Analysis of Checkpoint Marker Expression on Immune Cells Cultured with Cancer Cell Lines Using a 10-Color Assay on the BD FACSLyric™ Platform

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Abstract

The immune system plays a critical role in cancer progression. Recently, immune checkpoint-inhibiting drugs have demonstrated significant therapeutic benefit in the setting of melanoma. Much work is being done to explore the power of immune checkpoint inhibitors for treatment of additional cancer types. Immune checkpoint markers include programmed cell death-1 (PD-1, or CD279) and its ligand PDL-1 (CD274); the proliferation modulator CD152; the costimulatory molecules CD137, CD134, CD28 and its ligand CD86. Accurate measurement of marker expression is critical to patient selection and therapy evaluation. Here we demonstrate an elegant 10-color flow cytometry assay for quantitation of immune checkpoint markers on peripheral blood mononuclear cells (PBMCs) following ex vivo stimulation and co-culture with cancer cell lines of multiple lineage. Immune checkpoint surface expression was evaluated using the BD FACSLyric™ platform.

Cultured PBMCs exhibited robust increases in immune checkpoint marker expression levels in both the T-cell compartment and the non–T-cell compartment following stimulation via cell-to-cell interaction or by means of known soluble stimulants. The expression of these immune checkpoint markers is an inducible phenomenon that can be achieved by simulating the conditions of the tumor microenvironment and can be controlled through culture conditions or by the choice of stimulatory agent employed. Assessment of immune checkpoint markers may provide insights to help with the patient stratification, to help monitor treatment effectiveness, or to help design or optimize more effective immunomodulatory drugs.

FACSLyric is not currently available in the US. For research use only. Not for use in diagnostic or therapeutic procedures.

Results (1)

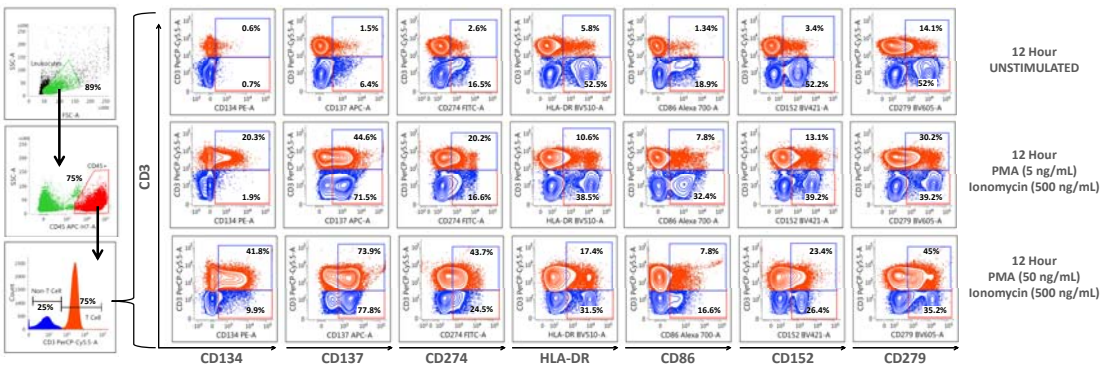

Figure 1. 10-color checkpoint panel gating strategy
Isolated PBMC cultures and PBMC cells co-cultured with MDA-MB-231 Cells demonstrated very similar results. A representative set of wells (PBMC cultured alone) is shown here. Cells were cultured for 12, 24, and 48 hours. At the conclusion of each time point, cells were stained with a 10-color flow cytometry panel. $CD45^+$ cells were divided into two main compartments based on CD3 expression. Expression of CD134, CD137, CD274, HLA-DR, CD86, CD152, and CD279 was enumerated on T cells ($CD3^+$) and Non-T cells ($CD3^-$).

Methods

PBMC Culture Setup and Stimulation
96-well plates were coated with PBS or anti-CD3 (Clone UCHT1) (BD Biosciences Cat. No. 555329) at concentrations of 0.5 µg/mL (Low), 2.0 µg/mL (Int), or 8.0 µg/mL (Hi). The plates were incubated at 37°C, 5% CO_2 for 2 hours and then placed in 4°C refrigerator overnight. The following day, all wells were rinsed twice with PBS and seeded with PBMCs at a concentration of 3×10^5 cells per well suspended in complete DMEM culture media. PBMCs were pooled from three different donors (all normal). After seeding with PBMCs, anti-CD3 coated wells were treated with soluble ant-CD28 at a concentration of 2 µg/mL. Wells coated with PBS alone (instead of anti-CD3) were left untreated (Unstimulated Controls) or were stimulated with Phytohemagglutinin (PHA) at concentrations of 1 µg/mL (Low), 3 µg/mL (Int), or 5 µg/mL (Hi), or alternatively with Phorbol 12-myristate 13-acetate (PMA) at concentrations of 5 ng/mL (Low), 50 ng/mL (Int), or 500 ng/mL (Hi). PMA-treated wells were additionally supplemented with 500 ng/mL of Ionomycin. Wells were set up in triplicate, and three identical 96-well plates were set up. 96-well plates were incubated at 37°C, 5% CO_2 for 12 hours, 24 hours, and 48 hours (data not shown).

Cell Culture and Co-Culture Set-up
MDA-MB-231 Cells (ATCC HTB-26), a human breast adenocarcinoma which was chosen based on their high expression of PDL-1 (CD274), were grown to 80% confluence in DMEM (Gibco, Ref#11995-065) supplemented with 10% heat-inactivated FBS and 1% Pen/Strep solution. Cells were harvested, washed and counted. MDA-MB-321 cells were mixed with PBMC at a ratio of approximately 3:1 (PBMC:MDA-MB-231). Co-cultures were stimulated exactly as described above using PBS. anti-CD3+CD28, PHA and PMA + Ionomycin. Co-cultures were harvested in parallel with the isolated PBMC cultures described above. Co-cultures were stained with a panel identical to that described in Table 1 (below) with the inclusion of CD326 for cancer cell line identification in place of HLA-DR.

Flow Cytometry
After incubation for each of the designated time periods (12, 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 PBS and recovered via centrifugation (7 minutes at 300*g*). Cells were then stained for 30 minutes with the 10-color antibody panel shown in **Table 1** (below). BD Horizon™ Brilliant Stain Buffer (BD Biosciences Cat. No. 659611). After staining, cells were again washed two times with PBS and recovered via centrifugation (7 minutes at 300*g*). Samples were analyzed on a BD FACSLyric system, and data analysis was performed using the BD FACSuite™ software platform.

	Fluorophore	Target	Alt Designation	BD Catalog #
1	APC-H7	CD45	PTPRC	560178
2	PerCp-Cy5.5	CD3	--	340949
3	BV421	CD152	CTLA-4	562743
4	PE-Cy7	CD28	--	560684
5	APC	CD137	4-1BB	550890
6	PE	CD134	OX40	555838
7	FITC	CD274	PD-1	558065
8	Alexa Fluor 700	CD86	B7-2	561124
9	BV605	CD279	PD-L1	563245
10	BV510	HLA-DR	--	563083

Table 1: 10-Color Checkpoint Panel

Results (2)

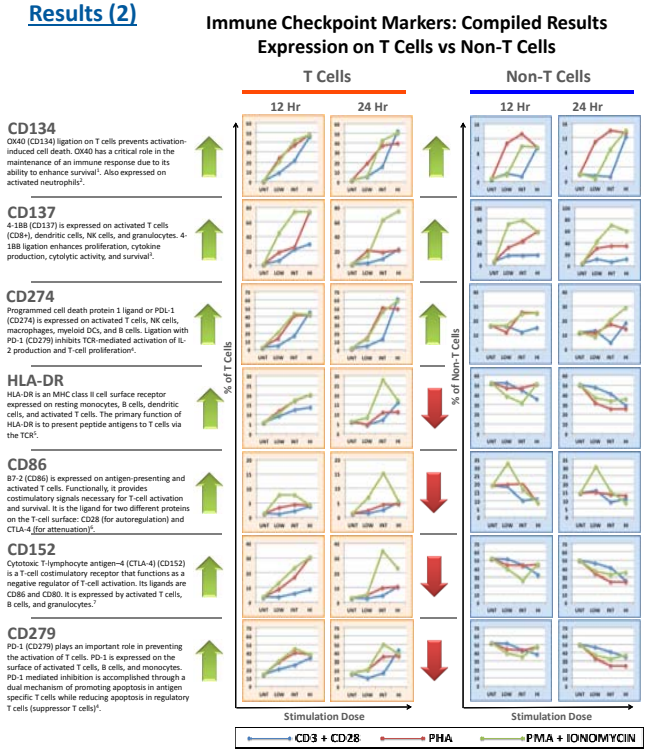

Figure 2. Expression of Checkpoint markers following prolonged stimulation
PBMC cultured alone were stimulated with anti-CD3 at concentrations of 0.5 µg/mL (Low), 2.0 µg/mL (Int), or 8.0 µg/mL (Hi) + CD28 (2 µg/mL) or with PHA at concentrations of 1 µg/mL (Low), 3 µg/mL (Int), or 5 µg/mL (Hi), or alternatively, PHA at concentrations of 5 ng/mL (Low), 50 ng/mL (Int), or 500 ng/mL (Hi). PMA-treated wells were supplemented with 500 ng/mL of Ionomycin. Stimulated cells were compared against unstimulated controls. Expression levels of all seven checkpoint markers increased in T cells, while the expression levels of these same markers showed increases and decreases in $CD3^-$ non-T cells.

Conclusions

- We were successfully able to use a 10-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, CD137, CD274, HLA-DR, CD86, CD152, and CD279.
- 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.
- Expression levels of immune checkpoint markers was increased in all states of stimulation (CD3 + CD28, PHA, and PMA + Ionomycin) on T cells
- Expression levels of CD134, CD137, and CD274 were increased in non-T cells following stimulation, while expression levels of HLA-DR, CD86, CD152, and CD279 demonstrated modest decreases in non-T cells following stimulation.
- PMA + Ionomycin consistently induced the most robust changes in immune checkpoint marker expression. The effects of PHA and CD3 + CD28 were less dramatic in most cases.
- Co-cultures of PBMC and the adenocarcinoma breast cancer cell line MDA-MB-231 which expresses high levels of PDL-1 (CD274) had no effect on the inducibility of checkpoint marker expression as co-cultures showed very similar results to PBMCs cultured alone.

References

1. The role of OX40-mediated co-stimulation in T-cell activation and survival. Redmond WL, Ruby CE, Weinberg AD. *Crit Rev Immunol*. 2009;29(3):187-201. Review. PMID: 19538134
2. Functional expression of CD134 by neutrophils. Baumann R, Yousefi S, Simon D, Russmann S, Mueller C, Simon HU. *Eur J Immunol.* 2004 Aug;34(8): 268-2275. PMID: 15259024
3. Biochemical and immunological characteristics of 4-1BB (CD137) receptor and ligand and potential applications in cancer therapy. Sica G, Chen L. *Arch. Immunol. Ther. Exp.* (Warsz.). 2000;47(5):275-279. PMID 10604232
4. The role of PD-1 and PD-L1 in T-cell immune suppression in patients with hematological malignancies. Shi L, Chen S, Yang L, Li Y. *J Hematol Oncol.* 2013 Sep 30;6(1):74. PMID: 24283718
5. HLA-DR: molecular insights and vaccine design. Stern LJ, Calvo-Calle JM. *Curr Pharm Des*. 2009;15(28):3249-3261. PMID: 19860674
6. IL-2 upregulates CD86 expression on human CD4(+) and CD8(+) T cells. Paine A, Kirchner H, Immenschuh S, Oelke M, Blaszcyk R, Eiz-Vesper B. *J Immunol.* 2012 Feb 15;188(4):1620-1629. PMID: 22246628
7. CTLA-4 is not restricted to the lymphoid cell lineage and can function as a target molecule for apoptosis induction of leukemic cells. Pistillo MP, Tazzari PL, Palmisano GL, et al. *Blood*. 2003 Jan 1;101(1):202-209. PMID: 12393538