

# The Where, the When, and the How of Immune Monitoring for Cancer Immunotherapies in the Era of Checkpoint Inhibition

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## Abstract

Clinical trials with immune checkpoint inhibitors have provided important insights into the mode of action of anticancer immune therapies and potential mechanisms of immune escape. Development of the next wave of rational clinical combination strategies will require a deep understanding of the mechanisms by which combination partners influence the battle between the immune system's capabilities to fight cancer and the immune-suppressive processes that promote tumor growth. This review focuses on our current understanding of tumor and circulating pharmacodynamic correlates of immune modulation and elaborates on lessons learned from human translational research with checkpoint inhibitors. Actionable tumor markers of immune

activation including CD8<sup>+</sup> T cells, PD-L1 IHC as a pharmacodynamic marker of T-cell function, T-cell clonality, and challenges with conduct of trials that ask scientific questions from serial biopsies are addressed. Proposals for clinical trial design, as well as future applications of peripheral pharmacodynamic endpoints as potential surrogates of early clinical activity, are discussed. On the basis of emerging mechanisms of response and immune escape, we propose the concept of the tumor immunity continuum as a framework for developing rational combination strategies. *Clin Cancer Res*; 22(8); 1865–74. ©2016 AACR.

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## Introduction

Since the approval of ipilimumab, surface receptors of immune cells have become validated targets in cancer therapy (1–4). Agents directed against the PD-L1/PD-1 pathway have shown promising efficacy in several indications, and agents such as nivolumab and pembrolizumab are now approved in melanoma and lung cancer (5–8). Despite these encouraging results, transformative clinical benefit is limited to a few patients. Spurred by the impressive efficacy of the combination of CTLA-4 and PD-1 blockade in melanoma (9), a large number of immunotherapy combinations are now being clinically evaluated. In addition, many established therapies, such as antiangiogenesis agents, targeted therapies, and standard-of-care chemotherapies, are being tested in combination with checkpoint inhibitors. The multitude of different approaches and combinations speaks to the fact that rational development of immunotherapy combinations has remained a challenge, owing to the incomplete understanding of the mode of action of immunotherapies and the lack of characterization of the immune effects of combination partners.

To improve our understanding of the immunologic responses to cancer treatments, measurement of pharmacodynamic markers

are frequently incorporated in clinical trials. These measurements aim to (i) increase our understanding and confirm the mode of action and establish whether biomarker modulations correlate with clinical efficacy; (ii) help define the dose–response relationship; and (iii) identify possible mechanisms of resistance to treatment or immune evasion. Here, we review the current literature on pharmacodynamic markers in the context of immunotherapies of cancer and highlight some of the key challenges and future directions for the field. While the PD-L1 diagnostic is an important marker for patient selection, particularly with 22C3 as the first approved IHC assay for pembrolizumab in non-small cell lung cancer, the diagnostic assays will not be the focus here.

## The Where: Immune Monitoring in the Tumor

Histologically, tumors can be broadly categorized as inflamed or noninflamed (10, 11). Inflamed tumors are characterized by the presence of tumor-infiltrating lymphocytes (TIL), high density of IFN $\gamma$ -producing CD8<sup>+</sup> T cells, expression of PD-L1 in tumor-infiltrating immune cells, possible genomic instability, and the presence of a preexisting antitumor immune response. In contrast, noninflamed tumors are immunologically ignorant and are poorly infiltrated by lymphocytes, rarely express PD-L1, and at the antipodal end of the tumor immunity continuum, are characterized by highly proliferating tumors with low mutational burden and low expression of antigen presentation machinery markers including MHC class I (refs. 12, 13; Fig. 1). Pediatric malignancies are a representative example of noninflamed tumors often driven by single gene mutational drivers (12).

To evade immune surveillance, tumors create an immunosuppressive microenvironment by recruiting myeloid-derived suppressor cells or secrete factors including TGF $\beta$ , which plays a

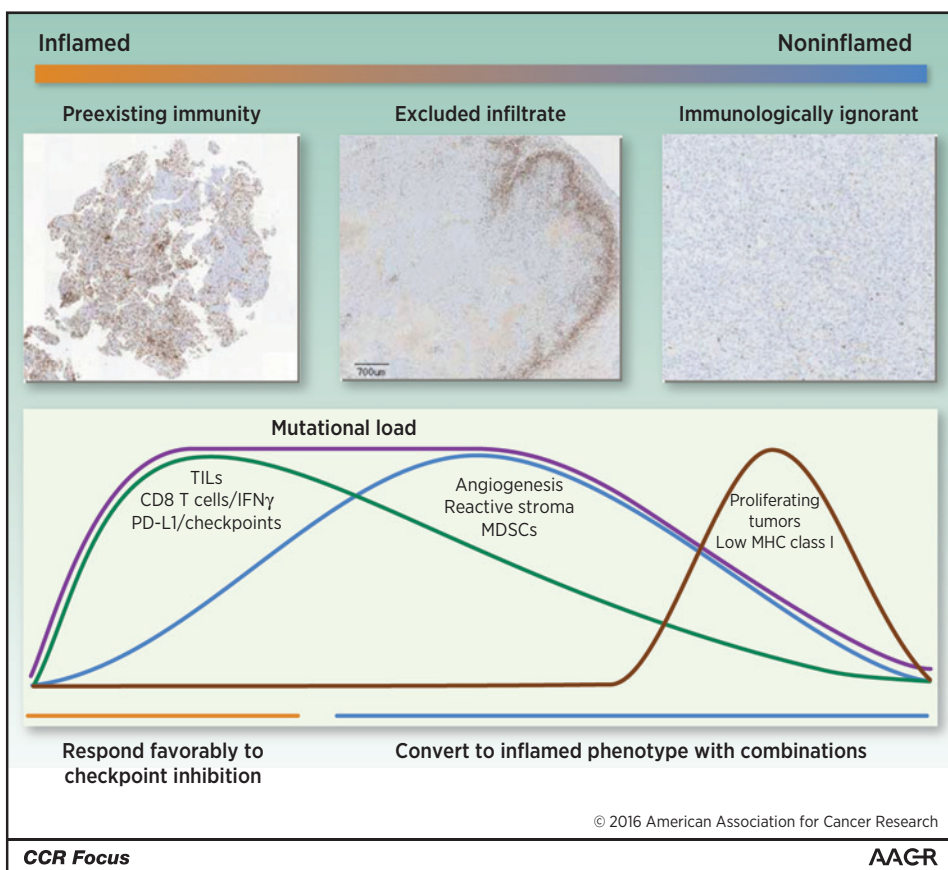
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**Figure 1.**

The tumor immunity continuum. Representative images of tumor CD8 IHC show three patterns of T cells associated with tumor cells. Tumors with preexisting immunity are represented by abundance of TILs, dense functional CD8<sup>+</sup> T-cell infiltration reflected by increased IFN $\gamma$  signaling, expression of checkpoint markers, including PD-L1, and high mutational burden. These characteristics reflect highly inflamed tumors. Despite high mutational burden, tumors with the excluded infiltrate or stromal T-cell phenotype are represented by increased influence of immunosuppressive reactive stroma, myeloid-derived suppressor cells (MDSC), and angiogenesis, all of which prevent infiltration of T cells into the tumors or suppress activation of T cells in the tumor milieu. Finally, immunologically ignorant tumors that contain very low infiltration of T cells are genomically stable with highly proliferating tumor cells. These are representative of noninflamed tumors.

dual role of inducing the expression of extracellular matrix genes and suppressing the expression of chemokines and cytokines required to facilitate T-cell infiltration into tumors (14). The resulting reactive stroma and dense extracellular matrix create a barrier to the infiltration of immune cells within the tumor which manifests into an excluded infiltrate phenotype with peritumoral or stromal T-cell localization (15). Tumors that exhibit high expression of gene signatures of reactive stroma or TGF $\beta$  signaling are often associated with lower expression of immune markers and are associated with poor outcomes across a number of cancer types, including ovarian cancer and colorectal cancer (16–18). The collective clinical evidence to date suggests that checkpoint inhibitors largely act by reinvigorating preexisting antitumor T-cell responses and are most effective in inflamed tumors as characterized by tumor PD-L1 positivity, high CD8<sup>+</sup> T-cell density, or the presence of a strong IFN $\gamma$  cytolytic T-cell signature (2, 6, 19, 20).

Clinical trials investigating checkpoint inhibitors are starting to yield scientific insight into the molecular and/or cellular features associated with response or immune escape. The best studied of these markers is the density of CD8<sup>+</sup> T cells by IHC as well as the measurement of tumor PD-L1. An active and functional antitumor T-cell response results in the production of IFN $\gamma$  (21, 22). PD-L1 is strongly induced by IFN $\gamma$ , resulting in adaptive expression on tumor cells and tumor-infiltrating immune cells as a negative feedback mechanism (23, 24). Biopsies in patients who respond favorably to PD-L1 inhibition have shown concomitant increases in CD8<sup>+</sup> T-cell densities with adaptive increase in tumor PD-L1 expression (2, 20), supporting PD-L1 expression as a surrogate for

an active CD8-driven antitumor T-cell response. Contextual evaluation of these markers has been informative in uncovering patterns of immune escape to checkpoint inhibition. For example, examination of serial on-treatment biopsies in patients treated with atezolizumab show three distinct patterns in the tumor immune microenvironment (2). Biopsies from responding patients are characterized by the phenotype described by preexisting immunity in the predose tissue with increased density of proliferating intra-epithelial CD8<sup>+</sup> T cells upon PD-L1 inhibition. These T cells are functionally active as represented by an increase in the IFN $\gamma$  signature and PD-L1 expression both in tumor cells and in TILs. In patients who do not respond to atezolizumab, two distinct patterns are observed. In one instance, treatment results in the lack of appreciable increase in CD8<sup>+</sup> T cells posttreatment. These tumors exhibit a nonfunctional immune response as seen in the immunologically ignorant phenotype (Fig. 1). In the second pattern, when predose tumors show the excluded infiltrate phenotype, PD-L1 inhibition results in increased functional CD8<sup>+</sup> T cells. However, the T cells are unable to infiltrate the tumor. Such excluded infiltrate patterns have been reported to be prevalent in liver metastases of colorectal cancer and melanoma (25, 26) and can be observed across a number of cancer types. These data provide clinical proof that preexisting immunity is necessary for an objective response to these agents (2). This finding is further supported by work from Tumeh and colleagues (20), where serial biopsies from melanoma patients treated with pembrolizumab were evaluated for the presence of CD8<sup>+</sup> T cells by IHC. Increased CD8<sup>+</sup> T-cell density was observed in posttreatment biopsies from

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responding patients. Conversely, tumors from nonresponders were characterized by low CD8<sup>+</sup> T-cell density in their predose biopsies with no appreciable increase in T-cell proliferation post-treatment. Furthermore, high baseline CD8<sup>+</sup> T-cell density both in the center and at the invasive margin was strongly predictive of clinical benefit. Similar observations have been made with ipilimumab in melanoma, in which elevated IFN $\gamma$  gene signatures in archival tumors are associated with improved clinical benefit (19). These data collectively point to the inflamed tumor being particularly sensitive to checkpoint inhibition. Moreover, they point to potential mechanisms of immune escape along the tumor immunity continuum wherein these agents are incapable of mounting an effective antitumor immune response in either the excluded immune infiltrate-associated immunosuppressed tumors or immunologically ignorant immune deserts (Fig. 1). Our understanding of these mechanisms of immune evasion is fundamental to the development of next-generation combination strategies.

There is now growing evidence for antigenic load of the tumor contributing to an effective antitumor immune response (27–30). Indeed, mutational load may be a particularly sensitive marker for RECIST responses to checkpoint inhibitors, independent of PD-L1 IHC (29). Highly mutated tumors are associated with an increased likelihood of generating mutated neoantigens that can be recognized by immune cells as foreign (13). Tumors harboring mismatch repair deficiencies even in cancers like colorectal cancer that often do not respond to monotherapy checkpoint inhibitors are particularly sensitive to anti-PD-1 therapy (30). In comparison with CD8 IHC, evaluation of an antigen-specific T-cell response constitutes a specific marker of antitumor immunity. One of the first technologies used to detect antigen-specific T cells in the tumor was MHC class I tetramer staining either applied in resected lesions made into a suspension or fixed tissues stained *in situ*. Using the former technology, high numbers of functional T cells could be identified in metastatic melanoma lesions either specific for the vaccine (31) or as a consequence of antigen spreading following vaccination (32, 33). Moreover, levels of antigen-specific T cells were also reported to relate to disease outcome in colorectal cancer (34). *In situ* MHC class I tetramer staining was also used to delineate the specificity of tumor-infiltrating T cells following vaccination with autologous dendritic cells loaded with tumor-derived peptides (35). One of the limitations of this technology is the sensitivity required to detect low frequency events in limiting amounts of tissue material. T-cell receptor (TCR) V $\beta$  repertoire analysis provides an alternative practical approach to demonstrate effective antitumor immunity. This approach uses the ability of T cells to recognize different peptides by the complementarity determining region 3 (CDR3) of the TCR and examines the overall changes in the TCR repertoire seeking to identify clonotypes that carry a given V $\beta$ -CDR3-J $\beta$  sequence (36). Although this method cannot elucidate the specificity of the detected T cells, it is reasonable to expect that following antigen recognition, clonal proliferation results in the amplification of T cells carrying the TCR sequence recognizing antigens present in the tumor microenvironment. To this end, Tumei and colleagues (20) reported that response to pembrolizumab was positively associated with a more restricted V $\beta$ -chain usage in predose melanoma tissues. Moreover, more than 10 times as many clones expanded after anti-PD-1 therapy in patients who responded favorably to checkpoint blockade, highlighting the potential utility of this biomarker as an on-treatment marker of response. Given these findings, renewed interest has been expressed in the

field of personalized cancer vaccines to assess the feasibility of this approach as a viable therapeutic strategy for the treatment of cancers whereby patients can be vaccinated with immunogenic neoantigens tailored to their unique immunogenic fingerprint. While still in the nascent phase of clinical testing, this strategy may provide one approach to converting noninflamed tumors to an inflamed phenotype. While CD8-driven T-cell responses have been well documented in the context of checkpoint inhibitors, the emerging role of CD4 T-cell responses against mutated neoantigens presented by MHC class II molecules on antigen-presenting cells may be central in monitoring responses to personalized cancer vaccines or adoptive cell therapies (37–40).

## The When: Optimal Time Points for Serial Biopsy Measurements

While serial biopsies from clinical trials are highly informative in elucidating the molecular mechanisms that determine a tumor's response, caution should be taken when interrogating data from such studies. Some of the key factors to consider include location of the serial biopsies, timing of the on-treatment biopsy, and interpretation of the tumor immune contexture. Location of the pre- and posttreatment biopsies is an important consideration in minimizing biologic variability. Organ-dependent differences in the tumor immune landscape can result in variable results if the pre- and posttreatment biopsies are not derived from tumor lesions located in the same organ. For example, liver metastases and lymph node metastases represent distinct tumor immune milieus that may confound interpretation of results if used to select biopsy pairs. Although challenging to implement, identifying a single lesion or lesions from the same organ may minimize the impact of biologic variability on drug treatment-related pharmacodynamic readouts.

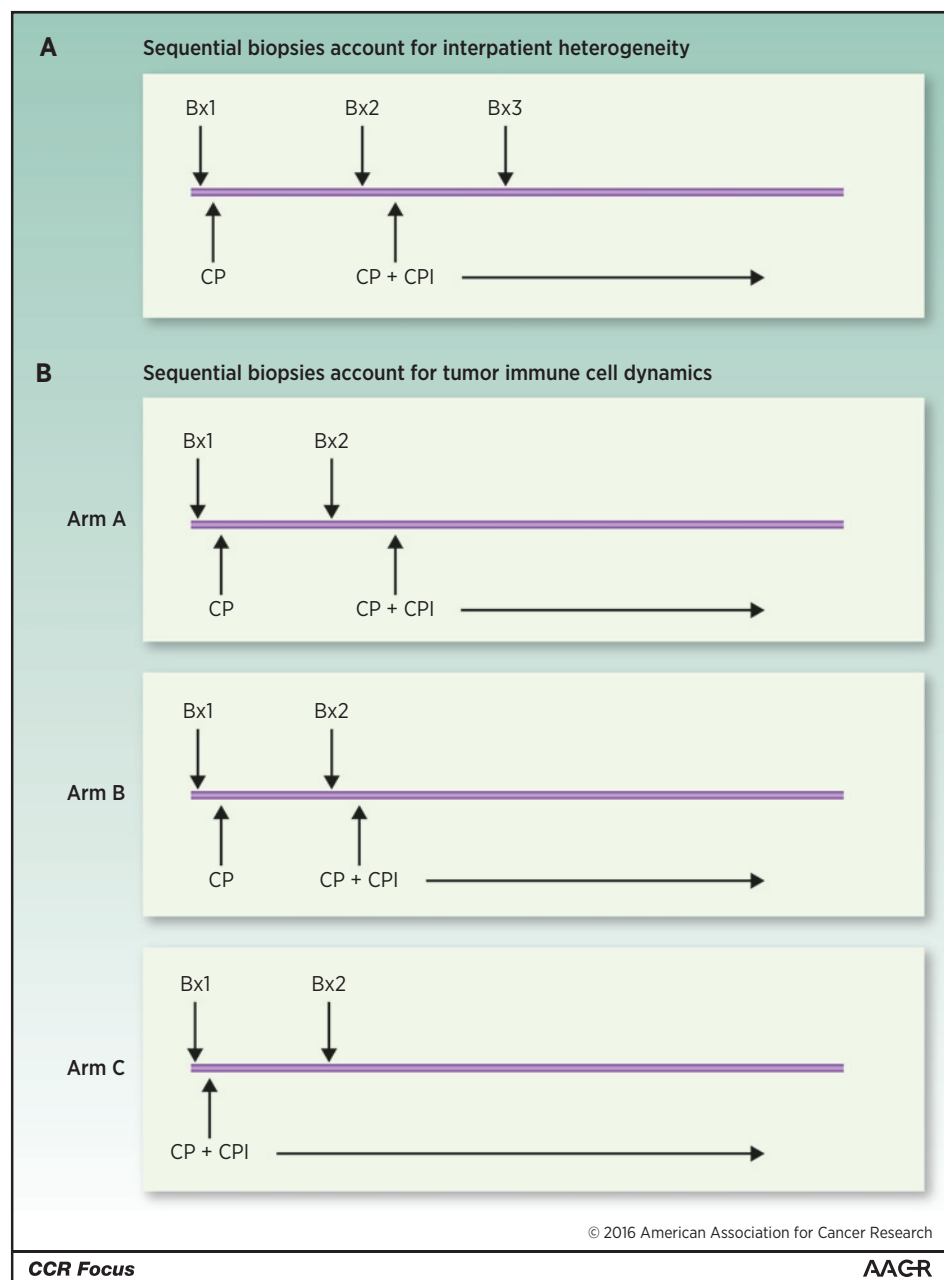
A second important consideration is the time point selected for the on-treatment biopsy. The time taken to mount an effective antitumor immune response by tumor immune-modulating agents is not well understood in humans. Clinical data suggest that the time to a RECIST response to a PD-L1/PD-1 targeted agent is typically 6 to 12 weeks in most cancer types studied to date (2, 6, 8). Using time to response as a gauge, posttreatment biopsies procured prior to an expected response ensure the presence of viable tumor cells for pathologic evaluation of the tumor area that is required for enumeration of immune cells as well as data normalization for downstream methods such as gene expression profiling or digital pathology. Thus, an optimal postdose time point may be 2–3 weeks after initiation of therapy. Yet another factor to consider with serial biopsies is that single lesions do not represent the general response patterns in an individual. Thus, dynamic radiologic measurements of the biopsied lesions may aid biologically meaningful interpretation of data. This is particularly true in patients who respond early to therapy. Often, the more accessible lesions in such patients may be the nonresponding lesions that can confound the biologic interpretation of biomarker data generated from such serial biopsies. The development of sophisticated imaging tools for lymphocyte infiltration like CD8 imaging may in the future allow more precise dynamic measurement of T-cell infiltration (41). Finally, while enumeration of CD8<sup>+</sup> T cells in the invasive margin and center of tumor confer independent prognostic significance (42), enumeration of T cells at the invasive margin in core-needle biopsies can be highly variable due to surgical sampling procedures. These factors need

to be taken into consideration while interpreting contextual data from serial biopsies.

### The How: Optimal Trial Designs for Interpretation of Biomarker Data

Anticancer agents hypothesized to convert noninflamed to inflamed tumors present as scientifically rational options to be studied as combination partners for checkpoint inhibitors. Enthusiasm for combinations also comes from compelling preclinical data supporting synergistic efficacy for established anticancer agents with immunotherapies (43–45). A bedside-to-bench reverse translational mechanistic dissection of the failure of the

combination of radiotherapy with anti-CTLA-4 therapy in melanoma patients was shown to be associated with upregulation of PD-L1 and subsequent T-cell exhaustion in preclinical models. This mechanism of resistance postulates a triple combination of radiotherapy with anti-CTLA-4 and a PD-L1/PD-1 inhibitor to overcome T-cell exhaustion and promote clonal expansion of T cells (45). Clinical interrogation of immune modulation of each therapy alone is an important step toward confirming proof of mechanism in human tumors. Tumor immune monitoring in trials designed with concurrent administration of the combination (46) or in trials designed with a run-in of one partner (47; Fig. 2A) is reasonable for interpretation of the combination biology. However, given the dynamic nature of tumor immune



**Figure 2.** Trial design considerations for combination therapies. A, a commonly employed trial design for interrogation of drug mechanism of action employs multiple biopsies (Bx) from the same individual with the combining partner (CP) alone or the combination of CP with checkpoint inhibitors (CPI). B, tumor immune modulation is a dynamic process. Trial designs that incorporate sequential biopsies keeping the time between biopsies constant for each agent enable comparison of the impact of each combining partner on tumor immune microenvironment.

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responses, studies that incorporate a fixed time point between biopsies to interrogate the biology of each combining partner may reduce the number of variables to account for while interpreting data from small translational trials. A three-arm trial in which a run-in with each combination partner with serial biopsies taken at a fixed time point after the first dose in both arms allows for a fair evaluation of biologic effects attributed to each combination partner alone (Fig. 2B). In Fig. 2B, Arm A and B represent biopsies taken prior to and after run-in with the combination partner (Arm A) or the checkpoint inhibitor (Arm B), after which combination therapy is administered in both arms of the study. Arm C represents serial biopsies procured before and after the combination, at the same time point as Arms A and B. As all patients on the trial eventually receive the combination therapy, all patients can be considered efficacy evaluable. Such trial designs can be useful for both biologic and clinical interpretation of immunotherapy combinations. As stated earlier, clinical advancement of imaging technologies such as anti-CD8 immuno-PET will provide further flexibility with respect to monitoring dynamic T-cell responses of immunotherapies (41).

An important consideration for pharmacodynamic markers relates to the use of biomarkers for dose selection. Unlike small-molecule-targeted agents such as Raf/MEK/PI3K inhibitors that block measurable signaling mechanisms in tumor cells, evidence for proximal pharmacodynamic biomarkers unequivocally demonstrating dose-dependent target inhibition for large-molecule immunotherapies is limited. In the absence of proximal biomarkers, peripheral receptor occupancy assays have been conducted for large molecules that bind surface receptors (48). For approved immunotherapies such as anti-PD-1 and anti-CTLA-4, the level of PD-1 or CTLA-4 expression on the surface of tumor-infiltrating T-cells is considerably high compared with circulating T cells (49). Incomplete receptor occupancy has been observed at the recommended dose of nivolumab (anti-PD-1) on TILs from melanoma patients compared with corresponding peripheral T cells, thus highlighting the importance of confirming peripheral pharmacodynamic changes in the tumor, particularly as they relate to dose selection (49).

## The Where: Blood as the Tissue Source for Immune Monitoring

Peripheral immune monitoring is attractive given the ease of sample collection and the ability to monitor pharmacodynamic activity over time. Early research on the effects of checkpoint inhibitors, mainly anti-CTLA-4, focused on analyses in blood for the identification of predictors or early indicators of response. However, the question arises whether these measurements have any relevance and reflect antitumor immunity. A large body of work now exists on peripheral markers of response to CTLA-4 blockade and vaccines. As this work was reviewed extensively elsewhere (50), a brief summary is presented here (Table 1). Remarkably, the effects of PD-1 or PD-L1 blockade on circulating immune cells are distinct from those of CTLA-4 inhibition. Absolute lymphocyte counts (ALC) were not predictive of response to nivolumab treatment in combination with or following ipilimumab treatment (51) and did not change after treatment with atezolizumab (2). Moreover, gene expression analysis of sorted peripheral monocytes and T cells from patients receiving ipilimumab or nivolumab or both drugs concurrently revealed distinct therapy-induced pharmacodynamic changes

(49). While CTLA-4 blockade induced a proliferation or cell-cycle-associated gene signature in transitional memory T cells, PD-1 blockade was associated with induction of several cytotoxic and NK-associated genes in T cells. Unlike anti-CTLA-4, anti-PD-1 therapy did not induce ICOS in T cells. While both anti-PD-1 and anti-CTLA-4 promote T-cell activation, these data provide a glimpse into differential mechanisms of anti-tumor or autoimmune effects of these agents. Whether these signals translate to the tumor bed remains to be studied.

Serum or plasma markers are particularly practical in biomarker research and have been investigated in the context of immunotherapies. High baseline levels of C-reactive protein (CRP) have been found to be negatively predictive of response to ipilimumab (52, 53), and a decrease in CRP and lactic dehydrogenase was associated with response in melanoma (54). In a separate study, elevated predose serum sCD25 (IL2R $\alpha$ ) levels have been associated with resistance to ipilimumab in metastatic melanoma (55). Circulating tumor burden markers can also be an early indicator of efficacy to immunotherapies. For example, early decreases in circulating carcinoembryonic antigen (CEA) have been associated with PFS in mismatch repair-deficient colorectal cancer patients treated with pembrolizumab (30). In this study, changes in circulating CEA occurred several weeks before median time to response was observed, signifying the utility of peripheral tumor burden markers as early markers of response to therapy.

Limited correlations have been observed between monitoring of antigen-specific T cells in the periphery and antitumor activity with vaccine-based therapies (Table 1; ref. 56). The rationale to investigate whether the combination of checkpoint inhibition with cancer vaccines can offer added clinical benefit stems from recent studies whereby, upon vaccination, upregulation of PD-1 on T cells specific for the vaccine (57) or induction of intratumoral PD-L1 expression as a consequence to T-cell infiltration (58) has been observed. In one of the first studies combining ipilimumab and tumor peptide vaccination (tyrosinase, gp100, and MART-1) in patients with advanced melanoma, antigen-specific T-cell responses were identified in peripheral blood of 47% of treated patients. Although no control arm was included, the authors concluded that the results differed from those observed (~20%) in similarly vaccinated patients with no ipilimumab (59). A randomized clinical trial that included gp100 vaccination alone or in combination with ipilimumab or ipilimumab alone, demonstrated that peptide vaccination did not improve overall survival in patients with previously treated metastatic melanoma, suggesting that the detection of antigen-specific T cells may be necessary but not sufficient for effective antitumor immunity (3). A second example includes 90 melanoma patients treated with nivolumab in combination with vaccination of the HLA-A2-presented peptides of gp100, MART-1, and NY-ESO-1, in whom no added clinical benefit was observed with the addition of the vaccine. While antigen-specific T cells could be detected in the periphery, their high levels at baseline were associated with disease progression (60). These preliminary observations highlight the need to further understand the role of peripheral antigen-specific T-cell monitoring as a biomarker of response for vaccine-based combinations.

Although most investigations focus on CD8 T-cell responses, the role of CD4 T cells should not be underestimated. Melanoma patients treated with ipilimumab and with evidence of clinical benefit following treatment had CD4 and CD8 T cells against

**Table 1.** Blood-based biomarkers described to be associated with clinical response to immunotherapies

Biomarker	Treatment	Indication	N	Sampling timepoints	Summary of findings	Reference
Absolute lymphocyte counts (ALC)	Ipilimumab (0.3, 3, or 10 mg/kg)	Melanoma	553	BL, every 3 weeks	Rate of ALC ↑ associated with clinical activity	(70)
	Ipilimumab (10 mg/kg)	Melanoma	53	BL, every 3 weeks	ALC after 2 doses >1,000/μL associated with clinical benefit and OS	(71)
	Ipilimumab (10 mg/kg)	Melanoma	27	BL, after weeks 4 and 7	ALC ↑ >1,500/μL at week 4 associated with response and OS	(72)
	Ipilimumab (0.3, 3, 10 mg/kg), various combinations	Melanoma	1,450	BL, every 3 weeks	Rate of ALC ↑ associated with OS, not ipi specific	(73)
Eosinophils	Ipilimumab (3 mg/kg)	Melanoma	73	BL, every 3 weeks	AEC ↑ >100/μL and ALC ↑ >1,000/μL associated with OS	(74)
	Ipilimumab (3 mg/kg)	Melanoma	123	BL	High BL AEC associated with OS	(75)
	Ipilimumab (3 mg/kg)	Melanoma	59	BL, every 3 weeks	AEC ↑ in cycle 1 associated with response	(76)
Neutrophil/leukocyte ratio	Ipilimumab (10 mg/kg)	Melanoma	27	BL, weeks 4, 7, and 10	Low N:L ratio at weeks 7 and 10 associated with OS	(77)
Regulatory T cells	Prostate GVAX/ipilimumab (0.3 to 5 mg/kg)	Prostate cancer	28	BL, every 4 weeks	Treg ↑ between BL and week 12 negatively associated with OS	(78)
	Ipilimumab (3 mg/kg)	Melanoma	95	BL, every 3 weeks	Treg ↓ between BL and week 6 associated with OS	(54)
	Ipilimumab (10 mg/kg)	Melanoma	35	BL, 6 weeks	Treg ↑ at week 12 associated with PFS	(79)
	Nivolumab with or without multipeptide vaccine (gp100, NY-ESO-1, MART-1)	Melanoma	90	BL, week 12	Treg ↑ associated with progression	(60)
Myeloid-driven suppressor cells (MDSC)	Ipilimumab (3 or 10 mg/kg)	Melanoma	26	BL, 6 weeks	Low monocytic MDSCs at BL associated with OS	(80)
	Ipilimumab (10 mg/kg)	Melanoma	35	BL, 6 weeks	MDSC ↓ associated with PFS	(79)
	Ipilimumab (3 mg/kg)	Melanoma	59	BL, every 3 weeks	Monocytic MDSC ↓ at cycle 1 associated with response	(76)
ICOS	Ipilimumab (3 mg/kg)	Melanoma	14	BL, weeks 7 and 12	CD4 <sup>+</sup> ICOS <sup>+</sup> ↑ associated with OS	(81)

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**Table 1.** Blood-based biomarkers described to be associated with clinical response to immunotherapies (Cont'd)

Biomarker	Treatment	Indication	N	Sampling timepoints	Summary of findings	Reference
	Tremelimumab (15 mg/kg) every 90 days	Melanoma	29	BL, days 14, 30, and 60	CD4 <sup>+</sup> ICOS <sup>+</sup> ↑ associated with OS	(82)
	Ipilimumab (10 mg/kg)	Melanoma	17	BL, weeks 4, 7, and 10	CD4 <sup>+</sup> ICOS <sup>+</sup> ↑ and CD8 <sup>+</sup> ICOS <sup>+</sup> ↑ associated with disease control and OS	(77)
Th17 cells	Tremelimumab (10 or 15 mg/kg) every 90 days, 6 pts in combination with DC vaccine (MART-1)	Melanoma	27	BL, between 30 and 60 days	Th17 ↑ associated with autoimmune toxicities; no association with response	(83)
	Ipilimumab (3 or 10 mg/kg), with multipeptide vaccine	Melanoma	75	BL, 6 months	Th17 inducibility ↑ associated with freedom from relapse	(84)
MART-1, Gp100, Tyros T-cell responses	Ipilimumab (3 or 10 mg/kg), with multipeptide vaccine	Melanoma	75	BL, 6 months	Antigen-specific T-cell induction assessed by ELISPOT could not be consistently associated with any added clinical benefit	(84)
NY-ESO-1, MART-1, gp100 T cells	Nivolumab with or without multipeptide vaccine (gp100, NY-ESO-1, MART-1)	Melanoma	90	BL, 3 months	High baseline antigen-specific CD8 <sup>+</sup> T cells associated with progression	(60)
Tyrosinase, gp100 and MART-1	Ipilimumab 0.3, 1.0, or 3.0 mg/kg	Melanoma	19	BL, 6 months	47% of treated patients generated specific T-cell responses	(59)
TCR Vb	Tremelimumab	Melanoma	21	BL, 1-2 months	Increase in unique T-cell clonotype not associated with clinical outcomes	(62)

Abbreviations: AEC, absolute eosinophil count; BL, baseline; Ipi, ipilimumab; N:L ratio, absolute neutrophils to lymphocytes; OS, overall survival; PFS, progression-free survival.

NY-ESO-1 at a higher frequency and functionality than did non-responding patients (61). These results are suggestive of the engagement of antigen-specific CD4 T cells upon checkpoint inhibitor treatment and attest to their contribution to effective antitumor immunity.

While TCR Vβ repertoire analysis has been conducted using peripheral blood, the association of the dynamic changes in Vβ usage in the periphery with antitumor immunity is unclear. Robert and colleagues (62) reported on the impact to T-cell clonal amplifications following treatment with tremelimumab in which nearly all patients receiving tremelimumab had an increase (19/21 patients) of unique T-cell clonotypes in peripheral blood. However, these changes did not differ between clinical responders and nonresponders. Similarly, CTLA-4 blockade in patients with metastatic castration-resistant prostate cancer and metastatic melanoma resulted in peripheral blood changes of the TCR Vβ repertoire (63). Both dynamic expansion and loss of clonotypes were identified, and although no association of these changes related to clinical outcome, improved overall survival was associated with high-frequency clones at baseline.

## The When: Optimal Time Points for Blood-Based Biomarker Measurements

Changes in peripheral markers with immunotherapies can be rapid and short lived analogous to T-cell differentiation following viral infections (64). Clonal expansion and differentiation into effector CD8<sup>+</sup> T cells can occur within days of viral challenge. While most of the effector T cells undergo programmed cell death and contract, the memory cells persist in the host for years. Similar dynamics have been observed for T cells after tumor vaccination or PD-L1 inhibition with atezolizumab (2, 65, 66). Administration of T-cell-stimulating agents such as aldesleukin also lead to a rapid and acute CD8<sup>+</sup> T-cell proliferation that is detectable days after administration going back to baseline levels by 3 weeks (67).

Early blood sampling is particularly important when acute toxicity is expected, such as the cytokine increase that is the hallmark of the cytokine release syndrome, the most common toxicity associated with T-cell engaging therapies such as chimeric antigen receptor (CAR)-modified T cells (68). Indeed, the observation that IL6 is strongly increased during cytokine release syndrome has led to the inclusion of anti-IL6 antibodies into the

risk management of T-cell engaging therapies. To capture the kinetics of the immunologic phenomena, extensive serial samplings of blood at every cycle with more frequent collections during the first cycle may be warranted.

## The How: Technologies for Blood-Based Biomarkers

The technologies for the analysis of blood samples are quite mature. As a method for the detection of antigen-specific T cells, tetramer staining is well established. In situations where material is limiting or where the antigen is unknown, analysis of the T-cell receptor repertoire may become a viable alternative. Flow cytometry for the characterization of T-cell receptor subsets is an effective and reproducible technology. Mass cytometry (69) is emerging as a technology that may allow an even more granular characterization of immune cell subsets, but is currently still limited in its applicability. Gene expression profiling can be applied successfully to detect changes in the global transcriptome to gain mechanistic insights into the activity or toxicity of immunotherapy agents (49). Different immunoassay formats are available for the reproducible and sensitive detection of soluble analytes such as cytokines that can help characterize untoward side effects of immune therapies.

## What Will the Future Look Like?

Significant effort has been made to characterize the mode of action of immunotherapies and identify markers predictive of response in humans. These investigations have provided important insights into the mechanisms of action of checkpoint inhibitors that bring us closer to being able to rationally select

combination partners. An important gap in our understanding is the lack of information about immunologic effects of "nonimmunotherapies" that may become promising combination partners for the established agents. The tumor cell does not survive or propagate in isolation but rather the immune microenvironment and the tumor mutanome coexist in a constant battle for supremacy. Thus, elucidating the intricate molecular and cellular interactions of such coexistence promises to offer significant insights in identifying optimal combination partners. As we learn more from such translational studies, one could envision a future with immunologic markers as primary endpoints of trials analogous to minimal residual disease in leukemia. With rapid technological advances in both tumor and peripheral immune monitoring, this future may not be distant.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

## Authors' Contributions

Conception and design: P.S. Hegde, S. Evers

Development of methodology: P.S. Hegde

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): V. Karanikas

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): V. Karanikas, S. Evers

Writing, review, and/or revision of the manuscript: P.S. Hegde, V. Karanikas, S. Evers

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