

Implications of the tumor immune microenvironment for staging and therapeutics

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Characterizing the tumor immune microenvironment enables the identification of new prognostic and predictive biomarkers, the development of novel therapeutic targets and strategies, and the possibility to guide first-line treatment algorithms. Although the driving elements within the tumor microenvironment of individual primary organ sites differ, many of the salient features remain the same. The presence of a robust antitumor milieu characterized by an abundance of CD8+ cytotoxic T-cells, Th1 helper cells, and associated cytokines often indicates a degree of tumor containment by the immune system and can even lead to tumor elimination. Some of these features have been combined into an ‘Immunoscore’, which has been shown to complement the prognostic ability of the current TNM staging for early stage colorectal carcinomas. Features of the immune microenvironment are also potential therapeutic targets, and immune checkpoint inhibitors targeting the PD-1/PD-L1 axis are especially promising. FDA-approved indications for anti-PD-1/PD-L1 are rapidly expanding across numerous tumor types and, in certain cases, are accompanied by companion or complimentary PD-L1 immunohistochemical diagnostics. Pathologists have direct visual access to tumor tissue and in-depth knowledge of the histological variations between and within tumor types and thus are poised to drive forward our understanding of the tumor microenvironment. This review summarizes the key components of the tumor microenvironment, presents an overview of and the challenges with PD-L1 antibodies and assays, and addresses newer candidate biomarkers, such as CD8+ cell density and mutational load. Characteristics of the local immune contexture and current pathology-related practices for specific tumor types are also addressed. In the future, characterization of the host antitumor immune response using multiplexed and multimodality biomarkers may help predict which patients will respond to immune-based therapies.

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The evolution of cancer is influenced by intricate interactions between tumor cells and the host immune response within the tumor microenvironment. Surgical pathologists are uniquely positioned

to evaluate the prognostic and predictive features of a cancer’s immune microenvironment. Several immune populations actively participate in tumor–immune regulation; however, our AJCC/UICC-TNM staging system follows a ‘tumor autonomous’ paradigm, whereby only features intrinsic to the tumor (depth of invasion, number of lymph nodes involved by metastases, etc.) are assessed as prognostic features. Indeed, cellular elements of both the innate and adaptive immune response impact tumor progression.^{1,2} Cytotoxic T cells, B cells, and macrophages can orchestrate tumor cell elimination, while other populations such as regulatory T cells (Tregs)

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and myeloid-derived suppressor cells can dampen the antitumor immune response and promote malignant cell growth and tissue invasion³ (Figure 1). Galon and colleagues demonstrated that the type, density, and location of immune cells within tumor samples is a superior prognostic biomarker in colorectal carcinoma when compared with current TNM staging. A potential clinical translation of the immune contexture into a prognostic marker has been established, designated the 'Immunoscore'.^{4,5} The Immunoscore is currently being investigated in a broad range of tumor types as a supplement to the current TNM staging system.

Immune checkpoints

The initiation of the adaptive immune response requires two signals, the first via antigen presentation between the major histocompatibility complex (MHC) and the T-cell receptor (Signal 1), and the second via CD28 costimulation with B7 (CD80 or CD86) (Signal 2). Once an immune response is initiated, the duration and amplitude of the response is modulated by a number of different checkpoints. One of these is CTLA-4, which has a much stronger affinity for the B7 molecules than CD28. CTLA-4 is upregulated approximately 48 h after T-cell activation and leads to attenuation of the immune response at the priming phase by checking early activation of naive and memory T cells via dominant-negative signaling. Another major checkpoint is the programmed death 1 (PD-1)/programmed death ligand-1 (PD-L1) pathway, which functions in the peripheral tissues in the effector phase of the immune response. Its role is thought to be to turn off the immune response and avoid potential autoimmune-type damage following long-term antigen exposure, such as during chronic viral infections.⁶

The PD-1/PD-L1 checkpoint is of particular interest because tumors can co-opt this pathway to dampen the local host immune response to tumor. PD-L1-mediated adaptive immune resistance was first described in melanoma.⁷ In this mechanism, surveilling T-cells recognize tumor neoantigens as foreign and become activated, upregulating PD-1 and secreting interferon-gamma (IFN- γ) (Figure 2a). In response to IFN- γ , the tumor cells and immune cells in the immediate tumor microenvironment express PD-L1. The PD-L1 then ligates PD-1, turning off the surveilling T-cells. PD-L1-mediated adaptive immune resistance by tumors has now been extended to a large number of tumor types, including non-small-cell lung carcinoma, Merkel cell carcinoma, anal squamous cell carcinoma, breast carcinoma, and hepatocellular carcinoma, among others.^{8–13} Oncogenic alterations have also been implicated in modulating PD-L1 expression, including *PTEN* loss,^{14,15} selective 9p24.1 gene amplification,¹⁶ and activation of the AKT-mTOR¹⁷ or JAK/STAT pathways.^{18,19} Some oncogenic events

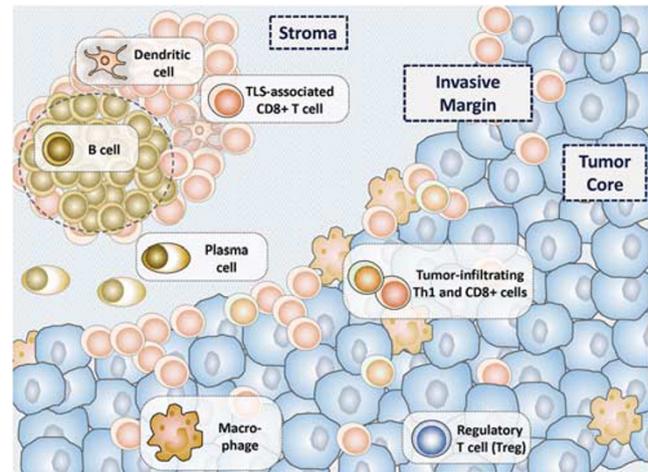


Figure 1 The immune contexture of a tumor dynamically shapes the tumor microenvironment in both a pro-tumorigenic and antitumorigenic manner. The antitumor immune milieu is characterized by an abundance of CD8+ cytotoxic T cells and type 1 helper (Th1) T cells. Tertiary lymphoid structures (TLS), when present, support a local antitumor immune attack. Emerging evidence also suggests that plasma cells have a role in tumor containment and potential elimination by the immune system. The pro-tumorigenic immune milieu is characterized by regulatory T cells (Tregs), type 2 helper (Th2, not shown) T cells, and specific macrophage subsets. Tumor cells may also demonstrate immune-evasion strategies, for example, the display of immune checkpoint molecules. For many tumor types, the interaction of the tumor with the host immune system occurs at the invasive margin of the tumor or surrounding intratumoral blood vessels. The balance between the pro-tumorigenic and antitumorigenic factors affects tumor development and ultimately patient survival.

may even lead to constitutive or 'innate' PD-L1 expression on the surface of tumor cells in the absence of an activated T-cell infiltrate²⁰ (Figure 2b). A combined (constitutive plus adaptive) pattern of PD-L1 expression may also be seen in some tumors.^{12,21}

Immune checkpoint inhibitors

Checkpoint inhibitor immunotherapies work by blocking the immune inhibitors CTLA-4 or PD-1/PD-L1, allowing the natural host antitumor immune response to eliminate a tumor and improve patient survival even in advanced cancers. Thus, unlike chemotherapy, radiotherapy and oncogene-targeted therapies, these agents work indirectly, ie, they are not necessarily directly antitumorigenic but work by 'releasing the brakes' to promote the host antitumor immune response.^{22,23} The kinetics of the responses are related to this distinct 'indirect' mechanism of action. Most responses occur between 2 and 12 weeks but are relatively delayed compared with the rapid changes seen with targeted therapies, eg, rapid reduction in tumor burden with targeted BRAF inhibitor therapy in patients with melanoma. Truly delayed responses have also been reported, with patients demonstrating the first indications of a response almost a year after starting therapy.²⁴

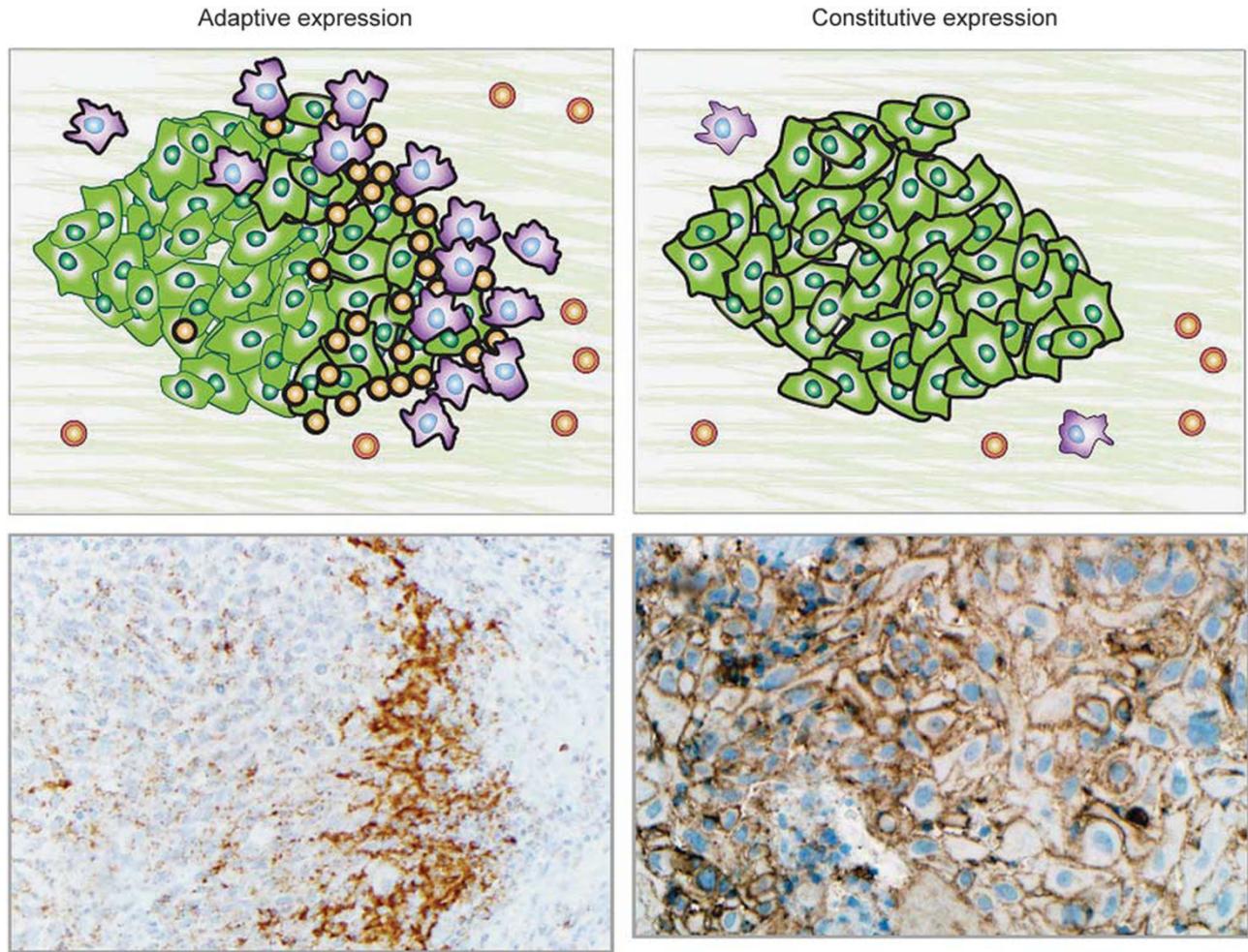


Figure 2 PD-L1 expression in the tumor microenvironment may demonstrate adaptive or constitutive expression patterns. Left panels: In adaptive PD-L1 expression, tumor and/or immune cell PD-L1 expression is geographically associated with tumor-infiltrating lymphocytes. This finding is consistent with cytokine (primarily IFN- γ) mediated adaptive immune resistance. Upper left panel: Schematic of PD-L1-mediated adaptive immune resistance. Tumor cells are shown in green, macrophages are purple, and TIL are orange. PD-L1 expression on the surface of the cells is denoted by a thick black line. Lower left panel: Photomicrograph shows a subcutaneous deposit of metastatic melanoma stained with immunohistochemistry for PD-L1 expression. PD-L1 expression is evident on tumor cells as well as immune cells at the host–tumor interface ($\times 200$, original magnification). Upper right panel: Schematic showing constitutive (non-immune cell associated/diffuse) PD-L1 expression on the surface of tumor cells. Such a pattern of PD-L1 expression is likely driven by tumor intrinsic factors, such as oncogenic pathways or PD-L1 gene amplification. Lower right panel: Photomicrograph of primary head and neck squamous cell carcinoma stained with immunohistochemistry for PD-L1 expression ($\times 250$, original magnification). PD-L1 expression is displayed on nearly every tumor cell and is independent of an immune infiltrate. Combined patterns of adaptive and constitutive expression may also be observed (not shown).

Perhaps most importantly, patients who demonstrate a response to therapy often show durable responses, even after treatment is halted; for example, a subset of patients with metastatic melanoma who responded to anti-CTLA-4 are still alive >10 years later.²⁵ This is in contrast to BRAF inhibitors in melanoma, where the median progression free survival is approximately 5–8 months.^{26,27} Also distinct to this class of agents, patients can be successfully reinduced with the same drug following relapse.²⁸ Another unusual response pattern with immunotherapy includes the radiographic picture of ‘pseudo-progression’, where tumors demonstrate apparent growth by scans before showing a reduction in size. This phenomenon is often attributed to effector immune cell infiltration into the tumor.²⁹

Many of the clinical successes with these agents have been pioneered in patients with melanoma. Responses to these agents have been reported to be ~20% for melanoma patients treated with anti-CTLA-4²⁵ and nearly double that for those treated with anti-PD-1.³⁰ As these two agents target distinct, non-redundant checkpoints, it was hypothesized that dual anti-PD-1 and anti-CTLA-4 blockade would have even greater antitumor activity. Indeed, response rates of ~60% have now been reported in melanoma patients receiving combination therapy, albeit with a large proportion of patients experiencing severe immune-related side effects.^{31,32} United States Federal Drug Administration (FDA) approvals have been secured for all three of these regimens for patients with melanoma (Figure 3). The FDA has also

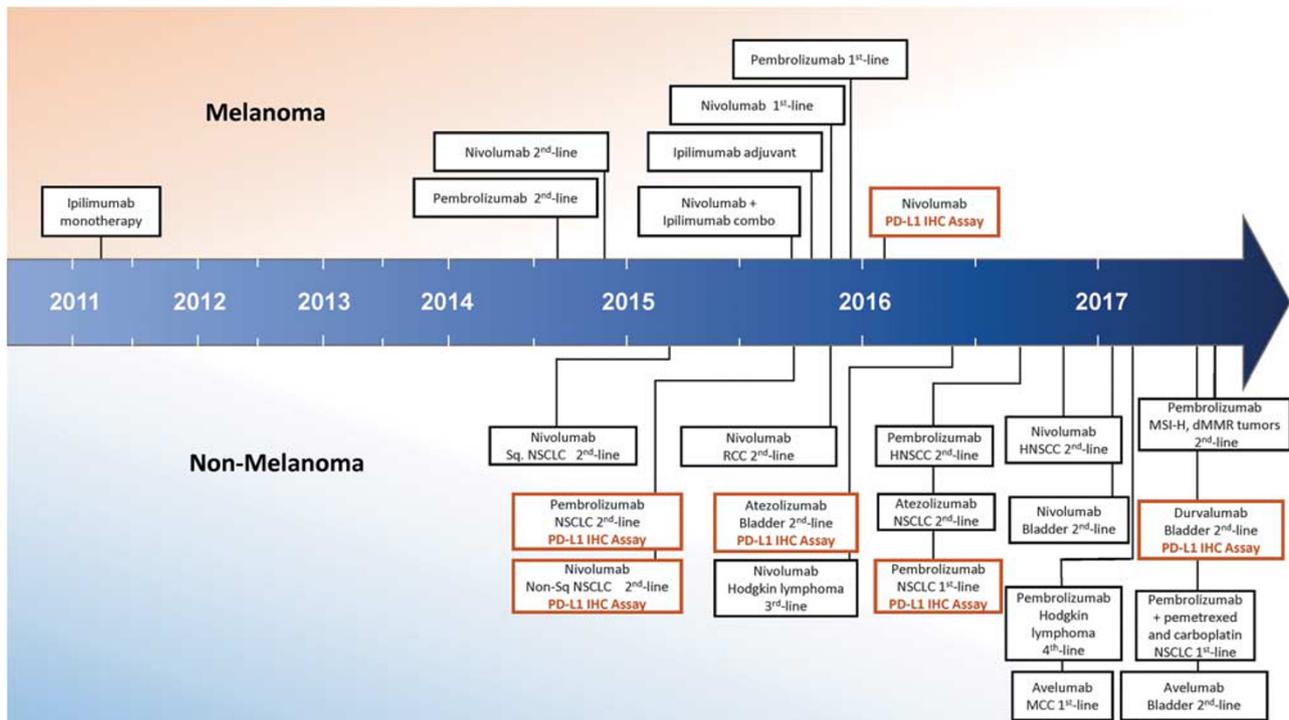


Figure 3 Timeline of FDA approvals for immune checkpoint blocking agents, including PD-L1 immunohistochemistry companion and complementary diagnostics. The earliest approvals were provided for patients with melanoma, including in 2011 for ipilimumab (anti-CTLA-4), in 2014 for nivolumab and pembrolizumab (anti-PD-1 agents) monotherapy, and in 2015 for combined ipilimumab and nivolumab. Anti-PD-1/PD-L1 agents for other tumor types first received approval in 2015, and the number of indications is rapidly expanding. Companion and complimentary PD-L1 immunohistochemistry (IHC) diagnostics were first approved in 2015 for patients with non-small cell lung carcinoma (NSCLC) in the second-line setting. In late 2016, however, pembrolizumab (anti-PD-1) and the companion PD-L1 IHC diagnostic secured approval as a first-line treatment for NSCLC. (Abbreviations: combo, combination; dMMR, mismatch repair deficient; HNSCC, head and neck squamous cell carcinoma; MCC, Merkel cell carcinoma; MSI-H, microsatellite instability-high; RCC, renal cell carcinoma; Sq, squamous).

approved anti-PD-1/PD-L1 agents for the treatment of both squamous and non-squamous non-small-cell lung carcinoma, bladder carcinoma, classical Hodgkin lymphoma, head and neck squamous cell carcinoma, and Merkel cell carcinoma, with anti-PD-1 therapy in non-small-cell lung carcinoma now considered a first-line approach. Most recently, the FDA approved pembrolizumab across multiple tumor types in cases with proven mismatch repair deficiency. Objective responses with anti-PD-1/PD-L1 monotherapy have also been reported in tumor types as diverse as hepatocellular carcinoma, glioblastoma, mesothelioma, gastric cancer, and gynecological tract malignancies. FDA-approved regimens now also include the combination of immunotherapy with a traditional chemotherapeutic regimen (pembrolizumab with pemetrexed and carboplatin) for the treatment of patients with previously untreated metastatic non-small-cell lung carcinoma.

Biomarkers of response to immune checkpoint inhibitors

Biomarkers predictive of response or resistance to these agents are highly sought after. The most well-studied biomarker is PD-L1 protein expression in the

tumor microenvironment. Immunohistochemical assays for PD-L1 are used as predictors of response to anti-PD-1/PD-L1 therapy and are currently the only FDA-approved companion or complimentary diagnostic for this class of agents. PD-L1 immunohistochemistry assays are only approved for specific clinical settings (Figure 3), and there are well-recognized limitations to PD-L1 as a predictive biomarker. Although PD-L1 expression clearly enriches for response to anti-PD-1/PD-L1 therapy, patients lacking PD-L1 expression may still derive clinical benefit.³³ Other potential biomarkers predictive of response include CD8 cell density and mutational burden.^{34–37} Tumor tissue-based markers of both innate and acquired resistance to these agents have also recently been reported,^{38–40} potentially expanding the assayable factors that may be combined in a multiplexed, multimodality assay with improved predictive value.

The purpose of this review is to introduce the concepts behind the role of the tumor immune microenvironment with a focus on the adaptive immune response and the direct clinical importance of these concepts for surgical pathologists (for reviews on the role of innate immune cells in the tumor microenvironment, see Gajewski *et al*¹ and Vesely *et al*²). The concepts emphasized in the review include: the fundamental characterization of the tumor

immune microenvironment, the use of immune based therapies in different tumor types, and the development of prognostic and predictive immune-based biomarkers. The surgical pathologist will undoubtedly have a critical role in furthering our understanding of the tumor immune microenvironment and in translating research findings into clinical care. Owing to the fact that most of these candidate biomarkers are assessed on tumor tissue, pathologists will be involved in continued biomarker investigations and the associated clinical assay development, as well as ultimately in their clinical use.

The Immunoscore

The Immunoscore represents an example of how elements of the immune microenvironment can be quantified in a standardized way and incorporated into a prognostic index that provides information more robust than the standard AJCC/UICC-TNM staging system. The standardized Immunoscore was based on the quantification (cells/mm²) of two lymphocyte populations (CD3 and CD8) within the central region and the invasive margin of colorectal carcinoma tumors and provides a scoring system ranging from Immunoscore 0 (I0) to Immunoscore 4 (I4) (Figure 4).⁴¹ Classification by the Immunoscore has been shown to have a prognostic significance superior to that of the classical TNM system for disease-free survival, disease-specific survival, and overall survival in patients with colorectal carcinoma.⁴ Several follow-up studies confirmed the strong prognostic value in patients with colorectal carcinoma at all stages of disease.^{42–44} Additional studies also showed that the Immunoscore is a stronger predictor of patient survival than microsatellite instability⁴⁵ and that the Immunoscore can also be of prognostic value when applied to brain metastases.⁴⁶

Based on these encouraging results, a worldwide consortium composed of International Expert Pathologists and Immunologists was formed in 2012.^{47,48} The main objectives were to assess the feasibility, reproducibility, significance, and robustness of Immunoscore. The consortium was initially composed of 23 centers from 17 countries and received support from the Society for Immunotherapy of Cancer. The statistical analysis plan was predefined, and all statistical analyses performed by external statisticians. The primary study end point was time to recurrence for patients classified by Immunoscore (High vs Low), and the end point was reached in the three cohorts of patients tested. Analyses were performed by Cox models stratified by enrolling center. The final results supported the significant prognostic value of the Immunoscore in 3855 patients with AJCC/UICC-TNM stage I/II/III colon cancers (presented in a plenary session at the ASCO annual meeting 2016).⁴⁴ The Immunoscore is currently being investigated in a broad range of tumor types as a supplement to the current TNM staging system.

Diagnostic assays

Companion and complimentary diagnostic tests aim to separate potential responders from non-responders for a known therapeutic, that is, the test should be able to predict which patients will respond to a given drug. In the best case scenario, such a diagnostic test has high sensitivity and high specificity for drug target that leads to high sensitivity and specificity for patient selection. For PD-1/PD-L1 axis therapies, there are at least five commonly used tests, four of which are FDA approved (Table 1). It is increasingly relevant for surgical pathologists to have an understanding of the available diagnostic tests, as well as the strengths and limitations of each, as we will have a role in ordering, performing, and interpreting these tests in the future.

As of the time of this publication, only one assay is a true companion diagnostic test (ie, required for the prescription of drug, in this case pembrolizumab), two are complementary diagnostic tests (ie, may be useful to inform prescribing practices but not required), one is a Class 1 diagnostic test, and one is a laboratory-developed test. Each assay has its own scoring system, contributing to the lack of harmonization across assays. Further confusion is caused by the fact that different thresholds for scoring a tumor as 'positive' are used, even within a single assay. For example, PD-L1 expression in >50% tumor cells is required to prescribe the anti-PD-1 agent pembrolizumab in the first line in lung cancer, but only >1% tumor cell staining is required for use in the second line.

Several studies have been conducted to assess the concordance of the available PD-L1 assay systems,^{49–52} while other studies have focused on only the anti-PD-L1 antibody clone itself, while essentially holding the remainder of the assay system constant. For the purpose of discussions here, when the term 'assay' is used, eg, SP142 assay, it refers to the entire marketed assay system, while, when the term 'antibody' is used, it only refers to the anti-PD-L1 clone used and not the remainder of the assay system (antigen retrieval, pH of buffer, type of amplification, etc). The Blueprint study⁴⁹ is perhaps the best-known example of an assay comparison study, as it represented the collaboration of the four leading companies with PD-1/PD-L1 axis therapies and the two companies that produced the FDA-cleared diagnostic assays. This study showed that the SP142 assay detected less PD-L1 expression on tumor cells compared with the other assays. A second study that was sponsored by NCCN/BMS was multi-institutional and statistically powered and included 13 pathologists from 7 institutions.⁵¹ This study revealed that the assay using SP142 was an outlier not only for tumor cell but also immune cell assessment, in both cases selecting only about half of the cases as PD-L1-positive compared with the other three assays. Pathologists were highly concordant in scoring the tumor cell PD-L1 labeling, but

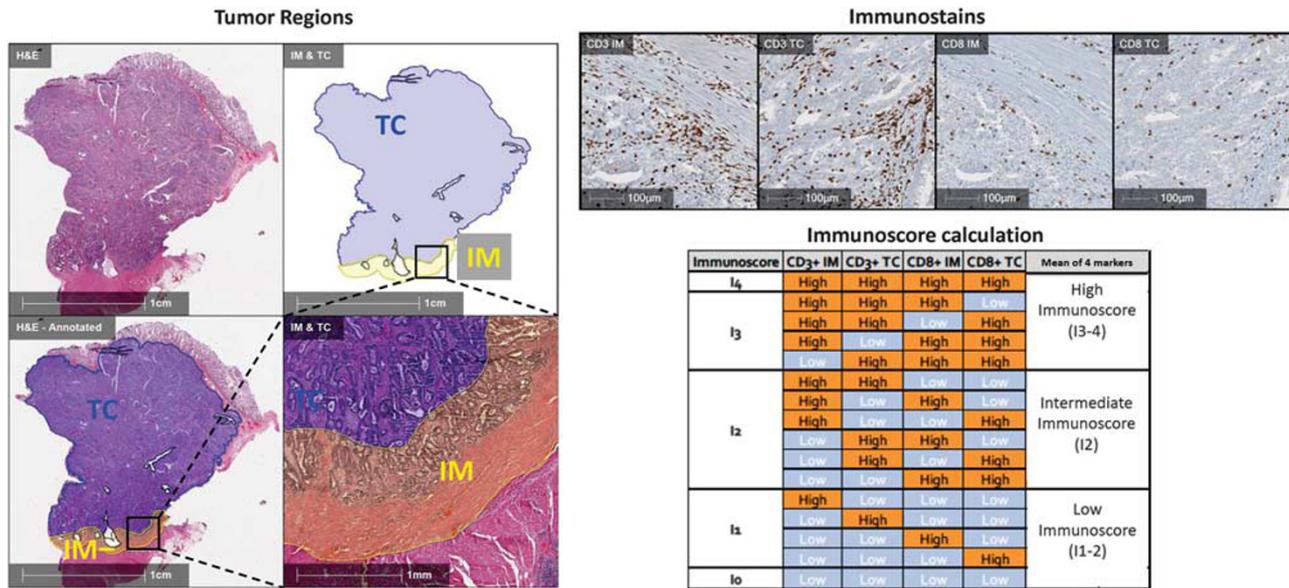


Figure 4 The Immunoscore is a standardized approach to characterizing T-cell infiltration of surgical pathology tumor specimen. Left panels: The border between the advancing tumor edge and normal tissue is annotated on a colorectal carcinoma specimen. A 500- μ m distance on either side of this border is designated the ‘invasive margin’ (IM, yellow region). The remainder of the tumor is designated as the ‘tumor core’ (TC, blue region). Upper right panels: Immunohistochemistry for CD3 and CD8 is used to quantify cell densities for each of these immune cell subsets in both the invasive margin and tumor core. Lower right panels: The density of each region is labeled ‘high’ or ‘low’ density for each marker. The mean percentile of the four immune parameters is calculated, resulting in a possible Immunoscore ranging from I0 to I4, or three categories (Low, Intermediate, High). (Abbreviations: IM, invasive margin; TC, tumor core).

Table 1 Comparison of PD-L1 immunohistochemical assays

Assay	22C3	28-8	SP142	SP263	E1L3N
Platform	Dako Link 48	Dako Link 48	Ventana Benchmark	Ventana Benchmark	LDT—Leica Bond
Drug	Pembrolizumab	Nivolumab	Atezolizumab	Durvalumab	None/any
Vendor	Merck	BMS	Genentech	Astra Zeneca	None/any
Tissue scored	Tumor cells	Tumor cells	Tumor cell and/or immune cells	Tumor cell and/or immune cells	Tumor cell and/or immune cells
Scoring system	> 50% > 1% 0%	> 10% > 5% > 1% 0%	Scoring system integrating TC and IC scores, eg, TC3 = > 50%; IC3 = > 10%; TC0 and IC0 = 0%, etc.	> 25% 1-25% 0%	No assay-specific scoring system
FDA status	Companion Dx in NSCLC	Complementary Dx in NSCLC	Complementary Dx in NSCLC and Bladder	Class 1 Dx	None (LDT)
Ratcliffe, <i>et al</i> —most and least concordant ^a (concordance)	Most: 28-8 (93.7%) Least: SP263 (91.1%)	Most: 22c3 (93.7%) Least: SP263 (91.7%)	Not tested	Most: 22c3 (91.7%) Least: 28-8 (91.1%)	Not tested
Blueprint Study—most and least concordant ^b (concordance)	Most: 28-8 (94.7%) Least: SP142 (86.8%)	Most: 22c3 (94.7%) Least: SP142 (81.6%)	Most: SP263 (65.8%) Least: 22c3 or 28.8 (63.2%)	Most: 22c3 or 28.8 (89.5%) Least: SP142 (86.8%)	Not tested
NCCN/BMS—% cases at > 50% and > 1% cut points ^c	> 50% = 19% > 1% = 64%	> 50% = 23% > 1% = 79%	> 50% = 6% > 1% = 32%	Not tested	> 50% = 24% > 1% = 72%

Abbreviations: Dx, diagnostic; LDT, laboratory-developed test.

^aOverall percentage agreement at > 1% cut point, taken from Table 1 of Ratcliffe *et al*.⁵⁰

^bConcordance between assays at positive–negative cut point for each test, taken from Table 2 in Hirsch *et al*.⁴⁹

^cPercentage of positive cases for tumor cells at two cut points, taken from Figure 2 of Rimm *et al*.⁵¹

they were unable to achieve concordance in scoring the immune cell PD-L1 labeling. The NCCN/BMS study did not include the SP263 assay but rather substituted a laboratory-developed test.

This laboratory-developed test performed statistically equivalent to the Dako 28-8 assay, indicating that a laboratory-developed test has the potential to match the characteristics of the FDA-approved test.

Also germane to laboratory-developed test use, two different studies in non-small-cell lung carcinoma and melanoma showed that many of the anti-PD-L1 antibodies themselves (including the SP142 antibody) have the potential to demonstrate similar analytic performance and that it is the assay conditions that drive the resultant differences.^{53,54}

To date, none of the studies comparing PD-L1 antibodies and assays have been performed on tissue from patients treated with PD-1 axis therapies. As a result, these studies have not been able to assess predictive value of the assays across therapies. Questions thus remain regarding which tests are required, which should be used, and whether they are all the same or if each has its own biological meaning. These questions are likely to be resolved in the near future. However, other issues inherent to stratifying patients as PD-L1-positive vs -negative by immunohistochemistry will remain. Perhaps the biggest issue is that, in nearly every cohort tested, there are patients with clinical benefit in the subgroup with no PD-L1 expression.³³ Although no test is perfectly sensitive, the sensitivity of the current immunohistochemical PD-L1 assays is lower than optimal. Recent efforts to increase sensitivity by including patients with lower cut points have failed,⁵⁵ whereas studies with higher specificity (potentially at the expense of sensitivity) resulted in superior performance of pembrolizumab when compared with chemotherapy in the first line in lung cancer.⁵⁶

There are a number of other issues that affect the results of PD-L1 assays that lack published scientific support. One key issue is the number of cells required for an adequate assay. The FDA label for the PD-L1 immunohistochemistry assay using antibody clone 22C3 requires that the specimen contain at least 100 tumor cells; however, there is no published scientific data supporting this cut point. A number of recent abstracts have suggested that cytology specimens may be adequate for PD-L1 assessment, but to date, none are published or associated with drug response data. Specimen cell number and specimen type are both dependent on inherent heterogeneity of the expression of PD-L1. PD-L1 expression within a tumor can be heterogeneous,⁵⁷ where different regions of the same section show different levels of expression. This has led to concerns that small biopsies may miss the region of the tumor with high PD-L1 expression. Rehman *et al*⁵⁸ further evaluated PD-L1 heterogeneity and noted that PD-L1 variation was greater within a single tumor slide than between tumor blocks, suggesting that core biopsies may be sufficient to sample heterogeneity across a tumor. However, while this assessment begins to define the heterogeneity of PD-L1 expression, this work does not define the minimum number of fields of view of PD-L1 required for a representative biopsy, as this data set was not supported by treatment outcome information.

Alternative predictive tests have focused on genomic tests, RNA signatures, or multiplexed fluorescence-based assessment of the tumor microenvironment. Whole-exome sequencing revealed that patients with high mutational load showed an increased likelihood of response to immune checkpoint inhibitors,³⁶ based on the concept that more mutations resulted in more neoantigens, which increased the likelihood of checkpoint-mediated immune inhibition. However, this predictive method has been limited by the fact that some patients with low mutational load also respond to PD-1 axis therapies. *PD-L1/PD-L2* amplification at chromosome 9p24.1 is a genetic basis for PD-L1 overexpression in a subset of lymphomas and solid tumors. This amplification has been shown to be predictive of improved clinical response among patients with classical Hodgkin lymphoma treated with PD-1 blockade and can identify novel tumor types with exquisite sensitivity to PD-1 blockade.^{59,60} However, the prognostic and predictive power has yet to be determined for additional tumor types that can harbor this genetic lesion.^{61,62} RNA signatures have also been designed to assess the immunological profile of the patients' immune response to the tumor. Early studies on the Nanostring platform suggest that an 18 gene panel can enrich for response to pembrolizumab with comparable sensitivity and specificity as PD-L1 immunohistochemical testing.⁶³ Finally, a number of groups have attempted to quantitatively assess the immune cell microenvironment using multiplex immunofluorescence.^{64,65} The studies have focused on T-cells combined with other markers (Figure 5), but it is too early to assess sensitivity and specificity of this approach.

Melanoma and other cutaneous malignancies

Studies on patients with advanced melanoma largely pioneered the use of checkpoint agents and associated biomarker discoveries. Ipilimumab (anti-CTLA-4) was the first checkpoint agent approved by the FDA for the treatment of advanced melanoma, based on an objective response rate of 11% as well as prolonged survival in 22%.^{25,66} Nivolumab and pembrolizumab (anti-PD-1) were the next agents to garner FDA approval, with patients with advanced melanoma showing increased objective response rates on the order of 30-40%.^{30,67,68} Most recently, dual anti-CTLA-4/PD-1 blockade has received FDA approval based on response rates as high as 55%, albeit with a notable side effect profile.⁶⁹ Each of these regimens is now considered standard of care for patients with advanced melanoma.

As with many other tumor types, PD-L1 expression is recognized as a biomarker for response to PD-1/PD-L1 checkpoint blockade. In fact, the adaptive resistance pattern of PD-L1 expression was first

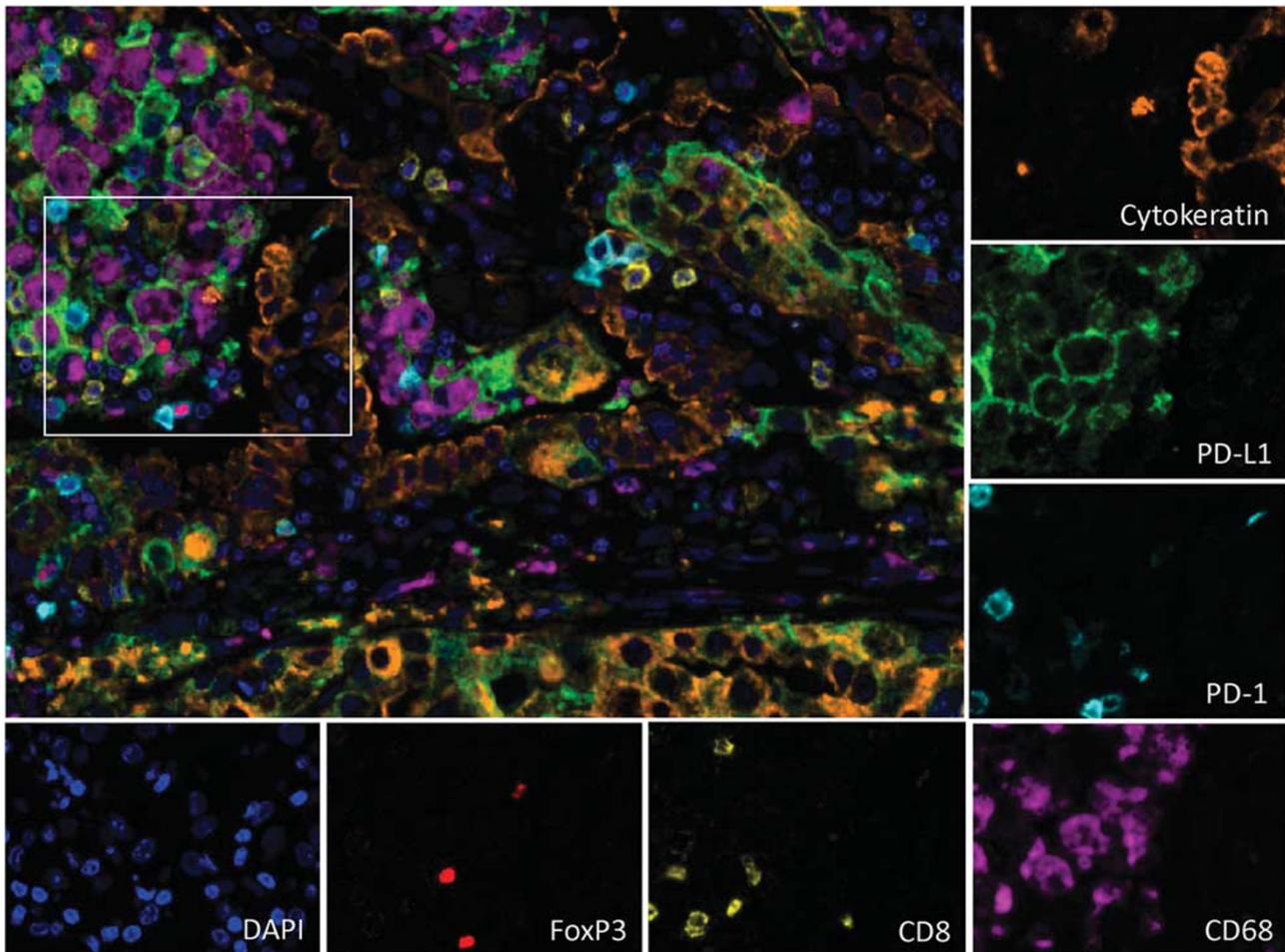


Figure 5 Multiplex immunofluorescence staining of a pulmonary non-small cell carcinoma (adenocarcinoma) in a formalin-fixed, paraffin-embedded section. Tumor cells (orange) and CD68+ macrophages (purple) both express PD-L1 (green). Rare CD8+ T-cells (yellow) are also present, some of which express PD-1 (cyan). Singular FoxP3+ regulatory T-cells (red) are also identified. The nuclei for each cell is stained with DAPI (blue) ($\times 200$, original magnification). The tumor in this example is expressing PD-L1 in a constitutive pattern, that is, independent of the degree of immune infiltrate.

described in melanoma.⁷ It is by far the dominant mechanism of PD-L1 display in this tumor type, with constitutive expression reported in only $\sim 1\%$ of cases. The adaptive pattern of PD-L1 expression also predominates in melanoma subtypes, such as ocular, desmoplastic, acral, and mucosal melanomas.⁷⁰ Adaptive PD-L1 expression is a positive prognostic feature when expressed by either tumor cells or tumor-associated immune cells.⁷ Both melanocyte and immune cell PD-L1 display is primarily IFN- γ -driven, though other cytokines such as interleukin-10 may preferentially contribute to macrophage as opposed to tumor cell PD-L1 expression.⁷¹ Importantly, PD-L1 expression is independent of the BRAF^{V600E} mutation, indicating that these two features should be considered separate biomarkers for therapeutic selection in patients with advanced disease.⁷²

PD-L1 expression is closely related to PD-L2 expression, the second ligand for PD-1. In fact, when whole transcriptome associations are performed, PD-L2 is the gene most closely associated with PD-L1

expression in melanoma.⁷³ Not surprisingly, PD-L2 expression is also a positive prognostic feature in these patients,^{73,74} likely because, similar to PD-L1, its expression in this tumor type tends to equate with an ongoing host response against tumor. The impact of PD-L2 expression as a predictive biomarker for anti-PD-1 blockade is not well understood at this time but is currently being explored by a number of investigative groups. Additional features of the PD-1/PD-L1 interaction in pretreatment specimens, such as CD8 density,³⁴ levels of PD-L1 expression,⁷⁵ and PD-1–PD-L1 interaction,⁷⁶ may have added value beyond the determination of whether PD-L1 is simply present or absent.

Other potential tissue-based biomarkers of response and resistance to checkpoint blockade in patients with melanoma include specific gene expression profiles and genomic features. Transcriptional signatures have been identified involving genes associated with wound healing and remodeling of the extracellular matrix, mesenchymal transition, and angiogenesis.³⁸ When expressed

simultaneously, these gene signatures have been proposed to confer an innate resistance to PD-1 therapy. Specific genetic resistance mechanisms to anti-PD-1 have also been identified in patients with melanoma, including *JAK1/2* mutations, influencing the response to IFN- γ , and B2 microglobulin loss, leading to a lack of MHC class I expression.^{39,40} Most recently, an increase in copy number alteration has been associated with decreased immune infiltration and a more resistant phenotype to sequential anti-CTLA-4, anti-PD-1 blockade.⁷⁷

Patients with other advanced cutaneous malignancies, including Merkel cell carcinoma, squamous cell carcinoma, and basal cell carcinoma, have also been treated with checkpoint inhibitors. Patients with Merkel cell carcinoma have shown response rates of 56 and 32% to anti-PD-1 and anti-PD-L1, respectively, the latter of which is now FDA approved.^{64,78} When biomarkers of response to anti-PD-1 were studied in patients with Merkel cell carcinoma, neither PD-L1 expression, mutational load, or viral status were predictive⁶⁴; however, preliminary results suggest that the density of PD-1+ cells within 15 μm of a PD-L1+ cell may be associated with response to therapy.⁷⁹ Early reports suggest that patients with aggressive squamous cell and basal cell carcinomas may also respond to immune checkpoint blockade therapy.^{80–82}

Non-small-cell lung carcinoma

Lung cancer represents one of the greatest sources of cancer-related morbidity and mortality worldwide.⁸³ Incremental gains in lung cancer survival have been driven by the use of combined platinum and pemetrexed chemotherapies,⁸⁴ as well as identification and directed therapy of oncogenic alterations in *EGFR*, *ALK*, and *ROS1*^{85,86} using tyrosine kinase inhibitors. However, the benefits to survival attributable to the evolution of chemotherapy strategies are small, and targeted therapies are applicable to only a minor subset of patients with lung cancer. For instance, fewer than one-third of lung adenocarcinoma patients in Western countries will qualify for *EGFR* or *ALK/ROS1* inhibitors. Therefore, clinical trials showing that immunotherapies confer a survival benefit over chemotherapy for patients with lung cancer have been greeted with great enthusiasm. As of May 2017, three immune checkpoint blockade inhibitors have been approved by the FDA for use in non-small-cell lung carcinoma, the anti-PD-1 antibodies pembrolizumab (first- and second-line setting) and nivolumab (second line) and the anti-PD-L1 antibody atezolizumab (second line).

Response rates vary based on drug, line of therapy, and histological subtype (squamous vs non-squamous non-small-cell lung cancer). Immunohistochemical staining for PD-L1 on tumor and immune cells has emerged as the dominant biomarker for selection of lung cancer patients for anti-PD-1 and

PD-L1 antibody therapy, with the previously discussed limitations. As noted above, only the PD-1 inhibitor pembrolizumab requires *a priori* knowledge of the PD-L1 protein expression status in clinical practice based on clinical trials (KEYNOTE) showing superior outcomes relative to chemotherapy. However, different PD-L1 expression cutoffs are used for pembrolizumab eligibility in the first- and second-line setting. Correlative biomarker analyses in the phase 1, KEYNOTE-001 trial (which included both untreated and previously treated patients) demonstrated that patients with PD-L1 labeling in at least 50% of tumor cells had a higher likelihood of response to pembrolizumab and longer progression-free and overall survival than those whose tumors had lower levels of PD-L1 expression.⁸⁷ The authors recognized, however, that some patients with lower levels of tumor PD-L1 expression also benefitted from immune checkpoint blockade. Indeed, subsequent phase 2 and 3 trials found that PD-L1 labeling in 1% of tumor cells also predicted improved survival with second-line pembrolizumab compared with standard-of-care chemotherapy in previously treated patients.⁸⁸ In the first-line setting, a phase 3 trial of pembrolizumab monotherapy showed striking improvements in progression-free and overall survival relative to platinum-based chemotherapy but was restricted to patients with at least 50% tumor cell staining with PD-L1.⁵⁶

Another PD-1 inhibitor, nivolumab, has also been approved for use in the second line; however, first-line trials (CHECKMATE-026) failed to show a benefit over chemotherapy when patients were stratified based on PD-L1 status.^{55,89} In addition to potential differences in the anti-PD-1 agents' activity, some other possible explanations for the disparate trial results include the different biomarker selection criteria, differing time intervals between specimen acquisition and treatment initiation, and statistical power of the studies to examine the correlation between different PD-L1 thresholds and response, as well as a history of possible prior radiation therapy.^{55,90} It is also important to note that patients with *EGFR* mutations and *ALK* rearrangements do not appear to benefit from immune checkpoint blockade and were excluded from trials in the first-line setting. As a result, patients with a new diagnosis of advanced-stage lung adenocarcinoma should undergo testing for *EGFR* mutations, *ALK* and *ROS1* rearrangements, and PD-L1 protein expression to determine eligibility for tyrosine kinase inhibitors and pembrolizumab therapy, respectively.

In addition to PD-L1 expression, the factors that may predict response to checkpoint blockade inhibitors in lung cancers include underlying oncogenic pathway activation, tumor mutation and/or neoantigen burden, and the tumor immune microenvironment. There is little doubt that a combination of these factors contributes to PD-1/PD-L1 axis engagement and influences the nature of the tumor cell-immune cell interaction. As with hypermutated,

microsatellite-high tumors of the colon, endometrium, and other sites, highly mutated lung cancers with smoking-related, transversion-high signatures appear significantly more likely to overexpress PD-L1 and to respond to PD-1 inhibition.^{36,91} Mechanistically, smoking-related transversion mutations in non-small-cell lung carcinoma contribute to neoantigen formation with resulting T-cell recruitment. IFN- γ release from tumor-infiltrating T cells may trigger adaptive upregulation of tumor PD-L1 and engagement with PD-1 expressing T cells, preventing immune attack. PD-1 inhibitors may be effective in unleashing the immune system in this scenario; however, a number of other tumor-specific factors are likely to influence the efficacy of this therapeutic strategy. Of note, reanalysis of the CHECKMATE-026 trial found that high tumor mutation burden, defined as >243 mutations by whole-exome sequencing, correlated with improved progression free survival and objective response rate in non-small-cell lung carcinoma patients receiving first-line nivolumab as compared with chemotherapy. Although tumor mutation burden or the combination of tumor mutation burden and high PD-L1 expression appeared to outperform PD-L1 alone as predictive biomarkers, the study was not powered for formal statistical analysis of these variables outperforming PD-L1 as a predictive biomarker in this setting.⁸⁹

Studies correlating oncogenic-driver mutations with the quality and quantity of immune infiltrates as well as the efficacy of PD-1/PD-L1 blockade have not been definitive. Some studies have failed to identify a correlation between tumor genotype and immunoprofile,⁹² while others have suggested that *KRAS/TP53* mutation status predicts for tumor adaptive immune resistance that may be amenable to PD-1 pathway blockade.⁹¹ *STK11* loss-of-function mutations, which occur in 10–15% of lung adenocarcinomas, tend to co-occur with *KRAS* mutations, appear to promote recruitment of neutrophils to the tumor and exclude T-cell infiltrates,^{93–95} and thus may confer relative resistance to immune checkpoint blockade. Therefore, it is likely that tumor genetics have an important role in defining the non-small-cell lung carcinoma tumor immune response.

Renal cell carcinoma

Immune infiltrates in renal cell carcinoma are prominent, and two main tumor immune microenvironment milieus have been described. In the majority of cases, the immune infiltrate is composed of poorly functional dendritic cells and anergic T cells.^{96–98} Renal cell carcinomas also rarely display peritumoral immune aggregates or tertiary lymphoid structures and therefore lack privileged sites for T-cell priming.^{99,100} In fact, renal cell carcinoma is one of the few cancer types where increased densities of CD8+, CD4+, and PD-1+ tumor-infiltrating T cells correlate with poor clinical

outcomes.^{101–103} The second type of microenvironment is more consistent with what is typically seen in other tumor types, namely, the CD8+ infiltrate is activated and represents an ongoing antitumor response that is associated with an improved prognosis. This phenotype has been characterized by using CD8 and Ki-67 double immunohistochemistry staining or the presence of an IFN- γ /Th1-type gene signature.¹⁰⁴ PD-L1 and PD-L2 expression has also been reported in renal cell carcinoma.^{73,103,105,106} However, the relationship between the two tumor immune microenvironments and the presence and pattern (adaptive vs constitutive) of PD-L1 and PD-L2 expression has yet to be thoroughly explored.

Renal cell carcinoma has long been recognized as an immunogenic tumor and indeed was one of the first cancer types that displayed objective responses after treatment with recombinant interleukin-2.^{107,108} Metastatic renal cell carcinoma is one of the tumor types that showed responses in early clinical trials for therapies blocking the PD-1/PD-L1 axis.^{109,110} Three clinical trials evaluating the efficacy of anti-PD-1 (nivolumab or atezolizumab) or anti-PD-L1 (MDX 1105) agents focused on patients with renal cell carcinoma have reported objective response rates ranging from 15 to 25%.^{111–113} PD-L1 expression by both tumor cells and immune cells has been investigated as a biomarker of response and, in general, trends toward an association. However, it has not shown as clear cut of a significance as a biomarker of response or progression-free survival, perhaps because PD-L1 expression may not be predominantly IFN- γ driven in this tumor type.⁷³

Urothelial carcinoma

The immune contexture in muscle-invasive urothelial neoplasms correlates with postcystectomy survival. Increased CD8+ infiltrates confer a favorable prognosis,¹¹⁴ whereas increased Treg infiltrates are associated with a worse prognosis.¹¹⁵ Tumor cell PD-L1 expression is observed in ~20% of muscle invasive urothelial carcinomas.^{114,116} Increased tumor cell PD-L1 expression is generally associated with higher-stage disease, but an association with postcystectomy survival has not been consistently observed across multiple cohorts.^{114,117–122} Many of the observed differences between these studies may be attributable to the use of different PD-L1 immunohistochemistry protocols, antibodies, scoring criteria, and sample sizes—again highlighting the perils of trying to compare across studies when there is a lack of assay harmonization and a unified scoring approach.

Urothelial carcinoma has a long-standing history of immune-based therapeutic approaches. In particular, the treatment of non-muscle invasive urothelial carcinoma with intravesical *Bacillus-Calmette-Guerin* (BCG) has proven to be an effective strategy with significant reduction in progression and

cancer-related death.¹²³ More recently, successful PD-1/PD-L1 blockade therapy in urothelial carcinoma has led to FDA approvals for certain clinical indications.^{124,125} More specifically, the early studies were performed in patients with muscle invasive carcinoma; however, trials are currently underway for non-muscle-invasive disease (NCT02451423, NCT02625961). When tumor cell and immune cell PD-L1 expression were evaluated as potential biomarkers of response to therapy in this tumor type, investigators found that immune cell PD-L1 expression is more predictive of response to PD-1/PD-L1 signaling inhibition than tumor cell PD-L1 expression.¹²⁵ This may be because there is a component of constitutive PD-L1 expression by tumor cells, as has been reported in tumors arising from squamous-type epithelium^{12,126} (Figure 2b). Notably, the immune contexture is also predictive of response to non-immune-based therapies such as conventional platinum-based neoadjuvant chemotherapy, whereby an increasing ratio of Treg to CD8 density is predictive of residual muscle-invasive tumor posttreatment.¹¹⁶

Hodgkin lymphoma and other hematologic malignancies

Hematological malignancies, much like certain solid tumors, have shown some early and encouraging results with PD-1 blockade. Classical Hodgkin lymphoma has been the most well studied thus far and has seen a relatively rapid progression from biomarker identification to clinical application. Specific chromosomal gains were identified in classical Hodgkin lymphoma, including 9p, by comparative genomic hybridization analyses of DNA from laser-capture microdissected Hodgkin Reed–Sternberg cells.¹²⁷ The same finding was uncovered using comparative genomic hybridization to test primary mediastinal large B-cell lymphoma, a neoplasm clinically and histomorphologically similar to classical Hodgkin Lymphoma.¹²⁸

More recently, integrated copy number data from high-density single-nucleotide polymorphism arrays were paired with transcriptional profiles and identified highly significant amplification specifically of 9p24.1 in classical Hodgkin lymphoma cell lines with resultant overexpression of the *PD-L1* and *PD-L2* genes.¹⁶ Increased *PD-L1* gene expression was additionally identified in 38% of microdissected Hodgkin Reed–Sternberg cells from primary nodular sclerosis subtype classical Hodgkin lymphomas, and this finding correlated tightly with increased PD-L1 protein expression by immunohistochemistry. In primary mediastinal large B-cell lymphoma, amplification of 9p24.1 also has been associated with increased PD-L2 expression by immunohistochemistry.¹²⁹ Interestingly, certain extranodal large B-cell lymphomas, including primary central nervous system lymphomas and

primary testicular lymphomas, also harbor 9p24.1/*PD-L1*/*PD-L2* copy number alterations.¹³⁰

To further investigate the PD-L1 protein expression in primary tumors, large-scale immunohistochemistry analyses of a variety of hematological neoplasms, including subtypes of classical Hodgkin lymphoma and related entities were pursued.¹³¹ Strong membranous PD-L1 labeling of Hodgkin Reed–Sternberg cells was identified in 84% of nodular sclerosis classical Hodgkin lymphoma, 88% of mixed cellularity classical Hodgkin lymphoma, and 100% of classical Hodgkin lymphoma—not otherwise specified. Of these, 82% showed at least 90% of the Hodgkin Reed–Sternberg cells to be positive for PD-L1. Additionally, in the majority of these cases, at least 20% of the total tumor cellularity, including nonmalignant tissue macrophages, showed strong membranous PD-L1 expression. In a subsequent study, a fluorescence *in situ* hybridization-based assay was employed to investigate the correlation between 9p24.1 alterations and clinical outcomes using 108 classical Hodgkin lymphoma biopsies.¹³² In this cohort, 97% of cases showed concordant alterations of *PD-L1* and *PD-L2* loci, with copy gain and amplification identified in 56 and 36% of cases, respectively; PD-L1 protein expression by immunohistochemistry correlated with copy number alterations. Most importantly, patients whose tumors showed 9p24.1 amplification were more likely to have advanced-stage disease and were associated with worse progression-free survival following standard induction therapy.

These biomarker-based studies have set the stage for a number of clinical trials aimed at examining the potential utility of PD-1 blockade in classical Hodgkin lymphoma. In one of the earliest such attempts, nivolumab was tested in 23 patients with relapsed or refractory Hodgkin lymphoma that had already received standard multi-agent chemotherapy.¹³³ In a subgroup of 10 patients with available pretreatment tissue biopsies, all tumors showed *PD-L1* and *PD-L2* copy number gains or amplifications by fluorescence *in situ* hybridization and membranous protein expression by immunohistochemistry on the Hodgkin Reed–Sternberg cells. An 87% overall response rate was seen in 23 patients, with 17% demonstrating a complete response, 70% a partial response, and 13% with stable disease; progression-free survival was 86% at 24 weeks. More recently, a trial was conducted in which 31 patients who had failed polytherapy (including brentuximab vedotin, an anti-CD30 antibody) received pembrolizumab.¹³⁴ In this cohort, 16% experienced a complete response and 48% had a partial response, producing an overall response rate of 65%. The progression-free survival was 69% at 24 weeks and 46% at 52 weeks. In these cases, as well, both PD-L1 and PD-L2 expression were found to be elevated. Importantly, in a small series of patients with relapsed/refractory primary central nervous system lymphomas and primary testicular

lymphoma, which are two tumor types with a high rate of 9p24.1 amplification, single-agent PD-1 blockade resulted in clinical responses in all patients.⁶⁰

The efficacy of PD-1 blockade in classical Hodgkin lymphoma and select other lymphoma types appears to be a direct result of 9p24.1 copy number alterations, whereas the majority of non-Hodgkin lymphomas have shown reduced clinical response rates, perhaps as a result of lacking these same chromosomal aberrations. In a clinical trial involving 81 patients with a variety of non-Hodgkin lymphomas, objective response rates of 40, 36, 15, and 40% were observed among patients with follicular lymphomas, diffuse large B-cell lymphoma, mycosis fungoides, and peripheral T-cell lymphoma, respectively.¹³⁵ Such results indicate that further exploration into the mechanisms behind effective PD-1 blockage are needed, particularly in hematological malignancies unrelated to classical Hodgkin lymphoma.

Head and neck squamous cell carcinoma

Head and neck squamous cell carcinoma is most often associated with either human papillomavirus (HPV) infection or tobacco/alcohol exposure. The HPV-positive tumors tend to occur in the oropharynx, are more sensitive to radiation therapy, and tend to have a better prognosis than their HPV-negative counterparts. The HPV-negative tumors tend to be associated with field cancerization and a higher mutational density, which may be attributable to their association with carcinogens and resultant p53 mutations.¹³⁶ The HPV-positive tumors were predicted to be responsive to immunotherapy, due to the presence of preexisting immunity against viral antigens as well as their typical location in the lymphoid-rich Waldeyer's ring. Somewhat surprisingly, clinical trials of anti-PD-1 therapy have shown comparable activity with overall response rates of approximately 10–20% and only slightly greater efficacy in HPV+ tumors.^{137,138} In 2016, the FDA approved nivolumab and pembrolizumab as second-line therapy for head and neck squamous cell carcinoma. Numerous phase III trials are ongoing, many of which are assessing the efficacy of combinatorial PD-1/PD-L1 blockade with either CTLA-4 inhibition or chemotherapy and/or radiation.¹³⁹

As with other tumor types, there is a high demand for biomarkers to guide patient selection. Estimates of PD-L1 prevalence in head and neck squamous cell carcinoma range from 30 to 70%, and patterns of both adaptive and constitutive PD-L1 expression in both HPV+ and HPV- tumors have been identified.^{126,140,141} When PD-L1 expression in this tumor type was explored in clinical trials for anti-PD-1 as a possible predictors of response and survival to anti-PD-1, the collective results have been somewhat ambiguous.^{138,142,143} Some of the

seemingly contradictory observations may be reflective of constitutive vs adaptive PD-L1 expression patterns in this tumor type. Further evaluation of PD-L1 as a biomarker response to monotherapy is ongoing in phase III trials (NCT02252042, NCT02358031), and the first results highlighting the potential additive value of assessing PD-L2 expression in addition to PD-L1 as a potential biomarker of response for head and neck squamous cell carcinoma were just published.¹⁴⁴

It is worth highlighting potential reasons why HPV-negative tumors may also respond to immune checkpoint-blocking agents. Although HPV-positive tumors tend to be more inflamed, HPV-negative tumors also contain immune infiltrates. Further, the relative proportion of immune cell subsets and checkpoint molecule expression is similar between virus-positive and -negative tumors.¹⁴⁵ Finally, mutational burden has also been associated with response to immune checkpoint blockade, and HPV-negative tumors have a higher mutational burden when compared with their virus-positive counterparts.

Colorectal carcinoma

Colorectal carcinoma is the third most common cancer type in men and women in the United States and represents a significant burden of disease. In a study of anti-PD-1 pembrolizumab in patients with colorectal carcinoma who failed conventional therapy, patients with microsatellite-unstable tumors (mismatch repair deficient) demonstrated a 40% objective response rate compared with 0% of patients with microsatellite-stable tumors (mismatch repair proficient).³⁵ Microsatellite-unstable tumors contain many features that foreshadowed potential responses to this class of agents. First, they contain thousands of mutations, resulting in a high rate of neo-epitope formation. These neo-epitopes provide ample substrate for immune cell targeting. Second, microsatellite-unstable tumors are characterized by abundant tumor-infiltrating lymphocytes, the majority of which are antitumor, cytotoxic CD8+ T cells. Finally, half of microsatellite-unstable colorectal carcinomas show the expression of PD-L1 in the tumor microenvironment, either on carcinoma or immune cells.¹⁴⁶

The high response rates of patients with microsatellite-unstable tumors is promising. In May 2017, the FDA granted accelerated approval for the anti-PD-1 pembrolizumab for all tumors with mismatch repair-deficient/microsatellite-unstable tumors, irrespective of the primary site of origin (Figure 3). Unfortunately, only 5–15% of colorectal cancers in the United States have this genotype. A small subset of patients with microsatellite-stable (mismatch repair proficient) colorectal tumors showed some disease control in the pembrolizumab trial,³⁵ and efforts are currently underway to

characterize the immune microenvironment of these tumors. Integrative analyses of large cohorts of colorectal cancer showed that the Immunoscore is a stronger prognostic marker of prognosis than microsatellite instability.⁴⁵ Studies are ongoing to determine whether it may also be a stronger predictor of response to various chemotherapeutic or immune-based therapies.¹⁴⁷

Breast carcinoma

Breast carcinoma is the most common cause of cancer in women in the United States and is second only to lung carcinoma in cancer-related deaths in women¹⁴⁸; thus the use of immunotherapy in breast carcinoma has the potential to impact a significant number of patients. Emerging evidence indicates the presence of a robust and active tumor immune microenvironment in a subset of breast carcinomas.¹⁴⁹ Indeed, the presence of an active

tumor microenvironment is demonstrated even in preinvasive ductal carcinoma *in situ*,¹⁵⁰ suggesting the possibility of exploring immunotherapy as a preventative strategy.

The majority of breast carcinoma immune microenvironment studies have been carried out in invasive ductal carcinoma.^{10,151,152} Triple-negative breast carcinomas (TNBC) and those with amplification of the human epidermal growth factor receptor-2 (HER-2) oncogene appear to be more immunogenic than luminal type, or estrogen receptor (ER) positive, breast carcinomas, both on the basis of higher degree of tumor-infiltrating lymphocytes and increased immune gene signatures in the former tumor types.¹⁴⁹ The potential to target the immune system in TNBC is particularly attractive, as these tumors not only tend to have a higher mutational burden (and thus increased potential neo-antigenicity) than other subsets of breast carcinoma but they also currently lack targeted treatment options.

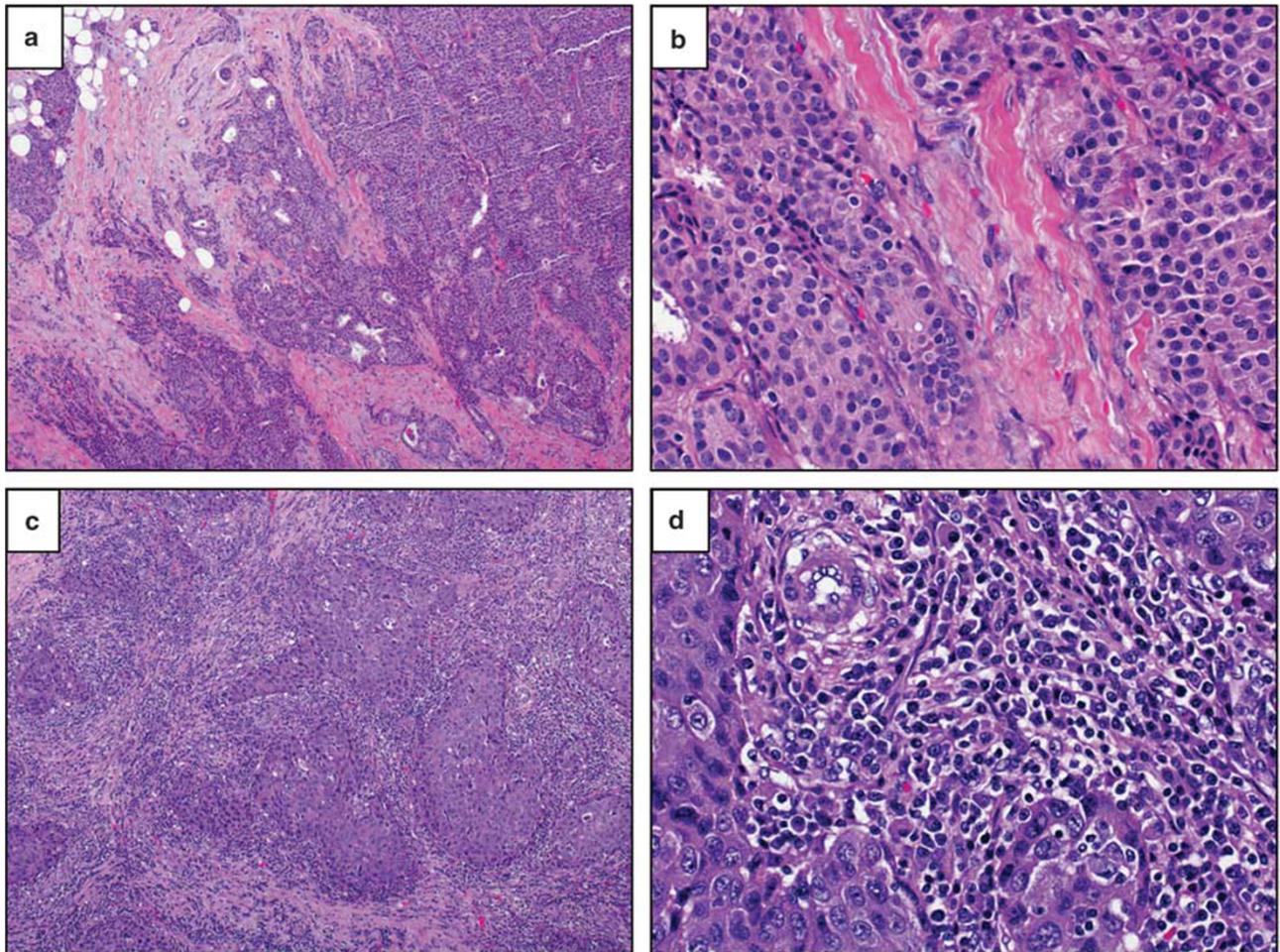


Figure 6 In breast carcinomas, tumor-infiltrating lymphocytes are scored as the percentage of stromal space within the tumor area occupied by mononuclear immune cells. The degree and composition of tumor-infiltrating lymphocytes varies across tumor subtypes and primary organ sites. (a, b) Here a primary luminal A-type breast carcinoma (ER+/PR+/HER-2-/Ki67-low) contains minimal tumor-infiltrating lymphocytes (< 5% of stromal area) (H&E, $\times 40$ and $\times 400$, original magnification). (c, d) In contrast, a primary HER-2+ breast carcinoma (ER-/PR-/HER-2+) contains brisk tumor-infiltrating lymphocytes (overall 80% of stromal area), qualifying as 'lymphocyte predominant breast cancer' (H&E, $\times 40$ and $\times 400$, original magnification).

The immune microenvironment in breast carcinoma has been largely defined by the presence and composition of tumor-infiltrating lymphocytes, which are most pronounced in TNBC and HER-2⁺ carcinomas and are emerging as promising prognostic and predictive biomarkers.^{151,152} An international working group proposed recommendations for assessing tumor-infiltrating lymphocytes in breast carcinoma on hematoxylin and eosin sections,¹⁵³ which will ideally lead to standardized scoring among studies. Tumor-infiltrating lymphocytes are assessed as a continuous variable of the percentage of the tumor stromal area occupied by lymphocytes (Figure 6), with a cutoff of 50–60% stromal lymphocytes used to define ‘lymphocyte-predominant breast carcinoma’.

Medullary carcinomas are a special subtype of breast carcinomas largely defined by the presence of a brisk lymphoplasmacytic infiltrate and have long been recognized to have a relatively favorable prognosis.¹⁵⁴ The presence of high tumor-infiltrating lymphocytes is also associated with improved prognosis in TNBC and HER-2⁺ invasive ductal carcinomas.^{151,152} In addition, the presence of brisk tumor-infiltrating lymphocytes in the tumor bed after neoadjuvant therapy is associated with improved prognosis in TNBC. Finally, the presence of tumor-infiltrating lymphocytes in the pretreatment tumor is predictive of a favorable response to neoadjuvant chemotherapy in TNBC, as measured by the degree of pathological complete response.¹⁵¹ Studies are conflicting regarding whether there is an association between tumor-infiltrating lymphocytes and response to neoadjuvant anti-HER-2 targeted therapy. Among tumor-infiltrating lymphocyte subsets, the presence of CD8⁺ cytotoxic T cells is favorable, whereas the presence of FoxP3⁺ regulatory T cells is unfavorable, with evidence that a high CD8/FOXP3 ratio after neoadjuvant chemotherapy predicts overall survival.¹⁵⁵

The role of immune checkpoint pathways is also under exploration in breast carcinoma. PD-L1 can be expressed by the carcinoma cells or immune cells, with increased expression seen in the TNBC and HER-2⁺ carcinomas.^{10,152} PD-L1 expression correlates with ER negativity and the overall degree of lymphocytes within the tumor in invasive ductal carcinomas, suggesting a component of adaptive immune expression of PD-L1.^{10,149} In contrast, in lobular carcinomas, PD-L1 expression is unrelated to the ER status or degree of tumor-infiltrating lymphocytes in the tumor,¹⁵⁶ suggesting a component of constitutive or innate expression of PD-L1 by the carcinoma cells. Studies are conflicting regarding the association between PD-L1 expression and prognosis in breast carcinoma, likely reflecting the heterogeneity in PD-L1 assays and scoring between studies.¹⁵⁷

Various immunotherapeutic strategies are under clinical investigation in breast carcinoma, including not only immune checkpoint inhibition but also peptide-based vaccines, immune cell transfer, and the

administration of costimulatory antibodies.^{149,152,158} Indeed, trastuzumab, a monoclonal antibody targeting HER-2, is itself an immunotherapy with at least a component of its clinical efficacy due to stimulation of the immune response.¹⁵⁸ Among checkpoint inhibitors, those targeting the PD-1/PD-L1 pathway have been most extensively studied, although agents targeting CTLA4 and LAG3 are also under investigation.^{149,152} Anti-PD-1/PD-L1 agents have shown promising results in patients with metastatic TNBC, with higher response rates in patients with PD-L1-positive tumors¹⁵²; however, a subset of patients with PD-L1-negative tumors also demonstrates clinical response. Clinical trials of immune checkpoint inhibitors in the neoadjuvant setting,¹⁵⁹ as well as coupling traditional therapeutic approaches such as chemotherapy with immune checkpoint inhibition, are also under investigation.^{152,158} As in other tumor types, the optimal biomarker for pathological prediction of response and trial inclusion remains unclear.

Conclusions and future considerations

The list of FDA-approved indications for immune checkpoint inhibitors as single agent or combinatory therapy is rapidly expanding, with anticipated approvals of new indications and new agents. With the expanding use of immunotherapy, there is a clear need for improved biomarker development. Biomarker development can be useful on not only on a per-patient basis but also in identifying tumor types most likely to respond to therapy. There are many solid tumor types such as thyroid or neuroendocrine tumors, which are not classically recognized as immune infiltrated. It is possible that if immune checkpoint blockade is to be effective tumor types such as these, it may be in the form of combination therapy, ie, an agent such as a tumor vaccine or even a small-molecule inhibitor could be used to incite a host response against tumor, followed by immune checkpoint blockade to protect and potentiate the resultant immune response. The preexisting paradigm for biomarker identification in the era of oncogenic targeted therapy, in which the presence of a given oncogene mutation is predictive of response, is not applicable to immunotherapeutic agents. There are numerous pathways dictating the interaction between a tumor and the immune cells, and distilling this interaction to a single marker is an oversimplification. Future biomarker strategies will undoubtedly include a more comprehensive assessment of both the innate and adaptive immune response to the tumor, as well as underlying features of the tumor itself.

The complexity of biomarker development for immune checkpoint blockade is exemplified by the development of PD-L1 immunohistochemistry as a diagnostic. Patients who are PD-L1-negative may demonstrate a clinical response to anti-PD-1/PD-L1 therapy, perhaps due to sampling error of a spatially

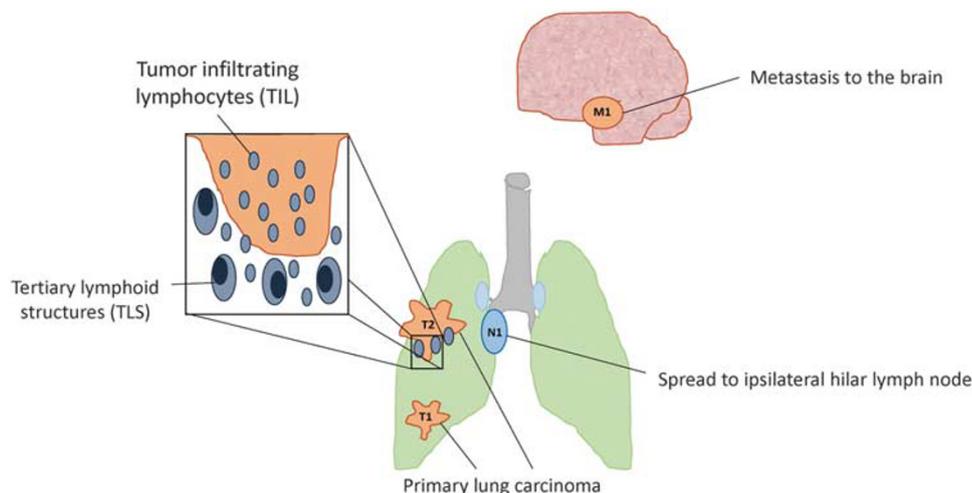


Figure 7 Future TNM staging systems for non-small cell lung carcinoma and other tumor types may include immune features. The current TNM staging system is based upon the degree of tumor invasion (T), presence and extent of nodal metastases (N), and presence of metastatic spread (M). Studies have shown that the presence and degree of tumor-infiltrating lymphocytes and tertiary lymphoid structures around the tumor bed may be favorable prognostic factors. Future incorporation of immune-based parameters as prognostic elements into the cancer staging system is likely in certain tumor types.

and temporally heterogeneous marker, issues with assay sensitivity, or simply the fact that PD-L1 may be an imperfect biomarker. Further underscoring the limitations of PD-L1 as a solitary marker, patients who are PD-L1-positive often do not respond to therapy. Additional enrichment for response in the PD-L1-positive population may be possible by the assessment of whether PD-L1 is expressed in an adaptive rather than a constitutive manner, and those investigations are currently underway. Additional efforts are clearly needed to standardize the assays and scoring systems used, including clarification as to the cell type expressing PD-L1 to be scored. Data from preclinical models suggests that PD-L1 expression on tumor cells and immune cells can modulate T-cell function in the tumor microenvironment to a similar degree.¹⁶⁰ Parallel studies in humans could help form the rational basis for a more unified PD-L1 immunohistochemistry scoring approach.

As the number of patients treated with checkpoint inhibition increases, the number of immune-related adverse events unique to this class of drugs will also increase. These include colitis, dermatitis, hepatitis, hypophysitis, myocarditis, pericarditis, and pneumonitis. Histopathological descriptions of many of these reactions have now been reported.^{161–165} The need for pathologists to maintain awareness of the histological manifestations of these drug-related events is critical for patient management, as is the importance of clear communication of clinical history by the treating physicians.

In conclusion, the recent success of immune-based cancer therapy is changing the practice of pathology. Surgical pathology specimens will be used for immune-based assays, including immunological and molecular biomarkers for therapeutic selection and monitoring. Elements of the tumor immune microenvironment are also recognized as

independently prognostic in certain tumor types, contributing to the possibility of incorporation of immune parameters into the TNM-based staging system in the future (Figure 7). Surgical pathologists will have a critical role in assessing specimens, performing assays, and addressing questions from treating physicians, oncologists, and patients regarding predictive and prognostic immune biomarkers.

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