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**eMaterial.** Supplemental Methods and Data

This supplementary material has been provided by the authors to give readers additional information about their work.
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Introduction

Immune therapies targeting programmed cell death 1 (PD-1) and its respective ligand, programmed cell death ligand -1 (PD-L1) have demonstrated objective clinical responses in a variety of advanced tumor types, including NSCLC (1-3). The PD-1/PD-L1 immune checkpoint axis is an important co-regulatory system in the immune response which leads to the inactivation/exhaustion of lymphocyte cells (4-6). Its natural function is to down-regulate the immune system response to self-antigens as evidenced by the development of significant autoimmunity in PD-L1 knockout mice (7). While both of the FDA-approved assays show a higher response rate in PD-L1 positive patients, substantial responses have been observed in PD-L1 negative patients (8-10). The definition of PD-L1 positive currently lacks standardization and prediction of response by IHC is limited by the subjective nature of the technique. Variable cutoffs for defining a positive case across trials have been utilized, for instance, the assay coupled with nivolumab uses categories of 0, >1, >5 and >10% cells with membranous staining, while the assay for pembrolizumab uses 0, 1-49, and >50% cells positive (1, 3). To further complicate the situation, atezolimab and avelumab also assess the expression of PD-L1 in the immune cells in the surrounding stroma (11-13).

PD-L1 is a single transmembrane protein with IgV-like and IgC-like extracellular domains, as well as a short 31 amino acid cytoplasmic tail at its C terminus (14). Antibodies against both intracellular and extracellular domains are available commercially, and it has been reported that antibodies targeting PD-L1’s cytoplasmic provide clearer membranous staining (15). This discrepancy could be explained by differential expression and/or function of PD-L1’s intra- and extracellular domains. For instance, an RNA splice variant lacking the extracellular IgV domain has been identified though endogenous expression of a corresponding protein isoform, but this has not been verified in any in vitro or animal models (16). Theoretically, cleavage of PD-L1 could account for differences in intracellular and extracellular PD-L1 staining. Unpublished data in our lab suggested antibodies directed at the extracellular domain revealed expression in some cases that were negative for the intracellular domain. However subsequent work suggested that antibody (E1J2J) showed lack of specificity in some common assay conditions and thus is was not included in this study.

Methods

Tissue Microarray Construction-Yale Index TMA: Retrospectively collected formalin fixed paraffin embedded tissue blocks from 30 patients were obtained from Yale Pathology Archives with a range of expression of PD-L1 as assessed in previous as yet unpublished studies. All cases obtained had signed consent for tissue use under approved human investigation committee protocol #9505008219. Cell line pellets were processed in an identical manner to tissue in the creation of cell line pellet blocks. Tissue and cell line blocks were prepared in a tissue microarray format containing 0.6 mm representative FFPE cores in two fold redundancy. Controls included, tissue controls; placenta and tonsil and cell line pellets H820, H441, HCC193, H1975, A431, H2882, Karpas 299, Ramos, parental and PD-L1 stably transfection MEL624. Cell lines were purchased from American Type Culture Collection or obtained from other labs (eFigure 1).

Antibodies: PD-L1 expression was evaluated by chromogenic immunohistochemistry (IHC) and quantitative immunofluorescence (QIF) using five monoclonal antibodies raised against the intracellular and extracellular domain. The intracellular antibodies included the following clones; E1L3N (Cell Signaling Technology, Inc. #13684), SP142 (Spring Bioscience Corp. M4420) and 9A11 (Cell Signaling Technology, Inc. #29122). The extracellular antibodies included; SP263 (Ventana Medical Systems, Inc. 790-4905), and 28-8 (Abcam plc. Ab205291). Antibodies deemed to pass validation showed positive membrane staining in Karpas 299, H820, H441, HCC193, H1975, A431, H2882 and transfected M624 with no expression in parental cells or Ramos; as well as membranous staining of the syncytiotrophoblast layer of the placenta, while showing absence in the stromal and vascular regions.

Quantitative Immunofluorescence: TMA slides were soaked in xylene twice for 20 minutes each. Slides were rehydrated in two 1-minute washes in 100% ethanol followed by one wash in 70% ethanol and rinsed in streaming tap water for 5 minutes. Antigen retrieval was performed in EDTA, pH 8 in the Lab Vision PT module. Buffers were preheated to 85 °C before slide addition and then heated to sub-boiling 97°C for 20 mins. Endogenous peroxidases were blocked by 30-minute incubation in 2.5% hydrogen peroxide in methanol. Nonspecific antigens were blocked using a 0.3% BSA in TBST for 30 minutes. Primary antibodies were incubated at concentrations described in Table 1 (main text) and 1:100 pan-cytokeratin (Clone AE1/AE3 Dako) overnight at 4 °C except for SP263 which was incubated for 20 minutes at 37 °C followed by 1hr incubation with pan-cytokeratin. Following this the slides were
incubated with Alexa 546-conjugated goat anti mouse secondary antibody (Life Technologies) 1:100 diluted in rabbit EnVision reagent (Dako K4009) for 1 hr at RT. Target signal was amplified using Cy5-Tyramide (Perkin Elmer SAT705A001EA) for 10 mins. Finally, TMAs were stained with 1:500 DAPI for 10 mins at RT and mounted with Prolong Gold antifade mounting reagent (Life Technologies P36394). Immunofluorescence was quantified using automated quantitative analysis (AQUA) on all regions of tissue on each slide as previously described (17). Briefly, fluorescent images of DAPI, Cy3 (Alexa 546-cytokeratin), and Cy5 (PD-L1) for each core were collected. Image analysis was carried out using the AQUAAnalysis software (Genoptix), which is generated for each compartment by dividing the sum of target pixel intensities by the area of the compartment in which the target is measured.

Quantitation of chromogenic IHC: Chromogenic IHC was quantified using the Aperio Positive Pixel Count based on the intensity of membrane staining of respective PD-L1 antibodies. Intensity as defined in this instance is proportional to the amount of light transmitted through the slide with values ranging from 0-255. Intensity values determined from Positive Pixel Count were subtracted from highest possible intensity (255) to create a directly proportional distribution of membrane intensity for each antibody.

Results

YTMA index array as a standardized PD-L1 array

In an effort to comparatively assess the measurement of PD-L1 across antibodies we constructed a PD-L1 specific index array called YTMA 337 using tumors and cell lines (eFigure 1). Both chromogenic and fluorescent immunostaining were performed. Representative DAB and QIF images of control tissue (placenta), cell lines (H820 and Ramos) and tumor tissue are shown in eFigure 2. Lack of staining in Ramos cell lines and Mel624 WT which are known negatives for PD-L1 was used to confirm specificity of all antibodies tested in this analysis. As expected all staining observed in the controls and tumor tissue was membranous in accordance with known cellular location of PD-L1. Positive staining of PD-L1 was observed in both tumor and stroma of lung cancer cases however only positive staining in tumor was used for determination of optimal antibody concentration.

To determine the optimal antibody concentration for each antibody, we used an algorithm which determines the adjusted signal to noise, based on QIF where signal to noise is calculated by dividing the to 10% of cases by the bottom 10% of cases (assumed to represent noise) then multiplied by the dynamic range as calculated by the difference between the mean of the top and bottom 10% of cases. The optimal concentrations of each of the antibodies in our QIF assay were determined by testing antibody concentrations ranging from one log above to one log below the manufacturers recommended concentration (except for SP263 which was only obtainable in prediluted form). Optimal concentrations, selected based on the adjusted signal to noise ratio were, 3.4, 0.077 and 1.0 µg/ml for E1L3N, SP142 and 9A11 respectively (eFigure 3). SP263 is currently only available as a prediluted antibody at 1.61 µg/ml, therefore we were limited in the dilutions we could perform however we tittered SP263 at three concentrations below the prediluted concentration. At 1.61 µg/ml SP263 demonstrated the highest adjusted signal to noise indicating that optimal concentration may be either the pre-diluted concentration or above, however we were unable to confirm due to lack of concentrated antibody. 28-8 showed a lack of reproducibility under QIF conditions in a standard QIF staining protocol (Day 1-3) with Kappa co-efficient ranging from 0.001 to 0.16 (eFigure 4A). We additionally included an assay performed on Bond Rx as per Abcam guidelines (Day 4 Operator 3) and regressed to SP142 (eFigure 4B) for cell lines and tumors. Regressions between SP142 and 28-8 tested on Bond Rx were 0.44 and 0.56 for tumor and cell line cores respectively. Representative Images from 28-8 immunostaining are shown in eFigure 4C. 28-8 results were reproducible under DAB staining of 15 Spot CLMA are described in main text. Representative examples of the DAB for all antibodies is shown in eFigure 5.

Chromogen-based assessment of PD-L1 antibody concordance

As demonstrated above 28-8 results were only reproducible under DAB staining conditions. To include this antibody in our comparisons, we utilized the Aperio Positive Pixel counter and assessed DAB staining of the Horizon 15 Spot CLMA array. All staining was carried out in a single run on the Bond Rx as described in materials and methods. Using this method, all five antibodies (E1L3N, SP142, 9A11, SP263 and 28-8) showed high levels of concordance (discussed in main text). A schematic of this array and representative images is shown in eFigure 5. Similarly, antibody 22c3 was evaluable only using the Dako Link 48 platform. A single CLMA slide was run on
this platform exactly according to manufacturer’s instructions. Quantification was the same as that used for 28-8 and the other DAB visualized specimens.

eReferences


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