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The Challenge for Development of Valuable Immuno-Oncology Biomarkers

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Abstract

The development of immunotherapy is an important breakthrough for the treatment of cancer, with anti-tumor efficacy observed in a wide variety of tumors. To optimize immunotherapy use, approaches must be developed to identify which patients are likely to achieve benefit. To minimize therapeutic toxicities and costs, understanding the ideal choice and sequencing of the numerous immuno-oncology agents available for individual patients is thus critical, but fraught with challenges. The immune tumor microenvironment (TME) is a unique aspect of the response to immuno-oncology agents and measurement of single biomarkers does not adequately capture these complex interactions. Therefore, multiple potential biomarkers are likely needed. Current candidates in this area include PD-L1 expression, CD8+ tumor infiltrating lymphocytes, tumor mutation load and neoantigen burden, immune related gene signatures and multiplex immunohistochemical assays that examine the pharmacodynamic and spatial interactions of the TME. The most fruitful investigations are likely to use several techniques to predict response and interrogate mechanisms of resistance. Immuno-oncology biomarker research must employ validated assays to ask focused research questions utilizing clinically annotated tissue collections

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Conflicts of Interest

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and biomarker focused clinical trial designs to investigate specific endpoints. Real time input from patients and their advocates into biomarker discovery is necessary to ensure that the investigations pursued will improve both clinical outcomes and quality of life. We herein provide a framework of recommendations to guide the search for immuno-oncology biomarkers of value.

Introduction

Checkpoint inhibitors are emerging as among the most effective anti-cancer agents in our armamentarium. These agents, which inhibit the immune checkpoint proteins programmed death cell protein-1 (PD-1) or cytotoxic T-lymphocyte associated antigen 4 (CTLA-4) are able to produce durable long-term remissions and are active in a broad range of tumor types, although not all patients respond (1–7). While antitumor activity is seen across a wide range of histologies, the response rate varies by tumor type, with responses to single agent PD-1 inhibitor therapy in the 50–60% range in Merkel cell carcinoma (8) and relapsed Hodgkin lymphoma (9), in the 30–40% range for melanoma and non small cell lung cancers (10,11), and in the 10–20% range for multiple solid tumors in early phase trials (12,13). Combinatorial strategies may improve response rates but also increase toxicity and should be approached with caution given the significant immune-mediated toxicities than can develop (11). Thus, biomarkers that predict response, resistance or toxicity are of paramount importance in order to effectively develop these agents. This paper will discuss the challenges of immune-related biomarkers that relate to the unique science of the dynamic immune microenvironment, the lack of available tissue collections with attention to specimen quality, strategies for incorporation of biomarker endpoints into clinical trials and the critical importance of the patient's perspective, accompanied by recommendations to provide a framework for further investigation and discussion.

An understanding of the challenges and limitations unique to immune-related biomarker research is necessary to determine the optimal selection of patients for therapy

The immune system is in constant flux, with targets that vary based on specific time points and locations within tumors, the presence of "microniches" (created by alterations in perfusion or oxygenation) and multiple different populations of immune infiltrating cells (14,15) (Figure 1). Thus, unlike the evaluation of certain classes of genomic targeted therapies which inhibit specific signaling targets, identifying critical immune interactions to measure at given points in this heterogenous environment may be difficult, rendering biomarker development uniquely challenging. In addition, immuno-oncology biomarkers collected at various time points in a disease course will serve different purposes, for instance, prediction of response versus monitoring for relapse; thus, different clinical scenarios confer different implications for biomarker development.

Candidate biomarkers include markers of a pre-existing anti-tumor immune infiltrate that is observed in certain developing tumors but also may be seen in response to both immune and cytotoxic therapies (14,15). Response to immunotherapy has been linked to an "inflamed" tumor microenvironment (TME), hallmarks of which include expression of interferon-

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inducible immunosuppressive molecules such as PD-L1 and indoleamine 2,3-dioxygenase (IDO) (Fig 1, items 1 and 6), interferon gamma production, M1 macrophages and a robust T-cell infiltrate (Figure 1, item 2) and may have fewer immunosuppressive cells such as M2 macrophages and myeloid derived suppressor cells (Fig 1, items 4, 5)(16,17). Gene signatures associated with T-cell inflamed tumors have also predicted response (Fig 1, item 7)(18). The presence of tumor infiltrating lymphocytes (TILs) in the TME is mechanistically a logical biomarker for PD-1/PD-L1 inhibitor response, as without these cells, no immune response will occur. Examining the TME from patients treated with CTLA-4 or PD-1/PD-L1 checkpoint blockade demonstrates an association between TIL counts and response both before and after treatment (19,20). However, these findings are not universal, and the dynamic and individual nature of the response renders these investigations difficult. TILs are known to be heterogenous and ideal methods of measurement can be a topic of debate, with hematoxylin/eosin (H and E) stain being a traditional method but with multiplex and molecular methods holding promise (21). Further work is needed to define optimal cut-points and other scoring metrics to determine the usefulness of TILs as a response biomarker.

Utilizing the Tumor Microenvironment to Guide Therapy

With several trials targeting the PD-1/PD-L1 checkpoint demonstrating that TME PD-L1 expression is positively associated with response (3,11,22), PD-L1 testing has begun to be incorporated into clinical algorithms. PD-L1 testing has been developed as a companion diagnostic per FDA guidelines for the treatment of non small cell lung cancer (23). However, implementation of widespread testing is limited by the magnitude of benefit, which varies considerably between trials and tumor histologies. It is important to note that the shortcomings of PD-L1 testing in current clinical practice are likely related to the variability of the assays and antibodies used to detect PD-L1, the thresholds used to define positivity, and the TME cell types that express PD-L1 (Fig 1, items 1, 6). Thus, while it seems obvious that PD-L1 on either tumor or immune cells (24,25) must be present for immune checkpoint therapy to be effective and tumors that are truly negative for PD-L1 should be resistant to therapy, the result from a PD-L1 test may be inaccurate and an apparently negative tumor may still respond (3,22,26,27). Ultimately, while PD-L1 expression may enrich for the potential of obtaining treatment response, it alone cannot adequately summarize the complexity of the tumor-immune system interactions and consistently predict patient benefit from immunotherapy. In tumors with constitutive PD-L1 (B7-H1) expression, the predictive value of PD-L1 may be improved by adding additional parameters such as infiltrating CD8+ T-cells or an IFN-gamma gene signature.

In dissecting the tumor microenvironment to understand which tumors may be most responsive to immunotherapy, the “Tumor-Immune Microenvironment (TIME)” classification system, developed by Zhang and Chen is helpful (28). This system builds upon prior classifications of tumors as T-cell inflamed, infiltrated with T lymphocytes and an interferon signature that may be primed for response to immunotherapy, versus non-inflamed tumors that lack robust T cell infiltration and are more resistant to immune approaches (17,29,30). TIME recognizes 4 separate phenotypes within the TME -- **T1** (B7-H1^{-}, TIL^{-}), **T2** (B7-H1^{+}, TIL^{+}), **T3** (B7-H1^{-}, TIL^{+}), and **T4** (B7-H1^{+}, TIL^{-}) -- that

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segregate tumors that are responsive from those that are resistant to checkpoint blockade; see Figure 2 (modelled after the figure in (28) for an illustration of these phenotypes). T2 tumors, which typically contain TILs and other immune cells, but are resistant to cell-killing by TILs due to B7-H1 expression, are thought to account for most anti-PD-1 responses. The T2 tumors that are resistant to anti-PD-1 therapy must have additional dominant immune pathways that are not fully understood and the source of active research. T1 and T4 tumors account for most tumors resistant to anti-PD-1 therapy, as they both lack TILs, but T4 differs from T1 in that it has intrinsic B7-H1 expression, likely due to the activation of oncogenic pathways (31). T3 tumors have TILs but lack B7-H1 expression, likely owing to the absence of IFN-γ production by T effector cells; similar to T1 tumors these are likely to be resistant to anti-PD-1 therapy and may require agents with other mechanisms of action to affect the TME.

Impact of genomic factors on response to immunotherapy

Tumor-specific genomic factors also play a role in response to immunotherapy (Fig 1, item 3). Mutational burden is the non-synonymous somatic mutational load of a tumor measured by DNA sequencing and serves as a surrogate for the number of mutated proteins within a tumor. This is thought to reflect the number of “non-self” antigens that the tumor contains, which is itself a surrogate for tumor antigenicity. The increased mutational burden of microsatellite unstable tumors is believed to account for their sensitivity to checkpoint inhibitors (32). In addition, tumors that harbor defects in enzymes such as DNA polymerase epsilon (*POLE*) may have an “ultramutator phenotype” that is especially vulnerable to checkpoint inhibition (33), a phenomenon similarly identified through investigation of exceptional responders. Furthermore, mutational burden has been associated with improved response to these agents in NSCLC (6) and melanoma (34–36) and recent data suggest that accounting for both the magnitude of mutations and their expression across tumor cell clones is more prognostic than mutational burden alone (37). The widespread expression of neoantigens across the heterogeneous tumor means that a T-cell response to that antigen is more likely to develop, and that a larger proportion of tumor cells are susceptible to attack by T-cells that recognize that antigen. Complicating issues with the applicability of this approach include ready availability of adequate tissue for sequencing, variability introduced by tumor location, whether the tumor is primary or metastatic and the impact of intervening treatments on sequencing results.

High mutation burden is not the only mechanism which induces local immune activation. Many human cancers are associated with viral infections such as Epstein-Barr virus (EBV) and human papilloma virus (HPV). Since viral proteins can be strongly antigenic and lead to a local immune response requiring induction of immune checkpoints for tumor survival, it stands to reason that viral associated cancers may be more immunogenic and more responsive to immunotherapy. Besides antigenicity, viral proteins can also influence the interaction between tumor and immune system through several other mechanisms. For example, patients with latent cytomegalovirus may have increased anti-tumor cytotoxic activity of natural killer cells (38). Likewise, EBV may drive the expression of PD-L1 (independent of 9p24.1 amplification) in Hodgkin’s lymphoma and other EBV+ cancers (39,40). Interestingly, Hodgkin’s lymphoma is one of the most responsive histologies to

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checkpoint blockade and expresses high levels of PD-L1 (41). In head and neck cancers, HPV+ tumors may have less PD-L1 expression but a higher proportion of T-cell infiltration (42). Thus, the presence of viral proteins could serve as an important biomarker for immunotherapy but the exact influence may be tumor- and virus-specific. To date there is little clinical data showing a clear relationship between immunotherapy response and the presence of a viral-associated cancer. In a recent clinical trial testing PD-1 inhibition for Merkel-cell carcinoma, PD-L1 expression was more frequent in Merkel-cell polyoma virus-positive tumors (71% vs 25%) and virus-positive tumors had a higher response rate (65% vs 44%), although the small patient number limits interpretation(8). More research is required to determine whether virus-associated cancers are more responsive to immunotherapy and whether viral proteins can be used as biomarkers for this therapy. It is possible that viral proteins may be useful surrogates for other biomarkers (eg, PD-L1, TIL density) that reflect the underlying mechanism whereby viral proteins influence the tumor – immune interaction.

Developing research focuses on novel biomarkers

Biomarkers of toxicity have been notoriously difficult to identify, however recent studies suggest that the immune microbiome plays a role in the development of toxicity (43–45). The microbiome may also be related to response to immune therapy, as seen in a recent report of a larger cohort of 228 patients with metastatic melanoma, nearly half of whom had been treated with anti-PD-1 therapy. In this study, patients who responded to therapy harbored gut microbiota with greater diversity, particularly greater amounts of clostridia bacterium, and tumors from these responding patients showed significantly increased immune infiltrates. In contrast, among saliva samples analyzed from patients with squamous carcinoma of the head and neck, no significant associations were detected among bacterial diversity with best overall response, tumor PD-L1 expression, or HPV16 status (46). These findings underscore the importance of taking both the tumor and host factors into consideration in the search for biomarkers(47), with further studies needed to determine the relevance of the immiune microbiome in predicting toxicity from and response to immunotherapy.

As immuno-oncology research continues to develop, many novel biomarkers are being explored. T-cell clonal diversity, as measured by T-cell receptor deep sequencing, is a measure of the breadth of the T-cell response that has been linked with response to checkpoint inhibition (20,48). Gene expression profiles, analyzed through proteomic nanostring or RNA-Sequencing approaches, may also be related to the immune response, as has been demonstrated with the use of interferon signatures in melanoma patients treated with pembrolizumab. These signatures correlated with improved clinical outcomes and thus may be ultimately useful for improved patient selection for therapy (18). Exploration of patient factors such as age, sex, and obesity, which may influence immune function, may identify additional biomarkers. As the sophistication and sensitivity of immune assays improve, exploration of peripheral blood immunophenotype and systemic cytokines may reveal prognostic and predictive signatures, and is particularly important given the advantages of blood based biomarkers over tumor biopsies that are invasive, sometimes difficult to procure, and limited by tumor heterogeneity. Proposed approaches include peripheral blood flow cytometry to analyze the immunome; examining PD-1/PD-L1

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exosomal material in plasma as a biomarker of immune evasion; and measuring circulating antibodies with specificity for tumor antigens as serologic markers of immune response (17,49).

While a detailed discussion is outside the scope of this paper, a brief mention of imaging biomarkers in development is important as a novel and developing field. De Vries et al. have utilized several imaging techniques to study early treatment related immune processes after myeloid dendritic cell vaccine injection. Dendritic cells have been tracked with both scintogrpahy (indium-111 labeled) and MRI (superparamagnetic iron oxide formulations) to study vaccine delivery and intranodal migration patterns of dendiritic cells (50). PET scans are also utilized which employ radiolabeled 3′-fluoro-3′-deoxy-thymidine (^{18}F-FLT), a thymidine analog that incorporates into the DNA of proliferating cells at different post vaccination time points after vaccination to visualize immune responses in the lymph nodes. Selective ^{18}F-FLT uptake may indicate a response parameter, with lack of uptake predicting a lack of response (see "Imaging Inflamation" in (49). Further study of these and other immune-PET techniques that are being developed preclinically (51) are of great interest.

To summarize, it is important to note that no one accurate immuno-oncology biomarker is available to select patients for therapy (Table 1). Perhaps a composite approach of multiple biomarkers will ultimately be implemented. While combining current developing biomarkers of immune monitoring may improve upon the current landscape, given the fluid nature of the immune milieu, reliable biomarkers are inherently difficult to identify. Further work is needed in these areas and is ongoing.

The search for valuable immuno-oncology biomarkers requires quality specimen acquisition as part of clinically annotated tissue collections from which information can be shared

While immuno-oncology biomarker discovery faces unique challenges, many parallels can be drawn between these efforts and the study of 'omics-based biomarkers in the setting of clinical trials (52,53). Conditions of specimen collection, processing and storage must be identified and specimen quality, quantity and composition rigorously evaluated. Feasibility studies are often necessary prior to incorporating biomarkers into clinical trials, or in settings where the tissue analyzed is irreplaceable. Standard operating procedures and quality assurance protocols must be developed that consider accuracy, precision, sensitivity and specificity, and turnaround time of assessments must be within acceptable timeframes (52–54).

The above considerations are important for rigorous evaluation of biomarkers that predict both response and primary resistance, as described above, as well as acquired (secondary) resistance. In examining biopsies of tumor tissue, first and foremost it is important to recall that the timing and location of such biopsies are criticial to remember in analyzing results and minimizing variability (17). In addition to an on treatment biopsy to assess parameters such as TIL infiltration, a biopsy may be timed before response is expected to measure viable tumor and changes in lymphocyte infiltration, with an expected appropriate timepoint

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of 2–3 weeks post treatment (17). Additionally biopsies repeated at the time of disease progression are highly informative, but often not completed. The reasons for this are multifactorial: patients whose tumors are progressing may be too distressed about the recurrence to consider another procedure and both physicians and patients may feel pressure to start a new therapy. In addition, many early phase immunotherapy studies have not requested or mandated these biopsies, a potential shortcoming. In part due to the restricitive nature of clinical trial eligibility (55), many patients who must receive immunotherapy as standard of care may have limited opportunities to participate in research directed at studies of resistance, as such studies may be infrequent or under-resourced. A creative solution to this has been the efforts of patient advocacy groups to begin international tissue bank consortia, for instance in melanoma (56). Finally, immuno-oncology agents induce unique changes in the TME that may lower the diagnostic yield and influence tissue quality of biopsies.

An example of the power of this approach is exemplified by work from the laboratories of Ribas and Lo with their seminal discovery of JAK-2 mutations in melanoma patients at the time of progression on pembrolizumab therapy (57). In this study, the authors performed whole-exome sequencing using the NimbleGen SeqCap EZ Human Exome Library followed by targeted gene expression of genes revealed by whole exome sequencing, using the nCountersystem (NanoString Technologies) to analyze biopsies from melanoma patients upon initiation of pembrolizumab therapy and at the time of development of resistance. Established human melanoma cell lines from patients were also used to analyze recognition by T-cell receptor transgenic T cells with the use of in vitro co-culture assays that detect antigen-induced release of interferon-γ. Resistance-associated loss-of-function mutations in the genes encoding interferon-receptor–associated Janus kinase 1 or 2 (*JAK1*or *JAK2*), concurrent with deletion of the wild-type allele, resulted in a lack of response to interferon gamma and insensitivity to its antiproliferative effect on tumor cells. A truncating mutation in the gene encoding the antigen-presenting protein beta-2-microglobulin (*B2M*) was also identified in a third patient and led to loss of surface expression of major histocompatibility complex class I. This elegant study suggests that tumors develop resistance to immune checkpoint blockade by unanticipated mechanisms and further emphasizes the importance of tumor biopsies at the time of progression in order to truly understand *in vivo* biology.

Newer approaches, including image-based *in vivo* detection, may allow further characterization of the TME and more comprehensive examination of pharmacodynamic effects (58) (Table 1). Multiplexed immunohistochemical assays that utilize novel microscopy and image analysis techniques can interrogate multiple antigens and their interactions within the TME. These analyses allow examination of the atypical dose-response relationships observed in immuno-oncology, where standard pharmacokinetic and pharmacodynamic parameters may not apply (59,60). Multiplex immunohistochemical techniques enable quantitative assessment of biomarkers in multiple cell types and their spatial relationships within tissue, facilitating the examination of multiple potential biomarkers simultaneously (e.g. IDO, LAG3, Tim-3, PD-L1, CTLA-4). The information provided by these techniques may reveal insights into the cell-cell interactions that are important for response to immune modulating agents and provide novel biomarkers for study in clinical trials. Particular attention to reagent validation and assay calibration is

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necessary to carry out these techniques, and the impact of tissue quantity and quality (i.e., liquid or small, fine needle aspirate biopsies versus core biopsies) cannot be underestimated (61,62).

In undertaking the above analyses, the availability of collections of clinically-annotated tissue for biomarker studies is a critical need, exemplified by the recommendations of the Cancer Moonshot panel (63). By sharing tissue collections from clinical trials and other sources, computational evaluation of multiple biomarkers using high dimensional correlative data to tease out patterns predictive of response or resistance may be of value (64). The cost and complexity of tissue acquisition and storage and clinical annotation of these collections with evolving clinical outcomes are major obstacles, and the lack of incentive to share resources is a significant limitation. The assembly of cancer immunotherapy networks, and education of patients and providers about the critical importance of tissue collection and analysis, provide important opportunities for advancement.

Biomarker endpoints must be strategically incorporated into immuno-oncology clinical trials

An important way to optimize the study of biomarkers for immunotherapy, given the challenges set forth above and the utilization of valuable tissues with finite availability, is the careful selection of biomarker endpoints to explore when designing clinical protocols. Prognostic biomarkers, which may be of use in defining a high-risk population in particular need of treatment, and predictive biomarkers that indicate whether a patient will benefit from a particular treatment (52,65–68), are needed. Choice of trial design must be carefully considered as there are a number of designs for incorporating biomarkers into phase 3 trials, depending on the predictive power of the biomarker(66,67,69–71). The simplest designs are the “enrichment” and “stratified” designs. If it is reasonably clear that treatment benefit is limited to the biomarker positive subgroup, the enrichment design is the most efficient and ethical. If there is doubt about whether the treatment benefit is limited to the biomarker subgroup, or there is doubt that the biomarker is accurate for that group, then the biomarker-stratified design is preferred, although it may increase the number of patients required. There should be sufficient power to detect the targeted treatment effect in the biomarker positive subgroup, but the biomarker negative subgroup is likely to be descriptive. The “biomarker strategy” design, whereby patients are randomized to be treated uniformly versus treated differentially according to biomarker status, is statistically inefficient as it includes the biomarker negative patients who are treated identically in both arms (66). For those reasons it is not preferred.

More complicated designs allow for integrating treatment and biomarker evaluation (70) in phase 3 trials. One example allows for testing the treatment effect separately in the marker positive and negative subgroups, as well as in the total patient population. The alpha (defined as the probability of incorrectly rejecting the null hypothesis) is split between the comparison in the marker positive subgroup and that in the over-all population (for example, .015 and .010, respectively), and the comparison is conducted first in the marker positive subgroup. If this test is positive, then the comparison is conducted at total alpha (.

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025) in the marker negative subgroup. On the other hand, if it is negative, then the comparison is conducted in the over-all population at the remainder of the alpha (.010). This approach allows for testing in both marker subgroups as well as including a back-up comparison in the overall population, in case the biomarker turns out to be non-predictive. An inappropriate variant of this approach is to conduct the comparison in the total population, rather than in the marker negative subgroup, if the comparison is positive in the marker positive subgroup. The serious flaw in this is that it can result in generalizing the recommendation for treatment to the marker negative subgroup, with high probability, if the effect is sufficiently strong in the marker positive subgroup or if that subgroup is a sufficiently large fraction of the whole, even if there is no treatment benefit in the marker negative subgroup(70).

Biomarkers can also be incorporated in phase 2 trials (65,68), to initially evaluate both the efficacy of the treatment and the potentially predictive value of the biomarker, to determine which phase 3 trial design is most appropriate. Freidlin et al. (68) give precise rules for distinguishing among the following:

1. The treatment is ineffective in the total population and it is ineffective in the biomarker positive subgroup, in particular, and, therefore, it should not be tested further.
2. The treatment is promising overall, with no predictive value to the biomarker, and should be tested in a phase 3 trial with no biomarker incorporation.
3. The treatment is promising in the biomarker positive subgroup only and should be tested in a phase 3 trial so restricted.
4. The treatment is promising, but the predictive value of the biomarker is unclear, and should be tested in a biomarker stratified phase 3 trial.

The above designs represent suggestions of different scenarios for biomarker driven investigations. Given the identified challenges in procuring tissue for biomarker research, it is critical that selected endpoints be part of focused research questions. Biomarker investigations in phase 0 and phase 1 trials are beyond the scope of this paper. While phase 0 trials generally have PD primary endpoints and phase 1 trials may have expansion cohorts to address PD secondary or exploratory endpoints, neither, in general, address the relation of potential biomarkers to clinical outcomes.

Biomarker selection must incorporate patient perspective and value assessments

While immunotherapy shows real promise for some patients, responses do not occur in most patients, although those who achieve responses, especially complete or near complete responses, may enjoy long term durable remissions. It is thus of critical importance to develop biomarkers that predict which patients are most likely to benefit. These efforts are enthusiastically supported by patient communities as long as they are involved in this process to ensure that future guidelines produce useful results for patients (72,73).

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Which biomarkers are patients most interested in?

Overall, predictive biomarkers contain the most promise for patients, whether they predict treatment response, adverse event profiles, or long-term remission. The most important biomarker, however, depends on each patient's situation. For instance, patients who do not have cancer are most interested in prognostic biomarkers of risk and actions they can take to avoid cancer. Patients with early stage cancers value prevention of recurrence, so predictive markers that help them identify valuable treatments are a priority. For patients with metastatic disease, pharmacodynamic measures of response to treatment, and validated predictive markers of response are most important. Common to all of these scenarios is a desire for tests that provide accurate, reproducible results that help them make treatment decisions. Unfortunately, few immuno-oncology biomarkers are available that meet these needs of patients. Currently available biomarkers that direct optimal selection and sequencing of therapy, such as PD-L1 expression, are imperfect at best given the variability of the assay as well as the inability to account for the complex response relationships of the TME. While an accurate, reliable biomarker is of highest priority in development, the value of blood based biomarkers that can reliably affect what is happening in the TME, compared with tumor that may be invasive to procure and limited by heterogeneity, is apparent.

When a biomarker recommends treatments of similar efficacy, measures of toxicity are of paramount importance to patients. For this reason, there is a clear need to develop biomarkers that predict toxicity in this setting. Patient-Reported Outcomes (PRO) and Quality of Life (QOL) studies may yield important insights and are discussed further by Anagnostou et al in this series (74). For example, a recent analysis of patient-reported outcomes from the CheckMate-067 trial showed differences in QOL, global health, and symptom burden between patients with melanoma treated with nivolumab, ipilimumab or the combination, which allows us to consider whether patients are willing to tolerate significant toxicities if survival is higher (75). In this setting, biomarkers of toxicity are of great value as they could predict the development of side effects for that patient group and can be used to guide treatment choices.

Finally, financial burden is an important consideration and biomarker tests that predict benefit from a specific treatment will help patient avoid the cost of therapy that is not likely to be effective (76). This makes it even more important to integrate biomarkers with patient outcome data to assess true value to patients (77). It is imperative to recall that impressive clinical trial results, if financially out of reach for the vast majority of patients, cannot be considered a true success.

Conclusions

With cancer immunotherapy likely to become a cornerstone of therapy for multiple cancer types, the importance of reliable biomarkers cannot be understated. It is important to remember that the development of immuno-oncology biomarkers is in its infancy, with few adequately validated assays that predict response to therapy. Given the complex and expensive nature of immuno-oncology biomarker discovery, focused research questions involving comprehensive tissue collections with carefully selected endpoints are a priority. In addition, incorporating patient preferences and perspectives is critical in the search for

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biomarkers of value. The future of immunooncology biomarker discovery shows great promise as early studies reveal novel biomarkers that are predictive of response and toxicity and these areas of research are being vigorously pursued.

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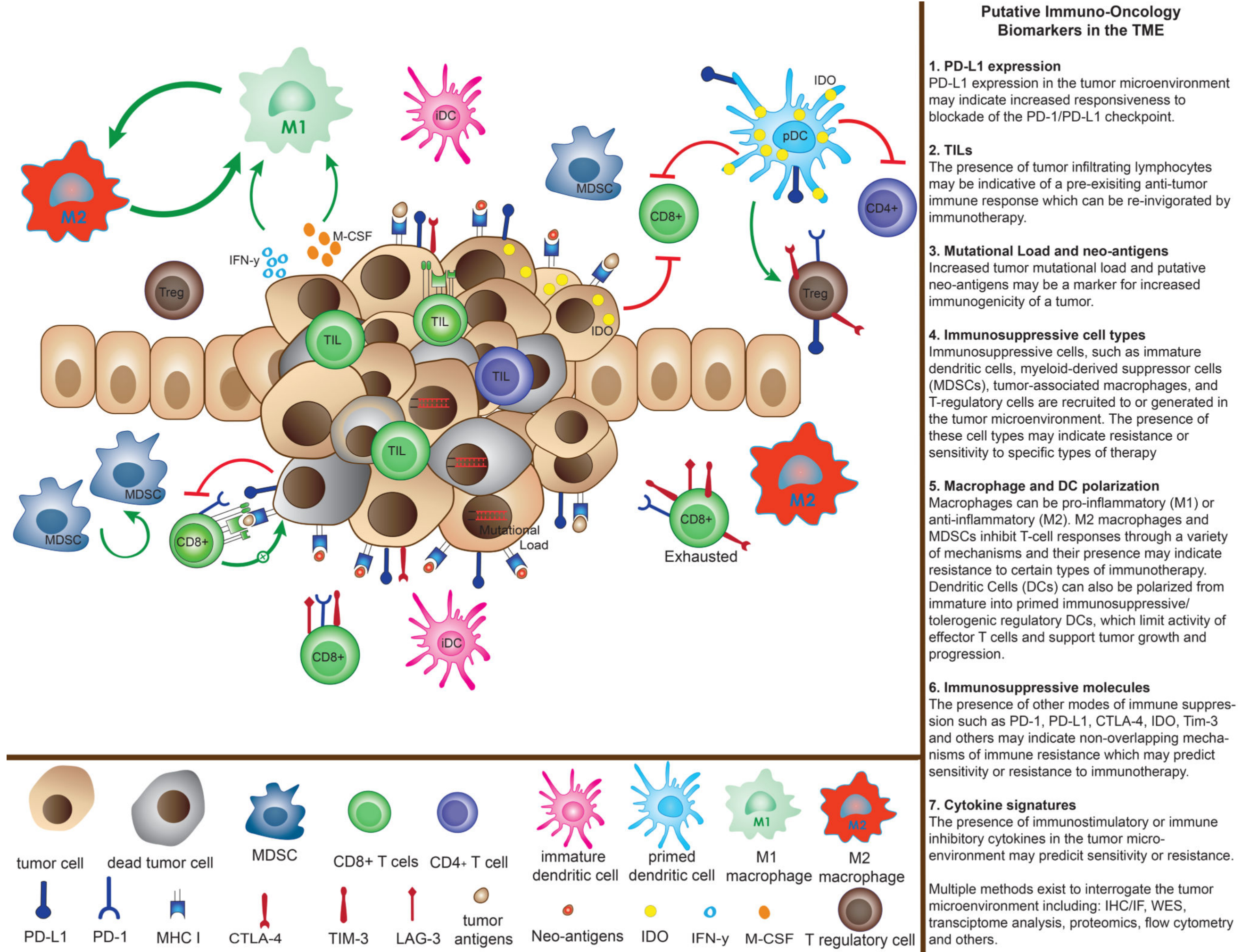

Figure 1. Putative Immuno-Oncology Biomarkers in the TME

1. PD-L1 expression PD-L1 expression in the tumor microenvironment may indicate increased responsiveness to blockade of the PD-1/PD-L1 checkpoint. **2. TILs** The presence of tumor infiltrating lymphocytes may be indicative of a pre-existing anti-tumor immune response which can be re-invigorated by immunotherapy. **3. Mutational Load and neo-antigens** Increased tumor mutational load and putative neo-antigens may be a marker for increased immunogenicity of a tumor. **4. Immunosuppressive cell types** Immunosuppressive cells, such as immature dendritic cells, myeloid-derived suppressor cells (MDSCs), tumor-associated macrophages, and T-regulatory cells are recruited to or generated in the tumor microenvironment. The presence of these cell types may indicate resistance or sensitivity to specific types of therapy. **5. Macrophage and DC polarization** Macrophages can be pro-inflammatory (M1) or anti-inflammatory (M2). M2 macrophages and MDSCs inhibit T-cell responses through a variety of mechanisms and their presence may indicate resistance to certain types of immunotherapy. Dendritic Cells (DCs) can also be polarized from immature into primed immunosuppressive/tolerogenic regulatory DCs, which limit activity of effector T cells and support tumor growth and progression. **6. Immunosuppressive molecules** The presence of other modes of immune suppression such

as PD-1, PD-L1, CTLA-4, IDO, Tim-3 and others may indicate non-overlapping mechanisms of immune resistance which may predict sensitivity or resistance to immunotherapy. **7. Cytokine signatures** The presence of immunostimulatory or immune inhibitory cytokines in the tumor microenvironment may predict sensitivity or resistance. Multiple methods exist to interrogate the tumor microenvironment including: IHC/IF, WES, transciptome analysis, proteomics, flow cytometry and others.

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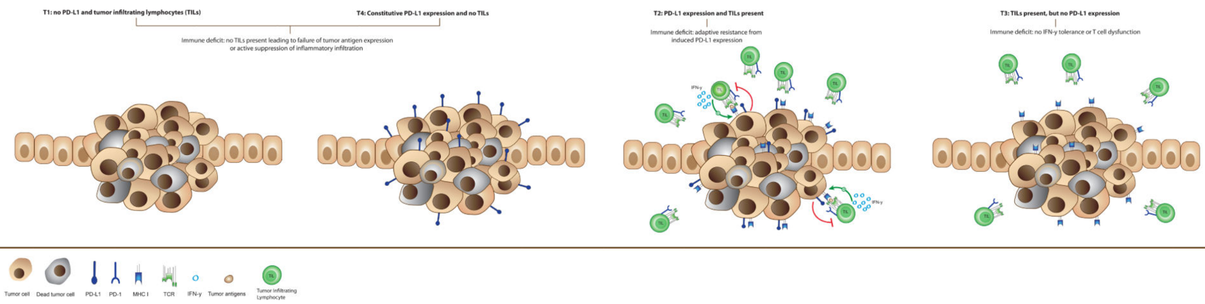

Figure 2. Subtypes of Tumor Immunity in MicroEnvironment (TIME) Classification

This figure, modelled after Zhang and Chen's (28), illustrates the four tumor subtypes of TIME; T1-T4.

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Table 1

Clinical significance and challenges of biomarkers for immunotherapy in development

Biomarker/ Technique	Clinical Significance	Challenges
All	Predict response, resistance, or toxicity to immune therapies	Dynamic immune microenvironment, Heterogeneity due to biopsy location, type, and primary versus metastatic lesion, Impact of intervening therapies
PD-L1 [3,10, 25]	Immunohistochemical approach to measure PD-L1 expression on tumor and immune cells	Variability in assays, antibodies, and TME cell types detected
$CD8^+$ T cells[11,19,20]	PD-1/PD-L1 expression on these cells predicts response to PD-1 blockade	Optimal cut-points, scoring metrics, and agreement on magnitude of change required for meaningful prediction of response
Tumor mutation load/WES[6,33–37]	High mutation load resulting from various factors (environmental insults, DNA repair defects) correlated with vulnerability to checkpoint inhibitor therapy in exceptional responders	Availability of adequate tissue for sequencing, WES costly with slower turnaround time than many clinical assays
Neoantigen burden[6,35–57]	Predict clinical benefit to ipilimumab and PD-1 blockade in lung cancer and melanoma	Dependent on WES data, with similar obstacles of cost and time
T cell clonal diversity[20, 48]	T-cell receptor deep sequencing to measure breadth of immune response	Availability of adequate tissue for sequencing, pre-identification of recognized antigens may be required for further investigations
Multiplex IHC[54,58,59]	Immunofluorescent detection of multiple immune cell and tumor phenotypes simultaneously as well as evaluation of spatial relationships within TME	Rigorous pre-assay calibration required; immuno-oncology agents may not affect all markers in the multiplex assay equally and primary markers must be selected
Microbiome modulation[44–47]	Components of the gut microbiota may facilitate the antitumor efficacy of immune checkpoint blockade	Inter-patient variability within the microbiome contributes to substantial heterogeneity in the mounting of immune responses
Gene expression profiling[18–20]	Interferon-gamma induced signatures especially may predict clinical benefit to checkpoint inhibition	Sizable tissue collections necessary to validate testing and training sets