

Biomarkers of Response to Immune Modulatory Therapies in Cancer

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Abstract

Immune modulatory antibody-based therapies serve to augment and direct the endogenous immune response against cancer. Significant efficacy has been demonstrated in multiple subtypes of solid and haematological malignancies. Despite great promise, responses are limited to a fraction of treated patients highlighting the need to decipher underlying mechanisms of response and resistance. Here we review progress in this area with a focus on the identification of candidate predictive biomarkers of response.

Keywords: Antibodies; Biomarkers; Cancer; CTLA-4; Immune modulation; PD-1

Introduction

The development and clinical application of immune modulatory antibody-based therapies continues to generate much excitement. In contrast to monoclonal antibodies (mAb) targeting cancer cells directly, immune modulatory mAb serve to direct and augment the endogenous immune response against cancer. This has translated into significant clinical activity in multiple subtypes of solid and haematological malignancies within randomised clinical trials [1-13]. At present, three therapeutic agents have received US Food and Drug Administration (FDA) approval. Ipilimumab, a humanised IgG1 monoclonal antibody (mAb), targeting cytotoxic T lymphocyte antigen-4 (CTLA-4), was the first drug to prolong survival in advanced melanoma, whilst nivolumab and pembrolizumab, both IgG4 mAbs targeting programmed cell death-1 (PD-1), have demonstrated significant clinical activity across multiple solid and, most recently, haematological tumour subtypes [3,5-7,9-13]. For responding patients, these agents offer the potential for durable remission and even cure. Response rates are, however, modest, engendering significant efforts to decipher underlying mechanisms of response and resistance. Significant evolution in the understanding of immune checkpoint modulation at both a cellular and molecular level has led to promising advances in the identification of candidate predictive biomarkers. Here we review this progress, with a focus on the current FDA-approved therapies employed in clinical practice.

CTLA-4 and PD-1 - Key Regulators of T cell Response and Function

CTLA-4 was first described as a novel B7 family member nearly three decades ago [14]. Highly conserved between species [15], it plays a critical role in immune regulation supported by the death of knockout (KO) mice by 3-4 weeks of age secondary to lymphoproliferative disease and associated multi-organ failure [16]. It

is a co-inhibitory cell surface molecule, closely related to CD28, also interacting with B7 molecules (CD80 and CD86) expressed on antigen presenting cells (APCs), but with greater affinity and avidity than CD28, thus negatively regulating T cell activation. T cell receptor (TCR) signalling in the presence of CTLA-4 inhibits T cell clonal expansion and initiation of effector functions such as IL-2 production [17]. It is thus a powerful negative regulator of T cell activation, recognised as an attractive therapeutic target on tumour-infiltrating lymphocytes (TILs).

PD-1 is also a B7 family member, related to CD28 and CTLA-4, demonstrated to negatively regulate TCR signalling upon engagement of its ligands programmed cell death ligand-1 (PD-L1) and/or programmed cell death ligand-2 (PD-L2) [18-23]. The PD-1 receptor was initially discovered as an upregulated gene in a T cell hybridoma undergoing cell death [24]. Signalling through PD-1 exerts its effects on cellular differentiation and survival through inhibition of the cell cycle and lymphocyte effector function and/or promotion of apoptosis; cellular events that are positively regulated by CD28 or interleukin-2 [25]. C57BL/6 and Balb/c mice KO for PD-1 develop late-onset glomerulonephritis and antibody-mediated cardiomyopathy respectively [26,27]. Furthermore, PD-1 loss in non-obese diabetic mice mediates accelerated insulinitis and pro-inflammatory T cell cytokine production [28]. Together, these findings demonstrate a key role of PD-1 in down-modulating immune responses and maintaining peripheral T cell tolerance.

Anti-CTLA-4 Therapy

Defining the mechanisms underlying the activity of anti-CTLA-4 therapy

Despite a number of elegant pre-clinical studies, a comprehensive understanding of the mechanisms underlying the activity of anti-CTLA-4 mAb has been lacking until recently. The initial hypothesis was that mAb targeting CTLA-4 would act to block co-inhibitory signals at the immune synapse, 'taking the brakes off' effector CD8 T

cell (Teff) responses. The subsequent demonstration that CTLA-4 is constitutively expressed on regulatory T cells (T_{reg}) raised the possibility of an additional impact on the T_{reg} compartment [29-32]. It was later demonstrated that for maximal anti-tumour activity, blockade of both Teff and T_{reg} compartments is required [33], although the specific impact of anti-CTLA-4 mAb on the T_{reg} compartment remained unclear. A consistent observation associated with anti-CTLA-4-mediated tumour rejection had been a positive shift in the intra-tumoural ratio of Teff to T_{reg} . A number of studies had demonstrated that anti-CTLA-4-mediated expansion of both Teff and T_{reg} in the blood and secondary lymphoid organs of mice [34-36]. It was therefore unclear how anti-CTLA-4 mAb act to preferentially expand Teff in the tumour whilst simultaneously expanding both populations in the periphery. Three pre-clinical studies subsequently demonstrated that anti-CTLA-4 mAb serve to preferentially deplete intra-tumoural T_{reg} leading to a shift in the Teff/ T_{reg} ratio correlating with tumour rejection [37-39]. Preferential depletion of T_{reg} occurs secondary to a higher relative density of expression of CTLA-4 on the T_{reg} versus Teff and a tumour microenvironment enriched for activatory Fc gamma receptor (Fc γ R) expressing tumour-associated macrophages (TAMs) with capacity for antibody-dependent cellular cytotoxicity (ADCC). These studies highlighted a previously unrecognised importance of antibody isotype, target molecule density and Fc γ R-expressing innate effector cells in dictating the final outcome of immune modulatory therapies.

Insights from clinical trials

In keeping with the described pre-clinical studies, Hodi and colleagues identified a striking linear relationship between the extent of tumour necrosis in post-treatment biopsies and the ratio of intra-tumoural CD8⁺ T cells and FoxP3⁺ T_{reg} in six patients with advanced melanoma and ovarian cancer undergoing CTLA-4 blockade with ipilimumab following GVAX therapy [40]. This raised the possibility of selectively targeting T_{reg} as a complementary strategy for combination therapy. In the same year, Sharma and colleagues conducted a neoadjuvant clinical trial of ipilimumab in patients with localised bladder cancer [41]. The study design allowed analysis of surgical specimens and peripheral blood mononuclear cells (PBMCs) prior to and following three infusions of ipilimumab. Anti-CTLA-4 therapy resulted in a consistent increase in CD4⁺ICOS⁺ T cells in both the periphery and tumour as well as a reduction in CD4⁺FoxP3⁺ cells in the tumour in all patients. A significant population of CD4⁺ICOS⁺ cells were identified as FoxP3⁻, IFN- γ -producing cells, demonstrated to recognise the tumour-associated antigen NY-ESO-1. The authors therefore described the observed shift in CD4⁺ICOS⁺ and CD4⁺FoxP3⁺ populations as a change in the balance of effector to regulatory T cells. Owing to the neoadjuvant nature of the study, the clinical relevance of the described immunological findings, specifically their predictive value, is yet to be determined.

In another small cohort of patients, Ribas and colleagues determined the impact of tremelimumab, an IgG2 mAb targeting CTLA-4, on the tumour microenvironment (TME) of patients with advanced melanoma [42]. Fifteen biopsies were performed in seven patients at variable timepoints pre- and post-therapy including both responding and non-responding lesions. Immunohistochemical analysis of the TME was performed with focus on CD8⁺ T cells, T_{reg} and the immunosuppressive enzyme indoleamine 2,3-dioxygenase (IDO). Responding patients were found to have a striking increase in granzyme B⁺ CD8⁺ T cells, these were absent at baseline and distributed throughout the tumour areas rather than in the periphery

as observed in pre-dosing biopsies. In contrast to the findings of Hodi and colleagues, no impact on T_{reg} or change in IDO expression by CD1a⁺ dendritic cells (DC's) was observed. This analysis was expanded within the setting of an early clinical trial, evaluating pre and post treatment biopsies in 32 patients with advanced melanoma undergoing therapy with tremelimumab [43]. CD8⁺ T cell infiltration was observed in response to therapy in both responding and non-responding patients. Functional analyses of HLA-DR, CD45RO and Ki67 on the described CD8⁺ TILs failed to differentiate between responders and non-responders. Analysis of FoxP3⁺ T_{reg} identified a trend towards higher infiltrates in responding lesions but nil significant.

In a similar manner, again within the context of a phase II trial, Hamid and colleagues attempted to prospectively identify candidate biomarkers from the TME associated with clinical response to ipilimumab in 82 patients with advanced melanoma [44]. Candidate biomarkers were evaluated in tumour biopsies collected pre-treatment and 24-72 hours after the second ipilimumab dose. In contrast to the findings of Huang et al., significant associations were observed between clinical activity and high baseline levels of FoxP3 and IDO. Baseline TIL scores did not significantly correlate with outcome, however, the increase in TILs between baseline and week 3 met significance. Based on the pre-clinical data, one might hypothesise that the observed relationship between baseline FoxP3⁺ cells and clinical outcome could be explained by depletion of T_{reg} by ipilimumab and consequent shift in the ratio of Teff to T_{reg} . The lack of similar findings with tremelimumab43 may be explained by its IgG2 isotype and resulting low affinity for Fc γ Rs on innate effector cells. With a lack of ADCC capacity, tremelimumab may therefore only benefit those with an existing, favourable baseline ratio of Teff to T_{reg} . Nevertheless, in the human setting, ipilimumab-mediated depletion of T_{reg} is yet to be demonstrated, although studies to date have largely focused on peripheral blood rather than tumour infiltrating lymphocytes [45].

Longitudinal sampling studies in patients with advanced cancer are notoriously difficult. Evaluation of mechanism is often less challenging in the neoadjuvant setting where samples are guaranteed before and after therapy. The drawback of these studies is the inability to determine the predictive value of any observed findings where surgical intervention is curative. A calculated approach was adopted by Tarhini and colleagues in evaluating the mechanistic activity of ipilimumab in patients with operable, regionally advanced melanoma [46]. Blood and tumour was assessed at baseline and then again at week 6 following two infusions of ipilimumab and surgical resection of disease. In keeping with pre-clinical studies, a significant increase in the percentage of circulating T_{reg} was observed and associated with improved progression free survival (PFS). Moreover, relative to baseline, an increase in activated (CD69⁺) tumour-infiltrating CD3⁺CD4⁺ and CD3⁺CD8⁺ T cells was observed. There was also a trend towards an inverse association between the change in intra-tumoural T_{reg} and clinical benefit, again in keeping with pre-clinical studies. Circulating and tumour-associated monocytic myeloid-derived suppressor cells (mMDSCs) were also assessed. Treatment was observed to mediate a reduction in both circulating and intra-tumoural mMDSCs associated with improved PFS.

Despite the enrolment of 35 patients, only 24 and 10 specimens were available for immunohistochemical and flow cytometric analysis respectively. This once again highlights the difficulties in tumour sampling in patients with advanced disease. In contrast to haematological malignancies, patients with advanced solid cancers

often have little or no readily accessible disease for biopsy. This group of patients are also commonly highly symptomatic of their disease and unfit to undergo more invasive sampling. Despite known dissociation in immune responses between tumour and peripheral blood [47], such difficulties have prompted efforts to identify biomarkers through non-invasive sampling.

Circulating biomarkers of response

Owing to the described mechanism of anti-CTLA-4 mAb, a number of studies have set out to evaluate the impact and possible predictive value of anti-CTLA-4 on circulating T lymphocytes. Ku and colleagues evaluated the change in peripheral absolute lymphocyte count (ALC) in relation to ipilimumab therapy in 53 patients with advanced melanoma [48]. Baseline ALC counts failed to predict response, however, an ALC of $\geq 2000/\mu\text{L}$ following two doses of ipilimumab (week 7) was associated with a significantly higher clinical benefit rate and median OS compared to those with an ALC of $<1000/\mu\text{L}$. Santeogoets and colleagues observed similar findings in ALC counts within a phase I/II dose escalation/expansion trial of GVAX plus ipilimumab in 28 patients with advanced prostate cancer. On further evaluation, however, no differences in overall frequencies of circulating CD3⁺CD4⁺/CD8⁺ T lymphocytes or CD3-CD56⁺ NK cells were observed [49]. A detailed analysis of pre-treatment frequencies of T_{eff} and T_{reg} subsets was performed as well as longitudinal analysis through therapy and correlation with clinical outcome. A number of findings were observed to correlate with median OS including baseline non-naïve CD8⁺, CD4⁺PD-1⁺ and CD4⁺ T cells. The strongest predictor of outcome, identified by unsupervised clustering, was baseline frequency of CD4⁺CTLA-4⁺ effector T cells. Interestingly, intracellular CTLA-4 was virtually undetectable in CD4⁺ T cells of healthy donors but abundant in both T_{reg} and CD4 effector T cells in prostate cancer patients. These data, in part, complement the described findings of Sharma et al. highlighting further a potential role for CD4 effector T cells in dictating the outcome of anti-CTLA-4 therapy. The study shed no further light on the predictive value of baseline or on therapy FoxP3⁺ T_{reg}, these were observed steadily to rise in response to therapy, with increases of greater than 50% associated with shorter OS.

With a specific aim of identifying novel biomarkers associated with both clinical benefit and ipilimumab-mediated toxicity, Wang and colleagues assessed baseline characteristics and changes in CD4⁺ and CD8⁺ T cells sorted from the peripheral blood of advanced melanoma patients receiving ipilimumab [50]. Microarray analysis of purified CD4⁺ and CD8⁺ T cells was performed to assess gene-profiling changes induced by ipilimumab in 75 patients. Thereafter, to verify changes in selected molecules, a flow cytometric study was undertaken with pre-treatment, 3 month and 6 month post-ipilimumab PBMC samples from expanded groups of 55, 25 and 37 patients respectively. Analysis of candidate biomarkers at baseline revealed a low percentage of Ki67⁺EOMES⁺CD8⁺ and EOMES⁺CD8⁺ T cells was significantly associated with relapse. Pre-therapy specimens were subsequently stratified by the median percentage of EOMES⁺CD8⁺ T cells. Patients with a higher baseline percentage of EOMES⁺CD8⁺ T cells had a significantly improved relapse-free survival; the same was true for Ki67⁺EOMES⁺CD8⁺ T cells. The authors proposed further validation of the predictive value of these markers in a prospective manner.

Although studies evaluating mechanisms of response and resistance to anti-CTLA-4 have largely focused on T lymphocytes, mMDSCs have been identified as a suppressor subset with capacity to impact on

outcome to anti-CTLA-4 therapy [51,52]. Meyer and colleagues collected peripheral blood samples from 49 patients with advanced melanoma undergoing ipilimumab therapy [51]. Lineage negative CD14⁺HLA-DR- mMDSC were enriched in the peripheral blood of melanoma patients relative to healthy donors. A trend towards higher frequencies of mMDSCs was observed in patients with a high burden of metastatic disease. Interestingly, significantly lower percentages of CD14⁺HLA-DR- mMDSCs were observed in patients responding to ipilimumab versus non-responders. Baseline values were therefore compared to mean values during and after treatment. A trend towards lower baseline values in responders was observed compared to non-responders. Patient numbers were small, possibly contributing to the lack of statistical significance. In parallel, Kitano and colleagues developed a computational algorithm-driven system for evaluation of mMDSC frequency for prediction of clinical outcomes. In a larger study of 68 patients with advanced melanoma treated with ipilimumab, a low pre-treatment mMDSC frequency, defined as less than 14.9%, was significantly associated with improved OS in both uni- and multi-variate analyses. In terms of mechanism, no relationship between ALC and mMDSC frequency was observed, however, a statistically significant inverse correlation between the percentage change in absolute CD8⁺ T cell number and mMDSC frequency at week 6 was demonstrated, in keeping with the described suppressor function of this myeloid subset.

Lactate dehydrogenase (LDH) emerged as a candidate biomarker following subgroup analysis of overall survival in a landmark phase III trial of ipilimumab in advanced melanoma [1]. Interestingly, the hazard ratio for ipilimumab versus the control arm was only significant in patients with baseline serum LDH values within normal range. Based on these data, Kelderman and colleagues retrospectively correlated baseline LDH in two separate cohorts of 'real world' melanoma patients treated within an expanded access programme (EAP) [53]. In a multi-variate model, LDH was found to be the strongest predictive factor for OS. Patients with a baseline LDH two times the upper limit of normal were highly unlikely to derive benefit from ipilimumab with a significantly lower median OS observed in this group. The absence of a control arm precluded discrimination between the prognostic and predictive value of LDH as a marker, nevertheless, in the absence of more robust biomarkers it was highlighted as a readily available marker to guide clinical decision-making.

In addition to its role as a key regulator of angiogenesis, vascular endothelial growth factor (VEGF) is a potent inhibitor of DC maturation and T cell responses [54,55]. Serum VEGF levels are known to correlate with melanoma stage, moreover, high circulating serum VEGF is a poor prognostic marker in patients with melanoma [56,57]. The prognostic and/or predictive value of serum VEGF in relation to immune modulatory therapy had, however, remained undetermined. Yuan and colleagues retrospectively analysed serum VEGF levels in 176 patients with advanced melanoma, before and after therapy with ipilimumab [58]. Baseline VEGF levels were associated with clinical response, patients with a baseline serum VEGF value greater than 43pg/mL, treated with either 3 or 10mg/kg of ipilimumab were less likely to derive clinical benefit. In addition, higher baseline serum VEGF levels were associated with a significantly poorer OS. Prospective studies were therefore called for in order to determine the predictive value. Such findings suggest potential synergy between anti-CTLA-4 and anti-VEGF therapies, indeed, combination therapy with ipilimumab and bevacizumab appears promising in early clinical trials [59].

Genetic determinants of response to anti-CTLA-4 therapy

Although the prognostic significance of TILs has been demonstrated for a number of solid cancers [60-65], their predictive value in relation to immune modulatory therapies is unclear. Ji and colleagues performed gene expression profiling on tumour biopsies collected from 45 patients with advanced melanoma, three weeks before commencement of ipilimumab, within a phase II clinical trial [66]. High baseline expression of immune-related genes was associated with favourable clinical outcome to therapy. Genes involved in the immune response increased in expression whilst those for melanoma-specific antigens and cell proliferation decreased. These findings highlighted the potential importance of an existing, endogenous anti-tumour immune response in dictating outcome to immune modulation. Adding another layer of complexity, recent advances in next generation sequencing techniques have enabled more detailed characterisation or 'immunoprofiling' of both the periphery and the tumour microenvironment allowing quantitative assessment of the clonality and repertoire of T cell receptors. In two studies evaluating T cell repertoire in response to anti-CTLA-4 therapy, a diversification of the T cell repertoire in the peripheral blood with an increased number of unique T cell receptor β chain complementarity determining region 3 (CDR3) sequences was observed. In a separate study, improved overall survival was seen in patients who maintained peripheral T cell clones that were present in high frequencies prior to anti-CTLA-4 blockade [67,68].

It remains to be elucidated whether the maintenance of a specific T cell receptor clonotype can be used as a prognostic and/or predictive biomarker in patients treated with immune modulatory antibodies. Furthermore, although T cell receptor sequencing provides information on the diversity and clonality of the T cell repertoire, limitations exist in the ability of this technique to provide detail regarding the antigen specificity of infiltrating lymphocytes. A study using peptide/MHC multimers and a panel of melanoma-derived neo-epitopes demonstrated a broadening of the peripheral melanoma-specific CD8 T cell repertoire following anti-CTLA-4 therapy with ipilimumab [69]. Interestingly, ipilimumab therapy did not appear to significantly affect the magnitude of the pre-existing melanoma-specific T cell peripheral responses suggesting anti-CTLA-4 therapy serves to prime rather than enhance pre-existing immune responses.

Until recently, identification of the molecular determinants driving tumour-infiltrating T cell responses has remained unclear. Based on the observations that somatic mutations can give rise to neo-epitopes and that these may serve as neoantigens [70], Snyder and colleagues conducted a study to determine whether the genetic landscape of a tumour impacts upon clinical benefit derived from anti-CTLA-4 therapies [71]. Whole exome sequencing was performed on pre-treatment tumour tissue and matched blood samples in 64 patients. With use of sequencing data and bioinformatic approaches, candidate neoantigens were identified. Thereafter, relevant mutated peptides were synthesized and tested for their ability to activate lymphocytes from ipilimumab-treated patients. Using a discovery set of 11 responding and 14 non-responding patients, a neo-antigenic repertoire, unique to responding patients, was defined and subsequently validated in a separate cohort of 39 patients. High mutational load was associated with a benefit from CTLA-4 therapy, however, this factor alone was not sufficient to impart a clinical benefit. Rather, there were specific somatic neo-epitopes shared by patients with a prolonged benefit and absent in non-responders. These observations require validation in a larger cohort of patients, however,

this study represents a major step forward, not only in the quest for predictive biomarkers, but for the entire field of tumour immunotherapy.

Anti-PD-1 Therapy

Defining the mechanisms underlying the activity of anti-PD-1 therapy

The programmed cell death-1 (PD-1) receptor-ligand interaction is a major pathway hijacked by tumours, promoting immune evasion and tumour escape. PD-1 is expressed on a variety of cellular subsets including activated T and B lymphocytes, natural killer (NK) cells, monocytes and dendritic cells [72]. In health, PD-1, expressed on the surface of activated T cells, acts to down-modulate unwanted or excessive immune responses, preventing autoimmunity and maintaining immunological tolerance to self-antigens. Expression patterns of PD-L1 and PD-L2 vary. PD-L1 is highly expressed on monocytes, but also at low levels on plasmacytoid and myeloid dendritic cell subsets as well as activated T lymphocytes [72]. Furthermore, PD-L1 expression can be induced by inflammatory cytokines including type I and type II interferons in non-haematopoietic cells of epithelial and endothelial origin [73]. In contrast, PD-L2 is expressed selectively within the myeloid compartment on macrophages and dendritic cells [72].

PD-L1 expression has been demonstrated in multiple solid tumours [74-76]. Two key mechanisms of tumour PD-L1 up-regulation and immune resistance have been described. Innate immune resistance refers to the constitutive expression of PD-L1 by tumour cells secondary to increased signalling via oncogenic pathways, independent of the cytokine milieu of the tumour microenvironment [75,77]. On the contrary, adaptive immune resistance is a process whereby tumour cells adapt to the endogenous immune response through aberrant upregulation of PD-L1 in the context of interferon gamma release by tumour infiltrating lymphocytes [78,79]. This mirrors the physiological role of PD-L1 upregulation, which serves to prevent excessive immune-mediated damage as a result of the immune response to infection [75,77].

In mouse models of chronic viral infection, continuous exposure to lymphocytic choriomeningitis virus (LCMV) was found to mediate functional dysregulation or 'exhaustion' of viral-specific CD8 T cells with associated increased PD-1 cell surface expression [80]. In vivo administration of antibodies blocking the PD-1/PD-L1 pathway restored viral-specific T cell function, leading to a substantial reduction in viral burden. Early studies in animal models provide support for the integral role of the PD-1/PD-L1 axis in tumour immunity. PD-L1 expression on tumour cells has been shown to inhibit T cell activation and lysis of tumour cells with increased tumour-specific T cell death [20,81]. In BALB/c PD-1 knockout mice, the growth of PD-L1 expressing murine myeloma cell lines was completely suppressed in contrast to rapid tumour cell growth in PD-1 positive controls [82]. Furthermore, PD-L1 expression on immunogenic P815 tumour cells associated with resistance to anti-4-1BB therapeutic antibody treatment was restored with anti-PD-L1 therapy [81]. Collectively, these data demonstrate the critical role of the PD-1/PD-L1 pathway in tumour immune evasion, highlighting its therapeutic significance in the treatment of cancer.

Insights from clinical trials

Several trials of monoclonal antibodies targeting the PD-1/PD-L1 pathway have demonstrated unprecedented rates of success with durable responses and survival benefit in patients with a variety of cancers [3-8,12,13,83]. In 2014, pembrolizumab and nivolumab, both fully humanised IgG4 mAbs targeting PD-1, received FDA approval for the treatment of patients with ipilimumab-refractory advanced melanoma. In the same year, nivolumab and an anti-PD-L1 inhibitor, MPDL3280A, achieved 'breakthrough designation' status for the treatment of subsets of refractory Hodgkin's lymphoma and metastatic bladder cancer respectively.

Whilst there is evidence to support PD-L1 expression as a predictor of response to anti-PD-1/PD-L1 therapy [3,6,8,12,13,83-85], the observed clinical activity in patients with PD-L1 negative tumours has questioned this hypothesis [5,6,11]. Initial support for tumour PD-L1 expression as a predictive biomarker arose from data generated within a phase I trial of nivolumab [3]. A response rate of 36% was reported in patients with PD-L1 positive tumours with no responses demonstrated in those harbouring PD-L1 negative tumours. The utility of PD-L1 as a predictive biomarker was later questioned however, following observations of clinical responses to nivolumab in PD-L1 negative melanoma in 17% of patients [85]. Furthermore, in a phase 1 trial of pembrolizumab, responses were also seen in PD-L1 negative melanoma and non-small cell lung cancer (NSCLC), albeit significantly lower than the PD-L1 positive subgroups [86]. Subsequent comprehensive analysis of tumour specimens obtained from 41 patients with a variety of advanced solid tumours treated with nivolumab, demonstrated that tumour PD-L1 expression was the factor most closely correlated with response to anti-PD-1 therapy, in keeping with findings from previous studies [87]. PD-L1 expression was significantly associated with tumour subtypes in which the majority of responses to anti-PD-1 therapy have been reported thus far, including melanoma, NSCLC and renal cell carcinoma. In a combination trial of ipilimumab and nivolumab, objective responses were reported in approximately 40% of patients with advanced melanoma treated with concurrent immunotherapy, irrespective of baseline tumour PD-L1 status, suggesting that tumour PD-L1 expression may be less relevant as a predictive biomarker for combination immunotherapy [6].

The dynamic, inducible nature of tumour PD-L1 expression, largely related to interferon gamma release by tumour-infiltrating lymphocytes may indeed explain the lack of consistency in correlation between tumour PD-L1 expression and response to anti-PD-1 therapy demonstrated in some clinical trials. Although, based on these studies, tumour PD-L1 expression as a predictive biomarker requires further evaluation, the observed clinical successes have led to the clinical evaluation of these agents in other highly positive PD-L1 expressing tumours such as Hodgkin lymphoma in which overexpression of PD-L1 on Reed-Sternberg cells occurs constitutively as a result of PD-L1 and PD-L2 gene co-amplification [8].

Comparison between and interpretation of the described clinical studies is limited by the use of varied staining antibodies and thresholds for determining PD-L1 positivity. Moreover, the observation that PD-L1 is expressed on tumour-infiltrating immune cells in addition to tumour cells, with some clinical trials including immune infiltrate PD-L1 expression in the cut off for PD-L1 positivity may be highly relevant [3,6,8,12,13,83-86]. Observed responses in PD-L1 negative patients in early stage clinical trials highlight the limited negative predictive power of PD-L1 expression as a biomarker of

response. Moreover, significant discordance in PD-L1 expression between primary tumours, metastases and intra-patient metastases was recently demonstrated in a study of advanced melanoma [88]. Discordance in PD-L1 expression in renal cell carcinoma between primary and metastatic lesions has also been described [89]. These findings may, at least in part, explain why PD-L1 is a poor negative predictor of response to treatment (Figure 1).

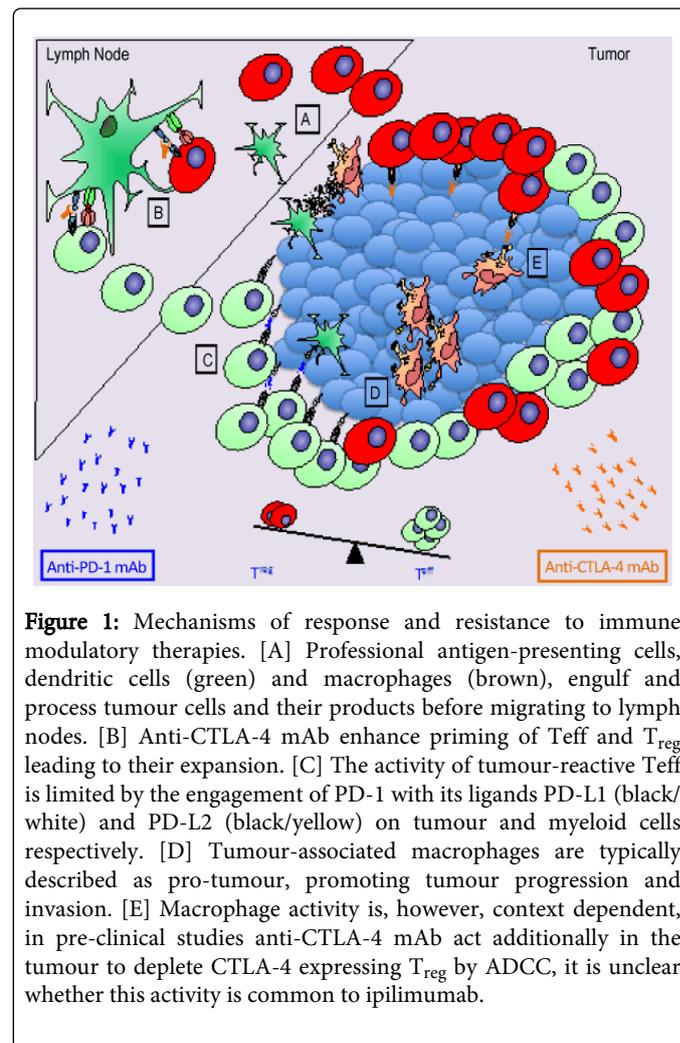


Figure 1: Mechanisms of response and resistance to immune modulatory therapies. [A] Professional antigen-presenting cells, dendritic cells (green) and macrophages (brown), engulf and process tumour cells and their products before migrating to lymph nodes. [B] Anti-CTLA-4 mAb enhance priming of Teff and T_{reg} leading to their expansion. [C] The activity of tumour-reactive Teff is limited by the engagement of PD-1 with its ligands PD-L1 (black/white) and PD-L2 (black/yellow) on tumour and myeloid cells respectively. [D] Tumour-associated macrophages are typically described as pro-tumour, promoting tumour progression and invasion. [E] Macrophage activity is, however, context dependent, in pre-clinical studies anti-CTLA-4 mAb act additionally in the tumour to deplete CTLA-4 expressing T_{reg} by ADCC, it is unclear whether this activity is common to ipilimumab.

Beyond tumour cell PD-L1 expression

In a series of recent publications, tumour-infiltrating immune cell rather than tumour PD-L1 expression was associated with clinical response to the anti-PD-L1 therapy MPDL3280A in patients with NSCLC and bladder cancer [83,90]. Responses were associated with baseline tumour gene expression of IFN γ , Granzyme-A, CD8 and EOMES, indicative of a Th1 type immune response. Increased CTLA-4 expression and the absence of chemokine CX3C motif ligand 1 (CX3CL1) expression was also seen in baseline tumour specimens of responding patients. Simultaneously, a study of pembrolizumab therapy in metastatic melanoma demonstrated higher numbers of CD8⁺PD-1⁺ and PD-L1⁺ positive cells within the tumour and at the tumour invasive margin in baseline tumour specimens obtained from responding patients. A predictive model of response to pembrolizumab therapy was developed based on CD8⁺ T cell

expression at the tumour invasive margin and was subsequently validated in a separate cohort of patients [84].

Collectively, these findings demonstrate the importance of acknowledging the interaction between multiple cellular subsets within the immune tumour microenvironment and the potential implications of these in dictating outcome. PD-L1 expression is likely only to form part of a predictive model or ‘immunoscore’ necessary for selecting patients expected to respond to anti-PD-1/PD-L1 therapy. In addition to PD-L1 expression, the geographical location and densities of various immune cell subsets appears to have predictive value [84,90]. Furthermore, the clinical significance of the relative expression of PD-L1 on tumour cells, myeloid cells and activated tumour-infiltrating lymphocytes is yet to be determined (Table 1).

| Anti-CTLA-4 therapies |
|---|
| Shift in intra-tumoural CD8/T _{reg} ratio [34] |
| Increase in circulating ICOS ⁺ CD4 ⁺ effector T cells [41] |
| Increase in Granzyme B ⁺ CD8 ⁺ T cells [42] |
| Rise in absolute lymphocyte count [48,49] |
| Baseline FoxP3 and IDO expression [44] |
| Baseline ‘immune active’ tumour microenvironment [66] |
| CTLA-4 ⁺ CD4 ⁺ effector T cells [49] |
| EOMES ⁺ CD8 ⁺ T cells [50] |
| Baseline circulating monocytic MDSCs [51] |
| Reduction in monocytic MDSCs in periphery and tumour [52] |
| Baseline serum VEGF [58] |
| Baseline serum LDH [53] |
| Maintenance of high frequency T cell clones in periphery [68] |
| Neo-antigenic repertoire [71] |
| Anti-PD-1/PD-L1 therapies |
| Tumour PD-L1 expression [3,6,8,12,13,84-86] |
| Tumour infiltrating immune cell PD-L1 expression [83] |
| Increased tumoural IFN γ , Granzyme-A, CD8, EOMES and CX3CL1 gene expression [90] |
| Increased CD8 ⁺ PD-1 ⁺ PD-L1 ⁺ cell density within tumour and at invasive tumour margin [84] |
| Increased clonality and reduced diversity of intra-tumoural T cell repertoire [84] |
| Higher somatic mutational burden and neo-antigenic repertoire [92] |

Table 1: Candidate biomarkers of response to immune modulatory therapy.

Genetic determinants of response to anti-PD-1 therapy

In a study of pembrolizumab therapy in patients with advanced melanoma, baseline tumour specimens from responding patients were found to have a more clonal and less diverse T cell repertoire [84]. Furthermore, a ten-fold increase in the number of expanded clones

following pembrolizumab therapy was seen in responding patients compared to those with disease progression [84]. The molecular determinants of relevant clonal T cell responses have, however, remained unclear until recently. Higher responses to anti-PD-1/PD-L1 therapy are typically seen in tumours associated with a high burden of somatic mutations such as melanoma, NSCLC and bladder cancer, highlighting the potential importance of the genomic landscape in predicting response [91]. Given the identification of a neo-antigenic repertoire, unique to responding patients undergoing CTLA-4 blockade, the same group studied patients with NSCLC undergoing pembrolizumab therapy [71,92]. Whole exome sequencing demonstrated a significant positive correlation between the level of somatic, non-synonymous mutational burden and improved response and progression-free survival [92]. The clinical efficacy of pembrolizumab was found to correlate with a higher burden of the identified neo-antigenic repertoire and a molecular signature characteristic of smoking-related mutagenesis. In a single patient with an observed rapid response to pembrolizumab, a CD8⁺ T cell response against a neoantigen resulting from a HERC1 P3278S mutation was observed in peripheral blood. This was only detectable following commencement of therapy (0.005%), three weeks post initiation of therapy the magnitude of response was 0.040% of CD8⁺T cells and this was maintained at day [44]. This study represents another key step in deciphering the mechanisms underlying response to immune modulatory therapies, furthermore, the observation that neo-antigen-reactive T cells could be detected in peripheral blood raises the possibility of blood-based/non-invasive sampling methods of monitoring response to therapy.

Conclusion

The identification of biomarkers of response to immune modulatory therapies is an area of high scientific and clinical priority. While many studies have failed to conclusively identify predictive biomarkers, the insights provided have and continue to be instrumental in advancing understanding. Undoubtedly, the recent demonstration of a specific neo-antigenic repertoire underlying response to CTLA-4 and PD-1 blockade represents a major step forward. Predictably, these studies raise a number of further questions and therapeutic challenges. The observation that a high burden of somatic mutations might in fact be used for therapeutic gain with immune modulation is transforming approaches within the field. The prospect of sequencing tumours on an individual patient basis, developing truly bespoke cellular therapies and optimising activity with appropriate immune modulation now appears both attractive and achievable. The success of immune modulation is the result of sound basic science brought to the clinic in an intelligent manner. Pre-clinical and clinical studies have served to inform each other, expanding understanding of the mechanisms underlying response and resistance. The challenge now is to exploit this understanding, translating it in durable benefit for the majority rather than a selected few.

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