PD-L1 Expression on Circulating Tumor Cells and Platelets in Patients with Metastatic Breast Cancer

Elizabeth P Darga
University of Michigan Rogel Cancer Center
Emily M. Dolce
University of Michigan Rogel Cancer Center
Fang Fang
University of Michigan School of Public Health
Kelley M. Kidwell
University of Michigan School of Public Health
Christina L. Gersch
University of Michigan Rogel Cancer Center
Steven Kregel
University of Michigan Rogel Cancer Center
Dafydd G. Thomas
University of Michigan Rogel Cancer Center
Anoop Gill
University of Michigan Rogel Cancer Center
Martha E. Brown
University of Michigan Rogel Cancer Center
Steven Gross
Menarini Siliconbiosystems
Mark Connelly
Menarini Siliconbiosystems
Michael Holinstat
University of Michigan Medical School
Erin F. Cobain
University of Michigan Rogel Cancer Center
James M. Rae
University of Michigan Rogel Cancer Center
Daniel F. Hayes (hayesdf@med.umich.edu)
University of Michigan Rogel Cancer Center https://orcid.org/0000-0003-3620-7575
Costanza Paoletti
University of Michigan Rogel Cancer Center

Research article

Keywords: Circulating Tumor Cell, CTC, PD-L1, Platelets, CTC PD-L1, Platelet PD-L1, CellSearch®

Posted Date: February 5th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-178272/v1

Version of Record: A version of this preprint was published at PLOS ONE on November 15th, 2021. See the published version at https://doi.org/10.1371/journal.pone.0260124.

Abstract

Background: Immune checkpoint inhibition (ICPi) is effective in several cancers. Expression of programmed death-ligand 1 (PD-L1) on circulating tumor or immune effector cells could provide insights into selection of patients for ICPi.

Methods: Whole blood (WB) was collected at serial timepoints from metastatic breast cancer (MBC) patients and healthy donors for circulating tumor cell (CTC) and platelet PD-L1 analysis using the CellSearch® assay. CTC PD-L1 was considered positive if detected on at least 1% of the cells; platelet PD-L1 was considered positive if ≥100 platelets per CellSearch frame expressed PD-L1.

Results: A total of 207 specimens from 124 MBC patients were collected. 52/124 (42%) samples at timepoint-1 (at or close to time of progressive disease) had ≥5 CTC/7.5ml WB. Of those, 21 (40%) had positive CTC PD-L1. In addition, platelet PD-L1 expression was observed in 35/124 (28%) at timepoint-1. Platelet PD-L1 was not detected in more than 70 specimens from 12 healthy donors. Platelet PD-L1 was associated with ≥5 CTC/7.5ml WB ($p=0.0002$), less likely in patients with higher red blood cell counts (OR=0.72, $p<0.001$) and a history of smoking tobacco (OR=0.76, $p<0.001$). Platelet PD-L1 staining was not associated with tumor marker status, recent procedures or treatments, platelet-affecting drugs, or CTC PD-L1 expression.

Conclusion: PD-L1 expression was found in MBC patients on both CTC and platelets in an independent fashion. Inter-patient platelet PD-L1 expression was highly heterogeneous suggesting that it is a biological event associated with cancer in some but not all patients. Taken together, our data suggest that CTC and platelet PD-L1 expression could play a role in predicting which patients should receive ICPi and as a pharmacodynamics biomarker during treatment.

Introduction

Immune checkpoint inhibition (ICPi) with antibodies to programmed cell death 1 (PD-1) and its ligand (PD-L1) is effective in several malignancies [1, 2]. PD-L1 expression on tumor or infiltrating immune cells in malignant tissue predicts benefit from anti-PD-L1/PD-1 therapies [3]. Other predictors of response to ICPi include presence of tumor infiltrating lymphocytes, human leukocyte antigen (HLA) status, high tumor mutation burden or surrogates of it, antigen presenting cells, and the host microbiome [4–6].

PD-L1 expression is dynamic. Tissues tested for PD-L1 are often collected at time periods long before the patient is treated with ICPi therapy [3]. Evaluation of circulating tumor biomarkers in blood, designated “liquid biopsies” may provide real-time estimates of tumor status [7]. In this regard, elevated circulating tumor cell (CTC) enumeration is prognostic in several metastatic epithelial cancers [8–11]. PD-L1 expression of CTC might provide additional information regarding potential clinical benefit from anti-PD-L1/PD-1 therapy [12–19].

In the process of evaluating PD-L1 expression on CTC with the CellSearch® CTC detection system [Menarini Silicon Biosystems (MSB)], we also observed PD-L1 staining of platelets. Therefore, we conducted a prospective preliminary study to determine the incidence of these findings in patients with metastatic breast cancer (MBC). We hypothesize both CTC and platelet expression of PD-L1 might be another mechanism of immune-checkpoint modulation, serving as additional predictive and monitoring factors for ICPi therapy.

Methods

Patient Accrual and Characteristics

Female patients with MBC of any subtype were enrolled into this prospective single-institution pilot study. Initial blood draws were at or close to the time the patient progressed on her most recent therapy. Serial blood draws were not at clinically relevant timepoints, but rather for additional experimentation and observation. Control blood was collected from male and female healthy donors. All subjects signed written informed consent approved by the Internal Review Board at the University of Michigan in accordance with the Declaration of Helsinki. Clinical characteristics were obtained by chart review and abstraction.

Blood Collection, Processing, CTC Enumeration, and Phenotyping

Whole blood (WB) was collected into 10ml CellSave tubes (MSB, Huntingdon Valley, PA) and processed through the CellSearch® (CXC kit) system (MSB, Huntingdon Valley, PA) as previously described [13, 20]. PD-L1 staining was performed using a phycoerythrin-labeled anti-human PD-L1 monoclonal antibody (Biolegend clone 29E.2A3; Cat# 329705, RRID:AB_940366; Biolegend, San Diego, CA) at 3.5 ug/ml, using methods similar to those previously described for other phenotypic markers [13, 14, 20, 21].

Quantifying Platelets from CellSearch® Enriched Product

The contents from CellSearch cartridges were extracted using gel loading tips coated in 2% BSA/PBS and placed into Eppendorf tubes. The number of platelets/ul was determined using the Hemavet® hv950 (Drew Scientific, Miami Lakes, FL).

Cell Culture for *in vitro* Experiments

Human breast cancer cell lines were cultured in a sterile incubator at 37°C with 5% CO_2. Characteristics for each cell line aredescribed in Supplementary Material (**Supplementary Table 1**). Expression status of ER, PgR, HER2, and PD-L1 for each cell line were previously described by Mittendorf et al. [22] (**Supplementary Table 1**). The cell lines used for *in vitro* experiments were used within two passages of defrosting. All cell lines were routinely tested for mycoplasma contamination and identified by Short Tandum Repeat profiling as previously described [20].

Platelet Characterization of CellSearch Enriched Product

Contents from CellSearch® cartridges were extracted, incubated for 30 minutes on ice with APC/Cy7 anti-human CD-42b and CD-41 monoclonal antibodies directed against platelets (clone HIP1 RRID: AB_2616778 Cat# 303920 and HIP8 RRID: AB_10896432 Cat#303715, respectively; Biolegend, San Diego, CA), centrifuged at 800g for 20 minutes, washed twice with 1% BSA/PBS, centrifuged for 8 minutes at 94g onto poly-lysine coated slides, then examined by fluorescence microscopy at 200X for platelet and PD-L1 expression.

Platelet Isolation from Whole Blood

Platelet rich plasma (PRP) was isolated from 7.5ml WB collected in CellSave tubes by 200g centrifugation for 10 minutes at room temperature (RT). Platelets were isolated by a second spin of 2,000g at RT and the platelet poor plasma (PPP) was centrifuged two additional times at 2,000g to remove residual platelets. The PPP and platelet pellet were each resuspended with dilution buffer to a total volume of 14ml. The sample tubes were taped to the 4ml mark to simulate the red blood cell layer, enabling the automated CellSearch® to process the sample. Because these samples lacked whole cells necessary to focus the CellTracks Analyzer camera, DAPI coated magnetic beads provided by MSB were added to each cartridge to permit scanning.

PD-L1 Knockdown in Cultured Human Breast Cancer Cells

Silencer® select pre-designed siRNA (s26547, s26548, s26549; Life Technologies, Carlsbad, CA) was used to knockdown PD-L1 expression in MDA-MB-231 cells. Two nonsense controls were used separately, one from Dharmacon and one from Silencer® select. The cells were transfected using Lipofectamine RNAiMax reagent (Invitrogen, Carlsbad, CA) in Opti-MEM medium with a final concentration of 25 pmol siRNA per well following manufacturer's instructions (Supplementary Materials and Methods).

Western Blot Analyses

PD-L1 protein expression in cultured human breast cancer cells was confirmed using Western blot analyses. For each sample, 35 ug of protein lysate was loaded onto 4-12% acrylamide gels. PVDF membrane was blocked with 5% milk/TBS-T and incubated with anti-PD-L1 rabbit primary monoclonal antibody (E1L3N Rabbit mAb; Cat# 13684, RRID:AB_2687655, Cell Signaling Technologies, Danvers, MA) and subsequently with horseradish peroxidase-linked anti-rabbit IgG secondary mAb

(Cat# 7074, RRID:AB_2099233, Cell Signaling Technologies, Danvers, MA). Horseradish peroxidase linked β-actin was used as a loading control (8H10D10, Cat# 3700, RRID:AB_2242334, Cell Signaling Technologies, Danvers, MA).

PD-L1 Gene Expression

Cell lysates were prepared using trizol and homogenized using a 23 gauge sterile syringe. RNA was extracted using the Qiagen mini-RNA extraction kit (Qiagen, Hilden, Germany) following manufacturer's instructions and included an Ambion DNA clean up kit (Invitrogen, Carlsbad, CA). Purified RNA was interrogated with the Promega Reverse Transcription Kit for RT-PCR (Promega, Madison, WI). Template cDNA was further amplified using sybergreen PCR with primer pairs designed for human PD-L1 (Integrated DNA Technologies, Coralville, IA). Samples were analyzed in triplicate using a CFX96 Real-Time PCR detection system (Bio-Rad, Hercules, CA). All samples were normalized to GAPDH as previously described [23] and measured as fold change of PD-L1 expression from untreated MDA-MB-231 cells. All primers were diluted to a working concentration of 250nM. PD-L1 primer pair sequences are as follows: primer pair 1 5'CTTCCGTTTAAGAAAAGGGAGAA3'/ 5'TTACGTCTCCTCCAAATGTGT3'; primer pair 2 5'CTGACATTCATCTTCCGTTTAAG3'/5'CGTCTCCTCCAAATGTGTATCA3'; primer pair 3 5'GACATTCATCTTCCGTTTAAGAAA3'/5'CGTCTCCTCCAAATGTGTATCA3'.

Statistical Analysis

The distribution of CTC PD-L1 and platelet PD-L1 expression in all blood samples was summarized using descriptive statistics. A marginal model with a logit link using generalized estimating equations (GEE) was fit to explore whether platelet PD-L1 positivity varied across the timepoints. To evaluate the association between timepoint-1 PD-L1 expression with timepoint-1 CTC and platelets, we performed a Pearson's chi-squared test and Fisher's exact test, respectively, each with significance level p=0.05.

Marginal models estimated using GEE assuming an independent working correlation structure were used to explore the clinical/pathological features associated with CTC and platelet PD-L1 expression. For CTC PD-L1 expression, the outcome consisted of the count of PD-L1 positive CTC using samples with >0 CTC/7.5ml WB. Platelet PD-L1 expression was dichotomized between negative (<100 PD-L1 positive platelets per frame) or positive (≥100 PD-L1 positive platelets per frame) expression. Associations between features of interest with PD-L1 expression were assessed singly. The false discovery rate approach was used to adjust the type I error to claim statistical significance with p-value ≤0.001.

Data management and analysis were conducted using Statistical Analysis System (SAS) statistical software, version 9.4 (Statistical Analysis System, RRID:SCR_008567, SAS Institute Inc., Cary, NC, USA).

Results

CTC PD-L1 Expression in Patients with MBC at Timepoint-1

Blood samples from 124 patients with MBC were assessed for CTC PD-L1 expression at timepoint-1 and subsequent timepoints in selected cases **(Supplementary Fig. 1)**. Timepoint-1 was at or close to any time that a patient was found to have progressive disease.

Of the 124 samples at timepoint-1, 52 (42%) had elevated CTC (≥ 5/7.5ml WB) (Table 1). Twenty-one (40%) of these 52 specimens had ≥ 1% CTC PD-L1 expression of 1–2+ [median 15.2% (range 1-100%); Fig. 1; Table 1], within a semi-quantitative grading system we developed (**Supplementary Fig. 2**). Although phenotyping data is typically reported only for patients with ≥ 5/7.5ml WB, for patients in this study with 1–4 CTC/7.5ml WB at least one PD-L1 positive CTC was also observed in 9/30 (30%) patients (Table 1).

Table 1
CTC PD-L1 expression at timepoint-1.

#CTC/7.5ml WB	CTC Enumeration[a]	PD-L1 Positive[b]
0 CTC	42	N/A
1–4 CTC	30	9 (30%)
≥ 5 CTC	52	21 (40%)
Total	**124**	**30 (24%)**
[a] Number of patients assessed for CTC/7.5ml WB at timepoint-1.		
[b] Number (%) of patients with ≥ 1% CTC PD-L1 expression at timepoint-1.		

Platelet PD-L1 Expression in Patients with MBC

In the classic CellSearch® system, leukocytes are identified by staining with fluoresceinated anti-CD45, and platelets are not visualized. During CTC PD-L1 expression analysis using CellSearch®, we observed PD-L1 staining on what visually appeared to be platelets. In extensive evaluations of WB using CellSearch® standard antibodies, as well as investigational studies using labeled antibodies against a number of other biomarkers, we have not observed platelet staining. Platelet PD-L1 was not observed in over 70 WB samples collected longitudinally from 12 healthy donors spiked with MDA-MB-231 cells and processed through CellSearch® and stained with the 29E.2A3 PD-L1 antibody. Therefore, we further investigated this curious finding.

In the CellSearch® system, a platelet specific marker cannot be used simultaneously with anti-PD-L1, since only one additional florescence channel is available for phenotyping. Therefore, using cytospins, we demonstrated that the PD-L1 positive, non-nucleated objects observed in CellSearch® co-stained with anti-platelet antibodies, confirming that they were indeed, platelets (Supplementary Materials and Methods, **Supplementary Fig. 3**). Platelet PD-L1 staining was not an artifact of the CellSave tube fixative (**Supplementary Fig. 4** and **Supplementary Table 2**). When PD-L1 positive platelets were visible, they were evenly distributed throughout the CellSearch® cartridge as is visualized in images of three different frames within one CellSearch patient cartridge (**Supplementary Fig. 5**). Additionally, we tested the specificity of the PD-L1 antibody we employed, 29E.2A3, by siRNA knockdown and cell line staining (**Supplementary Fig. 6–7**, see supplementary materials for details).

The CellSearch® system is designed to enrich for cells expressing the epithelial cell adhesion molecule (EpCAM). However, CellSearch® does not enrich epithelial cells to purification, and some leukocytes, which are EpCAM negative and CD-45 positive, are routinely carried-over during the enrichment process in all samples. Hence, the addition of fluoresceinated anti-CD45 in the CellSearch® system in order to distinguish the two. However, it was not previously recognized that platelets, as well, are carried over during the enrichment process, and since platelets would not mimic CTC, no added staining is included in the CellSearch® system. Therefore, we tested the carry-over of platelets in the CellSearch® assay.

For patient samples in which platelet PD-L1 staining was observed using the classic CellSearch® method (Fig. 2A), PD-L1 staining was maintained in the aliquot containing only the platelet pellet (Fig. 2B). Staining for CD-45 (APC) and CK (FITC) in this aliquot was negative, demonstrating that the sample was clear of white blood cell (WBC) and CTC carryover (Fig. 2B). Platelet poor plasma (PPP) did not have PD-L1 staining, confirming that the PD-L1 staining was on platelets (Fig. 2C). In both platelet pellet and PPP samples, the DAPI fluorescence was seen only on DAPI coated magnetic beads (Fig. 2B-E) added to permit scanning of the samples. For patient samples that did not have platelet PD-L1 staining present in the classic CellSearch® method, neither the platelet pellet nor PPP displayed PD-L1 staining (Fig. 2D-E). Taken together, these data confirmed that the non-CTC PD-L1 staining is on platelets and that platelets do indeed carry-over during the CellSearch enrichment process.

In a subset of patients, the number of platelets carried-over into the CellSearch cartridge was quantified using a Hemavet. Platelet PD-L1 staining was independent of number of platelets within the CellSearch® cartridge as well as routine clinical complete blood count determined on the same day as the research blood collection (**Supplementary Table 3**).

Inter-patient platelet PD-L1 expression was heterogeneous. Using a semi-quantitative scale (**Supplementary Fig. 8**), 41 (33%), 48 (39%), 24 (19%), and 11 (9%) of 124 samples at timepoint-1, had 0, < 100, 100-1,000, and > 1,000 PD-L1 positive platelets/frame of the CellSearch® cartridge, respectively (Table 2). Using an arbitrarily designated cutoff of ≥ 100 PD-L1 positive platelets/frame as positive, 35 (28%) samples were positive for platelet PD-L1 expression at timepoint-1.

Table 2
Platelet PD-L1 distribution in all samples

Platelet PD-L1 Score[a]	**Blood Draw Timepoints**				
	1	**2**	**3**	**4**	**5**
	N (%)	**N (%)**	**N (%)**	**N (%)**	**N (%)**
	124	**59**	**16**	**6**	**2**
Negative[b]	**89 (72%)**	**39 (66%)**	**9 (56%)**	**2 (33.3%)**	**0**
0	41 (33%)	16 (27%)	5 (31%)	0	0
< 100	48 (39%)	23 (39%)	4 (25%)	2 (33.3%)	0
Positive[b]	**35 (28%)**	**20 (34%)**	**7 (44%)**	**4 (66.6%)**	**2 (100%)**
100-1,000	24 (19%)	12 (20%)	5 (31%)	2 (33.3%)	0
> 1,000	11 (9%)	8 (14%)	2 (13%)	2 (33.3%)	2 (100%)

[a]Average platelet count/3 CellSearch Frames (see Supplementary Materials and Methods for details).

[b]Arbitrary classification of platelet PD-L1 staining (see Supplementary Materials and Methods for details). N = number.

At timepoint-1, platelet PD-L1 expression was associated with elevated CTC levels, but not with CTC PD-L1 expression. Twenty-four of the 52 (46%) samples with ≥ 5 CTC/7.5ml WB, but only 11/72 (15%) samples with < 5 CTC/7.5ml WB had PD-L1 positive platelets ($p = 0.0002$) (Table 3). Platelet PD-L1 expression was independent of CTC PD-L1 expression for both samples with ≥ 5 CTC/7.5ml WB ($p = 0.34$) and < 5 CTC/7.5ml WB ($p = 0.99$) (Table 3).

Table 3
Association of platelet PD-L1 score and CTC enumeration and PD-L1 expression at timepoint-1

		Association of Platelet PD-L1 Positivity with:										
		CTC Enumeration (≥5/7.5ml WB)[c]			CTC PD-L1 Expression [d, e]							
		N⁰ of CTC/7.5 ml WB				N⁰ of CTC/7.5 ml WB						
						0	1–4			≥5		
						N (%)	N (%)			N (%)		
		(0–4) N (%)	(≥ 5) N (%)	Total	CTC PD-L1 Score[b]	NA	NEG	POS	Total	NEG	POS	Total
PlateletPD-L1 score[a]	NEG (0 - <100)	61 (49%)	28 (23%)	89 (72%)		39 (54%)	15 (21%)	7 (10%)	61 (85%)	15 (29%)	13 (25%)	28 (54%)
	POS (100 - >1,000)	11 (9%)	24 (19%)	35 (28%)		3 (4%)	6 (8%)	2 (3%)	11 (15%)	16 (31%)	8 (15%)	24 (46%)
	Total	72 (58%)	52 (42%)	124		42 (58%)	21 (29%)	9 (13%)	72	31 (60%)	21 (40%)	52

[a]Arbitrary classification of platelet PD-L1 staining based on average platelet count/3 CellSearch Frames (see Supplementary Materials and Methods for details).

[b]Number (%) of patients with ≥ 5 CTC/7.5ml WB enumeration and < 1% (NEG) or ≥ 1% (POS) CTC PD-L1 expression.

[c]Chi-squared test for comparison Platelet PD-L1 NEG vs. POS according to CTC PD-L1 enumeration; p-value = 0.0002

[d]Chi-squared test comparison Platelet PD-L1 NEG vs. POS according to CTC PD-L1 expression if 0–4 CTC/7.5 ml whole blood; p-value = 0.34

[e]Fisher's exact test comparison Platelet PD-L1 NEG vs. POS according to CTC PD-L1 expression if CTC ≥ 5 p-value= 0.99

Association of CTC PD-L1 and Platelet PD-L1 Expression with Clinical/Pathological Features

We assessed associations of CTC PD-L1 and platelet PD-L1 expression with clinical and pathological features (**Supplementary Tables 4–8)**.

CTC PD-L1 and Clinical/Pathological Features. Only patients (n = 91) with ≥ 1 CTC/7.5ml WB at at least one timepoint were included in the analysis of association of CTC PD-L1 and clinical/pathological features. By univariable analysis, CTC PD-L1 positivity was less likely in patients with ER + compared to triple negative MBC at either the time of 1st clinical metastatic biopsy (RR = 0.33, p < 0.001) and at a subsequent, later metastatic biopsy (RR = 0.31, p < 0.001). Similarly, CTC PD-L1 was less likely in patients with HER2 + compared to triple negative MBC at the time of either metastatic biopsy (RR = 0.22, p < 0.001 for both) (**Supplementary Table 5**). CTC PD-L1 was also less likely in patients with bone only disease compared to patients without bone disease (RR = 0.14, p < 0.001). CTC PD-L1 was significantly increased in patients currently receiving or who had just progressed on either endocrine therapy (ET) (RR = 3.19, p < 0.001) or CDK4/6 inhibitors (RR = 4.11, p < 0.001) (**Supplementary Table 5**). CTC PD-L1 was not associated with anticoagulant drugs, although only 13 patients were on a dedicated anticoagulant medication (rivaroxaban, enoxaparin, apixaban, clopidogrel) (**Supplementary Table 5**).

In multivariable analysis, in contrast to the univariable analysis, CTC PD-L1 was significantly higher in patients with ER + compared to triple negative disease (RR = 2.56, p = 0.007) and HER2 + compared to triple negative disease (RR = 3.14, p = 0.04). In concert with the univariable analysis, it was associated with prior treatment or progression on CDK4/6 inhibitors (RR = 3.6, p = 0.008) (Table 4). Likewise, CTC PD-L1 was significantly lower in patients with bone only disease (RR = 0.09, p < 0.001) or with bone and other sites of disease (RR = 0.19, p < 0.001) (Table 4).

Table 4

Multivariable results of factors of interest with CTC PD-L1 positive rate [a] and Platelet PD-L1 positivity [b]

Characteristics	Category	Rate Ratio[c] or Odds Ratio[d] of PD-L1 Positivity (95% CI)	P-value [e]
CTC PD-L1			
Most recent met hormone status	(Overall)		0.03
	ER+, HER2- vs. Triple Neg	2.56 (1.298, 5.058)	0.007
	HER2 + vs. Triple Neg	3.14 (1.040, 9.492)	0.04
Disease Site	(Overall)		< 0.001
	Bone + other site vs. Other site (no bone)	0.19 (0.078, 0.462)	< 0.001
	Bone only vs. Other site (no bone)	0.09 (0.034, 0.243)	< 0.001
CDK4/6 inhibitor	Yes vs. No	3.60 (1.403, 9.238)	0.008
Platelet PD-L1			
RBC (M/ul)	Continuous variable	0.73 (0.642, 0.820)	< 0.001

[a] Only significant factors included in this table. See Supplementary Table 6 for full multivariable analysis.

[b] Only significant factors included in this table. See Supplementary Table 8 for full multivariable analysis.

[c] Rate ratio is calculated using Poisson GEE model assuming an independent correlation structure to explore the association between CTC PD-L1 and factors of interest.

[d] Platelet PD-L1 positivity is binary positive (≥ 100 PD-L1 positive platelets) or negative (< 100 PD-L1 positive platelets) by CellSearch, odds ratio is calculated using GEE model assuming an independent correlation structure to explore the association between Platelet PD-L1 and factors of interest.

[e] Statistical significance is any $p < 0.05$.

Platelet PD-L1 and Clinical/Pathological Features. By univariable analysis, platelet PD-L1 positivity was higher in patients who had increased numbers of CTC (OR = 1.03 for each 100 CTC/7.5ml increase, $p < 0.001$) and in patients with ≥ 5 vs. <5 CTC (OR = 1.45, $p < 0.001$), but as noted above was independent of CTC PD-L1 status (**Supplementary Table 7**). It was significantly lower in patients with increased number of red blood cell counts (univariable OR = 0.72 for each M/ul increase, $p < 0.001$; multivariable OR = 0.73, $p < 0.001$) (**Supplementary Table 7**, Table 4). Platelet PD-L1 was also statistically lower in patients who were current vs. passive/never smokers by univariable analysis (OR = 0.76, $p < 0.001$)(**Supplementary Table 7**). Anticoagulant drugs did not appear to affect platelet PD-L1 expression. However, since only a single patient was on clopidogrel and NSAID or aspirin use was taken on an as needed basis and often not recorded, no association with specific platelet-affecting agents could be drawn. Platelet PD-L1 expression was not associated with any other identifiable pathological or clinical features (**Supplementary Tables 7 and 8**).

No attempt to associate either CTC or platelet PD-L1 expression and outcomes was performed, since patients were enrolled based on convenience and represented a vast heterogeneity of breast cancer subtypes, treatments, lines of therapy, and follow-up.

Serial Specimen CTC PD-L1 and Platelet PD-L1 Expression

Serial CTC PD-L1. Of the 124 patients enrolled, 59 had specimens assessed for CTC PD-L1 and platelet PD-L1 at multiple subsequent timepoints, ranging from 1.5 weeks to 27 months after timepoint-1. Time between subsequent timepoints as well as whether the blood was drawn when the patient was on treatment or at progression is detailed in **Supplementary Table 9**. Of these 59, 35 (59%) had ≥ 1 CTC/7.5ml in two or more subsequent specimens and 14/35 (40%) were CTC PD-L1 negative at all timepoints (**data not shown**). Five (14%) patients maintained CTC PD-L1 positivity (Fig. 3A), 9 (26%) patients converted CTC PD-L1 status from negative to positive (Fig. 3B), 4 (11%) patients converted from positive to negative (Fig. 3C), and 3 (9%) patients had CTC PD-L1 expression fluctuating from negative to positive to negative again at subsequent timepoints (Fig. 3D).

Serial Platelet PD-L1. Platelet PD-L1 positivity varied significantly over time in some but not all patients ($p = 0.005$). Of the 13 patients who had platelet PD-L1 positive at timepoint-1, 10 (77%) maintained positivity at subsequent blood draws (Fig. 4A). In contrast, 2 (15%) patients converted from platelet PD-L1 positive to negative (Fig. 4B) and 1 (7%) patient had platelet PD-L1 status fluctuating from positive to negative to positive again among subsequent timepoints (Fig. 4C). Of the 46 patients who had platelet PD-L1 negative at timepoint-1, 31 (67%) maintained platelet PD-L1 negativity at subsequent blood draws (**data not shown**), whereas 15 (33%) patients converted from having platelet PD-L1 negative to positive at a later timepoint (Fig. 4D).

Discussion

In this study, using the CellSearch® assay system, we confirmed PD-L1 expression on CTC at timepoint-1 in 24% of patients with MBC. As expected, 42% of these patients had ≥ 5 CTC/7.5ml WB [9, 24, 25]. Of these, 40% were CTC PD-L1 positive. These data confirm previous reports of CTC PD-L1 expression and are very similar to the 37% CTC PD-L1 positivity reported by Jacot *et al* [17, 26]. However, we observed a lower percentage of CTC PD-L1 positivity than reported by other investigators, in which both the CTC isolation method and the anti-PD-L1 antibody differed from ours [27].

In multi-variable analyses, CTC PD-L1 expression was less likely to be observed in patients with bone metastases compared to those without bone metastases. By univariable analyses, CTC PD-L1 expression was more likely observed in patients currently receiving or who had recently progressed on ET, especially when given in combination with a CDK4/6 inhibitor, at the time of 1st blood draw. Enigmatically, in multi- but not uni-variable analysis, CTC PD-L1 was more likely to be observed in ER + and HER2 + versus triple negative MBC. The paradoxical finding of CTC PD-L1 association with ER negativity in uni-variable but with positivity in multi-variable analyses is unexplained, but might suggest that positivity is associated with some other feature, such as multiple prior lines of therapy and/or the cancer's becoming refractory to ET.

Importantly, we also observed platelet PD-L1 expression in 28% of the patients with MBC. Platelet PD-L1 staining was associated with elevated CTC levels, but was independent of CTC PD-L1 expression. Expression of platelet PD-L1 was highly heterogeneous among different MBC patients. Unlike CTC PD-L1 positivity, it was not associated with many distinct clinical or pathologic features, including tumor hormone receptor or HER2 status, apparent burden or site of disease, progressive or stable disease. Importantly, platelet PD-L1 positivity did not appear to be an artifact of causes that might be expected to activate platelets, such as recent surgery or other procedures within the preceding two months, presence of an intravascular indwelling device (port-a-cath), thrombocytopenia, or treatment. Platelet PD-L1 positivity was lower in patients who were current smokers and in those with increased red blood cell counts. For patients with serial blood draws, platelet PD-L1 expression remained stable in 69% of patients. Of the 18 patients that did have a change in platelet PD-L1, 15 (83%) changed from negative to positive over time.

Platelet PD-L1 expression has been previously described by flow cytometry, using the same PD-L1 antibody we employed [28, 29]. These reports have mostly, if not entirely, been within the context of comprehensive analysis of PD-L1 expression on all hematopoietic and immune-effector cells. Furthermore, the prior reports of CTC PD-L1 expression in patients with MBC have not included observations of platelet expression of PD-L1, [17, 26] raising concerns about the specificity of the individual antibodies used to evaluate this marker. Our extensive investigations demonstrated that a) the antibody (29E.2A3) used in our investigations was specific for PD-L1 and b) the elements that were staining for PD-L1 were clearly platelets (**Supplementary Materials and Methods**).

Platelets prevent hemorrhage, but have many other activities in normal hemostasis, wound healing, and immune function [30, 31]. We speculate that platelet PD-L1 expression might serve in the normal situation to protect epithelial cells from being innocent bystanders in the early immune response to infection. Further, as epithelial cells progress along the malignant continuum [32], some, but not all, may be associated with platelets expressing PD-L1 and commensurate immune suppression. Thus, platelet PD-L1 expression may be a mechanism of tumor escape from immune elimination [5, 33, 34]. More recently, Zaslavsky *et al* reported PD-L1 expression on platelets in metastatic cancer patients and demonstrated that platelets bind to cancer cells supporting the theory that platelets provide an immune escape mechanism [35]. In this paper, we provide evidence of substantial inter- and intra-patient heterogeneity of platelet-PD-L1 expression, consistent with the clinical observation of variable sensitivity of patient responses to immune checkpoint inhibition therapy to date [3, 36].

Taken together, these results strongly suggest that CTC PD-L1 and platelet PD-L1 expression are not due to technical or clinical artifacts, and might have clinical relevance in the treatment of patients with cancer. Several different putative predictive factors for anti-PD-L1 and anti-PD-1 therapies have been reported, including expression of PD-L1 itself, either on cancer cells, tissue or circulating immune effector cells [37, 38]. CTC PD-L1 has been reported to be an independent predictor of improved progression free survival in MBC patients who did not receive ICPi [26] as well as in melanoma patients starting pembrolizumab [19]. In addition to platelet PD-L1 identified in patients with metastatic cancer [35], platelet PD-L1 expression was also recently reported in four patients with lung cancer who benefited from atezolizumab [29]. Of interest, the association of CTC PD-L1 with CDK4/6 treatment in our study coincides with a report that CDK4/6 inhibitors upregulate PD-L1 expression [39, 40]. Therefore, determination and monitoring of CTC PD-L1 in patients receiving these agents provides insight into combination ICPi and CDK4/6 inhibitors. Additionally, studies have shown that radiotherapy transiently increases PD-L1 expression on CTC in NSCLC, suggesting ICPi can be used in conjunction with radiotherapy for added efficacy as well as further highlighting the importance of monitoring CTC PD-L1 [41].

Of note, the remarkable inter-patient heterogeneity suggests a potential biological, and perhaps pharmacodynamic, role for both CTC and platelet PD-L1 expression. The ease of identifying and quantitating CTC levels, CTC PD-L1 and platelet PD-L1 expression on a single analytical platform (CellSearch®) should facilitate further research in this area. In this regard, we are planning a pilot clinical study to determine if CTC or platelet PD-L1 expression might have a role in directing treatment with ICPi therapies.

Conclusion

In summary, we have demonstrated that PD-L1 expression can be reliably and quantitatively evaluated on CTC and platelets using the FDA-cleared CellSearch® assay in MBC patients. We speculate that CTC and/or platelet PD-L1 expression could predict benefit from ICPi therapies, particularly those directed towards the PD-1/PD-L1 pathway. Prospective studies of patients receiving anti-PD-1/PD-L1 therapies are warranted to further explore PD-L1 on both CTC and platelets in order to correlate findings with response rates and progression free survival to validate the use of PD-L1 as a predictive biomarker for liquid biopsies.

Declarations

Ethical Approval and Consent to participate:

All subjects signed written informed consent approved by the Internal Review Board at the University of Michigan in accordance with the Declaration of Helsinki.

Consent for publication:

All authors have reviewed the manuscript and have given consent for publication.

Availability of supporting data:

All relavant data is included in the manuscript. Clinical data is maintained in a secure database in the Hayes laboratory. To request access to clinical data, permission must be granted from the Internal Review Board at the University of Michigan.

Competing interests:

C.P. reports non-financial support (travel paid), contracts, and research support from Menarini Silicon Biosystems (MSB), the manufacturer of CellSearch® and, outside the submitted work, research funding from AstraZeneca, and Pfizer. C.P. is currently an employee of Eisai, Inc. D.F.H. reports research support from MSB during the conduct of the study. The University of Michigan (UM) holds patent US 8,790,878 B2 for which D.F.H. is designated as inventor, and that is licensed to MSB with annual royalties paid to UM and D.F.H. Outside the submitted work D.F.H. holds stock options from OncImmune LLC, InBiomotion, and serves on advisory boards for Cepheid, Freenome, CellWorks, Agendia, Salutogenic, EPIC Sciences and L-Nutra and UM receives research funding on his behalf from Merrimack, Eli Lilly, Puma Biotechnology, Pfizer, AstraZeneca. Outside the submitted work M.H. serves as a consultant and receives research support from Veralox Therapeutics. Outside the submitted work E.F.C. has served advisory/consulting roles for AstraZeneca, Biotheranostics, Ayala Pharmaceuticals and Athenex Oncology. The rest of the co-authors have nothing to disclose.

Funding:

This work was supported by Menarini Silicon Biosystems and the Fashion Footwear Charitable Foundation of New York/QVC Presents Shoes on Sale ™ (D.F.H.).

Author Contributions:

EPD: conceptualization, design, experiments, analysis, writing; EMD: experiments; FF, KMK: analysis; CLG, SK, DGT: experiments; AG, MEB: study coordinator; SG, MC, MH, EFC, JMR: conceptualization, design; DFH, CP: conceptualization, design, analysis.

Acknowledgements:

We would like to thank all of the patients that participated in this study.

References

1. Constantinidou A, Alifieris C, Trafalis DT: **Targeting Programmed Cell Death -1 (PD-1) and Ligand (PD-L1): A new era in cancer active immunotherapy**. *Pharmacol Ther* 2018.
2. Schmid P, Adams S, Rugo HS, Schneeweiss A, Barrios CH, Iwata H, Dieras V, Hegg R, Im SA, Shaw Wright G *et al*: **Atezolizumab and Nab-Paclitaxel in Advanced Triple-Negative Breast Cancer**. *N Engl J Med* 2018, **379**(22):2108-2121.
3. Davis AA, Patel VG: **The role of PD-L1 expression as a predictive biomarker: an analysis of all US Food and Drug Administration (FDA) approvals of immune checkpoint inhibitors**. *J Immunother Cancer* 2019, **7**(1):278.
4. Emens LA, Ascierto PA, Darcy PK, Demaria S, Eggermont AMM, Redmond WL, Seliger B, Marincola FM: **Cancer immunotherapy: Opportunities and challenges in the rapidly evolving clinical landscape**. *Eur J Cancer* 2017, **81**:116-129.
5. Lambert AW, Pattabiraman DR, Weinberg RA: **Emerging Biological Principles of Metastasis**. *Cell* 2017, **168**(4):670-691.
6. Carretero R, Romero JM, Ruiz-Cabello F, Maleno I, Rodriguez F, Camacho FM, Real LM, Garrido F, Cabrera T: **Analysis of HLA class I expression in progressing and regressing metastatic melanoma lesions after immunotherapy**. *Immunogenetics* 2008, **60**(8):439-447.
7. Pantel K, Alix-Panabieres C: **Circulating tumour cells in cancer patients: challenges and perspectives**. *Trends Mol Med* 2010, **16**(9):398-406.
8. Scher HI, Heller G, Molina A, Attard G, Danila DC, Jia X, Peng W, Sandhu SK, Olmos D, Riisnaes R *et al*: **Circulating tumor cell biomarker panel as an individual-level surrogate for survival in metastatic castration-resistant prostate cancer**. *J Clin Oncol* 2015, **33**(12):1348-1355.

9. Smerage JB, Barlow WE, Hortobagyi GN, Winer EP, Leyland-Jones B, Srkalovic G, Tejwani S, Schott AF, O'Rourke MA, Lew DL *et al*: **Circulating tumor cells and response to chemotherapy in metastatic breast cancer: SWOG S0500**. *J Clin Oncol* 2014, **32**(31):3483-3489.
10. Cohen SJ, Punt CJ, Iannotti N, Saidman BH, Sabbath KD, Gabrail NY, Picus J, Morse M, Mitchell E, Miller MC *et al*: **Relationship of circulating tumor cells to tumor response, progression-free survival, and overall survival in patients with metastatic colorectal cancer**. *J Clin Oncol* 2008, **26**(19):3213-3221.
11. Krebs MG, Sloane R, Priest L, Lancashire L, Hou JM, Greystoke A, Ward TH, Ferraldeschi R, Hughes A, Clack G *et al*: **Evaluation and prognostic significance of circulating tumor cells in patients with non-small-cell lung cancer**. *J Clin Oncol* 2011, **29**(12):1556-1563.
12. Paoletti C, Cani AK, Larios JM, Hovelson DH, Aung K, Darga EP, Cannell EM, Baratta PJ, Liu CJ, Chu D *et al*: **Comprehensive Mutation and Copy Number Profiling in Archived Circulating Breast Cancer Tumor Cells Documents Heterogeneous Resistance Mechanisms**. *Cancer Res* 2018, **78**(4):1110-1122.
13. Paoletti C, Muniz MC, Thomas DG, Griffith KA, Kidwell KM, Tokudome N, Brown ME, Aung K, Miller MC, Blossom DL *et al*: **Development of circulating tumor cell-endocrine therapy index in patients with hormone receptor-positive breast cancer**. *Clin Cancer Res* 2015, **21**(11):2487-2498.
14. Paoletti C, Li Y, Muniz MC, Kidwell KM, Aung K, Thomas DG, Brown ME, Abramson VG, Irvin WJ, Jr., Lin NU *et al*: **Significance of Circulating Tumor Cells in Metastatic Triple-Negative Breast Cancer Patients within a Randomized, Phase II Trial: TBCRC 019**. *Clin Cancer Res* 2015, **21**(12):2771-2779.
15. Boffa DJ, Graf RP, Salazar MC, Hoag J, Lu D, Krupa R, Louw J, Dugan L, Wang Y, Landers M *et al*: **Cellular Expression of PD-L1 in the Peripheral Blood of Lung Cancer Patients is Associated with Worse Survival**. *Cancer Epidemiol Biomarkers Prev* 2017, **26**(7):1139-1145.
16. Anantharaman A, Friedlander T, Lu D, Krupa R, Premasekharan G, Hough J, Edwards M, Paz R, Lindquist K, Graf R *et al*: **Programmed death-ligand 1 (PD-L1) characterization of circulating tumor cells (CTCs) in muscle invasive and metastatic bladder cancer patients**. *BMC Cancer* 2016, **16**(1):744.
17. Mazel M, Jacot W, Pantel K, Bartkowiak K, Topart D, Cayrefourcq L, Rossille D, Maudelonde T, Fest T, Alix-Panabieres C: **Frequent expression of PD-L1 on circulating breast cancer cells**. *Mol Oncol* 2015, **9**(9):1773-1782.
18. Janning M, Kobus F, Babayan A, Wikman H, Velthaus JL, Bergmann S, Schatz S, Falk M, Berger LA, Bottcher LM *et al*: **Determination of PD-L1 Expression in Circulating Tumor Cells of NSCLC Patients and Correlation with Response to PD-1/PD-L1 Inhibitors**. *Cancers (Basel)* 2019, **11**(6).
19. Khattak MA, Reid A, Freeman J, Pereira M, McEvoy A, Lo J, Frank MH, Meniawy T, Didan A, Spencer I *et al*: **PD-L1 Expression on Circulating Tumor Cells May Be Predictive of Response to Pembrolizumab in Advanced Melanoma: Results from a Pilot Study**. *Oncologist* 2020, **25**(3):e520-e527.
20. Paoletti C, Larios JM, Muniz MC, Aung K, Cannell EM, Darga EP, Kidwell KM, Thomas DG, Tokudome N, Brown ME *et al*: **Heterogeneous estrogen receptor expression in circulating tumor cells suggests diverse mechanisms of fulvestrant resistance**. *Mol Oncol* 2016, **10**(7):1078-1085.
21. Paoletti C, Schiavon G, Dolce EM, Darga EP, Carr TH, Geradts J, Hoch M, Klinowska T, Lindemann JPO, Marshall G *et al*: **Circulating biomarkers and resistance to endocrine therapy in metastatic breast cancers: correlative results from AZD9496 oral SERD Phase I trial**. *Clin Cancer Res* 2018.
22. Mittendorf EA, Philips AV, Meric-Bernstam F, Qiao N, Wu Y, Harrington S, Su X, Wang Y, Gonzalez-Angulo AM, Akcakanat A *et al*: **PD-L1 expression in triple-negative breast cancer**. *Cancer Immunol Res* 2014, **2**(4):361-370.
23. Gonzalez TL, Hancock M, Sun S, Gersch CL, Larios JM, David W, Hu J, Hayes DF, Wang S, Rae JM: **Targeted degradation of activating estrogen receptor alpha ligand-binding domain mutations in human breast cancer**. *Breast Cancer Res Treat* 2020, **180**(3):611-622.
24. Cristofanilli M, Budd GT, Ellis MJ, Stopeck A, Matera J, Miller MC, Reuben JM, Doyle GV, Allard WJ, Terstappen LW *et al*: **Circulating tumor cells, disease progression, and survival in metastatic breast cancer**. *N Engl J Med* 2004, **351**(8):781-791.

25. Bidard FC, Peeters DJ, Fehm T, Nole F, Gisbert-Criado R, Mavroudis D, Grisanti S, Generali D, Garcia-Saenz JA, Stebbing J *et al*: **Clinical validity of circulating tumour cells in patients with metastatic breast cancer: a pooled analysis of individual patient data**. *Lancet Oncol* 2014, **15**(4):406-414.
26. Jacot W, Mazel M, Mollevi C, Pouderoux S, D'Hondt V, Cayrefourcq L, Bourgier C, Boissiere-Michot F, Berrabah F, Lopez-Crapez E *et al*: **Clinical Correlations of Programmed Cell Death Ligand 1 Status in Liquid and Standard Biopsies in Breast Cancer**. *Clin Chem* 2020, **66**(8):1093-1101.
27. Schott DS, Pizon M, Pachmann U, Pachmann K: **Sensitive detection of PD-L1 expression on circulating epithelial tumor cells (CETCs) could be a potential biomarker to select patients for treatment with PD-1/PD-L1 inhibitors in early and metastatic solid tumors**. *Oncotarget* 2017, **8**(42):72755-72772.
28. Prestipino A, Emhardt AJ, Aumann K, O'Sullivan D, Gorantla SP, Duquesne S, Melchinger W, Braun L, Vuckovic S, Boerries M *et al*: **Oncogenic JAK2(V617F) causes PD-L1 expression, mediating immune escape in myeloproliferative neoplasms**. *Sci Transl Med* 2018, **10**(429).
29. Rolfes V, Idel C, Pries R, Plotze-Martin K, Habermann J, Gemoll T, Bohnet S, Latz E, Ribbat-Idel J, Franklin BS *et al*: **PD-L1 is expressed on human platelets and is affected by immune checkpoint therapy**. *Oncotarget* 2018, **9**(44):27460-27470.
30. Jenne CN: **Platelets: crossroads of immunity and hemostasis**. *Blood* 2014, **124**(5):671-672.
31. Nami N, Feci L, Napoliello L, Giordano A, Lorenzini S, Galeazzi M, Rubegni P, Fimiani M: **Crosstalk between platelets and PBMC: New evidence in wound healing**. *Platelets* 2016, **27**(2):143-148.
32. Hanahan D, Weinberg RA: **Hallmarks of cancer: the next generation**. *Cell* 2011, **144**(5):646-674.
33. Haemmerle M, Stone RL, Menter DG, Afshar-Kharghan V, Sood AK: **The Platelet Lifeline to Cancer: Challenges and Opportunities**. *Cancer Cell* 2018, **33**(6):965-983.
34. Szczerba BM, Castro-Giner F, Vetter M, Krol I, Gkountela S, Landin J, Scheidmann MC, Donato C, Scherrer R, Singer J *et al*: **Neutrophils escort circulating tumour cells to enable cell cycle progression**. *Nature* 2019, **566**(7745):553-557.
35. Zaslavsky AB, Adams MP, Cao X, Maj T, Choi JE, Stangl-Kremser J, Patel S, Putelo A, Lee SK, Nallandhighal S *et al*: **Platelet PD-L1 suppresses anti-cancer immune cell activity in PD-L1 negative tumors**. *Sci Rep* 2020, **10**(1):19296.
36. Doroshow DB, Sanmamed MF, Hastings K, Politi K, Rimm DL, Chen L, Melero I, Schalper KA, Herbst RS: **Immunotherapy in Non-Small Cell Lung Cancer: Facts and Hopes**. *Clin Cancer Res* 2019, **25**(15):4592-4602.
37. Wei XL, Wu QN, Chen DL, Zeng ZL, Lu JB, Liu ZX, Ju HQ, Ren C, Pan ZZ, Wang FH *et al*: **The Clinical and Biomarker Association of Programmed Death Ligand 1 and its Spatial Heterogeneous Expression in Colorectal Cancer**. *J Cancer* 2018, **9**(23):4325-4333.
38. Nabet BY, Esfahani MS, Moding EJ, Hamilton EG, Chabon JJ, Rizvi H, Steen CB, Chaudhuri AA, Liu CL, Hui AB *et al*: **Noninvasive Early Identification of Therapeutic Benefit from Immune Checkpoint Inhibition**. *Cell* 2020, **183**(2):363-376 e313.
39. Adams S, Gatti-Mays ME, Kalinsky K, Korde LA, Sharon E, Amiri-Kordestani L, Bear H, McArthur HL, Frank E, Perlmutter J *et al*: **Current Landscape of Immunotherapy in Breast Cancer: A Review**. *JAMA Oncol* 2019.
40. Zhang J, Bu X, Wang H, Zhu Y, Geng Y, Nihira NT, Tan Y, Ci Y, Wu F, Dai X *et al*: **Cyclin D-CDK4 kinase destabilizes PD-L1 via cullin 3-SPOP to control cancer immune surveillance**. *Nature* 2018, **553**(7686):91-95.
41. Wang Y, Kim TH, Fouladdel S, Zhang Z, Soni P, Qin A, Zhao L, Azizi E, Lawrence TS, Ramnath N *et al*: **PD-L1 Expression in Circulating Tumor Cells Increases during Radio(chemo)therapy and Indicates Poor Prognosis in Non-small Cell Lung Cancer**. *Sci Rep* 2019, **9**(1):566.

Figures

Figure 1.

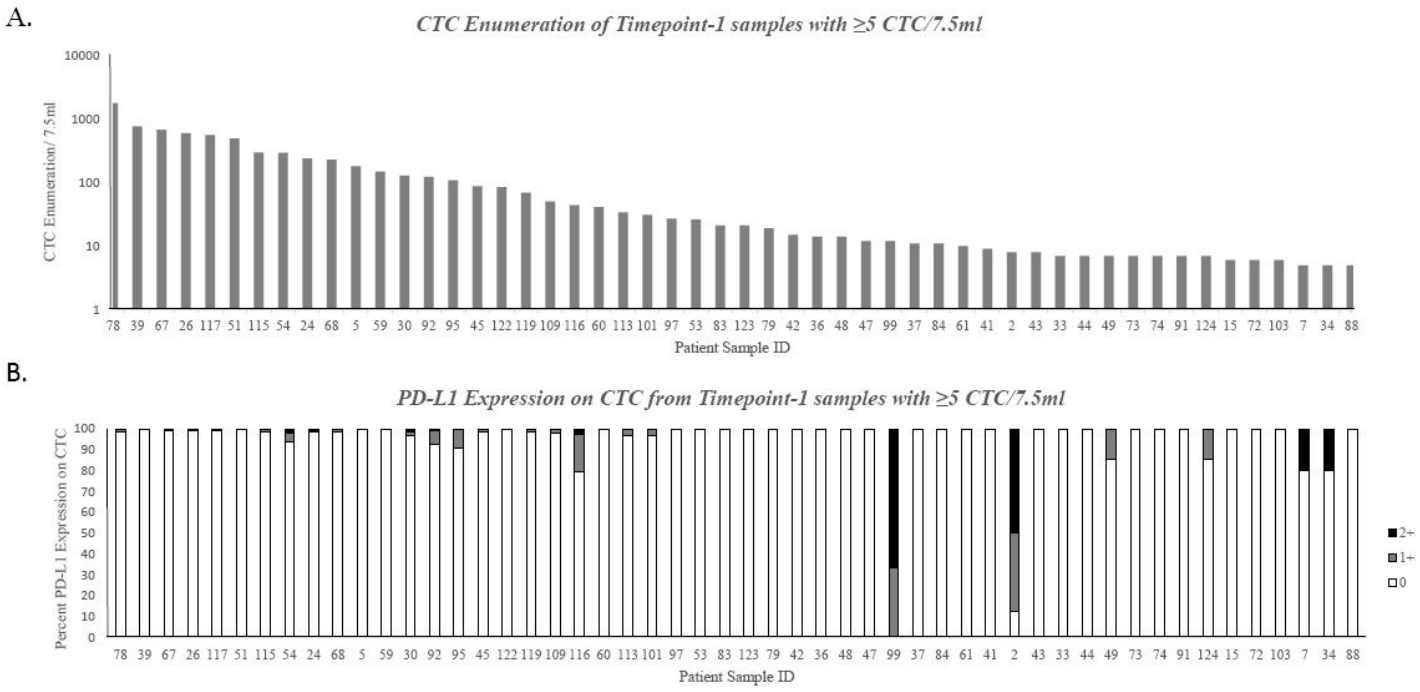

Figure 1

Please see the manuscript file for the full caption.

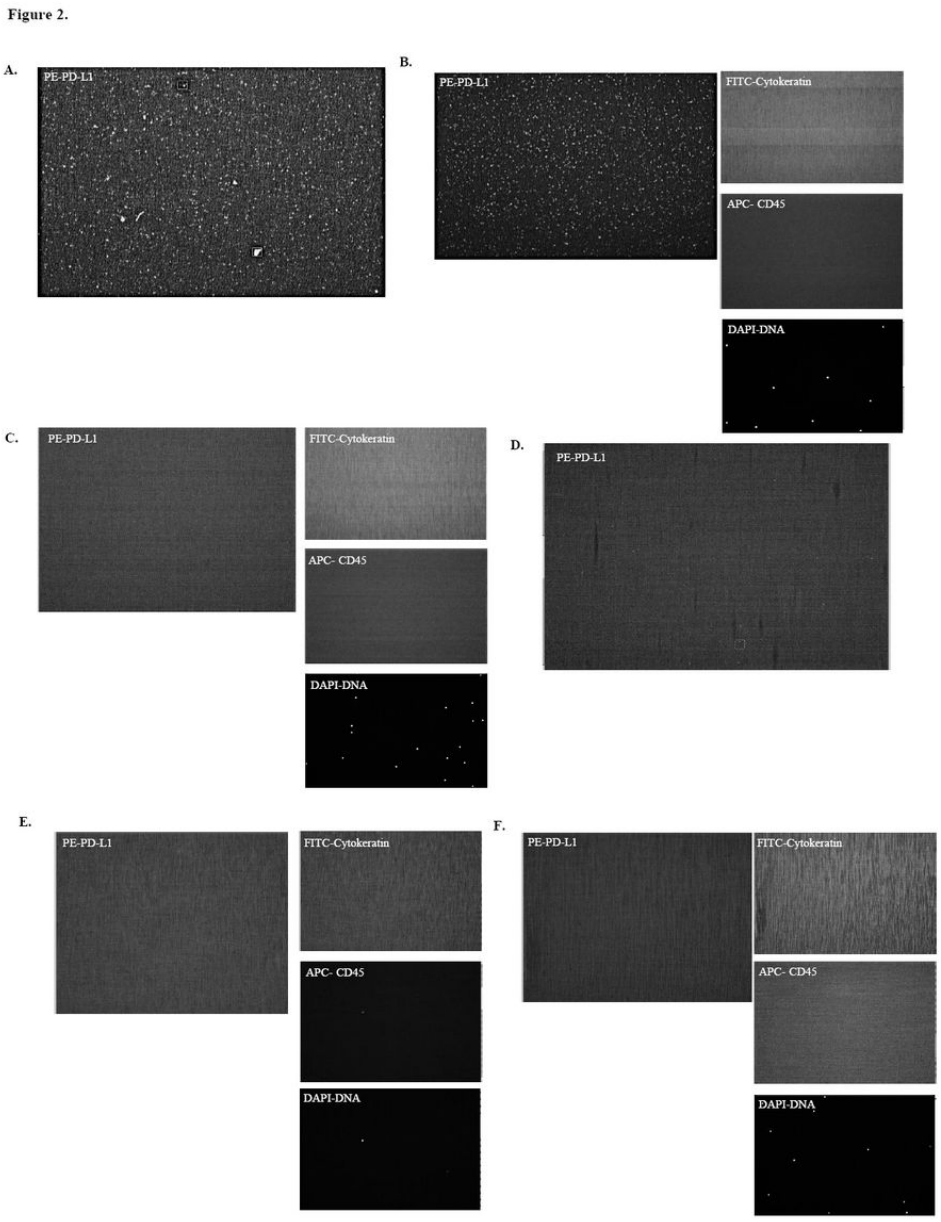

Figure 2

PD-L1 expression on platelets Whole Blood, a pure platelet pellet, and platelet poor plasma from two patients, one with positive platelet PD-L1(A-C) and one with negative platelet PD-L1 (D-F), was processed through CellSearch® in parallel with PD-L1 staining in the 4th channel. See Supplementary Methods text for details. A. Fluorescent image of positive platelet PD-L1 (PE conjugated) expression obtained from initial whole blood processed through CellSearch® using the classic method. Platelets staining for PD-L1 can be identified as white dots in the PE-PD-L1 frame. B. Fluorescent images of PD-L1 (PE conjugated), cytokeratin (FITC-conjugated), CD-45 (APC conjugated), and DNA (DAPI) for a platelet pellet from the patient in panel A with positive platelet PD-L1 processed through CellSearch®. Platelets staining for PD-L1 can be identified as white dots in the PE-PD-L1 frame. C. Fluorescent images of PD-L1 (PE conjugated), cytokeratin (FITC-conjugated), CD-45 (APC conjugated), and DNA (DAPI) for platelet poor plasma from the patient in panel A with positive platelet PD-L1 processed through CellSearch®. See Methods for details. No platelets staining for PD-L1 are identified in the PE-PD-L1 frame. D. Florescent image of negative platelet PD-L1 (PE conjugated) expression obtained from initial whole blood processed through CellSearch® using the classic method. No platelets staining for PD-L1 are identified in the PE-PD-L1 frame. E. Florescent images of PD-L1 (PE conjugated), cytokeratin (FITC-conjugated), CD-45 (APC conjugated), and DNA (DAPI) for platelet pellet from the patient in panel D with negative platelet

PD-L1 processed through CellSearch®. No platelets staining for PD-L1 are identified in the PE-PD-L1 frame. F. Florescent images of PD-L1 (PE conjugated), cytokeratin (FITC-conjugated), CD-45 (APC conjugated), and DNA (DAPI) for platelet poor plasma processed from the patient in panel D with negative platelet PD-L1 through CellSearch®. No platelets staining for PD-L1 are identified in the PE-PD-L1 frame.

Figure 3.

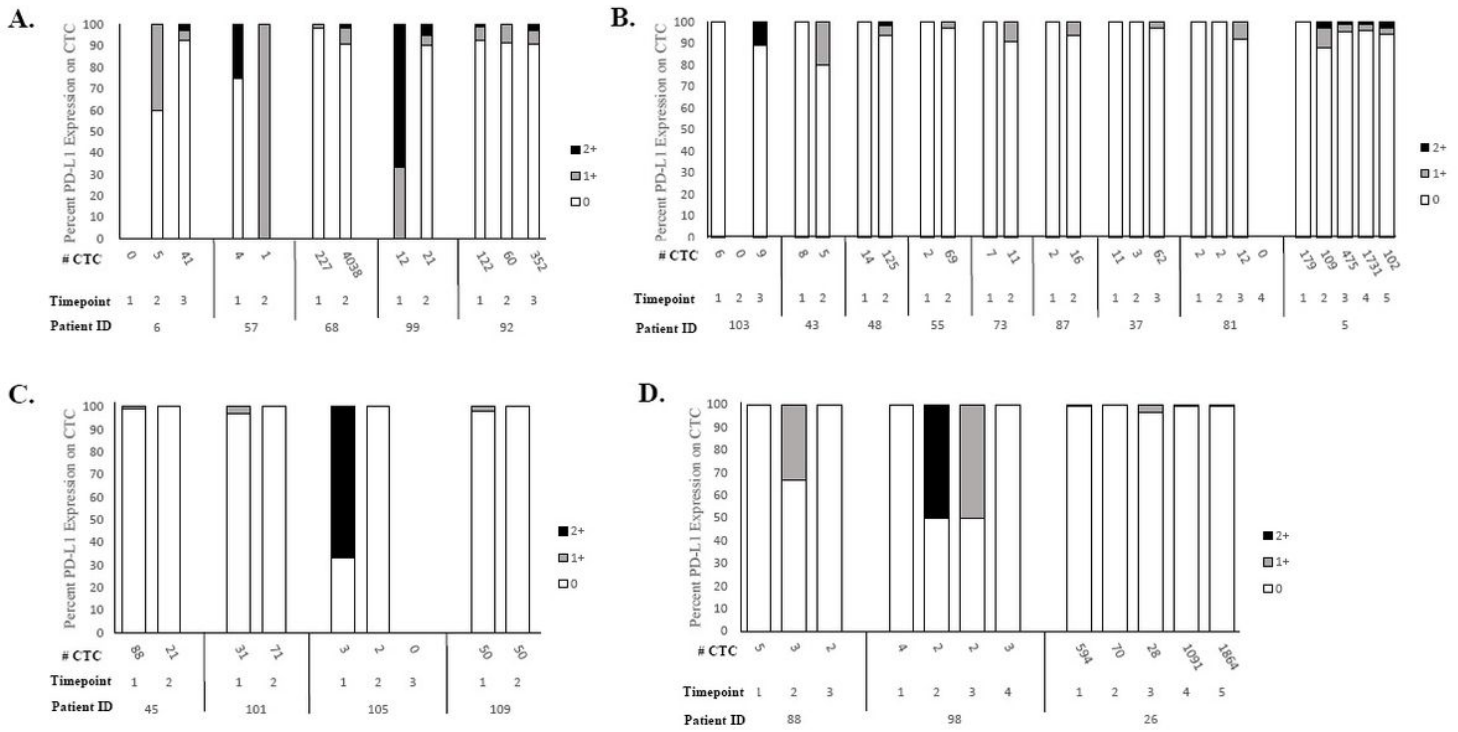

Figure 3

CTC PD-L1 expression at serial timepoints Whole blood from serial specimens from the same patients was processed through CellSearch® with PD-L1 staining in the 4th channel. Each stacked bar represents distribution of CTC PD-L1 staining as described in Figure 1. Patients in each category are separated by a vertical line. A. Patients (N=5) that maintained CTC PD-L1 positivity from timepoint-1 to subsequent timepoints. B. Patients (N=9) that converted CTC PD-L1 negative to positive from timepoint-1 to subsequent timepoints. C. Patients (N=4) that converted CTC PD-L1 positive to negative from timepoint-1 to subsequent timepoints. D. Patients (N=3) that had fluctuating CTC PD-L1 expression among multiple timepoints.

Figure 4.

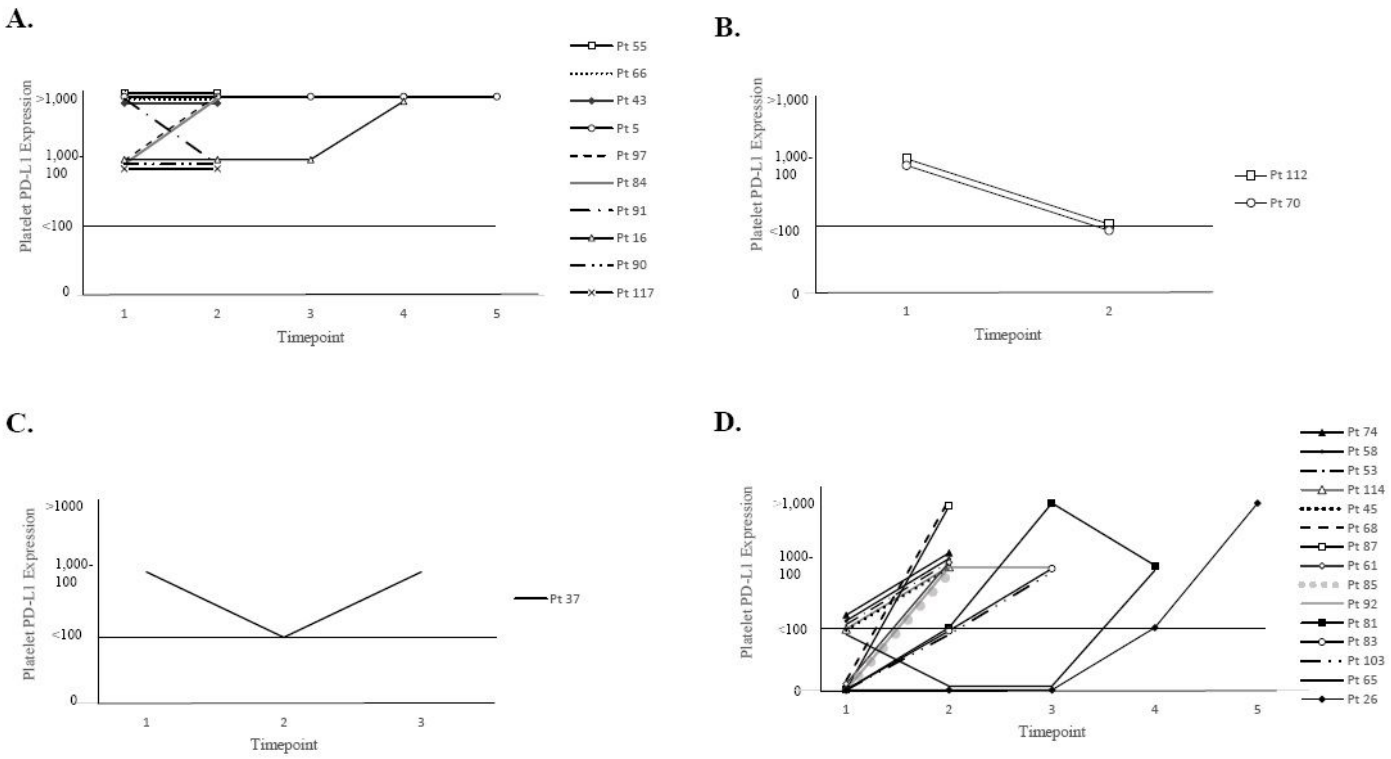

Figure 4

Platelet PD-L1 expression at serial timepoints Whole blood from serial specimens from the same patients was processed through CellSearch® with PD-L1 staining in the 4th channel. Each line represents platelet PD-L1 staining from separate patients. Any data point that falls at or below the solid black line is considered platelet PD-L1 negative (<100 PD-L1 positive platelets). A. Patients (N=10) that maintained platelet PD-L1 positive from timepoint-1 to subsequent timepoints. B. Patients (N=2) that converted from having platelet PD-L1 positive to platelet PD-L1 negative at subsequent timepoints. C. Patient (N=1) that had platelet PD-L1 expression fluctuating among multiple timepoints. D. Patients (N=15) that converted from platelet PD-L1 negative at timepoint-1 to platelet PD-L1 positive at subsequent timepoints.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Supplementarymaterialtablesandfigures.pdf