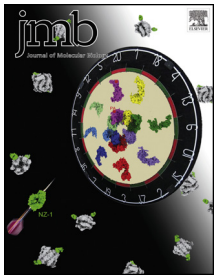

Keeping Tumors in Check: A Mechanistic Review of Clinical Response and Resistance to Immune Checkpoint Blockade in Cancer

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Abstract

Immune checkpoints are a diverse set of inhibitory signals to the immune system that play a functional role in adaptive immune response and self-tolerance. Dysregulation of these pathways is a vital mechanism in the avoidance of immune destruction by tumor cells. Immune checkpoint blockade (ICB) refers to targeted strategies to disrupt the tumor co-opted immune suppression to enhance anti-tumor immunity. Cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and programmed cell death 1 (PD-1) are two immune checkpoints that have the widest range of antibody-based therapies. These therapies have gone from promising approaches to Food and Drug Administration-approved first- and second-line agents for a number of immunogenic cancers. The burgeoning investigations of ICB efficacy in blood and solid cancers have underscored the importance of identifying the predictors of response and resistance to ICB. Identification of response correlates is made complicated by the observations of mixed reactions, or different responses in multiple lesions from the same patient, and delayed responses that can occur over a year after the induction therapy. Factors that can influence response and resistance in ICB can illuminate underlying molecular mechanisms of immune activation and suppression. These same response predictors can guide the identification of patients who would benefit from ICB, reduce off-target immune-relate adverse events, and facilitate the use of combinatorial therapies to increase efficacy. Here we review the underlying principles of immune checkpoint therapy and results of single-agent ICB clinical trials, and summarize the predictors of response and resistance.

Introduction

T-cell activation is a multilayered process involving cell-to-cell communication between antigen-presenting cells (APCs) and T lymphocytes (Fig. 1A). This model of T-cell activation is referred to as the two-signal model with a tertiary signal directing the type of immune activation. The initial signal comes from interactions between the T-cell receptor (TCR) on naïve CD4 or CD8 T cells and processed antigenic peptides via the major histocompatibility complex (MHC) on APCs [1]. Subsequently, the intracellular domains of the TCR are phosphorylated by Lck, leading to the recruitment and activation of Zap70, the major relay of TCR activation [2,3]. A network of anti-apoptotic and proliferative signals downstream of Zap70 provide the primary signal for activation of T cells [4–6]. The second signal, or co-stimulatory signal, comes via interaction between co-stimulatory receptors on T cells with their ligands on APCs. The most prominent co-stimulatory signal is the interaction of CD28 on the T cell with CD80 (B7-1) or CD86 (B7-2). This interaction leads to increased PI3K-mediated proliferative signaling and cytokine production [7,8]. A

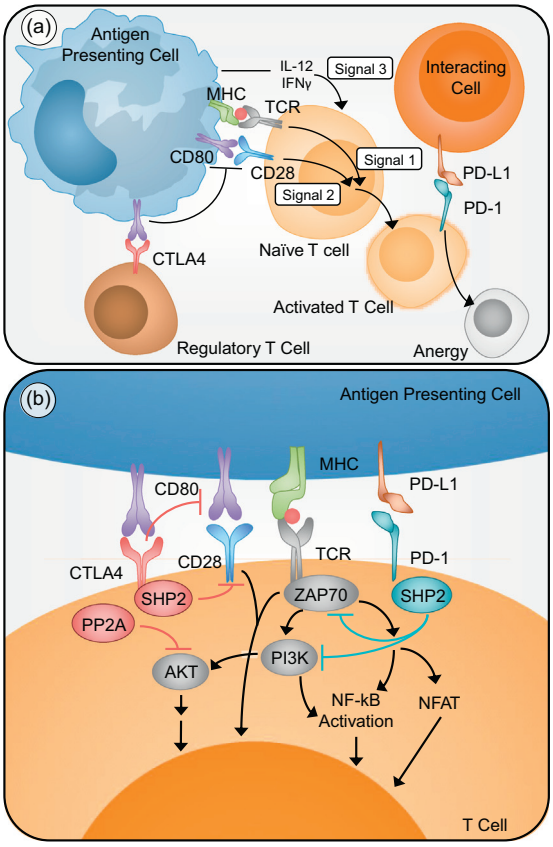

Fig. 1. T-cell activation and the role of immune checkpoints. (A) General schema of T-cell activation (see text for additional details). Signal 1 is provided by the TCR binding to the antigen presented on MHC. Signal 2 is the costimulation of the T cell by the interaction of CD28 on the T cell with CD80 or CD86. Cytokines act as a Signal 3 that directs the T-cell differentiation. (B) Mechanistic summary of TCR activation and points of CTLA-4-mediated (red) and PD-1-mediated (blue) inhibition.

tertiary level of signaling is provided by cytokines, directing lineage-specific expression of transcription factors and shaping the type of immune response [9]. The activation of T cells is balanced by co-inhibitory receptors on T cells, often upregulated upon TCR activation, which counterbalances co-stimulatory signals [10]. These co-inhibitory signals are referred to as immune checkpoints. Together, the signals from MHC-TCR, CD28-CD80/86, and cytokines determine the magnitude and effectiveness of T-cell responses to infection or cancer.

Immune evasion is recognized as one of the hallmarks of cancer [11], and therapies that elicit an anti-tumor immune response, either by directly stimulating an immunogenic response or by targeting inhibitory pathways, have long been sought after. Tumors can function to subvert adaptive immune activation by decreasing expression of MHC-I molecules to prevent the activating signal or through directing the T-cell differentiation or activity [12–15]. The latter is a diverse method involving the interaction of cells in the tumor microenvironment and can function by increasing infiltration of immunosuppressive regulatory T cells (Tregs), myeloid-derived suppressor cells, decreasing antigen presentation, the secretion of suppressive cytokines/chemokines, or the overexpression of ligands for immune checkpoint blockades (ICBs), like programmed death-ligand 1 (PD-L1) [14–16]. In this review, we will discuss the roles of immune checkpoints, cytotoxic T-lymphocyte-associated protein-4 (CTLA-4) and programmed cell death 1 (PD-1), in T-cell activation and immunotherapy. We summarize the clinical trial results for anti-CTLA-4, anti-PD-1, and anti-PD-L1 antibodies as monotherapies in cancer. The last portion will focus on the discussion of the markers for response and resistance to ICB.

Major targets of checkpoint blockade therapy

CTLA-4

CTLA-4 is a member of the CD28 family and is expressed exclusively by T lymphocytes. Upon T-cell activation, CTLA-4 within intracellular granules is translocated to the plasma membrane [17]. This fast translocation allows for CTLA-4-mediated regulation of the amplitude of T-cell response by regulating T-cell activation and priming. CTLA-4 binds to CD80 or CD86 costimulatory molecules, acting as a competitive antagonist with CD28 [18]. In addition, CTLA-4 works through protein tyrosine phosphatase 11 and 6 (also known as SHP-1/2) to dampen TCR signal [18]. CTLA-4 plays a role in one arm of peripheral tolerance, an immunological process to prevent self-reactive immune responses, by dampening T effector cell function and increasing immunosuppressive Treg activity [19]. Unlike effector cells, Tregs express CTLA-4 constitutively and acts as a major mechanism of suppression by Tregs [20]. CTLA-4 on Tregs competes with CD28 on effector T cells for binding with CD80/86 on APCs, thus suppressing T-cell activation. The higher level of CTLA-4 on Tregs also serves to preferentially deplete Tregs in tumors treated with anti-CTLA-4 therapies [21]. Increasing the binding affinity the Fc portion of the antibody enhances this depletion and response of tumors to anti-CTLA-4 therapy [22]. Due to the earlier inhibition mediated by CTLA-4, the blockade of CTLA-4 leads to non-specific immune cell activation and is associated with increased treatment-related adverse events (TRAEs) [23]. This nonspecific immune activation is underscored by the early death of *Ctla4*-null mice due to widespread lymphoproliferative disease within the peripheral T-cell compartment [24,25]. In humans, disrupted expression and localization of

CTLA-4 has been linked with type 1 diabetes, rheumatoid arthritis, systemic lupus erythematosus, and collagen-induced arthritis [26,27].

PD-1/PD-L1

PD-1, another member of the CD28 family, acts as a negative regulator of immune response preferentially in peripheral tissues through the interaction with PD-L1 or PD-L2. Part of a negative feedback loop after immune activation, PD-1 and PD-L1/2 plays a role in the maintenance of peripheral tolerance, letdown following immune activation and chronic infections. As a part of peripheral immune suppression, PD-1 is expressed on lymphocytes, monocytes, natural killer (NK) cells, and dendritic cells [28]. Importantly, PD-1 is absent on resting or naïve T cells and is transiently upregulated during the activation process. This upregulation is analogous to the development of T cells in the thymus, with increased PD-1 required for positive and negative selection of immature T cells following TCR activation [29]. Upon activation T cells upregulate the transcription of *PDCD1*, the gene locus for PD-1. The transcriptional regulation of PD-1 is different from CTLA-4, which localizes to the plasma membrane quickly after TCR signaling [17]. PD-L1 is broadly expressed, and the protein is easily to be induced by many cytokines, particularly type 1 and type 2 interferons [30–33]. In contrast to PD-L1, the second ligand, PD-L2, is exclusively expressed on APCs [32,34,35].

PD-1 suppression acts downstream of TCR activation through the stimulation of protein tyrosine phosphatases SHP1/2. In turn, SHP1/2 dephosphorylates ZAP70 and PI3K, the major targets of the costimulatory CD28 [36,37] (Fig. 1B). In addition, there are reports suggesting that PD-L1 and transforming-growth factor β (TGFβ) can convert naïve CD4 T cells into induced Tregs [38,39], which potentially provides a long-term immune suppression by PD-1/PD-L1 pathway [38,39]. The loss of PD-1 suppression is associated with antibody-mediated autoimmunity, like glomerulonephritis, dilated cardiomyopathy, and lupus-like autoimmunity [40–42]. Underscoring the importance of PD-1 in peripheral tolerance, single-nucleotide polymorphisms in PD-1 have also been associated with a wide range of autoimmune conditions, including systemic lupus erythematosus, type I diabetes, multiple scleroses, rheumatism, Graves' disease, and ankylosing spondylitis [43]. This link to antibody-related autoimmunity is likely a direct result of PD-1-mediated suppression of B cell activation [43].

Sustained expression of PD-1 on T cells is a marker for T-cell exhaustion [44]. Exhausted T cells arise from activated effector T cells that gradually become silenced due to persistent exposure to antigen [45]. Exhausted T cells become progressively dysfunctional due to increased expression of several inhibitory receptors, including PD-1 and CTLA-4, a gradual loss of effector cytokine secretion, and altered cellular metabolism [45,46]. T-cell exhaustion, which evolved as a mechanism to promote peripheral tolerance and prevent autoimmunity, is often co-opted by viruses during chronic infection and cancers as a mechanism of immune evasion [47,48] Effector T cells in the tumor microenvironment become exhausted due to constant exposure to tumor antigens and upregulation of PD-L1 on cancer cells and myeloid cells by oncogenic signaling and inflammatory cytokines [48–50]. It is thought that anti-PD-1 and PD-L1 therapies work in part by reversing or preventing T-cell exhaustion.

Other immune checkpoints under investigation

Outside of PD-1 and CTLA-4, there are a number of immune stimulatory and suppressive checkpoints currently under investigation as targets for cancer immunotherapies [23]. Similar to PD-1 and CTLA-4, other inhibitory checkpoints, LAG-3, TIM-3, BTLA4, VISTA, or TIGIT, can dampen the anti-tumor immune response by regulating T-cell activity [51,52]. Like PD-1, TIM-3, LAG-3, and BTLA are expressed on T cells subsequent to T-cell activation and have been implicated as markers of T-cell exhaustion in tumors [52–56]. TIM-3 also appears to shift the immune responses by negatively regulating Th1 CD4 T cells and cytotoxic CD8 T cells [57]. In contrast, VISTA is expressed by both APCs and T cells, with high expression on the suppressive myeloid-derived suppressor cells and Tregs, functioning in both myeloid cell activation and Treg function [58]. TIGIT is expressed by Tregs, T cells, and NK cells and bind poliovirus receptor on tumor cells or APCs. TIGIT appears to have direct and indirect suppressive effects, by acting as a competitive antagonist to CD226 binding by NK and T cells, but also leading to the recruitment of SHP1/2 and downstream inhibition of AKT signaling in T cells [59–62]. Furthermore, binding of poliovirus receptor to TIGIT on APCs and Tregs increases suppressive activity and release of inhibitory cytokines [60,63].

In contrast, immunostimulatory checkpoints, like ICOS, OX40, or CD40L, assist in the activation and maintenance of effector T cells. As a member of the CD28 family, ICOS binds B7H/B7RP-1 and can function in providing the second signal in immune activation [64]. Likewise, OX40 can act as a costimulatory signal, but the expression is induced upon T-cell activation and leads to the expression of anti-apoptotic factors BCL-2 and BCL-XL, which sustain the proliferative response in T cells [65,66]. OX40 is also constitutively expressed on Tregs and binding of the receptor to OX40L decreases Treg function [67]. The interaction of CD40 (on APCs) and CD40L on activated T cells promotes a proinflammatory immune responses via the induction of NF-κB signaling [68]. Unlike the inhibitory checkpoints where antibodies are used to physically obstruct interaction by the respective ligand,

stimulatory checkpoints can be targeted by ligand-expressing viral particles, recombinant ligand peptides, or agonistic monoclonal antibodies. As these emerging immune checkpoints are further developed, the wide range of mechanisms and targeting strategies will broaden efficacy of immunotherapies for patients by allowing physicians to better select therapies for individual tumors.

Results of using checkpoint blockade as monotherapies in cancer

CTLA-4 and PD-1/PD-L1 are the two most widely studied immune checkpoint pathways with monoclonal antibodies that target these two pathways having been approved by Food and Drug Administration (FDA) for cancer therapy. The success of these agents has spurred on development of other antibody-based or immunostimulatory drugs to be used as monotherapies or in combinatorial regimes. To date, there has been no comprehensive summary of single-agent ICB therapies. Analysis of these results and factors identified by these trials is the first step in understanding the patterns of response and resistance in patients receiving ICB. We performed a comprehensive summary of results for trials with at least one arm comparing monotherapy of anti-PD-1 (pembrolizumab, nivolumab), anti-CTLA-4 (ipilimumab), or anti-PD-L1 (atezolizumab). These four antibodies were selected based on trial results in multiple cancer types and accounted for 8069 patients across 43 clinical trials (Table 1). Inclusion criteria for summarized trials are the enrollment of clinical trial into ClinicalTrials.gov database and the measurement of objective response using clinical or radiographic data.

Based on the results of these trials, major predictors of response to ICB are the therapeutic targets and the tumor types. In general, agents targeting PD-1 have higher rates of response with lower toxicity, compared to anti-CTLA-4 antibody, ipilimumab. Evaluation of both the treatment efficacy and TRAEs of anti-CTLA-4 *versus* anti-PD-1 is limited by the broader use of ipilimumab in combination with other agents that are not included in our summary. Restricting to the 10 single-agent trials in melanoma, objective responses were 9.8%–19.1% compared to 24.1%–43.7% for patients on anti-PD-1 therapies [69–74]. Previous work has reported increased TRAEs with anti-CTLA-4 compared to anti-PD-1 therapy, which is seen in comparing 10 trials of melanoma with grade 3 or above TRAE for ipilimumab ranging from 19.6%–27% compared to 5.9%–16% for anti-PD-1 therapies [23]. Several additional complications exist comparing multiple trial results across single tumor types. For example, the high variability in objective responses to pembrolizumab (18.9%–44.8%) and nivolumab

Table 1. Comprehensive summary of clinical trials using pembrolizumab, nivolumab, atezolizumab, and ipilimumab as single-agent therapies

Cancer type	Agent	Target	Total	Objective response	TRAE 3+	Total 3+ AE	Citations
Melanoma	Pembrolizumab	PD-1	1179	24.1%–33.4%	5.9%–14%	19%–34%	[69–71]
	Nivolumab	PD-1	620	27.6%–43.7%	11.7%–16%	34%–43.5%	[72–74]
	Ipilimumab	CTLA-4	723	9.8%–19.05%	19.6%–27%	33.2%–55.6%	[69,72,75–78]
NSCLC	Pembrolizumab	PD-1	977	18.9%–44.8%	9.49%–26.6%	Not reported	[79–82]
	Nivolumab	PD-1	747	12.8%–26.1%	10%–18%	30.7%–46%	[74,83–85]
	Atezolizumab	PD-L1	566	13.6%–17%	12%–14.8%	44.4%–45%	[86,87]
UC	Atezolizumab	PD-L1	675	13.4%–26.3%	7%–16%	12.6%–54.4%	[88–90]
	Pembrolizumab	PD-1	266	21.1%	15%	52%	[91]
	Nivolumab	PD-1	256	19.6%	18	Not reported	[92]
RCC	Nivolumab	PD-1	607	20.8%–27.27%	11.3%–18.7%	Not reported	[74,93,94]
HL	Pembrolizumab	PD-1	241	65%–69%[a]	6.7%–100%	21.4%	[95,96]
	Nivolumab	PD-1	103	66%–87%[a]	31.3%–43.5%	76.3%–100%	[97,98]
Gastric Carcinoma	Nivolumab	PD-1	268	11.2%	10%	41.5%	[99]
HNSC	Pembrolizumab	PD-1	236	13.3%	13.1%	39.4%	[100]
HCC	Nivolumab	PD-1	214	19.6%	25%	60.4%	[101]
Sarcoma	Pembrolizumab	PD-1	80	5%–18%	7.1%–11.9%	54.8%–59.5%	[102]
	Nivolumab	PD-1	38	5.3%	28.6%	Not reported	[103]
MSI-H Tumors	Pembrolizumab	PD-1	81	25%–80%	20.24%	rported	[104]
TCA	Pembrolizumab	PD-1	40	22.5%	Not reported	77.5%	[105]
Anal Carcinoma	Nivolumab	PD-1	35	24%	16%	Not reported	[106]
Breast	Pembrolizumab	PD-1	27	18.5	15.6	Not reported	[107]
Merkel Cell Carcinoma	Pembrolizumab	PD-1	26	56%	15%	42.3%	[108]
CLL	Pembrolizumab	PD-1	25	12%	60%	Not reported	[109]
Hematological malignancies	Ipilimumab	CTLA-4	22	31.8%[a]	Not reported	86.4%	[110]
PMBCL	Pembrolizumab	PD-1	17	41%	11%	Not reported	[111]

TRAE 3+, grade 3 or above treatment-related adverse event; AE 3+, total grade 3 or above adverse event; NSCLC, non-small cell lung carcinoma; RCC, renal cell carcinoma; UC, urothelial carcinoma; HL, Hodgkin's lymphoma; HCC, hepatocellular carcinoma; PMBCL, primary mediastinal B-cell Lymphoma; HNSC, head and neck squamous cell carcinoma; CLL, chronic lymphocytic leukemia; TCA, thyroid carcinoma.

[a] Utilized different response criteria between studies.

(12.8%–26.1%) in non-small cell lung carcinoma (NSCLC) is likely partially a result of different inclusion criteria for patients based on PD-L1 immunohistochemical staining discussed later in this review [74,79–85]. Similarly, most of the trials use the criterion for objective response based on the reduction in tumor size greater than 30% as defined by the RECIST criteria [112]. Notably, each of the clinical trials in Hodgkin's lymphoma utilized different criteria to measure objective response [95–98]. Likewise, the definition of TRAE varies widely by tumor type with researchers reporting all TRAEs or only TRAEs that affect a certain percentage of the ICB-treated patients.

Tumor type is a predictive correlate of response with higher responses for both anti-PD-1 and anti-CTLA-4 agents in immunogenic tumors and lymphomas. Of note, in advanced, refractory Hodgkin's lymphoma responses range between 65% and 87% across both pembrolizumab and nivolumab [95–98]. High response rates were also seen by tumors with high microsatellite instability (MSI-H, 25%–80%, pembrolizumab) [104], Merkel Cell carcinoma (56%, pembrolizumab) [108], and primary mediastinal B-cell lymphoma (41%, pembrolizumab) [111]. Although beyond the scope of the inclusion criteria for the table, early phase trials included other solid tumors that did not demonstrate response to ICB, notably pancreatic cancer [113], castration-resistant prostate cancer [74], colorectal cancer [74], gastric cancer [114], and breast cancer [114].

Predictors of resistance and response for ICB therapy

The diverse clinical response of patients across multiple tumor types to ICB highlights the need to understand mechanisms that drive response and resistance. A variety of factors that influence immune response are directly or indirectly influenced by the tumor, providing permissive conditions for tumor growth and progression. For example, chronic inflammation can drive tumor growth by providing growth factors and preventing innate or adaptive anti-tumor immune responses [115,116]. In turn, the tumor can produce chemotactic agents and other factors that maintain the inflammation in a positive feedback loop [115,116]. The inflammation in cancer is influenced by systemic factors, like age, adiposity, genetics, or the gut microbiome. Like the factors that impact chronic inflammation and cancer, predictors of response to ICB can be divided into three categories by location: tumor cell intrinsic, tumor microenvironment, and systemic factors. Despite the tidy organization, many of the predictors of response interact at multiple levels and ultimately translate to modulating total infiltration and function of immune cells in the tumor before or after ICB therapy.

Tumor-intrinsic factors that predict response to ICB

Whole-exome sequencing has revealed the mutational load of tumors as a major predictor of ICB response [117–121]. Underlying these observations is the hypothesis that somatic mutations lead to tumor-specific neoantigens that can overcome self-tolerance (Fig. 2A); however, high mutational load is not necessary for ICB response. Notably, clear cell renal cell carcinoma (ccRCC) have greater than 20% objective response rates to anti-PD-1 therapy (Table 1) and a relatively low mutational load of 1 mutation/megabase, compared to the 10 to 400 mutations/megabase in melanoma or NSCLC [74,93,94,122]. Mutator phenotypes, like those with familial Lynch syndrome or other mismatch repair-deficient tumors, have objective response rates ranging from 25% to 80% for anti-PD-1 therapies [119]. This high response rate led to the accelerated approval in May of 2017 of pembrolizumab for unresectable or metastatic mismatch repair-deficient or MSI-H cancers regardless of their tissues of origin, a first such approval from the FDA that is based on molecular trait rather than cancer type. In NSCLC, increased C-to-A nucleotide transversions and decreased C-to-T transversions were found to be indicative of smoking status and may serve as a marker of response to ICB [120]. Like Lynch syndrome with mutations in DNA repair genes, most commonly *MLH1* and *MSH2*, mutations in *BRCA2*, *POLD1*, and *POLE* have also been identified in association with response to anti-PD-1 therapies [120,123]. BRCA2 is an adaptor for homologous recombination, while *POLD1* and *POLE* code for subunits of DNA polymerases that synthesize and repair DNA. Mutations in these three genes may also increase total mutational load in tumors. In a similar vein, a current open-label phase II clinical trial involving breast cancer patients with *BRCA1/2* germline mutations (NCT0302503) is being conducted and will assess if mutator phenotypes are a predictor of response outside of immunogenic tumors. Preclinical transgenic murine models of *BRCA1*-deficient breast tumors demonstrated that a combinatorial therapy of anti-CTLA-4 and anti-PD-1 with the DNA crosslinking agent, cisplatin, enhanced survival [124]. Unlike mutations, chromosomal copy number alterations and aneuploidy has been reported to be inversely associated with response to ICB due to the possible loss of immune mediators [125,126].

In the context of tumor-specific mutations, both quantity and quality play a role. Evaluating neoantigens on the basis of likelihood of presentation and T-cell recognition, termed neoantigen fitness, has been found to predict increased immunotherapy response [127]. Parsing neoantigen fitness is a basis for investigations into personalized neoantigen-based vaccines [127–131]. In combination with anti-PD-1 therapies, personalized-vaccine priming appears to work

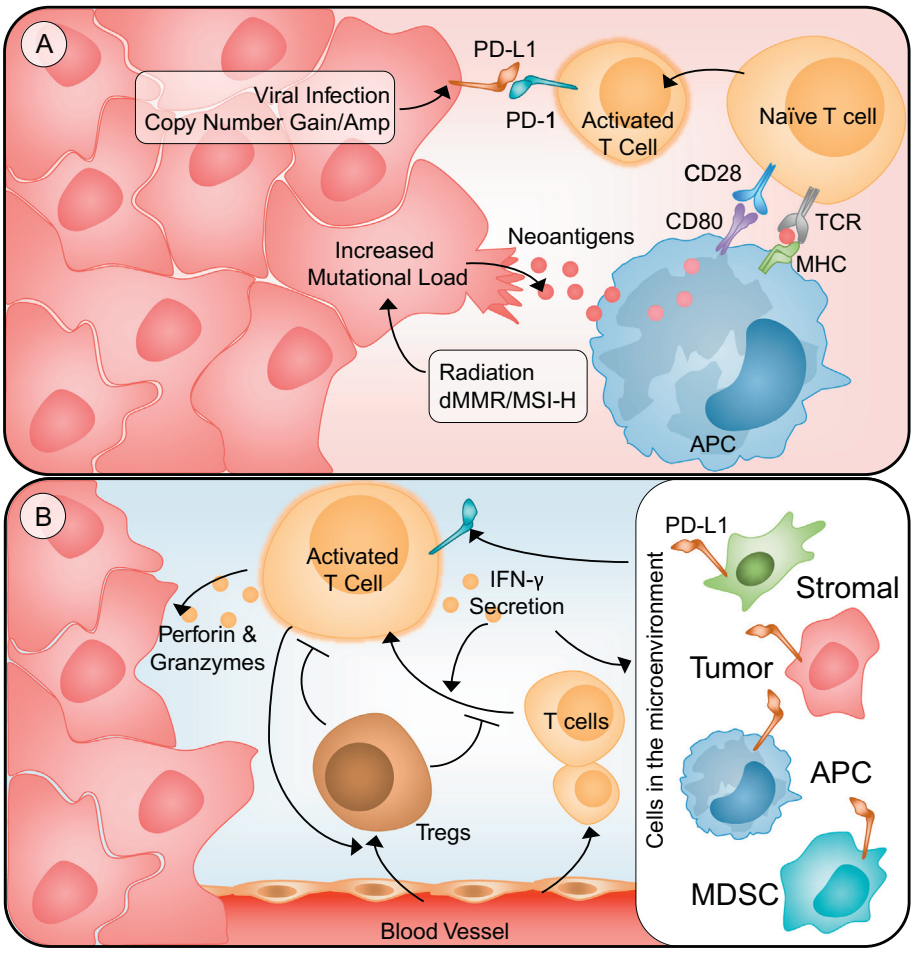

Fig. 2. Factors that influence response of tumors to immune checkpoint blockade. (A) Tumor intrinsic markers of response to therapy, focusing on increased mutation and expression of PD-L1 by tumor cells. (B) Tumor microenvironmental factors that influence response. Increased activated T cells lead to IFNγ that drives PD-L1 expression on other cells. Increased Tregs can suppress anti-tumor immune response.

synergistically in promoting antitumor immune response [128,131]. Anti-PD-1 therapy has been reported to contract the mutational load and cancer cell clonality in complete and partial responders; whereas nonresponders tend to have persistent mutational load and cancer cell clonality [132]. Beyond DNA repair-related genes, mutations in *SERPINB3* and *SERPINB4* mutations, a class of peptidase inhibitors, have been reported in responders to anti-CTLA-4 and anti-PD-1 [132,133]. Recent data from anti-PD-1 therapy in ccRCC found loss-of-function mutations in *PBRM1*, a chromatin remodeling subunit, to be associated with response and better overall survival [122]. Conversely, mutations in *JAK1* and *JAK2* kinases, major relays in interferon γ (IFN-γ) signaling in melanoma and epidermal growth factor receptor (*EGFR*) in NSCLC have been identified in progressors on ICB therapy [134,135].

In the context of anti-PD-1 therapies, PD-L1 immunohistochemical staining and gene expression in tumor cells have been proposed to predict response to PD-1 therapy in melanoma, NSCLC, renal cell carcinoma (RCC), and Hodgkin's lymphoma [85,97,136–142] (Fig. 2A). This corresponds to the proposed mechanisms of anti-PD-1 therapies, in which cancers with high levels of PD-L1 expression are suppressing anti-tumor immunity and the blockade overcomes the PD-1/PD-L1-mediated suppression. The use of PD-L1 staining in NSCLC using a cutoff of 5%–10% positive cells has mixed benefit [83,85,143,144] Using a higher cutoff of 50% PD-L1-positive cells in the Keynote-001 study of pembrolizumab in NSCLC, PD-L1^{+} patients had an objective response rate of 45% compared to 19.4% across all patients [81]. This predictive finding led to FDA approval of PD-L1 immunohistology as a companion diagnostic for pembrolizumab in 2015. Within melanoma, the staining for PD-L1 in greater than 5% of cells is more consistently associated with increased objective responses to anti-PD-1 therapy in a meta-analysis of 11 melanoma trials [144].

Interestingly, PD-L1 expression is not necessarily associated with regional or total lymphocytic infiltration [136]. These collective disparities may be a result of a greater association of immune infiltration with tumors containing PD-L1$^+$ immune cells compared to PD-L1$^+$ tumor cells alone [145].

Immune cells may drive PD-L1 expression on tumor cells through interferon signaling [30–33]. In melanoma, IFN-γ-mediated PD-L1 expression requires downstream activation of JAK/STAT, highlighting the role of previously mentioned mutations in *JAK1/JAK2* in resistance to anti-PD-1 therapies [32,134]. Immunogenic etiologies of increased PD-L1 expression have been reported in Merkel cell carcinoma with infection by Merkel cell polyomavirus or induced by the combinatorial ICB and oncolytic virus therapy [108,146]. Despite an association of Hodgkin's lymphoma with the Epstein–Barr virus, no association between infected patients and uninfected patients and PD-L1 expression has been reported [97]. Regardless of infection status, the Reed–Sternberg tumor cells of Hodgkin's lymphoma contain copy number gain or amplification of -L1 and PD-L2 genetic loci, *CD27*4 and *PDCD1LG* [97,147]. This may contribute to the up to 87% objective response rates reported in Hodgkin's lymphoma [97]. Interestingly, elevated PD-L1 expression on melanoma cells has been associated with poor response to combinatorial anti-CTLA-4 and radiation therapy, supporting the complementary, yet distinct roles of CTLA-4 and PD-1 in immune regulation [148].

Microenvironmental factors that predict response to ICB

A product of the immune-tumor microenvironment, immune response gene expression patterns have been linked to improved responses in both anti-PD-1 and anti-CTLA-4 therapies (Fig. 2B). Common among both of these therapies is activated CD8 T-cell signature and IFN-γ signaling. Principally, increased response has been associated with elevated expression of *CD8A*, *IFNG*, *PRF1*, *GZMA*, and *GZMB* [118,134,137,141,149]. The interface of tumor cells and immune cells plays a role in the activation of immune cells with mutations or copy number loses in both type 1 and type 2 interferons associated with decrease response or resistance to ICB [134,149]. Interestingly, immunosuppressive pathways, like IL-10, have had mixed observations, with *IL10* expression reported lower in responders before the induction of pembrolizumab [123]. Conversely, IL-10 is increased in PD-L1$^+$ melanomas [137] and copy number loss in the α subunit of the IL-10 receptor is has been seen in anti-CTLA-4 nonresponders [149].

Gene expression signatures are indirect measures of immune cell infiltration and activation in the tumor microenvironment. A major focus of prognostic forecasting for ICB response has been increased T-cell presence at the invasive margin or intratumorally [126,140,150]. More specifically, an increase in activated CD8 T cells and a decrease in immunosuppressive Tregs have been reported in responders to ICB (Fig. 2B) [140,150]. A hallmark of Lynch syndrome-associated hereditary nonpolyposis colorectal cancer and MSI-H sporadic colorectal cancer is lymphocytic infiltration, which may partially account for the increased response rates to ICB in these tumors [151,152]. In addition, MSI-H tumors have been associated with increased cytotoxic CD8 T lymphocytes and increased IFN-γ-secreting Th1 CD4 cells compared to microsatellite-stable tumors [153]. Work from Roh *et al.* [126] suggests that not only quantity of lymphocytic infiltration predicts better response, but also expanded TCR clonal populations before the induction of anti-PD-1 therapy are predictive. Similarly, after the induction of anti-PD-1 therapy, clonal expansion of T lymphocytes has been seen exclusively in patients with objective responses [132]. The clonal expansion of T cells, however, does not seem to be associated with the response to anti-CTLA-4 [126].

These works have led to attempts using combinatorial approaches to stimulate or prime the tumor microenvironment to increase T-cell infiltration and activity: notably, investigations into using ICB in combination with vaccines [154], anti-angiogenic molecules [155], oncolytic viruses [146], radiation [148,156,157], suppressors of TGFβ [158], and DNA methylation inhibitor [159]. The use of combinatorial radiation and ICB seems to function at multiple levels, by increasing lymphocytic infiltration, removing suppressive cells, and priming the microenvironment while also increasing mutational load [148,156,157]. Dysregulation following CD8 T-cell infiltration may contribute to resistances to ICB therapies by increasing PD-L1 expression by tumor cells, increasing Treg infiltration, and upregulating the suppressive indoleamine-2,3-dioxygenase (IDO) metabolite within the tumor microenvironment [32,160]. IDO itself may play a role in the resistance to anti-CTLA-4 therapy with clinical trials underway using IDO inhibitors with ICB [161,162].

Despite the focus on CD8 T-cell infiltration, response has been associated with a number of other immune cells. In PD-L1/TGFβ dual inhibition, increased infiltration of CD8 T cells, NK cells, dendritic cells, and M1 macrophages was seen in responders [132,158]. Although M1 macrophages have been observed to be increased with PD-L1/TGFβ dual inhibition, total macrophages may be decreased in responders to ICB monotherapy [132,158]. Macrophage polarization may play a larger role, with M1 polarization associated with PD-L1 expression and M2 polarization associated with PD-L1 and PD-L2 expression [163]. Beyond myeloid cells, PD-L1 staining in tumor cells and immune cells has been significantly associated with the presence of CD20$^+$ B cells and lymphoid aggregates [136]. Interestingly, PD-L1 staining on immune cells and tumor cells may have an additive effect in the percentage of intratumoral PD-1$^+$ CTLA-4$^+$ CD4 and

CD8 T cells [145]. Highlighting the difference in PD-L1 expression by tumor *versus* immune cells, limited evidence indicates that PD-L1 from immune cells is correlated with increased TIM-3$^+$ CD8 T cells in NSCLC [145].

Systemic factors that predict response to ICB

Systemically, there has been a focus on identify biomarkers of therapeutic response to ICB. Several groups have tested the idea of using circulating immune cells as a metric for response. In peripheral blood, increased total CD4 and CD8 lymphocyte count and clonal expansion of specific T lymphocyte have been reported in responders to ICB therapy [164–167]. A more detailed examination of T-cell dynamics in peripheral blood has demonstrated an increase at baseline in IL-4$^+$, GzmB$^+$, IFNγ$^+$, and GM-CSF$^+$ CD4 T cells and CTLA-4$^+$, GzmB$^+$, and IL-13$^+$ CD8 T cells among responders to anti-PD-1 therapy (Fig. 3) [168]. After the start of ICB therapy, responders had an increase in GzmB$^+$ and CTLA-4$^+$ CD8 T cells and PD-1$^+$, IL-4$^+$, IFNγ$^+$, IL-17A$^+$, and GzmB$^+$ CD4 T cells [148,168]. A greater percentage of exhausted PD-1$^+$ EOMES$^+$ CD8 T cells in the peripheral blood was associated with decreased response to anti-CTLA-4 and radiation combinatorial therapy [148]. Potential immune cell markers of response are not exclusive to T lymphocytes, with increases in myeloid populations being reported in responders [166,168]. Specifically, increases CD14$^+$CD16$^-$HLA$^-$DR^{hi} monocytes and eosinophils have been reported in responders after the induction of anti-PD-1 and anti-CTLA-4 therapies, respectively [166,168].

Human leukocyte antigens (HLAs) are a series of genes that encode the MHC that activate and regulate immune response. HLA typing represents a predictive factor at all three levels: expression on tumors cells, expression on immune cells in the microenvironment, and diversity of HLA molecules based on the genetics of an individual (Fig. 3). In a large analysis of patients, diversity of the both MHC-I (HLA-A,B,C), found on most nucleated cells in the body, and MHC-I (HLA-D$_n$), found on APCs, have been associated with increased response to ICB [169]. Homozygosity of HLA-B, HLA-A, HLA-DP, and HLA-DPB alleles was associated with poorer overall survival in patients receiving ICB therapy [169]. Specific alleles, like HLA-B62, was found to increase the risk of death on ICB, while HLA-B44 had significantly better overall survival [169]. Inoue *et al.* [141] found increased level of HLA-A in pre-treatment resections of patients with objective responses to nivolumab. Likewise, in the MHC-II family, HLA-DR IHC staining in melanoma patients receiving anti-PD-1 or anti-PD-L1 was associated with improved therapeutic response and infiltration of CD4/CD8 T cells [170]. Similar to response, TRAE from ICB has been linked with HLA-typing. An early report of anti-CTLA-4 trial data examining HLA-A*0201$^+$ *versus* HLA-A*0201$^-$ patients found no difference in response, but rather a significant elevation of immune-related adverse events in the HLA-A*0201$^+$ individuals [171]. The implication of off-target immune activation based on HLA genotype has also been seen in checkpoint-induced fulminant myocarditis and an association with HLA-DQ7 [172]. The off-target immune activation, although higher in anti-CTLA-4 therapies, is central to the debate of using ICB in patients with a history of autoimmunity or HLA-type predisposition [173].

Another emerging predictor of response to ICB is the gut microbiome (Fig. 3). Use of antibiotics has been associated with a reduction in efficacy of ICB in mice

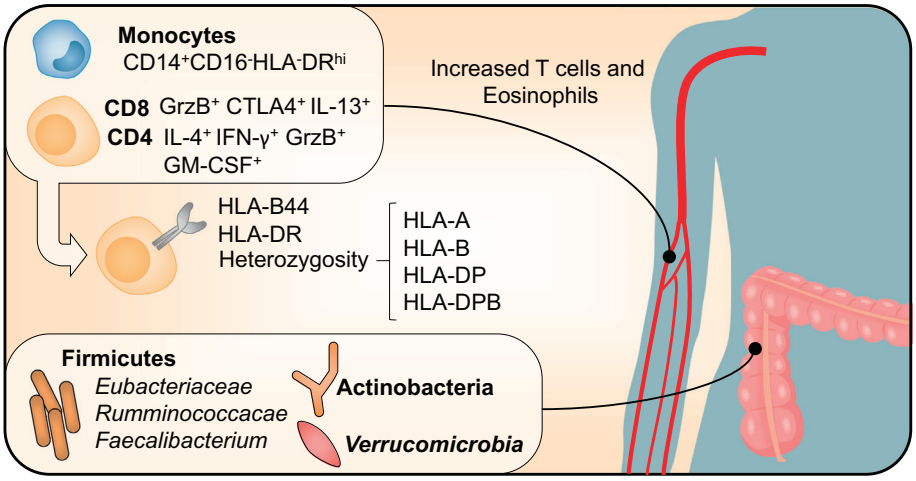

Fig. 3. Systemic factors that influence response to immune checkpoint. Factors can be divided into immune cells in the peripheral blood associated with better responses, HLA genotypes, and gut microbiome. Increased activated CD4 and CD8 T cells in the peripheral blood have been reported in responders. In addition, HLA diversity and HLA-B44/HLA-DR have been associated with better response. Recent reports of gut microbiome influences on ICB have made it an emerging predictive correlate for immunotherapy.

and humans [174,175]. Responders receiving PD-1 blockade have increased species in the *Firmicutes*, *Verrucomicrobia*, and *Actinobacteria* phyla [174, 176,177]. *Firmicutes*, gram-positive endospore-producing bacteria, form a large portion of the gut microbiome diversity. Within the *Firmicutes* phylum, responders have been observed to have elevated *Eubacteriaceae*, *Rumminococcacae*, and *Faecalibacterium* families in the *Clostridiales* order [174,176]. Additional reports on *Bacteriodetes*, a phylum consisting of gram-negative rods, vary and have been seen elevated in both responders and nonresponders of ICB, which may be a product of different collection time points of post- *versus* pre-treatment, respectively [174,176]. Initial preclinical assessment of supplementing *Firmicutes*, *Verrucomicrobia*, or *Bacteriodetes* species increased T-lymphocytic infiltration and response to ICB in murine models [174–176]. Similarly, oral gavage of fecal matter from human responders *versus* nonresponders in murine melanoma tumor models led to differential melanoma growth that recapitulate response to ICB in human patients [174,176–178]. Like HLA typing, the gut microbiome has been associated with TRAE. The use of ICB, more pronounced in anti-CTLA-4 therapy, can lead to colitis with mixed immune cell infiltration consisting of neutrophils, eosinophils, lymphocytes, and plasma cells within the lamina propria. This reactive colitis is also accompanied by elevated humoral responses to enteric flora and decreased in *Bacteriodetes* species [179,180]. Similar observations of immunoglobulin response and decreasing *Bacteriodetes* species have also been reported in inflammatory bowel disease [181]. The underlying mechanistic contribution of the gut microbiome to response to ICB is unknown and may be indirectly contributing to ICB response, modulating the metabolic profile of immune cells, or more directly acting as shared antigens between the tumor cells and gut microflora [182].

Challenges and future direction of immune checkpoint therapies

The development and use of immune checkpoint inhibitors for cancer therapy is an exciting advance in oncology. Central to this excitement is the premise that immune-based therapies offer a robust, long-term response or control in tumors, leading some groups to conclude the potential of curative therapies. Belaying this excitement is the small percentage of patients that respond to anti-CTLA-4 or anti-PD-1/PD-L1 monotherapies in solid tumors (Table 1, 5%–40%). Moreover, there is emerging evidence of adaptive resistance mechanisms to ICB that complement previously described predictors of response.

Similar to the predictors of response to ICB, the mechanisms of adaptive resistance function at multiple levels. Within the tumor, ICB appears to apply a selection pressure to reduce mutational load and prevent immune activation against tumor-specific antigens [132]. The same selection pressure may lead to mutations or copy number alterations in both type 1and type 2 interferons decreasing the efficacy of ICB by limiting immune activation in the tumor microenvironment [134,149]. In turn, characteristic changes in CD8 T cells are associated with resistance after the induction of ICB therapy. Elevation of exhaustion markers on CD8 T cells, like EOMES [148] and TIM-3[183], has been correlated with poorer response to ICB leading to the investigation of the use of combinations of checkpoint inhibitors. Notably the use of anti-PD-1 with anti-CTLA-4 [72,184], anti-TIM-3 [53,185], anti-TIGIT [186,187], and anti-LAG-3 [188] have shown additive/synergistic effects in terms of efficacy.

As challenging as it sounds, clinical trials combining different agents with ICB can be carefully designed to achieve maximal efficacy of ICBs. Many clinical trials with ICB combination, however, proceed with the lack of strong mechanism-based rationale and foreseeably fail as a result of their initial designs. For instance, radiotherapy and many chemotherapeutics, which are most commonly used in neoadjuvant, adjuvant, and/or combinatory settings, often lead to significant lymphodepletion. Simply combining these therapies with ICB may reduce the efficacy of ICB due to the significant loss of lymphocytes. It is thus imperative to determine the doses and duration of radiotherapy if combined with ICB, as well as the starting time for ICB administration. Another important concern is the mechanisms of action that independently promote an anti-tumor microenvironment. For example, a recent paper mentioned a potential combination of radiotherapy, anti-CTLA-4 and anti-PD-1 [148]. This combination seems to maximize the treatment efficacy due to complimentary mechanisms: (1) radiotherapy increases mutations and subsequent the diversity of TCR repertoire; (2) anti-CTLA-4 mainly depletes Tregs; and (3) anti-PD-1 reinvigorates exhausted CD8 effector T cells. Although these mechanisms may not be universal or unique, it provides a rationale for combination of different agents, by targeting multiple properties and maximizing cytotoxic effect of effector CD8 T cells. The safety of radiotherapy and ICB has been reported in prostate cancer, NSCLC, and melanoma [148,189,190], with a number of current clinical trials focusing on efficacy.

The use of chemotherapy with ICB can be efficacious. Such are the examples of the KEYNOTE-021 and KEYNOYE-189 trials, in which the addition of pembrolizumab to platinum- and folate-based chemotherapies increased response and progression-free survival compared to standard of care in NSCLC and led to the FDA-approval of the combination as a first-line therapy [191,192]. Importantly, the trials utilized carboplatin before the induction of ICB, while maintaining patients on pemetrexed and pembrolizumab

indefinitely [191,192]. These results demonstrate the safety and efficacy of ICB and combinatorial chemotherapy in NSCLC. However, the phase III KEYNOTE-189 had a reported objective response rate of 47.6% in the combination group, slightly above the range of previous single-agent anti-PD-1 trials (Table 1) [192]. The question that remains for these combinations is the efficacy of ICB as a single agent *versus* ICB and radio- or chemotherapy, with a paucity of clinical data to make any strong conclusions.

Moving beyond PD-1/PD-L1 and CTLA-4, the clinical applications of the ICB therapy are accelerating. At the time of submission, there are 1664 clinical trials on ClinicalTrials.gov enrolling patients or are actively treating patients using currently approved checkpoint inhibitors with or without combinations of recombinant cytokines, secondary checkpoint blockade, viral vectors, toll-like receptor agonists, radiation, or antibody–drug conjugates. These investigations will reveal new layers of complexity to anti-tumor immune response and eventually translate into better clinical outcomes for patients with cancer. A recent failure by combination of PD-1 inhibitor and IDO inhibitor (ECHO-301 phase III trial from Incyte and Merck) and subsequent cancellation of 12 other IDO-based trials further underscores the urgent and critical need for understanding the complex mechanism of these pathways to better predict if combinations will work and the patients who benefit the most from the combinations [193].

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Abbreviations used:
ICB, immune checkpoint blockade; CTLA-4, cytotoxic T-lymphocyte-associated protein; PD-1, programmed cell death 1; APC, antigen-presenting cells; TCR, T-cell receptor; MHC, major histocompatibility complex; Tregs, regulatory T cells; PD-L1, programmed death-ligand 1; TRAE, treatment-related adverse events; NSCLC, non-small cell lung carcinoma; MSI-H, high microsatellite instability; ccRCC, clear cell renal cell carcinoma; HLA, human leukocyte antigen.

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