Functional Flow Cytometry to predict PD-L1 Conformational Change and Improve Cancer Immunotherapy

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BACKGROUND

Multiple myeloma (MM) is characterized by the accumulation of malignant plasma cells in the bone marrow (BM) with an associated immunosuppressive BM microenvironment.

Programmed Death-Ligand 1 (PD-L1) has been shown to suppress immune responses through the interaction with Programmed Death-1 (PD-1). In MM, PD-L1 is overexpressed in MM plasma cells and in Myeloid Derived Suppressor Cells (MDSCs). PD-1 is expressed in T-cells. The interaction between PD-L1 and PD-1 decreases TCR-mediated proliferation and cytokine production.

PD-L1 plays an important role in tumor immune evasion and drug resistance, and is considered a therapeutic target. However, some MM individuals do not respond to treatments with PD-L1 or PD-1 inhibitors.

The **aim** of this study was to design and evaluate a direct functional screening assay to identify MM MDSCs PD-L1+ using flow cytometry, and its potential use in MM management.

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MATERIALS AND METHODS

Human bone marrow specimens anticoagulated with EDTA were used in this study. For functional analysis, no-lyse no-wash (NLNW) procedures were applied using Vybrant™ DyeCycle™ Violet to discriminate nucleated cells from erythrocytes and debris. Samples were acquired using the Attune™ NxT Flow Cytometer (Thermo Fisher). Samples were labeled with PE-PD-L1, APC-CD11b, PE-Cy7-CD33, and FITC-HLA-DR (Invitrogen™ eBioscience) to detect MDSCs using no-lyse no-wash methods. PD-L1 expression was studied in n=35 MM subjects, with and without bone marrow stimulation with PMA (Merck) for 10 minutes at 37ºC. PD-L1 cell surface expression was compared with cytoplasmic expression (n=11 subjects). For cytoplasmatic labelling, fixation and permeabilization were performed using 70% ethanol, after red blood cell lysis using ammonium chloride. Kinetics of PD-L1 expression were also studied over time. Competitive experiments in the presence of Durvalumab (0ng/µL to 250ng/µL) were used to study its interaction with PD-L1.

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RESULTS AND DISCUSSION

PD-L1 expression increases after marrow stimulation. PD-L1 was found dramatically increased after PMA stimulation (n=33 patients, 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 to PMA stimuli (PD-L1 fold-change ≤ 1) and a responding patient to PMA stimuli (PD-L1 fold-change > 1), with a fold-change value of 1 and 162, respectively. This heterogeneity could be associated with a differential response to immunotherapy among patients.

PD-L1 was not found at cytoplasmic level. Variation in PD-L1 fold-change among patients led us to consider the possibility that PD-L1 was expressed at cytoplasmic level, and after stimulation, translocated to the cell membrane, in the same manner as CD11b. PD-L1 and CD11b cytoplasmic levels were simultaneously studied in 11 patients. PD-L1 was found to be undetectable, in comparison with CD11b cytoplasmic reactive antigen (**Figure 2**).

PD-L1 is detected differentially depending on stimulation time. The fact that PD-L1 was not present at cytoplasmic level led us to investigate 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 showed different PD-L1 immunofluorescent profiles. When adding increasing concentrations of Durvalumab, PD-L1 detection by the fluorescent antibody showed different profiles among concentrations and patients. Both drug and monoclonal antibody bound to a similar PD-L1 site when the molecule had the proper conformation (**Figure 4**). Differences among patients could be related with structural modifications in PD-L1 molecule related with genetic mutations.

CONCLUSIONS

PD-L1 reactivity appears to result from complex interactions that can only be detected with minimal sample perturbation. Since this molecule is not found at 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 folding, as well as those targets having similar unexpected features, may help to develop a better treatment strategies or to predict therapy resistance. No-lyse no-wash methodologies in combination with functional assays show promise as an emerging strategy to model conformational changes in the target site.

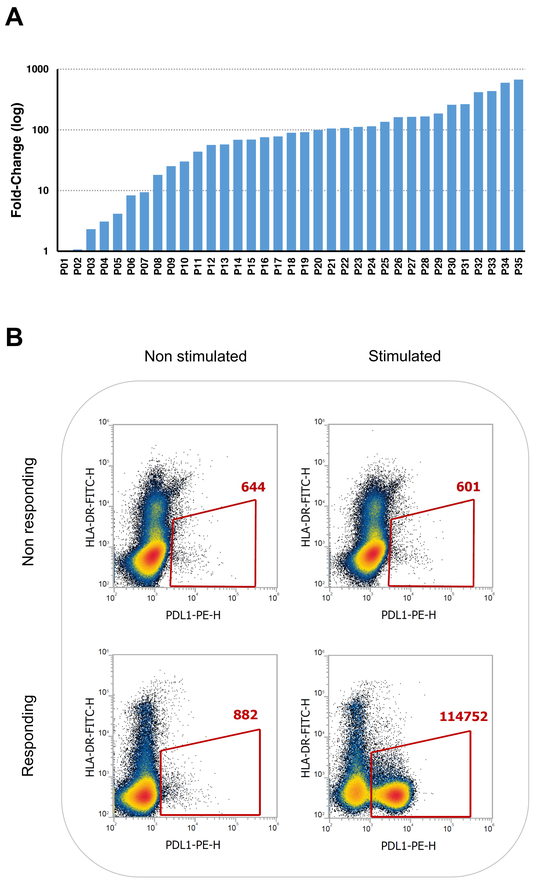
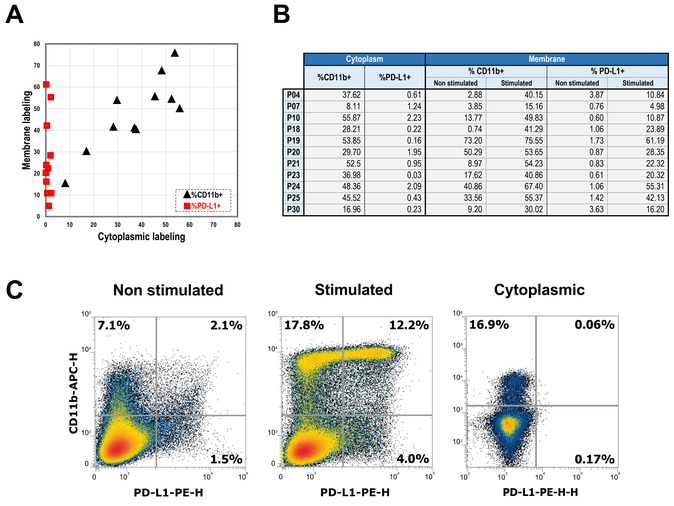


Figure 1. PD-L1 fold-change in a series of 35 multiple myeloma patients. (A) Fold-change was calculated as ratio of number of stimulated and non stimulated MDSCs PD-L1+. Patients P01 to P35 showed a wide fold-change variation, ranging from 1 (no variation) to 650. (B) Representative cases of a non-responding patient (P02) with PD-L1 fold-change ≤ 1 (upper row) and a responding patient (P26) with a PD-L1 fold-change > 1 (lower row). PD-L1+ cells are represented in terms of cell counts. Samples were acquired using the Attune™ NxT Flow Cytometer (Thermo Fisher).



	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	16.96	0.23	9.20	30.02	3.63	16.20

Figure 2. PD-L1 and CD11b surface and cytoplasmic levels determination. (A) Comparison of %CD11b+ and %PD-L1+ in surface and cytoplasm in a series of 11 multiple myeloma patients. (B) Results of %CD11b+ and %PD-L1+ obtained in cytoplasma and membrane (basal and stimulated). (C) Representative case (P30) of PD-L1 and CD11b surface determination non stimulated and stimulated and cytoplasmic determination. Samples were acquired using the Attune™ NxT Flow Cytometer (Thermo Fisher).

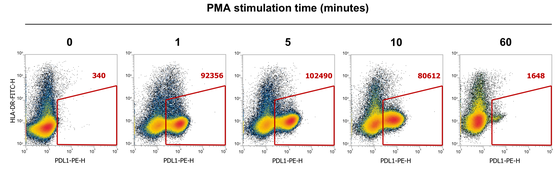


Figure 3. Kinetics of PD-L1 fold-change expression levels over time. PD-L1 was determined from 1 to 60 minutes of PMA stimulation. PD-L1+ cells are represented in terms of cell counts. Samples were acquired using the Attune™ NxT Flow Cytometer (Thermo Fisher).

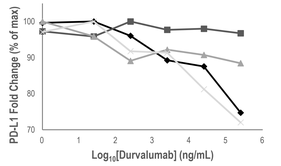


Figure 4. Competition experiments in presence of Durvalumab (immunotherapy drug) and PE-PD-L1 (monoclonal antibody). Cells were stimulated and incubated with 2.5ng/µL PE-PD-L1 and increasing concentrations of Durvalumab (0, 0.025, 0.25, 2.5, 25 and 250ng/µL). PE-PD-L1 expression levels were compared with non stimulated cells. Samples were acquired using the Attune™ NxT Flow Cytometer (Thermo Fisher).