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Biomarkers for Response to Immune Checkpoint Blockade

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Keywords

immune checkpoint blockade, PD-1, PD-L1, cancer therapy, biomarker

Abstract

Immune checkpoint blockade (ICB) has significant clinical activity in diverse cancer classes and can induce durable remissions in even refractory advanced disease. However, only a minority of cancer patients treated with ICB have long-term benefits, and ICB treatment is associated with significant, potentially life-threatening, autoimmune side effects. There is a great need to develop biomarkers of response to guide patient selection to maximize the chance of benefit and prevent unnecessary toxicity, and current biomarkers do not have optimal positive or negative predictive value. A variety of potential biomarkers are currently being developed, including those based on assessment of checkpoint protein expression, evaluation of tumor-intrinsic features including mutation burden and viral infection, evaluation of features of the tumor immune microenvironment including nature of immune cell infiltration, and features of the host such as composition of the gut microbiome. Better understanding of the underlying fundamental mechanisms of immune response and resistance to ICB, along with the use of complementary assays that interrogate distinct features of the tumor, the tumor microenvironment, and host immune system, will allow more precise use of these therapies to optimize patient outcomes.

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1. INTRODUCTION

Immune checkpoint blockade (ICB) with antibodies targeting PD-1, PD-L1, or CTLA-4 has been shown to induce clinically significant and sometime long-lasting responses in a broad range of human cancers (Hodi et al. 2010, Postow et al. 2015, Topalian et al. 2012). Responses are seen even in advanced, refractory cancers, and ICB is now approved for a rapidly growing number of tumor types and clinical indications. Response rates vary widely by tumor type, with high response rates (>50–60%) in certain cancers such as Hodgkin's disease and NK (natural killer) T cell lymphoma, but quite low response rates in others such as breast and prostate cancers (Ansell et al. 2015, Kim et al. 2018, Yarchoan et al. 2017). Although responses can be dramatic, only a minority of patients treated experience long-term clinical benefit, and treatment is associated with significant, sometimes life-threatening adverse reactions. Combination therapy of PD-1 antibodies with anti-CTLA-4 antibodies can increase response rates in some settings, but also significantly increases potential toxicity (Postow et al. 2015).

There is a pressing need to identify robust markers of response and resistance to ICB in order to identify which patients are most likely to benefit from ICB and which patients should be spared potential toxicity. Effective biomarker development has been challenging, as response to ICB is primarily observed in the clinic. The underlying mechanisms and immunologic targets activated by ICB are still under active investigation, and thus clear mechanistic-based biomarkers of clinical response remain to be validated.

One way to organize the approach to potential biomarker discovery is to consider four broad areas that likely impact response and resistance to ICB (**Figure 1**). The first is target assessment, which measures the presence and distribution of the molecular target of the therapeutic antibodies analyzed. For PD-1 antibodies, this has concentrated on expression of PD-L1, a ligand for PD-1 that can be expressed on both tumor cells and immune cells. The second category is tumor-intrinsic features, which include the mutational and transcriptional profile of the cancer at time of treatment. The third broad category comprises features of the tumor microenvironment. These include the composition and functional status of tumor-associated immune and inflammatory cells, as well as other stromal features. The fourth category is independent host features, such as immunologic history or microbiome, that may affect the systemic response to individual cancers. It is likely that key features of the tumor microenvironment may be driven by both tumor-intrinsic features and independent host features.

2. TARGET ASSESSMENT

As the antibodies used in ICB are thought to work by disrupting either the interaction of CTLA-4 and its ligands or PD-1 and its ligands, there has been significant focus on using the expression of these checkpoint proteins and ligands as biomarkers of response.

2.1. PD-L1 Expression

PD-1 and PD-L1 antibodies disrupt the interaction of PD-L1, which is expressed on tumor cells or nearby immune or inflammatory cells, and of the PD-1 receptor, which is expressed on T cells. There has been a great deal of effort investigating PD-1 expression on tumor cells or immune cells as a logical, mechanistic biomarker of response to immune checkpoint therapy. Currently, the most commonly utilized assay for PD-L1 expression is immunohistochemistry (IHC). Current data on the utility of PD-L1 IHC assays are quite mixed, and in part due to limited negative predictive value, there is limited understanding of the biological context and site of expression, as well as

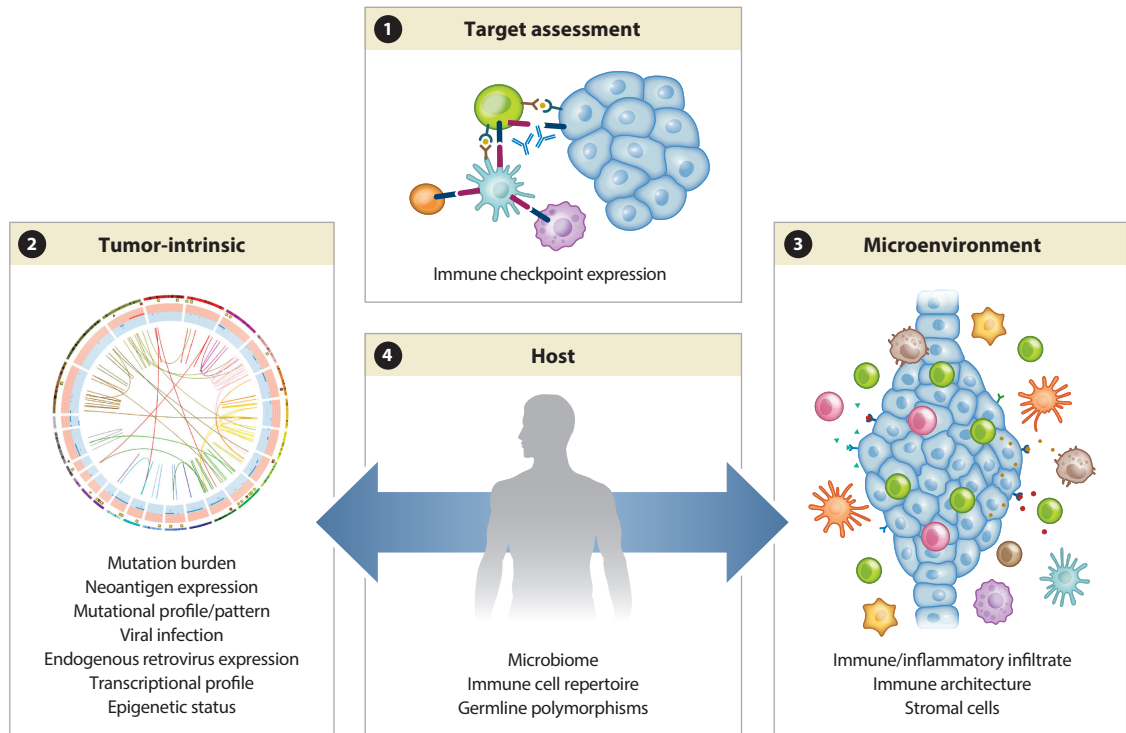


Figure 1

Major classes of potential biomarkers for response to immune checkpoint blockade. (❶) Target assessment: measuring the presence and distribution of the molecular target of the therapeutic antibodies. (❷) Tumor-intrinsic features: the mutational and transcriptional profile of the cancer at time of treatment. (❸) Tumor microenvironment: the composition and functional status of tumor-associated immune and inflammatory cells and other stromal cells. (❹) Independent host features, including immunologic history and composition of the gut microbiome.

technical limitations of assay performance and defining appropriate thresholds (Havel et al. 2019, Nishino et al. 2017, Yi et al. 2018).

Multiple studies have shown that patients with tumors that score positively for PD-L1 expression have higher rates of response and clinical benefit to ICB in multiple cancer classes, including non-small-cell lung cancer (NSCLC), urothelial cancer, triple-negative breast cancer, and others (Rosenberg et al. 2016, Schmid et al. 2018, Topalian et al. 2012). Assays for PD-L1 expression have been approved as companion diagnostics (CDx) for several indications. However, the presence and magnitude of the predictive value of PD-1 expression for response to ICB can vary widely among studies and agents used. For example, increasing PD-L1 expression in tumor samples of urothelial cancer was correlated with response to single-agent pembrolizumab (Balar et al. 2017), while no correlation with PD-L1 expression and response rates to nivolumab was seen in a similar group of patients with advanced urothelial cancer (Sharma et al. 2016). Similar conflicting results were initially seen in previously treated NSCLC, although in the first-line metastatic setting, patients with PD-L1-positive tumors (>1% or >50%) had better outcomes with pembrolizumab than with chemotherapy (Mok et al. 2019, Reck et al. 2016), whereas no such benefit over chemotherapy was seen in a separate trial in unselected patients treated with nivolumab (Carbone et al. 2017). These differing results in different settings may be in part due to different antibodies and cutoffs used in these studies (Ancevski Hunter et al. 2018, Hirsch et al. 2017, Nishino et al. 2017). Importantly,

while PD-L1 expression may enrich for response, there is still a significant response rate seen in PD-L1-negative tumors in several trials, demonstrating a relatively poor negative predictive value for this assay (Shen & Zhao 2018) and hesitation on the part of many clinicians to rely exclusively on this test to predict response.

Reasons for the variability in predictive power of PD-L1 testing may reflect both technical and biological limitations. In terms of technical limitations, since IHC is a nonlinear assay that depends on many variables, including fixation conditions and antigen retrieval steps, it is difficult to normalize between different labs and centers. Thus, different antibodies, thresholds, and cutoffs have been employed in different studies (Ancevski Hunter et al. 2018, Hirsch et al. 2017, Takada et al. 2018). As for biological limitations, PD-L1 expression is likely highly dynamic both temporally and spatially within the tumor microenvironment, and sampling error in single biopsies may not capture the true extent of expression. Physiologically relevant PD-L1 expression in some cases may be spatially distinct from the tumor mass and present in structures such as tertiary lymphoid structures or draining lymph nodes that may not be routinely sampled or assayed (Behr et al. 2014, Solinas et al. 2017). Similarly, PD-L1 expression may vary temporally, and expression at the time of the biopsy that was assayed may not reflect expression at the time of treatment. Finally, the impact of intervening therapies between the time a biopsy was taken and the time treatment initiated is uncertain.

2.2. New Approaches

Technical limitations in assessing PD-L1 expression could be overcome by alternative assays, such as mRNA (messenger RNA) measurement by RNA sequencing or reverse transcriptase polymerase chain reaction (RT-PCR) (Conroy et al. 2019, Erber et al. 2017); however, addressing potential biological limitations will remain challenging. New approaches for evaluating PD-L1 expression that are being developed include noninvasive nuclear imaging approaches, which may allow unbiased whole-body imaging of PD-L1 expression that can be followed over time (Kumar et al. 2019, Niemeijer et al. 2018). Initial studies using zirconium 89-labeled atezolizumab (Bensch et al. 2018) suggest that this approach may be more predictive than IHC- or RT-PCR-based assays.

As drugs are developed targeting other immune checkpoint targets, such as LAG-3 and TIM-1, it is likely that assays looking at expression of these targets will also be developed. Strict attention to the standardization of such assays and establishment of rational cutoffs will be required to optimize their clinical utility.

3. TUMOR-INTRINSIC BIOMARKERS

Several intrinsic features of the transformed cancer cell have been proposed as potential biomarkers of response to ICB and are detailed below (**Figure 2**).

3.1. Tumor Mutational Burden

The burden of nonsynonymous somatic small-variant mutations detected by next-generation sequencing (NGS) in tumor samples is correlated with response to ICB. Initially observed in melanoma (Snyder et al. 2014), this association between tumor mutational burden (TMB) and response to ICB has been demonstrated in multiple cancer types, including NSCLC (Hellmann et al. 2018, Rizvi et al. 2015), urothelial cancer (Rosenberg et al. 2016), and others. Examination of multitumor cohorts and meta-analyses of multiple studies support an overall correlation between TMB and response to ICB (Samstein et al. 2019, Yarchoan et al. 2017). It is hypothesized that

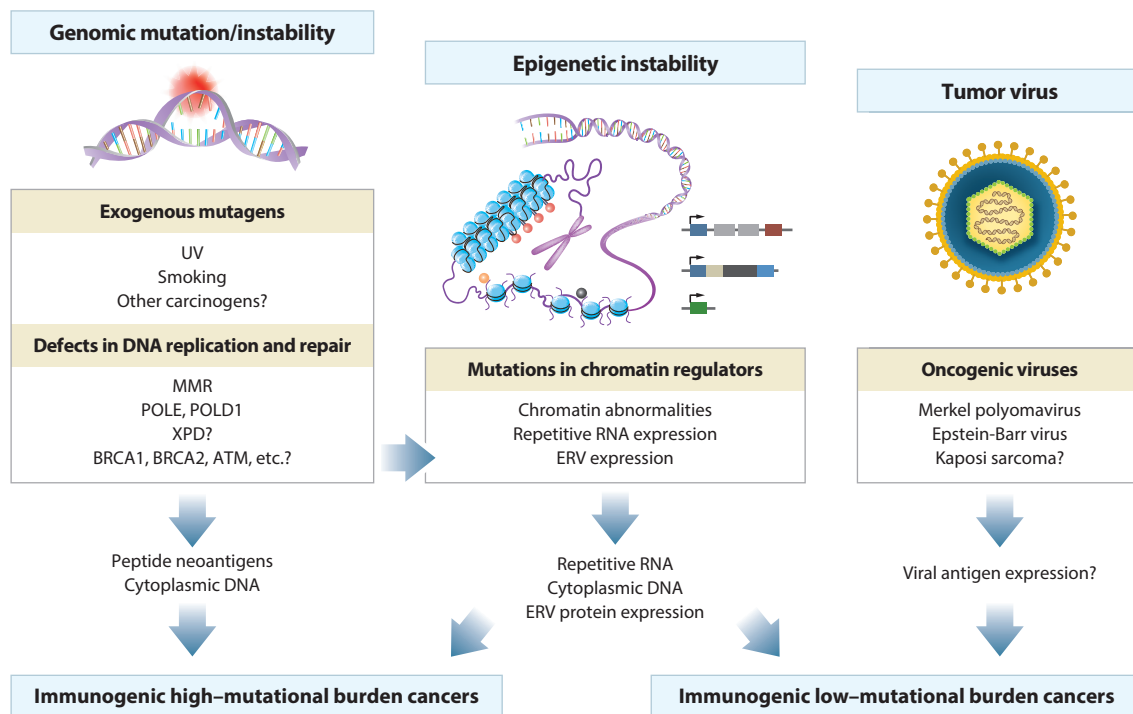


Figure 2

Potential intrinsic tumor features that may contribute to immunogenicity and sensitivity to immune checkpoint blockade in both high-mutational burden and low-mutational burden cancers. Exposure to exogenous mutagens or intrinsic defects in DNA repair and replication may lead to high-mutational burden cancers that are immunogenic. Defects in chromatin regulation leading to upregulation of repetitive elements, or infection with oncogenic viruses, may lead to immunogenic low-mutational burden cancers. Abbreviations: ERV, endogenous retrovirus; MMR, mismatch repair.

high TMB leads to increased production of so-called neoantigens that arise from alterations in the coding sequencing of expressed proteins in the tumor cell. These neoantigens can be recognized by the immune system, and tumors must evolve mechanisms to evade the immune response, possibly by upregulating immune checkpoints, and thus are vulnerable to ICB. Interestingly, high TMB either due to exogenous carcinogens, such as UV exposure in melanoma and squamous skin cancer or smoking in lung cancer, or arising from intrinsic defects in DNA repair or replication, as seen in cancers with mutations in mismatch repair (MMR) or proofreading defects in POLE or POLD1, are all associated with response to ICB. Initial studies used whole-exome sequencing to assess TMB, but several CLIA-certified hybrid capture-based gene sequencing panels, including the FoundationOne CDx panel and the MSK-IMPACT panel, can also estimate TMB accurately and have shown utility in predicting response to ICB (Chalmers et al. 2017, Goodman et al. 2017, Samstein et al. 2019).

Although TMB correlates with response to ICB in multiple cancer types, there are several challenges to using TMB as a biomarker. While high TMB does enrich for responders, there are clearly classes of low-TMB tumors, including clear cell renal cell carcinoma (ccRCC) and viral-driven tumors (see below) (Panda et al. 2018b, Yarchoan et al. 2017), that have significant response rates, suggesting that high TMB is only one of several potential markers of response. Similarly, it is not clear what the right TMB threshold should be to optimally identify potential responders, or

if TMB functions as a predictor of response only in some cancer classes. A pan-cancer analysis of TCGA (The Cancer Genome Atlas) data has demonstrated that an optimal TMB threshold that identifies tumors with evidence of lymphocytic infiltration and immune checkpoint gene expression may only be present in some cancer classes (Panda et al. 2017). Moreover, different cancer types may have different TMB cutoffs to optimally predict response (Panda et al. 2017, Samstein et al. 2019).

Different classes of mutation may also be more or less immunogenic, confounding attempts to simply count total TMB. Insertion/deletion mutations, and especially frameshift mutations that evade nonsense-mediated RNA decay (NMD) and generate truncated proteins containing a novel string of amino acids prior to the new stop codon, may be significantly more immunogenic than single missense mutations (Turajlic et al. 2017). The presence of gene fusions may also be immunogenic and unaccounted for (Yang et al. 2019). Similarly, truncal mutations present in all tumor cells may be more important predictors than subclonal mutations, and this difference may need to be accounted for when measuring TMB (McGranahan et al. 2016). TMB assays are also very sensitive to tumor purity of the samples sequenced, with low-purity samples tending to underestimate TMB.

3.2. DNA Repair Defects

Defects in DNA repair or replication that lead to a large TMB have emerged as potent predictors of response to ICB that may be histology independent.

3.2.1. Mismatch repair. MMR gene defects (MMRD) that arise due to gene mutation or epigenetic alterations are seen in a subset of human cancers, including a significant proportion of colorectal and endometrial cancers. MMRD can arise due to either germline mutations in MMR genes (Lynch syndrome) or biallelic somatic mutation or epigenetic silencing of these genes. Initial studies demonstrated that colon cancers harboring an underlying MMRD had significantly higher response rates to ICB than those with intact mismatch repair (Le et al. 2015). A large histology-independent trial demonstrated that cancers of diverse histology (12 types) with documented MMRD had an overall radiographic response rate of 53% when treated with pembrolizumab, leading to the first histology-agnostic approval for pembrolizumab in refractory MMRD solid tumors (Le et al. 2017). The mechanism by which MMRD predisposes to response to ICB is not established, but the neoantigen hypothesis is frequently invoked in this setting.

As MMRD can be seen in a small subset of a large variety of cancers (Hause et al. 2016), there is great interest in accurately identifying this phenotype. The most common methods to identify MMRD in tumor specimens are to survey for expression of specific proteins involved in MMR by IHC and to assay directly for the presence of microsatellite instability (MSI), an indirect marker of underlying MMRD, by DNA sequencing using PCR or NGS. The IHC methods can be difficult to score consistently and may have both false positives and negatives. Similarly, genomic assays can give a false negative in setting of low tumor purity, and the exact set of satellite repeats that are interrogated in MSI assays may not be optimal for all cancer types or may vary by the exact nature of the MMRD (Hause et al. 2016, Wang et al. 2017). Other methods to assay for the presence of MMRD, including the use of mutational signatures and TMB, may complement these assays and are being developed (Chalmers et al. 2017, Middha et al. 2017, Stadler et al. 2016).

3.2.2. Proofreading polymerase defects. Tumor-harboring mutations that disable the proofreading exonuclease activity of the main DNA polymerases POLE and POLD1 may also be highly

vulnerable to ICB. Such tumors are mostly microsatellite stable (MSS) but have an extraordinarily high TMB, with a characteristic mutation pattern (Heitzer & Tomlinson 2014, Park & Pursell 2019). POLE mutations are most commonly seen in endometrial cancers, where 9% of primary cancers show evidence of POLE mutations, but are also seen in a small subset of diverse solid tumors including lung cancer (Rayner et al. 2016). Several reports have demonstrated that POLE- and POLD1-mutant tumors that have evidence of immune infiltration and checkpoint gene expression tend to be exceptional responders to ICB (Mehnert et al. 2016, van Gool et al. 2015), and several studies are underway to directly test the hypothesis that polymerase-mutant tumors will respond to ICB (<https://www.clinicaltrials.gov/> identifiers NCT03428802, NCT03461952).

3.2.3. Other repair defects. Tumors harboring other defects in DNA repair that can give rise to high TMB, such as tumors arising in patients with xeroderma pigmentosa, may also be associated with response to ICB (Deinlein et al. 2017). Tumors with underlying defects in homologous recombination-mediated DNA repair, such as those with mutations in BRCA1 and BRCA2, do not have a very high burden of small nonsynonymous mutations, but do have a large number of structural variations and genomic rearrangements that are not well captured by exome sequencing but may be immunogenic (Davies et al. 2017, Yang et al. 2019). The underlying genomic instability may also lead to abnormal release of DNA fragments into the cytosol, which can activate the cGAS-STING and active innate immune response (Härtlova et al. 2015, Mouw et al. 2017, Woo et al. 2014). Some BRCA1/2-mutant solid tumors may have evidence of local immune infiltration (Lakhani et al. 1998, Strickland et al. 2016). There are few data on tumors with mutations in other BRCA1/2-related DNA repair proteins such as PALB2 and BRIP1 and other Fanconi anemia proteins. Prospective studies are required to determine if tumors with BRCA1/2 mutations or other mutations in DNA double-strand break repair genes are sensitive to ICB.

3.3. Mutation Pattern

In addition to quantity of mutations, the pattern of underlying mutations present in a cancer may also be a marker of response to ICB. Multiple mutational signatures have been identified based on the nucleotide context of the mutations, some of which are associated with exposure to particular carcinogens such as UV or tobacco (Alexandrov et al. 2013). In NSCLC, the presence of a mutational signature associated with smoking was as predictive of response to ICB as TMB (Rizvi et al. 2015). TMB and specific mutational signatures are not independent, as smoking signature is enriched in high-TMB NSCLC, while mutation patterns associated with MMRD and POLE mutations are enriched in high-TMB colorectal cancers and endometrial cancers (Panda et al. 2017). Other mutational signatures, including those associated with APOBEC3B activity and labeled kataegis, may also be associated with expression of PD-L1 and PD-L2 and mark a response to ICB (Boichard et al. 2017). As TMB can be confounded by low tumor purity, it may be possible to use both TMB and mutation pattern to develop classifiers that may function better than either parameter alone.

3.4. Neoantigen Load

The relationship between TMB/mutation pattern and response to ICB is postulated to be due to the presence of immunogenic neoantigens in high-TMB cancers. Bioinformatic algorithms have been developed that can predict potential immunogenic neoantigens based on the mutations present in an individual cancer and HLA phenotype, and theoretically these should function much better than TMB as predictors of response. Although initial reports hinted at the success

of such approaches (Chan et al. 2015, Snyder et al. 2014), multiple studies have not been able to demonstrate that current algorithms to predict neoantigen burden work any better than overall TMB in predicting response (Hellmann et al. 2018, Rizvi et al. 2015, Van Allen et al. 2015).

This failure of these neoantigen prediction methods to perform better than overall TMB may be due to multiple factors. These algorithms may not account for some key factors, such as prioritizing neoantigens produced by frameshift mutations that escape NMD (Turajlic et al. 2017), evaluating the clonality of neoantigens (McGranahan et al. 2016), and accounting for prior immunological exposures. More sophisticated approaches to predicting neoantigens may work better, including those that account for immune selection or that are better models of MHC (major histocompatibility complex) binding and evolutionary fitness, including those that predict the similarity of neoantigens to microbial pathogens (Balachandran et al. 2017, Luksza et al. 2017). Alternatively, it is possible that TMB may affect response to ICB through some mechanism other than the presence of peptide neoantigens.

3.5. Viral Infection

Cancers driven by oncogenic tumor viruses often have low somatic TMBs, as viral genes can act directly as oncogenes or by disrupting endogenous tumor-suppressor function. Some low-mutation cancers that have evidence of immune activation and high response rates to ICB are associated with presence of oncogenic tumor viruses (Rooney et al. 2015, Yarchoan et al. 2017), where viral proteins may be a source of immunogenic antigens.

3.5.1. Merkel cell polyomavirus. Merkel cell carcinoma (MCC) are high-grade small-cell cancers of the skin that can be caused by Merkel polyomavirus (Mpv) infection or UV damage and have high response rates to ICB (Kaufman et al. 2016, Nghiem et al. 2016). The Merkel cell cancers that are positive for Mpv have a very low somatic TMB (Goh et al. 2016); it is presumed that the integrated large T antigen functionally disables *RB1* and *TP53* in these cancers. The Merkel cell cancers that lack Mpv have a very high TMB (Goh et al. 2016), similar to squamous cell cancers of the skin, but with somatic mutations in *TP53* and *RB1* (Schadendorf et al. 2017). Thus, MCC all harbor loss of *RB1* and *TP53* function, either by the large T antigen of Mpv or by somatic mutation in the setting of a high UV TMB. Both Mpv infection in Mpv-positive MCC and the high TMB in Mpv-negative MCC may be immunogenic and render both subsets sensitive to ICB, although through separate mechanisms (Schadendorf et al. 2017).

3.5.2. Epstein-Barr virus. Epstein-Barr virus (EBV)-positive gastric cancer is a subset of low-TMB, MSS gastric cancers that have evidence of immune checkpoint activation and exceptional response to ICB (Panda et al. 2018b). An analysis of a phase II trial of pembrolizumab for advanced/refractory gastric cancers showed that of the 42 patients on trial, all 5 of those whose tumors were EBV positive had partial response to treatment (Kim et al. 2018). EBV-positive tumors were mutually exclusive with the MSI gastric cancers; thus, EBV-positive gastric cancers may define a subset of MSS gastric cancers that may be highly responsive to ICB. Other EBV-positive cancers such as NK/T cell lymphoma have also had high response rates to ICB, with response rates of 57–100% to pembrolizumab reported in refractory NK/T cell lymphoma in several series (Kim et al. 2018, Li et al. 2018). However, not all EBV-positive tumors have high response rates. In nasopharyngeal cancer (NPC), which is almost universally EBV positive, pembrolizumab in PD-L1-positive cases had a response rate of 27% (Hsu et al. 2017), while nivolumab treatment had a ~20% response rate, with no relationship to PD-L1 expression (Ma et al. 2018). The reason

for the relatively low response rate in EBV-positive NPC versus the other EBV-positive cancers is not clear.

3.5.3. Human papillomavirus. The relationship between human papillomavirus (HPV) infection, as seen in head and neck squamous cancers (HNSCC) and cervical cancers, and response to ICB is not straightforward. Nivolumab treatment led to better outcomes when compared to second-line chemotherapy in patients with HNSCC and was equally effective in HPV-positive and HPV-negative patients (Ferris et al. 2016). In a single-arm trial, the response rate to pembrolizumab was higher in HPV-positive HNSCC than in HPV-negative HNSCC (24% versus 16%) (Seiwert et al. 2016). In advanced cervical cancer, pembrolizumab treatment was associated with an overall response rate (ORR) of 12.2%. In this trial, the majority of patients were PD-L1 positive (83.7% had a combined positive score greater than 1), and all responses were in PD-L1-positive group, with a 14.6% ORR (Chung et al. 2019).

3.5.4. Other viruses. There are some reports that other classes of virally driven cancers may also respond well to ICB. ICB treatment had a 67% response rate in a small cohort of men treated for HIV-associated Kaposi sarcoma, a cancer driven by the HHV8 virus (Galanina et al. 2018). However, ICB treatment has been reported with rapid progression of HTLV-1-associated adult T cell leukemia/lymphoma (Ratner et al. 2018), suggesting that the exact role of PD-1 signaling in specific diseases may have a significant impact on the outcome of PD-1 pathway inhibition in virally induced cancers.

3.6. Expression of Repetitive RNAs

A significant portion of the human genome codes for classes of repetitive elements such as satellite repeats, retroelements including LINE and SINE elements, and endogenous retroviruses (ERVs). Almost all of these repetitive elements in the genome are constitutively epigenetically silenced and not transcribed. Several studies have suggested that a subset of human tumors have abnormal expression of these repetitive RNAs, including ERVs, which leads to the activation of innate immune signaling and upregulation of immune checkpoint genes (Rooney et al. 2015, Smith et al. 2018, Solovyov et al. 2018, Panda et al. 2018a). Our research group and others have recently found that ERV expression is associated with evidence of immune checkpoint gene expression and T cell infiltration in several cancers including ccRCC, a cancer with a significant response to immune checkpoint therapy despite having a relatively low TMB (Panda et al. 2018a, Rooney et al. 2015, Smith et al. 2018). Expression of ERVs was found to correlate well with response to ICB in patients with urothelial cancer and patients with ccRCC (Panda et al. 2018a, Solovyov et al. 2018). Intriguingly, ERV expression in cancers may be associated with alteration of chromatin-regulatory genes and can be induced by treatment by hypomethylating agents (Chiappinelli et al. 2015) and CDK4/6 inhibitors (Goel et al. 2017), suggesting potential rational combination treatment strategies.

3.7. Specific Somatic Mutations

The presence of specific mutations in cancers has also been associated with response and resistance to ICB. Mutations in SERPINB3 and SERPINB4 have been associated with improved outcomes in patients with advanced melanoma treated with anti-CTLA-4 antibodies, but are not prognostic in the absence of such therapy (Riaz et al. 2016). Truncating mutations in PBRM1, a member of the SWI/SNF PBAF chromatin remodeling complex, were also reported to induce the sensitivity

of tumor cells to T cell–mediated killing and were associated with response to ICB in a small cohort of ccRCC patients (Miao et al. 2018, Pan et al. 2018). No such association was seen in a larger analysis of a prospective study comparing the efficacy atezolizumab alone, atezolizumab plus bevacizumab, and sunitinib in ccRCC (McDermott et al. 2018). Mutations associated with resistance to ICB have also been reported. Lung cancers harboring both KRAS and STK11 mutations were associated with lack of response to ICB in several independent cohorts, and this association was independent of TMB (Skoulidis et al. 2018). Acquired mutations in JAK1 or JAK2, which may disable interferon signaling, and mutations in β 2-microglobulin (B2M), which may disable antigen presentation, have also been reported to be associated with acquired resistance to ICB (Zaretsky et al. 2016). However, the presence of B2M mutations in MSI colon cancer was not associated with resistance (Middha et al. 2019), suggesting that the role of B2M mutations may be context dependent and requires further validation.

3.8. Major Histocompatibility Complex Class I and II Expression

MHC class I and class II proteins play key roles in efficient antigen expression and are critical for T cell activation by tumor antigens (Gubin et al. 2014, Engels et al. 2013). A recent analysis of MHC class I and class II proteins in melanoma treated with ICB found that loss of MHC class I was associated with poor response to the anti-CTLA-4 antibody ipilimumab but did not predict resistance to the PD-1 antibody nivolumab or the combination of ipilimumab plus nivolumab (Rodig et al. 2018). Positive MHC class II expression was associated with better response to nivolumab treatment, but not to ipilimumab alone or to the combination of ipilimumab plus nivolumab. Tumor expression of MHC class I may be predictive of response to anti-CTLA-4 antibody treatment, and MHC class II expression is predictive of response to anti-PD-1 antibody treatment, but neither MCH class I nor class II is predictive of response to combined treatment. These findings suggest that there may be different biomarkers that are specific to CTLA-4 versus PD-1 targeting that reflect differences in underlying mechanisms of action of these agents (Wei et al. 2017).

3.9. Gene Expression Signatures

The transcriptional state of tumor cells may also impact their immunogenicity. Transcriptional profiling of bulk tumors can be difficult to interpret, as much of the relevant signatures may be dominated by stromal, nontumor cells. Single-cell sequencing approaches are one method to deconvolute the transcriptional signature and interrogate the profile of individual tumor cells. Single-cell sequencing of tumor cells from clinical melanoma samples has identified tumor-specific transcriptional programs that may be associated with T cell exclusion and resistance to ICB therapy (Jerby-Arnon et al. 2018). Transcriptional features associated with T cell exclusion and resistance to ICB included upregulation of CDK4/6 signaling targets, suggesting that CDK4/6 inhibitors could be employed to improve response to ICB. These findings are similar to other studies that have independently implicated CDK4/6 inhibitors as a potential strategy to increase tumor immunogenicity (Deng et al. 2018, Goel et al. 2017, Schaer et al. 2018, Zhang et al. 2018), and several clinical trials exploring the combination of CDK4/6 inhibitors with ICB are underway (NCT02779751, NCT03147287).

4. IMMUNE MICROENVIRONMENT

The composition of the tumor microenvironment and in particular the extent, nature, and functional status of immune and inflammatory cell populations have been postulated as key predictors

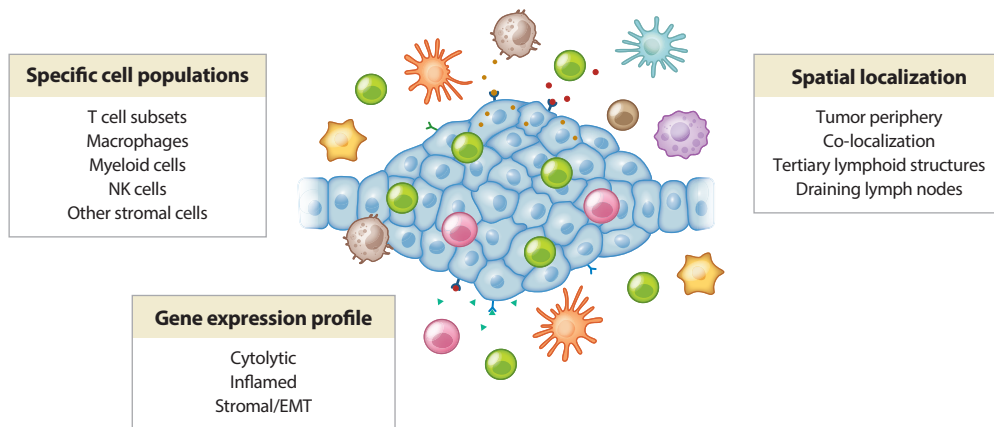


Figure 3

Components of the tumor immune microenvironment that could function as potential biomarkers for response to immune checkpoint blockade. These include the presence of specific immune cell populations, measurement of immune-related gene expression signatures, and analyses of spatial localization of immune cell populations. Abbreviations: EMT, epithelial-to-mesenchymal transition; NK, natural killer.

of response to ICB (**Figure 3**). An inflamed tumor microenvironment characterized by the presence of CD8⁺ cytotoxic T cells, a relative lack of CD4⁺ regulatory T cells, and activation of interferon-mediated signaling may be required for a therapeutic response to ICB (Spranger et al. 2013). Several methods to assay the functional composition of the tumor microenvironment have been proposed as biomarkers of response to ICB and are discussed below.

4.1. Tumor-Infiltrating Lymphocytes

The exact immune and inflammatory cells that mediate the therapeutic effect of ICB are still not well understood but are thought to involve CD8⁺ cytotoxic T cells, NK cells, and mature dendritic cells, possibly modulated by other cell types including macrophages and neutrophils (Chen & Mellman 2013). The presence of CD8⁺ tumor-infiltrating lymphocytes (TILs) is also associated with PD-L1 expression and is postulated to be a predictor of response to ICB. Initial studies showed that the presence of CD8⁺ T cells at tumor margins was associated with response to pembrolizumab in metastatic melanoma (Tumeh et al. 2014). The presence of TILs in breast cancer specimens, as assessed by a pathologist assessment of H&E (hematoxylin and eosin)-stained pathology sections, was correlated with response to single-agent pembrolizumab in the KEYNOTE-086 study (Loi et al. 2017). Similarly, increased TILs in pretreatment biopsy specimens of triple-negative breast cancer treated with neoadjuvant pembrolizumab plus chemotherapy in KEYNOTE-173 were associated with increased pathologic complete response and ORR (Loi et al. 2019).

Zhang and Chen (2016) have proposed a method to classify the tumor microenvironment in cancers based on expression of PD-L1 and TILs. They propose four classes of tumor immune microenvironment: T1 (PD-L1 and TIL negative), T2 (PD-L1 and TIL positive), T3 (PD-L1 negative and TIL positive), and T4 (PD-L1 positive and TIL negative). T2 cases are most likely to respond to ICB, while T1 cases are likely intrinsically resistant. T3 and T4 tumors may also have suboptimal response to ICB, but this could be overcome by rational combination strategies that aim to convert them to tumors with a T2 phenotype. T4 tumors have PD-L1 expression but lack TILs, so strategies to induce local cell death and lymphocyte infiltration, such as radiation therapy,

oncolytic viral therapy, or chemotherapy, may synergize with ICB (Zhang & Chen 2016). In T3 tumors, which have TILs but lack PD-L1 expression, interventions to induce PD-L1 expression by activating interferon pathways, such as the use of Toll-like receptor agonists or OX40 antagonists, may increase the response to ICB (Zhang & Chen 2016). Although this classification is compelling, it will need clinical validation.

4.1.1. Automated image analysis–based measurement of tumor-infiltrating lymphocytes.

Quantitation of TILs can be quite challenging, and although standardized methodologies such as Immunoscore are being developed (Pages et al. 2018), these may be observer dependent and require training and validation. To overcome variation, researchers have developed computer-assisted methods to identify and quantitate lymphocyte populations in high-resolution digital images of histology sections (Basavanahally et al. 2010, Johnson et al. 2018). Although encouraging results have been reported, further clinical validation will be required.

4.1.2. Flow cytometry–based profiling of tumor immune cells. The presence of other specific immune or inflammatory cells in the tumor microenvironment has also been postulated to affect response to ICB. For example, the presence of a specific subpopulation of tumor-resident CD103⁺/CD69⁺/CD8⁺ T cells has been associated with improved response to ICB in melanoma (Edwards et al. 2018). The presence of specific subsets of other immune cells, including polarized macrophages, activated NK cells, or specific T-regulatory populations (Heeren et al. 2019, Hsu et al. 2018, Su et al. 2018), has also been postulated to be a predictor of response to ICB.

4.1.3. Single-cell RNA sequencing of tumor-associated immune cells. Single-cell RNA sequencing (scRNA) of immune cells in the tumor microenvironment is an emerging powerful technique (Azizi et al. 2018). This approach has demonstrated that distinct CD8⁺ T cell states exist that may predict response or resistance to ICB. For example, expression of the transcription factor TCF7 identified a subset of CD8⁺/TCF7⁺ T cells enriched in melanomas that responded to ICB and were associated with improved survival, while a subset of CD8⁺ T cells enriched for T cell exhaustion markers was associated with nonresponse and poor outcome (Sade-Feldman et al. 2018).

4.1.4. Multiplex immunofluorescence. Methods utilizing multiplex immunofluorescence to identify and quantify immune cell subsets in tumor specimens are being actively investigated as potential predictors of response to ICB (Surace et al. 2019). These methods may preserve the spatial localization of lymphocytes within tumors and allow for multiparameter characterization of protein expression and precise cell counting; they have been characterized by some investigators as flow on a slide. Rigorous quantitation of CD8⁺ T cells using computer-assisted image analysis methods has been developed and correlates with response to ICB in melanoma (Wong et al. 2019).

Although the data on use of TILs, either alone or with other markers, are encouraging, there are significant obstacles to using TILs or other specific immune cell infiltrates as biomarkers of response. Methods to quantitate overall TILs or specific subsets of immune cells require standardization and prospective validation. Similarly, the spatial location and distribution of TILs may be as important as total number and thus may not be measured well in small biopsy specimens.

4.2. Gene Expression Assays

An alternative method to interrogate the immune microenvironment is to use RNA profiling of bulk tumor tissue. Although spatial localization may be lost, such assays do integrate the

microenvironment of the entire tumor sample analyzed. The overall idea is that RNA profiles that are both associated with an inflamed microenvironment and can integrate the presence of CD8⁺ T cell lymphocytes and the expression of immune checkpoints may be highly prognostic of response to ICB.

4.2.1. Cytolytic activity profile. The expression of granzyme A (GZMA) and perforin (PRF1) is associated with the presence of activated cytolytic immune infiltrate and immune checkpoint gene expression in multiple cancer types (Rooney et al. 2015, Balli et al. 2017). Gene expression analysis confirmed that GZMA and PRF1 expression in melanoma samples was enriched in responders to CTLA-4 blockade (Van Allen et al. 2015). Expression of GZMA and PRF1 has been incorporated into several gene expression signatures aimed at analyzing the immune microenvironment in cancer (Ayers et al. 2017, Narayanan et al. 2018).

4.2.2. T cell-inflamed gene expression profile. An 18-gene RNA panel that uses a Nanostring-based assay, can be implemented on standard formalin-fixed tissue, and includes genes related to markers of T cells, interferon gamma activity, NK cell activity, and cytolytic activity was developed using data from specimens obtained from clinical trials of pembrolizumab (Ayers et al. 2017). The gene expression profile (GEP) was then evaluated in a cohort of over 300 patients from 4 trials of single-agent pembrolizumab in 22 tumor types (enriched in melanoma and HNSCC), and its predictive value was compared with that of TMB and PD-L1 expression (Cristescu et al. 2018). Clinical responders had significantly higher GEP scores than nonresponders in the pan-tumor cohort ($p < 0.001$), with an AUROC (area under receiver operating characteristic) of 0.782. A similar analysis of over 400 patients from KEYNOTE-028, representing advanced cancers of 20 solid tumor types treated with pembrolizumab, also demonstrated that GEP scores were higher in responders than in nonresponders (Ott et al. 2019). In both studies, TMB, measured by whole-exome sequencing, was also strongly associated with response to pembrolizumab. Intriguingly, the correlation between GEP and TMB in both studies was significant but quite low [$r = 0.221$ and $p < 0.05$ (Cristescu et al. 2018) and $r = 0.20$ and $p = 0.007$ (Ott et al. 2019)], and both studies showed some added value in considering both GEP and TMB, with the highest response rates seen in patients with both high TMB and high GEP.

The relatively low correlation between TMB and GEP in these studies suggests that these may be independent predictors. Reasons for this include that the inflamed T cell response may be driven by other factors in some low-TMB cancers, such as viral infection (e.g., EBV, HPV) or chromatin abnormalities and ERV expression. Variations in tumor purity and sampling of the immune infiltrates in limited biopsy specimens may affect TMB and GEP assays differently, with low tumor purity leading to an underestimation of TMB, and high tumor purity leading to an underestimation of GEP score.

4.2.3. Other gene expression panels and methods. Other gene expression panels have been proposed as predictors of response to ICB (Nirmal et al. 2018, Prat et al. 2017). Stromal/EMT gene signatures may be associated with poor response to ICB in T cell infiltrated urothelial cancers (Wang et al. 2018). B cell signatures have also been identified as predictors of response to ICB in patients with malignant melanoma (Varn et al. 2019). Methods to deconvolute bulk gene expression data to impute the presence of specific lymphocyte subsets, such as CIBERSORT (Chen et al. 2018), have also been used to profile cancers.

5. HOST FACTORS

Independent features of the host may have a great impact on how individual tumors interact with the immune system and contribute to sensitivity or resistance to ICB. This includes composition of the host microbiome, features of the systemic immune system, germline polymorphism, and possibly other factors such as immune history and prior or coexisting infections.

5.1. Microbiome

The composition of the gut microbiota has multiple influences on the immune system, and its effect on both the efficacy of ICB and the spectrum of autoimmune side effects has begun to be elucidated (Gong et al. 2019, Zitvogel et al. 2018). There have been early observations that syngeneic mice housed in different facilities could have markedly different growth rates of implanted B16.SIY melanoma cell lines that were immune mediated (Sivan et al. 2015). These differences could be abrogated by cohousing or fecal transfer, implicating gut microbiota in modulating the tumor immune response. Intriguingly, oral administration of *Bifidobacterium* could induce an antitumor response and was synergistic with PD-1 antibody therapy, suggesting that manipulating the gut microbiome could influence response to ICB (Sivan et al. 2015). These observations in mice were extended to human studies, which demonstrated that composition of the human gut microbiome was associated with response to ICB in cancer patients. Responders had higher alpha diversity and an enrichment of the Ruminococcaceae family in one study focused on melanoma (Gopalakrishnan et al. 2018); in another melanoma study (Matson et al. 2018), responders had an enrichment of *Bifidobacterium*, *Collinsella*, and *Enterococcus* species, and *Akkermansia* species were enriched in patients in a third study that included diverse epithelial cancers (Routy et al. 2018). The reason for the differences in the microbial species associated with response in these studies is not clear but may reflect different methodologies, the regional heterogeneity of microbiota, or the instability of analysis given the large number of variables uncovered by microbiome sequencing. Perturbing the gut microbiome with antibiotics can adversely affect response to ICB in mouse models (Cremonesi et al. 2018), and association between prior antibiotic use and lack of response to ICB have been reported in patients (Derosa et al. 2018, Routy et al. 2018). However, a history of antibiotic use introduces many potential confounders that are associated with poor outcomes in cancer. Further independent prospective studies are required to validate these findings.

5.2. Systemic Immune Features

Features of the circulating immune and inflammatory cells are correlated with response to ICB. Elevated neutrophil/lymphocyte ratios and elevated lactate dehydrogenase are associated with poor response to ICB in several cancer types (Diem et al. 2016), while elevated eosinophil counts may be associated with increased response (Weide et al. 2016). The presence of increased levels of CD14⁺CD16⁺HLA-DR^{hi} monocytes, as measured by mass cytometry, are associated with increased response to anti-PD-1 therapy in patients with metastatic melanoma (Krieg et al. 2018). Increased regulatory T cells and relative lymphocyte counts were associated with increased response to ipilimumab in melanoma (Martens et al. 2016). Some studies have shown different predictors for response to anti-CTLA-4 and anti-PD-1 treatment, with CD4⁺ and CD8⁺ T cells being potential biomarkers for anti-CTLA-4 therapy, and NK cell subsets being correlated with response to anti-PD-1 therapy (Subrahmanyam et al. 2018). Increased peripheral T cell clonal diversity, measured by TCR sequencing assay, also correlated with response to ipilimumab in melanoma. Low clonal diversity was associated with increased response to anti-PD-1 treatment but with poor response to anti-CTLA-4 treatment, suggesting that different biomarkers may be required for specific ICB targets.

5.3. Germline Polymorphisms

Another host feature that may impact response to ICB is the presence of germline polymorphisms in immune genes. Host HLA-I group genes have been shown to impact ICB response in several ways. Diversity in the repertoire of HLA-I antigen-presenting molecules is driven by heterozygosity of the A, B, and C loci. Maximal heterozygosity of HLA-I loci was associated with increased clonal expansion of T cell repertoire and improved survival in a combined data set of patients with advanced solid tumors treated with ICB (Chowell et al. 2018). The effect of heterozygosity of HLA-I loci on survival was enhanced in high-TMB tumors. The presence of certain HLA-I supertypes, including HLAB44, are also associated with improved survival in melanoma treated with ICB (Chowell et al. 2018). Functional polymorphisms in other genes involved in antigen presentation or cytotoxic T cell effector function may also affect response to ICB.

6. SUMMARY

Overall there is at present no single, clinically validated biomarker of response to ICB that has both high-positive and high-negative predictive value. This is in part due to the limited understanding of the underlying mechanisms of susceptibility and response to ICB and to the fact that multiple tumor, microenvironmental, and host features may come into play in an individual patient. Different cancer types may have intrinsically different mechanisms of sensitivity and response to ICB, which may confound attempts to develop a universal biomarker for immunotherapy. As the field moves toward combining ICB with other active anticancer therapies, it will also be challenging to determine whether responses in individual patients are due to just one of the agents used, or if there is truly a synergistic or additive interaction. Further mechanistic insight into immune checkpoint inhibitors, the development of multimodal predictive models, and more sophisticated analyses of tumor, immune, and host components of patients undergoing ICB treatment will hopefully lead to better tools to guide the optimal use of ICB.

DISCLOSURE STATEMENT

S.G. consults for Novartis, Roche, Foundation Medicine, Inspirata, and Foghorn Therapeutics; he has equity in and patents licensed to Inspirata. S.G.'s spouse is an employee of Merck and has equity in Merck. J.M. consults for or receives honoraria from Genentech, EMD Serono, Merck, and Amgen; receives research funding from Merck, Sanofi, Novartis, Polynoma, Immunocore, Amgen, and AstraZeneca; and receives travel accommodation expenses from EMD Serono and Merck Sharp & Dohme.

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