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Biomarkers for Immunotherapy of Cancer

Methods and Protocols

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Biomarkers for Immunotherapy of Cancer

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Preface

Immunotherapies have emerged as highly promising approaches to treat cancer patients. The clinical efficacy of immunotherapy is limited to a minority of cancer patients. Thus, new approaches to improve the efficacy of immunotherapies for most patients are critically needed. Strategies to improve the efficacy of immunotherapy rely on both an enhanced understanding of the tumor/immune interface at the cellular and molecular level and an ability to select appropriate patients for a specific immunotherapy agent or combination therapy. Optimized biomarker strategies could help elucidate both of these areas and allow cancer immunotherapy to be tailored to the individual patient's disease.

This book evaluates the criteria currently used for the diagnosis and prognosis of cancer and for the prediction of its responsiveness to immunotherapy. Here, we endeavor to frame technical aspects within the boundaries of their suitability to address fundamental questions related to cancer immune responsiveness. We emphasize that methods should be attuned to the biology investigated and be gradually implemented from simplest to most complicated according to the proven need in the systematic quest to circumvent cancer immune responsiveness.

The positive reactions and feedback to the previous volume *Molecular Diagnostics for Melanoma* in 2014 that we edited for Springer have been appreciated and reinforced the importance of the biomarker focus to the disciplines of diagnosis and prediction in cancer. Melanoma has led the field of cancer in which immunotherapy has produced major clinical inroads. Despite their paradigm-shifting success in melanoma therapy, most patients still do not respond (or respond durably) to checkpoint inhibitors. A more complete understanding of the determinants of response, either from clinical or basic studies, could lead to more rationally targeted immunotherapies as well as novel ones. Why do some patients respond to immunotherapy while others do not? Speculation about this question is at the frontier of immunotherapy and immunobiology. Many questions remain about how best to select patients who will benefit from checkpoint inhibitors and how to optimally combine different complementary immunotherapy approaches with each other and with traditional cancer treatments. Therefore, the focus of the current volume has adjusted the focus to biomarkers for immunotherapy. The specific intent of this volume is to provide up-to-date information for the biomarkers and assays with the potential to predict responsiveness and the methods to assess them in clinical samples.

The critical importance of clinically applicable biomarkers based on the immunoprofiling prompted the addition of a new editor, Alessandra Cesano, MD, PhD, who is currently chief medical officer of the NanoString Technologies, Inc., has been a colleague for years, and has been extremely active member of the Society of Immunotherapy of Cancer. She is an extremely welcomed addition as the third editor of this book.

Based on broad needs and interest in enhancing the clinical results for immunotherapy, the chapters are focusing on methods for well-standardized assays that can be applied in research laboratory and have the potential to be translated into the clinic. It is reasonable to speculate that mutational load allows for more neoantigens and therefore more likelihood of response to checkpoint inhibitors. Indeed, mutation burden alone has been

correlated with the response of melanoma to ipilimumab and clinical benefit to anti-PD-1/PD-L1 inhibitors for NSCLC, bladder cancer, and head and neck cancers. The volume includes chapters on different aspects of tumor mutation burden analysis and interpretation. Computational and experimental approaches that consider the prediction of the optimal load of antigens on MHC molecules are also included.

There are chapters on the use of patients' samples of gene expression profiling to identify the differences between responders and nonresponders for immunotherapy. Such a strategy has the potential to more accurately apply drugs to patients who will benefit and avoid the cost and potential side effects for patients who won't benefit and need alternative therapies.

Several studies have linked the presence of tumor-infiltrating immune cells to prognostic and predictive benefit from immunotherapy. Clinical immunotherapy trials suggested that tumors with a high number of inflammation-causing T cells were more responsive to the immunotherapy-based drugs. Tumors with low inflammation, or low numbers of T cells, were less responsive to checkpoint inhibitors, highlighting the potential role of cytotoxic T-cell biomarkers such as CD8. Thus, in situ detection methods can have great potential value in patient selection and deserve systematic validation. Infiltrating immune cells into the tumor microenvironment are effectively captured through spatial and pictorial representations that inform on the antitumor immune activity. Mapping of the immune tumor microenvironment when applied in a systematic way provides the investigators a method to understand the tumor microenvironment activity and its interface with the immune system. Many IHC-based assays, including multiplex setup using different technical approaches, continue to emerge and have been discussed in this volume.

The range of information required to effectively select the best therapeutic combination for a patient has expanded enormously with the addition of many immune-oncology agents with different mechanisms of action. Because of the complexity of the immune response and tumor biology, it is unlikely that a single biomarker will be adequate to predict clinical response as demonstrated in multiple studies. Systems that systematically integrate each patient's morphological and molecular information that can be correlated to patient outcomes are needed. Thus, chapters focusing on the important role of integrating comprehensive research data for developing clinically relevant information were included in this book.

We hope that this book provides its audience with a deeper understanding of the broadening scope of the biomarker methods and needs to improve the outcome from immunotherapy. The editors made sure that the features input from experts in the field dedicated to translate scientific research from bench to bedside were included. The book provides not only details about the technical, standardization, and interpretation aspects of the methods but also introduces the reader to the background information. The complexities and intricacies of the tumor biology that justify the biomarker and assay development based on the scientifically rigorous research are also mentioned. The chapters' providers ensured that the highest standards are maintained, and each chapter contains hands-on, practical suggestions, illustrations, and examples throughout. We are proud of this book on so many aspects and hope that the commitment and the expertise of the contributors to this volume will be appreciated by the readers.

Bethesda, MD, USA — *Magdalena Thurin*
Seattle, WA, USA — *Alessandra Cesano*
Menlo Park, CA, USA — *Francesco M. Marincola*

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Part I

Scientific Questions and the Status of Inhibitory Receptors for Immunotherapy

Chapter 1

Status of Immune Oncology: Challenges and Opportunities

Alessandra Cesano, Francesco M. Marincola, and Magdalena Thurin

Abstract

This volume is intended to review the methods used to identify biomarkers predictive of cancer responsiveness to immunotherapy. The successful development of clinically actionable biomarkers depends upon three features: (a) their biological role with respect to malignant transformation and tumor progression; (b) the ability to detect them with robust, reliable, and clinically applicable assays; and (c) their prognostic or predictive value, as validated in clinical trials.

Identifying biomarkers that have predictive value for patient selection based on the likelihood of benefiting from anticancer immunotherapy is a lengthy and complex process. To date, few predictive biomarkers for anticancer immunotherapy have been robustly analytically and clinically validated (i.e., PD-L1 expression as measured by IHC assays and microsatellite instability (MSI)/dMMR as measured by PCR or IHC, respectively).

This introductory chapter to this book focuses on scientific and technical aspects relevant to the identification and validation of predictive biomarkers for immunotherapy. We emphasize that methods should address both the biology of the tumor and the tumor microenvironment. Moreover, the identification of biomarkers requires highly sensitive, multiplexed, comprehensive techniques, especially for application in clinical care. Thus, in this chapter, we will define the outstanding questions related to the immune biology of cancer as a base for development of the biomarkers and assays using diverse methodologies. These biomarkers will likely be identified through research that integrates conventional immunological approaches along with high-throughput genomic and proteomic screening and the host immune response of individual patients that relates to individual tumor biology and immune drugs' mechanism of action.

Checkpoint inhibitor therapy (CIT) is by now an accepted modality of cancer treatment. However, immune resistance is common, and most patients do not benefit from the treatment. The reasons for resistance are diverse, and approaches to circumvent it need to consider genetic, biologic, and environmental factors that affect anticancer immune response. Here, we propose to systematically address fundamental concepts based on the premise that malignant cells orchestrate their surroundings by interacting with innate and adaptive immune sensors. This principle applies to most cancers and governs their evolution in the immune-competent host. Understanding the basic requirement(s) for this evolutionary process will guide biomarker discovery and validation and ultimately guide to effective therapeutic choices. This volume will also discuss novel biomarker approaches aimed at informing an effective assay development from a mechanistic point of view, as well as the clinical implementation (i.e., patient enrichment) for immune therapies.

Key words Cancer immune resistance, Predictive biomarkers, Checkpoint inhibitors

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Abbreviations

CDR3	Complementarity determining region 3
CIT	Checkpoint inhibitor therapy
FDA	Food and Drug Administration
FFPE	Formalin-fixed paraffin-embedded
HLA	Human leukocyte antigen
ICD	Immunogenic cell death
ICR	Immunologic constant of rejection
IFN	Interferon
IHC	Immunohistochemistry
IL	Interleukin
IO	Immune oncology
MOA	Mechanism of action
MSI-H/dMMR	Microsatellite instability high/deficient mismatch repair
PD-L1	Programmed death-ligand 1
STAT	Signal transducer and activator of transcription
TCR	T cell receptor
TILs	Tumor-infiltrating lymphocytes
TIS	Tumor inflammation signature
TMB	Tumor mutation burden
TME	Tumor microenvironment

1 Introduction

> *In biology the answers pre-exist; it is the question that needs to be discovered*
> Jonas Salk, 1969 [1]

At no time this quote from Jonas Salk has been as pertinent as in this era of high-density data generation. Perhaps, the answer to the multifaceted question, "*Why do some patients and their cancer respond and others to not to immunotherapy?*" is already waiting in the meanders of large data pools and it is up to us to educate our queries to turn them into biologically dissectible elements.

Checkpoint inhibitor therapy (CIT) with anti-PD-1/PD-L1 and CTLA-4 inhibitors has proven to be a successful approach to anticancer immunotherapy because it has shown significant improvement in patient survival in multiple histologic types of advanced metastatic solid tumors [2]. In addition, several other immune oncology (IO) approaches such as adoptive cellular therapy and oncolytic virus-based products have shown promising results in specific indications [3–5]. However, the majority of advanced cancer patients receiving IO drugs do not benefit from these treatments. Many variables affect the efficacy of the response and additional inhibitory checkpoints can play a significant role in inhibiting anticancer response. Tumor heterogeneity at the steady

state is another obstacle to the success of cancer immunotherapy. Tumor escape mechanism(s) such as the development of cancer cell-resistant clones as well as antigen negative selection contribute to the therapeutic failures particularly in dynamic evolution of the tumor microenvironment (TME) in response to the selective pressure exercised by initially successful treatment [6]. Finally, several categories of circumstantial factors not directly related to the genetics of the host or the somatic evolution of the cancer cells such as environmental and behavioral factors, presence of comorbidities and respective non–cancer-related therapies and previous host immune status related to the age and the history of individual patients may affect responsiveness to therapy. An interesting circumstantial factor that may play a role in immune responsiveness to adoptive cell therapy is the quality of the cellular product.

Therefore, we emphasize that the quest for biomarkers that may predict responsiveness to help patient stratification should take into account several coordinates that determine the natural or treatment-induced evolution of cancer in the immune competent host including (1) the genetic background of the host; (2) the somatic genetic, epigenetic, and functional adaptation of cancer cells; (3) the circumstantial modifiers, and (4) the dynamic evolution in time determining immune escape, epitope spreading, and modifications induced by concomitant treatment [7].

These coordinates must be considered particularly when novel combinatorial approaches are sought based on diverse experimental evidence for their immune resistance [8, 9]. We recently assembled an inventory of mechanisms extracted from the public domain that have been proposed to be determinants of cancer immune resistance. We distillated them into a unified "*theory of everything*" [9]. We first segregated cancer immune landscapes into immune "active" versus immune "silent" according to the expression of a transcriptional signature termed "*the immunological constant of rejection*" (ICR) [10, 11]. The ICR defines the continuum of cancer immune surveillance bearing favorable prognostic and predictive connotation [12]. We then considered the unsupervised distribution of the expression of transcriptional signatures associated with immune regulatory properties in the cancer microenvironment [9]. These include other immune checkpoints [13], regulatory T cells [14], IL-23/IL-17 axis [15], myeloid suppressor cells [16], Indoleamine 2,3-dioxygenase 1 (IDO) [17], immunogenic cell death (ICD) [18], TAM family of tyrosine kinase receptors [19], hypoxia [20], cancer-associated fibroblasts [21], and barrier molecules [22]. In addition, oncogenic pathways associated with cancer immune landscapes were included such as the MAPK [11], the β-catenin [23], and the PI3K-γ [24] signatures. Self-organizing clustering assigned signatures to immune landscapes

and the significance was assessed by gene enrichment analysis. This approach demonstrated that all immune regulatory functions pertain to immune active cancers [9]. In addition, the PI3K-signature was preferentially expressed in the immune active landscape likely as a hallmark of myeloid cell function [25] rather than intrinsic cancer cell biology [26]. Immune silent cancers defined by lack of the expression of the ICR signature were depleted of all immune regulatory functions but were enriched with signatures related to specific oncogenic processes such as the activation of the β-catenin and of MAPK pathways [9]. In addition, we previously observed that silent cancers are characterized by a distinct mutational profile characterized by a low mutational burden resulting in decreased alterations of oncogene and tumor suppressor gene function [8, 27, 28]. Thus, silent cancers have a "cleaner" mutational footprint.

The observation that all immune regulatory functions, be it harbinger of immune effector or immune suppressive activity, were coexpressed led to the hypothesis that cancer evolution in the immune competent host faces a stochastic binary choice: some cancers orderly accrue a succession of genetic alterations that lead to essential growth advantages in avoidance of unnecessary functions similarly to the developmental process applied by stem cells in forming organs. When deviations occur from this orderly process, and cancer growth becomes dependent predominantly on genetic instability, a "trial-and-error" reshuffling of genetic traits gives growth advantage. The latter, however, appends the stochastic risk of gradually accumulating unnecessary functions such as tissue remodeling and chemoattraction that may trigger immune recognition [29]. In addition, it is possible that genetic instability may result in a disorderly cell cycle prone to ICD. Currently, the efficacy of immunotherapy is limited by mechanisms of resistance. These mechanisms of resistance not only define the outcomes and limit current immunotherapy but also point to future need to categorize cancer patients to facilitate antitumor immunity. Future studies should build on those trials and seek additional biomarkers that might improve the antitumor immune response, with the ultimate goal of increasing the rates of lasting responses to immunotherapy.

The enrichment of immune functions within the active landscape suggests that immune resistance to CIT in tumors is due to the presence of alternative compensatory regulatory mechanisms that surmount its effects. We refer to this mechanism as compensatory immune resistance. It is likely that the cancer immunity cycle described by Chen and Mellman [30] pertains particularly, and perhaps exclusively, to the cross talk of immune cells with cancer cells in immune active tumors.

Conversely, immune silent cancers are unlikely to respond to CIT because immune checkpoints are irrelevant to their natural history. We refer to this as primary immune ignorance. We may then refer to circumstantial immune resistance when factors extrinsic to the intrinsic biology of the host and its cancer play a modifiers role including the quality of the cell product in adaptive cell therapy approaches [31] .

In addition, it is possible that the immune responsive tumors may become resistant in response to selective pressure during successful therapy, thus developing escape mechanisms; we define this phenomenon as acquired or secondary immune resistance. Finally, we should refer to pseudo immune resistance when treatment cannot be completed due to limiting toxicity or requires administration of immune-suppressive drugs to control the autoimmune associated side effects that disable full potentiality of an IO approach.

2 From Immunology Back to Cell Biology

According to the current understanding of the role played by cancer genetics and its intrinsic cell biology in determining immune responsiveness, we propose that the primary questions to be addressed in the field of IO are whether:

1. Cancer is primarily a cell biology problem whereby malignant cells orchestrate changes in their surroundings or whether other environmental or germline factors play a significant role.
2. The immune response against cancer is primarily determined by innate immune mechanisms alerted by the release of damage-associated molecular patterns by cancer cells undergoing ICD or is primarily determined by self–nonself discrimination induced for instance by the expression of neoepitopes by the mutated cancer cells.

In this chapter, we postulate that the intrinsic biology of the cancer cell largely orchestrates its surroundings through the release of factor that stimulate the growth of a supportive stromal and vascular architecture in the developing new tissue as suggested by the Virchow's "healing wound" model [10, 29]. The cross talk with host cells may also result in varying degrees of chemoattraction of innate and adaptive immune cells, thus turning the cancer into a chronically inflamed tissue [29]. This collateral effect may not occur in all but only in the immune-active cancers, while the immune silent cancers follow a tissue remodeling biology closer to natural organ development rather than wound healing.

We propose in addition, that a stochastic process led by genetic instability encompasses an excessive accumulation of "trial-and-error" attempts that may destabilize the cell cycle and gradually degenerate into stress-associated ICD [32]. This notion is supported by the observation that the ICD signature is exclusively associated with the immune active phenotype [9], which in turn is characterized by genetic instability and increased mutational burden [33]. The relationship between immunogenicity and increased mutational burden has been ascribed to increased chances of developing neoantigens [34]. Here, we propose an alternative explanation related to the destabilization of the cellular life cycle resulting in the release of damage-associated molecular patterns more in line with Polly Matzinger's danger model [35]. Whether the former or the latter interpretations are correct remain to be defined in the context of human tumor biology, and these diverse, though not necessarily mutually exclusive, interpretations need to be taken into consideration when predictive and mechanistic biomarkers are sought.

At the end, despite elegant experimental models supporting either theory, the lead role played in humans by cancer cell biology and the response to it by adaptive and innate immune mechanisms remain to be defined.

3 A Systematic Quest Toward Understanding and Circumventing Cancer Immune Resistance

With the augmented interest in CIT and IO in general, the number of patients enrolled in clinical trials has increased exponentially including large randomized studies in which treatment efficacy in different patient populations can be evaluated. This provides an unprecedented opportunity to acquire precious samples to dissect the phenotype and genomic underpinning of immune responsiveness directly in human samples. Ayers et al. [36] used a transcriptional signature comparable to the ICR [9] termed "*the tumor inflammation signature*" (TIS) to define cancer immune landscapes that predicted immune responsiveness. They observed that the expression of interferon (IFN)-γ-related transcripts in pretreatment lesion is a strong predictor of immune responsiveness to CIT. This is very similar to previous observations in patients receiving systemic human recombinant interleukin-2 (IL-2), where the hallmark IFN-γ signaling signature of the ICR was the best predictor of complete responses [37]. Similarly, the expression of the IFN-γ-related chemokines CXCL-9, CXCL-10, CXCL-11, and CCL5 in melanoma lesion harvested for the expansion of tumor-infiltrating lymphocytes (TILs) representative of the ICR was a strong predictor of response to TIL therapy [38]. This and other comprehensive analyses

of pretreatment lesions using high-throughput technologies have better framed the biology of cancer immune responsiveness as discussed elsewhere [9, 28]. What is needed now is a systematic, hypothesis-driven design of future clinical trials that may expedite the collection of useful information to compare immune and tumor profiling data generated by different groups to integrate into database to be available for secondary analysis of determinants of cancer immune responsiveness [8, 28].

4 Framing the Question

What are the reasons that may determine human cancer immune resistance?

4.1 Predicting Immune Responsiveness and the Role of Pretreatment Biopsies

Most frequently, clinical trials include pretreatment tumor biopsies collection. These may provide important insights about the biology relevant to immune-responsiveness. In 2002 we published the result of a prospective molecular profiling of melanoma metastases in patients undergoing tumor antigen vaccination in combination with the systemic administration of IL-2 that suggested classifiers of immune responsiveness. We concluded that that "*immune responsiveness might be predetermined by a tumor microenvironment conducive to immune recognition*" [39]. Since then several studies have shown that tumors that are characterized by an immune active immune environment are more likely to respond to IO including CIT [36]. Interestingly, as previously described, the "immune signature" defining active tumors includes many transcripts that can be observed across several tumor types and includes, as described in the theory of everything, all immune effector and regulatory components [28]. The signature can be therefore, an indicator of immune activation status but it does not inform about the mechanisms of immune responsiveness. Although suboptimal but good example of the predictive biomarker is the assessment of the expression of the *Programmed-Cell Death Receptor (PD-1)-Ligand 1* (PD-L1/CD274) by cancer and/or immune cells at baseline, that is, before targeting the PD-1/PD-L1 pathway. It may be that the expression of the molecule targeted by CIT has little to do with the mechanistic interpretation that is frequently offered but rather it is a marker "associated" with an immune responsive phenotype as checkpoint inhibitors and their ligands are part of the extended signature of immune responsiveness where multiple rather than a single factors contribute to immune responsiveness in the context of compensatory immune resistance [28]. Although, overall, the extent of pretreatment and especially treatment-induced intratumoral T cell infiltration correlates with clinical responses, thereby supporting unleashing of tumor-specific T cells as the primary basis of anti-PD-1 therapy, the mechanistic basis for the variation in

response patterns or long-term clinical benefits (i.e., survival) remains poorly explained. Thus, a careful distinction must be made between predictive biomarkers that represent an association rather than having mechanistic significance even in cases when the latter would seem otherwise intuitive.

4.2 Systemic Effects of Therapy

There is currently little agreement over systemic parameters that are informative about treatment efficacy. Although associations have been proposed, most bear limited predictive value and mechanistic significance [40]. Most importantly, there is no known relationship between biomarkers accessible through the peripheral circulation and cancer immune landscapes although the latter have been shown to be closer predictors of responsiveness to IO approaches.

One of the salient goals in biomarker studies should address the alignment of systemic immune status with the corresponding tumor immune landscape. An example of a sound hypothesis-driven strategy is based on Peter Lee's seminal observation that circulating immune cells in patients with cancer display a dampened response to IFN stimulation compared with normal individuals. The assessment of signal transducer and activator of transcription (STAT)-1 phosphorylation ex vivo can reproducibly demonstrate this defect [41]. The deficiency is patient-specific and is dependent on tumor burden as dampening worsens progressively with advancing stages of disease. Yet a large proportion of patients is not affected and maintains normal phosphorylation of STAT-1 until the latest stages of disease progression. To our knowledge, no study has addressed the relationship between cancer immune landscapes and decreased STAT-1 phosphorylation, although we observed that overexpression of nitric oxide synthase 1 (NOS1) by melanoma cell lines could reproduce in vitro the dampening of IFN responsiveness in lymphocytes [42]. We suggest that the STAT-1 phosphorylation of circulating immune cells in response to IFN could be a specific biomarker of cancer immune landscapes. We also hypothesize that immune-active cancers are those most likely to induce such deficiency via compensatory immune regulatory activity that may reverberate in the peripheral circulation. The value of an easily accessible circulating biomarker representative of cancer immune landscapes could have important implications in understanding the mechanisms of immune responsiveness particularly during the evolving phases of therapy.

4.3 On-Target Effects of Therapy and the Critical Role of On-Treatment Biopsies: the Value of "Δ"

An obvious requirement for the understanding of immune responsiveness is validation of the mechanism of action (MOA) predicted for a given agent and the target organ and the defined metrics of changes compared with paired pretreatment biopsies. Intuitively, this seems a paramount requirement, yet clinical trials rarely include the collection of on-treatment biopsies and even less frequently paired pretreatment and on-treatment biopsies in which the "Δ"

changes from baseline can be accurately documented to assure that interpatient differences are truly due to distinct effects of treatment rather than intrinsic tumor heterogeneity.

We propose that validation of the MOA is critical for framing the definition of human cancer immune responsiveness. Various scenarios can be foreseen that define the algorithm of immune responsiveness:

1. The postulated MOA may never materialize in the target tissue, as the treatment does not reach its goal. It would be surprising to observe clinical regressions in such cases.
2. The MOA may differ from the predicted one. For instance, based on the observation that lymphocytes disappear from the circulation within minutes from the systemic administration of human recombinant IL-2 in association with a cytokine storm responsible for a massive capillary leak syndrome, it was assumed that IL-2 worked primarily by promoting trafficking of T cells to the tumor site. Serial biopsies of tumors performed during administration demonstrated that this was not the case: no lymphocytes appeared at the tumor site, but systemic administration of IL-2 induced a massive release of cytokines by IL-2-receptor bearing cells at the systemic level that in turn resulted indirectly in the polarization of tumor-associated macrophages toward an M1 phenotype [43].
3. The MOA is consistently observed independent of responsiveness. This observation suggests that additional modifiers are responsible for effectiveness and the MOA may be necessary but not sufficient. This could be the case for the lack of responsiveness to CIT observed in patients expressing the targeted checkpoints in an immune active landscape. Compensatory immune resistance may prevent effectiveness despite a positive pharmacodynamics outcome.
4. The MOA is not observed consistently, and its occurrence is tightly associated with outcome. This observation could provide mechanistic validation of the relevance of the MOA and at the same time provide reasons for immune resistance. This could be the case for primary immune resistance to CIT. The MOA is not being demonstrable in silent tumors in response to CIT simply because the targeted molecules are not expressed. In this case a link with causality can be established.
5. The MOA is not observed consistently but the phenotypic features are not associated with treatment outcome. This would question the significance of the MOA and several scenarios could be hypothesized:
   (a) No responses are seen in the absence of MOA.
   (b) Responses are seen exclusively in the presence of MOA but not consistently (suggesting other factors affecting the outcome beyond the MOA).

(c) Responses are not related with the presence of MOA, thus questioning its biological relevance.

6. The MOA can be observed consistently but with subtle quantitative or qualitative variations that can be identified, thus comparing responding vs. nonresponding patients.

We believe that this is a critical tactic to frame the quest to understand immune responsiveness and efforts should be encouraged to validate the MOA during clinical trials in relation to outcome. In addition, the assessment of pretreatment tumor biology and paired on-treatment qualitative and quantitative changes will be critical. The utility of both exome and transcriptome sequencing data generated from pretreatment tumor samples for the identification of potential determinants of response to anti-PD-1 should be highlighted.

4.4 Posttreatment Biopsies and Acquired (Secondary) Immune Resistance

Acquired (also referred to as secondary) immune resistance may or may not stem from successful treatment. However, it is likely that phenotypic alterations leading to immune resistance are more likely to occur under selective pressure. We have previously shown [39, 44] in the context of systemic IL-2 administration in combination with anticancer vaccines that lack of responsiveness is predominantly due to short-term and limited MOA rather than a selection mechanism of resistant tumor clones, while dramatic alterations, for instance, in antigen and/or *human leukocyte antigen* (HLA) expression most likely occur in recurring lesions after a preliminary response to therapy [44–47]. Most recently, interesting functional alterations in cancer cells related to responsiveness along IFN signaling have been described [48, 49]. Furthermore, ~30% of B-ALL patients successfully treated with anti-CD19 CAR-T cells relapse with CD19 negative disease [50]. Understanding secondary immune resistance not only can therefore enlighten about practical combinatorial approaches aimed at preempting and/or overcoming phenotypic changes but also may provide critical insights about the important mechanistic requirements for immune responsiveness similarly to the value of gene knockout in experimental systems.

5 Dissecting the Question

In the previous sections, we focus on the basic concepts relevant to the dissection of immune landscapes and their dynamic changes in relation to various mechanisms of immune resistance. However, cancer immune responsiveness is a multifactorial and complex phenomenon [51]. Thus, we propose in the context of the critical questions discussed above, that the correlative studies should be developed based on the specific elements according to biological

mechanism of different tumor types. Therefore, integration of multidisciplinary expertise (including tumor immunologists, geneticists, cell biologists, molecular biologists, biophysicists, computational analysts), is needed to guide scientific approaches and technologies for discovery and analytical validation of biomarker assays for clinical application. Below, we propose the following categories of questions should be addressed:

6 Host Germline Influence on Immune Responsiveness

It is likely that the host's immune status, whether determined by the genetic background or by environmental adaptations through the life time may influence the progression of cancer and its responsiveness to immunotherapy through an interplay of inherited and acquired factors similarly to the determinism of autoimmunity [52], since it is likely that cancer rejection is part of the broadly conserved phenomenon of immune-mediated tissue-specific destruction [10]. However, the germline contributions to immune responsiveness have not been systematically explored and establishing a link between host's genetic background and cancer immune phenotypes may guide biomarker discovery and contribute to the description of an immune-favorable patient phenotype within distinct therapeutic contexts. Several aspects of this complicated contribution to immune responsiveness will be discussed in the appropriate part of the book. Here, however, we would like to outline the postulated mechanisms by which the genetic background of the host may affect immune responsiveness.

The role of germline variants in cancer responsiveness to immunotherapy may determine:

1. Cancer immune landscapes.
2. Cancer immune responsiveness within distinct immune landscapes.
3. Intrinsic biology of cancer cells (since cancer cells incorporate in their genome functional variants that may affect their response to immunogenic stimuli).
4. Susceptibility to immune stimulation in relation to susceptibility to autoimmunity.
5. Genetic instability (i.e., BRCAness).
6. Modify cancer biology in association with somatic alterations.
7. Determine polymorphism of immune response receptors (i.e., CTLA-4).

7 Tumor Genetic Alterations and the Microenvironment

The accumulation of different genetic and epigenetic alterations is at the origin of intertumor and intratumor heterogeneity impacting cancer pathways, driving phenotypic variation, and posing significant challenges to personalized cancer medicine. Beyond these effects, an open question in IO is whether and how tumor intrinsic features affect the characteristic of the TME.

The following research questions addressing the role of genomic or nongenomic features that contribute to CIT response patterns should be asked to assess omics-scale features related to clinical response and survival patterns in order to gain insights into potential strategies for patient stratification and identification of CIT combinatorial therapies:

1. Mutational burden in determining immune landscapes and immune responsiveness.
2. Recurrent predicted neoepitope or experimentally validated neoepitopes derived from somatic nonsynonymous mutations that are critical for deriving clinical benefits from CIT therapy and HLA class I and II binding prediction.
3. Functional mutations (gain or loss of function) within cancer driver genes or tumor suppressor genes, respectively and their effects on the TME.
4. Genetic imbalances in determining immune landscapes and immune responsiveness.
5. Genetic rearrangements (chromosomal/locus or gene specific) in determining immune landscapes.
6. Numerical or structural genetic instability in determining immune landscapes.
7. Transcriptional signatures indicating differentially expressed genes between the responding versus nonresponding tumors.
8. Regulatory mechanisms in determining immune landscapes (β-catenin, MAP-kinases, the role of STAT-3.
9. Monoallelic expression in shaping TME.
10. Epigenetic regulation in shaping TME.

8 Role of the Environment

Circumstantial factors extrinsic to cancer cell biology or inherited genetic determinants may affect immune responsiveness to various degrees: this may include both environmental and behavioral factors and their interplay such as [53–55] the following:

1. Nutritional status and its effects on the immune function.
2. Microbiome and immune responsiveness.
3. Role of comorbidities in determining immune responsiveness.

These studies require a complex interaction of various disciplines spanning from metagenomics to epidemiology on one end and basic cellular immune biology on the other end that will be addressed in appropriate parts of this book.

9 Promising Approaches for IO Biomarkers

Research in the field of IO biomarkers already considers many aspects discussed above and seeks to characterize the relationship between the immune system, the tumor and its microenvironment, and the host. To identify IO biomarkers that measure the interplay between the immune system and the tumor, biomarker research and discovery is focusing on several key areas including markers of inflammation, tumor antigens and neoantigens, immune suppression markers, and host environment factors. The simultaneous evaluation and integration of multiple biomarkers may provide a more accurate and comprehensive assessment of the TME. This will help with achieving the goal of IO biomarker development to enable a more personalized approach to treatment by identifying patients who are likely to respond to specific immunotherapies.

The progress in fully realizing the potential of biomarker-driven assignment for anticancer approaches requires the development and implementation of novel clinical-grade biomarkers able to guide the selection of a single therapy agent or combination of drugs with complementary mechanisms of action targeting multiple mechanisms of response as well as of immune escape. Predictive biomarkers for immunotherapy differ from the biomarkers used for targeted therapies that are based on the presence of specific genetic aberration targeted by the drug (Table 1).

Table 1
Comparison of biomarkers for targeted and immunotherapy

	Driver mutations	IO Biomarkers
Examples	BRAF, EGFR, ALK, MET-specific mutations or fusions	PD-L1, tumor-infiltrating lymphocytes, MDSC, metabolic mediators, inflammation signatures
Target	Tumor	Tumor and TME
Presence	Constitutive	Dynamic and inducible
Metrics	Presence/binary decision	Level of expression (continuous variable), functional information, activation status

Biomarkers for immunotherapy require comprehensive approaches that encompass the complexity of the immune system and tumor biology which cannot be addressed by the use of a single analyte biomarker. Therefore, investigation of the biology and genomics of both the tumor and the host immune system is critical to recognize potential biomarkers. The availability of novel platforms and technologies should facilitate the integration of the

Table 2
Emerging tissue-based biomarkers predictive of immune-checkpoint inhibitor response

Platform	Biomarker	Assay examples
Immunohistochemistry (IHC)	PD-L1	Dako 22-8,[a] Dako 22C-3,[b] Ventana Assays[c] [58–61]
	TILs/CD8+ T cells	Higher baseline and posttreatment CD8+ T cell density [60–62]
	Panels of tumor and TME markers CD3, CD8, CD4, FoxP3, CD68, etc.	Multiplex immunohistochemistry (mIHC) [62–65]
	CD8+, CD3+ T cell density	Immunoscore; HalioDx Marseille, France [58]
	MSH2, MSH6, MLH, PMS2 expression	Deficient mismatch repair (dMMR) [66–69]
DNA sequencing	Total number of mutations per DNA coding region	Tumor Mutation Burden (TMB) [70–72]
	MSI markers (BAT25, BAT26, D2S123, D5S346, and D17S2720)	Microsatellite instability high (MSI-H) [66–69]
	Targeted DNA sequencing 324/468 gene mutations panel	Foundation One/MSK-IMPACT assays [73, 74]
DNA/RNA sequencing	Transcriptomic data filtered for putative neoantigens	Neoantigen burden [75–78]
	Quantification of complementarity-determining region 3 (CDR3) in the T cell receptor (TCR)	TCR clonality [62, 79]
Gene expression signatures	18-gene expression signature	Tumor Inflammation Score (TIS) [36]
	770-gene expression panel	PanCancer IO 360™ assay (NanoString Technologies Inc., [80]) [37–39]
	Gene expression profile	Teff Roche [81]

[a]DAKO 22-8: https://www.agilent.com/en-us/pd-l1-ihc-28-8-overview
[b]DAKO 22C-3: https://www.agilent.com/en/product/pharmdx/pd-l1-ihc-22c3-pharmdx
[c]Ventana: https://www.agilent.com/en-us/pd-l1-ihc-28-8-overview

molecular features of the tumor and the host factors for the development of multiplex profiles to guide personalized treatment in the future. However, before a candidate biomarker and/or new technology can be used in a clinical setting, rigorous steps to demonstrate the analytical and clinical validity of the biomarkers are required [56, 57]. Examples of technical platforms used for biomarker assays that have been already approved/cleared by U.S. Food and Drug Administration FDA or have shown preliminary evidence of an association with clinical benefit from immunotherapeutic interventions are presented below (Table 2).

10 Conclusions

This introductory chapter is meant to provide considerations for future systematic approaches for the understanding of human cancer immune responsiveness, by focusing on the delineation of fundamental biomedical questions that need to be addressed and the logical sequence in which they need to be considered. The idea is to weigh on the role that a transdisciplinary approach inclusive of genetics, genomics, computational biology, and cell biology may bear on the solutions for strategies to develop personalized approaches to immunotherapy for cancer.

Current work in immunotherapy continues to identify various tumor response and resistance mechanisms, and several promising biomarkers have been identified. However, future work is needed to develop biomarkers encompassing different mechanisms of tumor/host and different methods, in order to improve the efficacy of immunotherapy for the majority of patients.

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Chapter 2

Immunological Targets for Immunotherapy: Inhibitory T Cell Receptors

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Abstract

Tumor development is characterized by the accumulation of mutational and epigenetic changes that transform normal cells and survival pathways into self-sustaining cells capable of untrammeled growth. Although multiple modalities including surgery, radiation, and chemotherapy are available for the treatment of cancer, the benefits conferred are often limited. The immune system is capable of specific, durable, and adaptable responses. However, cancers hijack immune mechanisms such as negative regulatory checkpoints that have evolved to limit inflammatory and immune responses to thwart effective antitumor immunity. The development of monoclonal antibodies against inhibitory receptors expressed by immune cells has produced durable responses in a broad array of advanced malignancies and heralded a new dawn in the cancer armamentarium. However, these remarkable responses are limited to a minority of patients and indications, highlighting the need for more effective and novel approaches. Preclinical and clinical studies with immune checkpoint blockade are exploring the therapeutic potential antibody-based therapy targeting multiple inhibitory receptors. In this chapter, we discuss the current understanding of the structure, ligand specificities, function, and signaling activities of various inhibitory receptors. Additionally, we discuss the current development status of various immune checkpoint inhibitors targeting these negative immune receptors and highlight conceptual gaps in knowledge.

Key words Immunotherapy, Inhibitory receptors, PD-1, CTLA-4, TIM-3, TIGIT, LAG-3, BTLA, VISTA

1 Introduction

Cancer cells produce tumor antigens (TA) that are recognized by T cells and can induce tumor rejection [1]. The presence of CD8 tumor-infiltrating T lymphocytes (TIL) is usually a marker of good clinical outcome in multiple primary solid tumors [2–5]. However, spontaneous and vaccine-induced TA-specific T cells often fail to impede the growth of tumors in patients with advanced cancer [6, 7].

Multiple negative immunoregulatory pathways impede T cell–mediated tumor destruction in the tumor microenvironment (TME), contributing to the paradoxical coexistence of TA-specific CD8+ T cells and tumor progression in cancer patients. Among

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them, inhibitory receptors (IR) like PD-1 and CTLA-4 play a critical role in dampening T cell functions. Immunotherapies with immune checkpoint inhibitors directed against these immunoregulatory pathways provide long-term clinical benefits to patients with a growing range of solid tumors [8].

The development of monoclonal antibodies (mAb) targeting immune checkpoint receptors cytotoxic T lymphocyte associated antigen-4 (anti-CTLA-4) and programmed death 1 (PD-1) are proof of this therapeutic strategy. In this review, we discuss the preclinical and early clinical data supporting the rationale for current and future combinatorial therapeutic strategies targeting inhibitory immune checkpoints.

1.1 Inhibitory T Cell Receptors

1.1.1 Inhibitory T Cell Receptors: CTLA-4

CTLA-4: Structure and Ligands

Cytotoxic T lymphocyte-associated antigen-4 (CTLA-4, CD152) is an activation-induced glycoprotein that belongs to the immunoglobulin (Ig) superfamily. CTLA-4 is homologous to the T cell costimulatory protein CD28; but where CD28 provides the costimulatory signal required for antigen-specific T cell activation and expansion after the initial interaction between T cell receptor (TCR) and antigen presenting cells (APCs), CTLA-4 downregulates T cell responses [9–12]. CTLA-4 contains an extracellular V domain, a transmembrane domain, and a cytoplasmic tail. CTLA-4 cytoplasmic tail is structurally and functionally similar to CD28: it has no intrinsic catalytic activity but contains *both* a YVKM motif that can bind phosphatidylinositol 3-kinase (PI3K), protein phosphatase 2 A (PP2A) and SHP-2 *and* a separate proline-rich motif able to bind SH3 containing proteins [13].

CTLA-4 is constitutively expressed on regulatory T cells (Tregs), while expression on CD8+ T cells primarily occurs after initial activation. T regs primarily store CTLA-4 intracellularly within endosomes—providing a large intracellular pool that can be rapidly cycled to the cell surface upon activation. CTLA-4 has two natural ligands found on APCs: CD80 (B7.1) or CD86 (B7.2) [14–16].

CTLA-4: Signaling and Function

Unlike CD28 and PD-1 which are robustly expressed on cell surfaces, CTLA-4 is primarily distributed intracellularly where it is constitutively present as a homodimer [17, 18]. Although CTLA-4 signaling has been shown to be linked to phosphorylation of CD3ζ [19], disruption of ZAP-70 microclusters [20], and interaction with PI3K [21] or SHP-2 [22] or serine/threonine phosphatase PP2A [23], multiple other studies have shown that CTLA-4 inhibitory signaling was unrelated to each of these interactions [24–28]. Molecular imaging experiments have shown that both T regs and CD8+ T cells compete for the same ligands at the immune synapse in a cell-intrinsic fashion [29]. This suggests that upon antigen exposure, CTLA-4 binds CD80 and CD86 with greater affinity and avidity compared to CD28, enabling it to outcompete CD28

for ligand binding [30, 31] and argues that some measure of the inhibitory activity of CTLA-4 is due to ligand-dependent signaling. However, CTLA-4 inhibitory activity also results in ligand downregulation on APC via a transendocytic mechanism [32]. This mechanism is stimulated by TCR engagement, is cell-extrinsic, and has been observed in both T regs and CD8+ T cells [32]. Overall, these findings suggest that the primary inhibitory effect of CTLA-4 is to control access of CD28 to CD80/CD86 ligands and argues that the effects of CTLA-4 signaling are complex, contradictory and context-dependent.

Separately, other data suggest that some measure of CTLA-4's inhibitory effects on the T reg compartment is mediated by either intratumoral Treg depletion or reduced Treg suppressive activity [33–36]. CTLA-4 therapy is associated with an increase in the CD8 T cell–Treg ratio within tumors [37–43]. The effect of CTLA-4 blockade on the Treg compartment appears to be Fc-gamma receptor (Fc-γR) dependent and is associated with the presence of Fc-γR expressing macrophages [44, 45]. This effect is isotype dependent and antibodies with improved Fc effector function are associated with improved activity preclinically [46].

CTLA-4: Preclinical and Clinical Data

The discovery of the inhibitory function of CTLA-4 led to a series of experiments testing CTLA-4 inhibition in various murine tumor models. In 1996, Leach and colleagues demonstrated that antibody-mediated CTLA-4 blockade led to tumor rejection of transplantable mouse colon cancer and fibrosarcoma [47]. CTLA-4 blockade resulted in immunologic memory as previously challenged mice subsequently rejected implanted tumors without additional CTLA-4 blockade. CTLA-4 blockade was ineffective as a single-agent in B16 melanoma and SM1 mammary carcinoma [48, 49], although combining CTLA-4 blockade with GM-CSF-secreting vaccines resulted in tumor eradication [48, 49].

These results spurred the development of two anti-CTLA-4 mAb: ipilimumab (MDX-010; Medarex and Bristol-Myers Squibb) and tremelimumab (CP-675,206 or ticilimumab; Pfizer and Medimmune). Although both ipilimumab and tremelimumab are fully humanized mAb, ipilimumab belongs to the IgG1κ class and has a half-life of 12–14 days, while tremelimumab is a IgG2 mAb with a longer half-life of 22 days. The first clinical data came from a dose-escalation study in patients with advanced melanoma where authors reported two partial responses in a cohort of 17 patients treated with a single-dose of ipilimumab 3 mg/kg [50]. Subsequent studies tested a variety of doses and schedules in various diseases including melanoma [51] and lymphoma [52]. These early studies revealed three hallmark features: a clear *dose–response relationship* with greater responses at higher doses (albeit with a higher incidence of toxicity), a unique spectrum of "immune related adverse events" (irAE) that reflected