



REVIEW ARTICLE

Emerging predictors of the response to the blockade of immune checkpoints in cancer therapy

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Checkpoint blockade-based immunotherapy offers new options and powerful weapons for the treatment of cancer, but its efficacy varies greatly among different types of cancer and across individual patients. Thus, the development of the right tools that can be used to identify patients who could benefit from this therapy is of utmost importance in order to maximize the therapeutic benefit, minimize risk of toxicities, and guide combination approaches. Multiple predictors have emerged that are based on checkpoint receptor ligand expression, tumor mutational burden, neoantigen and microsatellite instability, tumor-infiltrating immune cells, and peripheral blood biomarkers. In this review, we discuss the current state and progress of predictors as aids in checkpoint blockade-based immunotherapy in cancer.

Keywords: Cancer immunotherapy; Checkpoint blockade immunotherapy; Predictive biomarkers; Precision oncology

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INTRODUCTION

Immunity in cancer patients is influenced by a complex network of tumor, host, and environmental factors that govern the threshold, strength, and timing of antitumor immunity.¹ There is clear evidence that the immune system can mount cytotoxic immune responses that can eradicate tumor cells as “foreign”, based on their unique and often extensive mutational profiles. However, the overriding natural balance between the immune system and tumor is tolerance, in which tumor cells are either not recognized by the immune system or have developed mechanisms to avoid immune surveillance.^{2,3} While the role of the immune system in cancer has remained unappreciated for many decades, the evasion of immune surveillance is considered one of the hallmarks of tumors. Cancer is able to effectively suppress antitumor immune responses by activating negative regulatory pathways (also called checkpoints) that are associated with immune homeostasis or by adopting features that enable them to actively escape detection (Fig. 1).^{4–7} To date, two such immune checkpoints, cytotoxic T-lymphocyte protein 4 (CTLA4) and programmed cell death protein 1 (PD-1), have garnered the most attention.⁸ Cancer vaccines,⁹ adoptive T cell transfer (ACT), and chimeric antigen receptor (CAR) T cells,^{10–12} bispecific antibodies,^{13,14} immune checkpoint blockades (ICBs),^{15,16} epigenetic therapeutics,^{17,18} and oncolytic viruses¹⁹ have come of age. Many immune-enhancing agents have recently emerged as powerful weapons in the oncological armamentarium.

Recent advances in ICBs have rekindled interest in the field of cancer immunotherapy. The era of ICBs has led to tremendous successes in cancer therapy, with long-term complete tumor regression in a proportion of patients with difficult-to-treat

malignancies, most notably melanoma, non-small-cell lung cancer (NSCLC), kidney cancer, bladder cancer, head and neck squamous cell carcinoma (HNSCC), solid tumors with microsatellite instability-high (hMSI) and mismatch repair deficiency (dMMR), Merkel cell carcinoma (MCC), and Hodgkin lymphoma.^{4,5,8,15} However, although these results are exciting, most patients with metastatic tumors do not respond to ICB therapy. For patients with metastatic melanoma or NSCLC for example, 19–45% of unselected, previously treated patients and 40–45% of patients with PD-L1-positive tumors in the frontline setting achieved an objective response to anti-PD-1 monotherapy.^{20–24} The combination of nivolumab plus ipilimumab in previously untreated patients with metastatic melanoma yielded a response rate of 72.1% among patients who were PD-L1-positive and 54.8% among patients who were PD-L1-negative; however, half of the patients experienced significant toxicity from the treatment regimen, and the survival benefit for this approach remains to be demonstrated.^{25,26} The prospect of broad therapeutic efficacy of ICBs across multiple tumor types remains elusive, such as in the treatment of pancreatic ductal adenocarcinoma and metastatic castration-resistant prostate cancer, which are largely resistant to checkpoint blockade-based immunotherapy.²⁷ Although there are myriad combinations of checkpoint blockades, co-stimulatory agonist antibodies, ACT, recombinant viruses, small molecules, epigenetic drugs, chemotherapeutic drugs, radiation and surgery that often result in further clinical improvements, these clinical advances lack validated biomarkers for prediction, prognostication, and mechanism of action that could unequivocally identify patients for enrollment and forecast the best combination of treatments.

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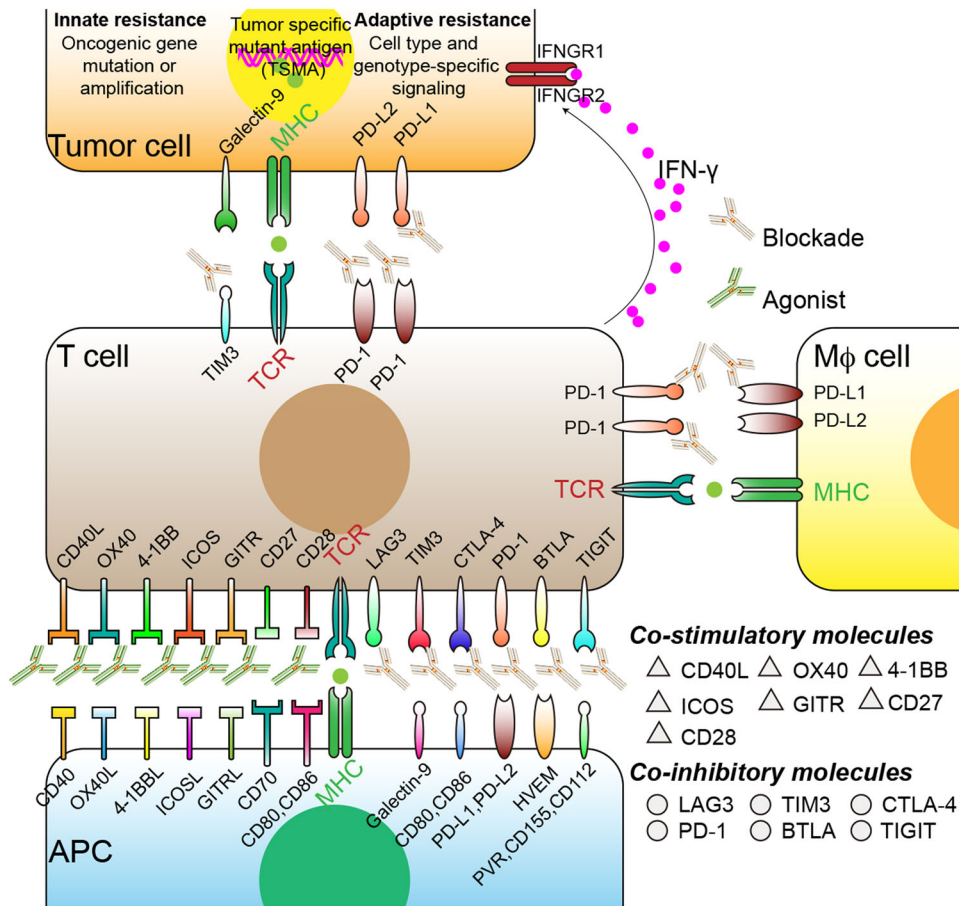


Fig. 1 Multiple co-stimulatory and co-inhibitory interactions between tumor cells and immune cells in the tumor microenvironment. An overview of the immune checkpoint molecules involved in the antitumor immune response

Clinical efficacies and the rapidly evolving landscape of treatment paradigms suggest that ICBs could become increasingly important and more broadly used for patients with advanced malignancies. In the new era of precision medicine, the establishment of predictors for checkpoint blockade-based immunotherapy is thus of utmost importance to identify which populations are more likely to respond to this therapy and to maximize the therapeutic benefit.^{28–32} Biomarkers that have high positive and negative predictive values are clearly needed to assist oncologists in selecting the best possible treatment (see Table 1). Significant efforts are currently underway to stratify patients based on genetic drivers in the tumor and on immune signatures in the tumor microenvironment (TME).^{28,29,33} Given the dynamic nature of immune responses to tumors and the complexity in the regulation of the expression of multiple immune checkpoints and their ligands, relying on any single immunologic biomarker to select patients for treatment may be difficult. Therefore, multiple components within the TME may need to be evaluated. Such effort may not only enable us to select patients who are more likely to respond but also provide potential non-responders with alternative, less expensive, and perhaps more efficacious therapeutic options. So far, strategies based on multiple biomarkers have emerged that focus on identifying aspects of the T cell-inflamed phenotype and tumor foreignness, reflected by tumor mutational burden (TMB), neoantigens, and hMSI/dMMR (Fig. 2). This review discusses the current state and progress in the identification of predictors for checkpoint blockade-based cancer immunotherapy.

PD-L1 STATUS

Since the early phase in the development of ICB therapy, the status of PD-L1 expression has been most widely studied as a potential biomarker in predicting which tumor subtypes are responsive to PD-1/PD-L1 blockade immunotherapies and in determining which individual patient may benefit from the therapy. However, these data are inconclusive.^{30–32} A phase I trial of nivolumab in patients with advanced melanoma, NSCLC, renal cell carcinoma (RCC), colorectal carcinoma (CRC), or castration-resistant prostate cancer supported a potential role for measuring PD-L1 expression in tumor biopsy specimens by immunohistochemistry (IHC).³⁴ Using a threshold of 5% PD-L1-positive tumor cells to define PD-L1 positivity, the overall response rate (ORR) of patients with PD-L1-positive tumors was higher than that of the patients with PD-L1-negative tumors. Subsequent trials of ICB have generally shown higher objective response rates to PD-1 axis blockade therapies in patient populations with PD-L1-positive diseases.^{20,23,35–38} Improved progression-free survival (PFS) and overall survival (OS) have also been shown in patients with advanced melanoma and NSCLC when comparing PD-L1-positive versus PD-L1-negative subgroups.^{25,31} Of note, although the results support a role for PD-L1 expression as a predictive biomarker, some patients with PD-L1-negative melanoma can also derive durable clinical benefit from PD-1 blockade.³⁵

PD-L1 can be expressed by tumor cells, but it is also expressed on infiltrating cells within the TME, such as macrophages, neutrophils, endothelial cells, and fibroblasts. Some studies have addressed whether the source of PD-L1 expression determines the

Table 1. A list of predictors for checkpoint blockade-based immunotherapy

Categories	Predictors	Description	Correlation with response to ICB therapy	Data type	Tumor type and agents tested	Refs
Gene expression status	PD-L1/ PD-L2	PD-L1/ PD-L2 mRNA expression	Positive correlation	RNA sequencing/IHC staining	<ul style="list-style-type: none"> Melanoma, NSCLC, renal cell carcinoma (RCC), castration-resistant prostate cancer, colorectal cancer, head and neck squamous cell carcinoma BMS-936558 (anti-PD-1 monoclonal antibody) Pembrolizumab (anti-PD-1) 	Topalian et al. ³⁴ , Yearley et al. ¹⁰⁶
	IFN- γ	IFN- γ gene expression	Positive correlation	RNA sequencing	<ul style="list-style-type: none"> Melanoma Pembrolizumab (anti-PD-1) 	Ayers et al. ¹⁰⁷
Genomic changes status		Loss of IFN- γ pathway genes in tumor cells	Negative correlation	Whole-genome sequencing/whole-exome sequencing/ RNA sequencing	<ul style="list-style-type: none"> Melanoma Ipilimumab (anti-CTLA-4) 	Gao et al. ¹⁰⁸
		IFN receptor-associated gene mutation	Negative correlation	Whole-genome sequencing/whole-exome sequencing/ RNA sequencing	<ul style="list-style-type: none"> Melanoma Pembrolizumab (anti-PD-1) 	Zaretsky et al. ⁹⁷
		Tumor mutation burden (TMB)	Positive correlation	Whole-genome sequencing/whole-exome sequencing	<ul style="list-style-type: none"> Melanoma Non-small cell lung cancer (NSCLC) Ipilimumab/tremelimumab (anti-CTLA-4) Pembrolizumab (anti-PD-1) 	Rizvi et al. ¹⁰⁹ , Snyder et al. ⁸⁹
Immune cell infiltration status		Evaluation of microsatellite sequences	Positive correlation	Whole-genome sequencing/whole-exome sequencing	<ul style="list-style-type: none"> Multiple tumor types Pembrolizumab (anti-PD-1) 	Le et al. ^{91,92}
		Tumor-infiltrating TCR clonality	Negative correlation	Whole-genome sequencing/whole-exome sequencing	<ul style="list-style-type: none"> Melanoma Pembrolizumab (anti-PD-1) 	Tumeh et al. ⁵⁰
Immune cell infiltration profile		Peripheral baseline TCR repertoire diversity	Positive correlation	Whole-genome sequencing/whole-exome sequencing/ RNA sequencing	<ul style="list-style-type: none"> Melanoma Ipilimumab (anti-CTLA-4) 	Postow et al. ¹¹⁰
		Neoantigen load; intratumor heterogeneity of neoantigens	Positive correlation	Whole-genome sequencing/whole-exome sequencing/ RNA sequencing	<ul style="list-style-type: none"> Melanoma NSCLC Ipilimumab (anti-CTLA-4) Pembrolizumab (anti-PD-1) 	Van Allen et al. ⁹⁰ , McGranahan et al. ⁸⁴
		Frameshift indel	Positive correlation	Whole-genome sequencing/whole-exome sequencing	<ul style="list-style-type: none"> Melanoma Ipilimumab (anti-CTLA-4) Pembrolizumab (anti-PD-1) 	Turajlic et al. ¹¹¹
Immune cell infiltration profile		Somatic copy number alterations (SCNA) score	Negative correlation	Whole-genome sequencing/whole-exome sequencing	<ul style="list-style-type: none"> Multiple tumor types Ipilimumab (anti-CTLA-4) 	Davoli et al. ¹¹²
		CD8 ⁺ T cell density	Positive correlation	RNA sequencing	<ul style="list-style-type: none"> Melanoma Pembrolizumab (anti-PD-1) 	Tumeh et al. ⁵⁰
Immune cell infiltration profile		PD-L1 expression level on TILs	Positive correlation	Positive correlation	<ul style="list-style-type: none"> Multiple tumor types MPDL3280A (anti-PD-L1) 	Herbst et al. ⁷⁷
		Fraction of tumor-infiltrating CD8 ⁺ T cells with expression of both PD-1 and CTLA-4	Negative correlation	Negative correlation	<ul style="list-style-type: none"> Melanoma Pembrolizumab (anti-PD-1) 	Daud et al. ⁷⁸

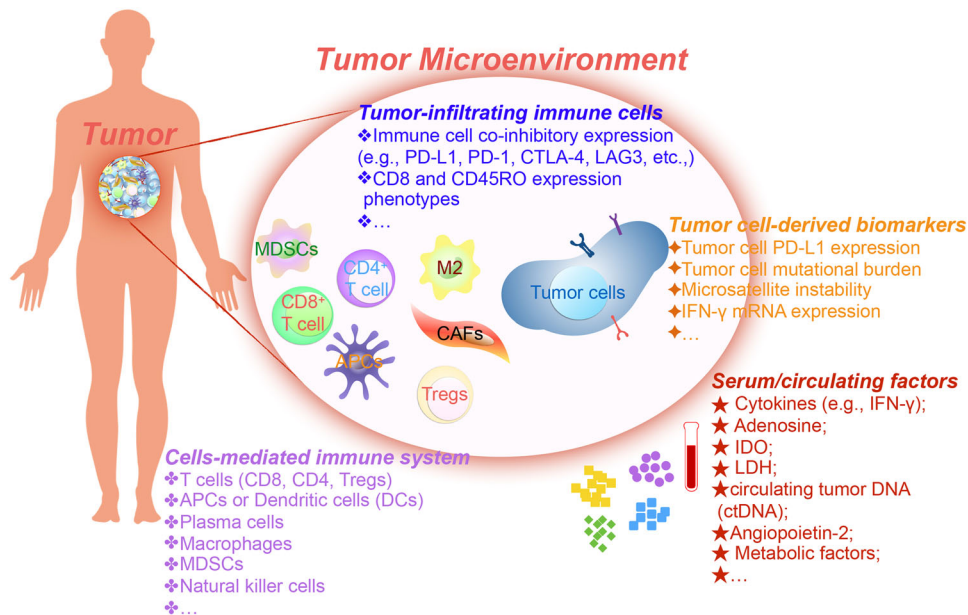


Fig. 2 Multifactorial biomarkers of clinical outcome of ICB therapy. Key elements in biomarker for ICB therapy. The tumor cell itself, TME, and the immune system must be considered in the ongoing efforts in biomarker development for ICB therapy

responsiveness to antibody blockade of the PD pathway (anti-PD therapy). PD-L1 expression by tumor cells was found to be positively correlated with objective response and with clinical benefit to the PD pathway blockade, while correlation of PD-L1 expression by infiltrating immune cells with objective clinical response did not reach statistical significance in multiple solid tumors.³⁹ However, in metastatic bladder cancer, PD-L1 in immune cells is the most predictive for the response to an anti-PD-L1 antibody.⁴⁰ In CRC patients with MSI, PD-L1 is expressed predominantly in immune cells rather than tumor cells.⁴¹ An association between high baseline PD-L1 expression in immune cells and improved response rate has been reported with atezolizumab,⁴² while no marked correlation between PD-L1 expression on tumor cells and outcome after treatment with nivolumab has been noted in metastatic urothelial carcinoma.⁴³ These conflicting observations could be related to several factors, including variations in PD-L1 expression by tumor cells and immune cells among tumor types, different PD-L1 cutoff points, intratumoral heterogeneity of PD-L1 expression, discordance of PD-L1 expression between primary tumor and metastases, and the likelihood of constant changes in PD-L1 expression owing to the dynamic nature of the TME (as described below). The mechanistic contribution of host and tumor PD-1/PD-L1 signaling to the therapeutic efficacy of PD pathway blockade remains elusive.^{44–46} For future use of PD-L1 as a predictive biomarker for therapeutic responsiveness, characterization of the expression pattern of PD-L1 within the TME is essential. Currently, two pre-clinical studies have provided some convincing proof of the relative importance of tumor-expressed versus stromal-expressed PD-L1 to this therapy response.^{45,46} These data raise the possibility that PD-L1 expressed in tumor cells is not a prerequisite for checkpoint blockade therapy and that PD-L1 expression on the host antigen-presenting cells (APCs), particularly dendritic cells (DCs) and macrophages in the TME and draining lymph nodes, rather than tumor cell-intrinsic PD-L1, may mechanistically account for the therapeutic efficacy of PD-L1 signaling blockade in multiple tumor-bearing mouse models and patients with tumors.

PD-L1 can also be a marker of immune activation. IFN- γ produced by effector T cells, soon after but not before the activation of an immune response,⁴⁷ is the major inducer of PD-L1 expression at the transcription level.⁴⁸ PD-L1 is induced on most

tumor cells in response to IFNs, predominantly IFN- γ , indicating an adaptive response to endogenous antitumor immunity.^{48,49} Supporting this, in metastatic melanoma samples, the densities of PD-L1-positive cells have been shown to significantly correlate with those of CD8⁺ T cells in the tumor and at the invasive tumor margin.⁵⁰ PD-L1 was induced by IFN- γ and TLR ligands through JAK, MEK, and MyD88 pathways.^{51–53} In Hodgkin lymphoma cells, several signaling pathways, including JAK2, MEK, and MAPK, play critical roles in PD-L1 expression.^{52,54} Moreover, activated immune cells, including DCs, macrophages, B cells, T cells, and natural killer cells, can also express PD-L1, which is mediated by the cytokine/chemokine and STAT3 pathways.^{55–57} Oncogenic pathways within tumor cells can also elevate PD-L1 expression by various mechanisms, for instance, the MAPK, PI3K, or AKT signaling pathways and some transcriptional factors, including HIF-1 α , STAT3, and NF- κ B, can transactivate PD-L1. In addition, the epigenetic writer EZH2 and epigenetic reader BET4 elevate PD-L1, whereas the epigenetic eraser histone deacetylase downregulates PD-L1 expression.^{58–62} Additionally, the loss of PTEN function and oncogenic activation of the PI3K pathway elevate PD-L1 expression post-transcriptionally.^{63,64} Moreover, CSN5, induced by NF- κ B/p65,⁶⁵ and novel CMTM6/4 transmembrane proteins decrease ubiquitination and stabilize PD-L1.^{66,67}

The assessment of intratumoral PD-L1 protein expression by IHC is the most popular method to date (Table 2). However, accurate measurement and scoring of PD-L1 protein expression are plagued by various technical and biological pitfalls (e.g., different antibodies for PD-L1 detection and manual versus automated staining techniques). Tumor heterogeneity, which exists both within the same tumor lesions and among different lesions within the same patient, is an inherent limitation of assessment approaches using tissue samples. Specimens can be obtained by various procedures, including surgical resection or needle biopsy, and focal intratumoral PD-L1 protein expression may be missed in small tumor specimens, resulting in a false-negative PD-L1 evaluation.⁶⁸ Thus, a tissue sample might not necessarily reflect the major immune phenotype of the tumor or the patient. Moreover, PD-L1 expression in tumor specimens collected days, months, or years earlier might not accurately reflect the PD-L1 status at the time of treatment initiation, and therapies given after biopsy but before administration of checkpoint inhibitors, such as

Table 2. PD-L1 expression as a predictor for response and outcome in selected clinical studies of anti-PD-(L)1 agents

Clinical trial identifier	Phase of clinical trial	Sample size (number of patients included)	Tumor type and agents tested	Cell type with IHC-stained surface	Cutoff values	PD-L1 positivity (%)	Association with response and outcome	Refs.
NCT01721759 (CheckMate 063)	Phase II	117 all	<ul style="list-style-type: none"> Advanced NSCLC; Nivolumab 	Tumor cells	≥5%	33% (25/76)	PD-L1 positive versus PD-L1 negative ORR: 24% versus 14%	Rizvi et al. ⁷²
NCT01927419 (CheckMate 069)	Phase III	142 all	<ul style="list-style-type: none"> Unresectable or metastatic melanoma treatment naive with measurable disease; Nivolumab plus Ipilimumab; Ipilimumab 	Tumor cells	≥5%	29.6% (35/118)	Nivolumab plus Ipilimumab group: PD-L1 positive versus PD-L1 negative ORR: 58.3% versus 55.4% Ipilimumab group: PD-L1 positive versus PD-L1 negative ORR: 18.2% versus 3.7%	Postow et al. ³⁶
NCT01721746 (CheckMate 037)	Phase III	631 all	<ul style="list-style-type: none"> Unresectable or metastatic melanoma after Ipilimumab or Ipilimumab and BRAF inhibitor if BRAF positive; Nivolumab 	Tumor cells	≥5%	49% (134/272)	Nivolumab group: PD-L1 positive versus PD-L1 negative ORR: 43.6% versus 20.3%	Weber et al. ¹¹³
NCT01642004 (CheckMate 017)	Phase III	272 all	<ul style="list-style-type: none"> Advanced squamous NSCLC Nivolumab versus docetaxel 	Tumor cells	≥1%, ≥5%, or ≥10%	<ul style="list-style-type: none"> 44% (119/272) for ≥1% respectively 30% (81/272) for ≥5% respectively 25% (69/272) for ≥10% respectively 	Nivolumab group: PD-L1 positive versus PD-L1 negative (≥1%, ≥5%, and ≥10%, respectively) ORR: 0.93, 0.29, and 0.64, respectively PFS: 0.7, 0.16, and 0.35, respectively OS: 0.56, 0.47, and 0.41, respectively	Brahmer et al. ²⁰
NCT01673867 (CheckMate 057)	Phase III	582 I	<ul style="list-style-type: none"> Advanced non-squamous NSCLC Nivolumab versus docetaxel 	Tumor cells	≥1%, ≥5%, or ≥10%	<ul style="list-style-type: none"> 42% (246/582) for ≥1% respectively 31% (181/582) for ≥5% respectively 28% (165/272) for ≥10% respectively 	Nivolumab group: PD-L1 positive versus PD-L1 negative (≥1%, ≥5%, and ≥10%, respectively) ORR: 0.002 (all) PFS: 0.02, <0.001, and <0.001, respectively OS: 0.06, <0.001, and <0.001, respectively	Borghaei et al. ⁷¹
NCT01844505 (CheckMate 067)	Phase III	945 I	<ul style="list-style-type: none"> Untreated patients with unresectable stage III or IV melanoma Nivolumab Nivolumab plus Ipilimumab 	Tumor cells	≥5%	23.6% (223/945)	Nivolumab group (PD-L1 positive versus PD-L1 negative): ORR: 57.5% versus 41.3%; Median PFS: 14 months versus 5.3 months; Nivolumab plus Ipilimumab group (PD-L1 positive versus PD-L1 negative): ORR: 72.1% versus 54.8%; Median PFS: 14 months versus 11.2 months; Ipilimumab group (PD-L1 positive versus PD-L1 negative): ORR: 21.3% versus 17.8%; Median PFS: 3.9 months versus 2.8 months;	Larkin et al. ²⁵
NCT01866319 (KEYNOTE-006)	Phase III	834 I	<ul style="list-style-type: none"> Melanoma Pembrolizumab versus Ipilimumab 	Tumor cells	≥1%	80.5% (671/834)	Pembrolizumab versus Ipilimumab: PFS: HR=0.53 and 0.52 in PD-L1 positive; HR=0.67 and 0.76 in PD-L1 negative;	Robert et al. ²⁶

Table 2 continued

Clinical trial identifier	Phase of clinical trial	Sample size (number of patients included)	Tumor type and agents tested	Cell type with IHC-stained surface	Cutoff values	PD-L1 positivity (%)	Association with response and outcome	Refs.
NCT02142738 (KEYNOTE-024)	Phase III	1653 all	<ul style="list-style-type: none"> • Treatment-naïve NSCLC • Pembrolizumab versus platinum-based chemotherapy 	Tumor cells	≥50%	30.2% (500/1653)	OS: HR=0.55 and 0.58 in PD-L1 positive; HR=0.91 and 1.02 in PD-L1 negative Pembrolizumab versus chemotherapy in PD-L1 positive patients: ORR: 44.8% versus 27.8%	Reck et al. ³⁷
NCT02000947	Phase Ib	118 all (102 eligible)	<ul style="list-style-type: none"> • NSCLC • Durvalumab plus tremelimumab 	Tumor cells	≥25%	21.5% (22/102)	Durvalumab 10–20 mg/kg every 2 or 4 weeks plus tremelimumab 1 mg/kg group: ORR: 22% in PD-L1 positive versus 29% in PD-L1 negative; Durvalumab 10–20 mg/kg every 2 or 4 weeks plus tremelimumab 3 mg/kg group: ORR: 40% in PD-L1 positive versus 12% in PD-L1 negative; Durvalumab 10–20 mg/kg every 2 or 4 weeks plus tremelimumab 10 mg/kg group: ORR: 0% in PD-L1 positive versus 0% in PD-L1 negative	Antonia et al. ¹¹⁴
NCT00730639	Phase I	129 all: 68 patients with PD-L1 expression	<ul style="list-style-type: none"> • Heavily pretreated advanced NSCLC • Nivolumab 	Tumor cells	≥5%	49% (33/68)	PD-L1 positive versus PD-L1 negative: ORR: 15% versus 14% Median PFS: 3.3 months versus 1.8 months Median OS: 7.8 months versus 10.5 months	Getting et al. ¹¹⁵
NCT01295827 (KEYNOTE 001)	Phase Ib	451 I	<ul style="list-style-type: none"> • Melanoma • Pembrolizumab 	Tumor and tumor-associated immune cells	≥1%: using MEL score: 0 (no membrane staining), 1 (>0%–<1%), 2 (≥1%–<10%), 3 (≥10%–<33%), 4 (≥33%–<66%), 5 (≥66%)	76% (344/451)	PD-L1 positive versus PD-L1 negative: PFS: HR=0.51 (median PFS: 5.6 months versus 2.8 months); OS: HR=0.5 (median OS: 29.9 months versus 12.6 months); Higher ORR in groups with higher PD-L1 expression.	Daud et al. ³⁵

radiation therapy, chemotherapy, or targeted therapy, may have altered PD-L1 expression. To date, different PD-L1 antibodies have been used for different ICB clinical studies. There is no validated antibody for IHC staining for this class of ICBs; therefore, each sponsor uses a different PD-L1 detection antibody, making comparisons of data across clinical trials difficult. Whereas PD-L1 protein expression can be membranous and/or cytoplasmic, only the membranous PD-L1 is functionally relevant by its contact with PD-1-positive T cells. Antibodies used for PD-L1 detection always result in both membranous and cytoplasmic staining, which may interfere with the positivity scoring of the tumor cell membrane. Additionally, PD-L1 can be expressed by diverse cell types within the TME, including tumor cells, activated lymphocytes, tumor-associated macrophages, and rare DCs, which poses additional challenges for scoring and interpretation. Due to current limitations in clinical sampling methods, the assessment of intratumoral PD-L1 protein expression before immunotherapy may be a useful but not definitive predictive biomarker of the response to the PD-1 pathway blockade.

Aside from copy number gains of the PD-L1 gene that potentially leads to constitutive expression, as seen in Hodgkin's lymphoma,⁶⁹ the levels of PD-L1 expression can be transient, and intrapatient and even intratumoral heterogeneity in PD-L1 tumor expression can exist.⁷⁰ Understanding the changes in PD-L1 expression in response to ICBs during therapy is of great interest; however, serial assessments of PD-L1 expression can be difficult owing to repeated tissue sampling. Positivity thresholds for PD-L1 expression for the studies vary, with some using a value of 1% or more of tumor cells and other using a value of 50% or more. A consensus in clinically relevant cutoffs for IHC assays for the use of PD-1 pathway blockade therapies remains urgently needed. The initial phase I trial showed an ORR to nivolumab in 36% (9/25) of the patients who tested positive for PD-L1 expression, including advanced melanoma, NSCLC, castration-resistant prostate cancer, RCC, and CRC. None of the patients who tested negative for PD-L1 demonstrated an objective response.³⁴ The CheckMate 057 (phase III) trial also showed a positive correlation between the treatment response and PD-L1 expression. When patients subjected to nivolumab treatment were divided according to pre-specified levels of tumor-membrane expression ($\geq 1\%$, $\geq 5\%$, and $\geq 10\%$) of PD-L1, significant increases were reported for OS and PFS at each expression threshold level. Additionally, increases in the ORR by PD-L1 expression were significant when using any of the expression threshold levels: $\geq 1\%$ (31% versus 9%; $P = 0.0019$), $\geq 5\%$ (36% versus 10%; $P = 0.002$), and $\geq 10\%$ (37% versus 11%; $P = 0.0021$),⁷¹ suggesting that even NSCLC patients with relatively low PD-L1 expression levels may respond remarkably to ICB therapy. The CheckMate 017 (phase III) trial evaluated the treatment responses to nivolumab at varying PD-L1 expression threshold levels ($\geq 1\%$, $\geq 5\%$, and $\geq 10\%$) according to the percentage of tumor cells expressing PD-L1.²⁰ Across the prespecified threshold levels ($\geq 1\%$, $\geq 5\%$, and $\geq 10\%$), PD-L1 expression was neither prognostic nor predictive of any of the efficacy end points. In the CheckMate 063 trial, of the samples evaluable for PD-L1 expression according to the percentage of tumor cells expressing PD-L1, 59%, 33%, and 33% of patients were characterized as PD-L1-positive at the $\geq 1\%$, $\geq 5\%$, and $\geq 10\%$ expression threshold levels, respectively.⁷² The ORRs were not significantly different when the patients were stratified by PD-L1 expression threshold level, despite the fact that rates of partial response (PR) and stable disease (SD) were consistently greater for PD-L1-positive patients than for the PD-L1-negative patients across all threshold levels: $\geq 1\%$ (PR, 31% versus 13%; SD, 22% versus 19%), $\geq 5\%$ (PR, 24% versus 14%; SD, 24% versus 20%), and $\geq 10\%$ (PR, 24% versus 14%; SD, 24% versus 20%). Overall, the findings from both studies (CheckMate 017 and CheckMate 063) concluded that the treatment response to nivolumab was not entirely dependent on the expression of PD-L1 (also see Table 2).

Thus, PD-L1 expression alone is a useful but not an ideal predictor because of its poor sensitivity and specificity. Another important aspect is that PD-L1 IHC alone does not evaluate factors that could impede the PD-1 axis blockade therapy response, such as whether active immune-cell engagement of the PD-1/PD-L1 axis occurs in the TME or whether other concurrent suppressive immune pathways are present. Notably, the value of predictors may change markedly with combination therapy. For instance, PD-L1 expression in melanoma biopsy samples obtained before treatment was not predictive of ORR in patients undergoing concomitant CTLA-4 and PD-1 blockade.^{25,36,38} Although PD-L1 IHC plays an important role in the stratification of patients included in PD-1/PD-L1 blockade therapy trials, it must be emphasized that testing for PD-1/PD-L1 expression in pretreatment biopsy samples provides only one timepoint static evaluation of immune expression and fails to capture dynamic changes that inevitably occur with immunotherapy treatment. Changes in biomarkers during clinical management still need to be explored. Based on these findings, PD-L1 IHC alone is not yet an adequate biomarker for routine clinical use in deciding which patients to offer PD-1 axis blockade therapy and which patients could benefit equally from monotherapy versus combination therapy. Other factors enabling response prediction should be incorporated in the process of patient selection for ICB to establish a paradigm of precision immunotherapy.

TUMOR-INFILTRATING IMMUNE CELLS

Beyond PD-L1 expression, additional intratumoral factors have been proposed as predictors for outcomes of ICB therapy. A significant number of non-neoplastic cells, including immune cells that are probably of biological significance, have been found within the TME.⁷³ Tumor-infiltrating lymphocytes (TILs) have gained increasingly wide attention as important players in the host immune response against tumors. Lymphocyte infiltration in tumor biopsy samples indicating a T cell-inflamed phenotype has been found to correlate with improved survival in retrospective studies of patients with a range of tumors, including NSCLC, melanoma, and CRC.³¹ Similarly, the presence of ectopic lymph node-like structures within solid tumor masses, such as CRC and melanoma metastases, might predict better patient survival.⁷⁴ Data have also shown that stage III NSCLC patients given chemotherapy and radiotherapy have longer PFS and OS when CD8⁺ T cell density is high in pretreatment biopsy samples than those with a low CD8⁺ T cell density.⁷⁵ CD8⁺ T cell density is associated with PD-L1 expression,⁷⁶ and CD8⁺ T cells can be more easily detected and quantified than PD-L1 expression on cell membranes in pathological specimens. The immune recognition of these tumors is thought to result in a host immune response or T cell-inflamed tumor phenotype, which improves disease control by immune mechanisms and might serve as a prognostic biomarker. The T cell-inflamed phenotype consisting of infiltrating T cells, chemokines, and type I interferon signature has also been shown to correlate with clinical benefit from immunotherapies, such as vaccines and IL-2.³ Therefore, further studies should also investigate whether density or composition of TIL infiltrates could be used as predictors for response to immunomodulatory therapy in cancer.

Moreover, a combination of biomarkers may have enhanced predictive power compared with individual markers. A combination of parameters, including the proximity of PD-1-positive to PD-L1-positive cells, CD8⁺ T cell activation, and IFN signaling pathway markers, has shown a higher predictive value. Since the checkpoints carry out effects targeting mostly T cells, TILs play an important role in response to checkpoint immunotherapies. In 46 patients with melanoma treated with pembrolizumab, higher numbers of immune cells expressing CD8, PD-1, or PD-L1 at the invasive tumor margin and inside tumors were detected in

pretreatment samples from responding patients than in patients who did not respond.⁵⁰ Additionally, a greater increase in CD8⁺ T cells in serial tumor samples during therapy correlated with a greater tumor regression on imaging. An increase in CD8⁺ T cell density was also seen in serial biopsy samples of tumors during anti-PD-1 treatment in the responding group but not in the disease progression group. Another study of patients with melanoma given PD-1 blocker therapy showed a modest association between CD3⁺CD8⁺CD45RO⁺ T cell densities in pretreatment samples of responders versus non-responders.³³ Despite the hints of clinical responses associated with several potential biomarkers, baseline CD8⁺ T cell density overlaps between the patients with a response and those with disease progression, which hinders the establishment of an absolute cutoff as a clinically useful predictor. Assessment of pretreatment tissue samples from patients with advanced malignancies treated with atezolizumab using IHC showed that the likelihood of response to atezolizumab was significantly correlated with higher levels of PD-L1 expression on TILs but not with PD-L1 expression on tumor cells.⁷⁷ However, in most patients with disease progression, no PD-L1 upregulation in tumor cells or TILs was detected, with three patterns observed: little or no TIL infiltration, the presence of intratumoral immune infiltrates with minimal to no expression of PD-L1, or the presence of an immune infiltrate solely around the outer edge of the tumor bed. A study using a multi-parameter flow cytometry assessment of freshly isolated pretreatment tumor samples from patients with metastatic melanoma showed that an increase in the fraction of CD8⁺ TILs with high levels of both PD-1 and CTLA-4 expression, which exhibit a partially exhausted T cell phenotype that is characterized by the ability to produce IFN- γ and the inability to produce TNF- α and IL-2, strongly correlated with response to pembrolizumab or nivolumab.⁷⁸

In addition to exploring the predictive and prognostic values of TILs, several studies also suggested the essential role of tumor-associated tertiary lymphoid structures (TLS), lymphoid aggregates that structurally resemble secondary lymphoid-like organs in the immune response against a tumor.⁷⁹ TLS are sites where T cells and B cells are contacting with APCs, such as DCs and macrophages, and immune reactions toward tumor-associated antigens are generated. Studies in human tumors support the utility of TLS as prognostic biomarkers in cancer, which can complement established immune prognostic factors that are based on the assessment of T cell density by IHC in the center and in the invasive margin of the tumors. Additionally, the presence of TLS-associated mature DCs appears to be required for the positive prognostic value of CD8⁺ TILs. In NSCLC, patients with a high CD8⁺ T cell density and a low TLS-associated mature DC density have a significantly higher risk of death than those patients with a high CD8⁺ T cell density and a high TLS-associated mature DC density.⁸⁰ Similarly, clear cell RCC enriched in CD8⁺ TILs but having a poor clinical outcome were found to be abundantly infiltrated with dysfunctional DCs and scarce TLS, indicating that immature DCs are a potential predictor for poor response to PD-1 axis blockade.⁸¹ It should be noted that DC-Lamp⁺ mature DCs are selectively found within lymphoid aggregates and may represent a specific marker of TLS within the TME in multiple tumor types. The mechanism of action of ICB takes advantage of the receptor–ligand interactions between tumor cells and immune cells; thus, increased awareness and further studies of the role of TILs are urgently needed for comprehensive biomarker development to predict the response to ICB therapy.

T CELL RECEPTOR CLONALITY

Antigens that are successfully presented on MHC molecules must be recognized by their cognate T cell receptors (TCRs). Using nucleotide level sequencing of the complementarity-determining

region (CDR3) domain, a signature corresponding to TCR clonality may be detected.⁸² Given the high accumulation of CD8⁺ T cells at the tumor bed among patients who responded to ICB therapy, further studies also investigated whether baseline TILs had a narrow TCR repertoire focused on a tumor-specific immune response and whether this narrow repertoire correlated with response to pembrolizumab.⁵⁰ Next-generation sequencing (NGS) was performed on pretreatment samples to capture all uniquely rearranged variable TCR β -chain regions. Of the 23 patients with available response and sequencing data receiving pembrolizumab treatment, 12 (52%) patients had an ORR, and 11 (48%) had disease progression.⁵⁰ The results showed that more restricted TCR β -chain diversity, and thus a more homogeneous T cell population, was significantly positively correlated with the response to pembrolizumab treatment. Moreover, pretreatment and posttreatment biopsy samples showed a significant increase in these clones after anti-PD-1 therapy in the responding group compared with that in the disease progression group, which implies a tumor-specific response to therapy for these patients. Notably, baseline TCR clonality did not highly correlate with TIL density, which suggests that some patients whose tumors have a low TIL density might still benefit from anti-PD-1 therapy if the TIL population has restricted TCR clonality specific to the tumor-associated antigen. This hypothesis needs to be further validated in a large patient population and might require identification of the recognized tumor antigens before such an approach could be used as a predictor. However, other studies have failed to replicate the correlation of TCR clonality with response to PD-1 blockade.⁸³ Another study examined CD8⁺ T cells in two patients with melanoma who responded to CTLA-4 blockade and identified CD8⁺ populations which were specific for tumor antigens.⁸⁴ TCR clonality and T cell antigen recognition, which are difficult to measure, have been inconsistent as biomarkers in prior studies, limiting their possible utility thus far.

TMB

Given that ICBs essentially “unleash” endogenous antitumor T cell responses, one could imagine that patients with a higher TMB, irrespective of driver mutations, would be more responsive to ICB. Accumulation of somatic mutations is a hallmark of tumors, but TMB varies dramatically both within and among tumor types, which reflects significant differences in the balance of DNA damage and DNA repair fidelity among tumors. Beyond their effects on the biological behavior of tumors, mutations in tumor cells can result in the generation of novel antigens, also termed neoantigens, which can be recognized as “foreign” by the immune system. Acquired mutations in the exome have the potential to manifest as changes at the protein level by two mechanisms: they create new sequences at the rearrangement, deletion or insertion junctions and, if out of frame, will create new amino acid sequences until a stop codon is reached.⁸⁵ Mutant proteins are processed by the proteasome, and the resultant neopeptides are bound by MHC molecules and presented on the cell surface. Because humans express three to six HLA I and three to six HLA II molecules, a single DNA alteration can generate multiple epitopes that are potentially recognized by T cells.^{86,87} Evidently, the antigenicity of neopeptides is not related to their function; passenger mutations with no functional role can be perfectly good tumor antigens, suggesting that tumors with a greater mutational load could possess more neoantigens, and thus, the patient may possess a larger repertoire of extant tumor-specific T cells. Several bioinformatics approaches have been developed to predict tumor neoantigens based on predicted MHC class I binding, TCR binding, and patient HLA type. Many clinical studies have established a correlation between mutational load and response to ICB therapy, which indicates that an increased overall mutational burden may result in greater neoantigen load and,

thus, is positively associated with clinical response and survival outcomes. Marked efficacy of ICB has been noted in melanoma and NSCLC patient tumors that are known to have higher numbers of somatic mutations than other tumors,⁸⁸ resulting in the generation of neoantigens, thereby enhancing T cell reactivity against the tumor, thus facilitating the efficacy of ICB. Melanoma, a carcinogen-induced cancer with one of the highest mutational loads among human tumors, has a particularly high response rate to anti-PD-1 therapy. Melanoma is also the only cancer in which anti-CTLA-4 monotherapy produces a significant response rate, and correlations between mutational load and anti-CTLA-4 response in melanoma have been reported.^{89,90} In contrast, tumors with relatively low median TMB, such as pancreatic and prostate cancer, have shown little response to PD-1 blocker therapy. Thus, whether TMB itself, besides specific oncogene or tumor suppressor gene mutations, will predict the response to anti-PD-1 therapy in a given tumor type remains to be studied.

Additionally, the relationship between the ability of tumor cells to correct intrinsic DNA errors and the response to immunotherapies has been under active investigation. A deficiency in DNA repair mechanisms can also lead to a high mutational burden in tumors, which can facilitate the efficacy of ICBs. Several reports have now linked a specific DNA damage exposure or a specific DNA repair pathway deficiency with ICB response.^{91–93} Evidence comes from the findings regarding PD-1 blockade in NSCLC, in which smokers had a higher response rate.²¹ Mutational load in smoking-associated lung cancer is known to be much higher than in non-smoking-associated lung cancer. An analysis of a small group of patients with lung cancer who were receiving pembrolizumab showed that patients deriving clinical benefit had higher mutational densities than those who did not benefit, and this genomic smoking signature was more predictive of ICB response than patient-reported smoking history. Moreover, several patients who achieved durable benefit from ICB had tumors with somatic mutations in genes involved in DNA replication or repair (such as *POLE*, *POLD1*, and *MSH2*), indicating that loss of DNA repair fidelity may have contributed to the increased mutational burden and ICB response in these tumors.⁹⁴

The most robust evidence for the correlation between DNA repair deficiency and response to ICB was obtained with tumors with dMMR machinery,^{92,93} dMMR is defined by defects in one or more of six genes that encode components of the MMR complex.⁹⁵ Functional loss of MMR genes in tumor cells thus result in many mutations in tumors, which are correlated with enhanced response to ICB therapy.^{31,95} Initial evidence for an interaction among dMMR, the immune microenvironment, and clinical outcomes based on IHC and NGS platforms has shown that a subset of CRC with an activated cytotoxic T lymphocyte (CTL)/Th1 microenvironment had improved prognosis and frequently harbored defects in the MMR machinery, as evidenced by MSI.⁴¹ The activated immune microenvironment within MSI tumors was counterbalanced by the selective elevation of multiple immune checkpoints, including PD-1, PD-L1, CTLA-4, and LAG3, raising the possibility that ICB may be selectively efficacious in the MSI subset of CRC.⁴¹ Based on this finding and the known correlation among MMR, high mutational burden, and prominent T cell infiltrate, a small phase II trial was initiated in patients with progressive metastatic carcinoma treated with pembrolizumab.⁹¹ Both PFS and ORR were higher in CRC patients with MMR deficiency than in those without this deficiency. Similar PFS and ORR were also noted in patients with MMR-deficient non-CRC. Whole-exome sequencing showed a significantly higher TMB in patients with MMR-deficient tumors compared with the TMB in those with MMR-proficient tumors; a higher somatic mutational burden was correlated with longer PFS.⁹¹ Notably, the Food and Drug Administration (FDA) recently granted its first tissue/site-agnostic approval to pembrolizumab for unresectable or metastatic, hMSI

or dMMR solid tumors that have progressed on prior treatment and who have no satisfactory alternate treatment options.

These studies present strong evidence for the use of TMB as a biomarker for ICB-based therapy. However, there are several limitations for using mutation landscape to identify potential patients that should be noted, including the small size of patient cohorts, the inclusion of patients with variable treatment history, and the use of tumor samples obtained at various timepoints;⁸⁹ the randomness of non-driver mutations in a tumor translates to the random generation of peptide epitopes that can be presented by host MHC molecules. Important exceptions were also noted in these studies, including patients with high mutational burden who did not respond to ICB and those with very low mutational burden with a good response to these agents. The mutation frequency varies in diverse tumors and even in one tumor type that is influenced by the exposure degree to the environmental mutagens, such as smoking. The large variability of somatic mutation makes it difficult to set the same cutoff point for mutation burden to predict the response to ICB therapy. Additionally, current computational algorithms are inadequate in predicting which mutations will generate epitopes recognized by T cells in the host. Whereas the application of current algorithms to specimens with high TMB, typical for melanomas and smoking-associated lung cancers, predicts hundreds of possible neoantigens, T cell responses are typically found against very few. Until these limitations can be resolved, the use of multiple types of data across platforms to predict mutational burden is probably the most practical approach to the development of biomarkers for the response to checkpoint blockade-based cancer immunotherapy.

IFN- γ

IFN- γ is a cytokine that was initially discovered to be crucial for the host response to viral infections and was recently found to play a key role in immune regulation and antitumor immunity.⁴⁹ IFN- γ is mainly produced by NK cells and NK T cells in innate immune response and by activated T cells in the setting of antigen-specific immunity. Loss of the IFN- γ signaling pathway is one of the main mechanisms of resistance to ICB.²⁷ However, the predictive value of IFN- γ to ICBs is still controversial. Notably, in a phase I study of the PD-L1 inhibitor atezolizumab, patients with advanced melanoma who responded to therapy had elevated expression of IFN- γ as well as IFN- γ -inducible genes (i.e., *IDO1* and *CXCL9*); however, this association was weak in patients with NSCLC or RCC.⁷⁷ Similarly, one study with a tumor-bearing mouse model revealed that the capacity of peripheral lymphocytes to produce IFN- γ may be a viable strategy for assessing patients' response to the onset of ICB and a valuable addition to existing immune monitoring portfolios.⁹⁶ Other studies have also examined the consequences of aberrations in downstream IFN- γ signaling. Loss-of-function mutations in genes encoding the IFN-receptor-associated tyrosine kinases JAK1 and JAK2 resulted in a lack of response to IFN- γ in tumor cells, indicating an additional role of IFN- γ in the development of an acquired resistance to ICB.⁹⁷ Given the necessity of IFN- γ signaling in response to antitumor immunity, further studies are urgently needed to determine whether the signaling status of IFN- γ is a reliable biomarker for response to ICB across different tumor types.

PERIPHERAL BLOOD BIOMARKERS

Although the assessment of the TME profiles is essential to identify robust predictors, clinical markers and morphological characteristics in patients and tumors can also be useful to stratify patients into subpopulations. Due to the ease of accessibility during therapy, peripheral blood predictors of response to ICB are highly sought-after. Blood is also more homogeneous than tumors, making the sampling of blood easier and more consistent.

Therefore, multiple peripheral blood biomarkers have been used to predict response and to monitor longitudinal changes during cancer immunotherapy.

Serum markers, such as lactate dehydrogenase (LDH) and immune cell counts, in routine blood analyses can be useful predictors of response to ICB. The predictive value of the combined assessment of these serum markers has been evaluated in patients with melanoma treated with pembrolizumab. Serum LDH is a standardized and simple marker that is easy to use in the clinic and is an established prognostic marker in melanoma, with elevated levels portending a worse prognosis.⁹⁸ Another study also confirmed the association of elevated baseline LDH with worse OS in patients with advanced melanoma who received anti-PD-1 therapy, further indicating that change in LDH during treatment was significantly correlated with response.⁹⁹ High LDH is also associated with poor prognosis in untreated patients and those receiving other therapies, such as chemotherapy.¹⁰⁰ Another potential serum biomarker for both baseline and early treatment changes is angiopoietin-2, a ligand of the receptor tyrosine kinase, which functions as a vessel-destabilizing molecule and is involved in resistance to anti-VEGF therapies.¹⁰¹ Angiopoietin-2 may also contribute to resistance to ICB therapy, which may contribute to its role in the recruitment of monocytes/macrophages into the TME and induction of PD-L1 expression on M2-polarized macrophages, which contributes to immunosuppression.¹⁰² High pretreatment serum angiopoietin-2 was associated with reduced OS in patients treated with CTLA-4 and PD-1 blockade.

Other peripheral blood cell markers have been studied as pretreatment predictors. In advanced melanoma patients treated with ipilimumab, a relative lymphocyte count (RLC) <10.5% was correlated with a 1-year survival probability of only 5%.⁹⁸ However, a low frequency of myeloid-derived suppressor cells (MDSCs), defined by flow cytometry as Lin⁻CD14⁺HLA-DR^{-/low}, was correlated with the highest probability of long-term survival.⁹⁸ In addition to a modest change of the lymphocyte compartment before therapy, a recent study using mass cytometry has identified an immune cell type, known as classical CD14⁺CD16⁻CD33⁺HLA-DR^{hi} monocytes, in the peripheral blood as a potential biomarker for response to PD-1 blockade therapy in metastatic melanoma.²⁸

Other predictors in peripheral blood are under investigation. For example, soluble PD-L1 (sPD-L1) has shown mixed results as a prognostic marker.¹⁰³ Changes in circulating sPD-L1 early after treatment could not distinguish responders from those with progressive disease, but a rise in sPD-L1 5 months after treatment initiation was correlated with partial responses to CTLA-4 or PD-1 blockade therapy.¹⁰³ Another potential predictor is circulating tumor DNA (ctDNA), which can be measured in peripheral blood and may be an accurate marker at baseline and during therapy. Patients with advanced melanoma who had a persistently elevated ctDNA on PD-1 blockade therapy had a poor prognosis, indicating that longitudinal assessment of ctDNA in advanced melanoma may help predict tumor response, PFS, and OS.¹⁰⁴ In addition, ctDNA was a better predictor of response and prognosis than LDH, disease burden, or performance status; however, ctDNA profiles were not accurate in patients with brain metastases. Additionally, there was a significant correlation between changes in ctDNA levels and tumor size at 8 weeks in a prospective study that evaluated patients with multiple tumor types treated with PD-1 blockades.¹⁰⁵ These results suggest that changes in ctDNA levels during therapy could be a promising tool for accurate monitoring of treatment efficacy with ICBs.

CONCLUSIONS AND PERSPECTIVES

Recent clinical advances in the blockade of immune checkpoints have brought about a paradigm shift in the treatment landscape of advanced malignancies. Despite these encouraging results,

clinical outcomes remain highly variable. Only a subset of patients benefits from ICB therapies, whereas the bulk of patients fails to respond. The development of robust pretreatment predictors and on-treatment monitors of response to ICBs is urgently needed. The development of reliable criteria that distinguish responders from non-responders before the initiation of treatment is a key next step for the further progress of this field toward personalized cancer immunotherapy. Despite the existence of numerous candidate predictors that have been shown to correlate with response in pre-clinical and clinical studies, no predictor is sufficiently robust in aiding patient selection for enrollment, and no predictor has been able to unequivocally identify the best combination of treatment. A combination of markers may enhance the predictive power. Thus, advances in translational predictor research are essential for personalized checkpoint blockade-based cancer immunotherapy as either a monotherapy or combinational therapy.

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ADDITIONAL INFORMATION

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