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ORIGINAL ARTICLE

Quantification of PD-L1 and PD-1 expression on tumor and immune cells in non-small cell lung cancer (NSCLC) using non-enzymatic tissue dissociation and flow cytometry

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Abstract
Objective We report a truly quantitative technology for PD-L1 expression in non-small cell lung cancer (NSCLC). In addition, we present a non-enzymatic technology that creates a cell suspension from fresh tumor tissue so that either fine-needle aspiration (FNA) or fresh tissue can be used in this assay.
Methods Non-enzymatic tissue homogenization (Incell-PREP; IncellDx, Menlo Park, California) was performed on 4-mm punch biopsies. An FNA was taken from the same tumor to create matched sample sets. Cells were labeled with antibodies directed against CD45, PD-1, and PD-L1 and then stained with DAPI to identify intact, single cells, and to analyze cell cycle.
Results Comparing the IncellPREP homogenization and FNA demonstrated a strong correlation (r^2 − 0.8) for expression of PD-L1. We compared PD-L1 expression by flow cytometry using a 1 % cutoff for positivity in the tumor cell population and a 1 % cutoff of cells with at least 1+ intensity in immunohistochemically stained tissue sections as positive. Ten of 12 lung tumor samples were concordant while 2 were discordant. PD-L1 expression by flow cytometry varied widely (1.2–89.4 %) even in the positive concordant cases. In addition, PD-L1 expression in the aneuploid tumor population did not necessarily agree with the expression in the diploid tumor population.
Summary Fine, unequivocal quantification of PD-L1 on tumor and immune cells in NSCLC may allow for better prediction of response to therapies. The present study also offers a technology that can create a universal sample type from either FNA or fresh tissue.

Keywords PD-L1 · Non-small cell lung cancer · Immune cells · PD-1 · Cell cycle

Abbreviation

AJCC	American Joint Committee on Cancer
DAB	Diaminobenzidine
DAPI	4′,6-Diamidino-2-phenylindole
DPBS	Dulbecco's phosphate-buffered saline
FFPE	Formalin-fixed, paraffin-embedded
FNA	Fine-needle aspiration
IHC	Immunohistochemistry
NSCLC	Non-small cell lung cancer
PD-L1	Programmed cell death ligand 1
RCC	Renal cell carcinoma
RPMI	Roswell Park Memorial Institute
TIL	Tumor-infiltrating lymphocytes

Introduction

Recently, a number of solid tumors including renal cell carcinoma (RCC), melanoma, ovarian cancer, and non-small cell lung cancer (NSCLC) have been shown to express programmed cell death ligand 1 (PD-1) which, through its receptor PD-L1, creates a local immunosuppressive microenvironment by evading immune checkpoints and results in a poorer prognosis [1–3]. A number of therapies block

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the PD-L1/PD-1 engagement and also activate the immune system to attack tumors [4–6], but the onus is now to determine which patients would benefit from these new pharmacologic agents. Furthermore, using diagnostic technology to quantify the expression of PD-L1 has the potential to spare patients from ineffective therapy and possible adverse autoimmune effects from these agents.

PD-1 is a protein encoded by the *PDCD1* gene and is a cell surface receptor expressed on T cells. PD-1 binds two ligands, PD-L1 and PD-L2. PD-1 functions as an immune checkpoint by preventing the activation of T cells, which reduces autoimmunity and promotes self-tolerance [3, 7]. Expression of PD-L1 in NSCLC has diagnostic and prognostic significance given the favorable response of tumors expressing this marker to immune checkpoint inhibitor therapy [8]. PD-L1 expression is currently determined by immunohistochemistry (IHC) to identify patients who would respond to immune checkpoint inhibition. PD-L1 IHC as a companion diagnostic to therapy has been problematic in determining which patients will be responsive to therapy. Issues with this method include subjectivity of the reviewer; processing variability; differences in semiquantitative cutoffs; and staining of tumor, immune cell, and even stromal cells. The range of PD-L1 IHC expression ranges from 14 to 100 % making predictive drug response decisions difficult [9–13]. Even more significant, PD-L1 consists of 2 hydrophilic binding sites [14], which makes the use of anti-PD-L1 antibodies in formalin-fixed, paraffin-embedded (FFPE) specimens challenging. All these variables contribute to variability in quantification of the PD-L1 expression on tumor samples.

Other factors present in the tumor microenvironment such as the quantity of tumor-infiltrating lymphocytes (TIL) may also contribute to therapeutic response [15, 16]; thus, a multiparameter approach to personalizing therapy is highly desirable. In the present study, we demonstrate a novel assay consisting of non-enzymatic sample preparation that creates a single-cell suspension of fresh cells from NSCLC tumors. From this single-cell suspension, we then quantified total number of immune cells and PD-L1/PD-1 expression on both immune cell subsets and on tumor cells. These data are compared to PD-L1 IHC.

Materials and methods

Samples

Fresh tissues were obtained from 12 NSCLC cases by Spectrum Health (Grand Rapids, Michigan) following informed consent. Date of collection, age, sex, ethnicity, diagnosis, primary tumor size, and American Joint Committee on Cancer (AJCC) classification were recorded. Tissues were excised and then stored in Roswell Park Memorial Institute (RPMI) 1640 medium at 2–8 °C prior to overnight shipment to IncellDx on cold packs.

Tissue dissociation using IncellPREP

Tumor biopsies of at least 2 cm were placed in RPMI for transport after which 4-mm punches were taken from each tissue, and placed in 2-mL Eppendorf tubes containing 800 μL Dulbecco's phosphate-buffered saline (DPBS). IncellPREP (IncellDx, Inc.) tissue homogenizers were inserted into each tube and set to run at 1 V until supernatant appeared cloudy (5–10 min). After tissue homogenization, a Cellometer (*Auto T4*, Nexcelom Bioscience, Inc.) was used to calculate cellular concentration, and cell counts were recorded. Supernatant was then removed and transferred to a separate tube before centrifugation at 300×*g* for 5 min. Following centrifugation, supernatant was aspirated, and cell pellet was resuspended in IncellPREP reagent at a concentration of 1×10^6 cells/mL and incubated at room temperature for 1 h prior to staining.

PD-L1/PD-1 staining of single-cell suspensions

Following fixation for 1 h in IncellFP (IncellDx, Inc.), 200 μL of sample equivalent to 200,000 cells was aliquoted to 12 × 75 mm tubes and subsequently washed with 1 mL of DPBS. Samples were then stained with PD-1 Alexa 488 (clone EH12.2H7, Biolegend, Inc.), PD-L1 PE (clone 29E.2A3, Biolegend, Inc.), and CD-45 PE/Cy7 (clone H130, Biolegend, Inc.)-conjugated antihuman antibodies in DPBS + 2 % bovine serum albumin (BSA) and then incubated for 30 min at room temperature in the dark. Next, 1 mL of DPBS + 2 % BSA was added to each tube and incubated at room temperature for 5 min, prior to centrifugation at 600×*g* for 5 min. Supernatant was aspirated, and a wash with DPBS + 2 % BSA was repeated once. Following this wash, 200 μL of 4′,6-diamidino-2-phenylindole (DAPI) at 1 μg/mL was added to each sample and incubated at room temperature for 30 min in the dark.

Flow cytometry

Cells were first analyzed on an EC800 Flow Cytometer (Sony Biosciences, Inc.) using a DAPI-Lin by DAPI-Peak-Lin density plot to set a gate on nucleated single cells. That gate was then applied to a CD45 by side scatter density plot to separate CD45+ cells (immune cells) from CD45− cells (putative tumor cells). Once those two populations were determined, each was analyzed for PD-1 and PD-L1 expression by forward scatter. Normal expression was determined by the level of expression on the CD45+ population, and gating was set. That gating was applied to the CD45− population, and PD-1 and PD-L1 expression above the normal cutoff was recorded.

Table 1 Comparison of cell yields from FNA and IncellPREP homogenization of fresh tumor tissue

Sample ID	FNA (cells/mL)	IncellPrep (cells/mL)
751	4.57E+05	4.17E+06
772	2.58E+05	7.83E+06
42	1.01E+06	3.40E+06
56	9.20E+05	5.02E+06
88	1.27E+06	5.78E+06
251	1.02E+06	4.13E+06
287	2.06E+06	3.76E+06
343	1.67E+06	5.79E+06
419	1.14E+06	8.37E+06
478	2.53E+06	4.77E+06
486	6.81E+06	5.07E+06
496	6.46E+06	3.79E+06
Mean	1.21E+06	4.90E+06

On average, greater than 10^6 cells/mL were recovered from both specimen types with a statistically significant increase in yield from the IncellPrep specimens

Immunohistochemistry

FFPE blocks were sectioned at 5 μm thickness. Slides were deparaffinized in a series of xylenes and progressively diluted alcohols to water. After rinsing with DI water, the slides were treated with citrate-based antigen retrieval solution that was preheated to 65 °C. Slides were incubated in the preheated citrate buffer and heated for 20 min at 99 °C and cooled down for 20 min before commencing IHC staining on the Dako autostainer. Endogenous peroxidase was quenched with KPL solution, followed by protein blocking and rabbit anti-PD-L1 antibody (clone SP142, Ventana, Inc.) incubation for 1 h at room temperature. Relevant non-specific isotype control was used as negative control. After rinsing the primary antibodies, the slides were incubated with biotin-conjugated secondary antibody and HRP-enzyme-conjugated streptavidin, followed by incubation with diaminobenzidine (DAB) to visualize the signal. Upon completion of the IHC staining, the slides were dehydrated in a series of alcohols and xylenes followed by coverslipping for microscopic evaluation.

Results

Cell recovery using IncellPrep

To determine the advantages of non-enzymatic cell homogenization compared with standard fine-needle aspiration, we performed counts of cell suspensions derived from a French technique fine-needle aspiration (FNA) and from IncellPREP non-enzymatic homogenization using a Cellometer cell counter. As demonstrated in Table 1, both techniques generated greater than 10^6 cells/mL using standardized techniques with the IncellPREP yields being 2.5 times the number of cells per preparation compared with FNA with the difference being statistically significant (Mann–Whitney, $p = 0.003$). The yields from both techniques were satisfactory for the downstream assays performed in this study.

Quantification of cell-type-specific PD-L1 and PD-1

To address the difficulty of quantifying immuno-oncology markers in tissue with heterogeneous cell populations, we labeled cells in suspension from both FNA and IncellPREP homogenization with biomarkers that distinguish between immune cells and tumor cells. We further multiplexed PD-L1 and PD-1 markers to unequivocally quantify the expression of these markers on the pre-identified immune or tumor cells (See Fig. 1). As demonstrated in Table 2, we found a range of expression levels between cell types and between samples using IncellPrep-prepared cell suspensions. PD-L1 expression ranged from 0 to 31 % on immune cells and from 0 to 89 % on tumor cells. The expression of PD-L1 was independent of tumor histology. PD-1 expression ranged from 0 to 10 % on immune cells and from 0 to 47 % on tumor cells. Similarly, PD-1 expression was independent of tumor histology. Comparing the IncellPREP homogenization to cells obtained by FNA yielded a strong correlation ($r^2 - 0.8$) for expression of the immuno-oncology response marker PD-L1 (see Fig. 2). Interestingly, the expression of PD-L1 in the aneuploidy population of a particular tumor was higher in all six aneuploidy cases as identified in Table 2 with a numeric DNA index for the aneuploid population. In the six cases, the range of PD-L1 expression on the aneuploidy tumor cell population was 9–70 % higher than the expression on all tumor cells taken together (data not shown).

Quantification of PD-L1 by flow cytometry compared to immunohistochemistry

To directly compare PD-L1 expression on lung tumors homogenized with IncellPREP with PD-L1 expression in tissue sections from the same tumor, we used standardized methodology for PD-L1 expression on tissue. Specifically, a characteristic positive stain shows a brown, rim pattern as shown in Fig. 3. Table 3 compares PD-L1 expression by flow cytometry using a 1 % cutoff for positivity in the tumor cell population and a 1 % cutoff of cells with characteristic rim staining in immunohistochemically stained tissue sections as positive. Using these criteria, 11 of 12 samples were concordant with the one discordant case being a

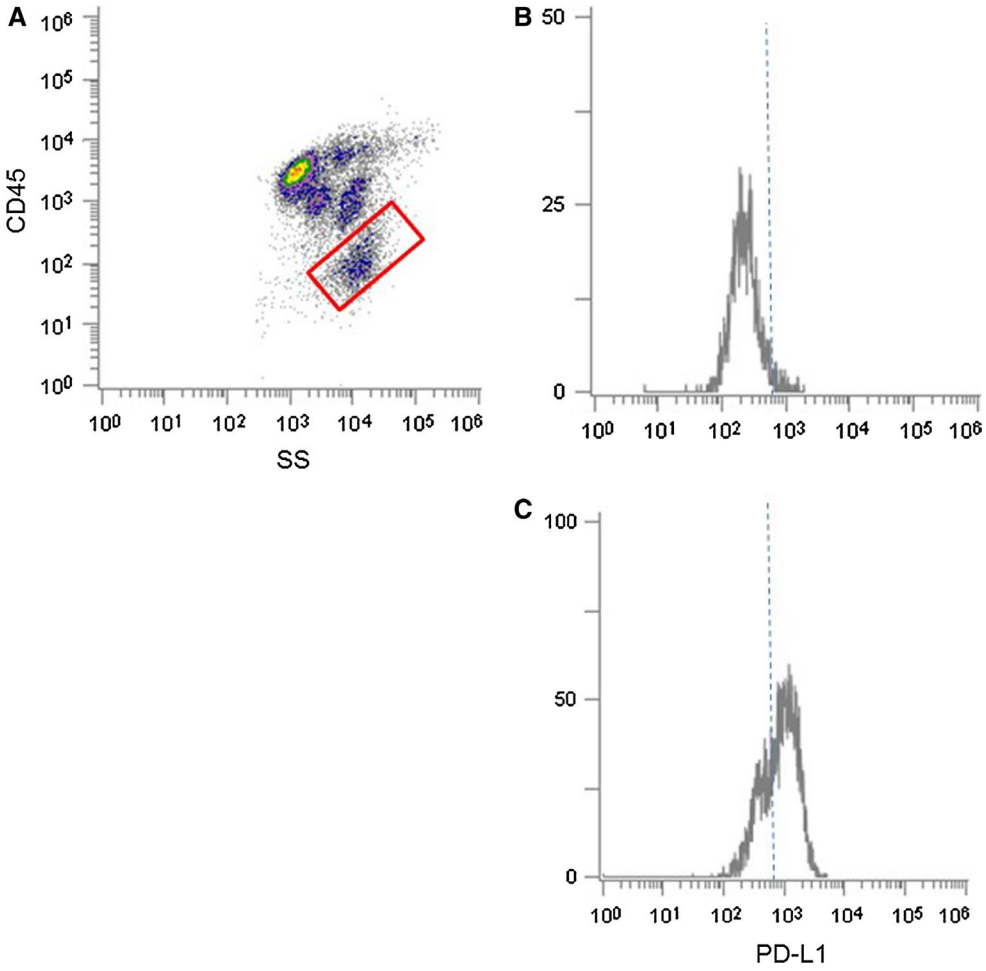

Fig. 1 PD-L1 quantification of IncellPREP-prepared cell suspension by flow cytometry. **a** Immune cells were electronically separated from tumor cells by CD45 antibody staining and side scatter (ss). Tumor cells (*red box*) were gated for PD-L1 expression in **b**, **c**. PD-L1 expression in tumor cells varied from low (**b**) to high (**c**) as listed in Table 2

Table 2 Expression of PD-L1 and PD-1 in tumor and immune cells from IncellPrep-prepared cell suspensions

Sample ID	Histology	% CD45+	% CD45− (tumor)	DNA Index CD45−	DNA Index CD45− aneuploid	CD45+ (immune) PD-L1 %	Tumor PD-L1 %	CD45+ (immune) PD-1 %	Tumor PD-1 %
751	SCC	74.07	12.17	0.94	1.64	2.63	3.97	0.56	0
772	AC	19	49.55	0.97	None	3.98	1.22	1.06	0.31
42	AC	22.61	65.58	0.9	2.18	30.69	63.98	2.04	6.39
56	AC	59.45	30.06	0.96	1.4	26.18	69.81	1.24	12.53
88	SCC	46.54	45.53	0.95	1.34	23.65	89.44	1.71	0.88
251	SCC	15.07	28.75	1.01	None	0.18	13.74	0	0
287	AS	23.34	58.62	0.93	None	2.42	0.77	0	0
343	SCC	25.72	47.84	0.94	None	0.97	0.52	0	0
419	AC	32.03	30.53	1.2	None	0.8	5.63	0	0
478	AC	20.8	70.8	0.87	None	0.47	0	0.54	0.86
486	AC	88.52	8.81	0.91	None	5.07	25.77	3.97	47.31
496	AC	37.25	44.74	0.96	1.48	0	0.06	0	0.06

SCC squamous cell carcinoma, *AC* adenocarcinoma, *AS* adenosquamous carcinoma

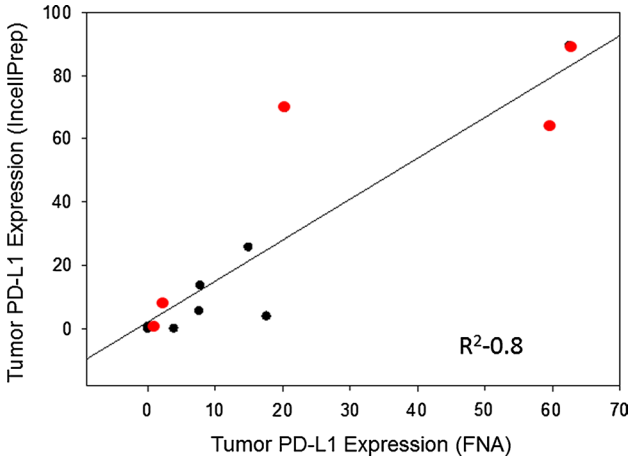

Fig. 2 Comparison of matched FNA and IncellPREP-prepared cells for PD-L1 demonstrated strong correlation ($r^2 - 0.8$) independent of the starting sample type. Aneuploid tumors are indicated with *red circles*

tumor with extensive necrosis giving a positive result by IHC and a negative result by flow cytometry.

Discussion

NSCLC accounts for the majority of lung cancer cases and includes various histologic types, including squamous cell carcinoma, adenocarcinoma, and large cell carcinoma [17]. The most common diagnostic sample types for NSCLC are FNA and biopsy [18, 19]. The majority of diagnoses are still performed by FNA because of the advanced stage and because of the widespread use of bronchoscopy [20–22]. The common use of FNA raises concern over the potential inequities of histologic typing of NSCLC and personalized diagnostics to inform treatment decisions that are based on immunohistochemistry on tissue blocks. Here, we present novel technology for creating equivalent cell suspension

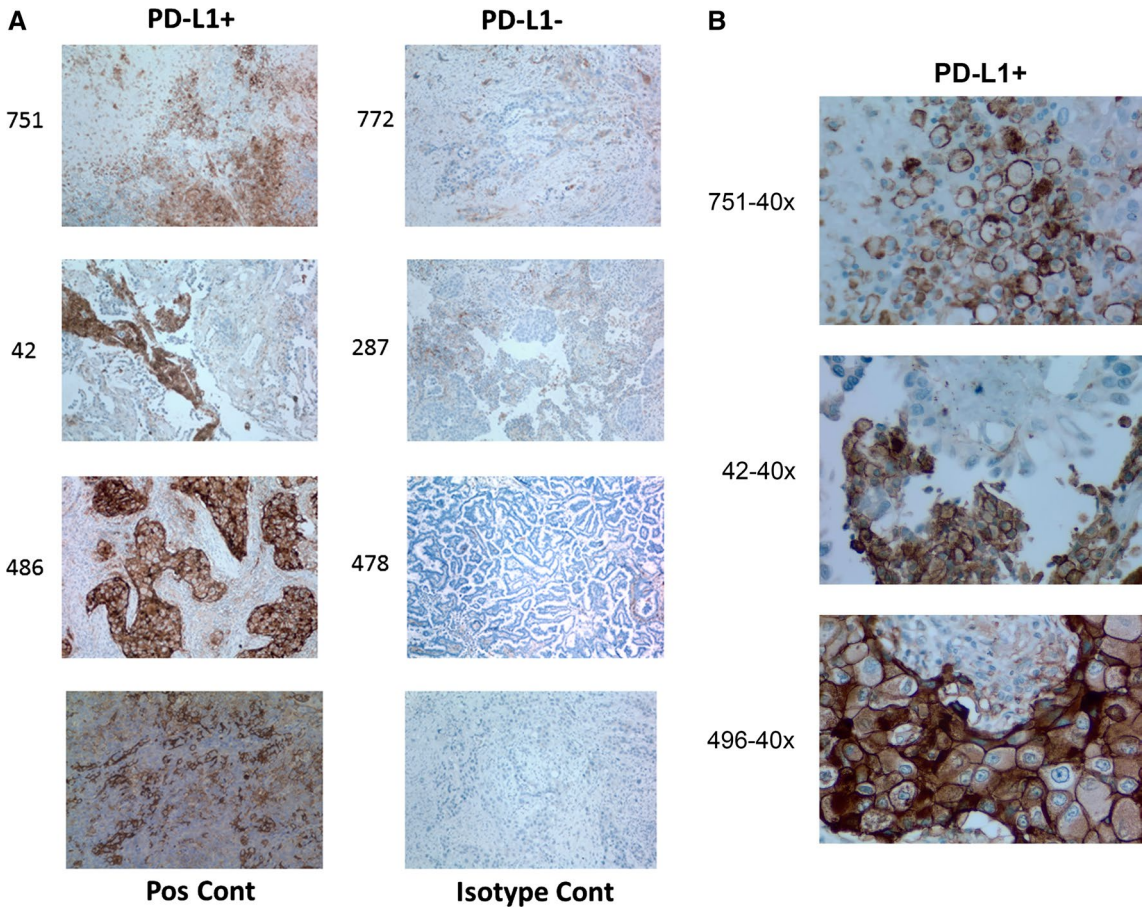

Fig. 3 **a** Immunohistochemical staining for PD-L1 in tissue blocks from matched flow cytometry samples. PD-L1-positive and PD-L1-negative tissues are shown (×200). Positive staining is indicated by a brown precipitate. A positive control tonsil sample and an isotype control (without primary antibody) are also shown. **b** High power view of characteristic rim pattern PD-L1 staining in tissues shown in **a**

Table 3 Determination of tumor expression of PD-L1 using published cutoffs for IHC and flow cytometric cutoffs as established in this study

Sample ID	IHC qualitative call (1 % CO)	Flow cytometry quantitative call (1 % CO)
751	Pos	Pos
772	Neg	Neg
42	Pos	Pos
56	Pos	Pos
88	Pos	Pos
251	Pos	Pos
287	Neg	Neg
343	Pos	Neg
419	Pos	Pos
478	Neg	Neg
486	Pos	Pos
496	Neg	Neg

samples from both FNA samples and homogenized whole biopsy samples. We demonstrated a correlation for PD-L1 expression of >0.8 for matched FNA and solid tumor samples that have been converted to a cell suspension using the IncellPREP kit. As expected, the tumor suspension yielded 0.5 logs more cells than a standard French method FNA, though both techniques generated more than enough cells to perform the flow cytometry-based analysis.

A possible concern with the two sampling approaches (FNA vs. biopsy) is the ability to determine the histologic subtype of NSCLC (e.g., squamous cell or adenocarcinoma), which has been shown in some studies to exhibit different responses to therapies such as pemetrexed [23]. Recent data on the accuracy of FNA cytology, however, demonstrated that cytologic and histological typing was concordant in 88 % of cases with statistical significance [24]. Further, as it relates to PD-L1 as a biomarker for PD-L1 antagonists, PD-L1-directed therapy is equally effective on all histologic subtypes of NSCLC [23]. In particular, a meta-analysis looking at PD-L1 expression in NSCLC found no correlation with a squamous cell or adenocarcinoma histologic type [25]. Similarly, in the present study, we did not see a correlation between PD-L1 expression on tumor cells and histologic type.

Current immunotherapies directed against PD-L1 focus on the use of IHC, which has been used for decades in anatomic pathology for qualitative assessment of cell lineage-specific markers. The pitfalls of IHC for quantification of antigens are numerous and well documented [9]. The sources of variability start in the pre-analytical phase of IHC which include tissue procurement from the operating room, time, and temperature prior to fixation, and, most importantly, the type and length of fixation. Cross-linking, aldehyde-based fixatives are still the overwhelming source of tissue fixatives used to create slides for morphologic assessment and slides for additional stains including IHC. During fixation, tissue antigens are cross-linked by the aldehydes, which can destroy the epitopes of interest for some applications often requiring antigen retrieval reagents (in order to recover staining of the target antigen) [8].

In our study, we present an assay system that is automated, fully quantitative, and capable of distinguishing expression of important biomarkers on the major cell types present (including both tumor cells and immune cells) within the tumor microenvironment. CD45+ immune cells ranged in percentage from ~15 to 88 %.

We demonstrated that the 1 % cutoff for both IHC and our flow-based PD-L1 assay correlates in 11 out of 12 cases. However, the percentage of tumor cells positive for PD-L1 expression using the truly quantitative flow method ranged from 0.5 to 89 %. Using the 1 % cutoff for both IHC and flow cytometry, we demonstrated overall a >0.9 correlation of tumor PD-L1 compared to IHC with the added attribute of quantification on immune cells and on aneuploidy populations of the tumor. These data suggest that a more quantitative assay could allow for more precise and more quantitative prediction of response to therapeutic modalities.

Most disturbing concerning the use of PD-L1 IHC are the cases of individuals with PD-L1 tumors including melanoma, renal cell, and NSCLC who respond to PD-L1 antagonist when the IHC is negative for tumor expression of PD-L1 [9]. Fine quantification of PD-L1 expression may allow for more precise establishment and less subjective cutoffs. Though use of the approach to PD-L1 quantification as presented in the current study awaits outcome studies to test the relevance of more quantitative information for response to treatment, studies have shown that PD-L1 expression aside from its quantification as an indication for therapy may be an independent prognostic marker [8].

The present study used clinically available instrumentation without the need for sophisticated image analysis, making clinical utility straightforward. Unlike IHC, a method for standardization of complex flow cytometric assays has been described that allows for interinstitutional proficiency and quality assurance. This process will be implemented to ensure the current assay results apply across laboratories [26].

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Compliance with ethical standards

Conflict of interest Amanda Chargin, Rian Morgan, Keith Shults, and Bruce K. Patterson are employees of IncellDx, Inc., Ellen Tsay was an uncompensated intern of IncellDx, Inc., Uma Sundram and Navneet Ratti declare that they have no conflict of interest.

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