 1).
- The expression of these immune checkpoint markers is an inducible phenomenon that can be controlled through culture conditions and by the choice of stimulatory agent (Figure 2).
- Inducible expression of checkpoint markers demonstrated a clear pattern of expression across donors. However, the degree of expression varied across the three donors tested.

References

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