Functional flow cytometry to predict PD-L1 conformational change and improve cancer immunotherapy

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CYTO VANCOUVER
June 22–26, 2019

BACKGROUND

Multiple myeloma (MM) is characterized by the accumulation of malignant plasma cells in the bone marrow (BM). Although new therapies have improved the results in the treatment of MM, today it remains incurable.

Programmed Death-Ligand 1 (PD-L1) has been identified as the responsible to suppress immune responses through the interaction with Programmed Death-1 (PD-1). In MM, PD-L1 is expressed in MM plasma cells, Myeloid Derived Suppressor Cells (MDSCs) and various non-hematopoietic cells. PD-1 is expressed in T-cells. The interaction between PD-L1 and PD-1 has been reported to decrease TCR-mediated proliferation and cytokine production. Thus, PD-L1 might play an important role in tumor immune evasion and drug resistance, being considered as a therapeutic target for this disease.

The aim of this study was to design and evaluate a direct screening assay to identify MM MDSCs PD-L1+ cells using flow cytometry, and to evaluate its potential use at the point of care diagnostic for MM.

MATERIALS AND METHODS

Human bone marrow specimens anticoagulated with EDTA were used in this study. For functional analysis, no-lyse no-wash (NLNW) procedures used Vybrant™ DyeCycle™ Violet to discriminate nucleated cells from erythrocytes and debris. Samples were acquired using the Attune™ NxT Flow Cytometer (Thermo Fisher).

PE-PD-L1 staining was combined with APC-CD11b, PE-Cy7-CD33, and FITC-HLA-DR (eBioscience) to detect MDSCs. PD-L1 expression was studied in a total of n=35 MM samples, with and without marrow stimulation. For stimulation, marrow cells were treated with PMA (Sigma Aldrich) for 10 minutes at 37ºC on a water bath. PD-L1 and CD11b cell surface expression was compared with cytoplasmic expression (on 11/35 patients). Kinetic analysis of PD-L1 expression was also studied over time (n=1). Competitive experiments in the presence of Durvalumab (0 ng/µL to 250 ng/µL), were used to study its interaction with PD-L1 (n=4 samples).

RESULTS

PD-L1 expression increases after marrow stimulation. PD-L1 was found dramatically increased after PMA stimulation (n=33 samples, 94.3%) ranging from 2 to 650 times (**Figure 1A**). **Figure 1B** shows PD-L1 levels with and without stimulation of a non-responding patient (PD-L1 fold-change ≤ 1) and a responding patient (PD-L1 fold-change > 1).

PD-L1 expression was undetectable at the cytoplasmic level. Variation in PD-L1 change among subjects led us to study PD-L1 cytoplasmic expression, before and after stimulation. PD-L1 and CD11b cytoplasmic levels were simultaneously studied in 11 subjects. PD-L1 was found to be undetectable, in comparison with CD11b cytoplasmic reactivity (**Figure 2**).

PD-L1 shows conformational changes after stimulation. Since PD-L1 was not present at the cytoplasmic level, we next investigated changes in PD-L1 expression over time. After stimulation, PD-L1 expression was found to be higher after 1 to 5 min, with a progressive decrease up to 1h (**Figure 3**).

Co-incubation with Durvalumab shows different PD-L1 immunofluorescent profiles. When adding increasing concentrations of Durvalumab, PE-PD-L1 expression decreased with increasing concentrations of the imunotherapy drug, suggesting that both drug and monoclonal antibody may react with similar antigenic sites (**Figure 4**).

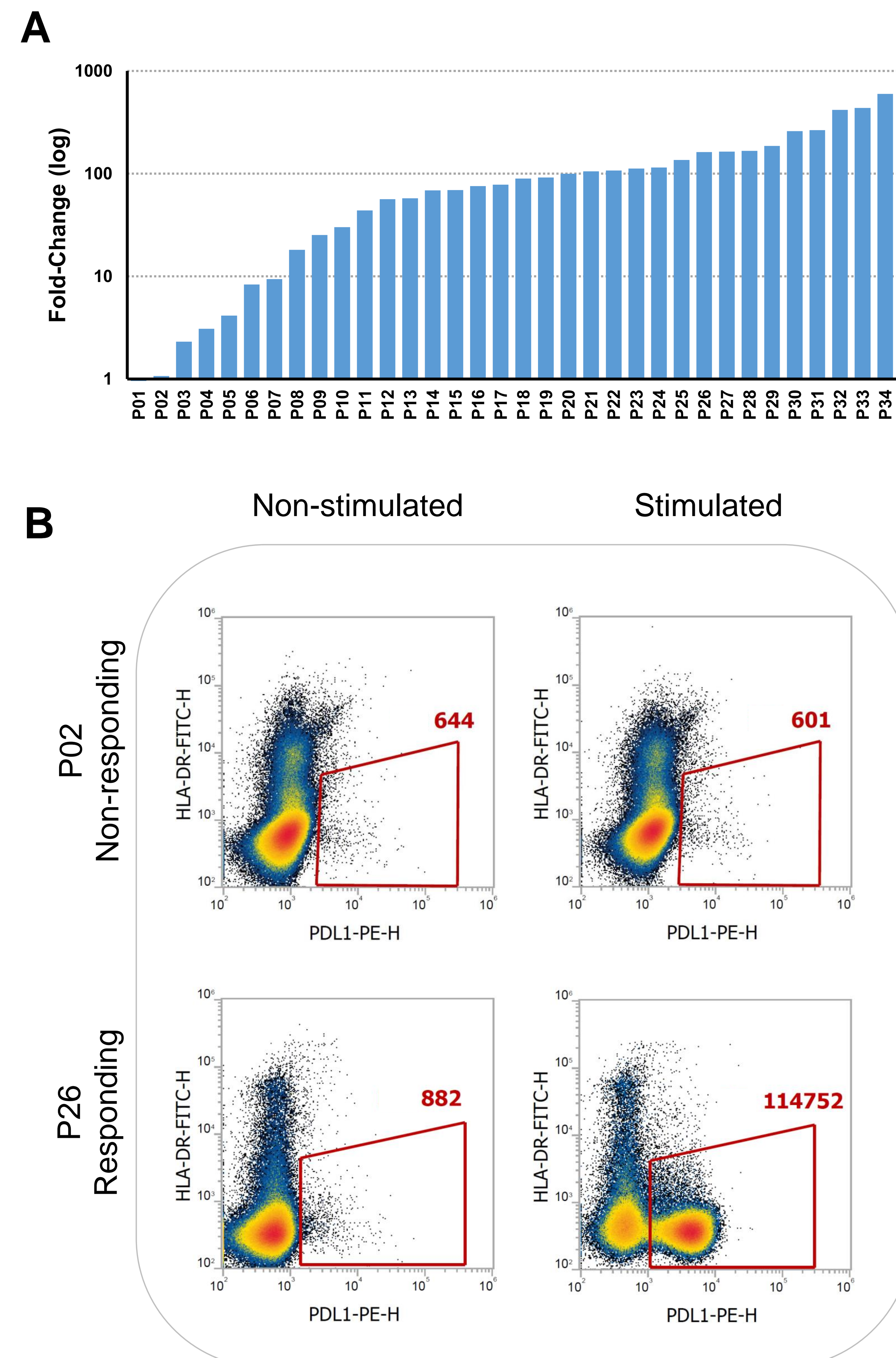
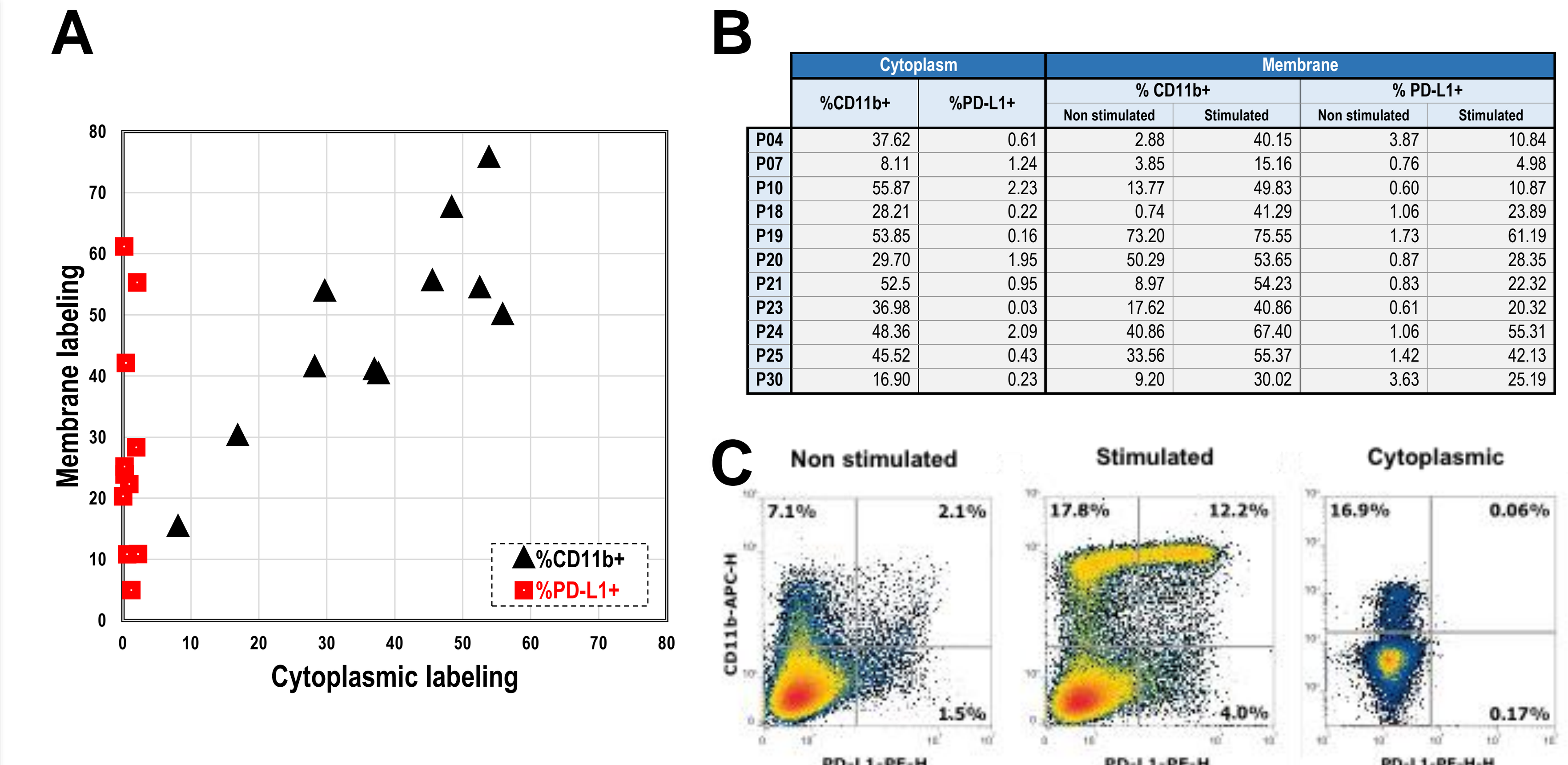


Figure 1. PD-L1 fold-change in a series of 35 multiple myeloma patients. (A) Fold-change was calculated as ratio of number of stimulated MDSCs PD-L1+ and non stimulated. Subjects P01 to P35 showed a wide fold-change variation, ranging from 1 (no variation) to 650. (B) Representative cases of a non-responding subject (P02) with PD-L1 fold-change ≤ 1 (upper row) and a responding subject (P26) with a PD-L1 fold-change > 1 (lower row).



	Cytoplasm		Membrane			
	%CD11b+	%PD-L1+	% CD11b+		% PD-L1+	
			Non stimulated	Stimulated	Non stimulated	Stimulated
P04	37.62	0.61	2.88	40.15	3.87	10.84
P07	8.11	1.24	3.85	15.16	0.76	4.98
P10	55.87	2.23	13.77	49.83	0.60	10.87
P18	28.21	0.22	0.74	41.29	1.06	23.89
P19	53.85	0.16	73.20	75.55	1.73	61.19
P20	29.70	1.95	50.29	53.65	0.87	28.35
P21	52.5	0.95	8.97	54.23	0.83	22.32
P23	36.98	0.03	17.62	40.86	0.61	20.32
P24	48.36	2.09	40.86	67.40	1.06	55.31
P25	45.52	0.43	33.56	55.37	1.42	42.13
P30	16.90	0.23	9.20	30.02	3.63	25.19

Figure 2. PD-L1 and CD11b surface and cytoplasmic expression. (A) Comparison of %CD11b+ and %PD-L1+ in surface and cytoplasm in a series of 11 multiple myeloma subjects. (B) CD11b+ and PD-L1+ expression results at cytoplasmic and cell surface level, with and without stumulation. (C) Representative results obtained from patient P30, showing simultaneous PD-L1 and CD11b expression data on non-stimulated and stimulated cells (cell surface and cytoplasmic determination).

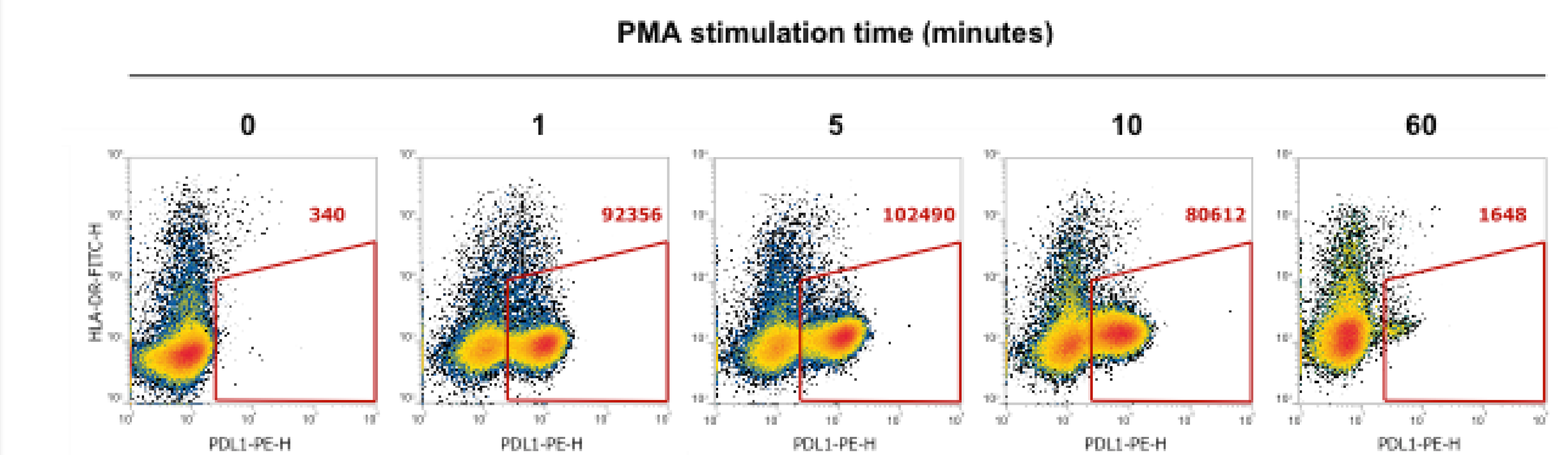


Figure 3. Kinetics of PD-L1 fold-change expression over time. PD-L1 was determined from 1 to 60 minutes, after PMA stimulation.

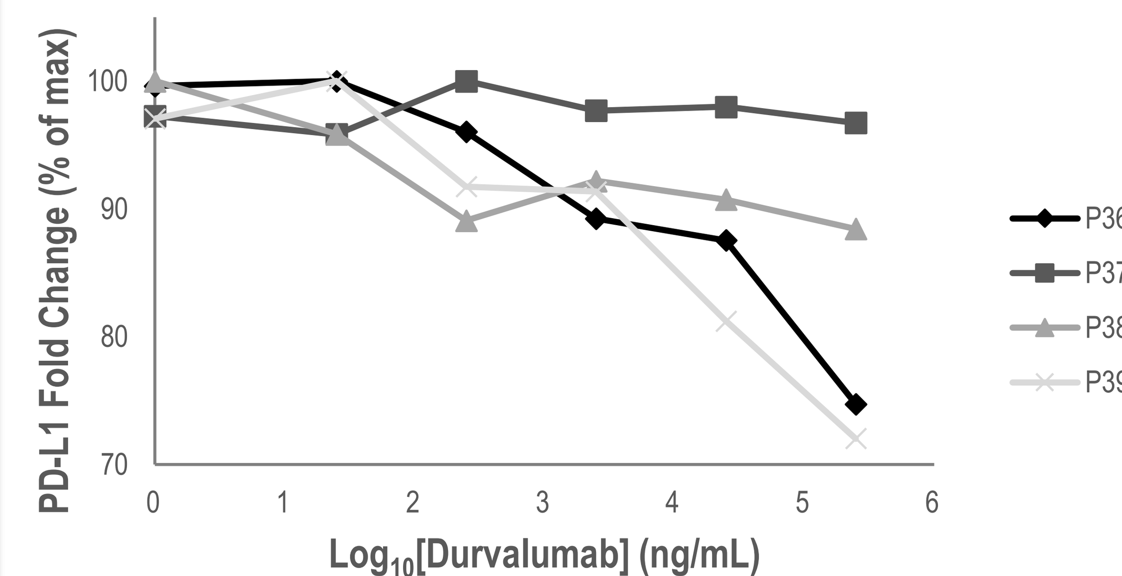


Figure 4. Competitive experiments in the presence of Durvalumab (immuno-therapy drug) and PE-PD-L1 (mono-clonal antibody). Cells were stimulated and incubated with 2.5ng/µL of PE-PD-L1 and increasing concentrations of Durvalumab (0, 0.025, 0.25, 2.5, 25 and 250ng/µL). PE-PD-L1 expression levels were compared with non stimulated cells.

CONCLUSIONS

PD-L1 reactivity appears to result from complex interactions that can only be detected with minimal sample perturbation. Since the PD-L1 molecule is not found at the cytoplasmic level, PD-L1 may reveal some steric changes in response to stimulation, even for a short period of time. This conformational change may be associated with a PD-L1 immunoregulatory mechanism that may affect therapies targeting the PD-1/PD-L1 checkpoint. Critical assessment of PD-L1 conformation, as well as those targets having similar unexpected features, may help to develop a better treatment strategy or to predict inhibitory therapy resistance. No-lyse no-wash methodologies in combination with functional assays may appear as an emerging strategy to model conformational changes in the target site.

ACKNOWLEDGEMENTS

2017 SGR 288 GRC

ThermoFisher SCIENTIFIC

Josep Carreras LEUKAEMIA Research Institute

ISAC INTERNATIONAL SOCIETY FOR ADVANCEMENT OF CYTOMETRY