Cancer Immunology Research

Predictive Biomarkers for Checkpoint Immunotherapy: Current Status and Challenges for Clinical Application

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Abstract

Immune-checkpoint blockade (ICB), in particular PD-1 inhibition, has rapidly changed the treatment landscape and altered therapeutic paradigms across many tumor types, with unprecedented rates of durable clinical responses in a number of cancers. Despite this success, only a subset of patients responds to ICB and, as a result, predictive biomarkers would be useful to guide the selection of patients for these therapies. This article highlights currently used biomarkers, as well as several promising novel candidates, and also discusses the challenges involved in establishing their analytic validity and clinical utility. Progress is being evaluated in melanoma and non–small cell lung cancer, for which PD-1 ± CTLA-4 inhibitors have become standard therapy, to other malignancies for which PD-L1 inhibitors remain investigational. Although single biomarkers have substantial limitations, a combination of biomarkers that reflect the interaction of host and tumor will likely be needed to provide a reproducible surrogate for the benefit of checkpoint modulation. *Cancer Immunol Res; 6(10); 1122–8. ©2018 AACR.*

Introduction

Progress in the field of immuno-oncology has changed traditional treatment paradigms, with immune-checkpoint blockade (ICB) now at the forefront of available therapies for a number of malignancies, with durable responses achieved even in advanced disease. Immune-checkpoint pathways are conserved mechanisms that regulate the immune response to maintain self-tolerance (1). In the cytotoxic T lymphocyte–associated antigen 4 (CTLA-4) pathway, T-cell activation requires both binding of the T-cell receptor (TCR) to the major histocompatibility complex (MHC), as well as binding of the costimulatory molecule CD28 on T cells to B7-1 and B7-2 ligands on antigen-presenting cells (APCs). CTLA-4, expressed on T cells, acts by competing with CD28 for binding to B7-1, resulting in diminished T-cell responses (2). Similarly, in the programmed death ligand-1 (PD-L1)/programmed cell death protein-1 (PD-1) pathway, binding of PD-L1 on tumor cells and APCs to the PD-1 receptor on T cells downregulates T-cell proliferation, effector function, and cytokine production (3). ICB therapies target these inhibitory pathways by blocking ligand–receptor interactions (Fig. 1), thereby augmenting the endogenous antitumor response (4).

With the advent of ICB, a subset of patients with treatment-refractory metastatic cancers has been able to achieve durable responses, although the majority of patients fail to respond. This underscores the need to identify predictive biomarkers for outcome. Biomarkers of response can help guide patient selection for single-agent or combination therapy as well as develop alternative treatment strategies (Fig. 2). Although rapid advances have been made in biomarker research, only a few biomarkers are clinically relevant such as PD-L1 expression, microsatellite instability (MSI), and mismatch-repair deficiency (dMMR). These biomarkers are now used to select patients for FDA-approved therapies such as pembrolizumab in the first-line treatment of patients with non–small cell lung cancer (NSCLC) with high PD-L1 expression, and pembrolizumab in the second-line setting for solid tumors that are MSI high or dMMR positive. Other biomarkers are not as well established and remain under investigation, though we briefly discuss them here.

Tumor Biomarkers

PD-L1 expression, tumor-infiltrating lymphocytes (TILs)

One of the earliest and most promising biomarkers is PD-L1 expression. Initial studies found that pretreatment PD-L1 expression on tumor cells (3, 5) and immune cells (6) predicted clinical outcomes to ICB across multiple tumor types. This also correlated with improved response rates, progression-free survival (PFS), and overall survival (OS) as seen in a large cohort of melanoma patients treated with pembrolizumab (KEYNOTE-001; ref. 7). Based on this landmark trial, pembrolizumab became the first PD-1 inhibitor granted FDA approval for patients with metastatic melanoma. In addition to tumor PD-L1 expression, it was demonstrated that preexisting $CD8^+$ TILs localized at the invasive tumor margin were predictive of melanoma response to PD-1 blockade (8). Another study also showed that $PD\text{-}L1^+$ melanocytes located next to TILs led to the secretion of interferon-gamma (IFNγ) leading to adaptive immune resistance (9).

Although baseline PD-L1 expression is a widely used biomarker, its expression is dynamic and may change over the course of clinical treatment, implying that baseline expression may not be useful as a sole predictive biomarker. For instance, in metastatic melanoma, responders had proliferation of cytotoxic T cells and $PD\text{-}1^+$ T cells within the tumor, as well as upregulation of tumoral

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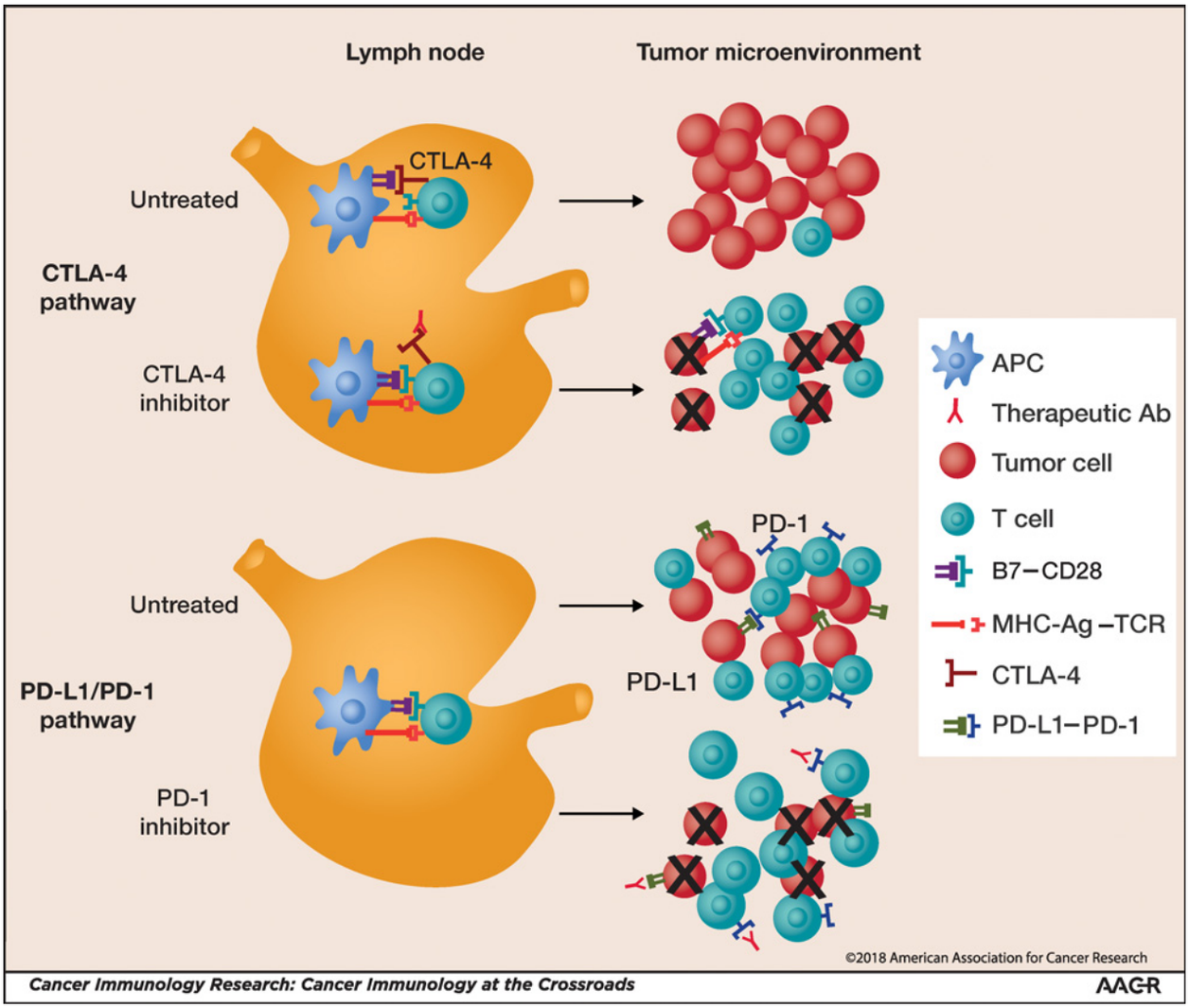

Figure 1.

Mechanisms of ICB through CTLA-4 and PD-L1/PD-1 pathways. In the CTLA-4 pathway, binding of the TCR to the MHC on APCs, in addition to CD28 on T cells to B7-1/B7-2 on APCs, results in T-cell activation. CTLA-4 acts by competing with CD28 for binding to B7-1, which abrogates T-cell activity. This interaction is targeted by CTLA-4 inhibitors, which restores T-cell activity. In the PD-L1/PD-1 pathway, PD-L1, which is located on APCs and tumor cells, binds to the PD-1 receptor on T cells, which downregulates T-cell responses. Similarly, PD-1 inhibitors prevent this ligand–receptor interaction, allowing for increased T-cell activity and enhanced immune response.

PD-L1 expression, shortly after their first dose of treatment (median of 11 days; ref. 10). Although baseline tumoral PD-L1 expression correlated with treatment response, induced intratumoral PD-1^+ T cells more strongly correlated with tumor shrinkage and PFS. Upon progression, immune-cell infiltrates and PD-L1 expression decreased, reflecting immune evasion, possibly a pharmacodynamic biomarker that could be used to dictate a change in treatment.

Although PD-L1 expression predicts the likelihood of response to ICB in several tumor types, it does not appear to correlate with response rates in other tumors and/or specific disease settings, highlighting the challenges of PD-L1 as a biomarker. This discrepancy could be attributed to its dynamic expression, assay variability, and cutoff values (11, 12), and disease-specific differences. For instance, in a large ICB trial in previously treated triple-negative breast cancer (TNBC; KEYNOTE-086), anti–PD-1 monotherapy was given to 170 patients, and response rates were not different between PD-$L1^+$ ($\geq$1%) and PD-$L1^-$ tumors (13). However, in the same study, pretreatment stromal TILs assessed on standard H&E-stained tumor sections were associated with response to ICB, with higher percentages correlating to better outcomes (14).

With increasing understanding of the complex interactions between tumor and host immunity in the tumor microenvironment (TME), two predictive classification schemas have been proposed. The first classifies the TME into four different types based on PD-L1 status and the presence or absence of TILs (15). Type I tumors are the most immunogenic with preexisting TIL^+/PD-$L1^+$ and are most likely to respond to ICB. Type II tumors are felt to constitute an immunologic desert, are TIL^-/PD-$L1^-$, and

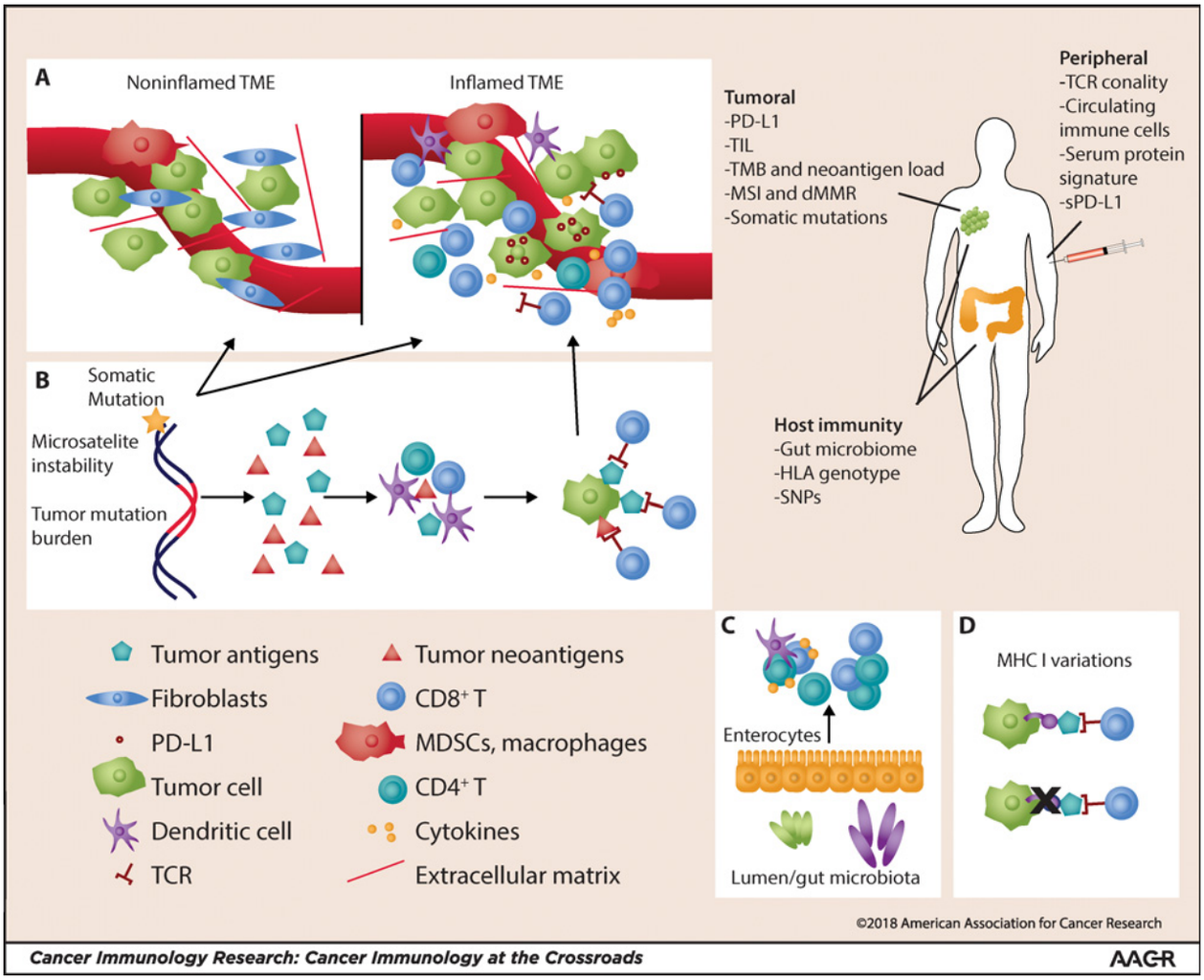

Figure 2.

Predictive biomarkers for response to ICB. **A,** Representative schema of the non-T cell–inflamed tumor microenvironment with dense stroma, as compared with the inflamed tumor microenvironment with a higher frequency of immune-cell infiltrates and concentration of cytokines. Note that tumor PD-L1 expression appears to be heterogeneous within and between tumors. **B,** Tumor mutation burden and neoantigen load. Tumors acquire new mutations, creating DNA sequences that can generate neoantigens that are recognized by T cells as immunogenic (**C**) gut microbiome. Presence of microbes, such as *Bacteroidales, Bifidobacterium, Collinsella,* and *Enterococcus* causes regulation of T-cell function, which can then infiltrate the tumor and modulate innate immunity. **D,** Variations within the HLA class I genotype may affect the antigen-specific T-cell response.

least likely to respond to ICB. Type III tumors are $TIL^-/PD\text{-}L1^+$, are "immune excluded," and presumably have a functional PD-L1 pathway but may require combination treatment in order to recruit lymphocytes into the tumor bed. Finally, type IV tumors are $TIL^+/PD\text{-}L1^-$ and may need to be treated with alternative strategies to target other immunosuppressive pathways.

An alternative framework classifies cancers into T cell–inflamed or "hot" tumors versus noninflamed or "cold" tumors (16). T cell–inflamed tumors are highly immune-cell infiltrated, characterized by expression of chemokines, which help recruit activated T cells into tumor sites, macrophage activation markers, and a type I IFN profile, all of which contribute to a higher probability of response to ICB. Additionally, following anti–PD-1 treatment, there is evidence for TIL expansion and proliferation correlating with tumor regression. This is in contrast to noninflamed tumors, which are less likely to respond to ICB. One area of active research is directed to determining how to transform a "cold" tumor into a "hot" tumor through the use of combination therapies.

MSI/dMMR, tumor mutation burden (TMB), and neoantigen load

In addition to PD-L1 expression, MSI is another clinically relevant biomarker used to predict outcome and select patients for ICB treatment. Approximately 5% of all solid tumors have been found to have dMMR, characterized either by the presence of MSI or by the absence of one or more MMR proteins by immunohistochemistry (IHC). dMMR cancers harbor hundreds to thousands of somatic mutations with increased generation of

neoepitopes, and MSI/dMMR has been shown to predict clinical benefit from ICB. In a phase II clinical trial of pembrolizumab, regardless of tumor type, patients with dMMR tumors had prominent TILs, higher response rates, and PFS, compared with MMR-proficient tumors (17). These findings were later expanded to evaluate PD-1 blockade solely in patients with dMMR across 12 tumor types (18). Objective response rates were observed in 53% of patients, and in patients achieving a complete response, median PFS and OS were not yet reached at the time of their publication. Correlative analyses showed *in vivo* expansion of T-cell clones against mutant neoantigens, suggesting increased recognition following pembrolizumab. Based on these findings, MSI has become the first biomarker used to select patients for ICB in a tissue/site-agnostic manner, with FDA approval of pembrolizumab for the second or later lines of therapy for metastatic, MSI-high tumors.

TMB is another biomarker similar in concept to MSI, though this is not FDA approved for clinical use and is still being investigated. When a tumor acquires mutations, it creates new DNA sequences that may encode neoantigens with immunogenic peptides that are recognized by T cells, allowing for increased sensitivity to ICB. Several studies demonstrated the association of the mutational landscape of tumors with responsiveness to PD-1 blockade in NSCLC (19) and anti–CTLA-4 therapy in melanoma (20, 21). These two malignancies often have a high TMB, one due to increased carcinogen exposure via cigarette smoke and the other due to exposure to DNA-damaging UV light.

In patients with metastatic NSCLC treated with pembrolizumab, a high nonsynonymous mutation burden above the median (209 mutations), correlated with higher durable clinical benefit, including objective response as well as PFS (19). When compared with PD-L1 expression, TMB appeared to be a better predictor of response, although some patients with high TMB did not respond, whereas others with low TMB responded well. Although neoantigen load is associated with an increased sensitivity to ICB, this may be more related to whether these neoantigens are clonal or "truncal" as opposed to subclonal or "branched" (22). Tumors enriched for clonal neoantigens were associated with an inflamed TME characterized by higher immune infiltration and PD-L1 expression. This also correlated with prolonged PFS and OS, as compared with tumors with larger subclonal populations or greater neoantigen heterogeneity.

Somatic mutations may also be associated with a high TMB and improved responses to ICB, initially observed in patients with NSCLC who were never-smokers (19). Mutations in genes involved in DNA repair, including DNA Polymerase Epsilon (*POLE*) or DNA Polymerase Delta 1 (*POLD1*), were associated with hypermutation and responses to ICB. Similar findings were observed in a patient with *POLE*-mutated endometrial cancer who had an increased TMB, upregulation of immune-checkpoint proteins, lymphocytic infiltration, and experienced a durable response to pembrolizumab (23).

Although TMB and neoantigen load have shown associations with ICB response, they have not been validated independently to predict response. Additionally, these assays are challenging to perform in everyday practice considering the cost and bioinformatics requirements involved in whole-exome sequencing and neoepitope prediction. Alternate methods predicting TMB, such as next-generation sequencing, which was associated with ICB benefit in melanoma and NSCLC (24, 25), are being studied.

Other potential biomarkers

With complex tumor-immune dynamics, compounded by the difficulty in establishing a single predictive biomarker, there is increased interest in the use of multiple biomarkers to better predict clinical outcomes with ICB. A number of studies have evaluated tumor gene-expression signatures, which integrate several tumor and immune-response parameters. An 18-gene pan-tumor signature using genes involved in IFNγ signaling correlated with clinical benefit to PD-1 inhibitors across distinct tumor types (26). This signature is associated with molecules involved in antigen presentation, chemokine expression, cytotoxic activity, and adaptive immune resistance. In general, a high IFNγ signature correlated with an inflamed TME and an increased response to PD-1 inhibitors, whereas a low IFNγ signature had a lower likelihood of response. However, there were some nonresponders with a strong IFNγ profile, suggesting the presence of underlying resistance mechanisms, and some responders with a low IFNγ signature, suggesting that other factors are involved. In contrast, resistance signatures such as the innate anti–PD-1 resistance (iPRES) transcriptional signature, seen in melanoma, are associated with an upregulation of genes involved in mesenchymal transition, cell adhesion, extracellular matrix remodeling, and general immunosuppression (27).

Peripheral Blood Biomarkers

TCR diversity and clonality

In metastatic melanoma patients treated with ipilimumab, peripheral blood TCR diversity was investigated by assessing richness (number of different V-J rearrangements) and evenness (similarity between the frequencies of V-J rearrangements), with high baseline TCR diversity correlating with lower tumor burden and prolonged stable disease (28). Similarly, in patients with metastatic urothelial cancers treated with atezolizumab, low peripheral TCR clonality, suggestive of peripheral expansion of dominant tumor TCR clones, correlated with improved PFS and OS (29).

Other potential biomarkers

Peripheral blood biomarkers, including circulating immune-cell subsets, serum protein signatures, and soluble PD-L1 (sPD-L1), have been studied, though these are less established. Peripheral myeloid-derived suppressor cells, which play a critical role in T-cell suppression, were found to have an inverse correlation with peripheral $CD8^+$ T-cell expansion and inferior outcomes with ipilimumab (30) and nivolumab treatment (31). In patients with metastatic melanoma, expansion of peripheral blood $CD38^+CD8^+$ T cells with an exhausted phenotype following pembrolizumab therapy, in addition to a lower baseline tumor burden, correlated with better clinical responses (32). This implies that reinvigoration of exhausted T cells is a key indicator of response to ICB, but may only work in patients with low tumor burden. Another peripheral biomarker involves a 209-protein signature, defined by mass spectrometry analysis of pretreatment serum from patients with metastatic melanoma treated with PD-1 antibodies (33). The serum proteins were involved in acute phase, complement, and wound-healing pathways and were able to stratify patients as "sensitive" and "resistant" with correlations with both PFS and OS. This may be used to identify patients who can benefit from combination treatments using inhibitors that target these pathways. Finally, sPD-L1 is a splice variant of PD-L1

secreted by tumor cells, as seen in melanoma patients treated with ipilimumab, with high concentrations of pretreatment sPD-L1 ($\geq$1.4 ng/mL) leading to inhibited T-cell responses and a trend toward rapid disease progression (34). This finding could be due to large tumor burden, increased aberrant splicing activity in the tumor, or a suppressed antitumor immune response.

Additional Host Immunity Biomarkers

Gut microbiome

Commensal microorganisms are necessary for the development, induction, and function of T cells to maintain host immune homeostasis. Studies suggest a role of the gut microbiome in antitumor immune responses and resistance to ICB. Utilizing genetically identical mice from two different facilities, which have different commensal microbes, it was found that anti–PD-L1 antitumor treatment effects differed by strain of mouse (35). These differences were eliminated by cohousing the animals, as well as with transfer of fecal material. Fecal transplantation also resulted in increased T-cell infiltration into the tumor and improved antitumor responses when given ICB. Through 16S ribosomal RNA sequencing, the authors found that *Bifidobacterium* was associated with these beneficial antitumor effects. Similar findings were demonstrated in another study using murine sarcoma models and by evaluation of patients with metastatic melanoma. Patients carrying a higher percentage of *Bacteroidales* species in the gut microbiome responded better to anti–CTLA-4 therapy (36). To confirm this finding, mice treated with broad spectrum antibiotics did not respond to anti–CTLA-4 therapy, as indicated by a decrease in $CD4^+$ T cells and TIL, although these effects were overcome when mice were inoculated with *Bacteroidales* species.

Additional studies support the finding that the microbiome affects treatment with checkpoint inhibitors in the clinical setting. Patients with NSCLC and renal cell carcinoma who were refractory to PD-1 therapy had low relative abundance of *Akkermansia muciniphila*, and fecal transplant of this bacterium to PD-1–resistant mice restored the efficacy of PD-1 blockade (37). In metastatic melanoma patients treated with anti–PD-1 therapy, responders had greater microbial diversity and relative abundance of *Ruminococcaceae*, as well as increased antigen presentation and effector T-cell activity, compared with nonresponders (38), whereas another group found a higher abundance of *Bifidobacterium longum*, *Collinsella aerofaciens*, and *Enterococcus faecium* (39). Altogether, these findings suggest that a variety of bacterial species are implicated in the antitumor response.

Human leukocyte antigen (HLA) genotype

HLA class I molecules present tumor antigens to $CD8^+$ T cells, which is a prerequisite for an effective antigen-specific adaptive T-cell response. This may be affected by variations within the HLA class I genotype (germline or somatic). In advanced melanoma and NSCLC patients treated with ICB, patients who were heterozygous at the HLA class I locus had improved OS, compared with those who were homozygous (40). This was also associated with higher TCR clonality and resultant T-cell clonal expansion following ICB. Patients with the HLA-B62 supertype or somatic loss of heterozygosity at HLA class 1 had poor outcomes, corresponding to impaired $CD8^+$ T-cell recognition of neoantigens, due to structural changes at the class I locus.

Germline single-nucleotide polymorphisms (SNP)

Polymorphisms of the PD-L1 gene in patients with NSCLC treated with nivolumab were found to be correlated with clinical outcomes (41). Using plasma DNA, seven SNPs thought to be functional based on prior studies were evaluated, and two were found to be associated with higher objective response rate and PFS, in particular, the C allele of PD-L1 rs4143815 and the G allele of PD-L1 rs2282055. As the sample size was small, this potential biomarker requires validation in a larger cohort of patients.

Challenges and Future Directions in Establishing a Biomarker

ICB has altered the treatment landscape for multiple types of cancers, allowing some patients to benefit from durable responses that otherwise may not have achieved with standard therapies. With the expanding scope of ICB, one of the major challenges is establishing predictive biomarkers to determine benefit with these drugs.

Although the "standard" and most widely used biomarker in clinical practice is tumor PD-L1 expression, several limitations make this an imperfect predictive marker. Currently, there are four diagnostic IHC assays that were developed with a specific PD-1/PD-L1 inhibitor (pembrolizumab, atezolizumab, nivolumab, and durvalumab), the first two of which are FDA approved as companion diagnostics (42). Each assay has different and/or multiple cutoffs for PD-L1 positivity. For example, in patients with NSCLC, PD-L1 expression is defined as $\geq$1%, $\geq$5%, or $\geq$10% for nivolumab, $\geq$1% or $\geq$50% for pembrolizumab, $\geq$25% for durvalumab, and $\geq$50% of tumor cells or $\geq$10% of immune cells for atezolizumab, respectively (11, 42, 43). This lack of standardization makes it very difficult to interpret results across clinical trials and hinders progress. Secondly, studies have demonstrated that PD-L1 expression is inducible and can change over the course of clinical treatment (10). PD-L1 expression has significant intertumoral and intratumoral heterogeneity and, thus, one tumor sample may not be an adequate representation (12). Although some studies have shown that patients with $PD\text{-}L1^-$ tumors can also derive benefit (5, 7), others have observed inferior outcomes for patients with PD-L1–low tumors when treated with PD-1/PD-L1 monotherapy. For example, in cisplatin-ineligible patients with metastatic urothelial cancer, decreased OS in patients with PD-L1–low status was noted in the phase III KEYNOTE-361 (pembrolizumab) and phase III IMvigor130 (atezolizumab; ref. 44). Finally, TILs have shown promise as a predictive biomarker in TNBC; guidelines for enumerating TILs in breast cancer and other malignancies are available from the International TIL Working Group (www.TILsinbreastcancer.org). Combinatorial biomarkers including PD-L1, TILs and/or effector T cells (IHC or gene signatures) are promising. IHC assays for diverse cell types and multiparameter immunofluorescence are currently being investigated.

Mutation burden ($\pm$ neoantigen load) is a promising biomarker that correlates with clinical outcomes in several malignancies, though there is no standardized cutoff for TMB, and ICB responses are observed even with low TMB. Additionally, this requires whole-exome sequencing, which is highly complex and costly and may not be readily available for routine clinical use. MSI or dMMR status, which is likely a surrogate for high TMB, is another solid tumor–based biomarker that qualifies

patients for pembrolizumab treatment via the first FDA approval of ICB in a cancer-agnostic setting.

Peripheral circulating immune cells, TCR diversity and sPD-L1 may be reflective of the TME, and have yet to be validated in clinical practice. Finally, though the gut microbiome is showing exciting promise as a marker of immune-checkpoint efficacy, its predictive value needs to be validated in larger clinical studies.

It is highly unlikely that any single biomarker can be used as a reproducible surrogate for the benefit of ICB. Instead, we will likely need to move toward harmonizing multiple biomarkers in order to better reflect the complex interactions between the host, tumor, and TME.

Disclosure of Potential Conflicts of Interest

J.S. Weber has ownership interest in Altor, Cytomx, and Biond; and is a consultant/advisory board member for Bristol-Myers Squibb, Merck, Cytomx, Novartis, Genentech, Astra Zeneca, Incyte, Celldex, Biond, Sellas, and GSK. S. Adams is a consultant/advisory board member for Merck and Genentech. No potential conflicts of interest were disclosed by the other author.

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