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PD-L1 (B7-H1) and PD-1 Pathway Blockade for Cancer Therapy: Mechanisms, Response Biomarkers and Combinations

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Abstract

PD-L1 and PD-1 (PD) pathway blockade is a highly promising therapy and has elicited durable anti-tumor responses and long-term remissions in a subset of patients with a broad spectrum of cancers. How to improve, widen, and predict the clinical response to anti-PD therapy is a central theme in the field of cancer immunology and immunotherapy. Oncologic, immunologic, genetic and biological studies focused on the human cancer microenvironment have yielded significant insight into this issue. In this Review, we focus on tumor microenvironment; evaluate several potential therapeutic response markers including the PD-L1 and PD-1 expression pattern, genetic mutations within cancer cells and neoantigens, cancer epigenetics and effector T cell landscape, microbiota, and their mechanisms of action and roles in shaping, being shaped and/or predicting therapeutic responses. We also discuss a variety of combinations with PD pathway blockade and their scientific rationales for cancer treatment.

Keywords

immune checkpoint; cancer immunotherapy; CTLA-4; PD-1; B7-H1; PD-L1; biomarkers; combination; regulatory T cell; IDO; myeloid derived suppressor cell; macrophage; regulatory T cell; dendritic cell; tumor microenvironment; objective response; cancer stem cell and cancer

Introduction

The tumor microenvironment is the primary location in which tumor cells and the host immune system interact. Characterization of the nature of immune responses in the human cancer microenvironment holds the key to understanding protective tumor immunity and improving and empowering current cancer immunotherapy. Accumulating evidence has

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revealed that the interaction between tumor cells and the host immune system fosters tumor immune evasion and ultimately results in tumor dissemination, relapse, and metastasis (1-3). Study of different cancer infiltrating immune cell subsets including CD4⁺Foxp3⁺ regulatory T cells (Tregs) (4), antigen presenting cells (APCs) (5, 6), myeloid derived suppressor cells (MDSCs) (7) and effector T cell subsets (8-13), and immune signature networks (3) have defined the nature of immune responses in the human cancer microenvironment and have allowed for elucidation of the critical importance of reversing immune suppressive mechanisms, including programmed cell death 1 ligand (PD-L1, B7-H1, CD274) and programmed cell death receptor 1 (PD-1, CD279) pathway (herein PD pathway) blockade (1, 2, 14, 15) to engender potent anti-tumor immunity. The identification of PD-L1 (16, 17), the finding that PD-1 is a receptor for PD-L1 (18), and the demonstration of the expression, regulation, and function of the PD pathway in the human cancer microenvironment (5, 14, 16, 17, 19-23) have provided scientific rationales and direct support for the current clinical application of PD pathway blockade (**Table 1**).

B7-H1 was cloned in 1999 (16). PD-1 has been subsequently identified as a counter-receptor for B7-H1 (18), and B7-H1 is therefore also known as PD-L1 to emphasize this receptor–ligand interaction. The expression profile of PD-L1 in human cancers has been previously reviewed (14). In addition to tumor cells (14, 17), high levels of PD-L1 protein expression have been observed in human tumor-associated APCs including tumor environmental dendritic cells (DCs), tumor draining lymph node DCs (5, 24), macrophages (20, 25), fibroblasts (26) and T cells (27, 28). PD-L1 expression can be induced or maintained by many cytokines (5, 17, 29, 30), of which interferon- γ (IFN γ) is the most potent. The association between tumor infiltrating T cells, IFN γ signaling genes, and PD-L1 expression suggests that effector T cell-derived IFN γ contributes to high levels of PD-L1 expression in the tumor microenvironment (31). Immune-induced tumor PD-L1 expression is considered to be an adaptive resistance mechanism for tumor cells in response to immune challenge (31-33). In addition, oncogenic phosphatase and tensin homolog (PTEN) loss results in enhanced PD-L1 expression in glioma (34) and triple negative breast cancer cells (35). In human T cell lymphoma, PD-L1 expression may depend on the expression and enzymatic activity of chimeric nucleophosmin (NPM) and anaplastic lymphoma kinase (ALK) (36). Thus, PD-L1 expression can also be regulated by intrinsic oncogenic pathways.

In addition to PD-L1, PD-L2 (B7-DC, CD273) also interacts with PD-1 with similar affinity to deliver a potentially suppressive signal. The immunologic function of this interaction in cancer immunity, however, may not be critical due to relatively rare expression of PD-L2 on cancer cells as well as its interaction with a potentially stimulatory receptor, repulsive guidance molecule b (RGMb) (15)(**Figure 1**).

PD-1 is absent on resting T cells and was initially found in activated mouse T cells upon TCR engagement (37) and subsequently in exhausted T cells in chronic infection murine models (38, 39). In patients with different types of cancer, high levels of PD-1 expression are detected in tumor infiltrating T cells including tumor antigen-specific T cells, presumably due to chronic antigenic stimulation. These human tumor associated PD-1⁺ T cells are functionally impaired and their biological activity can be partially recovered with PD-1 or PD-L1 blockade (19-23). A recent study reports that a subset of human melanoma

cells express PD-1 and melanoma cell-intrinsic PD-1 promotes melanoma cell growth (40). This finding, however, is inconsistent to previous result that PD-1 expression is predominant on tumor infiltrating lymphocytes but insignificant in melanoma based on immunohistochemistry analysis (41). The role of specific reagents and techniques in detecting PD-1 expression by tumor cells is important to further understand the biologic importance of the data. The magnitude of this surprising finding remains to be prospectively determined in additional studies across tumor types in patients. Thus, PD-L1 and PD-1 are expressed by various cellular components in the human tumor microenvironment, where they can inhibit anti-tumor T-cell immunity (**Figure 1**). This geographic expression profile is an important feature for PD pathway blockade.

In this review we focus on the human cancer microenvironment and PD pathway blockade. We propose that human anti-tumor immune responses are controlled and regulated by tumor somatic mutations, epigenetic alterations, and environmental cues. We discuss these three aspects and emphasize the relevant studies in the human cancer microenvironment and link scientific rationales to clinical combinational therapy with PD pathway blockade.

Mechanisms of action of the PD signaling pathway

How does PD signaling mediate dysfunctional tumor immunity? PD-L1⁺ cells, particularly PD-L1 expressing APCs and tumor cells, engage PD-1⁺ T cells, resulting in T cell dysfunction. Multiple modes of action are thought to explain T cell-immune evasion via the PD pathway. The engagement of PD-L1 and PD-1 may cause T cell apoptosis, anergy, exhaustion, and IL-10 expression (**Figure 1**). PD-L1 may function as a molecular "shield" to protect PD-L1⁺ tumor cells from CD8⁺ T cell-mediated lysis (14, 15). In addition to PD-1, an interaction between PD-L1 and CD80 has been demonstrated in mouse models (42, 43) (**Figure 1**). Activated T cells and APCs may express CD80, and CD80 may function as a receptor and deliver inhibitory signals when engaged by PD-L1 (42, 43). It has been shown that PD-L1 can function as a receptor to "back" transmit signals into T cells (44) and tumor cells (45) to affect their survival whereas the intracellular biochemistry of this "back" signaling is yet to be determined. Thus, PD-L1 could act as both ligand and receptor to execute immune-regulatory functions.

In addition to PD-L1, PD-1 is a receptor for PD-L2. RGMb is a binding partner for PD-L2 (46) (**Figure 1**). Thus, PD blockade may not be biologically identical and differing blockade may shift the balance in their interaction with their binding partners, leading to potential varied biological outcomes (**Figure 1**). Notably, the relationship between CD80, PD-1 and RGMb, PD-L1 and PD-L2 in their cellular expression profile, expression regulation, potential molecular interaction, and functional relevance in human tumor immunity and checkpoint immunotherapy have not been completely defined. Furthermore, as PD-L1 is expressed by different types of cells and mediates immune regulation via different mechanisms, it is not totally understood which major cellular and molecular mechanisms are correlated with clinical responses to PD pathway blockade in patients with cancer. Nonetheless, current clinical trials demonstrate similar clinical response patterns for anti-PD therapy (**Table 1**), suggesting that major cellular and molecular mechanisms associated with a clinical response may be shared in PD blockade. Future studies of the tumor

microenvironment in patients with or without immunotherapy will hopefully demonstrate the dynamics of these various interactions and the underlying cellular and molecular mechanisms, which are relevant for the further understanding of how immune elements shape and predict therapeutic response and non-response (**Figure 1**).

Clinical trials and response biomarkers of PD pathway blockade

Clinically, PD pathway blockade has demonstrated important activity across a spectrum of different tumor types spanning both solid tumors and hematologic malignancies including bladder cancer(47), breast cancer(48, 49), colorectal cancer(48, 50-53), diffuse large B-cell lymphoma(54), follicular lymphoma(55), gastric cancer(48), head and neck squamous cell carcinoma(49), Hodgkin lymphoma(56), melanoma(48-52, 57-65), ovarian cancer(48, 49), non-small-cell lung cancer (NSCLC) (48-51, 66-70), pancreatic cancer(48, 49), renal cell carcinoma (RCC) (48-51, 71), prostate cancer(50, 51), sarcoma(49), small-cell lung cancer (SCLC) (49), and uterine cancer(49). The objective response rates are varied from different cancer types in different clinical trials (**Table 1**). Based on current clinical data, bladder cancer(47), melanoma(48-52, 57-65), mismatch repair-deficient colorectal cancer(53) and certain hematopoietic malignancies (54, 56) may be among the most responsive cancer types. Specific features of particular clinical trials are summarized (**Table 1**) and discussed in the following sections.

Although head-to-head comparisons of antibodies to PD-1 and PD-L1 have not been done, levels of clinical response and toxicities appear to be generally consistent with either approach. Immune-related toxicities occur with PD pathway blockade but are much less frequent than those observed with cytotoxic T lymphocyte antigen-4 (CTLA-4) blockade (62, 63). The most frequently observed toxicity encountered with PD pathway blockade is fatigue, which often does not require treatment and does not necessarily limit duration of therapy. However, inflammatory pneumonitis has been observed, which may be fatal if not addressed promptly with corticosteroids or other means of immune suppression. Rare, high grade events like pneumonitis or interstitial nephritis may necessitate cessation of therapy. Yet, clinical responses remain durable despite cessation of therapy and even after immune suppression, implying that the optimal duration of therapy with PD pathway blocking agents remains to be determined. Given that PD pathway blockade induces a clinical response in subsets of cancer patients (**Table 1**), a central question is whether and how therapeutic responsiveness is predicted and/or is shaped by host and tumor components. There are many ongoing efforts to identify predictive biomarkers of PD pathway blockade. Recent studies have provided hints to associate clinical responses with several potential biomarkers (**Figure 2**).

Does expression level of PD-L1 predict clinical response (**Figure 2**)?

Tumor cells and APCs express high levels of PD-L1, and tumor associated PD-L1⁺ DCs mediate T cell suppression (5, 14). Tumor tissues of RCC (72), esophageal, gastric (73, 74), and ovarian (75) cancers show that PD-L1 expression is an indicator of poor prognosis for patient survival. It is reasonable to hypothesize that PD-L1 in tumor and/or APCs in the tumor microenvironment may predict or be associated with the clinical response of PD

blockade. In support of this, a correlation has been observed between the expression of tumor tissue PD-L1 and the likelihood of the response to anti-PD therapy in patients with melanoma (48, 51), NSCLC (66), and RCC (41). An 87% objective response is observed in patients with relapsed or refractory Hodgkin's lymphoma treated with anti-PD-1 (**Table 1**). In line with this, an amplification of PD-L1 and PD-L2 is detected in lymphoma cells in these patients (56). In addition to PD-L1 expression in tumor, expression of PD-L1 in tumor infiltrating immune cells, particularly myeloid APCs (macrophages and myeloid DCs), is correlated with clinical responses to anti-PD-L1 treatment in several types of cancer (**Table 1**) (49). In contrast, most progressing patients show a lack of PD-L1 upregulation by either tumor cells or tumor-infiltrating immune cells (49). However, the results in trials with anti-PD-1 (64) and the combination of anti-PD-L1 and anti-CTLA-4 suggest that melanoma patients can have a clinical response regardless of the tumor cell PD-L1 status (58, 76). Notably, the host immune cell PD-L1 expression, particularly PD-L1 expression in DCs in the tumor microenvironment and draining lymph nodes (5), has not been specifically examined in majority of clinical trials. Furthermore, activated T cells or innate immune cells can release type I and II IFN and stimulate de novo PD-L1 expression. Presumably, blocking newly induced PD-L1 can affect therapeutic responses. In support of this possibility, across multiple cancer types, responses to anti-PD-L1 therapy are frequent in patients with high PD-L1 expression in tumor-infiltrating immune cells, particularly macrophages and DCs, in the course of tumor regression (41, 47, 49). Hence, the relevance of host PD-L1 expression in shaping the clinical response to PD pathway blockade is important to consider.

Furthermore, PD-L1 expression may be clustered rather than diffuse in tumor tissues (17, 31) and is likely localized to the area where IFN γ ⁺ T cells infiltrate (31). Thus, current needle biopsy-based sampling of human tumor tissues may miss PD-L1 positive area and give false negative results. This problem may be overcome by an in vivo imaging method using radiolabeled high affinity PD-1 variants to assess PD-L1 expression in entire tumor as shown in tumor bearing mouse model (77). In addition, oncogene-driven expression of tumor PD-L1 may not correlate with tumor-infiltrating T cells. It remains to be determined whether the therapeutic efficacy of PD pathway blockade is similar between patients with oncogenic PD-L1⁺ tumors (31, 41, 78) and immune-driven PD-L1⁺ tumors.

With current limitations of clinical sampling methods, the expression of PD-L1 on the surface of tumor cells and immune cells prior to immunotherapy may be a useful, but not a definitive predictive biomarker of the response to the PD pathway blockade. Unlike the presence of oncogenic driver mutations, PD-L1 expression is a dynamic and inducible biomarker which is more of a relative indicator of the likelihood of response, rather than a binary predictor of response.

Do cancer neoantigens and/or somatic mutations predict a clinical response (Figure 2)?

The immune system can recognize developing cancers. Therapeutic manipulation of immunity can induce tumor regression. While tumor associated antigens (TAAs) are largely self-antigens, cancers contain somatic genetic mutations, which could be specific to the cancer. These mutation associated antigens can be tumor specific antigens and be presented to and recognized by T cells in patients with cancer. Aligned with this notion, the tumor

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mutational antigen (neoantigen) T cell response has been documented in a genetically engineered, autochthonous mouse model of sarcomagenesis (79), in a highly immunogenic methylcholanthrene (MCA) induced mouse sarcomas model (80), and in mouse vaccine models (81, 82). In patients with melanoma, tumor mutation-specific CD4⁺ (83, 84) and CD8⁺ (85) T cells are found, and these cells can mediate tumor regression. Vaccination with melanoma-derived mutant peptides augments T cell immunity directed at naturally occurring dominant neoantigens and subdominant neoantigens (86). Although the antigen specificity is unknown, the mismatch repair-defective subset of colorectal cancer displays high tumor infiltration of Th1-type T cells and CD8⁺ T cells along with high PD-L1 and PD-1 expression (87). Using large-scale genomic data sets of solid tumor tissues, the number of predicted MHC class I-associated neoantigens is correlated with the cytolytic activity of CD8⁺ T cells (88).

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Several lines of evidence support a link between PD pathway blockade and tumor mutation-derived antigen specific T cell responses. In the mouse MCA sarcoma model, treatment with anti-PD-1 and/or anti-CTLA-4 activates tumor mutational antigen specific T cells and results in tumor rejection (89). In patients with colorectal cancers, mismatch-repair status predicts clinical benefit of PD-1 blockade (53). In patients with NSCLC treated with anti-PD-1, high nonsynonymous mutation burden is associated with improved objective response, durable clinical benefit, and progression-free survival, and mutated antigen-specific CD8⁺ T cell responses parallel tumor regression (67). Lung cancer and melanoma are found to be clinically responsive in two early clinical trials with anti-PD therapy (51). These two types of cancer have high numbers of somatic mutations as a result of exposure to cigarette smoke and ultraviolet radiation. Biopsied specimens of regressing melanoma lesions are infiltrated by CD8⁺ T cells in patients treated with anti-PD therapy (57). A high objective response rate to anti-PD-1 therapy is observed in patients with microsatellite instable colorectal cancer, but not in patients with mismatch repair-proficient colorectal cancer (53). Similarly, in 29 stage IV NSCLC patients, there were 63% and 0% response rates respectively with high and low nonsynonymous mutation burden (67) (**Table 1**). The data suggest that the increased number of mutation-associated neoantigens may be associated with the enhanced responsiveness to PD pathway blockade in some cancer patients.

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Notably, we are at the beginning of our understanding of the relationship between mutant tumor neoantigens, their T cell responses, and cancer immunotherapy responses. Several observations are worth noting in this regard: (a) Melanoma and lung cancer naturally possess high levels of mutations. (b) High levels of mutations may not necessarily form high levels of immunogenic neoantigens; this may simply be a probabilistic phenomenon. (c) There is no direct evidence demonstrating that neoantigen specific T cells mediate tumor elimination in patients treated with PD pathway blockade. (d) Multiple factors including PD-L1 and PD-1 expression levels, effector T cell tumor trafficking, and environmental cues (microbiota) may contribute to immunotherapeutic responses. Nonetheless, recognizing the intrinsic ability of the immune system to specifically target the unique mutations potentially shared by many cancer types (90) is an important part of understanding the mechanism of cancer immunotherapy (91).

Do Th-1 type chemokines, tumor infiltrating T cells, and T cell clonality predict clinical responses (Figure 3)?

Th1-type chemokines are correlated with effector T cell density in some human tumors and are also positively associated with cancer patient survival (8, 10, 92). However, a crucial question is why some tumors are “inflamed” with effector T cell infiltration while others are not. Two research teams have proposed plausible mechanisms to address this question. In a murine melanoma model, tumor intrinsic β -catenin signaling negatively controls chemokine CCL4 expression. CCL4 mediates DC tumor trafficking. Thus, tumor β -catenin activation results in poor CCL4 expression and subsequently limits DC recruitment and DC-mediated T cell activation (**Figure 3**) (93). In human ovarian cancer (94) and colon cancer (95), poor T cell tumor infiltration is attributed to potent epigenetic silencing of tumor Th1-type chemokines CXCL9 and CXCL10, which mediate effector T cell and natural killer (NK) cell tumor migration (**Figure 3**) (94, 95). Polycomb repressive complex 2 (PRC2), the demethylase JMJD3-mediated histone H3 lysine 27 trimethylation (H3K27me3), and DNA methylation repress the expression of Th1-type chemokines and subsequently restrain effector T cell trafficking into the cancer microenvironment (94, 95). These studies suggest that intrinsic tumor oncogenic (93) and epigenetic (94, 95) pathways control T cell activation and/or migration. Both epigenetic silencing (96) and β -catenin signaling are intrinsic tumorigenic mechanisms and are associated with cancer stem cell properties. Thus, oncogenic genetic and epigenetic pathways may play dual biologic and immunologic roles in supporting tumor progression and limiting spontaneous and therapeutic-induced tumor specific T cell immunity (**Figure 3**).

PD-L1 expression is clearly important in the tumor microenvironment (5, 14, 15, 33). A major feature of PD pathway blockade is immune regulation specifically at the tumor site (15). Hence, it is reasonable to assume that pre-existing tumor infiltrating immune cells and Th1 type chemokines may correlate with clinical response to PD pathway blockade. In support of this possibility, recent clinical trials suggest that intra-tumoral T cell infiltration and Th-1 type gene expression and a clonal TCR repertoire are associated with improved clinical responses to anti-PD therapy (47, 97). The frequency of mutational antigen specific T cells and their functional status in the tumor infiltrating T cell populations remain to be investigated and compared prior to and post cancer immunotherapy. Given that oncogenic genetic (93) and epigenetic (94, 95) pathways control effector T cell tumor trafficking, epigenetic marks, enhancer of zeste homolog 2 (EZH2) and DNA methyltransferase 1 (DNMT1), are negatively associated with CD8⁺ T cells and patient survival (94, 95), and epigenetic reprogramming synergistically increases the effect of PD-L1 blockade(94), it will be interesting to explore whether tumor specific genetic and epigenetic marks are associated with the clinical response to anti-PD therapy (**Figure 2**).

Do microbiota contribute to clinical responses to PD pathway blockade?

Recent studies have shown that the gut microbiome can affect the outcome of cancer chemotherapy in murine models (98, 99). Chemotherapy induced Th1 and Th17 responses are enhanced by translocating commensals and contributes to tumor eradication (98, 99). This is in line with the anti-tumor role of polyfunctional Th17 cells in human ovarian cancer (11, 92). Similarly, commensal bacteria can alter the therapeutic effect of anti-PD-L1

therapy in mice bearing subcutaneous tumors, and the response to PD-L1 blockade is enhanced by supplementation with “good” bacteria, *Bifidobacterium*, during treatment (100). Interestingly, the antitumor effects of anti-CTLA-4 also depend on distinct *Bacteroides* species. In mice and patients, T cell responses specific for *B. thetaiotaomicron* or *B. fragilis* are associated with efficacy of CTLA-4 blockade (101). It appears that immune responses modulated by the gut microbiome can have systemic effects on tumor immunity and cancer therapy. It remains to be defined if the gut microbiome of cancer patients will have an important impact on PD pathway blockade including cancer neoantigen specific T cell responses and effector T cell tumor infiltration. Nonetheless, these studies raise the possibility that beneficial microorganisms may be an adjuvant for cancer immunotherapy. Thus, it will be scientifically and clinically interesting to profile patient gut microbiota and dissect the relationship with immune responses and clinical outcomes in the course of cancer immunotherapy.

We have discussed several biomarkers in shaping and predicting the clinical response to PD pathway blockade (**Figure 2**). Are there definite translational biomarkers for PD pathway blockade? Based on the immune profile, cancers may be classified into “inflamed” and “non-inflamed” types. The former is enriched with a Th1-type immune signature including Th1-type chemokines and effector T cells (presumably containing mutated antigen specific T cells) (94) and likely expresses a high amount of PD-L1. The latter is poorly immune infiltrated and likely expresses a limited amount of PD-L1. Recent clinical studies, largely from patients with melanoma, suggest that the “inflamed”, but not the “non-inflamed” tumor type, is highly responsive to PD pathway blockade (**Figure 2**). However, lymphocyte-rich regions may not be always associated with PD-L1 expression (41, 78, 102). Biologically, the “non-inflamed” tumor type may be closely associated with an epithelial-mesenchymal-transition (EMT) and stem-like type subgroup. In line with this possibility, the Th1-type immune profile is controlled by stem-like associated oncogenic and epigenetic pathways including β -catenin and PRC2 complex (93-95). Thus, immune “inflamed” cancers may be a “non-EMT/stem like type” and are more likely responders to PD blockade therapy. Analogously, the non-responders (or minimal responders) may be lacking T cell infiltration and Th1-type chemokines, less specific mutations and neoantigens, and enriched with multiple layers of immune suppressive mechanisms and potential EMT/stem-like types (**Figure 2**). An urgent next step is to define and develop combinatorial therapy to improve and enhance the clinical response in patients with different types of cancer.

Combinatorial regimens with PD pathway blockade

Because of the complexity of immune regulatory mechanisms and the heterogeneity of tumor and host, it is envisioned that combination immunotherapies will be required to efficiently treat a larger proportion of cancer patients (1). Continuing advances in our understanding of immune regulation and tumor immunity will allow for the development of new combination(s) for the treatment of different types of cancer. Based on particular limitations of single agent therapy and combinatorial scientific rationales, we have discussed a few examples of therapeutic combinations (**Figure 4**).

Enforcing effector T cell trafficking with epigenetic reprogramming drugs

Th1-type chemokines and effector T cell tumor infiltration are associated with therapeutic responses to PD pathway blockade (**Figure 2**). Histone modification and DNA methylation epigenetically repress tumor Th1-type chemokines and subsequently determine effector T cell trafficking into the tumor microenvironment (94, 95). It may be reasonable to surmise that cancer epigenetic reprogramming may remove Th1-type chemokine repressive marks and promote effector T cell trafficking into the tumor microenvironment and improve the therapeutic efficacy of PD pathway blockade. In support of this, treatment with cancer epigenetic reprogramming drugs including EZH2 inhibitors, DZNep (103), a selective inhibitor of EZH2 methyltransferase activity, GSK126 (104), and a DNMT inhibitor, 5-aza-2'-deoxycytidine (5-AZA dC), enhance tumor Th1-chemokine production and T cell trafficking into tumor (94, 95) and augment therapeutic effects of PD-L1 blockade and T cell therapy in a preclinical model (94). Furthermore, treatment with azacitidine up-regulates IFN signature genes in several human cancer cell lines (105, 106). 5-AZA dC treatment enhances the cancer and germline antigen NY-ESO-1 expression in human ovarian cancer cells (107) and promotes chemokine expression and T cell tumor trafficking in a mouse ovarian cancer model (108). Thus, epigenetic reprogramming can unlock the repressed Th1-type chemokines, IFN-signature genes, and tumor antigen expression, and may therefore condition tumor from poor T cell infiltration to rich T cell infiltration and ultimately potentiate PD blockade therapy (94, 95, 108).

Supplementation of effector T cells with adoptive T cell therapy (ACT)

There may be insufficient functional effector T cells in the tumor microenvironment. ACT, including in vitro expanded peripheral blood or tumor infiltrating T cells (TILs) and genetically engineered chimeric antigen receptor (CAR) T cells, is an important therapeutic approach. However, activated human TILs and TAA-specific T cells express PD-1 (19-23, 109). These data suggest a potential benefit for the combination of ACT and PD pathway blockade. In further support of the role of PD-1 blockade in ACT, melanoma TILs with zinc finger nuclease (ZFN)-mediated gene editing of PD-1 display increased effector function in vitro (110). New clinical trials will be warranted to further evaluate this strategy. Mechanistically, PD pathway blockade prior to ACT may prepare the specific “soil”, the less suppressive tumor microenvironment (1), for the transferred T cells to home and function. Given that transferred T cells are often activated and express PD-1 and immune activation can stimulate PD-L1 expression in tumor cells and APCs in the tumor microenvironment, it is assumed that concurrent or post PD pathway blockade to ACT may improve the functionality of the transferred T cells.

Promotion of T cell function via targeting TNF family and T cell metabolism

The tumor microenvironment is highly immunosuppressive in patients with advanced cancer. Targeting immunosuppressive mechanisms is considered an effective strategy to treat patients with cancer (1). On the other hand, it remains logical to directly stimulate and activate the immune cells in combination with PD pathway blockade (111). To this end, we can manipulate certain TNF family signaling ways in patients with cancer.

(a) CD40 and CD40L—The interaction of CD40 and CD40L delivers a potent costimulatory signal to T cells. The humanized CD40 agonist antibody CP-870893 in combination with chemotherapy is currently in clinical trials to treat patients with pancreatic cancer (112). Two other anti-CD40 antibodies, dacetuzumab and HCD122 are currently being tested in hematologic malignancies (113).

(b) OX40 and OX40L—The interaction of OX40 and OX40L may potentiate T cell activity (114) and agonistic anti-OX40 antibodies and OX40 ligands are currently being studied in clinical trials (115) (NCT02219724, NCT02410512, and NCT02221960).

(c) 4-1BB (CD137)—CD137 engagement may preferentially stimulate CD8⁺ T cells and NK cells. Administration of agonistic anti-4-1BB antibody improves T cell immunity in various murine tumor models (116). Recent clinical trials have shown promising results in single agent or in combination with anti-PD therapy for the treatment of advanced solid tumors (NCT02179918, NCT02554812).

(d) Targeting T cell metabolism—Recent studies reveal that abnormal metabolism impairs effector T cell function in the tumor microenvironment (13, 117, 118). Reprogramming tumor metabolism would be an interesting option in combination with PD pathway blockade (**Fig. 4**).

Thus, there are scientific rationales to support the combination with all of these agonistic antibodies and approaches with PD pathway blockade. Clinical studies will be needed to conclusively demonstrate the precise indications, effectiveness and side effects of given combination in treating specific type of cancer.

Subversion of immunosuppressive networks in the tumor microenvironment

Immunosuppressive networks are major obstacles for spontaneous and therapy-induced anti-tumor immunity (1). The clinical responses observed by blocking inhibitory B7 family members provides solid evidence to target additional immunosuppressive components in the tumor microenvironment.

(a) Targeting Tregs—Tregs actively inhibit T cell-mediated anti-tumor immunity in the human cancer microenvironment (4). Targeting Tregs is proposed as a therapeutic strategy to treat patients with cancer (119-121). Tregs express CTLA-4. Anti-CTLA-4 antibody may deplete Tregs in the tumor (122). Ipilimumab, a fully human anti-CTLA-4 mAb was approved by the U. S. Food and Drug Administration (FDA) in 2011 for the treatment of patients with advanced melanoma (123). Ipilimumab monotherapy produces clinical responses in 10-20% of patients and extends overall survival in metastatic melanoma, with 20% of patients surviving 3 years or longer (123, 124). In a phase I trial of anti-PD-1 combined with anti-CTLA-4 in patients with advanced melanoma, rapid and deep tumor regressions were observed in 53% of patients treated at the optimal dose level (58). Phase II and III clinical trials have confirmed these observations and further demonstrate the beneficial effects on the objective-response rate and the progression-free survival among patients with advanced melanoma who had not previously received treatment (63, 76) (**Table 1**). Interestingly, in patients with tumors demonstrating < 5% PD-L1 expression, this

combination appears to be more effective than either agent alone (63). However, the response rate for monotherapy with CTLA-4 blockade is generally lower than PD blockade in patients with melanoma (**Table 1**) (62, 63). Durability of responses with each approach is a topic of current study. Nonetheless, anti-CTLA-4 treatment may deplete Tregs and potentially increase the ratio between effector T cells and Tregs in the tumor microenvironment. Thus, this combination therapy has been approved by the FDA for treatment of BRAF V600 wild-type unresectable or metastatic melanoma. This treatment, however, may carry a relatively high frequency of immune-related toxicity. Therefore, a sequenced model with PD pathway blockade therapy first followed by anti-CTLA-4 later may be considered. In addition to anti-CTLA-4, other strategies targeting Tregs (121) including transforming growth factor (TGF) β signaling blockade may be used in combination with PD blockade (125). Tregs migrate into the human cancer microenvironment through CCL22 and CCR4 signaling pathway (4, 119). Blocking this pathway may enhance the effects of PD blockade. An anti-CCR4 antibody (mogamulizumab) can deplete circulating Tregs in patients with T cell leukemia and lymphoma (126). Ongoing studies are evaluating the efficacy of anti-CCR4 in combination with PD pathway blockade (NCT02301130). Human tumor associated Tregs express the ectonucleotidases CD39 and CD73, convert ATP to adenosine, and inhibit T cell activation by the adenosinergic pathway (92). A CD73-specific antibody has demonstrated an additive activity when combined with PD-1 antibodies in murine tumor models (127). Thus, CD39, CD73, adenosine and adenosine A2a receptor (ADORA2A) signaling blockade may be combined with PD pathway therapy to treat patients with cancer.

(b) Targeting myeloid cells—Human tumor associated MDSCs and inhibitory APCs including macrophages actively inhibit T cell-mediated anti-tumor immunity (5-7) and promote cancer stemness (7, 128) in the human cancer microenvironment. Given the roles of indoleamine-2,3-dioxygenase (IDO) in MDSC-mediated T cell suppression (129), the use of IDO inhibitor(s) (INCB024360) (32) with PD blockade is a potential option for combinatorial therapy.

(c) Targeting additional potentially immune inhibitory immunoglobulin superfamily molecules—The expression of B7-H3 (B7RP-2, CD276) and B7-H4 (B7x, B7S1) is found in different types of human tumor tissues (6, 14, 130, 131). In human hepatocellular carcinoma (HCC) B7-H3 expression is linked to limited T cell proliferation and IFN- γ production (132). B7-H3 blockade resulted in increased CD8⁺ T cell influx in murine pancreatic cancer (133) while some studies showed that B7-H3 could promote tumor immunity (132). Tumor-associated macrophages express B7-H4 and are able to suppress tumor-specific T cells (6). Therefore, immune modulatory role of B7-H3 and B7-H4 in different types of tumor or host cells is under debate (134, 135). Several clinical studies, however, have been initiated to test the effect of anti-B7-H3 antibodies in single agent (NCT02628535) or in combination with anti-CTLA-4 (NCT02381314) or with anti-PD-1 (NCT02475213) for treating solid tumors.

PD-1H (Dies1, VISTA, DD1 α) is a more recently identified B7-CD28 family molecule and was shown to be an immune inhibitory ligand in a mouse tumor model and in a mouse

experimental autoimmune encephalomyelitis (EAE) model (136). However, using PD-1H deficient mice and agonistic antibodies, this molecule was shown also to be an immune inhibitory receptor on T cells (137, 138). Thus, VISTA blockade in single agent or in the combination of anti-PD blockade may be potential regimens to be examined in future clinical trials once the necessary reagents are available.

The expression of T cell immunoglobulin mucin 3 (Tim-3) (139, 140), lymphocyte activation gene 3 protein (LAG3) (23), and T cell immunoglobulin and ITIM domain (TIGIT) (141, 142) are reported in human cancer infiltrating T cells. Blockade of Tim-3 (139, 140) and LAG3 (23, 143) and TIGIT (141, 142) with anti-PD antibody increases effector T cell function. Thus, the combinations of PD pathway with Tim-3, LAG3, and TIGIT blockade have been proposed and tested in clinical trials (NCT01968109, NCT02460224).

Targeting tumor specific antigen and antigen presentation

(a) Neoantigen vaccine—Traditional vaccines can activate T cells and induce immune responses to targets on tumor cells, but there is not substantial evidence of reproducible clinical responses in patients with established tumors (144). Interestingly, PD pathway blockade can increase the anti-tumor efficacy of conventional vaccines in animal models (145-147). As somatic genetic mutations could generate unique tumor specific neoantigens and neoantigen specific T cell responses (148), vaccination with immunogenic neoantigens has been examined in animal models (81, 82, 89). Mutated antigen specific T cell responses can be found in patients with cancer (67, 83-86). Thus, a novel vaccination platform will be likely incorporated with specific neoantigens and potentially in combination with PD pathway blockade. Ongoing research efforts are aimed at increasing the efficiency of producing personalized neo-epitope vaccines and also possibly identifying shared neoantigens for use along with immune modulating antibodies. As the tumor microenvironment is immunologically suppressive (1) and metabolically dysregulated (13, 117, 118), in addition to inducing and/or expanding neoantigen specific T cells via specific vaccination, it is also crucial to ensure that these T cells can efficiently traffic into and survive in the cancer microenvironment (**Figure 4**).

(b) Oncolytic viral therapy—Talimogene laherparepvec (T-VEC) is a herpes simplex virus type 1–derived oncolytic immunotherapy designed to selectively replicate within tumors and produce granulocyte macrophage colony-stimulating factor (GM-CSF) (149). The US FDA has approved T-VEC for the local treatment of unresectable cutaneous, subcutaneous, and nodal lesions in patients with melanoma recurrent after initial surgery. T-VEC may likely trigger DC differentiation and enhance antigen presentation, promote T cell activation and IFN- γ production, and induce PD-L1 expression. Thus, T-VEC may be a high priority agent for combination trials with PD blockade. A phase III study is currently exploring T-VEC with pembrolizumab (anti-PD-1) for unresected melanoma (NCT02263508).

Targeting inflammatory mediators with COX-2 inhibitor

Prostaglandin E2 (PGE2) and its key synthesizing enzyme cyclooxygenase 2 (COX2) can directly mediate pro-tumor activities and recruit and induce MDSCs in the tumor microenvironment (150). However, PD pathway blockade may increase the expression of PGE2 and pro-tumor inflammatory cytokines, which potentially offsets the therapeutic effects of this blockade (151). Several pre-clinical models demonstrate that inhibition of COX-2 synergizes with PD pathway blockade in eradicating tumors (151, 152), suggesting that COX inhibitors could be useful adjuvants for immune-based therapies including PD blockade in cancer patients.

Targeting innate immune signaling pathway

The innate immune system and its major signaling type I and II IFN contribute to the antitumor immune response (3, 153). Human tumor associated plasmacytoid DCs induce IL-10⁺ regulatory T cells (154, 155). However, after activation, these human tumor associated plasmacytoid DCs are capable of producing large amount of type-I IFN (154, 155). In tumor bearing mouse models, type I and II IFN signaling pathway is essential for therapeutic responses to chemotherapy (156, 157), radiation therapy (158, 159), and anti-HER2/neu therapy (160). In line with mouse studies, in women with metastatic breast cancer, response to anti-HER2/Neu therapy correlates with natural killer (NK) cell associated antibody-dependent cell-mediated cytotoxicity (ADCC) (161). Furthermore, PD-L1 expression can be potently stimulated through IFN signaling pathway. Thus, targeting innate immune signaling pathway in combination with PD pathway blockade is scientifically rationalized.

Targeting cancer cells

(a) Localized radiation—Radiation therapy is a well-recognized means to achieve local tumor destruction. It has been reported to trigger innate immune signaling pathways, impair regulatory T cells, activate CD8⁺ T cells, stimulate chemokine expression, and promote immune infiltration into tumors (158, 159, 162-164). However, radiation-induced inflammation (including IFN signaling) can enhance tumor PD-L1 expression (159, 163) which may reduce radiation-induced protective tumor immunity. Interestingly, increased PD-L1 expression may provide a window of opportunity for PD pathway blockade. In line with this notion, radiation therapy combined with anti-PD therapy can synergistically promote anti-tumor immunity in several tumor bearing mouse models (159, 163, 165, 166). Although there is a solid scientific rationale to support the combination of radiation and PD pathway blockade, radiation parameters including dose, site, and time may be critical to the success of such a combination and need to be further explored. Given the challenges in recapitulating human radiation fractionation regimens in animal models, clinical trials will be essential to carefully sort out the feasibility of this approach. Such clinical trials will also provide an opportunity to examine if radiation induces immunogenic mutation-associated neoantigens and whether the induced mutations are associated with treatment response.

(b) Chemotherapy—Mouse studies suggest that therapeutic responses to some chemotherapy agents including anthracyclines and oxaliplatin, may partially depend on

immune responses, particularly type-I IFN signaling mediated immunity (156, 167). Interestingly, PD-L1 can be induced by the IFN signaling pathway. Oxaliplatin treatment promotes PD-L1⁺ plasmacyte tumor infiltration in a mouse prostate cancer model (168). These preclinical studies suggest that PD pathway blockade may enhance the efficacy of chemotherapy. As chemotherapy induces genetic mutations, it is also reasonable to hypothesize that such combinations may induce mutation specific neoantigen-specific T cell responses and affect clinical outcomes. Nonetheless, chemotherapy also modulates the immune system and PD pathway blockade depends on ongoing immune responses, especially those in the tumor microenvironment (15). Future clinical studies will determine which therapeutic modality including agents, doses, and timing will increase clinical responses in combination with PD pathway blockade.

(c) Targeting oncogenic signals—Anti-HER2/neu antibody therapy and multiple receptor tyrosine kinases (RTK) inhibitors (sunitinib, imatinib) interrupt oncogenic signals and mediate tumor regression. Recent studies indicate that anti-HER2/neu therapy (160) and RTK inhibitors (169) can promote T cell activation and trafficking. PD pathway blockade in combination with anti-HER2/neu antibody or RTK inhibitors may be considered in the treatment of certain cancers. Careful attention will need to be paid to proper dose and scheduling of targeted therapies with immunotherapies to avoid potential suppression of T cell or APC activity given the physiologic role for some of the targeted pathways.

Other potential combinations

In addition to T cell and APC subsets and tumor cells, vascular endothelial cells, stromal fibroblasts, cancer stem cells, and microbiota may be targeted in combination with PD pathway blockade. Vascular endothelial growth factor A (VEGF-A) and CXCL12 (154, 170, 171) are highly expressed in the tumor microenvironment and mediate tumor angiogenesis. Targeting tumor stromal fibroblasts (172), CXCL12 and CXCR4 blockade (172), and VEGF targeted therapy (173) have been tested as combinatorial partners for immunotherapy in the literature. As human cancer microenvironmental immune cells including macrophages (128), MDSCs (7), Th22 cells (12) and inflammatory Tregs (174) and their associated cytokines IL-6 (128, 175, 176), IL-8 (177) and IL-22 (12) can promote and maintain the cancer stem cell pool, targeting cancer stem cell pathway with PD blockade may be an important option. The gut microbiota influence the host responsiveness to immunotherapy (100, 101). Beneficial bacteria inoculation or detrimental bacteria inhibition may be combined with PD pathway blockade in specific type of cancer. Additional potential combinatory strategies have been discussed in the literature (111).

Concluding remarks

PD pathway blockade has elicited durable clinical responses in patients with a broad spectrum of cancers with a reasonable toxicity profile (**Table 1**). This therapy largely relies on efficient T cell infiltration into tumor and effector T cell function in the tumor microenvironment. The human cancer immune microenvironment thus holds the key to understanding the nature of immunity in response to tumor progression and tumor immunotherapy (1, 15, 33). PD blockade may potentially induce and/or expand T cells

specific to mutated neoantigen in patients with cancer. Accumulating evidence points towards mechanism-based combination of various treatment regimens with PD pathway blockade to establish new standard of care for patients with cancer. Dynamic immunologic studies along with genetics and epigenetics in the human cancer microenvironment will guide the development of different combination therapies and generate novel insight into how the human immune system responds to and is shaped by a variety of tumor types.

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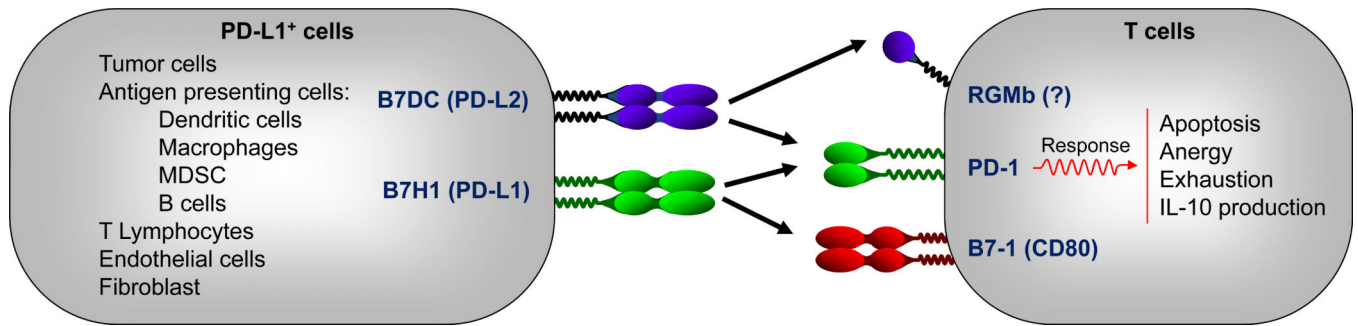


Figure 1. Mechanisms of action of the PD-L1 and PD-1 pathway. Tumor cells, APCs, and other cells express high levels of PD-L1. Engagement of PD-L1⁺ cells with T cells may induce T-cell apoptosis, anergy, functional exhaustion, or IL-10 production.

Active response to treatment

No or limited response to treatment

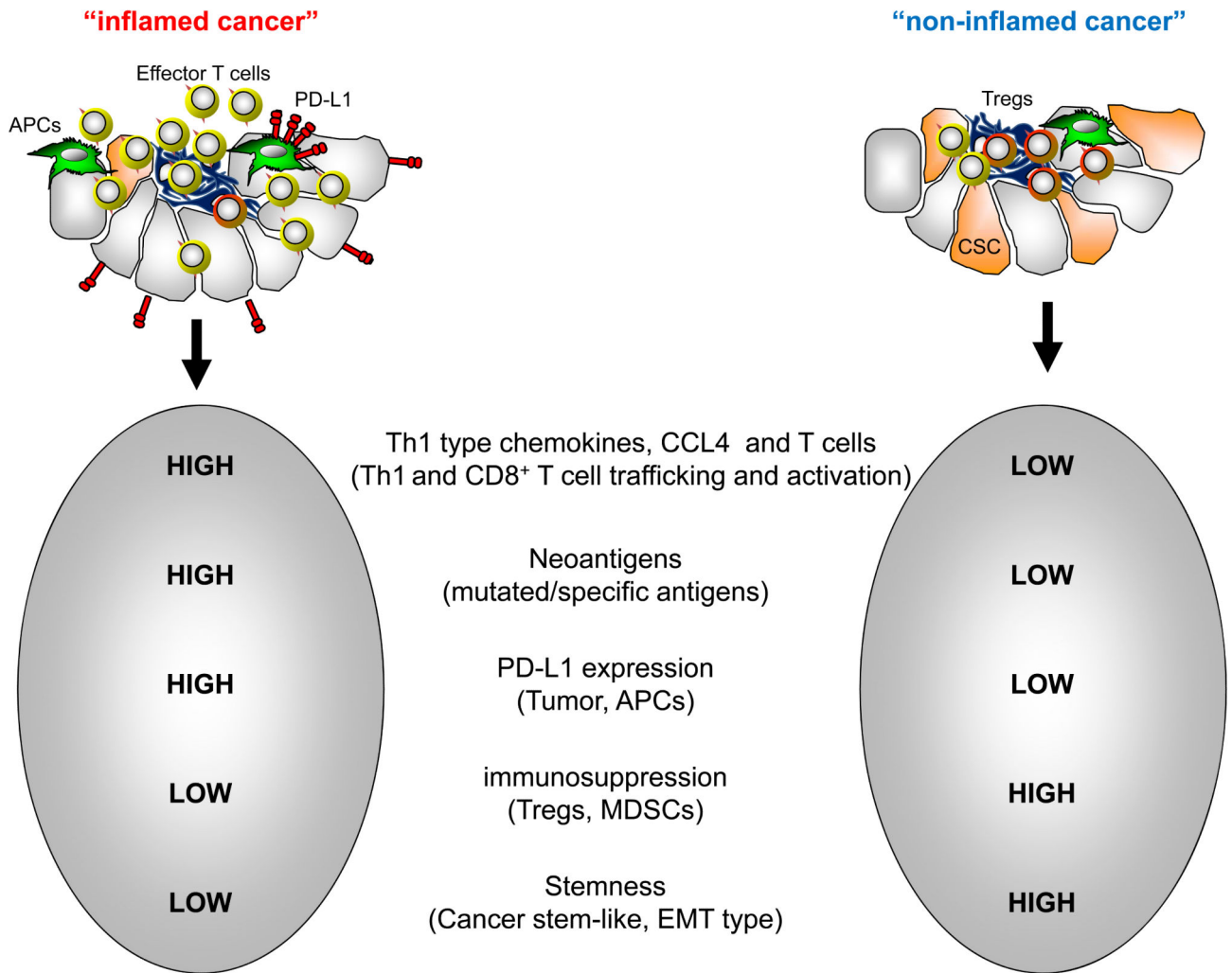


Figure 2. Proposed potential response biomarkers of PD pathway blockade. Several biomarkers including high levels of PD-L1 expression, Th1-type chemokines, infiltrating T cells, mutations, low levels of immune suppressive elements, and EMT/stem-like features may be associated with an active response to PD pathway blockade.

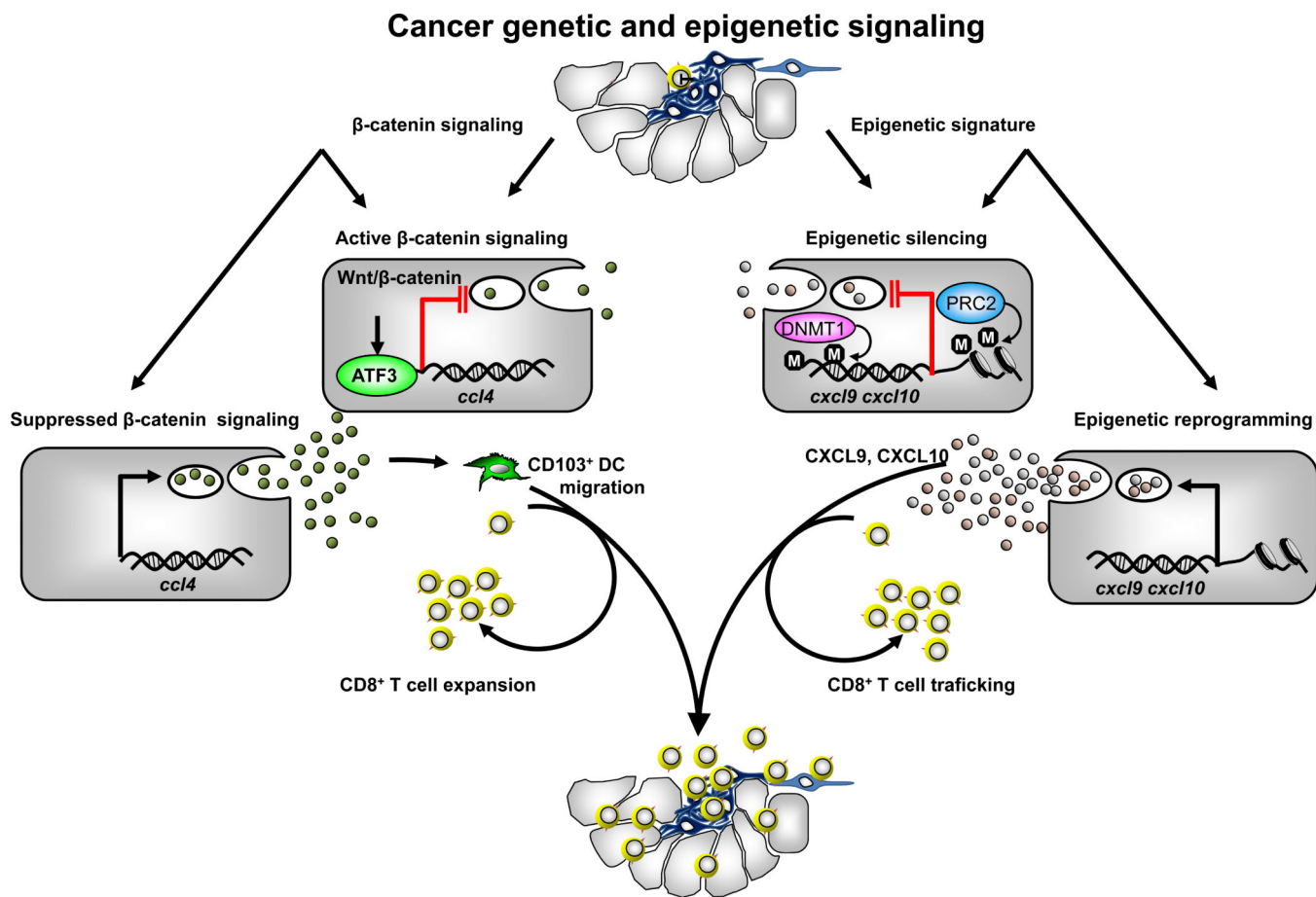


Figure 3. Mechanisms of poor tumor T cell infiltration. Active tumor β-catenin inhibits CCL4 expression and limits CD103⁺ DC recruitment and CD8⁺ T cell activation. Th1-type chemokines CXCL9 and CXCL10 are repressed by EZH2 and DNMT-mediated epigenetic silencing. Consequently CD8⁺ T cells poorly infiltrate tumor.

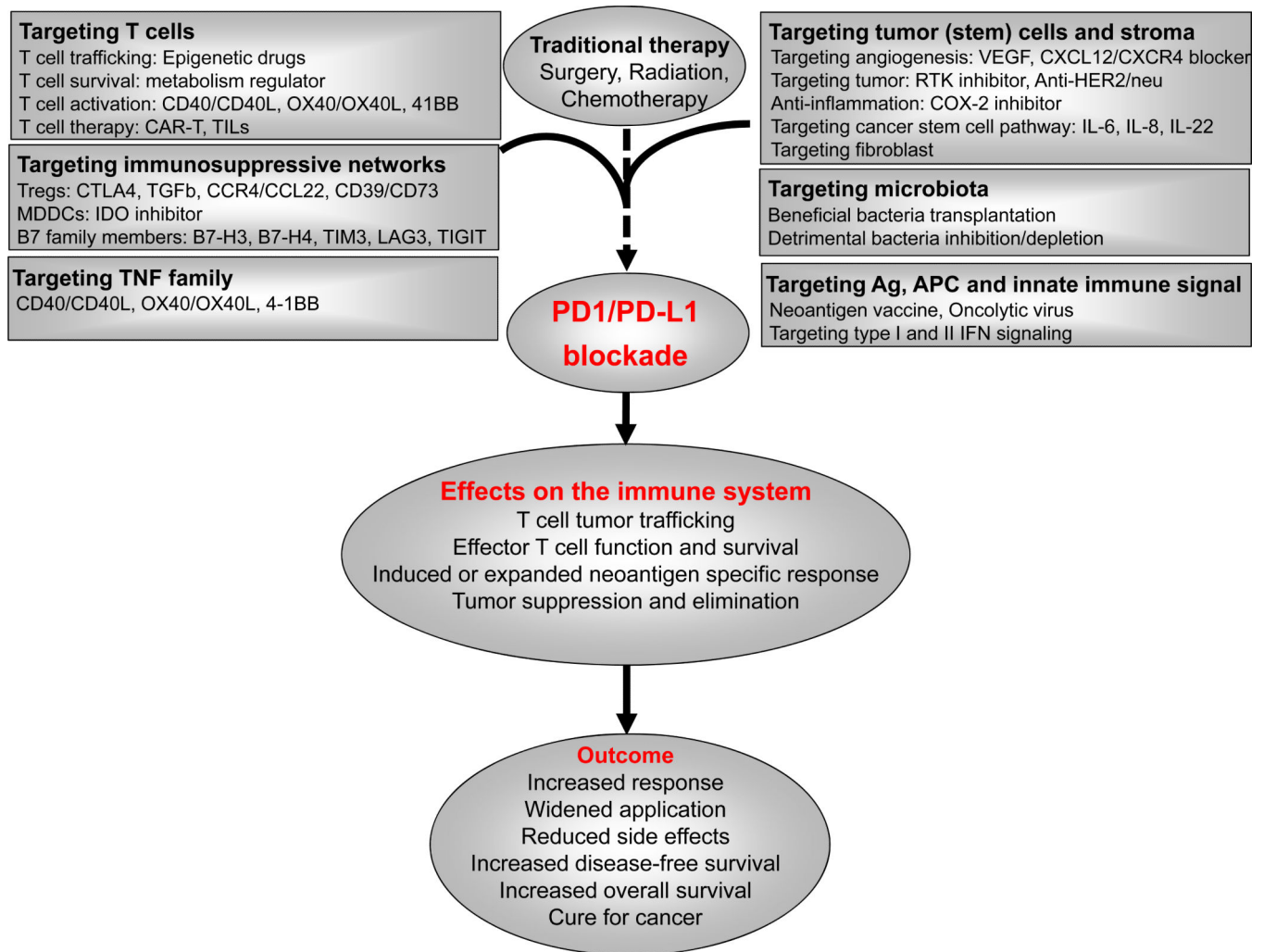


Figure 4. Scientific rationales of potential therapeutic combinations with PD pathway blockade. Multiple layers of immunosuppressive mechanisms, weak T cell activation, tumor intrinsic biological pathways contribute to cancer progression and therapy resistance. The different combinations with PD pathway blockade may yield a synergistic or additive clinical response.

Table 1

Examples of clinical trials with PD-1 and PD-L1 blockade

Target and drug information	Clinical response rate in different cancer types	Phase	Cases	References
Target: PD-1 Name: Nivolumab, Opdivo, BMS-936558, MDX-1106, ONO-4538 Isotype: Humanized IgG4a Source: Bristol-Myers Squibb, Ono Pharmaceuticals	12.8% in treatment-refractory metastatic melanoma, castrate-resistant prostate cancer, RCC, NSCLC, or CRC	I	39	(50)
	28% in advanced melanoma, 18% in NSCLC, 27% in RCC	I	296	(51)
	40% in melanoma treated with nivolumab + ipilimumab, 20% in nivolumab followed by ipilimumab	I	86	(58)
	87% in relapsed or refractory Hodgkin's lymphoma	I	23	(56)
	14.5% in refractory NSCLC	II	117	(70)
	31.7% in advanced melanoma progressed after anti-CTLA-4	III	405	(65)
	40% in previously untreated melanoma without BRAF mutation	III	418	(64)
	17% in previously treated advanced NSCLC	II	129	(69)
	29% in previously treated advanced RCC	I	34	(71)
	20% in advanced squamous-cell NSCLC	III	272	(68)
57.6% (nivolumab + ipilimumab) vs 19% (ipilimumab) vs 43.7% (nivolumab) in untreated stage III or IV melanoma	III	945	(63)	
Target: PD-1 Name: Pembrolizumab, Keytruda, MK-3475, lambrolizumab Isotype: Humanized IgG4 kappa Source: Merck	38% in melanoma	I	135	(57)
	26% in Ipilimumab-refractory advanced melanoma	I	173	(61)
	63% versus 0% in stage IV NSCLC patients with high and low nonsynonymous mutation burden	I	29	(67)
	19.4% in advanced NSCLC	I	495	(66)
	40% and 0% in mismatch repair-deficient/proficient CRC	II	41	(53)
	33% (pembrolizumab) and 11.9% (ipilimumab) in advanced melanoma	III	834	(62)
Target: PD-1 Name: Pidilizumab or CT-011 Isotype: Humanized IgG1 Source: CureTech Ltd	51% in diffuse large B-cell Lymphoma (after HSCT)	II	66	(54)
	66% in relapsed follicular lymphoma	II	32	(55)
Target: PD-L1 Name: MPDL3280A, RG7446 Isotype: Fc-modified human IgG1b Source: Genentech/Roche	21% overall response rate in advanced incurable cancer NSCLC, SCLC, melanoma, RCC, CRC, gastric cancer, head and neck squamous cell carcinoma, breast cancer, ovarian, pancreatic cancer, uterine cancer, sarcoma, pancreatoduodenal cancer	I	277	(49)
	52% in metastatic bladder cancer	I	68	(47)
Target: PD-L1 Name: BMS-936559, MDX-1105 Isotype: Fully human IgG4a Source: Bristol-Myers Squibb	17.3% in melanoma; 11.7% in RCC; 10.2% in NSCLC; 5.9% in ovarian cancer	I	207	(48)

Note: Clinical trials with less than 20 cases or published after October 1, 2015 were not included in the table. Abbreviations: RCC, renal cell carcinoma; NSCLC, non-small-cell lung cancer; CRC, colorectal cancer; HSC, Hematopoietic Stem-Cell Transplantation

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