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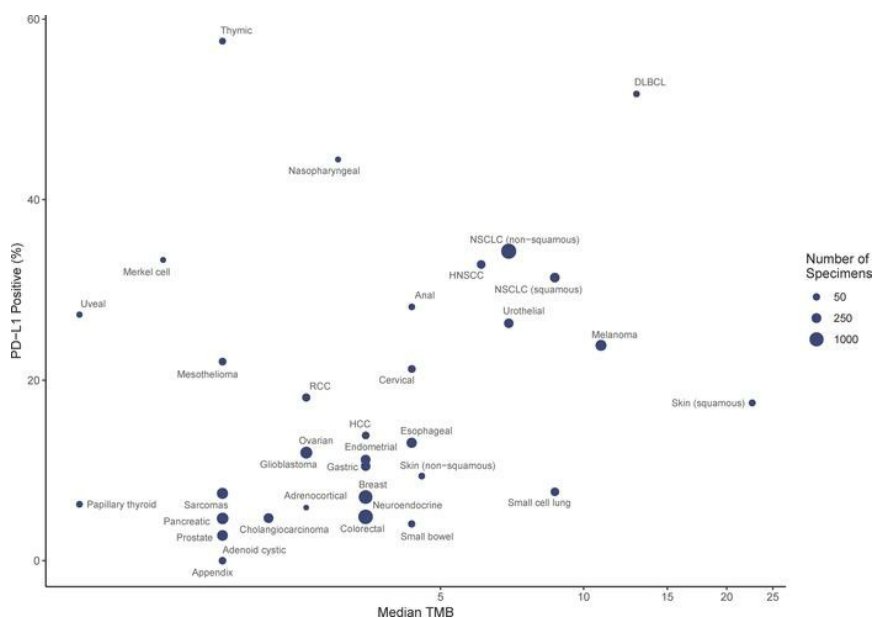
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### Graphical abstract



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# PD-L1 expression and tumor mutational burden are independent biomarkers in most cancers

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**BACKGROUND.** PD-L1 expression and tumor mutational burden (TMB) have emerged as important biomarkers of response to immune checkpoint inhibitor (ICI) therapy. These biomarkers have each succeeded and failed in predicting responders for different cancer types. We sought to describe the PD-L1 expression landscape across the spectrum of ICI-responsive human cancers, and to determine the relationship between PD-L1 expression, TMB, and response rates to ICIs.

**METHODS.** We assessed 9887 clinical samples for PD-L1 expression and TMB.

**RESULTS.** PD-L1 expression and TMB are not significantly correlated within most cancer subtypes, and they show only a marginal association at the tumor sample level (Pearson's correlation 0.084). Across distinct tumor types, PD-L1 expression and TMB have nonoverlapping effects on the response rate to PD-1/PD-L1 inhibitors and can broadly be used to categorize the immunologic subtypes of cancer.

**CONCLUSION.** Our results indicate that PD-L1 expression and TMB may each inform the use of ICIs, point to different mechanisms by which PD-L1 expression regulates ICI responsiveness, and identify new opportunities for therapeutic development.

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## Introduction

Immunotherapies targeting programmed cell death protein 1 (PD-1) or its ligand (PD-L1) can reverse immune tolerance and yield remarkable clinical responses for a subset of patients with cancer. Predictive biomarkers for immune checkpoint inhibitor (ICI) therapy have the potential to improve outcomes by identifying patients who are likely to benefit from ICI therapy while avoiding unnecessary toxicities in patients who are unlikely to benefit. The expression of PD-L1 on tumor cells or tumor-infiltrating immune cells by immunohistochemistry (IHC) has become the most widely used biomarker for selecting patients for ICI therapy (1). The utility of this biomarker was originally identified in the first clinical study of the PD-1 inhibitor nivolumab in 2010 (2). Since that time, the expression of PD-L1 has been interrogated in multiple large clinical trials of PD-1/PD-L1 inhibitors. These trials have confirmed that higher expression of PD-L1 can predict response to PD-1/PD-L1 inhibitors within multiple cancer types, including melanoma, non-small cell lung cancer (NSCLC), and urothelial cancer (3–5). The PD-1 inhibitor pembrolizumab has specific indications in PD-L1-expressing gastric cancer and NSCLC (4, 6, 7). However, PD-L1 expression has failed to enrich for responses to IPI therapy in other studies (8–10), and remains an imperfect biomarker that is insufficient to predict clinical benefit for all patients responsive to the different PD-1/PD-L1 inhibitors.

**Authorship note:** MY and LAA contributed equally to this work

**Conflict of interest:** LAA, MM, KM, and GMF are employees of and stockholders in Foundation Medicine.

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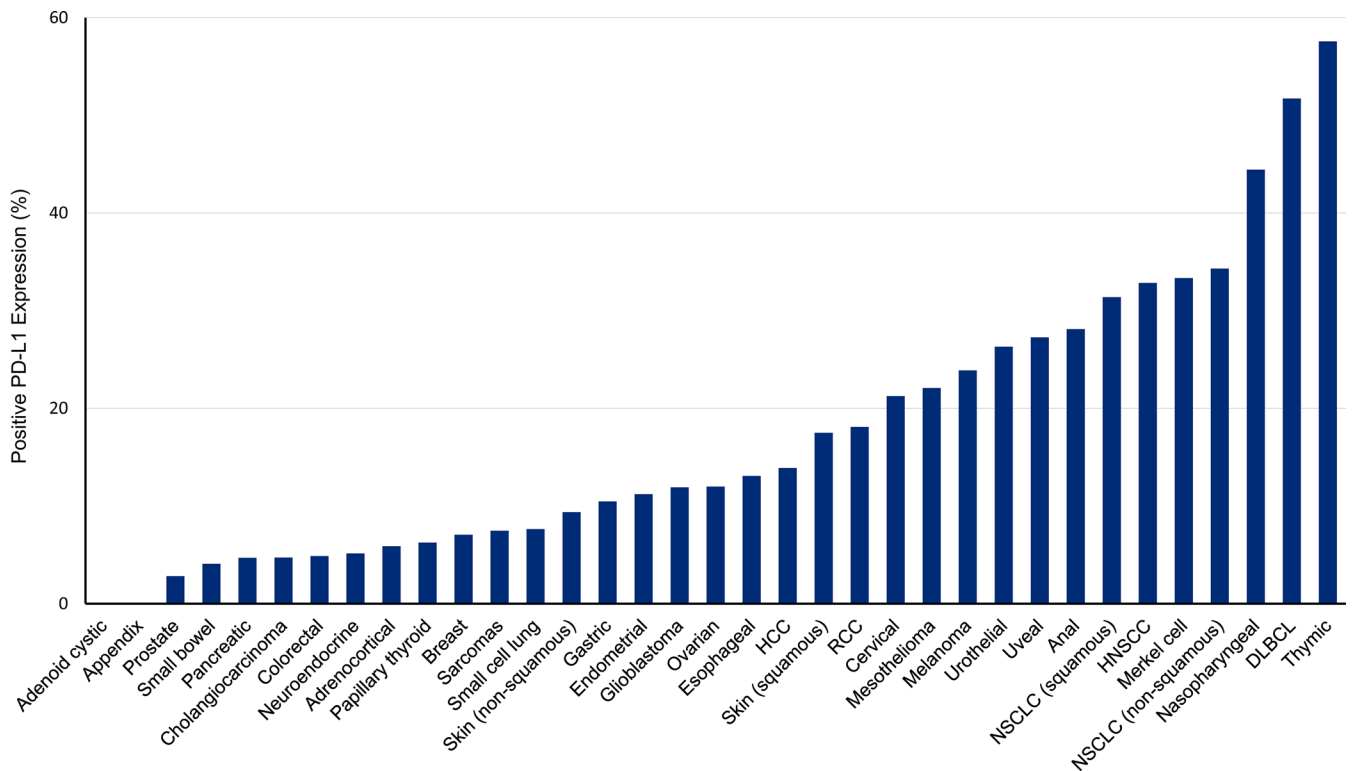
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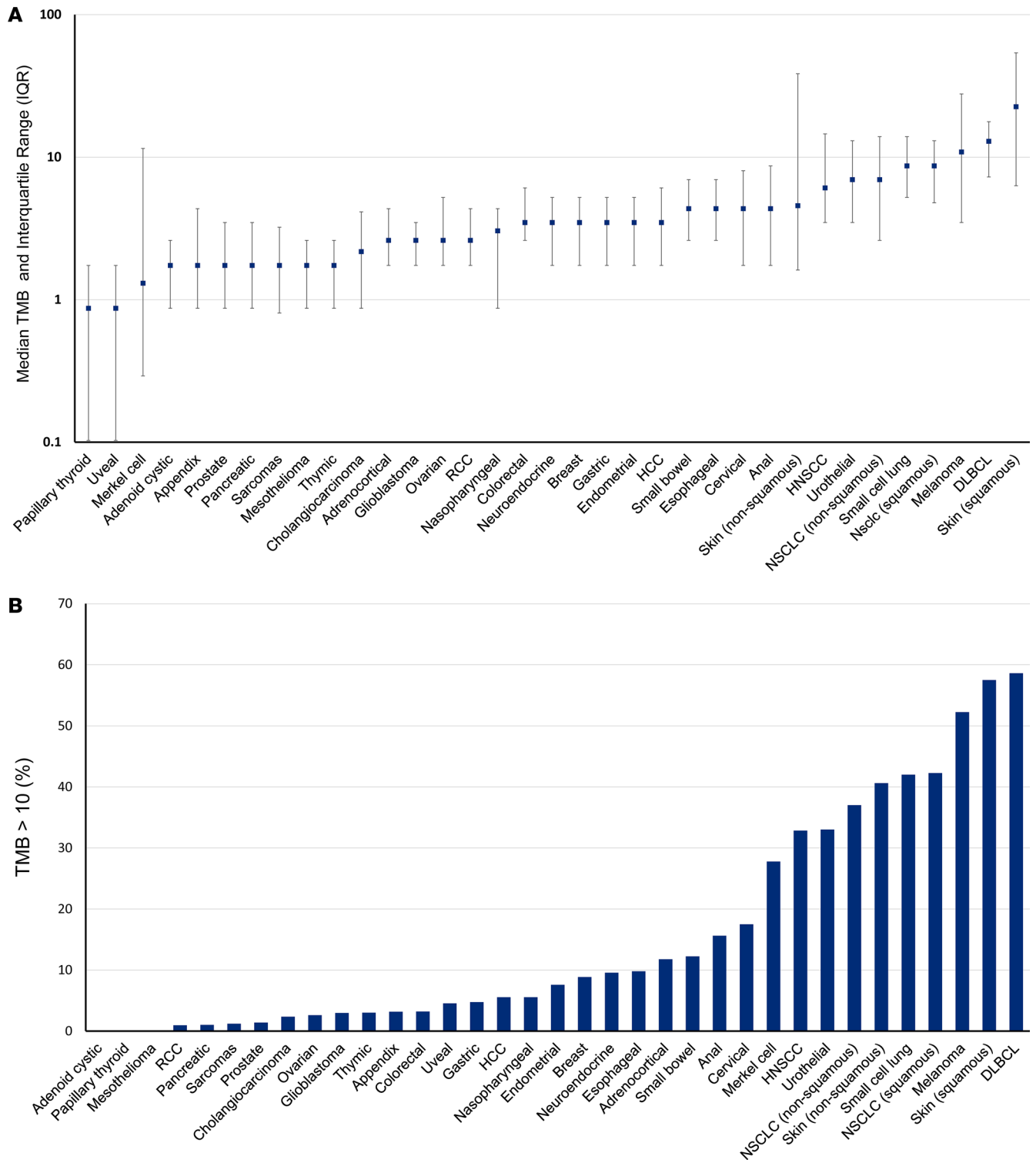
**Figure 1. Landscape of PD-L1 expression across the major tumor types.** Percentage of tumors with positive PD-L1 expression by IHC within 35 major tumor types, from the lowest frequency of positivity (left) to the highest frequency (right).

Another emerging clinical biomarker of response to ICIs is the tumor mutational burden (TMB), defined as the total number of mutations per coding area of a tumor genome. Each tumor mutation within a cancer cell has the potential to give rise to tumor-specific antigens (neoantigens), a subset of which will be processed and presented on HLA molecules on the cell surface of tumor cells and recognized by the immune system. The recognition of tumor antigens is thought to be a largely stochastic process, but each somatic mutation in the tumor DNA is proposed to increase the chance that the immune system will recognize and reject tumor cells in response to ICI therapy (11, 12). In support of this premise, the tumor types for which ICI therapy has proven to be most effective are those with a high TMB (13). Specifically, a higher TMB has also been shown to correlate with clinical benefit from ICI therapy within multiple tumors, including NSCLC (14, 15), small cell lung cancer (16), melanoma (17, 18), and colorectal cancer (19).

While both biomarkers are routinely obtained in the clinical care of oncology patients, their relationship across the spectrum of human cancers remains unclear. It is rational to hypothesize that a high TMB would induce a high density of neoantigen-specific tumor-infiltrating lymphocytes, leading to secretion of IFN- $\gamma$  and upregulation of PD-L1 on tumor cells (20, 21). Surprisingly, a recent analysis of TMB and PD-L1 expression in NSCLC patients who received combination anti-PD-1 and anti-CTLA-4 therapy found that these biomarkers may be uncorrelated (15). This suggests that PD-L1 and TMB may be independent predictive biomarkers that can each contribute to the identification of patients for immune checkpoint therapy. We sought to determine the relationship between PD-L1 expression and TMB across the entire spectrum of ICI-responsive human cancers, to use these predictive biomarkers to broadly define the immunologic subtypes of cancers, and to identify opportunities for therapeutics development.

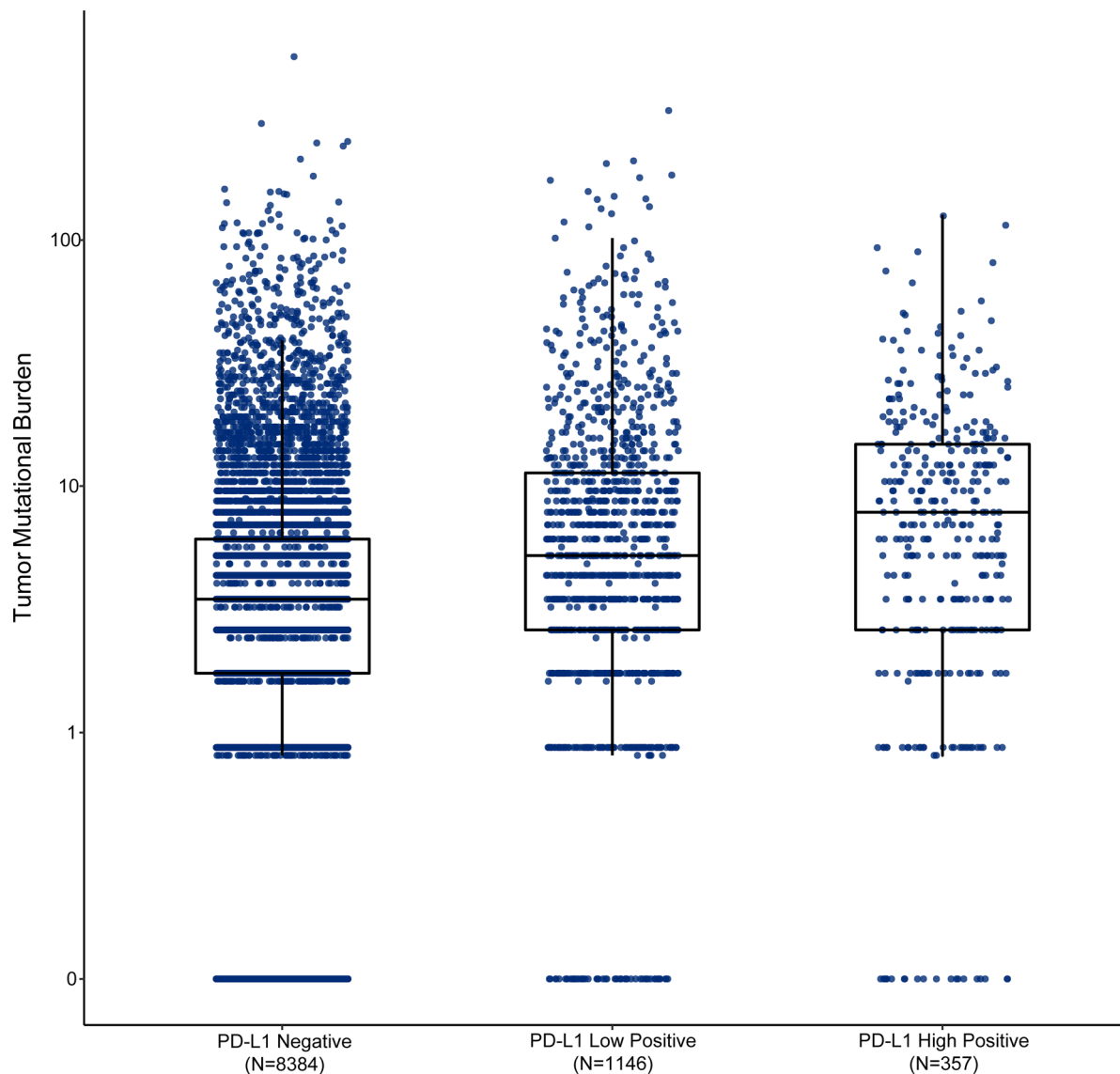
## Results

*PD-L1 expression and TMB landscape across tumor types.* Our cohort contained 9887 unique clinical samples for which paired comprehensive genomic profiling and PD-L1 expression were obtained during the course of standard clinical care. Summary PD-L1 qualitative IHC data for major tumor types are shown in Figure 1. Across all samples analyzed, 15.2% of samples were PD-L1 positive (defined as  $\geq 1\%$  tumor cells staining positive for PD-L1). Expression of PD-L1 varied widely among the tumor types examined. Thymic cancer



**Figure 2. The median TMB and interquartile range for 35 major tumor types. (A)** Tumors are ordered from the lowest median TMB (left) to the highest median TMB (right). **(B)** Percentage of tumor samples within 35 major tumor types with a TMB greater than 10 mutations/Mb.

and diffuse large B cell lymphoma had the highest frequency of PD-L1 positivity (51%–58%), whereas no adenoid cystic or appendiceal carcinoma samples were PD-L1 positive. Across all samples, 3.6% of specimens were PD-L1 high-positive (defined as  $\geq 50\%$  tumor cells staining positive for PD-L1). Among the distinct tumor types that were analyzed, nasopharyngeal and thymic carcinomas had the highest frequency of PD-L1-high clinical specimens.



**Figure 3. Relationship between TMB and PD-L1 expression.** The TMB for patients with PD-L1-negative tumors, PD-L1-low-positive tumors, and PD-L1-high-positive tumors is shown for all 9887 samples included in the overall cohort. The Pearson's correlation for PD-L1 expression and TMB across all samples was 0.084 ( $P < 10^{-16}$ ). The box plots show the 50th percentile, the 25th to 75th percentiles, and the farthest outlier or  $1.5 \times$  interquartile range, whichever is less.

The presence of viral antigens has previously been associated with PD-L1 expression in certain tumor types known to be viral-associated, which led us to examine PD-L1 expression across the spectrum of viral-associated cancers. In our cohort, the tumor types classically associated with the presence of viral antigens (anal carcinoma, cervical carcinoma, head and neck squamous cell carcinoma [HNSCC], hepatocellular carcinoma [HCC], Merkel cell carcinoma, nasopharyngeal) all demonstrated relatively high PD-L1 expression (13.9%–44.4% of samples expressing PD-L1). This was significantly higher than in non-viral-associated cancers (mean percentage PD-L1 positive: 29.0% viral vs. 14.7% nonviral,  $P = 0.015$ , Student's  $t$  test). To further investigate the relationship between viral antigen status and PD-L1 expression at the level of individual tumor specimens, we looked for the presence of HPV, EBV, Merkel cell polyomavirus, or HBV viral DNA in the appropriate tumor types. Viral DNA was detected in 100 tumor samples. Among these samples, 25 (25.0%) were PD-L1 positive by IHC, which was significantly higher than the 15.2% PD-L1 positivity rate across samples for which viral DNA was not detected ( $P = 0.011$ , Fisher's exact test comparing samples with detected vs. undetected viral DNA).

**Table 1. Classification of 35 major tumor types using the regression tree algorithm demonstrated in Figure 5C**

Noninflamed		Inflamed		Hypermutated
<7% Staining positive for PD-L1; TMB < 10	7%–13% Staining positive for PD-L1; TMB < 10	13%–33% Staining positive for PD-L1; TMB < 10	≥33% Staining positive for PD-L1; TMB < 10	Any PD-L1 TMB ≥ 10
Adenoid cystic, adrenocortical, appendix, cholangiocarcinoma, colorectal, neuroendocrine, pancreatic, papillary thyroid, prostate, small bowel	Breast, endometrial, gastric, glioblastoma, ovarian, sarcomas, skin (nonsquamous), small cell lung	Anal, cervical, esophageal, HCC, HNSCC, mesothelioma, NSCLC (squamous), renal cell carcinoma, urothelial, uveal	Merkel cell, nasopharyngeal, NSCLC (nonsquamous), thymic	DLBCL, melanoma, mismatch repair-deficient cancers, skin (squamous)

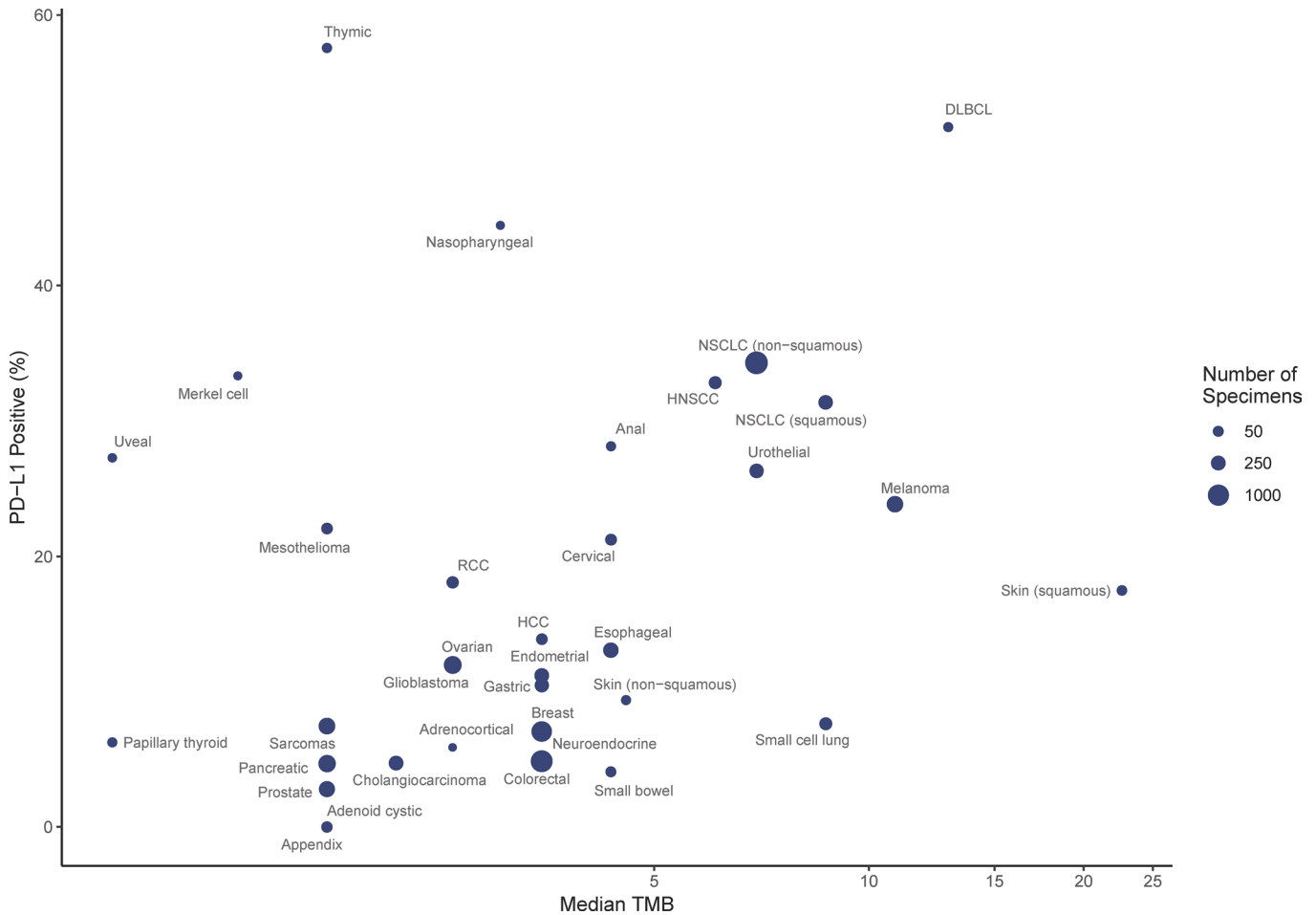
The median TMB across all samples was 3.48 mutations/Mb (IQR 1.74–6.96). Summary TMB data for 35 major and distinct tumor types are shown in Figure 2A. The median TMB for each tumor type ranged from 0.1 mutations/Mb in papillary thyroid cancer to 22.6 in skin squamous cell carcinoma. Consistent with prior reports, cancers associated with mutagens (lung cancers, skin cancers, urothelial cancer) generally had the highest TMBs of any tumor type. Merkel cell carcinoma and skin nonsquamous cancers demonstrated a broader range of TMBs than other tumor types. A TMB cutoff of >10 mutations/Mb has been used elsewhere to define the subset of tumors with a high TMB (15, 22). The percentage of tumors within each distinct tumor type with a TMB greater than 10 mutations/Mb is shown in Figure 2B. Across all sampled tumors, 16.4% of cancers had a TMB greater than 10 mutations/Mb, and 7.3% had a TMB greater than 20 mutations/Mb. These estimates of TMB are comparable to prior estimates of TMB in other cancer cohorts, including whole-exome studies (23).

*Relationship of PD-L1 expression and cancer genomics.* Figure 3 shows TMB for all PD-L1–negative, PD-L1–low-positive, and PD-L1–high-positive specimens. Across all individual specimens examined, there was a small but positive association between the PD-L1 expression and TMB (Pearson’s coefficient 0.084,  $P < 10^{-16}$ ). However, the relationship between these 2 biomarkers was not consistent across tumor types (Supplemental Table 1; supplemental material available online with this article; <https://doi.org/10.1172/jci.insight.126908DS1>). The association between TMB and PD-L1 was driven by a subset of the distinct tumor types that were sampled and was strongest within gastric cancers and endometrial cancers (Pearson’s coefficient all >0.3). There was also a weak but positive association between TMB and PD-L1 expression among melanoma, pancreatic, and NSCLC (Pearson’s coefficient all <0.15). However, TMB and PD-L1 expression did not correlate among most other major tumor types. Notably, TMB and PD-L1 expression demonstrated total independence among several tumor types for which PD-1/PD-L1 inhibitor therapy is effective and widely used in the clinical care of patients, including esophageal, HCC, HNSCC, Merkel cell carcinoma, renal cell carcinoma, squamous NSCLC, and small cell lung cancers.

We also examined the relationship between PD-L1 expression and TMB at the level of distinct tumor types. Summary PD-L1 expression and TMB data for 35 major and distinct tumor types are shown in Figure 4 and Supplemental Table 2. There was no relationship between the PD-L1 expression and median TMB for the major tumor types ( $r^2 = 0.06542$ ,  $P > 0.1$ ). Viral-associated tumor types for the most part clustered as PD-L1 high and TMB low or intermediate, whereas tumors types associated with mutagens were consistently TMB high but had variable PD-L1 expression.

*PD-L1 expression, TMB, and response to anti-PD-1 therapy.* We investigated the differences in utility of PD-L1 expression and TMB as clinical biomarkers by assessing the relationship of these biomarkers with clinical responses to PD-1/PD-L1 inhibitors across multiple cancer types. Through an extensive literature review, we identified 29 solid tumor types for which data regarding the objective response rate (ORR) to PD-1/PD-L1 inhibitor monotherapy have been reported. We excluded studies that exclusively selected for PD-L1 tumor expression or other immune-related biomarkers (see Methods for details).

To assess the correlation of median TMB and PD-L1 positivity with clinical outcomes across the 29 solid tumor types, each predictor was plotted against ORR (Figure 5) and a simple linear regression was performed. PD-L1 expression showed a significant correlation with ORR ( $r^2 = 0.204$ ,  $P = 0.0139$ ; Figure 5A), as did TMB ( $r^2 = 0.304$ ,  $P = 0.0019$ ; Figure 5B). The outlier tumor types with a higher response rate to PD-1/PD-L1 inhibitor monotherapy than would be anticipated from PD-L1 expression data (cutaneous squamous cell carcinoma, melanoma, and mismatch repair-deficient tumors) are all associated with mutagens



**Figure 4. Relationship between TMB and PD-L1 expression at the level of distinct tumor types.** There was no relationship between the PD-L1 expression and median TMB for the major tumor types.

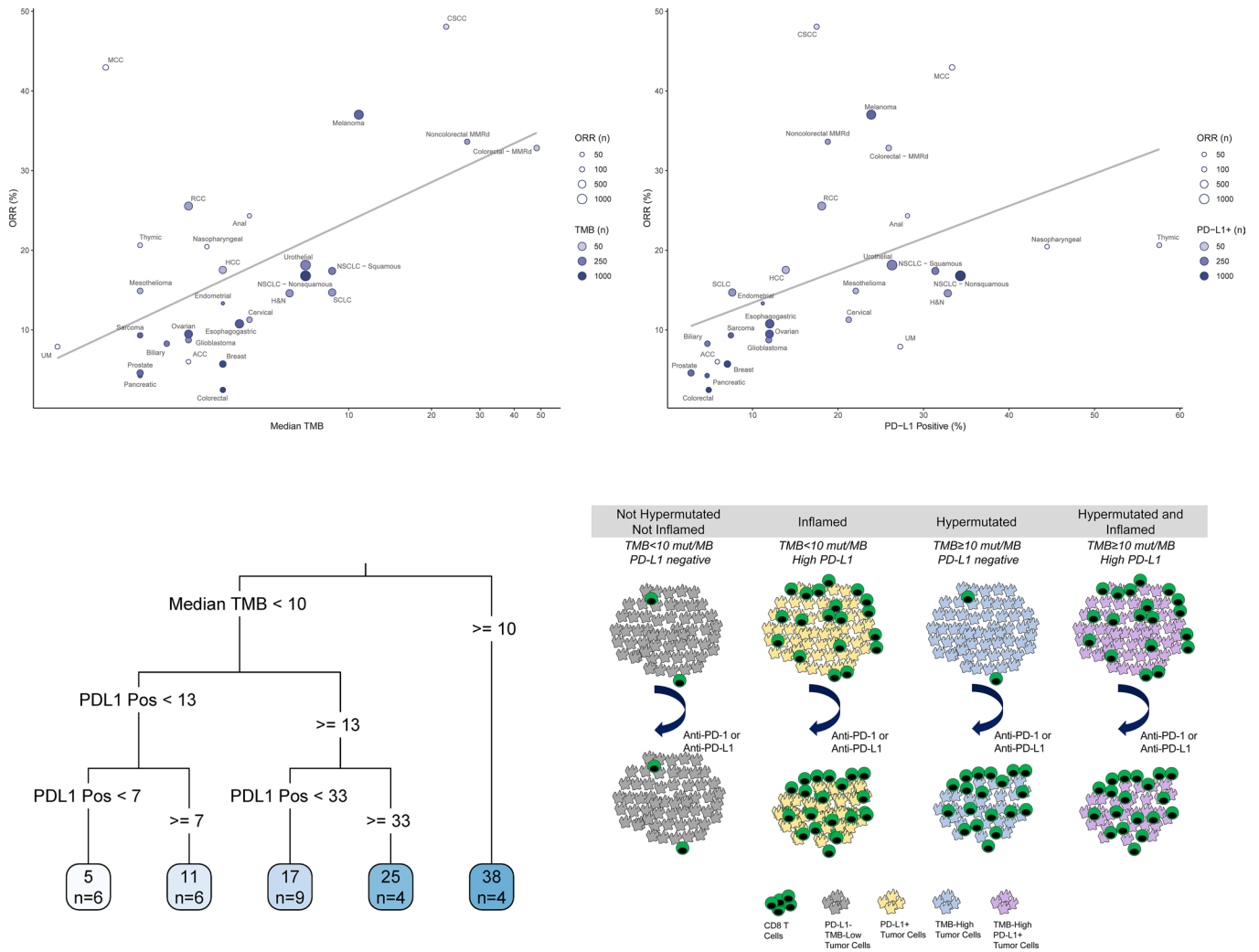
or genomic instability resulting in a high TMB. Merkel cell carcinoma also responded better than would be anticipated from the PD-L1 expression data, which was unsurprising because the nonviral subset of Merkel cell carcinoma is characterized by UV-mediated mutations and exhibits a high TMB (Figure 2B). These findings, together with the observation that PD-L1 positivity and TMB are independent variables at the level of distinct tumor types, led us to suspect that these two features of the tumor microenvironment may have non-overlapping effects on ORR. To test this, both variables and their interaction were used in a multiple linear regression to model ORR. This complete model performed better than either individual model, explaining approximately 53% of the variation in ORR ( $r^2 = 0.5256$ ,  $P = 0.00028$ ).

To explore the differing effects of PD-L1 positivity and TMB on the ORR, we used a regression tree algorithm that recursively identifies the most informative way to split the data. The regression tree (Figure 5C) demonstrates that tumor types with TMB greater than 10 have the best predicted ORR (38%) regardless of PD-L1 expression levels. Response rates for cancers with fewer than 10 mutations/Mb, however, are progressively higher as PD-L1 expression increases. This approach broadly characterizes tumors as hypermutated (TMB  $\geq 10$ ), inflamed (TMB  $< 10$ , PD-L1 positivity  $\geq 13\%$ ), and noninflamed (TMB  $< 10$ , PD-L1 positivity  $< 13$ ) (Figure 5D and Table 1). Using this classification, there are several cancers for which PD-1/PD-L1 inhibitor monotherapy has not yet been broadly studied that are unlikely to benefit from this therapy. These include adenoid cystic, cholangiocarcinoma, neuroendocrine, small bowel, and papillary thyroid cancers.

### Discussion

To our knowledge, this is the largest report of PD-L1 expression by IHC across multiple tumor types, and the first time that the relationship between PD-L1 expression by IHC and TMB has been broadly investigated





**Figure 5. TMB and PD-L1 expression broadly categorize the immunologic subtypes of cancer.** (A) There is a positive relationship between the PD-L1 expression positivity rate and the ORR for anti-PD-1 or anti-PD-L1 therapy for the 29 tumor types or subtypes for which data regarding the ORR are available. For each tumor type, we pooled the response data from the largest published studies that evaluated the ORR (see Methods). (B) There is also a positive relationship between the TMB and the ORR for anti-PD-1 or anti-PD-L1 therapy. (C) An unbiased regression tree algorithm recursively identifies that hypermutated tumor types with TMB ≥ 10 have the best predicted ORR (38%) regardless of PD-L1 positivity. Response rates for cancer types with fewer than 10 mutations/Mb, however, are progressively higher as PD-L1 positivity rates increase. The algorithm identified <7%, 7%–13%, 13%–33%, and >33% of tumors staining positive for PD-L1 as the 4 PD-L1 thresholds that would most informatively split the data. Table 1 details the major tumor types that belong to each of these categories. (D) The unbiased regression tree supports a model of anti-PD-1 therapy in which hypermutated tumor types as well as inflamed tumor types with high PD-L1 expression are likely to respond to anti-PD-1 or anti-PD-L1 therapy, whereas nonhypermutated tumor types with low PD-L1 expression are unlikely to respond. MMRd, mismatch repair-deficient; H&N, head and neck carcinoma; UM, uveal melanoma.

across the spectrum of human cancers. We find that while viral-associated cancers often have robust PD-L1 expression, PD-L1 expression is only marginally correlated with TMB across cancer types. Our results indicate that lack of spontaneous immune resistance through the PD-L1 pathway on tumor cells is unlikely to be due to a low number of mutational antigens resulting from a low TMB in most tumors. Furthermore, the relative independence of PD-L1 expression and TMB within most tumor types suggests that each biomarker can inform the use of ICI therapy in tumors with specific tumor microenvironments. In fact, these biomarkers can define the immunologic state of the tumor microenvironment as hypermutated, inflamed, or noninflamed.

The relative independence of PD-L1 expression and TMB, and the relationship of each biomarker with response to ICI therapy, indicate that these 2 biomarkers can broadly define the immunologic subtypes of cancer and identify opportunities for therapeutic development. Our data support a definition of PD-1/PD-L1 inhibitor-resistant tumor types as those with both low PD-L1 expression (<7% of samples with positive PD-L1 staining) and a median TMB of fewer than 10 mutations/Mb. These tumor types have thus far proven



to be resistant to single-agent ICI therapy, and provide an opportunity to explore novel combinatory strategies that overcome resistance to ICI monotherapy. By contrast, hypermutated tumor types, as well as tumor types with high PD-L1 staining, are likely to benefit from PD-1/PD-L1 inhibitor therapy. These data support the development of ICIs, even as single agents, in other tumor types with either high PD-L1 expression or a hypermutated phenotype.

Viral antigens resulting from viral open reading frames in the tumor DNA, as well as neoantigens resulting from non-synonymous mutations, insertions or deletions, and copy number gains and losses, should all be expected to contribute toward the immune recognition of tumor and the induction of adaptive immune resistance through immune checkpoint pathways (11). The strong association between viral antigens and PD-L1 expression, and the lack thereof for PD-L1 expression and TMB, are noteworthy and may indicate a difference in mechanisms of immune escape in TMB-high tumors as compared with viral-associated cancers. This finding builds on prior analyses of viral and nonviral tumors within multiple tumor types, which have consistently found a more infiltrated and inflamed tumor microenvironment in viral-associated tumor specimens (24–26). PD-1/PD-L1 inhibitor therapies have effects on the immune system both within and outside of the tumor microenvironment, and it is possible that reversal of PD-L1-mediated immunosuppression on the tumor cells themselves is a less relevant mechanism of action for PD-1/PD-L1 inhibitor therapies in high-TMB tumors.

Strengths of this investigation include the use of a single clinically validated assay for reporting PD-L1 expression and TMB across a large and representative number of clinical samples. A limitation is that aggregated clinical data were used for response data, and clinical response data were not from the same patients as the tumor specimens that were assessed. Our conclusions regarding clinical responses to ICIs require further validation at the level of individual patients. In conclusion, PD-L1 expression and TMB may inform the use of ICI therapy and identify the tumor types that are most likely to benefit from ICI therapy.

## Methods

**PD-L1 expression.** PD-L1 expression was obtained as part of routine cancer care from samples from 9887 patients who received tumor PD-L1 staining and genomic profiling through Foundation Medicine. PD-L1 status was determined through IHC performed on FFPE tissue sections, with the use of the commercially available SP142 PD-L1 antibody (Ventana). PD-L1 expression was performed using the Ventana Optiview DAB detection system on the Ventana Benchmark ULTRA platform. A pathologist determined the percentage of tumor cells with expression (0%–100%) and the intensity of expression (0, 1+, 2+). PD-L1 expression was reported as a continuous variable with the percentage of tumor cells staining with  $\geq 1+$  intensity. PD-L1 expression for each sample was also summarized as negative, low-positive, or high-positive PD-L1 expression. Negative expression was defined as  $< 1\%$  of tumor cells staining with  $\geq 1+$  intensity. Low-positive expression was  $\geq 1\%$  and  $< 50\%$  of tumor cells staining with  $\geq 1+$  intensity. High-positive expression was  $\geq 50\%$  of tumor cells staining with  $\geq 1+$  intensity. The pathology laboratory established performance characteristics for this assay per the requirements of the Clinical Laboratory Improvement Amendments (CLIA '88) and in accordance with College of American Pathologists checklist requirements and guidance (27).

**Genomic profiling.** TMB was assessed using a targeted comprehensive genomic profiling assay (FoundationOne) as previously described (28, 29). Briefly, hybridization capture of exonic regions from a panel of up to 405 genes that are commonly rearranged in cancer was applied to DNA extracted from FFPE clinical cancer specimens. All base substitutions, short insertions and deletions, copy number alterations, and gene fusions/rearrangements were initially recorded before filtering. Subsequent filtering for oncogenic driver events and germline mutations was performed using genomic databases, such as Catalogue Of Somatic Mutations In Cancer (COSMIC), The Short Genetic Variations database (dbSNP), and The Exome Aggregation Consortium (ExAC). TMB was defined as the number of unfiltered base substitutions (including synonymous mutations) per megabase of genome examined within the targeted genes. Prior validation studies have demonstrated that this targeted sequencing method is sufficiently accurate for TMB estimation as compared with whole exome sequencing approaches (28). Viral DNA detection was performed through Velvet de novo assembly of sequencing reads left unmapped to the human reference genome (hg19). Assembled contigs were competitively mapped by BLASTn to the NCBI database of 3.6 million viral nucleotide sequences, and positive viral status was determined by contigs  $\geq 100$  nucleotides in length with  $\geq 98\%$  identity to the BLAST sequence.

**Objective response rate across tumor types.** We conducted electronic searches of MEDLINE (January 1, 2012, to December 31, 2018) as well as abstracts presented at the American Society of Clinical Oncology, the European Society for Medical Oncology, and the American Association for Cancer Research (Annual Meetings 2012–2018) to identify the objective response rate (ORR) for anti-PD-1 or anti-PD-L1 therapy across the major and distinct solid tumor types for which PD-L1 and TMB data were available. The search terms used for this search were: nivolumab, BMS-936558, pembrolizumab, MK-3475, atezolizumab, MPD-L3280A, durvalumab, MEDI4736, avelumab, MSB0010718C, BMS-936559, cemiplimab, and REGN2810. In addition, we contacted disease experts at the Johns Hopkins Sidney Kimmel Comprehensive Cancer Center to identify additional studies that may have been missed in our electronic search. We excluded studies that included fewer than 5 participants, studies that investigated anti-PD-1 therapies only in combination with other agents, and studies that used PD-L1 expression cutoffs or other immune-related biomarkers as study entry criteria. For the remaining studies, only the largest published study for each anti-PD-1 therapy was included in the final assessment of pooled ORR for each cancer type or subtype. In tumor types for which large registrational studies were reported for at least 1 anti-PD-1 or anti-PD-L1 therapy, we also excluded other studies enrolling fewer than 40 patients or dose-finding studies with other anti-PD-1 agents. Only tumor types with a total of at least 10 total reported patient responses were included in our analysis.

**Statistics.** Across tumor types, median TMB and percentage PD-L1 positivity were compared with ORR and each other using the coefficient of determination ( $r^2$ ) derived from simple linear regressions. The relationship between both TMB and PD-L1 positivity and ORR was modeled using multiple linear regression, including a single interaction term. A regression tree was created to model ORR as a function of TMB and PD-L1 positivity using the *rpart* package (Therneau) for the R programming language, specifying a maximum depth of 3 nodes including the root node.

**Study approval.** Approval for the Foundation Medicine cohort, including a waiver of informed consent and a Health Insurance Portability and Accountability Act of 1996 waiver of authorization, was obtained from the Western Institutional Review Board (Puyallup, Washington, USA). The investigators were provided with only deidentified patient information in conducting this research.

## Author contributions

MY, ACH, and EMJ conceived and designed the study. MY, LAA, MM, KM, TTV, NZ, and GMF acquired data. MY, LAA, ACH, MM, KM, NZ, and GMF analyzed and interpreted data. MY, LAA, and ACH drafted the manuscript. GMF, NSA, DAL, and EMJ provided study supervision and critical revision. All authors read and approved the manuscript.

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