Cancer Biomarkers 36 (2023) 177–191
DOI 10.3233/CBM-220030
IOS Press

PD-L1 expression and CD8 positive lymphocytes in human neoplasms: A tissue microarray study on 11,838 tumor samples

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Received 26 January 2022
Accepted 1 January 2023

Abstract.
BACKGROUND: Programmed death ligand 1 (PD-L1) is the target of immune checkpoint inhibitor therapies in a growing number of tumor types, but a unanimous picture on PD-L1 expression across cancer types is lacking.
MATERIALS AND METHODS: We analyzed immunohistochemical PD-L1 expression in 11,838 samples from 118 human tumor types and its relationship with tumor infiltrating CD8 positive lymphocytes.
RESULTS: At a cut-off level of 10% positive tumor cells, PD-L1 positivity was seen in 85 of 118 (72%) tumor types, including thymoma (100% positive), Hodgkin's lymphoma (93%), anaplastic thyroid carcinoma (76%), Kaposi sarcoma (71%), sarcomatoid urothelial carcinoma (71%), and squamous cell carcinoma of the penis (67%), cervix (65%), floor of the mouth (61%), the lung (53%), and pharynx (50%). In immune cells, PD-L1 positivity was detectable in 103 (87%) tumor types, including tumors of haematopoetic and lymphoid tissues (75% to 100%), Warthin tumors of the parotid glands (95%) and Merkel cell carcinoma (82%). PD-L1 positivity in tumor cells was significantly correlated with the number of intratumoral CD8 positive lymphocytes across all tumor types as well as in individual tumor types, including serous carcinoma of the ovary, invasive breast carcinoma of no special type, intestinal gastric adenocarcinoma, and liposarcoma ($p < 0.0001$ each).
CONCLUSIONS: PD-L1 expression in tumor and inflammatory cells is found in a wide range of human tumor types. Higher rates of tumor infiltrating CD8 positive lymphocytes in PD-L1 positive than in PD-L1 negative cancers suggest that the antitumor immune response may trigger tumoral PD-L1 expression.

Keywords: PD-L1, CD8 positive lymphocytes, immunohistochemistry, tissue microarray, human cancers

1. Introduction

Immune checkpoint inhibitor (CPI) therapies targeting the programmed death 1/programmed death ligand 1 (PD-L1) pathway are increasingly employed in a growing number of tumor types [1]. However, not all patients react favorably to these drugs. PD-L1 immunohistochemistry is often applied to select patients

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with high likelihood to respond favorably to checkpoint inhibitors but criteria for "PD-L1 positivity" vary between tumor types and sometimes also between drugs. The proportion of PD-L1 positive tumor cells (tumor proportion score, TPS), the percentage of positive immune cells (immune cell score; ICS) or the combination of both (combined positivity score; CPS) are applied at different thresholds to define positive cases [2]. The significant role of PD-L1 for the immune microenvironment of tumors is illustrated by associations between PD-L1 expression in tumor cells and elevated numbers of intratumoral CD8 positive cytotoxic T-lymphocytes which were found in several tumor types [3–6].

More than 2,800 studies have analyzed cancers of various types for PD-L1 expression by immunohistochemistry. For most tumor types, however, the reported frequencies of PD-L1 positivity vary quite considerably. For example, the reported rate of PD-L1 positivity ranges from 0–92% in prostate cancer [7,8], 1.7%–75% in breast cancer [9,10], 5.5–89% in colorectal cancer [11,12], 22–68% in head & neck squamous cell carcinomas [13,14], 5.2–65% in stomach cancer [15,16], 3.9–63% in small cell lung cancer [17,18], 3.1–82% in liver cell carcinomas [19,20], 17–72% in malignant mesothelioma [21,22], 10–92% in malignant melanoma [23,24], 0–100% in chondrosarcoma [24,25], 0–100% in liposarcoma [24,26], and 7–100% in angiosarcoma [19,27]. Technical factors, staining protocols, antibodies used, definitions of thresholds to determine positivity, as well as a possible selection bias with respect to the analyzed tumors have been proposed as causes for these discrepancies. To better understand the relative importance of PD-L1 expression in different tumor types and its relationship with T-lymphocyte counts, a comprehensive study analyzing large numbers of tumors of different kinds under highly standardized conditions is required.

This study was designed to collect comparable data on the rate of PD-L1 expression in a broad range of different tissues using the same predefined scoring criteria. For this purpose, more than 14,800 tissue samples with preexisting data on intratumoral CD8 positive lymphocytes from 118 different tumor types and subtypes as well as 76 non-neoplastic tissue types were evaluated by immunohistochemistry in a tissue microarray (TMA) format.

2. Materials and methods

2.1. Experimental subjects

Tissue Microarrays (TMAs). The normal tissue TMA was composed of 8 samples from 8 different donors for each of 76 different normal tissue types (608 samples on one slide). The cancer TMAs contained a total of 14,897 primary tumors from 118 tumor types and subtypes. The composition of both normal and cancer TMAs is described in detail in the results section. All samples were from the archives of the Institutes of Pathology, University Hospital of Hamburg, Germany, the Institute of Pathology, Clinical Center Osnabrueck, Germany, and Department of Pathology, Academic Hospital Fuerth, Germany. Tissues were fixed in 4% buffered formalin and then embedded in paraffin. The TMA manufacturing process was described earlier in detail [28,29]. In brief, one tissue spot (diameter: 0.6 mm) was transmitted from a cancer containing donor block in an empty recipient paraffin block. The density of $CD8^+$ cells, as measured by IHC analysis and automated counting of $CD8^+$ tumor infiltrating immune cells (cells/mm^2), was available from an earlier study [30]. The use of archived remnants of diagnostic tissues for manufacturing of TMAs and their analysis for research purposes as well as patient data analysis has been approved by local laws (HmbKHG, §12) and by the local ethics committee (Ethics commission Hamburg, WF-049/09). All work has been carried out in compliance with the Helsinki Declaration.

2.2. Immunohistochemistry (IHC)

Freshly cut TMA sections were immunostained on one day and in one experiment. Slides were deparaffinized with xylol, rehydrated through a graded alcohol series and exposed to heat-induced retrieval for 5 minutes in an autoclave at 121°C in pH 9 Dako Target Retrieveal Solution™ (Agilent, CA, USA; #S2367). Endogenous peroxidase activity was blocked with Dako Peroxidase Blocking Solution™ (Agilent, CA, USA; #52023) for 10 minutes. Primary antibody specific for PD-L1 protein (rabbit recombinant, MS Validated Antibodies, Hamburg, Germany, clone MSVA-711R, cat.# 2083-711-R-1) was applied at 37°C for 60 minutes at a dilution of 1:150. Bound antibody was then visualized using the EnVision Kit™ (Agilent, CA, USA; #K5007) according to the manufacturer's directions. Slide scoring, including and distinction of tumor and immune cells and estimation of the fraction of stained tumor and immune cells, was performed manually by experienced pathologists using brightfield microscopy. Membranous PD-L1 staining of the cancer cells and immune cells was evaluated separately. In cancer cells, $\geqslant$ 10% of PD-L1 positive cells was considered PD-L1 positive. In

immune cells, PD-L1 staining was grouped into negative (no staining), few positive (few cells stained), and many positive (many cells stained) cells.

2.3. Antibody comparison

To evaluate the impact of antibody selection on PD-L1 immunohistochemistry data, staining properties of MSVA-711R, Cell Signaling Technology E1L3N, Roche SP142, and Roche SP263 were compared in normal tissues with known physiological PD-L1 expression as detailed in Supplementary Fig. S1. Immunohistochemistry protocols and automated staining systems were employed as recommended by the antibody vendors and are listed in Supplementary Table S1. To determine the sensitivity and specificity of each antibody, consensus sets of unequivocally PD-L1 positive and unequivocally PD-L1 negative tissue samples were identified from a tissue microarray with 352 high grade muscle invasive urinary bladder cancers. Consecutive sections were taken from the TMA and stained with the 4 antibodies. For maximal standardization of the PD-L1 status calling, neural network and digital image analysis were used as described in the Supplementary Methods. For MSVA-711R, the consensus set contained 96 cancers that were consistently positive with E1L3N, SP142, and SP263, and 188 cancers that were consistently negative with E1L3N, SP142, and SP263. For E1L3N, the consensus set contained cancers that were consistently positive ($n = 93$) or consistently negative ($n = 199$) with MSVA-711R, SP142, and SP263. For SP142, the consensus set contained cancers that were consistently positive ($n = 102$) or consistently negative ($n = 200$) with MSVA-711R, E1L3N, and SP263. For SP263, the consensus set contained cancers that were consistently positive ($n = 98$) or consistently negative ($n = 192$) with MSVA-711R, E1L3N, and SP142.

2.4. Statistics

Statistical calculations were performed with JMP® software 14 (SAS Institute Inc., NC, USA) [31] and R version 3.6.1 (The R foundation) [32,33]. The Pearson's correlation coefficient was used to measure the relationship between PD-L1 intensities and densities. ANOVA test was performed to search for associations between PD-L1 expression and $CD8^+$ cell density.

3. Results

3.1. Technical issue

A total of 11,838 (79.6%) of 14,879 tumor samples were interpretable in the TMA analysis. The remaining 3,059 (20.4%) samples were not analyzable due to the lack of unequivocal tumor cells or loss of the tissue spot during the technical procedures. On the normal tissue TMA, sufficient numbers of samples were always interpretable for each tissue to determine PD-L1 expression.

3.2. Antibody comparison

Representative images of our comparison of 4 anti-PD-L1 antibodies are shown in Supplementary Fig. S1. All antibodies showed the expected staining in normal tonsil epithelium, placenta, corpus luteum of the ovary, macrophages, and blood vessels. The comparatively low staining intensity observed with SP142 is in line with many earlier reports (reviewed in [34]). The results of the consensus set testing and the calculated sensitivity and specificity of each of the 4 antibodies are shown in Table 1. All antibodies proved to be highly specific and sensitive, with comparable performance.

3.3. PD-L1 staining pattern in normal tissue

A moderate to strong membranous PD-L1 immunostaining was found in alveolar macrophages of the lung, macrophages in the endometrium of the pregnant uterus and of the gastrointestinal tract, corpus luteum cells of the ovary, surface cell layers of the syncytiotrophoblast and chorion cells of the placenta, thymic epithelial cells, a fraction of squamous epithelial cells of the tonsil crypts as well as in dendritic cells and macrophages of lymphoid tissues. A weak to moderate PD-L1 staining was also observed in a fraction of epithelial cells of the adenohypophysis and in venous sinuses in the spleen (littoral cells). In addition, weak staining was found in fibrils of the anterior lobe of the pituitary gland. Representative images of PD-L1 positive normal tissues are shown in Fig. 1. PD-L1 staining was absent in epithelial cells of adrenal gland, thyroid gland, parathyroid gland, breast, respiratory epithelium, gastrointestinal tract, esophagus, gallbladder, pancreas, liver, cervix, endometrium, fallopian tube, epididymis, kidney, urinary bladder, prostate, seminal vesicle, testis, skin, as well as in muscle cells, fat, aorta, cerebellum, and the cerebrum.

3.4. PD-L1 in neoplastic tissue

If a cut-off level of $\geqslant$10% positive PD-L1 tumor cells was applied, PD-L1 positivity was observed in 1,691

Table 1
Sensitivity and specificity of 4 anti-PD-L1 antibodies. Consensus set: Tumors with unequivocal presence or absence of PD-L1 expression that were used to determine specificity and sensitivity (antibody performance) for each of the indicated anti-PD-L1 antibodies

		Antibody			
		MSVA-711R	E1L3N	SP142	SP263
Consensus set result	PD-L1 positive (n)	**96**	**93**	**102**	**98**
	PD-L1 negative (n)	**188**	**199**	**200**	**192**
Antibody performance	True positive (n)	92	92	92	92
	True negative (n)	187	187	187	187
	False positive (n)	1	12	13	5
	False negative (n)	4	1	10	6
	Sensitivity	**0.958**	**0.989**	**0.902**	**0.939**
	Specificity	**0.995**	**0.940**	**0.935**	**0.974**

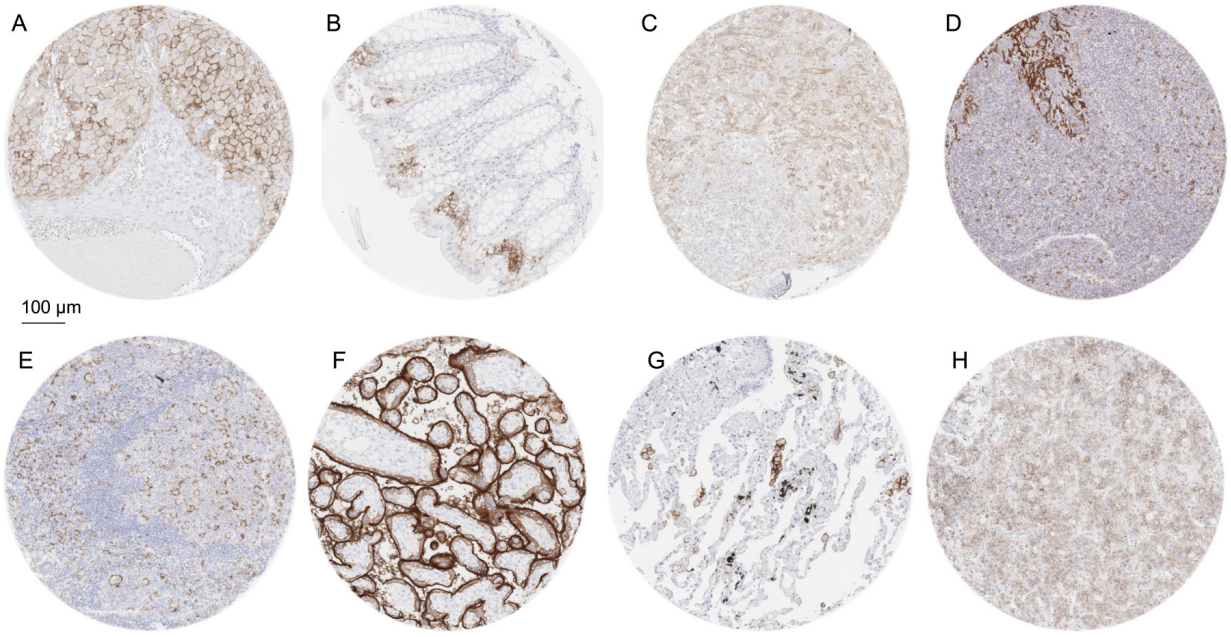

Fig. 1. PD-L1 immunostaining of normal cells using MSVA-711R. The panels show a membranous PD-L1 positivity of Corpus luteum cells in the ovary (A), macrophages in colon epithelium (B), small (littoral) blood vessels in the spleen (C), a fraction of crypt epithelial cells and macrophages of the tonsil (D), dendritic cells and macrophages in a lymph node (E), surface membranes of the syncytiotrophoblast in the placenta (F), alveolar macrophages in the lung (G) and of a fraction of epithelial cells in the adenohypophysis.

(14.3%) of 11,838 analyzable tumors. PD-L1 positivity was seen in cases from 85 of 118 (72%) tumor types. At least 50% PD-L1 positive cases were found in 10 (8.5%) tumor types, including thymoma (100%), Hodgkin lymphoma (93%), anaplastic thyroid carcinoma (76.3%), Kaposi sarcoma (71.4%), sarcomatous urothelial carcinoma (70.8%), as well as in squamous cell carcinomas of the penis (66.7%), cervix (64.5%), floor of the mouth (60.5%), lung (52.5%), and the pharynx (50.0%). PD-L1 was absent in tumor cells of all analyzed cases in 33 (28%) tumor categories, including non-Hodgkin lymphomas, germ cell tumors of the testis, mucinous carcinoma of the ovary, as well as tubular and mucinous carcinoma of the breast. Representative images of PD-L1 positive tumors are shown in Fig. 2. The staining in cancer cells was easy to identify in cases with a high number of positive tumor cells. In cases with few PD-L1 positive cells it was often difficult to decide whether positivity was caused by tumor cells or macrophages. In questionable cases, such cells were rather considered immune cells than tumor cells. In immune cells, PD-L1 staining was found in 3,630 (30.7%) cancers, including 15.3% cancers with few and 15.4% cancers with many positive stained immune cells. These positive cases were distributed among 103 of 118 tumor types (87.3%). The highest rates of PD-L1 positive immune cells were seen in tumors of haematopoetic and lymphoid systems (75% to 100%), seminoma (75.8%),

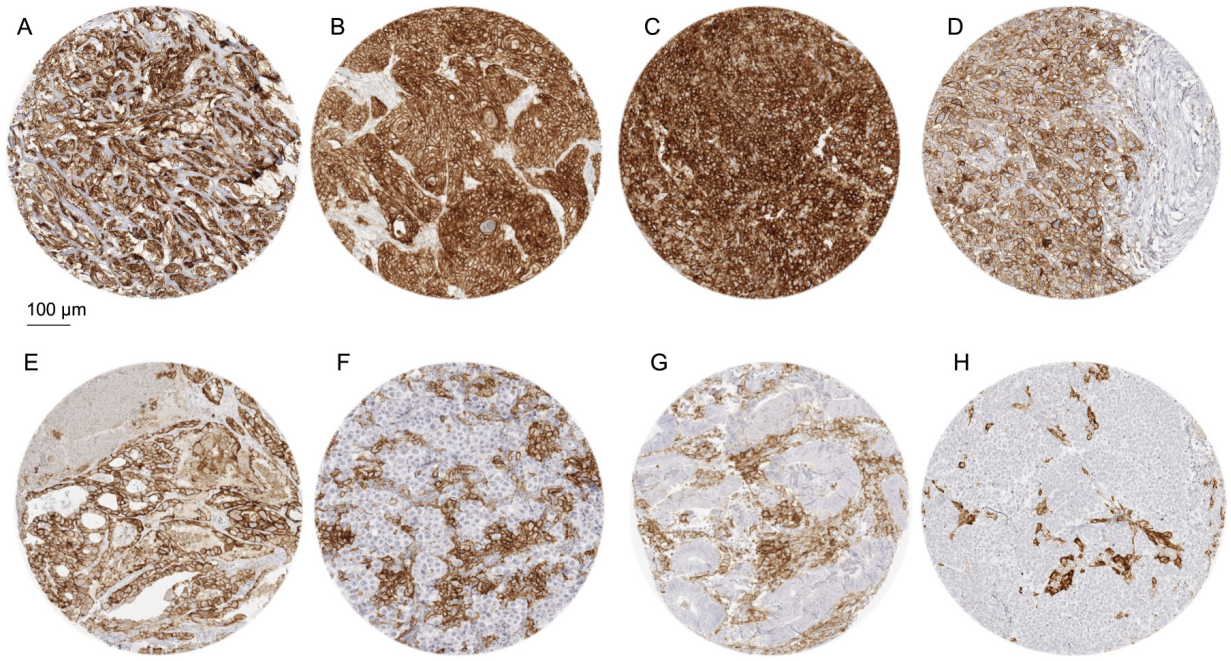

Fig. 2. PD-L1 immunostaining in cancer using MSVA-711R. The panels show a strong, predominantly membranous PD-L1 immunostaining of tumor cells in an epitheloid malignant mesothelioma (A), a muscle-invasive urothelial carcinoma (B), a squamous cell carcinoma of the oral cavity (C), and an anaplastic thyroid cancer (D). A papillary carcinoma of the thyroid shows a membranous staining of both cancer cells (strong intensity) and macrophages (moderate intensity) (E). Cases of seminoma (F), colorectal adenocarcinoma (G), and a Merkel cell carcinoma of the skin (H) do not show tumor cell staining but contain macrophages with intense PD-L1 positivity.

Warthin tumors of the parotid gland (95%), and Merkel cell carcinoma (82.2%). A detailed description of the immunostaining results in tumors is given in Table 2 and Fig. 3.

3.5. PD-L1 and CD8 expression

Data on intratumoral $CD8^+$ cell density was available for 5,500 (36.9%) of the tumors for which PD-L1 data were collected. Across all tumor entities, the intratumoral $CD8^+$ cell density was significantly higher in tumors with PD-L1 positive tumor cells (612.2 ± 22.9) than in PD-L1 negative tumors (254.2 ± 7.1; $p < 0.0001$). In a separate analysis of individual tumor categories, the relationship between PD-L1 expression in cancer cells and the density of $CD8^+$ cells reached significance in 10 of 33 analyzed tumor types/subtypes. Tumor entities with a significant association of PD-L1 positivity and a high density of $CD8^+$ cells included serous carcinoma of the ovary, invasive breast carcinoma of no special type, adenocarcinoma of the colon, clear cell renal cell carcinoma, intestinal gastric adenocarcinoma, and liposarcoma ($p < 0.0001$ each, Table 3).

4. Discussion

The analysis of more than 14,000 tumors in a highly standardized way enabled us to define the relative importance of PD-L1 expression across 118 important human tumor entities and to define its relationship with tumor infiltrating CD8 positive lymphocytes. A Medline Search using the terms "PD-L1 + cancer + immunohistochemistry" had identified 2,887 previous publications on October 13th, 2021. Even rare tumor types such as anaplastic thyroid cancer (4 studies), osteosarcoma (11 studies) and Merkel cell cancer (10 studies) have repeatedly been analyzed (e.g., [35–37]). However, the large number of studies has not led to a unanimous picture on PD-L1 expression in cancer as the results were highly variable in most tumor entities. Data from 907 studies on 72 different tumor entities are summarized in Fig. 4. These data show that criteria for defining PD-L1 positivity, including cutoffs ranging from 1% to 50% stained tumor cells as well as scores combining staining intensity and the fraction of stained tumor cells have contributed to the wide spread of data. Significant differences also exist, however, between studies employing identical definitions. For example, at a cut-off of 5%, the positivity rates varied from 6.1 to 45.9%

Table 2
PD-L1 in human tumor cells and immune cells

Tumor entity	On TMA (n)	Analyzable (n)	PD-L1 in tumor cells		PD-L1 in immune cells		
			Negative (%)	Positive (%)	Negative (%)	Few (%)	Many (%)
Tumors of the skin							
Pilomatrixoma	35	29	69.0	31.0	75.9	6.9	17.2
Basal cell carcinoma	88	68	95.6	4.4	67.6	8.8	23.5
Benign nevus	29	26	100.0	0.0	92.3	7.7	0.0
Squamous cell carcinoma of the skin	90	83	55.4	44.6	57.8	18.1	24.1
Malignant melanoma	46	39	87.2	12.8	66.7	17.9	15.4
Merkel cell carcinoma	46	45	97.8	2.2	17.8	28.9	53.3
Basal cell adenoma of the salivary gland	15	13	100.0	0.0	100.0	0.0	0.0
Tumors of the lung, pleura and thymus							
Adenocarcinoma of the lung	196	99	58.6	41.4	47.5	17.2	35.4
Squamous cell carcinoma of the lung	80	40	47.5	52.5	65.0	12.5	22.5
Small cell carcinoma of the lung	16	16	93.8	6.3	31.3	25.0	43.8
Mesothelioma, epitheloid	39	33	87.9	12.1	75.8	9.1	15.2
Mesothelioma, other types	76	71	64.8	35.2	85.9	5.6	8.5
Thymoma	29	25	0.0	100.0	56.0	28.0	16.0
Tumors of the female genital tract							
Squamous cell carcinoma of the vagina	30	29	65.5	34.5	65.5	24.1	10.3
Squamous cell carcinoma of the vulva	80	77	58.4	41.6	51.9	24.7	23.4
Squamous cell carcinoma of the cervix	80	76	35.5	64.5	43.4	19.7	36.8
Endometrioid endometrial carcinoma	186	146	94.5	5.5	77.4	9.6	13.0
Endometrial serous carcinoma	32	23	91.3	8.7	65.2	13.0	21.7
Carcinosarcoma of the uterus	48	37	97.3	2.7	78.4	5.4	16.2
Endometrial carcinoma, high grade, G3	13	7	85.7	14.3	14.3	42.9	42.9
Endometrial clear cell carcinoma	8	4	100.0	0.0	50.0	25.0	25.0
Endometrioid carcinoma of the ovary	73	53	84.9	15.1	73.6	18.9	7.5
Serous carcinoma of the ovary	509	398	84.2	15.8	58.0	16.1	25.9
Mucinous carcinoma of the ovary	70	48	100.0	0.0	79.2	16.7	4.2
Clear cell carcinoma of the ovary	50	40	77.5	22.5	72.5	17.5	10.0
Carcinosarcoma of the ovary	47	37	83.8	16.2	81.1	8.1	10.8
Tumors of the breast							
Invasive breast carcinoma of no special type	1345	1120	94.6	5.4	79.7	7.1	13.1
Lobular carcinoma of the breast	251	199	99.0	1.0	91.5	6.0	2.5
Medullary carcinoma of the breast	11	9	66.7	33.3	0.0	0.0	100.0
Tubular carcinoma of the breast	9	4	100.0	0.0	100.0	0.0	0.0
Mucinous carcinoma of the breast	36	24	100.0	0.0	87.5	12.5	0.0
Tumors of the digestive system							
Adenomatous polyp, low-grade dysplasia	50	43	97.7	2.3	51.2	25.6	23.3
Adenomatous polyp, high-grade dysplasia	50	46	95.7	4.3	23.9	23.9	52.2
Adenocarcinoma of the colon	1882	1408	96.2	3.8	52.0	37.4	10.6
Gastric adenocarcinoma, diffuse type	176	130	97.7	2.3	90.0	6.2	3.8
Gastric adenocarcinoma, intestinal type	174	131	76.3	23.7	48.1	24.4	27.5
Gastric adenocarcinoma, mixed type	62	53	84.9	15.1	66.0	17.0	17.0
Adenocarcinoma of the esophagus	83	60	90.0	10.0	46.7	28.3	25.0
Squamous cell carcinoma of the esophagus	76	48	54.2	45.8	33.3	31.3	35.4
Squamous cell carcinoma of the anal canal	89	84	63.1	36.9	47.6	26.2	26.2
Cholangiocarcinoma	113	94	91.5	8.5	76.6	11.7	11.7
Hepatocellular carcinoma	50	48	97.9	2.1	75.0	14.6	10.4
Ductal adenocarcinoma of the pancreas	612	448	89.1	10.9	79.9	16.1	4.0
Pancreatic/Ampullary adenocarcinoma	89	61	88.5	11.5	63.9	24.6	11.5
Acinar cell carcinoma of the pancreas	16	11	100.0	0.0	100.0	0.0	0.0
Gastrointestinal stromal tumor (GIST)	50	45	75.6	24.4	73.3	20.0	6.7
Tumors of the urinary system							
Non-invasive papillary urothelial carcinoma, pTa G2 low grade	177	148	99.3	0.7	87.2	6.8	6.1
Non-invasive papillary urothelial carcinoma, pTa G2 high grade	141	128	99.2	0.8	89.1	4.7	6.3
Non-invasive papillary urothelial carcinoma, pTa G3	187	150	93.3	6.7	64.0	17.3	18.7
Urothelial carcinoma, pT2-4 G3	1206	936	70.8	29.2	67.5	16.1	16.3
Small cell neuroendocrine carcinoma of the bladder	20	20	100.0	0.0	50.0	25.0	25.0

Table 2, continued

Tumor entity	On TMA (n)	Analyzable (n)	PD-L1 in tumor cells		PD-L1 in immune cells		
			Negative (%)	Positive (%)	Negative (%)	Few (%)	Many (%)
Sarcomatoid urothelial carcinoma	25	24	29.2	70.8	91.7	4.2	4.2
Clear cell renal cell carcinoma	857	665	95.0	5.0	91.4	5.6	3.0
Papillary renal cell carcinoma	255	199	84.4	15.6	85.4	8.0	6.5
Clear cell (tubulo) papillary renal cell carcinoma	21	15	100.0	0.0	93.3	0.0	6.7
Chromophobe renal cell carcinoma	131	100	85.0	15.0	100.0	0.0	0.0
Oncocytoma	177	141	68.8	31.2	93.6	5.0	1.4
Tumors of the male genital organs							
Adenocarcinoma of the prostate, Gleason 3 + 3	83	70	100.0	0.0	92.9	1.4	5.7
Adenocarcinoma of the prostate, Gleason 4 + 4	80	67	97.0	3.0	86.6	10.4	3.0
Adenocarcinoma of the prostate, Gleason 5 + 5	85	68	92.6	7.4	69.1	11.8	19.1
Adenocarcinoma of the prostate (recurrence)	258	210	96.7	3.3	94.3	3.3	2.4
Small cell neuroendocrine carcinoma of the prostate	19	17	100.0	0.0	52.9	35.3	11.8
Seminoma	621	475	100.0	0.0	24.2	24.0	51.8
Embryonal carcinoma of the testis	50	35	100.0	0.0	14.3	22.9	62.9
Yolk sac tumor	50	27	100.0	0.0	25.9	25.9	48.1
Teratoma	50	25	100.0	0.0	96.0	4.0	0.0
Squamous cell carcinoma of the penis	80	75	33.3	66.7	33.3	22.7	44.0
Tumors of endocrine organs							
Adenoma of the thyroid gland	113	99	84.8	15.2	96.0	0.0	4.0
Papillary thyroid carcinoma	391	345	69.0	31.0	78.8	13.0	8.1
Follicular thyroid carcinoma	154	130	67.7	32.3	97.7	1.5	0.8
Medullary thyroid carcinoma	111	90	84.4	15.6	94.4	4.4	1.1
Anaplastic thyroid carcinoma	45	38	23.7	76.3	57.9	15.8	26.3
Adrenal cortical adenoma	50	42	100.0	0.0	95.2	4.8	0.0
Adrenal cortical carcinoma	26	25	92.0	8.0	96.0	4.0	0.0
Phaeochromocytoma	50	50	68.0	32.0	82.0	14.0	4.0
Appendix, neuroendocrine tumor (NET)	22	14	92.9	7.1	92.9	0.0	7.1
Colorectal, neuroendocrine tumor (NET)	12	12	100.0	0.0	91.7	8.3	0.0
Ileum, neuroendocrine tumor (NET)	49	47	100.0	0.0	95.7	4.3	0.0
Lung, neuroendocrine tumor (NET)	19	18	94.4	5.6	100.0	0.0	0.0
Pancreas, neuroendocrine tumor (NET)	97	49	89.8	10.2	87.8	6.1	6.1
Colorectal, neuroendocrine carcinoma (NEC)	12	12	91.7	8.3	50.0	33.3	16.7
Gallbladder, neuroendocrine carcinoma (NEC)	4	4	100.0	0.0	25.0	25.0	50.0
Pancreas, neuroendocrine carcinoma (NEC)	14	12	100.0	0.0	75.0	16.7	8.3
Tumors of haemotopoetic and lymphoid tissues							
Hodgkin Lymphoma	103	43	7.0	93.0	0.0	2.3	97.7
Small lymphocytic lymphoma, B-cell type (B-SLL/B-CLL)	50	49	100.0	0.0	2.0	55.1	42.9
Diffuse large B cell lymphoma (DLBCL)	114	109	80.7	19.3	17.4	10.1	72.5
Follicular lymphoma	88	86	100.0	0.0	1.2	19.8	79.1
T-cell Non Hodgkin lymphoma	24	24	83.3	16.7	16.7	4.2	79.2
Mantle cell lymphoma	18	18	100.0	0.0	5.6	16.7	77.8
Marginal zone lymphoma	16	16	100.0	0.0	0.0	37.5	62.5
Diffuse large B-cell lymphoma (DLBCL) in the testis	16	16	87.5	12.5	12.5	12.5	75.0
Burkitt lymphoma	5	4	100.0	0.0	25.0	50.0	25.0
Tumors of soft tissue and bone							
Tendosynovial giant cell tumor	45	45	100.0	0.0	100.0	0.0	0.0
Granular cell tumor	53	48	97.9	2.1	100.0	0.0	0.0
Leiomyoma	50	41	100.0	0.0	100.0	0.0	0.0
Leiomyosarcoma	87	76	89.5	10.5	89.5	9.2	1.3
Liposarcoma	132	105	85.7	14.3	93.3	4.8	1.9
Malignant peripheral nerve sheath tumor (MPNST)	13	12	91.7	8.3	91.7	8.3	0.0
Myofibrosarcoma	26	26	69.2	30.8	84.6	7.7	7.7
Angiosarcoma	73	67	65.7	34.3	70.1	17.9	11.9
Angiomyolipoma	91	88	95.5	4.5	87.5	9.1	3.4
Dermatofibrosarcoma protuberans	21	16	100.0	0.0	100.0	0.0	0.0
Ganglioneuroma	14	11	81.8	18.2	100.0	0.0	0.0
Kaposi sarcoma	8	7	28.6	71.4	71.4	0.0	28.6
Neurofibroma	117	90	100.0	0.0	100.0	0.0	0.0

Table 2, continued

Tumor entity	On TMA (n)	Analyzable (n)	PD-L1 in tumor cells		PD-L1 in immune cells		
			Negative (%)	Positive (%)	Negative (%)	Few (%)	Many (%)
Sarcoma, not otherwise specified (NOS)	74	70	62.9	37.1	98.6	1.4	0.0
Paraganglioma	41	37	94.6	5.4	83.8	8.1	8.1
Ewing sarcoma	23	20	95.0	5.0	95.0	5.0	0.0
Rhabdomyosarcoma	6	6	100.0	0.0	100.0	0.0	0.0
Schwannoma	121	100	98.0	2.0	99.0	1.0	0.0
Synovial sarcoma	12	11	100.0	0.0	100.0	0.0	0.0
Osteosarcoma	43	32	100.0	0.0	96.9	3.1	0.0
Chondrosarcoma	38	19	68.4	31.6	100.0	0.0	0.0

Table 3
PD-L1 n human tumor cells and intratumoral CD8 positive ($CD8^+$) cells

	PD-L1 IHC in tumor cells	n	CD8+ cell density (mean ± SD)	P values
All cancers	Negative	5,016	254.2 ± 7.1	< 0.0001
	Positive	484	612.2 ± 22.9	
Mesothelioma, epitheloid	Negative	29	261.6 ± 54.3	0.0864
	Positive	4	537.9 ± 146.3	
Mesothelioma, other types	Negative	18	231.8 ± 82.4	0.1312
	Positive	10	446.7 ± 110.5	
Endometrioid carcinoma of the ovary	Negative	28	124.0 ± 62.6	0.9657
	Positive	5	117.0 ± 148.2	
Serous carcinoma of the ovary	Negative	279	142.0 ± 24.5	< 0.0001
	Positive	43	532.1 ± 62.4	
Clear cell carcinoma of the ovary	Negative	7	18.2 ± 7.7	0.7127
	Positive	2	11.9 ± 14.5	
Carcinosarcoma of the ovary	Negative	20	117.0 ± 46.6	0.5896
	Positive	4	54.5 ± 104.2	
Invasive breast carcinoma of no special type	Negative	997	294.6 ± 14.0	< 0.0001
	Positive	58	699.0 ± 58.0	
Lobular carcinoma of the breast	Negative	134	199.7 ± 22.3	0.7999
	Positive	1	134.0 ± 258.0	
Medullary carcinoma of the breast	Negative	6	1470.0 ± 485.9	0.1396
	Positive	3	2872.1 ± 687.2	
Adenocarcinoma of the colon	Negative	1229	259.3 ± 13.7	< 0.0001
	Positive	52	692.9 ± 66.8	
Clear cell renal cell carcinoma	Negative	568	435.6 ± 29.5	< 0.0001
	Positive	31	1164.2 ± 126.2	
Papillary cell renal cell carcinoma	Negative	127	233.9 ± 39.7	0.2765
	Positive	27	337.3 ± 86.0	
Oncocytoma	Negative	57	74.0 ± 18.1	0.3923
	Positive	31	100.2 ± 24.5	
Gastric adenocarcinoma, diffuse type	Negative	69	260.3 ± 50.5	0.7595
	Positive	2	352.8 ± 296.5	
Gastric adenocarcinoma, intestinal type	Negative	61	324.7 ± 62.3	< 0.0001
	Positive	15	1142.5 ± 125.7	
Gastric adenocarcinoma, mixed type	Negative	45	386.4 ± 94.7	0.1399
	Positive	8	751.9 ± 224.6	
Ductal carcinoma of the pancreas	Negative	351	222.2 ± 15.8	0.1014
	Positive	42	301.3 ± 45.5	
Pancreatic/Ampullary adenocarcinoma	Negative	34	268.8 ± 81.1	0.1313
	Positive	4	654.7 ± 236.4	
Sarcomatoid urothelial carcinoma	Negative	7	229.3 ± 342.1	0.2457
	Positive	17	714.1 ± 219.5	
Granular cell tumor	Negative	19	61.9 ± 11.1	0.0681
	Positive	1	158.0 ± 48.3	
Leiomyosarcoma	Negative	32	82.7 ± 28.6	0.1047
	Positive	3	245.5 ± 93.3	

Table 3, continued

	PD-L1 IHC in tumor cells	n	CD8+ cell density (mean ± SD)	P values
Liposarcoma	Negative	56	88.3 ± 53.9	< 0.0001
	Positive	10	1086.6 ± 127.6	
Malignant peripheral nerve sheath tumor (MPNST)	Negative	11	100.9 ± 61.3	0.0007
	Positive	1	1130.0 ± 203.2	
Myofibrosacroma	Negative	18	53.0 ± 272.6	0.0587
	Positive	8	1028.3 ± 408.9	
Angiosarcoma	Negative	22	105.6 ± 109.3	0.0042
	Positive	17	610.5 ± 124.4	
Angiomyolipoma	Negative	84	178.9 ± 45.9	0.9519
	Positive	4	165.9 ± 210.5	
Ganglioneuroma	Negative	9	32.4 ± 11.1	0.0339
	Positive	2	97.2 ± 23.5	
Kaposi sarcoma	Negative	2	304.3 ± 177.7	0.6885
	Positive	5	393.7 ± 112.4	
Sarcoma, not otherwise specified (NOS)	Negative	44	66.8 ± 100.4	0.0004
	Positive	26	677.0 ± 129.6	
Paraganglioma	Negative	35	150.6 ± 46.0	0.9313
	Positive	2	167.7 ± 192.4	
Primitive neuroectodermal tumor (PNET)	Negative	19	65.7 ± 23.7	0.5439
	Positive	1	0.0 ± 103.5	
Schwannoma	Negative	98	81.1 ± 15.9	0.3795
	Positive	2	180.4 ± 111.3	
Chondrosarcoma	Negative	5	481.4 ± 320.5	0.8664
	Positive	4	397.6 ± 358.4	

in colon cancer [38,39] , between 6.7% and 48.1% in gastric [40,41], or between 8.3% and 75% in non-small cell lung cancer [42,43]. The high concordance of the staining results and diagnostic performance obtained by 4 different anti-PD-L1 antibodies argues against a major role of antibody properties for these discrepant data. Various previous studies have also shown that the antibodies that are most commonly used for PD-L1 analysis can result in comparable data within studies [34,44–46], and that even the use of lab developed PD-L1 tests yield similar results as FDA approved companion diagnostics [47]. The comparison of data obtained from studies using identical antibodies also argues against a major role of antibody characteristics as drivers for the large bandwidth of PD-L1 data. For example, the antibody clone E1L3N has been used in more than 300 previous studies and resulted in PD-L1 positivity in 0–33% of clear cell renal cell carcinomas [23,48], 0–25% of colorectal carcinomas [23,49], 19-90% of lung adenocarcinomas [50,51], and 0–79% of pancreas carcinomas [23,52] at cut-off levels of 1% or 5% stained cancer cells to define positivity.

Rather underestimated causes for discrepant PD-L1 data include slide ageing and difficulties in the distinction of tumor associated macrophages from tumor cells. Others and we had earlier demonstrated that the immunostaining intensity on stored formalin-fixed tissue sections decreases over time [53,54] and that a significant reduction of staining may already occur 2 weeks after a tissue section has been taken [55]. This may be a relevant source of discrepant staining results particularly in clinical studies, where sections are often taken long before the analysis is made. In case of PD-L1, where macrophages often express the target protein at high levels, and where low thresholds are used for defining tumor cell positivity, it appears also likely that the quantity of tissue analyzed per patient and difficulties in the distinction of PD-L1 positive macrophages from cancer cells may have contributed to interpretation difficulties. That the analysis of larger tissue fragments more often leads to the perception of PD-L1 positivity than the analysis of small portions is shown by significant differences in data derived from TMA and from large section studies. For example, in 16 studies utilizing cut-off levels of 1% or 5% to define PD-L1 positivity in lung adenocarcinomas with the E1L3N antibody, the average positivity rate was 26% for TMA analyses but 41% for conventional large section staining. While these data might suggest that relevant PD-L1 findings are missed on TMAs, it is also evident that interpretation errors – such as mistaking macrophages for tumor cells – are more likely to occur on large sections [56]. Moreover, TMA studies comparing multiple samples per tumor versus only one sample per tumor have regularly found a significant relationship between the quantity of analyzed tissue and IHC positivity rate [56–59]. Only recently, it was shown that posttranslational glycolysation of the PD-L1 protein can negatively affect

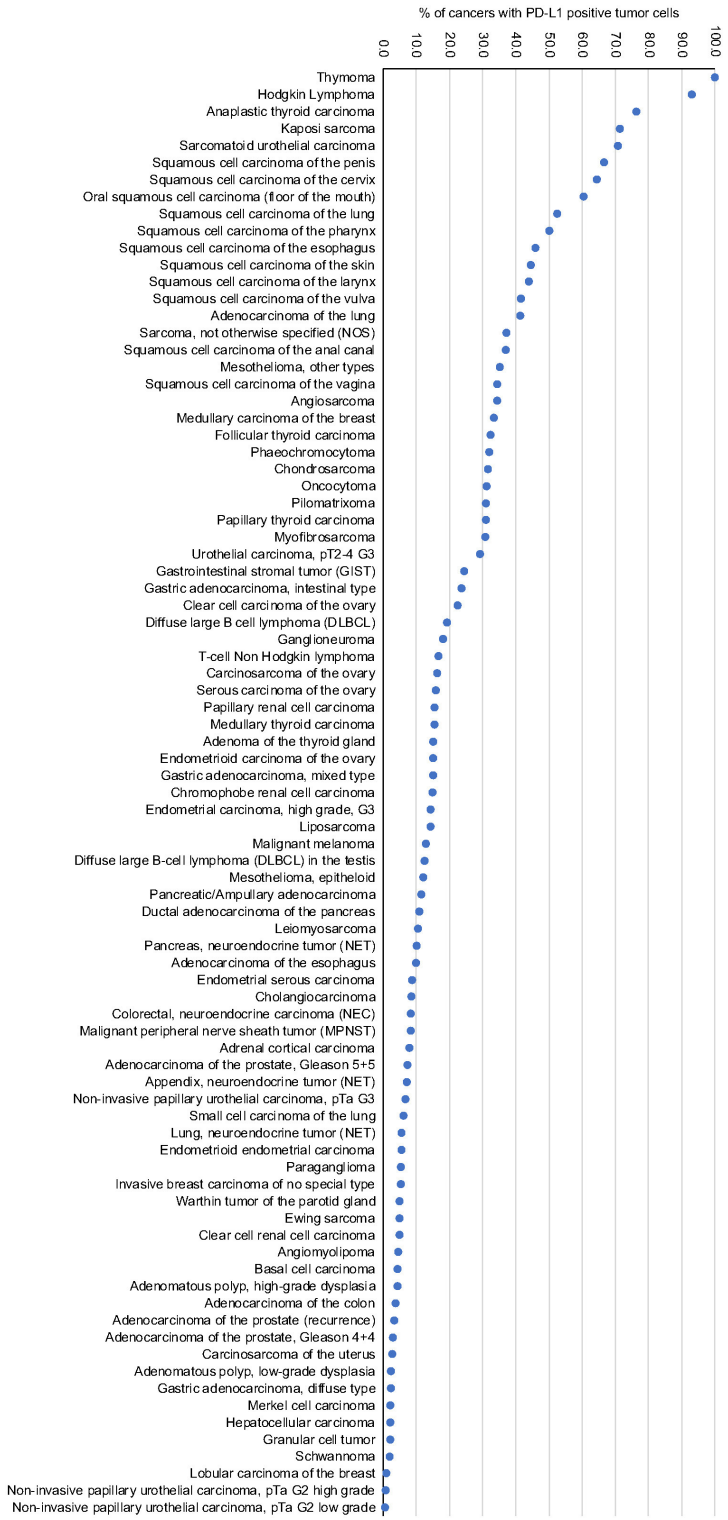

Fig. 3. Ranking order of PD-L1 immunostaining in human tumors. Only staining in tumor cells is shown.

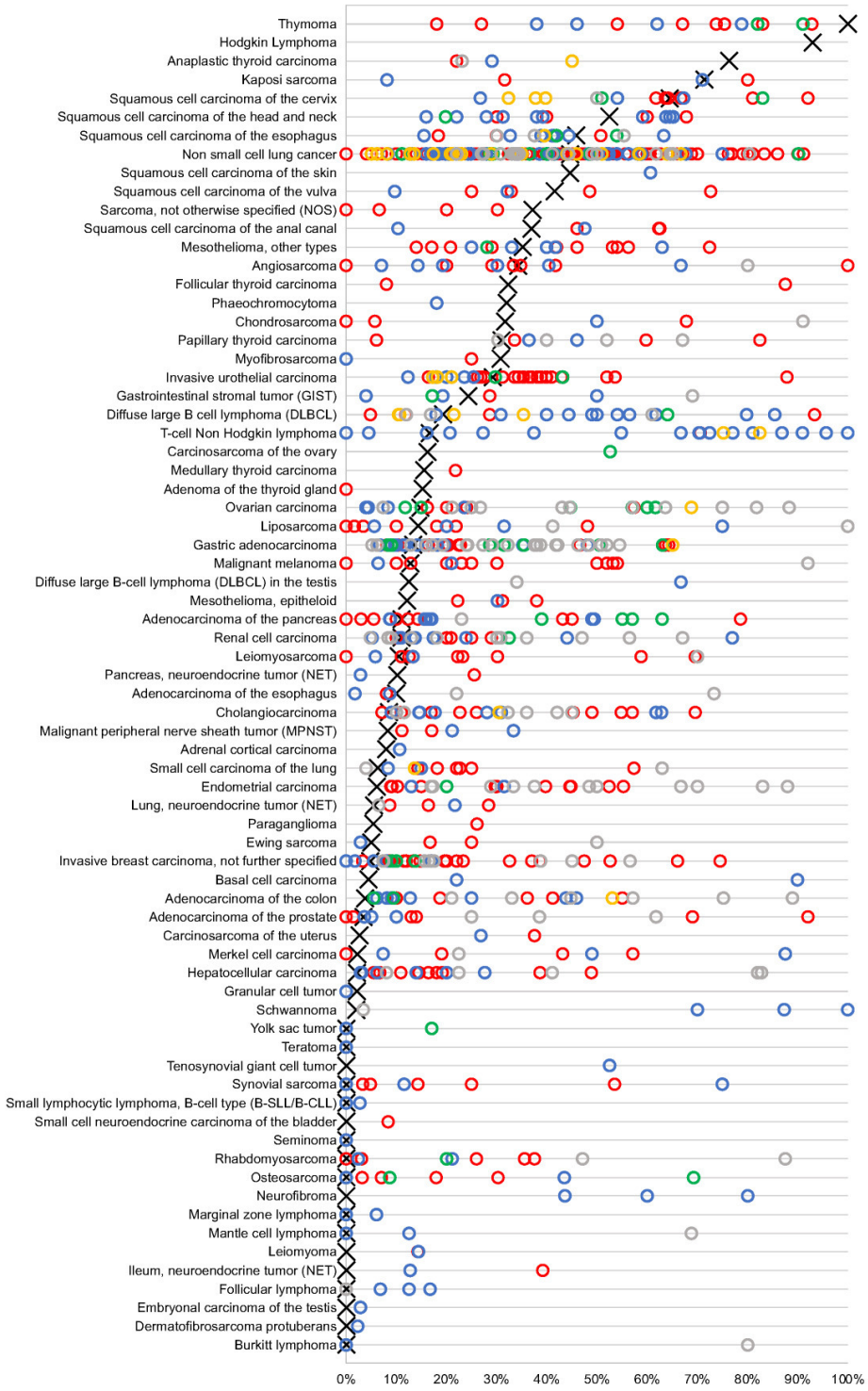

Fig. 4. Graphical comparison of PD-L1 data from this study (×) in comparison with the previous literature (circles). Color of circles indicates the threshold used to define PD-L1 positivity in these studies: red = 1%, blue = 5%, green = 10%, orange = ⩾ 25%, grey = other threshold. A list of studies used to build the figure is given in Supplementary Table S2.

binding of anti-PD-L1 antibodies in formalin fixed tissue samples [60]. Therefore, it has been suggested that tissue samples should be pretreated with deglycolysing reagents to reduce the risk of false-negative PD-L1 IHC findings. In our study, such a systematic change in staining protocol would potentially result in a higher overall number of PD-L1 positive tumors. However, because all tumor types would be equally affected, the relative ranking of PD-L1 positive tumor types would not change.

Groups of cancers that are of special interest based on our data include cancers with very high and very low rate of PD-L1 expression in cancer cells and tumors with a particularly high density of tumor associated PD-L1 inflammatory cells. The group of tumors with highest rates of PD-L1 positivity in tumor cells includes several tumor entities already approved for treatment with CPIs, such as Hodgkin lymphoma, squamous cell carcinomas of the head and neck, urothelial cancers and malignant mesothelioma. If the response to CPIs is indeed driven by tumoral PD-L1 expression in these tumors, cancers with a comparably high PD-L1 expression such as penile carcinoma, squamous cell carcinomas of the esophagus and the anal canal or anaplastic thyroid cancer should also represent premium targets for CPIs. Evidence for clinical responses already exists for anaplastic thyroid cancer [61], squamous cell cancers of the head and neck [62–65], oral cavity [66], esophagus [67,68] and skin [69], and a clinical trial is ongoing for squamous cell carcinoma of the cervix [70].

Cancers with a very low rate of tumoral PD-L1 expression for example include prostate cancer, a tumor known for is particularly poor response to CPIs [71] but also cancers such as Merkel cell carcinoma and small cell lung cancer which are both approved for CPI therapy. It is of note, that Merkel cell carcinoma (82.2%) and small cell lung cancer (68.7%) belong to these tumor types with the highest rates of PD-L1 positive immune cells in our analysis. These findings fit well with experimental data highlighting the particularly important role of PD-L1 expressing immune cells. For example, in colon and breast cancer mice models, anti–PD-L1 treatment changed the activity of tumor macrophages from an immune-suppressive to an immune-stimulatory state with an increase in activated CD8 positive cytotoxic T cells [72]. Triple negative breast cancer is the first tumor entity where the indication for CPI atezolizumab solely depends on the presence of intratumoral PD-L1 positive immune cells and is independent of whether tumor cells express PD-L1 [73,74].

Our data also show that an elevated density of CD8 positive intratumoral lymphocytes in PD-L1 expressing tumors is a general feature occurring across all cancer types. This observation is consistent with various reports describing associations between PD-L1 positivity in tumor cells and high numbers of tumor infiltrating lymphocytes in various individual cancer types [3–6]. Studies have also demonstrated that PD-L1 positivity is statistically linked to high mutation burden and microsatellite instability [75]. Altogether, these observations are well consistent with a model suggesting that PD-L1 is one of several immune-escape mechanisms that can be activated in highly immunogenic cancer cells in response to "lymphocyte attack".

In summary, the results of our study provide a ranking order of cancer types according to their PD-L1 expression in tumor and inflammatory cells. A consistently higher rate of tumor infiltrating CD8 positive lymphocytes in PD-L1 positive than in PD-L1 negative cancers corroborates the concept that tumoral PD-L1 expression is driven by a hostile immune environment.

Acknowledgments

We are grateful to Melanie Witt, Laura Behm, Inge Brandt, Maren Eisenberg, and Sünje Seekamp for excellent technical assistance.

Author contributions

Conception: KM, TK, RS, GS.
Interpretation or analysis of data: MK, EB, MJS, SDR, MK, CHM, NCB, TM, ML, AM, AML, DH, CF, NG, FJ, TSC, SS, EB, SM, AHM.
Preparation of the manuscript: KM, TK, GS.
Revision for important intellectual content: KM, TK, RS, GS, DH.
Supervision: KM, TK, RS, GS.
All authors agree to be accountable for the content of the work.

Conflict of interest

The PD-L1 antibody clone MSVA-711R was provided by MS Validated Antibodies GmbH (owned by a family member of GS).

Supplementary data

The supplementary files are available to download from http://dx.doi.org/10.3233/CBM-220030.

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