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Monitoring immune-checkpoint blockade: response evaluation and biomarker development

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Abstract

Cancer immunotherapy using immune-checkpoint blockade (ICB) has created a paradigm shift in the treatment of advanced-stage cancers. The promising antitumour activity of monoclonal antibodies targeting the immune-checkpoint proteins CTLA-4, PD-1, and PD-L1 led to regulatory approvals of these agents for the treatment of a variety of malignancies. Patients might experience clinical benefits from treatment with these agents, despite unconventional patterns of tumour response that can be misinterpreted as disease progression, warranting a new, specific approach to evaluate responses to immunotherapy. In addition, biomarkers that can predict responsiveness to ICB are being extensively investigated to further advance precision immunotherapy. Herein, we review the biological mechanisms underlying the unconventional response patterns associated with ICB, describe strategies for the objective assessments of such responses, and also highlight the ongoing efforts to identify biomarkers, in order to guide treatment with ICB. We provide state-of-the-art knowledge of immune-related response evaluations, identify unmet needs requiring further investigations, and propose future directions to maximize the benefits of ICB therapy.

Cancer immunotherapy with immune-checkpoint blockade (ICB) is based on the inhibition of the tumour- mediated suppression of anticancer immune responses, in contrast with therapeutic strategies that exert direct cytotoxic effects on tumour cells[1–4]. T cells have a major role in immune defense mechanisms against cancer: they recognize tumour antigens, consequently become activated, disseminate and, ultimately, eliminate cancer cells[1–4]. In this context, T-cell activation is regulated by the interplay of the stimulatory and inhibitory ligand–receptor interactions between T cells, dendritic cells, tumour cells, and macrophages in the tumour microenvironment (TME), with tumour cells acting as critical mediators of immunosuppression[2,5] (FIG. 1). Owing to their roles as regulators of T-cell activation, these

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receptor–ligand pairs are called 'immune checkpoints'. In addition to the TME, important interactions between T cells and antigen-presenting cells expressing immune- checkpoint molecules occur in secondary lymphoid tissues[5]. Agents targeting these checkpoints — that is, ICB agents — have been identified as promising treatment options for patients with cancer[2]. Immune-checkpoint inhibitors (ICIs) include, among others, monoclonal antibodies to the receptor cytotoxic T-lymphocyte antigen-4 (CTLA-4) expressed on T cells; programmed cell death protein 1 (PD-1), also expressed on T cells; or the PD-1 ligand (PD-L1), which is expressed by a variety of cell types, including some tumour cells. In 2010, the results of studies with the anti-CTLA-4 antibody ipilimumab in patients with advanced-stage melanoma[2,6] led to the regulatory approval of this agent 1 year after, and sparked a rapid increase of further studies of ICIs in patients with advanced-stage cancers. Subsequently, the anti-PD-1 antibodies nivolumab and pembrolizumab, and the anti-PD-L1 antibody atezolizumab, have shown marked therapeutic activity in various solid tumours and lymphomas, resulting in a number of regulatory approvals of these agents for the treatment of different malignancies.

The scope of ICB is expanding rapidly in the clinical oncology practice, and is expected to continue to grow further as new agents become available in the clinical setting. The comprehensive understanding of the benefits and risks associated with ICB is essential for those involved in the care of patients with cancer. In this Review, we provide an overview of the mechanisms of action of these agents, examine strategies for evaluation of immune-related response to ICB, and discuss ongoing efforts to develop predictive biomarkers of responsiveness. The unmet needs that require urgent attention are emphasized to provide directions for further studies.

Immune-related response evaluation

Immune-related response criteria (irRC) — a key concept

Owing to the unique antitumour mechanisms elicited by ICB, patients treated with these agents can have tumour response patterns that are not adequately captured using the conventional tumour-response criteria[3,4,7–10], such as the WHO criteria[11] and Response Evaluation Criteria in Solid Tumours (RECIST)[12,13]. For example, in a small subset of patients treated with ICIs, a response is detected after an initial increase in tumour burden (FIG. 2), or during or after the appearance of new lesions[3,4,10] (FIG. 3). According to the WHO criteria and RECIST, these events would be classified as tumour progression, and are therefore termed 'pseudoprogression'. With pseudoprogression, the apparent tumour-size increase detected upon imaging is thought to be caused by T-cell infiltration as a result of immune activation, rather than by tumour-cell proliferation[10]. In order to accurately assess these immune-related response phenomena, a series of workshops were held among 200 oncologists, immunotherapists, and regulatory experts, which resulted in the proposal, in 2009, of the immune-related response criteria (irRC)[3,4,10].

The key features of irRC include: firstly, the requirement for confirmation of disease progression on two consecutive scans (performed at least 4 weeks apart), such that the initial increase in tumour burden alone does not immediately define progression; secondly, the inclusion of new lesions in the sum of lesion measurements is considered to better reflect the

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total tumour burden — as opposed to WHO criteria and RECIST that define progression at the appearance of new lesions[3,4,10]. The irRC were first used to define the end points of a phase II trial of ipilimumab plus paclitaxel and carboplatin as first-line therapy in stage IIIB/IV non-small-cell lung cancer (NSCLC)[14]; this concept has been applied in subsequent clinical trials of ICIs[4,14–16].

The value of irRC was initially assessed retrospectively in patients with melanoma treated with ipilimumab in the first-line setting from whom post-progression scans were available, and did not initiate other anticancer therapies after disease progression[10]. Among 227 patients who received ipilimumab, 123 were described as having initial progression according to WHO criteria (which do not contemplate 'pseudoprogression') at the 12-week scan. Evidence of activity consistent with a response to this agent according to irRC was identified in 22 of 123 patients (18%; 10% of the whole cohort): five patients (23%) had an immune-related partial response, and 17 patients (77%) had immune-related stable disease[10]. These results indicate the irRC can be used to measure clinical benefit from ipilimumab for patients who have an initial increase in tumour burden and/or new detectable lesions. In those 22 patients with a response, overall survival was comparable to that of patients with a complete response, partial response, or stable disease defined using WHO criteria (median overall survival not reached (13.5 months–not reached) versus 31.2 months (27.8–31.2 months)), further emphasizing the importance of using specific criteria for the assessment of response to immunotherapy[10]. In a study published in 2016, the value of the irRC versus RECIST version 1.1 (RECIST1.1) in predicting patient outcome in response to the PD-1 inhibitor pembrolizumab was compared in 327 patients with advanced-stage melanoma[15]. The study reported that 24 (7%) of those 327 patients had atypical responses (defined as pseudoprogression by irRC). Importantly, overall survival was longer in the 84 patients (26%) who had progressive disease according to RECIST1.1, but non-progressive disease according to irRC, compared with that of 177 patients (54%) who had progressive disease according to both RECIST1.1 and irRC (median overall survival 22.5 months versus 8.4 months)[15].

Pitfalls of irRC to evaluate immune-related responses

Clearly, the irRC describes an important concept necessary for the assessment of immune-related responses; however, several pitfalls and issues related to irRC remain to be solved. One of them is a methodological issue related to tumour measurements: irRC is based on the WHO criteria[10,11], introduced in 1979, in which bidimensional measurements are performed and tumour burden is quantified as the product of the longest diameter and the longest perpendicular diameter[11,17]. Most clinical trials with patients with solid tumours conducted in the past decade, however, have followed RECIST measurement guidelines[18] — introduced in 2000 and revised in 2009 — to define trial end points and provide a basis for regulatory approvals for novel agents[12,13,17,19] (TABLE 1). In RECIST, unidimensional measurements are performed, using the longest diameters for non-nodal lesions, and the longest perpendicular diameters (short axis) for nodal lesions. Thus, the results from trials of ICIs evaluated using irRC (bidimensional measurements) cannot be compared directly with results from other trials that have used RECIST (unidimensional measurements)[8,20]. The comparison of irRC and RECIST-based assessments within the same trial is also challenging

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because divergent results might arise owing to the different measurement methods used, and not because of immune-related phenomena. Importantly, multiple prior studies have shown that bidimensional measurements are subject to larger measurement variability — and thus, have higher misclassification rates of response categories — than unidimensional measurements, resulting in inaccurate assessment of small changes in tumour burden[8,21–24]. Tumour response criteria were originally developed to serve as a common language to describe the outcomes of cancer treatment and thus, the above discussed methodological definition related to irRC requires further optimization[8,20].

Further aspects of the definition of pseudoprogression remain to be clarified. For example, the degree of tumour regression after an initial size increase that has been observed in patients who display pseudoprogression is variable. Of note, in many studies — including the first report of irRC[10] — patients did not need to meet the requirement of tumour reduction below the partial response threshold to be classified as having a response; instead, tumour regression in the range of stable disease (defined as neither sufficient shrinkage to qualify for partial response nor sufficient increase to qualify for progressive disease) was considered as evidence of therapeutic anticancer activity. This criterion is based, in part, on the concept that durable stable disease is a pattern of response to therapy. Indeed, results from a study in patients with melanoma treated with pembrolizumab demonstrated that increases in tumour burden of <20% from baseline (defined according to observations of tumour burden dynamics during ICB therapy using a spider plot) were associated with longer overall survival[25]. Prospective validations in other patient cohorts are needed but, notably, durable disease control (maintenance of a near baseline level of tumour burden measured at the time of initiation of ICB), even without a deep tumour reduction below the response threshold, can indicate benefit from treatment in the setting of ICB.

Moreover, in the irRC, pseudoprogression was defined mainly on the basis of tumour burden reductions after an initial increase observed around 12 weeks after initiation of therapy, a time point established according to the study design and follow-up intervals for patients with melanoma involved in trials of ICIs[26,27]. Results presented in 2017, however, indicate that pseudoprogression can occur later during the course of therapy and thus, a response after initial tumour size increase might be noted beyond the 4-week window between assessments defined in the irRC[25]. For example, in a study by Hodi and collaborators[15], ‘delayed pseudoprogression’ (detected after 12 weeks of therapy) was not confirmed as progressive disease at the next assessment (at least 4 weeks later) in 3% (9/327) of patients with melanoma treated with pembrolizumab. In another study involving patients treated with pembrolizumab, after immune-related progressive disease was confirmed on a subsequent scan, a therapeutic response was detected in 3% (3/107) of patients (median time to detection of initial immune- related progressive disease: 2.7 months)[25]. Indeed, the authors of the 2015 report from the Response Assessment for Neuro-Oncology (RANO) Working Group point out the lack of scientific rationale of the 4-week window defined in the irRC and, instead, recommend a 3-month period for confirmation of progressive disease in neuro-oncology trials, to avoid excluding pseudoprogression too early in the therapeutic course[28]. This recommendation from RANO was not, however, based on scientific data from studies specifically designed to define an adequate timeframe of confirmatory scans and, rather, was based on observations of spider plots of a few early trials of ICIs[28]. Therefore, the

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identification of the most optimal timeframe for confirmation of progressive disease will require further investigations.

Importantly, the generally low incidence of pseudoprogression should be acknowledged when monitoring patients treated with ICIs[18,20]. In studies of patients with melanoma receiving both CTLA-4 and PD-1 inhibitors, the incidence rate of pseudoprogression is approximately 10% (or often less)[10,15,25]. A lower incidence (5%; 6/129) was reported in patients with advanced-stage NSCLC in a phase I study of nivolumab; the incidence might be even lower in patients treated with standard-of-care immunotherapies[9,29]. Despite an increasing awareness of the pseudoprogression phenomenon, accumulating data indicate that this is a relatively uncommon event, and an increase of tumour burden is more likely to reflect true progression than pseudoprogression in most patients, raising a caution for optimism among health-care providers treating patients with tumour burden that increases in the setting of ICB.

Outstanding issues and future directions

The future directions of immune-related response evaluations are currently under active debate, with growing efforts to use standardized criteria that can improve upon the pitfalls and limitations of irRC. One proposed approach was to modify irRC to use unidimensional measurements and the same response categories as RECIST[15]. This modification was originally proposed in a study that evaluated the response to ipilimumab of 57 patients with advanced-stage melanoma in a phase II trial[8]. The study compared response assessments using the original, bidimensional irRC with those obtained using unidimensional measurement methods and adopting the threshold to define response categories according to RECIST. The key features of irRC (inclusion of new lesions and confirmation of pseudoprogression) were incorporated in both criteria. The response assessments were highly concordant: the evaluation of best immune-related response according to two measurements using both criteria was identical in 53 of 57 patients, whereas the estimated progression-free rate at 6 months was 70% for bidimensional irRC versus 81% for unidimensional irRC[8]. Estimates of the 25th percentile (time point at which a 75% progression-free survival (PFS) is reached) were 5.3 months (3.5 months–∞) by bidimensional assessment versus 9.1 months (3.7 months–∞) by unidimensional assessment; on the basis of the almost identical confidence intervals for this percentile, no evidence supports a difference in time to progression between both assessment methods[8]. Importantly, evaluation of the reproducibility of both measurement methods showed that the 95% limits of agreement of bidimensional measurements were twice as wide compared with unidimensional measurements (−31.3% to 19.7% versus −16.1% to 5.8%)[8]. This study provided a rationale to use a new method, termed immune-related RECIST (irRECIST), that incorporates RECIST-based measurement methods while maintaining the key features of irRC[4,7,20,30]. The results of these studies also provided a basis for the development of the iRECIST guidelines[31,32] for the evaluation of response in trials testing immunotherapeutics, which are largely based on RECIST1.1, but require confirmation of progression and do not define progression when new lesions appear. While iRECIST mostly follows the guidelines proposed by irRECIST, this method mandates new lesions to be measured and recorded separately instead of being added to the sum, and increase of new lesions or appearance of

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additional new lesions on the subsequent scan confirms progression (TABLE 1). Further efforts are ongoing to collect sufficient data to validate both irRECIST and iRECIST, which will contribute to further advance the immune-related response evaluation strategy.

Advances in the knowledge of immune-related responses have been challenged by the fact that only a few clinical trials have used the irRC or irRECIST as the primary criteria to define their end points[14,16,18]. Most trials continue to use RECIST1.1 to define the end points, mostly because regulatory agencies continue to base the approvals of novel agents on RECIST-defined outcomes. Of note, however, some trials have included irRC, irRECIST or similar modified criteria as secondary response criteria[14,16,33,34]. Whether the introduction of iRECIST contributes to wider and uniform application of immune-related response criteria in trials with ICIs remains to be confirmed.

Immuno-oncologists also face a dilemma in terms of treatment decision-making when tumour progression occurs during therapy[18,20]. In trials conducted in the past 9 years, patients have often been allowed to continue therapy beyond progression when they are deemed by investigators to be deriving continued therapeutic benefit[3]. Similarly, clinical providers base their treatment decisions on the overall assessments of clinical improvements and treatment tolerance. These approaches are subject to clinician's discretion, and objective guidelines establishing when to continue therapy or otherwise consider alternate options do not exist. Criteria for the evaluation of immune-related responses, including whether increases in tumour burden indicate progression and whether progression is confirmed, can provide information to guide treatment decisions; however, these criteria have essentially been developed for use in clinical trials of novel agents to describe the treatment results, and are not designed primarily to assist therapeutic decision-making in the clinical setting.

A preliminary observation[25] indicates that factors such as younger age might be associated with pseudoprogression (the median age of patients defined to undergo pseudoprogression is 46 years compared with 63 years for all other patients), but the contribution of this factor remains to be validated[25]. A possible approach to validation would be to evaluate clinical, biological, and radiographic features at baseline in large databases of patients treated with ICB, and identify predisposing factors for pseudoprogression. Information about the likelihood of disease progression could then be used by health-care providers to guide treatment decisions. Serial evaluations of serum factors (including cytokines) during therapy and how they correlate with radiographic tumour kinetics might also help to distinguish pseudoprogression from true progression. The use of radiographic strategies (combining both serial anatomic imaging and functional imaging) in immune-related evaluations should advance together with these efforts.

Biomarkers of immunotherapy response

Despite the remarkable success of clinical applications of immunotherapy reported in the past decade, the efficacy and effectiveness of these therapies varies greatly across individual patients and among different tumour types. A substantial unmet need is the development of biomarkers of response to immunotherapeutic agents, in order to identify, before initiation of treatment, which patients are likely to experience a response to and clinical benefit from

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such treatments. This aspect is particularly important in the management of tumours with low response rates, such as NSCLC (response rate ≤20%)[29,33,35–37], renal-cell carcinoma (RCC) and urothelial carcinoma (UCC), both with response rates ≤30%[38–42]. Herein, we present the immune-response- associated biomarkers that have been studied to date, discuss their strengths and weaknesses, and highlight ongoing efforts of biomarker development for use in precision cancer immunotherapy (FIG. 4).

PD-L1 and other immune-checkpoint molecules

Expression levels of PD-L1 on tumour cells (assessed by immunohistochemistry (IHC)) has been studied as a potential biomarker of response to ICB since the early phase of the development of these agents. In a phase I trial of nivolumab in patients with advanced-stage melanoma, NSCLC, RCC, castration-resistant prostate cancer, or colorectal cancer[43], the overall response rate (ORR) of patients with PD-L1-positive tumours (defined as ≥5% tumour cells expressing PD-L1) was higher overall than that of those with PD-L1-negative tumours[43] (TABLE 2). Subsequent trials of ICB, however, yielded contradictory results regarding the role of PD-L1 expression as a marker of response to treatment and clinical outcome because of the different criteria used to define PD-L1 positivity in order to determine the study cohorts[26,27,29,35–37,44–46] (TABLE 2). Of note, although the results support a role for PD-L1 expression as a predictive biomarker, some patients with PD-L1-negative melanoma can also derive durable clinical benefit from PD-1 blockade; thus, PD-L1 expression alone is not used for patient selection in this setting[45].

The predictive and prognostic values of PD-L1 expression in studies in patients with NSCLC have also been debated. In the exploratory analyses of some trials[29,44], no association was reported between PD-L1 expression and ORR, PFS, or overall survival; however, positive correlations have been reported in other trials[35,37] (TABLE 2). One of the limitations of these studies is the assessment of PD-L1 expression in archival tumour tissue, which might not reflect the PD-L1 expression status at the time of therapy initiation[29]. To this end, a phase I study of pembrolizumab in patients with advanced-stage NSCLC required contemporaneous tumour biopsy samples for the biomarker evaluation; a higher response rate and longer PFS and overall survival were observed in patients with PD-L1-positive tumours (defined as ≥50% of neoplastic cells showing membranous staining of PD-L1) compared with those with PD-L1-negative tumours, regardless of whether they had previously received treatment[36]. This observation was subsequently confirmed in a phase III study in treatment-naive patients with NSCLC receiving first-line pembrolizumab who were required to have ≥50% PD-L1 expression on tumour tissue samples[46] (TABLE 2). On the basis of the positive results of these studies, the FDA approved pembrolizumab for previously untreated metastatic NSCLC with PD-L1 expression ≥50%[47], expanding the application of PD-1 inhibitors for the first-line treatment of lung cancer. This approval also highlights the role of PD-L1 expression as a biomarker that enables an effective selection of the patients with advanced-stage NSCLC who are most likely to benefit from first-line ICB; the role of PD-L1 expression as a predictive biomarker in patients with tumours other than NSCLC remains to be established.

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Of note, the use of PD-L1 expression on IHC as a biomarker is associated with some issues. Most studies evaluated PD-L1 expression as the percentage of tumour cells showing cell-surface and/or membranous PD-L1 staining in a section containing at least 100 evaluable tumour cells; however, different assays and antibodies are currently used to detect PD-L1 staining, without standardization[48,49]. Moreover, variable cut-off values and different scoring methods have been used to define PD-L1 positivity on IHC (TABLE 2). The lack of standardized methods makes it difficult to collectively analyse the results from individual studies in order to reach robust overall consensus. Additionally, most anti-PD-L1 antibodies currently in use are directed to the extracellular domain of PD-L1 and their use results in a mixture of both cytoplasmic and membranous staining of tumour tissue on IHC[50]. Cytoplasmic staining obscures the interpretation of a positive result on the tumour-cell membrane, thus affecting the accuracy of the analysis[50].

A study using tissue samples from patients with NSCLC reported poor concordance in the assessment of PD-L1 protein expression with two different PD-L1 antibodies on conventional IHC (Cohen κ range: 0.124–0.340)[51]. Quantitative immunofluorescence showed an interassay discordance rate of 26.6%, demonstrating the heterogeneity of PD-L1 expression in serial sections of whole tissue from the same patient[51]. In the Blueprint PD-L1 IHC Assay Comparison Project[52], an industrial– academic collaborative partnership, four different PD-L1 IHC assays that have been used in clinical trials were evaluated. Using 39 NSCLC tumour samples, the study showed that the percentage of PD-L1-stained tumour cells was comparable between three of the four assays, but consistently lower with the fourth assay method. For all assays, greater variability in PD-L1 staining was found in immune cells than in tumour cells[52]. The study indicated that interchanging assay methods and cutoff values for PD-L1 positivity can lead to inconsistent classifications of PD-L1 status in some patients[52], highlighting the limitations of the current IHC approach to assessing PD-L1 expression in terms of reproducibility, as well as in sampling variability[49].

Tumour heterogeneity and sampling variability are inherent limitations of assessment approaches using tissue samples. Tumour heterogeneity exists both within the same tumour lesions and among different lesions within the same patient. Because of the invasiveness of tissue sampling, only one of multiple lesions is usually selected for sampling based on the accessibility, and only small samples might be obtained when needle biopsy is performed. Thus, a tissue sample might not necessarily reflect the major immune phenotype of the tumour or the patient. Additionally, PD-L1 expression levels can change over time and thus, specific requirements (such as the time of treatment with ICB or other agents) need to be clearly defined[49,53]. Understanding the utility of changes in PD-L1 expression in response to ICB during the course of therapy is of great interest; however, serial assessments of PD-L1 expression can be difficult because repeated tissue sampling is required. A substantial number of patients with PD-L1 positivity (at least 40–50%) do not achieve objective response to anti-PD-1–PD-L1 therapies; in addition, approximately 15% of patients negative for PD-L1 expression experience objective responses, in contrast to the initial report[53]. While some of the limitations discussed might, at least partially, explain the discrepancies observed in the assessment of PD-L1, further efforts are needed to refine the use of PD-L1 expression status as a robust biomarker for immunotherapy. In addition, genetic and non-

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genetic factors enabling response prediction should be incorporated in the process of patient selection for ICB in order to establish a paradigm of precision immunotherapy[53].

Tumour-infiltrating lymphocytes

A non- negligible amount of non-neoplastic cells are found within tumours; these cells include immune cells, which are probably of biological significance[54]. In particular, tumour-infiltrating lymphocytes (TILs) are increasingly recognized as important players in the immune response against cancer. A number of studies have demonstrated that increased TIL numbers are associated with better outcomes and longer survival for patients with a variety of malignancies[55]. For example, in 46 patients with melanoma treated with pembrolizumab, higher numbers of cells expressing CD8, PD-1, or PD-L1 at the invasive tumour margin and inside tumours were detected in pretreatment samples from responding patients, compared with patients who did not respond[56]. In addition, a greater increase in $CD8^+$ cells in serial tumour samples during therapy correlated with a greater tumour-size decrease on imaging (Spearman's correlation coefficient = −0.75; $P = 0.0002$)[56]. Herbst *et al.*[57] also highlighted the importance of PD-L1 expression assessment, not only on tumour cells but also on TILs. In their study, IHC assessment of pretreatment tissue samples from patients with advanced-stage cancer treated with atezolizumab revealed PD-L1 positivity on both tumour cells and TILs, with a higher percentage of PD-L1-positive TILs than PD-L1-positive tumour cells. PD-L1-positive TILs included myeloid cells (macrophages and dendritic cells) and T cells, whereas B cells were negative for PD-L1 expression[57]. The study also demonstrated that the likelihood of response to atezolizumab was significantly associated with higher levels of PD-L1 expression on TILs (ORR according to the percentage of PD-L1 positive cells per area: 46% (in 15/33 patients), 17% (4/23), 21% (7/34), 13% (8/60), and 8% (2/25), for ≥10%, ≥5%–<10%, 1%–<5%, <1%, and unknown, respectively; $P = 0.007$), but not with PD-L1 expression on tumour cells ($P = 0.079$)[57]. Serial tissue sampling during therapy showed that lesions regressing after treatment had a dense immune infiltrate and extensive tumour-cell necrosis. Nevertheless, in most patients with disease progression, no PD-L1 upregulation in tumour cells or TILs was detected, with three patterns observed: little or no TIL infiltration ('immunological ignorance'); the presence of intratumour immune infiltrates with minimal to no expression of PD-L1 ('non-functional immune response'); or the presence of an immune infiltrate solely around the outer edge of the tumour cell mass ('excluded infiltrate')[57].

A study using multiparameter flow cytometry assessment of freshly isolated pretreatment tumour samples from patients with metastatic melanoma showed that an increase in the fraction of tumour-infiltrating $CD8^+$ T cells with high expression of both PD-1 and CTLA-4 ($PD\text{-}1^{hi}/CTLA\text{-}4^{hi}$) strongly correlated with response to pembrolizumab or nivolumab[58]. In both the discovery and validation cohorts, patients with >20% $PD\text{-}1^{hi}/CTLA\text{-}4^{hi}$ cytotoxic T lymphocytes (CTLs) had higher response rates and longer PFS durations than those with ≤20% $PD\text{-}1^{hi}/CTLA\text{-}4^{hi}$ CTLs (ORR: 85.7% versus 0%, PFS: 31.6 months versus 9.6 months in the discovery cohort; ORR: 78.6% versus 0%, PFS: 15.9 months versus 9.9 months in the validation cohort). Functional analyses of $PD\text{-}1^{hi}/CTLA\text{-}4^{hi}$ CTLs demonstrated a partially exhausted T-cell phenotype, characterized by the ability to produce IFNγ and the inability to produce TNFα and IL-2 (REF.[58]). The mechanism of action of

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ICB involves the interaction between tumour cells and their TME, and the cellular immune reaction against tumours; therefore, increased awareness and further studies of the role of TILs are needed for comprehensive biomarker development to predict response to immunotherapy.

The 'immunoscore' as a biomarker for immunotherapy

Immune cells are scattered in both the core and in the invasive margin of tumours[54]. The analysis of the presence of TILs in both tumour regions improved the accuracy of prediction of survival for the different groups of patients with early-stage colorectal cancer, compared with that of single-region analysis[59], providing a rationale for conducting a systematic evaluation of immune cell infiltration in both the tumour core and the invasive margin. This approach, termed immunoscore, has been proposed as a method to characterize the immune contexture of the TME, and has been studied as a tool for tumour classification, prognostication, and prediction of response to therapy[54,59,60].

The immunoscore reflects the density of two lymphocyte populations (cytotoxic ($CD8^+$) and memory ($CD45RO^+$) T cells) in the core and in the invasive margin regions of tumours[54,61], resulting in a four-point scale score ranging from immunoscore 0 (I0; low densities of both cell types in both regions), to immunoscore 4 (I4; high densities of both cell types in both regions)[54,61]. The prognostic and predictive values of the immunoscore have been addressed mostly in the setting of colorectal cancer[60,62]. Mlecnik *et al.*[63] assessed the immunoscore of 599 specimens of stage I–IV colorectal tumours, and studied its relationship with the degree of extension of the primary tumour (according to the American Joint Committee on Cancer–International Union Against Cancer (AJCC–UICC) TNM classification system) and in relation to the recurrence rate[63]. The study demonstrated that the immunoscore was significantly associated with differences in disease-free, disease-specific, and overall survival, with a better prognosis for those patients classified as having a coordinated T-cell response. Multivariate analyses also showed the superiority of the immunoscore over the AJCC–UICC TNM classification system in predicting recurrence as well as survival[63]. Pages *et al.*[62] demonstrated that the immunoscore is an independent predictor of tumour recurrence and survival in patients with early-stage colorectal cancer, confirming that the extent of the immune reaction at the tumour site is directly correlated with better outcomes[62]. On the basis of these results[62,63], the value of the immunoscore is currently being validated internationally in routine clinical settings.

Given the association between the presence of $CD8^+$ T cells in the invasive margin regions and the expression of PD-1 and PD-L1 in the tumour and TME, the value of the immunoscore as a possible marker of response to ICB therapy is of great interest[54,56,64]. The application of the immunoscore in melanoma is currently under investigation; however, in this setting, the definition of immunoscore is a challenge because of the complex intratumoural immune reaction[60]. To date, the evaluation of the immunoscore in melanoma has been performed on tissue samples from metastatic lymph nodes, which are the most accessible and available malignant tissues in patients with melanoma. The immunoscore might facilitate treatment decisions, especially for patients with stage III disease who can benefit from adjuvant systemic therapy after tumour resection and lymph-node dissection[60].

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Concerns exist regarding the validity of the approach that focuses on lymph nodes alone[60], because lymph nodes are enriched in $CD3^+$ and $CD20^+$ lymphocytes and, in addition, lymph-node metastases can have different immune infiltration patterns compared with other metastatic lesions[60]. Further studies are ongoing to evaluate different immune populations and their relationships with response and benefit to ICB in patients with melanoma[60,64].

NSCLC is another tumour type in which the development of an effective biomarker is needed. The immunoscore remains to be prospectively evaluated in patients with NSCLC; however, in one study, tumour tissue samples from 536 patients with stage I–IIIA NSCLC were evaluated, revealing that a high density of PD-L1-positive immune cells in the stromal compartment, and of PD-1-positive intraepithelial TILs was associated with favourable disease-specific survival[65]. Conversely, low PD-L1-positive immune-cell density in the stromal compartment and low PD-1-positive intraepithelial TILs were associated with poor disease-specific survival across all disease stages (HR = 1.81; 95% CI 1.37–2.40; $P<0.001$). The contribution of both factors was shown to be significant in multivariate analyses, indicating that they are independent prognostic factors[65]. The utility of the immunoscore to predict tumour response to therapy of patients with advanced-stage NSCLC remains to be further investigated.

In addition to the scientific knowledge derived from ongoing research about immunoscore, technical barriers need to be overcome in order to translate this promising approach into routine clinical pathology and oncology practices. Robust software solutions are needed to enable automated detection of TILs in tumours, faster processing and turnaround, and accurate and efficient interpretation of a large volume of quantitative data on immune-cell densities[54,60]. As the concept of immunoscore becomes widely applied in different clinical contexts, the importance of developing and maintaining internationally agreed consensus of the definitions and strategies related to the immunoscore approach will increase.

IFNγ

IFNγ is a cytokine that plays a key part in immune regulation and anticancer immunity. IFNγ is mainly produced by natural killer cells and natural killer T cells in innate immune response, and by activated T cells in the setting of antigen-specific immunity[56,66]. The expression of PD-L1 on most tumour cells is induced by interferons, predominantly IFNγ; IFNγ signalling is necessary for an adaptive response to endogenous antitumour immunity[5,55,66,67]. In a phase I/II study of the PD-L1 inhibitor durvalumab[68], tumour samples were profiled for the expression of 100 preselected genes involved in immune activation[68]. The highest correlation with response was found for baseline IFNγ mRNA expression levels, with a response rate of 33% (14/43) in patients positive for IFNγ and 8% (6/79) in those negative for IFNγ expression. When combined with PD-L1 positivity, the highest response rate (46%, 10/22) was noted in those patients positive for both IFNγ and PD-L1 expression[68]. In a phase I study of the PD-L1 inhibitor atezolizumab, pretreatment tumour samples from patients with melanoma who responded to therapy had elevated expression of IFNγ and IFNγ-inducible genes (such as *IDO1* and *CXCL9*); however, this association was weak in patients with NSCLC or RCC[57]. Given the important role of IFNγ in the adaptive immune response, further studies are needed to determine whether the

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signalling status of this interferon is a reliable biomarker for response to ICB across different cancer types.

Additionally, in a study of mutations associated with acquired resistance to pembrolizumab therapy[69], loss-of-function mutations in the genes encoding the IFN-receptor-associated tyrosine kinases JAK1 and JAK2 were identified as relevant. Truncating mutations in *JAK1* and *JAK2* resulted in a lack of response to IFNγ in cell lines derived from tumours that were progressing[69]. These observations indicate an additional role of IFNγ in the development of acquired resistance to ICB, which remains to be further studied.

Mutational burden, neoantigens and microsatellite instability

Marked efficacy of ICB has been noted in patients with melanoma or squamous NSCLC, tumours that are known to have higher numbers of somatic mutations than other tumours[70]. These mutations in tumour cells can result in the generation of novel antigens, termed neoantigens, which can be recognized as non-self-epitopes by the immune system, thereby enhancing T-cell reactivity against the tumour, thus facilitating the efficacy of ICB[53,66,71]. Additionally, a deficiency in DNA-repair mechanisms can lead to a high mutational burden in tumours, which can lead to increased response to ICB[53,66,72].

In 2014, Snyder and collaborators had demonstrated the association between tumour mutational burden and the degree of clinical benefit in patients with melanoma treated with the CTLA-4 inhibitors ipilimumab or tremelimumab, and they identified a neoantigen landscape specific to tumours with a strong response to CTLA-4 inhibitors[73]. In this study, long-term clinical benefit was defined by radiographic evidence of freedom from disease, or evidence of stable or decreased volume of disease lasting >6 months. The mutational load (determined by the number of nonsynonymous mutations per exome) was significantly higher in patients with long-term clinical benefit in both the discovery set and validation set ($P = 0.01$ and $P = 0.009$, respectively). In a study by Rizvi *et al.*[74], a higher burden of somatic nonsynonymous mutations was associated with clinical efficacy in response to pembrolizumab in patients with NSCLC. In the discovery cohort ($n = 16$), the number of nonsynonymous mutations was higher in patients who derived durable clinical benefit (defined as partial or stable response lasting >6 months), compared with those without durable benefit: median of 302 versus 148 mutations; $P = 0.02$ (REF.[74]). Both ORR and PFS were higher in patients with an ‘elevated’ nonsynonymous mutational burden — defined as above 209, the median mutational burden of the cohort — compared with those with a ‘non-elevated’ burden (ORR: 63% versus 0%, $P = 0.03$; median PFS: 14.5 months versus 3.7 months, $P = 0.01$)[74]. In a validation cohort of 18 samples from patients with NSCLC treated with pembrolizumab, a higher rate of durable clinical benefit and longer PFS durations were noted in patients with a nonsynonymous mutational burden above 200 (median mutational burden of this cohort) compared with other patients (durable clinical benefit rate: 83% versus 22%, $P = 0.04$; median PFS: not reached versus 3.4 months, $P = 0.006$)[74].

The relationship between the ability of tumour cells to correct intrinsic DNA errors and a response to immunotherapies is another important concept that has been under active investigation in the past few years. Intrinsic errors in DNA replication occur at a rate of $1/1 \times 10^4$–10^5, and are corrected by the mismatch- repair (MMR) machinery[53]. Mutations in

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MMR genes in tumour cells therefore result in a large number of mutations that affect, among others, DNA microsatellite motifs[53]. Thus, microsatellite instability (MSI) is inversely correlated with the ability to repair DNA errors, and has been demonstrated to have a strong association with response to ICB[53,66]. The role of MMR deficiency (MSI) as a marker for clinical benefit from ICB has been studied in a phase II trial in patients with progressive metastatic carcinoma treated with pembrolizumab[72]. Both ORR and PFS were higher in patients with colorectal cancer with MMR deficiency than in those without (ORR: 40% (4/10) versus 0% (0/18); 20-week PFS: 78% (7/9) versus 11% (2/18))[72]. Similar ORR and PFS rates were noted in patients with MMR-deficient noncolorectal cancer (ORR: 71% (5/7); 20-week PFS: 67% (4/6))[72]. Whole-exome sequencing revealed a significantly higher tumour mutational burden in patients with MMR-deficient tumours compared with those with MMR-proficient tumours (mean number of somatic mutations per tumour: 1,782 versus 73; $P = 0.007$); a higher somatic mutational burden was associated with longer PFS ($P = 0.02$)[72].

These studies present promising evidence for the use of tumour mutational burden as a biomarker; however, several limitations should be noted, including the small size of patient cohorts, the inclusion of patients with variable treatment history, and the use of tumour samples obtained at various time points[73]. Important exceptions were also noted in these studies, including patients with high mutational burden who did not respond to ICB, and those with very low mutational burden with a good response to these agents[53]. Additionally, logistical barriers should be solved to widely apply tumour mutational burden as a biomarker using advanced genomic analysis techniques (such as whole-exome sequencing) in the clinical setting.

Moreover, results published in 2017 (REF.[75]), indicate that the importance of neoantigen load in predicting response to ICB is not as straightforward as had been initially anticipated. In this study[75], previously published cancer exome data from patients with melanoma treated with the CTLA-4 inhibitors ipilimumab and tremelimumab were re-analysed and combined with additional RNA-sequencing data. Both the total neoantigen and expressed neoantigen load were associated with a response to CTLA-4 blockade; however, neither was more predictive than somatic mutational burden[75]. In addition, the association between mutational burden and response to treatment was found only when samples obtained before treatment were analysed[75], whereas no association between mutation burden and treatment response was noted in patients whose tumour samples had been collected after initiating anti-CTLA-4 therapy. These findings reinforce the importance of multiple factors — ranging from IFNγ signalling to systemic factors — to the likelihood of a response to ICB treatment; these factors need to be analysed in an integrated way to improve the understanding of the immune-related response activated by those therapies and therapeutic resistance.

Serum markers

Although the assessment of the tumour and TME profiles is essential to identify robust biomarkers, clinical markers and morphological phenotypes of patients and tumours can also be useful to stratify patients into subpopulations. Serum markers, such as lactate dehydrogenase (LDH) and immune-cell counts, on routine blood analyses can be useful

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predictors of response to ICB. Indeed, these markers have shown potential utility for the prediction of response to ipilimumab. In patients treated with ipilimumab, high absolute-lymphocyte counts, low absolute-monocyte counts, and high relative-lymphocyte counts at baseline, and increasing absolute-lymphocyte counts during treatment were indicative of a favourable prognosis[76–78]. High relative eosinophil counts at baseline indicated favourable overall survival[76], and an early increase of the abundance of this cell type in the peripheral blood taken 18–19 days after the first ipilimumab infusion was associated with improved clinical responses to CTLA-4 blockade[79]. An increase of the absolute lymphocyte counts at 7 weeks of therapy after administering two doses of ipilimumab given 3 weeks apart was also shown to be associated with clinical benefit[2,80]. A study with results published in 2017 reported that, in patients with melanoma treated with ipilimumab, the baseline levels of $CD45RO^+/CD8^+$ T cells were significantly lower in patients without a response to therapy compared with those who had a response ($P<0.01$)[81]. In 80% (12/15) of patients with no response, the baseline levels of $CD45RO^+/CD8^+$ T cells were ≤25%, whereas all (6/6) patients with a response had ≥30% $CD45RO^+/CD8^+$ T cells[81]. In addition, elevated pretreatment levels of LDH were shown to be negatively associated with overall survival in patients with melanoma treated with ipilimumab or pembrolizumab[76–78,82,83].

The predictive value of combined assessment of these serum markers has been evaluated in 616 patients with melanoma treated with pembrolizumab[83]; high relative eosinophil counts and high relative lymphocyte counts, low LDH levels, and the absence of metastases in organs other than soft-tissue or lung were identified as independent baseline characteristics associated with favourable overall survival. The presence of all four favourable factors was associated with an excellent prognosis, with an ORR of 58.3% and a 1-year overall survival of 83.9%[83]. These serum markers have the advantage of being readily assessable in tests used routinely in clinical practice, and are also suitable for serial sampling over the course of treatment.

Radiographic markers

Radiographic assessments during therapy provide quantitative data on tumour dynamics that have been found to be associated with the response to and outcomes of precision cancer therapies[84–86], and might be useful to classify patients into subgroups according to different immune-related response patterns, with different prognostic implications[25]. Importantly, noninvasive functional imaging strategies need to be developed in the future to visualize and quantify target cells and molecules *in vivo*. For example, immuno-PET with the ^{89}Zr-desferrioxamine-labelled anti-CD8 cys-diabody (^{89}Zr-malDFO-169 cDb) enabled the noninvasive tracking of cytotoxic T cells in murine models of cancer immunotherapy[87]. Another study reported the use of small non-antibody therapeutics (the PD-1 ectodomain) as a high-affinity (110 pM) competitive antagonist of PD-L1; the ectodomain was radiolabelled and used as a PET-imaging tracer to distinguish PD-L1-positive tumours from PD-L1-negative tumours in mice[88]. Further development and application of these *in vivo* functional imaging approaches will enable investigators to address the issue of tumour heterogeneity and perform serial biomarker assessments over time, eventually overcoming the shortcomings of the current biomarkers, mostly derived from tissue samples.

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Future directions: multidisciplinary approach to establish robust biomarkers

A careful design of biomarker exploratory studies, based on a consensus from multiple parties, is essential to develop effective approaches to identify and validate robust markers for response and outcome of ICB. As evidenced by the exploratory analyses of PD-L1 expression as a possible marker, unified approaches and clear-cut guidelines are needed for the collection and processing of tissue specimens; as are detection methods (IHC or other bio chemical approaches) and evaluation strategies; and cut-off values to define positive results. A solution to the current controversies will be best found when such consensus and standardization are widely applied in prospective studies.

In order to achieve this goal and establish robust biomarkers for response and outcome of cancer immunotherapy, the US National Cancer Institute (NCI) has introduced important initiatives that encourage multidisciplinary approaches and public–private partnerships: the Partnership to Accelerate Cancer Therapies (PACT)[89] will bring multiple public and private stakeholders together to accelerate cancer research as part of the Cancer Moonshot Initiatives, focusing on the identification and validation of biomarkers of response and resistance to cancer therapies, with a special emphasis on immunotherapies. Related opportunities include the creation of Cancer Immune Monitoring and Analysis Centers (CIMACs)[90] and Cancer Immunologic Data Commons (CIDC)[91], which together will constitute the CIMAC–CIDC Network for correlative studies in clinical trials involving immunotherapy to identify immunotherapy biomarkers for optimizing the therapeutic strategies for patients. This network is expected to provide the infrastructure to perform correlative studies in clinical trials of cancer immunotherapy sponsored by the NCI[90,91].

As advocated in these initiatives, data sharing related to the collection and analysis of promising biomarkers among investigators will contribute to establishing large-scale databases, enabling validation of individual results, and subsequently translating biomarker discoveries into the clinical setting. Clinical trials going forward can then be designed in a biomarker-driven manner, and patients can be selected on the basis of biomarker findings, as has been successfully done for PD-L1 expression in the setting of first-line therapy for advanced-stage NSCLC. The establishment of a robust biomarker for patient selection, and the monitoring and management of decisions in ICB require a cohesive approach based on the active communication and collaboration among investigators from multiple disciplines, health-care providers, pharmaceutical industry and patients.

Conclusions

The advent of ICB has brought about a paradigm shift in the landscape of advanced-stage cancer treatment. ICB using these agents can result in unconventional response patterns and, thus, special strategies are required for the accurate evaluation of tumour responses and clinical outcomes. The development of robust biomarkers to monitor response to immunotherapy is a key next step for the further progress of this field towards precision immuno-oncology approaches.

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Key points

- A subset of patients receiving immune-checkpoint inhibitor therapy develop unconventional response patterns (termed ‘pseudoprogression’), in which tumour burden decreases after an initial increase, or during or after the appearance of new lesions
- The evaluation of pseudoprogression provides new challenges in treatment monitoring and therapeutic decision-making because it cannot be evaluated with the existing response-evaluation criteria
- The establishment of a standardized strategy to evaluate immune-related responses in patients receiving immune-checkpoint inhibitors is extremely important
- In addition, the development of robust biomarkers to assist prediction of response and clinical benefits of immune-checkpoint inhibitor therapy is essential to further advance the field as precision immuno-oncology
- The therapeutic activity of immune-checkpoint inhibitors is the result of a complex interplay between multiple factors in the tumour, tumour microenvironment, and immune system, requiring a collaborative approach to translate the emerging knowledge into the clinical context

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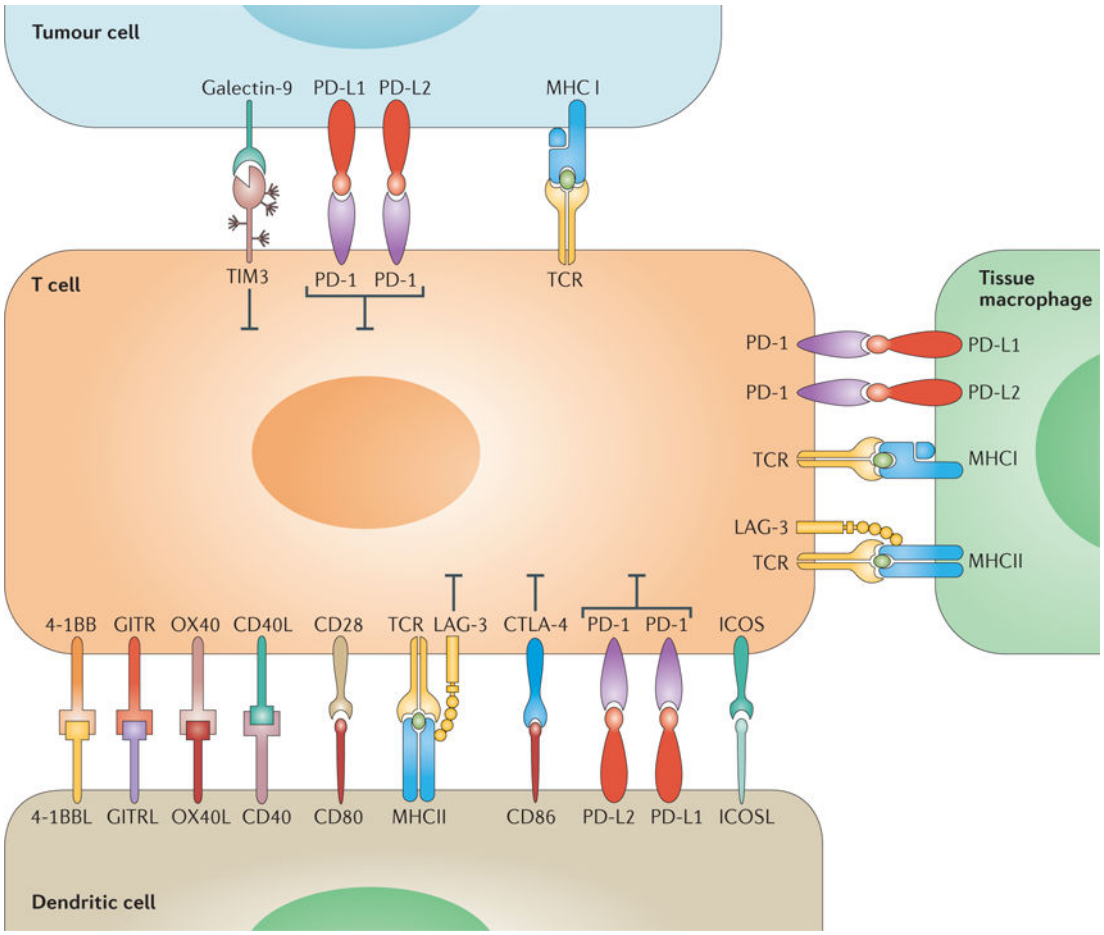

Figure 1. Ligand–receptor interactions between tumour cells and immune cells in the tumour microenvironment

An overview of the immune-checkpoint molecules involved in the regulation of the antitumour immune response.

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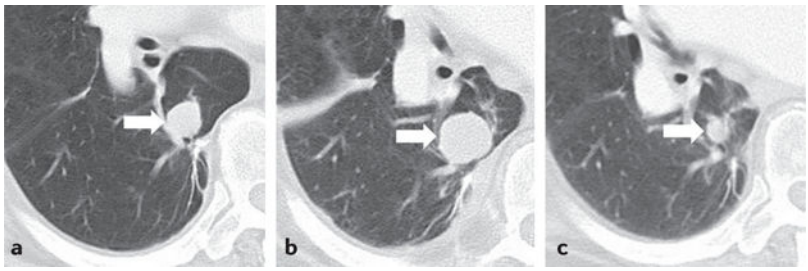

Figure 2. Response after initial increase in total tumour burden in a 77-year-old male with advanced-stage melanoma treated with ipilimumab

a | The baseline CT scan demonstrated a lung lesion (arrow) measuring 19 mm in the longest diameter. **b** | After 12 weeks of therapy, the lesion (arrow) measured 29 mm (53% increase compared with the baseline), indicating progressive disease by RECIST. **c** | The patient remained on therapy, and another follow-up CT scan (24 weeks after therapy initiation) showed a reduction in the size of the lesion (arrow), to 12 mm, indicative of an immune-related response to therapy. Reprinted from Nishino, M., Tirumani, S.H., Ramaiya, N.H. & Hodi, F.S. Cancer immunotherapy and immune-related response assessment: the role of radiologists in the new arena of cancer treatment, *Eur. J. Radiol.*, **84**, 1259–1268, Copyright (2015), with permission from Elsevier.

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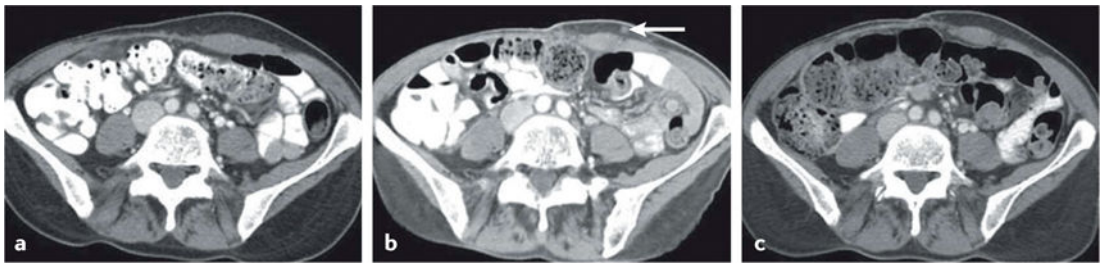

Figure 3. Response after appearance of a new lesion in a 56-year-old woman with metastatic melanoma treated with ipilimumab3

Contrast-enhanced CT scans of the abdomen a | before and b | 12 weeks after initiation of ipilimumab therapy revealed new subcutaneous nodule (arrow), suspected to be a new site of metastasis. c | A follow-up CT scan 24 weeks after initiation of therapy revealed resolution of the nodule, indicating response after appearance of a new lesion. Reprinted with permission from Nishino, M. *et al.* Personalized tumor response assessment in the era of molecular medicine: cancer-specific and therapy-specific response criteria to complement pitfalls of RECIST. *AJR Am. J. Roentgenol.* **198**, 737–745 (2012).

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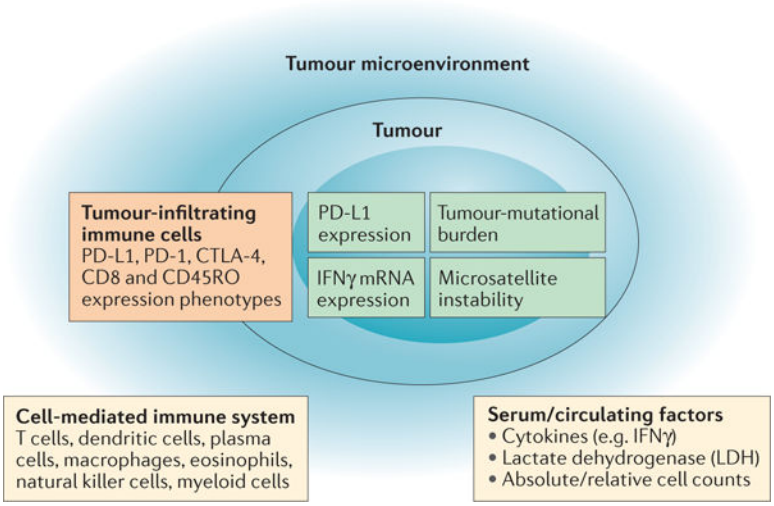

Figure 4. Key elements in biomarker development for immune-checkpoint inhibitor therapy

Consideration of the tumour, tumour microenvironment, and immune system must be incorporated in the ongoing efforts in biomarker development for immune-checkpoint inhibitor therapy.

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Table 1

Summary of the response assessment strategy using different criteria

	Bidimensional approach		Unidimensional approach		
Strategy	WHO (1979)[11]	irRC (2009)[7]	RECIST1.1 (2009, revised based on the original RECIST published in 2000)[12,13]	irRECIST (2013)[4,7–9]	iRECIST (2017)[31]
Measurement	LD × SD (cm^2)	LD × SD (cm^2)	• LD (cm) for lesions other than lymph nodes • SD (cm) for lymph nodes	• LD (cm) for lesions other than lymph nodes • SD (cm) for lymph nodes	• LD (cm) for lesions other than lymph nodes • SD (cm) for lymph nodes
Criteria for PR *	≥50% decrease	≥50% decrease	≥30% decrease	≥30% decrease	≥30% decrease
Criteria for PD ‡	≥25% increase, new lesion, or non-target PD	≥25% increase	≥20% and ≥5 mm increase, new lesion, or non-target PD	≥20% and ≥5 mm increase, or non-target PD	≥20% and ≥5 mm increase, or non-target PD
New lesions	Define PD	• Do not define PD • Included in the sum of measurements	Define PD	• Do not define PD • Included in the sum of measurements	• Result in unconfirmed PD • Confirmed PD only if additional new lesions appear or if an increase in size of new lesions is noted at the next assessment (≥5 mm for sum of new target lesion or any increase in new non-target lesion) • Recorded and measured separately and not included in the sum of measurements
Confirmation of PD	Not needed	Required on a consecutive scan (at least 4 weeks later)	Not needed	Required on a consecutive scan (at least 4 weeks later)	Required at the next assessment (4–8 weeks later)

*The percent change is calculated in comparison with the measurements at baseline.

‡The percent change is calculated in comparison with the measurements at the nadir (smallest tumour burden since baseline). LD, longest diameter; PD, progressive disease; PR, partial response; SD, short-axis diameter (longest perpendicular diameter).

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Table 2

PD-L1 expression as a biomarker for response and outcome in trials of anti-PD-1 agents

Study and phase	Tumour type and agents tested	Tissue specimen used and requirement for enrolment	Cell type with IHC-stained surface	Cut-off to define PD-L1 positivity	Percentage of PD-L1 positivity	Association with response and outcome
Topalian *et al.* (2012)[43] Phase I	• Advanced-stage solid tumours[*] • Nivolumab	• Archival/newly obtained pretreatment samples • Not required (42/296; 14%)[‡]	Tumour cells	≥5%	59.5% (25/42)	PD-L1-positive versus PD-L1 negative: • ORR: 36% (9/25) versus 0% (0/17); $P = 0.006$
Robert *et al.* (2015)[26] Phase III	• Melanoma • Nivolumab versus dacarbazine	• Tumour tissue from a metastatic or unresectable site • Required	Tumour cells	≥5%	35.4% (148/418)	Nivolumab versus dacarbazine: • ORR: 52.7% versus 10.8% in PD-L1-positive; 33.1% versus 15.7% in PD-L1-negative/ indeterminate • OS: HR = 0.3 (mOS: NA versus 12.39 months) for PD-L1-positive; HR = 0.48 (mOS: NA versus 10.22 months) for PD-L1 negative/ indeterminate
Robert *et al.* (2015)[27] Phase III	• Melanoma • Pembrolizumab versus ipilimumab	• Archival/newly obtained tumour samples • Required	Tumour cells	≥1%	80.5% (671/834)	Pembrolizumab (every 2 weeks or every 3 weeks) versus ipilimumab: • PFS: HR = 0.53 and 0.52 in PD-L1-positive; HR = 0.67 and 0.76 in PD-L1-negative • OS: HR = 0.55 and 0.58 in PD-L1-positive; HR = 0.91 and 1.02 in PD-L1-negative

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Study and phase	Tumour type and agents tested	Tissue specimen used and requirement for enrolment	Cell type with IHC-stained surface	Cut-off to define PD-L1 positivity	Percentage of PD-L1 positivity	Association with response and outcome
Daud *et al.* (2016)[45] Phase Ib	• Melanoma • Pembrolizumab	• Newly obtained tumour biopsy sample collected ≤60 days after first pembrolizumab dose • Required	Tumour and tumour-associated immune cells	≥1%§	76% (344/451)	PD-L1-positive versus PD-L1-negative: • PFS: HR= 0.51 (mPFS: 5.6 months versus 2.8 months) • OS: HR = 0.5 (mOS: 29.9 months versus 12.6 months) • Higher ORR in groups with higher PD-L1 expression (highest and lowest ORR: 57% in ≥33%–<66%-stained, and 8% in non-stained group; *P* <0.001)
Brahmer *et al.* (2015)[44] Phase III	• Squamous NSCLC • Nivolumab versus docetaxel	• Pretreatment archival or recent tumour-biopsy specimen • Required	Tumour cells	≥1%, ≥5%, or ≥10%	• 44% (119/272) for ≥1% • 30% (81/272) for ≥5% • 25% (69/272) for ≥10%	Nivolumab versus docetaxel: • PFS according to PD-L1 expression levels: HR = 0.66, 0.67, 0.75, 0.54, 0.7, 0.58, and 0.45 for <1%, ≥1%, <5%, ≥5%, <10%, ≥10%, and not quantifiable group, respectively • OS according to PD-L1 expression levels: HR = 0.58, 0.69, 0.7, 0.53, 0.7, 0.5, and 0.39 for <1%, ≥1%, <5%, ≥5%, <10%, ≥10%, and not quantifiable group, respectively Predictive value of PD-L1 expression (≥1%, ≥5%, and ≥10%) on response to nivolumab: • ORR: 0.93, 0.29, and 0.64, respectively

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Study and phase	Tumour type and agents tested	Tissue specimen used and requirement for enrolment	Cell type with IHC-stained surface	Cut-off to define PD-L1 positivity	Percentage of PD-L1 positivity	Association with response and outcome
						• PFS: 0.7, 0.16, and 0.35, respectively • OS: 0.56, 0.47, 0.41, respectively
Gettinger *et al.* (2015)[29] Phase I	• NSCLC • Nivolumab	• Archived tumour samples • Not required (68/129; 53%)[‡]	Tumour cells	≥5%	49% (33/68)	PD-L1 positive versus PD-L1-negative: • ORR: 15% (5/33) versus 14% (3/35) • mPFS: 3.3 months (95% CI 1.8–7.5 months) versus 1.8 months (95% CI 1.7–2.3 months) • mOS: 7.8 months (95% CI 5.6–21.7 months) versus 10.5 months (95% CI 5.2–14.8 months)
Rizvi *et al.* (2015)[35] Phase II	• Squamous NSCLC • Nivolumab	• Pretreatment archival tumour samples • Not required (86/117; 74%)[‡]	Tumour cells	≥5%	33% (25/76)	PD-L1-positive versus PD-L1-negative PD-L1: • ORR: 24% (6/25) versus 14% (7/51)
Borghaei *et al.* (2015)[37] Phase III	• Non-squamous NSCLC • Nivolumab versus docetaxel	• Pretreatment, archival or recent tumour-biopsy specimens • Required for use in biomarker analyses but not used in patient selection	Tumour cells	≥1%, ≥5%, or ≥10%	• 42% (246/582) for ≥1% • 31% (181/582) for ≥5% • 28% (165/582) for ≥10%	Nivolumab versus docetaxel: • mPFS according to PD-L1 expression levels (≥1%, ≥5%, and ≥10%): 4.2 versus 4.5 months (HR = 0.7), 5 versus 3.8 months (HR = 0.54), 5 versus 3.7 months (HR = 0.52), respectively • mOS according to PD-L1 expression levels (≥1%, ≥5%, and ≥10%): 17.7 versus 9 months

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Study and phase	Tumour type and agents tested	Tissue specimen used and requirement for enrolment	Cell type with IHC-stained surface	Cut-off to define PD-L1 positivity	Percentage of PD-L1 positivity	Association with response and outcome
						(HR = 0.58), 19.4 versus 8.1 months (HR = 0.43), 19.9 versus 8 months (HR = 0.4), respectively Predictive value of PD-L1 expression (≥1%, ≥5%, and ≥10%) on efficacy of nivolumab: • ORR: 0.002 (all) • PFS: 0.02, <0.001, and <0.001, respectively • OS: 0.06, <0.001, and <0.001, respectively
Garon *et al.* (2015)[36] Phase I	• NSCLC • Pembrolizumab	• Biopsy sample collected ≤60 days of first pembrolizumab dose with no intervening systemic therapy, or between last dose of previous systemic therapy and first pembrolizumab dose • Required	Tumour cells	≥50% //	• 23.2% (191/824) in all screened • 22.7% (146/643) in previously treated • 24.9% (45/181) in treatment-naive	PD-L1-positive versus PD-L1-negative (1–49% and <1%): • ORR: 34.2% (13/38) versus 9.3% (4/43) and 4/40 (10%) in training set; 45.2% (33/73) versus 16.5% (17/103) and 10.7% (3/28) in validation set • mPFS: 4.5 months versus 2.1 months (both groups) in training set; 6.4 months versus 4.1 months and 4 months in validation set • mOS: 13.7 months versus 5.9 months and 6.7 months in training set; not reached versus 10.6 months and 10.4 months in validation set

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Study and phase	Tumour type and agents tested	Tissue specimen used and requirement for enrolment	Cell type with IHC-stained surface	Cut-off to define PD-L1 positivity	Percentage of PD-L1 positivity	Association with response and outcome
Reck *et al.* (2016)[46] Phase III	• Treatment-naïve NSCLC • Pembrolizumab versus platinum-based chemotherapy	• Tumour samples obtained by core-needle or excisional biopsy, or from tissue resected at diagnosis of metastatic disease • Required[¶]	Tumour cells	≥50%	• 30.2% (500/1653) in screened patients • 100% in treated patients (*n* = 305)[¶]	Pembrolizumab versus chemotherapy in PD-L1 positive patients: • ORR: 44.8% versus 27.8% • mPFS: 10.3 months versus 6 months • 6-month OS: 80.2% versus 72.4% (HR = 0.6); *P* = 0.005

[*] 18 patients with melanoma, ten patients with NSCLC, seven patients with colorectal cancer, five patients with renal-cell cancer, and two patients with prostate cancer.

[‡] Percentage of patients with PD-L1 expression tested in total cohort.

[§] The study also used MEL score (MEL)[45]: 0 (no membrane staining), 1 (>0%–<1%), 2 (≥1–<10%), 3 (≥10%–<33%), 4 (≥33%–<66%), 5 (≥66%).

[‖] PD-L1 expression status evaluated using 'proportion score' (REF. [36]): <1%, 1–49%, and ≥50%; initially PD-L1 positivity was defined as ≥1%, but ≥50% was used to compare outcomes.

[¶] PD-L1 positivity (membrane staining ≥50%) was part of trial eligibility criteria. HR, hazard ratio; m, median; NA, not assessed; NSCLC, non-small-cell lung cancer; ORR, overall response rate; OS, overall survival; PFS, progression-free survival.